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Structural Determination of Biologically Active Peptaibol Metabolites of *Tolypocladium geodes* LP237

Sotiria Pischos

A Thesis

in

The Department

of

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Presented in Partial Fulfillment of the Requirements
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ABSTRACT

Structural Determination of Biologically Active Peptaibol Metabolites of *Tolypocladium geodes* LP237

Sotiria Pischos

Three antibiotic peptides, LP237-F8 (**1**), F5 (**2**) and F7 (**3**), were isolated from the liquid cultures of the fungus *Tolypocladium geodes*. Chemical shift assignments of the ^{1}H and ^{13}C NMR resonances and sequencing of these metabolites were achieved by extensive high-field 2D NMR spectroscopy. The N-terminal of peptides **1** and **2** is protected with an octanoyl (Oc) fatty acid unit, whereas that of peptide **3** is protected with a decanoyl (Dec) unit. The C-terminal of all three peptides is protected with the amino alcohol leucinol (Leol). All three metabolites contain the common amino acids Ala, Phe or Tyr, Pro and Gln, as well as the unusual amino acid α-aminoisobutyric acid (Aib). In addition, peptides **1** and **2** contain the amino acid α-amino-α-ethyl-*n*-pentanoic acid (α-ethylnorvaline, EtNor), which has not been previously reported as a constituent of a natural product. Metabolites **1**, **2** and **3** are new members of the class of natural products known as peptaibols.

To my parents, Constantina and Vassilios

and

to my sisters, Marigo and Andrianna

who have given me the love, courage, and support to face life's challenges.

To Tullio,

my friend, my love, my inspiration.....my soul mate

who has instilled in me the appreciation for knowledge.

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Abbreviations

A	angstroms
Ac	acetyl
Aib	α-aminoisobutyric acid
Ala	alanine
Asp	aspartate
ATP	adenosine triphosphate
ATPase	adenosine triphosphate synthetase
AZT	3'-azido-3'-deoxythymidine
BIRD	bilinear rotation decoupling
br	broad
°C	degree Celcius
cm	centimeters
COSY	correlated spectroscopy
d	doublet
D	deuterium
dd	doublet of doublets
Dec	decanoyl
DEPT	distortionless enhancement by polarization transfer
Diazald	*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
Et	ethyl
EtNor	ethylnorvaline
EtOAc	ethyl acetate
FAB	fast atom bombardment
g	gram
Gly	glycine
Gln	glutamine
Glu	glutamic acid
Glx	Glu or Gln

h	hour
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
Hyp	hydroxyproline
IC_{50}	inhibitory concentration
Ile	isoleucine
IR	infrared
Iva	isovaline
L	liter
LB	Luria-Bertani
Leu	leucine
Lol	leucinol
m	meter
m (in NMR tables)	multiplet
M	molar
μ	micron
MALDI	matrix-assisted laser desorption ionization
Me	methyl
MeOH	methanol
mg	milligrams
μg	microgram
MHz	megahertz
min	minute
ml	milliliter
μL	microliter
μM	micromolar
mm	millimeter
mmol	millimole
mol	mole
ms	mass spectrum
M. W.	molecular weight
n	normal
NBA	nitrobenzyl alcohol
ng	nanograms
nm	nanometers

NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
NOESY	NOE spectroscopy
Oc	octanoyl
ot	overlapping triplet
pét. ether	petroleum ether
Ph	phenyl
Phe	phenylalanine
Phol	phenylalaninol
ppm	parts per million
Pro	proline
ROESY	rotating-frame Overhauser enhancement spectroscopy
rpm	revolutions per minute
r. t.	room temperature
s	singlet
sec	second
Ser	serine
shd	shoulder
T	target
t	triplet
t	tertiary
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TMS	trimethylsilane
TOCSY	totally correlated spectroscopy
Trpol	tryptophanol
Tyr	tyrosine
UV	ultraviolet
v	volume
Val	valine

Chapter 1

INTRODUCTION

1.1 Characteristics of fungi

The word *fungus* has long been associated with discomforting thoughts. Perhaps this is due to the knowledge that fungi spoil food and they are plant, animal and human pathogens. However, fungi are an intricate part of all ecological systems. They play a very important role in decomposing dead organisms, thus recycling chemical elements vital to the environment. Even greater is the role they play for humans; not only do we eat fungi, such as mushrooms, but they are a major contributor to the pharmaceutical industry, where fungi are used to produce valuable drugs such as penicillin (from species of the genus *Penicillium*).

Fungi are eukaryotic organisms which can be either unicellular, for example yeast, or multicellular. They are composed of a mass of filaments, known as **hyphae**, which branch repeatedly to form a **mycelium**. The mycelium provides a large surface area which serves the fungus as a means of absorbing its nutrients.[1] The kingdom of fungi includes more than 100,000 species[1] and can be divided into four groups based on variations in sexual reproduction: zygomycetes, ascomycetes, basidiomycetes and deuteromycetes. For instance, zygomycetes, such as the black bread mold, ***Rhizopus stolonifer*** , are mostly terrestrial fungi in which sexual reproduction results in tough zygotes, called zygospores that can remain dormant when the environment is too harsh for growth of the fungus.

---

[1] Campbell, N. A. ; *In* Biology, The Benjamin Cummings Publishing Company, Inc Menlo Park, California, 1987, pp 586-599

1.2 *Tolypocladium geodes*

Tolypocladium geodes belongs to the group of fungi known as deuteromycetes. The fungi belonging to this group are also called imperfect because they have no known sex life cycle, and their conidia are formed asexually. The genus *Tolypocladium* was established by Gams in 1971 and includes 11 species: *T. balanoides*, *T. cylindrosporum*, *T. extinquens*, *T. geodes*, *T. inflatum* (or *T. niveum*), *T. microsporum*, *T. nubicola*, *T. parasiticum*, *T. terricola*, *T. trigonosporum*, and *T. tundrense*.

Tolypocladium species are Hyphomycetes with sparingly branched conidiophores, swollen phialides, and small one-celled conidia borne in slimy heads.[2a] *T. geodes* colonies (Figure 1.1) grow slowly, reaching a diameter of 10 - 15 mm after 10 days at room temperature. Colonies of *T. geodes* have a pronounced earthy odor and are distinguished by their phialides and conidia. Conidiophores are very sparingly branched and the phialides are usually solitary and often are borne directly on undifferentiated hyphae.[2a] This species has been found in Canada, The Netherlands, England, Denmark and Austria in soil at high altitudes. Although fungi of the *Tolypocladium* genus are commonly found in cold climates, *T. geodes* is a much more rare organism and is very sensitive to warm temperatures.[3] The *T. geodes* isolate LP237 was found in soil collected from the Pennine mountains in England at an altitude of 600 meters.[2b,c]

---

[2] a) Bissett, J *Can. J. Bot.* **1983**, *61*, 1311 b) *T. geodes* isolate LP237 was originally isolated by Dr P. Widden in 1982; Widden, P. *Mycologia*, **1987**, *79*, 298 c) The culture was deposited at the Biosystematics Research Institute (Ottawa, Canada) and its taxonomic identity was confirmed by J. Bissett d) Lundgren, B.; Baath, E.; Söderstrom, B I· *Trans. Brit. Mycol. Soc.* **1978**, *70*, 305

[3] Dowding, P. and Widden, P *In* Soil Organisms and Decomposition in Tundra. Holding, A. J. *Ed.* Tundra Biome Steering Committee, Stockholm, 1974, pp 123-150.

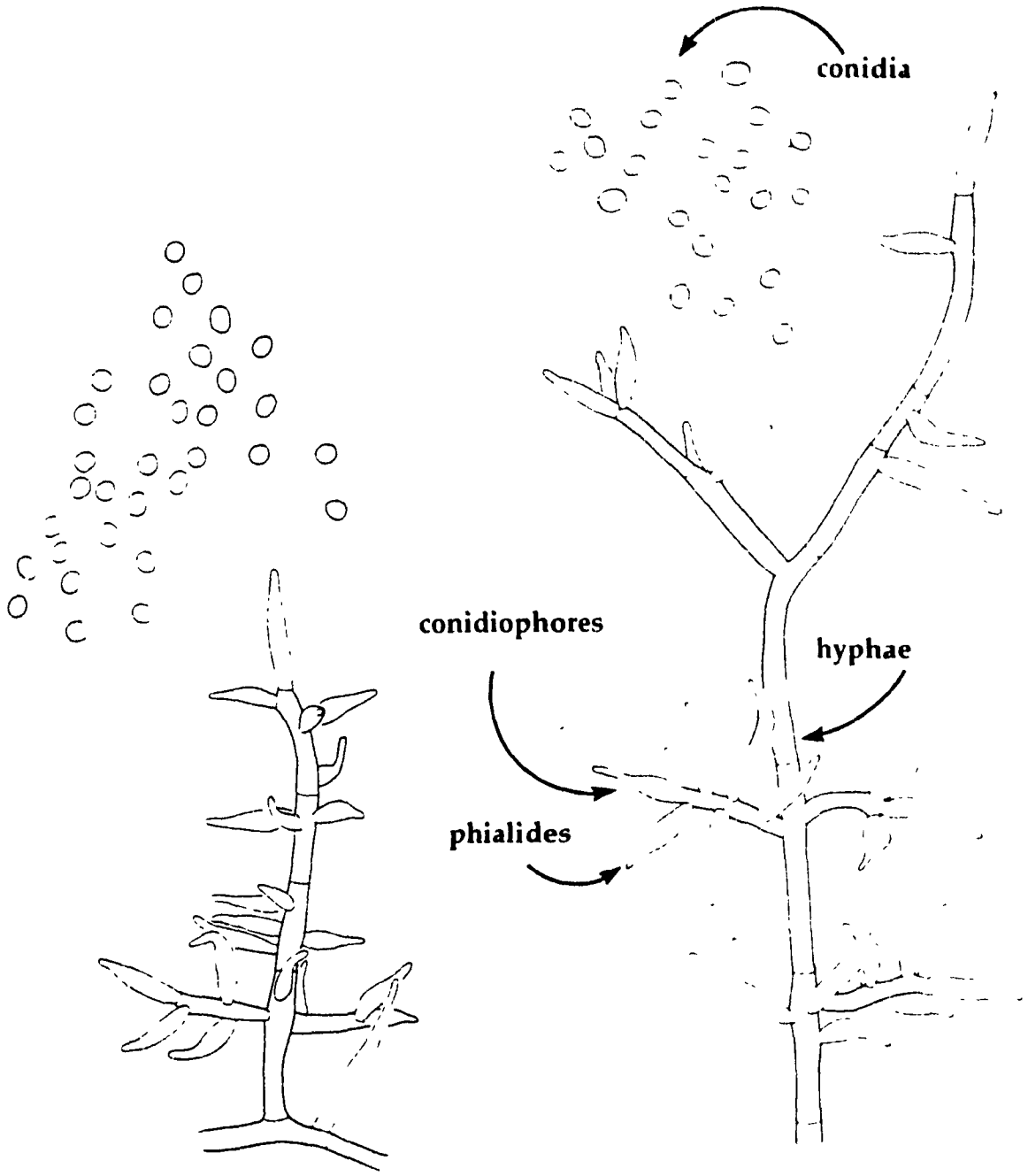


Figure 1.1 ***Tolypocladium geodes.***

1.3 Biologically active metabolites from the *Tolypocladium* genus

A multitude of secondary metabolites have been isolated from fungi, many of which are known to be peptides. In the past, several biologically active peptides have been isolated from *Tolypocladium* fungi, including the cyclosporins,[4] the efrapeptins[5] and the elvapeptins.[6]

Cyclosporins are interesting peptides which contain common amino acids, such as leucine and alanine, and an unusual amino acid which contains an olefinic side chain. Cyclosporin A (1), extracted from *T. inflatum*, was isolated by Traber and coworkers in 1977.[4b] It is a cyclic nonpolar undecapeptide used in organ transplant surgeries as an immunosuppressive agent. More specifically, cyclosporin A suppresses antigen-activated T-lymphocytes, which are known to initiate the immune response, and thus antibody formation is inhibited. It has also been shown that cyclosporin A suppresses delayed-type hypersensitivity and interferes with the release of inflammatory mediators in mice.[4c]

The efrapeptins are a complex mixture of peptide antibiotics also produced from the fungus *Tolypocladium niveum*. These peptides exhibit strong antifungal and insecticidal

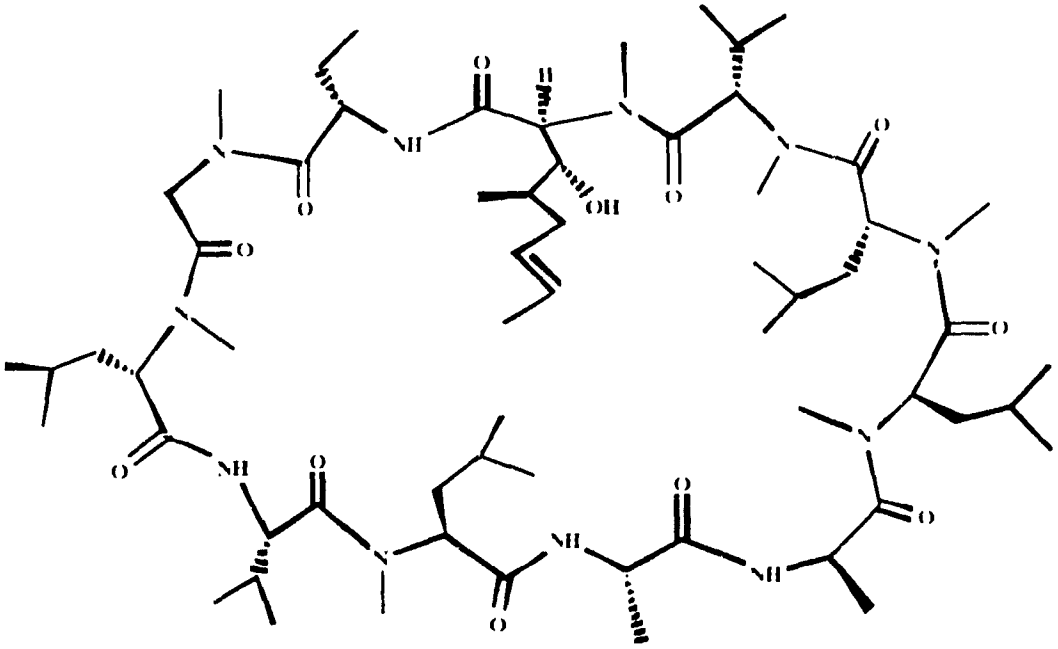
---

[4] a)Review Ovchinnikov, Y. A. and Ivanov, V. T. "The Cyclic Peptides: Structure, Conformation, and Function" *In* The Proteins Vol V, Neurath, H. and Hill, R. L. Eds Academic Press, New York, 1982, pp 547-555 b) Traber, R., Kuhn, M.; Loosli, H.-R.; Pache, W., Wartburg, A. *Helv. Chim. Acta*, **1977**, *60*, 1568. c) Colegate, S. M., Molyneux, R. J. *In* Bioactive Natural Products: Detection, Isolation and Structural Determination, CRC Press, Boca Raton, Florida, 1993, p12.

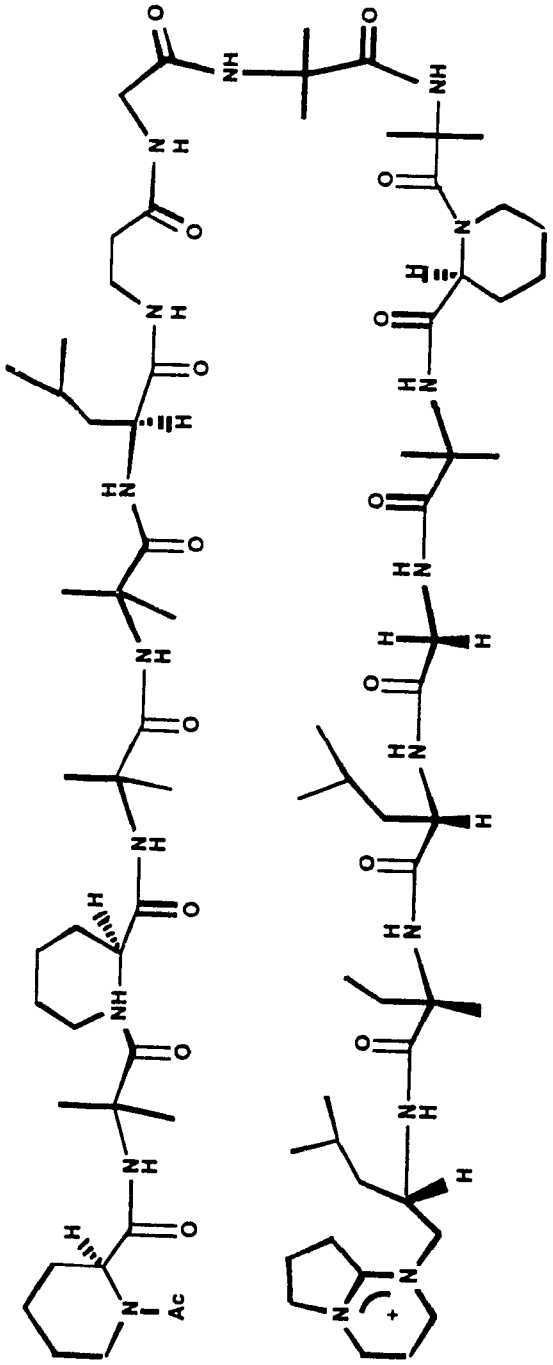
[5] a) Jackson, C. G., Linnett, P. E.; Beechey, R. B. and Henderson, P. J. F. *Biochem. Soc. Trans.* **1979**, *7*, 224. b) Bullough, D. A.; Jackson, C. G.; Henderson, P. J. F.; Cottee, F. H., Beechey, R. B. and Linnett, P. E. *Biochem. Int.* **1982**, *4*, 543 c) Gupta, S.; Krasnoff, S. B.; Roberts, D. W. and Renwick, J. A. A. *J. Am. Chem. Soc.* **1991**, *113*, 707. d) Krasnoff, S. B.; Gupta, S., St. Leger, R. J.; Renwick, J. A. A. and Roberts, D. W. *J. Invertebr. Path.* **1991**, *58*, 180 e) Gupta, S.; Krasnoff, S. B.; Roberts, D. W., Renwick, J. A. A., Brinen, L. S. and Clardy, J. *J. Org. Chem.* **1992**, *57*, 2306

[6] Bullough, D. A.; Jackson, C. G., Henderson, P. J. F.; Beechey, R. B. and Linnett, P. E. *FEBS Lett.* **1982**, *145*, 258

activity. For example, efrapeptin D (**2**) inhibits mitochondrial oxidative phosphorylation and ATPase activity in fungi and insects. It is believed that the efrapeptins act by binding competitively to the catalytic site of the soluble (F_1) component of mitochondrial ATPase,[5d] the enzyme that synthesizes ATP, thus blocking an essential arginine residue at the adenine nucleotide binding site. Unlike the cyclosporins, efrapeptin peptides are linear molecules that are hydrophobic and contain a C-terminal bicyclic amine blocking group, thought to be important in the biological activity of efrapeptins.[5c,e]



Cyclosporin A (1)



Efrapeptin D (2)

The elvapeptins are yet another family of antibiotics that have been isolated from *T. inflatum*. It has been reported that these peptides are produced when the cultures of *T. inflatum* are grown under limited aeration conditions,[5] and that they are potent inhibitors of mitochondrial ATPase activity. Bollough and coworkers[6] reported that although elvapeptin D is composed of the same amino acids (α-aminoisobutyric acid, isovaline, glycine, leucine, pipecolic acid, alanine and β-alanine) as efrapeptin D, the two peptides are clearly different. Analysis by FAB mass spectrometry revealed that the difference between the two antibiotics was in the nature of their C-terminus. The complete structure of elvapeptins still remains unknown.

1.4 The Peptaibols

Secondary metabolites belonging to the peptaibol family of antibiotics were isolated from the *T. geodes* isolate LP237. The isolation and structure determination of these natural products is reported in the following chapters. The peptaibols are a large group of membrane modifying peptides isolated from *Trichoderma* species such as *T. longibrachiatum*, *T. viride* and *T. harzianum*. All peptaibols have common characteristics, which include an amino acid sequence rich in α-aminoisobutyric acid, a protected amino terminus, and a C-terminal amino alcohol, such as leucinol,[7] phenylalaninol[8] or

[7] a) Rebuffat, S.; Prigent, Y., Auvin-Guette, C., Bodo, B. *Eur J Biochem* **1991**, *201*, 661 b) Auvin Guette, C.; Rebuffat, S., Prigent, Y., Bodo, B. *J Am. Chem. Soc.* **1992**, *114*, 2170 c) Matsura, K., Yesilada, A.; Iida, A.; Takaishi, Y., Kanai, M., Fujita, T. *J. Chem. Soc. Perkin Trans 1* **1993**, 381 d) Fujita, T.; Wada, S.; Iida, A.; Nishimura, T., Kanai, M., Toyama, N. *Chem. Pharm Bull.* **1994**, *42*, 489 e) Fujita, T.; Takaishi, Y. Shiromoto, T. *J Chem. Soc Chem. Comm.* **1979**, 413

[8] a) Pandey, R.C.; Cook, Jr., J.C., Rinehart, Jr., K.L. *J Am. Chem. Soc.* **1977**, *99*, 5205 b) Fujita, T., Iida, A.; Uesato, S., Takaishi, Y., Shingu, T., Saito, M., Morita, M. *J. Antibiot.* **1988**, *41*, 814 c) Iida, A., Okuda, M., Uesato, S., Takaishi, Y., Shingu, T., Morita, M., Fujita, T. *J. Chem. Soc., Perkin Trans 1* **1990**, 3249. d) Nagaoka, Y.; Iida, A., Fujita, T. *Chem. Pharm Bull* **1994**, *42*, 1258 e) Huang, Q., Tezuka, Y., Kikuchi, T.; Nishi, A., Tubaki, K., Tanaka, K. *Chem. Pharm. Bull.* **1995**, *43*, 223 f) Pandey, R.C.; Cook, Jr., J.C.; Rinehart, Jr., K.L. *J Am Chem Soc* **1977**, *99*, 8469

tryptophanol.[9] Alamethicin, trichopolyn, tricholongins (3), trichogin (4), trichorzianine (5), emerimicin (6), trichosporin and trichodecenins are some examples of antibiotic peptides that belong to the group of peptaibol fungal metabolites. Peptaibols have unique biological activities such as the formation of voltage-dependent ion channels[10] and induction of catecholamine secretion from adrenal chromaffin cells.[11] For example, it has been proposed that alamethicin I (7) forms voltage-dependent, ion channels via the aggregation of several molecules.[8d]

Ac-Aib-Gly-Phe-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Lol

Tricholongin BI (3)

Oc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol

Trichogin A IV (4)

Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-Ile-Gln-Gln-Trpol

Trichorzianine A IIIc (5)

---

[9] Bodo, B., Rebuffat, S ; El Hajji, M ; Davoust, D *J. Am. Chem. Soc.* **1985**, *107*, 6011.

[10] a) Mueller, P., Rudin, D. O. *Nature* (London), **1968**, *217*, 713. b) Sansom, M. S. P. *Prog. Biophys. Mol. Biol.* **1991**, *55*, 139.

[11] a) Fotenz, R. I., Lopez, M. G., Garcia-Sancho, J., Garcia, A. G *FEBS Lett.* **1991**, *283*, 89. b) Tachikawa, E., Takahashi, S., Furumachi, K., Kashimoto, T., Iida, A.; Nagaoka, Y., Fujita, T.; Takaishi, Y *Mol. Pharmacol.* **1991**, *40*, 790

Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Phol

Emerimicin IV (6)

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib Glu-Gln-Phol

Alamethicin I (7)

1.5 Project Objectives

In 1978, Lundgren *et al.* showed that of all the *Tolypocladium* species exhibit antagonistic properties towards other fungi,; *T. geodes* was found to be the strongest antagonist.[2d] In the course of our screening program for novel fungal metabolites exhibiting antitumor activity, the *T. geodes* isolate LP237 was found to produce metabolites exhibiting high levels of cytotoxicity and antibacterial activity. The focus of this research project was the isolation of biologically active metabolites from this culture. In addition to the complete separation and purification of each metabolite from the crude peptide mixture, NMR and mass spectral analysis lead to the structure determination of three metabolites, which proved to be novel natural products belonging to the family of peptaibols. Preliminary assessment of the biological activity of these compounds showed antitumor and antiviral activity.

Chapter 2

ISOLATION AND PURIFICATION OF PEPTIDES

2.1 Isolation and Purification Scheme

Liquid medium of 2% malt extract was inoculated with actively growing mycelium plugs of *T. geodes* and incubated at 20 °C on a rotary shaker for a period of 7 days. A bioassay-guided purification scheme was developed (Figure 2.1) for the isolation of the extracellular cytotoxic metabolites of *T. geodes* produced under these fermentation conditions. The presence of active metabolites in fractions collected fro each purification step was identified using the SOS Chromotest, a bacterial colorimetric assay which is widely used as a reliable test for the detection of genotoxic compounds (DNA-damaging compounds).[12]

[12] Review Quillardet, P. and Hofnung, M. *Mutation Research* , **1993**, *297*, 235.

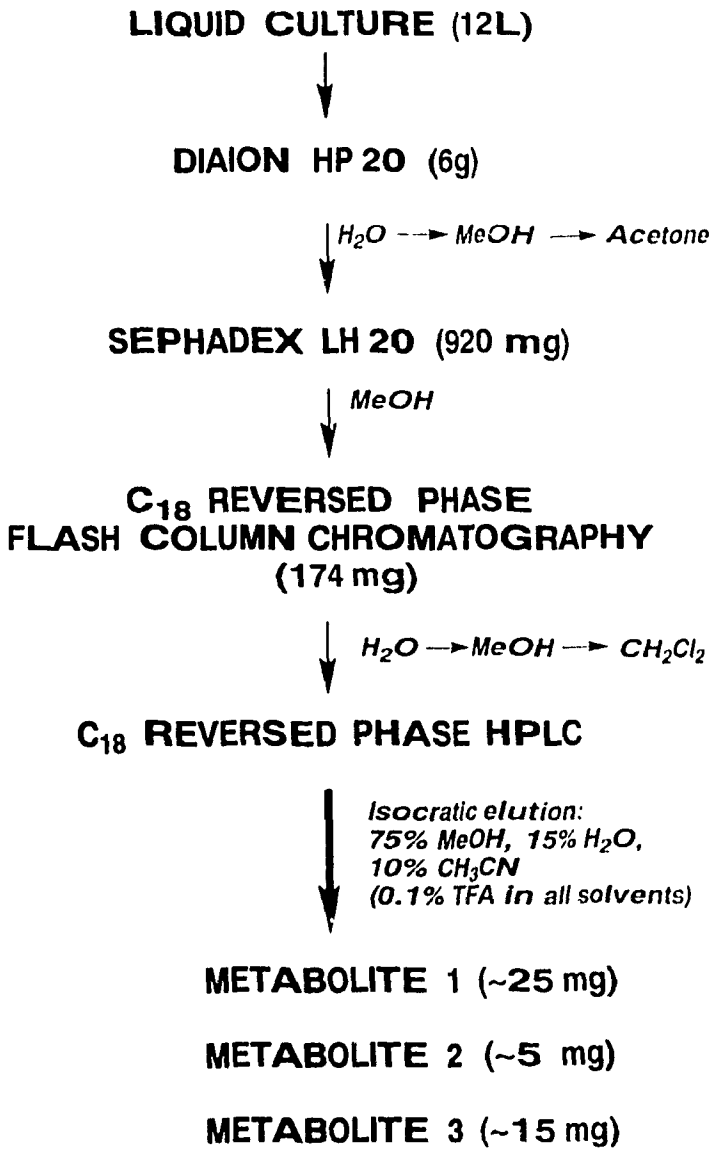


Figure 2.1 Isolation Scheme.

The fermentation broth was initially absorbed onto a column of Diaion HP 20 resin, which was eluted with a linear solvent gradient from H_2O to MeOH and then to acetone. This resin has large pores, about 500 - 700 m^2/g HP[13a], and a hydrophobic surface character made-up of styrene-divinylbenzene, as seen in Figure 2.2. This allows the resin to absorb the hydrophobic part of an organic molecule and to thus separate metabolites according to their hydrophobicity. The cytotoxic metabolites eluted from the column with aqueous MeOH (75-100%) and were further partitioned by size exclusion chromatography (Sephadex LH-20), using degassed, distilled methanol as the mobile phase. The exclusion limit of Sephadex LH 20 particles is equal to a molecular weight of 5000;[13b] thus any compound above this limit is washed out through the column at the beginning of the separation, and any metabolite below the limit gets separated according to its size.

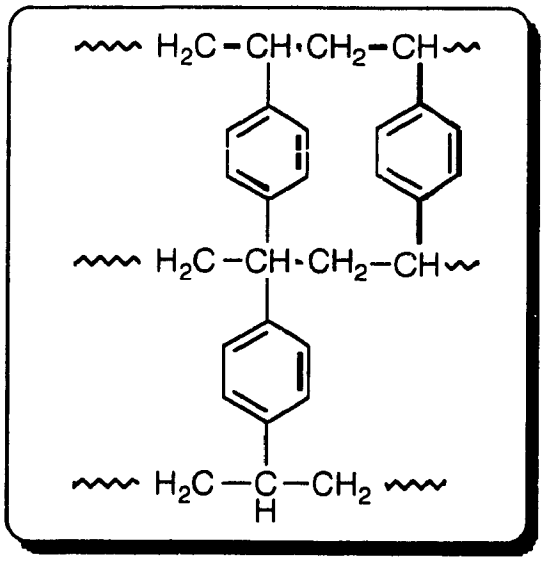


Figure 2.2 Hydrophobic surface of HP 20.

13 a)*In* Mitsubishi Kasei Corporation, How to Select the Best Type of Synthetic Adsorbent from "Diaion" and "Sepabeads", Distributed by Eicon Scientific, October 1986. b) *In* Pharmacia Biodirectory 1996, Biotech Uppsala, Sweden, Printed in the USA

Reverse phase, flash column chromatography[14] lead to the isolation of a strongly cytotoxic and genotoxic mixture of peptides, exhibiting an IC_{50} value in the range of 5-10 ng/ml with P388D1 murine leukemia cells and a positive SOS Chromotest. Reverse phase (C_{18}) silica gel was prepared from normal phase;[15] figure 2.3 illustrates the chemical reaction involved in the preparation of C_{18} reverse phase silica gel. Subsequently, the peptide mixture was absorbed onto the reverse phase silica and loaded on the column as a powder. Purification of the metabolites was accomplished using water, followed by a step gradient through methanol to dichloromethane. The solvent gradient used to elute the compounds from the reverse phase silica gel is shown in Appendix A, where the more polar compounds are eluted first and the less polar compounds are eluted at the end of the step gradient. The cytotoxic mixture of peptides eluted from the column with 85% to 95% aqueous methanol, and was further purified using high performance liquid chromatography (HPLC).

Mass spectral analysis (discussed in Chapter 3) of the cytotoxic mixture of peptides, by MALDI and ESI MS, indicated the presence of many different compounds ranging in molecular weight from *m/z* 1300-2000. Separation of these metabolites by C_{18} reversed phase semi-preparative HPLC, using a solvent mixture of MeOH : H_2O : MeCN (70:20:10 with 0.1% TFA in all solvents) allowed the isolation of five peptides. At a flow rate of 4 mL/min, metabolites LP237-F5 (**2**), F6, F7 (**3**), F8 (**1**) and F9 eluted from the HPLC column with retention times of 16.4 min, 17.2 min, 20.7 min, 23.6 min and 26.0 min, respectively (Figure 2.4). Each component was further purified by HPLC a second time, under the same conditions, in order to obtain the pure peptides as white amorphous solids. At the present time, the structural assignments of the three main components,

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[14] a)Blunt, J. W., Calder, V. L.; Fenwick, G. D., Lake, R. J., McCombs, J. D.; Munro, M. H. G. and Perry, N. B. *J. Nat. Prod.* **1987**, *50*, 290 b) Tsantrizos, Y. S., Ogilvie, K. K. and Watson, A. K. *Can. J. Chem.* **1992**, *70*, 2276.

[15] Evans, M. B., Dale, A. D., Little, C. J. *Chromatographia*, **1980**, *13*, 5

metabolites **1**, **2** and **3**, have been completed and those of peptides LP237-F6 and F9 are in progress.

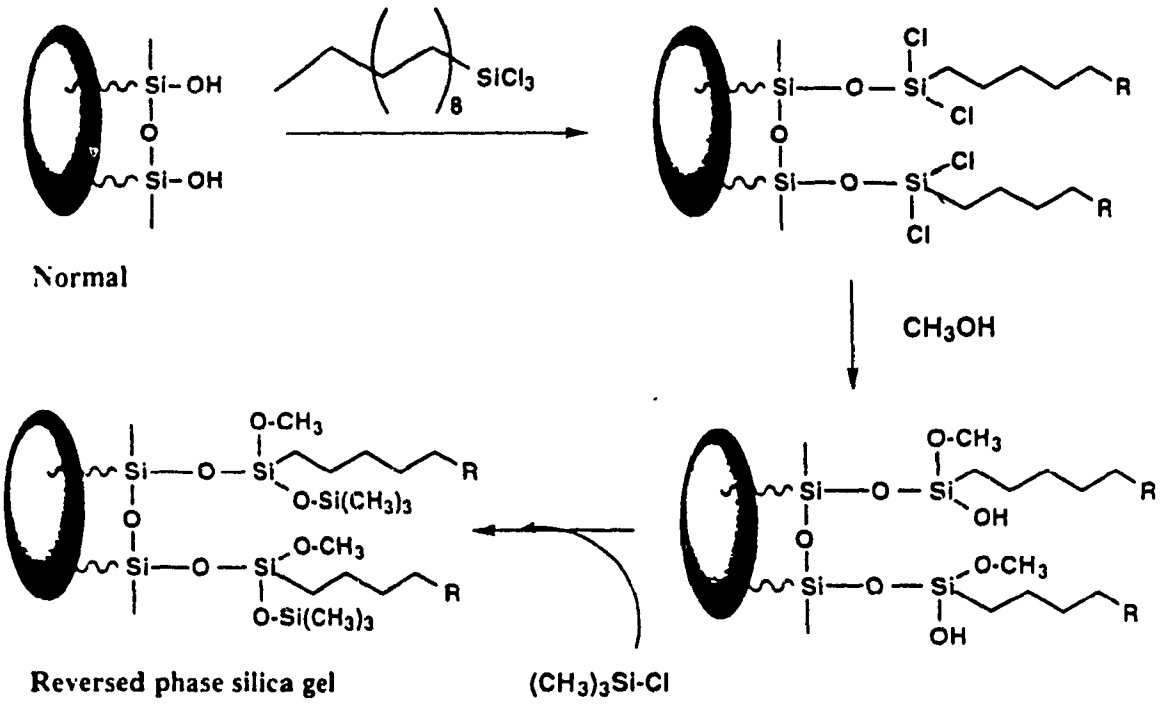

Figure 2.3 Preparation of reverse phase silica gel from normal silica gel.

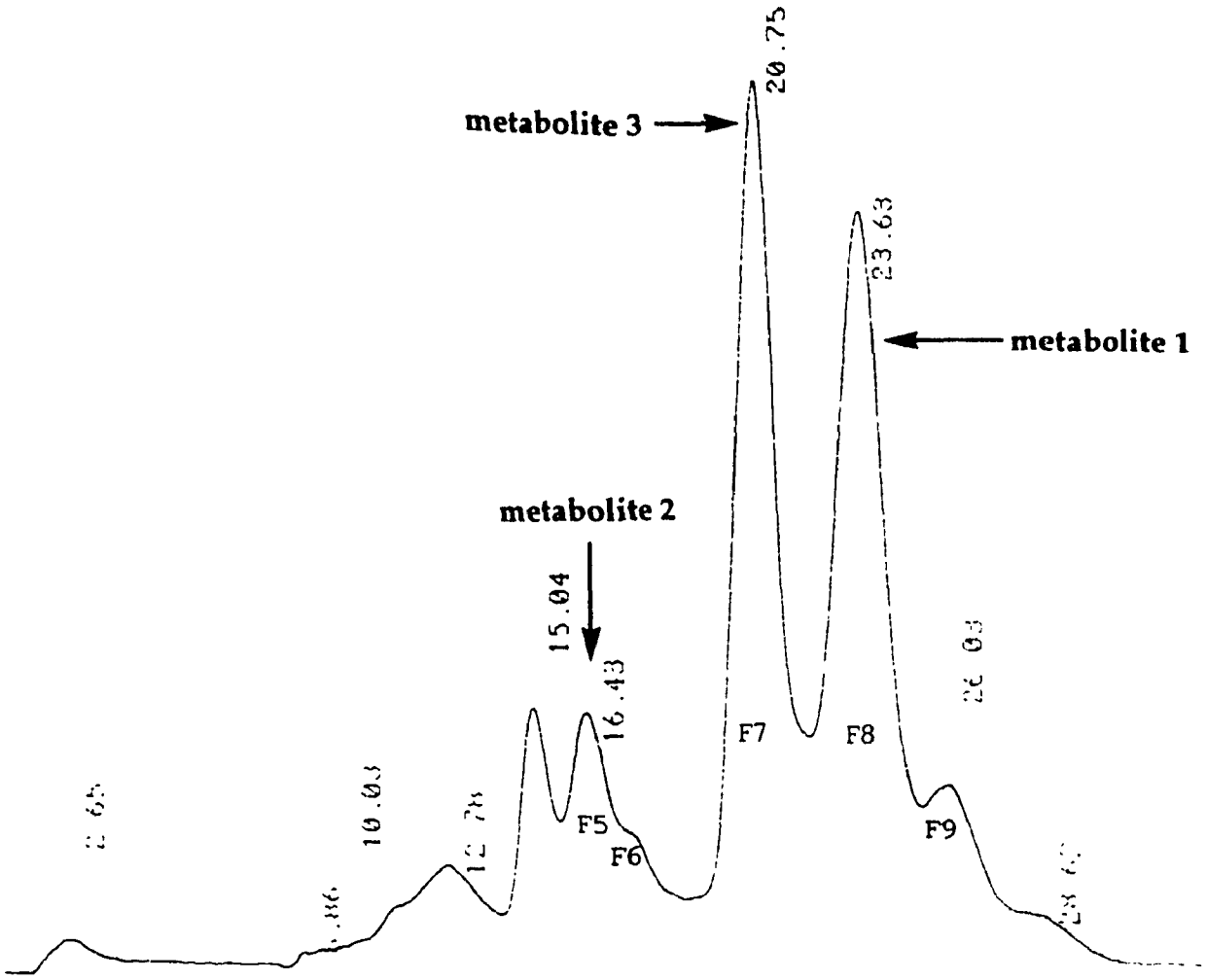

Figure 2.4 Reverse phase HPLC chromatogram of *T. geodes* LP 237 peptide mixture.

Chapter 3

STRUCTURE DETERMINATION OF METABOLITES LP237-F5, LP237-F7 AND LP237-F8

3.1 Introduction

The previous chapter provided details on how chromatography was used as a principal means by which biologically active components of a peptide mixture were partitioned according to their size and their polarity. The focus of this chapter is the use of spectroscopic techniques to identify the active metabolites of *T. geodes* LP 237 isolate. Spectroscopy constitutes a set of techniques that measure the response of a molecule to the input of energy. The resulting spectrum is a series of signals that show the magnitude of the interaction of a compound as a function of the incident energy. The energy source can be optical photons, as in ultraviolet, visible and infrared spectroscopy, or radio-frequency energy, as in nuclear magnetic resonance spectroscopy (NMR).[16] A somewhat different technique is mass spectrometry, where the mass of ions formed is determined when molecules are bombarded with high energy electrons, as in electron impact ionization.[17] More recently, the application of new techniques such as matrix-assisted laser desorption ionization (MALDI), which is based on laser desorption of organic ions, and electrospray ionization (ESI), where highly charged droplets are dispersed from a capillary in a strong electric field at atmospheric pressure, has made mass spectrometry a valuable tool in the

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[16] Ege, S. N.; *In* Organic Chemistry, Second Edition, D. C. Heath and Co., Toronto, Canada, 1989, pp 376-418.

[17] *Ibid* 16, pp 864-887

determination of biological molecules with larger molecular weights and more complex structures.

3.2 Mass Spectrometry

Mass spectrometry consists in *weighing* individual molecules by transforming them into ions and then measuring the response of their trajectories to electric and magnetic fields or both.[17] A mass spectrum is obtained by converting components of a sample into rapidly moving gaseous ions and resolving them on the basis of their mass-to-charge ratio (m/z).[17] Attempts to increase the sensitivity and accuracy of this method to the analysis of larger organic biomolecules have resulted in the development of electrospray and laser desorption ionization techniques. Prior to FAB (fast atom bombardment), MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization), the transformation of large molecules into gas-phase ions was very difficult and frustrating. The major concern was the inevitable decomposition of the molecules after vaporization.

The first of these techniques, FAB, uses beams of neutral atoms to ionize compounds from the surface of a liquid matrix, such as glycerol. In a condensed state, samples are ionized by bombardment with energetic xenon or argon atoms.[18] Part of a desorption process, both positive and negative analyte ions are sputtered from the surface of the sample. A beam of fast atoms is obtained by passing accelerated ions of argon or xenon from an ion gun through a chamber containing argon or xenon atoms at relatively high pressure. Speeding ions undergo an electron exchange reaction with the lower energy atoms in which charge neutralization occurs without substantial loss of initial kinetic

[18] Rinehart, K. L. *Science*, **1982**, *218*, 254

energy. Thus a beam of energetic atoms is formed. FAB of organic compounds usually produces significant amounts of the parent ion as well as ion fragments.[18]

In ESI, highly charged droplets are dispersed from a capillary in a strong electric field at atmospheric pressure.[19] Heat and/or dry gas are applied to the droplets to help evaporate the solvent. Multiply charged ions expelled from the droplets during the evaporation process are then drawn towards an inlet that admits them into the vacuum region of the spectrometer. An ESI mass spectrum of a large molecule is characterized by a series of peaks caused by ions carrying different numbers of charges and thus exhibiting different mass-to-charge values.[19]

MALDI is based on laser desorption of organic ions. The matrix used in MALDI is believed to function as a means of absorption of energy from the laser to prevent decomposition of the species analyzed, as well as a way to isolate biomolecules from each other to avoid aggregation that might otherwise interfere with analyses.[20] Unlike ESI, MALDI typically produces primarily singly charged ions.

In MS, individual ions are examined based on the atomic mass of individual isotopes. In low resolution mass spectrometry, the accuracy of the *m/z* can be measured to 0.2 mass units whereas in high resolution mass spectroscopy the accuracy of the *m/z* can be measured to 0.0001 mass units.

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[19] a) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science*, **1989**, *246*, 64. b) Dole, M.; Mack, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. D.; Alice, M. B. *J. Chem. Phys.* **1968**, *49*, 2240.

[20] a) Youngquist, R. S.; Fuentes, G. R.; Lacey, M.P.; Keough, T. *Rapid Commun. Mass Spec.* **1994**, *8*, 77. b) Danis, P. O.; Karr, D. E.; Simonsick, W. J.; Wu, D. T. *Macromol.* **1995**, *28*, 1229. c) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193.

3.3 Nuclear Magnetic Resonance

Nuclear magnetic resonance is perhaps the most valuable spectroscopic technique available to organic chemists, which can provide a complete picture of the carbon-hydrogen framework of an organic molecule. 1D and 2D NMR analysis was used extensively in the structural assignment of the three peptides produced by *T. geodes*. Among these, HMBC, HMQC, NOESY, COSY and DEPT were of valuable assistance in the structure determination of all three compounds and will be discussed in the following sections.

3.4 Structural Determination of Metabolite LP 237-F8 (1)

Both MALDI and FAB MS of metabolite **1** suggested a molecular formula of $C_{65}H_{108}N_{14}O_{15}$ [MALDI MS *m/z* 1348.4 $(M+Na)^+$, FAB MS (NBA+NaCl matrix) *m/z* 1347.74 $(M+Na)^+$, calculated mass for $C_{65}H_{108}N_{14}O_{15}Na$ 1347.80]. A strong absorption at 1649 cm^{-1}, together with an absorption at 3436 cm^{-1} and 1541 cm^{-1}, in the IR spectrum suggested the presence of a peptide linkage along with an intermolecularly hydrogen-bonded alcohol (3329 cm^{-1}) or a primary amide. The presence of 14 amide or ester moieties was confirmed by ^{13}C NMR (125 MHz). Complete acid hydrolysis and amino acid analysis[21] of LP237-F8 (**1**) indicated the presence of Phe, Ala, Pro and Glx in a ratio of 1:1:1:3. In amino acid analysis, a strong cation exchange column allows the acidic and straight chain hydroxy amino acids, such as Ser, Glu, Asp and Thr, to be eluted first, followed by the elution of small and neutral amino acids and finally basic and modified amino acids. Glx is the notation used to signify the presence of a Glu or Gln

21 Spackman, D. H.; Stein, W. H., Moore, S. *Anal. Chem* **1958**, *30*, 1190

unit, but not a Gly unit. Both Glu and Gln elute at the same time, thus there is no distinction between the two in amino acid analysis. Quantitative determination is done automatically by an instrument as it records the ninhydrin color value of the eluents of the ion exchange columns. A synthetic mixture of amino acids is injected into the instrument and is used as a standard.

Metabolite **1** was resistant to methylation with diazomethane and gave a negative ninhydrin test, suggesting the absence of free carboxyl and amino functionalities. Methylation of carboxylic acids is commonly done using diazomethane with significantly high yields at room temperature. The overall reaction is shown in Figure 3.1. Diazomethane also reacts with phenols to give the methylated product under relatively mild conditions.[22]

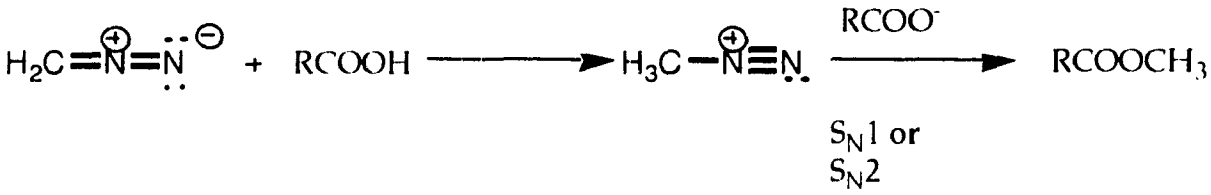

Figure 3.1 The diazomethane reaction with carboxylic acids.

The ^{1}H NMR (500 MHz) of **1** in DMSO-d_6 (Table 1) (Figure 3.2) clearly showed the presence of 16 NH resonances in the downfield region of the spectrum which are exchangeable in CD_3OD (NH δ 6.6-8.8, overlapping aromatic protons of Phe δ 7.16-7.22).

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[22] March, J. *In* Advanced Organic Reactions, Fourth Edition, John Wiley & Sons, Inc. New York, U.S.A. 1992, pp.388-400

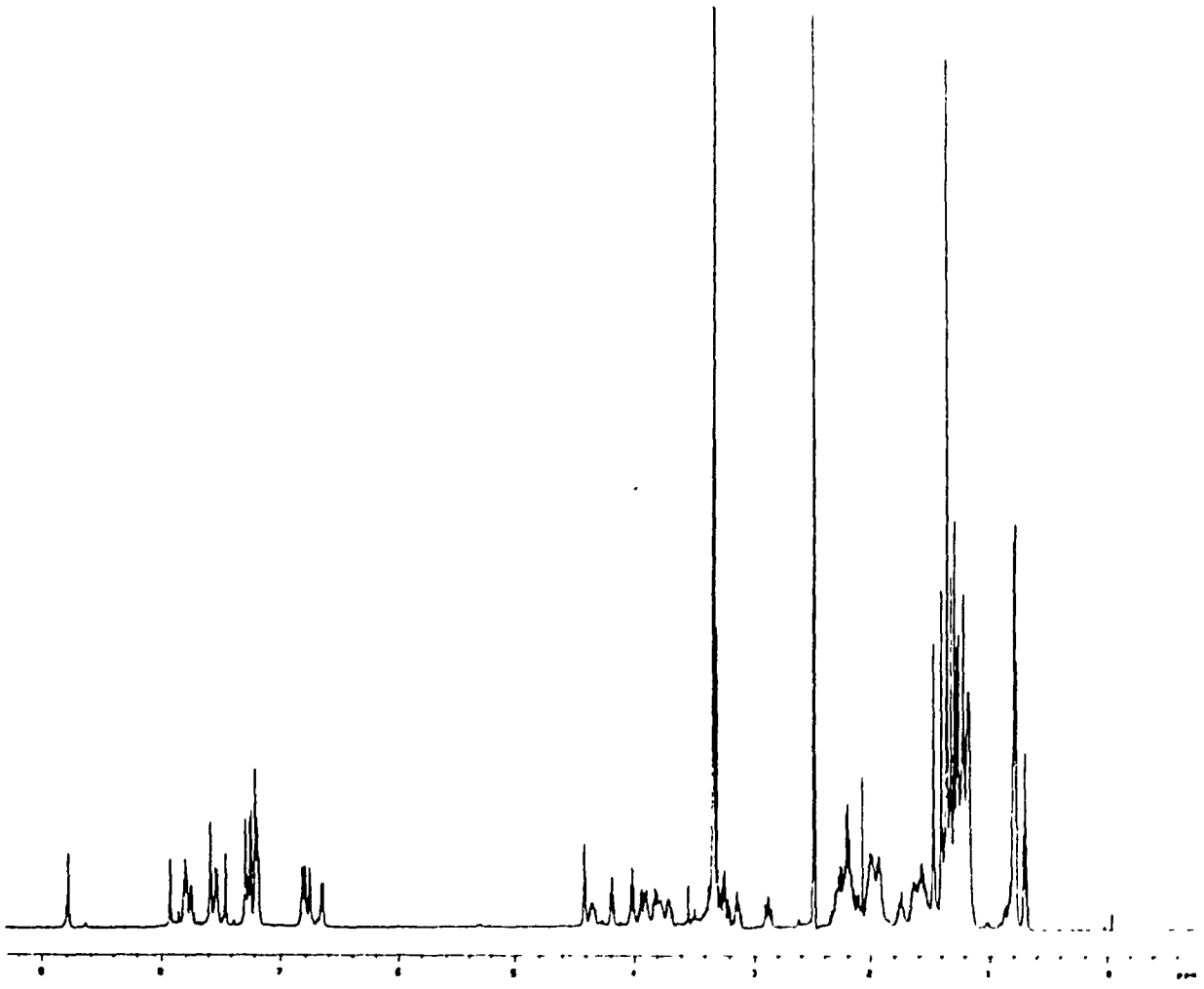

Figure 3.2 ^{1}H spectrum of LP237-F8 (1).

Chemical shift assignments for each amino acid unit were based on the combined ^{1}H, ^{13}C, COSY, DEPT, HMQC and HMBC NMR data (Table 1). For example, coupling of the α-H to the NH resonance of Phe (δ 4.35 to 7.81), Ala (δ 4.02 to 7.53), ^{6}Glx (δ 3.93 to 7.79), ^{7}Glx (δ 3.90 to 7.75), ^{3}Glx (δ 3.82 to 7.57) and leucinol (Lol, δ 3.77 to 6.65) were clearly observed in the COSY spectrum. COSY, correlation spectroscopy, is a homonuclear shift experiment in which protons are correlated to other protons on adjacent carbons due to *J* coupling between the two. Three other NH resonances at δ 6.76, 6.80 and 6.82 were observed which were coupled to those at δ ~7.16, 7.27 and 7.27 respectively and showed positive NOE effects (nuclear Overhauser effect, is a phenomenon which occurs between two spatially close protons when one of these protons is saturated by irradiation and the signal strength of the other is increased) on the γ-CH_2 resonances of the Glx units. Thus, these resonances were assigned to εNH_2 moieties of Gln units, establishing the identity of the three Glx units of LP237-F8 (**1**). Further analyses of the NMR data of **1** revealed the presence of one amino alcohol unit, leucinol (Lol), and three α-aminoisobutyric acid units (Aib), both of which are commonly found in peptaibol metabolites.

Based on the amino acid analysis and the overall MS and NMR spectral data of LP237-F8 (**1**), the unequivocal assignment of most amino acid units was achieved. The structural identity of the novel α-ethylnorvaline unit (EtNor, α-amino-α-ethyl-*n*-pentanoic acid) and the octyl fatty acid chain (Oc) proved to be the more challenging aspect of the structural assignment of metabolite **1**. Initially, two plausible structures were considered: (I) the structure contained an EtNor and an Oc chain as shown in Figure 3.3 and (II) an alternate structure having an isovaline amino acid in the place of the EtNor unit and a decanoyl fatty acid in the place of Oc. However, the alternate structure (II) was rejected after further analysis of the NMR data. For example, the ^{1}H NMR of LP237-F8 (Figure 3.1) clearly showed a total of five *shielded* methyl groups: four methyls between δ 0.76-

0.81, (assigned to the 2 δ-CH_3 of Lol, the C8-CH_3 of Oc and the δ-CH_3 of EtNor) and a fifth methyl at δ 0.696 (assigned to the γ'-CH_3 of EtNor). The expected chemical shift of a methyl group attached to an α-carbon, as in the case of isovaline, would be at δ ~1.3-1.4, whereas that of metabolite **1** is at δ ~0.8, consistent with the presence of the *n*-propyl group in EtNor. The methyl group of the fatty acid chain (C8-Oc) and the δ-CH_3 of EtNor nearly overlap at δ ~0.80 and they are both coupled to methylene protons at δ 1.20-1.25; a chemical shift of δ ~1.25 was assigned to the γ-CH_2 of EtNor based on the COSY and NOESY NMR data. NOESY is a two-dimensional experiment, analogous to the one dimensional transient NOE. It is a proton to proton correlation according to the spatial arrangement of the molecule. Protons up to 5 Å in proximity to each other may be correlated using NOESY. The HMBC (heteronuclear multiple bond correlation) spectrum of **1** shows clear correlation between the γ'-CH_3 and its neighboring methylene carbon (β'-C) at δ 26.09, as well as the quaternary α-carbon at δ 58.74. Neither of the two methyls at δ ~0.8 show a correlation to the same quaternary carbon, which strongly suggests (but does not prove) that those two methyls are located on carbon chains longer than an ethyl group. HMBC, as well as HMQC (heteronuclear multiple quantum correlation), are two-dimensional experiments in which protons are correlated to carbons due to *J* coupling, J_1 for HMQC and J_2,J_3 for HMBC.

Although distinct HMQC and HMBC NMR correlations between the protons and carbons of the propyl chain were somewhat difficult to confirm due to extensive overlapping of signals, the observed data were consistent with the proposed presence of an EtNor unit, located between ^{3}Gln and ^{5}Aib. Careful examination of the NOESY data associated with the EtNor unit showed NOE correlations between the δ-CH_3 and the γ'-CH_3, further suggesting that these two shielded methyls are part of the same amino acid. The presence of NOE correlations between the γ'-CH_3 of EtNor and its neighboring protons β-CH_2 (δ 1.31), β'-CH_2 (δ 1.63 & 2.187) and δ-CH_3 (δ 0.795) were clearly

observed. Finally, the EtNor-NH (δ 7.45) displays NOE interactions with neighboring NH groups (^{3}Gln and ^{5}Aib), the α-H of ^{3}Gln and some of the other protons within the EtNor unit. Thus, the structure of an α-amino-α-ethyl-*n*-pentanoic acid unit (α-ethylnorvaline, EtNor) was confirmed by the combined NMR data. This amino acid is exceedingly rare, found only in extraterrestrial sediments such as Murchison meteorite[24] It has also been synthesized and shown to be a competitive inhibitor of methionine.[25] To our knowledge, EtNor has not been previously reported as a constituent of a natural product.

Finally, the presence of an octanoyl (Oc) moiety at the N-terminal of the peptide was confirmed by the NMR data. DEPT NMR analysis of **1**, indicated the presence of 17 CH_2 [including the -CH_2-OH of ^{1}Lol (δ 64.11)], and 11 CH_3 carbons; the chemical shifts of the overlapping ^{13}C resonances (-CH_2- resonances of Oc overlapping at δ 1.17-1.22) were identified from the HMQC and HMBC experiments. DEPT (distortionless enhancement by polarization transfer) is an experiment where carbons can be categorized according to the number of protons bonded to them, giving a clear picture as to the number of CH, CH_2 and CH_3 groups in a molecule.

The sequence assignment of the peptide backbone was based primarily on the NOESY NMR data (Figure 3.3). Strong NOE effects were observed between amide protons and the α-protons of neighboring residues and also between amide protons and the side chains of the neighboring amino acids (β-, γ- and δ-protons) (Figure 3.3).

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[24] Cronin, J. R.; Pizzarello, S. *Geochim. Cosmochim. Acta*, **1986**, *50*, 2419

[25] Abshire, C. J.; Planet, G. *J. Med. Chem.* **1972**, *15*, 226

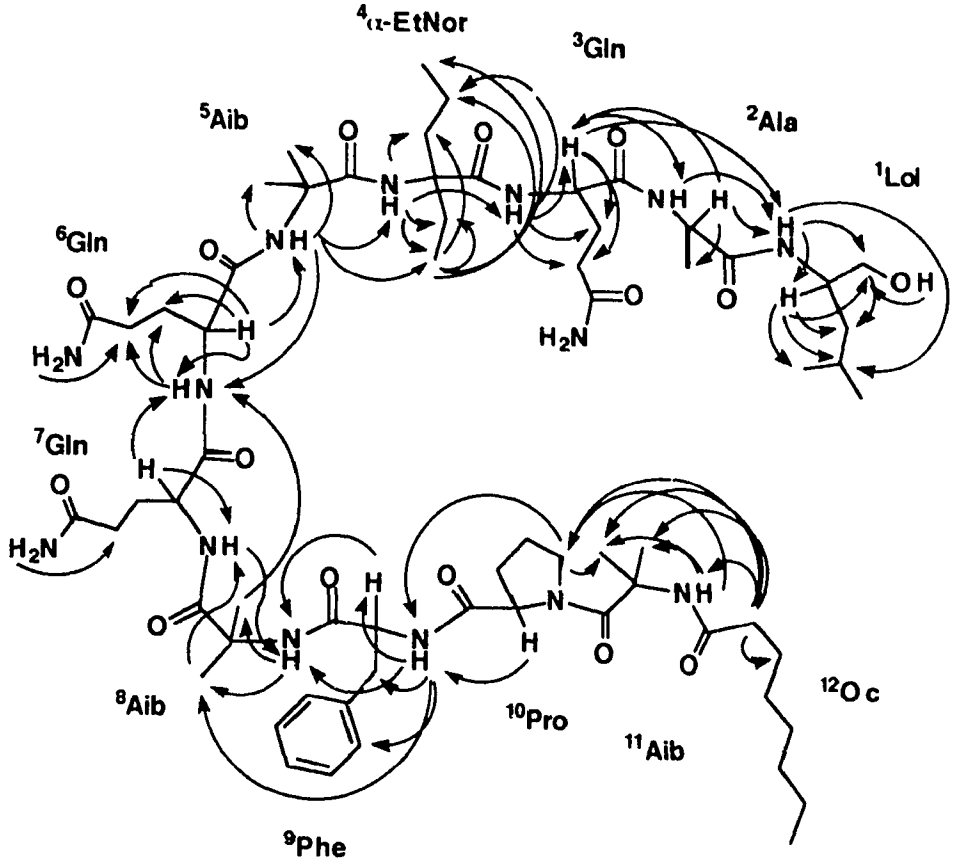


Figure 3.3 Structure of LP237-F8 (1) and NOEs observed in the NOESY NMR experiment.

3.5 Structure determination of Metabolite LP237-F5 (2)

Both MALDI and FAB MS of metabolite **2** suggested a molecular formula of $C_{65}H_{108}N_{14}O_{16}$ [MALDI MS (2,5-dihydroxy benzoic acid + NaCl or LiCl matrix) *m/z* 1365.1 $(M+Na)^+$ and 1347.8 $(M+Li)^+$, FAB MS (NBA+NaCl matrix) *m/z* 1363.5 $(M+Na)^+$, calculated mass for $C_{65}H_{108}N_{14}O_{16}Na$ = 1363.8]. LiCl was used as a competing cationizing agent with Na^+ and K^+ which are naturally found in the matrix, sample or on the sample slide.

The NMR spectral data of metabolite LP237-F5 (**2**) were very similar to those of the previously assigned peptaibol LP237-F8 (**1**). However, metabolite **2** could easily be methylated with diazomethane [MALDI MS (2,5-dihydroxy benzoic acid + LiCl matrix) *m/z* 1362.5 $(M+Li)^+$] whereas metabolite **1** was resistant to methylation under the same reaction conditions. Based on the NMR data, the difference in reactivity towards CH_2N_2 observed between **1** and **2** was attributed to the presence of a Tyr unit in **2** as opposed to a Phe unit in **1**. Complete acid hydrolysis of peptide **2** and amino acid analysis confirmed the presence of the amino acid Tyr, in addition to Ala, Pro and Glx in a ratio of 1:1:1:3.

The ^1H NMR spectrum (500 MHz in DMSO-d_6) of **2** showed the presence of 21 resonances in the downfield region (Table 2, Figure 3.4). The two doublets at δ 6.60 and 6.96 (J = 8.3 Hz, 2x2H) and the singlet at δ 9.18 (OH) were assigned to the Tyr unit. All of the remaining downfield protons could be exchanged in CD_3OD and they were assigned to 16 NH protons. The COSY NMR of **2** showed coupling between the NH resonances at δ 6.76, 6.80, 6.82 and those at 7.16, 7.27, 7.27, respectively (Figure 3.4). Thus, these signals were assigned to three ε-NH_2 groups of Gln. From the combined ^1H and COSY NMR data (Table 2), the α-H and the backbone NH protons could easily be assigned for the Tyr (δ 4.22, 7.73), Ala (δ 4.01, 7.52), ^{6}Gln (δ 3.92, 7.78), ^{7}Gln (δ 3.89, 7.73), ^{3}Gln (δ 3.81, 7.57) and Lol (δ 3.76, 6.65) units (Figure 3.4).

The remaining chemical shifts of each amino acid were assigned based on the combined ^1H, COSY, HMQC, HMBC and NOESY NMR data (Table 2). For example, coupling between the NH (δ 6.65) of Lol, the α-H resonance (δ 3.76) and the two non-equivalent -C<u>H</u>$_2$OH protons (δ 3.23 & 3.14) was clearly observed in the COSY NMR spectrum of metabolite **2**. The chemical shifts of the corresponding carbons were assigned

from the HMQC spectrum at δ 48.77 (α-C) and 64.38 (-CH_2OH).[27] Furthermore, coupling between the α-H resonance and the two β-H (δ ~1.30), as well as between the two δ-CH_3 groups (δ ~0.78), the β-H (δ ~1.30) and the γ-H (δ ~1.58), was also observed in the COSY spectrum of **2**. The ^{13}C chemical shifts of the β-CH_2 (δ 40.15) and the γ-CH (δ ~25.4) were obtained from the HMQC data, whereas the ^{13}C shifts for the two δ-CH_3 groups (δ 23.6 & 21.9) were obtained from the HMBC spectrum.[27]

The order of the amino acid sequence of peptide **2** was based primarily on the NOESY NMR data (Figure 3.5). Strong NOE effects were observed between the backbone amide protons and the α-protons of neighboring residues and also between amide protons and the side chains of the neighboring amino acids.

[27] Due to the very small amount of available material of metabolite **2** (~2 mg) a directly observed ^{13}C NMR spectrum was not obtained. However, the ^{1}H, COSY, HMQC, HMBC and NOESY NMR data of **2** was similar to that of metabolite **1** and consistent with the proposed structure.

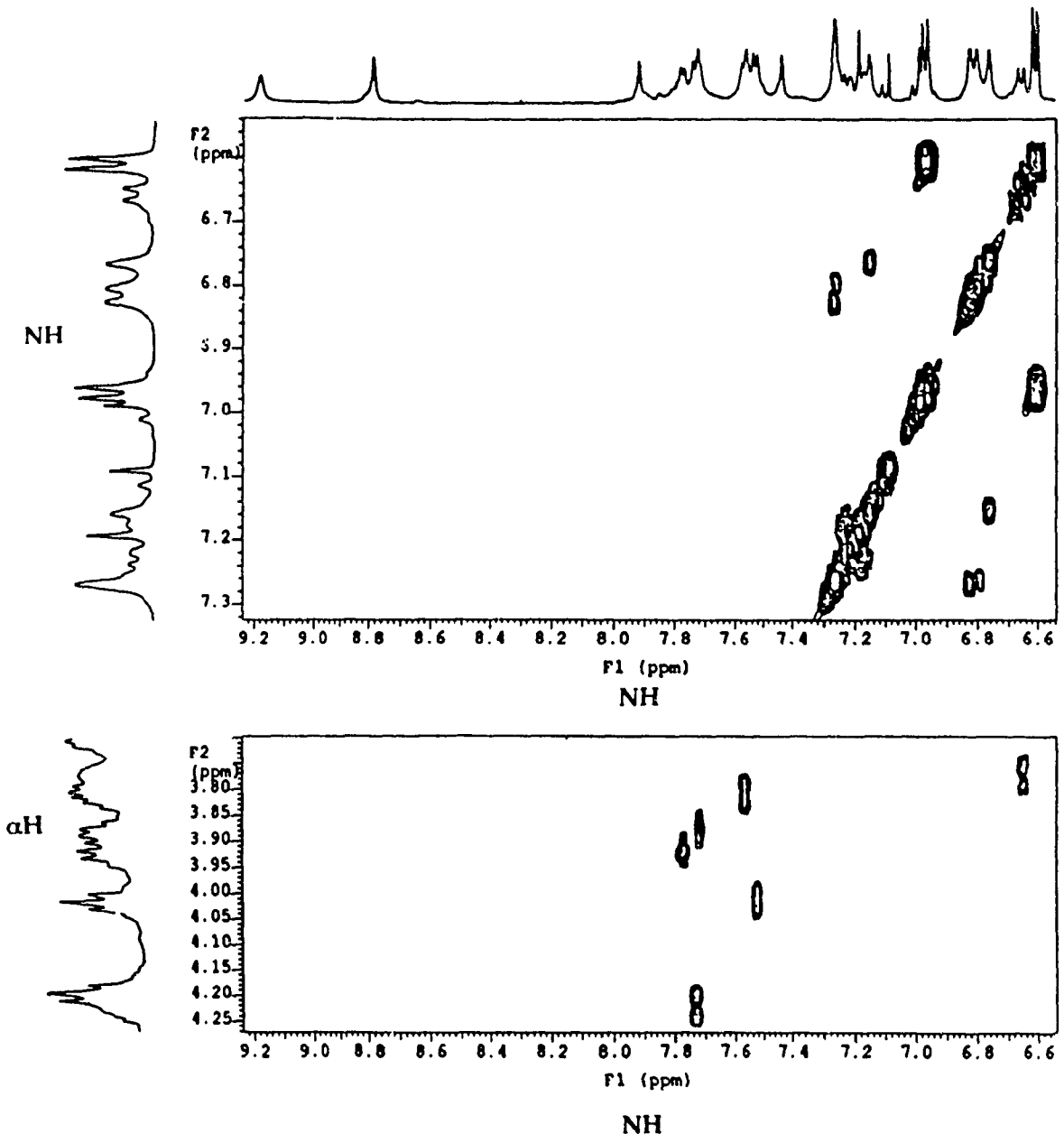


Figure 3.4 COSY NMR spectrum (NH and α-H region) of LP237-F5 (500 MHz, DMSO).

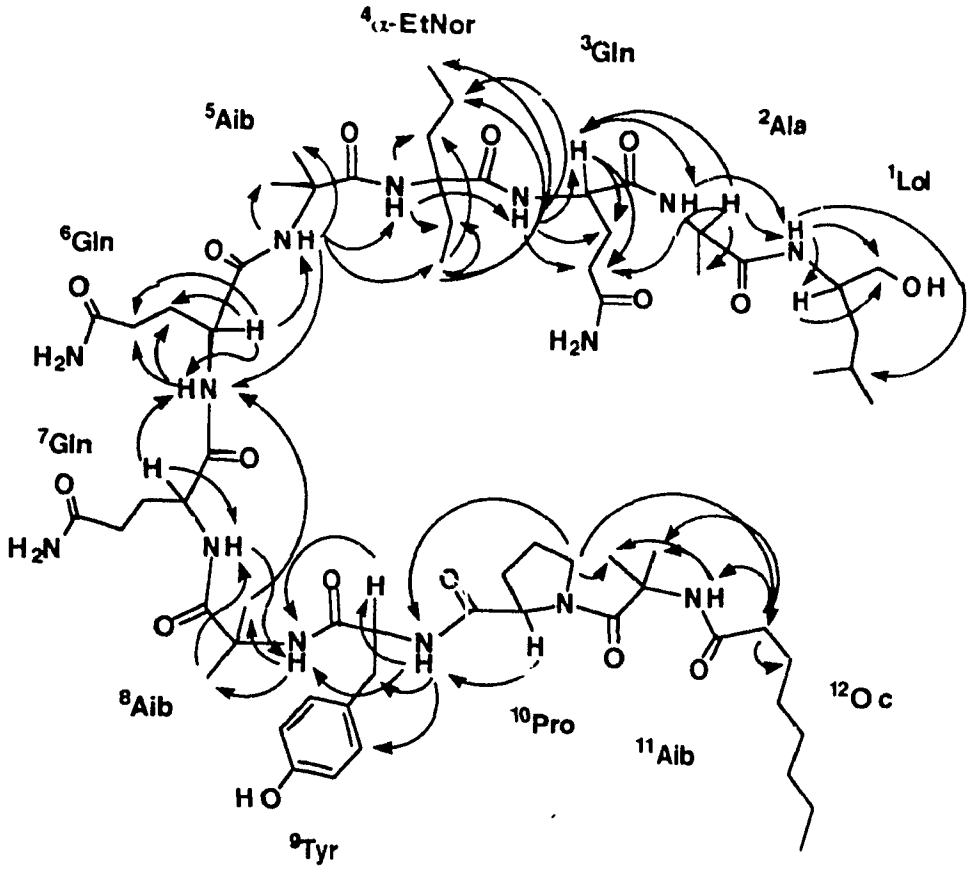


Figure 3.5 Structure of LP237-F5 (2) and NOEs observed in the NOESY NMR spectrum.

3.6 Structure determination of Metabolite LP237-F7 (3)

FAB MS of metabolite **3** suggested a molecular formula of $C_{64}H_{106}N_{14}O_{15}$ [(NBA+NaCl matrix) *m/z* 1333.8 $(M+Na)^+$, calculated mass for $C_{64}H_{106}N_{14}O_{15}Na$ = 1333.7859]. Amino acid analysis of **3** indicated the presence of the same common amino acid units as in metabolite **1**, Phe, Ala, Pro and Glx in a ratio of 1:1:1:3. Metabolite **3** was also resistant to methylation with diazomethane, suggesting the absence of free carboxyl or phenolic functionalities.

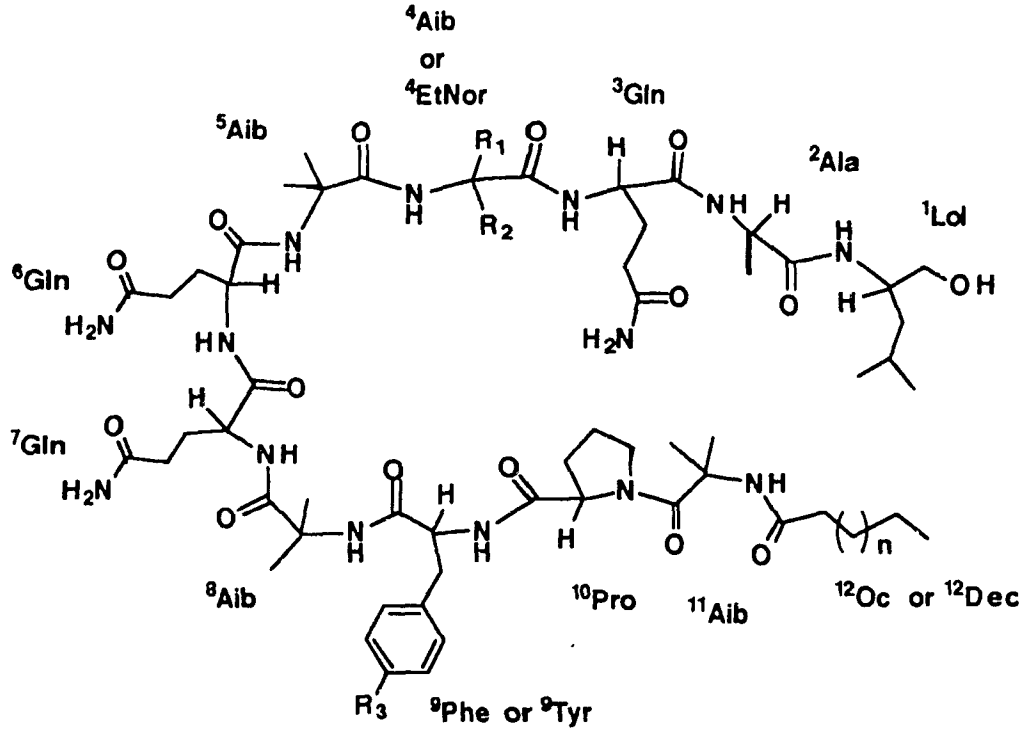
The ^1H NMR spectrum of metabolite LP237-F8 (**3**) was distinctly different from that of metabolite **1** in the upfield methyl region; a total of three methyl signals were observed at δ 0.7-0.9 in **3** as opposed to five in metabolite **1**. The two overlapping doublets observed (δ 0.887 & 0.893) were assigned to the two δCH_3 of Lol, and a triplet (δ 0.863) was assigned to the terminal methyl of the fatty acid unit. Thus, it was assumed that metabolite **3** did not contain the unusual amino acid EtNor which was found in **1** and **2**. This assumption was further supported by the absence of the expected correlations in the COSY spectrum of **3** between the methyl region and the methylene region for the EtNor unit, as had been observed previously in the COSY spectum of metabolite **1**. Furthermore, a total of eight singlets due to methyls of Aib units (δ 2x1.409, 1.458, 1.470, 1.474, 1.535, 1.539 & 1.604) were observed in the ^1H spectrum of **3**. In order to account for: (a) the similarities in the spectral data between **1** and **3**, (b) the difference in mass of 14 units, (c) the loss of the EtNor unit ($C_7H_{13}NO$, MW=127) and (d) the addition of a fourth Aib unit (C_4H_7NO, MW=85), it was proposed that peptide **3** contains a decanoyl (Dec) fatty acid instead of the Oc unit found in peptides **1** and **2**. The overlapping of many -CH_2- resonances in the ^1H NMR spectum of **3** did not permit exact chemical shift assignments for the Dec unit to be made, however, the presence of this unit

was confirmed from the ^{13}C and HMQC NMR data of this metabolite. The remaining amino acid units of peptide **3** were identical to those of peptide **1**, and their chemical shift assignments (Table 3) were consistent with the NMR data; the combined NMR data from the ^{1}H, ^{13}C, COSY, HMQC, HMBC, TOCSY, ROESY and NOESY spectra were used to confirm the proposed structural assignment. Two-dimensional ROESY, rotating-frame Overhauser enhancement spectroscopy, is an experimental NMR technique which measures homonuclear NOE effects under spin-locked conditions. TOCSY, totally correlated spectroscopy, generates a two-dimensional spectrum, where cross peaks exist between all the spins in a coupled system. This contrasts with COSY, where only cross peaks between spins that are directly coupled to each other are seen.

3.7 Conclusion

The structure of the main component in this mixture of peptides, antibiotic LP237-F8 (**1**), as well as the details on the isolation of several cytotoxic peptides from the liquid culture of *T. geodes* LP237 and the structure elucidation of two other metabolites, LP237-F5 (**2**) and F7 (**3**) has been described above. The complete ^{1}H and ^{13}C chemical shift assignments were achieved by extensive high-field 1D and 2D NMR spectroscopy. Metabolites **2** and **3** are structurally related to metabolite **1** (Figure 3.6). All three peptides (**1**, **2** and **3**) have both the N- and C-terminals protected with a short fatty acid unit and the amino alcohol leucinol (Lol), respectively. They contain common amino acids such as Ala, Phe or Tyr, Pro and Gln and the unusual amino acid α-aminoisobutyric acid (Aib), commonly found in peptaibols. Peptides **1** and **2** also contain the amino acid α-amino-α-ethyl-*n*-pentanoic acid (α-ethylnorvaline, α-EtNor), which has not been previously reported as a constituent of a natural product. Metabolites LP237-F5 (**1**), F7 (**2**) and F8

(3) are novel linear peptides belonging to the family of natural products known as peptaibols.



LP237-F8 (1), R_1 = Et, R_2 = *n*Pr (EtNor), R_3 = H (Phe), n = 4 (Oc)

LP237-F5 (2), R_1 = Et, R_2 = *n*Pr (EtNor), R_3 = OH (Tyr), n = 4 (Oc)

LP237-F7 (3), R_1 = R_2 = Me (Aib), R_3 = H (Phe), n = 6 (Dec)

Figure 3.6 Peptaibol metabolites of *T. geodes*.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 1 in DMSO-d_6

	Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, *J* (Hz)
^{1}Leu	α	48.83	3.772	1H, m
	β	40.32	1.319[23a]	2H, m
	γ	25.51	1.568[23a]	1H, m
	δ	23.61 & 21.99	0.775 & 0.793	2×3H, d, *J* = 6.3, *J* = 6.8
	$\underline{CH_2}$-OH	66.09	3.124 & 3.244	2H, m
	OH		4.411	3H, t, *J* = 5.86
	NH		6.655	1H, d, *J* = 9.3 Hz
^{2}Ala	α	49.80	4.015	1H, m
	β	17.59	1.259	3H, d, *J* = 7.3
	CO	172.17[23b]		
	NH		7.526	1H, d, *J* = 7.3
^{3}Gln	α	54.84	3.817	1H, m
	β	26.54	1.989[23a] & 1.929[23a]	2H, m
	γ	31.67	2.299[23a] & 2.196[23a]	2H, m
	δ CO	174.01[23b]		
	εNH_2		6.759 & 7.163	2H, br.s
	CO	172.17[23b]		
	NH		7.575	1H, d, *J* = 5.4
^{4}α-EtNor				
	α	58.75		
	β	30.57	1.310[23a]	2H, m
	γ	22.68	1.302[23a]	2H, m
	δ	14.22	0.795[23a]	3H, t, *J* = 6.8
	CO	176.81[23b]		
	NH		7.447	1H, s
	β'	26.54	2.187 & 1.628[23a]	2H, m
	γ'	7.33	0.696	3H, t, *J* = 7.5

	Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, J (Hz)
^{5}Aib	α	56.28		
	β	23.17	1.287	3H, s
	β	26.39	1.347	3H, s
	CO	175.51[23b]		
	NH		7.925	1H, s
^{6}Gln	α	55.57	3.933	1H, m
	β	26.54	1.972[23a]	2H, m
	γ	31.82	2.196[23a] & 2.101[23a]	2H, m
	δ CO	174.01[23b]		
	ε NH_2		6.802 & 7.266	2H, br. s
	CO	173.66[23b]		
	NH		7.785	1H, d, J = 6.6
^{7}Gln	α	55.57	3.899	1H, dt, J = 5.9, 8.3
	β	26.54	1.912[23a]	2H, m
	γ	31.82	2.187[23a]	2H, m
	δ CO	174.01[23b]		
	ε NH_2		6.819 & 7.275	2H, br. s
	CO	175.87[23b]		
	NH		7.748	1H, d, J = 5.4
^{8}Aib	α	56.14		
	β	23.76	1.347	3H, s
	β	26.25	1.466	3H, s
	CO	175.70[23b]		
	NH		7.580	1H, s
^{9}Phe	α	55.72	4.351	1H, ddd, J = 11.2, 8.3, 4.9
	β	36.36	3.210[23a] & 2.883	2H, m & dd, J = 13.7, 11.2
	1	137.61		

Assignment		^{13}C (δ)	^{1}H (δ)	int, mult, J (Hz)
	2,6	128.87	7.180	2H, m
	3,5	128.03	7.22[23a]	2H, m
	4	126.34	7.16	1H, m
	CO	175.88[23b]		
	NH		7.805	1H, d, J = 8.3
[10]Pro	α	64.70	4.191	1H, t, J = 7.5
	β	28.59	2.015, 1.216	2H, m
	γ	25.81	1.637, 1.731	2H, m
	δ	48.39	3.699, 3.278	2H, m
	CO	172.91[23b]		
[11]Aib	α	56.14		
	β	23.02	1.314	3H, s
	β	26.69	1.398	3H, s
	CO	173.28[23b]		
	NH		8.786	1H, s
[12]Oc	CO-1'	174.21[23b]		
	2'	35.00	2.26	2H, m
	3'	25.68	1.64	2H, m
	4'	29.06	1.22	2H, m
	5'	29.06	1.22	2H, m
	6'	31.41	1.17	2H, m
	7'	22.31	1.18	2H, m
	8'	14.02	0.81	3H, ot

23 *a* Due overlapping resonances in ^{1}H NMR spectrum of metabolite **1**, the exact chemical shift for each proton could not be determined, the values given were obtained from the HMQC or HMBC data
b Chemical shift assignment was based on the HMBC NMR data

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 2 in DMSO-d_6

Assignment	^{13}C[26b] (δ)	^{1}H (δ)	int, mult, J (Hz)
^{1}Leu α	48.77	3.760	1H, m
β	40.00	1.309	2H, m
γ	25.44	1.57[26a]	1H, m
δ	23.56 & 21.92	0.77[26a]	2x3H, d
CH$_2$-OH	66.09	3.124 & 3.244	2H, m
NH		6.652	1H, d, J = 9.3 Hz
^{2}Ala α	49.59	4.012	1H, m
β	17.59	1.259	3H, d, J = 7.3
NH		7.522	1H, d, J = 7.3
^{3}Gln, ^{6}Gln, ^{7}Gln			
α	54.80	3.805	1H, m
	55.34	3.922	1H, m
	55.62	3.886	1H,m
β	27.42-27.57	1.98-1.93[26a]	2H, m
γ	32.70	2.1-2.4[26a]	2H, m
	32.99		
	33.14		
NH		7.568	1H, d, J = 5.4
		7.730	
		7.775	
εNH$_2$		6.763 & 7.156	2H, br.s
		6.802 & 7.265	2H, br.s
		6.823 & 7.265	2H, br.s

[26] [a] Exact chemical shifts could not be determined due overlapping resonances in ^{1}H NMR spectrum. [b] Chemical shift assignment was based on the HMQC or HMBC NMR data; carbonyl and quaternary carbons could not be observed.

Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, J (Hz)
$^{4}\alpha$-EtNor			
α	60.27		
β	30.35	1.29[26a]	2H, m
γ	32.99	1.29[26a]	2H, m
δ	13.97	0.81[26a]	3H, t, J = 6.8
NH		7.433	1H, s
β'	26.54	2.19[26a] & 1.61[26a]	2H, m
γ'	7.12	0.694	3H, t, J = 7.5
^{5}Aib, ^{8}Aib, ^{11}Aib			
α	56.1-56.3		
β	25.6 & 26.6	1.30 & 1.35	2x3H, s
	26.2 & 26.0	1.35 & 1.46	
	~24 & 26.0	1.29 & 1.39	
NH		7.550	1H, s
		7.918	
		8.798	
^{9}Tyr			
α	56.44	4.218	1H, m
β	35.34	3.069 & 2.778	2H, m
1	---		
2,6	129.8	6.966	2H, d, J = 8.3
3,5	116.6	6.607	2H, d, J = 8.3
4	---		
NH		7.73[26a]	1H
^{10}Pro			
α	63.01	4.192	1H, t, J = 7.5
β	29.62	2.027, 1.291	2H, m
γ	26.83	1.68[26a], 1.75[26a]	2H, m

Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, *J* (Hz)
δ	48.49	3.698, 3.302	2H, m
$^{12}C_{8}$			
2'	35.07	2.25	2H, m
3'	25.68	1.66	2H, m
4', 5', 6', 7'	29.0-31.5	1.17[26a] -1.22[26a]	8H, m
8'	14.02	0.80[26a]	3H, t

Table 3. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of 3 in DMSO-d_6

Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, *J* (Hz)
^{1}Lol			
α	48.41	3.79	1H, br m
β	39.91	1.32[28a]	2H, m
γ	23.98[28c]	1.65[28a]	1H, m
δ	21.55 & 23.30	0.80[28a]	2x3H, d
CH2-OH	64.06	3.171 & 3.27[28a]	2H, dd, *J* = 7.8, *J* = 10.8 & m
NH		6.689	1H, d, *J* = 9.2 Hz
^{2}Ala α	49.26	4.039	1H, q, *J* = 7.3
β	17.13	1.278	3H, d, *J* = 7.3
CO	171.7[28b]		
NH		7.526	1H, d, *J* = 7.3
^{3}Gln, ^{6}Gln, ^{7}Gln			
α-6	55.01	3.935[28a]	1H, m
α-7	55.01	3.925[28a]	1H, m
α-3	54.45	3.826	1H, m
β-6	25.9[28b]	2.00[28a]	2H, m
β-7	25.9[28b]	1.95[28a]	2H, m
β-3	25.9[28b]	1.95[28a] & 2.00[28a]	2H, m
γ-6	31.71	2.10 & 2.22	2H, m
γ-7	31.35	2.15 & 2.21	2H, m
γ-3	31.35	2.20 & 2.30	2H, m
δ CO	~173-175[28b]		
εNH2		6.759 & 7.163	2H, br.s
CO	~173-175[28b]		
NH-6		7.81	1H
NH-7		7.732	1H, d, *J* = 5.5
NH-3		7.580	1H, d, *J* = 4.4

Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, *J* (Hz)
ε-NH_2		6.72, 6.76 & 6.79	3H, 3s
		7.17 & 7.25	1H & 2H, s
^{4}Aib, ^{5}Aib, ^{8}Aib, ^{11}Aib			
α	56.02		
	55.89		
	55.77		
	55.77		
β	26.4-25.6[28b]	1.307	3H, s
	23.5-22.59[28b]	1.335	3H, s
		1.359	6H, s
		1.371	3H, s
		1.397	3H, s
		1.411	3H, s
		1.478	3H, s
CO	~173-174[28b]		
NH-4		7.505	1H, s
NH-5		7.859	1H, s
NH-8		7.577	1H, s
NH-11		8.771	1H, s
^{9}Phe			
α	55.28	4.346	1H, m
β	35.86	3.220 & 2.904	2H, dd, *J* = 13.8, 4.2
1	137.60		
2,6	128.80	7.22	2H, m
3,5	127.97	7.22	2H, m
4	126.28	7.28	1H, m
CO	175.6[28b]		
NH		7.80[28a]	1H

Assignment	^{13}C (δ)	^{1}H (δ)	int, mult, J (Hz)
^{10}Pro			
α	62.49	4.205	1H, t, J = 7.2
β	28.04	2.02[28a], 1.22[28a]	2H, m
γ	25.19[28c]	1.65, 1.75	2H, m
δ	48.39	3.70, 3.30	2H, m
CO	171.7[28b]		
^{12}Dec			
CO-1'	~175.1[28b]		
2'	34.73[28d]	2.26[28d]	2H, m
3'	~25.1[28d]	1.64[28d]	2H, m
4'	28.51[28d]	1.22[28d]	
5'	28.57[28d]	1.22[28d]	
6'	28.57[28d]	1.17[28d]	
7'	28.73[28d]	1.17[28d]	
8'	31.11[28d]	1.17[28d]	
9'	21.94[28d]	1.18[28d]	
10'	13.79	0.81	3H, t, J = 7.1

[28] a)Exact chemical shifts could not be determined due to overlapping resonances in ^{1}H NMR spectrum
b)Although all of the expected carbonyl resonances (a total of 14 signals) were clearly observed in the ^{13}C NMR spectrum of metabolite **3**, the exact chemical shift of each amino acid unit could not be assigned due to extensive overlapping of the correlation signals in the HMBC spectrum.
c)Chemical shift assignment could be interchanged.
d)Chemical shift assignment was based on the HMQC NMR spectrum

Chapter 4

BIOLOGICAL ACTIVITY OF *TOLYPOCLADIUM GEODES* LP237 METABOLITES

4.1 Introduction

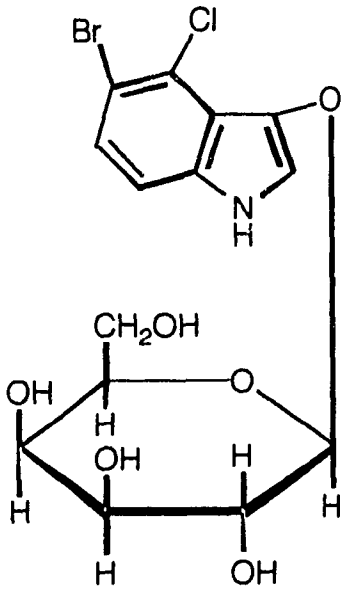
Preliminary studies on the biological activity of LP237-F8, F5 and F7, all isolated from *T. geodes*, as previously described, have revealed that these compounds may potentially be used someday as anti-cancer drugs. The activity of the metabolites was evaluated using the SOS Chromotest. In addition, various human and mouse cell lines were also used to test the antitumor activity of the *T. geodes* fungal metabolites.

4.2 The SOS Chromotest[29]

Throughout the various chromatographic steps in the isolation scheme which yielded the LP237 metabolites 1, 2 and 3 biological activity of the fractions collected was monitored using the SOS Chromotest. This assay is quick and fairly simple, and may be used quantitatively and/or qualitatively. It is a colorimetric assay system for the detection of DNA-damaging agents which induce cell filamentation. The test uses an *E-coli* strain (PQ37) which carries a *sfiA::lacZ* fusion and has a deletion for the normal *lac* region. *LacZ*

[29] a)Review: Quillardet, P. and Hofnung, M. *Mutation Res.* **1993**, *297*, 235. b)Mamber, S. W.; Okasinski, W. G., Pinter, C. D.; Tunac, J. B. *Mutation Res.* **1986**, *171*, 83. c)Quillardet, P. and Hofnung, M. *Mutation Res.* **1985**, *147*, 65

is the structural gene for β-galactosidase, which is now under control of the *sfiA* gene, an SOS function involved in cell-division inhibition. This means that β-galactosidase activity is strictly dependent on *sfiA* expression. Development of a dark blue color reaction surrounding the site of sample application, is indicative of β-galactosidase induction. Induction of β-galactosidase is seen by introducing X-Gal in the medium containing the PQ 37 cells and the various compounds. X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (**8**), is a sugar moiety attached to a chromophore which is also a substrate for β-galactosidase. When β-galactosidase is activated, it cleaves the chromophore from the sugar thus giving-off a blue color. Scoring of β-galactosidase activity was based upon the relative size and intensity of the color reaction (0 = negative, +1 = equivocal., +2 = weak positive, +3 = moderate positive, +4 = strong positive). A clear inhibition zone is observed within the color zone if samples are both cytotoxic, noted by T, and induce the SOS response. AZT was used as a control and given a score of +4T at a concentration of 250 μg/ml.



X-Gal (8)

In each of the chromatographic separations, various fractions were collected: fractions scoring the highest in the SOS chromotest were pooled together and further purified. It was observed that the fractions collected after the C_{18} reversed-phase silica gel column scored as high as +3T in the test. These were further purified by HPLC to yield the three metabolites described in the previous chapter, which were also tested with the SOS chromotest. In all three cases, the SOS response changed after purification; although the pure metabolites were still cytotoxic (that is they all scored T), β-galactosidase activity was given a score of +1 after HPLC purification of metabolites LP237-F5 and F7, and +2 for metabolite LP237-F8.

4.3 Determination of IC_{50}

High levels of cytotoxicity were observed in assays using P388D1 murine leukemia cells (IC_{50} = ~0.5 μg/ml) and a number of human carcinoma cell lines (lung A549, ovarian OVCAR3, colon SW620 and breast MCF7, IC_{50} = 0.2-0.5 μg/ml). Cytotoxicity using murine leukemic P388D1 cells was determined by adding different concentrations of compounds in media. Cells were incubated at 37°C and were counted after 48 h of incubation. IC_{50} values, concentration necessary to inhibit the growth of 50% of the total amount of initial cells, were obtained as %T/c ratios; %T is equal to the total number of cells after 48 h of incubation with the test compound minus the amount of initial cells, while c is equal to the total amount of cells in the control after 48 h minus the amount of initial cells. In general, the lower the value of IC_{50}, the more effective the drug.

In the assays described above, results concerning the biological activity of LP237 metabolites F8, F5 and F7 have been promising. Initially, the crude mixture had an IC_{50}

value equal to 0.5 µg/ml on P388D1 murine leukemia cells. After purification with the LH 20 column, the peptide mixture exhibited stronger cytotoxic activity (IC_{50} =52 ng/ml). Purification with the C_{18} reverse phase column resulted in fractions exhibiting IC_{50} values in the 3-5 ng/ml range. The cytotoxicity of metabolite **1** (IC_{50} =~30 µg/ml), **2** (IC_{50} =~10 µg/ml) and **3** (IC_{50} =~40 µg/ml) towards P388D1 murine leukemia cells were much lower than in the mixture of peptides. Therefore, it is reasonable to assume that the most potent component of the mixture remains to be identified or that there is a synergystic effect associated with the antitumor activity of the mixture. It is too soon to predict the activity of these metabolites. Further testing using the same techniques, as well as additional testing using various other types of assays may begin to clarify this issue. The biological activities of the *T. geodes* metabolites are currently under investigation.

Chapter 5

EXPERIMENTAL

5.1 Reagents and Chemicals

All chemicals used for the growth and maintenance of the *T. geodes* cultures, as well as chemicals used for the SOS chromotest (malt, yeast extract and agar) were obtained from BDH, with the exception of tryptone which was purchased from Difco and X-Gal, which was purchased from Gold Biotechnology Inc. AZT was used as a control in the SOS Chromotest. Diazald, 2-(2-ethoxyethoxy)ethanol, and the Diazald kit were bought from Aldrich. All deuterated solvents were purchased from Cambridge Isotopes Inc. and all HPLC grade solvents were bought from BDH. Octadecyltrichlorosilane and tetramethylsilane, used for the preparation of reversed-phase silica were obtained from Aldrich.

5.2 General Methods

5.2.1 Spectra:

Nuclear Magnetic Resonance spectra were obtained at 20-22°C using Varian Unity-500 spectrometer (operating at 499.84 MHz for ^{1}H and 125.7 MHz for ^{13}C) and Bruker-AMX 600 MHz instrument. ^{1}H and ^{13}C-NMR chemical shifts are quoted in ppm and are

referenced to the internal deuterated solvent downfield from tetramethylsilane (TMS). NOESY experiments (hypercomplex phase mode) were obtained using a mixing time of 400 and 800 ms with Varian's standard pulse program and 256 increments in t1. Zero-filled in the evolution domain and linear prediction to lengthen the evolution domain were used to obtain the final data size of the 2048 x 2048 complex matrix. The HMQC experiments with a preceding BIRD nulling period were used with hypercomplex phase mode and ^{13}C broadband waltz decoupling was applied during acquisition of the proton spectra. The HMBC experiment was acquired using similar conditions (without the null period and ^{13}C decoupling) and the t delay was set to 100 ms for long-range coupling. NOESY experiments performed on the Bruker AMX-600 spectrometer were obtained using a mixing time of 300 ms using Bruker's standard pulse program, 256 increments in t1, and a 2048 x 2048 complex matrix. The HMQC and HMBC NMR spectra were acquired using Bruker's standard pulse programs for these experiments.

All FAB mass spectra were performed using a ZAB-E4F instrument. A Kratos Kompact MALDI 3 V 4.0 instrument was used to record all MALDI mass spectra, while all ES mass spectra were run on a Finnigan SSQ 7000. The IR spectrum was obtained using a Nicolet, Magna-IR Spectrometer 550. All UV spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer instrument. Optical rotations were measured using a JASCO DIP-140 Digital polarimeter.

5.2.2 Chromatography:

Reverse phase flash column chromatography was carried out on silica gel (Silica Gel 60, 70-230 mesh) reacted with *n*-octadecyl-trichlorosilane. C_{18} reverse-phase silica

gel was prepared following literature procedures. All chromatographic solvents were fractionally distilled prior to use with the exception of HPLC grade solvents.

5.2.3 HPLC.

Doubly distilled H_2O was filtered through a 0.45 mm filter membrane and HPLC grade MeOH and MeCN, were filtered through a 0.5 mm filter membrane (Millipore Corp., Mississauga, ON) before they were used in HPLC. All solvents contained 0.1% TFA. Analysis and purification was carried out on a semi-preparative reverse phase, C_{18} column; Waters NOVA-PAK (25 mm x 100 mm, 6mm, 60Å) using a Waters instrument (pump model W600EP, UV/VIS detector model M486P, Rheodyne injector). All retention times were automatically recorded from the time of injection.

5.3 Preparation reversed-phase silica gel

Approximately 85 g of silica gel was dissolved in 400 ml of carbon tetrachloride. To this solution, 8 ml of octadecyltrichlorosilane was added using an oven-dried needle. The yellow mixture was allowed to stir at room temperature for 2 h. The solution was then filtered through an oven-dried sintered-glass funnel into a 500 ml flask. After washing the filter cake with 2x 200 ml methanol and 2x 200 ml dichloromethane, the powder was transferred into an oven-dried 500 ml round-bottom flask. This solid material was dissolved with 300 ml carbon tetrachloride and after adding 8 ml of tetramethylsilane to the solution, it was left to stir at room temperature for 2 h. The solution was filtered and

washed with excess dichloromethane. The filter cake was transferred into a clean, oven-dried 500 ml round-bottom flask and allowed to dry overnight under high vacuum

5.4 Preparation of biological media

The growth and maintenance of the *T. geodes* cultures requires the preparation of 2% malt solution and 2% malt agar solution. The 2% malt solution is prepared simply by adding 2 g of malt extract in 100 ml of distilled water while the 2% malt agar solution is prepared by adding 2 g of malt extract and 1.5 g of agar in 100 ml of distilled water. Both solutions need to be sterilized and are therefore autoclaved at 121°C for 20 min. LB broth, LB agar and 0.8% agar solutions are needed during the SOS test. The latter solution need not be sterile and is quickly prepared by adding 0.8 g of agar in 100 ml of distilled water. The solution is heated, using a microwave, until all the agar has dissolved. The LB broth is prepared by adding 5 g of tryptone, 2.5 g of yeast extract and 5 g of sodium chloride in 500 ml of distilled water. The solution is then autoclaved at 121°C for 20 min. The LB agar media is prepared as is LB broth with the addition of 6 g of agar before autoclaving.

5.5 General extraction of active metabolites

Stock cultures of *T. geodes* were maintained on 2% malt extract (Difco) plus 1.5% agar in slant tubes at 4°C. A small amount of mycelium was used to inoculate 2% malt extract agar plates which were then incubated in the dark, at room temperature for a period

of 14 days. Plugs (~15/flask) of actively growing mycelium were used to inoculate 2% malt extract liquid medium (6x500 ml culture in 3 L flasks). The fermentation flasks were then incubated at 20°C on a rotary shaker at 120 rpm for 7 days. The mycelium was removed by filtration, and the resulting filtrate was absorbed onto a Diaion HP 20 column (60 mm x 25cm), eluted using a linear step gradient from 100% H_2O to 100% MeOH to 100% acetone, at a flow rate of 10 ml/min. The cytotoxic fractions, which eluted from ~75% aqueous MeOH to 100% MeOH, were combined and evaporated to dryness to give ~1.5 g of a brown gum. The active crude was subsequently dissolved in 2-5 ml of MeOH, loaded on a Sephadex LH-20 column (25 mm x 100 cm) and partitioned into 90 fractions (~8 ml each) by eluting with degassed MeOH at a flow rate of 0.7 ml/min. Fractions 22-30 exhibited strong cytotoxic activity (IC_{50} = 52 ng/ml) and gave a strong positive SOS Chromotest. These fractions were combined, evaporated to dryness (~230 mg) and further purified by flash column chromatography on a C_{18} reverse phase column (20 mm x 15 cm), using a linear solvent gradient from 100% H_2O to 100% MeOH to 100% CH_2Cl_2 at a flow rate of 2ml/min. The active fractions eluted from ~85% aqueous MeOH to ~95% aqueous MeOH were strongly cytotoxic. These were combined and evaporated to dryness to give ~40 mg of active material, which was further analyzed by semi-preparative C_{18} reverse phase HPLC. HPLC purification of the above cytotoxic crude, using a solvent mixture of MeOH : H_2O : MeCN (70:20:10, with 0.1% TFA in all solvent), allowed the isolation of five biologically active peptides; two of these metabolites, LP237-F6 and F9, are currently under investigation. At a flow rate of 4 ml/min, metabolites **1**, **2** and **3** had a retention time of 23.6, 16.4 and 20.7 in respectively. All three peptides were purified by HPLC for a second time, using the same conditions, in order to isolate the pure compounds as amorphous white solids (~3.8 mg of **1**, ~1.2 mg of **2** and ~6.2 mg of **3**). Metabolite 1 UV (MeOH, nm): max 224, 258. $[a]_D$ +9.88 (c 0.0016, MeOH). NMR data given in Table 1. Metabolite 2 UV (MeOH, nm): max 226, 276. $[a]_D$ +9.50 (c 0.0027, MeOH). NMR data given in Table 2. Metabolite 3 UV (MeOH, nm): max 226, 258. $[a]_D$ +0.21 (c

0.0102, MeOH). NMR data given in Table 3. MS data for all three metabolites and IR data for metabolite **1** are reported in the discussion.

5.6 Bioassays against *E. coli* PQ 37 strain

Biological activity of metabolites **1** and **2** and **3** was determined using the *Escherichia coli* SOS Chromotest. 15 ml of warm LB agar is poured into a petri dish (100 x 15 mm) and allowed to cool. 200 µl of PQ 37 (incubated overnight in 60 ml of LB broth in a rotary shaker set at 220 rpm and 37°C) in 3 ml of 0.8 % agar is then poured into the dish. Wells were then manually cut through the solid agar; 15 µl of each metabolite and 10 µl of AZT were delivered into the appropriate well. The petri dishes were then incubated at 37°C for 24 h. The next day, 125 µl of X-Gal in 3 ml of 0.8% agar was poured in each plate and the dishes were incubated at 37°C for 20 min. Each plate was then scored according to the system described in the previous chapter.

5.7 Amino acid analysis

Metabolites **1**, **2**, and **3** were analyzed for their amino acid content. An aliquot of each metabolite was injected on a Beckman High Performance Analyzer, which utilizes a strong cation exchange column in a temperature gradient from 48°C to 65°C at 1°C/min.

Prior to injection, each sample is hydrolyzed using HCl, and it is actually the hydrolysate which is analyzed for the presence of amino acids. With the cationic exchange column, acidic and straight chain hydroxy amino acids are eluted first, followed by small and neutral amino acids; the basic and modified amino acids are eluted last with an increase in temperature and pH.

5.8 Methylation with Diazomethane

5.8.1 Diazomethane reaction

Dilute samples of metabolites **1** and **3** were prepared in 99% THF and 1% H_2O. Diazomethane was added to each of the samples until the solutions were saturated with the methylating agent and the characteristic yellow color of the diazomethane solution remained constant. The solutions were left at 25°C overnight and the solvent was evaporated off.

5.8.2 Preparation of diazomethane

For an alcohol-free ethereal solution, 28 ml of 2-(2-ethoxyethoxy)ethanol was added to 16 ml of ether and a solution of 5 g of potassium hydroxide in 8 ml of water in a 250 ml round bottom flask. A separatory funnel, containing 10g of Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) in 90 ml of ether was placed over the reaction vessel. The reaction flask was warmed to 65°C using a water bath and the Diazald solution was added over a period of 30 min. Preparation of diazomethane was carried out in glassware found in the Diazald kit, which is a set of distillation glassware with Clear-Seal joints. Plastic

tubings with the Diazald kit, as well as any solvent which may freeze should be avoided, as sharp edges of crystals formed may cause an explosion.

Please note that the generation and use of diazomethane is a hazardous procedure Diazald is a severe skin irritant and all skin contact should be avoided. Diazomethane is extremely toxic and highly irritating; it has been known to explode as a gas and in solution, upon contact with rough surfaces. Protective clothing, safety glasses and gloves should be worn at all times. All experimental procedure using diazald and/or diazomethane should be done in the fumehood using a safety shield.

Appendix A

Elution gradient for reversed phase silica gel column.

FRACTION NUMBER	AMOUNT OF WATER (%)	AMOUNT OF MeOH (%)	AMOUNT OF CH_2Cl_2 (%)	TOTAL VOLUME (ml)
1	100	0	0	100
2	95	5	0	100
3	90	10	0	100
4	85	15	0	100
5	80	20	0	100
6	75	25	0	100
7	70	30	0	100
8	65	35	0	100
9	60	40	0	100
10	55	45	0	100
11	50	50	0	100
12	45	55	0	100
13	40	60	0	100
14	35	65	0	100
15	30	70	0	100
16	25	75	0	100
17	20	80	0	100
18	15	85	0	100
19	10	90	0	100
20	5	95	0	100
21	0	100	0	100
22	0	50	50	100
23	0	0	100	100