ANTIBIOTIC SECONDARY METABOLITES OF BACTERIA
ISOLATED FROM THE MARINE ENVIRONMENT

by

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B.Sc., University of Alberta, 1992

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Department of Chemistry)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1997

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Department of Chemistry

The University of British Columbia
Vancouver, Canada

Date Sept 28, 1997
ABSTRACT

Bioassay guided fractionation of the organic extracts obtained from cultures of several bacteria strains from the marine environment led to the isolation of twelve new and nine previously described secondary metabolites. The structures of these metabolites were determined by extensive chemical and spectroscopic analysis. Stable isotope incorporation experiments were also performed using one of the isolated strains to investigate the biosynthetic origins of the atoms in the principle active secondary metabolite.

A culture of a *Bacillus* sp. isolated from the tissues of a marine worm collected near Loloata Island in Papua New Guinea produced a mixture of novel cyclic decapeptide antibiotics. Loloatins A (1), B (2), and C (3) were isolated and their structures were elucidated through NMR and mass spectrometric analysis. Peptides 1, 2, and 3 showed potent gram-positive antibiotic activity, including activity against drug resistant strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus* spp. Loloatin C (3) also showed strong Gram-negative antibiotic activity.

Massetolides A - H (4 - 11), novel cyclic depsipeptides, as well as the known compound viscosin (12), were isolated from cultures of two *Pseudomonas* sp. isolated from a marine alga and a marine tube worm each collected near Masset Inlet, B.C. and Moira Island, B.C. respectively. Massetolide A (4) and viscosin (12) exhibited *in vitro* antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*. 
The known compounds, AI77-B (13), AI77-F (14) as well as AI77-H (15), a new diastereomer of AI77-F(14), were isolated from several species of *Bacillus pumilus* isolated from various marine sources. AI77-B (13) exhibited cytotoxic and Gram-positive antibiotic activity. The absolute configuration of AI77-H (15) was determined by chemical modification and NMR analysis of the (R)- and (S)-α-methoxy-α-(trifluoromethyl)phenylacetate esters. Finally, stable isotope incorporation experiments were performed using a selected strain of *Bacillus pumilus* which demonstrated that AI77-B (13) is of mixed polyketide/amino acid biosynthetic origin.
L-Val  L-Orn  L-Leu  D-Tyr

L-aaR₂

L-Asn  D-Phe  L-aaR₁

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Al77-B (13)

Al77-F (14)

Al77-H (15)
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<tr>
<td>[α]_D</td>
<td>specific rotation at wavelength of sodium D line</td>
</tr>
<tr>
<td>[Θ]_λ</td>
<td>molar ellipticity at wavelength λ</td>
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<td>1D</td>
<td>one dimensional</td>
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<tr>
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<td>AHPPA</td>
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<tr>
<td>C₆D₆</td>
<td>benzene-$_d_6$</td>
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<td>coenzyme A</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
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<td>COSY</td>
<td>homonuclear correlation spectroscopy</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<td>δ</td>
<td>chemical shift in parts per million downfield from ((CH_3)_4Si)</td>
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<tr>
<td>D, Asp</td>
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<td>difference in mass</td>
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<td>4-N,N-dimethylaminopyridine</td>
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<td>DMSO</td>
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<tr>
<td>DSS</td>
<td>2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (NMR standard)</td>
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<td>ε</td>
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<tr>
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<td>50% effective dose</td>
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<td>Fourier transform</td>
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<td>Fourier transformation infra-red spectroscopy</td>
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<td>human immunodeficiency virus</td>
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<td>HMBC</td>
<td>heteronuclear multiple bond multiple quantum correlation spectroscopy</td>
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<tr>
<td>HMQC</td>
<td>heteronuclear multiple quantum correlation spectroscopy</td>
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</table>
HOHAHA  homonuclear Hartman Hahn correlation spectroscopy
HPLC    high performance liquid chromatography
HRDCIMS high resolution desorption chemical ionization mass spectrometry
HREIMS  high resolution electron impact ionization mass spectrometry
HRFABMS high resolution fast atom bombardment mass spectrometry
HRLSIMS = HRFABMS, high resolution liquid secondary ion mass spectrometry
i      NMR resonance due to impurity in sample
I, Ile  isoleucine
IC<sub>50</sub> 50% Inhibitory Concentration
IR     infra-red
J      scalar coupling constant in Hz
L, Leu  leucine
λ<sub>max</sub> wavelength at absorbance maximum
m      multiplet
M      molecular ion
m/z    mass-to-charge ratio
MAC    *Mycobacterium avium-intracellulare* complex
MAI    *Mycobacterium avium-intracellulare*
Me     methyl
Me<sub>2</sub>CO acetone
MeOH   methanol
MIC    minimum inhibitory concentration
MIS    Microbial Identification System
mp     melting point
MRSA   methicillin resistant *Staphylococcus aureus*
MTB  Mycobacterium tuberculosis
MTPA  (α-methoxy)(α-trifluoromethyl)phenylacetic acid - Mosher’s acid
N, Asn  asparagine
NMR  nuclear magnetic resonance
NOE  nuclear Overhauser effect
NP  normal phase
O, Orn  ornithine
ODS  octadecyl silyl
p  pentad
P, Pro  proline
P388  murine leukemia cell line P388
PFPA-IPA  pentafluoropropionyl amide isopropyl alcohol
Phe, F  phenylalanine
PTFE  poly tetrafluoro ethylene
ppm, PPM  parts per million
q  quartet
Q, Gin  glutamine
rf  radiofrequency
ROESY  rotating frame Overhauser effect correlation spectroscopy
RP  reversed phase
s  solvent
s  strong
s  singlet
S, Ser  serine
SCUBA  self-contained underwater breathing apparatus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>sp.</td>
<td>species (singular)</td>
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<tr>
<td>spp.</td>
<td>species (plural)</td>
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<td>triplet</td>
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<td>threonine</td>
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<td>tyrocidine A</td>
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<td>TB</td>
<td>tuberculosis</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<td>thin layer chromatography</td>
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<td>tryptophan</td>
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<td>Tyr, Y</td>
<td>tyrosine</td>
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<td>UV</td>
<td>ultra-violet</td>
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<td>V, Val</td>
<td>valine</td>
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<td>VRE</td>
<td>vancomycin resistant enterococci</td>
</tr>
<tr>
<td>W, Trp</td>
<td>tryptophan</td>
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<tr>
<td>Y, Tyr</td>
<td>tyrosine</td>
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</table>
ACKNOWLEDGEMENTS

I truly appreciate the support, encouragement, and direction offered by my research supervisor, Prof. Raymond Andersen.

I am indebted to Paul Haden for his tireless work over the years maintaining bacterial cultures and performing bioassays. I am also deeply indebted to the staff in Mass Spectrometry Services, specifically Dr. Eigendorf for his assistance and especially Chris Emond for his excellent work acquiring FABMS and MS-MS data on the peptide projects. The helpful staff in NMR services, Marietta Austria and Liane Darge are gratefully acknowledged for their assistance and aid in the use of the NMR spectrometers. The massetolides project was done in collaboration with Dr. Richard Lloyd, and his cooperation was appreciated, as were the cooperative efforts with Dr. Fangming Kong and Dr. Dilip de Silva. Special mention goes to Dr. David Williams for his suggestions and help over the past few years. I would like to thank Philip Johnson and Bill Goldring for their aid in computer molecular modeling simulations. Finally, I thank Bruno Cinel for his excellent editing skills.
DEDICATION

Robert Bruce Irving
(1970-1997)
You taught me to hold out for hope.

And

For Dad and Mom
Although second on this page,
First in my heart!
Chapter 1: Introduction

1.1 General Introduction

The discovery of penicillin in 1929 revolutionized natural products research. Until then, natural products research had focused largely on secondary metabolites from plants and the medicinal uses of these plant extracts. However, with the serendipitous discovery of penicillin came the realization that microorganisms were not only the cause of infection, but they also produced organic compounds antagonistic towards other microorganisms. Researchers then began to focus their attention on microorganisms as a source of clinically useful natural products. The result has been the identification to date of between 30,000 and 50,000 natural products originating from unicellular organisms, many of them clinically useful. However, most known antibiotics of microbial origin are derived from terrestrial Actinomycetes and recently it has become clear that the rate of discovery of novel secondary metabolites from this source is decreasing, and new sources of metabolites must be explored.

1.2 Drug Resistance in Bacteria

Despite the plethora of metabolites known and in use for treating bacterial infections, there is still a need for new antimicrobial agents. Almost immediately after penicillin was introduced as a clinical treatment for bacterial infections, bacterial strains that were resistant to penicillin developed. Since bacteria mutate, and are able to exchange chromosomal material between species via plasmids and transposons, it has been a constant battle to stay one step ahead of these pathogens. There are also several human pathogens to which there are still very few or no effective treatments. The emergence of human immunodeficiency virus (HIV) and acquired
immunodeficiency syndrome (AIDS) has particularly highlighted this problem, with AIDS patients succumbing to what were previously thought to be innocuous bacterial and fungal infections.³

One area where new drugs are desperately needed is in the treatment of antibiotic resistant strains of Gram-positive human pathogens. Methicillin resistant strains of *Staphylococcus aureus* (MRSA) cause infections that are refractory to standard anti-staphylococci antibiotics and in many cases vancomycin is the antibiotic of last resort. Infections due to enterococci have been difficult to treat for many years because these organisms are intrinsically resistant to many antibiotics. Ampicillin has been the mainstay for treatment of uncomplicated enterococcal infections, but many strains have now become resistant to ampicillin. Vancomycin is again the only effective treatment for these ampicillin resistant enterococcal infections. In the past few years, vancomycin resistant enterococcal strains (VRE) have begun to appear and they are rapidly spreading across North America. There are no effective antibiotics currently available for such organisms and the recent report of an outbreak of VRE with a 73% mortality rate has highlighted the seriousness of the situation.⁴ Furthermore, since bacterial species are able to “communicate” resistance to other species, there is a justified fear that methicillin and vancomycin resistant bacterial strains may develop, for which there would be no effective treatment.

Another group of bacteria that also causes several infections in humans are the mycobacteria - most notable of which are *Mycobacterium tuberculosis* (MTB) and *Mycobacterium avium-intracellulare* (MAI). Approximately one-third of the world's population harbors MTB the causative agent of TB, and is at risk for developing the disease.⁵ As a consequence, TB has become the leading cause of human death worldwide from infectious diseases. In North America, cases of TB steadily declined from 1882 until 1985 when the
incidence began increasing again. Coupled with the increasing incidence of TB has been the emergence of multidrug-resistant strains. One-third of all TB cases tested in a 1991 survey in New York were resistant to one or more drugs, a sobering result since the fatality rate for TB resistant to two or more antibiotics is equivalent to that of untreated TB (40 to 60%). 

*Mycobacterium avium-intracellulare* (MAI) results in a complex of opportunistic infections, MAC, that is associated with significant morbidity and mortality in AIDS patients. These infections are difficult to eradicate because MAI is intrinsically resistant to most antibiotics, including those usually active against MTB.

### 1.3 Marine Bacteria as a Source of Novel Secondary Metabolites

Due to the decrease in discoveries from terrestrial microbial sources and the pressing need for novel antibiotics, natural products chemists have searched for a new source of secondary metabolites. Working with the hypothesis that unique environmental habitats should yield novel microorganisms with new biosynthetic abilities, natural products chemists have turned to the ocean as an unexplored source of microorganisms. It has been assumed that marine microorganisms have developed unique metabolic and physiological capabilities to cope with extremes in salinity, pressure, temperature, and nutrient availability. The water column, marine sediments rich in organic materials, and the surfaces of marine plants and animals, provide a variety of micro-habitats for marine microorganisms. Hence, marine microorganisms offer the potential for the production of secondary metabolites not seen from terrestrial isolates.

The biomedical potential of marine bacteria was first recognized in 1947 by Rosenfeld and Zobell who showed that microorganisms present in seawater produced antimicrobial agents.
decade later and Grein and Meyers reported that almost one half of the actinomycetes isolated from the Florida coast exhibited antibiotic activity against both Gram-positive and Gram-negative bacteria. However, it was not until the early 1970's that chemical research on marine microorganisms began in earnest.

Over the past two decades microorganisms isolated from marine habitats have emerged as a promising source of new bioactive metabolites with potential for development into drugs for treating human diseases. Although this chapter is meant to introduce natural products isolated from marine bacteria (exclusive of marine fungi and microalgae) it is not meant as a comprehensive review of secondary metabolites from marine microorganisms. The reader is directed to any or all of the excellent reviews by Austin, Davidson, and Fenical, on this subject. This introduction only intends to provide a brief background to marine bacterial secondary metabolites and to discuss some selected examples which demonstrate the ability of marine bacteria to produce structurally novel and biologically active metabolites.

Throughout this thesis the terms “marine bacteria” and “marine microorganisms” refer to bacteria isolated from the marine environment. However, this does not necessarily indicate that these bacteria truly evolved in the marine environment. Some terrestrial species of bacteria with a high salt tolerance, notably actinomycetes and bacilli, are capable of forming stable endospores which may be washed into the oceans where they remain viable.
1.3.1 Distribution of Bacteria in the Marine Environment

Bacteria are generally recognized to belong to two different kingdoms, the eubacteria, and the more primitive archaebacteria. Most marine bacteria, including the cyanobacteria which are not treated in this thesis, are classified as eubacteria. These bacteria are further divided into two categories based on their cell wall structure. Eubacteria can be classified as either Gram-positive or Gram-negative depending on whether or not their cell wall retains a dye/iodine stain. It has been noted that like terrestrial soil, a large proportion of Gram-positive bacteria are found in marine sediments. The actinomycetes, previously mentioned as an important source of terrestrial secondary metabolites, and bacilli are common examples of Gram-positive eubacteria found in marine sediments. In contrast, water column isolates are comprised mostly of Gram-negative eubacteria including pseudomonads and vibrio bacteria.

The more primitive archaebacteria are also important bacterial inhabitants in the marine environment, and are often associated with extreme habitat conditions. Examples of archaebacteria associated with extreme environmental conditions are the halophilic archaebacteria which require high salt concentrations (12-24%) and thermoacidic archaebacteria which thrive in highly acidic solution and at temperatures near 90 °C. Unfortunately, there are very few reports in the chemical literature of secondary metabolites from these types of microbes.

1.3.2 Seawater Derived Bacteria

The water column contains a large proportion of Gram-negative bacteria. Furthermore, terrestrial isolates of Gram-negative micro-organisms have, in the past, been chemically uninteresting, yielding very few antibiotic or bioactive metabolites. However, the first bioactive
secondary metabolites isolated from marine microorganisms were isolated from a bacterium commonly present in the water column. This Gram-negative bacteria, first identified as *Pseudomonas bromoutilis*, but later reclassified to the *Alteromonas* genus, produced the highly brominated pyrrole antibiotic pentabromopseudilin (16). Pyrrole 16 proved to be more potent against Gram-positive bacteria than penicillin, with minimum inhibitory concentrations (MIC) ranging from 0.0063 to 0.2 μg/mL. Several years later Andersen and coworkers isolated several antibiotic metabolites including tetrabromopyrrole (17) and hexabromo-2,2'-bipyrrrole (18) from another *Alteromonas* sp. isolated from seawater. Tetrabromopyrrole (17) showed mild antibiotic activity against a wide range of Gram-positive and Gram-negative bacteria, as well as being autotoxic to the producing bacterium.

The discovery of these highly brominated pyrroles provided some of the first evidence that marine microbes, being in a unique environment, do posses unusual biochemistry since they are able to incorporate bromine ("abundant" in seawater but not terrestrially) into organic compounds.

*Alteromonas rubra*, another common seawater microbe, was shown to produce a series of C₁₆ aromatic acids (19, 19a, 20) that had no antibiotic activity but promoted smooth muscle relaxation in bronchodilator and neuromuscular assays.
The novel lactone oncorhyncolide (21), is a final example of an interesting secondary metabolite isolated from a Gram-negative seawater derived bacterium, *Serratia odorifera*\(^{26b}\). Although not possessing any known biological activity, 21 is of unusual biosynthetic origin. Needham and coworkers were able to show that this compound was derived through polyketide biosynthesis. Typically in polyketides methyl branches can occur in the polyketide chain by S-adenosyl methionine methylation at chain positions that were formerly C2 of acetate. However, the methyls at two sites in the carbon skeleton of the oncorhyncolide (21) skeleton were shown to be derived from C2 of acetate, and this branching occurred at positions in the polyketide chain that were formerly C1 of acetate.

![Chemical Structure of 21](image)

**21** * from C2 of acetate

### 1.3.3 Sediment Derived Bacteria

Bacteria are abundant in marine sediments and are comprised largely of actinomycetes and other Gram-positive bacteria. For example, Altermicidin (22) was obtained from cultures of the bacterium *Streptomyces sioyaensis* isolated from a marine sediment collected in the waters of Japan.\(^ {16}\) Compound 22 showed weak but selective Gram-negative antibiotic activity against *Xanthomonas* sp., and exhibited in vitro cytotoxicity against L1210 murine leukemia (IC\(_{50} = 0.85 \mu g/mL\).
Another example of bioactive metabolites of marine microbial origin was provided by Gustafson et al. who isolated macrolactins A - C (23 - 25) from a deep water sediment bacterium C-237. This Gram-positive bacterium could not be identified by standard methods, but produced 23 - 25 in liquid culture along with three other related macrolactins E - F. Macrolactin A (23), the major metabolite, showed modest antimicrobial activity, but more notably, inhibited several viruses including herpes simplex virus (IC₅₀ = 5.0 µg/mL) and HIV (IC₅₀ = 10 µg/mL).

Macrolactin A (23)  \( R = R' = R'' = H \)
Macrolactin B (24)  \( R = \text{glucose} \quad R' = R'' = H \)
Macrolactin C (25)  \( R = R' = H \quad R'' = \text{glucose} \)
The caprolactins (26, 27) are further examples of bioactive microbial metabolites produced by microorganisms obtained from marine sediments. These two related acyl aminolactams were characterized from an unidentified Gram-positive bacterium that was isolated from a deep sea core sediment sample. The caprolactams are mildly cytotoxic, and antiviral towards herpes simplex virus.¹⁸

Caprolactin A (26) R=H R₁=Me
Caprolactin B (27) R=Me R₁=H

A fourth example of interesting chemistry from marine microorganisms was recently published by Sitachitta et al., who described the isolation and structure elucidation of the structurally novel wailupemycins A - C (28 - 30) from a Streptomyces sp. cultured from a shallow water marine sediment.¹⁹ Wailupemycin A (29) exhibited antimicrobial activity against the Gram-negative bacterium *Escherichia coli*. 
Trischman et al. have recently reported a new cyclic acyldepsipeptide of the iturin class from a *Bacillus* sp isolated from a deep sea sediment.\(^\text{20}\) Halobacillin (31) is one of the few examples of a secondary metabolite from a marine bacilli reported in the chemical literature.\(^\text{21}\) Halobacillin (31) is closely related to the industrially important and much studied biosurfactant, surfactin (32). These two cyclic acyl depsipeptides differ only by the replacement of the glutamic acid in 32 with glutamine in 31, and illustrate how a seemingly minor modification in a peptide can radically affect the biological activity. Halobacillin (31) showed moderate human cancer cell cytotoxicity, but in contrast to surfactin (32) and the iturins, no antifungal or antibiotic activity. This microbe is an important example of a marine microorganism that produces a metabolite that is structurally similar to a secondary metabolite produced by a terrestrial microbe, however these metabolites have significantly different biological activity.

\[
\begin{align*}
\text{Halobacillin (31)} & \quad R=\text{NH}_2 \\
\text{Surfactin (32)} & \quad R=\text{OH}
\end{align*}
\]
1.3.4 Surface Associated and Symbiotic Bacteria

Like marine sediments, the surfaces of marine invertebrates and algae are also nutrient rich habitats. It is also becoming quite evident that bacteria form highly specific relationships with marine plants and animals. Recent studies have suggested that some of the bioactive compounds previously identified from marine invertebrates such as sponges and tunicates are truly of bacterial origin. This topic is well beyond the scope of this thesis, but the reader is directed to the reviews by Kogayashi and Ishibashi, or Schmitz on this subject. Nevertheless, a few selected examples of bioactive secondary metabolites of microorganisms isolated from the surfaces and tissues of marine macroorganisms are presented below.

Tetrodotoxin (33), the potent poison in the skin of the pufferfish, is probably the best known example of a bacterial secondary metabolite first isolated from a non-bacterial source. Pufferfish are considered a delicacy in Japan, and sushi chefs take extreme care in preparing the flesh of this toxic fish for consumption. Nevertheless, a few deaths are reported each year by tetrodotoxin poisoning. Tetrodotoxin (33) has also been isolated from a variety of other marine organisms including a red alga, a xanthid crab, molluscs, and flatworms. The wide diversity of sources for this toxin suggested a microbial source and it has been conclusively shown that 33 is produced by a variety of marine microbes spanning a diverse range of bacterial groups.
Salinamides A and B (33 and 34) are bicyclic depsipeptides produced by a streptomycete isolated from the surface of the jellyfish, *Cassiopeia xamachana*.\(^{25}\) Interestingly, the epoxide in 33 can be converted to the chlorohydrin 34 simply by reaction with HCl. Both 33 and 34 exhibit selective antibiotic activity against Gram-positive bacteria, as well as possessing anti-inflammatory properties. There has been some speculation that the selective activity against Gram-positive bacteria could be related to vancomycin’s known mode of action.

As a final example, Needham *et al.* isolated moiramides A - C (35 - 37) along with the known compound andrimid (38) from a solid agar culture of a *Pseudomonas fluorescens* sp. isolated from an unknown tunicate.\(^{26}\) Moiramide B (36) and andrimid (38) exhibited antibiotic activity against drug resistant Gram-positive bacteria. The absence of antibiotic activity by moiramide A (35) and by moiramide C (37) suggests that there is a strict requirement in the acylsuccinimide unit for antimicrobial activity.
1.4 Research Objectives

In collaboration with Dr. M. T. Kelly, we have established an ongoing program designed to screen microorganisms isolated from the oceans for the production of new antimicrobial agents. In cooperation with Dr. Kelly, bacteria have been isolated from uniquely marine habitats, inclusive of cold water and tropical locations. They have been isolated from seawater, marine sediments, and the surfaces and tissues of algae and marine invertebrates. All of the organisms were collected from coastal marine environments ranging from southern British Columbia to Alaska, as well as the tropical waters of Papua New Guinea. The objective of this thesis was to isolate by bioassay guided fractionation, novel biologically active secondary metabolites from these microorganisms isolated from the marine habitat. The bulk of this thesis focuses on the structure elucidation and characterization of the metabolites isolated during this study.
References


7 Rosenfeld, W. D.; Zobell, C. J. Bacteriol. 1947, 54, 393-398


10 Davidson, B. Current Opinion in Biotechnology 1995, 6, 284-291.


Chapter 2: Loloatins A - C (1 - 3)

2.1 Introduction

Dr. Kelly has isolated approximately 6000 marine microorganisms, and approximately half of them have been screened for production of antibiotics against drug resistant pathogens, including VRE and MRSA. Roughly 3000 of the screened bacterial isolates were cultured from the coastal waters of BC, and a further 300 of the screened isolates originated from the tropical waters of Papua New Guinea. Approximately 3.3% of the cold water isolates, and 3.7% of the warm water microbes exhibited antibiotic activity against one or more of the pathogens in the test panel. One of the tropical isolates, MK-PNG-276A, exhibited broad spectrum antibiotic activity against both Gram-positive and Gram-negative strains in the panel of human pathogens. This isolate was obtained from the tissues of an unidentified tube worm collected at -15 m off Loloata Island, Papua New Guinea and was identified as a Bacillus sp. by MIDI analysis of cellular fatty acids. MK-PNG-276A was therefore chosen as a candidate for further chemical studies in order to identify the bioactive metabolites produced by this microorganism.

<table>
<thead>
<tr>
<th>Test Panel of “Antibiotic Resistant“ Strains of Human Pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
</tr>
<tr>
<td>Pseudomonas cepacia</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.2 Results

2.2.1 Bioassay Guided Fractionation and Isolation

The antibiotic producing marine isolate MK-PNG-276A was grown in moderate scale culture in order to generate sufficient quantities of the secondary metabolites required for chemical identification of the active components. The isolate was cultured as lawns on trays of agar (24 cm long, 37 cm wide, 0.5 cm deep agar) using tryptic soy agar supplemented with NaCl to a concentration of 1%. The solid agar culture was harvested after four days incubation by gently scraping the cells from the agar. Cells and agar were then extracted separately with EtOAc in order to obtain crude intracellular (from the cells) and crude extracellular (from the agar) extracts.

The crude intracellular and extracellular extracts were tested for biological activity against the entire panel of human pathogens. The intracellular extract, which exhibited Gram-positive and Gram-negative antibiotic activity was fractionated by a modified Kupchan partitioning scheme. This involved partitioning the crude extract between organic solvents of increasing polarity and an aqueous methanol solution of varying concentrations. The fractions obtained after this solvent partitioning were tested against the sensitive human pathogens identified in the first bioassay. The EtOAc fraction, which was found to have MRSA and enterococci activity was further fractionated by a combination of size exclusion chromatography and reversed phase preparative chromatography. Throughout this fractionation procedure fractions were also screened by $^1$H NMR in order to identify NMR signals suggestive of novel chemistry.
The bioassay guided isolation procedure yielded a series of related bioactive peptides, which were named loloatins A - C (1 - 3) based on their elution order from reversed phase chromatography. Semi-pure fractions of loloatins A - C (1 - 3) were purified to chromatographic consistency (one peak of appropriate shape and retention time) using high performance liquid chromatography (HPLC). Loloatins A - C (1 - 3) showed potent antibiotic activity against Gram-positive methicillin resistant strains of *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus* sp. (VRE), and penicillin resistant *Streptococcus pneumoniae*. Loloatin C (3) also showed strong antibacterial activity against the Gram-negative bacterium, *Escherichia coli*.

![Chemical structure of loloatins A, B, and C](image-url)
2.2.2 Structure Elucidation of Loloatin B (2)

The structure of loloatin B (2), the most abundant of the three peptides, was determined by detailed analysis of one and two dimensional NMR spectra and mass spectrometric analysis of the natural product as well as the N-acetyl methyl ester derivative 39 of loloatin B (for a brief introduction into the NMR experiments used in this thesis see Appendix A). The structures of the individual amino acid residues were first determined by analysis of the COSY, HOHAHA, HMQC, and HMBC data and their configurations were determined by total acid hydrolysis, conversion of the derivatized amino acids to their pentafluoropropionyl amide isopropyl ester (PFPA-IPA) derivatives, and chiral GC analysis of the derivatized amino acids. Finally, the amino acid sequence in 2 was determined by analysis of the HMBC and ROESY data for both 2 and its derivative 39.
Loloatin B (2) was obtained as an optically active ([α]_b -79.5), amorphous beige solid that
gave a (M+H)^+ peak at m/z 1296.64232 in the HRFABMS. The \(^1H\) and \(^{13}C\) NMR spectra
(Figures 2.1 and 2.2) obtained for loloatin B (2) contained resonances that were characteristic of a
peptide. Thus, the \(^1H\) NMR spectrum (Figure 2.1) contained several resonances between δ 9.30
and 7.60 ppm assigned to protons attached to heteroatoms; several resonances between δ 7.60
and 6.50 ppm assigned to aromatic protons; resonances between δ 5.70 and 4.00 ppm belonging
to amino acid \textit{alpha} methines; as well as four superimposed aliphatic methyl resonances at δ 0.95
ppm. The \(^{13}C\) NMR spectrum (Figure 2.2) appeared to contain at least ten ester/amide carbonyl
(C=O) resonances between δ 169.0 and 174.0 ppm (Figure B.1); twenty aromatic carbon
resonances in the δ 110.0 to 166.0 region (Figure B.2); as well as 29 resonances more shielded
than δ 60.0 ppm (Figure B.3). Examination of the HMQC data suggested that peptide 2 was a
decapeptide with ten \textit{alpha}-CH resonances (Figure B.5). This information eliminated all possible
molecular formulas with fewer than 59 carbons, ten oxygens and ten nitrogens. Detailed analysis
of the COSY, HOHAHA, HMQC and HMBC data (Figures 2.3 - 2.7, B.4 - B.15, Table B.1),
followed by chiral GC analysis of the HCl hydrolysate of 2 (Table 2.1) revealed that peptide 2
contained two phenylalanine residues and one each of valine, leucine, proline, ornithine,
asparagine, aspartate, tyrosine and tryptophan. This data taken together with the HRFABMS
established the molecular formula as C_{67}H_{85}N_{13}O_{14} (ΔM 0.46 ppm).
Figure 2.1: 400 MHz $^1$H NMR Spectrum of Loloatin B(2) in DMSO-$d_6$
Figure 2.2: 100 MHz APT/\(^{13}\)C NMR Spectra of Loloatin B (2) in DMSO-\(d_6\)
Inspection of the HOHAHA spectrum of 2 suggested that the methyl envelope at δ 0.95 ppm belonged to two different aliphatic amino acid residues (Figure 2.4). Analysis of the HOHAHA and COSY data assigned an amide NH proton resonance at δ 7.92 to a leucine residue (standard three letter code: Leu, or the standard one letter code: L). HOHAHA correlations were seen between the amide NH (δ 7.92) and resonances at δ 4.55, 1.25, 1.35, 1.5 and 0.93 ppm. Examination of the COSY spectrum (Figure 2.3) assigned these resonances as the alpha-CH (δ 4.55), the beta-CH₂ geminal methylene resonances (δ 1.25 and 1.35), the gamma-CH (δ 1.5) and the two delta-CH₃'s at δ 0.93 ppm. Finally, HMBC correlations were observed between one of the geminal methylene resonances at δ 1.25 and the alpha-CH carbon resonance at δ 50.2 ppm (Figure B.7), as well as to an ester/amide carbonyl resonance at 171.8 ppm (Figure B.13).
A second amide NH resonance (δ 7.52), which in the HOHAHA spectrum (Figure 2.4), correlated into the methyl envelope, was assigned to the spin system of a valine residue, (Val or V). The HOHAHA data revealed correlations between this amide resonance (δ 7.52) and resonances at δ 4.56, 2.01 and 0.93 ppm. Analysis of the COSY spectrum (Figure 2.3) revealed that these resonances were the alpha-CH, beta-CH, and the gamma-CH$_3$'s respectively. Correlations observed in the HMBC spectrum between the beta-CH (δ 2.01) and the alpha carbon resonances (δ 57.0), and between the methyl envelope (δ 0.93) and this same alpha carbon resonance confirmed the assignment as a valine residue (Figure B.7). The carbonyl resonance (δ 169.9) was assigned by the observation of a correlation in the HMBC spectrum to the beta-CH proton resonance at δ 2.01 ppm (Figure B.13).
Examination of the aromatic region in the $^{13}$C and APT spectra for loloatin B (2) revealed twenty carbon resonances, seven of which were quaternary carbons (Figure B.2). This suggested that there were several different types of aromatic residues present in this peptide. The first aromatic residue recognized was a tyrosine (Tyr or Y). Two doublets at $\delta$ 6.98 and 6.61 ppm in the $^1$H spectrum of 2, each of which integrated to two protons, were assigned to a para-hydroxy substituted phenyl ring. Both of these proton resonances showed correlations in the HMBC to a deshielded quaternary aromatic carbon resonance at $\delta$ 156.2 (Figure B.11). Examination of the HMBC spectrum revealed a correlation between the ortho proton resonance at $\delta$ 6.98 and a methylene carbon resonance at $\delta$ 34.7 ppm (Figure B.9). This carbon's geminal methylene proton resonances ($\delta$ 2.81 and $\delta$ 2.70) showed correlations in the HOHAHA spectrum to an amide resonance at $\delta$ 9.21 and an alpha-CH at $\delta$ 4.22 ppm (Figure 2.4). Finally the ester/amide carbonyl could be assigned by the observation in the HMBC spectrum of correlations between the geminal methylene proton resonances ($\delta$ 2.70, 2.81) and the carbonyl resonance ($\delta$ 171.5 ppm) (Figure B.13).
A tryptophan residue was also identified from analysis of the NMR data (Trp, W). A strongly deshielded proton resonance at δ 10.81 ppm showed a correlation in the HMBC spectrum to three aromatic quaternary carbon resonances at δ 110.5, 136.0, and 127.0 as well as to an aromatic CH carbon resonance at δ 123.0 ppm (Figure B.12). These carbon resonances could be assigned to a trisubstituted pyrrole ring, and must therefore belong to the indole portion of a tryptophan residue. Unfortunately, the congestion of the aromatic signals in the ¹H NMR spectrum made it impossible to confidently assign the phenyl portion of this indole system; therefore, the remaining four CH's required for this aromatic system were assigned based on literature comparison.² Next, the geminal methylene resonances of the beta-CH₂ (δ 3.15) showed correlations in the HMBC spectrum to two quaternary carbons of the indole ring (δ 110.5 and δ 127.0) and the aromatic CH (δ 123.0 ppm) (Figure B.10). The HOHAHA data showed correlations between the amide NH proton resonance (δ 8.63), the alpha-CH (δ 4.5), and the beta-CH₂ resonance (δ 3.15) (Figure 2.4).
The assignment of a tyrosine and a tryptophan residue accounted for seven of the thirteen aromatic CH carbon resonances and five of the seven quaternary carbon resonances. Since an unsubstituted phenyl ring has one quaternary and only three aromatic CH carbon signals due to symmetry, the two remaining quaternary carbons and the remaining six aromatic CHs could easily be accounted for by two phenylalanine residues (Phe1, F; Phe2, F'). The first phenylalanine residue, Phe1 (F), had an amide resonance (δ 7.23) which in the HOHAHA spectrum showed correlations to the alpha-CH (δ 4.5) and beta-CH$_2$ proton resonances (δ 2.25 ppm) (Figure 2.4). The beta-CH$_2$ proton resonances showed a correlation in the HMBC spectrum to the ipso and ortho carbons of a phenyl ring (δ 138.5 and 128.8 ppm, respectively), thus establishing the assignment of the phenylalanine backbone (Figure B.10). Similarly, the HOHAHA spectrum revealed correlations from the amide NH of the second phenylalanine residue, Phe2 (F'), to the alpha-CH proton resonance at δ 5.57 and the beta-CH$_2$ geminal methylene resonances (δ 2.75 and 3.02 ppm) (Figure 2.4). This backbone was then connected to a phenyl ring by the observation of correlations in the HMBC spectrum between the beta-CH$_2$ geminal methylene resonances (δ 2.75 and 3.02) and the ipso (δ 137.4) and ortho (δ 129.2 ppm) carbon resonances of a phenyl ring (Figure B.10).
The presence of an asparagine or glutamine residue in peptide 2 was suggested by the observation of two proton resonances (δ 8.05 and 7.45 ppm) which correlated to each other in the COSY spectrum (Figure 2.3) and were assigned to primary amide protons. The presence of asparagine (Asn, N) was confirmed by analysis of the HOHAHA and HMBC data. One of the primary amide NH proton resonances (δ 8.05) showed a correlation in the HMBC spectrum to an ester/amide carbonyl at δ 173.0 ppm (Figure B.15). This carbonyl resonance, in turn, showed correlations in the HMBC to two geminal methylene proton resonances (δ 3.0, 3.37 ppm) (Figure B.13). Finally, the HOHAHA data showed that this beta-CH₂ belonged to the same spin system as an alpha-CH resonance (δ 4.46) and amide NH resonance (δ 9.03 ppm) (Figure 2.4).
A proline residue was also evident in the peptide (Pro, P). The most upfield proton resonance in the alpha-CH region of the $^1$H spectrum (δ 4.07 ppm) did not show any correlations in either the COSY spectrum or the HOHAHA spectrum to any amide NH resonance (Figures 2.3 and 2.4). Further, this alpha-CH resonance was seen to belong to a spin system having three contiguous methylene carbons (Figures 2.3 and 2.5). Thus, from the COSY and HOHAHA data the alpha-CH (δ 4.07), beta-CH$_2$ (δ 1.25 and 1.43), gamma-CH$_2$ (δ 1.07 and 0.41), and delta-CH$_2$ (δ 2.20, 3.30) proton resonances could be assigned unambiguously (Figures 2.3 and 2.5). The chemical shift of the delta-CH$_2$ (δ 45.8) suggested that this carbon was in turn bonded to a heteroatom, thus substantiating the assignment as a proline residue (Figure B.6). The observation of a correlation in the HMBC spectrum between the alpha-CH proton resonance and a carbon resonance at δ 169.2 established the assignment of the ester/amide carbonyl of this residue (Figure B.14).
Figure 2.3: 500 MHz COSY Spectrum of Loloatin B (2) in DMSO-$d_6$
Figure 2.4: 500 MHz HOHAHA Spectrum of Loloatin B (2) in DMSO-$d_6$ - NH Expansion
Figure 2.5: 500 MHz HOHAHA Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion
Figure 2.6: 500 MHz HMQC Spectrum of Loloatin B (2) in DMSO-$d_6$
Figure 2.7: 500 MHz HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$
Figure 2.8: 500 MHz ROESY Spectrum of Loloatin B (2) in DMSO-$d_6$. 
The uncommon amino acid, ornithine (Orn, O) was also present in peptide 2. Analysis of the HOHAHA and COSY data of 2 identified the alpha-CH (δ 5.27), beta-CH₂ (δ 1.8), gamma-CH₂ (δ 1.7), and delta-CH₂ (δ 2.8 and 2.9 ppm) proton resonances in this residue (Figures 2.3, 2.4, and 2.5). This residue was confirmed by examination of the NMR data for the derivative, N-acetyl loloatin B methyl ester (39) (Figures 2.9 - 2.15, B.16 - B.21, Table B.2). In the ¹H spectrum of the N-acetyl derivative, a new amide NH resonance at δ 7.45 ppm was assigned as the delta-NH of N-acetyl ornithine (Figure 2.9). In the HOHAHA spectrum of 39 both the alpha and delta-NH amide proton resonances (δ 8.81 and 7.45) showed correlations to proton resonances at δ 1.76 and 1.57 ppm (Figure 2.12). Inspection of the COSY data identified the alpha-CH (δ 5.36), beta-CH₂ (δ 1.76), gamma-CH₂ (δ 1.57), and delta-CH₂ (δ 3.03) resonances (Figure 2.11). Finally, correlations were observed in the HMBC spectrum from the delta-CH₂ resonance at δ 3.03 and the acetyl methyl resonance at δ 1.79 to the acetyl carbonyl resonance at δ 168.5 ppm (Figure B.19).

![N-Acetyl Orn in Loloatin B (2)](image1)

![N-Acetyl Orn in N-Acetyl Loloatin B Methyl Ester (39)](image2)
The HMBC and HOHAHA data also suggested the presence of an aspartate residue (Asp, D). An amide resonance at $\delta$ 8.32 ppm correlated in the HOHAHA spectrum to an alpha-CH ($\delta$ 4.28) and a set of geminal methylene resonances ($\delta$ 2.35 and 2.2 ppm) (Figure 2.4). The chemical shift of these geminal protons suggested they were attached to a carbon bonded to a deshielding group such as an aromatic ring or carbonyl functionality. This residue was confirmed to be aspartate by formation of the methyl ester in N acetyl loloatin B methyl ester 39 (Table B.2). In the derivative 39 a correlation in the HMBC spectrum was observed from the methyl ester proton resonances ($\delta$ 3.56) to the gamma carbonyl resonance of the aspartate residue ($\delta$ 170.3) (Figure B.20). This ester carbonyl resonance also showed a correlation in the HMBC spectrum to the geminal beta-CH$_2$ protons ($\delta$ 2.5 and 2.6 ppm) (Figure B.21). This observation provided evidence that the aspartic residue was involved in a typical alpha linkage, with the gamma carboxylate remaining free in the naturally occurring peptide.

Asp in Loloatin B (2)

Asp-Me ester in N-Acetyl Loloatin B Methyl ester (39)
As previously discussed, loloatin B (2) was converted to N-acetyl loloatin B methyl ester (39) to confirm the aspartate and ornithine amino acid residues. Thus 2 was acetylated in Pyr/Ac₂O followed by methylation with diazomethane to yield N-acetyl loloatin B methyl ester (39). Ester 39 was obtained as an amorphous white solid that gave a (M+H)^+ peak in the HRFABMS at m/z 1352.66940, appropriate for a molecular formula of C₇₀H₈₀N₁₃O₁₅ (ΔM 1.08 ppm). This molecular formula was consistent with the addition of one molecule each of MeOH and AcOH with the concomitant loss of two molecules of H₂O to the molecular formula of loloatin B (2). The ¹H and ¹³C NMR chemical shifts of the atoms of the amino acid residues in the derivative 39 were assigned in a similar fashion to loloatin B (Table B.2). Analysis of the COSY, HOHAHA, HMQC and HMBC data assigned the atoms in the derivative 39 (Figures 2.9 - 2.15, B.16 - B.21, Table B.2). However, it must be noted that NMR spectra of the derivative 39 were acquired with the addition of 5% C₆D₆ to the NMR solvent. The addition of C₆D₆ shifted some of the amide NH resonances in the δ 7.3 to 9.0 ppm region and the alpha-CH resonances in the δ 4.1 to 4.8 ppm region of the ¹H spectrum (Figure 2.9). This relieved some of the congestion and chemical shift degeneracy that was observed in loloatin B (2). Specifically, the Tyr and Phe2 amide proton resonances were well resolved in the mixed solvent, as were the Tyr and Asp alpha-CH proton resonances and the Asn alpha-CH proton resonance was resolved from the alpha-CH envelope around 4.65 ppm. The addition of benzene did not significantly alter the ¹³C chemical shifts of the carbon atoms in the peptide (Figure 2.10).
Figure 2.9: 400 MHz $^1$H NMR Spectrum of N-Acetyl Loloatin B Methyl Ester (39) in 20:1 DMSO-$d_6$/C$_6$D$_6$
Figure 2.10: 100 MHz $^{13}$C NMR Spectra of Loloatin B (2 - top, in DMSO-$d_6$) and N-Acetyl Loloatin B Methyl Ester (39 -bottom, in 20:1 DMSO-$d_6$/C$_6$D$_6$)
Figure 2.11: 500 MHz COSY Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-d$_6$/C$_6$D$_6$
Figure 2.12: 500 MHz HOHAHA Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-
$\text{d}_6$/C$_6$D$_6$
Figure 2.13: 500 MHz HMQC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-$d_6$/C$_6$D$_6$
Figure 2.14: 500 MHz HMBC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-$d_6$/C$_6$D$_6$
Figure 2.15: 500 MHz ROESY Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-
$\text{d}_2/C_6\text{D}_6$
Hydrolysis of 2 with 6N HCl containing thioglycolic acid (to reduce oxidation of tyrosine and tryptophan residues) and examination of the PFPA-IPA ester derivatives of the liberated amino acids via chiral GC analysis confirmed the presence of L-valine, L-ornithine, L-leucine, D-tyrosine, L-proline, L-phenylalanine, D-phenylalanine, L-tryptophan and L-aspartic acid (from Asp and Asn) (Table 2.1). The ten identified amino acid residues accounted for all of the atoms in the molecular formula of 2, and 31 of the 32 sites of unsaturation demanded by the molecular formula. Thus, loloatin B (2) had to be a monocyclic decapeptide.

### Table 2.1: Chiral GC Retention Times (in minutes) of Pentafluoropropionamide Isopropyl Ester Derivatives of Hydrolysates of Loloatins A - C (1 - 3) *

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Loloatin A (1)</th>
<th>Loloatin B (2)</th>
<th>Loloatin C (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Val</td>
<td>11.39</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>L-Val</td>
<td>11.74</td>
<td>11.60</td>
<td>11.57</td>
<td>11.59</td>
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<tr>
<td>D-Leu</td>
<td>15.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>L-Leu</td>
<td>15.88</td>
<td>15.69</td>
<td>15.65</td>
<td>15.67</td>
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<tr>
<td>D-Pro</td>
<td>18.39</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Pro</td>
<td>18.47</td>
<td>18.51</td>
<td>18.48</td>
<td>18.49</td>
</tr>
<tr>
<td>D-Asp</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Asp</td>
<td>22.19</td>
<td>22.19</td>
<td>22.14</td>
<td>22.15</td>
</tr>
<tr>
<td>D-Phe</td>
<td>26.81</td>
<td>26.70</td>
<td>26.67</td>
<td>26.68</td>
</tr>
<tr>
<td>L-Phe</td>
<td>27.08</td>
<td>26.97</td>
<td>26.93</td>
<td>-</td>
</tr>
<tr>
<td>D-Tyr</td>
<td>30.13</td>
<td>30.01</td>
<td>29.96</td>
<td>30.05</td>
</tr>
<tr>
<td>L-Tyr</td>
<td>30.41</td>
<td>30.24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Orn</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Orn</td>
<td>31.91</td>
<td>31.80</td>
<td>31.76</td>
<td>31.77</td>
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<tr>
<td>D-Trp</td>
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<tr>
<td>L-Trp</td>
<td>52.30</td>
<td>-</td>
<td>52.38</td>
<td>52.38</td>
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</tbody>
</table>

* All identifications were made by coinjection with authentic standards
The amino acid sequence in loloatin B (2) was determined by analysis of the HMBC and ROESY data for both 2 and 39. Correlations in the ROESY spectrum of 39 (Figure 2.15) were observed between amino acid alpha-CH protons and adjacent residue NH protons which unambiguously identified the following five amide bonds: Orn-CO/Leu-NH (δ 5.36/8.22), Asp-CO/Trp-NH (δ 4.40/8.72), Asn-CO/Asp-NH (δ 4.55/8.36), Phe2-CO/Asn-NH (δ 5.73/9.13) and Pro-CO/Phe1-NH (δ 4.18/7.39). A ROESY correlation observed between the Tyr alpha-CH resonance at δ 4.25 ppm and the Pro delta proton resonance at δ 3.30 in 39 identified the Tyr-CO/Pro-N amide bond, and a strong ROESY correlation between the Val-NH resonance at δ 7.52 and the Trp-NH resonance at δ 8.63 in 2 identified the Trp-CO/Val-NH amide linkage (Figure 2.8). The latter ROESY correlation suggests the possibility of a β-bulge in the Trp/Val region of the cyclic peptide.4
Observations of correlations in the HMBC spectrum from both the Val beta-CH proton resonance at δ 2.12 and the Orn-NH resonance at δ 8.81 to the well resolved carbonyl resonance at δ 169.6 in derivative 39 identified the Val-CO/Orn-NH amide bond (Figures B.19 and B.21). The HMBC correlations from the Leu beta-CH₂ proton resonance at δ 1.25 and the Tyr-NH resonance at δ 9.21 to the carbonyl resonance at δ 171.8 in 2 identified the Leu-CO/Tyr-NH amide bond (Figures B.13 and B.15). The final Phe₁-CO/Phe₂-NH amide bond was required to complete the macrocyclic ring.

HRFABMS and MS-MS studies supported the amino acid sequence derived from the NMR data (Table 2.2). The MS-MS data was consistent with initial cleavage of the ring at the Tyr-CO/Pro-N bond to give a linear decapeptide that sequentially loses Leu-Tyr (m/z 1019), Orn-Leu-Tyr (m/z 905) and Trp-Val-Orn-Leu-Tyr (m/z 621). FABMS peaks at m/z 245 and 377 could be assigned to the protonated fragments Pro-Phe₁ and Phe₂-Asn-Asp, respectively. The data for loloatin B (2) alone did not identify which of the two isomers of phenylalanine identified by GC analysis (Table 2.1) was at positions Phe₁ and Phe₂. This point was clarified with the subsequent characterization of loloatin C (3) (See Section 2.2.3).

Thus from the available data, and with the subsequent characterization of loloatin C (3), peptide 2 was determined to be a cyclic decapeptide. The peptide sequence is given in structure 2, and the molecule is a zwitterion in neutral solutions due to the presence of a free ornithine amino functionality and a free aspartic acid carboxylic group.
Table 2.2: FABMS Parent and Fragment Ions for Loloatins A - C (1 - 3)

<table>
<thead>
<tr>
<th>Fragment</th>
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<th>Loloatin B (2)</th>
<th>Loloatin C (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[P-aa1-F-N-D-aa2-V-O-L-Y]⁺</td>
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<td>1297</td>
<td>1336</td>
</tr>
<tr>
<td>[P-aa1-F-N-D-aa2-V-Q]⁺</td>
<td>998</td>
<td>1019</td>
<td>1060</td>
</tr>
<tr>
<td>[P-aa1-F-N-D-aa2-V]⁺</td>
<td>883</td>
<td>905</td>
<td>-</td>
</tr>
<tr>
<td>[P-aa1-F-N-D-aa2]⁺</td>
<td>784</td>
<td>807</td>
<td>-</td>
</tr>
<tr>
<td>[P-aa1-F-N-D]⁺</td>
<td>621</td>
<td>621</td>
<td>660</td>
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<tr>
<td>[P-aa1-F-N]⁺</td>
<td>506</td>
<td>506</td>
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</tr>
<tr>
<td>[P-aa1-F]⁺</td>
<td>-</td>
<td>392</td>
<td>431</td>
</tr>
<tr>
<td>[P-aa1]⁺</td>
<td>245</td>
<td>245</td>
<td>284</td>
</tr>
<tr>
<td>[F-N-D]⁺</td>
<td>377</td>
<td>377</td>
<td>377</td>
</tr>
</tbody>
</table>
Loloatin A (1) was obtained as an optically active \( ([\alpha]_D -88.0) \) white solid that gave a \((M+H)^+\) peak in the HRFABMS at \( m/z \) 1273.663082 appropriate for a molecular formula of \( C_{65}H_{85}N_{12}O_{15} \) (AM 4.00 ppm). This molecular formula differed from that of loloatin B (2) by the loss of \( C_2N \) and the addition of one oxygen atom. The \(^1\)H and \(^{13}\)C NMR spectra obtained for loloatin A (1) were similar to those of loloatin B (2) (Figures 2.16 and 2.17). However, the indole NH resonance in the \(^1\)H NMR spectrum of loloatin B (2) (\( \delta \) 10.81 ppm) was conspicuously absent in loloatin A (1). Additionally, the \(^1\)H NMR spectrum of loloatin A (1) seemed to have a less complicated set of resonances in the aromatic region of the spectrum (\( \delta \) 6.6 to 7.4 ppm), and significantly fewer aromatic resonances in the \(^{13}\)C NMR spectrum (Figure 2.18). In the aromatic region of the \(^1\)H NMR spectrum, the addition of two new doublets which
integrated to two protons apiece (δ 6.90 and 6.61 ppm) was observed. One of these doublets was apparently chemical shift degenerate with that of the meta proton resonances of the Tyr residue (δ 6.61) previously identified in 2. In the aromatic region of the 13C NMR spectrum only fourteen resonances were observed as opposed to the twenty observed in 2. Taken together, this evidence suggested that the tryptophan residue in loloatin B (2) was not present in loloatin A (1) and that a second tyrosine residue was present in 1.

Detailed analysis of the COSY, HOHAHA, HMQC and HMBC data confirmed that peptide 1 contained two phenylalanine residues, two tyrosine residues and one each of valine, leucine, proline, ornithine, asparagine and aspartate (Table B.3). Hydrolysis of 1 with 6N HCl containing thioglycolic acid3 and examination of the PFPA-IPA ester derivatives of the liberated amino acids via chiral GC analysis established the presence of L-phenylalanine, D-phenylalanine, D-tyrosine, L-tyrosine, L-valine, L-leucine, L-proline, L-ornithine and L-aspartic acid (from Asp and Asn) (Table 2.1). This evidence taken together firmly established that loloatin A had structure 1 and simply had a D-tyrosine residue in place of the D-tryptophan residue present in 2.

This structure was confirmed by mass spectrometric analysis (Table 2.2). Similar to loloatin B (2), the MS-MS data was consistent with initial cleavage of the ring at the Tyr-CO/Pro-N bond to give a linear decapeptide that sequentially loses Leu-Tyr (m/z 998), Orn-Leu-Tyr1 (m/z 883) and Tyr2-Val-Orn-Leu-Tyr1 (m/z 621). FABMS peaks at m/z 245 and 377 could be assigned to the protonated fragments Pro-Phe1 and Phe2-Asn-Asp, respectively.
Figure 2.16: 400 MHz $^1$H NMR Spectrum of Loloatin A (1) in DMSO-$_d_6$
Figure 2.17: 100 MHz APT/$^{13}$C NMR Spectra of Loloatin A (1) in DMSO-$d_6$
Figure 2.18: APT/$^{13}$C NMR Spectra of Loloatin A (1) - Aromatic Region Expansion
Loloatin C (3) was obtained as an optically active ([α]_D -75.5) white solid that gave a (M+H)^+ peak in the HRFABMS at m/z 1335.65267 appropriate for a molecular formula of C_{69}H_{87}N_{14}O_{14} (ΔM 0.04 ppm) which differed from that of loloatin B (2) by addition of C_2NH_2.

Once again, the ^1H and ^13C NMR spectra obtained for loloatin C (3) were very similar to those of loloatin B (2) (Figures 2.19 and 2.20). Unlike 2, there appeared to be two indole NH resonances in the ^1H NMR spectrum of loloatin C (3) (W11 - δ 10.64 and W11’ - δ 10.83 ppm). The ^1H NMR spectrum of loloatin C (3) also seemed to exhibit a more complicated set of aromatic resonances in addition to the characteristic tyrosine ortho and para proton resonances (δ 6.6 to 7.4 ppm), and the ^13C NMR spectrum showed at least twenty four aromatic resonances - nine of which were quaternary carbons (Figure 2.21). Subtraction of the eight quaternary carbons and eleven aromatic CH’s accounted for by two tryptophan and one tyrosine residues left one
quaternary and three aromatic CH resonances, thus suggesting a phenylalanine residue. Hence, the \(^1\)H and \(^{13}\)C NMR spectra and mass spectrometric analysis suggested that loloatin C (3) contained two tryptophan and one phenylalanine residues as opposed to loloatin B's (2) single tryptophan and two phenylalanine residues.

Detailed analysis of the COSY, HOHAHA, HMQC and HMBC data confirmed that peptide 3 contained two tryptophan residues and one each of valine, leucine, proline, ornithine, phenylalanine, tyrosine, asparagine and aspartate (Table B.4). Hydrolysis of 3 with 6N HCl containing thioglycolic acid\(^3\) and examination of the PFPA-IPA ester derivatives of the liberated amino acids via chiral GC analysis confirmed the presence of L-tryptophan, D-tyrosine, D-phenylalanine, L-valine, L-leucine, L-proline, L-ornithine and L-aspartic acid (from Asp and Asn) (Table 2.1).

Comparison of the \(^1\)H and \(^{13}\)C data suggested that the sequence in 3 was similar to the sequence of peptide 2. Analysis of the HMQC, HMBC and HOHAHA data (Table B.4) confirmed that the downfield alpha-CH resonance in the \(^1\)H spectrum of loloatin C (3) (\(\delta 5.60\) ppm) belonged to the D-phenylalanine residue present in 3. This evidence was used to assign the configuration in loloatin B (2) of the phenylalanine at this position in the sequence. Since the tyrosine alpha-CH in 3 resonated in the \(^1\)H spectrum at the same chemical shift as in 2 (\(\delta 4.20\) ppm), it followed that the second L-tryptophan residue observed in this peptide was at the position formerly occupied by L-phenylalanine in 2. MS-MS analysis supported this sequence, with initial cleavage of the ring at the Tyr-CO/Pro-N bond to give a linear decapeptide that sequentially loses Leu-Tyr (m/z 1060), Trp2-Val-Orn-Leu-Tyr (m/z 660). FABMS peaks at m/z 284 and 377 could be assigned to the protonated fragments Pro-Trp1 and Phe-Asn-Asp, respectively (Table 2.2).
Figure 2.20: 100 MHz APT/$^{13}$C NMR Spectra of Loloatin A (3) in DMSO-$d_6$
Figure 2.21: APT/$^{13}$C NMR Spectra of Loloatin C (3) in DMSO-$d_6$ - Aromatic Region Expansion
2.2.5 Biological Activity

Loloatins A - C (1 - 3) were tested for activity against a panel of human pathogenic bacteria including methicillin resistant strains of *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus* sp. (VRE), penicillin resistant *Streptococcus pneumoniae*, and *Escherichia coli* (Table 2.3).

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Loloatin A (1)</th>
<th>Loloatin B (2)</th>
<th>Loloatin C (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>2-4</td>
<td>4-8</td>
<td>0.5-1</td>
</tr>
<tr>
<td>methicillin resistant <em>S. aureus</em></td>
<td>2-4</td>
<td>4-8</td>
<td>0.5-1</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>2-4</td>
<td>4-8</td>
<td>1-2</td>
</tr>
<tr>
<td>vancomycin resistant <em>E. faecalis</em></td>
<td>2-4</td>
<td>4-8</td>
<td>1-2</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>2-4</td>
<td>4-8</td>
<td>2-4</td>
</tr>
<tr>
<td>vancomycin resistant <em>E. faecium</em></td>
<td>2-4</td>
<td>4-8</td>
<td>2-4</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>1-2</td>
<td>1-2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>penicillin resistant <em>S. pneumoniae</em></td>
<td>1-2</td>
<td>2-4</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>1-2</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em></td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
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<tr>
<td><em>Xanthomonas malophilia</em></td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>8-16</td>
<td>8-16</td>
<td>8-16</td>
</tr>
</tbody>
</table>
2.3 Discussion

Loloatins A - C (1 - 3) were isolated and characterized from a *Bacillus* sp. cultured from an unidentified tube worm found in the coastal waters of Papua New Guinea. The structures of loloatins A and C (1 and 3), as proposed in this thesis, have been confirmed by independent total synthesis.\(^5\) A search of the literature revealed that the loloatins share some structural features with the tyrocidines (40 - 44), which have been isolated from a *Bacillus brevis* species.\(^6\) The loloatins (1 - 3) and tyrocidines (40 - 44) are a family of antibiotic cyclic decapeptides containing four aromatic residues, two of which are present in the unusual D configuration. Unlike the tyrocidines however, the loloatins have zwitterionic character due to the presence of both ornithine and aspartic acid residues.

\[
\begin{array}{cccccc}
\text{Val} & \text{Orn} & \text{Leu} & \text{D-aa1} & \text{Pro} \\
\uparrow & & & & \\
\text{aa5} & \text{aa4} & \text{Asn} & \text{D-aa3} & \text{aa2}
\end{array}
\]

<table>
<thead>
<tr>
<th></th>
<th>aa1</th>
<th>aa2</th>
<th>aa3</th>
<th>aa4</th>
<th>aa5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loloatin A (1)</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
<td>Asp</td>
<td>Tyr</td>
</tr>
<tr>
<td>Loloatin B (2)</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
<td>Asp</td>
<td>Trp</td>
</tr>
<tr>
<td>Loloatin C (3)</td>
<td>Tyr</td>
<td>Trp</td>
<td>Phe</td>
<td>Asp</td>
<td>Trp</td>
</tr>
<tr>
<td>Tyrocidine A (40)</td>
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<td>Phe</td>
<td>Phe</td>
<td>Gln</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyrocidine B (41)</td>
<td>Phe</td>
<td>Trp</td>
<td>Phe</td>
<td>Gln</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyrocidine C (42)</td>
<td>Phe</td>
<td>Trp</td>
<td>Trp</td>
<td>Gln</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyrocidine D (43)</td>
<td>Phe</td>
<td>Trp</td>
<td>Trp</td>
<td>Gln</td>
<td>Trp</td>
</tr>
<tr>
<td>Tyrocidine E (44)</td>
<td>Phe</td>
<td>Phe</td>
<td>Phe</td>
<td>Gln</td>
<td>Phe</td>
</tr>
</tbody>
</table>
The physical and biochemical properties of the tyrocidines (40 - 44) have been extensively investigated. The conformation of tyrocidine A (40) has been studied in solution and in solid state, and has been shown to possess an anti-parallel β-sheet conformation with a type II' β-turn at D-Phe-Pro and a type I β-turn at Gln-Tyr. Comparison of the 1H chemical shift of the amide protons of tyrocidine A (40) and loloatin B (2) reveals that regardless of the side chain present, there is little variation in the actual chemical shifts of the amide protons between corresponding amino acids in each sequence (Table 2.4). This evidence along with the trans-annular ROESY correlations observed between ornithine and D-phenylalanine in loloatin B suggests that the loloatins (1 - 3) possess the same conformation as the tyrocidines in solution.

Table 2.4: Comparison of Amide NH 1H Chemical Shifts in Tyrocidine A (40) and Loloatin B (2), Recorded in DMSO-6

<table>
<thead>
<tr>
<th>Tyrocidine A (40)</th>
<th>Loloatin B (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δ (ppm)</td>
</tr>
<tr>
<td>Val</td>
<td>7.39 Val</td>
</tr>
<tr>
<td>Orn</td>
<td>8.85 Orn</td>
</tr>
<tr>
<td>Leu</td>
<td>7.88 Leu</td>
</tr>
<tr>
<td>D-Phe</td>
<td>9.26 D-Tyr</td>
</tr>
<tr>
<td>Pro</td>
<td>- Pro</td>
</tr>
<tr>
<td>Phe</td>
<td>7.18 Phe</td>
</tr>
<tr>
<td>D-Phe</td>
<td>9.01 D-Phe</td>
</tr>
<tr>
<td>Asn</td>
<td>9.00 Asn</td>
</tr>
<tr>
<td>Gln</td>
<td>8.71 Asp</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.40 Trp</td>
</tr>
</tbody>
</table>

The anti-parallel β-sheet of 2 is shown in Figure 2.22; hydrogen bonds are depicted between the Val-NH/Asn-CO, Val-CO/Asn-NH, Leu-NH/Phe1-CO, and Leu-CO/Phe1-NH, as well as the β bulge observed between the Trp-NH and Val-NH residues. This conformation also satisfies the observation of a strong ROESY correlation between Orn alpha-CH and Phe2 alpha-CH (Figure 2.8).
2.3.1 Biosynthesis

Microorganisms have been seen to produce a wide variety of bioactive peptides. These peptides may be cyclic and often contain unusual and/or modified amino acids and as well as amino acids of "unusual configuration." These peptides cannot be of ribosomal origin, since the RNA/ribosomal system is restricted to the twenty protein amino acids and possible modifications thereof. Of these bioactive peptides, tyrocidines (40 - 44) and gramicidin - S (45) have played critical roles in the understanding of non-ribosomal mediated peptide biosynthesis. An excellent review on the current state of research on peptide synthetases is provided by Kleinkauf et al.\textsuperscript{9}
Tyrocidine A (TA - 40) provides an excellent example of this non-ribosomal mediated peptide biosynthesis.7,10 A multifunctional enzyme synthetase (called a peptide synthetase) complex consisting of three enzymes designated the light, intermediate and heavy enzyme have been isolated and characterized in TA biosynthesis. The three enzyme subunits in TA have been shown to take mixtures of ATP and the eight amino acids present in TA and produce tyrocidine A (40). Furthermore, each of these enzyme subunits in the TA synthetase can be cleaved enzymatically into “modules” of approximately 70 kda per amino acid “activation site.” Thus each enzyme subunit in the synthetase consists of one or more “modules,” and the number of modules is directly proportional to the number of amino acids activated by that enzyme subunit. All modules contain a domain for activation of a specific amino acid and the different modules in each enzyme subunit are then responsible for taking its activated amino acid and adding it to a growing peptide chain. Moreover, it has been shown that there is an epimerase region associated with modules where D amino acids are added. In TA biosynthesis the light enzyme is responsible for activation of phenylalanine; the intermediate enzyme is responsible for elongation of the growing peptide chain with proline, L-phenylalanine and D-phenylalanine; and the heavy subunit is responsible for activation and addition of the remaining six amino acids in the peptide. In TA the light enzyme can accept either L or D phenylalanine, but ultimately the phenylalanine in the completed cyclic peptide is in the D configuration. In other peptide systems, the modules within the enzyme may contain domains capable of catalyzing substrate thiolation, epimerization or N-
methylation, and these modifications can occur before or after addition of the amino acid to the growing peptide chain.

\[
\text{enzyme-} \text{SH} + \text{aa} + \text{ATP} \xrightarrow{\text{PP}_i} \text{enzyme-} \text{SH} \xrightarrow{\text{AMP}} \text{enzyme-} \text{S-aa}
\]

**Amino Acid Activation and Thiol Esterification**

The first step performed by these modules in the enzyme subunits is the formation of a mixed anhydride between the amino acid and ATP. This initial aminoacyl adenylate is associated with, but not covalently bound to the enzyme module. The activated aminoacyl adenylate is then transferred to an acceptor thiol of a pantetheine arm that is associated with each activation site.

These pantetheine arms play crucial roles in knitting together the growing peptide chain, first by joining the amino acids to the head of the growing peptide chain and then transferring the peptide chain to the next module in the enzyme so the next amino acid can be added. As illustrated in the diagram, the pantetheine arm of the second module already has a dipeptide of the growing peptide chain attached. This arm interacts with the pantetheine arm of the third amino acid in the sequence and the activated "head" of the dipeptide then reacts with the acceptor amino acid (aa3) on the pantetheine arm of the third module. This elongation step is repeated at each of
the remaining modules in the enzyme complex until finally the synthetase cyclizes and releases the completed tyrocidine cyclic peptide.

Head to Tail Elongation of Peptide Chain

Given the structural similarities between the loloatins (1 - 3) and the tyrocidines (40 - 44), it is probable that the loloatins are biosynthesized by a multifunctional peptide synthetase nearly identical to the TA synthetase. If this peptide synthetase is similar, then the light enzyme subunit in the loloatin synthetase appears to be more specific towards tyrosine activation, as opposed to phenylalanine activation in the light subunit of the TA synthetase. The intermediate enzyme subunit seems to function in loloatin B biosynthesis in an identical fashion to that of TA. However, the “heavy” subunit of this proposed loloatin synthetase activates and adds asparagine, aspartate, tryptophan, valine, ornithine and finally leucine, whereas the TA synthetase activates and adds asparagine, glutamine, tyrosine, valine, ornithine and leucine.
2.3.2 Biological Significance

There have been extensive investigations into the role of the tyrocidines in the life cycle of *Bacillus brevis*. Evidence suggests that the tyrocidines play a regulatory role during sporulation of *B. brevis*. Tyrocidine A (40) also appears to relax superhelical chromosomal DNA, reducing torsional tension, thereby inducing "packaging" of the DNA when the bacteria enters sporulation phase.9b

The tyrocidines (40 - 44) have been shown to interact with phospholipid membranes, creating an ion channel through the membrane.12 This interruption of membrane function may explain the anti-microbial action of the tyrocidines against other species of bacteria. Examination of the MICs of Loloatin A - C (1 - 3) suggest they are at least as potent against Gram-positive bacteria as tyrocidine C (42) the most potent antibiotic in the tyrocidine family.6 There is no reported activity of the tyrocidines (40 - 44) against strains of Gram-negative bacteria. Loloatin C (3) appears to be the first member of this family to possess Gram-negative antimicrobial activity.
2.4 Experimental

NMR data were collected on either a Bruker AMX500, a Bruker WH400 or a Bruker AM400 spectrometer each equipped with a 5 mm probe. All spectra were obtained in DMSO-$d_6$. Proton spectra were referenced using internal residual DMSO-$d_6$ (δ 2.49) and carbon spectra were referenced to the DMSO methyl carbon resonance (δ 39.5). FABMS data were collected on a Kratos Concept IIHQ hybrid mass spectrometer with cesium ion secondary ionization and a magnetic sector mass analyzer. Samples were dissolved in a MeOH-thioglycerol matrix and spectra were obtained using a source voltage of 8 kV and a cesium ion gun voltage of 12 kV. Fragment ion peaks were confirmed via secondary MS-MS using a quadrapole mass analyzer. Infra-red spectra were measured on a Galaxy Series 3000 FT-IR spectrophotometer using polytetrafluoroethylene (PTFE) film plates. Optical rotations were measured on a Jasco J-710 spectropolarimeter (1 cm quartz cell).

Reversed-phase thin layer chromatography (TLC) was performed using Whatman MKC18F plates. Visualization was with UV (λ=254 nm) and/or ninhydrin spray reagents. Further information on the preparation of ninhydrin and other spray reagents can be found in Stahl. Sephadex LH-20 (bead size 25-100 μ) was used for size exclusion chromatography. Preparative reversed-phase chromatography was performed using reversed-phase silica prepared according to literature.

High performance liquid chromatography (HPLC) separations were done on one of two possible systems using either a Whatman Partisil 10 ODS-3 Magnum column or a Rainin Partisil 10-ODS column. The first system consisted of a Waters 600E HPLC pump/system controller
with a Waters 486 tunable absorbance detector. The second system consisted of a Waters 600E HPLC pump/system controller equipped with a Waters 996 photodiode array detector. Both systems were interfaced with a personal computer using Millenium™ 2010 chromatography software. Gas chromatography (GC) was performed on a Hewlett Packard 5880A Series GC with a flame ionization detector interfaced with a personal computer running Chrom Perfect™ v5.05 software.

The solvents used for extraction and for column chromatographies were Fisher reagent grade. HPLC solvents were Fisher HPLC grade which were filtered and degassed prior to use. All other solvents, reagents and standards were reagent or commercial grade and were used without further purification.

2.4.1 Isolation

The marine bacterial isolate MK-PNG-276A, identified as a *Bacillus* sp. by MIDI analysis of cellular fatty acids, was obtained from the tissues of an unidentified tube worm collected at -15 m off of Loloata Island, Papua New Guinea. MK-PNG-276A has been cryopreserved and deposited in the marine microbial culture collections at SeaTek and UBC. Moderate scale cultures of MK-PNG-276A were cultured on trays of solid tryptase soy agar supplemented with NaCl to a final concentration of 1%. Twenty six 400 mL trays (24 cm x 37 cm x 0.5 cm deep agar) were cultured for five days at 16 °C after which the cultures were harvested by gently scraping the cells from the agar surface. Lyophilized cells (61.5g dry weight) were exhaustively extracted with three 600 mL portions of MeOH that were combined, filtered, and reduced *in vacuo* to give a brown/gray tar. The tar was dissolved in 750 mL of MeOH/H₂O (1:4) and
sequentially extracted with hexanes (3 x 250 mL) and EtOAc (3 x 250 mL). The combined EtOAc extracts were reduced in vacuo to give a taupe/brown crystalline solid (5.5 g). Fractionation of the taupe/brown solid via Sephadex LH-20 chromatography (MeOH) gave an early eluting fraction that showed antibiotic activity against MRSA and Enterococci sp. Further purification of this fraction by preparative reversed-phase column chromatography and reversed-phase HPLC chromatography (9:1 MeOH/H2O containing 0.1% TFA) gave pure loloatins A (1) (281 mg), loloatin B (2) (1.87 g, 3% dry weight of cells) and loloatin C (3) (39 mg) as beige amorphous solids.

2.4.2 Total Acid Hydrolysis and GC Analysis

The acid hydrolysate mixtures of loloatin A (1), loloatin B (2) and loloatin C (3) were converted to the PFPA-IPA ester derivatives, and the GC retention times of the hydrolysate components were compared against the GC retention times of authentic standards on a chiral column. Thus, loloatin A, B and C (1.0 mg each) were hydrolyzed individually with 3 ml of 6 N HCL containing 1% thioglycollic acid (to reduce oxidation of tryptophan and tyrosine residues) for 8 h at 105 °C under N2 in a sealed vial. The hydrolysates were reduced to dryness using a stream of dry N2, and the reaction mixtures were then esterified with HCl/iPrOH at 100 °C for 45 min and reduced to dryness. The mixtures were then reacted with 50 μL of pentafluoropropionic anhydride in 250 μL of CH2Cl2 at 100 °C for 15 min in a sealed vial, the sample was then evaporated and redissolved in 500 μL CH2Cl2. Racemic mixtures as well as optically pure L-amino acid standards were derivatized in a similar fashion. The amino acid standards and the hydrolysate were analyzed on a 25 m chiralsil-Val Heliflex column with FID detection using the following conditions: He carrier, detector temp 275 °C, injector temp 250 °C, injector split ratio
25:1, initial oven temp 90 °C, initial time 5 min, program rate 4 °C/min, final oven temp 200 °C, final time 27.5 min.

2.4.3 Compound Summaries

Loloatin A (1), cyclic(L-asparaginyl-L-aspartyl-L-tyrosyl-L-valyl-L-ornithyl-L-leucyl-D-tyrosyl-L-proyl-L-phenylalanyl-D-phenylalanyl): isolated as a white solid (281 mg); mp 229-232 °C; IR (thin film on PTFE membrane) $\nu_{\text{max}}$: 3275 (br,m), 3032 (w), 3070 (w), 2958 (w), 1637 (br,s), 1537 (br,m), 1454 (br,w), 1251 (br,m); $[\alpha]_D -88.0$ (EtOH); UV (EtOH) $\gamma_{\text{max}}$ (e): 224 (21 000), 278 (3400); $^1$H and $^{13}$C NMR data see Table B.3; HRFABMS m/z (formula, ΔM ppm): 1273.63082 (M+H/C$_{65}$H$_{85}$N$_{12}$O$_{15}$, 4.00), 621.26866 (C$_{31}$H$_{37}$N$_{6}$O$_{5}$, 2.21), 506.24050 (C$_{27}$H$_{32}$N$_{5}$O$_{5}$, 0.31), 392.19821 (C$_{23}$H$_{26}$N$_{3}$O$_{3}$, 2.01) 377.14716 (C$_{17}$H$_{21}$N$_{4}$O$_{6}$, 2.78), 245.12933 (C$_{146}$H$_{17}$N$_{2}$O$_{2}$, 1.33).

Loloatin B (2), cyclic(L-asparaginyl-L-aspartyl-L-tryptophanyl-L-valyl-L-ornithyl-L-leucyl-D-tyrosyl-L-proyl-L-phenylalanyl-D-phenylalanyl): isolated as a white solid (1.87 g); mp 229-233 °C; IR (thin film on PTFE membrane) $\nu_{\text{max}}$: 3275 (br,m), 3070 (m), 3032 (w), 1637 (br,s), 1537 (br,m), 1454 (w); $[\alpha]_D -79.5$ (EtOH); UV (EtOH) $\gamma_{\text{max}}$ (e): 220 (43 000), 280 (5900); $^1$H and $^{13}$C NMR data see Table B.1; HRFABMS m/z (formula, ΔM ppm): 1296.64232 (M+H/C$_{67}$H$_{86}$N$_{13}$O$_{14}$, 0.46), 621.26944 (C$_{31}$H$_{37}$N$_{6}$O$_{8}$, 3.46) 506.24105 (C$_{27}$H$_{32}$N$_{5}$O$_{5}$, 1.39), 392.19848 (C$_{23}$H$_{26}$N$_{3}$O$_{3}$, 2.71) 377.14717 (C$_{17}$H$_{21}$N$_{4}$O$_{6}$, 2.80), 245.12922 (C$_{146}$H$_{17}$N$_{2}$O$_{2}$, 0.89).
Loloatin C (3), cyclic(L-asparaginyl-L-aspartyl-L-tryptophanyl-L-valyl-L-ornithyl-L-leucyl-D-tyrosyl-L-prolyl-L-tryptophanyl-D-phenylalanyl): isolated as a tan/white solid (39 mg); mp 239-243 °C; IR (thin film on PTFE membrane) $\nu_{\text{max}}$: 3273 (br,m), 3080 (w), 2960 (w), 1633 (br,s), 1537 (br,m), 1516 (br,m); $[\alpha]_D^{25.5}$ (EtOH); UV (EtOH) $\gamma_{\text{max}}$ ($\epsilon$): 223 (66 000), 280 (10 000); $^1$H and $^{13}$C NMR data see Table B.4; HRFABMS m/z (formula, $\Delta M$ ppm): 1335.65267 ($\text{M+H/C}_{69}\text{H}_{97}\text{N}_{14}\text{O}_{14}$, 0.04), 545.25262 ($\text{C}_{29}\text{H}_{33}\text{N}_{6}\text{O}_{5}$, 2.52), 431.20811 ($\text{C}_{23}\text{H}_{27}\text{N}_{4}\text{O}_{3}$, -0.48), 377.14580 ($\text{C}_{17}\text{H}_{21}\text{N}_{4}\text{O}_{6}$, -0.83), 284.14057 ($\text{C}_{16}\text{H}_{18}\text{N}_{3}O_{2}$, 2.37).

N-acetyl loloatin B methyl ester (39): Loloatin B (2) (100 mg) was acetylated under argon, at 23 °C for 16 hours, using 1 mL of Ac$_2$O and 2 mL anhydrous pyridine (freshly distilled). The solution was reduced in vacuo and the crude acetylated material was then loaded onto a reversed phase ODS Sep-Pak using 5 mL of 50% aqueous MeOH, followed by elution with 5 mL of MeOH. The MeOH eluent was reduced in vacuo, dissolved in 5 mL THF and reacted with diazomethane in a micromolar generator using a dry ice/Me$_2$CO bath to cool the THF solution. The reaction mixture was purified using reversed phase ODS HPLC with 17:3 MeOH/H$_2$O as eluent to yield 30 mg of N-acetyl loloatin B methyl ester (39). HRFABMS m/z (formula, $\Delta M$ ppm): 1352.66940 ($\text{M+H/C}_{70}\text{H}_{90}\text{N}_{13}\text{O}_{15}$, 1.08); $^1$H and $^{13}$C NMR data see Table B.2.
References


3 *CRC Handbook of Biochem. and Mol. Biol.* 1976, 2, 206.


5 Kelly, M. T., Seatek Biotechnology, Surrey, B.C., personal communication.


10 The reader is also directed to the excellent work on Gramicidin S biosynthesis, which further illustrates the modular system of peptide synthetases. See: Stachelhaus, T.; Marahiel, M. A. *J. Biol. Chem.* 1995, 270(11), 6163-6169


Chapter 3 : Massetolides A - H (4 - 11)

3.1 Introduction

This chapter describes the isolation and structure elucidation of a family of secondary metabolites produced by two different species of bacteria isolated from the coastal waters of British Columbia. Massetolides A - D (4 - 7) were isolated and characterized from the marine isolate MK90E85 obtained from the surface of an unidentified leafy red alga collected in Masset Inlet, B.C. The marine isolate MK91CC8, which was obtained from an unidentified tube worm that was collected near Moira Island, B.C., produced massetolides E - H (8 - 11) as well as the known compound viscosin (12). Massetolide A (4) and viscosin (12) exhibit in vitro antimycobacterial activity against Mycobacterium tuberculosis (MTB) and Mycobacterium avium-intacellulare. Isolation and characterization of secondary metabolites from MK91CC8 was accomplished in cooperation with Dr. Richard Lloyd.
<table>
<thead>
<tr>
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<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (4)</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH(CH₃)CH₂CH₃</td>
</tr>
<tr>
<td>B (5)</td>
<td>CH₂CH₃</td>
<td>CH₃</td>
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</tr>
<tr>
<td>C (6)</td>
<td>CH₂CH₂CH₃</td>
<td>CH₃</td>
<td>CH(CH₃)CH₂CH₃</td>
</tr>
<tr>
<td>D (7)</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₂CH(CH₃)₂</td>
</tr>
<tr>
<td>E (8)</td>
<td>CH₃</td>
<td>H</td>
<td>CH(CH₃)₂</td>
</tr>
<tr>
<td>F (9)</td>
<td>CH₃</td>
<td>H</td>
<td>CH₂CH(CH₃)₂</td>
</tr>
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<td>CH(CH₃)CH₂CH₃</td>
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<td>Viscosin (12)</td>
<td>CH₃</td>
<td>H</td>
<td>CH(CH₃)CH₂CH₃</td>
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</tbody>
</table>
3.2 Results

Marine isolate MK90E85 was obtained from the surface of an unidentifed leafy red alga collected in Masset Inlet, B.C. It was shown to belong to the genus *Pseudomonas* by fatty acid analysis, but it could not be confidently identified to the species level. Cultures of MK90E85 were grown as lawns on solid agar and were harvested by gently scraping the cells from the surface of the agar. Fractionation of the EtOAc-soluble materials from the cell extract in a manner similar to that described for the isolation of the loloatins (See Section 2.2.1) gave pure massetolides A (4), B (5), C (6) and D (7).

The marine isolate MK91CC8 was obtained from an unidentified tube worm that was collected near Moira Island, B.C. Once again, MK91CC8 was shown to be in the genus *Pseudomonas* by fatty acid analysis, but it also could not be confidently identified to the species level. Cultures of MK91CC8 were also grown on solid agar and harvested by gently scraping the cells from the surface of the agar. The agar from MK91CC8 cultures was extracted by soaking in ethyl acetate to give a crude extracellular extract. Fractionation of the extracellular extract gave pure massetolides E (8), F (9), G (10) and H (11) along with the known compound viscosin (12).
3.2.1 Structure Elucidation of Massetolide A (4)

The structure of 4 was determined by a detailed analysis of one dimensional and two dimensional NMR spectra and mass spectrometric analysis. The structures of the individual amino acid residues were determined by analysis of the COSY, HOHAHA, HMQC and HMBC data. The absolute configurations of these residues were determined by conversion of the liberated amino acids to their pentafluoropropionylamide isopropyl ester (PFPA-IPA) derivatives followed by chiral GC analysis. The sequence of the amino acid residues in 4 was determined by examination of the ROESY and HMBC data.

Massetolide A (4)  R=H
Massetolide A methyl ester (46)  R=Me

Massetolide A (4) was obtained as optically active ([α]D -45.9°, EtOH) colorless needles that gave a (M + H)+ peak at m/z 1140.7151 in the HRFABMS. The 1H and 13C NMR spectra obtained for massetolide A (4) contained resonances that were characteristic of peptides (Figures 3.1 and 3.3). The 1H NMR spectrum (Figure 3.1) contained nine resonances between δ 6.50 and
δ 9.00 ppm assigned to exchangeable protons attached to heteroatoms; several resonances between δ 3.50 and δ 5.50 assigned to protons attached to carbons bonded to a heteroatom; a methyl doublet resonance at δ 1.45 and a large envelope of methyl resonances between δ 0.84 and δ 1.03. The $^{13}$C NMR spectrum (Figure 3.3) appeared to contain at least eleven carbonyl (C=O) resonances (Figure C.1), and another 43 distinct carbon resonances more shielded than 72.0 ppm (Figures C.2 and C.3). From the APT and $^{13}$C NMR spectra two carbon CH resonances at 70.1 ppm could be assigned as carbinol methines (CHOR) (Figure C.2). Moreover, examination of the HMQCD spectrum (Figure 3.5) as well as the APT spectrum (Figure C.2) suggested that there were nine carbon resonances typical of amino acid alpha-methines as well as two hydroxy methylene carbon resonances (CH$_2$OR) in the 55 - 60 ppm region of the $^{13}$C spectrum. Massetolide A (4) tested negative to ninhydrin, which suggested that there was no free amino terminus. Roughly 80 protons could be accounted for by inspection of the integration data of the $^1$H spectrum (Figure 3.1) and inference from the APT and HMQC spectra (Figure 3.5). Consequently, it was established that the molecular formula of massetolide A (4) should have at least 53 carbons, 80 hydrogens, nine nitrogens, and twelve oxygens (since the possibility of two ether linkages, although unlikely, could not be ruled out at this point). This evidence taken together with the HRFABMS data established the molecular formula as C$_{55}$H$_{97}$N$_9$O$_{16}$ (ΔM 1.67 ppm).

Detailed analysis of the COSY, HMQC, HMBC and HOHAHA data (Figures 3.4 -3.7, Table C.1) revealed that peptide 4 contained one glutamic acid, one threonine, two isoleucine, three leucine and two serine residues. Hydrolysis of 4 with 6N HCl followed by chiral GC analysis of the PFPA-IPA esters of the individual amino acids confirmed the presence of D-serine, D-glutamic acid, L-isoleucine, D-allo-isoleucine, L-leucine, and D-allo-threonine (Table 3.1).
For clarity of this discussion, designations indicating a residue's placement in the final structure are used in this text to differentiate amino acids which are duplicated. The three leucine residues are therefore referred to as Leu1 (or the one letter code L), Leu5 (L'), and Leu7 (L''). The two isoleucine residues are referred to as Ile4 (I), and Ile9 (I'). The two serine residues are designated Ser6 (S) and Ser8 (S'). The $^1$H and $^{13}$C resonances for all the amino acids had to be assigned before final connectivity between the residues could be determined; thus only after having the complete structure 4 in hand was it possible to assign amino acid designations such as Leu1, Leu5 and Leu7. Even though the amino acid designations given in the following text appear straightforward, it must be remembered that the final determination of the amino acid composition, as well as the complete $^1$H and $^{13}$C assignment, was a complex problem with only one solution consistent with all the available data.
Figure 3.1: 500 MHz $^1$H NMR Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$
Figure 3.2: $^1$H NMR Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - alpha-CH expansion
Figure 3.3: 125 MHz $^{13}$C/APT NMR Spectra of Massetolide (4) in Me$_2$CO-$d_6$
Figure 3.4: 500 MHz COSY Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$
Figure 3.5: 500 MHz HMQC Spectrum of Massetolide A (4) in Me₂CO-d₆
Figure 3.6: 500 MHz HOHAHA of Massetolide A (4) in Me₂CO-d₆ - NH expansion
Figure 3.7: 500 MHz HMBC Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$
The HOHAHA spectrum revealed that the methyl envelope between $\delta$ 0.84 and $\delta$ 1.03 belonged to five different aliphatic amino acid residues (Figure 3.6). The first aliphatic spin system, starting with the most downfield amide NH resonance, was determined to be a leucine residue (Leu1, L). HOHAHA correlations were observed between the amide NH ($\delta$ 8.80) and resonances at $\delta$ 4.05, 1.85 and 0.95 ppm (Figure 3.6). Analysis of the COSY spectrum (Figure 3.4) assigned the Leu1 \textit{alpha}-CH ($\delta$ 4.05) and \textit{beta}-CH$_2$ ($\delta$ 1.85) resonances. An HMBC correlation was observed between the \textit{beta}-CH$_2$ resonance at $\delta$ 1.85 and a methine carbon at $\delta$ 25.4, whose proton resonated at $\delta$ 2.0 coincident with the solvent (Figure C.4). These \textit{beta}-methylene proton resonances also showed an HMBC correlation to two methyl carbon resonances at approximately $\delta$ 22.0 and 24.0 ppm (Figure C.4). The resonance belonging to the ester/amide carbonyl was identified through HMBC correlations observed between Leu1-CO ($\delta$ 175.5) and the proton resonances at $\delta$ 8.80 (Figure C.9), 4.05 (Figure C.8), and 1.85 ppm (Figure C.7).
The next aliphatic residue, another leucine residue (Leu5, L'), had an amide resonance at δ 8.50 that showed HOHAHA correlations to δ 3.68, 1.78, 2.0 and the methyl envelope at δ 0.91 ppm (Figure 3.6). Analysis of the COSY assigned the resonances to the Leu5 alpha-CH (δ 3.68), beta-CH₂ (δ 1.78) and gamma-CH (δ 2.0) protons (Figure 3.4). Since it was determined that there was a gamma-CH carbon, this should therefore be bonded to two methyl groups (δ 21.8, δ 23.8). The resonances for the methyl carbons in residues Leu1, Leu5 and Leu7 could not be assigned unambiguously and can be interchanged; however, the assignment of these methyls was not critical to the structure elucidation. Both the Leu5-NH (δ 8.50) and alpha-CH (δ 3.68) showed HMBC correlations to a carbonyl resonance at δ 171.1 ppm, thus establishing the identity of the ester/amide carbonyl (Figures C.8 and C.9).
The next residue to be assigned was an isoleucine residue (Ile4, I). The amide NH resonance at $\delta$ 8.05 showed correlations in the HOHAHA to an alpha-CH ($\delta$ 3.58), beta-CH ($\delta$ 2.0), gamma-CH$_2$ ($\delta$ 1.15 and 1.52), and methyl resonances at $\delta$ 1.09 and 0.94 ppm (Figure 3.6). HMBC correlations were observed between the Ile4 amide NH ($\delta$ 8.05) and the alpha- ($\delta$ 64.5) and beta-CHs ($\delta$ 36.0) (Figure C.6); between the alpha-CH ($\delta$ 3.58) and the beta-CH ($\delta$ 36.0) and gamma-CH$_3$ ($\delta$ 16.6) carbon resonances (Figure C.5); and between the gamma-CH$_2$ proton resonance at $\delta$ 1.15 and carbon resonances at $\delta$ 10.7 (delta-CH$_3$), 16.6 (gamma-CH$_3$), and 36.0 (beta-CH) (Figure C.4).

![Diagram of Ile4 residue]
Inspection of the HOHAHA and COSY data also identified a third leucine residue (Leu7, L”). The HOHAHA revealed correlations between the amide NH resonance (δ 7.62) and resonances at δ 4.20, 1.65, 2.0 ppm, and the methyl envelope at δ 0.93 and 1.00 ppm (Figure 3.6). Analysis of the COSY established these signals as the alpha-CH (δ 4.20), beta-CH₂ (δ 1.65), gamma-CH (δ 2.0), and the two delta-methyls (δ 0.93 and 1.00), (Figure 3.4).
A second isoleucine residue (Ile9, \( \Gamma' \)) was also deduced from the NMR data. However, assignment of the resonances of this residue proved to be difficult since the Ile9-NH resonance only showed correlations in the HOHAHA to resonances at \( \delta 4.60, 2.0, \) and the methyl envelope at 0.9 ppm (Figure 3.6), and unfortunately the resonance at \( \delta 4.60 \) was coincident with the resonance of the Ser8 alpha-CH, discussed further in the text. Nevertheless, the HMBC data revealed that this alpha-CH (\( \delta 4.60 \)) correlated to three carbon resonances at \( \delta 36.8 \) (beta-CH), \( \delta 25.1 \) (gamma-CH\(_2\)) and a gamma-CH at \( \delta 16.2 \) ppm (Figure C.5). The gamma-CH\(_2\) resonance at \( \delta 25.1 \) must in turn be bonded to one other substituent, which was assumed to be a methyl. This assumption was justified by total acid hydrolysis and analysis of the PFPA-IPA derivatives which revealed that there was at least two molecules of isoleucine present in massetolide A (4), L-isoleucine and D-allo-isoleucine (Table 3.1). Finally, the HMBC data revealed that the alpha-CH (\( \delta 4.60 \)) correlated to a carbonyl resonance at \( \delta 169.7 \) ppm (Figure C.8).
Examination of the HOHAHA data suggested that three of the four remaining amide resonances belonged to hydroxylated amino acids. The amide resonances at δ 7.18 and δ 8.13 each showed HOHAHA correlations to three other protons in the alpha-CH region of the spectrum (Figure 3.6). This could easily be explained by the presence of two serine residues (Ser6, S and Ser8, S'). Examination of the COSY data and the HMQC data firmly established the assignments of these residues (Figures 3.4 and 3.5). Ser6-NH (δ 7.18) showed a COSY correlation to an alpha-CH (δ 4.40), which in turn was bonded to a carbinol methylene with resonances at δ 3.85 and δ 4.20. One of the Ser6 beta-CH2 resonances at δ 3.85 also showed an HMBC correlation to a carbonyl resonance at δ 172.1 ppm (Figure C.8) firmly establishing the identity of the Ser6 ester/amide carbonyl. Similarly, for the other serine residue, the HOHAHA revealed correlations from the Ser8-NH (δ 8.13) to the alpha-CH (δ 4.60) and the two beta-carbinol methylene protons (beta-CH2OH) at δ 3.70 and 3.90 ppm. The Ser8 carbonyl resonance was identified by the observation that one of the geminal beta-CH2 protons (δ 3.70) was correlated to the carbonyl resonance at δ 171.7 in the HMBC (Figure C.8).
The amide resonance at δ 8.18 showed correlations in the HOHAHA to two protons in the \textit{alpha-CH} region of the spectrum, one of which was unusually deshielded, as well as showing a correlation to a methyl doublet at δ 1.45 (Figure 3.6). This spin system apparently belonged to a threonine residue (Thr, T), whose \textit{beta}-hydroxyl group was involved in an ester bond. Examination of the COSY assigned the \textit{alpha-CH} (δ 4.18) and the deshielded \textit{beta} carbinol methine (δ 5.38); the \textit{beta} carbinol methine was in turn correlated to the \textit{gamma-CH}\textsubscript{3} at 1.45 ppm (Figure 3.4).
The final amide resonance at δ 8.37 was shown to belong to a glutamic acid (Glu, E). The HOHAHA revealed that this amide NH proton resonance was correlated to resonances at δ 4.25, 2.08, and 2.51 ppm (Figure 3.6). Analysis of the COSY assigned these resonances to the alpha-CH and gamma-CH$_2$ protons respectively (Figure 3.4). An HMBC correlation from the alpha-CH (δ 4.25) to a carbonyl resonance at δ 176.5 established the identity of the amide carbonyl of this residue (Figure C.8). Esterification of massetolide A (4) with diazomethane gave the methyl ester at the glutamic residue. The $^1$H and $^{13}$C spectra for the methyl ester of massetolide A (46) are given in Figure 3.8 and Figure 3.9. Analysis of the HMBC for the methyl ester (46) showed that the new methyl singlet at δ 3.60 correlated to a carbonyl at δ 173.4 which in turn also showed a correlation to the gamma-CH$_2$ at δ 2.51 ppm (Figures 3.10 and 3.11). This evidence firmly established that the glutamate residue was involved in a typical α linkage with adjacent residues and existed as the free acid in native massetolide A (4).

\[ \text{Glu in Massetolide A (4)} \]

\[ \text{Glu Methyl Ester in Massetolide A Methyl Ester (46)} \]
Figure 0.1: 500 MHz $^1$H NMR Spectrum of Massetolide A Methyl Ester (46) in Me$_2$CO-$_d$6
Figure 0.2: 125 MHz $^{13}$C NMR Spectra of Massetolide A (4 - top spectrum) and Massetolide A Methyl Ester (46 - bottom spectrum)
Figure 3.10: 500 MHz HMBC Spectrum of Massetolide A Methyl Ester (46) in Me$_2$CO-$_{d6}$
Figure 3.11: HMBC Spectrum of Massetolide A Methyl Ester (46) in Me$_2$CO-$d_6$ - Expansion
Subtraction of the C, N, and O atoms accounted for by the nine identified amino acid residues (C₄₅N₉O₁₄) from the molecular formula of massetolide A showed that the remaining fragment of the molecule had to contain ten carbon and two oxygen atoms. The $^{13}$C NMR and HMQC data showed that the ten carbon atoms not accounted for by amino acid residues consisted of one aliphatic methyl (CH₃), one carbinol methine (CHOH), one ester/amide carbonyl (C=O), and seven aliphatic methylene (CH₂) carbons. This suite of carbons could only be accommodated by a linear ten carbon chain having the methyl and ester/amide carbonyl carbons at either terminus. The HMQC spectrum revealed that the carbinol methine carbon ($\delta$ 70.1) was attached to a proton that gave a $^1$H NMR resonance at $\delta$ 4.14 ppm (Figure C.5). In the COSY spectrum, the carbinol methine proton resonance ($\delta$ 4.14) was correlated to a pair of geminal methylene proton resonances at $\delta$ 2.56 and 2.63 (Figure 3.4). These methylene proton resonances were correlated in the HMBC spectrum to the ester/amide carbonyl carbon resonance ($\delta$ 174.7) that was at the terminus of the linear ten carbon chain (Figure C.7). Taken together, the above evidence suggested the presence of a $\beta$-hydroxydecanoyl fragment. The assignments of the carbons of this fatty acid unit were based on empirical calculations of $^{13}$C shifts in substituted alkanes³.
An HMBC correlation between the Leu1-NH resonance at δ 8.80 and the β-hydroxydecanoyl carbonyl resonance at δ 174.72 showed that the fatty acid fragment was linked to the Leu1 residue via an amide bond (Figure C.9). ROESY correlations between the Leu1-NH resonance at δ 8.80 and the β-hydroxydecanoyl alpha proton resonances at δ 2.56 and 2.63 (Figure 3.12) and the observation of an intense HRFABMS fragment at m/z 284 (Table 3.2) having a molecular composition of C₁₆H₂₀NO₃ corresponding to the FA-LEU1 unit confirmed this linkage.

The amino acid sequence in massetolide A (4) was determined from the HMBC data. HMBC correlations observed between the Glu-NH (δ 8.37) and the Leu1-CO (δ 175.5) resonances; between the Thr-NH (δ 8.18) and the Glu-CO (δ 176.5) resonances; and between the Ile4-NH (δ 8.05) and the Thr-CO (δ 174.4) resonances defined the partial sequence FA-Leu1-Glu-Thr-Ile4-CO (Figure C.9). The downfield chemical shift of the Thr carbinol methine proton resonance (δ 5.38) in massetolide A (4) indicated that the hydroxyl group was part of an ester linkage. An HMBC correlation between the Thr carbinol methine proton (δ 5.38) and the Ile9-CO (δ 169.7) resonances demonstrated that Ile9 was connected to the Thr via an ester linkage (Figure C.8). The Ile9-NH resonance (δ 6.64) showed an HMBC correlation to the Ser8-CO resonance (δ 171.7) indicating that the Ile9 amino group and the Ser8 carbonyl formed a peptide bond (Figure C.9). Additional HMBC correlations observed between the Leu7-NH (δ 7.62) and the Ser6-CO (δ 172.1) resonances and between the Ser6-NH (δ 7.18) and Leu5-CO (δ 171.1) resonances established the partial sequence HN-Leu5-Ser6-Leu7-CO (Figure C.9).
Figure 3.12: 500 MHz ROESY Spectrum of Massetolide A (4) in Me₂CO-d₆ - NH Expansion
The molecular formula of massetolide A (4) requires twelve sites of unsaturation. Since only eleven of these could be accounted for by the carbonyl functionalities identified in the component amino acid and fatty acid residues, the remaining site of unsaturation had to be present as a ring. The only way to join the two partial structures identified from the HMBC data together to form a ring and to leave the Glu delta carboxylic acid free was to link the Leu5 amino group to the Ile4 carboxyl and the Leu7 carboxyl group to the Ser8 amino group as shown in 4.

FABMS (Table 3.2) and ROESY data (Figure 3.12) supported the sequence assigned from the HMBC data. ROESY correlations observed between the Glu-NH (δ 8.37) and Leu1-alpha-CH (δ 4.05) resonances; between the Ile4-NH (δ 8.05) and Thr beta-CH (δ 5.38) resonances; between the Ile9-NH (δ 6.64) and Ser8-alpha-CH (δ 4.60) resonances; and between the Ser6-NH (δ 7.18) and Leu5-alpha-CH (δ 3.68) resonances (Figure 3.21) confirmed the HN-Leu1-Glu-CO, HN-Thr-Ile4-CO, HN-Leu5-Ser6-CO and HN-Ser8-Ile9-CO partial sequences identified from the HMBC data. The FABMS showed peaks at m/z 971 (MH - (FA)), 857 (MH - (FA-Leu1)), 728 (MH - (FA-Leu1-Glu)), 645 (MH - (FA-Leu1-Glu-Thr(-H2O)), 609 (MH - (FA-Leu1-Glu-Thr(-H2O)-Ile4)), 496 (FA-Leu1-Glu-Thr(-H2O)), 413 (FA-Leu1-Glu) and 284 (FA-Leu1) that were consistent with the proposed structure 4 for massetolide A. The data for massetolide A (4) alone did not identify which of the stereochemical forms of isoleucine, namely L-isoleucine or D-allo-isoleucine, were at the AA4 and AA9 positions. This point was clarified with the subsequent characterization of massetolide D (7) (See Section 3.2.3).
Once the structure of massetolide A (4) was in hand, it became apparent that it was related to viscosin (12)\textsuperscript{1,2} and the "white line inducing principle" (WLIP)\textsuperscript{4} isolated from \textit{P. viscosa} and \textit{P. reactans}, respectively. Since both viscosin (12) and WLIP contain D-\textbeta-hydroxydecanoyl residues, it has been assumed that the \textbeta-hydroxydecanoyl residue in massetolide A (4) also has the D configuration.

<table>
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<th>Table 3.1: Chiral GC Retention Times (in minutes) of Pentafluoropropionamide Isopropyl Ester Derivatives of Massetolides A - H (4 - 11) and Viscosin (12)\textsuperscript{a}</th>
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<tr>
<td>D-Thr</td>
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<tr>
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<tr>
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\textsuperscript{a} All identifications were made by coinjection with an authentic standard
Table 3.2: FABMS Fragment Ions for Massetolides A - H (4 - 11) and Viscosin (12)

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Table 3.3: $^1$H Chemical Shifts of the alpha-CH Resonances in Massetolides A - H (4 - 11) and Viscosin (12)

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<td>4.13 m</td>
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<td>4.10 m</td>
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<td>3.67 m</td>
<td>4.37 m</td>
<td>4.17 m</td>
<td>4.48 m</td>
<td>4.57 dd, 10.2, 3.2</td>
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<td>4.16 m</td>
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<td>3.69 m</td>
<td>4.40 dt, 8.6, 2.0</td>
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<tr>
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<td>4.08 m</td>
<td>4.23 m</td>
<td>4.20 m</td>
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<td>3.67 m</td>
<td>4.40 br d, 8.7</td>
<td>4.15 m</td>
<td>4.51 m</td>
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<td>4.18 m</td>
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<td>4.14 m</td>
<td>4.50 m</td>
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<td><strong>Viscosin (12)</strong></td>
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<td>4.18 m</td>
<td>3.46 dd, 11.2, 5.8</td>
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<td>4.14 m</td>
<td>4.57 m</td>
<td>4.58 dd, 10.0, 3.1</td>
</tr>
</tbody>
</table>
3.2.2 Structure Elucidation of Massetolide B (5) and Massetolide C (6)

Massetolide B (5) gave a (M + H)
\[^{+}\] peak at m/z 1154.73239 in the HRFABMS appropriate for a molecular formula of C\(_{56}\)H\(_{99}\)N\(_{9}\)O\(_{16}\) (ΔM 3.11 ppm) that differed from the molecular formula of massetolide A (4) simply by the addition of CH\(_{2}\). Comparison of the \(^{1}\)H NMR spectrum for massetolide B (5) with the \(^{1}\)H NMR spectrum of massetolide A (4) (Table 3.3) revealed that they contained the same amino acids and the FABMS data (Table 3.2) showed that they differed only in the \(\beta\)-hydroxy fatty acid residue. A peak at m/z 298 in the FABMS of 5, corresponding to the FA-Leu fragment A, required a fatty acid residue containing one additional methylene group compared with the \(\beta\)-hydroxydecanoic acid residue in 4. Hydrolysis of 5 with 6N HCl followed by chiral GC analysis of the PFPA-IPA esters of the individual amino acids confirmed the presence of the D-serine, D-glutamic acid, L-isoleucine, D-allo-isoleucine, L-leucine and D-allo-threonine residues also found in 4 (Table 3.1). The GC analysis also gave a peak corresponding
to the derivative formed from authentic β-hydroxyundecanoic acid under the hydrolysis and derivatization conditions, confirming that massetolide B had structure 5. A similar analysis of the data (Table 3.1 - 3.3) for massetolide C (6) (HRFABMS m/z 1168.74544, C_{57}H_{101}N_oO_{16}, ΔM 0.84 ppm) showed that it was simply the β-hydroxydodecanoic acid homologue of massetolide A (4).

3.2.3 Structure Elucidation of Massetolide D (7)

![Massetolide D (7) structure](image)

Massetolide D (7) gave a (M + H)^+ ion at m/z 1140.71379 in the HRFABMS that was appropriate for a molecular formula of C_{55}H_{97}N_{9}O_{16} (ΔM 0.56 ppm), identical with that of massetolide A (4). Hydrolysis of 7 with 6 N HCl followed by chiral GC analysis of the PFPA-IPA ester derivatives of the liberated residues identified D-allo-isoleucine, D-allo-threonine, D-serine, L-leucine, D-glutamic acid and β-hydroxydecanoic acid (Table 3.1). The hydrolysis results
combined with the MS data (Table 3.2) suggested that massetolide D (7) differed from
massetolide A (4) only by the replacement of isoleucine with either of the equal mass residues
leucine or D-allo-isoleucine. Comparison of the \( ^1H \) NMR data (Table 3.3) for massetolide D (7)
with that for massetolide A (4) revealed that the only detectable difference in the two spectra was
in the multiplicity of the alpha-CH resonance assigned to the AA9 residue in the spectrum of 7.
In the spectrum of massetolide A (4), the isoleucine AA9 alpha-CH resonance (Table 3.3 - I'2)
appeared as a doublet of doublets at \( \delta 4.60 \) (\( J = 10.1, 3.4 \) Hz) reflecting scalar coupling to the NH
and beta-CH protons. The corresponding resonance in the \( ^1H \) NMR spectrum of massetolide D
(7) appeared at the nearly identical chemical shift of \( \delta 4.59 \) but it was a doublet of triplets (\( J = 
4.0, 10.0 \) Hz) consistent with the presence of a leucine residue at the AA9 position and the D-allo-
iso-leucine at the AA4 position.
Figure 3.13: 500 MHz $^1$H NMR Spectra of Massetolides A - D (4 - 7) and Viscosin (12) in Me$_2$CO-$d_6$
Figure 3.14: 500 MHz $^1$H NMR Spectra of Massetolides A - D (4 - 7) and Viscosin (12) in Me$_2$CO-$d_6$ alpha-CH expansions
3.2.4 Structure Elucidation of Viscosin (12)

The major antimycobacterial component from extracts of the MK91CC8 cultures was the known compound viscosin (12). Even though the structure of viscosin had been in the literature for some time and the compound had been quite extensively investigated, the published NMR data was not sufficiently detailed to allow a direct comparison with the data collected on 12 isolated from the MK91CC8. Thus, an independent structure elucidation involving FABMS, NMR and hydrolytic analysis of MK91CC8 viscosin (12) was undertaken (Tables 3.1 - 3.3). This investigation confirmed that viscosin isolated from the MK91CC8 cultures indeed had the same structure as the known natural product.
3.2.5 Structure Elucidation of Massetolides E (8)

Massetolide E (8) gave a (M + H)$^+$ ion at m/z 1126.69708 in the HRFABMS appropriate for a molecular formula of C$_{53}$H$_{93}$N$_9$O$_{16}$ (ΔM 3.46 ppm) that differed from the molecular formula of viscosin (12) by the loss of CH$_2$. Hydrolysis of 8 with 6N HCl followed by chiral GC analysis of the PFPA-IPA esters of the individual amino acids identified the presence of L-valine, D-valine, D-serine, D-glutamic acid, L-leucine and D-allo-threonine residues (Table 3.1). Comparison of the fragment masses in the FABMS spectrum of 8 (Table 3.2) with those for viscosin (12) indicated that the molecules were identical in the sequence between the β-hydroxydecanoic acid through AA4. Examination of the $^1$H NMR spectrum of 8 (Table 3.3) revealed that the methine resonance at δ 4.55, which could be assigned on the basis of its chemical shift to the $\alpha$-CH proton (Table 3.3-I'2) of the AA9 residue by analogy with massetolides A (4), D (7) and viscosin (12), was a doublet of doublets (J = 3.4, 10.0 Hz). The multiplicity of the AA9 methine
resonance indicated that the second valine residue identified by the GC analysis was at this position and, therefore, that massetolide E had the structure 8.

3.2.6 Structure Elucidation of Massetolide F (9)

Massetolide F (9) gave a (M + H)$^+$ ion in the HRFABMS at m/z 1126.69708 appropriate for a molecular formula of C$_{54}$H$_{95}$N$_9$O$_{16}$ (ΔM 3.11 ppm), identical to that of viscosin (12). Hydrolysis of 9 with 6N HCl followed by chiral GC analysis of PFPA-IPA esters of the individual amino acids identified the presence of D-valine, D-serine, D-glutamic acid, L-leucine and D-allo-threonine residues (Table 3.1). The only difference in the MS and $^1$H NMR data (Tables 3.2 and 3.3) of viscosin (12) and massetolide F (9) was in the multiplicity of the AA9 alpha-CH resonance. In the $^1$H NMR spectrum of massetolide F (9), the methine resonance at δ 4.60 (Table
3.3-1'2), assigned to the AA9 residue, was a doublet of triplets \( J = 9.8, 4.4 \text{ Hz} \) which indicated the presence of a leucine at this position.

### 3.2.7 Structure Elucidation of Massetolide G (10) and Massetolide H (11)

Massetolides G (10) and H (11) were routinely shown to be the \( \beta \)-hydroxyundecanoic acid and \( \beta \)-hydroxydodecanoic acid analogs of viscosin (12), respectively, by analysis of their hydrolysis data, HRFABMS, and \(^1\text{H} \) NMR (Tables 3.1 - 3.3).
Figure 3.15: 500 MHz $^1$H NMR Spectra of Viscosin (12) and Massetolides E - H (8 - 11) in Me$_2$CO-$d_6$
Figure 3.16: 500 MHz $^1$H NMR Spectra of Viscosin (12) and Massetolides A - D (8 - 11) in Me$_2$CO-$d_6$ - alpha-CH expansions
3.2.8 Biological Activity

Massetolide A (4) and viscosin (12) were tested for activity against *Mycobacterium tuberculosis* and *M. avium-intracellulare* by using the proportion method on Middlebrook 7H10 agar. No activity was observed for either compound against a panel of other human pathogenic bacteria including *Escherichia coli* and *Staphylococcus aureus* (Table 3.4). Unfortunately there were insufficient quantities of the other massetolides for biological testing. A single intraperitoneal injection of 10 mg/kg of massetolide A (4) was found to be non-toxic to mice.

Table 3.4: Minimum Inhibitory Concentrations (μg/mL) of Massetolide A (4) and Viscosin (12)

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Massetolide A (4)</th>
<th>Viscosin (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus saprophyticus</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td><em>Enterococcus faecalis</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Xanthomonas maltophilia</em></td>
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<td>&gt;100</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>5-10</td>
<td>10-20</td>
</tr>
<tr>
<td><em>Mycobacterium avium-intracellulare</em></td>
<td>2.5-5</td>
<td>10-20</td>
</tr>
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</table>
3.3 Discussion

Massetolides A - H (4 - 11) and viscosin (12) were isolated and characterized from two pseudomonads isolated from the coastal waters of British Columbia. They comprise a family of cyclic depsipeptides with an alternating D and L amino acid sequence and a variable length (C10 to C12) fatty acid terminus. Ring closure of the depsipeptide is through an ester linkage between the hydroxyl of the third amino acid residue, D-allo threonine, and the carboxyl group an aliphatic amino acid, either L-valine, L-leucine, or L-isoleucine.

Viscosin (12) was first described in 1951 by Kochi et al.\textsuperscript{6} from Pseudomonas viscosa and a structure was later proposed in 1953 by Ohno et al.\textsuperscript{7} Synthesis of the proposed structure and comparison to authentic material later proved this proposal to be incorrect.\textsuperscript{8} A second proposal by Hiramoto et al.\textsuperscript{2} was confirmed by the total synthesis of seco-viscosin (the hydrolysis product of viscosin) and finally by solid phase synthesis of naturally occurring viscosin (12) in 1989.\textsuperscript{1} In 1991, Mortishire-Smith et al.\textsuperscript{4} reported the isolation, identification and three dimensional structure of “White Line Inducing Principle” (WLIP) from a species of Pseudomonas reactans. WLIP was shown to be identical in every respect to viscosin (12) except for the chirality of Leu5.

Massetolide A (4) differs from viscosin (12) by the replacement of the fourth amino acid in the sequence, D-valine in viscosin (12), by D-allo isoleucine in massetolide A (4). Massetolides B (5) and C (6) are simply the β-hydroxyundecanoic and β-hydroxydodecanoic acid versions of massetolide A (4). Similarly massetolides G (10) and H (11) are the β-hydroxy C11 and C12 fatty acid versions of viscosin (12). The L-isoleucine which forms the upsilon lactone linkage with D-allo threonine in massetolide A (4) and viscosin (12) is replaced by an L-leucine residue in
massetolides D (7) and massetolides F (9), respectively. Finally, massetolide E (8) has both D and L valine at positions four and nine in the peptide sequence.

The conformation of massetolide A (4) in solution is assumed to be similar to that of viscosin (12) since massetolide A (4) exhibits the same inter-residue NOEs as viscosin in solution. Mortishire-Smith et al. performed computational studies on the conformation of both viscosin and WLIP in solution and suggest that a type II β-turn may be formed by residues Val4 to Leu7 in viscosin (12). The stereochemistry of these residues (D L D L) removes the unfavorable 1,3 steric interactions between the carbonyl group of the second residue and the sidechain of the third residue in this type of turn. This unfavorable steric interaction is typically removed in proteins by the incorporation of a glycine in third position of the turn. They also showed that the lactone adopts a cis-conformation with the \textit{gamma}-CH$_3$ of the threonine residue being \textit{exo} to the peptide macrocycle.
3.3.1 Biological Significance

The discovery of this suite of compounds, along with known compound WLIP, suggests that these metabolites are biosynthesized by a non-ribosomal peptide synthetase similar to those that produce the tyrocidines (40 - 44) and loloatins (1 - 3) (see Section 2.3.1). Although this will not be discussed in great detail, an excellent review of the current state of research on peptide synthetases has been published by Kleinkauf et al.\textsuperscript{10} It is likely that the pseudomonad strains, MK90E85 and MK91CC8, both possess nearly identical peptide synthetase complexes. Although this is only speculation, since MK90E85 produces mainly the D-allo isoleucine analogues of the same metabolites produced by MK91CC8, it is not unlikely that MK90E85 is a valine mutant and is unable to biosynthesize valine. If unable to insert valine into the growing peptide chain, the enzyme complex responsible for the biosynthesis of viscosin (12) would substitute the amino acid which most resembles valine, namely isoleucine. Alternatively, it is possible that MK90E85 has a different synthetase than that in MK91CC8 which is selective for isoleucine instead of valine at the fourth amino acid position in the sequence. Either explanation would account for the suite of compounds we have observed in these two bacterial isolates.

Prior studies have shown that viscosin (12) is a powerful surfactant, possessing biological potency similar to industrially important surfactin (32).\textsuperscript{11} This is not unusual, considering both are peptidolipids which contain a fatty acid and several hydrophobic residues. These biosurfactants may play a role in the adhesion and/or detachment of bacteria to hydrophilic and/or hydrophobic substrates. Viscosin's biosurfactant property has also been implicated in facilitating the bacterial infection and the spread of decay on nonwounded broccoli florets.\textsuperscript{12}
Examination of the MICs for massetolide A (4) and viscosin (12) versus *Mycobacterium tuberculosis* (MTB) and *Mycobacterium avium intracellulare* (MAI) (Table 3.4) showed that variations in the nature of the aliphatic side chains of the component amino acids at the AA-4 and AA-9 positions resulted in a two fold increase in the potency of the compounds. There appears to be a general correlation between greater lipophilicity and increased potency in families of antimycobacterial compounds. This is consistent with the lower MIC's observed for massetolide A (4) versus viscosin (12) against MTB and MAI.

### 3.4 Experimental

NMR data were collected on a Bruker AMX500 spectrometer equipped with a 5 mm probe. Spectra of the massetolides were obtained in Me$_2$CO-$d_6$. Proton spectra were referenced using internal residual Me$_2$CO-$d_5$ ($\delta$ 2.04) and carbon spectra were referenced to the Me$_2$CO methyl carbon resonance ($\delta$ 29.8). FABMS data were collected on a Kratos Concept IIHQ hybrid mass spectrometer with cesium ion secondary ionization and a magnetic sector mass analyzer. Samples were dissolved in a MeOH-thioglycerol matrix and spectra were obtained using a source voltage of 8 kV and a cesium ion gun voltage of 12 kV. Fragment ion peaks were confirmed via secondary MS-MS using a quadrapole mass analyzer. Infra-red spectra were measured on a Perkin-Elmer 1600 FT-IR spectrophotometer using NaCl plates. Optical rotations were measured on a Jasco J-710 spectropolarimeter (1 cm quartz cell).

Normal and reversed-phase thin layer chromatography (TLC) was performed using Merck type 5554 aluminium-backed Kieselgel 60 F$_{254}$ and Whatman MKC18F plates, respectively. Visualization was with UV ($\lambda$=254 nm), vanillin/H$_2$SO$_4$/EtOH or ninhydrin spray reagents.
Further information on the preparation of vanillin, ninhydrin and other spray reagents can be found in Stahl.\textsuperscript{14} TLC plates of the massetolides were visualized by first spraying the plates with 5\% sodium hypochlorite solution, drying the plates in an oven at 60 °C, then visualizing the resulting chloramine derivative with a 4,4'-tetramethyldiamino-diphenylmethane / ninhydrin solution.\textsuperscript{15} Sephadex LH-20 (bead size 25-100 \( \mu \)) was used for size exclusion chromatography. Sigma type H TLC grade silica gel (10-40 \( \mu \), no binder, no fluorescent indicator) was used for preparative normal phase chromatography. Preparative reversed-phase chromatography was performed using reversed-phase silica prepared according to literature.\textsuperscript{16}

High performance liquid chromatography (HPLC) separations were done on one of two possible systems using a Whatman Partisil 10 ODS-3 Magnum column. The first system consisted of a Waters 501 HPLC pump equipped with a Perkin-Elmer LC-25 refractive index detector and a standard chart recorder. The second system consisted of a Waters 600E HPLC pump/system controller equipped with a Waters 410 differential refractometer detector interfaced with a personal computer using Millenium\textsuperscript{TM} 2010 chromatography software. Gas chromatography (GC) was performed on a Hewlett Packard 5880A Series GC with a flame ionization detector interfaced with a personal computer running Chrom Perfect\textsuperscript{TM} v5.05 software.

The solvents used for extraction and for column chromatographies were Fisher reagent grade. HPLC solvents were Fisher HPLC grade which were filtered and degassed prior to use. All other solvents, reagents and standards were reagent or commercial grade and were used without further purification.
3.4.1 Isolation

The marine isolate MK90E85 was obtained from the surface of an unidentified leafy red alga collected at -15 m in Masset Inlet, B.C. It was identified as a Pseudomonas sp. by fatty acid analysis (MIDI). MK90e85 has been cryopreserved and deposited in the marine microbial culture collections at SeaTek and UBC. Moderate scale cultures of MK90E85 were grown for four days at 16 °C as lawns on the surface of solid trypticase soy agar supplemented with NaCl to a final concentration of 1.5%. The cultures were harvested by gently scraping the cells from the surface of the agar. Lyophilization of the cells (41 g dry wt) followed by repeated extraction with MeOH gave a crude cell extract that was partitioned between EtOAc and 10% aqueous MeOH. The EtOAc soluble materials (1.2 g) were first fractionated via Sephadex LH-20 chromatography (MeOH), and subsequently by preparative RP chromatography (gradient CH$_3$CN/H$_2$O with 0.05% TFA). Repeated recycling on isocratic reversed-phase HPLC (7:3 CH$_3$CN/H$_2$O with 0.05% TFA) yielded pure massetolides A (4) (232 mg), B (5) (1 mg), C (6) (0.8 mg) and D (7) (1.9 mg).

The marine isolate MK91CC8 was obtained from an unidentified tube worm that was collected via SCUBA diving near Moira Island in Moira Sound, B.C. It was also identified as a Pseudomonas sp. by fatty acid analysis (MIDI). MK91CC8 has been cryopreserved and deposited in the marine microbial culture collections at SeaTek and UBC. Cultures of MK91CC8 were grown for four days at 16 °C as lawns on the surface of solid trypticase soy agar supplemented with NaCl to a final concentration of 1.5%. The cultures were harvested by gently scraping the cells from the surface of the agar and the agar was extracted by soaking in EtOAc, giving a crude extracellular extract (1.8 g from 20 trays). This extract was fractionated via
reversed-phase flash column chromatography (gradient CH$_3$CN/H$_2$O with 0.05% TFA) and isocratic reversed-phase HPLC (8/2 CH$_3$CN/H$_2$O with 0.05% TFA) to give pure massetolides E (8) (6 mg), F (9) (4 mg), G (10) (0.5 mg) and H (11) (0.5 mg) along with the known compound viscosin (12) (250 mg).

3.4.2 Total Acid Hydrolysis and GC Analysis

Massetolides A - H (4 - 11) and viscosin (12) were hydrolyzed and converted to the pentafluoropropionic amide isopropyl (PFPA-IPA) ester derivatives, and the GC retention times of the hydrolysate components were compared against the GC retention times of authentic standards on a chiral column (Table 3.2). Thus, the massetolide (0.5 mg) was dissolved in 6N HCl (1 mL) and heated at 110°C for a period of 3 days in a glass vial. The HCl was removed under a stream of N$_2$ gas. Isopropanol/HCl (250 μL) was added to the residue, the vial sealed and heated to 110°C for a further 45 minutes and then reduced to dryness using a stream of dry N$_2$. The residue was dissolved in CH$_2$Cl$_2$ (250 μL), pentafluoropropionyl anhydride (100 μL) was added, and the vial was sealed and heated to 110 °C for 15 minutes. Excess reagent was removed under a stream of dry N$_2$ and the derivatives were stored at -20 °C until ready for use. Racemic mixtures as well as optically pure L-amino acid standards were derivatized in a similar fashion. Standards of the hydroxy fatty acids were also prepared in this fashion using commercially available authentic β-hydroxydecanoic and β-hydroxydodecanoic acids. β-hydroxyundecanoic was prepared by Reformasky reaction of readily available trans-2-nonenal with ethyl bromoacetate according to literature. Subsequent hydrogenation with Pd (on C) yielded ethyl β-hydroxyundecanoate. The ethyl β-hydroxyundecanoate was purified by preparative NP column
chromatography (4:1 Hex/EtOAc), and the fatty acid ester was then subjected to the derivatization procedure.

The PFPA-IPA derivatized standards and the hydrolysates were analyzed on a 25 m Chiralsil-Val Heliflex column with FID detection using the following conditions: He carrier, detector temp 275 °C, injector temp 250 °C, injector split ratio 25:1, initial oven temp 90 °C, initial time 5 min, program rate 4 °C/min, final oven temp 200 °C, final time 27.5 min.

3.4.3 Compound Summaries

Massetolide A (4), N- [N- [N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxodecyl] -L-leucyl] -D-α-glutamyl] -D-allothreonyl] -D-alloisoleucyl] -L-leucyl] -D-seryl] -L-leucyl] -D-seryl] -L-isoleucine upsilon lactone: isolated as a white solid (232 mg); mp 237-238 °C (dec); IR (film) $\nu_{\text{max}}$ 3320 (br) 1640-1700 (several, s); $[\alpha]_D$ 45.9 (EtOH); NMR data see Table C.1; HRFABMS m/z (formula, $\Delta M$ ppm): 1140.71506 (M+H/C$_{55}$H$_{98}$N$_9$O$_{16}$, 1.67), 857.50061 (C$_{39}$H$_{69}$N$_9$O$_{13}$, 2.56), 728.45508 (C$_{34}$H$_{62}$N$_7$O$_{10}$, -1.01), 645.41899 (C$_{30}$H$_{57}$N$_6$O$_9$, 0.45), 609.38848 (C$_{31}$H$_{53}$N$_4$O$_8$, 3.51), 532.33710 (C$_{24}$H$_{46}$N$_5$O$_8$, 4.62), 496.30011 (C$_{23}$H$_{42}$N$_3$O$_7$, -4.36), 413.26492 (C$_{21}$H$_{37}$N$_2$O$_6$, -0.59), 284.22205 (C$_{16}$H$_{30}$NO$_3$, -1.84).

Massetolide A methyl ester (46): Massetolide A (4) (30 mg) was dissolved in 5 mL THF and reacted with diazomethane in a micro molar generator using a dry ice/Me$_2$CO bath to cool the THF solution. Massetolide A methyl ester (46) was purified using reversed phase ODS HPLC with 7:3 CH$_3$CN/H$_2$O as eluent to yield 30 mg of massetolide A methyl ester (46): isolated as a white solid; HRFABMS m/z (formula, $\Delta M$ ppm): 1154.72766 (M+H/C$_{56}$H$_{100}$N$_9$O$_{16}$, -0.99),
871.51271 (C_{46}H_{71}N_{6}O_{13}, -1.55), 728.45678 (C_{34}H_{62}N_{7}O_{10}, 1.32), 645.41887 (C_{26}H_{57}N_{6}O_{9}, 0.25),
623.39967 (C_{32}H_{55}N_{4}O_{8}, -3.72), 532.33559 (C_{24}H_{46}N_{3}O_{8}, 1.79), 510.31721 (C_{26}H_{44}N_{3}O_{7}, -1.40),
427.27895 (C_{22}H_{39}N_{2}O_{6}, -4.35), 284.22243 (C_{16}H_{30}NO_{3}, -0.50).

Massetolide B (5), N- [N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxoundecyl] -L-leucyl]
leucine uplson lactone: isolated as a white solid (1 mg); HRFABMS m/z (formula, ΔM ppm):
1154.73239 (M+H/C_{56}H_{100}N_{9}O_{16}, 3.11), 857.49931 (C_{39}H_{69}N_{8}O_{13}, 1.05), 728.45574
(C_{34}H_{62}N_{7}O_{10}, -0.10), 645.41762 (C_{30}H_{57}N_{6}O_{9}, -1.67), 623.39959 (C_{32}H_{55}N_{4}O_{8}, -3.85),
532.33390 (C_{24}H_{46}N_{3}O_{8}, -1.39), 510.31714 (C_{26}H_{44}N_{3}O_{7}, -1.55), 427.28130 (C_{22}H_{39}N_{2}O_{6}, 1.15),
298.23743 (C_{17}H_{32}NO_{3}, -2.66).

Massetolide C (6), N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxododecyl] -L-leucyl]
leucine uplson lactone: isolated as a white solid (0.8 mg); HRFABMS m/z (formula, ΔM ppm):
1168.74544 (M+H/C_{57}H_{102}N_{9}O_{16}, 0.84), 857.50031 (C_{39}H_{69}N_{8}O_{13}, 2.22), 728.45601
(C_{34}H_{62}N_{7}O_{10}, 0.26), 645.41920 (C_{30}H_{57}N_{6}O_{9}, 0.77), 637.41664 (C_{33}H_{57}N_{4}O_{8}, -1.58), 532.33621
(C_{24}H_{46}N_{3}O_{8}, 2.95), 524.33440 (C_{27}H_{46}N_{3}O_{7}, 1.56), 441.29674 (C_{23}H_{41}N_{2}O_{6}, 0.64), 312.25412
(C_{18}H_{34}NO_{3}, 0.81).

Massetolide D (7), N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxodecyl] -L-leucyl]-
leucine uplson lactone: isolated as a white solid (1.9 mg); HRFABMS m/z (formula, ΔM ppm):
1140.71379 (M+H/C_{55}H_{98}N_{9}O_{16}, 0.56), 857.49541 (C_{39}H_{69}N_{8}O_{13}, -3.50), 728.45430
(C_{34}H_{62}N_{7}O_{10}, -2.08), 645.41940 (C_{30}H_{37}N_{6}O_{9}, 1.09), 609.38486 (C_{31}H_{53}N_{4}O_{8}, -2.43), 532.3382 (C_{24}H_{46}N_{5}O_{8}, -1.54), 496.30480 (C_{23}H_{42}N_{7}O_{7}, 5.00), 413.26537 (C_{21}H_{37}N_{2}O_{6}, 0.50), 312.25412 (C_{16}H_{30}NO_{3}, -2.86).

Massetolide E (8), N- [N- [N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxodecyl] -L-leucyl] -D-α-glutamyl] -D-allothreonyl] -D-valyl] -L-leucyl] -D-seryl] -D-seryl] -L-valine \(\upsilon\) lactone: isolated as a white solid (6.0 mg); HRFABMS m/z (formula, ΔM ppm): 1112.68570 (M+H, C_{53}H_{94}N_{9}O_{16}, 3.46), 829.46409 (C_{37}H_{65}N_{9}O_{13}, -3.64), 700.42328 (C_{32}H_{58}N_{7}O_{16}, -1.76), 617.38734 (C_{28}H_{53}N_{6}O_{9}, -0.10), 595.36986 (C_{30}H_{51}N_{4}O_{8}, -1.40), 518.32089 (C_{23}H_{43}N_{5}O_{8}, 3.68), 496.30250 (C_{23}H_{42}N_{7}O_{7}, 0.46), 413.26607 (C_{21}H_{37}N_{2}O_{6}, 2.20), 284.22268 (C_{16}H_{30}NO_{3}, 0.37).

Massetolide F (9), N- [N- [N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxodecyl] -L-leucyl] -D-α-glutamyl] -D-allothreonyl] -D-valyl] -L-leucyl] -D-seryl] -D-seryl] -L-leucine \(\upsilon\) lactone: isolated as a white solid (4.0 mg); HRFABMS m/z (formula, ΔM ppm) 1126.69708 (M + H, C_{54}H_{96}N_{9}O_{16}, -0.37).

Massetolide G (10), N- [N- [N- [N- [N- [N- [N- [N- [(R)-3-hydroxy-1-oxoundecyl] -L-leucyl] -D-α-glutamyl] -D-allothreonyl] -D-valyl] -L-leucyl] -D-seryl] -D-seryl] -L-isoleucine \(\upsilon\) lactone: isolated as a white solid (0.5 mg); HRFABMS m/z (formula, ΔM ppm) 1140.71346 (M + H, C_{55}H_{98}N_{9}O_{16}, 0.26), 270.24298 (C_{16}H_{30}NO_{3}, -1.19).
Massetolide H (11), N-[N-[N-[N-[N-[N-[N-[N-[N-[(R)-3-hydroxy-1-oxodecyl]-L-leucyl]-D-α-glutamyl]-D-allothreonyl]-L-leucyl]-D-valyl]-L-leucyl]-D-seryl]-L-leucyl]-D-seryl]-D-isoleucine upsilon lactone: isolated as a white solid (0.5 mg); HRFABMS m/z (formula, ΔM ppm) 1154.72834 (M + H, C_{56}H_{100}N_{10}O_{16}, -0.40).

Viscosin (12), N-[N-[N-[N-[N-[N-[N-[N-[(R)-3-hydroxy-1-oxodecyl]-L-leucyl]-D-α-glutamyl]-D-allothreonyl]-D-valyl]-L-leucyl]-D-seryl]-L-leucyl]-D-seryl]-L-isoleucine upsilon lactone: isolated as a white solid (250 mg): mp 237-238 °C (dec); IR (film) \nu_{\text{max}} 3320 (br), 1640-1700 (several, s); HRFABMS m/z (formula, ΔM ppm): 1126.69695 (M+H, C_{54}H_{96}N_{9}O_{16}, -0.49), 843.48378 (C_{36}H_{67}N_{8}O_{13}, 1.21), 714.44249 (C_{33}H_{60}N_{9}O_{16}, 3.25), 631.40325 (C_{29}H_{55}N_{6}O_{9}, 0.31), 595.37154 (C_{30}H_{51}N_{4}O_{8}, 1.43), 496.30189 (C_{25}H_{42}N_{3}O_{7}, -0.79), 413.26502 (C_{21}H_{37}N_{2}O_{6}, -0.35), 284.22197 (C_{16}H_{30}NO_{3}, -2.12), 256.22745 (C_{15}H_{30}NO_{2}, -0.78).

(R,S) ethyl β-hydroxyundecanoate: isolated as a colourless oil (211 mg): IR (film) \nu_{\text{max}} 3453 (br), 2956, 2927 (s), 1737 (s); ^1H NMR (CDCl$_3$, 200 MHz) δ ppm: 4.11 (q, 1H, J=7.1), 3.96 (m, 1H), 2.93 (d, 1H, J=3.7), 2.41 (m, 2H), 1.4 (m, 2H), 1.25 (t, 3H, J=7.1), 1.25 (s, 12H), 0.96 (t, J=6.4); 13C NMR (CDCl$_3$, 50 MHz): δ ppm: 173.08 (C=O), 67.0 (CHOH), 60.61 (CH$_2$), 41.3 (CH$_2$), 36.51 (CH$_2$), 31.83 (CH$_2$), 29.50 (2 x CH$_2$), 29.20 (CH$_2$), 25.44 (CH$_2$), 22.63 (CH$_2$), 14.14 (CH$_3$), 14.05 (CH$_3$); HREIMS m/z (formula, ΔM ppm): 230.18820 (C_{13}H_{26}O_{3}, 0.7).
References


Chapter 4 : AI77 Type Compounds (13 - 15)

4.1 Introduction

During the course of this study several bacteria isolated from marine habitats were investigated which all produced the same biologically active metabolites. These isolates were identified as Bacillus spp. and extracts of their cultures exhibited significant antibiotic activity towards methicillin resistant Staphyloccocus aureus (MRSA). The major component isolated, AI77-B (13), which accounted for the MRSA activity, has previously been characterized in the literature.\(^1\) Another minor component present in the culture extracts, AI77-F (14) has also been formerly described.\(^1\) However, a previously undescribed minor constituent was also isolated from a culture of one of these marine isolates and the structure elucidation and stereochemical determination of this metabolite are presented herein. This metabolite proved to be the C9' epimer 15 of AI77-F (14), henceforth referred to as AI77-H (15). In addition, stable isotope incorporation experiments using \(^{13}\)C doubly labeled precursors were performed with one of the bacteria strains in order to determine the biogenetic origin of the atoms in this suite of related compounds.
4.2 Results and Discussion

Marine isolates, MK90A309, MK90X11, and MK90X55 were isolated from sediment samples taken in various locations throughout the coastal waters of B.C. These isolates were shown to be *Bacillus pumilus* spp. by MIDI fatty acid analysis. Further, the crude extracts of both the agar and liquid cultures of these isolates exhibited antibiotic activity towards MRSA. Thus, each of these cultures was grown in moderate scale liquid culture in order to provide sufficient quantities of the secondary metabolites for further investigation.

4.2.1 AI77-B (13) and AI77-F (14)

For clarity of this discussion, only one bacterial isolate, MK90A309, will be discussed further. The other isolates, MK90X11 and MK90X55, were treated in similar manners and yielded the same secondary metabolites. Isolate MK90A309 was grown in liquid shake culture at room temperature with tryptic soy broth medium containing 1% NaCl. Solid phase extraction of the liquid culture using XAD-4 (a non-ionic polymeric absorbent) followed by elution of these organics with methanol gave a crude extract exhibiting biological activity. This extract was then fractionated by bioassay guided fractionation yielding AI77-B (13), as well as two structurally similar but much less active components, AI77-F (14) and AI77-H (15).
Analysis of the MS and NMR data, followed by comparison with literature values identified the major secondary metabolite isolated as AI77-B (13) (Figure 4.1).\textsuperscript{1} AI77-B (13) showed antibacterial activity against \textit{Staphylococcus saprophyticus} and MRSA at concentrations of less than 20 µg/disc (paper disc diffusion assay), and appeared to account for most of the activity of the extract. AI77-B (13) also exhibited cytotoxicity against the murine P388 leukemia cell line with an ED\textsubscript{50} of 2.6 µg/mL. AI77-F (14) was isolated as a second minor metabolite (Figure 4.2). It too was identified by comparison of its NMR and MS data with literature values.\textsuperscript{1} AI77-F (14), exhibited significantly weaker activity against MRSA and \textit{S. saprophyticus} with effective concentrations of greater than 100 µg/disc (paper disc diffusion assay).

AI77-B (13) and AI77-F (14) were first isolated and identified by Shimojima \textit{et al.} from a terrestrial species of \textit{Bacillus pumilus} as Gram-positive antibiotics with unusual gastoprotective properties.\textsuperscript{1b} Another marine isolate, \textit{Bacillus pumilus} MK91Z50, investigated by Dr. Judy Needham of this research group, was shown to produce AI77 type compounds.\textsuperscript{2} This family of secondary metabolites has also been reported by a Russian research group from a marine \textit{B. pumilus} sp. cultured from a \textit{Dendrilla sp.} sponge.\textsuperscript{3} Structure activity relationship studies by Shimojima and Hayashi suggest that the amino acid moiety of AI77-B (13) significantly influences the activity, and the amino functionality is required for biological activity.\textsuperscript{1e} The relative stereochemistry of AI77-B (13) was determined by Shimojima \textit{et al.} through X-ray analysis, and the absolute stereochemistry identified by chemical degradation of 13 to L-leucine. Chemical interconversion of 13 to AI77-F (14), by formation of the γ-lactone followed by Hoffman elimination of the amino group, established the absolute stereochemistry of 14.\textsuperscript{1b}
Figure 4.1: 400 MHz $^1H$ NMR Spectrum of AI77-B (13) in DMSO-$d_6$
Figure 4.2: 400 MHz $^1$H NMR Spectrum of Al77-F (14) in CDCl$_3$
4.2.2 AI77-H (15)

A second minor metabolite 15 was also isolated by bioassay guided fractionation of the organic extracts of MK90A309. Isolated as a white amorphous solid, this compound gave a molecular ion in the HREIMS at m/z 389.14746 appropriate for a molecular formula of C_{20}H_{23}NO_7 (ΔM 2.1 ppm) identical to that of AI77-F (14). Comparison of the \(^1\)H (Figure 4.3) and \(^{13}\)C (Figure 4.4) NMR data obtained for 15 with the NMR data obtained for AI77-F (14) suggested they were diastereomers (Table 4.1).

Analysis of the COSY, HMQC, and HMBC data (Figures 4.5, 4.6, and 4.7) verified that 15 was a diastereomer of AI77-F (14). Further, the \(^1\)H and \(^{13}\)C NMR data for AI77-F (14) and AI77-H (15) differed only in the resonances assigned to the butenolide moiety of the molecule. Since the chemical shifts and coupling constants between 14 and 15 in the dihydroisocoumarin portion of the molecule were nearly identical, they were assumed to have the same relative stereochemistry in that moiety. In addition, AI77-F (14) and AI77-H (15) were assumed to have the same absolute configuration as AI77-B (13) in that portion of the molecule. However, in the butenolide portion of the metabolite the chemical shifts and \(^1\)H coupling constants at C8' and C9' differed significantly for the two diastereomers 14 and 15. Detailed analysis of the \(^1\)H-\(^1\)H coupling constants for C8' (15: δ 4.30, d, \(J_{H8'-H9'}=5.3\), 14: δ 4.54, d, \(J_{H8'-H9'}=4.6\)) suggested that 14 and 15 differed at either the C8' or C9' positions but not both. If both C8' and C9' had inverted stereochemistry at these centers in 15, these resonances would be expected to display the same coupling constant.
Table 4.1: \(^1\)H and \(^{13}\)C NMR Data for AI77-F (14) and its Diastereomer AI77-H (15) (CDCl\(_3\))

<table>
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<th>Atom #</th>
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<th>(^1)H (ppm)</th>
<th>(^{13})C (ppm)</th>
<th>(^1)H (ppm)</th>
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<th>HMBC</th>
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\* Acquired at 125 MHz.
\(b\) Acquired at 400 MHz.
\(c\) Acquired at 500 MHz.
Figure 4.3: 400 MHz $^1$H NMR Spectrum of Al77-H (15) in CDCl$_3$
Figure 4.4: 125 MHz $^{13}$C NMR Spectrum of Al77-H (15) in CDCl$_3$
Figure 4.5: 400 MHz COSY Spectrum of AI77-H (15) in CDCl$_3$
Figure 4.6: 500 MHz HMQC Spectrum of Al77-H (15) in CDCl₃
Figure 4.7: 500 MHz HMBC Spectrum of Al77-H (15) in CDCl$_3$
Mosher’s method using (S)- and (R)- (α-methoxy)(α-trifluormethyl)phenylacetic (MTPA) ester derivatives for determining the absolute configuration of secondary alcohols was used to determine the absolute stereochemistry of 15. A previous attempt at preparing the S-MTPA ester of AI77-H (15) directly using MTPA-Cl in CDCl₃ with triethylamine and DMAP proved unsuccessful and resulted in an apparent base promoted dehydration/rearrangement of the butenolide portion of the molecule to a substituted pyran-2-one. It was therefore evident that Mosher’s method could not be used on 15 directly. Thus, the unstable butenolide in 15 was hydrogenated and the product converted to its phenylmethoxy ether using MeI and K₂CO₃ in Me₂CO. This derivative was then converted to the (S)- and (R)- MTPA esters (47 and 48 respectively) according to literature procedures.
The chemical shifts of the protons for both the (R) - and (S) MTPA esters of the derivative were determined by analysis of the $^1$H and COSY data (Table 4.2). Computer simulations using the MacroModel molecular modeling program and MM3$^*$ force-field calculations$^4$ of the (R)- and (S) -MTPA esters indicated that there were no steric impediments to the MTPA ester carbonyl group adopting a synperiplanar conformation with the carbinol methine proton (H8'), as shown in Figure 4.8.

Figure 4.8 : Molecular Model of One of the Lowest Energy Conformations of 48
As shown in Figure 4.9 the $\Delta \delta$ values ($\Delta \delta = \delta_S - \delta_R$) for the derivative are positive on one side of the MTPA plane and negative on the other side. Notable exceptions were the 6'NH exchangeable amide resonance and H9' which should not be affected by the diamagnetic influence of the phenyl ring in the MTPA moiety. Thus, in accordance with the empirical rule established by Ohtani et al., the configuration at C8' in 15 was determined to be (S), which is the same configuration found in AI77-F (14). Therefore, AI77- H (15) must, a priori, be a C9' epimer of AI77-F (14).

Figure 4.9: $\Delta \delta$ (ppm) Values for the Mosher Ester Derivatives of Dihydro AI77- H (15)
Table 4.2: \(^1\)H NMR Data for S- and R-MTPA Esters of AI77-H Dihydro Derivative (47 & 48) (CDCl\(_3\))

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<tr>
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<td>1'</td>
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<td>~1.58 (1H, m)</td>
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<td>10'b</td>
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</tr>
<tr>
<td>11'a</td>
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<td>2.35</td>
<td>*</td>
</tr>
<tr>
<td>11'b</td>
<td>not obs.</td>
<td>not obs.</td>
<td>*</td>
</tr>
<tr>
<td>12'</td>
<td>MTPA OMe</td>
<td>3.513 (3H, s)</td>
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</table>

* Acquired at 400 MHz.
* Unable to determine these chemical shifts accurately.
Figure 4.10: 400 MHz $^1$H NMR Spectrum of S-MTPA Ester of AI77-H Dihydro Derivative (47) in CDCl$_3$
Figure 4.11: 400 MHz $^1$H NMR Spectrum of R-MTPA Ester of AI77-H Dihydro Derivative (48) in CDCl$_3$
4.2.3 Biosynthesis

Shimojima et al were the first to put forth a proposal for the biogenesis of the dihydroisocoumarin portion of the AI77 type compounds. They proposed that this portion of the molecule is of mixed polyketide/amino acid origin with four molecules of acetate and one molecule of L-leucine as a starter unit in a polyketide type pathway. However there has been no formal speculation on the biogenetic origins of the six carbon dicarboxylate portion of the molecule. This polyfunctionalized unit may be derived from a sugar. However, we hypothesize that this six carbon dicarboxylate portion is derived from L-aspartic acid homologated with one unit of acetate.

Precedence for amino acid homologation is found in the biosynthesis of (3S,4R)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA). In AHMHA an L-leucine residue is homologated by an acetate unit. This unusual amino acid is a constituent of both pepstatin A (51) and aphatinin E (52).
Another example of an acetate homologated amino acid is also found in aphatinin E (52), with the homologation of phenylalanine by acetate to 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA).\(^7\)

A third example of amino acid homologation can be found in the biosynthesis of the acylsuccinimide portion of andrimid (38). This acylsuccinimide moiety was shown to be derived from valine, glycine and acetate building blocks.\(^8\)
Thus, in order to investigate the biogenetic origins of the atoms in AI77-B (13) stable isotope incorporation experiments were performed. In biosynthetic experiments the use of stable isotopes has superseded the use of radioactively labeled isotopes, since stable isotopes provide more detailed biosynthetic information, illustrating the fates of C-H, C-C and C-O or C-N bonds. Doubly labeled $^{13}$C precursors have additional benefits to singly labeled precursors, since incorporation of intact doubly labeled precursors results in doublets flanking the natural abundance peaks in the $^{13}$C NMR spectrum of the secondary metabolite of interest. This is particularly important when specific incorporations are low. Variations in peak height for a selected carbon can vary from experiment to experiment due to uncontrollable factors, which can make it statistically impossible to determine singly labeled precursor incorporations of less than 0.2 % specific incorporation. Hence, two biosynthetic feeding experiments were performed with bacterial isolate MK90A309, the first using doubly labeled [1,2-$^{13}$C$_2$]acetate, the second experiment using doubly labeled [3,4-$^{13}$C$_2$]aspartic acid.

[3,4-$^{13}$C$_2$]aspartic acid was synthesized from [1,2-$^{13}$C$_2$]ethyl bromoacetate by a modified Gabriel-malonic ester synthesis using commercially available diethyl phthalimidomalonate. Standard conditions for this reaction involve heating the sodium or potassium salt of diethyl phthalimidomalonate in a large excess of ethyl bromoacetate which plays a dual role as both reagent and solvent, yielding a symmetrical imido adduct which is then decarboxylated under acidic conditions to yield aspartic acid (45% from diethyl phthalimidomalonate). Since $^{13}$C doubly labeled ethyl bromoacetate is expensive, the reaction conditions were modified and the reaction was performed in ethanol as solvent with less than one equivalent of [1,2-$^{13}$C$_2$]ethyl bromoacetate. However, the use of ethanol led to the solvolysis of the phthalimido ring giving a mixed phthalate
ester amide, which under acidic conditions still decarboxylated to aspartic acid (46% yield from [1,2-$^{13}$C$_2$]ethyl bromoacetate).

![Chemical diagram](image)

Figure 4.13: Synthetic Scheme for D,L-[3,4-$^{13}$C$_2$]Aspartic Acid

In two separate feeding experiments, one using [1,2-$^{13}$C$_2$]acetate (0.5g), the second using [3,4-$^{13}$C$_2$]aspartate (0.36 g), the labeled precursors were dissolved in water and filter sterilized before being added to the culture medium. Cultures were fed the labeled precursors immediately upon entering stationary phase, followed by a second feeding 24 hrs after that. The cultures were harvested by centrifugation after 96 hrs and AI77-B (13) was isolated and purified as described previously. In the $^{13}$C spectrum of AI77-B (13), the C8' and C9' carbons are chemical shift coincident (δ 71.4 and 71.5 ppm) making incorporation analysis difficult. This problem was easily remedied by conversion of 13 to its triacetate derivative (49) where C8' resonates at δ 73.4 ppm and C9' resonates at δ 83.4 ppm. In addition, the C10' and C11' resonances are much sharper in the $^{13}$C spectrum of the triacetate derivative 49 as opposed to the broad signals observed in 13.
The $^{13}$C spectrum from the doubly labeled acetate feeding experiment is shown in Figure 4.16. The carbon resonances in the AI77-B triacetate (49) carbon skeleton from the [1,2-$^{13}$C$_2$]acetate feeding experiment have all been normalized to the same peak height for the central singlet component, and plotted with a 1 ppm width in Figure 4.18. The resonances belonging to C1, C9, C8, C7, C6, C5, C10, C4, C7' and C8' all clearly show the natural abundance peak flanked by clear doublets resulting from specific incorporation of intact acetate units. As expected the $^{13}$C NMR signals for C3, C1', C2', C3', C4', C5', C9', C10', C11', and C12' as well as the $^{13}$C resonances due to the three acyl groups showed no apparent doublet flanking the natural abundance signal. Table 4.3 lists the carbon resonances and the % specific incorporation calculated for each peak, as well as the coupling constants (in Hz) for the doublet flanking the natural abundance signal. Numbers listed for specific incorporation = % enrichments above natural abundance = 1.1 $\times$ (combined integrated peak area of enriched satellites minus the combined theoretical peak area of the satellites due to natural abundance coupling)/(peak area of the natural abundance singlet plus the combined theoretical peak area of the satellites due to natural abundance coupling). Since the probability of observing natural abundance coupling between a pair of adjacent $^{13}$Cs is (1.1%)$^2$ = 0.000121, the combined area of the doublet components should be (0.000121/(0.011 - n x 0.000121)) x 100% = 1.1% of the intensity of the unenriched central singlet for each coupling interaction (n is the number of attached carbons and must have a value between 1 and 4 : therefore 0.011 - n x 0.000121 $\approx$ 0.011). Analysis of the coupling constants observed for the doublet components in the AI77 skeleton (Figure 4.18) revealed the pattern of intact acetate incorporation proposed in Figure 4.12. The intensity of the doublet components in the dihydroisocoumarin portion of 49 indicated an average incorporation of 1.34 % for the C1-C9, C8-C7, C6-C5 and C4-C10 acetate units. However, the C7'-C8' incorporation was significantly lower at 0.30%. This lower incorporation may suggest that the
biosynthesis of the six carbon dicarboxylate moiety of the molecule does not occur concurrently with the biosynthesis of the dihydroisocoumarin portion of AI77-B (13). Alternatively, this lower incorporation may reflect the possibility that acetate is first oxidized to glycolate (HOCH$_2$CO$_2$) before incorporation into the six carbon dicarboxylate unit.

The $^{13}$C spectrum from the [3,4-$^{13}$C$_2$]aspartate feeding experiment is shown in Figure 4.17. The carbon resonances in the AI77-B triacetate (49) carbon skeleton from this aspartate feeding experiment have all been normalized to the same peak height for the central singlet component, and all the resulting 1 ppm wide plots truncated to 20% of the normalized peak height (Figure 4.19). The C11' and C12' resonances clearly show incorporation of an intact $^{13}$C-$^{13}$C bond from labeled [3,4-$^{13}$C$_2$]aspartate, as proposed in Figure 4.14. Surprisingly, the resonances belonging to C1, C9, C8, C7, C6, C5, C10, C4, C7' and C8' all appear to show the natural abundance peak flanked by doublets resulting from specific incorporation of intact $^{13}$C-$^{13}$C bonds as well. This incorporation pattern suggests that [1,2-$^{13}$C$_2$]aspartate is metabolized to [1,2-$^{13}$C$_2$]acetate in vitro.

As expected the $^{13}$C NMR signals for C3, C1', C2', C3', C5', C9', C10', C11', and C12' as well as the $^{13}$C resonances due to the three acyl groups showed no apparent doublet flanking the natural abundance signal (C4' appears at first glance to be flanked by a doublet, but careful analysis suggested that this was simply due to an impurity present in the sample). Table 4.3 lists
the carbon resonances and the % specific incorporation calculated for each peak, as well as the
coupling constants (in Hz) for the doublet flanking the natural abundance signal. The intensity of
the doublet components in the labeled spectrum of 49 indicated an average incorporation of
0.21% for an intact [3,4-\(^{13}\)C\(_2\)]aspartate unit. A somewhat lower incorporation, 0.12 %, was
observed for the C1-C9, C8-C7, C6-C5, C4-C10 and C7'-C8' bonds arising from incorporation
of [1,2-\(^{13}\)C\(_2\)]acetate units derived from [3,4-\(^{13}\)C\(_2\)]aspartate.

Therefore, it would appear that the AI77 type compounds arise from a mixed biosynthetic
pathway which homologates L-aspartate with acetate and links this homologated amino acid with
L-leucine. L-leucine is homologated with four units of acetate to a pentaketide with L-leucine
replacing the first acetate unit in a typical pentaketide. A proposed biosynthetic pathway is
depicted in Figure 4.14, this system could be envisioned as a combination of the non-ribosomal
peptide biosynthetic pathway (see Section 2.3.1) and a polyketide type biosynthetic pathway.
However, it must be noted that no speculation is made on the actual timing of leucine
homologation, or on the timing of oxidation at C9' in the homologated aspartate unit. Leucine
may be modified before or after condensation with the homologated aspartate unit. Moreover,
oxidation at C9' may occur by oxidation of acetate to glycolate,\(^{10}\) or oxidation at this center may
take place later on in the biosynthesis.
Figure 4.14: Proposed Biosynthesis of AI77-B (13)
**Table 4.3: Specific Incorporation in N,O,O-triacetyl AI77-B (49) for [1,2-\(^{13}\)C\(_2\)]Acetate and [3,4-\(^{13}\)C\(_2\)]Aspartate Feeding Experiments**

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<th>([1,2-^{13}C_2]) Acetate feeding experiment</th>
<th>([3,4-^{13}C_2]) Aspartate feeding experiment</th>
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* These doublets were obscured by minor impurities
* acquired at 100 MHz in CDCl\(_3\)*

**N,O,O-triacetyl AI77-B (49)**
Figure 4.14: 400 MHz $^1$H NMR Spectrum of N,O,O-triacetyl AI77-B (49) in CDCl$_3$
Figure 4.15: 100 MHz $^{13}$C NMR Spectrum of N,O,O-triacetyl AI77-B (49) from [1,2-$^{13}$C$_2$]Acetate Feeding Experiment (in CDCl$_3$)
Figure 4.16: 100 MHz $^{13}$C NMR Spectrum of N,O,O-triacetyl AI77-B (49) from [3,4-$^{13}$C$_2$]Aspartate Feeding Experiment (in CDCl$_3$)
Figure 4.18: Normalized $^{13}$C Resonances for $[1,2-^{13}$C$_{2}]$Acetate Feeding Experiment of AI77-B triacetate (49) [100 MHz, CDCl$_3$, $\delta$ (ppm) - 1 ppm width]
Figure 4.19: Normalized and Truncated $^{13}$C Resonances for [3,4-$^{13}$C$_2$]Aspartate Feeding Experiment of AI77-B triacetate (49) [100 MHz, CDCl$_3$, $\delta$ (ppm) - 1 ppm width]
4.3 Experimental

NMR data were collected on either a Bruker AMX500, WH400, AM400 or AM200 spectrometer each equipped with 5 mm probes. Proton spectra were referenced either using internal residual mono-hydrogenated solvent resonances (CHCl₃ δ 7.24 or DMSO-d₆ δ 2.49 ppm), or in the case of D₂O, an external sample containing DSS (δ 4.63 ppm). Carbon spectra were referenced to either the internal solvent resonance (CDCl₃ - δ 77.0 or DMSO methyl carbon - δ 39.5 ppm), or in the case of D₂O, an external sample containing MeOH (δ 49.0 ppm). Low and High resolution EI mass spectra were recorded on Kratos AEI MS-59 and AEI MS-50 mass spectrometers respectively; low and high resolution CI mass spectra were collected on a Delsi-Nermag R10-10B DCI mass spectrometer with isobutane as ionization gas; and high resolution FABMS data were collected on a Kratos Concept IIHQ hybrid mass spectrometer with cesium ion secondary ionization and a magnetic sector mass analyzer. Circular dichroism (CD) spectra were obtained using a Jasco J-710 spectropolarimeter. Infra-red (IR) spectra were measured on a Galaxy series 3000 spectrophotometer using PTFE film plates.

Normal and reversed-phase thin layer chromatography (TLC) was performed using Merck type 5554 aluminium-backed Kieselgel 60 F₂₅₄ and Whatman MKC18F plates, respectively. Visualization was with UV (λ=254 nm), FeCl₃/HCl or ninhydrin spray reagents. Solid phase extractions were performed using XAD-4 non-ionic polymeric resin (20-60 mesh). Sephadex LH-20 (bead size 25-100 μ) was used for size exclusion chromatography. Sigma type H TLC grade silica gel (10-40 μ, no binder, no fluorescent indicator) was used for preparative normal phase chromatography. Preparative reversed-phase chromatography was performed using a reversed-phase 10g Waters Sep Pak.
High performance liquid chromatography (HPLC) separations were done on one of two possible systems using either a Whatman Partisil 10 ODS-3 or a Whatman Partisil 10μ Magnum column for reversed and normal phase separations respectively. The first system consisted of a Waters 501 HPLC pump equipped with a Perkin-Elmer LC-25 refractive index detector, a λ_{254} UV detector and a standard chart recorder. The second system consisted of Waters 600E HPLC pump/system controller with a Waters 486 tunable absorbance detector and a standard chart recorder.

The solvents used for extraction and for column chromatographies were Fisher reagent grade. HPLC solvents were Fisher HPLC grade which were filtered and degassed prior to use. [1,2-^{13}C_2] sodium acetate, and [1,2-^{13}C_2] ethyl bromoacetate were purchase from Cambridge Isotope Laboratories. All other solvents, reagents and standards were reagent or commercial grade and were used without further purification.

4.3.1 Isolation

The marine isolates MK90A309, MK90X11, MK90X55, and MK91Z50 were all isolated from sediment samples taken in various locations throughout the coastal waters of B.C., and they were identified as a *Bacillus pumilus* spp. by MIDI fatty acid analysis. All four strains have been cryopreserved and deposited in the marine microbial culture collections at SeaTek and UBC. Further discussion is limited to marine isolate MK90A309. Moderate scale cultures of MK90A309 were grown in either 7L fermentors at 16 °C or as liquid shake cultures at 16 °C (8 x
400 mL - 1L flasks) using trypticase soy supplemented with NaCl to a final concentration of 1.0%. The cultures were harvested after four days incubation by centrifugation.

The supernatant obtained after centrifugation was passed over XAD-4 to extract organic materials present, and the organics were then eluted with MeOH. The crude methanolic extract was then subjected to a modified Kupchan partitioning scheme. The crude MeOH extract was first extracted with hexanes, the MeOH layer was then diluted partially with H₂O (9:4 MeOH/H₂O), extracted with CHCl₃, and ultimately the aqueous fraction was reduced in vacuo to remove MeOH, and partitioned with EtOAc. The final aqueous fraction which exhibited antibiotic activity towards MRSA was then fractionated on a 10g reversed phase Sep Pak (gradient 100% H₂O - 100% MeOH.) The 60% to 100% MeOH fractions exhibiting MRSA activity were then combined and subjected to size exclusion chromatography on LH20 (MeOH). The fractions obtained were pooled according to their RP-TLC characteristics yielding semi pure AI77-B (13) (≈40 mg from a 3.2 L shaker flask culture). This sample was then further purified on reversed phase HPLC (6:4 MeOH/H₂O 50 mM KH₂PO₄ buffer pH 7), to yield AI77-B (13) (≈30 mg).

The EtOAc fraction from the Kupchan partitioning also exhibited slight MRSA activity, and was further purified by column chromatography on Sephadex LH-20 using a partitioning solvent system (8:2:1 EtOAc/MeOH/H₂O). This was then followed by preparative normal phase TLC (3:1 EtOAc/hexanes) yielding two interesting fractions by ¹H NMR, AI77-F (14) and AI77-H (15) (≈1.5 mg apiece from a 7L fermentation culture).
4.3.2 R- and S- MTPA Ester Derivatives of Dihydro AI77-H (15)

The R- and S- MTPA derivatives of dihydro AI77-H (15) were prepared by hydrogenation of 3 mg of 15 under an atm of H$_2$ and a small amount of Pd(C)5% in 5 mL EtOH for 16 hr ($\approx$10 mg). The reaction mixture was filtered, reduced in vacuo, and the residue heated at reflux with 1 mL CH$_3$I, 5 mL Me$_2$CO, and 3 mg K$_2$CO$_3$ for 24 hrs. The reaction mixture was reduced in vacuo, and the residue extracted with CH$_2$Cl$_2$. The crude product was then divided into two portions and each half was then esterified by heating at reflux for 24 hrs, 10 µL of either S- or R- (α-methoxy)(α-trifluoromethyl)phenylacetyl chloride, 10 µL Et$_3$N, 1 crystal of DMAP and 100 µL CHCl$_3$. The product thus obtained (47 or 48) was ultimately purified by preparative NP TLC (100% EtOAc).

4.3.3 Stable Isotope Feeding Experiments

A 7L fermentation culture of MK90A309 was fed in two portions 0.5 g [1,2-$^{13}$C$_2$] sodium acetate (in 2 x 5 mL portions of H$_2$O, filter sterilized) at 32 and 56 hrs, the culture was then harvested after the usual 96 hrs and AI77-B (13) isolated in the manner previously described (yield 212 mg). After the final RP HPLC purification of AI77-B (13), 50 mg of the product was acetylated with 2 mL pyridine and 1 mL Ac$_2$O for 16 hrs. The triacetate (49), obtained in nearly quantitative yield, was purified on NP HPLC (EtOAc) to yield N,O,O triacetyl AI77-B (49 -45 mg after HPLC purification).

[3,4-$^{13}$C$_2$]aspartate was prepared by a modification of the Gabriel-malonic ester synthesis of aspartic acid. Equimolar amounts of diethyl pthalimidomalonate (2.25 g, 7.4 mmol) and NaOEt (21% wt solution in EtOH - 0.402 g, 7.4 mmol) were stirred in 3 mL absolute EtOH. The
yellow solid that precipitated was then heated at reflux with 0.8 eq of [1,2-¹³C₂] ethyl bromoacetate (1.0 g, 5.9 mmol). After 24 hrs the mixture was diluted in 50 mL H₂O and extracted with three 50 mL portions of Et₂O. The ether extracts were combined and reduced in vacuo and purified by normal phase column chromatography (3:1 hexanes/EtOAc) to give 2.6 g 50. Intermediate 50 was then heated at reflux with 2 mL each of conc. HCl, glacial AcOH, and H₂O. After 8 hrs the reaction mixture was diluted with 100 mL H₂O, extracted with three 50 mL portions of EtOAc, and the aqueous fraction reduced in vacuo to yield 0.523 g of crude [3,4-¹³C₂]aspartic acid, which migrated with authentic aspartic acid on NP TLC (20:2:1 nBuOH/AcOH/H₂O). This crude product was further purified on Dowex 50W-X8 H⁺ cation exchange resin (50 -100 mesh) eluting with a H₂O-1M pyridine gradient. The ninhydrin positive fractions were pooled, and reduced in vacuo, to yield [3,4-¹³C₂]aspartic acid as a white solid (370 mg - 46% yield from [1,2-¹³C₂]ethyl bromoacetate).

A 3.2 L shaker flask culture of MK90A309 was fed in two portions 0.36 g [3,4-¹³C₂] aspartic acid (in 2 x 10 mL portions of H₂O, filter sterilized) at 32 and 56 hrs, the culture was then harvested after the usual 96 hrs and AI77-B (13) isolated in the manner previously described (yield 30 mg). After the final RP HPLC purification of AI77-B (13) the triacetate (49) was prepared and purified as described previously (26 mg after HPLC purification).

4.3.4 Compound Summaries

AI77-B (13): isolated as a white solid; mp 137-138 °C; IR (thin film on PTFE membrane) νmax:
3250 (br,s), 1668 (three ,s); CD (MeOH) θ₂₂₀ (e=8700), θ₂₂₈ (e=3600), θ₂₄₂ (e=7400), θ₂₅₉ (e=-7300); ¹H NMR (DMSO-d₆, 400 MHz): 7.86 (d, 1H, J=9.4), 7.46 (dd, 1H, J=8.3, 7.5), 6.84 (d,
1H, J=6.8), 6.80 (d, 1H, J=7.5), 4.68 (dm, 1H, J=12.5), 4.19 (m, 1H), 3.93 (d, 1H, J=7.3), 3.66 (m, 1H), 3.26 (m, 1H), 3.04 (dd, H, J=16.3, 12.5), 2.88 (dd, 1H, J=16.3, 2.4), 2.29 (dd, 1H, J=16.3, 3.5), 2.14 (dd, 1H, J=16.3, 9.6), 1.66 (m, 2H), 1.33 (m, 2H), 0.89 (d, 3H, J=6.3), 0.85 (d, 3H, J=6.3); $^{13}$C NMR δ ppm (DMSO- $d_6$, 100 MHz): 174.3 (C=O), 172.8 (C=O), 169.0 (C=O), 160.9 (CH), 140.7 (C), 136.3 (CH), 118.5 (CH), 115.2 (CH), 108.3 (C), 81.0 (CH), 71.9 (CH), 71.6 (CH), 50.3 (CH$_2$), 48.0 (CH), 39.0 (CH), 33.6 (CH$_2$), 29.0 (CH$_2$), 24.0 (CH$_2$), 23.3 (CH$_3$), 21.5 (CH$_3$); HRFABMS m/z (formula, ΔM ppm): 425.19245 (M+H/C$_{20}$H$_{29}$N$_2$O$_8$, 0.14).

AI77-F (14)$^1$: isolated as a white amorphous solid; IR (thin film on PTFE membrane) v$_{max}$ 3405 (br), 1792 (s), 1761 (s), 1678 (s), 1524 (s); UV (MeOH) λ$_{max}$ (ε): 245 nm (8800), 315 (5600); CD (MeOH) θ$_{220}$ (ε=-2900), θ$_{228}$ (ε=-8300), θ$_{242}$ (ε=7400), θ$_{259}$ (ε=-10 000); $^1$H and $^{13}$C NMR see Table 4.1; HREIMS m/z (formula, ΔM ppm): 389.14804 (C$_{20}$H$_{23}$NO$_7$, -1.5).

AI77-H (15): isolated as a white amorphous solid; IR (thin film on PTFE membrane) v$_{max}$: 3406 (br), 1790 (s), 1763 (s), 1673 (s), 1525 (s); UV (MeOH) λ$_{max}$ (ε): 244 nm (9000), 315 (5800); CD (MeOH) θ$_{214}$ (ε=52 000), θ$_{239}$ (ε=12 000), θ$_{259}$ (ε=-6700); $^1$H and $^{13}$C NMR see Table 4.1; HREIMS m/z (formula, ΔM ppm): 389.14746 (C$_{20}$H$_{23}$NO$_7$, 2.1).

(S)-(α methoxy)(α-trifluoromethyl)phenylacetyl dihydro AI77-H derivative (47): $^1$H NMR data see Table 4.2; HRFABMS m/z (formula, ΔM ppm): 622.22697 (M+H/C$_{31}$H$_{35}$NO$_9$F$_3$, 0.93).

(R)-(α methoxy)(α-trifluoromethyl)phenylacetyl dihydro AI77-H derivative (48): $^1$H NMR data see Table 4.2; HRFABMS m/z (formula, ΔM ppm): 622.22608 (M+H/C$_{31}$H$_{35}$NO$_9$F$_3$, -0.50).
N,O,O-triacetyl AI77-B \(\gamma\)-lactone (49): isolated as a white solid; IR (thin film on PTFE membrane) \(v_{\text{max}}\): 3540 (m), 1771 (three s), 1757(s), 1730(s); \(^1\)H NMR (CDCl\(_3\), 400 MHz): 7.51 (dd, 1H, \(J=7.9, 7.7\)), 7.23 (d, 1H, \(J=7.3\)), 7.18 (d, 1H, \(J=9.5\)), 7.15 (d, 1H, \(J=7.7\)), 7.00 (d, 1H, \(J=7.9\)), 5.35, (d, 1H, \(J=2.3\)), 4.71 (dd, 1H, \(J=2.3, 2.7\)), 4.66 (m, 1H), 4.44 (dt, 1H, \(J=11.6, 3.2\)), 4.28 (m, 1H), 3.66 (m, 1H), 3.26 (m, 1H), 2.99 (dd, H, \(J=16.3, 12.0\)), 2.95 (d, 1H, \(J=18.3\)), 2.88 (dd, 1H, \(J=16.3, 3.0\)), 2.49 (dd, 1H, \(J=18.3, 3.5\)), 2.29 (s, 3H), 2.10 (s, 3H), 1.76 (s, 3H), 1.55 (m, 2H), 1.34 (m, 1H), 0.89 (d, 3H, \(J=6.4\)), 0.84 (d, 3H, \(J=6.3\)); \(^{13}\)C NMR \(\delta\) ppm (CDCl\(_3\), 100 MHz): 175.4 (C=O), 170.9 (C=O), 169.8 (C=O), 169.6 (C=O), 166.1 (C=O), 162.0 (C=O), 151.7 (C), 141.3 (C), 134.8 (CH), 125.6 (CH), 122.9 (CH), 117.3 (C), 83.4 (CH), 80.0 (CH), 73.4 (CH), 49.4 (CH), 47.1 (CH), 39.9 (CH), 35.5 (CH\(_2\)), 30.9 (CH\(_2\)), 24.4 (CH\(_2\)), 23.0 (CH\(_3\)), 22.5 (CH\(_3\)), 21.6 (CH\(_3\)), 21.0 (CH\(_3\)), 20.6 (CH\(_3\)); HRFABMS m/z (formula, \(\Delta M\) ppm): 533.21382 (M+H/C\(_{26}\)H\(_{33}\)N\(_2\)O\(_{10}\), 0.56).

Gabriel-malonic ester synthesis of [3,4-\(^{13}\)C\(_2\)]aspartic acid intermediate (50): \(^1\)H NMR (CDCl\(_3\), 400 MHz): 7.84 (d, 1H, \(J=7.6\)), 7.48 (m, 3H), 7.33 (s), 4.28 (q, 2H, \(J=7.2\)), 4.27 (q, 4H, \(J=7.2\)), 4.12 (dq, 2H, \(^3J_{\text{CH}}=3.2, J=7.2\)), 3.67 (dd, 2H, \(^3J_{\text{CH}}=7.0, ^1J_{\text{CH}}=134.4\)), 1.31 (t, 3H, \(J=7.2\)), 1.26 (t, 6H, \(J=7.2\)), 1.23 (t, 3H, \(J=7.2\)); \(^{13}\)C NMR \(\delta\) ppm (CDCl\(_3\), 100 MHz): 170.34 (d, 100 x natural abundance, CH\(_2\), \(J=59.5\)), 168.0 (C=O), 166.7 (2xC=O), 166.0 (C=O), 136.8 (C), 131.7 (CH), 129.9 (2xCH), 129.8 (C), 127.6 (CH), 64.2 (d, C, \(J=41.2\)), 62.9 (CH\(_2\)), 61.3 (CH\(_2\)), 60.7 (CH\(_3\)), 37.4 (d, 100 x natural abundance, CH\(_2\), \(J=59.5\)), 14.1 (CH\(_3\)), 13.9 (2xCH\(_3\)); HRFABMS m/z (formula, \(\Delta M\) ppm): 440.18256 (M+H/\(^{12}\)C\(_9\)\(^{13}\)C\(_2\)H\(_{28}\)NO\(_9\), -1.27).
[3,4-$^{13}$C$_2$]aspartic acid: isolated as a white solid (360mg): mp >300 °C; $^1$H NMR (D$_2$O/DCI, 400 MHz): 3.66 (1H, m), 2.38(dm, 2H, J=132); $^{13}$C NMR δ ppm (D$_2$O/DCI, 100 MHz): 172.68 (d, 100 x natural abundance, C=O, J=55.4), 170.2 (C=O), 48.9 (d, CH, J=37.2), 33.4 (d, 100 x natural abundance, CH$_2$, J=55.4); HRCIMS m/z (formula, ΔM ppm): 136.05196 (M+H/$^{12}$C$_2$$^{13}$C$_2$H$_5$NO$_4$, 0.6).
References


2 MK91Z50 was investigated in Dr. Judy Needham’s Ph.D. Thesis, Secondary Metabolites of Bacteria Obtained from the Northeastern Pacific Ocean: Structure Elucidation and Biosynthetic Studies, University of British Columbia, Dec. 1993, pp 144-172.


11 There is increasing evidence for the “processive” mode of polyketide biosynthesis in which the oxidation state at the keto group generated after the addition of each acetate unit is adjusted prior to commencement of the next chain extension cycle. The polyketide depicted in Figure 4.14 is simply a formalism to help rationalize observed isotope incorporation results. See: Staunton, J.; Sutkowski, A. J. Chem. Soc. Chem. Commun. 1991, 1110-1112.

Chapter 5: Known Compounds Isolated from Marine Bacteria

5.1 Introduction

One of the realities of natural products isolation chemistry is the discovery of previously described compounds from new natural sources. This chapter serves to illustrate some of the known compounds, in addition to the AI77 type compounds (see Chapter 4), that were isolated in the progress of this study from various marine bacteria. Six previously described bioactive secondary metabolites were isolated from two different species of bacteria and two actinomycete species. All of these compounds have been isolated from terrestrial bacterial sources, and have been investigated thoroughly by other researchers. Hence, this chapter is just meant to provide a brief survey of the marine isolates studied, their metabolites and briefly mentions their biological activities, however it does not go into precise detail about the history and/or extent of research available on these compounds. If the reader is interested in more details, they are directed to the references listed herein.
5.2 Results and Discussion

5.2.1 Marine Isolate MK90A140

Marine isolate, MK90A140, cultured from a sediment sample taken in Quatsino Sound, B.C. and identified as *Pseudomonas chlororaphis*, was found to produce pyoluteorin (53) when grown in solid agar culture. Bioassay guided fractionation of the EtOAc extracts of the agar led to the isolation of a halogenated polycyclic aromatic compound which showed mild cytotoxicity against murine leukemia cells (ED$_{50}$ 7.42 µg/mL) and antibiotic activity towards MRSA (MIC 10 µg/disc) and *Pseudomonas cepacia* (MIC 20 µg/disc).\(^1\) Further, analysis of the $^1$H and $^{13}$C data and comparison with literature values confirmed this metabolite to be pyoluteorin (53).\(^2\) Pyoluteorin (53) is an antibiotic and phytotoxic metabolite previously reported from several terrestrial *Pseudomonas* spp.\(^3\)

![Pyoluteorin (53)](attachment:pyoluteorin.png)
5.2.2 Marine Isolate MKPNG8D

The marine isolate, MKPNG8D identified as a *Pseudomonas aeruginosa* species by MIDI fatty acid analysis, was isolated from the surface of an unidentified green sponge from Papua New Guinea. When grown on solid agar, this isolate was found to produce a cytotoxic and antimicrobial polycyclic aromatic metabolite. 1-phenazinecarboxamide (54) was isolated and characterized by bioassay guided fractionation of the methanolic extracts of the agar. This metabolite was mildly cytotoxic against murine leukemia (P388: ED$_{50}$ 5.98 μg/mL) and inhibited growth of MRSA (MIC 10 μg/disc). This polycyclic aromatic compound was identified by analysis of its $^1$H and $^{13}$C 1D and 2D NMR data, and comparison of the NMR data with literature values confirmed this identification. Compounds in the phenazine class have previously been isolated from several terrestrial species of *Pseudomonas chlororaphis* and *P. aeruginosa*, and are known to have antibiotic activity against phytopathogenic Gram-positive bacteria and fungi. Recently it was reported that 1-phenazinecarboxamide (54) and 1-phenazinecarboxylic acid were isolated from a *Pseudomonas* sp. associated with an Antarctic sponge.

![1-Phenazinecarboxamide (54)]
5.2.3 Marine Isolate MKQC73A

In cooperation with Dr. Fangming Kong, the secondary metabolites from a marine actinomycete, MKQC73A, isolated from a sediment sample taken in Esquimalt Harbour, B.C. were examined. MKQC73A when grown on solid agar appeared to exhibit cytotoxic and antibiotic activity. In an attempt to isolate and identify the active components in this culture, the isolate was grown in moderate scale on solid agar. Bioassay guided fractionation of the MeOH extracts of the agar cultures of this microorganism led to the isolation and structure elucidation of two macrolides, valinomycin (55) and tetranactin (56). Valinomycin (55) exhibited strong cytotoxicity against the P388 murine leukemia cell line (ED$_{50}$ 0.0011 µg/mL) and tetranactin (56) proved to be antibiotic towards MRSA and C. albicans (MIC's 1 µg/disc against both strains). These metabolites were first identified by NMR spectroscopy and mass spectrometric analysis and ultimately their structures were confirmed by comparison with published data.$^{6,7}$ Both valinomycin (55) and tetranactin (56) are ionophores previously isolated from several terrestrial Actinomyces and Streptomyces spp., and have significant broad spectrum antibiotic and insecticidal activity.$^8$
D-Hydroxyisovalerate

Valinomycin (55)

Tetranactin (56)
5.2.4 Marine Isolate MK-CLIN4723

A second actinomycete (MK-CLIN4723), isolated from a sediment taken in Von Donop Inlet, B.C. when grown on solid agar appeared to produce metabolites cytotoxic towards the murine P388 cell line. Thus, in cooperation with Dr. Dilip De Silva, the secondary metabolites from this marine actinomycete were investigated. The isolate was grown in moderate scale culture on solid agar and bioassay guided fractionation of the MeOH extracts of the agar led to the isolation and structure elucidation of two macrolides of the bafilomycin family, bafilomycin A$_2$ (57) and bafilomycin D (58). Bafilomycins A$_2$ and D (57 and 58) exhibited strong cytotoxicity against the P388 murine leukemia cell line (ED$_{50}$ 0.026 and 0.322 µg/mL respectively). These metabolites were first identified by extensive NMR spectroscopy and mass spectrometric analysis, but their structures were ultimately confirmed by comparison with published data.$^9$ The bafilomycins comprise a class of 16-membered diene macrolides previously isolated from several Actinomyces spp. They are reported to have broad spectrum antibiotic activity against Gram-positive bacteria, in addition to significant fungicidal, insecticidal and antiparasitic properties.
Bafilomycin A₁
Bafilomycin A₂ (57)
Bafilomycin B
Bafilomycin C
Bafilomycin D (58)
5.3 Experimental

NMR data were collected on either a Bruker AMX500, WH400, AM400 or AM200 spectrometer each equipped with 5 mm probes. Proton spectra were referenced using internal residual mono-hydrogenated solvent resonances (CHCl$_3$ δ 7.24 or Me$_2$CO-d$_5$ δ 2.04 ppm) and carbon spectra were referenced to the internal solvent resonance (CDCl$_3$ - δ 77.0 or Me$_2$CO methyl carbon - δ 29.8 ppm). Low and High resolution electron impact (EI) mass spectra were recorded on Kratos AEI MS-59 and AEI MS-50 mass spectrometers respectively. Negative ion FABMS data were collected on a Kratos Concept IIHQ hybrid mass spectrometer with cesium ion secondary ionization and a magnetic sector mass analyzer. Samples were dissolved in a MeOH/3-nitrobenzylalcohol matrix and spectra were obtained using a source voltage of 8 kV and a cesium ion gun voltage of 12 kV.

Normal and reversed-phase thin layer chromatography (TLC) was performed using Merck type 5554 aluminium-backed Kieselgel 60 F$_{254}$ and Whatman MKC18F plates, respectively. Visualization was with UV (λ=254 nm), vanillin/H$_2$SO$_4$/EtOH or ninhydrin spray reagents. Sephadex LH-20 (bead size 25-100 μm) was used for size exclusion chromatography. Sigma type H TLC grade silica gel (10-40 μm, no binder, no fluorescent indicator) was used for preparative normal phase chromatography. All solvents used for extraction and for column chromatographies were Fisher reagent grade.
5.3.1 Isolation

Marine isolates MK90A140, MKPNG8D, MKQC73A and MK-CLIN4723 have all been cryopreserved and deposited in the marine microbial culture collections at SeaTek and UBC. In a typical moderate scale culture, a selected isolate was grown for four days at 16 °C as lawns on the surface of solid trypticase soy agar supplemented with NaCl to a final concentration of 1.5%. The cultures were then harvested by gently scraping the cells from the surface of the agar. The agar was then extracted repeatedly with MeOH to give a crude extracellular extract that was then subjected to the Kupchan partitioning scheme. Thus, the crude methanolic extract was first extracted with hexanes, the MeOH layer was then diluted partially with H₂O (9:1 MeOH/H₂O), extracted with CCl₄, diluted again (9:4 MeOH/H₂O), extracted with CHCl₃, and ultimately the aqueous fraction was reduced in vacuo to remove MeOH, and partitioned with EtOAc.

5.4 Compound Summaries

MK90A140:

The EtOAc fraction obtained from the Kupchan partitioning scheme was first fractionated via Sephadex LH-20 chromatography (MeOH), and the antibiotic containing fractions were combined and further purified on normal phase silica (gradient 1:1 EtOAc/Hexanes - 100% EtOAc) to yield pyoluteorin (53), (4,5-dichloro-1H-pyrrol-2-yl) (2,6-dihydroxyphenyl) methanone: isolated as a red solid (15 mg); 13C NMR δ ppm (Me₂CO-d₆, 200 MHz): 7.14 (t, 1H, J=8.0), 6.78 (s, 1H), 6.46 (d, 2H, J=8.0), 2.65 (s, 2H); 13C NMR δ ppm (Me₂CO-d₆, 50 MHz): 183.9 (C=O), 158.1 (C), 133.4 (CH), 131.7 (C), 120.4 (C), 118.2 (CH), 113.8 (C), 111.4 (CH₂),
The cytotoxic CHCl₃ fraction obtained from the Kupchan partitioning scheme of the crude MeOH extract was subjected to size exclusion chromatography on Sephadex LH-20 (MeOH). A late eluting bright yellow fraction exhibiting mild cytotoxicity yielded yellow needles on standing. These active fractions were combined and reduced *in vacuo* to give 1-phenazinecarboxamide (54): isolated as bright yellow needles (MeOH) mp 234-235 (149 mg); ¹H NMR (CDCl₃, 400 MHz): 10.70 (bs, 1H), 8.99 (dd, 1H, J=7.1,1.4), 8.40 (dd, 1H, J=8.7, 1.4), 8.25 (m, 1H), 8.19 (m, 1H), 7.89 (m, 1H), 6.45 (bs, 1H); ¹³C NMR δ ppm (CDCl₃, 100 MHz): 166.6 (C=O), 143.5 (C), 143.2 (C),141.5 (C), 140.8 (C), 135.9 (CH), 134.3 (CH), 131.7 (CH), 131.0 (CH), 129.83 (CH), 129.79 (CH), 129.1 (CH), 129.0 (C); HREIMS m/z (formula, ΔM ppm): 223.07446 (C₁₃H₉N₃O₄, 0.4).

The hexane fraction obtained from the Kupchan partitioning scheme was subjected to bioassay guided fractionation (vs P388 murine leukemia cells) on normal phase silica (gradient 100% hexane - 100% EtOAc) yielded valinomycin (55): isolated as a yellow oil (38 mg); ¹H NMR (CDCl₃, 400 MHz): 7.79 (d, 1H, J=8.2), 7.68 (d, 1H, J=7.7), 5.27 (q, 1H, J=6.6), 4.99 (s, 1H), 4.12 (t, 1H, J=8.2), 3.97 (dd, 1H, J=7.7, 9.8), 2.32 (m, 2H), 2.22 (m, 1H), 1.43 (d, 3H, J=6.6), 1.06 (d, 3H, J=6.5), 1.02 (m, 3H), 0.97 (d, 3H, J=7.6), 0.95 (d, 3H, J=5.60), 0.93 (d, 3H, J=6.7); ¹³C NMR δ ppm (CDCl₃, 100 MHz), 172.3 (C=O), 171.6 (C=O), 170.7 (C=O), 170.0 (C=O), 78.6 (CH), 70.3 (CH), 60.3 (CH), 58.8 (CH), 30.2 (CH), 28.5 (CH), 28.3 (CH), 19.6
(CH₃), 19.4 (2xCH₃), 19.1 (CH₃), 19.0 (CH₃), 17.0 (CH₃), 16.6 (CH₃); HREIMS m/z (formula, ΔM ppm): 1110.63110 (C₃₄H₹₇N₂O₁₈, -2.7).

A second active fraction from this silica column yielded tetranaotactin (56), 5,14,23,32-tetrademethyl-5,14,23-tetraethyl-nonactin: isolated as a yellow oil (27 mg); ¹H NMR (CDCl₃, 500 MHz): 4.85 (dp, 1H, J=6.2, 1.0), 4.01 (q, 1H, J=7.2), 3.81 (p, 1H, J=6.7), 2.52 (p, 1H, J=7.2), 2.32, 1.96 (m, 1H), 1.88 (m, 1H), 1.74 (m, 1H), 1.70 (m, 1H), 1.59 (m, 1H), 1.58 (m, 1H) 1.53 (m, 1H), 1.45 (m, 1H), 1.07 (d, 3H, J=7.0), 0.85 (t, 3H, J=7.4); ¹³C NMR δ ppm (CDCl₃, 125 MHz): 174.5 (C=O), 79.8 (CH), 76.2 (CH) 73.3 (CH), 45.1 (CH), 39.9 (CH₂), 31.4 (CH₂), 28.0 (CH₂), 27.3 (CH₂), 12.9 (CH₃), 9.3 (CH₃); HREIMS m/z (formula, ΔM ppm): 792.50172 (C₄₄H₇₂O₁₂, -0.8).

**MK-CLIN4723:**

The CHCl₃ and CCl₄ extracts obtained from the Kupchan partitioning exhibited potent cytotoxicity against murine P388 leukemia cells. These extracts were combined and fractionated by column chromatography on normal phase silica (gradient 4:1 CH₂Cl₂/EtOAc - 100% MeOH) to yield five fractions, the third and fourth both exhibiting potent cytotoxicity. The third fraction was recycled on normal phase silica (5:1 - 3:2 CH₂Cl₂/EtOAc) to yield bafilomycin A₂ (57)⁶⁶: isolated as a clear glass (0.8 mg); ¹H NMR (CDCl₃, 500 MHz): 6.65 (s, 1H), 6.49 (dd, 1H, J=15.0, 10.8), 5.79 (d, 1H, J=10.5), 5.75 (d, 1H, J=9.2), 5.15 (dd, 1H, J=15.0, 9.3), 4.93 (d, 1H, J=7.8), 4.12 (m, 1H), 3.87 (dd, 1H, J=7.8, 9.3), 3.69 (m, 1H), 3.61 (s, 3H), 3.48 (bd, H, J=10.0), 3.47 (s, 3H), 3.22 (s, 3H), 2.52 (m, 1H), 2.28 (dd, 1H, J=4.7, 11.8), 2.13 (m, 1H), 2.12 (m, 1H), 1.97 (s, 3H), 1.93 (m, 1H), 1.92 (s, 3H), 1.90 (m, 1H), 1.86 (m, 1H), 1.74 (q, 1H, J=7.4), 1.14 (m, 1H), 1.05 (d, 3H, J=7.0), 1.02 (d, 3H, J=6.5), 0.92 (d, 6H, J=6.3), 0.91 (d, 3H, J=6.3), 0.88 (d, 3H, J=7.0), 0.81 (d, 3H, J=6.9), 0.75 (d, 3H, J=6.8); ¹³C NMR δ ppm (CDCl₃, 125 MHz):
167.3 (C=O), 143.0 (C), 142.7 (CH), 133.5 (CH), 132.9 (CH), 127.3 (CH), 125.3 (CH), 99.1 (C), 82.2 (CH), 81.2 (CH), 76.9 (CH), 75.8 (CH), 71.0 (CH), 70.6 (CH), 59.9 (CH), 55.6 (CH), 43.6 (CH$_3$), 42.1 (CH), 41.2 (CH$_2$), 41.0 (CH), 40.0 (CH), 37.2 (CH), 36.8 (CH), 21.7 (CH$_3$), 21.2 (CH$_3$), 20.1 (CH$_3$), 17.2 (CH$_3$), 14.3 (CH$_3$), 12.1 (CH$_3$), 9.9 (CH$_3$), 7.1 (CH$_3$); -ve ion HRLSIMS m/z (formula, $\Delta M$ ppm): 635.412.90 (M-H, C$_{35}$H$_{55}$O$_9$, -4.74).

The fourth fraction obtained from the silica column of the combined CHCl$_3$ and CCl$_4$ extracts was fractionated on Sephadex LH20 (MeOH). The second fraction collected from this column was then subjected to preparative normal phase silica chromatography (3:2 CH$_2$Cl$_2$/EtOAc) to yield bafilomycin D (58)$^{9c}$: isolated as a clear glass (1.6 mg): $^1$H NMR (CDCl$_3$, 500 MHz): 6.88 (dd, 1H, J=15.8, 8.2), 6.63 (s, 1H), 6.46 (dd, 1H, J=15.1, 10.0), 6.26 (d, 1H, J=15.8), 5.79 (d, 1H, J=10.3) 5.73 (d, 1H, J=10.0), 5.15 (dd, 1H, J=9.0, 15.1), 5.04 (d, 1H, J=8.0), 3.79 (dd, 1H, J=9.0, 8.0), 3.74 (m, 1H), 3.65 (s, 3H), 3.62 (bd, 1H, J=5.7), 3.28 (m, 1H), 3.21 (m, 1H), 3.20 (s, 3H), 3.16 (dd, 1H, J=5.9, 5.5), 2.95 (dq, 1H, J=6.0, 2.0), 2.50 (m, 2x1H), 2.19 (m, 1H), 2.03 (m, 1H), 1.96 (m, 1H), 1.96 (s, 3H), 1.89 (m, 1H), 1.89 (s, 3H), 1.70 (m, 1H), 1.50 (m, 1H), 1.28 (m, 1H), 1.25 (m, 1H), 1.18 (d, 3H, J=7.2), 1.09 (m, 1H), 1.07 (m, 1H), 1.06 (d, 3H, J=7.0), 1.04 (d, 3H, J=6.0), 1.02 (d, 6H, J=6.7), 0.90 (d, 3H, J=6.0); $^{13}$C NMR $\delta$ ppm (CDCl$_3$, 125 MHz): 203.1 (C=O), 166.4 (C=O), 148.5 (CH), 142.7 (C), 142.2 (C), 132.9 (CH), 132.7 (CH), 129.3 (CH), 127.1 (CH), 125.3 (CH), 83.2 (CH), 81.3 (CH), 79.8 (CH), 76.3 (CH), 72.6 (CH), 60.1 (CH), 55.7 (CH), 46.3 (CH), 41.3 (CH$_2$), 40.0 (CH), 39.7 (CH), 38.5 (CH), 36.8 (CH), 30.9 (CH), 21.9 (CH$_3$), 19.9 (CH$_3$), 19.7 (CH$_3$), 17.4 (CH$_3$), 16.8 (CH$_3$), 16.6 (CH$_3$), 14.0 (CH$_3$), 10.7 (CH$_3$), 10.2 (CH$_3$); -ve ion HRLSIMS m/z (formula, $\Delta M$ ppm): 603.39102 (M-H, C$_{35}$H$_{55}$O$_9$, 2.2).
References

1 Bioassay was performed using the paper disc diffusion assay method.


Chapter 6: Summary

Herein were presented the isolation and structure elucidations of two families of novel cyclic peptides with significant biological activities, as well as several other previously described secondary metabolites, all of which were obtained from the cultures of marine microbial isolates.

A culture of a Bacillus sp. isolated from the tissues of a marine worm collected near Loloata Island in Papua New Guinea produced a mixture of novel cyclic decapptide antibiotics. Loloatins A (1), B (2), and C (3) were isolated and characterized by extensive 1D and 2D NMR spectroscopy. The loloatins (1 - 3), which represent a new family of cyclic decapptides related to the tyrocidines (40 - 44), exhibited antimicrobial activity against antibiotic resistant Gram-positive pathogens, including drug resistant strains of Staphylococcus aureus, Streptococcus pneumoniae and Enterococcus spp.. Furthermore, even though loloatin C (3) is only different from loloatin B (2) by the replacement of phenylalanine in 2 by a tryptophan in 1, loloatin C (3) also exhibited strong Gram-negative antibiotic activity. This suggests that minor variations in the peptide sequence of the loloatins can produce significant differences in biological activity, and further structure activity relationship studies are warranted. Since there is a pressing need to develop antibiotics that will be effective against bacteria strains that become resistant to current therapies (such as a methicillin and vancomycin resistant Enterococci or Staphylococci sp.), one of the loloatins, or a variation thereof, may prove to be a effective treatment for such bacterial super-strains.
Massetolides A - H (4 - 11), novel cyclic depsipeptides, as well as the known compound viscosin (12), were isolated from cultures of two *Pseudomonas* sp. isolated from a marine alga and a marine tube worm each collected near Masset Inlet, B.C. and Moira Island, B.C. respectively. Massetolide A (4) and viscosin (12) exhibit *in vitro* antimicrobial activity against *Mycobacterium tuberculosis* (MTB) and *Mycobacterium avium-intracellulare* (MAI). Furthermore, since there is a two fold increase in potency of massetolide A (4) vs. viscosin (12) against these mycobacteria, further studies into the structure activity relationship of the peptide sequence are warranted. Viscosin (12) has been known for several decades and was discovered at a time when tuberculosis (TB) was believed to have been eradicated, consequently, interest in its anti-mycobacterial properties waned. Fortunately, the discovery of the massetolides coupled with the recent awareness of a need for anti-mycobacterial drugs effective against MTB and MAI, has led to further interest in this family of peptides as a potential TB and/or MAC treatment.

The known compounds, AI77-B (13) and AI77-F (14), as well as a previously undescribed C9' epimer 15 of AI77-F (14), were isolated from several species of *Bacillus pumilus* isolated from marine sediments. Stable isotope incorporation experiments were performed showing that AI77-B (13) is of mixed polyketide/amo acid biosynthetic origin, and currently there is considerable interest in bacteria that produce metabolites of mixed polyketide/amo acid origins. In the past decade, scientists have begun to mix and match genes in bacterial biosynthetic systems in order to produce new enzymatic systems capable of generating secondary metabolites of novel structure. This rational engineering of enzymatic systems has been applied to both polyketide type biosynthetic systems and non-ribosomal peptide biosynthetic systems.¹ The logical extension of this genetic manipulation would involve mixing characteristics of these two
different biosynthetic systems. Since marine isolate MK90A309 would appear to have a naturally occurring mixed enzymatic system, the dissection of this biosynthetic system would be very useful in future genetic engineering of other bacterial systems.

The secondary metabolites just described have all been purified from culture extracts of bacteria isolated from the marine environment. Two novel classes of cyclic peptides have been discovered with exciting possibilities as antimicrobial agents. Furthermore, biosynthetic studies on a third microbe, which produces a metabolite of mixed biosynthetic origin, has opened the door to new possibilities in genetic manipulation of enzymatic systems.

References

Appendix A: Nuclear Magnetic Resonance Spectroscopy

One-dimensional and two-dimensional Nuclear Magnetic Resonance (1D and 2D NMR) techniques have been used throughout this thesis in the structure elucidation of bacterial secondary metabolites. In particular, the HOHAHA, ROESY, and HMBC 2D NMR experiments played pivotal roles in this thesis in the sequence determination of two classes of cyclic peptides. Although high resolution mass spectrometry, X-ray analysis, chemical modification, and total synthesis play important roles in the structure elucidation of natural products, NMR techniques are now the standard tools in structure determination. 1D and 2D NMR spectra provide critical information on the nature and connectivities of protons and carbons in a molecule. In addition, insight can be obtained into the relative stereochemistry and conformation of molecules. Technological advances in superconducting magnets, new pulse programs, and inverse detection probes now enable NMR spectroscopists to determine the structures of small amounts of complex organic molecules. Herein are very simple explanations of the $^1$H, $^{13}$C, APT, COSY, HOHAHA, ROESY, HMQC, and HMBC NMR experiments employed in this thesis. The reader is directed to the excellent reviews on this subject, which provide more thorough discussions and detailed theoretical descriptions of 1D and 2D Fourier transform NMR.¹

A.1 One-Dimensional Fourier Transform NMR

NMR experiments are made possible by the magnetic properties of various nuclei. In general, a moving charge, such as spherically distributed positive charge on a nucleus, creates a magnetic field. $^1$H and $^{13}$C nuclei, have spherically distributed positive charge and spin in a
circular motion, and thus have an associated magnetic moment which can be effected by an external magnetic field. In the presence of a static magnetic field, $H_0$, nuclei, such as $^{13}\text{C}$ or $^1\text{H}$ with spin $\frac{1}{2}$, precess at their characteristic Larmor frequency, $\nu$. Further, the net magnetic moments of these nuclei will be aligned “with the field” or “against,” and there is an associated energy transition between these two spin states ($-\frac{1}{2} \rightarrow +\frac{1}{2}$). This transitional energy is given by Planck’s equation and is directly proportional to the static field $H_0$:

$$\Delta E = h \nu_0 = \gamma/2\pi (H_0)$$

where $\gamma$ is the gyromagnetic ratio of the nucleus

When a radiofrequency pulse, $\omega_1$, is applied corresponding to this transitional energy (i.e. $\omega_1 = 2\pi\nu_0$), the spin state of the nuclei is effected, and a detectable signal that decays over time is generated. This signal is called a free-induction decay (FID), and after some mathematical manipulation (Fourier transformation) this time-domain spectrum is converted into a “classic” one dimensional NMR frequency domain spectrum.

![Figure A.1: Conversion of the FID to a Frequency Domain NMR Spectrum](image)
In this introduction we have only considered the nuclei of isolated atoms, however all nuclei have electrons and these electrons are involved in different types of bonds with neighboring atoms. These electrons give rise to so called “shielding effects” which is directly proportional to the static magnetic field. These shielding effects cause nuclei in different bonding environments to experience slightly different “effective fields.”

\[ H_0 + H_{\text{elec}} = H_{\text{eff}} \]

Thus, in one dimension NMR the chemical shift (\( \delta \)) of the various atoms in the molecule is a measure of the resonance frequency of these nuclei in different chemical (electronic) environments under the influence of the applied field, \( H_0 \). In addition, the NMR signal of a nucleus can be “split” due to a through-bond energy transfer with neighboring nuclei, referred to as “scalar coupling.”

A quantum mechanical treatment of the behavior of nuclear spins in an external magnetic field is also possible, as opposed to the “classical” description just presented. In essence, the behavior of nuclear spins can be described by Hamiltonian operators which have well defined effects upon the magnetization of a given nucleus. Thus, the magnetization of nuclei can be described in terms of radiofrequency pulse, chemical shift, and scalar coupling operators. Although not dealt with in this discussion, this treatment of the behavior of nuclear spins provides a more thorough understanding of 2D NMR spectroscopy.\(^\text{1b} \)
A.1.1 A $^1$H NMR spectrum

The most common experiment in NMR spectroscopy is the 1D proton NMR spectrum (Figure A.2). Experimentally, this simply consists of a preparation time, $d_1$, to allow the nuclei in the sample to reach thermal equilibrium, followed by the application of a radiofrequency pulse to tip the bulk magnetization in the sample into the same plane as the detector of the NMR instrument (the $x$ - $y$ plane). This pulse ($\omega_1$) is often designated as an angle ($\theta = \omega_1 t$), where $t$ is the length of time the radio transmitter is left on. A short delay usually follows this pulse, allowing the transmitter coil to reach equilibrium, after which the FID is detected by the NMR instrument. This sequence is repeated several times, and the FIDs for each sequence are summed in order to improve the signal-to-noise ratio of the spectrum (an important benefit of FT-NMR). Ultimately, the summed FID is Fourier transformed in order to obtain the final 1D NMR spectrum.

$$\text{FID}$$

Figure A.2: Pulse Sequence for the Acquisition of a $^1$H NMR Spectrum

A.1.2 A $^{13}$C NMR spectrum

The pulse sequence for the acquisition of a $^{13}$C NMR spectrum is very similar to that used in obtaining a $^1$H NMR spectrum. However, $^{13}$C is a relatively insensitive nucleus compared to $^1$H, since the natural abundance of $^{13}$C is low (1.1%), and has a much smaller gyromagnetic ratio ($\gamma_{^{13}C} = 4\gamma_{^1H}$). Therefore, much longer acquisition times (typically 8 to 24 hours, depending on sample size) are required. To increase sensitivity of protonated carbons through heteronuclear
Overhauser enhancement, and to eliminate all $^1\text{H}-^{13}\text{C}$ couplings, the entire proton spectrum is irradiated during acquisition ("broad band decoupling").

A.1.3 The Attached Proton Test (APT) Spectrum

The attached proton test (APT) experiment exploits $^1\text{H}-^{13}\text{C}$ single bond scalar coupling to differentiate between carbons with even number of attached protons and carbons with odd number of attached protons. This is done instrumentally through gated decoupling to obtain modulation of the $^{13}\text{C}$ transverse magnetization due to one bond $^1\text{H}-^{13}\text{C}$ coupling. After the initial pulse the decoupler is switched off for a period equal to $1/J_{\text{CH}}$ (since $J_{\text{CH}} \approx 143$ Hz therefore $d_2 = 7$ ms), and the $^{13}\text{C}$ magnetization evolves under the influence of both chemical shift and scalar coupling. The second 180° pulse, and subsequent $d_2$ delay with proton decoupling, manipulates the carbon magnetizations such that quaternary and methylene carbons are 180° out of phase with methyl and methine carbons.
A.2 Two-Dimensional NMR Experiments

Two dimensional NMR spectroscopy exploits the power of the Fourier transform in converting a time domain spectrum into a frequency domain spectrum. Essentially, a series of FIDs (obtained as a function of $t_2$) are obtained with incremented evolution times ($t_1$) (Figure A.5). Fourier transformation of the FIDs in $t_2$ yields a series of spectra. However, during the evolution time $t_1$, the magnetization evolves under the influence of chemical shift, scalar coupling, or radiofrequency pulses. The series of spectra obtained in $t_2$ are therefore modulated with respect to these evolution phenomena which occur in $t_1$, and ultimately, a second Fourier transform over $t_1$ results in a 2D NMR spectrum as a function of two frequencies ($F_1$ and $F_2$).

![Figure A.5: Pulse Sequence for a Typical 2D NMR Experiment](image)

A.2.1 The $^1$H-$^1$H COSY Experiment

Homocuclear Correlation Spectroscopy is the most commonly used 2D NMR experiment. Experimentally the pulse sequence for obtaining a COSY spectrum (Figure A.6) consists of two pulses separated by an incremental delay, $t_1$. In very basic terms, magnetization which has been generated by the first pulse is modulated by the application of a second pulse (typically 90°). Thus, in the final spectrum off-diagonal peaks correlate spins (i.e. protons) that are coupled to each other. A series of intercoupled protons is designated a "spin system," and by analysis of the COSY spectrum we are able to trace correlations between coupled protons in a spin system.
A.2.2 The HOHAHA Experiment

The HOmonuclear HArtman HAhn (HOHAHA) experiment can be thought of as an alternative version of the COSY experiment. This technique relies on cross polarization coherence transfer, and this cross polarization can be obtained by applying a single coherent radiofrequency field to the sample. If the effective rf field experienced by two spins are identical, an oscillatory exchange of spin-locked magnetization (the Hartman-Hahn condition) can be obtained. The advantage of this cross-polarization transfer is the achievement of relay, and multiple-relay peaks in the final 2D NMR spectrum.

Consequently, a HOHAHA spectrum shows correlations between a proton resonance and other proton resonances in the same spin system, and a relay distance (dependent on the mixing (spin-lock) time - typically 2.5 ms) determines the number of proton resonances observed in a given spin system. Since the amino acids in a peptide are, in essence, small isolated spin systems, the HOHAHA technique is invaluable in the determining the type of amino acids present in a peptide.
A.2.3 The ROESY Experiment

Nuclear Overhauser Effect (NOE) experiments provide information on protons that are close together in space through observation of dipolar interactions between protons. The Rotating Frame Overhauser Enhancement Spectroscopy (ROESY) experiment provides information comparable to NOE experiments, and is simply a 2D version of the NOE experiment. The pulse program for the acquisition of a ROESY spectrum is identical to that used for the HOHAHA (Figure A.7). However, the rf pulse used to achieve the spin-lock is much smaller in the ROESY, and the transmitter carrier is usually placed in the upfield region of the spectrum. The combination of these two effects disables the Hartman-Hahn condition for oscillatory exchange of spin-locked magnetization, and thus prevents the appearance of HOHAHA crosspeaks in the ROESY spectrum. Moreover, ROESY crosspeaks appear with opposite phase to diagonal and HOHAHA correlations, and since the ROESY spectrum is obtained in the phase sensitive mode only NOE correlations appear on the positive side of the spectrum.

Experimentally, a 90° pulse is applied and the bulk magnetization in the sample evolves as a function of the evolution time ($t_1$). The second spin-locking pulse, is then applied for a mixing time (225 ms), during which the magnetization is “locked” and dipolar relaxation occurs. The resultant 2D NMR spectrum resembles a COSY spectrum, but instead of cross peaks representing scalar coupling, cross-peaks representing dipolar relaxation (through space coupled) are observed.

This experiment is particularly powerful in determining the amino acid sequence in a peptide, since it has been noted that irrespective of the secondary fold of a peptide or protein, at least one distance between NH, $\alpha$CH, and $\beta$CH protons between two adjacent residues is less than 3Å, and an NOE should be observed.6
A.2.4 The HMQC Experiment

The $^1$H detected Heteronuclear Multiple Quantum Coherence (HMQC) experiment is used to determine one bond $^1$H-$^{13}$C connections via correlations between the $^1$H resonances along the $F_2$ axis and $^{13}$C resonances along the $F_1$ axis. This experiment provides the same information as the $^{13}$C-detected HETeruclear CORrelation (HETCOR) experiment, however, it is much more sensitive since the more sensitive $^1$H nucleus is detected instead of the relatively insensitive $^{13}$C nucleus.

The pulse sequence for the HMQC is given in Figure A.8. An initial Bilinear Inversion, Rotational Decoupling (BIRD) pulse sequence removes signals from protons not directly coupled to $^{13}$C nuclei. The remainder of the pulse sequence is optimized for polarization transfer from one-bond $^1$H-$^{13}$C scalar coupling. Typical delay values used in this thesis were: $d_1 = 1.2 - 1.6$ s (relaxation delay); $d_2 = 3.5$ ms ($1/(2 J_{C,H}) \approx 143$ Hz); $d_4 = 3$ ms (instrument compensation factor); and $t_1 = 3$ ms (normal increment factor).

![Figure A.8: Pulse Sequence for the HMQC Experiment](image-url)
A.2.5 The HMBC Experiment

The inverse-detected Heteronuclear Multiple Bond multiple quantum Coherence experiment (HMBC) determines long-range (two or three bond) $^1$H-$^{13}$C connectivities, and is one of the most useful techniques in NMR structure elucidation. This experiment is similar to the HMQC experiment, however it is optimized for long range $^1$H-$^{13}$C scalar coupling.

The pulse sequence for the HMBC experiment is shown below. A low pass $J$ filter, consisting of a $^1$H-90° pulse, $d_2$, $^{13}$C-90° pulse sequence, removes most one-bond $^1$H-$^{13}$C correlations. However, the initial delays $d_1$ and $d_2$ are the same as those in the HMQC experiment, whereas $d_3$ is optimized for long range $^1$H-$^{13}$C $J$ values (since $J_{\text{CHR}} \approx 7$-8 Hz therefore $d_3 = 1/(2 J_{\text{CHR}}) = 60$ ms).

![Pulse Sequence for the HMBC Experiment](image)

This experiment is invaluable in determining the amino acid sequence in peptides and proteins, and usually proves to be complementary to the ROESY experiment. The assignment of a residue's carbonyl $^{13}$C resonance can often be established by intra-residue HMBC correlations to either the $\alpha$CH, $\beta$CH, or amide NH proton resonances. Connections between adjacent amino acids can then be established by observation of inter-residue HMBC correlations between the amide carbonyl resonance of a residue and the adjacent residue's NH or $\alpha$CH proton resonances.
References


Appendix B: Supplementary Materials to Chapter 2
Table B.1: NMR Data for Loloatin B (2) (DMSO-$d_6$)

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<th>Res.</th>
<th>Atom #</th>
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<th>$\delta^1H$ (ppm)</th>
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<th>HOHAHA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ROESY&lt;sup&gt;c&lt;/sup&gt;</th>
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\textsuperscript{a} Acquired at 100 MHz
\textsuperscript{b} Acquired at 400 MHz, J values measured in Hz
\textsuperscript{c} Acquired at 500 MHz
\textsuperscript{d} Chemical shift degenerate diastereotopic protons
\textsuperscript{e} Chemical shift coincident with solvent
\textsuperscript{f,g,h} May be interchanged
Figure B.1: $^{13}$C NMR Spectrum of Loloatin B (2) in DMSO-$d_6$ - Carbonyl Region Expansion
Figure B.2: APT/\textsuperscript{13}C NMR Spectra of Loloatin B (2) in DMSO-\textit{d}_6 - Aromatic Region Expansion
Figure B.3: APT/$^{13}$C NMR Spectra of Loloatin B (2) in DMSO-$d_6$ - Expansion
Figure B.4: HMQC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Aromatic Region Expansion
Figure B.5: HMQC Spectrum of Loloatin B (2) in DMSO-\textit{d}_{6} - alpha-CH Expansion
Figure B.6: HMQC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion
Figure B.7: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 1
Figure B.8: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 2
Figure B.9: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 3
Figure B.10: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 4
Figure B.11: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 5
Figure B.12: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 6
Figure B.13: HMBC Spectrum of Loloatin B (2) in DMSO-$d_6$ - Expansion 7
Figure B.14: HMBC Spectrum of Loloatin B (2) in DMSO-\textit{d}_6 - Expansion 8
Figure B.15: HMBC Spectrum of Loloatin B (2) in DMSO-\(d_6\) - Expansion 9
Table B.2: NMR Data for N-Acetyl Loloatin B Methyl Ester (39) (DMSO-\textit{d}_{6}/\textit{C}_{6}\textit{D}_{6} 20:1)

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<th>ROESY&lt;sup&gt;c&lt;/sup&gt;</th>
<th>HMBC&lt;sup&gt;c&lt;/sup&gt;</th>
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\(^a\) Acquired at 100 MHz
\(^b\) Acquired at 400 MHz, J values measured in Hz
\(^c\) Acquired at 500 MHz
\(^d\) Chemical shift degenerate diastereotopic protons
\(^e\) Chemical shift coincident with solvent
Figure B.16: HMQC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-$d_6$/C$_6$D$_6$ - Expansion 1
Figure B.17: HMQC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-$d_6$/$C_6D_6$ - Expansion 2
Figure B.18: HMQC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-\textit{d}_6/\textit{C}_6\textit{D}_6 - Expansion 3
Figure B.19: HMBC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-$d_6$/C$_6$D$_6$ - Expansion 1
Figure B.20: HMBC Spectrum of N-Ac Loloatin B Me Ester (39) - Expansion 2
Figure B.21: HMBC Spectrum of N-Ac Loloatin B Me Ester (39) in 20:1 DMSO-\textit{d}_6/C\textit{d}_6 - Expansion 3
Table B.3: NMR Data for Loloatin A (1) (DMSO-\(d_6\))

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\(^a\) From pyrene-deuterium cross peak
\(^b\) Assignments from aromatic region
\(^c\) Assignments from COSY and ROESY experiments

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<td>ROESY</td>
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*a* Acquired at 100 MHz  
*b* Acquired at 400 MHz, J values measured in Hz  
*c* Acquired at 500 MHz  
*d* Chemical shift degenerate diastereotopic protons  
*e* Chemical shift coincident with solvent  
*f* May be interchanged
Table B.4: NMR Data for Loloatin C (3) (DMSO-$d_6$)

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<th>Res.</th>
<th>Atom #</th>
<th>$\delta^{13}$C&lt;sup&gt;a&lt;/sup&gt; (ppm)</th>
<th>$\delta^1$H&lt;sup&gt;b&lt;/sup&gt; (ppm)</th>
<th>COSY&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HoHaHa&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ROESY&lt;sup&gt;c&lt;/sup&gt;</th>
<th>HMBC&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>V4, V5</td>
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<td>0.99 (3H, d)</td>
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\textsuperscript{a} Acquired at 100 MHz

\textsuperscript{b} Acquired at 400 MHz, J values measured in Hz

\textsuperscript{c} Acquired at 500 MHz

\textsuperscript{d} Chemical shift degenerate diastereotopic protons

\textsuperscript{e} Chemical shift coincident with solvent

\textsuperscript{f} May be interchanged
Appendix C : Supplementary Materials to Chapter 3
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<td>0.91 (3H, d, J=7)</td>
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<tr>
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<td>L'7 CO</td>
<td>171.1</td>
<td>-</td>
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<td>S2 αCH</td>
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<td>S1, S3a, S3b</td>
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<td>S1, S2</td>
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<tr>
<td></td>
<td>S3b γCH</td>
<td>42.0</td>
<td>4.20 (1H, m)</td>
<td>S2, S3a</td>
<td>S1, S2, S3a</td>
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<tr>
<td></td>
<td>S4 CO</td>
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<td>-</td>
<td>S2, S3a, L'1</td>
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<td>Ile9</td>
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<td>(δ\textsuperscript{1}3 env)</td>
<td>(δ\textsuperscript{1}3 env)</td>
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\*Acquired at 100 MHz
\*\*Acquired at 400 MHz
\*\*\*Acquired at 500 MHz, J values measured in Hz
\*dChemical shift degenerate protons
\*e,f,gMay be interchanged

Massetolide A methyl ester (46) shows an HMBC correlation between Glu-Me (δ 3.60, 3H, s), and Glu-δCO (δ 173.4), and a correlation between Glu-γCH\textsubscript{2} (δ 2.51, 2H, t, J=7) and Glu-δCO (δ 173.4).
Figure C.1: $^{13}$C NMR Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Carbonyl Region Expansion
Figure C.2: $^{13}$C NMR Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion
Figure C.3: $^{13}$C NMR Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion
Figure C.4: HMBC Spectrum of Massetolide A (4) in Me₂CO-\textit{d}_6 - Expansion 1
Figure C.5: HMBC Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion 2
Figure C.6: HMBC Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion 3
Figure C.7: HMBC Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion 4
Figure C.8: HMBC Spectrum of Massetolide A (4) in Me$_2$CO-$d_6$ - Expansion 5
Figure C.9: HMBC Spectrum of Massetolide A (4) in Me₂CO-d₆ - Expansion 6
Figure C.10: 500 MHz $^1$H NMR Spectrum of Massetolide B (5) in Me$_2$CO-$d_6$
Figure C.11: 500 MHz $^1$H NMR Spectrum of Massetolide B (5) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.12: 500 MHz $^1$H NMR Spectrum of Massetolide C (6) in Me$_2$CO-$d_6$
Figure C.13: 500 MHz $^1$H NMR Spectrum of Massetolide C (6) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.14: 500 MHz $^1$H NMR Spectrum of Massetolide D (7) in Me$_2$CO-$d_6$
Figure C.15: 500 MHz $^1$H NMR Spectrum of Massetolide D (7) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.16: 500 MHz $^1$H NMR Spectrum of Viscosin (12) in Me$_2$CO-$d_6$
Figure C.17: 500 MHz $^1$H NMR Spectrum of Viscosin (12) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.18: 500 MHz $^1$H NMR Spectrum of Massetolide E (8) in Me$_2$CO-$d_6$
Figure C.19: 500 MHz $^1$H NMR Spectrum of Massetolide E (8) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.20: 500 MHz $^1$H NMR Spectrum of Massetolide F (9) in Me$_2$CO-$d_6$
Figure C.21: 500 MHz $^1$H NMR Spectrum of Massetolide F (9) in Me$_2$CO-$_d_6$ - alpha-CH Expansion
Figure C.22: 500 MHz $^1$H NMR Spectrum of Massetolide G (10) in Me$_2$CO-$d_6$
Figure C.23: 500 MHz $^1$H NMR Spectrum of Massetolide G (10) in Me$_2$CO-$d_6$ - alpha-CH Expansion
Figure C.24: 500 MHz $^1$H NMR Spectrum of Massetolide H (11) in Me$_2$CO-$d_6$
Figure C.25: 500 MHz $^1\text{H}$ NMR Spectrum of Massetolide H (11) in Me$_2$CO-$d_6$ - alpha-CH Expansion