SYNTHESIS AND TESTING THE INTERMEDIACY OF DIKETOPIPERAZINES IN THE BIOSYNTHESIS OF ECTEINASCIDINS

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by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Russell G. Kerr, Department of Chemistry and Biochemistry, and has been approved by the members of her supervisory committee. It was submitted to the faculty of The Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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Ecteinascidin 743 is a trace secondary metabolite isolated from the marine tunicate, *Ecteinascidia turbinata*. Ecteinacidin 743 a most potent antitumor agent, is currently in Phase II clinical trials in Europe and in the USA. A cell-free extract of *Ecteinascidia turbinata* was used to investigate the biogenetic origin of the ecteinascidins. Incubation experiments with radiolabeled diketopiperazines indicated that the diketopiperazine of tyrosine is the first committed intermediate in the biosynthesis of ecteinascidins. Phenylalanine diketopiperazine was not transformed into the ecteinascidins indicating that this cyclic dipeptide is not an intermediate in the biosynthesis of ecteinascidins. The diketopiperazine of DOPA was used as a cold carrier demonstrating that the diketopiperazine of tyrosine is oxidized to DOPA diketopiperazine and then further transformed to the ecteinascidins.

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Chapter 1

Introduction

1.1 - Marine Natural Products as a Means of Drug Discovery

Nearly three quarters of the earth's surface is covered by the oceans. These vast tracts of water are home to a large variety of flora and fauna, including many phyla that have no counterparts on dry land.[1] Medicine benefits immensely from rich pharmacopoeia of our environment. According to a recent report, 57% of the 150 most prescribed drugs are either derived from natural products or are derivatives of them.[2] Marine organisms provide a quarter of about 10,000 different natural products; the rest comes from exotic leaves, barks, and other plant materials.[3] With little new ground gained in the war against the cancer and no cure for AIDS, and the looming threat of resistance to current antibiotics, modern researchers have become as intrigued by new natural pharmaceuticals as 15th century explorers were by spices.[3] Collectors search for these natural products in remote jungles, coral reefs, and depths of the ocean. Although it costs twice as much to get a specimen from the sea as it does to collect a terrestrial specimen, the effort is proving worthwhile. Until recently, this marine source remained largely untapped, especially compared with the number of natural products isolated from terrestrial plants. In 1940s SCUBA was developed and

with the development of improved underwater exploration equipment, the marine environment is finally giving up its secrets.[4] Ecologists estimate that as many as 30 million distinct species might exist worldwide; in the marine environment they are estimated to be 0.5-10 million, with the majority of them are still waiting to be discovered.[5] By1970s, the search for drugs from marine plants and animals was a vigorous endeavor that still continues today. But marine chemists say that they have only just begun to tap the chemical diversity of the sea. "In the Ocean there are an incredible number of organisms - and we don't know what their potential is" says chemist Dr. William Fenical of the Scripps Institution of Oceanography. According to Fenical "Natural products are 100 to 1000 times more likely to have biological effects than something cooked up in a laboratory". Nature has created compounds that can bind to proteins, confer chemical defenses, or function in communication; it's up to researchers to find the other uses of these compounds.[6]

In the last couple of years rapid molecular screening methods have allowed Fenical and other researchers in the marine research to cast a wider net by testing up to a thousand compounds a day.[3] And it is not only just US Pharmaceutical researchers who are looking at the oceans but also Japanese researchers also showing interest in the marine natural product research and Japan has become the world leader in the use of submersibles for collecting the deep water organisms.[3] Over recent years numerous patent applications have been filed for marine natural products, mainly for use as pharmaceuticals, food additives, cosmetics, novel enzymes and research tools for biomedical sciences.[7]

1.2 - Why Marine Organisms?

During the past 25 years, marine natural product chemists have been successful in discovering unique and new compounds with the potential biological activity. Approximately 1700 metabolites have been isolated from marine organisms from 1977 to 1985, and nearly 800 new compounds were isolated during 1987 and 1988.[8] History has now proven that marine organisms, especially invertebrates such as sponges, tunicates, and soft corals, produce secondary metabolites which are unprecedented within the terrestrial biosphere. Marine natural products, the secondary metabolites or nonprimary metabolites produced by the organisms that live in the sea, have received enormous attention for chemists and pharmacologists during the last two decades. For the first 10-12 years scientists were interested in isolating compounds with novel structures and did not show much emphasis on compounds with potential pharmaceutical activity, says marine chemist Dr. Ray Anderson, professor of chemistry and oceanography of the University of British Columbia.[9] The field of marine natural product chemistry is now becoming more sophisticated. Instead of searching for new metabolites researchers are looking for targeted compounds with pharmacologically useful activity.

The marine system is believed to be the original source of life on earth and many of the organisms in the aquatic world communicate with one another by way of signalling systems composed of primordial chemical messengers. Humans are connected through evolution to these marine organisms leading Colwell to

conclude that many of these primordial messengers from the marine environment have profound effects on human cells and tissues.[5] In addition to an evolutionary connection to humans the oceans have an enormous number and diversity of life forms. During the relatively few years that marine organisms have been mined for useful compounds, a large number of bioactive chemicals have been discovered, many of which have useful biotechnological applications either as research tools to understand other living systems or as potential drugs.[10] Based on this experience some of the most promising organisms for the discovery of future pharmaceutical drugs are believed to be marine invertebrates and microorganisms.

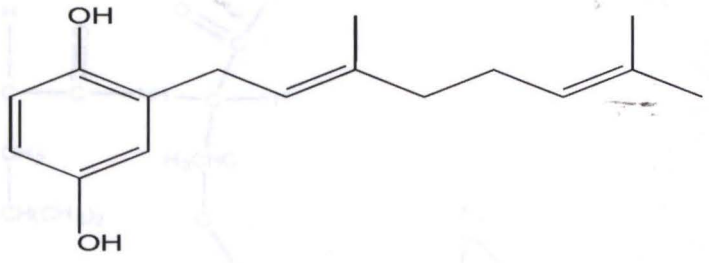
1.3 - Ascidians – Producers of Amino Acid Derived Secondary Metabolites

Many marine invertebrates are immobile, attached to the ocean floor and use highly evolved chemical compounds to attract food, block the growth of intruding neighbors or repel predators.[5] Between 1988 and mid 1992 approximately 165 new secondary metabolites have been isolated from marine ascidians.[5]

Ascidians belong to the phylum Chordata, which encompasses all vertebrate animals including mammals. Therefore, they represent the most highly evolved group of animals commonly investigated by marine natural products chemist. Members of the class Ascidiacea are commonly referred to as tunicates, because their body is covered by a cellulose type material called tunic, or as sea squirts, because many species expel streams of water through a siphon when

disturbed.[6] While the larvae have a primitive notochord, the adult ascidians are exclusively invertebrates and bear little resemblance to the other chordates. Adult ascidians are sessile filter feeders, either solitary or colonial and live preferentially in regions which are free from extensive wave shock but still receive free flowing sea water. Ascidian morphology is very diverse. Colonies of tunicates may grow upto 15 cm in length or as small as 1 cm. Colonial species grow on rocks or other hard surfaces.

Attention has been focused, more recently on ascidians because of their biologically active secondary metabolites. In 1974 Fenical isolated the first ascidian metabolite, geranyl hydroquinone (**1**) from *Aplidium* sp.[12]

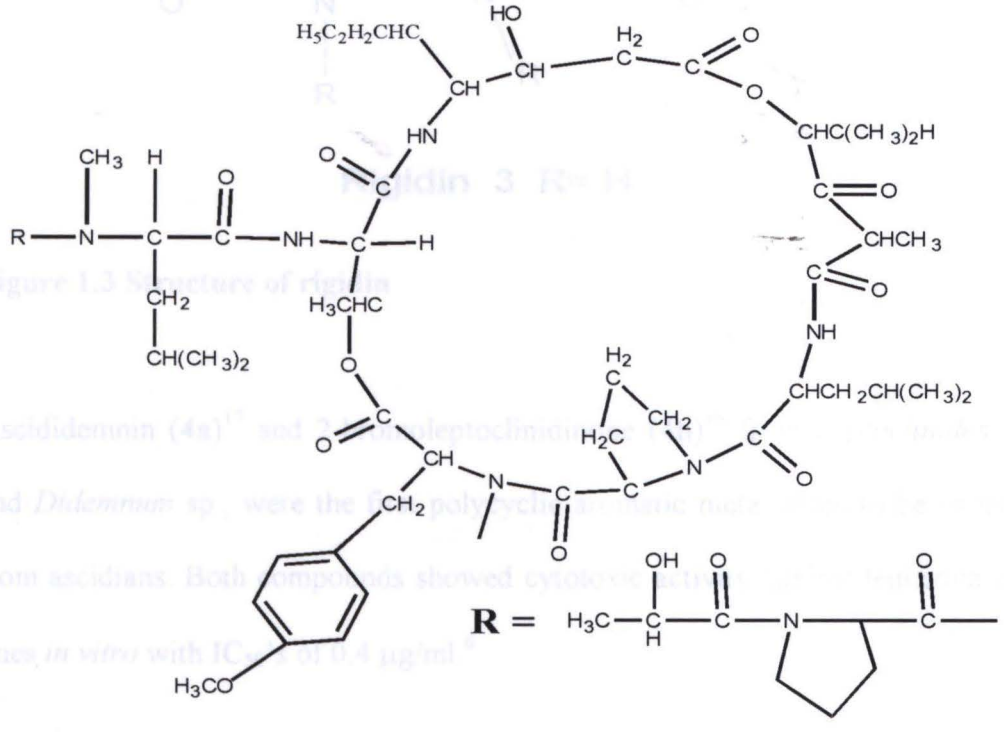


1: Geranyl hydroquinone

Figure 1.1 Structure of Geranyl hydroquinone

Geranyl hydroquinone has exhibited chemopreventive activity against some forms of leukemia and mammary carcinoma in test animals.[12] The occurrence of an anticancer compound in a marine organism coupled with the absence of neoplasms in ascidians provided a powerful incentive for study further of these organisms.[12]

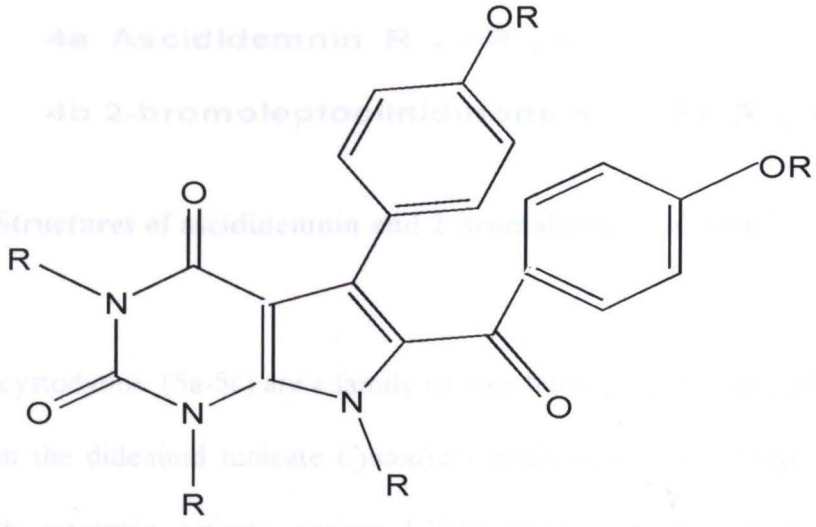
The cyclic depsipeptide didemnin B is the first marine natural product anti-cancer agent, isolated from the Caribbean tunicate *Tridemnum solidum,* to enter clinical trials.[13] Several new didemnins have been reported and their structures were determined in 1981 by Rinehart's group.[12] Didemnin B has demonstrated *in vivo* anticancer activity in mice against P388 murine leukemia and B16 melanoma. Didemnin B was tested against a number of tumors in a human tumor stem cell assay and a significant activity was observed after 1h of exposure at concentrations as low as 0.1 μg/ml in ovarian, kidney and breast cancer cells.[14]



2: Didemnin B

Figure 1.2 Structure of didemnin B

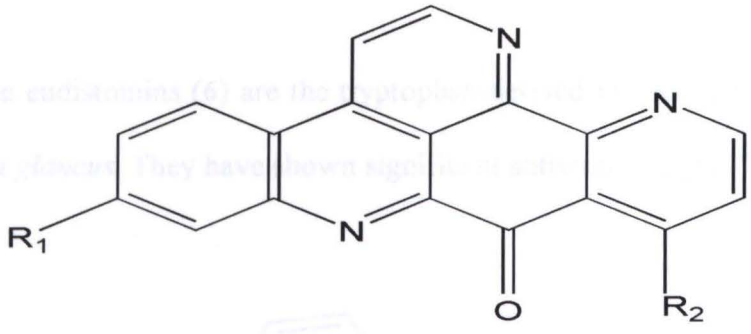
Another bioactive secondary metabolite rigidin (**3**) a novel pyrrolopyrimidine alkaloid with calmodulin antagonistic activity was isolated from the Okinawan marine tunicate *Eudistoma* cf. *rigida*.[15]



Rigidin 3 R= H

Figure 1.3 Structure of rigidin

Ascididemnin (**4a**)[17] and 2-bromoleptoclinidinone (**4b**)[16] from *Leptoclinides* sp. and *Didemnum* sp., were the first polycyclic aromatic metabolites to be isolated from ascidians. Both compounds showed cytotoxic activity against leukemia cell lines *in vitro* with IC_{50}'s of 0.4 μg/ml.[6]

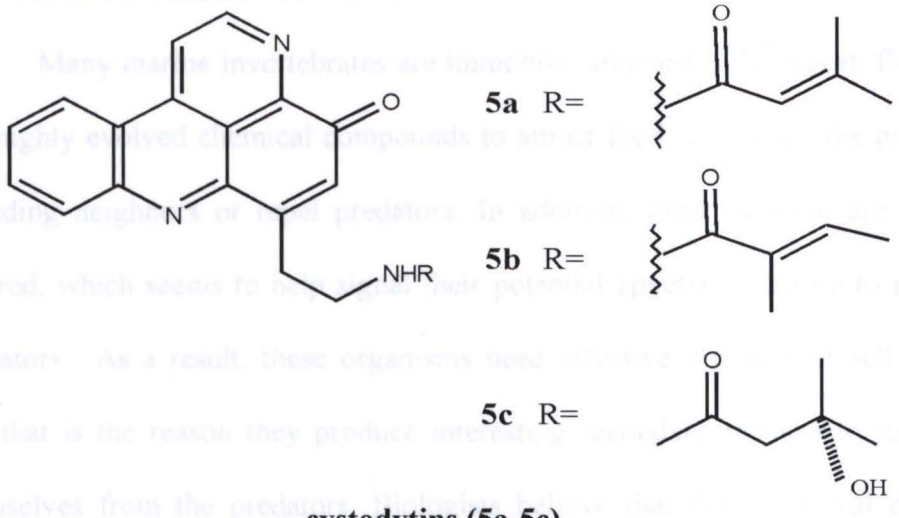


4a Ascididemnin $R_1 = R_2 = H$

4b 2-bromoleptoclinidinone $R_1 = Br, R_2 = H$

Figure 1.4 Structures of ascididemnin and 2-bromoleptoclinidinone

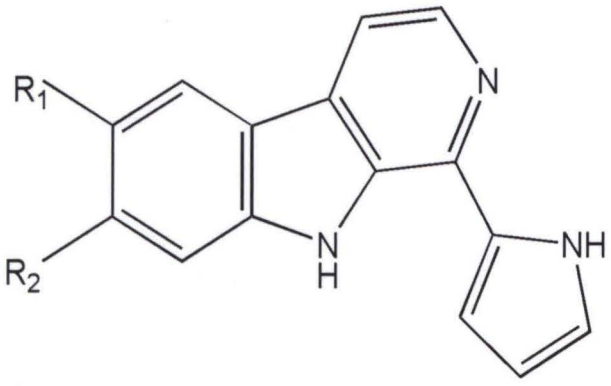
The cystodytins (5a-5c) are a family of nine tetracyclic aromatic alkaloids isolated from the didemnid tunicate *Cystodytes dellechiajei.*[17] Cystodytins A-C showed both cytotoxic activity against L1210 leukemia cells and powerful calcium-releasing activity in sarcoplasmic reticulum.



cystodytins (5a-5c)

Figure: 1.5 Structures of cystodytins

The eudistomins (**6**) are the tryptophan-derived metabolites isolated from *Eudistoma glaucus*. They have shown significant antiviral activity.[18]



$R_1 = OH$; $R_2 = Br$

6: eudistomin A

Figure 1.6 Structure of eudistomin A

1.4 - Chemical Defense Mechanism of Marine Invertebrates

Many marine invertebrates are immobile, attached to the ocean floor, and use highly evolved chemical compounds to attract food, and block the growth of intruding neighbors or repel predators. In addition, most of them are brightly colored, which seems to help signal their potential appetizing nature to potential predators. As a result, these organisms need effective methods of self-defense and that is the reason they produce interesting secondary metabolite to protect themselves from the predators. Biologists believe that these survival demands triggered the evolution of bioactive compounds.[5] Novel secondary metabolites that have been isolated from the marine invertebrates are most often assumed to

have a defensive mechanism. The sessile nature of many marine organisms has evolved a unique repertoire of chemicals used for defense, as well as communication and reproduction.[9]

Table 1.1 - Selected Marine Compounds Under Evaluation as Potential Anti-Cancer Drugs[5]

Compound	Source/Source for trials	Activity	Status
Bryostatin1	Bryozan; *Bugula neritina* **Collection**	Anti-cancer	Phase II & III clinical trials
Ecteinascidin-743	Tunicate; *Ecteinascidia turbinata* **Substantiable collection/aqua culture**	Anti-cancer; lung, breast and ovarian cancer; melanoma	Phase II clinical trials
Didemnin B	Tunicate; *Tridemnum solidum*	Anti-cancer; non-Hodgkin's lymphoma	Phase II clinical trials
Dolastatin 10	Opisthobrach mollusc; *Dolabella auricularia* **Synthesis**	Anti-cancer	Phase II clinical trials
Discodermolide	Marine sponge; *Discodermia dissoluta* **Synthesis**	Anti-cancer; immunosuppressant	Advanced pre-clinical studies
Eleutherobin	Soft coral; *Eceutherobia* sp. **Synthesis**	Anti-cancer	Pre-clinical studies
Halichondrin B	Sponge; *Halichondria okadai* **Aquaculture**	Anti-cancer	Pre-clinical studies

1.5 - Supply and Demand of Marine Secondary Metabolites

Once a useful bioactive compound is discovered, there can be a problem in obtaining the drug in sufficient yield for clinical trials. One possibility is to collect the secondary metabolite from the nature, which involves the harvesting the drug from the marine organism. In the 1940's SCUBA was developed and with the recent technology in submersible vehicles it became relatively easy to collect these organisms from the depths of the ocean. Some organisms are easy to obtain and also grow rapidly, making the natural organism a reasonable source for pre-clinical and even clinical studies. Marine secondary metabolites are often expressed in trace amounts in the source organisms. Some times there is a possibility that these secondary metabolites are not always present in the organism. Another possibility is to chemically synthesize the secondary metabolite. This approach can either be economically viable or not for large-scale production of these bioactive secondary metabolites.

Recent successes in the anticancer field have been derived from the marine invertebrates and the most advanced of these is bryostatin 1, isolated from Eastern Pacific bryozan *Bugula neritina* by G. R. Pettit of the Cancer Research Institute at Arizona State University.[19] Bryostatin 1 is an unusual anticancer compound because it is cytostatic, it inhibits the growth of tumors with very unusual and beneficial properties. Recent discovery by A. McGown's research group at the Paterson Institute for Cancer Research in Manchester, UK, indicated that bryostatin 1 improves the effectiveness of tamoxifen, a breast cancer therapeutic,

when given in combination *in vitro*.[4] The crucial problem with a compound such as bryostatin 1 is obtaining sufficient supply for testing and, if approved, for clinical use. Chemical synthesis of this drug has not yet been achieved and even it is completed it may not be the viable option for producing this drug on a large-scale. Sustainable wild collection of *B. neritina* are unlikely to be able to meet the projected demand of 50-100g/year.[4] In general, 1 g of dry *B. neritina* produces 7-8 μg of bryostatin 1. It was also found that there is a variation in the bryostatin 1 yield with location, season and genetic make-up of the bryozan.[4]

Another example is ecteinascidin-743 (Et-743), a potent anti-cancer agent isolated from marine tunicate *Ecteinascidia turbinata*.[20] Currently Et-743 is undergoing Phase II clinical trials and has proved to be active against breast, ovarian cancers.[20] Because it has a highly complex structure, there is not yet a commercially viable synthesis. For clinical trial use, 20-100 g per year would be needed; but for therapeutic use, 1-2 kg per year would be required from the harvest of as many as 20 million colonies.[5] Thus, harvesting the drug from collections of the tunicate would have a major impact on the ecosystem.

In order for marine-derived drugs to become useful in the market place, the supply problem needs to be overcome or circumvented. One possibility is to develop only those compounds for which commercially viable chemical synthesis is available. If we adopt this method then there is the real possibility of precluding the development of pharmacologically useful compounds, which possess complex structures. Another possibility could be large-scale cell culture or 'farming' of the

intact organism in aquaculture. Another possible solution is the use of genetic engineering to transfer the genes encoding the biosynthetic enzymes that produce the desired compound to microorganisms that can be in grown in huge quantities in fermentation facilities.

Marine ecosystems are enormously complex and the potential for marine natural products as drugs is still being exploited. Although marine natural products offer a potentially rich source of useful compounds, commercial development will require efficient methods of production. The structural diversity derived from marine organisms and the biological activity associated with them will ensure a continued interest in marine bioprospecting.

Chapter 2

Introduction to Ecteinascidins

2.1 - Background Information

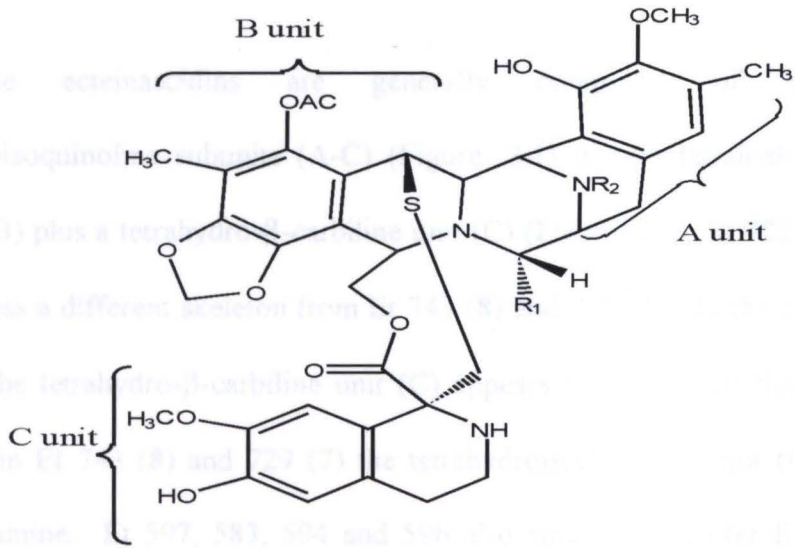
The ecteinascidins are a family of tetrahydroisoquinoline alkaloids present in trace quantities in the marine tunicate *Ecteinascidia turbinata*. A variety of ecteinascidins, ecteinascidins 729, 743, 745, 722, 759A, 759B, and 770, the tris (tetrahydroisoquinolines) with potent *in vivo* antitumor activity, have been isolated from this colonial tunicate (Figure: 2.1).[13]

Among the family of ecteinascidins, ecteinascidin 729 (Et 729) has best therapeutic ratio (*activity Vs toxicity*), yet Et 743 (**8**) is in Phase II clinical trials due to its slightly greater relative abundance ($IX10^{-4}\%$).[20] The clinical trials are being held in three European countries and in the United States. The patent for ecteinascidins is held by University of Illinois and it is licensed to Spanish based company Pharma Mar.

2.2 - Isolation and Structure Elucidation of Ecteinascidins

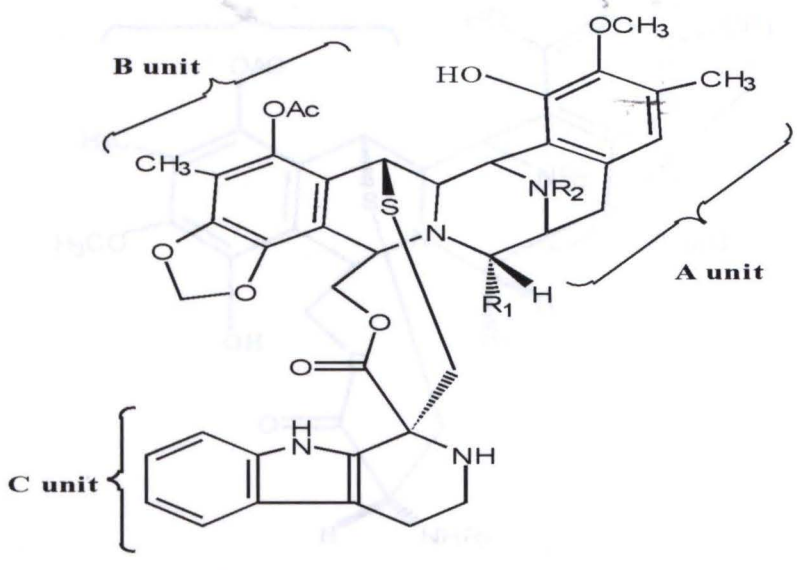
Ecteinascidins are exceedingly potent antitumor agents first isolated by Holt, from the marine tunicate *Ecteinascidia turbinata*.[21] A number of research groups had worked on the isolation of ecteinascidins, most notably the researchers

at the University of Illinois at Urbana – Champaign led by Professor Kenneth Rinehart.[21] Structures of ecteinascidins were elucidated independently by Dr. Amy Wright at Harbor Branch Oceanographic Institution, Ft. Pierce, Florida and by Dr. Rinehart at the University of Illinois.[20-22] Beginning from 1969 and continuing through 1983 many studies were conducted regarding the antitumor activity of extracts of *Ecteinascidia turbinata*, especially concerning it's activity against P-388 mouse leukemia.[12] In 1986 Rinehart's group isolated six ecteinascidins (Et 729, 743, 745, 759A, 759B, 770) (Figure: 2.1) from *Ecteinascidia turbinata*.[13] A number of bioassays were developed to assist the isolation of the ecteinascidins. Their standard isolation procedure involves the extraction with MeOH-toluene (3:1) followed by successive partitions with $CHCl_3$, EtOAc, and n-BuOH. The majority of the activity was found in $CHCl_3$ extract.[13] The key techniques employed for the isolation and characterization of ecteinascidins are centrifugal countercurrent chromatography, tissue culture bioautography and LC/FABMS.[20] The most abundant ecteinascidin, Ecteinascidin 743 showed a molecular ion at 744.2592 by HRFABMS, which agrees the molecular formula $C_{39}H_{42}N_3O_{10}S$.[20] The ^{13}C NMR spectrum confirmed the presence of 39 carbon atoms and electron spectroscopy for chemical analysis (ESCA) indicated a single sulfur atom.[20] Later in 1996 new ecteinascidins Et 597, 583, 594, and 596 were reported by Rinehart's group.[23] The presence of three tetrahydroisoquinoline units and their aromatic ring substituents were first identified by Holt *et al.*[21] and later substituent patterns were first corrected by Wright *et al.* [22] and also by Rinehart *et al.*[20]



7:	**Et 729**	**R_1 = OH,**	**R_2 = H**
8:	**Et 743**	**R_1 = OH,**	**R_2 = CH_3**
9:	**Et 745**	**R_1 = H,**	**R_2 = CH_3**
10:	**Et 759B**	**R_1 = OH,**	**R_2 = CH_3, S-oxide**
11:	**Et 770**	**R_1 = CN,**	**R_2 = CH_3**

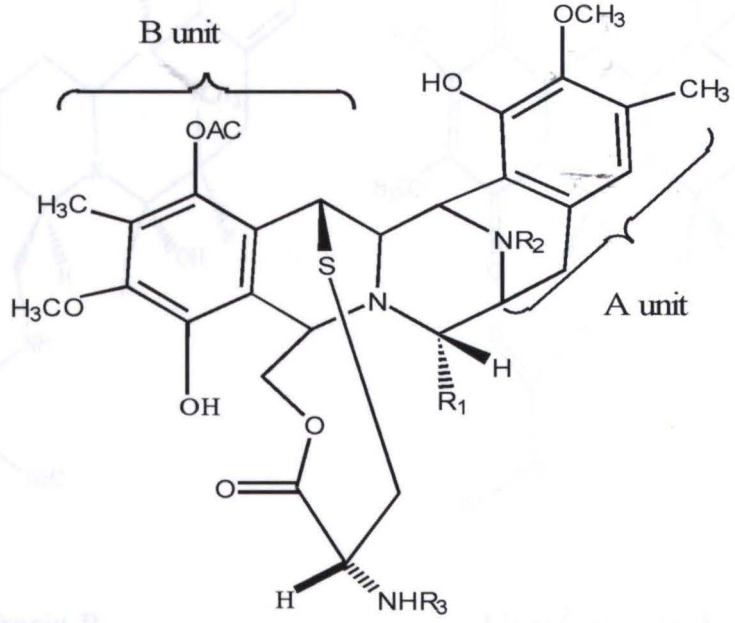
Figure 2.1: Structures of ecteinascidins 729, 743, 745, 759B and 770



12:	**Et 736**	**R_1 = OH,**	**R_2 = CH_3**
13:	**Et 722**	**R_1 = OH,**	**R_2 = H**

Figure 2.2: Structures of ecteinascidins 736 and 722

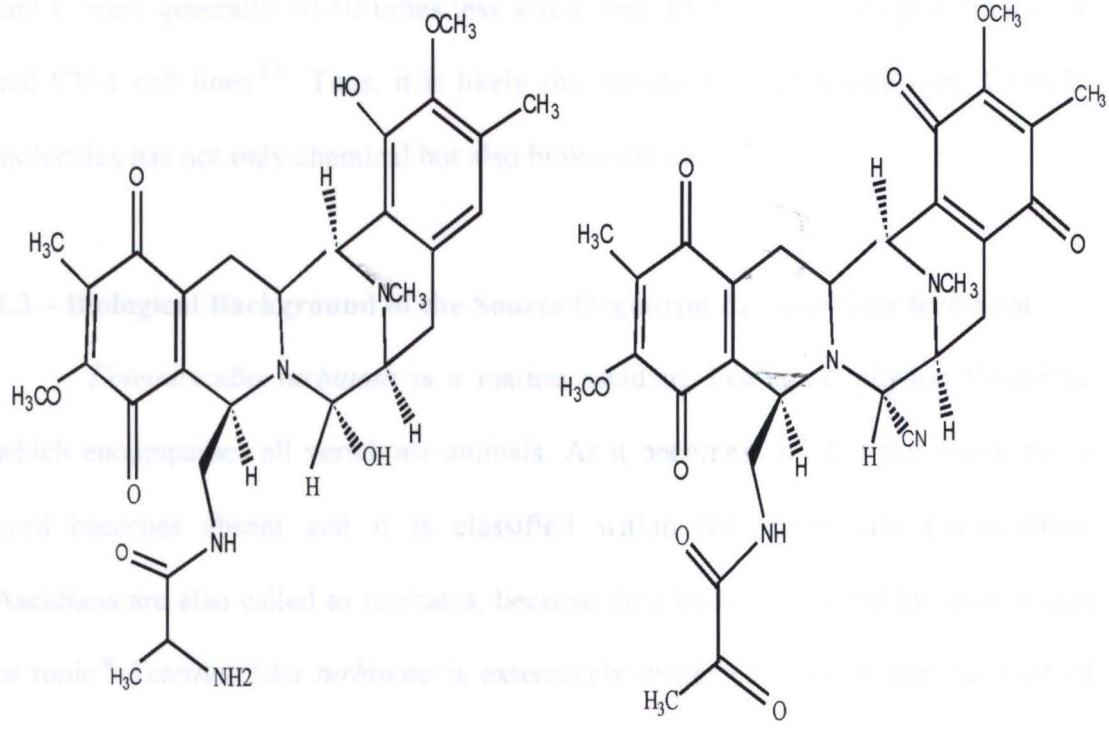
The ecteinascidins are generally composed of either three tetrahydroisoquinoline subunits (A-C) (Figure: 2.1) or two tetrahydroisoquinoline units (A, B) plus a tetrahydro-β-carbiline unit (C) (Figure: 2.2). Et 722 (**12**) and 736 (**13**) possess a different skeleton from Et 743 (**8**) and 729 (**7**). In the case of Et 722 and 736 the tetrahydro-β-carbiline unit (C) appears to be derived from tryptamine where as in Et 743 (**8**) and 729 (**7**) the tetrahydroisoquinoline unit (C) may come from dopamine. Et 597, 583, 594 and 596 also structurally differ from the other ecteinascidins. They lack the tetrahydroisoquinoline unit (C) in the southern hemisphere of the structure (Figure: 2.3).



14: Et 597 R_1 = OH, R_2 = CH_3, R_3 = H
15: Et 583 R_1 = OH, R_2 = H, R_3 = H

Figure 2.3: Structures of ecteinascidins 597 and 583

The carbon and nitrogen framework of the tetrahydroisoquinoline units (A-B) in ecteinascidins is similar to that of saframycins (**17**) and safracins (**16**), potential antitumor agents first isolated from cultured *Streptomyces lavendulae* species.[24] Ecteinascidins seems to share the biosynthetic origin of their tetrahydroisoquinoline units (A-B) with these saframycin compounds.[23] (Figure: 2.4)



16: safracin B **17: saframycin A**

Figure 2.4: Structures of safracin B and saframycin A

The tetrahydroisoquinoline or the tetrahydro-β-carboline (unit C) attached to the tetrahydroisoquinoline (unit B) by the 10-membered sulfide-containing lactone is a distinctive feature of the Et molecules, both structurally and biosynthetically.[23] Hence the most potent antitumor activity of Et's may at least in part, be attributed to the tetrahydroisoquinoline or tetrahydro-β-carboline (unit C). Saframycins, which lack the unit C, has lower efficacy than Et 743 & 729 in comparable tumor models.[23] It was reported that Et 597, 583, 594, and 596 (Figure: 2.3), which lack the aromatic unit C were generally 10-50 times less active than Et 743 and 729 against MEL 28 and CV-1 cell lines.[23] Thus, it is likely that tetrahydroisoquinoline (unit C) in Et molecules has not only chemical but also biological importance.[23]

2.3 – Biological Background of the Source Organism *Ecteinascidia turbinata*

Ecteinascidia turbinata is a marine ascidian, belong to phylum Chordata, which encompasses all vertebrate animals. As it becomes an adult the dorsal nerve cord becomes absent and it is classified within the subphylum Urochordata. Ascidians are also called as tunicates, because their body is covered by saclike case or tunic.[6] *Ecteinascidia turbinata* is extensively dispersed through out the Gulf of Mexico, the Bahamas, the Florida Keys, and the Caribbean Sea. They are filter feeders and live in shallow waters approximately 0.1 to six meters in depth.[25] They are normally found in areas possessing mangrove roots, turtle grass beds, and gorgonians, any hard substrate for the tunicate to attach.

2.4 - Biological Activity of Ecteinascidins

The cytotoxicity and antitumor activity of extracts from the Caribbean tunicate *Ecteinascidia turbinata* were first discovered in the late 1960s.[26] However, the purification of the active compounds was not completed until 1990. In 1969, Sigel *et al.* first discovered that ethanolic extracts from *Ecteinascidia turbinata* possesses *in vivo* activity against P388 murine leukemia and were also found to be powerful immunomodulators.[26] These extracts are capable of killing tumor cells *in vitro* and inhibit tumor growth *in vivo*.[26] Extracts of *Ecteinascidia turbinata* caused inhibition of mitogenic responses to splenocytes and was also able to induce splenomegaly and activation of T suppressor cells.[27] Et's activity has been reported against P388 leukemia, B16 melanoma, M5076 ovarian sarcoma.[27, 28] Et 743 can selectively alkylate guanine N2 from the DNA minor groove, and this alkylation is reversed by DNA denaturation.[29] Et 743 also interacts with the microtubule network and blocks cell cycle progression at late S/G_2 stages.[30] Thus Et 743 differs from other DNA alkylating agents that are currently in the clinical trials by both its biochemical activities and its profile of antitumor activity in preclinical models. In spite of the exceedingly potent activity of Et 743 *in vivo* against a variety of tumor models and responses in Phase I and II clinical trials, the mechanisms of antitumor activity of Et 743 have not yet been identified.[29]

2.5 - Sources of the Marine-Derived Drug Ecteinascidin 743

Ecteinascidin 743 (Et 743), a trace marine natural product with potent antiproliferative activity is currently undergoing phase II clinical trials. The current clinical trials necessitate a continuous and reliable source of this marine-derived drug. One method to obtain this drug is by collection from nature. Firstly, we have found that when careful analyses of individual colonies of the tunicate are performed, ecteinascidins are not always present. Secondly Et's are trace secondary metabolites and harvesting the drug from nature is therefore environmentally unsound and potentially problematic.

Dr. Shirley Pomponi at Harbor Branch Oceanographic Institute has estimated that for pre-clinical studies of the compound, approximately 5-10 g per year would be needed from 2.5-5 tonnes of the tunicate (or 50,000–100,000 colonies).[5] For clinical trials, 20-100 g per year would be needed, and for therapeutic use, 1-2 kg per year would be required from the harvest of as many as 20 million colonies per year.[5] Thus harvesting such a huge amount of drug would probably have a major impact on the ecosystem. So based on these estimates, a natural source for a therapeutic supply of Et 743 is clearly not realistic.

Another method to obtain this drug is by chemical synthesis. In 1996 Corey's group first synthesized the tetrahydroisoquinoline (unit C) (Figure: 2.1) of Et 743.[31] Later the total synthesis of Et 743 was successfully accomplished by Corey in mid 1996.[32] This is a complex multistep synthesis and is a very expensive method of production of Et 743. Several other groups including Akinori Kubo's group[33] at

Meiji Pharmaceutical University, Japan and Fukuyama's group[34] at University of Tokyo had worked on the partial and total synthesis of Et 743. All these methods involved multistep synthesis, which produced low yields of Et 743. Since the chemical synthesis is not economically viable, alternative methods to produce Et 743 in good yield should be approached.

2.6 - Alternative Sources for the Production of Ecteinascidin 743

Alternative sources used for the production of Et 743 are enzyme based methods and cell culture method. Enzyme based method include **biosynthetic studies,** which involves the identification of primary building blocks and advanced intermediates;[35] **enzymology**, the purification and characterization of enzymes responsible for the production of ecteinascidins and **genetics**, which involves isolation of the gene responsible to produce ecteinascidins and thereby incorporating the relevant biosynthetic genes into expression systems such as *Escherichia coli.*

A cell culture method would involve the culturing cells under a controlled environment. Tissue and cell culturing have become an attractive methods used by the natural product chemists for biosynthetic analysis. An advantage of using a cell culture technique is to determine whether an organism actually produces the secondary metabolite or a symbiotic organism produces the secondary metabolite.[36] Shirley Pomponi and co-workers at Harbor Branch Oceanographic Institution in Ft. Pierce, Florida have been successful in establishing viable replicative cell cultures of

marine sponges *Hymeniacidon heliophila*[37] and *Teichaxinella morchella*[38] and the marine tunicate *Ecteinascidia turbinata*.[39]

2.7 - Thesis Goal

The long-term purpose of the research program is to develop an enzyme based synthesis of the ecteinascidins, a family of trace antitumor agents present in the marine tunicate *Ecteinascidia turbinata*. The specific goal of this thesis project is to synthesize and test the intermediacy of the diketopiperazines of phenylalanine, tyrosine and dihydroxyphenylalanine (DOPA) as putative intermediates in the biosynthesis of ecteinascidin 743. The diketopiperazine of phenylalanine and tyrosine will be synthesized in the labeled form, and their conversion to Et 743 will be determined in a cell-free extract of *Ecteinascidia turbinata*. The diketopiperazine of DOPA will be utilized as a "cold carrier" to monitor its formation from the diketopiperazines of phenylalanine and tyrosine.

Chapter 3

Biosynthetic Analysis of the Ecteinascidins

3.1 - Biosynthetic Approach

In response to the growing number of structurally novel and biomedically promising compounds isolated from the marine organisms, investigations into biosynthetic origins of certain compounds have been initiated.[35] Many of the most promising drugs isolated from marine organisms present in trace quantities and their clinical development will be hampered by the supply problem. This supply problem can be addressed by a chemical synthesis or through a "biological" method such as aquaculture, cell culture or enzymatic means. Biosynthetic information can be used to facilitate all of the biological production methods. Biosynthetic studies include the identification of primary building blocks and the intermediates and thereby feeding them to a whole organism or enzyme extract and examination of the desired secondary metabolite produced to see if the compounds fed were used to in the formation of metabolite.[40]

3.2 - Biosynthetic Techniques

Biosynthetic experiments can be carried out using a variety of techniques. A common technique to investigate the secondary metabolite production is to conduct

the feeding experiment with a live organism (*in vivo*) or crude enzyme extract also called cell-free extract (*in vitro*).[35] The use of radiolabeled precursors or stable isotopes allows for the direct detection of precursors in the compound of interest. Radioisotopes (e.g. ^{14}C, ^{3}H) offer greatest sensitivity in detection and the radioactivity is assayed by scintillation counter.[41] Eventhough stable isotopes are not as sensitive as radioisotopes, they provide more detailed biosynthetic information, namely on the specificity of labeling, on the fate of C-H, C-C, C-O or C-N bonds and offer mechanistic information through the use of nuclear magnetic resonance (NMR) or mass spectrometry (MS).[41]

Biosynthetic experiments can be conducted either using *in vivo* or *in vitro* techniques. *In vivo* biosynthetic experiments using live organisms can be difficult to conduct, particularly with marine organisms, since it is difficult to mimic the marine environment in the laboratory. There is a possibility for the organism to undergo stress and can die during the experiment. The organism may utilize the precursor to synthesize other "less desirable" secondary metabolites more efficiently.[40] Restrictions such as lengthy incubation times, vitality of the organism, and limited uptake of certain precursors, reduce the efficiency of using the live organism for biosynthetic experiments.

In vitro biosynthetic experiments using either purified enzymes or crude enzymes (cell-free extracts) provide a convenient and rapid evaluation of biosynthetic problems.[40] In addition to being a common system in higher plants, the use of cell-free extracts has also received recognition as a valuable, biosynthetic tool

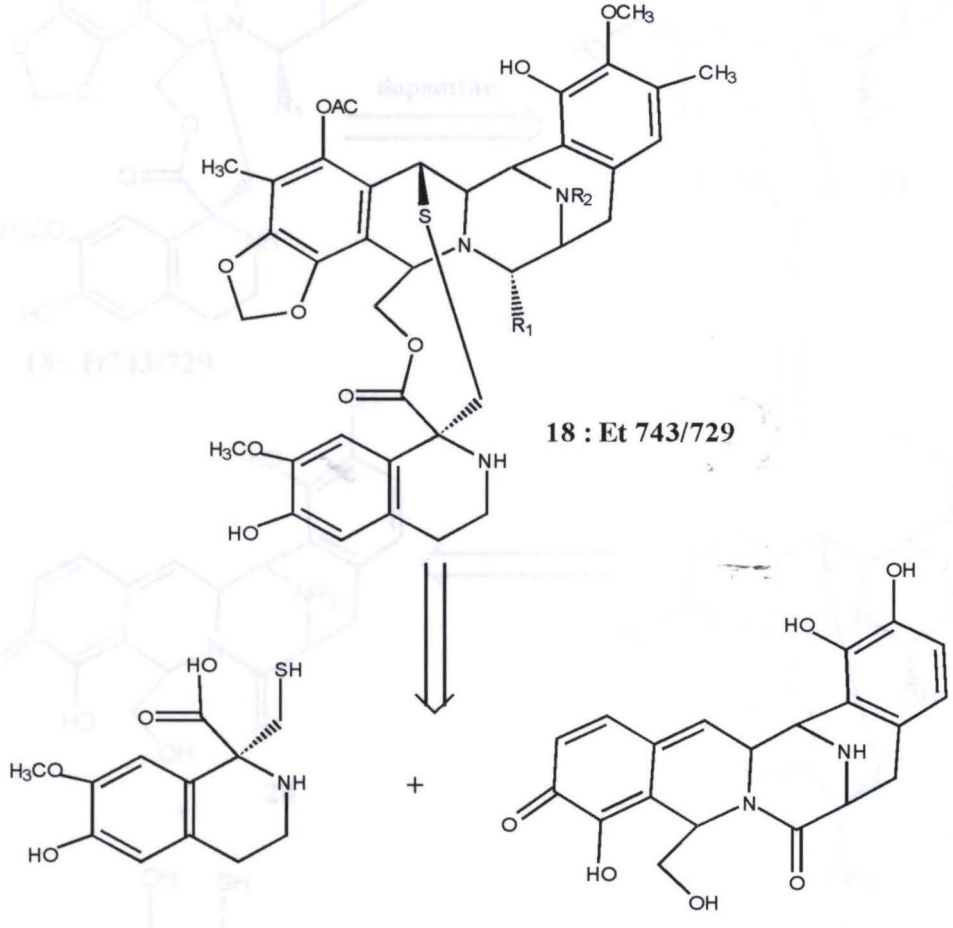
in marine invertebrates. Cell-free extracts are prepared by physically breaking open the cells of the organism and thereby releasing the cell's contents.[40] Various cofactors and protease inhibitors are often added to this extract to maintain the enzyme stability. Enzyme extracts can be stored at –80 ^{0}C thus providing a convenient research tool.[35] Since Et 743 is a trace secondary metabolite and it is not present in the tunicate all the time, biosynthetic experiments with cell-free extract are more convenient than working with live organism. Recent biosynthetic investigations by Kerr's lab of several bioactive secondary metabolites from a soft coral, [42] a tunicate,[43-45] and a bryozan[46] have demonstrated the effectiveness of using cell-free extracts to discern the metabolic origins of these metabolites in their respective organisms.

3.3 - Proposed Retro-Biosynthetic Analysis of the Ecteinascidins

The ecteinascidins are a family of closely related tetrahydroisoquinoline alkaloids with tremendous pharmaceutical potential. Et 743 and 729 are our prime targets since these are the predominant alkaloids produced by *Ecteinascidia turbinata*. A retro-biosynthetic analysis of Et 743/729 (**18**) reveals that there are many possible routes by which ecteinascidin biosynthesis may occur.

A proposed retro-biosynthetic analysis of the ecteinascidins is shown in Scheme 3.1. One can visualize that Et 743/729 (**18**) is structurally composed of two fragments, a tetrahydroisoquinoline unit (**19**), and a pentacyclic intermediate (**20**) (Scheme 3.1). The another proposed pathway by which ecteinascidin biosynthesis

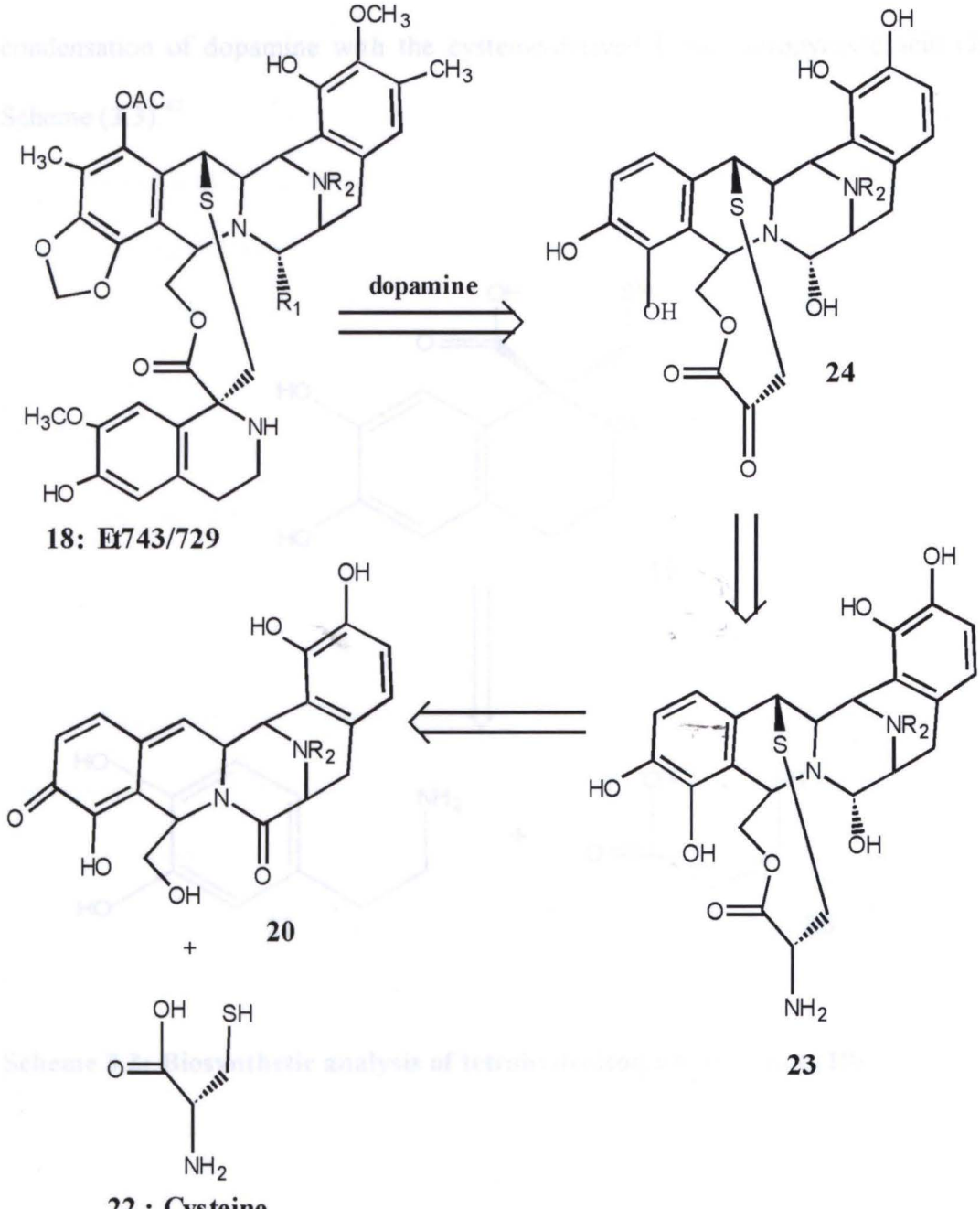
may occur is shown in the Scheme 3.2. In this pathway cysteine (**22**) or (β-mercaptopyruvic acid) (**25**) may combine with the pentacyclic intermediate (**20**) to give (**23**) which is oxidized to (**24**). The intermediate (**24**) may finally condense with dopamine (**21**) to yield Et 743/729 (**18**).



18 : Et 743/729

19 : tetrahydroisoquinoline **20 : pentacyclic intermediate**

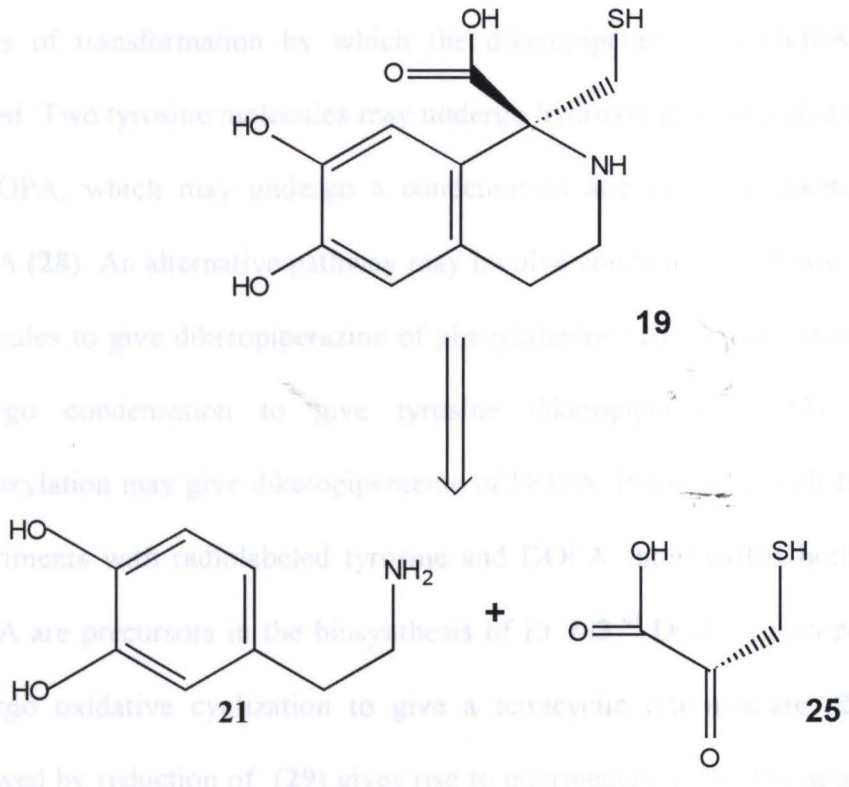
Scheme 3.1: Retro-biosynthetic analysis of Et 743/729



Scheme 3.2: Alternative biosynthetic pathway for Et 743/729

3.4 - Biosynthetic Analysis of the Tetrahydroisoquinoline Unit (19)

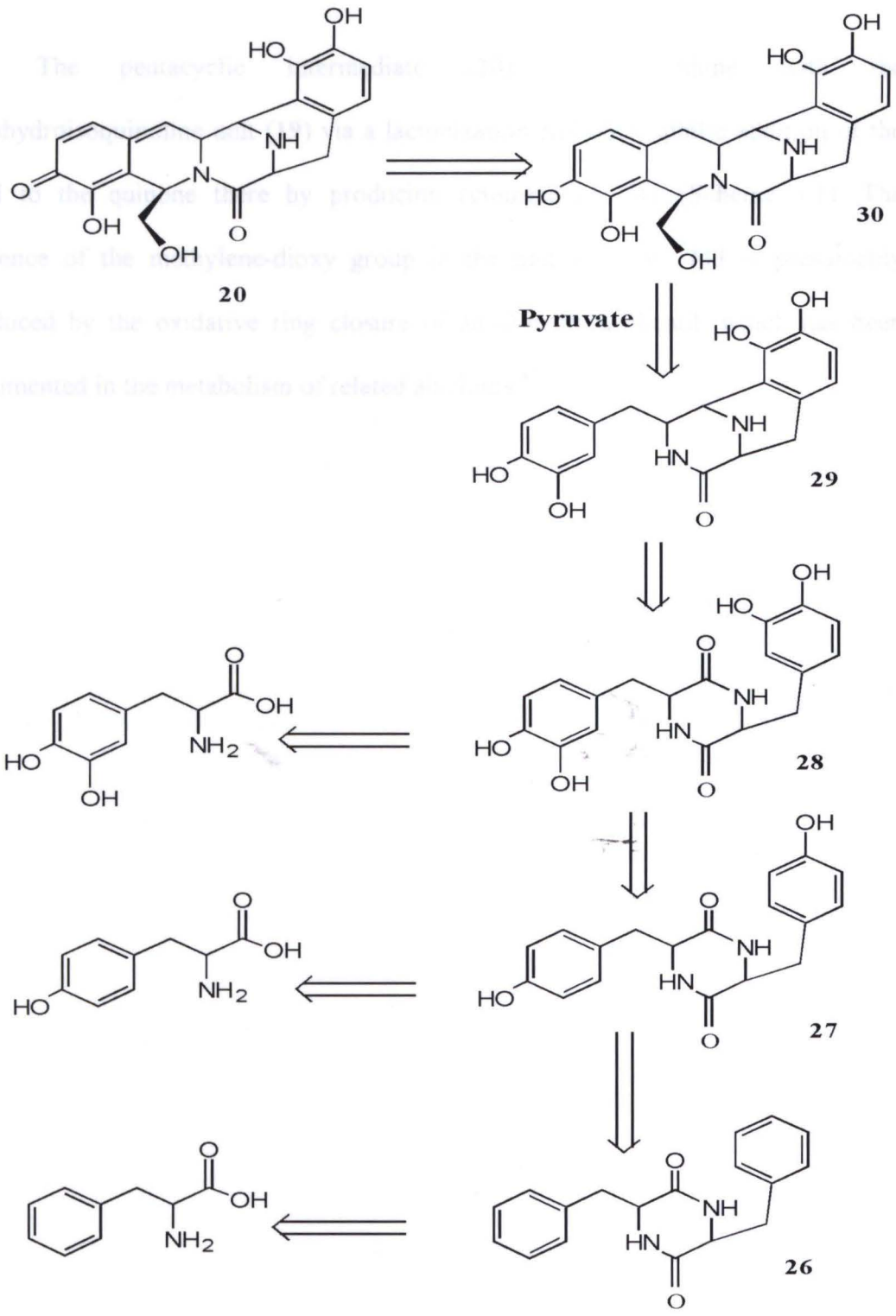
The tetrahydroisoquinoline unit (**19**) appears to be derived by the condensation of dopamine with the cysteine-derived β-mercaptopyruvic acid (**25**) Scheme (3.3).[47]



Scheme 3.3: Biosynthetic analysis of tetrahydroisoquinoline unit (19)

3.5 - Retro-Biosynthetic Analysis of Pentacyclic Intermediate (20)

Scheme 3.4 shows the biosynthetic steps to derive the pentacyclic intermediate (**20**). The pentacyclic intermediate appears to be derived from the diketopiperazine of DOPA or tyrosine. A variety of alkaloids such as echinulin[48], and brevianamide A[49], are produced via diketopiperazine intermediates which themselves are formed by the condensation of two aminoacids.[40] There are two modes of transformation by which the diketopiperazine of DOPA (**28**) can be formed. Two tyrosine molecules may undergo hydroxylation and give two molecules of DOPA, which may undergo a condensation and give the diketopiperazine of DOPA (**28**). An alternative pathway may involve condensation of two phenylalanine molecules to give diketopiperazine of phenylalanine (**26**) or two tyrosine molecules undergo condensation to give tyrosine diketopiperazine (**27**), which upon hydroxylation may give diketopiperazine of DOPA. Preliminary cell-free incubation experiments with radiolabeled tyrosine and DOPA showed that both tyrosine and DOPA are precursors in the biosynthesis of Et 743.[43] DOPA diketopiperazine may undergo oxidative cyclization to give a tetracyclic intermediate (**29**). Oxidation followed by reduction of (**29**) gives rise to intermediate (**30**). The source of the two carbon unit C_1-C_{22} in the conversion of (**29**) to (**30**) posses an intriguing biosynthetic question. Incubation studies with radiolabeled pyruvic acid showed that pyruvic acid is likely the source of the C_1-C_{22} unit of ecteinascidin.[44] Intermediate (**29**) would react with pyruvic acid to yield (**30**).[50]



Scheme 3.4: Retro-biosynthetic analysis of pentacyclic intermediate (20)

The pentacyclic intermediate (**20**) may combine with the tetrahydroisoquinoline unit (**19**) via a lactonization and nucleophilic addition of the thiol to the quinone there by producing ecteinascidin 743 (Scheme 3.1). The presence of the methylene-dioxy group in the unit A of Et 743 is presumably produced by the oxidative ring closure of an *O*-methoxyphenol, which has been documented in the metabolism of related alkaloids.[47]

Chapter 4

Synthesis of Diketopiperazines

4.1 - Introduction to Cyclic Peptides

Cyclic dipeptides, the 2,5-diketopiperazines, are widespread in nature, and have been isolated as natural products from higher animals, plants, marine organisms and microorganisms.[51] Diketopiperazines (DKPs), which are the smallest cyclic peptides, are a common motif in several natural products with therapeutic properties.[52] Furthermore DKPs have been shown to be useful scaffolds for rational design of several drugs.[53] DKPs are formed readily from peptides and proteins on thermolysis, and on acid or enzymatic hydrolysis, thus necessitating care to ensure that their presence in natural sources is not artefactual.[51] As an example, *cyclo*-L-His-L-Pro (**31**) has been identified in human blood.

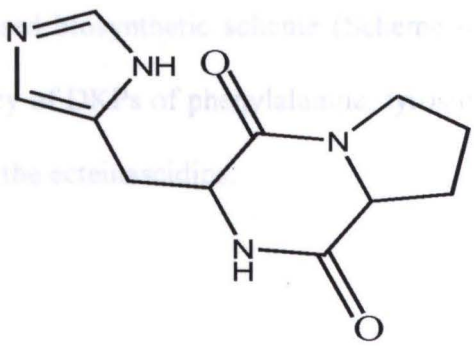


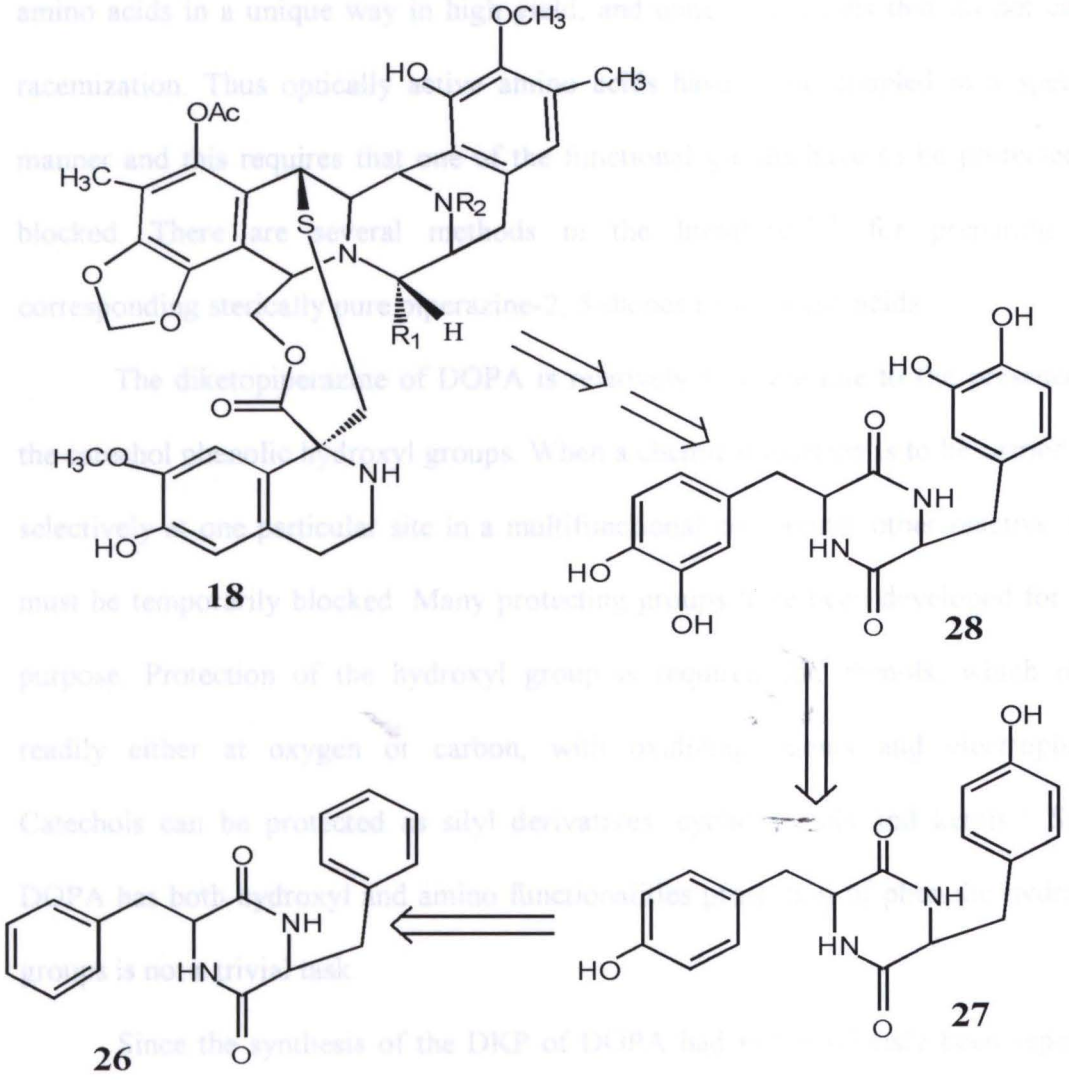
Figure 4.1: Structure of *Cyclo*-L-His-L-Proline (31)

While a number of diketopiperazines formed simply from condensation of L-amino acids are known, a majority of cyclic peptides are formed via considerable modification of a diketopiperazine skeleton, frequently involving N-methylation and oxidation processes, although it is not clear whether the modification occurs before or after the cyclization of amino acids.[51]

4.2 - Synthesis of Diketopiperazines of Phenylalanine, Tyrosine and DOPA

The biosynthetic analysis of ecteinascidins involves not only the identification of primary building blocks but also the putative intermediates. Preliminary experiments in which radiolabeled precursors incubated with cell-free extracts of *E. turbinata* showed that tyrosine, cysteine, dopamine and DOPA are the primary building blocks of ecteinascidis.[45] The pathway by which these aminoacids transformed into ecteinascidins is not yet known. However, biosynthetic mechanism can be elucidated by synthesizing the putative intermediates and assaying for their conversion to the target compound. Identification of these intermediates may also lead to the discovery of additional potent pharmaceutical compounds.

In our proposed biosynthetic scheme (Scheme 4.1), we have suggested the potential intermediacy of DKPs of phenylalanine, tyrosine and DOPA, which in turn may be converted to the ecteinascidins.



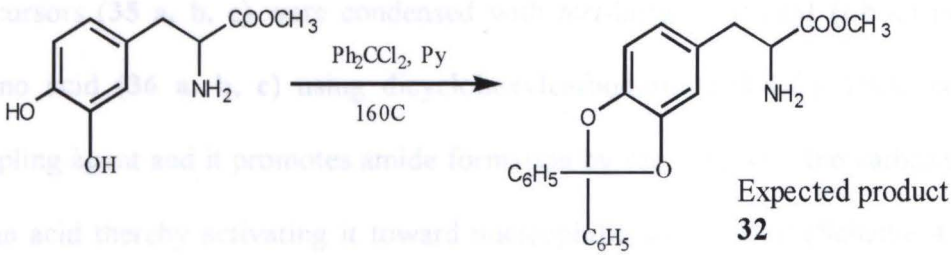
Scheme 4.1: Proposed intermediacy of diketopiperazines

The synthesis of cyclic peptides requires methods for assembling an array of amino acids in a unique way in high yield, and under conditions that do not cause racemization. Thus optically active amino acids have to be coupled in a specific manner and this requires that one of the functional groups have to be protected or blocked. There are several methods in the literature[54-56] for preparing the corresponding sterically pure piperazine-2, 5-diones from amino acids.

The diketopiperazine of DOPA is relatively unstable due to the presence of the catechol phenolic hydroxyl groups. When a chemical reaction is to be carried out selectively at one particular site in a multifunctional compound, other reactive sites must be temporarily blocked. Many protecting groups have been developed for this purpose. Protection of the hydroxyl group is required for phenols, which react readily either at oxygen or carbon, with oxidizing agents and electrophiles. Catechols can be protected as silyl derivatives, cyclic acetals and ketals. Since DOPA has both hydroxyl and amino functionalities protection of phenolic hydroxyl groups is not a trivial task.

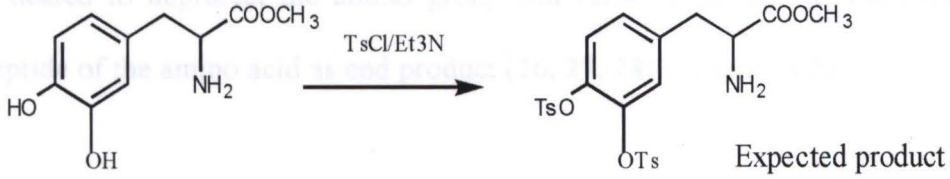
Since the synthesis of the DKP of DOPA had not previously been reported we felt it would be worth while to protect the catechol hydroxyls prior to the peptide coupling reactions. Following standard hydroxyl group protection methods, several methods to protect the hydroxyl groups of DOPA methyl ester were conducted (Scheme 4.2). Thin layer chromatography of reaction mixture of every protection method showed the formation of several products suggesting it was not worthwhile to purify and characterize each product.

Protection of hydroxyl group as ketal



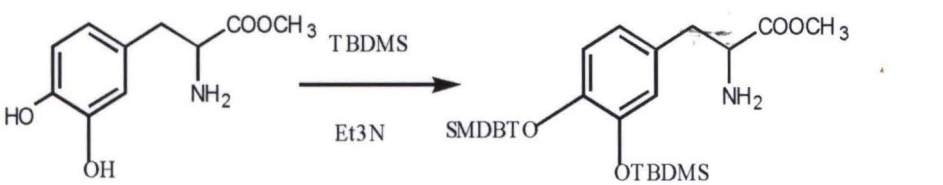
Expected product
32

Protection of hydroxyl group as tosylate



Expected product
33

Protection of hydroxyl group as silyl ether

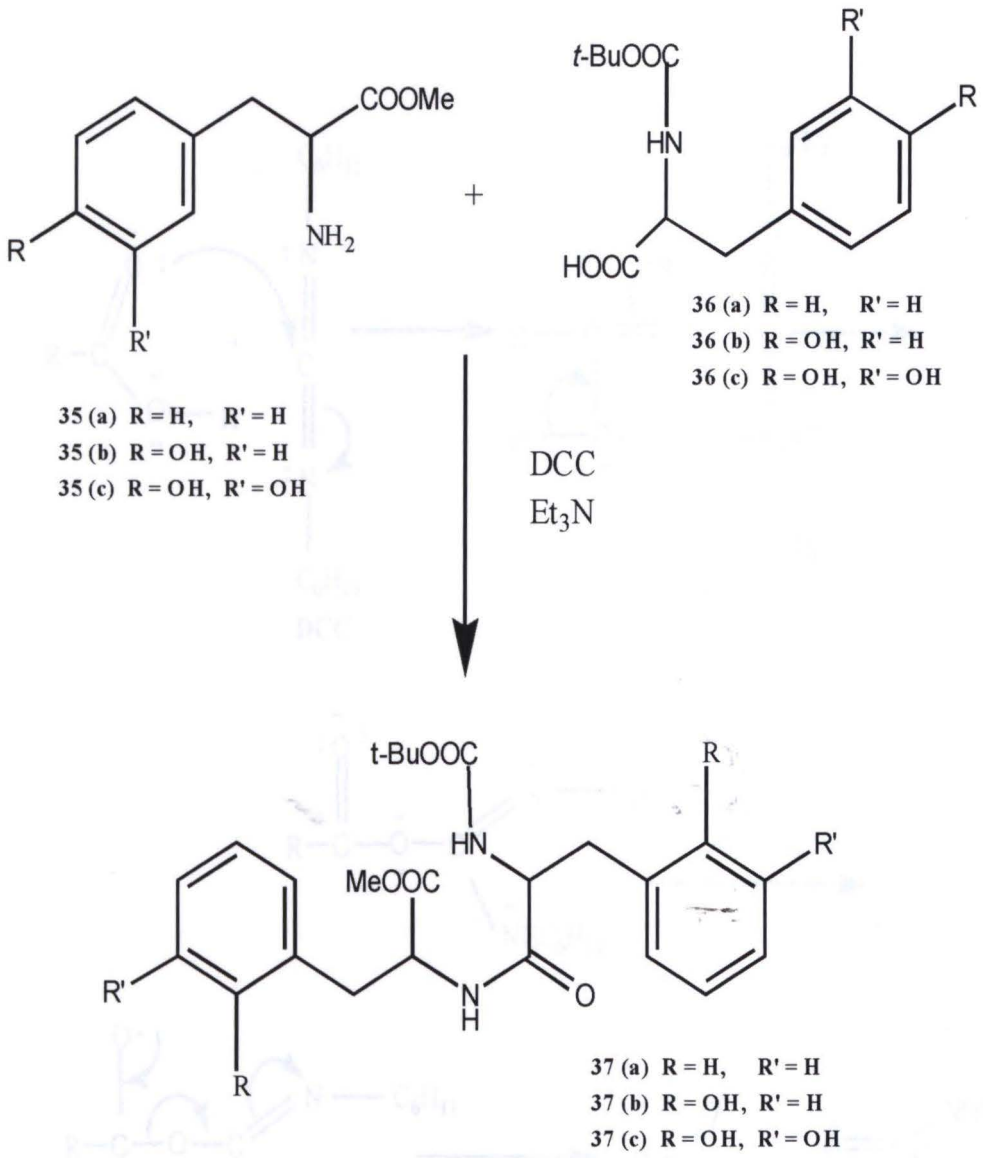


Expected product
34

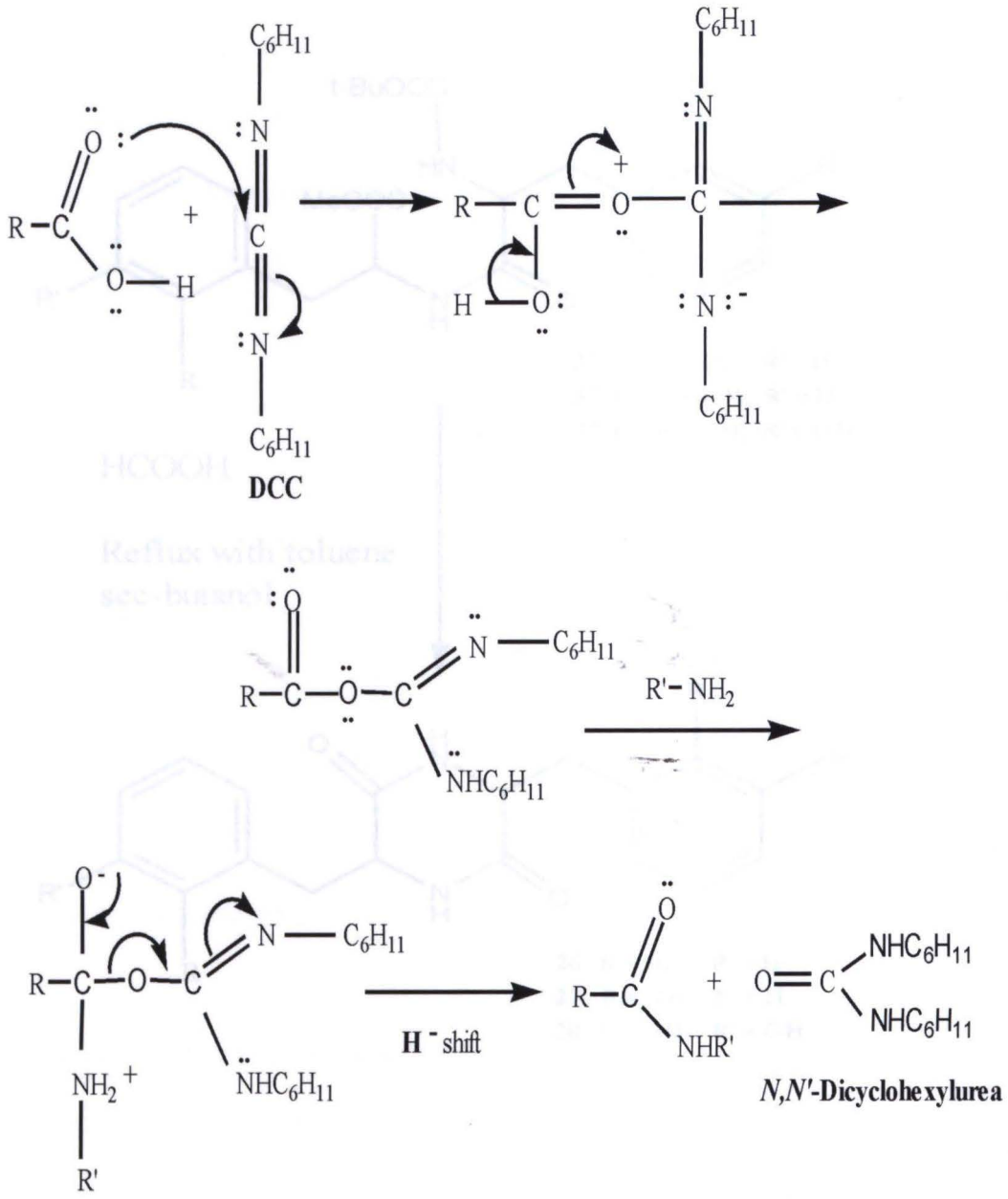
Scheme 4.2: Protection of hydroxyl groups of DOPA methyl ester

Thus, the synthesis of diketopiperazine of DOPA was conducted by carefully excluding oxygen and water all the times by performing all steps under argon.

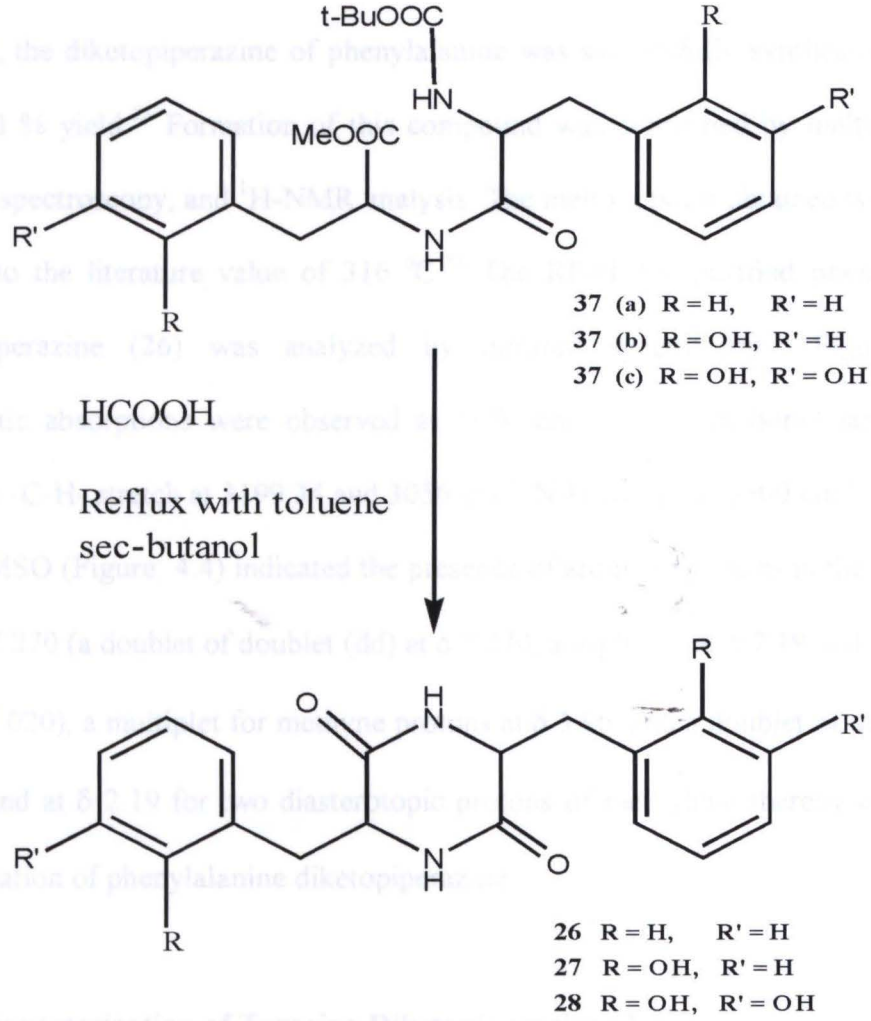
Diketopiperazines of phenylalanine (**26**), tyrosine (**27**) and DOPA (**28**) were synthesized as described in Scheme 4.3.[55, 57] The methyl ester form of the amino acid precursors (**35 a, b, c**) were condensed with *tert*-butoxycarbonyl (*t*-boc) protected amino acid (**36 a, b, c**) using dicyclohexylcarbodiimide (DCC). DCC acts as a coupling agent and it promotes amide formation by reacting with the carboxyl group of an acid thereby activating it toward nucleophilic substitution (Scheme 4.4). The acyclic peptide (**37 a, b, c**) formed in Scheme 4.3 was then treated with formic acid[58] and heated to deprotect the amino group and carboxyl groups to yield the cyclic dipeptide of the amino acid as end product (**26, 27, 28**) (Scheme 4.5).



Scheme 4.3: Formation of acyclic peptides of phenylalanine, tyrosine and DOPA



Scheme 4.4: A Mechanism for DCC – promoted amide synthesis



Scheme 4.5: Synthesis of diketopiperazines of phenylalanine, tyrosine, DOPA

4.3 – Characterization of Phenylalanine Diketopiperazine (26)

The diketopiperazine of phenylalanine was first synthesized in 1967 by Nitecki *et. al.*[55], however no spectral data has been reported. Following this protocol, the diketopiperazine of phenylalanine was successfully synthesized in our lab in 93 % yield.[55] Formation of this compound was confirmed by melting point, infrared spectroscopy, and ^{1}H-NMR analysis. The melting point obtained was 319 C, similar to the literature value of 316 ^{0}C.[55] The RP-HPLC purified phenylalanine diketopiperazine (**26**) was analyzed by infrared spectroscopy (Figure: 4.3). Diagnostic absorptions were observed at 1670 cm^{-1} (amide carbonyl stretch), an aromatic -C-H- stretch at 3199.34 and 3056 cm^{-1}, N-H stretch at 3300 cm^{-1}, ^{1}H-NMR in d_6-DMSO (Figure: 4.4) indicated the presence of aromatic protons in the region of δ 7.0 – 7.270 (a doublet of doublet (dd) at δ 7.270, a triplet (t) at δ 7.19 and a doublet (d) at δ7.020), a multiplet for methyne protons at δ 3.96, and a doublet of doublets at δ 2.25 and at δ 2.19 for two diasterotopic protons of methylene thereby confirmed the formation of phenylalanine diketopiperazine.

4.4 – Characterization of Tyrosine Diketopiperazine (27)

The diketopiperazine of tyrosine was first synthesized by Rohloff *et. al* [57], Following the protocol[57] the cyclic dipeptide of tyrosine was successfully synthesized in 96 % yield (Section 6.10). The formation of this compound was confirmed by melting point, infrared spectroscopy, ^{1}H-NMR, and ^{13}C-NMR analysis. The melting point obtained was 280 ^{0}C, similar to the literature value of 278 ^{0}C.[57]

Infrared spectral analysis (KBr pellet) was carried out on RP-HPLC purified tyrosine diketopiperazine Diagnostic absorptions were observed at 1671.14 cm^{-1} (amide carbonyl stretch) an aromatic –C-H- stretch at 3150 cm-1 and 2923.89 cm^{-1}, N-H stretch at 3400 cm^{-1} were observed (Figure: 4.6). A ^{1}H-NMR of tyrosine diketopiperazine was carried out in d_6-DMSO (Figure. 4.7). A singlet at δ 9.2 for OH protons, a broad singlet at δ 7.9 for NH protons, doublets at δ 6.8 and at δ 6.69 for aromatic protons, a multiplet at δ 3.89 for methyne protons, a doublet of doublets at δ 2.55 and at δ 2.139 for two diasterotopic protons of methylene indicated the formation of tyrosine diketopiperazine. ^{13}C-NMR/d_6-DMSO (Figure: 4.8) showed peaks for key functional groups that represent the molecule. An amide carbonyl at δ 166.50, methyne carbon at δ 56, and methylene carbon at δ 38.7 indicated the formation of tyrosine diketopiperazine.

4.5 – Characterization of DOPA Diketopiperazine (28)

The diketopiperazine of DOPA had not previously been synthesized and was produced in similar manner to that of the diketopiperazines of tyrosine and phenylalanine. Since the diketopiperazine of DOPA is relatively unstable, the synthesis was carried out carefully excluding water and oxygen and conducting the synthesis under argon. The product could not be crystallized and therefore the melting point of the product could not be determined. Synthesized DOPA DKP was purified by Reversed phase HPLC using a linear gradient with water (0.1 % TFA)

and acetonitrile using a UV/VIS detector. HPLC chromatogram showed (Figure: 4.9) several peaks. Each peak was collected and analyzed by ^{1}H-NMR.

According to the ^{1}H-NMR analysis it was determined the peak eluting at 13.75 minutes, as a single peak is the diketopiperazine of DOPA. ^{1}H-NMR in d_6-DMSO confirmed the presence of DOPA diketopiperazine. A broad singlet at δ 8.7 for hydroxyl protons, a singlet at δ 8.1 for NH protons, a doublet of doublets at δ 6.55 (J = 8.5, 1.5 Hz), a broad singlet at δ 6.51 and a broad doublet at δ 6.36 (J = 8.0 Hz) for aromatic protons, a multiplet at δ 4.13 for methyne protons a doublet of doublet at δ 2.75 (J = 13.5, 4 Hz), a multiplet at δ 1.92 for two diasterotopic methylene protons indicated the presence of DOPA diketopiperazine. NMR spectra also showed some peaks in the region of δ 1-1.7 and a triplet at δ 3.50. These peaks are due to the impurities from dicyclohexylurea, which is the byproduct of dicycyclohexyl carbodiimide the coupling agent used in the synthesis of acyclic dipeptide. Since cyclohexylurea is not UV sensitive it was not separated by HPLC and the diketopiperazine of DOPA eluted as a single peak. This impurity could not be separated by HPLC. Since this diketopiperazine was only required for use as a cold carrier, the impurity does not present a problem.

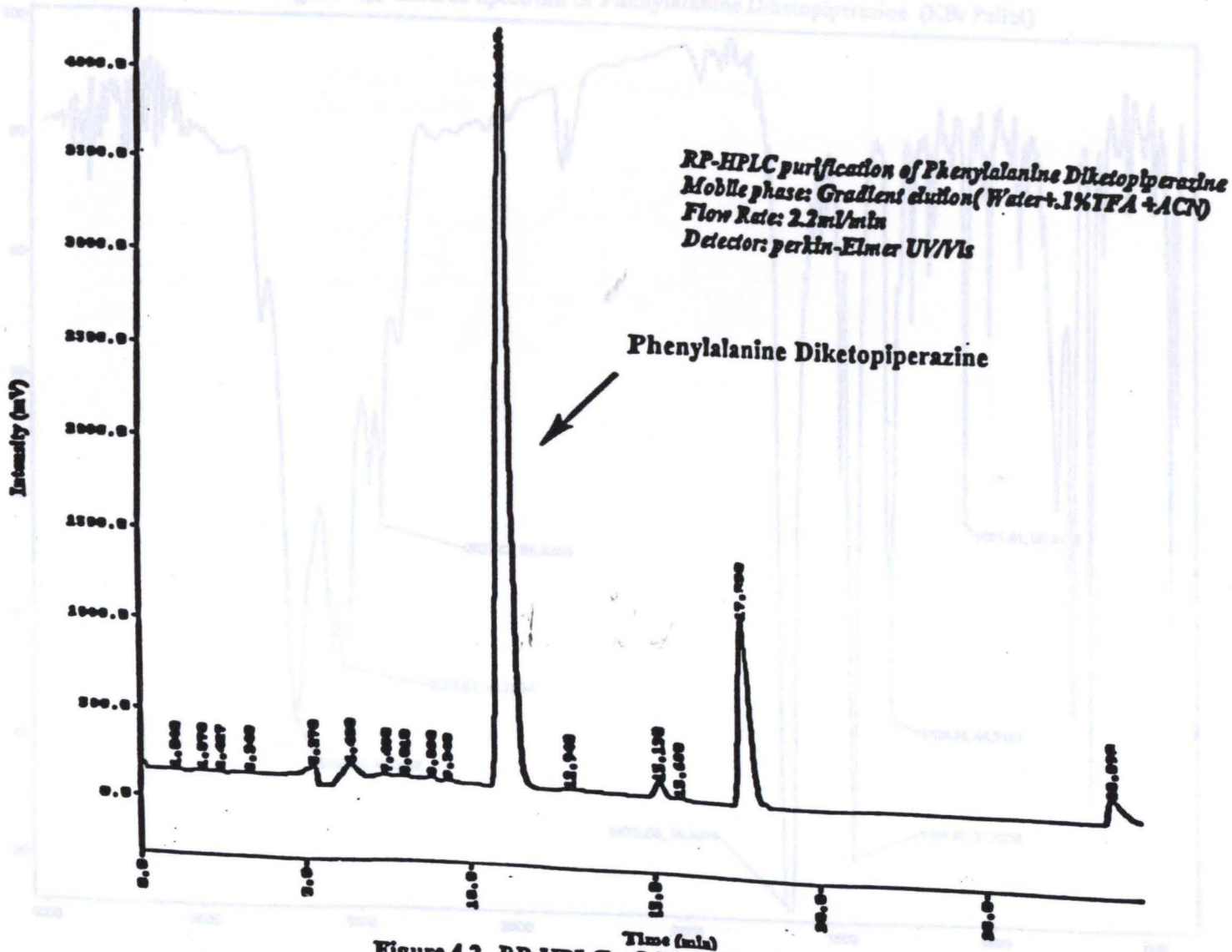
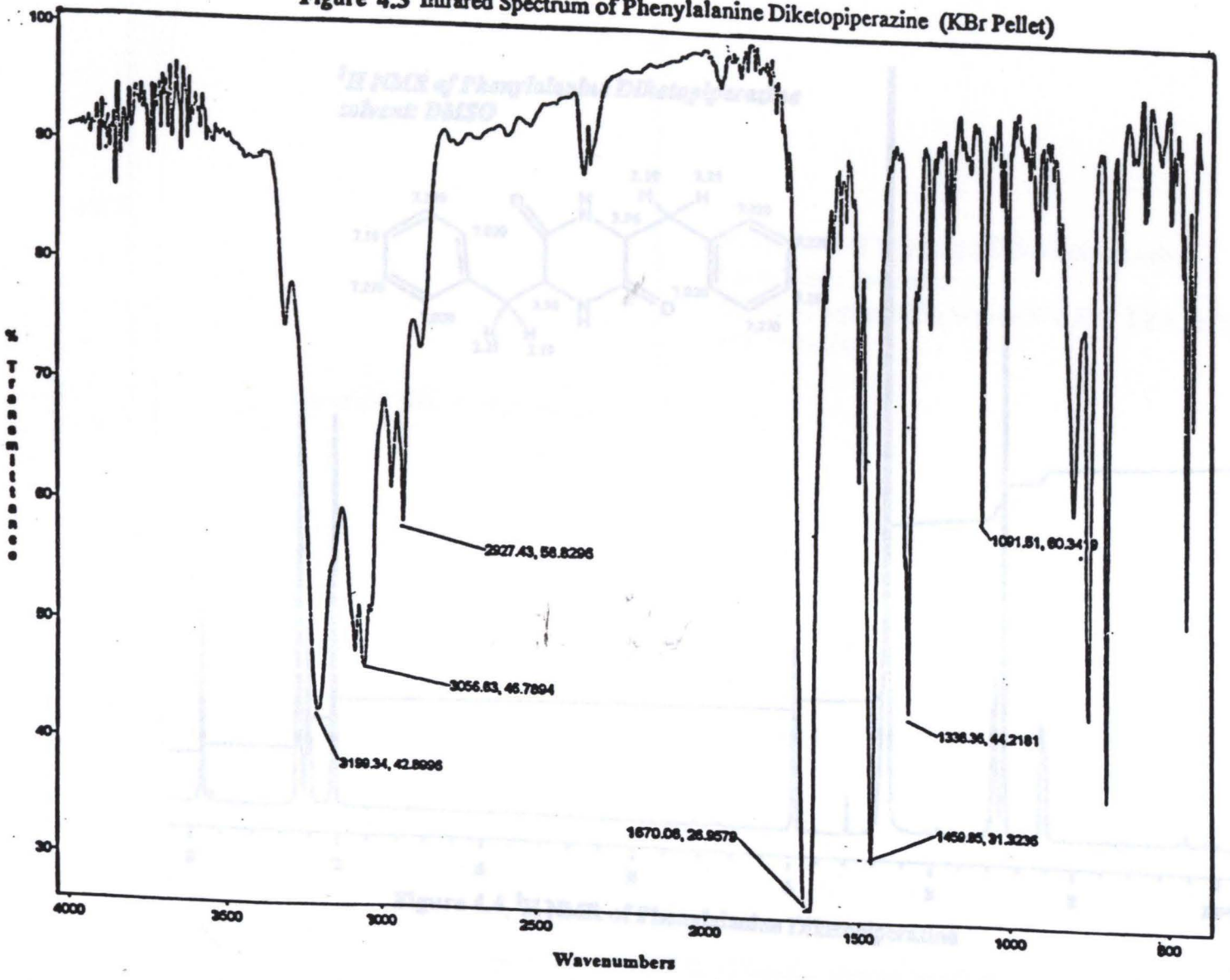


Figure 4.2 RP-HPLC of Phenylalanine Diketopiperazine

Figure 4.3 Infrared Spectrum of Phenylalanine Diketopiperazine (KBr Pellet)



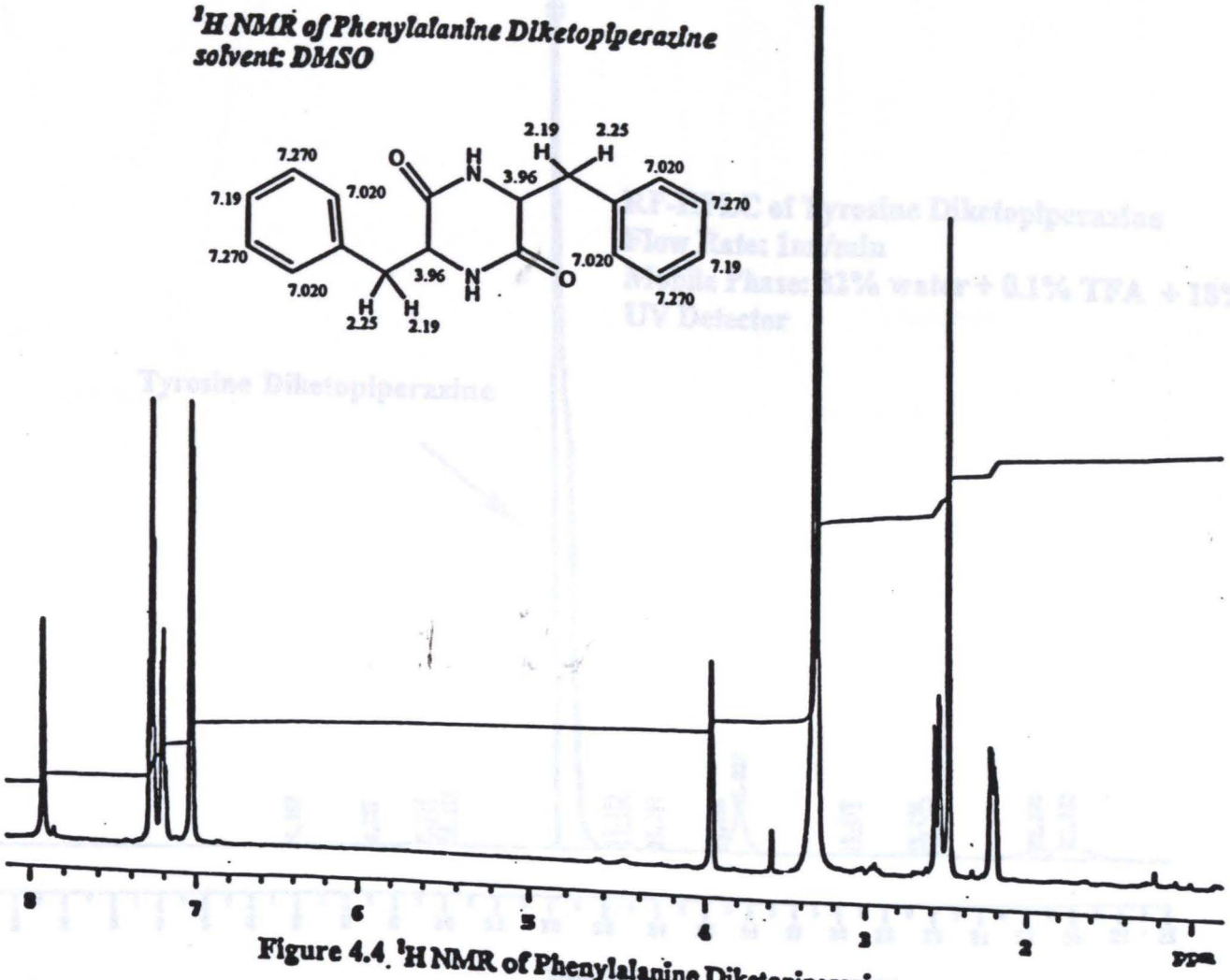


Figure 4.4 ^{1}H NMR of Phenylalanine Diketopiperazine

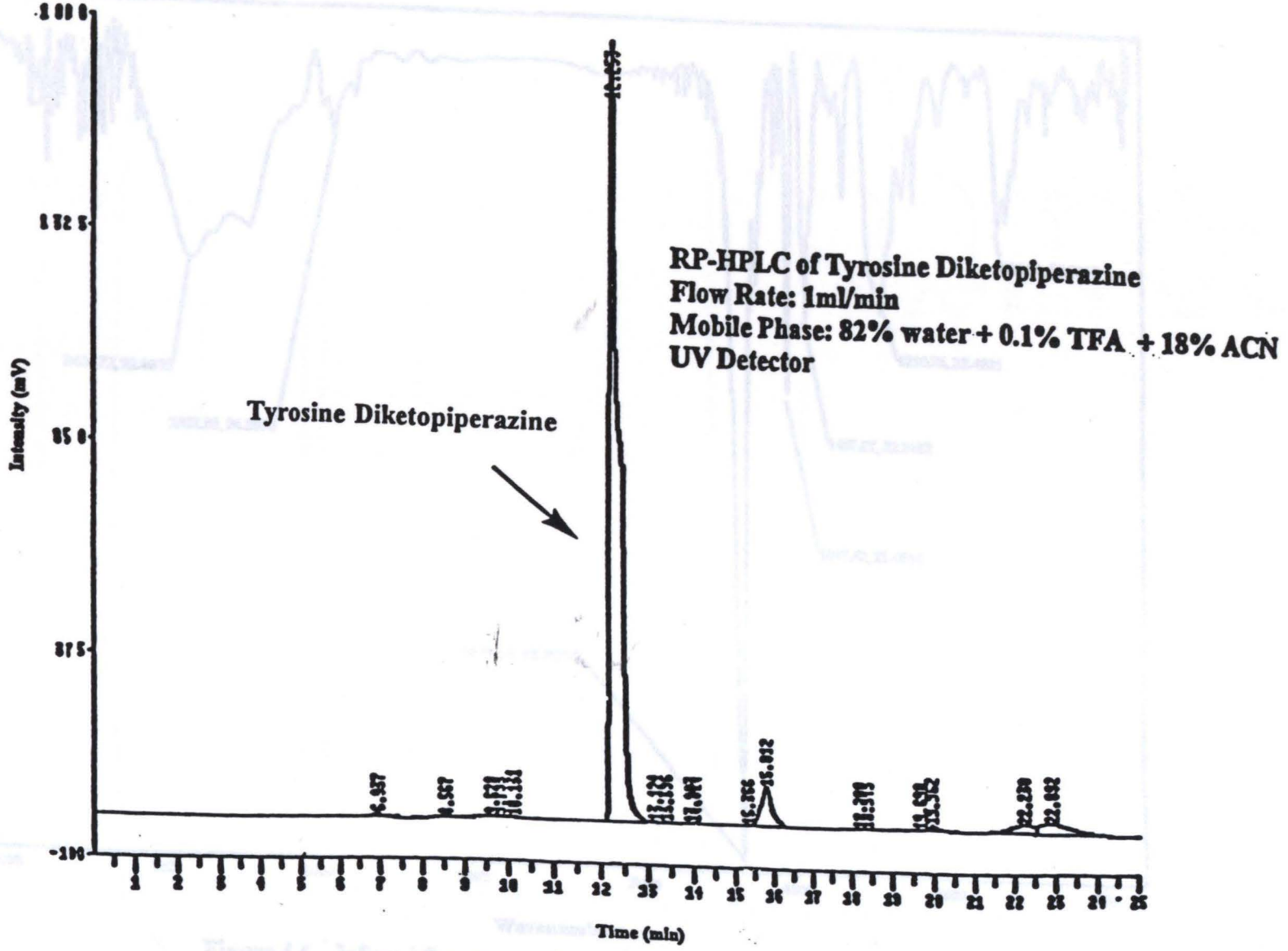


Figure 4.5 RP-HPLC of Tyrosine Diketopiperazine

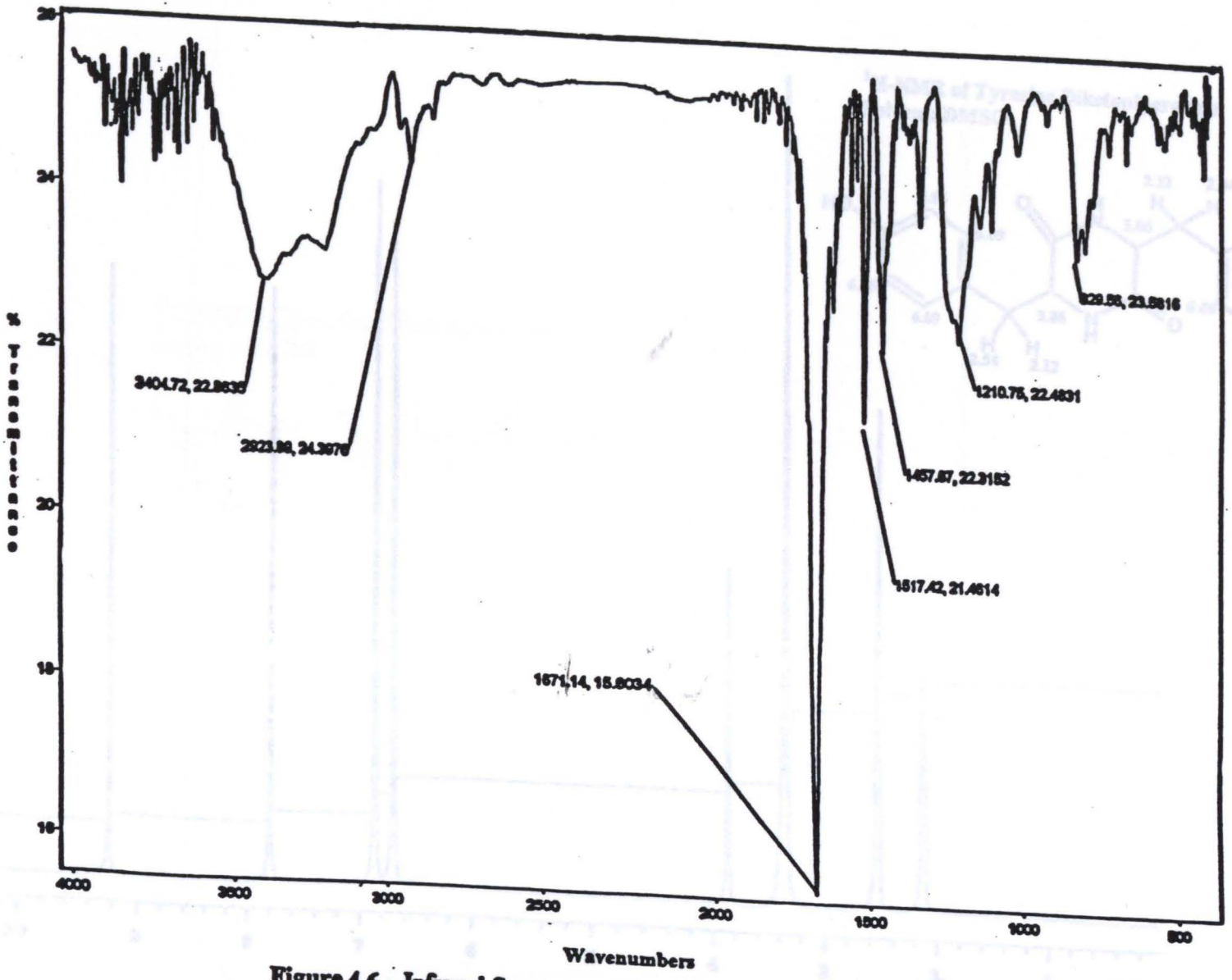


Figure 4.6 Infrared Spectrum of Tyrosine Diketopiperazine (KBr Pellet)

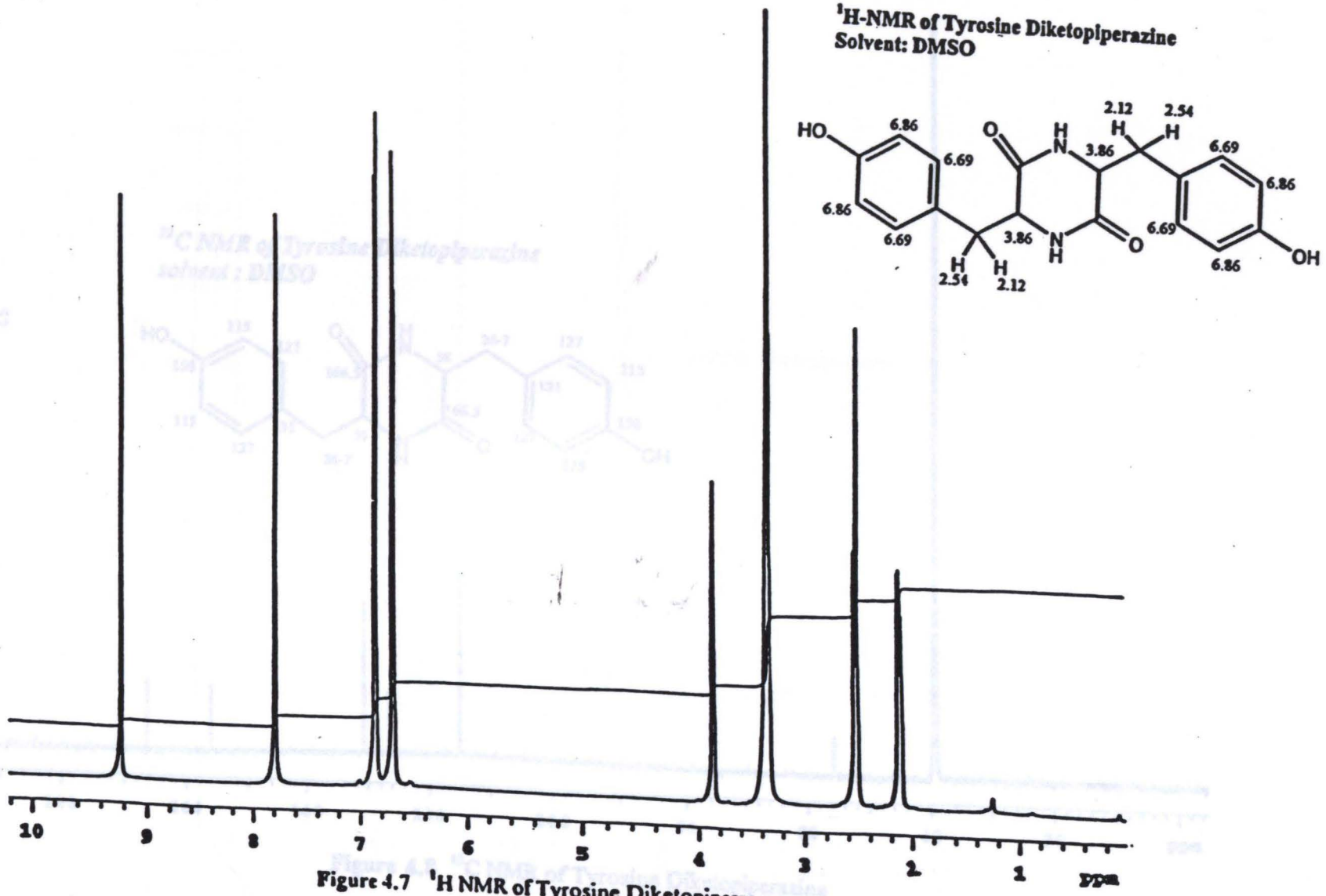


Figure 4.7 ^{1}H NMR of Tyrosine Diketopiperazine

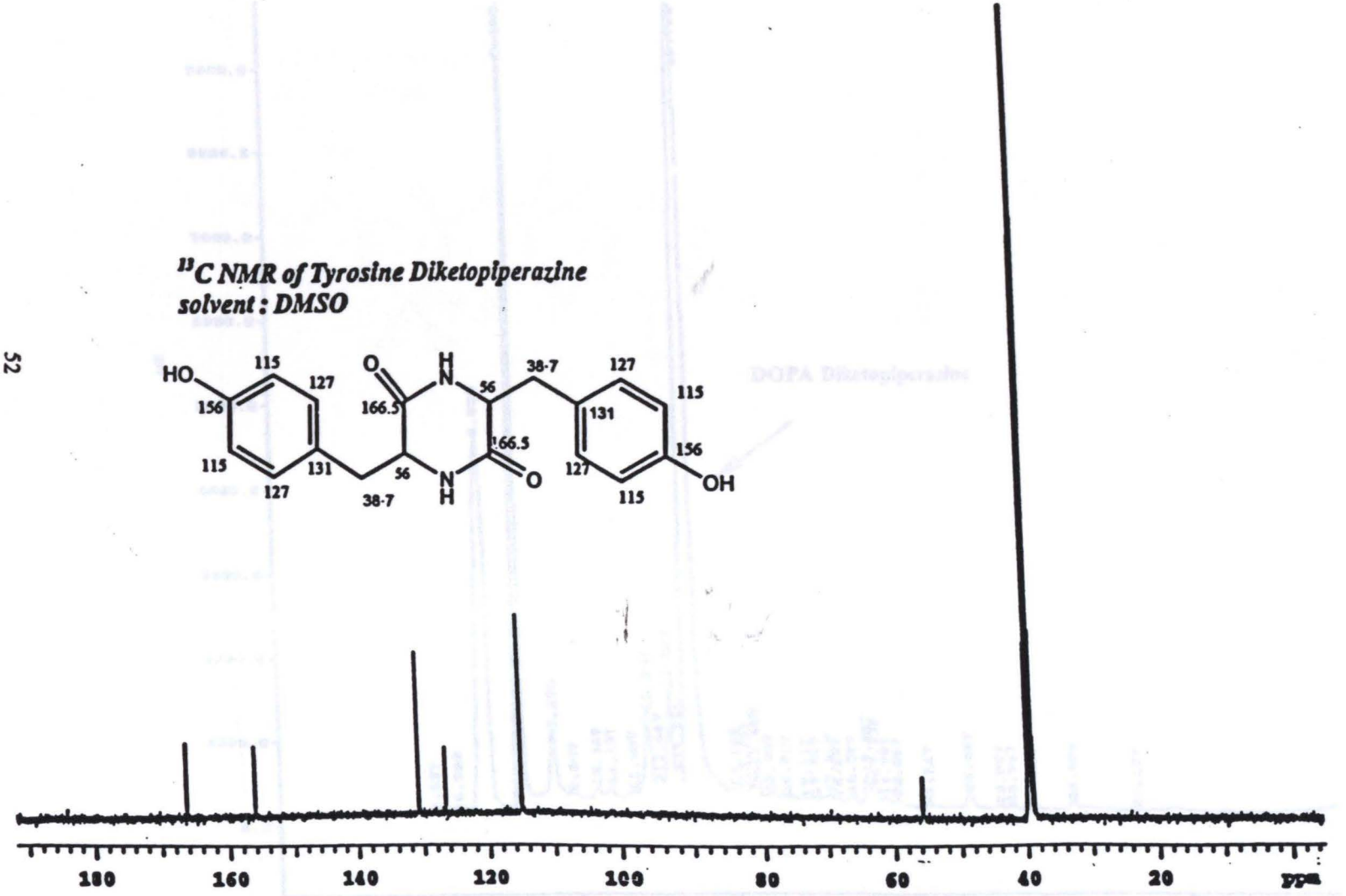


Figure 4.8 ^{13}C NMR of Tyrosine Diketopiperazine

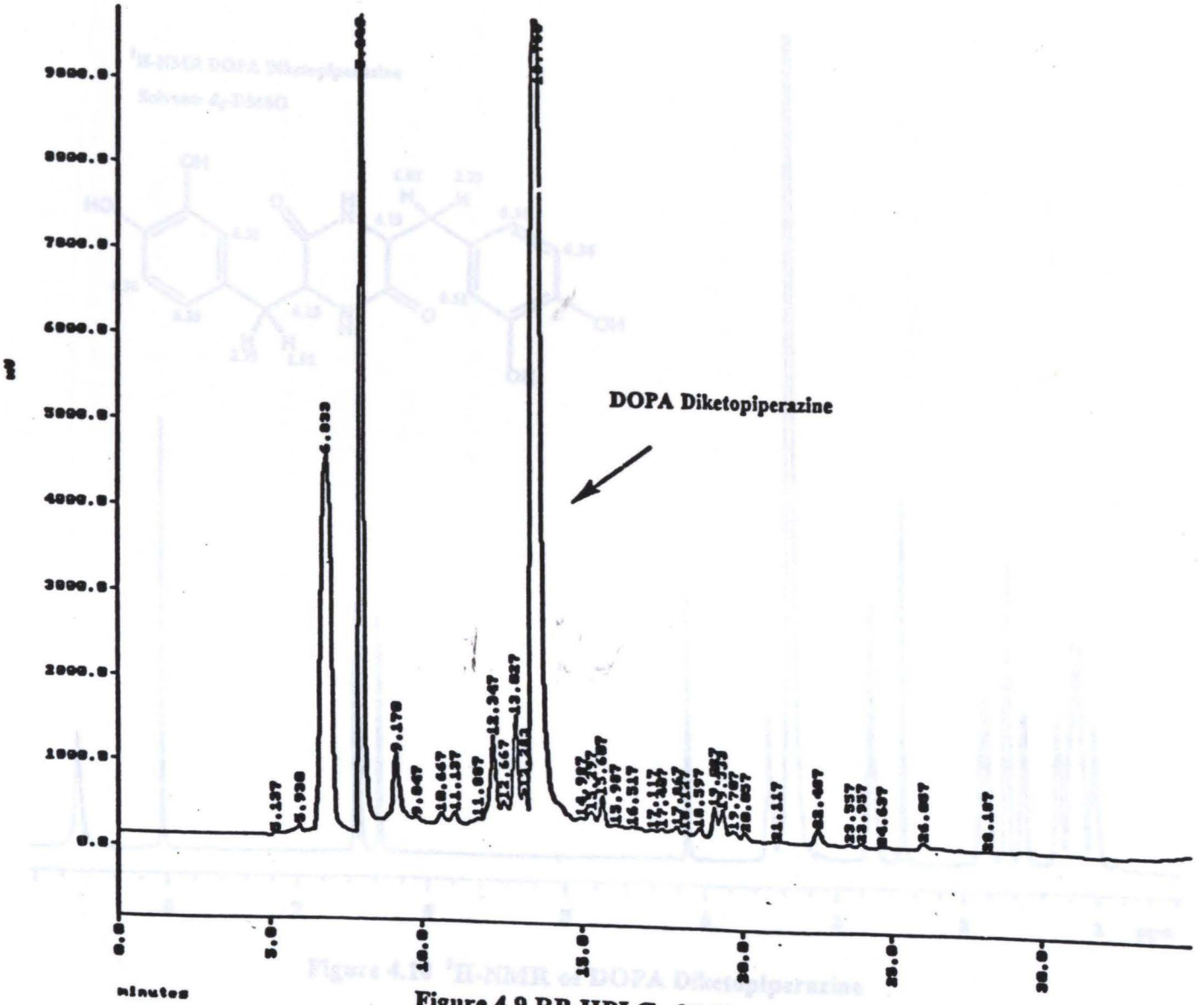


Figure 4.9 RP-HPLC of DOPA Diketopiperazine

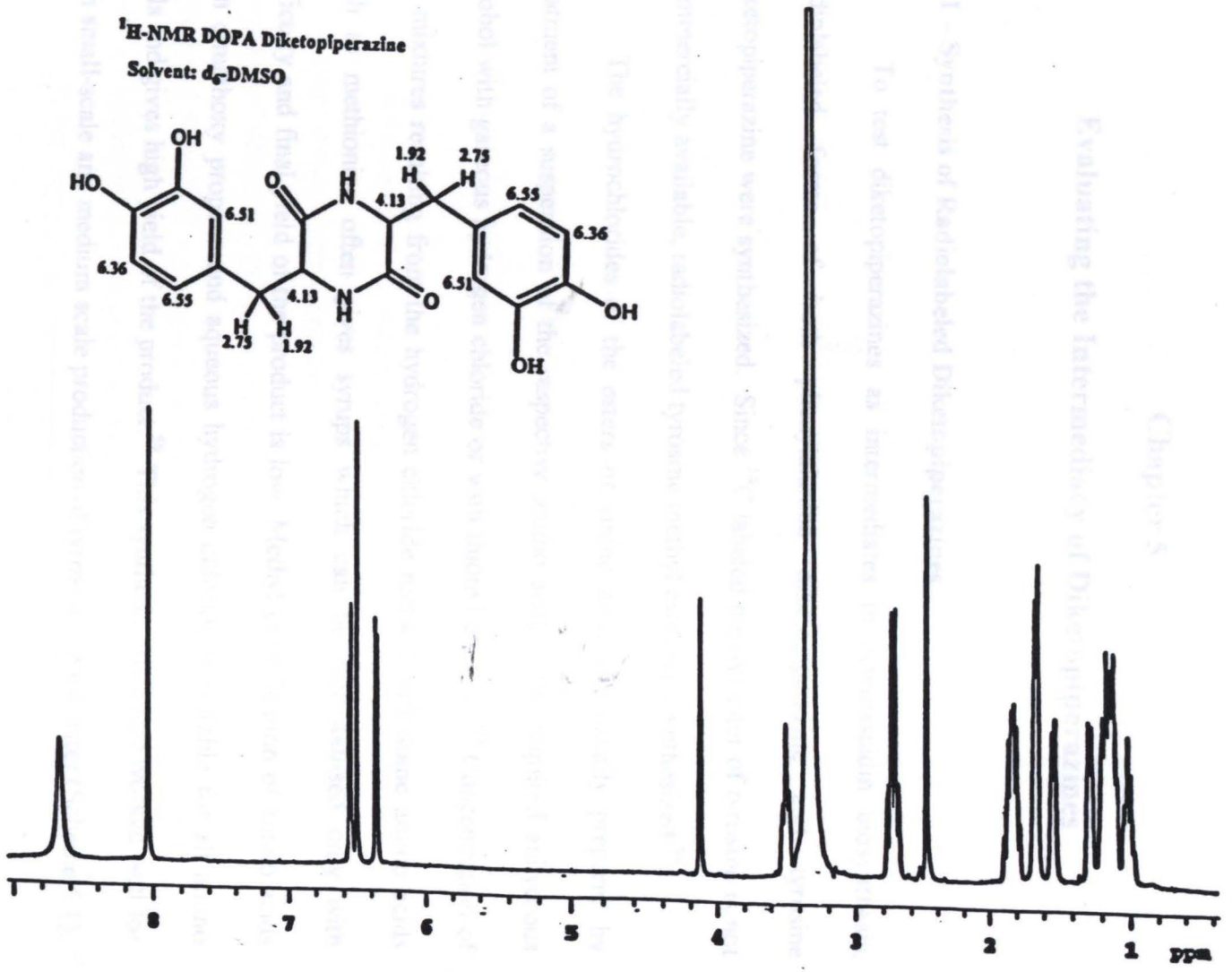


Figure 4.10 ^{1}H-NMR of DOPA Diketopiperazine

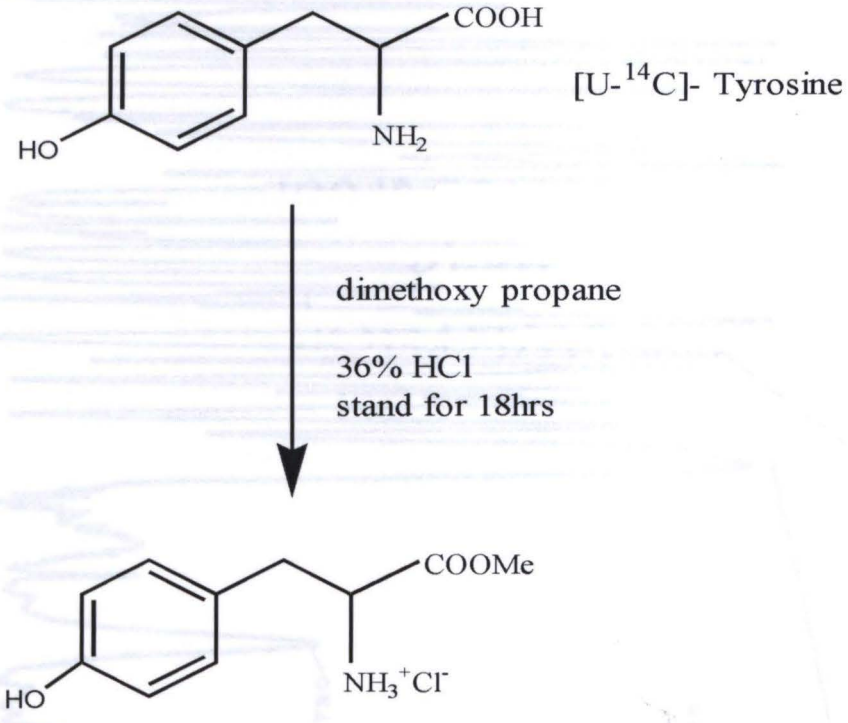
Chapter 5

Evaluating the Intermediacy of Diketopiperazines

5.1 – Synthesis of Radiolabeled Diketopiperazines

To test diketopiperazines as intermediates in ecteinascidin biosynthesis, radiolabeled forms of both phenylalanine diketopiperazine and tyrosine diketopiperazine were synthesized. Since ^{14}C labeled methyl ester of tyrosine is not commercially available, radiolabeled tyrosine methyl ester was synthesized.[59]

The hydrochlorides of the esters of amino acids are usually prepared by treatment of a suspension of the respective amino acid in the required anhydrous alcohol with gaseous hydrogen chloride or with thionyl chloride.[60] Concentration of the mixtures resulting from the hydrogen chloride method with some amino acids such as methionine often gives syrups which can be recrystallised only with difficulty and final yield of the product is low. Methyl esterification of amino acids with dimethoxy propane and aqueous hydrogen chloride is suitable for all amino acids and gives high yield of the product.[59] This synthetic procedure worked well for both small-scale and medium scale production of tyrosine methyl ester (Scheme 5.1).



Tyrosine Methyl Ester Hydrochloride (38)

Scheme 5.1: Methyl Esterification of Tyrosine

Formation of tyrosine methyl ester was confirmed by both melting point and by infrared spectroscopy (Section 6.11). Radiolabeled tyrosine (50-μCi) was successfully esterified (Section 6.12). The ethyl ester of phenylalanine in radiolabeled form (^{14}C - phenylalanine ethyl ester hydrochloride) is commercially available. Sequentially, radiolabeled diketopiperazines of phenylalanine (Section 6.9) and tyrosine (Section 6.13) were synthesized and were purified by RP-HPLC. The ^{14}C- phenylalanine DKP (**26**) was formed in 1.3 % radiochemical yield and the ^{14}C tyrosine DKP (**36**) formed in 1.2 % radiochemical yield.

Figure 5.1 Infrared spectrum of L-tyrosine

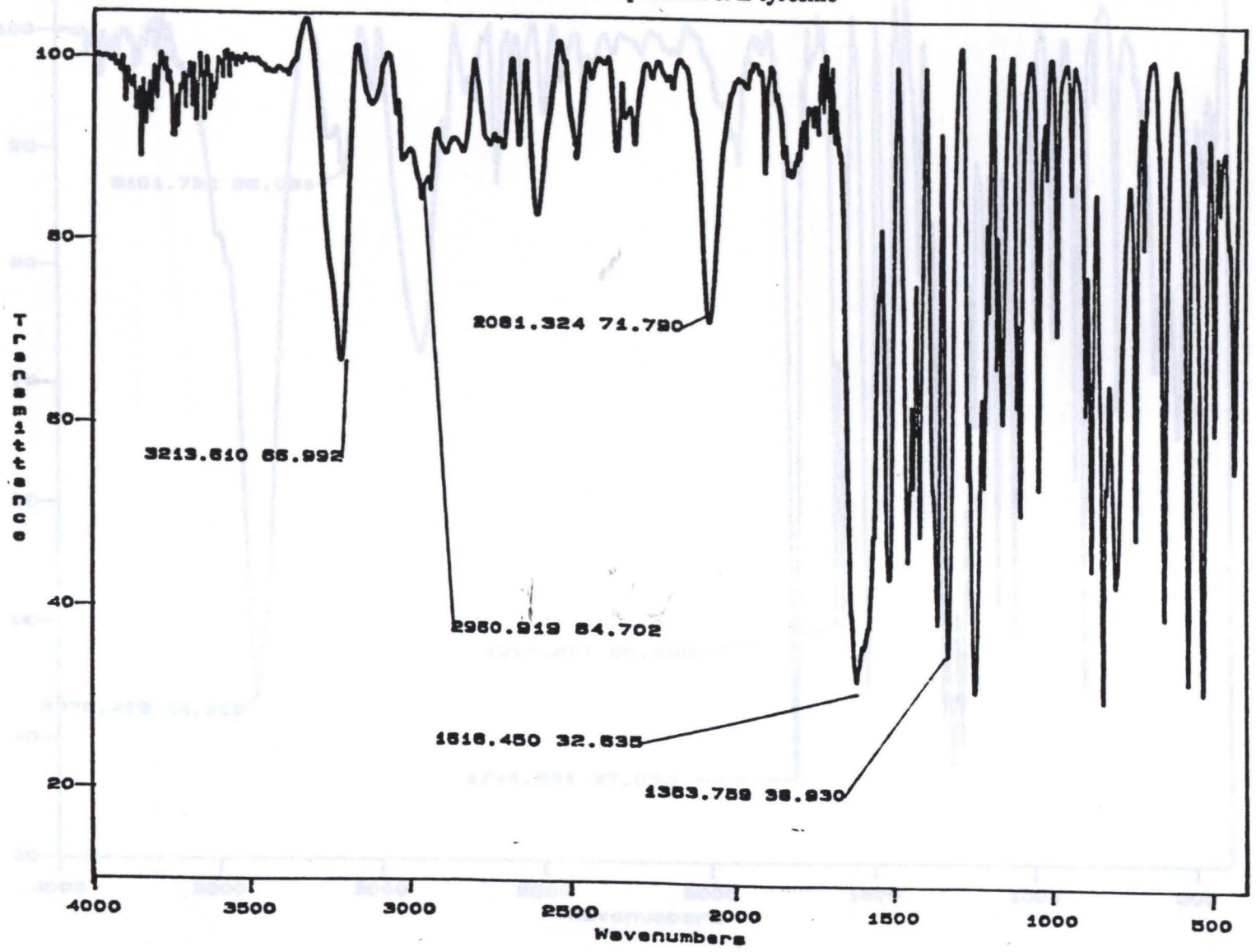
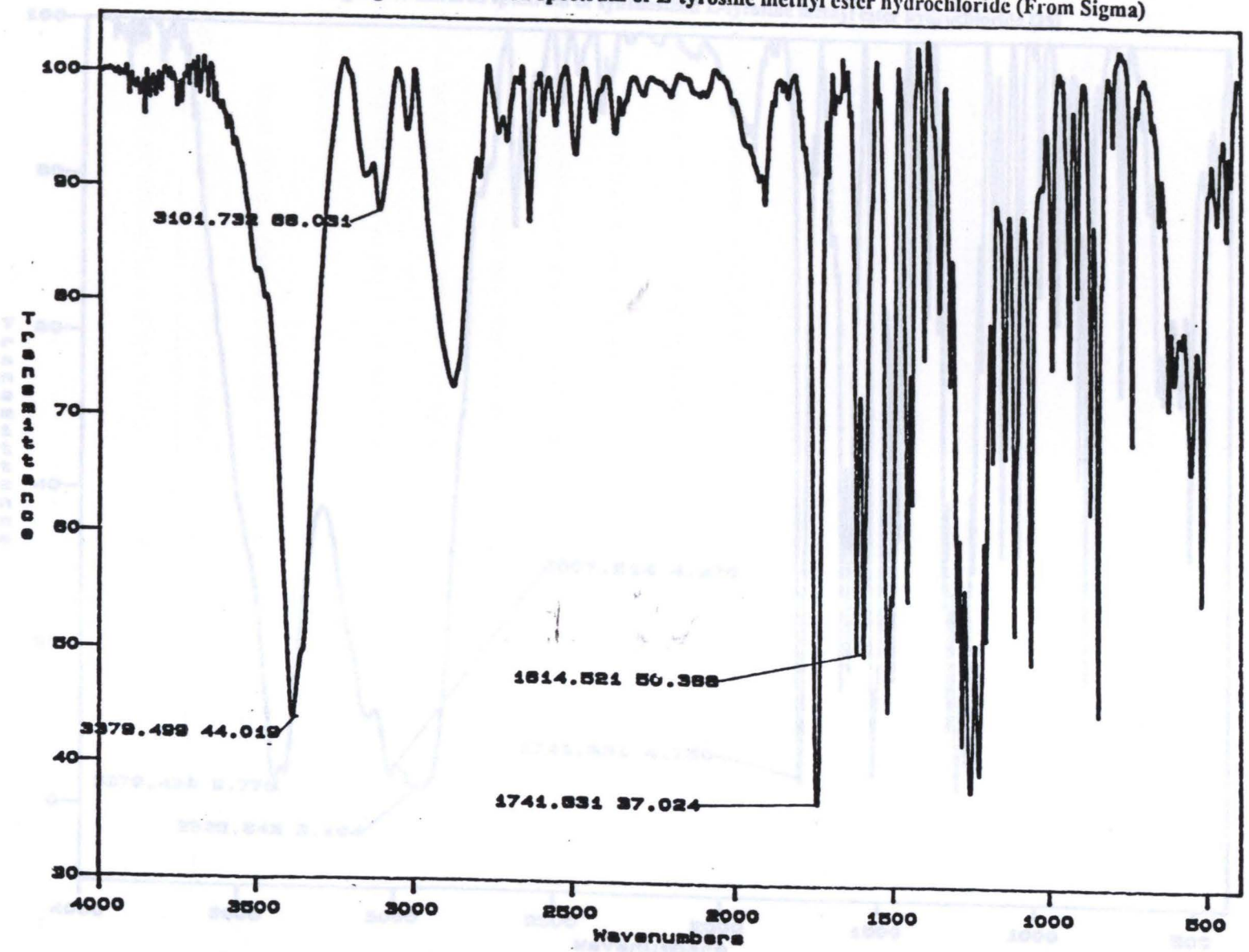


Figure 5.2 Infrared spectrum of L-tyrosine methyl ester hydrochloride (From Sigma)



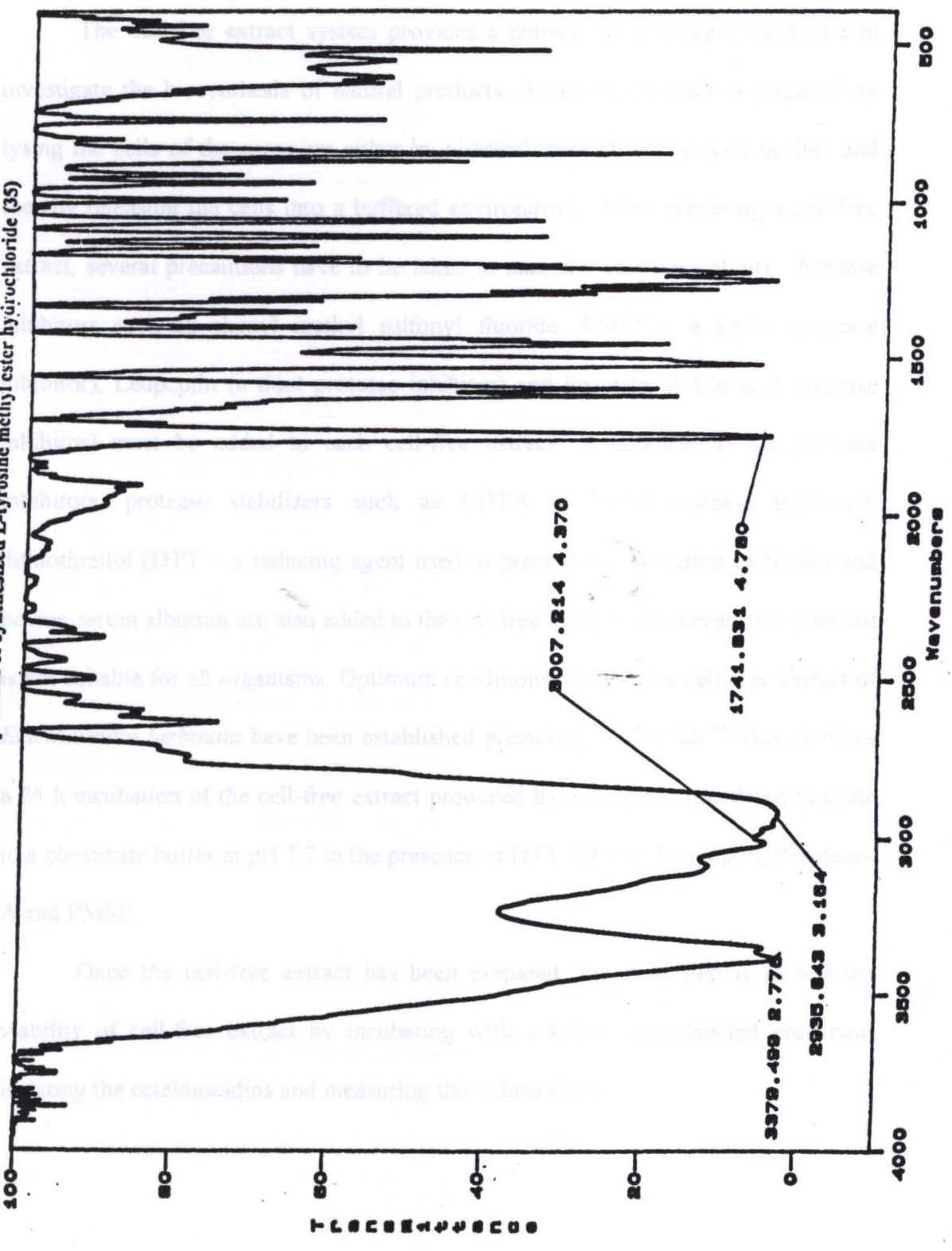


Figure 5.3 Infrared spectrum of synthesized L-tyrosine methyl ester hydrochloride (35)

5.2 The Cell-Free Extract

The cell-free extract system provides a convenient and rapid technique to investigate the biosynthesis of natural products. A cell-free extract is prepared by lysing the cells of the organism either by physical methods or by lysis buffers and thereby releasing the cells into a buffered environment. When preparing a cell-free extract, several precautions have to be taken to maintain enzyme stability. Protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF – a serine protease inhibitor), Leupeptin (a thiol protease inhibitor) and Pepstatin-A (an acid protease inhibitor) must be added to each cell-free extract. In addition to the protease inhibitors, protease stabilizers such as EDTA (a metalloprotease inhibitor), dithiothreitol (DTT – a reducing agent used to prevent the oxidation of thiols) and bovine serum albumin are also added to the cell-free extract. However, this protocol is not suitable for all organisms. Optimum conditions for a viable cell-free extract of *Ecteinascidia turbinata* have been established previously in our lab.[44] This involves a 24 h incubation of the cell-free extract produced by adding the powdered tunicate to a phosphate buffer at pH 7.7 in the presence of DTT, EDTA, Leupeptin, Pepstatin-A and PMSF.

Once the cell-free extract has been prepared, the next step is to test the viability of cell-free extract by incubating with a known radiolabeled precursor, isolating the ecteinascidins and measuring the radioactivity.

5.3 – Viability of the Cell-Free Extract

Before testing the intermediacy of diketopiperazines of phenylalanine and tyrosine in the biosynthesis of the ecteinascidins, the viability of the cell-free extract must be first determined. A cell-free extract of *Ecteinascidia turbinata* was prepared as described in the Section 6.6. Radiolabeled tyrosine was incubated with the cell-free extract (Section 6.7), and then the ecteinascidins were isolated and purified by vacuum flash column chromatography over C-18 and subsequently by RP-HPLC (Section 6.4) Ecteinascidins eluted at 1ml/min with a mobile phase of 82 % H_20 [0.1 % TFA] : 18 % CH_3CN. Ecteinascidins were eluted between 11.8 and 13 min. as a single peak. We have also observed that the retention time of ecteinascidin was dependent on the concentration of sample injection. The ecteinascidin peak (Figure: 5.5) was identified by comparison with the peak obtained from standard sample of Et 743 (Figure: 5.4). The fraction corresponding to ecteinascidins was tested for the recovered radioactivity. The peak corresponds to Et 743 showed the considerable recovered radioactivity (2000 dpm) confirming the viability of cell-free extract.

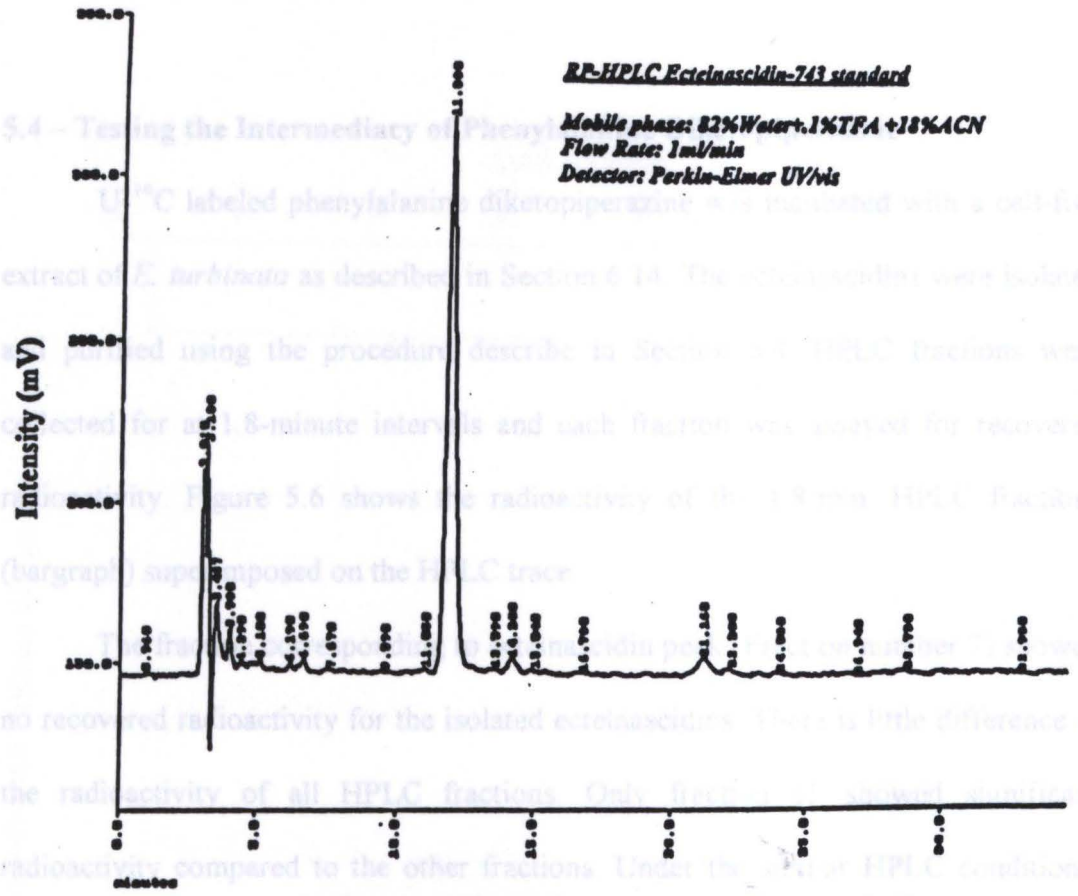


Figure 5.4 HPLC chromatogram of Et-standard

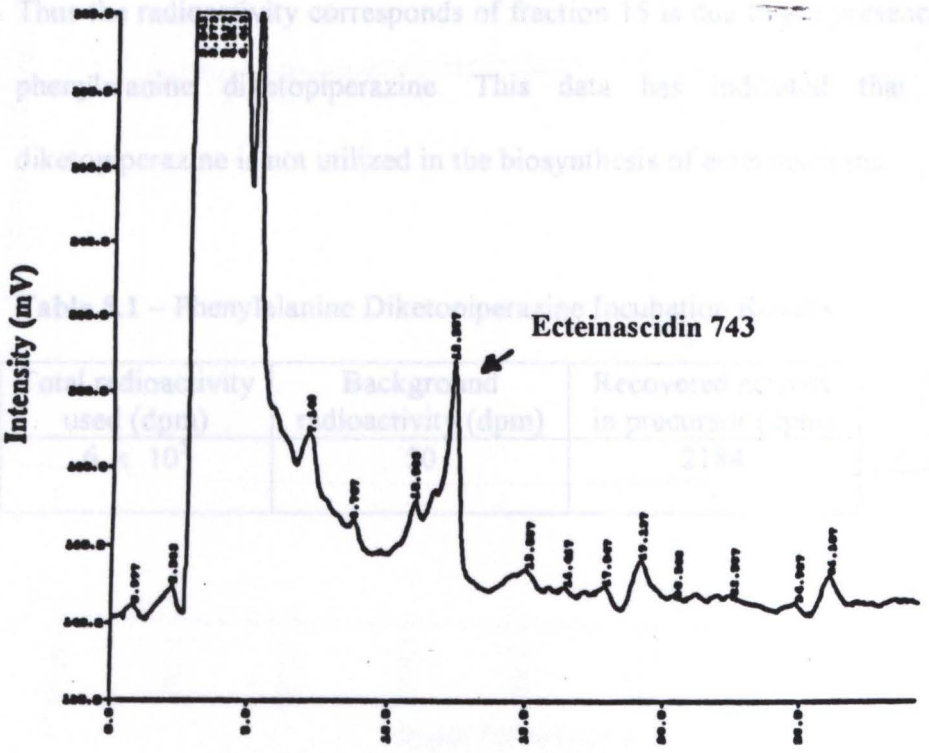


Figure 5.5 HPLC chromatogram of ^{14}C-tyrosine incubation studies

5.4 – Testing the Intermediacy of Phenylalanine Diketopiperazine

U-^{14}C labeled phenylalanine diketopiperazine was incubated with a cell-free extract of *E. turbinata* as described in Section 6.14. The ecteinascidins were isolated and purified using the procedure describe in Section 6.4. HPLC fractions were collected for at 1.8-minute intervals and each fraction was assayed for recovered radioactivity. Figure 5.6 shows the radioactivity of the 1.8-min. HPLC fractions (bargraph) superimposed on the HPLC trace.

The fraction corresponding to ecteinascidin peak (Fraction number 7) showed no recovered radioactivity for the isolated ecteinascidins. There is little difference in the radioactivity of all HPLC fractions. Only fraction 15 showed significant radioactivity compared to the other fractions. Under the similar HPLC conditions, phenylalanine diketopiperazine elutes at 26-28 minutes (Fraction 15) (Section 6.4). Thus the radioactivity corresponds of fraction 15 is due to the presence of unreacted phenylalanine diketopiperazine. This data has indicated that phenylalanine diketopiperazine is not utilized in the biosynthesis of ecteinascidins.

Table 5.1 – Phenylalanine Diketopiperazine Incubation Results

Total radioactivity used (dpm)	Background radioactivity (dpm)	Recovered activity in precursor (dpm)
6×10^5	50	2184

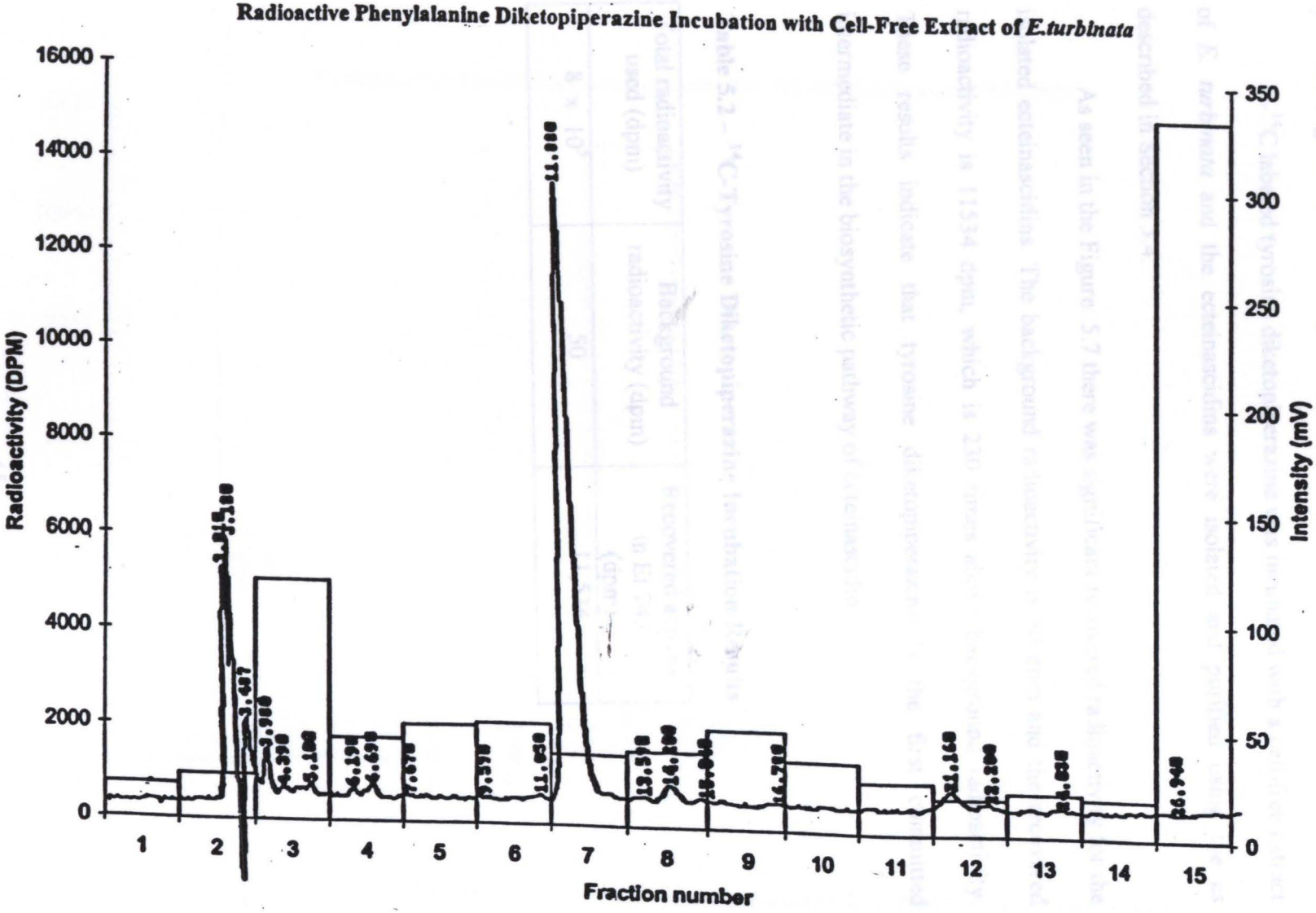


Figure 5.6 Recovered radioactivities of HPLC fractions superimposed on Et-743 standard HPLC profile

5.5 –Testing the Intermediacy of Tyrosine Diketopiperazine

U-^{14}C labeled tyrosine diketopiperazine was incubated with a cell-free extract of *E. turbinata* and the ecteinascidins were isolated and purified using the as described in Section 5.4.

As seen in the Figure: 5.7 there was significant recovered radioactivity for the isolated ecteinascidins. The background radioactivity is 50 dpm and the recovered radioactivity is 11534 dpm, which is 230 times above background radioactivity. These results indicate that tyrosine diketopiperazine is the first committed intermediate in the biosynthetic pathway of ecteinascidins.

Table 5.2 – ^{14}C-Tyrosine Diketopiperazine Incubation Results

Total radioactivity used (dpm)	Background radioactivity (dpm)	Recovered activity in Et 743 (dpm)
8 x 10^5	50	11,534

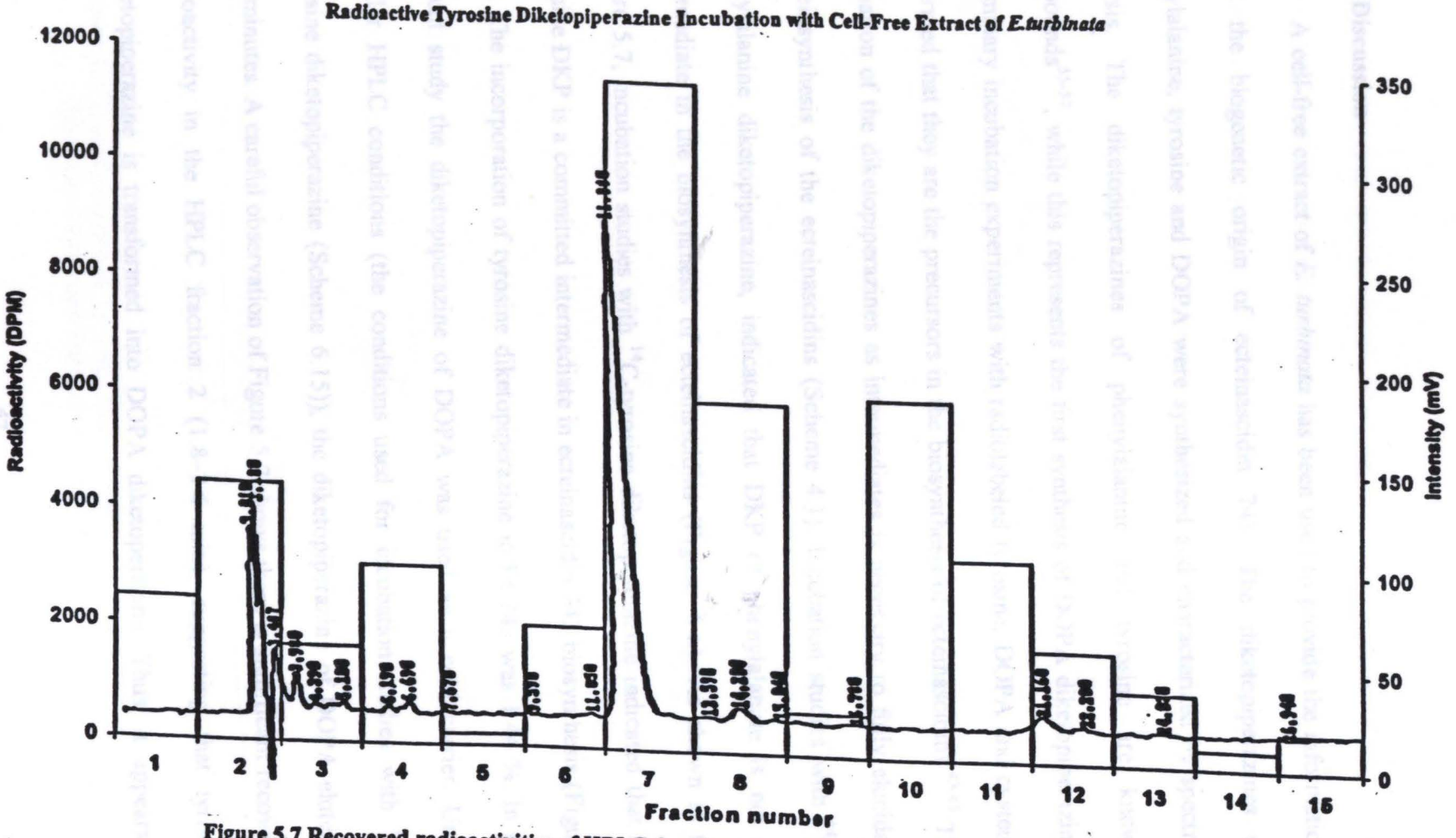


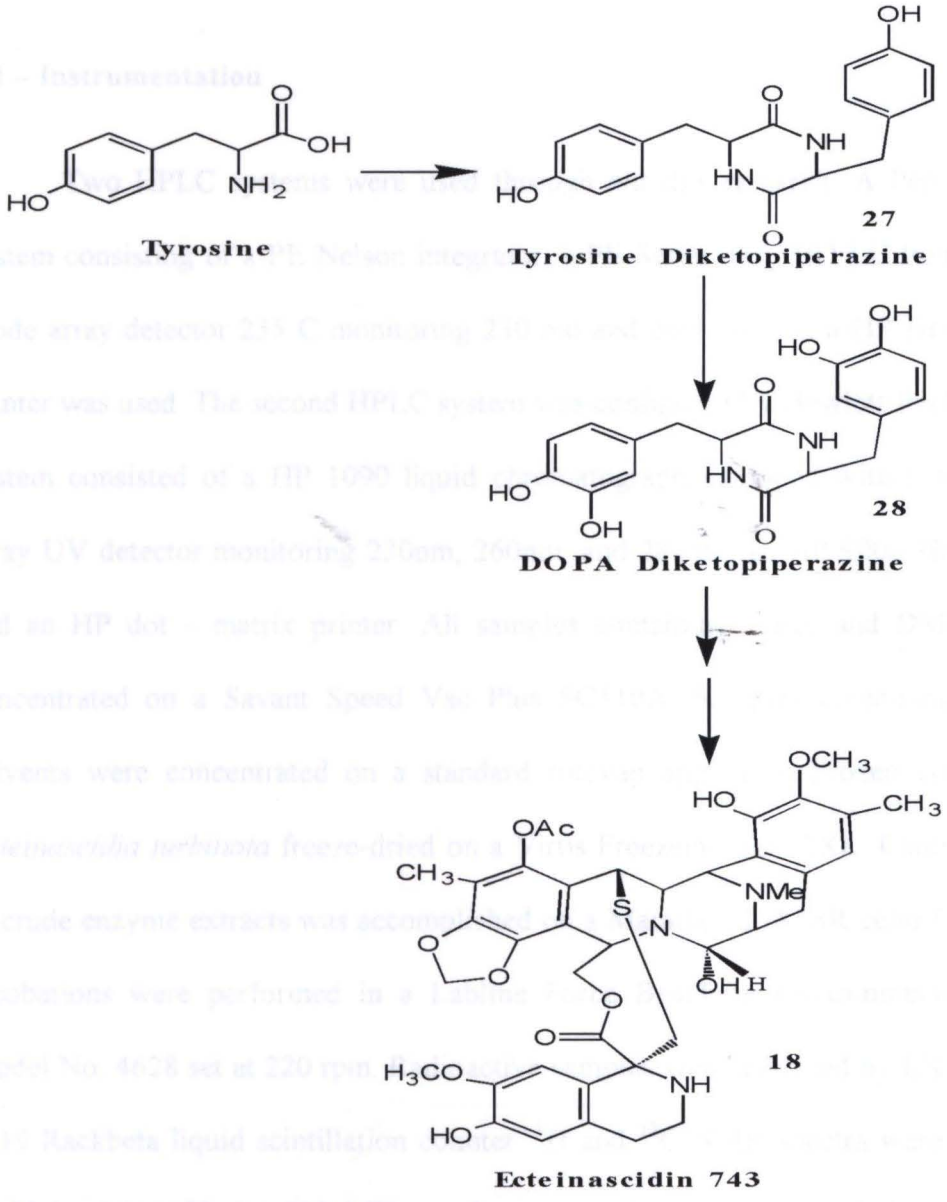
Figure 5.7 Recovered radioactivities of HPLC fractions superimposed on Et-743 standard HPLC Profile

5.6 – Discussion

A cell-free extract of *E. turbinata* has been used to provide the information about the biogenetic origin of ecteinascidin 743. The diketopiperazines of phenylalanine, tyrosine and DOPA were synthesized and characterized by spectral analysis. The diketopiperazines of phenylalanine and tyrosine are known compounds[55-57], while this represents the first synthesis of DOPA diketopiperazine. Preliminary incubation experiments with radiolabeled tyrosine, DOPA and cysteine confirmed that they are the precursors in the biosynthesis of ecteinascidins.[43-45] The evaluation of the diketopiperazines as intermediates is necessary to fully elucidate the biosynthesis of the ecteinascidins (Scheme 4.1). Incubation studies with ^{14}C-phenylalanine diketopiperazine, indicated that DKP of phenylalanine is not an intermediate in the biosynthesis of ecteinascidins (Figure: 5.6). As shown in the Figure 5.7, incubation studies with ^{14}C-tyrosine diketopiperazine indicated that the tyrosine DKP is a committed intermediate in ecteinascidin 743 biosynthesis (Figure: 5.7). The incorporation of tyrosine diketopiperazine in Et 743 was 1.44 %. In this present study the diketopiperazine of DOPA was used as a cold carrier. Under similar HPLC conditions (the conditions used for incubation studies with ^{14}C-tyrosine diketopiperazine (Scheme 6.15)), the diketopiperazine of DOPA elutes at 3.4 minutes. A careful observation of Figure 5.7 shows there is significant recovered radioactivity in the HPLC fraction 2 (1.8–3.6 min), suggesting that tyrosine diketopiperazine is transformed into DOPA diketoperazine. Thus, it appears as

though ecteinascidins are produced from the conversion of tyrosine to its diketopiperazine, followed by oxidation to DOPA diketopiperazine (Scheme 5.2).

To confirm the intermediacy of DOPA diketopiperazine, radiolabeled DOPA diketopiperazine must be synthesized and test with a cell-free extract.



Scheme 5.2 Transformation of Tyrosine Diketopiperazine to DOPA Diketopiperazine and Further to Ecteinascidin 743

Chapter 6

Experimental Section

6.1 – Instrumentation

Two HPLC systems were used through out this research. A Perkin Elmer system consisting of a PE Nelson integrator, a PE Series 410 BIO LC Pump. A PE diode array detector 235 C monitoring 230 nm and 260 nm, and a HP DeskJet 400 printer was used. The second HPLC system was configured by Hewlett-Packard. The system consisted of a HP 1090 liquid chromatograph equipped with a full diode array UV detector monitoring 230nm, 260nm, and 280nm, an HP 9000 Hard drive, and an HP dot – matrix printer. All samples containing water and DMSO were concentrated on a Savant Speed Vac Plus SC110A. Samples containing organic solvents were concentrated on a standard rotovap apparatus. Frozen colonies of *Ecteinascidia turbinata* freeze-dried on a Virtis Freezemobile 12XL. Centrifugation of crude enzyme extracts was accomplished on a Marathon 21K/BR centrifuge. CFE incubations were performed in a Labline Force Benchtop Environmental shaker Model No. 4628 set at 220 rpm. Radioactive samples were analyzed by LKB Wallac 1219 Rackbeta liquid scintillation counter. ^{1}H and ^{13}C NMR spectra were recorded on Unity Inova Varian-500 MHz nuclear magnetic spectrometer. Infrared spectra were obtained

on a Mattson 4020 Galaxy series FT-IR instrument. An Altex Ultraphere-ODS semi-preparative column (5 μm, 10 mm ID x 25 cm) was used for the purification of "cold" phenylalanine and tyrosine DKPs. A reversed phase C-18 Vydac (4.6 mm ID x 250 mm) analytical HPLC column was utilized for the purification of the Et standard, and for CFE incubation studies. A reversed phase C-18 Vydac (4.6 mm ID x 150 mm) analytical HPLC column was used for the purification of radiolabeled diketopiperazines.

6.2 – Materials

The reagents used in the synthesis of diketopiperazines and tyrosine methyl ester were obtained from Sigma and Aldrich Chemicals Co. All solvents used are optima or HPLC grade purchased from Fischer Scientific. Radiolabeled amino acids were purchased from American Radiolabeled Chemicals Inc. The 'Scintelene' scintillation fluid used to measure recovered radioactivity was purchased from FisherBiotech. EDTA, BSA, DTT, Leupeptin, Pepstatin A, PMSF, were purchased from Sigma Chemical Co. The flash column packing material was J.T. Baker Bakerbond C-18 silica 40 μm Prep LC packing.

6.3 – Collection of the Organism

Colonies of *E. turbinata* were collected at depths of 0.1m to 6 feet from a mangrove community, and Mayor's point located in Long Key, Florida. Freshly

collected tunicates were used in the preparation of the cell-free extract and which was prepared in the Keys Marine Laboratory (Florida Keys).

6.4 – Isolation of Ecteinascidins from Frozen Colonies of the Tunicate

Flash frozen tunicate (500 g) was lyophilized to dryness to yield 10 g of the dried organism. It was extracted by grinding with methanol (50 ml x 3) in a mortar and pestle. After filtration through celite, the methanolic extract was concentrated and separated through a C-18 flash column. A step gradient elution (100 % H_20 [0.1 %TFA] to 40 % CH_3CN, 20 % increments) of the extract afforded four fractions. The majority of the ecteinascidins were eluted with 60 % H_20 [0.1 % TFA] : 40 % CH_3CN. This fraction was then concentrated and ecteinascidins were purified by reversed phase HPLC. An analytical C-18 Vydac (4.6 mm ID x 250 mm) column was used on a Hewlett-Packard system monitoring 230nm. Ecteinascidins eluted at 1ml/min with a mobile phase of 82 % H_20 [0.1 % TFA] : 18 % CH_3CN. Ecteinascidins were eluted between 11.5 –13 min as a single peak. We have also observed that retention time of ecteinascidin was dependent on the concentration of sample injection. The ecteinascidin peak was detected at 230 nm by comparison with the peak obtained from standard Et 743 (Fig. 6.1).

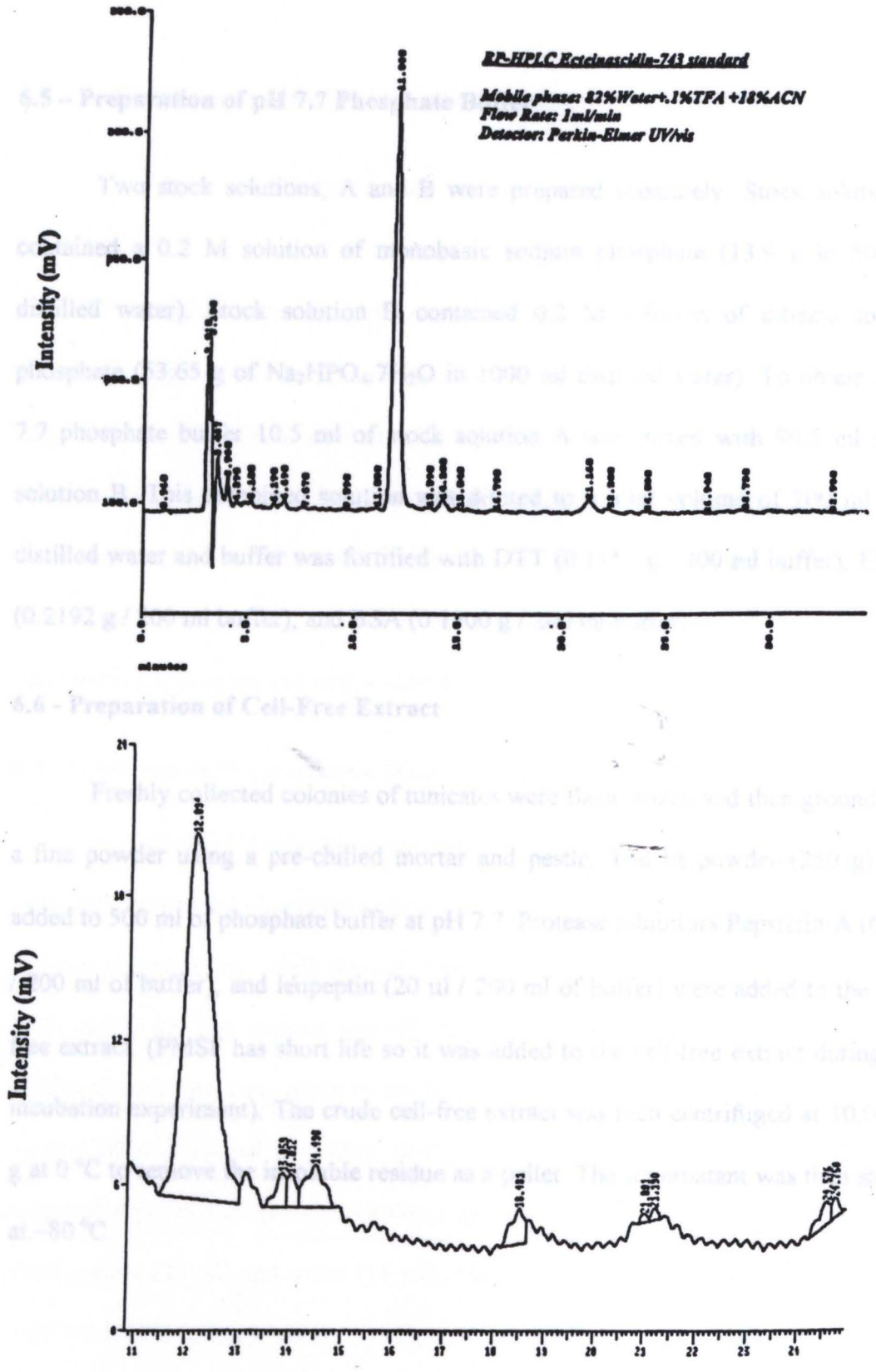


Figure 6.1 HPLC chromatograms of Et-standard and 40:60 (ACN : H2O [0.1 % TFA]) active fraction

6.5 – Preparation of pH 7.7 Phosphate Buffer

Two stock solutions, A and B were prepared separately. Stock solution A contained a 0.2 M solution of monobasic sodium phosphate (13.9 g in 500 ml distilled water). Stock solution B contained 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ in 1000 ml distilled water). To obtain a pH 7.7 phosphate buffer 10.5 ml of stock solution A was mixed with 90.5 ml stock solution B. This combined solution was diluted to a total volume of 200 ml with distilled water and buffer was fortified with DTT (0.1157 g / 200 ml buffer), EDTA (0.2192 g / 200 ml buffer), and BSA (0.1500 g / 200 ml buffer).

6.6 - Preparation of Cell-Free Extract

Freshly collected colonies of tunicates were flash frozen and then ground into a fine powder using a pre-chilled mortar and pestle. The Et powder (250 g) was added to 500 ml of phosphate buffer at pH 7.7. Protease inhibitors Pepstatin-A (60 µl / 200 ml of buffer), and leupeptin (20 µl / 200 ml of buffer) were added to the cell-free extract. (PMSF has short life so it was added to the cell-free extract during the incubation experiment). The crude cell-free extract was then centrifuged at 10,000 x g at 0 ^{0}C to remove the insoluble residue as a pellet. The supernatant was then stored at –80 ^{0}C.

6.7 – *In Vitro* Incubation of Radiolabeled Tyrosine with *E. turbinata*

To a 30-ml of cell-free extract of *E. turbinata*, 1 mg each of ATP Mg salt, NADP, NADPH and 45 µl of PMSF (1.74 mg in 50 µl ethanol) were added. 2.5 µCi of U-^{14}C tyrosine was incubated with the cell-free extract for 24 hours in a constant temperature bath at 27 °C. The reaction was quenched by adding equal amounts of ethyl acetate, which was subsequently removed. The extract was freeze-dried to remove excess water and the ecteinascidins were isolated using the step gradient elution of acetonitrile- water and purified by RP-HPLC as described in Section 6.4. The peak corresponding to the Et fraction was collected and the recovered radioactivity was measured with a scintillation counter.

6.8 – Synthesis of Phenylalanine Diketopiperazine (26)[55, 57]

A solution of L-phenylalanine methyl ester hydrochloride (0.323 g) and *N*-(*tert*- butoxycarbonyl)-L-phenylalanine (0.397 g) in DMF (3 ml) and acetonitrile (12 ml) was cooled on ice. With stirring, triethylamine (0.21 ml) was added followed by DCC (0.309 g). The mixture was allowed to stir at 0 °C for two hours. After the stirring the mixture was placed in the freezer overnight. The dicyclohexyl urea was filtered off and washed with ethyl acetate several times. The combined filtrate was evaporated under nitrogen gas leaving a gummy residue, which was extracted with ethyl acetate (25 ml) and water (15 ml). The organic layer was washed sequentially with 0.5 N HCl (15 ml), water (15 ml), 0.5N $NaHCO_3$ (15 ml) and brine (15 ml). The organic layer was then dried with sodium sulfate and the filtrate was evaporated

under nitrogen gas leaving a white solid. Purification by flash column chromatography (70% ethyl acetate/hexane) gave colorless foam of acyclic dipeptide of phenylalanine methyl ester.

The t-BOC- dipeptide methylester was then treated with formic acid (20 ml, 98 %) at room temperature for two hours with stirring. The formic acid was then evaporated leaving a residue of crude dipeptide ester formate. The dipeptide ester formate was dissolved in sec-butyl alcohol (40 ml) and toluene (10 ml) and the solution was refluxed under argon gas for two hours. The solution was concentrated to 5-10 ml and cooled to 0 ^{0}C, the product was then purified by C-18 column chromatography. A step gradient elution (100 % H_20 [0.1 %TFA] to 40 % CH_3CN, 20 % increments) of reaction product afforded four fractions. Diketopiperazine (**26**) was concentrated in the 100 % H_20 [0.1 %TFA] fraction. The phenylalanine diketopiperazine produced was 93% yield. The melting point obtained was 319 ^{0}C, similar to the literature value of 316 ^{0}C.[55] The phenylalanine diketopiperazine was then purified by RP-HPLC using an Altex Ultraphere-ODS semi-preparative column (5 μm, 10 mm ID x 25 cm). A linear gradient consisting of 75 % water (0.1 % TFA): 25 % CH_3CN to 100 % CH_3CN over 20 minutes at a flow rate of 2.2 ml/min afforded the diketopiperazine of phenylalanine (**26**) at 11.04 minutes. An IR analysis (KBr pellet, 1670 cm^{-1} (amide carbonyl), 3199.34 cm^{-1}, 3056.63 cm^{-1} (aromatic C-H stretch), 3300 cm^{-1} (N-H stretch)) and ^{1}H-NMR/d_6-DMSO (δ 7.27 (2H, dd), δ 7.19 (2H, t) δ7.020 (1H, d), δ 3.96 (2H, m), δ 2.25 (2H, dd), δ 2.19 (2H, dd) indicated

the formation of the diketopiperazine of phenylalanine.

6.9 – Synthesis of Radiolabeled Phenylalanine Diketopiperazine (26)

A solution of ^{14}C- labeled L-phenylalanine ethyl ester hydrochloride (50 μCi) and *N*- (*tert*- butoxycarbonyl)-L-phenylalanine (1.125 mg) in DMF (50 μl) and acetonitrile (450 μl) was cooled on ice. With stirring, triethylamine (10 μl) and DCC (1 mg) were added. The mixture was allowed to stir at 0°C for two hours. After the stirring, the mixture was placed in the freezer overnight. The dicyclohexyl urea was filtered off and washed with ethyl acetate several times. The reaction mix was washed with 0.5 N HCl (400 μl), water (400 μl), 0.5 N $NaHCO_3$ (400 μl) and brine (400 μl) and the organic layer evaporated under nitrogen gas. To the acyclic peptide of phenylalanine ethyl ester 2ml of formic acid was added to deprotect the carboxylic and amine groups. After 2 h of stirring the formic acid was evaporated and the reaction mix was dissolved in 1.5 ml of *sec*-butyl alcohol and toluene (1 ml). Then the solution was refluxed under argon for 2 hours. After evaporating the toluene and *sec*-butyl alcohol, the reaction mix was cooled to 0 °C and the crude radioactive phenylalanine diketopiperazine was purified by RP-HPLC. A linear gradient consisting of 80 % water (0.1 % TFA): 20 % CH_3CN to 100 % CH_3CN over 10 minutes at a flow rate of 1ml/min using a reversed phase C-18 Vydac (4.6 mm ID x 150 mm) analytical HPLC column afforded the radiolabeled phenylalanine diketopiperazine (**26**).

6.10 - Synthesis of Tyrosine Diketopiperazine (27) [55, 57]

A solution of L-tyrosine methyl ester hydrochloride (0.348 g) and *N*- (*tert*-butoxycarbonyl)-L-tyrosine (0.422 g) in DMF (3 ml) and acetonitrile (12 ml) was cooled on ice. With stirring, triethylamine (0.21 ml) was added followed by DCC (0.309 g). The mixture was allowed to stir at 0 °C for two hours. After the stirring the mixture was placed in the freezer overnight. The dicyclohexyl urea was filtered off and washed with ethyl acetate several times. The combined filtrate was evaporated under nitrogen gas leaving a pale yellow colored gummy residue, which was extracted with ethyl acetate (25 ml) and water (15 ml). The organic layer was washed sequentially with 0.5 N HCl (15 ml), water (15 ml), 0.5 N $NaHCO_3$ (15 ml) and brine (15 ml). The organic layer was then dried with sodium sulfate and the filtrate was evaporated under nitrogen gas leaving a yellow solid. Purification by flash column chromatography (R_f 0.40, 70% ethyl acetate/hexane) gave a color less foam of acyclic dipeptide of tyrosine methyl ester.

The t-BOC- dipeptide methylester was then treated with formic acid (20 ml, 98 %) at room temperature for two hours with stirring. The formic acid was then evaporated leaving a residue of crude dipeptide ester formate. The dipeptide ester formate was dissolved in *sec*-butyl alcohol (40 ml) and toluene (10 ml) and the solution was refluxed under argon gas for two hours. The solution was concentrated to 5-10 ml and cooled to 0 °C, the product was then purified by C-18 column chromatography. A step gradient elution (100 % H_20 [0.1 %TFA] to 40 % CH_3CN,

20 % increments) of reaction product afforded four fractions. Diketopiperazine was concentrated in (100 % H_20 [0.1 %TFA]) fraction. The tyrosine diketopiperazine produced was 96% yield. The melting point obtained was 280 ^{0}C, with a literature value of 278 ^{0}C. Then tyrosine diketopiperazine was then purified by RP-HPLC using an Altex Ultraphere-ODS semi-preparative column (5 μm, 10 mm ID x 25 cm). An isochratic gradient consisting of 82 % water (0.1 % TFA):18 % CH_3CN at a flow rate of 1ml/min afforded the diketopiperazine of tyrosine12.25 minutes. IR of the HPLC purified fraction (KBr) 1671.14 cm^{-1} (an amide carbonyl), 3150 cm^{-1} and 2923.89 cm^{-1} (aromatic -C-H- stretch), ^{1}H- NMR/(d_6DMSO) analysis (δ 9.2 (2 H s, OH), δ 7.9 (2H, br s NH), δ 6.86 (4 H, d), δ 6.69 (4H, d), δ 3.89 (2H, m), δ 2.55 (2H, dd), δ 2.139 (2H, dd) and ^{13}C-NMR / (d_6-DMSO) analysis (δ 166.50 (amide carbonyl), δ 156.1 (phenolic hydroxyl group), δ 56 (-CH-), and δ 38.7 (-CH_2-)) confirmed the formation of diketopiperazine of tyrosine (**27**).

6.11 – Synthesis of Tyrosine Methyl Ester (38)

L-tyrosine (0.181 g) was dissolved in 15 ml of 2,2-dimethoxy propane. To this reaction mixture 1ml of 36.5 % (12N) HCl was added and stirred for 18 h. The reaction mixture turned dark brown in color. The mixture was concentrated by vacuum and was dissolved in methanol. The methylester of tyrosine was recrystallised from methanol / ethyl ether. The tyrosine methyl ester produced was 95% yield. The melting point of the compound is 189 ^{0}C which matches the

literature value. IR spectrum of synthesized tyrosine methyl ester was compared with tyrosine methyl ester purchased from Sigma Co.

6.12 – Synthesis of Radiolabeled Tyrosine Methyl Ester

^{14}C-labeled L-tyrosine methyl ester is not available commercially. 50 μCi of L- tyrosine was dissolved in 2, 2 dimethoxy propane (1.5 ml). To this reaction mixture 100 μl of 36 % HCl (12N) was added and the reaction mix was allowed to stir for 18 h. The mixture was then purified by passing through a silica column. The purified product was then evaporated under nitrogen gas.

6.13 – Synthesis of Radiolabeled Tyrosine Diketopiperazine (27)

A solution of radiolabeled (^{14}C) tyrosine methyl ester and *N*- (*tert*-butoxycarbonyl)-L-tyrosine (1.125 mg) in DMF (50 μl) and acetonitrile (450 μl) was cooled on ice. With stirring, triethylamine (10 μl) and DCC (1 mg) was added. The mixture was allowed to stir at 0 ^{0}C for two hours. After the stirring the mixture was placed in the freezer overnight. The dicyclohexyl urea filtered off and washed with ethyl acetate several times. The reaction mix was added with 0.5 N HCl (400 μl), water (400 μl), 0.5 N $NaHCO_3$ (400 μl) and brine (400 μl). The organic layer was evaporated under nitrogen gas. To the acyclic dipeptide of tyrosine methyl ester 2ml of formic acid was added to deprotect the carboxylic and amine groups. After 2 h of stirring the formic acid was evaporated and the reaction mix was dissolved in 1.5 ml of *sec*-butyl alcohol and toluene (1 ml). Then the solution was refluxed under argon

for 2 hours. After evaporating the toluene and *sec*-butyl alcohol, the reaction mix was cooled to 0 °C and the crude radioactive tyrosine diketopiperazine was purified by RP-HPLC. An isochratic system consisting of 82 % water (0.1 % TFA): 18 % CH_3CN at a flow rate of 1ml/min using a reversed phase C-18 Vydac (4.6 mm ID x 150 mm) analytical HPLC column afforded the radiolabeled tyrosine diketopiperazine.

6.14 – Incubation of Radiolabeled Phenylalanine Diketopiperazine

A cell-free extract of *E. turbinata* was prepared (Section 6.6) in pH 7.7 phosphate buffers (Section 6.5) and incubated with radiolabeled phenylalanine diketopiperazine (0.3 μCi) following the procedure described in Section 6.7. The ecteinascidins were isolated and purified using the procedure described in Section 6.4. Fractions were collected for every 1.8 minutes and the recovered radioactivity of the ecteinascidins was measured using a scintillation counter.

6.15 - Incubation of Radiolabeled tyrosine Diketopiperazine (27)

A cell-free extract of E. turbinata was prepared (section 6.6) in pH 7.7 phosphate buffer (section 6.5) and incubated with radiolabeled tyrosine diketopiperazine (0.4 μCi) following the procedure described in Section 6.7. The ecteinascidins were isolated and purified using the procedure described in Section 6.4. Fractions were collected for every 1.8 minutes and the recovered radioactivity of the ecteinascidins was measured using a scintillation counter.

6.16 – Synthesis of DOPA Diketopiperazine (28) [55,57]

A solution of L-DOPA methyl ester hydrochloride (0.100 g) and *N*- (*tert*-butoxycarbonyl)-L-DOPA (0.193 g) in DMF (1.5 ml) and acetonitrile (8 ml) was cooled on ice. With stirring, triethylamine (0.21 ml) was added followed by DCC (0.90 g). The mixture was allowed to stir at 0°C for two hours. After the stirring the mixture was placed in the freezer overnight. The dicyclohexyl urea was filtered off and washed with ethyl acetate several times. The combined filtrate was evaporated under nitrogen gas leaving a pale yellow colored gummy residue, which was extracted with ethyl acetate (15 ml) and water (10 ml). The organic layer was washed sequentially with 0.5 N HCl (10 ml), water (10 ml), 0.5 N $NaHCO_3$ (10 ml) and brine (10 ml). All steps were conducted under continuous source of argon. The organic layer was then dried with sodium sulfate and the filtrate was evaporated under nitrogen gas leaving a yellow gummy material.

The t-BOC- dipeptide methylester was then treated with formic acid (10 ml, 98 %) at room temperature for two hours with stirring. The formic acid was then evaporated leaving a residue of crude dipeptide ester formate. The dipeptide ester formate was dissolved in *sec*-butyl alcohol (20 ml) and toluene (5 ml) and the solution was refluxed under argon gas for two hours. The solution was concentrated to 5-10 ml and cooled to 0 °C, the product was then purified by C-18 column chromatography. A step gradient elution (100 % H_20 [0.1 %TFA] to 40 % CH_3CN, 20 % increments) of reaction product afforded four fractions. Diketopiperazine was

concentrated in (100 % H_20 [0.1 %TFA]) fraction. The DOPA diketopiperazine produced was 83% yield. The product could not be crystallized and therefore the melting point of the compound could not be determined. The DOPA diketopiperazine was then purified by RP-HPLC using an Altex Ultraphere-ODS semi-preparative column (5 μm, 10 mm ID x 25 cm). A linear gradient system consisting of 75 % water (0.1 % TFA): 25 % CH_3CN to 100 % CH_3CN over 25 minutes at a flow rate of 2.0 ml/min afforded the diketopiperazine of DOPA (**28**) at 13.7 minutes. ^{1}H- NMR /(d_6-DMSO) analysis (δ 8.7(2 H, s, OH), δ 8.1 (2H, br s, NH), δ 6.55 (1 H, dd, J = 8.5, 1.5 Hz), δ 6.51 (1, br s), δ 6.36(1H, br d, J = 8.0 Hz), δ 4.15 (2H, m), δ 2.75 (2H, ddd, J = 4 Hz), δ 1.9 (2H, m) indicated the formation of diketopiperazine of DOPA. The NMR spectra also showed some peaks in the region of δ 1-1.7, and a triplet at δ 3.50. These peaks are due to the impurities from dicyclohexylurea, which is the byproduct of dicyclohexylcarbodiimide, the coupling agent used in the synthesis of acyclic dipeptide.

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