29th European Peptide Symposium

PEPTIDES 2006

Proceedings of the
Twenty-Ninth European Peptide Symposium
September 3-8, 2006, Gdansk, Poland

Edited by
Krzysztof Rolka • Piotr Rekowski • Jerzy Silberring

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www.29eps.com

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Preface

The 29th European Peptide Symposium was held in Gdansk, Poland from Sunday, September 3rd, to Friday, September 8th, 2006. The venue of the Symposium was Baltic Philharmonic Hall, conveniently located on the Olowianka Island in the centre of the town. The thousand-year-old city of Gdansk is situated on the Bay of Gdansk and the southern coast of the Baltic Sea. With its Hanseatic background, Gdansk played an important role in commercial relationships between countries in Europe. Gdansk is one of the most beautiful cities in Poland, famous for its history (including the three last decades of the past century) and architecture. The city is a perfect place to combine science, history and tradition.

Almost 900 scientists, accompanying persons and exhibitors from 42 countries attended the Symposium.

Following the tradition of the previous EPS meetings, Dr. Bert L. Schram Young Investigator's Mini-Symposium preceded the official opening of the conference. Twelve young researchers competed for two awards sponsored by the ESCOM Science Foundation. Co-chair persons of two scientific sessions Drs. Jirina Slaninova, Jean François Hernandez, Botond Penke, Piotr Mucha and David Andreu, the Scientific Affairs Officer of EPS, served as judges of this competition. The Dr. Bert L. Schram Awards were granted to Markus Muttenthaler (Australia) and Susana Gordo (Spain) plus a honorable mention to Nir Qvit (Israel). After welcome greetings by distinguished guests representing University of Gdansk and local administration; the official opening of the Symposium was followed by *in memoriam* of Prof. Bruce Merrifield presented by Prof. David Andreu. Then, Prof. Jean Martinez, Chairman of the EPS announced the laureates of EPS awards; the Joseph J. Rudinger Memorial Lecture Award was given as a shared prize to Profs. Ettore Benedetti and Claudio Toniolo, and the Zervas Award received Dr. Carlos Garcia-Echeverria. After presenting the awards to the winners (unfortunately Carlos Garcia-Echeverria was not present), Ettore Benedetti gave his lecture entitled: "Structure-activity relationships in peptides: from modelling to the rational drug design", followed by a talk by Claudio Toniolo entitled: "An adventure in peptide conformation." Carlos Garcia-Echeverria delivered his lecture entitled "Antagonists of intracellular protein-protein interactions: a new class of targeted anticancer agents" on Wednesday afternoon.

Keeping up the tradition of European Peptide Symposia, the 29th EPS covered all aspects of the latest developments in peptide research, providing researchers with different backgrounds an opportunity to present and exchange ideas. The overall quality of lectures and posters were excellent. As in Prague, the Program Committee had severe difficulties in selecting the speakers and to keep the proper balance between various European and other countries.

The first scientific session started Monday morning, whereas the last one was on Friday afternoon. The program was divided into fourteen sessions. Sixty-eight oral presentations were scheduled, including eight key-lectures delivered by Profs. Frank Bordusa, Jutta Eichler, Fred Nyberg, Claudio Toniolo, Shuguang Zhang and the Nobel Prize laureate Kurt Wuthrich. In addition, over 540 communications were presented as posters during three afternoon sessions. This very tense scientific program kept participants busy through the all days of the Symposium. The organizers hope that the social events: the philharmonic concert of Polish Baltic Philharmonic Orchestra (Monday evening), speakers' dinner (Tuesday evening), soccer game (Wednesday afternoon), and concert of Capella Gedanensis, followed by the Farewell Party at the Polish Maritime Museum in Gdansk (Thursday evening), helped participants to regenerate their physical strength for the next days of hard work. The

scientific program and abstracts of all presentations were published in the *Journal of Peptide Science, Vol. 12, Suppl., 2006*. Most of the results presented are published in the Proceedings, and now it is our pleasure to forward them to all participants.

One of the aims of the EPS symposia is to promote young researchers who are just at the beginning of their careers. Money collected in the Travel Grant Fund, allowed the organizers to refund partial expenses of 54 young participants. In addition, five researchers under the age of 30 or engaged in studies towards their Ph.D. degrees were also awarded for their poster presentations. These awards were sponsored by the ESCOM Science Foundation, the Rector of the University of Gdansk and *Thieme Chemistry*. The Poster Competition Committee consisting of 16 distinguished scientists, and headed by David Andreu, selected among over 100 presentations, the following winners: David Ireland (Australia), Kevin Pagel (Germany), Meritxell Teixido (Spain), Yi-Pin Chang (Taiwan) and Zvi Hayuka (Izrael). All young laureates received their prizes at the official part of the Farewell Party.

The Symposium in Gdansk contributed to the long tradition of the truly international meetings of researchers dealing with various aspects of peptides. The strength of the conference was (and is) a broad participation of young scientists who have a perfect opportunity to learn and exchange the ideas with top-researchers from Europe and all over the world. We do hope 29th EPS fulfilled the expectations of all participants.

We would like to thank the EPS Executive Committee and members of all Committees for excellent collaboration during preparation of the Symposium and for their invaluable help with selection of speakers, laureates, and young scientists who received travel grants and awards. It is also our pleasure to thank all sponsors and exhibitors, without whom the conference would not be possible to organize. Last but not least, we do appreciate help of all teams contributing to the success.

Finally, we would like to wish Hilkka Lankinen, a Chairperson of the next 30th EPS, all success in organization of the next meeting in Helsinki, which, we are sure, everyone will enjoy at both scientific and social levels.

Krzysztof Rolka

Piotr Rekowski

Jerzy Silberring

Chairpersons of 29th EPS: Jerzy Silberring (left), Piotr Rekowski (next from left, Krzysztof Rolka (right), together with the Nobel prize Winner, Kurt Wüthrich (second from right).

Twenty-Ninth European Peptide Symposium
September 3-8, 2006, Gdansk, Poland

Chairpersons:

Krzysztof Rolka	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Piotr Rekowski	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Jerzy Silberring	Faculty of Chemistry, Jagiellonian University, Krakow, Poland

Programme Committee:

David Andreu	Pompeu Fabra University, Barcelona, Spain
Ettore Benedetti	University Napoli Federico II , Napoli, Italy
Michael Bienert	Forschungsinstitut fur Molekulare Pharmakologie, Berlin, Germany
Chaim Gilon	The Hebrew University of Jerusalem, Jerusalem, Israel
Ferenc Hudecz	Eotvos Lorand University, Hungarian Acad. Sci., Budapest, Hungary
Jan Izdebski	University of Warsaw, Warsaw, Poland
Bernard Lammek	University of Gdansk, Gdansk, Poland
Hilkka Lankinen	University of Helsinki, Helsinki, Finland
Jean Martinez	CNRS, Montpellier, France
Piotr Rekowski	University of Gdansk, Gdansk, Poland
Krzysztof Rolka	University of Gdansk, Gdansk, Poland
Jerzy Silberring	Jagiellonian University, Krakow, Poland
Jirina Slaninova	Inst. of Organic Chemistry & Biochemistry, Prague, Czech Republic

Honorary Committee:

Paweł Adamowicz	Mayor of the City of Gdansk
Andrzej Ceynowa	Rector of the University of Gdansk
Jan Kozłowski	Marshal of Pomorskie Voivodeship
Brunon Synak	President of the Local Parliament
Lech Wałęsa	Former President of the Republic of Poland

Local Organizing Committee:

Anna Jaśkiewicz	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Anna Łęgowska	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Adam Lesner	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Hanna Miecznikowska	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Piotr Mucha	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Adam Prahl	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Piotr Rekowski	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Krzysztof Rolka	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Jarosław Ruczyński	Faculty of Chemistry, University of Gdansk, Gdansk, Poland
Jerzy Silberring	Faculty of Chemistry, Jagiellonian University, Krakow, Poland

Volunteer Team:

Eżbieta Bulak	Andrzej Sawuła
Dawid Dębowski	Adam Sieradzan
Tomasz Dyląg	Andrzej Sieradzan
Anna Kozłowska	Małgorzata Śleszyńska
Anna Kwiatkowska	Aleksandra Walewska
Bożena Kwiatkowska	Magdalena Wysocka
Brygida Lewandowska	Ewa Zabłotna
Mariola Olkowicz	

Twenty-Ninth European Peptide Symposium

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Applied Biosystems
Australian Peptide Association
Bachem AG
BCN Peptides S.A.
Bio-Synthesis
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The European Peptide Society
www.eurpepsoc.com

The European Peptide Society was founded in 1989. Its most important activity is the organisation in Europe of the biennial international symposium, which regularly attracts about 1000 participants from all over the world. The Society also supports financially smaller local meetings and workshops. It is an editor of the six-monthly Newsletter and the Society's official journal, the Journal of Peptide Science. The Society has a membership of about 1200 (from some 30 countries) who pay no subscription at present. The principle was established at the outset that there would be no subscription in order to ensure that all peptide scientists in Europe would be able to enrol. The Society administers the Josef Rudinger Memorial Lecture Award and the Leonidas Zervas Award, and a fund to assist younger members to attend symposia.

Travel Grant Committee:

David Andreu	Spain
Ettore Benedetti	Italy
Paul Cordopatis	Greece
Jerzy Silberring	Poland

Dr. Bert L. Schram Young Investigator's Mini-Symposium Committee:

David Andreu	Spain
Jean François Hernandez	France
Piotr Mucha	Poland
Botond Penke	Hungary
Jirina Slaninova	Czech Republic

Poster Competition Committee:

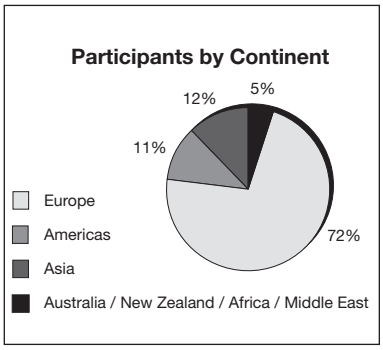
Paul Alewood	Australia
David Andreu	Spain - Chairman
Alex Eberle	Switzerland
Jutta Eichler	Germany
Assaf Friedler	Israel
Ronald Frank	Germany
Jan Jezek	Czech Republic
Ana-Isabel Jiménez	Spain
Yuji Kobayashi	Japan
Adam Lesner	Poland - Secretary
Gábor Mezö	Hungary
Alle Nervanen	Finland
Paolo Rovero	Italy
Costas Sakarellos	Grece
Masahatsu Tanaka	Japan
Cecille Unson	USA

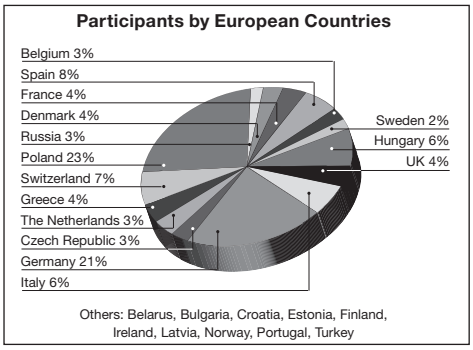
European Peptide Symposia

Symposium	Year	Location
First	1958	Prague, Czechoslovakia
Second	1959	Munich, GFR
Third	1960	Basel, Switzerland
Fourth	1961	Moscow, Russia
Fifth	1962	Oxford, UK
Sixth	1963	Athens, Greece
Seventh	1964	Budapest, Hungary
Eighth	1966	Noordwijk, The Netherlands
Ninth	1968	Orsay, France
Tenth	1969	Abano Terme, Italy
Eleventh	1971	Vienna, Austria
Twelfth	1972	Reinhardsbrunn, GDR
Thirteenth	1974	Kiriyat Anavim, Israel
Fourteenth	1976	Wépion, Belgium
Fifteenth	1978	Gdansk, Poland
Sixteenth	1980	Helsingør, Denmark
Seventeenth	1982	Prague, Czechoslovakia
Eighteenth	1984	Djurönäset, Sweden
Nineteenth	1986	Porto Carras, Greece
Twentieth	1988	Tübingen, GFR
Twenty-first	1990	Barcelona, Spain
Twenty-second	1992	Interlaken, Switzerland
Twenty-third	1994	Braga, Portugal
Twenty-fourth	1996	Edinburgh, UK
Twenty-fifth	1998	Budapest, Hungary
Twenty-sixth	2000	Montpellier, France
Twenty-seventh	2002	Sorrento, Italy
Twenty-eighth	2004	Prague, Czech Republic
Twenty-ninth	2006	Gdansk, Poland

International Peptide Symposia

First	1997	Kyoto, Japan
Second	2001	San Diego, USA
Third	2004	Prague, Czech Republic
Fourth	2007	Cairns, Queensland, Australia





Josef Rudinger Memorial Lecture Award

This Award was established by Ferring Pharmaceuticals in 1984, as a commemoration of Josef Rudinger's role in the foundation of the European Peptide Symposia and diverse contributions he made to peptide chemistry. Previous winners are listed below.

1986
Robert Schwyzer
(ETH Zürich, Switzerland)

1988
Erich Wünsch
(Max-Planck-Institute für Biochemie, Münich, Germany)

1990
R. Bruce Merrifield
(The Rockefeller University, New York, USA)

1992
Viktor Mutt
(Karolinska Institute, Stockholm, Sweden)

1994
Robert C. Sheppard
(MRC, Cambridge, United Kingdom)

1996
Ralph Hirschmann
(University of Pennsylvania, Philadelphia, USA)

1998
Shumpei Sakakibara
(Peptide Institute, Osaka, Japan)

2000
Bernard P. Roques
(INSERM, CNRS, Paris, France)

2002
Sándor Bajusz
(IVAX-Institute of Drug Research, Budapest, Hungary)

Kálmán Medzihradszky
(Department of Organic Chemistry, Eötvös L. University, Budapest, Hungary)

2004
Luis Moroder
(Max-Planck-Institute für Biochemie, Martinsried, Germany)

2006
Ettore Benedetti
Department of Biological Science & CIRPeB, Univerity of Naples "Federico II", Naples, Italy

Claudio Toniolo
Department of Chemistry, University of Padova, Italy

Claudio Toniolo

During more than 25 years Claudio Toniolo (C. T.) has been working on the synthesis in solution and 3D-structural determination of peptides based on C-alfa-tetrasubstituted alfa-amino acids. Inter alia, some of these residues characterise the sequence of membrane active peptaibols (the term peptaibol was introduced by C. T. in 1982). Other fields where this type of peptides studied by C. T. has found application involve their exploitation: (i) as spacers between chromophoric, photo-excitable and redox dyads, (ii) as templates for the construction of mini-receptors in supramolecular chemistry and in catalysis, and (iii) as analogues with reinforced structure of bioactive peptides. C. T. is author or co-author of more than 700 papers and is Editor or member of the Editorial Board of Chem. Eur. J., ChemBioChem, Chem. Biodiver., J. Pept. Sci., Chem. Biol. Drug Design, and Biopolymers (Pept. Sci.). C. T. has also recently been co-Editor of the five-volume series "Houben-Weyl, Methods of Organic Chemistry" on the synthesis of peptides and peptidomimetics. In 2005 he was awarded the gold medal "Piero Pino" for organic stereochemistry from the Italian Chemical Society (Division of Organic Chemistry).

C.T. has been Visiting Researcher (or Professor) at the Polytechnic Institute of Brooklyn, N. Y.; Portsmouth Polytechnic, U. K.; State University of New York at Binghamton, N. Y.; Indian Institute of Sciences, Bangalore, India; University of California at San Diego, CA; National University of Somalia, Mogadishu, Somalia; Osaka University, Osaka, Japan. He is currently Professor of Organic Chemistry in the Department of Chemistry, University of Padova.

Ettore Benedetti

Ettore Benedetti, born in Napoli (Italy) on June 7, 1940.
Professor of Chemistry, University of Napoli "Federico II".
Director of the Interuniversity Research Centre on Bioactive Peptides.

Ettore Benedetti (known to friends as "Bibi") received his Ph.D. in Chemistry in Napoli and he did postdoctoral studies at Polytechnic Institute of Brooklyn at New York with Prof. Murray Goodman, with whom he collaborate scientifically since then.

Bibi has served as Assistant Professor, and Associate Professor of Chemistry at the Science Faculty of the University of Napoli "Federico II", where in 1984 he was appointed Full Professor of Chemistry.

Bibi has been a Visiting Professor at Cornell University, a Brown and Williamson Professor at the University of Louisville, and a Fulbright Visiting Scholar at the University of California at San Diego.

Bibi is the organizing Chairman of the "Naples Workshops on Bioactive Peptides". In 2002, he was Chairman of the 27th European Peptide Symposium.

He also serves as member of the Advisory Board of Biopolymers and of the Editorial Board of Biopolymers–Peptide Science, of the Journal of Peptide Science, of Peptide Research and Therapeutics, and of Protein and Peptide Letters.

Bibi's research interests lie in peptide and protein chemistry: the relationships between structure and activity of numerous peptide and protein systems have been the object of his investigations, carried out with a variety of experimental and theoretical techniques, for the understanding of the mechanism of action of biologically relevant systems.

He has authored approximately 450 scientific articles in peer-reviewed journals, review articles and book chapters.

Leonidas-Zervas Award

This award was established by Bachem Inc. USA in 1984, in commemoration of Leonidas Zervas and the outstanding contributions he made to peptide chemistry. Previous winners are listed below.

1988
Alex Eberle
(University of Basel, Basel, Switzerland)

1990
Michal Lebl
(Czechoslovak Academy of Sciences, Prague)

Jean Martinez
(CNRS, Montpellier, France)

1992
Günther Jung
(University of Tübingen, Tübingen, Germany)

1994
Ernest Giralt
(University of Barcelona, Barcelona, Spain)
Fernando Albericio
(University of Barcelona, Barcelona, Spain)

1996
Morten Meldal
(Carlsberg Laboratory, Valby, Denmark)

1998
Annette G. Beck-Sickinger
(ETH Zürich, Switzerland)

2000
Antonello Pessi
(Istituto di Ricerche di Biologica Moleculare P. Angeletti, Pomezia, Rome, Italy)

2002
Thomas W. Muir
(Rockefeller University, New York, USA)

2004
Helene Gras-Masse
(Institut Pasteur de Lille, France)

2006
Carlos Garcia-Echeverria
Novartis Institutes for BioMedical Research, Basel, Switzerland

Carlos Garcia-Echeverria

Dr. Carlos Garcia-Echeverria received his Ph.D. degree in organic chemistry under the supervision of Profs. Fernando Albericio and Miquel Pons. After a 3-year post-doctoral stay at the University of Madison-Wisconsin with Prof. Daniel Rich, he joined the Exploratory Research Unit of Ciba-Geigy (now Novartis Institutes for BioMedical Research) in 1993, and the Oncology Research Group in 1995. He has been the medicinal chemistry sponsor and team head of different programs involving tumour cell growth control and apoptosis. Recently, he has been appointed Executive Director, Head Drug Discovery Oncology. His research activities have been mainly focused on the synthesis of phosphopeptides and the identification and development of inhibitors of protein and lipid kinases (e.g. IGF-IR, c-Met, PKB, PDK1 and PI3K), proteolytic enzymes (e.g. 20S proteasome) and protein-protein interactions (e.g. Grb2-SH2 and p53/hdm2). He is an inventor on 20 patents (issued or pending), and has published 10 book chapters and more than 100 articles and review papers. He has been honoured with the Novartis Leading Scientist Award (2002) and the Novartis Oncology President's Award (2003) for his seminal contributions to oncology drug discovery. He is Senior Editor of Drug Discovery and Chemical Biology and Drug Design. and a board member of Drug Design Reviews-Online, Expert Opinion on Therapeutic Targets, Journal of Peptide Research and Therapeutics, Current BioData, Recent Patent Reviews on Anti-cancer Drug Discovery and The Open Cancer Journal.

Dr. Bert L. Schram Young Investigator's Mini-Symposium:

Sponsored by ESCOM Science Foundation

Winners:

Markus Muttenthaler ~ University of Queensland, Brisbane, Australia

Susana Gordo ~ University of Barcelona, Barcelona, Spain

Honorable Mention:

Nir Qvit ~ Hebrew University of Jerusalem, Jerusalem, Israel

Young Investigator's Poster Competition:

Dr. Bert L. Schram Young Investigator's Poster Competition

Sponsored by ESCOM Science Foundation

David Ireland ~ University of Queensland, Brisbane, Australia

Kevin Pagel ~ Free University, Berlin, Germany

Young Investigator's Poster Competition:

Sponsored by the Rector of the University of Gdansk

Yi-Pin Chang ~ National Chung Cheng University, China-Yi, Taiwan

Meritxell Teixido ~ University of Barcelona, Barcelona, Spain

Young Investigator's Poster Competition:

Sponsored by Thieme Chemistry

Zvi Hayuka ~ Hebrew University of Jerusalem, Jerusalem, Israel

Twenty-Ninth European Peptide Symposium
Travel Grants

COUNTRY	NAME	LAB
Eastern Europe		
Belarus	Paulava	Martunova
Bulgaria	Minchev	Vezenkov
	Danalev	Vezenkov
	Todorov	Naydenova
Croatia	Gredicak	Horvat
	Matkovic	Mlinaric-Majerski
Czech Rep.	Ciencialova	Jiracek
	Sebestik	Hlavacek
Estonia	Säälik	Pooga
Hungary	Bánoczi	Hudecz
	Jakab	Mezö
	Hetényi	Fülöp
Latvia	Liepina	
Russia	Smirnova	Baratova
	Tereshkina	Shaitan
	Kuraeva	Kolesanova
	Moskalenko	Panarin
Australasia & S. America		
Australia	Muttenhaller	Alewood
Brasil	Hayashi	Kerkis
India	Vikram	Rahul Jain
Iran	Astaneh	Bolourtchian
Japan	Sohma	Kiso
Taiwan	Chang	Yen-ho Chu
Western Europe & Israel		
France	Upert	Condom
Sweden	Norgren	Arvidss
Belgium	Lukashuk	Tourwé
	Verzele	Madder
Britain	Jones	Howl
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Israel	Hayouka	Friedler
	Linde	Gilon
Italy	Paolini	Papini
	Guryanov	Toniolo
	Kieres	D'Ursi
	Ronga	Benedetti
	Rubini	Ruzza
Portugal	Henriques	Castanho
	Mano	Pedroso de Lima
Spain	De la Torre	Andreu
	Gordo	Giralt
	Quintanar-Audelo	Albericio
Germany	Coin	Beyermann
	Manea	Przybylski
	Rat	Fahrenholz
	Manzenrieder	Kessler
	Kusebauch	Moroder
	Pagel	Koksch
	Rennert	Beck-Sickinger
	Hampel	Heinzel
	Strijkowski	Eichler
	Weigelt	Sewald
Greece	Galanis	Cordopatis
	Galanakis	Spyroulias
Switzerland	Vadas	Rose

Twenty-Ninth European Peptide Symposium
Number of active participants per country

Australia	17
Belarus	4
Belgium	8
Brazil	2
Bulgaria	13
Canada	14
China	4
Croatia	3
Czech Republic	15
Denmark	8
Egypt	2
Estonia	2
Finland	8
France	21
Germany	74
Greece	36
Hungary	32
Iceland	1
India	6
Iran	18
Ireland	2
Israel	8
Italy	53
Japan	62
Latvia	3
New Zealand	1
Poland	87
Portugal	6
Russia	26
Russia/Chile	1
Singapore	1
South Africa	1
South Korea	1
Spain	26
Sweden	8
Switzerland	8
Taiwan	4
The Netherlands	5
Turkey	2
UK	12
Ukraine	1
USA	36

Twenty-Ninth European Peptide Symposium

and so it all converged in Gdansk in 2006…

AZIRIDINE-MEDIATED SYNTHESIS OF MULTITOPIC β-LACTAM SCAFFOLDS FOR β - AND γ-TURN STABILIZATION

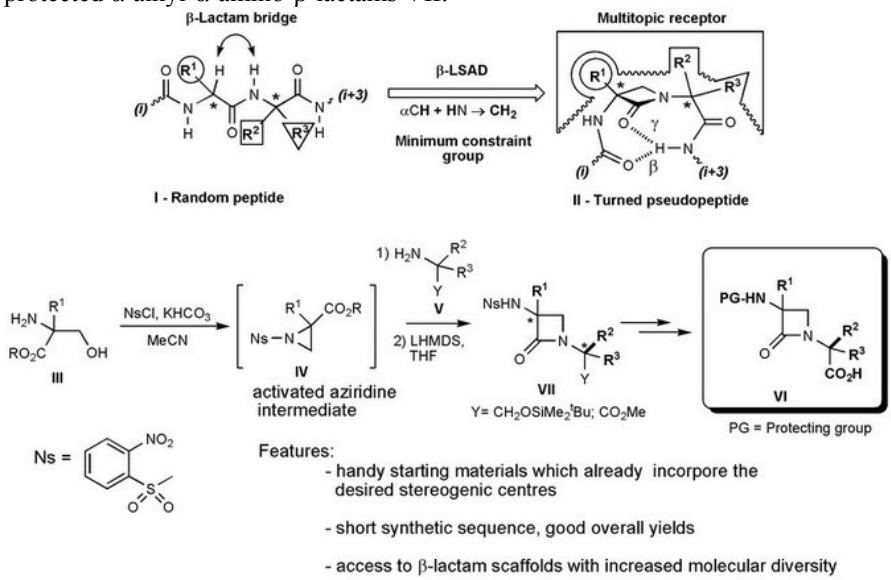
Claudio Palomo, Jesus M. Aizpurua, Jose I. Ganboa, Ana Benito, Raluca M. Fratila, Iraida Loinaz, Eva Balentova, Lourdes Cuerdo, Azucena Jimenez, Jose Ignacio Miranda and Joseba Oyarbide

Departamento De Quimica Organica-I, Facultad De Quimica, Universidad Del Pais Vasco, San Sebastian, Spain

Introduction

Of a methylene bridge between the C^{α}(i+1) and the N(i+2) atoms in an open peptide (I) to mimic simultaneously the C^{α}H(i+1) and HN(i+2) protons (β-Lactam Scaffold Assisted Design - β-LSAD) has proven to be a practical tool for the preparation of monotopic β-turn peptidomimetics (II, $R^2 = R^3 = H$), according to the principle of separation of constraint and recognition elements [1].

In this work we report a short, general, and stereocontrolled synthesis of multitopic β-lactam scaffolds of type VI. α-Alkyl serinates ββ III are converted into the corresponding enantiopure *N*-nosyl-aziridines IV which undergo *in situ* ring-opening with amino acids V. Subsequent base-promoted cyclization affords the N-protected α-alkyl-α-amino-β-lactams VII.



Incorporation of the novel scaffolds into linear and cyclic peptides and their conformational features are also presented, most of them showing stabilized β- and γ-turn conformations.

References

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ANGIOGENESIS INHIBITION ACTIVITY OF A CYCLIC RGD-B-LACTAM PENTAPEPTIDE

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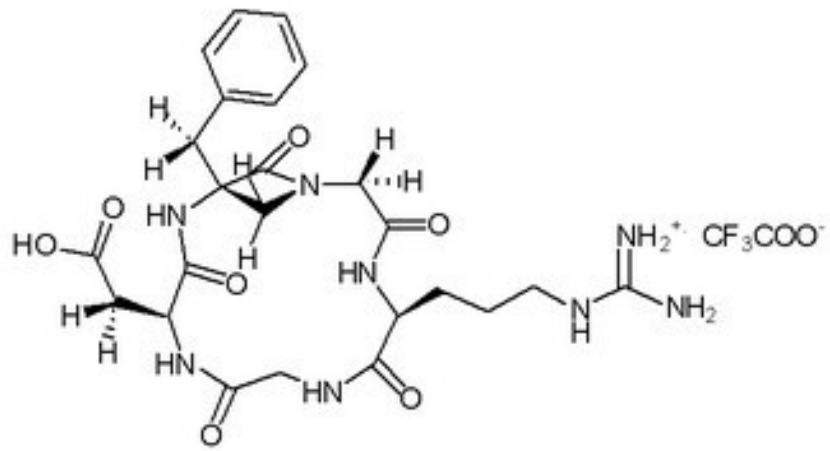
Thęαvβ3 integrin receptors play an important role in human tumor metastasis and growth. The inhibition of these receptors by antibodies or by cyclic peptides containing the Arg-Gly-Asp(RGD) sequence may be used as selectively treatment to suppress the disease [1].

Our research group has previously described that the formal introduction of a single carbon atom to bridge the C^{α} (i) and N(i+1) contiguous residues of a linear or cyclic peptide leads to α-amino-β-lactam peptidomimetics containing predictably placed β-turn and γ-turn motifs, respectively [2]. The combination of these results with the well-known capacity of RGD tripeptide for inhibition of the biological answer in integrin led us to the design of the following cyclic peptide.



The adhesion and cell-growth "*in vitro*" assays using human umbilical vein endothelial cells (HUVEC), as well as "*in vivo*" assays with xenograph mice revealed that the RGD peptidomimetic was active to micromolar concentrations, slightly better than the reference compound in this field: Cilengitide® [3, 4].

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CONFORMATIONAL STABILITY OF THE HUMAN CYSTATIN C α-HELICAL FRAGMENT

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Introduction

Human cystatin C (hCC), a monomeric cysteine proteases inhibitor belongs to the amyloidogenic proteins shown to dimerize through 3D domain swapping mechanism [1]. The hCC dimerization is preceded by an opening movement of L1 loop from β2-L1-β3 hairpin and separation of the β1-helix-β2 fragment from the remaining part of the molecule. However the analysis of the hCC amino acid sequence suggests that local interactions within and in the proximity of the N-terminal fragment of the α-helix might also promote partial loosening of the monomer structure contributing to the dimerization process. The N-terminal part of helix does not posses any of the preferred residues in the 12 α-helix unique positions [2]. Moreover, there are Glu19 and Glu20 that are classified as strongly avoided at N' and Ncap positions.

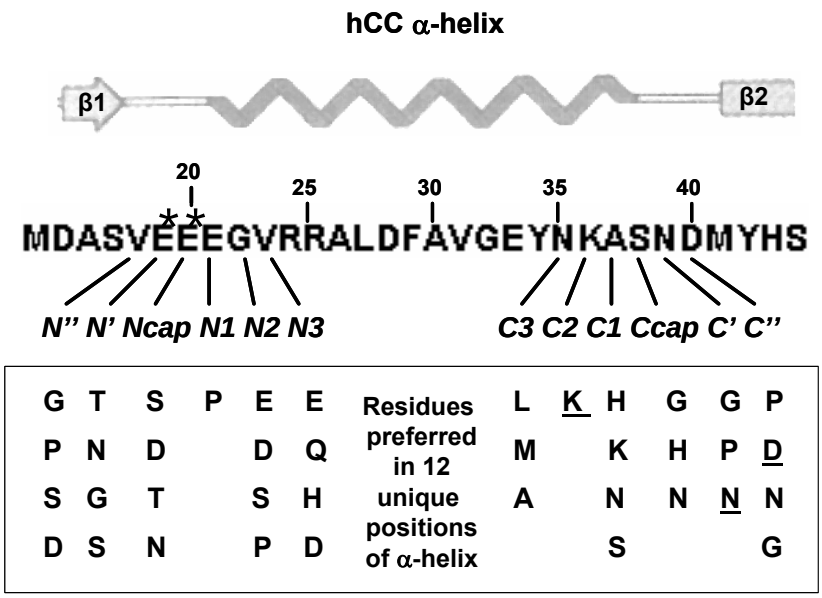

Fig. 1. Amino acid sequence within and around hCC α-helix (12 unique positions of helix are shown in italics).

Results and Discussion

The peptide fragment of hCC α-helix and its seven analogs were synthesized and studied using CD spectroscopy. The random coil was the predominant structure of the peptide corresponding to the α-helix and its N-truncated analogs. An increase of

α-helix content was observed only in conditions promoting the helical structure. Surprisingly, the attachment of Ser, known to be preferred in N-cap position of α-helix also didn't influence helix content. The replacement of Arg25 by Ala didn't change the helix content greatly. However, the substitution of Arg24 or Asp28 by Ala did significantly increase the helix content. These results indicate the presence of the electrostatic interactions between Arg24 and Asp28 which destabilize the helical structure of the studied hCC fragment.

Table 1. α-Helix content calculated from the CD spectra of peptide analogs of hCC helical fragment using the Selcon3 program [%].

peptide	PBS*	30% TFE in PBS*	20 mM SDS in PBS*	H_2O	20 mM SDS in H_2O
EGVRRALDFAVGEYNKA	11	39	53	6	55
RRALDFAVGEYNKA	11	30	35	4	22
RALDFAVGEYNKA	11	15	26	1	22
LDFAVGEYNKA	3	23	3	2	2
SEGVRRALDFAVGEYNKA	16	43	40	4	53
EGVARALDFAVGEYNKA	23	79	84	36	87
EGVRAALDFAVGEYNKA	15	25	45	5	17
EGVRRALAFAVGEYNKA	17	55	76	17	78

* 10 mM phosphate buffer, pH = 7,4

The distances between potential hydrogen side chain donors and acceptors (N...O) are as follows: 9.95 Å (Glu21←Arg24), 4.10 Å (Glu21←Arg25) and 3.09 (Arg24→Asp28) (PDB 1G96). The corresponding distances in hCC monomer taken from its model build on the bases of the crystal structure of dimer are 6.33, 4.30 and 8.60, respectively [3]. This data suggested the presence of ion-pair interactions between Glu21-Arg25 in monomer and dimer. However, in the dimer a new strong salt bridge seems to appear between Arg24 and Asp28. We suppose that electrostatic interaction between Arg24 and Asp28 could be one of the factors promoting the formation of the hCC dimmer.

Acknowledgments
Supported by UG-DS/8350-5-0131-6 and EFS ZPORR/2.22/II/2.6/ARP/U/2/05.

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N-METHYLATION OF C α-ALKYLATED LINEAR PEPTIDES: SYNTHETIC ASPECTS AND 3D-STRUCTURAL CONSEQUENCES

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Introduction

Peptides characterized by single or multiple N-methylated, *C^{α}-trisubstituted (e.g.*, protein) amino acids are of great interest in medicinal chemistry. Several naturally-occurring peptides, remarkably stable to enzymatic attacks, are based on N-methylated residues. The classical conditions (CH_3I/Ag_2O in DMF, 24 hours, room temperature) for N-methylation of the peptide function [1] are a useful tool for distinguishing solvent exposed from intramolecularly H-bonded –CO–NH– groups in *cyclic* peptides [2]. In this work we have extended this reaction to a set of Nα-acetylated, pivaloylated or *para*-bromobenzoylated, linear homo-peptides to the pentamer level based on the helicogenic *C^{α}-tetrasubstituted* α-amino acids Aib (α-aminoisobutyric acid) or (αMe)Nva (C^{α}-methyl norvaline) residues (Fig. 1) [3].

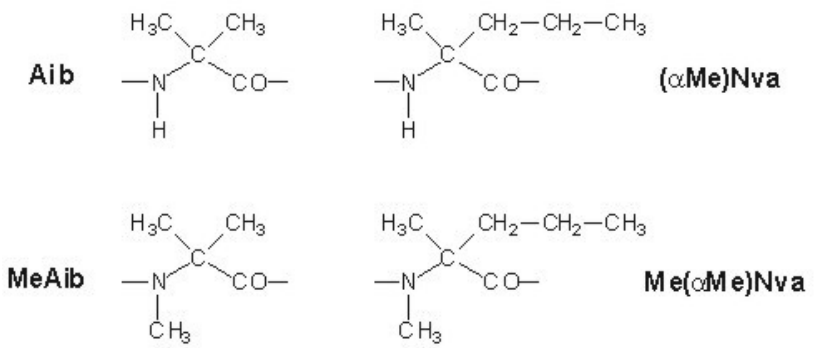

Fig. 1. The Cα-alkylated α-amino acids studied in this work.

Results and Discussion

Our results indicate that, in all peptide substrates investigated and under the classical conditions for N-methylation used, the N-terminal acylamide function is indeed modified, but this is not so for any internal or C-terminal peptide bond between two sterically demanding, C^{α}-alkylated, residues.

Our FT-IR absorption, NMR, and X-ray diffraction (Fig. 2) conformational analysis clearly shows that the N-terminally N-monomethylated peptides investigated are still β-turn folded or 3_{10}-helical, although with some distortion. In particular, the new tertiary amide bonds are *trans*. Conversely, the molecular packing in the

crystalline state is remarkably altered. These 3D-structural data are not surprising in view of the known observation that, for the formation of the *intra*molecular H-bonds stabilizing the β-turn and 3_{10}-helical structures, peptide molecules do not take advantage of the N(1)-H group as a donor, but rather that this functionality is massively involved in the *inter*molecular H-bonding schemes typical of the turn/helix aggregation modes.

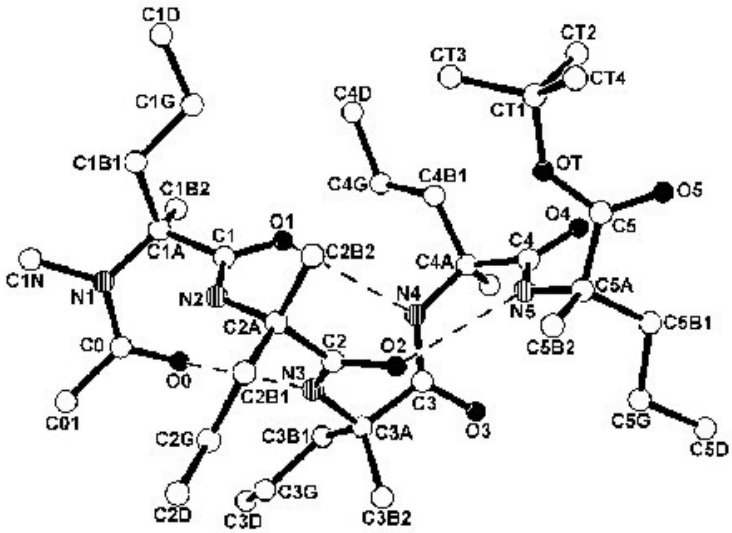

Fig. 2. X-Ray diffraction structure of Ac-L-Me(αMe)Nva-[L-(αMe)Nva]4-OtBu with atom numbering.

The present findings also suggest that this N-methylation approach cannot be exploited, as in cyclic peptides formed by coded amino acids, to detect the chemical reactivity and, indirectly, the preferred conformation of peptides based exclusively on C^{α}-alkylated α-amino acids. Finally, we confirmed previous findings that the peptide bond *following* the N^{α}-methylated amino acid is unstable even to mild acidic conditions.

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TWO-DIMENSIONAL INFRARED SPECTROSCOPY DISCRIMINATES BETWEEN 3_{10}- AND α-HELICES

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Introduction

Two-dimensional (2D) IR spectroscopy [1] is one of the most promising techniques for studying peptide 3_{10}- to α-helix transitions and equilibria because it can be used to obtain a cross-peak pattern of the amide-I (C=O stretching) modes. Cross-peaks appear in the spectra only if the vibrational modes are coupled, the coupling strength largely depending on the peptide secondary structure.

Furthermore, the time resolution of 2D IR spectroscopy is less than a few picoseconds for the amide-I bands of peptides, as determined by the time required to fully characterize the decay of vibrational coherences created and manipulated by femtosecond IR pulses. These unique characteristics have made 2D IR spectroscopy a powerful tool to elucidate structure and dynamics of peptides. In this work we applied femtoseconds 2D IR spectroscopy to the homo-octapeptide Z-[L-(αMe)Val]$_8$-O*t*Bu in solvents of diverging polarities to acquire spectral signatures that distinguish between 3_{10}- and α-helical structures.

Results and Discussion

We already described a slow 3_{10}- to α-helix transition in HFIP solution for this L-(αMe)Val octamer using far-UV CD [2, 3]. More recently, we showed that under these experimental conditions the conformational transition is accompanied by an acidolysis of the tert-butyl ester function (unpublished results). This peptide is much more chemically and conformationally stable in either $CDCl_3$ or TFE solution.

Suppression of diagonal peaks by controlling polarization of IR pulses clearly revealed cross-peak patterns that are crucial for peptide secondary structure determination. A doublet feature is observed when the peptide ester forms a 3_{10}-helix in $CDCl_3$ and TFE (Fig. 1a, 1b), and when it is at the initial stage of the 3_{10}- to α-helix transition in HFIP (Fig. 1c). This is the first report on the experimental 2D IR signature of a 3_{10}-helical peptide. By contrast, the 2D IR spectrum exhibits a multiple peak pattern after the peptide ester has acidolyzed and become an α-helix in HFIP (Fig. 1d). The time evolution of the 2D IR cross-peak pattern in HFIP highlights the change in the amide-I mode vibrational couplings that accompanies the 3_{10}- to α-helix transition.

These results, using a short model compound, demonstrate the powerful capability of 2D IR spectroscopy to discriminate between different peptide helical structures.

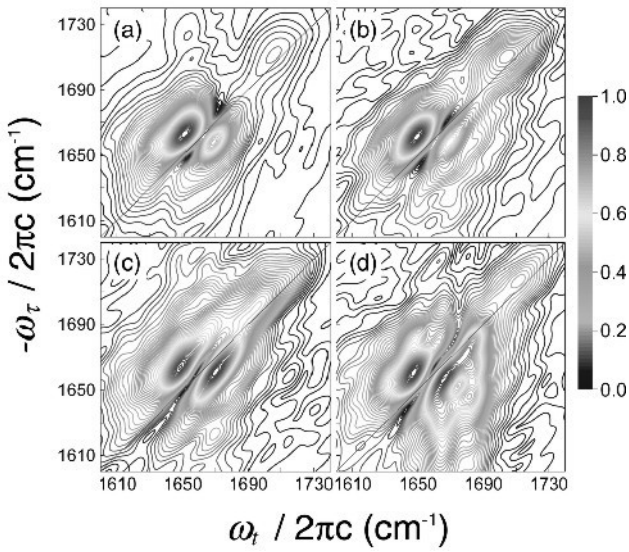

Fig. 1. 2D IR absolute magnitude spectra of Z-[L -(αMe)Val]$_8$-OtBu measured under <π/4, -π/4, Y, Z> polarization configuration in different solvents: (a) $CDCl_3$; (b) TFE; (c, d) HFIP. The spectra in HFIP were measured (c) immediately and (d) 34 days after sample preparation.

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ILE/ALLOILE DYAD:LACK OF SCREW-SENSE CONTROL OF THE 3_{10}-HELICAL STRUCTURE BY β-CARBON CONFIGURATION

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Introduction

Foldamers, including oligopeptides, represents a modern area of growing interest for fundamental and applied research activities because such compounds offer unique scaffolds for the development of molecular functions. However, the configurational factors controlling details of their 3D-properties, e.g. chirality of the side chain of the constituent monomeric unit, are, in general, still poorly understood. In this work we have contributed to a deeper knowledge of this problem by investigating the conformational properties of the four diastereomeric hexapeptide sequences -Aib-Xxx-$(Aib)_2$-Yyy-Aib-, heavily based on a host template rich in the strongly helicogenic, achiral, Aib residue, and each characterized by an L-Ile/L -*allo*Ile (Fig. 1) (Xxx and Yyy) guest dyad [1].

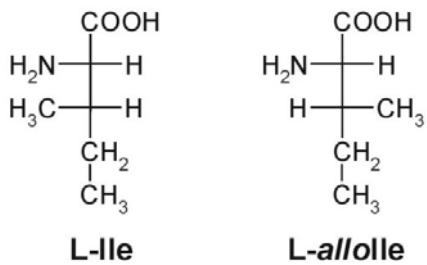

Fig. 1. Fischer's projections of L-Ile and L-alloIle.

Results and Discussion

A detailed FT-IR absorption, NMR, CD, and X-ray diffraction (Fig. 2) conformational study allowed us to validate our working hypothesis that all four peptides are folded in well-developed 3_{10}-helical structures.

In addition, the helical structures of all peptides investigated are found to privilege the right-handed screw sense, thus implying that the chirality of the amino acid α-carbon is more influential on this 3D-structural parameter than that of the β-carbon. These results should be compared with the published data on the Ile versus *allo*Ile homo-hexamers and heptamers, which clearly demonstrated a significantly higher stability for the self-associated β-sheet structure of the Ile oligomers [2]. Moreover, interestingly enough, here too the signs of the CD Cotton effects typical of the intermolecularly H-bonded β-sheet conformation reflect only the α-carbon configuration.

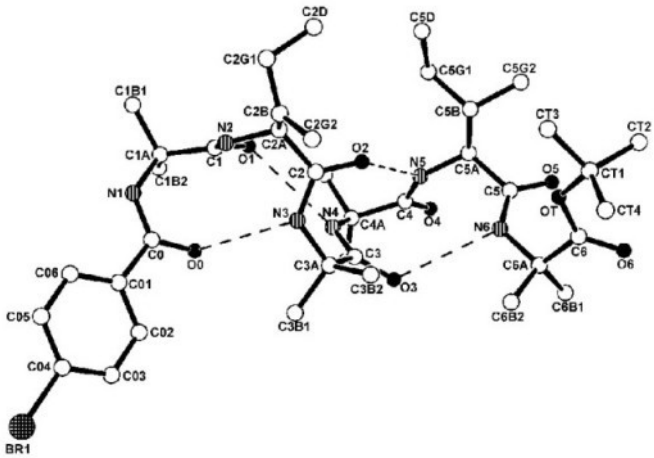

Fig. 2.X-Ray diffraction structure of the terminally protected, right-handed, 3_{10}-helical, hexapeptide Z-Aib-L -alloIle-$(Aib)_2$-L-Ile-Aib-OtBu with atom numbering. The four intramolecular C=O•••H-N H-bonds are represented by dashed lines. Only the major conformer [at the alloIle^2 C^{δ}-atom] is shown.

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ANTIMICROBIAL CATIONIC PEPTIDES: DESIGN, SYNTHESIS, CONFORMATIONAL AND BIOLOGICAL STUDIES

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Introduction

Antimicrobial peptides are used as the first defensive barrier of the organism against microbial infection. They possess positive charge, a substantial portion of hydrophobic residues and adopt amphipathic conformations when interacting with bacterial membranes [1]. Our approach is based on the design, synthesis and study of helical sequential polypeptides $(Arg\text{-}X\text{-}Gly)_n$, where X = Ala, Val, Leu and amphipathic Aib-containing peptide models of various chain-lengths, Ac-$(Aib\text{-}Arg\text{-}Aib\text{-}Leu)_n$-$NH_2$ (n = 1 - 4). The presence of Aib, which induces and stabilizes helical structures, in combination with the hydrophobic amino-acids and the positive charge of Arg side-chain, for the interaction with the negatively charged phospholipid membranes, target to the development of new antimicrobial peptides.

Results and Discussion

The sequential polypeptides $(Arg\text{-}X\text{-}Gly)_n$ were synthesized by polymerization of the pentachlorophenyl active esters of the appropriate tripeptides, while synthesis of the peptides Ac-$(Aib\text{-}Arg\text{-}Aib\text{-}Leu)_n$-$NH_2$ (n = 1 - 4) was performed by the Fmoc/*t*Bu strategy on a Rink-Amide AM resin [2]. Coupling reactions were performed using a molar ratio of amino-acid/HBTU/HOBt/DIEA/resin 3/3/3/9/1. Removal of Fmoc protective groups was achieved using piperidine/DMF and the acetylation was performed using acetic anhydride/pyridine. Peptides were cleaved from the resin using 95% TFA / 2.5% H_2O /2.5 % TIS, purified by HPLC and characterized by ESI-MS.

Table 1. Antimicrobial activity: minimal inhibitory concentration (MIC).

	MIC, μg/ml			
	$(Arg\text{-}Val\text{-}Gly)_n$	$(Arg\text{-}Leu\text{-}Gly)_n$	Ac-(Aib-Arg-Aib-Leu)$_3$-NH$_2$	Ac-(Aib-Arg-Aib-Leu)$_4$-NH$_2$
E.coli DH5α	–	150 μg/ml	250 μg/ml	150 μg/ml
P.aeruginosa	–	–	–	500 μg/ml
Z.mobilis 10988	–	–	–	500 μg/ml
M.smegmatis mc^{2}155	150 μg/ml	5 μg/ml	50 μg/ml	300 μg/ml
B. subtilis	–	–	50 μg/ml	5 μg/ml

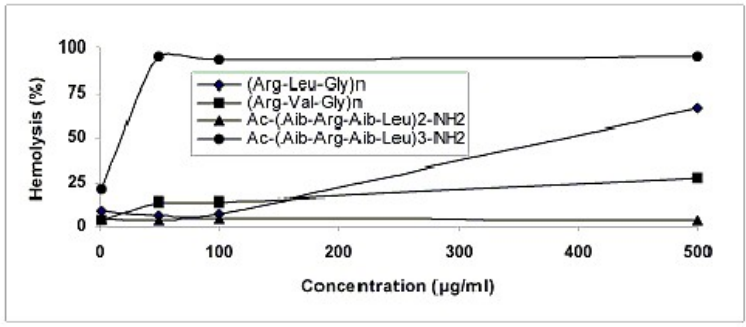

Fig. 1. Hemolytic activity.

Proteolytic stability

The enzymatic degradation of peptides with trypsin was carried out at 37 ^{o}C for 4h, proteolysis stopped by adding an appropriate trypsin inhibitor at different time points and the samples were tested for their residual antimicrobial activity.

Conformational studies

CD spectra of peptides (10^{-4} M) in 10^{-4} M TFE/H_2O (50:50), SDS and PBS indicate that the dimer, trimer and tetramer of Aib-Arg-Aib-Leu adopt helical characteristics.

Conclusions

The peptides Ac-(Aib-Arg-Aib-Leu)$_3$-NH_2 and Ac-(Aib-Arg-Aib-Leu)$_2$-NH_2 exhibit the best activity against bacteria and retain their antimicrobial activity after treatment with trypsin. The non-toxic polypeptides (Arg-X-Gly)$_n$, where X=Val, Leu exhibit antimicrobial activity, but become inactive after treatment with trypsin.

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BIOPHYSICAL PROPERTIES OF ALAMETHICIN F50/5 AND SELECTED ANALOGUES INSERTED IN ROD OUTER SEGMENT MEMBRANES

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Introduction

Peptides and proteins that form membrane-spanning pores comprise a diverse class of molecules ranging from short peptides, that are unregulated and create non-selective pathways, to large ion channel proteins, that are higly regulated and exhibit exquisite selectivity for specific ions. Numerous pore-forming peptides (native or re-engineered to create targeted and regulable cell-killing agents) are currently in development as antimicrobial agents, with additional potential applications as antiviral and antitumor agents. Moreover, pore-forming peptides are also being developed as biosensors, which could deliver a configurable binding site for analytes encoded in a readily measured electrical signal. However, the structural bases of pore formation and assembly have been determined experimentally only in a few cases.

In this work we investigated the biophysical characteristics and the pore formation dynamics of the naturally occurring major component of the neutral fraction (F50/5) of the peptaibol antibiotic alamethicin and two selected synthetic analogues forming membrane-spanning channels by using isolated red outer segments (OS) of reptilia and amphibia recorded in the *whole-cell* configuration. Once blocked the two OS endogenous conductances (the cGMP channels by light and the retinal exchanger by removing one of the transported ion species, i.e. K^+, Na^+ or Ca^{2+}, from both sides of the membrane), the OS membrane resistance (R_m) could be >5 GΩ. Therefore, any exogenous current can be studied down to the single channel level.

Results and Discussion

The amino acid sequences of alamethicin F50/5 and its two analogues [Glu(OMe)^{7,18,19}] and [Glu(OMe)^{18,19}] investigated in this work are listed in Fig. 1. Their syntheses by solution methods and their characterizations are described in reference [1].

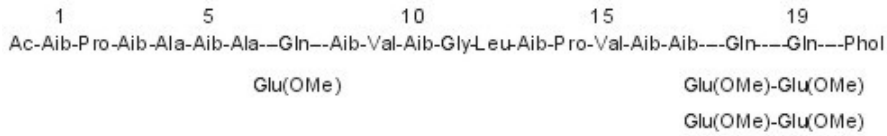

Fig. 1. Amino acid sequences of alamethicin F50/5 and its analogues exploited in this study.

Macroscopic currents of amplitude ~300 pA were recorded in symmetric K^+ or Na^+ (>100 mM) and Ca^{2+} (1 mM) concentrations from the commercially available alamethicin mixture, the synthetic alamethicin F50/5 and the two analogues applied at 1 μM concentration and at 20 mV. Once applied and removed the peptide, the current activates and deactivates with a time constant of about 160 ms. The synthetic analogues [Glu(OMe)^{7,18,19}] and [Glu(OMe)^{18,19}] produce a current of about 100 pA at 1 μM concentration and show a strong activation by hyperpolarization as alamethicin F50/5 itself. Clear single channel events were observed when the concentration of all of the alamethicin peptides was reduced to <250 nM.

These results indicate that the three Gln residues at positions 7, 18 and 19 of alamethicin F50/5 are not an absolute requirement for pore formation and its conduction properties. In general, pore assembly and disassembly are very fast and cooperative events.

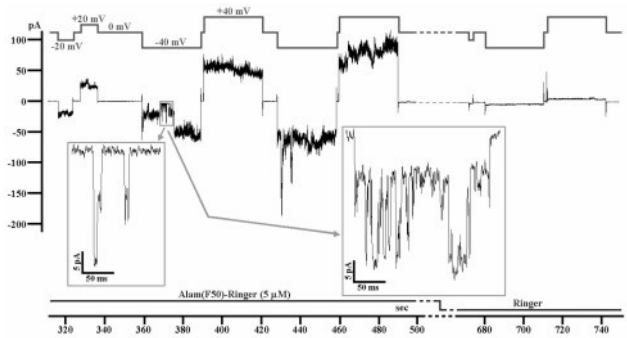

Fig. 2. Recorded macroscopic currents in the whole-cell configuration when a 5 μM solution of alamethicin F50/5 is applied and the potential is varied from +20 mV to 40 mV.

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EPR DISTANCE MEASUREMENTS IN A DOUBLY NITROXIDE-LABELLED HELICAL β-PEPTIDE

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Introduction

β-Peptide molecules possess interesting conformational characteristics and biological properties [1]. They represent a new class of foldamers potentially useful as templates and spacers. The three-dimensional structures of β-peptides have been experimentally investigated using X-ray diffraction and a variety of spectroscopic techniques, but they have never been doubly spin labelled and studied by EPR. To this aim, we took advantage of the β-amino acid residue trans-(*3R,4S*)-β-TOAC (Fig. 1), recently synthesized and spectroscopically characterized by our groups [2].

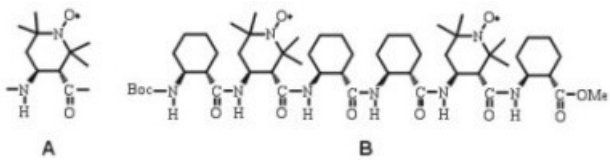

Fig. 1. Chemical structures of (A) the β-amino acid residue trans-(3R,4S)-β-TOAC and (B) the hexapeptide Boc-[(1S,2S)-ACHC-(3R,4S)-β-TOAC-(1S,2S)ACHC]$_2$-OMe.

Results and Discussion

We synthesized by classical solution methods a terminally protected hexapeptide, exclusively based on cyclohexyl β-amino acids, carrying four (*1S,2S*)-ACHC and two trans-β-TOAC residues at central *i, i+3* positions (Fig. 1) [3]. FT-IR absorption and far-UV CD studies strongly suggested that the β-hexapeptide is largely folded in the 3_{14} helical conformation in structure-supporting solvents ($CDCl_3$ and MeOH, respectively) [3].

The EPR spectra (Fig. 2A) are overwhelmingly produced by biradicals with strong exchange (J) and dipolar (D) interactions between the unpaired electrons. The calculated intramolecular distance d between the unpaired electrons, obtained from our experimental parameters, was found to be ca. 6Å, in good agreement with the expected distance (5.4 Å) derived from our model of the hexapeptide in the 3_{14}-helical conformation (Fig. 2B).

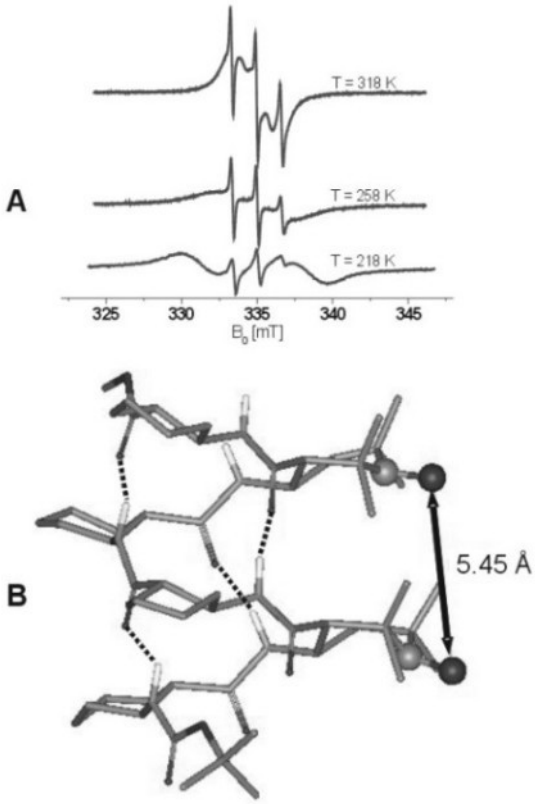

Fig. 2. (A) EPR spectrum of the hexapeptide in MeOH solution at different temperatures and (B) model of the hexapeptide in the 3_{14}-helical structure, highlighting the distance between the two nitroxyl probes, one on top of the other after a complete turn of the ternary helix.

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A NOVEL SUITE OF CYCLOTIDES: SEQUENCE VARIATION AND THE IMPLICATIONS FOR STRUCTURE, FUNCTION AND STABILITY

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Introduction

Cyclotides are a growing family of head-to-tail macrocyclic plant-derived peptides [1]. Typically 28-37 amino acids, they are defined by their unique knotted disulfide topology linking six conserved cystine residues and their characteristic cyclic backbone. This feature, referred to as the cyclic cystine knot (CCK) motif, engenders cyclotides with remarkable thermal, chemical and enzymatic stability [2]. Aside from a fundamental interest in their protein topology and their *in-planta* cyclization mechanism, their proposed native function as a combinatorial template for which to target an array of insect pests and pathogens together with their remarkable chemical properties suggest that they may have applications in drug design programs.

Results and Discussion

To further our understanding of the natural diversity of cyclotides and the implications of sequence variation on structure, function and stability, we set out to characterize the suite of cyclotides expressed in the plant *Viola odorata*. Cyclotides were isolated from winter and summer extractions using RP-HPLC, monitored by UV and mass spectrometry. Peptides were then sequenced by tandem mass spectrometry and their composition confirmed with quantitative amino acid analyses. We characterized six new bracelet and seven new Möbius cyclotides.

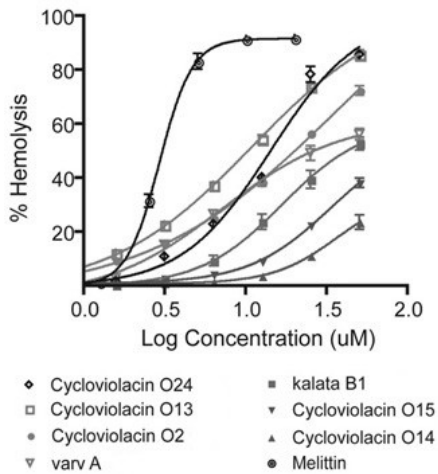

Fig. 1. Hemolytic activities of cyclotide naturalvariants from V. odorata relative to the well-known synthetic hemolytic agent melittin.

A selection of the new cyclotides was tested for their stability against proteolytic degradation to investigate how

tolerant the characteristic proetolytic resistance of the cyclotide framework is to sequence variation. Kalata B1 was included for comparison, as was a linear control peptide. No degradation by proteases was observed for any of the cyclotides tested while the linear control peptide was completely degraded.The insecticidal activity of cyclotides has been proposed to result from damage to membranes within the insect gut [3]. Accordingly, in this study we used hemolytic activity as a marker for bioactivity for a selection of the new cyclotides. Fig. 1 shows that the hemolytic activity of the various sequences does vary considerably.

The presence of the CCK motif in the new cyclotides was confirmed by determining the structure of cycloviolacin O14 in solution using NMR methods (Fig. 2). It was chosen as an example that is most different in properties than the other cyclotides, being the most hydrophilic, with the strategy being that if it adopts the CCK motif then the other new cyclotides that have more similar sequences to previously characterized cyclotides are also likely to adopt the motif.

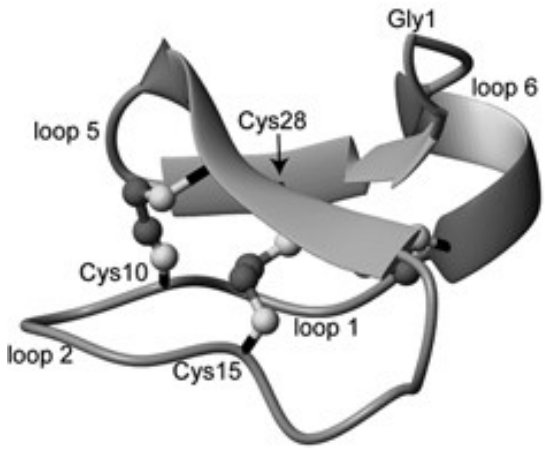

Fig. 2. Structure of cycloviolacin O14.

Acknowledgments

This work was supported in part by a grant from the Australian Research Council (ARC) and in part by the NHMRC.

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PEPTIDE ANALOGUES DERIVED FROM THE CYTOPLASMIC DOMAIN OF AIIBβ3 INTEGRIN RECEPTOR INHIBIT PLATELET AGGREGATION

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Introduction

Upon platelet activation by agonists the integrin receptor αIIbβ3 converts from a resting to an active conformation, resulting in platelet aggregation [1]. The membrane – proximal segments of the platelet integrin αIIb and β3 cytoplasmic domains are thought to directly interact to constrain the integrin in an inactive state [2]. Perturbation of this interaction by intracellular proteins, such as talin [3], results in αIIbβ3 activation (inside-out signaling). The aim of this work is to develop peptide analogues based on the cytoplasmic tail sequences of both αIIb and β3 subunits that could inhibit platelet aggregation by specifically disrupting the inside – out signaling pathway.

Results

Peptides were synthesized using the Fmoc/tBu protection strategy. The fluorescein-labeled peptides were generated by coupling *N*-hydroxysuccinimide fluorescein to the amino-terminal end of the resin-bound peptide. Palmitoylated (Pal) and carboxyfluorescein-labeled (CF) analogues were prepared by acylation of the α- and ε-amino groups of the N-terminal lysine by palmitic anhydrite and NHS-fluorescein respectively. The protein derived cell-penetrating peptide GRKKRRQRRRPPQ

(Tat fragment 48-60) was used in order to mediate the cellular internalization. All peptides were purified by RP-HPLC and the correct M.W. was confirmed by ESI-MS. In Table 1 are shown the synthesized peptide analogues.

Table 1. Peptide analogues of the cytoplasmic domain of integrin αIIbβ3.

Cytoplasmic Domain of $\alpha_{IIb}\beta_3$	Peptide Sequence
α_{IIb} 997-1003	RPPLEED
α_{IIb} 997-1003	CF- RPPLEED
α_{IIb} 997-1003	Pal-K-RPPLEED
α_{IIb} 997-1003	Pal-K(CF)-RPPLEED
α_{IIb} 1000-1008	LEEDDEEGE
α_{IIb} 1000-1008	CF- LEEDDEEGE
α_{IIb} 1000-1008	Pal-K-LEEDDEEGE
α_{IIb} 1000-1008	Pal-K(CF)-LEEDDEEGE
β_3 743-750	Tat-NNPLYKEA-NH_2
β_3 743-756	Tat-NNPLYKEATSTFTN-NH_2

Inhibition of the platelet aggregation was measured using Human Platelet Rich Plasma (PRP) and washed human platelets to a final concentration of 250.000 platelets/μl. The tested peptide was added to 500 μl of PRP or washed platelets. After 5 or 15 minutes of incubation, the agonist was added (ADP 2-5 μM, thrombin 0,5 U/ml). Inhibition effect was determined on the basis of the light transmittance changes. In Table 2 are summarized the biological results.

Table 2. Inhibitory activity and IC_{50} values.

Peptide Sequence	Incubation Time (min)		Concentration of the peptide (μM)		% Inhibition of the human platelet aggregation		IC_{50} (μM)	
	PRP	Washed	PRP	Washed	PRP	Washed	PRP	Washed
α_{IIb} 997-1003	5	15	1000	1000	29	16±13	-	1231
α_{IIb} Pal 997-1003	5	15	1000	1000	80 ± 6	87±11	445	105
α_{IIb} 1000-1008	5	15	1000	1000	68	96	625	770
α_{IIb} Pal 1000-1008	5	15	1000	1000	92±2	100	616	150
β_3 Tat 743-750	15	-	500	-	66±6	-	-	-
β_3 Tat 743-756	15	-	500	-	47±3	-	-	-

Discussion

The peptides derived from the cytoplasmic domains of the αIIbβ3 receptor can inhibit human platelet aggregation. The inhibition potency of the peptides increases upon palmitoylation probably due to increased internalization of the peptide into the platelets. The low IC_{50} value for the peptides Pal-KR^{997}PPLEED^{1003} and Pal-KL^{1000}EEDDEEGE^{1008} of the αIIb cytoplasmic domain, in the experiments with washed human platelets, indicates a specific interference to the mechanisms involving the platelet activation.

Acknowledgements

Acknowledgements to the GSRT (EPAN YB/88) and E.U. for the financial support.

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MOLECULAR DYNAMICS SIMULATIONS OF SIX POTENTIAL T CELL EPITOPES OF THE LA/SSB AUTOANTIGEN COMPLEXED WITH HLA-DQ7

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Introduction

In a previous study HLA-DQ2 and DQ7, highly correlated to autoimmune diseases as Sjogren's Syndrome (SS) and Systemic Lupus Erythematosus (SLE), were modeled on the basis of the crystal structure of HLA-DQ8 [1, 2]. By the combined application of Taylor, TEPITOPE and MULTIPRED common and/or similar T cell epitopes were identified. The best aligned epitopes were placed into the binding groove of the modelled DQ2 and DQ7 to perform energy minimization studies. Six epitopes were attributed to the HLA-DQ7 and nine to HLA-DQ2. In this study, molecular dynamics (MD) simulations were performed with TINKER molecular modeling software package to examine the binding of the modeled HLA-DQ7 complexed with six potential T cell epitopes of the La/SSB autoantigen. The proposed T cell epitopes, classified according to their binding efficiency, could act as immunotherapeutic drug-like molecules.

Result and Discussion

The six predicted T cell epitopes of the La/SSB autoantigen within the modeled HLA-DQ7 were subjected to molecular dynamics simulations in continuum solvation GB/SA at 300K of 150 ps duration and compared with the crystal structure of HLA-DQ8-insulin-B peptide complex used as template. The averages of the root mean square deviations (RMSD) and the radius of gyration were computed only for the Cα atoms of the complexes as a function of time and the averages of the solvent accessible surface areas (SASA) of the 9-mer central core of the predicted epitopes during the MD simulations within the binding groove of HLA-DQ7 were calculated (Table 1). The potential energy of interaction, ΔE_{int}, between the modeled HLA-DQ7 and each one of the potential T cell epitopes and the free energy of binding, ΔG_{bind}, of the complexes, using the LIE method were also measured (Table 2). According to the calculated averages the six predicted epitopes of the La/SSB autoantigen within the modeled HLA-DQ7 did not show any significant variation thus indicating their residence into the binding groove of the modeled HLA-DQ7. Furthermore, the potential energy of interaction, ΔE_{int}, and the free energy of binding, ΔG_{bind}, showed the thermodynamic stability of the HLA-DQ7/peptide complexes.

Table 1. Calculated averages of the radius of gyration (in Å), solvent asseccible surface area, SASA, (in Å²) and R.M.S.D (in Å) for the DQ-peptide complexes for 0-150 ps.

Complex	Radius of Gyration	SASA	RMSD
DQ8-peptide	23.891	1425.768	0.4160
DQ7-(68-79)	23.718	1386.478	0.3885
DQ7-(78-90)	24.371	1524.496	0.2586
DQ7-(149-161)	24.343	1433.783	0.5258
DQ7-(152-165)	23.788	1492.487	0.3672
DQ7-(154-167)	23.438	1330.979	0.2042
DQ7-(286-299)	24.411	1383.715	0.2592

Table 2. The calculated potential energy of interaction, ΔE_{int}, and the free energy of binding, ΔG_{bind}, of the DQ-peptide complexes, in Kcal/mol.

Complex	ΔE_{int}	ΔG_{bind}
DQ8-peptide	-74.778	-164.66
DQ7-(68-79)	-51.545	-197.42
DQ7-(286-299)	-69.358	-157.65
DQ7-(152-165)	-28.388	-146.23
DQ7-(78-90)	-72.785	-100.92
DQ7-(154-167)	-65.879	-68.94
DQ7-(149-161)	-122.63	-27.51

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STUDIES ON THE APOLIPOPROTEIN A-I REGION 104-117 AND ITS LIPOPEPTIDE ANALOGUE (SUBSTITUTION OF MET112 BY AMINO-TETRADECANOIC ACID) AS POTENTIAL ANTIATHEROGENIC.

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Introduction

Atherosclerosis is a multistep process and the underlying cause of heart disease and stroke in humans [1]. It is considered to be an immune-inflammatory disease, associated with LDL oxidation [2, 3]. The antiatherogenic properties of HDL are widely accepted [4, 5]. Apolipoprotein A-I (apoA-I), the major protein component of HDL, is a 243 amino acid amphipathic protein, and plays a crucial role in many mechanisms, such as promotion of reverse cholesterol transport [6 - 8]. In order to examine the antioxidant role of Met112 of human apoA-I, we have synthesized apoA-I helix 4 region 104-117, peptide (1) Ac-FQKKWQEEM^{112}ELYRQ-NH_2 and its lipopeptide analogue (Met112 → amino-tetradecanoic acid), peptide (2). Both peptides were tested for their ability to prevent human plasma LDL oxidation *in vitro*, induced by Cu^{2+}. LDL-associated PAF-AH activity was also measured in the presence of the peptides, in order to examine their protective role against the inactivation of PAF-AH during the LDL oxidation. Conformational studies of peptides (1) and (2) were also performed using CD spectroscopy.

Result and Discussion

Peptide syntheses. The peptides were synthesized manually by the solid-phase peptide synthesis and the Fmoc/tBu strategy, on a rink amide AM resin. The purity of the peptides was performed by semi-preparative HPLC and confirmed by analytical HPLC and ESI-MS.

Conformational study. CD experiments showed that both peptides adopt helical conformation in PBS, pH 7.4, and SDS comparable with that of apoA-I. In particular, peptide (2) exhibits pronounced helical characteristics ~50%.

Biological study. The LDL oxidation curves (dose-dependent experiments) in the presence of peptides (1) and (2) are presented in Fig. 1. PAF-AH activity was measured in the presence of peptides (1) and (2) before and after human plasma

LDL oxidation and the results are shown in Fig. 2 as the % reduction of PAF-AH activity after LDL oxidation.

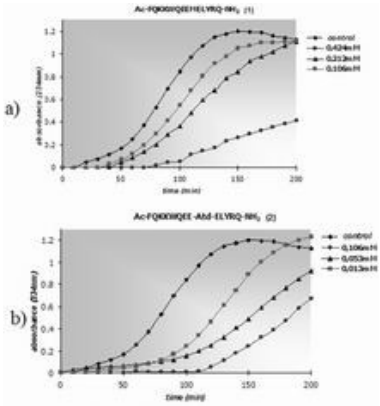

Fig. 1. Antioxydotant properties of peptides (1) and(2)

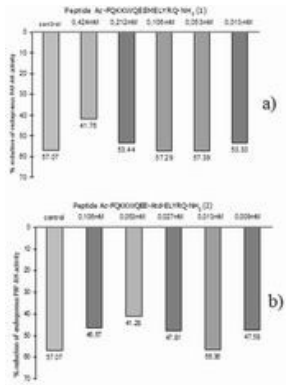

Fig. 2. PHF-AH activityin the presence of (1) and(2)

Both peptides inhibit human plasma LDL oxidation. Lipopeptide (2) is a better oxidant scavenger at 4-fold lower concentrations compared to (1), indicating that Met is not a prerequisite for the expression of the antioxidant activity.

It is assumed that both peptides, in accordance with their antioxidant properties, inhibit the PAF-AH inactivation.

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EXCITON COUPLED CIRCULAR DICHROISM OF INTRAMOLECULARLY INTERACTING BIS-PORPHYRIN CHROMOPHORES IN PEPTIDE CONJUGATES: DISTANCE AND ORIENTATION EFFECTS

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Introduction

When two chromophores are chirally oriented and close enough to one another in space, their excited states couple and become non-degenerate. This phenomenon, termed exciton coupling, produces a typical, bisignate, CD curve. The intensity of the CD couplet is dependent on the molar extinction coefficient and the distance between the interacting chromophoric moieties, while the sign is governed by the angle between the effective electron transition moments. In particular, exciton coupling over a long distance can be observed only with strongly absorbing chromophores, e. g. porphyrin derivatives, characterized by their extremely intense and sharp Soret band near 415 nm [1].

In this work we examined by the exciton coupled CD method the combined distance and angular dependencies, generated by the seven conformationally restricted β-turn and 3_{10} helical spacer peptides -L-Ala-[L-(αMe)Val]$_n$- (n = 1 - 7), on a system formed by two intramolecularly interacting 5-carbamido-5,10,15,20-tetraphenylporphyrin (TPP) chromophores (Fig. 1) [2, 3].

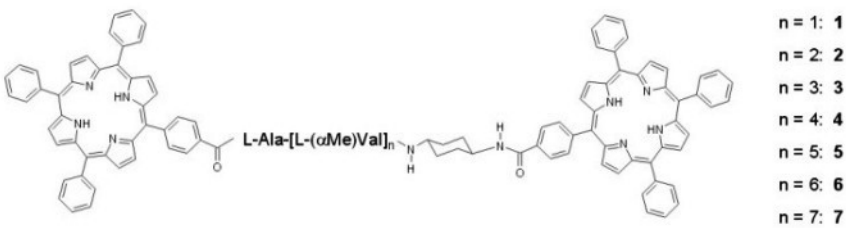

Fig. 1. The bis- and mono-porphyrin/peptide conjugates here described.

Results and Discussion

Our FT-IR absorption and 1H NMR results clearly show that the L-Ala/L-(αMe)Val peptide spacers 1 - 7 overwhelmingly adopt the β-turn and the 3_{10}-helix conformation in $CDCl_3$, a structure-supporting solvent.

The CD measurements in the visible region described in this work represent the first extensive experimental investigation of the exciton coupled CD phenomenon over a full set of sterically hindered oligopeptide spacers (from di- to octapeptide) with an ordered secondary structure of progressively increasing length (evolving from a single β-turn to a completely developed 3_{10}-helix). Our data show that the intramolecular porphyrin…porphyrin exciton coupling is still clearly detectable over the long interchromophoric distance of about 30 Å (compound 5). We also highlight a relevant role for the angular dependence between the effective transition moment directions of the two chromophores (compare the curves of compounds 3 and 5). Finally, our findings strongly confirm the view [1] that porphyrin derivatives are extremely promising reporter groups for extending the exciton chirality CD method to the still unexplored area of the conformational analysis of large biopolymers.

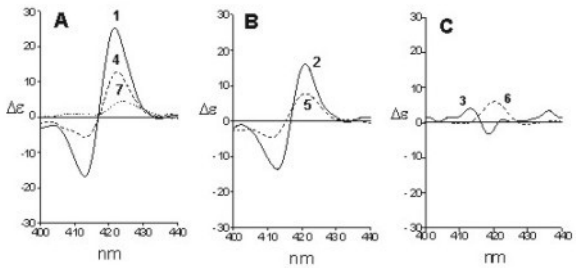

Fig. 2. CD spectra in the 400-440 nm region of TPP-CO-L-Ala-[L-(αMe)Val]$_n$-NHC$_6$H$_{10}$-NH-CO-TPP in CDCl$_3$ solution. (A): n = 1, 4 and 7 (1, 4, 7); (B): n = 2 and 5 (2, 5); (C): n = 3 and 6 (3, 6). Peptide concentration 1×10^{-6} M. The NH-C$_6$H$_{10}$-NH moiety refers to trans-1,4-diaminocyclohexane.

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THE PEPTIDE 3_{10}-HELIX AS A SPACER AND A TEMPLATE

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Introduction

Beside the classical α-helix and β-pleated sheet conformations, the only other principal long-range secondary structure that occurs significantly in peptides and globular proteins is the 3_{10}-helix [1]. A complete characterization of this helix is solution and in the crystal state has been achieved by our group using a number of physico-chemical techniques, thus allowing its unambiguous differentiation from the conformationally closely related α-helix [2].

Results and Discussion

More recently, we investigated this helical dimorphism in the crystal state and the solvent-driven equilibria between these two helical structures. Peptides with a specific main-chain length and amino acid sequence behave as molecular springs.

We applied rigidified β-turns (the basic unit of the 3_{10}-helix) and fully developed 3_{10}-helices to systems where the donor…acceptor groups are linked to the N- and C-termini of the peptide main chain [3]. By increasing the number of intervening residues in the peptide spacer, the donor…acceptor separation can be easily and precisely modulated.

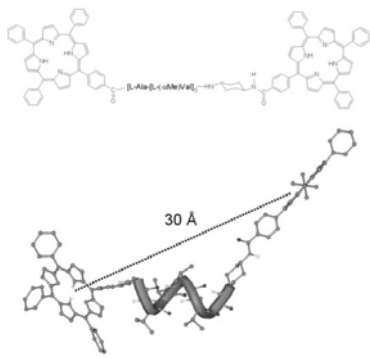

Fig. 1. The bis-porphyrin/peptide conjugate (top) with the longest peptide spacer still producing an observable CD exciton split phenomenon at the Soret band (415 nm)). In the computer model of the 3_{10}-helical conjugate (bottom) the centre-to-centre interchromophoric distance is indicated.

In particular, we analyzed the exciton split phenomenon arising from the interaction of two porphyrin chromophores by CD (Fig. 1), the electron and energy transfers between fluorophores and quenchers by photophysical techniques, and the

dissociative electron transfer between phthalimido and dialkyl peroxide moieties by electrochemical techniques.

We extended our analysis to peptide systems with appropriate side-chain decorations as templates for investigations in organic chemistry [e.g. macrocyclization (Grubbs' catalyzed ring-closing metathesis) (Fig. 2) and catalysis (enantioselective oxidation, kinetic resolution of a racemic alcohol, intramolecular transphosphorylation of an RNA model substrate], in supramolecular chemistry [e.g. molecular recognition of the [60]fullerene guest], and in physical chemistry, [e.g. donor-acceptor interactions (nitroxide…nitroxide, studied by EPR; fluorophore…nitroxide quencher studied by fluorescence)].

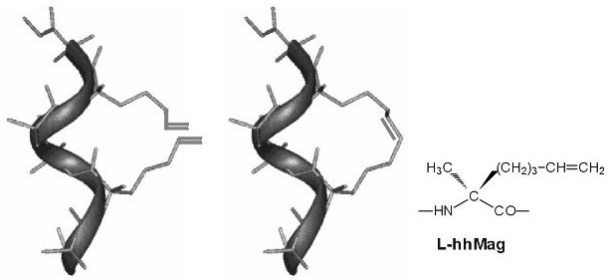

Fig. 2. Minimum energy models for the two hexapeptides Ac-Aib-L-hhMag-(Aib)$_2$-L-hhMag-Aib-NHMe (left) and its ring-closing metathesis trans(C=C) product (right). The unperturbed 3_{10}-helix is the template for the macrocyclization reaction.

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DEVELOPMENT OF DIAGNOSTICS AND VACCINE CANDIDATES FOR THE HIGHLY PATHOGENIC H5N1 INFLUENZA VIRUS

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Introduction

The Informational Spectrum Method (ISM) allows investigation of the periodicity of structural motifs with defined physicochemical characteristics determining biological properties of protein and DNA sequences [1, 2]. According to this concept, protein sequence is encoded into numerical sequence by representing every amino acid with a parameter, the electron-ion interaction potential (EIIP), which describes the average energy states of valence electrons in amino acids. By discrete Fourier transformation, this numerical sequence can be presented in the form of the informational spectrum (IS), representing the series of frequencies and their amplitudes. ISM analysis of different H5N1 and H5N2 isolates revealed that all contain a characteristic peak at frequency F(0.076) representing the information which is common for analyzed HA1 proteins and probably important for their biological function. In order to identify the domain of HA1 H5N1 with dominant contribution to the information represented by the frequency component F(0.076), its primary structure was scanned with peptides of different length. It was found that N-terminus of HA1 plays crucial role in determination of this information. Especially important is a 34 residues peptide representing the minimal part of HA1 whose amplitude is above the noise level in IS of protein. Informational spectrum of this peptide (denoted H5N1 HA1 N-terminus segment peptide) is presented in Fig.1.

Results and Discussion

The synthesis of the SOC_4-(H5N1 HA1 N-terminus segment)$_2$ conjugate was carried out by SPPS on a PAM-resin using the Boc/OBzl methodology. The crude product was dialyzed against water in a cut-off 1500 dialysis tybing and purified by semi-preparative RP-HPLC. The correct molecular mass was confirmed by ESI-MS. The H5N1 HA1 N-terminus segment, as well as the SOC_4 carrier were synthesized separately as control peptides.

The CD spectra of the H5N1 HA1 N-terminus segment peptide were recorded with Jasco 715 spectropolarimeter. Spectra were obtained with a 0.10 cm path length cell by signal averaging 3 scans from 180 to 260 nm with a scan speed of 50 nm/min. The CD spectra were smoothed after the subtraction of the solvent's CD. Analysis of the CD spectra with the CDNN calculation program gave a helix context about 15% under the conditions shown in Fig. 2 and equal amounts of β-sheet and random conformation.

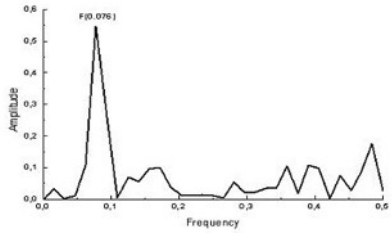

Fig. 1. The informational spectrum (IS) of the 34 residues peptide of the H5N1 influenza A virus.

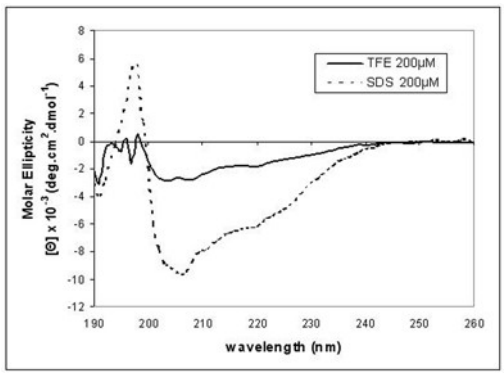

Fig. 2. CD spectra of the H5N1 HA1 N-terminus segment peptide in TFE and TFE/H_2O v/v 50/50, C=100 μM.

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INHIBITORS OF THE RAS/ERK SIGNALING PATHWAY INCORPORATING THE FXF MOTIF FOR FHE DEVELOPMENT OF ANTICANCER DRUGS

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Introduction

The RAS pathway is responsible for the regulation of the cell increase, sending periodically signals to the nucleus of the healthy or cancer cells.

The major objective of our study is the design, synthesis and study of peptide inhibitors that block the ERF phosphorylation [1] in the RAS pathway of the ERF/ERK interaction. Peptides which contain the interacting motif (FSF and FKF) of the ERF were synthesized. For comparison, modified analogs were synthesized and studied (substitution of the F by A). The sequence ERF (300-307): GSHFSFSP which contains the FSF motif, was conjugated to the Sequential Oligopeptide Carrier SOC_n-II in 3 copies in order to enhance the inhibitory activity of the FSF motif.

Results and Discussion

Peptide Synthesis

Peptides (1 - 4) were synthesized by the solid phase peptide synthesis on a Wang resin using the Fmoc methodology. The peptide (5) was synthesized by the solid phase peptide synthesis on a Pam resin using the Boc methodology.

1. SGGGSHFSFSPEDMK : ERF (297-311)

2. SSSPFKFKLQPPPLG : ERF (371-385)

3. SGGGSHASASPEDMK: [A^{303},A^{305}]ERF(297-311)

4. SSSPAKAKLQPPPLG : [A^{375},A^{377}]ERF(371-385)

5. $(Aib\text{-}Lys\text{-}Aib\text{-}Gly)_3$-$(GSHFSFSP)_3$: ERF (300-307)

Biological Assays

ERF1 and ERF2 peptides harboring the FXF motifs failed to inhibit more than 50% the *in vitro* ERF-Erk interaction even at 100 fold molar excess. This minimal inhibition appeared to be independent of the phenylalanines, since ERF3 and ERF4 gave comparable competition. These findings indicate that the FXF motifs are necessary but not the only interaction determinants.

Conformational Study

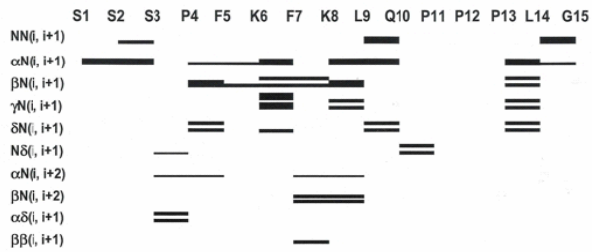
The ^{1}HNMR spectroscopy studies of the peptides were carried out in aqueous solutions H_2O/ D_2O 95/5 (v/v) (1D and 2D ^{1}HNMR). In Table 1 are given the

chemical shifts NH, $C^{\alpha}H$, $C^{\beta}H$, $C^{\gamma}H$, $C^{\delta}H$ and $C^{\zeta}H$ of the identified protons of ERF2 and the coupling constants for the peptide(2). Many and strong NOEs are observed in Table 2 suggesting a folded conformation in the central segment (4 - 10) of the peptide. Substitution by alanine at positions 5 and 7 (ERF4) resulted in a more flexible conformation.

Table 1. Coupling Constants (Hz, in brackets) and Chemical Shifts (ppm) of Assigned Proton Resonances of ERF2 Peptide in H_2O/D_2O (95/5 v/v)

Residue	$^3J_{H^{\alpha}}$	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	H^{δ}	H^{δ}	H^{ζ}	other
ERF2 peptide									
S1			4.21	4.02, 4.00					$^3J_{HH}$=12.1 Hz
(3.8, 5.4)									
S2	(7.0)	8.90	4.50	3.88, 3.86					$^3J_{HH}$=11.8 Hz
(4.6, 6.1)									
S3	(6.6)	8.61	4.79	3.85, 3.83					$^3J_{HH}$=11.8 Hz
P4			4.39	2.23, 1.95	1.79, 1.74	3.80, 3.67			
P5	(6.3)	8.27	4.53	2.99, 2.95		7.30	7.31	7.17	$^3J_{HH}$=13.3 Hz
(5.5, 5.4)									
K6	(7.4)	8.11	4.22	1.64, 1.60	1.29, 1.24	1.60	2.95	7.59	
F7	(6.7)	8.16	4.53	3.10, 3.01		7.27	7.37	7.17	$^3J_{HH}$=13.8 Hz
(6.5, 7.7)									
K8	(5.9)	8.28	4.25	1.76, 1.60	1.36	1.60	2.95	7.59	
L9	(6.6)	8.34	4.31	1.62	1.50	0.96, 0.91			
Q10	(7.1)	8.55	4.61	2.00, 1.94	2.39		7.67, 6.90		
P11			4.69	2.35, 2.02	2.00, 1.91	3.80, 3.64			
P12			4.73	2.36	2.06, 1.93	3.83, 3.65			
P13			4.42	2.3	2.05, 1.93	3.82, 3.66			
L14	(7.0)	8.49	4.34	1.69	1.63	0.94, 0.89			
G15	(5.8)	8.15	3.82						

Table 2. Depiction of NOEs of ERF2 from the NOESY spectrum in H_2O/D_2O (95/5 v/v).



Acknowledgements

This work was supported by the G.S.R.T and E.U. (Polish-Greek cooperation 2003-2006).

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MHC-DERIVED PEPTIDES AS IMMUNOMODULATORS IN AUTOIMMUNE DESEASES

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Introduction

The major histocompatibility complex (MHC) has a crucial role to initiate the immune response via the binding of the peptide fragments (epitopes) of foreign antigens and their presentation to the T-cell receptors (TCR). Co-receptor molecule CD4 enhances the binding TCR/MHC II. Small molecules that mimic surfaces of the MHC II molecule may lead to blockage of the autoimmune response and the development of drugs for immunotherapy [1]. The HLA-DQA1*0501/DQB1*0201 (DQ2) and HLA-DQA1*0501/DQB1*0301 (DQ7) are highly correlated to autoimmune diseases as Sjogren Syndrome (SS) and Systemic Lupus Erythematosus (SLE).

The major objective of our study is the precise determination of the non polymorphic β regions of the modeled HLA-DQ7, which are exposed to the solvent and may disrupt the interaction of DQ7 with $CD4^+$ T lymphocytes. It was found, using the GETAREA program that the regions 133-140 (Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr) and 59-66 (Glu-Tyr-Trp-Asn-Ser-Gln-Lys-Glu) display the highest solvent accessibility.

Result and Discussion

Peptide Synthesis

The peptides were synthesized on a Rink Amide resin using the Fmoc/OtBu methodology.

1. Phe-Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr-Gly-NH_2	p 131 (132-141)
2. Ac- Phe-Arg-Asn-Asp-Gln-Glu-Glu-Thr-Thr-Gly-NH_2	Ac-p 131 (132-141)
3. Ac-Ala-Glu-Tyr-Trp-Asn-Ser-Gln-Lys-Glu-Val-NH_2	Ac-p57 (58-67)
4. Ala-Glu-Tyr-Trp-Asn-Ser-Gln-Lys-Glu-Val-NH_2	p57 (58-67)

Biological Assays

The immunomodulatory activities of the peptides 1 - 4 were tested by the direct plaque-forming cell (PFC) test (*in vitro*) and the results are expressed as a PFC

number per 106 splenocytes. The results are summarized in Table 1. The peptides 1 and 2 are immunomodulators.

Table 1. PFC test

Preparation	µg/ml	PFCx10^6	± SE	P Student test	Suppression (%)
Control		1980	158.72		
p 131 (132-141)	1 10 100	2515 2485 2035	100.24 41.98 158.59	<0.05 <0.001 NS	-27.02 -25.51 -2.8
Ac-p 131 Ac (132-141)	1 10 100	2855 2435 2185	283.34 100.24 140.97	<0.05 NS NS	-44.19 -22.98 -10.35

The presence of β-turn was identified in the p131 peptide. The calculated, using the Swiss-Pdb Viewer program, φ,ψ and ω angle values, the distances between C^{α}Hs of i, i+3 residues (lower than 7Å) and O(i) and N(i+3) (lower than 3.5 Å) argue in favour of a type II΄ β-turn in the segment Arg^{133}NH - OC Gln^{136} (Tables 2 , 3).

Table 2. Values of the φ, ω, ψ angles of amino acids.

Amino	Ω	Φ	Ψ
132 PHE	176.29	-119.64	142.84
133 ARG	172.34	-122.44	101.33
134 ASN	-176.38	63.94	-108.52
135 ASP	179.54	-100.22	-5.57
136 GLN	-177.91	-111.70	145.60
137 GLU	175.13	-78.66	138.24
138 GLU	173.24	-86.09	119.82
139 THR	178.74	-104.14	-39.05
140 THR	-178.45	-98.86	158.07
141 GLY	-177.87	64.69	18.77

Table 3. Distances between C^{α}(i)- C^{α}(i+3)and O(i)-N(i+3)of amino acids.

C^{α} (i)	C^{α} (i+3)	DISTANCE	O (i)	N (i+3)	DISTANCE
PHE	ASP	6.89E	PHE	ASP	6.82E
ARG	GLN	5.49E	ARG	GLN	3.08E
ASN	GLU	8.16E	ASN	GLU	6.42E
ASP	GLU	9.82E	ASP	GLU	7.73E
GLN	THR	9.35E	GLN	THR	6.95E
GLU	THR	9.32	GLU	THR	6.13E
GLU	GLY	7.55E	GLU	GLY	6.89E

Acknowledgements

This work was supported by the G.S.R.T and E.U. (Polish - Greek cooperation 2003-2006).

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DEVELOPMENT OF A DIAGNOSTIC IMMUNOASSAY FOR THE DETECTION OF NATURAL ANTI-NTM/VIP ANTIBODIES OF HIV-1

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Introduction

Bioinformatic analysis revealed that a vasoactive intestinal peptide (VIP), with sequence AVFTDNYTLRLKQMAVKKYLN, although with little sequence similarity with NTM, RSANFTDNAKTIIVQLNESVEIV (280-306) of the C2 region of gp120, has very similar informational spectrum with that of NTM [1]. It was also demonstrated that human natural anti-VIP antibodies reactive with C2 region of gp120 play an important role in controlling the HIV disease progression.

A novel class of carriers termed Sequential Oligopeptide Carriers (SOCn) is successfully applied in our laboratory. SOCn is formed by the $(Lys\text{-}Aib\text{-}Gly)_n$ sequential motif, where n = 2 - 7, and adopts a predetermined secondary structure of 3_{10}-helix [2]. Taking advantage of the strong immunological cross reactivity between NTM and VIP peptides we propose to covalently attach multiple copies of the NTM/VIP peptide on Sequential Oligopeptide Carriers (SOCn) for the development of a sensitive, specific and prognostic ELISA assay for the detection of anti-NTM/VIP-derived antibodies and the progression of the disease.

Results and Discussion

With the aim to formulate an accurate, sensitive and reproducible ELISA for evaluating the occurrence of the anti-SOC_n-$(NTM/VIP)_n$ antibodies and correlate them with the progression of AIDS as a valuable tool for the clinicians to follow up HIV-infected individuals, the main core of NTM peptide (sequence FTDNAKTI), was conjugated, in four copies, to the Lys-$N^{\varepsilon}H$ groups of SOC_4. The Ac-SOC_4-$[FTDNAKTI]_4$ was synthesized manually by solid-phase synthesis using a Pam resin and the Boc/Bzl strategy, and was purified by RP-HPLC. The correct product was confirmed by analytical HPLC and ESI-MS.

In order to evaluate differences between titers of antibodies reactive with NTM and VIP in sera of patients long term non progressors (LTNP) and standard HIV+ individuals we tested the activity of peptide NTM (284-291) (FTDNAKTI) anchored to SOC_4 against HIV positive sera from HIV+ patients, sera from normal progressors patients and sera from HIV+ patients LTNP in ELISA (Fig. 1). According to the obtained results we conclude that sera of several HIV+ patients LTNPs are specifically recognized by Ac-SOC_4-NTM_4. These results are in accordance with previous findings suggesting the protective role of *anti*-NTM

antibodies in HIV infection. Further optimizations of experimental conditions are in progress.

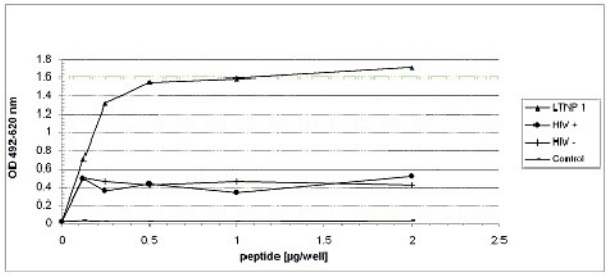

Fig. 1. Anti-Ac-SOC$_4$-NTM$_4$ ELISA against LTNP, HIV+, HIV- and control sera at different concentration of conjugate.

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DESIGN AND SYNTHESIS OF TWO CYCLIC ANALOGUES OF GONADOTROPIN RELEASING HORMONE (GNRH) FOR THE TREATMENT OF CANCER

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Introduction

Gonadotropin releasing hormone (GnRH) stimulates production of gonadotropin hormones (FSH and LH) through interaction with specific receptors triggering important biological functions. A vast number of linear analogues of GnRH has been synthesized and tested for several medicinal uses. Triptorelin, Leuprolide, Buserelin, Goserelin, Nafarelin, Histrelin, are used in cancer treatment and reproductive disorders [1]. In order to improve leuprolide's biological activity and bioavailability, two cyclic peptide analogues were designed and synthesized. Non natural amino-acid azetidine (Aze) at position 9 was used in order to reduce the proteolytic degradation of analogues. Moreover, substitution of Tyr^5 with Tyr(OMe) aimed at avoiding desensitization of GnRH receptors and introduction of D-Leu at position 6 intented to stabilize β-turn between aminoacids 5 to 8 (Tyr^5-Gly^6-Leu^7-Arg^8) of GnRH. Also, the usage of Pro and 3-aminobutyric acid at the N and C terminal respectively, simulates the important for activity residues pGlu and ethylamide of leuprolide (Fig. 1).

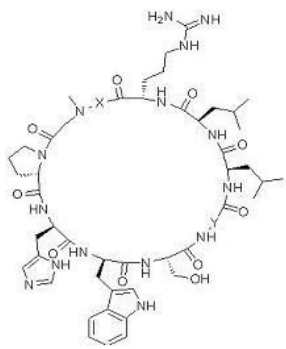
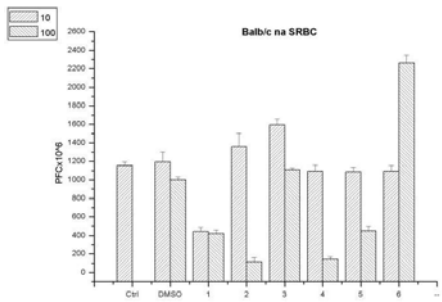


Fig. 1: Synthesized cyclic GnRH analogues [X: Pro, Aze and Y: Tyr or Tyr(OMe)].

Results and Discussion

The linear peptide was prepared on a 2-chlorotrityl chloride resin (0.7 mmol Cl^-/g) using solid-phase peptide synthetic method. The first N^{α}-Fmoc-protected amino acid [Fmoc-Pro-OH or Fmoc-Aze-OH (1 equiv)] was coupled to the resin in 1h in the presence of DIPEA (4.5 equiv) in DCM. The remaining peptide chain was

assembled by sequential couplings of the appropriate each time residue (2.5 equiv) in the presence of DIC (2.75 equiv) and HOBt (3.75 equiv) in DMF for 4 - 7h. The completeness of each coupling was verified by the Kaiser test and TLC (n-Butanol/Acetic acid/Water, 4:1:1). The protected peptide resin was then cleaved with the splitting solution DCM/TFE 7/3 (for 2h at RT). The cyclization was achieved by the use of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), HOAt and 2,4,6-collidine in DMF for highest yield reaction (80%) [2 - 4] (Fig. 2).



The use of HOAt speeds up the reaction rate of the cyclization when compared to common used HOBt. Usage of 2,4,6 collidine instead of DIPEA reduces racemization of the final product. The deprotection of the cyclic peptide was achieved with 70% TFA in DCM in the presence of 1,2-ethanedithiol (EDTH), anisole and water as scavengers (for 4h at RT). The purity of the final product was verified by RP-HPLC and its identification was achieved by ESI-MS.

Acknowledgements

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DESIGN AND SYNTHESIS OF A NOVEL NON-PEPTIDE MIMETIC OF PAR-1 THROMBIN RECEPTOR WITH FOUR PHARMACOPHORIC GROUPS

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Introduction

Thrombin receptors are attractive drug discovery targets because they mediate a variety of cellular actions of thrombin, such as thrombosis, hemostasis and inflammatory diseases. Thrombin acts on a specific receptor (PAR-1), a member of the seven transmembrane receptor family [1] and causes a limited proteolytic cleavage, leading to the formation of a new N-terminus capable of activating the receptor via intramolecular interaction [2 - 4].

Thrombin binds and cleaves the N-terminal exodomain region of $LDPR^{41}\downarrow S^{42}FLLR$ of PAR-1 generating a new N-terminus ($S^{42}FLLR^{46}$) pentapeptide which activates PAR-1 by binding to the body of the receptor. There were several reports that strongly suggest the potential utility of PAR-1 antagonist analogues for the treatment of thrombotic disorders [5].

Results and Discussion

Recent structure activity studies (SARs) in our laboratory have shown that a cluster of the two groups (phenyl, guanidine) together with an adjacent primary amino group is important for expression of maximum biological activity by thrombin receptor-derived analogues. In this report, we designed and synthesized a novel non-peptide mimetic, based on $S^{42}FLLR^{46}$ which carries the pharmacophoric features of Phe and Arg residues, incorporating with carboxyl and amino groups. The compound namely 1-[2'-amino-3'-phenyl-propanoyl]-4-[*N*'-(carboxy-methyl)-(7''-guanidyl-hexanoyl piperidine (NAT8), has four pharmacophoric groups (phenyl, guanidine, carboxyl and amino) aiming at improving the binding affinity with the PAR-1 receptor. As template, upon which the pharmacophoric groups are mounted, was chosen a rigid compound (piperidine) (Fig. 1).

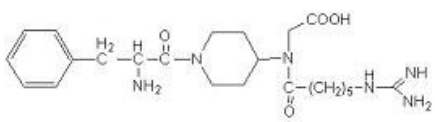

Fig. 1:1-[2'-amino-3'-phenyl-propanoyl]-4-[N'-(carboxy-methyl)-(7''-guanidyl-hexanoyl]piperidine (NAT8)

NAT8 has been synthesized using solid and solution phase organic synthesis. It had a strong relaxing effect upon the aortic rings pre-contracted with phenylephrine 1

μM. NAT8 induced a dose-dependent relaxing effect with a maximum efficiency (65.3%) at a concentration of 100 μM (Fig. 2).

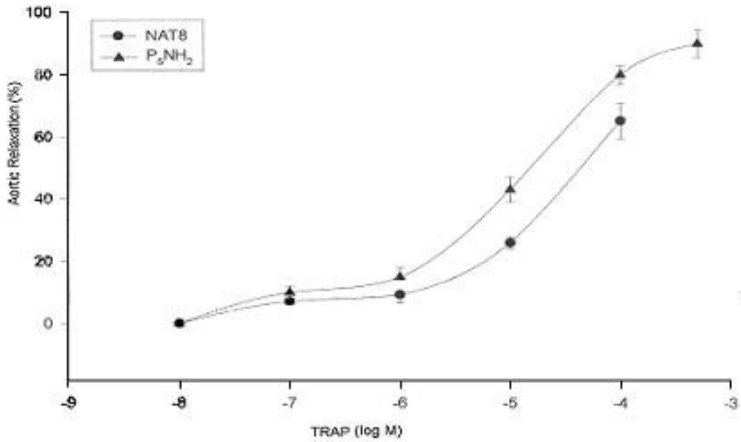

Fig. 2: Relaxant activities of NAT8 compared to P5NH2

Acknowledgements

This work was supported by National Grant PENED 2003, code 03ED827.

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POSITIONAL SCANNING LIBRARY OF AZO-PEPTIDES

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Introduction

Macrocyclic peptides may serve as artificial receptors for specific molecules. Incorporation of azo-bond into the cyclic backbone make it sensitive to conformation changes. One of important molecules for sensor development is cAMP [1]. In order to facilitate discovery of macrocyclic selective receptor, we have adopted positional scanning library method [2]. We would like to observe a spectral change of receptor, thought the receptor is in mixture of similar compounds with similar spectral properties, and thus special consideration must be taken during library design. If we assume that 3.5% spectral change allows receptor identification, a sub-library with three Xxx position has to have only three variable building blocks.

To obtain maximal possible diversity with this limitation, we have chosen acidic, basic and neutral but polar building blocks, such as Ser, Asp and Arg. Polarity is necessary to ensure solubility of receptor in aqueous media. A designed library has a general formula before azo cyclization H-Pap-Xxx-Xxx-Xxx-Xxx-(Tyr or His)-ε-Ahx-OH.

Results and Discussion

The first step in positional library synthesis is evaluation of relative reaction rates of individual building blocks on the resin intended for use. We compare resins such as H-Tyr(tBu)-ε-Ahx-OR and H-His(Trt)-ε-Ahx-OR and found similar coupling rates. Both aromatic amino acids are capable of azocoupling with diazonium salt derived from Pap. Acyclic sub-library precursors were prepared on Wang resin, after detachment and cyclization in solution, 2 x 3 x 4 (24) sub-libraries with 2 x 34 (162) compounds were obtained. Screening has not revealed any active substance – hit (Fig. 1), thus we have chosen a few randomly selected sequences and re-synthesized them. UV-VIS spectra did not indicate any change in presence of cAMP. Interaction with PGE1 was also evalueted with no positive result.

Conclusions

Positional scan was not successful for discovery of new cAMP receptor. This can be explained by both, measurement-on-the-edge phenomenon and/or by absence of active species. Further work will be aimed to macrocycles with higher count of amino acids (wider ring), and also to more hydrophobic macrocycles, where it is high probability for cAMP interactions.

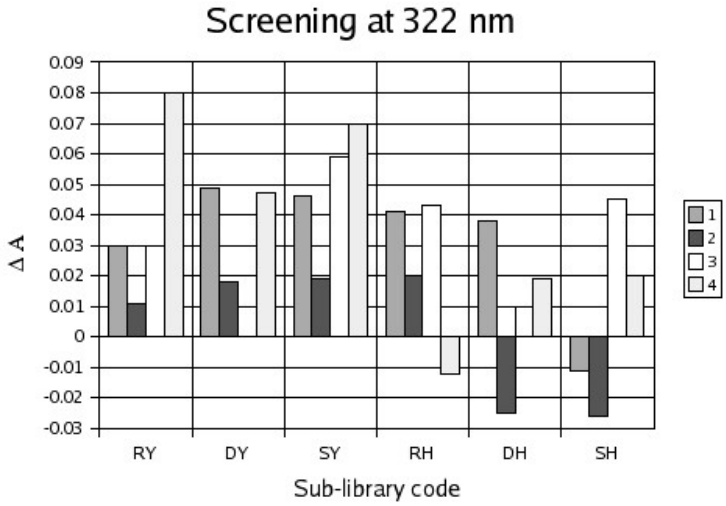

Fig. 1. Differences of sub-library absorbances in absence and in presence of 10 eq of cAMP.

Acknowledgements

This work was supported from grant of Grant Agency of CR 203/04/1421. I thank organizers for Travel Grant.

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DESIGN, SYNTHESIS AND CONFORMATIONAL ANALYSIS OF POTENT ANGIOTENSIN II LOSARTAN BASED ANTAGONIST

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Introduction

The octapeptide Angiotensin II (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH) is the major factor of the Renin – Angiotensin System (RAS) and plays a significant role in the regulation of arterial blood pressure. A number of AngII antagonist non-peptide mimetics (Irbesartan, Tasosartan) analogues is currently used for the treatment of hypertension. In the present study, a Losartan based analogue, 2-methoxy-carbonyl N^{π}-[(tetrazol-5´´-yl)phenyl-4´-yl] methylimidazol, was rationally designed and synthesized using the imidazole template on which phenyl, tetrazol and esteric groups are bound, according to AngII structure. The conformational analysis was carried out comparing the 3D structure of the synthesized analogue with the lowest energy 3D structure of [Sar^1, Ile^8]AngII (Sarilesin) and EXP3174 (bioactive metabolite of Losartan). The CHARMm force field was used for the simulations using the QUANTA 2005 (Accelrys) software on a Silicon Graphics O2 workstation. The scope of this study was the synthesis of a new analogue with fewer and cost effective synthetic steps and potent antagonist activity. The synthetic procedure includes: a) esterification of the urocanic carboxyl group, b) reduction of the aliphatic double bond, c) tritylation of the N^{τ} of imidazole, d) alkylation of N^{π} of imidazole with 5-(-4´-bromomethyl-phenyl) -2-triphenyl–methyl-tetrazol, and e) deprotection of trityl groups. This Losartan derivative was found to be potent in anesthetized rabbits.

Results and Discussion

The present work includes the design, synthesis and conformational analysis of losartan based analogue 6. This analogue was designed and synthesized based on Losartan and specifically its active metabolite, EXP3174, which was used as a drug lead, using the imidazole template. Figure 1 presents the synthesis of analogue 6 (Fig. 1). Molecular modeling and conformational analysis of [Sar^1]AngII (agonist), and [Sar^1,Ile^8]AngII (antagonist) [1, 2] peptides were carried out in order to determine the distances and interactions between the three aromatic rings of Tyr, His and Phe amino-acids as well as the C-terminal that are considered to be responsible for biological activity.

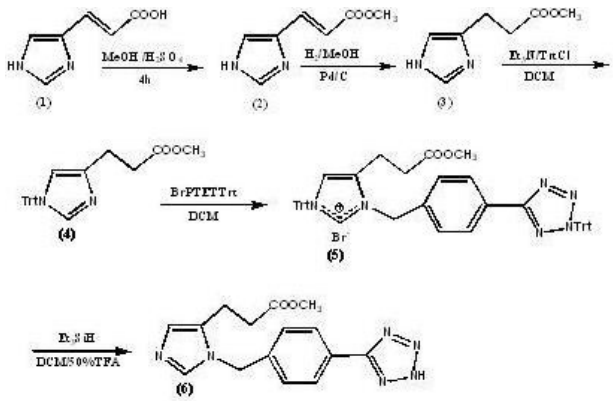

Fig. 1: Synthetic procedure of analogue (6).

The global energy minimum structure presented intramolecular distances which approached (± 2,5Å) the pharmacophore groups (imidazole, phenyl and tetrazol) of EXP3174 (Fig. 2) [3].

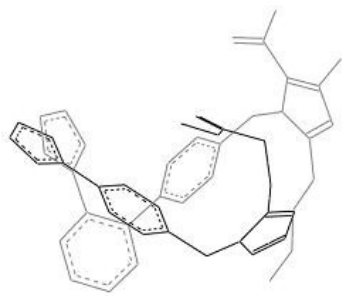

Fig. 2: Superimposition between EXP3174 and compound 6.

Acknowledgements

This work was supported by the Ministry of Energy and Technology of Greece (EPAN YB84).

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DETERMINATION OF POTENTIALS OF MEAN FORCE DEPENDENT ON ORIENTATION IN HYDROPHOBIC SYSTEMS MODELING SIDE-CHAINS OF BIOMOLECULES

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Introduction

Several studies have been carried out on the potentials of mean force (PMF) of systems modeling like-charged side chains of proteins [1 - 4], such as, e.g., guanidinium cations [1, 2] (modeling the side chains of arginine), between side-chains of two lysine residues [3] and a pair of acetate-guanidine [1, 4] ions in water to model interactions in proteins of the aspartic or glutamic acids with arginine side-chain. The PMFs of systems modeling the charged-nonpolar side-chains were also determined [1, 2]. The authors of papers [1 - 4] have determined PMFs averaged over all orientations. Very recently, Masunov and Lazaridis [5] calculated the PMFs of side chains models dependent on both distance and orientations. They performed a series of molecular dynamics simulations for the particular orientations as functions of distance between centers of interaction. However, these authors considered only selected orientations.

Results

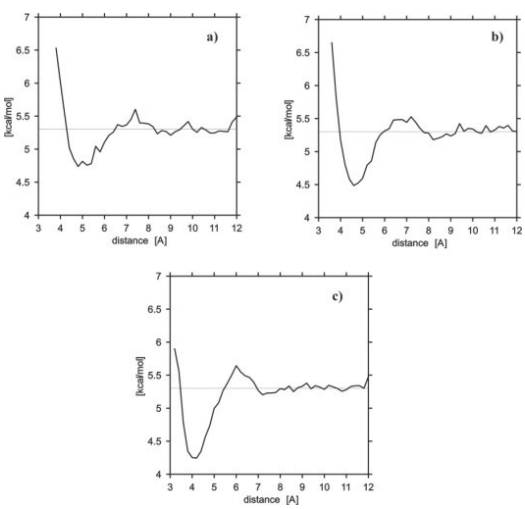

Fig. 1. Plots "a", "b" and "c" show potentials of mean force curves of two propane molecules modeling side chain - side chain interactions of two proline molecules. Curves "a", "b" and "c" refer to the "head-to-head", perpendicular and parallel orientations of the side-chains, respectively.

Discussion

The purpose of this study was to determine the potentials of mean force (PMF) of the interactions between models of nonpolar amino acid side chains in water. The potentials of mean force (PMFs) dependent on orientation were determined for systems forming hydrophobic and diagonal complexes composed of side-chain models of alanine, valine, leucine, proline and isoleucine, respectively, in water. For each hydrophobic pair in water a series of umbrella-sampling molecular dynamics simulations with the AMBER force field and explicit solvent (TIP3P water model) were carried out and the PMFs were calculated by using the Weighted Histogram Analysis Method (WHAM). In all cases a characteristic shape of PMF plots for hydrophobic association were found, which was manifested as the presence of contact minima and solvent separated minima.

Acknowledgments

This work was supported by grant the Polish Ministry of Science and Informatization (1 T09A 099 30).

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VALUATION OF TWO THEORETICAL METHODS TO ESTIMATE POTENTIOMETRIC-TITRATION CURVES OF PEPTIDES: COMPARISON WITH EXPERIMENT

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Introduction

In this work, we carried out potentiometric titrations of a model alanine-based peptide, Ac-XX$(A)_7$OO-NH_2 (XAO) (where X is diaminobutyric acid, A is alanine, and O is ornithine) in water, methanol and DMSO, respectively. We chose this sequence because only basic groups are present and, therefore, specific features of conformation (such as salt bridges) cannot influence the degree of ionization.

Results

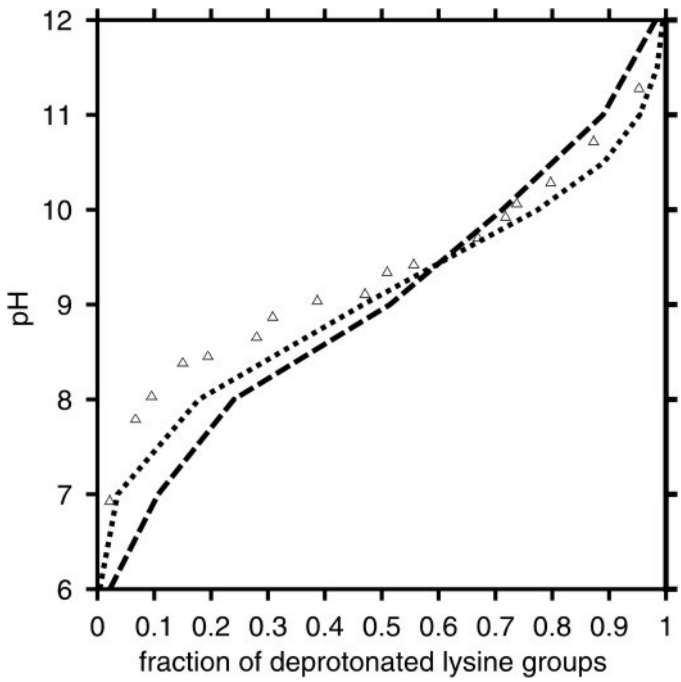

Fig. 1. Comparison of the experimental titration curve of polylysine (triangles); with the curve calculated by using the EDMC/PB/pH method (dashed line); and the MD/GB/pH method (dotted line).

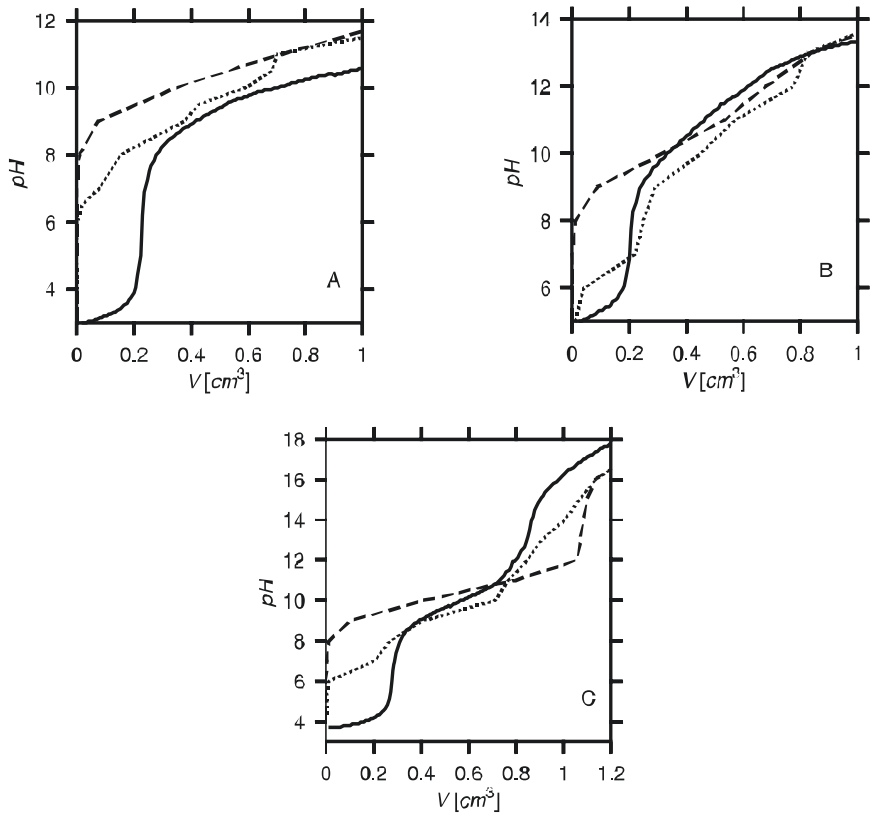

Fig. 2. Comparison of experimental (solid lines) and calculated titration curves of XAO by using the EDMC/PB/pH (dashed lines) and MD/GB/pH (dotted lines) in water (A), methanol (B), and DMSO (C).

Discussion

It can be seen that there is qualitatively good agreement between the theoretical titration curves and the experimental data. The calculated pKa corresponding to the pH at the 0.5 ionization degree agrees well with the experimental value. However, the experimental titration points form a steeper curve for lower pH values than the curve1 calculated by using the EDMC/PB/pH procedure or the curve calculated by using the MD/GB/pH procedure.

Acknowledgments

This work was supported by grant from the Polish Ministry of Science and Informatization 1 T09A 101 30 and a European Structural Founds stipend - ZPORR/2.22/II/2.6/ARP/U/2/05 (K. Bagińska).

SYNTHESIS AND OPIOID ACTIVITY PROFILES OF PEPTIDES CONTAINING 3,4-O-CARBORANYLENEPIPERIDINE AT THE C-TERMINUS

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Introduction

Recently, the synthesis of 3,4-*o*-carboranylenepiperidine (Cbp) was reported [1]. Cbp is an analogue of tetrahydroisoquinoline (Tiq). It is slightly bulkier and more lipophilic than Tiq. In the present study, we describe the syntheses and *in vitro* opioid activity profiles of a series of di-, tri- and tetra-opioid peptide analogues containing 2',6'-dimethyltyrosine (Dmt) at the N-terminus and Cbp at the C-terminus. Aside from being interesting probes for opioid receptor binding sites, Cbp-containing opioid peptides may have potential as Boron Neutron Capture Therapy (BNCT) agents in the treatment of cancer patients. The corresponding Tiq-containing peptides were also prepared and pharmacologically characterized for comparative purposes.

Result and Discussion

The peptides H-Dmt-Cbp (**1**), H-Dmt-Tiq (**2**), H-Dmt-D-Arg-Cbp (**3**), H-Dmt-D-Arg-Tiq (**4**), H-Dmt-D-Arg-Phe-Cbp (**5**) and H-Dmt-D-Arg-Phe-Tiq (**6**) were prepared either in solution or by a combination of solid-phase and solution techniques. The *in vitro* opioid activity profiles of the compounds were determined in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays and in receptor binding assays (Table 1).

The dipeptide H-Dmt-Cbp (**1**) was a δ antagonist with a K_e-value of 301 ± 76 nM in the MVD assay and a K_i^{δ} of 56.8 ±δ 8.3 nM in the receptor binding assay. Because of its very lipophilic character, compound **1** is likely to have good bioavailability and to be able to cross the blood-brain barrier. In comparison with the corresponding Tiq-analogue (**2**), compound **1** is about half as potent as a δ antagonist. Both dipeptides showed 6 - 7 - fold δ vs. μ selectivity in the binding assays. The tripeptide H-Dmt-D-Arg-Cbp (**3**) turned out to be a 5-fold more potent μ opioid agonist in the GPI assay than the corresponding Tiq^3-analogue (**4**). In contrast, **3** had 11-fold lower μ receptor binding affinity than **4**. This discrepancy may be due to the existence of different μ receptor subtypes in the GPI and in rat brain.

Table 1. In vitro opioid activity profiles of Cbp- and Tiq-containing opioid peptide analogues

No.	Compound	GPI	MVD		Receptor binding[c]		
		IC_{50}, nM	IC_{50}, nM	K_e, nM[a]	K_i^{μ}, nM	K_i^{δ}, nM	K_i^{δ}/K_i^{μ}
1	H-Dmt-Cbp	inactive		301	381	56.8	0.149
2	H-Dmt-Tiq	1450 (ED_{30})[b]		165	17.9	3.00	0.168
3	H-Dmt-D-Arg-Cbp	364	637		34.9	81.5	2.34
4	H-Dmt-D-Arg-Tiq	1890	7800		3.24	96.3	29.7
5	H-Dmt-D-Arg-Phe-Cbp	78.8	47.9		11.4	138	12.1
6	H-Dmt-D-Arg-Phe-Tiq	1.85	7.70		0.129	22.2	172
	[Leu^5]enkephalin	246	11.4		9.43	2.53	0.268

[a] Determined against deltorphin I. [b] Partial agonist (maximal inhibition of contractions = 60%). [c] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane binding sites.

The tetrapeptide H-Dmt-D-Arg-Phe-Cbp (**5**) was a μ agonist with low nanomolar μ receptor binding affinity (K_i^{μ} = 11.4 ± 0.3 nM) and considerable μ selectivity (K_i^{δ}/K_i^{μ} = 12.1). In comparison with the Tiq4-tetrapeptide (**6**), compound **5** had about 40-fold lower μ agonist potency in the GPI assay and was less μ-selective in the binding assays. All peptides had very weak binding affinity for κ receptors. In conclusion, we developed selective, Cbp-containing δ antagonists and μ agonists with significant potency.

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PEPTIDE CONJUGATES WITH 9-AMINOACRIDINES

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Introduction

9-Aminoacridine and its derivatives play an important role in medicinal chemistry. They are used for anti-cancer drug targeting and for treatment of severe diseases such as rheumatic arthritis, lupus erythrematosus, AIDS and prion diseases. The conjugation of them with peptide may improve their pharmacological properties such as availability, potency and harmlessness.

Results and Discussion

A class of 9-aminoacridine peptide conjugates was developed with aim to influence prion peptide DNA interaction (Figure 1). New compounds were designed, which DNA-binding constants are higher than that of prion peptide but their constants are kept as low as possible (for the second rule see also [1]). Peptide conjugates have been prepared by modified SPPS [2]. The library method was used for selection of the most potent DNA-binder of the set with formula Acr-Gly-Gly-Tyr-Gly-$NHCH_2CH_2NH$-Acr [3].

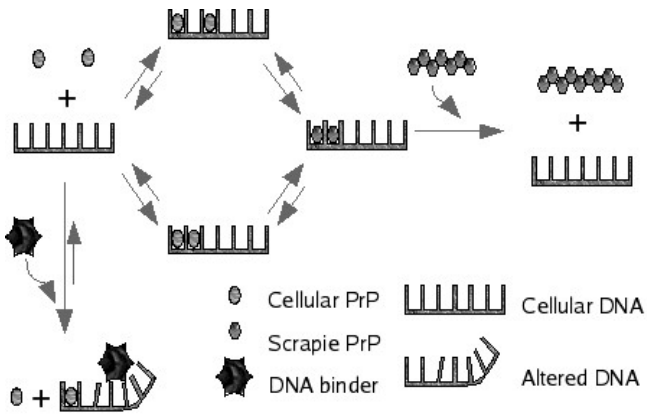
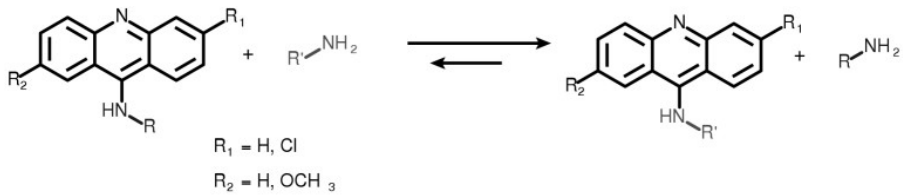


Fig. 1. Proposed mechanism of prion (PrP) – DNA interaction, where DNA serves as catalysts of prion nucleation process. Real catalyst of prion aggregation has not been known, yet, and thus it is called factor-X. Factor-X may be either protein, nucleic acid, lipid or saccharide. A pathway of possible inhibitor (DNA binder) of PrP-DNA interaction is also depicted. This scheme can be generalized, i. e. you have to influence either prion directly or factor-X in order to prevent prion toxicity.

Finally, we have described acridine exchange reaction, which could be responsible for pharmacological efficiency of FDA approved drug – Quinacrine (Scheme 1) [4].



Scheme 1. Acridin-9-yl exchange – a proposal for the action of some 9-aminoacridine drugs. The ε-amino group of lysine was suggested to serve as moderate acceptors of acridine moiety. Thus, the covalent modification of lysine residues side chain in hydrophobic core of prion protein aggregates could explain the discrepancy between ability of acridine drug quinacrine to reduce efficiently an incidence of prion protein in cell culture and its weak prion binding affinity [4].

Acknowledgements

This work was supported from grants GA CR 203/02/1379, 203/04/1421; GA AS CR A400550614. I thank organizers for Travel Grant.

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CYCLIC OPIOID PEPTIDE AGONISTS AND ANTAGONISTS OBTAINED VIA RING-CLOSING METATHESIS

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Introduction

The opioid peptide H-Tyr-c[D-Cys-Phe-Phe-Cys]NH_2 cyclized *via* a methylene dithioether (**13**) has recently been reported to be a potent and selective μ opioid agonist [1]. To assess the effect of replacing the sulfurs of this peptide with methylenes on the opioid activity profile, we prepared the dicarba analogues of **13** containing either Tyr or 2',6'-dimethyltyrosine (Dmt) in the 1-position (Fig. 1). In an effort to develop μ-selective opioid antagonists, we also prepared dicarba analogues of **13** containing 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) or (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(2*S*)-Mdp] in place of Tyr[1] [2].

Result and Discussion

The syntheses were performed on solid-phase (Rink amide NovaGel resin) by assembly of the linear precursor peptides and subsequent ring-closing metathesis between D-allylglycine[2] and (2*S*)-2-amino-5-hexenoic acid[5], using a second generation Grubbs catalyst. After cleavage from the resin, the olefinic peptides were obtained as mixtures of cis and trans isomers and subsequent catalytic hydrogenation yielded the saturated -CH_2-CH_2- bridged peptides. Opioid activities of the compounds *in vitro* were determined in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays and in receptor binding assays (Table 1).

H-Yyy-D-Gly-Phe-Phe-Gly-NH_2

CH_2-X-X-X-CH_2

N°	Yyy	- X - X - X -
1	Tyr	- HC = CH - CH_2 - (*cis*)
2	Tyr	- HC = CH - CH_2 - (*trans*)
3	Tyr	- CH_2 -CH_2 - CH_2 -
4	Dmt	- HC = CH - CH_2 - (*cis*)
5	Dmt	- HC = CH - CH_2 - (*trans*)
6	Dmt	- CH_2 - CH_2 - CH_2 -
7	Dhp	- HC = CH - CH_2 - (*cis*)
8	Dhp	- HC = CH - CH_2 - (*trans*)
9	Dhp	- CH_2 - CH_2 - CH_2 -
10	(2S)-Mdp	- HC = CH - CH_2 - (*cis*)
11	(2S)-Mdp	- HC = CH - CH_2 - (*trans*)
12	(2S)-Mdp	- CH_2 - CH_2 - CH_2 -
13	Tyr	- S - CH_2 - S -

Fig.1. Structural formulas of dicarba analogues of ***13***

Nearly all Tyr[1]- and Dmt1-cyclic olefinic and saturated cyclic peptides (**1 - 6**) showed subnanomolar μ and δ agonist potencies in the GPI and MVD assays, and subnanomolar or low nanomolar μ and δ receptor affinities in the binding assays. Thus, unlike the parent peptide **13**, these compounds turned out to be non-selective. These results indicate that replacement of the sulfurs in 13 with methylene groups

*Table 1. In vitro opioid activity profiles of dicarba analogues of **13***

	GPI		MVD	Receptor binding[d]		
No.	IC_{50}, nM	K_e, nM	IC_{50}, nM[a]	K_i^{μ}, nM	K_i^{δ}, nM	K_i^{δ}/K_i^{μ}
1	0.401		0.851	0.281	0.910	3.24
2	1.34		4.51	0.332	1.53	4.61
3	1.05		0.740	1.04	2.24	2.15
4	0.161		0.191	0.528	0.491	0.930
5	0.407		0.0444	0.438	0.286	0.653
6	0.509		0.0655	1.54	1.20	0.779
7		32.2	P.A. (e = 0.38)[b]	23.5	86.3	3.67
8		44.3	239	16.7	27.0	1.62
9		41.7	7500	8.95	39.2	4.38
10		32.4	P.A. (e = 0.24)[b]	7.44	7.67	1.03
11		157	P.A. (e = 0.33)[b]	323	35.3	0.109
12		25.7	K_e = 147 nM[c]	18.3	18.7	1.02

[a] Determined against DAMGO. [b] Partial agonist. [c] Antagonist; K_e determined against DPDPE. [d] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane binding sites.

has a pronounced effect on the opioid activity profile.The Dhp[1]- and (*2S*)-Mdp[1]-olefinic and saturated peptides (compounds **7 - 12**) turned out to be μ opioid antagonists with Ke values ranging from 25 to 157 nM. In the receptor binding assays μ antagonists **7 - 10** and **12** showed little μ vs. δ selectivity, whereas the trans olefinic compound **11**, surprisingly, was somewhat δ-selective (K_i^{μ}/K_i^{δ} = 9.2).

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PACAP PEPTIDES AS NEW A-SECRETASE ACTIVATORS

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Introduction

Proteolytic cleavage of the amyloid precursor protein (APP) by α-secretase within the Aβ sequence precludes formation of amyloidogenic peptides and leads to a release of soluble APPsα, which has neuroprotective properties (1).

Therefore, pharmacological upregulation of the α-secretase could be a possible approach for treatment of Alzheimer disease (AD).The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has neurotrophic, neuroprotective as well as anti-apoptotic properties and is involved in learning and memory processes (2). PACAP exists as a 38-residue form (PACAP-38), and as a shorter form corresponding to the N-terminal 27 amino acids of PACAP-38 (PACAP-27). The major form in tissues is PACAP-38, with the highest concentration found in the hypothalamus, cerebral cortex, hippocampus, posterior pituitary, testes and adrenal gland.Its specific G protein-coupled receptor PAC1 is expressed in several CNS regions including the hippocampal formation.

The main aim of the present study was to investigate a possible stimulating role of PACAP on the α-secretase activity and to elucidate the cellular signal transduction mechanisms involved in the activation of the α-secretase.

Result and Discussion

A major finding of the present study is that activation of the PAC1 receptor by its natural agonists PACAP-38 and PACAP-27 strongly increases the α-secretase activity.

The PACAP-induced APPsα secretion in human neuroblastoma cells was completely inhibited by the PAC1 receptor specific antagonist PACAP (6-38) (Fig.1A). In HEK cells stably overexpressing functional PAC1 receptors both natural PACAP forms stimulated the α-secretase activity to the same extent, whereas the related vasointestinal peptide VIP had no stimulatory effect (Fig. 1B).

The PACAP-induced release of APPsα was completely inhibited by two hydroxamate zinc metalloproteinase inhibitors (3).

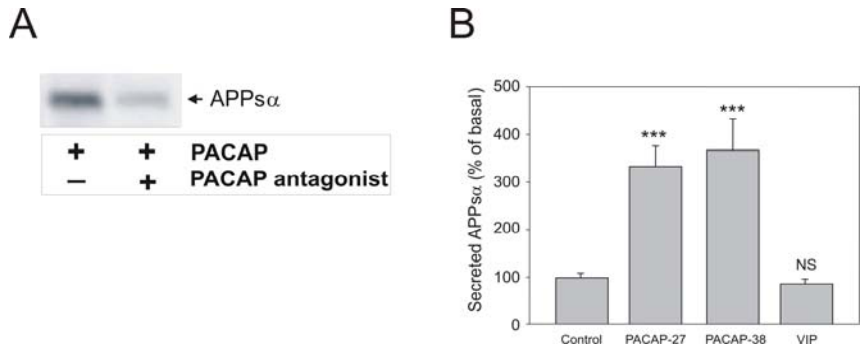

Fig. 1: PACAP induced secretion of APPsα from human neural SK-N-MC cells (A) and HEK cells overexpressing the PAC1 receptor (B).

Our *in vivo* studies show that the intranasal application of PACAP in a transgenic AD mouse model significantly increases the generation of neuroprotective APPsα and reduces the production of soluble Aβ peptides.

Conclusions

We have demonstrated *in vitro* and *in vivo* that PACAP acts as

α-secretase activator, therefore up-regulation of α-secretases by stimulation of the PAC1 receptor might be valuable approach for prevention or treatment of AD.

Acknowledgments

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NEUROPROTECTIVE PEPTIDES DERIVED FROM AMYLOID PRECURSOR PROTEIN AS TOOLS IN ALZHEIMER RESEARCH

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Introduction

The accumulation of the β-amyloid peptide (Aβ) in the brain is a central event leading to the development of Alzheimer's disease (AD). Aβ is a 40/42-residue fragment of the amyloid precursor protein (APP), processed by amyloidogenic pathway by two proteases known as β- and γ-secretase.

In the alternative non-amyloidogenic pathway APP is cleaved within the Aβ domain by α-secretase between amino acids 16(Lys) and 17(Leu) of the Aβ region [1]. The action of the α-secretase not only precludes the formation of pathological Aβ peptides but also induces release of a soluble large N-terminal APP fragment (APPsα). Secreted APPsα exerts proliferative properties in a variety of cell types as well as anti-apoptotic and neuroprotective effects. The existence of a membrane receptor for APPsα has been postulated for a long time, but at present the receptor protein is completely unknown. Direct identification of the binding sites of full APPsα will be difficult due to the binding of this multifunctional protein to several putative receptors responsible for distinct functions.

The aim of our work is to identify a receptor responsible for the neuroprotective effect of APPsα by using specific peptide ligands.

Result and Discussion

First we synthesized peptides of 10-(peptide I) and 20-(peptide II) amino acids with the C-terminal sequence of human APPsα (Table 1), which discriminates APPsα from APPsβ as the latter lacks neuroprotective function.

Table 1. Sequences of synthesized peptides.

Peptide	Sequence
Peptide I (human)	Ac-SEVKMDAEFR-NH_2
Peptide II (human)	Ac-SEVKMDAEFRHDSGYEVHHQ-NH_2
Peptide III (rodent)	Ac-SEVKMDAEFGHDSGFEVRHQ-NH_2 [a)]

a) species differences are underlined

Our results show the neuroprotective properties of these peptides against the toxicity of 6-hydroxydopamine (6-OHDA), $A\beta_{25-35}$ and $A\beta_{1-42}$ by viability assay in human neuroblastoma cells. The peptides protect human neuronal cells against

toxicity induced by 6-OHDA or Aβ peptides (Fig. 1) in a similar way as recombinant APPsα. However effects in rat neuronal cells could not be observed.

The human and rodent $A\beta_{40}$ peptide sequences differ in three amino acid residues [2] and therefore rodent 20-amino acid peptide (peptide III) was synthesized to determine the peptide specificity of neuroprotection. This peptide has neuroprotective properties only in rat neuronal cells whereas no effects were observed in human cells.

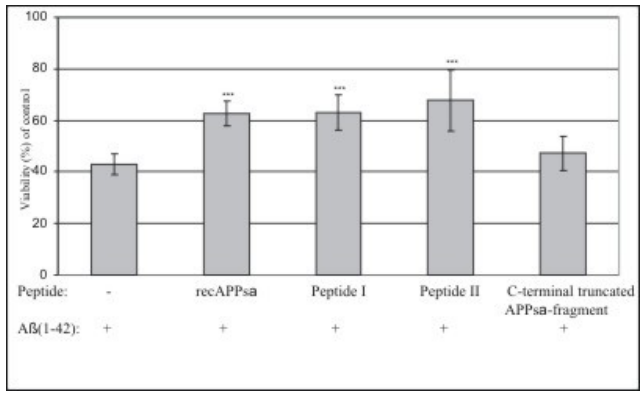

Fig. 1. Neuroprotective effects of peptides derived from APPsα C-terminus against $A\beta_{1-42}$ induced toxicity in differentiated human neuroblastoma cells (SH-SY5Y)

To identify an APPsα receptor by a classical ligand-binding assay, radioactive ligands were synthesized by a coupling reaction between the ε-amino group of lysine in the neuroprotective 20 amino acid peptide and tritium labeled N-succinimidyl [2, 3-^{3}H] propionate.

Conclusions

Our results suggest that peptides derivates from the C-terminus of APPsα could be a useful tool for identification of an APPsα receptor responsible for its neuroprotective effects.

Acknowledgments

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MODEL PEPTIDES MIMIC THE STRUCTURE AND FUNCTION OF THE N-TERMINUS OF THE PORE-FORMING TOXIN STICHOLYSIN II

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Introduction

Sticholysin II (StII), a water-soluble protein produced by the anemone Stichodactyla heliantus, binds to membranes, lysing cells through pore formation [1]. The monomer crystal structure has been solved [2], however, the toxin molecular mechanism of action is not fully understood. Similarly to equinatoxin II [3], StII´s N-terminal region is thought to play an important function in pore formation.

To investigate the role of StII´s N-terminal sequence in membrane binding and hemolysis, peptides corresponding to residues 1-30 (P1-30, ALAGTIIAGASLTFQVLDKVLEELGKVSRK), 11-30 (P11-30), and sequences where Leu/Ile were replaced by Trp (PW^2, PW^7, PW^{12}, PW^{17}, PW^{21}, and PW^{24}) were synthesized. The peptides functional, conformational, and topographical properties were evaluated by measuring permeabilization of erythrocytes and lipid vesicles, analysis of their CD spectra in solution (as a function of concentration, pH, ionic strength, and addition of the secondary structure-inducing solvent TFE) and upon binding to model membranes (micelles and small and large unilamellar vesicles - SUV and LUV), and fluorescence studies of their accessibility to a water soluble fluorescence quencher, respectively.

Results

All peptides exhibited hemolytic activity and permeabilized phospholipid vesicles, albeit to a lesser extent than the protein. P1-30, that contains the 1-10 hydrophobic stretch, showed higher activity than P11-30. Similarly to StII, the permeabilizing effect of P1-30 is cooperative.

CD spectra showed that while the more hydrophilic P11-30 displayed a random conformation, peptides containing residues 1-30 underwent aggregation with increasing concentration, pH, and ionic strength. In the presence of TFE and upon binding to micelles and bilayers, the peptides acquired α-helical conformation.

Fluorescence studies demonstrated that the first residues of StII´s N-terminus penetrate more deeply into the bilayer, whereas residues 14-26 are located more superficially.

Conclusions

The results provide a demonstration of the role of St II's N-terminus - the amphipathic α-helix and the initial hydrophobic segment - in the process of membrane insertion and pore formation. The data are in agreement with the formation of an amphipathic α-helix by residues 14-26, located at the membrane-water interface, and the deeper insertion of residues 1-10. Moreover, the data stress the contribution of the latter residues to the toxin's hemolytic activity. The combination of these functional-conformational-topographical data suggest that the mechanism of lysis is modulated by toroidal pore formation.

This process is very likely a common molecular mechanism of pore formation for actinoporins. This is the first study of model peptides that reproduce the pore forming activity of an actinoporin [4].

Acknowledgements

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THE "BIP METHOD" FOR ASSIGNMENT OF THE ABSOLUTE CONFIGURATION OF β-AMINO ACIDS: ICD IN THE BIPHENYL CORE OF BIP-β-XAA* DIPEPTIDES

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Introduction

The C^{α}-tetrasubstituted α-amino acid residue Bip possesses non isolable (*R*) and (*S*) conformationally labile atropoisomers [1]. We have previously reported that in the linear dipeptides Boc-Bip-α-Xaa*-OMe with α-Xaa* = L-Ala, L-Val, L-Leu, L-Phe, L-(αMe)Val and L-(αMe)Leu residues (Fig. 1), the onset of an equilibrium between two diastereoisomers could be observed by CD and ^{1}H NMR [2]. The phenomenon of induced circular dichroism (ICD) represents the basis for the "Bip method", an easy and fast configurational assignment of chiral α-amino acids. We have now investigated the Boc-Bip-β-Xaa*-OMe dipeptide series with β-Xaa* = L-β^3-HAla [3], L-β^3-HVal, L-β^3-HLeu, L-β^3-HPro and the cyclic β^{2,3}-amino acids (1*S*,2*S*)/(1*R*,2*R*)-ACHC and (1*S*,2*S*)/(1*R*,2*R*)-ACPC (Fig. 1).

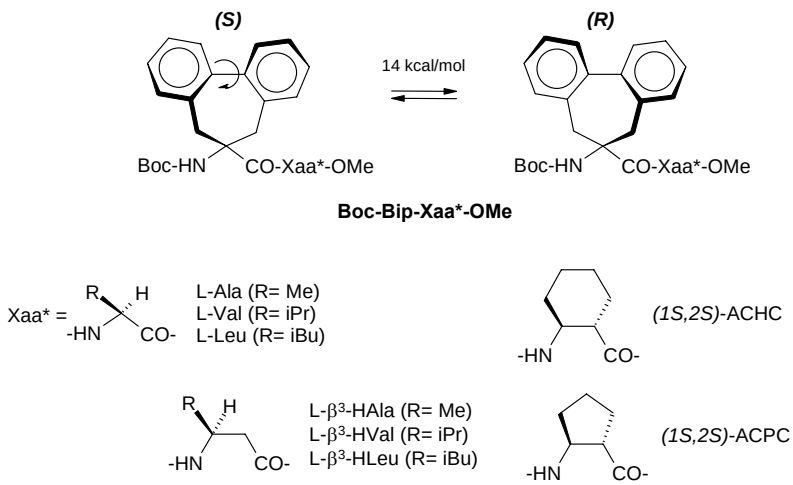

Fig. 1. Chemical structures and diastereoisomer equilibrium of the Boc-Bip-Xaa-OMe dipeptides discussed in this work.*

Result and Discussion

The terminally protected dipeptides were prepared in solution by coupling Boc-Bip-OH with the β-amino ester hydrochlorides HCl.β-Xaa*-OMe. The EDC/HOAt activation method was generally used for coupling at the sterically demanding C-terminus of Bip. In the same manner as previously observed [2], two sets of signals, corresponding to the presence of two diastereoisomers with unequal populations and exchanging slowly on the NMR time scale, were usually seen at low temperature. Significantly high d.r. values (ranging from 60:40 to 74:26) were observed at 233 K in CD_3OD for all combinations of Bip with both β^3- and cyclic $\beta^{2,3}$-amino acids.

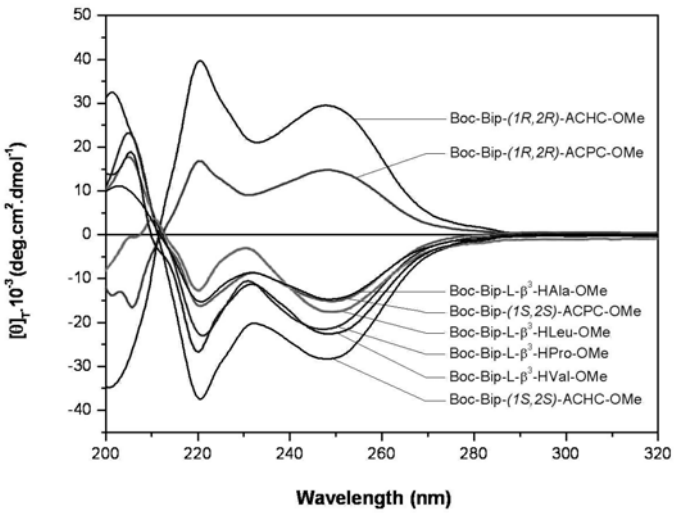

Fig. 2. Comparative CD spectra (200-300 nm range) of the terminally protected Boc-Bip-Xaa-OMe dipeptides in MeOH solution.*

Our analysis in MeOH solution allowed us to conclude that the ICD resulting from the induced axial chirality in the biphenyl core of the Bip residue gives clear information on the β-Xaa* configuration, for both β^3- and cyclic $\beta^{2,3}$-amino acids (Fig. 2). In all dipeptides, a P torsion of the biphenyl axial bond of Bip is preferentially induced by the L-β^3-Xaa* as well as by the cyclic (1*S*,2*S*)-$\beta^{2,3}$-Xaa* C-terminal residues. In conclusion, a substantial central-to-axial induction of chirality from the C-terminal β^3-Xaa*-OMe (β^3-HAla, β^3-HVal, β^3-HLeu, β^3-HPro) or cyclic $\beta^{2,3}$-Xaa*-OMe (ACHC, ACPC) amino ester residues to the N^{α}-Boc protected, pro-atropoisomeric, C^{α}-tetrasubstituted α-amino acid Bip has been unraveled in simple linear dipeptides, thus demonstrating that the Bip residue may be used as a convenient CD probe for the determination of the absolute configuration not only of α-amino acids, as previously reported [2], but of β-amino acids as well.

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PEPTIDE-MEDIATED DELIVERY OF SIRNA VIA COVALENT CONJUGATES

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Introduction

Improving the intracellular delivery of synthetic oligonucleotides and their analogs is an important goal in the development of small interfering RNA (siRNA) therapeutics for inhibiting gene expression in cell culture and *in vivo*. Previously, we have performed a systematic analysis of the ability of different structural classes of peptides to translocate across cell membranes and deliver siRNA into cells [1].

Lead candidates were conjugated to 21-mer siRNA and their ability to effectively knockdown TNF-α mRNA in activated human monocytes was evaluated. Previous studies have demonstrated that longer siRNAs that are processed by Dicer can result in more potent knockdown than the corresponding standard 21-mer siRNAs [2]. We reasoned that conjugation of delivery peptides to these longer siRNA may lead to greater potency. Dicer-substrate 25 - 27-mer siRNAs were conjugated via 5' end with the most effective peptides and their cell uptake properties were evaluated.

Results

The products resulting from *in vitro* digestion of peptide-conjugated RNA duplexes with recombinant human Dicer were identified using LCMS. Antisense strand (27 mer, CoP950 5'Asen) conjugated to the peptide via 5' end and sense strand (25 mer, CoP952 5'-sen) were cleaved to the desired 21 mer products in the same manner as non-conjugated duplex. Greater knockdown was observed when the delivery peptide was conjugated to the 5' end of antisense 27 mer strand versus conjugation to the 5' end of the sense strand (Fig. 1). We did not observe an improvement in knockdown when conjugates were transfected with lipofectamine.

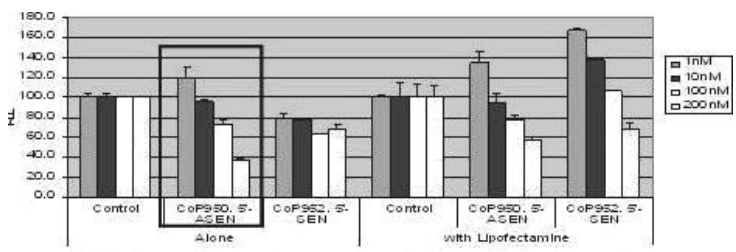

Figure 1. hTNF-α knockdown in human monocytes with peptide-siRNA conjugates

Discussion

According to the latest literature, the Dicer enzyme uses the overhang as a recognition feature and measures the cleavage site approximately 21 nucleotides away [3]. The asymmetric 25/27-mer siRNA are designed with only one overhang at the 3' end of the antisense strand which biases Dicer to cleave from that end only. By attaching the peptide to the 5' end of the antisense strand, which is opposite the overhang, the peptide does not interfere with this key recognition feature. A further benefit of this design is that after Dicer processing the peptide is cleaved from the 21/21-mer siRNA prior to RISC loading (Fig. 2). For the direct conjugation of delivery peptides to standard 21/21 siRNA, RISC must load the siRNA with the delivery peptide attached which may have a detrimental effect on activity. In summary, we have synthesized a new class of peptide siRNA conjugates which are capable of mediating TNF-αmRNA knockdown in activated macrophages.

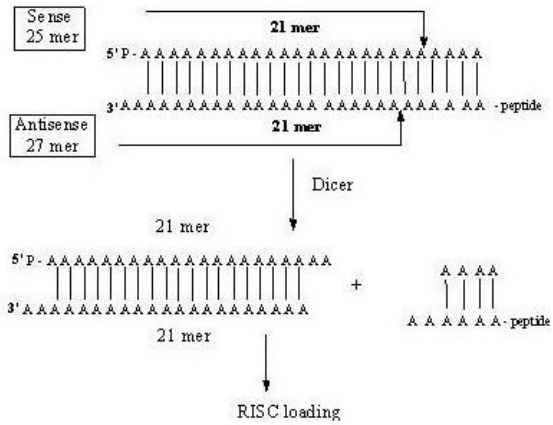

Figure 2. Products of *in vitro* dicer digestion.

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PLASMID DELIVERY AND TOPICAL ACTIVATION

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Introduction

Safe drug delivery technologies are pivotal for genetic interventiones. Nowadays all commonly used viral vectors are bearing the risk of inflammatory reaction. Some questions concerning the efficacy of delivery of the genetic substances, the desired topical gene activation and targeting have to be answered. We have attempted to develop a membrane non-perturbing delivery system which is qualified for transport of inactivated functional genes to come into cells *with smallest adverse reactions* [1]. Our concept bases on the use of peptide-nucleic-acids (PNAs) resistant against enzymatic digest. Oligonucleotide derivates, in which ribose-phosphate-bone has been replaced with ethylen-amin connected α-amino-ethyl-glycine-units [2]. The achiral character leads to more stabile PNA-DNA-complexes compared to DNA-DNA-complexes. These physico-chemical qualities combined with the optimal drug design of PNAs (clamp PNA) leads to stable compounds in physiological environment.

Experimental plan

Peptides were synthesized and the conjugates were composed according to the solid phase peptide synthesis and the protecting group chemistry strategies [3]. To avoid possible nonspecific binding the sequence of PNA was chosen excluding plasmids as well eukaryotic DNA sequences. The PNA peptides were also conjugated covalently non cleavable with a capronic acid spacer to the SV40 core NLS, PKKKRKV. The complete schematized sequence for the Clamp-PNA-BioShuttle is the $[TP\text{-}Spacer\text{-}Cys\text{-}S\text{-}S\text{-}Cys]_n$-PKKKRKV-Spacer-$[PNA_{clamp}]_2$.

The procedures for preparing the transmembrane peptide and the PNA were almost identical; in the case of the PNA synthesis we used a blocking step with acetic anhydride in every step.

The synthesis was carried out on a 0.05 mmol Tenta Gel R Ram (Rapp Polymers) 0.18 mmol/g of Substitution. As coupling agent 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used. For the synthesis 9-Fluorenylmethoxycarbonyl Fmoc-protected PNA monomers with the exocyclic aminogroups of the A, G and C bases being blocked with the benzhydroxyl (Bhoc) group were used. The protected PNA and peptide resins were treated with 20% piperidin in dimethylformamide for 5 minutes and further washed thoroughly with

dimethylformamide. Cleavage and deprotection of the resins were affected by treatment with 90% trifluoroacetic acid and 10% triethylsilan.

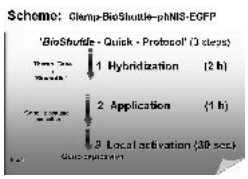
For the condensation we utilized Fmoc-Lys(Dde)-Wang resin.

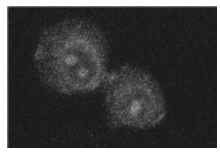
The hybridization of the 'BioShuttle' modules with the pDNA-EGFP-C3 (Clontech) was accomplished according to first description by Britten & Kohne.

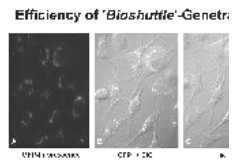
The mixture of the conjugate-pDNA hybrid was added into the culture medium of the cells in a final concentration of 100 pM. We tested several Tumor cells. The incubation time was over 60 min at Jurkat, AT1 and HeLa cells. The heading at 42°C leads to the dissociation of the conjugate/ pDNA hybrid und to the activation of the pDNA gene expression. Gene transfer into living cells and expression of the phNIS-EGFP were determined by CLSM, fluorescence reader and flow Cytometry methods.

Results

We found a rapid (after 60 min incubation time) and nearly 100% cellular uptake of pDNA into DU-145 carcinoma cells. By means of GFP-fluorescence, a period of 30 seconds was sufficient for gene activation (Fig. 2;3;4). After 24 h GFP was measured. Fig.1 shows the 'Quick-Protocol'. Non-activated control cells do not shown any fluorescence.







Discussion

'Clamp-PNA-BioShuttle' carriers could be a helpful tool for gene delivery and research in gene therapy. We intend to use it in the differentiation process of cells in the range of topically genetic interventiones.

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INDUCED AXIAL CHIRALITY IN THE BIPHENYL CORE OF 6,7-DIHYDRO-5H-DIBENZ[C,E]AZEPINE DERIVATIVES OF A- AND β-AMINO ESTERS

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Introduction

We have recently reported that the induced circular dichroism (ICD) of the biphenyl core of Boc-Bip-Xaa*-OMe dipeptides based on the conformationally labile Cα-tetrasubstituted α-amino acid residue Bip could allow an easy and fast configurational assignment for both α- and β-Xaa* amino acids [1]. In search for other biphenyl/Xaa* architectures in which a transfer of central to axial chirality could result in a potentially useful ICD, we considered N-substituted 6,7-dihydro-5H-dibenz[c,e]azepine (DAZ) derivatives as interesting candidates. Here we report the syntheses, and the ^{1}H NMR and CD analyses of a series of DAZ-Xaa*-OMe amino esters derived from α-, β^3-, and cyclic $\beta^{2,3}$-Xaa* residues, namely D-/L-Ala, D-/L-Val, L-Leu, L-Ile, L-Ser, L-β^3-HAla, L-β^3-HVal, L-β^3-HLeu, (1*S*,2*S*)/(1*R*,2*R*)-ACHC and (1*S*,2*S*)/(1*R*,2*R*)-ACPC (Fig. 1). The related amide derivatives Xaa*-DAZ have been recently investigated by Rosini et al. [2].

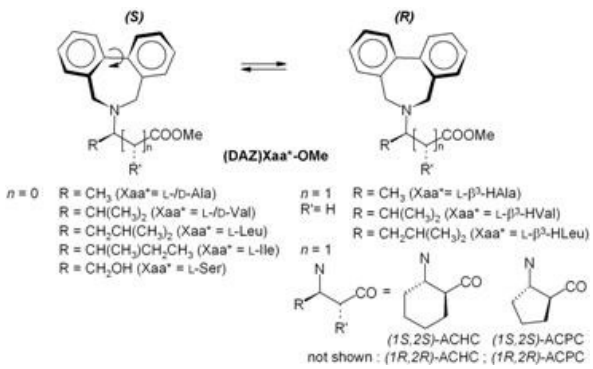

Fig. 1. Chemical structures and diastereoisomer equilibrium of the DAZ-Xaa-OMe amino esters discussed in this work.*

Results and Discussion

The DAZ-Xaa*-OMe derivatives were readily obtained by reaction of 2,2'-bis(bromomethyl)-1,1'-biphenyl with the amino esters H-Xaa*-OMe. The exclusive formation of the seven-membered ring system, already established in the 1950's [3], has been exploited for a long time until very recently [4]. The reaction,

conducted in THF at 60 °C in the presence of an excess of DIEA, afforded the derivatives shown in Fig. 1 in high yields. In ^{1}H NMR, two sets of signals corresponding to the presence of two diastereoisomers with diastereoisomeric ratios (d.r.) ranging from ca 50:50 to 66:34, were usually observed in CD_3OD at low temperature (ca 203 K) for all DAZ-Xaa*-OMe derivatives of α-, β^3-, and cyclic $\beta^{2,3}$-amino esters.

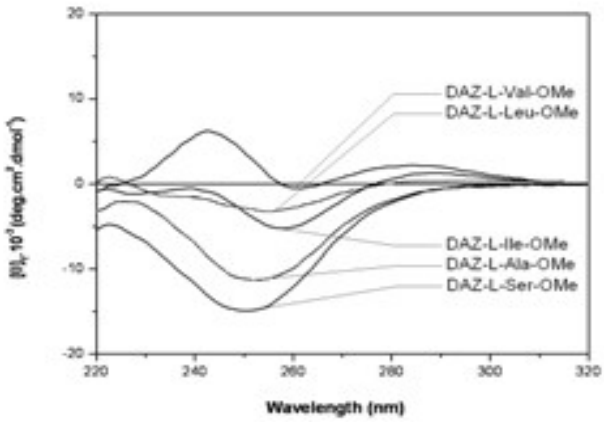

Fig. 2. Comparative CD spectra (220-320 nm range) of DAZ-L-α-Xaa-OMe derivatives in MeOH solution.*

CD analysis of the DAZ-Xaa*-OMe derivatives, performed in MeOH solution, indicated that an ICD results from the induced axial chirality of the biphenyl core of DAZ for L-Ala, L-Val, L-Ile, L-Ser, L-Leu (Fig. 2), as well as L-β^3-HAla, L-β^3-HVal, L-β^3-HLeu, (1*S*,2*S*)/(1*R*,2*R*)-ACHC and (1*S*,2*S*)/(1*R*,2*R*)-ACPC (not shown). In general, a negative sign of the Cotton effect at ca 250 nm, associated with a P torsion of the biphenyl axial bond, is preferentially induced by L-α-Xaa*-OMe (Fig. 2) as well as L-β^3-Xaa*-OMe and (1*S*,2*S*)-ACHC-OMe amino esters (not shown). Unexpectedly, however, both the wavelength maximum and the intensity of this Cotton effect are strongly dependent on the Xaa* residue. Therefore, more information on the factors governing the ICD is needed for the use of DAZ-Xaa*-OMe derivatives as spectroscopic probes for the determination of the absolute configuration of α-, β^3- and cyclic $\beta^{2,3}$-amino esters.

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APPLICATION OF PEPTOID METHODOLOGY FOR SYNTHESIS OF "DIFFICULT" PEPTIDES FREE OF ASPARTIMIDE AND RELATED PRODUCTS

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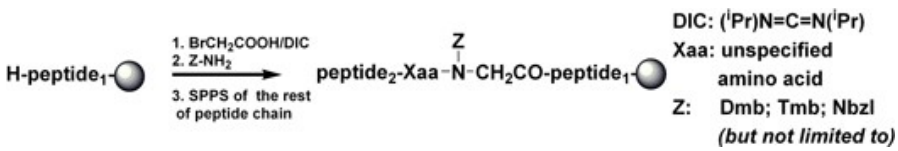
Introduction

The formation of aspartimide (aminosuccinimide, Asu) is the first step of the well-known degradation of Asp/Asn containing peptides and proteins at alkaline, neutral and acidic pH, both *in vitro* and *in vivo*. The strong bases used for deprotection in Fmoc-based SPPS promotes Asu formation especially at Asp-Gly sites and this results in a variety of rearranged and racemized products [1]. Only temporary protection of amide nitrogen carboxyl-terminal to Asp with Hmb or Hnb and analogues can eliminate completely the Asu formation [2]. However, the coupling of the residue next to Hmb-protected amino acid (except Gly) is often inefficient, accompanied by depsipeptide formation and this result an incomplete Hmb-deprotection. Available Hmb-protected building blocks are too expensive and limited to *t*Bu-side chain protection. Hmb-analogues are not commercially available and can only partially resolve the mentioned problems.

Results and Discussion

Dmb-backbone protection is efficient in preventing secondary structure formation at GG sites during standard Fmoc-SPPS [3]. In this work we explore the use of Dmb, Tmb and Nbzl protecting groups (Z) for the synthesis of "difficult"/Asu-prone peptides, in three different strategies: (A) Fmoc-Asn/Asp-(Z)Gly-OH dipeptides; (B) Fmoc-(Z)Gly-OH monomer building blocks and finally, (C) 'sub-monomeric' peptoid route [4] for synthesis of H-(Z)Gly on the resin (Scheme 1).



Scheme 1. Peptoid "sub-monomeric" approach for synthesis of "difficult" peptides and/or peptides free of Asu and related products.

We tested the new methods on model peptides VKD/NGYI, as well as on H-GLFGAIAGFIENGWEGMIDGGRKKRRQRRR-OH, a peptide containing HA21-20 and HIV-Tat(48-57) sequences, designed to deliver molecules of interest into

cells. When prepared with standard Fmoc-SPPS, both peptides showed extensive formation of Asu and piperidide as well as the presence of various racemized and isomerized products that makes product purification difficult or impossible.

The yield/purities of the products, prepared using strategies A-C were comparable with those obtained with the Hmb-protected dipeptide building block, and were found to be free of Asu/piperidides. Both the preformed building blocks (Strategies A and B) and the peptoid "sub-monomeric" approach (Strategy C) introduce proline-like structures, which can disrupt unwanted H-bonds during the synthesis of "difficult" peptides. Strategy B is useful for the synthesis of Xaa-Gly sequences (including DG) and potentially allows the incorporation of (Z)Gly at any point of the synthesis of 'difficult' peptides. The more general Strategy C is efficient, simple and flexible, it does not require separate or additional synthetic steps and is more cost effective than Strategies A, B.

The use of 2-nitrobenzylamine for bromine displacement (Strategy C) is a new method for the preparation of backbone-caged peptides. It can also be applied for the synthesis of cyclic peptides and - combined with orthogonal side chain protection of aspartic acid - for the synthesis of side chain modified Asx- peptides.

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DESIGNED 3_{14}-HELICAL β-PEPTIDES BASED ON CROWNED (S)- β^3-H-DOPA COMBINED WITH (1*S*, 2*S*)-ACHC RESIDUES

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Introduction

According to the well-documented ability of β-peptides to adopt a variety of helical secondary structures [1], different in nature and even more stable than those adopted by α-peptides, we found it interesting to introduce crown-ether receptors on the side chains of β-amino acids in view of their assembly in structurally well-defined β-peptide architectures of bis-crown compounds. In connection with the pioneering studies of Voyer et al. [2], who explored the use of peptide scaffolds based on crowned (L)-DOPA residues for the construction of molecular receptors and devices, we synthesized the (*S*)-β^3-H-DOPA analogue [3] and exploited its catechol function to prepare new series of (*S*)-β^3-H-DOPA[CROWN] amino acids carrying various crown-ether receptors on their side chains. Peptides based on these new crowned β-amino acids combined with (1*S*,2*S*)-ACHC (2-aminocyclohexanecarboxylic acid), known as a potent 3_{14}-helix inducer [1], to the hexamer level, with two crowned residues at the i and i+3 positions of the main chain (a representative example, the hexapeptide Boc-{(1*S*,2*S*)-ACHC-(*S*)-β^3-H-DOPA[21-C-7]-(1*S*,2*S*)-ACHC}$_2$-OMe 6b, is shown in Fig. 1), were synthesized by solution methods. Their conformational analysis was performed using FT-IR absorption, NMR and CD techniques.

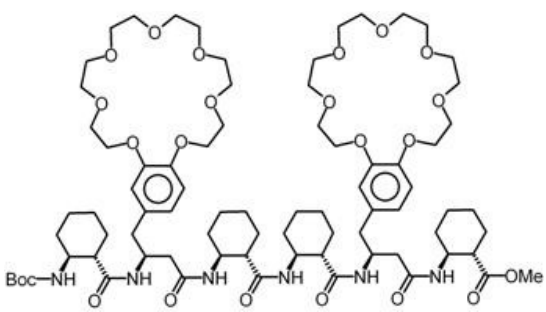

Fig. 1. Chemical structure of the hexapeptide 6b.

Result and Discussion

The catechol function of Boc-(*S*)-β^3-H-DOPA-OMe [3] was used to introduce various side-chain crown ethers by established procedures [2], to afford Boc-(*S*)-β^3-H-DOPA[CROWN]-OMe with [CROWN] = [15-C-5], [18-C-6] (a), [21-C-7] (b),

[Benzo-24-C-8] (c) and [(R)-Binol-20-C-6] (d). The four series of di-, tri-, tetra-, penta- and hexapeptides Boc-(*S*)-β^3-H-DOPA[CROWN]-(1*S*,2*S*)-ACHC-OMe 2a-d, Boc-(1*S*,2*S*)-ACHC-(*S*)-β^3-H-DOPA[CROWN]-(1*S*,2*S*)-ACHC-OMe 3a-d, Boc-{(1*S*,2*S*)-ACHC}$_2$-(*S*)-β^3-H-DOPA[CROWN]-(1*S*,2*S*)-ACHC-OMe 4a-d, Boc-(S)-β^3-H-DOPA [CROWN]-{(1*S*,2*S*)-ACHC}2-(*S*)-β^3-H-DOPA[CROWN]-(1*S*,2*S*)-ACHC-OMe 5a-d, and Boc-{(1*S*,2*S*)-ACHC-(*S*)-β^3-H-DOPA[CROWN]-(1*S*,2*S*)-ACHC}$_2$-OMe 6a-d were prepared in solution.

Fig. 2 shows the FT-IR absorption spectra in the N-H stretching (amide A) region in $CDCl_3$ solution for the representative, selected di- to hexapeptides 2b-6b. Only for the hexapeptide 6b is the low-frequency band at about 3300 cm^{-1} (attributed to H-bonded N-H groups) of much higher intensity than the high-frequency band at about 3430 cm^{-1} (attributed to free, solvated N-H groups). However, a second band (shoulder) present at about 3350-3370 cm^{-1} suggests the occurrence of two different kinds of intramolecular H-bonding.

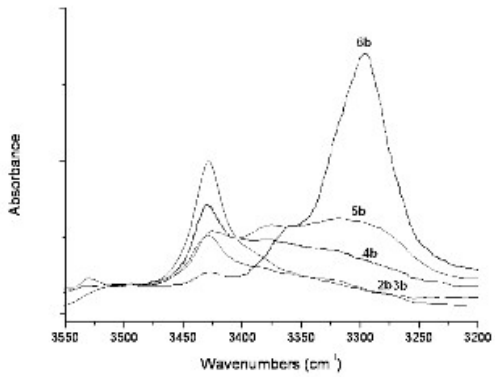

Fig. 2. FT-IR absorption spectra in the N-H stretching region of the 2b-6b series of peptides based on the (S)-β3-H-DOPA[21-C-7] and (1S,2S)-ACHC residues.

From temperature-dependent DMSO titrations of the hexapeptides, it appears that the NH^5 and NH^6 protons are more solvent exposed than the NH^{1-4} protons, as expected for a 3_{14}-helical peptide. The CD spectra of the 2a-6a, 2b-6b and 2c-6c series of peptides tend (for the highest oligomers) to a pattern characterized by an intense negative Cotton effect at about 215 nm followed by a positive Cotton effect centered near 200 nm. This dichroic spectrum is typically exhibited by (*M*)-3_{14}-helical peptide structures.

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SYNTHESIS AND 3D-STRUCTURAL ANALYSIS OF PEPTIDES BASED ON ANTAIB, AN ANTHRACENE-FUSED 1-AMINOCYCLOPENTANECARBOXYLIC ACID

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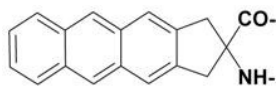
Introduction

Fluorescence spectroscopy has become a highly valuable technique for conformational study of biopolymers, development of peptide-based chemosensors and biochemical research in general [1]. We have designed a new fluorescent amino acid residue, antAib (2-amino-2, 3-dihydro-1*H*-cyclopenta[*b*]anthracene-2-carboxylic acid) (Fig. 1), which is based on a planar anthracene core and belongs to the class of cyclic, $C^{\alpha,\alpha}$-disubstituted glycines (β-turn and helix inducers in peptides) [2]. Peptides based on antAib combined with Ala residues have been synthesized and their conformation analyzed.



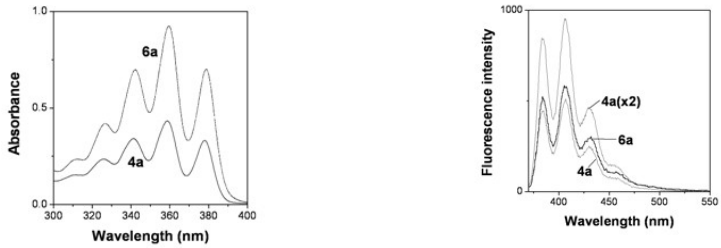
antAib

Fig. 1. Chemical structure of the antAib residue

Result and Discussion

The syntheses of the terminally protected derivatives Boc-antAib-OEt, Boc-antAib-OH, Fmoc-antAib-OtBu, and Fmoc-antAib-OH have been reported [2]. Peptides Boc-antAib-Ala-OMe 2a, X-Ala-antAib-Ala-OMe 3b (X = Fmoc) and 3a (X = Boc), Boc-antAib-$[Ala]_2$-OMe 3'a, X-$[Ala]_2$-antAib-Ala-OMe 4b (X = Fmoc) and 4a (X = Boc), Boc-antAib-$[Ala]_2$-antAib-Ala-OMe 5a, Boc-$[Ala]_3$-antAib-Ala-OMe 5'a, and Boc-antAib-$[Ala]_3$-antAib-Ala-OMe 6a were prepared by solution methods.

UV-Fluorescence analysis: Typical bands related to the anthracenyl chromophore [2] are exhibited by all antAib peptides (Fig. 2). As expected, the UV absorbance of the hexapeptide 6a (with two antAib residues) is double than that of tetrapeptide 4a (with one antAib residue). Evidence for marked autoquenching is provided by the fluorescence spectrum of 6a which is much less intense than that of a twice concentrated solution of 4a. CD analysis: CD bands of remarkable intensities are seen in the near-UV region of the anthracenyl chromophore, but only when there are two antAib residues in the peptide (6a; not shown).



A *B*

Fig. 2. (A) UV absorption spectra of peptides 4a and 6a in MeOH solution. (1mM). (B) Fluorescence spectra (λ_{exc}=358 nm) of peptides 4a and 6a in MeOH solution. Peptide concentrations: 0.001 mM for 4a and 6a, and 0.002 mM for 4a.

1H NMR analysis: The titration of the NH protons of hexapeptide 6a (by adding the perturbing agent DMSO, d6 to the $CDCl_3$ solution) is typical of a 3_{10}-helical peptide (with free NH^1 and NH^2 protons and H-bonded NH^3-to-NH^6 protons) (not shown).
FT-IR absorption analysis: Characteristic FT-IR absorption spectra (3500 - 3200 cm^{-1} region) for a set of 3_{10}-helical peptides are given by compounds 2a-6a (Fig. 3). Peptide self-association was shown to be of minor significance.

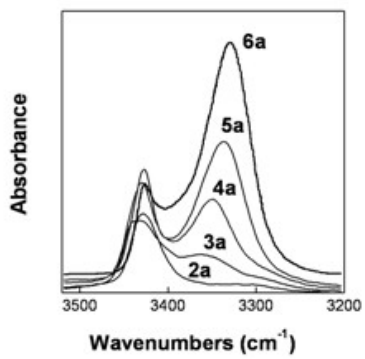


Fig. 3. FT-IR absorption spectra (N-H stretching region) of the peptide series 2a, 3a, 4a, 5a, and 6a in $CDCl_3$ solution. Peptide concentration: 1mM.

In conclusion, the achiral antAib residue exhibits useful UV, fluorescence, and induced CD properties in the interesting spectral region >300 nm. As other C^{α}-tetrasubstituted α-amino acids, including its Ac5c parent compound, antAib, tends to remarkably fold a peptide chain.

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HIGH N-METHYLATION OF PEPTIDES FOR IMPROVEMENT OF ACTIVITY AND ADME PROPERTIES

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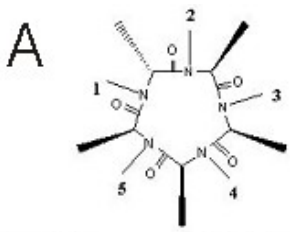
Introduction

Peptides are considered no good drug candidates due to enzymatic cleavage and lack of oral availability. Usually Lipinski's 'rule of five' is used as exclusion criterion in drug research, to have at least a chance of oral uptake [1]. But Cyclosporin violates all the rules and is orally available [2]. Thus we speculated that the oral availability of Cyclosporin probably owes to the high N-methylated peptide bonds. Thus an extensive N-methyl scan of Somatostatin hexapeptide analogue discovered by Veber et al. [3] was carried out. We were also interested in studying the detail conformational preference of the cyclic pentapeptides of general formula cyclo(-D-Ala-L-Ala_4-) imparted by higher N-methylation of the backbone. Extended N-methylation might lead to new conformations which can be employed as a template for the spatial orientation of the side chains and eventually be used for 'spatial screening' [4].



Site of N-methylation	Peptide bond
1	Always *trans*
2	Always *trans*
3	always *cis* except *cyclo*(-aAAAA-) (*trans*)
4	always trans except *cyclo*(-aAAAA-) (*cis*)
5	*cis* when 3 and/or 4 *N*-methylated *trans* when 1 and/or 2 *N*-methylated

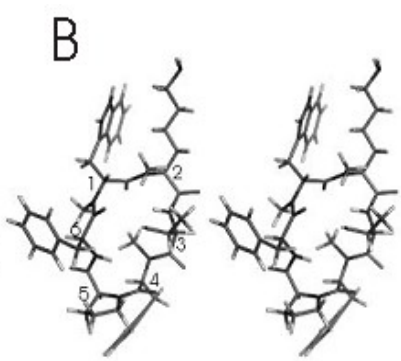


Fig. 1. (A) Correlation between site of N-methylation and conformation of the corresponding peptide bond.(B) Stereoview of 1.

Results and Discussion

The extended N-methylation of the pentapeptide cyclo(-D-Ala-L-Ala_4-), resulted in interesting conformations. We found that the N-methylation is allowed with retention of overall conformation only at selected sites, which results in the outward twist of the β-methyl groups to accommodate the bulky N-methyl group. At certain sites, the N-methylation results in 180° twist of the peptide bond plane and at others the N-methylation is in-tolerated completely and results in cis peptide bond. The detailed effect is depicted in Fig. 1A.

The higher N-methylated analogues of Somatostatin were tested for their binding affinities towards all the cloned receptors sst1-5, and seven out of thirty synthesized peptides revealed nanomolar affinities towards sst2 and sst5. All of these seven peptides were then tested for their oral availability in rats. Out of these seven compounds, cyclo(-MeTrp-MeLys-Thr-MePhe-Pro-Phe-) (**1**) showed significant uptake in the blood stream, with a plasma concentration of 242 ng/mL after 30 min and 151 ng/mL after 1 h, compared to 158 and 38 ng/mL at the same time points for the parent peptide. The detailed conformational analysis of **1** (Fig 1B) by Distance Geometry and Molecular Dynamics, reveals that the orientation of the side chains in the Veber peptide and **1** are similar (Fig 2), which results in the significant binding of the peptide. However, the N-methylation of the Phe^{11} results in the disruption of the βVI turn about Phe^{11}-Pro^{6} and along with the N-methylated D-Trp^{8}, the peptide is unable to adopt a 'folded' [5] conformation about Phe^{7} and Thr^{10}, as the possibility of forming two γ turns are nullified by these two specific N-methylated residues.

Acknowledgments

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PROTEASE-CATALYZED SEGMENT CONDENSATIONS USING CARBAMOYLMETHYL ESTERS AS ACYL DONORS

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Introduction

We have demonstrated the superiority of the carbamoylmethyl (Cam) ester as an acyl donor in the kinetically controlled peptide-bond formation mediated by α-chymotrypsin [1] and some microbial serine proteases [2, 3]. Thus, major drawbacks associated with protease-catalyzed peptide synthesis, *e.g.,* a narrow substrate specificity and the secondary hydrolysis of a growing peptide, can be overcome by employing this particular ester. We have also investigated the utilization of the Cam ester for the peptide synthesis catalyzed by a sulfhydryl protease, papain [4]. The couplings of a series of *N*-Z-amino acid Cam esters with a series of amino acid amides were examined in acetonitrile with low water content, the coupling efficiencies being dependent on the combination of amino acid residues. Some 2+1 segment condensations were also tried by this procedure. In continuation of our study, we report the papain-catalyzed synthesis of partial sequences of bioactive peptides through segment condensation employing Cam esters as acyl donors.

Results and Discussion

Employing the immobilized papain prepared from pH 8.0 phosphate buffer [4], the synthesis of partial sequences of some bioactive peptides was examined: substance P (7–11)-pentapeptide (-Phe-Phe-Gly-Leu-Met-), eledoisin (6–11)-hexapeptide (-Ala-Phe-Ile-Gly-Leu-Met-) and dermorphin (1–4)-tetrapeptide (-Tyr-D-Ala-Phe-Gly-). In the synthesis of substance P (7–11)-pentapeptide derivative, both 2 + 3 and 4 + 1 segment condensations were tried. The racemization-free authentic peptide was prepared by the EDC-HOBt-$CuCl_2$ method [5], while a mixture of epimeric peptides was prepared through the same segment condensation by the EDC method. The quantification of the aimed-at peptide, its epimer and the hydrolysis product of the carboxyl component were done by reversed-phase HPLC analysis. In both the segment condensations the desired peptide was obtained in ca. 80% yield (Table 1, Entries 1 and 2). Although the concomitant formation of a small amount of the hydrolysis product of the donor esters was inevitable, no epimeric peptides were detected on HPLC. The preparation of eledoisin (6–11)-hexapeptide derivative was examined through a 3 + 3 segment condensation (Entry 3). The desired peptide was obtained in ca. 90% yield. The synthesis of dermorphin (1–4)-tetrapeptide derivative, which contains a D-amino acid residue in the sequence, was examined through a 3 + 1 segment condensation (Entry 4). The

peptide yield was not high (57%) in this case, but no racemization of the Phe residue at the P1 position was detected.

In summary, the present results indicate the usefulness of the Cam ester as the acyl donor in the papain-catalyzed kinetically controlled segment condensations.

Table 1. Papain-catalyzed segment condensations (after 48 h).

Entry	Carboxyl component	Amino component	Peptide (%)
1	Boc-Phe-Phe-OCam	Gly-Leu-Met-NH_2	82.8
2	Boc-Phe-Phe-Gly-Leu-OCam	Met-NH_2	78.3
3	Boc-Ala-Phe-Ile -OCam	Gly-Leu-Met-NH_2	90.3
4	Boc-Tyr-D-Ala-Phe-OCam	Gl-NH_2	57.2

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DIRECTIONAL ELECTRON TRANSFER IN CONFORMATIONALLY CONSTRAINED, 3_{10}-HELICAL, OLIGOPEPTIDES

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Introduction

Directional control of electron transfer (ET) processes at the molecular level are precise requirements for the realization of electronic nanodevices. Peptide scaffolds functionalized by ET donor-acceptor (D/A) pairs show peculiar electron conduction properties, depending on the secondary structure and side-chain arrangements. More specifically, the electric macrodipole associated to helical peptides has been shown to generate an electrostatic field oriented from the N- (δ+) to the C-terminus (δ-), that remarkably affects the efficiency of the intramolecular ET process.

To further investigate this effect, we synthesized the following Nα-protected octapeptide amides:

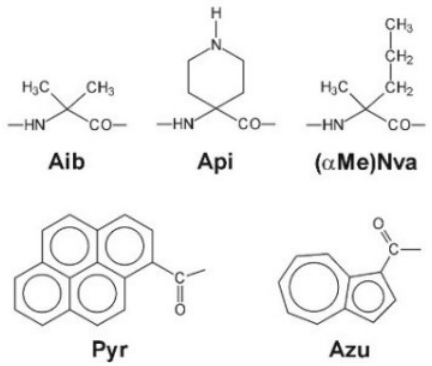
Z-Aib-Api(Pyr)-L-(αMe)Nva-Aib-[L-(αMe)Nva]$_2$-Aib-Api(Boc)-NH*t*Bu (P2)

Z-Aib-Api(Pyr)-L-(αMe)Nva-Aib-[L-(αMe)Nva]$_2$-Aib-Api(Azu)-NH*t*Bu (P2A8)

Z-Aib-Api(Azu)-L-(αMe)Nva-Aib-[L-(αMe)Nva]$_2$-Aib-Api(Pyr)-NH*t*Bu (A2P8)

[Aib = α-aminoisobutyric acid; Api = 4-aminopiperidine-4-carboxylic acid; (αMe)Nva: C^{α}-methylnorvaline; Pyr = 1-pyrenyl-carbonyl; Azu = 1-azulenyl-carbonyl].

P2A8 and A2P8 differ by the position of the donor (Pyr) and acceptor (Azu) pair in the chain. The two peptides, upon photoexcitation of the electron donor group, are expected to give rise to a charge-separated species, the polarity of which is oriented along the same direction (P2A8) or opposite to the electrostatic field direction (A2P8). P2 was synthesized as reference compound for photophysical studies.



Result and Discussion

FTIR absorption, NMR and CD studies indicate that in solution the peptides investigated overwhelmingly populate a 3_{10}-helical conformation, as expected from a sequence based exclusively on C^{α}-tetrasubstituted α-amino acids. Steady-state and time-resolved fluorescence experiments show that an excited state interaction between the excited Pyr chromophore and the Azu group actually takes place. In particular, the A2P8 time decay in acetonitrile (<kET>= $1.4x10^8$ s^{-1}) is definitely faster than that of P2A8 (<kET>= $6.7x10^7$ s^{-1}), as clearly shown by the time-resolved fluorescence experiments reported in Fig. 1. The reported rate constants are weighted averages over all of the populated conformers. This result confirms the 'helix dipole' effect on the efficiency of the ET process [1, 2] that stabilizes preferentially the charge-transfer state of opposite polarity, i.e. Azu(δ-)--Pyr(δ+). Experiments in DMSO support this conclusion. In this solvent the measured quenching rate constants are $6.3x10^7$ s^{-1} for P2A8 and $1.2x10^8$ s^{-1} for A2P8, suggesting that such a strongly interacting solvent does not affect the conformational equilibria in solution of these rigid peptides.

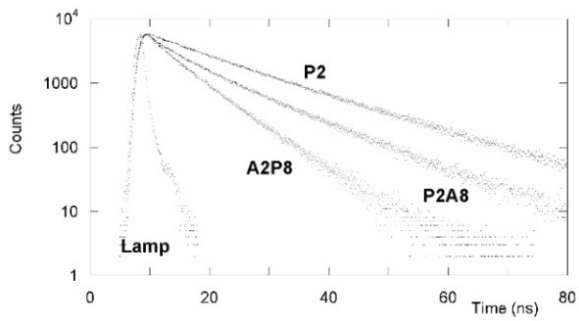


Fig. 1. Fluorescence time decay of P2, A2P8 and P2A8 in acetonitrile (λ_{exc}=341 nm; λ_{em}=410 nm).

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ENGINEERING BIOINSPIRED LUMINESCENT PROBES: ANTIMICROBIAL PEPTIDES CHELATING LANTHANIDE IONS

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Introduction

Lanthanide chelates are currently actively explored for potential applications in medical diagnostics, drug discovery, bioimaging and bioanalytical assays. On the other hand, oligopeptides are extremely interesting candidates as novel, biocompatible, smart materials, due to their ability to fold into specific structures, to store and transfer energy or electrons, to bind to membranes or proteins, and to transport ions and small organic molecules.

We are currently studying the ion binding properties of fluorescent analogues of trichogin GA IV, a natural peptide showing interesting antimicrobial activity. The primary structure of this peptaibol, denoted in the following as **F0**, is

Fmoc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe

where Aib is α-aminoisobutyric acid and Fmoc is fluoren-9-ylmethyloxycarbonyl, a well-known fluorescent group. Fluorescence and circular dichroism (CD) spectroscopies have been applied to investigate the **F0** binding properties with Gd(III) and Tb(III).

Results and Discussion

Recently, we have shown by spectroscopy and molecular mechanics studies that in solution trichogin GA IV analogues populate few ordered conformations, all characterized by a high content of helical structures and differing by the local arrangement of the Gly^5-Gly^6 central doublet [1]. CD titration experiments with Gd(III) and Tb(III) revealed that, upon ion binding, **F0** undergoes a conformational transition shifting the equilibrium from helical to helix-turn-helix structures. Notably, **F0** does not bind K^+, highlighting the selectivity of the peptide-ion interaction.

Interesting results were obtained by fluorescence measurements. It has been observed that upon ion binding the fluorescence of Tb(III) markedly increased, as clearly shown by the experiments reported in Fig. 1. The increase in the fluorescence intensity of Tb(III) upon complex formation can be ascribed to two concomitant factors: i) the release of water molecules from the coordination shell of the peptide and ii) an energy transfer process from the Fmoc excited state to Tb(III). The occurrence of a Förster energy transfer mechanism is suggested by the spectral

overlap between the emission of the donor molecule (Fmoc) and the absorption of the acceptor species [Tb(III)]. Excitation spectra carried out at the acceptor emission wavelength confirmed the transfer of excitation energy in the Fmoc→Tb(III) direction. From these measurements the Förster critical distance (R_0), i.e. the distance at which the energy transfer efficiency is 50%, can be easily obtained. For the peptide investigated here we found R_0=9.8 Å, suggesting that Tb(III) might bind in the vicinity of the Gly-Gly doublet.

These results confirm the ability of trichogin GA IV analogues to bind selectively lanthanide ions, suggesting the possibility to design a biocompatible, peptide-based, nanosensor supporting fluorescent [Tb(III)] and magnetic [Gd(III)] probes for simultaneous two-probes bioimaging.

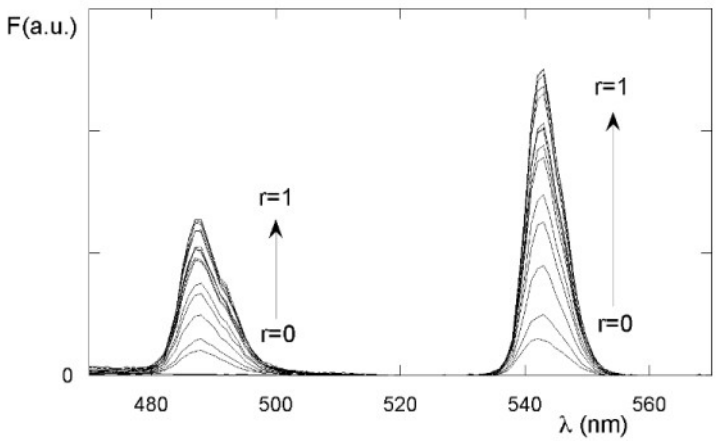

*Fig. 1. Enhancement of Tb(III) emission in acetonitrile by addition of **F0**. The peptide/ion ratio (r) varies from 0 to 1.*

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PHOTOCURRENT GENERATION BY SELF-ASSEMBLED PEPTIDE MONOLAYERS ON INTERDIGITATED GOLD MICROELECTRODES

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Introduction

Peptide self-assembled monolayers (SAMs) have shown exciting electron conduction properties in terms of long-range and directional electron transfer (ET) [1]. We have recently demonstrated that an hexapeptide, denoted in the following as SSA4WA, forms a densely-packed SAM on a gold surface with interesting growth dynamics and mobility properties [2]. The primary structure of SSA4WA, Lipo-$(Aib)_4$-Trp-Aib-O*t*Bu, comprises five helicogenic α-aminoisobutyric acid (Aib) residues and one tryptophan (Trp) residue, a fluorescent amino acid strongly absorbing in the near-UV region. The great Au affinity of the disulfide functionality of the N-terminal lipoyl (Lipo) group allows a covalent linking of the peptide to gold. Owing to the high content of Aib residues, the peptide adopts a rigid, helical, conformation despite the shortness of its main chain.

SSA_4WA was covalently linked to an interdigitated gold electrode (IDE) to study the photocurrent generated upon UV irradiation, thus measuring charge transport under steady-state conditions with much higher sensitivity than standard gold electrodes.

Results and Discussion

Cyclic voltammetry (CV) experiments confirmed that SSA4WA forms densely packed SAMs on the IDE. Photocurrent experiments in the Trp absorption region (irradiation at 280 and 270 nm) were performed on the peptide modified IDE using triethanolamine (TEOA) as the electron donor. The measured photocurrent intensity follows reversibly the on/off photoexcitation cycles without apparent degradation of the anodic current. Photocurrent measurements under the same experimental conditions on an IDE modified by an undecanethiol SAM were also performed as a reference (photocurrent generation was not observed in this case). When the peptide layer is irradiated in the UV range between 260 nm and 320 nm, the Trp singlet excited state gives rise to an ET process to the the gold electrode (Trp*→Au). Subsequently, TEOA transfers an electron to the $Trp^{+\bullet}$ radical cation, giving rise to a net, anodic, electronic current. Photocurrent intensity vs. applied potential voltage experiments were also carried out near the maximum absorption wavelength (λ=280 nm) of the Trp residue. For the peptide modified IDE a decrease in the anodic

photocurrent with an increase of the negative bias to the working electrode was observed, measuring a null photocurrent at a given, characteristic potential (zero-current potential). Very likely, the negative bias reduces the energy gap between the oxidation potential of the excited Trp group and the Au Fermi level, resulting in the decrease of the anodic photocurrent. It should be stressed that peptide SAM packing and homogeneity are not perturbed by photoirradiation, as demonstrated by CV measurements on the same sample before and after several cycles of photocurrent experiments.

These results emphasize the good electron mediating properties of a helical peptide spacer, -$(Aib)_4$-, and the role of an aromatic (indole) group in the photoinduced ET process, suggesting the possibility to develop bio-hybrid molecular devices based on the integration of photoactive peptide layers chemisorbed on conductive or semi-conductive supports.

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SYNTHESIS AND 3D-STRUCTURAL ANALYSIS OF PEPTIDES BASED ON C^{α}-TETRASUBSTITUTED α-AMINO ACIDS WITH BINAPHTHYL-CROWNED SIDE CHAINS

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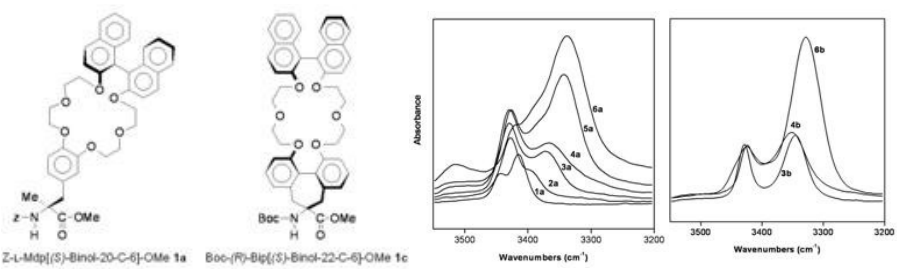
Introduction

Crowned Cα-tetrasubstituted α-amino acids with well-defined stereochemical properties are interesting targets, allowing control of the spatial organization of the crown-ether receptors in short-chain peptide structures for the construction of new molecular receptors and supramolecular devices using peptide frameworks [1]. We have shown previously that peptides based on L-Mdp[CROWN] residues derived from C^{α}-methyl-L-DOPA (Mdp) with [CROWN] = [15-C-5], [18-C-6] and [Benzo-24-C-8], have a strong propensity for folded/3_{10}-helical secondary structures [2]. In the same manner, peptides based on the more rigid, cyclic, Bip[20-C-6] residue have been investigated in our groups [3]. We envisioned that introduction of a binaphthyl unit as a part of the [CROWN] moiety would impart supplementary structural chiral recognition properties to these receptors [4]. We report here the synthesis of the terminally protected amino acids Z-L-Mdp[(*S*)-Binol-20-C-6]-OMe



(A) *(B)* *(C)*

Fig. 1. (A) Chemical structures of the binaphthyl-crowned amino acid residues Z-L-Mdp[(S)-Binol-20-C-6]-OMe ***1a*** *and Boc-(R)-Bip[(S)-Binol-22-C-6]-OMe* ***1c*** *(B) FT-IR absorption spectra in the N-H stretching region of the* ***1a-6a*** *and (C)* ***3b-6b*** *peptide series in $CDCl_3$ solution. Peptide concentration 1 mM.*

and Boc-(*R*)-Bip[(*S*)-Binol-22-C-6]-OMe (Fig. 1A), and the 3D-structural analysis of designed peptides to the hexamer level by FT-IR absorption and ^{1}H NMR techniques (Fig. 1B , 1C).

Results and Discussion

The catechol function of Z-L-Mdp-OMe was used to introduce the (S)-binaphthyl-crowned side chain, using Cs_2CO_3 as base and (-)-(*S*)-2,2'-bis(5-tosyloxy-3-oxa-1-pentyloxy)-1,1'-binaphthyl as alkylating agent to afford the desired Z-L-Mdp[(*S*)-Binol-20-C-6]-OMe **1a** (Fig. 1A). The same treatment applied to racemic Boc-(*R,S*)-Bip[OH]$_2$-OMe [3] afforded a mixture of Boc-(*R*)-Bip[(*S*)-Binol-22-C-6]-OMe **1c** and its isomer Boc-(*S*)-Bip[(*S*)-Binol-22-C-6]-OMe, which could be separated by chromatography. Four series of peptides based on these new amino acids, combined with Aib and/or Ala residues to the hexamer level, with two crowned residues at the i and i+3 positions of the main chain, Z-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **2a**, Fmoc-LAla-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **3a**, Z-{L-Ala}$_2$-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **4a**, Z-L-Mdp[(S)-Binol-20-C-6]-{L-Ala}$_2$-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **5a**, Fmoc-{L-Ala-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala}$_2$-OMe **6a**, Boc-Aib-L-Mdp[(*S*)-Binol-20-C-6]-OH **2b**, Boc-Aib-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **3b**, Fmoc-L-Ala-Aib-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala-OMe **4b**, Boc-{Aib-L-Mdp[(*S*)-Binol-20-C-6]-L-Ala}$_2$-OMe **6b**, Boc-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **2c**, Fmoc-L-Ala-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **3c**, Boc-{L-Ala}$_2$-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **4c**, Boc-(*R*)-Bip[(*S*)-Binol-22-C-6]-{L-Ala}$_2$-(*R*)-Bip[(S)-Binol-22-C-6]-L-Ala-OMe **5c**, Fmoc-{L-Ala-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala}$_2$-OMe **6c**, Boc-Aib-(*R*)-Bip[(*S*)-Binol-22-C-6]-OH **2d**, Boc-Aib-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **3d**, Fmoc-L-Ala-Aib-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **4d**, Boc-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-Aib-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala-OMe **5d**, and Boc-{Aib-(*R*)-Bip[(*S*)-Binol-22-C-6]-L-Ala}$_2$-OMe **6d**, were prepared in solution by stepwise and/or segment coupling(s).

The results of our FT-IR absorption analysis (for the series a and b, see Fig. 1A and Fig. 1B) indicate that the longest peptides of the various series are folded in highly intramolecularly H-bonded helical structures. The delineation of inaccessible (intramolecularly H bonded) NH groups of the hexapeptide 6b, carried out by evaluation of DMSO dependency of NH chemical shifts in ^{1}H NMR (not shown), confirmed our contention that these peptides preferentially adopt β-turn and 3_{10}-helix secondary structures.

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CYSTEINE PROTEASE-CATALYZED PEPTIDE SYNTHESIS

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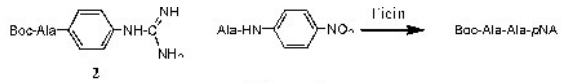
Introduction

Peptide synthesis using protease-catalyzed reverse reaction has been extensively studied with a variety of amino acids and peptide derivatives as coupling components [1 - 4]. It has been reported that the protease-catalyzed peptide synthesis is superior compared to the chemical coupling method due to the requirement of less side chain protection. The major drawback of the enzymatic method, however, is the respective substrate specificity. Among several enzymes already known, the cysteine protease ficin has proven to be a versatile low-cost biocatalyst. Recently, Hänsler et al. reported that with kinetically-controlled peptide synthesis in frozen aqueous solutions using Bz-Arg-OEt as acyl donor component it was possible to obtain satisfactory results [5].

Results and Discussion

Ficin-catalyzed coupling reaction of *tert*-butyloxycarbonyl-alanine *p*-guanidinophenyl ester (Boc-Ala-O*p*Gu) (2) and Ala-*p*NA to give Boc-Ala-Ala-*p*NA was examined (Scheme 1). The reaction was evaluated by changing the conditions such as the co-solvents (DMSO, DMF and acetonitrile), the pH of the medium and the concentration of the acyl acceptor (Ala-*p*NA). The best yield was obtained when the reaction was carried out using 1 mM of acyl donor (2), 35 mM of acyl acceptor (Ala-*p*NA) and 0.1U of ficin in 40% DMSO-GTA (50 mM, pH 9.0) at 37ºC.

A comparative study of cysteine protease and serine proteases (bovine trypsin, chum salmon trypsin and bovine thrombin) as the catalyst of peptide coupling led us to the conclusion that all substrates (1 - 8) (Fig. 1) behave as a good acyl donor. The coupling yields by cysteine protease (75 – 96%) are somewhat more significant compared with those of the serine proteases. The coupling time of cysteine protease is considerably faster than that of the serine protease.



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Scheme 1

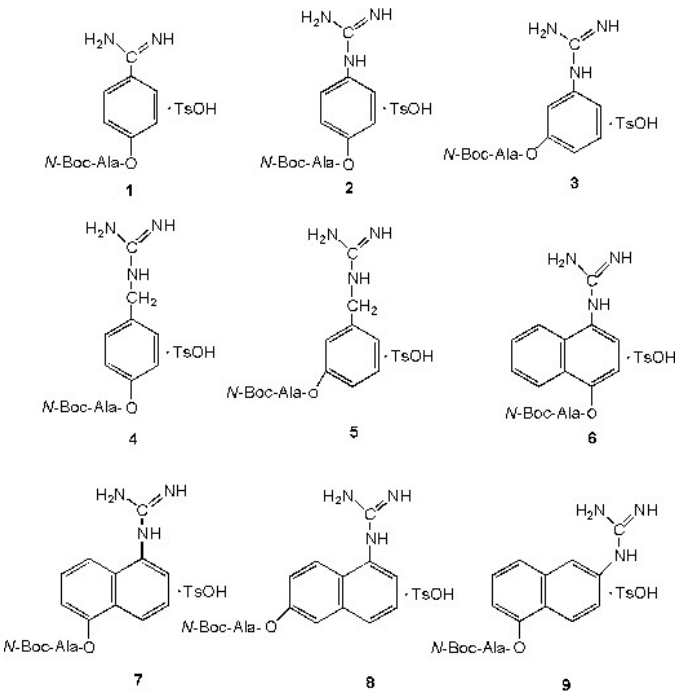

Fig1. Structure of Substrate

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SYNERGY BETWEEN ANTIBACTERIAL PEPTIDES AND ANTIBIOTICS

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Introduction

The short, proline-rich peptide family kills bacteria by selectively binding the 70 kDa bacterial heat shock protein (Hsp) DnaK and inhibiting protein folding [1]. Chimeric and combinatorial analogs exhibit 8-32 μg/mL MIC figures against a series of Enterobactericeae pathogens. The best peptide, A3-APO, kills β-lactam, trimethoprim, sulfamethoxazole, aminoglycoside and fluoroquinolone resistant Gram-negative strains [2]. β-Lactamases can be inactivated by compounds that bind the active site of these enzymes [3]. Since the proline-rich antibacterial peptides were shown to inactivate test bacterial enzymes, our idea was to use these peptides to inhibit all therapeutically important bacterial enzyme activity.

Results

We studied how sequential or simultaneous addition of A3-APO and amoxicillin to clinical Escherichia coli cultures, SEQ102 and BF1023, expressing the TEM-1 β-lactamase, influences the MIC (4). The control antibiotic was the aminoglycoside kanamycin as the main resistance mechanism proceeds through mutations in chromosomal genes and the pathogen was the kanamycin-resistant strain E. coli S5081. Ciprofloxacin and E. coli 045-849 were also included in the studies because fluoroquinolones induce DnaK production in *E. coli*. We wondered whether inactivation of the overexpressed Hsp would lead to increased sensitivity to ciprofloxacin.

Two concentrations of the peptide were used: one below the MIC and one just below the IC_{50} to ensure that the expected activity is dose dependent. After 1 h the antibiotics were added and growth inhibition was measured. Amoxicillin alone was inactive against *E. coli* BF1023 or SEQ102 but its activity could be fully recovered when the culture was pre-incubated with 8-16 μg/mL A3-APO. Synergy was not observed when the peptide and the small molecule antibiotic were added simultaneously indicating that the β-lactamase activity was manifested before the enzyme's folding could be inhibited. Likewise, no synergistic effect was observed at lower peptide concentrations. The experimental results were completely different with kanamycin and *E. coli* S5081 where no activity of the small molecule antibiotic could be recovered in any circumstance. Finally, results with ciprofloxacin and *E. coli* 045-849 indicated a trend that fell between β-lactam and aminoglycoside antibiotics. Preincubation of bacteria with 8 μg/mL peptide A3-APO in the synergy lane did result in lower absorbance readings than those

observed after ciprofloxacin or A3-APO administration alone but the effect was not seen with co-administration of the two antibiotics.

Discussion

Lowered peptide concentration did not result in dramatic recovery of ciprofloxacin activity, although some synergy was seen with or without preincubation, suggesting that the peptide derivative did potentate ciprofloxacin by eliminating a fraction of the active DnaK population that was induced by the fluoroquinolone. The mode of action of the synergy with β-lactams is likely inhibition of protein folding. As opposed to current β-lactamase inhibitors and combination therapies that work against only a limited number of strains, inhibition of all protein folding in bacteria is a universally applicable treatment option. Elimination of resistance to β-lactams by proline-rich peptide derivatives may give renewed life to these antibiotics for which large stockpiles are currently available.

Acknowledgements

The authors wish to thank Dr. Mare Cudic for helpful discussions.

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STRUCTURE-FUNCTION RELATIONSHIP OF INSULIN-LIKE PEPTIDE 3, A REGULATOR OF GERM CELL MATURATION

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Insulin-like peptide 3 (INSL3) is a member of the relaxin peptide family which is produced in testicular Leydig cells and ovarian thecal cells [1]. It comprises two peptide chains – A and B – that are held together in a characteristic insulin-like disposition. Gene knockout experiments have identified a key biological role in initiating testes descent during foetal development [2]. This action is mediated via a stimulation of gubunacular bulb swelling. This action is elicited via its recently-identified receptor, LGR8, a member of the leucine-rich repeat-containing G-protein coupled receptor family [3]. More recent studies have shown that in both male and female gonads, INSL3 and LGR8 represent a paracrine system important for meiosis induction in the ovary and male germ cell survival in the testis [4]. Thus INSL3 may have key clinical applications in fertility management. To identify the structural features that are responsible for the interaction of INSL3 with its receptor and to assist in the development of agonists and antagonists, a series of B-chain alanine substituted analogs were prepared and subjected to detailed *in vitro* receptor binding and activation assays. The results were analyzed in the light of the recently acquired NMR solution structure of INSL3.

The two chains of INSL3 analogs were prepared separately by conventional continuous flow Fmoc-solid phase synthesis. After cleavage and deprotection, these were subjected to subsequent regioselective disulfide bond formation [5]. The crude peptides were purified by RP-HPLC. The B-chain Ala-substituted INSL3 analogs that were prepared were: B^{12}, B^{16}, B^{19}, $B^{12,16}$, $B^{16,20}$, $B^{12,16,20}$ and $B^{12,16,19,20,27}$. The purity of each synthetic peptide was assessed by analytical RP-HPLC and MALDI TOF MS.

The peptides were assessed for both LGR8 binding and activation ability. The data showed clearly that five key residues within the B-chain of INSL3 are responsible for the interaction with the ectodomain of LGR8 [6]. These include Arg^{B16} and Val^{B19}, with His^{B12} and Arg^{B20} playing a secondary role, as evident from the synergistic effect on the activity in double and triple mutants involving these residues. Together, these amino acids combine with the previously identified critical residue, Trp^{B27} [7], to form the receptor binding surface. Correlation of this

information with the recently acquired solution structure of human INSL3 [6] showed that these residues form a principally continuous binding region within one face of the central B-chain α-helix. Work is in progress to exploit these findings in the design of mimetics of INSL3 with agonist and antagonists properties.

Acknowledgements

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SOLID-PHASE PEPTIDE SYNTHESIS USING NANOPARTICULATE AMINO ACIDS IN WATER

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Introduction

Solid-phase synthesis has been developed and automated in the field of peptide synthesis, and is currently being applied to general organic synthesis along with the spread of combinatorial chemistry. However this procedure requires a large amount of organic solvents. Because the safe organic solvent waste disposal is an important environmental issure, a method for peptide synthesis in water using low toxic reagents would be desirable. We developed solid phase peptide synthesis in water using water-soluble protected amino acids.[1, 2, 3] Here we develop a new technology for solid phase peptide synthesis using nanoparticulate amino acids in water.

Result and Discussion

9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids are poorly soluble in water, precluding their use for solid-phase synthesis in water. Grinding particles to nanosize can lead to increased specific surface area and homogenous mixing of multiple components. We therefore predicted that conversion of Fmoc-amino acids into homogenously water-dispersible nanoparticles would enable increased specific surface area and homogenous mixing with the resin in water, resulting in smooth progress of solid-phase reaction in water according to the Fmoc approach.

First, we selected Fmoc-Phe-OH, which has an aromatic ring in the side chain and is highly hydrophobic, to convert into water-dispersible nanoparticles. A dispersion additive poly(ethylene glycol) [PEG (mean molecular weight: 4,000)] was added to Fmoc-Phe-OH and the mixture was ground for 2 hours at room temperature in a planetary ball mill containing water with zirconia beads. Formulation consisted of 2.0 mmol of Fmoc-Phe-OH, 400 mg of PEG, 20 mL of water, and 80 g of zirconia beads. The size of the resulting water-dispersible nanoparticles was determined by a dynamic light scattering analysis (DLS) to be 265 ± 10 nm. A scanning electron microscope (SEM) image also revealed nanosize particles (Fig. 1B). Other Fmoc-amino acid nanosuspensions were prepared in the same way described above.

To evaluate nanoparticulate amino acids, Leu-enkephalin amide was synthesized on PEG-grafted Rink amide resin by the solid phase method in water. A water-soluble carbodiimide [WSCD, 1-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride] was used as a coupling reagent in the presence of *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB) and *N*,*N*-diisopropylethylamine (DIEA). Deprotection of Fmoc group was carried out with 0.1 M NaOH in 90% ethanol since fluorene compound derived from deprotection of Fmoc group was sparingly

soluble in water. Synthetic H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-PEG-grafted Rink amide resin was treated with trifluoroacetic acid and the resulting crude Leu-enkephalin amide was purified by HPLC. The overall yield calculated from the amino group content of the used resin was 67%. The present study proposes a solution to address the poor solubility of building blocks and offers a new solid-phase synthesis method using the "environmentally friendly solvent water". Reaction characteristics for nanosized building blocks will be explored to trigger a new methodology for synthetic reactions.

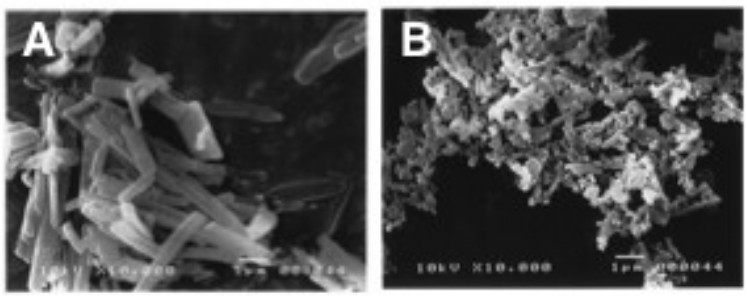

Fig. 1. (A) An SEM image of unprocessed Fmoc-Phe-OH, (B) An SEM image of water-dispersible nanoparticulate Fmoc-Phe-OH.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF CXCR4 ANTAGONISTS AS PHARMACEUTICAL AGENTS

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Introduction

A chemokine receptor CXCR4 has multiple critical functions in normal and pathologic physiology. CXCR4 is a GPCR that transduces signals of its endogenous ligand, CXCL12 (stromal cell-derived factor-1, SDF-1). The CXCL12-CXCR4 axis plays an important role in the migration of progenitors during embryologic development of the cardiovascular, hemopoietic, central nervous systems and so on. This axis has recently been proven to be involved in several problematic diseases, including HIV infection, cancer cell metastasis, leukemia cell progression, rheumatoid arthritis (RA) and pulmonary fibrosis. Thus, CXCR4 is a great therapeutic target to overcome the above diseases. Fourteen-mer peptides, T140 and its analogs, were previously found to be specific CXCR4 antagonists that were identified as HIV-entry inhibitors, anti-cancer-metastatic agents, anti-chronic lymphocytic/acute lymphoblastic leukemia agents and anti-RA agents [1]. Cyclic pentapeptides, such as FC131 [cyclo(D-Tyr-Arg-Arg-L-3-(2-naphthyl)alanine-Gly)], were previously found as CXCR4 antagonist leads based on pharmacophores of T140. Here is described the development of low molecular weight CXCR4 antagonists involving FC131 analogs and other compounds with different scaffolds including leaner-type structures.

Results

Several FC131 analogs, which have substitution for Arg2, Nal3 and D-Tyr5, were prepared and assessed for CXCR4-binding activity based on inhibitory activity against CXCL12 binding to CXCR4. Recently, a novel pharmacophore of T140-related CXCR4 antagonists, such as a 4-fluorophenyl moiety, was found in addition to the original pharmacophores of T140, Arg (x 2), Nal and Tyr. Since the phenol group of D-Tyr5 could not be replaced by the 4-fluorophenyl group with maintenance of high activity, as seen in the D-Phe(4-F)-substituted analog, we attempted to incorporate the 4-fluorophenyl group into the amino acid at position 1. [D-Phe(4-F)1, Arg5]-FC131 (FC602) showed potent activity, which is 10-fold more potent than that of [D-Tyr1, Arg5]-FC131 [2].

Next, a linear type of several low molecular weight CXCR4 antagonists were developed based on T140 analogs. Several compounds that were synthesized based on pharmacophores of T140 analogs showed significant anti-HIV activity and binding affinity for CXCR4. According to these results, two types of libraries based on the N-terminal region of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 were constructed to find effective lead compounds. Linear-type low molecular weight compounds obtained in this study are thought to be useful leads [3].

Previously, anthracene derivatives having two sets of zinc(II)-2,2'-dipicolylamine (Dpa) complex were identified as the first chemosensors that can selectively bind and sense phosphorylated peptide surfaces [4]. Several low molecular weight nonpeptide compounds having the dipicolylamine zinc(II) complex structure were identified as potent and selective antagonists of the chemokine receptor CXCR4 (Fig. 1). These compounds showed strong inhibitory activity against CXCL12 binding to CXCR4 [5].

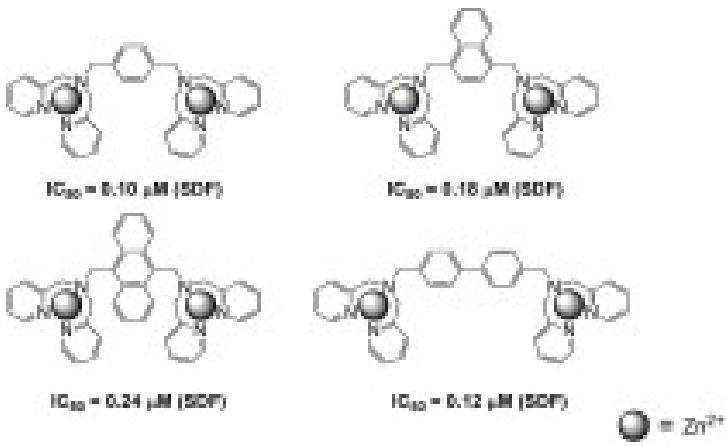

Fig. 1. A new class of CXCR4 antagonists having the dipicolylamine-zinc(II) complex structure

Since CXCR4 is involved in several problematic diseases, low molecular weight CXCR4 antagonists, involving zinc(II)-dipicolylamine unit-containing compounds, proved to be useful and attractive lead compounds for chemotherapy of these diseases.

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PURIFICATION OF SPARINGLY SOLUBLE PEPTIDES BY TEMPORARY SOLUBILIZATION

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Due to aggregation, even short peptides may be sparingly soluble in aqueous solvent systems impeding proper purification by RP-HPLC. For temporary solubilization, Englebretsen *et al*. developed "solubilizing tails" for the Boc-SPPS of poorly soluble peptides [1]. These specialized protecting groups are finally removed with aqueous base.

Solubilizing tails attached to the N-terminus may be introduced conveniently following SPPS. Based on an N^{α}-protecting group described by Tesser *et al*. [2] we developed an N-terminal solubilizing tail cleavable under milder basic conditions precluding damage to the purified peptide. To test this type of modification, the synthesis of the sparingly soluble HCV NS5B protein (407-419), SMSYTWTGALITP was performed.

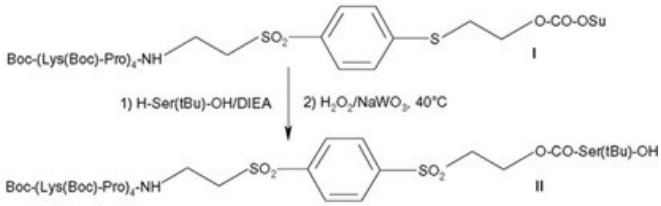
Peptides may also be temporarily solubilized by generating the isomeric depsipeptides (i.e., *O*-acyl isopeptidic bonds to Ser or Thr) [3] which rearrange to the desired peptide in the presence of bases. For comparison, this modification was also investigated in our model sequence. The results of both approaches will be discussed in this presentation.

Solubilizing Tail

Succinimidyl carbonate **I** was reacted with N-terminal H-Ser(*t*Bu)-OH followed by oxidation to obtain **II**, which was coupled to the peptide resin. I and II are very well soluble in most organic solvents. The protected tail Boc-(Lys(Boc)-Pro)$_4$-NH-CH_2-CH_2-SO_2-C_6H_4-SO_2-CH_2-O-CO- is smoothly removed by 20% piperidine/DMF. Nevertheless, **II** tolerates slightly basic conditions. Hence, it is compatible with routine coupling conditions applied during SPPS. Unfortunately, the tailed derivatives of Met and Cys cannot be obtained by this method due to their sensitivity during the oxidation step.

The outlined strategy was followed, since an activated derivative of the tail could not be obtained due to extreme base lability.

II (2 eq) was coupled to H-MSYTWTGALITP-ClTrt-® using TATU/collidine in DMF to produce the test sequence. A pure product (HPLC: 96%) was obtained from the crude water-soluble "tailed" peptide (HPLC:47%) by standard RP-HPLC followed by rapid cleavage of the tail with 2% aqueous piperidine. The desired peptide precipitated and the solubilizing tail-piperidine adduct remained in solution.



O-acyl Isopeptide

The motif Trp^{412}-Thr^{413} was chosen for generating the isopeptide link in the model peptide. The highly active TFFH [4], which mediates the acylation of alcohols in the presence of DMAP [5], turned out to be the most efficient coupling reagent evaluated in this study. The esterification of Fmoc-Trp(Boc)-OH to the β-hydroxyl moiety of terminal Boc-Thr^{413} applying TFFH/DIEA and 0.2 eq of DMAP (15h, at room temperature) resulted in a moderate yield, however in an excellent optical purity. The remaining hydroxyl groups were blocked by acetylation. Elongation of the peptide followed by acidolytic cleavage led to the isopeptide H-T(WTYSMS-H)GALIPT-OH. The soluble crude product (containing capped peptide (26%, HPLC) and traces of the D-epimer) was purified by RP-HPLC followed by ion exchange. In the presence of NH_3 at pH 8, the resulting isopeptide acetate readily rearranged to the native peptide.

Discussion

The sparingly soluble model peptide HCV NS5B (407-419) was readily purified by RP-HPLC following either tagging or conversion to the isopeptide. Both methods permit the specific regeneration of the target product. Albeit more laborious, the incorporation of the N^{α}-solubilizing tail *via* the linker to the N-terminal amino acid is more generally applicable and better scalable. For increasing its solubilizing efficiency, the tail may be elongated or modified. The isopeptide approach is more straightforward, but the necessity of Ser- or Thr- residues in the appropriate position limits this method. For a more efficient introduction of an isopeptide-modification, the synthesis of the corresponding buiding blocks is suggested [6].

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IDENTIFICATION OF THE VON WILLEBRAND FACTOR BINDING SITE IN COLLAGEN USING TRIPLE HELICAL PEPTIDES

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Introduction

The interaction of collagen with von Willebrand Factor (VWF) requires unique structural properties in both proteins [1]. Optimal haemostatic function requires multimerisation of up to fifty VWF monomers in circulating plasma; higher-order multimers bind collagen more tightly than smaller assemblies of VWF. Several collagens occur in the vessel wall, of which collagens I and III are considered most important in supporting platelet adhesion to the damaged vasculature [2]. We have identified the residues in the VWF A3 domain that bind collagen III, using site-directed mutagenesis guided by the crystal structure of the VWF A3 domain in complex with a monoclonal antibody (RU5) that inhibits its interaction with collagen [3]. However, the VWF-binding site(s) in collagen are unknown, although progress in understanding how collagen interacts with integrin α2β1 and GPVI has been made using short synthetic triple-helical peptide analogues of collagen [4], including the Collagen III Toolkit [5]. We used the same approach to identify the high-affinity VWF-binding site in human collagen III, information which may help to develop the collagen–VWF interaction as an anti-thrombotic target.

Results and Discussion

A single Collagen Toolkit peptide, #23 GPC-$(GPP)_5$-GPOGPSGPRGQ-OGVMGFOGPKGNDGAO-$(GPP)_5$-GPC-NH_2 (Fig 1), bound plasma–derived human VWF with similar affinity to the full-length collagen III. The binding interaction was abolished by a monoclonal antibody, RU5, directed against the collagen-binding site of VWF A3 domain. A non-helical derivative of peptide #23, with GAP rather than GPP repeats, did not bind VWF, indicating that a triple-helical structure is required for the recognition of collagen by VWF.

We identified RGQOGVMGF as the minimum sequence within collagen III required to bind VWF using truncated triple-helical derivatives of Col III-23 (Fig 2). An Ala-scan set was synthesised to determine the crucial residues in the minimum sequence, and we shown than R, O, V and F are essential.

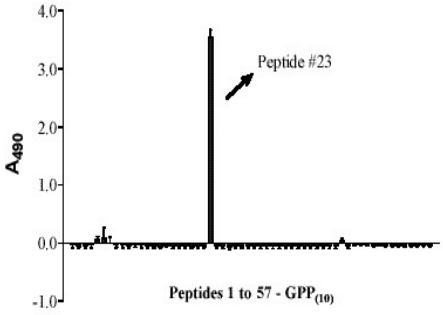

Figure1:The peptide of the collagen III Toolkit were each immobilizedon a 96 well plate and the adhesion of plasma-derived VWF was determined

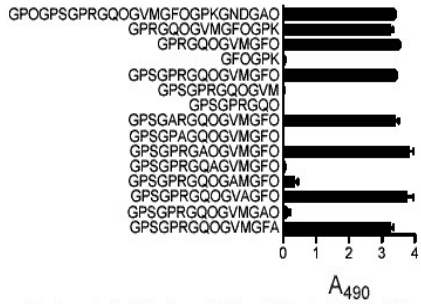

Figure2:The truncated and alanine-modified peptide derived from peptide 23 were immobilized on a 96-well plate, and incubated with plasma-derived VWF

The present work completes our understanding of the collagen–VWF interaction, providing information on the crucial sequences in collagen that perfectly complements our existing knowledge of the collagen-binding site in VWF. Our detailed knowledge may assist in targeting the collagen–VWF interaction for therapeutic purposes.

Acknowledgements

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INSECT KININ ANALOGS WITH CIS-PEPTIDE BOND MOTIF 4-AMINOPYROGLUTAMATE: OPTIMAL STEREOCHEMISTRY

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Introduction

Insect kinins share a highly conserved C-terminal pentapeptide sequence Phe-Xaa-Xbb-Trp-Gly-NH_2, where Xaa can be Tyr, His, Ser or Asn and Xbb can be Ala but is generally Ser or Pro [1]. They are potent diuretic peptides that preferentially form a *cis*-Pro, type VI β-turn. An insect kinin analog containing (*2S,4S*)-4-aminopyroglutamate (**APy**), a novel cis-peptide bond motif, has demonstrated significant insect diuretic activity [2]. Two susceptible hydrolysis sites in insect kinins [3] have been reported, with the primary site between the Pro^3 and the Trp^4 residues. In this study, we evaluate the **(*2S,4R*)-APy**, **(*2R,4R*)-APy**, and **(*2R,4S*)-APy** stereochemical variants of the insect kinin analog Ac-Arg-Phe-**APy**-Trp-Gly-NH_2 in order to identify the optimal motif stereochemistry for the design of peptidomimetic analogs with enhanced biostability.

Table 1. Cricket Malpighian tubule fluid secretion activity of insect kinin analogs with stereochemical variants of the Apy motif and a native achetakinin.

Peptide Analog		Cricket (*Acheta domesticus*) Malpighian Tubule Fluid Secretion	
		EC_{50} ($10^{-9}M$) [95% CL]	Maximum Response (%)
AK-III	Ala-Leu-Pro-Phe-Ser-Ser-Trp-Gly-NH_2	0.3	100 [3]
IK-APy	Ac-Arg-Phe-(***2S,4S***)-**APy**-Trp-Gly-NH_2	140 [136-149]	93±10
IK-apy	Ac-Arg-Phe-(***2R,4R***)-**APy**-Trp-Gly-NH_2	70 [32-172]	83±6
IK-Apy	Ac-Arg-Phe-(***2R,4S***)-**APy**-Trp-Gly-NH_2	7 [3.7-15.1]	93±6
IK-aPy	Ac-Arg-Phe-(***2S,4R***)-**APy**-Trp-Gly-NH_2	120 [113-136]	96±1

Results and Discussion

The insect kinin analogs **(*2R,4R*)-APy** and **(*2S,4R*)-APy** were found to stimulate cricket Malpighian tubule fluid secretion at EC_{50}'s of 7 and 12 x 10^{-8}M; values which are not statistically different from the activity (EC_{50} = 14 x 10^{-8}M) of the parent **(*2S,4S*)-APy** analog [3]. However, the diuretic activity of the (*2R,4S*)-APy stereochemical variant was found to be about 10-fold greater, with an EC_{50} of 0.7 x 10^{-8}M. All of the **APy** analogs approach the maximal diuretic response of the native insect kinins. NMR spectra were acquired [2] on a Bruker ARX-500 500 MHz instrument. Molecular modeling was performed [2] using TRIPOS Sybyl 6.3 software (Biopolymers). Based on ROESY data, the following non-trivial distance constraints were included in the molecular modeling calculations; 13 for **(*2S,4S*)-APy [3],** 15 for **(*2R,4R*)-APy**, 18 **for (*2S,4R*)-APy**, and 0 for **(*2R,4S*)-APy**. All four stereochemical **APy** analogs demonstrate significant diuretic activity, likely due to the ability of each to adopt an open '1 - 4' turn and an aromatic surface comprised of the Phe^{1} and Trp^{4} aromatic sidechains. The **(*2R,4S*)-APy** analog proved to be considerably more flexible than the other variants. The three more rigid **APy** analogs **(*2S,4S*), (*2R,4R*),** and **(*2S,4R*)** each feature a different stereochemistry at the **APy** moiety; and yet the diuretic activity is not significantly different. Therefore, the 10-fold greater potency of the **(*2R,4S*)-APy** analog over the other three variants may well be a function of its flexibility to adopt a better fit with the receptor. The **(*2R,4S*)** stereochemical **Apy** variant is the best **APy** scaffold for the design of biostable peptidomimetic analogs of this important class of insect neuropeptides.

Acknowledgements

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THERMODYNAMIC STUDY ON THE CALCIUM BINDING OF ASPARTIC ACID RICH CYCLOPEPTIDES

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Introduction

Ca^{2+} ions play an important role in biochemical pathways. They bind to enzymes and proteins in different processes. Aspartic (Asp, D) and glutamic (Glu, E) acid side chains are the main ligands of Ca^{2+}, but the contribution of the backbone carbonyl groups in the binding is also important. Generally, the binding sequence is the so-called EF-hand motif, containing 12 amino acids, but already known that some proteins with a non-EF-hand loop also bind Ca^{2+}. α-Lactalbumins (LAs) have a 10 amino acid long sequence (K^{79}FLDDDLTDD^{88}) for Ca^{2+} binding [1]. In this sequence 5 Asps are closer to each other than in EF-hand motif but only 3 Asp side chains take part in Ca^{2+} coordination.

Our aim is to understand the Ca^{2+} interaction with carboxylic group rich peptides. In the present study we focus on determining the importance of the conservative amino acids within the Ca^{2+} binding loop of this protein, using isothermal titration calorimetry (ITC).

Results and Discussion

At first we prepared a model with the decapeptide sequence of the calcium-binding loop of LA. In order to monitor the precise role of the binding Asp residues (82, 87–88) and of charge density created by the nonbinding Asp residues (83, 84) in the coordination of Ca^{2+}, we also designed models in which the Asp residues were substituted to Ala. In addition, some models in which Lys is deleted and substituted, respectively, were synthesized.

The ITC measurements have been performed under different conditions: besides of the different peptides, different solvents (Tris-HCl buffer at pH 7.4, methanol, ethanol, trifluoroethanol [TFE]) and different temperatures (10 °C, 25 °C and 40 °C) were used. Below we describe the most general trends observed.

In aqueous medium no heat effects appeared upon addition Ca^{2+}. With the increase the hydrophobic feature of the solvent (water<methanol<ethanol<TFE), an important and complex heat effects became visible. The results indicate that in aqueous medium Ca^{2+} does not bind to the decapeptide sequence; in apolar one peptide binds several Ca^{2+} ions. The poor binding tendency in aqueous medium is certainly due to a favorable cation-water interaction (hydration of Ca^{2+}-ions), however, also the interaction between the solvent and the peptide importantly will influence the ability to bind cations. Indeed, by circular dichroism (CD) measurements we previously demonstrated that in aqueous medium the peptides adopt an unordered secondary structure. In contrast, the CD spectra of the peptides

in TFE are representative of specific populations of turns [2]. The intramolecular organization becomes possible by the loss of overwhelming solvent interactions in the apolar medium.

In apolar solvents (methanol, ethanol, TFE) the first heat effect observed upon addition of small amounts of Ca^{2+}, is endothermic. This endothermic heat is more important when the apolar character of the medium is more pronounced. In methanol and ethanol, the endothermic peak is not followed by other heat effects and the titration curves fit to a cation/peptide binding ratio (rcat) of 1. In TFE solutions the titration profiles are more complicated and the endothermic effects observed at small Ca^{2+}/peptide ratios are followed by an exotherm effect when the Ca^{2+}/peptide ratios exceeds the ratio of 1. Moreover, depending upon de composition of the peptide the Ca^{2+} titration curve in TFE becomes more complex.

The results indicate that binding of Ca^{2+} to a peptide is strongly regulated by the polarity of the solvent and of the direct environment which surrounds the sites suited for the coordination of the Ca^{2+} ion.

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SYNTHESIS OF TRIFLUOROMETHYL KETONE-CONTAINING GLUTAMIC ACID AND GLUTAMINE PEPTIDES AS SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 3CL PROTEASE INHIBITORS

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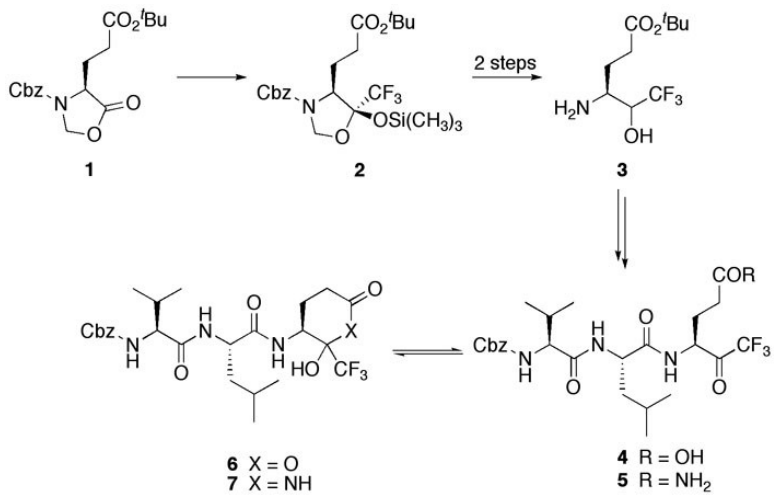
Introduction

Compounds containing a trifluoromethyl ketone moiety form an important group of biologically useful fluorinated molecules [1]. A trifluoromethyl group that is α to the carbonyl functionality increases the electrophilicity of the carbonyl carbon, and therefore makes it prone to nucleophilic substitution by the cysteine residue which is present in the active site of cysteine proteases such as the severe acute respiratory syndrome coronavirus (SARS-CoV). This would lead to the formation of a tetrahedral adduct that is believed to mimic the intermediate formed during peptide-bond hydrolysis [2] and suggests that compounds containing a trifluoromethyl ketone moiety may play an important role as SARS-CoV inhibitors . Based on these considerations, the synthesis of the peptides depicted in Scheme 1 (compounds 4 and 5) was embarked upon.



Scheme 1. Synthetic pathway to the targeted SARS-CoV protease inhibitors.

Result and Discussion

β-Amino alcohol 3 was synthesized in five steps starting from *N*-Cbz-L-glutamic acid. The first key step involved the introduction of a trifluoromethyl group to oxazolidinone 1 based on a reported method, 3 thus forming compound 2, that was converted in two steps to the desired substrate 3 (Scheme 1). β-Amino alcohol 3 was then coupled with the respective acids and further elaborated to form tri- and tetra- glutamic acid and glutamine peptides (here exemplified by compound 4 and 5). The target peptides were subjected to biological assays, which revealed that the compounds exhibited moderate activity against SARS-CoV. ^{1}H and ^{13}C NMR studies of the target compounds showed that they exist predominantly in the cyclic form (substrates 6 and 7) rather then the open chain form (compounds 4 and 5) in deuterated chloroform solution.

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NOVEL BENZOPHENON-GLUCOSIDES FOR THE STUDY OF MICROBIAL SUGAR RECOGNITION AND UPTAKE BY PHOTO-AFFINITY LABELING

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Induction

A series of photoactiveable benzophenon derivatives of arbutin were synthesized and characterized. Three compounds were studied as probes for BglF, the β-glucoside PTS transporter of Escherichia coli, and they demonstrated high affinity photolabling of the BglF. Since the PTS system is ubiquitous and unique to bacteria, these compounds may serve as leads for the development of novel antibacterial drugs.

The *Escherichia coli* BglF, a permease of the phosphoenolpyruvate-dependent-phosphotransferase system (PTS), catalyzes transport and phosphorylation of β-glucosides. The PTS is a multicomponent system that catalyzes vectorial phosphorylation of various sugars. The group translocators couples sugar transport to phosphorylation driven by the high phosphate transfer potential of phosphoenolpyruvate (PEP). PTS components are involved in complex signal transduction pathways including virulence. It is ubiquitous in bacteria but does not occur in animals and plants. Uniqueness and pleiotropic function make the PTS a target for new antibacterial drugs.

The PTS is composed of two general PTS proteins, EI and HPr, and several sugar specific enzymes II (EIIs). EIIs are composed of three domains. The domains EIIA and EIIB are involved in phosphorylation, whereas EIIC are membrane-bound and are active in sugar permeation across the membrane. The phosphate is transferred from PEP to the incoming sugar via EI, HPr, EIIA and EIIB. Despite extensive studies performed on the PTS permease, the nature of the sugar translocation channel is yet unknown.

To study the mechanism of the sugar translocation, BP group was used in order to produce an irreversible covalent binding of the Nirbutin with the BglF to probe the nature of the sugar translocation channel and recognition.

In order to map the binding site of Nirbutin-00 to BglF, the radiolabaled Nirbutin-01 was prepared. ^{14}C-Nirbutin-01 was utilized by *E. Coli*. Covalent labeling of the binding site of BglF with ^{14}C-Nirbutin-01 was achieved after BP irradiation. The labeled BglF will be used to map the active site after protein extraction, digestion and MS analysis.

In order to study the spatial structure of the BglF, especially the relationship between the sugar binding site and Cys24 that is essential to the function of BglF,

we have prepared a series of molecules with the general structure Nirbutin-spacer-NEM. The spacer will vary between 2 and 6 methylenes.

Results and Discussion

A series of novel BP derivatives of arbutin, were synthesized. Their biological activity and binding to the β-glucoside permease in *E. coli* were studied. Nirbutin is utilized as a β-glucoside substrate by BglF-producing *E. Coli* cells. Furthermore, using photolabeling and competitive inhibition experiments, we demonstrated that Nirbutin's are selective ligands for BglF and their binding follows saturation kinetics. Hence, Nirbutin's are of biologically active and are appropriate photoprobes for further characterizing of BglF.

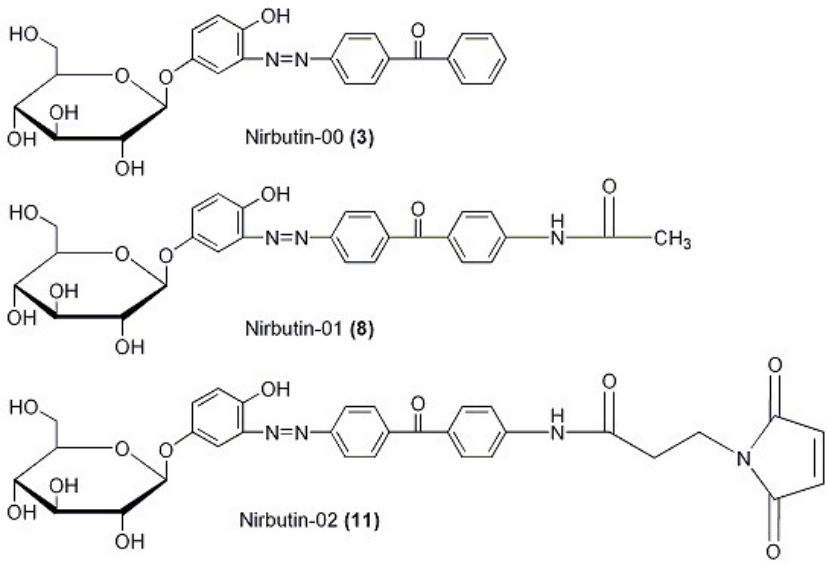

Figure 1 - Structures of the three arbutin BP derivatives: Nirbutin 00, Nirbutin 01 and Nirbutin 02.

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HIGHLY SENSITIVE FRET SUBSTRAATE FOR THE ASSAY OF MMPs

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Introduction

Matrix metalloproteinases (MMPs) are involved in the degradation of components of extracellular matrix and play an important role in apoptosis, embryogenesis, and reproduction tissue remodeling and repair. The development of agonists or inhibitors in controlling MMP activity continues to be of great interest for drug discovery and life science research.

FRET peptide-based MMP assays have been widely used for measuring MMP activity. However, most of these FRET peptides use Mca/Dnp or Edans/Dabcyl pairs [1], which have relatively weak fluorescence signals with short wavelengths. We designed and synthesized sixteen MMP substrates that incorporate 5-FAM and QXLTM520 by Fmoc solid phase synthesis method. QXLTM520 is an effective quencher for fluoresceins such as FAM and FITC. The new 5-FAM/QXLTM520 FRET peptides offer several advantages.

Results and Discussion

MMP Substrates (Table 1) were synthesized by Fmoc solid phase synthesis methods using Rink amide MBHA resin. For special amino acids [Dap(Mtt), Dab(Mtt), Cys(Me), Cha, Smc and D-Arg(Pbf)], couplings were performed with two-threefold excess of activated amino acids. Upon completion of the chain assembly, the peptide-resin was treated with 1 TFA and 3% TIPS in DCM to remove the Mtt group. QXLTM520 and 5-FAM were coupled to the side chain amino group of Dap, Dab and Lys using DIC:HOBt. Complete deprotection of the peptide and cleavage was performed with TFA:thioanisole:water:phenol:EDT. Crude peptides were purified to homogeneity by RP-HPLC.

Compared to EDANS and Mca, the extinction coefficient of 5-FAM is 13-fold higher and shows less interference from the short wavelength auto-fluorescence of drug candidates. Additionally, 5-FAM is much brighter and less sensitive to the environment than EDANS and Mca. These characteristics of 5-FAM prompted us to design 5-FAM containing MMP FRET peptide substrates in order to increase the sensitivity. QXL™520 serves as an excellent quencher for the 5-FAM, since its absorption spectrum perfectly overlaps with the emission spectrum of 5-FAM. Additionally, QXL™520 is a hydrophilic compound, which unlike DABCYL or Dnp is hydrophobic. This property of QXLTM520 increases the solubility of the peptide substrate in aqueous buffers, alleviating the problem caused by the hydrophobic nature of many fluorescent donors and quenchers.

Table 1. 5-FAM/QXL™520 FRET MMP substrates.

Seq# (Catalog No.)	FRET Peptide Sequence
#1 (60568-01)	QXLTM520-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-Lys(5-FAM)-NH_2
#2 (60569-01)	QXLTM520-Pro-Leu-Ala-Leu-Trp-Ala-Arg-Lys(5-FAM)-NH^2
#3 (60570-01)	QXLTM520-Pro-Leu-Gly-Cys(Me)-His-Ala-D-Arg-Lys(5-FAM)-NH_2
#4 (60571-01)	5-FAM-Pro-Leu-Ala-Nva-Dap(QXLTM520)-Ala-Arg-NH_2
#5 (60572-01)	5-FAM-Pro-Leu-Gly-Leu-Dap(QXL520 TM)-Ala-Arg-NH_2
#6 (60573-01)	QXLTM520-Pro-Leu-Gly-Met-Trp-Ser-Arg-Lys(5-FAM)-NH_2
#7 (60574-01)	QXLTM520-Pro-Tyr-Ala-Tyr-Trp-Met-Arg-Lys(5-FAM)-NH_2
#8 (60575-01)	QXLTM520-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(5-FAM)-NH_2
#9 (60576-01)	QXLTM520-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Lys(5-FAM)-NH_2
#10 (60577-01)	QXLTM520-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(5-FAM)-NH_2
#11 (60578-01)	5-FAM-Pro-Cha-Gly-Nva-His-Ala-Dap(QXLTM520)-NH_2
#12 (60579-01)	5-FAM-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(QXLTM520)-NH_2
#13 (60580-01)	5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(QXLTM520)-NH_2
#14 (60581-01)	QXLTM520-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2
#15 (60582-01)	QXLTM520-γ-Abu-Pro-Gln-Gly-Leu-Dab(5-FAM)-Ala-Lys-NH_2
#16 (60583-01)	QXLTM520-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Trp-Lys((5-FAM)-NH_2

Sixteen 5-FAM/QXL™520 FRET peptides were screened by MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14. Each of these peptides was found to be cleaved by certain MMPs. Seq# **14** was cleaved by all the MMPs tested and was shown to have the highest kinetic slope (RFU/min) among all the 5-FAM/QXL™520 FRET peptides listed in Table 1. Serial diluted MMP-7 was incubated with Seq# **14**. The results showed that seq# **14** can detect 62.5 pM of MMP-7 with the linear range up to 4 nM.

Two FRET peptides, Dabcyl-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Glu(Edans)-Ala-Lys-NH_2 and QXL™520-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2 (Seq# **14**), were tested by MMP-1(4 nM) for hydrolysis. The fluorescent signal was monitored at Ex/Em = 492 nm/518nm for FAM/QXL™520 pair or Ex/Em = 340nm/490 nm for Edans/Dabcyl pair. Seq# **14** showed higher sensitivity than Edans/Dabcyl based FRET peptide since it was more efficiently hydrolized by the same amount of MMP-1.

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REGIO- AND STEREOSELECTIVE SYNTHESIS OF (*Z*)-ALKENE *CIS*-XAA-PRO DIPEPTIDE MIMETICS

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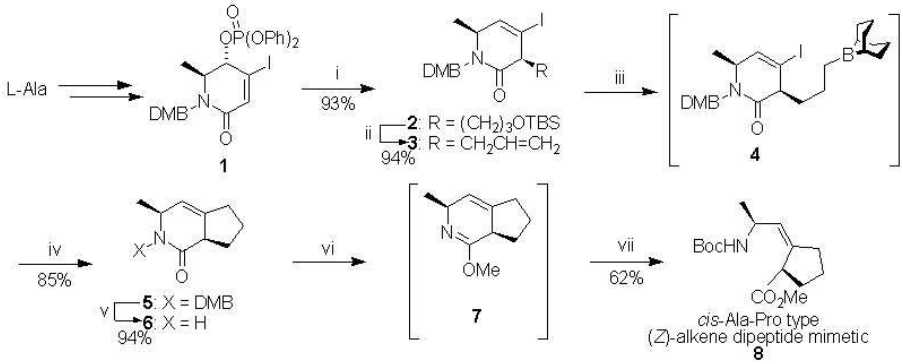
Introduction

Low energy barriers between *cis*-isomer and trans-isomers of an amino acid (Xaa)-Pro peptide bond allow cis-/trans-equilibrium of the bond, which exerts great influence on SAR of peptides and proteins. Therefore, restriction of the amide bond with (*Z*)- or (*E*)-alkene units should be one of useful means for elucidating the bioactive geometries. We have accomplished syntheses of (*E*)-alkene type Ser-Pro dipeptide mimetics using organocopper chemistry; however, we have yet to complete the stereoselective syntheses of (*Z*)-alkene containing counterpart. Recently, we have developed an efficient synthetic method for diketopiperazine mimetics by organocopper-mediated *anti*-SN_2' reactions [1]. These mimetics are potential precursors for (*Z*)-alkene containing dipeptide mimetics. Herein, we report the regio- and stereoselective synthesis of *cis*-Ala-Pro type (*Z*)-alkene dipeptide mimetic by the using the organocopper method.

Results

Scheme depicts the synthetic route for the desired *cis*-Ala-Pro type (*Z*)-alkene dipeptide mimetic where γ-phosphoryloxy-α,β-unsaturated-γ-lactam 1 from L-Ala is used as a key intermediate. Stereoselective introduction of a C3 unit onto the lactam was achieved by the treatment of 1 with $LiCu(CN)(CH_2)_3OTBS•2LiCl•LiI$ in THF. Next, we attempted to convert the incorporated α-alkyl moiety in the resulting anti-SN_2' reaction product to alkylborane moiety, which is amenable to the intramolecular Suzuki coupling for the construction of proline cyclic side chain moiety. To this end, the α-alkylated product was transformed to the olefin 3 by three steps reactions. The resulting olefin 3 was subjected to the hydroboration to give the alkylborane 4. After survey of the suitable reaction conditions, it was revealed that the treatment of the resulting alkylborane 4 with CsF in the presence of $PdCl_2$(dppf) as a catalyst gave the desired bicyclic lactam 5 in 85% yield from 3. After the deprotection of the DMB group on 5, ring opening of the resulting lactam 6 was achieved by O-methylation-mediated lactim ether formation followed by acid catalyzed hydrolysis. Without being purified, the resulting ester was treated with Boc_2O-Et_3N to yield the desired *cis*-Ala-Pro type (*Z*)-alkene dipeptide mimetic 8 in 62% yield from 6 [2].

Scheme. Synthesis of *cis*-(*Z*)-Alkene Containing Proline Dipeptide Mimetic.



Reagents: (i) $LiCu(CN)(CH_2)_3OTBS{\cdot}2LiCl{\cdot}LiI$, THF; (ii) a) H_2SiF_6, H_2O, CH_3CN, MeOH; b) 2-nitrophenylselenocyanate, PBu_3, pyridine; c) H_2O_2, H_2O, THF; (iii) 9-BBN-H, THF; (iv) CsF, $PdCl_2(dppf)$, DMF; (v) H_2O, TFA; (vi) a) $Me_3O{\cdot}BF_4$, 2,6-di-*tert*-butylpyridine, CH_2Cl_2; b) HCl, H_2O, then Boc_2O, Et_3N.

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SYNTHESIS AND BINDING PROFILES OF ENDOMORPHIN-2 ANALOGUES CONTAINING A CONFORMATIONALLY CONSTRAINED MOIETY

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Introduction

Endomorphin-2 (EM-2: H-Tyr-Pro-Phe-Phe-NH_2), an endogenous opioid peptide isolated from bovine and human brain, has high affinity and selectivity for the mu-opioid receptor and produces potent and prolonged analgesia in mice [1]. In this presentation, the synthesis of EM-2 analogues containing a conformationally constrained moiety and examination of their opioid receptor binding profiles were conducted in order to obtain more potent agonist or antagonist with stability against dipeptidyl peptidase IV (DPP IV). The ethylene-bridged Phe-Phe unit (eb[Phe-Phe]) or piperidine carboxylic acid (Pic) was employed in lieu of Pro.

Results and Discussion

The synthesis of eb[Phe-Phe] unit was achieved according to the procedure of Lammek B. *et al.* [2]. Protected peptides were synthesized by a solution method using Boc-chemistry. The receptor binding activity of peptides were assessed by radioligand receptor binding assay using mu- and delta-opioid receptors from COS-7 cell membranes expressing cloned opioid receptors.

The substitution of Pro in EM-2[K_i^{μ}=0.69 nM, K_i^{δ}=9,200 nM] and [Dmt (2',6'-dimethyl-L-tyrosine)[1]]EM-2 [K_i^{μ}=0.15 nM, K_i^{δ}=28 nM] with eb[Phe-Phe] gave H-Tyr-eb[Phe-Phe]-Phe-NH_2 [K_i^{μ}= 381 nM, K_i^{δ}=3,200 nM] and H-Dmt-eb[Phe-Phe]-Phe-NH_2 [K_i^{μ}= 5.66 nM, K_i^{δ}= 105 nM]), respectively, showing 550-37-fold less mu-receptor affinity relative to the corresponding parent peptide. The benzyl residue on the position 3 of piperazin-2-one ring either reduced the opioid-receptor affinities or the piperazin-2-one moiety could not impose enough conformational constraints to induce the proper spatial orientation of Tyr and Phe for ligand-receptor interaction.

The receptor binding activity (IC_{50} value) of EM-2 analogues containing Pic derivatives is summarized in Table 1. The Pic residue with its six-membered ring is

a mimic of Pro thereby reducing its rigidity. The substitution of Pro in EM-2 gave the following results: (i) Pic(4) decreased the mu-receptor binding activity (IC_{50} = 143 nM), which was 16-times lower than EM-2; (ii) on the other hand, substitution with L-Pic(2) retained a high mu-receptor binding activity (IC_{50}=2.13 nM). (iii) Furthermore, incorporation of Dmt enhanced the receptor binding activities: [Dmt^1, D-$Pip(2)^2$]EM-2 exhibited the highest mu-receptor binding activity (IC_{50}=0.21 nM), while [D-$Pip(2)^2$]EM-2 had a mu-receptor binding activity (IC_{50} = 7.61 nM) similar to EM-2. (iv) Substitution of Pro in [Dmt^1]EM-2 with Ala dropped the mu-receptor binding activity by half. It seems that the mu-receptor binding activity is improved by incorporating a conformationally constrained moiety, such as Pic(2).

Table 1. Binding activity of EM-2 analogues containing Pic derivatives

Peptide	Binding Activity [a] (IC_{50}:nM)	
	mu-Opioid Receptor[b]	delta-Opioid Receptor[c]
EM-2	8.65	>10,000
[Dmt^1]EM-2	0.35	367
[$Pic(4)^2$]EM-2	143	>10,000
[L-$Pic(2)^2$]EM-2	2.13	7,720
[D-$Pic(2)^2$]EM-2	7.61	1,250
[Dmt^1,Ala^2]EM-2	0.62	1,900
[Dmt^1,$Pic(4)^2$]EM-2	0.41	31
[Dmt^1,L-$Pic(2)^2$]EM-2	0.41	70
[Dmt^1,D-$Pic(2)^2$]EM-2	0.21	16

[a]COS-7 cell membrane preparations expressing mu- and delta-opioid receptors were used. [b][^{3}H]DAMGO was used as a radioligand. [c][^{3}H]deltorphin II was used as a radiolidand.

Acknowledgements

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AN NMDA RECEPTOR AGONIST: TETRAZOLYL-GLYCINE AS AN EFFECTIVE COPPER CHELATOR

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Introduction

NMDA receptors belong to ionotropic group of glutamate receptors. It has been postulated that disturbances in glutamatergic neurotransmission may contribute to neuropathologies and brain disorders [1]. The (*R,S*)-(Tetrazol-5-yl)glycine (**TG**) belongs to a highly potent NMDA (*N*-methyl-D-aspartic acid) receptor agonist with exitotoxic effects [2]. The aim of our studies was to investigate a chelating ability of **TG** towards copper(II) ions. Copper is widely distributed throughout the body with distinct concentration in the brain, where copper-mediated reactions play important roles. Copper enters cells as a ligand complex and seeks out targets that require it to function. For these reasons it was interesting to evaluate the stability and the structure of **TG** – copper(II) complexes.

Results and Discussion

The target of our studies were: (*R,S*)-(tetrazol-5-yl)glycine, 1,5-diamino-1*H*-1,2,3,4-tetrazole [3] and tetrazole aspartic acid analogues. The equilibrium and structural properties of complex species were characterized by pH-metric, UV-VIS and EPR methods. In the systems dimeric species dominante at acidic pH with Ntetr bridging elements. The bis chelated complexes were found at physiological pH. The two ligands are bound to Cu(II) via four nitrogen donors. This coordination mode results in strong metal-ligand interactions and the species is very stable and dominating in the wide pH range. Potentiometric and spectroscopic data suggest that: 1) **TG** is efficient ligand for copper(II) ions in acidic pH range whilst in the neutral and basic pH its affinity towards Cu(II) decreases in comparison to other ligands present in biofluids, e.g. histidine, 2) the insertion of 1,5-disubstituted tetrazole ring into amino acid molecule has a critical impact on the ligand chelating ability towards Cu(II) ions. The comparison of Cu(II) distribution between aspartic acid, 2-amino-3-(1*H*-tetrazol-5-yl)-propionic acid and 2-amino-3-(1-methyl-1*H*-tetrazol-5-yl)-propionic acid leads to the conclusion that the most efficient ligand for copper(II) ions is 2-amino-3-(1-methyl-1*H*-tetrazol-5-yl)-propionic acid. The results of our studies have revealed that the copper(II) ions binds to **TG** efficiently. This ligand can compete in copper transportation in cerebral fluids. It is very likely

that its copper complexes may affect both the ligand receptor affinity and/or the NMDA receptor activity as ligand-gated ion-channel.

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NOVEL BIOLOGICAL EFFECT OF PLANT PEPTIDE HORMONE PHYTOSULFOKINE-α (PSK-α) AND ITS SELECTED ANALOGUES

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Introduction

Continuing our studies on phytosulfokine-α (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH) (PSK-α) (I), a sulfated plant growth factor [1], its C-terminal truncated tetrapeptide PSK-β (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH) (II), and its analogues, we performed a further search for its biological properties in respect to plant and animals.

At the first stage of these investigation we undertook the studies *in vitro* on the influence of PSK-α and its analogues, such as: [Phe(4-NO_2)^{1,3}]- (III) [Tyr(PO_3H_2)^{1,3}]- (IV) and [Phe(4-F)^{1,3}]-PSK-α (V) on the growth and development of *Phoma narcissi* and *Botrytis tulipae*.

Five mm diameter plugs taken from 7-day-old culture of tested fungus were placed in the middle of 90mm Petri dishes containing PDA medium (potato-dextrose-agar) with PSK-α or its analogues at the concentration of 100, 136, 150, 200 or 400 $\mu g \cdot cm^{-3}$. Control plates contained the culture growing on PDA without any amendments. The diameter of fungi colony was measured within 4- or 5-day incubation at 25 ˚C in darkness. The data were subjected to analysis of variance and Duncans multiple range test.

In the second part, we performed studies to determine the antinociceptive effect *in vivo* of phytosulfokine-α and its selected analogues, such as: [Phe(4-NO_2)^1]- (VI), [D-Phe(4-NO_2)^1]- (VII), [Tyr^1]- (VIII), [Tyr^3]- (IX), and [Tyr^{1,3}]-PSK-α (X) in rats.

The study was carried out on adult Wistar female rats. On the day of experiment PSK-α and its analogues were injected icv through cannula at the same dose of 100nmol according to the method of Plech [2], and antinociceptive effect of injected peptides was determined by the method of a hot-plate test [3].

Peptides were synthesized by the solid phase method according to the Fmoc-procedure. The Tyr residue to be sulfated was introduced into the peptide chain as Tyr(OH). The partially protected peptide-resin was sulfated by DMF-SO_3 complex. Free peptide was released from the resin with 95% TFA in the presence of EDT.

All peptides were purified by HPLC on a C-18 (Beckman) column.

Results and Discussion

It was found that PSK-α has antifungal properties and inhibits *in vitro* the mycelium growth of *P. narcissi* and *B. tulipae* in a dose-dependent manner. PSK-α at the concentration of 400μg·cm-3, after a 4 days incubation, inhibited development of *P. narcissi* and *B. tulipae* at 42% and 57%, respectively. Moreover, compound V at the concentration of 136 $\mu g \cdot cm^{-3}$, after a 3 days incubation, inhibited growth of mycelium *P. narcissi* and *B. tulipae* at 32% and 92% respectively. PSK-β (II) showed inhibitory effect on the development of P. narcissi at 62%, whereas it was inactive in relation to *B. tulipae*. Other peptides were practically inactive in relation both to *P. narcissi* and *B. tulipae*.

In the second part of biological studies, we found that PSK-α induced a significant antinociceptive effect lasting 60 min. A prolonged antinociceptive effect of PSK suggests that the enzymatic degradation of this peptide in the rat brain is rather slow. It is similar to that observed for several other neuropeptides described in the literature [4, 5]. A similar effect was observed for peptides (VI - X). This effect is probably due to interaction of these peptides with the opiate receptors of the central nervous system of rats.

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INFLUENCE OF INSECT OOSTATIC PEPTIDE NEB-COLLOOSTATIN ON HEARTBEAT OF TWO INSECT SPECIES TENEBRIO MOLITOR AND ZOPHOBAS ATRATUS

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Introduction

Oogenesis in insects is a well studied and complex process. At present, three oostatic factors called Aea-TMOF (H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH) [1], Neb-TMOF (H-Asn-Pro-Thr-Asn-Leu-His-OH) [2], and Neb-colloostatin (H-Ser-Ile-Val-Pro-Leu-Gly-Leu-Pro-Val-Pro-Ile-Gly-Pro-Ile-Val-Val-Gly-Pro-Arg-OH) [3], isolated from Aedes aegypti (Aea-TMOF) and fleshfly *Neobellieria bullata* (Neb-TMOF and Neb-colloostatin).

The subject of our paper is synthesis of Neb-colloostatin (I) and its 29 analogues. Two analogues were modified in position 13 by Ala (II) or D-Pro (III) and other were oligopeptides with a shortened peptide sequence such as [1-13]-(IV), [14-19]-(V), [15-19]-(VI), [16-19]-(VII), [13-19]-(VIII), [12-19]-(IX), [11-19]-(X), [10-19]-(XI), [9-19]-(XII), [8-19]-(XIII), [7-19]-(XIV), [6-19]-(XV), [5-19]-(XVI), [4-19]-(XVII), [3-19]-(XVIII), [2-19]-(XIX), [1-11]-(XX), [1-4]-(XXI), [1-6]-(XXII), [1-8]-(XXIII), [1-9]-(XXIV), [3-9]-(XXV), [3-11]-(XXVI), [4-8]-(XXVII), [2-6]-(XXVIII) and [2-9]-colloostatin (XXIX).

Peptides were synthesized by the classical solid phase method according to the Fmoc or Boc-procedure. All peptides were purified by preparative HPLC.

Biological effects of the Neb-colloostatin and its analogues were evaluated *in vitro* by cardiotropic bioassays with the semi-isolated heart of Tenebrio molitor and Zophobas atratus and on contractions bioassay of the oviducts in both beetles according to the method Rosiński [4].

In this paper we discuss a cardiotropic effect of the investigated peptides.

Results and Discussion

We provide a first evidence that Neb-colloostatin exerts myotropic actions on the insect visceral muscles in two coleopteran species, *T. molitor* and *Z. atratus*. The peptide induces cardiostimulatory effect on the heart of T. molitor and stimulates contractions of the oviducts in both beetles at concentration of 10^{-7} M. However, the heart of *Z. atratus* is insensitive to Neb-colloostatin at concentrations up to 10^{-6} M which suggests species-specific cardiotropic action of this peptide The stimulation threshold for Neb-colloostatin on the *T. molitor* heart ranged from 10^{-9} to 10^{-8} M and the maximal response of the heart to this peptide was about 3 times smaller in

comparison to the effect induced by proctolin. Neb-colloostatin primarily causes a positive chronotropic effect without changes in amplitude contractions in the heart of Tenebrio. This is in contrast to proctolin, which induces a negative inotropic effect. Theses differences in the response of the heart to Neb-collooostatin and proctolin suggest that these peptides regulate myocardium by different mechanisms. The site of action of Neb-colloostatin on the heart of Tenebrio is not know, and the effect could be either on muscle fibers or on the pacemaker regions. Among of investigated Neb-colloostatin analogues, only peptides XVII, XVIII and XIX retained about 60% cardioexcitatory activity of native peptide in *T. molitor*. Other analogues of Neb-colloostatin were inactive. In bioassay on hearth of *Z. atratus* all analogues were practically inactive.

The question whether the myotropic actions of Neb-colloostatin on the heart and oviduct is physiologically relevant. Myostimulatory action of the peptide on these organs can be coordinated and may constitute a complex mechanism to increase the circulation in the abdominal cavity. On the other hand, Neb-colloostatin may control oviposition process in both beetles. It is possible that during increased contractions of the oviduct muscles after the peptide action the movement of eggs is initiated and females start oviposition.

Acknowledgements

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SYNTHESIS OF THE Mdm2 RING FINGER DOMAIN BY COMBINED CONVERGENT AND NATIVE CHEMICAL LIGATION METHODS

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Introduction

The RING finger domain of Mdm2, located at the C-terminal part of the protein, is necessary for the regulation of the tumour-suppressor protein p53 and therefore it is an important target for studying its interaction with small anticancer drug candidates. Due to the presence of multiple cysteine residues, the synthesis of such small proteins is difficult to be performed using recombinant techniques. The 48-residue Mdm2 peptide (Fig. 1) has been previously synthesized by sequential condensation of protected fragments on 2-chlorotrityl resin [1]. Here, we report the efficient synthesis of this peptide by applying a combination of Fmoc-based convergent synthesis (CPS) and native chemical ligation (NCL).

I^{1}EPCVICQGRPKNGCIVHGKTGHLMACFTCAKKLKKRNKPCPVCRQPI^{48}

Fig. 1. The primary sequence of the Mdm2-RING finger domain.

Results and Discussion

We divided the RING domain sequence into a 22-residue peptide containing an N-cysteine and a 26-residue thioester peptide. The 22 mer N-Cys peptide was prepared either by conventional SPPS on 2-chlorotrityl resin or by (27-32) + (33-48) fragment condensation. For the convergent synthesis of the thioester peptide we investigated two alternative routes, following a "post-assembly" thioesterification approach.

In route A, the 1-26 protected fragment was first prepared by the sequential condensation of the protected fragments 15-19, 10-14 and 1-9 on fragment 20-26, bound on 2-chlorotrityl resin. All Cys-residues were protected by the super acid-labile 4-methoxytrityl (Mmt) group. Fragment 1-26, after cleavage from the resin with a TFE/DCM (30:70) solution, was converted to the corresponding C-thioester by reacting with a 5-fold molar excess of either methyl mercaptoacetate or thiophenol using DIC/HOBt (5 eq.) as condensing agent in DCM. Thioesterification reaction was in both cases complete in 6 h at RT. The HPLC-analysis of the crude products obtained after total deprotection with TFA/TES/DCM (85:5:10), indicated purities 75 and 60% for the methyl mercaptoacetate and thiophenyl ester, respectively.

In route B, we followed a side-chain attachment approach. In this strategy the Fmoc-(23-26) fragment was directly attached through the side-chain of His on Trt-resin using the procedure previously described [2]. The resin-bound fragment (0.3 mmol/g resin), after carboxyl protection with the trityl group, was chain-elongated to the N-direction by three cycles of single amino acid addition and then by sequential condensation of fragments 15-19, 10-14 and 1-9, to obtain the Boc-(1-26) fragment. The on-resin thioesterification was performed, after removal of Trt-group with 0.5% TFA in DCM, by reacting with either methyl mercaptoacetate or thiophenol (5 eq.) using DIC/HOBt in DCM. Complete conversion to thioester was observed after a 2 h (two times) reaction at RT. Crude products obtained after cleavage from the resin and side-chain deprotection were of 65% purity.

HPLC-purified 1-26 thioesters, prepared by both routes A and B, were ligated with the N-Cys peptide in sodium phosphate buffer (pH 7.5), containing 2% thiophenol and 2% TCEP at RT. The reaction was fast, being essentially complete in 6 h (Fig 2). The yield of the HPLC-isolated RING(1-48) was high (70%, based on thioester).

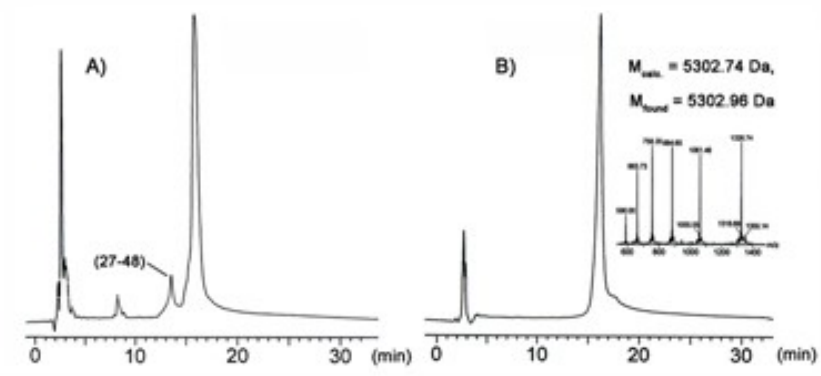

Fig. 2. (A) HPLC-analysis of crude RING(1-48) after 6 h ligation and (B) purified product.

Acknowledgments

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THE IMPORTANT ROLE OF 2,6-DIMETHYL-L-TYROSINE (DMT) FOR MANIFESTATION OF HIGH μ-OPIOID RECEPTOR AFFINITY IN OPIOIDMIMETICS

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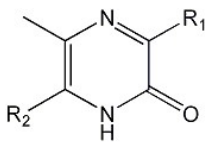
Introduction

The essential factor is N-terminal tyrosine residue in peptides to interact with opioid receptor, except for nociceptin, which has Phe at N-terminal. The 2',6'-dimethyl-L-tyrosine (Dmt) in lieu of the above Tyr residue dramatically enhanced receptor affinity and functional bioactivity and consistently altered receptor selectivity. The H-Dmt-NH-CH_3 was found to interact with μ-opioid receptor (K_i^{μ} = 7.45 nM) in the same range as morphine. However, the H-Tyr-NH-CH_3 did little interact with μ-opioid receptors (K_i^{μ} = 23,000 nM) [1]. The Tyr or Dmt were dimerized through diaminoalkanes [2] or 3,6-bis-(aminoalkyl)-2(1*H*)-pyrazinones [3] as spacer, in which Dmt dimers exhibited high receptor affinities and potent biological activities [3]. For further studies on the role of Dmt, Dmt monomeric pyrazinone derivatives were newly prepared by the general synthetic methods and their receptor affinities and biological activity were examined.

Results and Discussion

The structure of pyrazinone ring-containing opioid mimetics are shown in Fig. 1. Of the Tyr dimers, (**7**) exhibited the high μ-receptor affinity (K_i^{μ} = 7.58 nM), equal to that of morphine, and high receptor selectivity (K_i^{δ}/K_i^{μ} = 220) compared with other Tyr-pyrazinone derivatives (**1, 3, 5**: K_i^{μ} = 25.7 - 70.2 nM and K_i^{δ}/K_i^{μ} = 17 - 65) and substitution of Tyr with Dmt (**8**) enhanced μ-opioid receptor affinity by 361-fold (K_i^{μ} = 0.021 nM) with much higher selectivity (K_i^{δ}/K_i^{μ} = 1,519). Similarly, the affinity of **2, 4, 6** (K_i^{μ} = 0.041 - 0.114 nM) increased 537- 614-fold with replace of Tyr by Dmt, compared with the corresponding Tyr derivatives (**1, 2, 3**); however, in the case of 1,4-bis-[Dmt or Tyr -NH]butane, the relative activity (RA; the activity of Dmt derivative compared with the corresponding Tyr derivative) rose to 7,537, on the other hand, only 4.6-fold increases with respect to endomorphin-2. From the values of RA obtained, it was revealed that the degree of effects by replacement of Tyr with Dmt on μ-receptor affinity is depending on the structure of the parent molecule. The δ-opioid receptor affinity of (**5, 7**) are non-existent (K_i^{δ} = 2,770 and

924 nM, respectively), whereas the Dmt derivatives (**6, 8**) exhibited fairy high affinity (K_i^{δ} = 18.8 and 31.9 nM, respectively) with RA of 147 and 29, respectively, and they exhibited unique *in vitro* biological activity in MVD assay. Regarding Dmt-monomeric pyrazinone derivatives (**9, 10, 11, 12**), Dmt derivative (**12**) increased μ-receptor affinity by 313-fold relative to 11. Interestingly, although the compound (**10**) exhibited high μ-receptor affinity (K_i^{μ} = 0.62 nM) equal to that of endomorphins, it did not show agonistic activity in GPI assay. These results opened new possibility to design novel μ-opioid antagonists.



R_1	R_2
(1) H-Tyr-NH-$(CH_2)_3$	H-Tyr-NH-$(CH_2)_3$
(2) H-Dmt-NH-$(CH_2)_3$	H-Dmt-NH-$(CH_2)_3$
(3) H-Tyr-NH-$(CH_2)_4$	H-Tyr-NH-$(CH_2)_4$
(4) H-Dmt-NH-$(CH_2)_4$	H-Dmt-NH-$(CH_2)_4$
(5) H-Tyr-NH-$(CH_2)_3$	H-Tyr-NH-$(CH_2)_4$
(6) H-Dmt-NH-$(CH_2)_3$	H-Dmt-NH-$(CH_2)_4$
(7) H-Tyr-NH-$(CH_2)_4$	H-Tyr-NH-$(CH_2)_3$
(8) H-Dmt-NH-$(CH_2)_4$	H-Dmt-NH-$(CH_2)_3$
(9) H-Dmt-NH-$(CH_2)_4$	-CH_3
(10)H-Dmt-NH-$(CH_2)_4$	- H
(11)-CH_3	H-Tyr-NH-$(CH_2)_4$
(12)-CH_3	H-Dmt-NH-$(CH_2)_4$

Fig. 1. The structure of pyrazinone-containing opioidmimetics.

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DEVELOPMENT OF UNIQUE AND POTENT μ-OPIOID AGONISTS AND ANTAGONISTS CONTAINING DMT

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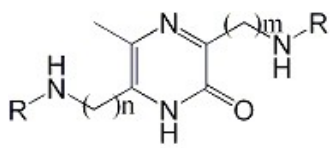
Introduction

It is well known that the N-terminal amino acid 2',6'-dimethyl-L-tyrosine (Dmt) in lieu of the natural N-terminal residue Tyr enhances binding affinity and bioactivity of numerous opioid peptide and opioid mimetic agonists and antagonists. C-Terminal modification of the Dmt by a methyl group, H-Dmt-NH-CH_3, exhibited μ-opioid receptor affinity (K_i^{μ} = 7.5 nM) similar to that of morphine; however, antinociception was only 0.64 - 0.85% relative to morphine [1]. Dmt plays an important role in the message domain to anchor opioid ligands into the active site of opioid receptors, but cannot trigger biological activity. Therefore, we attempt to prepare potent opioid agonists and antagonists, which resist enzyme degradation and pass through the epithelium membranes of the gastrointestinal tract and the blood-brain barrier. One approach entails dimerization of Dmt-containing pharmacophore through diaminoalkanes [2] or 3,6-bis-(aminoalkyl)-2(1*H*)-pyrazinone [3] (Fig. 1).



R: H-Dmt or H-Dmt-Tic (Tic = 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid)

Fig. 1. Structure of Dmt-containing pharmacophore dimers linked with pyrazinone ring.

Results and Discussion

Compounds **1** (R: Dmt, m = n: 3), **2** (R: Dmt, m = n: 4) and **3** (R: Dmt, m = 4, n = 3) exhibited high affinity for μ-opioid receptors (K_i^{μ} = 0.02 – 0.115 nM), agonism (GPI, IC_{50} = 1.3 – 1.9 nM), and antinociception in mice after systemic and oral administration, which verified passage through the epithelial membranes [3]. Furthermore, 1,4-bis-Dmt-NH-butane (**4**) and 1,6-bis-Dmt-NH-hexane (**5**) also exhibited high affinity for μ-opioid receptors (K_i^{μ} = 0.04 – 0.05 nM), agonism (GPI, IC_{50} = 3.0 - 5.3 nM), and antinociception in mice after systemic administration [2].

The tail to tail condensation of the δ-antagonist Dmt-Tic pharmacophore, which has high affinity (K_i^{μ} = 0.022 nM) and extraordinary selectivity relative to the μ-opioid receptor (K_i^{μ}/K_i^{δ} = 150,800), yielded potent δ-opioid receptor antagonists that exceeded that of the prototype (H-Dmt-Tic-OH: pA_2 = 8.43) by orders of magnitude (pA2 = 10.30 - 11.22) and *N,N*-dimethylation of 1,6-bis-[Dmt-Tic-NH]hexane (6) and 3,6-bis-[Dmt-Tic-NH-propyl]-pyrazinone (**7**) produced potent μ-/δ-opioid antagonists (pA_2 = 8.34 and 7.71 in GPI assay and pA_2 = 11.38 and 10.42 in MVD assay, respectively). μ-Opioid antagonists are important pharmacological tools not only to delineate critical biological, pharmacological and physiological roles played by these receptors, but also to serve as clinically and therapeutically relevant agents. *N*-Allylation converted the potent μ-opioid agonists [Dmt^1]endomorphin-1 and -2 into μ-opioid antagonists (**8** and **9**; pA_2 = 8.18 and 8.59, respectively). Compound (**9**) was a potent μ-opioid antagonist in dose-dependent manner against morphine, while 300 nM [*N*-allyl-Dmt^1]EM-1 (**8**) inhibited the spontaneous inhibitory post-synaptic currents (IPSC) elevated by 60 mM ethanol (Fig. 2), without affecting baseline activity. Thus, **8** might be a potential candidate in the alleviation of alcohol dependency.

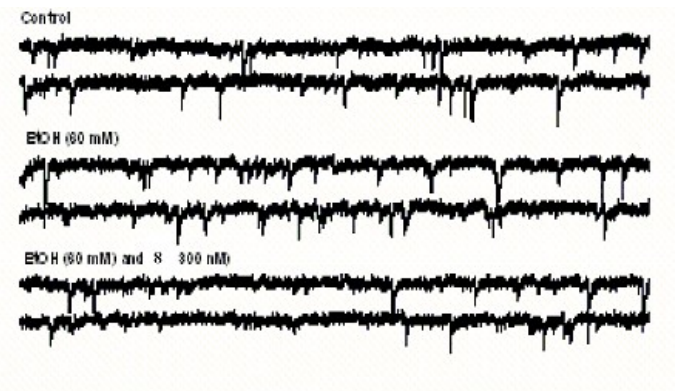


*Fig. 2. Changes in spontaneous IPSC frequency in the presence of ethanol and ethanol plus [N-allyl-Dmt^1]endomorphin-1 (**8**).*

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HLDF-6 HEXAPEPTIDE NEUROPROTECTIVE EFFECT ON RAT HIPPOCAMPAL NEURONS AT THE ALZHEIMER`S DISEASE MODEL IN VIVO AND *IN VITRO*

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Introduction

Alzheimer`s disease is one of the most widespread neurodegenerative disorders. Its main feature is an impairment of the brain cognitive functions. The presence of many extracellular amyloid (senile) plaques consisting mainly of β-amyloid peptides is the main biochemical hallmark of Alzheimer's disease.

The hexapeptide TGENHR (HLDF-6), which was first identified as a fragment of HLDF (Human Leukemia Differentiation Factor) molecule, displays a wide scope of biological activities *in vivo* and *in vitro* [1]. It protects the neurons of cerebellum from degeneration caused by sodium azide and prevents the disturbances of memory and cognitive functions of animals after intracerebellar introduction of this toxic agent. Although HLDF is expressed both in males and females, the neuroprotective and nootropic effects of HLDF-6 were shown only on males. We have demonstrated that HLDF-6 increase the testosterone level in male mice by stimulation of its biosynthesis.

The goal of our study is investigation of the protective effect of HLDF-6 on the neurons of rat hippocampus on the model of Alzheimer's disease *in vivo* and on the primary cultures of hippocampal neurons cultured in the presence of β-amyloid peptide *in vitro*. We also tried to study the mechanism of neuroprotective effect of HLDF-6.

Results and Discussion

The symptoms of Alzheimer's disease in male rats were induced by coinjection of β-amyloid peptide (25 - 35) and ibotenic acid into the rat hippocampus. HLDF-6 peptide precludes long-term memory loss and exploratory behavior activity decrease in such animals that was shown with Morris water maze learning test and passive avoidance test; furthermore, it reduces significantly the amount of pyknotic neurons in the CA1 area of hippocampus. The neuroprotective effect of this peptide was also demonstrated *in vitro* on the primary culture of rat hippocampal neurons upon β-amyloid caused toxicity. A significant increase in dihydrotestosterone (DHT) level was shown both in blood plasma of rats with Alzheimer's disease symptoms and the cultural medium of hippocampal neurons cultivating in the

presence of β-amyloid peptide. This was prevented by HLDF-6 peptide in both cases.

The results obtained allow us to suggest the next mechanism of the HLDF-6 neuroprotective action: β-Amyloid peptides decrease the fluidity of lipid membrane and activate the 5α-reductase. As a result the DHT concentration increased greatly. DHT at the same time stimulates the binding of *N*-methyl-D-aspartate (NMDA) to the receptors of pyramidal neurons in the CA1 area of hippocampus in males. Increase in the exitotoxicity mediated by NMDA receptors results in the death of the neurons of hippocampus. HLDF-6 increases the fluidity of lipid membrane decreasing the 5α-reductase activity and stimulates the neurosteroids genesis shifting the testosterone metabolism to the formation of estrogens. Thus HLDF-6 prevents the overactivation of NMDA receptors and protects the neurons of hippocampus from the death.

Acknowledgements

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TRANSLOCATION AND CELLULAR TRAFFICKING OF CELL PENETRATING PEPTIDES (CCP). DESIGN OF A MASS STABLE REPROTER TAG

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Introduction

Plethora of cell-penetrating peptides (CPP) has now been described. All the plausible mechanisms that allow transduction of one molecule from the extracellular to the intracellular medium have been stated to explain their cell-entry mechanism, which does not involve any chiral recognition [1]. The procedure we have developed to quantify by MALDI-TOF mass spectrometry the intracellular concentration of CPPs required a biotin with a linker composed of an isotope tag of either four [H,H]glycines (H-CPP) of four [^{2}H,^{2}H]glycines (D-CPP) [2]. Now, we report the design of a mass stable reporter (msr) in order to track the intracellular peptide and get insights into the cell-entry mechanism.

Results

The msr tag, trifluoroacetyl-(α,α-diethyl)glycine-Lys(N$^{\varepsilon}$biotin)-(D)Lys-Cys, required first the synthesis of the H- and D-form of trifluoroacetyl-(α,α-diethyl)glycine [3], starting from ethyl-*N*-((4-chlorophenyl)methylene)-glycinate. Dialkylation of this imine led to the H-(C_2H_5) or D-form (C_2D_5). Aldimine hydrolysis and saponification yielded (α,α-diethyl)glycine, which was trifluoroacetylated. The peptides were then obtained starting from a MBHA resin up to the N-ter cysteine. After the last coupling, the rest of the sequence was coupled manually with HBTU activation, except for the dialkylated residue, which required HATU activation.

This msr tag, introduced at the N-ter of $(RW)_9$, (^{msr}RRWWRRWRR-NH_2), a shorter analogue of the CPP (RW)16 [4], was shown to be stable to protease exposure (trypsin, chymotrypsin, pepsin and CHO-K1 lysates). The uptake into CHO-K1 cells of $^{msr}(RW)_9$ (7.5 µM) was found maximal after 30-60 min incubation and remained stable for at least 4 hours. The amount of internalized intact $^{msr}(RW)_9$ corresponds to 27 ± 3 µM (with 1.5 µL intracellular volume and 40.0 ± 4.6 pmoles of internalized peptide). No outflow of the full-length peptide was detected up to 18 hours.

No significant difference was detected both in the degradation fragments and in their relative intensities, when CHO cells were pre-incubated with NH_4Cl, which affects the acidic lysosomal pH, prior to the usual uptake experiment. Finally, KCl addition [5] to the incubation medium evoked a marked decrease of the cellular uptake of $^{msr}(RW)_9$, at 55 mM KCl the amount of internalized $^{msr}(RW)_9$ was reduced by 55%.

Discussion

Kinetic analysis of $^{msr}(RW)_9$ internalization showed that the steady-state is reached within 30-60 min with about 25 μM of peptide inside the cells, this concentration remaining stable for at least 4 hours. No saturation of the uptake process was detected when the extracellular concentration of the $^{msr}(RW)_9$ peptide was maintained at the same level (7.5 μM). Interestingly, these micromolar uptake concentrations are in the range that might be needed for a drug targeting an intracellular target with a micromolar affinity.

We showed that the membrane potential is a key element in the cell-entry mechanism. And, the analysis of the $^{msr}(RW)_9$ degradation as a function of time as well as the absence of action of NH_4Cl on the degradation profile, led us to conclude that once into cells the peptide should not be in lysosomes.

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TRICHOBREVINS, TRICHOCOMPACTINS, TRICHOCRYPTINS, AND TRICHOFERINS: NEW PEPTAIBIOTICS FROM PLANT-PROTECTIVE STRAINS OF THE TRICHODERMA BREVICOMPACTUM COMPLEX

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Introduction

Species of the fungal genus Trichoderma are commercially used as bioprotective agents against fungal plant diseases in agriculture. More than 400 strains were collected from their natural habitats and evaluated for biocontrol properties in viticulture. The biological activity towards Eutypa dieback and Esca, which are fungal diseases of grapevine trunks, was tested in plate bioassays. Seven of the most active isolates were classified as *Trichoderma brevicompactum*, or shown to be closely related to that species (*Trichoderma* cf. *brevicompactum*) [1].

Result and Discussion

Using recently described procedures [2 – 4], we could detect 68 novel peptaibiotics in these seven strains mentioned above (abbreviation and number of individual peptides produced in parentheses): 12-residue trichocryptins B (TCT-B; 14), 11-residue trichocryptins A (TCT-A; 12), 11-residue trichobrevins A and B (TBV; 19), 10-residue trichoferins (TFR; 6) and 8-residue trichocompactins (TCP; 17). Notably, all isolates also produced alamethicins F-30 [5]. The data support the hypothesis that peptaibiotics may partly be responsible for the established plant-protective activity of the Trichoderma strains tested. Representative major sequences of new peptaibiotics are listed in *Fig. 1*. Taken together, the differential patterns of peptaibiotic production and as well as the production of different trichothecene-type mycotoxins [6] clearly support DNA sequencing results [4]. Both molecular and chemotaxonomic approaches indicate the existence of two species within what has been called *Trichoderma brevicompactum*, so far.

	Residue											
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Peptaibiotic												
TCP IIIa	Ac	Aib	Gly	Ala	Lxx	Vxx	Gly	Lxx	Vxx			
TCT-A IIa	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol
TCT-B IIIb	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol
TBV-A IIa	Ac	Aib	Ala	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	
TBV-B IIIc	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	
TFR-A	MDA	Pro	AHMOD	Ala	Aib	Aib	Lxx	Ala	Aib	Aib	AMAE		

Figure 1. Representative major sequences of new peptaibiotics from strains of the T. brevicompactum complex. ***MDA****, α-* ***m****ethyl****d****ecanoic* ***a****cid;* ***AHMOD:*** *2-****a****mino-4-****m****ethyl-6-****h****ydroxy-8-****o****xo-****d****ecanoic acid,* ***AMAE****, 2-[(2'-****a****minopropyl)-****m****ethyl****a****mino]-****e****thanol.*

Acknowledgements

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NEW FRAGMENT ANALOGUES OF ANTISTASIN'S (ATS) ACTIVE SITE – DESIGN, SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP

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Introduction

In the recent years from the salivary glands of several bloodsucking animals like, teaks, leeches, vampire bats and so forth are isolated plenty of proteins and peptides with different molecular weight and well established anticoagulant activity. Many of the strongest anticoagulants isolated by bloodsucking animals are found in the extract of salivary glands of different kinds of leeches. Such leech is the Haementeria officinalis, from which is isolated the most active inhibitor of factor Xa (fXa) – ATS. This protein has molecular mass 15 kD consist of 119 amino acid residues including 20 cysteines. It consists of 2 domains (domain I - Arg^{32}-Cys-Arg-Val-His-Cys-Pro-His-Gly-Phe-Gln^{42} and domain II - Asn^{87}-Cys-Arg-Lys-Thr-Cys-Pro-Asn-Gly-Leu-Lys^{97}) which were interested us because of their similarity in the primary structure with many other later isolated peptides with high anticoagulant activity like bdellastasin, piguamerin, ghilantens, therostasin etc. In order to study the role of some amino acids in the process of interaction among peptides mimetics and the active site of fXa, some fragment analogues of ATS's active site were synthesized: 1. by removing of some amino acids 2. by replacement of some amino acids with the other with similar structure.

Result and Discussion

The synthesis of the following peptides Arg-Cys-Arg-Val-His-Cys-Pro-His-Gly-Phe-Gln; Asn-Cys-Arg-Lys-Thr-Cys-Pro-Asn-Gly-Leu-Lys; Mpa-Arg-Val-His-Cys-Pro-His-Gly-Phe-Gln; Mpa-Arg-Lys-Thr-Cys-Pro-Asn-Gly-Leu-Lys was realized by SPPS method and Fmoc-strategy on the Wang resin. The HBTU/DIPEA or DIPCDI methods were used for coupling of each amino acid.

The purification of peptides were carried out by RP-HPLC. The structures of the newly synthesized peptide were analyzed and proved by ES/MS.

The obtained peptides were studied for anticoagulant activity in respect to APTT and IC_{50} values were determined (Table 1). The data for APTT in the Table refer to values above Ref. t. 28.1 sec.

The accompanying differences in IC_{50} values, as measured with the APTT blood coagulation assay, suggest that the C-terminus of ATS (amino acids 109 - 119) is an important determinant of inhibitory potency [1]. In the previous our works we investigated different fragment analogues of C-terminus (109 - 116) of ATS. We observed that previously synthesized analogues of C-terminus of ATS have higher or similar anticoagulant activity than analogues of the active site of ATS [2].

Table 1. IC_{50} values of newly synthesized peptides

Peptide	IC_{50} (mol . 10^{-6})
Arg-Cys-Arg-Val-His-Cys-Pro-His-Gly-Phe-Gln	1.26
Asn-Cys-Arg-Lys-Thr-Cys-Pro-Asn-Gly-Leu-Lys	0.76
Mpa-Arg-Val-His-Cys-Pro-His-Gly-Phe-Gln	1.37
Mpa-Arg-Lys-Thr-Cys-Pro-Asn-Gly-Leu-Lys	7.06

Our investigation on the structure-activity relationship of a series of new analogues of domain I and domain II of ATS reveal that:

1. The availability of Arg32 or Asn87 isn't significant meaning for the anticoagulant activity

2. The chain shorting of N-terminus with an amino acid doesn't lead to significant decreasing of anticoagulant activity.

3. Replacement of Cys33 with Mpa haven't influence on the anticoagulant activity

4. Replacement of Cys88 with Mpa leads to 10 fold decreasing of anticoagulant activity.

Acknowledgements

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POTENTIAL METAL ATOM BINDING SITES IN INSECT KININ ANALOGS

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Introduction

Insect kinins share a highly conserved C-terminal pentapeptide sequence Phe-Xaa-Xbb-Trp-Gly-NH_2, where Xaa can be Tyr, His, Ser or Asn and Xbb can be Ala but is generally Ser or Pro. They are potent diuretic peptides that preferentially form a *cis*-Pro, type VI β-turn. An insect kinin analog containing (*2S,4S*)-4-aminopyroglutamate (APy), a novel *cis*-peptide bond, type VI β-turn motif has demonstrated significant insect diuretic activity [1, 2]. Metal ions act as important factors that influence the structure of oligopeptides and, therefore, may affect their bioactivity. In this work, the interaction of copper(II) ions with peptidomimetic insect kinin analogs, the stereoisomers Ac-Arg-Phe-apy-Trp-Gly-NH_2 and Ac-Arg-Phe-aPy-Trp-Gly-NH_2, have been studied. To determine the coordination mode of the metal ion in the peptide-metal complex, spectroscopic studies, along with computer modelling, have been performed.

Result and Discussion

The CD spectra, electronic absorption and EPR parameters allow assignment of stepwise bonding of nitrogen atoms to copper(II) ion in the metallopeptide molecule with increasing pH. Copper starts to bind the ligand at a pH 7. Once the copper ion is "anchored" to a peptide, it can displace protons, leading to the formation of complexes with four nitrogen donor atoms bounded. A CD signal located at 311 - 315 nm and the EPR parameters: $A_{\parallel}=208 \times 10^{-4}$ cm^{-1} and $g_{\parallel}=2.174$ confirm the 4N coordination in the equatorial plane. Electronic absorption spectra characterized by a wavelength of maximum absorption at 514 nm also support the four nitrogen donor atoms allocated in the coordination sphere of Cu(II) ion . One can conclude that the insertion of (*2R,4R* or *2S,4R*)-4-aminopyroglutamate (apy or aPy), a novel *cis*-peptide bond mimic influences the coordinating ability of the peptide studied. It stabilizes a folded peptide conformation that is more favorable for the formation of two five-membered chelating rings with a very likely $\{N^{-}_{apy/aPy}, 2N^{-}_{amide}, NH_{2Gly}\}$ coordination mode in the metallopeptide molecule. This bent conformation achieved may be essential for binding of the peptide at the receptor site.

Acknowledgements

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ANTITUMOR ACTIVITY OF NEW α-AMINOPHOSPHONIC ACIDS

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Introduction

Aminophosphonic acids constitute an important class of biologically active compounds, in view of their diverse biological effects together with insignificant toxicity towards mammalian cells. These compounds are effective in suppresing the tumour growth and are investigated as potential lead compounds with anticancer, antivirus and antibacterial activity [1, 2]. This study is a continuation of our previous work on the synthesis, genotoxic and antiproliferative effects of new α-aminophosphonic acids [3]. The correlation between the moderate clastogenic effect and the low values of mitotic index, obtained after the treatment with new compounds, gave us ground to carry out further more detailed investigations.

Result and Discussion

In order to evaluate the antitumor activity we synthesized new α-aminophosphonic acids **1 - 6** (Fig. 1). The phosphonopeptide analogue **7** was synthesized by SPPS (Solid Phase Peptide Synthesis) Fmoc-strategy. Wang resin was used as a solid-phase carrier, and TBTU/HOBt/DIEA was used as a condensing reagent.

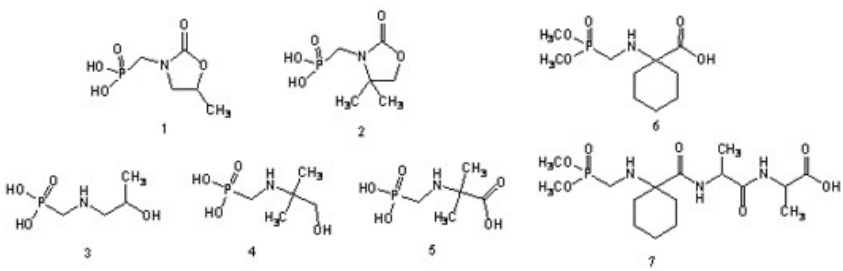

*Fig. 1. The structure of **1 - 7** was established by IR, NMR and MS spectroscopy.*

The cytotoxic activity of the tested compounds **1 - 7** was evaluated using the MTT-assay in a panel of human tumour cell lines, namely the acute myeloid leukemia HL-60, the chronic myeloid leukemias LAMA-84 and K-562, the non-Hodgkin lymphoma DOHH-2, the Hodgkin lymphoma HD-MY-Z and the urinary bladder carcinoma Ej. Cisplatin was utilized as reference compound (Table 1).

*Table 1. Cytotoxic activity of compounds **1 - 7** in a panel of human tumor cell lines as assessed by the MTT-dye reduction assay after 72 h treatment.*

Tested compounds	IC_{50} values (μM)					
	HL-60	K-562	LAMA-84	DOHH-2	HD-MY-Z	Ej
1	150.7	> 200	157.8	122.8	> 200	161.4
2	144.0	180.9	165.1	120.0	> 200	189.3
3	154.2	> 200	168.9	107.7	> 200	198.1
4	147.3	190.7	172.8	93.4	> 200	193.7
5	151.4	> 200	176.8	95.5	> 200	185.1
6	136.2	98.6	128.3	84.2	164.5	116.1
7	> 200	> 200	> 200	> 200	> 200	> 200
cisplatin	6.5	22.2	8.3	11.2	21.2	7.3

The aminophosphonates displayed cytotoxic activity at micromolar concentrations, whereby invariably compound 6 proved superior vs. the other analogues.

It is interesting that the inclusion of the dipeptide Ala-Ala into 1-(dimethoxyphosphoryl methylamino)cyclohexanecarboxylic acid **6** leads to significant loss of cytotoxic activity (Table 1).

The encountered cytotoxicity of the tested aminophosphonates *in vitro*, together with their previously established moderate clastogenic potential warrants further detailed exploitation of this structural scaffold, in order to define more precisely the structure activity rules and the mechanistic peculiarities for this novel class of cytotoxic agents.

Acknowledgements

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TRANSPORT OF PYRROLIDINE-BASED OXY-PEPTIDE NUCLEIC ACIDS INTO CYTOPLASM OF CHO CELLS

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Introduction

Peptide nucleic acids (PNAs) form stable hybrids with RNA because the neutral backbone of PNAs leads to stable hybrids with RNA [1]. But at the same time, the neutral backbone causes low solubility of the PNAs to form aggregates in aqueous solution. This undesirable property limits the use of PNAs in medicinal application [2]. To overcome the drawback, we synthesized new types of PNAs with improved water solubility by containing ether linkages and pyrrolidine rings in the main chain; pyrrolidine-based oxy-PNAs (POPNAs) [3]. The pyrrolidine ring possesses two chiral centers and there are four stereoisomers (*cis*-L-, *trans*-L-, *cis*-D-, and *trans*-D-configurations). Previously, we showed that, among the adenine homo-oligomers of the four different configurations, *trans*-L POPNA formed the most stable hybrid with the complementary RNA. In this study, we investigated cellular uptake and endosomal release of the *trans*-L-POPNA oligomers. The cellular uptake was achieved by mixing the POPNA oligomer with an N-terminal 23-mer peptide of the influenza virus hemagglutinin protein (HA2) that is covalently linked with an Arg heptamer.

Results and Discussion

The *trans*-d-POPNA oligomers were manually synthesized on solid phase peptide synthesis under Fmoc strategy. The sequences of the *trans*-L-POPNA oligomers were FAM-O-*TGG TGC GAA TTC*-K-NH_2 [FAM-PO(12)], FAM-O-*TGG TGC CTC*-O-RRRRRRR-NH_2 [FAM-PO(9)-R7], and FAM-O-*CAG TTA GGG TTA* G-G-NH_2 [FAM-PO(13)], where K, R, and G indicate Lys, Arg, and Gly, respectively. "O" indicates an AEEA linker. The purified oligomers were identified by MALDI-TOF mass spectroscopy.

Internalization of the *trans*-L-POPNA oligomer into CHO cells was observed with confocal laser-scanning microscopy. First, the CHO cells were cultured in the presence of 10 μM FAM-PO(12). No fluorescence was, however, observed with the CHO cells. In the case of FAM-PO(9)-R7, the FAM-fluorescence was detected. However, a closer look at the image indicates that the fluorescence is mostly confined in endosomes and even after 6 h incubation, the fluorescence image did not change. To release the POPNA oligomer from the endosomes, we added an HA2 that is labeled with a rhodamine fluorophore at the N-terminal and covalently linked with seven Arg at the C-terminal (Rho-HA2-R7). HA2 is known to disrupt endosomes [4]. The fluorescence images of the CHO cells after incubation with FAM-PO(13) in the absence and presence of Rho-HA2-R7 were observed.

Incubation with FAM-PO(13) alone, no internalization of the oligomer was observed. In the presence of Rho-HA2-R7, however, FAM-PO(13) was successfully internalized into CHO cells and, more importantly, the fluorescence spread over the whole cell. The fluorescence image indicates that the POPNA oligomer was internalized into cytoplasm within 1 h, when the oligomer was mixed with the HA2-R7 peptide. This indicates that the POPNA oligomers were taken up into endosomes together with the Rho-HA2-R7 and released into cytoplasm as the disruption of the endosomes by the HA2 peptide.

In this study, the POPNA oligomers were readily taken up into cytoplasm of CHO cells, when mixed with a HA2-R7 peptide. The POPNA is advantageous over the PNA, because of its wider applicability of base sequences and improve water solubility.

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THE CHANGES OF AMINO ACIDS CONTENT IN CONSEQUENCE OF STERILIZATION IN PROCESSED CHEESE

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Introduction

Combat rations are designed for boarding of soldiers and members of rescue system during their operation employment. The food components (including processed cheese) should have the shelf life at least 2 years at ambient temperature [1]. There is a possibility for reach the processed cheese durability at least of 2 years in its thermo-sterilization [2]). The basic materials for the processed cheeses production are the natural cheeses. The production is made by heating of prepared mixture of materials at particular under pressure and at continuous mixing to the reaching of homogenous matter of requested consistency [3].

By the previous studies [4] it was found, that the thermo-sterilization heating (117^{o}C for 20 minutes) causes the decrease of content of amino acids (usually to 10%). The aim of the work was to verify these results with the new batch of processed cheeses.

Results

Two groups of processed cheeses were analyzed (dry matter - 40% w/w, fat in the dry matter 45% w/w). There were evaluated crude protein content (Kjeldahl method), ammonia content (Conway method) and amino acids content (ionic chromatography, Na-citrate buffers, ninhydrin detection).

The effect of the sterilization heating 117 ^{o}C for 20 minutes on the crude protein content was not insignificant ($P \geq 0.05$). In both investigated groups there occurred, due to the sterilization, the significant decrease of the content of methionin, aspartic acid, serine, glutamic acid, valine, histidine, lysine and arginine ($P < 0.05$). At the second group of processed cheeses there also occurred the statistically significant decrease of cysteine a proline, which was not observed at the first group. The typical decreases ranged around 2% of the original amino acid contents in the non-sterilized processed cheeses. The largest losses occurred at serine (around 4%). It was found that the content of ammonia increases in both groups of processed cheeses due to sterilization (approx. 76 mg/kg^{-1}).

Discussion

At investigated groups of processed cheeses there occurred lower decreases, than it was found in previous study [4]. The losses of amino-acids could be caused, for example, by Maillard reactions, Strecker degradation of amino acids, deaminations, formation of lysinoalanine, etc. [5, 6]. In the work there was also proved, that one of the reaction products of processes, at which there occurs the decrease of amino acid contents, is the ammonia. There will occur, due to used sterilization heating, only a small decrease of nutrition value of processed cheeses (from the point of view of amino acids content), which is balanced by longer durability (years).

Acknowledgements

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STUDY OF THE PROBLEM OF ASPARTIMIDE FORMATION IN SOLID PHASE PEPTIDE SYNTHESIS USING ODMAB GROUP TO PROTECT SIDE CHAIN OF ASPARTIC ACID

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One of the best documented side reactions in the synthesis of aspartic acid-containing peptides is aspartimide formation. In this study we report the aspartimide problem during Fmoc-based SPPS of branched analogues of galanin fragment GWTLNSAGYLLGPDA (**1, 2**) using a hydrazine-labile 4{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyloxy group (ODmab) to protect the β-carboxy side chain group of Asp. Moreover to investigate the susceptibility of the Asp-Ala motif for Asi formation during SPPS shorter galanin fragment LGPDA (**3a-g**) and different conditions as shown in Table 1 were applied. All peptides were synthesized with the use of the standard coupling protocol (a 3-fold molar excess for 1,5 h) and conditions as shown in Table 1. Products of synthesis were analyzed by RP-HPLC and MALDI-MS.

Table 1. Relative content of main products of synthesis determined by HPLC peak area integration.

Lp.	Protecting group of Asp	Resin	Coupling method (molar ratio 1:1:1)	Fmoc cleavage (for 5+15 min)	Relative content [%]		
					Asp	Asi	Asp(OMe)
1	ODmab	TentaGel S RAM	AA:TBTU:HOBt	20% pipridine/DMF	11	72	17
2	O*t*Bu	TentaGel S RAM	AA:TBTU:HOBt	20% pipridine/DMF	100	–	–
3a	ODmab	TentaGel S RAM	AA:TBTU:HOBt	20% pipridine/DMF	46	32	22
3b	ODmab	TentaGel S RAM	AA:DIPCI:HOBt	20% pipridine/DMF	62	38	–
3c	ODmab	TentaGel S RAM	AA:TBTU:HOBt	2% DBU/DMF	–	52	48
3d	ODmab	TentaGel S RAM	AA:TBTU:HOBt	20% pipridine/DMF + 0,1 mM HOBt	44	35	21
3e	ODmab	TentaGel S RAM	AA:TBTU:HOBt	6% piperazine/DMF + 0,1 mM HOBt	80	20	–
3f	ODmab	TentaGel S PHB	AA:TBTU:HOBt	20% pipridine/DMF	66	34	–
3g	ODmab	Cl-(2'-Cl)Trt-PS	AA:TBTU:HOBt	20% pipridine/DMF	91	9	–

Analysis of products of synthesis of peptide **1** using Asp(ODmab) have shown the presence of aspartimide (Asi) as the main product of synthesis. Also small quantities of aspartyl methyl ester (Asp(OMe)) and aspartyl (Asp) peptide were found. Mentioned above problem we did not observed during synthesis of peptide **2** using Asp(O*t*Bu). In this case only desired aspartyl peptide was found as the main product of synthesis. Studies with the use of a shorter galanin fragment modified with Asp(ODmab) also showed rapid and quantitative process of Asi and Asi-related by-products formation (**3a**). Analysis of products of synthesis of peptides

3a-g showed the presence (in various quantities) of Asi, Asp(OMe) ester or target aspartyl peptide. α/β-Piperidides, reported as the main by-products result from Asi ring opening by piperidine, were not found in our study.

We have shown that the use of base stronger than piperidine (such as DBU) to Fmoc removal strongly promote Asi formation (**3c**). Also the use of TBTU/HOBt coupling method containing DIPEA in excess seems lead to Asi formation more efficiently than DIPCI/HOBt method (**3b**). Unexpectedly, application of a sensitive to mild acids Cl-(2'-Cl)Trt-PS resin (**3g**) or Fmoc cleavage mixture containing 6% piperazine (**3e**) caused a considerable decrease in content of Asi. As we found that Asp(OMe) ester formation results from Asi ring opening by the extensive use of MeOH, as a washing solvent during synthesis, we replaced MeOH with EtOH (**3e-g**). Alternatively we have shown that the removal of DIPEA from the synthesis protocol (**3b**) also solved this problem.

Our study suggests that the application of protocol based on Cl-(2'-Cl)Trt-PS resin, HOBt/DIPCI coupling method and Fmoc cleavage mixture containing 6% piperazine may lead to successful synthesis of Asi-free peptides using Fmoc-Asp(ODmab)-OH.

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PACLITAXEL COVALENTLY BOUND TO PEPTIDES AND SEQUENTIAL OLIGOPEPTIDE CARRIERS: SYNTHESIS AND ANTITUMOR ACTIVITY

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Introduction

Paclitaxel, the most important anticancer drug, isolated from the bark of the Pacific Yew tree Taxus brevifolia [1], is mainly used in the treatment of breast, lung, and ovarian cancer. Its anticancer activity is based on the ability of promoting microtubule assembly and inhibiting cell replication in the later G2 or M phase of the cell cycle [2]. A major problem for the clinical use of this drug is the low solubility in water and in most pharmaceutically acceptable solvents. In this work, we present the synthesis of three analogues in which paclitaxel was covalently bound to peptides or as multiple copies to synthetic carriers. The resulted peptide-paclitaxel derivatives possess greater solubility in water and inhibit the proliferation of human breast, prostate and cervical cancer cell lines.

Results and Discussion

The Ac-Cys-Arg-Gly-Asp-Arg-NH_2, and Ac-[Lys-Aib-Cys]$_n$-NH_2, n = 3, 4, peptides used for placlitaxel conjugation were synthesized on a Rink Amide resin by the solid phase peptide synthesis methodology using the Fmoc/tBut strategy. Reagent I (92,5% TFA: 2,5% TIS: 5% DMB) was used for the deprotection and cleavage of the peptides from the resin [3]. The chemoselective ligation method was applied for conjugation of Paclitaxel to peptides. For this purpose the Paclitaxel was chloro- or bromoacetylated to the C2' position.. The conjugation resulting in thioether linkage between the carrier and Paclitaxel was carried out by dissolving the chloro- or bromoacetylated Paclitaxel in MeCN/H_2O (2/1, v/v) under N_2 at 0°C and the pH was adjusted at ~7 with DIEA. The peptide or the sequential carrier was added to the solution from time to time in solid form. The target conjugates were isolated in overal yields varried between ~30 - 40%. Biological assays of the synthesized analogues were performed using three cell lines: HeLa: human epithelial cervical cancer cells, DU: human epithelial prostate cancer cells MCF-7: human epithelial breast cancer cells.

The analogue Ac-[Lys-Aib-Cys(CH_2CO-$C^{2'}$taxol)]$_4$-NH_2 when used at low concentrations inhibited cell proliferation more potently than paclitaxel.

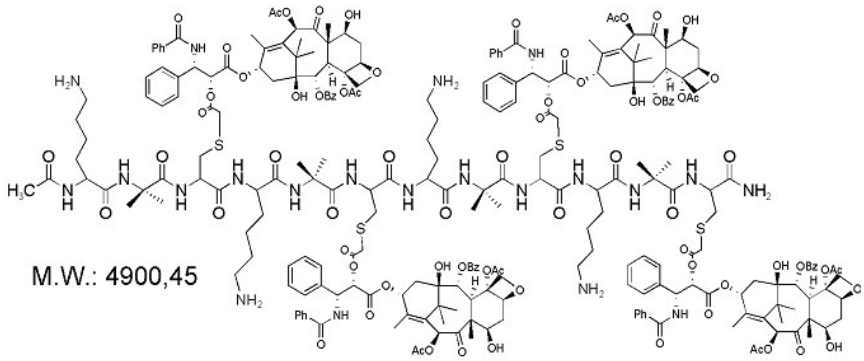

Fig. 1. Schematic representation of Ac-[Lys-Aib-Cys(CH_2CO-$C^{2'}$taxol)]$_4$-NH_2

In conclusion, three paclitaxel-peptide conjugates were successfully synthesized combining SPPS and chemoselective ligation methods. Two water-soluble paclitaxel-peptide conjugates showed similar biological activity to paclitaxel. The Ac-[Lys-Aib-Cys(CH_2CO-$C^{2'}$taxol)]$_4$-NH_2 derivative showed improved biological activity in comparison with paclitaxel in HeLa and DU cell lines.

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IN VITRO INVESTIGATION OF ANTIOXIDANT PROPERTIES OF SOME TYR-MIF-1 PEPTIDES IN ROS GENERATED SYSTEMS

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Introduction

Tyr-MIF-1 family peptides interact with opioid receptors and to non-opiate sites specific for each of the peptides [1, 2]. They could influence some maladies and have been recognized as modulators of reactive oxygen species (ROS) in mouse macrophages and human neutrophils [3]. So far no data about direct scavenger properties of Tyr-MIFs peptides about ROS were available. The aim of this study was to investigate the ability of Tyr-MIF-1 and MIF-1 to react with ROS: superoxide (O_2^-), hydrocyl radicals ($^{\bullet}OH$), hypochlorouse anion (OCl^-) and hydrogen peroxide (H_2O_2).

Results and Discussion

Four types of luminol-dependent chemiluminescence (CL) was used for ROS registration [4]. The ratio of CL in the presence and in the absence of the drug in percentage was termed CL scavenging index (CL-SI) [5]. All data is presented by CL-SI on Table 1.

Table 1. Scavenging properties of Tyr-MIF-1 and MIF-1 peptides against different ROS.

ROS Peptide concentration	Superoxide radical Assay I (CL-SI)	Hydroxyl radical Assay II (CL-SI)	Hypochlorouse anion Assay III (CL-SI)	Hydrogen peroxide Assay IV (CL-SI)
Tyr-MIF-1				
100 µmol/l	80.92 ± 4.85	65.82 ± 2.70	31.90 ± 2.83	52.36 ± 6.69
30 µmol/l	98.69 ± 6.62	91.61 ± 9.08	60.79 ± 4.87	74.21 ± 5.96
10 µmol/l	99. 45 ± 4.21	90.23 ± 7.41	78.02 ± 2.69	95. 45 ± 9.66
MIF-1				
100 µmol/l	106.10 ± 3.43	71.06 ± 4.43	37.83 ± 3.01	102.44 ± 9.42
30 µmol/l	101.55 ± 9.31	87.89 ± 8.21	60.95 ± 3.22	92.63 ± 11.81
10 µmol/l	101.03 ± 4.69	81.82 ± 4.99	78.69 ± 4.20	101.47 ± 8.20

The assay I was CL in a system of directly added superoxide - KO_2 [4]. The results are shown in Table 1-column 2. We found no significant changes of CL-SI for MIF-1 in all concentrations. Tyr-MIF-1 reduced CL-SI about 20% from control value at concentration of 100 µmol/l and had no significant effect at lower

concentration. These peptides do not exhibit a detectable scavenging effect for O_2^- in this system.

The two peptides have strongest •OH scavenging properties in this concentration. The effect was similar to Tyr-MIF-1 and MIF-1 (Table 1). They decreased chemiluminescent response about 35% and 30% respectively at 100 μmol/l. At 10 μmol/l the effect decreased about 10% from the controls. The higher scavenger properties of Tyr-MIFs toward •OH than O_2- were probably due to higher hydroxyl radicals' activity.

Assay III was CL in a system of NaOCl generated hypochlorite. At 100 μmol/l Tyr-MIF-1 and MIF-1 decrease CL-SI index by 3 times approximately the highest scavenger effect in all tested systems (Table-column 4). This effect remains significant in lower peptides concentration. This suggests a considerable ability for interaction with OCl-.

Assay IV was carried out in systems containing HPR (horse radish peroxidase) and H_2O_2. We investigated the ability of peptides to influence peroxidase dependent processes of H_2O_2 abolition. Tyr-MIF-1 decreased CL-SI in the system about 2 fold at 100 μmol/l. (Table 1–column 5). The effects decreased with lower concentrations.

MIF-1 had no interaction with this system.

The results suggest that scavenging properties of peptides should be taken into account in the free radical induced processes.

Acknowledgements

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FORMATION OF C-TERMINAL N-ALKYLATED AMIDE BYPRODUCTS IN FMOC-BASED SOLID PHASE PEPTIDE SYNTHESIS

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Introduction

The alkylation of sensitive amino acids (Trp, Tyr, Cys, Met, etc.) by carbocations resulted from the decomposition of the resin linker, is a common and well-documented phenomenon. This undesirable alkylation has been proved to occur in Fmoc-tBu strategy during removal of the peptide from the solid support. In order to minimize the occurrence of these side reactions a variety of multiple scavenger cleavage mixtures have been used in practice [1 - 3]. In our laboratory, using the Rink amide resin for the synthesis of various peptide amides by Fmoc strategy, we isolated, independently of the peptide sequence, a byproduct exhibiting an increase in molecular weight of 106 Amu compared to the target molecule. In this work we present the characterization and the procedure of preventing the formation of such byproducts.

Results and Discussion

The isolated byproduct, corresponding to a percentage of up to 35%, exhibited an absorbance at 280 nm in the UV detector despite the fact that aromatic residues were not present in the peptide sequence. The ESI-MS spectra revealed the presence of a molecular ion with an increased molecular weight of 106 Amu compared to the desired peptide.

As it is shown in Fig. 1 a C-terminal alkylated amide resulted from an inappropriate decomposition of the linker at positions **2** and **3** could explain the experimental results. This hypothesis was confirmed by recording the ^{1}H-NMR spectrum of the byproduct resulted during the synthesis of Gly-Ala-NH_2. The ^{1}H-NMR spectrum clearly demonstrated the presence of the p-hydroxy benzyl group covalently attached to the C-terminal amide group.

The possible cleavage positions shown in Fig. 1 prompted us to use the 1,3 dimethoxybenzene (DMB) as a component of the cleavage mixture. DMB represents the parent molecule of the resulted carbocation if the linker decomposition occurs at position 2. The use of DMB as component of the scavenger cocktail consisting of 92.5% TFA: 2.5% TIS:5% DMB (Reagent I) suppresses almost completely the alkylation of the C-terminal amide group during peptide cleavage from the Rink amide resin. Reagent I is suitable for the cleavage of a variety of peptides containing sensitive amino acids [4].

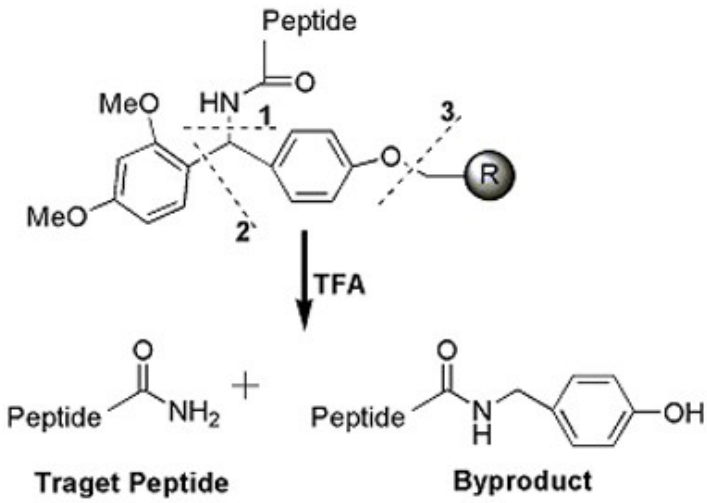

Fig. 1. Linker structure of the Rink amide resin. Dotted lines show the possible cleavage positions.

Conclusions

In this work we have demonstrated that decomposition of the Ring amide resin linker during TFA cleavage/deprotection step leads to the formation of C-terminal alkylated amide byproducts. The use of Reagent I as a cleavage mixture suppresses almost completely the formation of byproducts originated from the linker decomposition.

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SYNTHESIS AND ANTI-AGGREGATORY ACTIVITY OF (*S*,*S*) -CDC- AND AIIB 313-320 DERIVED HYBRID ANALOGUES

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Introduction

The -Arg-Gly-Asp- (RGD) motif of adhesive proteins and peptide analogues is recognized by the activated platelet integrin αIIbβ3 resulting in fibrinogen binding inhibition. Using strongly constraint cyclic (*S,S*) –CDC– containing compounds we were able to correlate the anti-aggregatory activity with the pseudo-dihedral angle, formed by the R-C^{ζ}, R-C^{α}, D-C^{α} and D-C^{β} atoms [1, 2]. On the other hand, in previous studies we attempted to determine the possible binding domains of αIIbβ3 in its activated state [3, 4]. The αIIb 313-320 sequence (YMESRADR) has experienced the highest anti-aggregatory activity. The synthetic octapeptide inhibits platelet aggregation and binds to immobilized fibrinogen [4]. However, the most interesting aspect is that both the cyclic (*S,S*) –CDC– containing compounds and the αIIb 313 - 320 analogue exhibit a non-RGD-like activity [2, 4]. Combining the available information from the –CDC– containing inhibitors and the sequence αIIb 313 - 320 we designed, synthesized and tested for their inhibitory activity, the new cyclic compounds **1-3** shown in Table 1.

Results and Discussion

The disulfide bridge formation of **1** and **3** was achieved by oxidation with $Tl(tfa)_3$ on solid support. For the synthesis of analogue **2**, after completion of the sequence of in solid phase, the Mtt side chain protecting group of Lys was selectively removed with 5% TFA, 1% TIS in DCM. The ε-amino group of the Lys was bromoacetylated using $BrCH_2COBr$ in DCM. All peptides were cleaved from the resin using 95% TFA, 2.5% DMB, 2.5% TIS at room temperature. The intramolecular thioether bond of **2** was generated in Tris/CH_3CN (3/1) solution at a concentration of ~ 0,5 mg/ml and pH 8.2. The IC_{50} values on ADP induced platelet aggregation of the tested analogues are given in Table 1.

Table 1. Inhibitory effect of the synthetic peptides on ADP induced platelet aggregation.

Peptide	M.W.		
	Expected	Found	IC_{50} (μM)
(S,S)YMESRCDCK-NH_2 (**1**)	1131.33	1131.10	80
Ac-Rc[K(NH)DC(S-CH_2CO)]R-NH_2 (**2**)	757.88	757.68	>>200
(S,S) Ac-CRDCR-NH_2 (**3**)	690.81	690.35	>>500
YMESRADR (**4**)	1027.13	1027.32	250
(S,S) Ac-RCDCR-NH_2 (**5**)	690.81	690.68	4.3±0.5
(S,S)PSRCDCR-NH_2 (**6**)	832.96	832.76	2.0±0.5

In conclusion, (i) the biological activity of the cyclic analogues is strongly affected by the size and the nature of the ring and (ii) the inhibitory activity of the αIIb 313-320 site can considerably be improved by incorporation of the -CDC- motif.

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SYNTHESIS AND RADIOLABELLING OF NEW ENDOMORPHIN ANALOGUES CONTAINING UNNATURAL α- AND β-AMINO ACIDS

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Introduction

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) are highly potent and selective endogenous μ-opioid receptor agonists (1). According to previous structure-activity studies, replacement of Tyr for Dmt (2', 6'-dimethyl tyrosine) resulted in potent but less selective endomorphins (2). Replacement of Pro by alicyclic β-amino acids as proline mimetics (*cis*-2-aminocyclopentanecarboxylic acid (Acpc) and 2-aminocyclohexenecarboxylic acid (Achc)) resulted in stable endomorphin analogues against proteolytic enzymes (3). This work focuses on new procedures of tritiated Dmt¹-endomorphin-2 isotopomers and Tyr-Acpc²/Achc²-endomorphin-2.

Result and Discussion

Precursors (Dmt¹-ΔPro²-Endomorphin-2, Tyr-*cis*-Acpc²-endoporphin-2, and Tyr-*cis*-ΔAchc²-endoporphin-2) and standard peptides (Dmt¹-endomorphin-2, Tyr-(*1S,2R*)Acpc²-endomorphin-2 and Tyr-(*1S,2R*)Achc²-endomorphin-2) were synthesized by SPPS. We used racemic Boc-*cis*-alicyclic β-amino acids (4) and diastereomer peptides were obtained. Diastereomers were separated by HPLC. Configuration of alicyclic β-amino acids in the peptides was determined after acidic hydrolysis by chiral TLC or GITC derivatization of the amino acid mixture and HPLC. The Rf and k' values of each alicyclic β-amino acid were compared to Rf and k' values of an enantiopure standard. The 3',5'I_2-Dmt¹-endomorphin-2 was prepared by iodination of Dmt¹-endomorphin-2 using chloramin-T. The four labelled endomorphin analogues were prepared in similar manner as we did earlier for parent tritiated endomorphins (5) using precursor peptides containing dehydro amino acids (ΔPro, Δ(*1S,2R*)Acpc or Δ(*1S,2R*)Achc), tritium gas and PdO/$BaSO_4$.

If 3',5'I_2-Dmt¹-endomorpnin-2 was used as precursor, catalytic dehalogenation occurred in the presence of tritium gas, PdO/$BaSO_4$ catalyst and triethylamine in a vacuum manifold. Specific radioactivity of ³H-labelled endomorphin analogues was very high (Table 1), practically theoretical values. In some cases (Dmt-³HPro-Phe-Phe-NH_2 and Tyr-³H-(*1S,2R*)Achc-Phe-Phe-NH_2) specific radioactivities were higher compared to the theoretical one, which means that exchange reaction occurred during the labelling.

Table 1. ³H-labelled new endomorphin analogues

Precursor peptides	Labelled peptides	Specific radioactivity
Dmt- ΔPro-Phe-Phe-NH_2	Dmt-^{3}HPro-Phe-Phe-NH_2	2.87 TBq/mmol
3',5'I_2Dmt-Pro-Phe-Phe-NH_2	3', 5'-^{3}HDmt-Pro-Phe-Phe-NH_2	1.95 TBq/mmol
Tyr- (1S, 2R)ΔAcpc-Phe-Phe-NH_2	Tyr-^{3}H(1S,2R)Acpc-Phe-Phe-NH_2	1.41 TBq/mmol
Tyr- (1S, 2R)ΔAchc-Phe-Phe-NH_2	Tyr-^{3}H(1S,2R)Achc-Phe-Phe-NH_2	2.35 TBq/mmol

Acknowledgements

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NOVEL TUMOR-TARGETED PEPTIDE-CAMPTOTHECIN CONJUGATES; SYNTHESIS AND BIOLOGICAL EVALUATION.

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Introduction

Small peptides have been used in literature, for targeting antitumoral drugs to specific membrane receptors over-expressed by tumor cells, such as somatostatin, bombesin, RGD peptide analogues and peptidomimetics. RGD peptides, that selectively recognize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, appear attractive candidates. Due to the role of integrins in the angiogenesis and tumor growing , tumor-targeted RGD peptide-cytotoxic drug conjugates are likely to exhibit a dual antitumor and antiangiogenic effect [1-3].

Results

We designed and synthesized new peptides containing the RGD sequence and bearing at 5-position a trifunctional amino acid with a carboxy-terminal side chain. Three peptides (Fig. 1) showing higher affinity to αv integrins, were selected as targeting devices. Analogues **P1-P3** were covalently attached with an amide bond to a Camptothecin derivative (CPT, **ST1968**, Fig. 2). The conjugates and peptides were prepared by classic methods of organic and peptide synthesis.

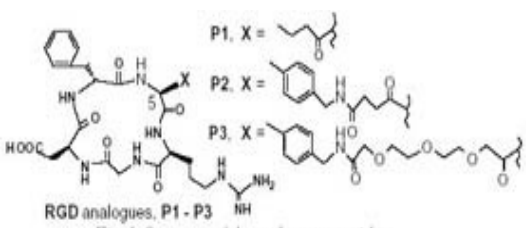

Fig. 1. Structure of the cyclopentapeptides

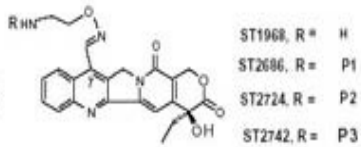

*Fig. 2. Structures of **ST1968** and conjugates*

Table 1. Binding of the peptides ***P1-P3*** *and the corresponding conjugates to the isolated* α_v *integrins and their effect on cell adhesionin the presence of vitronectin after 3 h treatment.*

	Binding assay, IC_{50} nM		Adhesion assay, IC_{50} μM		
Entry	$\alpha_v\beta_3$	$\alpha_v\beta_5$	PC3	A498	A2780
ST1968	no binding			no adhesion	
P1	28.6±0.76	0.17±0.01	38.8±8.5	6.5±0.6	5.2±0.2
ST2686	0.59±0.01	0.37±0.01	5.8±0.6	3.7±0.3	3.1±0.5
P2	37.6±0.99	5.1±0.07	33.7±7.8	34±2.2	9.5±1.9
ST2724	7.27±0.06	8.39±0.07	11.3±2.7	5.4±1.3	
P3	4.0±0.1	0.35±0.09	18.8±4.4	10.7±0.4	12.3±0.9
ST2742	12.5±2.1	6.5±0.03	8.3±1.0	3.1±0.3	7.1±0.3

Table2. Cytotoxicity on different tumor cell lines after 72 h treatment. IC_{50}, μM.

Entry	PC3	A498	A2780	H460[a]
ST1968	0.36±0.06	0.064±0.01	0.0049±0.001	1.07±0.18
ST2686	15±1.5	3.2±0.4	0.4±0.03	6.05±3.77
ST2724	4.7±0.4	4.3±0.1	0.16±0.001	16.4±10.9
ST2742	27±3.9	0.74±0.1	0 .18±0.01	3.53±0.11

[a] *after 1 h treatment.*

Binding to the isolated receptors and adhesion to the different tumoral cell lines were measured, showing that they effectively mediated the binding to the cell surface in a dose-dependent manner, whereas CPT did not bind the integrins or the cell surface (Table 1). We also demonstrated the internalization of our conjugates into tumor cells: After 72 h incubation, compounds were detected inside the cells by HPLC analysis with fluorometric detection. Table 2 reports the activities of the three conjugates compared with that of CPT alone.

Discussion

Differences within the conjugates can be explained by the composition of the linker that separates the integrin binding core from the cytotoxic molecule. The triethylene glycol chain seems to be a better mediator of the binding and activity. Considering together, cell adhesion and toxicity data, it is evident that the conjugates can efficiently bind to the membrane of the tumor cells. However, their cytotoxicity is more than one order of magnitude lower, if compared to the corresponding CPT alone, possibly due to the very slow release of the drug inside the cell. This behaviour must be attributed to the amide bond , between peptide and drug, too stable to be released in due time into the cell. On the other hand, cleavage assays revealed that the new analogues are potent Topo 1 poisons by themselves and share a common binding site with SN38, as documented by a similar pattern of DNA damage. Therefore, we can hypothesize that the cytotoxicity of the conjugates could be attributed to mixed effects, part to free CPT released and part to the whole conjugate, if able, to diffuse interacting with the enzyme.

In conclusion, the preliminary data presented prove the applicability of our targeting strategy. In vivo experiments are in progress.

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CHIMERIC MULTIFUNCTIONAL LIGANDS FOR THE TREATMENT OF PAIN

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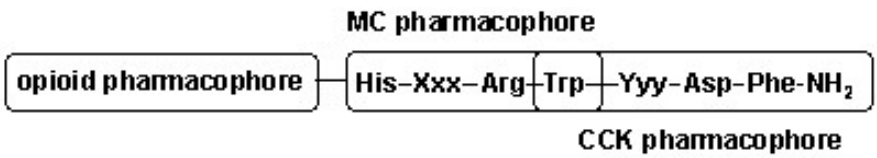
Introduction

Cholecystokinin (CCK) is known to give an anti-opioid effect and as a result, causes an increase of pain by inhibiting the opioid response [1 - 2]. Recent research has shown further that melanocortin (MC) receptors, mainly subtype MC-4R, produce an increase in response to pain stimuli [3 - 4]. Based on this previous work, we are developing chimeric ligands which will be of benefit to therapeutic pain treatment with enhanced opioid efficacy by acting as agonists at opioid receptors and antagonists at both CCK and MC receptors [5].



The design of the ligands was based on the hypothesis of targeting multiple receptors with overlapping pharmacophores. CCK and MC pharmacophores were overlapped by Trp, and different profiles of opioid pharmacophores were linked to the N-terminal of the MC pharmacophore.

Results and Discussion

The designed ligands showed moderate to high biological activity at both opioid and CCK receptors depending on their respective structures (Table 1). Preliminary data of some ligands also showed good affinity at the MC-4 receptor. From computer modeling experiments it was observed that the lowest energy conformation of LYS633 retains the turn structure of the MC pharmacophore. More interestingly, the aromatic group of the D-2Nal residue has the same predicted orientation as in SHU9119, a well known MC-4R antagonist. The topographical similarity is further enhanced when considering that the hydrophobic interaction between D-2Nal and Nle residues is conserved in both structures. The replacement of DPhe6 of LYS607 with D-2Nal6 reversed the selectivity from preferring the CCK-2 to the CCK-1 receptor, and the result is likely to be caused by the Nle hydrophobic interaction mentioned above. LYS614 preserved the high selectivity at the CCK-1 receptor over the CCK-2 receptor, while introducing an antagonist function of Dmt-Tic for the opioid receptor. LYS633 is a lead compound having good binding affinities at both the opioid and the CCK receptors, and good opioid

agonist and CCK antagonist functions in functional assays. In-vivo tests of LYS633 are in progress, and will demonstrate the validity of our working hypothesis.

Table 1. In-vitro biological activities for opioid and CCK receptors [5].

Ligand	Affinity (Ki, nM)				Opioid (IC_{50}, nM)		CCK(Ke, nM)
	hDOR [^{3}H]DPDPE	rDOR [^{3}H]DAMGO	hCCK-1 [^{125}I]CCK-8(SO_3)	hCCK-2	MVD(δ)	GPI(μ)	GPI/LMMP antagonist
LYS607	6.20	13.6	1400	18.0	40.2	132	None
LYS633	0.21	0.13	18.0	113	11.3	8.31	944
LYS614	0.91	4.65	15.0	200	13% at 1 μM*	5% at 1 μM	None

*Antagonist activity was observed.

LYS607 Tyr-D-Ala-Gly-Phe-His-D-PHE- Arg-Trp-Nle-Asp-Phe-NH_2

LYS633 Dmt-D-Ala-Gly-Phe-His-D-2Nal- Arg-Trp-Nle-Asp-Phe-NH_2

LYS614 Dmt-Tic-His-D-2Nal- Arg-Trp-Nle-Asp-Phe-NH_2

Acknowledgements

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DENDRIMERIC PEPTIDE INHIBITORS OF ANTHRAX LETHAL AND EDEMA FACTORS

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Introduction

Anthrax toxin consists of three proteins, the protective antigen (PA) and the two enzymes lethal factor (LF) and edema factor (EF), that are carried through the membrane of the target cell upon binding to membrane receptor-bound PA. No effective drug is currently available against anthrax intoxication. Here we describe the production of specific peptide inhibitors, obtained by selecting a large peptide phage library on recombinant PA and eluting specific binders by competition with LF. Such peptides inhibit the interaction of PA with LF and EF and neutralize anthrax toxins *in vitro* and *in vivo* [1]. Two peptides, TLPYWWLTPSNP (**p2**) and NVMTYWWLDPPL (**p3**), were selected and synthesized in the tetra-branched Multiple Antigen Peptide (MAP) form, inducing resistance to proteolytic degradation [2]. The two lead MAP2 and MAP3 peptides were systematically modified by alanine scanning, progressive shortening and residue randomization, to obtain an increase of peptide affinity and inhibitory efficiency. Affinity maturation of lead sequences led to selection of the MAP3V/A peptide, which is effective for *in vivo* neutralization of anthrax toxin activity. The same peptide also inhibits EF-mediated cAMP induction.

Result and Discussion

Anti-PA63 peptides were selected from the 12-mer peptide phage library Ph.D.12TM (NEB) by competitive panning, using recombinant PA as target and recombinant LF as competitor molecule. This enabled single step recovery of phages carrying peptides that interfere with PA63-LF binding. After three cycles of panning we selected two phage peptides, **p2** and **p3**, giving the highest inhibition of PA63-LF binding in a competition phage ELISA. The selected peptides shared the consensus motif YWWL, which is shared by previously described PA-binding peptides [3]. These peptides were synthesized both in linear and tetra-branched MAP form. Monomeric peptides were ineffective, whereas MAPs inhibited LF-PA63 binding in ELISA and were able to neutralize lethal toxin-induced murine macrophage cell death. The IC_{50} of MAP3 was about one log lower than that of MAP2. A second generation of MAPs was synthesized using alanine-scanning and progressive sequence shortening, to obtain peptides with higher affinity and inhibitory efficiency. Peptide with Ala in position 2 of MAP3 sequence (MAP3V/A) resulted in an evident improvement of MAP activity both in

competition ELISA and cytotoxicity inhibition. MAP3V/A inhibited also PA63-EF binding in ELISA and the IC_{50} value was comparable with that obtained for inhibition of PA63-LF binding (Fig. 1). Moreover, MAP3V/A was tested for *in vitro* neutralization of anthrax edema toxin, resulting able to completely inhibit EF-induced cAMP increase in different cell lines. MAP3V/A was tested also for *in vivo* neutralization of anthrax lethal toxin. Rats were injected with a mixture of purified PA83 and LF and died in about two hours. 1 mg of MAP3V/A injected 5 minutes after toxin completely neutralized lethality.

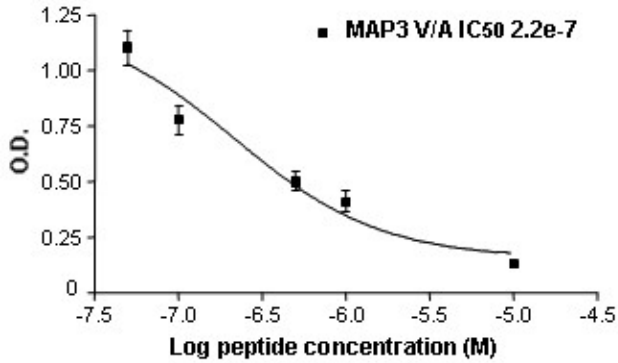

Fig.1 Inhibitory activity of MAP3V/A on EF/PA63 binding in ELISA

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HIV-TAT PEPTIDE-MODIFIED CHOLESTEROL PULLULAN: SYNTHESIS AND ACTIVITY AS A GENE VECTOR

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Introduction

Cell penetrating peptides have been known as a useful tool for nucleic acid, protein and drug delivery into cells. Cholesterol pullulan (CP), in which maltose moieties are partially modified by cholesterol, is unique in forming self-assembled nanoparticles (20-30 nm) in water [1]. Combination of these characteristics is considered to be promising for development of effective non-viral vectors without toxicity. A conjugate of HIV-Tat [2] and CP was synthesized and its gene expression efficiency was evaluated.

Results and Discussion

Fully protected HIV-Tat-(48-57)-Cys(Snm)-Gly-NH-R was obtained by conversion of the corresponding Cys(Acm) peptide which was synthesized by the solid-phase method [Snm: (*N*-methyl-*N*-phenylcarbamoyl)-sulfenyl] [3]. The sulfhydryl function was

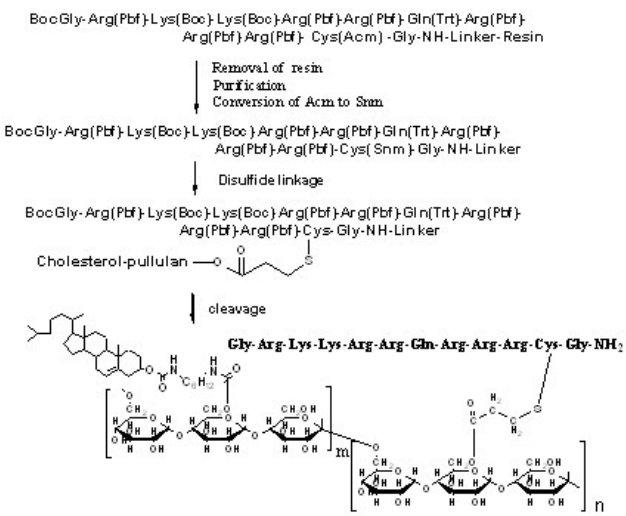

Fig. 1. Synthesis of cholesterol pullulan Tat.

introduced to the hydroxyl groups of CP by acylation with Trt-3-mercaptopropionic acid followed by acid treatment. Resulting 3-mercaptopropionyl-CP was coupled with Cys(Snm) peptide to form disulfide bridge and the protecting groups of the peptide were removed to give the CP-Tat conjugate (Fig. 1). CP-Tat and pCMV-Luc complex was transformed into COS7 cells and luciferase activity was analyzed after 24 h. CP-Tat elicited remarkable cytoplasmic luciferase activity and low toxicity. It showed particle diameter of approximately 80 nm and positive charge (+20 mV). These results suggest that CP-Tat is a safe and useful gene vector.

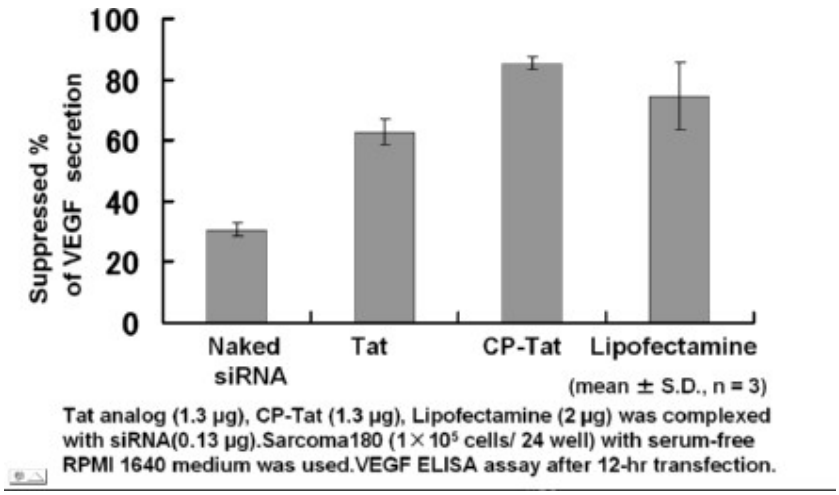

Fig. 2. Inhibitory effect of siRNA complexed with vector on VEGF secretion in S-180 cells .

CP-Tat was also effective as a siRNA carrier. The conjugate with siRNA was transfected to S-180 cells and the marked inhibitory activity of VEGF was observed compared with the conjugate with Lepofectmine (Fig. 2). CP-Tat showed also *in vivo* suppressive effect in tumor growth.

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PEPTAIBIOMICS: SURVEY OF THE FUNGAL GENUS HYPOCREA

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Introduction

Peptaibiotics are defined as a family of fungal peptides containing a high proportion of the nonproteinogenic amino acid Aib (α-aminoisobutyric acid) and showing biological activities. *N*-acetylated members of this group containing a C-terminal 1,2-amino alcohol are defined as peptaibols. Lipopeptaibols are acylated with a fatty acid at the N-terminus, and aminolipopeptides contain unusual heterocyclic residues at N- and/or C-termini. We apply peptaibiomics to fungal cultures grown on single agar plates. Here we present a survey of the peptaibiome of *Hypocrea* species. Peptaibiomics is the analytical methodology for the structural characterization of the totality of peptaibiotics expressed by filamentous fungi [1]. By comparing partial sequences deduced from analytical data with those compiled e.g. in the "Peptaibol Database" [2] the judgment is possible whether or not structures are novel, or related or identical to structures described.

Results and Discussion

Peptaibiomics comprises growth of species of the genus *Hypocre*a on potato-glucose-agar Petri dishes followed by treatment of mycelia with MeOH/DCM (1/1, v/v) and solid-phase extraction with Sep-Pak C-18 cartridges (1.5 cm x 1 cm). The purified peptides were analyzed by online coupling of HPLC with ESI-MS (for HPLC and ESI conditions see [1]). For scanning of molecular masses and fragments resulting from cleavage of the extremely labile Aib-Pro bond no collision induced dissociation (CID) energy was used, whereas application of a CID energy of 45% generated series of characteristic fragment ions [1]. The resulting partial structures were compared with structures in data bases [2]. Fig. 1 presents examples of partial sequences of *Hypocrea* species screened for peptaibiotics. In extracts of *Hypocrea semiorbis, H. vinosa, H. dichromospora, H. gelatinosa, H. nigricans, H. muroiana* and *H. lactea* a multitude of short-, middle- and long-chain Aib-containing peptides were characterized. Comparison of these sequences with peptaibiotics stored in [2] shows that *H. vinosa* and *H. lactea* produce peptides which are new analogs of the peptaibiotics trichogin and trichokingin from species of *Trichoderma.* For the other fragments and partial sequences no similarity could be validated. The data establish *Hypocrea* as a rich source of peptaibiotics.

***Hypocrea muroiana* MUCL 28442**		**MW**
1	[291]-Phe-Aib-Lxx-Aib-Lxx-[186]	1020
2	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Aib-Lxx-[842]	1950
3	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Vxx-Vxx-[816]	1924
4	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Aib-Lxx-196-Aib-Lxx-Gln-Gln-Pheol	1909

***Hypocrea nigricans* MUCL 28439**		**MW**
1	[157]-Vxx-Vxx-Vxx-Aib-[623]	1162
2	[270]-Vxx-Aib-Lxx-[623]	1190

***Hypocrea gelatinosa* CBS 724.87**		**MW**
1	[142]-Gln-Lxx-Lxx-Aib-[n.i.]	n.i.
2	[157]-Vxx-Lxx-Lxx-Aib-Vxx-[510]	1176
3	[142]-Gln-Lxx-Lxx-Aib-[623]	1204

***Hypocrea dichromospora* CBS 337.69**		**MW**
1	[184]-Ala-Vxx-Aib-Aib-Aib-Leuol	726
2	[184]-Ala-Lxx-Aib-Gly-Lxx-Leuol	740
3	[184]-Ala-Lxx-Aib-Ala-Lxx-Leuol	754
4	[255]-Lxx-Aib-Gly-Lxx-Vxx-[247]	969

***Hypocrea vinosa* CBS 247.63**		**MW**
1	[212]-Gly-Vxx-Aib-Gly-Gly-Vxx-Aib-Gly-Lxx-Leuol	1038
2	[212]-Gly-Lxx-Aib-Gly-Gly-Vxx-Aib-Gly-Lxx-Leuol	1052
3	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol	1052
4	[212]-Gly-Lxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol	1066

***Hypocrea semiorbis* CBS 244.63**		**MW**
1	[284]-Ala-Aib-Ala-213-Aib-Aib-Leu-Gly-Aib-[788]	1937
2	[284]-Ala-Aib-Ala-213-Vxx-Aib-Aib-Gly-Leu-[774]	1937
3	[284]-Ala-Aib-Aib-213-Vxx-Aib-Aib-Aib-Aib-[774]	1951
4	[284]-Ala-Aib-Aib-213-Ala-Leu-Aib-Gly-Leu-[788]	1965

***Hypocrea lactea* CBS 853.70**		**MW**
1	[212]-Gly-Lxx-Aib-Gly-Gly-Vxx-Aib-Gly-Vxx-Leuol	1038
2	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Vxx-Leuol	1038
3	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol	1052
4	[212]-Gly-Lxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol	1066

Fig. 1 Examples of partial sequences from species of Hypocrea analyzed for peptaibiotics; sequences containing less than 3 residues are not shown; abbreviations according to the standard three-letter nomenclature; Aib = α-aminoisobutyric acid; Lxx = Leu or Ile; Vxx = Val or Iva (isovaline); Leuol = leucinol; Pheol = Phenylalaninol; MW, molecular weight; n.i., not identified. Numbers in square brackets refer to not identified fragment ions.

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THE PEPTAIBIOME AND PEPTAIBOMICS. STRUCTURAL CHARACTERIZATION OF THE ENTIRETY OF PEPTAIBIOTICS EXPRESSED BY FUNGI

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Introduction

In analogy to the terms "proteome" and "proteomics" we define the "peptaibiome" as the entirety of fungal peptides containing the non-protein α-aminoisobutyric acid (Aib). Accordingly, "peptaibiomics" is the analytical methodology for the structural characterization of the entirety of peptaibiotics expressed in filamentous fungi [1]. A peptaibiotic is defined as fungal peptide containing Aib and exhibiting antibiotic or other bioactivities. Peptaibiotics consists of mixtures containing up to twenty residues peptides that have, in part, unusual N- and C-termini. We present a rapid and sensitive method for the detection and structural characterization of the peptaibiome expressed by fungal multienzyme complexes [2].

Results and Discussion

For peptaibomics the fungal cultures were grown for seven days on malt-extract agar in Petri dishes. Extraction was performed with three 5 ml portions of MeOH/dichloromethane (1/1, v/v). Extracts were cleaned up on a Sep-Pak C-18 cartridge (1.5 cm x 1.0 cm) [1]. Aliquots of 10 µl were analyzed by HPLC-ESI-MS using gradient elution with MeOH/MeCN/0.1% TFA mixtures (for HPLC and ESI conditions see [1]).

Use of a Kromasil KR100 column together with the gradient system provided best separations for medium-chain (11 - 17 residues) and long-chain (18 - 20 residues) peptaibiotics of varying lipophilicity. For scanning of molecular masses and fragments resulting from cleavage of the extremely labile Aib-Pro bond no collision induced dissociation (CID) energy was used, whereas a CID energy of 45% generated series of characteristic fragment ions [1]. From the mass differences (Δm) of fragment ions the presence of the marker amino acid Aib, characterized by Δm = 85.1 Da, as well as other constituents could be deduced.

Using peptaibomics, characteristic fragment ions were detected and the resulting partial structures were compared with structures in data bases [3]. Suitability of the method is demonstrated with the analysis of different *Trichoderma* strains. As an example, Fig. 1 shows of partial sequences of the mold *Trichoderma asperellum* (CBS 433.97). Comparison of these sequences with peptaibiotics already described in [3] shows that sequence no. 7 from *T. asperellum* might be identical with trichotoxin A 50E and sequence no. 9 might be trichotoxin A 50I from *Trichoderma* viride, strain NRRL 5242. For the partial sequences of peptides from some other

Trichoderma strains [1] no correspondence with known structures were found. Thus they are assumed to represent new micro heterogeneous peptaibiotics.

The peptaibomic technique applying HPLC-ESI-MS to extracts of molds cultured on agar plates, as described above, provides reliable diagnostic information on the totality of peptaibiotic production. Differentiation among new and already known structures is possible by comparison with sequences stored in relevant databases.

Trichoderma asperellum (CBS 433.97)

	MW
1 [227]–Aib–Lxx–Aib–Aib–Ala–Aib–763–Aib-Valol	1702
2 [156]–Ala–Aib–Vxx–Aib–Aib–Ala–Aib–[909]	1646
3 [227]–Aib–Lxx–Aib–Aib–Ala–Aib–[923]	1674
4 [270]–Lxx–Aib–Gln–Aib–Aib–Aib–158–Aib–210–Aib–Aib–Gln–Valol	1705
5 [270]–Lxx–Aib–Gln–Aib–Aib–Aib–172–Aib–210–Aib–Aib–Gln–Valol	1719
6 [270]–Lxx–Aib–Gln–Aib–Aib–Vxx–128–Aib–210–Aib–Aib–Gln–Valol	1689
7 [270]–Lxx–Aib–Gln–Aib–Aib–Aib–Ala–Ala–Aib–210–Aib–Aib–Gln–Valol	1689
8 [270]–Lxx–Aib–Gln–Aib–Aib–Aib–Ala–Aib–Aib–210–Aib–Aib–Gln–Valol	1703
9 [270]–Lxx–Aib–Gln–Aib–Aib–Aib–156–Aib–[625]	1717

Fig. 1 Examples of partial sequences from *Trichoderma asperellum* (CBS 433.97) analyzed for peptaibiotics; sequences containing less than 5 AA are not shown; abbreviations according to the standard three-letter nomenclature; Aib = α-aminoisobutyric acid; Lxx = Leu or Ile; Vxx = Val or Iva (isovaline); Valol = valinol; MW, molecular weight. Numbers in square brackets refer to not identified fragment ions.

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CARDIAC TROPONINS DERIVED CONJUGATES AS TOOLS FOR PRODUCING SPECIFIC ANTIBODIES: SYNTHESIS AND IMMUNE RESPONSE

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Intoduction

Troponin is a structural protein complex, which is responsible for the regulation of skeletal and cardiac muscle contraction. It consists of three components: troponin I (cTnI, 24 kDa), troponin C (cTnC, 18 kDa), and troponin T (cTnT, 37 kDa). Cardiac troponins are released into the bloodstream of patients after the onset of a cardiovascular damage [1]. Even minimal elevations over the normal values, of serum troponin T and I are being used for diagnosis of acute myocardial infarction. The development and commercialization of highly specific biological assays for the detection of cardiac troponins is based on the production of specific antibodies against the whole complex or individual subunits [2]. However, the specificity and sensitivity of these assays vary due to problems mainly originated from the fact that cardiac troponins have a high homology with the skeletal isoforms. The aim of this work is to select and synthesize appropriate regions of the cardiac isoforms of troponin I, C and T, suitable for the production of more sensitive and specific cardiac troponin detecting reagents. In order to construct the immunogenic complexes, the selected sequences were conjugated to the tetrameric Sequential Oligopeptide Carrier (Ac-SOC_4-OH) formed by the repetitive moiety Lys-Aib-Gly, either by the classic solid phase step-by-step methodology or by chemoselective ligation reactions. Using the carrier conjugated troponin sequences, anti-cardiac troponin complex specific antibodies in high titers were produced.

Result and Discussion

The R^{19}RRSSNVRAYATE^{31} (cTnI-19-31), the T^{118}KNITEIADLTQKI^{131} (cTnI-118-131), R^{185}EVGDWRKNIDA^{196} (cTnI-185-196), the S^{89}KGKSEEELS^{98} (cTnC-89-98) and the E^{21}EEEDWREDE^{30} (cTnT-21-30) regions have been chosen for production of antibodies. The selection was made on the basis of their predicted immunogenicity and the minimum homology compared to the skeletal isoforms. The protein-like molecules of branched architecture, suitable for immunization experiments, were produced by covalent ligation of four copies of the selected peptide sequences to sequential oligopeptide carrier Ac-[Lys-Aib-Gly]$_4$-OH [3]. Both, the step by step solid phase peptide synthesis (Boc and/or Fmoc strategies) and/or the chemoselective ligation methodologies were applied to synthesize the branched macromolecules of ~6 - 8 kDa molecular weights. Despite the fact that we paid special attention in order to avoid the progressive accumulation of by-products

during the SPPS, the final conjugates of cTnI-185-196 and cTnC-89-98 were not isolated, suggesting that the sequence of the epitopes strongly affects the quality and the yield of the target conjugate. To resolve this problem we successfully applied the chemoselective ligation method. To this aim the conjugated epitopes were synthesized bearing a Cys moiety at the C-terminus. On the other hand, the carrier was bromoacetylated at the side chain amino group of the four Lys. The conjugation resulting in thioether linkage between the carrier and epitope peptide was carried out by dissolving the sequential polypeptide containing bromoacetyl groups in 0.1 M Tris-buffer (pH 8.2)/MeCN (3/1, v/v) at 1 mg/mL concentration. 1.2 equivalents (calculated for the bromoacetyl content of the polypeptides)

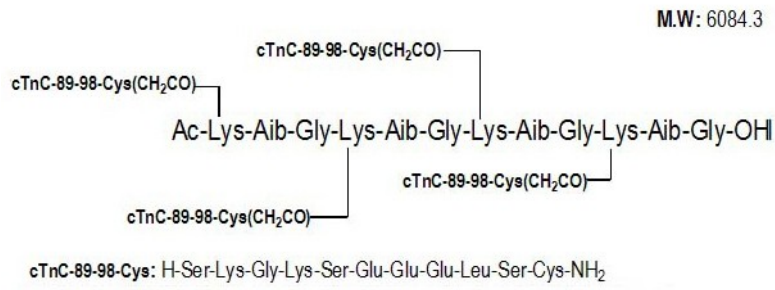

Fig.1. Schematic representation of Ac-[Lys(cTnC-89-98-Cys(CH_2CO))-Aib-Gly]$_4$-OH

of epitope peptide was added to the solution of bromoacetylated polypeptide from time to time in solid form. The mixtures were stirred in the dark under Ar for 8 – 10 h at RT. The synthesized conjugates, Ac-[Lys(cTnI-19-31)-Aib-Gly]$_4$-OH, Ac-[Lys(cTnI-118-131)-Aib-Gly]$_4$-OH, Ac-[Lys(cTnI-185-196-Cys(CH_2CO))-Aib-Gly]$_4$-OH, Ac-[Lys(cTnC-89-98-Cys(CH_2CO))-Aib-Gly]$_4$-OH and Ac-[Lys(cTnT-21-30-Cys(CH_2CO))-Aib-Gly]$_4$-OH (Fig. 1), were used as immunogens for releasing anti-troponin specific antibodies. Anti-cardiac troponin complex specific antibodies in high titers were produced.

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PEPTIDE/PROTEIN WASTE MATERIALS LIKE BINDERS OF FOUNDRY SANDS

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Introduction

The aim of this study was to research and develop a new foundry moulding mixture with new binder system based on proteins/peptides. At present, these moulds and cores containing resins, bentonite, cement or soluble glasses as binders of foundry sands are used. Furthermore, higher flexural and tensile strengths, good flowability of the sand, good surface property, easy shaking out under temperature 650 °C for aluminum casting and resistance of moisture absorption are also required. Water-soluble or water-swelling materials based on peptides form bonds with inorganic matrix, even though no chemical reactions take place. The experiments of some tested wastes based on proteins proved to be suitable binders for aluminum casting due to their environmental friendliness. To evaluate the quality and performance of molds with a new binder experimental casting has been performed.

Results

As a peptide/protein binder was used waste material from pharmaceutical industry (marked ZMK binder system). The determination of amino acids was made by automatic analyzer. The protein-based binder system was dissolved in hot water (about 75 °C) during mixing. Then, the solution was mixed into the hot foundry quartz sand until dry in the mixer. The coating process was analyzed using SEM. Prior to curing a core, the coated sand was mixed with water to evenly hydrate each coated particle. Binder addition 0.5 - 3% (based on sand weight) produces cores with adequate strength. Casting trials were performed using a core made with the new binder in the low-pressure hot box to evaluate shakeout and dimensional accuracy. These were verified and did not show any thermoplastic deformation. Surface photo of cast enabled to study the surface quality of aluminum cast which is very good, even without the refractory coating.

Discussion

As a binders of foundry moulding sands, some natural macromolecular biopolymers namely proteins wastes from pharmaceutical industry were tested. The preliminary results show that suitable protein binders of foundry sands can be based on supporting tissues, considering the content individual amino acids. Laboratory tests exhibited flexural strengths required for molding materials and can be used only 0.75 or 1% binder based on sand weight, dimensional accuracy was excellent. Bonds are accomplished by dehydrating protein in the sand mixture, no chemical

reaction takes place. SEM photos showed good migration of the binder to the contact points and the structure of quartz sand mould fixed by protein. Photos of surface of the cast showed suitable quality of the surface without refractory coating. One of the most important features of the binder is its water solubility. The binder behaves more or less plastically as water is added or removed. This allows the bonding mechanism to be reversed if water is added back to the protein. Any scrap core made with the binder can be recycled without having to add any additional binder. The binder does not require the use of toxic chemicals. Flawed cores can be reused and have not to be discharged.

Acknowledgements

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SIDE CHAIN AND BACKBONE CONFORMATIONAL PREFERENCES OF –CXC- CONTAINING PEPTIDES: EVIDENCED BY COMPUTATIONAL STUDIES

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Introduction

In this study, we expand our previous search1 of the influence of the (*S,S*)-CXC- motif on peptide's backbone conformation and side chain side chain interaction. We apply this investigation to a series of linear and cyclic (through disulfide bond) RGD peptide analogues (Table 1). Molecular Dynamics simulations were used to sample the conformational space of the peptides and trajectories received were analyzed. To characterize the backbone conformation we used the Φ/Ψ dihedral angles in order to assign a conformational state to each residue according to Zimmerman. In the sequences –XCYC- or –CXCY- (linear or cyclic), we used the the Pdoβ (pseudo dihedral angle of orientation) defined by the $C^{\beta}(X)$-$C^{\alpha}(X)$-$C^{\alpha}(Y)$-$C^{\beta}(Y)$ atoms, as a measure of the orientation of the side chains. This is similar to our previous work1 and to Stote [2].

Table 1. Linear and cyclic (through disulfide bond) peptides studied with molecular dynamics simulations.

peptide	sequence	peptide	sequence
ACAC	Ace^1-Ala^2-Cys^3-Ala^4-Cys^5-NH_2	cACAC	(S,S)Ace^1-Ala^2-Cys^3-Ala^4-Cys^5-NH_2
RCAC	Ace^1-Arg^2-Cys^3-Ala^4-Cys^5-NH_2	cRCAC	(S,S)Ace^1-Arg^2-Cys^3-Ala^4-Cys^5-NH_2
DCAC	Ace^1-Asp^2-Cys^3-Ala^4-Cys^5-NH_2	cDCAC	(S,S)Ace^1-Asp^2-Cys^3-Ala^4-Cys^5-NH_2
ACRC	Ace^1-Ala^2-Cys^3-Arg^4-Cys^5-NH_2	cACRC	(S,S)Ace^1-Ala^2-Cys^3-Arg^4-Cys^5-NH_2
ACDC	Ace^1-Ala^2-Cys^3- Asp^4-Cys^5-NH_2	cACDC	(S,S)Ace^1-Ala^2-Cys^3-Asp^4-Cys^5-NH_2
RCDC	Ace^1-Arg^2-Cys^3-Asp^4-Cys^5-NH_2	cRCDC	(S,S)Ace^1-Ala^2-Cys^3-Asp^4-Cys^5-NH_2
DCRC	Ace^1-Asp^2-Cys^3-Arg^4-Cys^5-NH_2	cDCRC	(S,S)Ace^1-Ala^2-Cys^3-Arg^4-Cys^5-NH_2
CACA	Ace^1-Cys^2-Ala^3-Cys^4-Ala^5-NH_2	cCACA	(S,S)Ace^1-Cys^2-Ala^3-Cys^4-Ala^5-NH_2
CRCA	Ace^1-Cys^2-Arg^3-Cys^4-Ala^5-NH_2	cCRCA	(S,S)Ace^1-Cys^2-Arg^3-Cys^4-Ala^5-NH_2
CDCA	Ace^1-Cys^2-Asp^3-Cys^4-Ala^5-NH_2	cCDCA	(S,S)Ace^1-Cys^2-Asp^3-Cys^4-Ala^5-NH_2
CACR	Ace^1-Cys^2-Ala^3-Cys^4-Arg^5-NH_2	cCACR	(S,S)Ace^1-Cys^2-Ala^3-Cys^4-Arg^5-NH_2
CACD	Ace^1-Cys^2-Ala^3-Cys^4-Asp^5-NH_2	cCACD	(S,S)Ace^1-Cys^2-Ala^3-Cys^4-Asp^5-NH_2
CRCD	Ace^1-Cys^2-Arg^3-Cys^4-Asp^5-NH_2	cCRCD	(S,S)Ace^1-Cys^2-Arg^3-Cys^4-Asp^5-NH_2
CDCR	Ace^1-Cys^2-Asp^3-Cys^4-Arg^5-NH_2	cCDCR	(S,S)Ace^1-Cys^2-Asp^3-Cys^4-Arg^5-NH_2

Results and Discussion

The main findings of our work are:

1. Pdoβ dihedral distributes almost uniformly in the [-180 , +180] region in all cases except those where two opposite charged residues are found in the sequence. Analogues RCDC, cRCDC, CRCD, cCRCD, DCRC, cDCRC, CDCR and cCDCR show strong peaks. Cyclization moves these peaks closer to 0o, thus facilitating the coplanar orientation of the side chains, which is believed to be the bioactive conformation [1, 2] Narrower distribution of PDOβ angle can result in less entropy loss upon folding in a "bioactive" conformation, so can explain, at least partly, bioactivity.
2. Backbone conformation of CY residues in –XCYC- motif are mostly influenced from the cyclization. Generally, conformational states A or B in linear analogues become C, D or E in cyclic analogues. This means that Ψ angle takes significantly bigger values (>120) in cyclic analogues. We did not observe any turn formation in peptide's backbone conformation in both linear and cyclic analogues.
3. Cyclization facilitates backbone stabilization, but charge-charge interactions also contribute to this stabilization.

Acknowledgements

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THE ROLE OF THE ARGININE SIDE CHAIN TO THE STRUCTURE STABILIZATION OF LHRH

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Introduction

Luteinizing hormone-releasing hormone (LHRH) is a linear hypothalamic decapeptide that is secreted from the hypothalamus and regulates the reproductive system. Like many hormones LHRH is a highly flexible molecule that can adopt many interconverting conformations in solution and the assignment of a predominant bioactive conformation is very difficult. Two type II β-turns placed in the central part of the molecule (-S^4-Y^5-G^6-L^7- and –Y^5-G^6-L^7-R^8-) have been proposed to stabilize the bioactive conformation [1, 2]. In this work, using various LHRH analogues and peptide models derived from the C-terminal sequence of the LHRH, we investigated the contribution of the Arg side chain to the structure stabilization of LHRH.

Result and Discussion

Peptide synthesis

The following analogues and peptide models were synthesized and studied: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (**1**), pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NHEt (**2**), pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt (**3**), pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt (4), Gly-Leu-Arg-Pro-Gly-NH_2 (**5**), Piv-Arg-Pro-[^{17}O]Gly-NH_2 (**6**), Piv-Nle-Pro-[^{17}O]Gly-NH_2 (7). Peptides **1, 5, 6** and **7** were synthesized by the SPPS methodology on a MBHA resin while the analogues **2, 3** and **4** on a EtNH-modified-Merrifield resin.

NMR experiments

The NMR experiments were performed at 295 - 355 K on a Bruker AMX 400 spectrometer. The detected NOEs connectivities as well as the high temperature coefficient values of almost all the amide protons either for the natural LHRH or for the analogues **2, 3** and **4** evidenced for their high backbone flexibility. Only two dNN (i, i+1) NOE effects between W^3/S^4 and S^4/Y^5 were detected. However, the most striking finding of this study was the high rotational freedom in the N-C^α bond of Gly6, which was estimated, to be greater than that observed for Gly^{10} although the later constitutes the C-terminal part of the molecule. These rotational states were evaluated on the basis of the $^3J_{N\alpha}$ and $^3J_{N\alpha'}$ values of Gly^6 and Gly^{10} (Fig. 1) [3]. According to the NMR data the restriced mobility of the C-terminal Gly can not be attributed to conformational restrictions imposed by the backbone conformation. A possible interaction of type side chain-backbone can be the origine of this behavior. The role of the arginine side chain in the structure stabilization is suported by ^{1}H and ^{17}O NMR conformational studies of the **5**, **6**, and **7** peptide models (Fig.1) [4]. The guanidinium group seems to participate in a

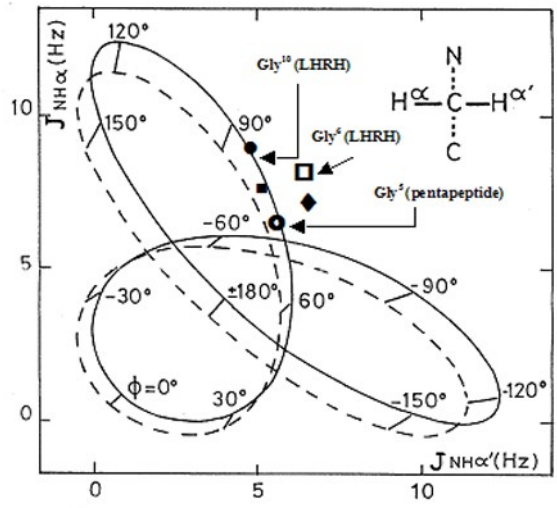

Fig. 1. Relation between $^3J_{N\alpha}$ and $^3J_{N\alpha'}$ coupling constants as a function of the rotational state φ of the N-C^{α} bond. Data situated inside the region between the solid line and the broken line are related to fixed φ, angles. Data for the Gly^6 (◘) and Gly^{10} (●) of the peptides pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, Piv-Arg-Pro-[^{17}O]Gly-NH_2 (■), Piv-Nle-Pro-[^{17}O]Gly-NH_2 (♦) and Gly-Leu-Arg-Pro-Gly-NH_2(◙).

hydrogen bonded interaction with the carbonylic oxygen of the C-terminal glycine. A similar interaction could explain the restricted mobility around the N-C^{α} bond of Gly^{10} of LHRH. The backbone conformation of the analogues in which Gly^6 was substituted by a D-amino acid and Gly^{10} by the –NHEt group seems to be similar to that of the natural LHRH.

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DESIGN AND SYNTHESIS OF NEW PEPTIDES WITH EXPECTED γ-SECRETASE AND AGGREGATION INHIBITORY ACTIVITY

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Introduction

The Alzheimer's disease (AD) is a neurodegenerative illness, which affects millions of people worldwide. AD is characterized by a progressive dementia of memory, intellectual, speech and brain disturbances.

In our previous work we proposed a new approach to the creation of potential inhibitors of γ-secretase by synthesizing new derivatives of the 7,8-dimethoxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine containing dipeptides [1, 2].

In the present work, we synthesized heptapeptides containing Lys-Leu-Val-Phe-Phe sequence bound to derivatives of *N*-(3,5-dichlorophenyl)-Ala-OH: *N*-[*N*-(3,5-dichlorophenyl)-D,L-Ala]-L-Phe-OH, *N*-[*N*-(3,5-dichlorophenyl)-D,L-Ala]-L-Val-OH, *N*-[*N*-(3,5-dichlorophenyl)-D,L-Ala]-L-Leu-OH, *N*-[*N*-(3,5-dichlorophenyl)-D,L-Ala]-Gly-OH. These heptapeptides were synthesized by a solid phase synthesis using Fmoc-strategy. By the synthesis of these compounds we intended to combine two important pharmacological effects for the treatment of the disease: inhibition of the γ-secretase by the *N*-(3,5-dichlorophenyl)-Ala-OH derivatives [3] and inhibition of the β-amyloid peptide (Aβ) aggregation by the pentapeptide sequence [4]. The new synthesized peptides were characterized by melting points, optical rotation, HPLC and MS.

Result and Discussion

We synthesized the new heptapeptides by a solid phase synthesis using Fmoc-strategy. As solid phase we used Wang-Resin (1% DVB) which is used to prepare free C-terminal carboxyl group peptides.

We synthesised the pentapeptide Lys-Leu-Val-Phe-Phe in a high yield (~100%) using 0.5 grams (0.5 mmol) resin and HBTU as a condensation agent, but after that we preferred TCTU as a more reactive agent. In every step we added 4 equivalents of the next amino acid, which was previously activated in DMF with 1.03 equivalents of TCTU and 1.05 equivalents of DIPEA at room temperature for 10 min. The synthesis of the amide bond was performed after that for 2 hours at room temperature. We carried out the Fmoc-groups deprotection by a treatment with 20% piperidine/DMF for 30 minutes. We followed the complete reaction of the Fmoc-group deprotection as well as the synthesis of the peptide bond by a standard Keiser test.

Thus obtained the pentapeptide was separated in 4 parts, which were after that condensed with the free acids mentioned above to prepare the heptapeptides as follows:

- [*N*-(3,5-dichlorophenyl)-D,L-Ala]-Val-Lys-Leu-Val-Phe-Phe /peptide 1/,
- [*N*-(3,5-dichlorophenyl)-D,L-Ala]-Leu-Lys-Leu-Val-Phe-Phe /peptide 2/,
- [*N*-(3,5-dichlorophenyl)-D,L-Ala]-Phe-Lys-Leu-Val-Phe-Phe /peptide 3/,
- [*N*-(3,5-dichlorophenyl)-D,L-Ala]-Gly-Lys-Leu-Val-Phe-Phe /peptide 4/.

The new synthesized compounds were mixture of diastereomers as shown by the MS and the HPLC data.

HPLC-profiles are as follows: peptide 1 - R_t= 2.49 and 2.90 min., peptide 2 - R_t= 2.80 and 3.17 min.; peptide 3 - R_t= 2.61, 2.89 and 3.14 min. and peptide 4 - R_t= 2.42 min.

MS-analysis show as follows: peptide 1 - M.W. = 968.08 $CHNCl_2$, found $[M+H]^+$ = 968.07 and 969.56; peptide 2 - M.W. = 982.10 $CHNCl_2$, found $[M+H]^+$ = 982.05 and 984.03; peptide 3 - M.W. = 1016.07 $CHNCl_2$, found $[M+H]^+$ = 1016.64 and 1017.50; peptide 4 - M.W. = 926.04 $CHNCl_2$, found $[M+H]^+$ = 925.95 and 927.94.

Biochemical and pharmaceutical data will be discussed in our future paper.

Acknowledgements

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STRUCTURE-ACTIVITY RELATIONSHIP OF PEPTIDE ANALOGUES DERIVED FROM THE INTEGRIN SUBUNIT αIIb

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Introduction

The $\alpha_{IIb}\beta_3$ receptor is the most abundant receptor on the surface of platelets and can interact with a variety of adhesive proteins including fibrinogen, fibronectin etc [1]. Fibrinogen binding on $\alpha_{IIb}\beta_3$ mediates platelet aggregation and thrombus formation. Mapping of the fibrinogen binding domains on α_{IIb} subunit suggested the sequence 313-332 as a putative binding site [2]. This region was restricted to sequence α_{IIb} 313-320 (YMESRADR), which inhibits ADP stimulated human platelets aggregation and binds to immobilized fibrinogen [3]. The contribution of each amino acid to the inhibitory activity was previously estimated [4]. Thus, substitution of either Y^{313} or M^{314} or E^{315} by A does not affect the activity of the parent octapeptide. Substitution of R^{317} or D^{319} by A has dramatically decreased the activity of the octapeptide.

In this study we present the conformational analysis of three α_{IIb} 313-320 synthetic analogues, Y**A**ESRADR (**A2**), YMES**A**ADR (**A5**) and YMESRA**A**R (**A7**), using NMR spectroscopy and distance geometry calculations.

Result and Discussion

Proton resonances were fully assigned through the combined use of TOCSY and NOESY experiments. The numerous nOe's detected concerned not only protons of successive residues in peptide sequence (i, i+1), but also long range correlations up to i, i+5 (in the case of **A5**). Several i, i+2 nOe effects were detected mainly between the side chain and the backbone protons. The NHs temperature coefficient values, the deshielding of $RN^{\varepsilon}H$ at position 5 and the broadening of $N^{\eta}{}_2H_4$ are in agreement with an interaction of guanidinium group of R^5 in both **A2** and **A7** peptides, however less intense in the case of **A7**. Simulated annealing molecular calculations were performed using nOe derived distances in order to illustrate plausible conformations of the peptides. Common structural characteristic of peptides **A2** and **A7** is the interaction between the side chains of R^5 and E^3, however in **A2** the guanidinium group of R5 seems to form salt bridges with both E^3 and D^7. Peptide **A5** is stabilized only by a week interaction between R^8 and E^3 side chains. The interactions between the residue side chains provoke different overall shape of the three molecules (Fig. 1).

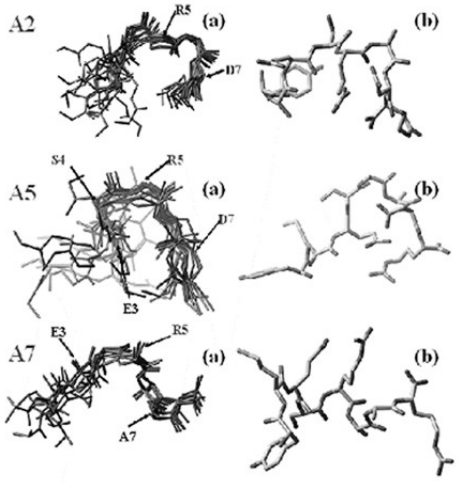

Fig. 1. Bundle of representative structures of the different conformational families resulted from the distance geometry calculations (a) and the energy minimized structure of the most populated family (b).

The fact he orientation of R and D side chains toward the same side of the molecule as well as the weakness of their ionic interaction contribute to increased biological activity [5] explains the retention of activity in the case of the peptide **A2**. The loss of activity in the case of **A5** and **A7** can be attributed either to complete alteration of the bioactive conformation or to the absence of the charged side chain group which is required for a direct contact with a complimentary group of the target protein (receptor or fibrinogen).

Acknowledgements

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HIGH COMPLEXITY PROTEASE AND KINASE PROFILING SOLUTION ASSAYS WITH READOUT ON RANDOMLY ASSEMBLED MICROARRAYS

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Introduction

We have developed miniaturized and multiplexed assays for the measurement of protease and kinase activities in complex samples. This technology will accelerate research in functional proteomics and enable biologist to carry out multiplexed protease and/or kinase inhibitor screens on a large scale. The assay readout is based on Illumina's universal Sentrix® BeadArrays [1, 2].

Results and Discussion

In our protease assay, unlike in the earlier techniques based on hybridizable peptide nucleic acid (PNA) tags readable on DNA arrays [3, 4], the peptide portion in our assay is C-terminally labeled with a biotin residue and contains a sequence of five histidine residues on the amino terminus. Upon protease cleavage, the portion of the peptide containing the biotin residue is detached from the oligonucleotide-peptide conjugate. Following the reaction, all biotin containing species are captured and removed by incubation with streptavidin beads. The cleaved conjugates that remain in solution are captured by hybridization of their oligonucleotide sequence to Sentrix BeadArrays and detected by a labeled antibody against the pentahistidine tag. We have generated multiple sets of oligonucleotide-tagged peptide substrates of different complexity (100 to 1000 substrates in a mixture) and have shown that the response of individual substrates is independent of the complexity of the mixture. Our results demonstrated the possibility to perform the protease assay in a multiplexed substrate environment with high sensitivity. Fig. 1 shows the response of various model proteases.

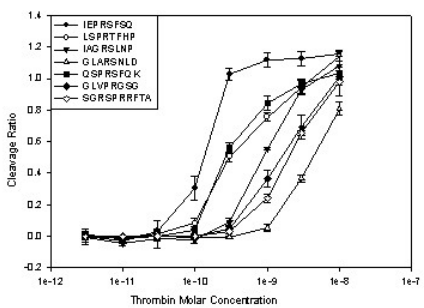

Fig. 1. Radial plots of 1000-plex substrate pool with model proteases.

We have used thrombin as a model enzyme for characterization of our assay using the 1000-plex substrate pool (substrate concentration ~0.25 nM each). The limit of detection (LOD) of thrombin activity using the best substrate in the pool (IEPRSFSQ) is 27 pM. An important characteristic of the enzymatic assay is the

linear dynamic range of enzyme concentrations which could be quantified. As can be seen from the Fig. 2, even though the linear dynamic range defined by an individual substrate is limited (roughly one order of magnitude), the use of various substrates combined can extend the dynamic range to over three orders of magnitude. Thus, the multiplexed assay provides a significant improvement over the assays utilizing only single substrate. The reproducibility of the assay allows the user to determine a measurable fold-change of <1.5 in thrombin concentration.

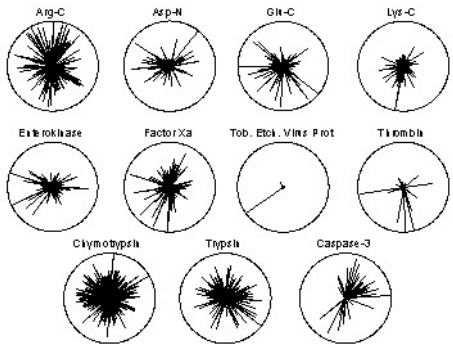

Fig. 2. Titration curves of the best thrombin substrates found in 1000-plex assay. Error bars indicate the standard deviation of three hybridization replicates.

To detect kinase-dependent phosphorylation, we developed a specific chemical modification of the electrophilic phosphate group with an amino-functionalized dye, utilizing 1-ethyl-3-[3-dimethylaminopropyl] carbodi-imide hydrochloride (EDC) as the coupling agent. We achieve selective phosphate labeling in a one-tube reaction, with a carboxylate blocking step (EDC, pH 6) prior to phosphate labeling as a key feature. Following labeling, the substrate mixture is deconvolved by hybridization to the complementary DNA sequences on a microarray. The approach is simple, mild, and labels all three phosphorylated amino acid residues in a variety of peptide substrates. Our substrate pool contains a diverse set of substrates targeted at over 50 kinases from all over the kinome. Similar to the protease assay, a broader range of enzyme concentrations can be quantified than possible with a single substrate. We characterized the phosphorylation signature for 26 different kinases acting on the mixture of 900 peptide substrates (~10 nM each), and showed that these signatures support detection of multiple kinase activities (at least 4) simultaneously. The ability to test kinase inhibition against multiple kinases at once provides economical screening of both novel and off-target inhibition by drug candidates. In addition, the analysis of kinase activities in a cell extract can be performed.

Acknowledgements

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TRITIUM LABELING OF BARUSIBAN (FE 200440), A POTENT OXYTOCIN ANTAGONIST

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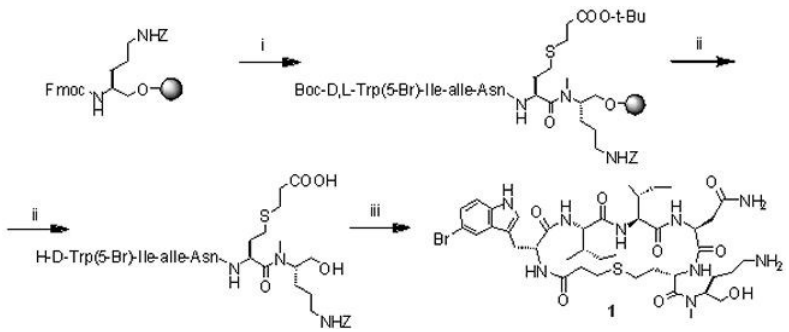
Introduction

Barusiban, [Mpa1,D-Trp^2,aIle^4,Abu^6,MeOrn-ol^7]OT-(1-7), is a potent, and selective oxytocin antagonist [1] and is currently undergoing Phase II clinical trials for pre-term labor. Studying the drug candidate distribution and metabolism necessitated preparation of a tritium-labeled peptide. Since barusiban contains an aromatic side chain in position 2, the replacement of a halogen atom by catalytic reduction with a hydrogen isotope in its halogenated precursor form, seemed to be the method of choice for preparation of the labeled tracer. We describe here a method of synthesis of monotritiated barusiban, comprising the preparation of the 5-bromotryptophan containing precursor and its subsequent catalytic reductive debromination.

Results and Discussion

Catalytic reductive dehalogenation has been utilized to synthesize numerous tritiated analogs of biologically active peptides (e.g. atosiban [2]). The labeling procedure requires the preparation of a halogenated precursor either by direct electrophilic iodination of a peptide or by introduction of a halogenated residue during peptide synthesis. Since barusiban contains the sensitive tryptophan residue, the preparation of the precursor 1 (Scheme 1) was achieved by the introduction of commercially available racemic 5 bromotryptophan during peptide assembly.



Scheme 1. Synthesis of brominated precursor 1; i: (1) 25% PIP, (2) o-NBS-Cl/2,4,6-collidine/DCM, (3) MeOH/TPP/DIAD/DME, (4) 2-mercaptoethanol/DBU/DMF, (5) Fmoc SPPS (DIC/HOBt, PIP); ii: (1) TFA/DCM/TIS, (2) HPLC separation; iii: (1) PyBOP/DIPEA/DMF, (2) TMSBr/thioanisole/ TFA, (3) HPLC, ion-exchange.

Thus, Fmoc-Orn(Z)-ol was first attached to 2-chlorotrityl resin in DCM containing pyridine. The Fmoc group was replaced with the o-NBS group and the resulting resin-bound sulfonamide was N methylated under Mitsunobu reaction conditions (MeOH/TPP/DIAD). The o-NBS group was subsequently removed with a 2-mercaptoethanol/DBU/DMF cocktail. The desired peptide was assembled by Fmoc chemistry using DIC or DIC/HOBt mediated couplings. The N-terminal amino acid was coupled as Boc-D,L-Trp(5-Br)-OH. The Z protected diastereoisomeric mixture of linear peptides was cleaved from the resin, and the two diastereoisomers were separated by preparative HPLC. The analogs were cyclized with PyBOP in DMF and the Z protecting group was removed with a TMSBr/thioanisol/TFA cocktail. The two diastereomeric peptides were purified by preparative HPLC, and the correct isomer was identified by its conversion to barusiban upon catalytic hydrogenation.

To optimize the bromine-tritium exchange step two solvents (MeOH and DMF) and two different catalysts (10 % Pd/C and 5 % $Pd/CaCO_3$, unreduced) were tested. The effect of bases on the course of the reaction was also examined. When Pd/C was used, the precursor was converted to the desired product but most of the compound was adsorbed by the catalyst. The use of $Pd/CaCO_3$ resulted in high yields and adsorption was not observed. Of the two solvents tested, DMF consistently provided higher reaction rates. The presence of base (DIPEA) did not influence the course of reaction in DMF.

The optimized conditions (DMF, 5% $Pd/CaCO_3$, 2:1 weight ratio of catalyst to peptide) were employed to test the efficiency of labeling with deuterium gas. Using this protocol, 75 - 85% deuterium atom incorporation was consistently achieved in multiple runs.

The actual tritiation was performed by Active Biotech, Lund, Sweden according to the deuteration procedure above. HPLC purification was performed using 50 mM ammonium acetate/ethanol buffer system. Ethanol was selected since it is a preferred solvent for storage of labeled materials and no solvent exchange was needed after HPLC purification. The specific activity of the obtained material was 18.5 Ci/mmol (about 66% of ^3H atom incorporation).

In conclusion, catalytic reductive debromination of a 5-bromo-substituted tryptophan containing precursor was found to be an excellent method for the synthesis of tritium-labeled barusiban.

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POLYPEPTIDE SYNTHESIS VIA THE N- TO S-ACYL SHIFT REACTION

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Introduction

A peptide thioester is a key building block for contemporary polypeptide synthesis based on ligation chemistry such as the thioester method [1] and native chemical ligation [2]. In this paper we describe a novel building block for the ligation, which does not contain the thioester moiety. We have already reported an intramolecular N to S acyl shift reaction under acidic conditions at the thiol containing residues such as a cysteine residue [3] and a thiol auxiliary for peptide ligation [4]. On the other hand, in 1985, Zanotti et al. reported that a diketopiperazine thioester, cyclo(-Cys($COCH_2Ph$)-Pro-) (1) was formed when a p-nitrophenyl (Np) ester, $PhCH_2CO$-Cys(S*t*Bu)-Pro-ONp (2), was treated with tributylphosphine under aqueous conditions [5]. The thioester 1 would be formed via the intramolecular N-S acyl shift reaction followed by diketopiperazine formation. Based on these observations, we designed an autoactivating unit, cysteinyl prolyl ester (CPE), for the peptide ligation, and found that in fact the CPE unit acted as an autoactivating unit for the ligation with a cysteinyl peptide, like the peptide thioester in native chemical ligation.

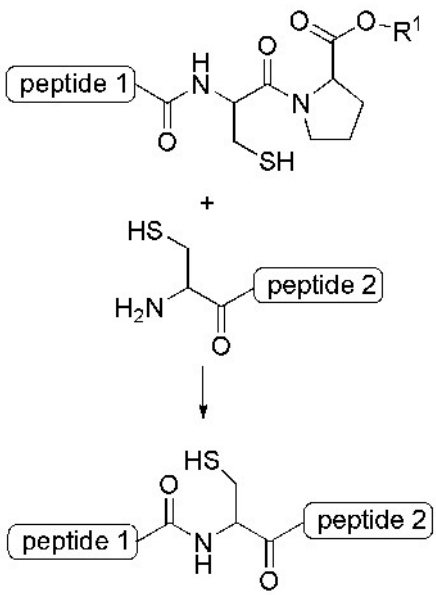

Fig. 1. CPE peptide ligation.

Results and Discussion

When a peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (3) was reacted with H-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (4) in a tricine buffer (pH 8.2) containing 20 mM tris(hydroxypropyl)phosphine and 6 M guanidine for 24 h, the ligated product, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (5), was formed and isolated by RP-HPLC in 60% yield. The glycine residue at the ligation site could be replaced by alanine, leucine, or valine residue, and ligated products were obtained in similar yields, respectively.

The peptide having the CPE unit can be prepared directly by standard Fmoc solid phase peptide synthesis, whereas the peptide thioester cannot because the thioester moiety is decomposed by piperidine treatment for the removal of the Fmoc group. Furthermore, the reactivity of the CPE autoactivating unit can be controlled by introducing a protecting group on the thiol of the cysteine residue. Therefore the direction of ligation reaction, toward the N or C terminus, can be easily controlled.

Acknowledgements

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SEMISYNTHESIS OF GLYCOPEPTIDE HORMONE PRECURSOR USING CHEMICAL LIGATION OF RECOMBINANT PEPTIDE THIOESTER

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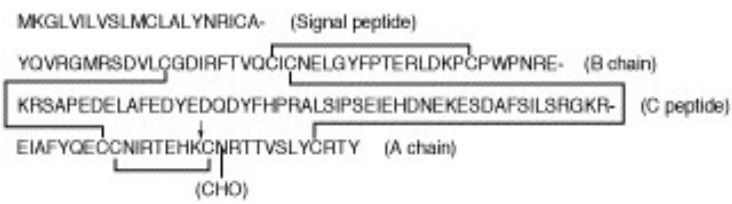
Introduction

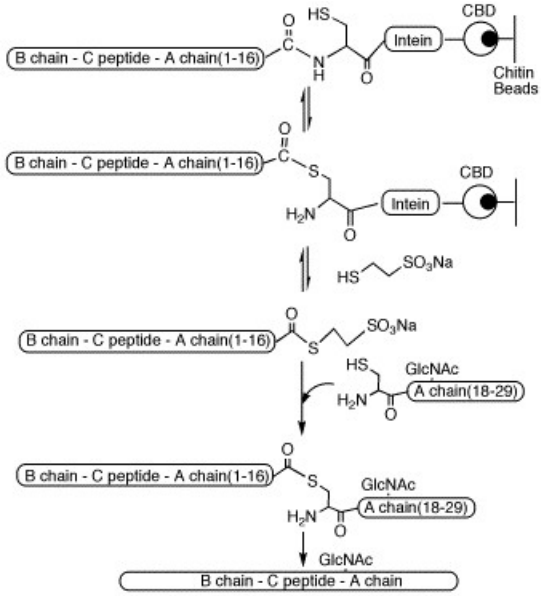
Androgenic gland hormone (AGH) is a heterodimeric glycopeptide hormone, which regulates sex differentiation in crustacea [1]. We previously achieved the chemical synthesis of AGHs carrying homogeneous carbohydrates [2]. First, A chain carrying GlcNAc, chitobiose, N-linked pentasaccharide or Man-GlcNAc-GlcNAc(Fuc) at Asn18 was prepared by the SPPS combining with our benzyl protection strategy at the carbohydrate portion. After B chain was also prepared by SPPS, two chains were crosslinked using selective disulfide bond formation to give AGHs carrying distinct carbohydrates. However, the biological activity was lower than the native AGH. A possible explanation of this problem is the failure in the folding of this glycopeptide. AGH is expressed as a single polypeptide chain and then processed into heterodimeric form by the removal of C-peptide [1].



Thus, we assumed that C-peptide, which connects two peptide chains in the proAGH, is essential for the proper folding of AGH. In this presentation, we prepared proAGH carrying homogeneous carbohydrate by the semi-synthetic approach.

Result and Discussion

The glycosylation site exists at the C-terminal region of proAGH. Thus, this polypeptide was constructed by the condensation of large N-terminal peptide, which was prepared by recombinant DNA technology, with short C-terminal synthetic glycopeptide as shown in figure below. The N-terminal portion was expressed in *E. coli* using intein-mediated peptide thioester preparation (IMPACT system). cDNA of AGH corresponding to B chain, C peptide and A chain (1-16) was cloned into the Nde I-Sap I-treated plasmid pTWIN1. The DNA was expressed as a fusion protein with Mex GyrA intein and carbohydrate binding domain (CRD) in *E. coli* ER2566 strain.



On the other hand, [Asn(GlcNAc)^{18}]-A chain (17-28) was chemically synthesized by the Fmoc method starting from Fmoc-Tyr(*t*Bu)-OCH_2-Wang-resin. Asn18 was introduced using Fmoc-Asn(GlcNAcBn)$_3$. After the completion of the chain assembly, the resin was treated with Reagent K, followed by the low-TfOH treatment [3, 4] to remove benzyl groups at the carbohydrate portion. After HPLC purification, the desired glycopeptide was obtained.

The ligation of two components was carried out in 0.1 M phosphate buffer (pH 6.9) containing 6 M guanidine hydrochloride and 2% thiophenol under nitrogen atmosphere. After overnight reaction, a new band, which shows slightly larger molecular weight than the N-terminal polypeptide thioester, appeared on SDS PAGE, indicating that the ligation reaction proceeded smoothly. The desired proAGH was purified by GFC. Currently, we are examining the folding of the obtained proAGH as well as the preparation of proAGH carrying more complex carbohydrate portion.

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"CLICK PEPTIDE": A NOVEL "*O*-ACYL ISOPEPTIDE METHOD" FOR PEPTIDE SYNTHESIS AND CHEMICAL BIOLOGY-ORIENTED SYNTHESIS OF ALZHEIMER'S DISEASE-RELATED Aβ ANALOGUES

Youhei Sohma[1], Atsuhiko Taniguchi[1], Yousuke Chiyomori[1], Fukue Fukao[1], Taku Yoshiya[1], Maiko Kimura[1], Takuma Okada[3], Keisuke Ikeda[3], Yoshio Hayashi[1], Tooru Kimura[1], Shun Hirota[2], Katsumi Matsuzaki[3] and Yoshiaki Kiso[1]

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In 2003, we had a surprising discovery that the isomerization of the peptide backbone from the *N*-acyl to *O*-acyl isopeptide structure, i.e., formation of one single ester bond, significantly changed the unfavorable secondary structure of the difficult sequence-containing peptides. Thus, this finding led to the development of the "O-acyl isopeptide method" as a novel method in the field of peptide science [1]. Interestingly, shortly after we disclosed the "*O*-acyl isopeptide method", Mutter et al. [2], Carpino *et al.* [3] and Börner et al. [4] also confirmed the efficacy of the method.

We further designed "O-acyl isodipeptide unit", e.g., Boc-Ser/Thr(Fmoc-Xaa)-OH. The use of isodipeptide units, in which the racemization-inducing esterification reaction could be omitted, allows the application of "*O*-acyl isopeptide method? to fully automated protocols for the synthesis of peptides/proteins.

In Chemical Biology-oriented research, we have successfully applied the "*O*-acyl isopeptide method" to the synthesis of Alzheimer's disease (AD)-related amyloid β peptide (Aβ) 1–42 analogues, leading to the development of "Click Peptide". The "Click Peptide" did not exhibit the self-assembling nature under physiological conditions because of one single ester, and could migrate to the original Aβ1–42 with a quick and easy one-way conversion reaction (so-called "click") via an O–N intramolecular acyl migration. The "Click Peptide" method would open doors for the investigation of the biological functions of Aβ1-42 in AD by inducible activation of Aβ1-42 self-assembly.

Moreover, very recently, we have developed a novel "racemization-free segment condensation" based on the "O-acyl isopeptide method". We hope that the strategy using the "*O*-acyl isopeptide method", in which a simple isomerization to an O-acyl isopeptide remarkably, temporarily changes the physicochemical properties of the native peptide, and an O–N intramolecular acyl migration triggers the native amide bond formation under physiological conditions, will further contribute to peptides and proteins studies.

This research was supported in part by The “Academic Frontier” Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) from the Japanese Government, and The 21st Century COE Program from MEXT. Y. S. and T. O. are grateful for Research Fellowships of JSPS for Young Scientists.

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APPLICATION OF O-N INTRAMOLECULAR CARBONATE-CARBAMATE MIGRATION: MIGRATION OF PROTECTIVE GROUPS IN AMINO ACIDS AND WATER-SOLUBLE PRODRUG STRATEGY

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Introduction

The introduction of anti-cancer agents, paclitaxel (Taxol) and docetaxel (Taxotere), has revolutionized the treatment of cancer. Moreover, other taxoids have been recently developed with improved antitumor potency. However, despite the promise that these agents have engendered, their poor water-solubility is a serious problem in intravenous administration. To overcome this problem we have developed new prodrug strategy based on, a well-known reaction in peptide chemistry, O–N intramolecular acyl migration. Prodrugs of paclitaxel and other taxoids, 2'-O-acyl isoforms of parent drugs, showed higher water-solubility and promising kinetics data [1 - 4].

Results and Discussion

Taxoids can be divided into two groups, 3'-*N*-acyl derivatives such as paclitaxel and 3'-*N*-alkoxycarbonyl derivatives such as docetaxel. Therefore, our prodrug strategy was also applied to carbamate-type taxoids. This study demonstrates for the first time the pure O–N intramolecular carbonate-carbamate migration reaction which proceeds under aqueous conditions with no side product formation [4]. The first successful application of this reaction in a prodrug strategy was demonstrated with the same promising data as for acyl migration. Thus, our prodrug strategy can be extended to carbamate derivatives of taxoids except for Boc. All prodrugs showed a significant increase in water solubility and promising kinetic data. These prodrugs, a 2'-*O*-isoform of taxoids, have no additional functional auxiliaries released during conversion to the parent drugs. This would be an advantage in toxicology and general pharmacology, since the detergent for solubilization, which has some side effects, can be omitted and the potential side effects caused by reported auxiliaries can be avoided. Therefore, we can recommend this strategy as a first choice for water-soluble prodrug design for other drugs if O–N acyl or carbonate-carbamate intramolecular migration is possible. In addition, the O–N intramolecular carbonate-carbamate migration can be provided under aqueous conditions and with no side product formation such as previously reported oxazolidinone or product of carbonate hydrolysis. This migration occurred as a common reaction of hydroxyamino acids, namely carbonate protective groups migrated to produce amino-protected carbamate derivatives of hydroxyamino acids with high efficiency and purity [5]. For example, a benzyloxycarbonyl (Z) group

migrated quantitatively from the oxygen to nitrogen atom under mild basic aqueous conditions. Therefore, this atom-economical reaction can provide a useful tool for peptide as well as organic chemists as typical and widely used protective groups can undergo this reaction.

Acknowledgements

This research was supported in part by the "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), and the 21st Century Center of Excellence Program "Development of Drug Discovery Frontier Integrated from Tradition to Proteome" from MEXT. MS is grateful for the Postdoctoral Fellowship of JSPS. YS is grateful for the Research Fellowship of JSPS for Young Scientists.

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PHOTO-TRIGGERED "CLICK PEPTIDE" BASED ON THE "*O*-ACYL ISOPEPTIDE METHOD": CONTROLLED PRODUCTION OF AMYLOID β PEPTIDE (Aβ)1-42

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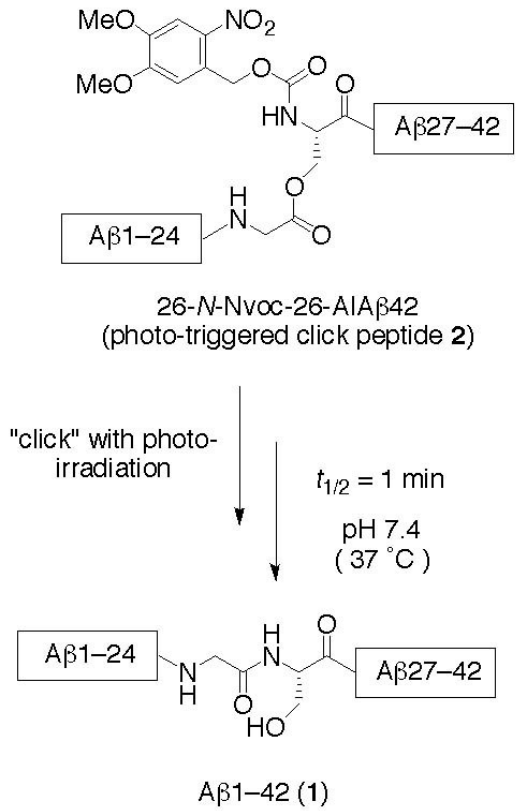
A clear understanding of the pathological mechanism of amyloid β peptide (Aβ) 1–42, a currently unexplained process, would be of great significance in the discovery of novel drug targets against Alzheimer's disease (AD). To date, it is known that the pathological mechanism is related to the Aβ1–42 dynamic events such as the folding, polymerization, and aggregation processes. However, the elucidation of these Aβ1–42 dynamic events is a difficult issue due to uncontrolled self-assembly, that also poses a significant obstacle in establishing an experimental system that clarifies the pathological function of Aβ1–42.

Recently, we have developed the "*O*-acyl isopeptide method" for peptide synthesis, in which a native amide bond at a hydroxyamino acid residue, e.g., Ser was isomerized to the ester bond, followed by an O–N intramolecular acyl migration reaction [1]. Moreover, we successfully applied this method to an efficient synthesis of Aβ1–42 (**1**) [2].

In this context, on the basis of the "*O*-acyl isopeptide method", we have developed a novel Chemical Biology-oriented analogue, photo-triggered "Click Peptide" of Aβ1–42 (**1**), e.g., "26-*N*-Nvoc-26-AIAβ42 (**2**)" in which a 6-nitroveratryloxycarbonyl (Nvoc) group was introduced at Ser^{26} in 26-*O*-acyl isoAβ1–42 (26-AIAβ42) [3].

From the results, 1) the click peptide **2** did not exhibit the self-assembling nature under physiological conditions due to one single modified ester; 2) photo-irradiation of the click peptide **2** and subsequent O–N intramolecular acyl migration afforded the intact Aβ1–42 (**1**) with a quick and one-way conversion (so-called "click"); and 3) no additional fibril inhibitory auxiliaries were released during conversion to Aβ1–42 (**1**).

This click peptide provides a novel system useful for investigating the dynamic biological functions of Aβ1–42 in AD.



This research was supported in part by the "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of the Japanese Government, and the 21st Century COE Program from MEXT. Y. S. and T. O. are grateful for Research Fellowships of JSPS for Young Scientists.

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"*O*-ACYL ISODIPEPTIDE UNIT" FOR THE EFFICIENT SYNTHESIS OF DIFFICULT SEQUENCE-CONTAINING PEPTIDES USING "*O*-ACYL ISOPEPTIDE METHOD"

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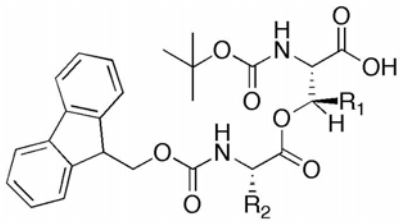
In regard to the synthesis of difficult sequence-containing peptides, we have recently disclosed an "*O*-acyl isopeptide method", in which a native amide bond at a hydroxyamino acid residue, e.g., Ser was isomerized to the ester bond, followed by an O–N intramolecular acyl migration [1].

In the synthesis of Ac-Val-Val-Thr-Val-Val-NH_2 (**1**) by *O*-acyl isopeptide method, Boc-Thr-OH was coupled to the H-Val-Val-NH-resin, and subsequent acylation with Fmoc-Val-OH to the β-hydroxyl group of Thr was performed using the DIPCDI/DMAP method in CH_2Cl_2 to obtain ester. After coupling with another Val residue, *N*-acetylation and TFA treatment, *O*-acyl isopeptide TFA×**2**, H-Thr(Ac-Val-Val)-Val-Val-NH_2 was obtained. However, a large amount of racemization (21%) of the esterified Val residue occurred in the DIPCDI/DMAP method [2].

To avoid this problem, we have developed a novel "*O*-acyl isodipeptide unit", Boc-Ser/Thr(Fmoc-Xaa)-OH. For the synthesis of **1** based on the *O*-acyl isopeptide method, the *O*-acyl isodipeptide Boc-Thr(Fmoc-Val)-OH was coupled to the H-Val-Val-NH-resin using the standard DIPCDI/HOBt method to obtain isopeptide-resin. After coupling with another Val residue followed by N-acetylation and 92.5% TFA treatment, *O*-acyl isopeptide TFA×**2** was obtained. HPLC analysis of crude **2** exhibited a high purity of the desired product **2** with no byproduct derived from the difficult sequence or racemization. Additionally, when TFA×**2** was dissolved and stirred in phosphate buffered saline (pH 7.4) at rt, quantitative O–N intramolecular acyl migration to the corresponding parent peptide 1 was observed with no side reaction.

A novel "*O*-acyl isodipeptide unit", Boc-Ser/Thr(Fmoc-Xaa)-OH has been successfully used for the efficient synthesis of difficult sequence-containing peptide based on the "*O*-acyl isopeptide method" in which racemization-inducible esterification could be omitted, suggesting that the use of *O*-acyl isodipeptide units allow the application of this method to fully automated protocols for the synthesis of long peptides or proteins.



R_1 = H; Boc–Ser(Fmoc–Xaa)–OH
R_1 = CH_3; Boc–Thr(Fmoc–Xaa)–OH

"*O*-acyl isodipeptide unit"

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N-S ACYL SHIFT REACTION ON PEPTIDE BACKBONE

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Introduction

It is recognized an *N-S* acyl shift reaction might occur at a cysteine residue in peptides to some extent under acidic conditions. However, the *N-S* acyl shift reaction has not been studied in details. In 1966, Sakakibara *et al*. referred to a possible *N-S* acyl shift reaction during the treatment of a cysteine-containing peptide with anhydrous hydrogen fluoride [1]. Vizzavona *et al.* recently reported that 4,5-dimethoxy-2-mercaptobenzyl (Dmmb) group-attached peptide generated a more polar compound on reversed-phase HPLC when the peptide was treated with a trifluoroacetic acid (TFA) solution [2].

Peptide thioesters are widely used as building blocks for polypeptide syntheses. An *N-S* acyl shift reaction is a key step in the protein splicing reaction [3, 4], which has been applied to the biological production of peptide thioesters [5]. If it were possible to accomplish an *N-S* acyl shift reaction of a peptide bond triggered by a chemical process such as TFA treatment, the reaction would offer a new basis for the development of a method for preparing peptide thioesters as well as for the chemical modification of proteins.

In this paper, we describe the evidence that an S-peptide is formed via an *N-S* acyl shift reaction in a TFA solution at a cysteine residue and also at the Dmmb group-attached amide bond in peptides. We also show that a 2-sulfoethyl thioester is formed in the reaction of the TFA-treated Dmmb-group-attached peptide with sodium 2-mercaptoethanesulfonate.

Results and Discussion

We analyzed products that were generated from thiol-containing peptides with a TFA solution. ^{13}C NMR spectroscopy indicated that 80% of Fmoc-Ile-Arg-Gly(1-^{13}C)-Cys-Arg-NH_2 (**1**) was transformed into a corresponding peptide thioester **2** in a TFA solution containing 29% $CDCl_3$ (v/v) after 900-h storage. An amide bond in a dipeptide, Fmoc-Gly(1-^{13}C)-D,L-(Dmmb)Ala-OMe (**3**), was also transformed into S-peptide **4** to the same extent in a TFA solution containing 14% $CDCl_3$ (v/v) after 48-h storage.

These results indicate that the *N-S* acyl shift reaction widely occurs under acidic conditions. S-peptide **4** was successively converted into a corresponding thioester, Fmoc-Gly(1-^{13}C)-$SCH_2CH_2SO_3H$ in the presence of sodium 2-mercaptoethane-sulfonate.

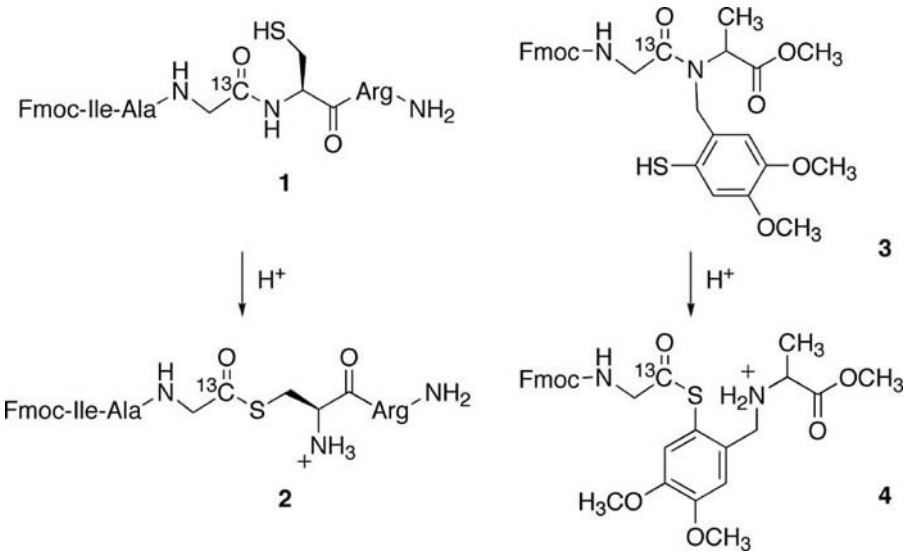

Fig. 1. N-S acyl shift reaction in a TFA solution.

Acknowledgements

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NOVEL THIO-DERIVATIVE OF PHENYLNORSTATINE AND ITS APPLICATION TO THE BACE1 INHIBITORS DESIGN

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Introduction

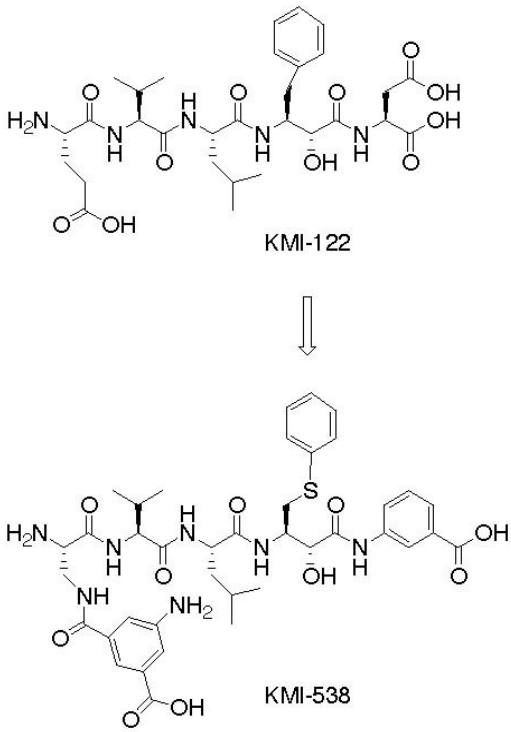
The accumulation of β-amyloid peptide (Aβ) is as a major factor in the pathogenesis of Alzheimer's disease. Aβ is formed by proteolytic processing of amyloid precursor protein (APP). Two enzymes, β-secretase (BACE1) and γ-secretase, are responsible for the sequential processing of APP. Since the cleavage of APP by β-secretase is the first step in Aβ formation, BACE1 plays a critical role in the progression of AD [1].

Results

In our previous study, we reported potent and small-sized BACE1 inhibitors containing phenylnorstatine [(*2R,3S*)-3-amino-2-hydroxy-4-phenylbutyric acid; Pns] at P1 position as a transition-state mimic. In developing more active compounds, we focused our attempts on the P1 position, where we replaced the Pns by its thio-derivative. Herein, we present the synthesis of a novel phenylthionorstatine [(2*R,3R*)-3-amino-2-hydroxy-4-(phenylthio)butyric acid; Ptns] as a P1 moiety, and then, an application to the BACE1 inhibitors design.We have synthesized Ptns starting from readily available *N*-benzyloxycarbonyl-serine and after multistep reaction (including Weinreb amide formation, thiophenyl group introduction, through cyanohydrin derivative the transformation into the 2-hydroxy ester and then acid). We have synthesized Ptns, and then the (*2R,3R*)-enantiomer was applied to SPPS (solid phase peptide synthesis). The peptide inhibitors, octa- and pentapeptide-type inhibitors of BACE1 containing Pns or Ptns at the P1 position, were adopted to enzyme assay using a recombinant human BACE1 and a fluorescence-quenching substrate, and showed high BACE1 inhibitory activity.

Discussion

A novel derivative of Pns, Ptns, was synthesized, and evaluated in comparison to corresponding Pns. Our attempts to replace Pns by its thio-derivative in octapetide type structure led us to KMI-172 (H-Glu-Val-Leu-Ptns-Asp-Ala-Glu-Phe-OH), an analogue of KMI-008 [2] (H-Glu-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH). Then, we reduced the size of inhibitors from octapeptides to pentapeptides. Compound KMI-122 (H-Glu-Val-Leu-Pns-Asp-OH) was a lead compound for pentapeptide series in our previous study [3]. Its Ptns counterpart possessed similar to Pns potency. After



modification at P1' position, where Asp was replaced by 3-aminobenzoic acid, and then combined with N-terminal optimization, we found out potent inhibitor KMI-538 with inhibitory activity 86% of BACE1 inhibition at 200 nM of enzyme concentration, while its Pns analogue KMI-494 possessed 80% of BACE1 inhibition at the same concentration of enzyme. This study suggests possibilities of the application of Ptns to develope inhibitors against other aspartyl proteases.

Acknowledgements

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DEVELOPMENT OF FIRST PHOTOACTIVATED PRODRUG OF PACLITAXEL VIA MODIFICATION OF PHENYLISOSERINE MOIETY

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Introduction

Paclitaxel (Taxol) is considered to be one of the most important drugs in cancer chemotherapy. However, this agent has no specificity for the target tumor tissues, which leads to systemic toxicity. A lot of prodrugs of paclitaxel have already been designed; however, none of them is available in clinical use. Photodynamic therapy (PDT) is used for cancer treatment. This technique is based on the administration of a sensitizer devoid of mutagenic properties, followed by the exposure of the pathological area to visible light. Photoactivation also affords a useful technique in life science to monitor biological processes by using so called "caged" compounds. These compounds are artificial molecules whose biological activity is masked by a covalently attached photocleavable group which can be selectively removed upon light activation to release parent bioactive molecules. Recently, we applied this caged strategy for a controlled generation of intact amyloid β peptide 1-42 (Aβ1-42) for the study of Alzheimer's disease [1].

Results and Discussion

Taking all those three strategies (prodrug, photodynamic therapy, and caged compound chemistry) into consideration, we designed a photoresponsive targeting prodrug of paclitaxel, namely phototaxel [2], which has a coumarin derivative conjugated to an amino group of isotaxel (*O*-acyl isoform of paclitaxel [3 - 5]). Phototaxel was prepared from 7-*N,N*-Diethylamino-4-hydroxymethyl coumarin (DECM) and 3'-*N*-debenzoylpaclitaxel. Upon visible light irradiation (430.6 nm) this prodrug released isotaxel ($t_{1/2}$ = 4.8 min), and subsequent spontaneous O–N intramolecular acyl migration (t = 15.1 min) formed intact paclitaxel. This delay of parent drug release after irradiation (related to migration of benzoyl group) is supposed to be short enough to avoid the intermediate (isotaxel) diffusion from the photoirradiated site before the parent drug is released. Moreover, we recently demonstrated much faster O–N intramolecular acyl migration in other highly potent taxoids [4 - 6]. Prodrug design based on the O–N intramolecular carbonate-carbamate migration reaction [7], exhibited even instantaneous conversion to a parent carbamate-type taxoid [5]. Phototaxoids derived from these type of taxoids would be more effective without risking diffusion from the irradiation site. In conclusion, we synthesized and evaluated a novel type of paclitaxel prodrug. This

prodrug showed promising kinetic data. Therefore, we believe that photoactivation can be promising novel strategy for design of tumor-targeting prodrugs.

Acknowledgements

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SYNTHESIS AND BIOLOGICAL EVALUATION OF SELECTED INSECT MYOTROPIC PEPTIDES

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[2]Department of Animal Physiology and Developmental Biology, A. Mickiewicz University, 61-701, Poznań, Poland

Introduction

Symbols and Special Characters Symbols and Special CharactersTo add a symbol to the paper body: Position the mouse-cursor where you wish to insert the symbol Click on the the symbol you would like to add In the last decade a number of insect peptides, structurally related to neuropeptide FMRF-amide has been identified. These peptides share the conserved X^1X^2RF-amide (where X^1 = R or F and X^2 = L) sequence at their C-terminal ends. Among them two peptides, Led-NPF-1 (ARGPQLRLRFamide) and Led-NPF-2 (APSLRLRF-amide) were isolated from the brain of *Lepitonotarsa decemlineata* [1] as well as Mas-MT-I, II and III (pEDVVHSFLRF-amide, GNSFLRF-amide, DPSFLRF-amide) which originated from the brain of *Manduca sexta* [3]. The object of our investigation was synthesis of two groups of peptides. There are: 1/ Led-NPF-1 (**I**), Led NPF-2 (**II**) and its 14 analogues with a fragment sequence such as: [2-10] - (**III**), [3-10] - (**VI**), [5-10] - (**V**), [6-10] - (**VI**), [7-10]- (**VII**) and [8-10] - (**IX**)-Led-NPF-1 and analogues where C-terminal Phe-amide was replaced by Phe-OH(**X**), and amides of D-Phe (**XI**), Tyr (**XII**), Phe(4-NO_2) (**XIII**) and Phe(4-N,N-di-Me) (**XIV**) and 2/ Mas-MT III (**XV**) and its analogues with shortened sequence [4-7]- (**XVI**), [3-7] - (**XVII**) and [2-7] - Mas-MT-III (**XVIII**).

Led-NPF-1, Led-NPF-2, Mas-MT-II and analogues were synthesized by the classical solid-phase method according to the Boc procedure. All peptides were purified by preparative HPLC on a RP-C-18 column.

Biological effect was evaluated by the cardiotropic test on the semi-isolated heart of two insect species: *Tenebrio molitor* and *Zophobas atratus* according to the method of Rosiński and Gäde [2]. In the preliminary investigation we found, that the Led-NPF-1 and its analogues show cardioinhibitory effect, while Mas-MT-III and analogues stimulated heartbeat in two insect species, similar to proctolin.

Results and Discussion

Both peptides (Led-NPF-1 and Led-NPF-2) induce a decrease in the heartbeat frequency and amplitude contractions and have the ability of inhibition of the endogenic contractile activity of the myocardium of *Tenebrio* and *Zophobas*. The heart of Tenebrio is inhibited by both peptides at the concentration range of 10^{-5} – 10^{-4} M, whereas the heart of Zophobas is inhibited by them at lower concentrations

Table 1. Myotropic effect on the heart of Led-NPF-1, Led-NPF-2, Mas-MT of their analogues in insect.

Peptide	Effect on the heart of insect relative to proctoline (%) (± SEM 7-10)			
	Z. atratus		*T. molitor*	
	at concentration		at concentration	
	10^{-6} inhibitory effect	10^{-9} stimulatory effect	10^{-7} stimulatory effect	10^{-8} stimulatory effect
Proctoline	100	100	100	100
I	-67	6	-	-
II	-95	6	-	-
III	-80	11	-	-
IV	-46	10	-	-
V	-53	17	-	-
VI	-45	10	-	-
XV	-	-	36	27
XVI	-	-	56	31
XVII	-	-	51	45
XVIII	-	-	31	21

(10^{-7} – 10^{-6}). Thus there seems to be a species-specific action of the NPF-related peptides which might depend on the sequence of these peptides. The differences in cardioinhibitory activity of both peptides can be in part attributed to differences in their potency and efficiency. Possibly, Led-NPF-1 and -2 are structurally more related to a native NPF-peptide of Zophobas (and thus better agonist) than to peptide(s) of *Tenebrio*. At present there is no information on the chemical structure of the native NPF-peptides of *Tenebrio* and *Zophobas.*

The carried out studies have been shown the cardiostimulatory action of Mas-MT-III and its analogues on semi-isolated heart of *Tenebrio molitor*. The peptides **XVI** and **XVII** have the strongest stimulatory activity, similar to the proctoline, however **XVIII** and **XV** are less potent cardiostimulators.

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MODIFYING PROTEIN p53 TETRAMERIZATION BY DESIGNED CALIX[4]ARENE COMPOUNDS

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Introduction

Protein p53 is one of the main targets in oncology research due to its high mutation frequency in human cancers. Some mutations affect its tetramerization domain compromising the ability to form the active tetramer. This is the case of R337H, associated to adrenocortical carcinoma [1]. Molecules which stabilize the tetrameric structure of p53 could rescue the functionality of those mutants. Pursuing this goal, calix[4]arene ligands have been structure-based designed and synthesized (Fig. 1 and 2). The results of a biophysical study of the interaction of ligands calix_bridge and calix[4]prop with the tetramerization domain of wild-type p53 (p53wt) and mutant R337H are here reported.

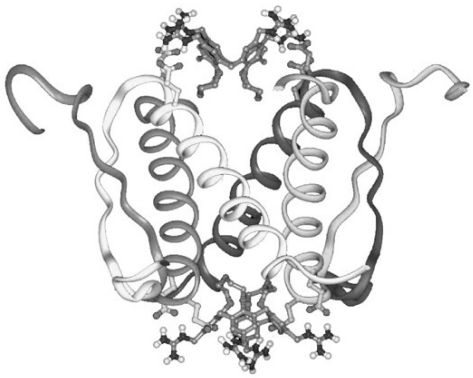

Fig. 1. Interaction model of calix[4]arens with p53 tetramerization domain

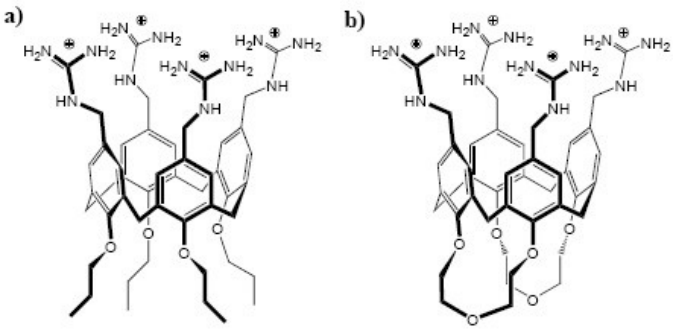

Fig. 2. Designed calix[4]arenes ligands: a) calix_bridge and b) calix[4]prop

Results and Discussion

a) Calix_bridge

Structural characterization of calix_bridge interaction has been done by NMR. ^{15}N-^{1}H-HSQC spectra show how protein signals shift in the presence of the calixarene, being the most affected those of residues near the designed binding region. Saturation transfer experiments (^{1}H-STD) on the ligand have allowed calix_bridge mapping, which completely agrees with the way it was designed.

Protein thermal stability has been evaluated by differential scanning calorimetry (DSC) and circular dichroism (CD). Calix_bridge increases R337H melting temperature by 20 ºC. Furthermore, CD spectra show that the calixarene stabilises the secondary structure of R337H. For p53wt no significant changes are observed.

Isothermal titration calorimetry (ITC) has determined that the interaction is endothermic, thus entropy driven. Data best fit models of sequential binding sites.

In conclusion, calix_bridge behaves as it was designed to.

b) Calix[4]prop

Calix[4]prop exhibits an amazing different behaviour, and results suggest that its interaction is causing not only thermal stabilization but also an structural change.

By ^{15}N-^{1}H-HSQC it has been observed that calix[4]prop decreases protein signal intensity, up to a point (around 1 equivalent) when they disappear and a whole new set of signals appears. Some of these new signals even shift when adding more calixarene.

CD spectra in the presence of the calixarene clearly show a change in the secondary structure of the protein, thus leading to hypothesize that new NMR signals may correspond to a new structural species. In spite of causing a lost in the α-helix component, the presence of calix[4]prop thermally stabilizes the proteins. By DSC and CD it has been found that R337H melting temperature increases by 30 ºC, while p53wt by 6 ºC.

Two energetic processes can be distinguished by ITC. A first endothermic process is observed at low stoichiometries. A second exothermic process becomes predominant at the same stoichiometries described for the NMR changes. Experimental data do not fit to any of the standard binding models, thus suggesting a more complicated interaction mechanism.

Many questions remain to be answered for calix[4]prop interaction. However, it is important to notice what a role plays the flexibility of the propyl groups in the calixarene ligand interaction.

Acknowledgments

S. Gordo and M. Martinell acknowledge the AGAUR for a predoctoral fellowship.

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SECONDARY STRUCTURAL ELEMENTS AND INTRAMOLECULAR H-BONDING PATTERNS OF POLY-(ALA) AND POLY-(GLN) PEPTIDES

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Introduction

Homopolymeric amino acids (HPAAs) are polypeptides consisting of the same amino acids. Some of them play a relevant role in the formation of several neurodegenerative diseases. Most probably the poly-(Ala) and poly-(Gln) are the best representatives of these peptides because of their important biological effects. Therefore, our aims were to perform conformational analysis and to investigate the secondary structural elements as well as intramolecular H-bonding patterns of these two HPAAs.

Results

To explore the conformational spaces of poly-$(Ala)_n$ and poly-$(Gln)_n$ peptides with different lengths (n = 7, 10 and 14), simulated annealing (SA) calculations were carried out with the AMBER 8 program [1]. For the SA simulations, the AMBER 99 force field [2] and the generalized Born solvent model were applied. Two different forms of the abovementioned HPAAs were modelled: either with charged N-terminal amino group and C-terminal carboxyl group or with the N- and C-termini blocked by acetyl and *N*-methyl amide groups, respectively.

For the conformers obtained from the SA calculations, the occurrence of various secondary structural elements (different types of β-turns, γ- and inverse γ-turns, α-helix, 3_{10}-helix, poly-proline II helix as well as parallel and antiparallel β-strands) was investigated. These results showed that for the poly-$(Ala)_n$ and poly-$(Gln)_n$ peptides, various secondary structures including mainly type I and type III β-turns, α-helix, 3_{10}-helix and antiparallel β-strand could be found along the entire sequence of molecules. Among the conformers of the HPAAs, several structures were observed, in which not only one, but two or more β-turns appeared either consecutively or in separated tetrapeptide units. Additionally, α- and 3_{10}-helical as well as antiparallel β-strand-like segments with different lengths were also identified in the investigated peptides.

For the conformers of both HPAAs, the intramolecular H-bonds formed between the backbone atoms were determined. Furthermore, the H-bonds evolved between the backbone and side-chain atoms as well as between the side-chain atoms of different residues were also identified for the poly-$(Gln)_n$ peptides. In accordance

with the presence of β-turns, 3_{10}- and α-helical segments, mainly i←i+3 and i←i+4 H-bonds formed between the backbone atoms were observed for both HPAAs. Moreover, in the case of poly-$(Gln)_n$ peptides, further different intramolecular H-bonds appeared by the participation of CO and NH groups of the Gln side-chains.

Discussion

The results of the conformational analysis pointed out that the poly-$(Ala)_n$ and poly-$(Gln)_n$ peptides can be characterized by different secondary structural elements, and together with their occurrences, characteristic H-bonding patterns could be observed for the HPAAs. In the case of terminally blocked form of peptides, larger populations of the abovementioned secondary structural elements were found compared to the zwitterionic form of molecules with the same length. For the conformers of the HPAAs, β-turns appeared in any segment consisting of four amino acids along the entire sequence of peptides. The periodic secondary structural elements (α-helix, 3_{10}-helix and antiparallel β-strand) evolved mainly in the tri- and tetrapeptide units, however, longer helices and strands also appeared in a smaller number.

Acknowledgements

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CONFORMATIONAL ANALYSIS OF THE ANTIMICROBIAL PEPTIDE, INDOLICIDIN CONTAINING CIS OR TRANS XXX-PRO PEPTIDE BONDS

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Introduction

Indolicidin is a 13-residue antimicrobial peptide (*H*-ILPWKWPWWPWRR-NH_2), which was isolated from bovine neutrophils [1]. This molecule possesses a wide spectrum of antibacterial, antifungal and antiviral activity, furthermore it has also haemolytic effect. Data derived from previous structural investigations led to considerably diverse conclusions regarding its secondary structure, therefore the aim of this study was to examine the effect of *cis-trans* isomerization on the conformational properties of this antimicrobial peptide.

Results

The conformational analysis of indolicidin containing cis or trans Xxx-Pro peptide bonds was performed by simulated annealing (SA) calculations with the use of AMBER 99 force field [2] and generalized Born solvent model. The SA simulations were carried out with the AMBER 8 program [3]. The indolicidin was modelled in two forms regarding the three Xxx-Pro peptide bonds: for either of them, the abovementioned peptide bonds were kept in trans configuration (all-trans), and for the other, the peptide possessed three *cis* Xxx-Pro peptide bonds (all-*cis*).

For the conformers of indolicidin with *cis* or *trans* Xxx-Pro peptide bonds, the evolving secondary structural elements were examined. In the case of all-trans isomer, type I and III β-turns were observed in certain tetrapeptide units, which contain Pro in the second position. Nevetheless, 3_{10}- and poly-proline II helical segments also appeared along the sequence of peptide possessing *trans* Xxx-Pro peptide bonds. For the all-*cis* isomer, type VI β-turns were identified in those tetrapeptide units, in which Pro is located in the third position. In the case of both isomers, β-turns and 3_{10}-helical segments occured in tetrapeptide units containing Pro in the first position, especially at the C-terminal part of peptides.

In the case of indolicidin, various intramolecular interactions may play an important role in stabilizing the structure of conformers. Therefore the presence of the H-bonds between backbone atoms, the aromatic-aromatic interactions between the side-chains of Trp amino acids and the proline-aromatic interactions between the side-chains of Trp and the pyrrolidine ring of Pro amino acids was investigated. According to the occurrences of β-turns and 3_{10}-helical segments, i←i+3 H-bonds formed between the backbone atoms were observed in certain tetrapeptide units. In

both isomers, aromatic-aromatic interactions were identified between the indole groups of Trp side-chains, however few types of them had large population. Proline-aromatic interactions formed between the Pro and the following Trp were found in both all-*trans* and all-*cis* isomers, while large populations of the interactions between the Pro and the preceding Trp were observed only for all-*cis* form.

Discussion

The conformational comparison of the peptides possessing *cis* or *trans* Xxx-Pro peptide bonds resulted in different secondary structural elements for both isomers. In spite of the differences, the C-terminal part of all-*trans* and all-*cis* forms showed partly similar conformational properties. The occurrences of various intramolecular interactions are in agreement with the observed secondary structures. The H-bonds play a role in the stablilization of type I and III β-turns as well as 3_{10}-helical segments, while the proline-aromatic interactions contribute to the stability of type VI β-turns.

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INFLUENCE OF MIF-1 AND TYR-MIF-1 ON THE DISTRIBUTION AND DENSITY OF NADPH-D AND TYROZINE HYDROXYLASE IN RAT BRAIN

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Introduction

The Tyr-MIF-1 family of peptides includes MIF-1, Tyr-MIF-1 are endogenous neuropeptides with opiate modulating and other effects in the central nervous system [1]. Tyr-MIF-1 is a novel peptide structurally related but immunoreactively different from MIF-1 [2]. Addressing this problem, we applied NADPH-d histochemistry and tyrosine hydroxylase and in order to determine distribution patterns of tyrosine hydroxylase (TH)-immunoreactive axonal elements and density of NADPH-d-reactive neurons in rat striatum treated with MIF-1 and Tyr-MIF-1 for similar properties.

Materials and Methods

We used Wistar rats treated intraperitoneally with MIF-1 and Tyr-MIF-1 (1 mg/kg) to study the histochemistry for NADPH-d and the immunocytochemistry for TH in the striatum. Morphometric analysis was performed using a microanalysis system (Olympus CUE-2). Data were compared by Student's t-test.

Results and Discussion

Different types of neurons are stained for NADPH-d such as large to small ovoid aspiny neurons in all parts of striatum. The staining patterns of NADPH-d-reactive neurons are comparable with those completed by the Golgi method. Their dendrites can be followed a long distance away from the cell body (Fig. 1).

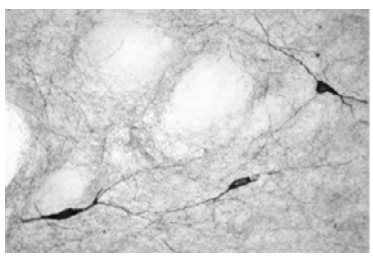

Fig. 1. The NADPH-d-reactive interneurons in the caudal division of the striatum. ×120.

Analyses of the quantitative data reveal that none of these two experiments altered the density of the NADPH-d-reactive neurons per mm2 in the striatum for rats treated with MIF-1 or Tyr-MIF-1 (Fig. 2).

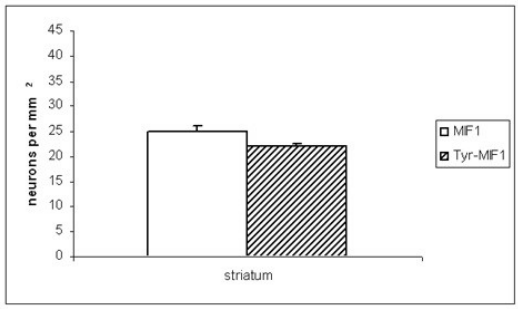

Fig. 2. Statistical analysis of NADPH-d-reactive neurons density measures in dorsal striatum revealed no significant differences for rats treated with MIF-1 or Tyr-MIF-1. ($P<0.1$). Values are presented as means ± standard error of the mean (S.E.M.).

The semiquantitative analysis revealed different representation of TH immunoreactive axonal elements. The highest concentration of TH immunoreactive axonal elements was found in the rostrally than caudally across its anteroposterior axis (Fig. 3).

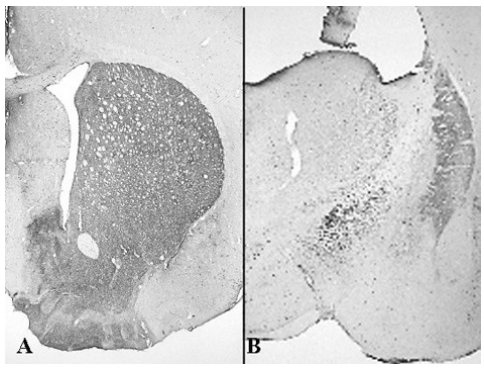

Fig. 3. TH immunoreactive fibers and axonal terminals were more abundant rostrally (A) than caudally (B). ×25.

In summary MIF-1 and Tyr-MIF-1 may affect distribution patterns of TH-immunoreactive axonal elements and density of NADPH-d-reactive neurons in the striatum.

Acknowledgements

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF AMINO ACID ESTER PRODRUGS OF ACYCLOVIR

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Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine (ACV) is an acyclic guanine nucleoside analogue that is widely used clinically as an antiherpetic agent. Its limited absorption (15% - 20%) in humans after oral administration prompted the search for prodrugs. A possible way to increase the bioavailability is by modifying the known antiviral drugs with various amino acids.

The aim of this study was to design and to synthesize of new thiazole containing amino acids (Val, Thz) ester prodrugs of acyclovir and to explore their activity on the HSV-1.

Synthesis of thiazole containing amino acid (Val, Gly) ester prodrugs of acyclovir involved formation of N-Boc protected amino acid anhydrides, coupling of the N-Boc protected amino acid anhydride with acyclovir, and deprotecting the amino group of the amino acid ester of acyclovir.

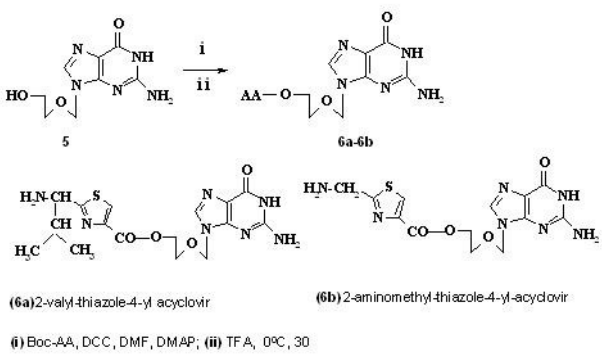
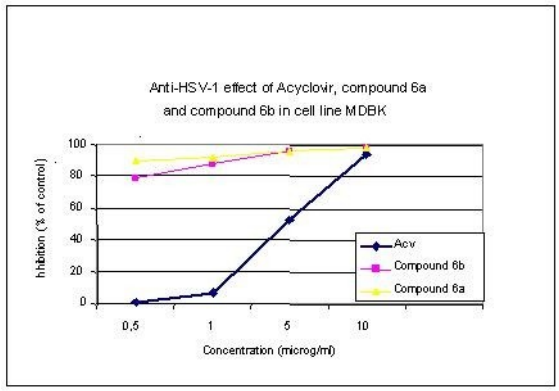


Figure 1.

The two examined derivatives and acyclovir as referent drug were applied in concentration 10, 5, 1 and 0.5 μg/ml. The Val-thiazole-4-yl-acyclovir and 2-aminomethyl-thiazole-4-yl-acyclovir shown insignificant effects on the herpesvirus replication – 20 and 10% inhibition respectively. Whereas the referent drug inhibited the viral replication completely in same dose (10 μg/ml).



These results suggest that Val-thiazole-4-yl-acyclovir and 2-aminomethyl-thiazole-4-yl-ACV may be attractive in higher concentrations for antiviral chemotherapy obligatory with lower cytotoxicity effect in comparison with the effective nucleoside analogs.

Design of amino acid prodrugs seems to be an attractive strategy to enhance the solubility of the otherwise poorly aqueous soluble compounds and also to afford a targeted and possibly enhanced delivery of the activedrug.

An implicit proof of this assumption is the fact that L-valyl ester of acyclovir (valacyclovir) shows bioavailability of 60%.

INFLUENZA A VIRUS PARTICLES AS A SUBSTRATE FOR BROMELAIN: REVEALING OF THE MATRIX M1 PROTEIN ACCESSIBILITY FOR LIMITED PROTEOLYSIS

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A method of Influenza A hemagglutinin (HA) spikes cleavage by enzyme bromelain in the presence of 50 mM β-mercaptoethanol [1] was initially employed for HA ectodomains crystallographic study [2]. The remaining spikeless subviral particles were used by us earlier for HA2 C-terminal fragment extraction and mass spectrometric (MS) study [3]. For structural investigation of HA2 C-terminal fragments and membrane-associated matrix M1 protein, we had to optimize conditions of the subviral particles preparing.

Among proteolytic enzymes probed (thermolysin, subtilisin Carlsberg, chymotrypsin, bromelain) bromelain (Sigma, Cat. No. 5144) has demonstrated the highest hydrolytic activity in relation to HA spikes. Bromelain activity was measured against high specific chromogenic substrate for cysteine proteases assay - Glp-Phe-Ala-*p*NA. The enzyme exhibited maximal activity in the presence of 1 - 5 mM β-mercaptoethanol, while 50 mM β-mercaptoethanol reduced enzyme activity dramatically. It was shown that 1.3×10^{-7}M E-64 (Sigma) was enough for complete inhibition of bromelain reaction.

Kinetics experiments with Influenza A virions revealed that most part of HA became digested by bromelain in the presence of 5 mM β-mercaptoethanol for 1.5 h; all HA was removed for 3h (Fig. 1A). In the absence of reducing agent, bromelain also removed HA spikes but slower: complete spikeless particles could be prepared for 6 h.

Several additional protein bands in a range of about 9 - 23 kDa were detected by SDS-PAGE analysis of the subviral particle preparations (Fig. 1B). These bands were analyzed by MALDI-TOF MS after in-gel trypsin hydrolysis and by N-terminal sequencing. They were shown to be fragments of M1 protein. These fragments didn't appear if virions were incubated at 37 °C with trypsin having no ability to digest HA, or without any enzyme. The M1 protein destruction was

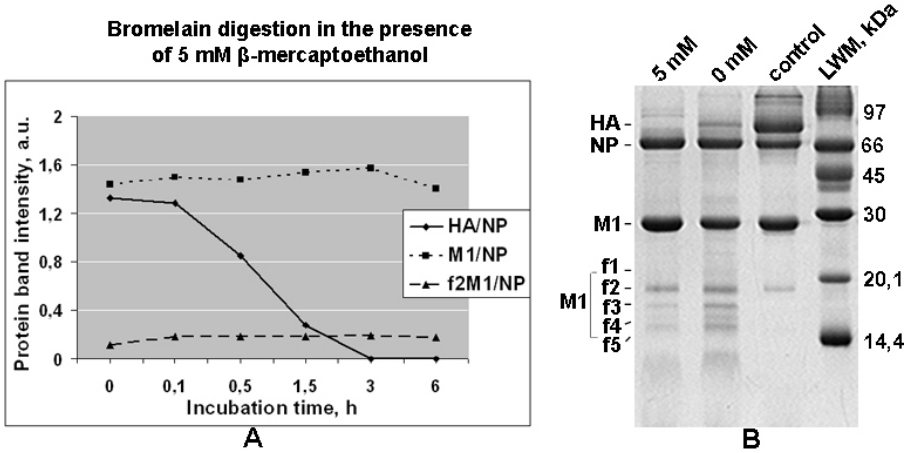

Fig. 1. Bromelain digestion of Influenza virions at 37 °C. (A) SDS-PAGE densitometry analysis of the subviral particles prepared during various periods of incubation with bromelain. (B) SDS-PAGE analysis of the control intact virions and subviral particles prepared for 3 h in the absence or presence (5 mM) of β-mercaptoethanol. Viral protein/Bromelain = 2:1 (w/w). HA-hemagglutinin; NP-nucleoprotein; M1-matrix protein; M1(f1-f5)-M1 protein fragments.

seriously reduced in the presence of β-mercaptoethanol. So, in the process of HA cleavage, M1 protein localized inside subviral particles became somehow accessible for limited proteolysis.

This work was supported by the ISTC #2816p and RFBR #06-04-48728.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SELECTED FRAGMENTS OF THE CRF-RELATED DIURETIC PEPTIDE OF LOCUSTA MIGRATORIA

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Introduction

Insect Diuretic Hormones are crucial for control of water balance in insects. Locusta Diuretic Hormone (Locmi-DH) is a neuropeptide, with 46 amino acids, that has been isolated from the locust Locusta Migratoria. It belongs to the CRF-related diuretic peptides and has the following amino acid sequence:

MGMGPSLSIVNPMDVLRQRLLLEIARRRLRDAEEQIKANKDFLQQI-*NH₂*

Locmi-DH(1-46)

Locmi-DH [1, 2] and its analogue[Hse(Me)^{1,3,13}]-Locmi-DH(1-46) [3], where methionines have been replaced by the isosteric amino acid methyl-homoserine, stimulate fluid secretion and cAMP production by locust Malpigian tubules *in vitro* and it is released into the haemolymph from corpora cardiaca. Structure-activity studies have shown that C-terminal fragment Locmi-DH(32-46) is active in the feeding behavioral assay in Locusta Migratoria while has very low response in stimulating cAMP production by Malpighian tubules from Scistocerca Gregaria [3].

In the present work we synthesized the C-terminal analogues Locmi-DH(34-46), Locmi-DH(36-46) and Locmi-DH(38-46) in order to determine whether the C-terminal fragment Locmi-DH(32-46) can be further truncated N-terminally with loss of its effects on feeding behavior in locust. We also synthesized the N-terminal fragments analogues [Hse(Me)^{1,3,13}]-Locmi-DH(1-30), [Hse(Me)^{13}]-Locmi-DH(6-30)-OH to study the role of the N-terminal part of Locmi-DH on biological activity.

Results and Discussion

The synthesis of C-terminal analogues Locmi-DH(34-46), Locmi-DH(36-46) and Locmi-DH(38-46) was performed in the solid phase in the stepwise manner and on 2-chlorotrityl-chloride resin (2-CLTR), on which linker Rink-Bernatowitz was anchored [4], using Fmoc/tBu method and DIC/HOBt in DMF as the coupling agent. The full deprotection was carried out using TFA/DCM and appropriate scavengers [1]. The strategy for the synthesis of the N-terminal fragments analogues [Hse(Me)^{1,3,13}]-Locmi-DH(1-30) and [Hse(Me)^{13}]-Locmi-DH(6-30)-OH involved the solid phase synthesis, on the 2-CLTR resin [5], of the protected acid fragments Fmoc-Locmi-DH(1-5)-OH, Fmoc-Locmi-DH(6-12)-OH, Fmoc-Locmi-DH(13-23)-OH and these were successively coupled to the and Locmi-DH(24-30) which was anchored on 2-CLTR resin using 3 mmol excess of the Fmoc-protected

fragments over the N-componenet. As condensing agent was used DIC/HOBt in DMSO. Each condensation reaction was performed in three short-lasting fragment couplings of 3 h and the applied concentration of the C-component was 0.1 M . In that way the peptide fragment did not remain activated in solution for a long time thus minimizing racemization. The crude peptides analogues were purified by semi-preparative HPLC.

The purity of the peptide analogues was estimated by reverse-phase HPLC and their structures were identified by ESI-MS. The C-terminal peptide fragments when tested for feeding behavior had no significant activity compared to the Locmi-DH(32-46) which is active. These results indicate that the Locmi-DH(32-46) is the minimum sequence required for activity in the feeding behavior assay. For the N-terminal fragments preliminary results showed also no significant activity in the above assay.

Acknowledgments

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FTIR SPECTROSCOPIC STUDIES ON HELICAL INTERMEDIATE STAGE DURING AGGREGATION PROCESS OF THE B-AMYLOID 11-28 FRAGMENT AND ITS VARIANTS

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Introduction

The amyloid β peptide (Aβ) is the major component of the amyloid plaques in the brains of Alzheimer's disease patients. Monomeric, physiological Aβ is benign, but by an unknown mechanism it becomes aggregated and neurotoxic. Recent evidence suggests that Aβ oligomers, not the final, insoluble fibril, constitute the main cause of Aβ neurotoxicity. Studying rare genetic cases associated with the early, familial Alzheimer's disease led to the hypothesis that familial AD mutations facilitate Aβ assembly into neurotoxic oligomers [1]. Despite many studies on clinically relevant Aβ variants, the mechanisms by which the single mutations cause diseases with diverse pathological presentation are not fully understood. To shed some light on details of structural changes accompanying the initial stage of the aggregation process, we have studied the Aβ(11-28) fragment and its mutation-related variants using FTIR spectroscopy. Our recent CD and aggregation studies on this Aβ fragment proved it to be a good model for structural studies [2].

Results and Discussion

FT IR studies of Aβ(11-28) and its clinically relevant variants (Fig. 1) in hexafluoroisopropanol (HFIP) and its mixtures with D_2O enabled us to compare the conformational and aggregational behaviour of the Aβ mutant fragments in terms of structural changes provoked by water addition.

Aβ(11-28) (native): $E^{11}VHHQKLFF\underline{A^{21}E^{22}D^{23}}VGSNK^{28}$-*amide*

Variants: Flemish (A21G), Arctic (E22G), Italian (E22K),

Dutch (E22Q), Iowa (D23N)

Fig. 1. The sequence of the studied Aβ(11-28) peptides.

The spectroscopic data revealed similarities between the native and the Italian mutant fragments and between the Arctic and Dutch Aβ(11-28) variants. The wild type fragment and its E22K counterpart were found to be the most helical ones. The spectra in pure HFIP, a strong β-helix inducer, revealed an unexpectedly high amount of β-sheet conformations (~1625 cm^{-1}) in all variants studied. Moreover, in the case of the Arctic and Dutch variants the band was split and a weak high-

frequency component (~1685 cm^{-1}) was observed, suggesting their profound propensity towards antiparallel β-sheet formation [3].

The unequivocal spectral analysis of the spectra in HFIP/D_2O mixtures was slightly impeded by the lack of reference IR data in these solvents; nevertheless, some general observations could be made.

For the majority of the peptides the increase of the D_2O content resulted in the transient increase of a helical conformation share supporting the idea of an α-helix-containing intermediate in the aggregation process [4].

In the spectra of the slow-aggregating Aβ(11-28) E22K and A21G, besides peaks typical of an α-helical conformation (1652 - 1659 cm^{-1}), there are unique maxima at ~1666 cm^{-1} which can be attributed to 3_{10}-turns/3_{10}-helix, which may imply that this conformation is a folding intermediate to a well-structured α-helix. In the case of the Italian variant a strong maximum at 1666 cm^{-1} disappears after further addition of water and the emergence of a peak characteristic of an α-helix (1653 - 1655 cm^{-1}) is observed.

The spectra of the Flemish variant imply that the aggregation of this Aβ variant proceeds via unstructured conformations, leading probably to a different pathological mechanism.

The presence of bands between 1613 and 1633 cm^{-1} and their "high-frequency counterparts" (1685 - 1691 cm^{-1}) reflects the presence of different forms of β-sheet-forming structures. With the increase of the D_2O content, a pronounced increase of the band at the lowest frequency (1613 - 1620 cm^{-1}) is observed manifesting a progressive formation of multistrand, antiparallel β-sheet.

Acknowledgements

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TWO EFFICIENT WAYS TO MASK DESTABILIZING INTERHELICAL ELECTROSTATIC REPULSIONS IN α -HELICAL COILED COIL-PEPTIDES

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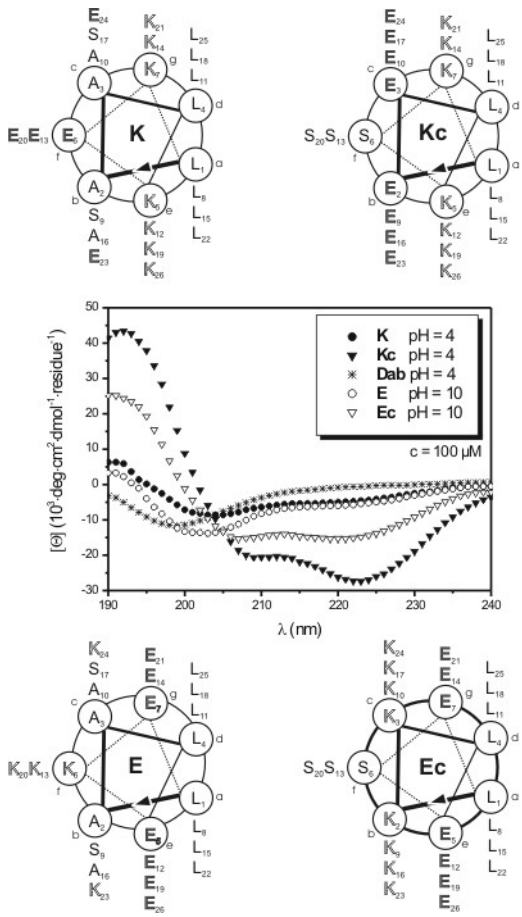
The α-helical coiled coil structural motif consists of two to five α-helices which are wrapped around each other with a slight left-handed superhelical twist. Its sequence is characterized by a heptad repeat pattern of seven amino acids denoted $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$. Positions a and d are mostly occupied by apolar amino acids (Leu, Ile and Val) which form the interhelical hydrophobic core by *knobs into holes* packing [1].



Many of the factors that contribute to coiled coil stability are very well understood [2, 3]. However, the role of the amino acids not being a discrete part of one of the interhelical recognition domain has not been systematically investigated. To unveil the impact of amino acids in positions b and c on conformation and coiled coil stability four model peptides were examined using temperature dependent circular dichroism spectroscopy (CD) at different concentrations and pH values.

All model peptides have a pH-dependent interhelical electrostatic repulsive recognition domain (positions e and g) composed exclusively of Lys (peptides K and Kc) or Glu (peptides E and Ec). In peptides Kc and Ec positions b and c are occupied by charged amino acids, while in peptides K and E these positions are mostly occupied by unpolar residues like alanine and serine. Therefore, the formation of intrahelical ionic interactions is only possible in case of peptides Kc and Ec. As expected, pH induced interhelical electrostatic repulsion predominantly resulted in random coil formation of the peptides K (at pH 4) and E (at pH 10). In contrast, the peptides Kc as well as Ec exhibit an α-helical conformation over a wide pH range (pH 4 - 10). Considering the identical amino acid composition within positions a, d, e and g of the corresponding peptides K/K_c and E/E_c, respectively, these observations are rather surprising. According to these findings, coiled coil stability also depends on the amino acids that are not part of the interhelical recognition domains. These results suggest that conformational instability due to interhelical repulsions can be compensated by intrahelical attractive interactions between positions e and b as well as between c and g, respectively.

The possibility of positions e and g to exhibit also hydrophobic interactions with the hydrophobic core have been discussed [4]. We could show, that a reduction of the alkyl side chain length by one ethylene unit through replacement of all lysines in peptide K by the non-natural amino acid L-α,γ-diaminobutyric acid (peptide Dab) leads to a dramatic destabilization of the coiled coil. This finding proves that the hydrophobic part of the amino acid side chains in positions e and g additionally take part in formation of the hydrophobic core by extending it and protecting it from the aqueous solvent.

Therefore, the repulsion of equally charged amino acids in positions e and g can be overruled by involving them either into attractive intrahelical electrostatic interactions or into hydrophobic core formation.

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STRUCTURE-ACTIVITY STUDIES OF RGD-CONTAINING PEPTIDES DERIVED FROM ADHESIVE PROTEINS. APPLICATION OF STRUCTURAL CRITERIA EVALUATED FROM BIOACTIVE SYNTHETIC RGD PEPTIDE ANALOGUES

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Intoduction

Integrins are cell surface receptors, mediating cell adhesion to extracellular matrices [1]. Of the known integrins, half of them bind to the Arg-Gly-Asp (RGD) motif as the primary recognition sequence of their ligands. Among the numerous processes involving the integrins and adhesive proteins is platelets aggregation. The most critical integrin for the aggregation phenomenon is the $\alpha_{IIb}\beta_3$ via its binding to fibrinogen. However, the ability of an integrin to distinguish between different RGD-containing extracellular matrix proteins is believed to be partially due to the variety of RGD conformations. Structural studies by NMR and molecular dynamics simulations suggested the presence of a well defined Gly-Asp type II β-turn as a prerequisite for integrin binding while selectivity of ligands has been correlated with the distances between either the C^{β} atoms or the opposite charged centers of Arg and Asp residues and/or the pseudo-dihedral angle (*pdo*) (formed by the Arg C^{ζ}, Arg C^{α}, Asp C^{α} and Asp C^{γ} atoms) which defines the relative orientation of the Arg and Asp side chains [2]. The results of a previous study indicated a very good correlation between the pseudo-dihedral angle and the biological activity and the fulfillment of the criterion $-45^{o}<pdo<+45^{o}$ in combination with the longer distance between the opposite charged centers was proved to be a prerequisite for an analogue to exhibit activity [3].

In the present study, we examined the above mentioned structural criteria in combination with the anti-aggregatory activity of RGD-containing 15-peptides, derived from the active sites of the adhesive proteins fibrinogen, fibronectin, vitronectin and osteopondin as well as, from the cryptic RGD site of von Willebrand factor. The peptides H-NIMEILRGDFSSANN-OH and H-SSTSYNRGDSTFESK-OH (108-122 and 585-599 of the α chain of fibrinogen, respectively), H-VDTYDGRGDSVVYGL-OH (153-167 of osteopondin), H-VYAVTGRGDSPASSK-OH (1518-1532 of fibronectin), H-VLYMDERGDC(Acm)VPKAQ-OH (692-706 of vWF) and H-C(Acm)KPQVTRGDVFTMPE-OH (58-72 of vitronectin) were synthesized and studied by ^{1}H NMR and molecular dynamics simulations.

Results and Discussion

Among the six 15-peptides, the one derived from the segment 58-72 of human vitronectin exhibited the higher anti-aggregatory activity. All the other 15-peptides, even those derived from fibrinogen, which is the main ligand of $\alpha_{IIb}\beta_3$, proved to be less potent than the Ac-RGD-NH_2. A possible explanation for this result is either the absence of possible synergistic sites, which exist, in the native proteins and/or the conformational flexibility of the linear 15-peptides compared to the conformation of the same fragments in the native chains of the proteins.

The conformation of the peptides derived from the sequences of the above adhesive proteins, was studied by ^{1}H NMR spectroscopy in DMSO-d_6 solution and torsion angle dynamics structure calculations. Apart from overall structures, we focused on the local RGD conformational features in order to evaluate the structure-activity relationship of RGD peptides, which inhibit the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$. Our results have shown that the backbone secondary structure of the RGD fragment of these peptides exists in similar conformations in solution and as it has been also shown for some native proteins, the common conformational feature is a turn, centered at Arg-Gly or Gly-Asp residues. This conformation allows the opposite charged side chains of Arg and Asp to be exposed from the core of the molecule and therefore be able to bind to integrins. However, the differences in activity of compounds can only be correlated with the distance and the relative orientation of the Arg and Asp side chains. Application of structural criteria regarding the two opposite charged centers has shown that a distance greater than 7 Å between them is a common feature for the most active compounds, but this cannot stand as a unique criterion since other inactive compounds also fulfill this distance. A better correlation of the structure-activity relationship is provided by the pseudo-dihedral angle (*pdo*) formed by the two charged centers [2]. *pdo* Values in the range of -45° to +45° are characteristic of the active analogues and when this criterion is fulfilled, longer distances than 7 Å between the opposite charged centers are indicative of the most potent compounds.

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CHARACTERIZATION OF PACAP ANALOGS RESISTANT TO DIPEPTIDYL PEPTIDASE IV

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 27- or 38-amino acid peptide initially identified for its ability to stimulate cAMP formation in anterior pituitary cells [1]. Recent studies indicate that PACAP acts as a potent neuroprotective factor in various *in vivo* and *in vitro* pathophysiological models [2]. In particular, an i.v. infusion of PACAP after middle cerebral artery occlusion appeared to be efficient to reduce the infarct volume in the injured brain and to improve neurological functions. Despite its therapeutic potential in diverse neurodegenerative diseases, the short *in vivo* half-life of PACAP impairs its use as a clinical agent. PACAP is mainly metabolized by dipeptidyl peptidase IV (DPP IV), an ubiquitous amino-terminal dipeptidase [3]. Furthermore, the degradation of PACAP by DPP IV leads to the formation of metabolites that may behave as antagonists towards the PAC1 receptor, which is responsible for the neuroprotective effects of PACAP. Therefore, the development of PACAP agonists resistant to degradation by DPP IV represents a first step in the design of a neuroprotective drug.

Results

PACAP analogs of both isoforms (27- and 38-amino acids) were synthesized using solid phase peptide chemistry. The chemical modifications were targeted on position 2 (Ser) and the N-terminal His moiety. PACAP analogs (10^{-5} M) were incubated with DPP IV (5 mU) to assess their stability against this enzyme. RP-HPLC and mass spectrometry were used to determine the half-lives of the peptides. All peptides were also tested for their ability to induce rat pheochromocytoma PC12 cells differentiation, an effect mediated through the PAC1 receptor [4]. In this assay, PC12 cells were exposed to increasing concentrations of peptides (from 10^{-12} to 10^{-7} M) and the number of cells was counted and compared to the control group. The EC_{50} and maximal efficacy are shown in Table 1.

Discussion

Acylation (acetyl or hexanoyl) of the N-terminus or substitution of the Ser2 residue with D-Ser prevented the metabolic action of DPP IV for at least 4 hours. Similarly, incorporation of a-aminoisobutyric acid (Aib) increased the peptide stability but did not inhibit completely the enzymatic action of this peptidase. In accordance with previous studies, PACAP27 analogs that were labile towards DPP IV showed a higher resistance compared with their PACAP38 counterparts [5]. All modified

Table 1: Biological activity and stability against DPP IV of PACAP analogs.

Peptides	*DPP IV assay*	*PC12 cell differentiation bioassay*	
	Half-lives (min)	$EC_{50} \pm S.E.M$ [a] *(nM)*	$E_{max} \pm S.E.M.$ [b] *(%)*
PACAP38	19	0.79 ± 0.14	100 ± 4
[N-α-acetyl]PACAP38	> 240*	1.40 ± 0.16	98 ± 6
[N-α-hexanoyl]PACAP38	> 240*	2.21 ± 0.15	85 ± 4
[D-Ser^2]PACAP38	> 240*	0.96 ± 0.14	91 ± 3
[Aib^2]PACAP38	55	1.05 ± 0.15	104 ± 5
PACAP27	28	0.56 ± 0.13	99 ± 2
[N-α-acetyl]PACAP27	> 240*	0.74 ± 0.14	95 ± 3
[N-α-hexanoyl]PACAP27	> 240*	1.93 ± 0.17	85 ± 4
[D-Ser^2]PACAP27	> 240*	0.82 ± 0.15	89 ± 3
[Aib^2]PACAP27	191	0.46 ± 0.14	99 ± 3

[a] Concentration producing 50% of the maximum effect.
[b] Percentage of efficacy compared with the maximal value obtained with PACAP38.
* No degradation was observed after 240 minutes of treatment.

peptides were highly potent to inhibit PC12 cell proliferation and to induce their differentiation, except for the (N^{α}-hexanoyl)PACAP38 and (N^{α}-hexanoyl)-PACAP27 analogs, which were slightly less effective. Because (N^{α}-acetyl)PACAP derivatives remained active, the decrease in biological activity observed with the N^{α}-hexanoyl analogs most likely comes from steric hindrance produced by the aliphatic chain rather than from the absence of the free amine function. Thus, this study showed that it is possible to increase the resistance of PACAP against DPP IV while preserving the biological activity of the peptide. The improved stability of those new peptides should help to demonstrate the potential of PACAP analogs for the treatment of neurodegenerative diseases.

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MEASUREMENT AND APPLICATION OF RESIDUAL DIPOLAR COUPLINGS IN MOLECULAR MECHANICS SIMULATIONS ON EFRAPEPTIN C

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Introdruction

The fungus Tolypocladium niveum and other members of this genus roduce the efrapeptins as a mixture of closely related sequence analogues. They are peptide antibiotics and consist predominantly of the nonproteinogenic amino acids α-aminoisobutyric acid (Aib), isovaline (Iva), β-alanine (βAla) and pipecolic acid (Pip), have an acetylated N-terminus, and bear an unusual cationic C-terminal head group. Jost *et al.* established the first total synthesis of efrapeptin C [1].

The crystal structure of F_1-ATPase in complex with efrapeptin C was determined by Abrahams et al. [2]. Here, efrapeptin C adopts a 3_{10}-helical secondary structure between the amino acids 1-6 and 9-15 with an unusual cis-peptide bond between Leu6 and βAla7. The efrapeptins are competitive inhibitors of F_0F_1-ATPase, insecticides and active against the malaria pathogen *Plasmodium falciparum*. An anti-proliferative effect was also reported.

Results and discussion

α- and 3_{10}-Helical peptides have similar CD-band shapes in the far UV. For the differentiation between α- and 3_{10}-helix a ratio $R = \theta_{230}/\theta_{208}$ can be calculated. For efrapeptin C in 2,2,2-trifluoroethanol, the ratio R is 0.3 indicating 3_{10}-helical or β-bend-ribbon structure elements. In combination with VCD data, differentiation between α- and 3_{10}-helix is possible. Here, a weakly negative absorption for the amide II-band would hint towards a α-helix, while the strong negative absorption indicates a 3_{10}-helix for efrapeptin C.

Conformational analysis based on NMR structural data was started to derive structure-activity relationships. Improved assignment and a sufficient number of restraints were achieved by measurement and application of residual dipolar couplings (RDC). The RDC-values were determined by HSQC spectra without decoupling during acquisition in weakly crosslinked poly(dimethylsiloxane) (PDMS) as alignment medium. The quadrupolar deuterium splitting indicated the quality of the gel [3].

A DG/SA approach generated the starting structures based on NOE and ^3J coupling constants as restraints. The lowest energy conformation with NOE violations less than 0.05 nm was used as starting structure for the subsequent RDC based SA-refinement. The initial alignment tensor was calculated with the powder pattern method and then until convergence with the program Pales [4, 5]. Molecular

dynamics were performed with GROMOS96 starting with the best energy conformation from the prior SA. The secondary structure derived from RDC values contains 3_{10}-helical and β-bend-ribbon structure elements between residues 1-6 and 9-16. Further, it is stabilised by hydrogen bonds between CO^{-i} and $NH^{(i+4)}$. PDMS influences the structure of efrapeptin C obviously only by interacting with Gly13 resulting in a high field shift of its ^{1}H resonances. This could result in a reduced helix breaking effect of Gly and an extended helical structure at the C-terminus.

The structure shows higher dynamics in the central and terminal region and is nearly fixed in the helical regions. The preference for a right- or left-handed helix is partly not clearly assignable. Aib as an achiral secondary structure inducer shows no preference and results in φ and ψ torsion angles adopting positive and negative values of helices and β-turns [6].

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IANUS PEPTIDE ARRAYS: A NEW METHOD FOR DETECTION OF PROTEIN-LIGAND INTERACTION SITES

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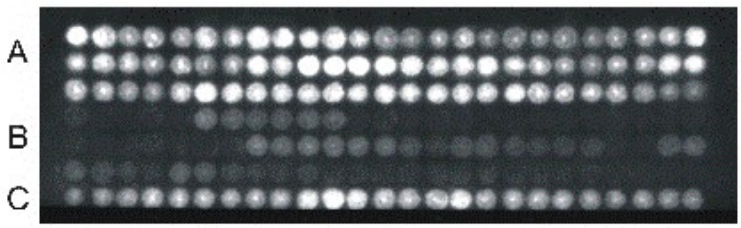
Introduction

Protein-protein and protein-ligand interactions are between few of the most important processes in living organisms. The understanding of those interactions gives us insights into fundamental events of the life cycle. Among others, peptide libraries prepared by spot synthesis on different solid carriers are powerful biotechnological tools for the study of these interactions [1]. It is well known that the tertiary structure of proteins is essential for their biological functions, a property which is lacked by smaller peptides. However, it has already been shown that small peptide fragments can (i) adopt a well-defined secondary structure and (ii) retain some of the activity of the parent protein. In this work we are showing that protein-ligand interactions can be represented by and investigated as peptide-peptide interaction using peptide pairs that are immobilized on a solid support (IANUS peptide arrays) [2].

Results and Discussion

As a model system is the well studied Streptavidin/*Strep*-tag II complex applied (K_d = 72 μM) [3]. Using a standard spot-technique, the streptavidin scans are synthesized on to one side of the orthogonal protected linker, which is attached to the derivatised cellulose membrane, and the *Strep*-tag II peptide (SNWSHPQFEK) on the another side. Subsequently, peptide pairs attached to the cellulose membrane are probed with streptavidin and any bound streptavidin is detected. The IANUS hypothesis assumes that two peptides representing the (i) interacting epitope of the protein and (ii) the corresponding ligand are able to interact when synthesized on a solid support. Hence, the potential binding of *Strep*-tag II to soluble streptavidin probe should be decreased due to the existence of competitive interactions with the neighboring immobilized peptide epitope derived from streptavidin. The key amino acids involved in the binding of *Strep*-tag II to streptavidin either involved in hydrogen bonds and salt bridges like Ser45, Ala46, Val47, Glu51, Ser52, Tyr54, Arg84, Ser88, Thr90 or hydrophobic interactions like Trp79, have been identified by this array. It is also shown that substituting IANUS positive interacting pairs at a single amino acid position in each peptide chain gave a clear indication for the specificity of the method. A protein free method for detection of matrix bound peptide-peptide interaction in-between each peptide pair is developed. The each

peptide chain in pair is labeled with dansyl (as fluorescence donor) or fluorescein (acceptor). After irradiation of the membrane with UV light of 360 nm, peptide-peptide interactions are analyzed by measuring the fluorescence intensity at 520 nm of dye labels. The interacting peptide pairs are identified as highly fluorescence spots. However, the densitometry analysis of each spot is made and the fluorescence intensity of peptide pair is calculated by dividing of peptide pair fluorescence with sum of fluorescence of each single chain ($I = I_{df}/(I_d+(I_f)_{mean})$. If the membrane is incubated in biotin solution (native streptavidin ligand $K_d = 10^{-15}$) the fluorescence of interacting peptide pairs derived from binding pocket of streptavidin decreased significantly.



A) Streptavidin scan (12,2)-dansyl:Strep-tag II-fluorescein I_{df}
B) Streptavidin scan (12,2)-dansyl:No second chain I_d
C) No first chain:Strep-tag II-fluorescein I_f

The Ianus-peptide array provides a novel high-throughput method for a preliminary mapping of unknown protein-ligand interaction sites. The array allow as detection of protein interacting sites even without use of soluble protein. Also, screening for inhibitors of protein-ligand or protein-protein interaction is possible using this method.

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ABOUT EPIMERIZATION OF PEPTIDE ALDEHYDES – A SYSTEMATIC STUDY

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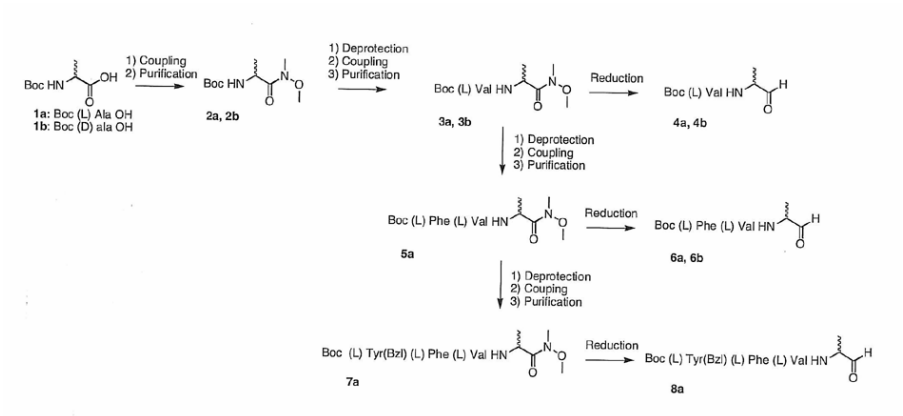
Introduction

Various peptide aldehydes have been found to be potent enzyme inhibitors [1 - 4]. These peptide aldehydes can also be used in a wide range of chemistry including pseudo-peptide bond [5] or chemical ligation [6] Whatever the chosen synthetic route, the obtained peptide aldehydes are known to be very sensitive to epimerization on the C-terminal α carbon. To our knowledge, no systematic study has been done on this phenomenon. We propose in this study a simple method allowing detection of the optical purity on a model dipeptide aldehyde. We also studied various purification conditions of di-, tri-, and tetrapeptide aldehydes with the aim to find a free epimerization method.

Results

As the Weinreb amide [7] can be used both as a C-terminal-protecting group during peptide elongation and as a precursor for the aldehyde function, we decided to perform the synthesis in solution, which allows the purification of all intermediates before reduction (Scheme 1).



Scheme 1. Synthetic route to peptide aldehydes.

We considered our previous model tripeptide Boc-Phe-Val-Ala-H, and we chose to add at the N-terminus Boc-Tyr(Bzl)-OH for its hydrophobic character allowing easy purification of the Weinreb precursor on silica gel column. The syntheses were performed in parallel starting from L-Ala and L-Ala to get the authentic diastereoisomers corresponding to the di-, tri-, and tetrapeptides. After reduction the

crude peptide aldehydes were analyzed by ^{1}H NMR spectroscopy in $CDCl_3$, and revealed only one proton aldehyde peak, showing once again that the Weinreb amide reduction in these conditions was racemization-free. We then placed the peptide aldehydes in various purification conditions: solvent in the presence of silica gel, solvent containing 0.1% pyridine in the presence of silica gel and solvent in the presence of alumina, the last two conditions supposed to suppress the acid-catalyzed epimerization by avoiding enolization of the aldehyde moiety (*Table 1*).

Table 1. Determination of the epimerization of the C-terminal residue by ^{1}H NMR under various purification conditions and after storage of the crude peptide aldehyde in solution.

Compounds	% [a] crude of reduction		% D[a] SiO_2	% D[a] SiO_2/π	% D[a] Al_2O_3	% D[a] on crude after one week
	% D	% L				
4a	0	100	41	49	32	35
4b	100	0	71	68	57	83
6a	0	100	49	30	48	30
6b	100	0	75	59	52	68
8a	0	100	25	34	45	28

Discussion

We have demonstrated that epimerization of the adjacent carbon to the aldehyde function in peptide aldehydes can be easily checked by ^{1}H NMR in $CDCl_3$ not only in tripeptide, but also in dipeptide and tetrapeptide aldehydes. Furthermore, as it can be seen in Table 1, the three different conditions tested for purification were not free of epimerization, either at level of the dipeptide or at the levels of tri- and tetrapeptides. No correlation could be found regarding the epimerization rate in the three purification systems. On the other hand, it could also be observed that the peptide aldehydes epimerized when they were stored in $CDCl_3$ solution (even on storage for only a week at room temperature). We can conclude that in the tested conditions, no free epimerization purification method exists for peptide aldehydes. We propose in this study a model dipeptide aldehyde Boc-(L)-Val-(L)-Ala-H to check by ^{1}H NMR the safety of new synthetic methods of peptide aldehyde epimerization.

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NMR STUDY OF UROTENSIN-II RECEPTOR STRUCTURAL DOMAINS

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Introduction

Human urotensin-II (hU-II) [1] and urotensin-II-related peptide (URP) are potent vasoconstrictors and this action is mediated through a specific receptor identified as UT [2, 3]. This receptor is expressed abundantly in the mammalian cardiovasculature and effects of U-II and URP can be blocked with urantide, a selective antagonist. Recently, we have shown, using the surface plasmon resonance technology, that both U-II and URP bound extracellular loop-2 (EC2) and -3 (EC3) with similar affinities, whereas none of these two ligands were able to interact with the extracellular loop-1 (EC1). Moreover, the binding of urantide was observed only with EC2. The absence of binding of urantide to EC3 suggested that this loop would be involved in the signal transduction process [4]. Therefore, the structural characterization of the extracellular domains of UT will help in understanding the specificity of ligand binding. In this study, we solved the solution structures of the EC2 and EC3 domains in order to further investigate the interaction of U-II, URP and urantide to UT receptor.

Results

Synthetic receptor fragments EC2 (L182-L212) and EC3 (A281-T300) were studied using NMR spectroscopy in the presence of DPC micelles. The association of the peptides with micelles was clearly shown by the increase in the line width of the 1H signals. All proton resonances were assigned using a combination of COSY, TOCSY and NOESY experiments. Analysis of the NMR conformational parameters (NOEs and Halpha chemical shift) indicated the presence of helices in the N-terminal and C-terminal regions of EC2, and in the C-terminal region of EC3. From NOESY spectra, a total of 386 and 261 distances restraints were generated for EC2 and EC3 respectively. These restraints were used for structure calculations with CNX program. EC2 presented an α-helix at both extremities involving residues P183-R192 and P205-Y211, whereas for EC3, a short α-helical structure was observed between residues H284 and L288 followed by a long α-helical structure between residues P290 and T300.

Discussion

Structural evaluations of GPCRs showed that the membrane domains of the receptors adopt frequently a similar geometry. Thus, we analyzed a number of structural characteristics of synthetic UT extracellular fragments involved in the binding of agonists and antagonist of UT. In DPC micelles, which served to mimic

the biological membrane environment, we showed that EC2 and EC3 adopted helical conformations. This structuration might be essential for selectivity and affinity of the ligands.

Acknowledgements

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STRUCTURAL DETAILS OF BACTERIAL LIPOPEPTIDES DETERMINING RECOGNITION BY TOLL-LIKE RECEPTOR DIMERS

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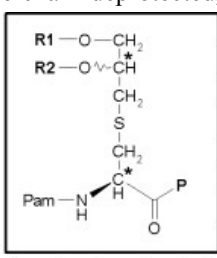
Introduction

Toll-like receptors (TLR) are expressed on cells of the innate immune system and respond to microbial constituents. Eleven different mammalian TLRs have been identified so far. Lipoproteins (LP) which are strong immune modulators of the innate immune system [1] are part of the outer membrane of bacteria, *Rhodopseudomonas viridis*, and mycoplasma. Until recently, it was generally assumed that triacylated LP and their synthetic analogs like $Pam_3Cys\text{-}SK_4$, are recognized by TLR2/TLR1 heteromers, whereas diacylated LP, like FSL-1, induce signalling through TLR2/TLR6 heteromers. To analyse more closely the structural basis of triacylated LP in TLR heteromer usage a variety of synthetic LP analogs was synthesized (Fig. 1) and tested using cells of TLR2-, TLR6-, and TLR1-deficient mice [2, 3].

Results

The synthesis of triacyl-lipopeptides was carried out by fully automated solid phase peptide synthesis and Fmoc/*t*Bu chemistry on chlorotrityl-resins. The peptidyl-resin was finally elongated with the unusual lipoamino acid *N*-palmitoyl-*S*-(2,3-dihydroxy-2(*R,S*)-propyl)-(*R*)-cysteine (Fmoc-Dhc) [4] followed by esterification of the diol on solid phase with fatty acid (10 eq), DIPEA (10 eq) DMAP (1 eq) and DIC activation (10 eq). The triacyl-lipopeptides were cleaved from the resins and side-chain deprotected, purified by precipitation and analysed by ESI-MS. Using our general method for the synthesis of triacyl-lipopeptides seven different carboxylic acids were attached to the resin-bound palmitoyl-lipopeptide PamDhc-$SSNASK_4$ (Fig. 1).



R1 = R2	Lipoprotein analog
Hexanoyl-	$Pam Hex_2 Cys\text{-}P$
Octanoyl-	$Pam Oct_2 Cys\text{-}P$
Nonanoyl-	$Pam Pel_2 Cys\text{-}P$
Decanoyl-	$Pam Dec_2 Cys\text{-}P$
Dodecanoyl-	$Pam Dod_2 Cys\text{-}P$
Tetradecanoyl-	$Pam Myr_2 Cys\text{-}P$
Hexadecanoyl-	$Pam Pam_2 Cys\text{-}P$

Pam: Hexadecanoyl residue (palmitoyl)
P: Peptide sequence $SSNASK_4$

Fig. 1. Core structure of the newly synthesised triacyl analogs of the bacterial lipoproteins.

Polyclonal activation of B-lymphocytes from spleen cells of TLR1-/- and the corresponding wild type mice was performed as described [5]. 4×10^5 spleen cells/200 µl in 96-well flat bottom cell culture dishes were stimulated for a total culture time of 48 h and incubated with ^{3}H-TdR for the final 24 h of culture. $PamDec_2C$-$SSNASK_4$, $PamDod_2C$-$SSNASK_4$, $PamMyr_2C$-$SSNSSK_4$- and $PamPam_2C$-$SSNASK_4$ were strong stimulators of B-lymphocyte proliferation from wild time mice, whereas $PamOct_2C$-$SSNASK_4$ and $PamPel_2C$-$SSNASK_4$ had only moderate bioactivity. The LP analog $PamHex_2C$-$SSNASK_4$ exerted only low stimulatory activity. In TLR1-deficient B-lymphocytes no responses were observed after stimulation with LP analogs with short and moderate length (6 up to 10 carbon atoms) of the ester-bound fatty acids. However, $PamDod_2C$-$SSNASK_4$, $PamMyr_2C$-$SSNASK_4$, and $PamPam_2C$-$SSNASK_4$ stimulated TLR1-deficient B-lymphocytes with about 50% activity in comparison to the responses in wild-type cells (Fig. 2). A similar structure-activity relationship of these LP analogs was found for stimulation of IL-6 and TNF-a-release in bone-marrow-derived macrophages (data not shown).

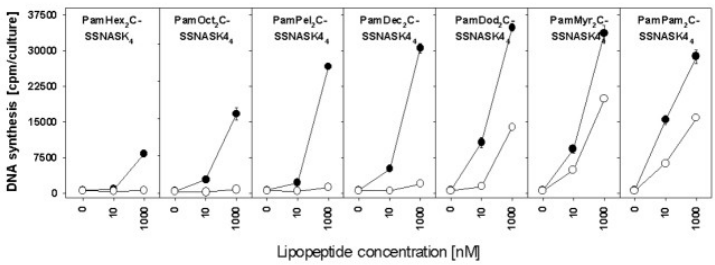


Fig. 2. Stimulation of cells from TLR1-deficient and wild-type mice with various Pam_2C-$SSNASK_4$ analogs. Murine spleen cells were stimulated with synthetic LP at various concentrations. ● wild type cells; O TLR1- deficient cells.

Discussion

In accordance to our previous discovery of TLR6-independent diacylated LP we demonstrate here that not the acylation pattern alone defines the coreceptor usage but also the nature of the acyl chains. Triacylated LP containing fatty acids of chain length from 12 to 16 also stimulate cells in a TLR1-independent manner. Furthermore, we could establish triacylated LP with short-length ester-bound fatty acids (like $PamOct_2C$-$SSNASK_4$), which induce no response in TLR1-deficient cells.

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EN ROUTE TO SYNTHETIC ANTIFREEZE GLYCOPEPTIDES

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Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) allow organisms to survive in polar and subpolar seas where the water temperatures decline below the colligative freezing point of their body fluids. Both protein families adsorbe on the surface of embryonic ice crystals to inhibit their growth. The antifreeze activity of these compounds has been proven by different experimental observations as thermal hysteresis, change of crystal shape, suppression of recrystallization and ice nucleation. In contrast to AFP, the function of AFGP has not yet been examined in detail due to the structural complexity and numerous isoforms isolated from natural sources and difficulties in the synthesis of homologous peptides with high sugar density.

AFGPs usually consist of a varying number of repeating units of $(Ala\text{-}Ala\text{-}Thr)_n$ with minor sequence variations and the disaccharide α-D-galactosyl-(1-3)-β-*N*-acetyl-D-galactosamine attached as a glycoside to the hydroxyl oxygen of the threonine residues.

The first step to such glycopeptides is a high yield synthetic approach to the disaccharide with an acetamido moiety in the C-2 position and the preparation of the glycosylated amino acid. Different routes from the literature have been evaluated regarding the anomeric ratio in the glycosylation and the yields in multigram synthesis. These reactions include different orthogonal protecting group as well as glycosylation strategies. All disaccharide precursors have been generated from easily accessible D-galactose pentaacetate. Classical glycosylation methods [1] such as the Koenigs-Knorr and the trichloroacetimidate method have been tested.

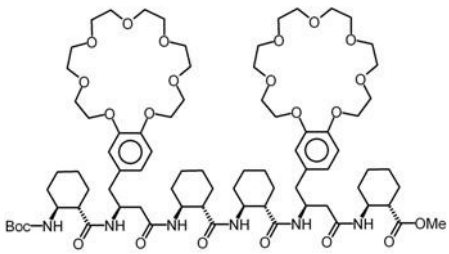
The preparation of the disaccharide succeeded in 85% with a diastereomeric ratio of 9:1 favouring the desired β-anomer using a trichloroacetimidate donor. The glycosylation of the amino acid using the Koenigs-Knorr method and the trichloroacetimidate method yields between 50 - 80%, but usually gives poor α-selectivity (1:1 to 4:1).

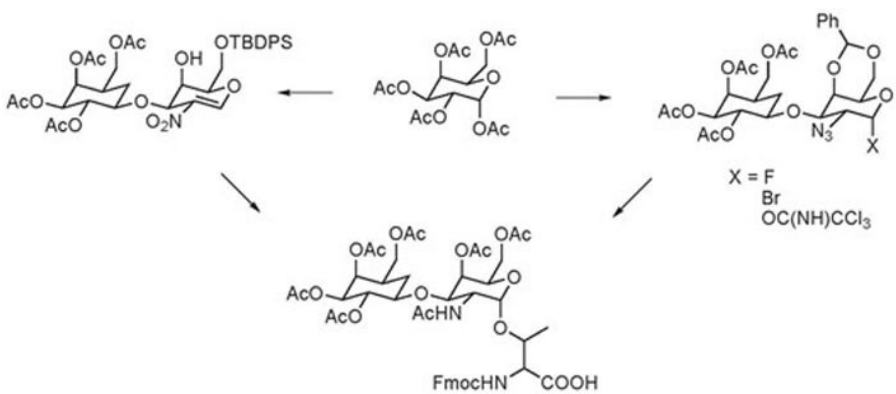
Alternatively, a nitroglycal in a Michael-like reaction was employed to obtain the glycosylated amino acid [2].

Different routes lead to the desired product, but the diastereoselectivities and overall yields are quite different. Especially the reactions using a Koenigs-Knorr donor for the formation of an α-glycosidic bond to the amino acid give poor anomeric ratios. The trichloroacetimidate method is very useful for the preparation of β-linked carbohydrates. The most promising strategy for the preparation of the

glycosylated amino acid is the nitroglycal assembly. The different glycosylation steps give very good anomeric ratios, though the protecting group strategy needs optimisation.

After successful preparation of the amino acid, it will be used in peptide synthesis using an Fmoc-strategy. The conformational analysis of the peptides with various spectroscopic methods and the microphysical analysis are important for a deeper understanding of the molecular requirements of antifreeze activity and structural optimisation.





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DNA-PEPTIDE INTERACTION FORCES ON THE SINGLE MOLECULE LEVEL

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Interactions between proteins and DNA are important to all living organisms. The goal is to investigate the molecular recognition between DNA and epitopes of the transcription factor PhoB from *E. coli* on the single molecule level and to identify specific amino acids required for DNA binding.

The protein PhoB controls the expression of proteins involved in phosphorus transport and metabolism. It is composed of a transactivation domain (amino acids 1 - 127) and a DNA binding domain (DBD, amino acids 123 - 229), that binds to specific DNA sequences (*pho* boxes) containing a TGTCA sequence using a *helix-turn-helix*-motif. Phosphorylation of the

transactivation domain changes the conformation and activates PhoB. The isolated DBD binds to DNA without activation [1]. Chemical synthesis of peptide epitopes present in the DNA binding domain of PhoB (amino acids 190 - 209) [2] and isolation of the full DBD (amino acids 127 - 229) of PhoB were performed. The protein was purified using intein mediated protein purification [4]. An additional cysteine residue was ligated to the protein using intein mediated ligation reactions. The cysteine can be used for immobilisation and labeling.

Mutated peptides and proteins, in which strategic amino acids (Arg193, His198 and Arg203) were replaced by alanine residues have also been examined to reveal the contributions of single residues to molecular recognition. CD spectra do not indicate structural differences between mutant and wildtype proteins.

The binding contribution of the protein is determined by electrophoretic mobility shift assays (EMSA) using Cy3-labeled *pho* box DNA. With *expG* DNA as negative control no shift is visible. For the mutants, no activity was detected in EMSA-experiments.

In single molecule force spectroscopy (AFM) experiments [2] it has been shown that a peptide with a native PhoB-sequence (PhoB(190 - 209)) as well as the recombinant protein PhoB(127 - 229) bind to DNA (see Table 1). Compared to the peptide the binding of the protein is increased 1000-fold. In the wildtype protein, the three changed amino acids, Arg193, His198 and Arg203, are important for unspecific DNA-binding by forming salt bridges with the phosphate backbone [3]. For both, peptide PhoB(190 - 209) and protein PhoB(127 - 229), the corresponding

mutant R203A did not display any activity. Since the CD spectra did not show obvious differences, Arg203 seems to be crucial for binding. In contrast, the mutants R193A and H198A have shown different activities in peptides and proteins. Both proteins exhibited less binding than the wildtype protein, for the mutant PhoB(127 - 229) R193A a stronger binding was measured than for mutant PhoB(127 - 229) H198A.

Peptide PhoB(190 - 209) H198A displayed less activity than the peptide with native sequence. Surprisingly, the peptide PhoB(190 - 209) R193A displayed stronger binding than the native peptide. This result can presumably be attributed to an enhanced α-helical conformation of the peptide in solution [1]. In all cases, competition experiments were performed to prove specific DNA binding. In future experiments on-rates will be measured in surface plasmon resonance and fluorescence correlation spectroscopy experiments.

Table 1: AFM results

Mutation	k_{off} [s^{-1}] **peptide PhoB (190-209)**	τ [s]	k_{off} [s^{-1}] **protein PhoB (127-229)**	τ [s]
wildtype	$3,1 \pm 2,1$	0,32	$0,0025 \pm 0,0021$	400
R193A	$0,071 \pm 0,053$	14	$0,012 \pm 0,008$	83
H198A	$49,5 \pm 21,2$	0,20	$0,760 \pm 0,250$	1
R203A	–	–	–	–

Acknowledgements

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FEEDING-RELATED EFFECTS OF CART (COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT) PEPTIDES AND CHOLECYSTOKININ IN MOUSE OBESE MODELS

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Introduction

CART (cocaine- and amphetamine-regulated transcript) peptides are neurotransmitters involved in feeding, stress and endocrine regulation [1]. CART is expressed in high amounts in about half of the vagal afferent neurons, together with CCK-A receptor which mediates satiating effect of cholecystokinin (CCK). Broberger et al [2] showed that all CCK-A expressing cells contained CART and that CART and CCK-A receptor mRNA were parallely regulated. These finding suggest a possible co-operation and synergistic effect of CART and CCK on satiety similarly as it was found for CCK and leptin [3]. In this study, we used intact, monosodium glutamate (MSG) induced obese and diet-induced obese (DIO) mice to search for relationship between CART peptides and CCK in food intake regulation.

Results and Discussion

Male C57Bl/6 mice were used in experiments. MSG obesity was induced to newborn mice by repeated subcutaneous administration of monosodium glutamate [4]. Control mice were intact. For diet-induced obesity (DIO) development, mice were fed with high-fat diet (HF, 60% calories as fat). MSG obesity had an early onset accompanied with lesions in hypothalamic arcuate nucleus and distorted food intake regulation. Consumption of HF diet caused late-onset obesity. Both MSG and DIO mice did not differ in their body weights from controls, but their fat to body weights were substantially enhanced compared with controls. MSG obese mice had massively increased leptin concentration. Mice with DIO developed significant hyperglycemia and mild hyperleptinemia and hyperinsulinemia. Feeding experiments were performed after overnight fasting, lean control, MSG or DIO mice were injected intraperitoneally (IP) with cholecystokinin (CCK-8) or devazepide (CCK-A receptor antagonist), intracerebroventricularly (ICV) with CART(61 - 102) fragment or their combination. Cumulative food intake was then measured. Anorectic effect of CART(61 - 102) was enhanced by IP injection of a subtreshold dose of CCK-8, while CCK-A antagonist devazepide blocked the lowering effect of CART(61 - 102) on food intake (see Fig. 1).

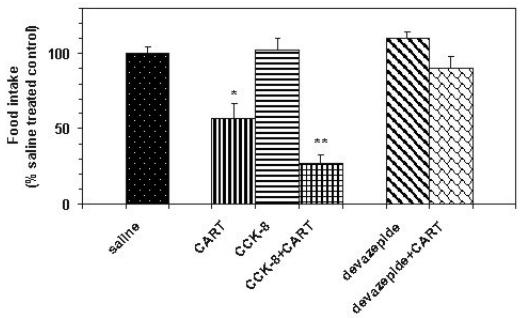

*Fig. 1. Synergistic effect of CART and CCK-8 on food intake of fasted control mice 45 min after injection: saline (IP), CART(61 - 102) 0.5µg/mouse (ICV), CCK-8 4 µg/kg (IP), CART(61-102) 0.5 µg/mouse (ICV)+CCK-8 4 µg/kg (IP), devazepide 1 mg/kg (IP), devazepide 1 mg/kg (IP)+CART 0.5 µg/mouse (ICV) (*p<0,05, **p<0,01 vs. saline-treated group, n = 7 - 10).*

Fasted MSG mice were hypophagic, their food intake was influenced neither with CART(61 - 102) nor CCK-8. In DIO mice, synergistic effect of CART(61 - 102) and CCK-8 was preserved, even though DIO mice were less sensitive to CART than lean controls. Analogously, devazepide suppressed anorectic effect of CART(61 - 102) in DIO mice. In conclusion, CART peptide and CCK-8 acted synergistically both in lean and DIO obese fasted male mice lowering their food intake. The anorectic effect of CART was blocked by CCK-A antagonist devazepide. If CART signaling in hypothalamus was disturbed because of lesions in the MSG mice, neither CART nor CCK were effective in decreasing food intake. It means that for CCK satiety effect in hindbrain, CART signaling is necessary.

Acknowledgements

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PHOTO-CONTROL OF THE COLLAGEN TRIPLE HELIX

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Introduction

Collagens are known to fold into a highly ordered rod-shaped triple helix with stretches of lower and higher suprastructural stability and even disruptions to modulate recognition by other proteins that interact with the extracellular matrix [1]. To increase understanding of folding and stability of the collagen triple helix, we have addressed the design of photocontrolled collagenous peptides. Our aim was to crosslink two side chains of the repetitive (Pro-Hyp-Gly) sequence motifs of collagenous peptides with Pro/Hyp replaced regioselectively by two cysteine or mercaptoproline residues via reaction with an azobenzene chromophore in analogy to our previous studies on photomodulation of the conformational preferences of cyclic peptides and more recently of hairpin-peptide model systems [2].

Results and Discussion

By molecular modeling appropriate sequence positions were selected for crosslinking the Cys and Mpc residues with the light-switchable azobenzene-4,4'-*N*-(4-iodo-2-butynenyl)carboxyamide. While Cys residues in positions X and/or Y are known to weaken considerably the triple-helix stability [3], a 4-mercapto or 4-thioether substituent in the pyrrolidine rings was expected to affect to lesser extents this type of fold. Indeed peptide **1** consisting of seven triplets and containing two (*2S,4S*)-mercaptoproline residues retained sufficient thermal stability to be used as parent compound for incorporation of the azobenzene clamp and production of **2**.

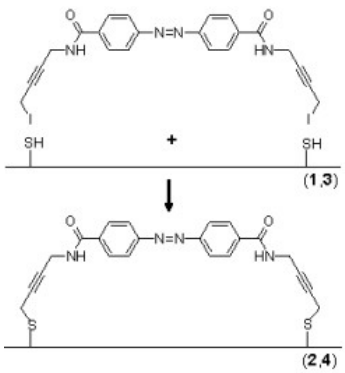

*Fig. 1. Reaction of the collagenous peptides Ac-(Gly-Pro-Hyp)$_2$-Gly-(4S)-Mpc-Hyp-Gly-Pro-Hyp-Gly-Pro-(4S)-Mpc-(Gly-Pro-Hyp)$_2$-Gly-Gly-NH$_2$ (**1**) and Ac-(Gly-Pro-Hyp)$_3$-Gly-Cys-Hyp-Gly-Pro-Hyp-Gly-Pro-Cys-(Gly-Pro-Hyp)$_5$-Gly-Gly-NH$_2$ (**3**) with the azobenzene diiodo derivative to produce the side-chain crossbridged peptides **2** and **4**, respectively.*

Conversely, for the Cys-peptide series an extension to the undecarepeat peptide **3** was required to achieve sufficient structural stability for its conversion to the azobenzene-peptide **4**. Since both azobenzene-peptides **2** and **4** are not sufficiently soluble in water, their structural characterization in the trans- and cis-azobenzene

isomeric states was performed in MeOH/0.1 M AcOH (4:1), i.e. under conditions known to strongly stabilize triple-helical structures of collagenous peptides [4]. Indeed, both CD spectra and NMR NOESY and FHSQC experiments clearly confirmed a triple-helical fold of the trans-azobenzene isomers. Conversely, light induced *trans*-to-*cis* isomerization at 27 °C leads in the case of peptide **2** to unfolding and thus, to a trimer-monomer transition [5], while for peptide **4**, as graphically outlined in Fig. 2, it provokes local unfolding of the side-chain clamped bis-cysteinyl sequence including the N-terminally flanking $(Gly\text{-}Pro\text{-}Hyp)_3$ triplets with retention of the triple-helical fold in the C-terminal $(Gly\text{-}Pro\text{-}Hyp)_5$ portion of the molecule (unpublished results). Rate-limiting effects in triple helix formation are i) the intermolecular association into trimers which can be partly overcome operating at high concentrations (1 mM), ii) correct registration of the chains and iii) the *cis*-to-*trans* isomerization of the Gly-Pro- and Xaa-Hyp/Pro bonds [6]. While time-resolved CD and IR-spectroscopy of compound **2** should allow to monitor at different time scales the first steps of folding including self-association kinetics, compound **4** could well serve for analyzing local folding/unfolding of collagen triple helices.

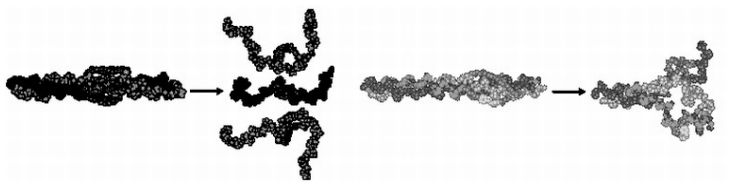

*Fig. 2. Unfolding of peptide **2** accompanied by trimer-to-monomer dissociation (left) and partial unfolding of compopund **4** upon trans-to-cis photoisomerization of the azobenzene clamp (right), as assessed by CD and, particularly, NMR spectroscopy.*

Acknowledgements

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CONFORMATION AND *CIS-TRANS* ISOMERIZATION OF NONPROLYL AND PROLYL RESIDUES

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Introduction

In proteins, the peptide bond is known to prefer dominantly the *trans* conformation. The analyses of X-ray protein structures showed that the *cis* populations are ~0.04% for the nonprolyl peptide bond and ~6% for the prolyl peptide bond [1]. It has been reported that the *cis-trans* isomerization of the X-Pro bond is often involved in the rate-determining steps for folding and refolding of various proteins [2]. Although nonprolyl *cis* peptide bonds are rare in proteins, they often occur in regions near the active sites of proteins, and contribute to regulate the biochemical properties and binding modes [3]. There are only a limited number of works reported on the kinetics and thermodynamics of the nonprolyl *cis-trans* isomerization for peptides [4, 5]. The conformational study on the alanine and proline dipeptides is carried out to explore the differences in the backbone conformational preference and the *cis-trans* isomerization for the nonprolyl and prolyl residues. All ab initio HF and density functional B3LYP calculations were carried out using the Gaussian 03 package [6]. The computational methods are described elsewhere in detail [7, 8].

Result and Discussion

The calculated results are summarized in Table 1. For the alanine and proline dipeptides, the populations of the conformation tC with an intramolecular C_7 hydrogen bond significantly decrease, and those of the polyproline II-like conformation tF and the α-helical conformation tA increase with the increase of solvent polarity, which is in good agreement with the results from CD and NMR experiments. For both the dipeptides, the relative free energy of the *cis* conformer to the *trans* conformer decreases and the rotational barrier to the *cis-trans* isomerization increases as the solvent polarity increases. It is found that the *cis-trans* isomerization proceeds in common through only the clockwise rotation with $\omega' \approx +120°$ about the nonprolyl and prolyl peptide bonds in the gas phase and in solution. The pertinent distance d(N···H-N) between the prolyl nitrogen and the following amide hydrogen can successfully describe the increase in the rotational barriers for the nonprolyl and prolyl *trans-cis* isomerization as the solvent polarity increases, and the higher barriers for the nonprolyl residue than the prolyl residue, as seen in experimental and calculated results. By analyzing the contributions to rotational barriers, the *cis-trans* isomerizations for the nonprolyl and prolyl peptide bonds is proven to be entirely enthalpy-driven in the gas phase and in solution. This is consistent with the experimental results on proline-containing peptides, determined kinetically as a function of temperature [9]. The calculated *cis* populations and rotational barriers to the *cis-trans* isomerization for both the dipeptides in chloroform and/or water accord with the experimental values [4, 5, 8].

Table 1. Populations of Backbone Conformations, Rotational Barriers, and Relative Free Energies of Cis Conformers for Ac-Ala-NHMe and Ac-Pro-NHMe Calculated at the B3LYP/6-311++G(d,p) Level in the Gas Phase and in Solution

	backbone populations[a]						rotational barrier[b,c]		rel energy[b,d]
solvent	C	E	A	F	cis[e]	exptl cis[e]	$\Delta G^{\ddagger}_{tc}$	$\Delta G^{\ddagger}_{ct}$	$\Delta G_{c/t}$
					Ac-Ala-NHMe				
gas phase	42.2	51.9	0.1	0.0	0.1		19.66	15.63	4.03
chloroform	20.7	55.4	7.9	15.2	0.1		21.61	17.66	3.95
water	0.0	37.1	26.8	33.5	0.4	0.4[f]	23.03	20.24	2.79
							(21.8[f])	(18.5,[f] 17.9[g])	(3.3[f])
					Ac-Pro-NHMe[h]				
gas phase	97.4		2.5	0.0	2.5		19.15	16.99	2.16
chloroform	54.9		12.2	32.9	9.0	15 ± 4	19.32	17.68	1.64
water	0.0		10.1	89.9	23.3	27 ± 3	21.61	20.93	0.68
							(20.4)	(19.8)	(0.6)

[a] Conformations C, E, A, and F are equivalent to C_7^{eq}, C_5, α_R, and P_{II} structures, respectively. The populations (%) were computed using the relative Gibbs free energy of local minima. [b] Units in kcal/mol. Experimental values are listed in parentheses. The lowest Gibbs free energy for each of trans, cis, and transition state conformations was used for these calculations. Free energies were calculated at 25 °C. [c] $\Delta G^{\ddagger}_{tc}$ and $\Delta G^{\ddagger}_{ct}$ represent the barriers for the trans-to-cis and cis-to-trans rotations for the Ac-Ala and Ac-Pro peptide bonds. [d] $\Delta G_{c/t}$ is the relative Gibbs free energy of the cis conformer to the trans conformer. [e] Cis Ac-Ala or Ac-Pro peptide bonds. [f] Average values for Ala-Phe, Phe-Ala, Tyr-Ala, and Ala-Tyr peptides at 298 K from ref 4. [g] Average values for Gly-Gly, Gly-Ala, Ala-Gly, and Ala-Ala peptides at 298 K from ref 5. [h] From ref 7.

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FMOC SOLID-PHASE SYNTHESIS OF PEPTIDE THIOESTERS USING AN INTRAMOLECULAR *N,S*-ACYL SHIFT [1]

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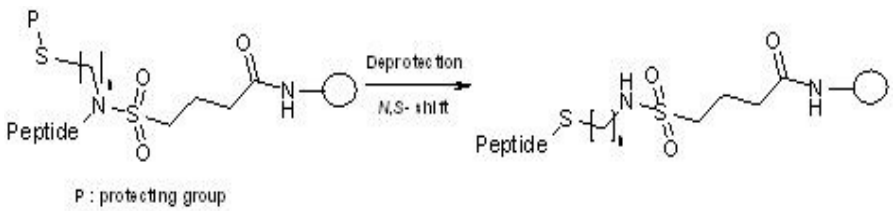
Chemoselective ligation methods, such as native chemical ligation [2], allow the assembly of proteins of moderate size and provide ready access to natural as well as modified proteins. Native chemical ligation is based on the reaction of a peptide bearing a C-terminal thioester group with an N-terminal cysteinyl peptide, leading to the formation of an amide bond at the amino acid-Cys junction.

The key starting materials for native chemical ligation are unprotected C-terminal thioester peptides [2c]. Thioester peptides can be prepared by Boc/benzyl solid-phase peptide synthesis. However, the widespread use of the Fmoc/*tert*-butyl chemistry [3] for peptide synthesis has stimulated the development of methods for preparation of thioester peptides that are compatible with the basic treatments used to remove the Fmoc α-amino protecting group. We report here a novel method for thioester peptide synthesis that is based on the use of the Kenner sulfonamide linker [4].

The strategy adopted in this work is summarized in the Scheme. After peptide elongation on a 3-carboxypropanesulfonamide linker by Fmoc/*tert*-butyl solid-phase chemistry, Mitsunobu alkylation of the acylsulfonamide group is performed with a protected mercaptoethanol or mercaptopropan-1-ol derivative. Removal of the thiol protecting group gives supported intermediate, which features a thiol nucleophile in close proximity to the activated carboxyl group. It was found to rearrange spontaneously through an intramolecular *N,S*-acyl shift to give a protected thioester peptide still attached to the solid support.



Final deprotection of the peptide chain is performed in TFA as usual. The use of a Rink linker between the 3-carboxypropanesulfonamide arm and the solid support leads to the liberation of the thioester peptide in solution. Alternately, the use of a TFA-resistant linker results in the formation of a deprotected thioester peptide still attached to the resin. It was engaged successfully in a native chemical ligation from the solid phase.

The results show that the alkylation of the sulfonamide doesn't depend on the nature of the first amino acid (Ala or Val) directly attached to the sulfonamide linker, probably because the site of alkylation is far enough from the C^{α}. Alcohols derived from mercaptoethanol or mercaptopropan-1-ol were examined. Better results were obtained with the mercaptopropan-1-ol derivative. The method is fully compatible with His(Trt) residues, to the contrary of the standard method using diazomethane or iodoacetonitrile as reagents for alkylation of the acylsulfonamide.

The thioesters were useful for native chemical ligations in solution or from the solid support.

Acknowledgements

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SYTHESIS OF MODIFIED *N*-ACYLHOMOSERINELACTONES

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Introduction

Quorum sensing (QS) is a cell-density dependent phenomenon of bacterial colonies. This system enables them to sense the excess of a critical cell concentration. Small amounts of permanently produced effector molecules get enriched and initiate a specific gene expression. In gram-negative bacteria *N*-acyl-homoserinelactones (AHLs) serve as signal molecules, while for gram-positive bacteria post-translationally modified peptides were observed in this function. Some bacteria have even more than one quorum sensing system, as it was found for the soil bacterium Sinorhizobium meliloti. Both systems are based on AHLs and can be distinguished by the length of the acyl chain of the effector molecules [1].

Results

L-Homoserinelactone was coupled to fatty acids with side chains between 7 and 18 carbon atoms. In analogy to naturally occurring variations an 3-oxo group as well as an unsaturated bond was introduced to some of the fatty acids.

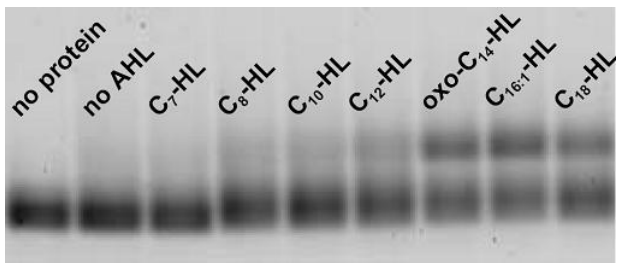

Fig. 1. EMSA studies about the AHL-dependent DNA binding of the receptor protein. Components were employed in the binding reaction at 10 μM (AHL), 8 μM (protein) and 250 μM (DNA).

After acylation their biological activity was tested. The addition of AHL leads upon binding to an activation of the reporter protein. In the presence of the DNA domain a complex is formed and can be seen as a mass shift in the electrophoretic mobility shift assays (EMSA, Fig. 1).

In order to attach a spacer unit to the effector molecule a hydroxylated homoserinelactone was synthesised. Follwing the route of Rapoport and Afzali-Ardakani, L-methionine was used as the starting material to prepare

enantiomerically pure L-vinylglycine.[2] After dihydroxylation and cyclisation *cis* and *trans*-3-hydroxy-L-homoserinelactone was obtained.

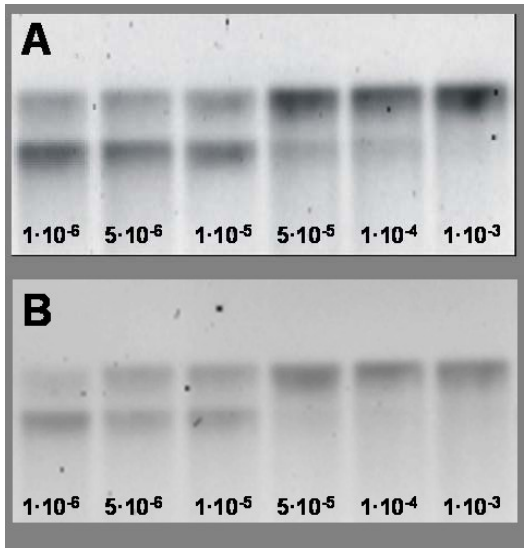

Fig. 2. EMSA analysis to reveal the effector concentration dependent DNA binding of receptor protein. Concentrations of the effector molecules AHL (A) and β hydroxy AHL (B) are given in mol/l.

The diastereoisomers were acylated with palmitoleic acid and compared to the corresponding unmodified AHL. The described test system was used at constant protein and DNA concentration but varying amounts of the effectors to determine the minimum concentration necessary for activation.

Discussion

It was shown that the fatty acid chain length is crucial to the quorum sensing system. Receptor binding of AHL may occur with shorter acyl groups but AHL mediated protein DNA interaction for the long chain system of S. meliloti requires a minimum chain length of ten to twelve carbon atoms.

A homoserinelactone with an additional β-hydroxyl function at the ring was proven to be still active in *S. meliloti* quorum sensing. A further modified AHL with an introduced spacer unit currently is under investigation. Such a probe facilitates a more detailed investigation of quorum sensing systems (SPR, AFM, fluorescence studies).

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SMALL UBIQUITIN-BINDING PEPTIDES

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Introduction

Ubiquitination [1], the covalent attachment of one or multiple polymerize dubiquitins, is a post-translational modification of proteins, which has manifold functions [2]. It mainly determines proteins for degradation, but also activation, deactivation or substrate alteration. Due to its ubiquitinous distribution in all eukaryotes no high-affinity antibodies could be originated until now [3]. Therefore ubiquitin interacting peptides are of interest for the detection of ubiqutination.Until now 6 ubiquitin-interacting enzyme families (E1 to E6) with more than 1,000 proteins are known. Ubiquitin carboxy hydrolases are a subfamily of the E6-familiy and detach single ubiquitins from poly-ubiquitin chains with a nanomolar binding constant. Based on crystal structures short peptides were selected (Fig. 1) and analysed for interacting with ubiquitin by four physically different methods.

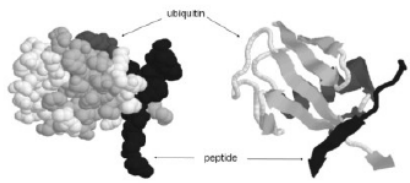

Figure 1: Visualisation of potential "interacting" peptide derived from UCH-L3 (pdb file 1XD3)

Results and Discussion

Selected peptides were synthesized by Fmoc/tBu strategy as peptide amides or N terminally elongated with aminohexanoic acid and 5/6-carboxy-fluorescein, the Cy5-like S0387 or cystein. Fluorescently labeled peptides were used in Fluorescence Correlation Spectroscopy (FCS) [4] for fast detection of interaction with ubiquitin. All peptides which showed a significant shift in the FCS-signal were monitored and quantified with label free detection based on Reflectometric Interference Spectroscopy (RIfS) [5]. From the on- and off-kinetic of signal increase at different ubiquitin concentrations K_D values were derived. The dodecapeptide **DPDELRFNAIAL** derived from UCH-L3 showed a specific signal (Fig. 2) after incubation with ubiquitin (in comparison to the unspecific signal obtained with ovalbumin) and a KD value of 13 ± 3 µM was calculated. These results were confirmed by further experiments with labeled and non-labeled peptides in Isothermal Calorimetry Measurements (ITC). Finally $^{1}H,^{15}N$-NMR chemical shift

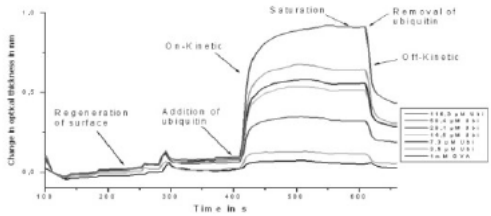

Figure 2: RIfS analysis with covalently bound peptide Cys-Ahx-DPDELRFNAIAL titrated with ubiquitin.

analyses with ^{15}N-labeled ubiquitin were carried out with peptides with the highest affinity. The chemical shift perturbations (Fig. 3A) were very specific and allowed the localisation (Fig. 3B) of the interaction of ubiquitin with the peptide.

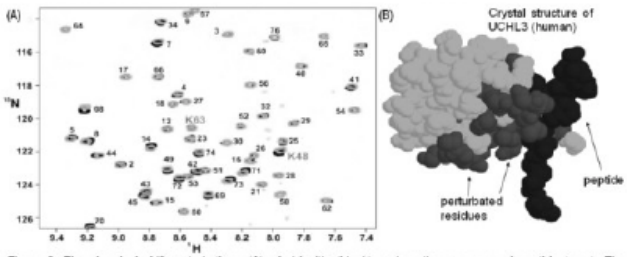

Figure 3: The chemical shift perturbations (A) of ubiquitin (black) and on the presence of peptide (grey). The affected residues are located next to the peptide (B). It is obvious that the dodecapeptide DPDELRFNAIAL assembles a similar position on ubiquitin as in the crystal structure of UCH-L3.

The results revealed DPDELRFNAIAL and DELRFNAI as ubiquitin-binding peptides. Both peptides showed fast equilibria within 30 s and the binding constants are in the low micromolar range. The NMR results showed that the binding site is similar to Lys48 polymerized ubiquitin and open the perspectives for the discrimination of Lys48 and Lys63 polymerized ubiquitin chains.

Acknowledgements

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ADDRESSING THE PROBLEM OF CANCER DETECTION AND TREATMENT WITH MULTIMERIC LIGANDS THAT ADDRESS THE CELL SURFACE: CANCER VS. NORMAL CELLS/TISSUES

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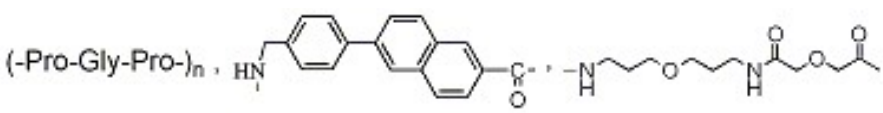
Introduction

Cancers are multifactorial diseases with many gene changes and with multiple phenotypes. We are utilizing genomics and proteomics to evaluate the differences in typical cancer cells, in normal vs. cancerous tissue with particular emphasis on differences in cell surface expressed proteins. We are then utilizing these differences at the cell surface to design multivalent ligands that will recognize cancer cells vs. normal cells as an approach to early detection and treatment. This approach provides a new paradigm for developing multimeric ligands for imaging and as therapeutic agents [1].

Results and Discussion

Since our primary interests are in the detection and treatment of melanoma and pancreatic cancers, we chose as initial targets for our multivalent ligands, the melanocortin, opioid and cholecystokinin (CCK) receptors. The structure activity relationships (SAR) for the peptide ligands for these receptors are well established for both agonists and antagonists. The advantages of both homomeric and heteromeric multivalent ligands is now well established [e.g. 1 - 3]. From molecular modeling of the G-protein coupled receptors (GPCRs) and molecular dynamic studies, the optimal three dimensional spacing for individual ligands in the multimers were suggested to be about 25 - 45 Å. The scaffold design involved the use of a variety of linkers such as those shown in Fig. 1 which were chosen for their variations in rigidity and flexibility, and for their biocompatible chemical properties. Cells which expressed the receptors both homogeneously and heterogeneously were developed to evaluate the synergy that might be obtained, and were used in a new high throughput assays system we have recently developed [4] using time resolved fluorescence to examine the binding affinity of both homodimeric ligands and heterodimeric



ligands. For the heterodimeric ligands such as NDP-α-MSH(7)-(Pro-Gly)$_9$-NDP-α-MSH(7) vs. the monomeric version (only one NDP-α-MSH(7) moiety) binding synergies of 5 - 20 fold generally were seen when the estimated distances between ligands were 15-30 Å. For heterodimeric ligands such as NDP-α-MSH(7)-(Pro-Gly)$_9$-Deltorphin II synergies of 10 - 20 fold generally were seen, but again only when the estimated distance between ligands was 15 - 50Å.

These results strongly suggest that utilization of multivalent ligands using both homomeric and heteromeric ligands with appropriate linkers can lead to enhanced affinity and synergy to cancer cells with the appropriately expressed receptors/acceptors on the cell surface. Work is in progress to demonstrate that these ligands can be used to preferentially image cancer vs. normal cells in animal models.

Acknowledgements

This work was supported by grants from the U.S. Public Health Service, National Cancer Institute R33 CA95944 and RO1 CA97360 and BIO5 (U of A).

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SIDE-CHAIN CHIRAL CENTERS OF AMINO ACIDS AND HELICAL-SCREW HANDEDNESS OF THEIR PEPTIDES

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Introduction

Helices shown in proteins, as a secondary structure, almost always form right-handed screw sense. The right-handedness of the helix is believed to result from the chiral center at the α-position of proteinogenic L-α-amino acids [1]. Among proteinogenic amino acids, L-isoleucine and L-threonine possess an additional chiral center at the side-chain β-carbon besides the α-carbon. However, only little attention has been paid as to how the side-chain chiral centers affect the secondary structures of their peptides [2]. Recently, we have reported that side-chain chiral centers of chiral cyclic α,α-disubstituted amino acid (*S,S*)-Ac_5c^{dOM} affected the helical secondary structure of its peptides, and the helical-screw direction could be controlled by the side-chain chiral centers without a chiral center at the α-carbon atom (Fig. 1) [3]. Herein we synthesized a chiral bicyclic α,α-disubstituted amino acid, (1*R*,6*R*)-8-aminobicyclo[4.3.0]non-3-ene-8-carboxylic acid {(*R,R*)-$Ab_{5,6=}c$}, and its analogs. Also, we prepared its homopeptides, and studied the relationship between the side-chain chiral centers and the helical-screw handedness of their peptides.

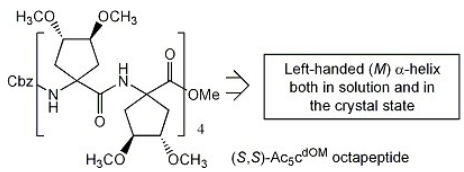

Fig. 1. (S,S)-Ac_5c^{dOM} Octapeptide forming an α-helix.

Results and Discussion:

We designed and synthesized an optically active bicyclic α,α-disubstituted α-amino acid; (*R,R*)-$Ab_{5,6=}c$, in which the α-carbon atom is not a chiral center but the asymmetric centers exist at the side-chain bicyclic skeleton. The amino acid (*R,R*)-$Ab_{5,6=}c$ was synthesized from (*S,S*)-cyclohex-4-ene-1,2-dicarboxylic acid **1** [4] as shown in Fig. 2. The acid (*S,S*)-**1** was converted to a diiodide **2** by reduction and subsequent substitution with iodide. Then, ethyl isocyanoacetate was bisalkylated with **2**, followed by acidic hydrolysis and protection with Boc_2O to give amino acid Boc-[(*R,R*)-$Ab_{5,6=}c$]-OEt (**3**). The olefin in the amino acid **3** could be easily

converted to several functional groups. Ozonolysis of the olefin in **3**, followed by reduction with $NaBH_4$ afforded a dihydroxy amino acid **4** and by oxidation with Oxone® gave a dicarboxylic amino acid **5**, and by reductive amination with $BnNH_2$ produced a bicyclic seven-membered ring amino acid **6**. Furthermore, hydrogenation of the olefin in **3** afforded saturated amino acid Boc-[(*R,R*)-$Ab_{5,6}$c]-OEt **7**. Homopeptides Boc-[(*R,R*)-$Ab_{5,6=}$c]n-OEt (n = 3, 6, 9) were prepared by solution-phase methods, and the six olefin functions in (*R,R*)-$Ab_{5,6=}$c hexapeptide **8** were hydrogenated by H_2/20% $Pd(OH)_2$-C in one step to afford the saturated peptide Boc-[(*R,R*)-$Ab_{5,6}$c]6-OEt **9** in 70% yield.

The IR, ^{1}H NMR, CD spectra, and the X-ray crystallographic analysis revealed that the (R,R)-$Ab_{5,6=}$c hexapeptide having twelve chiral centers at the side chain forms both diastereomeric right-handed (P) and left-handed (M) 3_{10}-helices. These results are in contrast with the left-handed (M) (*S,S*)-Ac_5c^{dOM} homopeptides controlled by side-chain chiral centers, and suggest that the side-chain chiral environments (bulkiness or flexibility) might be important for the control of the helical-screw handedness [5].

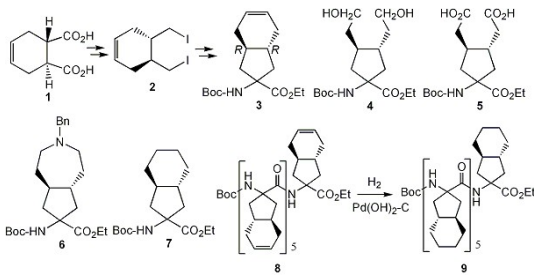

Fig. 2. Synthesis of chiral bicyclic and cyclic α,α-disubstituted α-amino acids, and their peptides.

Acknowledgements

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CYCLIC PEPTIDES COMPRISING B-AMINO ACIDS AS INTEGRIN $\alpha_4\beta_1$ LIGANDS

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Integrins are heterodimeric transmembrane proteins and constitute an important family of cell adhesion molecules. Currently, 24 different combinations of α and β subunits with diverse functionality are known. Integrin $\alpha_4\beta_1$ is involved in inflammatory processes, leukocyte migration and tumor angiogenesis. VCAM-1, a natural ligand of $\alpha_4\beta_1$, has been structurally characterized by X-ray crystallography, including the putative binding loop with the amino acid sequence TQIDSPLN. While linear peptides based on this sequence do not show any inhibitory effect in competiti ve binding assays, cyclic octapeptides with micromolar IC_{50} values have been reported [1]. Smaller, thus conformationally more restrained cyclic peptides might bind more tightly because of entropic reasons.

We conducted a spatial screening for high affity integrin $\alpha_4\beta_1$ ligands using a series of penta- and hexapeptides based on TQIDSPLN subsequences. β-Homoamino acids, i.e. β^3-amino acids with proteinogenic side-chains, have been incorporated as structure inducers to enforce formation of distinct secondary structure. Although β^3-amino acids are supposed to prefer the central posi tion of pseudo-γ-turns (Ψγ-turns) [2], less data exist on their structure inducing potential than for e. g. D-amino acids. Apart from the structural characterization of potential high affnity ligands for integrin $\alpha_4\beta_1$, a major goal of this work is to provide a better understanding of the influence of β^3-amino acids on the structure of cyclic peptides. NMR spectroscopy followed by DG/SA and MD calculations has been used to elucidate the structures of three pentapeptides, c-(SPLND) **2**, c-(SPL$^{\beta h}$ND) **3**, and the model peptide c-(V$^{\beta h}$AFLI) **1**, as well as three hexapeptides c-(SPLNVD) **4**, c-(S$^{\beta h}$PLNID) **5** and c-($^{\beta h}$SPL$^{\beta h}$NID) **6**. A novel torsion angle clustering procedure has been integrated into the structure determination workflow, with substantially improved results compared to previous approaches. The results substantiate the Ψγ-turn forming propensity of β^3-amino acids in cyclic pentapeptides. The all-α-peptide **2** shows a classical arrangement with a γ^i-turn around Asp facing a βI-turn around Pro and Leu. Exchange of Asn against β-homoasparagine leads to 3 and results in a drastic conformational change: the Ser-Pro bonds changes its configuration to *cis*, accordingly Pro is now found in position i+2 of a βVIa-turn, and the β-amino acid occupies the central position of a Ψγ-turn. The model peptide **1** also comprises a Ψγ-turn around β-homoalanine, the only structure inducing building block incorporated in this peptide. Furthermore, our results point towards a preference of β-homoproline for the i+1 position of Ψβ-turns in hexapeptides, which is in agreement with previous investigations [3, 4]. More details concerning the

structural studies and the aforementioned torsion angle clustering will be discussed elsewhere [5].

A comparison with the X-ray structure of VCAM-1 shows that the binding loop has been approximated quite well by some of the peptides, especially **2**, **4** and **5**, so one might expect a high affinity for $\alpha_4\beta_1$ in these cases. However, none of the peptides shows a significant inhibitory effect in competive binding assays. This is also true for several other cyclic penta- and hexapeptides we have tested which are based on the TQIDS(P) and QIDSP(L) subsequences. In reference to the premise, these results are quite surprising. Therefore, we also synthesized the reportedly active octapeptides [1] and subjected them to our affinity assay in order to validate the starting point of the screening. Though, we did not find any of the octapeptides to have a significant affinity for $\alpha_4\beta_1$. We conclude that TQIDSPLN is at most a weak recognition sequence for $\alpha_4\beta_1$, and that the results of the previous work are at least doubtful.

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NEW SYNTHETIC APPROACHES TO POLYPROLINE-BASED DENDRIMERS

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Introduction

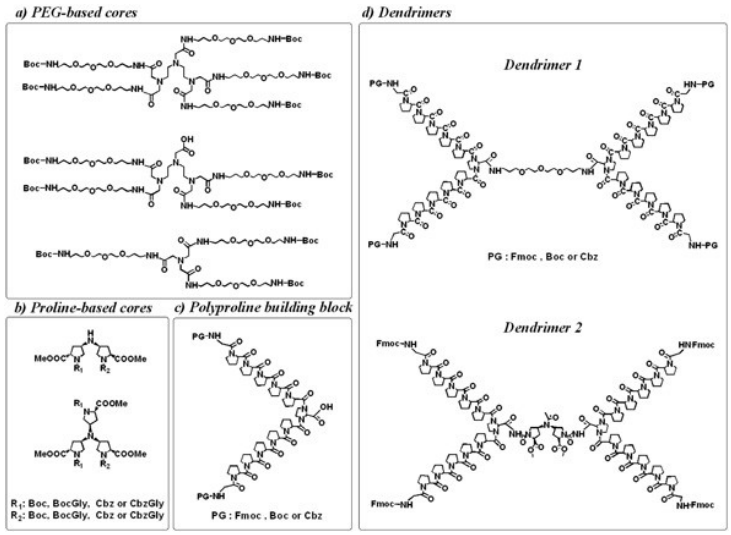
The development of new biopolymer materials for applications such as gene carriers and drug delivery systems is of great interest to biomedical sciences. In this field, a number of types of biopolymers have been developed. Dendrimers, a class of biopolymers, receive particular attention because of their properties; they are highly branched polymers with a well-defined chemical composition and structure. In addition, they have a compact globular shape, monodisperse size and controllable surface functionalities [1]. Dendrimers are macromolecular compounds obtained by an iterative sequence of reaction steps, in which series of branched building blocks are coupled around an inner core. Ideally, this methodology permits the development of monodisperse macromolecules with a regular and highly branched three-dimensional architecture [1]. Peptide dendrimers are of special interest as they incorporate amino acids in their structures and offer additional features such a biocompatibility and biodegradability, which are crucial in biomedical applications [1]. In previous studies performed by our laboratory, we described the synthesis of polyproline-based dendrimers by solid phase methodology [2 - 3]. In these, the complexity to achieve polyproline branched peptides was demonstrated and the main drawbacks identified were the use of large excesses of reagents and low building block incorporation [4 - 6]. Despite these synthetic handicaps, these biopolymers have the capacity to cross the mammalian cell membrane and moderate toxicity [7], thereby opening up the use of these compounds as new drug delivery systems.

Results and Discussion

To design the synthesis of versatile polyproline dendrimers, here we studied methodology that involved a combination of solid-phase and solution techniques. With this aim, diverse multivalent PEG- and proline-based cores were synthesized, as shown in Fig. 1a-b. Imidazolidine-2-carboxylic acid (Imd), a symmetrical non-natural amino acid, was used as branching unit. Glycine residues in the N-terminal position of the building block were crucial to accomplish the synthesis of these polyproline dendrimers. Three amino-protecting groups (Fmoc, Boc and Cbz) were explored in this position (Fig. 1c).

In preliminary studies, dendrimers were synthesized by coupling bivalent PEG- or proline-based cores and diverse N-terminal-protected (Fmoc, Boc and Cbz)

polyproline building blocks. The effect of these protecting groups on dendrimer synthesis was evaluated (Fig. 1d).



All the protected building blocks allowed us to obtain the corresponding final protected dendrimer; however, when the Fmoc-protected building block was used, partial Fmoc removal during the coupling reaction was observed. In the case of the synthesis performed with Cbz-protected building block, it was not possible to obtain the final unprotected dendrimer. Nevertheless, the use of Boc protection rendered better results in the coupling reaction and removal of the final Boc-protecting group.

Acknowledgements

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PEPTOMERIC ANALOGUES OF TRYPSYN INHIBITOR SFTI-1

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Introduction

Sunflower trypsin inhibitor SFTI-1 is the smallest and the most potent known peptidic trypsin inhibitor from the Bowman-Birk class of proteins [1]. This head-to-tail-cyclized 14-amino-acid peptide contains one disulfide bridge and a Lys5 residue present in the P1 position, which is responsible for inhibitor specificity.

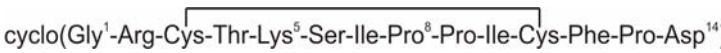

Fig. 1. Primary structure of SFTI-1.

As was reported by us [2] and other groups, SFTI-1 analogues with one cycle only retain trypsin inhibitory activity. Very recently we have shown [3] that introduction of N-substituted glycine residues mimicking Lys and Phe (denoted as Nlys and Nphe) in the P_1 position of monocyclic SFTI-1 containing the disulfide bridge only yielded potent trypsin and chymotrypsin inhibitors, respectively. This novel class of proteinase inhibitors contains completely proteolytic resistant P_1-P_1'reactive site. Here we report a chemical synthesis of 10 new analogues of SFTI-1 modified in the P1 position by these peptoid monomers (Nlys and Nphe). Each of the synthesized peptomeric (peptide-peptoid hybrid polymer) SFTI-1 analogues contains one of the following cycles: head-to-tail, disulfide bridge formed by Cys, by Pen and by Cys/Pen residues.

Results and Discussion

All peptides were synthesized using Fmoc chemistry. N-Substituted glycine derivatives were introduced in the peptide chain by the submonomeric approach using bromoacetic acid and primary amines. The association equilibrium constants (K_a) were determined as described previously [2]. The obtained results are summarized in Table 1. Our studies show that from 10 synthesized peptomers 9 display either trypsin or chymotrypsin inhibitory activity. We find it very interesting that among two completely acyclic analogues one (analogue **4**) is a potent chymotrypsin inhibitor while the second (analogue **2**) that differ in P_1 position only is inactive. We postulate that in the case of analogue 2 the lack of activity is a result of a cleavage of N-terminal dipeptide Gly-Arg by trypsin, whereas in analogue **4** this peptide bond is resistant against chymotrypsin.

Table 1. Physicochemical properties and association equilibrium constants (K_a) of peptomeric SFTI-1 analogues.

Inhibitor	MW calc/found	K_a [M^{-1}]	
		Bovine β-trypsin	Bovine α -chymotrypsin
SFTI-1 (monocyclic)	1531.2/1531.8	$9.9x10^9$	$4.9x10^6$
[Phe^5]SFTI-1 (monocyclic)	1550.2/1550.5		$2x10^9$
1. cyclo(Gly-Arg-**Abu**-Thr-**Nlys**-Ser-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp)	1479.8/1479.8	$1.9x10^8$	
2. Gly-Arg-**Abu**-Thr-**Nlys**-Ser-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp	1497.8/1497.6	inactive	
3. cyclo(Gly-Arg-**Abu**-Thr-**Nphe**-Ser-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp)	1498.8/1498.5		$7.0x10^7$
4. Gly-Arg-**Abu**-Thr-**Nphe**-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp	1516.8/1516.6		$6.2x10^7$
5. Gly-Arg-cyclo(Cys-Thr-**Nlys**-Ser-Ile-Pro-Pro-Ile-Cys)-**Nphe**-Pro-Asp	1531.8/1531.6	$6.1x10^7$	
6. Gly-Arg-cyclo(Cys-Thr-**Nphe**-Ser-Ile-Pro-Pro-Ile-Cys)-**Nphe**-Pro-Asp	1550.8/1551.0		$9.3x10^7$
7. Gly-Arg-cyclo(**Pen**-Thr-**Nlys**-Ser-Ile-Pro-Pro-Ile-**Pen**)-Phe-Pro-Asp	1587.9/1587.8	$1.5x10^8$	
8. Gly-Arg-cyclo(**Pen**-Thr-**Nphe**-Ser-Ile-Pro-Pro-Ile-**Pen**)-Phe-Pro-Asp	1606.9/1606.4		$1.2x10^8$
9. Gly-Arg-cyclo(Cys-Thr-**Nlys**-Ser-Ile-Pro-Pro-Ile-**Pen**)-Phe-Pro-Asp	1559.9/1559.6	$3.6x10^8$	
10. Gly-Arg-cyclo(Cys-Thr-**Nphe**-Ser-Ile-Pro-Pro-Ile-**Pen**)-Phe-Pro-Asp	1578.9/1579.0		$1.4x10^8$

Interestingly, the K_a values measured for analogues 7 - 10 in which one or both Cys residues were replaced with Pen residues and for analogues that comprise two N-substituted glycine residues in position P_1 as well as in place of Phe12 are, within experimental error, the same as that of reference compounds.

Acknowledgements

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INFLUENCE OF TERMINAL FRAGMENTS IN SELECTED LOW-MOLECULAR COMPOUNDS ON THEIR ANTIMICROBIAL ACTIVITY

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Introduction

Increased resistance of bacterial pathogens to currently employed antibiotics has resulted in efforts to develop antimicrobial compounds with new mechanisms of action. Previously, we have synthesized some highly potent antimicrobial compounds based upon the N-terminal binding fragment of human cystatin C [1, 2]. Some derivatives of the general structure: X-Arg-Leu-NH-alkyl-NH-Y (where X and Y were acyl groups with aromatic carbocyclic system) have displayed the broad antibacterial spectrum and high activity against several clinically important Gram-positive pathogens, including multi-resistant staphylococci. We have found that the presence of an unsubstituted, carbocyclic aromatic system at the end of both the X- and Y-acyl groups is absolutely essential for their antibacterial activity.

This work is focused on the influence of the X- and Y-acyl group structure on the bactericidal activity of the tested compounds.

Results

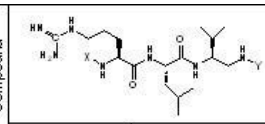
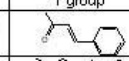
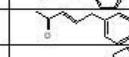
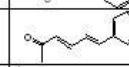
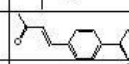
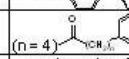
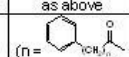
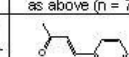
Nine new peptidyl derivatives (**3** – **11**), structurally based on two most potent antibacterial compounds (**1**, **2**) found earlier in our laboratory [1], were synthesized and investigated for antibacterial activity. In the first group of the series the *trans*-cinnamoyl group in **1** was replaced by selected substituents of the general structure: C_6H_5-$(CH_2)_n$-CO- (where n = 4 – 7) or Ar-$(CH{=}CH)_n$-CO- (where n = 1 or 2) resulting in **5** – **8** and **3** - **4**, respectively. The second group consisted of three analogues (**9** – **11**) of **1** in which C_6H_5-$(CH_2)_n$-CO- moiety (where n = 1 – 3) substituted the benzyloxycarbonyl. Structures of the discussed compounds are presented in Table 1.

Discussion

Three of nine new derivatives, **4**, **7** and **8**, displayed *in vitro* activity against all tested bacterial species (*S. pyogenes, S. agalactiae, S. epidermidis*, group C *streptococci, E. faecalis, S. aureus*). Analogues **5**, **6**, **10** and **11** have not been tested against group C *streptococci* and *S. aureus*, yet. The compound **9** showed no antibacterial activity against any of six bacterial species tested. The growth of *S. pyogenes, S. agalactiae* and *S. aureus* was suppressed in higher degree by peptidyl derivatives containing the acyl chains with the length comparable to **1** and **2**

(compounds **5**, **6** and **10**, where n = 4, 5 or 2, respectively). Surprisingly, a definite activity against *E. faecalis* was noted for the compounds **5** and **6**, whereas our lead substances, **1** and **2**, were practically inactive. Compounds **7**, **8** and **11**, having the longer backbone then **1** and **2**, had a minor effect on the growth of the tested bacterial species. None of the tested analogues inhibited the growth of *S. pneumoniae, S. oralis* or two tested Gram-negative species, *E. coli and H. influenzae*.

Table 1. Antibacterial effects of 1-11.*

Compound			Inhibition zones [3]					
	X group	Y group	*S. pyogenes*	*S. agalactiae*	Group C *Streptococci*	*E. faecalis*	*S. aureus*	*S. epidermidis*
1			++	++	+	(+)	++	++
2	as above		++	+	++	+	++	+++
3	as above		++	++	+	-	++	++
4	as above		+	+	+	(+)	+	++
5	as above		++	++	nd	++	++	nd
6	as above	as above (n = 5)	++	++	nd	++	++	nd
7	as above	as above (n = 6)	+	+	+	(+)	+	++
8	as above	as above (n = 7)	(+)	(+)	+	(+)	(+)	+
9			-	-	-	-	-	-
10	as above (n = 2)	as above	++	++	nd	nd	+	nd
11	as above (n = 3)	as above	+	+	nd	nd	+	nd

*Inhibition zone diameter: - = no zone, (+) = 6-7 mm, + = 8-11 mm, ++ = 12-15 mm, +++ = >15 mm, nd = not done (in agar well diffusion tests after dissolution to 1 mg/ml in both PBS and PBS-DMSO).

In conclusion, a double bond in the acyl group on the C-terminus and a urethane-type bond on the N-terminus are not necessary for preserving the antibacterial spectrum and the activity. Furthermore, an appropriate length of both, the peptide fragment and the X- and Y- groups seems to be an important requirement.

Acknowledgements

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USE OF POLYMER-SUPPORTED BASES FOR THE PREPARATION OF HIGHLY PURE PROLINE NCA

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Polymers made out of amino acids (polyamino acids, peptide copolymers) are emerging as promising therapeutic compounds. These polymers are finding widespread application in the field of drug delivery [1]. A milestone example of therapeutic polyamino acid carriers is paclitaxel polyglumex [2], a biologically-enhanced version of taxol conjugated to a polyglutamate polymer. In this context, polyproline is also attracting much attention for its therapeutic potential. It has been used to solubilize poorly water-soluble proteins [3]. Polyproline polymers have also found used in affinity chromatography for the purification of platelet profilin [4], and more recently, in the design of dendrimers [5]. Polyamino acids are most conveniently synthesized by polymerization of the corresponding amino acid N-carboxyanhydride (NCA). However, the case of proline is unique among coded amino acids as it has the α-amino group bound to the side chain yielding a cyclic secondary amine and showing some conformational restrictions. These features probably underlie the poor synthetic yields obtained using current available methods for α-amino acid NCA formation.

Amino acid NCA are obtained by the method described by Fuchs [6]. In the case of proline, the N-carbamoyl intermediate does not cyclise spontaneously as it takes place with other amino acids, and the use of a non-nucleophilic base, typically a tertiary amine, is required (Fig. 1). Triethylamine, is commonly used but it renders a low conversion of the N carbamoyl chloride to the expected Pro-NCA, together with the presence of the Pro-Pro diketopiperazine byproduct. Polymer-supported bases have been used instead of triethylamine (Fig. 2). The use of resin-bound tertiary amines provided much higher yields of Pro-NCA together with very low percentages of the diketopiperazine byproduct and, obviously, the total absence of tertiary amine contamination. The resin DEAM-PS was the one that provided virtually no diketopiperazine byproduct. Resins DMAP-PS and TBD-PS furnished very low reaction yields. The characteristic α-proton signals of Pro carbamoyl chloride, Pro-Pro DKP, and Pro-NCA were assigned and used to estimate the yield of each product. Another advantage of using polymer-supported bases is the ease of crystallization of the desired Pro-NCA. Since the base hydrochloride is removed by filtration, purities above 99% were regularly obtained for the different batches of Pro-NCA.

The polymer-supported base can be also regenerated for further use. A washing step of the resin with piperidine in DMF (1:4) neutralizes the ammonium salt and the yields obtained with recycled resin were reproducible.

In conclusion, an efficient procedure for the obtention of proline N-carboxyanhydride in high yield and purity using polymer-supported tertiary amines is reported. The resin can be recycled by regenerating the free base after a neutralization step with piperidine in DMF and a filtration of the resin. This efficient method would facilitate the preparation of polyproline-based polymers.

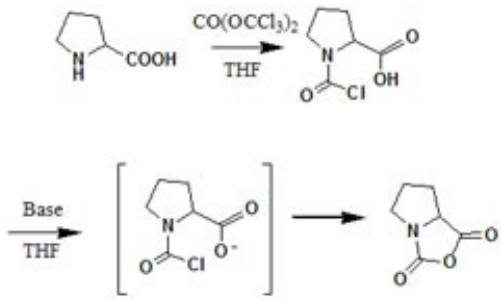

Figure 1

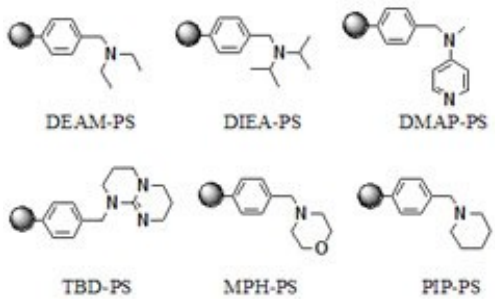

Figure 2

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OXYTOCIN, DIVALENT CATIONS AND MECHANISM OF ACTION

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Introduction

It has been known for a long time that divalent cations, especially Mg^{2+} and Mn^{2+}, influence significantly binding to receptors and biological activity of oxytocin (OT) [1]. There is very low specific binding of [^{3}H]OT to OT receptors (OTR) in the absence of Mg^{2+} or Mn^{2+}. Recently an article appeared showing formation of a complex: divalent cation-OT and stressing the importance of the N-terminal amino group of OT for binding and activity [2]. However deamino-analogues of OT, i.e. analogues lacking the N-terminal amino group, have high biological potency, higher than OT, and their binding to the receptors is also influenced by divalent cations. Here we have studied formation of the complexes of not only OT, however also of deaminooxytocin (dOT) and an OT antagonist (OTA) with Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} by means of electrospray MS and binding of the above mentioned peptides to human OTR in the presence or absence of the ions.

Materials and Methods

OT was from Ferring, dOT was synthesized in IOCB ASCR, OTA ([d$(CH_2)_5^1$,Tyr(Me)^2 Thr^4,Orn^8,Tyr^9-NH_2]vasotocin) was from Bachem, Iodogen was from Pierce, all other chemicals were from Sigma Aldrich or Serva. [^{125}I]OTA was prepared using Iodogen procedure and purified using radioHPLC to get monoiodinated substance. Determination of binding affinity to human OTR was performed using [^{125}I]OTA (0.05 nM), crude membrane preparation of HEK OTR cells (cells stable-expressing human OTR kindly donated by Dr. G.Gimpl [3]) and various concentrations of peptides (0.1 – 10000 nM) for 30 min at 35 °C in a volume of 0.25 ml. Buffer used was 50 mM HEPES, pH 7.6, containing different concentration of chlorides of Mg^{2+}, Mn^{2+}, Zn^{2+}, or Ca^{2+} and 1mg/ml bovine serum albumin. The reaction was terminated by quick filtration on a Brandel cell harvester. The electrospray spectra were recorded using Q-Tof micro (Waters, Milford, MA, USA) mass spectrometer. Samples dissolved in water solution of the salts were mixed with acetonitrile (1:1) and injected to the mobile phase flow (water/acetonitrile 1:1, 7 μL/min) using a manual injector (10 μL). The ion source operating parameters were as follows: spray voltage 3.5 kV, sample cone voltage 40 V, desolvation temperature 150 °C , and source temperature 80 °C. The positive ions were recorded in the mass range 150-1500 u. Spectra were processed using MassLynx software (Waters).

Results and Discussion

Electrospray-MS study of OT, dOT and OTA (50 μM peptide alone or after incubation with 200 μM of the relevant ion) shows that all these compounds form molecular adducts with Zn^{2+}, Mg^{2+}, Mn^{2+} and Ca^{2+}. In binding experiments using [^{125}I]OTA, the quantity of tracer bound to human OTR strongly depends on the character and concentration of divalent ion. IC_{50} values for OTA (antagonist) were independent of the divalent ion character (it corresponded to 2.5 - 4 nM) or its absence, however for OT and dOT (strong agonists), the IC_{50} values were low in the presence of Mg^{2+} and Mn^{2+} (nM range) and almost 1000 times higher in the presence of $Zn^{2+}$and Ca^{2+}. All this argues against the role of the N-terminal amino group of OT in the binding to the receptor. The high uterotonic activity of OT and dOT in the *in vitro* test in the absence of Mg^{2+} in the medium further points to the idea that the divalent ions do work on the membrane site of the receptors. Active conformations of OT and vasopressin are still not described. The role of Mg^{2+} and Mn^{2+} in the mechanism of signal transduction was not elucidated. More information is necessary. Theoretical molecular modeling calculation should be performed with caution taking into consideration binding affinity to receptors in the presence and absence of Mg^{2+} and Mn^{2+} and biological activity of not only OT but also different analogues of OT.

Acknowledgements

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PRION PROTEIN HELIX-2 CONFORMATIONAL PROPERTIES: IMPLICATIONS FOR FULL LENGTH PROTEIN FOLDING AND STABILITY

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Introduction

The aetiology of neurodegenerative prion diseases is currently associated to the unfolding of the cellular prion protein, PrP^{C}, and to its conversion into an oligomeric β-pleated, but still structurally mysterious "scrapie" variant (PrP^{Sc}) [1]. Knowledge of the molecular basis of such a mechanism can provide key clues for the rational structure-based drug design of compounds able to block or prevent disease. We have previously reported on the ambivalent structural behavior of the 173-195 protein fragment, corresponding to the helix 2, which could play a role in the nucleation process of protein misfolding and oligomerization [2, 3]. The helix-2 amino acid sequence, comprising residues 173 - 195, is invariant across a wide variety of species. A group of four threonine residues within this segment is a good candidate to promote a local α→β transition, because of its high intrinsic β-propensity. In order to further shed light on the structural properties of this intriguing protein domain and on the influence that a disease-associated mutation can have on its relative stability, we have undertaken a comparative NMR study on peptides corresponding to the full length helix 2, PrP[173-195], to the shorter C-terminal region, PrP[180-195], and to the mutant, PrP[173-195]D178N, bearing the most important mutation occurring in CJD [4]. Given the strong aggregation tendency of the above peptides in aqueous solution at mM concentrations, the NMR analysis has been performed in absolute TFE. Further structural details on the conformational landscape of this PrP helical domain were obtained by MD simulations in water as well as in aqueous SDS buffer. Further MD simulations were performed on the D178N mutated full length C-globular prion domain in vacuo and in SDS solution.

Results and Discussion

Conformational ambiguity makes the structural arrangement of the segment corresponding to helix 2 strongly dependent on the environment. Evidence supports the view that the single Asp178 residue, that is the most important point mutation occurring in CJD [4], plays a key role in determining the structural properties of the PrP globular domain. We have found that the wild type peptide is significantly affected by the replacement of the negatively charged Asp178 by a neutral Asn residue. The consequent structural rearrangement leads to the formation of two short helices separated by a kink centered on Lys185 and Gln186. MD simulations performed in presence of SDS are in full agreement with experimental evidence coming from CD data and show that all peptides are in a β-type organization.

After substituting Asp178 with an Asn residue, the C-globular prion structure was optimized and subjected to MD, both in vacuo and in water. Overall, these data strongly suggest that the role played by the helix 2 domain is not to be considered neutral in the misfolding mechanism of the cellular prion protein to the scrapie isoform. In fact, MD simulations carried out on the mutated C-globular prion domain result in elongation of the existing β-strands and weakening of the global helical content. These data indicate that the D178N mutation is able to induce a significant conformational change in the entire protein and the partial or complete unfolding of helices 2 and 3, which we suggest to participate in the PrP^{C} to PrP^{Sc} conversion.

Acknowledgments

This work has been supported by funds from the Ministero dell'Istruzione, dell'Università e della Ricerca (M.I.U.R., FIRB 2001 RBNE01ARR4 and FIRB 2003 RBNE03PX83) and by the Università Italo-Francese (Programma VINCI 2005).

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SINGLE AMINO ACID SUBSTITUTION ANALOGUES OF THE ANTIBACTERIAL PEPTIDE [A7]-ANOPLIN

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Introduction

Anoplin, GLLKRIKTLL-NH_2, is an antibacterial peptide isolated from the solitary spider wasp, *Anoplius samariensis* [1].

Previously, we have reported a structure-activity study of anoplin based on 37 analogues [2]. Alanine positional scanning showed restrictions on 6 out of the 10 residues, and improved MIC values for the remaining 4 residues, including [A^7]-anoplin. In the present paper, we report a series of analogues of [A^7]-anoplin in which Ala was replaced by Val, Leu, Ile, Phe, Trp and Asn.

Results

Peptide synthesis was accomplished manually, using a TentaGel S RAM resin and Fmoc solid-phase peptide chemistry. Amino acids (4 equiv.) were coupled employing TBTU (4 equiv.), HOBt (4 equiv.) and DIEA (6 equiv.) for 60 minutes.

The resin was washed and drained and a one hour recoupling was performed. Following synthesis, the peptide resins were washed, dried and cleaved using TFA:H_2O:triisopropylsilane. Finally, all analogues were purified by preparative HPLC and characterized by LC-MS. A stock peptide solution in 1% DMSO was prepared and the peptide concentration of each peptide was determined by amino acid analysis.

The anoplin analogues were tested for antibacterial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 was determined using a broth microdilution assay modified from the method of Hancock [3].

Briefly, stock solutions of the peptides were dissolved in 1% DMSO to a concentration of 1 mg/mL. The MIC of each peptide was read as the lowest concentration of peptide that inhibited visible growth of the bacteria after 24 h of incubation at 37 ^{0}C. All MIC determinations were performed in triplicate, and corrected following amino acid analysis.

Ampicillin was used as control. Furthermore, the cytotoxity towards red blod cells was evaluated using a hemolytic activity assay.

Table1. Characteristics and biological activity of Anoplin Analogues

ID	RT[a]	G+[b]	G-[c]	EC_{50}[d]	<H>[e]
[I^7]	19.3	2	2	38	0.070
[L^7]	19.3	2	2	30	0.050
[F^7]	18.5	2	2	29	0.058
[W^7]	18.3	2	2	36	0.034
[V^7]	17.8	2	2	101	0.051
[A^7]	17.3	5	5	203	0.022
[N^7]	15.4	>47	47	N.H.[f]	-0.067

Legends. a: RP-HPLC retention time b: *S. aureus* (µg/ml) c: *E. coli* (µg/ml); d: concentration (µg/ml) where 50% of the red blood cells are lysed e: Mean hydrophobicity. f: N.H. not hemolytic.

Discussion

The [V^7]-, [I^7]-, [L^7]-, [F^7]- and [W^7]-anoplin analogues displayed improved MIC activities towards *S. aureus* and *E. coli* (2 µg/ml both strains) as compared with [A^7]-anoplin (5 µg/ml both strains). However, the analogues also turned out to be more hemolytic. The anoplin analogues [I^7], [F^7], [L^7], [W^7] showed 50% hemolysis at concentrations ranging from 29 - 38 µg/ml, while [V^7]-anoplin and [A^7] displayed EC_{50} values of 101 µg/ml and 203 µg/ml, respectively.

In agreement with helical wheel predictions, [N^7]-anoplin did not show any significant antibaterial (47 µg/ml both strains) or hemolytic activity. Finally, we found a good correlation between HPLC retention times and Eisenberg´s mean hydrophobicity [4].

Conclusions

In conclusion, the results presented here suggest that anoplin analogues are promising lead structures for developing future antibacterial agents.

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PEPTIDE DERIVATIZED LIPOSOMES AS TARGET SPECIFIC MRI CONTRAST AGENTS

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Introduction

Magnetic Resonance Imaging is one of the most powerful non-invasive diagnostic methods in medicine. It gives very resolved images but, due to its very low sensitivity, needs higher concentration (10^{-4} M) of contrast agents such as paramagnetic Gd(III) complexes.

We used two different strategies for the development of a new generation of contrast agents in MRI having, at the same time, high contrast activity and high target selectivity.

The first strategy uses bioactive peptides functionalized with one or more bifunctional chelating agents and their Gd(III)-complexes.

The second strategy is based on the use of supramolecular aggregates containing both an high number of Gd(III) complexes and surface exposed moieties of a bioactive peptide. In fact, Gadolinium complexes containing supramolecular aggregates (micelles and liposomes), present two interesting properties: an enhanced ability to increase solvent proton relaxation rates; and a longer life time of the contrast agent in the circulating blood by avoiding the typical extravasation. The target specifity of the contrast agents could be obtained by labelling the supramolecular aggregates with bioactive peptides able to address them on the specific biological target overexpressed by cancerous cells.

Results and Discussion

By using the first strategy, one or more, up to ten, bifunctional chelating agents (DTPAGlu) and their Gd(III)-complexes, DTPAGlu-Gd(III), have been covalently attached to the N-terminal end of the bioactive peptide CCK8. This peptide is able to recognize with nanomolar affinity the two cholecyctokinin receptors (CCKA and CCKB) overexpressed by cancer cells. The chelating agents are linked to the bioactive peptide by using an amino acidic spacer. Unfortunately, even if the relaxivity of each Gd(III) complex is higher than the relaxivity of an isolated DTPAGlu-Gd(III), the number of Gd(III) complexes delivered by the CCK8 peptide on the target receptors, is not enough to give good contrast in MRI and therefore these compounds are ineffective as target selective contrast agents.

In the case of the second strategy, we have assembled supramolecular aggregates (cylindrical micelles, vesicles or liposomes) by mixing two different monomers:

one of them containing a Gadolinium complex ($(C18)_2$DTPAGlu-Gd(III)) and the other the bioactive CCK8 peptide (DSPE-PEG2000-CCK8). Liposome aggregates were prepared by mixing the compounds in water solution and providing energy to the system by sonication and extrusion procedures.

Both monomers were synthesized according to solid phase protocols based on Fmoc strategy. DSPE-PEG_{2000}-CCK8 was obtained by coupling DSPE-PEG_{2000}-OH to the αNH2 of CCK8 peptide linked to the solid support. Reaction was performed in NMP, by using 2 equivalents of DSPE-PEG_{2000}-OH and PyBOP/HOBt. The yield (95%) was monitored by colorimetric phosphorous test. The crude was purified by dialysis and its purity and identity were confirmed by analytical RP-HPLC and by MALDI. The $(C18)_2$DTPAGlu synthesis and its Gd(III) coordination process were carried out as previously described [2]. Aggregates were prepared at different molar ratio between the two components, according to the following scheme: Sample **A** = DSPE-PEG_{2000}-CCK8/$(C18)_2$DTPAGlu-Gd 100/0; **B** = 70/30; **C** = 50/50; **D** = 20/80; **E** = 0/100.

Features of the aggregates have been obtained through the Atomic Force Microscopy (AFM) and Small-Angle Neutron Scattering (SANS) techniques.

In sample A, containing pure DSPE-PEG2000-CCK8, liposomes are present. The aggregates are substantially unperturbed by the introduction of moderate amount of $(C18)_2$DTPAGlu-Gd (sample B and C). Their thickness is 80 Å. Further addition of the $(C18)_2$DTPAGlu-Gd (sample D) to the system produces a drastic change in the structure of the aggregates, and a liposome-micelle transition is observed. These latter are rod-like micelles of (~40 Å of radius and length larger than 200 Å. Finally, in sample E the system is dominated by micelles [2].

The average size of bright spots (660 ± 250 Å), determined by AFM technique at high magnification are compatible with SANS data.

In order to obtain a better understanding of the solution dynamics of the aggregates (sample C, D and E) foreseeing for their MRI application, relaxivity measurements were performed. The proton relaxivity at 20 MHz and 298 K resulted to be exactly the same (17.2 $mM^{-1}s^{-1}$) for both C and D preparations though in the two cases its value is the result of the combination of different local and global contributions.

The new peptide derivatized liposomes for the high relaxitivity value and for the presence of a surface exposed peptide are very promising candidates as target selective MRI contrast agents.

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LOOKING AT THE DYNAMICS OF PEPTIDE-RECEPTOR INTERACTION WITH TEMPERATURE DEPENDENT METHIONINE PROXIMITY ASSAY ON THE HUMAN ANGIOTENSIN II TYPE 1 RECEPTOR

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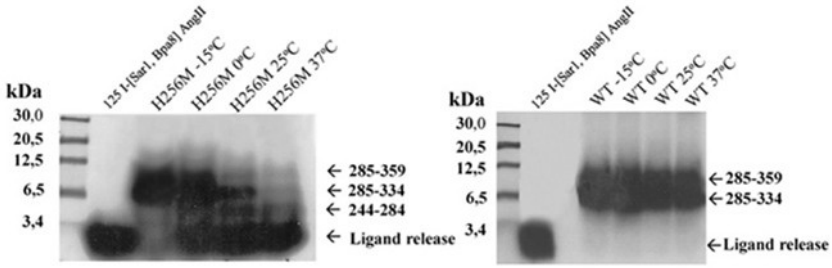
The molecular structures of G Protein Coupled Receptors (GPCRs) are difficult to determine by classical protein structure determination. The human Angiotensin II type 1 receptor (hAT1), is responsible for vascular tonus among other functions. On this GPCR the Methionine proximity Assays (MPA) has recently been developed to elucidate ligand binding environment and its general molecular structure [1 - 3]. This technique in combination with other methods enabled us to refine the structural model of the hAT1 receptor.

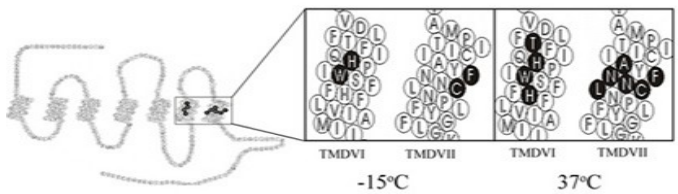
MPA is a mutagenesis study of the receptor with a photolabile ligand having a photochemical selectivity for methionines. Contact points are determined through changes in fragmentation patterns observed after chemical digestion of labelled receptors [1 - 3]. We introduce temperature variation to MPA to understand conformational dynamics of hAT1. The positive contact points obtained in previous studies were incubated at room temperature as previously described [1] and then photolabelled at temperatures ranging from -15 ºC to 37 ºC. This enables us to better understand the structural dynamics of the receptor by observing temperature altered ligand contact points and to determine the binding pocket of the most probable minimal energy conformation. The results show an unexpected intra-receptor dynamic permitting multiple receptor conformations not accessible at low temperatures; these receptor dynamics are probably responsible for attaining an active conformation necessary for GPCR signalling.

The MPA positive contact point mutants utilised were F249M, W253M and H256M in the 6^{th} transmembrane domain (TMDVI) and F293M, N294M, N295M, C296M and L297M in the 7^{th} (TMDVII), all showing comparable biological properties to WT AT1 except N294M that showed binding but no efficacy [1]. CNBr digestion digestion of labelled TMDVI mutants showed temperature dependently altered contact points compared to WT. At low temperatures in the H256M mutant, the ligand contact points are primarily in TMDVII (7.2 kDa and 10.0 kDa) and this is opposed to higher temperatures where the ligand labels primarily the 256M mutation in TMDVI (5.8 kDa).



The mutants F249M and W253M also showed less intense but similar temperature dependant shifts. Photolabelling of TMDVII at lower temperatures showed that only two residues can be labelled at -15 ºC, as opposed to five in the more dynamic 37 ºC receptor conformations. Thus temperature induced mobility would widen the Bpa action radius allowing outlying Met tagging. In a low energy conformation, the H256M and W253M mutant receptors can still be photolabelled but with much less Met specific tagging.

The "cold" conformation shows a more limited binding pocket, the basic contact points in the WT receptor are at 293F and 296C in the minimal energy conformation since the N294M, N295M and L297M mutants did not photolabel significantly at -15 ºC. In the "hot" conformation however a more than complete helical turn of contact points are continuously accessible, ranging from 293 - 297 in TMVII, suggestive of an unravelling of the helical structure [1].



The herein introduced temperature variation has broadened the horizon for the MPA approach; it allows looking at dynamic structural changes and the thermodynamics of ligand-receptor interactions. Eventually, this tool might permit to study the conformational aspects of GPCR activation and thermodynamics.

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UTILIZATION OF CLEAR-OXTM, POLYMER SUPPORTED OXIDANT FOR DISULFIDE BRIDGE FORMATION IN PEPTIDES.

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Introduction

Disulfide bridge formation in synthetic peptides is a difficult process that often outcomes low efficiencies of the final product or its misfolding. There are several oxidation strategies, among them spontaneous (air oxidation) or selective approaches utilizing orthogonal protection of the thiol groups of Cys redisues . Very recently Darlak *el al*. [1] proposed to use a cross-linked ethoxylate acrylate resin with attached 5,5'-dithiobis(2-nitrobenzoic acid) (named as CLEAR-OX^{TM}) as a fast and efficient reagent of disulfide bridge formation. Herein we report results of disulfide bridge formation in three peptides:(**1**) analogue of Sunflower trypsin inhibitor SFTI-1, (**2**) the analogue of α-conotoxin ImI and (**3**) trypsin inhibitor isolated from seeds of garden four-o'clock (MJTI). The listed peptides differ in disulfide bridge number and their sequences are given below:

[Val^5] SFTI-I: GRCTVSIPPICFPD (**1**)

[Sar^9] ImI: GCCSDPRCSarWRC-NH2 (**2**)

MJTI-I: EDEECAKTDQICPPNAPNYCCSGSCVPHPRLRIFVCA (**3**)

Results and Discussion

Multiple oxidation methods have been applied for all peptides including: air oxidation, DMSO oxidation, H_2O_2 oxidation and CLEAR-OX^{TM} oxidation. Oxidation process was monitored using RP-HPLC while the molecular weight of all compounds was determinated by MALDI TOF mass spectrometer. In case of peptide **1** all methods yielded the final product as judging by its molecular weigh and trypsin activity. The only difference was the period of time required to complete the oxidation process. In case of air or DMSO oxidation the linear non oxidized peptide was observed up to 24 h. Utilization of CLEAR-OX^{TM} resin completed the process after 10 minutes.

All oxidation methods of conotoxin [Sar^9]ImI yielded to multiple oxidation products which are in a good agreement with the results described by Nielsen *et al.* [2]. Two peaks on chromatograms were observed but only one corresponds with biologically active peptide. The oxidation efficiency of conotoxin analogue measured as area of peak of biologically active compound is presented in Fig. 1. Two methods DMSO and CLEAR-OX^{TM} outcome high yield of properly folded peptide. However DMSO oxidation requires more time for clean up procedure than the of application of CLEAR-OX^{TM} resin

Oxidative folding of trypsin inhibitor MJTI was successful only in case of air oxidation. All other methods applied resulting not properly folded peptide.

Aim of this work was to display oxidation potency and properties of CLEAR-OX^{TM} resin in spectrum of wildely used oxidation methods. Based on results obtained for peptide containing two (peptide **1**) or four (peptide **2**) cystein residues the efficiency of oxidation using CLEAR-OX^{TM} reagent is similar to DMSO method. The advantage of the first one is that it requires no clean up work, so it should be considered as reagent of choice when oxidative folding requires more powerful methods.

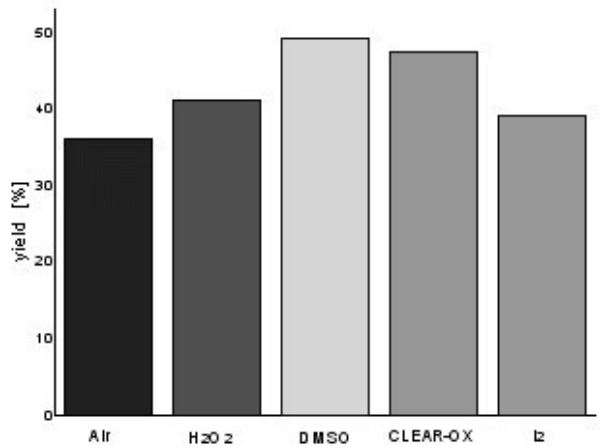

Fig. 1. Yield of oxidation of conotoxin [Sar^{9}]ImI.

Acknowledgments:

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CHROMOGENIC SUBSTRATES OF SERINE PROTEASES DESIGNED BY THE COMBINATORIAL CHEMISTRY METHODS

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Introduction

Peptides with p-nitroanilide (*p*NA) at their C-termini introduced by Erlanger in the sixties of the last century are first choice chromogenic substrates for determination of proteinases activity [1]. The attachment at the C-terminus of peptide 5-amino-2-nitrobezoic acid ($Anb^{5,2}$) that is the *p*NA analogue carrying additional carboxyl moiety, allowed to use of a standard solid phase method for the synthesis of chromogenic substrates.

In our previous work [2] we described the synthesis of chromogenic substrate library of bovine β-trypsin containing $Anb^{5,2}$-NH_2. Herein we are reporting the synthesis of $Anb^{5,2}$-NH_2 peptide library, which was designed to select chromogenic substrates of bovine α-chymotrypsin and human leukocyte elastase(HLE). General formula of synthesized library is as follows:

$$X_4\text{-}X_3\text{-}X_2\text{-}X_1\text{-}Anb^{5,2}\text{-}NH_2$$

Where in position X_1 = Lys, Arg, Ala, Val, Leu, Nle, Ser, Phe, Tyr; in X_2 all proteinogenic amino acid except Cys, X_3 = Ala, Pro, Val, Phe, Ser, Lys, Arg, Asp, X_4 = Phe, Lys, Val, Ile, Abu, Ala, Asp.

Such library consists of 9576 tetrapeptides.

Results and Discussion

The solid-phase synthesis of the chromogenic substrate library was achieved using portioning-mixing method. Deconvolution of the peptide library synthesized was performed by the iterative method in solution. The most active chymotrypsin and elastase sequences were compounds **1** and **15**, respectively. Such sequences were used to design a second generation substrates, modified at N-and C-termini of selected substrates. The obtained results (Table 1) indicate that the optimal residue in position X_1 that is responsible for the proper recognition by enzyme is Tyr in case of chymotrypsin and Val for HLE. The presence of the protecting groups at the N-terminus increase the values of substrate specificity constants (k_{cat}/K_M) towards its cognate enzyme. The highest activity is displayed for the substrates with Z group at their N-termini. Significantly more active appeared substrates containing amide moiety attached to the C-terminal chromophore. As a consequence of such modifications, two most acive substrates were selected. Compound **4** is one of the best chymotrypsin pNA based substrate described up to date [3]. Compound **24** was selected for HLE.

Table 1. Physical properties and kinetic parameters of chromogenic substrates against bovine chymotrypsin and human leukocyte elastase.

Substrate sequence Y_1-Phe-X-Thr-Tyr-Anb^{3,2}-OY_2	MW calc. (found)	R_t (HPLC) [min]	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [$M^{-1} \times s^{-1}$] × 10^3
Bovine α-chymotrypsin					
1. H-Phe-Ala-Thr-Tyr-Anb^{3,2}-NH_2	663.0 (664.0)	15.72	21.2 ± 2.0	136.9 ± 12.0	15.3 ± 0.1
2. Ac-Phe-Ala-Thr-Tyr-Anb^{3,2}-NH_2	705.1 (705.2)	19.28	22.7 ± 1.5	59.6 ± 2.6	38.6 ± 0.9
3. Tos- Phe-Ala-Thr-Tyr-Anb^{3,2}-NH_2	817.2(818.2)	25.84	4.0 ± 0.3	18.1 ± 3.0	21.7± 1.9
4. Z- Phe-Ala-Thr-Tyr-Anb^{3,2}-NH_2	**796.0(796.0)**	**24.77**	**16.7 ± 0.9**	**17.7 ± 2.1**	**93.1 ± 6.0**
5. H-Phe-Ala-Thr-Tyr-Anb^{3,2}-OH	663.9 (665.0)	17.23	15.0 ± 1.0	235.0 ± 20.0	6.4 ± 0.1
6. Ac-Phe-Ala-Thr-Tyr-Anb^{3,2}-OH	705.2 (706.0)	21.03	20.1 + 0.9	105.0 + 8.7	19.1 − 0.7
7. Tos- Phe-Ala-Thr-Tyr-Anb^{3,2}-OH	817.2(817.2)	26.36	2.0 ± 0.1	63.8 ± 3.8	3.1 ± 0.1
8. H-Phe-Arg-Thr-Tyr-Anb^{3,2}-NH_2	748.0 (749.2)	14.46	22.0 ± 1.9	80.6 ± 13.0	27.3 ± 2.0
9. Ac-Phe-Arg-Thr-Tyr-Anb^{3,2}-NH_2	790.0 (791.0)	17.22	14.4 ± 1.0	29.2 ± 3.1	47.9 – 1.8
10. Tos-Phe-Arg-Thr-Tyr-Anb^{3,2}-NH_2	902.3(903.3)	21.95	9.9 ± 1.3	179.2 ± 35.0	5.5 ± 0.4
11. Z- Phe-Arg-Thr-Tyr-Anb^{3,2}-NH_2	881.0(883.4)	23.04	9.2 ± 0.5	51.0 ± 4.6	18.0 – 0.6
12. H-Phe-Arg-Thr-Tyr-Anb^{3,2}-OH	746.7 (750.4)	15.69	17.0 + 1.2	82.8 + 6.2	20.5 − 0.1
13. Ac-Phe-Arg-Thr-Tyr-Anb^{3,2}-OH	790.0 (792.0)	18.81	29.9 ± 2.0	56.9 ± 5.1	35.1 ± 0.8
14. Tos-Phe-Arg-Thr-Tyr-Anb^{3,2}-OH	902.0 (904.4)	23.05	4.8 ± 0.8	91.4 ± 6.3	5.3 ± 0.6
Human leukocyte elastase					
15. H-Phe-Phe-Ala-Val-Anb^{3,2}-NH_2	646.6 (646.3)	19.80	5.5 ± 0.1	368.4 ± 31.0	1.49 ± 0.09
16. Ac-Phe-Phe-Ala-Val-Anb^{3,2}-NH_2	688.6 (688.3)	24.30	4.8 ± 0.1	124.4 ± 4.8	3.86 ± 0.04
17. Tos-Phe-Phe-Ala-Val-Anb^{3,2}-NH_2	800.7 (801.0)	18.61*	0.5±0.1	254.7 ± 20.1	1.96 ± 0.32
18. Z-Phe-Phe-Ala-Val- Anb^{3,2}-NH_2	779.7 (780.0)	19.17*	0.7±0.1	74.1 ± 5.8	0.14 ± 0.01
18. H-Phe-Phe-Ala-Val-Anb^{3,2}-OH	646.5 (647.3)	21.65	1.2 ± 0.1	123.5 ± 18.0	0.97 ± 0.11
19. Ac-Phe-Phe-Ala-Val-Anb^{3,2}-OH	688.5 (688.3)	26.25	1.1 ± 0.1	45.0 ± 6.3	2.44 ± 0.32
20. Tos- Phe-Phe-Ala-Val-Anb^{3,2}-OH	800.7(801.0)	19.84*	0.8 ± 0.1	327.5 ± 28.3	0.24 ± 0.12
21. H-Phe-Phe-Pro-Val-Anb^{3,2}-NH_2	672.6 (672.3)	20.15	5.0 ± 0.1	197.0 ± 12	2.54 ± 0.09
22. Ac-Phe-Phe-Pro-Val-Anb^{3,2}-NH_2	714.6 (714.3)	24.01	8.5 ±0.3	105.2 ± 4.2	8.08 ± 0.09
23. Tos-Phe-Phe-Pro-Val-Anb^{3,2}-NH_2	826.8 (827.2)	18.63*	1.0 ± 0.1	167.4 ± 10.2	0.59 ± 0.12
24 Z- Phe-Phe-Pro-Val-Anb^{3,2}-NH_2	**805.7 (806.1)**	**19.15***	**0.7±0.1**	**5.1 ± 0.3**	**13.73±1.03**
24. H-Phe-Phe-Pro-Val-Anb^{3,2}-OH	672.6 (672.3)	22.17	3.6 ± 0.1	140.0 ± 11	2.57 ± 0.13
25. Ac-Phe-Phe-Pro-Val-Anb^{3,2}-OH	714.6 (714.3)	26.43	6.9 ± 0.1	126.1 ± 7.2	5.47 ± 0.23

HPLC analysis was performed on the Pro Star system (Varian, Australia) equipped with Kromasil 100 C_8 column (8×250 mm) (Knauer, Germany) and UV-VIS detector was used. A linear gradient from 10% B to 90% B within 30 minutes was applied (A: 0.1 % TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm. For compounds marked * the linear gradient 30% B to 95% B within 30 minutes was applied. Mass spectra were recorded using Biflex III MALDI TOF mass spectrometer (Bruker, Germany).

Acknowledgments

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ONE MODEL PEPTIDE TO ELUCIDATE PH AND CONCENTRATION DEPENDENCY OF AMYLOID FORMATION

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Protein aggregation is known to be the pathogenic factor of many neurodegenerative diseases such as Alzheimer's disease. A common feature of the concerned proteins is the transition from the native, mostly α-helical conformation beyond ß-sheet rich intermediates to pathological amyloid fibers. The underlying conformational change can be triggered by protein concentration or environmental changes such as pH values.

Therefore, our aim was to generate a *de novo* designed model peptide that contains structural elements for both, stable α-helical folding as well as ß-sheet formation. The design is based on the well studied α-helical coiled coil folding motif and follows three design features (Fig. 1a) [1]. (1) Leucine in positions **a** and **d** and the arrangement of Lysine and Glutamic acid in positions **e** and **g** lead to a stable interhelical interaction domain. (2) To generate a pH dependency of coiled coil stability, Lysine and Glutamic acid are arranged in adjacent positions of the helix. Lysine residues destabilize helix formation at acidic pH, whereas Glutamic acid residues cause intramolecular repulsion at highly basic conditions. (3) Positions **b**, **c**, and **f**, having a minor effect on coiled coil dimerization, have been used to introduce Valine as ß-sheet inducing amino acid. In the case of a destabilization of the helix by pH, the peptide is able to adopt ß-sheet rich structures at sufficiently high concentrations.

The conformation and structure of the resulting aggregates was characterized by CD spectroscopy and by cryo transmission electron microscopy after 72 hours, when all spectra became invariable (Fig. 1b). Concentrations of 250 µM at pH 4.0 yield globular particles of the unfolded peptide with a typical size of 2.5 nm (Fig. 1c). At concentrations above 300 µM and pH 4.0, defined ß-sheet containing aggregates are formed that are characterized by helically twisted ribbons with a typical width of 8 nm (Fig. 1d). In contrast, at concentrations higher than 250 µM but pH 7.4, the peptide forms highly ordered α-helical fibers of three- or four-stranded assemblies with an estimated diameter of 2.5 nm (Fig. 1e). Furthermore, concentrations above 250 µM and pH 9.0 result in ß-sheet rich tubular structures of two fibers twisted around each other with an overall diameter of 8 nm (Fig. 1f).

In conclusion, we successfully generated a model peptide that, without changes in its primary structure, can be directed to adopt different defined secondary structures at will by adjustment of pH or peptide concentration. Thus, this system allows to

systematically study now the consequences of the interplay between peptide primary structure and environmental factors for conformation on a molecular level.

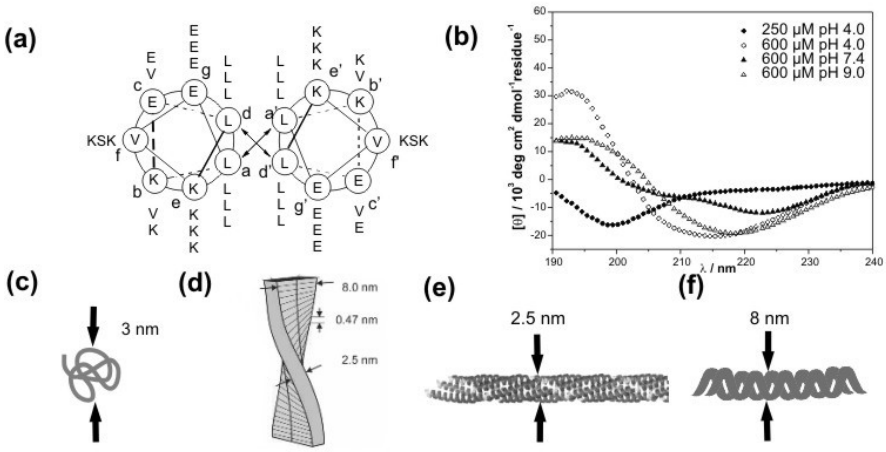

Figure 1. (a) Helical wheel of the model peptide. (b) CD spectra of the model peptide at different concentrations and pH values taken 72 hours after sample preparation, when all spectra became invariable. Structural models estimated by cryo electron microscopy are shown for the following conditions: (c) 250 µM at pH 4.0, (d) 600 µM at pH 4.0, (e) 600 µM at pH 7.4 and (f) 600 µM at pH 9.0.

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IDENTIFICATION OF CARDIOPROTECTIVE PEPTIDES FROM THROMBIN: DESIGN, SYNTHESIS AND PHARMACOLOGICAL INVESTIGATIONS

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Introduction

Thrombin plays a key role in various disorders such as arterial thrombosis, restenosis, atherosclerosis, and inflammation. Recent studies have shown that thrombin system, over-activity of which during myocardial ischemia contributes to the progression of heart failure, is important in maintaining cardiac function. Insights into the way in which thrombin interacts with its many substrates and cofactors have been clarified by crystal structure and site-directed mutagenesis analyses in the past decades, but until recently there has been little consideration of how its non-proteolytic functions are performed. In this study we proceeded from the observation that cleavage at the autolysis loop-1 of α-thrombin (Arg77, Arg75, and Arg67), leading to forming of β- and γ-thrombins [1], generates neo-domain containing the first of two angiotensin-like motifs, TALP-I [2], while cleavage of this region did not lead to a major reorganization of the folded structure of enzyme. To elucidate a proposed role of TALP-1 in regulatory activity of thrombin series of thrombin-derived peptides were designed, prepared, and pharmacologically evaluated.

Results

Series of compounds, including the proline- and RGD-containing peptides, starting sequences of which were multiple modified into linear or cyclic peptides for antagonistic properties, were prepared using both the classical method of peptide synthesis in solution and solid phase method. Compounds were purified by preparative reversed-phase HPLC on a Waters HPLC system and characterized by analytical RP HPLC, NMR, and FAB MS. Results of pharmacological studies of functional status and morphology (light microscopy and electron microscopy methods) on rat heart underwent prolonged ischemia-reperfusion revealed significant activity of three synthesized peptides LP-mc, TP-H7* and TP-L13 on cardiovascular system. These compounds caused decreasing of blood pressure, possessing cardioprotective properties, reduction of the zone of myocardium with necrosis, improving recovery from ischemia.

Discussion

Structural bases for the design of synthetic peptides were provided by numerous crystallographic data on thrombin [3 - 10], in which TALP-1 sequence forms extended structures that resembled conformation of angiotensin bound to AT1 and AT2 receptors, however, differences in conformations should be taken into account [11 - 12]. A review of the known structures of thrombin and other serine proteases leads us to a new concept that in addition to parent enzymes proteolytic derivatives might be involved in regulation cardiovascular disease-related processes. Although no systematic examination regarding regulatory role of α-thrombin proteolytic derivatives (β-, γ-, δ-, and ζ-thrombin) has been made, it is known that limited proteolysis causes exposition of new N- and C-terminal sites at its molecule surface. The obtained data allow hoping that TALP-I containing neo-domain of thrombin can contribute to non-proteolytic responses of α-thrombin associated with myocardial ischaemia as "angiotensin-like tethered ligands", probably through mechanisms involving activation of non-cleaved PARs and/or non-PARs pathway. Conclusions: Three novel cardioprotective peptides have resulted from thrombin structure-based design and subsequent improvement of the initial lead molecule.

Acknowledgements

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ENANTIOSEPARATION OF β-AMINO ACIDS

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Introduction

The aim of the present work was to evaluate HPLC methods for the separation of enantiomers of eighteen 3-amino-3-aryl-substituted propanoic acids (β-amino acids); the structure was **H_2N-CHX-CH_2-COOH** where X was different aryl substituent (Table).

Direct separations were carried out on different macrocyclic glycopeptide based stationary phases, such Chirobiotic **T2** containing teicoplanin, Chirobiotic **TAG**

Chromatographic data, retention factor of the first eluting enantiomer (k'_1), separation factor (α) and resolution (R_S) of the enantioseparation of *beta-amino acids with the structures H_2N-CHX-CH_2-COOH (1-18) on teicoplanin* (**T2**), *teicoplanin aglycone* (**TAG**) *and on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based* (**crown ether**) columns

Compound X	Column	Eluent (v/v/v)	k'_1	α	R_S
1,	T2	100/0.01/0.01, a	2.08	1.17	1.00
	crown ether	80/20, b	2.27	1.30	1.44
2,	T2	100/0.01/0.01, a	2.13	1.13	0.95
	crown ether	50/50, c	2.86	1.27	1.46
3,	T2	100/0.01/0.01, a	1.93	1.23	1.09
	crown ether	50/50, c	3.52	1.51	1.97
4,	T2	100/0.01/0.01, a	2.66	1.13	0.90
	crown ether	50/50, c	2.92	1.31	1.73
5,	crown ether	50/50, c	3.83	1.31	1.73
6,	T2	100/0.01/0.01, a	1.97	1.13	0.80
7,	TAG	100/0/0, a	5.46	1,06	0.75
	crown ether	50/50, c	4.43	1.28	1.88
8,	T2	100/0.01/0.01, a	2.45	1.20	1.15
	crown ether	80/20, b	2.84	1.42	1.97
9,	T2	100/0.01/0.01, a	2.72	1.18	1.10
	crown ether	50/50, c	3.18	1.45	1.73
10,	T2	100/0.01/0.01, a	2.21	1.15	0.90
	crown ether	80/20, b	5.40	1.50	1.80
11,	crown ether	20/80, b	3.34	1.14	1.10
12,	T2	100/0.01/0.01, a	2.85	1.11	0.85
	crown ether	50/50, c	6.27	1.22	1.90
13,	T2	100/0.01/0.01, a	3.00	1.18	1.10
	crown ether	50/50, c	3.56	1.48	1.90
14,	T2	100/0.01/0.01, a	2.35	1.14	1.00
	crown ether	80/20, b	5.78	1.53	1.87
15,	T2	100/0.01/0.01, a	1.99	1.27	1.65
	crown ether	20/80, d	8.42	1.32	2.45
16,	TAG	10/90, e	4.53	1.07	0.80
	crown ether	50/50, c	7.52	1.46	2.66
17,	TAG	10/90, e	10.26	1.18	1.10
	crown ether	50/50, f	8.37	1.89	4.46
18,	T2	100/0.01/0.01, a	2.33	1.15	0.95
	crown ether	20/80, d	7.05	1.34	2.50

Chromatographic conditions: mobil phase, **a**, MeOH/acetic acid/triethyl amine = 100/0.01/0.01 (v/v/v), **b, c** and **d**, H_2O / MeOH = 80/20, 50/50 and 20/80 (v/v), 10 mM acetic acid, **e**, 0.1% aqueous triethylammonium acetate (pH 4.1)/MeOH = 10/90 (v/v), **f**, H_2O / MeOH = 50/50 (v/v), 0.423 mM trifluoroacetic acid; flow rate, 0.5 ml min^{-1}; detection, 205 nm

containing teicoplanin aglycon, and on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based **crown ether** column.

Results and Discussion

Investigations on *macrocyclic glycopeptide* columns revealed that substitution on the aromatic rings of the β-amino acids (compounds **2** - **14**) did not result in a significant change in the retention factors of the first-eluting enantiomers. At a constant mobile phase composition, MeOH/AcOH/TEA (100/0.01/0.01 v/v/v) on the Chirobiotic T2, k_1's ranged from 1.93 to 3.08. For analogs **4** - **7** or **9** - **12**, having the same substituents but in different positions, the para-substituted compounds had larger retention factors, and generally larger α values. On the T2 column, for analogs **8**, **9**, **13** or **10**, **14** (having the halogen substituents in the same position), larger retention factors were obtained for the bromo-substituted analogs than for the chloro- or fluoro-substituted ones, while the α values did not change significantly. In the process of chiral recognition the primary "docking" interaction (between the amine of the CSP and the carboxylate of the analyte) should be similar. H-bonding, hydrophobic, steric etc. interactions should be taken into account. Our results shed light on the importance of steric and hydrophobic interactions in these separations on macrocyclic glycopeptide-based columns. For the *crown ether-based* CSP, the most important interaction of analytes containing a primary amino group is complex formation between the protonated primary amino group and the oxygen atoms in the crown ether ring. As concerns a comparison of the retention factors obtained for the analytes containing the same substituents in different positions on the aromatic ring, analytes **4** - **7** and **9** - **12** revealed that the *meta*-substituted analogs were more strongly retained than the *para*-substituted ones, and *ortho* position of the substituents was unfavorable regarding the enantioseparation. The enantiomers of analyte **6** were not separated. All of these data may be explained by a separation mechanism in which, besides complex formation there may be an intermolecular H-bonding interaction. It seems that the H-bonding effect is favorable when electron-donating atoms (oxygen or halogen) are in the *meta* or *para* position, and unfavorable when electron-donating atoms are in the *ortho* position.

Conclusions

HPLC methods were developed for the separation of the enantiomers of 3-aryl-substituted β-amino acids. Baseline separation was achieved in most cases. The crown-ether based column proved more suitable than the macrocyclic glycopeptides-based columns for the separation of these type of β-amino acids. Elution sequences were different. On macrocyclic glycopeptides S<R and on crown-ether-based columns R<S elution sequences were found in most cases.

Acknowledgements

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HIGHLY SPECIFIC ANTIBODIES FOR USE IN SANDWICH-TYPE ANTIBODY MICROARRAY ANALYSES OF COMPLEX BIOLOGICAL SAMPLES

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Introduction

Antibody microarrays are the protein complement of DNA microarrays for proteome research and medical protein diagnostics. Antibodies represent ideal protein capture molecules due to their high robustness as well as their availability in almost unlimited diversity. However, a high specificity against the protein antigen is a prerequisite for use in antibody microarrays [1]. Even subtle cross-reactivities to other proteins may severely limit the performance of this analytical tool, particularly when complex biological samples are to be analysed. This is even more eminent when two antibodies for capture and detection of the same analyte protein are required in a sandwich-type assay. To address this need and also to open new sources for highly specific antibodies we decided to exploit the comprehensive and diverse antibody pools available from polyclonal sera of immunized animals.

Results and Discussion

Our concept takes advantage of experiences in peptide-based epitope mapping. A series of overlapping antigen-derived peptide fragments is synthesized in a macroarray format on a cellulose membrane by SPOT synthesis [2]. Antibodies from a polyclonal serum (pAb) bind their specific linear epitopes (peptide fragment) and thereby separate into epitope specific antibody pools (Fig. 1). Bound antibodies were eluted from the peptide spots and then systematically evaluated under real assay conditions by screening the respective pools as detection antibodies on a microarray of capture antibodies together with the protein sample to be analysed (Fig. 2). The most suitable pool/s then are selected for purification in larger quantities by peptide affinity chromatography.

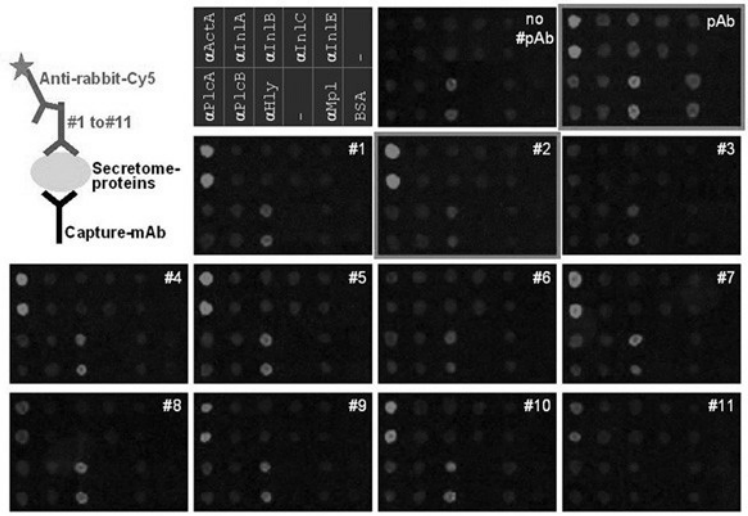

Fig.1. A series of 191 overlapping 15-mer peptide fragments covering the complete sequence of ActA from Listeria monocytogenes was synthesized on a cellulose membrane and probed with a rabbit polyclonal anti-ActA (pAb-ActA) serum followed by detection with AP-conjugated goat anti-rabbit Ab. Regions #1 to #11 were excised from the membrane, saturated with serum, and the antibodies eleuted. #8 serves as negative control.

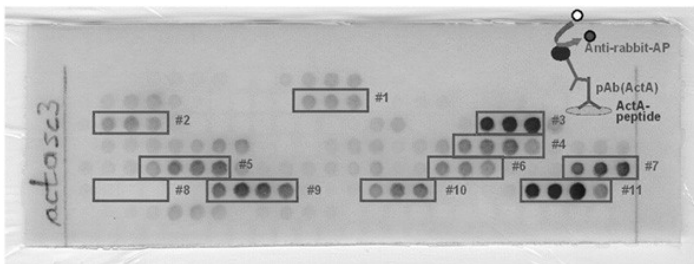

Fig. 2. Small microarrays printed in duplicate with a set of 9 monoclonal antibodies (capture-mAb) against different listeria proteins onto glass chips were assayed with a sample extract of listeria (secretome with ~200 proteins) and the Ab pools #1 to #11 plus the pAb-Acta. #2 shows the highest selectivity for ActA.

Acknowledgements

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HIGH-LEVEL EXPRESSION OF GLUCAGON AND GLUCAGON-LIKE PEPTIDE 1 RECEPTORS IN TETRACYCLINE-INDUCIBLE STABLE HEK293S GNTI- CELLS

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Introduction

The lack of high-resolution, three-dimensional information is an important factor that limits our understanding of the molecular basis of peptide ligand binding and subsequent development of potential drugs. Despite insights from structure-function studies a detailed 3-d structure of receptor-bound peptide hormone will ultimately come from NMR and X-ray analysis. These studies require relatively large amounts of purified receptor and most GPCRs are notoriously difficult to overexpress and isolate in functional form. We have established and characterized a novel tetracycline-inducible system in stable HEK293S GnTI$^-$ cells for the high-level expression of both human glucagon (hGR) and glucagon-like peptide 1 (hGLP-1R) receptors [1]. Using a strategy frequently used in bacterial expression systems, the membrane proteins are expressed by induction of the desired gene only after the cells have grown to near-maximum density. In addition, these cells lack N-acetylglucosaminyltransferase I activity and do not allow formation of heterogeneous N-glycans that may complicate crystallization protocols and NMR analysis. To facilitate single-step, immunoaffinity purification of hGR and hGLP-1R, the rhodopsin 1D4 peptide tag TETSQVAPA was engineered at the extreme C-terminus of each receptor.

Results and Discussion

HEK293S GnTI- cells were transfected with tetracycline-inducible expression plasmids pACMV-*tetO*-hGR and pACMV-*tetO*-hGLP-1R, followed by selection with Geneticin (200 μg/ml). Receptor expression was induced with growth medium supplemented with tetracycline (2 μg/ml) and sodium butyrate (5 mM). The cells were incubated for 48 hours and harvested for functional assays.

Human GR and human GLP-1R appeared as sharp bands even before treatment with N-Glycosidase F, indicating receptors with a single glycan species. A quantitative analysis of the optical densities of the protein bands show a theoretical yield each of 1.27 μg hGR and 0.81 μg hGLP-1R per milligram total membrane protein. The effective concentration at 50% stimulation (EC_{50}) in a typical dose-dependent adenylyl cyclase assay was 0.42 nM for hGR and 0.22 nM for hGLP-1R expressing cells. Membranes of cells expressing hGR bound ^{125}I-glucagon with an apparent dissociation constant of between 40 - 60 nM. Membranes prepared from hGR-expressing cells were incubated in the presence of Bpa-glucagon and photolyzed. A band corresponding to the correct molecular weight was detected

with an antibody to glucagon. This is the first photoaffinity labeling of recombinant glucagon receptor.

Membrane fractions were treated with varying concentrations of detergents CHAPS and n-dodecyl-β-maltoside (DM) and pelleted. Western blots of the supernatants showed that

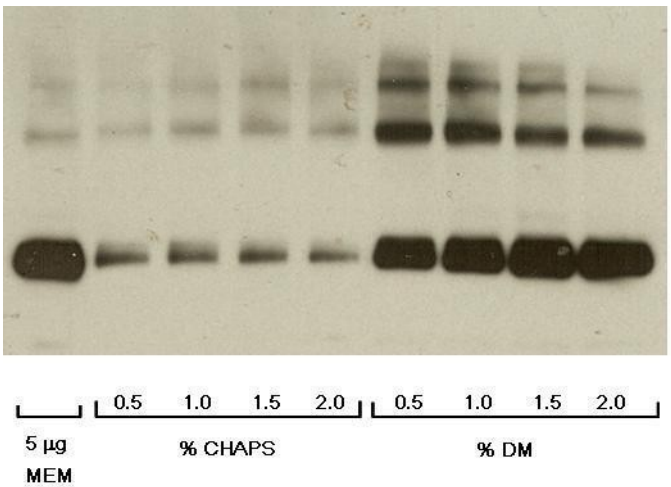

Fig. 1. Detergent extraction of stably expressed human glucagon receptor.

DM was more efficient than CHAPS in extracting out GR and GLP-1R from cell membranes (Fig. 1). More importantly, greater than 90% receptor binding activity was retained in the DM-soluble fraction. This encouraging observation indicates that solubilization and subsequent affinity purification of expressed hGR and hGLP-1R should be feasible. After conditions for cell growth and receptor expression are optimized in a bioreactor, large-scale production of GR and GLP-1R is now underway. Expression of family B GPCRs using this novel system should provide a reliable and sufficient source of receptor protein for structural and biophysical studies.

Acknowledgements

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FENTEPHALINES: DIMERIC BIOACTIVE PEPTIDES BASED ON AMINO ACIDS COUPLED TO 4-ANILINO-N-PHENETHYL-PIPERIDINE

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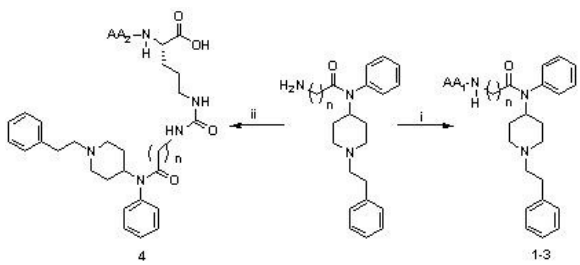
Introduction

4-Anilidopiperidines represent the most powerful synthetic analgesics, which include fentanyl and related compounds. Fentanyl is a μ-selective synthetic analgesic which is 50 - 100 times more potent than morphine, has a short duration of action and an onset of action almost immediately after *iv* administration. Fentanyl, sufentanyl, and alfentanyl are used for analgesia in clinical practice despite the side effects such as respiratory depression, physical dependence, and rapid tolerance. Our work constitutes the synthesis of chimeric opiates which contain the N-terminal tetrapeptide (Tyr-D-Ala-Gly-Phe) as the message moiety and a 4-anilinopiperidine moiety as the address portion. The 4-anilinopiperidine part is expected to assist transport and binding of the peptide portion to the opiate receptors. In electrically stimulated preparations of ileum and vas deferens, the inhibitory effect of our previously synthesized opioid agonist, H-Tyr-D-Ala-Gly-Phe-β-Ala-ANPP, showed nanomolar binding affinity [1] and a rapid onset of analgesia, even when low concentrations were used, which suggests that the novel class of opioids merits further development.



Scheme 1. (i) Stepwise chain elongation (n=1, AA_1: H-Tyr-X-Gly-Phe, X: *D*-Ala, *D*-Phe, *D*-Abu); (ii) n=2, AA_2: H-Tyr-*D*-Ala-Gly-Phe-Leu-Arg, SPPS using Fmoc-AA-OH on loaded BocLys(Fmoc) Merrifield resin.

Results

We previously reported that coupling to 4-anilino-N-phenethyl-piperidine (ANPP) can be efficiently mediated by using phthaloyl amino acids in the presence of DIPEA, followed by cleavage with ethanolamine (n = 1) or hydrazine hydrate (n = 2). The designed analogues (Scheme 1) were prepared in good yields by solution phase synthesis using Pht/Boc chemistry and tested for *in vitro* opioid activity. One of the analogues was synthesized on a solid support using Boc-Lys(Fmoc)OH

loaded Merrifield resin. Interestingly, in this case coupling of Leu[5] did not lead to sequence deletion or diketopiperazine formation and the target peptide was obtained in quantative yield.

Table 1. Functional analysis & affinity for opioid receptors.

	Competitive Binding		GTP Binding			
	NG108-10DhDOR	*CHOrMOR*	*NG108-10DhDOR*		*CHOrMOR*	
Drug	[^{3}H] DPDPE	[^{3}H] DAMGO	EC_{50} (nM)	Emax (%)	EC_{50} (nM)	Emax (%)
(1) H-Tyr-*D*-Ala-Gly-Phe-Leu-Gly-ANPP	26.6	13.3	n.d.	n.d.	n.d.	n.d.
(2) H-Tyr-*D*-Abu-Gly-Phe-Leu-Gly-ANPP	2.8	0.43	5.8	22	35	77
(3) H-Tyr-*D*-Phe-Gly-Phe-Leu-Gly-ANPP	11.9	0.09	36.5	49	44.6	43
(4) H-Tyr-*D*-Ala-Gly-Phe-Leu-Arg-Lys(β-Ala-ANPP)-OH	11.5	0.35	n.d.	n.d.	n.d.	n.d.
(5) H-Tyr-*D*-Ala-Gly-Phe-Leu-Arg-OH*	4.79	0.63				
(6) H-Tyr-*D*-Ala-Gly-Phe-β-Ala-ANPP	1.1	0.9	62	141	60	142

* *Data according to reference [2]; n.d. not determined.*

Compounds **1**, **2**, **3** and **4** were evaluated for their binding affinities at the μ, and δ opioid receptors following reported procedures. The results of our biological tests show that substitution of the propionyl moiety of fentanyl with opioid peptide ligands leads to compounds possessing high affinity for opioid receptors.

Discussion

The inherent μ-opioid receptor selectivity of fentanyl and its analogues allowed introduction of μ-selectivity into corresponding opioid peptide analogues. Incorporation of D-Phe in to the opioid peptide sequence (entry **3**), produced an analogue with a very high μ-opioid receptor affinity but low potency in GPI (K_i = 952.0 ± 134.8 nM), and MVD (K_i = 177.0 ± 17.8 nM) bioassays, which suggests that this peptide acts as an antagonist at the μ-opioid receptor. Incorporation of D-Abu (entry **2**) into the novel peptides proved to be more advantageous compared to D-Ala (entry **1**). Attachment of the 4-anilinopiperidine moiety to the side chain of lysine via urea bond preserved the free carboxy terminus of the peptide, and had a positive effect on the potency: compound 4 showed K_i = 8.557 +/- 1.710 nM in MVD compared to K_i = 273 +/- 35 nM of the parent compound, dalargin [3].

Acknowledgments

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FURTHER DEVELOPMENT OF A PRACTICAL PRODUCTION SYSTEM FOR LABELED PEPTIDE ARRAYS FOCUSING ON HIGH THROUGHPUT PROTEIN DETECTION

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Introduction

The novel high throughput protein detection system using designed peptide arrays has been successfully demonstrated using a novel "protein-chip", in which the interaction between proteins and designed peptide arrays are visualized as a "protein fingerprint" generated from fluorescent intensity differences [1, 2]. The deposited peptide solution can be dried with or without covalent immobilization. The resulting arrays are subsequently exposed to protein solution. These chips have distinct storage and delivery advantages over conventional immobilized protein chips [3]. The present paper describes recent developments focusing on practical production systems for such peptide-array chips.

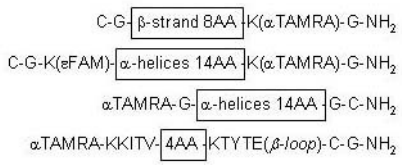

Fig. 1. Architecture of designed labeled peptide libraries. Boxes indicate diversity.

Results

Several hundred peptides incorporating α-helices, β-strands and β-loops, which include Cys residues for immobilization, were prepared. TAMRA and FAM were chosen from practical considerations [1, 4]. We have confirmed that the FRET system using two dyes gives highly sensitive results, and have shown that a single dye gives similar results. Thus the majority of designed peptides were labeled with TAMRA. Fig. 1 indicates the architecture of designed peptide libraries synthesized in the present work.

Requirements for both mechanical and chemical stability necessitated the development of a novel chip material based on amorphous carbon, which has significant advantages over glass or polymer plates, as it shows no self-fluorescence and is easy to manufacture (precise and high throughput processing). Additionally, chip-plates with few nanoliter wells have been prepared using a YVO4-laser. These chips can not only be used for the present purpose but also in other bio-chip cell- or

bead-arrays. The surface has been derivatized through the remote plasma method, using argon plasma with aryl amine or by ammonia irradiation. Conditions have been optimized through characterization by X-ray photoelectron spectroscopy (XPS). Aryl amine graft-polymerization gave better results over ammonia plasma. Although XPS data do not directly correlate with reaction stoichiometry, an ion analysis using an electric conductivity detector has been developed. The results revealed that the loading amounts on the present amino-carbon plate was 4.4 pmol/mm², which is 1.5 - 2 fold higher than commercial slide-glass.

Peptides dissolved in PBS-DMF mixture were deposited on the surface of these chips covalently through 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester or non-covalently using a non-contact microarraying system, Piezorray™ (Perkin Elmer, MA), which delivers 350 picoliter amounts of labeled peptide solution to give ca. 200 μm diameter spot. The resulting arrayed chips were characterized using a modified fluorescent scanner, CRBio IIe (Hitachi Software Engineering, Tokyo). Additionally, fluorescent microscopy in combination with a CCD-camera was also evaluated.

Discussion

Labeled peptides arrayed on the novel chip surface afford a high throughput protein detection and characterization system. The practical manufacturing process of peptide-arrays has been developed both with respect to production economics and throughput. As designed peptide microarrays are more stable than those consisting of proteins/antibodies, they have significant advantages for industrial production.

Acknowledgements

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SH2 DOMAIN DIRECTED EFFECTORS OF SHP-1 PHOSPHATASE ACTIVITY

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Introduction

The tandem SH2 domain (N-SH2, C-SH2) containing protein tyrosine phosphatase SHP-1 regulates signal transduction pathways mediated by a variety of hematopoietic receptors [1]. In the inactive state the N-terminal SH2 domain interacts with the PTP-domain. Catalytic activity of SHP-1 is stimulated through binding of a pY-ligand to the N-SH2 domain [1-3]. Synthetic ligands based on a natural binding site from receptor tyrosine kinase Ros (1) have been shown to bind to SHP-1 N-SH2 with considerably high affinity and thus are interesting tools to modulate SHP-1 activity [4]. We therefore focussed our interest on the determination of the recognition requirements of pY-ligands for binding to the SH2 domains of SHP-1.

Result and Discussion

In order to obtain information about the mechanism of SHP-1 activation we previously investigated the conformational preferences of SHP-1 SH2 ligands based on the consensus sequence class I (Table 1) [4]. The most promising candidates from this study (2, 3) displayed a high binding affinity but partially inhibited SHP-1 activation. These peptides were used as a template for the new compounds 4-9. These peptides contained optimal residues at positions pY+1 and pY+3 that were suggested to significantly influence the process of dissociation of the N-SH2/PTP-complex upon pY-ligand binding [4, 5]. All ligands (4-9) showed an increased binding affinity and also stimulated SHP-1 activity better than the lead compound (1). The sequences related to consensus sequence class II (6, 9) were comparable to Ros pY2267 (1). Thus, for our ligands class I consensus was preferred with respect to binding and SHP-1 activation. In general, the binding affinities of all ligands were higher for the N-SH2 than for the C-SH2 domain [5]. Furthermore, we examined the SH2 domain specificity beyond pY+3 using a combinatorial peptide library (AA(L/I/V/T)NpY(A/T/V)Q(L/I/V)XXX) [6]. We found that the N-SH2 domain prefers both hydrophobic and positively charged amino acids at the positions pY+4 and pY+5. In contrast, a wide variety of amino acids was accepted at pY+6. The incorporation of the preferred amino acids in peptides 11-13 resulted in an increased binding affinity compared to the control (10). Peptides with the highest binding affinity of both studies were chosen for further investigations [5, 6].

The informations that were obtained from ligands 1-13 will help to find scaffolds for SHP-1 inhibitor design targeting SH2 domain-protein interactions.

Sequence (No)	BC_{50} [µM]	K_d [µM] (N-SH2)	K_d [µM] (C-SH2)	Ref
LKpYMQMF (Class I)	230	2.4 ± 0.4	n.d.	3
LYpYANTLI (Class II)	85	0.65 ± 0.05	2.0 ± 0.1	3
EGLMpYMVL (1)	160	1.44 ± 0.45	2.44 ± 0.97	5
EGLc[X(COCH$_2$NH)pYMD]L (2)	n.a.	0.11 ± 0.01	1.01 ± 0.19	5
EGLc[X(COCH$_2$NH)pYME]L (3)	n.a.	0.21 ± 0.07	1.35 ± 0.13	5
EGLMpYFVNle (4)	17	0.09 ± 0.01	n.d.	5
EGLMpYMleVNle (5)	47	0.11 ± 0.15	n.d.	5
EGLMpYMVL (6)	124	0.39 ± 0.07	1.00 ± 0.48	5
EGLc[X(COCH$_2$NH)pYFD]Nle (7)	31	0.06 ± 0.01	6.43 ± 1.16	5
EGLc[X(COCH$_2$NH)pYMleD]Nle (8)	49	0.06 ± 0.01	3.33 ± 1.18	5
EGLc[X(COCH$_2$NH)pYAD]L (9)	123	0.07 ± 0.12	1.41 ± 0.61	5
AALMpYAQLAAA (10)	100	10 ± 1	26 ± 4	5
AALMpYAQLWYG (11)	31	0.28 ± 0.03	18 ± 3	5
AALMpYAQLWAA (12)	n.d.	0.41 ± 0.05	11 ± 4	5
AALMpYAQLMleFF (13)	23	0.17 ± 0.03	9.8 ± 1.3	5

n.d. = not determined; n.a. = not active; linkage between X and D = -COCH$_2$NH-.

Acknowledgment

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STRUCTURAL REQUIREMENTS OF SUBSTRATES FOR THE PTP DOMAIN OF EYA PROTEINS

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Introduction

Eyes absent (Eya) proteins are involved in the regulation of cell differentiation and organ development (e.g. eye, inner ear, muscle, kidney) and known to function as nuclear transcription factors interacting with Six (sine oculis) and Dach (dachshund) proteins [1]. Recently, it has been reported that the C-terminal domain of Eya proteins has a phosphatase like activity with a specificity for phosphotyrosine (pY). In contrast to classical protein tyrosine phosphatases, Eya proteins have been suggested to employ an aspartate residue as the nucleophile in a metal-dependent reaction [1, 2]. Since the C-terminal domains in animal and plant Eya proteins share high sequence homology we used the Eya homologue of *Arabidopsis thaliana* (AtEya) to investigate the structural requirements of substrates for Eya proteins. We prepared a combinatorial peptide library (AAXXXXPmpXXXX-linker-Tentagel) containing phosphono-methyl-phenyl-alanine (Pmp) as pY-mimetic to screen for binding to the Eya domain. Secondly, a peptide (**1**) derived from Src Y^{419} [1, 2] was chosen as a template for truncation and replacement studies. The contribution of individual residues on both sides of pY to binding and catalysis was assessed by kinetic analysis using a spectrophotometric assay.

Results and Discussion

Using the peptide library approach [4] we found that sequences containing positively charged (H, R, K) and/or large hydrophobic (F, Y) residues N-terminal to pY were preferably recognized by AtEya. The selectivity at the C-terminal positions was different in that the preferred amino acid at +1 and +2 was H, and at +3 a slight preference for D, Q, H, F, and Y was found. At position pY+4 hydrophobic residues (V/I/L/Nle/F/Y) were detected. To evaluate the peptides from the library screening and the Src Y^{419} analogues as AtEya substrates we determined the kinetic constants (Table 1). The substrate specificity constants (k_{cat}/K_M) for the phosphopeptide conversions did not vary extremely for comparable peptide length. In comparison to pNPP, with only few exceptions the K_M values slightly decreased for the peptide conversions, while k_{cat} values strongly depended on the respective substrate constitution. Obviously, AtEya displays a differential recognition of pY residues in a distinct primary structure context. This can be exemplified by comparing lead peptide **1** with library-derived peptides **17-19**. Further studies will reveal more details about the substrate binding, specificity and mechanism of action. Due to the fact that physiological substrates of Eya proteins await

identification, our investigations are useful to support the search and characterization of proteins that are interactions partners and/or substrates of Eya proteins.

Sequence (No)	K_M (mM)	k_{cat} (s^{-1})	10^{-6} x k_{cat}/K_M (M^{-1} s^{-1})
pNPP	2.65 ± 0.29	6676 ± 260	2.52
pY	2.61 ± 0.50	6693 ± 509	2.56
EDAEpYAARG-NH_2 (**1**)	2.01 ± 0.10	17875 ± 915	8.89
ADAEpYAARG-NH_2 (**2**)	1.99 ± 0.06	10143 ± 311	5.10
EAAEpYAARG-NH_2 (**3**)	1.95 ± 0.05	10067 ± 272	5.16
EDAApYAARG-NH_2 (**4**)	2.05± 0.06	4995 ± 156	2.44
EDAEpYAAAG-NH_2 (**5**)	1.89 ± 0.19	11057 ± 1095	5.85
EDAEpYAARA-NH_2 (**6**)	1.85 ± 0.06	11241 ± 368	6.08
DAEpYAARG-NH_2 (**7**)	2.69 ± 0.10	11020 ± 356	4.10
AEpYAARG-NH_2 (**8**)	2.91 ± 0.28	15141 ± 1435	5.20
EpYAARG-NH_2 (**9**)	2.92 ± 0.37	7578 ± 971	2.59
pYAARG-NH_2 (**10**)	2.31 ± 0.08	2296 ± 79	0.99
EDAEpYAAR-NH_2 (**11**)	2.06 ± 0.08	10443 ± 401	5.07
EDAEpYAA-NH_2 (**12**)	2.07 ± 0.09	13838 ± 621	6.68
EDAEpYA-NH_2 (**13**)	1.89 ± 0.11	11169 ± 652	5.91
EDAEpY-NH_2 (**14**)	1.78 ± 0.06	11657 ± 423	6.55
EDAEpSAARG-NH_2 (**15**)	n.d.	n.d.	n.d.
EDAEpTAARG-NH_2 (**16**)	n.d.	n.d.	n.d.
ARRApYVAAA-NH_2 (**17**)	1.37 ± 0.08	3602 ± 219	2.63
AAKRpYIRRA-NH_2 (**18**)	1.18 ± 0.06	4083 ± 220	3.46
HHRKpYHHFV-NH_2 (**19**)	1.19 ± 0.02	4986 ± 87	4.19

pNPP, *p*-nitrophenylphosphate; pY, phosphotyrosine; n.d., not dephosphorylated.

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NOVEL APPROACHES TO STUDY DRUG DELIVERY TO THE BRAIN

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Neurodegenerative diseases such as Parkinson and Alzheimer disease, schizophrenia, epilepsy, brain tumors and HIV are pharmaceutical targets located inside the brain. In many cases there are promising compounds for their treatment, however owing to their blood-brain barrier (BBB) transport problems > 98% of these potential drugs do not go to drug development stage.

The BBB is a natural defense mechanism designed to keep harmful substances out of the brain. The anatomical bases of the BBB are the tight junctions at the endothelial cells of the brain capillaries.

The prediction of a peptide's ability to cross the BBB is not a simple task; a rational study of the relevant factors that affect the movement across this physiological barrier is needed.
Different transport mechanisms occur at the BBB, this work has focused on the passive diffusion mechanism. To evaluate this mechanism we chose the PAMPA assay.

Parallel Artificial Membrane Permeability Assay (PAMPA) originally introduced by Kansy uses an artificial membrane in the form of filter supported phospholipids bilayers. The phospholipid membrane mimics the cell membrane, but has no means for active or paracellular transport. It is a very convenient tool to evaluate the transport of compounds by passive diffusion. This technique allows the evaluation of pure compounds, mixtures and even plant extracts.
Here we present two new approaches to study drug delivery to the brain:
Firstly, the evaluation of compound mixtures on PAMPA assay. This approach increases the throughput of the study and structure–activity relationships governing the passage across the BBB can be easily establish.

A first library consisting on 15 diketopiperazines (DKP: *N*-MePhe-X were: X: PyrAla, Gly, Pro, Tyr, PheGly, Phe, HomoPhe, Tic, Pro(Phe), NMeTrp restricted, Cha, 2Nal, Oct, 1PyrenylAla, NMePhe) was prepared on solid-phase.
After the synthesis and characterization, a mixture of the 15 DKPs was prepared and evaluated by PAMPA assay.

A set of six side-chains showing good transport properties (%Transport4h between 20 - 40%) were chosen (X: Phe, HomoPhe, Cha, 2Nal, Oct, 1PyrenylAla) and the corresponding di-*N*-methylated diketopiperazines (DKP: *N*-MePhe-*N*-MeX) were prepared in solid-phase. The evaluation of this second library of DKPs help us to elucidate the effect of the methyl groups on the passive diffusion.

We have also evaluated the two libraries of DKPs using a chromatographic technique, IAMC (immobilized artificial membrane chromatography) developed by Pidgeon. This technique uses phospholipid molecules covalently immobilized to silica particles at high density as the stationary phase. It has been used to predict transport across biological barriers, it exhibits a good correlation with *in vitro* cell based assays and it is very convenient in terms of high throughput. IAMC

interactions include ionic, lipophilic and hydrogen bonding interactions. Our PAMPA and IAMC results show a nice correlation.

The second approach presents the use of DKPs as BBB-shuttles. The best DKPs, which have the ability to cross the BBB, have been modified in order to attach drugs or other substances, referred as cargoes, which can not cross the BBB by themselves. As an example of cargo, Baicalin, a flavanoid isolated from the traditional Chinese medicinal plant *Scutellaria Baicalensis Georgi*, was chosen.

It is known that the brain might serve as an occult reservoir for HIV viral replication because of the failure of anti-retroviral drugs to cross the BBB. Baicalin shows interesting HIV replication inhibition properties but it can not cross the BBB. The library of DKPs with the baicalin attached (DKP Phe (*p*-NH-CO-Baicalin)-N-MeX, X: Phe, HomoPhe, Cha, 2Nal, Oct, 1PyrenylAla) was also prepared and evaluated in the PAMPA assay, showing positive transport for these BBB-shuttles, being the DKP Phe (*p*-NH-CO-Baicalin)-N-MeX were: X = 2Nal the most promising one (%Transport4h higher than 7%).
These two new approaches allow assaying compound's permeability in the early stages of a drug development project, and then designing novel analogues with improved BBB transport properties or using blood-brain-barrier shuttles for their delivery.

PAMPA assay has show to be a useful tool to evaluate compounds mixture and even plant extracts. Synthetical methodology for the synthesis of mono and di-N-methylated DKPs and DKP-baicalin constructs has been established. The DKPs have shown to be promising compounds as BBB-shuttle for cargoes not able to cross the BBB by themselves. Some rules that govern the passage of compound through the BBB by passive diffusion can be established and could be used to design new compounds: Incorporation of *N*-methyl groups and the presence of aromatic or aliphatic side chains in the DKP scaffold represents an improvement in transport, being careful to don't achieve a completely insoluble and retained in the membrane molecule. In the other hand, the presence of aromatic nitrogens or hydroxilic groups is very deleterious for the passive diffusion.

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COVALENT CAPTURE OF PEPTIDES WITHOUT N-TERMINAL CYS OR THR

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Introduction

Covalent capture purification involves immobilization of N-terminal Cys- or Thr-polypeptides on an aldehyde support, followed by washing and finally release (Fig. 1) [1]. This process was successfully applied to the purification of chemically synthesized polypeptides and larger ones produced by recombinant DNA means. Recently it has been shown with 2-mercapto-4,5-dimethoxybenzyl (Dmmb) and N^{α}-(1-phenyl-2-mercaptoethyl) that this technique is suited for the purification of polypeptides used in native and extended chemical ligation [2].

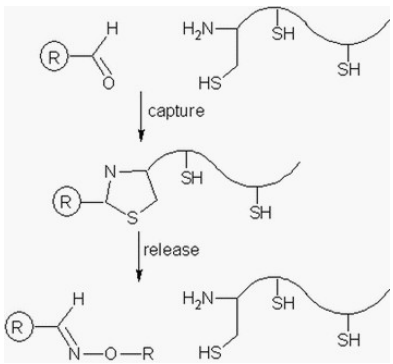

Fig. 1. Covalent capture

Here we describe new linkers which make it possible to use covalent capture for purification of peptides having Gly at the N terminus. The linkers have an N-terminal cysteine as capture functionality and a spacer group. The group is linked to the $^{\alpha}N$ of the polypeptide chain via an O-N bond which may be chemoselectively cleaved by Zn after purification [3] has been achieved by covalent capture.

Results and Discussion

For the formation of -O-NH-CH_2-CO- moiety, reaction between –O-NH_2 and Br-CH_2-CO- funcionalities was exploited [3] (Fig. 2). Based on the literature *tert*-butyl(3-aminopropoxy)carbamate (**I**) [4] was synthesized and acylated by Fmoc-Cys(Meb)-OSu. After removing the Boc group, bromoacetylated peptide-resin was reacted with the linker using different conditions, but no satisfactory yield was achieved.

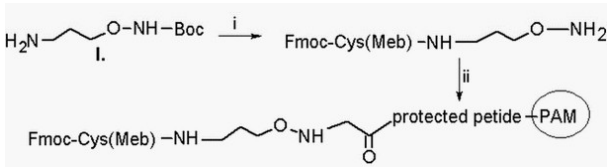

Fig. 2. i.) Fmoc-Cys(Meb)-OSu,TFA; ii.) Br-CH_2-CO-protected peptide on resin

To improve the synthesis oxime formation between –O-NH_2 compound and glyoxylic acid followed by reduction was attempted to form the -O-NH-CH_2- moiety (Fig. 3). In this case each reaction went to completion.

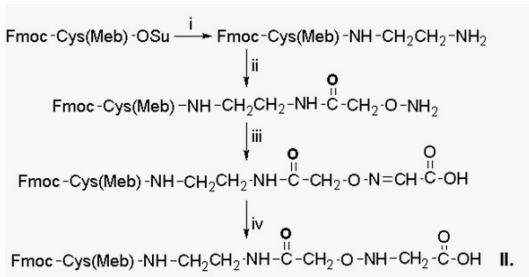

Fig. 3. i:) Boc-NH-CH_2CH_2-NH_2, TFA; ii.) Boc-NH_2-O-CH_2-CO-OSu, TFA; iii.) HCO-COOH; iv.) $NaBH_3CN$

The glycine derivative (**II**) was introduced by standard coupling methods to a protected peptide on Pam resin.

In both case the protecting groups were cleaved and the peptides were removed at the same time from the resin by liquid HF. Both synthetic procedures resulted in product which were applied for covalent capture. After Zn reduction in glacial acetic acid both of them gave the desired peptide in excellent yield without any side-reaction.

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CONFORMATIONAL STUDIES OF SELECTED BENZYLSULPHONYLACROYL PEPTIDES – PUTATIVE INHIBITORS OF CATHEPSIN B

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Introduction

Our work is focused on the search of a dominate conformation in solution for selective peptidyl inhibitors of cathepsin B acylated with the (*E*)-3-(benzylsulphonyl)acroyl group (Bsa). The double bond, embedded in the Bsa moiety is activated by two electron-withdrawing groups and may be a good target for the Michael-type addition of the catalytically active -SH group. Previously, we found two peptidyl derivatives of the structures: Bsa-Phe-Asn(EA-Bz)-OH (**1**) (where EA-Bz is $-CH_2-CH_2-NH-CO-C_6H_5$) and Bsa-Ile-Pro-OH (**2**) which showed high inhibitory activity and selectivity towards cathepsin B (K_i = 14.2 nM and 2.9 nM for **1** and **2**, respectively). The peptide derivative Dabcyl-Arg-Leu-Val-Gly-Phe-Asp(Edans)-OH, was also found as the selective and potent substrate for cathepsin B. The dipeptide H-Phe-Asp(Edans)-OH, released during its proteolysis, was taken as our lead compound to design the inhibitor **1**. The peptidyl part in **2** was taken from the inhibitor of cathepsin B, known as CA074 [1]. It should be noted that all our peptidyl derivatives with Bsa moiety show a competitive and reversible mode of an inhibition towards enzymes from the papain-like family.

Results

Using 1D and 2D ^{1}H-NMR (TOCSY, COSY, ROESY) spectroscopy along with theoretical calculations we determined the conformational properties of the inhibitors **1** and **2**. The NMR spectra were measured in DMSO-d_6 at following temperatures: 22, 25, 30, 35 and 40 ºC. MD simulations were carried out using AMBER force field at 303 K in a periodic box of the constant volume. Calculations were started from extended conformations in DMSO. In the structure calculations, we used time-averaged distance restraints derived from the NMR spectroscopy.

Discussion

Analyzing the NMR spectra of **1** we have seen only one set of proton resonances with all peptide bonds in *trans* configuration. In opposite, the NMR spectra of **2** due to the *cis*/*trans* isomerization displayed two distinct sets of proton resonances. Furthermore, five sets of proton resonances of HN Ile^2 were observed in the fingerprint region of the NMR (TOCSY). Two of them corresponded to *trans* and *cis* isomers, whilst remaining suggested flexibility of the side chain of Ile^2, despite

the fact that it is located in the middle part of molecule. The $^{3}J_{H\alpha\text{-}C=C\text{-}H\beta}$ coupling constant in Bsa residue (15.1 Hz) confirmed the existence of the *trans* isomer only. Therefore, the multiplicity of Ile^{2} resonances could not be caused by the presence of *cis* and *trans* isomers of Bsa residue. The exchange cross peak *trans*-H^{α}(Pro)-*cis*-H^{α}(Pro) suggests the *cis*/*trans* isomerization on Ile^{2}-Pro^{3} peptide bond. Moreover, the Hα(Ile^{2})-Hδ2(Pro^{3}) and Hα(Ile^{2})-Hδ1(Pro^{3}) ROE effects indicate the presence of both *trans* and *cis* isomers, respectively. Based on the integration of nonoverlapping signals, namely Hα of Pro, we have determined a ratio of *cis*/*trans* isomers at 303 K to be 7:93.

The temperature coefficients of all amide protons were in the range $3.5 \leq -\Delta\delta/\Delta T \leq 5.4$ ppm/K. That points at weak intermolecular hydrogen bonds.

A superposition of the structures of both molecules obtained from 3.2 to 4.0 ns of MD simulations with time-averaged distance restraints is shown in Fig. 1a-b. We have observed that after adding the time-averaged distance restraints 2 maintained its conformation for the whole MD simulations, whilst the conformation of **1** changed significantly during the experiment time.

Fig. 1c shows the comparison of the averaged structures of both peptides. They displayed high similarity of the structure along the backbone, but the location of their N-termini as well as side chains was noticeable different. The differences in the C-terminal fragment of the molecules resulted from the presence of the Pro residue at the position 3 of **2**, as it effectively fixes the backbone dihedral φ angle at the -60^{o}. This limited the possible conformations of C-terminal part of **2** contrary to the Bz-EA residue in **1**. No intermolecular hydrogen bonds have been found in any of the calculated structures. That may suggest an expanded conformation.

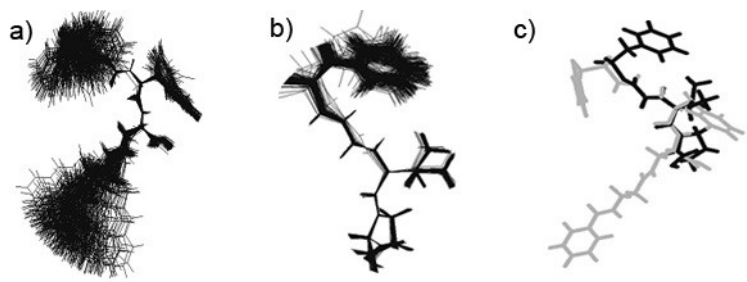

*Fig. 1. Superposition of: conformations of (a) **1** and (b) **2** during MD, (c) averaged structures of **1** and **2**.*

Acknowledgements

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IN SEARCH OF SYNTHETIC LOW-MOLECULAR PEPTIDE DERIVATIVES WITH ACTIVITY AGAINST FUNGAL PATHOGENS

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Introduction

Antimicrobial peptides are considered one of the most innovative class of anti-infective agents that have been discovered over the last two decades and therefore, a source of inspiration for novel drug design. A lot of them possess a form of β-motif within the backbone which frequently is crucial for high activity against particular species. Earlier, we have found a series of low-molecular peptide derivatives of the general structure: X-Arg-Leu-NH-CH(R)-CH_2-NH-Y (where X and Y were acyl groups with aromatic carbocyclic system) displaying high antimicrobial activities against several clinically important Gram-positive pathogenic bacteria, viruses and fungi [1, 2]. Our preliminary studies indicated that all of them prefer to exist in one stable conformation. This dominating form includes a β-turn which is located within a diamine residue in the position 3 and is stabilized by the strong hydrogen bond between C=O group from Leu residue and 2-NH group of the diamine. In addition, the phenyl ring from Y residue and the guanidyl moiety of Arg are located very close one to each other in the most potent compounds. The compound 1, of the structure Z-Arg-Leu-ValΨ[CH_2-NH]-NH-CO-CH=CH-C_6H_5, was found as a highly active agent with the widest antimicrobial spectrum.

Results

In our studies **1** was chosen as a lead compound. We have synthesized and investigated for the antifungal activity its thirteen analogues (**2** – **14**) of the general structure: Z-AA_1-AA_2-AA_3Ψ[CH_2-NH]-NH-X, where X was cinnamoyl (Cin) or 3-benzylacroyl (Bac). The particular structural motifs which allow forming the desirable intramolecular β-turn have been built-in to the backbone of all the investigated compounds. *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231) were chosen as the tested fungal pathogens in the antimicrobial assays.

Discussion

We have found that substitution of a diamine residue derived from Val in **1** with a vicinal diamine derived both from more bulky Leu (compound **12**) and smaller Gly (compound **13**), did not influence the antifungal activity significantly. The compound **11**, possessing the reversed direction of diamine acylation is less active

against *C. albicans* (MIC 256 mg/ml) than **1** (MIC 64 mg/ml). The analogues **6**, **7**, **8** and **9** have been designed to enhance aqueous solubility. In comparison with **1** they possess more polar side chains (Ser and Thr) or are characterized by lower hydrophobicity (Gly and Sar) than Leu residue in the position 2. Unfortunately, these modifications resulted in drastic decrease of the activity against all the tested fungal pathogens. The compounds **4**, **7** and **9** have the position 2 occupied by selected amino acids which are known to create *cis*-peptide bond with their acylating residue (Pro in **4**, Gly in **7** and Sar in **9**). There is a high possibility that such modification can diametrically change the conformation of the backbone, especially its N-terminus. Considerable decrease of the antifungal activity of 4 against both *C. albicans* and *A. niger* (MIC 512 mg/ml for both species), **7** (MIC 512 mg/ml for both species) and **9** (MIC 1024 mg/ml for *C. albicans* and 512 mg/ml for *A. niger*) in comparison with the the activity of the lead compound **1** can be an argument certifying our assumption on the significance of the phenyl ring and the guanidyl moiety interlocation for preserving the activity. The series of analogues of **1**, including the compounds **2**, **3** and **10**, was designed as antifungals with an increased resistance to a proteolytic degradation. Unfortunately, all of these D-amino acid-containing derivatives are characterized by much worse inhibition ability towards both *C. albicans* and *A. niger* growth than **1**. Especially, the inversion of the configuration on the conserved Arg residue (comp. **2**) leads to the undesirable antifungal effects (MIC 1024 mg/ml for both species). We have also synthesized two analogues, **5** and **14**, possessing positions 2 and 4 substituted by more bulky but with similar hydrophobicity residues than those ones in **1**. The presence of Bac residue on the C-terminus of **14** is valuable for preserving high antifungal activity (MIC 64 mg/ml for *C. albicans* and MIC 128 mg/ml for A. niger for both **1** and **14**), whereas substitution of Leu with Phe leads to the increase of the MIC values to 256 mg/ml for *C. albicans* and up to 1024 mg/ml for *A. niger*.

Acknowledgements

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HEXAFLUOROACETONE AS A VERSATILE PROTECTING/ACTIVATING REAGENT FOR BETA-HYDROXY ACIDS, SERINE AND THREONINE

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Introduction

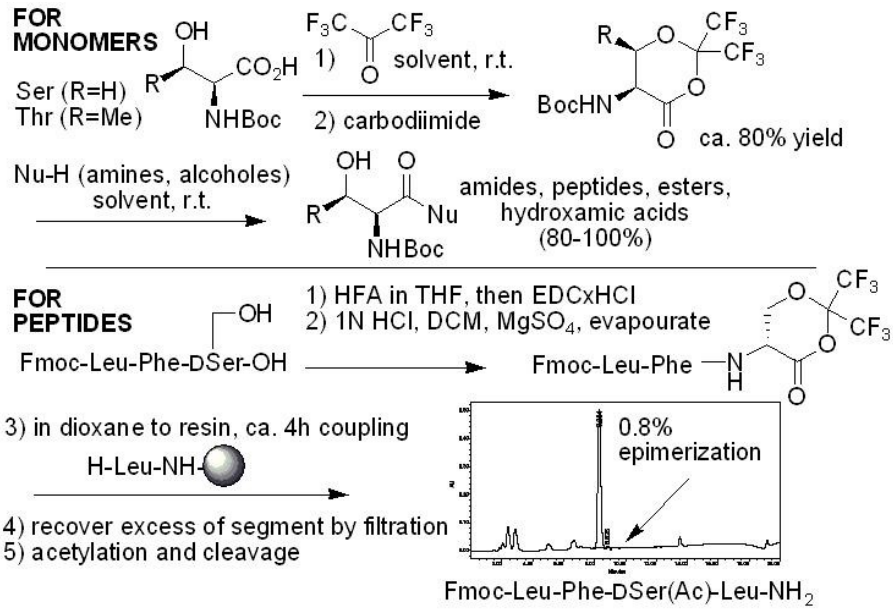
In convergent solid-phase peptide synthesis (CSPPS), peptide segments prior synthesized on solid support are coupled to solid-phase bound peptides [1]. However, this technique has several specific complications. Firstly, because of the risk of epimerization at the C-terminus via the oxazolone mechanism, peptide segments are often chosen so as to have either Gly, Pro or pseudoprolines [2] at this position. Secondly, it is often difficult to achieve acceptably high concentrations of peptide segment in the coupling medium. This tends to lower the yield of the coupling reaction making it necessary to repeat it. Thirdly, an excess of peptide segment whose synthesis may have required considerable investment in time and effort is required in order to drive the coupling to completion. This excess is normally not recoverable. Here we present a new strategy that addresses these limitations.

Results and Discussion

Hexafluoroacetone (HFA) undergoes with α-hydroxy acids a cyclocondensation to give five-membered lactones in one step. These compounds represent 1-carboxy-activated/α-hydroxy-protected α-hydroxy acids. They readily react with N and O-nucleophiles to the corresponding carboxy-derivatives [3]. We found that this protecting/activating strategy can also be applied to β-hydroxy carboxylic acids. The formation of six-membered lactones proceeds in two steps via formation of the ketal followed by intramolecular ring closure mediated by carbodiimides to give 2,2-bis(trifluoromethyl)-1,3-dioxan-4-ones in good yields. Like their five-membered counterparts, these six-membered lactones react with amines and alcoholes at room temperature to give the corresponding amides and esters, as demonstrated for the Ser and Thr derivatives. No racemization and no acylation of the concomitantly deprotected β-OH function are observed.

Also peptide segments with C-terminal Ser or Thr residues can be activated with HFA and coupled to solid-phase bound peptide residues. Epimerization can widely (to less than 1%) surpressed performing the activation step in THF as solvent. The activated segments are well soluble in organic solvents due to the lipophilic character of the trifluormethyl groups. In comparison with the pseudoproline-technique [2] or the "*O*-acyl isopeptide method" [4], which proceed epimerization-

free for segments bearing Ser and Thr as C-terminus, the HFA-protocol offers the unique option of easily recovering of unreacted excess by filtration after complete coupling. The HFA-activated segments are sufficiently stable if stored at low temperatures and can be used for subsequent couplings to give products of the same purity. Further results will be published elsewere.



Acknowledgements

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NOVEL HETEROCYCLIC AMINO ACIDS - POST-ASSEMBLY ON-RESIN MODIFICATION OF PEPTIDES

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Introduction

The application of novel nonproteinaceous heterocyclic amino acids to peptide synthesis results in new compounds with interesting structural, physicochemical and biological properties. The modified structures offer much more possibilities in terms of conformation, stability and activity, however their design, synthesis and analysis require more attention.

Our research is focused on nitrogen containing heterocycles: imidazoles, benzimidazoles and quinoxalines because of their well known biological activity and complexing abilities. Recent reports describe DNA affinity of 2,3-bis(2-pyridyl)quinoxaline (DPQ) complexes with transition metals [1], cyclophilin A inhibition by quinoxaline derivative [2], antihistamine action and RNA binding of benzimidazoles [3], antiinflammatory properties and MAP kinase inhibition by imidazoles [4].

Therefore we investigate the synthesis of novel heterocyclic amino acids through formation of „privileged structures" after the classical peptide synthesis on solid support. The transformation of amino acid functional groups after on-resin peptide assembly is a convenient method of generating modified peptides as new potential biological agents. At the same time the method utilizes the full scope of solid phase organic synthesis and combinatorial chemistry.

Results

We investigated the formation of imidazole, benzimidazole and quinoxaline moieties through condensation with various aldehydes and α-dicarbonyl compounds. The imidazole synthesis utilizes the N-terminal or side chain amino group of amino acids, whereas a derivate of phenylalanine, β-(4-amino-3-nitrophenyl)-alanine, was developed for benzimidazole and quinoxaline synthesis [5,6]. The structure of diketopiperazine formed from D-Phe and L-Dpqa (2,3-di(pyridine-2-yl)quinoxalin-6-yl-alanine), was examined thoroughly by NMR to confirm the optical purity of quinoxaline derivative. The chemical shift of H^{α} proton of L-Dpqa was 3.47 ppm whereas in diasteromeric diketopiperazine cyclo(L-Dpqa-L-Phe) the shift was 4.16 ppm. In our case the imidazole formation on Wang resin according to the method of Sarshar [7] gave poor yields, which prompted us to use more acid-stable Merrifield support.

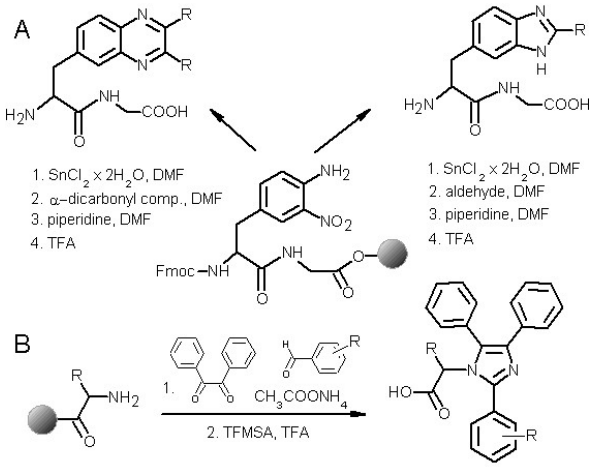

Fig. 1. On-resin synthesis of (A) quinoxaline, benzimidazole and (B) quinoxaline scaffolds.

Discussion

We have developed a set of efficient and straightforward methods of heterocyclic moieties formation in peptides attached to solid support. On-resin modification of peptides allows generation of combinatorial libraries due to broad range of commercially available aldehydes and α-dicarbonyl compounds. The advantage of solid support condensation step is the simplification of by-product removal, especially important in imidazole synthesis. The side chain based condensation occurs outside the chiral centres, therefore reducing the risk of racemization. In case of N-terminal imidazole formation, two HPLC-separable forms of modified peptides were observed. The optical purity of new phenylalanine analogue, Fmoc-β-(4-amino-3-nitrophenyl)-alanine was confirmed by X-ray [6] and diketopiperazine analysis.

Acknowledgements

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HOMOARGININE-CONTAINING OPIOID PEPTIDE ANALOGUES

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Introduction

A survey of opioid peptide precursors reveals that pairs of basic amino acids are primary sites of proteolytic cleavage. However, the rate of cleavage at each pair is different and several resulting biologically active peptides still contain one or more pairs of basic amino acids within the sequence.

These peptides could be precursors of smaller biologically active peptides or could be transformed into inactive peptides. For this reason, it is difficult to attribute the observed biological effects to the intrinsic activity of the examined peptide or to its enzymatic degradation products in *in vivo* studies.

Moreover, peptides containing pairs of basic amino acids are poor candidates for medical applications because of their increased susceptibility to enzymatic cleavage. We recently obtained several peptides resistant to trypsin-like enzymes by substituting homoarginine (Har) for Arg and Lys [1]. The aim of the present work was to elaborate a convenient method for the synthesis of selected opioid peptide analogues in which Arg was replaced by Har.

Results and Discussion

Two novel Har derivatives, Boc-Har{ω,ώ-[Z(2Br)]$_2$}-OH and Boc-Har{ω,ώ-[Z(2Cl)]$_2$}-OH, were prepared by guanidinylation of Boc-Lys-OH using *N*,*N*'-[Z(2Br)]$_2$-*S*-methylisothiourea and *N*,*N*'-[Z(2Cl)]$_2$-*S*-methylisothiourea [2], respectively. These derivatives were used for the synthesis of homoarginine-containing peptides.

For comparison, native peptides were synthesized using previously described procedures.

Examination of the products by HPLC and ESI-MS revealed that the purity of the crude materials obtained with the use of the new derivatives was higher than that obtained with the use of Boc-Arg(Tos)-OH.

Table 1. Opioid peptides.

Peptide	Sequence
dynorphin A(1-13)	H-TyrGlyGlyPheLeuArgArgIleArgProLysLeuLys-OH
[Har^{6,7,9}]dynorphin A(1-13)	H-TyrGlyGlyPheLeuHarHarIleHarProLysLeuLys-OH
α-neoendorphin	H-TyrGlyGlyPheLeuArgLysTyrProLys-OH
[Har^{6}]α-neoendorphin	H-TyrGlyGlyPheLeuHarLysTyrProLys-OH
[Har^{6}]β-neoendorphin	H-TyrGlyGlyPheLeuHarLysTyrPro-OH
[Har^{6,7}]β-neoendorphin	H-TyrGlyGlyPheLeuHarHarTyrPro-OH
[Har^{6,7,9,19,23,26}]dynorphin-32	H-TyrGlyGlyPheLeuHarHarIleHarProLysLeuLys-TrpAspAsnGlnLysHarTyrGlyGlyPheLeuHarHar-GlnPheLysValValThr-OH

The peptides were tested in the guinea-pig ileum (GPI) and mouse vas defrens (MVD) assays.

Table 2. GPI and MVD assays of opioid peptide.

	GPI		MVD		MVD/GPI
Coumpound	IC$_{50}$[nM][a]	rel. potency	IC$_{50}$[nM][a]	rel. potency	IC$_{50}$ratio
dynorphin A(1-13)	0.535 ± 0.062	460 ± 53	7.45 ± 0.39	1.53 ± 0.008	13.9
[Har^{6,7,9}]dynorphin A(1-13)	1.17 ± 0.23	210 ± 41	16.4 ± 1.1	0.695 ± 0.047	14.0
α-nependorphin	11.6 ± 0.8	21.2 ± 1.5	31.7 ± 1.6	0.360 ± 0.018	0.366
[Har^{6}]α-neoendorphin	20.3 ± 1.2	12.1 ± 0.7	35.4 ± 1.3	0.322 ± 0.012	0.573
[Har^{6}]β-neoendorphin	244 ± 22	1.01± 0.09	79.2 ± 11.0	0.144 ± 0.020	0.32
[Har^{6,7}]β-neoendorphin	145 ± 6	1.70 ± 0.07	75.5 ± 9.5	0.151 ± 0019	0.52
[Har^{6,7,9,19,23,26}]dynorphin-32	6.36 ± 0.53	38.7 ± 3.2	146 ± 26	0.078 ± 0.014	23.0
[Leu^{5}]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a]Mean of 3-6 determinations ± SEM

The modified peptides were fairly potent in these assays. The activities were similar to those of the corresponding native peptides. It should be noted that the analogue of dynorphin-32 containing six Har residues is also an active compound. These results indicate that substitution of Har residues for Lys or Arg is of interest for the design of biologically active peptides with increased resistance to degradation by trypsin-like enzymes.

Acknowledgements

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A NOVEL PEPTIDE VECTOR FOR EFFICIENT INTRACELLULAR DELIVERY OF PROTEINS

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Introduction

The use of protein- and peptide-based drugs is highly limited by their low uptake by cells. Cell penetrating peptides (CPP) such as HIV-1 Tat, *Drosophila Antennapedia*, HSV VP22 and others are used to resolve this problem [1 - 3]. However the need of covalent binding of the peptide vector to a cargo molecule restricts the use of these systems. Recently another peptide Pep1 that is able to form noncovalent complexes with proteins and to transfer them through the membrane was described [4].

Here we report a new peptide vector for the intracellular delivery of foreign proteins (P10C) based on the sequence of the well-known antibiotic peptide gramicidin A. Some studies on the model planar lipid bilayer and liposomal membranes were performed to investigate peptide-membrane interactions.

Results

Hydrophobic membrane active sequence of gramicidin A was modified by the lysine-rich motif on the C-terminal to improve intracellular uptake and solubility in aqueous solutions. The N-terminal formyl-group was substituted by the acetyl one to reduce the ion-channel forming activity.

The uptake of the P10C/protein complexes was observed on the model protein β-Gal. Varying peptide/β-Gal molar ratio from 1 to 1000 the optimal P10C concentration was determined and it was 10 times less than those of Pep1.

On different mammalian cell lines (HeLa, NIH 3T3, COS-1, 293, CHO, L-929, Jurkat, Sp2/0) it was shown that the transduction activity of P10C doesn't depend on the cell type.

Cytotoxic concentration of P10C was estimated by means of MTT test and the lack of toxicity was observed at concentrations up to 5.5 μM, while the optimal working concentration of peptide lied in the range of 0.3-3 μM. It is interesting to note that the formylated analogue of P10C produced the cyctotoxic effect at the concentration of 0.2 μM.

We then examined the protein-peptide complexes uptake at 4 ^{o}C and demonstrated that highly reduced but definite protein internalization occurred even at low temperature.

Studies on the intracellular localization of P10C by itself were performed in live cells by means of confocal fluorescent microscopy. Acquired images demonstrated that the peptide was associated with the cell membrane. The electrical current measurement on the planar lipid bilayer revealed the ability of P10C to form the ion-conducting channels with parameters close to those of gramicidin. Addition of H_2O_2 to the buffer solution led to the appearance of the channels characterized by the longer life-time. P10C induced the carboxyfluorescein efflux from liposomes composed of diphytonoylphosphatidylcholine. At the same time liposomes remained intact. Earlier the same effect was demonstrated on the similar amphipathic analogue of gramicidin [5]. It was shown also that neither gramicidin nor polycationic motif by themselves induced the carboxyfluorescein leakage.

Discussion

A new amphipathic analogue of gramicidin A (P10C) was shown to be able to deliver efficiently foreign proteins in different cell lines. On the model protein β-Gal we demonstrated that it was active at lower concentrations than Pep1. No toxicity was observed in the range of working concentrations of P10C whereas the formylated analogue was much more toxic due to ion-channel forming activity. Protein-peptide complexes internalization at low temperature denoted the participation of endocytosis independent mechanisms in cellular uptake process. At the same time peptide by itself was associated with the cell membrane. So to elucidate the protein penetration through the cellular membrane some experiments on the model bilayers were performed. Current measurement on the planar lipid membrane revealed the ability of P10C to open gramicidin-like ion channels, whereas in oxidizing conditions channels with the longer life time were observed that was the result of the disulfide bounds formation between two peptide molecules being close by each other within the monolayer. Carboxyfluorescein efflux from liposomes indicates on the formation of large transmembrane pores apparently composed of few molecules of P10C. Based on all these data we proposed that the same mechanism of pore-formation can be responsible for protein internalization also.

Acknowledgements

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SDF GAMMA: SYNTHESIS AND HEPARIN BIDING SITES IDENTIFICATION

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Chemokines are small proteins which promote directional migration of leukocytes through binding to specific G-protein coupled receptors. Increasing evidences proved the involvement of these proteins in pathogenesis of immune and inflammatory processes, morphogenesis and tissue repair or tumour development. Chemokines also bind to heparan sulphate (HS), a complex polysaccharide found on most cell surface and within extracellular matrix. Binding to HS is thought to be functionally important.

The specific aim of this work is to identify the Heparin Binding Sites (HBS) of the CXCL12/SDF 1 (Stromal cell Derived Factor 1) chemokine isoform γ as previously done for the α isoform [1]. The new findings will implement and complete our knowledge on the role played by HS in SDF functions.

SDF γ sequence (98 AAs) consists of SDF1α sequence (68 AAs) extended by a 30 AAs Cter peptide:

KPVSLSYRCPCRFFESHIARANVK^{24}HLK^{27}ILNTPNCALQIVARLKNNNRQVC IDPKLKWIQEYLEKALNK^{68}GRREEKVGK77K^{78}EK^{80}IGK^{83}K^{84}KR^{86}QK^{88}K^{89}R K^{91}AAQKRKN^{98}.

The *wt* chemokine was synthesised and 10 mg of pure folded protein were obtained (yield 4%). The correct folding of the synthetic chemokine was confirmed by chemotaxis experiments on Jurkat cells (50% transwell migration at 10 nM concentration). SDF γ heparin "affinity" was then evaluated on a HiTrap Heparin column. As anticipated from the AAs sequence, the Heparin column results confirm the presence of additional Heparin Binding Sites in SDF γ as compared to SDF α (1.01 M NaCl *versus* 0.59).

In order to determine those additional HBS, we postulated that the Cter peptide could be study as an « independent » module. HBS usually (but not only) consist in BBXB motives (B for basic AAs). Looking to the Cter sequence, four putative HBS were identified and three Cter peptides were synthesised, where basic residues were mutated to Ser.

	G R R E E K V G K K E K I G K K K R Q K K R K A A Q K R K N
Site1	B B X B
Site2	B B X B
Site3	B B X B
Site4	B B X B

Heparin column results shown that combined mutations of sites 1+2+4 drastically reduce the heparin « affinity » as compared to the wt Cter peptide (0.28 M NaCl *versus* 0.88).

These mutations were then introduced in the full-length chemokine (SDF γ mut2) in conjunction with Lys24 and Lys 27 mutations to Ser, HBS previously identified in SDF 1α [1]. A second mutant (SDF γ mut1) was also synthesised in which Lys24 and Lys27 were not mutated. Heparin column results shown that SDF γ mut2 affinity is drastically reduced as compared to SDF γ *wt* (0.49 M NaCl *versus* 1.01). SDF γ mut1 affinity is partially maintained (0.69 M NaCl *versus* 1.01).

Preliminary BIAcore results shown that SDF γ mut2 has lost the majority of its Heparin binding capacity whereas mut1 still binds to heparin sensorchip.

In conclusion, total solid-phase peptide synthesis of SDF isoform γ (98 Aas) was achieved, affording 10 mg of pure folded protein. Correct folding of the synthetic chemokine was confirmed by chemotaxis experiments. As for the α isoform (1), the major Heparin Binding Sites of SDF γ were identified. AAs involved in the binding to Heparin are: Lys 24, 27, 77, 78, 80, 83, 84, 88, 89, 91 and Arg 86. Biological activity studies of the non-heparin binding SDF γ mut2 are in progress.

Acknowledgements

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NOVEL PEPTIDE-DERIVED ENEDIYNE STRUCTURES

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Introduction

Enediyne antitumour antibiotics (neocarzinostatin, the esperamicin family, the calicheamicin family, the dynemicin family) represent a new class of compounds with complex architecture, high biological activity and the unique reaction mechanism. In spite of differences in their structure, the central part of the molecule occupies a *Z*-1,5-diyn-3-en unit (the enediyne moiety) embedded within the 9- or 10-membered ring. When an enediyne molecule anchors to the minor groove of the DNA, a cascade of reactions is initiated, leading to the cycloaromatization of enediyne unit and the formation of the 1,4-benzenoid diradical (Bergman cyclization, Fig. 1.). The highly reactive diradical strips two hydrogen atoms from the sugar-phosphate skeleton of the DNA, causing bursting of both DNA strands [1].

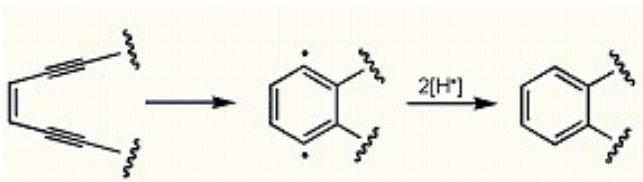

Fig. 1. The mechanism of Bergman cyclization.

Results

As a result of their complex structure, along with poor selectivity, there is an urge of synthesizing simpler, easily accessible and more selective enediyne-related compounds. Peptides could be good carriers of the enediyne unit, since they allow variations in conformational constrains and acid-base properties by simple amino acid replacement and can also carry certain degree of selectivity towards the certain tissues. The aim of presented research is preparation of cyclic peptide-enediyne molecules suitable for the study of Bergman cyclization triggered by thermal or photo-activation. We have prepared a model compound, Gly-Phe dipeptide with an enediyne N→N bridging unit.

Discussion

As presented in Fig. 2, C-terminus of both Gly and Phe amino acids was protected by the ethyl ester group. Amino group was activated by conversion into the sulfonamide group, by the treatment with 2-nitrobenzenesulfonyl chloride (2-NBS). N-terminus activation was achieved to facilitate *N*-alkylation reaction in the following step. In glycine derivative, ethyl ester is hydrolyzed with 1 M NaOH in MeOH, forming **1**. Phenylalanine derivative was *N*-alkylated with propargyl bromide in the presence of K_2CO_3, which leads to the formation of **2**. There is a

great number of reports dealing with the synthesis of enediyne moiety. Generally, Pd/Cu catalyzed cross-coupling reaction between terminal acetylenes and aryl or alkenyl halides is applied (Sonogashira reaction) [2]. However, reaction conditions strongly depend on the nature of reactants involved in the reaction. Optimal conditions for the preparation of chloroenyn derivate **3** involve dry, inert atmosphere and a careful sequence of reactant addition. Deprotection of 2-NBS group using 2-mercaptoethanol yielded secondary amine **4**. Dipeptide **5** was formed from **1** and **4** by the mixed anhydride method. Gly part of the dipeptide was then *N*-alkylated with propargyl bromide as described above, yielding **6**, while enediyne-bridged dipeptide **7** was formed under Sonogashira reaction conditions. It is expected that, after deprotection, **7** will afford a suitable model for the studies of Bergman cyclization, binding to then suitable carrier and it's interaction with the DNA. There is also a possibility of embedding **7** into the peptide sequence.

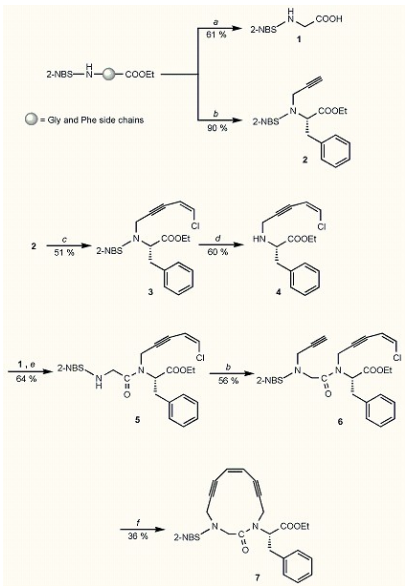

Fig. 2. Synthesis of a Gly-Phe dipeptide with an enediyne bridging unit: a 1M NaOH, MeOH; b HC≡C-CH_2Br, K_2CO_3, DMF; c cis-ClCH=CHCl, Bu-NH_2, Pd($C_6H_5CN)_2Cl_2$, CuI, THF; d HS-CH_2CH_2-OH, DBU, DMF; e ClOOCiBu, NMM, THF; f Bu-NH_2, Pd($C_6H_5CN)_2Cl_2$, CuI, THF.

Acknowledgements

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CARBOHYDRATE-INDUCED MODIFICATIONS OF OPIOID PEPTIDES - COMPARATIVE CD AND FTIR SPECTROSCOPIC STUDIES

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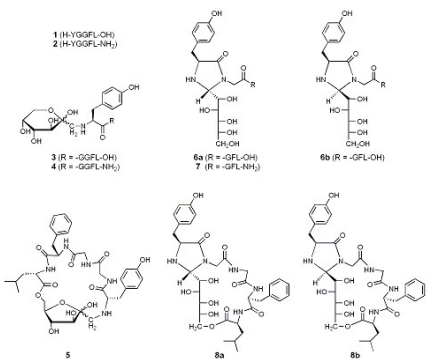
Introduction

The variety of vital functions played by glycoproteins in many physiological and pathological processes inspired the design of glycopeptides and the study of the effect of glycosylation on conformation. We have prepared linear and cyclic Amadori and imidazolidinone-type glycopeptides 3-8, glucose-modified analogs of the opioid peptides, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu, **1**) and Leu-enkephalin amide (**2**) [1 - 3]. This work reports comparative CD and FTIR spectroscopic studies on glycopeptides **3** - **8** in comparison with spectroscopic data of the non-modified flexible parent peptides **1** and **2**.



Results

The CD and FTIR measurements of compounds **1** - **8** were performed in different solvents in order to expose the structural and conformational differences caused by a keto-sugar, a rigid 5-membered imidazolidinone ring and/or cyclization.

CD spectra were recorded on a Jasco Dichrograph J-810 at room temperature. A quartz cuvette with 0.2 mm path length was used for far-UV (250 – 200 nm) measurements and a quartz cuvette with 10.0 mm path length for near-UV (310 – 250 nm) measurements. CD spectra were taken in 2,2,2-trifluoroethanol (TFE), water and in 1:1 mixture of these solvents. The sample concentrations ranging between 0.2 - 0.4 mg/mL. FTIR spectra were recorded on a Bruker Equinox 55 spectrometer at room temperature in a CaF_2 cell of 0.2 mm path length. The sample concentration in TFE was 2 mg/mL in each measurement.

Discussion

The combined application of CD and FTIR spectroscopy allowed to evaluate the relative amount of the extended and folded conformers of Amadori compounds (**2** - **5**) as well as imidazolidinones (**6** - **8**).

In general, the CD and FTIR spectra of the zwitterionic peptides **1**, **3** and **6a** reflect in TFE solution more expressed foldedness than the corresponding amide analogs **2**, **4** and **7**.

Based on the CD spectra, the keto-sugar anomers attached to the N-terminus of enkephalins do not have a significant effect upon the conformer equilibrium of the parent peptides [4]. The spectral features of the bicyclic Amadori derivative **5** are compatible with high population of a type II β-turn.

The CD spectra of imidazolidinones **6** - **8** are determined both by the foldedness of the peptide backbone and the position of the aromatic chromophore.

The imidazolidinone moiety incorporated into the structure of compounds **6** - **8** does not change the positive sign of the broad ^1L_b band of the parent peptides **1** and **2**. The opposite, negative sign of the ^1L_b band in the CD spectrum of the Amadori compounds suggests strong interaction between the Tyr side chain and the sugar moiety.

The N-substituted lactam subunite of all imidazolidinone compounds (**6** - **8**) has a characteristic FTIR spectral contribution below 1700 cm^{-1}. The *cis* lactam band at ~1690 cm^{-1} in the spectrum of the *trans* and *cis* isomers **8a** and **8b** shows a great difference in position and intensity indicating different population of folded and extended conformers. Furthermore, in the long-wavelength far-UV region of imidazolidinones CD spectra, all *trans* derivatives possess negative Cotton effect (CE) while the corresponding *cis* isomers have positive CE of band corresponding to the $n \rightarrow \pi^*$ lactam transition. Those findings allow for stereochemical correlation and makes possible to distinguish between *trans*- and *cis*-substituted imidazolidinones.

Acknowledgements

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EXTENDED ACCESS TO CHIRAL α,α-DISUBSTITUTED α-AMINO ACIDS VIA TRANSFORMATION OF α-ALKYLSERINE TO β-LACTONES

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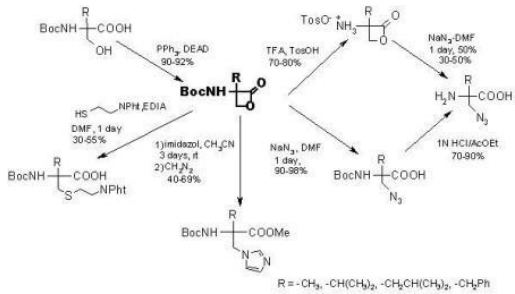
Introduction

N-Protected β-lactones of serine, threonine and cysteine are useful and versatile intermediates in the enantioselective synthesis of β-substituted-α-amino acid via ring opening by various nucleophiles. A large variety of carbon, nitrogen, oxygen, sulfur, and halogen nucleophiles were used to attack chiral *N*-protected serine β-lactones at the β-carbon yielding optically pure *N*-protected β-substituted alanines [1]. On the other hand, building blocks such as α,α-disubstituted amino acids have become important in medicinal chemistry and biochemistry. Incorporation of these units into peptides results in conformational restrictions and increased constraints of the peptidomimetic structures. α,α-Disubstituted amino acids have been successfully used to force peptides into their biologically active conformations, often resulting in peptidomimetics with remarkable resistance to enzymatic degradation. An easy access to α-hydroxymethylamino acids provided by general method developed in our laboratory [2] have prompted us to perform their further transformation into multifunctional α,α-disubstituted amino acids. Recently we have published the transformation of α-alkylserines into α-alkylcysteines *via* α-alkyl-β-lactones [3]. The starting Boc-3-amino-3-alkyl-2-oxetanones can be readily obtained by cyclization of *N*-Boc-α-alkylserines under Mitsunobu reaction conditions [4]. In the present study we report the ring opening of β-lactones α-alkylserines with various nuclephiles to obtain protected and free noncanonical amino acids, α-alkyl-β-substituted alanines.

Results and Discussion

Chiral α-alkylserines were synthesized by selective α-hydroxymetylation of oxazolones derived from coded amino acids and resolved into enantiomers by fractional crystallization. These compounds were used as starting materials for preparation of α-alkyl-β-substituted alanines. *N*-Protected α-alkylserine β-lactones (Boc-3-amino-3-alkyl-2-oxetanones) were readily formed under modified Mitsunobu conditions and undergone the ring-opening reaction with a variety of nucleophiles (Scheme 1). The chemical synthesis of β-azido-L-alanine was achieved via ring-opening of tosylate salt of (*S*)-3-amino-2-oxetanone (β-lactone of serine) [5]. Our attempts to follow this procedure for the conversion of 3-amino-3-methyl-2-oxetanone (β-lactone of α-methylserine) p-toluenesulfonic acid salt into α-methyl-β-azidoalanine were less efficient (50%). Reactions carried out with protected β-lactones of α-alkylserines as starting materials and NaN_3 in DMF,



Scheme 1. Ring-opening of Boc-α-alkylserine β-lactones

after 24 hours gave Boc-α-alkyl-β-azidoalanines in 90 - 98% yield. After careful evaporation of solvent *in vacuo* at 30 ºC, the products β-lactone ring-opening were separated and Boc-group was removed using 1N HCl in AcOEt at room temperature. The crude final products were purified by ion exchange chromatography. Free α-alkyl-β-azidoalanines were obtain in 70 - 90% yield. *S*-(Aminoethyl)-L-cysteine hydrochloride was obtained in reaction of (*S*)-3-amino-2-oxetanone, p-toluenosulfonic acid salt with 2-mercaptoethylamine hydrochloride in 85% yield [5]. Application of this procedure for ring-opening of α-alkyl-α-amino acid β-lactones due to steric hindrance needs prolonged reaction times. To avoid nucleophilic attack of solvents like water and free amino group of cysteamine we used *N*-phtaloylcysteamine in anhydrous DMF under argon for ring-opening reaction. After the reaction was completed (24 hours), the products were purified by silica gel chromatography. The desired *S*-(*N*-phtaloylaminoethyl)-α-alkyl-cysteines were obtained in the range of yield 30 - 55%. *N*-Phtaloylcysteamine is easily available from cysteamine hydrochloride and *N*-etoksycarbonylphtalimide in the presence of Na_2CO_3 under argon atmosphere. β-Imidazol-1-yl-alanine, analog of histidine is an useful replacement of coded amino acids in bioactive peptides. *N*-Boc-3-imidazol-1-yl-alanine methyl ester was prepared via ring-opening of *N*-(*tert*-butyloksycarbonyl)-L-serine β-lactone with imidazol in acetonitrile in 61 yield [6]. We applied similar conditions to open *N*-Boc-α-alkylserine β-lactone, but due to steric hindrance of the substrates the reaction time has been prolonged to 3 days. After reaction was complited the excess of diazomethane was removed by adding acetic acid and the reaction mixtures were chromatographed on silica gel. The synthesis *N*-Boc-β-imidazol-1-yl-α-alkylalanines methyl ester proceeded in overall yield 40-60%. The structures of all new α-alkyl-β-substituted alanines were confirmed by ^1H and ^{13}C NMR spectroscopy.

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PROCESSING OF hSARS-CoV SPIKE PROTEIN AND HEPTAD REPEAT DOMAINS OF CLEAVED C-TERMINAL FRAGMENT IN SARS INFECTION

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Introduction

Human Severe Acute Respiratory Syndrome Coronavirus (hSARS-CoV) is the causative agent for SARS infection. Its spike protein (S) is a target for SARS therapeutics [1, 2]. S-protein contains a proteolytic cleavage site and two interacting heptad repeat regions HR-N and HR-C. Following cleavage by host protease/s, S-protein generates N-terminal S1 and C-terminal S2-fragment. The latter is responsible for its fusion with host membrane via its interacting HR-N and HR-C domains that form coiled coil 6-helix bundle [3, 4]. Recently we reported that S-protein is cleaved by proprotein convertase (PC) furin at $RNTR^{761}\downarrow EV$ site [5]. However other PCs like PC5 and PC7 may also be involved. Here our goal is to compare *in vitro* the role of furin, PC5 and PC7 in S-protein cleavage and study the interaction of HR-N and HR-C domains.

Results and Discussion:

To study S-protein cleavage, we synthesized an intramolecularly quenched fluorogenic peptide QSARS [Abz-EQDRNTR$^{761}\downarrow$EVFA-Tyx, Abz = 2-amino benzoic acid and Tyx = 3-nitro tyrosine] [5] containing the cleavage site The peptide was digested with recombinant furin, PC5 and PC7 with similar enzymatic activity towards pyroERTKR-MCA (MCA = 4-methyl coumarin 7-amide). HPLC and mass spectra indicated that it is cleaved at $RNTR^{761}\downarrow EV$ by all three PCs, furin being the most effective enzyme with ~24- and ~10-fold more potency than PC5 and PC7 respectively. This cleavage can be blocked in by PC-inhibitor, α1Pdx [6] (Fig. 1), suggesting its potential therapeutic implication in SARS infection.

To study interaction between HR-N and HR-C domains, we synthesized fluorescent and non-fluorescent peptide (892-931) from HR-N domain which is implicated in the interaction. To identify segment of HR-C domain critical for interaction we selected peptides from segments (1153-1189), (1173-1189), (1153-1172) and (1163-1182) (Table 1). When fluoresecent HR-N peptide is incubated with HR-C peptides we observed fluorescence maximum shift and quenching suggesting that entire (1153-1189) domain may be required for binding with HR-N. Using gel electrophoresis we noted formation of high oligomers especially with HR-C (1153-1189) peptide. Our data indicated that furin and PC5 but not PC7 is involved in SARS-co-V S-protein processing and PC-inhibitors block this cleavage. Moreover the HR-N and HR-C domain interaction takes place via the segments (892-931) and (1153-1189) of spike protein.

Fig 1 RP-HPLC of furin digests of QSARS in absence and presence of varying concentrations of α1Pdx, a potent inhibitor of furin.

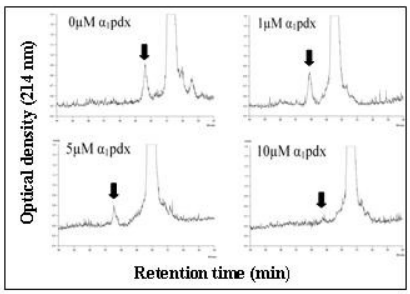

Fig 2. Excitation fluorescence spectra of fluorescent HR-N peptide (Fl-892-931) in absence (A) and presence of various HR-C peptides in increasing amounts (B=1:0.25, C=1:0.5, D:1:0.75, E:1:10, molar ratio), (λem= 535 nm),

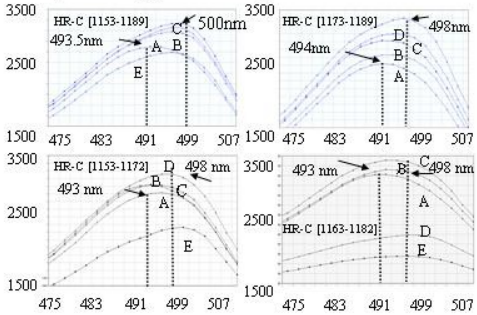

Table 2 HR-N and HR-C peptides, Fl=fluorescein moiety, Ahx=ε-amino hexanoic acid)

HR-N peptides	[892-931]	GVTQNVLYEN QKQIANQFNK AISQIQESLT TTSTALGKLQ
	[Fl-892-931]	Fl-Ahx_2-GVTQNVLYEN QKQIANQFNK AISQIQESLTTTSTALGKLQ
HR-C peptides	[1153-1189]	GINASVVNIQ KEIDRLNEVA KNLNESLIDL QELGKYE
	[1173-1189]	KNLNESLIDL QELGKYE
	[1153-1172]	GINASVVNIQ KEIDRLNEVA
	[1163-1182]	KEIDRLNEVA KNLNESLIDL

Acknowledgements

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CHEMICAL SYNTHESIS AND KINETIC STUDIES OF DIMERIC ANALOGUES OF TRYPSIN INHIBITOR SFTI-1 AND ITS TWO ANALOGUES CONTAINING A CARBONYL BRIDGE

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Introduction

SFTI-1 (Gly-Arg-Cys-Thr-Lys^5-Ser^6-Ile-Pro-Pro-Cys-Phe-Pro-Asp, disulfide bridge and head to tail cyclization) is the smallest and the most potent peptidic trypsin inhibitor known so far. For this reason it became an attractive object for studying enzyme-inhibitor interaction. SFTI-1 displays high sequential and structural homology with the binding loop of the family of Bowman-Birk inhibitors (BBIs). Lys^5-Ser^6 is the inhibitor reactive site (P_1-P_1'). Here we report the chemical synthesis and kinetic studies of a series of SFTI-1 analogues based on double sequence of the wild inhibitor. The question, whether such dimeric analogues can combine features from both, BBI and SFTI-1, such as the ability of the BBIs to allow dual inhibition, and the small size and constrained nature of SFTI-1, has been asked here. The influence of different cyclic elements on the inhibitory activity of our dimeric analogues was also investigated. Thus, two monomeric analogues **7** and **8** of SFTI-1 with the disulfide bridge substituted with the carbonyl one are presented here. Since the carbonyl bridge has not been previously introduced into proteinase inhibitor, its impact on the activity and proteolitic stability of such modified analogues had to be investigated.

Results

All peptides were synthesized manually by solid phase using Fmoc chemistry. The head to tail cyclization was performed using PyBop/DIPEA in DMF. The formation of disulfide bridges was achieved using I_2 in MeOH. The carbonyl bridges were formed by bis(4-nitrophenyl)carbonate and DIPEA in DMF [1]. The association equilibrium constants (K_a) were determined as described previously [2]. The stoichiometry of the complex formation in the case of analogues **5** and **6** with trypsin or chymotrypsin was determined using chymotrypsin competitive binding assay studies at 25 ^oC in the presence of trypsin. In this experiment a proper amount of trypsin (2 fold excess of inhibitor in each cuvette) was added to the increasing amount of inhibitor. After a suitable incubation time the constant amount of chymotrypsin was added.

Table 1. Association equilibrium constants (K_a) and the cyclic elements of SFTI-1 analogues. Errors in determination of K_a values never exceed 10%., ch-in the presence of chymotrypsin, t-in the presence of trypsin.

N	Sequence of analogue	Type of cyclization	Ka [M^{-1}]	
			trypsin	chymotr.
1	wild SFTI-1	disulfide bridge, head to tail	1.1×10^{10}	4.9×10^{6}
2	bisSFTI-1	two disulfide bridges	1.1×10^{9}	
3	bisSFTI-1	two disulfide bridges, head to tail	9.1×10^{8}	
4	[$Abu^{11,17}$]bisSFTI-1	disulfide bridge, head to tail	4.4×10^{8}	
5	[$Abu^{11,17}K^{19}$]bisSFTI	disulfide bridge	4.4×10^{8}	
6	[$Abu^{11,17}F^{19}$]bisSFTI	disulfide bridge	2.6×10^{8} - 1.2×10^{10} **ch**	8.7×10^{8}- 5.3×10^{9} **t**
7	[$Dap^{3,11}$]SFTI-1	carbonyl bridge	3.5×10^{8}	
8	[$Dap^{3,11}$]SFTI-1	carbonyl bridge, head to tail	8.1×10^{8}	

Discussion

All analogues presented in Table 1 display trypsin inhibitory activity. K_a values with bovine β-trypsin for all analogues synthesized, including **7** and **8** ones containing carbonyl bridge, appeared to be one order of magnitude lower than obtained for wild SFTI-1. However, they are still potent inhibitors. The most interesting results were obtained for analogue **6**, which displayed inhibitory activity against trypsin and chymotrypsin. This 28 amino acid peptide inhibited both enzymes simultaneously and independently. Moreover, preincubation of this analogue with trypsin or chymotrypsin not only retains but even significantly increases inhibitory activity towards the second enzyme. Similar investigations were very recently published by Jaulent and Leatherbarrow [3] where the design of double-headed inhibitors based on the binding loop of "classical" BBI was shown. Such 16-amino acid inhibitor has inhibited both proteinases independently but not simultaneously. The authors concluded it was to small to accommodate two molecules of enzymes.

Acknowledgements

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DEVELOPMENT OF A DEVICE HAVING 96-CHANNEL COLUMN-LIKE REACTORS FOR SIMULTANEOUS PURIFICATION AND PRODUCTION OF OLIGOPEPTIDES

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Introduction

We are currently constructing a large number of peptides, which have fluorescent dye(s) and are arrayed for protein detection [1]. Therefore, high throughput separation is required. Recently, we have developed a 96-channel pressurized device (AB96) and specially designed a column-like reactor (LibraTube®) [2] which can be used for high throughput (pre-)purification of labeled peptide libraries. Additionally, the present device could be used for fast oligopeptide syntheses.

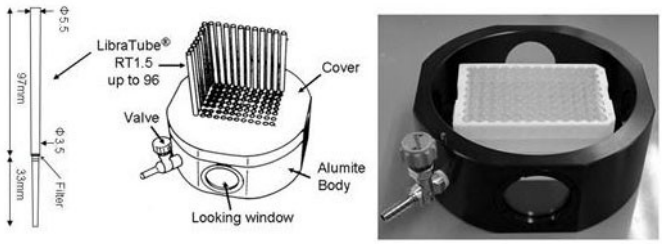

Fig. 1. LibraTube® and AB96 unit. The unit contains a recovery 96 well plate, MultiTiter™ (consisting of 2 mL removable glass vials) instead of the conventional deep-well plate.

Results

Fig. 1 shows the pressure stable vessel, AB96, which is alumite coating and stable against 10 mM HCl, organic acids and bases generally used for SPPS, and the column-like LibraTube® (volume ca 1.5 mL) with a flit. LibraTube® is polypropyrene and designed to be leakage free and to stand upright. Reaction mixtures of TAMRA-peptides are applied on the column packed with Sephadex LH-20, rapidly eluted and the colored fractions pooled, concentrated and purified by the single step RP-HPLC. Numerous packing materials can be used. Additionally, pre-packed silica-based materials (12 nm, 20 micron silica, length 45 mm) have been prepared and used for optimization of separation conditions. A crude synthetic sample is applied and allows rapid determination of the best combination of elution solvents.

Nowadays peptides and nucleotides are synthesized by automated solid-phase synthesis. One of the critical points is the complete mixing of acyl-component with resin to avoid formation of deletion peptides. Thus, numerous techniques such as rotation, vortex mixing, gas bubbling and continuous flow have been employed in commercial synthesizers. We have found that oligopeptides can be easily and rapidly obtained using our unit without any agitation or mixing, as activated acyl-components are passed drop-wise through the LibraTube®. The drop-rate is controlled by the pressure. Piperidine solutions or washing solvents can be rapidly removed from reactors under reduced pressure. A higher-throughput can be realized by the use of a dispensing system. The present simple system has been used for the rapid construction of various peptide libraries. Cleavage can also be accomplished in the same reactor-columns. Antioxidative high quality tripeptide [3] libraries have been prepared and directly used for several assays without purification. A 96 well format MultiTiter™ can be placed inside the unit for recovery and an octa-channel pipette can be used for simultaneous addition of solvents/reagents. This unique system is useful for high-throughput screening, drug discovery assays, combi-chem syntheses, and various analyses using autosamplers. Disposable parts are only glass vials thus affording low running costs with environmental advantages.

Discussion

The present system provides economical synthesis and purification of compound libraries. Partial purification affords prolonged HPLC column life. The present device has also been successfully used for coupling in solution using resin bound reagents such as DCC-bound resins and scavenger-resins popular in combinatorial chemistry.

Acknowledgements

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STRUCTURE-ACTIVITY STUDIES OF CYCLIC AZA-β^3-RGD PEPTIDE ANALOGS

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Introduction

Integrins are a family of membrane receptors, which participate in important cell–cell and cell–extracellular matrix interactions in many normal biological and pathophysiological processes like thrombosis, osteoporosis, and tumor metastasis [1]. Small peptide containing the RGD (Arg-Gly-Asp) sequence is a common cell-recognition motif for a wide variety of integrin receptors. Among them, RGD cyclic pentapeptides are an exciting class of $\alpha_v\beta_3$ integrin antagonists [2]. The γ-turn present in RGD sequence was found to contribute to the specific recognition by $\alpha_v\beta_3$ integrin receptor .

Mixed α-aminoxy acid, α-amino acid form a γ-turn initiated by the following N-O turn an eight-membered-ring intramolecular hydrogen bond [3]. We have demonstrated that aza-β^3-peptides are structured by the formation of N-N turn, a bifidic intramolecular eight membered hydrogene bonded interaction, very similar to N-O turn [4]. Hybride peptides composed of alterning aza-β^3-amino acids and α-amino acids could be a new strategy to induce a γ-turn initiated by the N-N turn. The aim of this study was to investigate the effects of replacement of amino acids by aza-β^3-amino acid analogs in RGD-peptides and to observe if the RGD motif resides in position i to i+2 of a regular γ-turn.

Results and Discussion

RGD mimetics was synthesized by coupling Fmoc-protected-amino acids on a preloaded resin or on resin where the first aza-β^3-amino acid was attached. Coupling cycles for the solid phase synthesis need a longer time for aza-β^3-amino acids than for α-amino acids. Deprotection cycles, cleavage from the resin, cyclisation and removal of the protecting groups are identical to those for peptides. Structure of RGD mimetics was then investigated. To obtain the best affinity and selectivity The RGD motif must occupy positions i to i+2 of a γ-turn and position i+1 to i+3 of a βII' turn. Moreover the distance between the C^β of the Asp and Arg must be lower than 7 Å. ^{1}H NMR studies and 2D ROESY experiments of hybrid

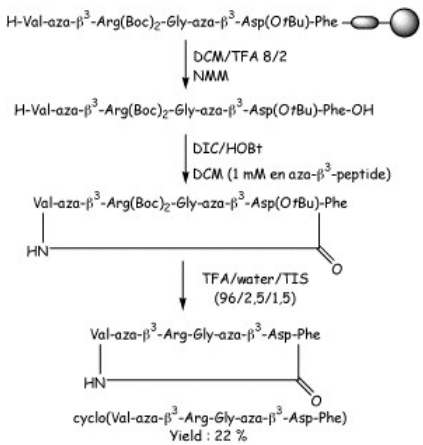

Figure 1. Synthesis of Linear and cyclic RGD peptide analogues

peptides were carried out in D_2O and in DMSO. In DMSO, a downfield chemical shift of the hydrazidic N-H was observed as shown in Fig. 2. This downfield chemical shift could reveal the formation of seven-membered-ring intramolecular hydrogen bonds (γ-turn) between hydrazidic Arg $C=O_i$ and Asp NH_{i+2}.

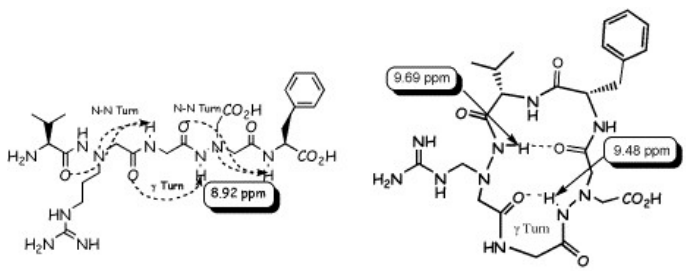

Figure 2. Linear and cyclic RGD peptide analogues

Peptides composed of alterning aza-β^3-amino acids and α-amino acids seem to be a new strategy to induce a γ-turn. This γ-turn could be initiated by the N-N turn or hydrazino turn, an eight-membered-ring intramolecular hydrogen bond induced by an aza-β^3-amino acid, Antimetastatic effects of these RGD mimetics are under investigation.

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OXYTOCIN ANALOGUES MODIFIED WITH GLYCOAMINO ACIDS

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Introduction

Carbohydrate moieties of glycopeptides play different decisive roles in various biological phenomena [1].

The conformation and solubility of proteins are influenced by the oligosacharide chain that may result in inhibition of proteolytic degradation as well as may influence the transport of the drug through biological membranes [2].

This means, that the synthesis of glycopeptides is very attractive for an understanding of the mutual interactions between peptide and sugar parts and for their biological effect [3 - 5].

We examined different possibilities of transformations of Fmoc-protected serine and threonine derivatives into O-glycosylated precursors suitable for SPPS. We prepared eight oxytocin analogues modified with O-linked glycoside in position 4 (Fig. 1).

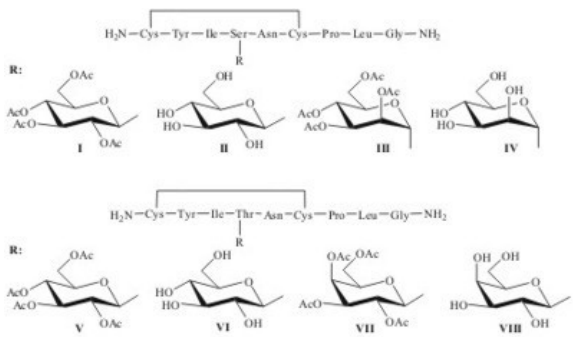

Fig. 1. Structures of new OT analogues.

We determined the biological potencies of the new analogues in classical bioassay. In addition we determined the affinity of the analogues to human OT receptors and tested the influence of glycosylation on the stability of oxytocin toward enzymatic degradation.

Results

Table 1. Pharmacological properties of new analogues of OT.

Analogue	Activity*			Affinity to human
	Uterotonic *in vitro*		Pressor	OT receptor
	no Mg^{2+} (IU/mg)	1 mM Mg^{2+} (IU/mg)	(IU/mg)	IC50 [nM]
OT	500	450	3.9	2.5
I	0	0.04 ± 0.01	0	40000
II	0.22 ± 0.06	0.84 ± 0.26	0	770
III	0.02	0.21 ± 0.01	0	-
IV	0.36 ± 0.08	1.8 ± 0.2	0	-
V	0	<0.1	0	>10000
VI	0.48 ± 0.16	0.9 ± 0.1	0	265 ± 15
VII	0	<0.1	0	>10000
VIII	0.25 ± 0.03	1.0 ± 0.1	0	466 ± 51

* The activity was determined in rats. The antidiuretic activity was not observed up to a dose of 0.1 mg/kg.

The glycopeptides **I** – **VIII** were designed in order to obtain OT analogues with changed biochemical profile. We expected that they may show lower but prolonged activity, due to probable slow enzymatic degradation. Results of biochemical evaluation of analogues **I** – **VIII** together with relevant values for OT are presented in Table 1. The influence of glycosylation on susceptibility toward proteolytic degradation was investigated using a model enzyme α-chymotrypsin. Oxytocin and glycosylated analogues were incubated with chymotrypsin and their degradation was monitored using HPLC.

Table 2. Degradation of OT and its analogues I – VIII.

Peptide	OT	I	II	III	IV	V	VI	VII	VIII
$t_{1/2}$ [min]	16	50	27	27	36	98	60	114	64

$t_{1/2}$ is the time in which half of the original peptide is degraded.

The stability of the glycopeptides is presented in Table 2 with OT as reference. The increase in half-life was observed for all glycosylated analogues. The analogue **V** displayed more than 6-fold and analogue **VI** more than 7-fold higher stability than OT. In general, O-acetylated glycopeptides (**I**, **V**, **VII**) displayed 2-fold higher stability than deacetylated (**II**, **VI**, **VIII**) ones.

Discussion

All analogues **I** – **VIII** showed uterotonic activity much lower than that of OT. The activity was slightly higher in the presence of magnesium. Also the affinity of the analogues to the oxytocin receptor was low, even if higher than we would expect from the biological activity. This however may be due to the species difference. In the uterotonic test *in vitro* in the used arrangement we are not able to judge any prolongation of the effect. None of the analogues showed any activity in the pressor test. There was no sign of possible formation of an active substance in time. The baseline of the blood pressure of the animals was stable.

Acknowledgements

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SEARCH FOR IMMUNOSUPPRESSANTS WITH ENHANCED BIOAVAILABILITY BASED ON THE SEQUENCE OF CYCLOLINOPEPTIDE X

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Introduction

Cyclolinopeptide X (CLX), isolated from flax seed, has a sequence cyclo-(XPPFFILL). X is a nonproteinaceous amino acid, (2*S*,4*R*) 4-amino-*N*-methylproline and plays a role of dipeptide moiety with a nonplanar *cis* peptidomimetic bond. CLX exerts immunosuppression comparable to that of cyclolinopeptide A (CLA). Analogues with γ-aminobutyric acid (γAbu) substituted for X revealed immunosuppressive activity, however, lower than that of CLA [1].

We have suggested that the Phe-Phe dipeptide is removed from the CLX sequence in an oxygen-induced natural conversion process to form a new cyclic hexapeptide cyclo-(XPPILL) [2]. Now, a series of "converted cyclolinopeptide X" analogues conjugated to nonaarginine, Tat peptide, and other cell-permeable sequences was synthesized on Rink amide resin:

1. γAbu-PPILL-Ahx-RRRRRRRRR-NH_2
2. (γAbu-PPILL-Ahx-)$_2$Lys-RRRRRRRRR-NH_2
3. Aib-GPPILL-Ahx-RRRRRRRRR-NH_2
4. Aib-GPP-Chg-Cha-Cha-Ahx-RRRRRRRRR-NH_2
5. γAbu-PPILL-Ahx-GRKKRRQRRRPQ-NH_2
6. γAbu-PPILL-Ahx-RRRRNRTRRNRRRVR-NH_2
7. γAbu-PPILL-Ahx-GRKRKKRT-NH_2
8. γAbu-PPILL-Ahx-PKKKRKV-NH_2
9. γAbu-PPILL-Ahx-RRRRRRRRR-NH_2
10. Aib-Chg-PPILL-Ahx-RRRRRRRRR-NH_2

The influence of the peptides on the concanavalin A and pokeweed mitogen-induced T- and B-cell proliferation, humoral immune response (AFC *in vitro* test) and microbial activity was studied using standard tests [3, 4].

Results and Discussion:

Synthesized peptides, in contrast to CLX, were well soluble in solvents used in the biological tests.

In cases of peptides **2**, **4** and to a lesser degree compound **9**, a significant suppression of lymphocyte proliferation was correlated with strong inhibition of the AFC number *in vitro*. No such a correlation was observed with peptide **7**. Peptides **3** and **10** strongly suppressed the AFC number with no effect on cell proliferation (Fig. 1).

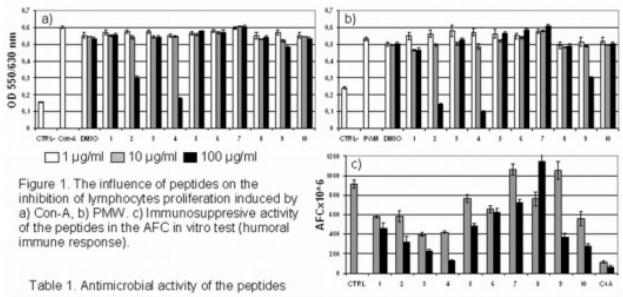

Figure 1. The influence of peptides on the inhibition of lymphocytes proliferation induced by a) Con-A, b) PMW. c) Immunosuppresive activity of the peptides in the AFC in vitro test (humoral immune response).

Table 1. Antimicrobial activity of the peptides

Peptides	Minimal inhibitory concentration [µg/ml]							
	B. subtilis ATCC 6633	*E. faecalis* PCM 896	*S. epidermidis* ATCC 14990	*S. aureus* ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*P. vulgaris* NCTC 4635	*C. albicans* ATCC 10231
1	4	32	2	64	128	1024	512	128
2	4	16	4	64	32	32	1024	128
3	8	64	2	128	128	1024	512	128
4	4	16	2	16	64	128	512	64
5	64	512	4	512	512	>1024	>1024	512
6	16	32	2	128	128	>1024	>1024	256
7	128	1024	8	1024	1024	>1024	>1024	512
8	1024	1024	128	>1024	1024	>1024	>1024	512
9	4	32	2	64	64	64	512	128
10	4	32	1	32	64	32	512	128

The compounds exhibited antimicrobial activity mostly against Gram-positive bacteria. The least active were compounds **5**, **7** and **8**. The remaining peptides were highly active over the concentration range 1 – 128 µg/ml. Against the Gram-negative strains the most active were peptides 2, 9 and 10. The remaining compounds exhibited a moderate antimicrobial activity (Table 1). All the studied compounds exhibited antifungal activity. The most active in this regard was peptide **4**. High activity of peptide **2** may result from the duplication of the "converted CLX" sequence. Highly active peptide **4**, with its cyclohexyl residues next to the Pro-Pro pair, resembles the sequence of known immunosuppressory fragment Pro-Pro-Phe-Phe found in CLA and other cyclopeptides [5].

Acknowledgements

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NEW ERYTHROPOIETIN RECEPTOR (EPOR) AGONISTS

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Introduction

Erythropoietin (EPO) is a hormone of 166 amino acids that controls proliferation and differentiation of red blood cells. It is therapeutically used for the treatment of anemia.

Some 13-20 residue peptides, EMP (EPO mimetic peptide), were discovered that compete with EPO on EPOR, although they share no sequence similarity with the hormone [1]. Another peptide, ERP (EPOR derived peptide), was reported to activate the receptor through an alternative binding site and these two peptides were shown to have synergic action *in vitro* [2].

We synthesized new multivalent erythropoietin analogues: EMP homo-dimers and EMP-ERP hetero-dimers. Monodisperse PEG-based polyamide of various lengths [3] were used to link the different peptides and oxime chemistry allowed conjugation of EMP through the N- or C- terminus [4]. Combination of monodisperse linkers and chemoselective oxime ligation resulted in homogeneous biomolecules.

Results and Discussion

Based on multivalency, we synthesized new EPOR agonist dimers. EMP peptides were synthesized by SPPS using *in situ* neutralization Boc chemistry on MBHA resin. An aminooxy moiety was manually coupled as its *N*-hydroxysuccinimide ester (Boc-AoA-OSu) after synthesis completion (N-EMP). For the C-terminal EMP derivative (C-EMP), Boc-Lys(Fmoc) was used to initiate the synthesis to allow Boc-AoA-OSu coupling on the ε-amine after selective deprotection. Disulfide bond was formed with hydrogen peroxide.

To conjugate the peptides, monodisperse PEG-based polyamides were prepared as described in [3] (Fig. 1). Three different linker lengths were synthesized to vary the distance between two monomers. Terminal Ser residues were oxidized with periodate to obtain dialdehyde linkers. Finally, the aminooxy EMPs were reacted with dialdehyde linkers to form dimers. Conjugating the peptides to the linkers by the chemoselective oxime reaction allowed the synthesis of homogenous constructs for comparison with previous EPO analogues [5], with the possibility to attach the EMP through their N- or C-terminus.

EMP-ERP heterodimers were also synthesized. ERP was first synthesized by SPPS on MBHA resin (Fig. 2). Manual addition of five “PEG-succ” units to the growing chain prolonged ERP with a flexible linker [3]. As for the dialdehyde linker, a Ser residue was coupled at the end of the linker. After HF cleavage, Cys was alkylated

with iodoacetamide to avoid sulfonic acid formation and Ser was oxidized with periodate to a glyoxylyl function. The reaction of N-EMP or C-EMP with the latter molecule formed heterodimers by oxime ligation. Targeting the EPO receptor on two distinct sites with one single molecule, moreover with peptides that have synergic activity, is promising for biological activity.

Further details of this work are in press [6], and biological characterization of the molecules is currently under investigation.

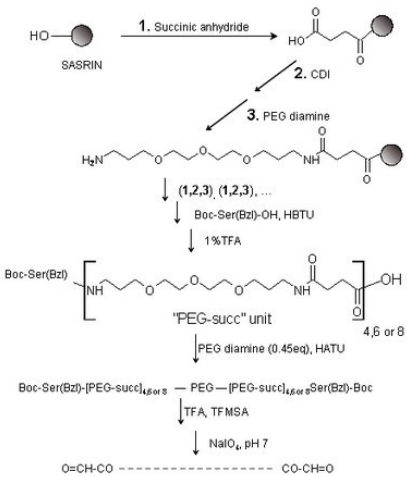

Figure 1. Synthesis of monodisperse dialdehyde linker

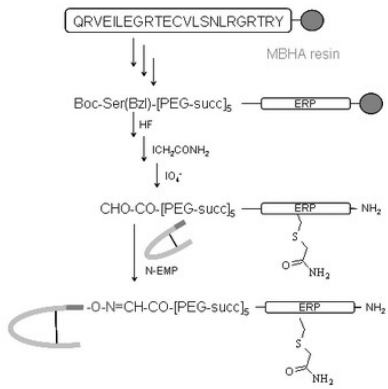

Figure 2. Synthesis scheme of N-EMP-ERP heterodimer

Acknowledgements

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UNEXPECTED DIFFERENT DISULFIDE CONNECTIVITIES OF CYSTEINE-RICH PEPTIDES WITH IDENTICAL CYSTEINE PATTERNS

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Introduction

Synthetic cysteine-rich replicates of naturally occurring peptides such as hormones, neurotransmitters, enzyme inhibitors, defensins and toxins often can be oxidatively folded in high yields to their native structures. The presence of identical cysteine patterns in the sequence generally leads to identical disulfide connectivities and homologous spatial structures despite significant variability in the non-cysteine positions. The consensus evolved to attribute unknown disulfide connectivities on the homology basis of cysteine patterns.

Results and Discussion

Minicollagen-1 from the wall of *Hydra* nematocysts (Mcol1) is a trimeric protein containing a central collagen characteristic sequence consisting of $(Gly\text{-}Xaa\text{-}Yaa)_n$ repeats. This is flanked by two poly-proline stretches of different length which connect the collagen portion with N- and C- terminal cysteine-rich domains most probably involved in the assembly of an intermolecular disulfide network [1, 2]. Structural analysis of the synthetic peptides corresponding to these folded domains by NMR revealed a remarkable exception to above mentioned general rule. Despite an identical cysteine sequence pattern, they form different disulfide bridges and exhibit distinctly different folds (Figs. 1 and 2) [3, 4]. In addition to the six common cysteine residues, the two sequences share a proline which in the C-terminal domain is in *trans* and in the N-terminal domain in *cis* conformation. Comparative analysis of the oxidative folding revealed for the C-terminal domain a fast and highly cooperative formation of a single disulfide isomer [1], while the N-terminal domain refolds mainly via a fully oxidized intermediate with non-native disulfide connectivities that in the initial folding phase result from the fast quasi-stochastic disulfide formation according to the proximity rule [5]. In this intermediate the Cys^{22}, Cys^{26} loop with its uneven number of intervening residues favours the *trans*-to-*cis* isomerization of the Ala^{23}-Pro^{24} bond according to MD simulations and in full agreement with recent studies on model peptides [6]. This unexpected, but favoured isomerization has to play a key role in the different spatial arrangement of the two cysteine-rich peptides, which rather surprisingly exhibit an inverse succession of almost identical structural motifs: β-turn-β-turn-γ-turn-α-helix in the N-terminal and α-helix-γ-turn-β-turn-β-turn in the C-terminal domain. Such asymmetric arrangement of two domains in one polypeptide strand raises the question of its functional role in the assembly of the collagen molecules during maturation and compaction of the nematocyst wall.

To our knowledge, these two cysteine-rich domains which most probably derive from gene duplication represent one of the few cases where two short peptides with identical cysteine patterns fold uniquely and with high yields into defined, but differing spatial structures. Therefore, such cysteine-rich domains may well represent ideal targets for *ab initio* structure calculations to learn more about the elementary information encoded in such primordial sequences.

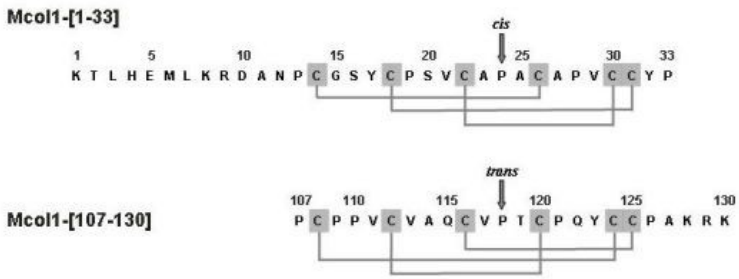

Fig. 1. Disulfide connectivities of the N- and C-terminal domains of Mcol1.

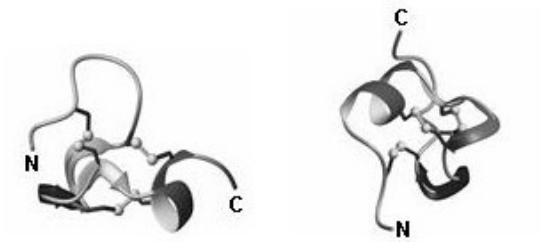

Fig. 2. Spatial structures of the N-terminal (left) and C-terminal domain (right) of Mcol1.

Acknowledgements

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DESIGN AND SYNTHESIS OF PEPTIDE – MACROLIDE CONJUGATES

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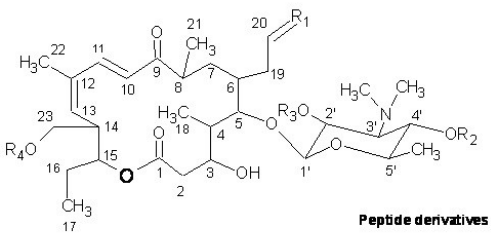
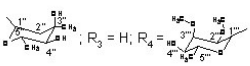
Introduction

Macrolides, widely known translation inhibitors, bind to ribosomal tunnel (RT) in a way that their lactone ring is located orthogonally to the long axis of the RT preventing the passage of nascent polypeptide chain through the tunnel [1]. Peptide – macrolide conjugates are of interest both as antibacterial agents and potential probes for investigation of nascent peptide chain topography in the RT [2]. Two types of such conjugates are possible: with peptide fragments directed to peptidyl transferase center or to the exit of the RT. The goal of this study was to develop the synthesis of peptide derivatives of tylosin (Tyl) and desmycosin (Des) at position C20 and O-micaminosyltylonolide (OMT) at position C23 of lactone ring (Fig.).



Tyl: R_1 = O; R_2 =  ; R_3 = H; R_4 =

Des: R_1 = O; R_2 = R_3 = H; R_4 = 

OMT: R_1 = O; R_2 = R_3 = R_4 = H

Ac_2OMT: R_1 = O; R_2 = R_3 = Ac; R_4 = H

Peptide derivatives

R_1 = $NOCH_2CO$-Ala_5-OMe;
$NOCH_2CO$-GPGPGP-OMe;
$NOCH_2CO$-AAFAAFK(Z)-OMe;

R_2 = H;

R_3 = H;

R_4 = 

R_1= O; R_2 = R_3 = H;

R_4 = Boc-AAF; Boc-FG; Form-MLF

Results and Discussion.

The peptides: Boc-PheGly-OH (**1**), Boc-AlaAlaPhe-OH (**2**), Form-MetLeuPhe-OH (**3**), H-Ala_5-OMe (**4**), H-$(GlyPro)_3$-OBzl (**5**), H-AlaAlaPheAlaAlaPheLys(Z)-OMe (**6**), were used for modification of antibiotics. Peptides **1** - **3** were obtained by HOBt/DCC-method in solution, peptides **4** - **6** were synthesized by Boc based SPPS on the Merrifield resin using DCC or HBTU as coupling reagents. Protected peptides **1** - **3** were coupled with C^{23}-hydroxyl group of OMT or 2',4'-di-Ac-OMT

(R_2 = R_3 = Ac) by using DCC/DMAP or HBTU as activating reagents. The target peptide – OMT conjugates 7 (R_4= Boc-PheGly-), **8** (Boc-AlaAlaPhe-), **9** (Form-MetLeuPhe-) prepared were purified by silicagel column chromatography using chloroform - methanol elution systems and identified with the help of MALDI mass spectrometry.

The HPLC analysis of these compounds with use Mixed-Mode Retention Mechanism columns Primesep B2 (RP-AX) and Primesep 100 (RP-CX) permitted to develop the method of the prediction of retention time and optimization of their separation conditions (software ACD/Method Development Suite, version 10.0, from ACD/Labs). The application of this method for such peptide – macrolide conjugates will allow in future to detect them in different biological tissues during their antibiotic activities investigation.

The peptide esters **4** - **6** first were *N*-acylated by Boc-(aminooxy)acetic acid to yield Boc-(aminooxy)acyl peptides esters: Boc-$NHOCH_2CO$-Ala_5-OMe (**10**), Boc-$NHOCH_2CO$-$(GlyPro)_3$-OMe (**11**) and Boc-$NHOCH_2CO$-AlaAlaPheAlaAlaPheLys(Z)-OMe (**12**). Peptide derivatives **10** - **12** after N-deprotection were conjugated with C20-aldehyde groups of Tyl and Des to give target peptide – Tyl conjugate **13** (R_1= $NOCH_2CO$-Ala_5-OMe) and peptide – Des conjugates **14** (R_1= $NOCH_2CO$-Ala_5-OMe), **15** (R_1= $NOCH_2CO$-$(GlyPro)_3$-OMe) and **16** (R_1= $NOCH_2CO$-AlaAlaPheAlaAlaPheLys(Z)-OMe) which were purified by preparative TLC and identified with MALDI mass spectrometry.

Acknowledgements

This study was supported by the Russian Foundation for Basic Researches (grants 04-04-49480_a and 05-04-08131-ofi_a).

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INHIBITION OF HUMAN TRYPTASE BY CYCLOTHEONAMIDE ANALOGS

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Introduction

Cyclotheonamides are inhibitors of trypsin-like serine proteases that are characterized by an extended peptide conformation which is stabilized by macrolactamization. The X-ray structure of cyclotheonamide A in complex with trypsin has shown that this particular conformation allows to address in a substrate-like manner beside the S1 pocket also the S'1, S2, and in a less pronounced manner the S3 pocket. The S_1 ligand, (*S*)-3-amino-6-guanidino-2-oxo-hexanoic acid, interacts via its guanido function with Asp189 at the bottom of the S_1 pocket. In addition, the ketone covalently modifies the γ-oxygen of Ser195 by hemiketal formation [1]. Recently, two novel cyclotheonamides have been isolated from a marine sponge of the genus *Ircinia*. One of them, cyclotheonamide E4 (Fig. 1), is a potent inhibitor of human tryptase [2].

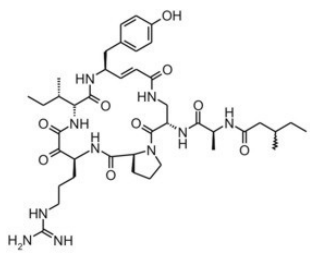

Fig. 1 Structure of cyclotheonamide E4.

Results

The design of a first generation analog of cyclotheonamide E4 was mainly guided by the idea to convert the parent compound into a fully reversible acting inhibitor. Accordingly, the S1 ligand was structurally replaced by β-homolysine. A solid phase approach based on the Fmoc/*t*Bu strategy was applied for the synthesis that allows for an on-resin macrolactamization (Fig. 2). The α-amino group of (*S*)-2,3-diamino propanoic acid was selected as anchoring point. The functionalization with different P3 residues at the anchoring site was performed in solution upon cleavage of the fully protected cyclic core molecule. Using an acetyl group as a model residue for the S3 pocket, a first generation analog of cyclotheonamide E4 was obtained fully characterized by RP-HPLC and HRMS. Preliminary studies have revealed that this compound exhibits weak inhibitory activity against trypsin (K_i 230 μM) and tryptase (K_i 380 μM).

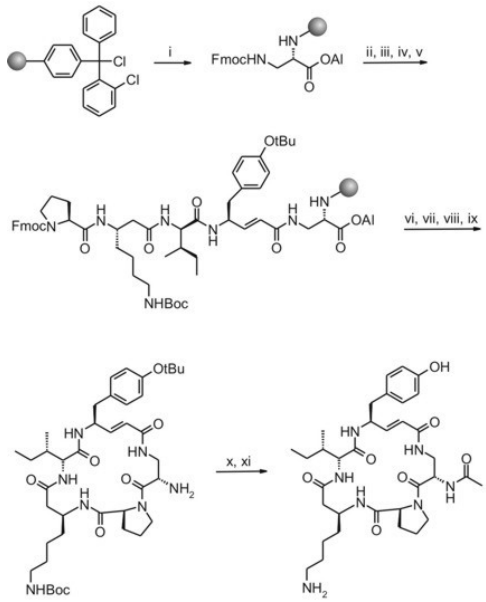

Fig. 2 Synthesis of a first generation analog of cyclotheonamide E4. Reaction conditions: i) a. H-Dpr(Fmoc)-OAlxHCl (2 eq.), DIEA, DCM, b. MeOH/DCM, DIEA, Loading: 0.36 mmol/g; ii) a. piperidine/DMF (v/v, 1:4), b. Fmoc-vTyr(tBu)-OH/HBTU/HOBt/DIEA (1:1:1:1, 2x1 eq.), DMF; iii) a. piperidine/DMF (v/v, 1:4), b. Fmoc-D-allo-Ile-OH/HBTU/HOBt/DIEA (1:1:1:1, 4 eq.), DMF; iv) a. piperidine/DMF (v/v, 1:4), b. Fmoc-β-Homolys(Boc)-OH/HBTU/HOBt/DIEA (1:1:1:1, 2x1 eq.), DMF; v) a. piperidine/DMF (v/v, 1:4), b. Fmoc-Pro-OH/HBTU/HOBt/DIEA (1:1:1:1, 4 eq.), DMF; vi) Pd(PPh$_3$)4, PhSiH$_3$, DCM; vii) piperidine/DMF (v/v, 1:4); viii) PyBOP/HOBt/DIEA (1:1:2, 3 eq.), DMF; ix) TFA/TIS/DCM (1:1:98, v/v/v), over eight steps 90%; x) Ac$_2$O/pyridine (1:1, 10 eq.), DCM, 83%; xi) TFA/H$_2$O (95:5, v/v), 87%.

Discussion

The developed solid phase approach gives access to focused libraries of cyclotheonamide analogs with positively charged β-homo amino acid as ligands for the S1 binding pocket. Moreover, the synthetic strategy allows for a probing of different P'1, P2 and P3 residues in a systematical manner and, thus to establish a reliable SAR profile for different trypsin-like serine proteases among them in particular for tryptase.

Acknowledgements

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EFFECTS OF SOME PROKINETICS ON PLASMA CHOLECYSTOKININ-LIKE IMMUNOREACTIVE SUBSTANCE LEVELS IN HEALTHY HUMAN

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Cholecystokinin (CCK) is widely distributed in mainly brain and small intestine. In gastrointestinal system, important actions of CCK are contraction of gall bladder, acceleration of bile transport to duodenum and stimulation of pancreatic exocrine function. On the other hand, in both central and peripheral nervous system, CCK acts as neurotransmitter. Recently, CCK is focused at modulation effects on feeding especially. In this study, we tried to establish a sensitive and specific enzyme immunoassay (EIA) for detecting CCK and to investigate the effect of some dopamine D receptor antagonists (domperidone (D2>D1), metoclopramide (D1>D2), and itopride). CCK has many molecular forms and its C-terminal sequence is the same as gastrin I. Therefore, long-term specific quantitative analysis could not be established. Since the development of specific antibodies for CCK in the 1980s, RIA methods for CCK have been widely used although those methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA system while minimizing the disadvantages. Our EIA was sensitive (2.0 pg) and specific for CCK, and the sharp inhibition curve obtained was linear between 10 and 625 pg/ml (Fig. 1). The CCK antibody YP030 was found to cross-react with CCK-8 and have little cross-reactivity with only gastrin I among the other endogenous peptides.

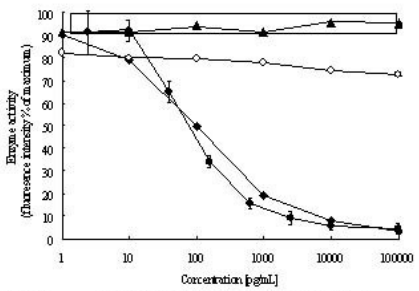

Fig 1. Inhibition curves of CCK (●), CCK-8 (◆), CCK-4 (▲), gastrin I (□), and other endogenous peptides (□) in the EIA by competition between CCK-12 conjugated with β-Gal to wed antiserum YP030.

Molecular heterogeneity in human plasma was examined by HPLC. The main CCK-like immunoreactive substance (IS) in plasma was eluted at the same elution time as the synthetic human CCK with several unknown peaks. However, CCK-IS

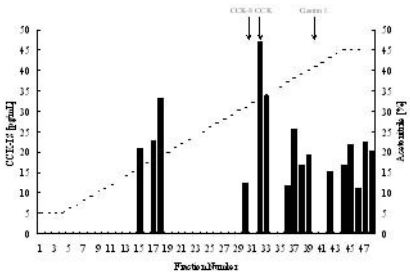

Fig 2. HPLC elution profiles of CCK-IS in human plasma. The dotted line indicates the acetonitrile gradient.

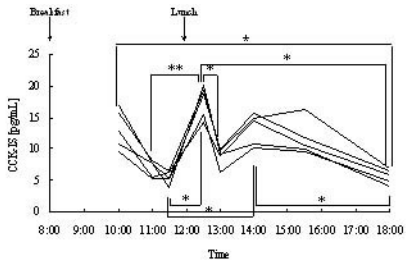

Fig 3. Circadian rhythms of plasma CCK-IS level profiles of five healthy human subjects during daytime. Times of intake of food and drink (breakfast and lunch) are indicated by arrows. */* < 0.05, and **/* < 0.01 significantly different from the placebo.

in plasma was not eluted at the elution time of synthetic gastrin I and CCK-8. Therefore, we thought that the CCK antibody YP030 recognized the CCK C-terminus except for the common sequences (same as CCK-4) with gastrin I, and that those unknown peaks might be due to CCK fragments (Fig. 2). We applied the novel EIA to detect CCK-IS in human plasma.

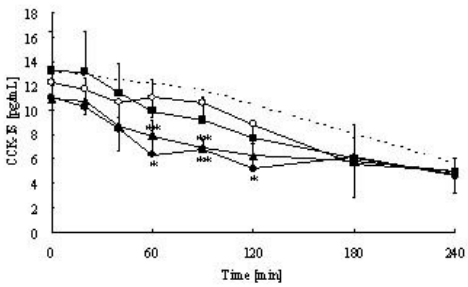

Fig 4. Effects of metoclopramide (●), domperidone (◆), Itopride (■), or placebo (○) on plasma CCK-IS levels. Each value represents the mean ± s.d., n = 5. */* < 0.05, and **/* < 0.01 and compared significantly different from the placebo.

The circadian rhythms of CCK-IS in the daytime were investigated for clinical use for human plasma. After a meal, CCK-IS rose significantly, then fell and rose again 2 h after the meal. The two-phase increase of CCK-IS was observed. At the first peak, CCK-IS might stimulate pancreatic exocrine responses and inhibit gastric emptying. At the second peak, CCK-IS might stimulate pancreatic digestive enzyme secretion or promote peristaltic reflex (Fig. 3). We revealed that domperidone and itopride caused significant decreases in plasma CCK-IS levels but metoclopramide did not (Fig. 4). We thought itopride was more specific dopamine D1 receptor from the viewpoint of changes of CCK-IS. We established a sensitive and specific EIA for CCK. urthermore, using the EIA, we analyzed

pharmacological effects of some prokinetics on plasma CCK. Our EIA may be also useful for diagnosis or analysis of diseases.

SYNTHESIS AND BIOLOGICAL EVALUATION OF OXYTOCIN ANALOGUES CONTAINING TETRAZOLE DERIVATIVES OF AMINO ACIDS

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Introduction

Unnatural amino acids are useful building blocks in drug discovery efforts because they can provide interactions with receptors or enzymes that are not possible with the 20 coded amino acids. Among structural units that mimic pharmacophoric functional groups, the acidic 5-tetrazolyl group is widely used in medicinal chemistry. There is now a large number of tetrazole compounds reported specifically for biological activity [1, 2]. In this work we present synthesis of oxytocin analogues containing tetrazolyl derivatives of amino acids.

Syntheses of amino acids

We synthesized derivatives of the following amino acids: aspartic **1**, glutamic **2**, and α-aminoadipic **3** acid containing tetrazole ring in side chains. Compounds **1** and **2** were obtained from L-asparagine and L-glutamine. Compound **3** was obtained in racemic form as well as in L-form. For the structures, see Fig. 1.

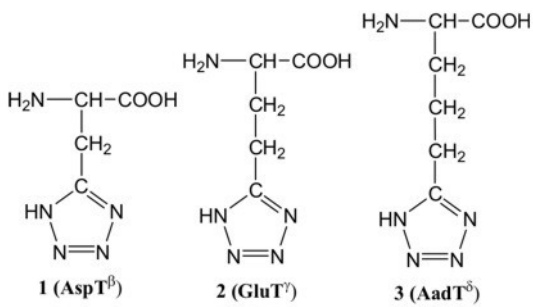

Figure 1. Structures of tetrazolyl derivatives of amino acids.

Syntheses of analogues of oxytocin

Structure-activity relationship studies of oxytocin showed that modification of glutamine in position 4 could result in very active analogues. Continuing work in that field, we incorporated compounds **1**, **2** and racemic **3** in position 4 obtaining oxytocin analogues **II**, **III** and enantiomeric mixture of **IV** and **V**, respectively. The individual diastereomers **IV** and **V** were separated using HPLC. To confirm which diastereisomer contained L-form of **3**, we synthesized oxytocin with L-form of

compound **3** and then compared HPLC retention times. Additionally we incorporated amino acid 1 in position 5, obtaining oxytocin analogue **I**. Structures of oxytocin analogues are depicted in Table 1.

Biological evaluation

We have determined the biological potencies of the new analogues in classical bioassays [3 -7]. In addition we have determined the affinity of the analogues to human OT receptors. Results of biological evaluation together with relevant values for oxytocin are presented in Table 1

Table 1. Biological activity of oxytocin analogues.

Analogue		Activity*			Affinity to human OT receptor IC_{50}[nM]
		Uteronic *in vitro*		Pressor IU/mg	
		IU/mg (no Mg^{+2})	IU/mg (1mM Mg^{+2})		
OT		500	450	3.9	2.5
I	Cys-Tyr-Ile-Gln-AspTβ-Cys-Pro-Leu-Gly·NH_2	0.08±0.02	1.18±0.40	0	945±85
II	Cys-Tyr-Ile-AspTβ-Asn·Cys-Pro-Leu-Gly·NH_2	17.3±4,5	158±35	0	18.5±2.5
III	Cys-Tyr-Ile-GluTγ-Asn·Cys-Pro-Leu-Gly·NH_2	8.9±1.5	22.5	0	16
IV	Cys-Tyr-Ile-AadTδ-Asn·Cys-Pro-Leu-Gly·NH_2	20.1±1.9	80±20	0.19±0.07	21
V	Cys-Tyr-Ile-D-AadTδ-Asn-Cys-Pro-Leu-Gly NH_2	0.29±0.06	1.8±0.2	0.10±0.02	1400

*The antidiuretic activity was not observed up to a dose of 0.1 mg/kg

These results confirm conclusion drawn from SAR analyses for oxytocin [8, 9] that asparagine in position 5 is more important for biological activity than glutamine in position 4 and should not be modified. All examined analogues show uterotonic activity lower than OT but analogue **I**, possessing tetrazolyl amino acid in position 5, shows hardly any activity. Peptides **I** - **III** posses no pressor activity and peptides **IV** and **V** only negligible one.

Acknowledgements

EFS stipend (ZPORR/2.22/II/2.6/ARP/U/2/05) and grant No. Z40550506 (JS)

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PSEUDO AND BRANCH-PEPTIDE INHIBITORS OF SUBTILISIN KEXIN ISOZYME-1: DESIGN AND BIOCHEMICAL APPLICATIONS

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Introduction

Subtilisin Kexin Isozyme-1 (SKI-1) or Site1 Protease (S1P) is a membrane bound Ca^{+2}-dependent mammalian subtilase of pyrolysin subtype [1, 2]. It cleaves peptide bonds at the consensus motif **Arg-X-Leu/Ile/Val-Leu/Thr/Gly/Lys⇓** where X = any amino acid except Cys. Clinically it has been linked to lipid metabolism and cholesterol homeostasis [1, 2]. It plays role in infections caused by hemorrhagic fever viruses via its role in the activation of viral glycoproteins [3]. Owing to these reasons interest has grown to develop specific and potent inhibitors of SKI-1 [4 - 6]. Here the main objective is to develop small molecule inhibitors of SKI-1. Two strategies were adopted: (i) multi-branch peptide assembly and (ii) pseudopeptide.

Results and Discussion

For multibranch approach we selected a peptide h(human)SKI-$1^{128\text{-}137}$ from its prodomain. This contains SKI-1 secondary activation site $K^{137}⇓Y$ which is crucial for binding with SKI-1 enzyme. We synthesized linear, 2- and 4-branch peptides (Table 1) where Ahx = ε-Amino hexanoic acid; Aoaa = Aminooxy-acetic acid; Adoa = 8-Amino-3,6-dioxa-octanoic acid, ⇓ = SKI-1 cleavage site, Ymut = Tyrosine mutant; residues underlined are unnatural amino acids substituting in one or more positions

Linear SKI-$1^{128\text{-}137}$ (**I** and **Ia**) and branch peptides (**II** - **IV**) inhibit SKI-1 activity when measured against Dabcyl-CMV$^{638\text{-}648}$-Edans substrate (Dabcyl-RGVVNA⇓SSRLA-Edans) (4). IC_{50} values (Table 2) revealed that 4-branch peptide is the most efficient SKI-1 inhibitor with IC_{50} = 0.9 μM. This is ~1.3- and 8.6-fold more potent than the corresponding 2-branch and linear peptides respectively. This suggests that assembly of multiple inhibitory peptides in a single unit promotes enzyme inhibition. Interestingly linker containing 2-branch peptide is ~3-fold more potent than that without the linker. Also presence of hydrophobic Fmoc group at P10 position is detrimental for inhibitory activity.

For pseudopeptide strategy, an oxymethylene function was used. Thus, unnatural amino acid Aoaa or Adoa was introduced at P1-P1' position of hSKI-$1^{172\text{-}179}$ and hSKI-$1^{169\text{-}179}$ peptides, which contain primary SKI-1 cleavage site L175↓R. A P7-Tyr-mutant was also prepared because of SKI-1's preference [5, 6]. This peptide (X) exhibited higher SKI-1 inhibitory property than wild type (Table 2). Circular dichroism (CD) spectra indicated a strong interaction of this peptide with SKI-1 (Fig. 1) compared to wild type, consistent with the observed inhibition profile.

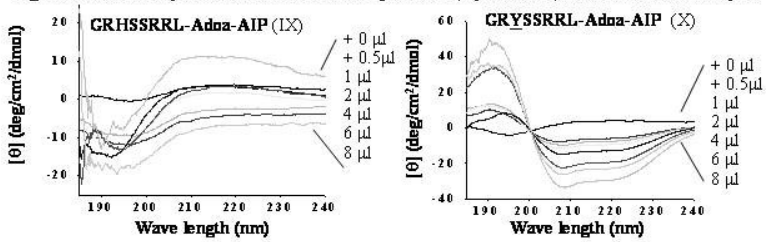
Table 1 List of peptides and analogs as SKI-1 inhibitors. The position numbers are based on prodomain sequence of hSKI-1.

	Peptide Name	Amino acid sequence
Branch/linear peptides based on secondary proSKI-1 processing site		
I	Fmoc-hSKI-1$^{128\text{-}137}$	Fmoc^{128}PQRKVFRSLK^{137}
Ia	hSKI-1$^{128\text{-}137}$	^{128}PQRKVFRSLK^{137}
II	hSKI-1$^{128\text{-}137}$-2-branch	(^{128}PQRKVFRSLK^{137})$_2$-KA
III	hSKI-1$^{128\text{-}137}$-Ahx-2-branch	(^{128}PQRKVFRSLK^{137}-Ahx)$_2$-KA
IV	hSKI-1$^{128\text{-}137}$-Ahx-4-branch	(^{128}PQRKVFRSLK^{137}Ahx)$_4$-K$_2$KA
Pseudopeptides based on primary proSKI-1 processing site		
V	hSKI-1$^{183\text{-}190}$	^{183}RRLL ⇓ RAIP^{190}
VI	hSKI-1$^{183\text{-}190}$-Aoaa	^{183}RRLL(Aoaa)RAIP^{190}
VII	hSKI-1$^{183\text{-}190}$-Adoa1	^{183}RRL(Adoa)AIP^{190}
VIII	hSKI-1$^{183\text{-}190}$-Adoa2	^{183}RRL(Adoa)IP^{190}
IX	hSKI-1$^{178\text{-}190}$Adoa	^{178}GRHSSRRL(Adoa)AIP^{190}
X	hSKI-1$^{178\text{-}190}$Adoa-Y$_{mor}$	^{178}GRYSSRRL(Adoa)AIP^{190}

Table 2 Inhibitory constants using 100μM Dab-CMV$^{638\text{-}648}$-Edans (S1) & Q-GPC$^{251\text{-}263}$ (S2) substrates.

Inhibitor	Substrate	IC_{50} (μM)	K_i (μM)
(I)	S1	14.8 ± 3.1	-
(Ia)	S1	7.9 ± 1.8	-
(II)	S1	4.4 ± 0.6	-
(III)	S1	1.2 ± 0.3	-
(IV)	S1	0.92 ± 0.04	-
(VI)	S2	34 ± 5.1	17 ± 3.0
(VII)	S2	54 ± 8.5	28 ± 2.7
(VIII)	S2	250 ± 14.2	110 ± 9.5
(IX)	S2	239 ± 16.2	109 ± 10.4
(X)	S2	49 ± 3.9	24.8 ± 0.8

Fig 1 Effects on CD upon incubation with increasing amounts (top to bottom) recombinant SKI-1 enzyme



Acknowledgements

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CELL SIGNALING OF THE MELANOCORTIN RECEPTOR 1 BY MASS SPECTROMETRIC ANALYSIS

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Introduction

The melanocortin receptors and their ligands have been found to modulate a wide range of physiological functions [1]. The particular function of the receptor is associated with the type of receptor and the kind of cell it is expressed in. There is a great interest in elucidating the mechanism of the function of these receptors to aid in the development of novel ligands to be used as pharmacological agents. The role of phoshorylation must be investigated to understand this process. So far, it is postulated that phosphorylation occurs after agonist binding [2], thereby leading to internalization of the receptor and the down regulation as an agonist effect on the cell. The experiments results here focused on investigating agonist induced phosphorylation of the human melanocortin receptor 1 (hMC1R), which has been found to modulate skin pigmentation.

Results

The human melanocortin receptor 1 (hMC1R) was constructed to contain a FLAG epitope and a hexahistidine tag at the amino-terminus as well as at the carboxyl terminus to facilitate purification. Stably transfected hMC1R in human embryonic kidney (HEK293) cell lines that expressed the receptor resulted in a K_D value of 0.1 and 0.2 nM respectively in each case when the super potent agonist MTII was competed with [^{125}I]NDP-α-MSH. Treatment of the tagged receptors in the HEK293 cells with agonist resulted in down-regulation which indicates that these tagged receptors retain their biological functions. The hMC1R was solubilized from cell membranes with n-dodecyl-β-D-maltoside [3, 4] and purified at a Nickel chelating resin and a newly constructed affinity column. The purified hMC1R was a glycoprotein that migrated on SDS/PAGE with a molecular mass of 58 kDa. The results from (MALDI-TOF) mass spectrometry was used to identify and characterize peptides derived from the hMC1R following in-gel digestion with chymotrypsin (Table 1). The phosphorylation sites were identified on the purified human melanocortin receptor 1 with agonist treatment with MTII (Fig. 1).

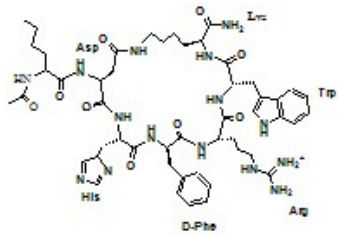

Fig. 1. Structure of MTII

Table 1. Agonist mediated phosphorylation of the hMC1R. Upper: Chymotrypsin digest derived some of peptides from the hMC1R. Lower: Chymotrypsin digest derived some of peptides from hMC1R pretreated with MTII.

Residues	*Sequence*	*mass*	*Exp mass*
27-41	AANQTGARCLEVSIL	1602.8274	1602.7897
57-69	TGSVEVIAKNRNL	1400.7862	1400.6154
107-116	GVDRYISIF	1068.6530	1068.4948
139-148	GVDRYISIFY	1232.6315	1232.6096
159-168	RAVVLPRAAF	1099.6740	1099.4548
297-305	IILFHSQEL	1099.6152	1099.4548

Residues	*Sequence*	*mass*	*Exp mass*
91-101	EICSVNTLLL	1248.6510	1248.6653
90-100	LSEICSVNTLL	1245.6510	1248.6653
114-127	QQLDDCLCANVSSF	1656.6998	1656.4839
114-127 +1PO4	QQLDDCLCANVSSF	1736.6661	1736.5976
114-127 +2 PO4	QQLDDCLCANVSSF	1816.6324	1816.4815
121-127+ 2PO4	CANVSSF	944.2626	944.5453
128-143 +2PO4	IVISSLAITMLGVDRY	1910.9104	1910.6401
278-285	NACIIKLL	944.5603	944.5453
289-296	NFIAPAFL	892.4933	892.3791

Discussion

The purification scheme for the human melanocortin receptors (hMC1R) proved successfully in gaining purified receptors for mass spectrometric analysis. Mass spectrometric analysis of human melanocortin receptor 1 activated by peptide vs non-peptide agonists reveal some of the different phosphorylation sites (data not shown), which provide evidence of different cell signaling and trafficking at the melanocortin system by using peptide vs nonpeptide agonists.

Acknowledgements

Supported by Grants from the USPHS DK 17420 and DA 06284

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CONFORMATIONAL AND METABOLIC CONSEQUENCES OF SUBSTITUTION OF PHENYLALANINE WITH β³-HOMOPHENYLALANINE

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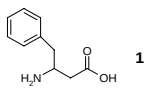
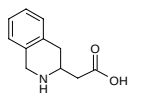
Introduction

Endogenous opioid peptides have long acted as model compounds for the development of new analgesic drugs. This peptides interact with different subtypes of opioid receptors, such as δ,μ,κ. Endomorphin-1 and -2 are opioid peptide agonists with high selectivity for μ-opioid receptors [1]. In search for more potent μ-selective ligands, tetrapeptide analogue Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) has been synthesized [2].



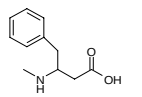
1

β³-*homo*-phenylalanie (β³-*h*Phe)



2

β³–*homo*-1,2,3,4-tetrahydroiso quinoline -3-carboxylic acid (β³-*h*Tic)



3

β³-*N*-methyl-*homo*-phenylalanine (*N*-Me-β³-*h*Phe)

In recent years much attention has been focused on various β-amino acids and β-peptides. The substitution of α-amino acids for their β-isomers in biologically active peptides may result in an increased enzymatic stability and also in strong influence on peptides conformations.

In the present study, to evaluate role of both, metabolic and conformational factors on biological activity of model opioid peptide Tyr-D-Ala-Phe-Phe-NH_2 (TAPP), we have synthesized short library of analogues in which phenylalanine residues have been replaced with β³-homophenylalanine or its derivatives (**1** - **3**)

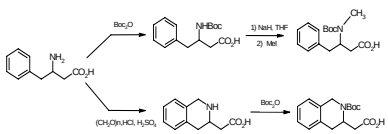
Results and Discussion

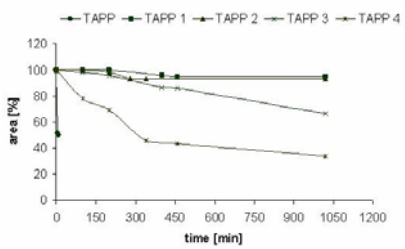
The β³-hPhe was prepared from Phe based on the homologation of *N*-Boc-Phe-ol. The synthesis of β³-hTic and *N*-Me-β³-hPhe by homologation of Tic and *N*-MePhe respectively, failed. The β³-hTic and *N*-Me-β³hPhe were prepared from β³-hPhe according to the Scheme. The target peptides were prepared in solution by fragment condensation of Boc-Tyr-D-Ala-OH with proper *C*-terminal dipeptide containing β³-amino acid. We used conventional Boc chemistry, using TBTU/HOBt in the presence of DIEA. All new TAPP analogues were tested for μ-opioid receptor affinity (Table). Radioreceptor binding assays have been performed by the method described previously [3]. In this series of analogues only TAPP 2 displayed significant affinity for μ-opioid receptors. Replacing of phenylalanine residues with β³-hPhe or its derivatives in position 3 strongly decreased affinity to μ-opioid receptor. Low affinity of peptide analogues containing β³-homoamino acids in this

position may suggest that introduction of additional methylene group destabilized proper μ active conformation due to different topographical location of aromatic ring.

Analogues of TAPP were examined in the field of their resistance to enzymatic degradation. TAPP has Phe-NH_2 at the C-terminus, and this amide bond is apparently very sensitive to deamidation by α-chymotrypsin. To verify the effect of residue substitution, we examined the hydrolysis of peptides under the same condition (Tris buffer, pH 7.4; 37 ºC). Hydrolysis results were collected in graphics (Fig.): the amount of remaining starting peptide vs time, given in area % of the initial value calculated from HPLC [4]. Analysis of the metabolites were performed using HPLC /EI-MS. The digestions of tetrapeptides with α-CT were followed over two days. After 10 min the amounts of deamidated TAPP, were around 50%, while TAPP 1-TAPP 4 analogues were completely intact. After 7 hours only about 10% of investigated analogues were digested by α-CT and in the metabolites small amounts of Tyr and C-terminal tripeptides were found.

In conclusion the C-terminal amide of TAAP 1-TAPP 4 is almost fully resistance against α-chymotrypsin.





Peptides	IC_{50}[nM] (*versus* [^{3}H]Naltrexone)
Tyr-D-Ala-Phe-Phe-NH_2 (**TAPP**)	15,5 (±6,33)
Tyr-D-Ala-**β^3-*h*Phe**-Phe-NH_2 (**TAPP 1**)	>1000
Tyr-D-Ala-Phe-**β^3-*h*Phe**-NH_2 (**TAPP 2**)	91,2 (±5,54)
Tyr-D-Ala-***N*-Meβ^3-*h*Phe**-Phe-NH_2 (**TAPP 3**)	not active
Tyr-D-Ala-**β^3-*h*Tic**-PheNH_2 (**TAPP 4**)	407 (±5,49)

Acknowledgements

We thank TriMen Chemicals Poland for EI-MS spectra.

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MOLECULAR MODELING-BASED STUDY OF NEW VASOPRESSIN ANALOGS AFFINITY TOWARD HUMAN NEUROHYPOPHYSEAL HORMONE RECEPTORS

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Introduction

The neurohypophyseal hormone arginine vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; AVP) is a cyclic nonapeptide that plays several important physiological roles, such as the control of blood pressure, adrenocorticotropin hormone secretion and the regulation of water balance, mediated via three different subtypes of vasopressin receptors: V_{1a}, V_{1b} and V_2 (V_{1aR}, V_{1bR} and V_{2R}) and to a small extent via oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2; OT) receptors (OTR). In this study, nine vasopressin analogs have been investigated: analog **1**: [Aic^2]AVP, analog **2**: [Mpa^1,Aic^2]AVP, analog **3**: [Aic^2,D-Arg^8]VP, analog **4**: [Mpa^1,Aic^2,D-Arg^8]VP, analog **5**: [Aic^2,Val^4]AVP, analog **6**: [Mpa^1,Aic^2,Val^4]AVP, analog **7**: [Aic^3]AVP, analog **8**: [Mpa^1,Aic^3]AVP, analog **9**: [Aic^3,D-Arg^8]VP, (Aic: 2-aminoindane-2-carboxylic acid). To determine their presumable biological activity docking and molecular modeling methods have been used. Result of this investigation will be compared with the results of functional assays (currently unavailable) and if they compatible, the molecular modeling methods could be an alternative for more expensive and time-consuming clinical tests.

Results

The theory about the relationship between the value of dihedral angle between aromatic rings Aic and Phe (analogs **1** ÷ **6**) or Aic and Tyr (analogs **7** ÷ **9**) (Fig. 1) cannot be interchangeable verified. The initial 15 ÷ 25% of conformations with aromatic rings of residues in positions 2 and 3 of AVP analogs interacting with each other (Aic-Ar angle less than 90 degrees; present mainly in analog **7**, analog **8** and analog **9**) are changing into conformations with Aic, Phe or Tyr aromatic rings pointed outside. This change, in our opinion, is due to very limited space in the receptor binding cavities. In high temperatures simulation (Constrained Simulated Annealing; CSA) the conformational changes of the analogs are restricted by the surrounding sidechains of receptor amino acid residues – the Aic and Ar rings pointed outwards are inclined to occupy smaller space.

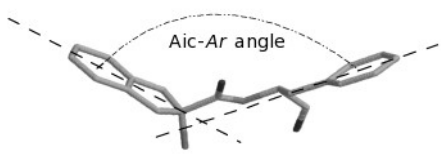

*Fig. 1. Measured AicAr angle. Ar is Phe (analogs **1** ÷**6**) or Tyr (analogs **7**÷ **9**) analog residue.*

Analogs **7** ÷ **9** seem reveal high affinity and low selectivity since inactive models of receptors readily interact with them. Low percentage of occurrence of analogous complexes with activated forms suggest that these analogs might have antagonistic properties. Analog **1** is as good agonist of OTR and V_{1R}. Analog **4** is antagonist of OTR and V_{2R}. Furthermore analog **1** – in inactive forms of receptors – showed very low affinity to all types of receptors and high for activated OTR and V_{1R}, which could be a hint that this analog is good agonist. Analog **2** shows very low affinity to the OT and V_1 receptors in inactive forms and moderate in the case of activated V_{1R} – which could prove its pressor activity. The C-terminal part of all of tested analogs is highly hydrophilic. Additional substitution of Cys1 for Mpa1 (analogs: **2**, **4**, **6** and **8**) enhances much hydrophobic properties of the N-terminus of the peptide. We have taken this dual polarity into account in selecting receptors-analogs complexes for optimization after docking procedure as well as for selection of final complexes. In this approach, ligands oriented with their hydrophilic fragments towards the entrance of the binding pocket of the receptor were preferred. The receptor amino acid residues forming the binding pocket and, in our opinion, essential for ligand binding, have been identified on the basis of the distance, frequency-of-occurrence criteria and general electrostatic rules such as possibility of occurrence interaction of any kind. We have chosen all amino acid residues whose the nearest atom was situated not further than 2.5 Å off any amino acid residue of the analyzed analog.

Conclusions

In the process of designing new analogs, knowledge of regions of ligand binding is critical because of the possibility of designing new ligands of a desired geometry. Moreover the results of other AVP analogs docking [1], being in accordance with the experimental data show, that such a "probabilistic-criterion" is a very good tool in recognizing specificity of ligand binding.

Acknowledgements

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NEW SYNTHETIC PROBES FOR THE ANALYSIS OF THE CELL MEMBRANE PROTEOME AND INTERACTOME

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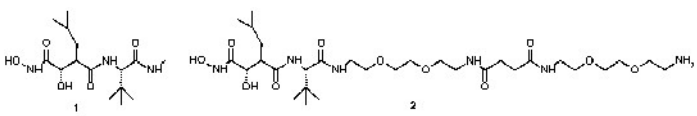
Introduction

Despite the fact that many technological advances are currently involved in proteome analysis, like two-dimensional gel electrophoresis and mass spectrometry, there is still a great need for the development of novel engineered chemical probes for proteomics and interactomics. Here, we describe our approach concerning the study of proteome and interactome. It relies on the use of a small synthetic inhibitor chemically modified to allow for its immobilisation to magnetic beads or affinity chromatography materials. Proteins will be detected together with their native interaction partners because of non-denaturing conditions. This general procedure is applied to the enrichment of metalloproteinases, especially matrix metalloproteinases, which are potential targets in tumour therapy.

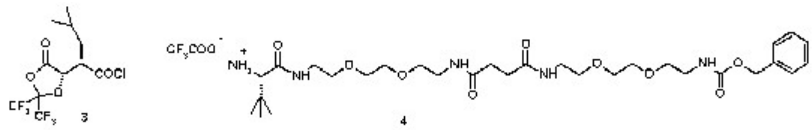
Hydroxamic acids are known to be potent inhibitors of metalloproteinases. Marimastat **1** is a reversible inhibitor with a good potency and shows activity towards a wide range of metalloproteinases. The parent compound has been modified with a spacer to allow immobilisation on a solid surface to give new marimastat derivative **2**.



1 2

Results and Discussion

The new probe **2** has been synthesised successfully in 7% total yield. The key step of the synthesis is the coupling of acid chloride **3**, which can be obtained starting from (-)-diethyltartrate, to L-tert-Leucinamide derivative **4**. Binding studies with human recombinant MMP-2 were performed using surface plasmon resonance, and show that MMP-2 binds specifically to marimastat derivative **2**. Fishing studies with magnetic beads coated with probe **2** show that it is possible to fish MMP-2 out of the buffer, and that proteins can be released in the media by elution with an EDTA containing buffer. Preliminary studies with placenta extract using marimastat derivative **2** immobilised on an affinity column demonstrate that creation of a subproteome is possible starting from a complex mixture. In principle, modified binding and elution conditions should provide interaction partners of the target proteins.



3 4

Acknowledgements

This research was supported through European Community Marie Curie Fellowship. Thanks to Prof. K. Niehaus and N. Kuepper for the MALDI-TOF MS measurements for protein identification.

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CYCLIC DERMORPHIN AND DELTORPHIN ANALOGUES

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[1]Department of Chemistry, Warsaw University, Warsaw, Poland and [2]Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, QC, Canada

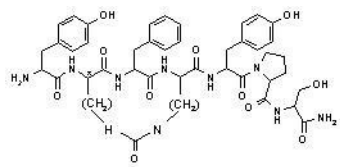
Introduction

Recently, we described the synthesis of several cyclic opioid analogues that contained the N-terminal sequence 1-4, common to dermorphin and deltorphin. Some of them showed very high agonist potency in the GPI and in the MVD assays [1, 2].

In this work, we designed new analogues in which the sequences were elongated at the C-terminal to obtain the full sequences of dermorphin and deltorphin.

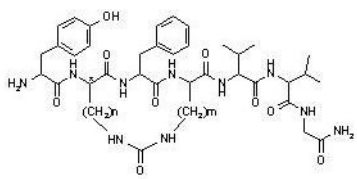
Results and Discussion

Six cyclic dermorphin and six cyclic deltorphin analogues have been prepared. Cyclization was accomplished by reaction of linear precursor with bis(4-nitrophenyl) carbonate.



		Ring size
1	{[H-Tyr-D-Lys(&1)-Phe-Orn(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	17
2	{[H-Tyr-D-Lys(&1)-Phe-Dab(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	16
3	{[H-Tyr-D-Orn(&1)-Phe-Orn(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	16
4	{[H-Tyr-D-Lys(&1)-Phe-Dap(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	15
5	{[H-Tyr-D-Orn(&1)-Phe-Dab(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	15
6	{[H-Tyr-D-Orn(&1)-Phe-Dap(&2)-Tyr-Pro-Ser-NH2][&1CO&2]}	14

Fig. 1. Dermorphin analogues.



		Ring size
1	{[H-Tyr-D-Lys(&1)-Phe-Lys(&2)-Val-Val-Gly-NH2][&1CO&2]}	18
2	{[H-Tyr-D-Orn(&1)-Phe-Lys(&2)-Val-Val-Gly-NH2][&1CO&2]}	17
3	{[H-Tyr-D-Lys(&1)-Phe-Dab(&2)-Val-Val-Gly-NH2][&1CO&2]}	16
4	{[H-Tyr-D-Lys(&1)-Phe-Dap(&2)-Val-Val-Gly-NH2][&1CO&2]}	15
5	{[H-Tyr-D-Orn(&1)-Phe-Dab(&2)-Val-Val-Gly-NH2][&1CO&2]}	15
6	{[H-Tyr-D-Orn(&1)-Phe-Dap(&2)-Val-Val-Gly-NH2][&1CO&2]}	14

Fig. 2. Deltorphin analogues.

All peptides were tested in the GPI and in the MVD assays.

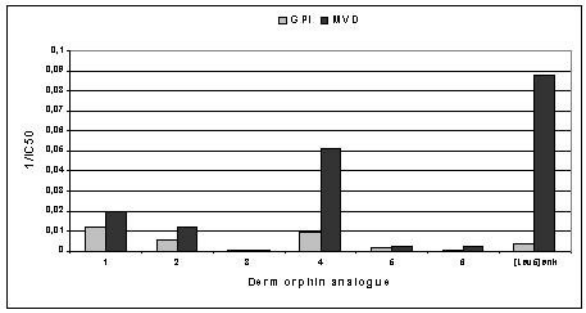


Fig. 3. GPI and MVD assay of cyclic dermorphin analogues.

In most cases, dermorphin (**1** - **7**) analogues were less potent than previously described dermorphin (**1** - **4**) analogues. The only exception, were analogues containing Lys^2/Orn^4 and Lys^2/Dap^4 which were equipotent in the MVD assay.

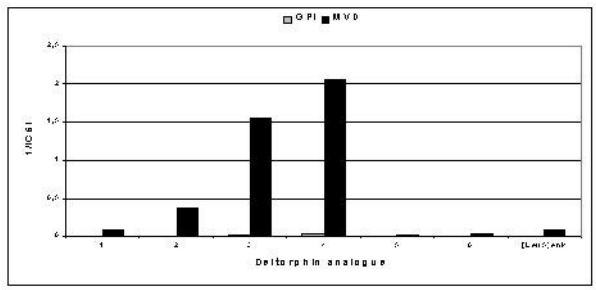


Fig. 4. GPI and MVD assay of cyclic deltorphin analogues.

The activities of the deltorphin (**1** - **7**) analogues were dramatically lower in the GPI assay than those of the (**1** - **4**) analogues and they were more potent in the MVD assay than the (**1** - **4**) analogues.

As a result, these analogues turned out to be δ selective opioids (the MVD/GPI ratio of **3** was 0.98×10^{-2}, and the ratios of **2** and **6** (0.27×10^{-2}) were even lower).

Acknowledgements

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NMR-BASED CONFORMATIONAL STUDIES OF VASOPRESSIN ANALOGUES MODIFIED AT POSITION 2 WITH 1-NAPHTHYLALANINE ENANTIOMERS

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Introduction

It is believed that appropriate orientation of aromatic side chains of vasopressin (AVP) analogues is crucial for binding to the receptors. Replacement of L-amino acid by its D enantiomer is a simple modification inducing changes in orientation of the side chain. The AVP analogues investigated by us turned out to be potent V_{1a} antagonists. In turn, inversion of configuration of the residue at position 2 converted a weak antidiuretic agonist, **[Cpa^1,L-1-Nal^2]AVP (I)**, into a highly potent antidiuretic antagonist, **[Cpa^1,D-1-Nal^2]AVP (II)** [1].

Results and Discussion

The NMR spectra of both analogues displayed two distinct sets of residual proton resonances, which is a result of *cis/trans* isomerization of Cys^6-Pro^7 peptide bond. The proportion of the *cis* isomer is 7% and 6% for **I** and **II**, respectively. The appropriate NOE effects in cyclic part of molecules suggest β-turns at positions 2,3 and 3,4. Moreover, in both cases, the temperature coefficient of the amide proton of Asn^5 (0.7 (**I**) and 1.7 (**II**) ppb/K) reveals existence of the NH^5-CO^2 hydrogen bond, which may stabilize β-turn at position 3,4. Similarly, the temperature coefficient of the amide proton of Gln^4 (1.9 (**I**) and 0.5 (**II**) ppb/K) shows that this proton may be involved in a hydrogen bond either with CO^1 (β-turn at position 2,3) or with CO^2 (γ- or an inverse γ-turn at position 3). The three-dimensional structures were generated by MD calculations with simulated annealing algorithm using the AMBER [2] program with time-averaged distances and dihedral angle restraints obtained from nuclear Overhauser effects and vicinal coupling constants, respectively. Finally 300 energy-minimized conformations were obtained for each peptide. Thirteen and six families for **[Cpa^1,L-1-Nal^2]AVP** and **[Cpa^1,D-1-Nal^2]AVP**, respectively, were found with an rms deviation cut-off of 3Å.

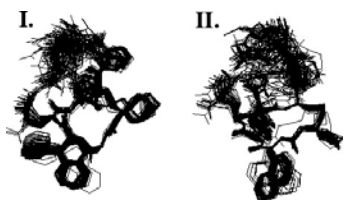

*Fig. 1. Superposed conformations from the family with the highest number of structures of [Cpa^1,L-1-Nal^2]AVP(I) and [Cpa^1,D-1-Nal^2]AVP(II). RMSD $_{1\text{-backbone}}$= 0.233 and 0.148 Å for **I** and **II**, respectively.*

Main structural features of both peptides are β-turns at position 3,4. With the former analogue, either type I or IV of β-turn occurs, whereas with the latter, the βIII-turn

seems to dominate. Furthermore, the conformations of both peptides have also another common feature, they form β-turn at position 2,3. It should be emphasized that the conformations of both analogues are also stabilized by the network of hydrogen bonds between the guanidine group of Arg^8 and the carboxamide group of Gln^4 and Asn^5 side chains. As a result, the C-terminal part of the molecules is in a close contact with the tocin ring.

The antipressor properties of both analogues may be due to the lack of a hydroxyl group in the aromatic side chain of the residue at position 2. In turn, the differentiated activities of both analogues towards V_2 receptors could be attributed to mutual arrangement of the aromatic side chains, still more as it has been established that these residues play a very important role in recognition and binding with receptors. For instance, we have found that the L-1-Nal2 and Phe3 side chains of **[Cpa^1,L-1-Nal^2]AVP** stick out on the same side of the cyclic part of the molecule and lie over each other but are not parallel. In turn, the aromatic rings of D-1-Nal2 and Phe3 in **[Cpa^1,D-1-Nal^2]AVP** are directed in opposite side of the pressin ring plane.

Conclusions

The change in chirality of the residue at position 2 did not affect considerably secondary structure of the peptides. This is, however, one of the modifications that can alter orientation of the side chain. Mutual orientation of the side chains, especially in the cyclic part of the molecule, affects location of the peptide inside the receptor cavity and may determine activities of the analogues.

Acknowledgements

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MOLECULAR MECHANISM OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRI'S) INTERACTIONS WITH SEROTONIN TRANSPORTER

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Introduction

Serotonin selective reuptake inhibitors (SSRIs) are currently among the most frequently prescribed therapeutic agents in depression. Their therapeutic use also includes obsessive-compulsive disorder, panic disorder and bulimia. The serotonin transporter (SERT), which consists of 12 transmembrane helices (TMH) with the amino and carboxy terminals localized intracellularly [1, 2], is the molecular target of SSRIs. The understanding of the mechanism of action of SERT remains a primary goal in the search for developing novel treatments for diseases associated with serotonergic dysfunction.

In the present study the molecular interactions of a series of inhibitors (buspirone analogues) with SERT were theoretically investigated by molecular modeling techniques. The SERT binding affinity of these ligands has previously been measured experimentally.

A 3D model of SERT based on the crystal structure of a bacterial homologue of Na^+/Cl^- -dependent neurotransmitter transporters from *Aquifex aeolicus* [3, 4] was used for the calculations. SERT inhibitors were docked into the model using a flexible Monte Carlo docking procedure as implemented in the Internal Coordinate Mechanics (ICM) software [5]. The binding site was defined based on the information about familiar conversation among the neurotransmitter symporter family (SNF) and site-directed mutagenesis studies of neurotransmitter transporters [6]. Different low-energy conformations of the ligands in the conformational stack accumulated during the flexible docking process were examined to select conformations showing favorable interactions with amino acid residues inside the binding site. For each ligand, the best pose was selected, using the visual inspection of the interactions and the scoring function implemented in ICM program [5], Xscore program [7] and Drugscore program [8].

Results and Discussion

In the present study, favorable interactions between the buspirone analogues and SERT in a pore formed between TMHs 1, 3, 6, 8 were observed. All the ligands interacted with the residues: Tyr95, Ala96, Asp98, Gly100, Asn101 (TMH1); Ala169, Ile172, Ala173, Tyr176 (TMH3); Phe335, Ser336, Gly338, Phe341,

Val343 (TMH6) and Ser438, Thr439, Gly442, Leu443 (TMH8). Recently, site-directed mutagenesis studies have confirmed the involvement of amino acids residues in TMH1 and TMH3 in the ligand binding to SERT [9]. The binding mode of the buspirone analogous suggested by the present study is important for understanding the mechanism of action of these ligands, and for development of new ligands with improved potency and selectivity that might have a therapeutic potential.

Acknowledgements

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SILAPROLINE-CONTAINING ODN ANALOGS

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Introduction

The octadecaneuropeptide (ODN) is a potent inhibitor of food intake [1] and the anorexigenic effect of ODN is blocked by the antagonist cyclo[D-Leu5]OP. *In vitro*, ODN increases intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured rat astrocytes through activation of a GPCR coupled to PLC [2, 3]. The action of ODN on $[Ca^{2+}]_i$ is mimicked by the C-terminal octapeptide RPGLLDLK (OP), partially antagonized (40%) by the linear analog [D-Leu^5]OP, and totally suppressed by the head-to-tail cyclic analog cyclo[D-Leu^5]OP [4, 5]. Silaproline (Sip) is a silylated proline-surrogate which influences both the physicochemical and pharmacodynamic properties of peptides without affecting their native 3D-structure [6]. Since the Pro2 residue of OP and [D-Leu^5]OP is involved in the bioactive conformation of these peptides [5], the aim of the present study was to evaluate the bioactivity of [Sip^2]OP and [Sip^2,D-Leu^5]OP on Ca^{2+} mobilization on cultured rat astrocytes by microfluorimetry.

Results and Discussion

[Sip^2]OP and [Sip^2,D-Leu^5]OP were synthesized by the conventional solid phase methodology on a 433A peptide synthesizer (Applied Biosystems) by using the standard Fmoc manufacturer's procedures and the structure of the peptides was verified by MALDI-TOF mass spectrometry analysis. Application of [Sip^2]OP (10^{-8} M) in the vicinity of single astrocytes provoked a transient increase in $[Ca^{2+}]_i$ with an amplitude significantly higher than that induced by ODN at the same dose (Fig. 1A). Repeated pulses of [Sip^2]OP (10^{-8} M) resulted in a sequential increase of $[Ca^{2+}]_i$ with gradual attenuation of the response suggesting a receptor desensitization phenomenon as previously reported for iterative applications of ODN and its agonist cycloOP [5]. Preincubation of astrocytes with the antagonist cyclo[D-Leu^5]OP (10^{-6} M) partially abolished the effect of [Sip^2]OP (Fig. 1B). Finally, [Sip^2,D-Leu^5]OP (10^{-8} and 10^{-6} M), which did not affect by itself basal $[Ca^{2+}]_i$, reduced by 37% the ODN-evoked $[Ca^{2+}]_i$ increase (Fig. 2) in very much the same way as its non-silylated counterpart, the [D-Leu^5]OP analog [4].

In conclusion, these data indicate that the substitution of Pro2 by Sip enhances the efficacy of OP and does not affect the antagonistic properties of [D-Leu^5]OP. Owing to their better stability and enhanced hydrophobicity, silylated ODN analogs

may cross more easily the blood-brain barrier and thus may prove useful for the development of novel appetite-regulating drugs.

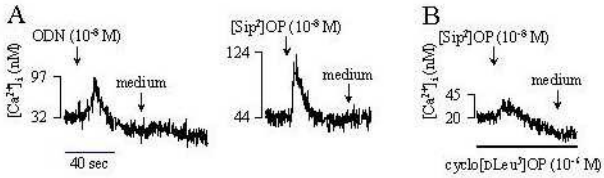

Fig. 1. Effect of [Sip^2]OP (10^{-8} M) in the absence (A) or presence of 10^{-6} M cyclo[D-Leu^5]OP (B) on $[Ca^{2+}]_i$ in singl e astrocytes.

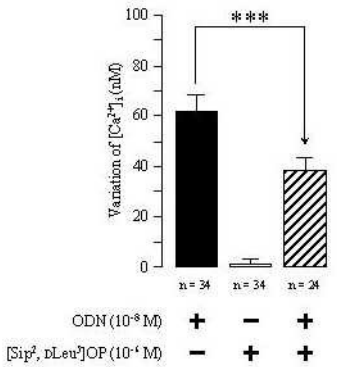

*Fig. 2. Effect of ODN (10^{-8} M), [Sip^2,D-Leu^5]OP (10^{-6} M) or both on $[Ca^{2+}]_I$ in single astrocytes. n, number of cells tested.***p<0.001 vs ODN (Student's t-test)*

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AGONIST AND ANTAGONIST ACTIVITIES OF PEPTIDES RELATED TO 26RFA, THE LAST MEMBER OF THE RFAMIDE PEPTIDE SUPERFAMILY

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Introduction

The term RFamide-related peptides (RFRPs) designates a family of biologically active peptides that possess the common signature Arg-Phe-NH_2 at their C-terminal extremity [1]. 26RFa, a novel RFRP characterized in our laboratory, is the endogenous ligand of the orphan receptor GPR103 [2 - 4]. The structure of 26RFa has been strongly preserved from amphibians to mammals [2], indicating that this peptide subserves important biological functions. The genes encoding the 26RFa precursor and GPR103 are both actively expressed in hypothalamic nuclei involved in the control of energy homeostasis [5], suggesting that 26RFa may regulate feeding behavior. Indeed, *icv* administration of 26RFa induces a potent orexigenic effect in mouse [5, 6]. It has been recently shown that GPR103-deficient mice exhibit a kyphotic hump and suffer from osteopenia [7]. Analysis of the human 26RFa (*h*26RFa) precursor indicates that it may generate several additionnal RFRPs including an N-terminally extended form (43RFa), a truncated form ($26RFa_{(20-26)}$) and a 9-amino acid peptide located upstream of 43RFa (h9RFa). Molecular modeling under ^{1}H-NMR constraints of h26RFa shows that the Pro^4-Arg^{17} region encompasses an α-helix flanked by two N- and C-terminal disordered extremities [8]. The aim of the present study was to investigate the Ca^{2+}-mobilizing activity of a series of human and rodent 26RFa-related peptides in a CHO cell line expressing human GPR103 and the $G\alpha_{16}$ protein subunit.

Results and Discussion

*h*26RFa and *h*43RFa (10^{-11} to 10^{-5} M) induced a dose-dependent increase in $[Ca^{2+}]_i$ with potencies in the nanomolar range (EC_{50} = 6.05 ± 0.73 nM and 5.16 ± 1.45 nM, respectively) (Fig. 1A). The rodent counterparts, *r*26RFa and *r*43RFa, also provoked a concentration-dependent increase in $[Ca^{2+}]_i$ (EC_{50} = 2.22 ± 0.82 nM and

3.52 ± 0.47 nM, respectively) (Fig. 1B). Conversely, *h*9RFa, only present in human, and 26RFa$_{(20-26)}$, despite of a strictly conserved sequence from amphibians to mammals [2], were about 400 times less potent than *h*26RFa, indicating that these peptides do not act as endogenous ligands of GPR103 (EC_{50} = 2910 ± 653 nM and 2320 ± 534 nM, respectively) (Fig. 1 A and B). Although the N-terminal domain of 26RFa is well structured, *h*26RFa$_{(1-16)}$, *h*26RFa$_{(8-16)}$, *h*26RFa$_{(4-17)}$, *r*26RFa$_{(1-16)}$ and *r*26RFa$_{(8-16)}$ were totally devoid of agonistic and antagonistic activities indicating that the C-terminal part of the peptide plays a crucial role for receptor activation (data not shown). In conclusion, these structure-activity relationship data constitute the first step towards the development of new GPR103-ligands that could prove useful for the treatment of feeding or bone mineralization disorders.

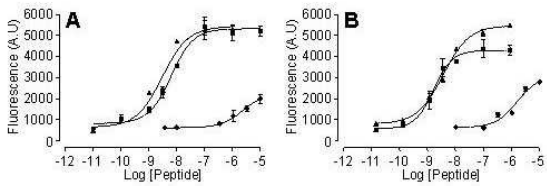

Fig. 1. Effects of graded concentrations of (A) h26RFa (■), h43RFa (▲) and h9RFa (♦) and (B) r26RFa (■), r43RFa (▲) and 26RFa$_{(20-26)}$ (♦) on $[Ca^{2+}]_i$ in CHO GPR103-transfected cells. Each value represents the mean amplitude (± SEM) of the calcium response calculated from at least 6 different experiments.

Acknowledgements

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HUMORAL RESPONSE AGAINST MYCOBACTERIAL HEPARIN-BINDING HEMAGGLUTININ: THE ROLE OF METHYLATION STUDIED USING PEPTIDE MICROARRAYS

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Infection with *M. tuberculosis* most frequently results in a latent form of tuberculosis (TB), without any clinical manifestation. Approximately two billion persons are estimated to be currently infected with the tubercle bacillus, and thus are at risk to develop disease. However, the vast majority of the infected individuals will remain healthy, and TB disease is an exception, despite the fact that around 2 million persons die from TB each year.

M. tuberculosis produces on its surface a methylated protein antigen named HBHA (for heparin binding haemagglutinin) which binds heparan sulfate glycoaminoglycans on the surface of epithelial cells [1], This binding is required for escaping from the lung to deeper organs, and may be responsible for the induction of latent TB.

The methylation pattern of native HBHA (nHBHA) is very complex and involves the last 41 amino acids of nHBHA. This region, called peptide C, is functionally important, as it contains 15 lysines that are responsible for the binding of the protein to sulfated glycoconjugates. The methylation of this functionally important domain protects it against proteolytic degradation. The role of methylation on the cellular response, but not on the humoral response against nHBHA has been studied in detail. We present here the results of a peptide microarray study on the role of the methylation in the humoral response against nHBHA.

Results

The sequence of peptide C is:

LPK**K**AAPA**KK**AAPA**KK**AAPA**KK**AAA**KK**APA**KK**AAA**KK**VTQK

The 13 lysines in bold are modified as mono or dimethyllysine. Consequently, peptide C in nHBHA is a complex combinatorial mixture of about 2^{13} = 8192 polypeptides. $Lys(Me)_2$ is the most frequent amino acid for the 5 first modified Lys residues and statistically as high as 11% of the polypeptide chains present 3 $K(Me)_2AAPAK(Me)_2$ repeats. We thus examined whether these repeats could be the target of antibodies that are produced after immunization with nHBHA.

Peptide C was synthesized as a combinatorial mixture (HBHA 1 CM) by Fmoc/*tert*-butyl SPPS. For each modified position, mixtures of Fmoc-L-Lys(Me)-OH and Fmoc-L-Lys(Me)$_2$-OH were coupled to the peptidyl resin. We have also synthesized unmodified peptide C (HBHA 1) which was treated in solution with formaldehyde in the presence of $NaCNBH_3$ and $NiCl_2$ to give fully methylated peptide C (HBHA 1 M).

These peptides, nHBHA, recombinant HBHA, produced in *E. coli* (rHBHA) and rHBHADC (HBHA without the peptide C domain), as well as tag controls for specificity (HA, Flag, Myc) were used to prepare polypeptide microarrays. These microarrays were used to analyze a pool of sera from mice immunized with nHBHA (Fig. 1) or from mice that receive only the adjuvant (data not shown).

The data show that the capture is specific and requires methylation, since the unmodified HBHA 1 gave a signal close to the background of the microarray. The fully methylated peptide HBHA 1 M and the mixture HBHA 1 CM gave similar fluorescence intensities, showing that HBHA 1 M is an interesting alternative to the combinatorial HBHA 1 CM for the capture of antibodies that are directed against the methylated domain of HBHA.

Further studies using shorter peptides derived from peptide C and sera from mice infected with *M. tuberculosis* are in progress.

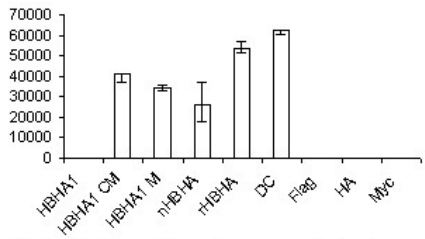

Fig. 1. Peptide-protein microarray analysis of a pool of sera from mice immunized with nHBHA. Microarrays (n = 3) were incubated with the pool of sera, washed and then incubated with tetramethylrhodamine labelled anti-murine IgG antibodies. Data (median, interquartile range) are expressed in arbitrary units (532 nm).

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A 1,2-AMINO ALCOHOL DERIVATIVE OF FMOC AMINO ACIDS: A MASKED ALDEHYDE FOR USE IN SEQUENTIAL CHEMOSELECTIVE LIGATION

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Introduction

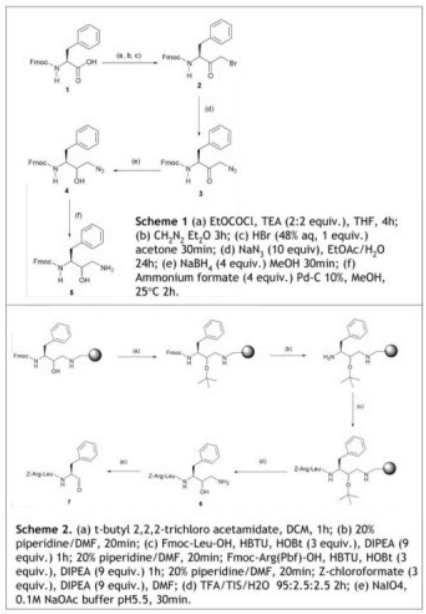
Peptides synthesised by solid phase peptide chemistry have a sequence dependent limit of approximately 100 amino acids. Chemoselective ligation of unprotected peptide fragments in solution utilising the selective reaction of a peptide with a C-terminal aldehyde with another peptide exhibiting an N-terminal hydroxylamine or hydrazine is one method available for the synthesis of larger peptides and proteins [1]. The generation of long peptide fragments by Fmoc chemistry, bearing a C-terminal aldehyde is problematic. Current methods include the attachment of an Fmoc amino aldehyde to threonine resin via an oxazolidine ring, followed by peptide synthesis, and a two-stage deprotection and cleavage [2], or by the reduction of a peptide-Weinreb amide [3]. Both methods are only applicable to the formation of short peptide aldehydes, and also produce aldehyde peptides directly from cleavage from the resin making them inappropriate for sequential chemoselective ligation. One method for the synthesis of long C-terminal aldehyde peptides is by periodate oxidation of a C-terminal 1,2-diol [4]. Masking an aldehyde as a diol has been previously employed in the sequential chemoselective ligation of several long peptide fragments [5]. However, this technique had a number of limitations associated with the amino acid diols, and have led us to the development of a new amino acid masked aldehyde derivative.

Results

A restraint with the diol system was the low level of substitution achieved with chlorotrityl resin with any other derivative than Gly-diol, contributing to low yields of product. Periodate oxidation of a 1,2-amino alcohol is an alternative route to an aldehyde [6], and the amino functionality is significantly more reactive towards chlorotrityl resin than an alcohol. We therefore have developed a synthetic route for the preparation of 1,2-amino alcohol derivatives (Scheme 1), starting from commercially available Fmoc protected amino acids. The small peptide aldehyde Z-RLF-H, a chymostatin analogue, was synthesised using standard Fmoc peptide chemistry (Scheme 2). This small peptide was chosen as a proof of principle, and to allow characterisation of the aldehyde. ^{1}H NMR showed a small amount of epimerisation of the aldehyde following purification by RP-HPLC, which can be avoided in future by purifying the peptides prior to periodate oxidation.

Discussion

The new 1,2-amino alcohol derivatives of Fmoc protected amino acids allow synthesis of peptides bearing a C-terminal amino alcohol in high yield. Unmasking of the amino alcohol to aldehyde by periodate oxidation proceeds smoothly to produce C-terminal peptide aldehydes. Our future goal is the synthesis of long peptide fragments, for sequential chemoselective ligation. Using 1,2-diol derivatives, masked aldehyde peptide fragments had only been produced in small quantities, due to the low substitution of the diol to chlorotrityl resin between 0.01 and 0.05 mmol/g. The substitution levels obtained with the amino alcohol derivatives has been between 0.2 to 0.4 mmol/g, which is optimum for long peptide fragment synthesis. In summary, this new amino alcohol derivative not only allows the facile synthesis of peptides containing C-terminal aldehydes, but will also enable the production of masked aldehyde fragments in high yield, for sequential chemoselective ligation, allowing several peptide fragments to be ligated, and providing access to the chemical synthesis of large peptides and proteins.



Scheme 1 (a) EtOCOCl, TEA (2:2 equiv.), THF, 4h; (b) CH_2N_2 Et_2O 3h; (c) HBr (48% aq, 1 equiv.) acetone 30min; (d) NaN_3 (10 equiv), EtOAc/H_2O 24h; (e) $NaBH_4$ (4 equiv.) MeOH 30min; (f) Ammonium formate (4 equiv.) Pd-C 10%, MeOH, 25°C 2h.

Scheme 2. (a) t-butyl 2,2,2-trichloro acetamidate, DCM, 1h; (b) 20% piperidine/DMF, 20min; (c) Fmoc-Leu-OH, HBTU, HOBt (3 equiv.), DIPEA (9 equiv.) 1h; 20% piperidine/DMF, 20min; Fmoc-Arg(Pbf)-OH, HBTU, HOBt (3 equiv.), DIPEA (9 equiv.) 1h; 20% piperidine/DMF, 20min; Z-chloroformate (3 equiv.), DIPEA (9 equiv.), DMF; (d) TFA/TIS/H2O 95:2.5:2.5 2h; (e) NaIO4, 0.1M NaOAc buffer pH5.5, 30min.

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STRUCTURAL AND FUNCTIONAL DIVERSITY OF ENDOGENOUS REGULATORY OLIGOPEPTIDES

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Introduction

Regulatory oligopeptides generally do not exceed ~50 amino acid residues [1]. For more than a century, natural oligopeptides, have attracted scientific attention as biochemical regulators. The very first such oligopeptide, carnosine (β-Ala-His), was discovered by Gulevitch & Amiradzhibi in 1900 in Russia [2]. Since that time, thousands oligopeptide regulators have been described, and now almost 500 new natural oligopeptides emerge annually, out of a literature of more than 20,000 publications each year. To date, the chemical structures of nearly 6000 oligopeptides have been identified from more than 1000 organisms representing all the biological kingdoms. Scientists usually believe that natural oligopeptides may regulate many vital processes.

Results

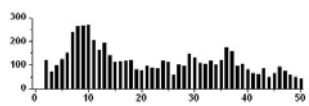
We have compiled the known physical, chemical, and biological properties of the natural regulatory oligopeptides – whether synthesized on ribosomes or by non-ribosomal enzymes – and have constructed an internet-accessible database, EROP-Moscow (Endogenous Regulatory OligoPeptides), which resides at http://erop.inbi.ras.ru. Most peptides included in EROP-Moscow are formed by ribosomal synthesis. This database enables users to perform rapid searches and to carry out statistical analysis of all the available information. EROP-Moscow provides extensive links with the SwissProt-TrEMBL peptide-protein database, as well as with the PubMed biomedical bibliographic database. Many EROP-Moscow oligopeptides are absent from other convenient databases. EROP-Moscow database presents multilevel bioinformation via an html-based interface. This interface includes several pages. All of them contain internal EROP-Moscow links as well as links to external databases SwissProt, PIR, PDB, and PubMed. A special set of Statistics pages is devoted to the overall characteristics of data on oligopeptides listed in EROP-Moscow. They present graphic and tabular information on:

- A chronological diagram, by years, for decoding chemical structures of new oligopeptides;
- Size distribution of oligopeptides (number of amino acid residues);
- Current numerical yield of oligopeptides per taxonomic group;
- Total amino acid residue content of all oligopeptides;
- Relative contributions of international scientists, by home country, to the discovery of new oligopeptide structures;
- Organisms covered (>1000), and tissues and organs (>500);
- Functional classes of oligopeptides (~100);

– Primary literature sources (>300); and
– Authors of the original publications devoted to decoding of oligopeptide chemical structures (>8000).

Discussion

Statistical information of EROP-Moscow database demonstrates the great structural and functional diversity of regulatory oligopeptides. The results of this analysis demonstrate that few integrated biological processes are known which are not regulated, or at least modulated, by oligopeptides. Such roles are especially well known in the regulatory organ systems, viz., nervous, endocrine, and immune systems [3], but their functions extend well beyond the bounds of single organ systems or even of single biological species. Neuropeptides, oligopeptide hormones, antimicrobial agents, and toxins represent the largest functional classes. Antimicrobial oligopeptides produced by prokaryotes themselves, for example, regulate competition for ecological niches and simultaneously function as signaling molecules for species-specific intercellular communication [4]. And even eukaryotic oligopeptide toxins seem to play important roles in regulating interspecies reactions [5]. Thus, apparently natural oligopeptides can be considered as regulators of nearly all vital processes. In addition to solving true informational problems, EROP-Moscow can serve as a basis for new research and for elucidating general principles of structural and functional organization for oligopeptides. For example, the current size distribution of oligopeptides, by number of amino acid residues, shows a numerical peak around ~7 - 10 residues, but this peak has no proper rationale at present. Study of structurally homologous families should facilitate prediction of the functional properties of newly found oligopeptide molecules, should provide bases for classifying newly discovered molecules, and in turn should promote creation of novel, highly efficient pharmaceutics derived from the natural regulatory oligopeptides. Users of EROP-Moscow database are asked to cite this paper in their relevant published research.



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CELLULAR UPTAKE OF S4(13)-PV CELL PENETRATING PEPTIDE: AN ENDOCYTOSIS-INDEPENDENT PROCESS FOLLOWING PEPTIDE CONFORMATIONAL CHANGES INDUCED BY PEPTIDE-MEMBRANE INTERACTIONS

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Introduction

Cell penetrating peptides have been successfully used to mediate the intracellular delivery of a wide variety of molecules *in vitro* and *in vivo*, although the mechanisms by which cellular uptake occurs remain a matter of extensive debate [1, 2]. Recently, we conducted a systematic analysis of the mechanism underlying the cellular uptake of the S4(13)-PV cell penetrating peptide, a chimeric peptide that results from the combination of a 13 amino acid sequence, derived from the Dermaseptin S4 peptide, with the nuclear localization signal of the SV40 large-T antigen (Table 1) [3, 4]. We reported that the S4(13)-PV peptide accumulates inside cells very efficiently through a rapid, dose-dependent and non-toxic process. Studies addressing the effect of several drugs, as well as experiments involving overexpression of a dominant-negative mutant of dynamin, consistently excluded endocytosis as the mechanism responsible for the cellular uptake of this peptide. Comparative analysis of peptide uptake by mutant cells lacking heparan sulfate proteoglycans demonstrated that the presence of these components at the cell surface facilitates peptide uptake [4]. Overall, these results suggest that the cellular uptake of the S4(13)-PV peptide results from its direct interaction with target membranes, which motivated a detailed biophysical characterization of the interaction of the peptide with model membranes.

Table 1. Sequences of the S4(13)-PV and scrambled peptides. The nuclear localization signal of SV40 large T antigen NLS is underlined in the sequence of the S4(13)-PV peptide.

peptide	sequence
$S4_{13}$-PV	ALWKTLLKKVLKA<u>PKKKRKV</u>C–NH_2
scrambled	KTLKVAKWLKKAKPLRKLVKC–NH_2

Results and Discussion

To evaluate the interaction of the S4(13)-PV peptide with membranes, the intrinsic fluorescence of the peptide (which results from a single tryptophan residue located at the N-terminus, Table 1) was analyzed in the presence of lipid vesicles of different charge density (neutral POPC vesicles, and negatively charged POPC:POPG (4:1), POPC:POPG (1:1) or POPG vesicles). Upon interaction with negatively charged membranes, significant changes in the intrinsic fluorescence of the S4(13)-PV peptide were observed, namely a shift of the wavelength of maximal fluorescence emission towards shorter wavelengths and an increase in fluorescence intensity, suggesting that the peptide becomes localized in a highly hydrophobic environment, most likely inserted into the lipid bilayer. In agreement with these results, quenching experiments aimed at evaluating the exposure of the tryptophan residue demonstrated that, upon interaction with negatively charged membranes, the S4(13)-PV peptide becomes less exposed to the aqueous environment.

Circular dichroism analysis demonstrated that upon interaction with negatively charged membranes the S4(13)-PV peptide also undergoes significant conformational changes that are consistent with the formation of helical structures. Comparative analysis using a scrambled peptide (Table 1) showed that such conformational changes are dependent on the peptide sequence.

Analysis of the extent of cellular uptake and subcellular localization of the two peptides demonstrated that the S4(13)-PV peptide is distributed throughout the cytoplasm and nucleus of cells, whereas the scrambled peptide presents a punctated cytoplasmic distribution, characteristic of an endocytic uptake mechanism. These results clearly indicate that the secondary structure acquired by the S4(13)-PV peptide upon interaction with membranes is intricately related to its capacity to translocate across biological membranes and efficiently accumulate inside cells.

Taken together, our data support that the cellular uptake of the S4(13)-PV cell penetrating peptide is a consequence of its direct translocation through cell membranes following conformational changes induced by peptide-membrane interactions.

Acknowledgements

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INTERACTION OF HEME- AND CHLOROPHYLL-BASED COFACTORS WITH *DE NOVO* SYNTHESIZED PEPTIDES

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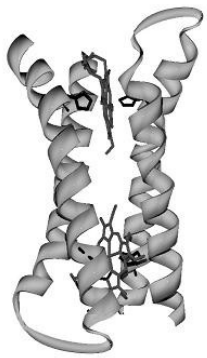
Introduction

De novo synthesized peptides are important for studying and mimicking



Four-helix bundle of peptide M2

the structure-function relationship of native proteins. The light harvesting complexes of higher plants or photosynthetic bacteria absorb and transport light energy to the reaction center, converting light energy to an electrochemical potential. This process includes many electron transfer steps between pigments: (bacterio)chlorophylls, quinones, hemes, etc. The motivation and aim of the present work is to construct small model complexes, e.g., cofactor binding peptides forming four-helix bundles. Hemes or zinc chlorins were incorporated into such peptides which were designed based on the cytochrome *b* subunit of the cytochrome bc_1 complex [1 - 3]. Furthermore, an exogenously, non-covalent bound quinone was added to the peptide-bound zinc chlorins to obtain an artificial peptide-based donor-acceptor system as a first step towards the creation of a synthetic photosystem.

Material and Methods

The chemical synthesis of two amphiphilic peptides containing 62 amino acids was performed with a multiple peptide synthesizer using the Fmoc/*t*-Bu strategy [4] on a PAL-PEG-PS resin. The peptides consist of an α-loop-α-motif. In peptide M1 two homodimeric α-helices are linked through cysteine residues at the C-terminus of each helix, forming a disulfide bridge. In contrast, the loop region of peptide M2 is formed by eight glycine residues. The peptides were purified by HPLC and identified by MALDI-TOF or ESI mass spectrometry. Chlorophyll *a* was isolated

from *Spirulina platensis* and further chemically modified to give Zinc-Pheophorbide *a* (ZnPheid*a*). As a hydrophilic cofactor Zinc-Chlorin *e6* (ZnC*e6*) was applied, which was synthesized from C*e6* by addition of zinc acetate in a buffer solution. Both zinc chlorins were purified by HPLC and characterized by standard methods. For obtaining the peptide-cofactor complexes, the peptides were dissolved in a buffer solution and the cofactors were added. After stirring at 4 °C under green light the samples were purified on a Sephadex G-25 column.

Results and Discussion

The gel filtration chromatography showed the successful generation of four-helix bundles in a buffered solution, in which the pigments are ligated through the histidine residues of the peptides. A stepwise titration of the pigments into the peptides showed, that two cofactors are incorporated into the four-helix bundles. A sharp Soret band at 412 nm and an a and b band (563 and 533 nm, respectively) were detectable originating from the peptide bound heme group. X-band EPR and Resonance Raman spectra showed a six-coordinated low spin Fe(III)-protophorphyrin IX in the four-helix bundle. A redox potential of Em (pH 7) = – 198 mV vs. NHE was determined by redox titration, which gives evidence to two identical heme groups and an antiparallel topology of the dimeric peptides, see Fig. above. The UV-Vis spectra of ligated ZnPheid*a* and ZnC*e6* showed a shift of the Soret as well as of the Qy band in comparison with their unbound species. Excitation in the Soret band of the zinc chlorin peptide complexes resulted in a shift of the Qy band in the fluorescence spectra, as well. Resonance Raman spectroscopy gave evidence for five-coordinated zinc chlorins. The triplet spectrum of the ZnPheid*a*-M1/M2 complex, characterized by light-induced Transient-Electron Paramagnetic Resonance spectroscopy, gave an eaeaea-polarization pattern, whereas that of ZnC*e6*-M1/M2 was *aaaeee*. Following a suggestion from Razeghifard [5], a non-covalently bound quinone was added to the peptide bound zinc chlorins and the electron transfer rate constants for quenching by benzoquinone of $k_{BQ} = (0.7 - 0.8)\times10^{9}\ M^{-1}s^{-1}$ were determined. Furthermore, a time-resolved spin polarized radical pair, $^{3}(ZnCe6^{\bullet+}\ BQ^{\bullet-})$, from the peptide M2 bound pigment was detectable by Transient-EPR.

Acknowledgements

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SYNTHESIS AND CONFORMATIONAL ANALYSIS OF MIXED CYCLIC α/β²-TETRAPEPTIDES

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Introduction

Short linear peptides exhibit a high degree of conformational flexibility which makes them less functional for biological applications [1]. Cyclization is a common methodology for reducing this conformational freedom, enhancing not only the stability but also the bioavailability and metabolic stability of a peptide [2]. However, cyclic peptides as short as tetramers most often still exists as multiple conformers in polar solvents such as water or DMSO [3].

In similarity to D-amino acids and proline, β-amino acids are proven to be turn-inducing residues with potential to enhance structural stability of short peptides [4]. Sewald *et al.* [5] and Fairlie *et al.* [6] have reported studies concerning the incorporation of a β³-amino acid residue into short cyclic peptides, otherwise composed of only α-amino acids, investigating how β³-amino acids influence the conformational stability.

In a similar study, we have investigated the effect of incorporating a β²-amino acid residue into cyclic tetrapeptides.7

Results and Discussion

The position as well as the stereochemistry of the β²-amino acid was of high interest, leading to the synthesis of four mixed cyclic tetramers (**2a,b** and **3a,b**) and a α-analogue (1). Structure **2a** (cyclo(-(*R*)-β²hPhe-D-Pro-Lys-Phe-)) and **2b** (cyclo(-(*S*)-β²hPhe-D-Pro-Lys-Phe-)) have the turn-inducing residues D-Pro and β²hPhe at i and i-1 position, respectively, assuming a stabilizing effect in the D-Pro-β²hPhe sequence whereas **3a** (cyclo(-Phe-D-Pro-Lys-(*R*)-β²hPhe-)) and **3b** (cyclo(-Phe-D-Pro-Lys-(*S*)-β²hPhe-)), have these residues separated in an i and i+2 relation. The (*R*)-isomer has relative configuration as natural α-amino acids.

The synthetic strategy includes standard Fmoc solid phase synthesis of the linear peptide using Barlos resin, followed by cleavage of the linear peptide from the resin under mild conditions (DCM/TFE/AcOH (3:1:1)), and final cyclization in solution.

Initially, all five cyclo-peptides were analyzed by ^{1}H NMR spectroscopic studies in different solvents (D_2O/H_2O (1:9), CH_3OH/ CD_3OD (1:9), and DMSO) and at variable temperatures (DMSO). An X-ray crystallographic study on the side-chain protected (Boc) **2a** revealed the solid-state structure of this peptide.

The structure of **2a** extracted from 2D NMR analysis used as restraints in a computational study features two hydrogen bonds, involving amide protons on the β²hPhe residue, as predicted from the data gained from temperature studies in

DMSO. Likewise, the amide proton of the β^2hPhe **2b** was predicted and found to be involved in hydrogen bonding. The calculation for peptide **3a** is seen to involve a hydrogen bond involving the β^2hPhe residue; again in line with the temperature coefficient. The solution structure of peptide **3b** in water was not calculated due to the presence of two conformers, but the temperature coefficient suggested involvement of the lysine residue in hydrogen bonding.

In short, we could conclude that peptides **2a** and **3a**, both containing (*R*)-β^2hPhe, possessed higher degree of conformational stability than **2b** and **3b**, containing (*S*)-β^2hPhe. The position of the β-amino acid did not affect the stability, but the side-chain chirality of β^2hPhe did.

Acknowledgements

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SUBTILISIN-CHITOSAN BIOCOMPOSITE CAPABLE TO CATALYZE PEPTIDE BOND FORMATION IN LOW WATER MEDIA

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Introduction

One of the main sectors of modern peptide chemistry is synthesis of oligopeptides containing different chromogenic and fluorogenic moieties, peptidomimetics, which are substrates for different proteases. Chemoenzymatic production of such a peptide derivatives is the favorable way. Nowadays hydrolases, particularly proteases and lipases, are intensively used in various laboratory and industrial processes in water, aqueous-organic mixtures as well as in nonaqueous media [1, 2]. Fixing of enzymes on/in suitable insoluble supports has many advantages such as high operational stability, possibility of recycling and improved activity in low water media [3, 4].

Results

Two types of the composite materials (gel and film) were prepared by mixing of chitosan and subtilisin solutions. Re-precipitation was then effected by one of several means: 1) evaporation 2) addition of NaOH 3) addition of glutaraldehyde (GA) crosslinker, 4) addition of tripolyphosphate 5) addition of Na_2SO_4. It was found that all these techniques gave composites containing enzyme, although the levels of incorporation differed drastically, as did specific activity (Table 1).

Table 1. Properties of subtilisin-chitosan biocomposites.

Sample preparation, additive	Loading, mg protein/g biocatalyst	activity/g biocatalyst
film	0.81	0.0389
Gel (NaOH)	0.61	0.0474
Gel (TPP)	8.1	0.06
Gel (1%GA+NaOH)	22.3	0.42
Gel (2.5%GA)	20.2	0.07
Gel (2%GA)	35.8	1.1
Fibers (Na_2SO_4)	20	8.7

The loading increased along with enzyme concentration up to 15mg/mL (fig. 1).

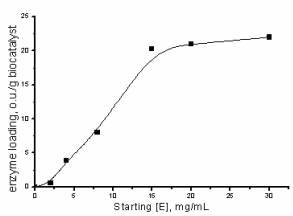

Fig. 1. Dependence of loading on starting subtilisin concentration

After optimization of preparation technique we choose several samples possessing high activity and capable of 60 - 92% formation of the tetrapeptide Z-Ala-Ala-Leu-Phe-pNA in mixture of dimethylformamide/acetonitrile (6/4) (Table 2).

Table 2. Properties of subtilisin/chitosan films, obtained using GA.

Sample preparation	GA concentration, %	Loading, mg protein/g biocatalyst	activity /g biocatalyst	Z-Ala-Ala-Leu-Phe-pNA yield, **%**
Sandwich	0.5	44.34	0.64	0
GA addition before drying	0.25	0.343	3.1	25
	0.5	65	0.84	92
GA addition after drying	0.25	0.42	3.5	80
	0.5	27	0.5	85
Lyophilization	0.5	50	12.7	60

Discussion

Subtilisin immobilized on PVA cryogel, hydrophilic polymeric support, was active and stable in aqueous and low water media [5 - 8]. Chitosan is natural hydrophilic polymer, which can be used as a support for enzyme immobilization [3]. The specific activity (on Z-Ala-Ala-Leu-*p*NA) of subtilisin-chitosan composites, obtained by different techniques varied in a broad range. Most active were fibers formed by Na_2SO_4, but subtilisin leached off this biocatalyst very easily as enzyme was not fixed on the support; fibers were very soft and inconvenient in use.

Treatment by GA gave a material with good mechanical properties and high activity. The dependence of loading on starting enzyme concentration showed on Fig. 1 reach a plateau at [E] = 15 mg/mL revealing a limit of subtilisin incorporation in the chitosan gel.

Films from chitosan/subtilisin biocomposite, crosslinked with GA, were obtained by: 1) treatment of chitosan film by GA and then by subtilisin solution (sandwich), 2) mixing subtilisin and chitosan solutions and then addition of GA before drying, 3) the same as 2) but GA was added after drying and 4) same as 3) but film was lyophilized (Table 2). All samples were active in hydrolysis, especially “sandwich”, but in low water media it was inactive probably due to exposition of enzyme into organic solvent. Other samples catalyzed peptide bond formation in nonaqueous media with product yields from 15 to 92%. The best biocatalyst was film from subtilisin, chitosan and 0.5%GA mixture. Thus subtilisin-chitosan biocatalytic films characterized by preparation simplicity, activity and stability in different media.

Acknowledgements

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A NOVEL APPROACH TO ALZHEIMER'S DISEASE THERAPY: INHIBITION OF Aβ42 OLIGOMERIZATION BY C-TERMINAL Aβ42 FRAGMENTS

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Introduction

Alzheimer's disease (AD) is the major cause of dementia among the elderly. A key event in AD etiology is the assembly of amyloid-β protein (Aβ) into neurotoxic oligomers.

Aβ is produced *in vivo* in two predominant forms comprising 40 (Aβ40) or 42 (Aβ42) amino acid residues. Both peptides aggregate into insoluble fibrils, yet their early assembly occurs via distinct pathways. Aβ42 preferentially forms pentamers, hexamers, and dodecamers, whereas Aβ40 assembles predominantly into dimers, trimers, and tetramers [1]. The oligomers of Aβ42 exhibit substantially greater neurotoxicity than those of Aβ40 [2]. Several studies indicate that the extended C-terminus of Aβ42 plays a key role in the oligomerization process. Molecular dynamics simulations [3] and limited proteolysis/mass spectrometry experiments [4] suggest the existence of a stable turn conformation at the C-terminus of Aβ42, which is not formed by Aβ40.

Here we present an approach for the development of Aβ42 oligomerization inhibitors based on the hypothesis that molecules with high affinity for the C-terminus, such as peptides derived from the C-terminus itself, will disrupt the assembly of Aβ42.

Results

A series of C-terminal fragments (CTFs) of Aβ42, Aβ(x-42) with x = 28 - 39, has been prepared to study their potential to inhibit Aβ42 oligomerization and neurotoxicity.

The inhibition of Aβ42 oligomerization was studied by quantification of Aβ42 hexamers in the presence of increasing CTF concentrations. The amount of Aβ42 hexamer was determined using a cross-linking assay followed by SDS-PAGE and silver staining. The hexamer intensity relative to the entire lane was measured by densitometry. The short CTFs (x = 36 - 39), and control peptides Aβ(30-40) and Aβ(34-40) had no inhibitory effect at concentrations >100 μM. Aβ(x-42), x = 28, 30-35, caused a decrease of the relative hexamer abundance, with Aβ(29-42) as the most active peptide (IC_{50} = 0.16 μM).

The toxicity of Aβ42 CTFs towards differentiated PC-12 (rat pheochromocytoma) cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay to evaluate residual cell metabolism. Except for Aβ(28-42), which was more toxic than Aβ42 and mild toxicity observed for Aβ(32-42), the CTFs were non-toxic up to a concentration of 20 μM. In addition, the MTT assay was used to study the activity of Aβ42 CTFs as inhibitors of Aβ42-induced neurotoxicity. Differentiated PC-12 cells were incubated with a mixture of Aβ42 (5 μM) and each CTF (50 μM) for 15 h. All CTFs displayed inhibitory activity. The most potent inhibitor was Aβ(31-42), which completely abolished Aβ42-induced cytotoxicity.

Dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy were used to study the aggregation and conformational changes of the CTFs over 96 h. Aβ(39-42) through Aβ(34-42) did not show significant scattering at concentrations >100 μM, whereas Aβ(33-42) (128 μM) aggregated readily reaching an average hydrodynamic radius of ~200 nm at 48 h. The aggregation of Aβ(33-42) was accompanied by the formation of ~50% β-sheet structure after 4h, whereas the shorter CTFs remained unstructured. Longer CTFs followed similar aggregation patterns at lower concentrations and with faster kinetics.

Discussion

Based on structural predictions, Aβ42 CTFs were prepared and found to be potent inhibitors of Aβ42 hexamer formation and neurotoxicity. Surprisingly, inhibition of Aβ42 oligomerization and toxicity may be unrelated. Initial observations of fast aggregation of and β-sheet formation by longer CTFs suggest that inhibition of Aβ42 toxicity by these peptides may be related to their propensity to aggregate.

Acknowledgements

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VASOPRESSIN AND OXYTOCIN RECEPTORS INTERACTIONS WITH AGONISTS AND ANTAGONISTS – MOLECULAR MODELING STUDY

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Introduction

The vasopressin and oxytocin receptors (V_{1aR}, V_{2R} and OTR) are membrane–embedded proteins belonging to the large family A of G protein-coupled receptors (GPCRs) [1]. GPCRs are built of single polypeptide chains that traverse the membrane seven times forming the transmembrane domain consisting of seven α-helices (TM), respectively, connected with extracellular (EL) and intracellular (IL) loops [2]. Vasopressin and oxytocin receptors are involved in crucial physiological functions such as regulation of water metabolism, control of blood pressure and stimulation of labor and lactation, mediated via V_{2R}, V_{1aR} and OTR, respectively. As such, they are involved in a number of pathological conditions and are important drug targets. Understanding their inhibition and activation mechanisms may improve design of ligands capable of selective stimulation or blockade of respective receptors presenting therapeutic targets. To investigate the receptor–agonist and receptor–antagonist interactions, five ligands, both endogenous and synthetic agents showing different biological activities have been studied: the natural hormones oxytocin and arginine vasopressin (CYIQNCPLG-NH_2, OT and CYFQNCPRG-NH_2, AVP, respectively); as well as desmopressin ([1-deamino,D-Arg^8]AVP, dDAVP) – highly selective and potent V_{2R} agonist; atosiban ([Mpa^1,D-$Tyr(Et)^2$,Thr^4,Orn^8]OT, AT) – an OTR/V_{1aR} antagonist; and barusiban (Mpa^1,D-Trp^2,Ile^3,*allo*-Ile^4,Asn^5,Abu^6,MeOrn-ol^7,BA) – a potent and selective OTR antagonist. Thirty computer models of receptor–ligand complexes have been modeled *via* docking and molecular dynamics (MD) and analyzed in details.

Results

The relaxed receptor-ligand complexes have been obtained. The model of receptor-ligand interactions, involving both the highly conserved and non-conserved residues has been proposed. In particular, the recurrent appearance of the highly conserved residues strongly indicates their role in ligand binding. Thus, in all complexes, the same highly conserved residues form the ligand binding pocket: Val2.53 [footnote], Gln2.57, Gln2.61, Gln3.32, Met3.36 and Phe6.51. To determine the differences in agonist *versus* antagonist binding to the respective receptors, the receptor-antagonist complexes (OTR–BA, OTR–AT and V_{1aR}–AT) have been set against the receptor–agonist complexes (OTR–OT, V_{1aR}–AVP, V_{2R}–AVP and V_{2R}–dDAVP). The following residues: Asp2.50, Ser3.39, Phe/Tyr6.44 (in OTR and V_{1aR} respectively), Asn7.49 and EL2 Asp186/202 (OTR/V_{1aR}) have been found to interact only with antagonists, whereas Glu1.35, Val3.28, Lys3.29 and

Phe/Phe/Tyr3.37 (in OTR, V_{1aR} and V_{2R} respectively) have been found to be involved exclusively in agonists binding. The non-conserved residues have been determined as crucial for selectivity of binding. The residues located at the extracellular ends of helices have been found to be involved mainly in specific ligand recognition. The agonists binding pockets overlap only partially and different receptor residues have been proposed to control any specific receptor-ligand pair selectivity: Phe185, Val294 and Val314 for OTR–OT; Asn196, Arg201 and Thr333 for V1aR–AVP; Gln180, Arg181 and Arg202 for V_{2R}–AVP; Asp103, Gln180 and Thr190 for V_{2R}–dDAVP.

A hypothesis on a role of the second extracellular loop (EL2) have been put forth. Accordingly, EL2 may provide an anchor for the C-terminal polar part of the ligand approaching from the extracellular side. In the next step, the ligand enters the binding pocket of the receptor and EL2 covers the binding cavity. This binding is supported by a strong attraction of the N-terminal hydrophobic part of the ligand by hydrophobic receptor residues in the lower part of the binding pocket. Moreover, the residues interacting with G protein have been identified and a role of the internal water molecules in ligand binding has been proposed. The water molecules observed in the binding pocket appear to be involved in the receptor–ligand interactions by forming additional hydrogen bonds at their contact surface.

Conclusions

In summary, identification of residues responsible for agonist *versus* antagonist binding, as well as the mechanism of ligand recognition might significantly facilitate the rational design of new vasopressin and oxytocin analogs useful in several pathological conditions pertinent to their receptors. Moreover, given structural homology among family A GPCRs, presented results might be useful in design of drugs acting via other family A members.

Footnote. The TM receptor residues are identified using the Ballesteros-Weinstein (B-W) numbering scheme [Ballesteros J.A. and H. Weinstein, Methods Neurosci 25 (1995) 366] or with the absolute numbers (the non-conserved residues responsible for selectivity toward the respective receptor). In the B-W scheme, the most conserved residue in the TM helix 'N' has been given the number 'N'50, and each residue is numbered according to its position relative to this reference. Residues placed in loops are identified with the loop name and residue absolute number.

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A CELL-BASED APPROACH FOR BIOSYNTHESIS/SCREENING OF CYCLIC PEPTIDE LIBRARIES AGAINST BACTERIAL TOXINS

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Introduction

Available methods for developing and screening drug-like molecules able to knock-out toxins or pathogenic microorganisms have serious limitations. In order to be useful, these new methods must provide high-throughput analysis and identify specific binders in a short period of time. To meet this need, we are developing an approach that uses living cells to generate libraries of small biomolecules, which are then screened inside the cell for activity. Our group is using this new, combined approach to find highly specific ligands capable of disabling Anthrax Lethal Factor (LF) as proof of principle. Key to our approach is the development of a method for the *in vivo* biosynthesis of libraries of cyclic peptides, and an efficient screening process that can be carried out inside the cell. We have used two natural disulfide-containing cyclic peptides, the cyclotides Kalata B1 (KB-1) and MCoTI-II, and the trypsin inhibitor (SFTI-1) as templates for library design. Cyclic peptide libraries based upon these molecular scaffolds have been biosynthesized using engineered protein splicing units in *E. coli* cells. At the same time, we have developed a FRET-based protein reporter that is able to detect LF activity *in vivo*. Both LF and its FRET-based substrate reporter have been co-expressed in *E. coli*.

Results

Our approach for the biosynthesis of circular polypeptides is based on the use of an intramolecular version of the native chemical ligation combined with the use of a modified protein splicing unit [1, 2] (Fig. 1). In the case of cyclotide MCoTI-II and SFTI-1 peptide, the corresponding circular reduced polypeptide were able to fold spontaneously in the cytoplasm to adopt the native structure after cyclization (Fig. 1A and 1B). Using this biosynthetic approach we have also produced a small library based on the KB1 and SFTI-1 scaffolds (Fig. 1C). Structural characterization for the cyclotides KB1 and MCoTI-II was accomplished using standard 2D-homonuclear NMR techniques [3]. The biological activity of MCoTI-II and SFTI-1 was tested using a trypsin inhibitory assay. Our *in vivo* FRET-based reporter for LF was designed to contain the natural substrate of LF flanked by two fluorescent proteins, CFP and YFP [4]. This reporter protein was readily expressed in *E. coli* showing high levels of FRET *in vivo*. The FRET signal was significantly reduced when LF and the FRET-based reporter protein were sequentially expressed in *E. coli*.

In summary we report here the first biosynthesis, using DNA recombinant techniques, of different circular Cys-containing peptides in *E. coli*, including the cyclotide MCoTI-II and the Bowman-Birk sunflower trypsin inhibitor (SFTI-1). We have also shown that our biosynthetic approach can be used to generate cyclotide- and SFTI-based libraries that could be screened *in vitro* or *in vivo*. Finally, we have also developed a new cell-based FRET reporter for the *in vivo* screening of LF inhibitors. Future work will focus in the screening of cyclotide- and SFTI-based libraries against LF using our FRET-based reporter *in vivo*.

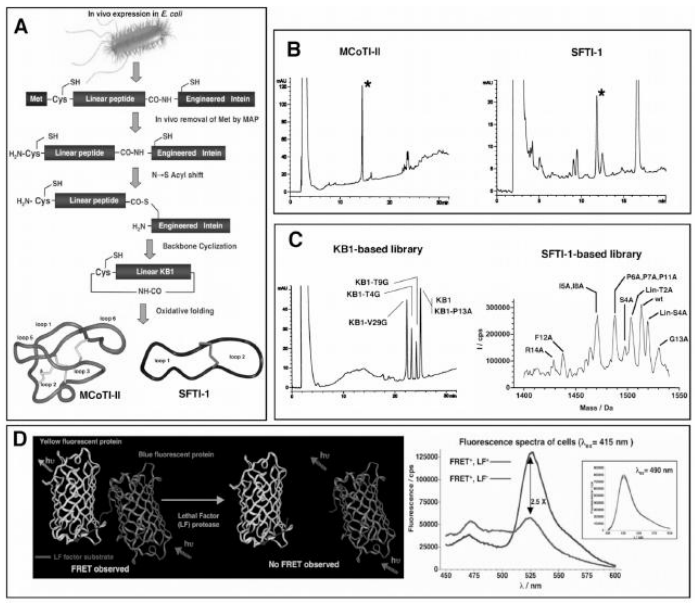

Figure 1. A. *In vivo* biosynthesis of backbone cyclized polypeptides using engineered protein splicing units. **B.** In vivo biosynthesis of cyclotide MCoTI-II and trypsin inhibitor SFTI-1. Analytical HPLC traces of different cellular lysates after being purified on a trypsin-column. The asterisk indicates the corresponding oxidized cyclic product. **C.** *In vivo* biosynthesis of cyclotide- and SFTI-based libraries. Left panel, analytical HPLC trace of a KB1-based library. Right panel, ES-MS trace of a SFTI-1 library encoding different Ala mutants. **D.** FRET-based reporter to screen for LF activity in side living *E. coli* cells.

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DISCOVERY OF NOVEL LOW-MOLECULAR-WEIGHT GPR54 AGONISTS

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Introduction

Metastin (kisspeptin-54) is a product of the metastasis suppressor gene KiSS-1. This C-terminally amidated peptide was identified as the endogenous ligand for the G-protein coupled receptor GPR54 (hOT7T175, AXOR12) [1]. The residues of metastin critical for GPR54 agonistic activity are located at the C-terminal decapeptide amide, kisspeptin-10/metastin(45-54). It has been reported that metastin-GPR54 signaling regulates gonadotropin secretion and negatively regulates cancer metastasis. Therefore, GPR54 agonists could be promising therapeutic agents for hypogonadotropic hypogonadism and metastatic cancers. In order to develop potent low-molecular-weight GPR54 agonists, structure-activity relationship (SAR) study on kisspeptin-10 was conducted.

Results and Discussion

Previously, it was demonstrated that 5- and 7-residue neuropeptides derived from invertebrates possess GPR54 agonistic activity [2]. Metastin and these neuropeptides contain common RW- or RF-amide motifs at the C-terminal. On the basis of the structural similarity between these peptides, several peptides having a basic modification group at the N-terminus were designed and synthesized by standard Fmoc-based solid-phase peptide synthesis and on-resin modification of the N-terminus. GPR54 agonistic activities of the synthetic peptides were assessed by Flipr assay, in which the increase of intracellular Ca^{2+} ion induced by GPR54 stimulation was detected. As a result, we found that two novel pentapeptides showed high level potency and efficacy for GPR54 [FM052a: BisPy-Amb-FGLRW-NH_2; FM053a: Gmb-FGLRW-NH_2; BisPy = *N,N*-bis(2-pyridinylmethyl), Amb = 4-(aminomethyl)benzoyl, Gmb = 4-(guanidinomethyl)benzoyl] [3]. These peptides suppressed the migration of pancreatic cancer cell lines and stimulated phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) [4]. Furthermore, we investigated what functional groups could substitute for the N-terminal and side-chain of the constituent residues [5]. Systematic modification using a variety of aromatic acyl groups such as substituted benzoyl groups afforded a novel GPR54 agonist, TOM80 [4-fluorobenzoyl-FGLRW-NH_2], that is equipotent to the parent kisspeptin-10.

The classical quantitative structure-activity relationship (QSAR) study on eight peptides having a 4-substituted benzoyl group at the N-terminus gave the following equation:

$$-\log Q = 1.13(\pm 0.39)F + 0.37(\pm 0.17)E_s - 0.23(\pm 0.16)$$

$$n = 8,\ s = 0.089,\ r^2 = 0.92\ [Q = EC_{50}(\text{peptide})/EC_{50}(\text{kisspeptin-10})]$$

This equation indicated that inductively electron-negative and small substituting groups are preferred for the position 4 on the benzoyl group. This was in agreement with the observed bioactivity of the peptides. 3D-QSAR using CoMFA of 25 peptides also supported the equation.

In conclusion, through the SAR study on kisspeptin-10 and the down-sized analogues, the N-fluorobenzoyl pentapeptide TOM80 was identified as a novel GPR54 agonist. This SAR data rationalized by classical and 3D-QSAR analysis could be useful for design of more potent GPR54 agonists and antagonists.

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SYNTHESIS AND CONFORMATIONAL STUDIES OF GLYCOSYLATED β-PEPTIDES

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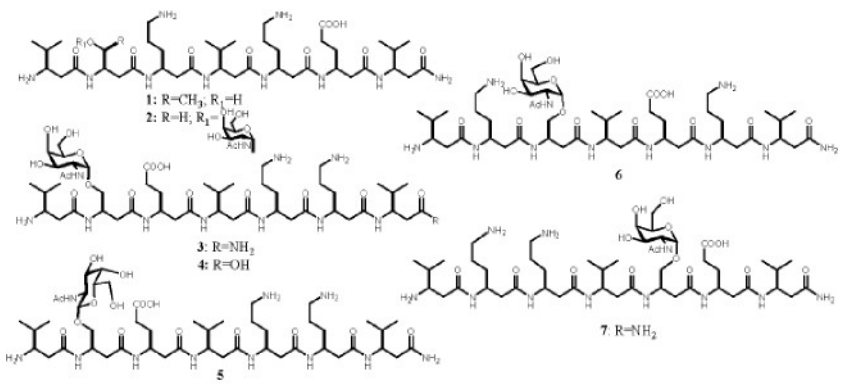
Introduction

β-Peptides are unnatural backbone modified peptides which are stable towards both metabolic and proteolytic degradation [1]. They have the ability to form defined and stable secondary structures with as few as six residues.2 One of the most studied secondary structure is the 3_{14}-helix formed when the β-peptide is composed of acyclic proteinogenic β^3-amino acids.

A majority of proteins in nature are post-translationally modified and the most abundant post-translational modification is protein glycosylation. This modification affects both the biological as well as the structural properties of the peptide.

By linking monosaccharide moieties synthetically to β^3-peptides, we think that enhanced knowledge about the properties of β-peptides foldamers can be gained.



Results and Discussion

Initially, a linear β^3-peptide (**1**) and its glycosylated analogue (**2**) were synthesized by standard Fmoc solid phase peptide synthesis. The conformations were first investigated by circular dichroism (CD) spectroscopy in both methanol and buffer solution (pH 6.9). If the peptide adopts a left-handed 3_{14}-helix it should give rise to a CD pattern with minimum, zero crossing, and a maximum at 215, 208, and 198 nm, respectively.

By comparing the intensity of the absorption at 215 nm it was seen that the incorporation of a carbohydrate moiety on β^3-hSer in position 2 of the peptide leads to a slight destabilization of the 3_{14}-helical conformation in methanol solution. In water, both β^3-peptides shows a dramatic decrease of absorption suggesting that they only partially adopt this conformation in aqueous solution.

Additionally, detailed ^{1}H and 2D NMR studies were performed in CH_3OH/CD_3OD (9:1), using the information retrieved as parameters in a Macro Model conformational search. From these investigations it was concluded that the unglycosylated **1**, as expected, adopts a stable 3_{14}-helical secondary structure whereas **2** forms 2 sets of conformational families due to the possibility of both Orn-5 and Glu-6 to coordinate to the *N*-acetyl group of the monosaccharide.

In according with design strategies developed Schepartz *et al*. [3] we designed and synthesized five new glycosylated β-peptides (**3 -7**). Conformational studies of these was initiated by CD spectroscopy of **2 - 7** in methanol and PBC buffer (pH 7) (c = 0.1 mM at 25.0 °C). In methanol, all glycosylated β^3-peptides adopt 3_{14}-helical structures with equal intensity, except **6** which has noticeably lower intensity. Interestingly, in PBC buffer (pH 7) β^3-peptides **3**, **4** and **7** gives rise to 3_{14}-helical structures possessing higher stability compared to the others. Concerning the stability of **3** and **4** it was concluded that by changing the position of the two ion-bridging residues β^3-hOrn and β^3-hGlu as compared to **2** resulted in enhanced stability. The use of a free C-terminus in this sequence does not give rise to further stabilization, but is not acting destabilizing either. The GalNAcβ^3-hSer position in β^3-peptide **7** is favorable, but it is still left to investigate whether a free C-terminus in this sequence would give rise to even further stabilization.

Acknowledgements

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SYNTHESIS AND CIRCULAR DICHROISM SPECTROSCOPIC INVESTIGATIONS OF LINEAR AND CYCLIC β-PEPTOIDS WITH α-CHIRAL SIDE CHAINS

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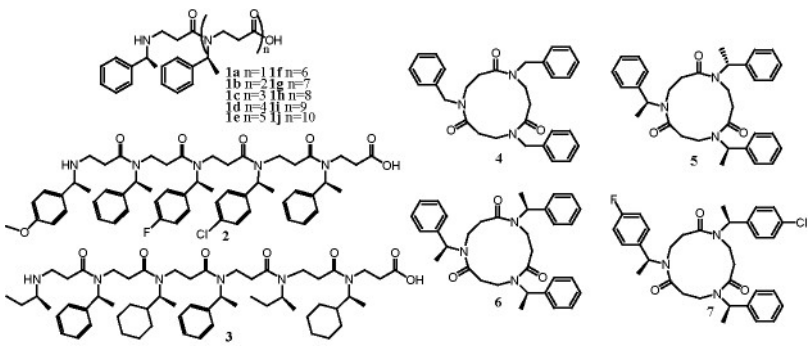
Introduction

α-Peptoids, i.e. oligo-*N*-substituted glycines, with α-chiral substituents on the nitrogen atom are known to fold into helical structures [1] and several interesting biological applications have been reported based on this property [2].

The synthesis of short β-peptoids with non-chiral substituents, i.e. oligo-*N*-substituted β-Ala, was first described by Hamper *et al.* in 1998 [3]. We developed a solid-phase procedure for preparing β-peptoids with α-chiral aromatic *N*-substituents and here we describe the folding propensity studies by CD spectroscopy [4].

Results and Discussion

β-Peptoids with α-chiral *N*-substituents are expected to be difficult to synthesize due to low reactivity of the secondary amine in the acylation step, as well as sterical repulsions during the nucleophilic addition of the α-branched side-chain. Optimal condition for solid-phase synthesis of β-peptoids was concluded to be the use of Tentagel resin in high water content solution.



With the optimized procedure at hand, the solid phase synthesis of *N*-chiral ((*S*)-*N*-1-phenylethyl) β-peptoids **1a-j** was initiated. Additionally, β-peptoids **2** and **3**, with variations in the α-chiral side-chains, and four cyclic tri-β-peptoids (**4-7**) were synthesized.

The ability of the *N*-(*S*)-1-phenylethyl β-peptoids (**1a-j**) to adopt an ordered secondary structure was evaluated by CD spectroscopy. The folding propensities of the β-peptoids **1a-j** were first analyzed in methanol (c = 0.1 mM, 25 °C). All ten β-

peptoids gave rise to CD spectra almost identical in both shape (double minima near 204 and 218 nm) and intensity with those arising from right-handed helical secondary α-peptoid analogues recorded in acetonitrile [5]. The CD spectra of **1b**, **d**, **f**, **h** and **j** in acetonitrile (c = 0.1 mM, 25 °C) gave rise to similar results. Further, the influence of temperature (-5 - 75°C) as well as solvent was studied using β-peptoid **1d**. TFE are known to stabilize helical secondary structures in polypeptides, but have marginal effects for α-peptoids [6]. Neither did we observe a significant change in the CD pattern as compared to the spectra observed in methanol and acetonitrile. Concerning the temperature studies, a decrease of CD intensity was observed with increasing temperature.

The ability of β-peptoids **2** and **3** to fold was investigated by CD spectroscopy in (c = 0.1 mM, 25 °C). As comparison with the CD spectra of pentamer **1d** both **2** and **3** gave rise to CD spectra with lower intensities.

In conclusion, despite the similarity between the CD spectra of **1a-j**, **2** and **3** recorded in methanol and acetonitrile (only **1b**, **d**, **f**, **h** and **j**) and those reported for *N*-(*S*)-1-phenylethyl α-peptoids; **5a** our results do not show any proof that our β-peptoids form ordered secondary structures. Nonetheless, longer chain β-peptoid with α-chiral side-chains is still an interesting class of biomimetic oligomers which may be applicable in areas that do not necessarily require secondary structure formation in order to function.

Acknowledgements

This work was supported by Vetenskapsrådet (the Swedish Research Council), the Carl Trygger Foundation, and Magnus Bergvall's Foundation. S.Z. is grateful to the Wenner-Gren foundation for a fellowship 2003-2004.

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INTRA-AND INTERMOLECULAR FLEXIBILITY: A NEW VIEW ON PROTEIN-PROTEIN INTERACTIONS

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Introduction

While proteinases are highly beneficial, they carry on a potential danger in living cells, thus put under strict control both in time and place. Several man-made drugs are potent inhibitors such as Captopril, regulating hypertension by acting on metalloproteases with a nanomolar inhibition constant ($K_i \approx 4$ nM) [1], or Melagatran, a μM inhibitor of a serine protease [2] controlling blood coagulation However, natural inhibitors such as BPTI (bovine pancreatic trypsin inhibitor) can exceed the above K_i values and reach the sub picomolar range (e.g. for BPTI $K_i <$ 0.1 pM on bovine trypsin). Half a decade ago, a pair of inhibitors were isolated in our laboratory from the desert locust, *Schistocerca gregaria*: SGCI (*Sch. gregaria* chymotrypsin inhibitor) and SGTI (*Sch. gregaria* trypsin inhibitor) [3]. The biological significance of these inhibitors lies in their – yet unresolved – connection with locust invasions, a threat even these days in Africa and the Middle East [4]. Understanding the structure-activity relationship of these inhibitors could not only have biological and agricultural significance, but they may also serve as prototypes to decipher the mechanism of how Mother Nature designed such potent inhibitors during evolution.

Results

The solution state structure of the above two inhibitors determined by NMR spectroscopy [5] reveals that these 35 amino acid residue long peptides behave like small proteins sharing a scaffold composed of an antiparallel β-sheet stabilized by three disulfide bridges and organized around a hydrophobic core. Unlike peptide hormones of comparable size, the success of these inhibitors strongly depends on their 3D structure. The common view on canonical inhibitors is that the rigidity of their proteinase binding loop is the key to inhibition [6]. These inhibitors, while having a unique 3D-structure also "preserve" considerable local flexibility (peptide-like feature) needed for adaptivity and specificity.

To deduce the 'minimal' scaffold responsible for biological activity, we have designed and synthesised cyclopeptide type peptidomimics [7]. Our smallest models incorporating both the entire binding loop and additional subsets of the original inhibitor, are weak inhibitors. Larger and more "sophisticated" models became sub μM inhibitors ($K_i \sim 10^{-7}$ M). In parallel, the small models show no well defined 3D structure [8] unlike the longer ones presenting a better defined overall scaffold.

Beside structure, backbone dynamics also have a role in the inhibition mechanism. By NMR methods using isotope-labeled inhibitors, we have established that the two inhibitors, SGCI and SGTI have distinguishable dynamics properties. With some exaggeration, we could say that differences between the two natural entities are much more pronounced in terms of dynamics than in terms of shape [9]. X-ray analysis of the complex of the arthropod trypsin with SGTI [10] showed that the binding site is extended both toward the C- and the N-terminus, with segments showing conformational differences. In fact, residues that are involved in the binding due to the extended binding site are those that have different backbone fold in the free and in the complex state.

Discussion

Therefore, the biological specificity and the very tight binding is due to the revealed extended interactions. If so, the adaptive interfaces have to have specific dynamic properties. Very tight binding coupled with specificity requires not only a suitable scaffold with all required functional groups (structural properties), but also special dynamic features. In conclusion, we have demonstrated that not only structural but also flexibility issues have to be addressed to explain full biological efficiency. Thus, the „Emil Fisher's lock-and-key" concept, while fruitful in interpreting the action of canonical inhibitors, should be refined by including dynamical properties of the partners. In the design of peptidomimetics for other proteinases, or perhaps in the case of other drug candidates, the consideration of mobility (flexibility-rigidity) beside structural features could be of great significance.

Acknowledgements

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THE STABILITY OF SYNTHETIC STATHERIN IN EXTRACTS OF HUMAN PAROTID, SUBMANDIBULAR AND SUBLINGUAL GLANDS

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Introduction

Whole saliva is composed of secretions from parotid, submandibular and sublingual glands, and smaller ones from saliva of minor salivary glands (e.g. palatal and labial). Saliva contains a variety of proteins and polypeptides. One of them is statherin, a low-molecular weight, acidic, phosphorylated miniprotein, rich in tyrosine, proline and glutamine [1]. Statherin RNA was detected in parotid and submandibular glands [2, 3]. Statherin molecular weight is 5380 Da, and consists of 43 amino acid residues. Statherin is a multifunctional molecule that possesses a high affinity for hydroxyapatite, and functions as a boundary lubricant on the enamel surface. This miniprotein can substantially inhibit the primary and secondary precipitation of calcium phosphate salts. Some microbes present in the oral cavity (e.g. *Porphyromonas gingivalis, Fusobacterium nucleatum*) display a tendency to adhere to the molecule of statherin adsorbed on the surface of hydroxyapatite. In addition, statherin may take part in the transport of calcium and phosphate during secretion in the salivary glands. Statherin plays a role in processes of mineralization in the oral cavity, and takes part in the formation of acquired dental pellicle.

The aim of our study was to investigate the stability of statherin in the extracts of the major salivary glands.

Results and Discussion

Statherin was synthesized manually by the SPPS method using the Fmoc technique and purified and analyzed by the RP-HPLC [4]. Submandibular, sublingual and parotid gland tissues were obtained at autopsy 12 h after death. Samples of the gland tissues were homogenized, centrifugated (30,000 g, 30 min., 4 °C) and the supernatants were frozen and stored at –70 °C prior to analysis. Synthetic statherin was dissolved in the 10 mM Tris-HCl buffer of pH 8.2 (concentration 0.5 mg/ml) and was added to supernatants before analysis (45 μg/ml). The samples were mixed and divided into two parts which were stored at room temperature (RT) and -30°C and were analyzed for the presence of statherin by the MALDI-TOF MS technique and SDS PAGE. The MS analyses were performed at the beginning of experiment, after 4 h of storage and later every 24 h. The products of decomposition of statherin at RT were observed in the MS spectra after 4 h. After 24 h of storage at RT the peak of statherin was missing in the spectra of samples of the synthetic statherin in

extracts of submandibular and sublingual glands. In the case of the extract of parotid gland, statherin was not detected after 48 h. In all samples that were frozen, statherin was present even after one year. The prepared samples stored at RT were also analysed by SDS PAGE using a 15% gel at the beginning of the experiment and later every 24 h. Synthetic statherin was used as standard. In all prepared extracts of the major salivary glands the statherin bands were missing after 24 h of storage.

In conclusion, statherin has been found to be decomposed not only in extracts of the parotid and submandibular glands where RNA coding this peptide was detected, but also in the extract of sublingual glands. Both the storage time and temperature have been found to affect the stability of synthetic statherin.

Acknowledgments

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A NEW FAMILY OF SERINE PROTEINASE INHIBITORS: PURIFICATION, AMINO ACID SEQUENCE, TOPOLOGY OF DISULFIDE BRIDGES AND CHEMICAL SYNTHESIS OF INHIBITORS ISOLATED FROM *Mirabilis jalapa* AND *Spinacia oleracea* SEEDS

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Serine proteinase inhibitors of a common canonical mechanism of action are divided into at least twenty families based on amino acid similarities, topology of disulfide bonds and localization of reactive site [1].

From the seeds of garden four-o'clock and spinach we isolated two serine proteinase inhibitors (MJTI I - *Mirabilis jalapa* trypsin inhibitor and SOTI I - *Spinacia oleracea* trypsin inhibitor), which are probably the representatives of a new family of inhibitors. They were found to be a single polypeptide chain proteins of molecular weight 3593 Da (SOTI I) that consist of 35 amino acid residues and 4060 Da MJTI I (37 amino acids) respectively. The purification procedures of these inhibitors included affinity chromatography on immobilized methylchymotrypsin in a presence of 5 M NaCl, ion exchange chromatography and/or preparative electrophoresis and finally RP-HPLC on C_{18} column.

Primary structures of SOTI and MJTI (Fig. 1) differ from well-known trypsin inhibitors, but show significant similarity to one another, as well as to the antimicrobial peptides isolated from the seeds of *Mirabilis jalapa* (MJ-AMP1, MJ-AMP2), *Mesembryanthemum crystallinum* (AMP1) and *Phytolacca americana* (AMP-2 and PAFS-S) and hemolymph-origin of *Acrocinus longimanus* (Alo-1, 2 and 3).

The association equilibrium constants (K_a) for MJTI I and SOTI I were determined by the Green-Work method modified in the laboratory of M. Laskowski [2]. For both peptides K_a with bovine β-trypsin were found to be about 10^7 - 10^9 M^{-1}.

Both inhibitors were synthesized by the solid-phase method, using Fmoc-chemistry. Tenta Gel S AC-Gln(Trt)-Fmoc and Tenta Gel S AC-Ala-Fmoc (substitution of amino acid 0.2 meq/g, Rapp Polymere, Germany) were used as a support. The syntheses were carried out manually. Disulfide bridges for synthesised peptides were formed after appling air oxidation. The synthesized inhibitors and inhibitors isolated from plants have similar properties. The disulfide bridge pattern in both

isolated inhibitors was established after digestion with thermolysine followed by proteinase K, and MALDI-TOF analysis [3]. The disulfide bridges pattern for both peptides is as follow: Cys^{1}- Cys^{4}, Cys^{2} - Cys^{5} and Cys^{3} - Cys^{6}.

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MJTI I  EDEECAKTDQIC-PPNAPNYCCSGSCVPHPRLRIFVCA
SOTI I     KCSPSGAICSGFGPPEQCCSGACVPHPILRIFVCQ
MJ-AMP1   <ECIGNGGRCNENVGPPYCCSGFCLRQPGQGYGYCKNR
MJ-AMP2     CIGNGGRCNENVGPPYCCSGFCLRQPNQGYGVCRNR
PAFP-S    AGCIKNGGRCNASAGPPYCCSSYCFQIAGQSYGVCKNR
AMP-2      ACIKNGGRCVASGGPPYCCSNYCLQIAGQSYGVCKKH
AMP1      AKCIKNGKGCREDQGPPFCCSGFCYRQVGWARGYCKNR
Alo-1       CIKNGNGCQPDGSQGNCCSRYCHKEPGWVAGYCR
Alo-2       CIANRNGCQPDGSQGNCCSGYCHKEPGWVAGYCR
Alo-3       CIKNGNGCQPNGSQGNCCSGYCHKQPGWVAGYCRRK
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Fig. 1. Sequence alignment of sequences of MJTI I and SOTI I with homological proteins.

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A NOVEL POLYHISTIDINE TYPE LIGAND FOR ZINC(II) AND COPPER(II) BINDING

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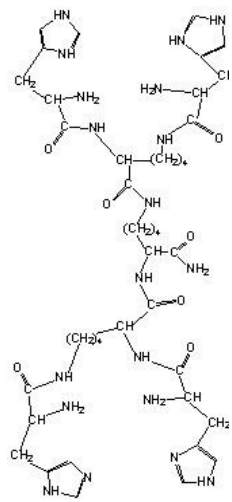
Introduction

Histidines play essential role in binding of biological metal ions, either in small or macromolecular chelating molecules, e.g. in metalloenzymes. Therefore the low molecular weight polyhistidine type ligands are of potential importance for use as model substances. The imidazole group in the histidine side chain is one of the most effective metal ion binding site in metalloproteins. In most cases it is supposed to bind in a monodentate way to the metal ions, however, some examples are already known of bidentate or chelate type coordination. Our earlier studies on the copper(II) complexes of various hexapeptides revealed that histidine offers a broad variety of metal ion binding sites [1].

Results and Discussion



Continuing our investigations on a novel branched oligopeptide type ligand - $(His)_4(Lys)_2Lys\text{-}NH_2$ (as shown in Fig.) - prepared by solid phase peptide synthesis - we investigated the metal ion binding properties with zinc(II) and copper(II).

Such multihistidine type ligands are used as molecular recognition agents, but they are also sensitive to metal ions. Beside the modelling of the biological system we can also expect, that such ligands will bind strongly and selectively the different metal ions, depending on the pH of the solutions. Such a behaviour would be of great value in the environmental application of these ligands attached to a solid support.

The eight primary metal-binding sites are the four imidazole and four ammine groups on the ligand. pH-potentiometric titrations revealed, that up to pH 8 all these donor atoms loose their protons on increasing pH. The competition between the protons and the metal ions results in the decrease of pK values to about 1-3 in the case of copper(II) and to about 4-6 in the case of zinc(II) ions. This reflects the higher stabilities of the complexes formed with copper(II) in spite of the weak axial coordination that seems to occur in zinc(II) complexes. A solid substance is precipitated in both case above pH 8. This is a neutral complex with copper(II) and

a mixed hidroxo or a hydroxo complex with zinc(II). In addition to ammine and imidazole nitrogens, in the presence of copper(II) ions the amide nitrogens may loose protons, as well, that results in a clear solution above pH 10 again. Combined potentiometric, spectrophotometric, CD and NMR spectroscopic methods were utilized to investigate the speciation and the structure of the complexes formed in aqueous solution.

The investigations revealed, that the main binding mode of the ligand up to pH 8 is the bis-histamine type coordination. In the binding process the various branches of the ligand behave independently from each other, but the formation of the histamine type chelates occur in a cooperative way. The CD spectra of the copper(II) complexes show clearly that in the bis-histamine type coordination mode the two imidazole and the two amine donor groups are in *cis* position to each other, respectively. This will result in a sterical constraint between the two bulky imidazole rings and hence they are twisted out of the equatorial coordination plane giving rise to enhanced chirality of the complex. The significant change of the CD spectra in the alkaline medium also proves the rearrangement of the donor atoms around the metal ion, which is due to the deprotonation and the parallel coordination of the peptide nitrogen donor atoms. The subsequent deprotonation will occur on the shortest branch of the ligand, where there is a possibility of formation of fused five membered chelate ring. This type of coordination rearrangement is commonly observed among the histidine containing short peptides, with the histidine at the N-terminus [3].

The prepared Cu(II) complexes cleaved DNA, but it is not known whether in oxidative or in hydrolytic manner. Because of this ambiguity further studies with Zn(II) complexes will be undertaken.

Acknowledgments

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SYNTHESIS AND BIOLOGICAL PROPERTIES ANALOGUES OF CYCLOLINOPEPTIDE A CONTAINING HOMOPHENYLALANINE OR α-HYDROXYMETHYLPHENYLALANINE

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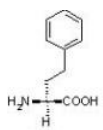
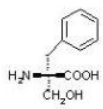
Introduction

The search of new immunosupressants, exhibiting the mechanism of action characteristic for cyclosporine A (CsA) and FK-506 is an important challenge for medicinal chemistry. Cyclolinopeptide A (CLA) natural cyclic nonapeptide [cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe)] possesses a strong immunosuppressive activity comparable with that of CsA in low doses. The possibility of practical application of CLA as a therapeutic agent is limited due to its high hydrophobicity. It has been suggested that the tetrapeptide sequence Pro^6-Pro^7-Phe^8-Phe^9 is responsible for the interaction of the CLA molecule with the proper cellular receptor. In order to evaluate the role of this tetrapeptide unit for biological activity of native peptide, we decided to modified this fragment. We present linear and cyclic CLA analogues in which phenylalanine residues have been replaced with homophenylalanine (HoPhe) or amphiphilic α-hydroxmethylphenylalnine (HmPhe).



HoPhe



HmPhe

Results and Discussion

Racemic α-hydroxymethylphenylalanine was obtained via selective α-hydroxymethylation of *N*-benzoylphenylalanine oxazolone [1], and resolved by fractional crystallization of diastereoisomeric salt of its *N*-Bz derivative with (-)quinine [2]. Peptides were synthesized by the manual solid-phase method. Standard *N*-Boc-protected amino acids and homophenylalanine were obtained from commercial sources. Boc-HmPhe was synthesized as previously described [3]. For 'difficult' coupling, when HmPhe was acylated or used as an acylating component, prolonged reaction times (24 and 16 h for repeated coupling) were necessary. The crude linear peptides were cyclized (PPAA or EDC/HOAt) and purified by preparative reversed-phase HPLC (purity >95%). Molecular weights were confirmed by mass spectrometry (Table 1).

Table 1. Physicochemical data for new analogues of LA and CLA

No	Peptide Structure	HPLC t_R	MALDI Formula	MW	[M+Na]⁺
1	(*R*)-**HmPhe**-Phe-Leu-Ile-Ile-Leu-Val-Pro-Pro	11.78[a]	$C_{58}H_{98}N_8O_{11}$	1110.68	1088.41
2	Pro-(*R*)-**HmPhe**-Phe-Leu-Ile-Ile-Leu-Val-Pro	13.63[a]	$C_{58}H_{98}N_8O_{11}$	1110.65	1088,38
3	(*R*)-**HmPhe**(*R*)-**HmPhe**-Leu- Ile-Ile-Leu-Val-Pro	13.03[a]	$C_{59}H_{100}N_8O_{11}$	1140.69	1118.65
4	Leu-Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**-Phe	10.33[a]	$C_{58}H_{92}N_8O_{12}$	1072.35	1095.12
5	**HoPhe**-Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe	8.43[a]	$C_{58}H_{92}N_8O_{12}$	1072.35	1095.12
6	Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**-**HoPhe**-Leu	5.75[b]	$C_{58}H_{94}N_8O_{12}$	1086.42	1109.42
7	c[Pro-Pro-Val-Leu-Ile-Ile-Leu-**Phe**^{D}-(*R*)-**HmPhe**]	15.95[a]	$C_{58}H_{92}N_8O_{10}$	1070.41	1092.62
8	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**^{L}-**Phe**^{D}]	16.53[a]	$C_{58}H_{92}N_8O_9$	1054.38	1076.11
9	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**Phe**^{L}-**HoPhe**^{D}]	16.37[a]	$C_{58}H_{92}N_8O_9$	1054.38	1076.12
10	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**^{L}-**HoPhe**^{D}]	16.73[a]	$C_{58}H_{94}N_8O_9$	1048.41	1090.63

Biological investigations (Table 2) showed that among modified HoPhe peptides, compounds **8** and **9** displayed similar inhibitory properties in the assays of mitogen-induced B-cell proliferation and the humoral immune response *in vitro*. Surprisingly, **8** strongly inhibited T-cell proliferation at 100 ug/ml concentration whereas in this assay **3** was stimulatory.

Table 2. Immunological activities of the peptides

No	Peptide Structure	Immunological characteristics
1	(*R*)-**HmPhe**-Phe-Leu-Ile-Ile-Leu-Val-Pro-Pro	relatively strong inhibitor of antibody production, effects on cell proliferation not tested
7	c[Pro-Pro-Val-Leu-Ile-Ile-Leu-**Phe**^{D}-(*R*)-**HmPhe**]	relatively strong inhibitor of antibody production, effects on cell proliferation not tested
8	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**^{L}-**Phe**^{D}]	exceptionally strong suppressor of cell proliferation and antibody production
9	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**Phe**^{L}-**HoPhe**^{D}]	stimulator of T-cell proliferation but inhibitor of B-cell proliferation and antibody production
10	c[Leu-Ile-Ile-Leu-Val-Pro-Pro-**HoPhe**^{L}-**HoPhe**^{D}]	stimulator of T-cell proliferation, weak inhibitor of B-cell proliferation and antibody production

Peptide **10** showed a moderate inhibitory property in the T-cell proliferation and the humoral immune response *in vitro* but a stimulatory action in the T-cell proliferation assay. HmPhe peptide (**7**) exhibited a moderate suppressive activity in the humoral immune response *in vitro*, their effects were, however, not strictly dose-dependent as in the case of **8** and **9**, i.e. similar degree of suppression was achieved with low and high concentration of these peptides. Of importance, **8** and **10** were devoid of cell toxicity at 10 and 100 ug/ml concentrations. Therefore, peptide **8**, an universal suppressor, appears to be attractive to search for its potential therapeutic applications

Acknowledgements

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CONFORMATIONAL STUDIES OF TWO NEW CYCLIC PEPTIDES WITH INHIBITORY ACTIVITY ON LIGAND-INTEGRIN-BINDING

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Binding of ligands to integrins plays a major role in cell adhesion, migration, and signal transduction of cells. These interactions are important not only for normal cell functions, but also in pathogenic processes. The integrin $\alpha_V\beta_3$ for example is involved in tumor cell adhesion and osteoporosis. The association of ligands is specific and requires minimal recognition sequences. Therefore, suppression of integrin activity using competitive inhibitors bears great pharmacological potential.

The tripeptide sequence RGD is a prominent recognition sequence of integrin ligands. Two new cyclic pentapeptides were synthesized containing the binding sequence RGD as well as 3-amino-cyclopropane-1,2-dicarboxylic acid monomethyl ester (ACC) and valine varying only with respect to the stereochemistry of ACC. Both the (+) (all *R*) and (-) (all *S*) isomers of ACC were incorporated. Cell adhesion assays were performed with the new peptides. The interaction of K562 cells and fibronectin is mainly mediated by integrin $\alpha_5\beta_1$, while the interaction of WM115 cells and vitronectin is mediated by integrin $\alpha_V\beta_3$. In these assays, c-(-Arg-Gly-Asp-(+)-β-ACC-Val-) **1** shows a higher binding activity to integrin $\alpha_V\beta_3$ (IC_{50} = 0.02 μM than c-(-Arg-Gly-Asp-(-)-β-ACC-Val-) **2** (IC_{50} = 0.63 μM). Both peptides display a higher affinity to integrin $\alpha_V\beta_3$ than to integrin $\alpha_5\beta_1$ (IC_{50} = 1.48 μM for peptide **1**, IC_{50} = 1.78 μM) for peptide **2**).

In order to derive a structure-activity relationship of these two isomers, solution structures in DMSO-D_6 were investigated by NMR spectroscopy. Subsequently, structural information was obtained by applying distance restraints derived from the NMR spectra in distance geometry/simulated annealing, generating 1000 structures. These were clustered according to their backbone torsion angles [1] and used as starting structures in restrained molecular dynamics calculations whichlead to structural proposals for the peptides. In **1**, glycine occupies the central position of a γ-turn and (+)-β-ACC is in the i+1 position of a pseudo-β-turn, which is indicated by the distance of 578 pm between the C^{α}-atoms of Arg and Asp. In the structure of **2**, Asp is in the central position of a γ^{i}-turn. Positioned between the amino acids (-)-β-ACC and Gly, it contains a turn with torsion angles of a 3_{10}-helix, formerly referred to as β^{III}-turn.

Comparison of the structure of the peptides to peptides from previous studies [2] reveals that the turns in **1** are at the same positions as in c-(-Arg-Gly-Asp-D-Phe-Val-) **3**, a peptide with a high affinity to integrin $\alpha_V\beta_3$. Both also show a short

distance between the C^{β} of Arg and Asp **1**: 706 pm, **3**: 650 pm [3],indicating a non-stretched RGD-sequence. On the other hand, **2** resembles c-(-Arg-Gly-Asp-Phe-D-Val-) **4**, a peptide which is also less active than c-(-Arg-Gly-Asp-D-Phe-Val-). Peptides **2** and **4** have a longer distance between the C^{β} of Arg and Asp **2**: 826 pm, **4**: 905 pm [3]. Therefore, they contain a more stretched RGD-sequence. It has been shown previously, that peptides with a non-stretched RGD-sequence display higher affinity to integrin $\alpha_V\beta_3$. The results of the structural studies in comparison with the biological tests thus confirm the structure-activity relationship known up to now.

Acknowledgements

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COMPARATIVE CONFORMATIONAL STUDIES OF *Z-RLR(aza)GIV-OMe* – THE POTENT AND SELECTIVE CATHEPSIN B INHIBITOR AND *Z-RLRGIV-OMe* – ITS PARENT PEPTIDE

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Introduction

Cathepsin B is a cysteine protease of the papain family acting mainly as a component of the intracellular protein degradation system [1]. Extracellular cathepsin B has been implicated in asthma and bone disorders. The activity of all cathepsins *in vivo* is regulated *inter alia* by their specific inhibitors, cystatins [2]. Recently, a great interest in the design of low molecular mass cysteine protease inhibitors as possible agents for treatment of various diseases has been observed [3]. A number of peptidyl derivatives structurally based on the inhibitory sites of cystatins has been synthesized but these compounds are prone to proteolytic degradation and are rapidly excreted and poorly bioavailable. These problems can be overcome by use of respective peptidomimetics. Among them we can mention azapeptides, peptide analogs in which α-CH group of one or more amino acid is replaced by a nitrogen atom [4]. In this work we do conformational studies in DMSO using NMR of the peptides Z-RLRGIV-OMe and Z-RLR(aza)GIV-OMe, where (aza)G stands for aza-glycine. All syntheses, NMR studies and MD simulations were carried out as already described [5], except that for the NMR measurements Varian Unity 500 Plus Spectrometer 500 MHz at 303 K was used in this work, see Ref. [5] for more details.

Results

The representative ^{1}H NMR spectra of both peptides are given in Fig. 1, top The parent peptide exists as a mixture of 4 distinct on the time-scale components. Their measured distribution is: 31%, 20%, 30% and 19% using NH-αH Gly signals as a probe. In the major conformations all peptide bonds are *trans*. Big values of the temperature coefficients suggest no hydrogen bonds for NH protons and subsequently a flexible structure of the peptide. The calculated structures of Z-RLRGIV-OMe appear irregular and extended (Fig. 1, bottom). All conformations are stabilized by several β-turns at various sites. The aza-mimic exists as a mixture of at least 2 distinct on the time-scale components. All peptide bonds in the major conformation are *trans*. Small values of the temperature coefficients suggest strong hydrogen bonds for NH protons of Leu, (aza)Gly and Ile. The N- and C-terminal fragments of Z-RLR(aza)GIV-OMe in the major conformation are very close to each other, thus indicating a bent structure in the central part of the molecule. All conformations are stabilized by β-turns involving R1-(aza)G4 and L2-I5. The side

chains of the inhibitor's amino acid residues are exposed to the solvent. In the major conformation the side-chains of R1, R3 and I5 are situated on one side of the molecule while the bulky Z and I5 parts are closely oriented to form a hydrophobic cluster (Fig. 1, bottom).

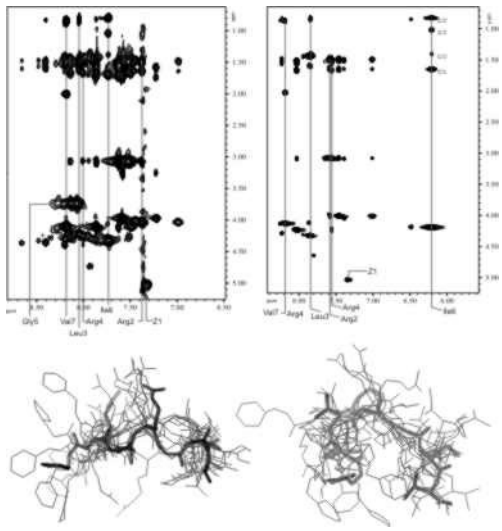

Fig.1. Top: A diagnostic region of TOCSY spectra in DMSO-d_6 (T = 303 K) of Z-RLRGIV-OMe, left, and Z-RLR(aza)GIV-OMe, right. Bottom: C^{α} overlap of low-energy conformations of Z-RLRGIV-OMe, left, and Z-RLR(aza)GIV-OMe, right.

Discussion

Both studied peptides Z-RLRGIV-OMe and Z-RLR(aza)GIV-OMe consist of mixtures of major and minor conformations in the DMSO-d_6. The replacement of the α-CH in Gly by an N atom leads to a more rigid and bent structure in the azapeptide. On the contrary, the structures of the parent peptide are less ordered and flexible. Azapeptides are known to reversibly inactivate cysteine proteases, the current aza-analog is a selective inhibitor of cathepsin B. Just like in the solution, it also adopts bent conformations in the complex with the enzyme, as found in the MD simulation, not shown.

Acknowledgements

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ANGIOTENSIN I-CONVERTING ENZYME - SUBSTRATE INTERACTIONS STUDIED THROUGH MOLECULAR DYNAMICS SIMULATIONS

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Introduction

Angiotensin Converting Enzyme (ACE) is a type-I membrane-anchored dipeptidyl carboxypeptidase essential for blood pressure regulation and homeostasis through the renin-angiotensin-aldosterone system. ACE falls into the group of metallo-peptidases with the characteristic His-Glu-X-X-His zinc-binding motif (*M2 gluzincins family*) [1, 2]. *In vivo*, ACE converts angiotensin I (ANG) decapeptide into the vasopressor ANG II by removal of the C-terminal His-Leu and hydrolyses bradykinin (BRK). Inhibition of ACE prevents formation of hypertensive agent ANG II and potentiates the hypotensive BRK thereby lowering the blood pressure. Inhibitors of ACE are widely used for cardiovascular diseases with annual sales over US $6 billion. ACE exists in two isoforms that are transcribed from the same gene in a tissue-specific manner: the somatic form in somatic tissues and the germinal form in sperm cells. The protein domain organization of the two isoforms of ACE is shown on the right. Determination of the crystal structures of tACE and sACE along with the inhibitor lisinopril gave insight into the key interactions at the S2, S1, S1' and S2' pockets and a basis for domain-specific inhibitor design [3 - 4].

Results and Discussion

In vitro, ANG and BRK are hydrolyzed at comparable rates by both sites of ACE, but *in vivo* ANG-I is predominantly hydrolyzed by the C-domain, a process which is highly dependent on the chloride concentration. The hydrolytic reaction proceeds via a mechanism similar to the one proposed for thermolysin: Nucleophilic attack of a H_2O/-OH to the C=O of the scissile bond via a tetrahedral gem-diolate intermediate. Some physiologically important peptides such as luliberin (LH-RH) are preferentially hydrolyzed by the N-domain that cleaves the C-terminal amidated tripeptide. In order to gain additional information on the enzyme-substrate interactions we have simulated the binding of several ACE substrate peptides into the catalytic site of both domains of ACE, in such a configuration that the C=O of the scissile bond is coordinated to Zn(II).

Determination of the binding modes of the substrates has been performed by dividing the central binding cavity into two parts and by docking one half of the peptide in each part. The docking conformations were calculated using the program AutoDock and the initial geometries of the enzyme-substrate complex were refined with AMBER (Fig. 1). The docked conformation of each peptide into the catalytic channel of ACE represents only one possible model of the enzyme-substrate

interaction. To introduce the effect of the protein flexibility that is very important and to sample the conformational space of the complex, molecular dynamics simulations were performed with explicit solvent representation. In this way we can explore the protein reorganization upon substrate binding and monitor the local motions at the binding interface.

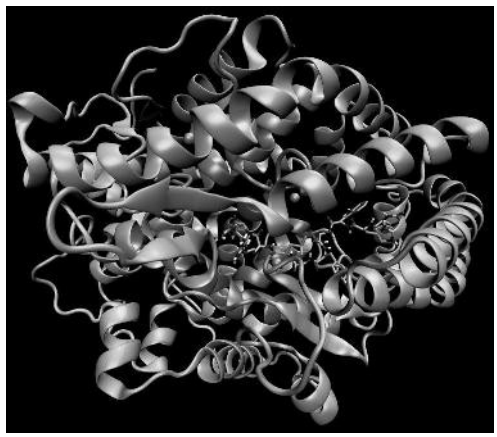

Fig. 1. Docked conformation of tACE–LHRH complex after energy minimization with AMBER.

Additionally, calculations in explicit solvent representation introduces the effect of the polar water on the protein conformation and the interactions between the enzyme and substrates. Examination of the trajectories reveals several characteristics of the interaction between the peptides and ACE, such as the protein flexibility at specific sites via the atomic fluctuations per residue, as well as investigation of specific geometrical features of their interactions (distances, angles and dihedrals). As shown above we can monitor as a function of time the hydrogen bonding network of the complex, in addition to the position of the solvent molecules that mediate such interactions. Major hydrophobic interactions are also examined extensively [5].

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CONFORMATIONALLY CONSTRAINED CCK8 ANALOGUES AS LIGANDS FOR CHOLECYSTOKININ RECEPTORS

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Introduction

Small radiolabelled compounds such as peptides are very attractive tools for the diagnosis of several different pathologies. Among the possible biological targets for radiolabelled compounds, the cholecystokinin receptors CCK_A-R and CCK_B-R are very promising, due to their overexpression in many tumours: CCKA-R is overexpressed in pancreatic cancer, while CCK_B-R is found in small cell lung cancer, colon and gastric cancers, medullary thyroid carcinomas, astrocitomas and stromal ovarian tumors [1]. These receptors belong to the GPCR superfamily and are localized in the cell membrane. Both CCK_A-R and CCK_B-R have been thoroughly investigated with the aim of characterising the molecular basis of their interaction with the CCK peptide hormone. The NMR structures of two complexes formed by the natural ligand CCK8 with the N-terminal fragment of CCKA receptor and with the third extracellular loop (EL3) of CCKB receptor have been recently published [2, 3]. We used these structures as a starting point to design receptor selective CCK8 analogues.

Results and Discussion

The structural details of the CCK8/CCK_A-R(1-47) allowed us the design of new CCK8 analogues, characterized by a cyclic skeleton introduced to stabilize the bioactive conformation and to increase the enzymatic stability of the peptide [4]. Some of them are also characterised by the presence of modified natural amino acids which allow the ligand to be better accommodated in the binding site and to meet the topochemical array of the pharmacophore model. (Fig.1).

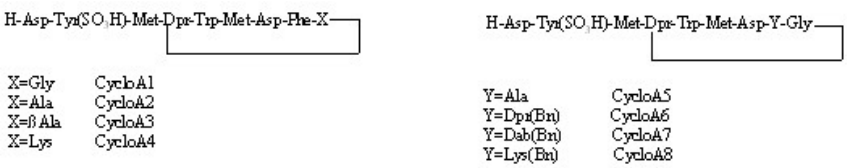

Fig. 1. Cyclic peptides as ligands for CCK_A receptor

Preliminary biological assays were performed on all synthesized analogues, in order to verify their binding ability towards CCK_A receptor expressed in cultured cells.

We have used a similar rational approach, based on the structural details of the complex between CCK8 and the CCK_B receptor fragment, to design a library of peptide ligands endowed with specific binding properties toward CCKB-R. The rational design of peptidomimetic analogues was done according to the following criteria: i) to preserve the major interactions between the Trp^{30}-Phe^{33} tract of the ligand and the CCKB-R(352-379) receptor fragment, ii) to stabilise the bioactive turn-like conformation in the Trp^{30}-Phe^{33} region and iii) to preserve as far as possible any further interactions between the ligand and the whole receptor. As a result, we have designed the four classes of cyclic peptidomimetic molecules that are sketched in Fig. 2.

The binding affinity of the new ligands for CCKB-R was assessed by displacement experiments of ^{111}In-radiolabeled CCK8 in cells overexpressing the CCKB receptor.

In conclusion, the finding that all peptide analogues obtained, for CCKA-R as well as for CCKB-R, are less active than parent CCK8 indicates that the cyclic constraint introduced in the CCK8 C-terminus to stabilise the bioactive conformation might also partially hinder its binding to the CCK receptors, likely because of sterical effects.

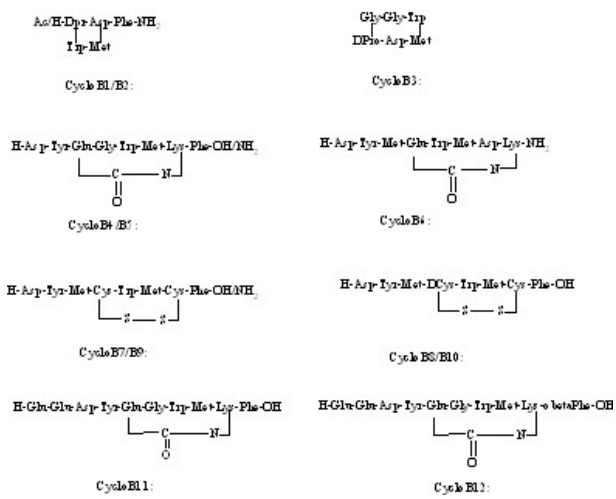

Fig. 2. Cyclic peptides as ligands for CCK_B receptor

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STRUCTURE-FUNCTION RELATIONSHIP OF ANALOGUES OF PTH(1-11) CONTAINING A COMBINATION OF AIB AND (αMe)VAL

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Introduction

Parathyroid hormone (PTH) is an 84 amino acid peptide hormone. Produced in the parathyroid glands, it acts primarily on bone and kidney to maintain extracellular calcium levels within normal limits. It has been shown that the first 34-amino acid fragment of PTH is sufficient to bind and activate the PTH type I receptor (PTH1R). The study of reduced-size PTH agonist and antagonist analogues has been the subject of extensive research [1, 2], for the development of safer and non-parenteral bone anabolic drugs. Recent investigations focusing on the interaction of N-terminal fragments of PTH with PTH1R showed that certain modifications can increase signalling potency in peptides as short as 11 amino acids (e.g. $S^3 \rightarrow A^3$, $N^{10} \rightarrow Q^{10}$, $L^{11} \rightarrow R^{11}$).

This work represents our continuing effort to investigate the role of the side chain of Valine at position 2 in the interaction of short PTH fragments with PTH1R. We synthesized and conformationally and biologically characterized a series of PTH(1-11) analogues containing sterically hindered and helix-promoting C^{α}-tetrasubstituted amino acids. In fact, it has been demonstrated that the structural order of residues 1-4 plays a significant role in PTH action [3] and this is the reason why in this series we constrained the N-terminal backbone conformation with α-amino isobutyric acid (Aib) at the first positions. Moreover, we inserted α(Me)Val at position 2, preserving the hydrophobic side chain of V^2, which appears to be critical for receptor interaction. Synthesized analogues and cAMP Stimulation Response in C20 Cells (Luciferase Assay) are reported in the following table:

		EC_{50} (nM)
I	[(αMe)Val^2, Aib^3, Nle^8, Arg^{11}]-rPTH(1-11)NH_2	>10^5
II	[Aib^1, (αMe)Val^2, Nle^8, Arg^{11}]-rPTH(1-11)NH_2	>10^5
III	[(αMe)Val^2, Aib^4, Nle^8, Arg^{11}]-rPTH(1-11)NH_2	>10^5
IV	[Aib^1,(αMe)Val^2, Aib^3, Nle^8, Arg^{11}]-rPTH(1-11)NH_2	$7 \cdot 10^4$
V	[Aib^1, DVal^2, Aib^3, Nle^8, Gln^{10}, Har^{11}]-rPTH(1-11)NH_2	$7 \cdot 10^3$(*)
VI	[Aib^{1,3}, Gln^{10}, Har^{11}]-rPTH(1-11)NH_2	1.1

(*) preliminary data

Results and Discussion

The peptides were synthesized by SPPS employing Fmoc-protected amino acids. Fmoc-α(Me)Val-OH was protected using Fmoc-Cl/TMS-Cl/Dipea. We combined the HBTU/HOBt/DIPEA and the acyl fluoride coupling methods [4]. The latter was used to incorporate the Cα-tetrasubstituted amino acids.

The CD spectra of the six analogues show a clear tendency to fold in a helical structure. Analogue VI shows the highest helix content estimated at 60%. In all analogues, the chemical shift differences of αCH protons with respect to the corresponding random coil values identify a helical segment spanning the sequence from residue 1 to Nle[8] (Fig. 1). A number of αH(i)-NH(i+3), αH(i)-βH(i+3) and αH(i)-NH(i+4) connectivities typical of the α-helix in the ROESY spectra indicate that the single substitution with Aib at position 3 or 4 is more effective to promote helicity that the single substitution at position 1.

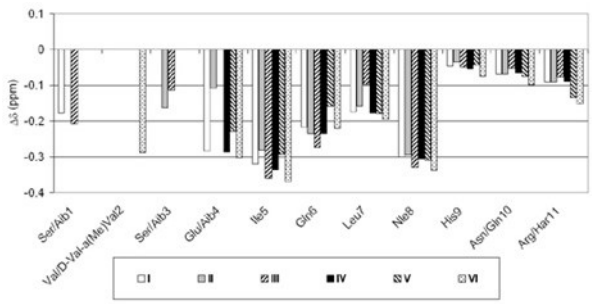

*Fig. 1. Secondary chemical shifts of the αH protons of analogues **I-VI**. Δδ<-0.1 ppm indicates the preference for α-helix structure.*

Superimposition of the ensembles of the low energy structures resulting from simulated annealing MD calculations generally indicated a clear convergence towards the helical structure in the region 4-8. Analogues **IV** and **VI** did not show the same behavior, probably because of some slight differences in the ROESY spectra due to overlapped peaks.

We have shown that substitutions with Aib at positions 1 and 3 increase helix stability, and this can be correlated with better EC_{50} values for analogues **IV** and **V**. The strategical role of V^2 in the interaction with the PTH1R receptor is confirmed: analogues **V** and **VI** differ only for the chirality of Val^2 and exhibit a very similar secondary structure, but have a very different behavior in terms of biological activity. Moreover, binding tests carried on analogues **I** - **V** did not show any antagonistic activity and this confirms the C-terminal α-helical segment (from R^{20} to V^{31}) of PTH to be the principal receptor binding domain.

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RETROINVERSO PEPTIDES INHIBIT AMYLOID NEUROTOXICITY AND ARE THERAPEUTIC AGENTS AGAINST ALZHEIMER'S DISEASE

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Beta-amyloid peptide (Aβ) is the major protein component of senile plaques and cerebrovascular amyloid deposits in the brains of Alzheimer disease (AD) patients. Aggregates of Aβ are neurotoxic and a likely cause of the cognitive decline seen in Alzheimer's patients [1]. Recently it has been demonstrated that soluble low-molecular oligomeric Aβ and protofibrils are more damaging to synaptic function than insoluble fibrils [2]. Prevention of aggregation of Aβ is thus a therapeutic target.

Methods. An ELISA has been described that measures the extent of conversion of Aβ into soluble oligomers [3]. In this ELISA we measure the adhesion of Aβ epitopes as it aggregates to form soluble, low molecular weight oligomers, by using the same antibody twice in a sandwich ELISA. The second coating of antibody bears a biotin moiety, allowing measure of the aggregate. As our ELISA assay gives an accurate read-out of the formation of toxic forms of Aβ, it provides an accurate way of measuring the efficacy of inhibitory compounds likely to be useful for preventing cognitive deterioration in Alzhemier's patients. Aβ 40 and 42, were synthesised as described [4]. Monomeric Aβ(1-40) and Aβ(1-42) (200 μM) were prepared by triple recycling in TFA and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), and drying under a slow stream of dry N_2. Dissolution of the target to 200 μM was with sterile distilled water. Aliquots (200 μl each) were stored at -20 ºC until used. The peptide rGffvlkr-amide containing the retro-inverse recognition sequence in Aβ was synthesised on Fmoc PAL-PEG-Polystyrene resin (Applied Biosystems))(0.2mmoles; 0.22mmole/g), coupling in succession, D-Arg, D-Lys, D-Leu, D-Val, D-Phe, D-Phe, Gly, D-Arg, with PyBOP and HATU. rGffvlkr-pentadiamine was synthesised by reductive amination of formyl polystyrene resin 4-{4-formyl-3-methoxyphenoxy}butyric acid (FMBP) NovaGel TM with mono-1-butoxycarbonyl, 1,5-diminopentane (tosyl salt) (Novabiochem). The target peptides were cleaved and deprotected in TFA, triisopropylsilane (5% by vol) and water (5% by vol), triturated in ether, and purified by HPLC on a column of Dynamax C8, in 0.1% TFA with an acetonitrile gradient. Purity was verified by MALDI mass spectrometry on a Shimidazu-Kratos Axima MALDI mass spectrometer. Other inhibitors were obtained from Bachem. Peptides were incubated together with Aβ in sterile 0.1 M Tris-HCl (pH 7.4), to obtain soluble oligomers.

Results Fig. 1. showed that the retroinverso peptide rGffvlk-NH_2 and rGffvlk-pentadiamine were more effective than the native sequences KLVFF-NH_2 at preventing the formation of oligomers of Aβ40 and 42, by co-incubation in 0.1M Tris (pH 7.4).rGffvlkr-NH_2 prevented formation of fibrillar Aβ in plaques was shown by the disappearance of fibrils, seen by TEM, after long periods of incubation (up to 12 days) (Fig. 2). rGffvlkr-NH_2 was more potent than native L-peptides KLVFF-NH_2 over the same time period of incubation at the same molar ratios.

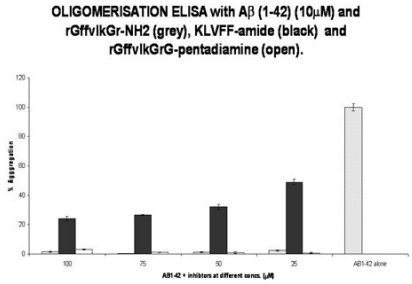

Fig. 1. Double ELISA measure of soluble oligomers from Aβ42 (0.1 mM) incubated in 0.1 M Tris (pH 7.4) with rGffvlkGr-NH_2 (diagonal hatch), rGffvlkGrG-pentadiamine (verticle hatch) and KLVFF-NH_2 (solid bar).

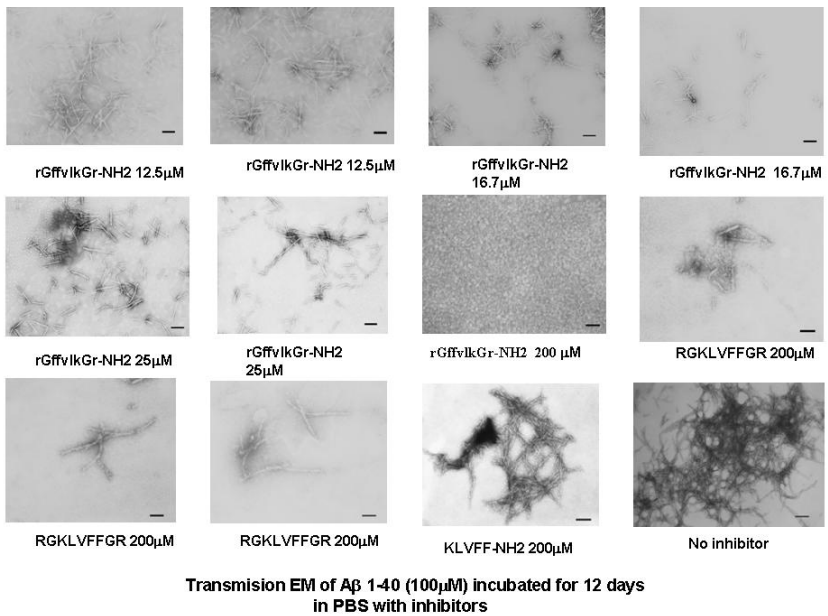

Figure 2. Transmision EM of Aβ 1-40 (100μM) incubated for 12 days in PBS with inhibitors. Retroinverso inhibitors are in lower case.

Discussion Thus retroinverso sequences would therefore appear to be more potent than peptide inhibitors based on the recognition sequence in the natural L-configuration, and thus possess superior properties as potential therapeutic agents to target Alzheimer's Disease.

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IDENTIFICATION OF NEW NON NATURAL ANALOGUES OF B DOMAIN OF STAPHYLOCOCCAL PROTEIN A, USING A "ONE-BEAD ONE-COMPOUND" COMBINATORIAL STRATEGY

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Introduction

The B-domain is one of the five nearly homologous domains of staphylococcal protein A. This domain contains three α-helices which are assembled in an antiparallel three-helix bundle [1]. The B domain binds the Fc region of mammalian immunoglobulin through the N-terminal fragment that contains two α-helices [2]. The C-terminal helix does not interact with Fc but it is necessary for the correct folding and immunoglobulin recognition of the B-domain. A ¨one-bead one-compound¨ library of 300 peptides has been designed to find new analogue peptides of the C-terminal helix that bind to the N-terminal fragment (Fig. 1.).

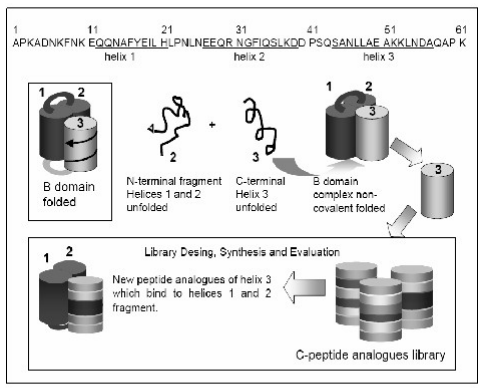

Fig. 1. System description.

Results and Discussion

The “one-bead one-compound” library of 300 different peptides was designed based on the sequence of the C-terminal helix. The library was prepared by solid phase chemistry using the Boc/Bn strategy, with the base labile *N*-[(9-hydroxymethyl)-2-fluorenyl]succinamic acid as linker anchored to a PEGA resin [3, 4]. Positively stained beads were detected by visualisation after incubation with the N-terminal fragment and rabbit IgG labelled with fluorescein. 9 New analogues of the C-terminal peptide were found and their sequence identified by MALDI-TOF MS/MS.

The two most representative active peptides were synthesized using Fmoc/*t*Bu chemistry. N-terminal fragment and C-peptides mixed in equimolar amount were analyzed by CD spectroscopy. Both analogues showed a CD spectrum characteristic of α-helical peptides.

The interaction between rabbit IgG and the non-covalent complexes of B domain were studied by surface plasmon resonance. The N-terminal fragment was immobilised on CM5 sensor chip surface. The C-terminal peptides were co-injected with of IgG at different concentration. The different dissociation behaviours suggest formation of a complex between C and N terminal fragment that increases the stability of the complex with IgG.

The wild type B domain and analogues were synthesized by Fmoc/*t*Bu chemistry. The domains were structurally characterized by CD and NMR spectroscopy. By CD both the mutants and wild type showed the characteristic spectra of α–helices. NMR Studies showed that all three compound adopted globular well-folded structures. The binding between human Fc and the B domain and its mutants was evaluated by surface plasmon resonance. The human Fc was immobilized on surface of CM5 sensor chip. Although the kinetics of binding for the mutants are different that for the wild type, both mutants are also able to bind the immunoglobulin with high affinity

Conclusions

New analogues of C-terminal helix of B domain of staphyloccocal protein A have been identified using a combinatorial approach. The new analogues have been shown to form non-covalent complexes with B domain N-terminal peptide using CD and surface plasmon resonance. The complete domains with the C-terminal mutations have been synthesized and structurally characterized by CD and NMR spectroscopy. The wild type and the new mutants adopt predominantly an α-helical structure. The mutants have been able to bind to the immunoglobulin with high affinity.

Acknowledgements

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A RHEGNYLOGIC STRATEGY FOR THE SYNTHESIS OF SIGNAL TRANSDUCTION MODULATORY, CELL PENETRATING PEPTIDES

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Introduction

Many cell-penetrating peptides (CPP) have been utilised as biologically inert vectors.

A majority of these studies employ sychnologically organised constructs in which a bioactive cargo (*message*) is chemically conjugated to the CPP (*address*). Previously, we have adopted a sychnologic strategy to modulate intracellular signal transduction. Using chimeric constructs composed of the CPP transportan 10, conjugated to partial sequences that correspond to functional domains of signal transduction proteins, we have selectively modulated a variety of cellular activities including secretion and activation of p42/p44 mitogen-activated protein kinases [1, 2]. However, a QSAR-based algorithm can now be used to predict CPP that reside within the primary sequences of proteins [3].

We have adapted this strategy to identify CPP within signal transducing proteins including functional domains that govern protein-protein interactions.

Data presented herein indicate that it is now feasible to identify rhegnylogic sequences, containing vectoral-independent discontinuously organised pharmacophores, that are cell penetrant modulators of signal transduction pathways.

Results and Discussion

Using our rhegnylogic concept, we identified a 20 AA fragment (H-RKLTTIFPLNWKYRKALSLG-NH_2), within the first intracellular loop of the human type (a) calcitonin receptor ($hCTR_{(a)}$). This sequence, $hCTR_{(a)}{}^{174\text{-}193}$, includes a splice variant 16AA insert that modulates the pharmacology of hCTRs by inhibiting receptor-stimulated inositol phosphate metabolism, but facilitating the synthesis of cAMP [4] . To establish whether our rhegnylogically-organised CPP modulated hCTR pharmacology we used the human ECV304 cell line that endogenously expresses the $hCTR_{(a)}$ isoform [5]. $hCTR_{(a)}{}^{174\text{-}193}$ (1 μM) independently stimulated cAMP formation in ECV304 cells and augmented $hCTR_{(a)}$-stimulated cAMP by the application of salmon calcitonin (sCT) (Fig. 1. left panel). Moreover, preliminary investigations indicate that $hCTR_{(a)}{}^{174\text{-}193}$ directly activated heterotrimeric G proteins as measured by the initial rate of binding of [^{35}S]GTPγS to rat brain cortical membranes (Fig. 1. right panel). Confocal microscopy images captured on living cells confirmed that fluorescein-labelled $hCTR_{(a)}{}^{174\text{-}193}$ (1 μM) efficiently translocated the ECV304 plasma membrane.

Our rhegnylogic strategy was also applied to cytochrome C (CytC), a signalling protein integral to apoptotic events. This relatively small protein of 104 AAs was introduced into the QSAR prediction algorithm to identify putative CPP. Two rhegnylogic CPPs from the C-terminal helix of CytC, $CytC^{77\text{-}101}$ (H-GTKMIFVGIKKKEERADLIAYLKKA-NH_2) and $CytC^{86\text{-}101}$ (H-KKKEERADLIAYLKKA-NH_2) reduced the viability of U373MG astrocytoma by 39.3% and 34.9% respectively, at a peptide concentration of 30 μM.
In situ TUNEL staining confirmed that peptide-induced cell death was mediated by apoptotic mechanisms, thus eliminating necrosis and membrane perturbation commonly associated with high concentrations of CPP. Interestingly, confocal images indicated the differential sub-cellular distributions of the two peptides (Fig. 2). Rhodamine-labelled $CytC^{77\text{-}101}$ (5 μM) translocated U373MG plasma membranes to assume a perinuclear distribution (Fig. 2a), whereas $CytC^{86\text{-}101}$ (5 μM) more specifically located within the nucleus (Fig. 2b). Thus, $CytC^{86\text{-}101}$ may prove to be a useful CPP to affect the specific nuclear delivery of bioactive cargoes (peptide and nucleic acid). Future co-localisation studies will more definitively establish the intracellular ultrastructures with which these new CPPs associate. In conclusion, a rhegnylogic strategy is an effective approach to identify novel signal modulatory CPPs that influence eukaryotic cell biology.

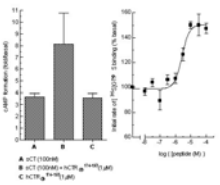

Fig. 1. Biological activities of the rhegnylogic CPP $hCTR_{(a)}^{174\text{-}193}$

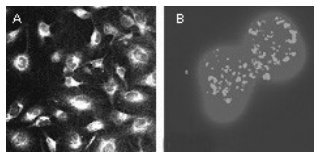

Fig. 2. Differential subcellular distribution of the rhegnylogic CPPs $CytC^{77\text{-}101}$ (A) and $CytC^{86\text{-}101}$ (B, nuclei were counterstained with DAPI, dark grey).

Acknowledegments

Keith Holding, University of Wolverhampton, U.K. provided excellent technical assistance.

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SYNTHESIS AND CONFORMATIONAL ANALYSIS OF A PRO-AIB HYDROXYETHYLENE DIPEPTIDE ISOSTERE

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Introduction

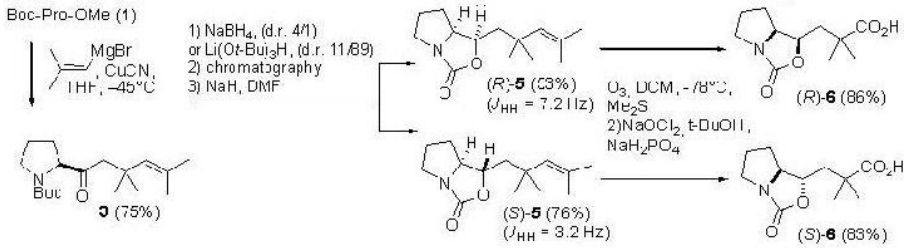
β-Turns play important roles in molecular recognition events of peptides and proteins in biological systems. Hydroxyethylene amide isosteres have served as transition-state mimics of amide bond hydrolysis in inhibitors of aspartyl peptidases designed to treat hypertension, AIDS, and Alzheimer's disease [1]. Extended structures appear to be unaffected by the hydroxyethylene replacement [2]. Because their application in turn conformations has yet to be well studied, Pro-Aib hydroxyethylene isostere 6 was made and incorporated into model β-turn sequence Boc-Phe-Prp-Aib-NH(Me)Bn **11** [3].

Results and Discussion

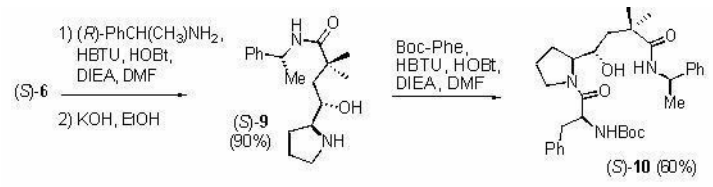
δ-Amino γ-hydroxy acid **6** was made by cascade addition of a substituted vinyl Grignard reagent to Boc-Pro-OMe **1** [4], with ketone reduction and olefin oxidation of the resulting homoallylic ketone **3** (Scheme 1). 2-Methyl-1-propenylmagnesium bromide (400 mol%) and CuCN (40 mol%) were premixed prior to addition of **1** (100 mol%) to give ketone **3** in 75% yield after chromatographic purification on silica gel. Alcohols **4** were prepared diastereoselectively by reduction of ketone **3** using $NaBH_4$ in EtOH or $LiAl(Ot\text{-}Bu)_3H$ in THF at 0 °C to provide as major diastereomer respectively, *syn*- and *anti*-alcohol (*S*)- and (*R*)-**4** after chromatography. Oxazolidinones (*R*)- and (*S*)-**5** were made quantitatively from alcohols (*R*)- and (*S*)-**4** using NaH in DMF, and their configurations were assigned based on their respective vicinal coupling constants [5]: J_{HH} = 3.2 Hz and 7.2 Hz. Ozonolysis of the olefin, and, without further purification of the corresponding aldehyde, oxidation with an aqueous solution of NaH_2PO_4 and $NaClO_2$ gave δ-amino γ-hydroxy acids (*R*)- and (*S*)-**6** in 86% and 83% overall yields respectively. Diasteromeric peptides (*R*)- and (*S*)-**10** were respectively made from oxazolidinone acid **6** as described for (*S*)-**10** in Scheme 2. The ^{1}H-NMR spectrum of peptide (*S*)-**10** in $CDCl_3$ at 25 °C showed sets of resonances consistent with a 37:63 mixture of *cis* and *trans* isomers about the Pro residue, while diastereomeric peptide (*R*)-**10** exhibited only one pattern of signals that were consistent for the *trans* isomer in the ^{1}H-NMR and NOESY spectrum in $CDCl_3$.

In the NOESY spectra in $CDCl_3$ of (*R*)-**10** and *trans*-(*S*)-**10** NOE correlations existed between the NH(Me)Bn proton and the methylene protons of the amide isostere. The methine proton CH(OH) shared NOE with the β-H of the prolyl ring and the methyl protons of the Aib residue; also the methyl protons of the Aib

residue share NOE with the β-H of the prolyl ring. These correlations were characteristic of a folded structure similar to that adopted by the parent peptide **11**.



Scheme 1



Scheme 2

Conclusions

Pro-Aib hydroxyethylene isosteres **6** were made, introduced into model peptides **10**, and evaluated by IR and NMR-NOESY spectroscopy that demonstrated compact conformations were maintained that were similar to the parent peptide **11**.

Acknowledgments

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CAPILLARY ELECTROPHORESIS ANALYSIS OF INTERACTION OF NUCLEOBASE-CONTAINING TAT PEPTIDES WITH TAR RNA HIV-1

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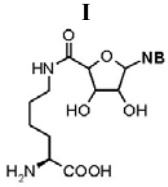
Introduction

Binding of Tat peptide to HIV-1 TAR RNA is mediated by a 9-amino-acid, highly basic Arg^{49}-Lys-Lys-Arg^{52}-Arg-Gln-Arg-Arg-Arg^{57} sequence of the ARD (Arginine Rich Domain). The key role in these interactions is played by Arg52.

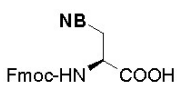
Results and Discussion

We prepared analogues of the Tat peptide containing NBAs (nucleobase amino acids) of three general types: NBAs containing nucleobase residues connected directly to carbon β of alanine **I** or to carbon γ of 2-aminobutyric acid (homoalanine) **II** or to ε-amino group of Lys *via* an acetic acid linker **III**. NBAs of types **I** and **II** were synthesised before peptide synthesis and used as pre-made building blocks. Alanine derivatives **I** (purine-base ones only) were synthesised by means of the Mitsunobu reaction of serine derivatives with alanine and 2-amino-6-chloropurine (guanine precursor), respectively. Homoalanine derivatives **II** were synthesised by means of nucleophilic substitution by individual nucleobases in 2-amino-4-bromobutyric acid in alkaline conditions [1].

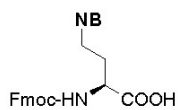
I



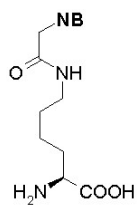
II



III



IV



In the case of NBAs of type **III**, the Lys residue was incorporated into the peptide and after peptide synthesis was selectively deprotected and reacted with an acetic acid derivative containing a nucleobase residue in position α. NBAs of this type are henceforth designated as Lys(NBAc). NBAs containing nucleoside residues (type **IV**) connected to nitrogen ε of Lys via a carboxyl group were created by oxidising the hydroxyl group in position 5' of ribose. In these cases the Lys residue was incorporated into the peptide and after peptide synthesis was selectively deprotected and reacted with a nucleoside derivative with hydroxyl groups in positions 2' and 3' protected by an acetone acetal moiety [2] and with the $–CH_2OH$ residue oxidised to a carboxyl group [3]. NBAs of this type are henceforth designated as Lys(NBC).

All peptides were synthesized by solid phase peptide synthesis utilising the Fmoc strategy [4]. Tat – TAR interactions were observed using CEMSA (capillary electrophoresis mobility shift assay).

Table 1. Primary structures of synthesized peptides.

Amino Acid Sequence
RKK**A(βA)**RQRRR-NH_2
RKK**A(βG)**RQRRR-NH_2
RKK**X(γA)**RQRRR-NH_2
RKK**X(γG)**RQRRR-NH_2
RKK**X(γT)**RQRRR-NH_2
RKK**X(γC)**RQRRR-NH_2
Ac-RKK**K(AAc)**RQRRR-NH_2
Ac-RKK**K(CAc)**RQRRR-NH_2
Ac-RKK**K(GAc)**RQRRR-NH_2
Ac-RKK**K(TAc)**RQRRR-NH_2
Ac-RKK**K(UAc)**RQRRR-NH_2
Ac-RKK**K(AdC)**RQRRR-NH_2
Ac-RKK**K(GuC)**RQRRR-NH_2
Ac-RKK**K(UrC)**RQRRR-NH_2
Ac-RKK**K(CyC)**RQRRR-NH_2

All the peptides modified with alanine and homoalanine NBAs show very similar interactions with TAR RNA. This seems surprising in view of the fact that even slight modifications of the Tat Arg52 residue (like shortening the side chain by one methylene group or converting the guanidine moiety to an urea derivative) result in drastic loss of complex formation ability [6].

All Tat peptide analogues containing nucleobase residues attached to Lys52 side chain *via* an acetic acid linker as well as nucleoside moieties attached by the oxidised carboxyl group in position 5' of ribose exhibit very similar behaviour towards TAR RNA. They all form relatively stable complexes in a concentration-dependent manner, with the exception of the guanosine derivative, which does not react with the RNA strand at all. This may suggest that the observed complex formation is different in character than in the case of the native Tat peptide, which is very sensitive to even slight modifications of the key arginine residue in position 52 [6].

Acknowledgements

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INFLUENCE OF PEPTIDE MIMIC TURN STEREOCHEMISTRY ON ALLOSTERIC ANTAGONISM AT THE PROSTAGLANDIN F2α RECEPTOR

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Introduction

Prostaglandins (PG) are involved in a large number of biological activities with tissue specificity mediated by their G-protein coupled receptors (GPCRs). $PGF_{2\alpha}$ receptors, found specifically in uterine muscles, initiate parturition and labor. They play a key role in preterm labor, for which medical costs are estimated at $9 billion per year in the USA [1]. Peptide mimics developed in our laboratory [2, 3] serve as allosteric antagonists of the $PGF_{2\alpha}$ receptor [4, 5]. The importance of the turn geometry of the central residue in these peptide mimics has now been investigated using enantiomeric indolizidin-2-one (Iaa) β-turn mimics [6].

Results and Discussion

Mimicry of a potent lead octapeptide 1 of all D-configuration, led to a relatively low molecular weight analog *S*-2 exhibiting high efficacy (>80%), specificity, and exerting negligible toxicity (Fig. 1) [2, 3]. Considering the Iaa as a mimic of a turn centered at Gly-His, the role of β-turn stereochemistry for biological activity has been investigated by replacement of the (*S*,*S*,*S*)- for (*R*,*R*,*R*)-Iaa isomer, which amounts to switching from type II' to type II conformation.

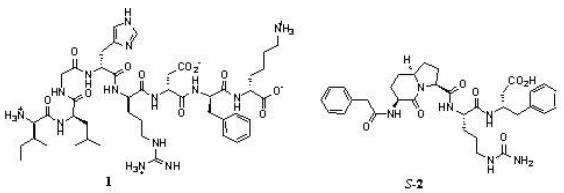

Fig. 1. Octapeptide 1 and mimic S-2.

Peptide mimics *S*- and *R*-2 were synthesized using a Boc-protection strategy on oxime resin (Fig. 2) [7]. Boc-Cit-OH was first coupled to the resin, using DCC and ethyl 2-(hydroxyimino)-2-cyanoacetate (EACNox) in DCM. Deprotection of the Boc group was performed with 20% TFA in DCM. The enantiomeric Boc-Iaa-OH residues [8] and phenylacetic acid were sequentially coupled to the resin using TBTU and DIEA in DMF. Couplings were monitored by Kaiser test, and LC/MS analysis of product from cleavage of a resin aliquot with methoxyethylamine in chloroform. The resin was cleaved by displacement with H-βPhe-OBn (100 mol%) in the presence of DIEA (100 mol%) and AcOH (100 mol%) in DCM. After purification of the benzyl ester by preparative HPLC, acids

S- and *R*-**2** were liberated by hydrogenolysis in EtOH and isolated by preparative HPLC for biological testing.

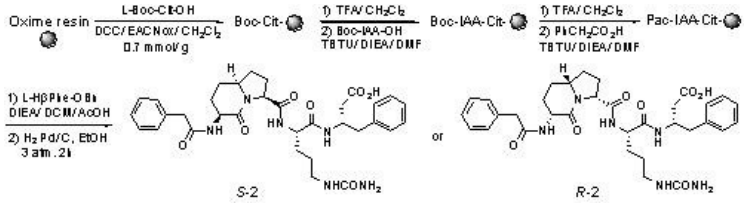

Fig. 2. Solid phase synthesis of peptide mimics.

The effect of peptide mimics *S*- and *R*-**2** on uterine contractility in the process of labor was tested against $PGF_{2\alpha}$ responses on myometrial strips from pregnant mice [2] (Fig. 3). Compared to 1, neither *S*- nor *R*-**2** blocked spontaneous myometrial contractions. However, S-**2** dissipated robust $PGF_{2\alpha}$-induced contractions of mouse myometrium, such that $PGF_{2\alpha}$-induced mean tension (5 g) was concentration-dependently reduced to 2.5 g with 10 μM *S*-**2**. In contrast, the enantiomer *R*-**2** did not inhibit contractile activity of $PGF_{2\alpha}$ on uterine smooth muscle.

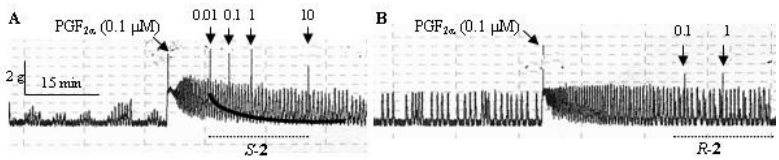

*Fig. 3. Tracings of spontaneous and $PGF_{2\alpha}$-induced myometrial contraction of pregnant mice tissue treated with A. S-**2** ; B. R-**2**.*

In conclusion, examination of the relationship between turn geometry and activity in allosteric antagonists of the $PGF_{2\alpha}$ receptor was performed using peptide mimics *S*- and *R*-**2**. Preliminary studies support the importance of a type II' β-turn mimicked by the (3*S*,6*S*,9*S*)-Iaa residue as a requirement for inhibition of uterine contractions induced by $PGF_{2\alpha}$.

Acknowledgments

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NOVOKININ, AN ANGIOTENSIN AT_2 AGONIST PEPTIDE, DECREASES FOOD INTAKE IN MICE

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Introduction

There are two receptor subtypes for angiotensin II: AT_1 and AT_2 receptor, which is known to mediate hypertensive and hypotensive activity, respectively. Novokinin (RPLKPW) is an agonist for AT_2 receptor, which has been designed by replacing 4 amino acids in a vasorelaxing hexapeptide ovokinin(2-7) (RADHPF) derived from ovalbumin [1, 2]. We previously reported that novokinin relaxed blood vessels and reduced blood pressure after oral administration through AT_2 receptor [2]. Recently, we also found that novokinin stimulates hair growth and prevents alopecia induced by anti-cancer drug. In this study, we found that novokinin and angiotensin analogues suppress food intake via AT_2 receptor.

Results and Discussion

An AT_2 agonist novokinin suppresses food intake after oral administration

Novokinin suppressed food intake after intracerebroventricular administration in a dose-dependent manner (30 - 100 nmol/mouse) in fasted male mice. Orally administered novokinin also decreased food intake at a dose of 100 - 300 mg/kg. Novokinin has higher affinity for AT_2 receptor (K_i = 0.30 μM) than that of AT_1 receptor (K_i = 4.1 μM). Pretreatment with an AT_2 selective antagonist PD123319 (6.5 nmol/mouse) inhibited novokinin-induced anorexigenic activity after central administration at a dose of 30 nmol/mouse, suggesting that novokinin suppresses food intake through AT_2 receptor.

Angiotensin II and III suppress food intake via AT_2 receptor

Next, we tested whether angiotensin II and III suppressed food intake. Centrally administered angiotensin II having affinity for both AT_1 and AT_2 receptors suppressed food intake. The angiotensin II-induced anorexigenic effect was inhibited by AT_2 antagonist PD123319 but not by AT_1 antagonist TCV-116. Angiotensin III, which is more selective for AT_2 than AT_1 receptor, decreased food intake after central administration, and the anorexigenic effect was blocked by pretreatment with AT_2 antagonist. These results suggest that angiotensin II and III decreases food intake via AT_2 receptor.

Prostaglandin E2-EP4 receptor is activated downstream of the AT_2 receptor

We focused on the mechanism of anorexigenic activity of angiotensin agonists downstream of the AT_2 receptor. Anti-hypertensive and anti-alopecia activities of novokinin were blocked by cyclooxygenase (COX) inhibitor [3]. Then, we examined the effect of COX inhibitor indomethacin on the anorexigenic action of novokinin. Centrally administered novokinin-induced suppression of food intake was blocked by indomethacin, indicating that novokinin suppressed food intake by

way of COX metabolites. Among COX products, prostaglandin (PG) E2 is known to suppress food intake after central administration. Recently, we have reported that PGE2 decreases food intake via EP4 receptor among four receptor subtypes (EP1-EP4) for PGE_2 [4]. The anorexigenic effect of novokinin was also blocked by an EP4 receptor antagonist ONO-AE3-208. Furthermore, the suppression of food intake after angiotensin II was also blocked by EP4 antagonist. Taken together, novokinin, angiotensin II and III decrease food intake via AT_2 receptor, which is coupled to PGE_2 production and EP4 receptor activation. These signal transductions are a novel pathway regulating food intake.

Acknowledgement

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PEPTOID-SCAN IN ANTIMICROBIAL PEPTIDES: SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF [NARG]PEPTIDE-PEPTOID HYBRIDS OF APIDAECIN IB

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Introduction

Insect apidaecins [1], a family of short proline-arginine rich antimicrobial peptides, act principally on Gram-negative bacteria throught a non-membranolytic mechanism involving multiple cellular targets [2]. Both aspects of their mechanism of action make these peptides very attractive for pharmaceutical and biotechnological applications. The existence of multiple cellular targets is expected to slow down the appearance of the cationic antimicrobial resistance and the ability of the peptides to entry the cell without destroying the cellular membrane makes them potential molecular transporters. Following our investigation on the structure-activity relationships of proline-arginine peptides [3], we decided to prepare a library of peptide-peptoid hybrids to identify residues crucial for the cell penetrating ability and/or the antibacterial activity. Peptoid residues represent a relative conservative example of peptidomimetics in which the amino acid side chains have been shifted from the α-carbon to the α-nitrogen, to give *N*-substituted glycine residues. In view of the importance of the arginine residues on both the antibacterial properties of cationic antimicrobial peptides and the ability of Arg-rich peptides to translocate across the membranes, we firstly performed a peptoid-scan on each arginine residue present in the apidaecin Ib sequence. The trisubstitued hybrid was also prepared (Fig. 1).

1: H-Gly-Asn-Asn-Arg^{4}-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg^{12}-Pro-Pro-His-Pro-Arg^{17}-Leu-OH
2: H-Gly-Asn-Asn-**Narg^{4}**-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg^{12}-Pro-Pro-His-Pro-Arg^{17}-Leu-OH
3: H-Gly-Asn-Asn-Arg^{4}-Pro-Val-Tyr-Ile-Pro-Gln-Pro-**Narg^{12}**-Pro-Pro-His-Pro-Arg^{17}-Leu-OH
4: H-Gly-Asn-Asn-Arg^{4}-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg^{12}-Pro-Pro-His-Pro-**Narg^{17}**-Leu-OH
5: H-Gly-Asn-Asn-**Narg^{4}**-Pro-Val-Tyr-Ile-Pro-Gln-Pro-**Narg^{12}**-Pro-Pro-His-Pro-**Narg^{17}**-Leu-OH

Fig.1. Amino acid sequence of Apidaecin Ib and its synthetic peptide-peptoid hybrids.

Results and Discussion

Peptide-peptoid hybrids were automatically assembled on solid phase, using the Wang-resin and the Fmoc/*t*Bu chemistry. HBTU or HATU were used as coupling reagents in the acylation of proteinogenic and peptoid residues, respectively. Analogue **5** was synthesized using Fmoc-Narg(Pmc)-OH prepared in solution from

3-guanidino(Pmc)propanamine and ethyl bromoacetate. The hybrid **5** was cleaved from the resin and fully deprotected by treatment with a mixture of TFA/H_2O/TIS, purified and characterized by HPLC and ESI-MS. Analogues **2 - 4** were obtained by on resin guanidinylation of the corresponding [Norn]peptide-peptoid hybrids, easily prepared by a modification of the Zuckerman's sub-monomer method [4]. The bromoacetic acid residue was introduced in the desired position of the peptide chain and 1,3-propanediamine was used for the nucleophilic displacement of the halogen. Selective protection, as Dde derivative, of the side chain amino group of the resulting *N*-aminopropylglycil residue allowed us to continue the peptide chain assembling. The last residue was introduced as Boc derivative. Selective removal of the Dde group from the Norn residue (3% hydrazine), followed by guanidinylation. (bis-Boc-guanylpyrazole, 2 eq, overnight) and cleavage from the resin, yielded hybrids **2 - 4** (30-45% yield after purification) which were characterized by HPLC and ESI-MS. The CD spectra of apidaecin Ib and analogues **2 - 5** in aqueous solution suggest the presence of largely unordered structures. However the existence of a polyproline II-like secondary structure can not be excluded. CD Spectra of peptides 1-4 in 90% aqueous TFE are indicative of the presence of β-turns but the spectrum of the analogue **5** is largely unaffected by the organic solvent. The lack of amide hydrogens in the three Narg residues reduces the possibility of intra-chain hydrogen bonds. The antimicrobial activity of peptides **1-5** was tested by the broth microdilution assay in ¾ MHB, against *E. coli* ATCC2 5922, *P. aeruginos*a ATCC 25668 and three strains of *S. aureus* (ATCC BAA44, ATCC 29213 and 6538P). As the apidaecin Ib, the peptide-peptoid hybrids **2** and **3** were only active against *E. coli,* with a MIC value respectively comparable with or higher than that of the native peptide. Hybrids **4** and **5** were fully inactive indicating that the Arg/Narg substitution in the C-terminal part of the peptide is detrimental for the antimicrobial activity. These findings suggest that a defined conformation of this part of the molecule is probably needed to interact with its intracellular target. On the contrary Arg/Narg substitution in the N-terminal part of the sequence is well tolerated and this point should be considered in drug design based on this peptide family.

Acknowledgments

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THE EFFECTS OF N-TERMINAL MODIFICATION OF ARGININE VASOPRESSIN (AVP) ANALOGUES WITH 2-AMINOINDANE-2-CARBOXYLIC ACID. HIGHLY POTENT V_2 AGONISTS

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Introduction

Many of the arginine vasopressin (AVP) agonists and antagonists have been designed and synthesized in the course of extensive investigations of the structure – activity relationships [1 - 3]. All these efforts resulted in probably the best understanding of such relationship among peptide hormones. However, the design of analogues that are very active and truly selective for AVP receptors, still remains an area of great interest.

We have designed, synthesized and determined some pharmacological properties of nine new analogues of AVP substituted at positions 2 or 3 with 2-aminoindane-2-carboxylic acid (Aic). In addition to the reduction of flexibility, this new amino acid should enhance the resistance of the resulting peptides to enzymes. We synthesized the following analogues: [Aic^2]AVP (**I**), [Mpa^1,Aic^2]AVP (**II**) (Mpa, 3-mercaptopropionic acid), [Aic^2,D-Arg^8]VP (**III**), [Mpa^1,Aic^2,D-Arg^8]VP (**IV**), [Aic^2,Val^4,D-Arg^8]VP (**V**), [Mpa^1,Aic^2,Val^4,D-Arg^8]VP (**VI**), [Aic^3]AVP (**VII**), [Mpa^1,Aic^3]AVP (**VIII**) and [Aic^3,D-Arg^8]VP (**IX**).

Results and Discussion

The nine new analogues of AVP (**I - IX**) were synthesized by Fmoc strategy, purified and characterized. The values of the molecular ions were as expected and the purity was higher than 97%. The activities of the new analogues were determined in the *in vitro* rat uterotonic test in the absence of magnesium ions, the rat pressor test, and in the antidiuretic assay the conscious rats were used (for details concerning all tests, see ref. [4]). The results of pharmacological evaluation of peptides **I - VI**, together with relevant data for AVP and some related peptides, are presented in Table 1. The analogues **VII – IX** were devoid of any activity and thus are not included in the Table 1. Peptides modified at position 2 with Aic exhibited weak (**II** and **III**), moderate (**I** and **IV**) or strong (**V** and **VI**) antiuterotonic activities. Two analogues (**I** and **II**) showed weak pressor agonism whereas compounds **IV** and **VI** were weak pressor antagonists. Peptides **III** and **V** were inactive. As regards antidiuretic activity, new peptides were either potent (**I - V**) or highly potent (**VI**) agonists with prolonged action. Analogues substituted with Aic at position 3 (**VII - IX**) resulted in complete removal of all the activities

studied. The results presented in Table 1 show that the antidiuretic potency of analogues **I**, **II**, **IV** and **V** is similar to that of AVP if calculated on the basis of the threshold doses. Yet the analogues have steeper dose/response curves thus having prolonged activity.

In our opinion, these interesting peptides deserve further investigations for changes in three dimensional shape of the molecules using NMR, CD and theoretical molecular modelling methods and, optionally, crystallography. These useful tools may contribute to the explanation of closer relations between the backbone structure and functional groups. Summing up, our studies provide new, useful information about structure – activity relationships and open up new possibilities for designing potent V_2 agonists.

Table 1. Pharmacological properties of new analogues of AVP (IU/mg or pA_2)

Compound		Activity		
		Uterotonic *in vitro* no Mg^{2+}	Pressor IU/mg or pA_2	Antidiuretic IU/mg[b] $t_{1/2}$ 60 ($t_{1/2}$ 200)
AVP[a]		17	412	465
[Mpa^1]AVP[a]		27-63	346-370	1300-1745
[D-Arg^8]VP[a]		0.4	4.1	114-257
[Mpa^1,D-Arg^8]VP[a]		1.5-5.1	~0.39	800-50 000
[Val^4]AVP[a]		-	32	738
[Mpa^1,Val^4]AVP[a]		-	51	1150
[Aic^2]AVP	**I**	pA_2 = 7.27	9.4	~450 (45 000)
[Mpa^1,Aic^2]AVP	**II**	pA_2 = 6.50	5.3	~450 (45 000)
[Aic^2,D-Arg^8]VP	**III**	pA_2 = 6.86	0	~45 (4 500)
[Mpa^1,Aic^2,D-Arg^8]VP	**IV**	pA_2 = 7.31	pA_2 = 5.60	~450 (45 000)
[Aic^2,Val^4,D-Arg^8]VP	**V**	pA_2 = 7.93	0	~450 (45 000)
[Mpa^1,Aic^2,Val^4,D-Arg^8]VP	**VI**	pA_2 = 8.06	pA_2 =6.25	~4 500 (450 000)

[a] *values taken from [3];*
[b] *the activities obtained by comparing doses of the analogues and AVP resulting in an antidiuresis time of $t_{1/2}$=60 min. in arbitrary units; in parentheses, the activities obtained by comparing doses of the analogues and AVP resulting in an antidiuresis time of $t_{1/2}$=200 min.*

Acknowledgements

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INFLUENCE OF CONFORMATIONALLY CONSTRAINED AMINO ACIDS REPLACING POSITIONS 2 AND 3 OF ARGININE VASOPRESSIN (AVP) AND ITS ANALOGUES ON THEIR PHARMACOLOGICAL PROPERTIES

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Introduction

It is believed that the Phe residue at position 3 of AVP is involved mainly in recognition of this hormone and its binding to the receptors [1]. In turn, the Tyr residue at position 2 seems to be involved in initiating the pressor response of AVP [1]. It has been shown that modifications of these positions alone or in combination with other suitable changes yield analogues with very interesting biological properties [2 - 4]. Only limited information is available on the accommodation of the aromatic or cyclic units into the peptide backbones formed by α-amino acid sequences [5, 6]. It has been shown that such modifications are of considerable importance for designing drugs as it increases stability of these compounds to enzymatic degradation [7]. All the above mentioned findings highlighted importance of the conformation of the N-terminus of AVP analogues and prompted us to continue our studies by introduction new sterically restricted units into this part of the molecule. We have designed and synthesized thirteen new analogues of AVP and determined some of their pharmacological properties. We substituted two amino acid residues at positions 2 and 3 with one sterically constrained amino acid, such as 4-aminobenzoic acid (Abz), *cis*-4-aminocyclohexanecarboxylic acid (ach) and *trans*-4-aminocyclohexanecarboxylic acid (Ach). In addition to the reduction of flexibility, these amino acids should enhance resistance of the resulting peptides to enzymes. We synthesized the following analogues: [Abz^{2,3}]AVP (**I**), [ach^{2,3}]AVP (**II**), [Ach^{2,3}]AVP (**III**), [Mpa^{1},Abz^{2,3}]AVP (**IV**), [Mpa^{1},ach^{2,3}]AVP (**V**), [Mpa^{1},Ach^{2,3}]AVP (**VI**), [Mpa^{1},Abz^{2,3},D-Arg^{8}]VP (**VII**), [Mpa^{1},ach^{2,3},D-Arg^{8}]VP (**VIII**), [Mpa^{1},Ach^{2,3},D-Arg^{8}]VP (**IX**), [Mpa^{1},Abz^{2,3},Val^{4},D-Arg^{8}]VP (**X**), [Mpa^{1},ach^{2,3},Val^{4},D-Arg^{8}]VP (**XI**), [Mpa^{1},Ach^{2,3},Val^{4},D-Arg^{8}]VP (**XII**) and [Cpa^{1},Abz^{2,3}]AVP (**XIII**).

Results and Discussion

Thirteen new analogues of AVP (**I - XIII**) were synthesized manually, using Boc or Fmoc chemistry and characterized. Their physicochemical properties are presented in Table 1. The purity of each peptide was determined by HPLC and the values of the molecular ions were as expected. The activities of the analogues were determined in the *in vitro* rat uterotonic test in the absence of magnesium ions, in

the rat pressor test, and in the antidiuretic assay on conscious rats (for details concerning all tests, see ref. [8]). Unfortunately, in our case, incorporation of either the Abz residue or its aliphatic counterparts, ach and Ach, into the short flexible peptide molecules resulted in inactive compounds. However, we believe that even due to this disappointing result in terms of biological activity, it was worthwhile to learn how our unusual modifications would affect pharmacological properties of AVP and its agonists and antagonist. Our results, while not impressive in terms of biological activities of the reported peptides, offer important information for structure-activity considerations and for designing AVP analogues.

Table 1. Physicochemical properties of peptides I-XIII.

Analogue		Formula	HPLC (t_R)[a]	Molecular ion[b] Calculated	Found
[Abz^{2}]AVP	I	$C_{39}H_{51}N_{11}O_{10}S_2$	9.4	892.9	893.2
[ach^{2}]AVP	II	$C_{39}H_{53}N_{11}O_{10}S_2$	10.9	898.9	899.5
[Ach^{2}]AVP	III	$C_{39}H_{53}N_{11}O_{10}S_2$	10.6	898.9	899.4
[Mpa^{1},Abz^{2}]AVP	IV	$C_{39}H_{51}N_{11}O_9S_2$	11.6	877.9	878.4
[Mpa^{1},ach^{2}]AVP	V	$C_{39}H_{53}N_{11}O_9S_2$	11.8	883.9	884.4
[Mpa^{1},Ach^{2}]AVP	VI	$C_{39}H_{53}N_{11}O_9S_2$	11.9	883.9	884.2
[Mpa^{1},Abz^{2},D-Arg^{8}]VP	VII	$C_{38}H_{51}N_{11}O_9S_2$	11.7	877.9	878.3
[Mpa^{1},ach^{2},D-Arg^{8}]VP	VIII	$C_{38}H_{53}N_{11}O_9S_2$	12.1	883.9	884.1
[Mpa^{1},Ach^{2},D-Arg^{8}]VP	IX	$C_{38}H_{53}N_{11}O_9S_2$	12.1	883.9	884.2
[Mpa^{1},Abz^{2},Val^{4},D-Arg^{8}]VP	X	$C_{38}H_{51}N_{11}O_8S_2$	10.2	848.9	849.3
[Mpa^{1},ach^{2},Val^{4},D-Arg^{8}]VP	XI	$C_{38}H_{53}N_{11}O_8S_2$	12.8	854.9	855.2
[Mpa^{1},Ach^{2},Val^{4},D-Arg^{8}]VP	XII	$C_{38}H_{53}N_{11}O_8S_2$	12.3	854.9	855.4
[Cpa^{1},Abz^{2}]AVP	XIII	$C_{38}H_{48}N_{10}O_9S_1$	11.3	945.9	946.3

[a] The purity of the peptides was determined on a Vydac C_{18} column (5 μm, 4.6×250 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile/0.1% aqueous TFA (80:20 v/v). A linear gradient from 1% to 40% of solution [B] was applied for peptides I-IX and from 10% to 60% of solution [B] for X-XIII for 20 min at a flow rate of 1 mL/min.

[b] The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer.

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SYNTHESIS OF PEPTIDES DERIVED FROM GnRH AND THEIR ANALYSIS AND CHARACTERIZATION BY CAPILLARY ZONE ELECTROPHORESIS

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Introduction

Rapidly increasing knowledge of new gonadotropin-releasing hormones (GnRHs) of different species of the animal kingdom induces the need to prepare new synthetic derivatives and fragments with higher potency and metabolic stability and suitable for the formulation of new immunogens. The species related differences in the sequence of the native mammalian GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2, concern predominantly the positions 5, 7 and 8, particularly Tyr^5 is replaced for His or Leu, Leu7 by Val or Trp, and Arg8 is substituted by Lys, Ser, Asn or Gln.

Results and Discussion

Chemical synthesis of GnRH peptides and their fragments was carried out by SPPS. The most frequent protection strategy was Fmoc/tBu mostly in combination with Boc/Bzl strategy. The way of liberation of the peptide chain reflected the type of carrier used. The product was purified by RP-HPLC. Synthetic GnRHs and their analogs and fragments were qualitatively and quantitatively analyzed by CZE as cations in acidic background electrolytes (BGE I, 2 M acetic acid, pH 2.18; BGE II, 50 mM Tris, 100 mM H_3PO_4, pH 2.25; BGE III, 100 mM iminodiacetic acid, pH

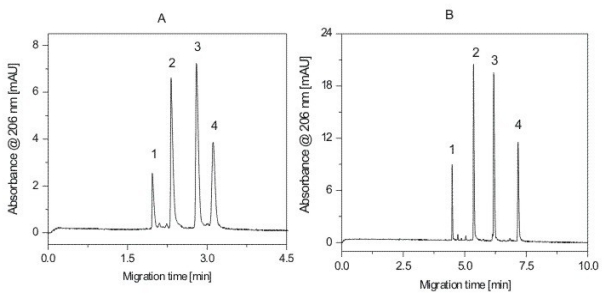

Fig. 1. CZE separation of structurally related hGnRH and its fragments in (A) BGE V, (B) BGE IV, 1 – fragment of hGnRH 4-10, 2 – hGnRH, 3 – fragment of hGnRH 1-4, 4 – [Des-Arg-Pro-Gly-NH_2]hGnRH.

2.30; BGE IV, 200 mM iminodiacetic acid, pH 2.32; BGE V, 500 mM acetic acid, pH 2.50). Analyses were carried out in a Beckman MDQ apparatus equipped with uncoated fused silica capillary (I.D. 50 μm, effective/total length 28.8/39.0 cm) and UV-photometric detector set at 206 nm.

The purity degree of the crude and HPLC purified synthetic GnRHs and their analogs and fragments was determined as relative corrected peak area using only picomole to femtomole amounts of peptides in nanoliter applied sample volume. The values of purity degrees reached 88 - 99%. The fastest separation of structurally related hGnRH and its fragments was achieved in BGE V, 500 mM acetic acid, pH 2.50 (Fig. 1A) and the best efficiency and resolution of peptides were achieved in isoelectric BGE IV, 200 mM iminodiacetic acid, pH 2.32 (Fig. 1B). Effective electrophoretic mobilities, m_{ep}, of analyzed peptides and their effective charges, q, were determined in all BGEs; their values in the fastest and the more efficient BGEs IV and V are shown in Table 1.

Table 1 Calculated effective charges, q, and CZE determined effective electrophoretic mobilities, mep, corrected to standard temperature, 25 °C, of analyzed peptides in best BGEs.

Peptide	q [e]		m_{ep} (10^{-9} $m^2V^{-1}s^{-1}$)	
	BGE IV	BGE V	BGE IV	BGE V
Human GnRH (hGnRH)	2.00	2.00	12.87	16.85
[Des-Arg-Pro-Gly-NH_2]hGnRH	0.92	0.91	7.66	9.55
Fragment of hGnRH 1-4	0.92	0.91	10.13	11.94
Fragment of hGnRH 4-10	2.00	2.00	16.86	21.99

Acknowledgements

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ANALOGUES OF ARGININE VASOPRESSIN (AVP) MODIFIED IN THE N-TERMINAL PART OF THE MOLECULE WITH A CONFORMATIONALLY CONSTRAINED *cis*-PEPTIDE BOND MOTIF

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Introduction

Biologically active peptides exhibit multiple conformations in solution. Thus, the synthesis of conformationally restricted analogues is a valuable approach for determining structure-activity relationships. Restrictions can be imposed e.g. by formation of cyclic structures within the peptide framework either through disulfide and lactam bridges or by substitution of chosen amino acid residues or building blocks with sterically restricted fragments that limit conformational freedom, forcing the peptide backbone and/or side chains to adopt specific orientations.

In our laboratory we have shown that such an approach could result in analogues with very interesting pharmacological properties [1 - 3]. Recently, continuing our efforts in this direction, we replaced the residues at positions 2 or 3 of AVP and some of its analogues with 1-aminocyclohexane-1-carboxylic acid (Acc) and 1-aminocyclopentane-1-carboxylic acid (Apc) [4, 5]. Our results have shown that both the Acc^3 and Apc^3 modifications were deleterious to interaction with V_1, V_2 and oxytocin receptors, except for [Mpa^1,Acc^3,Val^4,D-Arg^8]VP, which is potent and selective antidiuretic agonist. In the case of the Acc^2 and Apc^2 substitution the situation was different, as it selectively modified interaction with the aforementioned receptors.

Bearing all this in mind, we have designed, synthesized and determined some pharmacological properties of four new analogues of AVP with two amino acid residues substituted at positions 2 and 3 or 3 and 4 with one amino acid residue, either a (2*S*, 4*S*) (APy) or (2*R*, 4*S*) (Apy) diastereomer of 4-aminopyroglutamic acid, our novel *cis*-peptide bond motif. Computer modelling data suggest that this modification, e.g. short-range cyclization, should stand for β-turn type VI mimetics [6]. Introduction of such motifs into bioactive peptides might be used to explore the impact of β-turn geometry on their potency and selectivity. Moreover, this modification, apart from reducing the flexibility, also changed the character of a fragment of the molecule from aromatic to aliphatic. In the next four peptides we combined the above modification with a de-amino modification (3-mercaptopropionic acid, Mpa) at position 1.

Results and Discussion

The eight new analogues of AVP (**I - VIII**) were synthesized manually using Fmoc chemistry and characterized. Their physicochemical properties are presented in Table 1. The purity of each peptide was determined by HPLC and the values of the molecular ions were as expected.

The activities of the analogues were determined in the *in vitro* rat uterotonic test in the absence or in the presence of 1 mM magnesium ions, in the rat pressor test, and in the antidiuretic assay on conscious rats (for details concerning all tests, see ref. [4, 5]). Unfortunately, all the new analogues were inactive in all assays performed. These results, while not impressive in terms of the biological activities of the reported peptides, appear to offer new information about the structure-activity relationships useful for designing AVP analogues.

Table 1. Physicochemical properties of peptides I-VIII

Analogue		Formula	HPLC (t_R)[a]	Molecular ion[b] Calculated	Found
[APy^{2,3}]AVP	I	$C_{39}H_{52}N_{12}O_{11}S_2$	10.5	899.9	900.7
[Mpa^1,APy^{2,3}]AVP	II	$C_{39}H_{52}N_{12}O_{11}S_2$	12.7	884.9	886.1
[APy^{3,4}]AVP	III	$C_{39}H_{50}N_{14}O_{11}S_2$	11.1	934.9	935.2
[Mpa^1,APy^{3,4}]AVP	IV	$C_{39}H_{39}N_{14}O_{11}S_2$	14.3	919.9	920.5
[Apy^{2,3}]AVP	V	$C_{39}H_{39}N_{12}O_{11}S_2$	10.2	899.9	900.5
[Mpa^1,Apy^{2,3}]AVP	VI	$C_{39}H_{52}N_{14}O_{11}S_2$	11.5	884.9	885.9
[Apy^{3,4}]AVP	VII	$C_{39}H_{50}N_{14}O_{11}S_2$	10.9	934.9	935.5
[Mpa^1,Apy^{3,4}]AVP	VIII	$C_{39}H_{39}N_{14}O_{11}S_2$	14.3	919.9	920.4

[a] The purity of the peptides was determined on a Vydac C_{18} column (5 µm, 4.6×250 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile:0.1% aqueous TFA (80:20 v/v). A linear gradient from 1% to 80% of solution [B] for 20 min was applied at a flow rate of 1 mL/min.
[b] The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer.

Acknowledgements

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SOLID STATE NMR STRUCTURE ANALYSIS OF MEMBRANE BOUND ANTIMICROBIAL AND CELL PENETRATING PEPTIDES

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Introduction

In nature most organisms produce antimicrobial peptides (AMPs) in response to microbial invasion, which kill the bacteria by permeabilizing their cellular membrane. Cell penetrating peptides (CPPs), on the other hand, maintain the membrane integrity while traversing the lipid bilayer. Cationic CPPs can carry cargo such as drugs, nucleic acids or nanoparticles. The functional mechanism of AMPs and CPPs is still not clear, and their membrane interactions (perturbation and translocation) are subject of intense investigation. Solid state NMR is a suitable tool to investigate the structure and dynamic behaviour of AMPs and CPPs in their functionally relevant lipid environment. We have characterized various ^{19}F-labeled peptides by highly sensitive ^{19}F-NMR, supporting these data with non-perturbing labels ^{2}H and ^{15}N. Their effect on phospholipid membranes was examined by ^{31}P-NMR. Here, we studied the AMPs Gramicidin S (*cyclo*-[P<u>V</u>O<u>L</u>^{D}F]$_2$), PGLa (GMASKAG<u>AIA</u>GK<u>IA</u>KVALKAL-NH_2), K3 (KIAGKI<u>A</u>K<u>IAG</u>KIAKIAGKIA-NH_2), as well as the different CCP types HIV-TAT (GRKKRRQRRRPPQ) and MAP (KLALK<u>L</u>A<u>L</u>KA<u>L</u>KAA<u>L</u>KLA-NH_2). The underlined positions were selectively labelled with ^{19}F-amino acids, and the other isotope-labelled positions are shown in italics.

Results

Peptide analogues were synthesized containing one specific ^{19}F-labeled amino acid as a local structural reporter, such as 4-fluoro-phenylglycine (4F-Phg) or 4-trifluoromethyl-phenylglycine (4CF_3-Phg). Their functional integrity was validated by testing their biological activities. NMR samples were prepared by reconstituting the peptides into model membranes and their three-dimensional backbone structures were calculated from a set of ^{19}F-NMR orientational constraint as previously described [1]. The alignment and dynamics of a peptide in the bilayer is defined by three parameters: the tilt angle of the peptide τ between its main symmetry axis and the membrane normal, the azimuthal angle ρ which defines the rotation of the amphiphilic faces, and the molecular order parameter S_{mol} which describes the molecular wobble.

Gramicidin S is a symmetric cyclic decapeptide, in which we substituted two leucines (or valines) by 4F-Phg (or 4CF_3-Phg). It was studied in oriented DMPC

membranes as previously described [1, 2]. At a low peptide to lipid ratio (P/L $\leq$ 1:80), gramicidin S shows a single ^{19}F-resonance at 119 ppm, indicating that both 4F-Phg labels are aligned in symmetric way. Data analysis shows that the peptide is surface-bound and highly mobile (τ = 0º, $S_{mol} \approx 0.3$). At high concentration (P/L $\geq$ 1:20) two resonances at -65 and -80 ppm appear, suggesting a complete re-alignment of gramicidin S. The new structure corresponds to an upright orientation of the cyclic backbone ($\tau \approx 80º$, S_{mol} = 1.0). The value of ρ was determined independently as -45º, using ^{15}N labeled leucines (or valines). The upright orientation is likely to be stabilized by formation of an oligomeric pore.

An analogous ^{19}F-NMR analysis of the helical peptide PGLa also showed a concentration dependent two-state re-alignment [3] At low peptide concentration a surface-bound state was observed (τ = 89º, ρ = 106º, S_{mol} = 0.6), while at high concentration a tilted state was found (τ = 123º, ρ = 85º, S_{mol} = 0.6). The tilted state appears to be stabilized by the formation of dimers, as have been previously reported for the related peptide K3. Using non-perturbing ^{2}H-NMR, the alignment of PGLa was confirmed and refined for both peptide concentrations [4]. In the case of the CPPs MAP and HIV-TAT a different picture emerged, as MAP causes significant disorder in the lipid bilayer at higher concentration. HIV-TAT, on the other hand, gives rise to an isotropic signal in DMPC. This was interpreted in terms of an inverted micellar morphology, which possibly forms via bidentate complexes between the arginine side chains and the phosphate head groups of the lipid [5].

Discussion

^{19}F-NMR has proven to be a powerful method for investigating peptide structures and their lipid interactions. In several systems we have observed a concentration-dependent re-alignment and self-assembly of the peptides in the membrane, accompanied by substantial effects on the lipid bilayer. We conclude that the peptide concentration in the membrane plays a key role in the mechanism of action, and the respective threshold concentrations can be readily determined by sensitive ^{19}F-NMR and accurate ^{2}H-NMR.

Acknowledgements

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SYNTHETIC STUDY ON DERIVATIVES OF DIMERIC PEPTIDE FROM HUMAN IgG1 HINGE REGION

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Introduction

The central part of protein from the surface region of human immunoglobulin-IgG1 is occupied by the dimeric structure **1**, $(H\text{-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH})_2$ representing a suitable core peptide for design and synthesis of various peptide conjugates with regard to their immunochemical applications [1, 2]. Its synthesis by classical approach in solution [3, 4] utilized the orthogonal protection of Cys residues with a selective closure of disulfide bonds or random oxidation affording over 90% of parallel dimer that could be purified by chromatography.

Results and Discussion

We have developed simplified syntheses of the above peptide and its bis-N-protected derivatives **2** - **5** in solution or on soluble (PEG) or insoluble (PS-DVB) polymer supports, to be used in segment condensation with variety of peptides possessing antigenic properties. In the solution approach, the truncated decapeptide dimer **2** $(H\text{-Cys-Pro-Pro-Cys-Pro-NH}_2)_2$ [5] was prepared using bis-N^{α}-Boc-cystine-bis-pentafluorophenyl ester. After the cleavage of N^{α}-Boc-groups, the proline residues were added successively to both the amino groups of the cystine

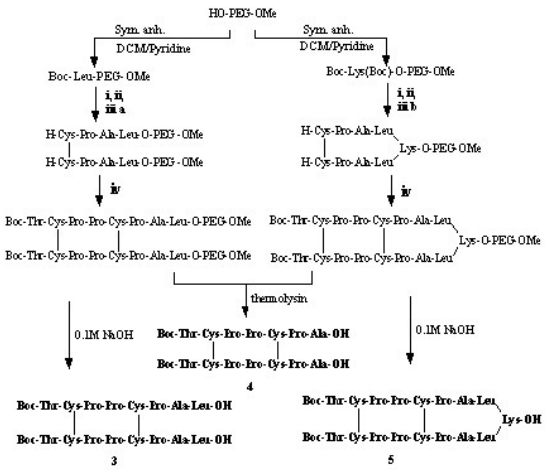

Fig. 1. i- 50% TFA/DCM; **ii**- 20% DIEA/DCM; **iii a**- $(Boc\text{-}Ala)_2O$, $(Boc\text{-}Pro)_2O$, $(Boc\text{-}Cys\text{-}OPfp)_2$ in DCM/DIEA; **iii b**- $(Boc\text{-}Leu)_2O$, $(Boc\text{-}Ala)_2O$, $(Boc\text{-}Pro)_2O$; $(Boc\text{-}Cys\text{-}OPfp)_2$ in DCM/DIEA; **iv**- 2x $(Boc\text{-}Pro)_2O$, $(Boc\text{-}Cys\text{-}OPfp)_2$, Boc-Thr-OPfp in DCM/DIEA

residue, followed by addition of the second cystine and cleavage of N^{α}-Boc-protected derivatives **3** - **5**, performed on soluble polymer-PEG-OMe [6] in CH_2Cl_2 (Fig. 1). Thus, also in the PEG-OMe synthesis the parallel arrangement of both the peptide sequences was achieved unequivocally. Using this polymer, also the lysine was introduced as the first amino acid and the peptide sequences was allowed to grow on both the amino groups of this residue. After the Lys residue, Leu was attached as enzymatically cleavable linker. Parallelly to common detachment of peptides from the PEG by a mild saponification, we used also the thermolysine catalysis, after the C-terminal proline residue in **1** was replaced for the leucine one. In the SPPS on PS-DVB polymer, the Fmoc/*t*Bu and Cys(Trt) protection was applied and both the disulfide bridges between the linear peptides were closed by action of pure oxygen under mildly elevated pressure. Such a modification shortened the random oxidation four times in comparison with the air oxidation and there were no traces of antiparallel or monomer disulfides of hinge peptide 1 detected, there. Finally, we optimized the preparation of polypeptides containing the hinge structures in the central part of corresponding molecules, as the conjugates with antigenic peptides, e.g. EBNA-20-peptide (Fig. 2). ELISA test will be used to study the ability of the hinge structure to present this antigenic peptide in comparison with other carriers.

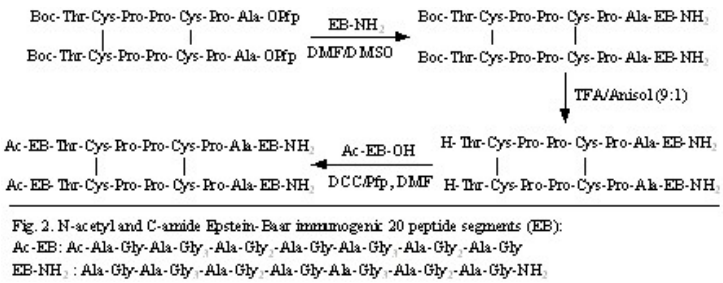

Fig. 2. N-acetyl and C-amide Epstein-Baar immunogenic 20 peptide segments (EB):
Ac-EB: Ac-Ala-Gly-Ala-Gly$_3$-Ala-Gly$_2$-Ala-Gly-Ala-Gly$_3$-Ala-Gly$_2$-Ala-Gly
EB-NH$_2$: Ala-Gly-Ala-Gly$_3$-Ala-Gly$_2$-Ala-Gly-Ala-Gly$_3$-Ala-Gly$_2$-Ala-Gly-NH$_2$

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PROTECTION OF NEUROTOXICITY OF A-BETA BY RETROINVERSO PEPTIDES

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Introduction

Aggregates of Aβ are neurotoxic and a cause of cognitive decline seen in Alzheimer's patients [1]. KLVFF, containing the 16-20 sequence of full-length Aβ, disrupts fibril formation, and inhibits fibril formation at a 10-fold molar excess. We have developed potent inhibitors of Aβ aggregation and toxicity by synthesis of the reverse of the recognition sequence within Aβ, KTVFF as ffvlk, using D-amino acids rather than L found in the native sequence. The retroinverse configuration gives rise to peptides with increased resistance to proteolytic degradation, but amino acid side-chains are in the same orientation with respect to each other as in the native sequence. Termini were substituted with Arg to induce dispersal of the amyloid oligomers, and C-terminal amide to increase transport into brain.

Methods

Aβ40 and 42, were synthesised as described [2]. Monomeric Aβ40 and Aβ42 solutions were prepared by triple recycling in TFA and HFIP, and drying under dry N_2. The peptide rGffvlkr-amide (lower case = D-amino acids) containing the retroinverso recognition sequence in Aβ was synthesised on PAL-PEG-Polystyrene resin (Applied Biosystems 0.22 mmole/g). The target peptide was cleaved and deprotected in TFA, TIPS (5% by vol) and water (5% by vol), triturated in ether, and purified by gradient HPLC on a column of Dynamax C8 in 0.1% TFA. Purity was verified by MALDI mass spectrometry. Other inhibitors were obtained from Bachem. Peptides were incubated together with Aβ in sterile 0.1 M Tris-HCl (pH 7.4) (25 μM) and added to SHSY-5Y cells for culture for 16 hours. Cells remaining were quantitated by MTT [3].

Results

The MTT staining in Fig. 1 showed that the retroinverso peptide *rGffvlk-NH_2* was more effective than the native sequences LVFFG or RGKLVFFGR-amide at preventing Aβ-induced cell death.

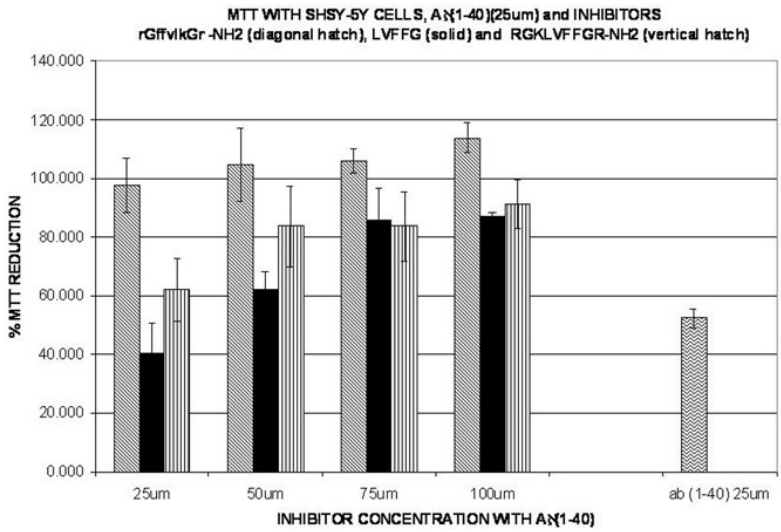

Fig.1. MTT dye uptake into living SHSY-5Y cells after incubation for 16 hrs with aggregated Aβ40 (25 μM) and inhibitors rGffvlk-NH_2 (diagonal hatch), LVFFG (solid bars) or RGKLVFFGR-NH_2 (verticle hatch). The % MTT reduction was the dye stain as % of the stain of cells incubated with no addition.

Discussion

The KLFF-sequence (Aβ16-20) is the region in Aβ that most efficiently bings to itself, and this sequence is necessary for fibril formation. Aβ16-20 binds to the homologous region in Aβ in an antiparallel manner. A molecular model of aggregated Aβ [3] indicates it forms antiparallel β-sheets, in keeping with solid state NMR spectra [4]. KLVFF sequences in adjacent monomers iteract, the Phe side chains in particular interact by π-orbital overlap. The disposition of side chains in retroinverso ffvlk maintain these key interactions. In view of the increased stability of D-residues against mammalian protease, it is reasonable that expect that effective therapeutics against AD will comprise retroinverso amyloid-binding sequences, with additional groups that disperse amyloid aggregates and increase access to brain.

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INTERACTION BETWEEN PENETRATIN AND PHOSPHOLIPIDS BILAYERS

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Introduction

Penetratin (RQIKIWFQNRRMKWKK) is a cell-penetrating peptide. Its translocation mechanism is not clear, but the first step may be the interaction between the peptide and the lipid bilayer [1]. Recent studies have shown that penetratin interacts with negatively charged liposomes [2]. The interaction depends on the ratio of lipid to peptide [3]. In order to analyse the effect of the charges of the phopholipid bilayer on the penetration-liposome interaction we aimed to analyse the influence of penetratin on liposome preparation of two different phospholipids (DPPC/DPPG 2:8 - 8:2) using fluorescence spectroscopy.

Results

In the first set of experiments, liposome labelled with fluorescent markers (1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene p-toluensulfonate (TMA-DPH) or 8-anilino1-sulfonaphthalene (ANS)) were incubated with penetratin and the fluorescence polarisation of dyes was determined as a function of the temperature (Fig. 1.). In the range of 15 - 200 mol/mol phospholipid/penetratin ratio, no change in the transition temperature was observed indicating that penetratin has no influence on the membrane structure.

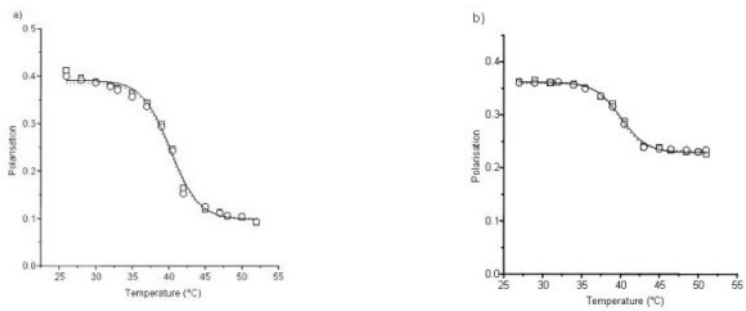

Fig. 1. Polarisation value vs temperature curves recorded for DPPC:DPPG = 80%:20% bound DPH (a) and TMA-DPH (b) in the presence or absence of penetratin (control: solid , penetratin: dot). L/P = 20.

Next, we have analysed the interactions between phospholipids and penetratin by monitoring changes in the intrinsic fluorescence of the peptide due to the presence

of two Trp residues in its sequence. Comparing the emission spectra corresponding to penetratin in aqueous medium or in presence of vesicles one can clearly appreciate a blue shift (Fig. 2.). This indicates that Trp residues are mainly exposed to a hydrophobic environment. Analysis of the main band shows low values of polarization suggesting a free motion of the peptide backbone. On the contrary polarization determined for penetratin mixed with liposome results in higher values (Fig. 3.). This indicates that hydrophobic residues, like Trp, are inserted into the bilayer and their motion is restricted.

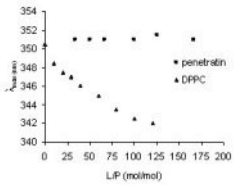

Fig. 2. λ_{max} of the Trp emission as a function of lipid-peptide ratio. (0.1 M sodium acetate buffer, pH 7.4)

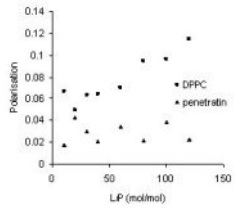

Fig. 3. Fluorescence polarisation of Trp as a function of lipid-peptide ratio. (0.1 M sodium acetate buffer, pH 7.4)

Discussion

The penetratin and other cell-penetrating peptides are useful molecular tools for the drug delivery. Better understanding of their translocation mechanism could be helpful to design appropriate drug-conjugates for delivery. Data based on fluorescence polarisation of liposome bound dyes show that binding of penetratin does not cause major alteration in the structure of the bilayers composed of different phospholipids studied. Perhaps, penetratin as a small molecule can interact only with hydrophilic head group of a few phopholipid molecules on the surface, but this is not enough to alter the membrane structure. Changes in the fluorescence of intrinsic Trp residues also confirm that penetratin interact with neutral DPPC liposome. These results suggest that the hydrophobic region of penetratin, including the Trp residue(s), could be involved in the liposome interaction, but the electrostatic repulsion between peptide molecules hinder the binding of large number of peptides.

Acknowledgements

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CYCLOLINOPEPTIDE A ANALOGUES CONTAINING β^2-ISOPROLINE AND β^3-HOMOPROLINE

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Introduction

Cyclolinopeptide A (CLA), a natural cyclic nonapeptide: cyclo(-Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-), isolated from linseed oil [1], possesses a strong immunosuppressive activity comparable at low doses with that of cyclosporin A [2]. However, despite lack of cytotoxicity and relatively strong, universal immunosuppressive properties, it has not found application in therapy. It has been postulated that the tetrapeptide sequence Pro-Pro-Phe-Phe is responsible for the interaction of the CLA molecule with a proper cellular receptor [3].

Results and Discussion

Our early studies showed that the replacement of phenylalanine residues by the dipeptide unit, containing ethylene link bridging two phenylalanine nitrogens, gave CLA analogues with opposite biological action [4]. Now we present CLA analogues in which proline residues were replaced by β^2-isoproline and β^3-homoproline residues, respectively (Fig. 1). The immunosuppressive activity of these new compounds remains on the level of the native peptide.

1. cyclo(Leu^1-Val^2-βhPro^3-Pro4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

2. cyclo(Leu^1-Val^2-Pro^3-βhPro^4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

3. cyclo(Leu^1-Val^2-βhPro^3-βhPro^4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

4. cyclo(Leu^1-Val^2-βiPro^3-Pro^4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

5. cyclo(Leu^1-Val^2-Pro^3-βiPro^4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

6. cyclo(Leu^1-Val^2-βiPro^3-βiPro^4-Phe^5-Phe^6-Leu^7-Ile^8-Ile^9)

The synthesis of β^2-isoproline was performed according to the known procedure [5]. The starting 4-hydroxyproline was decarboxylated followed by the hydroxy activation. Substitution by cyanide and hydrolysis gave the desired β-amino acid. β^3-Homoproline was obtained in the similar manner [6], starting from Boc-prolinol. The linear peptides were prepared manually using standard solid-phase procedure on the Wang resin, using Fmoc group for N-amino protection and TBTU as a coupling reagent. The cyclization of linear precursors has been made in the solution using EDC/HOBt coupling reagents.

To evaluate the immunosuppressive activity of the newly synthesized CLA

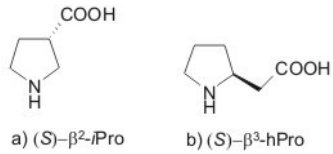

Fig.1. Structures of a) β^2-isoproline (β^2-iPro) and b) β^3-homoproline (β^3-hPro).

analogues, the mitogen-induced splenocyte proliferation and the humoral immune response *in vitro* assays were employed [7]. The effect of the peptides on the humoral response was tested by determination of the plaque forming cell number (PFC) (Fig. 2). Toxicity was determined with respect to WEHI 164.13 cells [8]. All peptides displayed differential, predominantly suppressive activities and they exhibited none or relatively low cell toxicity at higher dose (100 μg/ml) (not shown). Some of them, like **3**, needs application of other experimental models, such as mixed lymphocyte reaction and graft rejection due to the lack of toxicity, the strong inhibition of T and B-cell proliferation (not shown) and no suppressive effect in the humoral immune response. Such properties are important in the prevention of allograft rejection.

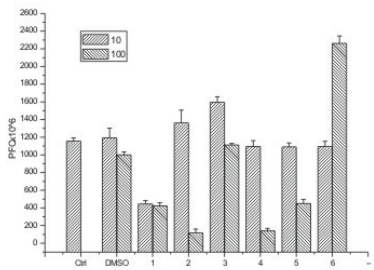

Fig.2. Determination of the humoral immune response in vitro. PFC number in splenocytes of BALB/c immunized by SRBC and treated with two doses of peptides 1-6.

Acknowledgments

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ONE POT NATIVE CHEMICAL LIGATION OF THREE SEGMENTS

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Introduction

The N-terminal ectodomain of the CRF receptor is a 120 amino acids sequence which is difficult to synthesize with traditional SPPS synthesis even by the use of pseudo prolines, depsipeptide or protected segments coupling strategies. The stepwise native chemical ligation seems to be the method of choice to accomplish the synthesis of the N-terminal ectodomain of the CRF receptor because it contains 6 cysteines. Nevertheless intermediate purification steps of this method can be a drawback with regard to the overall yield of the synthesis. The thiazolidine protecting group is used as temporary protection for cysteine [1] which is easily removed by methoxyamine×HCl and allows the sequential ligation of three or more segments without intermediate purification steps. Here we present one pot chemical ligation of the following peptide which is a part of the N-terminal ectodomain of the CRF Receptor.

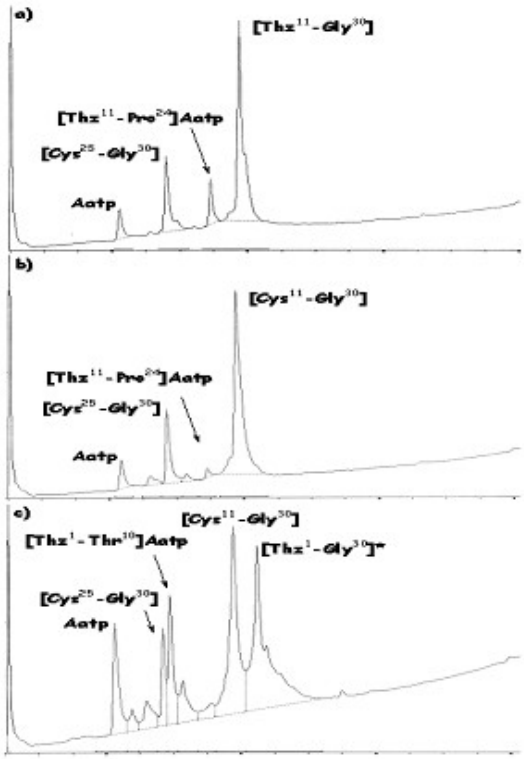
(Thz)C^{1}NASVDLIGTC^{11}WPRSPAGQLVVRP$^{24}C^{25}$PAFFYG^{30}

Results

The peptide thiol esters were made according to our previously published procedure [2] by solid-phase peptide synthesis methods using Fmoc strategy. The thiazolidine was coupled as a Boc protected amino acid. The ligation was performed in 6 M Gn·HCl, 0.1 M Na_2PO_4, pH 7.5 with TCEP as reducing agent. The first ligation of [Thz^{11}-Pro^{24}]thioester and [Cys^{25}-Gly^{30}] went to completion after 5h (Fig. 1a). Methoxyamine×HCl (0.2 M) was then added to the crude ligation mixture, the pH dropped consequently to 4. Overnight reaction at ambient temperature resulted in the complete removal of the protecting group which was confirmed by a LC-MS analysis (Fig. 1b). The pH was carefully readjusted to 7.5 by adding a solution 1 M Na_2HPO_4 to the mixture. Addition of the third peptide segment [Thz^{1}-Thr^{10}] to the crude ligation mixture resulted in the formation of the desired product (Fig. 1c) which was confirmed by a LC-MS analysis.

Discussion

The use of thiazolidine as intermediate protecting group makes possible the ligation of three segments without an intermediate purification step. It should also be possible to couple more than three segments after optimisation of the process.



Aknowledgments

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CYCLIC PEPTIDES AS INHIBITORS OF INTEGRINS - LIGAND INTERACTION

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Introduction

Integrins constitute a family of transmembrane cell surface receptors. They are involved in cell-cell and cell-extracellular matrix interactions. Thus, they participate in many physiological and pathophysiological processes and are of crucial importance for the living organism. Integrins possess two non-covalently bound subunits, α and β, that jointly participate in ligand binding. These dimeric proteins show very high specificity in recognition of natural ligands. For example, $\alpha_4\beta_1$ integrin recognizes VCAM-1 (vascular cell adhesion molecule 1) and fibronectin through binding the amino acid motifs TQIDSLPN and LDV, respectively. On the other hand, fibronectin is a classical ligand for $\alpha_5\beta_1$ integrin with the recognition motif RGD. As shown, identification of the integrin ligands occurs through small recognition amino acid sequences (often tripeptides). Thus, small cyclic peptides possessing a recognition motif in the appropriate three-dimensional conformation are able to interfere with the integrin-ligand interactions and act as inhibitors. Introduction of β-amino acids into the peptide leads to stabilization of the secondary structure and an enhancement of the activity and selectivity [1].

The aim of this investigation is the characterization of small cyclic peptides containing the RGD motif and a new building block $^{\beta}$Acc. Selectivity and specificity of newly synthesized peptides c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) and c(Arg-Gly-Asp-(-)-$^{\beta}$Acc-Val) were investigated in biological assays and binding studies with surface plasmon resonance.

Results and Discussion

The biological activity of the cyclic peptides were determined in cell adhesion assays. WM115 and K562 cells were used as the model cell lines. Vitronectin and fibronectin were used as the natural ligands for the integrins expressed on the surface of WM115 and K562 cells, respectively. The adhesion of the WM115 cells to vitronectin is predominantly mediated by integrin $\alpha_v\beta_3$, whereas the adhesion of K562 to fibronectin is mainly mediated by integrin $\alpha_5\beta_1$ [2, 3]. In both cases RGD amino acid binding epitope is involved in molecular recognition. The peptides known from the literature, c(Arg-Gly-Asp-D -Phe-Val) and c(Arg-Gly-Asp-D-Phe-$^{\beta}$Ala-Val), were used as the control [2, 4].

The experiments with living cells have shown that peptide c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) is the new active and selective inhibitor of integrin $\alpha_v\beta_3$ (IC_{50} value of 20 nM). The peptide c(Arg-Gly-Asp-(-)-$^{\beta}$Acc-Val) has shown the lower activity (IC_{50} value of 630 nM) im comparison to plus diastereomer c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-

Val). The high activity of c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) was also demonstrated in surface plasmon resonance experiments with membrane extracts from WM115 cells. The results show that introduction of the new building block $^{\beta}$Acc has led to enhancement of the activity and specificity of the c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) to integrin $\alpha_v\beta_3$.

The structure analysis of the peptides c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) and c(Arg-Gly-Asp-(-)-$^{\beta}$Acc-Val) has revealed the differences of both diastereomers. Peptide c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) possesses a βIV-turn between Asp and Arg and a γ-turn between Arg and Asp. The reference peptide c(Arg-Gly-Asp-D-Phe-Val) shows high similarity to c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) with the βII'-turn and γ-turn localized in the same positions [4]. The peptide c(Arg-Gly-Asp- Asp-(-)-$^{\beta}$Acc-Val) possesses a βIII-turn with Val in i+1 position between (-)-$^{\beta}$Acc and Gly. The distance between C^{α} of Asp and Arg of the literature peptide c(Arg-Gly-Asp-D-Phe-Val) is 668 pm [4]. The same distance for peptide c(Arg-Gly-Asp-(+)-$^{\beta}$Acc-Val) and c(Arg-Gly-Asp-(-)-$^{\beta}$Acc-Val) is 706 pm and 826 pm, respectively. This explains the differences in the activity of both peptides.

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NEW BRADYKININ ANALOGUES SUBSTITUTED IN POSITIONS 7 AND 8 WITH STERICALLY RESTRICTED 1-AMINOCYCLOPENTANE-1-CARBOXYLIC ACID

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Introduction

Major improvements in the design of B_2 antagonists were made as soon as conformationally constrained amino acid residues were incorporated in their C-terminal ends. In our laboratory, we discovered that acylation of the N-terminus of many B_2 antagonists with bulky acyl groups consistently improved the antagonistic potency in the blood pressure test [1]. Recently, we demonstrated that antagonistic activity at the B_2 receptor might be attributed to peptides having at position 7 suitable achiral, non-aromatic, conformationally constrained amino acid, *e.g.* 1-aminocyclohexane-1-carboxylic acid (Acc).

Having in mind successful manipulations consisting in substitution of sterically restricted amino acids in the C-terminal part of BK analogues that resulted in highly potent and selective B_2 antagonists , we have decided to use 1-aminocyclopentane-1-carboxylic acid (Apc) as a substituent. In comparison to the Acc residue used previously, Apc is smaller (the cyclic side chain ring consists of five atoms) and more restricted. Similar to the Acc substitution, it should reduce the flexibility of the peptide backbone by restricting conformational freedom. This type of modification is an example of the C_α-C_α cyclization, whereby a dialkylated glycine residue is converted into a cyclic side chain (1-aminocycloalkane-1-carboxylic acid). As in our previous studies, we have used [D-Arg^0,Hyp^3,Thi^{5,8}, D-Phe^7]BK, the B_2 antagonist previously synthesized in Stewart's laboratory, as a starting structure and we have substituted either position 7 or 8 with Apc. In the next two peptides we combined this modification with acylation of the N-terminus with 1-adamantaneacetic acid (Aaa) that had previously been shown to improve the antagonistic properties.

Table 1. Pharmacological properties of new analogues of BK

Peptide	Uterotonic Potency % of activity of BK or pA_2	Vasodepressor potency		
		ED_{20} (mg/min)	ED_{50} (mg/min)	ED_{90} (mg/min)
[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK	pA_2=6.88±0.08[a]	1.73±0.43[a]	-	124.17±27.04[b]
I [D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7] BK	0.25%	13.49±4.25	166.9±63.16	8302±4782
II Aaa[D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7] BK	0	2.83±0.36	46.03±12.66	2899±1187
III [D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK	pA_2=6.77±0.05	0.35±0.05	3.45±0.37	91.04±18.69
IV Aaa[D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK	pA_2=7.30±0.11	0.14±0.03	0.98±0.06	17.57±3.37

a value taken from ref. [2], b value taken from ref. [3].

Results and Discussion

From the results presented in Table 1 it is obvious that substitution of Apc in position 7 (similar to Acc^7 modification) is disadvantageous for B_2 antagonistic potency of the resulting analogues. Nevertheless, we could once more obtain analogues substituted in position 7 with an achiral, non-aromatic, conformationally constrained amino acid of antagonistic activity. Until recently, the presence of aromatic D-amino acid residue in position 7 was considered to be necessary for B_2 antagonism. This finding is another example supporting our previous results which have shown that the D-amino acid residue in position 7 can be replaced, together with an amino acid occupying position 8, by an appropriate sterically restricted unit [4]. From our previous data we learned that acylation of the N-terminus of BK antagonists with bulky groups consistently improved antagonistic potency in the BPT. In view of the present data, this appears to be valid for both pairs of analogues. However, the enhancing effect is weaker than that previously reported. It is also interesting to note that peptides substituted in position 7 with Apc which showed a moderate or a weak antagonistic potency in BPT were completely inactive in the rat uterus assay. The same effect was observed for analogues substituted in position 7 with the Acc residue. This seems to suggest once more the possibility of the presence of different subtypes of B_2 receptors in the uterus and blood vessels, postulated by various investigators.

Acknowledgments

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NEW BRADYKININ ANALOGUES ACYLATED ON THEIR N-TERMINUS: EFFECT ON RAT BLOOD PRESSURE AND RAT UTERUS

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Introduction

Our previous studies suggested that acylation of the N-terminus of several known B2 antagonists with various kinds of bulky acyl groups consistently improved their antagonistic potency in rat blood pressure assay [1]. On the other hand, our earlier observations seem to suggest that effects of acylation on the contractility of isolated rat uterus depend substantially on the chemical character of the acyl group, as we observed that this modification might either change the range of antagonism or even transform it into agonism [2, 3].

Bearing all this in mind, we decided to synthesize seven new analogues of bradykinin by N-terminal acylation with various acyl groups (4-hydroxy-3-metoxybenzoic (**X_1**), 4-hydroxybenzoic (**X_2**), 3-hydroxy-4-methylbenzoic (**X_3**), 3-(2-hydroxyphenyl)propionic (**X_4**), 3-(4-hydroxyphenyl)propionic (**X_5**), 1-hydroxy-2-naphthoic (**X_6**) and 2-nornbornaneacetic acids (**X_7**)) of a moderately potent B_2 antagonist, previously synthesized by Stewart's group, D-Arg-Arg-Pro-Hyp-Gly-Thr-Ser-D-Phe-Thi-Arg. The analogues were tested *in vitro* for their blood pressure-lowering and uterotonic activities.

Table 1. Pharmacological properties of new analogues of BK.

Peptide	Uterotonic Potency	Vasodepressor potency		
	pA_2	Ed_{30} (mg/min)	ED_{30} (mg/min)	ED_{60} (mg/min)
[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.88±0.08[a]	1.73±0.43[b]	-	124.17±27.04[b]
Aaa[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	7.43±0.11[a]	0.84±0.09[b]	-	13.94±1.69[b]
(I) X_1[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	5.82±0.30	0.18±0.02	1.35±0.13	20.17±2.39
(II) X_2[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.51±0.19	0.46±0.08	2.89±0.31	36.36±6.33
(III) X_3[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.89±0.29	2.04±0.37	11.70±1.34	122.62±24.28
(IV) X_4[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.38±0.25	2.36±0.59	10.97±1.72	97.40±17.00
(V) X_5[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.26±0.10	0.29±0.04	1.38±0.11	13.47±2.87
(VI) X_6[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.77±0.25	0.39±0.07	5.41±0.60	222.55±54.81
(VII) X_7[D-Arg^1,Hyp^3,Thi^5,D-Phe^7,Thi^8] BK	6.40±0.14	3.32±0.50	18.94±1.92	225.08±51.35

[a]value taken from ref. [3], [b]value taken from ref. [4]

Results And Discusion

In the present work we substituted seven new acyl groups in the N-terminus of the model antagonist. As seen in Table 1, it is clear that the proposed modifications preserved (analogues **III** and **IV**), increased (analogues **I**, **II**, **V**) or decreased (analogues **VI**, **VII**) the antagonistic potency in the rat blood pressure test. Moreover, the extent of the antagonism does not seem to depend on the acid strength, but rather on substitution of the hydroxyl group to the aromatic ring and the distance of the bulky fragment of the acyl substituent from the main peptide chain. All analogues acylated with 4-hydroxylated compounds are much more active than the Stewart's peptide and almost as active as its Aaa-acylated counterpart. It is also interesting to note that the distance of the substituted aromatic fragment of the acyl group from the main peptide chain influenced the antagonistic activity of the analogues. In the case of analogue II, in which the aromatic fragment is linked directly to the carboxyl group, its activity is a few times lower than that of analogue V.

On the other hand, all substituents differently influenced the interaction with the rat uterine receptors. In this case, five of our modifications resulted in less active B_2 blockers (**I**, **II**, **IV**, **V** and **VII**), while two of them (**III** and **VI**) preserved antagonistic activity of the parent peptide. Moreover, it is very interesting that two of our most active B_2 antagonists in the blood pressure test are the weakest antagonists in the rat uterus test. This finding seems to suggests once more different type of interactions with B_2 receptors localized on both types of tissues. Finally, looking at the activity of analogue **VII**, we have demonstrated how important for the activity of BK antagonists is bulkiness of an acid used for acylation of the N-terminus of the molecule. Thus, peptide **VII**, acylated with 2-norbornaneacetic acid, was a few times weaker antagonist than the model peptide and much less active that its acylated counterpart in both tests. In summary, our studies provided new information on the structure-activity relationship of bradykinin analogues that may have an impact on designing selectively acting bradykinin antagonists.

Acknowledgments

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SYNTHESIS OF BIOLOGICALLY ACTIVE PEPTIDE-PNA CONJUGATES AND ARTIFICIAL RECEPTORS BY SORTASE-MEDIATED LIGATION

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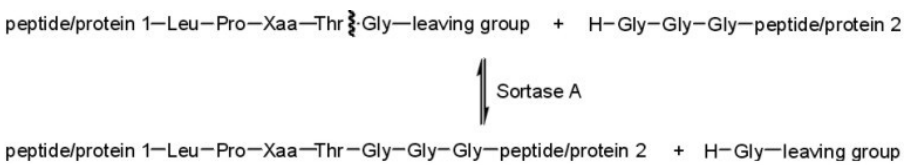
Introduction

Chemical ligation allows almost selective modification of unprotected peptides or proteins and thereby offers the opportunity for the synthesis of non-natural constructs[1]. Even more selective than chemical may be enzymatic ligations.

Sortases are transpeptidases found in Gram-positive bacteria. The *Staphylococcus aureus* sortase isoform SrtA (Sortase A) cleaves proteins at a LPXTG-motif between threonine and glycine, and subsequently transfers the acyl-fragment to a N-terminal oligoglycine. *In vivo*, this reaction serves for the covalent attachment of proteins to the surface of the bacterium [2]. Recently, sortase-mediated ligation was introduced as a new method for peptide and protein ligation [3].



Results and Discussion

We could show that independently from the starting point the reaction mixture ends up in the same composition. The sortase-mediated ligation is therefore an equilibrium reaction which is important when using this reaction for synthesis.

By synthesis of two peptide libraries (Dns-LPKTGX^{1}RR-NH_2 and Dns-LPKTGX^{2}RR-NH_2) which were subjected with the same nucleophile and sortase, we could show that the residue X^1 following the recognition motif has an influence on the reaction. While glycine in this position gives the most rapid and highest conversion (72%), proline gives almost no conversion after 24 h. The residue X^2 seems not to be recognized by the enzyme and therefore has no influence on the equilibrium.

Application 1: Synthesis of peptide nucleic acid – cell-penetrating peptide (PNA-CPP) conjugates

We applied the ligation to the synthesis of a PNA-CPP conjugate. The 18-mer PNA sequence is targeted to the aberrant splice site of a mutated β-globin intron 2, which interrupts the coding sequence of a Luciferase reporter gene [4]. The PNA was C-terminally extended by the optimized LPKTGG-motif and coupled to the cell-

penetrating peptide MAP [5] bearing three additional N-terminal glycine residues. However, ligation of the two molecules gave only a conversion of 38%, showing that the recognition motif is important but not the only determinant for the equilibrium constant. In order to push this equilibrium reaction to the product side, the small leaving group could be easily removed by dialysis. Indeed, reaction under dialysis conditions gave conversions of 61% and 94% at molar ratios of 1/1 and 1/5 (PNA/peptide), respectively. The product was readily separable by semi-preparative HPLC and was homogeneous according to analytical HPLC and MS.

The biological activity of the conjugate was investigated using Kole's Splicing-correction assay [4] showing an enhanced antisense activity in a dose-dependent manner, whereas the PNA alone remained ineffective in restoring the aberrant splicing. A conjugate with a scrambled PNA sequence did not show any activity confirming the sequence specificity.

Application 2: Synthesis of an artificial receptor construct

The CRF_1-receptor is a 7TM G protein-coupled receptor which is important in stress response. The extracellular domains are mainly responsible for ligand binding, consisting of the N-terminus, which contains three disulfide bridges, and three loops. In order to perform binding studies with potential ligand libraries, a soluble receptor construct is desirable.

A chemically prepared 3-loop construct bearing an N-terminal Gly_3-motif was by means of sortase A coupled to a N-terminus obtained by overexpression in *E. coli* C-terminally elongated with LPKTGGRR. The identity of the 23 kDa construct was shown by MS and its binding properties were evaluated in a scintillation proximity assay. The natural ligand Urocortin displaces radioactively labelled Sauvagine with high affinity (EC_{50} ~80 nM).

Acknowledgements

We thank A. Klose, G. Vogelreiter, H. Lerch and D. Krause for excellent technical assistance. This work was supported by the European Commission (QLK3-CT-2002-01989) and the DFG (HA 2694/3-1).

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SYNTHESIS OF 4-AMINO-3-OXO-TETRAHYDROAZEPINO[3,4-*b*]INDOLES: NEW CONFORMATIONALLY CONSTRAINED TRP ANALOGS

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Introduction

The use of conformational restriction of bioactive peptides is a well established strategy to improve their pharmacological profile. A local constraint is obtained by using cyclic analogs of amino acids [1, 2]. The six-membered ring derivatives of Phe and Trp (Tic, Tcc) have been extremely successful. The alternative conformational constraint obtained by linking the aromatic ring of Phe or Tyr to the nitrogen atom of the succeeding amino acid, resulted in the seven-membered benzo-lactam derivatives (Aba, Hba) [3 - 6]. We present the synthesis of corresponding Trp analogs which contain the aminoazepinone skeleton (Aia).

Results

The most general synthetic route towards benzo-substituted aminoazepinones uses o-formyl-phenylalanine as a starting material [6]. Therefore we focused our attention on the preparation of the corresponding 2'-formyltryptophan, and its conversion into the desired Aia analogs (Fig. 1).

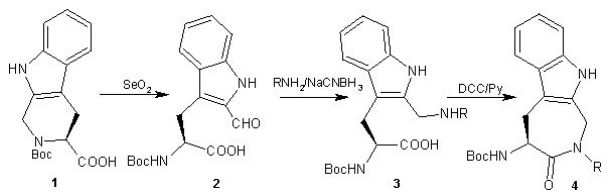
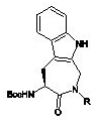


Fig. 1. Synthesis of Aia analogs

Boc-2'-formyl tryptophan **2** was obtained through the oxidation reaction of Boc-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid **1** with SeO_2 [7]. Reductive amination was performed in solution using $NaCNBH_3$ and an appropriate amine or amino acid esters. Intermediates **3** were not isolated, but the crude mixtures were used in the following cyclization reaction using DCC as the coupling agent. The crude products **4** were purified by flash chromatography. The yields reported in the Table 1 were calculated over 2 steps. Boc-protected Aia-Gly, Aia-Ala, Aia-Phe and Aia-Aha ethyl and benzyl esters were obtained in satisfactory overall yield.

Table 1. Yields of Aia analogs.

Entry		Yield (%)
4a	Boc-Aia-Bn R= $CH_2C_6H_5$	48
4b	Boc-Aia-Gly-OEt R= $CH_2COOCH_2CH_3$	42
4c	Boc-Aia-Gly-OBn R=$CH_2COOCH_2C_6H_5$	45
4d	Boc-Aia-Ala-OMe R=(*S*)$CH(CH_3)COOCH_3$	61
4e	Boc-Aia-Ala-OBn R= (*S*)$CH(CH_3)COOCH_2C_6H_5$	53
4f	Boc-Aia-Aha-OMe R= (*S*)$(CH_2)_5COOCH_3$	54
4g	Boc-Aia-Phe-OMe R= (*S*)$CH(CH_2C_6H_5)COOCH_3$	31

After Boc-deprotection of 4g, the Aia-Phe-OMe derivative has been incorporated into the endomorphin-1 opioid peptide sequence by segment coupling with Boc-Tyr-Pro-OH, to probe the bioactive conformation. The receptor affinities of the obtained endomorphin-1 analogs (Tyr-Pro-Aia-Phe-NH_2 and Tyr-Pro-Aia-Phe-OH) for μ- and δ-opioid receptors were determined. The Aia containing endomorphins show a loss in μ-opioid receptor affinity, and no δ-affinity. This indicates that the type of conformational restriction that is imposed by the Aia residue in position 3 is not tolerated by the opioid receptors.

Discussion

The easy preparation of Boc-2'-formyl-Trp gives an easy access to the 4-amino-3-oxo-tetrahydroazepino[3,4-*b*]indole skeleton. The procedure allows the preparation of a variety of constrained dipeptide analogs. Moreover we have demonstrated that these dipeptide analogs can be introduced into bioactive peptides using standard methodology. Such peptide analogs can provide interesting information about the receptor-bound conformation of Trp-containing peptides.

Acknowledgements

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A PyBOP INDUCED SIDE-REACTION IN PNA SYNTHESIS

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Introduction

Peptide nucleic acids (PNAs) have been derived from oligonucleotides by substituting the phosphate-sugar backbone by *N*-(2-aminoethyl)glycine units [1]. PNAs are resistant to enzymatic degradation by nucleases and peptidases and form very stable duplexes and triple helix structures with complementary DNA or RNA. Therefore, PNAs gained broad attention as interesting oligonucleotide analogues in antisense/antigene experiments and as diagnostic tools.

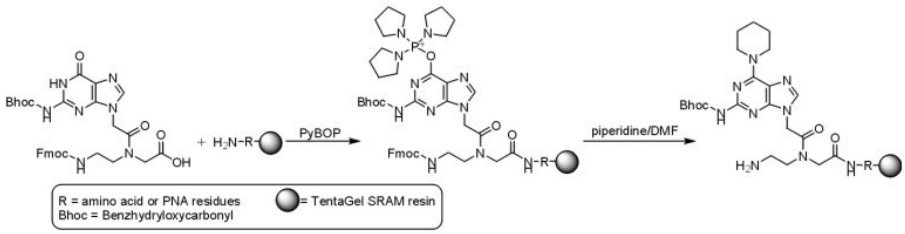
In principle, the activation of the PNA monomers can be performed with several activating reagents known from peptide synthesis. Especially, HATU or PyBOP are often used. Synthesis with guanidinium-derived coupling reagents like HATU is more laborious, because preactivation is needed in order to avoid guanidinylation of the N-terminus, a side reaction, occasionally observed in peptide synthesis, but more frequently in the synthesis of PNAs [2].

When using PyBOP or similar phosphonium compounds (like BOP, PyBroP, PyAOP, *etc*.), no chain-terminating side reactions at the amino terminus have been detected [3]. Thus, preactivation should not be needed, which is especially useful in automated synthesis.

Results and Discussion

Surprisingly, in the PyBOP-mediated syntheses of PNA oligomers in our lab the obtained products showed molecular masses 67 Da above the expected in mass spectrometry, whereas the synthesis with HATU gave the desired products. Detailed analysis of the acetylated termination sequences by mass spectrometry revealed, that the modification occurred at the only guanine residue in the sequence, and was not observed in guanine-free sequences.

In order to further characterize the side reaction, a short fragment containing adenine and guanine was synthesized using HATU and PyBOP activation. While HATU with preactivation gave the desired product, the phosphonium ion of the PyBOP reagent reacted with the enolizable keto group of the guanine base to a considerable extent. The thus activated alcohol is substituted by the nucleophile piperidine during Fmoc cleavage, yielding the final product. Both products are stable during TFA cleavage and could be characterized by MS/MS spectrometry. This side reaction also takes place during subsequent coupling steps yielding completely modified guanine residues [4].



Our results show, although we suspect this side reaction to be sequence dependent, that care must be taken when synthesizing PNAs with PyBOP activation. In ongoing studies we will investigate the influence of different amines on the phosphonium-evoked modification. This reaction may offer an opportunity to synthesize guanine derivatives modified in position 6 by treatment of the phosphonium intermediate with a variety of nucleophiles. Investigations on the coupling efficiency of other coupling reagents for PNA synthesis (e.g. HBTU, PyAOP, etc.) and their reactivity towards the nucleobase side chains are currently underway.

Acknowledgements

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SYNTHESIS OF 5-SUBSTITUTED-URACIL PNA MONOMERS BY Pd-CATALYZED CROSS-COUPLINGS

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Introduction

Peptide nucleic acids (PNAs), containing *N*-(2-aminoethyl)glycine backbone instead of sugar-phosphate moiety of the natural nucleic acids, belong to the most powerful and promising DNA mimics due to their strong hybridization ability with DNA and RNA and complete resistance to nucleases and proteases.

In oligonucleotides 5-C-substitution of uracil base, among other things, makes possible the introduction of fluorophores and spin labels. In addition, PNA-DNA chimeras [1] as well as normal DNA oligomers containing 5-propynyl-uracils in place of thymines have higher duplex stability [2] and increased mismatch recognizing ability [3]. Incorporation of different 5-heteroaryl-uracil moieties into short oligodeoxynucleotides also results in enhanced thermal stability of the modified DNA:RNA duplexes compared to that of the thymine-containing counterpart.

On the basis of increased π-π stacking interactions between the neighbouring bases, similar incorporation of 5-aryl- and 5-alkynyl-uracils into PNA-s is also expected to increase the stability of PNA:DNA and PNA:RNA complexes that probably should be accompanied by higher mismatch penalty, as well. Besides, some hydrophobic aryl or aralkynyl substituents are likely to improve the cell-permeability too, which is rather poor in the case of unmodified PNAs.

Results and Discussion

A number of protected PNA monomers containing 5-alkynyl- and 5-aryl-uracil bases have been synthesized using the following Pd-catalyzed cross-coupling reactions.

Stille couplings: Due to stronger metallic character of tin relative to boron, aryl-trialkylstannanes are better aryl group donors than the corresponding aryl-boronic acids. Coupling of the base-unprotected 5-iodo-uracil PNA monomer with some aryl-tributylstannanes afforded the required 5-aryl analogues in good yields. However, since only very few aryl-trialkylstannanes are commercially available in addition most of them are toxic, further studies on alternative methods like Suzuki coupling seemed to be reasonable.

Sonogashira couplings: Although couplings of the base-unprotected 5-I-U PNA monomer with 3 different terminal alkynes gave high overall yields but proportions of the unrequired furano[2,3-*d*]pyrimidine by-products were significant (30 - 40%) in all cases. To prevent the intramolecular cyclization p-methoxybenzyl (PMB) protection of the lactam function was necessary. Starting from this N^3-PMB-

protected monomer the required 5-alkynyl derivatives, as sole products, were isolated by nearly quantitative yields.

Suzuki couplings: Unfortunately, attempted couplings of the base-unprotected 5-I-U monomer even with the most reactive 2-thienyl-boronic acid under various conditions were unsuccessful. Since in the presence of bases there must be a negative charge on the 4-O atom, which is in close proximity with the Pd atom thus can lower its electrophilic character, thereby the transmetallation step is likely to be inhibited. It was confirmed by couplings of the N^3-PMB-protected monomer with more aryl-boronic acids which gave from acceptable to good yields for the required 5-aryl-substituted products. In two investigated cases the efficiency of couplings further increased when boronic acid pinacol esters were used instead of the free acids.

UV spectra of all the investigated 5-aryl- and alkynyl-uracil PNA monomers show large bathochromic shifts relative to that of the thymine-containing reference compound which refers to a near planar orientation between the uracil base and the 5-substituents. It is expected to enhance the base-stacking interactions of adjacent base pairs, leading to higher duplex stability.

Acknowledgements

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CONTROLLING THE HELICAL SECONDARY STRUCTURE OF HETEROPEPTIDES USING CHIRAL CYCLIC α,α-DISUBSTITUTED AMINO ACIDS

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Introduction

Helical structures in proteins almost always form a right-handed (*P*) helical-screw sense, which is believed to result from the asymmetric center at the α-position of L-α-amino acids [1]. Besides an asymmetric center at the α-position, L-Ile and L-Thr possess an additional chiral center at the side-chain β-position. However, only a little attention has been paid as to how the asymmetric center at the side chain affects the secondary structure of peptides. We have previously reported that chiral cyclic α,α-disubstituted amino acid (*S,S*)-Ac_5c^{dOM}, in which the α-carbon is not a chiral center but the asymmetric centers exist at the side chain cyclopentane, could control the helical-screw direction of its homopeptides into the left-handedness [2]. Herein we synthesized heteropeptides containing (*S,S*)-Ac_5c^{dOM} in Aib sequences, and also peptides containing four various disubstituted amino acids in L-Leu sequences. The Aib is an achiral amino acid, and thus does not have a bias for the helical-screw handedness, while the L-Leu has an asymmetric center at the α-position and has a property for β-sheet or helix formation [3]. Furthermore, we studied the preferred secondary structures of these peptides, and effect of the Ac_5c^{dOM} on the conformation.

Fig. 1. X-ray structure of (S,S)-Ac_5c^{dOM} octapeptide

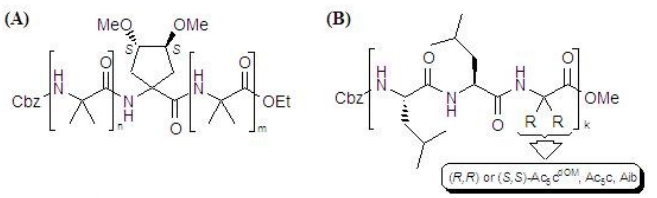

Fig. 2. Structure of heteropeptides; (A) Aib heteropeptides and (B) Leu heteropeptides

Results and Discussion

Both enantiomers of chiral cyclic α,α-disubstituted amino acids Ac_5c^{dOM} were synthesized starting from dimethyl L-(+)- and D-(-)-tartrate, according to our previous report [2]. The chiral cyclic amino acid was incorporated into Aib sequence by solution-phase methods; the (*S,S*)-Ac_5c^{dOM} was introduced to the N-terminal, to the C-terminal, and at the center position of Aib peptides. Conformational analysis by using the ^{1}H NMR, FT-IR, and X-ray crystallographic analysis revealed that dominant conformation of the Aib peptides containing a chiral cyclic (*S,S*)-Ac_5c^{dOM} was 3_{10}-helix both in solution, and in the solid state. However, the control of helical-screw handedness by one chiral (*S,S*)-Ac_5c^{dOM} in Aib sequences seemed to be difficult. Also, we incorporated the achiral or chiral disubstituted amino acids into L-Leu sequences. Conformation analysis by using CD, ^{1}H NMR, and FT-IR spectra disclosed that the dominant conformation of heteropeptides containing the chiral cyclic Ac_5c^{dOM} in L-Leu sequences was the right-handed (*P*) helical structure, due to the chiral centers at the α-position of L-α-amino acid. The detailed conformation analysis including the X-ray crystallographic analysis will be reported elsewhere.

Acknowledgements

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NEW MELANOCORTIN SEQUENCE HFRW MIMETICS

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Introduction

Melanocortin receptors (MCRs) are involved in multiple physiological processes. Agonists and antagonists for MCRs have a potential for treatment of diseases and health disorders such as overweight and sexual dysfunctions. The natural agonists for the MCRs are melanocortins. We have earlier reported investigations on active core mimics of melanocortins such as reductive amination products [1], *N*-alkylaminoacids and their derivatives [2], as well as *N*-alkylated dipeptide amides and related structures [3]. Here we present a study of 4-component melanocortin active core His-Phe-Arg-Trp non-peptide imitations.

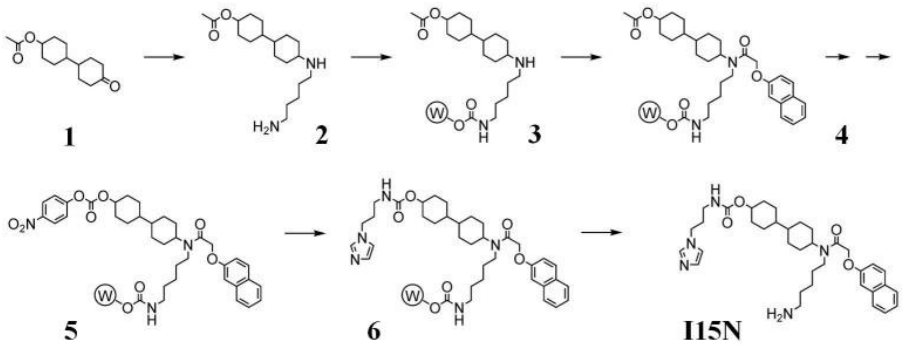
Results and Discussion

A series of melanocortin active core tetrapeptide HFRW nonpeptide imitations were prepared using combinations of solution and solid phase synthesis. Most of the compounds included residue of 3-(1-imidazolyl) propylamine or histamine as substitutes of histidine. Phenylalanine residue, which is included in melanocortins was replaced by residues of derivatives of 4,4'-disubstituted isopropylidenedicyclohexane, 4,4'-disubstituted bicyclohexane, 1,4-disubstituted cyclohexane, 1,5-disubstituted cyclooctane, and 1,2-, 1,3- or 1,4-disubstituted benzenes. Instead of arginine residue, residues of 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 2-butyl-2-ethyl-1,5-pentanediamine, 4,4'-methylene-bis(cyclohexylamine), and 4,4'-diamino-diphenylmethane were introduced. Finally, as replacement of the tryptophan residue served 2-naphtyloxyacetyl-, (4-1*H*-indol-3-yl)-butyryl-, 2-phenylethanesulfonyl-, and naphthalene-2-sulfonyl- groups.

Chemical synthesis included mono-*O*-acetylation of diol (e. g. commercial 4,4'-bicyclohexanediol in DMF solution was treated with one equivalent of acetic anhydride in the presence of 4-dimethylaminopyridine). Swern oxidation (dimethylsulfoxide, oxalyl chloride, triethylamine, -78 ^{o}C) followed yielding corresponding ketone (**1**). This compound was then introduced into reaction with diamine in CH_2Cl_2 in the presence of $TiCl_4$. The Schiff base formed was further reacted with $NaCNBH_3$ without isolation. Compound (**2**) thus obtained was attached to 4-nitrophenyloxycarbonylated Wang polymer, forming product (**3**). 2-Naphthoxyacetylgroup was then introduced activating carboxylic acid with PyBroP. Further, to remove *O*-acetyl protection, the product containing structure (**4**) was treated with LiOMe in methanol-CH_2Cl_2 solution. Treatment with 4-

nitrophenylchloroformate in CH_2Cl_2 in presence of DIEA followed, giving activated compound (**5**), which was introduced into reaction with a primary amine containing an imidazole ring. Reaction of the polymeric product (**6**) with trifluoroacetic acid based cleavage cocktail terminated the synthesis.

Shown below is the preparation of mimetic **I15N**, as a typical example:



Preparative HPLC allowed isolating of two isomers for most of end products. By NMR study including DQF-COSY, NOESY, TOCSY, sensitivity-enhanced ^{13}C-HSQC and ^{13}C-^{1}H HMBC approaches they were identified as derivatives of O-*trans*, N-*cis* or *trans*, *trans* forms of 4,4'-disubstituted bicyclohexane or 4,4'-isopropylidenedicyclohexane. Formation of these forms could be explained taking into account mixture of isomers present in commercial diols and different reactivity of their *cis* or *trans* hydroxy groups.

When tested in binding assays on melanocortin receptor subtypes 1, 3 - 5, our active core imitations exhibited a micromolar affinity. In most cases the first (O-*trans*, N-*cis*) isomer of isopropylidenedicyclohexane derivatives turned out to be a slightly more active than the second (*trans, trans*) one. Similarly, comparing affinities of all compound pairs, differing only by the presence of a histamine or 3-(1-imidazolyl)-propylamine residue, we found that histamine derivatives were slightly better binders. The structure-activity data and methods and approaches described herein will be useful for further peptidomimetics studies.

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AMADORI-MODIFIED PEPTIDES - A SITE-SPECIFIC STRATEGY FOR SOLID PHASE PEPTIDE SYNTHESIS

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Introduction

Non-enzymatic glycosylation, also called glycation, is a common modification in living organisms formed by the reaction of carbohydrates with free amino groups of peptides and proteins. Amadori products are early markers for ageing, diabetes mellitus and Alzheimer's disease. Here we describe a strategy to glycate specifically amino groups on partially protected resin bound peptides by a global post synthetic approach.

Results

The Amadori modifying reagent was synthesized starting from beta-D-fructose (**1**) introducing acetonide protecting groups (**2**) (Fig. 1) [1]. The protected sugar was oxidized to the aldehyde (**3**) in a Swern-like oxidation [2]. 2,3-4,5-Di-*O*-isopropyliden-aldehydo-beta-D-arabino-hexos-2-ulo-2,6-pyranose was obtained in an overall yield of 38%. All peptides were synthesized by Fmoc/*t*Bu-chemistry using carbodiimide activation. The methyltrityl group of the lysine to be modified was selectively cleaved with 1% TFA in dichloromethane. All five unmodified peptides with different hydrophobic, hydrophilic and sterical environments were obtained in high yields and purities (Table 1). After glycation by reductive amination in a mixture of methanol, isopropanol and water (2:2:1 by vol.) using a ten-fold molar excess of 2,3-4,5-di-*O*-isopropylidene-aldehydo-beta-D-arabino-hexos-2-ulo-2,6-pyranose and $NaBH_3CN$ for 18 h at 70 °C [3] the unprotected Amadori-modified peptide (**5**) was obtained after TFA cleavage (5% H_2O, 2 h) at average yields of about 50%. The major by-products resulted from an incomplete cleavage of the sugar protecting groups. The amount of these mono- and bisprotected by-products was reduced by a second cleavage of the precipitated peptide with fresh TFA (5% H_2O) for 1.5 h. Thereby, the overall yields of the glycated peptides were increased to 70 - 80% independent of the sequence and approximately 50% for the N-terminal modification. The sugar protected by-products were well separated by RP-HPLC. All Amadori peptides were characterized by MALDI and ESI mass spectrometry confirming the expected mass shift of 162 Da and their characteristic fragmentation pattern [4].

Discussion

Compared to a recently published solid phase approach [5] for Amadori-modified peptides using D-glucose in DMF at 110 °C, this new strategy resulted in higher yields and purities. Importantly, no oxidized by-products were obtained due to the sugar protecting groups. The relatively low yield for the N-terminal modification

was mostly related to a decreased cleavage of the sugar protecting group, which might be sequence dependent and needs to be further investigated using different model peptides. The presented global post synthetic approach appears to be a new useful tool to routinely synthesize glycated peptides in high yields and purities.

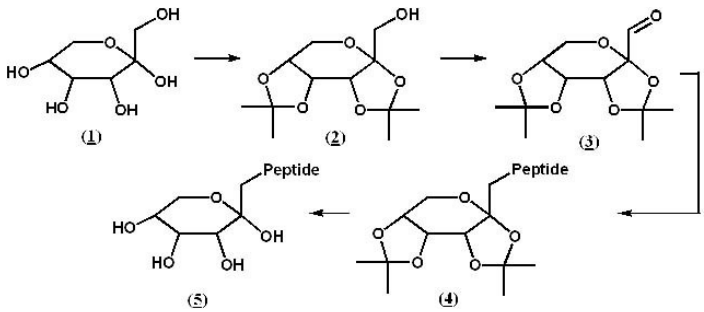

Fig. 1. Synthesis of 2,3-4,5-di-O-isopropylidene-aldehydo-beta-D-arabino-hexos-2-ulo-2,6-pyranose and coupling to the peptide.

Table 1. Peptide sequences and yields of Amadori modified peptides.

Peptide	peak area %		
Sequence	Amadori modified Peptide	Unmodified Peptide	partly Protected Amadori-product
AGGK*AAFL	82,1	0,9	16,9
ASK*ASKFL	81,5	1,2	16,0
AK*ASADFL	77,4	1,9	18,7
AK*DSASFL	76,7	3,6	18,8
*H_2N-AKASASFL	52,2	22,1	22,5

**modified position*

Acknowledgements

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N-AMIDINO-PROLINE DERIVATIVES AND THEIR APPLICATION IN PEPTIDE SYNTHESIS

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Introduction

Unnatural amino acids represent valuable tool for the enhancement of peptide activity, bioavailability and stability to enzymatic degradation. Therefore, synthesis of new amino acids and their derivatives with protected functional groups belongs to promising directions of contemporary peptide chemistry. We selected *N*-amidino-L-proline [1, 2] as a hybrid structure, modelling key features of Arg-Pro sequence localised at N-termini of many natural peptide hormones. Possibility of its practical application implies synthesis of derivatives with protected amidino group to ensure solubility in organic solvents and avoid side reaction in the course of activation.

The objective of presented study was estimation of protected *N*-amidino-proline derivatives utility in classical and solid phase peptide synthesis.

Results and Discussion

N-amidino-proline (**1**) can be easily synthesized from proline and cyanamide. Due to the hydrophilic nature it possesses extremely poor solubility in DMF and other organic solvents. Non-covalent protection of amidino group using *p*-toluenesulfonic acid improves solubility of *N*-amidino-proline in DMF, however is insufficient to prevent side reaction during DIC/HOBt activation. Similar side product formation was observed in the course of *N*-amidino-proline trifluoroacetilation by means of $(TFA)_2O$. Data of ESI MS, ^{1}H-NMR and ^{13}C-NMR spectra evidence for intramolecular cyclization resulted in bicyclic structure (**2**) (Fig. 1).

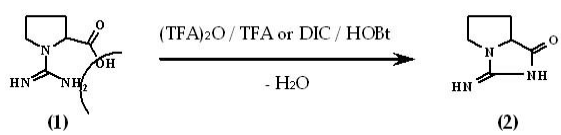

Fig.1. Cyclization of N-amidino-proline

Synthesis of mesitylenesulfonyl-protected (Mts) *N*-amidino-L-proline was achieved by two methods [3] and its optical purity has been determined by RP-HPLC separation of diastereomeric peptides: *N*-amidino-L-Pro-L-Ala-OH and *N*-amidino-D-Pro-L-Ala-OH.

Investigation on Mts-protected *N*-amidino-proline utility in SPPS was performed during the parallel synthesis of RGDF and its analogue (**3**) (Fig. 2). In case of *N*-amidino-proline (route **a**) the coupling stage demands prolonged reaction time as

compared to Arg(Mts) or application of more efficient condensation agents, due to the steric hindrance.

In alternative synthesis of RGD analogues we have performed one-pot modifications of N-terminal secondary or primary amino group of peptidyl-polymer. Guanidilation of Gly by *N*,*N*'-di-*tert*-butoxycarbonyl-1*H*-benzotriazole-1-carboxamidine (route **c** [4]) proved to take place significantly easier as compared to Pro (2 h and 19 h respectively). On the other hand we have failed with Mukaiyama's reagent in the case of Pro (route **b** [5]). Instead of desired product we have observed formation of a complex between peptide and reagent.

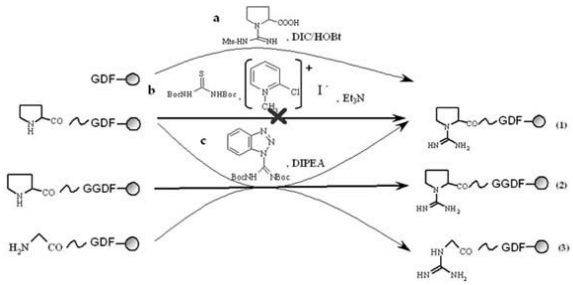

Fig. 2. Schemes of SPPS.

It is known that Arg-Gly bond is cleaved by several enzymes including thrombin, trypsin and clostripain. Investigation on stability to enzymatic degradation demonstrates that substitution of arginine residue in natural RGDF sequence by *N*-amidino-proline significantly increases the half-life time of tested peptide in human blood plasma.

Conclusions

In summary we demonstrated that unprotected *N*-amidino-proline is prone to cyclization under the dehydrating agents treatment, while its Mts-protected derivative can be successfully applied both in classical and solid phase peptide synthesis. At the same time solid phase guanidinylation by protected *N*,*N*'-di-*tert*-butoxycarbonyl-1*H*- benzotriazole-1-carboxamidine seems to be preferred method for the synthesis of short peptides modified by *N*-amidino-proline.

Acknowledgements

We thank Prof. Mark A. Lipton and Prof. Luis Moroder for valuable discussion.

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THE DEPSIPEPTIDE METHODOLOGY: APPLICATION TO EXCEPTIONALLY DIFFICULT SEQUENCES AND DEVELOPMENT OF AN AUTOMATED TECHNIQUE VIA DEPSIDIPEPTIDE UNITS

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Introduction

A peptide sequence that is difficult to synthesize because of aggregation phenomena can be more easily obtained through its depsipeptide analogue, which is assembled by extending the growing peptide chain, from a suitable point on, *via* the β-hydroxyl function of a Ser/Thr residue. The discontinuity introduced in the regular pattern of amide bonds by the presence of a depsi unit affects the tendency of the peptide to fold, thus providing in most cases an improvement in synthetic efficiency. Moreover, after cleavage of the peptide from the solid support, each depsipeptide unit provides an additional ionizable moiety, thereby increasing solubility and facilitating purification. The conversion to the target amide peptide is smoothly achieved through an *O,N*-acyl shift, which occurs quantitatively under mildly basic conditions over a short period of time [1].

Results and Discussion

At first, the general stability of depsipeptides toward the standard reagents involved in solid phase synthesis (20% piperidine for Fmoc removal and TFA for cleavage) was assessed. By taking as a model the homooligopeptide $(VT)_{10}$, and building a depsi bond at each Thr site, a poly-depsipeptide chain was assembled, and obtained as a unique product [2].

The depsipeptide method was then applied to the synthesis of the difficult sequence $G^{1}ATAV\underline{S}^{6}EWTEYK\underline{T}^{13}ANGK\underline{T}^{18}YYYNNRTLE\underline{S}^{28}TWEKPQELK^{37}–NH_2$, the N^{15}-amide analogue of the WW domain FBP28, which was impossible to obtain using standard SPPS (Fig. 1A). At first two depsi units were introduced into the chain (Fig. 1B), using for esterification DIC/NMI activation in DCM (2 x 2h). In the raw product, fragments apparently derived by cleavage at the depsi bonds were identified, and shown to be due either to undesired additional loading onto the amide resin arising under the strong activation used for *O*-acylation (they do not appear, if a capping step under *O*-acylation conditions is performed after the linking), or to diketopiperazine formation during Fmoc removal from the second residue following a depsi bond. Diketopiperazine formation was circumvented using at this position a more base-labile a-amino protecting group, the Bsmoc residue (removed with 2% piperidine, 3 x 1min).

With these precautions a tetra-depsi isomer of [N^{15}]FBP28-amide was easily assembled (Fig. 1C), and then smoothly shifted to the native amide form (Fig. 1D). For this product the occurrence of epimerization of the amino acid activated during *O*-acylation was also evaluated. The extent of D-enantiomer formation was 0.13% for Glu, 1.21% for Val, and 1.66% for Lys (present in two depsi units) [2].

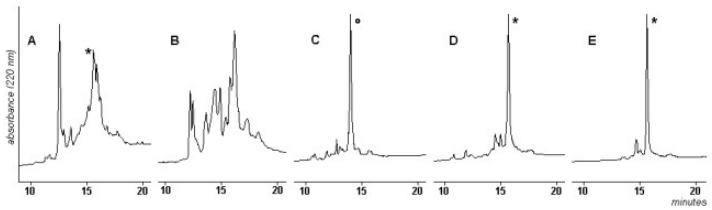
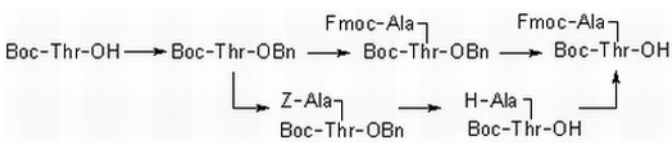


*Fig. 1. HPLC profiles of the crude [Asn^{15}]FBP28-NH$_2$. **A**) standard synthesis ; **B**) synthesis via two depsi units (ES 27/28, KT 17/18); **C**) tetra-depsi analogue (ES 27/28, KT 17/18, KT 12/13, VS 5/6) obtained via the optimized procedure; **D**) shifted tetra-depsi analogue, **E**) synthesis via pseudoproline [ES 27/28, KT 12/13 (coupled via HATU, 4h), VS 5/6]. *target product.*

In addition, a strategy was developed for the synthesis of Fmoc derivatives of depsidipeptide units suitable as building blocks [2]. The scheme below shows two alternative methods for the synthesis of the Ala/Thr depsipeptide block, which was used for the completely automated synthesis of depsi analogues of the C-terminal segment of the protein Crambin, C^{16}RLPGTPEALCATYTGCIIIPGATCPGDYAN^{46}, leading to products of excellent purity.



Finally, most of the peptides examined in this work were resynthesized using pseudoproline units [3] in the same positions where depsipeptide units were used. The synthetic efficiency of the two techniques was comparable (see Fig. 1E).

Acknowledgments

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SYNTHESIS OF PRO-RICH PEPTIDE ANALOGUE CONTAINING PIPECOLIC ACID: INFLUENCE ON PEPTIDE BOND STABILITY AND CONFORMATION

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Introduction

Pipecolic acid (Pip, piperidine-2-carboxylic acid) a naturally occurring, nonproteogenic α-imino acid, is a six-membered, piperidine ring based, proline analogue. It has been extensively used as proline substitute in numerous synthesis of peptido-mimetics and it has also been incorporated into a variety of bioactive peptides. Pipecolic acid has also been incorporated into proteins as a proline homologue in order to probe the role of ring size in structure and function [1]. With the aim to study the influence of the ring steric hidrance in the Src homology 3 (SH3) mediated substrate recruiment we replaced all Pro residues into the HPK1 394-403 proline-rich sequence (PPPLPPKPKF), named P2, by pipecolic acid residues (Pip6). Surprisingly, this peptide is sensitive to the acidic conditions (90% TFA) used to cleavage the peptide from the Wang resin and to deprotect the Lys side chains. This is in stark contrast to acidic cleavage of proline-containing peptides accomplished under the same conditions. Our research aims to investigate the influence of the pipecolic residue on peptide bond stability to acidolysis, as well as and on peptide conformation.

Results and Discussion

In previous work we demonstrated that the peptide P2 was stable towards TFA treatment, adopting a PPII helix conformation at 5 °C in aqueous solution [2]. On the contrary, the Pip6 peptide, containing pipecolic acid instead of proline residue, was readily hydrolyzed by TFA treatment yielding two by-products: H-Pip-Pip-Pip-Leu-Pip-OH (fragment A) and H-Pip-Lys-Pip-Lys-Phe-OH (fragment B). The treatment of peptide-resin under different acidic conditions (TFA/DCM 1%, r.t., 60 min.; TFA/DCM 50%, r.t., 60 min.; TFA/anisole/water/TIPS 90:5:4:1, r.t., 60 min.; HCl 0.1 M, r.t., 60 min., 24h - 72h; HCl 0.1 M, r.t., 10d - 24d) yielded either complete or partial peptide degradation, and the amount of by-products was strongly related to the reaction conditions. To explain the degradation of Pip6 we propose a mechanism of cleavage via the formation of oxazolone-like intermediate [3] from a specie protonated at the carbonyl of the pipecolic acid residue involved in the hydrolizable amide bond.

The drawing-force of this mechanism is the charge density on the oxygen of the carbonyl involved in the nucleophilic attack (Fig. 1) that in this peptide is increased by the inductive effect of the alkyl side chain group of the leucine residue, and the steric interactions induced by the different ring size of the pipecolic residue.

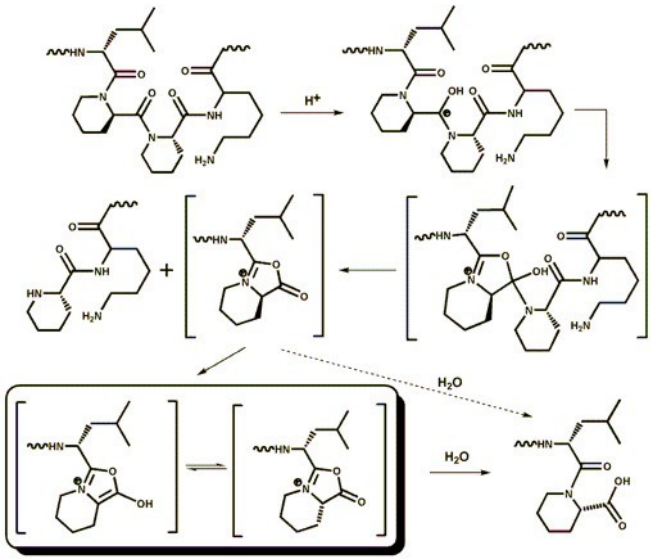

Fig.1. Proposed mechanism of the acidic hydrolysis.

This mechanism of hydrolysis is confirmed by ESI-MS studies. Indeed, the analysis of the by-products obtained by treatment of Pip6-resin peptide with deuterated trifluoroacetic acid showed the incorporation of a deuterium atom in the fragment A, confirming the presence of the proposed oxazolone-like intermediates and its keto-enol tautomerization.

The preliminary CD studies of Pip6 were carried out in aqueous buffer solution (Tris-HCl 20 mM, pH 7.5) and in 95% v/v *n*-propanol/buffer solution a favorable condition to induce PPI helix conformation [4].

The CD spectra showed that the introduction of pipecolic acid in the peptide sequence strongly decrease the propensity of Pip6 peptide to adopt a PPII helix conformation also a low temperature (5 °C). On the other hand, the presence of pipecolic residue does not a stable PPI helix conformation on Pip6 peptide.

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SUGAR AMINO ACIDS – SYNTHESIS AND CONFORMATIONAL ANALYSIS OF DIMETHYL 3-AMINO-2,3-DIDEOXYHEXOPYRANOSIDURONATES

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Introduction

Sugar amino acids (SAAs) are sugar moieties containing at least one amino and one carboxyl group[1]. Such compounds are important class of polyfunctional scaffolds where the carboxyl, amino and hydroxyl termini allow to create structural diversities akin to biologically active molecules[2]. The rigid pyranose ring system of monosaccharide amino acid can be used as molecular template to display pharmacophoric groups in well defined spatial orientation [3, 4]. Additionally, sugar amino acids have been used extensively in the area of peptidomimetic studies[1 - 3].

Results

Starting from 6,3-lactone of D-glucuronic acid, dimethyl 3-amino-2,3-dideoxyhexopyranosiduronates, new SAA monomers, with α-D-*arabino*, β-D-*arabino*, α-D-*ribo* and β-D-*ribo* structures were synthesized. All new SAAs, as well as their precursors (3-azido and 4-*O*-acetyl-3-azido analogues) were characterized by means of IR, NMR and MS. In order to define three dimensional spatial arrangement of substitiuents, conformational analysis of synthesized SAAs and their precursors, based on ^{1}H NMR studies, is provided. Additionally, configuration and conformation of dimethyl 4-O-acetyl-3-azido-2,3-dideoxy-β-D-*arabino*-hexopyranosiduronate was confirmed by the single-crystal X-ray diffraction analysis.

Discussion

Among the SAAs described here only these with D-arabino configuration adopt the $^4C_1$ conformation in solution (CD_3OD). This is demonstrated by the coupling constants $J_{2a,3}$ = 12.21, $J_{3,4}$ = 9.77 and $J_{4,5}$ = 9.77 Hz, which indicate the axial orientation of the H-2_a, H-3, H-4 and H-5 protons. The $^4C_1$ conformation is optimal for these compounds since all the groups are equatorially oriented. The opposite configuration of the C-3 carbon atom in SAAs with D-ribo configuration causes a deviation from $^4C_1$ form, which is due to the unfavorable axial orientation of the 3-amino group. This is confirmed by the coupling constants $J_{2a,3}$ = 3.91, $J_{3,4}$ = 4.39 and $J_{4,5}$ = 8.30 Hz (α-D-*ribo*) and $J_{2a,3}$ = 10.25, $J_{3,4}$ = 3.42 and $J_{4,5}$ = 3.42 Hz (β-D-ribo). We assume that the conformational equilibrium exists between the $^4C_1$ and $^1C_4$ forms in the case of SAAs with D-*ribo* configuration. It is also possible that these SAAs adopt one of the skew-boat forms.

The coupling constants of obtained by us SAA precursors, allow us to draw the same conclusions. All precursors with D-*arabino* configuration adopt the $^4C_1$ conformation in solution whereas these with D-*ribo* configuration adopt other than the $^4C_1$ form.

Our previous findings showed that the analogous compounds with D-*arabino* and D-*ribo* structures, having 5-CH_2X group (X = OH, OAc, OTs, I) in place of 5-$COOCH_3$ group, adopt solely the $^4C_1$ conformation [5]. This means that introduction of the carboxyl group onto C-5 carbon atom fundamentally change the conformational preferences of the pyranose ring.

Acknowledgements

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SYNTHESIS OF AN AUXILIARY GROUP FOR CHEMICAL LIGATION AT THE X-GLY SITE

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Introduction

Native Chemical Ligation (NCL) is one of the most powerful methods for protein synthesis [1]. The possibility to synthesize longer polypeptides, by the solid-phase methodology upgrade, followed by assembly to proteins allows to assess functional aspects of interesting domains. However, for the classical approach, a crucial Cys residue at the N-terminal of one of the fragments is required to achieve ligation at an X-Cys site [2]. In order to generalize this approach, glycine analogues have been developed to promote S to N acyl transfer in similar way to native chemical ligation with N-terminal cysteine residues. Here we report the synthesis of a Gly-derivative starting from 2-mercapto-4,5-dimethoxybenzylamine in which the thiol group is protected as 4-methoxybenzyl (Mob) derivative. Although this group is too TFA-labile for the synthesis of large peptides according to the Boc/Bzl strategy, it has found successful application in Fmoc strategy. The Mob group should be removed by treatment with mercury (II) trifluoroacetate in 80% aqueous acetic acid. This new auxiliary group, orthogonally protected toward C-terminal thioester synthesis conditions, in the Fmoc/*t*Bu chemistry, is then designed for the chemoselective peptide ligation.

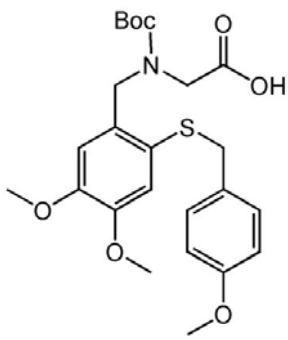

Fig. 1. The new Gly derivative, designed for chemoselective peptide ligation

Results and Discussion

Starting from commercially available 4,5-dimethoxy-2-mercaptobenzylamine hydrochloride (DMMBA), we synthesized the corresponding Gly-derivative in four steps. DMMBA was suspended in dichloromethane under nitrogen atmosphere. Then, after dissolution with TFA, was condensed with 4-methoxybenzylchloride. The obtained product was added of methyl 2-bromoacetate and DIEA to obtain the Gly methyl ester derivative. The two last steps involved the saponification of the

carboxyl moiety and the protection of the α-amino group of Gly. The proposed synthesis allows to obtain the desired Boc protected final compound with good yield and purity. The next goal of our work wil be to test the glycine derivative for the chemoselective peptide ligation involving three or more peptide fragments. The building block will be, with a standard coupling reaction, added to a peptide chain making this approach compatible with regular Fmoc chemistry.

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EFFECT OF NEAR-UV IRRADIATION ON A SINGLE CHAIN VARIABLE FRAGMENT OF THE MONOCLONAL ANTIBODY 82D6A3 AND ITS MUTANTS

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Proteins are targets for photo-destruction due to absorption of incident light by endogenous chromophores. The near-UV light can induce changes in the protein structure due to the disruption of disulfide bonds upon excitation of a nearby Trp-residue [1]. Mass spectroscopic data presented evidence that structural modification observed upon irradiation of goat alpha-lactalbumin at 290 nm results from tryptophan (Trp) mediated cleavage of disulfide bonds [2, 3].

Most of the proteins of the immunoglobulin superfamily contain a so-called triad, consisting of two S atoms, forming a disulfide bridge, and a single Trp in their close vicinity [4]. This characteristic makes immunoglobulins well suited agents to explore the protein degeneration induced by near-UV excitation of Trp-residues. We focussed our study on the variable domains of an Ig. The V_{Light} and V_{Heavy} domains of the monoclonal antibody 82D6A3 were linked *via* $(Gly_4Ser)_3$ to obtain a single chain variable fragment (scFv). The scFv specifically interacts with the A3-domain of VWF [5] as the entire monoclonal antibody 82D6A3 does, but contains only two triads and does not contain other disulfide bridges.

This study is directed to define the degree to which the variable domains of the scFv are photosensitive. We also searched to clarify the contribution of each Trp-disulfide triad within these variable domains to the process of photo-degradation. For this purpose, we constructed three Trp mutants of the wild-type scFv. In two mutants, either W35 adjacent (3.6 Å) to disulfide bond C23-C88 in V_{Light} (scFv-W35F(V_L)) or W36 adjacent (4.9 Å) to C22-C96 in V_{Heavy} (scFv-W36F(V_H)) was substituted by a Phe. In the third mutant, scFv-W35F(V_L)/W36F(V_H), both above mentioned Trp residues were changed to Phe.

Results and discussion

Upon illumination with near UV-light the scFv and its mutants partially lose their capacity to bind to VWF. This indicates that the structure, orientation or accessibility of the paratope is changed. Accordingly, in our study disulfide bonds are broken upon illumination of scFv-wt and of the mutants scFv-W35F(V_L) and scFv-W36F(V_H). The amount of free thiols after 1 hour of illumination is small

compared to the loss of affinity observed after the same illumination time. In addition, we observed that upon illumination of wild-type scFv and of the single Trp mutants, dimers and higher aggregates are formed of which a larger part is linked by reducible bonds. Therefore, the new intermolecular disulfide bonds seem be formed by recombination of intermediately formed thiyl radicals following irradiation of wild-type scFv and of single Trp mutants.

In contrast to the findings for wtscFv and for its single mutants no light-induced formation of free thiols could be detected in the double mutant scFv-W35F(V_L)/W36F(V_H). Indeed, the important fluorescence increase obtained upon simultaneous substitution of W35 (V_L) and of W36 (V_H) confirms that the three Trp residues resulting in scFv-W35F(V_L)/W36F(V_H) do not transfer an appreciable amount of their excitation energy to the disulfide bonds. Surprisingly, the simultaneous substitution of W35(V_L) and of W36(V_H) did not lead to a preservation of affinity for VWF upon illumination. Therefore, in addition to the effects which are induced by light absorption of W35(V_L) and of W36(V_H), light absorption of W47(V_H), W50(V_H) and/or W108(V_H) has to create specific alterations in the paratope of V_L and V_H domains of the scFv and presumably also of the monoclonal antibody 82D6A3.

These results indicate that the excited Trp residues in scFv initiate different photochemical reactions which result in the loss binding capacity of the paratope. Photolysis of disulfide bonds is initiated by Trp35(V_L) and Trp36(V_H). However, replacement of these Trp residues does not significantly protect the scFv against irradiation induced loss of affinity.

Acknowledgements

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BACKBONE-MODIFIED AMYLIN DERIVATIVES: IMPLICATIONS FOR AMYLOID INHIBITOR DESIGN AND AS TEMPLATE FOR SELF-ASSEMBLED BIONANOMATERIALS

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Introduction

Uncontrolled aggregation of proteins/polypeptides leading to the formation of amyloid fibrils is a major cause of a number of diseases. Examples of these so-called amyloid-diseases are Alzheimer's disease, Parkinson's disease, transmissible spongiform encephalopathies and diabetes type II [1]. The latter is characterized by deposits of islet amyloid polypeptide (IAPP, or amylin) which are present in the pancreatic islets. Amyloid fibrils are characterized by the (anti)parallel organization of β-pleated sheets which lead to a reduced solubility of the protein/polypeptide and to the formation of deposits of amyloid plaques [2]. A well accepted approach to interfere with β-sheet formation is the design of soluble β-sheet peptides to disrupt the hydrogen bonding network which ultimately leads to the disassembly of the amyloid fibrils [3]. Although we have shown that mutation of a single amide bond into the corresponding ester, peptoid or *N*-butylated amino acid, at position 28 of the human IAPP9(20-29) peptide: H-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-NH_2, was able to inhibit amyloid formation [4], these modified IAPP(20-29) derivatives, however, were not able to inhibit/retard fibril formation of the full-length amylin or to resolubilize preformed fibrils. However, amide bond modifications at alternate positions have been used successfully with the Aβ(16-22) peptide in order to obtain aggregation inhibitors [5]. Therefore, we have designed and synthesized amylin(20-29) derivatives in which the amides at position 24, 26 and 28 have been modified by *N*-butylation, or by incorporation of peptoid residues or by ester moieties (Fig. 1) [6].

Results and Discussion

Amylin(20-29) with three alternate ester moieties formed a gel after dissolution in 0.1% TFA in H_2O. However, typical amyloid fibrils were not observed by transmission electron microscopy (TEM), which was confirmed by FTIR since the typical absorption for β-sheets (1630 - 1625 cm^{-1}) was absent. Instead fibrils, large helical ribbons (length: 7.4 μm; width: 170 nm) and even tube-like supramolecular structures were observed. In case of depsipeptides this morphology of helical ribbons as final-stage folding assemblies has not been described before.

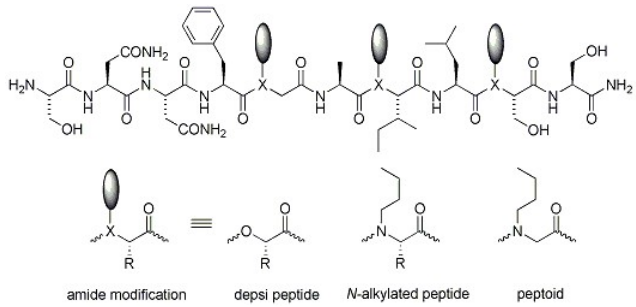

Fig. 1. Amylin derivatives in which three alternate amide bonds have been modified by either an ester bond, N-butyl amino acid- or peptoid moiety.

As expected, a solution of the N-butylated peptide in 0.1% TFA/H_2O formed a clear solution and gel formation did not occur. Fibril formation as judged by TEM and FTIR was absent. Surprisingly, helical ribbons (length: 1.5 μm; width: 250 nm) were observed by TEM in this case. Similarly, the peptide-peptoid hybrid rapidly dissolved in 0.1% TFA/H_2O and gel formation was absent. In addition, no fibrils could be detected by either TEM or FTIR. However, this amylin derivative did not assemble into helical supramolecular structures and was found to be an effective inhibitor of fibril formation of native amylin(20-29) in contrast to the depsipeptide and the *N*-butylated amylin(20-29) derivative. None of the newly designed amylin(20-29) derivatives were able to inhibit fibril formation of full length amylin(1-37) and preformed fibrils were also unaffected by these amylin derivatives. The increased hydrophobicity of the depsipeptide and the *N*-butylated peptide might be the driving force for the self-assembly into the observed helical ribbons. Absence of any supramolecular structures formed by the peptid-peptoid hybrid could be explained by the increased flexibility of the tertiary amides and the absence of crucial side chains (Ile26).

Conclusions

Modification of the peptide backbone allows the control of aggregation behavior of an amyloid peptide into fibrils, helical ribbons or peptide nanotubes [7]. Self-assembly of these modified amylin derivatives into structures other than amyloid fibrils might be important for the design of peptide-based bionanomaterials. These investigations were supported by CW-NWO.

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BINDING STUDIES OF HPK1 PRO-RICH PEPTIDES TO THE CORTACTIN SH3 DOMAIN.

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Introduction

The hematopoietic progenitor kinase 1 (HPK1), a mammalian hematopoiesis-specific Ste20 kinase, implicated in the regulation of MAP kinase is a potent stimulator of the stress-activated JNK/SAPK protein kinase cascade. HPK1 consists of an N-terminal Ser/Thr kinase domain, followed by a central region harboring four proline-rich motifs called P1, P2, P3 and P4, respectively, and a presumably regulatory C-terminal tail. HPK1 proline-rich motifs play an important role mediating interactions with signaling proteins containing polyproline binding domains and in particular with SH3 domains. Previous studies showed that P1, which contains the canonical PxxPxR motif, and P2 and P4 with the canonical PxxPxK motif interact with the C-terminal SH3 domain of hematopoietic lineage cell-specific protein 1 (HS1) with different affinity, P2>P1>P4 [1].

HS1 protein shows the highest similarity to the cortactin protein. This protein was initially identified as one of the most prominent tyrosine phosphorylated proteins in v-Src infected chicken embryo fibroblasts. Overexpression of cortactin frequently occurs in several human carcinomas and correlates with lymph node metastasis and increased mortality. Elevated expression of cortactin increases cell motility, invasion and metastasis [2]. While cortactin and HS1 proteins display an overall similarity of about 51%, the sequences corresponding to the SH3 domain and the 37-amino-acids repeat domain reach a similarity of about 86%.

Results and Discussion

The HPK1 proline-rich motifs [P1 (PELPPAIPRRIR), P2 (PPPLPPKPKF), P3 (PPPNSPRPGPPP) and P4 (KPPLLPPKKE)] were synthesized by solid-phase peptide synthesis either on Wang (P1, P2 and P4) or on 2-chloro-tritil resin (P3) using Fmoc/HBTU chemistry to investigate their affinity to cortactin SH3 domain.

Using Non-Immobilized Ligand Interaction Assay by Circular Dichroism (NILIA-CD) we evaluated the binding properties of the cortactin SH3 domain to HPK1 proline-rich motifs. The proline-rich modules adopt an extended left-handed helix of polyproline of type II (PPII) conformations upon binding to the cognate binding sites. Common features of the SH3 proline-rich binding sites are the conserved Trp and Tyr side-chain residues that clamp the two proline residues (P_i and P_{i+3}) of the PPII turn of the ligand in a zip type fashion. Upon peptide addition, the binding was

monitored by the CD changes of the Trp side-chains of the conjugate GST-SH3cortactin. The dissociation constant K_d was determined analyzing the CD data at 290 nm using a non-linear regression method [3]. The results show that the four HPK1 Pro-rich peptides are not equivalent. P2 appears to have the highest affinity (K_d = 0.8 ± 0.1 μM) than P1 (K_d = 9.6 ± 1.0 μM) and P4 (K_d = 33.3 ± 3 μM) whilst P3 does not interact.

A P2 analogue, depleted of the C-terminal KF residues, was then synthesized and its interaction with the conjugate GST-SH3cortactin protein was analyzed. Results showed that this depletion is not tolerated by the SH3 domain of cortactin suggesting the importance of the C-terminal Lys residue in the substrate recruitment, as confirm by preliminary in silico studies, which show as both Lys residues are oriented in an optimal position to interact with Glu and Asp residues of the SH3 domain.

Acknowledgements

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FALLAXIN: OPTIMIZATION OF CLEAVAGE CONDITIONS FOLLOWING SOLID-PHASE SYNTHESIS

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Introduction

The antibacterial peptide amide "fallaxin", GVVDILKGAAKDIAGHLASKVMNKL-NH_2, was recently isolated from the West-Indian mountain chicken frog "leptodactylus fallax" [1]. Fallaxin has been shown to inhibit the growth of the Gram-negative bacteria *E. coli*, *P.aeruginosa, E.cloacae*, *K. pneumoniae* with relatively low potency (MIC ≥20 mM). Fallaxin shows almost no toxicity towards erythrocytes with a hemolytic activity of HC_{50}>200 mM [1] and is therefore a promising candidate for a structure-activity study.

The peptide contains some sensitive side-chains, including methionine, which is easily oxidized to methionine-sulfoxide [Met(O)] during acidolytic cleavage [2 - 4].

As part of an ongoing structure-activity study of fallaxin, we have investigated three different cleavage cocktails for the deprotection of fallaxin. The following reagents were used. Reagent R: trifluoroacetic acid-1,2-ethanedithiol-anisole-thioanisole (90:3:2:5), Reagent L: trifluoroacetic acid-triisopropylsilane-dithiothreitole-water (88:2:5:5) and Reagent X: trifluoroacetic acid-triisopropylsilane-1,2-ethanedithiol-water (94:1:2.5:2.5). According to literature reports, all three reagents are able to suppress the formation of methionine-sulfoxide and alkylated by-products [2 - 3].

Synthesis of fallaxin was performed manually using standard Fmoc-chemistry on 100 mg Tenta Gel S RAM resin. The following side-chain protections of amino acid derivatives were used: Trt for Asn and His; Bu^t for Ser and Asp; Boc for Lys. Protected amino acids were coupled in three-fold excess employing a protocol with HATU/DIEA (1:1.5) activation, and NMP as solvent [4]. Final cleavage and deprotection were carried out using either, Reagent R, Reagent L or Reagent X for 2h under nitrogen atmosphere.

Results

Following workup, the crude peptide products were analyzed by RP-HPLC and MALDI-TOF MS.

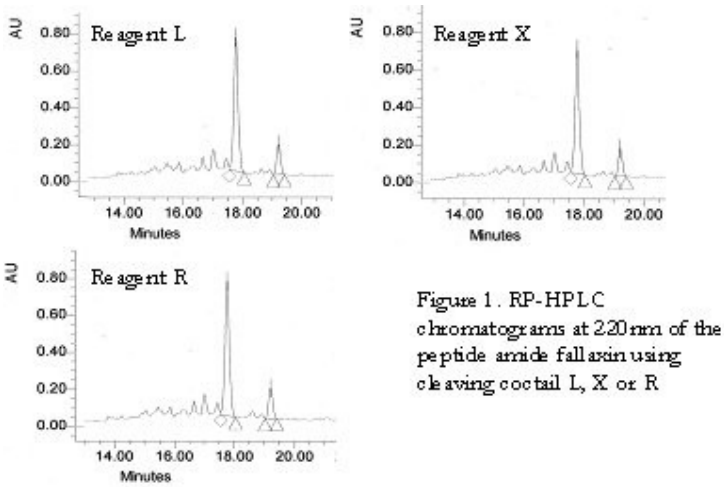

Figure 1. RP-HPLC chromatograms at 220 nm of the peptide amide fallaxin using cleaving cocktail L, X or R

Discussion

Surprisingly, the analytical RP-HPLC and MALDI-TOF MS chromatograms showed no major differences. All three cleavage cocktails gave crude fallaxin in good purity. Reagents L, X and R were all able to suppress the acid catalyzed Met oxidation and the formation of alkylated by-products. In conclusion, we present a procedure for an efficient preparation of fallaxin in good yield and purity.

Acknowledgements

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SYNTHESIS OF TETRAHYDRO-β-CARBOLINE-CONSTRAINED TRYPTOPHAN DIPEPTIDE MIMETICS

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Introduction

Tryptophan is often a key pharmacophore which determines the affinity of peptide ligands for their receptors. Therefore cyclic analogues which introduce local constraints, reduce the flexibility of the peptide chain and stabilize the bioactive conformation are very valuable tool in peptidomimetic research . The Pictet-Spengler (P-S) reaction has been widely applied to prepare analogs in which the α-nitrogen has been linked to the aromatic ring through a methylene unit [1]. A new stereogenic center is formed in the P-S reaction when using substituted aldehydes. The use of chiral carbonyl components (for example α-aminoaldehydes) can influence the configuration of that stereogenic center [2]. We now report the diastereoselectivity studies of the P-S reaction between L-Trp-OMe and α-aminoaldehydes derived from L and D amino acids and the further constraining of the resulting β-carbolines by introducing additional 5 or 6-membered ring. Such compounds may be interesting scaffolds for peptidomimetic design [3 - 5].

Results

Aminoaldehydes **2** were synthesized via the Fehrentz and Castro method. The Pictet-Spengler reactions were carried with Trp-OMe and Cbz-protected L and D amino aldehydes (Fig. 1), at -30 °C for 5 h and at RT overnight.

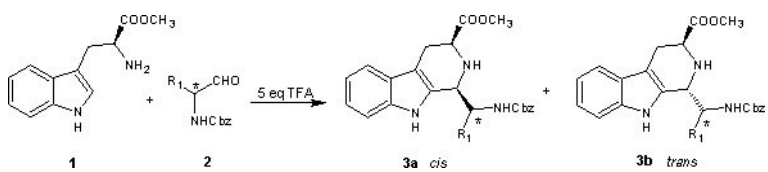

Fig. 1. The Pictet-Spengler cyclization.

The ratio of diastereomers **3a**, **3b** was determined before purification by NMR (Table 1). The reaction yields were between 50 - 80%. The configuration of the new created stereogenic center was assigned by 2D NMR spectra (ROESY). Reactions with L-aminoaldehydes preferentially formed the *trans*-isomers, whereas reactions with D-aminoaldehydes selectively led to the *cis*-isomers.

Substrate	*cis/trans* ratio determined by NMR [%]	Substrate	*cis/trans* ratio determined by NMR [%]
Cbz-L-ValH	16/84	Cbz-D-ValH	100/0
Cbz-L-LeuH	27/73	Cbz-D-LeuH	100/0
Cbz-L-IleH	18/82	Cbz-D-IleH	100/0
Cbz-L-PheH	25/75	Cbz-D-PheH	100/0
Cbz-L-NleH	29/71		

Table 1. The ratio of cis/trans isomers determined by NMR

The separated diastereomers **3a** and **3b** were further restricted (Fig. 2) via introduction of an additional 5 or 6-membered ring (C=O **4**, CH_2 **5**, $CH_2C=O$ **6** bridge). **3a** were also intramolecularly cyclized **7**.

The separated diastereomers **3a** and **3b** were further restricted (Fig. 2) via introduction of an additional 5 or 6-membered ring (C=O **4**, CH_2 **5**, $CH_2C=O$ **6** bridge). **3a** were also intramolecularly cyclized **7**.

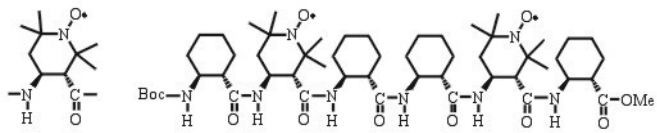

Fig. 2. Further restriction of the P-S products.

Discussion

The ratio of the formed P-S products depends mainly on the structure and chirality of the aldehyde component. That significant difference testifies that there is chirality transfer from the optically active carbonyl reagent to the newly generated stereocenter. It is possible to further restrict of the P-S products and these highly restricted fused tetracyclic peptidomimetic motifs could be incorporated into peptides to rigidify their conformation, or they can be used as peptidomimetic scaffolds.

Acknowledgements

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FLUORIMETRIC INVESTIGATION AND MOLECULAR DYNAMICS SIMULATION ON INTERACTION BETWEEN PRION PROTEIN HELIX 2 AND TETRACYCLINE

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Introduction

Though the physiological role of the ubiquitary prion protein (PrP) is still largely unknown, it is currently accepted that its isomerization from a prevailingly α- to a predominantly β-conformation is the basic mechanism governing the onset of transmissible spongiform encephalopathies [1]. Hypothesis on the unusual PrP structure instability, include the presence of one or more weakness points that, under particular cellular conditions do succumb provoking a structure collapse toward a an aggregated, but more stable β-conformation. Recently, it has been demonstrated that the PrP subdomain encompassing helix 2 (H2) residues displays a conformational duality, being able to adopt both α-helical and β-structure conformations, and that such a behaviour could be imputed to the highly conserved threonine-rich C-terminal region [2, 3]. The region is therefore gaining an increasing interest for the study of the PrP misfolding mechanism and as an "hot spot" for the rational design of compounds able to block or prevent the prion diseases. Previous studies have pointed out that the well characterized antibiotic tetracycline (TC), with low toxicity and favourable pharmacokinetics, binds to and disrupts PrP peptide aggregates, preventing prion infectivity [4, 5] and decreasing considerably the proteinase resistance of prion fibrils [6]. We demonstrate here by an integrated spectroscopical and computational study that tetracycline can interact with model peptides derived from the C-terminal globular domain of the prion protein, PrP[173-195], and that interaction concerns residues within the C-terminal half of the helix 2 with a significant involvement of the threonine rich region.

Results

Firstly, a preliminary investigation of the conformational properties of C-terminally amidated and N-terminally fluoresceinated prion peptides PrP[173-195], PrP[180-195] and PrP[106-126] has been undertaken by CD spectroscopy, either isolated and in presence of TC. As a first result, we observe that the N-terminally fluoresceinated PrP[173-195] and PrP[180-195] adopt an α-helix conformation, while the fluoresceinated PrP[106-126] does not. Further, TC does not influence the structure of the peptides in solutions, even when added in very large excess. Thus, in order to investigate a possible TC-peptides interaction we have utilized steady-state fluorimetry, by titrating the peptides with increasing amounts of antibiotic and

recording the fluorescence quenching. As negative control, the titration was also carried out on β[25-35], another representative amyloidogenic peptide [7]. The fluorescence intensities were used to evaluate the fraction of tetracycline bound by $\alpha = \Delta F/\Delta F_{max}$, where ΔF is the fluorescence change observed after each addition and ΔF_{max} refers to 100% binding. The apparent dissociation constant ($K_{D'}$) was evaluated by the one-site binding equilibrium equation [8] $\alpha=\{K_{D'}+P_0+T_0-[(K_{D'}+P_0+T_0)^2 - 4P_0T_0]^{1/2}\}/2P_0$. Here, P_0 is the initial concentration of peptide (0.1 mM) and T_0 is the concentration of added Tetracycline (0 ÷ 1 mM). While the prion peptides PrP[173-195] and PrP[180-195] display an apparent dissociation constant of 189 ± 7 nM and 483 ± 30 nM for the complexes TC-PrP[173-195] and TC-PrP[180-195] respectively, the control peptide β[25-35] and the PrP[106-126] peptide, already described as able to interact with TC, are unable to bind the antibiotic in the explored concentration range. The smaller dissociation constant observed for the longer peptide can be explained by a contribution from residues outside the 180-195 region or to a higher stability of its helix conformation. Accordingly, the PrP[173-195] CD spectrum shows more intense bands, suggestive of a higher helical content compared with PrP[180-195]. Both automated docking calculations (AutoDock package) and MD simulations (GROMACS package) have been carried out to predict that TC exhibits a strong preference for the coordination to the C-terminal part, encompassing aminoacids 183-195, of hPrP[173-195] (see Fig.1).

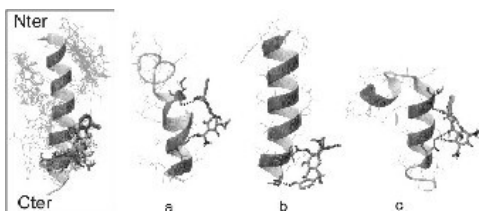

Fig.1. Docked structures obtained by AutoDock calculations. The three energetically most stable clusters of docked structures are in stick. (Bordered image). Representative frames extracted during the a) MD1, b) MD2 and c) MD3 molecular dynamics simulations are displayed too.

Acknowledgements

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STRUCTURAL MIMICRY OF BIOLOGICAL PROTEIN SURFACES USING CLIPS TECHNOLOGY

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Introduction

Structure-based design of synthetic peptide-based molecules that mimic the functional site of proteins nowadays plays an important role in drug discovery. Applications are widespread, ranging from synthetic anti-viral, anti-fertility or anti-tumor vaccines to therapeutic agents able to mimic or disrupt protein-protein interactions. The total synthesis of such complex structures is demanding, which limits their application and emphasizes the need for high-efficiency synthetic strategies.

As part of our research program on mapping and reconstruction of discontinuous binding sites, we developed the CLIPS™ technology (Chemically Linked Peptides on Scaffolds) that makes use of a conformational fixation of linear peptides. Unprotected peptides, containing two cysteines were shown to react fast with small aromatic scaffolds like α,α'-dibromo-*m*-xylene to form the cyclic product in high yield.

Results

Two monoclonal antibodies that block the biological activity of Follical Stimulating Hormone (FSH) were tested in pepscan against all overlapping 12-, 15- and 18-mer peptides from FSH. These peptides, linked via their C-terminus to a functionalized polypropylene 455-well plate, were cyclized via N- and C-terminally added cysteines using α,α'-dibromo-m-xylene. In contrast to earlier scans with linear peptides from FSH, even short 12-mer cyclic peptides from the ß3-loop were found to give a high response. Based on these results, the most reactive 12-mer loop peptide was synthesized by cyclization of the linear peptide with α,α'-dibromo-m-xylene via two additional cysteines at the termini. The native cysteine residue in RVPGCAHHADSL was replaced by Asp or Ala. Addition of extra flanking amino acids to this cyclic peptide generated CLIPS peptides that were highly active in a competition assay against FSH (2 μM). Immunization of rats using the CLIPS peptides generated high peptide antibody titres for 16-mer peptides and longer. However, the 32-mer CLIPS peptide generated a high anti-FSH titre and induced neutralization against native FSH.

Table 1. Serological results of immunization of rats with CLIPS peptides. All peptides were cyclized via their cysteines by α,α'-dibromo-m-xylene, and were N-terminally acetylated and C-terminally amidated. Underlined is the 12-mer core-sequence. Anti-peptide and -FSH titres are expressed as –log(serum dilution), NT = neutralization titre against FSH, expressed as lowest possible dilution still active in the Y1-cell bioassay.

Peptide sequence	Rat #	ELISA		FSH-NT
		peptide	FSH	
CRVPGDAHHADSLC	10.1	1.3	<1,0	-
	10.2	<1.0	<1,0	-
CVRVPGAAHHADSLYC	10.9	3.5	<1,0	-
	10.10	1.8	<1,0	-
YETCRVPGAAHHADSLCTYP	10.13	4.2	<1,0	-
	10.14	>4.3	<1,0	-
TFKELVYETCRVPGAAHHADSLCTYPVA-	11.1	4.1	1.5	-
-TQAH	11.2	>4.3	4.0	1/64
KIQKTATFKELVYETCRVPGAAHHADSL-	11.7	>4.3	<1,0	-
-CTYPVATQAHAGK	11.8	>4.3	<1,0	-
mAb 6602, 1 ug/mL start				1/125

Discussion

Small aromatic scaffolds like α,α'-dibromo-*m*-xylene were found to be highly efficient reagents to produce cyclic peptides in mapping studies. These short cyclic CLIPS peptides were also found to react with monoclonal antibodies that did not respond in standard scans. In this way it becomes much easier to locate reactive core sequences in loop stuctures of proteins. These short core peptides did not show competition against native FSH, however, when the sequence is extended at both sides with flanking amino acids, these CLIPS peptides gave competition at 2 μM level. After immunization of rats, neutralization and high anti-FSH titres were found for the 32-mer CLIPS peptide.

In conclusion, the reaction of the α,α'-dibromo-*m*-xylene scaffold with peptides containing two cysteines is fast and generates almost exclusively intramolecular loop structures. These CLIPS peptides are very efficient to determine reactive core sequences in scanning experiments. Further, the scaffold induces a loop structure in FSH peptides which leads to a product that competes with FSH against monoclonal antibodies and generates antibodies that neutralize the bioactivity of the native protein.

Acknowledgements

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IDENTIFICATION OF INHIBITORS OF PLGF/ FLT-1 INTERACTION BY THE SCREENING OF PEPTIDE COMPOUNDS LIBRARIES

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Introduction

Blood vessel formation largely contributes to the pathogenesis of numerous diseases, including ischemia and cancer [1]. In this regard therapeutic strategies aim to stimulate vascular growth in ischemic tissues and suppress their formation in pathologies. Placental Growth Factor (PlGF), a close homolog of Vascular Endothelial Growth Factor (VEGF), stimulates angiogenesis and collateral growth in ischemic heart and limb. Whereas VEGF exerts its biological function through the binding to both VEGF receptor-1 (VEGFR-1or Flt-1) and VEGFR-2 (or KDR), PlGF binds specifically to Flt1 and is able to replace VEGF in the binding with this receptor. The complex PlGF/Flt-1 constitutes a potential candidate for therapeutic modulation of angiogenesis and inflammation [2]. The PlGF/Flt-1 binding has multipunctual features [3] and potential antagonists must have a sufficient molecular surface to reach distant contact points. To identify new molecules able to inhibit the Flt-1/PlGF binding, we have successfully screened several peptide combinatorial libraries using a competitive ELISA assay and tested the resulting hits in a cellular assay.

Results

A peptide library built using a branched tetrameric structure, $(NH_2\text{-}X_1\text{-}X_2\text{-}X_3)_4\text{-}Lys_2\text{-}Lys\text{-}Gly$, was designed to obtain original molecules with a high recognition surface. 30 different unnatural building blocks (except a Glycine), were utilized to construct a random library with a complexity of $30^3 = 27.000$ molecules, arranged in 30 different sub-pools. Libraries were synthesized by SPPS following Fmoc chemistry and the portioning-mixing method to achieve randomization. Libraries were thus screened in a competition ELISA assay with coated Flt-1 receptor and PlGF as ligand (mouse variants). In the first screening sub-library 4 was selected for further investigation. Then sub-libraries $(NH_2\text{-}4\text{-}B_2\text{-}X_3)_4\text{-}Lys_2\text{-}Lys\text{-}Gly$ were synthesized and screened in the same assay format identifying the monomer 23 as the best working in the second position. 30 single tetrameric peptides $(NH_2\text{-}4\text{-}23\text{-}B_3)_4\text{-}Lys_3\text{-}Gly$, were finally produced and assayed. At least two different peptides carrying monomers 5 and 23 in position 3, were selected as the most active. The inhibition properties of 4-23-23 and 4-23-5 tetrapeptides were confirmed on the human variants of PlGF and receptor and they, after purification, showed a 5-10 μM overall IC_{50}.

After peptide immobilization on microtiter plates, we evaluated their binding capacities to either Flt-1, KDR and PlGF, finding out that only Flt-1 was effectively bound. The relative contribution of single residues and of tetrameric structure to activity was evaluated by using Ala-mutated peptides and dimeric or trimeric variants. Furthermore the specific activity of the two selected peptides $(NH_2\text{-}4\text{-}23\text{-}5)_4\text{-}Lys_2\text{-}Lys\text{-}Gly$ and $(NH_2\text{-}4\text{-}23\text{-}23)_4\text{-}Lys_2\text{-}Lys\text{-}Gly$ was assayed in a capillary tube formation assay performed with Human Umbilical Vein Endothelian Cells (HUVEC). The molecules inhibit the formation of a capillary-like network induced by PlGF on primary human endothelial cells, at a concentration of about 4 μM.

Discussion

By using an ELISA-like assay, we have identified two tetrameric peptides able to antagonize the Flt-1/PlGF complex, binding to Flt-1. Peptide affinities are in th μM range and depend on both sequence and structure, as any amino acid substitution or structure semplification, affects binding capacity. The selected peptides are able to block the receptor activity in a cellular assay of capillary tube formation in HUVEC at a concentration of about 4 micromolar.

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TRP-RICH ANTIBACTERIAL PEPTIDES AND THEIR ACTION ON MEMBRANES

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Introduction

Among a great number of antibacterial peptides the group of Trp-rich peptides is of special interest. For the present study we have synthesized several Trp-rich peptides:

ILPWKWPWWPWRR-NH_2 - indolicidin [1] (I)

PITWPWKWWKGG-NH_2 - 3B3 [2] (II)

PLSWFFPRTWGKR-NH_2 - GSP-1a [3] (III)

FPVTWRWWKWWKG-NH_2 - puroindoline [4] (IV)

VRRFPWWWPFLRR-NH_2 - tritrypticin [5] (V)

and investigated their antimicrobial and hemolytic activities as well as their action on outer and inner microbial membranes of *E. coli* ML35p.

Results

The peptides were synthesized by a solid phase method using Boc-technology and purified by HPLC. Their purity and individuality were confirmed by HPLC, amino acid analysis and MS. Antimicrobial activity of the peptides was estimated by a radial diffusion assay [6]. A set of microorganizms used for the testing included Gram-negative bacteria *Escherichia coli*, Gram-positive bacteria *Listeria monocytogenes* and fungi *Candida albicans*. Hemolytic activity was tested towards human erythrocytes. The ability of the peptides to permeabilize *E. coli* membranes (outer and inner) was estimated by spectrophotometric and fluorometric procedures [7], using 3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid and *o*-nitrophenyl-β-D-galactopyranoside as substrates for β-lactamase and β-galactosidase correspondingly. All obtained Trp-rich peptides exhibited potent antimicrobial activity against microorganisms listed above with MICs ranged from 1 to 10 μg/ml. While peptides I and V were substantially toxic for human erythrocytes, peptides II - IV had no hemolytic effect.

Table 1. Antimicrobal activity of Trp-rich peptides.

Species	MICs, μg/ml				
	I	II	III	IV	IV
E. coli ML-35p	1.8	7.8	2.3	2.4	1.8
L.monocytogenes EGD	1.3	4.8	2.4	1.6	1.5
C. albicans 820	2.4	13.7	4.4	2.9	2.1

Discussion

Although all peptides synthesized possessed antimicrobial effect they varied in their ability to permeabilize outer and inner *E.coli* ML35p membranes, demonstrating different membrane-lytic activity. Indolicidin permeabilized both outer and inner E.coli membranes, but permeabilization was not as rapid as in case of protegrin-1(PG-1). In contrast to indolicidin, three other investigated Trp-rich peptides (3B3, GSP-1 and tritrypticin) increased the permeability of outer membrane only, without affecting the inner membrane. Lack of hemolytic activity in concentrations 10 - 100 times above MIC and decreased ability to affect bacterial inner membrane, allowed us to suggest, that the mechanism of 3B3 and GSP-1a action differs from the action of distinct membrane-lytic peptides (PG-1, for example) and the destruction of bacterial membranes is not the base of their antimicrobial action.

Acknowledgements

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SOLID PHASE SYNTHESIS OF ARGININE BASED PEPTOIDS AS INHIBITORS OF TRPV1 CHANNEL

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Inflammatory pain begins when noxious stimuli (thermal, chemical or mechanical) excite sensorial neurons called nociceptors. The activation of these nociceptors is due to depolarization of the cell membrane generated by the opening of some ionic channels. One of these channels is TRPV1 (Transient Receptor Potential Vanilloid 1) which is directly implied in thermal hyperalgesia associated to inflammation [1,2]. The activation of TRPV1 results in a rapid increase of intracellular Ca^{2+} levels. The channel is activated specifically at temperatures higher than 42 ºC, acidic pH and by the vanilloid capsaicin [3]. It has been found that peptoid H-Arg-15-15C inhibits the activation of TRPV1 at the micromolar level by blocking the pore entrance and avoiding calcium flow [4]. Our aim is the design and synthesis of new, more potent, TRPV1 antagonists with anti-inflammatory and analgesic properties.

Based on the structure of the peptoid H-Arg-15-15C (Fig. 1) we designed a small library of novel compounds with some chemical modifications: incorporation of different residues positively charged to evaluate the effect of the basicity; substitution of 2,4-dichlorophenethyl group for a different hydrophobic planar group such as the indole ring; suppression of the acetyl on the C-terminal; modification of the N-terminal by substitution with an amine bond and incorporation of a cyclic amino acid like proline on the structure. The synthesis of the library was performed in solid phase [5]. The anchorage was performed on Rink amide MBHA or BAL-MBHA resins, depending of the C-terminal. The synthesis of the backbone of the peptoids consists in the acylation of a secondary amine with chloroacetic acid in the presence of DIPCDI and thereafter the amination of the chloromethyl intermediate with a hydrophobic amine in basic media. All compounds were released from the resin using TFA/H_2O (95:5) mixtures. Comparative blockade activity of peptoids was first evaluated by voltage clamp in Xenopus oocytes expressing TRPV1. The channels were activated with capsaicin 10 μM and recorded at a holding potential of -40 mV. The selectivity of peptoids was evaluated comparing their activity in NMDA (*N*-methyl-D-aspartate) channels, which were activated with Glu–Gly 100 μM - 20 μM [6, 7]. Based on these results we conclude that best blockade activity was obtained with those molecules containing a hydrophobic moiety close to a basic group such as a guanidinium or a primary amine, incorporated on the lateral chain of the N-terminal amino acid. The combination of two indole groups and the elimination of the acetyl group on the C-terminal increased the selectivity for TRPV1 versus NMDA. The more selective

and active compounds (1-5, 19 and 21, see Fig. 1) are being tested in animals models to evaluate their analgesic and anti-inflammatory activity.

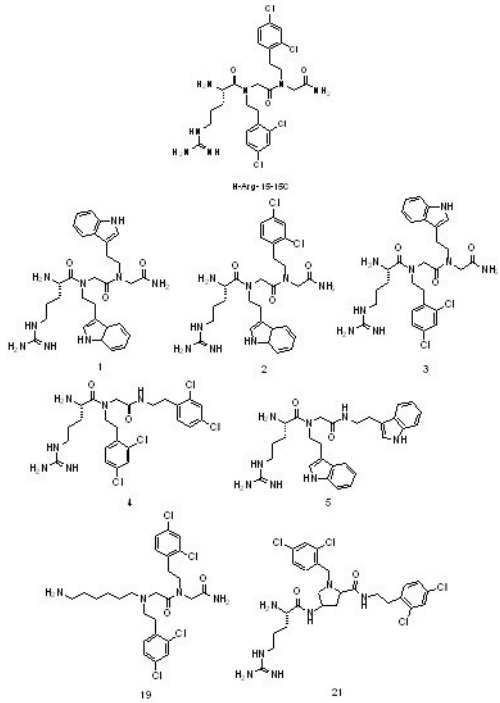

Figure 1

Acknowledgements

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SYNTHESIS OF ARENICIN-1 – AN ANTIMICROBIAL PEPTIDE FROM COELOMOCYTES OF MARINE POLYCHAETA ARENICOLA MARINA

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Introduction

Recently new 21-residue antimicrobial peptides - arenicins were isolated from coelomocytes of marine polychaeta Arenicola marina and their sequences were determined [1].

There are two isoforms of arenicins which are differed only with single amino acid. These peptides have no structure similarity to any previously identified antimicrobial peptides.

We have synthesized arenicin-1 and estimated its antimicrobial properties.

Results

The linear arenicin-1 was prepared by solid phase method using Boc-technology without any problem.

However the cyclization caused the appreciable difficulties. The following methods of oxidation were used: oxygen of air, $K_3Fe(CN)_6$ and hydrogen peroxide in aqueous or aqueous/organic media.

The best results were obtained by using hydrogen peroxide in methanol and air/charcoal, but even in these cases the yield of the final peptide did not exceed 5-7%. Antimicrobial activity of synthetic arenicin-1 was estimated in radial agar-diffusion assay [2].

Discussion

Synthetic arenicin had the same HPLC profile and MS MALDI-TOF spectra as a natural molecule.

The peptide showed an antimicrobial activity against Gram-positive bacteria: Listeria monocytogenes EGD, Staphylococcus aureus MR ATCC 33591, Gram-

negative bacteria: *Escherichia coli* ML-35p, *Pseudomonas aeruginosa* and fungi *Candida albicans* 820. Minimal inhibitory concentrations of synthetic arenicin-1 against all tested strains were less then 1 mkM and equal to those of natural arenicin-1.

Acknowledgements

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ANTIBACTERIAL PROLINE-RICH PEPTIDES – SYNTHESIS AND ANTIBACTERIAL ACTIVITY

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Introduction

The family of antibacterial proline-rich peptides, originally isolated from insects, shows remarkable activity against diverse bacterial and fungal pathogens. While more and more bacterial pathogens become resistant to common drugs, this part of insect innate immunity provides a new promising approach to develop future peptide-drugs. Short proline-rich peptides, *e.g.* drosocin (fruit fly) [1, 2], formaecin (red bulldog ant) [3] and metalnikowin (green shield bug) [4], possess a significant sequence homology and share a common mechanism of action. The combination or insertion of sequence-regions from different native antibacterial peptide sequences may offer several advantageous effects including further reduction of toxicity and broadening of the antimicrobial activity. The aim of this study was to synthesize different proline-rich 15 to 22 mer peptides taken from the literature, as well as combinations with C- and N-terminal fragments of drosocin and formaecin.

Results

Peptide sequences derived from the short proline-rich peptide families drosocin, formaecin, and metalnikowin with antimicrobial activities were synthesized on solid phase using the Fmoc/*t*Bu-strategy. After purification by reversed phase HPLC the peptides were obtained in high purity and their masses were confirmed by MALDI-MS. The antibacterial activities of the peptides were tested in agar diffusion assays with the Gram-negative *Escherichia coli* and the Gram-positive *Micrococcus luteus* bacteria strains in triplicates (Table 1). Unglycosylated drosocin, a well-investigated antibacterial peptide from *Drosophila melanogaster*, showed the highest activity against both strains among the tested peptides. Amidation of the C-terminus decreased its activity significantly for both strains. In contrast, formaecin inhibited only *E. coli* at a lower rate. Replacement of the N-terminal 10 residues of formaecin by the corresponding drosocin sequence increased its activity for both strains significantly, but it was still much lower than the drosocin activity. Interestingly, the C-terminal amide was more active than the free acid. A mixed hybrid of the N-terminal formaecin and the C-terminal drosocin sequence was highly active against *M. luteus* but not all against *E. coli*. The investigated metalnikowin sequences inhibited the tested bacteria only slightly, whereas heliocin was specific for *M. luteus*.

Table 1: Agar diffusion assays of the synthetic peptides

Peptide	Sequence	Inhibition zones in cm	
		Escherichia coli BL21AI	*Micrococcus luteus ATCC10240*
Drosocin	GKPRPYSPRPTSHPRPIRV	18	36
Drosocin amide	GKPRPYSPRPTSHPRPIRV-NH_2	10f	27
Formaecin 1	GRPNPVNNKPT(GalNAc)PHPRL	10	-
Drosocin (N) + Formaecin (C)	GKPRPYSPRPT*PHPRL*	13p	15
Drosocin (N) + Formaecin (C) amide	GKPRPYSPRPT*PHPRL*-NH_2	14p	22
Formaecin (N) + Drosocin (C)	*GRPNPVNNKP*TSHPRPIRV	-	28
Metalnikowin IIB amide	VDKPDYRPRPWPR*NMI*-NH_2	15	-
Metalnikowin III amide	VDKPDYRPRPWPR*PNM*-NH_2	10f	10f
Metalnikowin I amide	VDKPDYRPRP*RPPNM*-NH_2	10f	-
Heliocin amide	JRFIHPTYRPPPQPRRPVIMRA-NH_2	-	28

The antibacterial activities were tested by spotting 5 µg purified peptide on a bacteria coated agar plate. After an incubation time of 24 h at 37 °C the activity was determined by measuring the diameter of the inhibition zones on the plate. p partial inhibition, f faint partial inhibition, – no visible inhibiton,

Discussion

Compared to drosocin all the tested short proline-rich peptides were significantly less active against the two tested bacterial strains. Generally, a higher potency for peptides containing a C-terminal acid was obtained. Interestingly the hybride sequences from formaecin and drosocin showed a high specificity for either of the two tested bacteria indicating that one hybrid might carry a sequence targeting Gram-positive strains and the one a sequence targeting Gram-negative strains. This will be further investigated.

Acknowledgements

Financial support by the European Fond for Regional Structure Development (EFRE, European Union and the Free State Saxonia) is gratefully acknowledged.

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VIBRATIONAL AND CHIROPTICAL SPECTROSCOPIC INVESTIGATIONS ON *CIS*-2-AMINOCYCLOPENTANECARBOXYLIC ACID OLIGOMERS

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Introduction

Incorporation of conformationally restricted cyclic β-amino acid residues in β-peptides permits a rational control over helix and strand secondary structures. It was shown earlier that homo-oligomers composed of (1*R*,2*S*)-2-aminocyclopentanecarboxylic acid residues [(1*R*,2*S*)-*cis*-ACPC] form a self-stabilizing Z6-strand secondary structure in the solution phase [1], while those composed of (1*R*,2*R*)-*trans*-ACPC residues are known to adopt a highly stable H12 helix conformation [2]. Thus, inversion of the relative configuration (from *trans* to *cis*) of the ACPC residues enabled the preferred periodic structure to be switched from a helix to a non-polar strand. Herein we report an electronic circular dichroism (ECD), Fourier-transform infrared (FTIR) and vibrational circular dichroism (VCD) spectroscopic study of recently synthesized tetra- and hexamers consisting of alternating enantiomers of *cis*-ACPC residues [3], H-[(1*S*,2*R*)-*cis*-ACPC-(1*R*,2*S*)-*cis*-ACPC]$_n$-NH_2 (**1**, n = 2; **2**, n = 3).

Result and Discussion

The ECD spectra of **1** - **2** in methanol were recorded with a Jasco J-810 dichrograph in a 0.02 cm quartz cell, using concentrations of ~1 mM. The spectra in methanol, showing a positive couplet with the positive band at ~207 nm and the negative band around 189 nm, were significantly different from those of homo-oligomers having Z6-strand-like structure [1].

Molecular modeling was started with a standard restrained simulated annealing protocol, using molecular mechanics. Final optimizations and vibrational analysis of the lowest-energy conformers wer performed with the Gaussian03 program at the B3LYP/6-31G* level of theory.

The calculations predicted the H10/12 helix as the prevailing structure. This helix is stabilized by alternating C10 and C12 intramolecular H-bonds, with the exception of the C-terminus, capped by a C8 intramolecular H-bond.

FTIR and VCD spectra at a resolution of 4 cm^{-1} were recorded in dichloromethane and DMSO-d_6 solution with a Bruker PMA 37 VCD/PM-IRRAS module connected to an Equinox 55 FTIR spectrometer, using a CaF_2 cell with a path length of 0.207 mm and sample concentrations of 10 mg/ml.

The FTIR spectra of **1** - **2** in CH_2Cl_2 showed the highest-intensity amide I band at ~1659 and ~1649 cm^{-1}, respectively, compatible with a helical structure. These bands were accompanied by medium-intensity bands/shoulders at ~1632 and ~1673

cm^{-1}, having contribution from the δ_s HN_2 vibration of the N-terminal amino group, and the amide I vibration of the C-terminal $CONH_2$ group, respectively. DMSO-d_6, disrupting the more accessible C8 H-bond of the C-terminal residue and solvating the N-terminal NH_2 group, shifted especially the latter two IR bands, while the amide I bands having more contribution from the inner part of the helical sequence were less affected.

Combined with quantum chemical calculations, VCD has become a powerful technique for the stereochemical and conformational analysis of chiral molecules [4]. The theoretical VCD curves were simulated from the calculated wavenumber and rotatory strength data by using Lorentzian band shape and a half-width at half-height value of 6 cm^{-1}. The calculated vibrational frequencies were scaled by a factor of 0.963. The VCD spectra recorded in CH_2Cl_2 are in excellent agreement with the calculated spectra (Fig. 1), providing a final proof for the right-handed H10/H12 helical structure. The intensities of the VCD spectra in DMSO-d_6 are considerably lower, however, the overall amide I and II band shape is very similar. This means that the formed helix is mostly preserved in DMSO.

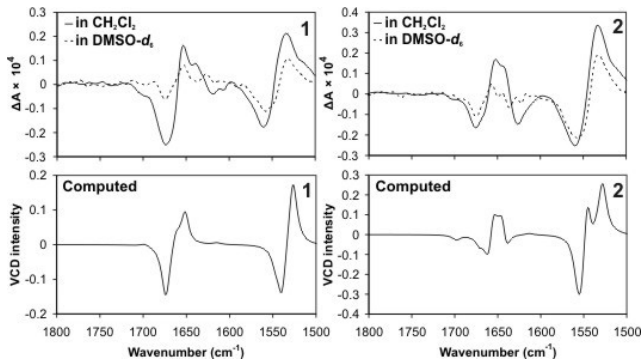

*Fig. 1. Experimental and computed VCD spectra of **1** - **2**.*

Acknowledgements

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SYNTHESIS OF BUILDING BLOCKS FOR PEPTIDE SYNTHESIS BY USING 4-(4,6-DIMETHOXY-[1,3,5]TRIAZIN-2-YL)-4-METHYL-MORPHOLINIUM TOLUENE-4-SULFONATE

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Introduction

There is an increasing demand for new building blocks necessary for efficient synthesis of complex biomolecules. The most attractive are those, prepared from less reactive substrates or formed under reaction conditions too harsh for the final synthetic target.

Result and Discussion

4-(4,6-Dimethoxy-[1,3,5]triazinyl)-methylmorpholinium toluenesulfonate [1] was found useful for the synthesis of allyl, pentafluorophenyl, N-hydroxysuccinimide and 4-nitrophenyl esters.

Table 1. Synthesis of ester building blocks by means of 4-(4,6-dimethoxy-[1,3,5]triazinyl)-methylmorpholinium toluenesulfonate.

Entry	Ester	Yield [%]	Purity [%]
1	Fmoc-L-Glu-(OtBu)-OAll	77	93
2	Fmoc-L-Ser(tBu)-OAll	82	93
3	Fmoc-L-Lys(Boc)-OAll	96	96
4	Fmoc-L-Asp-(OtBu)-OAll	86	99
5	F-moc-L-Asp-(OtBu)-OPfp	88	93
6	Fmoc-L-Ala-OPfp	73	70
7	Fmoc-L-Ala-ONSu	66	95
8	Z-Leu-Leu-ONP	85	99

Very efficient formation of amide bond was observed in the synthesis of chromogenic substrates **1** - **5** and glycosylated amino acids **6** - **7**.

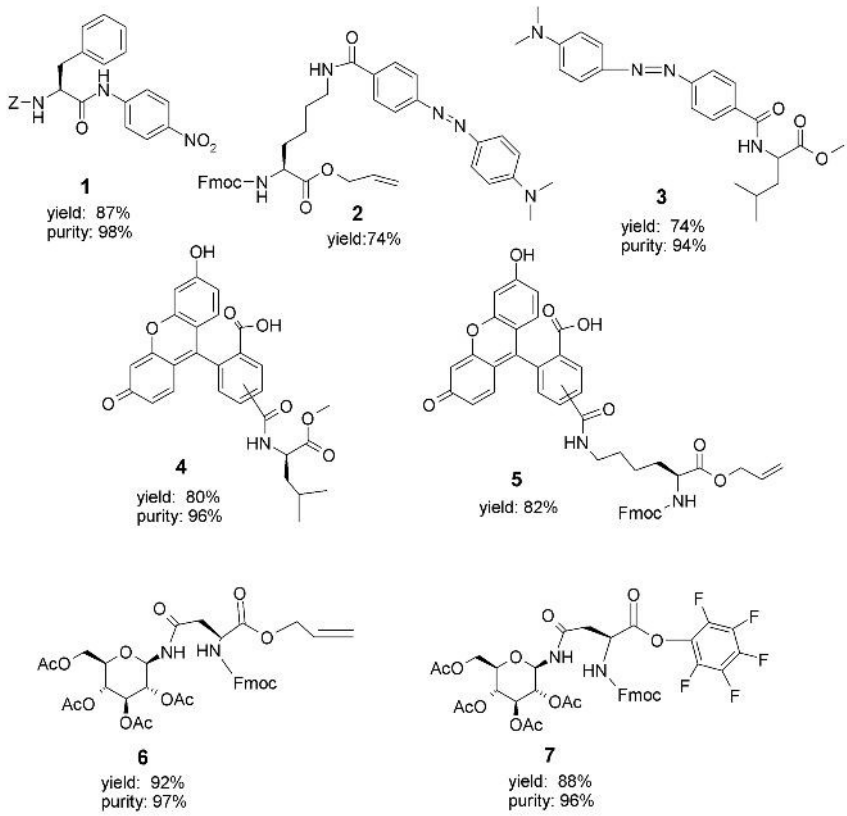

Fig. 1. Synthesis of amide building blocks by means of 4-(4,6-dimethoxy-[1,3,5]triazinyl)-methylmorpholinium toluenesulfonate.

Activation of chromogenic carboxylic components bearing diazo-, amine-, phenol and/or lactone functional group proceed selectively, therefore no additional protecting group were needed in synthesis.

Acknowledgements

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IDENTIFICATION, SYNTHESIS AND CHARACTERIZATION OF AGGREGATION INDUCING PEPTIDES DERIVED FROM GPBP PROTEIN

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Introduction

Goodpasture (GP) disease is an exclusive human disorder characterized by altered renal and pulmonary functions caused by deposits of autoantibodies along the glomerular and alveolar basement membranes. The pathogenic antibodies arise against the non-collagenous C-terminal (NC1) region of the α3 chain of collagen IV (α3(IV)NC1), also called the GP antigen [1]. The origin of the GP antigen resides in conformational changes rather than in mutations of the collagen molecules. Goodpasture antigen-binding protein (GPBP) is a non-conventional Ser/Thr protein kinase that has been cloned for its capacity to bind the N-terminal region of the human Goodpasture antigen [2]. GPBP is highly expressed in several autoimmune conditions suggesting that its activity may be important in human autoimmune pathogenesis [1]. GPBP interacts *in vivo* and *in vitro* with α3(IV)NC1 [1]. Interaction of GPBP with other proteins may induce conformational changes that could trigger the response of the immune system. An anomalous protein conformation could lead to protein aggregation.

Our objective is to characterize a nine amino acid-long sequence (p1) with very high tendency to form aggregates that we have identified using the algorithm TANGO [3]. Furthermore, an alignment of GPBP sequences was performed to identify homologous p1 sequences in other species. As a result, five peptides (p3-p7) of potential interest were identified. Invertebrate species showed a lower propensity to aggregate according to TANGO. Since His7 is conserved in all invertebrate sequences, a V7H mutant derivate of p1 (p2) was also designed for further studies. The potential role in protein aggregation of these peptides will be studied.

Results

Circular dichroism spectroscopy. At pH 2, peptides 1 and 4 showed a CD spectra characteristic of beta structure whose intensity increased after a month of incubation.

Tioflavin-T. It is well established that ThT interacts with the crossed-beta-sheet structure present in amyloid fibrils. Peptides p1 and p4, at pH 2, tested positive for ThT binding. These data suggest that peptides p1 and p4 form amyloid fibrils.

Electron microscopy. Amyloid fibrils formation was studied by electron microscopy. In agreement previous experiments, only peptides p1 and p4 formed amyloid fibrils at pH2.

Influence of pH in the aggregation of p2. At pH 2, the charge of the His residue could prevent aggregation. Thus, structural changes for p2 were monitored by circular dicroism spectroscopy at pH 6. Deprotonation of His allowed beta structure formation suggesting that hydrophobicity of residue 7 may be important for aggregation.

Peptide-protein interaction. It is well established that the rate of formation of fibrils by amyloidogenic proteins is enhanced by the addition of preformed fibrils, a phenomenon known as seeding. We performed experiments to assess the capability of p1 to induce protein aggregation. In the presence of pre-formed fibrils of p1, GPBP acquired a stable beta conformation. This change in conformation did not take place in the absence of pre-formed fibrils even in the presence of fresh p1 (Fig. 1).

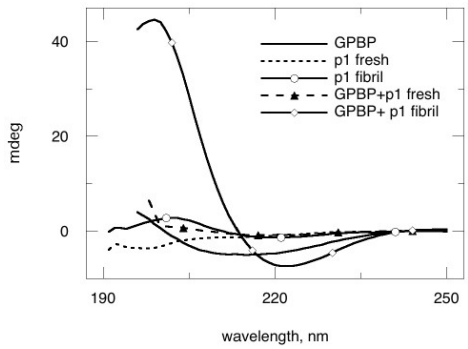

Fig. 1. Seeding experiments. Far UV-CD spectra of 1 mM GPBP in the absence or in the presence of 10 % of a solution of 0.6 mM p1, fresh or fibril.

Discussion

It has been confirmed that GPBP contains a sequence with high tendency for beta-aggregation. At pH 2, peptides p1 (human) and p4 (zebrafish) formed amyloid fibrils. This fibril formation was sequence dependent being hydrophobicity of residue 7 an important determinant. Furthermore, p1 was able to induced a stable beta conformation in GPBP.

Aknwoledgement

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ACCESS TO ALL STEREOISOMERS OF THE CYCLOALKANE ANALOGUES OF PHENYLALANINE IN ENANTIOMERICALLY PURE FORM

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Introduction

Constrained analogues of proteinogenic amino acids are useful tools in the design of peptides with improved pharmacokinetics as well as in the establishment of structure-activity relationships. In this context, the biological significance of phenylalanine, which is located in the pharmacophoric regions of many bioactive peptides and acts often as a key residue in peptide-receptor recognition, has stimulated the synthesis of a wide variety of phenylalanine surrogates.

We have been working on the synthesis of a family of phenylalanine derivatives obtained by linking the α and β carbons through an alkylidene bridge of variable length. This modification gives rise to the series of 1-amino-2-phenylcycloalkanecarboxylic acids (Fig. 1), that we call c_nPhe, with n representing the size of the ring formed. In these phenylalanine derivatives, rotation about the Cα–Cβ bond is prohibited and the orientation of the phenyl side-chain is dictated by both the ring size and the stereochemistry at the α and β carbons.

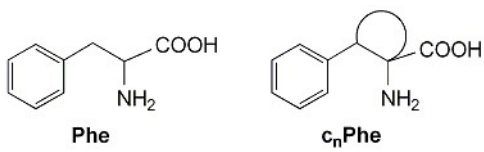

Fig. 1. Phenylalanine and its cycloalkane analogues

Result and Discussion

We have developed efficient methodologies for the synthesis of the cyclopropane (n = 3), cyclobutane (n = 4), cyclopentane (n = 5) and cyclohexane (n = 6) analogues of phenylalanine suitably protected for incorporation into peptides (Fig. 2) by combination of racemic synthetic routes with chromatographic resolution [1].

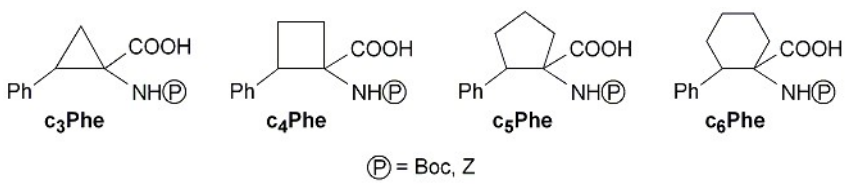

Fig. 2. Structure of the phenylalanine analogues synthesised (c_nPhe)

The preparation of the cyclopropane surrogate c_3Phe was based on a cycloaddition process between phenyldiazomethane and an α,β-dehydroalanine derivative [2, 3], whereas for the synthesis of the cyclobutane analogue c_4Phe dialkylation of a glycine equivalent was used [4]. The cyclopentane surrogate c_5Phe was obtained by Strecker reaction on 2-phenylcyclopentanone [5], in the same way that 2-phenylcyclohexanone served as starting material for the synthesis of the *trans* derivatives of c_6Phe [6]. For the *cis* isomers of this amino acid, a Diels-Alder reaction between 1,3-butadiene and (*Z*)-2-phenyl-4-benzylidene-5(4*H*)-oxazolone proved very efficient [7].

It should be noted that for a given c_nPhe derivative, four different stereoisomeric forms are possible (Fig. 3): the (*R,R*) and (*S,S*) stereoisomers bear the phenyl and amino groups in a *cis* relative disposition, whereas these groups are *trans* in the (*R,S*) and (*S,R*) derivatives.

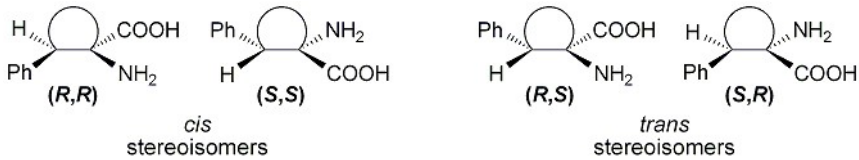

Fig. 3. Four different stereoisomers have been obtained for each c_nPhe derivative

Racemic *cis* and *trans* compounds were separately obtained either by using selective synthetic pathways or by separation by column chromatography. Enantiomerically pure products were then isolated from racemic mixtures by preparative HPLC resolution using chiral stationary phases.

Acknowledgments

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A CYCLOPROPANE AMINO ACID INDUCES A DOUBLE γ-TURN

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Introduction

Turns are important elements of secondary structure in peptides and proteins. Different types of turns are distinguished according to the number of residues involved [1]. The most abundant is the β-turn (Fig. 1), which involves four consecutive amino acids with the CO at position i being hydrogen-bonded to the i+3 NH. The γ-turn is centred at a single residue and is generally stabilized by a hydrogen bond between the i CO and the i+2 NH (Fig. 1).

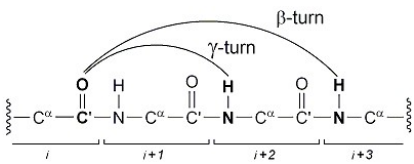

Fig. 1. Schematic representation of the main types of peptide turns

In the course of our investigations on model dipeptides of general structure RCO-L-Pro-Xaa-NHR', we have synthesised a compound of this series, incorporating a cyclopropane amino acid, namely (*R*)-1-amino-2,2-diphenylcyclopropane-carboxylic acid (D-c_3Dip). We present here the solid-state structure of this dipeptide solved by X-ray diffraction analysis, together with the results of theoretical calculations.

Result and Discussion

Model dipeptides RCO-L-Pro-Xaa-NHR' are the smallest systems able to adopt the β-turn conformation, which is favoured by the presence of proline at position i+1. Thus, dipeptide Ac-L-Pro-D-c_3Dip-NHMe (Fig. 2) was expected to accommodate a β-turn in the solid state.

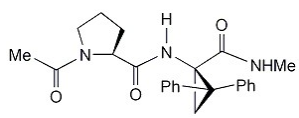

Fig. 2. Structure of dipeptide Ac-L-Pro-D-c_3Dip-NHMe

However, this was not the conformation observed in the crystalline structure of this compound [2]. Instead, two consecutive γ-turns were found (Fig. 3), with the (φ,ψ) torsion angles of both L-Pro (–82,61) and D-c_3Dip (–72,47) lying close to the typical

values of a γ-turn [1]. To stabilise the turn, the L-Pro CO and methylamide NH form a strong intramolecular hydrogen bond (N•••O distance 2.81 Å; N–H•••O angle 147°), as do the acetyl CO and D-c_3Dip NH groups (N•••O distance 2.78 Å; N–H•••O angle 152°).

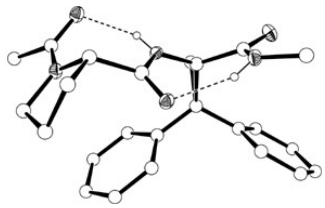

Fig. 3. X-Ray diffraction structure of Ac-L-Pro-D-c_3Dip-NHMe exhibiting two γ-turns

This is the first example of an L-Pro-Xaa dipeptide accommodating a folded conformation different from a β-turn. Moreover, the double γ-turn encountered is unique among crystalline short linear peptides. Indeed, proline was never before found to accommodate a γ-turn in the crystalline structure of a linear oligopeptide. Accordingly, the γ-turn disposition adopted by proline in this dipeptide should be ascribed to the presence of the contiguous D-c_3Dip residue. This consideration is of special relevance since proline is the proteinogenic amino acid with the strongest conformational preferences.

Theoretical calculations also show the high preference of this cyclopropane amino acid for the γ-turn conformation [3]. Thus, on exploring the conformational preferences of Ac-c_3Dip-NHMe, the γ-turn was found to be the global minimum not only in vacuo, but also in the presence of polar solvents like water (Table 1).

Table 1. Calculated minima for Ac-L-c_3Dip-NHMe in water

minimum	ϕ	ψ	relative E (Kcal/mol)
1	72	–46	0.0
2	–77	96	4.6
3	–82	–20	4.8
4	65	–172	4.3

B3LYP/6-311G(d) level

Acknowledgments

Financial support from Ministerio de Educacion y Ciencia (project CTQ2004-5358) and Gobierno de Aragon (research group E40) is gratefully acknowledged.

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INVESTIGATING THE BIOCHEMICAL REASONS FOR SENSITIVITY OF SOME PATIENTS TO ANTICOAGULANT WARFARIN

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Introduction

Warfarin is the most widely prescribed anticoagulant drug for the prevention and treatment of arterial and venous thromboembolic disorders[1]. Because of large interpatients variability in the dose-anticoagulant effect relationship and a narrow therapeutic index careful dosage adjustment based on INR (International Normalized Ratio) is essential1. Warfarin is available as a racemic mixture of two enantiomers, (*S*)-and (*R*)-warfarin. In contrast to (*R*)-warfarin, which is metabolized by multiple cytochrome P450s (CYPs), including CYP1A2 and CYP3A4,(*S*)-warfarin, is predominantly metabolized to 7-hydroxywarfarin by polymorphic CYP2C9. Since the potency of (*S*)-warfarin is much higher than that of (*R*)-warfarin, about 3-to 5-fold,any change in the activity of CYP2C9 gene is likely to have a significant influence on the anticoagulant response. Previous in vitro findings revealed that certain variants in the CYP2C9 gene are associated with large interindividual differences in the pharmacokinetic and pharmacodynamic outcomes of warfarin therapy. Three major alleles have been found to date in humans: Arg144/Ile359, and Cys144/Ile359 and Arg144/Leu359, which have been designated CYP2C9*1 (wild-type), CYP2C9*2, and CYP2C9*3, respectively [2]. The aim of this study was to determine the pharmacogenetic effect of CYP2C9 polymorphisms on warfarin sensitivity in some Iranian patients.

Patiens and methods

A total of 58 patients from Imam khomeini Hospital, in which warfarin therapy was initiated were taken into analysis. The study group consisted of 28 males, and 30 females, aged 14-85 with average 49 old years, and selected solely on the basis of their weekly warfarin dose. For each patients we provided a form considering (sex, age, weight, height, warfarin daily dose, concurrent medications, INR,). At the first time, a blood sample was taken for PT, INR and blood clotting factors (FII, FV, FVII, FVIII, FIX) measurements and CYP2C9 genotyping. Genomic DNA was isolated from whole blood using DNG^{TM} PLUSE solution. For detection of CYP2C9*2, and CYP2C9*3 variants, a protocol based on PCR technique and endonuclease digestion with, Eco47I(Ava II) and KpnI, RFLP was used. PCR was performed with primers, 5′TACAAATACAATGAAAATATCATG 3′(forward), for exon 3 and 5' CTAACAACCAGACTCATAATG 3′ (reverse) and 5'TGCACGAGGTCCAGAGGTAC3' (forward) and 5′ACAAACTTACCTTGGGAATG AGA3′ (reverse) for exon 7. A forced mismatch was included in forward primer create a restriction site for kpnI digestion.

Results

According to following definition we divided patients in two groups: with normal response and with sensitive response. Sensitive response group were patients with:

1- Weekly warfarin dose less than 10.5 mg and INR in therapeutic range.
2- With normal warfarin dose had above range PT after one week without vitamin K.
3- Above range PT after 3 days with vitamin K.

The 58 patients were genotyped for both the CYP2C9*2 and CYP2C9*3 polymorphisms. Patients homozygous for wild-type allele had 164 and 527- bp fragments, patiens homozygous for CYP2C9*2 had 691-bp and patients heterozygous for CYP2C9*2 had 691, 164, 527-bp fragments. The frequency of genotypes in two patients groups are shown in Table 1.

Table 1: crosstabulation genotypes

			Genotypes			Total
			*1*1	*1*2	*2*2	
Group	Normal Response	count	3	16	3	22
		%	13.6	72.7	13.6	100.0
	Sensitive Response	count	26	9	1	36
		%	72.2	25.0	2.8	100.0
Total		count	29	25	4	58
		%	50.0	43.1	6.9	100.0

Discussion

The interindividual variability in anticoagulation response to warfarin is multifactorial. Patient and environmental factors including age, body size, dietary vitamin K status, concurrent diseases, and drugs have been shown to affect anticoagulant response to warfarin[3,4]. This study has confirmed an association between CYP2C9 genotype and warfarin sensitivity, high INRs and bleeding complications.

We identified 4 (2*2*), 25 (1*2*), 29 (1*1*) and no 3* variants in our patients group. In sensitive response group about 90% and in normal response group about 27% of patients had variant genotypes. There were some exceptions that need more inspections.

Acknowledgements

This work was supported by a research grant from Imam Khomeini Hospital.

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FREIDINGER LACTAM SCAN OF GONADOTROPIN RELEASING HORMONE (GNRH) USING FMOC-PROTECTED CYCLIC SULFAMIDATE METHYL ESTER

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Introduction

α-Amino γ-lactams, commonly named "Freidinger lactams" [1], can constrain the backbone conformation in structure-activity relationship studies of biologically active peptides (Fig. 1). Step-wise synthesis of lactam-bridged peptides is desired and in development using cyclic sulfamidate esters as lactam precursors [2]. Using Fmoc-protection on solid-phase, the synthesis of a set of five lactam-peptide analogs of gonadotropin releasing hormone (GnRH:**1**, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was accomplished by placing lactams at respectively the residues pGlu1: **2**, Ser4: **3**, Gly6: **4**, Leu7: **5** and Pro9: **6**.

Result and Discussion

Dioxathiazinane **7** was synthesized from homoserine (Hse, **8**) [3] in up to 22% overall yield by a 4 steps approach (Fig. 1).

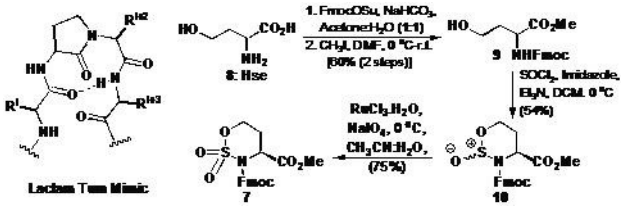

Fig. 1. α-Amino γ-lactam in generic peptide and synthesis of cyclic sulfamidate **7***.*

GnRH stimulates the anterior pituitary gland to release the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in a process that eventually leads to gonadal steroidogenesis and gametogenesis. Freidinger and coworkers demonstrated that an active turn conformation was situated at the Tyr-Gly-Leu-Arg residue in GnRH by replacing the Gly-Leu residue by an α-amino γ-lactam-Leu dipeptide moiety which produced an analog exhibiting 8.9 times higher potency than the native hormone for releasing LH in pituitary cells [1]. With this precedent as a positive control, lactams **2** - **6** were synthesized on Rink resin using standard Fmoc protocols for coupling and deprotection [4]. *N*-Alkylation of resin-bound peptides was performed using cyclic sulfamidate **7** (200 mol%) in THF at rt. Lactam cyclization was then performed by treating the resin with 1 mol% AcOH in

DMF (0.5 M) at 70 ºC for 50 h. Deprotection and elongation of the peptide were performed as described. Final cleave was performed with a freshly prepared cocktail of TFA/H_2O/TIS (9.5/2.5/2.5, v/v/v, 20 mL/g resin) for 2 h at rt. The filtrate was concentrated to about 1 mL and treated with Et_2O. The resulting lactam bridged peptide precipitate was filtered, washed with Et_2O, dissolved in acetonitrile/H_2O (1:1) and lyophilized to a foam or powder, which was purified by RP-HPLC.

Table 1: Yields and purities of lactam-bridged peptides

	CH_3OH [a] tr (min)	CH_3CN [a]	Crude Purity % [b]	Purified Yield % [c]
1	5.40	3.27	33.90	23.45
2	5.13	3.01	17.83	10.63
3	5.64	3.37	17.36	9.35
4	5.55	3.45	16.14	9.11
5	4.94	3.87	22.21	17.6
6	5.42	3.34	10.53	9.5

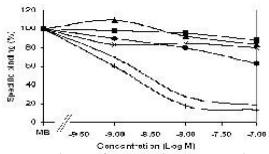

Fig. 2: Binding of GnRH lactam analogs 2-6

a Analytical HPLC on C18 column (4.6 x 50 mm, 5 µm), flow rate = 0.5 mL/min over 4 min with a linear gradient from water (0.1% TFA) to CH_3OH (0.1% TFA) or from water (0.1% TFA) to CH_3CN (0.1% TFA). b HPLC purity at 214 nm. c Yields of 99% pure peptide after purification by RP-HPLC are based on manufacturer's reported loading for Rink resin.

The displacement (%) of specifically bound ^{125}I[D-Lys^6]GnRH from pituitary membranes of pro-estrous rats was determined by adding increasing concentrations of unlabeled GnRH analogs **2, 3, 4, 5**, and **6** (Fig. 2). Membranes were incubated for 90 min at 4 ºC with ^{125}I[D-Lys^6]GnRH and with unlabeled peptides. Non-specific binding was defined as binding of the labeled ligand in the presence of 1 µM [D-Lys^6]GnRH and was extracted from the total binding for the calculation of specific maximal binding (MB). Results are the mean of an experiment carried out in duplicates.

Conclusions

The synthesis of the Freidinger lactams on solid support offers potential for performing positional scanning to identify turn secondary structures for peptidic activity. Although no other analogs were active, as predicted by precedent **1**, lactam **4** had better binding than D-Lys-GnRH peptide.

Acknowledgements

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THE STUDIES ON THE OPTIMIZATION OF THE SOLID PHASE SYNTHESIS OF PEPTIDE-DERIVED AMADORI PRODUCTS

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Introduction

The reaction of amino groups of proteins and peptides with glucose yields Amadori products considered to be the key intermediates in the formation of advanced glycation end products. Peptide-based Amadori products could be used as markers of diabetes mellitus, aging, and Alzheimer disease which makes them the subject of interest in clinical chemistry. Recent reports suggest also a significant influence of the glycation process on the function of relatively short-lived hormones and regulatory peptides [1, 2] Unfortunately, the methods of synthesis of Amadori-modified peptides reported in literature are limited to the "in solution" strategy which makes them time consuming. Previously we investigated the solid-phase synthesis of peptide-derived Amadori products based on the direct reaction of ε-amino groups of lysine with a solution of glucose in DMF at 60 - 70 °C [3] A similar method was developed by Frolov *et al*. however, these methods have several drawbacks [4]. They require high temperatures (70 °C or 110 °C) and long reaction times, while the yields are relatively low and sequence-dependent

Result and Discussion

For the synthesis of Amadori derivatives of peptides we used 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose **1** [5] This reagent can be obtained from fructose in a two-step procedure. **1** was used previously in the solution phase synthetic protocols [6, 7]. The optimization of reaction conditions was performed on the model peptide Fmoc-Lys(Mtt)-Ala-Ala-Phe attached to the Wang resin. After deprotection (1% TFA/DCM) the ε-amino group of Lys was alkylated by **1** in the presence of sodium cyanoborohydride. The reaction was performed using various concentrations of **1** and $NaBH_3CN$. After the reaction was complete Fmoc group was removed using 20% piperidine in DMF, and the reaction product was cleaved from the resin using TFA containing 5% of water.

To test whether this procedure is suitable for site-specific synthesis of peptide-derived Amadori products, we obtained two possible derivatives of the fragment of BSA, Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu glycated on the ε-amino groups in the side-chains of Lys7 and Lys9. The scheme of synthesis is presented in Fig. 1.

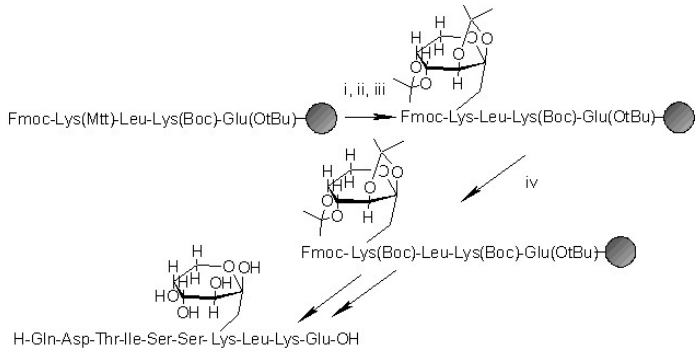

Fig. 1 Reagents and conditions: (i) 1 %TFA in DCM 10 min at rt 7×; (ii) 2 M DIEA in DMF, 10 min at rt; (iii) **1** *2 eq, $NaBH_3CN$ (5eq) in 1 % solution AcOH in DMF, 2 h at rt.; (iv) Boc_2O (20 eq), DIEA in DMF 12 h at rt.*

The identity and homogeneity of the obtained peptides were verified by HPLC and ESI-MS. The proposed solid phase procedure for the synthesis of peptide–derived Amadori products does not require high temperature and gives good yields and relatively high purities. To our best knowledge, this is the first method allowing the routine synthesis of this group of compounds.

Acknowledgements

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CONFORMATIONAL STUDIES OF ANTIMICROBIAL PROTEGRIN-1 ANALOGUES, BM-1 AND BM-2, BY USING NMR SPECTROSCOPY

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Introduction

Protegrin-1 (PG-1) is an 18-amino acid peptide with an amidated C-terminus, which forms an antiparallel β-sheet, constrained by two disulfide bridges [1, 2, 3]. The native sequence of PG-1 is highly cationic, containing six positively charged arginine residues. It was found that the structural features such as amphiphilicity, charge and shape are important for the cytolitic activity of PG-1 [1].

In this study, we investigate the Structure Activity Relationship of two PG-1 analogues: RGLCYCRGRFCVCVG-NH_2 (BM-1) and RGLCYRPRFVCVG-NH_2 (BM-2) (Fig. 1). Our antimicrobial activity studies of these peptides show that the BM-1 peptide is active against microbic species as well as the native PG-1, whereas BM-2 is completely inactive. The BM-1 analoque is shorter than the native PG-1 and contains only three arginine residues, therefore it is much cheaper in the chemical synthesis, this being advantage of this antimicrobial peptide.

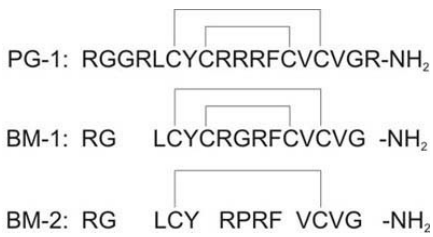

Fig. 1. The sequence of PG-1 peptide and its analogues, BM-1 and BM-2.

Results

The peptides were synthesized by solid-phase procedures using 9-fluorenylmetoxycarbonyl (Fmoc) methodology. The structures of BM-1 and BM-2 were investigated using one- and two-dimensional ^{1}H NMR spectroscopy in dimethyl sulfoxide (DMSO-d_6). All NMR data were processed and cross-peak volume calculations were performed with VNMR and XEASY [4] programs. All calculations were based on generating the peptides' structure using DYANA [5] and XPLOR [6] programs with simulated-annealing (SA) and simulated-annealing refinement algorithms. Using the TOCSY spectra the proton chemical shifts were recognised and using the NOESY spectrum the sequence of two PG-1 analogues was confirmed.

Our conformational studies show that BM-1 forms a regular β-sheet structure, which is very similar to that of the native PG-1 peptide. The BM-1 structure consists of two antiparallel β-strands (Fig. 2a). The peptide occurs as one stable isomer with all-*trans* geometry of the peptide bonds and it is the most flexible on the N- and C-terminus. The disulphide bridges are distributed in the other direction than side chains of hydrophobic amino acid residues.

The shorter analogue, BM-2, is much more flexible and forms major and minor conformations (Fig. 2b, Fig. 2c).

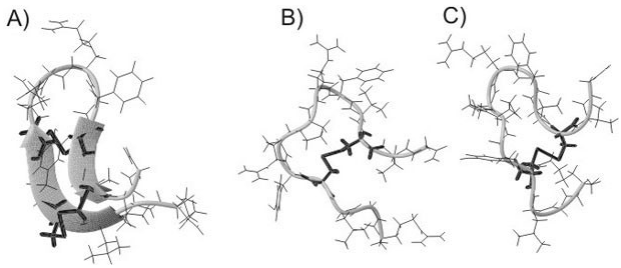

Fig. 2. Peptides' structures: a) β-sheet structure of BM-1, b) major conformation of BM-2, c) minor conformation of BM-2.

Discussion

Removal of amino acid residues from the PG-1 structure: Gly3, Arg4 and Arg18 and replacement of Arg10 for a glycine residue does not change peptide's conformation and allows to retain the peptide's biological activity as it is for the BM-1 peptide.

In comparison with BM-1, absence of two cysteine residues in the sequence of BM-2 could be the main reason for unstable conformation and the antimicrobial inactivity of the BM-2 analogue. Thus the presence of two internal disulphide bridges is an important structural feature to stabilize the β-sheet structure of the PG-1 analogues and their biological activity.

Acknowledgments

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CHARACTERIZATION OF THE EPITOPE FOR ANTI HUMAN RESPIRATORY SYNCYTIAL VIRUS F PROTEIN MONOCLONAL ANTIBODY 101F USING SYNTHETIC PEPTIDES AND RECOMBINANT F PROTEIN MUTANTS

Marian Kruszynski[1], Sheng Jiun Wu[1], Albert Schmidt[1], Eric J. Beil[1], Nicole D. Day[3], Patrick J. Branigan[3], Changbao Liu[3], Lester L. Gutshall[3], Ping Tsui[2] and Alfred M. Del Vecchio[3]

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Introduction

Human respiratory syncytial virus (HRSV) is a membrane-enveloped virus that encodes two major surface glycoproteins (G and F). Monoclonal antibodies (mAbs) that interact with RSV fusion (F) protein reduce infectivity by blocking viral and cell membrane fusion. Chimeric (ch) 101F is a potent mouse anti-RSV F neutralizing mAb with a human IgG1 Fc region that recognizes antigenic region IV/V/VI of the F protein. We measured the binding of ch101F to a series of synthetic peptides corresponding to truncations or point mutations within amino acids 422-438 of the HRSV F protein, which encompasses most sequences of antigenic site IV, V, VI. The results were complemented using ELISA to measure binding of 101F to a series of F protein mutants with single amino acid substitutions at positions 427, 429 and 433 expressed recombinantly at the cell surface.

Result and Discussion

Peptides were synthesized using a Symphony Multiple Peptide Synthesizer SMPS-110. A spacer of four ethylenoxy units (PEG_4) was inserted between the biotin and the α-amino group of peptides. Products were characterized by an analytical HPLC, capillary electrophoresis (CE) and mass spectrometry (MS). Peptides were coated on MSD ELISA plates and incubated with serial dilutions of either ch101F or humanized mAb, palivizumab (Synagis®), as a control. The binding results are shown in Fig. 1. The truncation peptide (422-436) [CTASNKNRGIIKTFS] bound to ch101F with the highest affinity and a minimum peptide sequence (423-436) was sufficient for recognition. As demonstrated, the R^{429} and K^{433} were critical for binding of 101F mAb, while another basic residue, K^{427} showed a minor contribution to the binding. The critical role of Lys433 was further confirmed by ELISA binding of ch101F to the recombinant F protein mutants with single amino acid substitutions at positions 427, 429 and 433 expressed on cell surfaces as presented in Fig. 2. These data also confirmed ch101F recognizes a distinct epitope from mAb Palivizumab (Synagis®).

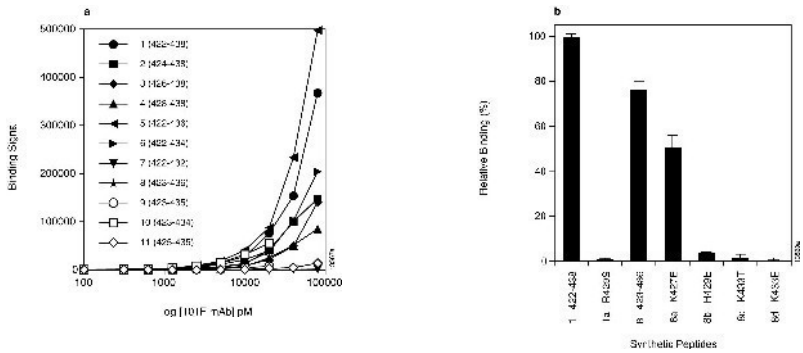

Fig. 1. a. ELISA binding of ch101F mAb to truncated peptides; b. Relative binding affinity of ch101F mAb to synthetic substitution peptides. Values are presented as binding relative to peptide 1 (422 - 438) (100%). 5 ml of 100 mg/ml for each peptide was coated in each well and 10 nM of ch101F mAb was dispensed in each well. Each data point represents the mean ± standard deviation of triplicate experiments. Binding of Synagis® for all peptides was <0.5

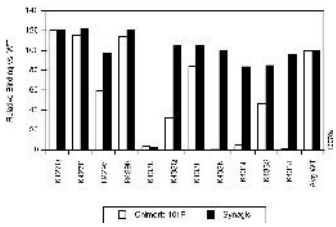

Fig 2. To determine mAb binding to native RSV F expressed on a cell surface, 293T cells in a 96-well plate were transfected with plasmids expressing either RSV F wild-type or HRSV F containing a single amino acid change. At 20 - 24 hr post-transfection, cells were fixed with 0.05% glutaraldehyde and assayed for binding of either chimeric 101F or Synagis® (Palivizumab) by ELISA. Binding activity is represented relative to wild-type RSV F protein (100%).

In summary, ELISA assays of ch101F binding showed identical specificity for the synthetic peptides and recombinant HRSV F protein mutants expressed on the surface of transfected cells. The structural basis of antigen-antibody binding may be explored by mapping and characterizing the epitopes for HRSV neutralizing antibodies. The goal is to provide a rational for the design of high affinity antibodies and peptide antagonists with potential for therapeutic use *in vivo*.

Acknowledgements

The parental HRSV neutralizing mAb 101F was generated in the laboratory of Dr. Jose A. Melero in Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Majadahonda 28220, Madrid, Spain.

NMR STRUCTURAL STUDY OF CFC CRIPTO DOMAINS

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Introduction

Cripto is a membrane protein now recognized as an important therapeutic intervention target in several widespread cancers, including breast, colon and lung carcinomas [1]. The protein displays all the features of an oncogene, being able to support survival, transformation, migration and proliferation in a large variety of cell lines; it is also highly overexpressed in many tumors, while it is poorly detectable in normal tissues. Structurally, Cripto is composed by two adjacent cysteine-rich motifs, the EGF-like and the CFC, by an N-terminal signal peptide and by a C-terminal hydrophobic region involved in cell membrane attachment. The EGF-like and the CFC motifs are small domains of about 40 residues, compacted by three internal disulfide bridges that are deemed to fulfill distinct functional roles. ELISA assays carried out with synthetic domains have shown that substitution of Trp107 with Ala in mouse CFC totally abolishes binding to Alk4, while changing His104 only partially suppresses receptor recognition [2]. Therefore, the knowledge of the CFC structure and of the structural determinants for Alk4 and Activin recognition is of primary importance for the development of new tumor suppressive agents and for further elucidation of the molecular mechanisms underlying the Cripto-dependent tumor formation and progression.

Results

The NMR analysis of Cripto CFC domains was carried out on synthetic polypeptides prepared and refolded as described [2]. A preliminary structural diagnosis was obtained by comparing the CFC and Trp107Ala-CFC alphaCH proton chemical shifts to random coil values for each residue. Most residues show positive deviations from random coil values, more marked in the central part of the molecule, suggesting the presence of extended structures. NOE contacts for cysteines indicate a C1-C4, C2-C6, C3-C5 pattern, consistent with previous studies. At pH 3, the molecular model of CFC shows an ellipsoidal compact shape with approximate dimensions of 24 Å × 20 Å × 18 Å. The molecular model of W107A-CFC also shows an ellipsoidal prolate shape, but with approximate dimensions of 30 Å ×14 Å × 16 Å. In the CFC molecular model, the side chains of His104 and Trp107 residues, involved in the binding with the receptor are exposed to the solvent and surrounded by positively charged residues above (Lys96, His98,

His119) and below (Lys110, Lys111). In addition, a large hydrophobic patch, spanning residues 122-130, which is believed to be responsible of the interaction of Cripto with the cellular membrane or to be part of the interface with the adjacent EGF-like domain, is found opposite to the binding site. At pH 6, the alphaCH chemical shift deviations from random coil values versus residue number show a trend similar to those measured for both peptides at pH 3. However, the deviations at pH 6 are very large, indicating a higher percentage of ordered conformers at pH 6 than at pH 3. The folding of both molecules is globally extended with the presence of three anti-parallel strands linked by the disulfide bridges and connected through loops larger than those observed at acid pH. The first and the central strands are more regular than the third one. NOEs indicate a higher percentage of ordered conformations at pH 6 than at pH 3, as expected, and, although the total NOE number is higher at pH 3 than at pH 6, the percentage of long-range NOEs increases at physiological conditions for both sequences (27% *vs*. 7% for CFC and 16% *vs*. 15% for the Trp107Ala variant). Moreover, the decrease of total NOE number at pH 6 is manly due to the lack of several NH resonances and to the related backbone NOE effects.

Discussion

All the determined structures for the two Cripto CFC variants show a globally extended folding and, as expected, they appear generally more structured at physiological pH values than under acidic conditions. Both the wild type and the Trp^{107}Ala mutant display quite dynamic structures characterized, on the edges, by large flexible loops restrained by the disulfide bridges and, in the central core, by mostly non canonical strands. The calculated structures for both molecules, even at acidic pH, show that Trp107 side chains are solvent exposed, and so is the His104 side chain in wild type polypeptide. In this region several positively charged residues are also gathered that could thereby contribute to stabilize protein-protein interactions. Structural data also confirm the presence of a previously predicted [3] hydrophobic patch on the molecule side that is opposite to that of the Alk4 binding site.

Acknowledgements

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SYNTHESIS OF NEW ANTIFOLATE DRUG PEPTIDE CONJUGATES

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Introduction

Antifolate drugs are inhibitors directed to interfere with folate metabolic pathway. Methotrexate (MTX) and pemetrexed (Alimta®, Pem) are known folic acid analogues used mainly in the treatment of cancer. In order to increase efficacy various peptide conjugates of MTX have been prepared for intracellular delivery [1] We have reported earlier the synthesis of octaarginine conjugates in which one of the carboxylic groups of MTX was attached to the N-terminal of the peptides [2] However, we found that the substitution of carboxylic groups resulted in no improved biological effect of MTX. Now we describe the synthesis of peptide conjugates of folic acid analogue (Pem) in which the carboxylic groups are untouched. Modified octaarginine, penetratin or cyclo(CGNKRTRGC) peptide, capable to deliver covalently attached cargo molecule into the lymphoid system [3] were used as delivery moiety.

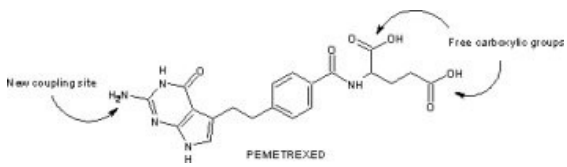


Fig. 1. Cytostatic effect of Pem and Mtx on human leukemia (HL-60) cells.

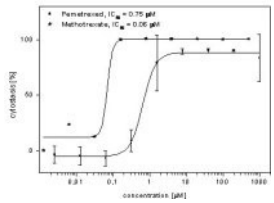
Results

For the preparation of peptide conjugates of Pem, first peptides were synthesized by solid phase peptide technique. Ac-CR8, Ac-CRQIKIWFQNRRMKWKK (Ac-Cys-penetratin) and cyclo(CH_2CO-GGNKRTRGC)-Ahx-C-NH_2, where Ahx is for 6-aminohexanoic acid, were produced by Fmoc/*t*Bu or Boc/Bzl strategy using DIC/HOBt coupling agents in DMF. In the linear form of the cyclic peptide the N-terminal Cys, present in the native sequence, was replaced by $ClCH_2CO$-Gly residue and the C-terminal was elongated with Ahx-Cys(Acm) dimer. After cyclization in 0.1 M Tris buffer, pH 8, the Acm group was removed by silver trifluoromethanesulfonate in trifluoroacetic acid containing 1% anisole. The crude products were purified by RP-HPLC and used for conjugation with Pem in solution. The carboxylic groups of Pem were protected as methyl esters and the aromatic amino group was chloroacetylated by chloroaceticacid pentachlorophenyl ester in

DMF. The conjugation was performed between the chloroacetyl group of Pem and the thiol group of peptides. The reaction was monitored by RP-HPLC. The crude product was purified by RP-HPLC and was characterized by mass spectrometry (Table 1). Cytostatic effect of Pem was evaluated on human leukemia (HL-60) cells and compared with that of methotrexate using MTT assay (Fig. 1).

Table 1. Characteristics of Pem-peptide conjugates and their components.

Compound	MS (M + H$^+$) calculated	MS (M + H$^+$) measured	R_t(min)
ClAc-Pemetrexed-methyl ester	532.9	532.1	35.9
Ac-CRRRRRRRR-NH_2	1412.7	1412.4	20.2
Ac-CRQIKIWFQNRRMKWKK-NH_2	2391.9	2391.7	31.1
cyclo(CH_2CO-GGNKRTRGC)AhxC-NH_2	1203.8	1204.3	21.4
ClAc-GGNKRTRGC-Ahx-C(Acm)-NH_2	1309.6	1310.6	21.6
Ac-C(CH_2CO-Pem(OMe)$_2$)RRRRRRRR-NH_2	1909.1	n.t.	28.0
Ac-C(CH_2CO-Pem(OMe)$_2$) RQIKIWFQNRRMKWKK-NH_2	2888.3	n.t.	33.6
cyclo(CH_2CO-GGNKRTRGC)AhxC(CH_2CO-Pem(OMe)$_2$)-NH_2	1700.2	n.t.	30.1



Discussion

Pem was coupled to peptides (R8, penetratin or modified cyclopeptide) containing Cys through thioether bound using the aromatic amino group of the molecule. The carboxylic groups of Pem were protected as methyl esters and chloroacetyl group was introduced at the aromatic amino function. Conjugation reactions were performed between the chloroacetylated and protected Pem and the thiol group of peptides. Chloroacetylated Pem was solved in 0.1 M Tris buffer, pH 8 – DMF solution. Stochiometric amount of peptides were slowly added to the Pem containing solution and the reactions were monitored by RP-HPLC. The products formed in a few hours at room temperature.

Cytostatic activity of Pem was evaluated on human leukemia (HL-60) cells and characterized by IC_{50} value. We found that Pem was active (IC_{50} < 1 μM), but less efficient than methotrexate (IC_{50} = 0.06 μM).

Acknowledgements

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FOLDING NUCLEUS OF SHEEP PRION PROTEINS

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Prion diseases are a group of fatal neurodegenerative diseases. According to the prevailing hypothesis, the misfolding of a naturally occurring prion protein (PrP) from its normal cellular form (PrPC) to a virulent scrapie form (PrPSc) is responsible for prion pathogenesis [1]. Structural studies indicate that PrPC contains a flexible N-terminal domain and a structured C-terminal domain that includes three α-helices, one two-stranded antiparallel β-sheet, and one disulfide bond [2, 3]. In contrast, the structure of the scrapie form PrPSc remains poorly understood. FTIR and CD studies suggest that PrPSc has much more β-sheet content (43%) than does PrPC (3%) [4].

PrP's sole disulfide bond may be involved in its conformational change [5, 6]. A recent kinetics study of PrP refolding identified an intermediate that may be a precursor of the PrPSc isoform [7]. We hypothesized that local structure around the PrP disulfide bond was present in this folding intermediate. To test our hypothesis, we measured the local structure in a peptide model of the protein segments flanking the disulfide bond using CD and hydrogen/deuterium exchange (HDX) monitored by MALDI-TOF. We and others have observed such structure in other peptide models of folding nuclei [8, 9]. However, no secondary structure was observed in any of our PrP peptide models at neutral pH, although other solution conditions (such as lowered pH) remain to be tested. Our results suggest that the folding intermediate must involve other segments of the PrP protein.

Results

Construction of Peptide Models

Multiple sequence alignment was performed to identify key residues around the PrP disulfide bond. Two peptide sequences were selected: YSNQNNFVHDCVN ITVKQH (residues 169-187, denoted P1), and MERVVEQMCITQYQRE (residues 206-221, denoted P2), which are involved in α-helix 2 and 3, respectively, in folded PrPC.

Determinations of Secondary Structure of Peptide Models

P1 and P2 were synthesized by SynPep Corporation, and purified by RP-HPLC. Their N- and C-termini were blocked by acetylation and amidation, respectively. MALDI-TOF was performed to confirm their identities. Because P1 and P2 each have one cysteine, they can be disulfide-bonded to form P1P1 and P2P2 homodimers and P1P2 heterodimer. We prepared these three dimeric model peptides (P1P1, P2P2, and P1P2) using air-oxidization, followed by RP-HPLC purification and MALDI-TOF identification. CD spectra of these five model peptides (P1, P2, P1P1, P2P2, and P1P2) were acquired on a Jasco J-810 CD

spectropolarimeter from 190 nm to 250 nm at 4 °C, pH 7.5. All of them exhibited random coil. HDX has also been carried out on P2 and P2P2, and we found that P2P2 didn't show any protection against HDX compared to P2, which agrees with our CD measurements.

Discussion

If local structure around the PrP disulfide bond is sufficient to form the PrP folding intermediate, the P1P2 model peptide should be able to adopt stable secondary structure, e.g., the native α-helical structure of PrPC or the unknown secondary structure of PrPSc. We have conducted secondary structure measurements on the five model peptides (P1, P2, P1P1, P2P2, and P1P2), and compared the structures in the monomeric and the dimeric peptides to determine the effect of the PrP disulfide bond on the structures of the dimeric peptides. Since our preliminary data suggest that P1P2 is unable to form stable secondary structure at pH 7.5, we conclude that local structure around the disulfide bond is not sufficient to form the PrP folding intermediate at neutral pH. More experiments will be carried out to confirm this conclusion. Furthermore, we will measure the secondary structure of our five model peptides at pH 4 - 6, because the structural conversion of PrP is believed to occur under acidic conditions [10, 11].

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A RATIONAL APPROACH TO EVALUATING PEPTIDE PURITY

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Introduction

Synthetic peptides invariably contain impurities that are analogs of the main product.

Generic analytical methods such as the ubiquitous 0.1% TFA/water/MeCN HPLC mobile phase system [1, 2] using a single, broad gradient, often fail to detect closely eluting impurities. As a result, many pharmaceutical development projects erroneously use cocktails of related peptides rather than pure substances.

This paper describes the use of a series of different buffer systems coupled with a simplified method for gradient adjustment to rapidly develop the best analytical system. The approach is demonstrated using model peptides, ACTH(1-39) and ACTH(22-27) [3] and their deamidation products.

Results

The following HPLC mobile phases were used:

System TFA: Mobile Phases: (A) 0.1% TFA in water; (B) 90% MeCN/10% water/0.09% TFA. System TEAP: (A) 0.1 M Triethylammonium Phosphate, pH 2.3; (B): MeCN. System $NaClO_4$: (A) 0.1 M $NaClO_4$, pH 2.7; (B) MeCN. System IEX: (A) 5 mM KH_2PO_4, pH 3.0 (or 7.0), 25%(v/v) MeCN; (B) 5 mM KH_2PO_4, pH 3.0 (or 7.0), 0.25M KCl, 25% (v/v) MeCN.

Reverse phase column: Supelco Discovery HS C18, 150 x 4.6 mm, 3 µm, 120Å

Ion-exchange column (IEX): Polysulfoethyl A, 200 x 4.6 mm, 5 µm, 200Å

Flow rate: 1 mL/min, detection at 220 nm.

Analytical development was started with System TFA using a reversed phase column. A broad gradient of 5 to 65% Mobile Phase B in 30 minutes at 60°C (φ = 2%/min) was run. The retention times of the main peak (t_R) and the void volume (t_0) were noted. If the main peak did not elute during the run, then the chromatogram was rerun using 35 to 95% Mobile Phase B in 30 minutes (however not System TEAP due to salt precipitation risk).

After subtracting the dwell time (about 3 minutes) and using the retention time of the main peak, the % Mobile Phase B, at which the main peak eluted (B_R), was calculated as:

$$B_R = (t_R - t_0) \times \varphi + B_0$$

where (B_0) is the initial % Mobile Phase B.

An extended gradient of 0.5% Mobile Phase B/min was used, starting at 10% below B_R and continuing to B_R +10%, over a time of 40 min.

The above procedure was repeated for each of the buffer systems and columns and each chromatogram was examined for shoulders or asymmetry around the main peak, which could be an indication of further, unresolved impurities.

Further optimization of the method depends on the development phase of the product and may involve (a) adjusting the column temperature in 10 °C increments (but not exceeding the boiling point of the mobile phase), (b) reducing the gradient to 0.25% or 0.1%/min, and (c) changing the pH of the elution buffer to 7, or close to the isoelectric point of the peptide (but not exceeding the operating specifications for the column).

The results, in terms of chromatographic resolution obtained for the model peptide mixtures are summarized in Tables I and II.

Table I. Separation of ACTH(22-27) and [Asp^{25}]-ACTH(22-27) –"easy separation"

	Buffer system	Gradient	Resolution
1a	TFA, 20°C	5 to 65% (B) in 30 min	1.2
1b	TFA 60°C	5 to 25% (B) in 40 min	5.7
1c	TEAP 60°C	1 to 21% (B) in 40 min	8.5
1d	$NaClO_4$, 60°C	5 to 25% (B) in 40 min	5.3
1e	IEX, 60°C, pH 3.0	3 to 23% (B) in 40 min	3.5

Table II. Separation of ACTH(1-39) and [Asp^{25}]-ACTH(1-39)-"difficult separation"

	Buffer system	Gradient	Resolution
2a	TFA, 20°C	5 to 65% (B) in 30 min	None
2b	TFA 60°C	27 to 47% (B) in 40 min	0.7
2c	TEAP 60°C	21 to 41% (B) in 40 min	1.2
2d	$NaClO_4$ 60°C	31 to 51% (B) in 40 min	<0.5
2e	IEX, 60°C, pH 7.0	5 to 65% (B) in 40 min	6.7

Conclusions

A single peak in the chromatogram of a peptide sample is not proof of a pure compound. Successful (baseline) separations of model peptide mixtures are demonstrated using a rapid, systematic approach to HPLC method development with reversed-phase and ion-exchange methods.

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SYNTHESIS AND ANTIBODY RECOGNITION OF CYCLIC CONSTRUCTS OF EPITOPE PEPTIDE 9-22 OF HERPES SIMPLEX VIRUS GD

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Introduction

Glycoprotein D represents a major immunogenic component of the virion envelope of herpes simplex virus and able to induce high titres of neutralizing antibodies. One of its optimal epitopes is the 9-22 region (^{9}LKMADPNRFRGKDL^{22}) of which we synthesised linear and cyclic variants [1]. Several cyclic peptides possessing thioether bond and different ring size have been prepared and some of them were conjugated with tetratuftsin derivative (Ac-$[TKPKG]_4$-NH_2) by thioether bond formation using selectively removable Cys protecting groups. Antibody binding data suggested that the size of the cycle has considerable influence on antibody recognition, however, the replacement of Met in position 11 by Nle is permitted. Conjugation of cyclic peptide might increase the antibody binding depending on the structure and/or conjugation site of the cyclic peptide. Conjugates of different carrier constructs showed increased binding capacity to A16 monoclonal antibody [2].

In this project our goal was to prepare a new group of conjugates capable of increasing the production of antibodies using tetratuftsin $[TKPKG]_4$ carrier. Conjugate containing cyclic peptide (c[CH_2CO-LKNleADPNRFRGK(Ac-CGFLG)DLAhxC]; cHSV) with the best binding capacity (7.2 pmol/100 μL) as well as the conjugate containing the linear (H-LKNleADPNRFRGKDLC-NH_2) epitope (0.7 pmol/100 μL) were selected for this study [3] To enhance the antigenicity of the tetratuftsin conjugate we also introduced a promiscuous T cell epitope peptide derived from tetanus toxoid (YSYFPSV).

Results

The peptides were prepared by solid phase peptide synthesis, with Boc/Bzl and mixed Boc/Bzl - Fmoc/*t*Bu techniques. The promiscuous epitope peptide was attached to both amino groups of lysine residue coupled to the N-terminus of the carrier (Ac-YSYFPSV-K(Ac-YSYFPSV)-$[TKPK(ClAc)G]_4$-NH_2). Cyclopeptide cHSV was obtained from its semiprotected linear precursor through thioether bond formation in a 0.1 M Tris buffer, pH 8 solution. After cyclization the Cys protecting group Acm was removed by the silver triflate method. The linear and cyclic epitope

peptides were conjugated to the carrier in a 0.1 M Tris buffer solution at pH 8 during 48 hours. The reaction was followed by analytical RP/HPLC and ESI-MS. Each reaction step was followed by semipreparative RP-HPLC purification, and the products were characterized by ESI-MS and analytical RP-HPLC. Conformation in solution was analysed by CD spectroscopy, in water, water-TFE (1:1) and TFE solvents.

Discussion

The conjugates were synthesised with a good yield (30 - 40%) and purity (>90%). The CD spectra did not show significant differencies between the conformation of the conjugated and free peptides. This suggests that attachment to the modified carrier does not alter the conformation and thus the recognition of free peptides containing the epitopes, similar to previously used carriers [4].

Acknowledgements

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COMPUTATIONAL STUDY ON CONFORMATION OF OLIGOPEPTIDES CONTAINING CHIRAL CYCLIC α,α-DISUBSTITUTED

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Introduction

Prediction of the conformation of peptides using computational simulation is an interesting challenge for the design of functionalized and bioactive peptides. We have shown the Monte Carlo conformational search using MacroModel is useful for conformational study of oligopeptides prepared from α,α-disubstituted α-amino acids. Moreover, we have studied conformational analysis of oligopeptides containing chiral α,α-disubstituted α-amino acids to predict the helical screw sense of helical structures (α-helix, 3_{10}-helix). Here we report computational study on conformation of oligopeptides containing cyclic α,α-disubstituted α-amino acids with side-chain chiral centers.

Fig. 1. Helical structures of oligopeptides

Result and Discussion

Conformational search calculations of oligopeptides 1, 2, containing chiral cyclic α,α-disubstituted amino acids, have performed using the Monte Carlo method of MacroModel (ver. 8.1, Schrödinger, Inc.). When AMBER* force field was used, the global minimum energy conformation of peptide 1 was a left-handed α-helix, which was more stable than a left-handed 3_{10}-helix by 4.2 kcal/mol. The results

were in agreement with its X-ray structure, which showed a left-handed α-helix [1] The global minimum energy conformation of peptide 2 was a right-handed 3_{10}-helix, which was more stable than a left-handed 3_{10}-helix by 1.6 kcal/mol. Same results were obtained by STO-3G level molecular orbital calculation. The difference of energies was small. There were both right- and left-handed 3_{10}-helices in the solid state [2, 3]. These results indicated computational simulation using conformational search calculations could predict the helical screw sense of oligopeptides containing chiral cyclic α,α-disubstituted amino acids.

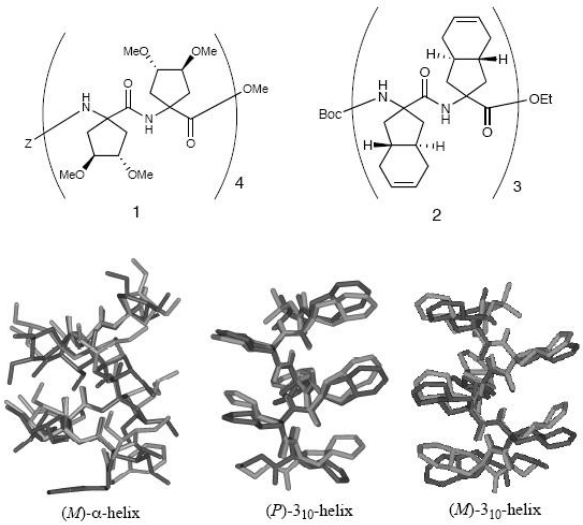

Fig. 2. Modeled structures (light) and X-ray structures (dark) of oligopeptides 1, 2.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NEW GNRH-III DERIVATIVES

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Introduction

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH or LH-RH) is a key hormone in the regulation of mammalian reproduction. GnRH might function as a modulator of the activity of diverse systems in the brain and many organs. About 50% of breast, 70% of ovarian and 80% of endometrial cancer cells express GnRH and its receptors [1]. Native GnRH and its agonists were found to inhibit the proliferation of human breast- ovarian- and endometrial cancer cell lines in a dose- and time-dependent manner. GnRH-III is a GnRH agonist isolated from the sea lamprey (*Petromyzon marinus*) and it has also an antiproliferative effect without significant activity on LH secretion, therefore it can be regarded as selective antitumour agent [2].

Human GnRH (Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and GnRH-III (Pyr-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) differ in four amino acid residues. Synthetic peptides according to both sequences were studied in this experiment.

Our aims were to design different GnRH-III derivatives in order to investigate the receptor binding and internalisation into MCF-7 human breast carcinoma cell line.

Results

For investigation of cellular uptake the 5,(6)-carboxyfluorescein (CF) labeled GnRH-III (Pyr-HWSHDWK(X-GFLGC)PG-NH_2), $[GnRH\text{-}III(C)]_2$ dimer ([Pyr-HWSHDWK(X-C)PG-NH_2]$_2$) and $[GnRH\text{-}III(CGFLG]_2$ dimer ([Pyr-HWSHDWK(X-CGFLG)PG-NH_2]$_2$) were synthesised (X means 5(6)-carboxyfluorescein). In case of dimers only one arm was labeled with CF. The synthesis of peptide derivatives were carried out by mixed Boc/Bzl and Fmoc/*t*Bu strategy on MBHA resin. The peptides were cleaved with HF in the presence of scavengers, and they were purified by RP-HPLC and characterized by ESI-MS.

The cellular uptake was studied on MCF-7 human breast carcinoma cell line. Cells were treated in serum free medium and they were incubated at 37 ^oC or 4 ^oC 3 μM; 6 μM; 12 μM solutions of GnRH-III(CF-GFLGC-NH_2).

In case of competition studies cells were incubated in different concentration of the hGnRH (14.1 μM, 70.5 μM, 141 μM, 282 μM) for 30 minutes, then GnRH-III(CF-GFLGC-NH_2) was added in 12 μM concentration and the incubation was continued for 1 or 6 hrs.

CF-[GnRH-III(C)]$_2$ and CF-[GnRH-III(CGFLG)]$_2$ dimers were added to the cells in different concentrations for 6 hours. Cells were pretreated with hGnRH and the suitable unlabeled dimers for 30 minutes in competition studies.

Cells were harvested with trypsin or they were scraped off in order to get one-cell suspension for flow cytometry. 10^4 cells were measured in a BD LSR II cytometer.

We found that GnRH derivatives were internalised by MCF-7 cells and the uptake is time and concentration dependent. There is a difference in the localization of the labeled peptide between the living and the dead cells because the living cells bind peptides mainly on their surface; in contrast to living cells, labeled peptides could be detected inside the dead cells.

Human GnRH influenced the uptake of GnRH-III(CF-GFLGC) in concentration dependent manner. We found that the dimers are taken up by MCF-7 cells more effectively than monomer and the human GnRH has no influence on the internalisation of the dimmers. In case of CF-[GnRH-III(CGFLG)$_2$] the unlabeled dimer inhibited the uptake in contrast to GnRH-III(CGFLG) dimer the GnRH-III(C) dimer had no effect on the uptake of labeled GnRH-III(C) dimer. At 4 $^\circ$C there was no any uptake.

Discusion

We can assess that the internalization of compounds was carried out by time and concentration dependent manner, however, there were differences between the cellular uptake of GnRH-III(CF-GFLGC) and the labeled GnRH-III dimers by MCF-7 cell line. 12% of dead cells taken up the monomer during 6 hours incubation. 45% of dead cells internalized the dimers in six hours. In competition studies human GnRH significantly stimulated the uptake of monomer (34% of the dead cells) and the CF-[GnRH-III(CGFLG)]$_2$ (60% of the dead cells). But hGnRH had no effect on the cellular uptake of labeled GnRH-III(C) dimer. Monomer and the dimer might have different process in the internalization. In virtue of flow cytometrical results the planned peptide structures, both monomer and the dimmers, are good models for the internalisation studies and they will be new multifunctional conjugates for the targeted tumour therapy.

Acknowledgements

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CHEMOTACTIC PEPTIDE BASED DRUG TARGETING OF METHOTREXATE

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Introduction

Recently, development in the field of drug delivery systems (DDS) facilitating more specific and efficient therapy in cancer research. Water-soluble drug conjugates, which consist of a targeting sequence and drug molecule attached with or without a carrier molecule, offer specific cellular uptake pathway by receptor mediated endocytosis. Targeting sequences can be e.g. hormone or chemotactic peptides recognized by receptors that are overexpressed on tumour cells.
In this study, the synthesis as well as the chemotactic activity of bioconjugates developed for chemotactic drug targeting (CDT) will be presented. In these conjugates, a tetratuftsin derivative ($[TKPKG]_4$) were used as carrier [1] and formyl-tripeptides or tuftsin derivatives (For-MLF, For-NleLF, TKPR, For-TKPR, TKPKG, Ac-TKPKG) as chemoattractant targeting sequences were investigated. Methotrexate (Mtx) (L-4-amino-*N*-10-methylpteroyl-glutamic acid) was applied as anticancer agent and was attached to the carrier *via* an enzyme (e.g. Catepsin B) labile pentapeptide (GFLGC) spacer. The drug-spacer conjugate was synthesised followed by conjugation with the chloroacetylated carrier through thioether bond [2].

Results

The tetratuftsin derivative was built up on MBHA resin using standard Boc chemistry. The following side chain-protected amino acids were used: Thr(Bzl), Lys(Z(2Cl)) in position 2 and Lys(Fmoc) in position 4 of the repeat units. After the synthesis of the carrier backbone, a Boc-Lys(Boc)-OH was attached to the N-terminus. The Fmoc groups were removed and the formyl peptides, as chemoattractant targeting sequences, were built up on every second Lys using standard Fmoc strategy [2]. However, Fmoc/Bzl strategy was carried out for the incorporation of tuftsin sequences. After the removal of the last Fmoc group from the N-terminus of the branches, a formyl (in case of For-MLF, For-NleLF and For-TKPR) or acetyl group (in case of Ac-TKPKG) was attached to the free α-amino groups. After completion of the synthesis of the chemotactic branches, the Boc-groups from N-terminal Lys were removed and chloroacetylation was performed. The chloroacetylated peptides were removed from the resin with liquid HF in the presence of appropriate scavengers. The crude products were purified by RP-HPLC and the pure compounds were characterized by analytical HPLC and ESI-MS.

The Mtx-containing GFLGC peptide was synthesised on Rink Amide MBHA resin using Fmoc chemistry. Trityl group was used for the side chain protection of Cys. The peptide was cleaved from the resin with TFA in the presence of scavengers. The crude products were purified by RP-HPLC and the pure compounds were characterized by analytical HPLC and ESI-MS [2].
The chloroacetylated peptides were dissolved in 0.1 M Tris buffer (pH 8.2) and Mtx-GFLGC-NH_2 was added to the solution in solid form at regular intervals and at room temperature. The conjugation reaction was monitored by analytical HPLC. At the end of the reaction the mixture was purified by RP-HPLC and the pure compounds were characterized by analytical HPLC and ESI-MS [2].
Six conjugates containing Mtx were prepared:
(Mtx-GFLGC{CH_2CO}-NH_2)-K(Mtx-GFLGC{CH_2CO}-NH_2)-[TKPK(X)G]$_4$-NH_2, where X=chemotactic peptides: For-MLF, For-NleLF, TKPKG, Ac-TKPKG, TKPR, For-TKPR.
The conjugates and their components as controls were studied in chemotaxis on THP-1 monocytes.
Cellular uptake of the compounds was studied by using fluorescent-labelled analogues. Bioimaging using confocal laser scanning microscopy (CLSM) showed accumulation of the conjugates by THP-1 monocyte cells. The labelled analogues were found in the cytoplasm of the cells.

Discussion

In case of conjugates containing tuftsin derivatives in the branches (TKPR, For-TKPR, TKPKG and Ac-TKPKG) the chemotactic activity was decreased in contrast to their components in certain cases, but all of the conjugates maintained the chemoattractant effect.
Conjugates with formyl-peptides in the branches (For-MLF and For-NleLF) conserved the chemotactic effect, moreover at micromolar concentration they proved to be extraordinary chemoattractive.
The results of the biological tests confirmed the feasibility of this new chemotactic drug targeting strategy for increasing the efficacy and specificity of cancer chemotherapy.

Acknowledgements

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INVESTIGATING MELANOCORTIN PEPTIDES CONFORMATIONS IN DIFFERENT ENVIRONMENTS

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Introduction

The melanocortin receptors are involved in many physiological functions, including pigmentation, sexual function, feeding behavior, and energy homeostasis, making them potential targets to treat obesity, sexual dysfunction, *etc* [1]. Understanding the conformational basis of the receptor-ligand interactions is crucial for the design of potent and selective ligands for these receptors.

Result and Discussion

The conformational preferences of the cyclic melanocortin agonists and antagonists MTII, SHU9119, [Pro^6]MTII, and PG911 were comprehensively investigated by solution NMR spectroscopy under different environmental conditions. In particular, water and water/DMSO (8:2) solutions were used as isotropic solutions, and a 200 mM aqueous solution of DPC (dodecylphosphocholine) was used as a membrane mimetic environment. NMR parameters indicate structural flexibility for all analyzed compounds in water and water/DMSO solutions. However, weak $d_{\alpha N(i,\ i+2)}$ NOE contact between His6 and Arg8, observed in the NOESY spectra performed at temperatures below 283 K, is in accordance with a β-turn structure previously found for MTII [2]. In DPC solution peptides spectra indicate the presence of stable structures. In particular, $^3J_{HN\text{-}H\alpha}$ coupling constants and Hα CSI (chemical shift index) values clearly point to a helical structure of the N-terminal residues (4 - 7) and extended conformation of residues 8 - 9.

Peptide	Sequence*
MTII	Ac-Nle^4-c[Asp^5-His^6-DPhe^7-Arg^8-Trp^9-Lys^{10}]-NH_2
SHU9119	Ac-Nle^4-c[Asp^5-His^6-DNal^7-Arg^8-Trp^9-Lys^{10}]-NH_2
[Pro^6]MTII	Ac-Nle^4-c[Asp^5-Pro^6-DPhe^7-Arg^8-Trp^9-Lys^{10}]-NH_2
PG911	Ac-Nle^4-c[Asp^5-Hyp^6-DNal^7-Arg^8-Trp^9-Lys^{10}]-NH_2

* Nle = Norleucine; Nal = 2'-Naphtylalanine; Hyp = γ-Hydroxyproline.

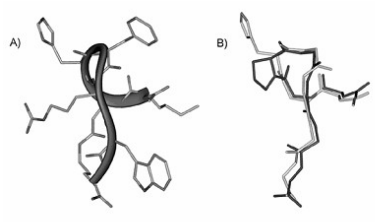

Fig. 1. Structures of [Pro^6]MTII and MTII.

NMR data obtained in DPC solution were used as input for a restrained molecular dynamic calculation. Lowest energy conformer of MTII is shown in Fig.1. Two beta-turns that involve Nle4 to D-Phe7 (distorted type VIII) and Asp5 to Arg8 (distorted type II), can be identified in MTII. Conversely, residues 8 to 10 are in an extended conformation. Peptide surface has amphipathic nature. In fact, considering the pseudo-plane defined by the backbone atoms (ribbon, Fig. 1.A) hydrophobic residues Nle4, D-Phe7 and Trp9 lie on one side (right side) while positively charged residues His6 and Arg8 lie on the other side. Structure of [Pro^6]MTII is very similar to that obtained for MTII (Fig.1 B, black and grey, respectively). Structure computation of compounds SHU9119 and PG911 is currently in progress.

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ASSAYING THE PRESENCE OF SOME HISTONE-LIKE PROTEINS (FOR EXAMPLE HU) IN HALOBACILLUS KARAJENSIS

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Introduction

Bacteria contain small, basic, abundant proteins that share some properties like amino acid sequence, electrostatic charge, low molecular weight and binding to DNA with eucaryotic histones and therefore are called histone-like proteins (HLPs) (1). The amount of HLPs associated with the Escherichia coli DNA vary during different growth phases [1]. Among the proteins associated with the DNA HU, IHF, H-NS, Fis and StpA have been mainly studied. The *Escherichia coli* HU is a small (9 kDa), basic (pI 8.57) heterodimer [1]. It introduces negative supercoiling in relaxed DNA, participates in initiation of replication, transcription and site specific recombination. The genes coding this protein are hup α and hup ß [2]. It resembles H1 and H2B in its amino acids sequence [1].

In this study we assayed the presence of some histone-like proteins specially HU in *Halobacillus karajensis* which is a gram positive, novel moderately halophilic bacteria, recently isolated from surface saline soils of Karaj region in Iran [3].

Result and Discussion

Before preparing protein extracts from *H.karajenis* and *E.coli* the growth curves for both bacteria were drawn in 24 hours at 620 nm. The protein extraction was carried out during the exponential phase where HU is most abundant.

Protein extraction by Ammonium sulfate precipitation (75 & 80%) was carried out [5]. The results were loaded on SDS PAGE 15%. After staining with comassie blue a 9.5 kDa protein with almost the same electrophoretic pattern as HU in Bacillaceae was observed (Fig 1A).

(A) (B)

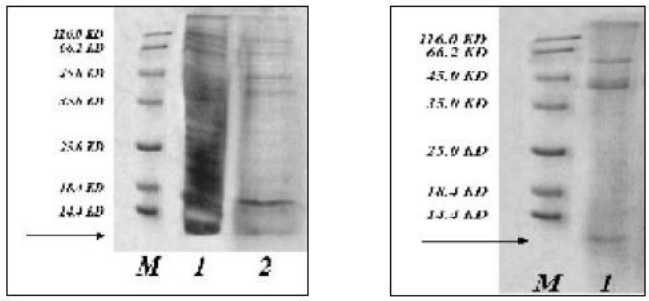

Fig 1 (A) - Protein extracts from H. karajenis obtained by ammonium sulfate percipitation 75% (1) and 80% (2). Standard protein marker is Fermentas Sm# 0431. (B) - Protein extraction from H.karajenis by Johns method (1).

Considering the fact that HU shares amino acid similarities with H1 eukaryotic histone [1], PCA 5% method which is specifically used for extracting H1 from mammalian cells was used for protein extraction from *Halobacillus karajensis* [4]. More over since HU protein has been completely studied in *E.coli* it was selected as a positive control which surely has the histone-like protein HU. Afterwards the protein extracts were loaded on SDS PAGE 15% and stained with commasie blue. The electrophoretic pattern was similar to that of HU in and the presence of a 9.5 kDa protein was estimated by drawing the protein standard curve for the gel (Fig. 1B).

Acknowledgments

We thank IBB biochemical lab for providing anti H1° antibody.

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STRUCTURAL STUDIES OF GLUTEN - PEPTIDE ANALOGS

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Introduction

Tissue transglutaminase (TG2) is an enzyme that plays a key role in the pathogenesis of the celiac disease. TG2 is the main autoantigen recognized by the anti-endomysium antibodies and, furthermore, catalyzes the deamidation of strategic glutamine to glutamic acid within the sequence of immunodominant gliadin epitopes [1] Recently, another unexpected role for surface TG2, in the innate immune response in the celiac disease, has been suggested [2] It follows that TG2 inhibitors might represent a potential attractive pharmacological alternative to the gluten-free diet that, nowadays, is the only possible therapy for celiac patients [3]. Starting from the sequence of the heptapeptide PQPQLPY, known to be a high affinity substrate of human TG2, we have synthesized new analogs replacing Pro3 with different constrained amino acids (D-Pro, Pip, Chg, Ind, Deg, Inp, Hyp, Thz) with the aim to develop specific inhibitors of TG2. Actually, proline residues present in the gluten epitopes are important in determining the immunogenicity of the epitopes and the specificity of TG [2, 3].

Herein, we describe the preliminary conformational studies of the synthesized analogs by NMR spectroscopy and molecular modeling methods.

Results and Discussion

To evaluate, preliminarily, the activity of TG2 on synthesized peptides, proton spectra in absence and in presence of TG2 were acquired. To perform protonic spectra in presence of TG2 a phosphate buffer solution of peptide containing $CaCl_2$ (5 mM) and TG2 (0.08 U) was used. The disappearance of the peak corresponding to the amide protons of the side chain of Gln4, due to the catalytic activity of TG2, was checked in the time.

Interestingly, the proton spectrum of [Thz]-peptide doesn't show any modification in the glutamine resonances region even after two weeks from the addition of TG2.

TOCSY and ROESY spectra of [Thz]-peptide were performed in water and HFA/water 50/50 v:v solution, on a Varian INOVA 700 at 298 K. Complete assignments of the proton resonances were obtained by standard procedures using SPARKY software package [4].

ROESY spectra in two different environments don't show significant differences in the number of diagnostic NOE effects so the following conformational analysis was based on NMR data in water.

Three-dimensional structures were calculated by simulated annealing in torsion angle space, and restrained molecular dynamics methods based on NOE restraints, using DYANA software package. Among 200 calculated structures, the 50 ones with the lowest value of target function were subjected to further minimization procedure by Discover module of InsightII [Accel].

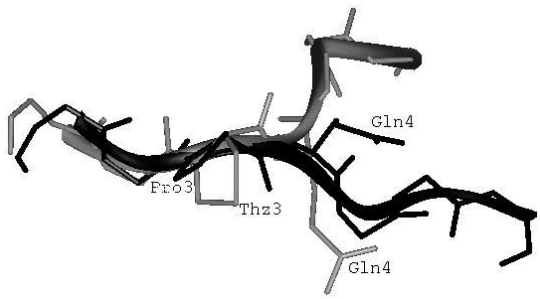

Fig. 1. Shows the superposition of the mean structures of D-Pro- (black) and Thz- (grey) peptides, based on NOE restraints in water. D-Pro was chosen as representative of peptides whose proton spectrum is modified in the presence of TG2. It is noteworthy that the side chains of residues in position 3 and in position 4 exhibit different orientation in two peptides. This condition could explain the different behaviour of [Thz]-peptide that doesn't react in presence of TG2.

Given the role of the domain PQPQLPY in the gliadin proteins, structural analysis on its analogs are of considerable interest. The results of our studies might be useful to clarify the role of the proline residues in the interaction of the gluten epitopes with TG2 and, consequently, to gain new insight in the molecular mechanism of celiac disease.

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DRUG DELIVERY BASED ON GNRH-III AS TARGETING MOIETY

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Introduction

Chemotherapy is still one of the primary modalities for treatment of cancer. However, the application of free chemotherapeutic agents has some drawbacks e.g. the lack of selectivity, the fast elimination from the blood circulation and toxic side effects resulting from high doses necessary for efficacy. The use of chemotherapy is also restricted by acquired or intrinsic multidrug resistance (MDR) of cancer cells. To overcome these disadvantages chemotherapeutic agents can be attached to carrier or targeting molecules. The higher selectivity of the conjugates might be based on the increased endocytotic properties of tumour cells or/and the presence of tumour specific or overexpressed receptors on the plasma membrane of the cancer cells. GnRH receptors are expressed on tumours in higher concentrations than on most normal cell types. Recently, several drug delivery systems based on GnRH derivatives as targeting moiety were developed to minimize the uptake of the drug by normal cells and enhance their influx in cancer cells [1, 2]. GnRH-III isolated from sea lamprey (Petromyzon marinus) has direct antitumour activity on many type of tumour cells, but shows significanly less potency on LH and FSH releasing compared to human GnRH (hGnRH) [3]. The preparation of GnRH-III derivatives available for drug targeting and the investigation of the antiproliferative effect of the compounds were the major goals of the present studies. Prior to the attachment of drug molecules to the targeting sequences, 5(6)-carboxyfluorescein (CF) labeled GnRH-III derivatives were prepared. Cellular uptake by MCF-7 (human breast) and HT-29 (human colon) cancer cells was investigated in recent experiments.

Results

GnRH-III (GlpHWSHDWKPG-NH_2) (1), its dimer derivatives and truncated versions were prepared and labeled with CF for cellular uptake studies. The linear peptides were synthesised on MBHA resin by mixed Boc/Fmoc chemistry that allowed the incorporation of a branch on the side chain of Lys8, that can be modified without the loss of biological activity [4, 5]. Dimer derivatives of GnRH-III were prepared in solution (0.1 M Tris buffer; pH 8.0) via disulfide bond formation using air oxidation ([GlpHWSHDWK(H-C)PG-NH_2]$_2$ (2) and [GlpHWSHDWK(Ac-C)PG-NH_2]$_2$ (3)). CF was attached directly to the peptides by the application of its pentachlorophenyl ester derivative ([GlpHWSHDWK(X-C)PG-NH_2]$_2$ (5) and [GlpHWSHDWK(X-CGFLG)PG-NH_2]$_2$ (6) or GlpHWSHDWK(CF)PG-NH_2 (7) and truncated versions Ac-HWSHDWK(CF)PG-NH_2 (8), Ac-WSHDWK(CF)PG-NH_2 (9), Ac-SHDWK(CF)PG-NH_2 (10)). In case of the dimer derivatives only one arm of the dimer peptides was substituted by CF. Cys containing spacer (CF-GFLGC-NH_2) was conjugated to

the chloroacetylated GnRH-III (GlpHWSHDWK(ClAc)PG-NH_2) via thioether linkage resulting in peptide GlpHWSHDWK(CF-GFLGC)PG-NH_2 (4). The compounds were characterized by RP-HPLC and ESI-MS.
Antiproliferative effect of GnRH-III derivatives was determined on MCF-7 and HT-29 cancer cell lines. According to Western blott analysis both cell lines contain GnRH-I receptors. The dimers (2, 3) showed increased antiproliferative effect on both cell lines(40% and 45% on MCF-7 and 30% and 50% on HT-29, respectively at 50μM peptide concentration) than GnRH-III (18% and 19%). The dimer derivative (3) had also lower potency on LH-releasing compared to the GnRH-III (136 ± 12 *vs*. 405 ± 19, respectively at 1μM peptide concentration). The cellular uptake of the CF-labeled GnRH-III and its derivatives was studied by flow cytometry. Data suggested that the dimer derivatives (5, 6) were taken up by MCF-7 cells faster (3 hrs) and in a higher amount according to the fluorescent intensity than the monomer derivative (6 hrs) (4). However, the cellular uptake of the compounds was carried out in a time and concentration dependent manner in all cases. We also observed that the preincubation of MCF-7 cells with human GnRH increased the uptake of the GnRH-III(CF-GFLGC), while the preincubation of cells with the entire dimer derivative decreased the cellular uptake of the dimer. We could also detected that the truncated peptides were taken up by HT-29 cells similarly to GnRH-III (7 - 10).

Discussion

The results of the biological studies indicate that dimer derivatives of GnRH-III have increased antiproliferative activity and less LH-releasing potency. Next to this enhanced tumour selectivity, the dimers might be better candidates for drug delivery, because of their faster and higher cellular uptake by MCF-7 cells. The N-terminal truncation of GnRH-III did not decrease the cellular uptake of the compounds. This observation indicate, that the enzymatic degradation of the compounds might result in derivatives which are still available for drug targeting. The antiproliferative activity of the truncated compounds will be further studied.

Acknowledgements

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SYNTHESIS AND STUDIES OF A POLY-HISTIDINE SPECIFIC CYSTEINE BASED FLUORESCENT LABEL.

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Introduction

Site specific labeling of biomacromolecules with different probes such as spin labels or fluorophores enables application of sensitive spectroscopic methods for the study of their structure, function and localization. The major limitations of many currently available labels are low yield of the labeling process, irreversibility of the tagging and low selectivity, which becomes a particular bottleneck in the case of studies conducted *in vivo*. Multivalent transition metal complexes with ligands like nitrilotriacetic acid (NTA) are very well suited candidates as new labels. Transition metal ions bind relatively tightly and specifically to cysteine and/or histidine [1, 2], amino acids that occur rather rarely in protein structure, thus providing selectivity of the tag. Moreover, coordinate interactions are in most cases completely reversible upon addition of competing ligand. Finally, simple chemistry of many multivalent chelators enables synthesis of desired tags bearing many different functionalities. We have designed a new fluorescent, selective and reversible affinity probe showing high affinity to poly-histidine tagged proteins. Amino acid-based affinity labels in current use exploit trifunctional compounds like aspartic or glutamic acid and lysine [3, 4]. These linkages result in a relatively long and flexible spacer between the tag and the protein backbone. We have used cysteine as an amino acid component to shorten the spacer; bimane was chosen as a fluorophore that can be cross-linked to two cysteine derivatives [5].

Results

The new dye was synthesized using simple and established chemistry [6] with some minor modifications. Modification of the α-amino group of cysteine yielded the NTA "head", and reaction of the side chain thiol group with dibromobimane provided a new small, neutral, water-soluble fluorescent reagent, BM-[Cys$(NTA)_2$]. The dye shows fluorescence quenching upon addition of nickel ions similar to that observed for analogous fluorescein and lysine-based tags [3], but the extent of quenching is smaller and enables application of the compound as a fluorophore. Steady state fluorescence and anisotropy measurements confirmed binding of the dye to purified proteins carrying hexa- or deca-histidine tags with affinity in the micromolar range (for 6-His-tag K_d = 40 μM, whereas for 10-His-tag K_d = 3.6 μM). The dissociation rate is slow since the dye is not competed off by binding of the labeled protein to Ni-NTA immobilized on agarose beads. The long wavelength emission maximum of the dye (λ_{ex} = 385, nm λ_{em} = 475 nm) makes it a suitable

FRET donor for fluorescein-based fluorophores. Double labeling of cellular retinoic acid binding protein (CRABP I) with the BM-[Cys(NTA)]$_2$ and the fluorescein-derived bi-arsenical dye FlAsH [7] indeed led to intramolecular energy transfer. Additionally the dye can be also used for fast detection of the His-tagged proteins on blots with sensitivity up to 150 ng of purified protein. Labeling selectivity towards His-tagged proteins was also observed in the whole cell lysates. Our preliminary attempts have confirmed that the dye is capable of entering mammalian cells (HeLa cell line) thus it can be also used for *in vivo* visualization of His-tagged proteins.

Discussion

Application of cysteine as a template for the synthesis of a small molecule affinity dye yielded a new, water soluble compound, capable of selective binding of poly-histidine stretches with affinity in the micromolar range. The dye can be used as a donor in fluorescence-based experiments, as well as an economical dye for pre-screening of protein blots. The greatest advantages of the compound are its water solubility, small size, simple design strategy and low cost of preparation. The utility of the dye was confirmed in vitro, and the observed cell internalization opens the possibility of its application in protein studies conducted *in vivo*.

Acknowledgements

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PEPTIDE BASED RADIOPHARMACEUTICALS FOR IMAGING OF TWO DIFFERENT RECEPTORS

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Introduction

G-protein coupled receptors (GPCR) with high affinity for peptides are known to be overexpressed concomitantly on membrane of tumour cells and are thus interesting targets for tumour diagnosis and therapy. One of the challenges in nuclear medicine will be to take advantage of the peptide receptors co-expression for multireceptor tumour targeting [1]. A prerequisite for development of multireceptor tumour targeting *in vivo* is, however, the availability of opportune radioisotope coordinated by ligands able to bind bioactive molecules. Bifunctional chelating agents (BFCAs) able to give *in vivo* stable complexes of ReO^{3+} and TcO^{3+} were designed and characterized. Our goal was the design and synthesis of new scaffolds containing a radioactive metal complex conjugates to two peptides able to recognize two receptors at the same time. The 3 + 1 mixed ligand approach, based on the simultaneous action of a dianionic tridentate ligand and of a monodentate thiol (co-ligand) on metal precursor, offers easy and rational access to neutral metal complexes [3].

Results and Discussion

The peptides selected to design the new radioactive multireceptor ligand were CCK8 and vapreotide (RC160). The first is able to recognize CCKA/CCKB receptors and the second is a somatostatin analogue which bind SSTR2/SSTR5. Both these receptors are overexpressed in many tumours.

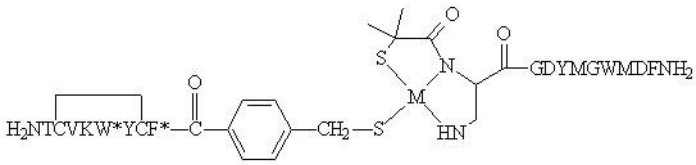
Molecular modelling studies indicate that the CCK8 peptide adopts the right conformation for cholecystokinin receptor binding, and that modifications on its N-terminal end, obtained by introducing chelating agents or its metal complexes, should not affect the interaction with CCK receptors [4]. In the same manner, the chelating agent on the N-terminal of the vapreotide, somatostatin analog, does not affect the interaction of the peptide with SSTR2 and SSTR5 [5].

Labelling with the ReO^{3+} and TcO^{3+} moiety requires ligands able to fill four coordination position in order to obtain *in vivo* stable complexes. In our approach a tridentate chelating agent coordinates the metal by a thiolate sulphur and two amide nitrogens (SNN) while the monodentate ligand uses the thiolate sulphur (S). The preparation of complexes requires the simultaneous action of the dianionic tridentate ligand and of the monoanionic monodentate thiolate on a suitable metal precursor. The tridentate ligand peptide conjugate was built up on the N-terminus of

CCK8 on the solid phase following Fmoc strategy, and coupling Fmoc-Dap(Mtt)-OH and 2-methyl-2-thio *p*-methoxybenzyl propanoic acid.

The peptide conjugate monodentate ligand is obtained by coupling the *p*-thiomethyl-phenyl succinamide on the N-terminal of the cyclic vapreotide (somatostatin analog) on solid phase. Both ligands were purified by HPLC chromatography and characterized by NMR and ESI-MS.

Different protocols were developed to obtain the Rhenium and Technetium complexes: the most promising need the simultaneous addition of the tridentate and monodentate ligands to the metal precursor. The Rhenium complex was characterized by ^{1}HNMR, ESI-MS, IR. Its *in vivo* application may be extremely attractive as a means to improve the efficacy of peptide targeting in tumours; the concomitant application of multiple radioligands will selectively increase the radioactivity accumulation in tumours, an advantage not only for diagnostic but specially for radiotherapeutic pourposes. The multi-target probes can play a crucial role in this task giving the possibility to bind more receptors with one molecule.



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SYNTHESIS OF A SPIROBENZAZEPINONE AS A β–TURN MIMIC IN BRADYKININ

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Introduction

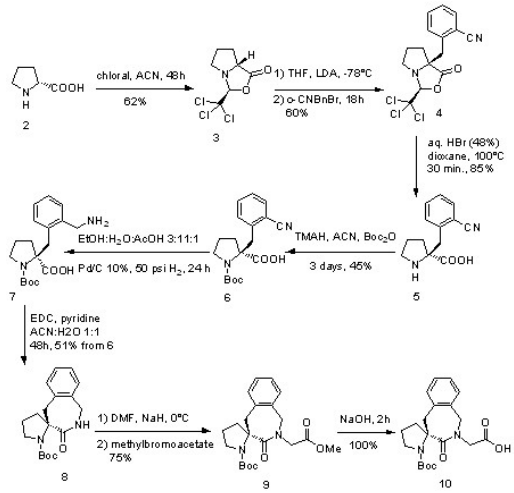
Turn structures are important recognition elements of peptides and proteins. Structures that mimic or induce turns are attractive from a medicinal point of view. Spirobenzazepinone 10 (Scheme 1) can be considered as a conformationally constrained analog of the Pro-Phe dipeptide. Such spirolactams, when incorporated into bioactive peptides, are shown to induce a β-turn conformation [1] The high affinity of bradykinin analogs for the B2 receptor has been related to their propensity to adopt a C-terminal β-turn conformation. Amblard *et al.* substituted the D-Tic-Oic dipeptide sequence in HOE140 by the benzothiazepinone template D-BT to obtain potent B2 agonists [2].

In analogy with the results we now report the result of this substitution by spirobenzazepinone **10** and the its enantioselective synthesis starting from D-Pro.

Result and Discussion

Starting from D-proline **2** and chloral, oxazolidinone **3** was prepared with 62% yield [3] The next reaction involves an alkylation with *o*-cyanobenzylbromide. The oxazolidinone **4** was then cleaved using aqueous HBr in dioxane at 100 °C. For the Boc-protection of **5**, tetramethyl ammonium hydroxide and Boc anhydride were used. The yield of 45% corresponds to literature results for similar compounds [4].

Then the cyano group was hydrogenated in order to obtain the aminomethyl compound **7** which underwent an intramolecular coupling reaction using EDC. The overall yield from **6** to **8** is 51%. The resulting benzazepinone ring could then be *N*-alkylated using NaH and methyl bromoacetate. Finally, methyl ester **9** was converted to the corresponding acid **10** by a saponification reaction with NaOH.



*Scheme 1. Enantioselective synthesis of **10**.*

In order to determine the enantiomeric excess of **5**, it was derivatized with (S)-NIFE and an HPLC-analysis of the resulting diastereomers was carried out [5]. The racemic mixture and enantiopure compound **5** were both derivatized and after comparison of the HPLC-spectra, the ee was determined. A value of 99% was obtained with a detection limit of 1%.

Racemic **10** was incorporated into the HOE140 sequence by SPPS. The resulting diastereomeric peptides were separated by preparative HPLC.

Binding affinities for the bradykinin B2 receptor were determined for these derivatives and were 25 nM (K_i(A)) and 3.2 nM (K_i(B)). Both compounds were B2 antagonists in the guinea pig smooth muscle contractility assay. The absolute configuration of the most potent analog was determined as (*R*) by incorporation of enantiomeric pure **10** and subsequent HPLC analysis.

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TOTAL CHEMICAL SYNTHESIS OF INSULIN VIA BIOMIMETIC DISULFIDE PAIRING AND FOLDING

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Introduction

Insulin is a peptide hormone used in treatment of diabetes. Several insulin analogues have recently been introduced clinically for improved treatment of diabetes. Industrial production of such insulins are based on microbial expression systems, which are highly efficient, but generally limited to the 20 proteogenic amino acids. Also, some sequences form inclusion bodies or fail to express.

Insulin consists of an A-chain with one intramolecular disulfide bond and a B-chain. These two peptide chains are connected by two intermolecular disulfide bonds. Total chemical synthesis of insulin in research scale was a landmark achievement in peptide science. However, the most common methods rely on recombination of A- and B-chains under "random" folding [1 - 3] and pairing of the three disulfide bridges or by an orthogonal protection scheme pairing the six cysteines [4]. These folding and pairing steps are difficult and low yielding. A general approach using a removable auxiliary which can direct correct formation of disulfide bridges is highly desirable.

In the pancreas as well as in microbial expression systems, insulin and insulin analogues are prepared and folded as single chain precursors, with a C-peptide connecting the A- and B-chains. The C-peptide helps direct the orientation of the A and the B chains in obtaining the correct disulfide pairing and overall peptide folding. Physiological proinsulin has a 35-mer C-peptide, however a shorter C-peptide has been introduced and used in microbial expression systems [5, 6]. Our goal was to chemically synthesize a linear sequence of this 53 amino acid precursor, fold in vitro and then enzymatically remove the C-peptide to obtain DesB30 insulin (Fig. 1).

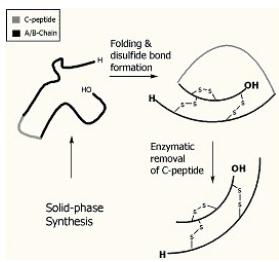

Fig. 1. SPPS of linear MI3, followed by disulfide pairing and folding. The shorter C-peptide was removed enzymatically to give DesB30 insulin

Results and Discussion

The shorter C-peptide consists of three amino acids (KAA) and is enzymatically removed by *Achromobacter Lyticus* Protease (ALP) [7]. ALP cleaves after the two lysine residue thereby producing DesB30 insulin. The linear synthesis of mini-proinsulin (MI3, H-B-chain-AAK-A-chain-OH) was performed by Fmoc solid-phase peptide synthesis. The peptide was synthesised through the side chain of A21N. The final cleavage mixture was not soluble in neutral buffer but fully dissolved in a 6 M GnHCl phosphate buffer at pH 7.5 with excess amounts of a reducing thiol. The final peptide could not be characterised in reduced form. However MI3 was identified and isolated upon folding of the crude product by dialysis against a TRIS-buffer, pH 8.5, containing cysteine/cystine (8:1). Following purification MI3 was enzymatically cleaved to give DesB30 insulin.

This method opens a new route to insulin variants with abiotic building blocks for use as molecular probes or potential therapeutic agents.

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MAPPING OF SIGNAL SEQUENCE BINDING BY THE PROKARYOTIC SIGNAL RECOGNITION PARTICLE USING A CROSS-LINKING APPROACH

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Introduction

Signal sequences target proteins for secretion or for integration into the cytoplasmic membrane. Interestingly, signal sequences share general properties but not primary structure; their features include a charged N-terminal 'n' region, a hydrophobic core of 8 to 20 residues (the 'h' region), and a less hydrophobic 'c' region of 6 to 10 residues [1]. In eukaryotes, signal sequences are recognized by the 54 kDa subunit of the signal recognition particle (SRP), a complex of six proteins and a 7S RNA. E. coli SRP, termed Ffh, consists of a protein that is highly homologous to the 54 kDa subunit of mammalian SRP and a 4.5S RNA [2]. Both Ffh and SRP54 are made up of two domains: the N-terminal NG domain, and the C-terminal M domain. Initial cross-linking results led to a model in which the signal sequence is bound by the M domain [3]; however, in previous work from our laboratory [4], the G domain was implicated in direct binding of the signal sequence. Here, we report further examination of the fascinating puzzle of signal sequence recognition.

Results

To map the signal peptide binding site on *E. coli* SRP we applied two cross-linking methods that have differing chemistries. In both cases, we utilized a modified form of the signal peptide from alkaline phosphatase, MKQKKKLALLLLLLLLTPV TKAC-NH_2, which was biotinylated at the N-terminus to enable monitoring of cross-linked products by enhanced chemiluminescence (ECL). In the first cross-linking method, PICUP (photo-induced crosslinking of unmodified proteins) [5] a direct covalent bond is created between amino acids side chains, and the reactive side chains must include a residue from which an electron can be extracted to form a radical, and a nucleophile. In the second method, benzophenone serves as a reactive photoprobe [6]. Benzophenone was introduced at positions 2, 10 - 14 and 23 of the signal peptide by modification of a cysteine residue with 4-(maleimido)benzophenone, which creates a linker with length of ca. 12 Å (the MBP series) or by using 4-(benzoyl)phenylalanine, creating a ca. 6 Å long linker (the BP series). Identification of the domain to which signal peptide cross-linked was achieved by enzymatic of chemical cleavage of the cross-linking products. In PICUP cross-linking, all peptides cross-linked to the NG-domain. Positional variation in the efficiency of cross-linking can be associated with different

accessibility or orientation of the particular Cys towards reactive groups on the Ffh surface.

In benzophenone-mediated cross-linking, however, signal peptides cross-linked predominantly to the M-domain, regardless of the probe position or linker length. Cross-linking efficiency for both MBP and BP series varied significantly with the position of the photoprobe: C12>C13=C11>C10>C14; roughly consistent with a helical conformation of the SRP-bound signal peptide.

When the isolated NG and M domains were subjected to PICUP cross-linking in the presence of C12-Cys MBP signal peptide, followed by the irradiation of the sample with UV light to trigger benzophenone cross-linking, a particle with molecular weight appropriate for cross-linked intact Ffh molecule (ca. 55 kD) was observed. Based on its mass, its reactivity with anti-His-tag antibody and its detection by ECL, this particle contains the NG-domain, the M-domain, and the signal peptide.

Discussion

Our results are consistent with a model in which SRP binds signal peptides in the interdomain space between NG- and M-domains. The NG-domain may serves as a primary binding site via interactions with the central, hydrophobic domain and the polar ends of the signal peptide. Binding to the M-domain appears to be mediated largely by hydrophobic interactions. A similar pattern of M domain cross-linking efficiency for MBP and BP series peptides provocatively suggests malleability of the binding pocket, as needed for the binding of a wide array of signal peptides of varying sequence but conserved hydrophobic character. Our hypothesized interdomain cross-linking mode was supported by double cross-linking experiments with separated domains. Further experiments to explore the binding pocket in greater detail are in progress.

Acknowledgements

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β–HOMO–AMINO ACID SCAN OF ANGIOTENSIN IV

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Introduction

Angiotensin IV (Ang IV, H-Val-Tyr-Ile-His-Pro-Phe-OH) is a biological active peptide fragment of Ang II. It improves memory acquisition and has vascular and renal actions1. It was proposed that Ang IV may exert its effects by binding to AT4 receptors2. Those were recently identified as cystinyl aminopeptidase (EC3.4.11.3, CAP, also denoted as IRAP), a membrane-associated zinc-dependent metallopeptidase of the M1 family. Since the aminopeptidase N (EC3.4.11.2, AP-N, M1) activity is also susceptible to inhibition by Ang IV, it might represent an alternative target for Ang IV. It is still not clear how Ang IV exerts its effects, through inhibition of the activity of these enzyme or through activating their receptor function 3. The incorporation of β–amino acids has been successful in creating peptidomimetics that not only have potent biological activity, but are also resistant to proteolysis. In this study the amino acids of Ang IV were respectively replaced by both β^2- and β^3-homo-amino acids.

Results

Synthesis of all peptides was carried out by SPPS using (Boc) or (Fmoc) N-terminal protected amino acids, on Merrifield (0.57 mmol/g), Wang (0,76 mmol/g) and 2-chlorotrityl chloride resin (1.5 mmol/g) resin. β^2-AA's were used as racemic mixture and after synthesis two diastereoisomeric peptides were obtained ("a" or "b" or mixture "ab") after HPLC purification.

Enzyme assays:

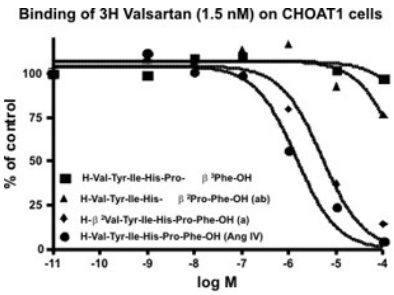
Catalytic activity was measured in membrane homogenates of HEK293 cells transient transfected with human CAP or AP-N in the presence of different concentrations of compound.

Sequence	pKi IRAP	pKi APN
H-β³Val-Tyr-Ile-His-Pro-Phe-OH	5,25	
H-Val-β³Tyr-Ile-His-Pro-Phe-OH	6,74	5,31
H-Val-Tyr-β³Ile-His-Pro-Phe-OH	6,81	5,02
H-Val-Tyr-Ile-His-β³Pro-Phe-OH (a)	6,97	5,38
H-Val-Tyr-Ile-His-β³Pro-Phe-OH (b)	7,11	5,59
H-Val-Tyr-Ile-His-Pro-β³Phe-OH	7,68	5,53

Sequence	pKi IRAP	pKi APN
H-β³Val-Tyr-Ile-His-Pro-Phe-OH (a)	7,58	5,36
H-β³Val-Tyr-Ile-His-Pro-Phe-OH (ab)	7,41	5,33
H-Val-β³Tyr-Ile-His-Pro-Phe-OH (ab)	5,97	4,99
H-Val-Tyr-β³Leu-His-Pro-Phe-OH (a)	6,70	5,23
H-Val-Tyr-β³Leu-His-Pro-Phe-OH (ab)	6,75	5,84
H-Val-Tyr-Ile-His-β³Pro-Phe-OH (ab)	7,21	5,40
H-Val-Tyr-Ile-His-Pro-β³Phe-OH (ab)	6,95	5,51

Stability experiments:

Stability experiments were performed in membrane homogenates of CHO-K1 cells which contain endogenous CAP.

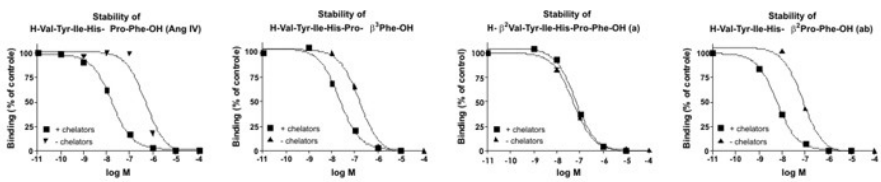


Sequence	EC_{50}
H-VYIHP β^3F-OH	$9{,}3*10^{-4}$
H-VYIH β^2PF-OH	$2{,}3*10^{-4}$
H-β^2VYIHPF-OH	$5{,}1*10^{-6}$
H-VYIHPF-OH	$1{,}1*10^{-6}$

Only H-β^3VYIHPF-OH shown no degradation in a presence of enzyme inhibitiors.

Binding to the AT1 receptor:

Affinity of the compounds to the AT1 receptor was tested on intact CHO-AT1 cells. Cells were incubated with different concentrations of compound and ^{3}H Valsartan (1.5 nM) for 40 min at 37 ºC.



Conclusions

At least 3 peptides are IRAP selective (100-fold more than APN) and more selective than Ang IV (EC_{50} for Ang IV: IRAP – 7.25, APN – 6.08). H-β^2VYIHPF-OH is more stable than Ang IV. H-β^2VYIHPF-OH and H-VYIHβ^2PF-OH are AT4 selective, they do not bind to AT1 receptor

Acknowledgements

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^{99m}Tc LABELING OF CCK8 PEPTIDES BY USING AMINODIPHOSPHINE COLIGANDS: IN VITRO AND IN VIVO EVALUATION FOR CHOLECYSTOKININ-B RECEPTOR IMAGING

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Introduction

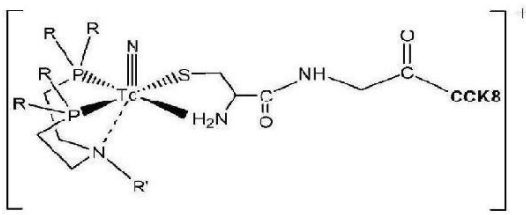
Radiolabeled compounds based on peptides, able to recognize protein membrane receptor, play an important role in diagnosis and therapy of neoplastic diseases [1]. The cholecystokinin receptors (CCKA-R and CCKB-R) are overexpressed in many malignant tumours. In the last years receptor interaction with the cholecystokinin peptide conjugates, modified on N terminal sequence with chelating group to coordinate several radioactive metals, were chemically and biologically investigated [2]. All conjugates save their affinity with receptor, but the stability and the biodistribution *in vivo* is highly dependent on the chelating moiety. These results stimulate to improve the inertness of metal complexes and the biodistribution properties. Herein we describe the preparation and the biological characterization of the ^{99m}Tc(N)(NS-Cys-Gly-CCK8)(PNP3)$^{+}$ peptide conjugate complex. In addition, *in vivo* receptor targeting and biodistribution were performed in xenograft-bearing nude mice.

Result and Discussion

Nonsulfated octapeptide CCK8 was selected as target vector for CCKB-R targeting . A glycine residue as spacer and a cysteine able as bidentate chelating group metal centre were coupled by amide bond on N terminal moiety. The choice of cysteine position on peptide skeleton sequence is based on previous results of the molecular modelling studies (quantum mechanics and molecular mechanics). Therefore the amino acid sequence of the Cys-Gly-CCK8 peptide, with the amidated C-terminus as in the natural peptide, was: Cys-Gly-D^{26}Y^{27}M^{28}G^{29}W^{30}M^{31}D^{32}F^{33}NH$_2$, according to the numbering of cholecystokinin-33. The peptide was obtained by solid-phase peptide synthesis, performed under standard conditions using 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The compound was purified by HPLC and the identity confirmed by mass spectroscopy (MALDI TOF). The Cys intermolecular bridge was not detected. The preparation of the asymmetrical complex was conduced in hydroalcholic solution following two different ways. A two-steps procedure (method 1) involved the preliminary production of a mixture of

^{99m}Tc-nitrido precursors, all containing the $[Tc{\equiv}N]^{2+}$ core, through the reduction of pertechnetate with tin(II) chloride in the presence of SDH as donor of the nitrido nitrogen atom. In the second step, the diphosphine and the bidentate NS-Cys-Gly-CCK8 ligand were simultaneously added to the reaction vial to afford the final monocationic compound in high yield. Alternatively, a one step procedure, method 2, can be used to obtain the final asymmetrical compound in the same radiochemical yield. The compound structure is reported in Fig. The PNP3 ligand satured three position and the cystein moiety fill two coordination positions.

The radiolabeling efficiency and purity of the complexes were evaluated by TLC and HPLC chromatography: efficiency and purity were higher than 90%. The resulting ^{99m}Tc(N) mixed compound has been evaluated with regard to the following criteria: a) ease of radiosynthesis and labeling efficiency b) stability toward transchelation with Cys and GSH, and c) stability toward degradation with human serum, mouse serum and mouse liver homogenates. ^{99m}Tc(N)(NS-Cys-Gly-CCK8)(PNP3) showed high specific binding to A431-CCKB-R cells, with typical saturation binding characteristics. The apparent K_d for the receptor was estimated to be 19 ± 4.6 nmol/L (mean ± SE) and the number of binding sites was in the order of 106 per cell. similar to values found in other CCK8 derivatives previously described [3]. These experiments were performed at 4 °C to block receptor internalization and thus measure only ligand-receptor interaction. In vivo experiments were carried out in nude mice bearing control and CCKB-R overexpressing cells. The uptake is higher in overexpressing cells than in control cells. The only organs showing higher uptake were kidneys and gastrointestinal tract, while uptake is negligible in the other organs and similar to others conjugates. Receptor specific targeting has been shown to be easily obtained and much of the future development efforts will be focused on reducing non-target organ accumulation



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SITE-SPECIFIC N-TERMINAL MODIFICATION OF PROTEINS USING A RATIONALLY DESIGNED TRYPSIN VARIANT

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Introduction

Site-specific covalent modification of proteins is a fundamental tool in medicine, biology and biochemistry. Despite the extraordinary interest in selectively modified proteins, there is however a lack of generally usable and flexible methods, which restore full biological function of the modified proteins in all cases. In the present contribution, we describe the suitability of the genetically optimized trypsin variant K60E/N143H/E151H/D189K for N-terminal modifications of proteins with synthetic functionalities under native state conditions. The trypsin variant possesses a strongly reduced proteolytic activity combined with an artificial metal binding site within the S'-subsite region of the biocatalyst. The latter was shown to mediate highly specific histidine complexations via zinc ions leading to an increased specificity of the enzyme towards histidine-containing peptide sequences [1]. Placing of such sequences at the N-terminal region of respective proteins via standard mutagenesis provides suitable precursor targets for site-specific modification. Mediated by the acceptance of substrate mimetics by the optimized biocatalyst, which allows for the coupling of non-specific as well as non-peptidic acyl moieties [2], N-terminal dansylation reactions of proteins such as *E. coli* Parvulin 10 or human Pin1 proceed efficiently with total yields of isolated modified proteins of about 40%. Importantly, no undesired cleavage reactions could be detected leading to highly active modified protein products.

Results and Discussion

The trypsin variant K60E/D189K, which was used as a starting point for our studies, is known to catalyze 4-guanidinophenyl (OGp) ester-mediated peptide ligations with significantly decreased proteolytic site reactions [3]. For further improvement, we equipped this trypsin species with an extended specificity for P_2'-histidine moieties by introducing a metal binding site into the corresponding S_2'-subsite of the enzyme. After expression and purification, the synthetic utility of the respective trypsin K60E/N143H/E151H/D189K to couple OGp esters and P_2'-histidine-containing peptides in the presence of zinc ions was estimated by using Bz-Gly-OGp and Ala-His-Ala-Ala-Gly as model reactants. Quantitative analysis of these reactions revealed an increase of product yields for P_2'-histidine sequences from 20 to 100% compared to trypsin K60E/D189K [4].

To evaluate the function of the optimized trypsin species to mediate N-terminal modifications of real proteins, the peptidyl prolyl *cis*/*trans* isomerases (PPIases) *E. coli* Parvulin 10 and human Pin1 were recombinantly expressed with an additional

histidine residue at their N-termini. The introduction of an N-terminal histidine is known to avoid the *in vivo* cleavage of the initial methionine [5] and thus, leads to P_2'-histidine-containing protein species, which can be directly used as substrates for the designed trypsin variant. The modification reactions themselves were performed in aqueous buffer at a pH of 8.0 using Dansyl-Gly-Lys(Biotin)-Gly-OGp as acylating reagent (Fig. 1). The efficiency of the reactions were quantitatively analyzed by RP-HPLC and revealed for Parvulin 10 and Pin1 yields of 40 and 38%, respectively. Mass spectroscopy and limited proteolysis indicated a clear course of catalysis with single acylated proteins at their N-termini. Enzyme kinetic studies have finally proven full catalytic activity of the two modified PPIases and thus, illustrate the biocompatibility of the novel catalyst.

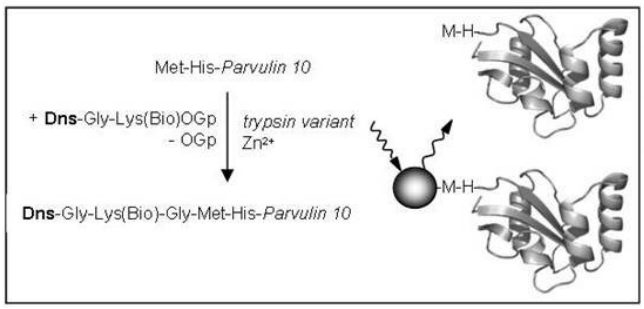

Figure 1: *N*-terminal modification of *E. coli* Parvulin 10 in the presence of zinc ions catalyzed by the trypsin variant K60E/N143H/ E151H/D189K. Conditions: HEPES buffer pH 8.0, 20 µM trypsin variant, 200 µM MH-Parvulin10, 2 mM ester, 60 µM $ZnCl_2$

Acknowledgements

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ASSAYING OF THE PRESENCE OF H-NS PROTEIN IN A NATIVE STRAIN OF HALOMONAS SPECIES

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Introduction

H-NS is one of the most abundant nucleoid-associated proteins and has been implicated in the compact organization of the nucleoid structure. It is widely distributed within gram-negative bacteria [2]. H-NS is a small protein that binds DNA in a sequence-independent manner but recognizes curved DNA [6]. Recently, H-NS homologues have been identified in most gram-negative bacteria. Many discovered homologues have low sequence identity with each other (especially at N-terminal domain) and cannot be identified on the basis of sequence homology [1]. The H-NS protein forms oligomers via its N-terminal part and binds DNA fragments via its C-terminal domain, both essential properties of H-NS family proteins [5]. The role of the H-NS proteins in bacterial physiology remains unknown. But it seems to be involved in the regulation of various genes involved in adaptation to environmental challenges [7].

The increasing number of investigations has revealed the existence of several H-NS-related proteins in gram-negative bacteria with different life styles, but there is no study about the existence of histone-like proteins especially H-NS in halophilic bacteria. According to the essential role of H-NS protein in nucleoid structure and bacterial physiology, in this study we tried to determine the presence of this protein in a gram negative halophilic bacterium belong to *Halomonas genus* that was recently isolated from surface saline soil of Karaj region, Iran. Studying on its 16SrRNA demonstrated that this bacterium is highly probable *Halomonas elongata*. To do so, we extract H-NS from it and compare its electrophoretic pattern with that of *E. coli*.

Results and Discussion

Since the presence of H-NS protein in E.coli was confirmed with different studies, this bacterium was chosed as a control bacterium. H-NS protein was expressed during mid-log phase, so to identify this time, growth curves of both bacteria within 24 hours was achieved by measurement of the absorbance at 620 nm.

E. coli and *Halomonas* were grown and harvested at the mid-log growth phase and then protein extraction was carried out. Because H-NS is a histone-like protein and has some similarities with eukaryotic histones; we used a specific method for protein extraction that was previously described by [3] for histone isolation. In this procedure basic proteins was dissolved in cold perchloric acid (PCA) 5% and then precipitated with acetone. Protein extract from *E.coli* and Halomonas were analyzed

with SDS-PAGE 15%. All identified H-NS like proteins have molecular weight around 15 kDa. This band wasn't seen in SDS-PAGE patterns of PCA extractions of both bacteria. Since the presence of H-NS in different strain of *E.coli* was confirmed frequently, it seems that this procedure isn't capable to isolate H-NS protein. So, we used a specific method which was suggested by [8] to isolate H-NS from E.coli using Ammonium sulfate (40% to 60%). The result of protein extraction was determined with SDS-PAGE (15%) and is shown below in Fig. 1. According to the standard curve of molecular weight a 15 kDa band was seen in electrophoresis pattern of both bacteria. So, we concluded that this protein probably exists in studied bacterium. To prove this result, further researches are needed that is carried out by us.

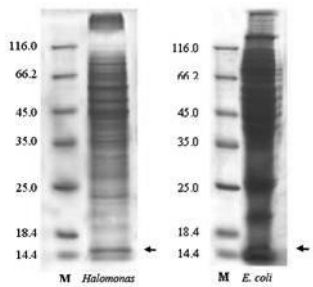

Fig.1. SDS-PAGE patterns of E. coli (right) and Halomonas (left) protein extract with ammonium sulfate. Gels were stained with Coomassie Brilliant Blue. The arrow shows H-NS like protein.

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ENGINEERING OF TRIMERIC COILED COILS OF HIV GP41 PROTEIN HR1 REGION FOR COVALENT STABILIZATION AND FURTHER CONJUGATION

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Introduction

In the HIV mechanism of membrane fusion for viral entry the HR1 and HR2 regions of HIV transmembrane gp41 subunit form a fusogenic 6-helix-bundle structure, in which three a-helices formed by HR2 peptides pack in an antiparallel manner against a central three stranded coiled coil formed by the HR1 peptides. The fusion intermediate is the target of both synthetic C- and N-peptides, which inhibit viral infection by preventing formation of the 6-helix bundle. While HR2 peptides are potent inhibitors of viral fusion [1], peptides from the HR1 region of gp41 protein have poor inhibitor activity because of their low tendency to form a trimeric coiled coil. Accordingly, chimeric peptides, consisting of a designed trimeric coiled coil (IZ) fused to gp41 HR1 sequences are potent fusion inhibitors as reported for IZN17 [2]. We recently showed that covalent stabilization of the designed coiled coil, $(CCIZN17)_3$, by interchain disulfide bonds, yielded an extremely potent and broad inhibitor of viral infection [3].

We have now developed an alternative synthetic strategy to obtain a covalently-linked, structurally stable, IZN17 trimer, Cys-thio$(IZN17)_3$, which allows the presence in the molecule of a free thiol for subsequent chemoselective reactions (Fig.1)

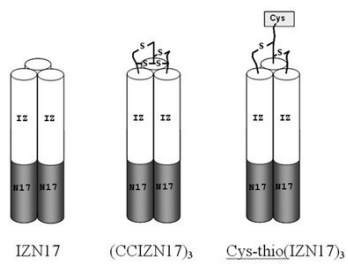

Fig. 1: A schematic model of the designed IZN17, $(CCIZN17)_3$ and Cys-thio$(IZN17)_3$ homotrimeric coiled coils. Each chimeric N peptide consists of an N-terminal designed trimeric coiled coil (white) fused to a segment of the gp41 HR1 region, N17(grey). In $(CCIZN17)_3$ the three helices are covalently stabilized by three interchain disulfide bridges while in Cys-thio$(IZN17)_3$ they are covalently held together by two interchain thioether bonds.

Result and Discussion

First, we showed that stable interchain thioether bonds can be effectively substituted for the disulfides. Second, we devised an orthogonal cysteine protection scheme which allows formation of the thioether bonds, while leaving an extra free cysteine on demand. The chemical strategy to assemble the covalent trimer relied on the following steps:

1) Fmoc-solid phase synthesis of N-terminal bromoacetylated IZN17 and of Cys(P) CCIZN17 peptide precursors (P = protecting group).

2) Chemoselective thioether conjugation between one Cys(P)-CCIZN17 chain and two Br-acetyl-IZN17 chains.

3) Removal of the protecting group P.

The orthogonal protecting group (P) was chosen on the basis of the compatibility of its deprotection procedure with the chemical stability of the thioether trimeric precursor. The acetamidomethyl group (Acm) best suited for this purpose, as it was completely removed by 10 min incubation with 100 eq of silver trifluoromethanesulfonate (AgOTf) in acetic acid, 2% anisole, without damage to the interchain thioether linkages. The Cys-protected covalent trimer was purified from the unreacted monomeric precursors by gel filtration chromatography. Deprotection of the Acm group with AgOTf yielded the final product, Cys-thio$(IZN17)_3$, which was purified from the reaction mixture by gel filtration chromatography.

Circular Dichroism analysis [3], thermal denaturation experiments [3], mAb D5 competition assay [4], and HIV neutralization assay [3,4] demonstrated that the thioether-stabilized trimer retains the same structural and functional properties of the parent Cys-stabilized construct, as summarized in the Table below.

Peptide	T_m (2M GdnCl), °C	D5 competition IC_{50}(nM)	Antiviral Potency IC_{50}(nM)
IZN17	61.5	193.4	1.7
$(ccIZN17)_3$	> 90	12.1	0.1
$(thioIZN17)_3$	> 90	28.2	0.2

In conclusion, we have described a strategy to engineer a trimeric coiled coil to obtain a covalently linked, structurally stable construct endowed with a free thiol for further derivatization. This extra functionality allows conjugation of this and similar constructs to a variety of moieties, such as PEG, lipids, carrier proteins and Toll-like receptor ligands, with the aim of improving either the pharmacokinetic profile for drug use, and/or to enhance immunogenicity for use as subunit vaccines.

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ANALOGUES OF [MPA[1], D-(ET)TYR[2]] OR [MPA1, D-NAL(1)[2]] OXYTOCIN CONTAINING NON NATURAL AMINO ACIDS IN POSITION 3

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Introduction

Oxytocin (OT) is a hypothalamic cyclic nonapeptide that is released into the general circulation from the posterior lobe of the pituitary. Generally known is its capability of inducing uterine contractions and facilitation of milk ejection during lactation. However widespread distribution of OT receptors in the brain and specific behavioral effects of centrally applied OT have firmly established OT role as a neurotransmitter modulating reproductive and social behaviors [1, 2]. The role of OT in preterm labor led to the search for and design of synthetic peptide antagonists as potential tocolytic agents. The design of new OT antagonists is based on data from structure-activity studies. Antagonistic activity depends on the configuration and the hydrophobicity of the amino acid at position 2. Based on these findings and for investigation of the role of position 3 in biological potency, we synthesized twelve new analogues of [Mpa[1], D-Tyr[2](Et)] or [Mpa[1], D-Nal(1)[2]]OT containing α-amino-isobutyric acid [Aib], L/D-α-*t*-butylglycine [Gly(Bu^t)], L/D-β-(2-thienyl)-alanine [Thi] and D-3-pyridylalanine [(Pal(3)] in position 3.

Results

The analogues were synthesized by Fmoc solid phase methodology [3] utilizing a Rink Amide MBHA [4] and Sieber Amide resin [5] as solid support to provide the peptide amide. Stepwise synthesis of a peptide analogue was achieved with DIC/HOBt as coupling agents in DMF [6, 7]. The cyclization was performed in DMSO/H_2O (1:4, v/v) for 24 - 48h or alternatively in AcCN/CCl_4 using tetrabutylammoniumfluoride (TBAF) [8]. All the analogues were purified by gel filtration chromatography and the final purification was achieved by reversed-phase HPLC. The identification of the analogues was performed by ES-MS.

The analogues were tested for their potency in two pharmacological assays: a) uterotonic *in vitro* test in the absence of Mg^{2+} on an isolated strip of rat uterus [9] and b) in the pressor test on phenoxybenzamine treated male rats [10]. Parallel the binding affinity of the analogues to cloned human oxytocin receptors was determinated. The results of biological evaluation of the new analogue (I–XII) are summarized in Table 1. The activities of OT and Atosiban are also cited in Table 1 for comparison and reference.

Discussion

The replacement of Ile3 by non natural amino acids combined with D-Tyr(Et)^2 or D-Nal(1)^2 and Mpa1 modifications influenced biological activities significantly. The substitution of Tyr^2 with D-Nal(1) leads to analogues with higher antioxytocic uterotonic activity in comparison to analogues with D-Tyr(Et). In addition, the analogues with the L-stereoisomers in position 3 showed strikingly higher potency in comparison with those with D-stereoisomers, while analogues VIII and XI with D-Thi^3 and D-Pal(3)^3, respectively, were entirely inactive.

Table 1. Biological activities of new oxytocin analogues

	Analogues	Biological Activity		
		Uterus in vitro (pA_2)	Pressor	Binding Affinity IC_{50} (nM)
	OT	546 (IU/mg)	3.1	6.5
	Atosiban	8.29±0.05	0.02±0.02	
I	[Mpa^1,D-Tyr(Et)^2,Aib^3]OT	7.42±0.07	0	911±100
II	[Mpa^1,D-Nal(1)^2,Aib^3]OT	7.15±0.07	0	372
III	[Mpa^1,D-Tyr(Et)^2,Gly(Bu^t)^3]OT	7.77±0.11	0	972±7
IV	[Mpa^1,D-Tyr(Et)^2,D-Gly(Bu^t)^3]OT	5.67±0.05	0	7 479±732
V	[Mpa^1,D-Nal(1)^2,Gly(Bu^t)^3]OT	8.34±0.30	0	33.1±2.4
VI	[Mpa^1,D-Nal(1)^2,D-Gly(Bu^t)^3]OT	<5.60	0	>10 000
VII	[Mpa^1,D-Tyr(Et)^2,Thi^3]OT	7.09±0.13	0	223
VIII	[Mpa^1,D-Tyr(Et)^2,D-Thi^3]OT	0	0	>10 000
IX	[Mpa^1,D-Nal(1)^2,Thi^3]OT	8.50±0.24	pA_2=6.10	5.2±0.7
X	[Mpa^1,D-Nal(1)^2,D-Thi^3]OT	6.17±0.22	0	3 226±93
XI	[Mpa^1,D-Tyr(Et)^2,D-Pal(3)^3]OT	0	0	>10 000
XII	[Mpa^1,D-Nal(1)^2,D-Pal(3)^3]OT	6.16±0.30	0	3 608±568

All the analogues are inactive in the pressor test, with the exception of analogue IX which showed a weak antagonistic activity (pA_2 = 6.10). As far as the binding affinity to the human OT receptor is concerned, the analogues V and IX with L-Gly(Bu^t)^3 and L-Thi^3, respectively, exhibited high binding affinity (IC_{50} = 33.4 and 5.1 nM), while the replacement of Ile^3 by Aib^3 or the D-stereoisomers decreased the affinity remarkably.

Acknowledgements

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A NEW SYNTHETIC APPROACH TO UNSATURATED AND SATURATED DICARBA-ANALOGUES OF OCTREOTIDE

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Introduction

Somatostatin is a cyclic tetradecaptide able to inhibit the release of GH, binding to receptors expressed in approximately 90% of carcinoid tumours [1]. However, the clinical use of Somatostatin is limited by its short half-life *in vivo*. To overcome this severe drawback, many analogues have been prepared and tested. Among them, the cyclic octapeptide octreotide is still used in clinical protocols. The amino acid sequence of octreotide retains the –S-S- bridge of the parent Somatostatin, sensitive to oxidizing and reducing agents. This prompted us to search new cyclic analogues of similar size, carrying the pharmacophore motif of octreotide (Phe-D-Trp-Lys-Thr) but bridged by means of a more robust tether. At the same time, the new bridge had to preserve the β-II' turn conformation found in the active sequence.

Results

We previously synthesized the dicarba-analogue **1** containing a -CH=CH- bridge in substitution of the -S-S- chain [2].The synthesis was performed on Rink Amide resin and afforded the pure Z-isomer in 21% yield. Later on, because of the role of lypophilic residues (i.e. $Phe^{6,7,11}$) of the native hormone [3], we prepared two other unsaturated dicarba-analogues substituting Thr^6 residue with Phe^6 (**2**) and $Tyr(Bzl)^6$ (**3**), respectively (Fig. 1) in the aim of favouring the binding to the Somatostatin receptors [4]. The synthesis was carried out on H-Thr-ol(*t*Bu)-2-chlorotrityl resin. The cyclization between the two allylglycine residues was performed on resin by RCM, comparing the action of 1st and 2nd generation Grubb's catalysts [5].

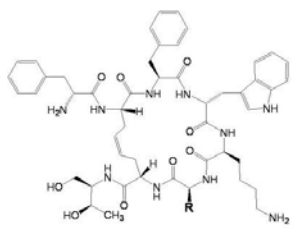

Fig. 1 R= CH_3-(CH)-OH (1), R= CH_2-Phenyl (2), R=CH_2- 4-Benzyloxyphenyl (3).

The unsaturated –CH=CH- bridge of **1** and **3** was then reduced in order to further improve the stability of the analogues and the flexibility of the entire structure. Compound **1** was reduced with H_2, (15 - 20% $Pd(OH)_2/C$ as catalyst), affording the saturated cyclopeptide **4** in 35% yield. Compound **3**, was reduced using a Wilkinson's catalyst (60 psi H_2 pressure) [6], saving the Bzl group. This reduction was conveniently performed on resin, overcoming one purification step. The saturated derivative **5** was obtained in 15% yield.

Discussion

The new synthetic route we used consists of a minor number of steps respect to the synthesis of the analogue 1. In addition, the synthesis was stopped to the allylglycine residue, in order to overcome steric problems in the cyclization reaction, due to D-Phe[1]. The N-terminal residue was anchored after the cyclization step. The use of the 2nd generation Grubbs catalyst helved the reaction time and increased the yield of about 10% respect to the cyclization with the 1st generation one. The unsaturated analogues were obtained as *E*/*Z* isomers (20/80). Ruthenium contaminants were removed for the most part by SPE, making easier the purification performed by RP-HPLC. Compound **3**, **4** and **5** were analyzed by NMR in H_2O/DMSO-d_6 at 277 K. Inter-chain NOEs between $C_\beta H$'s of the residue 2 and $C_\gamma H$ of the residue 7 and between $C_\beta H$'s of residue 7 and of residue 2, established the *Z* geometry of the isolated compound **3**. ^1H NMR of compounds **4** and **5** showed the presence of the saturated bridge. Compound **4** was further analyzed by 1D and 2D techniques (DQF-COSY, TOCSY and ROESY). The preferred conformations were obtained from the analysis of the NMR derived experimental data (NOEs, $^3J_{H\alpha\text{-}HN}$ coupling constants, and temperature coefficients of amide protons) suggesting the presence of a β-turn about the residues 3 - 6 as previously established for the analogue **1**.

Acknowledgements

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EFFECT OF HYDRATION ON THE THERMAL STABILITY OF THE COLLAGEN TRIPLE HELIX

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Introduction

The collagen triple helix has the characteristic repeating sequence $(X\text{-}Y\text{-}Gly)_n$, where X and Y are often imino acids, Pro or HypR (4(*R*)-hydroxyproline). Although it is well known that HypR at the Y position plays an important role in stabilizing the collagen triple helix, the stabilizing mechanism has not been resolved. So far, it had been believed that $(Pro\text{-}HypR\text{-}Gly)_{10}$ acquires high thermal stability because of a hydrogen bond possibly formed between the hydroxyl group of HypR and water molecule. Our thermodynamic analysis supported the significance of hydrogen bonds in the thermal stability of the triple helical structure for $(Pro\text{-}HypR\text{-}Gly)_{10}$ [1].

Recently, from the study of the double substituted model peptides, we reported that the transition temperature of $(HypR\text{-}HypR\text{-}Gly)_{10}$ is as the same as that of $(Pro\text{-}HypR\text{-}Gly)_{10}$ [2]. Furthermore, we also showed that both ΔH and ΔS of the thermal transition from the triple helix to the single coil of $(HypR\text{-}HypR\text{-}Gly)_{10}$ are obviously smaller than those of $(Pro\text{-}HypR\text{-}Gly)_{10}$. To explain the mechanism of thermal stability of $(HypR\text{-}HypR\text{-}Gly)_{10}$, we performed X-ray crystallographic analysis and volumetric study.

Result and Discussion

The full-length structure of $(HypR\text{-}HypR\text{-}Gly)_{10}$ was determined and refined at 1.5 Å resolution. The overall structure of $(HypR\text{-}HypR\text{-}Gly)_{10}$ appears to be similar to those of the other collagen model peptides with 7/2 helical symmetry and same Rich and Crick II hydrogen bond patterns [3, 4]. An asymmetric unit of the crystal of $(HypR\text{-}HypR\text{-}Gly)_{10}$ contains 177 water molecules which were involved in water bridges stabilizing the peptide conformation as in the cases of other collagen model peptides. The typical profile of networks of water bridges observed in $(HypR\text{-}HypR\text{-}Gly)_{10}$ is demonstrated, along with that of $(Pro\text{-}HypR\text{-}Gly)_n$ in Fig. 1. Interestingly, it is indicated that the increase in the number of HypR residues in the tripeptide unit does not necessarily lead to increase in the number of hydrated waters. This result is also confirmed by the measurements of the hydration volume

(V_{hyd}), which is the difference between the molecular volumes observed in solution and calculated ones from the crystal structure, of these model peptides (Table 1).

On the other hand, as shown in Table 1, the degrees of hydration of (Pro-HypR-Gly)$_{10}$ in the single-coil state are almost the same whereas that of (HypR-HypR-Gly)$_{10}$ is obviously larger. Because hydration reduces the enthalpy due to the formation of hydrogen bond with water molecule and diminishes the entropy due to the restriction of water molecules surrounding a peptide molecule, we concluded that the thermal stability of (HypR-HypR-Gly)$_{10}$ is able to be described by its high hydration in the single-coil state.

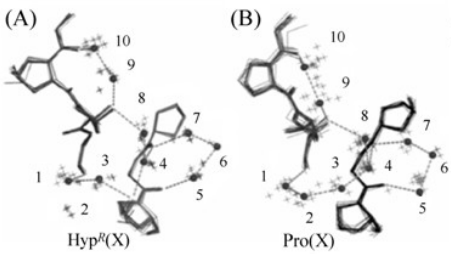

Figure 1. Distribution of water molecules which participate in the water bridge observed in (Hyp^R-Hyp^R-Gly)$_{10}$ (A) and (Pro-Hyp^R-Gly)$_n$ (B). Dotted lines designate the hydrogen bonds.

Table 1. Observed partial molar ($\bar{V}_{obs}$), calculated (V_{calc}), and hydration volumes (V_{hyd}) of the collagen model peptides in the triple-helix state (t) and single-coil state (s).

peptides	state	$\bar{V}_{obs}$ /cm^3 mol^{-1} [a]	V_{calc} /cm^3 mol^{-1} [a]	V_{hyd} /cm^3 mol^{-1} [a]
(Pro-Pro-Gly)$_{10}$	t[b]	169.4	179.0	-9.6
	s[c]	177.4	180.0	-2.6
(Pro-Hyp^R-Gly)$_{10}$	t[b]	164.7	183.3	-18.6
	s[d]	178.2	182.2	-4.0
(Hyp^R-Hyp^R-Gly)$_{10}$	t[b]	171.7	190.7	-19.0
	s[d]	176.9	184.2	-7.3

[a]Expressed per tripeptide unit. [b]Observed at 10°C. [c]Observed at 70°C. [d]Observed at 80°C.

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NEW ANALOGUES OF [ARG^8]-VASOPRESSIN CONTAINING L-α-*t*-BUTYLGLYCINE IN POSITION 9

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Introduction

Arginine vasopressin (AVP) is a neurohypophyseal nonapeptide hormone, containing a 20-membered ring (from Cys-1 to Cys-6) and an acyclic tripeptide tail (Pro^7-Arg^8-Gly-NH$_2$^{9}). It elicits a variety of responses both centrally and peripherally by acting on three known G-protein coupled receptors: V1$_a$, V1$_b$ and V2 and on oxytocin (OT) receptor [1]. In addition to its well-known antidiuretic activity, AVP has also complex cardiovascular actions [2] and adrenocorticotropic hormone (ACTH) releasing activity. The configuration and the hydrophobicity of the aromatic amino acid in position 2 are important factors for the antagonistic activity [3], while elimination of the N-terminal amino group plays an important role in prolongation of the activity. Furthermore the C-terminal Gly-NH$_2$ can be deleted or replaced by a wide variety of substituents with excellent retention of V1-antagonistic potency [4]. On the basis of these findings, we set out the synthesis and pharmacological investigation of six analogues containing mercapto propionic acid (Mpa) in position 1, Tyrosine(*O*-Methyl) [Tyr(Me)] or 2-Naphtylalanine [Nal(2)] in position 2 and L-α-*t*-butyglycine [Gly(Bu^t)] in position 9. We also studied the impact of modified C-terminal amide on biological potency of the new AVP analogues.

Results

The new analogues were synthesized by Fmoc/Bu^t solid phase methodology utilizing the Rink Amide MBHA resin [5], the [3-((Ethyl-Fmoc-amino)-methyl)-1-indol-1-yl]-acetyl AM resin [6] and the 2-chlorotrityl-chloride resin. The cyclization was performed in DMSO/H$_2$O (2:8, v/v) for 24-36h. All the analogues were purified by gel filtration chromatography. Final purification was achieved by preparative HPLC on reversed-phase support C-18. Electrospray MS was in agreement with the expected results. The analogues were tested for their potency in three pharmacological tests, i.e. uterotonic in vitro test [9, 10], in the pressure test [11] and for their antidiuretic potency [12]. Parallel determination of binding affinity of the analogues to cloned human oxytocin receptors on HEK cell membranes [13] was performed. The results of biological evaluation are summarized in Table 1.

Discussion

In the pressor test, all the analogues showed very weak antagonistic activity, except for analogue VI which was inactive.

Table 1. Biological Activities of New AVP Analogues

Analogues		Biological Activity			
		Anti-pressor (pA_2)	Antidiuretic (per cent of activity of AVP)	Anti-Uterotonic *in vitro* (pA_2)	Binding Affinity to Human OTR IC_{50} (nM)
I	[Mpa^1,$Tyr(Me)^2$,$Gly(Bu^t)^9$-NH_2]AVP	6.51 ± 0.12	0.5-2	7.87 ± 0.10	340 ± 46
II	[Mpa^1,$Nal(2)^2$,$Gly(Bu^t)^9$-NH_2]AVP	6.07 ± 0.12	0.5-2	8.23 ± 0.33	12.3 ± 0.8
III	[Mpa^1,$Tyr(Me)^2$,$Gly(Bu^t)^9$-NHEt]AVP	6.10 ± 0.27	0.5-2	8.25 ± 0.17	304 ± 29
IV	[Mpa^1,$Nal(2)^2$,$Gly(Bu^t)^9$-NHEt]AVP	<5.8	0	8.23 ± 0.09	32.8 ± 1.3
V	[Mpa^1,$Tyr(Me)^2$,$Gly(Bu^t)^9$-OH]AVP	<5.8	0.5-2	7.98 ± 0.25	200 ± 30
VI	[Mpa^1,$Nal(2)^2$,$Gly(Bu^t)^9$-OH]AVP	0	0	7.91 ± 0.15	32.3 ± 6.7
	AVP	-	100	-	5.5±4
Activity of AVP 465 IU/mg taken as 100 %, 0 means no activity up to the dose of 0.1mg/kg of exp. animal					

The modifications decreased significantly antidiuretic activity of the analogues. In pilot experiments analogues I, II, III and V exhibited very low agonistic V2 activity, and analogues IV and VI were inactive up to the dose 0.01 mg/kg of experimental animal.All analogues exhibited comparably high antiuterotonic potency (pA_2= 7.87 - 8.25). Analogues III and IV with N-ethylamide in the C-terminal part of the molecule showed the highest anti-OT activity, but the differences are not significant.Additional modification at the C-terminal amide influenced only slightly the biological potency of the analogues having replacement of the amino acids at positions 1, 2, and 9 with Mpa^1, $Tyr(Me)^2$ or $Nal(2)^2$ and $Gly(Bu^t)^9$, respectively. However, analogue II displayed the highest binding affinity to human oxytocin receptors, followed by analogues VI and IV, all having 2-Naphtylalanine in position 2.

Acknowledgements

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IMPROVEMENT OF AUTOANTIBODY DETECTION IN AUTOIMMUNE DISEASES BY INNOVATIVE SOLID-PHASE GLYCOPEPTIDE BASED TECHNOLOGIES

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Introduction

Measurement of a variety of disease specific biomarkers, such as antibodies, is an important tool for the diagnosis, the follow-up and future prevention of specific autoimmune diseases. In fact, sera from patients suffering from autoimmune disorders often contain multiple types of autoantibodies. Some autoantibodies can be exclusive of a disease and thus used as biomarkers for diagnosis; others fluctuate with disease exacerbations or remissions and are extremely valuable in the follow up of patients. In this scenario, identification of autoantibodies, as disease biomarkers, should be achieved using native antigens in simple biological assays. The role and predictive value of antibodies has been demonstrated in our laboratory in the field of Multiple Sclerosis [1]. We have recently developed CSF114(Glc), a structure based designed glucosylated peptide, characterized by a β-turn structure, as the first Multiple Sclerosis Antigenic Probe accurately measuring high affinity autoantibodies (biomarkers of disease activity) in sera of a statistically significant patients' population. The ELISA diagnostic test, MSPepKit, based on the glycopeptide CSF114(Glc), has been developed to recognize specific autoantibodies in Multiple Sclerosis patients' sera.

Result and Discussion

Detection of autoantibodies by synthetic peptide antigens in solid phase assays requires immobilisation of the post-translationally modified (PTM) peptides as synthetic probes. CSF114(Glc) has been selected as a specific Multiple Sclerosis Antigenic Probe because of its binding properties to autoantibodies putatively involved in the pathogenesis of MS.

The aim of this study is to evaluate the specificity, sensitivity, matrix effects and analysis time of different screening technologies SP-ELISA, BIAcore and the laser bead autoantigen microarrays (BioPlex) in the analysis of antibody profiles in MS patients' sera, in order to improve turn around times and meet budget constraints.

As alternative techniques of ELISA, we selected a biosensor technology based on surface plasmon resonance and Bio-Plex suspension array system, BioRad. The

biosensor technology and Bio-Plex suspension array system offer advantages such as rapid analysis, and high sensitivity for a high throughput screening. Immobilisation of PTM peptides was based on different strategies that are anchoring the synthetic antigen on different solid supports, such as polystyrene well plates (ELISA), dextran coated gold chip (BIAcore), and carboxy-coated polystyrene beads (Bio-Plex). Optimisation of the different techniques was performed with anti-CSF114(Glc) autoantibodies isolated using affinity chromatography from MS patients' sera. The analytical parameters such as specificity, sensitivity, and matrix effect were evaluated. The different technologies have been used for a high throughput screening of MS sera.

The CSF114(Glc)–based immunosensor was tested in BIAcore using positive and negative control sera. The positive controls, from MS patients' sera were compared with healthy blood donors. The sensor was able to discriminate between MS patients and the healthy blood donors. A Bio-Plex ELISA test was carried out using MS patients' and healthy blood donors' sera. CSF114(Glc) and CSF114 (as a negative control) were immobilised via amide bond on colour specific beads. The test can distinguish between the negative and positive controls.

The results indicate that all technologies investigated may offer interesting future applications for the prognosis of autoimmune diseases in particular in the MS diagnostic field.

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N-TRIAZINYLAMMONIUM SALTS IMMOBILIZED ON SOLID SUPPORT AS COUPLING REAGENTS

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Introduction

An application of solid support bound reagents facilitate the isolation of products and work-up procedures. Therefore solid supported reagents were found exceedingly useful is all syntheses involving excessive amounts of substrates, for protein modification and labeling.

Results and Discussion

Immobilized 2-chloro-4-methoxy-1,3,5-triazines **1a-e** anchored on cellulose, silica, Wang, BHA, and TG HMBA resin were prepared by the treatment of 2,4-dichloro-6-methoxy-1,3,5-triazine with appropriate solid support in the presence of a base. Stable, immobilized, triazine coupling reagents **3a-e** were obtained in reactions of **1a-e** (Fig. 1) with *N*-methylmorpholinium *p*-toluenesulfonates in the presence of sodium bicarbonate or DIPEA [1].

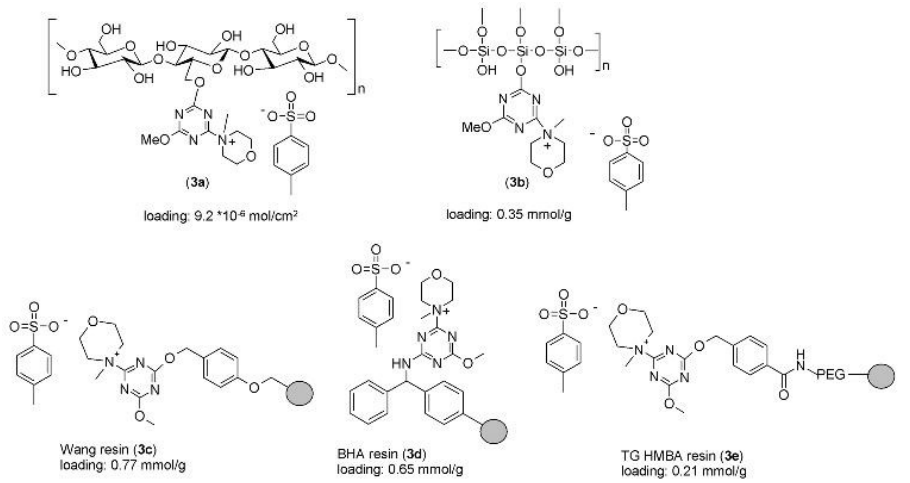

*Fig. 1. Solid-supported N-triazinylammonium coupling reagents **3a-e**.*

The loading of the solid carriers was calculated from N, S contents determined by microanalysis. A synthetic value of triazine reagents **3a,b** was confirmed by dipeptide synthesis. Activation of carboxylic components proceeded under conditions similar to the standard synthesis in solution. Treatment with amino component gave chromatographically homogenous peptides in 72 - 91% yield (Table 1).

*Table 1. Synthesis of dipeptides by means of **3a,b**.*

Dipeptide	Coupling reagent	Method A		Method B	
		Yield [%]	Purity [%]	Yield [%]	Purity [%]
Z-Phe-Ala-OMe	**3a**	77	91	72	93
Z-Ala-Leu-OMe	„	81	89	77	91
Z-Phe-Aib-OMe	„	84	92	81	94
Z-Aib-Ala-OMe	„	75	88	70	90
Z-Aib-Phe-OMe	„	72	90	70	91
Fmoc-Ala-Ala-OMe	„	85	95	83	97
Fmoc-Val-Ala-OMe	„	81	96	79	98
Fmoc-Leu-Ala-OMe	„	84	94	82	95
Fmoc-Phe-Ala-OMe	„	79	97	76	98
Z-Ala-Leu-OMe	**3b**			88	97
Boc-Ala-Leu-OMe	„			79	96
Fmoc-Ala-Leu-OMe	„			91	99

Triazine reagents supported on cellulose and on silica were found efficient in the synthesis of Z-, Boc, or Fmoc protected dipeptides. Moreover, experiments involving activation of sterically demanding Aib confirmed that an access to the reactive centers of immobilized reagents remains principally unrestricted, although slightly lower yield and purity of respective peptides were noticed in this case.

Acknowledgements

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DESIGN AND SYNTHESIS OF SHORT PEPTIDES FOR THE TREATMENT OF ALZHEIMER'S AND PARKINSON'S DISEASES

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Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive neurodegenerative disorders which are marked by neuronal accumulation of toxic misfolded proteins which form amyloid plaques [1]. The main components of the plaques are β-amyloid peptides (Aβ[1-40] and Aβ[1-42]) and α-synuclein [2]. The key process in the pathology of AD and PD is the formation of fibrils and aggregates of Aβ peptides and α-synuclein [3,4]. We have previously shown that small peptides structurally related to the sequence of Aβ[1-42] protect against the neurotoxicity of Aβ peptides [5-8]. Recent studies by other groups have shown that β-synuclein can counteract the aggregation of α-synuclein in the neurodegenerative process of PD [9], hereby might protect the central nervous system from the neurotoxic effects of alpha-synuclein [9]. A drug discovery program based on fragments and peptide derivatives of β-synuclein was initiated and found that a tetrapeptide (KEGV) decreased expression of Aβ peptides and protected against its neurotoxicity *in vivo* [10]. Our aim was to find and synthesize short peptides which inhibit the neurotoxic effects of Aβ peptides. Since β-synuclein inhibits the aggregation of α-synuclein, we compared the sequences of α- and β-synuclein [11] and found four common sequences. These peptides have been synthesized in amide forms at their C-termini as putative neuroprotective agents. The effects of these peptides were investigated by using in vitro and *in vivo* assays.

Results

The following peptides have been synthesized by manual solid phase synthesis using Boc chemistry: Lys-Glu-Gly-Val-NH_2 (KEGVa), Lys-Glu-Gly-Val-OH (KEGV-OH), Lys-Glu-Gln-Val-NH_2 (KEQV), Lys-Glu-Gln-Ala-NH_2, (KEQA), Arg-Glu-Gly-Val-NH_2 (REGV), Lys-Gln-Gly-Val-NH_2 (KQGV). The peptides have been investigated in MTT test (12) and transmission electron microscopy [8] (TEM) in vitro and in electrophysiological test on rats using multibarrel electrodes [13] *in vivo*.

1) Among the four new tetrapeptides, KEQA protected most effectively (91%) against the neurotoxic effect of Aβ[1-42] in the MTT assay (Fig. 1).

2) None of the new tetrapeptides inhibited the formation of fibrils of Aβ[1-42] in the TEM experiments (data not shown).

3) KEGVa partially protected against the enhanced NMDA triggered firing response of Aβ[1-42] in the *in vivo* electrophysiological tests when they were administered in mixture.

Discussion

1) The β-synuclein fragment KEQA protects SH-SY5Y neuroblastoma cells from the neurotoxic effect of Aβ[1-42] in the MTT assay.
2) Since none of the tetrapeptides inhibited the fibril formation of Aβ[1-42] (TEM), establishment of the mechanism of neuroprotective effect of KEQA needs further work.
3) The MTT assay in vitro and the electrophysiological measurements *in vivo* seem to be suitable methods for testing the neuroprotective effects of new peptides designed against AD or PD.
4) KEQA can be a starting point in further design for potential drugs which could protect neurons from toxic misfolded proteins in AD or PD.

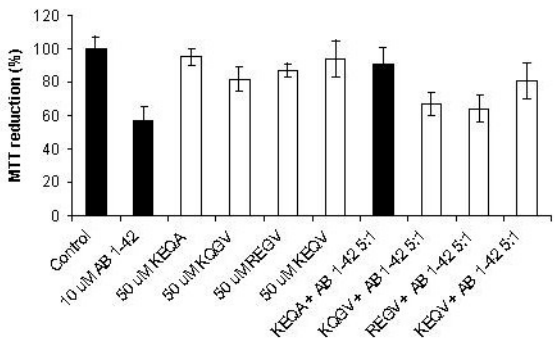

Fig. 1. Neurotoxic activity of Aβ[1-42] and short peptides investigated alone or together with Aβ[1-42] in MTT test on differentiated human neuroblastoma cells (SH-SY5Y) (n=14, ANOVA Post Hoc Test, Bonferroni). Cell viabilities are expressed as percentages of untreated control.

Acknowledgements

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CONFORMATIONAL BEHAVIOUR OF TEMPORIN A AND TEMPORIN L BY NMR SPECTROSCOPY IN DIFFERENT ENVIRONMENTAL CONDITION

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Introduction

Temporins are among the smallest antimicrobial peptides so far described, together with the 13-residue peptide indolicidin and the cyclic dodecapeptide bactenecin. Temporin L (TL) is a small, basic, highly hydrophobic peptide amide (FVQWFSKFLGRIL-NH_2) found in the skin of the European red frog, *Rana Temporaria*. This peptide shows an important antimicrobial activity against a broad spectrum of microorganism, including clinically important strains. Temporin A (TA) is the most well known among thetemporins. It is a strongly hydrophobic, 13-amino-acid peptide (FLPLIGRVLSGIL-HN_2) that exhibits antibacterial (mainly against gram-positive cocci) and antifungal activities (against yeast-like *Candida albicans*). The N-terminal residue, Arg7, and two Ile residues (5 and 12) are described as the critical ones responsible for the antibacterial activity. TA exerts its antimicrobial activity by its ability to form a transmembrane pore via a barrel-stave mechanism or to form a 'carpet' on the membrane surface via the 'carpet-like' model. Mangoni *et al.* reported that the ability of temporins to destroy microbial cells is independent of membrane composition, since they lysed artificial vesicles built from zwitterionic and acid phospholipids as well.

In particular, temporin A is preferentially active against Gram-positive bacterial strains while temporin L has the highest activity among all temporins studied to date against human erythrocytes, fungi, and bacteria, including Gram-negative strains.It is very important to understand the behavior and the mechanism of action of temporins for the development of new antimicrobial agent, because in the last decade the problem of resistance has been born. So we investigated the preferential conformation of TL and TA in SDS solution.

FVQWFSKFLGRIL-NH_2 Temporin L

FLPLIGRVLSGIL-NH_2 Temporin A

Results and Discussion

Temporin L was studied by solution NMR in membrane mimetic environment (SDS solution). NMR spectra were acquired on a Varian INOVA 700 at 298 K (Fig. 1). NMR parameters were used in a TAD restrained molecular dynamic calculation.

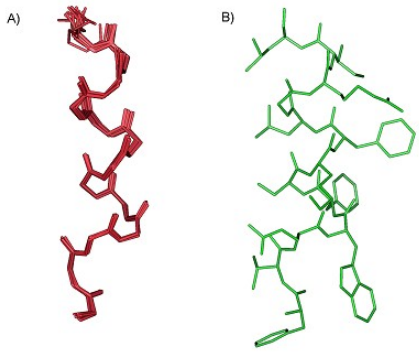

Fig. 1. Superposition of the best 20 calculated conformations of temporine L (A), the lowest energy conformer of temporine L was shown with its side chains. It showed high tendency to the a-helix formation (B).

Aim of this study was to investigate by NMR the structural–conformational features of Temporin A and L, two short peptides belonging to an interesting class of natural substances known to be active mainly against Gram-positive/negative bacteria and fungi. Experimental results indicate that temporin L shows a higher propensity, with respect to temporin A, in forming a-helical structures. These results are in according with a previous study carried out by a circular dihchoism (CD) and molecular dynamics (MD) simulation. The results will be used to design and synthesis of rational synthetic compounds with improved activity.Understanding the basis of the interactions of temporins with lipids could be crucial in discovering of potent antimicrobial agents.

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PEPTIDE-CONJUGATED NANOPARTICLES FOR TUMOUR-SELECTIVE IMAGING AND DRUG DELIVERY

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Introduction

Quantum Dot nanoparticles (Qdots) are highly fluorescent nanodevices. The particles are nanometer-scale (roughly protein-sized) atom clusters of a semiconductor material (cadmium mixed with selenium or tellurium), which has been coated with an additional semiconductor shell (zinc sulfide) to improve the optical properties of the material. Qdot products combine the high fluorescence inherent in the nanocrystal structure with a highly customizable surface that can be modified by conjugation to a wide range of molecules of interest including peptides.

The utility of targeted nanoparticles as fluorescent probes for tissue imaging has recently been subject to widespread interest. An exciting prospect is the development of nanoparticles conjugated to targeting peptides and cytotoxic cargoes, which preferentially bind to specific tissues to provide effective tools for drug delivery. These multifunctional nanodevices combine both diagnostic and therapeutic functions by acting as fluorescent probes that offer targeted delivery of therapeutic agents.

Our recent studies have covalently modified the surface of highly fluorescent QDots with cell-penetrating peptides (CPPs) to label U251MG cells.

Methods

QDots were initially coupled to polyethylene glycol(PEG) linkers via carboxyl functionalities on their surface. The coupling was facilitated by (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) that couples the carboxyl groups to free amines on the PEG linkers. PEG spacers, as well as acting as linkers, have the additional advantage of imparting favourable properties such as increased solubility.

Poly-arginine peptides of varying lengths were synthesised and coupled to the polyethylene glycol linkers using EDC as a linking agent. The inherent cell penetrating nature of poly-arginine peptides ensured that their coupling to the construct would conferr a cell penetrating capacity on the modified QDot.

20 nM, 30 nM and 40 nM solutions of the QDot construct were then applied to the human glioblastoma cell line U251M in 24 well plates. The cells were incubated overnight with the construct to allow binding and tranfer of the QD construct across the plasma membrane. The cells were counter-stained with DAPI to highlite the cell nuclei and imaged by confocal microscopy.

Results

Cells incubated with conjugated QDots showed a bright fluorescent green signal at concentrations of 20 nm, 30 nm and 40 nm, indicating effective internalisation of the modified nanoparticles (Fig. 1). The signal was absent from all control experiments, QDots did not exhibit any cell penetrant activity in the absence of covalently bound poly-arginine peptides.

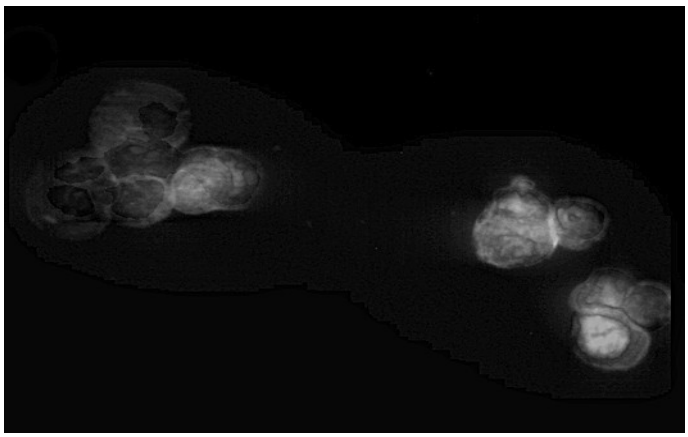

Fig. 1. The image shows U251MG cells incubated overnight with a peptide-conjugated QD (green) at 30nm indicating that the conjugate has entered the cytoplasm of the cell. The cells have been stained with Dapi, a nuclear counterstain (blue).

Discussion

The QDots used in this study emit a bright green fluoresence and presence of the sigal in U251M cells incubated with the modified QDot constructs confirms that cell penetrating peptides can be utilised to conferr a cell penetrating capacity to Qdots. We will further develop QDots as GBM-selective delivery systems. An exciting prospect is the development of QD conjugated to tumour homing peptides and cytotoxic cargoes, which preferentially bind to specific tissues to provide effective tools for drug delivery. One further important application of these particles could employ their fluorescent properties to demarcate the periphery of tumour masses, thereby allowing greater accuracy in the de-bulking procedures aimed at removing the tumour mass.

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THE NEW REARRANGEMENT OF *N*-ACYLATED 2-HYDROXYMETHYL-2-AMINO ACIDS TO 4,5-DIHYDRO-1,3-OXAZOLE-4-CARBOXYLIC ACIDS

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Introduction

Several derivatives of oxazole and thiazole carboxylic acids and analogues with partially hydrogenated heterocyclic ring were identified as components of natural products. Many of them were found pharmaceutically interesting as antibiotic with strong antiviral and cytostatic activity, immunomodulators, antiinflamatory drugs and many others. In most of the cases these heterocyclic carboxylic acids were prepared in the multistep procedures by modification of appropriate derivatives of serine or substituted serine analogues [1].

Result and Discussion

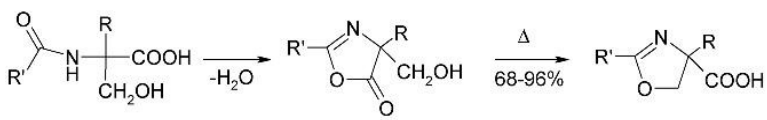
Recently, we found the new simple and efficient method of preparation of 4,5-dihydro-1,3-oxazole-4-carboxylic acids **3** (Table 1).

*Table 1. Synthesis of 4,5-dihydro-1,3-oxazole-4-carboxylic acids **3a-g**.*

3	R	R'? C=O	yield [%]	mp [°C]	$[\alpha]_D^{25}$
rac-**3a**	CH_3	C_6H_5	70	115	-
S-**3a**	CH_3	C_6H_5	72	111	+3.9 (5.0; MeOH)
rac-**3b**	C_3H_6	C_6H_5	80	110-111	-
rac-**3c**	$CH(CH_3)_2$	C_6H_5	80	155-157	-
rac-**3d**	$CH_3CH(CH_3)_2$	C_6H_5	68	84-85	-
rac-**3e**	$C_6H_5CH_2$	C_6H_5	96	126-127	-
S-**3e**	$C_6H_5CH_2$	C_6H_5	90	120-121	+59.5 (2.5; MeOH);
R-**3e**	$C_6H_5CH_2$	C_6H_5	88	123-124	-67.0 (2.5; MeOH)
rac-**3f**	CH_3	Z-Aib	91	102-103	-
rac-**3g**	CH_3	Z-Ach*	92	134-136	-

*)

Ach – 1-aminocyclohexanecarboxylic acid



1a-g **2a-g** **3a-g**

The procedure is based on the rearrangement of 4-hydroxymethyl-1,3-oxazolin-5-one **2** readily accessible by cyclodehydration of ***N***-acylated serines **1** (Scheme 1).

Synthesis of **2a-g** was carried out conveniently by the treatment of **1a-g** with 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) in the presence of *N*-methylmorpholine (NMM). This procedure was found advantageous because any side products were easily removed by simple washing-out work-up, affording almost quantitatively crude **2a-g** sufficiently pure for the next synthetic step without additional purification.

1,3-Oxazolin-5-one **2a-g** rearranged subsequently, when heated in boiling m-xylene, into the final product **3a-g** in 68 - 96% overall yield (see Table 1). Oxazolin-5-ones **2f,g** derived from N-protected dipeptides with 2-substituted serine residue in C-terminal position **1f,g** rearranged noticeably less readily although appropriate **3f,g** were also isolated in high yield.

Determination of an enantiomeric purity of **3a,e** by HPLC on optically active stationary phase confirmed that rearrangement of optically active **1a,e** proceeded without loss of optical homogeneity.

Coupling of racemic **3** with rac-H-Ala-OBn by means of triazine condensing reagents, gave appropriate peptides as mixture of diastereomers in 94 - 97 % yield (see Table 2).

Table 2. Synthesis of peptides from 4,5-dihydro-1,3-oxazole-4-carboxylic acids ***3b-d****.*

3	R	R'?C=O	N-component	yield [%]	mp [°C]
rac-**3b**	CH_3	C_6H_5	*rac*-H-Ala-OBn	94	oil
rac-**3c**	$CH(CH_3)_2$	C_6H_5	"	97	oil
rac-**3d**	$CH_2CH(CH_3)_2$	C_6H_5	"	96	oil

Conclusions

This opened the convenient access for the application of 1,3-oxazole-4-carboxylic acids **3** as sterically hindered building block with severely reduced conformational flexibility of the peptide chain and building blocks for polyazole antibiotics and DNA bonding oligopeptides synthesis.

Acknowledgements

The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25.

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SYNTHESIS OF NEW CYCLIC RGD PEPTIDES FOR SPECIFIC TUMOR CELL TARGETING

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Introduction

The short RGD tripeptide binds specifically to αV-β3 integrins which are overexpressed in several types of cancer cells. Therefore, integrins constitute a potent target for drug delivery. Moreover the RGD sequence is more efficient when included in constrained cyclic peptides [1] Our purpose is to design a new class of tetracyclopeptides closed through an urea bond to increase the constraints. By reducing the number of aminoacids and by introducing a urea bond, we expect this cyclic targeting peptide to improve its selectivity and/or its affinity for αV-β3 receptors.

Materials and Methods

Peptide synthesis: The linear RGDK peptide was synthesized manually on solid phase following the Fmoc chemistry. The N-terminal Fmoc group and the Alloc protecting group on the ε-amine of the lysine residue were cleaved successively. The semi-protected peptide then was detached from the chlorotrityl resin (see Fig. 1), and the cyclisation between the two amines through an urea bond using *N,N'*-carbonyldiimidazole (CDI) was allowed for 12 - 16 h. The peptide was then labelled with a fluoresceine maleimide after the insertion of a S-protected cysteamine linker as previously described [2]. The pentacyclopeptide (cRGDfE) [1] and a control peptide (cRGEfE) showing a reduced binding affinity for targeted receptor were also synthesized as previously described [2].

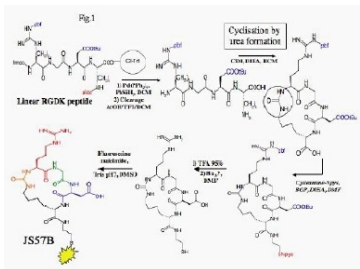

Fig. 1. Synthesis scheme of the cRGDK tetracyclopeptide.

Biological evaluation: The level of expression of αV-β3 receptors was first confirmed on expressing and non-expressing cells (HuVEC and A549 respectively) using either a CD51/CD61 or LM-609 antibody anti αV-β3. Both HuVEC and

A549 cells were incubated for 4 h or 24 h in their respective culture media with 1 μM of labelled peptide. Cells were then harvested, washed and analyzed by flow cytometry (FACS). Results are expressed in fluorescence arbitrary units. The pentacyclopeptide cRGDfE was also used for comparison [1].

Results

HPLC profile showed the quantitative cyclisation of the protected tetracyclopeptide (data not shown), thus avoiding purification. This allows its direct labelling onto a fluorochrome (this study) or its chemical ligation onto various type of amine containing structures (under development in our group) upon formation of an amide bond (see Fig. 1).

Using FACS analysis, the new tetracyclo peptide (JS57b) shows a better uptake (+17%) in αV-β3 cells than the initial pentacyclo-RGDfE peptide (P1) after 4h incubation (Fig. 2a). This increase of the fluorescence associated to HUVEC cells is more significant (+67%) after a 24h incubation period (Fig. 2b).

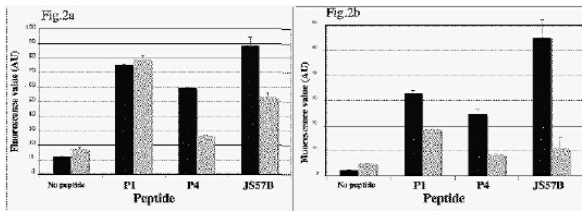

Fig. 2. FACS analysis of the two cell lines (HUVEC in black, A549 in grey) incubated for 4 h (2a) or 24 h (2b) with different peptides (P1: cRGDfE, P4: RGEfE, JS57B: cRGDK with the urea bond).

Discussion

The tetracyclopeptide JS57B shows a better uptake in αV-β3 expressing cells compared to the cRGDfE pentacyclopeptide. This is likely due to a better affinity and/or a better specificity towards the targeted αV-β3 receptors. This will have to be confirmed using more accurate technique such as BiaCore with purified αV-β3 receptors. Based on this strategy, several tetracyclopeptides containing the RGD sequence are being synthesized and will be further evaluated for their selectivity and affinity for αV-β3 receptors expressing cells in order to vectorize various antitumoral drugs into cancer cells.

Acknowledement:

L.R. received a fellowship from ARC and J.S. from “la Ligue Régionale contre le Cancer”. We are also grateful to INSERM, ARC and the CAMPLP for financial support.

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A PHOTOSWITCHABLE PEPTIDE MIMETIC

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Introduction

The β-hairpin is a peptide secondary structure motif involved in many biological and physiological processes. Some examples are DNA and RNA-binding proteins, processes related to Alzheimer's disease, and the interaction between antigens and antibodies.

Result and Discussion

Here we present the development of a stilbene-type peptide mimetic capable of light-triggered conformational changes between an unfolded random coil and a folded β-hairpin like conformation [1]. The peptide sequence used for this study originates from the S4 region of the TATA-box binding protein which is known to form a β-sheet [2].

In our study trans isomers of both cyclic and linear peptide mimetics were prepared by conventional SPPS. A stilbene unit capable of reversible *cis* - *trans* isomerization was incorporated into the peptide. By irradiation of a DMSO solution of the peptidomimetics at one of the absorption maxima of the stilbene unit (λ_{max}= 300 or 280 nm), photoisomerization was obtained. Thus, irradiation of the *trans* isomer of a linear peptide mimetic at 300 nm for 3 h resulted in a photostationary mixture containing 63% of the *cis* isomer, while for the cyclic peptide mimetic irradiation at 300 nm for 1.5 h produced 80% of the cis isomer (Fig. 1). The outcome of the photochemical studies was investigated by NMR spectroscopy and CD measurements. Our analytical data was then used as distance constrains in molecular modelling.

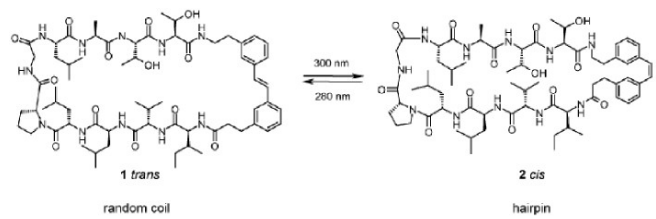

Fig. 1. The structure of the cyclic β-hairpin mimetics upon irradiation.

In contrast with its linear analogue, interstrand NOEs could be observed for the *cis* isomer of the cyclic peptidomimetic 2. This provides a strong evidence for its ability to form a β-hairpin. The concentration independency of the chemical shifts

(of the amide protons) along with diffusion studies confirmed the absence of aggregation under the conditions applied. The temperature coefficient ($\Delta\delta/\Delta T$) is a qualitative indicator for the involvement of amide protons in intramolecular hydrogen bonding and provides information about the secondary structure of the peptide. For the cyclic peptide mimetic 2, these temperature coefficients were observed to be alternating, a fact indicating ß-hairpin conformation.

Translational self-diffusion measurement (PGSE NMR) is an excellent tool for investigation of peptides and proteins. The translational diffusion coefficient (Dt) is proportional to the ratio of the molecular mass and the hydrodynamic radius of a compound [3]. Our PGSE NMR experiments revealed a small but significant change of the diffusion coefficient upon photoisomerization of the cyclic peptide mimetic (*trans* 1, Dt = 1.8 x 10^{-6} vs. *cis* 2, Dt = 1.4 x 10^{-6} cm^2/s), but not of the linear peptide mimetic. This change of Dt might indicate a decrease of the hydrodynamic size [4]. The larger diffusion coefficient of the folded *cis* isomer most probably originates from lower solvent accessibility of its intramolecular hydrogen-bonded amide protons, a fact confirming the conclusion derived from variable temperature ^{1}H NMR studies of the amide chemical shifts.

In addition, alteration of the CD spectrum of the cyclic petidomimetic was observed upon photoirradiation.1 The negative amide band for the *trans* isomer shifts from 214 nm to 220 nm wavelength upon isomerization to *cis*, a change likely to originate from a β-turn to β-hairpin transition.

Acknowledgements

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SULFONATES OF N-TRIAZINYLAMMONIUM SALTS AS HIGHLY EFFICIENT, INEXPENSIVE AND ENVIRONMENTALLY FRIENDLY COUPLING REAGENTS FOR PEPTIDE SYNTHESIS IN SOLUTION

Beata Kolesińska[1], Justyna Kolesińska[1], Anna Frymarkiewicz[1], Kamil K. Rożniakowski[1], Giuseppina Sabatino[2,3], Fabio Rizzolo[2,3], Anna M. Papini[2,3] and Zbigniew J. Kamiński[1]

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Introduction

Tetrafluoroborate 4-(4,6-dimethoxy-[1,3,5]triazin-2-yl)-4-methylmorpholinium has been found useful in synthesis of linear and cyclic peptides in SPPS and in step by step and/or fragment coupling in solution [1]. Herein we have attempted to expand this family of new generation triazine based coupling reagents by including inexpensive and environmentally friendly *N*-triazinylammonium salts of sulfonic acids [2].

Result and Discussion

4-(4,6-Dimethoxy-[1,3,5]triazin-2-yl)-4-methylmorpholinium toluene-4-sulfonate (**3a**) was prepared by treatment of 2-chloro-4,6-dimethoxy-1,3,5-triazine (**1**) with 4-methyl-morpholinium toluene-4-sulfonate (**2a**) in the presence of sodium bicarbonate (Fig. 1) [2]. Other analogues **3b-r** were prepared from salts of methanesulfonic acid (**2b**), trifluoromethanesulfonic acid (**2c**), camphorosulfonic acid (**2d**), and amidosulfonic acid (**2e**) with *N*-methylpiperidine, quinuclidine and DABCO.

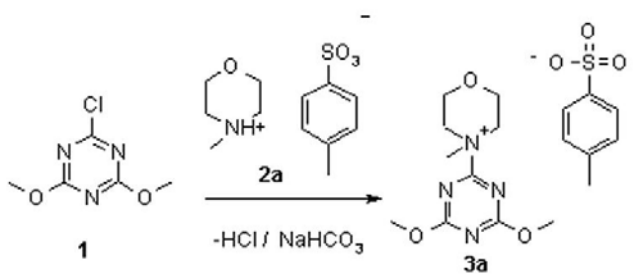

*Fig. 1. Synthesis of 4-(4,6-dimethoxy-[1,3,5]triazin-2-yl)-4-methylmorpholinium toluene-4-sulfonate (**3a**).*

A participation of triazine "superactive ester" **5** as active species in condensations mediated by **3a-d** has been documented in the model experiments [3]. All triazine

coupling reagents **3a-d** has been found versatile due to superior stability, increased solubility in typical solvents used in peptide synthesis and easy removable side-product **8** (Fig. 2).

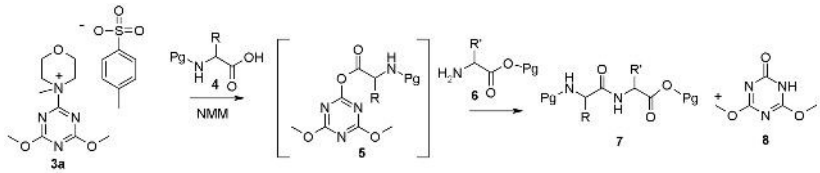

*Fig. 2. Synthesis of peptides by using of **3a**.*

Reagent **3a** has been found useful in the coupling involving Z-, Boc-, Fmoc-protecting groups affording di- pentapeptides in high yield is step by step approach and in fragment coupling.

Acknowledgements

The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25.

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AN NMR STUDY OF THE INTERACTION BETWEEN BRADYKININ AND ANGIOTENSIN-I AND SYNTHETIC PEPTIDE-BASED ANGIOTENSIN-I CONVERTING ENZYME CATALYTIC SITE MAQUETTES (CSM)

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Introduction

Angiotensin-I converting enzyme (ACE) belongs to the M2 family of the MA clan of zinc metallopeptidases and can act either as a dipeptidyl carboxypeptidase, or as an endopeptidase. Among the ACE peptide substrates, the most distinguished are angiotensin I (AI) and bradykinin (BK) due to their role in blood pressure regulation [1].

Despite the fact that biological data strongly suggest that the two ACE active sites exhibit different selectivity and activity towards physiological and exogenous substrates none experimental evidence for the interaction of AI and BK with ACE catalytic sites, is available so far. As previously described [2] we synthesized by solid-phase synthesis 36 - 46 residues ACE catalytic site maquettes (CSM) bearing the native sequence of the two ACE somatic form catalytic domains. High-resolution multinuclear NMR spectroscopy was applied to analyze the conformational features of ACE CSMs and ACE substrates AI and BK. Then titration experiments were conducted and ACE CSMs were titrated by AI/BK peptides, monitored by NMR.

Result and Discussion

Solution Structure of ACEC-36 CSM and Comparison with ACE testis Crystal Structure.

The NMR solution structure of the Zn(II)-loaded ACEC-36 maquette is present in Fig. 1B. The peptide construct exhibits three regions, one at each terminus and one in the intermediate spacer, having α-helix structure. These data are fully consistent with the pattern of sequential NOEs (data not shown) [2 - 4]. Consequently, NMR data suggest that both Zn-binding motifs are found in helical (C-terminal motif) or in helix-like (N-terminal motif) conformation as has been observed for other gluzincin metallopeptidases crystal structures [2].

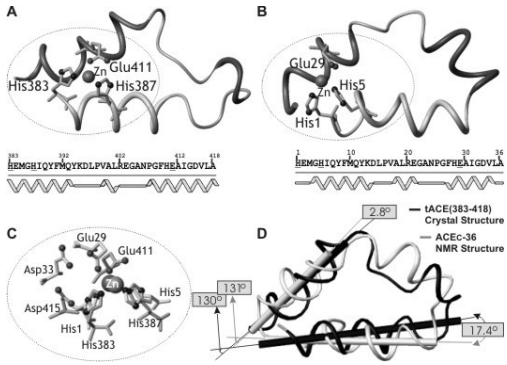

Fig. 1. Domain of the active zinc-binding site of ACE testis crystal structure (A); respective model of the family of 30 NMR structures of ACEc-36 CSM (B), and schematic representation of helical fragments. (C) Best fit of the Zn(II) ligands. (D) Overlapping of NMR and X-ray structure and angles comparison among the helix motifs axis.

The ACEC-36 NMR solution structure indicates remarkable similarities when compared to the homologous fragment of the ACE testis crystal structure (Fig. 1A) [5]. In particular, the three helical fragments of ACE synthetic peptides are also found in testis ACE crystal structure. In Fig. 1, a schematic representation of the helical fragments of the X-ray and NMR C-terminal active site models is presented.

Interaction between ACEs' CSM and AI/BK peptides studied through NMR

The interaction between of both ACE catalytic sites maquettes with the two most functionally important native substrate of ACE (AI and BK) have been studied in solution. Titration of ACE constructs with AI and BK peptides have been monitored by 1D and 2D NMR spectroscopy in order to determine the stoichiometry, affinity and ACE residues which play important role in substrate recognition and binding (data not shown).

Conclusions

The catalytic sites of somatic isoform differ slightly in amino acid composition, exhibiting in some cases different specificity and activity against natural substrates as AI and BK. Our studies concerning ACE somatic form catalytic sites structure and theirs interaction with AI and BK may aid in the design of novel and specific inhibitors of ACE, aiming to effectively blood pressure control.

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SYNTHESIS AND *IN VITRO* EFFECT OF PEPTIDES AND PEPTIDE-CONJUGATES FROM 16 kDa PROTEIN OF MYCOBACTERIUM TUBERCULOSIS ON IFN-γ PRODUCTION

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Introduction

The development of a quick and cost effective test to diagnose the infection with *Mycobacterium tuberculosis* is urgently needed. Synthetic peptides can be as efficient in detecting T-cell response as recombinant proteins with the advantage of faster production and lower costs. The 16 kDa protein of *Mycobacterium tuberculosis* provokes specific immune response, therefore related epitope peptides and peptide-conjugates can be considered as potential diagnostics. In our previous study we have determined the functional human T-cell epitope within the 91-110 region [1]. Based on these results we synthesised (i) native and non-native (alanine and β-alanine) flanking region elongated variants, (ii) 91-104 peptides with alanine substitution at different position according to the HLA DR and TCR binding sites [1] and (iii) C-terminal cysteine elongated variants of 91-106 peptide. Covalent conjugation of epitope peptides to macromolecular carriers can increase the immunogenicity by their repetitive presentation.

The long-term aim of the study is to develop a peptide-based candidates to improve the immunodiagnosis of tuberculosis.

Results

Peptides were prepared by solid phase synthesis using Boc/Bzl or Fmoc/*t*Bu strategy. Primary structure and homogenicity of synthetic peptides were characterised by analytical RP-HPLC, ESI MS and amino acid analysis.

Three different types of carrier molecules were prepared: (i) sequential oligopeptide (tetratuftsin derivative, (H-[Thr-Lys-Pro-Lys-Gly]$_4$-NH_2), (T20)), (ii) lysine dendrimer (H-Lys-Lys(H-Lys)-Arg-Arg-β-Ala-NH_2) (MAP) and (iii) branched chain polypeptides with lysine backbone poly[Lys(Ser$_{0.9}$-D,L-Ala$_{3.5}$)] (SAK), poly[Lys(Glu$_{1.0}$-D,L-Ala$_{3.5}$)] (EAK). 91-106-Cys peptide was conjugated via thioether bond and the obtained peptide-conjugates were carefully characterised [2,3].

We studied the T-cell response and IFN-γ production after treatment with the peptides on PBMC (Peripherial Blood Mononuclear Cells) from patients and healthy (PPD positive and negative) donors. We found that PPD negative, healthy

subjects did not response to peptides (Fig. 1/a). Radiologically and microbiologically diagnostised TB patients showed high level of IFN-γ production to the peptides (Fig. 1/b). T-cell response decreased during the antitubercular treatment of patients with active tuberculosis (Fig. 1/c). The carrier molecules did not influence the IFN-γ production, there was not unspecific stimulation of T-cells (Fig. 1/d).

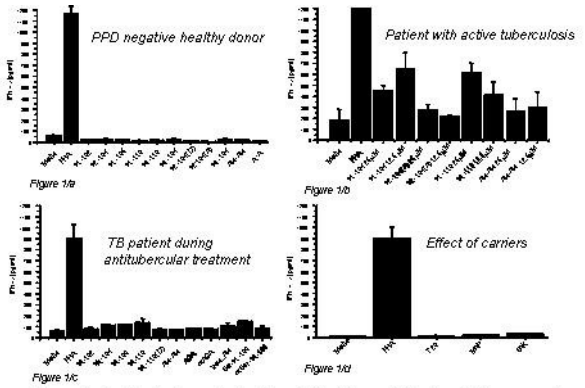

Figure 1/a,b,c,d *In vitro* analysis of T-cell stimulatory activity of synthetic compounds on PBMC from patients and healthy subjects.

Discussion

Modified T-cell epitope peptides provoke specific T-cell response and IFN-γ production. Based on the functional studies, promising peptide candidate was conjugated to different carrier molecules for more efficient antigen presentation. We can distinguish the profile of T-cell response of healthy controls and TB patients. Carrier molecules do not stimulate the T-cells to produce IFN-γ. The peptide-conjugates will be evaluated on PBMC and on whole blood from different donors.

Candidates were found for the development of a peptide based reagent to diagnose *Mycobacterium tuberculosis* infection.

Acknowledgements

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SECONDARY STRUCTURE ANALYSIS OF TWO RECOMBINANT PEPTIDES FROM ACTIVE SITES OF HUMAN ANGIOTENSIN CONVERTING ENZYME (ACE)

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Introduction

The two major abilities of Angiotensin Converting Enzyme is the conversion of Angiotensin-I to the highly potent vasoconstrictor Angiotensin-II [1] and the inactivation of the vasodilatory peptide bradykinin [2,3]. These activities render ACE through the renin–angiotensin–aldosterone system. There are two isoforms of ACE that transcribed from the same gene in a tissue-specific manner. Somatic ACE, which is present in brush-border epithelial cells and endothelial cells, exists as a glycoprotein composed of a single, large polypeptide chain of 1,306 amino acids, whereas in sperm cells it is a lower-molecular-mass glycoform of 732 amino acids. The somatic form consists of two homologous domains (N and C domain). Each domain contains an active site with a conserved HEXXH—24-aa spacer---EA**IGD** zinc binding motif [4]. Here we report the biotechnological production of two peptides of somatic ACE. These peptides are Ala_{361}-Gly_{468} and Ala_{958}-Ser_{1065} and correspond to N-terminal and C-terminal active sites of the enzyme.

Results

Expression vector pET-3a was used to subclone the DNA sequences of above mentioned peptides of human so-matic ACE. The resulting constructs, were used to transform competent *E. coli* cells.

Cells were cultured at 37 °C in 1L Luria Broth (LB) supplemented with 100 mg/ml ampicillin and induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for 6h. ACE peptides were expressed in insoluble inclusion bodies and were solubilized in a 8 M urea buffer. Soluble peptides were purified by gel filtration. Elution was occurred with the same buffer and 1ml fractions were collected and monitored by absorbance at 278 nm. The molecular mass of peptides was verified by Electrospray ionization-mass spectrometry. 12 mg of $ACE_{361\text{-}468}$ and 9 mg for the $ACE_{958\text{-}1065}$ were obtained per 1 L culture. The molecular mass of both recombinant protein fragments were in accordance with their theoretical calculations based on DNA sequences.

CD spectra were recorded at room temperature using a Jasco-J810 spectropolarimeter (Tokyo, Japan). A 0.1 cm optical path length quartz cell was used to record spectra of proteins in the far ultraviolet region (200 – 260 nm) at a protein concentration of 1.0 mg/ml. Spectra were signal-averaged over eight scans.

The value for the α-helical content was 35.8%, when $ACE_{361-468}$ was diluted in 66% 2,2,2-trifluoroethanol in a Tris buffer pH 7. This compares well with the theoretically calculated (Fig. 1A).

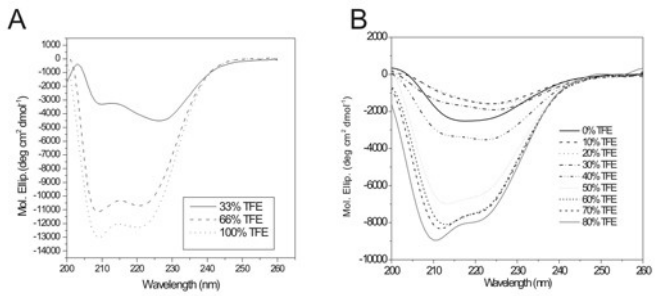

Fig.1. Circular Dichroism spectra of (A) $ACE_{361-468}$, (B) $ACE_{958-1065}$.

$ACE_{958-1065}$ peptide (Fig.1B) shown that at 70% of 2,2,2-trifluoroethanol the content of α-helix was 41.1%. This is also in consistence with the one found in the crystal structure of testis ACE, indicating that the backbone poly-peptide chain of the purified protein had an almost identical conformation [5].

Discussion

Overall, the expression system proved to be advantageous in speed and facility of purification. The peptides were more than 99% pure with a single step of purification. The in vitro folded recombinant proteins had almost identical secondary structural features compared with the homologous domain of testis ACE. This result indi-cates that the followed procedure did not the final structure of recombinant proteins. Despite the already resolved structure of testis ACE using X-ray crystallography [5], NMR spectroscopy study may be an alternative method for solving its structure in solution in the presence and in the absence of its substrate contributing in the design of new more selective inhibitors that might effectively control blood pres-sure. For this purpose the use of ^{15}N and/or ^{13}C labelled nutrition media will provide these peptides labelled.

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MODULATION OF ACTIVITY OF NKR-P1A AND NKR-P1B RECEPTORS ON NK CELLS BY COMBLIKE GLYCODENDRIMERS

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Introduction

NKR-P1 receptor in rodents plays an important role in the potency of natural killer cells to kill tumour, or infected, or stressed cells. Several isoforms of NKR-P1 have been identified: NKR-P1A (activation receptor), and NKR-P1B, NKR-P1D (inhibitory receptors). Natural ligands for these receptors are complicated oligosaccharide structures [1]. Simple oligosaccharides and some *N*-acetylated hexosamines have been recently identified as substitutes of these complex structures [2]. When expressed on dendrimeric structures, β-D-GlcNAc or α-D-Man monosaccharides were shown to significantly discriminate between NKR-P1A and NKR-P1B receptors, the behaviour not observed for simple monosaccharides [3]. We prepared comblike glycodendrimers bearing Tn antigen (α-D-GalNAc) and evaluated their ability to modulated activity of these receptors [4].

Result and Discussion

Comb-like dendrimers with 1, 2 and 3 copies of Tn antigen of each branch (**1-3**) were prepared by a chemoselective ligation using nucleophilic addition of cysteine-modified glycopeptides to an activated double bond (3-maleimidopropanoate-modified linear peptide: Ac-(Lys(Mal)Gly)$_4$-NH$_2$). Ligation was carried out in 15 mM phosphate buffer (pH 6.7) at RT for 1 - 24 h.

Compounds **1-3** effectively inhibited the binding of the anti-Tn monoclonal antibody 83D4 to plates coated with asialo-OSM in sugar substitution-dependent manner: IC_{50} values ranging from 10^{-5} M for 1 up to 10^{-8} M for **3**. In binding studies with NKR-P1A and NKR-P1B rat receptors (inhibition of binding of the receptor to β-D-GlcNAc$_{23}$BSA neoglycoprotein-coated plates) compounds **1** and **2** behaved almost identically: **1** had inhibitory activities comparable with the standard ligand, D-GlcNAc ($IC_{50} = 10^{-7}$ M). Compound **2** showed to be much better inhibitor with IC50 of 10^{-10} M, ranking this compound itself among the high affinity ligands for NKR-P1A. Different behaviour was observed for **3**: for NKR-P1A receptor **3** inhibited less than the negative control, D-mannose; for NKR-P1B receptor **3** showed specific binding with $IC_{50} = 3 \times 10^{-11}$ M.

In cytotoxicity assays we tested the influence of the individual compounds on natural killing of the NK sensitive tumour cell line YAC-1 and NK resistant rat cell

line P815, respectively. At all concentrations and effector: target rations tested, we found that **1** and **2** enhance and **3** inhibits natural killing of these cells. We also found that **3** inhibited natural killing of P815 cell line even in the equimolar presence of **1** or **2**, or even their combination, Fig. 1A. To evaluate the immunological effects of the dendrimers from the standpoint of their potential use in human therapies, we repeated this test using the human NK resistant cell line RAJI and obtained very similar results, Fig. 1B.

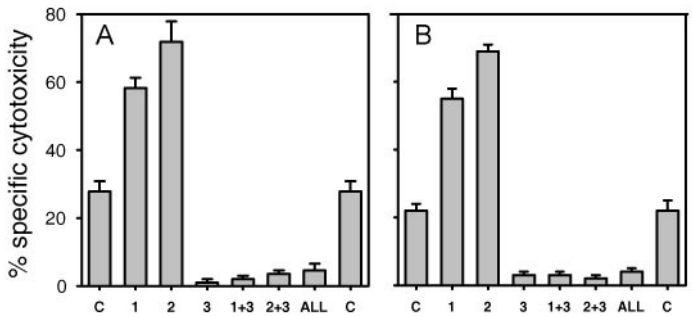

Fig. 1. Combined effects of mixtures of compounds **1+3**, **2+3** and **1+2+3** on enhancement/inhibition of natural killing of NK resistant mouse tumor cell line P815 (A) and human tumor cell line RAJI (B).

Acknowledgements

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DIASTEREOSELECTIVE SYNTHESIS OF (Z)-FLUOROALKENE DIPEPTIDE ISOSTERES BASED ON ORGANOCOPPER-MEDIATED REDUCTION/DIRECT ALKYLATION VIA TRANSMETALATION

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Introduction

Replacement of native hydrolyzable peptide bonds with non-hydrolyzable mimetics is an established approach toward overcoming their major drawbacks including rapid proteolysis. Furthermore, conformationally restricted analogues represent attractive structural approaches toward more effective agents. In particular, alkene-type dipeptide isosteres containing (*E*)-alkene or (*Z*)-fluoroalkene units are thought to be potential dipeptide mimetics. (*Z*)-Fluoroalkene dipeptide isosteres are structurally similar to (*E*)-alkene dipeptide isosteres (EADIs), but differ in their electrostatic nature. This plays a significant role in their intra- and inter-molecular interactions. Of note, due to the presence of the highly electronegative fluorine substituent, (*Z*)-fluoroalkene dipeptide isosteres more faithfully resemble native peptides than do EADIs.

Recently, we disclosed a new synthetic approach to (*Z*)-fluoroalkene dipeptide isosteres utilizing organocopper- or SmI2-mediated reduction of δ-amino-γ,γ-difluoro-α,β-enoates *via* a single electron transfer mechanism [1]. However, stereoselective incorporation of α-substituents had yet to be achieved. Herein, we describe the diastereoselective synthesis of (*Z*)-fluoroalkene isosteres utilizing an efficient one-pot reaction involving consecutive organocopper-mediated reduction/transmetalation/asymmetric alkylation.

Results and Discussion

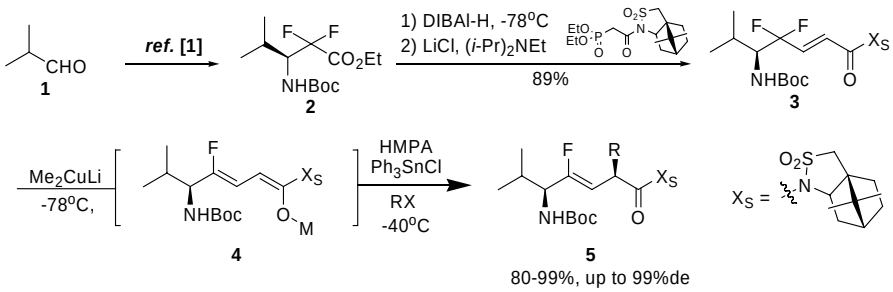
To develop an efficient methodology, we envisioned a one-pot construction of the fluoroalkene isostere framework by organocopper-mediated reduction of γ,γ-difluoro-α,β-unsaturated carbonyl compounds carrying a chiral auxiliary. This would be followed by trapping of the dienolate species with alkyl halides regio- and stereoselectively.

The α,α-difluoro-β-amino ester **2** was reduced to the aldehyde and then subjected to Horner-Wadsworth-Emmons type coupling with (*S*)-*N*-diethoxyphosphono-acetylcamphorsultam [2] to give *N*-enoyl sultam **3** in *E*-selective manner. This served as a substrate for organocopper-mediated reduction/transmetalation/ asymmetirc alkylation.

N-enoyl sultam **3** was subjected to organocopper-mediated reduction via successive two-electron transfer to provide the stable Li or Cu dienolate intermediate **4**. Transmetalation technique of **3** from Li or Cu dienolate to more reactive tin

dienolate [3], followed by trapping the dienolate by various alkyl halides gave α-substituted fluoroalkene isosteres **5** in high chemical yields and with good to excellent diastereoselectivities (90–99% de). The optical purity of each product was determined by RP-HPLC. Fluoroolefinic geometries of all products were established by ^{1}H NMR [4], and the absolute configurations of the alkyl groups at a-position were determined by circular dichroism measurements with use of an empirical rule after converting to the corresponding methyl esters [5].

In conclusion, we have developed the new synthetic methodology for the highly diastereoselective synthesis of functionalized (*Z*)-fluoroalkene dipeptide isosteres. The key utility of the newly developed methodology can be found in its one-pot reduction/transmetalation/asymmetric alkylation methodology. Further studies to apply fluoroalkene isosteres to bioactive peptides are now in progress.



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IMMUNOENZYMATIC ASSAY WITH PEPTIDE ANTIGENS IMMOBILIZED ON CELLULOSE: EFFECT OF THE LINKER ON ANTIBODY RECOGNITION

Beata Kolesińska[1], Sebastian Grabowski[2], Iwona Konieczna[3], Wiesław Kaca[2,3], Elisa Peroni[4,5], Anna M. Papini[4,5], Paulo Rovero[4,6] and Zbigniew J. Kamiński[1]

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Introduction

Synthetic peptides are largely used as antigens in solid-phase immunoenzymatic assays (ELISA) for recognition of antibodies (Abs) in biomedical research and, most importantly, in the set up of diagnostic methods. It is well known that the method of peptide immobilization on the solid support is very important for a correct Ab recognition. This aspect is crucial when the antigens are covalently linked to the support, as in the SPOT assay, based on peptides covalently linked to cellulose [1]. Therefore in our studies on the pathogenic relevance of auto-antibodies in Multiple Sclerosis (MS) and antibodies in patients infected with *Helicobacter pylori* (Hp) we synthesized appropriate oligopeptides immobilized on cellulose via N- or C-termini, using standard β-alanine linkers as well as a new linker, developed for this particular studies, based on isocyanuric acid derivatives [2].

Results and Discussion

We previously developed the structure-based designed glycopeptide CSF114(Glc), as antigenic probe to detect sugar-specific autoantibodies (correlating with disease activity) by ELISA in a consistent population of MS patients [3]. CSF114(Glc) was immoblized on cellulose *via* C-terminus directly (m = 0) or using β-alanine spacer (m = 1, 2, 3) **1**, or isocyanuric linker **2** [2] and *via* N-terminus (Fig. 1) using *N*-triazine linker **3** [4].

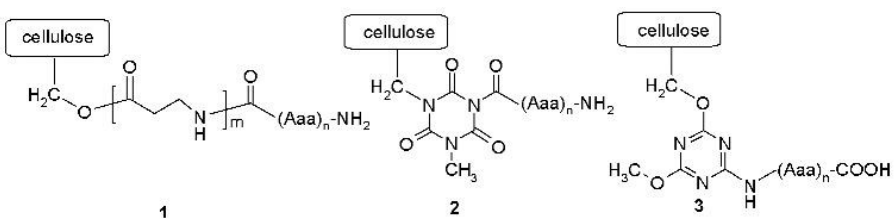

Fig. 1. Peptides anchored to cellulose via C-, and N-terminus.

The immunological assays were performed on the glycopeptide synthesized on cellulose versus the non glycosylated one using MS sera as positive control and normal blood donor sera as negative control.

The results let us to asses that only the glycopeptide immobilized via C-terminus was able to discriminate between positive and negative sera. Moreover we further investigated the role of the different linkers. Preliminary results showed that CSF114(Glc) linked to cellulose *via* βAla_3 spacer **1** was able to better discriminate between positive and negative sera in comparison with the glycopeptide linked via isocyanuric linker **2** or peptide linked N-terminally **3**.

In order to study the recognitions of Hp UreB F8 epitope: Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe and UB-33 epitope: Cys-His-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile were synthesized directly on the cellulose plate using triazine based condensing reagent. 321-339 Hp urease fragments were anchored on the cellulose plate directly *via* C-terminus or *via* βAla_3 linker **1**, *via* izocyanuric acid **2** and N-terminally *via* triazine **3**.

In the case of UreB F8 Hp urease smallest epitope (SIKEDVQF), and epitope UB-33 (321-339 Hp fragment: CHHLDKSIKEDVQFADSRI) the strongest and the most specific reactions with of Hp suffering patients sera were obtained for isocyanuric acid linker **2**. Less specific reactions were obtained with epitopes anchored to the cellulose support N-terminally via triazine linker **3**. Surprisingly, no reaction with sera Hp suffering patients were obtained for epitopes linked directly to the cellulose support with carboxylic function or *via* βAla spacer **1**.

Acknowledgements

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SYNTHESIS OF EFRAPEPTINS AND SIMILAR PEPTAIBIOTICS

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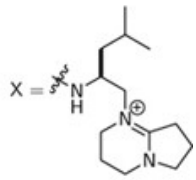
Peptaibiotics are non-ribosomally produced linear peptides which have a high content of α,α-dialkylated amino acids like α-amino isobutyric acid (Aib) or L and D-isovaline (Iva) and cyclic amino acids like 2-amino-2-cyclopropyl carboxylic acid (Acc), pipecolic acid (Pip) or β-methyl proline ($^{\beta Me}$Pro) [1]. They can be isolated from fungi, marine sponges or bacteria and often have interesting biological or medicinal properties. Efrapeptins are a class of sequence-homologous peptaibiotics (efrapeptins C-G) that are produced by fungi of the species *Tolypocladium* [2,3]. They are inhibitors of F_1-ATPase [4] show insecticidal and antiproliferative properties [5] and are also active against the malaria pathogen *Plasmodium falciparum* [6]. Efrapeptins contain an unusual cationic head group derived from leucinol at the C-terminus, while the N-terminus is acetylated [7]. Adenopeptin is a peptide antibiotic which is produced by fungi of the species *Chrysosporium*. It shows apoptotic properties in cells oncogenically transformed by adenoviruses. It consists mostly of the α,α-dialkylated amino acids Aib and Iva, has the same cationic head group as the efrapeptins and the N-terminus is also acetylated [8]. Efrapeptins C-G, adenopeptin and an adenopeptin analogue were synthesised chemically for the first time by applying a convenient method for the introduction of Aib and Iva residues [7]. The incorporation of the Aib and Iva residues in solid phase syntheses succeeds smoothly using the highly reactive azido acid chloride for acylation of the resin bound amino component [9] followed by reduction of the resulting azide to the amine using Vilarrasa's reagent $[Et_3NH]^+[Sn(SPh)_3]^-$ [10]. The full peptide sequences are assembled from two fragments and the cationic head group. The N-terminal fragment and the central fragment are synthesised by solid phase methods using the 2-chlorotrityl resin. The C-terminal fragment is obtained by solution techniques from leucinol and DBN. The following segment condensations succeed using *N*-HATU as the coupling reagent. A special challenge is the enzyme based synthesis of L-H-Iva-OH as starting material.

Ac-Pip-Aib-Pip-Xaa-Aib-Leu-$^{\beta}$Ala-Gly-Aib-Aib-Pip-Aib-Yaa-Leu-Zaa-X

	Xaa	Yaa	Zaa
efrapeptin C	Aib	Gly	Aib
efrapeptin D	Aib	Gly	Iva
efrapeptin E	Iva	Gly	Iva
efrapeptin F	Aib	Ala	Iva
efrapeptin G	Iva	Ala	Iva



Ac-Pro-Iva-Iva-$^{\beta}$Ala-Gly-Aib-Aib-Iva-Aib-Xaa-Aib-Yaa-Iva-X

	Xaa	Yaa
adenopeptin	Aib	Pip
adenopeptin analogue	Pip	Aib

The enzymatic resolution of the α-azido carboxamide using *Mycobacterium neoaurum* yields L-H-Iva-OH with an enantiomeric excess of 98 %. The amino acid is subsequently converted to the azido acid chloride via diazotransfer followed by reaction with thionyl chloride. CD-spectroscopic data give information about the three-dimensional structure of efrapeptins and adenopeptins. In the case of efrapeptins C-G the obtained data indicate a 3_{10}-helical structure. The exchange of Aib against Iva or Gly against Ala has only small effects on structural aspects as the CD-spectra are quite similar for all efrapeptins. CD-spectroscopic data for adenopeptin and the adenopeptin analogue give completely different results though there are only marginal changes in the amino acid sequences of these two peptides. The interchange of Aib10 and Pip12 leads to strong changes in the structural properties which is displayed by the CD-spectroscopic data. In the case of the adenopeptin analogue the expected 3_{10}-helical structure can be assumed from the obtained spectrum, while in the case of adenopeptin no clear spectrum was obtained. The presented synthetic strategy enables the synthesis of a vast variety of efrapeptin and adenopeptin analogues for conformational and biological studies. As efrapeptins and adenopeptins are considered to be potential drugs these conformational studies could provide new lead structures for the improvement of biological and medicinal properties.

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SYNTHESIS OF GLP-1 ANALOGUES AS POTENTIAL AGENTS FOR BLOOD GLUCOSE CONTROL

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Introduction

Type II diabetes, which represents about 90% of all cases, is fast becoming an epidemic in many societies, with 170 million cases diagnosed worldwide to date (WHO). Glucagon-like peptide 1 (GLP-1) (7-37), a 31 amino acid peptide incretin hormone secreted by the lower intestine in response to feeding, naturally stimulates pancreatic insulin secretion to control glucose levels [1]. Exogenous administration of GLP-1 and related analogues has shown great promise as a major component of an anti-diabetes therapeutic regimen.

One of the major obstacles to development of GLP-1-based therapeutics is the short circulating half-life of a few minutes, due to proteolytic cleavage and renal clearance. In particular, the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) cleaves the N terminal His^{7}-Ala^{8} dimer to yield the GLP-1 (9-37) fragment, which has antagonistic properties. Thus, effective therapeutics must incorporate derivatives stabilized against proteolysis while being packaged in an extended release format to retard renal clearance and thus avoid daily injections. GLP-1 analogues with affinity for serum albumin exhibit extended circulating half-lives but have reduced potency. Others stable against DPP-IV-mediated proteolysis (e.g. Exendin-4), having reached the market (Byetta, Amylin-Lilly), are only about 50% homologous to GLP-1, raising questions of immunogenicity when incorporated into controlled release formulations. Thus, there remains strong interest and need for development of potent, stable and long-acting GLP-1 analogues as anti-diabetic agents. We have synthesized a number of GLP-1 analogues stabilized against DPP-IV proteolysis and report their ability to stimulate insulin release in a cell culture assay.

Result and Discussion

All peptides were synthesized by solid phase methodology using Fmoc chemistry on WANG resins and purified by RP-HPLC. Each analogue was stabilized to DPP-IV hydrolysis by a combination of amide bond replacement between Ala8 and Glu9 and/or amino acid substitution of Ala8. The native sequence is given below

GLP-1 (7-37) HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG

The following GLP-1 analogues were synthesized: SP001, SP002, SP005, SP006, SP007 and SP008. Their DPP-IV stabilities were measured by incubating each analogue with DPP-IV at pH 7.5, 37 °C for the stated times, and monitoring the cleavage by RP-HPLC [2]. Only the SP007 analogue was susceptible to DPP-IV hydrolysis, with the N terminal dipeptide completely hydrolyzed within 24 hr. The others were completely stable to DPP-IV when compared to non-enzymatic degradation as control.

Peptide	Activity of each peptide as **percentage** of insulin release induced compared to cells only (100%)	
	15 µM	**3 µM**
Native GLP-1 7-37	196 ± 36	110 ± 20
SP001	126 ± 74	92 ± 28
SP002	**254 ± 70**	**192 ± 38**
SP005	**358 ± 128**	**171 ± 53**
SP006	**330 ± 89**	**154 ± 25**
SP007	235 ± 91	113 ± 65
SP008	170 ± 48	112 ± 44
Native GLP-1 9-37 control	123 ± 17	ND
Exendin-4 (Exenatide)	172 ± 57	ND

Table 1 GLP-1 analogue-induced insulin release in TC-6 cells

The ability of each analogue to stimulate glucose-dependent insulin secretion in a murine- derived, pancreatic beta-TC-6 cell line in culture was next examined. The data is shown in Table 1, with levels of peptide-stimulated insulin release in the presence of 5 mM glucose shown as a percentage of insulin release by cells in the presence of glucose alone. The SP002, SP005, and SP006 analogues at both 15 µM and 3 µM concentrations were able stimulate insulin secretion at 1 – 2 fold levels above that seen for native GLP-1 (7-37). Furthermore, at 15 µM each of these 3 analogues stimulated insulin secretion at levels comparable or greater than that induced by exendin-4, which is being prescribed for treatment of Type II diabetes. These results have led to further trials evaluating them for their ability to induce insulin release in isolated pancreatic islets, as well as control of hyperglycemia in a murine model.

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DIRECT ANTIPROLIFERATIVE EFFECT ON BREAST CANCER CELLS OF NEW LEUPROLIDE ANALOGUES WITH MODIFICATIONS IN POSITIONS 3 & 6

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Introduction

Analogues of GnRH have been widely used in oncology and gynaecology to induce reversible chemical castration [1, 2]. In addition to the classic hypophysiotropic action of GnRH, it has been shown that many malignant cells, such as breast cancer cells, secrete GnRH and express the GnRH receptor/s [2, 3]. Leuprolide is a synthetic GnRH agonist and active pharmaceutical ingredient of commercially available drugs. In order to study the effect of modifications in positions 3 and 6 of leuprolide on breast cancer cell proliferation, we synthesized fifteen new conformationally restricted analogues. D-Leu^6 of Leuprolide was substituted by α,α dialkyl amino acids (Aib: α-aminoisobutyric acid, Deg: diethylglycine), D-Gly(*t*Bu) and β-cycloexyl-D-alanine (D-Cha) and/or Trp^3 by D–Trp, D- and L-1,2,3,4,-tetrahydro-isoquinoline-3-carboxylic acid (Tic) (Table 1).

Results

The overall yield of the synthesis of the new GnRH analogues was in the range of 41-55%. Of α,α–dialkyl amino acids (I and V) or D- /L- Gly(*t*Bu) (IX and XI) in position 6 resulted in a decrease of binding affinity in comparison to leuprolide (Table 1). However, the pituitary binding affinities of analogues I and IX are higher than that of GnRH. GnRH and analogues I and XIV had no significant effect on breast cancer cell proliferation, despite their high binding affinity to the pituitary GnRH receptor. Leuprolide and analogue V significantly inhibited MCF-7 proliferation in comparable potencies, but analogues IX and XI had higher activity.

Modification of Trp^3 with non natural aromatic amino acids deteriorates pituitary binding affinity and increases antiproliferative activity in analogues containing α,α-dialkyl amino acids or keeps the same activity in the other analogues (Table 1). This divergence of activity might be explained by binding of the GnRH analogues to different binding sites on the high affinity GnRH receptor on breast cancer cells and/or the presence of a low-affinity receptor (maybe, a receptor for GnRH II or alternative GnRH receptor splice variants) on breast cancer cells which selectively binds GnRH analogues having modifications in position 3 [4,5]. Certainly these assumptions deserve further investigation and it cannot be ruled out at present that increased potency may be due to a reduced enzymic rate of degradation. It should be noted that the inhibitory effect of IV, VII & VIII was higher than that of leuprolide at the significance level of $p<0.05$ by 48%, 68% & 96%, respectively.

Table 1: GnRH receptor binding affinity of new GnRH analogues in αT3-1 cell membranes using [^{125}I-Tyr^5]GnRH as radioligand; GnRH and leuprolide are included as control. The

inhibitory effect on MCF-7 cell proliferation after 48 h incubation in the presence (at 30 μM concentration) or absence of test analogues (control) is also presented.

Code	Peptide	Binding Affinity IC_{50}s, nM	Inhibition (% of control)
	GnRH	5 ± 0.1	9 ± 5
	[D-Leu^6, *des*Gly^{10}]GnRH-NHEt /Leuprolide	0.65 ± 0.08	25 ± 5*
I	[Aib^6, *des*Gly^{10}]GnRH-NHEt	4.16 ± 0.1	9 ± 4
II	[D-Trp^3, Aib^6, *des*Gly^{10}]GnRH-NHEt	694 ± 0.5	32 ± 7*
III	[L-Tic^3, Aib^6, *des*Gly^{10}]GnRH-NHEt	700 ± 0.5	29 ± 8*
IV	[D-Tic^3, Aib^6, *des*Gly^{10}]GnRH-NHEt	188 ± 0.3	37 ± 5*
V	[Deg^6, *des*Gly^{10}]GnRH-NHEt	20 ± 0.08	26 ± 6*
VI	[D-Trp^3, Deg^6, *des*Gly^{10}]GnRH-NHEt	<1000	32 ± 5*
VII	[L-Tic^3, Deg^6, *des*Gly^{10}]GnRH-NHEt	7.8 ± 0.2	42 ± 7*
VIII	[D-Tic^3, Deg^6, *des*Gly^{10}]GnRH-NHEt	358 ±0 .4	49 ± 8*
IX	[Gly(^{t}Bu)^6, *des*Gly^{10}]-GnRH-NHEt	1.1 ± 0.1	40 ± 9*
X	[D-Tic^3, Gly(^{t}Bu)^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	41 ± 8*
XI	[D-Gly(^{t}Bu)^6, *des*Gly^{10}]-GnRH-NHEt	260 ± 3.2	44 ± 7*
XII	[D-Trp^3, D-Gly(tBu)^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	44 ± 7*
XII	[D-Tic^3, D-Gly(^{t}Bu)^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	34 ± 9*
XIII	[D-Tic^3, D-Gly(tBu)^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	34 ± 9*
XIV	[D-Cha^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	10 ± 5
XV	[D-Trp^3, D-Cha^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	16 ± 6
XVI	[D-Tic^3, D-Cha^6, *des*Gly^{10}]-GnRH-NHEt	nt[#]	3 ± 5

One asterisk (*) indicates significant difference from control at the level of $P<0.05$, nt, not tested compounds

Conclusions

The inhibitory effect on the proliferation of human breast cancer cells greatly depends from the nature of the substituted amino acid in position 6 and is usually increased by simultaneously substitutions of Trp^3 with non natural aromatic amino acids. Furthermore, we obtained six new analogues of leuprolide which have higher antiproliferative effect than leuprolide.

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STRUCTURAL VARIATION IN E3 LIGASES UBIQUITINATION PLATFORM

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Introduction

p53 is a tumor suppressor protein, called also "The Guardian of the cells and genome" and one of the most effective defensive weapons of human body against carcinogenesis [1]. Activation and/or inactivation of p53 is a critical step in the tumor suppression/formation process, respectively. The suppression of p53 levels is being indirectly regulated by the protein itself, which activates the expression of the oncogene mdm2 (murine double minute 2), which expresses the HDM2 protein [2]. HDMX [3], a HDM2 homologue, is also being implicated in p53 suppression. The C-terminal regions of HDM2 and HDMX are RING finger domains and catalyze the last stage of protein signaling for proteolysis, through the ubiquitin pathway acting therefore as E3 ubiquitin ligases. The activity of p53 as a transcription factor is strongly coupled to HDM2 protein overexpression which is a molecular mechanism of the cell towards p53 ubiquitinylation, degradation and inactivation.

Ubiquitination of proteins occurs through a pathway that involves E1, E2 and E3 enzymes [4]. The E1 enzyme binds, and through the synergistically action of ATP, activates ubiquitin, a protein which is capable of forming an isopeptide bond with a side chain lysine on a target protein. E2 ubiquitin conjugating enzyme associates with ubiquitin which is trans-esterified to a conserved cysteine of E2 enzyme. E2 act as a transporter of ubiquitin to the target protein. E3 enzyme couples with the E2 to bind the substrate and assemble a multi-ubiquitin chain on the substrate catalyzing the formation of a covalent bond between ubiquitin and substrates.

Results and Discussion

HDM2 and HDMX Ring fingers possess a non-typical Ring finger Zn-binding motif, where a Cys of the Zinc binding C_3HC_4 ring finger motif, has been replaced by a Threonine (Thr25, Thr455 in native sequence).

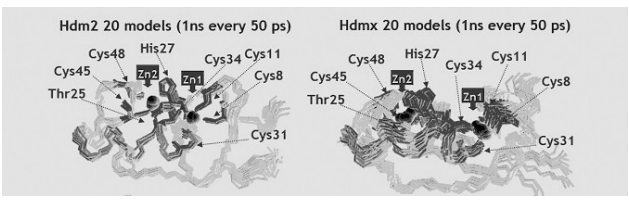

Fig. 1. HDM2 and HDMX RING finger models extracted every 50 ps during the 1 ns MD course.

This threonine seems to be conserved among HDM2 orthologs, and HDMX. Since no other RING finger sequence with a TGH Zn-binding motif is known so-far, its role to Zinc chelation is debated [5] An overview of experimentally determined 3D structure RING fingers structures, suggest that sequence and topological features, with apparent impact on folding and architecture of Ring fingers, suggest that this TGH motif is highly plausible to be the second Zinc(II) binding motif in HDM2 and HDMX Ring fingers. It is noteworthy that metal binding to TGH sequence suggests a rather similar fold to other Ring fingers rather than the fold adopted when metal ion is bound to CIVH motif. TGH motif is found to the loop between the first and the second (β1 and β2) antiparallel β-sheets, which comprise the core of the typical ββαβ fold of the Ring finger structures.

The structure and the stability of HDM2 structure has been monitored through Molecular Dynamics Simulations (Fig. 1) using AMBER8.0. MD data suggest that models where the TGH motif is involved through Thr25 and His27 to metal binding the typical ββαβ Ring finger fold remains, but it collapses when CIVH sequence bind to Zn.

Without any doubt the importance of homo- and hetero-dimeric complexes in ubiquitin ligase signaling is of immense interest and new approach against cancer targets to the E3 ligases and their interaction with other E3 ligases and/or E2 enzymes [6] It is also well established that HDM2-HDMX interaction regulates the ubiquitination not only of p53 protein but also the (auto)ubiquitination of HDM2 and RING domains determine the stabilization or destabilization of these proteins.

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LC-MS/MS DETECTION AND IDENTIFICATION OF NINE NODULARINS, CYCLIC PENTAPEPTIDES PRODUCED BY THE CYANOBACTERIUM *NODULARIA SPUMIGENA*

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Introduction

Nodularin ([6(Z)Adda^3]NOD) is a cyclic pentapeptide hepatotoxin (LD_{50} = 50 μg kg^{-1}) synthesised by a bloom-forming planktonic cyanobacterium *Nodularia spumigena*. The toxicity of nodularin is associated with inhibition of eukaryotic protein phosphatase type 1 and 2A [1]. The structure of nodularin is cyclo[D-erythro-β-methylAsp(iso-linkage)-L-Arg-Adda-D-Glu(iso-linkage)-2-(methyl-amino)-2(*Z*)-dehydrobutyric acid], where Adda is the C20 β-amino acid, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid. The configuration of the Adda - Glu part of the toxins is essential for their activity [2].

In the current study the liquid chromatography ionspray mass spectrometry (LC/ISP-MS) instrument with quadrupole analyser associated with time-of-flight (TOF) was applied to characterize nodularin variants present in bloom and culture samples of *N. spumigena* from the Gulf of Gdańsk (Baltic Sea).

Results

Extracts of bloom and culture *N. spumigena* cells were analysed by hybrid quadrupole-time-of-flight liquid chromatography mass spectrometry/mass spectrometry (TOF LC-MS/MS) with ionspray (ISP) and collission induced dissociation (CID). Amino acid structures and sequence of the analysed peptides were derived from the fragmentation pattern of their $[M+H]^+$ ions (Fig. 1). Nodularin [6(*E*)Adda^3]NOD with the molecular ion at m/z 825 $[M+H]^+$ was detected as the main component among nine variants extracted from *N. spumigena* cells. In TIC, a second peak of m/z 825 was observed. Since its fragmentation pattern was the same as [6(*E*)Adda^3]NOD, it might be concluded that both compounds are geometrical isomer.

Additionally, ISPMS/CID analyses revealed a presence of linear NOD ($[M+H]^+$ = 843), which is considered to be a biogenic precursor of the compound. In the extracts three demethylated variants have been found, as well. The sites of demethylation were located on aspartic acid [Asp^1]NOD, Adda residue [DMAdda^3]NOD, and dehydrobutyric acid [dhb^5]NOD. In three other nodularin variants an additional methyl group is located in Adda [MeAdda]NOD, Glu [Glu^4(OMe)]NOD and MeAsp [MeAsp^1(OMe)]NOD residues.

Discussion

In *N. spumigena* from New Zealand the linear NOD, [6(E)Adda^3]NOD, [6(*Z*)Adda^3]NOD, [D-Asp^1]NOD and [DMAdda^3]NOD were identified [3] Additionally, Rinehart *et al.* [2] reported the detection of [Glu^4(OMe)]NOD. Until now, in *N. spumigena* from the Baltic Sea only the unmodified nodularin and the [D-Asp^1]NOD have been detected.

In the current study, the ISPMS/CID analyses of the Baltic *N. spumigena* cell extracts showed that the variety of nodularin analogues produced by the species was larger than was previously thought. Generally, the same nodularin variants were produced in culture and in the field, but they were present in the cell in different proportions. Three of the total nine nodularin variants characterised in the present study, [dhb5]NOD, [MeAdda]NOD and [MeAsp^1(OMe)]NOD have not been identified in *N. spumigena* from New Zealand [3].

In fact, all the modifications in nodularin structure, except for an additional methyl group in Adda residue, have been characterized for microcystins.

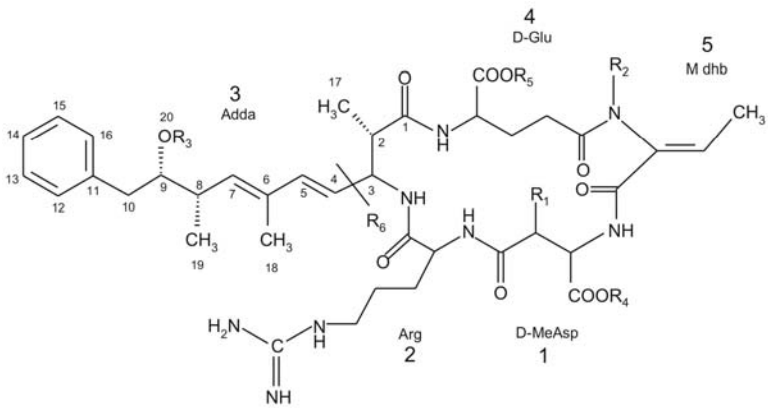

Fig. 1. Structures of the analysed peptides.

Acknowledgements

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BETA-AMYLOID INTERACTIONS WITH METALS

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Introduction

Extracellular aggregates of beta-amyloid (Abeta) are thought to be neurotoxic agents in Alzheimer's disease (AD) [1]. Disruption of metal homeostasis resulting in the accumulation of abnormally high concentrations of metals (Cu, Fe, Zn, Al) within the Abeta plaques has been found in the brains of AD patients [2]. Abeta deposits in the AD are associated with markers of oxidative stress [3]. Additionally, Abeta in the senile plaques is oxidatively modified. Redox active metals like copper and iron bound by Abeta are significant sources of reactive oxygen species (ROS). Ability of Abeta to bind both redox active and redox inactive metals like zinc and aluminum may modify the redox equilibrium, regarded as one of the mechanisms of the peptide toxicity.

In this work we describe the changes of oxidative and hydrolytic properties of Abeta (1-40) (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) that are affected by the peptide folding and metal binding.

Results

Metal binding by Abeta induces changes in the CD spectra that are metal- and concentration-dependent. CDNN analysis of the spectra [4] revealed that the metal binding affects mainly random coil content or a-helix in the secondary structure of Abeta, leaving the β-sheet relatively unchanged. Generally, during the first two hours of a complex formation a decrease of α-helix and an increase of β-sheet content take place. Changes in the concentration of random coil are metal dependent. After 24 hour of metal-peptide complex incubation further increase of random coil and β-sheet content are observable with a simultaneous decrease of a-helix concentration. Abeta-metal complexes demonstrated lower Thioflavine T fluorescence [5] than free Abeta.

Abeta is able to decompose a series of fluorescein esters with increasing number of carbon atoms in the ester moiety, being the most active towards butyrate. After 24 hour incubation of Abeta in PBS before ester addition, a decrease of ester hydrolysis was observed. Our earlier studies with metal chelators (EDTA, EGTA, desferrioxamine, phenanthroline) demonstrated reduction of ester hydrolysis by Abeta, suggesting that the reaction may be metal-dependent [6]. Therefore we decided to study the influence of metals on butyrate hydrolysis. Suprisingly, none of the metal studied increased hydrolysis by fresh Abeta. On the contrary, a decrease of hydrolysis in the presence of all studied metals was observed. The strongest effect of inhibition was noticed in the presence of copper. Similarly to free Abeta after 24 hour incubation of the metal-Abeta complex a decrease of hydrolysis was observed. Again, in the presence of copper hydrolysis was lower, while iron-Abeta complex slightly increased the hydrolysis reaction.

Oxidation of non-fluorescent 2',7'-dichlorodihydrofluorescein (H_2DCF) to highly fluorescent 2',7'-dichlorofluorescein (DCF) (exc. 495 nm, em. 525 nm) was used to measure oxidative properties of Abeta [6]. Among studied by us Abeta-metal complexes, only Abeta-copper complexes demonstrated significant increase of H_2DCF oxidation. Soluble complexes with other metals did not potentiate H_2DCF oxidation. Incubation of Abeta as well as Abeta-metal complexes in solution resulted in a very fast reduction of its oxidative properties. After 2 hour incubation, complex Abeta-copper preserved about 30% of its oxidative activity, loosing it completely after a prolonged incubation.

Disscusion

The biology of Abeta(1-40) is linked to its interactions with metals. The peptide subsequent neurotoxicity is also likely to be mediated through metal redox alterations. The data indicate that interactions with metals occur at structural as well as functional levels, affecting both conformation and oxidative and/or hydrolytic properties of the peptide. Changes in the peptide structure promoted by the metal ions reduced strongly its oxidative abilities and limited hydrolytic properties. Moreover, observed changes of Abeta properties occur fast within first hours of Abeta-metal complex formation. Characterization of Abeta metallobiochemistry may be useful for optimization of new therapeutic approaches of metal pharmacology in AD.

Acknowledgements

This work has been supported by the KBN grant 3 PO5F 00524.

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MODIFIED CYCLIC PENTAPEPTIDES FOR TARGETING CXCR4 CHEMOKINE RECEPTOR EXPRESSION

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Introduction

Several diseases like HIV-1 infection, cancer metastasis, rheumatoid arthritis, and chronic lymphocytic B-cell leukaemia are linked to the CXCR4-chemokine receptor [1]. Antagonising its natural ligand SDF-1α, is a promising way of treating these diseases.

Fujii *et al.* developed the highly affine peptide cyclo[-Arg[1]-Arg[2]-Nal[3]-Gly[4]-D-Tyr[5]] [2]. Herein we describe further enhancement towards metabolic stability, oral bioavailability and chemical versatility to attach radiolabels for tumour diagnosis via PET imaging.

Results

Replacing an amide proton by a *N*-methyl group increases proteolytic stability and oral bioavailability.

15 peptides were synthesised using an optimised procedure [3] for fast, on-resin *N*-methylation compatible with Fmoc SPPS.

Mono-*N*-methylation of Arg1 or Arg2 yielded interesting peptides still retaining 6 - 8 fold reduced affinity.

Only two of the ten di-*N*-methylated peptides showed measurable affinity.

Searching for a site where prosthetic groups for radiolabelling could easily be attached the arginine side chains were substituted by citrulline.

On the one hand side Arg1 could be replaced without dramatic loss of activity while exchange of Arg2 led to complete diminishment of activity.

Arg1 was replaced by ornithine and its side chain acylated focusing on fluorinated acids that could introduce an 18F radiolabel for later PET imaging (Fig.1.).

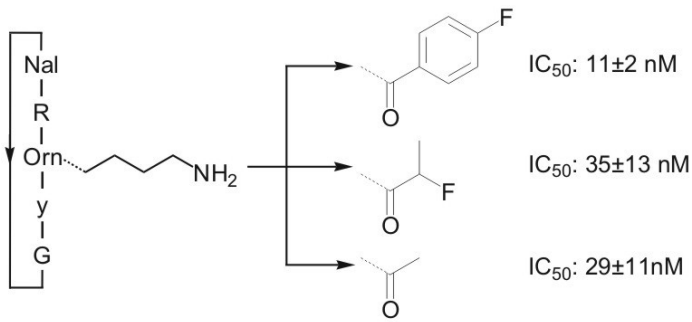

Fig. 1: Affinity of peptides acylated on the ornithine side chain

The bulkiest acid showed the highest affinity apparently benefiting from hydrophobic stabilisation.

A first test has been conducted, successfully radiolabelling the ornithine-peptide with *p*-[^{18}F]-benzoate and subsequent PET imaging in a non-tumour-bearing mouse.

Discussion

Based on a cyclic pentapeptide as lead sequence, we developed potential peptidic radioligands for imaging CXCR4 positive tumours by PET. Furthermore we synthesised various cyclic peptides with *N*-methylated amide bonds and replaced one functionality to enhance chemical versatility while still retaining good affinity. Additionally these investigations yielded information about the importance of the arginine guanidino functionalities and their chemical surroundings on the CXCR4 receptor. Beyond that successful radiolabelling and PET imaging with the most active peptide was achieved.

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COUPLING REAGENTS FOR INCORPORATION OF EXTREMELY DIFFICULT SUBSTRATES INTO THE PEPTIDE CHAIN

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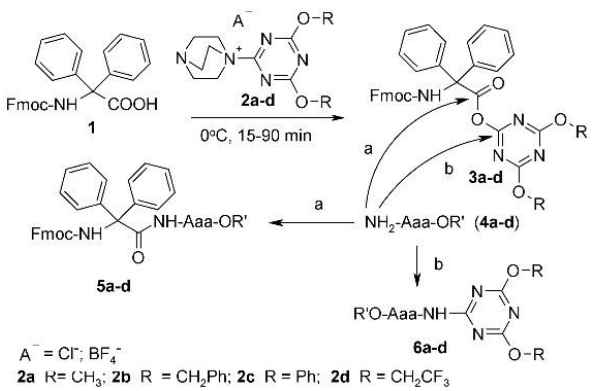
Introduction

It is well known that incorporation of α,α-disubstituted amino acids very efficiently introduces a conformational constrain into the peptide backbone. Therefore several peptides bearing α,α-disubstituted glycines such as α,α-dimethyl-, α,α-diethyl-, or α,α-dipropyl glycine etc. have been successfully prepared and examined for conformational behavior and biological activity. However, increasing the size of side chains of α,α-disubstituted amino acid, severely reduces its reactivity, causing serious synthetic difficulties. Thus, attempts for incorporation into peptide chain of numerous of very interesting, more sterically demanding amino acids, such as for example α,α-diphenyl glycine (Dpg), which impart rigidity to the peptide backbone, but also acting as a useful vehicle for studying π-π interactions, involved construction of peptides from non-amino acids fragments [1] because limited efficiency of already known coupling reagents.

Result and Discussion

In the search for a coupling reagents useful for incorporation of difficult substrates derived from Dpg into the peptide chain we verified an utility of *N*-triazinylammonium salts with different tertiary amine, different substituents in triazine ring, and counter-anion. It has been found, that carboxylic function of *N*-Fmoc protected α,α-diphenyl glycine is most vigorously activated by salts **2** derived from DABCO.



In this case activation of *N*-Fmoc protected α,α-diphenyl glycine with different triazine reagents proceeded in 15 - 90 min. and the reactivity of reagents increased steadily with electron withdrawing effect of substituent in the triazine ring. Thus, for the most reactive triazines **2** the rate of Fmoc-Dfg-OH activation leading to triazine superactive esters **3** were comparable to the rate observed for standard proteinogenic substrates.

Less advantageous, however, were acylation experiments because of formation of the expected dipeptides (route a) was accompanied by side-product formation in attack of amino-component on aromatic sp^2 carbon of triazine ring (route b). The intensity of side- reaction was suppressed when the less substituted triazine **2a**; (R=CH_3) or **2b** (R=$CH_2C_6H_5$) were used in the synthesis, but neither addition of HOBt nor DMAP diminished formation of the side - product **6**. Under advantageous conditions expected dipeptide **5** were formed in 42 - 93% yield.

Acknowledgements

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ANTHRAX LETHAL FACTOR - SUBSTRATE INTERACTION THROUGH MOLECULAR DYNAMICS SIMULATIONS

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Introduction

Anthrax is a disease of animals and humans, caused by the bacterium *Bacillus anthracis*. Anthrax toxin (AT) consists of three proteins, one of which is the anthrax lethal factor (ALF) [1]. ALF is a gluzincin Zn-dependent highly specific metalloprotease (~90.000 kDa), which belongs to the M34 family of the MA clan of zinc metalloproteases. ALF cleaves most isoforms of mitogen-activated protein kinase (MAPK)-kinases (MEKs) close to their amino termini, leading to the inhibition of one or more signaling pathways [2]. No data are available on the enzyme-substrate interaction at the molecular level. Therefore, classical molecular dynamics simulations have been performed on the entire protein structure of ALF and the solvent waters in order to clarify the binding and the specific substrate-protein interactions of MEKs/MKKs to the active site of ALF. These simulations reveal the key role of specific hydrophobic interactions between the substrate and the active site pocket of the enzyme. These simulations may be of help in the design of new bioactive compounds with possible inhibitory activity on ALF protease.

Result and Discussion

The X-ray structure of ALF was taken from the RCSB Protein Data Bank (accession code 1PWU), where LF represents the LF E687C mutant. Due to the large size of MKK4, we have considered only the residues next to the cleavage site:

Subsequently, 2 ns long classical molecular dynamic simulation has been performed on the complete solvate enzyme, and snapshot structures were extracted for every 1 ps. Due to the large size of ALF (~776 residues) and since domain I (residues 1-263) does not play an active role in the catalytic process, in our model we have considered only domains II, III, IV. In addition, we haven't considered the water molecule that is bound to zinc. The system is composed by the protein (II, III and IV domains – 513 residues), the substrate (12 residues) and a shell of water of 10 Å for a total of ~67000 atoms. The system temperature and the total energy of each simulation model were monitored during the MD simulation and found to converge to stable values. The system temperature was coupled at 300 K and the total energy fluctuated within less than 0.2%. The root-mean-square deviation (RMSD) from the starting structure is an important criterion for the convergence of

the system. The RMSDs of the C_α atoms are shown in Fig. 1, indicating that the whole simulation system appears to have been stable after 250 ps of equilibration.

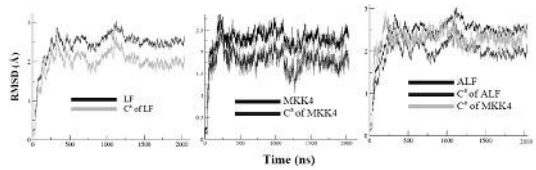

Fig. 1. Time dependence of the RMSDs from the starting structure of ALF (a), MMK4 (b) and ALF-MKK4 (c) for C_α atoms , and during the 2 ns MD simulation.

Analysis is being carried out in order to probe protein-substrate interactions. Several key distances indicative of conformational changes were monitored throughout the simulation: (1) Lys2(MKK4) - Glu70(ALF), (2) Lys2(MKK4) – Ser409(ALF), (3) Thr4(MKK4) - His391(ALF), (4) Arg6(MKK4) - Val412(ALF), (5) Thr8(MKK4) - Asn469(ALF), (6) Asn10(MKK4) - Ser63(ALF) (Fig. 2).

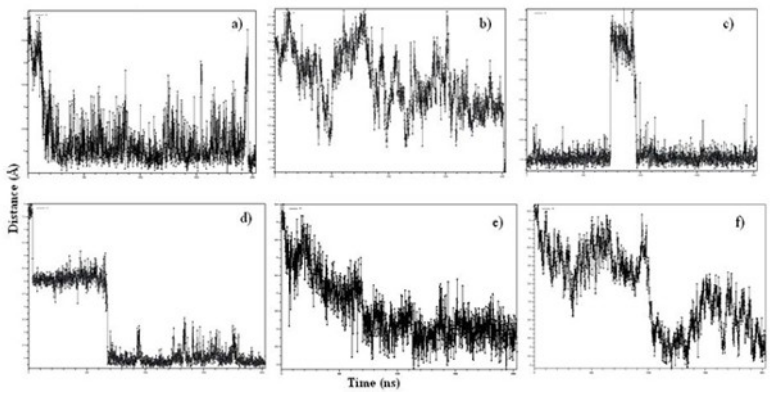

Fig. 2. Some characteristic distances in the MD simulations of ALF-MKK4.(a) (a) Lys2(MKK4) - Glu70(ALF), (b) Lys2(MKK4) – Ser409(ALF), (c) Thr4(MKK4) - His391(ALF), (d) Arg6(MKK4) - Val412(ALF), (e) Thr8(MKK4) - Asn469(ALF), (f) Asn10(MKK4) - Ser63(ALF).

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N-TERMINAL DIMERIZATION OF BIOLOGICALLY ACTIVE PEPTIDES ON SOLID SUPPORT

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Introduction

Receptor dimerization is an essential part of cellular signal transduction. Molecules that induce or stabilize the association of receptor macromolecules are of potential therapeutic value. Bivalent ligands, having two pharmacophores linked by a spacer, could connect vicinal receptors and thereby possess enhanced agonistic or antagonistic potencies [1]. Dimeric analogs of biologically active peptides with a specific spacer length may also strongly interact with proteins containing multiple binding sites. Human leukocyte antigens (HLA) present antigenic peptides to helper T cells and alert the immune system to infectious attack. It was found that the exposed nonapeptide frgament of HLA-DQ (164 - 172, TPQRGDVYT), bearing a close resemblance to thymopentin (RKDVY), possesses immunosuppressory activity [2], which even increases for the cyclic analog of the peptide [3]. The corresponding nonapeptide fragment of the HLA-DR molecule, located in the exposed loop of the β chain (164 - 172) and having the sequence VPRSGEVYT, also suppresses the immune response [4]. It is known that the HLA-DR molecules interact with their T-cell receptors and coreceptors as superdimers [5], suggesting that properly designed dimeric analogs of VPRSGEVYT may interfere with the adhesion stronger than their monomeric analogs. On the basis of the three-dimensional structure of the HLA-DR superdimer [6], we designed new dimeric analogs in which the VPRSGEVYT peptides are linked through their N- and/or C-termini by linkers able to mimic the dimeric nature of the immunosuppressive fragments of HLA class II molecules.

Results

Dimeric peptides were synthesized using standard Fmoc solid-phase strategy (Fig. 1). The crosslinking of N-terminal amino groups was achieved on solid support through the reaction with pentafluorophenyl esters of poly(ethyleneglycol)biscarboxylic acid $PEG(COOPfp)_2$ (polydispersed for library synthesis, n = 6 - 19 oxoethylene units; or monodispersed, n = 12). The pentafluorophenyl esters of dicarboxylic acids $(CH_2)_n(COOPfp)_2$ (n = 3, 4, 8, 10, 12) were used for the dimerization of HLA-DQ fragment RGDVY. For the C-terminal linkage, the Fmoc-Lys(Fmoc)-OH derivative was used, and the resulting peptide dimer was assembled from a specific number of Gly residues [7] followed by the required peptide sequence. The cyclodimer synthesis utilized both these dimerization methods. The identity of purified (HPLC) peptide dimers was analyzed by HPLC, ESI-MS and ESI-MS/MS.

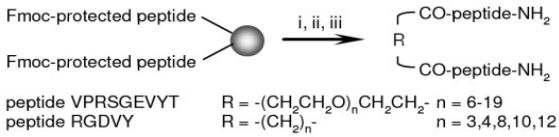

Fig. 1. The N-terminal dimerization of peptides on solid phase. MBHA resin, (i) piperidine, 20% in DMF, (ii) R(COOPfp)$_2$, HOBt, DMF, 48 h, rt; (iii) TFA, thioanisole, phenol, TIS, H_2O, 5 h, rt.

The influence of PEG dimerization on immunomodulatory activity of peptides was examined in AFC *in vitro* experiment. All the examined PEG dimers of VPRSGEVYT peptide were more potent than the peptide itself. The N-dimer library clearly exhibited a dose-dependent inhibition of the immune response with the strongest effect in the whole series at the dose of 100 μg/ml, however, at the lowest dose the N,C-cyclodimer exhibited the strongest immunosuppression.

Discussion

We developed a new and straightforward method of direct N-terminal chemically induced dimerization of peptides on solid support. The procedure using the pentafluorophenyl esters of poly(ethyleneglycol)biscarboxylic and dicarboxylic acids is compatible with solid-phase peptide synthesis. Practical aspects of the method were evaluated through synthesis of peptide dimers of HLA-DQ and HLA-DR fragments [8]. The N-dimers with PEG-derived linkers were much more water-soluble than the N-dimers linked by dicarboxylic acids or the C-dimers with oligo-Gly linker. The evaluation of the influence of PEG-linked dimers on antigen presentation and immune response involving HLA resulted in new compounds with significantly increased biological activity. The approach used for the N,C-cyclodimer synthesis exemplifies a new strategy for parallel cyclodimerization on solid support.

Acknowledgements

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NMR STRUCTURAL ANALYSIS OF THE HIV-1 GP120 V3 – CCR5 CO-RECEPTOR N-TERMINAL INTERACTION

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Introduction

One of the most severe consequences of Acquired Immunodeficiency Syndrome (AIDS) has been its epidemic spread all around the world [1, 2] . HIV epidemic has exceeded all predictions made a decade ago and has caused more than 22 million deaths [3]. Until recently, treatment of the disease was based on nucleoside analogue reverse transcriptase and protease inhibitors but the cost of such combined antiviral therapy is remarkably high. Targeting the virus-cell interaction by inhibiting the virus attachment and entry process offers an attractive alternative approach [4]. The main cell population affected by the HIV-1 infection belongs to the $CD4^+$ T lymphocyte family. Recent convincing evidence indicates that the majority of these cells that die due to HIV-1 are not actually infected by the virus. Instead, these cells are being led to programmed cell death after activation of apoptotic mechanisms by the virus. A potential mechanism by which the virus appears to deregulate the physiological function of these cells has been identified, using synthetic peptides [2]. Ionic interactions between the variable V3 domain of the HIV-1 coat glycoprotein gp120 and the amino terminal of the chemokine receptor CCR5 play a prominent role in this process3.

Result and Discussion

Standard multidimensional and multinuclear NMR spectroscopy was applied to probe the structural and physicochemical determinants of three representative peptides from V3 domain of the HIV-1 and a 22-residue peptide, representing the amino terminal of the chemokine receptor CCR5, in their free or interacting state. The fact that during titration only one peak, which is the weighted average of the corresponding peptide cross-peak in its free and bound state, is observed in HSQC spectra after each addition of the V3 peptides to CCR5 peptide, suggests that there is a fast exchange of the interacting peptides between their free and bound-state. Preliminary analysis of the ^{1}H -^{15}N HSQC spectra that were recorded during CCR5 titration with the V3 peptides revealed that as far as LAI peptide is concerned Ser3, Ile4, Arg5 and Ile6 exhibited the most important chemical shift differentiation, while for the MN and SF2 peptides Ile4, His5, Ile6 and Gly7 as well as to Gly7,

Arg10 and Ala11, respectively. The HSQC spectra comparison for CCR5 peptide, when in its free or interacting (with LAI, MN and SF2 peptides) state, displays a similarity of the results, with Tyr3, Gln4 and Tyr10 being the most affected by the titration amino acids.

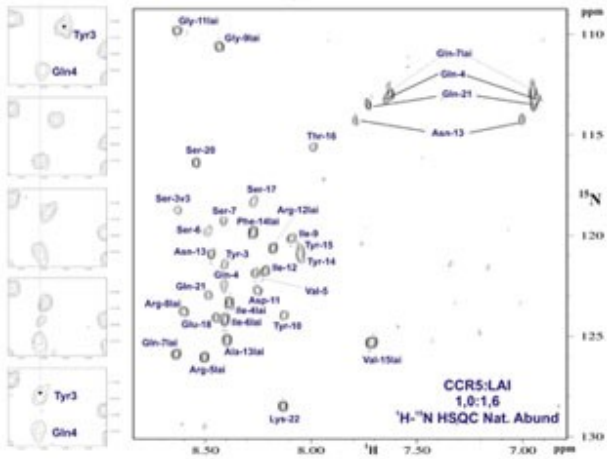

Fig. 1. Chemical shift changes of CCR5 Tyr3 and Gln4 ^{1}H-^{15}N cross peak during CCR5 titration with LAI peptide.

The fact that chemical shift changes in ^{1}H-^{15}N cross peaks were observed also for the middle of the V3 MN sequence could be rationalized by larger conformational changes of MN His5 compared to LAI Arg5 (manifested by larger ^{1}H-^{15}N cross peak shift of His5) [5]. The above conformational changes at position 5 in V3 MN could affect the Gly7 in the middle of the sequence, which is also the first residue of the GPGR "V3-loop". However, this is not the case in V3 LAI peptide which apart from the smaller shift changes of Arg5 cross peak is also two residues longer in sequence than the V3 MN peptide. Conformational variations involving the glycoprotein's GPGR V3-loop might be of great interest since this peptide segment is considered as one of the structure determinants of the gp120- receptor interacting interface [6].

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DEVELOPMENT AND IMMUNOANALYTICAL CHARACTERISATION OF BIOCONJUGATES COMPRISING A ß-AMYLOID PLAQUE SPECIFIC EPITOPE

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Introduction

New immunotherapeutic approaches have been developed for treatment of neurodegenerative diseases of the Alzheimer's dementia (AD) type. The identification of a β-amyloid-plaque specific epitope, Aβ(4-10) (FRHDSGY) [1], recognised by therapeutically active antibodies from transgenic AD mice, provides the basis for development of AD vaccines and for molecular diagnostics. Our work was focused on the design, structural and immunoanalytical characterisation of bioconjugates comprising the β-amyloid (4-10) epitope as new vaccine lead structures against Alzheimer's disease.

Results

In order to produce branched antigenic polypeptides the Aβ(4-10) epitope was attached via thioether linkage to synthetic carriers with well-defined structures [2], such as tetratuftsin derivatives (Ac-[TKPK(ClAc)G]$_4$-NH_2, Ac-[TKPK(ClAc-GFLG)G]$_4$-NH_2, Ac-FFLLTRILTIPQSLD-[TKPK(ClAc)G]$_4$-NH_2); sequential oligopeptide carrier (Ac-[Lys(ClAc)-Aib-Gly]$_4$-OH) and lysine dendrimer (ClAc-Lys(ClAc)-Lys(ClAc-Lys(ClAc))-Arg-Arg-βAla-NH_2). The chloroacetylated carriers were prepared by solid phase peptide synthesis according to Boc/Bzl strategy and the Aβ(4-10) epitope peptides containing one cysteine residue with free thiol group either at the C- or N-terminus were synthesised by Fmoc/*t*Bu chemistry (H-FRHDSGYC-NH_2, Ac-CFRHDSGY-NH_2, H-FRHDSGYGGGGGC-NH_2, Ac-CGGGGGFRHDSGY-NH_2). The conjugation reactions were performed in solution under slightly alkaline conditions (0.1 M Tris buffer, pH 8.1) and monitored by analytical RP-HPLC and MALDI-TOF mass spectrometry. Structures and molecular homogeneities of all epitope peptides, carriers and conjugates were ascertained by HPLC, MALDI- and ESI-FTICR-MS.

Comparative binding studies of the conjugates with a mouse anti-Aβ(1-17) monoclonal antibody were performed by indirect enzyme linked immunosorbent assay (ELISA). The β-amyloid(4-10)-tetratuftsin derivative conjugates in which the epitope was coupled to the carriers through its N-terminus had higher binding to the antibodies in comparison with the conjugates in which the attachment site of the epitope to the carrier was the C-terminus. The presence of the pentaglycine spacer between the carrier and the epitope peptide led to increased binding, due to higher

accessibility of the epitope to the antibodies. The introduction of an additional GFLG tetrapeptide spacer did not influence significantly the antibody recognition. The application of the tetratuftsin carrier elongated by a promiscuous helper T-cell epitope of a hepatitis B surface antigen (Ac-FFLLTRILTIPQSLD-$[TKPK(ClAc)G]_4$-NH_2) resulted in conjugates which bound to the antibodies with lower intensities.

Similar results with respect to the influence of the epitope topology on the antibody recognition were obtained in the case of bioconjugates containing the Aβ(4-10) epitope attached to the sequential oligopeptide carrier and to the lysine dendrimer. The highest binding intensity was obtained when the epitope was attached to the carriers through its N-terminus and a pentaglycine spacer was present between the carrier and the epitope peptide. Investigations of the influence of the carrier type on the antibody binding did not show a pronounced effect on the binding affinities of the conjugates.

Conformational preferences of the synthesized compounds in water and in TFE were determined by circular dichroism (CD) spectroscopy. In water, all conjugates adopt random coil conformation independent on their primary structure. The CD spectra of the conjugates recorded in TFE showed pronounced differences depending strongly on the attachment site of the β-amyloid(4-10) epitope to the carrier and not on the chemical nature of the carrier. Conjugates in which the epitope was coupled to the carrier through its C-terminus showed less ordered structure than the conjugates in which the attachment site of the epitope was at the N-terminus. The presence of the pentaglycine spacer did not influence the conformational preferences of the conjugates.

Discussion

Bioconjugates with well-defined structures in which the β-amyloid(4-10) epitope was attached to various carriers were successfully prepared by chemoselective ligation (thioether linkage approaches). The antigenicity of the compounds was determined by indirect ELISA and it was found that the major factors which influenced significantly the antibody recognition were represented by the epitope topology and the presence of a pentaglycine spacer between the epitope peptide and the carrier. The attachment site of the epitope to the carrier had also a marked effect on the solution conformtion of the conjugates.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Hungarian Research Fund (OTKA) No: T 32425 and T 43576.

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TANDEM NATIVE LIGATION AT X-GLY AND X-CYS POSITIONS BY AUXILIARY GROUP PROTECTED ORTHOGONALLY TO ACID

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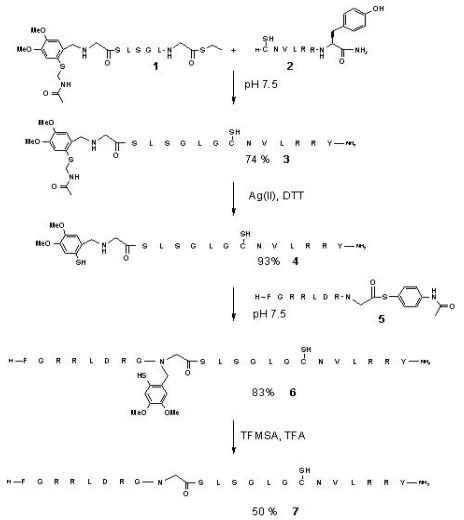
Introduction

Native chemical ligation allows the splicing of unprotected peptides and proteins by amide bonds via reaction of peptide C-terminal thioesters with peptide N-terminal cysteins [1, 2]. The original require-ment for N-terminal cystein has recently been circumvented by introduction of various auxiliary groups, such as 1-phenyl-2-mercaptoethyl and 4,5-dimethoxy-2-mercaptobenzyl (Dmmb) [3 - 8]. These auxiliary groups have during SPPS been protected by acid-labile groups. Accordingly, the auxiliary mercapto group is deprotected simultaneously with the overall peptide deprotection. Acidic deprotections of the auxiliary mercapto groups however limit the methods to ligation of two peptide fragments, because sequences containing combinations of thioesters and unprotected mercapto auxiliary groups will be internally reactive.

The present work reports the preparation of *S*-acetamidomethyl-4,5-dimethoxy-2-mercaptobenzylamine hydrochloride, abbreviated Dmmb(Acm), along with the use of Dmmb(Acm) in peptide synthesis and tandem ligation [9, 10] in a combination of X-Cys and X-Gly positions.

Results and Discussion

4,5-Dimethoxy-2-mercaptobenzylamine [11] is commercially available and was *S*-protected with acetamidomethyl by treatment with *N*-hydroxymethylacetamide in aqueous hydrochloric acid [12]. Dmmb(Acm) was isolated in 94% yield. While Acm-cleavage from alkyl mercaptan (cysteine) is well established, Acm-cleavages from aromatic mercaptyls are scarcely described [13]. Encouragingly, Dmmb(Acm) could be deprotected smoothly with aqueous Ag(II) at pH 2-3.



Native ligation using Dmmb(Acm) was tested as outlined in the scheme. Peptide thioester 1 was pre-pared from trithioortho ester peptide [14] via N-terminal bromoacetylation and reaction with Dmmb(Acm). Peptides **1** and **2** were ligated, and the Acm group removed with Ag(II) to provide Dmmb-peptide **4**. Peptide thioester **5** was prepared by the method of Von Eggelkraut-Gottanka *et al.* [15], and reacted with peptide **4** to provide tandem ligation product **6**. Removal of Dmmb with TFMSA-TFA gave the final product **7**. Equilibriums between N- and S-acyl species have previously been described for Dmmb-mediated ligation intermediates [6]. However, the major product in the present case, involving Gly-Gly link at the Dmmb site, was the desired product **7**.

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ENZYMATIC SYNTHESIS OF HIGH SPECIFIC SUBSTRATES FOR CYSTEINE PROTEASES ASSAY

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Introduction

Cysteine proteases, hydrolytic enzymes of different origin, play very important role in biochemical processes. They have found a number of applications in many areas, such as biotechnology, medicine, food industry and agriculture. Activity determination of cysteine proteases is necessary for practical as well as research purposes. For convenient activity assay short synthetic peptides with specific amino acid sequence contained chromogenic or fluorogenic moieties should be used. [1]

Enzymatic peptide synthesis is a good tool for obtaining different biologically active peptides. Immobilized enzymes proved to be a convenient biocatalyst in this case [2]. We used serine proteases, subtilisin *Carlsberg* and α-chymotrypsin immobilized on poly(vinyl alcohol) cryogel (PVA-cryogel), for synthesis of high specific substrates for cysteine proteases assay.

Results

The highly specific chromogenic substrate for cysteine proteases assay Glp-Phe-Ala-*p*NA was obtained using subtilisin Carlsberg and α-chymotrypsin immobilized on PVA-cryogel. Immobilized enzymes were used as the catalysts at the final coupling stage of different substrates syntheses:

Glp-Phe-OMe + Xaa-*p*NA → Glp-Phe-Xaa-*p*NA,

where Xaa = Ala, Cys-Bzl, Glp – the residue of pyroglutamic acid, *p*NA – *p*-nitroanilide.

The influence of components concentrations, the reaction mixture composition, the biocatalyst amount and time on product yield was studied. All products were characterized by HPLC and amino acid analysis; MS data were obtained for Glp-Phe-Ala-AMC.

Table. Product yields in syntheses of chromogenic and fluorogenic substrates using proteases immobilized on poly(vinyl alcohol) cryogel.

Substrate	Yield after 24 h, %	Protease immobilized on poly(vinyl alcohol) cryogel
Glp-Phe-Ala-pNA	95	Subtilisin Carlsberg
	88	α-Chymotrypsin
Glp-Phe-Ala-AMC	100	α-Chymotrypsin
Glp-Phe-Cys(Bzl)-pNA	40	Subtilisin Carlsberg
	47	α-Chymotrypsin

Following the proposed scheme the highly specific fluorogenic substrate for cysteine proteases assay Glp-Phe-Ala-AMC (AMC – 4-amino-7-methylcoumaride) was obtained using α-chymotrypsin immobilized on PVA-cryogel as the catalyst.

Kinetic parameters of hydrolysis of Glp-Phe-Ala-AMC by papain were studied. The initial reaction rates were determined fluorometrically at 380 nm and 460 nm according to the 4-amino-7-methylcoumarine formation. The catalytic constant k_{cat} and Michaelis constant K_M were determined by analyzing the reaction rate–substrate concentration dependence using double reverse Lineweaver–Burk coordinates. It was found that $k_{cat}/K_M = 267 \pm 7$ $sec^{-1}mM^{-1}$.

Discussion

Optimization of enzymatic stage in substrates synthesis was carried out. It was shown that the best conditions were: anhydrous dimethylformamide/acetonitrile mixture 20/80 (v/v), initial concentrations – 85 mM, and enzyme-to-substrate ratio 1:3900. Under these conditions several specific substrates for cysteine proteases assay Glp-Phe-Ala-*p*NA, Glp-Phe-Cys(Bzl)-*p*NA and Glp-Phe-Ala-AMC were obtained with high product yields (up to 100% in 24 h) using subtilisin and chymotrypsin immobilized on PVA-cryogel. The quantitative product yields, guaranteed optical purity of substrates as well as isolation and purification facility of target peptides were provided by the enzymes used at the last condensation stage.

The obtained substrates were tested for the papain assay. Glp-Phe-Ala-AMC was found to be a high specific and high selective substrate of papain. The activity of papain by hydrolysis of Glp-Phe-Ala-AMC was found to be 121.8 U. At the same time the activity of papain by hydrolysis of standard substrate Bzl-Arg-*p*NA was 0.02 U, indicating strong Glp-Phe-Ala-AMC binding and high conversion rate at the papain active site.

Acknowledgments

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SEMISYNTHESIS OF CAGED PHOSPHORYLATED AND FLUORESCENTLY LABELED STAT6 PROTEINS

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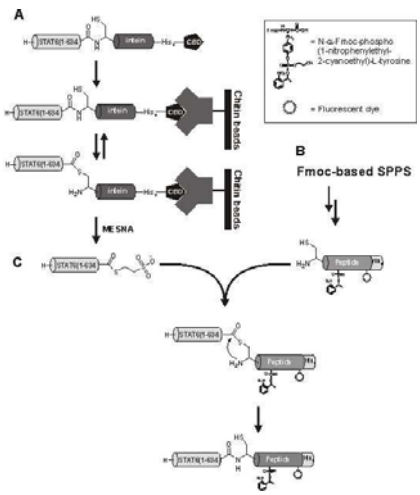
Introduction

Signal transducer and activators of transcription (STAT) proteins comprise a family of 7 human transcription factors that reside in the cytoplasm in their non-active state. STAT6, a 847 amino acid multi-domain protein, is a member of this family and is intimately involved in cellular response to stimulation by cytokines such as IL-4 and IL-13 [1]. Binding of these interleukins to their respective receptors leads to phosphorylation of STAT6 by Janus-kinases (JAK) at tyrosine 641 and the subsequent formation of STAT dimers. Homodimerization occurs by reciprocal binding of two phosphorylated STAT6 monomers via their respective SH2 domains to their phosporylated tyrosine residues at position 641. These dimers translocate into the nucleus where they bind to DNA promoter regions and induce expression of specific genes. This signal transduction pathway plays a key role in the TH2-immune response and related to this in viral infections, inflammatory processes, allergic reactions and certain types of cancer [2]. Our aim is to study dimerization and DNA binding of STAT6 *in vitro* as well as its localization on the cellular level by releasing the phospho group on tyrosine 641 from a caged phospho-tyrosine building block by irradiation with light at 365 nm.

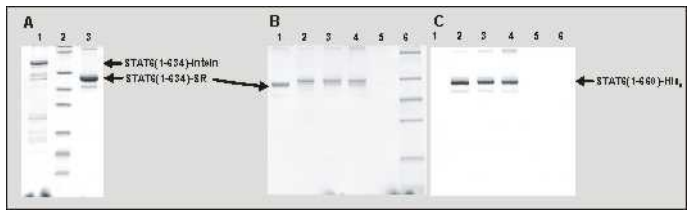
This goal is achieved by establishing semisynthetic access to C-terminally truncated STAT6 variants that carry a caged phospho-tyrosine residue and/or a fluorescent label (Fig. 1).



Results and Discussion

A variety of phosphorylated, caged phosphorylated and fluorescently labeled peptides comprising amino acids 635 to 660 of STAT6 with amino acid 635 changed from a glycine into a cysteine an additional hexa-histidine tag were synthesized by Fmoc-based SPPS (Fig. 1B). Caged phospho-tyrosine equipped with a light removable nitrophenylethyl group was prepared according to a procedure described by Rothman *et al.* [3, 4]. These peptides were identified by electrospray mass spectrometry and obtained with isolated yields of 10 - 20 % in high purity.

Recombinant expression of STAT6(1-634) as a fusion construct with the GyrA intein, a His6-tag and a chitin binding domain produced 5 - 10 mg of protein per liter *E. coli* culture (Fig. 1A). Cleavage of the intein and generation of STAT6(1-634)-thioester was induced by addition of an excess of mercaptoethansulfonate (Mesna) (Fig. 2A).



The resulting STAT6-thioester was linked to synthetic peptides carrying the caged phospho group and a fluorescent dye by native chemical ligation (Fig. 2 B/C) [5]. Isolated yields were between 15 and 30% depending on the peptide used and the obtained ligation products were tested for biological activity with regard to DNA binding in gel shift assays as well as in *in vivo* measurements.

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OPTIMIZATION OF THE TOTAL SOLID-PHASE SYNTHESIS OF LIPODEPSIPEPTIDE ANTIBIOTIC FUSARICIDIN A

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Introduction

Naturally occurring cyclic depsipeptides are compounds of great interest because of their diverse range of biological activities [1]. Traditional methods to prepare cyclic peptides and therefore depsipeptides involve solid-phase synthesis of the selectively protected linear precursor and cyclization in solution under high dilution conditions [2-4] As an attractive alternative, cyclization could be performed while peptides still remain anchored to the resin. Taking into consideration the greater reactivity of the amino group, and therefore minimal possibility of side reactions, we chose macrolactamization for depsipeptide ring closure. Although this synthetic strategy appears to be better choice for solid-phase depsipeptide ring closure, undesired intramolecular *O*→*N* acyl shift may occur if basic conditions were to be used [5]. To test this possibility and to optimize solid-phase synthesis of cyclic lipodepsipeptides, we have synthesized cyclic fusaricidin A's analogue 1 as a model compound (Fig. 1).

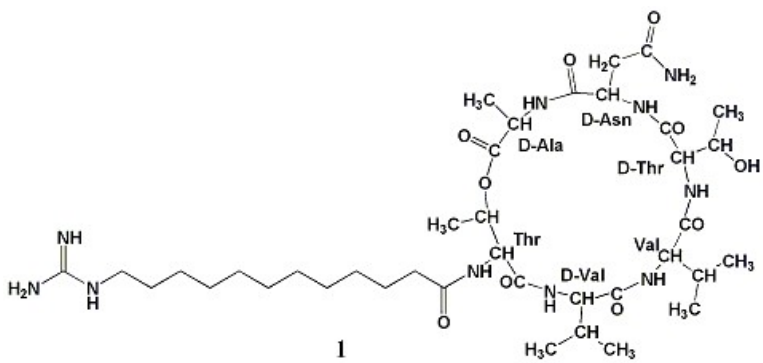

Fig. 1. Fusaricidin A analogue.

Results

In order to optimize solid-phase synthesis of fusaricidin A we synthesized analogue 1 which differs from fusaricidin A in that D-*allo*-Thr is replaced by D-Thr and 15-guanidino-3-hydroxypentadecanoic acid is substituted by 12-guanidinododecanoic acid since that building blocks are commercially available. For the synthesis of analogue 1 three different amide resins were tested especially when the depsipeptide bond formation in concerned: Rink Amide MBHA (Novabiochem), Tentagel S RAM (Advanced ChemTech) and PAL-ChemMatrix (Matrix

Innovation). Our most successful synthetic approach included attachment of first amino acid through its side chain, successful combination of four quasi-orthogonal protecting groups (Fmoc/*t*Bu/Allyl/Alloc), stepwise solid-phase synthesis of linear peptide, lipid tail attachment using pentafluorophenol ester derivative followed by depsipeptide bond formation (DIC/DMAP method) and on-resin cyclization [6]. The Mukiyama's reagent, combined with *N,N*-bis(tert-butoxycarbonyl)thiourea, was found to be superb for the transformation of ω-amino group in the lipid tail fragment into guanidino group [7] in comparison to 1*H*-pyrazole-1-carboxamidine method [8]. The best results for lipodepsipeptide 1 cleavage from the solid support were obtained with Reagent K. The final product 1 was purified by RP-HPLC and characterized by MALDI-TOF MS.

Discussion

For the synthesis of 1 three solid supports were tested. Among them PAL-Chemmatrix and Tentagel S RAM gave the best results. Ester (depsipeptide) bond formation using DIC/DMAP method was favored when dichloromethane was used as a solvent. When DMF was present in reaction mixture the yields of esterification were either very low or reaction did not proceed at all. The *O*→*N* acyl shift was observed in cyclic depsipeptide during standard Fmoc removal. The incorporation of lipid tail prior to esterification and cyclization resulted in complete inhibition of *O*→*N* acyl shift reaction. Coupling of *N*-Fmoc-12-aminododecanoic acid to the L-Thr was performed using its pentafluorophenyl ester derivative. In our case couplings using DIC/DMAP, HBTU/HOBt, and HCTU were completely unsuccessful. Transformation of ω-amino group into guanidino group was examined using two methods: 1*H*-pyrazole-1-carboxamidine and DIEA in DMF or using *N,N*-bis(*tert*-butoxycarbonyl)thiourea together with Mukaiyama's reagent. The latter method gave excellent results after just 2 hours. When cyclic lipodepsipeptide 1 was submitted to selective basic hydrolysis of ester bond (ring opening reaction) the corresponding linear lipopeptide product was obtained, confirming the existence of ester bond within the peptide loop. Assessment of biological activity of 1 is in progress and will be reported elsewhere.

Acknowledgements

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SYNTHESIS OF β^2-HOMO AROMATIC AMINO ACIDS AND THEIR APPLICATION IN ENDOMORPHIN ANALOGUES

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Introduction

The use of non-protein amino acids to overcome the limitation of susceptibility to proteolytic degradation of the natural biologically active peptides is a popular strategy. The application of the β-amino acids is one of the approaches. The availability of unnatural β-amino acids creates limitation of such approach. The most popular β-amino acids are commercially available β^3-amino acids.

We have developed a convenient method for the synthesis of β^2-aromatic amino acids. We applied this method for synthesis of β^2-homo-tyrosine and β^2-homo-phenylalanine. These amino acids were incorporated into the endomorphin-2 sequence and its D-Ala^2-analogue (TAPP) for structure–activity relationship study.

Endomorphin-2 H-Tyr-Pro-Phe-Phe-NH_2

TAPP H-Tyr-D-Ala-Phe-Phe-NH_2

Results and Discussion

The *N*-Boc-protected β^2-aromatic amino acid esters (**D**) were obtained in two-step reaction. The α-cyanocinnamate esters (**C**), products of the first step, were synthesized via the Knoevenagel condensation of methyl cyanoacetate (**A**) and aromatic aldehydes (**B**) [1, 2]. The second step, which involves reduction of α-cyanocinnamate esters and then N-protection, was done in "one-pot", where $CoCl_2$-$NaBH_4$ combination was used as reducing agent in MeOH as solvent (Fig. 1).

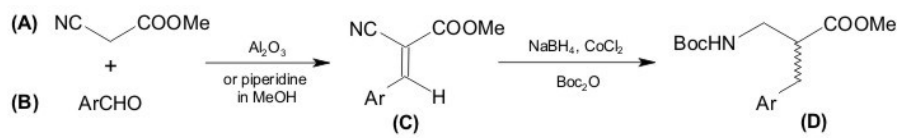

Fig. 1. Synthesis of N-Boc-protected β^2-aromatic amino acid esters.

The β^2-aromatic amino acids were incorporated as racemic mixtures into the endomorphin-2 opioid peptide sequence and its D-Ala^2-analogue (TAPP). All of peptides were synthesized in the solution (fragment condensation strategy) with TBTU or HBTU as coupling reagent. The final analogues were isolated by HPLC method. In two cases (analogues 2 and 12) we were not able to separate the diastereoisomers and bindings assays were performed for diastereomeric mixtures.

The receptor affinities of the obtained endomorphin-2 and TAPP analogues containing β-amino acids for μ-opioid receptor were measured and compared to those of endomorphin-2 and TAPP (Table 1) [3].

Table 1. Binding affinities of endomorphin and its analogues

No	Peptide sequence		IC_{50} μ ± S.E.M. [nM][a]
1	Tyr-Pro-Phe-Phe-NH_2 **Endomorphin-2**		10,9±3,95
2	(*R,S*)β[2]**h-Tyr**-Pro-Phe-Phe-NH_2		707,9±3,14
3	Tyr-Pro-(*R* or *S*)β[2]**h-Phe**-Phe-NH_2	(more polar)	97,7±4,45
4	Tyr-Pro-(*S* or *R*)β[2]**h-Phe**-Phe-NH_2	(less polar)	>1000
5	Tyr-Pro-Phe-(*R* or *S*)β[2]**h-Phe**-NH_2	(more polar)	134,9±2,64
6	Tyr-Pro-Phe-(*S* or *R*)β[2]**h-Phe**-NH_2	(less polar)	194,9±4,37
7	Tyr-D-Ala-Phe-Phe-NH_2 **TAPP**		5,1±3,50
8	(*R* or *S*)β[2]**h-Tyr**-D-Ala-Phe-Phe-NH_2	(more polar)	>1000
9	(*S* or *R*)β[2]**h-Tyr**-D-Ala-Phe-Phe-NH_2	(less polar)	77,6±2,42
10	Tyr-D-Ala-(*R* or *S*)β[2]**h-Phe**-Phe-NH_2	(more polar)	107,1±4,27
11	Tyr-D-Ala-(*S* or *R*)β[2]**h-Phe**-Phe-NH_2	(less polar)	38,9±4,35
12	Tyr-D-Ala-Phe-(*R,S*)β[2]**h-Phe**-NH_2		9,1±2,07

[a] μ-ligand: [^{3}H]naloxone.

The conclusions from the current studies are:

1. The chirality of the β^2-homo tyrosine in position 1 is as much important as for α- tyrosine in native opioid sequence.

2. Substitution of phenylalanine in position 4 with racemic β^2-homo phenylalanine in the TAPP sequence resulted in analogue(s) with the relative high affinity to the μ receptors. Our initial studies showed that this analogue is more enzymatic stabile compare to the parent compound.

3. Incorporation of racemic β^2-homo phenylalanine in position 3 in endomorphin-2 and TAPP sequences resulted in analogues, with similar reduction of the binding affinity to the μ receptors (8 - 20-fold).

4. Obtained analogues with the incorporation of the racemic mixtures of β-amino acids possess similar properties which make them sometimes impossible to separate. Therefore the next step of our study will be the separation of isomers of β-amino acids and incorporation them to the peptide sequence in the pure enantiomeric form.

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TARGETING $\alpha_v\beta_3$ INTEGRIN RECEPTOR BY A NEW AND SELECTIVE PEPTIDE ANTAGONIST

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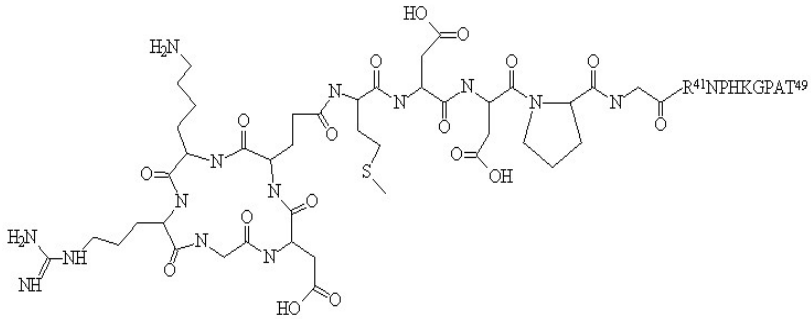
Introduction

The process of new blood vessel formation (angiogenesis) is known to be essential for the tumor growth and metastatic spread ensuring the supply of oxygen and nutrients to proliferating tumor cells. Angiogenesis is characterized by the overexpression of the so-called "angiogenic markers" that normally are not present in resting cells. Among these, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins belonging to a large family of heterodimeric transmembrane cell surface receptor family, play an important role in cell-cell and cell-extracellular matrix adhesion processes [1]. Clinical studies indicate that antagonists able to prevent $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-natural ligand bond can block angiogenic process. Moreover while $\alpha_v\beta_5$ is widely expressed by many malignant tumor cells, $\alpha_v\beta_3$ has a relatively limited cellular distribution compared with that of $\alpha_v\beta_5$. Therefore, in order to target $\alpha_v\beta_3$-mediated processes for diagnostic or therapeutic purposes, the development of new compounds able to block integrin-natural ligand interaction and discriminate between $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is required. To date, various therapeutic candidates, including antibodies, small molecules peptidomimetics, and cyclic peptides have been clinically evaluated and shown to successfully modulate $\alpha_v\beta_3$–mediated processes. So far, the pentapeptide cyclo (-Arg-Gly-Asp-D-Phe-NMeVal-), referred to as c(RGDf[NMe]V), is one of the most active $\alpha_v\beta_3$ antagonists reported in the literature.

Results and Discussion

The crystal structures of the extracellular segment of integrin $\alpha_v\beta_3$ in its unligated state and in complex with c(RGDf[NMe]V) and the docking studies on $\alpha_v\beta_3$ integrin ligands [2, 3]. have shown that the main interactions are between the positively charged arginine and the α-subunit and between the anionic aspartic acid and the β-subunit, and that selectivity between different subunits is achieved by the RGD sequence conformation. Previous studies also reported that echistatin, the smallest (49 residues) of the viper (*Echis carinatus*) disintegrins, is a potent antagonist of the integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ and that the amino acids adjacent to the RGD motif together with the 41- 49 C-terminal residues appear to be critical for the selective recognition of integrins. Mutation and photoaffinity cross-linking experiments, and NMR conformational analysis combined with docking studies

have provided evidence that the C-terminal region of echistatin binds to a site within the β_3 subunit of the $\alpha_v\beta_3$ receptor.



Starting from this structural information, we have designed a bifunctional chimeric molecule made up of a cyclic portion derived from c(RGDf[NMe]V) peptide and a sequence corresponding to 28 - 30 and 41- 49 Echistatin C-terminal tail covalently linked by Pro-Gly spacer. The designed peptide was evaluated and compared to c(RGDfV), a Cilengitide analogue, for its ability to inhibit cell adhesion to vitronectin in human erythroleukemia K562 cells overexpressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. The obtained result indicated RGDechi shows towards αvβ3 receptor affinity comparable to c(RGDfV) one, but it reveals more selective indicating lack of cross-reactivity with $\alpha_v\beta_5$ overexpressing cells [4].

Our findings indicate that the RGDechi chimeric peptide is a novel and selective ligand for $\alpha_v\beta_3$ integrin. This sequence represents the lead compound to develop novel anticancer drugs and/or new class of diagnostic noninvasive tracers as suitable tools for $\alpha_v\beta_3$-targeted therapy and imaging.

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SOLID –STATE ISOTOPIC EXCHANGE WITH SPILLOVER-HYDROGEN IN PROTEINS AND PEPTIDES

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Introduction

The present report summarizes the latest data devoted to theoretical and experimental investigation of the high temperature solid state catalytic isotope exchange reaction (HSCIE) that takes place in peptides and proteins by the action of deuterium and tritium [1- 3]. The available MS-procedures, designed to estimate the amount of protein, are aimed at derivatization at different stages of sample preparation, and as the best result, it is only possible to achieve quality comparison of the objects involved. The HSCIE reaction allows the production of evenly deuterium labelled proteins and peptides, and their application makes it possible to create a qualitative mass spectrometry method for protein analysis.

Results

The HSCIE reaction makes it possible to produce uniformly deuterium labelled amino acids. The opioid 6-member peptide dalargin with deuterium and tritium label was produced with high isotope substitution using the HSCIE reaction. Tritium distribution in [G-^{3}H]dalargin was analysed with ^{3}H-NMR. [G-^{2}H]dalargin was analysed with MS. Deuterium labelled dalargin has been produced, with average incorporation of 4.4 and 5.9 deuterium atoms (Fig. 1). Deuterium labelled peptide can be used for the creation of sensitive procedures for *in vivo* and *in vitro* quantitative assessment with MS. To solve this task of quantitative analysis in proteomics, employment of evenly deuterium labelled proteins produced by the reaction of high temperature solid-state catalytic isotope exchange (HSCIE) with gaseous deuterium has been suggested. Introduction of specified amounts of these deuterium labelled proteins to biological objects prior to isolation, separation and tripsinolysis will make it possible to collect, with the help of chromato-mass spectrometry, quantitative information about the presence of proteins and polypeptides under study in the tissues.

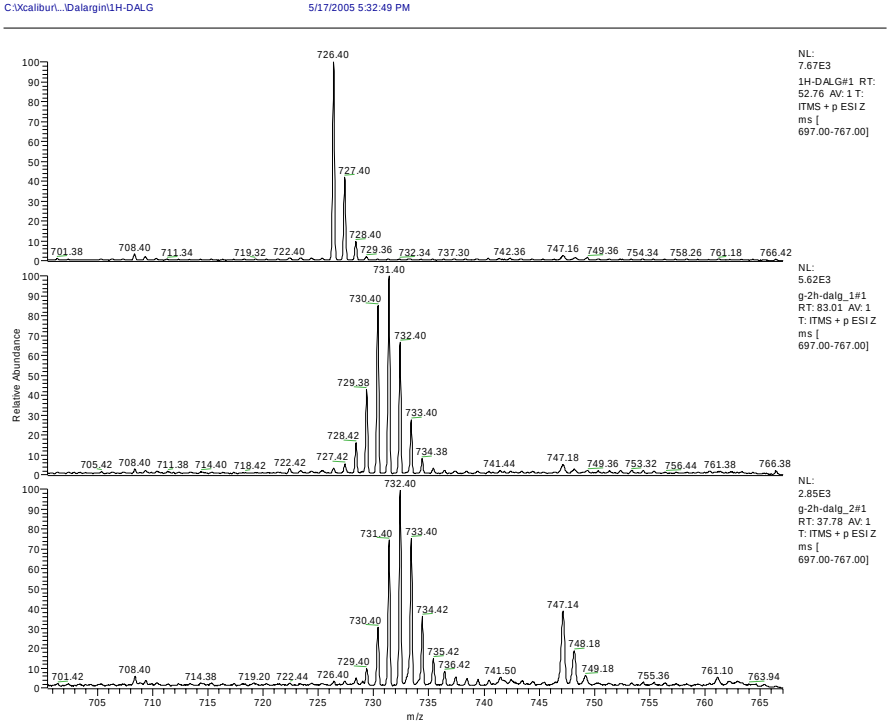

Fig. 1. MS analysis of [G-^{1}H]DALG(a) and [G-^{2}H]DALG(b,c) with average incorporation of 4.4 and 5.9 deuterium atoms, respectively.

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HOW TO MAKE PEPTIDE MIMETICS

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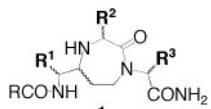
The idea of making scaffold constrained peptide mimetics was first proposed more than 27 years ago [1, 2]. Since then a large amount of effort has been expended attempting to make drug-like mimetics by rational means from peptide hormones. The results of this effort have not been impressive. There are few rationally designed scaffold based non-peptide low nm mimetics known [3] and none appear to have entered clinical trials [4]. Given the idea of making non-peptide mimetics and optimising them into drugs appears reasonable, then this failure needs explaining.

In our work on peptide turn mimetics we sought to develop syntheses based on the following principles:

- the peptide side-chains at all positions are included
- the synthetic approach is general for different side-chains
- chirality is retained and controlled
- relative positioning of the side-chains is maintained



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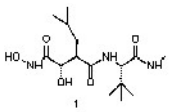
Results

We have developed relatively simple and flexible syntheses of the diazacycloheptane system 1 that follow the principles above [5]. Our methods produce tripeptide mimetics with a wide range of side-chains based on the 1,4-diazacycloheptane scaffold. These mimetics contain up to four chiral centres and our methods allow for the selective synthesis of all sixteen possible diastereomers. In addition we have demonstrated that the ability to control chirality is highly valuable in the process of optimising the biological activity and selectivity of mimetics. Application of the methods to hormone mimetics have produced compounds with activity at a number of hormone receptors including somatostatin, urotensin II, C5a and melanocortin receptors. Melanocortin-5 receptor antagonists with 3 nm affinity and selectivity of 700+ fold relative to the other melanocortin receptors have been obtained. The scheme describes the simplest of the methods giving rise to a tripeptide mimetic having two of three sidechains plus N- and C-termini in only four steps from Boc-Glycine Weinreb [6] amide 2. The Michael ketone 3 was prepared by a similar method to that of Rapoport [7]. Conjugate addition under mild conditions gave the aminoketone 4. The hindered amine in this compound was best acylated by the use of an amino acid fluoride [8]. Hydrogenation of the Cbz group frees the amine to cyclise to the imine and undergo reductive amination in the same reaction to give the target 7.



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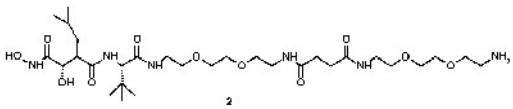

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Discussion

Largely due to synthetic expediency the suggested principles for peptide mimetic design have rarely been followed. This reflects the difficulty of doing chemistry with the necessarily highly functionalised systems – compromise is the rule rather than the exception. This factor may have contributed to the poor record achieved in bringing scaffold based peptide mimetics into the clinic, let alone to market.

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4. Myriad Pharmaceuticals has recently started a phase I trial of a thrombin inhibitor based on a scaffold developed at Molecumetics, the former peptide mimetics company founded by Michael Kahn. However this is an enzyme inhibitor rather than a hormone mimetic and is based on a beta strand conformation. Peptide substrate derived enzyme inhibitors have been a notable success of peptide mimetic design – many HIV protease inhibitors arguably fall into this class.
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EFFECT OF SHORT PEPTIDES ON Aß(1-42) FIBRILS

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Introduction

It is widely accepted that the aggregates of ß-amyloid peptides, which are formed during the enzymatic cleavage of amyloid precursor protein has a pivotal role in the progression of Alzheimer disease. Short peptides like H-Leu-Pro-Phe-Phe-Asp-OH (LPFFD-OH) and H-Leu-Pro-Tyr-Phe-Asp-NH_2 (LPYFD-NH_2) can influence the structure and aggregation of ß-amyloid peptides. Soto's pentapeptide LPFFD-OH has been published as a ß-sheet breaker (BSB) [1]. These pentapeptides must bind to amyloid fibrils for being able to behave as a BSB or disturb the structure of the fibrils. Such peptides can inhibit the progression of Alzheimer disease [1,2]. Our aims were to check if these peptides has a protective effect against Aß induced cellular responses and to investigate if LPFFD-OH and LPYFD-NH_2 bind to fibrillar ß-amyloid peptides.

Results

MTT assay [3] revealed that Aß(1-42) reduces the viability of SH-SY5Y neuroblastoma cells by 50% and this reduction can be attenuated by LPYFD-NH_2 and LPFFD-OH pentapeptides. When LPYFD-NH_2 and LPFFD-OH was added 90% and 70% of the cells retained their viability, respectively.

In vivo electrophysiology experiments [2] showed that Aß(1-42) increased the maximum values of NMDA evoked responses of rat hippocampal neurons to 214 % of control. When Aß(1-42) was mixed with LPYFD-NH_2 and LPFFD-OH these responses were 134 and 169% of control, respectively.

Radioligand binding experiments showed that tritiated LPYFD-NH_2 bind to fibrils of synthetic Aß(1-42) and the bound labeled peptide can be replaced by non-labeled LPYFD-NH_2 and LPFFD-OH in a concentration dependent manner (Fig. 1).

Big aggregates of Aß(1-42) (>10 nm) are non-visible for NMR spectroscopy. The signal intensities of the pentapeptides decreased upon addition of fibrillar Aß(1-42) (Fig. 2).

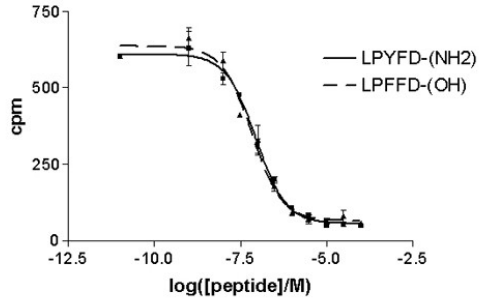

Fig. 1. Competition experiments between unlabeled peptides and [^{3}H]-LPYFD-NH_2 on aggregated Aß.

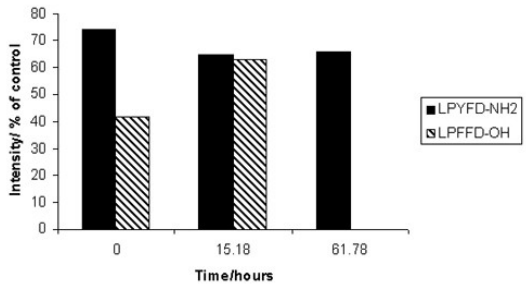

Fig. 2. Decline of the intensity of the NMR signals of the pentapeptides.

Discussion

MTT assay and electrophysiology experiments showed that the examined pentapeptides can inhibit Aß(1-42) induced processes both '*in vitro*' and '*in vivo*', and LPYFD-NH_2 has a greater protective effect than LPFFD-OH. Relaxation of 1H signals of pentapeptides occur upon binding to Aß aggregates. Thus the decrease in the intensities of signals reveal binding to such structures. The result of the radioligand binding experiments also supported that both pentapeptides bind to fibrillar Aß(1-42) in a replacable manner.

Acknowledgements

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Radiolabeled peptide was prepared by Dr. Géza Tóth and his coworkers.

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SHORT PEPTIDE FRAGMENTS WITH ANTIULCER ACTIVITY FROM A COLLAGEN HYDROLYSATE.

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Introduction

The glyproline family of regulatory peptides [1] includes Pro-Gly-Pro, Gly-Pro, Pro-Gly, and also Hyp peptides. A distinctive feature of these peptides is that they exhibit a broad spectrum of biological activities: the inhibition of some constituents involved in blood clotting and thrombosis [2], the maintenance of gastric mucosa homeostasis [3], the survival of cells under the conditions of oxidative stress [4]. The possibility of glyproline action through the central nervous system was confirmed, in the stress model of ulcer formation, by the presence of antiulcer effects upon the normalization of stressogenic behavioral disorders [5]. Glyprolines are believed to be the endogenous peptides that may be formed in the synthesis or catabolism of collagen [2]. The addition of gelatin (partially hydrolyzed collagen) as a food supplement for rats reliably reduces the lesions induced by ethanol in stomach [6]. This protective effect can be caused by such collagen peptides as PG, GP, and PGP, and also by Hyp containing petides, exhibiting some antiulcer activity [5].

Results

HPLC analysis of the peptide hydrolysate showed that it consists mainly of short glyprolines. Thirty individual peptides with molecular masses in the range of 174 - 420 amu were isolated by HPLC from the extract. The 2 – 4-aa peptides are predominantly present, including the peptides PG, GP, PGP, and HypGP.

The intragastric introduction of the extract at doses of 1 and 0.1 mg/kg resulted in a significant reduction of the ethanol and stressor lesions in gastric mucosa. It is well known that the pathogenesis of ethanol-induced lesions is mainly determined by peripheral mechanisms, whereas the stress-induced lesions, by central mechanisms [3]. The data obtained indicate that collagen extract may affect both central and peripheral mechanisms. Such duality of such action fully correlates with the data we previously obtained for the individual peptides PG, GP and PGP [3]. Thus, the results of the above described experiments indicate that the extract enhances the stability of gastric mucosa toward the action of such ulcerogenic factors, as ethanol and stress due to its protective antiulcer properties. It should be emphasized that PHC is active not only at a dose of 1 mg/kg, but at a dose that is lower by one order

of magnitude. When used in the ethanol model at a low dose, PHC tends to reduce the lesion area. In the stress model of ulcer formation at a dose of 0.1 mg/kg, PHC has the efficiency similar to that observed at the higher dose and causes two-fold reduction of the ulcer area. The same high activity of PHC at a dose of 1 mg/kg was observed in the acetate and alcohol models of ulcer formation. In the acetate model, which corresponds by histomorphological and temporal characteristics to the stomach lesions observed upon the development of peptic ulcer in human, the introduction of PHC for 3 days to animals with the fully developed ulcer resulted in a significant reduction of the area of lesions in comparison with control. This means that PHC accelerate the ulcer healing, *i.e*., exhibits a therapeutic (healing) antiulcer effect.

It should be noted that the therapeutic effect is exhibited upon both the intraperitoneal and intragastric administration. Moreover, a tendency to faster ulcer healing was observed in the last case (>50% as compared to >40%).In our experiments PHC exhibits both protective and healing antiulcer effects comparable with the effects of individual PGP, which is known now as the most potent glyproline [1].

Thus, we have found that PHC is effective in all the models of ulcer formation and leads to a normalization of the parameters of stomach homeostasis. Probably, not only the above-mentioned glyprolines but also other still unknown short peptides are involved in the antiulcer activity of PHC This presumption is supported by the fact that PHC totally containing less than 10% of the peptides PGP, PG, GP, and HypGP exhibited in some ulcer models the activity, which is even somewhat higher than that of the individual PGP. In order to develop the most promising protective and healing antiulcer preparations, it seems reasonable to study the antiulcer effects of individual glyproline constituents of PHC.

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CPHV-1 VACCINES BASED ON PEPTIDE CONJUGATES: SYNTHESIS AND CHARACTERIZATION

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Introduction

Peptide based vaccines provide more advantages than disadvantages when compared to vaccines based on recombinant proteins, DNA constructs, attenuated bacterial or viral vectors, of being selective, chemically defined and safe [1]. In addition, large quantities of chemically purified peptide vaccines can be prepared. A number of report indicates that general peptides incorporating helper T cell determinants and B cell epitopes in multiple copies in branched architecture were better immunogens [2]. To achieve this result, epitope peptides were conjugated to an appropriate carrier molecule, chosen on the basis of the size, the number of reactive groups and the solubility

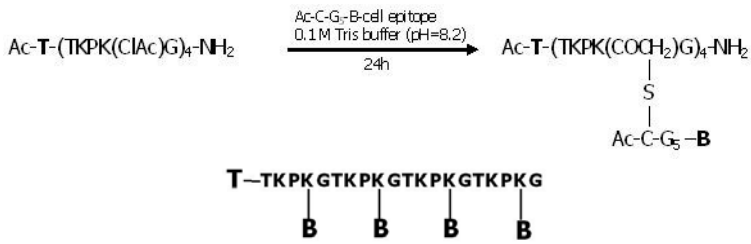
Our goal is to prepared epitope based vaccines against caprine herpesvirus 1 (CpHV-1). This virus which belongs to the family *Herpesviridae*, sub-family *Alphaherpesvirinae*, is responsible for a generalized disease characterized by high morbidity and mortality and severe gastroenteric lesions in goat kids aged 1- 2 weeks [3]. The use of a vaccine could provide a powerful tool for the control of CpHV-1 infection. A classical inactivated vaccine was prepared to assay the efficacy in the prevention of CpHV-1 infection in goats [4]. This experimental vaccine did not induce any adverse effects, either local or genital and, therefore, appears to be safe, but the vaccine was not able to completely prevent infection by the vaginal route.

Result and Discussion

For the rational design of synthetic vaccines, a potential immunogen must contain a carrier and covalently bond selected epitopes As carrier was considered a tetratuftsin derivative, consisting of four repeated units TKPKG pentapeptide [5]. In order to localize immunogenic epitopes, glycoproteins B, C and D of CpHV-1 were analyzed with various prediction programs and the data were compared. The TEPITOPE and the CTL prediction programs were used to identify the possible T cell epitopes while PHD and Chou-Fasman prediction methods were applied to recognize the potential B cell epitopes. The potential T cell epitope $gB_{453\text{-}465}$ (VVAFRPLLSNELA), was synthesized, step by step, on the N-terminus of the

carrier $[TKPK(ClAc)G]_4$. Possible B cell epitopes $gC_{186-194}$ (FRSSRGDAE) and $gB_{328-336}$ (GYYSRDLTS), on solid phase methodology, were elongated at the N-terminal with Boc-Cys(Bzl)-OH after the coupling of Gly spacer. The cysteine is used to form the thioether bond with a lysine ε-haloacetylated amino group of the carrier. In order to moderate the level of dimer formation, α-amino groups of Cys were acetylated and the compounds were proved to be suitable for conjugation. Compounds were cleaved from the resin with HF and successively were purified by semi-preparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS.

For the preparation of goat vaccines, B cell epitopes were conjugated via thioether bond with the choroacetylated N^{ε}-amino groups of lysine residues of the T cell-tetratuftsin derivatives in 1 M TRIS buffer (scheme 1). After 24 h the reaction was completed, the solution was acidified by adding TFA to achieve pH 3. Conjugates $Ac\text{-}gB_{453-465}\text{-}[TKPK(AcCG_5gB_{328-336})G]_4\text{-}NH_2$ and $Ac\text{-}gB_{453-465}\text{-}[TKPK(AcCG_5gC_{186-194})G]_4\text{-}NH_2$ containing four copies of the attached peptide (Fig. 1) were separated by semi-preparative RP-HPLC and characterized by ESI-MS.



The results of ELISA show strong IgB antibody binding of T cell epitope-tetratuftsin conjugate with caprine sera (CpHV positive). High reactivities of the sera with the biotinylated peptides (biot-G_5-$gB_{328-336}$ and biot-G_5-$gC_{186-194}$) suggest that chosen peptides correspond to an immunodominant region. ELISA data demonstrate that the B cell epitopes and the conjugates T cell-tetratuftsin induce epitope-specific and antibody responses. On the basis of ELISA the selected T cell and B cell epitopes are good hapten-candidate in vaccines. *In vitro* and *in vivo* experiments are currently being performed in order to analyse the activity of our synthetic vaccines.

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NEUROPEPTIDE Y (NPY) ANALOGUES AS POTENTIAL PEPTIDE CARRIERS FOR CHEMOTHERAPEUTICS

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Introduction

Recently, many human tumours have been shown to selectively overexpress receptor molecules. These receptors represent potential targets for cancer therapy, since their peptide ligands could act as target specific drug carriers [1]. Neuropeptide Y (NPY) is a peptide hormone consisting of 36 amino acids, that binds to so-called Y receptors. A strong predominance of Y1 receptors have been found in breast carcinomas compared to Y2 receptors in normal breast tissue, which suggest a switch in the NPY receptor expression from Y2 to Y1 during the neoplastic transformation [2]. Accordingly, the targeting of breast cancer for therapy with Y1 specific NPY analogues is suggested. Herein, we studied the binding and cytotoxic properties of several NPY analogues that might be qualified for breast tumor targeting in human breast cancer MCF-7 cells.

Result and Discussion

The investigated peptide amides have been synthesised by automated Fmoc/t-Bu solid-phase strategy as described previously [3]. Pig NPY (pNPY) was modified by introducing the cathepsin B cleavage site GFLG with an N-terminal Cys that serves as coupling point for chemotherapeutics either by a disulfide bond or nucleophilic addition. The lysosomal protease cathepsin B frequently is expressed in breast cancer tissue. Thus, the cleavage site is thought to improve the drug release inside the cells. To evaluate a favourable position for the modification, GFLG was added either N_{α}-terminally by using a spacer, or to the N^{ε} of Lys^{4} or to a non-native Lys^{15}, respectively. These positions have been shown to be not important for the ligand binding at the receptor [4].

As summarised in Table 1, all analogues were tested for binding affinity at MCF-7 cells. Compared to native pNPY, the modified peptides show ~ 8.5-fold lower binding affinities, except of peptide [4]. The reason is most probably due to steric hindrance.

Furthermore, possible cytotoxic effects of all analogues have been investigated by using a Resazurin-based cell viability assay. MCF-7 cells have been incubated with several concentrations of NPY analogues for 24 h. Approximately 90 % of the cells survived the treatment with up to 100 µM peptide concentration for all analogues except of peptide [4]. This peptide showed some cytotoxicity at 100 µM concentration (survival 75%) (data not shown).

Table 1. Sequences and binding affinity (IC50 values) of pNPY analogues.

peptide		sequence	IC_{50} [nM]
(1)	pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	16.2
(2)	CGFLG-$(\beta Ala)_2$-pNPY	CGFLG-$(\beta Ala)_2$-YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2	136.0
(3)	[K^4(CGFLG)]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2 ^{L}GLFGC	138.9
(4)	[K^{15}(CGFLG)]-pNPY	YPSKPDNPGEDAPAKDLARYYSALRHYINLITRQRY-NH_2 ^{L}GLFGC	74.0

By conjugating chemotherapeutics to the novel NPY analogues, smart peptide-drug complexes can be formed. With the help of the GFLG sequence the effective drug concentration should be increased inside the cells. Furthermore, by using a Y1 receptor preferring NPY analogue, *e.g.* [Phe^7,Pro^{34}]-pNPY, the complex could be applied as a peptidic drug carrier for effective and selective breast cancer targeting.

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SYNTHESIS AND HIGH RESOLUTION MASS SPECTROMETRIC STRUCTURAL CHARACTERIZATION OF POLYUBIQUITIN CONJUGATES

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Introduction

Modification of proteins by covalent attachment of ubiquitin plays a fundamental role in the control of many biological processes including cell cycle regulation, transcription, DNA repair, and apoptosis [1, 2]. Ubiquitin contains 7 lysine residues each of which can be used for polyubiquitin conjugation and chain formation. Recent evidence indicates that the actual lysine residue of ubiquitin used for ubiquitin-ubiquitin conjugation determines the biochemical function and cellular destination of the respective polyubiquitin chain. The synthesis and structural characterization of chemically defined polyubiquitin chains that differ by the lysine residues used for ubiquitin-ubiquitin attachment have been the major goal of the present study.

Results

The synthetic concept pursued makes use of ubiquitin derivatives, in which the specific lysine residue of an "acceptor" ubiquitin to be conjugated is activated by chloroacetylation, whereas in a "donor" ubiquitin the C-terminal glycine is substituted with a cysteine residue. Ubiquitin partial peptide building blocks were first synthesized using conventional orthogonal solid phase peptide synthesis (SPPS) that contain the specific ε-amino-chloroacetylated lysine residue in the "acceptor" peptide sequence. The side chain conjugation to the "donor" ubiquitin sequence was performed by *S*-alkylation to a C-terminal cysteine residue introduced in addition to and/or in place of the terminal glycine-76 of ubiquitin [3]. In a first variant recombinant "donor" ubiquitin containing a C-terminal cysteine was prepared by bacterial expression according to established protocols, and conjugated to yield a diubiquitin. Initial studies showed that reaction of the N^{ε}-chloroacetylated peptides with C-terminal thiol-peptides was relatively slow under slightly basic conditions, and required long reaction times, increasing the risk of side reactions. Therefore, we investigated the increase of reactivity by substituting the N^{ε}-chloroacetyl peptides with the corresponding - bromo or –iodo derivatives. First conjugation reactions were performed in solution between a Lys^{63}-chloroacetylated ubiquitin peptide and second ubiquitin elongated by a cysteine residue at the C-terminus, by treatment with a saturated KI solution containing 0.1% TFA, under alkaline conditions (0.1 M Tris buffer, pH 8.7). The conjugation reaction was monitored by analytical RP-HPLC on a C18 column. The resulting conjugation

product of G^{76}-Ubiquitin with in situ iodo-acetylated ubiquitin (54 - 76) was characterized by ESI-FT-ICR-MS and ESI-Ion trap-MS [4] (Fig. 1).

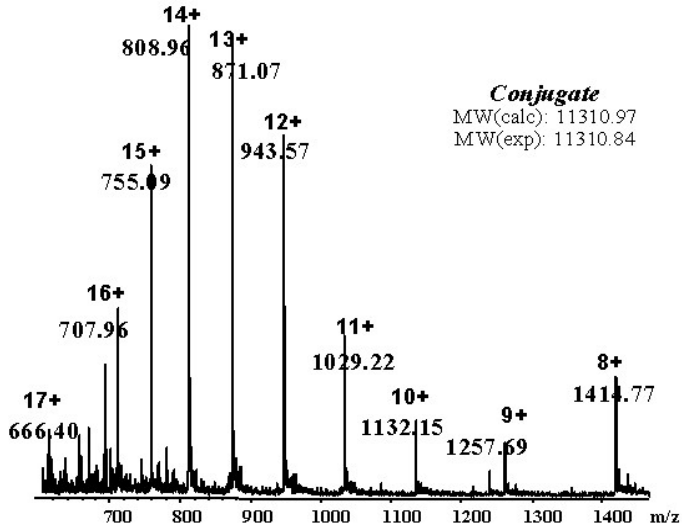

Fig. 1. ESI-Ion trap-MS of the conjugation product of iodoacetylated Ubiquitin(54-76) with Cys^{76}-Ubiquitin.

Discussion

This procedure developed here presents several advantages compared to conventional conjugation, particularly (i) specific linkage to each of the lysine residues of ubiquitin, (ii) specific and chemically stable conjugation of ubiquitin chains, and (iii) the possibility to build up oligomeric ubiquitin conjugates that contain authentic lysine-peptide linkages as branching sites. Furthermore, this approach is feasible to conjugation of complete ubiquitin sequences, as well as specific partial ubiquitin peptides. Corresponding comparative studies of the biochemical activities are expected to give important information on structural requirements and recognition structures of the lysine-branched conjugates.

Acknowledgements

This study has been supported by grants from the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (M.P., M. Sch).

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SYNTHESIS AND BINDING ACTIVITIES FOR THE OPIOID RECEPTORS OF ENDOMORPHIN AND MORPHICEPTIN ANALOGS CONTAINING CONFORMATIONALLY CONSTRAINED 1-AMINOCYCLOALKANE-1-CARBOXYLIC ACIDS

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Introduction

Endomorphin-1 (EM-1) and endomorphin-2 (EM-2) are very potent endogenous opioid peptides, which exhibit high affinity and selectivity for the μ-opioid receptor [1]. Previously, we have synthesized endomorphin analogs replacing Pro in position 2 with various α,α-disubstituted glycines, such as Aib and 1-aminocycloalkane-1-carboxylic acid [Ac_nc (n = 3, 5, 6); n indicates a ring size]. Among them, [Ac_3c^2]-EM-1 and [Ac_3c^2]-EM-2 containing 1-aminocyclopropane-1-carboxylic acid (Ac_3c) exhibited 3- and 6-fold higher affinities than those of EM-1 and EM-2, respectively, for the μ-opioid receptor. Therefore, the substitution of Ac_3c for Pro in position 2 of EM can be useful for analogs to adopt bioactive conformation [2]. This is also the case for morphiceptin (MC) analogs containing an α,α-disubstituted glycine instead of Pro in the 2-position [3]. We report here the synthesis of [Ac_nc^2]-analogs of bioactive EM and MC analogs, which contain 2',6'-dimethyl-L-tyrosine (Dmt) and L-phenylalaninol (Pheol) in position 1 and 4, respectively, which has been reported in the literatures [4 - 6], and their structure-activity relationship.

Result and Discussion

Endomorphin analogs were prepared by the EDC-HOBt method. The purity was verified by HPLC. The receptor binding potency of various analogs summarized in Table 1 was assessed by the radio-ligand receptor binding assay using COS-7 cells expressing μ or δ opioid receptors. [Ac_3c^2]-EM-1, [Ac_3c^2]-EM-2 and [Ac_4c^2]-EM-2 exhibited higher binding potency than EM-1 and EM-2 for μ-opioid receptor. They showed fairly high selectivity for the μ over δ receptors. Every analogs of [Dmt^1, Ac_nc^2]-EM-2 exhibited much higher binding affinity than EM-1 and -2. Among them, [Dmt^1,Ac_3c^2]-EM-1 and [Dmt^1,Ac_4c^2]-EM-2 exhibited the highest potency

Table 1. Binding potency of endomorphin analogs to μ- and δ-opioid receptors.

Peptides	Binding potency (IC_{50} : nM)	
	μ-Receptor	δ-Receptor
Tyr-Pro-Trp-Phe-NH_2 (EM-1)	2.65 ± 0.67	>10,000
Tyr-Pro-Phe-Phe-NH_2 (EM-2)	3.61 ± 0.46	>10,000
Tyr-Ac_3c-Trp-Phe-NH_2	2.06 ± 0.20	3,030 ± 427
Tyr-Ac_3c-Phe-Phe-NH_2	0.660 ± 0.045	1,390 ± 129
Tyr-Ac_4c-Phe-Phe-NH_2	1.55 ± 0.13	4,110 ± 433
Tyr-Ac_5c-Phe-Phe-NH_2	7.73 ± 1.87	8,480 ± 305
Tyr-Ac_6c-Phe-Phe-NH_2	26.0 ± 4.9	4,820 ± 102
Dmt-Pro-Phe-Phe-NH_2 [a]	0.15 [a] ± 0.04	28.2 [a] ± 8.1
Dmt-Ac_3c-Trp-Phe-NH_2	0.173 ± 0.05	27.3 ± 3.8
Dmt-Ac_3c-Phe-Phe-NH_2	0.210 ± 0.07	11.5 ± 0.2
Dmt-Ac_4c-Phe-Phe-NH_2	0.332 ± 0.092	68.5 ± 5.9
Dmt-Ac_5c-Phe-Phe-NH_2	0.627 ± 0.108	254 ± 16
Dmt-Ac_6c-Phe-Phe-NH_2	0.808 ± 0.09	245 ± 84
Tyr-Pro-Phe-Pro-NH_2 (MC) [b]	87.6 ± 15.4	N.B.
Tyr-Aib-Phe-Pro-NH_2	325 ± 25	>10,000
Tyr-Ac_3c-Phe-Pro-NH_2	58.8 ± 17.2	>10,000
Tyr-Ac_4c-Phe-Pro-NH_2	66.1 ± 9.1	>10,000
Tyr-Ac_5c-Phe-Pro-NH_2	619 ± 41	>10,000
Tyr-Ac_6c-Phe-Pro-NH_2	199 ± 27	>10,000
Tyr-Pro-Phe-Pheol [c]	2.55 ± 0.42	>10,000
Tyr-Aib-Phe-Pheol	43.4 ± 8.1	>10,000
Tyr-Ac_3c-Phe-Pheol	1.23 ± 0.32	2780 ± 540
Tyr-Ac_5c-Phe-Pheol	30.5 ± 7.4	>10,000

a) Ref. 5. b) Ref. 4. c) Ref. 6.

for the μ-opioid receptor. However, the replacement of Tyr1 by Dmt reduced δ/μ-selectivity. Although [$Pheol^4$]-EM-2 showed slightly higher affinity than EM-2, [Ac_3c^2,$Pheol^4$]-EM-2 exhibited higher affinity than the [$Pheol^4$]-analog for the μ-opioid receptor. [Ac_3c^2]-MC and [Ac_4c^2]-MC also exhibited 1.5- and 1.3-fold higher affinity, respectively, than MC itself for the μ-opioid receptor. In conclusion, the replacement of Pro to Ac_3c and Ac_4c seems to be efficient to make these analogs adopt bioactive conformation and exhibit higher affinity for the μ receptor, even in EM analogs in which an amino acid in position 1 or 4 are replaced by the other amino acid.

Acknowledgements

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SYNTHESIS AND INVESTIGATION OF NEUROPEPTIDE Y ANALOGUES CONTAINING AN ARTIFICIAL PYRIDONE DIPEPTIDE MIMETIC

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Introduction

More than two decades after its first isolation from pig brain [1] neuropeptide Y (NPY) still attracts significant attention, which might be due to its wide distribution throughout mammalian brain. NPY consists of 36 amino acids, is C-terminally amidated and suggested to exhibit an N-terminal left-handed polyproline type II helix (residues 1- 9), a β-turn (residues 10 - 13), and an amphiphilic α-helix from position 14 to the C-terminally flexible loop structure. The C-terminal segment has been shown to play an important role in binding to all receptor subtypes [2] and is structurally well defined by various NMR investigations. Therefore, structure-affinity/activity-relationship studies have been significantly focused on this part of the NPY molecule [3]. In contrast, less information is available on the N-terminal segment.

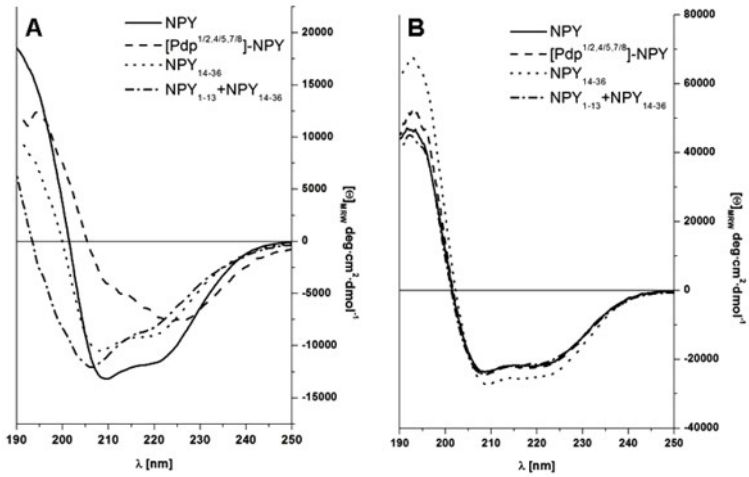
Result and Discussion

To investigate the influence of the N-terminus on the structure of NPY a conformationally constrained building block (Pdp) has been introduced into NPY at positions 1/2, 4/5, and 7/8 substituting three natively occurring proline residues. This bicyclic pyridone exhibits an N-terminal dehydroalanine moiety twofold linked to a C-terminal thiaproline and is N-terminally completely planar (φ = 180°, ψ = 180°) [4]. Automated SPPS using Fmoc/*tert*-butyl strategy has been applied for peptide assembly of the C-terminal segment of NPY [5], HATU/DIEA for manual coupling of the Fmoc-protected building block and the directly following amino acids. Full length NPY as well as N- and C-terminally truncated analogues (NPY_{1-13} and NPY_{14-36}) have been synthesised for comparative CD studies.

	Sequence
NPY (porcine)	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2
[$Pdp^{1/2,4/5,7/8}$]-NPY	ΔS-ΔD-ΔGEDAPAEDLARYYSALRHYINLITRQRY-NH_2
NPY_{1-13}	YPSKPDNPGEDAP-NH_2
NPY_{14-36}	AEDLARYYSALRHYINLITRQRY-NH_2

CD studies showed that NPY, [$Pdp^{1/2,4/5,7/8}$]-NPY as well as an equimolar mixture of NPY_{1-13} and NPY_{14-36} exhibit equal mean-residue-weight molar ellipticities ([Θ]MRW) in 50% TFE (Fig. 1 B). This suggests that all peptides are able to form an α-helix in a helix-stabilising environment. The α-helical, C-terminal segment NPY_{14-36} exhibits higher $[\Theta]_{MRW}$ values, as expected. In contrast, in 10 mM phosphate buffer pH 7 (Fig. 1A) the stability of the C-terminal α-helix is influenced

by the N-terminus. Deletion of the N-terminal segment resulted in lower $[\Theta]_{MRW}$ values that indicate a loss in stability as the CD spectrum of NPY is not identical to the spectrum of the mixture of both NPY fragments. Threefold introduction of the pyridone dipeptide in the N-terminal segment of NPY resulted in a significantly destabilised α-helix. The CD spectra clearly showed that the α-helix is influenced by the N-terminus but still can be formed and stabilised by TFE or in a membrane environment prior to binding to the receptor.



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AN UNIVERSAL PEPTIDE SCAFFOLD TO DEVELOP ANTIGENIC PROBES SPECIFIC FOR AUTOIMMUNE DISEASES

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Introduction

We demonstrated, for the first time, that *N*-glucosylation is a Post-Translational Modification (PTM) possibly triggering autoantibody response in Multiple Sclerosis (MS). This was possible because of an innovative chemical "reverse approach", which led to CSF114(Glc), a structure-based designed glycopeptide. This glycopeptide is the first Multiple Sclerosis Antigenic Probe accurately measuring high affinity autoantibodies (biomarkers of disease activity) in sera of a statistically significant patients' population, compared to other autoimmune diseases [1]. Therefore, our "reverse approach" can be extended to other autoimmune conditions, such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE), proposing the β-hairpin structure of CSF114 [2] as an "Universal Peptide Scaffold" to be modified with a series of glycosyl amino acids (different in sugars and linkages), in the aim of developing personalized diagnostic/prognostic tests.

Results

In order to detect and characterize autoantibodies as biomarkers, it is necessary to optimize properly defined synthetic peptide epitopes bearing a selected specific PTM.

In the field of Rheumatoid Arthritis, PTMs heavily affect the immunogenicity of Collagen II in murine models. Galactosylation of specific lysine residues plays a critical role for T cell recognition of Collagen II [3]. In fact the Collagen sequence (259-273) bearing one or two galactose residues was recognized as dominant epitope in the T cell response of RA patients [4].

Starting from these data, we undertook the synthesis of *N*- and *O*-galactosyl amino acids to be introduced in the right position of our universal peptide scaffold. We synthesized the galactosyl building blocks Fmoc-Asn(GalOAc$_4$)-O*t*Bu (**1**) and Fmoc-Ser(GalOBz$_4$)-OPfp (**2**), orthogonally protected for solid-phase peptide synthesis (SPPS), and we introduced them in the CSF114 sequence to obtain the *N*-galactocopeptide [Asn^7(Gal)]CSF114 (**I**) and the *O*-galactocopeptide

[Ser^7(Gal)]CSF114 (**II**), respectively. The galactopeptides were purified and tested by solid-phase ELISA (SP-ELISA) in RA patients' sera.

Our "reverse approach" was extended to the study of SLE disease. The etiology of SLE is still unknown, but many evidences support the idea that mannosylation of epitopes could be associated with the disease progress in the murine model [5]. Therefore, we decided to perform the synthesis of peptides bearing mannosyl moieties in the β-hairpin structure of CSF114, to be used as putative autoantigens in SLE.

Fmoc-Asn(ManOAc$_4$)-O*t*Bu (**3**) and Fmoc-Ser(ManOBz$_4$)-OPfp (**4**) were synthesized and employed in the preparation of the *N*-mannopeptide [Asn^7(Man)]CSF114 (**III**) and the *O*-mannopeptide [Ser^7(Man)]CSF114 (**IV**), respectively.

Discussion

The biological results of the galactopeptides showed that the *O*-galactopeptide [Ser^7(Gal)]CSF114 (**II**) recognizes autoantibodies in 30% of RA analyzed sera, and let us to hypothesize the importance of *O*-galactosylation in antibody recognition in this disease.

Biological tests on mannopeptides will confirm our hypothesis about their involvement in triggering SLE.

In conclusion, our "reverse approach", successfull in MS, has been extended to other autoimmune conditions, as RA and SLE, proposing CSF114 as an "Universal Peptide Scaffold" to be modified for the development of specific biomarkers. Therefore, the CSF114 β-turn structure, exposing at the best the aberrant PTM specific for antibody-mediated forms of other autoimmune diseases, will lead to a family of Synthetic Antigenic Probes to be used in diagnostic/prognostic immunoassays possibly useful for monitoring response to therapies.

Acknowledgements

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A STABLE CONFORMER OF PROLINE TRIPEPTIDE WITH ACE-INHIBITORY ACTIVITY PROVED BY SOLID-STATE IR-LD SPECTROSCOPIC AND THEORETICAL ANALYSIS

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Introduction

In contrast to the vast information on the production and characterization of antihypertensive peptides, there is very limited information on the conformation-bioactivity relationships [1].

The aim of this study is a structural prediction and IR-characteristic bands assignment of antihypertensive tripeptide Valyl-Prolyl-Proline (H-Val-Pro-Pro-OH) by means of lineardichroic infrared (IR-LD) spectroscopy of oriented solid sample as a nematic liquid crystal suspension [2]. The data are supported by theoretical ones obtained by ab initio calculations at Hartree-Fock level of theory and 6-31++G** basis set.

The geometry parameters are compared with known crystallographic ones, indicating a good correlation. The clearance of IR-spectroscopic character of the bands, the IR-data of the tripeptide have been assigned on the basis of IR-LD spectroscopic results in solid-state of amino acids zwitterions L-Valine (L-Val) and L-Proline (L-Pro), respectively.

Result and Discussion

Theoretical analysis. The optimized geometry of tripeptide studied shown in Fig.1 corresponds to the most stable conformer with Erel equal to 0.1 kJ×mol^{-1} obtained by preliminary conformational analysis generated by a minimization of an energy varying the dihedral angles. The amide fragments are with transoide conformation due to the theoretical predicted dihedral C=O-NC angles of 171.4^{o} and 174.4^{o}, respectively. The other conformers with Erel lower than 5 kJ×mol^{-1} are 12 conformers with E_{rel} values within 0.1- 3.2 kJ×mol^{-1} The optimized values of H-Val-Pro-Pro-OH (Fig. 1) agree well with those refined by X-ray diffraction for similar peptide systems as, H-Pro-Pro-Pro-OH and H-Pro-Val-OH [3 - 4].

Conventional and linear-polarized IR-spectroscopic data. The characteristic IR-bands of tripeptide Val-Pro-Pro are supported by detailed IR-LD spectroscopic analysis of the pure amino acids L-Val and L-Pro. According to single crystal X-ray data of Pro, the unit cell contains four molecules mutually similarly oriented by

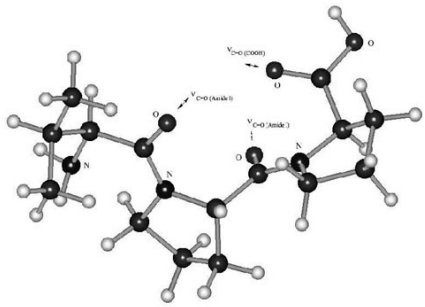

Fig.1. Optimized geometry of tripeptide studied.

pairs thus assumed the near to co-linear orientation of δNH_2^+ and $\nu^{as}COO^-$ in the frame of these neighboring pairs of molecules. For that reason the obtained IRLD reduced spectrum illustrates the strong reduction of 1567 cm^{-1} peak with the 1626 cm^{-1} one. Due to the last procedure second pairs of observed maxima at 1612 cm^{-1} and 1552 cm^{-1} correspond to δNH_2^+ and $\nu^{as}COO^-$ of a second differently oriented molecule of L-Pro in the frame of the unit cell.The multiple character of 1750–1500 cm^{-1} region is determined as well , with applied deconvolution and curve-fitting procedure resulting in the series of peaks. Comparing the solid-state IR-spectra of all the systems studied, it is interesting to note that in contrast to pure amino acids stabilized as H_3N^+-R-COO^- (L-Val) and H_2N^+-R-COO^- (L-Pro) zwitterions with characteristic IR-spectral bands of -NH_3^+, -NH_2^+ and -COO^- groups, the tripeptide H-Val-Pro-Pro-OH is characterized by a series of peaks assigned to $\nu^{as}NH_2$, $\nu^{s}NH_2$ and νC=O (COOH) bands. The consequent elimination of 1733 cm^{-1}, 1675 cm^{-1} and 1640 cm^{-1} in different dichroic ration confirms adequately the different orientation of the corresponding νC=O stretching and amide I transition moments in the molecule of H-Val-Pro-Pro-OH. According to our theoretical data their mutual orientation is at an angle of 87.9°, 72.0° and 82.6°, respectively Fig.1. The chemically synthesized tripeptide H-Val-Pro-Pro-OH is spectroscopically and structurally elucidated by a series of methods starting with ^1H and ^{13}C-NMR spectroscopy in solution, solid-state linear polarized IR-spectroscopy based, on an orientation technique as a nematic liquid crystal suspension and quantum chemical calculations at HF/6-31++G**. In contrast to other peptide systems studied, where the neutral form is characterized by zwitterionic NH_3^+-R-COO^- form, H-Val-Pro-Pro-OH exist as NH_2-R-COOH form. These data are an important part of systematic *in vitro* investigations leading to a clearance of the relationship between structure and biological activity.

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SOLID-PHASE SYNTHESIS OF PROLINE TRIPEPTIDES WITH ACE- INHIBITORY ACTIVITY

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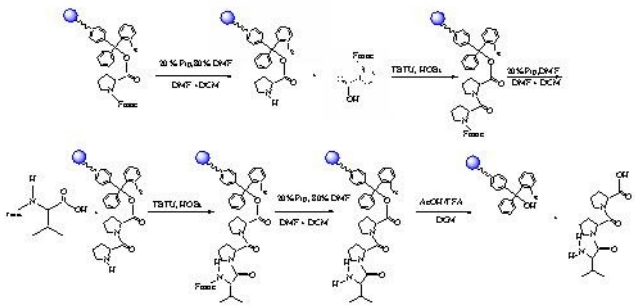
Introduction

Angiotensin-converting enzyme (ACE) inhibitors are among most important therapeutic agents for treating hypertension, reducing the development of type 2 diabetes in persons with essential hypertension and for anti stress. Many ACE inhibitory peptides have been discovered after enzymatic hydrolysis of different food proteins. This kind of peptides can successfully be used as nutrient additives to functional foods with antihypersensitive activity. Some tripeptides having Pro or Trp as C-terminal amino acid , have been reported to be real inhibitors and to show antihypertensive activity *in vivo* [1]. Ile-Lys-Trp from chicken muscle, H-Val-Pro-Pro-OH and H-Ile-Pro-Pro-OH from the serum of a variety of yoghurts including Bulgarian yoghurt as well are examples of these real inhibitors.

The aim of the present study is the chemical synthesis of proline -containing peptides H-Val-Pro-Pro-OH and H-Ile-Pro-Pro-OH for structural and kinetic studies

Result and Discussion

Although Val-Pro-Pro and Ile-Pro-Pro were discovered in 1995 there is no information cocerrning their successful classical chemical or solid phase peptide synthesis (SPPS).The chemical synthesis of Pro- peptides was achieved realized by the SPPS method and Fmoc-strategy. The HBU/DIPEA method was used for coupling of each amino acid. We didn't succeed to obtain Fmoc-Pro-Pro-OH, because of a side reaction which leads to diketopiperasine [2]. The peptides containing the very difficult C-terminal Pro-Pro, Val-Pro, Tyr-Pro sequences could be obtained with Wang resin in less than 5% yield. For the synthesis of the target peptides with free C-terminal group 2-chlorotrityl chloride resin (CLTR) was used successfully by us.



The chemically synthesized H-Val-Pro-Pro-OH and H-Ile-Pro-Pro-OH were purified by high performance liquid chromatography (HPLC). A Biotronic (Germany) HPLC system equipped with Hibar 300 SB - C8 10 µm column (9.4 x 250 mm) and UV detector at 214 nm were used. IPP and VPP were eluted by a linear gradient of solvent A (0.1% TFA-H_2O) to 32% of solvent B (0.1% TFA-AcCN) at a flow rate of 1.5 ml×min^{-1}. Peaks for VPP 17 min and IPP 19 min were detected and the peak area of each peak was measured.

Some preliminarily kinetic measurements were carried out using plant amino peptidase isolated from chick-pea (*Cicer Arietinum L*). Aminipepdidase activity was monitored at 405 nm by measurement of the cleavage of H-Leu-*p*-nitroanilide (Leu-*p*NA) in 50 mM sodium-phosphate buffer, pH 7.0. It was fount that chemically synthesized VPP and IPP are not substrates but exhibit considerable inhibitory activity to plant dipeptidases.

By using SPPS strategy we chemically synthesized Pro-peptides H-Val-Pro-Pro-OH and H-Ile-Pro-Pro-OH in both high yield and purity. We found conditions for the purification and crystallization of the peptides with antihypertensive action for structural and kinetic studies.

It is reported for the first time that H-Val-Pro-Pro-OH and H-Ile-Pro-Pro-OH are inhibitors of plant aminopeptidases.

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SITE SPECIFIC IMMOBILISATION OF THE HUMAN ALDO/KETO REDUCTASE AKR1A1

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Introduction

The major drawback of common enzyme immobilisation techniques is a lack in specificity. Crosslinking between the enzymes and formation of multilayers results in a great loss of activity. The aim of this work was to modify an enzyme by site-directed immobilisation and thereby forming well defined monolayers with orientated active sides. One approach is the labeling of a protein with biotin for the immobilisation on a streptividin coated carrier. We chose the human aldo/keto reductase AKR1A1, a monomer consisting of 324 amino acids. It has a well characterised $(\alpha/\beta)_8$-barrel structure and the N- as well as the C-terminus of the AKR1A1 are freely accessible for modifications. The enzyme catalyses the NADPH dependent reduction of various aliphatic and aromatic aldehydes and ketones to the corresponding alcohols. Such an immobilised AKR1A1 may find may be used in various applications in the biosensor field [1].

Result and Discussion

To produce monofunctionalised enzymes, the strategy of Expressed Protein Ligation (EPL) [2] was applied. By using the IMPACT®-system, a C-terminal fusion protein of AKR1A1 with an intein/chitin binding domain was generated. Subsequent intein mediated splicing led to the C_α-thioester [3,4]. The thioester was monitored by mass spectrometry and coupled in a further step to a short biotin containing peptide (Cys-Lys(-Ahx-Ahx-Biotin)-NH_2). The ligation product was detected by SDS-PAGE/Western blot and subsequent biotin specific staining. This AKR1A1-Cys-Lys(Ahx-Ahx-Biotin)-NH_2) segment can now be immobilised on streptavidin coated polystyrene plates. Tryptic digestion followed by mass spectrometry analysis showed a peptide mass finger print according to the profile of the aldo/keto-reductase and streptavidin. Analysing the surface with ELISA confirmed the specific binding. Saturation experiments revealed a maximum amount of 180 pmol AKR1A1 per well compared to 300 pmol free binding positions per well.

The activity of the enzyme was investigated by photometry at 340 nm measuring the decrease in absorbance of NADPH during the catalysed reaction. Drawing a comparison between the unmodified, the biotinylated and the specifically immobilised enzyme, the affinity to the substrate (Michaelis-Menten constant) remained nearly unchanged. The turnover number declined in the immobilisation process by one order of magnitude. However, compared to an adsorptive, unspecific immobilisation the turnover number is 20-fold increased.

The site specific monolabeling with biotin presents therefore a useful tool for an orientated immobilisation of enzymes.

Acknowledgements

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SUGAR BUILDING-BLOCKS FOR SOLID-PHASE GLYCOPEPTIDES SYNTHESIS

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Introduction

For the first time, by a structure-based design, we selected CSF114(Glc) as a synthetic glycopeptide, containing a β-glucosyl moiety linked to an Asparagine residue at position 7 [1, 2]. We demonstrated that *N*-glucosylation is fundamental for autoantibody recognition in Multiple Sclerosis patients' sera [1-3] and Asn(Glc) is the minimal epitope. Therefore, CSF114(Glc) can be used as a specific synthetic Multiple Sclerosis Antigenic Probe (MSAP).

To further characterize the autoantibody recognition in MS, we further investigated the importance of *N*-glycosylic bond and of the sugar moiety.

Results

Previous results let us to hypothesize that an aberrant *N*-glucosylation could trigger a pathogenetic autoantibody response in MS. To verify if the increased stability of this bond to chemical and enzymatic cleavage is relevant in autoantibody recognition in MS, we synthesized the Fmoc-protected *p*-glucosylphenylalanine with the C-C linked glucose mimic in ß-configuration [4]. In fact, the C-glycopeptides are not subject to deglycosylation *in vivo* [5].

The CSF114-type glycopeptide was synthesized using TBTU/HOBt/NMM as coupling reagents in Fmoc/*t*Bu SPPS on a manual synthesizer. The building block was introduced in the peptide sequence of CSF114 at the position 7. After purification and characterisation the glycomimetic containing peptide was tested in comparison with CSF114(Glc) on positive MS patients'sera and negative controls, in competitive (Fig. 1) and non-competitive (data not shown) solid-phase ELISA.

Ribose is produced *in vivo* from glucose, and it plays a fundamental role in cellular energy metabolism and cellular signaling. Therefore, following our hypothesis of an aberrant glucosylation triggering autoimmunity in MS, we could assume that a ribose moiety (instead of glucose) may be transferred to a target protein. We

synthesized the building block Fmoc-Asn(ßRib)-OH that will be introduced at position 7 in the peptide sequence of CSF114.

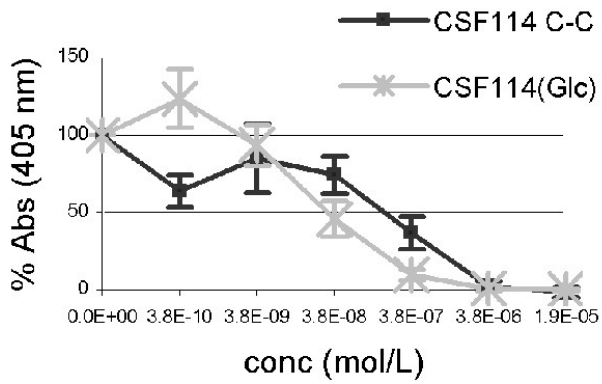

Fig. 1. Competitive solid-phase ELISA.

Discussion

Biological results showed that CSF114(Glc) is the only glycopeptide able to detect autoantibodies in MS patients' sera and confirm, up to now, the importance of the *N*-glycosylic bond between the sugar and the amino acid in autoantibody recognition in MS.

Acknowledgements

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CONFORMATIONAL ANALYSIS OF THE NEW OBESITY RELATED PEPTIDE OBESTATIN

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Introduction

Obestatin, is a 23 aminoacid ghrelin-associated peptide, early identified as the endogenous ligand of GPR39 orphan receptor [1]. Obestatin and Ghrelin originate from a common ancestor of 117 residues, propre-ghrelin. Many experimental data show that the peptides bind distinct receptors belonging to subgroup of type A GPCRs. An increasing number of functional tests [2] show that Obestatin has a functional anorexigenic role related to the interference with gastric emptying and jejunal motility. On the other hand ghrelin physiologically stimulate weight gain by binding to growth hormone segretagogue receptor (GHSR).Here, we present the synthesis and NMR study of Obestatin and its biologically active 13-mer C-terminal fragment Ob(11-23). The work was aimed to characterize the structural motifs of Obestatin as the starting point for the design and the development of new anti-obesity molecules.

Result and Discussion

CD and NMR experiments of Obestatin and Ob(11-23) were recorded in water and in mixed micelle solutions made up of Dodecylphosphocholine (DPC) and Sodium dodecylsulphate (SDS) 10% [3]. CD spectra recorded in water solutions are typical of random coil structures, indicating that both the peptides are characterized by high flexibility in aqueous solution. On the contrary, CD spectra recorded in micelle solution, evidence a significant preference of both the peptides to assume turn-helical structures (Fig.1).

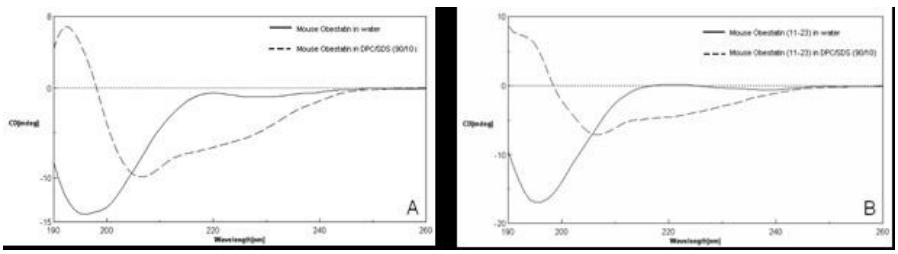

Fig.1. CD spectra of obestatin (A) and Ob(11-23) (B) in water (continued line) and in DPC/SDS micelle solution (dotted line).

TOCSY and NOESY NMR spectra of Obestatin and Ob(11-23) were recorded in water and in DPC/SDS micelles at 600 MHz. The data collected in water suggest the presence of unfolded disordered structures. In membrane mimicking environment short and medium range NOEs are consistent with the presence of turn helical structure in the C-terminal portion. 3D structures of Obestatin and Ob(11-

23) were calculated on the basis of NOE interprotonic distances, using DYANA software (Fig.2). The structure boundles of both the peptides evidence a good fitting among the conformers in the regions 11 - 22 and 13 - 22 respectively, where the dihedral angle are consistent with the presence of helical structures. Moreover, the analysis of Obestatin and Ob(11 - 23) NMR structures reveals the tendency of side chains to occupy defined conformational spaces.

Fig. 2. Structure boundle of Obestatin (left) and Ob(11-23)(right) calculated by DYANA software according to DPC/SDS micellar solution data.

Conclusions

NMR conformational analysis of Obestatin and Ob(11 - 23) shows that no relevant presence of ordered secondary structure is observable in water for both the peptides. In membrane mimicking solutions the peptides assume a turn helical secondary structure in the C-terminal portion. The α-helix is characterized by residues of medium polarity, whereas positive charge moieties are located at the beginning and at the end of the structure.

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CONFORMATIONALLY DRIVEN RATIONAL DESIGN OF GLYCOPEPTIDES AS SYNTHETIC PROBES FOR THE DETECTION OF AUTOANTIBODIES, BIOMARKERS OF MULTIPLE SCLEROSIS

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Introduction

The glycopeptide CSF114(Glc) is the first synthetic Multiple Sclerosis Antigenic Probe (MSAP) able to detect with a high-sensitivity specific biomarkers correlating with an antibody-mediated disease form of Multiple Sclerosis (MS) [1]. The presence of anti-CSF114(Glc) antibodies is in agreement with the hypothesis of an aberrant glucosylation of native myelin antigen(s) triggering an antibody-mediated MS, in which MSAP is possibly a mimetic of glucosylated native antigen(s) recognized by autoantibodies. Therefore, to unravel the pathogenetic mechanism triggering autoantibodies in MS, we are focusing our efforts on native antigen(s) identification, targets of anti-CSF114(Glc) autoantibodies involved in inflammation and demyelination process. We have previously shown that key elements for autoantibody recognition by MSAP are the β-D-glucopyranosyl moiety linked to an Asn residue by an *N*-glycosylic bond, and the β-hairpin motif between residues 2 and 14 [2].

Results

Protein domains selected by a tertiary structure alignment were synthesized by Fmoc/*t*Bu SPPS. The glycopeptides [Asn^{179}(Glc)]NogoR(173-191), [Asn^{192}(Glc)]OMgp(186-204), [Asn^{53}(Glc)]hMOG(47-67), and [Asn^{104}(Glc)]hMOG(97-117) were purified by HPLC, characterized by ESI-MS, and tested in competitive ELISA. According to the residue preference for the formation of the different main classes of β-turns [2], we modified the CSF114(Glc) peptide sequence introducing type I and II' β-turns. Therefore, we synthesized by Fmoc/*t*Bu SPPS the glucosylated peptides [Pro^{7},Asn^{8}(Glc),Thr^{10}]CSF114 and [Gly^{7},Asn^{8}(Glc),Thr^{10}]CSF114 designed to respectively contain a type II' and I β-turn. The glycopeptides were purified by HPLC, characterized by ESI-MS, and tested in competitive ELISA (Fig. 1).

Discussion

To identify native antigens involved in an antibody-mediated MS, we undertook a bioinformatic analysis to select myelin proteins characterized by a primary or a tertiary structure similar to the CSF114(Glc) one. Interesting results were obtained by a tertiary structure alignment of CSF114(Glc) with protein domains of the nervous system containing a β-hairpin. By the conformational alignment with CSF114(Glc), we selected protein domains of Nogo Receptor (NogoR), Oligodendrocyte Myelin Glycoprotein (OMgp), and Myelin-Oligodendrocyte Glycoprotein (MOG). NogoR and OMgp are proteins involved in myelin sheath formation and in inhibition of axon regeneration after a lesion. MOG is one of the most important candidate autoantigen in MS. The MOG and NogoR glucosylated peptides, selected by the conformational alignment, show high affinity for anti-CSF114(Glc) antibodies. In particular, [Asn^{179}(Glc)]NogoR(173-191) has a biological activity similar to CSF114(Glc) [IC_{50} = 21.1 nmol/L], while [Asn^{53}(Glc)]hMOG(47-67) and [Asn^{104}(Glc)]hMOG(97-117) [IC_{50} = 3.84 nmol/L] are stronger inhibitors than CSF114(Glc). However, a direct involvement of MOG and NogoR, as native autoantigens mimicked by CSF114(Glc), has to be further investigated. To better understand the role of the peptide structure in autoantibody recognition, we undertook a conformationally driven rational design of the glucosylated synthetic probes. In particular, as CSF114(Glc) is characterized by a β-hairpin motif with a type I' β-turn, containing Asn^{7} and Gly^{8} as central residues [3], we decided to investigate the role of the β-turn type in antibody recognition.

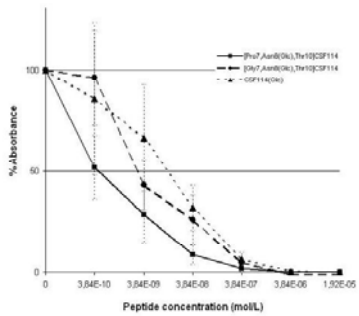

Fig. 1. Inhibition of antibodies binding by competitive ELISA.

[Pro^{7},Asn^{8}(Glc),Thr^{10}]CSF114 [IC_{50} = 0.384 nmol/L] recognizes autoantibodies with a higher affinity than[Gly^{7},Asn^{8}(Glc),Thr^{10}]CSF114 [IC_{50} = 3.84 nmol/L] and CSF114(Glc), demonstrating the importance of the peptide sequence. Therefore, by this conformationally driven rational design we succeeded in selecting [Pro^{7},Asn^{8}(Glc),Thr^{10}]CSF114 as a more potent and selective MSAP with a high affinity for autoantibodies, as disease biomarkers in MS.

Acknowledgements
We thank Fondazione Ente Cassa di Risparmio di Firenze and FIRB (RBIN04TWKN2006) for the financial support to the Laboratory of Peptide & Protein Chemistry & Biology.

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INHIBITION OF Aß(25-35) AGGREGATION BY NICOTINE AND ITS SYNTHETIC ANALOGUES

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Introduction

The β-amyloid peptides are the predominant protein component of senile plaques in Alzheimer Desease (AD). Depending upon conditions, these peptides undergo conformational transition from random coil or alpha helical monomers to the highly toxic β-sheet oligomers which form the mature fibrils. Inhibitors of amyloidosis would bind to the more soluble α-helical structure and slow down or prevent the conversion from α-helix to ß-sheet structure. Many lines of evidence suggest that the natural compound nicotine, a major component of cigarette smoke, decreases β-amyloidosis process [1 - 2]. However, the exact mechanism by which nicotine interacts with the amyloid ß-peptides is not yet well understood. In the present work, we performed NMR, EPR and CD conformational analysis of the Aβ(25-35) amyloid peptide, that is a biologically active region of Aß amyloid exhibiting large β-sheet aggregates [3], in presence of nicotine and correlated thiazolidine compounds.

Result and Discussion

CD experiments put in evidence the capacity of L(-)-nicotine in stabilizing the random coil monomers of Aß(25-35) amyloid in an α-helical conformation. On the contrary, in fibrillar conditions L(-)-nicotine was unable to induce conformational changes in the ß-sheet oligomers of the peptide. These results suggested a preventing, but not a disaggregating, effect of nicotine on the amyloid aggregation process. Upon the same experimental conditions, CD spectra of Aß(25-35) in water in presence of thiazolidine derivatives (Fig. 1) were also investigated. In this case, we found that only the compound **3** was able to induce appreciable conformational changes in the random coil structure of the peptide.

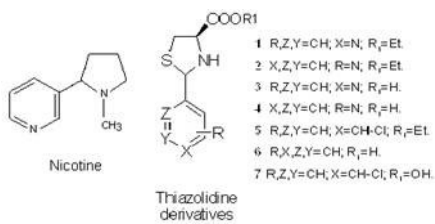

Fig.1. Structure of nicotine and Thiazolidine compounds.

^{1}H NMR spectra (600Mz) of the Aß(25-35) peptide in H_20/D_20 (90:10) (Fig. 2) were recorded in absence and with increasing amounts of nicotine. Interestingly, in

presence of nicotine, the ^{31}Ile and ^{35}Met residues in the peptide sequence showed changes in chemical shifts, indicative of binding. Analogously, ^{1}H NMR spectra (600Mz) of the Aß(25-35) peptide in H_20/D_20 (90:10) in presence of compounds **3**, at 300 K showed the capability of compound **3** to induce changes in chemical shifts of the ^{31}Ile and ^{35}Met residues in the peptide sequence.

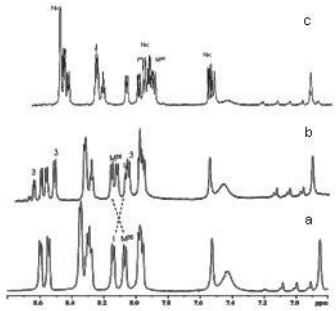

Fig. 2. 1H NMR of Aß(25-35) peptide a) in water, b) in presence of compound 3, c) in presence of nicotine.

Conclusions

CD and NMR experiments evidence that a direct interaction between the monomeric Aß(25-35) peptide and (-)-nicotine takes place. In the main, nicotine stabilizes the α–helical structure of the peptide, preventing the transition to the toxic b-sheet oligomers. Upon same experimental conditions, thiazolidine compounds sharing different structural features with nicotine are mainly unable to affect the conformational behaviour of the peptide, except for compound 3. These findings suggest that nicotine/amyloid interaction could be specific.

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DESIGN AND SYNTHESIS OF HEME PEPTIDES FOR BIOREMEDIATION

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Introduction

Synthetic dyes (azo/anthraquinonic), used extensively in textile industry, are extremely pollutants whereas their degradation is even more difficult. These dyes are soluble only in organic solvents, thus being a liability for water purification. Their degradation by-products contribute to the aggravation of the enviroment. The objective of our research is the development of substances able to degrade these dyes efficiently, with the minimum burden on nature. A class of enzymes called peroxidases, and specifically the versatile peroxidases (VP), is able to degrade the dyes with great efficiency. In particular, the activity presented by a specific VP, the one of *Pleurotus Eryngii* fungus, is very important for the creation of miniaturized degrading systems. Therefore, the design and the synthesis of peptides sustaining the biological activity of the VP enzyme, the natural production of which is really limited, is extremely challenging to set up bioremediation methods.

Results

On the basis of the molecular models of VP [1], we designed peptides able to coordinate the heme, necessary for the biological activity of the enzyme, optimizing the conformation by HyperChem, ChemDraw and Chem3D. The linear peptide (**I**) and the corresponding head-to-tail cyclopeptide structure derived from HyperChem were selected. The design of new peptides was further optimized, based on the x-ray structure of VP [2]. A new shorter peptide sequence (**II**) was modeled for the coordination of the heme and of the manganese as well. Additional features on this peptide was the incorporation of the Long Range Electron Transfer Pathway (LRETP). The conformation was once again affirmed by HyperChem (Fig. 1).

The linear peptide **I** (FHPPRPSFPFKVFGNKDFFVPEG) was synthesized by a Fmoc/*t*Bu solid-phase strategy on a TCT resin. The peptide **I** was purified by RP-HPLC and characterized by ESI-MS ($[M+3H]^{3+}$ found 898.7, calc. 898.5 m/z). Peptide **III** (WLCAHCDHDPANEHE) was synthesized by a microwave-assisted Fmoc/*t*Bu SPPS, purified by RP-HPLC and characterized by ESI-MS ($[M+2H]^{2+}$ found 889.0, calc. 888.8 m/z).

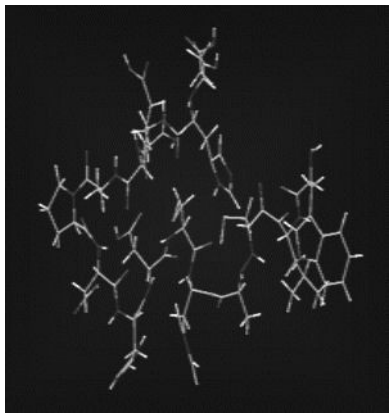

*Fig. 1. Molecular modelling of peptide **III**.*

Discussion

Miniaturized degrading systems mimicking the biological activity presented by the specific VP enzyme from *Pleurotus Eryngii* can be very important tools to set up bioremediation techniques. The VP enzyme contains three characteristic features important for its biological action: (i) the LRETP, (ii) the Mn(II) coordination site, and (iii) the Heme coordination site. Therefore, to synthesize peptide-based compounds characterized by these structural motifs we are undertaking a structure-activity relationship study.

Starting from a molecular modelling we selected and optimized three different peptide scaffolds. While the structure of peptide **I** and of the corresponding head-to-tail cyclopeptide **II** were selected by the the molecular model of VP, peptide **III** (Fig. 1) was designed on the basis of the X-ray structure [2]. Moreover, peptide **III** was built to allow the incorporation of the Heme and Mn(II) coordination sites, as well as to contain the LRETP. Therefore, peptide **III** contains the amino acid residues involved in the biological activity of the VP enzyme.

To further optimize the peptide scaffold for a structure-activity relationship study, peptides **I** and **III** were synthesised by Fmoc/*t*Bu SPPS. Peptides **I** and **III** will be further tested for their ability to coordinate the heme. Moreover, peptide **III** will be also tested for its ability to coordinate Mn^{2+} Coordination studies will be instrumental for the development of miniaturized peptides for dye degradation in water purification.

Acknowledgements

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AN EFFICIENT MICROWAVE-ASSISTED SOLID PHASE SYNTHESIS OF GRAMICIDIN A FOR STUDIES IN BILAYER-LIPID MEMBRANE

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Introduction

Linear gramicidins represent the most investigated family of antibiotic peptides forming ionic channels. The hydrophobic Gramicidin A, HCO-VGAlAvVvWlWlWlW-NHCH$_2$CH$_2$OH, is characterized by the presence of an N-terminal L-valine residue *N*-formylated and a C-terminal tryptophan linked to the amino group of ethanolamine. Its synthesis is well recognized as a compendium of difficult couplings. Infact, very poor results in terms of yield and purity of crude Gramicidin A are obtained using conventional SPPS. It is possible that problems in its SPPS are due to slow couplings, intermolecular aggregation or steric interferences between protecting groups, generating deletion sequences. Microwave technology applied to SPPS [1] has been recently proposed as valid support to the enhancement of coupling rates. Therefore, we optimized a solid-phase synthesis of Gramicidin A by microwave-assisted technique.

Result and Discussion

Synthesis of Gramicidin. The synthesis of Gramicidin A was performed by Liberty^{TM} Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC), an additional module of Discover^{TM} (CEM Corporation, Matthews, NC) that combines microwave energy and SPPS. Gramicidin A was synthesized on glycinol 2-chlorotrityl resin (0.51 mmol/g) manually functionalized with the first amino acid residue Fmoc-Trp(Boc)-OH. We undertook deprotection of the N-terminal function by 15% piperidine in DMF, coupling reactions with 0.5 M TBTU in DMF and 2 M DIEA in NMP. Deprotections (two cycles of 30 and 180 sec) and coupling reactions (one cycle of 300 sec) were microwave assisted. The final cleavage and side-chain deprotections were performed with a TFA/TIS/H_2O solution (95:2.5:2.5) for 3 h at room temperature. By a microwave-assisted SPPS, purity in crude Gramicidin A (determined by RP-HPLC) was 72%, while by conventional SPPS the purity was lower than 20%. In conclusion, the use of microwave energy in SPPS represents an important breakthrough in the difficult synthesis of Gramicidin A, which can be highlighted in the following points: dramatic reduction of reaction times, higher yields (possibly due to the enhancement of kinetic and thermodynamic reactions,

including deprotection, coupling and cleavage steps), reproducible reaction conditions (due to the control mechanism of reaction parameters, *i.e*. temperature and pressure), significant cost savings, because of the use of less excess of reagents required to drive each amino acid coupling reaction to completion.

Electrochemical studies in biomimetic membrane. The membrane-spanning gramicidin channels, selective for univalent cations, are dimers formed by transmembrane association of two single-stranded right-handed helical monomers, which are joined at their formyl N-termini. Since the length of a Gramicidin A monomer matches the lipid monolayer, just like a gramicidin dimer matches the lipid bilayer, we studied this peptide in a dioleoylphosphatidylcholine (DOPC) monolayer self-assembled on hanging mercury drop electrode. Incorporation of Gramicidin A in DOPC-coated mercury electrode from its methanol solution in aqueous 0.1 M KCl increases the capacity of the monolayer throughout the potential range of stability of the film, while leaving its resistance practically unaltered (Fig. 1). The lipid monolayer, which is not permeable to Tl(I) ions in the absence of Gramicidin A, gives rise to a well-defined cyclic voltammogram due to the Tl(I)/Tl(Hg) couple in its presence, thus confirming the membrane modifying properties of this peptide (data not showed).

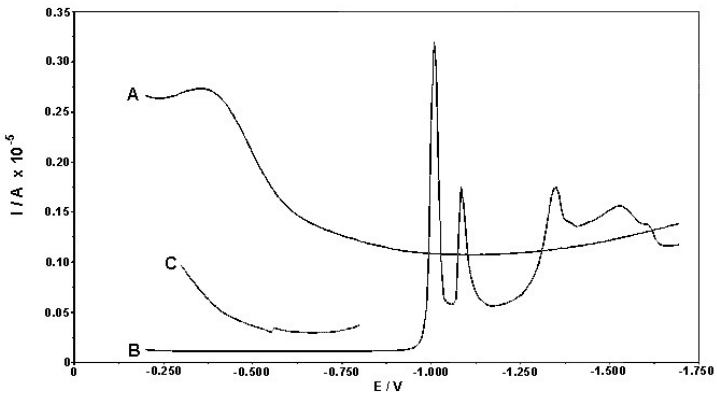

Fig. 1. AC voltammograms of bare (A) and DOPC-coated (B) mercury in contact with a 0.1 M KCl aqueous solution. (C) curve differs from the (B) one by the presence of Gramicidin A incorporated in the DOPC monolayer.

Acknowledgements

We thank Fondazione Ente Cassa di Risparmio di Firenze and PRIN 2004 for the financial support.

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SYNTHESIS OF PEPTOID-PEPTIDE ANALOGS OF SUBSTANCE P FRAGMENTS INCORPORATING D-AMINO ACIDS

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Introduction

C-Terminal analogs of Substance P (SP) have been studied for their ability to prevent tumor growth or the proliferation of several cancer cell lines. The incorporation of D-amino acids into the sequence of SP and N-methylation of peptide bonds have shown to protect SP from the action of plasma and tissue peptidases [1, 2].

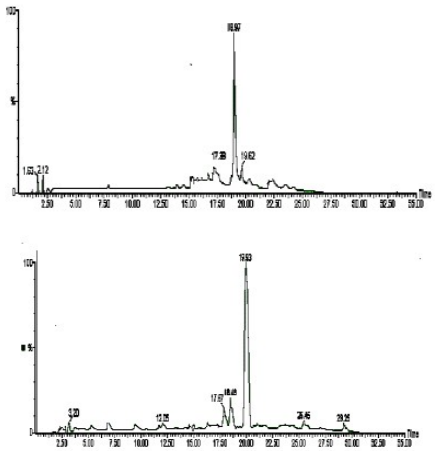
Aiming to design and prepare more potential antagonist of cancer cells proliferation and taking into account that all the metabolites of the C-terminal hexapeptide analog [Arg^{6}, D-$Trp^{7,9}$, $MePhe^{8}$]SP_{6-11} (antagonist G) [3] possess the N-Me group and D-Trp residue, we proceeded to the synthesis of N-Me peptides and peptoid-peptide hybrids. The latter are oligomeric peptido-mimetics containing the residue [-N(Bzl)-CH_2-CO-]=(NPhe). The incorporation of *N*-substituted glycine in peptide chains has been proved to improve their stability against proteases and to give biologically active peptides [4].

Result and Discussion

Based on the synthesis of tetrapeptoid-peptide hybrids [5,6] H-Arg^{1}-D-Trp^{2}-$NPhe^{3}$-D-Trp^{4}-OH and H-D-Trp^{1}-$NPhe^{2}$-D-Trp^{3}-Leu^{4}-OH, corresponding to metabolites of antagonist G we proceeded to the synthesis of the N-methylated peptides Glp^{1}-D-Trp^{2}-$MePhe^{3}$-D-Trp^{4}-$Glu(OBzl)^{5}$-NH_2 (I), Glp^{1}-D-Trp^{2}-$MePhe^{3}$-D-Trp^{4}-Leu^{5}-$Glu(OBzl)^{6}$-NH_2 (II) and the hexapeptoid-peptide hybrids Glp^{1}-D-Trp^{2}-$NPhe^{3}$-D-Trp^{4}-Leu^{5}-$Glu(OBzl)^{6}$-NH_2 (III) and Glp^{1}-D-Trp^{2}-$NPhe^{3}$-D-Trp^{4}-Leu^{5}-$Glu(OBzl)^{6}$-OH (IV). All these analogs have incorporated the amino acid residues Glp at their N-terminal and Glu(OBzl) at the C-terminal end. This incorporation has shown to give the analogs increased resistance and biological activity. All the products were purified by HPLC and identified using ESI-MS (Table 1). Their biological properties and activity against the cancer cells proliferation are under investigation.

Table 1. Purification (HPLC) and identification (ESI-MS) of the analogs

Analogs	M.W.	t_R (min)
Glp[1]-D-Trp[2]-MePhe[3]-D-Trp[4]-Glu(OBzl)[5]-NH_2	881.21	17.67
Glp[1]-D-Trp[2]-MePhe[3]-D-Trp[4]-Leu[5]-Glu(OBzl)[6]-NH_2	994.28	19.47
Glp[1]-D-Trp[2]-NPhe[3]-D-Trp[4]-Leu[5]-Glu(OBzl)[6]-NH_2	980.25	19.93
Glp[1]-D-Trp[2]-NPhe[3]-D-Trp[4]-Leu[5]-Glu(OBzl)[6]-OH	980.91	18.60



RP-HPLC of analog II. 5%(A), 95%(B) → 100%(A), 0%(B) in 30min
A: 0.08% TFA in CH_3CN, B: 0.08% TFA in H_2O.
RP-HPLC of analog III. 5%(A), 95%(B) → 100%(A), 0%(B) in 30min
A: 0.08% TFA in CH_3CN, B: 0.08% TFA in H_2O.

Acknowledgments

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SYNTHETIC PEPTIDES OF 558-565 LOOP OF THE A2 SUBUNIT OF FVIIIA OF BLOOD COAGULATION CASCADE AND THEIR BIOLOGICAL ACTIVITY

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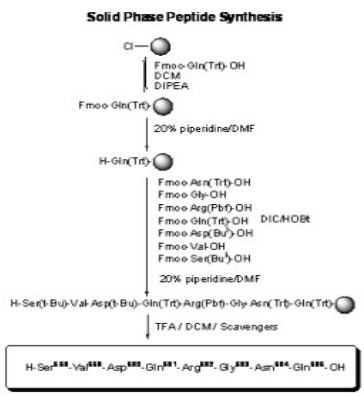
Introduction

The generation of a fibrin clot is mediated by the regulated activation of a series of serine proteases and their cofactors. Factor VIII in its activated form, FVIIIa, acts as a cofactor to the serine protease FIXa, in the conversion of the zymogen FX to the active enzyme FXa. Both FVIII and FIX are essential for normal coagulation, deficiencies of either are associated with the bleeding diatheses hemophilia A and B, respectively. The role of FVIIIa is to bind factor IXa, generating the phospholipid-dependent intrinsic factor Xase complex. The Ser^{558}-Gln^{565} region within the A2 subunit has been shown to be crucial for VIIIa-IXa interaction [1, 2, 3].

Results

In an attempt to study this interaction, we synthesized a series of fifteen peptide analogs of 558-565 loop of the A2 subunit. The syntheses were carried out by using SPPS and Fmoc/But methodology (Fig. 1).



Using the same methodology we synthesize peptides incorporating Asp or Asp derivatives instead of Asn564. The afforded compounds were purified by RP-HPLC and lyophilized to give white fluffy solid, identified by ESI-MS (Table 1).

Table 1 : Synthesized Analogs of the Peptide Sequence Ser558- Gln565

No	Synthesized Analogs	ESI-MS
1.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}-OH	903.64
2.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}-OH	945.22
3.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp^{564}-Gln^{565}-OH	903.61
4.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp^{564}-Gln^{565}-OH	945.91
5.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OMe)^{564}-Gln^{565}-OH	917.23
6.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OMe)^{564}-Gln^{565}-OH	959.47
7.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OBzl)^{564}-Gln^{565}-OH	993.78
8.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OBzl)^{564}-Gln^{565}-OH	1035.36
9.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}-NH_2	902.44
10.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}-NH_2	944.39
11.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp^{564}-Gln^{565}-NH_2	902.42
12.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp^{564}-Gln^{565}-NH_2	944.39
13.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OMe)^{564}-Gln^{565}-NH_2	916.43
14.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OMe)^{564}-Gln^{565}-NH_2	958.24
15.	H-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OBzl)^{564}-Gln^{565}-NH_2	994.52
16.	Ac-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asp(OBzl)^{564}-Gln^{565}-NH_2	1036.72

Biological Assays

All the synthesized peptide analogs are under investigation for their inhibitory activity on human platelet aggregation *in vitro* and also for their activity on thrombin production during the intrinsic pathway of blood coagulation cascade.

Acknowledgements

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THE INFLUENCE OF SIDE CHAIN GLYCOSYLATION AND FLANKING REGION MODIFICATION ON THE ANTIBODY RECOGNITION OF A MUC2 EPITOPE

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Introduction

Mucin glycoproteins, produced by epithelial cells, can be overexpressed and/or underglycosylated in case of carcinomas. Consequently, the hindered protein backbone in the normal glycoproteins becomes accessible for the immune system. This phenomenon could be useful in tumour diagnosis and immunotherapy. The mucins are built up of tandem repeat units, the sequence of that of the MUC2 mucin found in the gastrointestinal tract is ^{1}PTTTPITTTTTVTPTPTPTGTQT^{23} [1]. Our research group has been studying the epitope structure of the MUC2 repeat unit using monoclonal antibody MAb 996, raised against the antigenic part of the MUC2 tandem repeat [2]. This antibody recognises samples of human colon carcinoma. We have demonstrated that the minimal epitope recognised by this antibody is the PTGTQ sequence [3]. Using elongated epitope peptide libraries of AX^{1}PTGTQAA and AAPTGTQX^{2}A we have found that in position X^{1} apolar [4], in position X^{2} aromatic residues [5] could enhance the antibody recognition. Our aim was to produce modified PTGTQ MUC2 epitopes with maintained or even enhanced specificity and to clarify the effect of different modifications on MAb 996 antibody binding. Accordingly, we have studied the effect of A) amino acid changes in the flanking region to produce "superantigens", or B) glycosylation in the epitope core and flank to determine possible differences between the antibody recognition of normal and tumourous mucin.

Results

We have prepared X^{1}PTGTQX^{2} peptides; and glycopeptides derived from peptide ^{16}PTPTGTQ^{22} glycosylated in positions 17, 19, 21 or all three positions. Peptides were prepared on Wang resin using Fmoc/*t*Bu strategy. Fmoc groups were removed with piperidine, coupling was performed either with DIC/HOBt, PyBOP/DIEA or with Fmoc-Thr(GalNacα)-dimethylphosphinothioic mixed anhydride. The peptides were cleaved from the resin with TFA/water, TFA/1,4-buthanedithiol/water or TFA/1,4-buthanedithiol/triethylsilane, and after isolation were purified with RP-HPLC. We have identified and characterised the peptides with amino acid analysis, mass spectrometry and analytical RP-HPLC. The ability of the peptides to inhibit the binding of MAb 996 monoclonal antibody to the (K^{12}VTPTPTPTGTQTPT^{25})-BSA target antigen was measured in competitive ELISA experiments. The ELISA

plates were coated with the target antigen, then MAb 996 and peptides in different concentrations were added. The binding was monitored with peroxidase labeled goat anti-mouse Ig and ABTS - H_2O_2. The developing colour was detected at λ = 405 nm. All X^1PTGTQX^2 peptides have been recognised by MAb 996 antibody, with different efficiency. In agreement with our previous findings, N-terminal Ile and Val, and C-terminal Phe or Tyr indeed resulted in stronger antibody binding then the native Thr (TPTGTQT: IC_{50} = 378 μmol/L, IPTGTQF, IPTGTQY, VPTGTQF: IC_{50} = 144, 162, 160 μmol/L, respectively); also, Pro and Asp in the same positions weakened the binding (PPTGTQD: IC_{50} = 586 μmol/L). On the other hand, native TPTGTQ peptide lacking C-terminal flanking amino acid showed stronger antibody binding (IC_{50} = 138 μmol/L) than even IPTGTQF. Glycosylation of peptide ^{16}PTPTGTQ^{22} in the flanking region, on Thr^{17} did not significantly influence the binding of MAb 996 (IC_{50} = 39 and 25 μmol/L, respectively), but interestingly, on Thr^{19} (part of the MAb 996 epitope) the presence of *N*-acetyl-galactosamine increased the antibody recognition by nearly one order of magnitude (IC_{50} = 6.4 μmol/L). Glycosylation on Thr^{21} completely diminished MAb 996 binding, indicating the importance of the Thr^{21} side chain in the antibody recognition.

Discussion

Although it was possible to enhance the MAb 996 antibody binding of the native TPTGTQT peptide by replacing the N- and C-terminal Thr-s with hydrophobic and aromatic amino acids, respectively; this approach is not suitable to achieve stronger antibody recognition, because any additional C-terminal residue reduces the binding, compared to the native TPTGTQ peptide, indicating the necessity of free α-carboxy group on the C-terminal glutamine. According to our expectations, while glycosylation in the flanking sequence of PTPTGTQ peptide did not influence the antibody binding, glycosylation within the epitope core, on Thr^{21} of ^{16}PTPTGTQ^{22}, completely demolished it. Interestingly, on Thr^{19} which is also part of the epitope, glycosylation slightly but consistently increased the strength of peptide – antibody interaction.

Acknowledgements

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SYNTHESIS AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF NATURAL ANTIMICROBIAL PEPTIDES AND CONVENTIONAL ANTIBIOTICS

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Introduction

The purpose of this study was to investigate the *in vitro* activity of natural antimicrobial peptides: citropin 1.1 (GLFDVIKKVASVIGGL-NH_2), piscidin 1 (FFHHIFRGIVHVGKTIHRLVTG-NH_2), protegrin 1 (RGGRLCYCRRRFCV-CVGR-NH_2), temporin A (FLPLIGRVLSGIL-NH_2), uperin 3.6 (GVIDAAKKV-VNVLKNLF-NH_2) and three chemically engineered analogues of antimicrobial peptides: iseganan IB-367 (RGGLCYCRGRFCVCVGR-NH_2), omiganan MSI-226 (ILRWPWWPWRRK-NH_2) and pexiganan MBI-78 (GIGKFLKKAKKFG-KAFVKILKK-NH_2) [1].

Result and Discussion

The peptides were synthesized by the solid phase method using Fmoc chemistry and purified by HPLC [2]. The aim of microbiological study was to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of eight analyzed peptides against reference strains of bacteria. The MIC was determined by broth microdilution method with use polypropylene 96-well plates. The MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculum. For comparison, in the research we included chloramphenicol, rifampicin, piperacillin and vancomycin. Experiments were performed in triplicate.

Both the natural antimicrobial peptides and the analogues inhibited the growth of bacteria, but at higher concentrations than did conventional antibiotics. Nevertheless, both natural origin of antimicrobial peptides and their low toxicity constitute a considerable advantage and this is an argument for considering the antimicrobial peptides as good candidates for medicines.

Table 1. Antimicrobial activity of tested compounds

Antimicrobial agent	MIC/MBC (microgram/ml)						
	Gram-positive bacteria				*Gram-negative bacteria*		
	B. subtilis ATCC 6633	*E. faecalis* PCM 896	*S. epidermidis* ATCC 14990	*S. aureus* ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*P. vulgaris* NCTC 4635
Citropin	16/16	16/16	8/8	8/32	128/128	256/256	256/512
Piscidin	4/4	8/8	2/2	8/16	32/32	128/256	128/512
Protegrin	1/1	0.5/1	0.5/1	4/4	4/4	8/8	32/32
Temporin	8/16	64/128	8/16	8/16	512/512	512/512	512/1024
Uperin	4/4	32/64	16/16	16/32	128/128	256/256	256/256
Iseganan	1/1	0.5/1	0.5/1	16/16	16/16	32/32	128/128
Omiganan	8/8	32/32	8/8	12/64	32/64	128/128	1024/>1024
Pexiganan	8/8	8/8	2/8	4/8	4/8	32/128	1024/1024
Vancomycin	0.125/2	0.25/1	0.25/2	0.25/2	-	-	-
Rifampicin	0.032/0.032	4/8	0.032/0.064	0.032/0.064	-	-	-
Piperacilin	0.25	4/4	1/4	1/4	0.5/2	2/8	1/64
Chroramphenicol	2/4	32/128	2/16	4/32	64/256	64/128	2/16

MIC - minimal inhibitory concentration, MBC - minimal bactericidal concentration

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BIOTIN-DERIVATIVES WITH ENHANCED SOLUBILITY FOR THE LABELLING OF IGG FC PEPTIDES

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Introduction

The biotin – avidin interaction is the strongest non-covalent interaction known (K_d $\sim 10^{-15}$) [1]. Several biological assays apply peptides or proteins attached to biotin. These can be used with avidin-coated ELISA plates or Biacore microchips as target in binding assays, or can be utilised as labelled entities for capturing enzyme- or fluorophore-labelled avidin, *e.g.* in FACS. To avoid the blocking of the peptide's/protein's active sites with the avidin, a spacer of 6-amino-hexanoic acid is usually used between the biotin and the compound to be labelled [2]. In some cases the biologically active peptides are poorly soluble or insoluble in water. Also, the biotinylated peptides have lower solubility than unlabelled peptides, making the purification difficult. Our aim was to prepare biotinylating reagents containing oligo-ethyleneglycol spacer of different length, for enhanced solubility.

Results

Boc-EDA-TEG building block was prepared from Boc-ethylenediamine (EDA) and asymmetrically butyl-protected 3,6,9-trioxaundecanedioic acid (TEG). The dicarboxylic acid was transesterificated with butyl-formate using Dowex ion-exchange resin as a mobile heterogenous catalyst [3], which is capable to separate the product from the reagent, and the solvent mixture prevents the formation of by-product diester. The asymmetric ester was coupled to Boc-ethylenediamine with dicyclohexyl-carbodiimide in dichloromethane, and then the butyl group was removed by basic hydrolysis.

Due to the difficulty of preparing Fmoc-EDA, Fmoc-protected linker was synthesized with the reaction of unprotected EDA and 3,6,9-trioxaundecanedioic acid anhydride. In organic solvent the EDA-TEG zwitterion precipitated, preventing the second acylation [4]. The amino group of the resulting EDA-TEG was protected with Fmoc, using FmocOSu.

The biotin-$(EDA\text{-}TEG)_n$ reagents (n = 1, 2, 3) were built up on solid phase, either by Boc and Fmoc chemistry. The protected EDA-TEG was coupled to Merrifield or Wang resin. The desired length of spacer was achieved with stepwise solid phase synthesis using DIC/HOBt coupling. The Boc or Fmoc groups were removed with trifluoracetic acid or piperidine, respectively. Biotin was attached with BOP/HOBt/DIEA reagents; the success of the coupling was monitored with ninhydrin. The biotinylating reagents were cleaved from the resin with HF or trifluoracetic acid/water.

After the synthesis of the reagent and its attachment to a CVVVDVSHEDP-MBHA resin we have observed that the amide bond between the peptide and the reagent was cleaved in the HF cleavage procedure. When we coupled the reagent to preloaded Gly-*p*-alkoxybenzylalcohol (Wang) resin to model the coupling to resin-bound peptide, we could isolate the product after TFA cleavage. Therefore we have applied a β-alanine to the biotinylating reagent, to stabilize the bond in HF conditions between the resin-bound CP peptide and biotinylating reagent. We have synthesized a new set of biotinylating reagents with a β-alanine part, on solid support, with the method described above.

The biotinylated peptide conjugate was synthesized on solid phase. On the resins containing the CVVVDVSHEDP and VVDVSHEDP peptides with free amino groups the biotinylating reagents biotin-$(EDA\text{-}TEG)_n$-β-Ala were coupled with PyBOP/HOBt/DIEA in DMF. After monitoring the success of the coupling with ninhydrin assay, the conjugate was cleaved from the resin with liquid HF, resulting in the labelled peptide.

We have studied the water solubility of CVVVDVSHEDP peptide labelled with different biotin derivatives. We have observed that while biotin or biotinyl-6-aminohexanoic acid lowered the solubility of this peptide, the biotin-EDA-TEG-β-Ala labelled peptide was even more soluble than the unlabelled peptide.

Discussion

We have prepared a new class of biotinylating reagents with step-wise synthesis on solid phase. These biotin derivatives are highly soluble in water and also in solvents usually used in peptide synthesis, and can be attached to resin-bound peptides in the same method as an amino acid, and enhance the solubility of the peptides. These compounds could be useful for avidin based binding assays.

Acknowledgements

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DETERMINATION OF HEPCIDIN CONTENT OF URINE USING ELISA AND MASS SPECTROMETRY

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Introduction

Hepcidin is a liver-expressed cysteine-rich cationic 25-mer peptide (DTHFPICIFCCGCCHRSKCGMCCKT) which plays an important role in the regulation of iron metabolism. Human urine contains two predominant forms, comprised of 20 and 25 amino acids, that differ only by N-terminal truncation [1]. To date, only specific immuno-dot assay [2] and mass spectrometric methods to measure hepcidin level in urine has been documented [3, 4]. Measurements of hepcidin concentrations could therefore be useful in diagnosis of iron disorders and would provide further insight into hepcidin regulation *in vivo*. High-throughput assays for hepcidin detection and quantification in plasma and urine have not been generally available, and the development of reagents has been hampered by technical difficulties. The production of synthetic hepcidin in its native conformation or the isolation of hepcidin from urine involves complex, time-consuming procedures [3]. Here we report new synthetic peptide derivatives of native 25-mer hepcidin which compounds are promising standards in mass spectrometric or immuno-based assays.

Results

Five hepcidin derived linear peptides corresponding to 1-7, $(Gly)^5$-1-7, 1-25, 13-25 regions were synthesised using Fmoc/*t*Bu chemistry on Tenta Gel resin with DIC/HOBt coupling [5]. These linear hepcidin-peptides were modified with biotin and *N*-(+)-biotinyl-6-aminocaproic acid using PyBOP/HOBt/DIEA coupling methods for ELISA studies. The homogeneity and the primary structure of peptides and biotinylated peptides were verified by analytical RP-HPLC, amino acid analysis and ESI-MS (data not shown). The peptides were obtained as linear products of good quality. The ELISA assay was carried out using rabbit anti-human hepcidin IgG (Fig. 1 A, B). The immuno-dot assay was carried out on Immobilon-P PVDF membrane using rabbit anti-human hepcidin IgG as 1st antibody and goat anti-rabbit-HRPO labelled antibody with ECL Advanced Chemiluminescent Reagent as developer. As standards we applied synthetic 25-mer hepcidin, in 10-250 ng range per dot (Fig. 1 C).

Hepcidin-25 peptide from urine samples was detected by MALDI-TOF mass spectrometry. Experiments were performed on a Voyager DE-Pro MALDI-TOF mass spectrometer equipped with wavelength λ = 337 nm nitrogen laser, in positive, linear acceleration mode. Urine samples were purified on NP20 ProteinChips (Ciphergen), mimicking normal phase silicate functionality. The surface of the ProteinChips was equilibrated using 0.1% TFA solution. 15 μL urine sample (containing 0.1% TFA) was placed onto the surface, and incubated for 1 min. Then the surface was washed with 0.1% TFA to remove any non-binding components. Peptides were eluted by adding 2 μL matrix solution, placed onto the MALDI target, and allowed to crystallize on air. α-Cyano-4-hydroxycinnamic acid (CCA)

was used as MALDI matrix (10 mg/mL in acetonitrile:water = 1:1, 0.1% TFA). Quantitative determination of hepcidin-25 in urine will be performed using the N-terminal acetylated hepcidin peptide as an internal standard. The acetylation of the 25-mer peptide does not influence the MALDI response, and the peaks can be detected individually (no overlapping was observed) (data not shown).

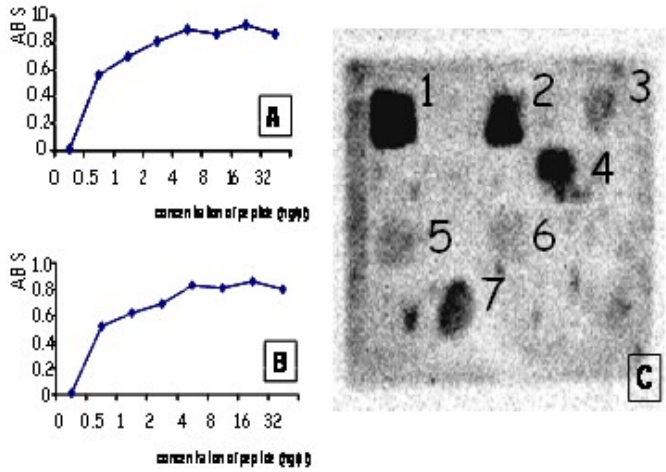

Fig. 1. Recognition of the rabbit anti-human hepcidin IgG (A) biotynil-^{1}*DTHFPICIFCCGCCHRSKCGMCCKT2*^{5} *and (B) biotynil-*^{1}*DTHPPIC*^{7} *peptides (C) qualitative immono-dot assay of (1-3) synthetic 25-mer peptides 250 - 100 - 5 ng/dot (4) commercially available hepcidin (Alpha diagnostic International, 100 - 10 ng/dot (6) urine sample No. 10 (7) positive control (rabbit sera 1:1000 v/v).*

Discussion

We have synthesised linear form of 25-mer hepcidin and its truncated versions and their biotinylated derivatives. The biotinyl-^{1}DTHFPIC^{7} peptide and biotinyl-^{13}CCHRSKCGMCCKT^{25} peptide were stabile, no dimerisation or polymerisation were observed. The biotinylated peptides are promising antigens to replace 25-mer hepcidin standard in ELISA measurements.

Mass spectrometric identification and quantification of hepcidin peptides from urine samples was performed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. We have detected protonated molecular ions of the 25-mer hepcidin in urine samples.

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ROLE OF THE C-TERMINAL CONSERVED SEQUENCE MOTIF IN THE FUNCTION OF dUTPase FROM MASON-PFIZER MONKEY RETROVIRUS

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Introduction

dUTPases (deoxyuridine 5'-triphosphate nucleotidohydrolase) have an essential role in regulating cellular dUTP/dTTP ratios by catalyzing the hydrolysis of dUTP into the dTTP precursor dUMP and pyrophosphate. Lack of enzymatic activity leads to high levels of incorporation of deoxy- uridine moieties into DNA. All free living organisms, as well as several DNA and retroviruses encode dUTPase. Betaretroviral genomes contain the dUTPase gene adjacent to the gene of the nucleocapsid polypeptide. dUTPase from Mason-Pfizer monkey retrovirus (MPMV dUTPase) is a betaretroviral member of the dUTPase enzyme family. The homotrimeric organization characteristic of dUTPases is retained in this bifunctional fusion protein [1]. The fusion protein nucleocapsid-dUTPase is present in virions of Mason-Pfizer monkey betaretrovirus and in virus-infected cells where it potentially contributes to RNA/DNA folding and reverse transcription [2, 3]. In addition to trimeric dUTPase core, the protein possesses flexible N- and C-termini consisting of the nucleocapsid segment and a peptide motif conserved in dUTPases. In the case of Mason-Pfizer monkey betaretrovirus the structural background responsible for the low catalytic efficiency:

– between the 4. and 5. conserved motif the amino acid sequence of the arm is shorter than in other dUTPases, the function of this arm is folding over the active site

– structure and amino acid sequence of MPMV dUTPase shows the difference between betaretroviral proteins as compared to other dUTPases

To analyze the function of the flexible C-terminal peptide segment, reconstitution experiments were designed with the truncated enzyme lacking the C-terminal 14-mer peptide (PYRGQGSFGSSDIY) and the 14-mer synthetic peptide with the same sequence.

Results

Synthesis: the 14-mer peptide was synthesized by solid phase peptide synthesis using Fmoc/*t*Butyl strategy on 2-chlorotrityl-chloride resin. The Fmoc protecting groups were removed with 2% DBU+2% piperidine (v/v). Coupling was performed with HOBt/DIPCDI method. The peptide was cleaved from the resin by 0.2% TFA/DCM. The side chain protecting groups were cleaved from the peptide with TFA containing TIS and H_2O. The peptide was characterized by RP-HPLC and MS.

Crystallization: the synthetic 14-mer peptide was solved in protein buffer (50 mM TrisHCl buffer containing 200 mM NH_4Cl, 5 mM DTT, 0.5 mM phenylmethyl sulfonil fluoride) and dialyzed overnight against the same buffer to remove any residual chemicals from the synthesis. The truncated dUTPase protein was mixed in 1:2 molar ratios with the synthetic peptide representing the C-terminus of the enzyme. Substrate analogue ligand of the

dUTPase enzyme (α,β-imino-dUTP) was added together with 10 mM $MgCl_2$ to occupy the enzyme's active site and facilitate binding of the peptide. Substrate as well as substrate analogue binding has been previously shown to support localization of the C-terminus in dUTPases from various sources [4, 5]. Crystallization attempts were carried out using the crystallization conditions optimized for the full length dUTPase [6]. In the crystallization drops the dUTPase: peptide complex solutions were mixed with equal volumes of crystallization solution containing 8% PEG 8000 and 100 mM TrisHCl pH 8.5. The drops were equilibrated against the reservoire (crystallization) solution in 3ml closed wells.

Discussion

The fusion protein nucleocapsid-dUTPase is present in virions of Mason-Pfizer monkey beta retrovirus and in virus-infected cells where it potentially contributes to RNA/DNA folding and reverse transcription. The truncated enzyme proved to be practically inactive. Addition of the synthetic 14-mer at 100 fold molar excess resulted in partial complamentation of the catalytic activity (to 10% of original). We conclude that the C-terminal 14-mer is essential for catalytic activity.

A mixture of the truncated enzyme and the 14-mer peptide (this latter at 2 fold excess) was put to crystallization trials. Crystallization attempts were carried out using the crystallization conditions optimized for the full length dUTPase [6]. Small crystals were observed by slow saturation of the crystallization drops via vapor diffusion. Crystals were tested at synchrotron beamline PX11 EMBL/DESY, Hamburg and they diffracted to 3.4 Å resolution. At this resolution, details of sidechain interactions cannot be visualized and even the straightforward localization of flexible segments is challenging; therefore improvement of the crystal quality is desired prior to data collection and structure determination.

Acknowledgements

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LONG-TERM INTERACTION BETWEEN β-AMYLOID FIBRILS AND SHORT PEPTIDES

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Introduction

Aggregates of ß-amyloid peptide (Aß) play central role in Alzheimer's disease (AD). Soto's pentapeptide LPFFD and our LPYFD-amide (LPYFD-NH_2) are effective neuroprotective agents against Aß assemblies both *in vitro* and *in vivo* [1, 2]. These short peptides can interact with Aß peptides [1] but the nature of the interaction has not proved yet (ß-sheet breakers, molecular surface covering substances or other effects?).

The main aims of these studies were to follow the aggregation process of $Aß_{1-42}$ from oligomers /monomer to matured fibrils, to study the interaction between short peptides (LPFFD and LPYFD-NH_2) and $Aß_{1-42}$, and to understand the neuroprotective effect of Soto's LPFFD and our LPFYD-NH_2 pentapeptides.

Results

As revealed by circular dichroism experiments, the conformation of $Aß_{1-42}$ changes gradually during "aging" in water from unordered conformation to high ß-sheet content. After 120 hrs aging distorted (twisted) ß-sheet structures (fibrillization) appear.When $Aß_{1-42}$ was coincubated with the pentapeptides for a week, LPFFD and LPYFD-NH_2 have only small influence on the conformation changes. LPFFD slightly increases the formation of twisted ß-sheet conformation (fibrillization).When Aß was aggregated for one week and after that 2 molar excess of LPFFD or LPYFD-NH_2 were added, a rapid and significant change of the conformation of aged $Aß_{1-42}$ (fibrils) has occured. Both pentapeptides increased the amount of twisted ß-sheets, this indicates aggregation of $Aß_{1-42}$.

Dinamic light scattering measurements in H_2O showed the aggregation of $Aß_{1-42}$ to big assemblies in a few days. The pentapeptides LPFFD and LPYFD-NH_2 in 5-times molar excess can not prevent Aß-aggregation in H_2O (Fig. 1, 2).

Transmission electron microscopy experiments show that $Aß_{1-42}$ peptide aggregates rapidly to fibrils (c = 0.5 mg/ml) and neither LPFFD, nor LPYFD-NH_2 can prevent Aß-aggregation in H_2O, when the pentapeptides are in 5-times excess.

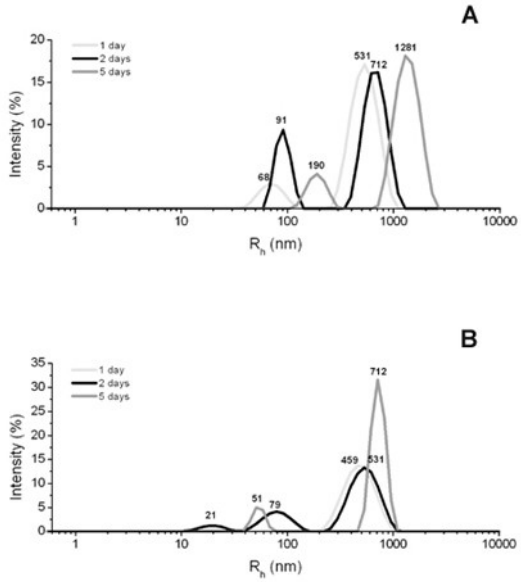

Fig. 1. Dynamic light scattering measurements. ***A****, $A\beta_{1-42}$ is incubated alone,* ***B****, Aß is incubated with LPFFD.*

Discussion

Aggregation of $A\beta_{1-42}$ in H_2O starts with a conformational change and gradually results in highly ordered distorted (twisted) ß-sheet structure. Soto's LPFFD and our LPYFD-NH_2 (both are neuroprotective) can not prevent conformational changes and aggregation of $A\beta_{1-42}$. The above mentioned pentapeptides rather increase the conformational changes and fibrillization of $A\beta_{1-42}$. We suppose that these short peptides bind to Aß-fibrils and can cover their surface.

Acknowledgements

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CONJUGATION OF A CYCLIC RGD DERIVATIVE TO BRANCHED CHAIN POLYMERIC POLYPEPTIDE: SYNTHESIS AND BIOLOGICAL STUDY OF AK-[CYCLO(RGDFC)]

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Introduction

The interaction of $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors with their ligands is selectively implicated in many important biological processes, including angiogenesis, bone-formation, tumor genesis and tissue-genetic migration of embryonic cells [1]. The receptors react with a number of extracellular matrix components (fibronectin, vitronectin, osteopontin, etc…). As a common motif, responsible and inevitable for the binding of $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors the RGD tripeptide was identified. By now there are several synthetic cyclic RGD pentapeptides (such as Kessler-peptide) are known as selective ligands for $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors [2].

The aim of this study was to develop RGD-coated surfaces for cell adhesion assays. Therefore we have produced RGD conjugate with polycationic carrier considering its beneficial binding to the surface of plastic plates used for biological studies. This new conjugate contains a macromolecular carrier, the branched chain polycationic polypeptide: poly[Lys(D,L-Ala)$_{4.5}$], known as AK [3] and the cyclo(RGDfC) derivative.

Results

Synthesis: To avoid the racemisation occurs in "head-to-tail" cyclisation, glycine as C-terminal amino acid residue was introduced. The linear side chain protected precursor peptide DfCRG was built up on 2-Cl-trityl chloride resin by Fmoc/*t*Bu method using and DIPCI/HOBt coupling reagents. After cleavage of side chain protected peptide (H-Asp(O*t*Bu)-D-Phe-Cys(Trt)-Arg(Pbf)-Gly-OH) from the resin cyclisation was achieved by BOP/HOBt/DIEA reagents in a diluted DMF solution. After cleavage of side chain protecting groups by reagent K the cyclopeptide was analysed by analytical RP-HPLC and ESI-MS prior to conjugation reaction. Coupling of the cyclopeptide to poly[Lys(D,L-Ala)$_{4.5}$], the AK polymer was carried out by thioether linkage. For development of thioether bond between the polymeric polypeptide and the cyclic peptide, the AK polymer was chloroacetylated using Cl-CH_2-CO-OPcp. Conjugate was characterised by amino acid analysis.

Cell-adhesion assay: On AK-[cyclo(RGDfC)] conjugate coated surfaces (plastic/glass petridish) one-cell suspension of different cell lines: A431 (human

carcinoma), A7r5 (rat embryonic aorta smooth muscle), MDCK (dog kidney epithelium), NE-4C (mouse embryonic ectoderm), E14,5 primary neuron and E14,5 mouse primary fibroblast were tested. Qualitative (morphological) analysis was performed of the differently spread cells by monitoring the adhesion by microscope/video microscope. The round, suspended, singular cells could be well distinguished from the cells are in different stages of spreading and attachment. Our observations are summarized in Table 1.

Table 1. Attachment of different cell types to the AK-[cyclo(RGDfC)] conjugate coated surfaces

Cell lines	Attachment of cells to the AK-[cyclo(RGDfC)] conjugate coated surfaces
A431 human carcinoma	excellent attachment either after a short (15 min) and long (24 hours) period of time, on either glass or plastic surfaces coated with the conjugate
A7r5 rat embryonic aorta smooth muscle	
MDCK dog kidney epithelium	
NE-4C mouse embryonic ectoderm	
E14,5 primary neuron	attachment was not observed
E14,5 mouse primary fibroblast	similar attachment behaviour in case of conjugate and control on every tested surfaces

Discussion

Immobilizing the AK-[cyclo(RGDfC)] conjugate to either glass, or plastic was found to support cell-attachment in case of various cell types. As a speciality, all investigated cell lines - also the primary neural cells - attached to, spread and survived on the AK-[cyclo(RGDfC)] coated surface in the absence of serum. Construction and immobilization of appropriate cyclic RGD - polypeptide conjugates represent an additional tool to investigate selective cell adhesion and may provide a novel scaffold-material for directed cell-seeding.

Acknowledgements

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PREPARATION AND USE OF AN OPTIMIZED CLEAR-OX FOR MULTI-GRAM SYNTHESES OF DISULFIDE-BRIDGED PEPTIDES UNDER MILD CONDITIONS

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Introduction

The major approaches to form disulfide bridges in peptide synthesis include solution oxidations (which is the most widely used), oxidations of resin-bound peptides, and uses of a polymer-bound oxidant. Polymer-supported reagents are increasing in popularity, since they combine the advantages of solid-phase chemistry with the versatility of solution-phase reactions. We previously reported that Ellman's reagent attached to CLEAR supports [1] resulted in the successful reagent, CLEAR-OX [2]. For the present studies, CLEAR-OX was produced by an alternative synthetic route, which was modified and scaled-up from a previously reported procedure [3]. This newer approach is preferred because it is simpler and avoids solid-phase transformations, such as an oxidation step. Commercially available Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) = DTNB] was converted, in solution, to its preactivated bis-*N*-hydroxysuccinimide ester, and then reacted with lysine, again in solution. The resultant preformed cyclic DTNB-lysine derivative was covalently attached to CLEAR polymeric support with a β-alanine spacer to yield the final CLEAR-OX [4]. Therefore, oxidations performed using CLEAR-OX prepared in this fashion cannot be the result of residual oxidant adsorbed to the resin. The model peptides selected as targets for oxidation include common, naturally occurring peptides or their analogs, and feature in some cases incorporation of a sterically hindered penicillamine residue, as well as inclusion of Trp and Met (prone to side reactions during solution oxidations). The goal of these studies was to probe the effectiveness of monomeric intramolecular disulfide formation mediated by CLEAR-OX, and to compare results with those from solution oxidations.

Result and Discussion

All peptide sequences used for oxidation studies were prepared via manual or automated solid phase synthesis (Milligen 9050) using Fmoc/*t*-Butyl strategies on either polystyrene or CLEAR resins. Peptides were cleaved from the resins with TFA: phenol: water: triisopropylsilane (88:5:5:2, v/v/v/v) for 2 h at room temperature under inert atmosphere, and were subjected directly to oxidations. All solution oxidations were performed with peptide concentrations of 1 mg/ml in either degassed 0.1 M ammonium acetate buffer at pH 6.8 using $K_3Fe(CN)_6$ as the oxidant, or in degassed 0.1 M ammonium bicarbonate buffer in acetonitrile (1:1,

v/v) at pH 8.5 with H_2O_2 as the oxidant, or in methanol:water (4:1, v/v) at pH 4 (AcOH) with a 10 mM solution of iodine in methanol as the oxidant. Oxidations with CLEAR-OX resin were performed with peptide concentrations of 5 - 7 mg/ml and 3-fold molar excess of CLEAR-OX in degassed 0.1 M ammonium acetate buffer in acetonitrile (1:1, v/v) at pH 6.8. Oxidation products were analyzed by RP-HPLC and ESI-MS.

Table I. Selected CLEAR-OX Mediated Oxidation Results

Peptide Sequence	Scale	Purity of Cleaved Dithio	Crude Yield	Purity of Oxidized Crude	Yield*	Purity*
1 H-Asp-[Pen-Phe-Trp-Lys-Tyr-Cys]-Val-OH	2.01 g	59 %	1.16 g (57.7 %)	56 %	0.109 g (9.4 %)	96.5 %
2 H-Asp-[Pen-Phe-D-Trp-Orn-Tyr-Cys]-Val-OH	2.01 g	59 %	1.91 g (95.6 %)	63 %	0.318 g (16.6 %)	99.1 %
3 H-Asp-[Pen-Phe-D-Trp-Dab-Tyr-Cys]-Val-OH	1.91 g	45 %	1.86 g (97.2 %)	74 %	0.305 g (16.4 %)	96.9 %
4 Cpa-[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Cpa-NH_2	0.57 g	80 %	0.6 g (>100 %)	81 %	0.121 g (20 %)	99.5 %

Cpa = 4-Chlorophenylalanine, Dab = 2,4-Diaminobutyric Acid, Pal = 4-Pyridylalanine * Purifications were performed in two-stage reversed-phase chromatography processes

Results

Data for selected peptides are presented in Table I. CLEAR-OX mediated oxidations were simple and easy to perform. Products were separated from the polymer-bound oxidant by filtration. In the majority of the tested peptides, oxidations resulted in the expected disulfide products. Oxidations using CLEAR-OX were carried out at considerably higher concentrations than solution oxidations, thus leading to reduced solvent use for larger scale reactions. Oxidations were carried out with as low as two-fold excess of CLEAR-OX to obtain satisfactory yields, and were complete within 1 - 2 hours. The optimum pH values were found to be below 7. Solubility problems of the reduced peptides were overcome by the addition of acetonitrile to the CLEAR-OX cyclization mixtures. In the case of a peptide containing two penicillamine residues (DPDPE), the substrate bound to CLEAR-OX irreversibly without generating the expected cyclic product. However, in the majority of cases, CLEAR-OX-mediated oxidations gave yields and purities that were better than, or comparable to, traditional methods. In sequences that contain oxidation-sensitive residues (Met, Trp, Tyr), CLEAR-OX oxidations were found to be superior to other methods.

Acknowledgements

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SYNTHESIS OF dUTPase DERIVED PEPTIDES FOR GENERATION OF ENZYME SPECIFIC ANTIBODIES

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Introduction

The recently identified uracil-DNA specific nuclease is the first representative of a new family of nucleases (Vertessy et al, submitted). This enzyme contributes to survival of multiresistant cancer cells, while its inhibition induces thymine-less cell death in this way a promising anti-cancer strategy [1, 2]. To analyze the physiological function of this protein, peptide conjugates were prepared to serve as synthetic antigens for the generation of antibodies against various isoforms of dUTPase.

Transgenic mammalian plasmid vectors containing the *Drosophila melanogaster* (*D. mel*.) dUTPase cDNA were transfected into human cancer cell lines of different origin.

In *Drosophila melanogaster* (*D. mel*.), two physiological isoforms of the enzyme were identified, but only the full-length isoform contains the putative NLS sequence. Our aim was to produce antiserum specific for the N-terminal NLS containing dUTPase isoform. For this reason we prepared conjugates constructed from peptides derived from the N-terminal NLS sequences of *Drosophila* or human dUTPase proteins (Table 1.) and from a macromolecular carrier [3].

Results

Synthesis: We used poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK) as a synthetic polymeric branched polypeptide [3] and bovine serum albumin (BSA) as a natural macromolecular carrier. C-terminal Cys was added to the native sequences of selected peptides (Table 1.) for incorporation of SH group to develop thioether bond between the peptide and the carrier. The Cys^2 amino acid in peptide derived from human dUTPase was prepared in Acm-protected form to prevent reactions during biological experiments. Peptides were prepared by Fmoc/*t*Bu solid phase method utilizing Syro2000 peptide synthesizer, using Rink-amide MBHA resin and DIPCI/HOBt coupling reagents. For the establishment of thioether bond between the SAK polymer and Cys containing peptides, chloroacetyl group was introduced into the side chain terminal amino acid of SAK using Cl-CH_2-CO-OPcp. In case of BSA conjugates BSA was activated prior to coupling reaction by maleimido-benzoyl-*N*-hydroxysuccinimide ester [5]. Peptides were analysed by analytical RP-HPLC and ESI-MS prior to conjugation reaction. Conjugates were characterised by amino acid analysis.

Table 1. N-terminal sequences of the Drosophila (1) and the human (2) dUTPases, and the the sequence of the corresponding synthetic peptide derivatives (P_KC and P_SC)

(1) H-Pro[1]-Ser[2]-Thr[3]-Asp[4]-Phe[5]-Ala[6]-Asp[7]-Ile[8]-Pro[9]-Ala[10]-Ala[11]-Lys[12]-Lys[13]-.....

P_KC:

H-Pro[1]-Ser[2]-Thr[3]-Asp[4]-Phe[5]-Ala[6]-Asp[7]-Ile[8]-Pro[9]-Ala[10]-Ala[11]-Lys[12]-Lys[13]-Cys[14]-NH_2

(2) H-Pro[1]-Cys[2]-Ser[3]-Glu[4]-Glu[5]-Thr[6]-Pro[7]-Ala[8]-Ile[9]-Ser[10]-.....

P_SC: H-Pro[1]-Cys(Acm)[2]-Ser[3]-Glu[4]-Glu[5]-Thr[6]-Pro[7]-Ala[8]-Ile[9]-Ser[10]-Cys[11]-NH_2

Immunization and immunoblot analysis of serums: Wistar rats were immunized with the conjugates. After 8 weeks, blood samples were collected from the immunized rats. Serums used diluted in Western blot on nitrocellulose membranes were tested for immunoreactivity against NLS-containing dUTPase isoforms by immunoblot analysis. Serum samples were reacted with proteins derived from HT-29 cell line (wild type or transfected with a plasmid containing full-lenght *D. mel.* dUTPase cDNA), and with total protein content of *D.mel*. 'Schneider 2' cells.

Discussion

In the case of serum samples from SAK-conjugates immunized rats binding was not detectable even at 1:5,000 dilution. In the case of serum samples derived from BSA-P_SC conjugate immunization several bands were visible at 1:5,000 dilution, showing that there is no specific binding to the full length isoform dUTPase. On the immunoblot with BSA-P_KC conjugate immunized serum only one band was detected in the case of total protein of D. mel. Schneider 2 cells and transfected HT-29 cells, at 1:15,000 dilution. On the line of wild type HT-29 cells, which do not produce *D. Mel*. dUTPase, band was not detectable.

In conclusion we succeeded in preparing polyclonal antibodies recognising only the full-length, NLS containing *Drosophila melanogaster* dUTPase isoform.

Acknowledgements

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MOLECULAR DYNAMICS STUDIES OF BETA2-L1-BETA3 HUMAN CYSTATIN C FRAGMENT AND ITS MUTANTS

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Introduction

The human cystatin C (hCC) is a one of known domain swapping proteins [1]. During the process one of β-hairpin loops (L1 loop) of human cystatin C changes its conformation which leads to destabilization of the monomer structure and to formation of domain-swapped dimmer. The alleviation of distortions of the L1 loop Val 57 residue was assumed to be the causative force for changing the loop conformation [2]. Following the above assumption and our previous results of conformational studies of the human cystatin C β-hairpin (β2-L1-β3) peptide [3], we investigate conformations of the wild type peptide (WT) and its mutants: V57D, V57P and V57N. We performed conformational studies by means of molecular modeling methods. The study revealed that the residue in the loop 57th position has a great impact on the β2-L1-β3 peptide conformation stability.

Results

Molecular dynamics studies performed with AMBER 8.0 package showed the differences in stability of β-hairpin conformation of the wild-type and mutated peptides. The occurrence of the hydrogen bonds during dynamics indicates that V57N and V57D mutants have the strongest tendency to remain in the β-hairpin conformation during simulations. The mean distance between Cα atoms of 56^{th} and 60^{th} residues was 4.512 Å and 5.414 Å in the case of V57N and V57D peptides respectively, whereas in the case of WT and V57P peptides has higher values (7.601 Å and 9.471 Å respectively). The standard deviation of the distance value is the highest in the case of WT peptide.

The PMF (Potential of Mean Force) free energy profile calculated along the distance between Cα atoms of 56^{th} and 60^{th} residues, reveals the energy minimum around 5 Å for V57N,V57P and V57D mutants and the minimum around 5.5 Å for the WT peptide as well as the presence of the more shallow minima at 6.4 Å and 8 Å for V57P mutant and 7.4 Å for and WT peptide.

Discussion

The loop residues are known to be very important for β-hairpin folding and stability. The Val branched residue is non-typical β-hairpin loop residue. Nevertheless the Val 57 in hCC protein is important for inhibitory activity of cystatin C. The presented results prove that the loop residue at 57^{th} position has an impact on the stability of the β-hairpin conformation of β2-L1-β3 hCC fragment.

The loop of the wild type peptide tends to wider in comparison with the starting β-hairpin structure during molecular dynamics simulation. In addition the large standard deviation value and the minimum and maximum values of the distance between Cα atoms of 56^{th} and 60^{th} residues (4.374 Å and 11.297 Å) reveal that WT peptide structure fluctuates between two energetically low conformations. The V57D and V57N mutants are more stable than the wild type β-hairpin, and such mutations are potentially useful in the further studies of the hCC monomeric structure stabilization.

Acknowledgements

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NOVEL BICYCLIC TEMPLATES FOR PEPTIDOMIMETICS TARGETING GPCRS

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Introduction

Interest in understanding and modulating the functions and pharmacology of peptide-activated G Protein-Coupled Receptors (GPCRs) has led to increased demand for new ligands which target them. Having developed many of the most important unnatural peptide ligands for certain receptor subfamilies, a new generation of compounds employing peptidomimetic scaffolds was pursued [1].

Methods and Results

New synthetic methodology was developed to provide general templates. The choice of synthetic route and starting materials, including amino acids, allows structural and stereochemical variations in the template, as well as an array of substitutions to the core structures. For example, Fig. 1 illustrates that, from Boc-phenylalanine, compound **1** can be produced in five steps, and compound **2** in six steps.

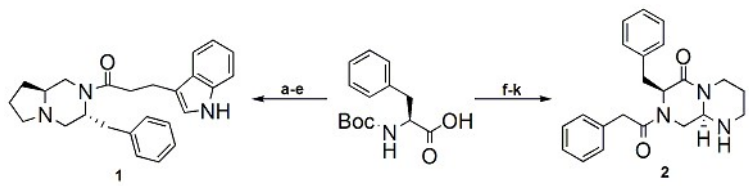

Fig. 1. a. H-Pro-OMe, PyBOP, HOBt, DIEA, DMF b. TFA c. Et_3N, MeOH d. $LiAlH_4$, THF e. Indolepropionic acid, PyBOP, HOBt, DIEA, DMF f. Cbz-NH-$(CH_2)_3$-NH_2, BOP, HOBt, NMP g. TFA(25%)/DCM h. $(MeO)_2CHCHO$, Et_3N j. Phenylacetic acid, CIP, HOAt, DIEA, DCM k. HCOOH.

Ligands were rationally designed to target specific receptor systems based upon structure-activity relationships (SAR) and molecular modeling using structural data from known ligands. Where this design was based on peptides, mimicry of key amino acid sequences known to adopt β-turn structures was the focus. First targeted were the melanocortin receptors (MCRs), for which the standard peptide agonist is MT-II. Computational models of **1** and **2**, as well as other potential members of each compound class, were superimposed on an NMR-derived structure for MT-II.

Assays of the ligand series including **1** for binding to human MCRs (transfected to HEK293 cells) and subsequent cAMP accumulation indicate that these compounds are high-affinity antagonists, with many exhibiting remarkable selectivity for

particular MCR subtypes. Five hMC5R-selective antagonists have been discovered. The previously noted **1** is an antagonist at hMC1R and hMC3R.

Whether the binding and antagonism occurs via an orthosteric or allosteric site remains unresolved at this time, but binding efficiencies—defined as the displacement of radioligand relative to MT-II—of less than one hundred percent suggest that allosterism is at work in at least some cases.

Besides providing additional selective antagonists, attempts are being made to achieve agonism with these structures. Additional substituents containing basic nitrogens can be incorporated into these structures by the use of novel amino acid starting materials. Development of the asymmetric synthetic methods to make these amino acids was a crucial innovation. It is hoped that the additional functional group can provide the molecular recognition element necessary for agonism.

The success of this series bodes well for compounds such as **2**, and the application of the novel structural templates to other peptide-mediated GPCR targets. An interest in pain research makes the opioid and neurokinin systems particularly attractive, and new ligands have been designed using a similar molecular modeling approach.

Discussion

The role of β-turn secondary structure in molecular recognition for peptide-activated GPCRs has been well established [2]. This is the conceptual framework within which these new heterocyclic scaffolds have been designed. Functionally, at least one of these new compound classes has been validated as a peptidomimetic. Further studies will establish whether the mode of interaction is mechanistically similar to that found with peptide ligands.

Regardless, new ligands in either series will be useful in studying the biology of these systems. MCRs, for example, have been found to play a role in pigmentation, nociception, inflammation, appetite, energy balance, and a host of other processes. This particular array of biological activity has also made these receptors a target for drug discovery in a number of therapeutic areas, including obesity, cachexia, cancer, erectile dysfunction, and pain. These new structures will be pharmacological tools and potential leads in drug discovery efforts.

Acknowledgements

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MULTI-EPITOPIC LIPID CORE PEPTIDE VACCINE WITH BROAD PROTECTIVE IMMUNE RESPONSES AGAINST GROUP A STREPTOCOCCUS

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Introduction

Short peptides as antigens are generally unable to elicit effective antibody responses following immunization without carrier proteins and adjuvants. These additives suffer undesirable problems in practice: carrier proteins induce suppression of antibody responses; many adjuvants are unsuitable for human use. The multiple antigen peptide (MAP) system showed enhancement of immunogenicity and reduction in need of carrier proteins. The use of T-helper cell epitopes generates the proliferation of T cells and can eliminate the use of protein carriers [1]. Lipidation of peptide antigens may elicit functional antibody responses without co-administration of adjuvants. The authors have established the usefulness of the sequence Laa-Gly-Laa-Laa-Gly (Laa; lipo amino acid) as an adjuvant [2]. To find more effective vaccine system using Laa, the authors made the library of peptide vaccines, which has following three essential in single construct: (1) T cell epitope derived from the fusion protein of morbillivirus canine distemper virus [1], (2) J14 derived from the M-protein conserved region of Group A Streptococca as a B cell epitope, and (3) lipid function using Laa as an adjuvant, as shown in Fig. 1.

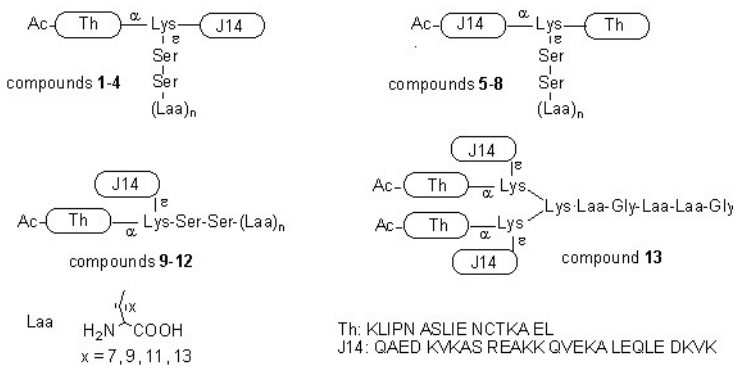

Fig. 1. Structure of peptide vaccine candidates.

Results

Peptide vaccine candidates were synthesized by manual SPPS method and mice (5 per group) were immunized with them. The serum antibody productions on 20 - and 30 - days after immunization were evaluated by ELISA. Most of our compounds showed immune responses without co-administration of adjuvants (Fig. 2). Compounds bearing the lipid function at their C-terminus showed stronger antibody titers than compounds in which the lipid moieties are attached on Lys ε amine.

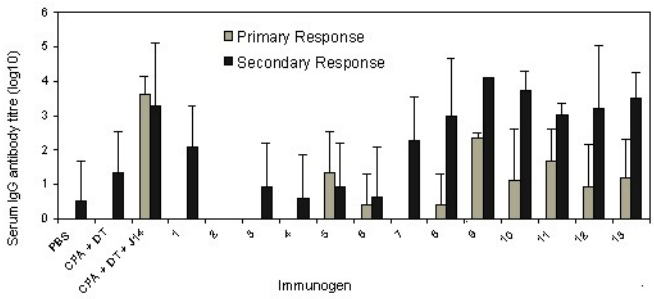

Fig. 1. Serum IgG antibody responces in mice.

Discussion

It was reported that the location of lipid function within peptide vaccine influenced their solubility and immunogenicity; the branched lipopeptide is more potent than the liner lipopeptide [3]. This study demonstrated that within branched construct the configurations of each three components, B-cell epitope, T-helper epitope and lipid, affected the immunogenic property of lipopeptide vaccines.

Acknowledgements

This work was supported by funding from the National Health and Medical Research Council (Australia) and National Heart Foundation (Australia).

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ENDOGENOUS INHIBITOR OF DYNORPHIN CONVERTING ENZYME IN HUMAN CEREBROSPINAL FLUID

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Dynorphin converting enzymes are a recently identified group of proteinases capable of converting the dynorphin-related opioid peptides to enkephalins. These enzymes convert the kappa-receptor specific dynorphins to delta-receptor specific enkephalins, and may play an important role by releasing ligands mediating analgesic activity, and act in drug dependence mechanisms. One of these enzymes is a serine peptidase present in human and rat cerebrospnal fluids (CSF). Little is known about the regulation of the activity of neuropeptide peptidases in the CNS. Recently, we described our preliminary finding of a protein in the CSF possessing inhibitory activity against a dynorphin converting enzyme isolated from CSF. Here, we present a more detailed study on the identification and properties of this inhibitor, which also inhibited other serine proteases such as trypsin, chymotrypsin and plasmin.

hCSF was purified on a Sepharose-anhydrotrypsin column. The material, containing highest inhibitory activity, was subjected to the reversed phase chromatography. The chromatographed material was subjected to SDS/PAGE. Visualization of protein by gel staining revealed one major band with an apparent molecular mass of 16 kDa, similarly to the data obtained by MALDI-TOF MS. This band was excised and subjected to N-terminal sequencing, which yielded 15 amino acid residues (Fig. 1, bold letters). Computer analysis of the obtained sequence using Fasta 3 showed that it was identical with part of the sequence of the bikunin moiety (underlined residues) of the alpha-1-microglobulin/ bikunin precursor.

The kinetic parameters for the enzyme-substrate and enzyme-inhibitor interactions were K_M = 9 μM and K_i = 1.7 nM. The interaction between dynorphin convertase and bikunin fulfills the competitive model of the inhibition. Similar data were obtained when material from human urine was applied (data not shown).

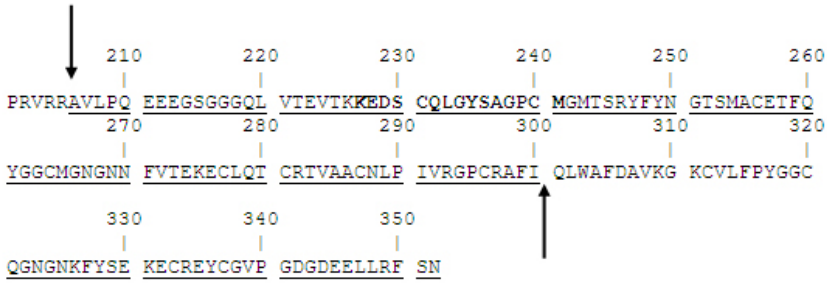

Fig. 1. The obtained sequence, which is shown by bold letters, can be fitted into that of the alpha-1-microglobulin/bikunin precursor, the sequence of which is partially shown. The two proteins are released from the precursor by a proteolytic cleavage. Bikunin is released at the sites indicated by arrows.

The sequence obtained by us indicated that the isolated protein was truncated twenty one amino acid residues downstream of the N-terminus. This result is consistent with the apparent molecular mass of about 16 kDa obtained by SDS/PAGE and MALDI-TOF MS. The hydrodynamic radius of bikunin is similar to that of albumin and using gel filtration, we found that the size of the inhibitor in unfractionated CSF was similar to that of albumin (data not shown). Thus, it seems that bikunin has been truncated during the purification procedure. This cleavage has occurred betwen two basic amino acid residues which is characteristic of the action of trypsin. This trypsin might have leaked from the gel used for the isolation of the inhibitor.

This paper provides evidence that a neuropeptide convertase derived from the CNS might be regulated by an endogenous protein inhibitor, co-existing in the same fluid. It is unknown if this comprises a regulatory mechanism, thus partially decreasing the enzymatic activity of DCE, or whether an inactive enzyme pool exists in the fluid, being activated when necessary, or is it a typical mechanism of inactivation of the enzyme activity which should be terminated after its release from the tissue. Further work would be necessary to provide a complete answer to these questions. Nevertheless, such finding may shed more light on the mechanism of inhibition of neuropeptide convertases, to serve as a lead for synthesis of other compounds, and can also be useful for enzyme purification by affinity chromatography.

Acknowledgements

This work was supported by the ICGEB (Trieste, Italy) grant CRP/POL05/02.

OPTIMIZATION OF THE OXIDATIVE FOLDING REACTION AND DETERMINATION OF THE DISULFIDE STRUCTURE FOR HUMAN α- AND β-DEFENSINS

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Introduction

To assess the biological activities of human α-defensins and β-defensins (hBDs), extreme care is required in the chemical synthesis to avoid ambiguity in quality because disulfide isomers without the native pairing have been demonstrated to exhibit similar antimicrobial activity to that of the native defensins [1, 2]. In the present study, we chemically synthesized human α-defensin 1, 2, 3 and 5 [*i.e.* human neutrophil peptide (HNP) 1, 2, 3 and HD5], and hBDs 1-4 (Fig. 1), and determined the optimal conditions for folding the respective reduced peptides preferentially into a native conformation. The synthetic human α-defensins and hBDs of high homogeneity were confirmed to have the respective native disulfide structures.

```
HNP1          ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP2           CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP3          DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HD5         ATCYCRHGRCATRESLSGVCEISGRLYRLCCR

hBD1       DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
hBD2    GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
hBD3  GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
hBD4      ELDRICGYGTARCRKK-CRSQEYRIGRCPNTYA-CCLRK
```

Fig. 1. Structures of human α-defensins and hBDs.

Results and Discussion

In order to determine the optimal conditions for folding the reduced α-defensins and hBDs assembled by employing Boc chemistry, each reduced peptide was subjected to oxidative folding performed in 0.1 M NH_4OAc (pH 7.8) containing GSH/GSSG (peptide : GSH : GSSG = 1 : 100 : 10) at a peptide concentration of 1 x 10^{-5} M at 25ºC under combinations of the following reaction conditions: 1) varying the salt and its concentration; 2) lowering the reaction temperature from 25 ºC to 4 ºC; 3) conducting the reaction in the presence or absence of denaturant, GnHCl; 4) varying the organic solvent and its concentration.

α-defensins: The α-defensin molecule in both reduced and oxidized forms raised a solubility problem in aqueous folding media due to its hydrophobic compositions. Aggregation could be prevented by adding GnHCl or various organic solvents. Addition of MeCN was effective not only to improve the solubility but also to accelerate the folding reaction.

hBDs: Addition of GnHCl or organic solvents more or less interfered with the folding reaction rather than accelerating it. In addition, no significant improvements in the folding were observed by changing the ammonium salts as in the case of α-defensins. Therefore, we focused on two factors (*i.e.* NH_4OAc concentration and reaction temperature) to lower the electrostatic repulsions in highly positively charged molecules, such as hBDs. Under several sets of optimal conditions, hBDs were recovered in more than 80% yield.

Next, to determine the disulfide structure, the synthetic peptides were digested with appropriate enzyme(s) to obtain cystine segments. Since the molecules contain two adjacent Cys residues, the enzymatic digestion resulted in yielding their respective sets of two cystine segments consisting of one disulfide linkage and two disulfide linkages. The latter segments, for which two possible disulfide modes could be considered, were subjected to Edman degradation to assign their disulfide structures with the guidance of the cycles detecting diPTH cystine. As for HNP1-3, however, the Edman degradation could not discriminate between two possible disulfide modes because diPTH cystine could be detected at the same cycles in both structures. To avoid this problem, the acetyl group was introduced to the N-terminus of the parent molecule prior to enzymatic digestions. This provided a cystine segment having three peptide chains linked by two disulfide bonds, in which the peptide chain originating from the N-terminus was tagged with the acetyl group. This procedure was successfully applied to discriminating two possible structures by the Edman degradation. All the synthetic human α-defensins and hBDs were confirmed to have the respective native disulfide pairing (*i.e.* Cys^1-Cys^6, Cys^2-Cys^4 and Cys^3-Cys^5, and Cys^1-Cys^5, Cys^2-Cys^4 and Cys^3-Cys^6).

In conclusion, all the reduced human α-defensins and hBDs could be efficiently oxidized to the α- and β-defensin-type disulfide structures, respectively, under the conditions determined in the present study. These synthetic peptides of high homogeneity were used to accurately assess the antimicrobial activity against *E. coli.*

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PLANT PHOTORECEPTOR PHYTOCHROME A AND B HAVE DIFFERENT DIMERIZATION MECHANISMS

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Introduction

Light is one of the most important environmental factors that challenge the developing plant. Light acts not only as an energy source for plants but as an environmental signal that regulates plant development. Plants can sense the intensity, wavelength, direction, and timing of illumination using diverse photoreceptors: cryptochromes and phototropins for UV-A/blue light and phytochromes for red and far-red light. Phytochromes are large, soluble chromoproteins that exist as dimmers in solution. The phytochrome molecule consists of an N-terminal photosensory domain with covalently linked chromophore and a C-terminal domain that contains signaling motifs such as a kinase and two PAS domains. The N- and C-domains are connected by a proteolytically-sensitive hinge region that includes phosphorylatable serine. The phytochrome function is regulated mainly by three molecular events: (1) photo-interconversion between two conformational states, (2) dimerization and (3) reversible phosphorylation within the hinge region. The C-terminal domain together with the hinge is responsible for the last two events. Previous studies using size-exclusion chromatography (SEC) experiments and λ repressor-based *in vivo* assays suggest that two regions, residues 599 - 683 in oat PhyA corresponding to 601- 685 in rice PhyA within the hinge-PAS1 region and/or a large portion of HKLD at the C-terminus, are capable of mediating dimerization [1, 2]. However, the exact dimerization site has not been identified yet. Here we report comprehensive studies of the PAS1 domains with the N-flanking hinge region of PhyA and PhyB, two major phytochromes out of three in rice. These proteins show high similarity in their primary structure but have distinct physiological functions.

Result and Discussion

The self-association property of $PhyA^{601-740}$ was examined by combined use of analytical ultracentrifugation and NMR spectroscopy. The molecular weight determined by the sedimentation equilibrium experiment was 16750 ± 300 which is in good agreement with the theoretical value of the monomeric $PHYA^{601-740}$ (15388). In addition, the ^{1}H-^{15}N HSQC spectra measured at various protein concentrations from 25 μM to 1.6 mM showed no changes in signal positions and spectral widths. These results lead to conclusion that the rice $PHYA^{601-740}$ exists as a monomer in the concentration range examined. In contrast, $PhyB^{647-782}$, the PhyB fragment corresponding to $PhyA^{601-740}$, formed a homodimer in concentration dependent manner as revealed by the analytical SEC and NMR spectroscopy.

PhyB$^{647-782}$ exists as a dimer at the protein concentration above 1 mM while as a monomer at concentration below 25 μM.

Deletion of the first 19 residues from PhyB$^{647-782}$ resulted in the PhyB fragment which adopts only the monomeric form in the entire concentration examined. Interestingly, the ^{1}H-^{15}N HSQC spectrum of PhyB$^{666-782}$ is nearly superimposable with that of PhyB$^{647-782}$ at the monomer-predominant concentration (25 μM). These results strongly demonstrate that the part of the hinge region, L^{647}-M^{665}, plays a critical role in the dimer formation of PhyB but does not affect conformation of the rest of the PhyB$^{647-782}$ molecule in the monomeric state.

Comparison of amino acid sequences within the hinge region between PhyA and PhyB reveals that hydrophobic residues are well conserved whereas several charges residues are substituted with opposite charged ones. To assess specific elements necessary for the dimerization of PhyB$^{647-782}$, we characterized self-association property of several site-directed mutants. The PhyB$^{647-782}$ mutants examined involve three from-PhyB-to-PhyA mutations, E651K, R653D and R663S, and three charge-inversion ones, D656R, R663D and R653D/R663D within the hinge region. One of the from-PhyB-to-PhyA mutants, R663S, displayed greatly reduced dimerization ability. Little amount of the dimer was detected at the protein concentration of 0.5 mM and approximately 75% of R663S exists as a monomer even at 1.7 mM. The replacement of the same residue with a negatively charged Asp resulted in further reduction of the dimerization ability. The R653D mutant showed an attenuated dimer formation and approximately 60% of the protein exists as a monomer at 0.5 mM. The double mutant, R653D/R663D, is not able to dimerize in the entire concentration range examined (< 2 mM). On the other hand, no drastic change in dimerization profile was observed for the negative-to-positive charge-inversion mutants, E651K and D656R. Our mutation studies clearly demonstrate that the positively charged arginine residues within the hinge region of PhyB are important for its dimerization, and the R663 residue has a predominant effect. This idea is supported by the fact that R663 is conserved in all PhyBs, but not found in PhyA at all. The difference in dimerization ability of the PAS1 domain with the N-flanking hinge region between PhyA and PhyB may participate in the difference of their physiological functions.

Acknowledgements

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INFLUENCE OF NALOXONE IN THE ANTINOCICEPTIVE EFFECTS OF TYR-MIF-1'S ANALOGUES

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Introduction

The Tyr-MIF-1 family of peptides (Tyr-MIF-1`s) includes MIF-1, Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1, which have been isolated from bovine hypothalamus and human parietal cortex tissue [1]. Members of this family of peptides have been shown to have opiate modulating effects [2, 3]. Tyr-MIF-1 has antinociceptive effect by binding to m-opioid receptors as well as to their specific non-opiate receptors in the brain [4].

The aim of this study was to investigate the analgesic effects of modified at position 1 analogues of Tyr-MIF-1 during acute pain - *N*-(Me)-Tyr-MIF-1, D-Tyr(Me)-MIF-1, Tyr(Cl_2)-MIF-1, Tyr(Br_2)-MIF-1.

The experiments were carried out on male Wistar rats (180 - 200 g) housed at 12 h light/dark cycle. Food and water were available ad libitum. All experiments were carried out between 09.00 a.m./12.00 p.m. Each group included 8 - 10 rats.

Antinociceptive effects were evaluated using the paw pressure (PP) test. The changes in the mechanical nociceptive threshold of the rats were measured by the using an analgesiameter (Ugo Basile). The pressure was applied to the hind-paw and the pressure (g) required to elicit nociceptive responses such as squeak and struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Tyr-MIF-1 and naloxone (all in dose 1 mg/kg, i.p.) were obtained from (Bachem AG). The analogues of Tyr-MIF-1 - *N*-(Me)-Tyr-MIF-1, D-Tyr(Me)-MIF-1, Tyr(Cl_2)-MIF-1, Tyr(Br_2)-MIF-1 (all in dose 1 mg/kg, *i.p.*) were synthesized according the procedure previously reported [5]. All the peptides and naloxone were dissolved in sterile saline (0.9% NaCl) solution and were injected intraperitoneally (*i.p.*).

The experimental procedures were carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes.

The results were statistically assessed by one-way analysis of variance (ANOVA).

Result and Discussion

The investigation started 15 min after *i.p.* injection of peptides. All newly synthesized analogues (1 mg/kg, *i.p.*) applied alone exerted analgesic effects compared to the control group. The antinociceptive effect of Tyr(Br_2)-MIF-1 was higher compared to all investigated peptides (Fig.1).

To determine whether analgesic activity of the synthesized Tyr-MIF-1 analogues are mediated by the opioid system a competitive antagonist of opiate receptors as naloxone (Nal) was used. Naloxone was injected 20 min. before each of investigated peptides. The results showed that Nal decreased significantly their effects (Fig.1).

In conclusion it could be suggested that in analgesic effects of newly synthesized analogues of Tyr-MIF-1 during acute pain is involved opioidergic system.

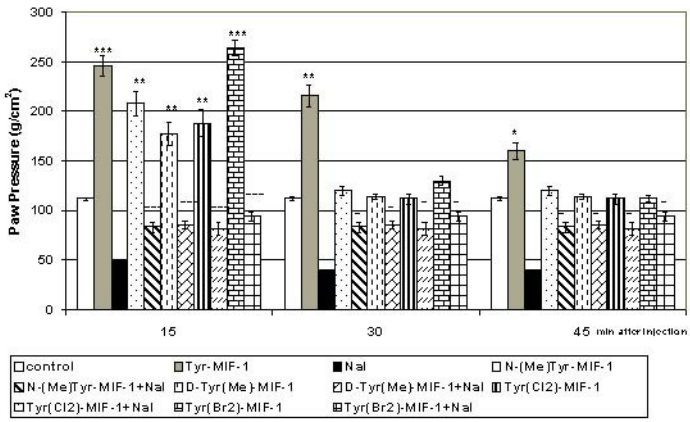

*Fig.1. Effects of Tyr-MIF-1 and analogues (1 mg/kg, i.p.) on nociception Data are presented as mean ± S.E.M.; *P<0.01, ** P<0.01 versus control; $^{+}$P<0.01, $^{++}$ P<0.01 versus each of peptides.*

Acknowledgements

For the support of this work we are grateful to the National Found for Scientific Research of Bulgaria (Contracts VUL-04/05 and X-1201).

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SOLID PHASE SYNTHESIS OF SYNAPOYL-PEPTIDE AMIDES

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Introduction

In recent years, peptides have extensively been modified in order to find new therapeutic agents for pharmaceutical applications. Such modifications are required because natural peptides are often excreted rapidly or do not pass biological membranes resulting in a poor bioavailability. In that respect, non-protein amino acids are particularly interesting. Another important modifying element is the phenolic acids. It was well documented that phenolic acids and their derivatives exhibit a variety of biological effects, including antiviral, antimicrobial, antitumour, analgesic and antioxidant activity [1, 2]. Herein we report the synthesis of a series of tri- and tetrapeptide mimetics containing non-protein amino acids canavanine (**Cav**) and **sLys** and sinapic acid (**SA**), as analogues of analgesic peptides MIF-1 and Tyr-MIF-1: **SA**-Pro-Leu-Gly-NH_2 (**1**); **SA**-Tyr-Pro-Leu-Gly-NH_2 (**2**); **SA**-Tyr-Pro-**sLys**-Gly-NH_2 (**3**); **SA**-Tyr-Pro-**Cav**-Gly-NH_2 (**4**); **SA**-Pro-**Cav**-Gly-NH_2 (**5**); Pro-Leu-Gly-**Eda**-**SA** (**6**); Pro-Leu-Gly-**Pda**-**SA** (**7**). The purpose of this study, therefore was to investigate the analgesic effects of newly synthsized analogues of MIF-1 and Tyr-MIF-1 during acute pain.

Result and Discussion

Based on previous SAR [3] new cinnamoyl amides of analgesic oligopeptides were designed. Manual stepwise solid phase techniques using both Fmoc- and Boc-strategy was applied. The first modification included MIF-1 and Tyr-MIF-1 analogues **1 - 5**, where sinapic acid was attached to the N-terminus of the peptide chain, and Leu was replaced with **Cav** or **sLys**. The peptides were synthesized on a Rink-amide resin using Fmoc-strategy with DIC/HOBt activation. Next, the analogues **6** and **7** modified at C-terminus with biogenic amines and sinapic acid were obtained on a Merrifield resin using Boc-strategy with DCC/HOBt activation. The crude peptides were purified by preparative TLC and their purity was checked by analytical HPLC. The precise molecular mass was confirmed by ESI-MS.

Antinociceptive effects were evaluated using the paw pressure (PP) test. The experiments were carried out on male Wistar rats (180 – 200 g), treated with *i.p.* doses of 1 mg/kg,. SA injected alone exerted significant analgesic effect compared to Tyr-MIF-1 and MIF-1. Peptides **1, 2**, **6**, and **7** showed analgesic effect similar to

MIF-1 (Fig. 1). Among all analogues, **1** and **7** showed the most pronounced analgesic effects. The analogues **3**, **4** and **5** exerted analgesic effect comparable to the control group. The analogue **5** showed significant analgesic effect higher than MIF-1, but lower than SA. Peptides **3** and **4** had significant analgesic effects, which were lower than Tyr-MIF-1 and SA (Fig. 2). The results suggest certain structure-activity relationships within new series: a) substitution of **SA** and **Cav** in MIF-1 enhances analgesic activity; b) substitution of **SA** and **Cav** and **sLys** in Tyr-MIF-1 reduces analgesic activity.

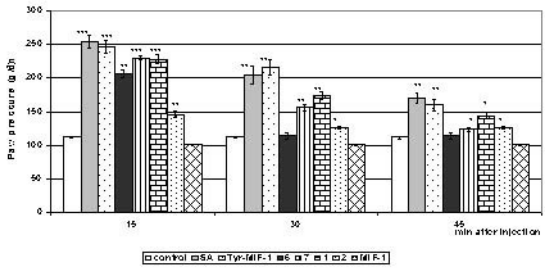

*Fig.1. Effects of Sin, Tyr-MIF-1 and analogues (1 mg/kg, i.p.) on nociception. Data are presented as mean ± S.E.M.; *P<0.01, **P<0.01, ***P<0.01 versus control; P<0.01, $^{++}$P<0.01 versus MIF-1 or Tyr-MIF-1.*

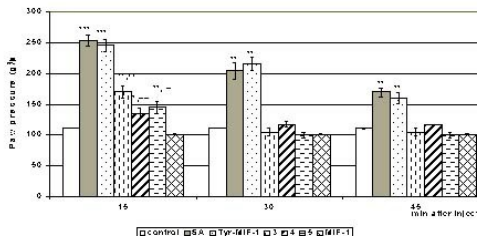

*Fig. 2. Effects of Sin, MIF, Tyr-MIF-1 and analogues (1 mg/kg, i.p.) on nociception. Data are presented as mean ± S.E.M.; *P<0.01, **P<0.01, ***P<0.01 versus control; $^{+}$P<0.01, $^{++}$P<0.01 versus MIF-1 or Tyr-MIF-1.*

Acknowledgements

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C-ACYLATIONS OF POLYMERIC PHOSPHORANYLIDENE ACETATES FOR C-TERMINAL VARIATION OF PEPTIDE CARBOXYLIC ACIDS

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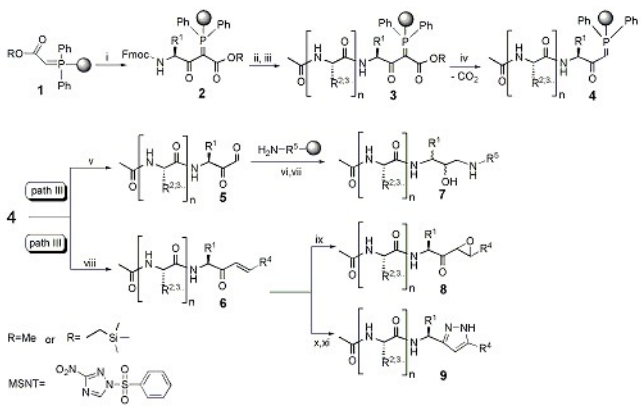
Introduction

We have recently demonstrated an efficient C-acylation of polymer-supported 2-phosphoranylidene acetonitriles which could be further employed in Fmoc-based peptide chemistry eventually yielding a library of norstatines as transition state isosteres [1, 2]. In continuation we now further report that the acylphosphorane **2** can be efficiently synthesised by C-acylation of yet another class of linker reagents, the polymer-supported 2-phosphoranylidene acetates **1**. The phosphorane **2** was employed in standard peptide synthesis and could be cleaved of the resins by three alternative methods leading to various C-terminal variations of peptide carboxylic acids including peptidyl 4-amino-2,3-dioxo butanoates (peptidyl-2,3-diketoesters), peptidyl-1-amino-3-buten-4-ones **6**, and peptidyl-3-amino-2-oxo-propanals **5** (peptidyl-ketoaldehydes) [3, 4]. These compounds are excellent intermediates for the synthesis of pharmaceutically important compounds such as 1,5-diamino-3-hydroxy-propane **7**, and for fast synthesis of peptidyl heterocycles such as epoxides **8**, pyrazoles **9** *etc*. on the C-terminus.



Scheme 1: Chemical diversity through the C-acylation of phosphoranes

Reagents and reaction conditions; i, 5 eq Fmoc-AA-OH, 5 eq MSNT, 5 eq 2,6-lutidine, DCM, rt, 12 h; ii, 20% piperidine/DMF; iii, Fmoc-AA-OH 3 eq, DIC 3 eq, HOBt 3 eq, DMF, rt, 3 h, repeated for R^2, R^3; iv, for R=$C_2H_2Si(CH_3)_3$: 3 eq (TAS-F) tris-(dimethylamino)-sulfonium-difluorotrimethylsilicate, DMF, rt, 3 h; v,

DMDO/acetone, 0° C, 30 min; vi, 1 eq pre loaded TCP-resin, 2.0 eq *N*-peptidyl 3-amino-2-oxo-propanals, 3.0 eq $NaCNBH_3$, 3 eq $NaBH_4$, DMF/1% AcOH, rt, 3 h; vii DCM/AcOH:TFE (8:1:1, v:v:v); viii, 3 eq R^4CHO, THF, rt, 12 h; ix, DMDO/acetone, DCM, rt, under N_2, 12 h; x, 3 eq $RNHNH_2$, THF, 80°C, 8 h; xi, 1,2 eq, DDQ, toluene, rt, 8 h.

Result and Discussion

The synthetic strategy and reaction conditions are illustrated in Scheme 1. The polymer supported reagent **1** was coupled with Fmoc- amino acid using MSNT as coupling reagent to furnished **2**. The Fmoc-cleavage and subsequent coupling with another Fmoc amino acid followed by Fmoc deprotection and subsequent acylation yielded compound **3**. **3** was subjected to saponification and decarboxylation to leading to peptidyl ketoaldehyde **5** or the peptidyl α,β-unsaturated ketones **6**. As depicted in Scheme 1, compounds **5** and **6** serve as important electrophilic building blocks and can be converted into various compounds of biological interest.

In summary, we have developed a protocol for preparing various building blocks such as peptidyl ketoaldehydes **5**, α,β-unsaturated ketones **6** and diketoesters on solid phase using phosphoranes as a linker reagent. These building blocks were further converted into useful compounds of pharmaceutical importance *e.g.* peptidyl ketoaldehydes, diamino propanols , and peptidyl heterocycles **8**, **9** *etc*.

Acknowledgements

We sincerely thank Dr. Pradeep Kumar for helpful discussion and his support.

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THIOAMIDE SOLID-PHASE PEPTIDE SYNTHESIS T-SPPS: A NOVEL SYNTHESIS METHOD FOR PEPTIDE THIOESTERS

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Introduction

The total synthesis of longer peptides or even proteins still challenges modern chemistry. Due to the possible accumulation of side reactions and consequently failure sequences, commonly used SPPS protocols may only yield in peptides of 50-60 amino acids in length. To circumvent these problems, chemical and enzymatic approaches for the ligation of peptide fragments have been developed. The commonly used native chemical ligation [1], Staudinger ligation [2] and enzymatic ligation technology [3] characteristically start from peptide α-thioesters as valuable building blocks for the ligation rection.

Here we present a novel route to the synthesis of peptide α-thioesters which is compatible with a slightly modified Fmoc/*t*Bu-chemistry. The key feature of this approach is a thioxo amino acid moiety coupled to an amine functionalized polymeric carrier, e.g. MBHA resin. Based on Harrowven's *et al.* finding that thioamides can be converted to the respective thioesters via a specific alkylation of the thioxo function [4] we adapted this approach to amino acids and peptides.

Material and Methods

The starting compound Fmoc-Xaa-Ψ[CS-NH]-MBHA can be generated via direct coupling of pre-activated N^{α}-Fmoc protected thioxo amino acid nitrobenzo-triazolides [5] to the amino function of the polymeric carrier. Alternatively, standard resin-bound N^{α}-Fmoc protected amino acids are easily converted into the thioxo amino acid counterpart by using P_4S_{10} or Lawesson's reagent [6], respectively.

Following an automated solid-phase peptide synthesis, the peptide thioester is liberated by the key reaction of the approach that involves the alkylation of the sulphur which gives the thioimidic acid ester intermediate that is further hydrolysed by water to the respective thioester (Fig. 1).

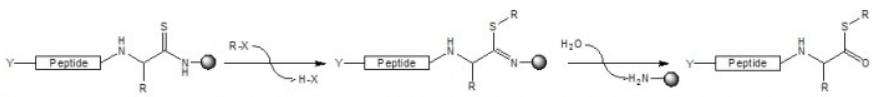

Figure 1: Key reaction of the T-SPPS. After alkylating the thioxo sulphur, the thioimidic acid ester formed can be hydrolyzed to the respective peptide α-thioester.

Results

After optimising the conditions of the thioester release regarding reaction time, water and TFA concentration the yields of Fmoc-Xaa-SMe as well as the rate of epimerization were determined (Table 1).

Table 1: Yields of Fmoc-Xaa-SMe under optimized conditions (5% water, 10% TFA, 30 °C, 24 h) using iodomethane as alkylating reagent. [1)] Fraction of D-epimer that is build up during the thioester synthesis as determined using enzymatic hydrolysis assays. Detection limit (d.l.): 0.01%.

Fmoc-Xaa-SMe	Yield in %	D-Epimer in % [1)]
Gly	81	-
Val	47	n.d.
Leu	72	2.3
Ile	53	n.d.
Pro	65	n.d.
Ala	77	1.7
Thr(*t*Bu)	43	n.d.
Ser(*t*Bu)	54	2.0
Tyr(*t*Bu)	54	3.0
Lys(Boc)	62	<d.l.
Arg(Pbf)	69	1.1
Gln(Trt)	61	n.d.
Asn(Trt)	51	n.d.
Glu(O*t*Bu)	50	2.0
Asp(O*t*Bu)	50	2.1
Trp(Boc)	57	1.0
His(Trt)	53	<d.l.
Phe	59	3.0

Figure 2: Mass spectrum of Z-KLALDLLE-SMe, calcd.: 1077 found: 1078 (m/z)

Finally, the new approach was transferred successfully to the synthesis of the Z-KLALDLLE peptide methylthioester (Fig. 2).

Generally it can be noted that this method is not only restricted to methylthio-esters. Based on the reaction mechanism, also other halides like ethyl bromide, benzylbromide or bromoacetic acid act as efficient alkylating agents yielding the respective thioester products.

Acknowledgements

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ENGINEERING PROTEASES AS COUPLING REAGENTS IN PROTEIN SEMISYNTHESIS

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Introduction

Chemical protein synthesis is a powerful method for assembling selectively modified proteins, providing a freedom for protein engineering inaccessible by standard site-directed mutagenesis. This benefit comes, however, together with the need for developing approaches that allow for the selective, universal and racemisation-free incorporation of chemically modified peptide fragments into the protein targets. Efforts in this direction pioneered the substrate mimetics-mediated protease catalysis as a novel ligation method that apparently breaks with the popular rule whereupon the specificity of an enzyme leads necessarily to a decrease in the method's flexibility. In fact, the approach tolerates a high structural diversity at the ligation site despite the originally pronounced substrate preferences of the biocatalyst. Unwanted peptide bond cleavages based on the native proteolytic activity of wild-type proteases, however, still remain as a serious side reaction limiting the universality of this biocatalytic approach.

Results

The proteolytic activity represents an inherent catalytic behavior of proteases manifested in their individual protein structures. Thus, manipulations of the latter should yield effects in the former which finally may result in biocatalysts with minimized undesired cleavage activities but full 'synthetic' ligation functions. By screening a library of more than 60 rationally designed trypsin species, covering enzyme variants with only one mutation site but also those bearing 2, 3 and up to 4 mutated amino acid positions, a double mutant could be identified which indeed shows almost no proteolytic activity while the enzyme's ligase function is still remained. After basic characterization, the synthetic behavior of the optimized trypsin species has been successfully evaluated on example of the synthesis of the peptidyl prolyl-*cis*/*trans*-isomerase parvulin 10 from *E. coli* by the ligation of 43 amino acid containing peptide thioester substrate mimetic obtained via intein methodology with a full synthetic 49-mer peptide fragment. Kinetic characterization of the protein synthesized revealed a fully functional parvulin 10 species being as active as the respective wild-type isomerase. Furthermore, the same enzyme in combination with the substrate mimetics approach could be likewise used to mediate site-specific modifications of native proteins at their N-termini without any risk of proteolytic side reactions and interestingly, without affecting the ε-amino functions of lysine moieties. By incorporation of two

additional mutations, i.e. of two histidine moieties, the trypsin double mutant was further converted into a 'restriction transamidase' which selectively recognizes the amino acid sequence Tyr-Arg-His being synthetically active between tyrosine and arginine. Incorporation of this tripeptide sequence at the C-terminus of proteins was found to enable site-specific modifications at the respective terminus leaving the N-terminal moiety of the protein target untouched. Vice versa, placing the recognition sequence at the protein's N-terminus allows for highly efficient N-terminal modification reactions. The latter profits from the selective cleavage activity of the biocatalyst which allows for an easy combination of the modification reaction with the commonly used tag-based protein purification technologies. Thus, combined use of the cleavage and synthetic activity of the enzyme variant enables specific cleavage of the purification tag and subsequently, selective N-terminal modification reactions in an efficient and easy to use one-pot approach. Finally, the general power of the optimized biocatalyst to mediate selective reactions at both the N- and C-terminus of a native protein simultaneously could be initially illustrated by linking two different fluorophores to the two termini of RNase T1 (Fig. 1).

Fig. 1: *C-* and *N*-terminal modification of RNase T1 mediated by the genetically optimized trypsin variant (A) and MS-spectrum of the modified protein product (B).

Discussion

The results convincingly show the general power of enzyme and substrate engineering in the biocatalytic synthesis and modification of peptides and proteins. Combined use of both technologies improves the performance of the classical protease-mediated peptide synthesis approach significantly enabling not only the synthesis but also the site-selective modification of proteins at termini with remarkable efficiencies.

Acknowledgements

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TAT-PNA CHIMERIC CONSTRUCTS FOR GENE TRANSFER DETECTION BY IN VIVO IMAGING TECHNIQUES

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Non-invasive imaging of gene expression has promising applications such as identifying disease-related genes, monitoring gene therapy, or detecting gene doping by athletes. Positron-emission and single- photon emission computerized tomographies (PET, SPECT) are the most sensitive techniques in this regard. We report here the preliminary results of a pilot study (IMAGENE) where ectopic gene transfer in mice has been detected by image-based techniques. A key tool in our approach are peptide-PNA constructs (Fig. 1) integrating, in a single molecule, i) a cell-penetrating Tat(48-60) sequence; ii) a PNA sequence complementary to erythropoietin (EPO) RNA, and iii) a Cys residue to which either a fluorophore or an isotopically labeled unit is attached (^{131}I for SPECT, ^{11}C for PET).

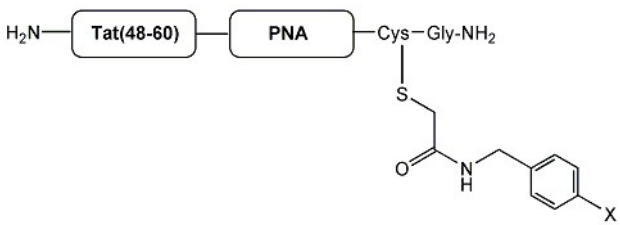

Fig. 1. Tat-PNA chimeric constructs for image-based detection of gene transfer. R refers to either a fluorophore or to an [^{131}I] 4-iodobenzylcarbamoylmethyl group for SPECT.

A search of the murine EPO RNA for minimal length nucleotide sequences with zero homology to the remaining murine genome, aided by structure prediction programs (RNA Fold, Vienna RNA package, etc.), yielded three 14-base sequences with optimal possibilities which, converted into the complementary PNA sequences, were assembled by stepwise solid phase synthesis, followed in tandem by the Tat(48-60) peptide sequence. Boc/Z and Fmoc/*t*Bu chemistries [1] were respectively used for the PNA and peptide sections of the hybrids, which were satisfactorily characterized by reverse phase HPLC and MALDI-TOF mass spectrometry. Melting curves with complementary DNA oligonucleotides gave T_m >80ºC in all cases.

In order to evaluate the cellular uptake of the Tat-PNA constructs, C2C12 muscle-derived mouse cells were transfected with the pCMVmEpo expression vector and, once EPO production was confirmed by ELISA in cell culture supernatants, cells were incubated with fluorescein-labelled versions of the Tat-PNA hybrids. At 5 µM concentration, uptake levels nearing 100% were observed by fluorescence microscopy for one of the hybrids (Fig. 2). Concomitant with this finding, a >50% inhibition of EPO expression by the cells was observed at the same PNA concentration. This Tat-PNA hybrid was selected for further *in vivo* studies.

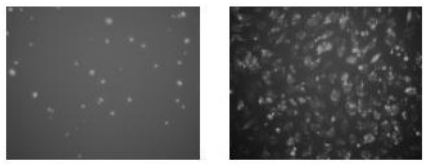

Fig. 2. Cell uptake of Tat-PNA construct by EPO-expressing C2C12 mouse muscle cells. Left panel: PNA sequence alone, 5 µM; right panel: Tat-PNA hybrid, 5 µM.

Having satisfactory confirmed the cellular uptake of the Tat-PNA constructs, we next investigated the possibility of using the constructs to monitor ectopic gene transfer. To this end, the EPO gene was transferred to the right hind limb of BalbC mice by electroporation with 50 µg of the corresponding plasmid DNA. Successful transfer of the EPO gene was verified by ELISA monitoring of serum EPO levels. In parallel, EPO expression was directly confirmed by hematocrit measurement. Next, the Tat-PNA constructs, labeled with 10 - 12 MBq of ^{131}I at the 4-iodobenzylcarbamoyl)methyl moiety, were injected in the tail vein of the mice and real-time imaging of the animals by SPECT was initiated. Differences in radioactivity between left and right hind limbs of the transfected mice within 30-60 min after Tat-PNA administration were measured in a Siemens Orbiter 750 gammacamera, and used to define a standard uptake ratio [=(right – left) / left]. A value of 0.5 was found for mice injected with the Tat-PNA construct, while for control animals the uptake ratio was predictably around zero.

Taken together, the above results confirm that EPO antisense PNAs integrated into suitable tandem constructions with the Tat(48-60) sequence can be successfully incorporated *in vivo*, and that they cause a partial antisense effect on EPO expression, thus demonstrating PNA penetration and hybridization with mRNA. Furthermore, the pilot experiment in gene-transferred mice indicates that some differential detectability is possible also *in vivo*. Further refinement of this methodology is expected to provide higher sensitivity and resolution, eventually allowing external diagnosis of gene therapy in different contexts, including the control of gene doping.

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SYNTHESIS OF OCTREOTIDE ACCORDING TO DIORASSP®

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Introduction

Three years ago Diosynth presented its newly developed method for the large-scale manufacturing of peptides in solution, called DioRaSSP – Diosynth Rapid Solution Synthesis of Peptides [1, 2]. This method combines the advantages of the homogeneous character of classical solution-phase synthesis with the generic character inherent to the solid-phase approach. Processes according to this highly efficient synthesis method consist of repetitive cycles of coupling and deprotection in a permanent organic phase and are further characterized by the fact that intermediates are not isolated. The processes are easy to scale up and yield products of reproducible high purity, which is guaranteed by a new quenching method for residual activated compounds, applying an anion-forming amine such as a β-alanine ester. The inherent scalability of DioRaSSP implies the same process and impurity profile throughout all stages of development, that is, from the first laboratory sample to production batches, combined with intrinsically short process times.

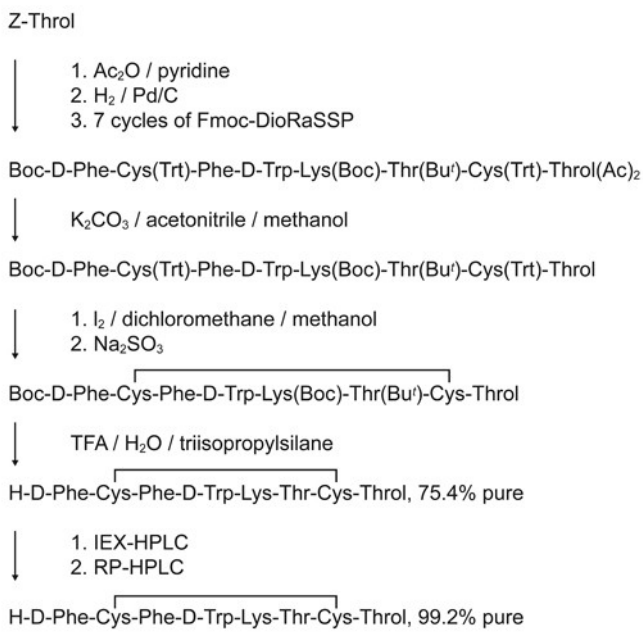
DioRaSSP is essentially independent of the applied protecting scheme, allowing the application of relatively cheap, commercially available amino acid derivatives, and offering maximum flexibility with the applied (mild) chemistry, depending on the sequence of the actual peptide. In Z-DioRaSSP, the benzyloxycarbonyl (Z) function is applied for temporary amino protection. This function is removed by hydrogenolysis in each cycle of the process, using formate as the preferred hydrogen donor. The application of the 9-fluorenylmethyloxycarbonyl (Fmoc) function for temporary amino protection enables the incorporation of sulfur-containing residues. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) is used to effect fast cleavage of this function in ethyl acetate, while morpholine is added to scavenge the arising alkene.

Results

A novel synthesis route towards Octreotide was recently developed at our laboratories applying Fmoc-DioRaSSP. The synthesis started from commercially available Z-threoninol (Throl). This compound was first acetylated using acetic anhydride in pyridine in order to impede acylation of the alcohol functions during the further assembly of the sequence, and to ensure anchoring of the growing peptide in the organic phase. Upon hydrogenation, all subsequent amino acid derivatives were coupled as the N^{α}-Fmoc protected derivatives with the exception of the N-terminal D-Phe, yielding the protected precursor Boc-D-Phe-Cys(Trt)-Phe-D-Trp-Lys(Boc)-Thr(But)-Cys(Trt)-Throl$(Ac)_2$. Next, the acetyl functions were

removed through mild saponification with K_2CO_3, during which no elimination was observed. Subsequent disulfide bond formation took place by iodolysis in a mixture of dichloromethane and methanol. Finally, acidolysis led to crude Octreotide of 75.4 a/a% purity in an overall yield of 56% based on starting Z-threoninol. The crude product was purified using a combination of ion-exchange and reversed-phase HPLC to a purity of 99.2 a/a%.

DioRaSSP has already proven its advantages in the preparation of linear peptides of up to 20 residues in terms of time-to-market, manufacturing efficiency, quality assurance and the environment. Based on the positive outcome of the topical synthesis of Octreotide, it may be concluded that DioRaSSP is also the method of choice for the efficient manufacturing of disulfide-containing peptides.



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STRUCTURE ACTIVITY STUDIES ON HIGHLY POTENT VASOPRESSIN HYPOTENSIVE PEPTIDE

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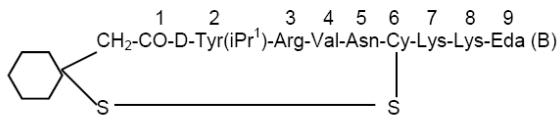
Introduction

Besides its well known antidiuretic, vasopressor, ACTH releasing and uterine contracting effects, mediated respectively by the V_2, V_{1a}, V_{1b} and OT (oxytocin) receptors, vasopressin (VP) also causes vasodilation (for references see 1). While studying the effects of a broad series of position three changes in a non-selective VP antagonist, we serendipidously discovered the first analog of VP, which exhibits selective hypotensive activity. This peptide, d$(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP (A) (Table 1) is devoid of VP antagonistic activities and of the characteristic agonistic activities of VP [1]. Peptide (A) exhibits a hypotensive potency, measured as its vasodepressor effective dose (ED) = 4.66 µg/100g. (For definition of ED see Footnotec Table 1). We recently reported that the D-Tyr(iPr)^2,Lys^7,Lys^8,Eda^9 (ethylenediamine) analog of (A), d$(CH_2)_5$[D-Tyr(iPr)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP B) exhibits an ED = 0.15 µg/100 g; a 30-fold increase in hypotensive activity relative to (A) [2]. (B) has the following structure:



In this study, we investigated the effects of modifications of (B) at positions 3: with Lys and Nar (peptides 1, 2, Table 1), 4 with Abu, Nva, Leu, Nle, Cha, Thr and Har (peptides 3-9, Table 1), 3 and 9; with Nar (Norarginine) and Eda-G (1-amino, 2-guanadinoethane) (peptide 10, Table 1) and 7 and 10 with Arg and Eda retro Tyr (peptide 11, Table 1). The vasodepressor potencies of the resultant 11 analogs of (B) are given in Table 1.

Results and Discussion

Peptides 1-11 (Table 1) were synthesized by the Merrifield solid phase method as previously described [2] and were assayed for agonism and antagonism in VP vasopressor, antidiuretic, oxytocic (in vitro) and VP vasodepressor assays as described [1, 2]. All peptides exhibit negligible agonistic or antagonistic, antidiuretic, vasopressor and oxytocic activities. While none of these new peptides are more potent than peptide (B), nonetheless, all peptides exhibit significant enhancements in vasodepressor potency relative to the original hypotensive peptide

(A) (Table 1). Thus it is clear that peptide (B), tolerates a broad spectrum of structural changes at positions 3, 4, 7 and 10 with retention of hypotensive activity.

Conclusions

We report here a structure-activity relationship investigation on the effects of single modifications at positions 3 and 4, and combined modifications at positions 3 and 9, 7 and 10 of the lead hypotensive peptide d(CH_2)$_5$[DTyr(iPr)^2,Arg^3,Val^4,Lys^7,Eda^9]-LVP (B).

Table 1. Vasodepressor Potencies of Analogues of d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP (B) with single and combined modifications.

No.	Peptide	Vaso-depressor ED[d], μg/100g
A	d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP(A)[a]	4.66
B	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Val^4,Lys^7,Eda^9]LVP[b]	0.15
1	d(CH_2)$_5$[D-Tyr(iPr^i)^2,Nar^3,Val^4,Lys^7,Eda^9]LVP[c]	0.23
2	d(CH_2)$_5$[D-Tyr(iPr)^2,Nar^3,Val^4,Lys^7,Eda^9]LVP[c]	0.30
3	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Cha^4,Lys^7,Eda^9]LVP[c]	0.22
4	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Nle^4,Lys^7,Eda^9]LVP[c]	0.36
5	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Leu^4,Lys^7,Eda^9]LVP[c]	0.45
6	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Abu^4,Lys^7,Eda^9]LVP[c]	0.54
7	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Nva^4,Lys^7,Eda^9]LVP[c]	0.79
8	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Thr^4,Lys^7,Eda^9]LVP[c]	0.78
9	d(CH_2)$_5$[D-Tyr(iPr)^2,Arg^3,Har^4,Lys^7,Eda^9]LVP[c]	1.92
10	d(CH_2)$_5$[D-Tyr(iPr)^2,Nar^3,Val^4,Lys^7,EdaG^9]LVP[c]	0.21
C	d(CH_2)$_5$[DTyr(iPr)^2,Arg^3,Val^4,Lys^7,Eda←Tyr^{10}]LVP[c]	0.14
11	d(CH_2)$_5$[DTyr(iPr)^2,Arg^3,Val^4,Arg^7,Eda←Tyr^{10}]LVP[c]	0.25

[a]Data from reference[2]. [b]This presentation. [c]ED, effective dose (in μg 100g^{-1}i.v.) is the dose that produces a vasodepressor response of 5cm^2 AUC in the 5-min period following injection of test peptide. AUC, area under the vasodepressor response curve.

These studies show that all changes in (B) are well tolerated, leading to peptides which, like the parent peptide (B), exhibit significant enhancement in hypotensive potencies relative to (A). These findings offer promising clues to the design of more potent VP hypotensive agonists, and to the design of radioidinatable and fluorescent ligands as probes of the putative VP vasodilating receptor. Besides being of value as new research tools for studies on the multifaceted roles of VP in the regulation of cardiovascular physiology and pathophysiology [1, 2], these findings may be of benefit for the development of a novel class of antihypertensive agents for therapeutic use.

Acknowledgements

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DESIGN OF POTENT AND SELECTIVE AGONISTS FOR BOTH HUMAN AND RAT VASOPRESSIN V_{1b} RECEPTORS

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Introduction

We recently reported a breakthrough in the search for vasopressin (VP) agonists which are selective for the human V_{1b} (pituitary) receptor with respect to the V_{1a} (vasopressor), V_2 (renal) and OT (oxytocin) receptors [1]. This was accomplished by replacements of the Gln4 residue in [deamino] arginine-vasopressin (dAVP) with Cha^4, Leu^4, Orn^4 and Arg^4. The resulting peptides, d[Cha^4]AVP (**A**), d[Leu^4]AVP (**B**), d[Orn^4]AVP (**C**) and d[Arg^4]AVP (**D**) [1], were subsequently shown to exhibit potent antidiuretic activities in rat bioassays (see Table 1 for antidiuretic activities of (**A**) and (**B**)). They are thus not selective for the rat V_{1b} receptor with respect to the rat V_2 receptor [2]. Peptides **A-D** served as excellent leads to the design of selective agonists for the rat VP V_{1b} receptor [3]. Replacement of the Arg8 residue in **A-D** by Lys, Orn, Dab and Dap led to the first potent and selective agonists for the rat V_{1b} receptor [3]. We report here the human VP receptor affinities of the most promising of these: d[Cha^4,Lys^8]VP (**1**), d[Cha^4,Dab^8]VP (**2**), d[Leu^4,Lys^8]VP (**3**) and d[Leu^4,Dap^8] (**4**).

Result and Discussion

The rat antidiuretic activities and human V_2, V_{1b}, V_{1a} and OT receptor affinities of peptides **1-4** together with those of (**A**), (**B**), AVP and dAVP are given in Table 1. Peptides **1-4** exhibit nanomolar (peptide **1**) and subnanomolar affinities (peptides **2-4**) for the human V_{1b}R. They exhibit very weak affinities for the human V_2R. They also exhibit weak affinities for the hV_{1a}R and hOTR. These four peptides were previously shown to be highly selective agonists for the rat V_{1b}R with respect to the rat V_2R, V_{1a}R and OTR (**3**). We now report that they also exhibit high affinity and selectivity for the human V_{1b}R.

Table 1. Antidiuretic (ADH) activities and binding properties of Lys^8,Orn^8,Dab^8, Dap^8 analogues of (A) and (B) for human VP and OT receptors[a]

			Affinity (K_i) nM)			
No.	Peptide	ADH (U/mg)	$hV_{1b}R$	hV_2R	$hV_{1a}R$	hOTR
	AVP[c]	323	0.68	1.2	1.1	1.7
	dAVP[c]	1745	0.37	5.0	3.8	-
A	d[Cha[4]] AVP[c]	133.6	1.2	750	151	240
1	d[Cha[4], Lys[8]]VP[b]	0.82	2.2	11484	283	141
2	d[Cha[4], Dab[8]]VP[b]	1.04	0.52	7337	176	349
B	d[Leu[4]] AVP[c]	378	0.23	245	44.1	211
3	d[Leu[4], Lys[8]]VP[b]	10.51	0.51	6713	69.3	29
4	d[Leu[4], Dap[8]]VP[b]	0.75	0.60	8730	91.7	83

[a]Bindings assays were performed on plasma membranes from CHO cells stably transfected with the human VP/OT receptors. K^i values are the mean ± SEM of at least three independent experiments each performed in triplicate. [b]This presentation. [c]Data from refs. (1 and 2).

Conclusions

Peptides (**A**) and (**B**), the first selective agonists for the human $V_{1b}R$ [1] were subsequently shown not to be selective for the rat $V_{1b}R$ [2]. By contrast, we show here that peptides (**1-4**), the first selective agonists for the rat $V_{1b}R$ (**3**) are also selective for the human $V_{1b}R$. These are the first ligands which exhibit potent and selective agonism for both the human and the rat $V_{1b}Rs$. Besides their value as probes for studies on the human and rat $V_{1b}Rs$, peptides **1-4** are promising new leads for the design of peptide antagonists for the rat and human V_{1b} receptors and of radiolabelled and fluorescent ligands for the rat and human V_{1b} receptors.

Acknowledgements

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THE FIRST BIVALENT LIGANDS FOR OXYTOCIN AND VASOPRESSIN RECEPTORS

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Introduction

The bivalent ligand approach has been an invaluable tool for the design of potent and selective agonists and antagonists for receptors of a wide variety of biologically active peptides and non-peptides [1 and references therein]. To-date, the bivalent ligand approach has not been utilized in the neurohypophysial peptide design field [2, 3 and references therein]. We now report the syntheses and some preliminary pharmacological properties of the first bivalent ligands for the human (h) oxytocin (OT) and vasopressin (VP) V_{1a} and V_{1b} receptors. Suberic acid, utilized as reported in [4], served as the spacer joining Orn or Lys residues in an OT/V_{1a} antagonist $d(CH_2)_5$[Tyr(Me)$_2$]OVT (A) [5], and two linear VP V_{1a}/OT antagonists, HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2 (B), and HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Lys-NH_2 (C) to give the dimers (1), (2) and (3) Table 1. The peptide monomers (A), (B) and (C) were synthesized by the Merrifield solid phase method as previously described [5, 6]. The suberyl-bis-peptide dimers (1), (2) and (3) were synthesized as described in [4]. The affinity constants of (1), (2) and (3) were obtained by heterologous competition experiments performed on monkey kidney COS7 cells transiently transfected by means of electroporation with human OTR, V_{1a}R and V_{1b}R cDNAs as previously described [7].

Result and Discussion

The affinities of the bivalent ligands (1), (2) and (3) for the human OT, V_{1b} and V_{1a} receptors, together with those previously reported for atosiban resynthesized in our laboratory [2], are presented in Table 1. With affinities for the human OTR of 26.8 nM, 4.03 nM and 4.63 nM, the dimeric peptides 1- 3 clearly have higher affinities for the hOTR than atosiban (K_i = 76.4 nM). Peptides (1) and (2) exhibit lower and equal affinities respectively for the hV_{1a}R than Atosiban.

Conclusions

This preliminary study shows that the dimeric peptides (1) and (2) have higher affinities for the hOTR, lower and equal affinities respectively for the hV_{1a}R than Atosiban. They thus may possess advantages over atosiban as tocolytics for the

Table 1. Human OTR, V_{1a}-R and V_{1b}-R affinities of the dimeric cyclic and linear OT/VP antagonists (1-3) compared with those of Atosiban[a]

		Affinity (K_i)[a] (nM)		
No.	Peptide	hOT-R	hV_{1b}-R	hV_{1a}-R
	Atosiban[b] d[D-Tyr(Et)^2,Thr^4]OVT	76.4[b]		5.1[b]
1	Sub^8-[d$(CH_2)_5$[Tyr(Me)^2,Orn^8]VT]$_2$[c]	26.8	>1000	52
2	Sub^8-[HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys-NH_2]$_2$[c]	4.03	3.6	5.15
3	Sub^9-[HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Lys-NH_2]$_2$[c]	4.63	n.d.[a]	0.40

[a]Competition binding experiments were performed on cell homogenates of transiently transfected COS7 cells. K_i values for the OTR were obtained by means of displacement of [^{3}H]OT. K_i values for the V_{1a}R and V_{1b}R were obtained by means of displacement of [^{3}H]AVP [b]Data from ref. 2. [C]This presentation. [d]n.d. = not determined.

treatment of premature birth. This preliminary study further shows that the bivalent ligand approach [1], utilized here for the first time in the neurohypophysial peptide field, merits further investigation for the design of OT/V_{1a} antagonists [2, 3, 6].

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FUNCTIONAL CRYPTIC PEPTIDES AND THEIR PHYSIOLOGICAL ROLES

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Introduction

Endogenous functional peptides such as hormones or neurotransmitters regulate various tissues and organs. These peptides are produced by the specific cleavages of their precursor proteins that per se have no biological functions. It is widely thought that the kind of peptides having physiological functions is limited and that many peptides present in our bodies are merely metabolites of various proteins and have no physiological functions. Recently, we have isolated and identified novel neutrophil-activating peptides (active at nanomolar concentrations) which are presumably produced by proteolytic cleavages of mitochondrial proteins. And we postulated the possibilities that many of unidentified endogenous peptides produced on various occasions such as inflammation and regeneration of tissues, transmit physiological signals [1, 2]. We therefore named such functional cryptic peptides that are hidden in protein sequences “cryptides” and specifically designated cryptides derived from mitochondrial proteins “mitocryptides” [2]. Among them, mytocryptide (MCT)-1, formerly we designated COSP-1, activated not only neutrophils but also G_i type of G proteins directly [2, 3]. In the present study, we predicted functional cryptic peptides that activate G proteins by analyzing the distribution of charged and hydrophobic residues and examined whether they actually activate neutrophils. Receptors for these peptides were also investigated by the direct cross-linking experiments between peptides and their target proteins.

Result and Discussion

Recently, we identified MCT-1 and -7 which are functional cryptic peptides derived from mitochondrial proteins and which induce migration and activation of neutrophil-like cells [1- 3]. They also activate the G_{i2} type of G proteins directly. These peptides have features, in common, in their distributions of charged and hydrophobic amino acid residues, but homologies in their primary structures were not apparent [3]. In the present study, we predicted functional peptide fragments that activate G proteins in mitochondrial protein sequences utilizing the protein sequence database SWISS-Prot, and the prediction is based on the distribution of charged and hydrophobic amino acid residues. Several peptides such as MCT-3, and -5 whose primary structures were not related to each other were predicted as functional peptides. In fact, these peptides activated G proteins in the membrane fraction of HL-60 cells differentiated into neutrophil-like cells (Fig. 1). They also

stimulated not only chemotaxis of but also β-hexosaminidase release from the differentiated HL-60 cells.

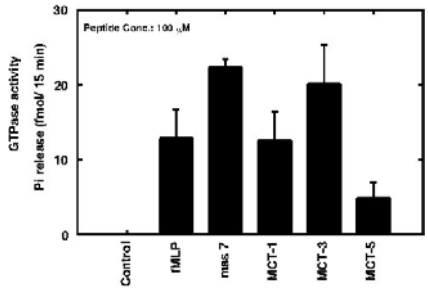

Fig. 1. Effects of various peptides on the activity of G proteins in the membrane fraction of HL-60 cells differentiated into neutrophil-like cells. The activation of G proteins was quantified by the stimulation of GTPase activity.

Since MCT-3 and -5 have been identified as neutrophil-activating peptides, we tried to identify receptors of MCT-3 and MCT-5 in order to characterize the peptides' signaling pathways in neutrophils. Biotinylated MCT-3 and MCT-5 were incubated with the membrane fraction of differentiated HL-60 cells and the bound molecules were purified using streptavidin beads. An 83-kDa protein was found to bind to biotinylated MCT-3, and unlabeled MCT-3 peptide competed with the labeled peptide for the binding to this protein. Biotinylated MCT-5 also bound to the 83-kDa protein and competed not only with its unlabeled peptide but also with the MCT-3 peptide for the binding to the protein, suggesting the possibility that this 83-kDa protein is the receptor molecule for both MCT-3 and MCT-5. Characterization of the 83-kDa protein is now in progress.

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CAPILLARY ELECTROPHORESIS ANALYSIS OF POST-TRANSLATIONAL MODIFICATIONS IN PEPTIDES

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Introduction

The purpose of controlled post-translational modifications (PTMs) is to increase the diversity of functional groups of amino acid residues of proteins. PTMs control ligand recognition, enzymatic activity, subcellular localization or a lifetime of most eukaryotic proteins. Thus, if there would be about 25 000 human genes, there may be hundreds thousands to millions PTM-protein isoforms [1]. Detection, characterization and understanding of these subtle modifications present of formidable challenge for analytical techniques [2]. Identification and understanding of roles of PTM-proteins will be probably more challenging than decoding of the human genome. Because of enormous complexity of cellular protein profile expression, sophisticated methods have to be used for PTMs detection.

Results

Capillary electrophoresis (CE) has become one of the most powerful techniques of modern chemistry. High performance and sensitivity of CE allow for effective separation of complex protein mixtures. Protein separation is usually followed by enzymatic digestion. Following a peptide map is separated and analyzed. A separation efficiency of PTM-peptides may be strongly enhanced by modifying phosphate separation buffer with organic additives (Fig. 1).

Generally, complex mixtures of PTM-peptides are not resolved in typical buffers (like phosphate) used in free zone capillary electrophoresis (FZCE) (Fig. 1A). Modification of such buffers with trifluoroethanol (TFE) significantly increases the separation efficiency. Protein separation is usually followed by enzymatic digestion, and a peptide map is separated and analyzed.

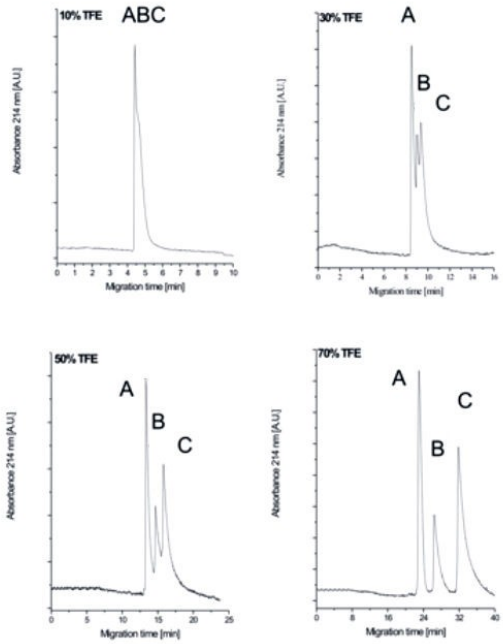

Fig. 1. TFE concentration-dependent CE separation of Tat analogues. A- [Ac-Arg^{52}(Me)2]Tat(52-57)NH2, B- [Arg^{52}(Me)2]Tat(52-57)NH2, C- Tat(52-57)NH2

Conclusions

Using TFE-modified buffer it was possible to separate methylated and acetylated Tat-peptide analogues (Fig. 1). Usually basic peptides or proteins are difficult to characterize. Effective CE separation of PTM-peptides or proteins should allow to detect and characterize a role of PTMs in the living cell. The superposition of modification events in diseased and healthy cells may help to identify diagnostic markers of disease states.

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ENDOSTATIN AS A NOVEL MARKER FOR HCC

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Introduction

The tumor angiogenesis is critical for growth and metastasis of cancers. Endostatin, inhibits proliferation of endothelial cells and induces its apoptosis [1]. Endostatin ,a proteolytic cleavage outcome of collagen XVIII, shows long forms: expressed by the tumor hepatocytes, and short forms expressed by cholangiocarcinoma. The short form of endostatin forms the tumor extra-cellular matrix in primary and metastatic liver cancers [2].

Results

Endostatin was elevated in 2 of 8 patients with GIT cancers with no metastasis , a marker for hepatic metaplasia [3].

Table 1:Distribution of sonographic findings, serum AFP, CEA, Ca19.9, and Endostatin levels .

Parameter	control	GIT/meta	GIT/no met	HCC
sonographgy				
malignant		8	8	20
Normal liver	10	-	-	-
Single lesion		-	8	6
Multiple lesions		8	-	14
AFP				
Median	2.00	475.0	14.0	116.50
5th percentile	0.10	130.0	8.0	12.00
95th percentile	3.00	500.0	20.0	469.00
CEA				
Median	1.0	86.1	3.3	2.1
5th percentile	0.50	78.0	1.2	0.8
95th percentile	3.00	88.0	4.0	87.8
Ca19.9				
Median	7.4	196.0	33.0	43.0
5th percentile	6.5	144.0	13.5	8.3
95th percentile	12.0	225.0	54.0	216.4
Endostatin				
Median	53.0	59.5	107.5	467.2
5th percentile	28.0	33.0	11.3	230.01
95th percentile	145.0	470.0	480.00	1350.0

met.= metastasis

Table 2: P values of different measured parameters using T Test.

Group#Grou	AFP	CEA	Ca19.9	Endostatin
1#2	0.000	0.000	0.000	0.004
1#3	0.000	0.002	0.000	*0.060(non-sig*
1#4	0.004	*0.157(non-sig*	0.003	0.000
2#3	0.000	0.000	0.000	*0.504(non-sig*
2#4	0.001	0.000	0.000	*0.071(non-sig*
3#4	0.014	*0.271(non-sig*	*0.158(non-sig*	0.026

Group1:control; Group 2: GIT cancer with metastasis; Group 3 : GIT cancer without metastasis; Group 4: HCC.

Table 3: Cut off values obtained by Receiver Operating Characteristic curve (ROC), sensitivities and specificities.

Parameter	Group	Cut off point	Sensitivity	Specificity
AFP	GITca+meta.	66.5ng/ml	100%	100%
	GITca no met	5.5 ng/ml	100%	100%
	HCC	7.5ng/ml	100%	100%
CEA	GITca+met	40.5 µg/L	100%	100%
	GIT no met	1.15 µg/L	100%	100%
	HCC	1.15 µg/L	90%	80%
Ca19.9	GITca+met	78.0 U/ml	100%	100%
	GIT ca no met	12.75 U/ml	100%	100%
	HCC	12.5 U/ml	95%	100%
Endostatin	GITca + met	71 ng/ml	87.5%	90%
	GITca no met	52.5 ng/ml	62.5%	50%
	HCC	187.5 ng/ml	100%	100%

Met.= Metastasis

Discussion

CEA and CA19.9 were equally efficient in differentiating between GIT cancer with and without metastasis. AFP differentiates between primary and secondary liver affection by 100% efficiency at cut-off points (66.5 ng/ml for GIT cancer with metastasis, and 7.5 ng/ml for HCC) . Endostatin had a sole value in diagnosing primary HCC . It gives 100% efficiency in diagnosing HCC, detects metastasis, even before sonography.

Conclusions

AFP is done for diagnosis, and for determining primary-or secondary transformation . Endostatin confirms the diagnosis of primary HCC, and detects liver metastasis.

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THE CHALLENGE OF SYNTHESIZING COMPLEX PEPTIDES: THE SYNERGY BETWEEN CHEMMATRIX RESIN® AND PSEUDO-PROLINE BUILDING BLOCKS

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Recent years have marked an increasing demand by academic and pharmaceutical industry research groups for a more efficient methodology for the preparation of complex peptides [1] One of our ongoing projects is focused on the RANTES (1-68) chemokine. The chemokine family comprises large structured small cytokines which up-regulate inflammation processes. RANTES is an important target due to its high anti-HIV activity. Thus, a stepwise synthesis of this chemokine using Fmoc chemistry will speed up research in this field as well as in the preparation of analogues. The methodology described here is based on the simultaneous application of two strategies.

The first strategy, recently developed by our groups, uses an amphiphilic resin that significantly fine tunes the synthetic strategy of complex and aggregating peptides [2] The use of ChemMatrix® resin is particularly suitable for the preparation of hydrophobic, highly structured, and poly-Arg peptides, as compared to polystyrene (PS) resins. In the most remarkable example, the stepwise solid-phase synthesis of the extremely complex β-amyloid (1-42) reached a crude material of 91% purity.

The second strategy is based on the incorporation of pseudoproline (ΨPro) dipeptides into the peptide sequence. The incorporation of ΨPro building blocks into the sequence disrupts the aggregation thought to be responsible for the problems encountered during peptide assembly [3, 4] In the case of RANTES (1-68), by using classical Fmoc peptide synthesis on polystyrene resin, only the fragment RANTES Arg^{44}-Ser^{68} (25 amino acids) was obtained. On the other hand, the synthesis using ΨPro dipeptides progressed more smoothly, but unfortunately only the peptide until Ile24 (45 amino acids) was achieved.

For this kind of complex peptide, the combination of ΨPro dipeptides and ChemMatrix® resin is the strategy of choice. For RANTES (1-68), this synergy rendered the desired peptide. To evaluate the benefits of the ΨPro, two syntheses were performed in parallel. In addition to the incorporation of 4 ΨPro dipeptides in strategic points of the structured peptide, the introduction of only 3 ΨPro dipeptides was also tested. In both cases the desired peptide was obtained, giving an improved crude in the former case, thereby confirming that the approach that combines the full PEG resin, ChemMatrix®, and ΨPro dipeptides disrupts backbone interactions.

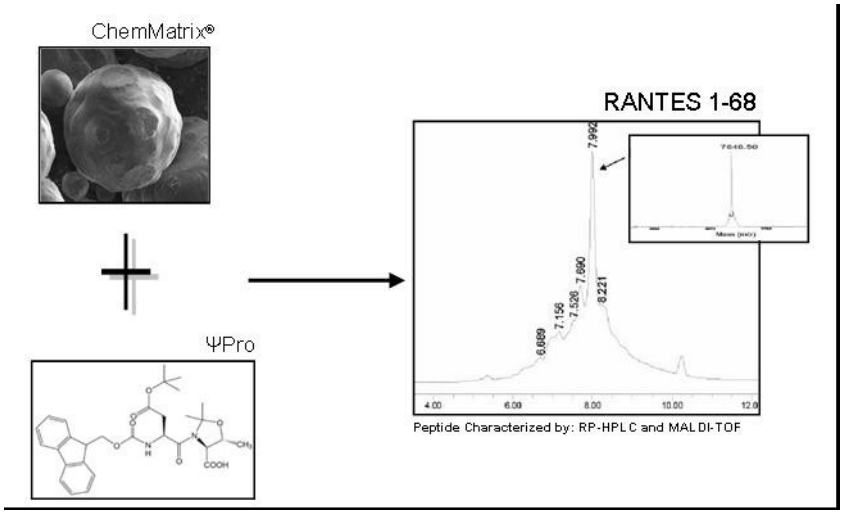

Fig. 1. The combination of the two strategies renders the complex chemokine RANTES (1-68).

In conclusion, an efficient stepwise synthesis of a complex peptide has been successful performed using this combined strategy. We are confident that this methodology will also be applicable for the preparation of biological peptides of interest, thereby contributing to the development of more peptide-based drugs.

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PROTEIN EPITOPE MIMETICS: AN INNOVATIVE APPROACH TO THE DISCOVERY OF SELECTIVE AND HIGHLY POTENT SERINE PROTEASE INHIBITORS

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The Protein Epitope Mimetics (PEM) technology, developed by Polyphor Ltd. in collaboration with Prof. John Robinson at the University of Zurich, provides access to β-hairpin [1] and α-helix mimetics [2] of proteins. PEM are cyclic peptide-like (1 - 2 kDa) β-hairpin-like molecules and modulate protein-protein interactions. We successfully applied PEM for lead finding and optimization where highly selective lead compounds for pharmaceutical development are notoriously difficult to establish, *e.g.* chemokine receptor antagonists [3] and antibiotics. Proteases are important targets pursued by the pharmaceutical industry in a broad range of indications [4]. Here we report the discovery of new selective and reversible serine protease inhibitors. The crystal structure analysis of sunflower trypsin inhibitor (SFTI) revealed a β -hairpin structured backbone cyclic disulfide bridged 14-mer peptide (Fig. 1). The PEM technology was used to produce libraries of trypsin inhibitors using the primary structure of the SFTI. A good starting point for the design of PEM-libraries against cathepsin G and human neutrophil elastase (HNE) are *e.g.* X-ray structures such as: 1CGH, 1AU8 and structure activity data [5]. In particular, residues of SFTI remote from the active site were systematically varied.

Table 1. K_i-values (nM) of 11 serine proteases (n.d. = not determined).

Ki (nM)	POL7010	POL6014	POL9428
Cathepsin G	31	88889	8889
HN Elastase	10000	1	8389
Tryptase	10000	50000	8
Trypsin	8333	83000	4500
Chymotrypsin	10000	100000	10000
Chymase	8333	83000	8333
Thrombin	n.d.	83000	41667
Factor Xa	10000	100000	50000
Plasmin	n.d.	n.d.	1018
Urokinase	n.d.	100000	100000
Kallikrein	n.d.	n.d.	50000

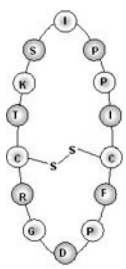

Fig. 1. Structure of SFTI.

All peptides were synthesized by an optimized solid-phase/liquid-phase process and purified by an automated high-throughput LC-MS method. Inhibitory potencies toward the target and additional 9 pharmacologically relevant proteases were measured with the standard chromogenic substrate enzyme assays. Hit compounds were further profiled in human and rat *in vitro* ADME-assays. Various primary hit compounds were optimized in iterative cycles and resulted in our final lead compounds POL7010 and POL6015 (see Table 1 and 2). A weak inhibitor for tryptase was identified from a panel of 110 compounds selected from the existing Cathepsin G and HNE inhibitors. 120 PEM molecules were synthesized in 2 additional optimization rounds within 7 months including syntheses and all biological assays. The resulting compound POL9428 had an excellent selectivity and ADMET profile (Table 1 and 2).

Table 2: Summary of in vitro ADMET- data

In-vitro ADMET Properties		POL7010	POL6014	POL9428
	Inhibitors for:	Cathepsin G	Elastase	Tryptase
Plasma stability (human)	% remaining 4h	93	98	95
Plasma stability (rat)	% remaining 4h	97	93	97
Liver microsomal metabolism (human)	% remaining 1h	99	98	100
Liver microsomal metabolism (rat)	% remaining 1h	100	100	100
Protein binding (human plasma)	(%)	42	53	63
Protein binding (rat plasma)	(%)	29	60	49
Cytotoxicity Hela	GI_{50} (µM)	>50	>50	46
Cytotoxicity Cos-7	GI_{50} (µM)	>50	>50	>50

We have established an integrated discovery platform for protease inhibitors. PEM-Technology enabled us to discover highly potent (K_i values between 1 and 31 nM) and selective (>1:1000) reversible cathepsin G, human neutrophil elastase (HNE) and tryptase inhibitors. Interestingly, important contributions of inhibitor-target interactions distant to the active site could be confirmed by NMR and X-Ray studies (to be published). We were able to fine-tune potency, selectivity and pharmacological properties by integrating our SAR and ADMET-properties data based on over 1`000 PEM molecules with structural data and homology modeling.

PEM molecules showing high stability (> 90% after 4h) in rat and human plasma, liver microsomes and no toxicity in cell lines were selected for *in vivo* analysis. Oral bioavailability in rats was demonstrated with a selected cathepsin G inhibitor. Pharmacokinetic results indicate that PEM protease inhibitors have a sufficiently long-live to be further progressed.

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STUDY ON THE SYNTHESES AND LC/ESI-MSN ANALYSES OF THE GLUTATHIONE CONJUGATES OF BILE ACIDS

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Introduction

In human body, drugs and biologically active substances having a carboxyl group in the molecules have been known to transform into various metabolites. For example, non-steroidal anti-inflammatory drugs (NSAIDs) or bile acids (BAs) such as cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA) are converted into the corresponding active intermediate like a coenzyme A (CoA) thioester prior to the conjugation with amino acids. The acyl-adenylate has been also noted as an intermediate of CoA ligation [1], and react *in vitro* with proteins resulting in acylated proteins [2]. In addition to the protein adducts, drugs such as NSAIDs [3] and clofibric acid [4] have shown to be metabolized to a thioester-linked glutathione conjugate via the acyl-linked metabolites. However, no evidence is so far obtained to confirm such metabolism related to BAs, since there are no suitable standard samples and a reliable method for determination.

Results and Discussion

To study a novel metabolic pathway of BAs via conjugation with GSH, we synthesized GSH conjugates (**1** ~ **5**) of five major BAs in human body as shown in Fig. 1. For the syntheses of these conjugates, we first prepared the protected glutathione derivative by conventional solution method as follows. Troc-Cys(Acm)-OH was coupled with AcOH. H-Gly-OBut by HOSu ester method. After the removal of the Troc group with Zn/AcOH, the amine component thus obtained was coupled with Boc-Glu-OBut by DCC/HOBt method to give fully protected glutathione derivative. The Acm group for the thiol protection was then removed with $(AcO)_2Hg$ in 50% AcOH, and a subsequent treatment with 2-mercaptoethanol to remove Hg^{2+} produced the thiol free peptide. Next, each BA was converted into the corresponding succinimidyl ester, and the thus-obtained active ester was coupled with the thiol free peptide under basic conditions. The Boc and But groups in the product were finally removed to give desirable BAs-GSH, i.e., **1**~ **5**.

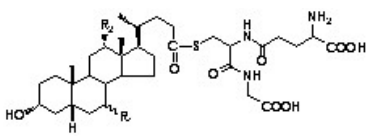

Fig. 1. The structures of synthetic BAs-GSH.

The structures of BAs-GSH were confirmed by ^{1}H NMR and ESI-MS^{2} in positive and negative ion modes. A typical CID spectrum of protonated molecule $[M+H]^{+}$ (**1**) of CA-GSH is shown in Fig. 2. The spectrum shows the dehydrated ion (**A**) at m/z 680.3 as base peak along with five core peaks **B** ~ **F** derived from **1** as a result of the cleavage at positions depicted by dotted line. Further, many dehydrated ions arising from CA part in the fragments **A** ~ **E** were observed.

On the basis of ESI-MS^{2} study, we examined the non-enzymatic formation of GSH conjugates *in vitro* using reactive metabolites of CA as substrate. In the experiment, CA-AMP and/or CA-CoA [4], which are known as reactive intermediates in conjugation with amino acids, were incubated with GSH in the 10 mM phosphate buffer (pH 7.5), respectively. Analysis of the incubation mixture by means of LC/ESI-MS^{n} confirmed the formation of CA-GSH from both intermediates. These results suggest that other BAs-GSH are also produced via BA-AMP or BA-CoA intermediate. The investigations to confirm the biotransformation of BAs into GSH conjugate in liver and the presence in urine and bile of humans are currently being undertaken, and the results will be reported elsewhere.

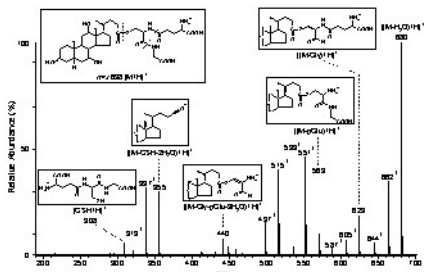

Fig. 2. CID spectrum of $[(CA\text{-}GSH) + H]^{+}$ ion (m/z 698). The asterisked fragments are due to the ions based on dehydration at CA part.

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DESIGN AND SYNTHESIS OF HISTONE DEACETYLASE INHIBITORS CONTAINING THIOETHER MOIETY AS THE FUNCTIONAL GROUP

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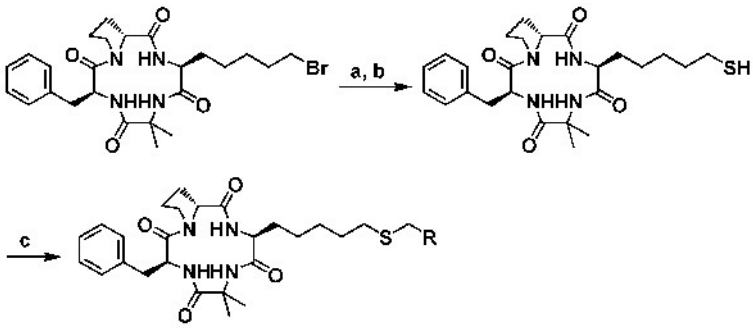
Introduction

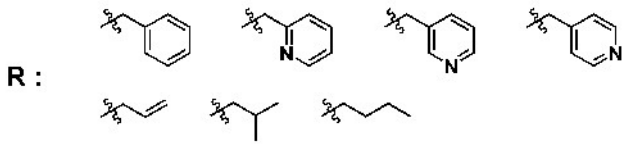
Histone deacetylase (HDAC) has metalloprotease-related mechanism in its catalytic activity. Published structural data for HDAC-like protein, a bacterial enzyme sharing high homology to the HDACs in its active site, confirmed that this protein contains a zinc in the active site [1]. For the discovery of specific HDAC inhibitors, a number of hydroxamic acids and related compounds have been designed based on the ligating function to the zinc atom. The mechanism also involves an appropriate nucleophile in the active site. On the other hand, thiol group has been introduced into inhibitors of various metalloproteases for instance, angiotensin-converting enzyme [2] and thermolysin [3]. Since, the thiol group is generally a useful ligand toward the zinc atom in hydrolases, we introduced it into the reactive amino acid in cyclic tetrapeptide to find out novel HDAC inhibitors [4]. The thiol group was protected as disulfide. The disulfide is assumed to be reduced easily to release mercaptan in the living cells. In the course of research, we newly designed a number of thioethers, in which thiol group is alkylated in variety. In order to figure out the necessary structure of thioether, in which S would not be the strong ligand to zinc atom, we designed and synthesized cyclic tetrapeptide corresponding to chlamydocin. These compounds were profiled by the inhibition of HDAC1, HDAC4, and HDAC6. The p21 promoter assay was also employed to evaluate cell-level effects.

Result and Discussion

In order to Fig. 1 out the necessary structure of thioether, in which S would not be the strong ligand to zinc atom, we designed and synthesized cyclic tetrapeptide corresponding to chlamydocin. The intermediate, cyclo(-L-Ab7-Aib-L-Phe-D-Pro-) (Ab7 is 2-amino-7-bromoheptanoic acid) was synthesized according to the literature [4]. The bromide was converted to the thioacetate and subsequently to mercaptans. The thiol group was reacted with various alkyl bromide and the products containing the thioether were purified by silica gel chromatography (Scheme 1). The products were analyzed by HPLC and HR-FAB MS. Benzyl and 3 isomers of pyridyl methyl thioethers showed potent activities in order of 4-pyridyl > benzyl > 2-pyridyl > 3-pyridyl. This fact suggests that there is a broad space beyond the active site where zinc atom exists. The thioether with medium size alkyl group showed potency between methyl thioether and benzyl thioether.



Reagents : (a) AcSK, DMF; (b) Methylamine, DMF; (c) Br-R, Et_3N, DMF.



Scheme 1. Synthesis of cyclic tetrapeptide containing thioether moiety.

Acknowledgements

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SYNTHESIS AND EVALUATION OF FLUORINATED P-BORONOPHENYLALANINE DERIVATIVES AS BORON CARRIER AND MRI PROBE

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Introduction

Recently, boron neutron capture therapy (BNCT) based on the selective accumulation of compounds with ^{10}B atom in tumor cells and the subsequent irradiation with thermal neutron is highly noted as one of useful techniques for treatment of cancer [1]. So far *p*-(^{10}B)boronophenylalanine (^{10}Bpa) (**1**) and *p*-(^{10}B)boronophenylalaninol (^{10}Bpa-ol) (**2**) have been developed as valuable ^{10}B carriers for BNCT [2]. In addition, the magnetic resonance imaging based on the measurement of ^{19}F atom (^{19}F MRI) is noted as a quite plausible method for diagnosis of cancer [3].

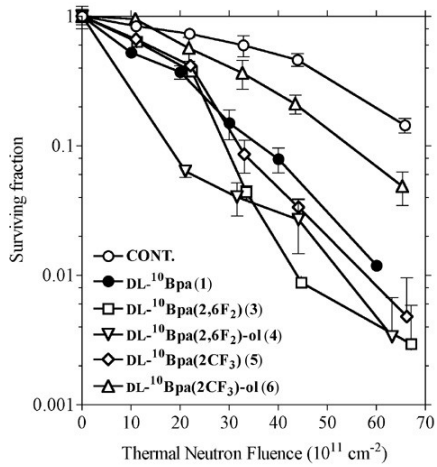
To develop the practical material utilizing not only as the ^{10}B carrier but also as the ^{19}F MRI probe, we synthesized four fluorinated ^{10}Bpa derivatives such as *β*-[4-(^{10}B)borono-2,6-difluorophenyl]alanine [$^{10}Bpa(2,6F_2)$] (**3**), *β*-[4-(^{10}B)borono-2,6-difluorophenyl]alaninol [$^{10}Bpa(2,6F_2)$-ol] (**4**), *β*-[4-(^{10}B)borono-2-trifluoromethylphenyl]alanine [$^{10}Bpa(2CF_3)$] (**5**) and *β*-[4-(^{10}B)borono-2-trifluoromethylphenyl]alanine [$^{10}Bpa(2CF_3)$-ol] (**6**) [4].

In the present paper we focus on the determination of the incorporated amount of fluorinated ^{10}Bpa derivatives **3** ~ **6** into several kinds of cancer cells, and the ^{19}F NMR measurement of these compounds as well.

Results and Discussion

We first examined the tumor cell killing effects of **1** and fluorinated ^{10}Bpa derivatives **3** ~ **6** against C6 cells (rat glioma) by neutron irradiation to estimate the incorporated amount of these compounds into cancer cells. For this purpose, C6 cells incorporating **1** or fluorinated Bpa derivatives **3** ~ **6**, respectively, were irradiated by thermal neutron, and the generated colonies after incubation for 9 days were counted (Fig. 1). As a result, we confirmed that **3** ~ **5** are also usable as the ^{10}B carrier like **1**.

We next examined the detection-sensitivity in the measurement of ^{19}F NMR of these fluorinated ^{10}Bpa derivatives in deuterium saline to elucidate the applicability of compounds **3** ~ **6** to ^{19}F MRI. As shown in Fig. 2, we confirmed that **3** ~ **6** are sensitive enough to detect them incorporated into cancer cells, since intracellular concentration of these compounds can be estimated to be *ca*. 1 ~ 20 mM. In particularly, the derivatives **5** and **6** containing the CF_3 group seem to be quite plausible candidates as the ^{19}F MRI probe.



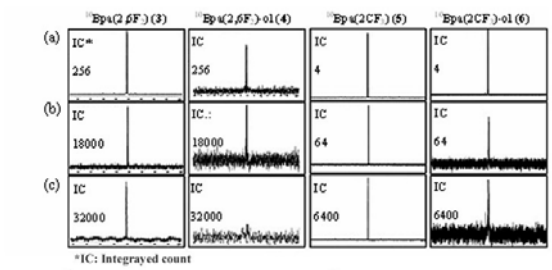


Fig. 2. ^{19}F NMR measurement of fluorinated ^{10}Bpa derivatives. (a): 2.4 mM, (b) 0.24 mM, (c) 0.024 mM.

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ASSEMBLING OF THE EXTRACELLULAR CRF RECEPTOR DOMAINS BY MEANS OF CHEMICAL AND EMZYMATIC LIGATIONS

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Introduction

Ligand binding is the initial step of stimulation for G protein-coupled receptors (GPCRs), transmitting signals from the extracellular environment to the interior of the cell. Understanding how ligands bind to GPCRs at the molecular level is hampered by a lack of appropriate proteins for high resolution structural analysis, and the search for new ligands is limited by the need of cellular systems which exhibit appropriate receptors. Extracellular receptor domains (ECDs) play often a major role for ligand binding and can be, therefore, interesting tools to screening potential ligands and obtain first structural information on ligand binding mechanisms. Whilst single ECDs are easily available, chemically or by expression in *E. coli*, a construct of the four ECDs of a 7tm-GPCR has not been achieved yet. Here, we report on the first semi-synthesis of such a 23 kD-construct, consisting of ECD1-4 of the 7tm-GPCR Corticotropin-Releasing Factor receptor [1].

Results

The assembly was accomplished by the combination of protein expression in *E. coli* (ECD1), peptide synthesis (ECD 2, 3, 4), and chemical (loops to template) as well as enzymatic (3-loop construct to ECD1) ligations. At first, the receptor loops have been prepared as the corresponding linear peptides, bearing C-terminally an additional glycine and N-terminally cysteinyl-glycine, using standard Fmoc-chemistry on a Cl-trityl resin. After cleavage from resin with acetic acid/ TFE/ DCM and repeated lyophilization, the protected peptide acids were activated in the presence of *p*-acetamidothiophenol to form the corresponding thiol esters, which were subsequently deprotected with TFA and finally purified by HPLC [1]. The purified, linear peptide thiol esters gave by treatment in aqueous solution at pH 8.5 the corresponding cyclic peptides by native chemical ligation (NCL), which were ready for chemical ligation to a peptide template bearing maleimido groups via their single thiol groups at the cysteine used for NCL. The stepwise incorporation of the three loops was accomplished by the subsequent incorporation of maleimido-hexanoic acid residues into the peptide template that contained differently protected lysine residues which were selectively deprotected.

The peptide template contained a N-terminal H-$(Gly)_3$-motif for the sortase A-mediated ligation [2] to the receptor N-terminus which was prepared by means of

expression in E. coli and folding forming a characteristic disulfide pattern [3]. For the ligation the receptor N-terminus was C-terminally elongated by the recognition motif of sortase A (LPKTG-RR). The sortase-mediated ligation of the 3-loop construct (10 kD) and the receptor N-terminus (13 kD) led to the desired product (23 kD) consisting of all four extracellular receptor domains, which showed, in contrast to the soluble receptor N-terminus which exhibits a high-affinity binding only to the natural ligand urocortin I, a significant affinity also for the natural ligand sauvagine.

Discussion

The preparation of complex, biologically interesting proteins as tools for structural investigation or ligand screening requires significant methodological improvements. One of the improvements may consist in the combination of different techniques, such as the expression of specifically modified proteins with an enzyme-mediated ligation step to couple an already complex protein having 3 disulfide bridges to another complex protein chemically prepared by means of chemical ligation techniques. With the preparation of a protein consisting of all ectodomains of a G protein-coupled receptor we demonstrated a route to obtain such artificial receptor constructs, which shows a remarkable affnity for natural ligands.

Acknowledgements

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PHOSPHINO PEPTIDES AS INHIBITORS OF HUMAN β-SECRETASE (BACE1)

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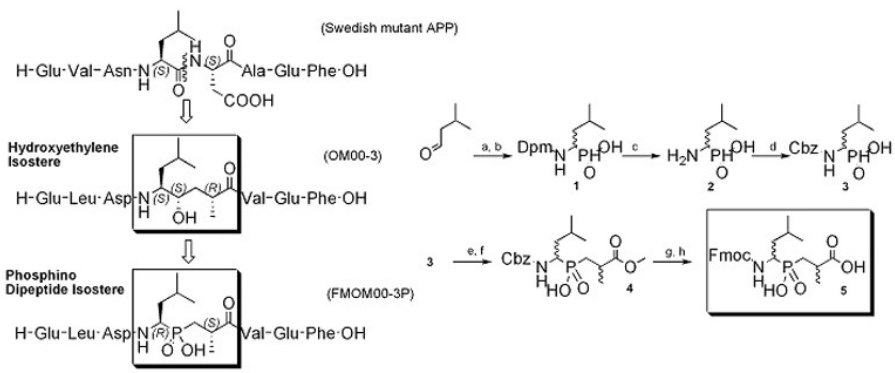
Introduction

The octapeptide OM00-3 [1] containing a hydroxyethylene (HE) isostere is an inhibitor of BACE1 based on the sequence of Swedish mutant β-amyloid precursor protein (APP). Since BACE1, a unique member of the pepsin family of aspartyl proteases, initiates the pathogenic processing of APP by cleaving at the N-terminus, it is a molecular target for therapeutic intervention in Alzheimer's disease (AD) [2]. The phosphinic acid moiety is an excellent mimic of the tetrahedral transition state of amide bond hydrolysis. Therefore we used the sequence of inhibitor OM00-3 and replaced the HE isostere by a phosphino dipeptide (PDP) isostere to generate a phosphino peptide (PP) (FMOM00-3P) as inhibitor of BACE1 (Fig. 1).



Reagents and conditions: (a) 1 eq. diphenylmethylamine, reflux, 12 h (toluene); (b) 1 eq. H_3PO_2, reflux, 1h (ethanol), 25% (2 steps); (c) 47% aq. HBr, reflux, 2h, 57%; (d) 1.1 eq. Cbz-Cl, 1N $NaHCO_3$/dioxane, 73%; (e) 5 eq. HMDS, 100-110°C, 2h (Ar); (f) 60°C, 1.25 eq. methacrylacidmethylester, 85-90°C, 3h (Ar), 91% (2 steps); (g) 57% aq. HI, 2h; (h) 1.2 eq. FmocCl, 0°C, 12h, 40% Na_2CO_3/dioxane, 74% (2 steps)

Fig. 1. Reaction scheme for the synthesis of Fmoc-LeuΨ[POOH-CH_2]-Ala-OH **5.**

Results and Discussion

Our strategy to obtain Fmoc-LeuΨ[POOH-CH_2]-Ala-OH (**5**) (Fig. 1) as key fragment for peptide synthesis of FMOM00-3P starts with the synthesis of phosphinic acid (**2**) as described by Baylis *et al.* [3]. Usually PDP isosteres are synthesized according to Boyd *et al*. [4]. Full deprotection of **4** could be achieved by treatment with 50% HI solution. After synthesis of key fragment (**5**) the amino

acid sequence H-Val-Glu(O*t*Bu)-Phe-OH was built up on trityl chloride polystyrene resin (TCP) using standard Fmoc solid-phase conditions. Compound (**5**) was dissolved in DCM and coupled by activation with PyBOP® in the presence of DIPEA. The amino acids sequence was completed and the PP was cleaved and purified by semi-preparative reversed phase HPLC. Unfortunately only 3 baseline separated fractions FMOM00-3P (a), FMOM00-3P (b), FMOM00-3P (c) could be isolated instead of **4** diastereomers. Analytical HPLC-MS showed that FMOM00-3P (b) consisted of 2 diastereomers while the others were optical pure compounds.

The inhibitory effects of the PP against BACE1 were examined by an *in vitro* assay. The fraction containing 2 diastereomers had an activity of 675 nM. While the 2 pure diastereomers had activities of 12 nM for FMOM00-3P (a) and 2.0 μM for FMOM00-3 P (c). FMOM00-3 P (a) is therefore the most potent inhibitor of the 4 diastereomers.

Compared to the published activity of 0.3 nM [1], OM00-3 has under these assay conditions an activity of 6 nM. Therefore by using a PDP isostere we lose only a factor of two in activity. This is still highly active compared to other isosteres, and gives good perspectives for a further optimization of the amino acid sequence for PDP isosters as well as for the development of new peptide mimetica using PDP isostere as central motive.

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AN EVALUATION OF PEG-BASED SOLID SUPPORTS FOR THE SYNTHESIS OF DIFFICULT AND/OR LARGE PEPTIDES

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The polymer support plays an often decisive role in the outcome of solid phase synthesis [1]. Among the different types of supports available, polyethyleneglycol (PEG)-based materials are finding increasing application for peptide synthesis. In particular, demonstrable advantages over classic polystyrene (PS) resins have been reported for amphiphilic resins such as PEG-PS [1, 2]. More recently, cross-linked resins entirely based on PEG, such ChemMatrix®, have been introduced. We have run a comparative study of their performance using as test models several sequences with known synthetic difficulties when made on PS or PEG-PS supports.

Our first instance has been a 15-residue cecropin-melittin antibiotic peptide which even under "optimized" Fmoc synthesis conditions (Rink amide PS, HBTU, ABI 433A synthesizer) leads to unexpectedly complex crude materials. The main HPLC peak, corresponding to the target sequence, typically amounts to barely 50% of the final product. In contrast, the same peptide assembled on Rink amide-functionalized ChemMatrix® resin gives a practically homogeneous (>93%) product.

Secondly, a 28-residue sequence partially reproducing the HIV-1 gp41 ectodomain [3] gives an intractable product when made on PS; on PEG-PS, with several recoupling steps, a complex crude with <30% of the target material is obtained. In contrast, the ChemMatrix®-based synthesis using single couplings leads to a very clean (>85%) crude from which the target peptide is easily purified (Fig. 1).

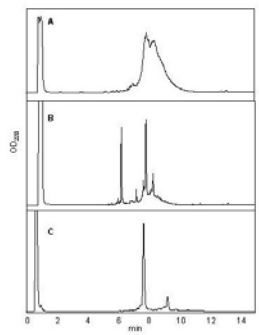

Fig. 1. Effect of the solid support on the quality of HIV-1 gp41 ectodomain peptide. Fmoc synthesis on A: Rink amide-PS; B: Rink amide-PEG-PS with nine (out of 28) recoupling steps; C: Rink-amide ChemMatrix®, single couplings throughout.

Our next trial involved two 21-residue peptides, each with an N-terminal Arg_{11} extension. Synthetic difficulties in sequences with consecutive Arg residues are well-known and may be attributed to steric conflicts among bulky Pbf side chain protecting groups, or perhaps to the cumulative effect of detrimental δ-lactam fomation. In our hands, however, the synthesis on ChemMatrix® yielded crude products of remarkable quality (>85%), by-products consisting mainly on Pbf adducts that could be minimized by longer cleavage times.

The touchstone of our study were CCL4L2 and CCL4L, two 64- and 69-residue cytokines [4]. The 64-mer was readily assembled on ChemMatrix® to give a crude of very good quality from which bioactive material was obtained after purification. A parallel synthesis on PS was abandoned after 35 cycles in view of the very poor quality of the product thus far assembled. Interestingly, with a mere 5 extra residues, the synthetic outcome for CCL4L was totally different, even on the ChemMatrix® resin, and made additional refinements necessary. Use of a Ser(*t*Bu)-Ser($^{\Psi Me,Me}$pro) dipeptide unit turned out to be crucial for the success of the synthesis.

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THE EFFECTS OF A SYNTHETIC PEPTIDE WITH ESTROGEN-LIKE ACTIVITY ON BONE DEVELOPMENT IN MICE

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Introduction

Estrogen is a key regulator of skeletal mass. However the use of estrogen or selective estrogen receptor (ER) modulators in treatment of osteoporosis is limited due to substantial risks for breast cancer.

Recently, we developed peptides with estrogen-like activity as potential estrogen-based new drugs [1,2]. Peptide EMP-1 (Estrogen Mimetic Peptide-1; VSWFFE), inhibits the binding of ^{3}H-estradiol to ERα and ERβ with low potency (IC_{50} = 100 μM) and possesses estrogen-like activity *in vitro* and in-vivo [2]. The present study was undertaken to test potential estrogen-like activities of EMP-1 in bone, in the presence and absence of estrogen.

Results

EMP-1 (VSWFFE) was prepared by conventional solid-phase peptide synthesis and purified by RP-HPLC (96% purity). Whole femora, including cartilage-growth plates were analyzed by micro-computed tomography (μCT).

Administering EMP-1 daily, 5-50 μg/day for 11 weeks to intact sexually mature female SJL mice did not affect the femoral dimensions or trabecular density and architecture remain unchanged. By contrast, administration of 50 μg/day EMP-1 for 10 weeks to ovariectomized (OVX) animals significantly affected their longitudinal and radial growth rates. Whereas OVX animals injected with vehicle (10W/OVX/VEH) and Sham-OVX controls (10W/Sham) exhibited normal femoral elongation, the longitudinal growth of the EMP-1 treated animals (10W/OVX/EMP-1) was restrained by approximately 40% with reference to either 1W/Sham or 1W/OVX mice (Fig. 1). The femoral-diaphyseal diameter in the peptide treated group was also smaller compared to non treated animals (data not shown). Actually, EMP-1 completely arrested the femoral growth.

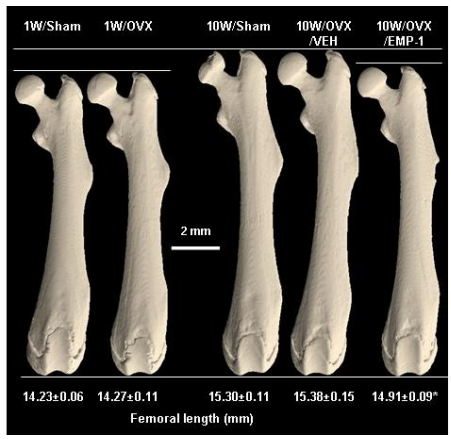

*Fig. 1: Representative pictures of femur (median length) of each group in the OVX animals experiment and length of each group (mean ± SE). Two reference groups are animals one week after sham operation (1W/Sham) and one week after ovariectomy (1W/OVX). *P<0.05 vs. 10W/OVX/VEH.*

Because a significant effect of EMP-1 was on femoral elongation, and since longitudinal growth is primarily a function of growth plate activity, we carried out a μCT analysis of the distal femoral growth-plate. Indeed, in the EMP-1 treated animals the non-calcified part of the plate, as well as the zone of provisional calcification, were thinner as compared to non-treated animals.

Discussion

Peptide EMP-1 had a dramatic effect on the femoral bone and growth plate architecture in OVX mice. These changes included overall reduction of longitudinal (by 40%) and radial growth rates, reduction of growth plate thickness and thickness of the zone of provisional calcification. EMP-1 had no effect on the trabecular bone. Longitudinal bone growth occurs through enchodral bone formation and cartilage matrix mineraliztion. Growth-plate chondrocytes express both ERα and ERβ, receptors that are targeted by EMP-1. In OVX animals, activation of these receptors by estrogen leads to enhanced growth plate fusion, which results in the inhibition of longitudinal growth [3]. Hence, EMP-1 inhibited femoral elongation by activation of ERs in growth plate chondrocytes, similarly to estrogen. EMP-1 may be used for development of drugs to treat skeletal overgrowth diseases, such as acromegaly and Sotos Syndrome. In addition, EMP-1 can be used as research tool to study growth mechanisms in skeletal tissues.

Acknowledgements

We are grateful to Aviva Kapitkovsky and Sara Rubinraut (Weizmann Institute of Science) for assistance in peptide synthesis, to Batia Gayer and to Tikva Kulik for technical assistance, and to Yeda (Weizmann Institute of Science) for financial support.

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ELECTRON TRANSFER TO FLAT PEPTIDES

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Introduction

We already reported the synthesis and characterization of a set of Nα-para-bromobenzoylated α,β-didehydroalanine (ΔAla) homo-peptide esters to the hexamer level [1]. They were found to adopt the fully-extended (2.0_5-helix) conformation. In the ΔAla homo-peptides this novel peptide structure is stabilized by N_i-H…O_i=C'$_i$ and C^{β}_{i+1}-H…O_i=C'$_i$ intramolecular H-bonds. More recently, we described the synthesis and chemical characterization of N^{α}-*para*-cyanobenzoylated (*p*CN-Bz) ΔAla homo-peptide esters from monomer to pentamer [2], more suitable for an in-depth investigation of the mechanism of electron transfer across peptide bridges [3].

Result and Discussion

In the present work we have first examined the 3D-structural propensities of the *p*CNBz-(ΔAla)$_n$-OMe homo-peptides by use of X-ray diffraction (Fig. 1), FT-IR absorption (Fig. 2A), and NMR (Fig. 2B).

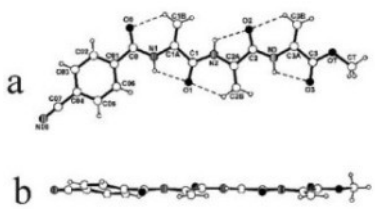

Fig. 1. Two orthogonal views (a and b) of the X-ray diffraction structure of pCNBz-(ΔAla)$_3$-OMe.

All oligomers are found in a completely flat, highly intramolecularly H-bonded, fully-extended (multiple C_5) conformation.. The position of the IR absorption maximum (3380 cm^{-1}) for an internal ΔAla residue and the extremely limited sensitivity of the NH proton chemical shifts to increasing temperature confirm the conformational conclusions extracted from the X-ray diffraction analysis.

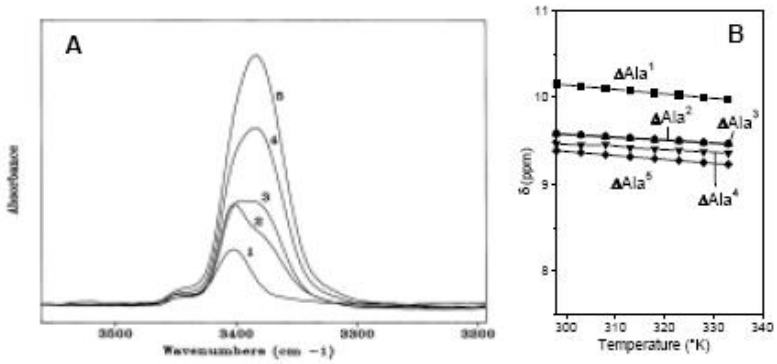

Fig. 2. (A) FT-IR absorption spectra in the N-H stretching region of the homo-oligomers pCNBz-(ΔAla)$_n$-OMe (n = 1-5) in $CDCl_3$ solution. (B) Plot of the NH proton chemical shifts of pCNBz-(ΔAla)$_5$-OMe in DMSO,d_6 solution as a function of heating.

A major outcome of our subsequent cyclic voltammetry study is that the initial one-electron uptake by all ΔAla homo-oligomers occurs at the same potential. This value is significantly less negative than that measured for the $(Aib)_n$ peptide spacers [1]. We believe this is due to a different delocalization of the LUMO (and SOMO). While for the $(Aib)_n$ spacers the LUMO is localized on the redox moiety, for the $(\Delta Ala)_n$ peptides the LUMO expands onto the diffuse π-system offered by the N-terminal residue. Interestingly, the reduction potential of the *p*CNBz-NH- moiety is unaffected by peptide main-chain lengthening. We conclude that the peptide bridge does not include a stabilization of the LUMO of the donor through a cooperative increase of the strength of the intramolecular H-bonds, as previously observed for the $(Aib)_n$ spacers. In agreement with the electrochemistry data, the results of our theoretical study show that the SOMO delocalization only involves the C=C bond of the N-terminal ΔAla unit and that the orbital shape and energy are independent of peptide length.

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SHAPE OF DE NOVO DESIGNED PROTEINS IN SOLUTION: SMALL ANGLE X-RAY SCATTERING

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Introduction

De novo design and total chemical synthesis of proteins provides powerful approaches for biological and biophysical studies, which will be important for construction of molecular probes, material science, and nanobioscience. We have previously described helix bundle carboproteins, where carbohydrate templates were used to direct the organization of the attached peptide helices. However, characterization by NMR is made difficult by the structural economy often used in *de novo* design, with several copies of the same peptide sequence used. Furthermore, if *de novo* designed proteins fail to crystallize, options for structural characterizations are limited. SAXS measurements of proteins are fast and straightforward and new powerful methods for data analysis allow shape determination with 1-2 Å resolution [1].

Results

Here we present a comparative SAXS study of four *de novo* designed helix bundles. The synthesis of these helix bundles by oxime ligation of 2-4 copies of amphipatic C-terminal hexadecapeptide aldehydes to tetra-aminooxyacetyl functionalized templates has previously been described [2]. The Table 1. below lists the investigated carboproteins:

Table1. Ac-YEELLKKLEELLKKAG-H; Peptide B: Ac-D-Pal-EELLKKLEELLKKAG-H; Galp-s-s-Galp: disulfide linked Galp dimer; Altp: D-altropyranoside Galp: D-galactopyranoside, cDTE: cyclodithioerythritol; Pal, Pyridylalanine.

	Gal*p*-s-s-Galp	Alt*p*	Gal*p*	cDTE
Peptide A		Carbo 2	Carbo 3	Carbo4
Peptide B	Carbo 1			

The pair distance distribution functions (PDDF) were derived from the scattering data by indirect Fourier transformation. For SAXS, proteins can be regarded as homogenous, which makes the interpretation of the PDDF fairly easy, because it can be viewed as a histogram of the distances within the single particles. As can be seen from Fig. 1, the p-(r) functions of the four carboproteins clearly contain similar features. The maximum peak (i.e. the most populated distance) is found around 19 Å, however, carboprotein 1 has a slightly lower maximum. Also, the maximum distances are found in a narrow range from 65 to 70 Å. The most

pronounced differences are found at intermediate distances form 25 to 50 Å showing that carboproteins 1-4 do have different structures.

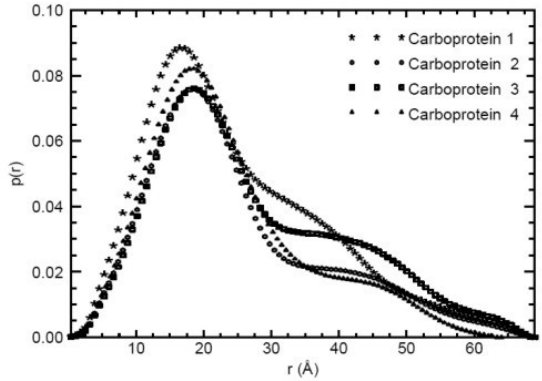

Fig. 1. Pair distance distribution functions (PDDF): p-(r) functions of carboproteins 1-4.

Discussion

Molecular weights calculations from SAXS show that carboproteins 1-3 are monomeric in solution, whereas carboprotein 4 forms a dimer as designed. The *p*-(*r*) functions of carboproteins 2 and 4 were similar in the whole Å-range showing that the 2+2 helix dimer (4) adopts practically the same solution structure as the 4-helix monomer. Thus, this observation points towards that the template does not influence the overall fold of the protein. On the other hand, when one compares carboproteins 2 and 3 it is obvious that the templates, in this case, do impact on the fold. However, this template effect was expected because the D-galactopyranoside template has all hydroxy groups in the same plane whereas D-altropyranoside has one hydroxy group protruding in the opposite plane making it increasingly difficult to align the helixes. Hence, this observation illustrates that protein structural topology can be manipulated through template distance-geometry design. The high values of Dmax found in all carboprotiens exclude the possibility of a true 4-helix bundle. Instead, data is more consistent with 3+1 helix folding, which would suggest that peptide sequences A and B encode 3-helix formation and not 4-helix formation as expected. In line with this interpretation, structure elucidation with ab initio modeling pointed towards 3+1 helix folding.

Acknowledgements

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NANOSTRUCTURES FORMED FROM LIPIDATED OLIGOPEPTIDES IMMOBILIZED ON CELLULOSE AS ARTIFICIAL RECEPTORS

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Introduction

Structures formed from N-lipidated oligopeptides immobilized in the regular pattern on the cellulose surface are capable for selective binding of ligand molecules, thus acting like artificial receptors [1]. Due to the conformational flexibility of lipidated oligopeptide chains, the supramolecular structure is highly flexible, forming the cavities with the shape and prosperities adjusted most effectively to requirements of the guest molecule.

Result and Discussion

The receptor layer was prepared in the stepwise process involving functionalization of cellulose with 1,3,5-triazine derivative followed by reaction with *m*-fenylenediamine, *N*-Fmoc aminoacids, deprotection of N-terminus and completed by bonding of carboxylic acid. Thus, according to the concept, the receptor pockets were formed from the neighboring, identical lipidated aminoacids (lipidated peptides), spaced in the regular pattern as depicted below on Fig. 1.

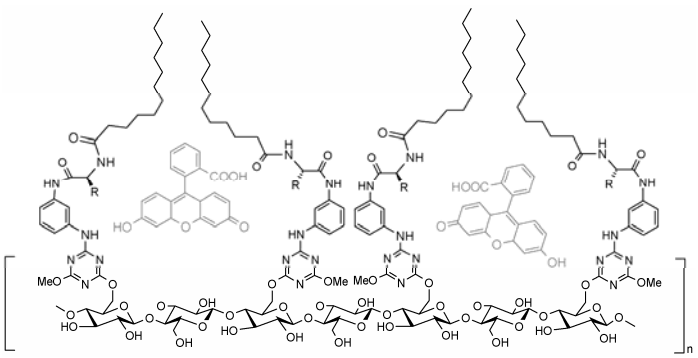

Fig. 1. Structure and proposed mechanism of binding by receptors

In the previous studies we observed that binding of analyte by the receptor depends on the structure of lipopeptide fragment. Moreover, interactions of any colorless analyte with an array were successfully visualized by the processes involving competitive adsorption-desorption of appropriate dye [1].

In order to study the mechanism of molecular recognition an array of receptors was prepared, treated with fluoresceine and fluoresce for excitation at 496 nm has been measured. It has been expected that charge distribution inside the receptor pocket should alter the fluorescence. It has been found that difference in λ_{max}, curvature and intensity of fluorescence depended on the structure of the peptide motif and lipidic fragment of receptor (Fig. 2).

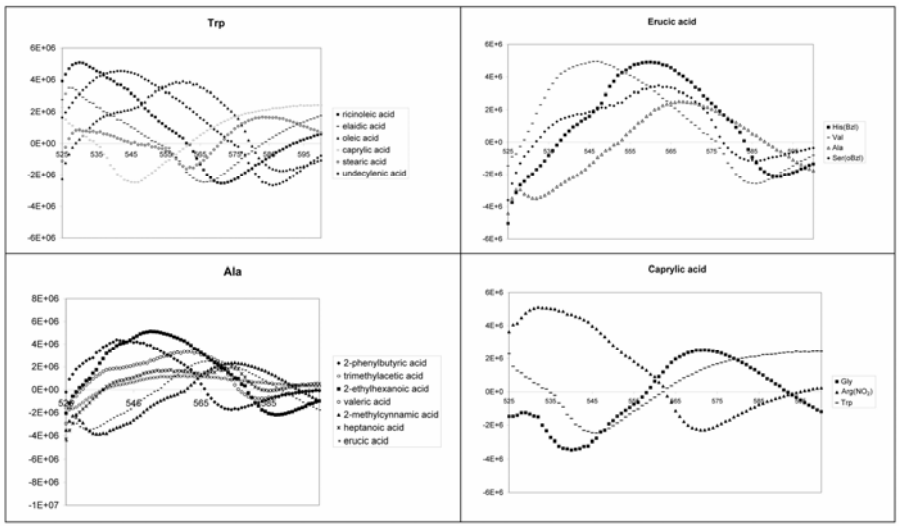

Fig. 2. The influence of amino acid (left side) and lipidic fragments (right side) on fluorescence (presented data of 4 randomly selected sub-arrays).

This is indicative for a dissimilar environment of the fluoresceine molecule and strongly suggest the differences in the polarity, shape and conformation of the receptor pocket.

Acknowledgements

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PREDICTION OF FIBRIL CORE REGIONS IN AMYLOID FORMING PROTEINS

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Introduction

The formations of amyloid fibrils have been reported for various amyloidosis. Up to date several structural models of fibrils are proposed for respective proteins. However, their common basic structures and universal features to induce amyloid fibril formations are not known in detail. Previously, we examined intermolecular interactions among the several amino acid residues in barnase, of which is known to form amyloid-like fibril. Based on the experimental results using a series of mutant barnase, we have succeeded in identifying the essential interactions for amyloid formation. The results indicated that the most essential interaction is the hydrophobic interaction between amino acid residues effectively operating in inter-strand, inter-molecular, and inter-sheet manners, as shown in Fig.1 [1]. In the present paper, we describe a novel prediction method for core regions of various fibril-forming proteins and show the verification of the assumption that the fibril formation is caused by the alignment of hydrophobic residues and hydrogen-bonding side-chains in the direction of fibril axis.

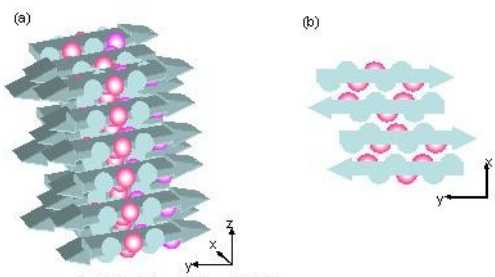

Fig.1. Structual model of amyloid fibrils.
(a) Antiparallel alignment of b-strands forming hydrophobic arrays.
(b) Cross-sectional view of the amyloid fibril.

Result and Discussion

The fibril formation is caused by the alignment of hydrophobic residues in the direction of fibril axis. Our fibril model requires that the paired hydrophobic residues exist in both sides of a β-sheet. At first, we calculated the score quantifying interactions between side-chains in the antiparallel orientation of β-strands. The total score of hydrophobic interactions between the paired β-strands is estimated by the summation of the individual hydrophobic scores, as shown in Fig.2 [2]. Next, the peptides with predicted sequences of fibril cores, which are a couple of high-

scored regions with a designed turn moiety to induce a hairpin-like form, were chemically synthesized. As a result, most of peptides showed enhanced fluorescence intensities of amyloid-sensitive thioflavin T, suggesting the formation of amyloid fibrils. Therefore, our score system was proved to be effective in predicting the formation of amyloid fibrils, as shown in Fig.3.

In addition, we also applied this method to prion protein, we could predict four possible β-strands with hetero-paired orientation. The peptides including predicted sequences were synthesized and examined by electron microscopy. Some synthetic peptides involving these strands were proven to have fibril-forming ability. Thus, we have developed a novel method to predict the core regions that induce amyloid fibrils. The method described in this paper is basically applicable to both antiparallel and parallel β-sheet formation with not only homo but also hetero pairing.

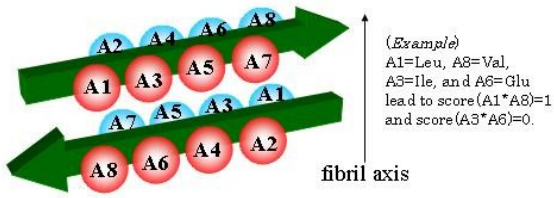

Fig.2. Model of the calculation of fibril-forming propensit

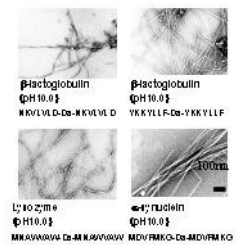

Fig3. Electron microscopic observation of incubated peptides with high-scored sequences

Acknowledgments

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SPECIFIC RNA BINDING OF IMMOBILIZED PNA'S

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Introduction

To study structure and function of various RNAs, *in vitro* RNA transcripts can be employed as the substitutes for the native RNAs in many cases [1]. However, to study RNAs possessing post-transcriptional modifications [2], it is necessary to use native RNAs isolated from living organisms. Previously, a sequence-specific selection method using a solid-phase DNA has been devised [3]. Although this method is easier than traditional methods that are based on column chromatographies, it needs high temperature treatment, which might assist hydrolysis of RNA strand and might impair heat labile modifications. PNA-RNA hybrid has been known to be much more stable than DNA-RNA hybrid [4]. Thus PNA-based RNA purification method seems to be able to improve the DNA-based method. In this study, we attempted to isolate a tRNA and a noncoding RNA by using biotynlated PNAs and streptavidin-beads.

Result and Discussion

Base-protected PNA monomers were purchased from Applied Biosystems. PNAs were manually synthesized by a solid-phase method with an Fmoc N-protecting group as described [5]. Fmoc-NH-SAL-PEG resin (Watanabe Chemicals, Japan) was used as a supporting resin. The N-terminus was attached with a biotin linker (biotin-X, SE, Molecular Probes).

We first attempted to isolate $tRNA^{Leu}_{GAG}$ from *E. coli* tRNA mixture (Sigma-Aldrich). We used a biotinylated PNA (9mer) complementary to 3'-end of the tRNA without the CCA-end. To bind the PNA and the tRNA, the PNA/tRNA mixture was heated at 43-55 ºC for 5min. Then the mixture was mixed with streptavidin agarose (Novagen) at 25 ºC, and elution of the tRNA from the immobilized PNA was performed at 37 ºC. After the purification procedure using the 9-mer PNA, the eluate mainly includes the tRNA having the same length as the $tRNA^{Leu}_{GAG}$ transcript. The heat treatment at 55 ºC led to the best purity of the tRNA, and it was obtained also by the heat treatment at 43 ºC. Damage of the RNA throughout the procedure seems to be ignorable by this method because it takes a short time and does not need high temperature procedure. The tRNA obtained was sequenced by the Donis-Keller's method [6], and it was confirmed that the tRNA was *E. coli* $tRNA^{Leu}_{GAG}$.

We also attempted to isolate a noncoding RNA (*oxyS* RNA) [7], which is induced by oxidative damage in *E. coli*, from *E. coli* RNA mixture. *E. coli* RNA mixture was prepared from *E. coli* pellet using ISOGEN (NIPPON GENE). After the

purification procedure using 10-mer PNA complementary to *oxyS* RNA, an RNA having the same length as *oxyS* RNA was obtained.

In this study we could successfully isolated one of *E. coli* tRNAs and one of *E. coli* noncoding RNAs using biotinylated-PNA. Thus, it is suggested that this method enable us to purify a variety of RNAs without high temperature treatment.

Acknowledgements

We thank C. Kumano, T. Manabe, and M. Arita (Okayama Univ.) for their help.

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STUDIES ON DEPROTECTION OF THE FMOC-GROUP WITH DIFFERENT BASES

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Introduction

The standard reagent for Fmoc-deprotection in solid phase peptide synthesis is 20% piperidine in DMF or NMP. Alternative reagents for piperidine are described in the literature [1-5]. In view to the Fmoc-deprotection reaction, basicity (pK_a–value) is necessary to form the dibenzofulvene (DBF). The nucleophilicity of the reagent is important to build up the Fluorenylmethyl-base adduct (Fm-base adduct). Stronger deprotection reagents like DBU and DBN [5] with a higher pK_a than piperidine are used for difficult Fmoc-cleavage. These class of reagents are not able to react with the dibenzofulvene and are not eliminating the DBF out of the reaction equilibrium. Smoother reagents with a lower pK_a like morpholine are described to avoid side reactions for base sensitive sequences and suppression of aspartimide formation [6].

Due to the fact that piperidine is used for illegal production of psychotropic drugs, the government controls the distribution with additional paperwork.

For this purpose we investigated homologous and analog derivatives of piperidine by kinetic studies.

A comparison of alternative bases was carried out by UV kinetic measurement on Fmoc-amino acid. Conductivity monitoring on a 433A peptide synthesizer was used to create kinetic data under real synthesis conditions. Peptide synthesis was performed with different bases under standard conditions on a 433A.

Results and Discussion

The kinetic results, compared to piperidine as a standard, are showing that the pK_a value of the deprotection base is in correlation with the reaction rate. The CH_2-cyclic analogs are showing reaction rates in the order Pyrrolidine >Piperidine >Hexamethylenimine.

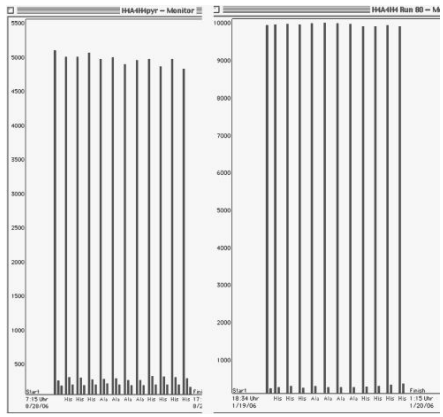
Comparing the Dialkylamines (Ethyl, Propyl-and Butylamine) the reaction rate becomes slower with the length of the alkyl-group. A sterical hindrance is observed with Diisopropylamine, which results in a very slow kinetic. The reaction rate of the Methyl-substituted piperidines is in order: 4-Methyl- > 3-Methyl- > 2-Methyl-piperidine.

Hetero-Analogs, i. e. derivatives of the piperidine with an additional N or O, are reacting in order: Piperazine > 1-Methyl-piperazine > Morpholine. This is reflecting the influence of the pK_a-value of the base. Morpholine with a pK_a of 8.33, the weakest base tested in this study, was reacting very slow compared to the bases

with higher pK_a values. Kinetic data with conductivity monitoring are showing that the deprotection of

Fmoc-Gly-OH and Fmoc-Gly-HMP-resin is completed by cyclic bases in 2 min.

Pyrrolidine and Hexamethylenimine are a good substitutes for piperidine as demonstrated for the synthesis' of the peptide $H_4A_4H_4$ (Fig.1).



A: Pyrrolidine *B: Hexamethylenimine*

Fig. 1. UV- monitoring traces of the synthesis of $H_4A_4H_4$ -peptide using A: Pyrrolidine B: Hexamethylenimine as base. 433A chemistry: UV Fastmoc 0.10, S200

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MOLECULAR DYNAMICS STUDY OF TWO ABL-SH3 DOMAIN PEPTIDES TOWARDS AMYLOID FORMATION

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Introduction

Amyloid formation and deposit is connected with Alzheimer's disease, Parkinson's disease, the type II diabetes, the prion-related diseases and Finnish familial amyloidosis. After protein misfolding short peptide sequences act as "hot spots" providing the driving force for protein aggregation in amyloid fibrils. Previously we have characterized in detail one of these "hot spots" in the diverging turn of PI3-SH3 domain, one of the most extensively studied amyloidogenic proteins not related to any disease [1, 2]. Based on homology search we have identified an aggregation prone region in the same structural element of the related Abl-SH3 domain of *Drosophila* DLSFMKGE (MK) whereas the human homologous region DLSFKKGE (KK) is predicted to be less amyloidogenic. The possible reason for the difference of amyloid formation propensities of the two peptides was investigated by molecular dynamics (MD) of two and ten strand β-sheet structures at 30K, 170K/190K – for two and ten strand β-sheets respectively, and at 300 K.

Results and Discussion

The antiparallel alanine flat β-sheets consisting of two and ten strands were constructed, minimized, and mutated to the sequences DLSFMKGE and DLSFKKGE. All four systems: 1) DLSFMKGE – two strands (2xMK) (Fig. 1. a-d), 2) DLSFKKGE – two strands (2xKK) (Fig. 1. e-h), 3) DLSFMKGE – ten strands (10xMK) (Fig. 1 i-l), 4) DLSFKKGE – ten strands (10xKK) (Fig. 1. m-p), were surrounded by 10 Å layer of water molecules over the solute and subjected to MD, Amber 8.0 force field, NTP protocol. The MD runs were started at the temperature of 10 K and the temperature was elevated stepwise by 10 degrees till 300 K. Longer MD runs were done at 30 K (78 ns), 170 K (140 ns) and 300 K for the two strand systems, and at 30 K (47 ns), 190 K (55 ns) and 300 K for the ten strand systems. The MD results show considerably higher hydrogen bond percentage for DLSFMKGE than that one for DLSFKKGE during the course of the simulation, thus suggesting that DLSFMKGE is a potential fibril-maker, but DLSFKKGE is not. These results might explain why most SH3 domains posses two conserved basic residues at the diverging turn, which my act as gate-keepers to avoid aggregation. The increased stability of the β-sheet formed from the 10xMK system arises because of the interactions between the methionine and the phenylalanine residues of the neighboring strands. Replacement of Met by Lys removes that

stability factor. Two strand β-sheet systems were stable until 170 K. The ten strand β-sheets appears to be more stable. The fact that with the course of the simulation one β-sheet systems both two-strand and ten strand, partly or completely melts at higher temperatures suggests, that to form fibrils they should be stabilized by parallely placed other β-sheets.

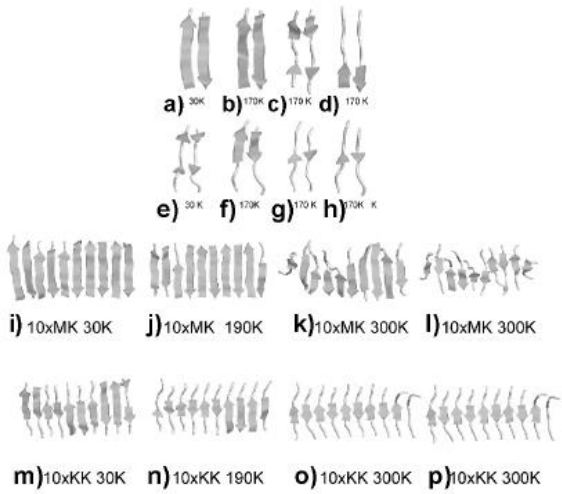

Fig. 1: MD snapshots of DLSFMKGE two strand system : a) at 30K, 23159 ps, b) at 170K, 42359 ps, c) at 170 K, 91467 ps, d) at 170 K, 99126 ps ; MD snapshots of DLSFKKGE two strand system: e) at 30K, 19941 ps, f) at 170K, 42880 ps, g) at 170K, 92658 ps h) at 170K, 98969 ps ; MD snapshots of DLSFMKGE ten strand system: i) at 30 K, 15343 ps, j) at 190 K, 58023 ps, k) at 300K, 58489 ps, l) at 300 K, 64419 ps; MD snapshots of DLSFKKGE ten strand system: m) at 30 K, 15950 ps, n) at 190K, 58588 ps, o) at 300 K, 58466 ps, p) at 300 K, 64038 ps.

Acknowledgments

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STRUCTURAL ANALYSIS OF A PEPTIDE MIMICKING THE BINDING SITE OF HYAP-WW DOMAIN FOR PROLINE-RICH LIGANDS

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Introduction

The binding site for proline-rich ligands of the WW domain of human Yes-associated protein (hYAP-WW) is located within the β2 and β3 strands of a three-stranded antiparallel beta sheet in the C-terminal part of the protein, as demonstrated by structure analysis of a hYAP-WW – ligand complex (Fig. 1) [1]. This part of the molecule contains the primary contact residues (Y28, L30, H32 and W39) for the interaction with the ligand. Together with threonine methyl groups, these residues form a hydrophobic patch on the protein surface. A cyclic peptide covering the hYAP-WW binding site has been previously synthesized and found to mimic the binding of hYAP-WW to a proline-rich ligand [2]. In this study, NMR spectroscopy was used to explore the solution conformation of this cyclic mimetic peptide, in order to gain insight into the structural basis of functional mimicry.

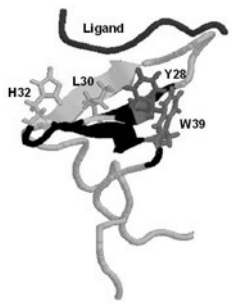

Fig. 1. 3D structure of a complex of hYAP-WW with the peptide ligand GTPPPPYTVG [1]. The fragment shown in black represents the peptide synthesized to mimic the hYAP-WW binding site containing the primary contact residues of hYAP-WW (Y28, L30, H32 and W39).

Result and Discussion

The cyclic peptide mimicking the hYAP-WW binding site (cyclo[succ-RYFLNHIDQTTTWQ-Lys]-NH_2) was structurally characterized using two-dimensional ^{1}H NMR spectroscopy. In addition to the NOEs that were expected based on sequential proximity, such as HN(i+1)-Hα(i), Hα(i+1)-Hα(i), HN(i+1)-HN(i), several long-ranging NOEs between spin systems of sequentially distant amino acids were observed, in particular between the Cα proton of F29 and a Cγ proton of Q40, as well as between the amide proton of N31 and the Cα proton of T37 (Fig. 2).

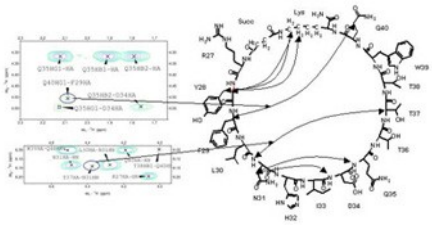

Fig. 2. Long-ranging NOEs in the 2D ^{1}H NMR spectra of cyclo[succ-RYFLNHIDQTTTWQ-Lys]-NH_2). Left: Sections of the NOESY spectrum. Right: Graphical presentation of the cyclic peptide. Residue numbering was kept consistent with hYAP-WW.

These long-ranging NOEs indicate a conformation of cyclo[succ-RYFLNHIDQTTTWQ-Lys]-NH_2), in which W39 and T37, respectively, are in spatial proximity to the other contact residues (Y28, L30 and H32), thus possibly enabling formation of a hydrophobic patch similar to that in the hYAP-WW – ligand complex. 3D structure calculation for the peptide, based on these results, is in progress.

Acknowledgements

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SYNTHESIS OF TRITIATED β-AMYLOID PEPTIDES AND THEIR USE IN BIOLOGICAL STUDIES

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Introduction

β-Amyloid (Aβ) peptides play a crutial role in starting Alzheimer.s disease. The exact molecular mechanism of action of these peptides has not yet been perfectly understood. Aβ peptides may interact with cell membrane proteins and/or intracellular structures, proteins or cell organelles. Application of radiolabelled Aβ peptides in biological experiments may help to answer the question, which interactions are most important in starting neuronal dysfunction and cell death. Proteomical methods could help to identify protein networks interacting with Aβ peptides. Therefore our aims were: a./ The synthesis of tritium labelled Aβ peptides; b ./ In vivo study of the fate of these peptides in the brain (localization); c./ Identification of Aβ binding proteins using the method of proteomics.

Result and Discussion

We present here the synthesis of six Aβ peptides including Aβ[^{3,4}ΔLeu^{34}(31-35)], Aβ[^{3,4}ΔLeu^{34}(25-35)] and Aβ[$^{3,5'}$-diiodoTyr^{10}(1-40)] for tritium labelling [1]. The synthesis of peptides were carried out with Boc- and Fmoc- chemistry using standard manual solid phase methods. Aβ[Ile-Ile-Gly-^{3,4}ΔLeu^{34}-Met] was synthetized by Fmoc-protected amino acids on Sasrin resin, which can be cleaved with 1 % TFA/DCM. Tritium labelling of precursor peptides were carried out by catalytic saturation or dehalogenation method, yielding Aβ[^{3}H-Leu^{34}) and Aβ(^{3}H-Tyr) with specific activity of around 120 and 35 Ci/mmol respectively. The labelled peptides were purified by RP-HPLC.

In *in vivo* experiment: Aβ(31-35), Aβ(25-35) and the tritiated Aβ peptides were incorporated into the motor cortex of the anesthetised cat and the result were investigated by light and electronmicroscope. The motor cortices (area 4) were exposed on both sides and covered with thin filter paper containing the radiolabelled peptide (4x5 mm, 2 μM peptide with 5.6 μCi activity). The cortices were treated in situ for 1 h, dissected and fixed in Bouin (for light microscopic study), and in Karnovsky (for electromicroscopic study) fixatives. The labelled peptides have expressed significant incorporation in all layers of the neocortex after one hour epicortical exposition *(*Fig.1. A and Fig.1. B). The type, number and intensity of labelling in stimulation and control conditions have indicated differential incorporation at cellular and subcellular levels.

Search for Aβ binding proteins [2]: extract of synaptic plasma membrane protein of rat forebrain supernatant was incubated overnight with Aβ(1-42) aggregates for co-

precipitation. Analysis of the co-precipitated proteins was carried out in-gel digestion after electrophoresis. The identification of proteins were by MALDI-TOF MS. Some of the identified cytoplasmic proteins areas as follows: glyceraldehyde-3-phosphate dehydrogenase, tubulin alfa and beta chain, P 130Cas-associated protein, gamma-enolase, elongation factor 1- alpha [1].

The most important results of our experiments as follows: 1./ Aβ(31-35) can enter the neurons and bind to different proteins of cytoplasm and cell organelles 2./ Labelled Aβ peptites can bind to the nucleus, nucleolus and cytoplasm of neurons and glial cells of the brain cortex (each layer). The labelling of neuropil shows postsynaptic localization (about 1/3 of the synapses counted was found labelled.) 3./ Excessive cationic entry, osmotic swelling and hints of apoptotic-like neurodegenerative changes were found (LM and EM studies) after Aβ administration. 4./ Identification of Aβ-binding proteins shows that key enzymes of glycolysis and oxidative phosphorylation can bind Aβ fibrils. Aβ peptides entering the neurons could decrease ATP synthesis and destroy the microtubular system causing cell death.

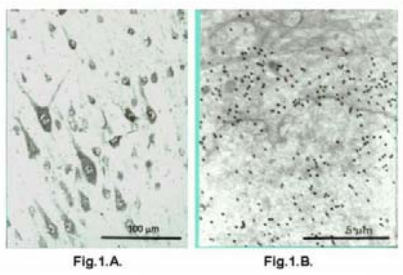

Fig.1. Light microscopic studies (cat cortex, in vivo) using Aβ(25-35) and Aβ(31-35) peptides (A). Electromicroscopic autoradiographic study with Aβ[^{3}H-Leu34(31-35)] pentapeptide (B).

Acknowledgements

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EFFECTS OF HISTONE DEACETYLASE INHIBITORS BASED ON CHLAMYDOCIN TOWARD KK-A^{y} DIABETES MODEL MICE

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Introduction

Histone deacetylases (HDAC) have important roles in the regulation of the cell cycles by the cooperation with histone acetyl transferase. The HDACs involved in class-I and II have metalloprotease-related mechanism in its catalytic activity. These enzymes could be inhibited by small molecules bearing various zinc ligands such as hydroxamic acid [1, 2] and mercaptan [3]. Based on the structure of chlamydocin, which has a cyclic tetrapeptide framework, cyclo(-L-Aoe-Aib-L-Phe-D-Pro-), where Aoe is (*2S,9S*)-2-amino-8-oxo-9,10-epoxydecanoyl, we have developed potent HDAC inhibitors.

In the present study, we examined the chlamydocin hydroxamic acid and SS-hybrid toward the diabetes model mice, KK-A^{y}.

Result and Discussion

Chlamydocin hydroxamic acid (1) exhibited satisfactory effect for the decreases of both blood glucose and blood insulin with the reference of pioglitazone. The SS-hybrid (2) (Fig. 1.), which is expected to be reduced inside of cells to generate the corresponding thiol-containing cyclic tetrapeptide, also showed a significant effect but less than (1).

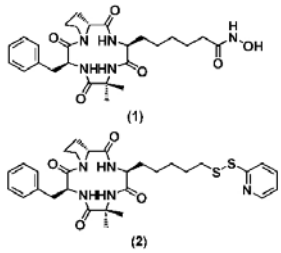

Fig. 1. Chlamydocin hydroxamic acid (1) and SS-hybrid (2).

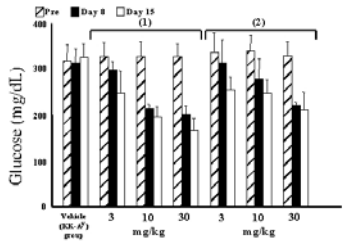

Fig. 2. Effect of HDAC inhibitors on the blood glucose of KK-A^{y} mice.

The effect was dose-dependent from 3 mg/kg to 30 mg/kg (Fig. 2). The effect of HDAC inhibitors were also confirmed by the observation of hyperacetylation of histones by Western blot experiments.

Acknowledgments

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NEW HIGHLY POTENT PEPTIDE BRADYKININ B-1 RECEPTOR ANTAGONISTS

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Introduction

Bradykinin and its homolog kallidin (KD) are endogenous peptides and have two major classes of receptors, B-1 and B-2. B-1 ligands typically lack the C-terminal Arg residue. There is growing evidence that the B-1 receptor, in addition to the B-2 receptor, plays an important role in various pathophysiological states including chronic inflammation, pain, trauma and cancer. Therefore, there is current interest in development of highly potent bradykinin B-1 receptor antagonists. Much effort was aimed at development of bradykinin antagonists as therapeutic agents (Table 1). The first full chain bradykinin antagonist which showed high BK antagonism both on B-1 and B-2 receptors, B9430 (DR-R-P-Hyp-G-Igl-S-DIgl-Oic-R, Hyp: *trans*-4-hydroxyproline; Igl: α-(2-indanyl)glycine; Oic: octahydroindole-2-carboxylic acid), was developed by Gera at the Stewart laboratory a decade ago [1]. The des-Arg9-analog of B9430, B9858, had remarkably high B-1 antagonist activity [2]. An extensive structure-activity relationship study resulted in a highly active and selective B-1 antagonist, B9958 (Table 1). In this paper we describe the development of new, highly potent and N-terminal enzyme resistant analogs of B9958.

Result and Discussion

The peptides were synthesized by solid-phase technology on Merrifield resin using conventional Boc procedures. Amino acids were coupled using BOP/HOBt and Boc-protecting groups were removed by trifluoroacetic acid. Cleavage of the peptides from the resin was done by HF and finally the peptides were purified by HPLC. The peptides were characterized by TLC, analytical HPLC and laser desorption mass spectroscopy (LDMS).

Aminopeptidase N (APN: CD13, EC3.4.11.2) is more critical for the inactivation of the peptide BK B1 receptor antagonists *in vivo* than other peptidases (angiotensin-converting enzyme and neutral endopeptidase). APN limits the potency and lifetime of the B1 antagonists, which typically have N-terminal lysine or arginine residues. Protection by acylation can seriously degrade potency but interestingly N-terminal acylation of our highly potent and selective B1 antagonist, B9958 with the bulky, hydrophobic 2,3,4,5,6-pentafluorocinnamic acid (F5c) as in B10324 gave good inhibition *in vivo* of both lung (86%) and prostate (43%) cancers [3]. To overcome the above limitation several new bradykinin B-1 antagonists have been designed and synthesized having N-terminal basic residues in the D-configuration (D-Arg, D-Lys, D-Orn) to circumvent the inactivation problem while keeping an acceptable

affinity. A competition assay for radioligand binding to smooth muscle cells was used to evaluate the affinity of the analogs (K_i values in Table 1). In both human and rabbit smooth muscle cells, B9958 had the highest affinity. The analogs were 1.2- to 20-fold less potent in that respect. Inclusion of amastatin, an aminopeptidase N inhibitor, increases its

Table 1. *Structures and activities of selected BK peptides for mammalian B-1 receptors and aminopeptidase N*

Number	Structure	Receptor Affinity		Enzyme Affinity	
		Human	Rabbit	Human	Rabbit
		K_i, nM		K_i, μM	
des-R^9-BK	R-P-P-G-F-S-P-F	>166	24	--	--
des-R^{10}-KD	K-R-P-P-G-F-S-P-F	0.1	0.14	--	--
B9430	DR-R-P-Hyp-G-Igl-S-DIgl-Oic-R	12.6	63	--	--
B9858	K-K-R-P-Hyp-G-Igl-S-DIgl-Oic	0.08	5.4	--	--
B9958	K-K-R-P-Hyp-G-CpG-S-DTic-CpG	0.055	0.144	4.87	0.96
B10350	K-K-R- P-Hyp-G-Igl-S-DTic-CpG	--	0.74	--	1.89
B10352	DK-K-R-P-Hyp-G-CpG-S-DTic-CpG	0.143	0.41	NI	8.73
B10354	DOm-K-R-P-Hyp-G-CpG-S-DTic-CpG	0.196	0.66	NI	43.80
B10356	DR-K-R-P-Hyp-G-CpG-S-DTic-CpG	0.065	0.40	2.20	0.86
B10358	DR-DR-R-P-Hyp-G-CpG-S-DTic-CpG	1.122	1.06	NI	2.21

Abbreviations: Cpg: α-cyclopentylglycine; Tic: tetrahydroisoquinoline-3-carboxylic acid; NI: no inhibition.

apparent potency in rabbit aorta contractility assay. Analogs of B9958 having D-Lys (B10352) or D-Arg (B10356) replacements of the N-terminal residue were slightly less potent than the parent peptide, B9958, in receptor binding, but were more potent in the contractility assay. Their action on rabbit aorta was not potentiated by amastatin. Remarkably, B10356 was found to be a competitive inhibitor of aminopeptidase N for hydrolysis of L-alanine-*p*-nitroanilide (L-Ala-*p*Na), a standard aminopeptidase N chromogenic substrate; it potentiates vasoactive peptides susceptible to this peptidase, such as angiotensin III. Thus, B10356 is both a B-1 receptor antagonist and an aminopeptidase N inhibitor at micromolar concentrations, unlike B10352, which retains a purer profile (with ~10-fold less affinity for the aminopeptidase of rabbit cell membranes and an even larger gap for the human enzyme). These results also suggest that the Lys residue in the second position in B10356 is important for aminopeptidase N affinity.

COMPARISON OF THE ANTIBODY RESPONSE IN MICE IMMUNIZED WITH DIFFERENT PRESENTATIONS OF JY1 PEPTIDE

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Introduction

Nowadays, considerable attention has been focused on the development of peptide-based vaccines as an alternative to conventional formulations. Peptide vaccines have obvious advantages such as: high safety, low cost and easy handling and storage. In contrast to classical vaccines, they can be tailor-made against specific targets including antigenic sites not easily accessible to the immune system. However, they suffer also from limitations such as the usually low immunogenicity of small peptides and the vulnerability to proteolytic degradation. Many of these problems can be addressed by improved forms of presentation of the synthetic epitope to the immune system, including formulations such as multiple antigen peptide systems (MAP), polymerization, conjugation to carriers such as proteins or synthetic polymers [1, 2].

Herein, we have compared different strategies to increase the immunogenicity of an antigenic HIV peptide as a vaccine candidate. Our selected B-cell epitope comprises 15 amino acids (317-331) of the V3 region of HIV-1, JY1 isolate, and is in tandem with a T-helper epitope corresponding to the 830-844 region of *tetanus* toxoid. Several presentations, including oligomerization, MAP dendrimer, conjugation to dextran beads or to other macromolecular carriers, have been synthesized and evaluated.

Result and Discussion

Free and oligomeric versions

It consisted of the JY1 epitope (RQSTPIGLGQALYTT) in tandem with the T-cell epitope of *tetanus* toxoid (TT, QYIKANSKFIGITEL), with an intervening Lys pair designed as a cathepsin-like cleavage site, and with flanking Cys-Gly and Gly-Cys residues at each end to promote oligomerization. The peptide was synthesized by solid phase methods, with the two Cys residues protected with the HF-stable Acm group. After cleavage and characterization, the Acm-protected monomer was treated with iodine to remove the two Acm groups and oligomerization was performed in AcOH : H_2O, where the monomer was highly soluble. The reaction

proceeded quickly and a high conversion to oligomer was obtained. ESI-MS analysis of the main fractions of the oligomerization mixture allowed identification of the tetra-, tri- and dimeric species.

Multiple Antigenic Peptide System

Two multivalent (MAP-like) dendrimeric presentations of the JY1 peptide with defined composition and varying number of B- and T-cell epitopes were also designed and synthesized. The JY1-MAP4 immunogen, containing four B and two Th epitopes, resulted from dimerization of a monomer with a single Lys branching point, while the JY1-MAP8, with eight and four epitopes, respectively, contained three Lys branching units in each monomer. The monomers were prepared by Boc/benzyl solid phase chemistry and dimerized. To evaluate the quality of each synthesis, an aliquot of each final dimer was characterized by RP-HPLC.

Peptide on dextran beads

Cross-linked dextran beads were chemically modified with a sub-stoichiometric amount of Fmoc-βAla to give an amine-functionalized support suitable for solid-phase peptide synthesis. On this dextran support, the JY1-LysLys-TT sequence was assembled by Fmoc chemistry, using HOBt and DIPC-mediated couplings with relatively high excess of all components. The quality of the dextran-bound peptide was evaluated by RP-HPLC. The main product of the chromatogram was satisfactorily characterized as the target peptide by ESI-MS.

Peptide and MAP conjugates

To further enhance the immunogenicity of the anti-peptide response, both the monomeric and the two dendrimeric versions of the JY1-TT peptide were conjugated to recombinant HBsAg protein. The conjugations of JY1-TT monomer, JY1-MAP4 and JY1-MAP8 to HBsAg were done by means of succinic anhydride. The first step was succinylation of HBsAg under mildly alkaline conditions, followed by dialysis to remove excess succinic anhydride and thus avoid unwanted MAP-MAP and/or peptide-peptide cross-linking. The carboxyl groups on the modified HBsAg were next activated with EDAC and allowed to react with the free amino groups of either linear monomer or JY1-MAP4 or JY1-MAP8. The conjugates were purified by gel filtration to avoid aggregation.

Analysis of the antibody response

Groups of eight Balb/c mice were inoculated subcutaneously with different presentations of the JY1 peptide antigen and their anti-peptide antibody responses after four doses of the epitope were evaluated by ELISA. Both HBsAg-JY1-MAP4 and HBsAg-JY1-MAP8 conjugates elicited the highest titers against JY1. The superior immunogenicity of these conjugates may be attributed to the combination of large size and epitope multiplicity, plus the known T-cell response of the carrier protein.

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CONFORMATIONAL STUDIES OF A SHORT PEPTIDE CORRESPONDING TO THE C-TERMINAL β-HAIRPIN STRUCTURE OF THE B1 DOMAIN OF PROTEIN G

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Introduction

Three types of regular secondary structure are recognized in proteins: α-helix, β-sheet and turns. Secondary structure elements such as α-helices, β-hairpins, and β-turns are often folding-initiation sites. This observation prompted us to focus on model systems in which a well-defined secondary structure occurs in the absence of a tertiary structural context. These systems provide a way to separate the intrinsic source of secondary structural stability from tertiary context effects [1]. A model protein, which contains these three types of regular secondary structures, is the immunoglobulin binding protein G (PDB ID code 1IGD) [1] from *Streptococcus sp*. 1IGD contains 61 amino-acid residues, and consists of two antiparallel-packed β-hairpins and an α-helix in the middle of the sequence packed to the β-sheet. The second β-hairpin was found to be stable in isolation, protected from H/D exchange early in folding [2]. This fragment is therefore likely to be the first folding initiation site of the protein which could provide an adequate nucleation center on which the rest of the polypeptide chain would find a favorable environment to fold [3]. Thus, among two β-hairpins, the 48-59 fragment of 1IGD corresponding to the C-terminal β-hairpin was synthesized and investigated.

Results

We investigated the effect of different environmental and temperature conditions on formation of the 48-59 β-hairpin structure. The NMR spectra in aqueous solution (pH 5.7) at temperature 10 ^{o}C show that this peptide exists in equilibrium between a β-hairpin and an unordered structure. Calculations of the structure from the NMR data indicate that, at 10 ^{o}C and pH 5.7, the IG(48-59) peptide adopts a population containing a native-like β-hairpin structure with the native conformation of the β-turn and flexible N- and C-termini whereas, at 32 ^{o}C, the peptide forms a β-hairpin in the Ala^{53}-Phe^{57} region with nonlocal hydrogen bonds involving the backbones of Lys55 and Thr56. Our NMR studies show that the 48-59 fragment at temperatures lower than exists in an equilibrium of two conformations – a regular β-hairpin and a statistical coil (sc). Although increasing temperature resulted in shifting the equilibrium in the direction of the statistical-coil structure, the overall β-hairpin shape of the 48-59 fragment was maintained. The CD spectra show that the statistical-coil conformation is the predominant structure of IG(48-59) in water and in phosphate buffers, regardless of pH (25 - 90% sc). The presence of CF_3CH_2OH

results in an increase of ~ 30 % in the amount of ordered structure of this fragment of 1IGD with respect to the amount of statistical-coil conformation. The IG(48-59) fragment of 1IGD exhibits a tendency to form a β-hairpin as in native 1IGD. FT-IR spectra show that the peptide forms mostly a disordered or β-sheet conformation in the solid phase. The amount of disordered conformation of the IG(48-59) peptide is 45% and 81% in water and CF_3CH_2OH, respectively. The amount of β-sheet conformation is 50% and 15.5% in water and CF_3CH_2OH, respectively. DSC measurements suggest that this peptide has a tendency to aggregate at low temperatures (at about 10^oC).

Discussion

The experimental studies of the IG(48-59) fragment show a tendency toward β-hairpin formation but, at low temperatures, the β-hairpin structure could be induced by aggregation. Our NMR results suggest that the folding of a β-hairpin is initiated from the β-turn, then "zips up" the remaining native hydrogen bonds. A turn stabilized by hydrogen bonds positions the aromatic residues so that they are poised to pack into a hydrocarbon cluster.

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THE RECOGNITION MECHANISM OF THE N-TERMINAL REGIONS OF HISTONE H2A AND H3 BY PEPTIDYLARGININE DEIMINASE

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Introduction

Peptidylarginine deiminase 4 promotes a catalytic reaction in Arg residues of peptides and proteins, such as histone and the eukaryotic translation initiation factor 4G1, resulting in the production of the citrullinated proteins [1-3]. Recently, it is reported that the citrullination of cellular proteins might cause rheumatoid arthritis [4]. Therefore, the histone citrullination is thought to be related to rheumatoid arthritis. However, the citrullination reaction of histones by PAD4 has not been studied yet in detail. In order to investigate the N-terminal acetylation effect on the PAD4 recognition, the acetylated or non-acetylated N-terminal peptides (20 amino acid residues) of histones H2A and H3 were chemically synthesized, as shown in Fig. 1. In this paper, the PAD4 reactivity of each Arg residue in the N-terminal peptides of H2A and H3 was estimated *in vitro*.

H2A-N20: SGRGKQGGKARAKAKTRSSR
Ac-H2A-N20: Ac-SGRGKQGGKARAKAKTRSSR
H3-N20: ARTKQTARKSTGGKAPRKQL
Ac-H3-N20: Ac-ARTKQTARKSTGGKAPRKQL

Fig. 1. Amino acid sequences of the acetylated or non-acetylated N-terminal peptides (20 amino acid residues) of histone H2A and H3

Result and Discussion

PAD4 recognizes the Arg3 residue in histone H2A as well as the Arg3 residue in histone H4 *in vivo* [5, 6]. In addition, it is reported that the Arg17 residue in histone H3 was predominantly citrullinated and the Arg8 was also partially citrullinated [7]. In these studies, N-terminal acetylated H2A and H4 and N-terminal non-acetylated H3 were examined on the PAD4 recognition since the N-terminal Ser residue in histone H2A was predominantly acetylated *in vivo* and the N-terminal Ala residue in histone H3 was not acetylated *in vivo*. However, the N-terminal acetylation effect of histone subunits on PAD4 recognition has not been studied yet. In addition, N-terminal non-acetylated H2A was also reported and the N-terminal analysis of histone H3 has not been proceeded in detail. Therefore, in order to investigate the N-terminal acetylation effects of histone H2A and H3 on PAD4 recognition, a series of the N-terminal peptides of H2A and H3 was prepared by the solid phase peptide synthesis. We observed two major products containing citrulline residue(s) by treatment of non-acetylated H2A-N20 with PAD4 *in vitro*. MALDI-TOF/MS

analysis indicated that the Arg3 residue in non-acetylated H2A-N20 is citrullinated as well as Ac-H2A-N20 [8]. However, another Arg residue was also citrullinated by PAD4. This result suggests that the acetylation at the N-terminal amino group affects the PAD4 recognition on histone H2A. PAD4 recognizes the Arg3 residue, which is localized at the second position of the type II β-turn structure, of histone H2A [9]. The Arg3 residue is closely localized at the N-terminal moiety. Therefore, one can speculate that the N-terminal acetyl group stabilizes the β-turn structure for PAD4 recognition and the de-acetylation caused destabilization of the β-turn structure at the N-terminal moiety, resulting in the loss of specific recognition. The N-terminal acetylated H3 peptide was also examined the citrullination by PAD4. Surprisingly, the N-terminal acetylation of H3 dramatically affected the PAD4 recognition. The H3-N20 peptide was citrullinated at the Arg17 and Arg8 *in vitro* as well as *in vivo* experiments of H3 [7]. However, Ac-H3-N20 was predominantly citrullinated at the Arg2 residue *in vitro*. The Arg residue is localized at the second position of the type II β-turn structure in the crystal structure of H2A peptide and PAD4 complex, as described above. Therefore, our results indicates that the acetylation of the N-terminal Ala residue (Ac-H3-N20) induces the β-turn formation at the N-terminal moiety of histone H3, resulting in the dramatic effect on PAD4 recognition. This result also suggests that the Arg 17 originally may not be a suitable substrate residue of PAD4 since the –Pro-Arg-Lys-Gln- moiety can not form the type II β-turn structure [9]. In conclusion, the N-terminal acetylation is important for PAD4 recognition of histone H2A and H3. The de-acetylation of H2A causes the PAD4 recognition at the Arg3 and another Arg residue *in vitro*, and the acetylation of H3 causes the PAD4 recognition at the Arg2 residue.

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LHRH ANALOGS CONTAINING PALMITOYL MOIETY POSSESS CYTOTOXIC ACTION ON TUMOR CELLS *IN VITRO*

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Introduction

Last years it was shown that LHRH receptors are overexpressed in the most of adenocarcinoma cells in contrast to their low content in normal tissues except for hypophysis [1]. These data along with peculiarities of intracellular metabolism and signal transduction in tumor cells create the basis for LHRH analogues application in therapy of breast, ovary, prostate, lung, intestine, liver and kidney cancers. In this case the effect of peptide may be complex including inhibition of steroid hormones level, induction of apoptosis and, possibly stimulation of immune system. The direct effect of LHRH analogues on tumor cells is low. Both agonists and antagonists are widely applied as vectors for the targeting of cytotoxic agent, which permit a reduction of effective dose and side effects [2]. The advantages of this approach are the action on hormone-dependent and hormone-independent cells and the prevention of tumor transition into the state insensitive to hormonal therapy. However, some difficulties during the preparation of hybrid compounds stimulate the search for the LHRH analogues that themselves exhibit a cytotoxic effect.

Result and Discussion

Our previous results demonstrated a high antitumor effect of peptide (1) on prostate carcinoma model *in vivo* and its *in vitro* inefficiency. At the same time, efficiency of peptide (2) modified with a palmitoyl residue both *in vitro* and *in vivo* suggests its direct cytotoxic action. In present study we investigated the influence of N-terminal modification on cytotoxicity of LHRH analogues using different human adenocarcinoma cell lines. Peptides (3)-(26) were synthesized by solid phase method using the Boc/Bzl-strategy. The hydrophobic N-terminal group was attached by DIC/Cl-HOBt method using free palmitic, lauric and trimethylacetic acid or the corresponding derivatives of N-terminal amino acid residues. The peptides were desalted by gel filtration on a Sephadex G-15 column and further purified by means of SPE and HPLC. The cytotoxic activity of LHRH analogues was studied on various lines of human adenocarcinoma cells (ATCC), including that of mammary gland, prostate, ovaries, intestine, and liver, which contained LHRH receptors. The F-89 normal fibroblasts were used as control cells. The efficiency of analogues (at concentration $2\text{-}5 \times 10^{-5}$ M) was evaluated according to staining of the viable cells (Hemacolor assay). The modification of peptide antigens by the residue of palmitic acid is widely used for the enhancement of immune response. However, there are many reports indicating apoptosisinducing effect of palmitic acid and its selective cytotocxic action on tumor cells [3, 4]. Therefore, we

decided to use the N-terminal palmitoyl group for increasing the antitumor activity of LHRH analogues. In addition, this modification could facilitate peptide penetration through the blood-brain barrier. A comparison of peptides efficiency confirms the importance of the palmityl residue for the cytotoxic action. Shortening of the carbon chain of N-terminal group or the use of branched trimethylacetic acid result in a dramatic decrease in the effect. Moreover, none of the examined analogues, except the palmitoyl derivatives, exhibit any cytotoxic activity. It was demonstrated that the nature and number of amino acid residues located between the palmitoyl group and position 1 in LHRH structure have a significant influence on the analogues efficiency. Thus the incorporation of Pro-Gly and D-Pro-Gly sequences into the structure (2) decreases the activity. A similar effect is observed in the case of Lys residue attachment to the N-terminal Pro in peptide (11). At the same time, truncation of amino acid sequence and/or application of D-Lys result in highly effective compounds. Significant influence of point modifications on the cytotoxic effect of the analogues (substitution of Ala for Pro and Gly residues) suggest the specificity of peptides action and possible participation of LHRH receptors in this process. The absence of cytotoxic effect on the normal human fibroblasts for peptides applied at doses lethal for the tumor cells additionally confirms this proposal. Peptide efficiency *in vitro* can be substantially increased (2-6 times) by the incorporation of nuclear localization sequence (NLS). Further biological studies emphasize important role of NLS position in analogue structure. Thus, it was demonstrated that attachment of palmitoyl moiety produce LHRH analogues possessed antitumor action *in vitro*. These data in conjunction with those for NLScontaining peptides are in favour of supposed apoptose-inducing activity.

H-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (1)
Pam-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (2)
Piv-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (3)
Lau-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (4)
H-Pro-D-Nal-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (5)
Pam-Pro-D-Nal-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (6)
H-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt (7)
Pam-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt (8)
H-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (9)
H-Gly-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (10)
Pam-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (11)
Lau-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (12)
Hx-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (13)
Pam-Gly-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (14)
Pam-Pro-Gly-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (15)
Pam-D-Pro-Gly-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (16)
Pam-Lys-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (17)
Pam-Lys-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (18)
Pam-D-Lys-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (19)
Pam-D-Lys-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (20)
Pam-D-Pro-Gly-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (21)
Pam-Pro-Pro-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (22)
Pam-D-Pro-Pro-Pro-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (23)
Pam-Pro-Gly-D-Leu-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (24)
Pam-Pro-Gly-D-Phe-Ala-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (25)
Pam-Pro-Ala-D-Phe-Pro-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 (26)

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THE UNEXPECTED BIOLOGICAL ACTIVITY OF MODIFIED CYCLOLINOPEPTIDE A

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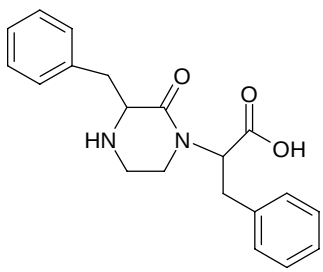
Introduction

Cyclolinopeptide A, (CLA, 1), a cyclic nonapeptide cyclo(-Pro[1]-Pro[2]-Phe[3]-Phe[4]-Leu[5]-Ile[6]-Leu[8]-Val[9]-), possesses strong immunossupresive and antimalarial activity as well as the ability to inhibit cholate uptake into hepatocytes [1]. The mechanism of cyclolinopeptide A activity is similar to that of cyclosporine A. On the basis of the comprehensive structure-activity studies the following factors were found to be important for the immunossupresive activity of CLA: presence of the Pro-Pro *cis* amide bond, the "edge-to-face" interaction and distance between the aromatic rings. Recently, a (Phe-Phe)[8,9]-CLA, 2, analogue was synthesized with the Phe-Phe fragment constrained by anethylene linker [2]:



To evaluate the immunosuppressive activity of this new CLA analogue the mouse splenocyte proliferation assay has been employed. The action of the new CLA analogue is immunomodulatory: stimulatory at low doses (0.1 - 1 μg/mL) and inhibitory at high doses (10 μg/mL) [2].

Result and Discussion

In order to determine changes in the molecular structure of 2 in comparison with the native CLA 1, which may be responsible for the switch of bioactivity, NMR and theoretical studies were carried out.

NMR spectra were recorded on Bruker spectrometers (DPX 250, DRX 500 and 600) in DMSO-d_6 or $CDCl_3$ solutions. For signal assignment 2D NMR experiments were applied: COSY DQF, TOCSY, NOESY and HSQC (^{1}H-^{13}C, ^{1}H-^{15}N), recorded at 298 K and 214 K. The temperature dependent spectra were measured in $CDCl_3$ between 298 and 214 K, in 15 K steps.

The very broad proton signals, even at high field (600 MHz) and at low temperature (214 K), evidence the existence of several conformers in intermediate chemical exchange. The analysis of spectra recorded in $CDCl_3$ indicates the presence of at least two isomers even at 214 K. Under the same conditions unmodified 1 exists as only one isomer [3]. The NH signals are spread over the range of 2.0 ppm. Majority of them exhibits temperature coefficients larger than -2.4 ppb/K. The most up-field signal at -0.12 ppm was assigned as one of the β protons of Pro2 in the major isomer. The up-field shift can be accounted for the ring current of the aromatic ring of Phe3. This conclusion is supported by the NOE between β Pro2 and Phe3 ortho protons, recorded at 214 K.

We were able to assign diagnostic signals for the geometry of Xxx-Pro bonds in CDCl3 at 298 K. The NOE between the two α protons of Pro1 and Pro2 is consistent with the *cis* Pro^1-Pro^2 peptide bond. The trans geometry of Val^9-Pro^1 was concluded from the NOE correlation between Val9 α proton and δ protons of Pro1.

Theoretical calculations were carried out for 2-*cis* isomer with *cis* amide bond between Pro1 and Pro2 and for all-*trans* peptide bonds isomer 2-*trans*. Molecular dynamics calculations were performed with NAMD (CHARMM27 all-atom force field) and GROMACS (GROMOS 53a6 united atom force field). CHARMM27 parameters for ethylene bridge were created by analogy to the existing atom types. Starting coordinates were prepared on the basis of 1 X-ray structure [3].

The analysis of NMR and computational data led us to the following conclusions; although the introduction of the ethylene bridge between Phe3 and Phe4 should constrain the mobility of this fragment of the modified cyclolinopeptide A 2, the overall flexibility of the molecule increases due to smaller number of the hydrogen bonds. MD calculations for the all-*trans* isomer 2-*trans* predict it more rigid than 2-*cis*. The proximity of Pro2 and Phe3 rings in 2-*cis* isomer was predicted theoretically and confirmed by NMR spectroscopy at low temperature.

Acknowledgements

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BIOCHEMICAL ANALYSIS OF VKORC1 POLYMORPHISMS IN SOME IRANIAN WARFARIN RESISTANT PATIENTS

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Introduction

Warfarin is still one of the most widely used oral anticoagulants for the treatment of venous and arterial thrombosis. However, warfarin treatment is problematic because the dose requirement for warfarin is highly variable [1, 2].

It can be influenced by many factors such as pharmacokinetic factors (due to differences in absorption or metabolic clearance of warfarin induced by drug interaction or patient base situation such as gender), and pharmacodynamic factors (due to differences in the hemostatic response to given concentrations of warfarin) [3]. Warfarin exerts its anticoagulant effect by inhibiting the vitamin K epoxide reductase enzyme complex (VKOR) that recycles vitamin K1 2,3-epoxide to vitamin K1 hydroquinone.

Cytochrome P450, subfamily IIC, polypeptide 9 (CYP2C9) is a liver enzyme that catalyzes the hydroxylation of warfarin [4, 5], and its genetic polymorphisms is responsible for warfarin sensitivity. These genetic variants can not explain all of inter- individual differences in warfarin dosage and suggest that maybe additional factors contribute to this variability. Recently, a component of the VKOR termed VKORC1 has been identified as a therapeutic target site of warfarin [6, 7], and its mutations were found in some warfarin- resistant patients [7, 8].

Due to the importance of this drug in clinical approaches, we studied the relationship between warfarin maintenance dose and pharmacodynamic factors in warfarin resistant Iranian patients [9]. The aim of this work is to evaluate a relationship between warfarin maintenance dose and VKORC1 genotype in warfarin resistant Iranian patients.

We had 6 Iranian patients, who were prescribed oral warfarin with high dose, were recruited from Imam Hospital (Tehran, Iran). They received more than 10 mg warfarin per day. The blood DNA of all patients were extracted with DNGTM-plus solution.

DNA samples were amplified by polymerase chain reaction (PCR) in a final volume of 100 μL, consists of 0.5μM from each primer, 0.2 mM deoxynucleoside triphosphate (dNTP), 1μg genomic DNA, 2.5 U Taq polymerase (Cinagen taq DNA polymerase, Iran) in 1x PCR buffer. The PCR were done in 35 cycles that in each one denaturation occurred 45 sec in 93 ºC, annealing occured 30 sec in 58 ºC for exon 1 and 2 and 56 ºC for exon 3, and elongation occured 90 sec in 72 ºC. PCR products were digested with Hin6I, MspI and Eco72I in order to find previous

reported mutations. We used conformational sensitive gel electrophoresis (CSGE) for screening samples with mutation(s) in their VKORC1 gene. In the other study we determined warfarin level in blood of patients with HPLC and the result encouraged us for sequencing all the exons of one of the patients who received 100 mg warfarin per day.

Results and Discussion

According to all these methods, all of the samples were wild type. Data obtained from HPLC showed high concentration of warfarin in one of the patients who needs more than 100 mg of warfarin per day. Although we didn't find any mutation using RFLP and CSGE but we decided to sequence every 3 exons. Sequencing of exons showed that the patient had no mutation in VKORC1 gene.

These results indicated that maybe other genes, at least in this patient, are responsible for resistance to warfarin.

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N-ALKYLATION, A KEY STEP FOR A NEW SYNTHESIS OF NICOTIANAMINE UNNATURAL ANALOGUES

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Introduction

Nicotianamine (NA) which was first isolated from the leaves of *Nicotiana Tabacum L*. [1], is known as a key biosynthetic precursor of phytosiderophores. Various studies have proved that NA plays a significant role in plants physiology as an iron, nickel, zinc transporter [2].

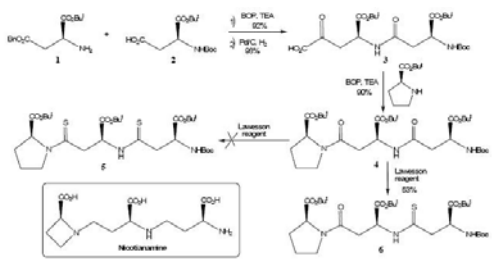
The aim of our study was to synthesize NA unnatural analogues via peptide intermediates, to investigate the mechanisms of metal transport and accumulation within the plant.

There are mainly two synthetic strategies described in the literature for the synthesis of nicotianamine: reductive amination of intermediate protected aldehydes [3] and more recently reduction of amide bonds via thioamide [4].

We intended to synthesis a first analogue by replacing azetidine ring with proline (Scheme 1), using the thioamide strategy.

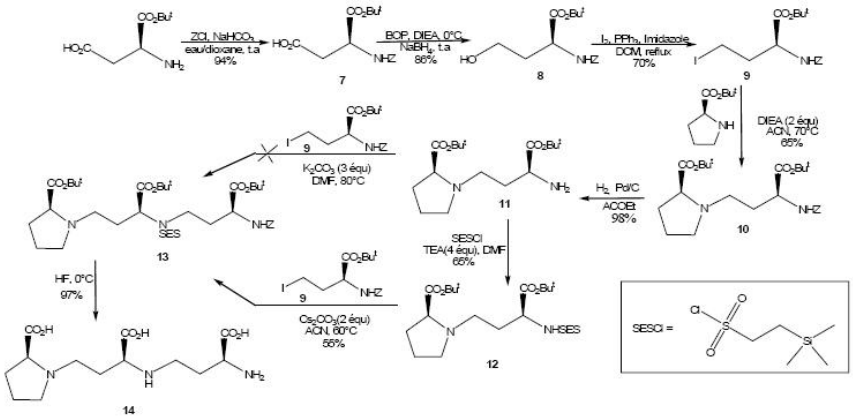
Result and Discussion

The reductive thioamidation approach was chosen because of the easy preparation of buildings blocks. As shown in Fig.1 HAsp(OBn)O*t*Bu (1) was reacted with aspartic acid (2), then hydrogenolys is afforded the dipeptide (3). A Coupling step with proline in the presence of TEA gave the tripeptide (4). In this case, contrary to what happened for NA synthesis, using Lawesson's reagent did not allow convertion of the tripeptide into the thioamide (5). In fact, the thioamidation of the secondary amide did not occur, and compound (6) was obtained.



Therefore, a new strategy was designed to reach the target. The principle of this new method is based on the nucleophilic substitution of iodine by amine. As shown

in Fig. 2, benzyl 2- (*S*)-((*tert*-butoxycarbonyl) amino)-4-iodobutanoate (9), the key precursor, was obtained after three steps in 58% overall yield [5].



Commercially avaible L-aspartic-α-tertiobutyl ester was N-protected using benzyl chloroformate (7). Treatment of (7) with BOP/DIEA then $NaBH_4$ gave the homoserine derivative which was converted into iodide (9) using iodine, triphenylphosphine and imidazole. *N*-alkylation of (9) with proline *tert*-tiobutyl ester in the presence of DIEA gave the pseudopeptide (10) in good yield. Hydrogenolysis of (10) using 10% Pd/C and H_2 occurred quantitatively. Direct *N*-alkylation of the primary amine (11) with iodide (9) was unsuccessful. The 2-trimethylsilylethylsulfonylgroup (or SES) was used to promote base-activated alkylation and protecting the primary amine as a sulfonamide (12). Under the conventional conditions (ACN, Cs_2CO_3, 60 °C) the sulfonamide (12) was alkylated in moderate yield. Finally, removal of the protecting groups in a HF single step afforded the proline analogue (14) after lyophilisation as a white powder.

In conclusion, the new synthesis developed to produce the proline analogue is very practical, short and has several advantages: no racemisation, good overall yield, reproducibility. It will be applied to improve natural NA synthesis.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF GRAMICIDIN S ANALOGUES CONTAINING CONSTRAINED PHENYLALANINES

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Development of new classes of antibiotics to counteract bacterial resistance has been intensely pursued in recent years. The search for new molecules has led to the study of naturally occurring antimicrobial peptides since, as most of these molecules target the cell membrane, development of resistance is judged to be unlikely. The cationic antimicrobial peptide gramicidin S (GS, Fig. 1A), isolated from *Bacillus brevis*, is active against a wide range of bacteria and fungi. Unfortunately, GS exhibits a high haemolytic activity, limiting its use as an antibiotic for topical applications. This peptide is a C2-symmetric cyclic decamer that adopts a rigid β-structure, in which the Val, Orn and Leu residues align to form the antiparallel β-strands and D-Phe and Pro induce type II' β-turns. There is a wide interest in the generation of new GS analogues in order to dissociate the antimicrobial and haemolytic activities. In addition, GS provides a suitable model to study the structural preferences of non-proteinogenic amino acids.

A **B**

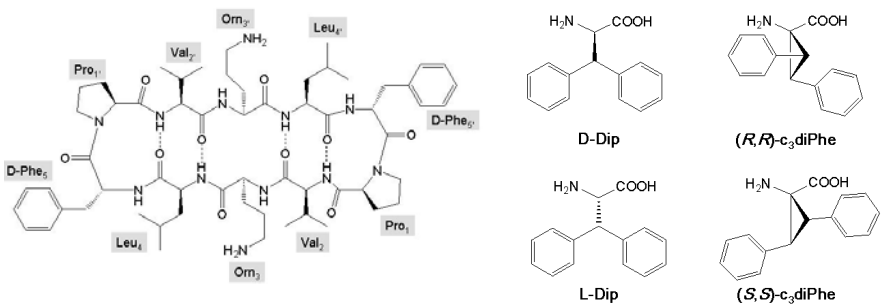

Fig. 1. Panel A: Structure of gramicidin S. Panel B: Conformationally constrained amino acids used as replacements of L- and D-Phe.

We have modified GS by replacing both D-Phe residues of the β-turn region by conformationally restricted D-diphenylalanine (D-Dip) (analogue 1, Fig. 1B). In addition, we have explored the effect of replacing the native D-Phe-Pro dipeptide in GS by D-Pro-Phe, a well-known type II' β–turn forming dipeptide [1] (analogue 2), and in a further step we have replaced the Phe residue of this dipeptide by constrained amino acids such as L-Dip (analogue 3), (*R,R*)-$c_2$diPhe (analogue 4) [2], (*S,S*)-$c_2$diPhe (analogue 5) [2], or 1-aminocyclopropanecarboxylic acid (Ac_3c,

analogue 6). All peptides were prepared from a linear precursor assembled by Boc SPPS, cyclized in solution (HBTU/HOBt, DIEA, DMF, 1 h) [3], deformylated at the two Orn residues (20% HCl in methanol, 37 ºC) [3], purified by HPLC and characterized by MALDI-TOF mass spectrometry.

The biological activity of all GS analogues was tested on bacteria (*e.g., Staphylococcus aureus*) and on the promastigote form of *Leishmania donovani*, a protozoan parasite. On the first organism, analogues 1, 3 and 4 paralleled the activity of GS, while the remaining analogues were clearly inactive. On *Leishmania*, the same analogues plus analogue 2 were active, in the order 1 > 3 > GS > 4 > 2. Further studies on eukaryotic microorganisms are under way to determine their mechanism of action. We are also currently investigating the solution structure of the active analogues by 2D NMR in order to establish structure-activity relationships.

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PHOTOPROBE-PEPTIDES FOR THE SYSTEMATIC CHARACTERIZATION OF THE INTERACTIONS OF ANGIOTENSIN II WITH ITS RECEPTOR AT1

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Introduction

Seven transmembrane domain (TMD) G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors and are implicated in various physiopathologies. Approximatively 50 % of all current market drugs act via these receptors, making them privilegeous targets in pharmacotherapy. The rational design of drugs targeting GPCRs requires a molecular-based knowledge of those proteins that may be acquired by identification of ligand-receptor interactions through photoaffinity labelling. This approach allows to directly map the ligand-receptor interface by covalent bonding of the radio-labelled photoprobe ligand within the immediate molecular surroundings of its cognate receptor. This information may then be used in conjunction with computational molecular modeling procedures, based on the X-ray crystallography of bovine rhodopsin, to build and validate homology molecular models of liganded receptors.

Results

In the present contribution, a systematic incorporation of both biradical-ketone-generating amino acid photoprobe p-benzoyl-L-phenylalanine (Bpa) and carbene-generating amino acid photoprobe p–[3–(trifluoromethyl)–3H–diazirin–3–yl]–L–phenylalanine (Tdf) into the N-terminal part of the octapeptide angiotensin II (AngII) sequence was carried out to produce two series of three different radio-labelled photoprobe AngII peptides: 125I[Bpa1]AngII, 125I[Sar1,Bpa2]AngII, 125I[Sar1,Bpa3]AngII as well as 125I[Tdf1]AngII, 125I[Sar1,Tdf2]AngII and 125I[Sar1,Tdf3]AngII. The two photopeptide series were tested for their affinities and for their intrinsic activities on a peptidergic GPCR, the human AngII type 1 receptor (hAT1), as well as on a constitutively active mutant (CAM), the [N111G]hAT1 receptor (hAT1-CAM). All analogues displayed AngII-like properties on the CAM receptor model: i.e. low nM affinities with agonistic properties. In order to facilitate identification of photoprobe incorporation sites by CNBr cleavage (C-terminal Methionine (Met)-specific), hAT1-CAM with several single X→Met mutations ([N111G/I11M], [N111G/A21M], [N111G/I172M], [N111G/L195M], [N111G/I258M] and [N111G/L268M]hAT1) were used. All hAT1-CAM/Met-mutants showed pharmacological properties similar to hAT1-CAM.

In photoaffinity labelling experiments, all AngII analogues produced an excellent photoaffinity labeling yield with the exception of 125I[Tdf1]AngII. 125I[Bpa1]AngII photolabelled all Met-mutants and CNBr cleavage confirmed interactions with the N-terminus (residues 2-21), the extracellular loop (ECL) 2 (1) and the ECL3 of hAT1-CAM. However, 125I[Tdf1]AngII photolabelled practically no proteinaceous material due to the putative quenching of the photogenerated carbene by extracellular water (2). Both 125I[Sar1,Bpa2]AngII and 125I[Sar1,Tdf2]AngII photolabelled all Met-mutants but with important differences. 125I[Sar1,Bpa2]AngII evidenced interactions mostly with the ECL2 and less with the ECL3 of hAT1-CAM, whereas 125I[Sar1,Tdf2]AngII demonstrated interactions mostly with the N-terminus (residues 12-21), in proportion similar to

125I[Sar1,Bpa2]AngII / ECL2, and less with the ECL3 of hAT1-CAM. Both 125I[Sar1,Bpa3]AngII and 125I[Sar1,Tdf3]AngII also photolabelled all Met-mutants but with identical labelling patterns: they showed only interactions with the ECL2, as previously described (3).

Discussion

The combination of the more selective biradical-ketone-generating amino acid photoprobe Bpa in conjunction with the highly reactive, non-selective carbene-generating amino acid photoprobe Tdf allows for a more thorough characterization of the binding site of GPCRs than with the use of a single photoprobe. Previous photoaffinity labelling studies on hAT1 have shown an extended β-strand ligand orientation of AngII within and parallel to the TMD bundle (4): the N-terminal part of AngII interacts with ECL2 (1,3) and its C-terminal part interacting with residues from a pocket formed by TMD 3, 6 & 7 (5). The present contribution sheds more light on the interaction of the AngII N-terminal part with hAT1. ECL2 still appears to be the major interaction region but we now show that also the N-terminus (residues 2-21) as well as ECL3 also contribute to this interaction. Moreover, the putative quenching of the photogenerated carbene of 125I[Tdf1]AngII suggest that water is present in this outer receptor area. Furthermore, the AngII-hAT1 interactions narrow down to an exclusive ECL2 interaction at position 3 of AngII. The relatively permissive pharmacology of hAT1-CAM will allow for the systematic elucidation of AngII-hAT1 interactions, whereas WT-hAT1 is not accessible due to prohibitively low affinities of the mid-sequence photoprobes of the present series.

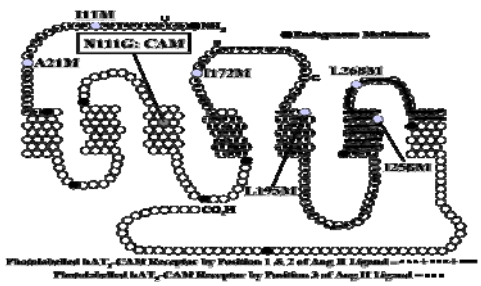

Figure 1. Schematic representation showing the interactions of positions1, 2 and 3 of AngII with its receptor hAT1.

Acknowledgments

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