Chemoenzymatic synthesis of sugar-related polyhydroxylated compounds, iminocyclitols and their derivatives as glycosidase inhibitors

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Instituto de Química Avanzada de Cataluña (IQAC)
Consejo Superior De Investigaciones Científicas (CSIC)

Facultad de Química, Universidad de Barcelona,
Departamento de Química Orgánica, Programa de Química Orgánica

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)
INSTITUTO DE QUÍMICA AVANZADA DE CATALUÑA (IQAC)

UNIVERSIDAD DE BARCELONA
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Memoria presentada por Alda Lisa Concia para optar al Título de Doctor por la Universidad de Barcelona

Dirigida por:
Dr. Jesús Joglar Tamargo
Dr. Pere Clapés Saborit

Tutor:
Dr. Francesc Rabanal Anglada

Doctoranda:
Alda Lisa Concia

A mio papà,
per avermi insegnato
l'importanza del sapere
e avermi trasmesso
la sua curiosità.

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Summary

Section 1 (**Introduction**) is a comprehensive review of the subjects discussed in this thesis: biocatalysis, aldolases and iminocyclitols. It contains a description of the application of dihydroxyacetone phosphate (DHAP) and dihydroxyacetone (DHA) utilizing aldolases to the chemoenzymatic synthesis of bioactive compounds and an introduction to the structure, biological activities and synthesis of iminocyclitols. Recent bibliographic references are included at the end of the section.

Section 2 (**Objectives**) outlines the aims of this thesis.

Section 3 (**Results and discussion**) describes the studies carried out in this thesis. Bibliographic references are included as an overview of previous results and to support our statements at the end of each chapter.

- **Section 3.1** deals with the application of D-fructose-6-phosphate aldolase in organic chemistry. It describes the chemoenzymatic preparation of polyhydroxylated compounds, sugars 1-deoxy-D-xylulose and 1-deoxy-D-*ido*-hept-2-ulose and iminocyclitols 1-deoxynojirimycin, 1-deoxymannojirimycin and *N*-alkylated derivatives, 1,4-dideoxy-1,4-imino-D-arabinitol and 1,4,5-trideoxy-1,4-imino-D-arabinitol. An unprecedented methodology, having as a key step a novel enzymatic aldol addition reactions catalyzed by D-fructose-6-phosphate aldolase, is presented.

- **Section 3.2** presents the cascade chemical-enzymatic synthesis of a collection of novel 1,4-dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB) 2-aminomethyl derivatives including 2-oxopiperazine conjugates which have an interest as potential glycosidase inhibitors.

- **Section 3.3** describes the chemoenzymatic synthesis of novel polyhydroxylated pyrrolizidines of the family of casuarines by means of an asymmetric strategy based on cascade aldol additions catalyzed by dihydroxyacetone phosphate (DHAP) and dihydroxyacetone (DHA) aldolases.

- **Section 3.4** describes the methodology and results of the preliminary *in vitro* assays for glycosidases inhibition activity of some of the compounds synthetized during the course of this thesis.

Section 4 (**Experimental**) describes the procedure for the experimental work carried out in this project. ^{1}H and ^{13}C NMR spectra can be found in the attached CD.

Contents

Publications

- Concia, A. L.; Lozano, C.; Castillo, J. A.; Parella, T.; Joglar, J.; Clapés, P., D-Fructose-6-phosphate aldolase in organic synthesis: cascade chemical-enzymatic preparation of sugar-related polyhydroxylated compounds. *Chem. Eur. J.* **2009,** *15* (15), 3808-3816.
- Concia, A. L.; Gomez, L.; Bujons, J.; Parella, T.; Vilaplana, C.; Cardona, P. J.; Joglar, J.; Clapés, P., Chemo-enzymatic synthesis and glycosidase inhibitory properties of 1,4 dideoxy-1,4-imino-D-arabinitol (DAB) and LAB 2-aminomethyl derivatives. *Org. Biomol. Chem.* **2012,** under submission.

Frequently used abbreviations

Cbz: Benzyloxycarbonyl

CFTR: Cystic fibrosis transmembrane conductance regulator

DAB: 1,4-Dideoxy-1,4-imino-D-arabinitol

DHA: Dihydroxyacetone

DHAP: Dihydroxyacetone phosphate

DMF: *N,N*-Dimethylformamide

DMJ: 1-Deoxymannojirimycin

DNJ: 1-Deoxynojirimycin

E. coli: Escherichia coli

ER: Endoplasmic reticulum

FSA: D-Fructose-6-phosphate aldolase

FucA: L-Fuculose-1-phosphate aldolase

GO: Glycolaldehyde

GP: Glycogen phosphorylase

HA: Hydroxyacetone

HPLC: High performance liquid chromatography

IBX: 2-Iodoxybenzoic acid

LAB: 1,4-Dideoxy-1,4-imino- L-arabinitol

LSDs: Lysosomal storage disorders

NJ: Nojirimycin

NMR: Nuclear magnetic resonance

RhuA: L-Rhamnulose-1-phosphate aldolase

TEA: Triethylamine

t-Bu or ^{t}Bu: *Tert*-butyl

wt: wild type

1. INTRODUCTION

1.1. BIOCATALYSIS AND CHEMOENZYMATIC PROCESSES:GENERAL CONSIDERATIONS

Biocatalysts are proteins (i.e., enzymes) evolved by nature to achieve the speed and coordination of all the chemical transformation necessary for life. The fact that isolated enzymes can also catalyze these reactions outside the living systems and accept unnatural substrate enables their application in synthetic chemistry.[1,2] Owing to the high efficiency and exquisite selectivity (either regio- and enantioselectivity) resulting from the chiral nature of enzymes, biocatalytic reactions are particularly suitable for the synthesis of highly functionalized chiral molecules with a strongly increasing demand. Enzyme-catalyzed reactions offer the possibility to build molecular complexity from simple starting materials often sidestepping the need for cumbersome protection/deprotection loops. Furthermore the mild condition required in enzymatic transformations and the use of water as solvent makes biocatalysis one of the greenest technologies which is able to fulfill the ongoing need for economically and environmentally more sustainable industrial processes.[3] Hence the safety, energy saving, raw material cost and environmental concerns in manufacturing of pharmaceutical and fine chemicals prompted the interest in discovery and development of novel biocatalytic reactions, particularly when no direct functional group transformation is known or as alternative to the classical synthetic procedures that lack selectivity or sustainability.[3] The advantages of using enzymes in organic synthesis are obviously accompanied by some disadvantages.[4] Not all substrates are readily soluble in aqueous solvent, the intrinsic specificity of enzyme limits the number of substrates of possible transformations and finally the enzymes can be costly and unstable. For these reasons many efforts have been devoted at a research scale to screen catalytic activity of natural enzymes with a broad range of substrate, to optimize the reaction conditions and to elucidate the precise catalytic mechanisms. Since the early studies in this area several classes of enzyme have been used in synthetic chemistry (e.g. Hydrolases, Oxydoreductases, Lyases, Glycosyltranferases, Transaminases) and a multitude of optimized reactions have been described.[2,4,5]

During the past two decades many biocatalytic steps have been introduced in large scale production with significant cost reduction and process simplification.[6-8] For instance the synthetic route of cortisone acetate was simplified from 31 to 5 steps by the introduction of biocatalytic transformations with the reduction of product cost from $200 to $6 per gram.[9]

In addition to the discovery of new natural enzyme by exploration of Earth's biodiversity, many studies are nowadays focused on the development of new biocatalysts by genetic engineering. In this sense directed evolution has emerged as a technology to overcome limitations, such as specificity or stereoselectivity, and to enhance the rate and conversion or to find new catalytic activities.[10] Recently a number of recombinant enzymes have been isolated significantly widening the toolbox for biotransformations.

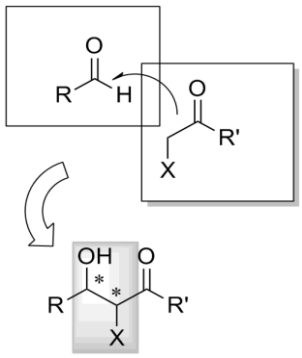
Among enzymes, aldolases are one of the most promising class of biocatalysts being able to construct chiral molecular frameworks from simple precursors. The features and applications of aldolases will be fully described in chapter 1.2.

1.2. ALDOLASES

1.2.1. Application of aldolases in organic synthesis

Aldolases are a specific group of lyases evolved by nature to catalyse aldol and retroaldol reactions in carbohydrate and amino acid metabolism.

The aldol reaction consists in the addition of an enolizable carbonyl compound to another carbonyl compound that is either an aldehyde or a ketone with the formation of one or two new chiral centres (Scheme 1).[11]



Scheme 1 Aldol addition.

The aldol addition reaction is one of the most powerful methods for carbon-carbon bonds formation that is of the utmost importance in organic chemistry since it enables the concomitant functionalization and creation of stereogenic centres. The control on the absolute configuration of the newly formed stereogenic centres has been a challenging goal in this methodology. Therefore a number of methods for the catalytic asymmetric aldol addition have been developed.[12]

In this context aldolases, being mild and selective, represent one of the greenest tools for the construction of molecular frameworks with a number of stereogenic centres also advantageous with regard to molecular economy (i.e., use of underivatized substrates).

Aldolases catalyse the reversible formation of C-C bonds by the aldol addition of a nucleophilic donor, typically a ketone enolate (or analog), onto an electrophilic aldehyde acceptor. Simultaneously, in most cases the stereochemistry at the newly formed stereocentre(s) is strictly controlled by the enzyme. Therefore, biocatalytic asymmetric aldol reactions result especially

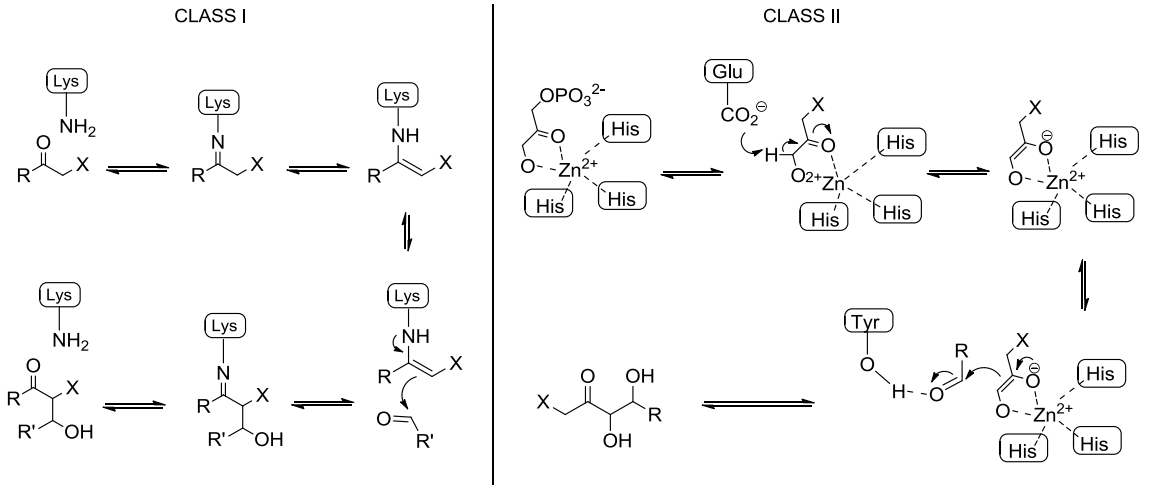
suitable for the synthesis of biologically relevant classes of compound, typically polyfunctional and water soluble such as aminoacids and sugars among other polyhydroxylated compounds.

For this reason a growing interest in synthetic application of aldolases has been fostered during the last three decades resulting in several dozen of natural aldolases identified, many of which are easily accessible by recombinant techniques on a scale sufficient for preparative applications.[13,14]

1.2.2. Classes of aldolases and their applications

Mechanistically, the aldolase catalysed aldol reactions involve the formation of a nucleophilic carbon from a carbonyl compound by stereospecific α-deprotonation and the attack to an electrophilic carbonyl carbon.

Depending on the nature of the active site and consequently on the mechanisms to activate the nucleophilic component, natural occurring aldolases are divided into two main groups: class I and class II (Scheme 2).

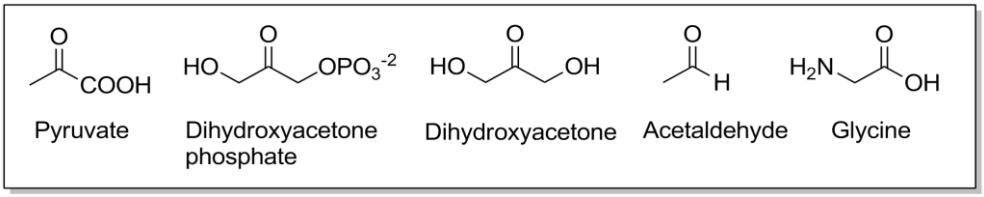


Scheme 2 Mechanism of Class I and II aldolases.

In the Class I aldolases, an enamine is produced by covalent binding to a conserved lysine residue, whereas in the Class II aldolases, an enediol is formed by chelating coordination to a transition metal cation (mostly Zn^{2+}), which acts as a Lewis acid promoter.[14]

Most aldolases can accept a broad structural variety of aldehyde acceptors, whereas they tolerate only small structural, isosteric modifications in the donor. Natural aldolases have been thus early classified according to their donor specificity and grouped into five categories (Scheme 3):

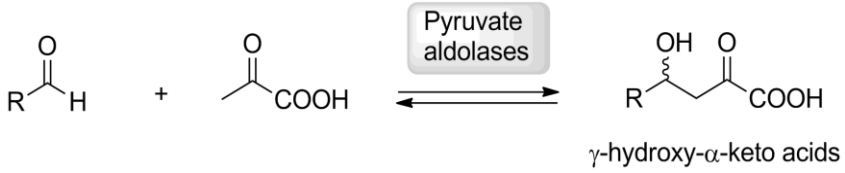
1) Pyruvate/(and phosphoenolpyruvate)–dependent aldolases
2) dihydroxyacetone phosphate(DHAP)-dependent aldolases
3) dihydroxyacetone (DHA)-dependent aldolases
4) acetaldehyde-dependent aldolase
5) glycine-dependent aldolase



Scheme 3 Nucleophilic donor substrates.

Even though this initial classification is still valid, other nucleophilic donors have been reported to be substrates for certain aldolases, such as hydroxyacetone, hydroxybutanone, glycoladehyde, 2-oxobutyrate, alanine and oxalacetate. Moreover the number of substrates is increasing thus varying significantly the concept of the strict donor specificity of aldolases.

Pyruvate dependent aldolases belong to a large family of enzymes that reversible catalyze the aldol addition of pyruvate to a variety of polyhydroxylated aldehydes yielding γ-hydroxy-α-keto acids (Scheme 4). They are usually type I aldolases that form an enamine intermediate with the pyruvate. There exist also type II (i.e. metal cofactor, enolate formation) pyruvate aldolases but have received less attention from the synthetic point of view.[15,16]

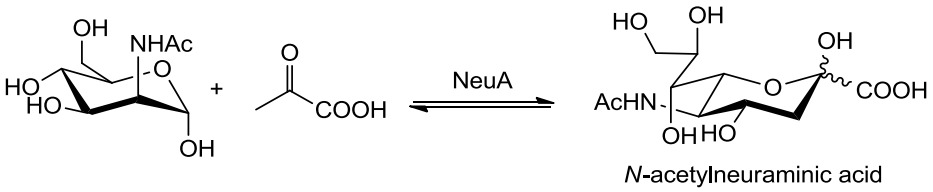


Scheme 4 General reaction catalyzed by pyruvate dependent aldolases.

In vivo, pyruvate-dependent lyases mostly serve a catabolic function in the degradation of sialic acids and their derivatives as well as other acidic carbohydrates. In spite of their catabolic physiological function, aldol additions catalyzed by pyruvate-dependent aldolases are reversible processes so that the equilibrium can be driven toward the aldol product by an excess of pyruvate and exploited for synthetic purpose.[13]

Examples of aldolase belonging to this family are the *N*-acetylneuraminic acid aldolase (NeuA), the 2-keto-3-deoxy-D-manno-octosonate aldolase (KdoA), the 2-keto-3-deoxy-6-phospho-D-gluconate (KDPGlc aldolase or GlcA) and 2-keto-3-deoxy-6-phospho-D-galactonate aldolases (KDPGal aldolase or GalA). These enzymes form a stereocomplementary set of pyruvate dependent aldolases each one with its acceptor preference profile.

Among pyruvate aldolases one of the most studied is *N*-acetylneuraminic acid (NeuA) also known as sialic acid adolase. This enzyme catalyzes the reversible addition of pyruvate to *N*-acetyl-D-mannosamine in the catabolic pathway of sialic acid (Scheme 5). It is highly specific for donor but tolerates a variety of acceptor and unlike most aldolases the stereochemical outcome depends on the structure of the substrate.

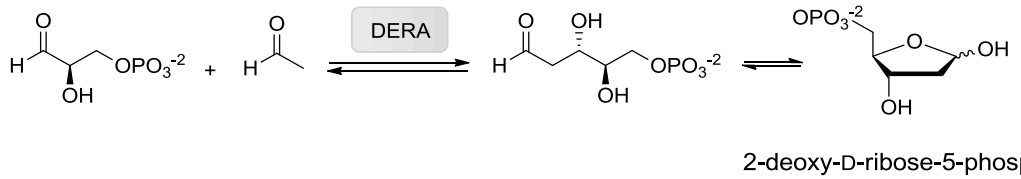


Scheme 5 Addition of pyruvate to *N*-acetyl-D-mannosamine catalyzed by NeuA.

The importance of natural occurring sialic acid-containing structures in a number of pathological processes, from bacterial and virus infection to tumor metastasis[17], prompted the interest in the application of NeuA for synthetic purpose.[18]

Many efforts have been directed toward the genetic evolution of NeuA to obtain mutants that tolerate substrate modifications with the aim of widening its utility as a catalyst.[19-22] NeuA and its mutants has been exploited to the stereoselective synthesis of various sialic acid mimics as neuraminidase inhibitors that possess anti-Influenza virus activity and is currently used for the industrial preparation of the antiviral Zanamivir.[23,24]

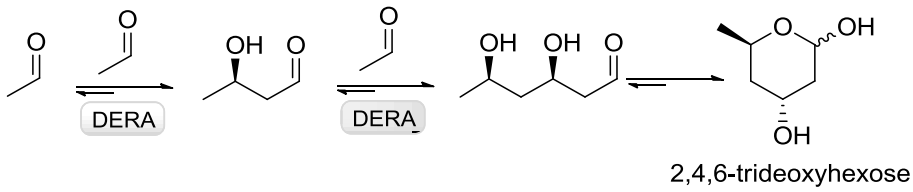
In the family of **acetaldehyde-dependent aldolases** there is currently only one member: 2-deoxy-D-ribose-5-phosphate aldolase (RibA or DERA). In vivo, it catalyzes reversibily the aldol addition of acetaldehyde to D-glyceraldehyde-3-phosphate to yield 2-deoxy-D-ribose-5-phosphate in the deoxyribonucleosides catabolic pathway (Scheme 6).



Scheme 6 DERA-catalyzed the reversible aldol addition of acetaldehyde to D-glyceraldehyde-3-phosphate.

In addition to acetaldehyde, propanal, acetone and fluoroacetone are also accepted as donor substrates but a very low rate. The enzyme shows a relatively broad range of tolerance towards unnatural acceptor substrates (2-hydroxyaldehydes, azidoaldehydes, thio substituted aldehydes) even though often at reduced catalytic rates.[25,26]

Furthermore DERA has been the first aldolase to perform self and cross-aldol reaction of two aldehydes. Synthetically, this is one the most attractive feature of DERA since the the first aldol addition furnishes another aldehyde that can serve again as an acceptor. Thus, it can be used by DERA, or in combination with other aldolases, for cascade aldol reactions (Scheme 7).[27-29]



Scheme 7 Two sequential aldol additions of acetaldehyde to acetaldehyde catalyzed by DERA.

Some successful examples have been reported in literature illustrating the utility of these enzymes in asymmetric synthesis. Synthetic applications of DERA range from preparation of various sugar derivatives, such as 2-deoxy, thio and aza sugars to the synthesis of key fragments of the anticholesterolemic atorvastatin[30] and anticancer agents epothilone A/C.[31]

Glycine dependent aldolases, namely serine hydroxymethyltransferase (SHMT) and threonine aldolases (ThrA) are pyridoxal-5'-phosphate dependent enzymes involved in biosynthesis and degradation of hydroxylated amino acids that catalyze the aldol addition of glycine/alanine to aldehydes.[32-35]

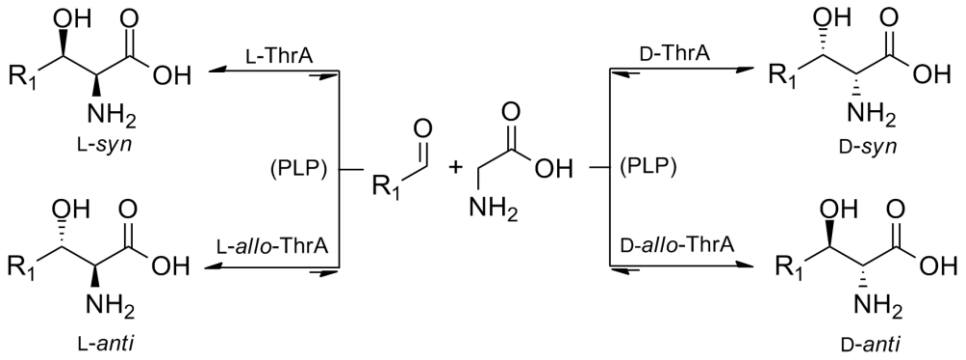
Since two new stereogenic centers are formed, four possible products can be formally obtained from a single aldehyde, depending on the specificity of the aldolase at the α- and β-carbons (Scheme 8).[36,37] With respect to α-carbon threonine aldolases have been divided into two types: L- and D-specific threonine aldolases.[32,36,37] L-specific threonine aldolases can further be divided into three sub-types according to their specificity at the β-carbon of threonine: L-threonine aldolase (LThrA), which preferentially cleaves L-threonine (L-Thr); L-allo-threonine aldolase (L-*allo*-ThrA), which cleaves L-allo-threonine (L-*allo*-Thr); and L-low specificity threonine aldolase which

accepts both L-Thr and L-*allo*-Thr as substrates. Regarding the D-series up to now only D-low specificity threoniβne aldolase (DTA) have been idendified in nature.[33]

Threonine aldolases tolerates a range of acceptor aldehydes with a complete control of the α-carbon stereochemistry but often with low stereoselectivity at the β-carbon that leads to a mixture of *syn*- and *anti*- products.[37,38]

A recent study by Griengl and coworkers has reported two new natural threonine aldolases that tolerate D-alanine, D-serine and, to a lower extend, D-cysteine (but not the L enantiomers) as donor substrates. The enzymes are L-allo-threonine aldolase from *Aeromonas jandaei* and D-threonine aldolase from *Pseudomonas sp.* and catalyze stereoselectively the retroaldol reaction of β-hydroxy-α-methylthreonine, an β-hydroxy-α,α-dialkyl-α-amino acid, to produce lactaldehyde and D-alanine.[39]

Although the natural retroaldolization was fully stereoselective, when both enzymes were investigated as catalysts for the aldol addition to a wide variety of aldehydes they showed moderate to low streoselectivity. This is a general drawback of known threonine aldolases.



Scheme 8 Threonine aldolases catalyzed reactions.

The synthetic application of threonine aldolases provides interesting routes to β-hydroxy-α-amino acids as well as for the chemical resolution of β-hydroxy-α-amino acid racemates.[38,40-42] β-Hydroxy-α-amino acids are an important class of compounds with an extensive pharmaceutical interest by virtue of their own biological activity and their presence in many naturally occurring complex compounds with antibiotic and immunosuppressant properties.[36,38,40,43] Furthermore they are excellent intermediates for the synthesis of several biologically relevant compounds and useful building blocks for other structures of biological interest.[44-48]

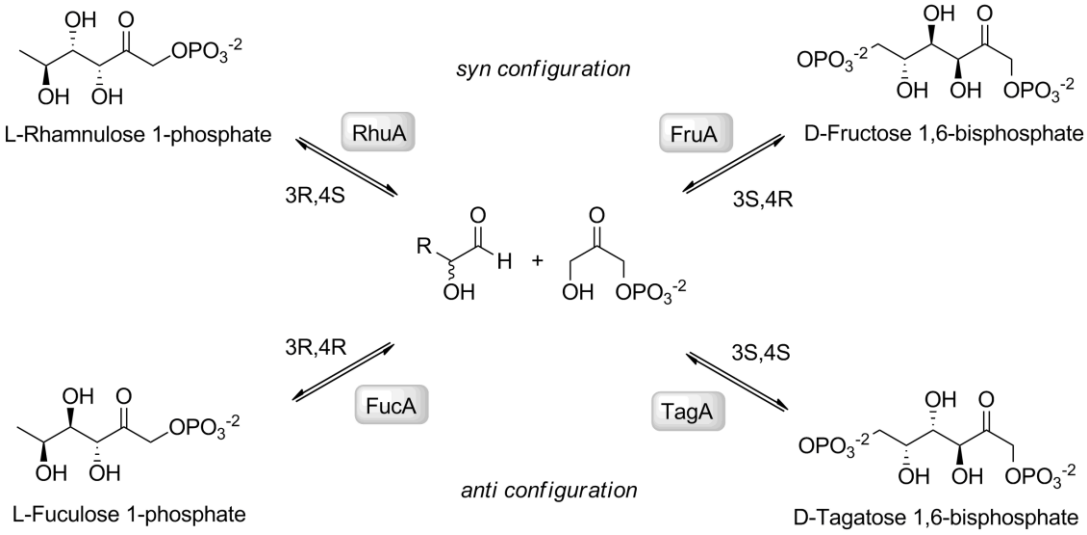
A very successful example for the application of L-ThrA as biocatalyst has been recently reported for the preparation at industrial scale of L-threo-3,4-dihydroxyphenylserine (DOPS), used for the treatment of Parkinson's disease.[49,50]

1.2.3. DHAP-dependent aldolases

Dihydroxyacetone phosphate (DHAP)-dependent aldolases comprise a family of lyases that catalyse the reversible aldol cleavage of ketose-1-phosphate or 1,6-diphosphate, a fundamental reaction in the catabolic pathway of mammalian and microbial carbohydrates.

Nature evolved four specific types of aldolases to cleave the four possible stereochemical combinations of the vicinal diol of ketose (Scheme 9):

- L-fuculose-1-phosphate aldolase (FucA)
- L-rhamnulose-1-phosphate aldolase (RhuA)
- D-fructose-1,6-bisphosphate aldolase (FruA)
- D-tagatose-1,6-bisphosphate aldolase (TagA)



Scheme 9 Stereocomplementary set of DHAP-aldolases and their natural substrates.

In the direction of synthesis these enzymes can catalyze the aldol addition of DHAP to a large variety of aldehyde acceptors. As a result, two new asymmetric centres are formed whose stereochemical configuration can be formally controlled by choosing one out four stereocomplementary DHAP-aldolases.[35] The high stereoselectivity and broad acceptor tolerance resulted especially attractive for asymmetric synthesis, particularly for the stereocontrolled synthesis of polyoxygenated compounds. Indeed, DHAP-aldolases have been successfully employed for the preparation of various sugar mimic such as deoxysugars and iminocyclitols.[34,51]

The stereoselectivity of the reaction catalyzed by DHAP-aldolase is mostly high and the C3/C4 configuration of aldol adducts follows that of the natural substrates, particularly at C3. However, the stereoselectivity at C-4 (i.e., the one generated from the addition to the aldehyde) may

depend on the acceptor structure and, in some instances, may be inverted generating thus diasteromeric mixture of products.[52]

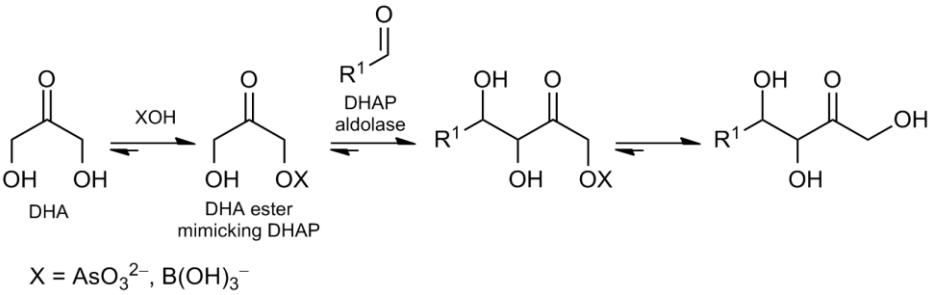
In spite of their tolerance for different acceptor substrate these enzymes are highly selective for the nucleophilic substrate DHAP, tolerating only few isosteric modifications.[16] This strict requirement for the phosphate moiety represents the major drawback for several reasons: DHAP is chemically unstable and difficult to prepare. It decomposes at basic pH and room temperature into inorganic phosphate and methyl glyoxal, both of which may inhibit the aldolase. DHAP degradation, can be considerably reduced by lowering the reaction temperature to 4°C, which constitutes an optimum between residual aldolase activity and minimum rate of DHAP loss.[53] Furthermore the phosphate group is generally not desired in the final product and has to be removed by enzymatic hydrolysis with acid phosphatase.

Many chemical methods for preparation of DHAP are available but most of them remain quite expensive and tedious often suffering from either low yields, complicated work-up, or toxic reagents or catalysts.[35,54]

In order to overcome this problem several enzymatic approaches for *in situ* generation of DHAP have been developed.[35,54,55] However, enzymatic routes have the drawbacks: to render more complex product mixtures, and to need the control over the activity of multiple enzymes within one reaction scheme.

Alternative strategies focused on eliminating the need for DHAP either by reaction engineering, directed evolution or exploitation of newly discovered enzymes have been explored to some extent.

Isosters of DHAP have been investigated in order to allow the acceptance of the inexpensive dihydroxyacetone (DHA) by existing DHAP-aldolases thus reducing costs and improving atom economy (Scheme 10).



Clapés, Fessner **2011**, Science of Synthesis, Vol. 2, pp 677-734.

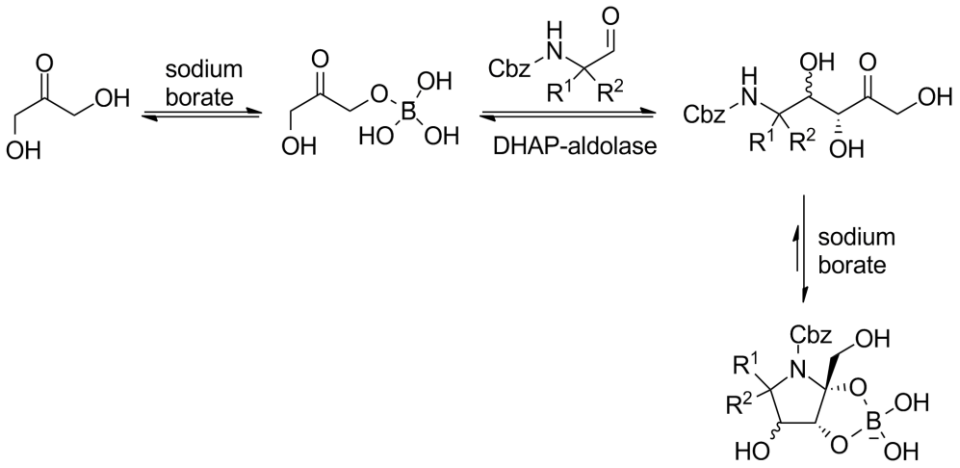
Scheme 10 In situ generation of dihydroxyacetone phosphate mimics from dihydroxyacetone.

Arsenate an vanadate esters of DHA, generated *in situ* with the inorganic salts, have been successfully used as donor for FucA, RhuA and FruA catalyzed reactions.[56,57] However, due to the chemical decomposition of vanadate esters and the toxicity of arsenate salts they are not

ideal alternatives. Moreover, the performance of these DHAP mimics was kinetically much lower than that of the original substrate.

The use of DHA-borate ester as phosphate ester mimic resulted much more convenient and was fully investigated. RhuA was the first DHAP-aldolase described to accept DHA as a donor substrate in the presence of borate buffer for the synthesis of L-sugars and L-iminocyclitols by reversible *in situ* formation of DHA-borate ester.[58]

Recent studies in our group reported that RhuA catalyzes with good conversion and stereoselectivity the aldol addition of DHA-borate ester to various *N*-benzyloxycarbonyl (*N*-Cbz)-aminoaldehyde.[59] Furthermore borate buffer seems to improve aldolase-catalyzed reaction yields by trapping products as borate complexes thus preventing the retroaldol cleavage (Scheme 11).



Scheme 11 Borate is able to improve reaction yields by *in situ* product trapping. The formation of vicinal borate diesters with the cyclic form of aldol adducts shift the reaction equilibrium.

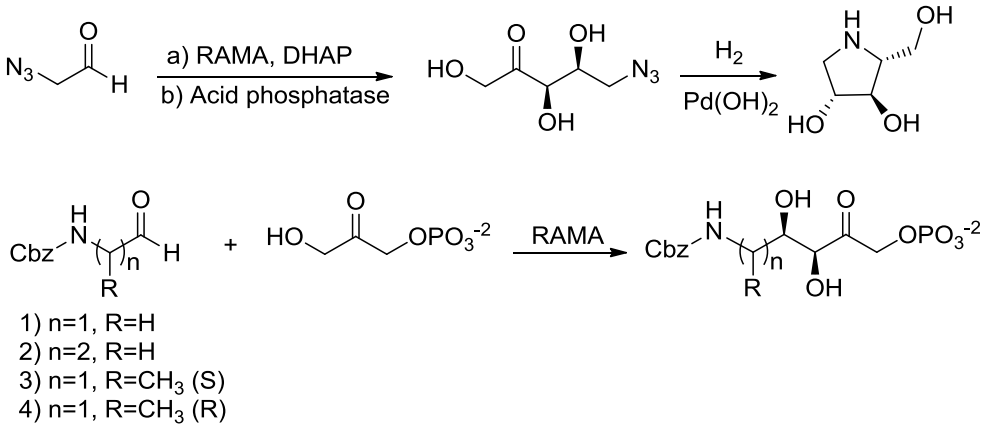
FucA from *E. coli* had no detectable activity with DHA-borate while accepted arsenate esters. Despite the same attempt to use DHA-borate ester with FucA failed, it was found that the use of boric buffer for FucA catalysis with DHAP improves both yield and speed reaction confirming the trapping role of boric salt in shifting the equilibrium position of the aldol addition.[59]

In the present work the use of boric buffer for the aldol addition of DHA to *N*-Cbz-glycinal with RhuA has been successfully applied for the synthesis of 1,4-dideoxy-1,4-imino-L-arabinitol (LAB) as described in section 3.2.

D-Fructose-1,6-bisphosphate aldolase (FruA or FDP) catalyses in vivo the reversible aldol addition of DHAP to D-glyceraldehyde-3-phosphate to form D-fructose-1,6-diphosphate with *3S/4R* stereochemistry. Both class I and class II enzyme were isolated from several mammalian and microbial sources and have been extensively investigated.[60] The class I FruA from rabbit muscle (RAMA) was the first assayed and one of the most studied DHAP-aldolase. It has been employed to catalyse the aldol addition of DHAP to various aldehydes with a high stereoselectivity

and it was the first DHAP-aldolase used for the preparation of iminocyclitols from azidoaldehydes (Scheme 12).[61-64]

In our research group RAMA was successfully employed to catalyzed aldol addition between DHAP and *N*-Cbz-aminoaldehydes (Scheme 12) in emulsion systems which is the key step for the preparation of structurally diverse polyhydroxylated pyrrolidines derivatives (See section 1.3.5).[65]



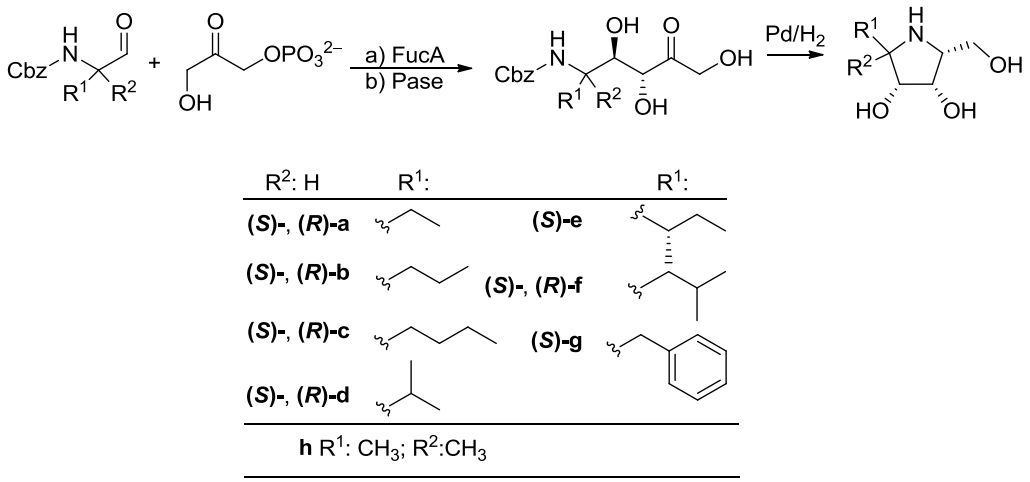
Scheme 12 RAMA catalyzed synthesis of 1,4-dideoxy-1,4-imino-D-arabinitol from azidoacetaldehyde[62] (top) and aldol addition between DHAP and *N*-Cbz-aminoaldehydes (bottom).[65]

L-Fuculose-1-phosphate aldolase (FucA) is a class II aldolase involved in the catabolism of L-fucose, a deoxysugar present in *N*-linked glycans in mammals, insects and plants. This enzyme has been extensively studied in aldol additions with a broad variety of aldehydes, and it has demonstrated to be a very stereospecific catalyst with most of the assayed acceptors.[66]

In our group, FucA from *E. coli* has been applied to the synthesis of a collection of iminocyclitols with inhibitory activity toward a series of glycosidases (Scheme 13).[67,68] The catalyst was assayed for the reaction between DHAP and a collection of alkyl and aryl substituted α-*N*-Cbz-amino aldehydes with different concentrations and reaction media (water-dimethyl formamide mixtures or emulsion). While C-α-alkyl linear substitutions on the acceptor aldehyde were tolerated, branched alkyl substitutions or the structurally restrained aldehydes *N*-Cbz-*(R/S)*-prolinal were not accepted. Dimethyl and benzyl substitutions were also tolerated with poor conversion. FucA was fully stereoselective regardless of the configuration of the aminoaldehyde.

To overcome the acceptor tolerance limitations of FucA, our group carried out the active site structure-guided engineering directed to facilitate the accommodation of sterically demanding acceptor aldehydes.[69] This work rendered a mutant, FucA Phe131Ala, which tolerates most of the sterically hindered aldehydes assayed as acceptors, and significantly improves the reaction rates with previously tolerated *N*-Cbz-amino aldehydes. FucA Phe131Ala has been thus applied the synthesis of a collection of polyhydroxylated pyrrolizidines from *N*-Cbz-prolinal derivatives.[69]

In the present work FucA Phe131Ala was successfully employed for the preparation of iminocyclitols of casuarine type which will be described in section 3.3.

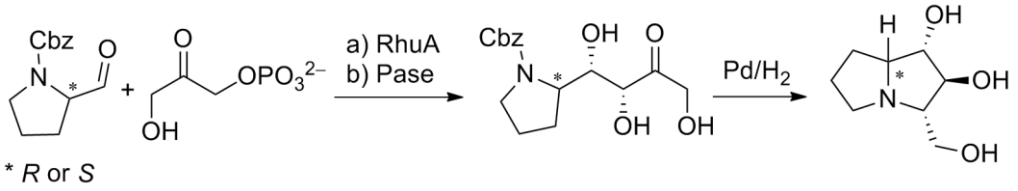


Scheme 13 Collection of alkyl an aryl substituted α-*N*-Cbz-amino aldehydes screened as substrates for FucA.[68] Pase: Phosphatase.

L-Rhamnulose-1-phosphate aldolase (RhuA) in vivo catalyzes the reversible cleavage of L-rhamnulose 1-phosphate, with *3R/4S* stereochemistry, to L-lactaldehyde and DHAP. This enzyme resulted thus a catalyst stereocomplementary to FruA. RhuA is a type II enzyme found in several microorganisms and structurally related to other bacterial carbohydrate-processing enzymes such as FucA from *E. coli.*

RhuA demonstrate to be a versatile catalyst and to accept a large structural diversity of acceptor including bulky aldehydes. As FucA, RhuA has been used in our lab for the preparation of iminocyclitols with inhibitory activity toward glycosidases through the catalysis of the aldol addition of DHAP to different *N*-Cbz-amino aldehydes (Scheme 13).[65,68] While the substrate tolerance was very high, the diasteroselectivity resulted dependent on both the structure and stereochemistry of the acceptor aldehyde. In fact, RhuA gave the expected *syn* products with the *(S)*-aminoaldehyde and mixtures of *syn*/*anti* configured aldol adducts with the *(R)*-aminoaldehyde.

Glycosidase inhibitors of the hyacinthacine and alexine (i.e., polyhydroxylated pyrrolizidines) types have also been prepared in our group by a concise two-step aldol addition of DHAP to *N*-Cbz-protected prolinal, catalyzed by RhuA from *E. coli*, followed by a reductive amination (Scheme 14).[70]



Scheme 14 RhuA mediated the synthesis of polyhydroxylated pyrrolizidine type iminocyclitols.

As mentioned earlier, RhuA was used with unphosphorylated DHA as donor in presence of borate buffer first by Wong and co-workers with α-hydroxy- and azido-aldehydes[58] and then by our group with *N*-Cbz-amino aldehydes.[59]

N-Cbz-amino aldehydes appeared more suitable acceptors for RhuA catalyzed addition to DHA-borate ester providing high yields (e.g. 70–90%) with excellent stereoselectivity (>98 : 2 syn/anti) for most *S* or *R* configured acceptors which resulted improved respect to the reactions performed with DHAP. The stereochemical outcome depended on the *N*-Cbz-amino aldehyde enantiomer: the *S* acceptors gave the syn *(3R,4S)* aldol adduct whereas the *R* ones gave the anti *(3R,4R)* diastereomer.

It was also reported by us that RhuA can accept DHA as donor without the addition of borate buffer. However, the presence of borate enhanced the reactivity by ~10-fold.[71] In order to improve the reactivity of RhuA toward DHA without the need of borate we endeavor to alter the donor substrate specificity by means of protein engineering. Therefore a collection of mutants of the phosphate binding site of RhuA was explored. The main residues interacting with the phosphate group of DHAP were replaced independently for aspartate, with the aim to establish new polar contacts that may stabilize DHA. Among the five mutants assayed RhuA Asn29Asp was found hyperactive for the DHA addition to *N*-Cbz-amino aldehydes.[71]

D-Tagatose-1,6-bisphosphate aldolase (TagA) is responsible for the degradation of D-tagatose-1,6-bisphosphate to give D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

The aldolase accepts a range of unphosphorylated aldehydes as substrates but yields diastereomeric mixtures only. Having the poorest stereocontrol among all DHAP-aldolases its synthetic utility is limited.[16] However, with suitable protein engineering, this stereoselectivity may be improved in the future, and this improvement would allow the generation of all four possible C3/C4 diastereomeric adducts by these four aldolases.

1.2.4. DHA-dependent aldolases

The selectivity for the nucleophilic substrate DHAP is one of the main drawbacks of the DHAP-aldolases that hamper their practical preparative application, particularly at industrial scale. In this regard the discovery of new aldolases that naturally accept unphosphorylated substrates is of paramount importance.

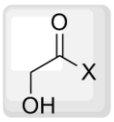
D-Fructose 6-phosphate aldolase (FSA) from *E.coli* is the first and unique natural dihydroxyacetone-utilizing aldolase described to date. This enzyme, first reported by Schurmann and Sprenger in 2001[72], catalyses the reversible formation of fructose-6-phosphate from DHA and 3-phosphoglyceraldehyde. Its physiological role is not yet clear while its structure and biochemical properties have been thoroughly studied.[73] Because of the striking advantage of

avoiding the use of phosphorylated nucleophiles FSA has appeared since its finding as an extremely promising catalyst.

The use of DHA as donor for the preparation of unphosphorylated targets simplifies the synthetic strategy avoiding the manipulation of the phosphate moiety and fostering the methodology to industrial application.

FSA has found several synthetic applications and showed to accept a number of structurally diverse acceptor aldehydes exhibiting an exquisite D-*syn* stereochemical outcome for all reported substrates. One of the first applications was described by our group in 2006 with the synthesis of D-fagomine[74] and many other products have been obtained by aldol reactions catalyzed with FSA.[75,76]

Further studies, including the one that will be described in this thesis, reported that FSA tolerates ketone donors different from DHA such as hydroxyacetone (HA) and hydroxybutanone (HB) while keeping unaltered the stereoselectivity (Scheme 15).[76]



X = H, CH_3, CH_2OH, CH_2CH_3

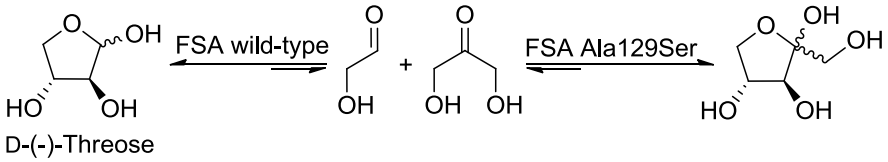
Scheme 15 Requirement of the hydroxymethylcarbonyl moiety for diverse FSA donors.

The donor promiscuity of FSA is not limited to ketones. Glycoaldehyde (GO) was indeed found to act as nucleophile for the aldol addiction with various aldehydes thus widening the possibility for synthetic applications.[77] This novel activity of FSA allowed the synthesis of aldose-type sugars by asymmetric self and cross-aldol addiction of glycolaldehyde in water, an unprecedented reaction in both bio- and organocatalytic reactions. Moreover, being the products aldehydes itself, they can be used as substrates for cascade reactions with further aldol additions.

Comparing the conversions of aldol additions and FSA reactivity for the donors they appeared higher for HA and GO while DHA was the worse donor. The low affinity for DHA was demonstrated also by the only product formed when DHA and GO were mixed in the presence of FSA which was self aldol product of GO (i.e., D-threose) (Scheme 16). FSA variants have been explored in order to improve the catalytic performance and broaden the range of possible substrates.[78-80]

Ala129 was identified as putatively involved in the binding site of the C-1 hydroxy group of the donor substrate.[73] In the course of investigations on the FSA function and mechanism it was found that the FSA mutant Ala129Ser exhibited improved tolerance towards DHA and turned out an efficient tool in the preparation of carbohydrates and analogues.[79] The aldol addition of DHA

to GO to give to D-xylulose with 80% aldehyde conversion also demonstrate the higher affinity for DHA of Ala129Ser respect to FSA wt (Scheme 16).



Scheme 16 Self and cross aldol addition of GO and DHA catalyzed by FSA wt and FSA Ala129Ser, respectively.

In our lab, site-directed mutagenesis of the acceptor binding site of FSA wild type and Ala129Ser was oriented to improve the tolerance towards *N*-Cbz-aminoaldehydes for the preparation of iminocyclitols.[80] The mutant FSA Ala165Gly was found to enhance the activity with previously tolerated acceptors and made possible the reaction with α-substituted *N*-Cbz-aminoaldehydes. This mutant has been successfully employed in this work for the optimized synthesis of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) described in section 3.2.

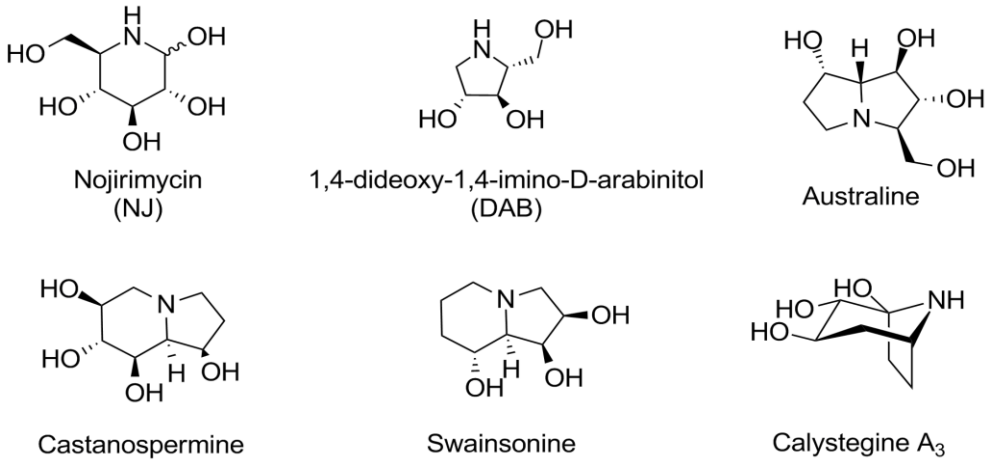
A double mutant FSA Ala129Ser/Ala165Gly was also prepared in our group and resulted to have an activity between 5- to >900-fold higher than that of wild-type for reactions with both HA and DHA donors and *N*-Cbz-aminoaldehyde derivatives. Further works to ascertain the substrate scope of FSA Ala129Ser/Ala165Gly and other mutations in the acceptor binding site are currently in progress in our group.

Another enzyme possessing DHA-dependent aldolase activity was found as a result of a mutagenic study on the transaldolase B (TalB) from *E. coli.*[81] This transaldolase catalyzes the reversible transfer of a dihydroxyacetone moiety from fructose-6-phosphate to erythrose-4-phosphate, yielding sedoheptulose-1,7-bisphosphate. Despite it has a high homology to FSA it can only transfer a dihydroxyacetone moiety from a ketose donor onto an aldehyde acceptor but doesn't have direct aldolic activity. Sprenger and coworkers reported a mutant of this enzyme, the TalBF178Y which is able to use DHA as a substrate directly in aldol reactions with an efficiency similiar to FSA.[81] Recent mutagenic studies aimed at improving the affinity of this variant towards unphosphorylated acceptor aldehydes by modification of the acceptor binding site.[82-84]

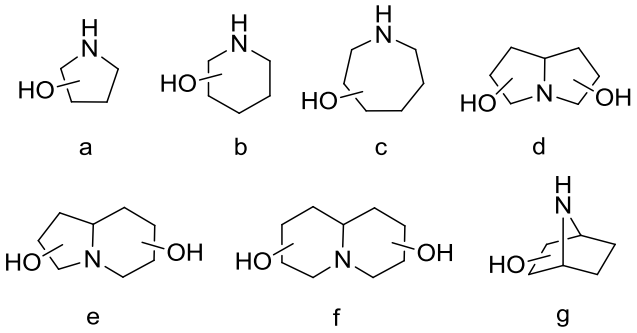
1.3. IMINOCYCLITOLS

1.3.1. Definition and historical background

Iminocyclitols, alternatively known as azasugars or iminosugars, are polyhydroxylated cyclic compounds mimicking sugars in which the endocyclic oxygen is substituted by a nitrogen atom. Due to the structural similarity with natural mono- and disaccharides they can easily interact with a wide range of sugars-handling processes thus providing a great opportunity to broaden our knowledge and comprehension about the role of carbohydrate in functioning of biological systems.[85]



Scheme 17 Examples of naturally occurring iminocyclitols.



Scheme 18 Common structural motifs of natural and synthetic iminosugars a: pyrrolidines; b: piperidines; c: azepanes; d: pyrrolizidines; e: indolizidines; f: quinolizidines; g: notropanes.

The increasing understanding in glycobiology during the past 20 years together with the isolation of many naturally occurring iminocyclitols fuelled the extensive research in this area. Early studies in this field dates 40 years ago when the polyhydroxylated alkaloid 5-amino-5-deoxyglucopyranose was isolated from *Streptomyces nojiriensis* (Scheme 17). After characterization it was given the name of nojirimycin (NJ) and was found out to have important

inhibitory activity towards glycosidases.[86] To date over 100 compounds with large variety of monocyclic and bicyclic structures have been isolated from natural sources like plants, bacteria and fungi. Based on the core structural skeleton they are classified into seven classes: polyhydroxylated pyrrolidines, piperidines, azepanes, indolizidines, quinolizidine, pyrrolizidine and nortropanes (Scheme 18).

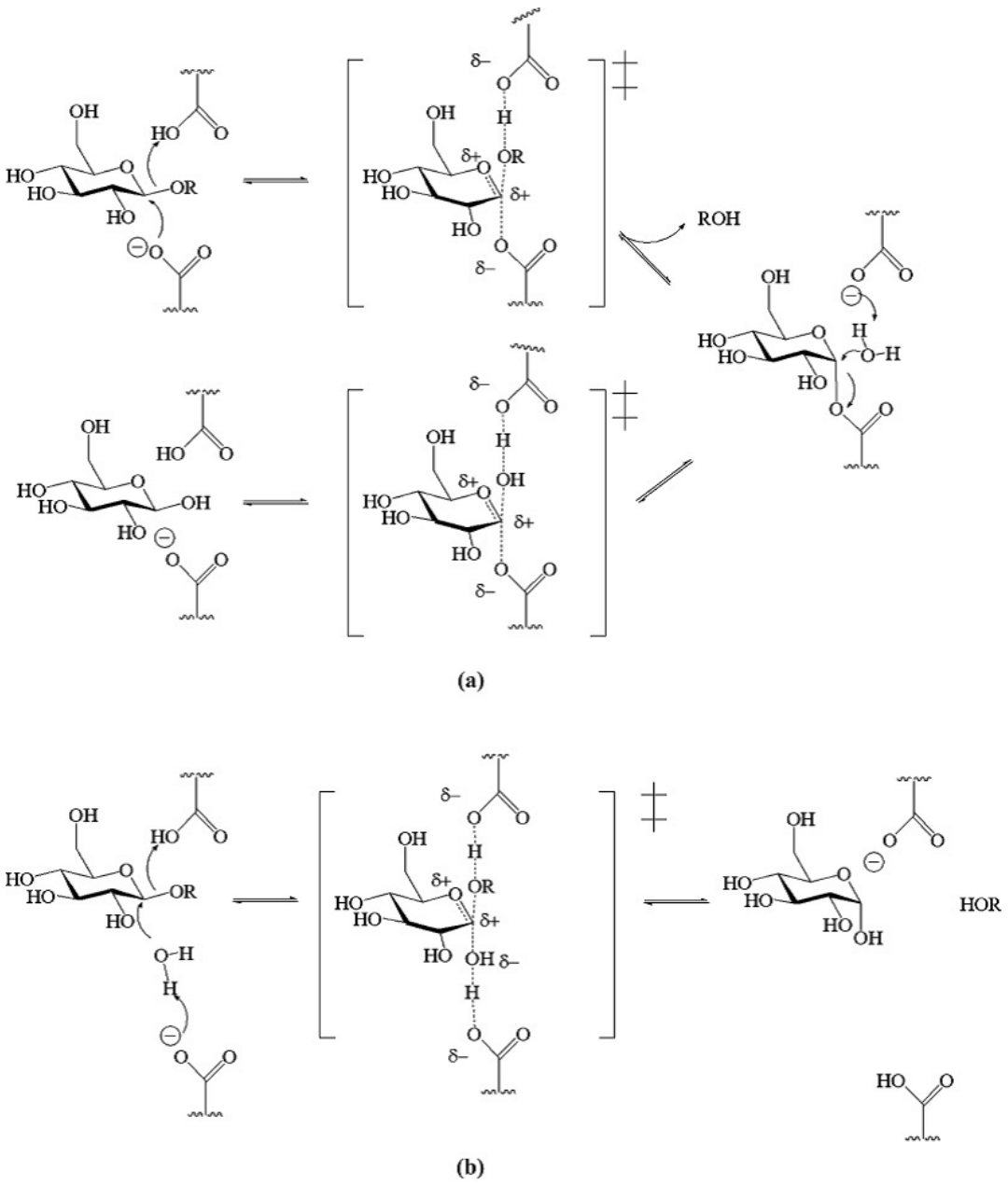
1.3.2. Biological properties

Iminocyclitols are an attractive class of glycomimetics. Most of them show powerful inhibition or modulation of the activity of several carbohydrate-processing enzymes such as glycosidases and glycotranferases and interference with the function of other carbohydrate-recognizing protein.[85,87]

Glycosidases are a group of enzymes responsible for the specific hydrolysis of the glycosidic bond in di-, oligo- and polysaccharides and glycoconjugates. The general mechanism of action of glycosidases (and glycotransferases) was first proposed by Koshland in 1953[88] and, with only few exceptions[89-91], has been confirmed by vast amount of biochemical investigation.[92-94] The hydrolysis of glycosides (and the transglycosylation) can be regarded as a nucleophilic displacement reaction at the anomeric carbon atom of the glyconmoiety.[95] The reaction can proceed with two possible stereochemical outcomes: inversion or retention of the anomeric configuration of the product respect to the substrate. Both mechanisms involve oxacarbenium-ion-like transition states and a pair of carboxylic acids (although there are exceptions) at the active site of the enzyme (Scheme 19).[96-98] In inverting glycosidases, the reaction occurs *via* a single-displacement mechanism wherein one carboxylic acid acts as a general base and the other as a general acid. In retaining enzymes, the reaction proceeds *via* a double displacement mechanism with the formation of a glycosyl enzyme intermediate and two consecutive inversions.

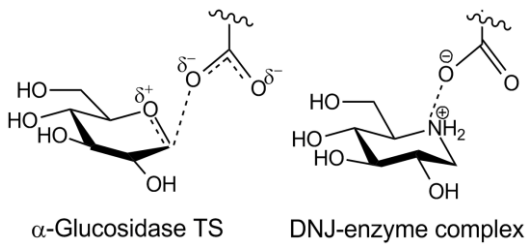
Iminocyclitols activity toward glycosidases lay in the capacity to mimic the hydrolysis transition state of sugar moieties during glycosidase catalysis due to protonation of endocyclic nitrogen atom at physiological pH conditions.[98] The configuration of the hydroxyl groups as well as position of the cationic centre, geometry and charge distribution, ring size and flexibility are determinant for selectivity of iminosugars for different enzymes (Scheme 20).[95]

Even though the proposed model remains largely unchanged, the precise mechanism and the nature of the transition state for each glycosidase are still under study. The evaluation of effects of systematic structural variations on inhibitory potency and specificity of glycomimetics, as iminocyclitols, is extremely profitable to advance our understanding of mechanisms of catalysis on an empiric basis. Hence, iminocyclitols as transition state-mimics constitute valuable probes in biochemical studies of glycosidase mechanism.[92]



Rempel, B.P. and S.G. Glycobiology, **2008** 18(8) 570-586

Scheme 19 (a) Mechanism for a retaining β-glucosidase. (b) Mechanism for an inverting β-glucosidase.



Scheme 20 α–Glucosidase transition state mimicry of deoxynojirimycin (DNJ).

Glycosidases are crucially important in a plethora of biochemical pathways, such as carbohydrate digestion in intestinal tract, lysosomal catabolism of glycoconjugates and post-translational glycoprotein processing. It is estimated that more than 50% of human identified glycosidases are involved in diseases and metabolic pathologies. Glycosilation disorders affect cell-cell communication, cell-matrix interaction and immunological response and are related to diseases such as cancer, viral infections, non-insulin-dependent (type II) diabetes, cystic fibrosis, rheumatoid arthritis, lysosomal storage disorders among others. For this reason iminosugars are regarded as extremely useful tools for basic glycosylation studies and therapeutic purpose.

1.3.3. Therapeutic applications and mode of action

The medical application of iminocyclitols goes back to ancient times and traditional phytomedicines in which the use of plants extremely rich in polyhydroxylated alkaloids was recommended for the treatment of several diseases.[99,100] As mentioned earlier the large number of carbohydrate involving pathologies confers to iminosugars an enormous therapeutic potential.

Although the biological effects of iminocyclitols are largely due to the inhibitory activity towards glycosidases, new enzymatic targets are continuously emerging. Indeed, in some instances the observed biological activity of iminosugars could not be totally elucidated by glycosidase inhibition. Additional modes of action are often uncertain therefore the study of biological effects unrelated to glycosidase inhibition is of utmost importance to the emergence of new therapeutic options and to the comprehension of less well understood pharmacological pathways. For example the traditional targets for the use of iminosugars in oncology were enzymes involved in cell-surface glycoconjugates processing such as glucosidases, mannosidases and fucosiltranferases. Many efforts have been devoted in searching for more potent and selective inhibitors of these glycoprocessing enzymes. Nevertheless the number of anticancer effects observed for different iminocyclitols (e.g., growth suppression, antimetastatic effect, immunological regulation, antiangiogenic activity, apoptosis induction) suggested the existence of different modes of action that may or may not be directly related to glycosidase inhibition. New targetable pathways for iminosugar cancer therapy are currently under study such as extracellular matrix degradation[101] or immune system response.[102,103]

The increasing amount of carbohydrate-related targets for iminosugars, such as soluble proteins, inositol processing enzymes, carbohydrate receptors and polar active sites, together with the emergence of new mechanisms different form inhibition such as carbohydrate-recognizing proteins folding assistance, are continuously widening the range of their potential medical applications.[104]

Furthermore water solubility, chemical stability, good absorption, due to the possibility to take advantage of carbohydrate cellular transport mechanism,[105] and highly oxygenated structure

that confers metabolic stability are advantageous intrinsic properties that endow iminosugars with a favourable position as drug candidates.[104]

Hence, iminocyclitols as potential therapeutic agents have gained the interest of pharmaceutical industry. Using naturally occurring alkaloid derivatives found in plants as lead compounds, chemical libraries were created with the aim to discover new therapeutically beneficial molecules, in some instances culminating in clinical trials in humans.

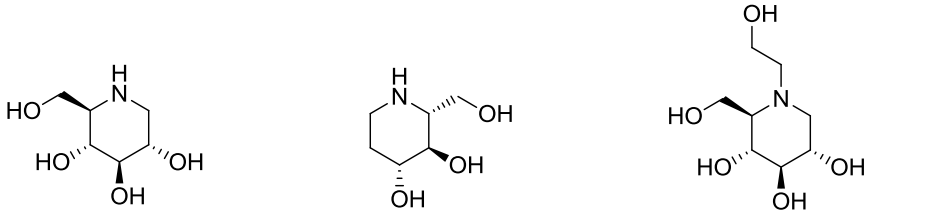
The main therapeutic applications of iminocyclitols are described here below:

a) Diabetes

Compounds that inhibit digestive glucosidases can establish glycemic control over hyperglycemia and they are potential candidates as oral anti-diabetic drugs.

Persistent hyperglycemia from carbohydrate abuse may lead to insulin resistance that may develop into type 2 diabetes, elevated fasting blood glucose and excess weight. Insulin resistance, increased waist circumference, dyslipidaemia and high blood pressure are factors in the so-called metabolic syndrome.[106]

Some natural iminosugars like deoxynojirimycin (DNJ) (Scheme 21) and castanospermine (Scheme 17) can reduce glucose absorption from the gut as a consequence of inhibition of intestinal disaccharidases (e.g., sucrose, lactase, maltase) and thus delay post-prandial hyperglycemia.[107] Recently our research group described that D-fagomine (Scheme 21) lowers postprandial glycaemia in rats following the intake of sucrose and starch probably by delaying saccharide hydrolysis by brush-border glycosidases.[108] However, any effect on postprandial glycaemia after intake of glucose neither on insulin secretion was observed.



Scheme 21 Iminosugars with antihyperglycemic properties.

Other iminocyclitols are able to regulate blood glucose level by controlling glycogenolysis through the modulation of the activity of glycogen-processing enzymes such as glycogen phosphorylase (GP), amylo-1,6-glucosidase and glycogen synthase.[109-112] 1,4-Dideoxy-1,4-imino-D-arabinitol (DAB), which chemo-enzymatic synthesis is object of this thesis, is the powerful α-glucosidase inhibitor. DAB has been also reported to inhibit GP, the enzyme responsible for the breakdown of glycogen into glucose monomer in muscle and liver tissues, and it has been investigated as potential anti-diabetic agent.[112-114] The synthetic derivative *N*-hydroxymethyl-DNJ (Miglitol,

Glyset®, Scheme 21) inhibits intestinal α-glucosidases and glycogenesis and is an approved marketed drug currently used for treatment of diabetes type 2.[115]

Beside, recent research in the regulatory role of glycosphingolipids in pathogenesis of insuline resistance, suggest that tailored iminosugars can act as anti-diabetic with others mechanisms related to modulation of lipid metabolism.[116-118]

b) Cancer

Cell surface glycoconjugates such as glycoprotein and glycolipids are involved in diverse cell function including modulation of cell-cell communication and recognition, cell adhesion, differentiation and transport. It is well established that alterations in cellular repertoire of *N*-glycans on surface glycoprotein are directly associated with malignant transformation and tumour progression.[119] Tumour aberrant glycosylation can be modified by inhibition or modulation of carbohydrate processing enzyme inside the Golgi apparatus and endoplasmic reticulum. Glycosidases and glycotrasferases involved in *N*-glycans biosynthesis (e.g., Glucosidase I and II, Golgi mannosidase I and II, *N*-acetylglucosaminyltransferase, fucosyltransferase) represent thus a potential target for cancer therapy and their inhibition may provide a basis for the use of iminosugars as anti-tumoral agents.

Some specific processing glycosidase inhibitors have been evaluated in preventing tumour growth and colonization through targeting this pathway. For instance the α-glucosidase inhibitor castanospermine (Scheme 17) exhibited anticancer effects like inhibition of tumour cell growth, metastasis and angiogenesis.[120] Swainsonine (Scheme 17), a potent Golgi α-mannosidase II inhibitor, has also been widely described to prevent tumour cell invasion and metastasis and has shown numerous other anticancer effects.[121] It is currently thought that the antimetastatic activity is a combination of different mechanisms in addition to the known interference with *N*-glycan synthesis, especially the enhancement of cell mediated killing through the innate immune system. The research on swainsonine anticancer activity thus opened a new possibility for antineoplastic use of iminosugars based on tumour immunological regulation.[122,123]

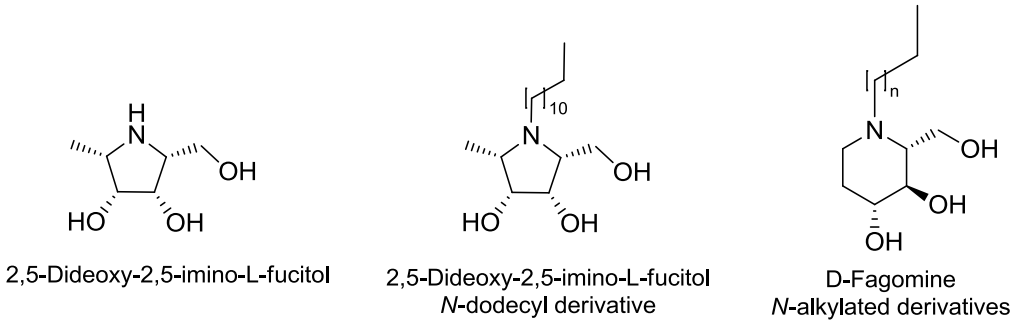
The enzymes involved in extracellular matrix degradation by tumor cells, an event which occurs at various stages of the metastatic process, have also been identified as target for iminocyclitols. Examples of these glycosidases are heparanase[124,125], heparan sulfate 2-O-sulfotransferase[126], metalloproteinases[127,128] and some hexosaminidases (i.e., β-*N*-acetyl-glucosaminidase, α-*N*-acetyl-galactosaminidase).[129] Inhibitors of these enzymes demonstrated to have effective anticancer effects.[130,131]

L-Fucose residue in sialyl Lewis X oligosaccharide, which is overexpressed on the surface of some tumor cells, is essential for their adhesion to the endothelial-leukocyte adhesion molecules. High activity and aberrant distribution of α-fucosidases have also been observed in cancer cells.[132-134] It was suggested that fucosidase in invasive human carcinoma cell mediates degradation of the

subendothelial extracellular matrix.[135,136] Therefore, fucosidation patterns are associated to the metastasic process.

Iminocyclitols that inhibit fucosidase and fucotransferase can be useful to avoid the metastasis of cancer cells. Several pyrrolidine and piperidine type iminosugars were described as inhibitors of α-L-fucosidase.[137-145] Recently our research group has reported the synthesis and biological evaluation of several pyrrolidinic iminocyclitols with inhibitory activity toward α-L-fucosidase from bovine kidney.[67,68] Among all, the most potent inhibitor was 2,5-dideoxy-2,5-imino-L-fucitol (Scheme 22).

In a further study 2,5-dideoxy-2,5-imino-L-fucitol and *N*-dodecyl derivative as well as D-fagomine (Scheme 21) and *N*-alkylated derivatives, that have been previously sinthesized by our group[74], were tested in several human cancer cell lines.[146] The cytotoxic effect and the associated phenotypic alterations were evaluated. Furthermore the activities of specific glycosidases on cell lysates were analyzed in order to verify the inhibitory effect. Results indicate that the longer *N*-alkylated chain attached to the iminosugar induced the higher cytotoxicity and the higher inhibition on glycosidase activities in cell lysates, although the glycosidase specificity for each compound remains unaffected.



2,5-Dideoxy-2,5-imino-L-fucitol

2,5-Dideoxy-2,5-imino-L-fucitol
N-dodecyl derivative

D-Fagomine
N-alkylated derivatives

Scheme 22 Iminocyclitols with inhibitory activity toward α-L-fucosidase

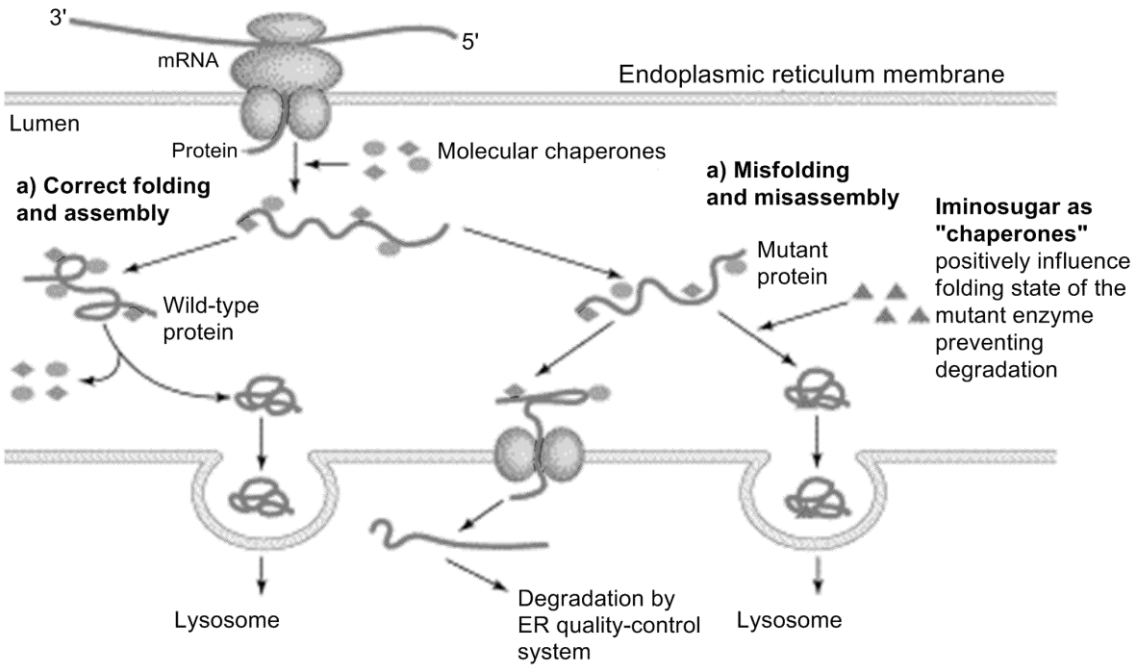
c) Lysosomal storage disorders

Lysosomal storage disorders (LSDs) are a relatively rare group of inherited metabolic diseases resulting from the anomalous catalytic activity of enzymes responsible for degradation of macromolecules such as glycogen, glycosphingolipids and glycoproteins.[147]

Genetic defects in enzymes of catabolic pathways lead to lysosomal storage of undegraded material that produces various symptoms depending upon the nature of accumulated substance and affected tissues.[148] More than 50 human disorders related to genetic enzymatic defects have been identified and characterized among which some glycosphingolipidoses (GSLs) caused by mutations in catabolic glycosidases can be treated with iminocyclitols.[149]

Different approaches have been followed in the pursuit for therapy for LSDs such as enzyme replacement therapy, bone marrow transplantation and substrate reduction therapy.[150] Iminocyclitols have first found therapeutic utility in substrate reduction therapy based on the control of glycolipids accumulation by inhibition of enzymes involved in their biosynthesis. One of

these key enzymes is the ceramide glucosyltransferase that is responsible for the synthesis of many glycosphingolipids and can be inhibited by different *N*-alkylated iminosugars.[151] *N*-butyl-deoxynojirimycin (miglustat, Zavesca®) (Scheme 23) is approved for treating type I Gaucher's and Nieman-Pick type C disease (NPC).



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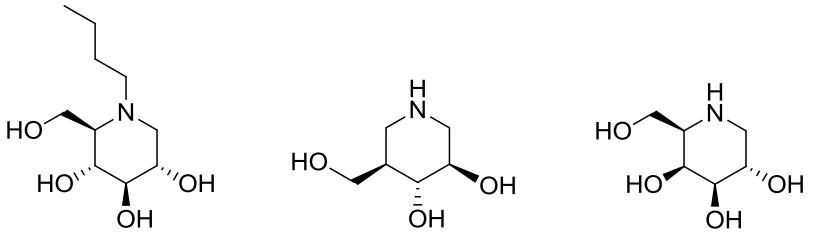


Scheme 23 Top: Folding and trafficking pathways of proteins in ER and iminosugars intervention as pharmacological chaperones. Bottom: Iminocyclitols with Active-Syte-Specific-Chaperone activity for LDSs treatment.

An alternative approach for the reduction of the threshold concentration of GSL in the lysosome to non-pathological level is correcting the folding and trafficking pathways of the mutant protein in order to avoid the degradation quality control system in the endoplasmic reticulum and thereby restore or partially increase their activity (Scheme 23). This strategy has been named Chaperon Mediated Therapy. Some iminosugars at sub-inhibitory concentration have shown the capacity to bind lysosomal hydrolases and enhance the stability of the mutant, thus inducing the functional conformation of misfolded enzymes. This Active-Syte-Specific-Chaperone activity of iminocyclitols is the base for the development of a second generation candidates for LDSs treatment some of

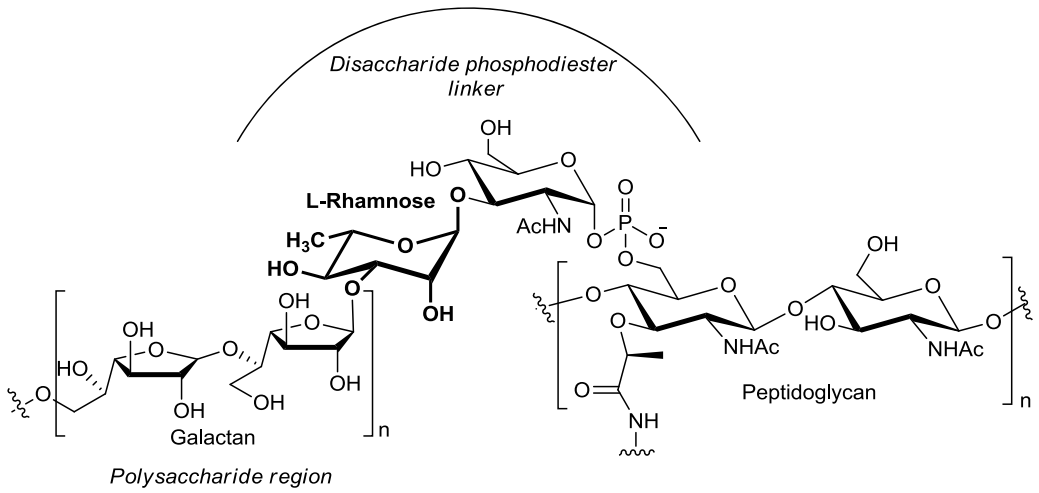
which are demonstrating favorable pre-clinical and clinical results: Iso-fagomine (Plicera®) for Gaucher's disease and 1-deoxygalctonojirimycin (Migalastat, Amigal®) for Fabry's disease (Scheme 23).[152]

d) Infections

Like eukaryotic cells carbohydrates and glycoconjugates play an important role in life cycle of pathogenic organisms and mediate a wide variety of infective processes such as host cell invasion and immune system evasion. The considerable differences between mammalian and microbial carbohydrates provide several possibilities for therapeutic intervention. Anti-infective therapy takes advantage of these differences by specific inhibition of enzymes expressed in an infective organism but not in the host.

Several natural and synthetic iminocyclitols have shown antibacterial properties through inhibition of essential microbial biosynthetic pathways or suppression of cytotoxic effects of associated toxins.[153] Examples of specific enzymes are bacterial transglycosilase (TGase), UDP-galactopyranosemutase (Gal-mutase) and naringinase, all of which have been targeted by iminosugar-based inhibition.[154-156]

The mycobacterial cell wall consists of an outer lipid layer and an inner peptidoglycan layer essential for cell growth and survival in the host.[157] The outer layer is composed of lipids known as mycolic acids that are esterified to the non-reducing terminal arabinosyl residues of the polysaccharide arabinogalactan. The reducing end of arabinogalactan polysaccharide is connected to the peptidoglycan via the disaccharide phosphodiester linker, containing an L-rhamnosyl residue (Scheme 24).



Scheme 24 *Mycobacterium tuberculosis* cell-wall glycans and linker region.

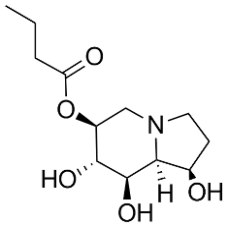
It has been demonstrated that the synthesis of this linker region would be essential for mycobacterial viability.[158] Since L-rhamnose is not found in mammalian cells, inhibition of

L-rhamnose-processing enzymes is a striking drug target opportunity for the treatment of mycobacterial infections such as tuberculosis.[159]

Some iminocyclitols have shown to possess rhamnomimetic property and to inhibit L-rhamnose processing enzymes such as naringinase.[156,160-163] In chapter 3.2 we described the synthesis and characterization of 2-aminomethyl-polyhydroxylated pyrrolidines that demonstrated inhibitory activity toward α-rhamnosidase from *Penicillinium decumbens*. It has been suggested that iminocyclitol derivatives inhibitors of α-L-rhamnosidase could have the ability to inhibit thymidine diphosphate-(dTDP)–rhamnose biosynthesis or the incorporation of α-rhamnose into the cell wall of *Mycobacterium tuberculosis*, the causative agent of tuberculosis.[162,164] Since the compounds described interfere with mycobacterium biosynthesis of the bridging disaccharide region they constitute potential chemotherapeutic agents for this disease and they were thus assayed in mycobacterial systems.

Antiviral activity of iminosugar is mainly based on modulation of viral glycoprotein by specific inhibition of *N*-glycans processing enzymes.

1-Deoxynojiromycin (Scheme 21), a potent α-D-glucosidase inhibitor, has anti-HIV activities by the inhibition of the HIV entry, although the complete mechanism has not been fully characterized.[165] The glucosidase inhibitor castanospermine (Scheme 17) also demonstrates to inhibit Dengue virus *in vitro* and *in vivo*[166] and its ester derivative Celgosivir® (Scheme 25) has been proposed for treatment of Hepatitis C virus.[167] Both compounds have also been shown to be active against HIV replication in vitro.[168-170]



Scheme 25 Celgosivir®.

Studies of the effects of *N*-alkylated piperidinic iminosugar highlighted the possibility for new targets and mechanisms for antiviral iminosugar therapy.[104,171,172] Like in the case of anticancer activity, antiviral properties of these synthetic analogs are probably a combination of effects like interference with ion channels critical for the release of virions as in the case of hepatitis C virus (HCV)[173] or stimulation of host defense through activation of glycolipids receptors.[174,175]

e) Cystic fibrosis

Iminosugars have also been recently investigated as therapeutic agents for the treatment of Cystic Fibrosis (CF). CF is a genetic disorder that involves all epithelial cells and is characterized by a multitude of symptoms caused by dysfunction in various organs like lungs, sinuses,

pancreas, liver, bile ducts, intestines, bone and sweat glands, reproductive tract. The most serious manifestation is a respiratory disease resulting from frequent lung infections.

The biological cause of CF is a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene resulting in malfunctioning of the CFTR-protein. This protein is a chloride channel involved in the control of ion and water movement through the epithelial cells thereby regulating the components of sweat, digestive juices and mucus. Many mutations can affect the function of CFTR-protein among which the most common one is F508-del that cause inefficient trafficking of the protein to the apical membrane of many epithelial cells. The misfolded CFTR-protein retains some channel functions but fails to escape the endoplasmic reticulum (ER) and is not transported properly to the plasma membrane.

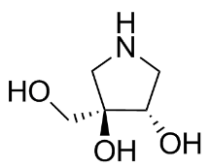
In the ER a quality control system monitors the folding and assembly of proteins and ensures that only folded proteins proceed along the secretory pathway[176], otherwise activating protein degradation. The entry in the degradation pathways is mediated by *N*-glycosylation and by the interaction of newly synthesized glycoprotein with the chaperone system.[177-179] Specifically it seems that the interaction between CFTR-protein and the chaperone calnexine mediated by glucosilation is main cause of retention of the protein in the ER.

A possibility for therapeutic intervention is to suppress the ER degradation of delF508 protein and the correct the protein folding and transport. It has been hypothesized that the disturbance of the ER quality control system by inhibiting the deglucosylation of delF508 protein in the ER might prevent its entry in the degradation pathway and partially restore its function.[180]

Miglustat (Scheme 23) has been investigated as corrector agent in many CF experimental models and is currently under clinical trial evaluation.[180-182] Inhibition of α-glucosidase and reduction of the interaction between delF508 protein and calnexine has been suggested as the main mechanism of CFTR corrector effect of miglustat.[181]

However, uncertainty exists regarding its mode of action. The relationship between defective CFTR rescue by miglustat and glycosidase inhibition has not been firmly established and the precise mechanism of action is not clearly understood. It has been recently observed that miglustat has an anti-inflammatory effect in bronchial cells independently of the correction of F508del-CFTR.[183]

The presence of a non-inhibitory mode of action has also emerged from a study on a novel series of branched iminocyclitols. The pyrrolidine Iso-1,4-dideoxy-1,4-imino-L-arabinitol (Iso-LAB) (Scheme 26) lacks glycosidase inhibitory activity but has shown to be effective corrector of F508del-CFTR trafficking probably acting as pharmacological chaperone.[184]



Scheme 26 Iso-LAB.

f) Neurodegenerative diseases

As described earlier, iminocyclitols can act as pharmacological chaperons in folding dysfunction associated with lysosomal storage disorder which are well known to be neuropathogenic.

The majority of glycosphingolipidoses, such as type II and III Gaucher's disease or GM1 and GM2 gangliosidoses, strongly involve the central nervous system and cause severe neurological alterations. The connection between lysosomal dysfunction and neurodegeneration is thus well documented.[185,186] This fact has highlighted the possible therapeutic intervention of iminosugar in neurophatogenic pathways prompting out investigations in this area. Hence, the potential use of iminosugar as chaperone agents for treatment of neurodegenerative diseases associated with folding disorders, including Alzheimer's and Parkinson's diseases, is currently being investigated.[104] Besides, an additional therapeutic opportunity for the iminosugar based treatment of neurodegeneration comes from the recently found inhibitory property against *O*-linked-*N*-acetylglucosaminidase (OGA), an enzyme involved in the etiology of Alzheimer's disease.[187] Recent reports disclose the utility of certain natural and synthetic iminocyclitols as inhibitors of OGA even if the design of more selective and potent analogue is necessary.[188]

1.3.4. Polyhydroxylated pyrrolidines 1,4-dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB)

1,4-Dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB) are respectively natural and synthetic polyhydroxylated pyrrolidines with a vast biological activity. Both D- and L-enantiomers of 1,4-dideoxy-1,4-iminoarabinitol have a considerable potential as glycosidase inhibitors by virtue of their structural similarity with natural five-membered sugars (and their respective glycosylcations). In fact both compounds demonstrate to be inhibitors of a number of carbohydrate-processing enzymes, each one with a different range of specificity.

The natural occurring enantiomer 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) was first isolated from the fruits of *Angylocalyx boutiqueanus*[189] and subsequently found in many species of plants[190,191] as well as in marine sponges.[192] Among other sugar mimic alkaloids it is considered responsible for the biological activity of many plants extracts. It was found as 2-*O*-β-D-glucoside conjugate in mulberry leaves (*Morus alba L.*)[193,194], traditionally used to cure the "Xiao-ke" (diabetes) in Chinese medicine and whose extracts were reported to have a potent antihyperglicemic activity in streptozocin (STZ)-induced diabetic mice.[195]

The corresponding unnatural enantiomer LAB synthesized first from D-xylose[196] is also known to have a broad inhibitory activity toward glycosidases.

DAB was described as a strong competitive inhibitor of yeast α-glucosidases, Golgi α-mannosidase I and II and threalase in addition to various α-glucosidases, suggesting that it has a versatile superimposition on the various glycosilcations[197], while weaker activity was early observed for LAB.[196,198]

Despite preliminary results the synthetic enantiomer was discovered to be more potent inhibitor than the natural compound towards mice gut saccharidases, expecially isomaltase[107] and moderate inhibitor of Golgi α-mannosidase[197] and α-L-rhamnosidase.[69] Further studies elucidated that LAB, as other L-enantiomer of furanose and pyranose-mimicking iminocyclitols,[199] were much more potent inhibitors of a series of α-glucosidases (other than yeast α-glucosidase) than their D-enantiomers. From kinetic analysis it was found that L-enantiomers act in a non-competitive manner (binding the enzymes to the site different from the active site) whereas the D-enantiomers are competitive inhibitors.

Another important activity of DAB is the powerful inhibition of glycogen phosphorylase (GP), the enzyme responsible for the breakdown of glycogen into glucose monomer and for the control glycogenolysis in muscle and liver tissues. Glycogen-degrading enzymes have attracted much interest as targets of anti-diabetic drugs since it is well established that the increased hepatic glucose production in type 2 diabetes significantly contributes to hyperglycemia. Thus, the inhibition of glycogen breakdown through inhibition of GP is a possible way to suppress hepatic glucose output and lower blood glucose.[200]

DAB was reported to be a potent inhibitor of basal and glucagon-stimulated glycogenolysis in primary rat hepatocytes[113] and to have antihyperglycemic effect in vivo in obese mice.[114] It was shown to strongly inhibit various mammalian glycogen phosphorylases (extracted liver and muscle rabbit GP)[109,112] in a noncompetitive manner but secondary effects on glycogen synthase[201] and debranching enzyme amylo-1,6-glucosidase[111] may contribute to the reported anti-hyperglycemic effect. No activity toward glycogen-degrading enzyme was observed for LAB except a weak inhibition of amylo-1,6-glucosidase.

Both compounds have also been screened as potential inhibitors of HIV replication and LAB was found a powerful inhibitor of the cytopatic effect of the virus in infected T-lymphocytes.[202]

In this thesis the stereodivergent asymmetric chemo-enzymatic synthesis of both DAB and LAB and of a collection of their 2-aminomethyl derivatives is described (section 3.2).

1.3.5. Chemo-enzymatic synthesis of iminosugars and derivatives

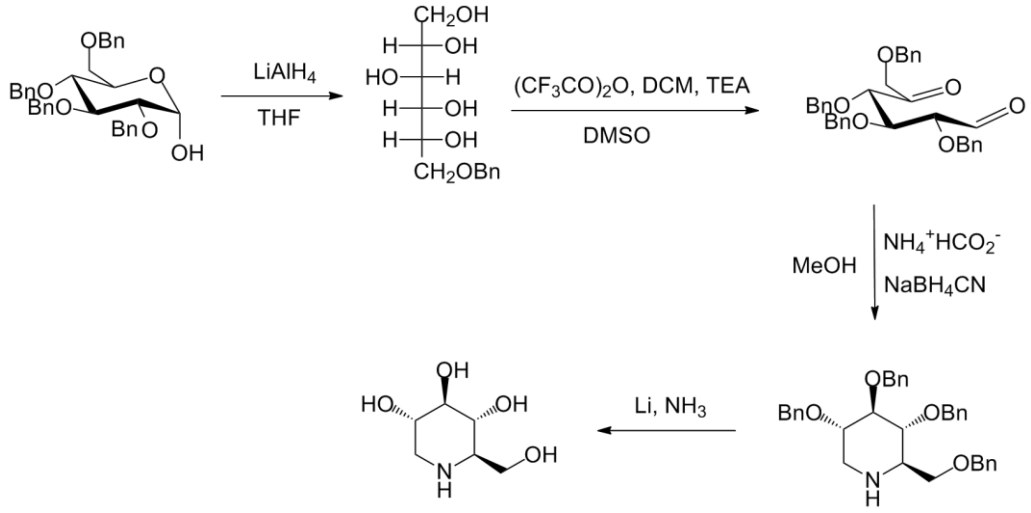
For years preparation of iminosugars has attracted the synthetic community's attention because their wide-ranging biological activities and promising drug profiles. As a result a great number of different synthetic approaches have been proposed to face the challenging features of this class of compounds: the high functionalization and the number of chiral centers.

Two different strategies have been applied for their synthesis: chemical and chemo-enzymatic.

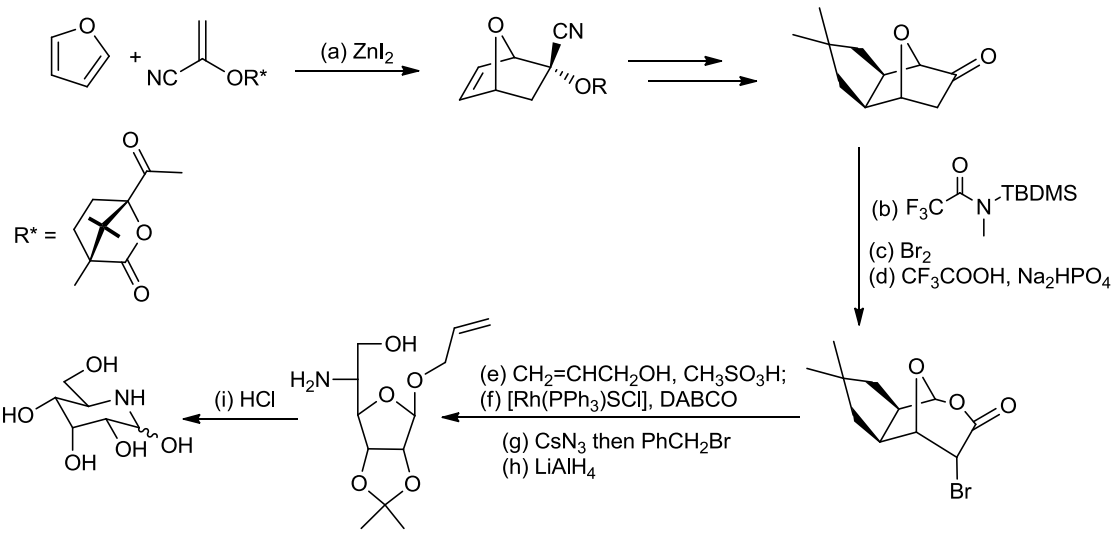
Chemical synthetic approaches can be broadly divided into methods that use chiral starting material, typically monosaccharides or alditols, and others that start from achiral non-glycidic precursors.

The first methodologies are mainly based on the modification of readily available natural sugars, such as D-glucose[203,204], D-fructose[205], D-gluconolactona[206], D-ribosa[207], D,L-xylose or D-sorbitol[208] by the introduction of an amino function and the subsequent cyclization (Scheme 27). The nitrogen was introduced in the sugar skeleton by amination of the anomeric centers or via chain amination. The generation of the piperidine or pyrrolidine ring is subsequently achieved by reductive amination of azidoketones, intramolecular amino group attack on leaving groups and activated double bonds.[209]

In methods using non-carbohydrate starting material the skeleton of iminosugars is stereoselectively built up by chemical condensations [210,211] or cycloaddition reactions (Scheme 28).[212,213]

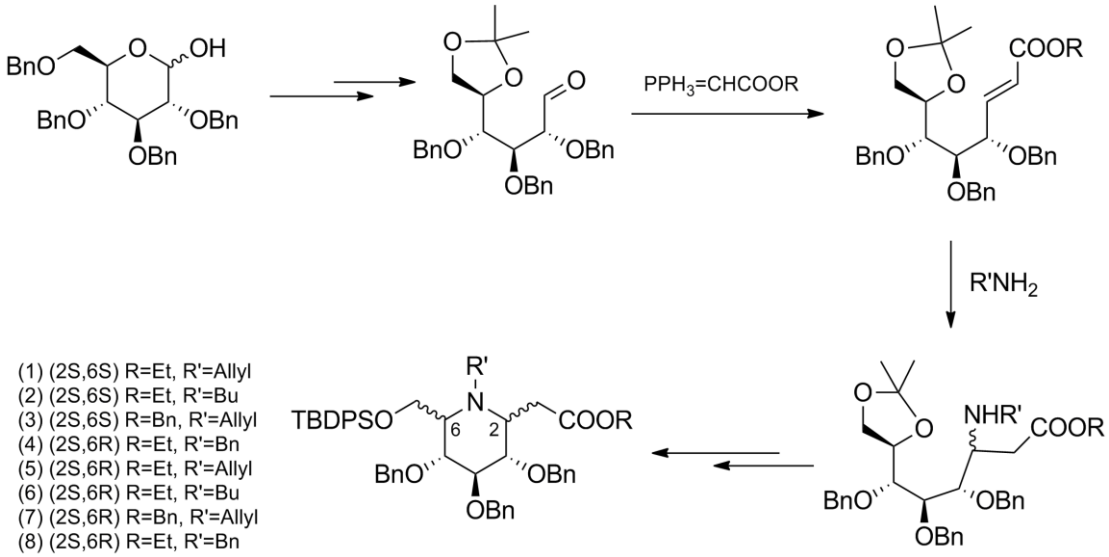


Scheme 27 Synthesis of 1-deoxynojirimycin from protected α-glucopyranose.[203]



Scheme 28 Total synthesis of allo-nojirimycin starting with Diels-Alder adduct of furan and 1-cyanovinyl-(1*S*)-camphanate.[213]

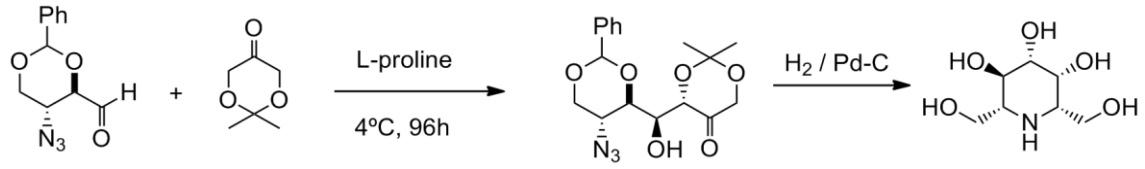
Due to the number of synthetic manipulations required for the preparation of iminosugars many efforts have been made to establish valuable synthetic approaches which allow the synthesis of a library of compounds by a common synthetic pathway instead of concentrate in a single target molecule (Scheme 29).[214]



Scheme 29 Small library of eight iminosugar scaffolds starting from a commercially available protected glucopyranose.[214]

Overall, chemical methods using both chiral and achiral starting material are complexes and expensive. They indeed involve extensive protecting group manipulation and cumbersome activation reactions that lead to difficult and long synthetic sequences. Consequently moderate to low global yield are achieved.

In last few years some alternative enantioselective routes based on asymmetric catalysis have been reported. The key steps of these methods are organocatalytic condensations such as asymmetric proline catalyse daldol reaction[215,216] (Scheme 30), catalytic asymmetric Mannich–Wittig olefination reaction and a subsequent catalytic diastereoselective dihydroxylation[217] or organocatalytic anti-Michael-anti-Aza-Henry reactions.[218] Respect to the previous chemical methods these novel organocatalytic routes are much more selective and concise. Nevertheless, they generally require protected substrates and organic solvent media that are important drawback in comparison with biocatalytic procedures.



Scheme 30 Organocatalytic synthesis of β-homomannojirimycin by proline catalysed aldol reaction of dioxanone with α-azidoaldehyde.[215]

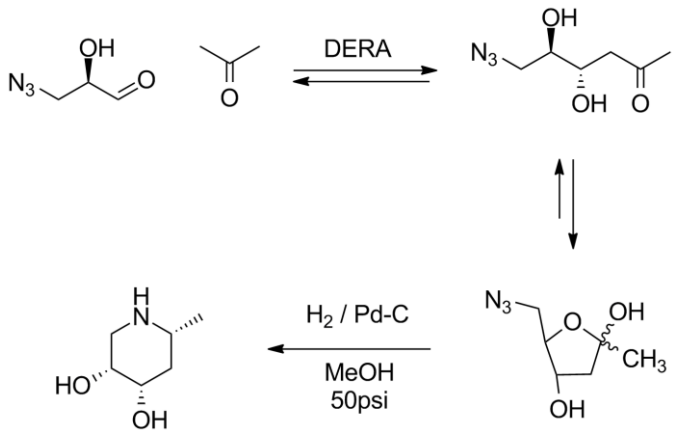
As mentioned, an important objective for the researchers in the area of iminocyclitols preparation has been to simplify key synthetic steps to significantly improve the commercial potential of this chemistry class.

In this regard chemo-enzymatic approaches offer some advantages. These methods are based on the use of aldolase for the construction of the polyhydroxylated skeleton of iminosugar followed by chemical cyclization. Aldolases employed in the synthesis of iminocyclitols are the 2-deoxyribose-5-phosphate aldolase (DERA)[25] and various dihydroxyacetone phosphate(DHAP)-dependent aldolases.

The key steps of these methods are the stereoselective aldol addition of a ketone donor to an aldehyde bearing an amino group (or a synthetic equivalent such azide) and the subsequent cyclization by reductive amination. The first step permits to build up the molecule often with fine control of the absolute configuration of the newly formed chiral centres. The occurrence of a set of stereocomplementary natural biocatalysts allows the construction of different stereoisomers.

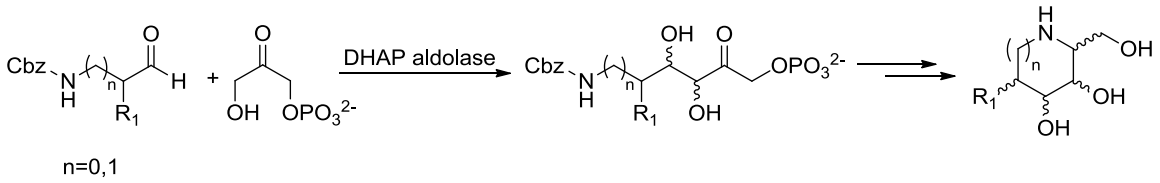
DERA was used to the preparation of 2-deoxyiminosugars by addition of different donors (propanal, acetone and fluoroacetone) to D-3-azido-2-hydroxypropanal. The azidoketo sugar intermediate was subsequently reduced to form a transient imine further reduced to the iminosugar. An example of deoxyiminosugar synthesis employing DERA is illustrated in scheme 31.

Azidoaldehydes have been used also as substrates of DHAP-dependent aldolases taking advantage of the acceptor substrate tolerance of this class of enzymes. Fructose-1,6-diphosphate aldolase (FruA, FDP or RAMA), fuculose-1-phosphate aldolase (FucA) and rhamnulose-1-phosphate (RhuA) were extensively used to catalyze stereoselective aldol addition of dihydroxyacetone phosphate to various α- and β-azidoaldehydes (e.g., 2-azido-3-hydroxypropanal and 3-azido-2-hydroxypropanal)[63,64] to provide iminocyclitols after enzymatic dephosphorylation and catalytic reductive amination.



Scheme 31 Chemoenzymatic synthesis 1,4,5,6-tetradeoxy-1,5-imino-D-lyxitol using DERA.[25]

In our research group DHAP was added to different *N*-Cbz-2 and 3-aminoaldehydes and C-α-substituted *N*-Cbz-2-aminoaldehydes using RAMA, RhuA and FucA generating thus a collection of pyrrolidines and piperidine-type iminosugars (Scheme 32).[65,67,68]



Scheme 32 Synthesis of iminocyclitols library using DHAP-dependent aldolase and α-substituted *N*-Cbz-aminoaldehydes.

These efficient and selective synthetic routes to iminosugars have the advantages to circumvent protection-deprotection loops needed by chemical approaches and to produce fewer impurities which results in easier purification and improvement of global yields. Moreover the simple and achiral starting material and the mild conditions required contribute to the scalability of the procedures.

On the other hand the main bottleneck of chemo-enzymatic approaches is the need of dihydroxyacetone phosphate as donor which is expensive and difficult to prepare and handle.

For this reason the discovery of aldolases that accept DHA instead of DHAP as substrate, fully described in section 1.2.3, widely improved the efficiency of these methods.

The chemo-enzymatic synthesis of iminocyclitols which have, as key step, the FSA-mediated aldol reactions of dihydroxyacetone and hydroxyacetone to different aldehydes are part of the present work and are described in section 3.1 of this thesis. Considering the extensive biological activity and pharmaceutical industry interest of iminocyclitols, the efficiency and scalability of the synthetic processes for their preparation is of paramount importance.

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2. OBJECTIVES

This thesis was devoted to the synthetic application of dihydroxyacetone and dihydroxyacetone phosphate dependent aldolases for the preparation of polyoxygenated chiral complex bioactive compounds. This goal was focused on four specific objectives:

1) D-Fructose-6-phosphate aldolase (FSA) is an innovative biocatalyst with the outstanding advantage of using non-phosphorylated donor substrates. The main goals of this section were:

- To explore the synthetic potential of FSA by assaying novel aldol addition reactions.
- To assess the stereoselectivity and acceptance of non-natural substrates for the enzyme and study its reactivity with regard to the aldehyde acceptor and donor used.
- To demonstrate its utility as catalyst for the alternative preparation of chiral complex polyhydroxylated structures (**Section 3.1**).

2) To synthetize chemoenzymatically two polyhydroxylated pyrrolidines, with important biological properties (1,4-dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB)), with the aim to introduce new functional groups that can modulate their inhibitory properties against glycosidases. Both polyhydroxylated pyrrolidines will be conveniently protected and modified to install new fuctional moieties. To this end, we will explore a cascade reaction approach, with minimum intermediate chemical steps, to obtain unprecedented 2-aminomethyl derivatives of DAB and LAB with potential novel biological activity (**Section 3.2**).

3) In previous studies in our lab, a collection of polyhydroxylated pyrrolizidines was obtained using dihydroxyacetone phosphate aldolases (RhuA and FucA) to catalyze the key aldol addition of DHAP to *N*-Cbz-pyrrolidinecarbaldehyde.
In this connection another goal will be to explore a chemoenzymatic cascade reactions strategy for the synthesis of new polyhydroxylated pyrrolizidines derived from the polyhydroxylated pyrrolidine building blocks used in Section 3.2. Towards this goal we will investigate the aldol additions of DHAP or DHA to (2*S*,3*R*,4*R*)- and (2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehydepyrrolidine catalyzed by different DHAP y DHA-dependent aldolasas (**Section 3.3**).

4) To investigate the preliminary inhibitory properties of the 2-aminomethyl DAB and LAB derivatives obtained during the course of this thesis. The compounds are to be assayed as inhibitors against a panel of commercial glycosidases with the aim to establish the potential therapeutic targets where they can be directed (**Section 3.4**).

3. RESULTS AND DISCUSSION

3.1. D-FRUCTOSE-6-PHOSPHATE ALDOLASE IN ORGANIC SYNTHESIS: CASCADE CHEMICAL-ENZYMATIC PREPARATION OF SUGAR-RELATED POLYHYDROXYLATED COMPOUNDS

Aldol additions catalyzed by D-fructose-6-phosphate aldolase from *Escherichia coli* (FSA)[1] are emerging as a key enzymatic reaction in the synthesis of enantiopure complex carbohydrates, sugar derivatives and analogues among other polyhydroxylated products.[2-4] This is because FSA has the striking advantage of accepting unphosphorilated DHA analogues as donors instead of the expensive and synthetically time consuming dihydroxyacetone phosphate that DHAP-dependent aldolases need, maintaining the complete stereocontrol over the asymmetric centers at the newly formed C-C bond.[3,4] Thus, FSA has the ability to stereoselectively catalyze aldol additions of dihydroxyacetone (DHA), hydroxyacetone (HA) and hydroxybutanone (HB) to a variety of aldehydes.[2-4] Moreover, glycoaldehyde has been recently described by our group to be accepted as donor for various aldol addition catalysed by FSA.[5]

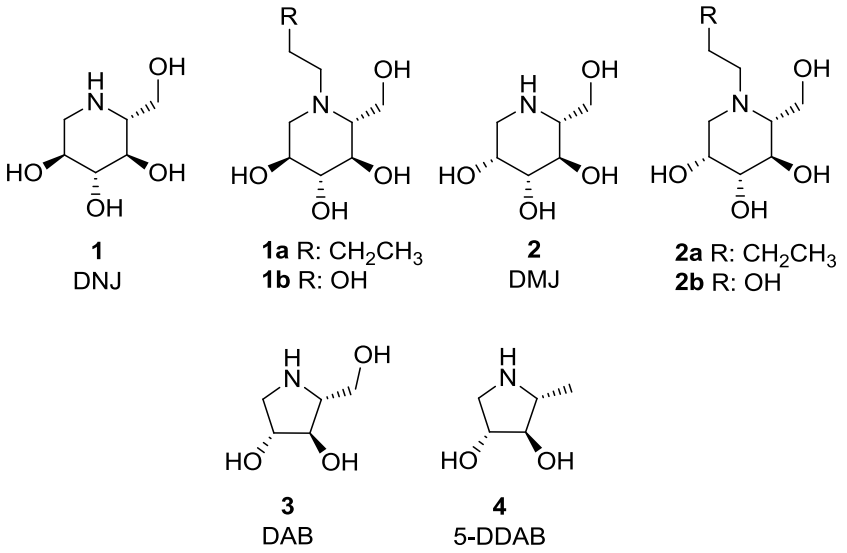
The first example of the synthetic capabilities of FSA was described in our lab with the synthesis of D-fagomine, a naturally occurring D-iminosugar.[3] In parallel to our work, Wong and co-workers[4] have further contributed to exploit the synthetic possibilities of FSA using azido and other *N*-Cbz-aminoaldehydes as acceptors. They found that both HA and HB were excellent donors for their enzymatic aldol addition reactions, but with limitations in scope using DHA.

In this chapter novel aldol addition reactions of dihydroxyacetone and hydroxyacetone to different aldehydes catalyzed by D-fructose-6-phosphate aldolase (FSA) are described. Furthermore a chemical-enzymatic cascade reaction approach for the synthesis of iminosugars and carbohydrates from aldol adducts is presented.

3.1.1. Chemo-enzymatic preparation of iminosugars

A chemical-enzymatic cascade reaction approach to the synthesis of 1-deoxynojirimycin (DNJ) (**1**), 1-deoxymannojirimycin (DMJ) (**2**), and their *N*-alkylated derivatives **1/2a-b**, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (**3**), 1,4,5-trideoxy-1,4-imino-D-arabinitol (5-DDAB) (**4**) is presented.

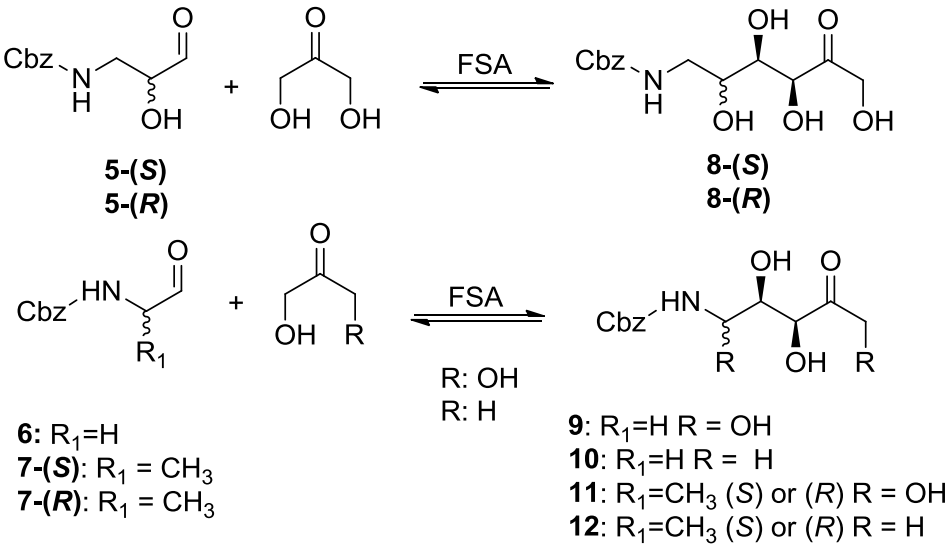
1-Deoxynojirimycin[6] is a potent α-glucosidase inhibitor and the intermediate for the synthesis of *N*-butyl-DNJ (miglustat) **1a**, which it is used to treat type I Gaucher disease, and *N*-(2-hydroxyethyl)-DNJ (miglitol) **1b**, an oral antidiabetic drug primarily used in type 2 diabetes treatment. 1-Deoxymannojirimycin (DMJ) is a potent inhibitor of Golgi α-mannosidase I[7] with a therapeutic potential as antiviral[8,9] and in tumor treatment.[10] 1,4-Dideoxy-1,4-imino-D-arabinitol (DAB) is a potent α-glucosidase inhibitor[11,12] and, a promising potential therapeutic candidate as anti-hyperglycemic agent (Section 1.3.3. and 1.3.4).[13-15] 1,4,5-Trideoxy-1,4-imino-D-arabinitol (5-DDAB) was found to have inhibitory properties against α-, β-mannosidase from jack beans and snail, respectively, and α-, β-glucosidase[4] and its corresponding enantiomer (5-DLAB) is an inhibitor of α-L-rhamnosidase from Penicillium decumbens.[16]



Scheme 1 Iminosugars obtained by chemical-enzymatic cascade routes.

The chemo-enzymatic synthesis of iminocyclitols have as key step the FSA-catalyzed aldol addition of dihydroxyacetone and hydroxyacetone to different protected aminoaldehydes followed by deprotecion/intramolecular reductive amination (Section 1.3.5).

To this end novel aldol addition reactions of dihydroxyacetone and hydroxyacetone to a variety of *N*-benzyloxycarbonyl-aminoaldehydes catalyzed by D-fructose-6-phosphate aldolase (FSA) were assayed (Scheme 2, Table 1).



Scheme 2 *N*-Cbz-aminoaldehydes assayed as acceptor for FSA and their aldol adducts.

(*S*)-3-(*N*-Cbz)-Amino-2-hydroxypropanal (**5-(*S*)**), its enantiomer **5-(*R*)** and *N*-Cbz-glycinal (**6**) were acceptors for FSA catalyzed reaction whereas neither (*S*) nor (*R*) Cbz-alaninal (**7**) were tolerated as substrates (Scheme 2, Table 1). For all the accepted aldehydes the enzymatic aldol addition was fully stereoselective. As described in section 1.2.4, both (*S*) and (*R*) Cbz-alaninal would be accepted as substrate by a variant of FSA expressed in our lab in which the residue Ala165 in the active site of FSA was replaced by a glycine. This substitution allowed thus more space and flexibility to allocate the aldehydes, especially those C-α substituted.[17]

Acceptor	Donor	Reaction Conversion (%)[a]	Aldol product	Isolated Yield (%)
5-(*S*)	DHA	100	**8-(*S*)**	ni[b]
5-(*R*)	DHA	100	**8-(*R*)**	ni[b]
6	DHA	60	**9**	40
6	HA	96	**10**	75
7-(*S*)	DHA	-	**11-(*S*)**	-
7-(*S*)	HA	-	**11-(*S*)**	-
7-(*R*)	DHA	-	**12-(*R*)**	-
7-(*R*)	HA	-	**12-(*R*)**	-

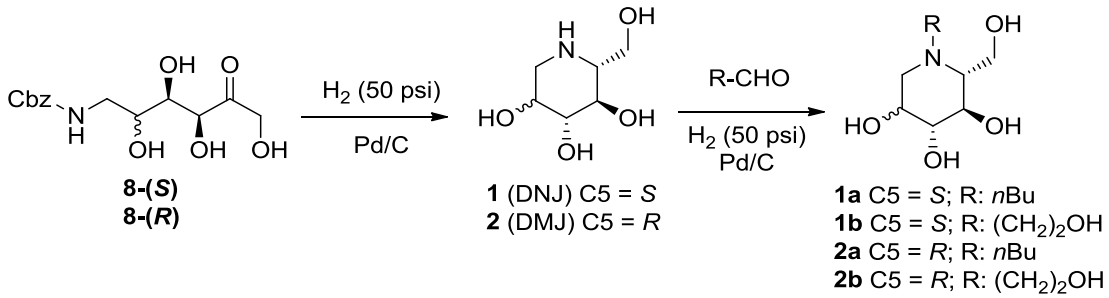
Table 1. FSA-catalyzed aldol addition reactions with *N*-Cbz-aminoaldehyes. Summary of the results obtained for each reaction and acceptor/donor assayed. [a] Molar percent conversion to aldol adduct with respect to limiting substrate. Values are measured from the crude reaction mixture by HPLC using the external standard method calibrated with purified compounds. The reaction media consisted in aqueous buffer 100% or mixtures dimethylformamide/buffer 1:4 depending on the solubility of the aldehyde (see experimental section). [b] Not isolated.

N-Cbz-aminoaldehydes were prepared by 2-iodoxybenzoic acid (IBX) oxidation of the corresponding *N*-Cbz-alcohols, whose unprotected precursors are readily commercially available.[18,19] *N*-Cbz-glycinal and *N*-Cbz-alaninal were successfully isolated and assayed as substrate for aldol additions while (*S*)- and (*R*)-3-(*N*-Cbz)-amino-2-hydroxypropanal did not survive to several attempts to isolate it as revealed by NMR analysis. During IBX oxidation of (*R*)-3-(*N*-Cbz)-amino-2-hydroxypropanol a major product was observed by TLC concomitant with several minor side-product formations. The oxidation was considered finished when the major spot on TLC-analysis reached a maximum (4h). At this point there was still some starting material and other side-products. The yield was estimated to be around 50-60%. Then a simple aqueous work-up was performed (i.e. washing the ethyl acetate reaction solution with brine containing sodium bicarbonate (5% w/v)) and the remaining **5-(*S*)** or **5-(*R*)** in ethyl acetate was added straight, in a cascade chemo-enzymatic transformation, to an aqueous solution containing both FSA and DHA. Then, the liquid-liquid two-phase system mixture was placed in a rotary evaporator

and the ethyl acetate eliminated in vacuum with gentle agitation at 25ºC. Interestingly, under these conditions **5-(*S*)** and **5-(*R*)** were accepted as substrates by FSA in the aldol additions with DHA and full conversion of the aldehyde was reached after 24 h (Table 1). The aldol adducts were not isolated and used as crude mixture to obtain the corresponding cyclic derivatives. DNJ (**1**) and DMJ (**2**) were thus obtained by reductive amination of the products **8-(*S*)** or **8-(*R*)** rendered by the aldol addition of DHA to (*S*) and (*R*) 3-(*N*-Cbz)-amino-2-hydroxypropanal, respectively.

The crude aldol reaction mixture was filtered through Celite® and submitted to reductive amination with H_2 in the presence of Pd/C and the corresponding products purified by ion exchange chromatography on a weak-type anion resin (Scheme 3).

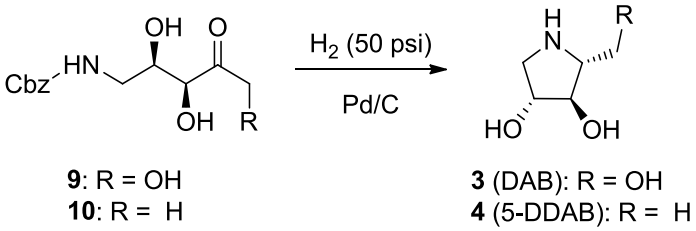
Pure products **1** and **2** were treated with butanal or 2-benzyloxyethanal and H_2 (50 psi) in the presence of Pd/C furnishing **1a** (miglustat), **1b** (miglitol), **2a** (*N*-butyl-1-deoxymannojirimycin) and **2b** (*N*-hydroxyethyl-1-deoxymannojirimycin), respectively (Scheme 3).



Scheme 3 Synthesis of DNJ, DMJ and derivatives from FSA aldol adducts intermediates.

Following a similar strategy, DAB (**3**) and the corresponding 5-deoxy analog **4** were thus obtained (Scheme 4). *N*-Cbz-glycinal (**6**) was substrate of FSA but reacted more efficiently with HA (95% conversion) than with DHA (60% conversion). These differences may be related to the distinct affinity of FSA for the donor substrate[2,4] or due to the different equilibrium position of the reaction. Attempts to improve the conversion with DHA by either increasing the enzyme concentration or with consecutive additions of the aldehyde failed. In this case, aldol adduct **9** was extensively purified in order to remove the unreacted donor and aldehyde thus avoiding an excess of byproducts formation during the Cbz deprotection–reductive amination step which might complicate the final purification steps. The product **9** was obtained with only a 40% isolated yield. The optimization of the aldol addition of DHA to *N*-Cbz-glycinal would have been possible employing as catalyst a variant of FSA developed in our research group.[17] The mutant FSA Ala165Gly allowed to reach a complete conversion by HLPC and to increase significantly the final product yield. This will be discussed in section 3.2. Aldol adduct **10** was also purified and obtained in 75% isolated yield.

As ascertained by NMR analysis and by comparison with previous data,[19] FSA catalyst was stereoselective for the aldol additions of *N*-Cbz-glycinal to both HA and DHA affording the expected 3*S*,4*S* configuration of the two newly formed stereogenic centers. The reductive amination and purification by ion exchange chromatography furnished pure **3** and **4** in 80% and 68% isolated yield (24% and 56% from the aldehyde), respectively (Scheme 3). It is noteworthy that the reductive amination of **10** gave the expected *cis* relation between the methyl at C2 and the hydroxyl at C4 as a major product but with *ca* 17% of the corresponding *trans* relation (i.e. epimeric product at C2) which could not be separated by ion exchange chromatography.[20]



9: R = OH
10: R = H

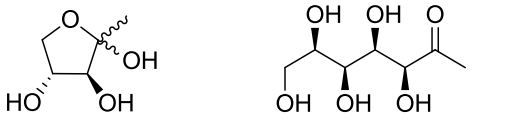
3 (DAB): R = OH
4 (5-DDAB): R = H

Scheme 4 Synthesis of DAB and 5-DDAB from FSA aldol adducts of DHA/HA and Cbz-glycinal.

3.1.2. Chemo-enzymatic preparation of polyhydroxylated compounds and carbohydrates

Novel aldol addition reactions of dihydroxyacetone and hydroxyacetone to hydroxyaldehydes, thioaldehyde and an aldose, catalyzed by D-fructose-6-phosphate aldolase (FSA) were assayed with the aim to gain insight into the enzyme reactivity toward different donors/acceptors.

The aldol additions described in this section provide intermediates potentially useful for the preparation of polyhydroxylated compounds such as sugars, deoxysugars and thiosugars. As example of the valuable synthetic potential of FSA for the construction of chiral complex polyhydroxylated sugar-type structures, the preparation of 1-deoxy-D-xylulose (**24**) and 1-deoxy-D-*ido*-hept-2-ulose (**28**) are presented.



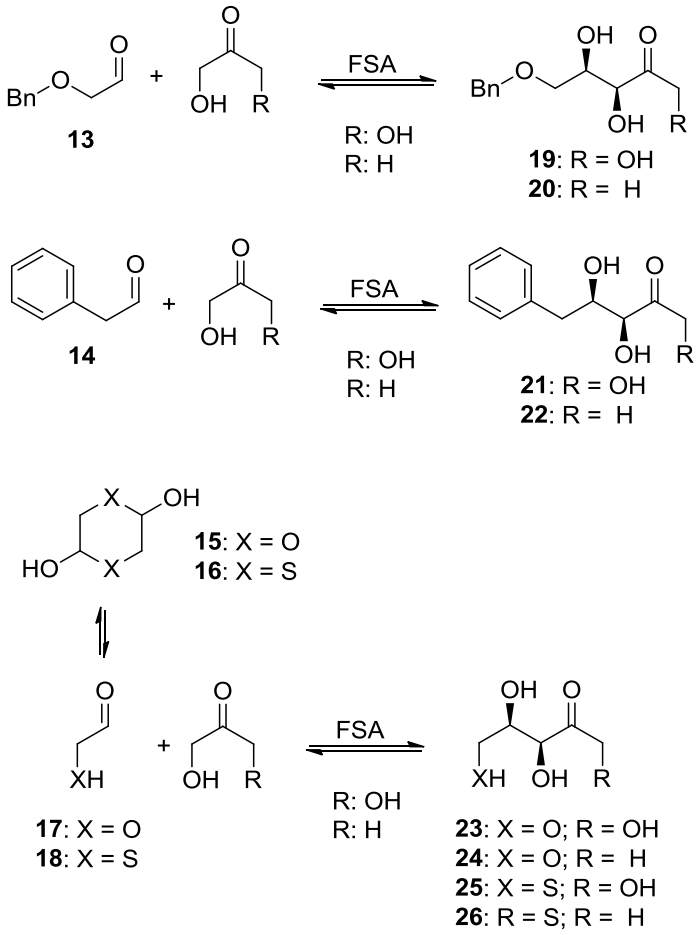
1-deoxy-D-xylulose

1-deoxy-D-*ido*-hept-2-ulose

Scheme 5 Deoxysugars prepared by aldol addition catalyzed by FSA.

Analogues and derivatives of 1-deoxy-D-xylulose may be valuable molecules as inhibitors of DXP reductoisomerase and potential antibacterial and anti-malarial agents.[21-23] Furthermore, 1-deoxy-D-xylulose itself is easily incorporated into the mevalonate independent methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis, occurring in bacteria and plant plastids.[24-26] 1-deoxy-D-xylulose and other deoxysugars are important because the great number of roles in

many physiologically significant reactions such as cellular adhesion, cell-cell interactions, immune response, fertilization and target recognition of toxins, antibiotics and microorganisms.[27,28]



Scheme 6 FSA-catalyzed aldol addition reactions aldol addition of DHA and HA to different aldehydes.

2-Benzyloxyethanal (**13**), 2-phenylethanal (**14**), 2-hydroxyethanal (**17**) and 2-mercaptoethanal (**18**) were assayed as acceptors for aldol additions of DHA and HA catalysed by FSA (Scheme 6 Table 2). The reaction conversions for the aldol additions assayed are summarized in Table 2.

2-Benzyloxyethanal (**13**) was tolerated as substrate for FSA but, as for *N*-Cbz-glycinal, the outcome of the aldol addition depended strongly on the donor: a poor 30% conversion was achieved with DHA while HA reacted almost quantitatively (98% conversion) (Table 2). The corresponding aldol adducts **19** and **20** were obtained in 28% and 71% isolated yields, respectively and 99% purity by HPLC in both cases (Table 2). Remarkably, the enzymatic aldol addition was stereoselective and, under the detection limits of high field NMR, no other diasteromers than those of *3S,4S* configuration were detected.

2-Phenylethanal (**14**) was also substrate for FSA (Scheme 6) and, interestingly, compared with 2-benzyloxyethanal and *N*-Cbz-glycinal acceptors, in this case both DHA and HA were good donor substrates (Table 2). This is likely due to the fact that **14** is a good acceptor aldehyde and

therefore FSA gave good conversions with both DHA and HA. Furthermore, it may be that in this case the conversion was not limited by the equilibrium.

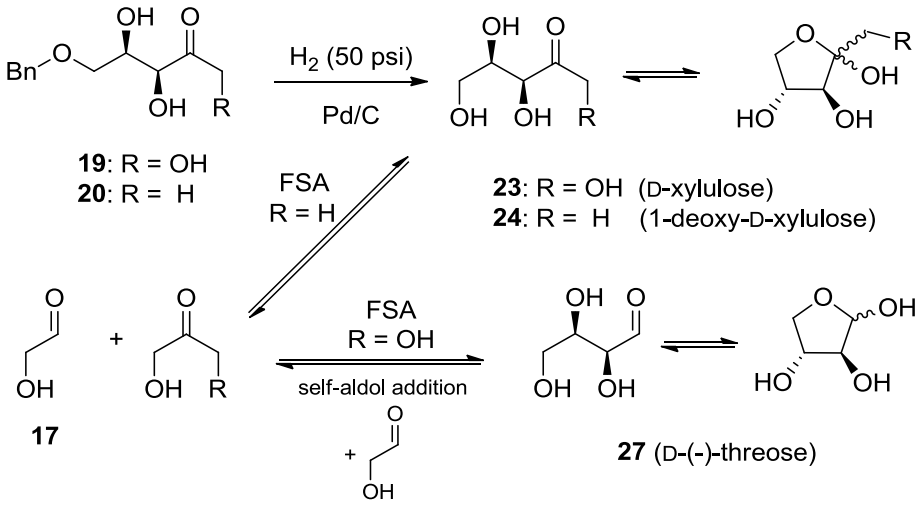
Acceptor	Donor	Reaction Conversion (%)[a]	Aldol product	Isolated Yield (%)
13	DHA	35	**19**	28
13	HA	95	**20**	71
14	DHA	77	**21**	46
14	HA	70	**22**	48
15	DHA	-	**23**[b]	
15	HA	100[c]	**24**[d]	51[e]
16	DHA	-	**25**	-
16	HA	-	**26**	-
27	HA	90[c]	**28**[c]	58

Table 2 FSA-catalyzed aldol addition reactions. Summary of the results obtained for each reaction and acceptor/donor assayed. [a] Molar percent conversion to aldol adduct with respect to limiting substrate. Values are measured from the crude reaction mixture by HPLC using the external standard method calibrated with purified compounds. The reaction media consisted in aqueous buffer 100% or mixtures dimethylformamide/buffer 1:4 depending on the solubility of the aldehyde (see experimental section). [b] Product different from the expected (see text section 3.1.2). [c] Estimated by TLC. [d] Existing in equilibrium in the acyclic and cyclic forms. [e] Mixture of *threo* (major, 3*S*,4*R* in the aldol adduct) and *erythro* (minor, 3*R*,4*R* in the aldol adduct) isomers.

2-Mercaptoethanal (2-mercaptoacetaldehyde; **18**), supplied as the corresponding thiohemiacetal dimer (1,4-dithiane-2,5-diol; **16**), was also attempt as substrate for FSA. The reaction was performed at 40ºC and mild acid pH (pH=6 and pH=5). The reaction didn't give aldol adduct in any of the conditions assayed likely because thiohemiacetals are generally difficult to hydrolyze.

2-Hydroxyethanal (glycolaldehyde; **17**) and D-(-)-threose (**27**) were acceptor substrates for FSA and will be discussed later. The aldol additions of DHA and HA to 2-benzyloxyethanal provide an alternative enzymatic route to the synthesis of D-xylulose (**23**) and 1-deoxy-D-xylulose (**24**), respectively (Scheme 7).

The aldol adducts **19** and **20** may be valuable building blocks for the synthesis of analogues of these compounds. 5-*O*-Benzyl-D-xylulose (**19**) is a key intermediate in the synthesis of some polyhydroxylated piperidines.[29] Moreover, the presence of a benzyl moiety provided an additional tactical advantage for purification purposes.[30]



Scheme 7 Synthetic routes to D-xylulose and 1-deoxy-D-xylulose and FSA-catalyzed self-aldol addition of 2-hydroxyethanal.

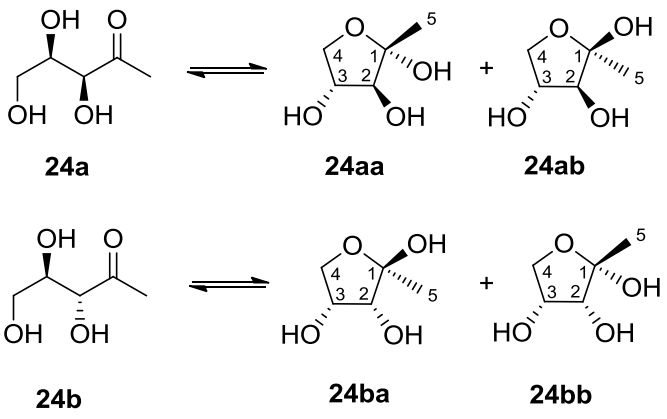
Removal of the benzyl group in **20** by H_2 (50 psi) in the presence of Pd/C was carried out to furnish **24** with a purity of 98% without any further purification steps (71% isolated yield). This chemo-enzymatic method to 1-deoxy-D-xylulose synthesis provides a clean, highly selective, and concise alternative for the preparation of this compound by comparison with existing classic or enzymatic methods (Scheme 7).[30-34]

We also attempted to prepare D-xylulose and 1-deoxy-D-xylulose by starting with the unprotected 2-hydroxyethanal (glycolaldehyde, **17**; Scheme 7). The reactions with HA and DHA differed significantly. Under the conditions assayed, no D-xylulose was detected in the aldol addition of DHA to **17** in the presence of FSA. In a further research by our group this reaction was found to give the product of self-addition of 2-hydroxyethanal (D-(–)-threose, Scheme 7). FSA *wild type* was thus reported to accept 2-hydroxyethanal as donor[5] as described in section 1.2.4 (see Scheme 16 Section 1.2.4).

The aldol addition of DHA to 2-hydroxyethanal to furnish **23** (D-xylulose) would have been possible only with the variant of FSA Ala129Ser which demonstrated to have a higher affinity for DHA than for 2-hydroxyethanal (Scheme 16 Section 1.2.4).[35]

On the other hand, FSA catalysed aldol addition of HA to 2-hydroxyethanal in >98% yield after 24h. As ascertained by NMR analysis, the purified material revealed the expected 3*S*,4*R threo* aldol adduct along with around 20% of the *erythro* isomer. A detailed NMR analysis of the enzymatic reaction course revealed that FSA was fully stereoselective in this reaction. Therefore, the formation of the *erythro* isomer was not due to a lack of stereoselectivity by FSA but to the conditions of the crude reaction mixture: lyophilization was performed prior to purification by flash chromatography on silica. 1-Deoxy-D-xylulose was found to epimerize upon lyophilization at pH>6.0, whereas no diastereomer was detected when adjusting the solution to pH 5. Therefore,

the introduction of a benzyl moiety onto the hydroxy group at C5 appears to be tactically useful (51 vs. 71% isolated yield), first, by avoiding complications during the work-up and purification steps and, secondly, by facilitating product isolation.



Scheme 8 Isomeric species furnished by the aldol addition of HA to 2-hydroxyethanal catalyzed by FSA upon lyophilization at pH≥6.

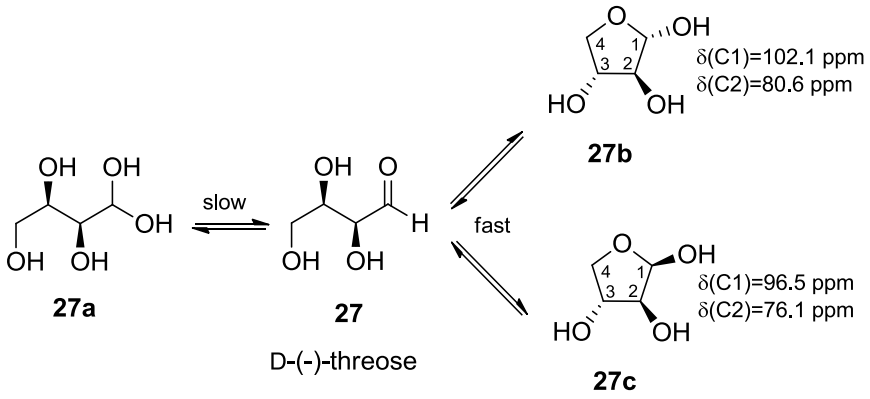
Compounds	δ (ppm)			
	C1	C2	C3	C5
24aa	106.3	81.5	77.0	20.5
24ab	102.6	81.7	76.0	23.7
24ba	106.0	76.3	70.9	21.3
24bb	102.0	75.0	70.3	23.1

Table 3 Observed ^{13}C NMR chemical shifts of the compounds depicted in Scheme 8.

The NMR spectra of the *threo/erythro* mixture of aldol product **24** showed the presence of six different species: Two acyclic derivatives (**24a** and **24b**) and four isomeric cyclic forms (**24aa**, **24ab**, **24ba** and **24bb**) (Scheme 8). The stereochemistries were assigned by comparing the ^{13}C NMR chemical shifts of the cyclic species, as shown in Table 3. The ^{1}H NMR chemical shifts and NOE data also confirmed the presence of these structures. The observed chemical shifts of C1 and C5 shown in Table 3 indicate the relative configurations at the C1,C2 stereogenic centers. An important 2–3 ppm shielding effect of C2-OH on the C5 methyl chemical shift is clearly observed for isomers **24aa** and **24ba** as compared with **24ab** and **24bb**, respectively. Furthermore, the upfield chemical shifts of C2 and C3 for **24aa** and **24ab** in comparison with those for **24ba** and **24bb** also confirm the *trans/cis* disposition of each compound, respectively. Hence, because the 3*S*,4*R* configuration did not change during the course of the enzymatic reaction, the 3*R*,4*R* stereochemistry is expected for the *erythro* aldol adduct, 1-deoxy-D-erythro-2-pentulose **24b** (Scheme 8).

As previously mentioned it was also observed that D-(–)-threose is a good acceptor substrate for FSA. Hence, we attempt the aldol addition of HA to aldoses such as D-(–)-threose as a way of accessing other deoxysugars.

Hexoses, pentoses, and tetroses were found to be poor acceptors for DHAP dependent aldolases like D-fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA) probably due to both the cyclic hemiacetalic form and the hydration of the carbonyl group which may hamper its reactivity.[36,37]

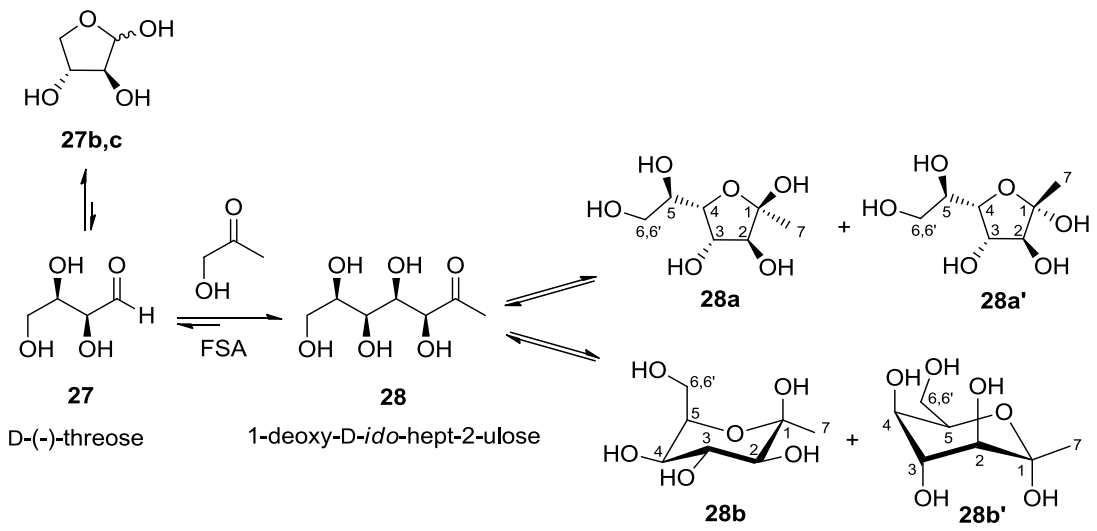


Scheme 9 Equilibria of D-(–)-threose by NMR analysis**.**

NMR analysis of D-(–)-threose in D_2O revealed that on the NMR timescale two cyclic products, **27b** and **27c**, are in fast equilibrium with the open form **27** and that this is in slow equilibrium with the corresponding hydrate **27a** (Scheme 9). This statement can be made on the basis of the chemical exchange cross-peak observed in the NOESY spectrum between the aldehydic H1 proton of **27** resonating at 9.71 ppm and the corresponding H1 protons of species **27b** and **27c** (resonating at 5.25 and 5.40 ppm, respectively), but not with H1 of **27a** (resonating at 5.03 ppm). Moreover, from the NMR analysis of the cyclic forms it is important to note that upfield shifts for both C1 (anomeric) and C2 were observed for **27c** (Scheme 9) in comparison with those of **27b**, which indicates the relative *cis/trans* configuration of the OH substituents, respectively, in good agreement with the NMR data obtained for compounds **24aa**, **24ab**, **24ba** and **24bb**.

Hence, as ascertained by NMR spectroscopy, a small amount of aldehyde **27** existed in aqueous solution and therefore a priori it may be qualified as a poor acceptor aldehyde. The addition of HA to D-(–)-threose catalyzed by FSA furnished 1-deoxy-D-*ido*-hept-2-ulose (**28**; Scheme 10) in an isolated yield of 58% and a diastereomeric excess of >98%. Interestingly, the conversion to heptulose was >90% by visual inspection of the TLC plate. On the other hand, no aldol adduct was detected when DHA was used as the donor substrate.

This example suggests that a variety of biologically interesting 1-deoxy sugars can be synthesized by FSA-catalyzed aldol addition of HA and/or HB to aldoses.



Scheme 10 FSA-mediated enzymatic synthesis of 1-deoxy-D-*ido*-hept-2-ulose.

Analysis of the resulting isolated compound using a complete suite of 1D and 2D NMR experiments allowed complete characterization of the resulting acyclic **28**, the five-membered cycles **28a** and **28a'**, and the six-membered cycles **28b** and **28b'**. The structures of the five-membered ring isomers were established through a three-bond crosspeak in the HMBC spectrum between H4 and C1, whereas HMBC cross-peaks between H5 and C1 were observed with the six-membered-ring isomers. The relative configuration of the C1 center in each of the two five-membered-ring isomers was established from the characteristic downfield effect on the anomeric C1 and on the methyl C7 chemical shifts. Furthermore, structural elucidation of the six-membered derivatives was based on the relative configuration between the methyl C7 and the hydroxymethylene C6 groups. In **28b'**, both groups are in an equatorial disposition, whereas in **28b**, the methyl is predominantly in an equatorial position with C6 in an axial position. The ^{1}H NMR chemical shift of the proton H_2 is a good indicator of this equatorial/axial disposition and NOE data confirm such conformational behavior.

In summary FSA resulted to be a robust and stereoselective catalyst for aldol additions of HA and DHA to a variety of aldehydes, with the great advantage of using non-phosphorylated donor substrates. From the results reported from other authors and those obtained in this work it can be concluded that FSA possesses a remarkable stereoselectivity regardless of the aldehyde acceptor employed.

Concerning the acceptor substrate FSA demonstrates to accept a broad variety of aldehydes including that bearing hydroxyl group at 2-position. However, FSA wild type did not tolerate neither *(S)* nor *(R)*-*N*-Cbz-alaninal, which bears a methyl group at 2-position. Thus, the polarity of the 2-substitution could be also a factor to consider in the substrate selectivity of FSA.

Concerning the donor substrate, it was observed that HA was the best one regardless of the aldehyde acceptor used in a good agreement with previous studies, whereas the reactivity of DHA depended on the aldehyde acceptor.

The products obtained by FSA-catalyzed aldol additions are valuable building blocks for the synthesis of more complexes polyhydroxylated compounds as demonstrated with the preparation of iminosugars and deoxysugars. Importantly, we have found that FSA accepts simple sugars as acceptors using HA as donor. This may open new synthetic possibilities for the preparation of relevant novel complex sugar related compounds. Further studies are currently being carried out in our lab in this direction.

All these features make FSA a key biocatalyst for alternative synthetic approaches of biologically active products with known therapeutic and research interest or novel structures relevant for drug discovery

References:

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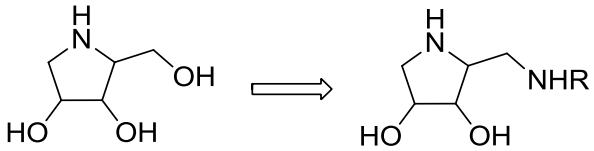
3.2. CASCADE CHEMICAL-ENZYMATIC SYNTHESIS OF DAB, LAB AND 2-AMINOMETHYL DERIVATIVES

1,4-dideoxy-1,4-imino-D-arabinitol (DAB) is a natural occurring polyhydroxylated pyrrolidine with an exceptional biological activity.[1] Its corresponding enantiomer 1,4-dideoxy-1,4-imino-L-arabinitol (LAB) has been synthetized and also showed to have biological properties.[2,3] Both iminocyclitols exhibit strong inhibitory effect on various glycosidases such as α-glucosidases, α-mannosidase as well as various intestinal disaccharidases and liver glycogen phosphorylase.[4-6] Due to their remarkable biological activity, fully described in section 1.3.4, DAB and LAB have been regarded as potential therapeutic agents for the treatment of glycosidases involving pathologies such as type 2 diabetes and HIV infection among others.

Structural modification of a lead compound is a powerful strategy for studying relationships of structure to activity with the aim of drug discovery. The synthesis of derivatives of naturally occurring iminocyclitols with biological properties may improve their activity or may redefine the inhibitory profile, especially, with regard to specificity. The synthesis of novel molecules from a lead compound may also contribute to the understanding of the targeted pathological pathways and to the optimization of the therapeutic strategy.

Structural modification of DAB and LAB has led to novel derivatives with unprecedented activities. For instance, preliminary experiments suggest that 1,4-dideoxy-2-hydroxymethyl-1,4-imino-L-threitol (isoLAB) may have a role in studies of the mechanisms of chaperoning the folding of CFTR of cystic fibrosis.[7] 2-Acetamido-1,4-imino-1,2,4-trideoxy-L-arabinitol (LAB*N*Ac) and its *N*-benzyl derivative (*N*Bn-LAB*N*Ac) were found potent non-competitive inhibitors of D-hexosaminidase, that may lead to new strategies for the treatment of diseases such as cancer, arteriosclerosis and some lysosomal storage diseases.[8,9] Moreover, it has been reported that modification of the hydroxyl moiety to generate 3,4-dihydroxypyrrolidin-2-yl derivatives have been performed, with stereochemistries in positions 2, 3 and 4 different from DAB or LAB, furnishing potent inhibitors of α-mannosidase from jack bean, drosophila GMII (dGMII) and α-mannosidases II from LN18 and LNZ308 human glioblastoma cell lines.[10-15]

Hence, we have considered interesting the investigation on the synthesis and inhibitory properties of new DAB and LAB 2-aminomethyl derivatives (Scheme 1). To this end, the hydroxymethyl functionality on DAB and LAB was substituted by aminomethyl moieties (Scheme 1) leading to a collection of conjugates with different molecules: aromatic amines (Section 3.2.3), aminoacids (Section 3.2.4), aminoalcohols (Section 3.2.5) and an iminocyclitol (D-fagomine, Section 3.2.5).



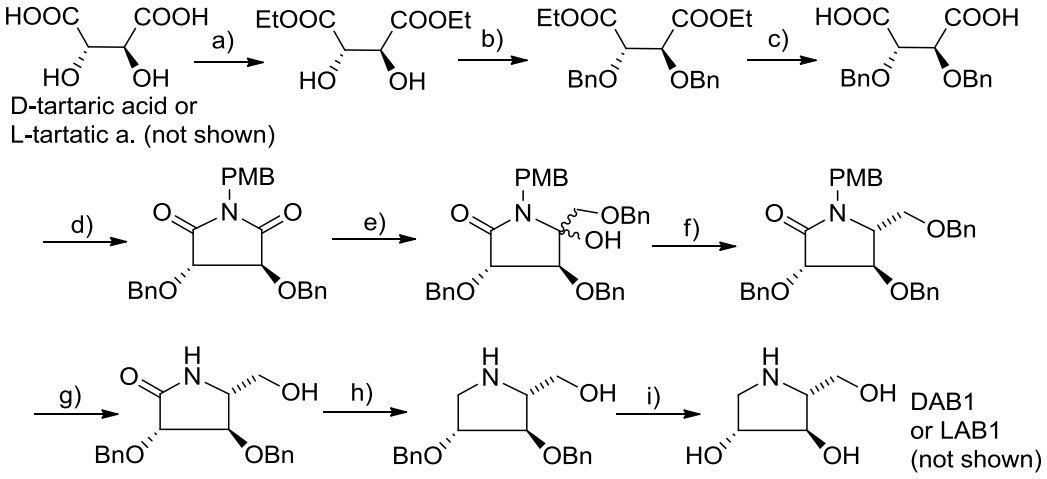
1: (2*R*,3*R*,4*R*) DAB1
2: (2*S*,3*S*,4*S*) LAB1

Scheme 1 Proposed DAB and LAB structural modification at the hydroxymethyl moiety.

3.2.1. Optimized chemical-enzymatic preparation of Cbz-DAB and LAB

Polyhydroxylated pyrrolidine DAB (**1**) and LAB (**2**) demonstrated a remarkable biological activity and thus many efforts have been devoted for their preparation. Chemical approaches to the preparation of DAB and LAB usually need chiral building blocks as starting materials. The synthesis from D-xylose requires 11-12 steps with overall yields of about 23 to 30% for DAB and LAB, respectively.[16-18] Another method started with an elaborated aldehyde derived from D-(–)-diethyltartrate furnishing DAB and LAB in ca 18% yield after 8-9 steps.[19] DAB and LAB were also prepared in 9 steps employing as starting material D and L-tartaric acid, respectively, with a 30-35% isolated yield (Scheme 2).[20]

Another synthetic strategy includes a combination of solid and solution-phase synthesis to generate rapidly a library of polyhydroxylated pyrrolidines in a combinatorial approach.[21] Recently, the synthesis of LAB was accomplished in 19% overall yield after 10 steps from L-serine using the Sharpless asymmetric dihydroxylation as the key step (Scheme 3).[22]



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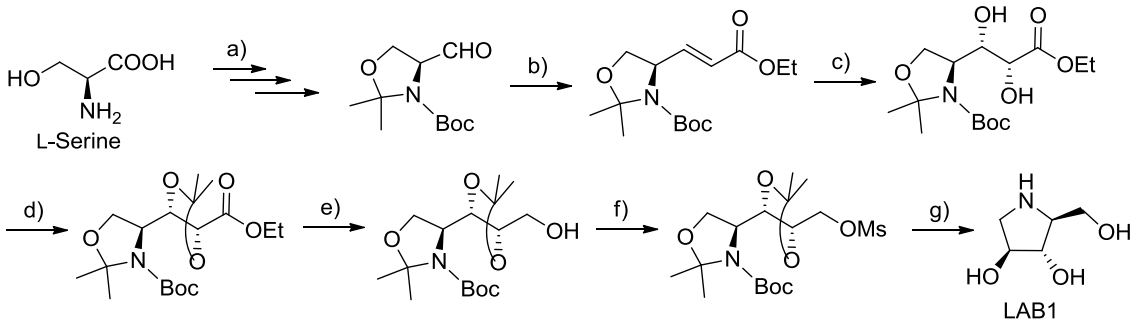
Scheme 2 Example of a chemical method for the synthesis of DAB and LAB. Reagents and conditions: a) EtOH, $SOCl_2$; b) NaH, BnBr, DMF, -20 to 0ºC; c) LiOH aq., EtOH, 0-5º; d) PBM-NH_2, CH_2Cl_2, AcCl reflux; e) $BnOCH_2Cl$, Mg, $HgCl_2$ (cat.), THF, -78 °C, 9 h; f) Et_3SiH, BF_3OEt_2, CH_2Cl_2, -78 °C to rt; g) $(NH_4)_2Ce(NO_3)_6$, CH_3CN:H_2O=9:1, 0 °C, 4 h; rt, 1.5 h; h) $LiAlH_4$, THF, 60 °C, 12 h; i) 10% Pd/C, HCOOH, MeOH, rt, 24 h; HCl; PMB = *para*-methoxybenzyl.



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Scheme 3 Recently reported enantioselective synthesis of LAB. Reagents and conditions: (a) (i) Boc_2O, 1 M NaOH, dioxane, H_2O, 0 °C to r.t., 3.5 h; (ii) MeI, K_2CO_3, DMF, 0 °C to r.t., 2 h, 86%; (iii) 2,2-DMP, acetone, $BF_3{\cdot}OEt_2$, 2 h, 90%; (iv) DIBAL-H, –78 °C, anhyd. toluene, 1–2 h, 75%; (b) $Ph_3P{=}CHCO_2Et$, anhyd. THF, 60 °C, 5 h, 90%; (c) OsO_4, $(DHQ)_2PHAL$, $K_3Fe(CN)_6$, K_2CO_3, $MsNH_2$, *t*-BuOH–H_2O, 0 °C, 24 h, 92%; (d) 2,2-DMP, TsOH, anhyd. toluene, reflux, 30 min, 96%; (e) $LiAlH_4$, anhyd. THF, 0 °C to r.t., 1 h, 75%; (f) MsCl, Et_3N, DMAP, anhyd. CH_2Cl_2, 0 °C,2 h, 81%; (g) 2 M HCl, EtOAc, 0 °C, 3 h, then sat. $NaHCO_3$ soln. (to pH 8), 1 h, 69%.

In section 3.1.1 we described the chemo-enzymatic synthesis of DAB starting from simple and achiral materials. This two-step strategy is based on the aldol addition of dihydroxyacetone to *N*-Cbz-glycinal catalyzed by D-fructose-6-phosphate aldolase wild type (FSA wt) followed by a one pot Cbz removal and reductive amination by means of H_2 in the presence of Pd/C. DHA reacted with *N*-Cbz-glycinal in the presence of FSA wt with a conversion of 60% and low isolated yield of final product was achieved (24%).

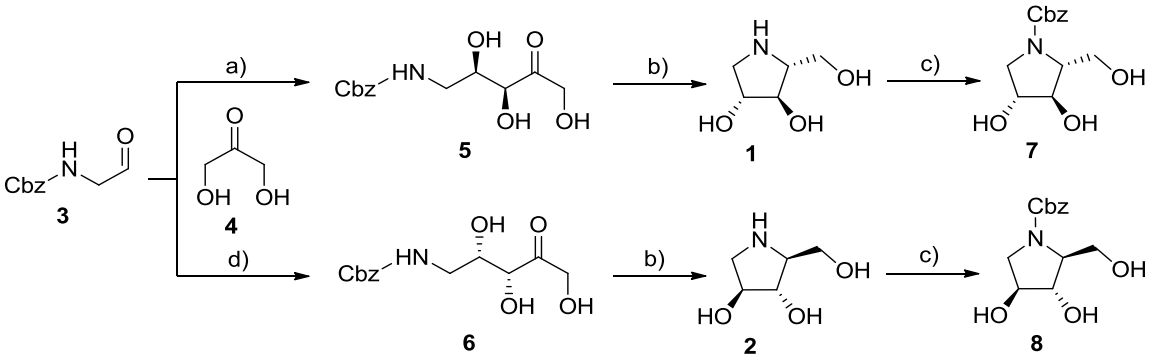
During the experimental period of the present thesis, a development on the structure oriented FSA modification led to a mutant, FSA Ala165Gly, which afforded the complete conversion for the aldol addition of dihydroxyacetone to Cbz-glycinal and enabled to obtain DAB in 70% isolated yield.[23]

Similarly, in another research in our lab was found that the precursor of LAB could be prepared by using L-rhamnulose-1-phosphate aldolase in the presence of 200 mM aqueous borate buffer (Section 1.2.3). LAB was thus obtained in 35% isolated yield (90% pure compound) (Scheme 4).[24]

These findings allowed us to develop a new chemoenzymatic and asymmetric steroedivergent methodology using the inexpensive and achiral DHA donor and Cbz-glycinal acceptor for the synthesis of DAB and LAB derivatives mediated by D-fructose-6-phosphate aldolase mutant Ala165Gly and L-rhamnulose-1-phosphate aldolase, respectively.

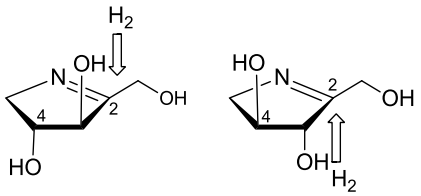
To optimize the synthetic route to *N*-Cbz protected DAB and LAB (*N*-Cbz-(2*R*,3*R*,4*R*)- and (2*S*,3*S*,4*S*)-2-hydroxymethyl-3,4-dihydroxypyrrolidine, **7** and **8**, Scheme 4), that are the key intermediates for the preparation of DAB and LAB 2-aminomethyl derivatives, we developed a

cascade methodology in which sequential reactions were performed on the aqueous crude residue without need for intermediate isolation and purification.



Scheme 4 Chemoenzymatic synthesis of DAB and LAB derivatives. Reagents and conditions: a) DHA, D-fructose-6-phosphate aldolase from *E. coli* mutant Ala165Gly b) H_2 (50 psi) Pd/C, c) benzyl chloroformate, dioxane/water 1:1, d) DHA, L-rhamnulose-1-phosphate aldolase from *E. coli* , 200 mM aqueous borate buffer.

After the aldol additions of DHA to *N*-Cbz-glycinal, a simple work up/purification was carried out. Reaction mixtures (crudes) were diluted with H_2O up to a double volume and loaded onto a glass column packed with Amberlite™ XAD™16 stationary phase. The column was washed with H_2O to efficiently remove the enzymes, the excess of DHA and the salts and the aldol adducts **5** and **6** were eluted with H_2O/EtOH. Pure fractions were collected and the solvent reduced in vacuum. The crudes were therefore treated overnight with H_2 (50 psi) in the presence of Pd/C to obtain DAB and LAB in aqueous solution. The reductive amination with Pd/C was highly diastereoselective and the stereochemistry observed at C-2 was controlled exclusively by the configuration at C-4 (Scheme 5). Indeed, no diasteroisomer at C4 was observed upon hydrogenation of the crude. In this case, the hydrogenation took place from the face opposite to the C-4 hydroxyl group, as reported previously by other authors and by our group.[25-31]



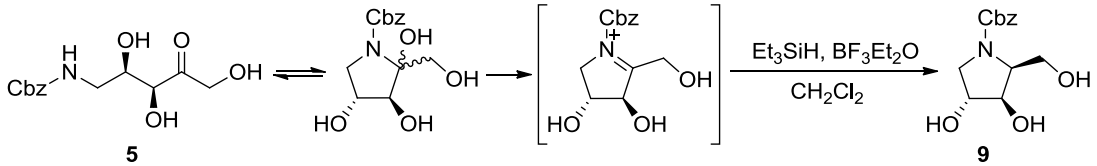
Scheme 5 The intramolecular diastereoselective hydrogenation of the imine took place from the face opposite to the C-4 hydroxyl group.

DAB and LAB (**1** and **2**) were protected without purification by treatment of the aqueous solution, diluted with 1,4-dioxane, with benzyl chloroformate at 0 °C. *N*-Cbz-DAB and *N*-Cbz-LAB (**7** and **8**) were finally purified by flash column chromatography on silica and obtained in

46% and 53% isolated yields, respectively. This optimized strategy compares favorably with other reported procedures (between 18-30% for the underivatized DAB and LAB).[16-22,32]

Moreover, with the aim to simplify the procedure, we attempted to perform the cyclization without need to remove the benzyloxycarbonyl protecting group on the amine function (Scheme 6).

In previous works, we have observed that the aldol adducts formed from the aldol addition of DHAP, DHA or glycolaldehyde to *N*-Cbz-aminoaldehydes, consist of a mixture of an acyclic and cyclic hemiaminal compounds in equilibrium (Scheme 6).[31,33-35] The percentage of the hemiaminal was particularly significant for the five member ring owing to the minimal transition state strain energy for its formation as compared with the other ring sizes.[31,33-35] Therefore, we envisaged to reduce the imine generated under acidic conditions from the hemiaminal of the cyclic form of *N*-Cbz-protected aldol adduct which exists in equilibrium with the corresponding open chain ketone. The aldol adduct from FSA catalyst was taken as example. Reduction with sodium cyanoborohydride and sodium borohydride failed in our hands. Reduction with triethylsilane in the presence of boron trifluoride diethyl etherate[36] proceeded in 68% isolated yield. However, the reaction furnished as major product *N*-benzyloxycarbonyl-1,4-dideoxy-1,4-imino-L-xylitol (*N*-Cbz-(2*S*,3*R*,4*R*)-2-hydroxymethyl-3,4-dihydroxypyrrolidine, **9**) with the C-2 configuration inverted as compared with the one obtained with the Pd/C catalyst (*cis* 2-4 instead of *trans* 2-4 stereochemistry). As ascertained by NMR data from the resulting deprotected iminocyclitol **9'** the diasteromeric ratio was 4:1 C2*S*:C2*R*.



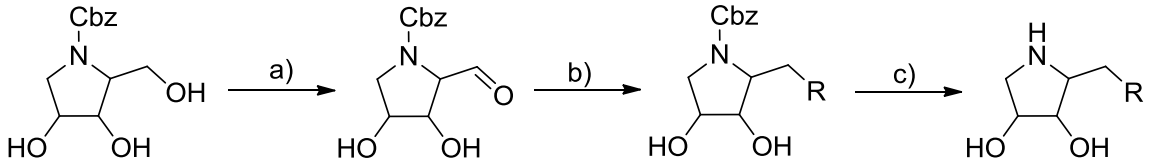
Scheme 6 Intramolecular reductive amination/cyclization of aldol adduct without removing amine protection.

Interestingly, this result encompasses more general applicability in the chemo-enzymatic strategy for the synthesis of iminocyclitols providing a useful potential stereocomplementary method for the reductive animation.

3.2.2. Synthesis of a collection of novel DAB and LAB 2-aminomethyl derivatives: general synthetic strategy

The synthetic strategy for the preparation of 2-aminomethyl derivatives of DAB and LAB (2*R*,3*R*,4*R*)- and (2*S*,3*S*,4*S*)-3,4-dihydroxy-2-aminomethylpyrrolidin derivatives, (Scheme 7), is based on sequential cascade reactions of oxidation and reductive amination of the *N*-benzyloxycarbonyl protected DAB and LAB (**7** and **8**, Scheme 7).

Aldehydes **10** and **11** ((2*S*,3*R*,4*R*)- and (2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehydepyrrolidine) were obtained by treatment of *N*-Cbz DAB (**7**) and LAB (**8**) with 2-iodoxybenzoic acid (IBX) at reflux in ethyl acetate. The oxidation reached complete conversion after 4h and these aldehydes were freshly and readily used without any further purification step. After filtration of the IBX the reaction solvent was thus changed to anhydrous methanol and the aldehydes reacted with an excess of amines at 20 ºC under acidic conditions (pH=5), providing the corresponding imines that were reduced *in situ* with sodium cyanoborohydride. The procedure provided *N*-Cbz-2-aminomethyl derivatives in 40-67% isolated yields. Deprotection of Cbz group by hydrogenolysis in the presence of Pd/C gave the corresponding DAB and LAB 2-aminomethyl derivatives, respectively (Scheme 7). The specific reactivity of each amine will be described later on.



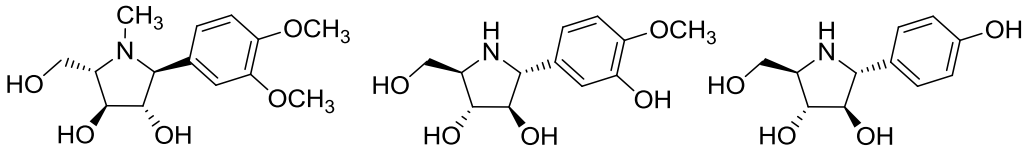
7: (2*R*,3*R*,4*R*)
8: (2*S*,3*S*,4*S*)

10: (2*S*,3*R*,4*R*)
11: (2*R*,3*S*,4*S*)

Scheme 7 Synthetic strategy for the preparation of DAB and LAB 2-aminomethyl derivatives from Cbz protected DAB and LAB. Reagents and conditions: a) 2-Iodoxybenzoic acid (IBX), AcOEt reflux, b) amine R-NH_2, CH_3COOH, $NaBH_3CN$, c) H2 (22 psi) Pd/C.

3.2.3. Reductive amination with aromatic amines

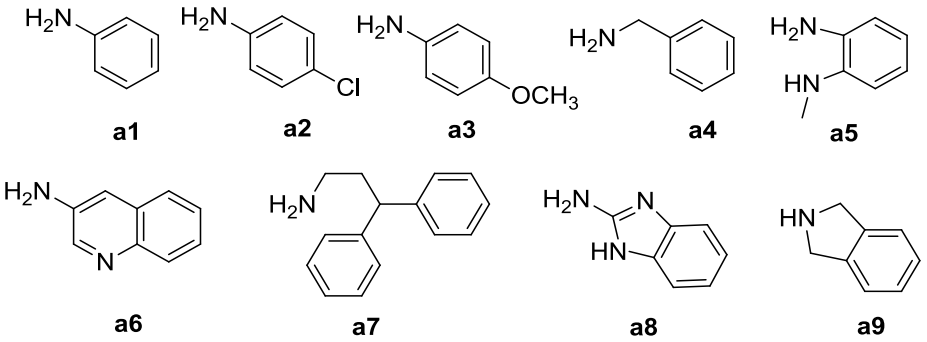
It was found that (2*R*,3*R*,4*S*)- and (2*S*,3*R*,4*S*)-2-(aminomethyl)pyrrolidine-3,4-diol derivatives with aromatic moieties lead to potent and selective competitive inhibitors of α-D-mannosidase from jack bean and from almonds.[11,12] The parent iminocyclitols, 1,4-dideoxy-1,4-imino-D-mannitol and 1,4-dideoxy-1,4-imino-D-ribitol with the two vicinal *cis*-oriented hydroxyl groups mandatory for effective inhibition, possessed already inhibitory activity against mannosidase.[37-39] Furthermore, a class of polyhydroxylated pyrrolidine derivatives namely codonopsinol[40] and radicamines A and B isolated from *Codonopsis clematidea* and *Lobelia chinensis Lour*, respectively[41,42] were uncovered, in which an aryl moiety is directly attached to the C-2 position of the pyrrolidine ring (Scheme 8).



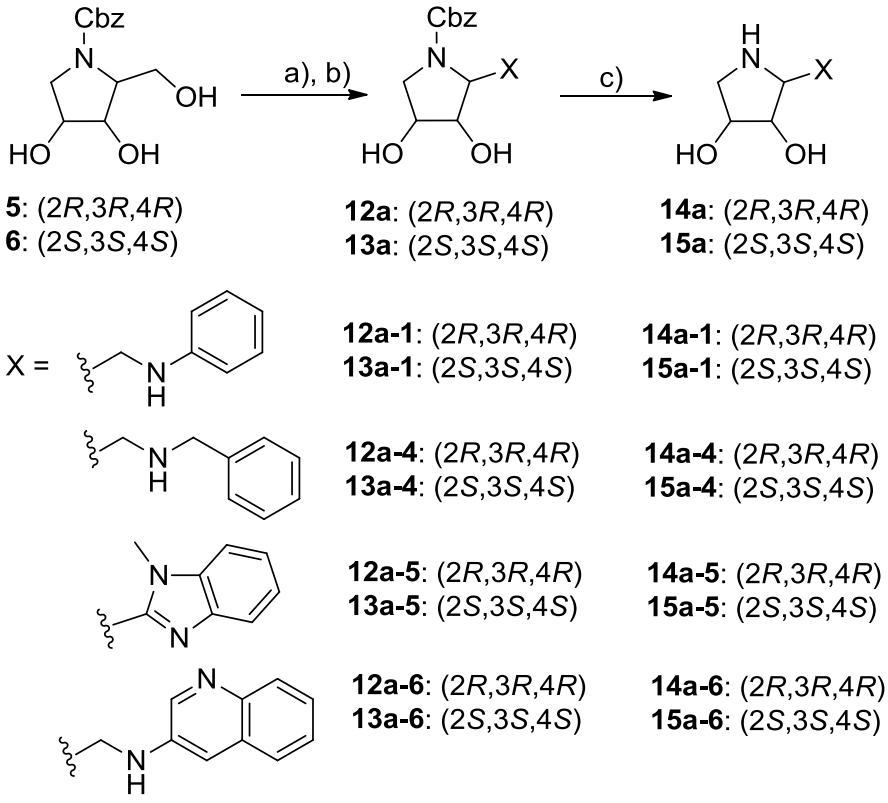
Scheme 8 Structures of codonopsinol and radicamines A and B isolated from *Codonopsis clematidea* and *Lobelia chinensis Lour*, respectively.

These compounds were found to be inhibitors of α-glucosidase at micromolar range. Recently, a library of 2-aryl polyhydroxylated pyrrolidine derivatives and their enantiomers, analogues of codonopsinol and radicamines, bearing different substituents on the aryl ring were reported, some of them with a strong inhibitory activity against α-glucosidases.[43] Inspired by these results we envisaged that similar modifications in DAB and LAB iminocyclitols may improve their inhibitory profile in terms of potency and/or selectivity.

Aromatic amines **a1-9** (Scheme 9) were therefore assayed as reagents for the reductive amination of aldehydes **10** and **11** ((2*S*,3*R*,4*R*)- and (2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehyde-pyrrolidine) in the presence of acetic acid. 4-Chloroaniline (**a2**), *p*-anisidine (**a3**), 3,3-diphenylpropylamine (**a7**) and 2-aminobenzimidazole (**a8**) didn't react with the aldehydes while isoindoline (**a9**) showed low reactivity leading to a poor 20% reaction conversion. Reductive amination with aniline (**a1**) and benzylamine (**a4)** followed by hydrogenolysis afforded the desired products **14a-1**, **15a-1** and **14a-4**, **15a-4**, respectively, with an isolated yield around 40% (Scheme 10, Table 1).



Scheme 9 Amines assayed as reagent for reductive amination.

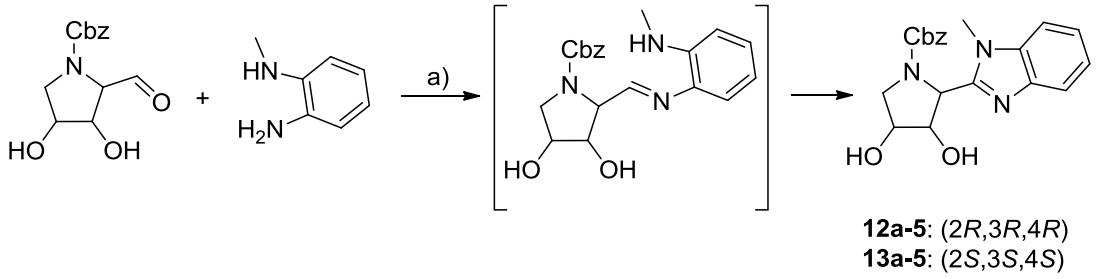


Scheme 10 Reagents and conditions: a) 2-Iodoxybenzoic acid (IBX), AcOEt reflux, b) amine **a1-9**, CH_3COOH $NaBH_3CN$, c) H_2 (22 psi) Pd/C

[a]	[b]	[c]	[d]	[e]	[f]
a1	(2*R*,3*R*,4*R*)	**12a-1**	58%	**14a-1**	38%
	(2*S*,3*S*,4*S*)	**13a-1**	40%	**15a-1**	34%
a4	(2*R*,3*R*,4*R*)	**12a-4**	56%	**14a-4**	44%
	(2*S*,3*S*,4*S*)	**13a-4**	48%	**15a-4**	40%
a5	(2*R*,3*R*,4*R*)	**12a-5**	67%	**14a-5**	52%
	(2*S*,3*S*,4*S*)	**13a-5**	55%	**15a-5**	40%
a6	(2*R*,3*R*,4*R*)	**12a-6**	55%	**14a-6**	40%
	(2*S*,3*S*,4*S*)	**13a-6**	57%	**15a-6**	54%

Table 1 Summary of results obtained for reductive amination reactions with aromatic amines and hydrogenolysis of furnished *N*-Cbz protected conjugates. [a]= Amine reagent, [b]= absolute configuration, [c]= *N*-Cbz-derivatives, [d]= oxidation/reductive amination isolated yields calculated from alcohols **7** and **8**, [e]= final derivatives [f]= global isolated yields for amine derivatives yields calculated from alcohols **7** and **8**.

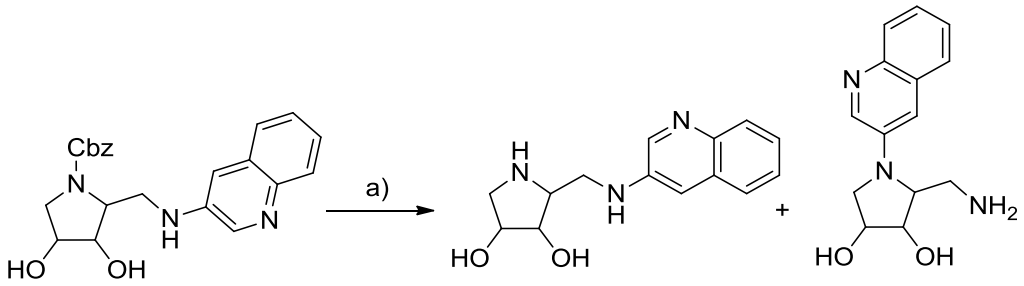
The reaction with *N*-methyl-1,2-phenylenediamine (**a5**) didn't proceed *via* reductive amination but expectedly *via* oxidative cyclocondensation (Scheme 11).[44,45] The reaction indeed occurred without reducing agent furnishing the corresponding Cbz-protected benzimidazole derivatives in 67% and 55% isolated yields, respectively (Scheme 10, Table 1). After deprotection by hydrogenolysis product **14a-5** and **15a-5** were obtained in a global isolated yield of 52% and 40%.



Scheme 11 Cyclocondensation with *N*-methyl-1,2-phenylenediamine. Reagents and conditions: a) CH_3COOH at 25ºC, spontaneous oxidation.

Reductive amination with 3-aminoquinoline (**a6**) provided the desired *N*-Cbz derivatives. However, unexpected products **14a-6'** and **15a-6'** were isolated after the final hydrogenolysis in almost 50% ratio respect to the desired products (Scheme 12). The attack of the incoming amine of the pyrrolidine to the position C3 of the quinolein moiety could be favored by the protonation of the aminomethyl groups which switches the electrophilic positions in the ring. The displacement of the aminomethyl functionality could thus occur in acidic conditions. We observe

indeed that the side reaction could be minimized by keeping the pH of the hydrogenolysis at 6-7. Therefore, only a 5% of byproducts **14a-6'** and **15a-6'** were obtained performing the final Cbz deprotection step at neutral pH.



12a-6: (2*R*,3*R*,4*R*)
13a-6: (2*S*,3*S*,4*S*)

14a-6: (2*R*,3*R*,4*R*)
15a-6: (2*S*,3*S*,4*S*)

14a-6': (2*R*,3*R*,4*R*)
15a-6': (2*S*,3*S*,4*S*)

Scheme 12 Reagents and conditions: a) H_2 (22 psi), Pd/C.

Products **14a-1(4,5,6)** and **15a-1(4,5,6)** were screened as inhibitors against a panel of commercial glycosidases (Section 3.4).

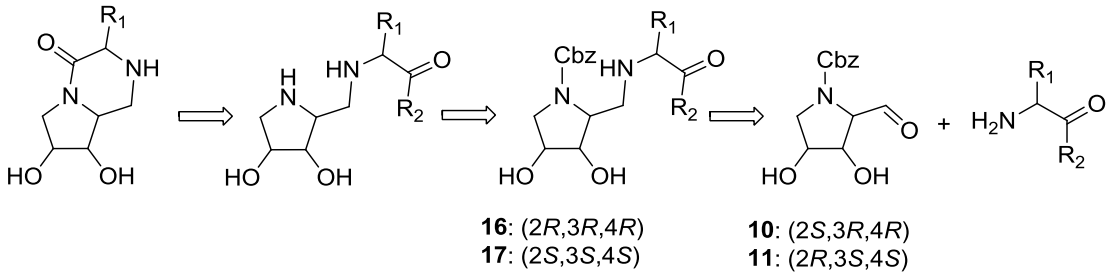
3.2.4. Reductive amination with aminoacids: synthesis of 2-oxopiperazine conjugates

The piperazine ring is an important pharmacophore in medicinal chemistry[46] since it is found in a vast array of biologically active molecules and in many positive hits during biological screens.[47] 2-Oxopiperazines are common structural elements of a number of natural compounds and used as peptidomimetic moieties for the discovery of novel, bioactive small molecules.[46,48] Therefore, piperazines and their keto analogues, such as 2-oxopiperazines, have been recognized as amongst the most important scaffolds in medicinal chemistry and drug discovery.

The biological significance of the piperazine ring and iminocyclitols prompted us to study the conjugation of these two moieties to obtain 2-oxopiperazine iminocyclitol fused derivatives namely hexahydropyrrolo[1,2-*a*]pyrazinone derivatives (Scheme 13).

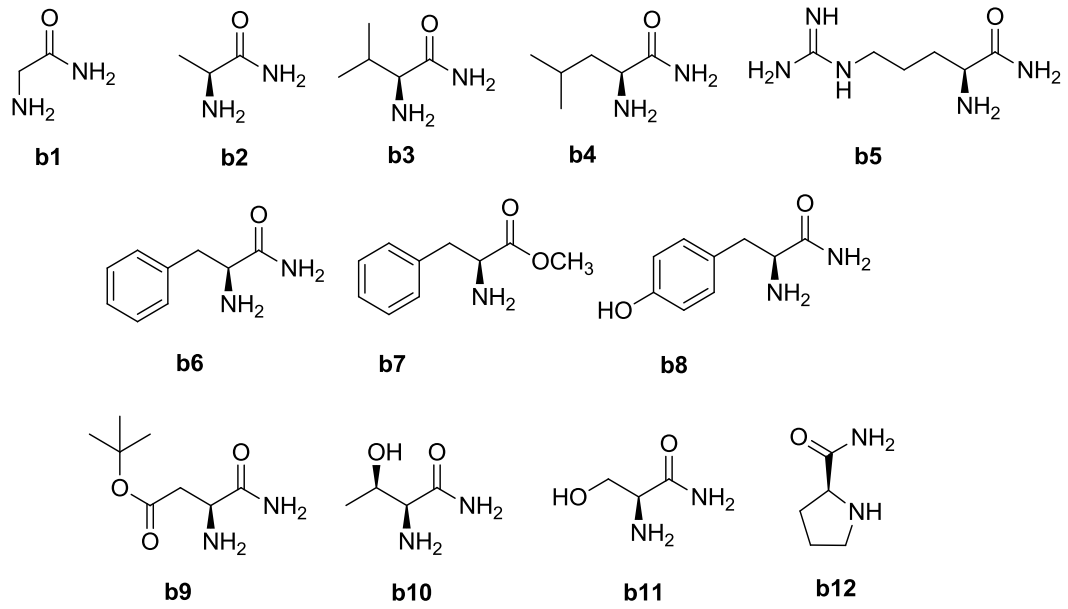
2-Oxopiperazines moiety could be retrosynthetically derived from Cbz unprotected 2-aminomethyl DAB or LAB aminoacidic derivatives (**16** and **17**) by intramolecular lactamization, in analogy with the undesired diketopiperazine formation that occur during solid phase peptide synthesis (Scheme 13).[49-51]

Indeed, after deprotection by hydrogenolisis, the intramolecular attack of the free amine of the pyrrolidine to the carbonyl in aminoacidic moiety generated an oxipiperazine ring fused to the iminocyclitol.



Scheme 13 Retrosynthetic strategy for the preparation of hexahydropyrrolo[1,2-*a*]pyrazinone derivatives.

Therefore, following the general methodology described above, we envisaged to conjugate DAB and LAB with aminoacids **b1-12** (Scheme 14) by reductive amination of aldehyde **10** and **11** (Scheme 15).

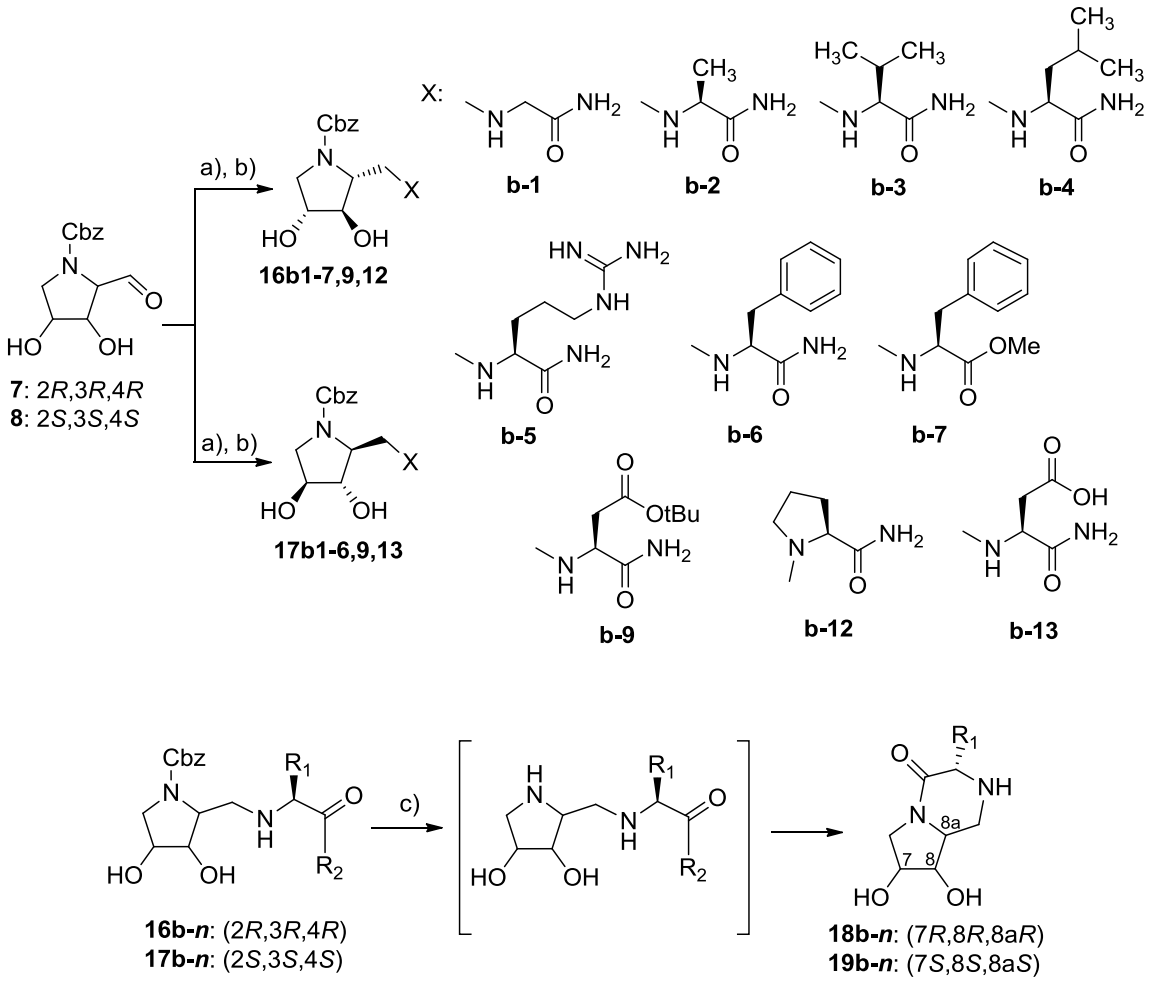


Scheme 14 Aminoacids used for the preparation of DAB and LAB 2-aminomethyl derivatives.

Aminoacids amides gave good isolated yields (51-70%) and were the reagents of choice. Aminoacid esters were the most obvious choice but it was discouraging to find out that only the Phe-OMe (**b7**) was readily capable to form the corresponding **18b-6** derivative. Furthermore, the β-carboxylate of the aspartic acid amide (**b9**) must be protected to make the reaction proceed towards the final compounds **18b-9** and **19b-13**.

L-Tyrosinamide (**b8**), L-threoninamide (**b10**) and L-serinamide (**b11**) didn't react with the aldehyde. Reductive aminations with glycinamide (**b1**), L-alaninamide(**b2**), L-valinamide (**b3**), L-leucinamide(**b4**), L-argininamide (**b5**), L-phenylalaninamide (**b6**), L-phenylalanin methylester (**b7**), *O-tert*-butyl-L-aspartamide (**b9**) and L-prolinamide (**b12**) furnished the desired

corresponding Cbz- DAB and LAB 2-aminomethyl derivatives (**16b** and **17b**) which were purified by HPLC and fully characterized (Scheme 15, Table 2).



Scheme 15 Reagents and conditions: a) 2-Iodoxybenzoic acid (IBX), AcOEt reflux, b) aminoacid **b1-12**, CH_3COOH $NaBH_3CN$, c) H_2 (22 psi) Pd/C; R_1= see Table 2.

After Cbz deprotection by treatment with H_2 in presence of Pd/C, we observed that only some of the afforded aminoacidic derivatives spontaneously cyclized to give the corresponding **18b** and **19b** desired products. Intramolecular lactamization was spontaneous for most of the aminoacidic derivatives even though only DAB derivative from Gly-NH_2 (**b-1**) and LAB derivatives form Gly-NH_2, Ala-NH_2 and Leu-NH_2 (**b-2** and **b-4**) underwent rapid and complete cyclization at room temperature.

The rest of DAB and LAB conjugates, unless derivative from Val-NH_2 (**b-3**), gave partial spontaneous cyclization and afforded mixtures of products. Therefore, we attempted to force the intramolecular lactamization by increasing the temperature.

LAB conjugates with Arg-NH_2 (**b-5**), Phe-NH_2 (**b-6**) and Asp(O*t*-Bu)-NH_2 (**b-9**) furnished the corresponding 2-oxopepirazineiminocyclitol derivative (**19b-5**, **19b-6** and **19b-13**) by heating at 40ºC. The aspartic acid derivative **17b-9** gave the corresponding intramolecular product

(**19b-13**) in quantitative yields upon removal of β-*t*-Bu protecting group. Derivative from Val (**b-3**) was the only LAB derivative that did not undergo complete lactamization reaction at 40ºC nor by increasing temperature at 100ºC.

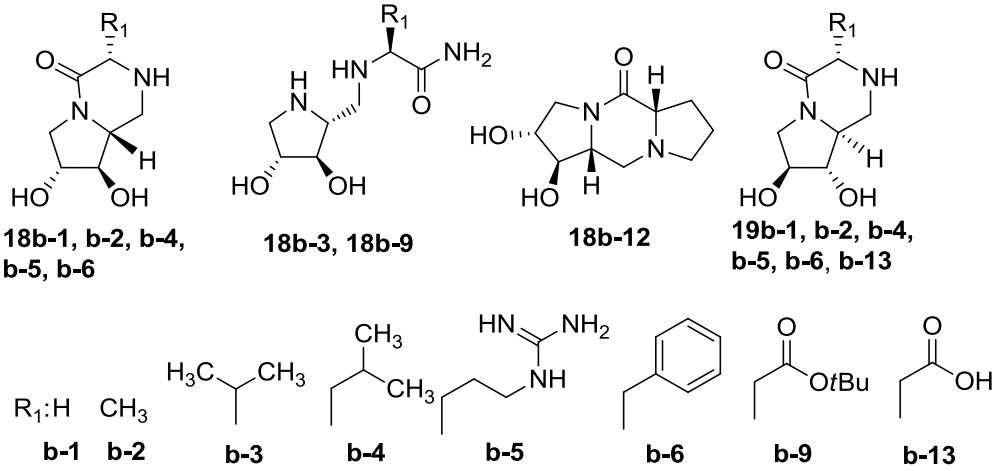
With regard to DAB derivatives the cyclization was partially spontaneous only for Ala-NH_2 and Pro-NH_2 conjugates, which afforded the desired product **18b-2** and **18b-12** by heating at 40ºC overnight. Arg-NH_2 (**b-5**) and Leu-NH_2 (**b-4**) DAB conjugates required refluxing in water overnight (100ºC) whereas, as in the case of LAB conjugate, Val-NH_2 derivative (**b-3**) did not give any bicyclic product in any of the conditions assayed, probably due to steric effects of the isopropyl group.

The phenylalanine amide derivative (**16b-6**) from DAB furnished only 50% of the bicyclic product (**18b-6**) even refluxing in water overnight, whereas quantitative yield were obtained at 40ºC from the corresponding acid of methyl ester derivative (**16b-7**).

The Asp(O*t*-Bu) conjugate (**16b-9**) did not form the cyclic species under mild conditions, while a number of unidentified byproducts appear when it was submitted to reflux in water (100ºC). Removal of the β-*t*-Bu group did not help to the lactamization reaction and it did not survive to the reaction conditions.

The conditions of lactamization of each derivative are summarized in Table 2.

This resulted in a new class of hexahydropyrrolo[1,2-*a*]pyrazinone derivatives (i.e. 2-oxopepirazine derivatives) that might be considered analogues of indolizidines (Scheme 16). These compunds were assayed as inhibitor of a series of commercial glycosidases (Section 3.4).



Scheme 16 DAB and LAB 2-oxopiperazine iminocyclitol fused derivatives and amino acid conjugates.

[a]	R_1 =	R_2 =	[b]	[c]	[d]	[e]	[f]	[g]
b1	-H	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-1**	58%	**18b-1**	25ºC	53%
	-H	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-1**	60%	**19b-1**	25ºC	60%
b2	$-CH_3$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-2**	58%	**18b-2**	40ºC	55%
	$-CH_3$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-2**	44%	**19b-2**	25ºC	44%
b3	$-CH(CH_3)_2$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-3**	51%	**18b-3***	-	44%
	$-CH(CH_3)_2$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-3**	50%	-	-	-
b4	$-CH_2CH(CH_3)_2$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-4**	62%	**18b-4**	100ºC	60%
	$-CH_2CH(CH_3)_2$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-4**	50%	**19b-4**	25ºC	50%
b5	$-(CH_2)_3NH-C=NH(NH_2)$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-5**	55%	**18b-5**	100ºC	50%
	$-(CH_2)_3NH-C=NH(NH_2)$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-5**	48%	**19b-5**	40ºC	48%
b6	-Bn	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-6**	70%	**-**	-	-
	-Bn	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-6**	55%	**19b-6**	40ºC	55%
b7	-Bn	$-OCH_3$	(2*R*,3*R*,4*R*)	**16b-7**	70%	**18b-6**	40ºC	66%
b8	$-CH_2PhOH$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-8**	-	-	-	-
	$-CH_2PhOH$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-8**	-	-	-	-
b9	$-CH_2COOtBu$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-9**	56%	**18b-9***	-	52%
	$-CH_2COOtBu$ (-COOH)	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-9 (17b-13)**	47%	**19b-13**	40ºC	47%
b10	$-CH(OH)CH_3$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-10**	-	-	-	-
	$-CH(OH)CH_3$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-10**	-	-	-	-
b11	$-CH_2OH$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-11**	-	-	-	-
	$-CH_2OH$	$-NH_2$	(2*S*,3*S*,4*S*)	**17b-11**	-	-	-	-
b12	$-CH-(CH_2)_3-$	$-NH_2$	(2*R*,3*R*,4*R*)	**16b-12**	63%	**18b-12**	40ºC	62%

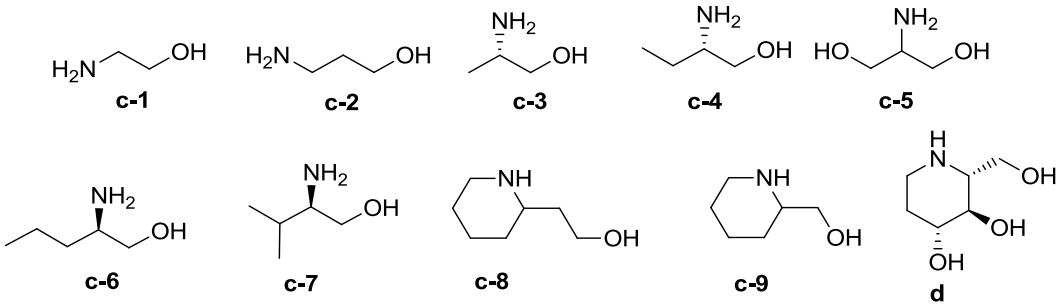
Table 2 Summary of results obtained for reductive amination reactions with aminoacids and deprotection/lactamization of furnished conjugates. [a] = Aminoacid reagent, [b] = absolute configuration, [c] = *N*-Cbz-derivatives , [d] = oxidation/reductive ammination isolated yields calculated from alcohols **7** and **8**, [e] = bicyclic derivatives or amino acid conjugates, * not cyclized (see Scheme 16) cyclized, [f] = lactamization conditions [g] = global isolated yields for bicyclic derivatives or amino acid conjugates calculated from alcohols **7** and **8**.

3.2.5. Reductive amination with aminoalcohols

In analogy with the 2-aminomethylpyrrolidine derivatives described in sections 3.2.3 and 3.2.4 we envisaged to prepare DAB conjugates with a series of aminoalcohols. 2-Aminomethyl derivatives of DAB with aminoalcohols can be regarded as analogues of 1,4-dideoxy-1,4-iminoalditols in which the corresponding polyhydroxyl chain is substituted by an aminoalcohol though a C-N bond.[52-59] Moreover, the introduction of different functionalities might increase the recognition sites of the molecule, that might improve its activity and selectivity.[60]

In order to evaluate the effect on biological activity of different aminoalcohols chain length, chain branch and absolute configuration at C7, we selected aminoalcohols **c1-9** (Scheme 17) that were therefore assayed as reagent for the reductive amination with aldehyde **10**.

Furthermore, the DAB 2-aminomethyl derivative with the secondary aminoalcohol D-fagomine (**d**) was prepared aiming to assess the result, on global biological properties, of the conjugation of two bioactive iminocyclitols.



Scheme 17 Aminoalcohols assayed for reductive amination of aldehyde **7**.

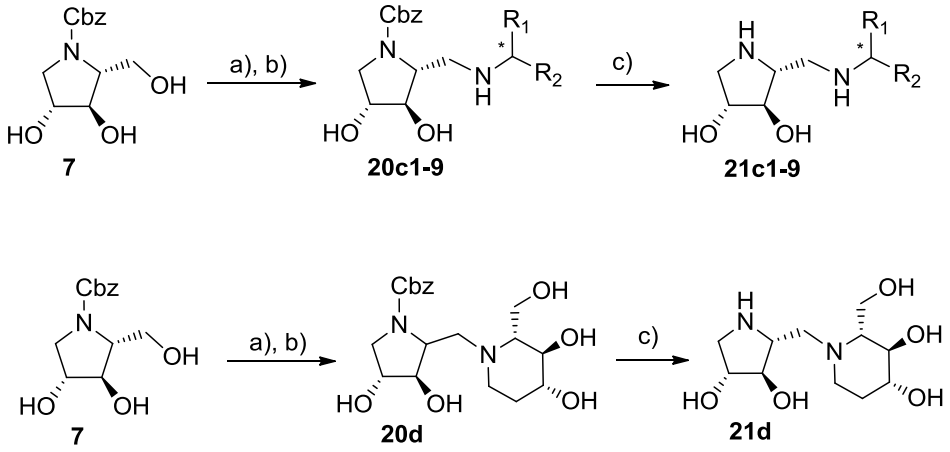
Ethanolamine (**c-1**), 3-aminopropanol (**c-2**), (*S*)-2-amino-1-propanol (**c-3**), (*S*)-2-amino-1-butanol (**c-4**), 2-amino-1,3-propanediol (**c-5**), (*R*)-2-amino-1-pentanol (**c-6**), (*R*)-2-amino-3-methyl-1-butanol (**c-7**) reacted with aldehyde **10** affording the corresponding *N*-Cbz-pyrrolidin derivatives **20c1-7** (Scheme 18, Table 3). After hydrogenolysis, products **21c1-7** were obtained in 45-66% isolated global yields.

D-Fagomine, also reacted with the aldehyde **10** affording the corresponding iminocyclitol biconjugate **21d** in 41% isolated yield (Scheme 18, Table 3). 2-Piperidinethanol (**c-8**) and 2-piperidinmethanol (**c-9**) were not reactive probably because of a combination of low nucleophilicity and steric hindrance of secondary amines.

The NMR analysis showed that for some conjugates a secondary product corresponding to 2-aminoalcohol 1,4-dideoxy-1,4-imino-L-xylitol derivative was identified. This resulted from the epimerization of the DAB at C2 that probably occurred during the imine formation. The amount varied depending on the aminoalcohol employed e.g. ethanolamine 25:75 C2*S*:C2*R*, propanolamine 1:9 C2*S*:C2*R*, 17:83 C2*S*:C2*R* for **c-3** and **c-4** and not observed for aminoalcohols

c5-7 and **d**. Separation of both compounds was accomplished by cation exchange chromatography under conditions employed in a previous work.[61]

Needless to say that an identical methodology can be followed for the LAB conjugates



Scheme 18 Reagents and conditions: a) 2-iodoxybenzoic acid (IBX), AcOEt reflux, b) Amines **c1-7** and **d**, CH_3COOH, $NaBH_3CN$, c) H_2 (22 psi) Pd/C.

[a]	[b]	R_1 =	R_2 =	[c]	[d]	[e]	[f]
c-1	-	H	CH_2OH	**20c-1**	40%	**21c-1**	40%
c-2	-	H	CH_2CH_2OH	**20c-2**	70%	**21c-2**	64%
c-3	(*S*)	CH_3	CH_2OH	**20c-3**	72%	**21c-3**	66%
c-4	(*S*)	CH_2CH_3	CH_2OH	**20c-4**	64%	**21c-4**	58%
c-5	-	CH_2OH	CH_2OH	**20c-5**	66%	**21c-5**	65%
c-6	(*R*)	$(CH_{2)2}CH_3$	CH_2OH	**20c-6**	57%	**21c-6**	51%
c-7	(*R*)	$CH(CH_3)_2$	CH_2OH	**20c-7**	47%	**21c-7**	44%
c-8	(*R*)/(*S*)	$-(CH_2)_4-$	$(CH_2)_2OH$	-	**-**	-	-
c-9	(*R*)/(*S*)	$-(CH_2)_4-$	CH_2OH	**-**	**-**	-	-
d	-	-	-	**20d**	55%	**21d**	41%

Table 3 Summary of results obtained for preparation of aminoalcohols and D-fagomine DAB conjugates. [a] = Aminoalcohols reagent, [b] = absolute configuration of C7, [c] = *N*-Cbz-derivatives , [d] = oxidation/reductive amination isolated yields calculated from alcohol **7**, [e] = final product derivatives [f] = global isolated yields for final derivatives yields calculated from alcohol **7**.

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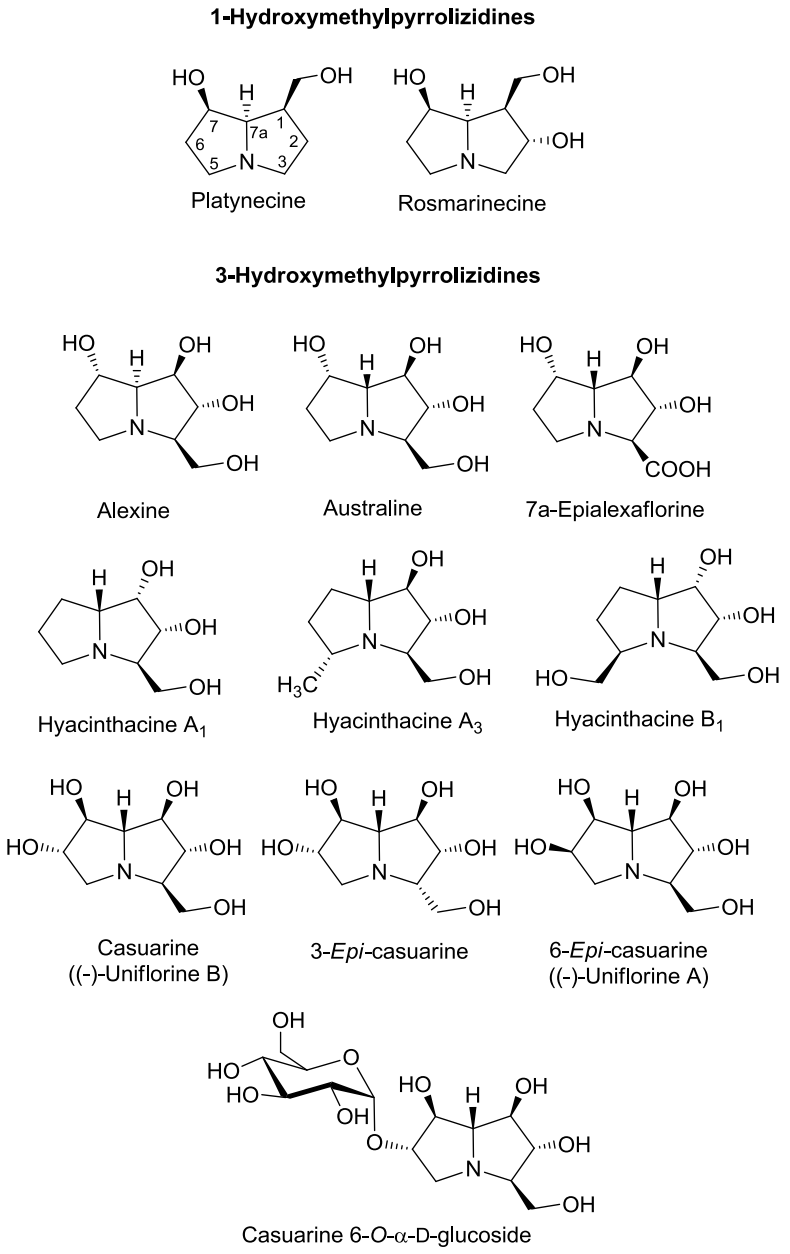
3.3. CHEMICAL-ENZYMATIC ASYMMETRIC SYNTHESIS OF NOVEL POLYHYDROXYLATED PYRROLIZIDINES

Polyhydroxylated pyrrolizidines are a class of bicyclic iminocyclitols with two five-membered rings fused together. These bicyclic alkaloids have a less obvious structural relationship to monosaccharides but in each case the configuration of hydroxyl substituents on the ring can be compared to those of sugars. Some of them possess a remarkable biological activity as inhibitors of glycoprocessing enzymes. Most of the pyrrolizidine alkaloids have been isolated from natural sources and exhibited inhibitory activity against various glycosidases and glycosyltransferases.[1] Therefore, polyhydroxylated pyrrolizidines, along with others alkaloid sugar mimics, have been regarded with interest as potential antidiabetic, anticancer and antiviral agents, as has been fully described in section 1.3 of this thesis.



Scheme 1 Examples of naturally occurring polyhydroxylated pyrrolizidines.

The great diversity of polyhydroxylated pyrrolizidines in the number hydroxyl substituent and their absolute configuration provide a broad spectrum of bioactivity. Most of naturally occurring pyrrolizidines possess a hydroxymethyl substituent at C-1 (e.g. necines, such as platynecine, Scheme 1)[2,3], however a relatively new group of pyrrolizidines with a carbon branch at C-3 (e.g., 3-hydroxymethylpyrrolizidine, Scheme 1) has been uncovered.[1]

Alexine and its 7a epimer, Australine, were the first alkaloids of this group that were isolated from *Alexa leiopetala* and *Castanospermum australe*, respectively.[4-6] Other stereoisomers of alexine and the related amino acid 7a-epialexaflorine, found in *Alexa grandiflora*[7] have also been identified.[1,6,8-12] Most of the alexines exhibit significant glycosidase inhibition, especially against amyloglucosidase, and antiviral properties.[9,13-16]

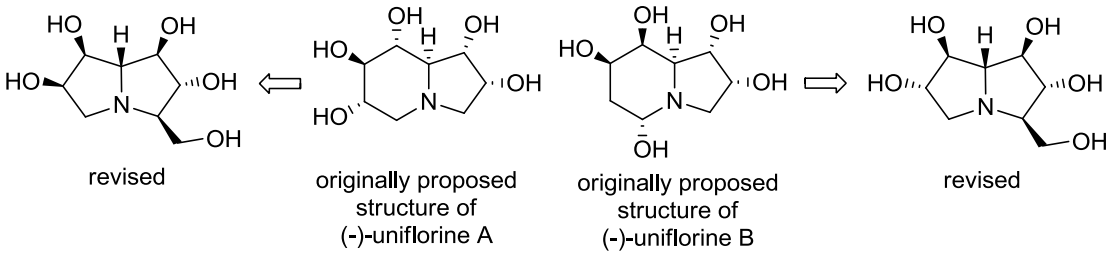
In 1999 a new family of pyrrolizidines alkaloids with carbon subtituents both at C-3 and C-5 were isolated from the Hyacinthaceae family plants and named hyacinthacines.[10-12,17,18] Hyacinthacines A1, A2, A3 and B3, have been isolated from the bulbs of *Muscari armeniacum*[11] and hyacinthacines B1, B2 and C1, from *Hyacinthoides non-scripta*[10] and *Scilla campanulata*[10]. Hyacinthacines B7, C2, C3, C4, and C5 have been recently found in *Scilla socialis*.[12,18,19] Hyacinthacines were found to be good inhibitors against various glycosidases such as rat intestinal lactase[10], α-L-fucosidase[11], amyloglucosidase[11], β-glucosidase and β-galactosidase form different sources.[18]

Highly oxygenated pyrrolizidines, e.g. casuarine (6-hydroxy-7-*epi*-australine, 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine, Scheme 1) and its 6-*O*-α-glucoside have been isolated from *Casuarina equisetifolia* (Casuarinaceae) and *Eugenia jambolana* (Myrtaceae).[20,21] Bark extracts of *Casuarina equisetifolia* have been claimed to be useful for treatment of diarrhea and colic and have been prescribed for the treatment of cancer in Western Samoa. Infusions prepared from *Eugenia jambolana* and *Eugenia uniflora* were also used in natural Paraguayan and Indian medicine as antidiarrheic, diuretic, antirheumatic, antifebrile, and antidiabetic preparations.[22-24]

Casuarine possesses 6 adjacent stereogenic centers and a potential 63 stereoisomers. Two diastereomers were isolated from natural sources, namely 3-*epi*-casuarine from the shrub *Myrtus communis L.* (Myrtle)[25], and 6-*epi*-casuarine from *Eugenia uniflora*.[22] The isolation of these diasteromers from natural sources indicates that, similar to the alexines and hyacinthacines, many stereoisomers may exist in Nature.

6-*Epi*-casuarine was originally named (−)-uniflorine A and erroneously given the structure of pentahydroxylated indolizidine, depicted in Scheme 2. Total synthesis of putative (−)-uniflorine A revealed that NMR data were not in agreement with those reported for the natural product and that the original structural assignment was incorrect.[26,27] Moreover, it was observe that NMR data and optical rotation of (−)-uniflorine B, also found in *Eugenia uniflora* extracts, correspond to those of the known alkaloid casuarine.[28]

The structures of (–)-uniflorine A and B were therefore revised to 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidines and the natural compounds were revealed to be actually casuarine and 6-*epi*-casuarine, respectively (Scheme 1). Further total synthesis of 6-*epi*-casuarine and non-natural enantiomers supported this structural hypothesis.[29-31]



Scheme 2 Original erroneous structures of (–)-uniflorine A and B.

Casuarines, as the other pyrrolizidine alkaloids, have remarkable biological properties. It is a potent and specific inhibitor of α-glucosidase (IC_{50}=1.2 μM againts α-glucosidase from rice) and amyloglucosidase from *Aspergillus niger* (IC_{50}=0.7 μM).[16,20] It is also able to inhibit rat intestinal maltase, isomaltase and sucrase.[16,22] Moreover casuarine exhibits powerful inhibition of glycoprotein processing glucosidase I (72% inhibition at 5 μg/mL) but no effect on glycoprotein processing in culture cells.[32] It has been recently reported that casuarine inhibits human maltase-glucoamylase (MGAM) more strongly than acarbose, a tetrasaccharide analogue currently on the market as an antidiabetic drug (Glucobay, Precose), and thus it may be a promising candidate as lead compound for the development of novel anti-diabetic drugs.[33]

Casuarine 6-*O*-α-D-glucopyranoside was shown to be active against fungal amyloglucosidase, porcine kidney trehalase and β-glucosidase but showed no inhibition towards α-glucosidases.[16] 6-*Epi*-casuarine ((–)-uniflorine A) exhibited moderate inhibitory activity against α-glucosidases from *Saccharomyces cerevisiae* and *Bacillus stearothermophilus*, rat intestinal maltase and sucrase, and amyloglucosidase from *Aspergillus niger* [22,34] whereas 3-*epi*-casuarine selectively inhibited β-glucosidases.[25]

As a result of these potentially useful biological activities, synthetic endeavors on the casuarines and their unnatural stereoisomers have been reported.[25,31,32,34-36] Furthermore, the unambiguous synthesis of casuarine diastereomers may shed light on structural characterization of newly isolated bioactive molecules, which is usually not trivial as it was the case of the uniflorines. At the same time, it may allow the identification of natural products in complex mixtures as well as furnish samples for the unequivocal determination of the biological properties of such materials.

The changes in configuration at one, or more, of the stereogenic centers of casuarines and other polyhydroxylated pyrrolizidines can cause a dramatic change in the conformation of the bicyclic system and in the biological properties.[37,25] It is not easy to predict the glycosidase inhibition profile from the configuration of the hydroxyl groups and the difference of the substituent on the pyrrolizidine ring. Therefore, it would be of interest for structure–activity relationship studies to

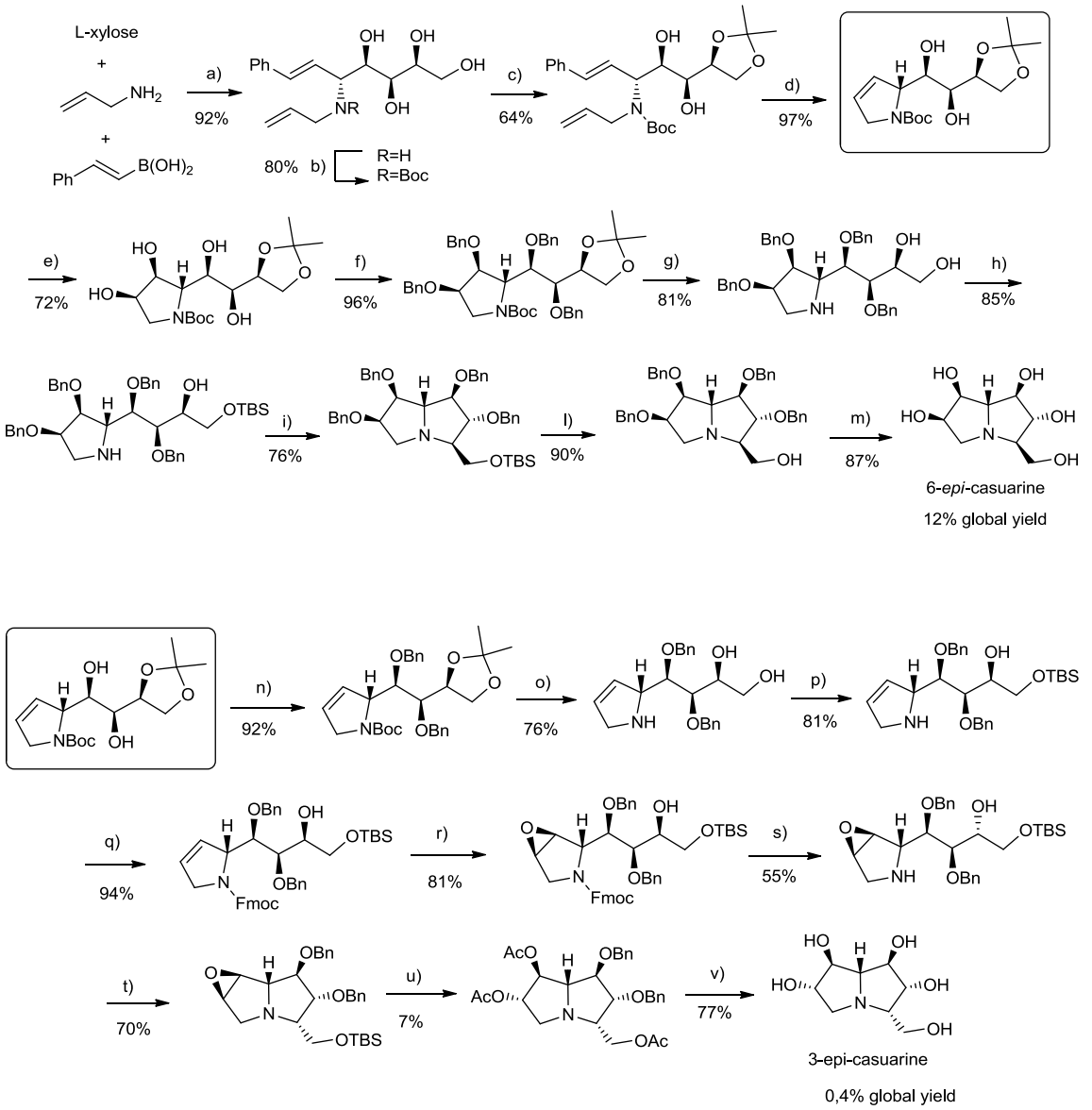
have a variety of highly oxygenated or substituted pyrrolizidine alkaloids in order to understand the structural requirements for glycosidase inhibition.[11]

The stereochemical richness of these alkaloids provides a considerable challenge for their efficient synthesis in amounts needed for biological evaluation. Their chemical syntheses usually involve chiral starting materials and cumbersome protection-deprotection schemes that lead to moderate stereoselection and global yields.[31,34] A recent example of chemical synthesis of casuarine isomers is shown in Scheme 3. Therefore, devising stereocontrolled synthetic routes that allow easy access to casuarine stereoisomers is of value.

In previous works of our group, we developed alternative chemoenzymatic methodologies for the preparation of polyhydroxylated pyrrolizidines of the hyacinthacine and alexine types. These procedures are based on aldol addition reactions of dihydroxyacetone phosphate (DHAP) to *N*-Cbz-pyrrolidine carbaldehyde derivatives from proline and 3- and 4-hydroxyproline catalyzed by L-rhamnulose-1-phosphate aldolase (RhuA) and L-fuculose-1-phosphate aldolase Phe131Ala mutant (FucA Phe131Ala) both from *E. coli*.[38,39]

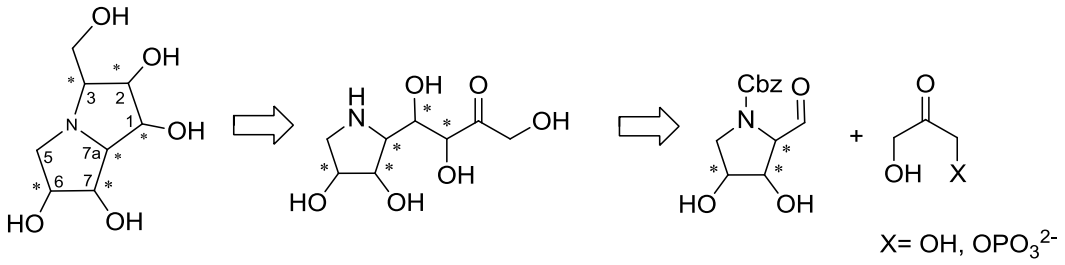
Inspired by our previous works, in this section we explore the chemo-enzymatic synthesis of highly polyhydroxylated pyrrolizidines of the casuarine type using FSA and DHAP-aldolases.

This approach did not require protection of the hydroxyl groups, minimizes the intermediate chemical steps thus reducing the purification steps, costs and wastes. Thus, a straightforward chemo-enzymatic route to casuarine stereoisomers, namely 2-*epi*-casuarine, 2,3-*epi*-casuarine, *ent*-casuarine and *ent*-3-*epi*-casuarine, is described.



Scheme 3 Recent chemical syntheses of polyhydroxylated pyrrolizidines with common intermediate from chiral starting material.[31,34] Reactions and conditions: a) EtOH, rt, ion-exchange; b) $(Boc)_2O$, Et_3N, MeOH, rt; c) DMP, PPTS, acetone, rt, 20h; d) Grubbs' I cat., CH_2Cl_2 reflux, 18h; e) $K_2OsO_4 \cdot 2H_2O$, NMO, acetone/H_2O, rt, 18h; f) NaH, BnBr, *n*-Bu_4NI, THF, rt, 1d; g) HCl/MeOH, rt, 18h; h) TBSCl, DMAP, imidazole, CH_2Cl_2, rt, 2d; i) DIAD, PPh_3, Et_3NHCl, py, rt, 3d; l) HCl/MeOH, rt, 18h; m) $PdCl_2$, H_2 (1 atm), MeOH, 1d, ion-exchange. n) NaH, BnBr, *n*-Bu_4NI, THF, rt, 18h; o) HCl/MeOH, rt, 30h; p) TBSCl, DMAP, imidazole, THF, rt, 1d; q) FmocCl, THF, sat Na_2CO_3, 0ºC, 3h; r) CF_3COOH, oxone, $NaHCO_3$, MeCN/H_2O, 0ºC, 2h; s) 1) *p*-NO_2ArCO_2H, DIAD, PPh_3, tol., rt, 5h, 2) K_2CO_3, MeOH, rt, 1d; t) DIAD, Ph_3P, tol., 80ºC, 3d; u) (1) $NaHSO_4$, CH_2Cl_2, 50ºC, 7d, (2) Ac_2O, py, DMAP, 24h; v) $PdCl_2$, H_2 (1 atm), MeOH, 4d, ion-exchange.

As shown in the retrosynthetic analysis (Scheme 4), we envisaged that 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine could be derived by two-step asymmetric aldol addition and reductive amination reactions from *N*-Cbz-pyrrolidine carbaldehyde derivative of 1,4-dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB) which were obtained chemoenzymatically as described in Section 3.2 of this thesis.



Scheme 4 Retrosynthetic analysis to obtain 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine.

Therefore the key steps of the synthetic strategy are: the stereoselective enzymatic aldol addition of DHAP or DHA to the aldehydes **3** and **4** (*N*-Cbz-(2*S*,3*R*,4*R*)- and (2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehydepyrrolidine) catalysed by DHAP- or DHA-dependent aldolases and the one pot hydrogenolysis reductive amination reactions (Scheme 5).

The absolute configurations of C-6, C-7 and C-7a of the pyrrolizidines are fixed by the starting adelhydes. The stereochemistries at C-1 and C-2 are controlled by the DHAP(DHA)-dependent aldolases, whereas the configuration at position C-3 depends on the reductive amination with Pd/C.

To this end we assayed the reactions of DHAP with the *N*-Cbz-pyrrolidine carbaldehyde intermediates **3** and **4** catalysed by the enzymes: L-fuculose-1-phosphate aldolase wild type, L-fuculose-1-phosphate aldolase from *E. coli* mutants Phe131Ala, Phe131Ala/Phe206Ala, Phe131Ser, Phe131Glu and L-rhamnulose-1-phosphate aldolase wild type from *E. coli*.

With the aim of avoiding the use of DHAP and the need for dephosphorylation step, we also attempted the enzymatic aldol additions of DHA to the same intermediates using L-rhamnulose 1-phosphate aldolase wt in the presence of borate buffer and D-fructose-6-phosphate aldolase (FSA) mutant Ala165Gly, both from *E. coli*.

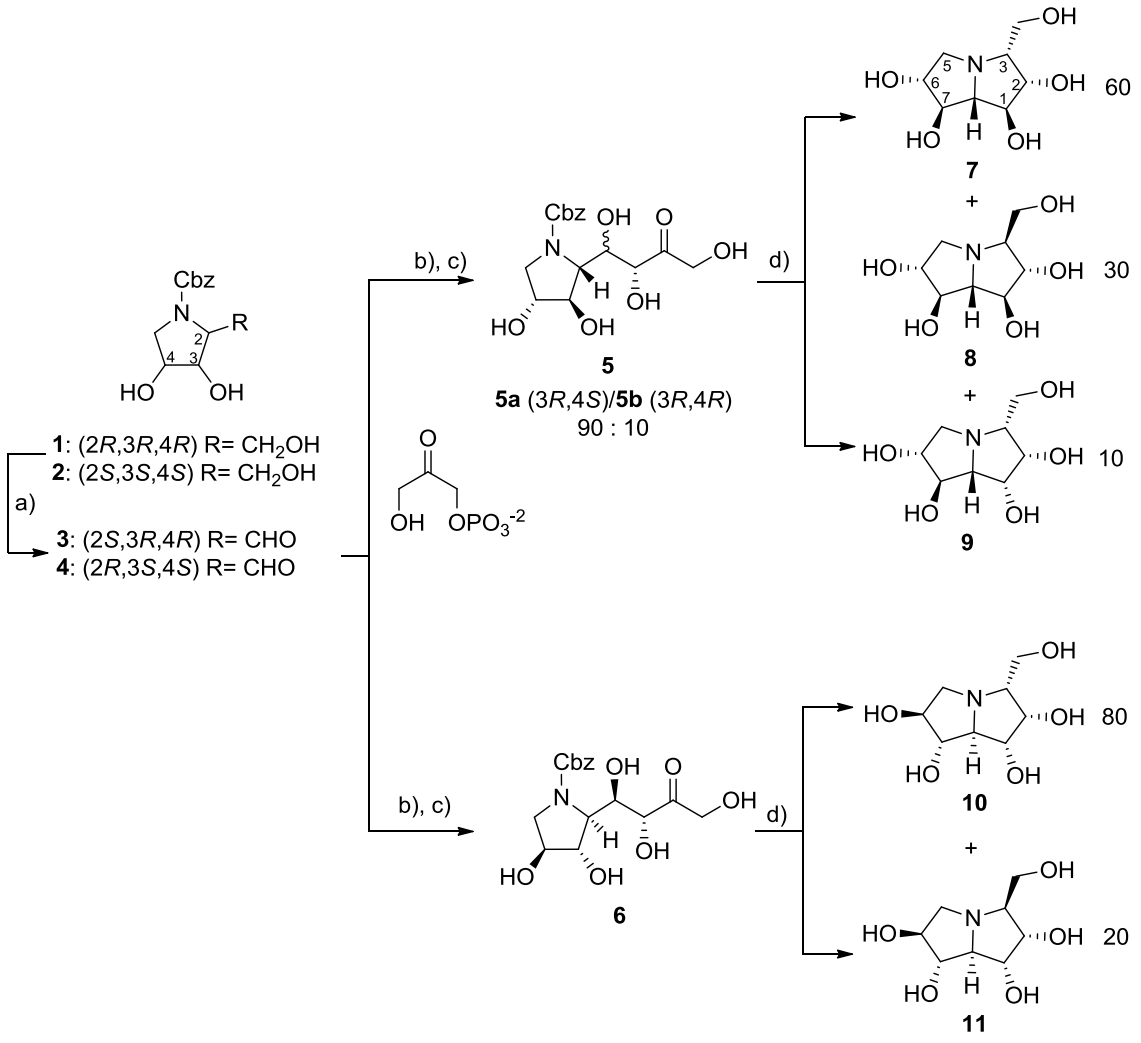
The reactions with L-fuculose-1-phosphate aldolase mutant Phe131Ala furnished the aldol adducts **5** and **6** in 50% conversion, whereas no aldol adduct products were detected for all the other enzymes in any of the conditions assayed.

The methodology for the preparation of the *N*-Cbz-polyhydroxylated pyrrolidines **1** and **2** (*N*-Cbz-DAB and LAB), and the corresponding *N*-Cbz-polyhydroxylated pyrrolidine carbaldehydes **3** and **4**, was described in section 3.2. After the oxidation of **1** and **2** with IBX, the solid was removed by filtration and the reaction mixtures were washed with $NaHCO_3$ 5% aqueous solution.

The aldol addition reactions with L-fuculose-1-phosphate aldolase mutant Phe131Ala were performed at 4ºC in DMF/buffer (5mM KCl, 50mand IC_{50} (μM) TEA, 1:4) and monitored by HPLC. After 24 h peak of the aldol adduct was constant with the time at 50% conversion. After workup, the aldol adducts from the two enantiomeric aldehydes were directly dephosphorylated with acid phosphatase without any prior purification.

The furnished unphosphorylated intermediates **5** and **6** were thus purified by HPLC and obtained with an isolated yield of around 20% in 3 steps.

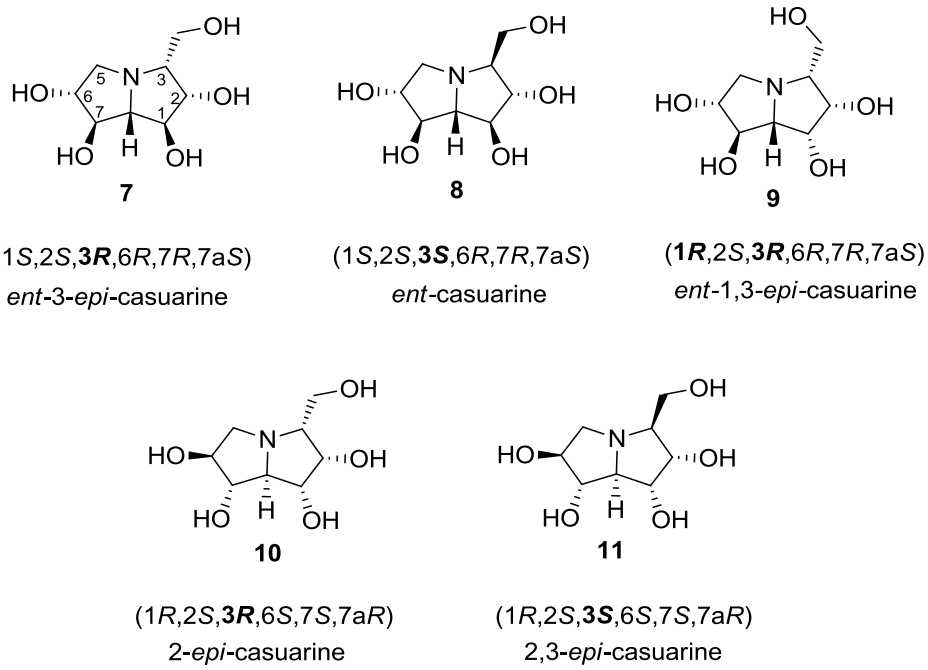
The one pot removal of *N*-Cbz protecting group/intramolecular reductive amination by catalytic hydrogenation with Pd/C furnished a material that was fully characterized by NMR as a mixture of casuarines stereoisomers: **7**,**8**,**9** from aldehyde **3** in 6:3:1 ratio and **10**,**11** in 4:1 ratio from aldehyde **4** (Scheme 5).



Scheme 5 Chemoenzymatic synthesis of four stereoisomers of casuarine. Reagents and conditions: a) 2-Iodoxybenzoic acid (IBX), AcOEt reflux, 4h; b) DHAP, L-fuculose-6-phosphate aldolase from *E. coli* mutant Phe131Ala, DMF:H_2O 1:4, 5 mM aq KCl, 50 mM aq TEA, 4ºC, 24h. c) Acid phosphatase, 50 mM citrate buffer pH 5; d) H_2 (50 psi) Pd/C.

Ion-exchange chromatography on a CM-sepharose in the NH_4^+ form eluted isocratically with 7 mM NH_4OH, gave an excellent separation of the two diastereoisomers that were fully characterized except **9** that was not detected after purification. From the NMR analysis resulted that the major product (**7**) from DAB-carbaldehyde derivative (**3**) had 1*S*,2*S*,3*R*,6*R*,7*R*,7a*S* configuration and the minor product (**8**) was its epimer at C3 with 1*S*,2*S*,3*S*,6*R*,7*R*,7a*S* configuration corresponding to the enantiomers of 3-*epi*-casuarine and casuarine, respectively (Scheme 6).

Similarly, from LAB-carbaldehyde derivative (**4**) we obtained compound (**10**) as major product with 1*R*,2*S*,3*R*,6*S*,7*S*,7a*R*, configuration that match the casuarine stereoisomer 2-*epi*-casuarine. The configuration of the minor product (**11**) was 1*R*,2*S*,3*S*,6*S*,7*S*,7a*R* which corresponded to the C-3 epimer of casuarine, 2,3-*epi*-casuarine.



Scheme 6 Casuarine stereoisomers obtained chemoenzymatically.

From these results we can conclude that, with regard to the stereochemistry of the aldol reactions, the (*S*)-pyrrolidinecarbaldehyde gave a mixture of the *syn/anti* configured aldol adducts in 9:1 ratio (**5**), which is the inverse stereochemistry in C4 as compared with the natural reaction (e.g. aldol adduct of DHAP to L-lactaldehyde), whereas its (*R*) enantiomer furnished exclusively the *anti* aldol adduct (**6**). Therefore, reactions catalyzed by the FucA Phe131Ala were highly stereoselective and the stereochemical outcome was governed by the structural features of the aldehydes, that is in good agreement with observations previously reported by our group.[39]

The formation of the two diasteromers was thus the result of the lack of stereoselectivity of the reductive amination catalysed by Pd/C. This reaction in fact gave mixtures of two diasteroisomers epimeric at C-3 that can be easily separated by ion-exchange chromatography.

Casuarine stereoisomers obtained are potential glycosidases inhibitor as described earlier. The full exploration of their inhibitory activities against commercial glycosidases and rat intestinal disaccharidases (sucrase, lactase and maltase) is currently in progress in our lab. The preliminary inhibitory properties found are summarized in Table 1.

	8	**7**	**10**	**11**
α-D-glucosidase[b]	5	0	7	2
α-D-glucosidase[c]	37	2	23	17
β-D-glucosidase[d]	n.i	n.i	60	70
β-D-galactosidase[e]	70	55	30	50
α-L-rhamnosidase[f]	30	70	n.i	n.i
α-D-mannosidase[g]	n.i	n.i	65	n.i
starch processing glycosidases[h]	40	6	22	29
maltase	35	4	19	27
lactase	n.i	55	30	57
sucrase	19 (203.7)	8 (2.3)	16 (104.4)	20 (248.1)

Table 1 Enzyme activities (%) at 1 mM and IC_{50} (μM) (in parenthesis) of the compounds synthesized, against rat intestinal saccharidases.[a] [a] The experiments were performed in triplicate for each set of saccharidases obtained from one rat. [b] From Baker's yeast. [c] From rice. [d] From sweet almonds. [e] From bovine liver. [f] From Penicillium decumbens. [g] From jack beans, Genus canavalia. [h] Inhibition of the glucose release from the hydrolysis of starch by the gut mucosal suspension from rat intestine. In this case starch can be hydrolysed by the amylases and amyloglucosidases releasing glucose and other reducing oligosaccharides, which can be further hydrolysed by the presence of disaccharidases.[39-41] n.i.: no inhibition.

The percentage of enzyme activity in the presence of inhibitor at 1 mM concentration has been measured and the IC_{50} has been determined for rat intestinal sucrase. The IC_{50} for the other enzymes will be determined in due course in our lab.

From these preliminary results it can be deduced that all the four products are good and selective inhibitors of α-D-glucosidase, especially from Baker's yeast. The *ent*-3-*epi*-casuarine is the most potent inhibitor of both α-D-glucosidase from Baker's yeast and rice whereas is not active against β-D-glucosidase as its enantiomer.[25]

Concerning the inhibitory properties against the rat intestinal disaccharidases all the products have moderate to good activity except the *ent*-casuarine that did not inhibit lactase.

Insterestingly, the *ent*-3-*epi*-casuarine is a potent inhibitor of maltase, sucrase (IC_{50}=2.3 μM) and glucose release from starch hydrolysis in a good agreement with the inhibition profile exhibited against commercial α-D-glucosidases. As selective inhibitors of α-D-glucosidases these products are potentially useful for treatment of diabetes type 2.

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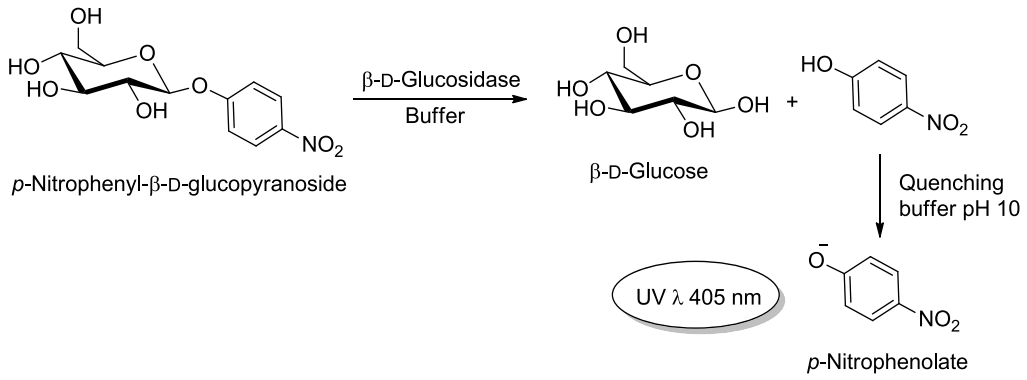
3.4. GLYCOSIDASES INHIBITION ASSAYS

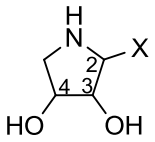
The novel 1,4-dideoxy-1,4-imino-D- and -L-arabinitol 2-aminomethyl derivatives (Scheme 2), prepared using the methodologies detailed in Section 3.2 were tested as inhibitors against a panel of commercial glycosidases. This was considered a preliminary screening to establish their inhibitory properties and the potential therapeutic targets that they can be directed to. We examined the enzyme inhibition activities against α-D-glucosidase from baker's yeast and rice, β-D-glucosidase from sweet almonds, β-D-galactosidase from bovine liver, α-L-rhamnosidase from *Penicillium decumbens*, α-D-mannosidase from jack beans (*Genus canavalia*) and α-L-fucosidase from bovine kidney.

The enzyme inhibition activities for commercial glycosidases were evaluated according to the methodology previously reported in our lab with minor modifications.[1-3] The experimental details are described in Section 4.5. A colorimetric method based on the use of commercial enzymes and *p*-nitrophenyl substrates was used.

Glycosidase solutions were prepared with the appropriate buffer and incubated with the test compound (i.e., inhibitor) at different concentrations. After addition of the corresponding *p*-nitrophenyl substrate solution, incubations were continued for different time periods depending on the enzymatic inhibition assay, then the reaction was stopped by addition of a quenching solution and its absorbance at 405 nm was recorded using a microplate reader. Upon treatment with quenching buffer (basic pH=10) the *p*-nitrophenol liberated in each assay is converted to *p*-nitrophenolate anion whose amount was measured by UV/VIS spectrophotometry at 405 nm (Scheme 1).

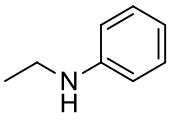


Scheme 1 Schematic of glycosidase inhibitory activity assay (example for β-D-glucosidase).

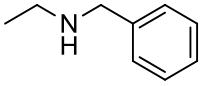


14a-1, a-4, a-5, a-6: (2*R*,3*R*,4*R*)
15a-1, a-4, a-5, a-6: (2*S*,3*S*,4*S*)

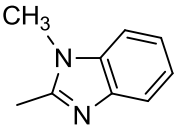
X:



a-1



a-4



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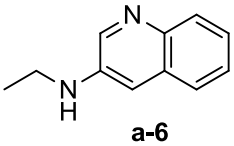
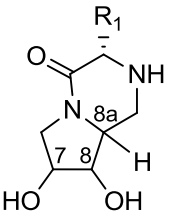
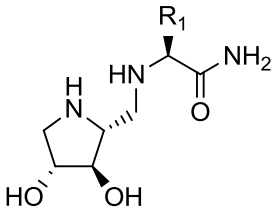


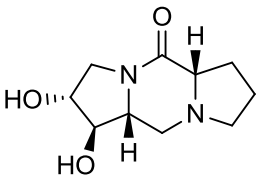
a-6



18b-1, b-2, b-4, b-5, b-6: (7*R*,8*R*,8a*R*)
19b-1, b-2, b-4, b-5, b-6, **b-13**: (7*S*,8*S*,8a*S*)

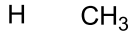


18b-3, 18b-9

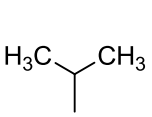


18b-12

R^1:



b-1 **b-2**



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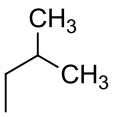

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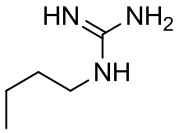

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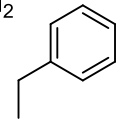

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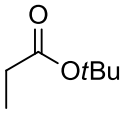

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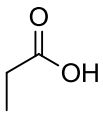
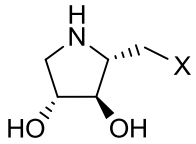
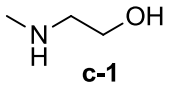


b-13

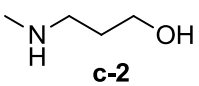


21c-1, c-2, c-3, c-4, c-5, c-6, c-7, d

X:



c-1



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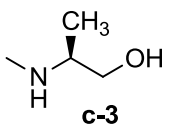

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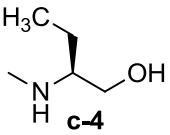
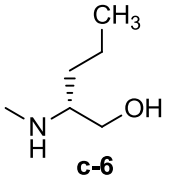
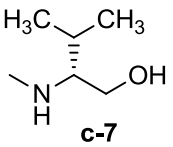


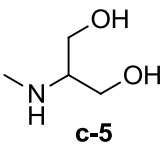
c-4



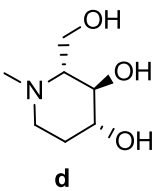
c-6



c-7



c-5



d

Scheme 2 Structures of iminocyclitol conjugates tested for glycosidase inhibition activity.

The inhibitory activity was compared with that of the parent compounds DAB and LAB, measured in our laboratory in a previous work.[2] The results of inhibitions assays are shown in Table 1 and are described here below. The inhibitory profiles of the new derivatives differ considerably from those of DAB and LAB:

α-D-Glucosidase from baker's yeast and rice: The aromatic **14a-1**,**a-4**,**a-6** and **15a-1**,**a-4**,**a-5**, **a-6** derivatives have much lower inhibitory properties against α-D-glucosidase from baker's yeast than the parent compounds DAB and LAB (Table 1). Only compound **15a-1** showed moderate noncompetitive inhibition against this glucosidase. Among the amino alcohol and amino acid conjugates and 2-oxopiperazine-iminocyclitol derivatives synthetized, compounds **21c-6**, **18b-4** and **18b-9** were the best competitive inhibitors of α-D-glucosidase from baker's yeast with comparable activities. Compound **18b-3** was the best noncompetitive inhibitor of this glucosidase. Although the structure of the substituent differs considerably, all of them have a DAB configuration (Scheme 2). The aromatic derivatives and amino alcohol conjugates were mostly inactive against α-D-glucosidase from rice. Among the 2-oxopiperazine-iminocyclitol derivatives, compound **19b-4** was the best inhibitor among the derivatives tested, while **18b-2** and **18b-5** showed inhibitory properties comparable to the parent compound DAB.

β-D-Glucosidase from sweet almonds: Compound **15a-4** was a moderate inhibitor of β-D-glucosidase from sweet almonds while the parent compound LAB and its enantiomer DAB were weak inhibitors of this glycosidase. The rest of the evaluated compounds had either weak activities or were completely inactive against this glycosidase.

β-D-Galactosidase from bovine liver: Aromatic derivatives **14a-1**, **14a-6** and **15a-6** were weak inhibitors while the parent DAB and LAB were inactive towards this glycosidase. It appears that the presence of an aromatic moiety is necessary for the inhibitory activity.[4-6] Indeed, among the 2-oxopiperazine-iminocyclitol derivatives only **19b-6**, derived from phenylalanine, showed some activity. The rest of derivatives were not inhibitors of this glycosidase.

α-L-Rhamnosidase from Penicillium decumbens: Among the aromatic substitutions only those with LAB configuration, **15a-1-15a-6**, were moderately to weakly active against α-L-rhamnosidase; consequently, the orientation of the hydroxyl groups and the amine moiety is a strong factor that determines the right interaction with the glycosidase. It has been suggested that the α-L-rhamnosidase inhibition shown by some pyrrolidines can be rationalized in terms of stereochemical similarities with α-L-rhamnose.[6,7] Thus, it is apparent that the stereochemistry of **15a-1-15a-6** (2*S*,3*S*,4*S*) matches that of the rhamnose moiety at C-3, C-4 and C-5. This would place the 2-aryl containing substituent on the same location as the 5-methyl group of rhamnose. The best inhibitor was compound **15a-1** whose structure is similar to those reported by Chapman et al. (e.g. (2*S*,3*S*,4*S*)-2-benzylpyrrolidine-3,4-diol).[6] Moreover, Kim et al. also proposed a role as aglycone for hydrophobic substituents at C-2 of pyrrolidines.[7] This is consistent with the absence

of inhibitory properties in the amino alcohol and 2-oxopiperazine derivatives, where basically non-aromatic moieties are present.

α-D-Mannosidase from jack beans Genus canavalia and α-L-fucosidase from bovine kidney: It appears that a *cis* arrangement of the hydroxyl groups at C-3 and C-4, which parallels that of the hydroxyl groups at C-2 and C-3 of α-D-mannose and other known pyrrolidine inhibitors like mannostatin A, is a strong structural requirement for the inhibition of α-D-mannosidase by pyrrolidine iminocyclitols.[8-15] The fact that the compounds synthetized do not match this C-3 and C-4 configuration is probably the reason for their inability to inhibit this glycosidase.
Similarly to α-D-mannosidase, α-L-fucosidase has also a strong stereochemical demanding active site.[15] Pyrrolidine derivatives with stereochemistry (3*S*,4*R*,5*S*) were reported as strong inhibitors of α-L-fucosidase,[15-18] whereas diasteromeric analogues were usually moderate to weak inhibitors.[19] The derivatives obtained in the present work have (3*R*,4*R*) or (3*S*,4*S*) configuration, therefore they showed no inhibition activities.

A preliminary exploration of the inhibitory activities against rat intestinal disaccharidases (sucrase, lactase, maltase) have also been performed in our lab and will be part of the doctoral thesis of Ms Livia Gómez Cortés. It has been suggested that the iminocyclitol derivatives which are inhibitors of α-L-rhamnosidase from *P. decumbens* could have the ability to inhibit deoxythymidine diphosphate (dTDP)-L-rhamnose biosynthesis on *Mycobacterium tuberculosis*, and therefore constitute potential chemotherapeutic agents for the tuberculosis.[20-22] We have established collaboration with Dr. Cristina Vilaplana and Prof. Dr. Pere Joan Cardona from the Unitat de Tuberculosi Experimental. Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol of the Universitat Autònoma de Barcelona, CIBERES. The inhibitors of α-L-rhamnosidase found in this work have been also assayed in mycobacterial systems, particularly against *Mycobacterium tuberculosis* H37Rv laboratory strain, in the lab of Prof. Dr. Cardona and Ms Gómez from our lab was in charge to carry out the experiments.

Table 1 Activities, IC_{50} (μM) and K_i (μM) (in parenthesis), of the compounds synthesized against commercial glycosidases.[a]

Product	α-D-glucosidase[b]	α-D-glucosidase[c]	β-D-glucosidase[d]	β-D-galactosidase[e]	α-L-rhamnosidase[f]	α-D-mannosidase[g]	α-L-fucosidase[h]
14a-1	n.i.	n.i.	370 ± 11 (215 ± 18) C	155 ± 19 (406 ± 128) NC α = 1	n.i	n.i	n.i
15a-1	40 ± 6 (44 ± 13) NC α = 1	620 ± 29 (236 ± 61) C	n.i.	n.i	15.6 ± 0.5 (38 ± 12) NC α = 1	n.i	n.i
14a-4	n.i.	n.i	832 ± 105 (338 ± 116) C	n.i	n.i	n.i	n.i.
15a-4	136 ± 13 (208 ± 102) NC α = 1	n.i.	38 ± 4 (41 ± 13) C	n.i	274 ± 35 (699 ± 112) NC α > 1	n.i	n.i.
15a-5	n.i.	n.i.	n.i.	n.i	320 ± 130 (331±19) NC α = 1	n.i	n.i.
14a-6	165 ± 44 (170 ± 95) C	n.i	n.i.	263 ± 50 (308 ± 55) NC α = 1	n.i	n.i	n.i.
15a-6	247 ± 26 (100 ± 66) NC α = 1	n.i.	n.i.	401± 66	132 ± 31 (132 ± 27) C	n.i	n.i.
18b-2	n.i.	76 ± 19 (39 ± 2) NC α = 1	n.i.	1116 ± 28	n.i.	n.i.	n.i.
18b-3	**1.1 ± 0.1** **(54 ± 20)** **NC α > 1**	448 ± 214 (190 ± 33) C	n.i.	n.i.	n.i.	n.i.	n.i.

18b-4	**9.2 ± 1.5 (7.1 ± 1.5) C**	208 ± 23 (261 ± 29) C	285 ± 15 (394 ± 19) C	n.i.	n.i.	n.i.	n.i.
18b-5	n.i.	60 ± 19 (21 ± 7) C	n.i.	n.i.	n.i.	n.i.	n.i.
18b-9	**3.9 ± 3.1 (16.7 ± 7.8) C**	685 ± 110 (451 ± 170) C	n.i.	n.i.	n.i.	n.i.	n.i
19b-1	401 ± 35 (323 ± 65) NC $\alpha > 1$	n.i	n.i	n.i.	n.i	n.i	n.i.
19b-2	239 ± 9 (323 ± 64) NC $\alpha > 1$	n.i	n.i.	n.i.	n.i	n.i.	n.i.
19b-4	340 ± 25 (337 ± 84) NC $\alpha > 1$	14 ± 2 (13 ± 2) C	n.i.	n.i.	n.i.	n.i.	n.i.
19b-5	140 ± 44 (124 ± 13) NC $\alpha = 1$	n.i.	n.i.	n.i.	n.i.	709 ± 56	n.i.
19b-6	50 ± 14 (76 ± 8) UC	148 ± 50 (87 ± 25) C	n.i.	690.5 ± 85.9	n.i.	174 ± 12 (103 ± 27) NC $\alpha = 1$	n.i.
19b-13	41 ± 8 (181± 24) UC	382 ±41 (176 ± 4) C	n.i.	n.i.	n.i.	n.i.	n.i.
21c-1	273 ± 31 (358 ± 52) NC $\alpha > 1$	n.i	n.i	n.i.	n.i	n.i	n.i.

21c-2	158 ± 25 (153 ± 8) NC α > 1	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
21c-4	n.i.	n.i.	260 ± 100 (734 ± 19) NC α > 1	n.i.	n.i.	n.i.	n.i.
21c-6	**4.6 ± 1.6** **(2.1 ± 0.8)** C	342 ± 90 (144 ± 90) C	150 ± 71 (223 ± 9) NC α > 1	n.i.	n.i.	n.i.	n.i.
21c-7	110 ± 13 (96 ± 10) NC α > 1	n.i.	460 ± 353 (561 ± 30) NC α > 1	n.i.	n.i.	n.i.	n.i.
21d	n.i.	466 ± 64 (186 ± 21) C	n.i.	n.i.	n.i.	n.i.	n.i.
DAB	0.33 ± 0.02 (0.17 ± 0.01) C	218 ± 3 (104 ± 75) C	276 ± 25 (100 ± 64) C	n.i.	n.i.	286 ± 27 (111 ± 60) NC α > 1	20 ± 1 (5 ± 1) C
LAB	1.8 ± 0.1 (0.8 ± 0.1) NC α = 1	0.05 ± 0.01 (0.040 ± 0.003) NC ·> 1	685 ± 112 (1014 ± 81) NC α > 1	n.i.	56 ± 5 (98 ± 5) NC α = 1	n.i	n.i.

[a] Data are means of triplicate experiments± standard error of the mean (SE). [b] From Baker's yeast. [c] From rice. [d] From sweet almonds. [e] From bovine liver. [f] From *Penicillium decumbens*. [g] From jack beans, *Genus canavalia*. [h] From bovine kidney. C: competitive inhibition. NC: noncompetitive inhibition.[1] n.i.: no inhibition, i.e. $IC_{50} \geq 1$ mM.

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4. CONCLUSIONS

The following general conclusions correspond to the four objectives previously described in Section 2.

1) D-Fructose-6-phosphate aldolase (FSA) has been assayed as catalyst for aldol additions of DHA and HA to a variety of aldehydes.
Concerning the selectivity it can be concluded that FSA tolerates a variety of non-natural substrates and possesses a remarkable stereoselectivity regardless of the aldehyde acceptor employed. It was observed that the reaction performance depended strongly on the donor substrate. HA was found to be the best donor, whereas the reactivity of DHA depended on the aldehyde acceptor.
In the course of our investigations, it was found that FSA can accept simple sugars as acceptors using HA as donor, opening new synthetic possibilities for the preparation of important novel complex carbohydrate-related compounds from aldoses.
FSA showed to be a robust and stereoselective biocatalyst for alternative synthetic approaches of biologically active products, with the great advantage of using non-phosphorylated donor substrates, a rather unprecedented quality among the known DHAP-dependent aldolases. In this regard a chemoenzymatic cascade reaction strategy for the synthesis of polyhydroxylated compounds, sugars and iminocyclitols was described.

2) An efficient chemo-enzymatic procedure for the synthesis of 2-aminomethyl derivatives of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and its enantiomer (LAB), was developed.
The preparation of *N*-Cbz-polyhydroxylated pyrrolidine intermediates was optimized. The mild and selective oxidation of these intermediates with IBX, followed by a reductive amination step, provides an easy access to a number of new derivatives with aromatic amines, aminoalcohols or amino acids. These later leading to the formation of unprecedented piperazine-iminocyclitol fused compounds.
Moreover, an alternative method to generate the *N*-Cbz-iminosugar derivative was tested, leading to 1,4-dideoxy-1,4-imino-L-xylitol and opening new possibilities for obtaining novel stereocomplementary derivatives epimers at C2 to those from DAB and LAB.

3) A straightforward chemo-enzymatic route to novel polyhydroxylated pyrrolizidines of casuarine type was described. The chemoenzymatic procedure was based on the stereocontrolled cascade enzymatic aldol addition of dihydroxyacetone or dihydroxyacetone phosphate to *N*-Cbz-pyrrolidinecarbaldehydes derived from 1,4-dideoxy-1,4-imino-D-arabinitol (DAB and LAB), catalyzed by DHA or DHAP-dependent aldolases and the subsequent one pot hydrogenolysis-reductive amination.

The aldol reactions with L-fuculose-6-phosphate aldolase mutant Phe131Ala furnished four unprecedented casuarine stereoisomers. The stereoselectivity of the aldol reactions was high and dependent on the structural features of the aldehydes while the reductive amination gave mixtures of two diasteroisomers epimeric at C3 in different proportions.
The aldol addition of DHAP to (*S*)-*N*-Cbz-pyrrolidinecarbaldehydes from DAB affords the *syn* aldol adduct leading to the formation of *ent*-casuarine and *ent*-3-*epi*-casuarine. From the (*R*)-enantiomer was obtained the *anti* aldol adduct and the two isomers 2-*epi*-casuarine, 2,3-*epi*-casuarine.

4) The enzyme inhibition activities of some compounds synthesized in this thesis have been tested against a panel of commercial glycosidases.
The inhibitory profiles were compared with those of the parent compounds 1,4-dideoxy-1,4-imino-D- and -L-arabinitol (DAB and LAB), and a preliminary analysis on the correlation between biological activities and structural motifs of derivatives has been carried out.
It results that the inhibitory properties of the new DAB and LAB derivatives against the panel of commercial glycosidases differed considerably from those of the parent compounds DAB and LAB. The aromatic aminomethyl LAB derivatives are moderate to good inhibitors of α-L-rhamnosidase. Particularly aniline derivative from LAB has better inhibitory properties than its parent LAB. The aminoalcohol, aminoacid and oxopiperazine derivatives are selective inhibitors of α-D-glucosidases, particularly DAB conjugates with (*R*)-2-amino-1-pentanol and *O*-*tert*-butyl-L-aspartamide were selective towards α-D-glucosidase from baker's yeast.

5. EXPERIMENTAL SECTION

5.1. GENERAL METHODS AND INSTRUMENTS:

NMR SPECTROSCOPY:

Routine, ^{1}H (400-500 MHz) and ^{13}C (101 MHz) NMR spectra of compounds were recorded with a Varian Mercury-400 and Varian Anova-500 spectrometers, respectively. High-field ^{1}H and ^{13}C NMR analyses were carried out by using a Bruker Avance 500 spectrometer equipped with a high-sensitive CryoProbe for D_2O and CD_3OD solutions (studies performed by Dr. Teodor Parella, NMR service, Autonomous University of Barcelona). Full characterization of the described compounds was performed using typical gradient-enhanced 2D experiments: COSY, NOESY, HSQC and HMBC, recorded under routine conditions. When possible, NOE data was obtained from selective 1D NOESY experiments by using a single pulsed-field-gradient echo as a selective excitation method and a mixing time of 500 ms. When necessary ^{1}H NMR and NOESY spectra were recorded at different temperatures to study the different exchange phenomena and to avoid the presence of false NOE cross-peaks that make difficult both structural and dynamic studies.

HPLC ANALYSES:

HPLC analyses were performed on a LaChrom Elite-HPLC (VWR-Hitachi) with a RP-HPLC XBridge® C18, 5µm, 4.6 x 250 mm column from Waters (Massachusetts, USA). Samples (50 µL) of the reaction were withdrawn from the reaction medium, dissolved in MeOH and analyzed by HPLC. The solvent system used was: solvent (A): H_2O 0.1 % (v/v) trifluoroacetic acid (TFA) and solvent (B): CH_3CN/H_2O 4/1 0.095 % (v/v) TFA, gradient elution from 10 % to 70 % B in 30 min or from 2 % to 62 % B in 30 min, flow rate 1 mL min^{-1}, detection 215 nm.

POLARIMETRY:

Specific optical rotations ($[\alpha]_D^{22}$) were measured with a Perkin Elmer Model 341 (Überlingen, Germany) polarimeter (Na lamp, 589nm).

SPECTROPHOTOMETRY:

Absorbances were detected and recorded with a UV/VIS Spectramax Plus (Molecular Devices Corporation) spectrophotometer/microplate reader.

ION-EXCHANGE CHROMATOGRAFY:

Ion-exchange chromatography was performed on a FPLC system with a glass column (450–25 mm) packed wth CM-Sepharose CL-6B (Amersham Pharmacia) stationary phase in NH_4^+ form.

5.2. PREPARATION OF IMINOCYCLITOLS AND SUGARS:

5.2.1. Materials:

Butanal (99%), 2-hydroxyethanal dimer (purum) as a mixture of stereoisomers (98.0%), palladium over charcoal (10% Pd), benzyl chloroformate (technical grade; 95%), hydroxyacetone (HA), (S)- and (R)-3-amino-1,2-propanediol and 2-benzyloxyethanal (97%), 2-phenylethanal (90%), and slightly calcined Celite were obtained from Sigma-Aldrich. Dihydroxyacetone (DHA) and aluminium oxide (90 active neutral) were purchased from Merck. All the reagents were used straight from the bottle without any prior purification. D-Fructose-6-phosphate aldolase (FSA) lyophilized powder (0.42 U mg^{-1} protein measured by the Bradford method, activity determined by using a spectrophotometric assay as described by Schürmann and Sprenger[1] was prepared as described previously.[2] *N*-Benzyloxycarbonyl-2-aminoethanal (*N*-Cbz-glycinal) was prepared following the methodology described.[2] D-(–)-Threose was prepared according to the method previously described by our group.[3]

Deionized water was used for preparative HPLC and Milli-Q grade water for analytical HPLC. All the other solvents used were of analytical grade.

5.2.2. Methods:

(*S*)- and (*R*)-3-[*N*-(Benzyloxycarbonyl)amino]-2-hydroxypropanal (5-(*S*) and 5-(*R*)):

The title compounds were prepared from the corresponding (*S*)- and (*R*)-3-[*N*-(benzyloxycarbonyl)amino]-1,2-propanediol, which were synthesized from commercial (*S*)- and (*R*)-3-amino-1,2-propanediol and benzyl chloroformate according to the published procedure.[4] The primary alcohol of both (*S*)- and (*R*)-3-[*N*-(benzyloxycarbonyl)amino]-1,2-propanediol was oxidized by IBX in ethyl acetate at reflux. IBX (5 g, 18 mmol) was added to a solution of the (*S*)- or (*R*)-3-[*N*-(benzyloxycarbonyl)amino]-1,2-propanediol (2 g, 9 mmol) in EtOAc (200 mL) and the mixture was heated at reflux for 5–6 h. The resulting (*S*)- or (*R*)-3-[*N*-(benzyloxycarbonyl)amino]-2-hydroxypropanal were not isolated, but used directly in the enzymatic aldol addition reaction. The estimated yield was around 50–60%.

1-Deoxynojirimycin and 1-deoxymannojirimycin (1 and 2):

In a round-bottomed flask, dihydroxyacetone (1.9 g, 21.1 mmol) and FSA aldolase powder (1.2 g, 312 U) were dissolved in water (200 mL) adjusted to pH 7 with $NaHCO_3$. The aldehyde **5-(*S*)** or **5-(*R*)** (2 g, 9 mmol, estimated) dissolved in EtOAc (200 mL) was then added to this solution. The reaction mixture was then placed in a rotary evaporator at 25ºC under vacuum and with gently agitation until all the EtOAc was evaporated. Finally, the reaction was placed in a reciprocal shaker (120 rpm) at 25ºC. After 24 h, the TLC showed that all the aldehyde was consumed and a

second portion of the aldehyde was added following the same procedure. After 40 h (i.e., 16 h after the second aldehyde addition) the TLC showed that the aldehyde was nearly consumed. At this point, MeOH (40 mL) was added to the mixture to stop the reaction, adjusted to pH 3 with diluted HCl and filtered through Celite. The filtrate was treated with H_2 (50 psi) in the presence of Pd/C (1.6 g) at room temperature during 24 h. After removal of the catalyst by filtration through 0.45 µm nylon membrane filter, the filtrate was adjusted to pH 5 with AcOH and purified by ion-exchange chromatography on a FPLC system.

Bulk stationary phase CM-Sepharose fast flow (Amersham Pharmacia) in NH_4^+ form was packed into a glass column (45x2.5 cm) to a final bed volume of 220 mL. The flow rate was 3 mL min^{-1} during loading and changed to 5 mL min^{-1} for elution. The CM-Shepharose was equilibrated initially with H_2O. Then, an aqueous solution (200 mL) of the crude material at pH 5 was loaded onto the column. Minor colored impurities were washed away with H_2O (2-3 column volumes). Then, 1-deoxynojirimycin was eluted with 2.5 mM NH_4OH (500 mL), the same eluent composition and volume was employed in the case of 1-deoxymannojirimycin. The operation was repeated until the whole crude mixture (600 mL; 200 x 3) of each compound was consumed. The fractions were analyzed by NMR. Pure fractions were pooled and lyophilized affording 1-deoxynojirimycin (**1**; 717 mg, 49 %) and 1-deoxymannojirimycin (**2**; 200 mg, 14 %)

D-1-Deoxynojirimycin (1): $[\alpha]_D^{22}$ = + 34.4 (*c* = 0.96 in H_2O) (lit.[5] $[\alpha]_D$ = + 40.3 (*c* = 1.47 in H_2O)); ^{1}H NMR (400 MHz, D_2O): δ = 3.84 (A of AB system, *J* = 3.0 Hz, 11.7, 1H), 3.64 (B of AB system, *J* = 6.3 Hz, 11.6, 1H), 3.50 (ddd, *J* = 5.2, 9.1, 10.8 Hz, 1H), 3.33 (t, *J* = 9.1 Hz, 1H), 3.24 (t, *J* = 9.4 Hz, 1H), 3.13 (dd, *J* = 5.2 Hz, 12.4, 1H), 2.55 (m, *J* = 2.9, 6.2, 9.4 Hz, 1H), 2.47 (dd, *J* = 10.8 Hz, 12.2, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 80.9, 73.9, 73.3, 63.7, 63.1, 51.1.

D-1-Deoxymannojirimycin (2): $[\alpha]_D^{22}$ = – 36.1 (*c* = 1.3 in H_2O) (lit.[6] $[\alpha]_D$= – 40.2 (*c* = 0.33 in H_2O)); ^{1}H NMR (400 MHz, D_2O): δ = 3.99 (brdd, *J* = 2.9, 4.4 Hz, 1H), 3.76 (d, *J* = 3.9 Hz, 2H), 3.60 (t, *J* = 9.3 Hz, 1H), 3.56 (dd, *J* = 3.1, 9.6 Hz, 1H), 3.01 (dd, *J* = 2.7, 14.3 Hz, 1H), 2.75 (dd, *J* = 1.2, 14.3 Hz, 1H), 2.47 (dt, *J* = 3.8, 9.4 Hz, 1H); ^{13}C NMR: (101 MHz, D_2O) δ = 77.0, 71.6, 70.7, 63.1 (2C), 50.8.

***N*-Butyl-1-Deoxynojirimycin (1a; miglustat):**

Butanal (260 mg, 3.2 mmol) and **1** (100 mg, 0.61 mmol) were dissolved in EtOH/H_2O (1:1, 15 mL), and Pd/C (200 mg) was added. The mixture was shaken under H_2 (50 psi) overnight at room temperature. After removal of the catalyst by filtration through 0.45 µm nylon membrane filter, the solvent was evaporated under reduced pressure. The brown oily residue obtained was purified by ion exchange chromatography on a FPLC system as described above with 50 mL bed

column. The product was eluted with 10 mM NH_4OH (500 mL) to yield the pure title compound (60 mg, 60 %). $[\alpha]_D^{22}$ = – 9.7 (*c* = 1.13 in H_2O) (lit.[7] $[\alpha]_D$ = – 15.9 (*c* = 0.77 in H_2O)); ^1H NMR (500 MHz, D_2O): δ = 3.89 (dd, *J* = 2.4, 12.8 Hz, 1H), 3.81 (dd, *J* = 2.7, 12.7 Hz, 1H), 3.51 (ddd, *J* = 4.9, 9.5, 10.7 Hz, 1H), 3.35 (t, *J* = 9.5 Hz, 1H), 3.23 (t, *J* = 9.3 Hz, 1H), 3.00 (dd, *J* = 5.0, 11.5 Hz, 1H), 2.72 (m, 1H), 2.58 (m, 1H), 2.27 (t, *J* = 11.2 Hz, 1H), 2.23 (dt, *J* = 2.6, 9.9 Hz, 1H), 1.44 (m, 2H), 1.26 (m, 2H), 0.88 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 81.0, 72.7, 71.5, 67.7, 60.1, 57.9, 54.5, 27.7, 22.8, 15.9.

***N*-2-(hydroxyethyl)-1-deoxynojirimycin (1b; miglitol)**:

Benzyloxyacetaldehyde (50 mg, 0.3 mmol) and **1** (15 mg, 0.09 mmol) were dissolved in MeOH (10 mL) and Pd/C (100 mg) was added. The mixture was shaken under H_2 (50 psi) overnight at room temperature. The reaction mixture was worked up and purified by ion-exchange chromatography as described for miglustat. Miglitol was eluted with 5 mM NH_4OH (190 mL) to yield the pure title compound (10 mg, 67 %). $[\alpha]_D^{22}$ = – 7.7 (*c* = 0.26 in H_2O); ^1H NMR (500 MHz, D_2O): δ = 3.92 (A of AB system, *J* = 2.4, 12.8 Hz, 1H), 3.82 (B of AB system, *J* = 3.0, 12.9 Hz, 1H), 3.74 (td, *J* = 2.9, 6.2 Hz, 2H), 3.53 (m, 1H), 3.35 (t, *J* = 9.5 Hz, 1H), 3.25 (t, *J* = 9.2 Hz, 1H), 3.08 (dd, *J* = 4.9, 11.6 Hz, 1H), 2.94 (dt, *J* = 6.6, 13.2 Hz, 1H), 2.72 (dt, *J* = 5.9, 14.2 Hz, 1H), 2.36 (t, *J* = 11.1 Hz, 1H), 2.31 (dt, *J* = 2.7, 9.7, 12.5 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ = 80.9, 72.6, 71.4, 68.2, 60.5, 60.2, 60.1, 58.7, 55.4.

***N*-Butyl-1-deoxymannojirimycin (2a):**

The title compound was obtained by the methodology described above for the preparation of miglustat. In this case the amounts of reactants were as follows: **2** (35 mg, 0.21 mmol), butanal (81 mg, 1.1 mmol), and Pd/C (50 mg). The reaction mixture was worked up and the product needed no further purification. $[\alpha]_D^{22}$ = – 47.4 (*c* = 0.76 in H_2O); ^1H NMR (500 MHz, D_2O): δ = 4.00 (s, 1H), 3.93 (A of AB system, *J* = 2.5, 12.8 Hz, 1H), 3.87 (B of AB system, *J* = 2.6, 12.7 Hz, 1H), 3.71 (t, *J* = 9.6 Hz, 1H), 3.50 (dd, *J* = 3.5, 9.6 Hz, 1H), 3.08 (dd, *J* = 3.1 Hz, 13.1, 1H), 2.82 (m, 1H), 2.70 (m, 2H), 2.36 (d, *J* = 8.6 Hz, 1H), 1.47 (m, 2H), 1.27 (m, 2H), 0.89 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 76.7, 70.0, 68.1, 59.8, 57.3, 54.9, 27.6, 22.6, 15.8.

***N*-(2-Hydroxyethyl)-1-deoxymannojirimycin (2b):**

The title compound was obtained by the methodology described above for the preparation of miglitol. 2-Benzyloxyethanal (50 mg, 0.3 mmol) and **2** (15 mg, 0.09 mmol) furnished **2b** (16.2 mg, 85%). $[\alpha]_D^{22}$ = – 76.9 (*c* = 0.26 in H_2O)**;** ^1H NMR (500 MHz, D_2O): δ = 3.95 (m (A of AB system + H-5), 2H), 3.84 (B of AB system, *J* = 2.8, 12.6 Hz, 1H), 3.71 (m, 2 H), 3.64 (t, *J* = 9.5 Hz, 1 H), 3.48 (dd, *J* = 3.5, 9.6 Hz, 1 H), 3.04 (dd, *J* = 3.5, 12.9 Hz, 1H), 3.02 (ddd, *J* = 6.3, 7.2,

13.8 Hz, 1H), 2.62 (dt, *J* = 5.4, 14.2 Hz, 1H), 2.59 (dd, J = 1.6, 12.9 Hz, 1 H), 2.26 (dt, 3J=2.6, 9.4 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 77.0, 70.4, 68.7, 60.7, 60.6, 57.9, 55.9.

(3*S*,4*R*)-5-[*N*-(Benzyloxycarbonyl)amino]-5-deoxypent-2-ulose (9):

DHA (4.5 g, 49 mmol) and FSA aldolase powder (3 g, 1260 U) were dissolved in 50 mM boric/borate buffer (pH 7.0, 480 mL). *N*-Cbz-glycinal (**6**; 8 g, 41 mmol) dissolved in DMF (120 mL) was added to this mixture. The reaction mixture was then placed in a reciprocal shaker (120 rpm) at 25ºC. After 24 h (50% conversion), MeOH (200 mL) was added to the mixture to stop the reaction, which was then filtered through Celite. Excess DHA was removed by preparative HPLC as follows. The crude of **9** was loaded onto a preparative column (47 x 300 mm) filled with Bondapack C18 (Waters, 300 Å, 15-20 µm stationary phase). DHA was washed out with water and the product was eluted with a H_2O/CH_3CN gradient from 0 % to 30 % CH_3CN in 40 min**.** The flow rate was 95 mL min-1 and the products were detected at 215 nm. The fractions were analyzed under isocratic conditions (33% of solvent B) by analytical HPLC. The pure fractions were pooled and lyophilized. Then the excess aldehyde was eliminated by flash chromatography on silica with ethyl acetate/hexane (9:1) and the product was eluted with ethyl acetate to give **9** (3.3 g, 30 %) as a white solid. A complex NMR spectrum was recorded that was consistent with the previously described formation of two cyclic diastereomeric hemiaminal compounds and their conformational equilibrium.[8] ^{1}H NMR (500 MHz, D_2O): δ = 7.40 (dd, *J* = 7.4, 10.7 Hz), 5.17 (m), 4.52 (dd, *J* = 19.4, 51.6 Hz), 4.33 (d, *J* = 1.4 Hz), 4.18 (dt, *J* = 9.4, 18.8 Hz), 4.08 (ddd, *J* = 2.0, 5.9, 7.8 Hz), 3.94 (m), 3.61 (m), 3.28 (m), 3.06 (t, *J* = 9.6 Hz), 2.99 (t, *J* = 9.7 Hz).

1,4-Dideoxy-1,4-imino-D-arabinitol (3; DAB):

(3*S*,4*R*)-5-[*N*-(benzyloxycarbonyl)amino]-5-deoxypent-2-ulose (1 g) was dissolved with water/ethanol (1:1, 300mL) and treated with H_2 (50 psi) in the presence of Pd/C (0.25 g, 10% Pd). The crude material (500 mg) was purified by ion-exchange chromatography (200 mL column volume) on CM-Sepharose fast flow (Amersham Pharmacia) in NH_4^+ form as follows. An aqueous solution of the crude material (250 mg in 10 mL) at pH 5 was loaded onto the column. Minor colored impurities were washed away with H_2O (2-3 column volumes). Then, 1,4-dideoxy-1,4-imino-D-arabinitol was eluted isocratically with 0.01 M NH_4OH; eluted peak from 263 to 473 mL. The operation was repeated until the whole crude mixture of each compound was consumed. The fractions were analyzed by NMR spectroscopy. Pure fractions were pooled, adjusted to pH 5.2 and lyophilized to afford the title compound (400 mg, 80 %, 24 % from the aldehyde). $[\alpha]_D^{22}$ = +35.2 (*c* = 1.0 in MeOH), $[\alpha]_D^{22}$ = +26.2 (*c* = 0.9 in H_2O) (lit.[9] $[\alpha]_D^{20}$ = +7.8 (*c* = 0.46 in H_2O) as a free base; $[\alpha]_D^{20}$ = +37.9 (*c* = 0.53 in H_2O) as hydrochloride); ^{1}H NMR (500 MHz, D_2O): δ = 4.35 (dt, *J* = 2.7, 5.1 Hz, 1H), 4.11 (t, *J* = 3.3 Hz, 1H), 3.97 (dd, *J* = 4.6, 12.2 Hz, 1H), 3.85 (dd,

J = 8.3, 12.2 Hz, 1H), 3.63 (dd, *J* = 3.8, 7.9 Hz, 1H), 3.59 (dd, *J* = 4.6, 12.3 Hz, 1H), 3.37 (dd, *J* = 2.6, 12.5 Hz, 1H). ^{13}C NMR (101 MHz, D_2O): δ = 78.4, 77.0, 69.3, 61.7, 52.7.

(3*S*,4*R*)-5-[*N*-(Benzyloxycarbonyl)amino]-1,5-dideoxypent-2-ulose (10):

HA (0.118 g, 1.44 mmol) and FSA aldolase powder (0.130 g, 55 U) were dissolved in 50 mM boric/borate buffer pH 7.0 (12 mL). *N*-Cbz-glycinal (**6**; 0.233 g, 1.2 mmol) dissolved in DMF (3 mL) was added to this mixture. The reaction mixture was then placed in a reciprocal shaker (120 rpm) at 25ºC. After 24 h, MeOH (200 mL) was added to the mixture to stop the reaction, which was then filtered through Celite. The crude material was purified by flash chromatography on silica and the product eluted with hexane/ethyl acetate (4:6) to yield (243 mg, 75 %) as a white solid. Complex NMR spectra were recorded that were consistent with the above observations. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.34 (m), 5.10 (m), 4.22 (m), 4.09 (m), 3.58 (t, *J* = 5.9 Hz, 1H), 3.28 (ddd, *J* = 5.9, 12.7, 15.3 Hz), 2.22 (br s), 1.34 (m), 0.93 (m); ^{13}C NMR (101 MHz, CD_3OD): δ = 213.2, 160.5, 139.7, 130.8, 130.4, 130.2, 80.4, 73.0, 70.4, 68.9, 63.2, 62.9, 46.0, 45.6, 41.5, 33.0, 31.5, 27.9, 26.3, 25.4, 15.8, 12.8.

1,4,5-Trideoxy-1,4-imino-D-arabinitol (4; 5-DDAB):

The title compound was obtained from (3*S*,4*R*)-5-[*N*-(benzyloxycarbonyl)amino]-1,5-dideoxypent-2-ulose (**10,** 200 mg, 0.75 mmol) by a similar methodology described for the synthesis of DAB (conditions: water/ethanol 1:1 (60 mL) and Pd/C (50 mg, 10% Pd)). The crude product was purified by ion-exchange chromatography on CM-Sepharose fast flow (Amersham Pharmacia) as described above for DAB. The title compound was eluted isocratically with 0.01 M NH_4OH (eluted peak from 577 to 772 mL) and was obtained as a white solid (60 mg, 56%). $[\alpha]_D^{22}$ = +8.0 (*c* = 1.0 in H_2O)(lit.enantiomer[10] $[\alpha]_D^{22}$ = –7.13 (*c* = 1.08 in H_2O)); ^{1}H NMR (500 MHz, D_2O): δ = 4.17 (dt, *J* = 3.5, 5.9 Hz, 1H), 3.74 (dd, *J* = 3.8, 5.2 Hz, 1H), 3.26 (dd, *J* = 5.7, 12.6 Hz, 1H), 3.15 (p, *J* = 6.7 Hz, 1H), 3.00 (dd, *J* = 3.2, 12.6 Hz, 1H), 1.30 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 86.4, 80.3, 61.9, 53.1, 19.8.

Minor diasteroromer epimer at C2 (**4a**; 17% by NMR) arising from reductive amination reaction: ^{1}H NMR (500 MHz, D_2O): δ = 4.08 (m, 1H), 3.82 (t, *J* = 3.0 Hz, 1H), 3.68 (dd, *J* = 6.0, 11.7 Hz, 1H), 3.57 (qd, *J* = 2.6, 6.6 Hz, 1H), 3.22 (dd, *J* = 3.6, 11.7 Hz, 1H), 1.25 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 84.1, 77.1, 62.2, 54.3, 20.6.

5-*O*-Benzyl-D-xylulose (19):

The title compound was obtained by the methodology described above for **9**. The amounts of reactants used were the following: DHA (0.09 g, 1 mmol) and FSA aldolase powder (0.09 g, 38 U) dissolved in 50 mM boric/borate buffer pH 7.0 (12 mL) and benzyloxyacetaldehyde (0.150 g, 1

mmol) dissolved in DMF (3 mL). After 24 h the conversion was 35%. Purification on silica gel (ethyl acetate/hexane 85:15) yielded 70 mg, (28 %) of **19**. $[\alpha]_D^{22}$ = +2.2 (*c* = 1.0 in $CHCl_3$)(lit.[11] $[\alpha]_D^{22}$ = +2.8 (*c* = 0.25 in $CHCl_3$)); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.31 (m, 5H), 4.54 (s, 2H), 4.50 (dd, *J* = 19.3, 45.8 hZ, 2H), 4.31 (d, *J* = 2.2 Hz, 1H), 4.13 (dt, *J* = 2.2, 6.4 Hz, 1H), 3.59 (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 213.7, 139.7, 129.5, 129.0, 128.8, 77.3, 74.4, 72.2, 71.7, 68.0.

5-*O*-Benzyl-1-deoxy-D-xylulose (20):

The title compound was obtained by the methodology described above for **10**. The amounts of reactants used were the following: HA (0.68 g, 9.2 mmol) and FSA aldolase crude powder (0.113 g, 47 U) dissolved in 50 mM boric/borate buffer pH 7.0 (80 mL) and benzyloxyacetaldehyde (1.16 g, 7.7 mmol) dissolved in DMF (20 mL). After 24 h the conversion was 99%. The reaction mixture was extracted with ethyl acetate (3x80 mL), dried over anhydrous Na_2SO_4 and the solvent evaporated to dryness under vacuum. Further purification was accomplished on silica gel (ethyl acetate/hexane from 1:4 to 1:1) to yield 1.2 g, (71 %) of **20.** $[\alpha]_D^{22}$ = +58.2 (*c* = 1.12 in CH_2Cl_2)(lit.[12] $[\alpha]_D^{20}$ = +52.5 (*c* = 1.17 in CH_2Cl_2)); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.30 (m, 5H), 4.54 (s, 2H), 4.21 (d, *J* = 1.9 Hz, 1H), 4.16 (td, *J* = 2.0, 6.2 Hz, 1H), 3.64 (AB system, *J* = 6.5, 9.5 Hz, 1H), 3.55 (AB system, *J* = 6.2, 9.5 Hz, 1H), 2.22 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 212.1, 139.7, 129.5, 129.0, 128.8, 78.9, 74.5, 72.1, 72.0, 26.8.

1-Deoxy-D-xylulose (24) by procedure A:

Compound **20** (1.0 g, 4.5 mmol) was dissolved in MeOH (100 mL), 10% Pd/C catalyst (100 mg) was added, and the mixture was stirred at room temperature under H_2 (50 psi) overnight. Then the mixture was filtered through 0.45 μm nylon membrane filter, the solvent was evaporated, and the residue dissolved in water and lyophilized to obtain **20** (600 mg, 99%) as a mixture of two cyclic and one acyclic form as a white solid. $[\alpha]_D^{22}$ = +54.1 (*c* = 1.11 in H_2O) (lit.[13] $[\alpha]_D^{20}$ = +22.4 (*c* = 1.10 in H_2O); $[\alpha]_D^{22}$ = +8.8 (*c* = 1.9 in CH_3OH)(lit.[14] $[\alpha]_D^{20}$ = − 1.7 (*c* = 1.8 in CH_3OH)); Acyclic isomer: ^{1}H NMR (500 MHz, CD_3OD): δ = 4.22 (m, 1H), 3.99 (td, *J* = 2.1, 6.5 Hz, 1H), 3.66 (dd, *J* = 6.7, 10.9 Hz, 1H), 3.58 (dd, *J* = 6.3, 10.9 Hz, 1H), 2.24 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 212.3, 74.1, 72.2, 62.4, 25.2. Cyclic form, the α-anomer: ^{1}H NMR (500 MHz, CD_3OD): δ = 4.29 (m, 1H), 4.15 (m, 1H), 3.72 (m, 1H), 3.59 (dd, *J* = 4.4, 9.3 Hz, 1H), 1.49 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 102.6, 81.7, 76.0, 70.0, 23.7. Cyclic form, β-anomer: ^{1}H NMR (500 MHz, CD_3OD): δ = 4.16 (m, 1H), 3.86 (m, 2H), 3.86 (m,1H), 3.84 (m, 1H), 1.45 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 106.3, 81.5, 77.0, 72.0, 20.5.

1-Deoxy-D-xylulose (24) by procedure B:

The title compound was also synthesized from 2-hydroxyethanal (glycolaldehyde) (3.0 g, 50 mmol) and hydroxyacetone (4.63 g, 62.49 mmol). FSA aldolase crude powder (0.312 g, 75 U) dissolved in 50 mM $NaHCO_3$ adjusted to pH 7.8 (50 mL) was added to a solution of the reactants in the same buffer (100 mL, total final volume 150 mL). After 24 h no 2-hydroxyethanal was detected by TLC ($CHCl_3$/MeOH, 5:1) and the reaction was stopped by adding MeOH (100 mL). The mixture was filtered through activated charcoal and the filtrate adjusted to pH 7 and lyophilized. Purification on silica gel eluted with $CHCl_3$/MeOH from 1:0 to 17:3 yielded in three runs 3.35 g (51%) of **24**. The compound recovered was not pure with 20% of the epimer at C2 being detected: 1-Deoxy-D-*erythro*-2-pentulose (**24b**).

The NMR spectra showed the presence of six species: Two acyclic and two cyclic forms corresponding to both diastereomers. The ^1H and ^{13}C NMR data of the major diastereomer (a mixture of two cyclic and one acyclic form) were identical to those of 1-deoxy-d-xylulose (**24**) listed above. Minor (20%) product 1-deoxy-D-*erythro*-2-pentulose. Acyclic isomer: ^1H NMR (500 MHz, CD_3OD): δ = 4.16 (d, *J*= 6.0 Hz, 1H), 3.90 (q, *J* = 5.5 Hz, 1 H), 3.72 (dd, *J* = 4.4, 11.3 Hz, 1H), 3.63 (dd, *J* = 6.0, 11.3 Hz, 1H), 2.30 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 212.3, 73.1, 72.0, 62.1, 25.9. Cyclic form, the α anomer: ^1H NMR (500 MHz, CD_3OD): δ = 4.23 (m, 1 H), 4.10 (m, 1H), 3.66 (m, 1H), 3.52 (dd, *J* = 4.4, 9.3 Hz, 1 H), 1.43 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 102.0, 75.0, 71.2, 70.3, 23.1. Cyclic form, the β anomer: ^1H NMR (500 MHz, CD_3OD): δ = 4.27 (m, 1H), 3.81 (m, 2H), 3.78 (m, 1H), 1.38 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 106.0, 76.3, 70.9, 70.6, 21.3.

D-(–)-Threose (27):

D-(–)-Threose was used as supplied and in this work an extensive NMR analysis was performed. The NMR spectra showed the presence of four species in equilibrium: Two cyclic species corresponding to the α and β anomers and two acyclic ones corresponding to the aldehyde and its hydrate. Acyclic form: ^1H NMR (500 MHz, D_2O): δ = 5.01 (d, *J* = 6.3 Hz, 1H), 3.90 (m, 1H), 3.69–3.65 (m, 1H), 3.47 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 89.7, 73.2, 70.8, 62.9. Cyclic form, the α anomer: ^1H NMR (500 MHz, D_2O): δ = 5.40 (d, *J* = 4.2 Hz, 1H), 4.30 (m, 1H), 4.08 (m, 1H), 4.19 (m, 1H), 3.65 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 96.5, 76.1, 74.8, 70.4. Cyclic form, the β anomer: ^1H NMR (500 MHz, D_2O): δ = 5.25 (d, *J* = 0.9 Hz, 1 H), 4.23 (m, 1H), 4.21 (m, 1 H), 4.06 (m, 1 H), 3.96 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 102.0, 80.6, 75.0, 73.0.

1-Deoxy-D-*ido*-hept-2-ulose (28):

D-(–)-Threose (**27**; 0.49 g, 4.1 mmol) and hydroxyacetone (0.37 g, 5 mmol) were dissolved in 50 mM $NaHCO_3$ (20 mL) previously adjusted to pH 7.7 with HCl. FSA aldolase crude powder (0.104 g, 25 U) was added to this solution. After 24 h most of D-(–)-threose has been consumed as detected by TLC ($CHCl_3$/MeOH 5:1) and the reaction was stopped by adding MeOH (20 mL). The mixture was filtered through activated charcoal and the filtrate adjusted to pH 5.02 and lyophilized. Purification on silica gel eluted with $CHCl_3$/AcOEt (1:1)/MeOH from 1:0 to 4:1 yielded **28** (463 mg, 58%). $[\alpha]_D^{22}$ = + 3.3 (*c* = 1.22 in H_2O). The NMR showed the presence of five isomeric forms: Two five-membered (**28a** and **28a'**) and two six-membered (**28b**, **28b'**) cyclic species corresponding to the α, β anomers and one acyclic corresponding to the open chain form (**28**). **28**: ^{1}H NMR (600 MHz, D_2O) δ = 4.51 (d, *J* = 2.1 Hz, 1 H), 4.24 (dd, *J* = 2.2, 7.1 Hz, 1H), 3.87 (m, 1 H), 3.80 (m, 1H), 3.72–3.70 (m, 2 H), 2.23 (s, 3H); ^{13}C NMR (151 MHz, D_2O) δ = 212.6, 77.2, 71.7, 71.3, 70.9, 62.9, 25.8. **28a**: ^{1}H NMR (600 MHz, D_2O) δ = 4.36 (t, *J* = 6.5 Hz, 1H), 4.17 (dd, *J* = 3.7, 7.0 Hz, 1H), 3.93 (d, *J* = 3.5 Hz, 1H), 3.92 (m, 1H), 3.70 (m, 1H), 3.63 (m, 1 H), 1.51 (s, 3H); ^{13}C NMR (151 MHz, D_2O) δ = 101.3, 80.4, 75.4, 76.5, 69.9, 62.7, 23.6. **28a'**: ^{1}H NMR (600 MHz, D_2O) δ = 4.26 (m, 1 H), 4.15 (m, 1H), 4.07 (d, *J* = 2.8 Hz, 1 H), 3.98 (m, 1 H), 3.73 (m, 1H), 3.62 (s, 1H), 1.46 (s, 3H); ^{13}C NMR (151 MHz, D_2O) δ = 105.8, 81.0, 75.9, 80.4, 71.0, 62.7, 20.8. **28b**: ^{1}H NMR (600 MHz, D_2O) δ = 4.10 (m, 1 H), 3.96 (m, 1H), 3.91 (m, 1H), 3.86 (m, 1 H), 3.84 (m, 1H), 3.38 (d, *J* = 8.6 Hz, 1H); ^{13}C NMR (151 MHz, D_2O) δ = 98.3, 75.6, 74.8, 70.7, 70.2, 61.2, 24.7. **28b'**: ^{1}H NMR (600 MHz, D_2O) δ = 4.26 (m, 1H), 3.96 (m, 1H), 3.79 (m, 1H), 3.78 (m, 1 H), 3.62 (m, 1 H), 1.45 (s, 3H); ^{13}C NMR (151 MHz, D_2O) δ = 98.7, 70.6, 70.6, 69.0, 68.7, 61.2, 24.0.

(3*S*,4*R*)-1,3,4-trihydroxy-5-phenylpentan-2-one (21):

The title compound was obtained by the methodology described above for **9**. The amounts of reactants used were the following: DHA (0.135 g, 1.5 mmol) and FSA aldolase crude powder (0.130 g, 55 U) dissolved in 50 mM boric/borate buffer at pH 7.0 (12 mL) and 2-phenylethanal (0.200 g, 1 mmol) dissolved in DMF (3 mL). After 24 h the conversion was 77%. The product was purified by reversed-phase HPLC using a preparative column (47 x 300 mm) filled with Bondapack C18 (Waters, 300 Å, 15-20 µm stationary phase), which was eluted with a H_2O/CH_3CN gradient from 0 to 20% CH_3CN in 40 min. The flow rate was 95 mL min^{-1} and the products were detected at 215 nm. Lyophilization of the pure fraction pool yielded 141 mg (46%) of **21**.

$[\alpha]_D^{22}$ = +35.0 (*c* = 1.0 in $CHCl_3$) (lit.[15] $[\alpha]_D^{20}$ = +26.7 (*c* = 1.07 in $CHCl_3$); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.23 (m, 5H), 4.48 (q, *J* = 19.3 Hz, 2H), 4.14 (dd, *J* = 6.2, 8.2 Hz, 1H), 4.04 (d, *J* = 2.0 Hz, 1H), 2.94 (AB system, *J* = 7.1, 13.4 Hz, 1H), 2.85 (AB system, *J* = 7.4, 13.3 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 214.3, 139.9, 130.6, 129.6, 127.5, 78.3, 75.1, 68.0, 40.8.

(3*S*,4*R*)-3,4-dihydroxy-5-phenylpentan-2-one (22):

The title compound was obtained by the methodology described above for **10**. The amounts of reactants used were the following: HA (0.111 g, 1.5 mmol) and FSA aldolase crude powder (0.130 g, 55 U) dissolved in 50 mM boric/borate buffer at pH 7.0 (12 mL) and 2-phenylethanal (0.200 g, 1.5 mmol) dissolved in DMF (3 mL). After 24 h the conversion was 77%. Purification by reversed-phase HPLC using a preparative column (47 x 300 mm) filled with Bondapack C18 (Waters, 300 Å, 15-20 μm stationary phase). The product was eluted with a H_2O/CH_3CN gradient from 0 to 30% CH_3CN in 40 min. The flow rate was 95 mL min^{-1} (10 mL min^{-1})?? and the products were detected at 215 nm. Lyophilization of the pure fraction pool yielded 70 mg (28%) of **22**. $[\alpha]_D^{22}$ = +29.8 (*c* = 1.0 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.23 (m, 5H), 4.16 (td, *J* = 1.8, 7.2 Hz, 1H), 3.94 (d, *J* = 1.9 Hz, 1H), 2.95 (AB system, *J* = 7.1, 13.3 Hz, 1H), 2.86 (AB system, *J* = 7.4, 13.4 Hz, 1H), 2.18 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD): δ = 212.6, 140.0, 130.7, 129. 6, 127.5, 79.8, 74.9, 41.1, 26.6.[16]

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5.3. PREPARATION OF DAB AND LAB 2-AMINOMETHYL DERIVATIVES:

5.3.1. Materials:

Aniline, benzylamine, 3-aminoquinoline and *N*-methyl-1,2-phenylenediamine, amino alcohols and amino acid derivatives were purchased from Sigma-Aldrich. Dyhydroxyacetone (DHA) was purchased from Merck. *N*-Cbz-2-amino ethanal (*N*-Cbz-glycinal) used was synthesized in our lab using procedures published in previous works.[1,2] L-Rhamnulose-1-phosphate aldolase [Co^{II}] (RhuA) (3.8 U mg^{-1} protein) (1 Unit (U) catalyzes the cleavage of 1 μmol of L-rhamnulose-1-phosphate per minute at 25ºC and pH 7.5 (100 mM Tris·HCl + 150 mM KCl)) was produced in our lab using standard procedures.[3,4] FSA Ala165Gly (0.9 U mg^{-1} protein; 1U catalysed the formation of 1 μmol of D-fructose-6-phosphate (D-F6P) per minute from DHA (300 mM) and D,L-glyceraldehyde-3-phosphate at 30ºC in 50 mM glycylglycine buffer pH 8.5, containing 1 mM DTT) was produced in our lab using standard procedures.[5]

Aqueous borate solutions were prepared by adjusting the desired pH of a solution of boric acid with 2 M aq. NaOH. Water for analytical and preparative HPLC and for the preparation of buffers was obtained from an Arium® Pro Ultrapure Water Purification System (SartoriusStedim Biotech). All other solvents used were of analytical grade.

5.3.2. Methods:

***N*-Benzyloxycarbonyl-1,4-dideoxy-1,4-imino-D-arabinitol (7):**

FSA Ala165Gly (150 U, 150 mg, 1.06 U mg^{-1}) and dihydroxyacetone DHA (1.3 g, 14.5 mmol) were dissolved in 0.1 M triethanolamine buffer, pH 7.0 (135 mL). This solution was added to *N*-Cbz-glycinal (2.25 g, 11.6 mmol) dissolved in *N,N*-dimethylformamide DMF (15 ml) and the mixture was shaken at 25°C. After 24 h (99 % conversion) the crude was diluted with H_2O up to a volume of (150 mL) and loaded onto a glass column (5 cm diameter x 20 cm length, volume 400 mL) packed with Amberlite™ XAD™ 16 (Rohm and Haas) stationary phase, which was previously equilibrated with plain H_2O. After loading, the column was washed with H_2O (800 mL, 2 column volumes). This step removed efficiently the enzyme, the excess of DHA and the salts. The aldol adduct was then eluted with H_2O/EtOH 4:6 (800 mL, 2 column volumes). The fractions containing the product were pooled and the solvent reduced to about 300 mL in vacuum. The aqueous residue was diluted with EtOH (200 ml). The solution was divided into two batches (250 mL) and each one was treated overnight with H_2 (50 psi) in the presence of Pd/C (0.4 g, 10 % Pd). The catalyst was removed by filtration and EtOH/water was evaporated in vacuum. NMR data and physical properties matched those already published using the same methodology.[5,6] The aqueous solution (200 mL) was diluted with 1,4-dioxane (200 mL) and solid $NaHCO_3$ (1.9 g, 23.2 mmol) was added under stirring. After cooling to 0 °C, benzyl chloroformate (1.8 mL, 12.7 mmol) was added dropwise. After the addition, the reaction mixture was allowed to warm up to

room temperature and it was stirred overnight. Dioxane was removed under vacuum, the aqueous residue was diluted with saturated $NaHCO_3$ solution (100 mL) and the product extracted with ethyl acetate (4 x 150 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuum. The crude material was purified by flash column chromatography on silica (EtOAc/MeOH from 100:0 to 9:1) to yield the title compound (1.4 g, 46% yield). HPLC analysis: gradient elution from 10% to 70% B in 40 min; t_R = 14.5 min, 98% purity by HPLC. $[\alpha]_D^{22}$ = – 28.0 (*c* = 1.0 in MeOH) (lit.,[7] $[\alpha]_D^{22}$ = – 28.9 (*c* = 1.9 in MeOH); lit.,[8] $[\alpha]_D^{22}$ = – 29.7 (*c* = 0.3 in MeOH)); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.32 (m, 5H, arom), 5.13 (m, 2H, $OC\underline{H_2}Ph$), 4.17 (s, 1H, H-3), 4.12 (s, 1H H-3-rotamer), 4.04 (br s, 1H, H-4), 3.82 (m, 4H, H-2-H-5-H-6-H-6'), 3.38 (m, 1H, H-5') (complex spectrum due to the existence of rotamers as described also by Fleet et al.[8]); ^{13}C NMR (101 MHz, CD_3OD): δ = 157.3 (C=O), 157.1 (C=O rotamer), 138.0 (C-Ar), 137.9 (C-Ar rotamer), 129.5 (C-Ar), 129.1 (C-Ar), 129.1 (C-Ar), 128.9 (C-Ar), 128.8 (C-Ar), 79.4 (C-3), 78.7 (C-3 rotamer), 76.2 (C-4), 75.6 (C-4 rotamer), 68.6 (C-2), 68.3 (C-2 rotamer), 68.1 ($C\underline{H_2}Ph$), 68.0 ($C\underline{H_2}Ph$ rotamer), 61.8 (C-6), 61.6 (C-6 rotamer), 54.8 (C-5), 54.4 (C-5 rotamer).

***N*-Benzyloxycarbonyl-1,4-dideoxy-1,4-imino-L-arabinitol (8):**

RhuA (400 U, 32 mL of NH_4SO_4 suspension, 0.35 mg protein mL^{-1}, 12.5 U mL^{-1}) and dihydroxyacetone DHA (1.35 g, 15 mmol) were dissolved in sodium borate 0.25 M, pH 7.0 (160 mL). This solution was added to *N*-Cbz-glycinal (2.33 g, 12 mmol) dissolved in DMF (40 ml) and the mixture was shaken at 25°C. After 24 h (90% conversion) the crude was diluted with H_2O up to a volume of 200 mL and loaded onto a glass column packed with Amberlite™ XAD™ 16 (Rohm and Haas) following an identical procedure described above with minor modifications: The aldol adduct was then eluted with H_2O/EtOH 4:6 (1000 mL, 2.5 column volumes). The fractions containing the product were pooled and the solvent reduced to about 400 mL in vacuum. The aqueous residue was diluted with EtOH (400 ml). The solution was divided into two batches (400 mL) and each one was treated overnight with H_2 (50 psi) in the presence of Pd/C (0.6 g, 10 % Pd).

The catalyst was removed by filtration and EtOH/water was evaporated in vacuum. NMR data and physical properties matched those already published using the same methodology and are.[9] The aqueous solution (*ca* 150 ml) was diluted with 1,4-dioxane (150 ml) and solid $NaHCO_3$ (1.8 g, 21.6 mmol) was added under stirring. After cooling to 0 °C, benzyl chloroformate (1.7 mL, 11.8 mmol) was added dropwise. After the addition, the reaction mixture was allowed to warm up to room temperature and it was stirred overnight. Dioxane was removed under vacuum, the aqueous residue was diluted with saturated $NaHCO_3$ solution (100 mL) and the product extracted with ethyl acetate (4 x 150 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuum. The crude material was purified by flash column chromatography on silica (EtOAc/MeOH from 100:0 to 9:1) to yield the title compound (1.73 g, 53% yield, 98% purity by HPLC). $[\alpha]_D^{22}$ = + 25.0 (*c* = 1.2 in MeOH). HPLC retention time and NMR data identical to that of *N*-Cbz-DAB.

1,4-Dideoxy-1,4-imino-L-xylitol (9'):

The aldol adduct (500 mg, 1.77 mmol) obtained from the aldol addition of DHA to *N*-Cbz-glycinal catalyzed by D-fructose-6-phosphate aldolase mutant Ala165Gly (see above) was dissolved in anhydrous CH_2Cl_2 (12 mL) and cooled down to 0ºC. A solution of BF_3Et_2O (0.22 ml, 1.77 mmol, 1 eq) and subsequently Et_3SiH (0.57 ml, 3.54 mmol, 2 eq) were added under N_2 and the reaction mixture was left to reach room temperature under stirring. After 2 h the reaction was quenched to pH=7 with saturated $NaHCO_3$ (5 mL). The aqueous layer was diluted with brine (5 mL), and the mixture extracted with EtOAc, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/CH_3OH 30:1) to give the corresponding Cbz protected 1,4-dideoxy-1,4-imino-L-xylitol (**9**) as an oil (320 mg, 68% yield). $[\alpha]_D^{22}$ = + 20.0 (*c* = 1.0 in MeOH). Purity 98 % by HPLC. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.33 (m, 5H, arom), 5.14 (m, 2H, O*CH*$_2$Ph), 4.14 (m, 2H, H-3-H-4), 3.89 (m, 3H, H-2-H-6-H-6'), 3.65 (m, 1H, H-5), 3.40 (m, 1H, H-5'); ^{13}C NMR (101 MHz, CD_3OD): δ = 157.76 (C=O), 157.35 (C=O rotamer), 138.04 (C-Ar), 137.94 (C-Ar rotamer), 129.51 (C-Ar), 129.06 (2C-Ar), 128.86 (2C-Ar), 77.96 (C-3), 77.53 (C-3 rotamer), 75.21 (C-4), 74.86 (C-4 rotamer), 68.28 (CH_2Ph), 68.15 (CH_2Ph rotamer), 62.81 (C-2), 61.66 (C-2 rotamer), 60.85 (C-6), 60.27 (C-6 rotamer), 53.26 (C-5), 53.10 (C-5 rotamer). The presence of rotamers makes difficult to obtain the relative stereochemistry. Moreover, some important signals were overlapped making difficult to analyze the NOE experiments. Therefore it was decided to remove the Cbz group by treatment of a sample of the *N*-Cbz-1,4-dideoxy-1,4-imino-L-xylitol (40 mg, 0.14 mmol) with H_2 (22 psi) in the presence of Pd/C (20 mg). After removal of the Pd/C by filtration and lyophylization, the title compound (20 mg, 0.14 mmol) was obtained in quantitative yield. RMN and physical data matched those reported using other procedures.[10,11] $[\alpha]_D^{22}$ = – 3.0 (*c* = 1.0 in H_2O) (lit.,[10] $[\alpha]_D^{22}$ = – 4.0 (*c* = 0.10 in H_2O); lit.,[11] $[\alpha]_D^{22}$ = – 4.4 (*c* = 0.04 in CH_3OH)); ^{1}H NMR (400 MHz, D_2O): δ = 4.25 (dt, *J* = 4.9, 1.9 Hz, 1H, H-4), 4.20 (dd, *J* = 4.0, 1.7 Hz, 1H, H-3), 3.88 (dd, *J* = 11.5, 6.0 Hz, 1H, H-6), 3.76 (dd, *J* = 11.5, 7.3 Hz, 1H. H-6'), 3.51 (ddd, *J* = 7.2, 6.1, 4.1 Hz, 1H, H-2), 3.41 (dd, *J* = 12.7, 5.0 Hz, 1H, H-5), 2.93 (dd, *J* = 12.8, 2.0 Hz, 1H, H-5'); ^{13}C NMR (101 MHz, D_2O): δ = 76.14 (C-4), 75.87 (C-3), 61.46 (C-2), 59.05 (C-6), 50.59 (C-5). HRMS (ESI-TOF): m/z $[M+H]^+$ for $C_5H_{12}NO_3^+$ calculated 134.0817; observed 134.0819.

Oxidation reaction:

N-benzyloxycarbonyl-1,4-dideoxy-1,4-imino-D-arabinitol (**7**) or its enantiomer **8**, (1 eq.) was dissolved in AcOEt (50 mL) at 40°C. 2-Iodoxybenzoic acid (IBX) (4 eq.) was added to this solution and the reaction mixture was stirred under reflux. After 3.5 h the reaction mixture was cooled down to room temperature, the solid removed by filtration and the solvent evaporated under reduced pressure. This product was used in the next step without further purification and it was not characterized.

Reductive amination reactions:

Synthesis of (2*R*,3*R*,4*R*)- or (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-3,4-dihydroxypyrrolidine-2-derivatives. The crude from the oxidation reaction was dissolved in dry MeOH (10-50 mL; to have an estimated concentration of the corresponding aldehyde between 0.016-0.025 M), treated with the amine (5 equiv mol^{-1} aldehyde) in glacial acetic acid (5 equiv mol^{-1} aldehyde) and stirred for 2h at room temperature. Then, $NaBH_3CN$ (1.5 equiv mol^{-1} aldehyde) was added and the reaction mixture was stirred overnight. The solvent was removed under reduced pressure, and the crude product was purified either by flash chromatography on silica or preparative HPLC. Purification by HPLC was performed as follows (general procedure): The crude was dissolved in MeOH and loaded onto a semi-preparative X-Terra Prep MS C-18, 10 µm, 19 x 250 mm column. The solvent system used was: solvent (A): aqueous trifluoroacetic acid (TFA) (0.1 % (v/v)) and solvent (B): TFA (0.095 % (v/v)) in CH_3CN/H_2O 4:1 or plain MeOH. Salts and solvents were washed out with 100% A during 10 min. The product was eluted with a gradient of B (see below in each case). The flow rate was 10 mL min^{-1} and the products were detected at 215 nm. The fractions were analyzed by HPLC. Fractions containing the product were pooled and lyophilized.

N-Cbz removal reactions:

The Cbz-protected DAB and LAB 2-aminomethyl derivative (115-170 mg, aromatic amines; 74-200 mg amino alcohols; 104-165 mg amino acids DAB conjugates; 64-205 mg amino acids LAB conjugates) was dissolved with ethanol and treated with H_2 (22 psi) in the presence of Pd/C (10% Pd, 37-100 mg) during 24 h at room temperature. The catalyst was removed by filtration and the solvent evaporated under vacuum. For amines, the crude was purified by preparative HPLC see conditions in each case.

For amino alcohols, when necessary, the product was further purified by cation exchange chromatography on CM-Sepharose CL-6B (Amersham Pharmacia) stationary phase in NH_4^+ form. The stationary phase was packed into a glass column (450–25 mm) to provide a final bed volume of 220 mL. The flow rate was 4 mL min^{-1}. The CM-Sepharose-NH_4^+ was washed initially with H_2O. An aqueous solution of the crude material at pH 5 was then loaded onto the column. Minor colored impurities were washed away with H_2O (440 mL, 2 bed volumes). Products were eluted with aqueous NH_4OH (0.01 M) (400 mL). Pure fractions were pooled and lyophilized.

For amino acids after the Cbz-group removal the product was left at room temperature without solvent or dissolved in water and heated at 40 or 100ºC depending on the derivative until the intramolecular aminolysis was completed. Then the product was assayed (see Section 3.4) without any further purification.

*DAB and LAB aromatic 2-aminomethyl derivatives **14a** and **15a**.*

(2*R*,3*R*,4*R*)-3,4-dihydroxy-2-((phenylamino)methyl)pyrrolidine (14a-1):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((phenylamino)methyl)pyrrolidine (**12a-1**; 170 mg, 58% yield) was purified by flash chromatography on silica (AcOEt/Hexane 3:2). HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 25.0 min. The title compound (100 mg, 66% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 40 min; t_R = 13.3 min. $[\alpha]_D^{22}$ = + 46.4 (*c* = 1.4 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.15 (dd, *J* = 8.5, 7.4 Hz, 2H), 6.70 (m, 3H), 4.24 (m, 1H), 4.11 (d, *J* = 0.8 Hz, 1H), 3.72 (ddd, *J* = 9.4, 5.4, 1.5 Hz, 1H), 3.58 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.53 (dd, *J* = 14.0, 9.6 Hz, 1H), 3.48 (dd, *J* = 12.0, 3.7 Hz, 1H), 3.34 (d, *J* = 11.9 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 148.8, 130.2, 119.0, 114.1, 78.5, 76.2, 67.2, 52.2, 45.0. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{11}H_{17}N_2O_2^+$ calculated 209.1285; observed 209.1276.

(2*R*,3*R*,4*R*)-2-((benzylamino)methyl)-3,4-dihydroxypyrrolidine (14a-4):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-((benzylamino)methyl)-3,4-dihydroxypyrrolidine (**12a-4**; 165 mg, 56% yield) was purified by flash chromatography on silica (AcOEt/MeOH 9:1). HPLC analysis: gradient elution from 2% to 62% B in 40 min; t_R = 10.6 min. The title compound (81 mg, 78% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 25.0 min. $[\alpha]_D^{22}$ = + 14.3 (*c* = 1.0 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.49 (m, 5H), 4.28 (s, 2H), 4.24 (m, 1H), 4.21 (s, 1H), 3.82 (t, *J* = 6.1 Hz, 1H), 3.58 (dd, *J* = 12.2, 5.0 Hz, 3H), 3.41 (d, *J* = 12.0 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 132.8, 131.0, 130.6, 130.2, 79.3, 75.9, 64.9, 53.1, 53.1, 48.5. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{12}H_{19}N_2O_2^+$ calculated 223.1441; observed 223.1431.

(2*R*,3*R*,4*R*)-3,4-dihydroxy-2-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)pyrrolidines (14a-5):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)pyrrolidine (**12a-5**; 184 mg, 67% yield) was purified by flash chromatography on silica (AcOEt/Hexane 7:3). HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 22.2 min. The title compound (136 mg, 78% yield) was prepared according to the general procedure described above. HPLC purification: gradient elution from 0 to 60% B in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 40 min; t_R = 12.0 min. $[\alpha]_D^{22}$ = – 2.4 (*c* = 1 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.70 (d, *J* = 8.3 Hz, 1H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.39 (m, 1H), 7.33 (m, 1H), 5.03 (d, *J* = 3.1 Hz, 1H), 4.53 (t, *J* = 2.5 Hz, 1H), 4.37 (dt, *J* = 4.7, 2.4 Hz, 1H), 3.95 (s, 3H), 3.75 (dd, *J* = 12.0, 4.6 Hz, 1H), 3.59 (dd, *J* = 11.9, 2.3 Hz, 1H); ^{13}C NMR (101

MHz, CD_3OD): δ = 149.2, 137.2, 125.2, 124.6, 119.4, 111.6, 80.8, 76.6, 62.3, 52.8, 30.9. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{12}H_{16}N_3O_2^+$ calculated 234.1237; observed 234.1235.

(2*R*,3*R*,4*R*)-3,4-dihydroxy-2-((quinolin-3-ylamino)methyl)pyrrolidine (14a-6):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((quinolin-3-ylamino)methyl)pyrrolidine (**12a-6**; 167 mg, 55% yield) was purified by flash chromatography on silica (AcOEt/MeOH 19:1). HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 22.6 min. The title compound (110 mg, 70% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 50% B in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 40 min; t_R = 11.2 min. $[\alpha]_D^{22}$ = + 22.6 (*c* = 0.5 in MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ = 8.70 (d, *J* = 2.8 Hz, 1H), 7.98 (ddd, *J* = 5.4, 4.9, 2.8 Hz, 3H), 7.71 (m, 2H), 4.29 (m, 1H), 4.20 (d, *J* = 1.1 Hz, 1H), 3.81 (dd, *J* = 13.4, 3.4 Hz, 3H), 3.56 (dd, *J* = 12.0, 3.6 Hz, 1H), 3.41 (d, *J* = 12.0 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 143.77, 138.36, 135.37, 131.65, 130.61, 130.10, 128.14, 122.92, 119.63, 78.62, 76.30, 66.67, 52.78, 44.54. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{14}H_{18}N_3O_2^+$ calculated 260.1393; observed 260.1381.

(2*R*,3*R*,4*R*)-2-(aminomethyl)-1-(quinolin-3-yl)pyrrolidine-3,4-diol (14a-6'):

The title compound (7 mg, 5% yield) was obtained after treatment of the precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((quinolin-3-ylamino)methyl)pyrrolidine (**12a-6**) with H_2 in the presence of Pd/C. HPLC purification: gradient elution from 0 to 60% B in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 40 min; t_R = 10.9 min. $[\alpha]_D^{22}$ = – 28.0 (*c* = 0.5 in MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ = 8.84 (d, *J* = 2.9 Hz, 1H), 8.13 (d, *J* = 2.8 Hz, 1H), 8.05 (m, 2H), 7.75 (m, 2H), 4.37 (d, *J* = 3.8 Hz, 1H), 4.35 (s, 1H), 4.15 (dd, *J* = 7.1, 1.8 Hz, 1H), 3.79 (m, 2H), 3.67 (dd, *J* = 13.8, 7.2 Hz, 1H), 3.37 (dd, *J* = 13.9, 2.0 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 143.5, 135.6, 134.95, 131.5, 130.7, 130.7, 128.4, 122.7, 122.4, 80.7, 75.3, 66.6, 57.8, 39.5. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{14}H_{17}N_3O_2^+$ calculated 260.1399; observed 260.1389.

(2*S*,3*S*,4*S*)-3,4-dihydroxy-2-((phenylamino)methyl)pyrrolidine (15a-1):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((phenylamino)methyl)pyrrolidine (**13a-1**; 115 mg, 40% yield) was purified by HPLC: gradient elution from 30 to 80% B in 30 min. HPLC analysis: identical to that **14a-1**. The title compound (60 mg, 85% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. $[\alpha]_D^{22}$ = – 41.5 (*c* = 1.1 in MeOH); ^{1}H NMR (600 MHz, MeOD): δ = 7.15 (dd, *J* = 8.6, 7.4 Hz, 2H), 6.70 (m, 3H), 4.24 (m, 1H), 4.11 (d, *J* = 1.0 Hz, 1H), 3.72 (ddd, *J* = 9.5, 5.4, 1.6 Hz, 1H), 3.58 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.53 (dd, *J* = 14.0, 9.7 Hz, 1H), 3.48 (dd, *J* = 12.0, 3.7 Hz, 1H), 3.34 (d, *J* = 12.4 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 148.8, 130.2,

119.0, 114.11, 78.5, 76.3, 67.3, 52.3, 45.1. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{11}H_{17}N_2O_2^+$ calculated 209.1285; observed 209.1276.

(2*S*,3*S*,4*S*)-2-((benzylamino)methyl)-3,4-dihydroxypyrrolidine (15a-4):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-((benzylamino)methyl)-3,4-dihydroxypyrrolidine (**13a-4**; 133 mg, 48% yield) was purified by HPLC: gradient elution from 0 to 70% B in 40 min. HPLC analysis: identical to that **14a-4**. The title compound (64 mg, 85% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. $[\alpha]_D^{22}$ = – 14.4 (*c* = 1.3 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.49 (m, 5H), 4.27 (s, 2H), 4.24 (m, 1H), 4.20 (s, 1H), 3.82 (t, *J* = 6.4 Hz, 1H), 3.57 (dd, *J* = 9.2, 5.1 Hz, 3H), 3.40 (d, *J* = 12.1 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 132.2, 131.1, 130.8, 130.3, 79.3, 75.7, 64.62, 53.1, 53.0, 48.4. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{12}H_{19}N_2O_2^+$ calculated 223.1441; observed 223.1440.

(2*S*,3*S*,4*S*)-3,4-dihydroxy-2-(1-methyl-1H-benzo[*d*]imidazol-2-yl)pyrrolidine (15a-5):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)pyrrolidine (**13a-5**; 152 mg, 55% yield) was purified by HPLC: gradient elution from 10 to 70% B in 40 min. HPLC analysis: identical to that **14a-5**. The title compound (58 mg, 74% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. $[\alpha]_D^{22}$ = + 2.6 (*c* = 0.9 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.68 (d, *J* = 8.0 Hz, 1H), 7.57 (d, *J* = 8.1 Hz, 1H), 7.37 (m, 1H), 7.31 (m, 1H), 5.01 (d, *J* = 3.2 Hz, 1H), 4.51 (t, *J* = 2.6 Hz, 1H), 4.36 (dt, *J* = 4.8, 2.4 Hz, 1H), 3.94 (s, 3H), 3.74 (dd, *J* = 12.0, 4.7 Hz, 1H), 3.58 (dd, *J* = 11.9, 2.4 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 149.6, 141.6, 137.7, 125.0, 124.3, 119.9, 111.4, 81.0, 76.7, 62.7, 52.7, 30.8. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{12}H_{16}N_3O_2^+$ calculated 234.1237; observed 234.1230.

(2*S*,3*S*,4*S*)-3,4-dihydroxy-2-((quinolin-3-ylamino)methyl)pyrrolidines (15a-6):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((quinolin-3-ylamino)methyl)pyrrolidine (**13a-6**; 170 mg, 57% yield) was purified by HPLC: gradient elution from 0 to 65% B in 35 min. HPLC analysis: identical to that **14a-6**. The title compound (540 mg, 95% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 50% B in 40 min. $[\alpha]_D^{22}$ = – 35.0 (*c* = 1.0 in MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ = 8.72 (d, *J* = 2.7 Hz, 1H), 7.98 (m, 3H), 7.71 (m, 2H), 4.29 (m, 1H), 4.21 (d, *J* = 0.9 Hz, 1H), 3.82 (m, 3H), 3.57 (dd, *J* = 12.0, 3.7 Hz, 1H), 3.42 (d, *J* = 12.0 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 143.76, 137.88, 134.73, 131.64, 130.64, 130.16, 128.14, 122.47, 119.95, 78.62, 76.27, 66.46, 52.62, 44.47. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{14}H_{18}N_3O_2^+$ calculated 260.1393; observed 260.1404.

(2S,3S,4S)-2-(aminomethyl)-1-(quinolin-3-yl)pyrrolidine-3,4-diol (15a-6'):

The title compound (28 mg, 5% yield) was prepared according to the general procedure described above. HPLC Purification: gradient elution from 0 to 60% B in 40 min. $[\alpha]_D^{22}$ = + 17.7 (*c* = 0.7 in MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ = 8.87 (d, *J* = 2.9 Hz, 1H), 8.20 (d, *J* = 2.8 Hz, 1H), 8.07 (m, 2H), 7.77 (m, 2H), 4.38 (d, *J* = 4.1 Hz, 1H), 4.36 (s, 1H), 4.17 (dd, *J* = 7.1, 1.8 Hz, 1H), 3.80 (m, 2H), 3.68 (dd, *J* = 13.9, 7.3 Hz, 1H), 3.37 (dd, *J* = 13.9, 2.0 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD): δ = 143.3, 137.3, 137.3, 131.3, 130.0, 129.5, 128.1, 124.3, 120.43, 80.8, 75.3, 66.5, 57.7, 39.7. HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_{14}H_{17}N_3O_2^+$ calculated 260.1399; observed 260.1390.

*DAB and LAB 2-oxopiperazine fused derivatives and amino acid conjugates **18b**, and **19b**.*

(7*R*,8*R*,8a*R*)-7,8-dihydroxyhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (18b-1):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-((glycineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-1**; 140 mg, 58% yield) was purified by HPLC: gradient elution 0% MeOH for 5 min, then from 0% to 70% MeOH in 35 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 16.9 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.47 (m, 5H, arom), 5.22 (m, 2H, O*CH*$_2$Ph), 4.26 (broad s, 1H, H-4), 4.13 (m, 2H, H-2-3), 3.88 (m, 3H, H-5-7-7'), 3.48 (m, 3H H-5'-6-6'); ^{13}C NMR (101 MHz, D_2O): δ = 168.2 ($CONH_2$), 157.9 (COBn), 156.7 (COBn-rotamer), 135.8 (C-Ar), 135.4 (C-Ar-rotamer), 128.8 (C-Ar), 128.8 (C-Ar), 128.5 (C-Ar), 128.5 (C-Ar), 127.9 (C-Ar), 78.3 (C-3 rotamer), 77.6 (C-3), 73.6 (C-4), 73.1 (C-4 rotamer), 68.4 (CH_2Ph), 68.0 (CH_2Ph rotamer), 62.1 (C-2), 61.9 (C-2 rotamer), 53.0 (C-5 rotamer), 52.4 (C-5), 49.6 (C-6), 48.7 (C-6 rotamer), 48.0 (C-7). The title compound (78 mg, 92% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 2.3 (*c* = 1.4 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.33 (dd, *J* = 13.8, 7.0 Hz, 1H), 3.96 (t, *J* = 6.7 Hz, 1H), 3.83 (m, 5H), 3.49 (m, 1H), 3.15 (t, *J* = 11.3 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 164.3, 77.6, 72.7, 57.9, 48.8, 44.4, 43.5. HRMS-ESI: *m*/*z* calcd for $C_7H_{13}N_2O_3$ 173.0926 $[M+H^+]$; found: 173.0923.

(3*S*,7*R*,8*R*,8a*R*)-7,8-dihydroxy-3-methylhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (18b-2):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-alanineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-2**; 110 mg, 58 % yield) was purified by HPLC: gradient elution 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 13.5 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.47 (m, 5H, Ar), 5.21 (m, 2H, OCH_2Ph), 4.25 (br s, 1H, H-4), 4.18 (s, 1H, H-3rotamer), 4.12 (br s, 2H, H-3-H-2), 4.05 (m, 2H, H-2-rotamer-H-7), 3.91 (m, 1H, H-7-rotamer), 3.84 (td, *J* = 12.2, 5.0 Hz, 2H, H-5;H-5-rotamer), 3.52 (d, *J* = 12.5 Hz, 1H, H-5'), 3.48 (d, *J* = 12.6 Hz, 1H, H-5' rotamer), 3.44 (br s, 2H, H-6-H-6 rotamer), 3.36 (br s, 2H, H-6'-H-6' rotamer), 1.53 (d, *J* = 7.0 Hz, 3H, H-8), 1.43 (d, *J* = 7.0 Hz, 3H, H-8-rotamer); ^{13}C NMR (101 MHz, D_2O): δ = 172.2 ($CONH_2$), 157.8 (COBn), 156.6 (COBn-rotamer), 135.9 (C-Ar), 135.5 (C-Ar-

rotamer), 128.9 (C-Ar), 128.8 (C-Ar), 128.5 (C-Ar), 128.5 (C-Ar), 127.9 (C-Ar), 78.1 (C-3-rotamer), 77.4 (C-3), 73.6 (C-4), 73.1 (C-4-rotamer), 68.3(CH_2Ph-rotamer), 68.1 (CH_2Ph), 62.2 (C-2), 62.0 (C-2-rotamer), 56.2 (C-7-rotamer), 56.2 (C-7), 52.9 (C-5), 52.3 (C-5-rotamer), 47.6 (C-6), 46.9 (C-6-rotamer), 15.2 (C-8). The title compound (63 mg, 95% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 22.2 (*c* = 1.2 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.34 (dd, *J* = 13.7, 6.4 Hz, 1H), 4.22 (q, *J* = 7.3 Hz, 1H), 4.04 (m, 1H), 3.85 (m, 3H), 3.51 (dd, *J* = 12.6, 6.3 Hz, 1H), 3.40 (m, 1H), 1.61 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 166.1, 77.5, 72.8, 57.3, 51.2, 49.2, 40.5, 14.2. HRMS-ESI: *m*/*z* calcd for $C_8H_{15}N_2O_3$ 187.1083 [M+H^+]; found: 187.1089.

(3*S*,7*R*,8*R*,8a*R*)-7,8-dihydroxy-3-isobutylhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (18b-4):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-leucineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-4**; 132 mg, 62 % yield) was purified by HPLC: gradient elution 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 18.3 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.46 (m, 5H, Ar), 5.20 (m, 2H, OCH_2Ph), 4.24 (m, 1H, H-4), 4.18 (s, 1H, H-3-rotamer), 4.10 (br s, 2H, H-2-H-3), 4.03 (br s, 1H, H-2-rotamer), 3.94 (t, *J* = 7.0 Hz, 1H, H-7), 3.82 (m, 2H, H-5-H-7-rotamer), 3.50 (m, 2H, H-5'-H-6), 3.36 (m, 2H, H-6'-H-6-rotamer), 3.20 (dd, *J* = 13.1, 6.6 Hz, 1H, H-6'-rotamer), 1.62 (m, 3H, H-8-H8'-H-9), 0.94 (m, 6H, CH_3); ^{13}C NMR (101 MHz, D_2O): δ = 171.3 ($CONH_2$), 158.0 (COBn), 156.6 (COBn-rotamer), 135.8 (C-Ar), 135.3 (C-Ar-rotamer), 128.9 (C-Ar), 128.8 (C-Ar), 128.6 (C-Ar), 128.5 (C-Ar), 127.9 (C-Ar), 78.3 (C-3 rotamer), 77.5 (C-3), 73.4 (C-4), 73.0 (C-4 rotamer), 68.4 (CH_2Ph rotamer), 68.1 (CH_2Ph), 62.2 (C-2), 62.1 (C-2 rotamer), 59.8 (C-7 rotamer), 59.6 (C-7), 53.0 (C-5 rotamer), 52.3 (C-5), 48.5 (C-6), 47.4 (C-6 rotamer), 38.9 (C-8), 38.7 (C-8 rotamer), 24.0 (C-9 rotamer), 24.0 (C-9), 21.8 (C-10 rotamer), 21.5 (C-10), 21.3 (C-11), 21.0 (C-11 rotamer). The title compound (94 mg, 96% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 9.0 (*c* = 1.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.32 (dd, *J* = 13.3, 6.1 Hz, 1H), 4.00 (m, 2H), 3.81 (m, 1H), 3.50 (dd, *J* = 12.7, 5.9 Hz, 1H), 3.30 (m, 1H), 1.81 (m, 1H), 0.98 (t, *J* = 5.5 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 166.6, 77.7, 72.9, 57.7, 53.6, 49.3, 41.1, 37.9, 24.3, 21.7, 20.6. HRMS-ESI: *m*/*z* calcd for $C_{11}H_{21}N_2O_3$ 229.1552 [M+H^+]; found: 229.1538.

1-(3-((3*S*,7*R*,8*R*,8a*R*)-7,8-dihydroxy-4-oxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)propyl)guanidine (18b-5):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-arginineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-5**) (129 mg, 55% yield) was purified by HPLC: gradient elution 0% MeOH for 5 min, then from 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 12.4 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.45 (m, 5H, Ar), 5.20 (m, 2H, OCH_2Ph), 4.25 (m, 1H, H-4), 4.18 (s, 1H, H-3 rotamer), 4.10 (br s, 2H, H-2-H-3), 4.06

(br s, 1H, H-2 rotamer), 3.97 (t, *J* = 6.3 Hz, 1H, H-7), 3.85 (m, 2H, H-5-H-7 rotamer), 3.44 (m, 3H, H-5'-H-6-H-6'), 3.19 (m, 2H, H-10-H-10'), 1.93 (m, 1H, H-8), 1.64 (m, 3H, H-8'-H-9-H-9'); ^{13}C NMR (101 MHz, D_2O): δ = 170.4 ($CONH_2$), 158.1 (COBn), 156.7 (C=NH), 135.9 (C-Ar), 135.4 (C-Ar rotamer), 128.9 (C-Ar), 128.8 (C-Ar), 128.6 (C-Ar), 128.5 (C-Ar), 127.8 (C-Ar), 78.2 (C-3 rotamer), 77.4 (C-3), 73.4 (C-4), 73.0 (C-4 rotamer), 68.3 (CH_2Ph rotamer), 68.1 (CH_2Ph), 62.2 (C-2), 62.0 (C-2 rotamer), 60.4 (C-7 rotamer), 60.2 (C-7), 53.0 (C-5 rotamer), 52.3 (C-5), 48.6 (C-6), 47.6 (C-6 rotamer), 40.2 (C-10), 26.8 (C-8), 26.7 (C-8 rotamer), 23.3 (C-9 rotamer), 23.3 (C-9). The title compound (100 mg, 92% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 2.0 (*c* = 1.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.32 (dd, *J* = 12.6, 6.5 Hz, 1H), 4.03 (dd, *J* = 11.9, 5.7 Hz, 2H), 3.82 (m, 3H), 3.53 (dd, *J* = 12.7, 5.7 Hz, 1H), 3.26 (m, 3H), 2.14 (m, 1H), 1.91 (m, 1H), 1.78 (m, 2H); ^{13}C NMR (101 MHz, D_2O): δ = 165.8, 156.7, 77.7, 72.9, 57.8, 54.7, 49.3, 41.6, 40.4, 26.4, 24.8. HRMS-ESI: *m*/*z* calcd for $C_{11}H_{22}N_5O_3$ 272.1723 [M+H^+]; found: 272.1726.

(3*S*,7*R*,8*R*,8a*R*)-3-benzyl-7,8-dihydroxyhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (18b-6):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-phenylalanineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-6**, 165 mg, 70% yield) was purified by HPLC: 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 20.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.33 (m, 10H, Ar), 5.06 (q, *J* = 12.3 Hz, 2H, OCH_2Ph), 4.15 (t, *J* = 7.0 Hz, 1H, H-7), 4.05 (br s, 1H, H-4), 3.93 (br s, 2H, H-2-H-3), 3.70 (dd, *J* = 11.6, 4.3 Hz, 1H, H-5), 3.43 (m, 3H, H-5'-H-6-H-6'), 3.19 (ddd, *J* = 38.2, 14.1, 7.0 Hz, 2H, CH_2Ph); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.6 ($CONH_2$), 159.0 (COBn), 137.5 (C-Ar), 135.0 (C-Ar), 130.6 (2C-Ar), 130.11(2C-Ar), 129.6 (C-Ar), 129.3 (C-Ar), 129.1 (C-Ar), 128.9 (C-Ar), 79.4 (C-3), 75.11 (C-4), 68.9 (OCH_2Ph), 64.3 (C-2), 63.4 (C-7), 54.2 (C-5), 51.2 (C-6), 37.9 (CH_2Ph). The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((((*S*)-1-methoxy-1-oxo-3-phenylpropan-2-yl)amino)methyl)pyrrolidines (**16b-7**, 230 mg, 70% yield) was purified by HPLC: gradient elution 10% to 80% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 23.5 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.30 (m, 10H, Ar), 5.12 (m, 2H, CH_2Ph), 4.29 (t, *J* = 6.6 Hz, 1H, H-7), 4.04 (br s, 1H, H-4), 3.93 (m, 2H, H-2-H-3), 3.77 (s, 3H, OCH_3), 3.73 (dd, *J* = 11.6, 4.5 Hz, 1H, H-5), 3.48 (dd, *J* = 13.0, 2.8 Hz, 1H, H-6), 3.43 (d, *J* = 12.0 Hz, 1H, H-5′), 3.36 (m, 1H, H-6′), 3.22 (d, *J* = 6.6 Hz, 2H, H-8-H-8'); ^{13}C NMR (101 MHz, CD_3OD): δ = 173.87 ($CONH_2$), 158.95 (COBn), 137.62 (C-Ar), 135.15 (C-Ar), 130.44 (2C-Ar), 130.14 (2C-Ar), 129.60 (2C-Ar), 129.32 (2C-Ar), 129.06 (2C-Ar), 79.54 (C-3), 75.32 (C-4), 68.89 (CH_2Ph), 64.86 (C-2), 62.83 (C-7), 54.50 (C-5), 53.53 (OCH_3), 50.85 (C-6), 37.20 (C-8). The title compound (100 mg, 95% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 11.4 (*c* = 1.2 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 7.37 (m, 5H), 4.41 (dd, *J* = 7.5, 5.5 Hz, 1H), 4.28 (dd, *J* = 12.3, 6.1 Hz, 1H), 3.80 (m, 3H), 3.65 (m, 1H), 3.52 (dd, *J* = 12.8, 5.8 Hz, 1H), 3.43 (dd,

J = 15.1, 5.3 Hz, 1H), 3.31 (dd, *J* = 15.1, 7.7 Hz, 1H), 2.91 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 164.6, 134.0, 129.3, 129.2, 128.1, 77.7, 72.8, 57.2, 55.8, 49.3, 41.5, 34.5. HRMS-ESI: *m*/*z* calcd for $C_{14}H_{19}N_2O_3$ 263.1396 [M+H$^+$]; found: 263.1377.

(1*R*,2*R*,5a*S*,10a*R*)-1,2-dihydroxyoctahydrodipyrrolo[1,2-a:1',2'-d]pyrazin-5(1*H*)-one (18b-12):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-prolineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-12,** 128 mg, 63% yield) was purified by HPLC: gradient elution 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 18.0 min. Assigned the major rotamer: ^{1}H NMR (500 MHz, CD_3OD): δ = 7.44 (m, 5H, Ar), 5.23 (m, 2H, OCH_2Ph), 4.28 (s, 1H, H-7), 4.16 (m, 2H, H-3-H-4), 4.00 (t, *J* = 5.6 Hz, 1H, H-2), 3.87 (br s, 1H, H-10), 3.75 (m, 2H, H-5-H-6), 3.53 (m, 2H, H-5'-H-6'), 3.38 (m, 1H, H-10'), 2.59 (m, 1H, H-8), 2.23 (m, 1H, H-9), 2.12 (m, 2H, H-8'-H-9'); ^{13}C NMR (101 MHz, CD_3OD): δ = 171.0 ($CONH_2$), 159.8 (COBn), 137.8 (C-Ar), 130.1 (C-Ar), 129.9 (C-Ar), 129.6 (C-Ar), 129.3 (C-Ar), 129.1 (C-Ar), 78.8 (C-3), 76.0 (C-4), 70.5 (C-7) 68.7 (OCH_2Ph), 63.7 (C-2), 59.5 (C-6), 55.4 (C-10), 54.1 (C-5), 30.3 (C-8), 24.2 (C-9). The title compound (90 mg, 97% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 19.0 (*c* = 1 in MeOH) of the mixture; (major) ^{1}H NMR (400 MHz, D_2O): δ = 4.38 (m, 1H), 4.33 (dd, *J* = 13.7, 6.9 Hz, 1H), 3.99 (m, 1H), 3.93 (dd, *J* = 12.1, 2.7 Hz, 1H), 3.86 (m, 1H),3.85 (m, 1H), 3.83 (m, 1H), 3.47 (dd, *J* = 12.6, 6.8 Hz, 1H), 3.39 (m, 1H), 3.23 (t, *J* = 11.4 Hz, 1H), 2.55 (m, 1H), 2.24 (m, 1H), 2.20 (m, 1H), 2.09 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 165.7, 77.0, 72.6, 62.1, 58.2, 56.9, 50.6, 48.7, 27.4, 21.8. (minor) ^{1}H NMR (400 MHz, D_2O): δ = 4.40 (m, 1H), 4.22 (m, 1H), 4.07 (m, 1H), 4.04 (m, 1H), 3.83 (m, 1H), 3.58 (m, 1H), 3.52 (d, *J* = 14.0 Hz, 1H), 3.47 (m, 1H), 3.33 (m, 1H), 3.32 (m, 1H), 2.53 (m, 1H), 2.10 (m, 1H), 2.11 (m, 1H), 1.98 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 168.2, 74.1, 68.1, 61.0, 52.7, 51.8, 51.6, 48.5, 29.5, 23.6. HRMS-ESI: *m*/*z* calcd for $C_{10}H_{17}N_2O_3$ 213.1239 [M+H$^+$]; found: 213.1226.

(2*R*,3*R*,4*R*)-2-(((*S*)-valineamidyl)methyl)-3,4-dihydroxypyrrolidine (18b-3):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-valineamidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-3**, 104 mg, 51% yield) was purified by HPLC: gradient elution: 0% MeOH for 5 min, then from 10% to 80% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 16.5 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.35 (m, 5H, Ar), 5.19 (s, 2H, OCH_2Ph), 4.09 (br s, 1H, H-4), 3.99 (m, 2H, H-2-H-3), 3.75 (dd, *J* = 11.5, 4.3 Hz, 1H, H-5), 3.68 (d, *J* = 5.8 Hz, 1H, H-7), 3.49 (m, 2H, H-5'-H-6), 3.32 (m, 1H, H-6'), 2.20 (td, *J* = 13.5, 6.8 Hz, 1H, H-8), 1.07 (m, 6H, $2CH_3$); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.1 ($CONH_2$), 159.3 (COBn), 137.5 (C-Ar), 129.6 (2C-Ar), 129.3 (C-Ar), 129.1 (2C-Ar), 79.5 (C-3), 75.0 (C-4), 69.1 (OCH_2Ph), 68.0 (C-7), 64.4 (C-2), 54.3 (C-5), 51.8 (C-6), 31.1 (C-8), 18.6 (CH_3), 18.5 (CH_3). The title compound (65 mg, 87% yield) was prepared according to the general procedure described

above. $[\alpha]_D^{22}$ = + 8.0 (*c* = 1.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.35 (dt, *J* = 4.4, 2.2 Hz, 1H), 4.14 (t, *J* = 2.3 Hz, 1H), 3.60 (m, 2H), 3.41 (dd, *J* = 12.6, 1.7 Hz, 1H), 3.05 (m, 2H), 2.97 (dd, *J* = 13.3, 8.5 Hz, 1H), 1.93 (tt, *J* = 13.6, 6.8 Hz, 1H), 0.98 (dd, *J* = 8.7, 6.9 Hz, 6H); ^{13}C NMR (101 MHz, D_2O): δ = 179.0, 77.1, 74.5, 67.9, 65.6, 50.3, 47.3, 31.0, 18.4, 18.1. HRMS-ESI: *m*/*z* calcd for $C_{10}H_{22}N_3O_3$ 232.1661 [M+H$^+$]; found: 232.1642.

(2*R*,3*R*,4*R*)-2-(((*S*)-aspartic(β*tert*butyl)amidyl)methyl)-3,4-dihydroxypyrrolidine (18b-9):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((*S*)-aspartic(β^{t}Bu)amidyl)methyl)-3,4-dihydroxypyrrolidine (**16b-9**, 138 mg, 56% yield) was purified by HPLC: gradient elution 10% to 80% MeOH in 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 20.8 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36 (m, 5H, Ar), 5.18 (m, 2H, OCH_2Ph), 4.07 (m, 2H, H-4-H-7), 3.99 (br s, 2H, H-2-H-3), 3.76 (dd, *J* = 11.6, 4.5 Hz, 1H, H-5), 3.42 (m, 3H, H-5'-H-6-H-6'), 2.87 (m, 2H, H-8-H-8'), 1.48 (s, 9H, $3CH_3$); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.2 ($CONH_2$), 170.1 (COO^{t}Bu), 158.9 (COBn), 137.5 (C-Ar), 129.6 (C-Ar), 129.6 (C-Ar), 129.4 (C-Ar), 129.3 (C-Ar), 129.0 (C-Ar), 84.1 (*C*-CH_3), 79.4 (C-3), 75.3 (C-4), 68.8 (OCH_2Ph), 64.4 (C-2), 58.4 (C-7), 54.3 (C-5), 51.0 (C-6), 36.3 (C-8), 28.2($3CH_3$). The title compound (95 mg, 93% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 16.6 (*c* = 0.9 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.36 (dt, *J* = 4.4, 2.3 Hz, 1H), 4.14 (t, *J* = 2.3 Hz, 1H), 3.62 (m, 3H), 3.42 (dd, *J* = 12.6, 1.8 Hz, 1H), 3.13 (dd, *J* = 13.3, 5.4 Hz, 1H), 3.05 (dd, *J* = 13.3, 9.0 Hz, 1H), 2.74 (d, *J* = 6.3 Hz, 2H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, D_2O): δ = 177.7, 172.3, 83.5, 77.0, 74.5, 65.4, 57.7, 50.3, 46.4, 37.8, 27.3. HRMS-ESI: *m*/*z* calcd for $C_{13}H_{26}N_3O_5$ 304.1872 [M+H$^+$]; found: 304.1869.

(7*S*,8*S*,8a*S*)-7,8-dihydroxyhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (19b-1):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-((glycineamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-1**, 147 mg, 60% yield) was purified by HPLC: gradient elution 0% MeOH for 5 min, then from 0% to 70% MeOH in 35 min. HPLC analysis: identical to that **16b-1**. ^{1}H and ^{13}C NMR matched those listed above for compound **16b-1**. The title compound (100 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 2.5 (*c* = 2.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.31 (dd, *J* = 14.3, 7.1 Hz, 1H), 3.94 (t, *J* = 6.2 Hz, 1H), 3.83 (m, 4H), 3.47 (dd, *J* = 12.5, 6.9 Hz, 1H), 3.18 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 163.3, 77.5, 72.6, 57.4, 48.8, 43.9, 43.3. HRMS-ESI: *m*/*z* calcd for $C_7H_{13}N_2O_3$ 173.0926 [M+H$^+$]; found: 173.0930.

(3*S*,7*S*,8*S*,8a*S*)-7,8-dihydroxy-3-methylhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (19b-2):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-alanineamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-2**, 50 mg, 44 % yield) was purified by HPLC: gradient elution 0% to 70% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 13.4 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.30 (m, 5H, Ar), 5.05 (d, *J* = 5.7 Hz, 2H, OCH_2Ph), 4.11 (m, 1H, H-4), 4.08 (m, 1H, H-4-rotamer), 4.02 (s, 1H, H-3-rotamer), 3.93 (m, 3H, H-2-H-3-H-7), 3.77 (q, *J* = 7.0 Hz, 1H, H-7-rotamer), 3.68 (dt, *J* = 11.0, 5.3 Hz, 1H, H-5), 3.36 (m, 2H, H-5'-H-6), 3.15 (ddd, *J* = 25.4, 13.1, 7.1 Hz, 1H, H-6'), 1.41 (d, *J* = 7.1 Hz, 3H, CH_3), 1.24 (d, *J* = 7.1 Hz, 3H, CH_3 rotamer); ^{13}C NMR (101 MHz, D_2O): δ = 172.0 (CONH2), 171.8 (CONH2 rotamer), 158.0 (COBn), 156.5 (COBn-rotamer), 135.8 (C-Ar), 135.3 (C-Ar-rotamer), 128.9 (C-Ar), 128.7 (C-Ar), 128.6 (C-Ar), 128.5 (C-Ar), 127.9 (C-Ar), 78.2 (C-3-rotamer), 77.6 (C-3), 73.5 (C-4-rotamer), 73.0 (C-4), 68.4 (OCH_2Ph-rotamer), 68.1 (CH_2Ph), 62.1 (C-2), 62.1 (C-2-rotamer), 56.4 (C-7-rotamer), 56.2 (C-7), 53.1 (C-5-rotamer), 52.3 (C-5), 48.3 (C-6), 47.4 (C-6-rotamer), 15.4 (C-8), 15.2 (C-8-rotamer). The title compound (45 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 26.0 (*c* = 1.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.34 (dd, *J* = 14.2, 7.3 Hz, 1H), 4.09 (q, *J* = 7.2 Hz, 1H), 3.91 (m, 3H), 3.82 (dd, *J* = 12.7, 8.0 Hz, 1H), 3.50 (dd, *J* = 12.7, 6.9 Hz, 1H), 3.31 (m, 1H), 1.59 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 165.9, 77.5, 72.8, 57.4, 52.6, 49.1, 43.3, 14.4. HRMS-ESI: *m*/*z* calcd for $C_8H_{15}N_2O_3$ 187.1083 [M+H$^+$]; found: 187.1085.

(3*S*,7*S*,8*S*,8a*S*)-7,8-dihydroxy-3-isobutylhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (19b-4):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-leucineamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-4**) (45 mg, 50 % yield) was purified by HPLC: gradient elution 0% to 70% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 18.5 min. ^{1}H NMR (400 MHz, D_2O): δ = 7.47 (d, *J* = 13.8 Hz, 5H, Ar), 5.21 (m, 2H, OCH_2Ph), 4.26 (m, 1H, H-4), 4.15 (m, 2H, H-2-H-3), 4.10 (br s, 1H, H-3-rotamer), 4.02 (m, 1H, H-7), 3.84 (m, 2H, H-5-H-7-rotamer), 3.47 (m, 2H, H-5'-H-6), 3.32 (m, 1H, H-6'), 1.75 (m, 3H, H-8-H-8'-H-9), 1.55 (m, 2H, H-8-rotamer-H-9-rotamer), 0.94 (m, 6H, CH_3); ^{13}C NMR (101 MHz, D_2O): δ = 171.08 (CONH2), 158.19 (COBn), 156.45 (COBn-rotamer), 135.74 (C-Ar), 131.96 (C-Ar-rotamer), 129.03 (C-Ar), 128.95 (C-Ar), 128.74 (C-Ar), 128.54 (C-Ar), 127.97 (C-Ar), 78.27 (C-3), 77.51 (C-3 rotamer), 73.42 (C-4), 72.93 (C-4 rotamer), 68.53 (CH_2Ph), 68.18 (CH_2Ph rotamer), 62.01 (C-2), 59.80 (C-7), 59.42 (C-7 rotamer), 53.07 (C-5), 52.35 (C-5 rotamer), 48.92 (C-6), 48.26 (C-6 rotamer), 38.91 (C-8), 38.62 (C-8 rotamer), 23.96 (C-9), 21.92 (C-10), 21.86 (C-10 rotamer), 21.12 (C-11), 20.92 (C-11 rotamer). The title compound (20 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 3.8 (*c* = 0.9 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.33 (dd, *J* = 14.3, 7.2 Hz, 1H), 4.06 (dd, *J* = 10.0, 3.5 Hz, 1H), 3.96 (t, *J* =

7.5 Hz, 1H), 3.88 (m, 2H), 3.80 (dd, *J* = 12.5, 7.8 Hz, 1H), 3.52 (dd, *J* = 12.6, 6.8 Hz, 1H), 3.29 (t, *J* = 11.6 Hz, 1H), 1.96 (m, 1H), 1.79 (m, 2H), 0.98 (t, *J* = 5.7 Hz, 6H); ^{13}C NMR (101 MHz, D_2O): δ = 165.7, 77.4, 72.6, 57.1, 54.8, 49.2, 43.3, 38.6, 23.7, 22.1, 19.9. HRMS-ESI: *m*/*z* calcd for $C_{11}H_{21}N_2O_3$ 229.1552 [M+H$^+$]; found: 229.1545.

1-(3-((3*S*,7*S*,8*S*,8a*S*)-7,8-dihydroxy-4-oxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)propyl)guanidine (19b-5):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-arginineamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-5**, 49 mg, 48% yield) was purified by HPLC: gradient elution 0% to 60% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 12.2 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36 (m, 5H, Ar), 5.15 (m, 2H, OCH_2Ph), 4.10 (d, *J* = 3.6 Hz, 1H, H-4), 4.03 (br s, 3H, H-2-H-3-H-7), 3.75 (dd, *J* = 11.7, 4.3 Hz, 1H, H-5), 3.49 (m, 2H, H-5'-H-6), 3.35 (m, 1H, H-6'), 3.23 (t, *J* = 5.8 Hz, 2H, H-10-H-10'), 1.98 (m, 2H, H-8-H-8'), 1.66 (m, 2H, H-9-H-9'); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.7 ($CONH_2$), 159.2 (COBn), 158.7 (C=NH), 137.6 (C-Ar), 129.7 (C-Ar), 129.6 (C-Ar), 129.4 (C-Ar), 129.2 (C-Ar), 129.0 (C-Ar), 79.4 (C-3), 75.3 (C-4), 68.9 (OCH_2Ph), 64.4 (C-2), 61.1 (C-7), 54.5 (C-5), 50.9 (C-6), 41.7 (C-10), 28.8 (C-8), 25.0 (C-9). The title compound (60 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 16.9 (*c* = 1.2 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.33 (dd, *J* = 14.4, 7.1 Hz, 1H), 4.00 (dd, *J* = 8.3, 4.4 Hz, 1H), 3.96 (m, 1H), 3.88 (m, 2H), 3.81 (dd, *J* = 12.6, 7.8 Hz, 1H), 3.52 (dd, *J* = 12.6, 6.9 Hz, 1H), 3.26 (m, 3H), 2.18 (ddd, *J* = 15.6, 10.2, 4.8 Hz, 1H), 1.91 (m, 1H), 1.77 (m, 2H); ^{13}C NMR (101 MHz, D_2O): δ = 166.8, 156.7, 77.5, 72.7, 58.4, 56.3, 49.1, 43.7, 40.3, 27.2, 24.2. HRMS-ESI: *m*/*z* calcd for $C_{11}H_{22}N_5O_3$ 272.1723 [M+H$^+$]; found: 272.1716.

(3*S*,7*S*,8*S*,8a*S*)-3-benzyl-7,8-dihydroxyhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one (19b-6):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-phenylalanineamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-6**, 54 mg, 55% yield) was purified by HPLC: gradient elution: 0% to 70% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 19.4 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.32 (m, 10H, Ar), 5.20 (m, 2H, OCH_2Ph), 4.23 (dd, *J* = 7.4, 6.1 Hz, 1H, H-7), 4.06 (br s, 1H, H-4), 4.00 (m, 1H, H-2), 3.95 (s, 1H, H-3), 3.73 (dd, *J* = 11.7, 4.3 Hz, 1H, H-5), 3.40 (m, 3H, H-5'-H-6-H-6'), 3.21 (ddd, *J* = 21.5, 13.9, 6.8 Hz, 2H, CH_2Ph); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.3 ($CONH_2$), 159.1 (COBn), 137.6 (C-Ar), 135.0 (C-Ar), 130.6 (2C-Ar), 130.0 (2C-Ar), 129.6 (C-Ar), 129.3 (C-Ar), 129.2 (C-Ar), 128.9 (C-Ar), 79.4 (C-3), 75.3 (C-4), 68.9 (OCH_2Ph), 64.3 (C-2), 62.8 (C-7), 54.5 (C-5), 50.7 (C-6), 37.8 (C-8). The title compound (34 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = – 5.7 (*c* = 1.1 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 7.42 (m, 5H), 4.34 (m, 2H), 3.95 (m, 1H), 3.84 (m, 3H), 3.61 (dd, *J* = 14.8, 4.2 Hz, 1H), 3.56 (dd, *J* = 12.7, 6.9 Hz, 1H),

3.26 (t, *J* = 12.1 Hz, 1H), 3.14 (dd, *J* = 14.9, 10.1 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 164.2, 133.9, 129.3, 129.2, 128.1, 77.4, 72.7, 58.0, 57.1, 49.2, 43.5, 35.0. HRMS-ESI: *m*/*z* calcd for $C_{14}H_{19}N_2O_3$ 263.1396 [M+H$^+$]; found: 263.1399.

2-((3*S*,7*S*,8*S*,8a*S*)-7,8-dihydroxy-4-oxooctahydropyrrolo[1,2-*a*]pyrazin-3-yl)acetic acid (19b-13):

The precursor (2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-aspartic(β^{t}Buester)amidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-9**, 64 mg, 47% yield) was purified by HPLC: gradient elution 0% to 70% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 20.6 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36 (m, 5H, Ar), 5.19 (s, 2H, OCH_2Ph), 4.19 (m, 1H, H-7), 4.09 (br s, 1H, H-4), 4.02 (br s, 2H H-2-H-3), 3.75 (dd, *J* = 11.6, 4.1 Hz, 1H, H-5), 3.47 (m, 3H, H-5'-H-6-H-6'), 3.00 (t, *J* = 4.8 Hz, 2H, H-8-H-8'), 1.48 (s, 9H, CH_3); ^{13}C NMR (101 MHz, CD_3OD): δ = 170.0 (2C-$CONH_2$-COO^tBu), 159.0 (COBn), 137.6 (C-Ar), 129.7 (C-Ar), 129.6 (C-Ar), 129.4 (C-Ar), 129.2 (C-Ar), 129.1 (C-Ar), 84.1 (*C*-CH_3), 79.5 (C-3), 75.2 (C-4), 68.9 (OCH_2Ph), 64.4 (C-2), 58.4 (C-7), 54.4 (C-5), 51.8 (C-6), 36.7 (C-8), 28.2 ($3CH_3$). The precursor (2*S*,3*S*,4*S*)--*N*-benzyloxycarbonyl-2-(((*S*)-asparticamidyl)methyl)-3,4-dihydroxypyrrolidine (**17b-13**) (55 mg, 47% yield) was prepared by removing the *t*Bu ester of the β-carboxylate group. *Deprotection of the tBu group*: **17b-9** (60 mg, 0.13 mmol) was dissolved in trifluoroacetic acid (TFA) (7 mL) and left to stand at room temperature for 6 h. After that time, no starting material was detected by HPLC. The derivative was purified by HPLC using a gradient elution: 0% to 70% B during 40 min. HPLC analysis: gradient elution from 10% to 70% B in 30 min; t_R = 13.3 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.35 (m, 5H, Ar), 5.20 (m, 2H, OCH_2Ph), 4.21 (m, 1H, H-7), 4.09 (br s, 1H, H-4), 4.03 (br s, 2H, H-2-H-3), 3.75 (dd, *J* = 11.7, 4.3 Hz, 1H, H-5), 3.56 (dd, *J* = 13.0, 2.6 Hz, 1H, H-6), 3.45 (m, 2H, H-5'-H-6'), 3.05 (dd, *J* = 9.2, 5.8 Hz, 2H, H-8-H-8'); ^{13}C NMR (101 MHz, CD_3OD): δ = 172.5 ($CONH_2$), 170.2 (COOH), 158.9 (COBn), 137.6 (C-Ar), 129.6 (2C-Ar), 129.3 (C-Ar), 129.1 (2C-Ar), 79.6 (C-3), 75.3 (C-4), 68.9 (OCH_2Ph), 64.5 (C-2), 58.5 (C-7), 54.5 (C-5), 51.6 (C-6), 35.5 ($CONH_2$). The title compound (32 mg, 99% yield) was then prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 46.6 (*c* = 0.8 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.35 (dd, J = 14.3, 7.1 Hz, 1H), 4.29 (m, 1H), 3.95 (m, 3H), 3.84 (dd, J = 12.6, 7.9 Hz, 1H), 3.48 (dd, J = 12.6, 6.8 Hz, 1H), 3.39 (t, J = 11.8 Hz, 1H), 3.23 (dd, J = 18.4, 5.5 Hz, 1H), 3.07 (dd, J = 18.4, 3.7 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 173.8, 164.3, 77.4, 72.7, 57.2, 53.1, 49.1, 43.8, 33.6. HRMS-ESI: *m*/*z* calcd for $C_9H_{15}N_2O_5$ 231.0981 [M+H$^+$]; found: 231.0995.

(2*S*,3*S*,4*S*)-*N*-benzyloxycarbonyl-2-(((*S*)-valineamidyl)methyl)-3,4-dihydroxypyrrolidine (17b-3):

The title compound (60 mg, 50% yield) was prepared according to the general procedure described above and purified by HPLC: gradient elution 0% to 70% B during 40 min. HPLC

analysis: gradient elution from 10% to 70% B in 30 min; t_R = 16.4 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.37 (m, 5H, Ar), 5.18 (m, 2H, OCH_2Ph), 4.08 (br s, 1H, H-4), 4.05 (d, *J* = 7.0 Hz, 1H, H-2), 4.00 (br s, 1H, H-3), 3.83 (d, *J* = 4.5 Hz, 1H, H-7), 3.75 (dd, *J* = 11.7, 4.3 Hz, 1H, H-5), 3.48 (d, *J* = 11.8 Hz, 1H, H-5'), 3.43 (d, *J* = 2.2 Hz, 1H, H-6), 3.35 (m, 1H, H-6'), 2.26 (m, 1H, H-8), 1.10 (m, 6H, $2CH_3$); ^{13}C NMR (101 MHz, CD_3OD): δ = 169.7 ($CONH_2$), 159.3 (COBn), 137.6 (C-Ar), 129.6 (2C-Ar), 129.3 (C-Ar), 129.1 (2C-Ar), 79.5 (C-3), 75.3 (C-4), 69.0 (OCH_2Ph), 66.8 (C-7), 64.4 (C-2), 54.5 (C-5), 51.4 (C-6), 31.1 (C-8), 18.8 (CH_3), 17.8 (CH_3). This compound could not be converted to the corresponding bicyclic (3*S*,7*S*,8*S*,8a*S*)-7,8-dihydroxy-3-isopropylhexahydropyrrolo[1,2-*a*]pyrazin-4(1*H*)-one. No further characterization was conducted.

*DAB 2-aminomethyl alcohol derivatives **21**.*

(2*R*,3*R*,4*R*)-2-(((2-hydroxyethyl)amino)methyl)pyrrolidine-3,4-diol (21c-1):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-(((2-hydroxyethyl)amino)methyl)pyrrolidine (**20c-1**, 95 mg, 40% yield) was purified by HPLC: gradient elution 0% MeOH for 5 min, then from 0% to 70% MeOH in 35 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 16.7 min. The title compound (64 mg, 98% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 4.7 (*c* = 1.0 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.21 (m, 1H), 3.97 (t, *J* = 3.5 Hz, 1H), 3.76 (t, *J* = 5.3 Hz, 2H), 3.45 (m, 1H), 3.37 (dd, *J* = 12.3, 5.2 Hz, 1H), 3.24 (d, *J* = 4.9 Hz, 1H), 3.15 (dd, *J* = 13.2, 9.1 Hz, 1H), 3.06 (m, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 78.9, 75.6, 62.1, 57.7, 50.7, 49.8, 48.9. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_7H_{17}N_2O_3^+$ calculated 177.1239; found 177.1226.

(2*R*,3*R*,4*R*)-2-(((3-hydroxypropyl)amino)methyl)pyrrolidine-3,4-diol (21c-2):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-(((3-hydroxypropyl)amino)methyl)pyrrolidine (**20c-2**, 172 mg, 70% yield) was purified by HPLC: gradient elution from 0% to 60% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 17.0 min. The title compound (103 mg, 91% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 6.7 (*c* = 0.9 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.21 (dt, *J* = 5.5, 3.4 Hz, 1H), 3.96 (t, *J* = 3.8 Hz, 1H), 3.68 (t, *J* = 6.1 Hz, 2H), 3.42 (dt, *J* = 8.9, 4.5 Hz, 1H), 3.34 (ddd, *J* = 13.1, 10.7, 5.2 Hz, 2H), 3.22 (dd, *J* = 13.1, 8.7 Hz, 1H), 3.12 (m, 2H), 3.02 (dd, *J* = 12.3, 3.3 Hz, 1H), 1.89 (m, 2H); ^{13}C NMR (101 MHz, D_2O): δ = 78.78, 75.31, 61.03, 58.85, 50.64, 48.68, 46.05, 27.92. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_8H_{19}N_2O_3^+$ calculated 191.1395; found 191.1375.

(2*R*,3*R*,4*R*)-2-((((*S*)-1-hydroxypropan-2-yl)amino)methyl)pyrrolidine-3,4-diol (21c-3):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-(((((*S*)-1-hydroxypropan-2-yl))amino)methyl)pyrrolidine (**20c-3**, 175 mg, 72% yield) was purified by HPLC: gradient elution

from 0% to 60% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 18.0 min. The title compound (100 mg, 92% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 14.0 (*c* = 1.5 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.39 (dt, *J* = 4.1, 1.9 Hz, 1H), 4.30 (t, *J* = 2.4 Hz, 1H), 3.94 (td, *J* = 6.4, 2.9 Hz, 1H), 3.87 (dd, *J* = 12.7, 3.6 Hz, 1H), 3.66 (m, 4H), 3.53 (ddd, *J* = 10.2, 6.6, 3.4 Hz, 1H), 3.48 (d, *J* = 12.7 Hz, 1H), 1.33 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 77.55, 73.70, 62.03, 60.93, 56.70, 51.24, 44.53, 12.63. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_8H_{19}N_2O_3^+$ calculated 191.1395; found 191.1375.

(2*R*,3*R*,4*R*)-2-((((*S*)-1-hydroxybutan-2-yl)amino)methyl)pyrrolidine-3,4-diol (21c-4):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((((*S*)-1-hydroxybutan-2-yl)amino)methyl)pyrrolidine (**20c-4**, 122 mg, 64% yield) was purified by HPLC: gradient elution from 10% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 19.9 min. The title compound (64 mg, 91% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 16.1 (*c* = 1.0 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.23 (m, 1H), 3.98 (t, *J* = 3.5 Hz, 1H), 3.79 (dd, *J* = 12.5, 3.7 Hz, 1H), 3.64 (m, 1H), 3.44 (dt, *J* = 8.9, 4.6 Hz, 1H), 3.38 (dd, *J* = 12.3, 5.3 Hz, 1H), 3.26 (dd, *J* = 13.2, 5.0 Hz, 1H), 3.17 (dd, *J* = 13.2, 8.4 Hz, 1H), 3.02 (m, 2H), 1.62 (m, 2H), 0.93 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 78.8, 75.5, 61.8, 60.6, 59.2, 50.4, 46.0, 21.0, 9.1. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_9H_{21}N_2O_3^+$ calculated 205.1552; found 205.1547.

(2*R*,3*R*,4*R*)-2-(((1,3-dihydroxypropan-2-yl)amino)methyl)pyrrolidine-3,4-diol (21c-5):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-2-(((1,3-dihydroxypropan-2-yl)amino)methyl)-3,4-dihydroxypyrrolidine (**20c-5**, 126 mg, 66 yield) was purified by HPLC: gradient elution from 10% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 17.1 min. The title compound (78 mg, 99% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 15.2 (*c* = 1.2 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.29 (dt, *J* = 5.0, 2.6 Hz, 1H), 4.08 (t, *J* = 2.7 Hz, 1H), 3.70 (dd, *J* = 11.8, 4.4 Hz, 2H), 3.59 (m, 3H), 3.52 (dd, *J* = 12.6, 4.8 Hz, 1H), 3.30 (d, *J* = 2.4 Hz, 1H), 3.25 (dd, *J* = 13.4, 5.0 Hz, 1H), 3.12 (dd, *J* = 13.4, 8.9 Hz, 1H), 2.93 (m, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 77.5, 74.5, 64.3, 59.9, 59.8, 50.2, 45.9. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_8H_{19}N_2O_4^+$ calculated 207.1316; found 207.1333.

(2*R*,3*R*,4*R*)-2-((((*R*)-1-hydroxypentan-2-yl)amino)methyl)pyrrolidine-3,4-diol (21c-6):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((((*R*)-1-hydroxypentan-2-yl)amino)methyl)pyrrolidine (**20c-6**, 112 mg, 57 yield) was purified by HPLC: gradient elution from 10% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30

min; t_R = 22.0 min. The title compound (59 mg, 89% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 1.05 (*c* = 0.9 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.31 (m, 1H), 4.16 (s, 1H), 3.85 (m, 1H), 3.70 (m, 2H), 3.53 (m, 2H), 3.43 (dd, *J* = 13.5, 8.3 Hz, 1H), 3.29 (m, 2H), 1.62 (dd, *J* = 15.2, 7.5 Hz, 2H), 1.37 (m, 2H), 0.90 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, D_2O): δ = 78.0, 74.4, 62.0, 59.9, 58.6, 50.9, 45.1, 29.2, 18.1, 12.9. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_{10}H_{23}N_2O_3^+$ calculated 219.1709; found 219.1691.

(2*R*,3*R*,4*R*)-2-((((*R*)-1-hydroxy-3-methylbutan-2-yl)amino)methyl)pyrrolidine-3,4-diol (21c-7):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl-3,4-dihydroxy-2-((((*R*)-1-hydroxy-3-methylbutan-2-yl)amino)methyl)pyrrolidine (**20c-7,** 94 mg, 47% yield) was purified by HPLC: gradient elution from 10% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 21.6 min. The title compound (50 mg, 94% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 18.3 (*c* = 0.9 in MeOH); ^{1}H NMR (500 MHz, D_2O): δ = 4.27 (dt, *J* = 5.1, 2.7 Hz, 1H), 4.08 (t, *J* = 2.8 Hz, 1H), 3.83 (dd, *J* = 12.5, 3.9 Hz, 1H), 3.69 (dd, *J* = 12.5, 6.8 Hz, 1H), 3.62 (dt, *J* = 8.2, 4.2 Hz, 1H), 3.48 (dd, *J* = 12.5, 5.0 Hz, 1H), 3.40 (dd, *J* = 13.5, 4.8 Hz, 1H), 3.31 (dd, *J* = 13.5, 8.5 Hz, 1H), 3.20 (dd, *J* = 12.5, 2.4 Hz, 1H), 2.95 (m, 1H), 2.00 (dq, *J* = 13.4, 6.8 Hz, 1H), 0.96 (dd, *J* = 25.3, 6.9 Hz, 6H); ^{13}C NMR (101 MHz, D_2O): δ = 78.2, 74.8, 64.8, 62.4, 58.1, 50.6, 46.2, 27.1, 18.1, 17.0. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_{10}H_{23}N_2O_3^+$ calculated 219.1709 ; found 219.1691.

(2*R*,3*R*,4*R*)-1-(((2*R*,3*R*,4*R*)-3,4-dihydroxypyrrolidin-2-yl)methyl)-2-(hydroxymethyl)piperidine-3,4-diol (21d):

The precursor (2*R*,3*R*,4*R*)-*N*-benzyloxycarbonyl 2-(((2*R*,3*R*,4*R*)-3,4-dihydroxy-2-(hydroxymethyl)piperidin-1-yl)methyl)-3,4-dihydroxypyrrolidine (**21d**, 102 mg, 52% yield) was purified by HPLC: gradient elution from 0% to 70% MeOH in 40 min. HPLC analysis: gradient elution from 2% to 62% B in 30 min; t_R = 17.3 min. The title compound (60 mg, 79% yield) was prepared according to the general procedure described above. $[\alpha]_D^{22}$ = + 35.4 (*c* = 0.9 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.30 (s, 1H), 4.09 (s, 1H), 3.94 (dd, *J* = 12.3, 3.5 Hz, 1H), 3.70 (m, 2H), 3.51 (m, 2H), 3.32 (d, *J* = 12.9 Hz, 1H), 3.20 (t, *J* = 9.4 Hz, 1H), 3.11 (dd, *J* = 14.7, 5.4 Hz, 1H), 2.98 (m, 2H), 2.57 (m, 2H), 1.85 (dd, *J* = 13.2, 4.6 Hz, 1H), 1.62 (ddd, *J* = 25.0, 12.7, 4.0 Hz, 1H); ^{13}C NMR (101 MHz, D_2O): δ = 77.0, 74.8, 73.0, 71.4, 65.2, 64.2, 58.3, 50.3, 48.9, 48.2, 29.1. HRMS (ESI-TOF): *m*/*z* [M+H$^+$] for $C_{11}H_{23}N_2O_5^+$ calculated 263.1607; found 263.1592.

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5.4. PREPARATION OF CASUARINE TYPE POLYHYDROXYLATED PYRROLIZIDINES

5.4.1. Materials:

The cyclic dimer precursor of dihydroxyacetone phosphate (DHAP), 2,5-diethoxy-*p*-dioxane-2,5-dimethanol-*O*-2^1-*O*-5^1-bisphosphate, was synthesized in our lab using a procedure described by Jung et al. with slight modifications.[1] DHAP was obtained from the precursor by acidic hydrolysis at 65°C. L-Fuculose-1-phosphate aldolase mutant FucA PHE131ALA (0.1 U mg^{-1}) was obtained as previously described by our group (1 U cleaves 1 µmol of L-fuculose-1-phosphate per minute at 25°C and pH 7.5 (100 mM Tris·HCl + 150 mM KCl)).[2,3] Acid phosphatase from potato (lyophilized powder 1.9 U mg^{-1}) was purchased from Sigma-Aldrich. Aqueous borate solutions were prepared by adjusting the desired pH of a solution of boric acid with 2 M aq. NaOH. Deionized water was used for the preparation of buffers and solutions and Milli-Q grade water, obtained from an Arium® Pro Ultrapure Water Purification System (SartoriusStedim Biotech), for analytical and preparative HPLC. All other solvents used were of analytical grade.

5.4.2. Methods:

N-Cbz-(2*S*,3*R*,4*R*)- and -(2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehydepyrrolidine (**3** and **4**) were prepared as described in Section 3.2 of this thesis.

Before using for enzymatic aldol reactions, the crudes of the oxidation were washed with aqueous $NaHCO_3$ 5% in order to eliminate byproducts and ethyl acetate removed under reduced pressure.

Synthesis of 7+8+9:

Enzymatic aldol addition:

Reactions at preparative scale (21 mL total volume) were carried out in 100 mL Erlenmeyer flasks with screw caps. *N*-Cbz-(2*S*,3*R*,4*R*)-3,4-dihydroxy-2-carbaldehydepyrrolidine **3** (320 mg 1.2 mmol, 60 mM final concentration) was dissolved in DMF (4.2 mL 20% v/v of total amount) and cooled to 0ºC. A freshly prepared solution of DHAP at pH 6.9 (1.2 mmol, 14.2 mL of a 89 mM solution, 60 mM final concentration), KCl buffer (0.52 mL of a 200 mM solution, 5 mM final concentration) and TEA buffer (2.1 mL of a 500 mM solution, 50 mM final concentration) were added 4ºC under vigorous agitation. Finally, FucA Phe131Ala solution (10 mg per mL reaction mixture) was added and mixed again. The reaction was placed on a horizontal shaking bath (100 rpm) at constant temperature of 4ºC. The reactions were monitored by HPLC until the peak of the aldol adduct was constant with the time (24 h, 50% conversion). The enzymatic reactions were stopped by addition of fresh MeOH (20 mL) and the solid removed by centrifugation (3000 rpm 15 minuts). Then, the methanol was evaporated and the aqueous solution washed with

ethyl acetate (2x15 mL) to remove the unreacted *N*-protected aminoaldehyde. The aqueous layer was collected, the remaining ethyl acetate removed under reduced pressure.

Dephosphorylation:

The aqueous solution was diluted with citric buffer pH 5 (11.2 mL of a 400 mM solution, 50 mM final concentration) and plain water until a final volume of 90 ml. Acid phosphatase (0.3 U per mmol phosphorylated substrate) was added to this solution. The reaction was followed by HPLC until no starting material was detected. Then, the reaction mixture was filtered through a 0.45 µm cellulose membrane filter. The filtrate was loaded onto semi-preparative X-Terra Prep MS C-18, 10 µm, 19 x 250 mm column and eluted with a gradient of CH_3CN in plain water. The solvent system used was: solvent (A): aqueous trifluoroacetic acid (TFA) (0.1 % (v/v)) and solvent (B): TFA (0.095 % (v/v)) in CH_3CN/H_2O 4:1. Salts and solvents were washed out with 100% A during 5 min and the product was eluted with a gradient of B (0 to 50% over 30 min). Pure fractions were pooled and lyophilized obtaining a pale brown solid (95 mg, 22% overall yield).

Removal of Cbz group and reductive amination:

Pd/C (50 mg) was added to a solution of the aldol adduct obtained above (95 mg) in H_2O/MeOH 4:1 (25 mL). The reaction mixture was shaken under hydrogen gas (50 psi) overnight at room temperature. After removal of the catalyst by filtration through Celite™, the pH of the filtrate was adjusted to pH 5.5, the solvent was evaporated under reduced pressure and then lyophilized. The diasteromeric mixture **7**-**8**-**9** (41 mg, 75 %) was afforded as a brown solid.

The two diastereoisomers were separated by on exchange chromatography on a CM-sepharose in the NH_4^+ form, eluted isocratically with 7mM ammonium hydroxide. Characterization of the lyophilized, products, except **9**, was accomplished by NMR spectroscopy.

Synthesis of 10+11:

The same procedure was followed starting with the *N*-Cbz-(2*R*,3*S*,4*S*)-3,4-dihydroxy-2-carbaldehydepyrrolidine (**4**) obtaining the unphosphorylated aldol adduct **6** (93 mg, 21 % overall yield) and the mixture of diasteromers **10**+**11** as pale brown solid (27 mg, 50%).

Purification by ion exchange chromatography:

The diasteromeric mixture **7**+**8+9** (41 mg) was separated by ion exchange chromatography on a FPLC system. CM-Sepharose CL-6B (Amersham Pharmacia) in the NH_4^+ form as stationary phase was packed into a glass column (450–25 mm) to a final bed volume of 220 mL. The flow rate was 4 mL min^{-1}. The CM-Shepharose-NH_4^+ was washed initially with H_2O. Then, an aqueous solution of the crude material at pH 7 was loaded onto the column. Minor colored impurities were washed away with H_2O (440 mL, 2 bed volumes). The retained compounds **7** and **8** were eluted at 320

mL and at 240 mL of aqueous 7mM NH_4OH, respectively. Pure fractions were pooled and lyophilized affording **7** (17 mg) and **8** (8 mg).

The diasteromers **10** and **11** were separated using the same procedure as above. The diasteromeric mixture **10**+**11** (27 mg) was loaded onto the column packed with CM-Shepharose-NH_4^+. After washing with plain water, the retained compounds were eluted isocratically with aqueous 7mM NH_4OH. Retained compounds were eluted at 320 mL of eluent affording **10** (12 mg) and at 288 mL affording **11** (7 mg).

(1*S*,2*S*,3*R*,6*R*,7*R*,7a*S*)-1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine (7) (ent-3-*epi*-casuarine)

$[\alpha]_D^{22}$ = - 5.9 (*c* = 0.85 in MeOH) (lit.[4] of the enanatiomer $[\alpha]_D^{25}$ = +2 (*c* = 0.04 in H_2O)); (lit.[5] of the enanatiomer $[\alpha]_D^{23}$ = +5.7 (*c* = 0.5 in H_2O));
^{1}H NMR (400 MHz, D_2O): δ = 4.51 (s, 1H, H-1), 4.38 (d, *J* = 2.3, 1H, H-2), 4.28 (m, 2H, H-6-H-7), 4.10 (dd, *J* = 7.1, 3.8, 2H, H-8-H-8'), 4.03 (m, 1H, H-3), 3.82 (d, *J* = 8.1, 1H, H-7a), 3.72 (dd, *J* = 10.9, 6.2, 1H, H-5), 3.58 (t, *J* = 10.7, 1H, H-5'); ^{13}C NMR (101 MHz, D_2O): δ = 76.72 (C-1), 76.69 (C-2), 75.91 (C-7), 75.73 (C-7a), 72.81 (C-6), 67.39 (C-3), 54.98 (C-8), 50.37 (C-5). HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_8H_{16}NO_5^+$ calculated 206.1022 ; observed 206.1011.

(1*S*,2*S*,3*S*,6*R*,7*R*,7a*S*)-1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine (8) (ent-casuarine)

$[\alpha]_D^{22}$ = -0.8 (*c* = 0.8 in MeOH) (lit.[6]of the enanatiomer $[\alpha]_D^{23}$ = +18.1 (*c* = 1.0 in H_2O)); lit.[7] of the enantiomer $[\alpha]_D^{24}$ = +16.9 (*c* = 0.8 in H_2O)); ^{1}H NMR (400 MHz, D_2O): δ = 4.54 (d, *J* = 8.0 Hz, 1H, H-7), 4.52 (d, *J* = 2.5 Hz, 1H, H-6), 4.43 (t, *J* = 8.6 Hz, 1H, H-1), 4.04 (m, 2H, H-2-H-8), 3.92 (dd, *J* = 13.1, 5.2 Hz, 1H, H-8'), 3.81 (m, 2H, H-5-H-7a), 3.69 (ddd, *J* = 10.2, 5.1, 3.2 Hz, 1H, H-3), 3.61 (d, *J* = 13.2 Hz, 1H, H-5'); ^{13}C NMR (101 MHz, D_2O) δ = 76.65 (C-6), 76.4 (C-7), 75.2 (2C-1-7a), 73.1 (C-2), 71.1 (C-3), 58.6 (C-5), 56.5 (C-8). HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_8H_{16}NO_5^+$ calculated 206.1022 ; observed 206.1011.

(1*R*,2*S*,3*R*,6*S*,7*S*,7a*R*)-1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine (10) (2-*epi*-casuarine)

$[\alpha]_D^{22}$ = - 20.8 (*c* = 1.2 in MeOH); ^{1}H NMR (400 MHz, D_2O): δ = 4.37 (dd, *J* = 8.8, 4.0 Hz, 1H, H-1), 4.26 (m, 2H, H-2-H-6), 4.21 (t, *J* = 3.0 Hz, 1H, H-7), 3.85 (m, 1H, H-8), 3.71 (dd, *J* = 11.2, 6.4 Hz, 1H, H-8'), 3.31 (m, 3H, H-3-H-5-H-7a), 2.94 (dd, *J* = 12.3, 3.7 Hz, 1H, H-5'); ^{13}C NMR (101 MHz, D_2O) δ = 78.5 (C-7), 77.9 (C-6), 74.9 (C-1), 73.6 (C-7a), 73.0 (C-2), 70.3 (C-3), 59.7 (C-8), 57.9 (C-5). HRMS (ESI-TOF): *m*/*z* $[M+H]^+$ for $C_8H_{16}NO_5^+$ calculated 206.1022; observed 206.1007.

(1*R*,2*S*,3*S*,6*S*,7*S*,7a*R*)-1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine (11) (2,3-*epi*-casuarine)

$[\alpha]_D^{22}$ = -12.8 (*c* = 0.7 in H_2O); ^1H NMR (400 MHz, D_2O): δ = 4.23 (m, 1H, H-1), 4.16 (m, 2H, H-2-H-6), 3.92 (m, 3H, H-7-H-8-H-8'), 3.36 (m, 3H, H-3-H-5-H-7a), 3.00 (t, *J* = 9.4 Hz, 1H, H-5'); ^{13}C NMR (101 MHz, D_2O): δ = 77.83 (C-7), 74.40 (C-6), 74.31 (C-7a), 73.01 (C-1), 70.23 (C-2), 64.48 (C-3), 58.21 (C-8), 50.03 (C-5). HRMS (ESI-TOF): *m/z* $[M+H]^+$ for $C_8H_{16}NO_5^+$ calculated 206.1022 ; observed 206.1007.

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5.5. ENZYMATIC INHIBITION ASSAYS:

5.5.1. Materials:

Ketamine chlorhydrate and Imalgene 1000 were from Merial Laboratorios S.A. (Barcelona, Spain). Xylacine and Rompun 2% were from Química Farmacéutica S.A. (Barcelona, Spain). Starch from potatoes, starch azure, 4-hydroxybenzoic acid, 4-aminoantipyrine, Glucose oxydase Type II-S from *Aspergillus niger*, Peroxidase Type II from horseradish, α-D-Glucosidase from *Saccharomyces cerevisiae*, α-D-glucosidase from rice, β-D-glucosidase from sweet almonds, β-D-galactosidase from bovine liver, α-D-mannosidase from jack beans, *Genus Canavalia*, α-L-rhamnosidase from *Penicillium decumbens*, α-L-fucosidase from bovine kidney and the synthetic substrates *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-rhamnopyranoside, and *p*-nitrophenyl-α-D-fucopyranoside were purchase from Sigma-Aldrich.

5.5.2. Methods:

Enzymatic inhibition assays against commercial glycosidases:

Commercial glycosidase solutions were prepared with the appropriate buffer and incubated in 96-well plates at 37ºC without (control) or with inhibitor (1.0 mM to 2.0 nM) during 3 min for α-D-glucosidase from *Saccharomyces cerevisiae*, β-D-glucosidase, α-D-mannosidase, α-L-rhamnosidase, α-L-fucosidase and 5 min for β-D-galactosidase. After addition of the corresponding substrate solution, incubations were prolonged during different time periods: 10 min for α-D-glucosidase from rice, 3 min for β-D-glucosidase, 6 min for α-D-mannosidase, 5 min for α-L-rhamnosidase, 7 min for α-L-fucosidase and 16 min for β-D-galactosidase and stopped by addition of Tris solution (50 μL, 1 M) or glycine buffer (180 μL, 100 mM, pH 10), depending on the enzymatic inhibition assay. The amount of *p*-nitrophenol formed was determined at 405 nm with UV/VIS Spectramax Plus (Molecular Devices Corporation) spectrophotometer. α-D-Glucosidase from *Saccharomyces cerevisiae* activity was determined with *p*-nitrophenyl-α-D-glucopyranoside (1 mM) in phosphate buffer (100 mM; pH 7.2). α-D-Glucosidase from rice activity was determined with *p*-nitrophenyl-α-D-glucopyranoside (1 mM) in sodium acetate buffer (50 mM, pH 5.0). β-D-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucopyranoside (1 mM) in sodium acetate buffer (100 mM, pH 5.0). β-D-Galactosidase activity was determined with *p*-nitrophenyl-β-D-galactopyranoside (1 mM) in sodium phosphate buffer (100 mM, 0.1 mM $MgCl_2$, pH 7.2). α-D-Mannosidase activity was determined with *p*-nitrophenyl-α-D-mannopyranoside (1 mM) in sodium acetate buffer (50 mM, pH 5.0). α-L-Rhamnosidase activity was determined with *p*-nitrophenyl-α-D-rhamnopyranoside (1 mM) in sodium acetate buffer (50 mM, pH 5.0). α-L-Fucosidase activity was determined with *p*-nitrophenyl-α-D-fucopyranoside (0.15 mM) in sodium acetate buffer (50 mM, pH 5.0). The commercial glycosidase solutions were prepared as

follows: α-D-glucosidase from *Saccharomyces cerevisiae* (0.15 mg mL^{-1} buffer); α-D-glucosidase from rice $(NH_4)_2SO_4$ suspension (100 μL) in buffer (5 mL); β-D-glucosidase: (0.1 mg mL^{-1} buffer), β-D-galactosidase from *Aspergillus oryzae* (0.5 mg mL^{-1} buffer), α-L-rhamnosidase (naringinase) (0.3 mg mL^{-1} buffer); α-D-mannosidase $(NH_4)_2SO_4$ suspension (25 μL) in buffer (10 mL); β-D-galactosidase from bovine liver (0.1 mg mL^{-1} buffer), and α-L-fucosidase $(NH_4)_2SO_4$ suspension (33 μL) in buffer (10 mL).

Kinetics of inhibition:

The nature of the inhibition against enzymes and the K_i values were determined from Lineweaver–Burk plots.[1]

References:

1. Copeland, R.A. and Editor, *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide to Medicinal Chemists and Pharmacologists* **2005**. 296 pp.

ANNEX I

Resumen

1) INTRODUCCIÓN:

Los biocatalizadores son proteínas (i.e. enzimas) que la naturaleza ha evolucionado para facilitar todas las transformaciones químicas necesarias a la vida. El hecho de que enzimas aisladas puedan catalizar reacciones *in vitro* y aceptar substratos distintos de los fisiológicos ha permitido su aplicación en la síntesis química.[1]

Los biocatalizadores, por su naturaleza quiral intrínseca, son una herramienta poderosa para la síntesis asimétrica lo que ha originado un campo de investigación creciente dentro de la química sintética.

La propiedades más atractivas de los enzimas son su elevada eficiencia y su selectividad (regio- y estereoselectividad). Por esa razón las reacciones biocatalíticas son especialmente útiles para la síntesis de compuestos quirales altamente funcionalizados cuya demanda esta en continuo aumento.

Además, las suaves condiciones de reacción utilizadas en las transformaciones enzimáticas y la utilización de agua como solvente hacen de la biocatálisis una de las tecnologías mas atractivas capaz de satisfacer la creciente necesidad de procesos industriales siempre más sostenibles del punto de vista ambiental y económico.[2-4] A lo largo de los últimos 20 años, se han introducido en la producción sintética a escala industrial varias etapas biocataliticas con una significante reducción de costos y simplificación de procesos. Desde los primeros estudios en este área diversas clases de enzimas han sido aplicadas en química sintética (e.g., hidrolasas, oxidorreductasas, liasas, glicotransferasas, transaminasas) describiéndose una gran cantidad de reacciones optimizadas.[5,6] Las ventajas de la utilización de los enzimas en química orgánica están, obviamente, acompañadas por algunas importantes desventajas como problemas de solubilidad de los sustratos en solventes acuosos o la especificidad intrínseca de las enzimas que limita en muchas ocasiones la tolerancia por sustratos estructuralmente muy dispares para los que el enzima ha evolucionado. Cabe decir que sus limitaciones no van mas allá de los que tienen los métodos químicos convencionales, por ejemplo el hecho de que existan más de un centenar de métodos para la formación del enlace amida indica que no existe un método ideal aplicable a todos los casos.[7]

Por otro lado, actualmente el descubrimiento de nuevos enzimas naturales por exploración de la biodiversidad del medio ambiente es objeto de una intensa investigación. Así, se está invirtiendo un enorme esfuerzo en el desarrollo de nuevos biocatalizadores mediante ingeniería genética, especialmente por evolución dirigida, con la finalidad de superar las limitaciones de selectividad, optimizar la eficiencia o descubrir nuevas actividades catalíticas.[8]

Entre los biocatalizadores existentes aplicables en síntesis orgánica, las aldolasas son unos de los más prometedores ya que permiten la construcción de estructuras moleculares quirales a partir de precursores simples no quirales.

Las **aldolasas** constituyen un grupo específico de liasas que catalizan reacciones aldólicas y retroaldólicas en el metabolismo de carbohidratos y aminoácidos.

La reacción aldólica consiste en la adición de un compuesto carbonílico enolizable a otro compuesto carbonílico (aldehído o cetona) con formación de uno o dos nuevos centros quirales.

La reacción aldólica es uno de los métodos mas poderosos para la creación de enlaces carbono-carbono y la construcción de estructuras quirales complejas, ya que permite al mismo tiempo funcionalización e introducción centros estereogénicos.[9] El control de la configuración absoluta de los nuevos centros quirales generados ha sido un reto para este método y, en general, para la síntesis orgánica. Por ello, se han desarrollado diversos métodos catalíticos de adición aldólica asimétrica.[9,10]

En este contexto las aldolasas representan un grupo de enzimas muy importantes para el desarrollo de procesos sintéticos que involucran la formación asimétrica de enlaces carbono-carbono. La principal ventaja de las aldolasas es su estereoselectividad, o sea su capacidad de controlar eficazmente la estereoquímica de la adición aldólica, donde la configuración de uno de los nuevos centros estereogénicos creados depende exclusivamente de la enzima y es, en principio, independiente de los reactivos de la adición aldólica. Su empleo ofrece una herramienta sintética en sintonía con los principios de la química verde para la síntesis asimétrica de productos polihidroxilados con estereoquímica definida como carbohidratos, aminoácidos e iminociclitoles.

Las aldolasas han demostrado ser especialmente útiles para la producción de compuestos enantiopuros biológicamente activos, ya que a menudo su síntesis química convencional implica múltiples y complejas secuencias de protección/desprotección, dificultades en el control de la estereoquímica, en el aislamiento y la purificación del producto. Por todo ello, en los últimos años, estos enzimas han sido objeto de numerosos estudios que demuestran su utilidad como catalizadores en síntesis orgánica asimétrica.[11]

Las aldolasas naturales pueden ser divididas en dos grupos de acuerdo con su mecanismo catalítico que a su vez depende de la naturaleza del sitio activo de la enzima y, consecuentemente, del mecanismo de activación del componente nucleófilo (dador). Desde el punto de vista del mecanismo, las reacciones catalizadas por aldolasas implican la formación de un carbono nucleófilo desde un compuesto carbonílico por α-desprotonación estereoespecífica y subsecuente ataque a un carbono carbonílico electrófilo (aceptor). Las aldolasas de Clase I utilizan el grupo amino de una lisina en el centro activo de la enzima para formar una enamina intermedia y activar el dador aldólico. En las aldolasas de Clase II, en cambio, se forma un enolato por coordinación con un catión de un metal de transición divalente (generalmente el Zn^{2+}) en el centro activo de la enzima que actúa como ácido de Lewis.[12]

La mayoría de las aldolasas toleran como aceptores electrófilos una amplia variedad de aldehídos mientras que aceptan solamente pequeñas modificaciones estructurales o isostéricas en el

sustrato dador. Debido a su especificidad por el sustrato dador las aldolasas han sido clasificadas también según este criterio y agrupadas en cinco categorías (Figura 1):

1) aldolasas dependientes de *piruvato/fosfoenolpiruvato*
   (que proporcionan 3-deoxi-2-cetoácidos)

2) aldolasas dependientes del *fosfato de dihidroxiacetona* (DHAP)
   (que proporcionan cetosas-1-fosfato)

3) aldolasas dependientes de *dihidroxiacetona* (DHA)
   (que proporcionan cetosas)

4) aldolasas dependientes de *acetaldehído*
   (que proporcionan 3-hidroxialdehídos)

5) aldolasas dependientes *glicina*
   (que proporcionan β-hidroxi-α-aminoácidos)

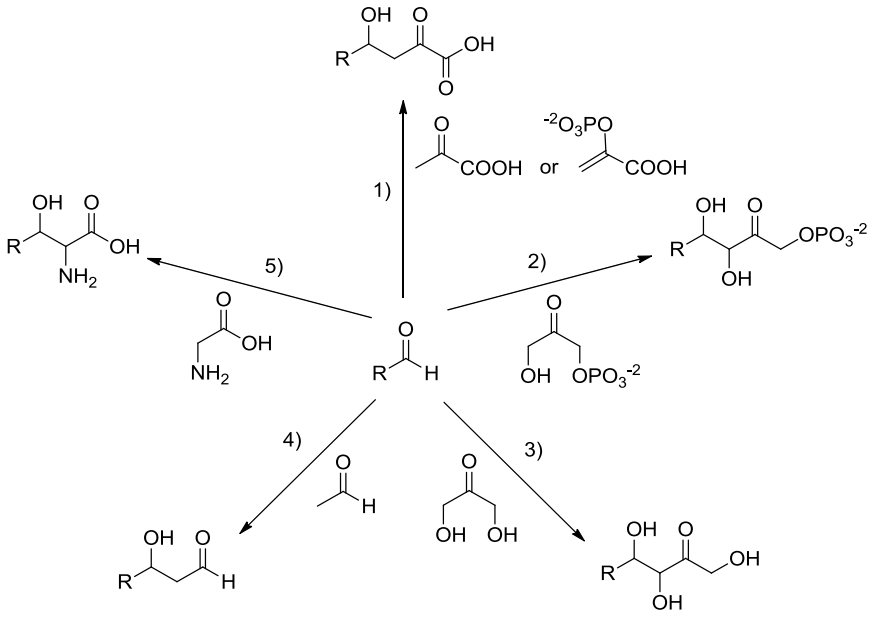


Figura 1 Grupos de aldolasas según dependencia por el sustrato dador.

Aunque esta clasificación inicial es fundamentalmente valida, algunas aldolasas presentan también tolerancia para otros dadores estructuralmente relacionados tales como la hidroxiacetona, hidroxibutanona, glicolaldehído, oxalacetato, 2-oxobutirato y alanina. Además, el número de dadores que aparecen como sustratos de aldolasas convencionales va en aumento con lo cual el concepto de dependencia y especificidad de las aldolasas por el dador está en fase de continua revisión.

En esta tesis se han utilizado aldolasas dependientes de DHAP y DHA.

Las **aldolasas dependientes del fosfato de dihidroxiacetona (DHAP)** catalizan reversiblemente *in vivo* la reacción retro-aldólica de cetosas-1-fosfato o 1,6-difosfato, fundamental en catabolismo de los carbohidratos en mamíferos y microbios.

La naturaleza ha desarrollado cuatro tipos específicos de aldolasas para procesar las cuatro combinaciones posibles del dioles vecinales de las cetosas:

- D-fructosa 1,6-bifosfato aldolasa (FruA)
- D-tagatosa 1,6-bifosfato aldolasa (TagA)
- L-fuculosa 1-fosfato aldolasa (FucA)
- L-ramnulosa 1-fosfato aldolasa (RhuA)

En la dirección de síntesis, estas enzimas catalizan la adición reversible de DHAP a D-gliceraldehído 3-fosfato o L-lactaldehído generando cada una un producto único cuya estereoquímica en C3 y C4 resulta complementaria (Figura 2).

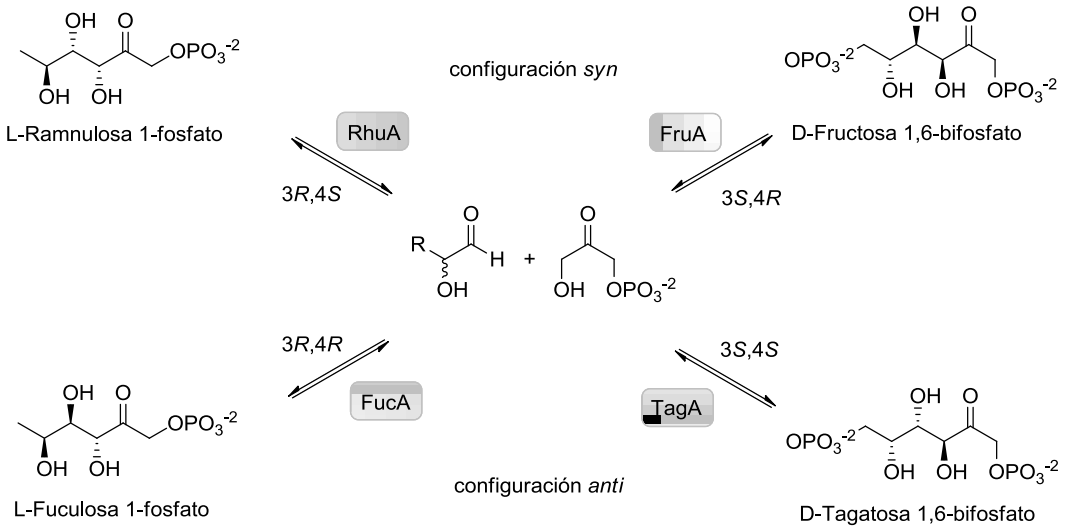


Figura 2 Estereoquímica complementaria de las aldolasas dependientes de DHAP.

De la misma manera pueden catalizar la adición aldólica de DHAP a una gran variedad de aldehídos aceptores originando dos nuevos centros estereogénicos cuya configuración absoluta está formalmente controlada, salvo excepciones, por la elección de una de las cuatro DHAP-aldolasas estéreo complementarias.[13]

La alta estereoselectividad y la tolerancia por un amplio rango de aldehídos aceptores son características particularmente atractivas para la síntesis asimétrica especialmente de compuestos quirales polioxigenados. Por lo tanto las DHAP-aldolasas han sido empleadas extensivamente en la preparación de glicomiméticos tales como desoxiazúcares, fluoroazúcares e iminociclitoles.[14]

Como se ha mencionado la estereolectividad de las reacciones catalizadas por DHAP-aldolasas es generalmente alta y la configuración C3/C4 de los productos sigue la de la reacción fisiológica

particularmente en C3. Sin embargo la configuración en C4 en algunos casos puede depender de la estructura del aceptor generando, en determinadas ocasiones, mezclas epiméricas en C4.[12]

La tagatosa-1,6-bisfosfato aldolasa es la menos esteroselectiva de las aldolasas DHAP dependientes y en la mayoría de reacciones estudiadas no presenta control de la estereoquímica sobre la reacción de adición aldólica cuando se emplean aldehídos distintos al natural por lo que tiene una utilidad sintética limitada.[12]

En nuestro grupo de investigación las aldolasas dependientes de DHAP han sido aplicadas con éxito a la preparación quimioenzimática de iminociclitoles mediante reacciones de adición aldólica de DHAP a *N*-benciloxicarbonilaminoaldehídos.[15-19]

A pesar de su amplia tolerancia por el sustrato aceptor estas enzimas son altamente específicas por el sustrato dador, aceptando pocas variaciones estructurales.[20] La estricta dependencia de la DHAP representa, sin embargo, el mayor inconveniente de estas enzimas por varias razones. La DHAP es un producto costoso, difícil de sintetizar e inestable ya que se degrada a pH básicos a metilglioxal y fosfato inorgánico, ambos inhibidores de aldolasas.

Además, en el producto final generalmente no se desea el grupo fosfato que tiene que ser eliminado por hidrólisis enzimática con fosfatasa ácida.

En la literatura existen muchas metodologías para preparar la DHAP aunque la mayoría son costosos y complejos, con rendimiento bajo y toxicidad de los reactivos empleados.[13,21]

Para intentan superar estas limitaciones se han aplicado diversas estrategias: el desarrollo de diferentes metodologías para generar *in situ* la DHAP, el empleo de miméticos de DHAP y la eliminación directa de la necesidad de DHAP. En la literatura se han descrito algunos métodos enzimáticos para la generación y utilización *en situ* de DHAP los cuales siguen teniendo algunos inconvenientes como, la formación de mezclas más complejas de productos, la posibilidad de reacciones cruzadas que afecten al rendimiento final y la necesidad del control sobre la actividad de varios enzimas en un único esquema de reacción.[13,21,22]

Como alternativa se ha investigado también la utilización de algunos isósteros del éster de fosfato (como arsenatos y vanadatos) que permitan el empleo de DHA con las DHAP-aldolasas existentes. Arsenatos y vanadatos de DHA, formados *in situ*, han sido utilizados como miméticos de la DHAP en reacciones catalizadas por FucA, RhuA y FruA.[23,24] Las principales limitaciones a la aplicabilidad sintética de esta estrategia son la toxicidad del arsenato, incompatible con el concepto de química verde de los procesos enzimáticos, la inestabilidad del vanadato y el bajo rendimiento de la reacciones respecto al sustrato original.

Recientemente se ha descrito la utilización de borato de DHA como mimético de DHAP.[25] Su aplicabilidad ha sido demostrada con L-ramnulosa 1-fosfato aldolasa (RhuA) que acepta como dador DHA en presencia de buffer bórico por formación *in situ* de éster borato. Además el uso de buffer bórico parece mejorar el rendimiento de las reacciones por complejación del producto con el borato evitando de esta manera la reacción retroaldólica. La utilización de esta estrategia en síntesis de iminociclitoles permite el empleo de DHA en lugar de DHAP y simplifica la síntesis

eliminando la introducción y eliminación posterior del grupo fosfato. Nuestro grupo de investigación ha descrito recientemente adiciones aldólicas entre borato de DHA y varios *N*-benciloxicarbonil-aminoaldehídos catalizadas por RhuA wt.[26]

En esta tesis el empleo de buffer bórico para la adición aldólica de DHA con *N*-Cbz-glicinal catalizada por RhuA ha sido aplicada con éxito a la síntesis de 1,4-dideoxi-1,4-imino-L-arabinitol (LAB) como se describe mas adelante. Los esfuerzos mas recientes apuntan a la eliminación directa de la necesidad de DHAP, por mutagénesis dirigida, evolución dirigida o búsqueda de nuevos enzimas naturales.

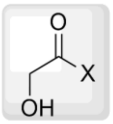
La D-Fructosa 6-fosfato aldolasa (FSA) es la primera y única aldolasa natural **dependiente de dihidroxiacetona (DHA)** descrita hasta el momento. Esta enzima, que fue publicada por primera vez por Schürmann y Sprenger en el 2001,[27] cataliza reversiblemente la adición aldólica de dihidroxiacetona (DHA) a D-gliceraldehído 3-fosfato para formar D-fructosa 6-fosfato. Su rol fisiológico aún no está claro mientras su estructura y sus propiedades bioquímicas han sido ampliamente investigadas.[28]

Gracias a su destacada capacidad de aceptar DHA y evitar el uso de sustratos dadores fosforilados, la FSA ha sido considerada de desde su descubrimiento un biocatalizador extremadamente prometedor. El uso de la DHA como sustrato dador para la preparación de compuestos no fosforilados simplifica la estrategia sintética evitando manejar la función fosfato e impulsando el aplicación industrial de las reacciones aldólicas enzimáticas.

La FSA ha sido utilizada con éxito como herramienta sintética en adiciones aldólicas de una amplia variedad de aldehídos estructuralmente diferentes exhibiendo para todos los sustratos descritos una impecable estereoquímica D-*syn*. Estudios adicionales, incluido el que será presentado en esta tesis, han descrito que la hidroxiacetona (HA) y la hidroxibutanona (HB) también pueden actuar como dadores, manteniendo inalterada la estereoselectividad.[29]

Una de las primeras aplicaciones fue descrita por nuestro grupo en 2006 con la síntesis de la D-fagomina.[30] A partir de aquí han sido obtenidos otros productos por medio de reacciones aldólicas catalizadas por FSA con diversos aceptores y donadores proporcionando una amplia variedad estructural de aductos aldólicos útiles en la síntesis de productos naturales y estructuras inéditas.[29,31]

La promiscuidad de la FSA frente a los sustratos dadores no está limitada a las cetonas (Figura3). Nuestro grupo descubrió recientemente que el glicolaldehído (GO) puede actuar como nucleófilo en adiciones aldólicas con varios aldehídos, ampliando así las posibilidades sintéticas.[32] Esta actividad inédita de la FSA ha permitido la síntesis de aldoazúcares a través de autoadición o adición de glicolaldehído en agua, una reacción sin precedentes ni en biocatálisis ni en organocatálisis. Adicionalmente, siendo el producto de la reacción un aldehído, éste puede utilizarse en cascada como sustrato aceptor para ulteriores adiciones aldólicas.



X = H, CH_3, CH_2OH, CH_2CH_3

Figura 3 Requisito estructural (hidroximetilcarbonilo) de diferentes dadores de FSA.

Comparando las conversiones de las adiciones aldólicas y la reactividad de la FSA hacia los dadores, HA y GO han resultado más activos que la DHA. La baja afinidad por DHA se ha demostrado también mediante reacciones de competencia, monitorizando las reacciones de adición de DHA a GO catalizadas por FSA en las que el único producto originado fue el resultante de la autoadición aldólica de GO (i.e. D-treosa) (Figura 4). De cara a superar estas limitaciones, se han explorado variantes de la FSA con la finalidad de mejorar la eficiencia catalítica y ampliar la gama de posibles sustratos.[33,34]

El residuo de Ala129 ha sido identificado como uno de los sitios probables de unión del grupo hidroxilo del C1 del sustrato dador.[28] En el curso de las investigaciones sobre la FSA, la mutante de FSA Ala129Ser, obtenida por mutagénesis dirigida, presentó un incremento importante de afinidad hacia DHA y resultó ser una herramienta eficiente para la preparación de carbohidratos y análogos, como se ha demostrado por la adición aldólica de DHA a GO proporcionando D-xilulosa con un 80% de rendimiento (Figura 4).[33]

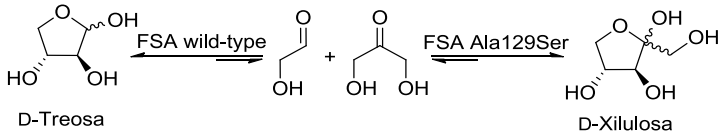


Figura 4 Autoadición de GO y adición de DHA a GO catalizadas por FSA wt y FSA A129S, respectivamente.

En nuestro laboratorio se intentó el rediseño racional del sitio de unión del sustrato aceptor de la FSA wt y de la FSA Ala129Ser por mutagénesis dirigida, con la finalidad de mejorar su eficiencia catalítica hacia *N*-Cbz-aminoaldehídos.[34] La mutante FSA Ala165Gly resultó mas activa con los aceptores ya tolerados por la FSA wt y permitió la reacción con *N*-Cbz-aminoaldehídos α-sustituidos. Esta mutante ha sido utilizada en este trabajo para la síntesis optimizada de 1,4-dideoxi-1,4-imino-D-arabinitol (DAB) descrito en la sección 3.2.

En esta tesis se han obtenido compuestos polihidroxilados, iminoazúcares y nuevos análogos inhibidores de glicosidasas y glicotransferasas mediante el desarrollo de nuevas metodologías quimoenzimáticas que emplean biocatalizadores de la clase de las aldolasas dependiente de DHA y DHAP, descritas anteriormente. A continuación se explicará la importancia de esta clase de productos.

Los **iminociclitoles** o iminoazúcares son análogos de azúcares hemiacetálicos, en los cuales el oxigeno endocíclico está reemplazado por un átomo de nitrógeno.

Los alcaloides polihidroxilados son iminociclitoles de origen natural aislado de plantas y microorganismos.[35,36]

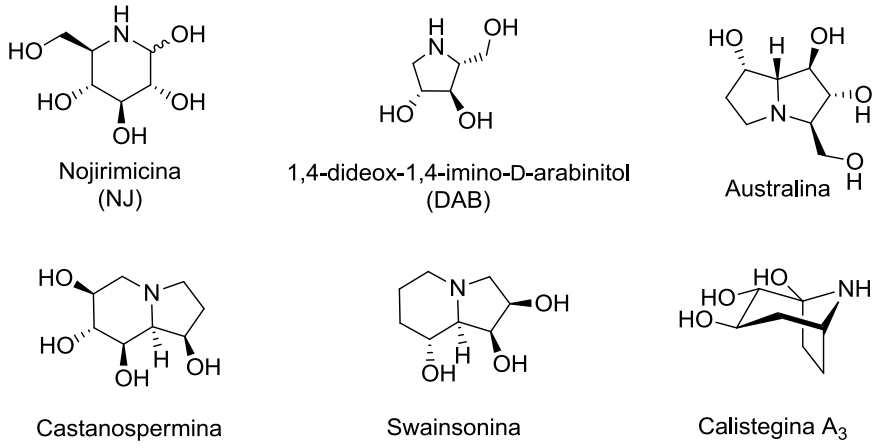


Figura 5 Ejemplos de iminociclitoles naturales.

A lo largo de los últimos 20 años se han identificado más de 100 alcaloides polihidroxilados con diferentes estructuras (Figura 5). Los más conocidos se clasifican dentro de 7 clases diferentes de estructuras: pirrolidinas, piperidinas, azepanos, pirrolizidinas, indolizidinas, quinolizidines y nortropanos (Figura 6). Muchos de estos compuestos son biológicamente activos especialmente como inhibidores de glicosidasas y glicosiltransferasas.[37]

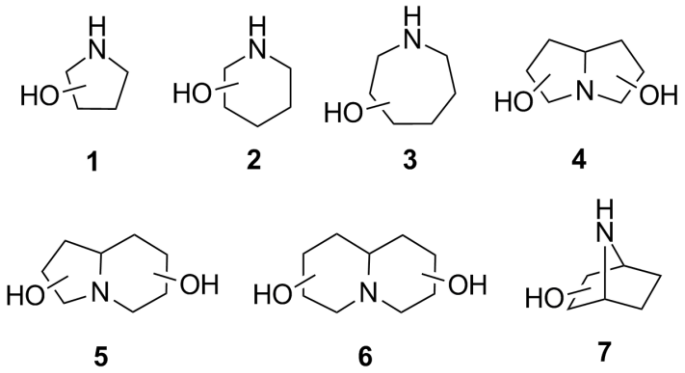


Figura 6 Clases estructurales de iminoazúcares naturales y sintéticos **1**: pirrolidinas; **2**: piperidinas; **3**: azepanos; **4**: pirrolizidinas; **5**: indolizidinas; **6**: quinolizidinas; **7**: notropanos.

Debido a su analogía estructural con los carbohidratos naturales, los iminoazúcares pueden actuar como glicomiméticos modulando o inhibiendo los enzimas que procesan los carbohidratos como glicosidasas y glicosiltransferasas o interfiriendo con la función de otras proteínas de reconocimiento molecular que involucran carbohidratos.[38]

Las glicosidasas son enzimas responsables de la hidrólisis de enlaces glicosídicos de oligosacáridos y glicoconjugados que están involucradas en un amplio rango de procesos biológicos cruciales como digestión intestinal, procesamiento post-translacional de glicoproteínas, sistemas de control de calidad en el retículo endoplasmático y los mecanismos de degradación asociada y catabolismo lisosomal de glicoconjugados.

Una amplia variedad de procesos que están relacionados con el reconocimiento y la comunicación celular, tanto fisiológicos como la regulación de la conectividad tisular y la respuesta inmune como patológicos, como infección viral y bacteriana, dependen del estado de glicosilación de proteínas.

Por estos motivos los inhibidores de glicosidasas son compuestos muy atractivos en química médica. Por un lado sirven como herramienta biológica para el estudio de las funciones biológicas de los oligosacáridos y para la comprensión de patologías en las que intervienen carbohidratos. Por otra parte tienen un potencial terapéutico para el tratamiento de una gran variedad de enfermedades como diabetes de tipo 2, infecciones virales, cáncer, desórdenes de almacenamiento lisosomal, entre otras.[39]

Como ya se ha mencionado la principal actividad biológica de los iminociclitoles es la potente inhibición de glicosidasas. Esta actividad se justifica por su similitud con el estado de transición de la reacción de hidrólisis enzimática de carbohidratos (Figura 7).[40,41]

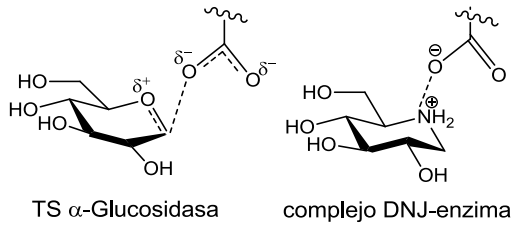


Figura 7 Semejanza entre estado de transición de α-glucosidasa con glucosa y deoxinojirimicina (DNJ).

El átomo de nitrógeno del anillo, ya que está protonado a pH fisiológico, es capaz de mimetizar el ión oxonio generado durante la hidrólisis. Esta analogía con el estado de transición de la reacción de hidrólisis enzimática es tanto mayor cuanto mas semejante sea la configuración del iminoazúcar a la del monosacárido que es sustrato natural de cada glicosidasa y, en teoría, mayor será su capacidad de inhibición de esa enzima.

Otra importante propiedad biológica de los iminociclitoles es la capacidad de actuar como chaperonas químicas,[42] o sea, promover el correcto plegamiento y estabilización de proteínas mutantes cuya inactivación es la causa de diversas enfermedades y desórdenes metabólicos genéticos.[43] Algunos trastornos lisosomales hereditarios, como la enfermedad de Gaucher, la enfermedad de Fabry, la gangliosidosis GM1 y otras enfermedades debidas a mutaciones de

proteínas, como la fibrosis quística, pueden ser tratados con iminociclitoles que actúan como chaperonas farmacológicas.

Las 1,4-Dideoxyi-1,4-imino-D- y -L-arabinitol (DAB and LAB, Figura 8) son dos pirrolidinas polihidroxiladas, respectivamente natural y sintética, que poseen una amplia actividad biológica.
Ambos enantiómeros son potenciales inhibidores de glicosidasas gracias a su semejanza estructural con azúcares de cinco miembros (y sus respectivos glicosil-cationes). De hecho estos dos compuestos han demostrado inhibir varias glicosidasas, cada uno con su perfil de especificidad.

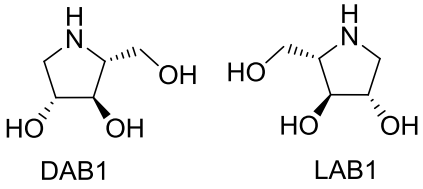


Figura 8 Enantiómeros de 1,4-dideoxi-1,4-imino-arabinitol.

El 1,4-Dideoxyi-1,4-imino-D-arabinitol (DAB) ha sido aislado de diferentes plantas[44-46] así como de esponjas marinas[47] y ha sido descrito como potente inhibidor de varias α-glucosidasas, de Golgi α-mannosidasa I y II y de trehalasa.[48-51] Otra propiedad importante de DAB es la eficaz inhibición de la glicógeno fosforilasa y el consecuente efecto antihiperglucémico in vivo.[52-55] Esta última enzima es responsable de la degradación del glucógeno a glucosa y de la regulación de la glucogenolisis en hígado y músculos. Su inhibición puede tener efecto antihiperglucémico y por eso es considerada una diana de fármacos antidiabéticos.[56]
El enantiómero sintético LAB, por otra parte, es un potente inhibidor no competitivo de una serie de α-glucosidasas (diferentes de la de levadura), de disacaridasas intestinales en ratas, especialmente isomaltasa,[57] y moderado inhibidor de α-mannosidasa de Golgi[50] y de α-L-ramnosidasa[58], mientras no se ha detectado actividad frente a enzimas que degradan de glucógeno.
Adicionalmente los dos compuestos han sido ensayado como potenciales inhibidores de la replicación del virus HIV y, en particular, LAB ha resultado ser un potente inhibidor del efecto citopático en linfocitos T infectados por el virus.[59]
En esta tesis se ha desarrollado una estrategia quimioenzimática para la síntesis de DAB, LAB y de una colección de sus derivados 2-aminometílicos con potencial actividad inhibidora de glicosidasas.

2) OBJETIVOS:

El objetivo principal de esta tesis ha sido la aplicación de aldolasas dependientes de dihidroxiacetona y dihidroxiacetona fosfato a la síntesis de compuestos quirales bioactivos. Este objetivo principal se ha dividido en cuatro objetivos específicos:

1) La D-fructosa 6-fosfato aldolasa (FSA) es un biocatalizador novedoso que tiene la ventaja de utilizar sustratos dadores no fosforilados. Los objetivos principales de este apartado han sido:
- Explorar las potencialidades sintéticas de la FSA ensayando nuevas reacciones de adición aldólica.
- Evaluar su estereoselectividad y su tolerancia hacia sustratos no naturales y estudiar su reactividad respecto a los aldehídos y los dadores utilizados.
- Demostrar su utilidad como biocatalizador para la síntesis alternativa de complejos compuestos quirales polihidroxilados (**Sección 3.1**).

2) Sintetizar quimioenzimáticamente dos pirrolidinas polihidroxiladas con importantes propiedades biológicas (1,4-dideoxi-1,4-imino-D- y -L-arabinitol (DAB y LAB)), con la finalidad de introducir nuevos grupos funcionales que puedan modular sus propiedades inhibitorias frente a glicosidasas. Ambos compuestos serán convenientemente protegidos y modificados para instalar nuevos grupos funcionales. Con este objetivo se desarrollará una estrategia de síntesis en cascada, con el menor número posible de etapas intermedias, para obtener nuevos derivados 2-aminometílicos de DAB y LAB con nueva actividad biológica potencial (**Sección 3.2**).

3) En estudios precedentes en nuestro grupo, se ha obtenido una colección de pirrolizidinas polihidroxiladas utilizando aldolasas dependientes de dihidroxiacetona fosfato (RhuA y FucA) para catalizar la adición aldólica entre DHAP y *N*-Cbz-pirrolidincarbaldehídos.
A este respecto, otro objetivo es explorar una estrategia quimioenzimática de síntesis en cascada para la preparación de nuevas pirrolizidinas polihidroxiladas derivadas de los mismos intermedios pirrolidínicos utilizados en Sección 3.2. Con esta finalidad se estudiará la adición aldólica de DHAP y DHA a (2*S*,3*R*,4*R*)- y (2*R*,3*S*,4*S*)-3,4-dihidroxi-2-carbaldehídopirrolidinas catalizadas por diferentes aldolasas dependientes de DHAP y DHA (**Sección 3.3**).

4) Investigar las propiedades inhibitorias preliminares de los derivados 2-aminometílicos de DAB y LAB obtenidos a lo largo de esta tesis. Los compuestos serán ensayados como inhibidores frente a glicosidasas comerciales con la finalidad de individuar potenciales dianas terapéuticas (**Sección 3.4**).

3) RESULTADOS

3.1 D-FRUCTOSA-6-FOSFATO ALDOLASA EN SÍNTESIS ORGÁNICA: PREPARACIÓN QUIMIOENZIMÁTICA DE COMPUESTOS POLIHIDROXILADOS, CARBOHIDRATOS E IMINOCICLITOLES

En esta sección se describen nuevas adiciónes aldólicas estereoselectivas de dihidroxiacetona (DHA) e hidroxiacetona (HA) a diferentes aldehídos catalizadas por la enzima D-fructosa-6-fosfato aldolasa (FSA), biocatalizador muy prometedor con la ventaja de utilizar sustratos dadores no fosforilados, como se ha descrito en la introducción. Asimismo, se describe una metodología quimioenzimática para la obtención de iminoazúcares, carbohidratos y otros compuestos quirales polihidroxilados.

La D-fructosa-6-fosfato aldolasa (FSA) se ha ensayado como catalizador para las reacciones de adición aldólica de DHA y HA con *N*-benciloxicarbonilaminoaldehídos, preparados por oxidación con IBX a partir de los correspondientes *N*-benciloxicarbonilaminoalcoholes.

Los aldehídos ensayados como aceptores han sido (*S*)-3-(*N*-Cbz)-amino-2-hidroxipropanal **5-(*S*)** y su enantiómero **5-(*R*)**, *N*-Cbz-glicinal **6**, (*S*) o (*R*) *N*-Cbz-alaninal **7-(*S*)** y **7-(*R*)** (Figura 1).

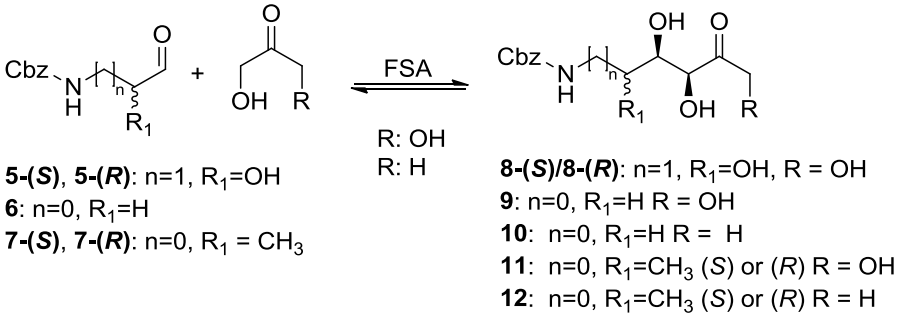


Figura 1 *N*-Cbz-aminoaldehídos ensayados como aceptores.

Todos los aldehídos, menos el (*S*) y (*R*)-*N*-Cbz-alaninal, fueron tolerados como sustratos aceptores con completa estereoselectividad, siendo la reactividad de FSA mayor cuando el dador es HA.

Los aductos aldólicos obtenidos han sido sometido a hidrogenolísis en presencia de Pd/C que mediante desprotección y concomitante aminación reductora han proporcionado los iminociclitoles 1-deoxinojirimicina (DNJ) (**1**), 1-deoximannojirimicina (DMJ) (**2**), 1,4-dideoxi-1,4-imino-D-arabinitol (DAB) (**3**), 1,4,5-trideoxi-1,4-imino-D-arabinitol (5-DDAB) (**4**) (Figura 2).

La 1-deoxinojirimicina (DNJ) (**1**), 1-deoximannojirimicina (DMJ) (**2**) también fueron tratados con butanal o 2-benciloxiethanal e H_2 (50 psi) en presencia de Pd/C generando los derivados *N*-alquilados **1/2a-b** (Figura 2).

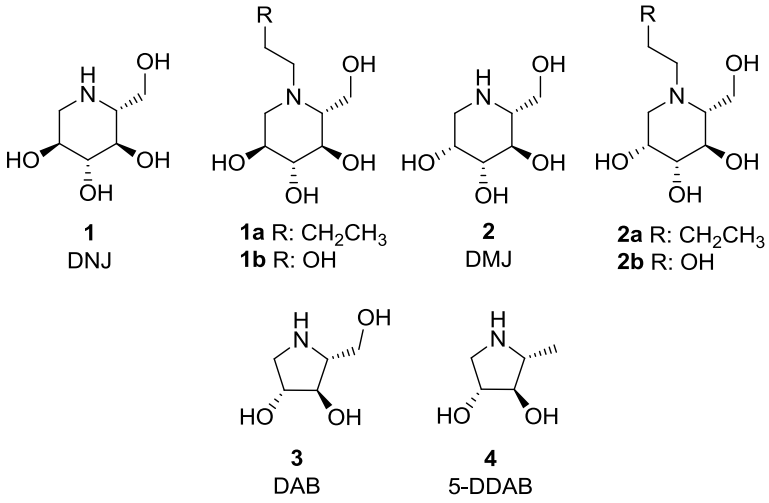


Figura 2 Iminociclitoles obtenidos quimioenzimaticamente.

Los aldehídos 2-benciloxietanal, 2-feniletanal, glicolaldehído (2-hidroxietanal) y 2-mercaptoetanal fueron también ensayados como aceptores para reacciones aldólicas con DHA y HA catalizadas por FSA. El 2-benciloxietanal y 2-feniletanal proporcionaron los correspondientes aductos aldólicos con conversiones que dependieron del sustrato dador. La desprotección del grupo bencilo por hidrogenolísis de 2-benciloxietanal con HA proporcionó el deoxiazúcar 1-deoxi-D-xilulosa (**24**) (Figura 3). Con el 2-mercaptoetanal, utilizado como dímero tiohemiacetálico hidrolizado *in situ* a 40ºC, no se observó ningún producto en las condiciones de reacciones ensayadas.

La reacción de DHA con glicolaldehído, utilizado también como dímero hemiacetálico, no generó D-xilulosa sino el producto de autoadición de glicolaldehído, D-treosa como ya fue observado en nuestro en nuestro grupo en estudios anteriores.[32]

Por otra parte, la reacción de HA con glicolaldehído fue completa en 24h generando estereoselectivamente 1-deoxi-D-xilulosa (**24**).

Adicionalmente, se descubrió que la FSA puede tolerar azúcares sencillos como sustratos aceptores, utilizando HA como dador. Por ejemplo, la D-treosa, que en solución acuosa está presente parcialmente en forma aldehídica, fue sustrato aceptor para la adición aldólica de HA catalizada por FSA, generando esteroselectivamente el azúcar 1-deoxi-D-*ido*-hept-2-ulosa (**28**) (Figura 3).

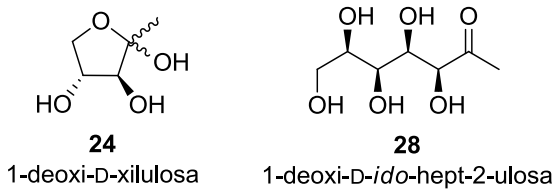


Figura 3 Carbohidratos obtenidos quimioenzimaticamente.

3.2 SÍNTESIS QUIMIOENZIMÁTICA EN CASCADA DE DAB, LAB Y DERIVADOS 2-AMINOMETILICOS

En esta sección se ha desarrollado una metodología quimoenzimática para la síntesis de derivados 2-aminometílicos de 1,4-dideoxi-1,4-imino-D-arabinitol (DAB, **1**) y su enantiómero (LAB, **2**) con aminas aromáticas, aminoalcoholes, aminoácidos y el iminociclitol fagomina.

En primer lugar se ha optimizado la obtención de los intermedios *N*-Cbz-polihidroxipirrolidinicos **7** y **8** según la metodología descrita en Figura 1.

Los dos compuestos han sido obtenidos mediante reacción aldólica entre *N*-Cbz-glicinal y la dihidroxiacetona catalizada por las enzimas D-fructosa-6-fosfato-aldolasa (FSA) y L-ramnulosa-1-fosfato-aldolasa (RhuA) respectivamente, subsiguiente hidrogenación catalítica y protección del nitrógeno endocíclico con grupo benciloxicarbonil (Cbz) (Figura 1).

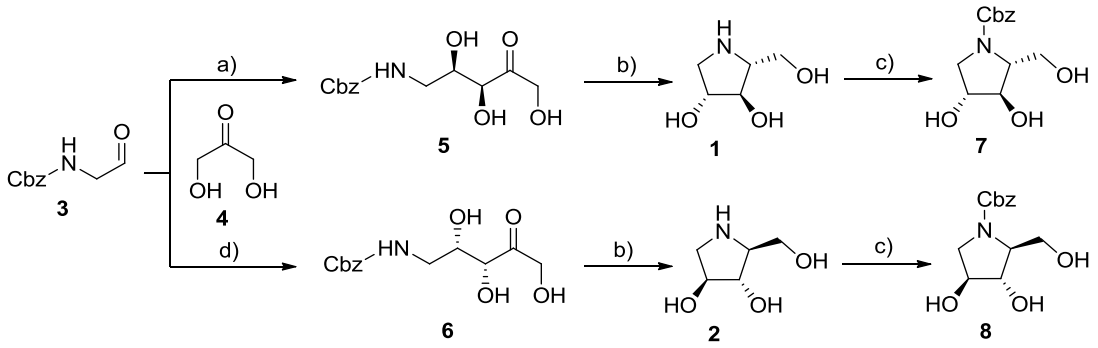


Figura 1 Síntesis quimioenzimática de *N*-Cbz-derivados de DAB y LAB. Reactivos y condiciones: a) DHA, D-fructosa-6-fosfato aldolasa de *E. coli* mutante Ala165Gly b) H_2 (50 psi) Pd/C, c) cloroformiato de bencilo, dioxano/agua 1:1, d) DHA, L-ramnulosa-1- fosfato aldolasa de *E. coli*, 200 mM buffer bórico.

La estrategia de síntesis de los derivados 2-aminometílicos consta de una oxidación suave y selectiva con IBX los intermedios **7** y **8** y subsiguiente aminación reductora con la amina y cianoborohidruro de sodio en medio acido (pH 5). La última etapa es la desprotección del grupo Cbz por hidrogenación catalítica (Figura 2).

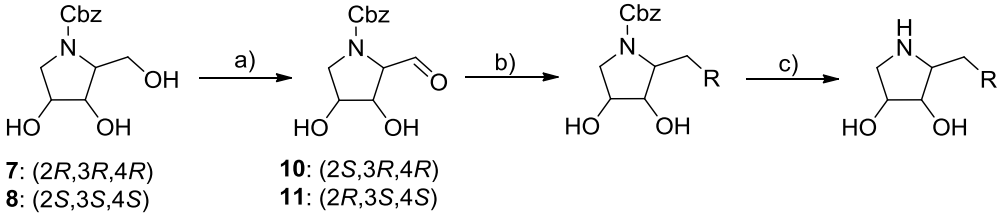


Figura 2 Estrategia de síntesis para la preparación de derivados 2-aminometílicos de DAB y LAB. Reactivos y condiciones: a) ácido 2-yodoxibenzoico, AcOEt reflujo, b) amina R-NH_2, CH_3COOH, $NaBH_3CN$, c) H_2 (22 psi) Pd/C.

La metodología ha sido ensayada con los siguientes reactivos amínicos:

- Aminas aromáticas: benzilamina, anilina, 3-aminoquinolina, *N*-metil-1,2-fenilendiamina, 4-cloroanilina, *p*-anisidina, 3,3-difenilpropilamina, isoindolina y 2-aminobenzimidazol.
- Aminoalcoholes: propanolamina, etanolamina, (*R*)-2-amino-1-pentanol, (*R*)-2-amino-3-metil-1-butanol, (*S*)-2-amino-1-butanol, (*S*)-2-amino-1-propanol, 2-amino-1,3-propanodiol, 2-piperidinetanol y 2-piperidinmetanol.
- Derivados de aminoácidos: glicinamida, L-alaninamida, L-argininamida, L-valinamida, L-leucinamida, L-fenilalaninamida, éster metílico de la L-fenilalanina, L-prolinamida, L-tirosinamida, L-treoninamida, L-serinamida y éster *O*-*tert*-butílico de la L-aspartamida.
- Iminociclitol: fagomina.

Todas las reacciones proporcionaron los intermedios 2-aminometílicos *N*-protegidos con benciloxicarbonilo, a excepción de las aminas: 4-cloroanilina, *p*-anisidina, 3,3-difenilpropilamina, 2-aminobenzimidazol, isoindolina, 2-piperidinetanol, 2-piperidinmetanol, L-tirosinamida, L-treoninamida y L-serinamida.

En el caso de derivados de aminoácidos, las desprotecciones finales del grupo *N*-benciloxicarbonilo llevaron a la formación de compuestos inéditos con dos anillos, iminociclitol y 2-oxo-piperazina, fusionados (Figura 3). En algunos casos la reacción fue espontánea y en otros necesitó calentamiento a diferentes temperaturas en agua.

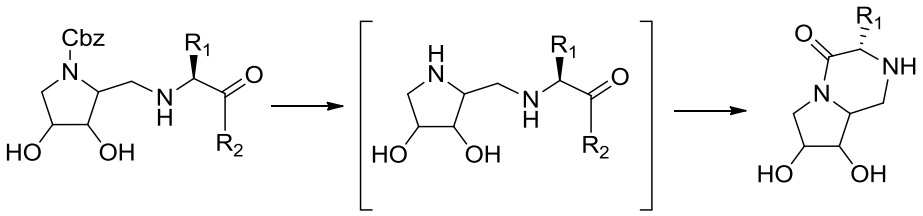


Figura 3 Formación anillo 2-oxo-piperazínico fusionado.

Todos los productos obtenidos son potenciales inhibidores de glicosidasas y glicosiltransferasas y sus propiedades biológicas han sido ensayadas como se describe en la Sección 3.4.

3.3 SINTESIS QUIMIOENZIMÁTICA DE PIRROLIZIDINAS POLIHIDROXILADAS ESTEREOISOMEROS DE LA CASUARINA:

La casuarina es un alcaloide pirrolizidínico natural con amplia actividad biológica.[60] Es un potente inhibidor de α-glucosidasa y amiloglucosidasa de *Aspergillus niger*.[60,61] Sus estereoisómeros, algunos de los cuales han sido aislados de plantas, y su 6-*O*-α-D-glucopiranosido también posen actividad inhibidora de glicosidasas.[61-63]

Debido a su estructura altamente oxigenada y a la presencia de seis estereocentros la caracterización de la casuarina y de algunos isómeros ha sido objeto de varios estudios.[64-67]

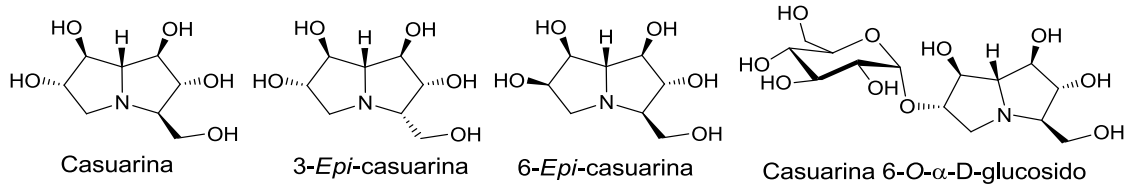


Figura 1 Casuarinas de origen natural

En esta sección se ha puesto a punto la preparación de isómeros de casuarina mediante metodología quimoenzimática.

Desde el punto de vista retrosintético, la estructura de las casuarinas (**a**) puede ser obtenida por aminación reductora intramolecular entre el carbonilo y la amina segundaria del compuesto **b** (Figura 2). El compuesto **b** puede, a su vez, ser derivado de los *N*-Cbz-pirrolidincarbaldehídos **c** por adición aldólica de dihidroxiacetona o dihidroxiacetona fosfato. Los aldehídos **c** se obtienen de las pirrolidinas polihidroxiladas 1,4-dideoxi-1,4-imino-D- y L-arabinitol (DAB y LAB) cuya preparación ha sido descrita en la Sección 3.2.

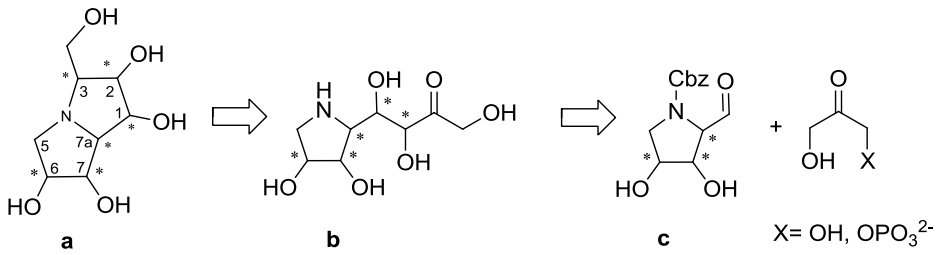


Figura 2 Análisis retrosintética

En la figura 3 se ilustra la estrategia de síntesis. La primera etapa es la oxidación con ácido 2-yodoxibenzoico (IBX) del hidroxilo primario de los iminociclitoles DAB y LAB en los cuales la amina secundaria es convenientemente protegida con el grupo carbobenciloxi (**1** y **2**).

Los aldehídos **3** y **4** obtenidos se han sometido a reacciones aldólicas enzimáticas en cascada.

Se han ensayado las adiciones aldólicas de DHAP utilizando L-fuculosa-6-fosfato aldolasa (FucA) wild type, mutantes de L-fuculosa-6-fosfato aldolasa Phe131Ala, Phe131Ala/Phe206Ala, Phe131Ser, Phe131Glu y L-ramnulosa-1-fosfato aldolasa wild type.

También se ha ensayado la adición aldólica de DHA a los mismos intermedios utilizando L-ramnulosa-1-fosfato aldolasa wt en presencia de buffer bórico y D-fructosa-6-fosfato aldolasa (FSA) mutante Ala165Gly.

La mutante FucA Phe131Ala proporcionó los compuestos **5** y **6** tras desfosforilación con fosfatasa acida, mientras que no se detectó aducto utilizando otra aldolasa en las condiciones de reacción ensayadas.

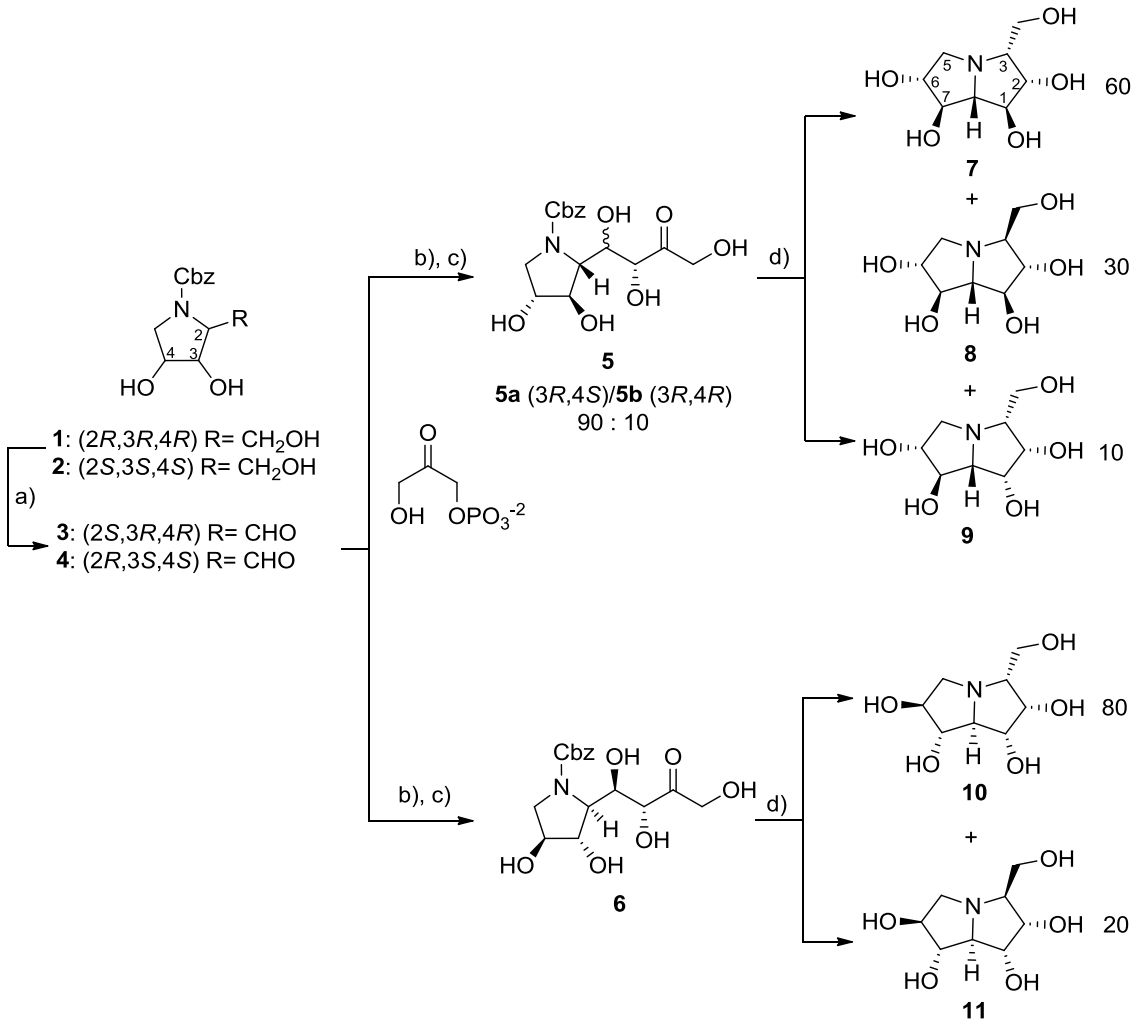


Figura 2: Sintesis quimoenzimática de cuatro estereoisómeros de la casuarina. Reactivos y condiciones: a) IBX, AcOEt reflujo, 4h, b) DHAP, L-fuculosa-6-fosfato aldolasa de *E. coli* mutante Phe131Ala, DMF:H_2O 1:4, 5 mM aq KCl, 50 mM aq TEA, 4ºC, 24h. c) fosfatasa acida d) H_2 (50 psi) Pd/C

Finalmente la desprotección del grupo amino y concomitante aminación reductora intramolecular por hidrogenación catalítica proporcionó un crudo que, tras análisis por RMN, resultò ser la mezcla de estereoisomersos de la casuarina: **7**,**8,9** a partir del aldehído **3** en proporción 6:3:1 y **10**,**11** en proporción 4:1 a partir del aldehído **4** (Scheme 5).

Con estos resultados se puede concluir que la diferente estereoquímica de los dos compuestos **5** y **6** fue determinada por la enzima y por el sustrato. En el caso del aldehído **4** (LAB-carbaldehído con configuración 2*R*) la estereoquímica *anti* sigue la del producto natural de la enzima (L-fuculoso-1-fosfato). En cambio en el caso del aldehído **3** derivado de DAB (configuración 2*S*) la configuración de los dos nuevos centros quirales fue *syn* (90%) y *anti* (10%). Por otra parte la formación de diasterómeros epímeros en C-3 indica que la hidrogenación catalítica no fue estereoselectiva.

Los productos fueron separados con éxito por cromatografía de intercambio cationico y fueron caracterizados por RMN, a excepción de **9** que no fue detectado tras la purificación.

El producto mayoritario (**7**), derivado de DAB-carbaldehído, resultó tener configuración absoluta 1*S*,2*S*,3*R*,6*R*,7*R*,7a*S* y el producto minoritario (**8**), su epímero en C3, con configuración 1*S*,2*S*,3*S*,6*R*,7*R*,7a*S*, correspondientes a los enantiómeros de la 3-*epi*-casuarina y casuarina, respectivamente.

Asimismo, a partir del derivado LAB-carbaldehído (**4**) se obtuvieron el compuesto mayoritario (**10**) con configuración 1*R*,2*S*,3*R*,6*S*,7*S*,7a*R*, que coincide con la 2-*epi*-casuarina y el producto minoritario (**11**) con configuración 1*R*,2*S*,3*S*,6*S*,7*S*,7a*R* que corresponde a la 2,3-*epi*-casuarina.

Estos compuestos tienen también potencial actividad biológica como inhibidores de glicosidasas, especialmente sacaridasas intestinales.

3.4 ENSAYOS DE INHIBICIÓN ENZIMATICA DE DERIVADOS 2-AMINOMETILICOS DE DABY LAB

En esta Sección se han investigado las propiedades inhibitorias preliminares de los derivados 2-aminometílicos de 1,4-dideoxi-1,4-imino-D- y -L-arabinitol obtenidos a lo largo de esta tesis y se ha comparado el perfil de inhibición con el de los compuestos originales, DAB y LAB. Los compuestos han sido ensayados como inhibidores frente a las siguientes glicosidasas comerciales:

- α-D-glucosidasa de levadura
- α-D-glucosidasa de arroz
- β-D-glucosidasa de almendras dulces
- β-D-galactosidas de higado bovino
- α-L-ramnosidasa de *Penicillium decumbens*
- α-D-mannosidasa de alubia negra *Genus canavalia*
- α-L-fucosidasa de riñon bovino

Muchos de los nuevos derivados son activos frente a glicosidasas comerciales con un perfil de inhibición que difiere considerablemente de las de los compuestos originales DAB y LAB. Los derivados 2-aminometílaromáticos de LAB son desde moderados a buenos inhibidores de α-L-ramnosidasa. Los derivados con aminoalcoholes, aminoácidos y oxopiperazinas son inhibidores selectivos de α-D-glucosidasas.

4) CONCLUSIONES:

Las conclusiones generales, correspondientes a los objetivos tipificados en el apartado **OBJETIVOS** se resumen a continuación:

1) La D-fructosa 6-fosfato aldolasa (FSA) ha sido ensayada como biocatalizador para la adición aldólica de DHA y HA a una variedad de aldehídos.
Con respecto a la selectividad se puede concluir que la FSA tolera una amplia variedad de aceptores no-naturales y posee una excelente estereoselectividad independientemente del aldehído utilizado. Por otra parte ha sido observado que la reactividad depende fuertemente del sustrato dador. Se observó que la HA fue el mejor sustrato dador, mientras que la reactividad de la DHA dependió del aldehído aceptor.
En el curso de la investigación se descubrió que la FSA puede tolera azúcares sencillos como sustrato aceptor pero utilizando HA como dador. Lo cual abre nuevas posibilidades sintéticas para la preparación de importantes carbohidratos complejos empleando aldolasas.
La FSA se ha demostrado un biocatalizador robusto y altamente estereoselectivo para estrategias alternativas de síntesis de compuestos orgánicos biológicamente relevantes con la remarcable ventaja de utilizar dadores no fosforilados, prerrogativa inédita entre las aldolasas conocidas dependientes de DHAP.
Por lo tanto ha sido descrita estrategia quimioenzimática de reacciones en cascada para la síntesis de compuestos polihidroxilados, azúcares e iminociclitoles.

2) Se ha desarrollado una eficiente metodología quimioenzimática para la síntesis de derivados 2-aminometílicos de 1,4-dideoxi-1,4-imino-D-arabinitol (DAB) y su enantiómero (LAB). Se ha optimizado la obtención de los intermedios *N*-Cbz- polihidroxipirrolidinicos.
Tras la oxidación suave y selectiva con IBX de estos intermedios, una etapa de aminación reductora con aminas aromáticas, aminoalcoholes y aminoácidos ofrece fácil acceso a una variedad de nuevos derivados 2-aminometílicos. Los correspondientes derivados con aminoácidos llevaron a la formación de compuestos con dos anillos fusionados de iminociclitol y piperazina, inéditos hasta el momento.
Además, se examinó una metodología alternativa para generar *N*-Cbz-imínociclitoles que proporcionó 1,4-dideoxy-1,4-imino-L-xilitol y podría abrir nuevas posibilidades para la obtención de nuevos derivados estereocomplementarios, epímeros en C2 de los derivados obtenidos a partir de DAB y LAB.

3) Se ha descrito una ruta quimioenzimática directa para la síntesis de nuevas pirrolizidinas polihidroxiladas de la familia de las casuarinas.
La metodología quimioenzimática estaba basada en las adiciones aldólicas enzimáticas, estereocontroladas multietapa de dihidroxiacetona o dihidroxiacetona fosfato a *N*-Cbz-

pirrolidincarbaldehídos derivados de 1,4-dideoxi-1,4-imino-D- y -L-arabinitol (DAB and LAB), catalizada por aldolasas dependientes de DHA o DHAP y la subsiguiente reacción *one pot* de hidrogenolisis-aminación reductora.

Las reacciones aldólicas con L-fuculosa 1-fosfato aldolasa (FucA) mutante Phe131Ala proporcionaron cuatro estereoisomeros inéditos de la casuarina. La estereoselectividad de la reacción aldólica fue alta y dependiente de las características estructurales de los aldehídos mientras la aminación reductora originó en diferentes proporciones dos diasterómeros epímericos en C3.

La adición aldólica de DHAP a (*S*)-*N*-Cbz-pirrolidincarbaldehídos derivados de DAB proporcionó el aducto aldólico *syn* que lleva a la formación de la *ent*-casuarina y *ent*-3-*epi*-casuarina. A partir del enantiómero (*R*) se produjeron los aductos aldólicos *anti/syn* en proporción 9:1 y se obtuvieron los dos isómeros 2-*epi*-casuarina, 2,3-*epi*-casuarina.

4) Se ha ensayado la actividad de inhibición enzimática frente diferentes glicosidasas comerciales de algunos derivados obtenidos en esta tesis. El perfil de inhibición de estos derivados ha sido comparado con el de los compuestos originales, 1,4-dideoxi-1,4-imino-D- y -L-arabinitol (DAB and LAB), llevando a cabo un análisis preliminar de la correlación entre actividad biológica y características estructurales de los derivados.

Resultó que las propiedades inhibitorias frente glicosidasas comerciales de los nuevos derivados de DAB y LAB difieren considerablemente de las de los compuestos originales DAB y LAB.

Los derivados 2-aminometíl aromáticos de LAB son desde moderados a buenos inhibidores de α-L-ramnosidasa. Especialmente el derivado de LAB con anilina posee mejor efecto inhibitorio del compuesto original LAB. Los derivados con aminoalcoholes, aminoácidos y oxopiperazinas son inhibidores selectivos de α-D-glucosidasas, particularmente los conjugados de DAB con (*R*)-2-amino-1-pentanol y *O-tert*-butil-L-aspartamide son selectivos para α-D-glucosidasas de levadura.

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