BIOACTIVE MARINE NATURAL PRODUCTS

by

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ABSTRACT

The chemical exploration of extracts from cultures of the marine bacterial isolate PNG-276 yielded the novel antibiotic tauramamide (2.13), a non-ribosomal peptide active against cultures of Enterococcus sp. and methicillin-resistant Staphylococcus aureus (MRSA). A study of extracts of the marine sponge Spirastrella coccinea yielded the novel macrolide methylspirastrellolide C (3.14), which is active against protein phosphatase 2A (PP2A). A third study examined sponge extracts active in a cannabinoid receptor assay, yielding two known compounds, an A-nor-steroid derivative (4.10) and bengamide A (4.11). Neither purified compound was active in the cannabinoid receptor assay, although in both cases this is the first report of these compounds being isolated from Stylissa massa and Hemiasterella aff. affinis sponges, respectively.
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<tr>
<td>°</td>
<td>-degree(s)</td>
</tr>
<tr>
<td>1D</td>
<td>-one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>-two-dimensional</td>
</tr>
<tr>
<td>$[^{25}\alpha]_{D}$</td>
<td>-specific rotation at wavelength of sodium D line at 25°C</td>
</tr>
<tr>
<td>Ac</td>
<td>-acetate</td>
</tr>
<tr>
<td>AcOH</td>
<td>-acetic acid</td>
</tr>
<tr>
<td>Arg</td>
<td>-arginine (three letter abbreviation)</td>
</tr>
<tr>
<td>b</td>
<td>-broad</td>
</tr>
<tr>
<td>bs</td>
<td>-broad singlet</td>
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<td>-methylene</td>
</tr>
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<td>CH$_3$</td>
<td>-methyl</td>
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<td>HMQC</td>
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<tr>
<td>HPLC</td>
<td>-high-performance liquid chromatography</td>
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<tr>
<td>hr</td>
<td>-hour(s)</td>
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<tr>
<td>HRESIMS</td>
<td>-high-resolution electrospray ionisation mass spectrometry</td>
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HSQC - two-dimensional heteronuclear single quantum coherence spectroscopy
Hz - hertz
I - impurity
J - coupling constant in hertz
L - leucine (single letter abbreviation)
Leu - leucine (three letter abbreviation)
LRESIMS - low-resolution electrospray ionisation mass spectrometry
m - multiplet
M - molar concentration
M+ - molecular ion
Me - methyl
MeCN - acetonitrile
MeOH - methanol
mg - milligram(s)
MHz - megahertz
MIC - minimum inhibitory concentration
mL - millilitre(s)
mm - millimetre(s)
mmol - millimol(s)
mRNA - messenger ribonucleic acid
MRSA - methicillin-resistant Staphylococcus aureus
MS - mass spectrometry
m/z - mass to charge ratio
N - normal
N2 - nitrogen
NaCl - sodium chloride
nm - nanometre(s)
NMR - nuclear magnetic resonance
NP - normal phase
NRPS - non-ribosomal peptide synthase
"PrOH - 1-propanol
PCP - peptidyl carrier protein
4'-PP - 4'-phosphopantethiene
ppm - parts per million
PyBOP - benzotriazol-1-yl-oxytritylridinosophosphonium hexafluorophosphate
q - quartet
R - arginine (single letter abbreviation)
ROESY - rotating frame Overhauser enhancement spectroscopy
RP - reversed-phase
s - singlet, solvent (on spectrum)
S - serine (single letter abbreviation)
SAR - structure-activity relationship
SCUBA - self-contained underwater breathing apparatus
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<td>sp³ hybrid orbital</td>
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DEDICATION

For Erin
Chapter 1: Marine Natural Products Chemistry

1.1 Marine Natural Products Chemistry: A Multidisciplinary Approach

"Why in the world do you want to go down into the sea?" is a riddle we are often asked by practical people. George Mallory was asked why he wanted to climb Mt. Everest, and his answer serves for us, too. "Because it is there," he said. We are obsessed with the incredible realm of oceanic life waiting to be known. The mean level of habitation on land, the home of all animals and plants, is a thin tissue shorter than a man. The living room of the oceans, which average twelve thousand feet in depth, is more than a thousand times the volume of the land habitat.

J. Y. Cousteau

Marine natural products (MNP) chemistry is a relatively young science, made practical only after the widespread availability of the demand regulator driven Aqua Lung invented by Jacques-Yves Cousteau and Emile Gagnan in 1943, which allowed semi-autonomous exploration of the undersea realm. However, a wide range of chemical structures have been identified in the relatively short time since its inception. The many different types of studies carried out on marine organisms and their chemical constituents are representative of the different groups of scientists who study this vast subject. Scientists with biological backgrounds have directed studies into the chemical ecology or biosynthesis of secondary metabolites. Those with a chemistry background have focused upon novel chemical structures (chemical prospecting) or potential applications of these compounds as chemotherapeutics (drug
discovery). As is often the case, discoveries made in one stream of study frequently lead into another.

The main goal of the research contained herein is that of drug discovery, although the natural biological role of compounds and their methods of biogenesis are also addressed. The diversity of biological activities and structural classes of these compounds is testament to the diversity of chemical structures and their concomitant biological functions in the marine environment.

1.2 Drugs From The Sea

Natural products have historically been strong contributors to the pool of available drugs.\(^7\,^8\) Secondary metabolites are compounds produced by organisms which are arguably unnecessary for the base functioning of the organism.\(^9\,^†\) The vast structural diversity of secondary metabolites in nature makes them an ideal resource for the discovery of new drugs.\(^10\) It is unbiased by human imagination or ease of synthesis (unlike combinatorial chemistry efforts), and thus is a rich resource for new chemistry. Research on the secondary metabolites of terrestrial organisms has been ongoing for many years, with a great number of successes (Figure 1.1). Although the natural product itself is occasionally the compound used therapeutically, more often it is a lead from which more pharmaceutically appropriate compounds are derived. Nevertheless, the drug is of natural origins.

\(^{†}\) This is in contrast to primary metabolites such as amino acids, nucleic acids, sugars and lipids, which are necessary for the functioning of all cells.
Despite past successes in the terrestrial realm, the search for new and more potent drugs continues. In addition, novel molecular targets with therapeutic potential are continually being unveiled as biochemists study diseases in greater detail than was previously possible. In contrast to terrestrial studies, which have been ongoing for a great many years, marine studies have only been practical for the last 30 or 40 years. With the relative ease of collection of marine organisms since the advent of modern SCUBA, it has become possible for chemists to assemble libraries of crude extracts from marine organisms and, working in close collaboration with biochemists, to study these libraries with the objective of isolating and identifying new and interesting bioactive secondary metabolites. This, in essence, is the study of marine natural products chemistry.

A wide range of organic chemical structural classes have been observed in extracts of marine organisms, many of which have been found to be uniquely
marine in origin. From this wide spectrum of structural classes a large number have been found to be bioactive. Some of these (or modifications thereof) have even been found to be active enough to warrant expensive and time consuming clinical trials. Ara C (1.5) was developed following the discovery of spongothymidine (1.6) and spongouridine (1.7), and is used in the treatment of leukemia. Ziconotide (1.8), marketed under the band name Prialt®, is an ω-conotoxin MV-IIA derived from the Conus magus, a marine fish-hunting cone snail. This small (25 residue) peptide is currently used as an analgesic due to potent activity on voltage-gated Ca\(^{2+}\) channels.

![Chemical structures](image)

**Figure 1.2** Structures of Ara-C (1.5), spongothymidine (1.6), spongouridine (1.7) and ziconotide (1.8)

Ecteinascidin 743 (ET-743, Yondelis, 1.9) was isolated from the marine tunicate Ecteinascidia turbinata, and identified as possessing strong anti-tumor activity following an initial observation of cytotoxicity in the crude extracts. The
structural complexity of 1.9 has led to a number of synthetic studies,\textsuperscript{20} whereas the biological activity has given rise to a number of biological studies, including advanced clinical trials.\textsuperscript{21} It is currently approved for use for treatment of soft-tissue sarcomas, and is being studied for its effects on ovarian cancer. Finally, pseudopterosins A (1.10) and C (1.11) were isolated from the Caribbean sea whip \textit{Pseudopterogorgia elisabethae} in the mid 1980s and were found to possess potent analgesic and anti-inflammatory activities.\textsuperscript{22,23} They are currently used by Estee Lauder as a topical anti-inflammatory agent in cosmetics.\textsuperscript{24} A great many more marine-derived compounds are currently in clinical and preclinical trials.\textsuperscript{13}

\textbf{Figure 1.3} Structures of ET-743 (1.9), pseudopterosin A (1.10) and E (1.11)
A survey of the literature published up to the end of June 2005 indicates several trends in marine natural products research. The most productive source of chemical structures is attributed to phylum Porifera (sponges), from which a number of interesting discoveries continue to be made today (Figure 1.4). There are a growing number of studies aimed at determining whether compounds isolated from sponges are in fact synthesized by the sponge, or by microorganisms living on or within the sponge host. This question is far from resolved, although microorganisms appear to be a more important source than the relatively small number of compounds attributed to them in the literature indicate. In a typical research laboratory, while many projects may continue to focus on sponges, bacterial studies are becoming increasingly commonplace.

![Figure 1.4](Proportion of compounds in Marinlit database, grouped by phyla of origin. Total 17068 structures.)

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† Search conducted using MarinLit Marine Literature Database, Version pc13.4.
1.3 Marine Microorganisms as an Emerging Resource

Many of the terrestrial drugs currently in use have been isolated from microorganisms (penicillin G, (1.2); erythromycin (1.12), adriamycin (1.13), and vancomycin, (1.14) are a few of note).\textsuperscript{30-34} It is only in recent years, however, that the vast resource of marine microorganisms has become readily accessible, thus the study of the chemistry of these fascinating organisms has only been possible for a relatively short while. It is useful to note that the term marine microorganisms used in the discussion to follow refers to heterotrophic organisms (primarily bacteria and fungi). The chemistry of autotrophic marine microorganisms such as blue-green or red algae has been studied in much more detail than that of the heterotrophs, due in part to ease of isolation, relative prevalence, as well as ease of collection and cultivation.\textsuperscript{35,36} In essence, one major difference between autotrophs and heterotrophs is that the latter derive their energy from nutrition, whereas the former derive their energy from photosynthesis.
Figure 1.5 Structures of some drugs derived from terrestrial microorganisms: Erythromycin (1.12), from *Streptomyces erythreus*, adriamycin (1.13), from *Streptomyces peucetius*, and vancomycin (1.14), from *Streptomyces orientalis*

It has been often reported that there are greater than one hundred thousand microbial cells per mL of seawater, and recent studies indicate even higher concentrations of microbial diversity in unique biospheres such as deep sea vents.\textsuperscript{37,38} While genetic studies have constantly increased the number of identified unique species present in the ocean, the ability to culture these organisms has improved much more slowly.\textsuperscript{39} However, recent studies indicate that major hurdles have been overcome in the culturing of these intriguing and chemically productive organisms.\textsuperscript{40-43}

The research by Dr. William Fenical and Dr. Paul Jensen at Scripps Institute of Oceanography is testament to the possibilities of the culturing of
marine microorganisms. Azamarone (1.15) is one of many novel structures identified from their investigations of the secondary metabolites from marine bacteria.\cite{44} In this case, the novel meroterpenoid was isolated from a saline culture of a *Streptomyces* strain CNQ776. The same culture was found to produce actinofuranones A and B (1.16 and 1.17, respectively), novel polyketides which, like 1.15, showed weak (20-40 µM) cytotoxicity against mouse splenocyte T-cells and macrophages.\cite{45} Further studies on cultures of the newly discovered genera *Salinospora* and *Marinospora* by the same group has yielded a number of interesting structures (see Chapter 2).\cite{46-48}

Another approach to the cultivation of marine bacteria is to isolate organisms directly from the surface of marine invertebrates. A prime example of this is the isolation of guangamides A and B (1.18 and 1.19, respectively) from an unidentified fungus which was isolated from a marine *lanthella* sp. sponge.\cite{49} Compounds 1.18 and 1.19 were found to be weakly toxic against *Staphylococcus epidermis* and *Enterococcus durans* (MIC = 100 µg/mL).
One appealing feature of the study of marine bacteria is that it provides an answer to the supply issue. Studies of marine invertebrate chemistry are often plagued by the low natural abundance of metabolites in the organism. By obtaining stable cultures of marine microorganisms, researchers can feasibly culture indefinite amounts of microbes, and thus potentially produce unlimited amounts of compound. One such culture is PNG-276, a remarkably productive microbe which has been the focus of much study in our laboratory. Further studies of this organism are discussed in Chapter 2.

1.4 Marine Sponges as a Productive Resource

As mentioned previously, marine sponges have been a significant contributor to marine natural products chemistry. It should be noted that for the purposes of this discussion no differentiation will be made between compounds produced by the sponge and compounds possibly produced by symbiotic
microorganisms (*vide supra*). Unless the culture has been grown apart from the sponge, it will be treated as a sponge metabolite.

Irrespective of their origin, a huge number of compounds have been isolated from sponges. Early investigations focussed upon fascinating multi-halogenated compounds such as the carbodiimine dichloride 1.20, as well as peptides such as jaspamide (1.21) and cyclotheonamide A (1.22). Entire families of compounds were discovered in sponge extracts, such as the oroidin alkaloids, of which oroidin (1.23), dibromophakellin (1.24) and palau’amine (1.25) are just a few.\(^{59-62}\)

**Figure 1.8** Examples of compounds isolated from marine sponges
One of the underlying themes of marine natural products chemistry is that diversity in chemical structures corresponds to a diversity of biological activity. This is particularly the case with compounds produced by marine sponges. Manoalide (1.26) was isolated from the sponge *Luffariella variabilis* collected in Palau, and found to possess potent anti-inflammatory activity. Discodermolide (1.27) is a potent cytotoxin isolated from the deep-water sponge *Discodermia dissoluta* found in the Bahamas, the activity of which will be discussed in more detail in Chapter 3. Bioassays have been focussed primarily on anticancer activities, which is due both to the relative prevalence of funding in cancer research, and also the high occurrence of cytotoxic compounds in marine extracts and readily cultured cancer cells to test these extracts. Nevertheless, a survey of the literature shows that antibiotic, antifungal, immune active (either activation or repression), antiviral, antiparasitic and antifouling activities are also common for marine natural products.† With this in mind, it remains to outline the particular projects which are summarized herein.

![Figure 1.9 Structures of manoalide (1.26) and discodermolide (1.27)](image)

† The listed categories collectively account for 46% of reported activities, whereas cytotoxicity and anticancer activity accounts for the remaining 54% of reported activities (Total 11238 reported activities). Search conducted using MarinLit Marine Literature Database, Version pc13.4.
1.5 Antibiotic Resistance

Antibiotic resistance is a problem that is rapidly approaching crisis proportions. A number of excellent reviews explore this subject, in particular a recent issue of *Chemical Reviews*[^66-74] and a special issue of *Biochemical Pharmacology*.[^75-81] While it is not necessary to exhaustively report the history of antibiotic resistance, it is worthwhile to look at a few of the major developments in the field of antibiotic therapy.

The discovery of the antibiotic producing fungus *Penicillium* and subsequent isolation of penicillin by Fleming and coworkers in 1940 ushered in the golden era of antibiotic usage.[^30,82] A period of rapid expansion followed, culminating in the late 1960s with the discovery of many of the classes of antibiotics currently in use (Figure 1.10). Although there have been important developments in antibiotic research since then, there have in fact been few new chemical classes discovered. The majority of the research to develop new antibiotics has focused upon the development of new versions of the classic structural types.[^83]
Figure 1.10 Some major classes of antibiotics currently in use. Shown are penicillin G (1.2), erythromycin (1.12), vancomycin (1.14), sulfamethoxazole (1.28), chloramphenicol (1.29), tetracycline (1.30) and streptomycin (1.31)

Exposure to antibiotics in the environment during the last 60 years has created an evolutionary selective pressure that favors microorganisms that are able to survive in the presence of antibiotics. Coupled with the slowdown in discovery of new antibiotics, this has led to large increases in the severity and prevalence of outbreaks of antibiotic resistant bacteria. There is an urgent need for the discovery and development of the next line of antibiotics.
This is an area of research ideally suited to marine natural products drug discovery. Whereas soil organisms are the principal source of most current antibiotics, marine microorganisms may well be a source of new antibiotics. Microorganisms in the marine environment maintain a complex balance with their competitors through the use of a number of techniques, one of which may be chemical warfare. The compounds produced for this purpose could well be the next generation of antibiotics if isolated and characterized.

1.6 Cancer Chemotherapeutics

Approximately 153,100 new cases of cancer and approximately 70,400 deaths from cancer are predicted in Canada in 2006. Traditionally, natural products have contributed greatly to the search for new anti-cancer drugs. Classes of compounds such as the vinca alkaloids and the taxanes (see Chapter 3) are currently on the front line of cancer therapies. This strong contribution from terrestrial sources has extended into the field of marine natural products, with several successful research programs underway, one in which the Andersen lab participates.

The efforts of several leading marine natural products groups have yielded a number of clinical candidates, several of which are in advanced clinical trials. Bryostatin 1 (1.32), isolated from the bryozoan *Bugula neritina* has been studied in a number of human clinical trials for its unique and selective interaction with protein kinase C isozymes, although this activity seems to be
most useful when combined with other cytotoxic compounds. Subsequent to this, the use of the natural product may be limited by supply, where it would be prohibitively expensive and/or destructive to collect the necessary amount of organisms to provide the compounds needed. As such, related synthetic compounds are often pursued due to the increased feasibility of using a synthetically attainable analogue. For example, the combined structures of the marine natural product psammaplin A (1.33) with information gleaned from trichostatin A (1.34) and trapoxin B (1.35) led to the development of NVP-LAQ824 (1.36), an anticancer clinical candidate having powerful histone deacetylase activity. Compound 1.36 is not clearly derived from any of compounds 1.33, 1.34 or 1.35, but is rather a synthetic derivative which possesses the pharmacophore of each of them.
One of the keys to a successful drug discovery program is the range of bioassays available to the chemists. Our laboratory has had a long and fruitful collaboration with the laboratory of Michel Roberge, also located at UBC. A particularly productive assay has been an antimitotic assay, which tests for compounds that arrest cells in mitosis using a TG-3 monoclonal antibody. Using this assay, desmethyleleutherobin (1.37) and five related novel analogues, were identified from the octocoral *Erythropodium caribaeorum*. Further studies on antimitotic extracts of the Caribbean sponge *Spirastrella coccinea*, also identified using this assay, are outlined in Chapter 3.
1.7 Cellular Signaling/Cannabinoid Receptors

A relatively recent area of study for our laboratory is a collaboration with the lab of Tom Grigliatti, also at UBC. A pilot screen of a subset of our marine extract library against insect cell cultures transfected with human G-protein-coupled receptors yielded a number of hits. The investigation of two of these hits is outlined in Chapter 4. This particular project focussed on two cannabinoid (CB) receptors, CB₁ and CB₂, and looked at both agonist and antagonist effects. Both CB₁ and CB₂ are membrane bound G-protein-coupled receptors, although CB₁ is concentrated primarily in the central nervous system, whereas CB₂ is concentrated in the immune system.

Although little has been done to research new CB active compounds, the CB receptors have been implicated in a number of disease states ranging from multiple sclerosis and cancer to schizophrenia and post-traumatic stress disorder. They are also thought to be involved in pain management and appetite control. As such, compounds which activate or deactivate these
receptors could be of use for therapy or further study of these receptors and their function.

The representative compounds which interact with CB receptors are anandamide (1.38) and (-)-$\Delta^9$-tetrahydrocannabinol (1.39). Compound 1.38 is an endocannabinoid, which is a molecule synthesized within the body that interacts with CB receptors. Compound 1.39 is one of a number of related cannabinoids found in the plant Cannabis sativa which interact with CB receptors in the human body. Beyond these two structure types, very few other small molecules have been found to interact with these receptors. The chemical diversity present in the marine environment is proposed to be a good place to start looking for new CB active compounds. Chapter 4 contains the results of the study of two sponge extracts using the CB assay.

![Figure 1.13 Structures of anandamide (1.38) and (-)-$\Delta^9$-tetrahydrocannabinol (1.39), two CB active compounds](image)

**1.8 Summary**

One important area of marine natural products research involves the examination of the secondary metabolites of marine organisms for novel and
bioactive compounds. This natural source of chemical creativity has already yielded a number of effective compounds, and research is ongoing on a number of promising lead structures. The studies described herein focus on three specific areas of research. In the second chapter the isolation and structure elucidation of a new antibiotic lipopeptide from a marine bacterium is described. In the third chapter the isolation and structure elucidation of a novel antimitotic macrolide from a marine sponge is described. The structure elucidation of this compound helps solve questions on the relative configuration of a related analogue. The final chapter describes the preliminary investigation of two sponges which showed interesting activity in the cannabinoid receptor assay.
Chapter 2: Tauramamide, A Novel Antibacterial Lipopeptide from the Marine Bacterial Isolate PNG-276

2.1 Introduction

Historically, the field of marine natural products had been dominated by compounds from sponges, coelenterates, bryozoans, molluscs, tunicates, echinoderms and algae. It is reasonable to conclude that this is due to their complex chemistry and ecology, as well as their relative ease of collection. Conversely, marine microorganisms were only rarely examined in the early days, primarily due to the difficulty in culturing these organisms. A number of studies, however, began to indicate the possibility that some secondary metabolites thought to be biosynthesized by the host organism (e.g. the sponge) are in fact metabolites produced by the vast array of microorganisms living in and on the sponge. Recent advances in sample collection and microorganism cultivation have allowed researchers to study these organisms in greater detail, accessing the huge potential of marine microbial compounds.

Marine bacteria are a vast and largely untapped resource of chemical diversity. Nevertheless, there are a growing number of success stories present in the literature. A large body of work on the culturing of chemically interesting marine microorganisms has been done by the research group of Dr. William Fenical, located at Scripps Institute of Oceanography, in La Jolla, California. Focusing largely upon the isolation and cultivation of marine actinomycetes, a number of new genera have been characterized, including the
marinomycin (2.1) producing genus *Marinospora* and salinosporamide A (2.2) producing genus *Salinospora*.\textsuperscript{109,110} Compound 2.1 was found to possess both antimicrobial activity and selective cytotoxic activity against cancer cell lines, whereas 2.2 was found to be highly cytotoxic against cancer cells, specifically by inhibiting proteasome activity. The proteasome is a multiunit enzyme complex that is responsible for most protein degradation in the cell apart from the lysozome, and is thought to be a good target for drug discovery due to its role in degrading regulatory proteins. Compound 2.2 shares this activity with omuralide (2.3), which possesses similar beta lactone functionality, although 2.2 is significantly more active than 2.3.\textsuperscript{110}

![Chemical structures of marinomycin A (2.1), salinosporamide A (2.2) and omuralide (2.3)](image-url)

**Figure 2.1** Marinomycin A (2.1), salinosporamide A (2.2) and omuralide (2.3)

Our research laboratory has also had a number of successes in the pursuit of marine natural products derived from marine bacterial isolates. Massetolide D (2.4) was one of a family of analogues isolated from cultures of marine pseudomonads MK90e85 and MK91CC8.\textsuperscript{111} These compounds were
found to be active against both *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* (MICs 2.5-10 μg/mL). Holyrines A (2.5) and B were isolated from cultures of marine actinomycete N96C-47. Holyrines are intermediates in the biosynthesis of the antifungal and anticancer compound staurosporine (2.6). 

**Figure 2.2** Massetolide D (2.4), holyrine A (2.5) and staurosporine (2.6)

The marine isolate PNG-276 is a particularly chemically prolific organism cultured from the tissues of an unidentified tube worm collected off the coast of Loloata Island, Papua New Guinea. Initial bioassays of crude extracts from
cultures of this isolate indicated strong antibiotic activity against a range of human pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), *Escherichia coli*, and *Candida albicans*. The bioassay-guided fractionation of PNG-276 culture extracts yielded a series of cyclic decapeptide antibiotics, loloatins A (2.7) -D. Of particular note is their strong antibacterial activity against MRSA, VRE and penicillin-resistant *Streptococcus pneumoniae* (MIC = 0.5-4 µg/mL), and lesser activity against the yeast *C. albicans* (MIC = 8-16 µg/mL). They possess many common features of nonribosomal peptides including D-amino acids and unusual amino acids (eg. hydroxyproline in loloatin D).

![Figure 2.3 Loloatin A (2.7)](image)

Further analysis of PNG-276 extracts yielded bogorol A (2.8), a novel cationic peptide antibiotic. This compound exhibits strong activity against MRSA and VRE (MICs of 2.0 and 10 µg/mL, respectively). As with the loloatins,
it too possesses D-amino acids and unique amino acids (in this case ornithine, and 2-amino-2-butenoic acid), and thus is also likely nonribosomal in origin. Unlike the loloatins, however, bogorol A is classified as a linear cationic peptide, and thus is thought to act primarily upon the cell membrane of target organisms.\(^\text{121}\) The presence of N-H NOESY correlations in bogorol A between adjacent amino acid residues supports the proposal of an α-helix secondary structure common among other linear cationic peptides. Cationic peptides are found in nature as part of the innate immune system, and may be potential tools against antibiotic resistant organisms.\(^\text{122,123}\)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{bogorol_a.png}
\caption{Bogorol A \((2.8)\)}
\end{figure}

Two antifungal compounds, basiliskamides A and B \((2.9, 2.10)\), were subsequently isolated from cultures of PNG-276, as were two acyldipeptides, tupuseleiamides A and B \((2.11, 2.12)\), with no known activity.\(^\text{124}\) Basiliskamides A and B were both strongly active against \(C.\ albicans\) (MICs of 1.0 and 3.1 \(\mu\)g/mL, respectively). Further testing against fresh cultures of \(C.\ albicans\)
demonstrated that 2.9 was as active as amphotericin B, a common benchmark for antifungal activity. Whereas 2.9 and 2.10 are polyketide in origin, 2.11 and 2.12 are of mixed polyketide and peptide origin.

![Structural formulas of compounds 2.9, 2.10, 2.11, and 2.12]

**Figure 2.5** Basiliskamides A and B (2.9 and 2.10), and tupuseleinamides A and B (2.11 and 2.12)

A survey of the structural classes produced by PNG-276 indicates that the majority of compounds are produced via nonribosomal peptide synthesis (NRPS). In standard ribosomal peptide synthesis, genetic information is translated from DNA via RNA, and leads to the assembly of peptides and proteins based upon the genetic code in the DNA sequence. This employs the 20 standard amino acids, all of which are in the natural L configuration. NRPS occurs via a completely different process, however, on which a number of excellent reviews have been written.\(^1\)\(^2\)\(^5\)\(^6\) Instead of being assembled on the ribosome, as standard peptides, nonribosomal peptides are assembled on large, multiunit enzymes or multi-enzyme complexes (Figure 2.6).
Figure 2.6 Schematic of peptide synthesis in a typical NRPS (adapted from Schwarzer et al.)\textsuperscript{126} A) The 4'-phosphopantetheine (4'PP) cofactor, bound covalently to the peptidyl-carrier protein (PCP) is, in its unloaded state, strongly attracted to the adenylation (A) domain, which selects the amino acid to be loaded. B) The loaded aminoacyl-S-4'PP is then attracted to the acceptor position of the upstream condensation-(C)-domain, and remains until the peptide bond is formed. C) After the peptide bond is formed, the peptidyl-S-4'PP becomes attracted to the donor position of the downstream C domain, setting the stage for the subsequent peptide bond to be formed.

As it does not rely upon the 20 amino acids in the genetic code, unique amino acids can be added to the peptide as it is formed. Instead, amino acids are selected for each step by the adenylation domains of the NRPS enzyme (Figure 2.6). The enzyme, much like an assembly line, passes the growing peptide between condensation domains. The “code”, as it were, is in the NRPS itself, not read off mRNA as occurs with traditional ribosomal biosynthesis.
The ability to incorporate novel amino acid substrates is not the only unique feature that NRPSs have at their disposal. Natural L amino acids can be epimerized to the D configuration on special epimerization (E) domains. Polyketide or fatty acid subunits can also be introduced due to the similarity between NRPS synthases and polyketide synthases. Small heterocyclic rings may be formed through cyclization of threonine, serine or cysteine residues on to the peptide backbone on heterocylizase (Cy) domains. Methylation of nitrogen on the amide backbone is also common, via S-adenosylmethionine (SAM) and a N-methylase (N-Mt) domain. All of these modifications to the standard peptide features engender new functions and stability to proteolysis in the resulting products.

It is reasonable to predict that studies of marine bacteria will continue to produce novel and potentially useful antibiotics. The relatively high numbers of bacteria present in the ocean ( >10^5 cells/mL seawater)^127, coupled with their unique environment and genetic diversity present optimal conditions for producing a large variety of secondary metabolites.^108 Indeed, recent studies indicate that marine microbial numbers and diversity may be even higher than previously estimated.^128 One feature of complex mixtures of competing organisms is the presence of a number of strategies for gaining an advantage over competitors. These range from differing growth rates, differing nutrient requirements and methods for sequestering limiting nutrients, to the use of and defense against inhibitory metabolites in the environment. If even a small fraction of these organisms have adapted secondary metabolites to gain
advantage over their competitors, there should be a wealth of antibiotics to discover. With this in mind, our research group continues to study bacteria isolated from marine habitats for new antibiotics.

2.2 Isolation and Characterization of Tauramamide Methyl Ester

The marine bacterial isolate PNG-276 was cultured from an unidentified tube worm collected at a depth of 15 m near Loloata Island, Papua New Guinea. The initial rationale for this study of PNG-276 cultures was to isolate more of the basiliskamides (2.9 and 2.10) for an unrelated project. The culture conditions and extraction techniques were therefore based largely upon those used to isolate 2.9 and 2.10. Subsequent analysis of $^1$H NMR of resultant C. albicans active fractions however identified tauramamide (2.13) as a novel and bioactive compound. This compound is named after Taurama point, which is adjacent to Loloata Island, Papua New Guinea, in the fashion of the loloatins, bogorols and tupuseleiamides. The isolation and structure elucidation of this lipopeptide became the primary focus of the project.
2.2.1 Isolation of Tauramamide Methyl Ester

PNG-276 was grown as confluent lawns on trays of solid tryptic soy agar supplemented with 1% NaCl to simulate marine conditions. Cells were collected by scraping the agar surface, and lyophilized to yield a brown powder (~30 g), prior to exhaustive MeOH extraction (3 X 250 mL). The combined methanol extract was concentrated in vacuo to yield a brown gum (3.8 g) and then partitioned between H₂O and EtOAc. The organic partition (1.0 g) was active against C. albicans. Lipophilic size exclusion chromatography (Sephadex LH-20®) in MeOH followed by subsequent partitioning size exclusion chromatography (Sephadex LH-20®) in 20:5:2 EtOAc/MeOH/H₂O yielded a number of fractions which were pooled based upon bioassay information, NMR and TLC characteristics. The most active fraction was then subjected to reversed-phase C-18 chromatography (Waters 2 g Sep-Pak®) eluting with a stepwise gradient from H₂O to MeOH. Bioassay, TLC and ¹H NMR were again used to pool the resultant fractions. The active material from this
chromatography (92.8 mg) was then separated by reversed-phase HPLC (40% MeCN/H₂O + 0.1 % TFA, CSC-Inertsil® ODS2 column) yielding tauramamide methyl ester (2.14, 7.4 mg, 0.02% dry cell weight).

2.2.2 Structure Elucidation of Tauramamide Methyl Ester

Tauramamide A methyl ester was isolated as a clear pale yellow solid that gave a [M+H]⁺ ion at m/z 878.5181 in high-resolution electrospray ionization time-of-flight mass spectrometry (HRESI-TOFMS), indicating a molecular formula of C₄₅H₆₇N₉O₉ (C₄₅H₆₈N₉O₉ calculated for m/z 878.5185) requiring 17 degrees of unsaturation. Analysis of ¹H and ¹³C 1D and 2D NMR (COSY, HMQC, HMBC, ROESY) data identified several amino acid fragments, which were assigned as follows. For clarity of discussion, the numbering scheme is shown in Figure 2.8. Each amino acid is numbered separately for greater ease of discussion.

![Structure of tauramamide methyl ester (2.14)](image)

Figure 2.8 Structure of tauramamide methyl ester (2.14)
Figure 2.9 500 MHz $^1$H NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$
Figure 2.10 150 MHz $^{13}$C NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$
Figure 2.11 500 MHz COSY NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$
Figure 2.12 500 MHz ROESY NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$. 
Figure 2.13 500 MHz HMQC NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$
Figure 2.14 500 MHz HMBC NMR spectrum of tauramamide methyl ester (2.14) recorded in DMSO-$d_6$
Table 2.1 1D and 2D NMR data for tauramamide methyl ester (2.14), recorded at 500 MHz (\(^1H\)) and 100 MHz (\(^13C\)) in DMSO-\(d_6\)

<table>
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<tr>
<th>C#</th>
<th>(^1C)</th>
<th>(^1H) (J)</th>
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<th>ROEESY</th>
<th>HMBC ((^1H) to (^13C))</th>
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<td>-</td>
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<td>-</td>
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<td>NH</td>
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<tr>
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<td>R4</td>
<td>-</td>
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<tr>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>0.71 d (6.3), 3H</td>
<td>L3</td>
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<td>L3, L4'</td>
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<td>4'</td>
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<td>Serine, Ser, S</td>
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<tr>
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<td>Y4/Y8, Y6</td>
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<td>Y5/Y8</td>
<td>Y6-OH</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>6-OH</td>
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<td>9.13 s</td>
<td>Y5/Y7</td>
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<td>OA3, OA5</td>
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<td>1.14 m, 2H</td>
<td>OA4, OA5</td>
<td>-</td>
<td>-</td>
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<td>1.07 m, 2H</td>
<td>OA5, OA7</td>
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<tr>
<td>7</td>
<td>27.0</td>
<td>1.44 bm (6.7)</td>
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<td>8</td>
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<td>0.83 d (6.7), 3H</td>
<td>OA7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8'</td>
<td>22.5</td>
<td>0.83 d (6.7), 3H</td>
<td>OA7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Analysis of the NMR data suggested that the molecule was a peptide based upon the readily observable α-methines producing resonances at δ 4-5 in the proton spectrum, as well as the backbone amide N-H protons which produced resonances at δ 7.5-8.5 in the proton spectrum which were correlated in a pairwise fashion in the COSY spectrum (Figure 2.15). Discussion of the spectra will thus first examine each individual amino acid residue, followed by connection to the adjacent residue.

Figure 2.15 Expansion of COSY NMR spectrum of tauramamide methyl ester (2.14), showing Hα-NH correlations

A proton which resonates at δ 4.29 (R1) showed a COSY correlation to an amide proton resonating at δ 8.39 (R-NH). Further sequential COSY correlations
from R1 to δ 1.67 (R2) and δ 1.77 (R2') establish the adjacent methylene protons. Both R2 and R2' subsequently correlate in the COSY spectrum to the methylene protons at δ 1.52 (R3), which in turn correlate to the methylene protons at δ 3.11 (R4). The R4 protons correlate in the COSY spectrum to δ 7.50 (R-NH'), which completes the side chain ¹H spin system. HMBC correlations from both δ 3.11 (R4) and δ 7.50 (R-NH') to δ c 156.7 (R5), identified the guanidinium carbon, and HMQC correlations assigned all other carbon signals as δ c 51.3 (R1), δ c 27.5 (R2), δ c 25.0 (R3) and δ c 40.6 (R4), respectively. A weak HMBC correlation from δ 4.29 (R1) to a carbon at δ c 172.1 (R-CO) indicated a carbonyl, which is established as a methyl ester by observation of an HMBC correlation from δ 3.62 to the same carbonyl at δ c 172.1 (R-CO). ROESY correlations are consistent with the COSY assignments, confirming the presence of an arginine residue, which accounts for two degrees of unsaturation. A ROESY correlation from δ 8.39 (R-NH) to δ 4.53 (W1) connects the arginine spin system to the adjacent amino acid. A summary of the data used to elucidate the arginine residue can be found in Figure 2.16.
Figure 2.16 COSY NMR spectrum, proton and carbon assignments, as well as select 2D correlations for arginine residue in tauramamide methyl ester (2.14)

The proton resonance at δ 4.53 (W1) showed a COSY correlation to a resonance at δ 8.14 (W-NH), as well as δ 2.89 (W2) and δ 3.15 (W2'). A ROESY correlation between δ 2.89 (W2) to δ 7.11 (W10) indicated an aromatic system proximate to the amino acid backbone. The proton resonance at δ 7.11
(W10) showed a COSY correlation to δ 10.76 (W-NH'), appropriate for an indole NH. This in turn showed a ROESY correlation to a proton resonance at δ 7.30 (W8), which correlated in the COSY spectrum to δ 7.04 (W7), and onwards in the COSY spectrum to δ 6.96 (W6), terminating with a correlation to δ 7.60 (W5). This spin system is also consistent with the presence of an indole. The protonated carbon signals were assigned using HMQC data as δ_c 123.9 (W10), δ_c 118.4 (W5), δ_c 118.0 (W6), δ_c 120.7 (W7) and δ_c 111.1 (W8), respectively. An HMBC correlation from δ 6.96 (W6) assigned δ_c 127.1 (W4), similarly an HMBC correlation from δ 7.04 (W7) assigned δ_c 136.0 (W9). No protons correlated to δ_c 109.8 (W3) in the HMBC spectrum, however it could be assigned by process of elimination following assignment of the tyrosine residue (vide infra). The carbonyl of the tryptophan residue remains unassigned due to insufficient data in the HMBC spectrum, however, the number of carbonyl resonances in the 13C spectrum (Figure 2.10) are consistent with the proposed structure. The completion of the assignment of the tryptophan residue accounts for seven degrees of unsaturation. A ROESY correlation from δ 8.14 (W-NH) to δ 4.20 (L1) connected the tryptophan residue to the next amino acid. A summary of the data used to elucidate the tryptophan residue can be found in Figure 2.17.
The methine proton resonance at δ 4.20 (L1) showed a COSY correlation to δ 7.75 (L-NH), as well as δ 1.06 (L2). Subsequent COSY correlations from L2 to δ 1.25 (L3), and on from L3 to δ 0.71 and δ 0.68 (L4, L4') complete the leucine \(^1\)H spin system. Protonated carbon resonances were assigned as δ c 51.7 (L1),
δc 40.5 (L2), δc 23.7 (L3), δc 22.8 (L4) and δc 21.5 (L4') using HMQC data, allowing assignment of the leucine residue, and accounting for one degree of unsaturation. A ROESY correlation from δ 7.75 (L-NH) to δ 4.22 (S1) connected the leucine residue to the following amino acid. A summary of the data used to elucidate the leucine residue is found in Figure 2.18.

Figure 2.18 COSY NMR spectrum, proton and carbon assignments, as well as select 2D correlations for leucine residue in tauramamide methyl ester (2.14)
The proton resonance at δ 4.22 (S1) showed a COSY correlation to δ 8.03 (S-NH), as well as δ 3.47 (S2) and δ 3.52 (S2'). COSY correlations were evident from δ 3.47 (S2) and δ 3.52 (S2') to a resonance at δ 4.83 (S-OH), completing the serine ¹H spin system. Protonated carbon signals were assigned as δc 55.1 (S1) and δc 61.7 (S2) using HMQC data, confirming the presence of a serine residue, and accounting for one degree of unsaturation. A ROESY correlation from δ 8.03 (S-NH) to δ 4.41 (Y1) connected the serine to the adjacent amino acid. A summary of the data used to elucidate the serine residue is found in Figure 2.19.
The proton resonance at $\delta$ 4.41 (Y1) showed a COSY correlation to $\delta$ 7.96 (Y-NH) as well as $\delta$ 2.64 (Y2) and $\delta$ 2.85 (Y2'). ROESY correlations from $\delta$ 2.64 (Y2) and $\delta$ 2.85 (Y2') to $\delta$ 7.02 (Y4/Y8) connected the aromatic spin system to the aliphatic spin system. A COSY correlation from $\delta$ 7.02 (Y4/Y8) to $\delta$ 6.62 (Y5/Y7) identified the vicinal protons while a ROESY correlation from $\delta$ 6.62 (Y5/Y7) to $\delta$ 9.13 (Y6-OH) completed the tyrosine spin system, which contains
an element of symmetry. Protonated carbon resonances were assigned as δ_c 54.5 (Y1), δ_c 36.5 (Y2), δ_c 130.0 (Y4/Y8) and δ_c 114.7 (Y5/Y7) using HMQC correlations. An HMBC correlation from δ 7.02 (Y4/Y8) to δ_c 155.7 (Y6), and an additional HMBC correlation from δ 6.62 (Y5/Y7) to δ_c 127.8 (Y3) established the quaternary carbon centres. The final amino acid is thus identified as tyrosine, which accounts for five degrees of unsaturation. A summary of the data used to elucidate the tyrosine residue is found in Figure 2.20.

Figure 2.20 COSY NMR spectrum, proton and carbon assignments, as well as select 2D correlations for tyrosine residue in tauramamide methyl ester (2.14)
A ROESY correlation from δ 7.96 (Y-NH) to a methylene at δ 2.01 (OA2) connects the tyrosine residue an aliphatic system via an amide linkage. Subsequent COSY correlations into an aliphatic envelope in the ¹H NMR spectrum are due to a lipid chain, which terminates in a geminal dimethyl at δ 0.83 (OA8/OA8'), and requires one degree of unsaturation. The lack of functionality evident in the 2D spectra, coupled with the mass spectral evidence indicated the lipid to be 7-methyloctanoic amide (Figure 2.8). Careful inspection of the COSY spectrum, as well as comparison with the 600 MHz data from tauramamide ethyl ester yielded the proton and carbon assignments found in Table 2.1. The remaining signal (δ 3.62, δC 51.8 (ME1)) is thus placed at the C-terminus of the peptide as a methyl ester, resulting in the complete constitution of tauramamide methyl ester (2.14), and contains the requisite 17 degrees of unsaturation (Figure 2.8). A summary of the ROESY data used to connect the amino acids in the chain is found in Appendix I.

2.2.3 LSIMS Information

Given the peptide nature of 2.14, sequence information was sought from liquid secondary ion mass spectrometry (LSIMS), a comparatively soft ionization technique which can frequently yield sequence information in peptides.¹ Full sequence information was not forthcoming, as the sample did not fragment as well as expected, however a few key ions were observed which were consistent with the proposed structure. The intact molecular species was observed both at

¹ Measurement performed by UBC Mass Spectrometry Service.
m/z 878.2 [M]$^+$ and m/z 879.2 [M+H]$^+$. Cleavage of the serine-tyrosine amide bond yields a smaller fragment at m/z 327 [F1+Na]$^+$ (Figure 2.21). Cleavage of the tyrosine residue adjacent to the benzylic carbon yields an intense fragment at m/z 197 [F2]$^+$. The complete LSIMS spectrum of 2.14 can be found in Appendix II.

![Fragment structure](image)

**Figure 2.21** Fragments observed in LSIMS spectrum of tauramamide methyl ester (2.14)

### 2.2.4 Comments on Tauramamide Methyl Ester

Although the 2D NMR data of the 2.14 was sufficient to determine the constitution of tauramamide, there were still some gaps which existed in the data due to the relative paucity of information from the HMBC. More material was needed for complete bioassay and configurational analysis (*vide infra*), thus an extraction of a second bacterial culture was performed.

Of particular interest in the structure of tauramamide methyl ester is whether or not the C-terminal ester is natural or an isolation artifact. Methyl
esters of this kind are occasionally formed as a result of repeated treatment with MeOH during extraction and chromatography. To test the hypothesis that the methyl ester was an artifact of the isolation conditions, the entire extraction was repeated on fresh cells using EtOH in place of MeOH during extraction and chromatography. Tauramamide ethyl ester was isolated from this second extract, illustrating that the methyl esters are in fact isolation artifacts and are not the true natural products themselves.

2.3 Isolation and Characterization of Tauramamide Ethyl Ester

2.3.1 Isolation of Tauramamide Ethyl Ester

The isolation of the ethyl ester proceeded using an abbreviated method from that used to purify tauramamide methyl ester. Cells were grown as confluent lawns on tryptic soy agar supplemented to 1% NaCl to simulate marine conditions. Cells were collected by scraping the agar surface, and lyophilized (~28g) prior to exhaustive EtOH extraction (3 X 250 mL). The ethanolic extract was dried \textit{in vacuo} to yield a brown gum and then partitioned between H$_2$O and EtOAc. The EtOAc soluble material was separated by lipophilic size exclusion chromatography (Sephadex LH-20$^\text{®}$) in EtOH and fractions were pooled based upon $^1$H NMR and TLC characteristics. The ethyl ester containing fraction (230.4 mg) was then subjected to reversed-phase C-18 HPLC (50% MeCN/H$_2$O + 0.1% TFA gradient to 70% MeCN/H$_2$O + 0.1% TFA over 40 minutes, CSC-
2.3.2 Structure Elucidation of Tauramamide Ethyl Ester

1D ($^1$H and $^{13}$C) and 2D NMR (COSY, ROESY, HSQC, HMBC) experiments were performed and the data is summarized in Table 2.2. The availability of a 600 MHz spectrometer equipped with a cryoprobe was a definite advantage for data acquisition, as correlations not observed using the 500 MHz instrument were now readily observed using comparable amounts of material. The structure of 2.15 differs from that of 2.14 only by an extra methylene in the ester, and thus the NMR data are quite similar, so the discussion of the structural elucidation is abbreviated. All one bond $^1$H-$^{13}$C correlations were established using HSCQ data. For clarity of discussion, the numbering scheme used during the structural elucidation of 2.15 is found in Figure 2.22.

![Figure 2.22](image)

**Figure 2.22** Constitution of tauramamide ethyl ester (2.15)
Figure 2.23 600 MHz $^1$H NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-$d_6$
Figure 2.24 150 MHz $^{13}$C NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-$d_6$
Figure 2.25 600 MHz COSY NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-$d_6$
Figure 2.26 600 MHz ROESY NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-$d_6$. 
Figure 2.27 600 MHz HSQC NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-\(d_6\)
Figure 2.28 600 MHz HMBC NMR spectrum of tauramamide ethyl ester (2.15) recorded in DMSO-$d_6$
Table 2.2  1D and 2D NMR data for tauramamide ethyl ester (2.15), recorded at 600 MHz ($^{1}$H) and 150 MHz ($^{13}$C) in DMSO-$d_6$

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<th>$^{1}$H</th>
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<th>ROESY</th>
<th>HMBC ($^{1}$H to $^{13}$C)</th>
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<td>1</td>
<td>14.1</td>
<td>1.18 t (7.2), 3H</td>
<td>EE2</td>
<td>EE2</td>
<td>EE2</td>
</tr>
<tr>
<td>2</td>
<td>60.5</td>
<td>4.09 m, 2H</td>
<td>EE1</td>
<td>EE1</td>
<td>EE1, R-CO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arginine, Arg, R</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>51.8</td>
<td>4.25 bs</td>
<td>R2, R-NH</td>
<td>R2, R2', R3, R4, R-NH</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.7 m</td>
<td>1.77 m</td>
<td>R1, R3</td>
<td>R1, R-NH</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25.1</td>
<td>1.53 m, 2H</td>
<td>R2, R2', R4</td>
<td>R1, R-NH'</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40.3</td>
<td>3.11 m, 2H</td>
<td>R3, R-NH'</td>
<td>R1, R-NH</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>156.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.43 d (7.7)</td>
<td>-</td>
<td>R1</td>
<td>R1, W1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.49 m</td>
<td>-</td>
<td>R4</td>
<td>R2, R2', R3, R4</td>
</tr>
<tr>
<td>7</td>
<td>172.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>171.7</td>
<td>4.54 bs</td>
<td>W2, W2', W-NH</td>
<td>W2, W2', W5, W10, W-NH, R-NH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>27.6</td>
<td>2.88 m</td>
<td>W1</td>
<td>W1, W10, W5, W-NH</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.16 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>118.4</td>
<td>7.62 d (7.7)</td>
<td>W6</td>
<td>W2', W1, W6</td>
<td>W3, W7, W9</td>
</tr>
<tr>
<td>6</td>
<td>118.1</td>
<td>6.96 dd (7.2, 7.7)</td>
<td>W5, W7</td>
<td>W5</td>
<td>W4, W6</td>
</tr>
<tr>
<td>7</td>
<td>120.7</td>
<td>7.04 m</td>
<td>W6, W8</td>
<td>W6</td>
<td>W5, W9</td>
</tr>
<tr>
<td>8</td>
<td>111.2</td>
<td>7.30 d (8.2)</td>
<td>W7</td>
<td>W7, W-NH'</td>
<td>W4, W6</td>
</tr>
<tr>
<td>9</td>
<td>136.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>123.9</td>
<td>7.11 d (1.5)</td>
<td>W-NH'</td>
<td>W1, W2, W-NH, W-NH'</td>
<td>W3, W4, W9</td>
</tr>
<tr>
<td></td>
<td>8.17 bd (8.2)</td>
<td>-</td>
<td>W1</td>
<td>W, W2, W10, L1, L-NH</td>
<td>-</td>
</tr>
<tr>
<td>NH</td>
<td>10.77 s</td>
<td>-</td>
<td>-</td>
<td>W10</td>
<td>W3, W4, W9, W10</td>
</tr>
<tr>
<td>NH'</td>
<td>7.76 c (8.2)</td>
<td>-</td>
<td>L1</td>
<td>L2, S1, S-NH, W-NH</td>
<td>L1, S-CO</td>
</tr>
<tr>
<td>CO</td>
<td>171.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>51.2</td>
<td>4.23 bs</td>
<td>L2, L-NH</td>
<td>L3, L4', W-NH</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>40.7</td>
<td>1.08 m, 2H</td>
<td>L1, L3</td>
<td>L-NH</td>
<td>L-CO</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.7</td>
<td>1.23 m</td>
<td>L2, L4, L4'</td>
<td>L1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22.8</td>
<td>0.70 d (6.6), 3H</td>
<td>L3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>21.5</td>
<td>0.67 d (6.7), 3H</td>
<td>L3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.62 c (8.2)</td>
<td>L1</td>
<td>L2, S1, S-NH, W-NH</td>
<td>L1, S-CO</td>
</tr>
<tr>
<td>CO</td>
<td>169.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>55.0</td>
<td>4.24 bs</td>
<td>S2, S2', S-NH</td>
<td>S2, S2', S2-OH, S-NH, L-NH</td>
<td>S-CO, S2</td>
</tr>
<tr>
<td>2</td>
<td>61.7</td>
<td>3.46 m</td>
<td>S1</td>
<td>S1, S2-OH, S-NH</td>
<td>S-CO</td>
</tr>
<tr>
<td>3</td>
<td>3.51 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-OH</td>
<td>4.84 t (5.4)</td>
<td>S2, S2'</td>
<td>S1, S2, S2'</td>
<td>S1, S2</td>
<td></td>
</tr>
<tr>
<td>NH</td>
<td>8.05 d (7.7)</td>
<td>S1</td>
<td>S1, S2, S2', S2-OH, L-NH, Y1</td>
<td>S1, Y-CO</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>171.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>54.5</td>
<td>4.42 bs</td>
<td>Y2, Y2', Y-NH</td>
<td>Y2, Y2', Y4/Y8, Y-NH, S-NH</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>36.6</td>
<td>2.63 m</td>
<td>Y1</td>
<td>Y1, Y4/Y8, Y-NH</td>
<td>Y1, Y3, Y4/Y8, Y-CO</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28.8</td>
<td>2.84 m</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4, 8</td>
<td>130.0</td>
<td>7.02 d (8.2), 2H</td>
<td>Y5/Y7</td>
<td>Y1, Y2, Y2', Y5/Y7, Y-NH</td>
<td>Y2, Y4/Y8, Y5/Y7, Y6</td>
</tr>
<tr>
<td>5, 7</td>
<td>114.7</td>
<td>6.61 d (8.2), 2H</td>
<td>Y4/Y8</td>
<td>Y4/Y8, Y6-OH</td>
<td>Y3, Y5/Y7, Y6</td>
</tr>
<tr>
<td>6</td>
<td>155.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6-OH</td>
<td>9.14 s</td>
<td>-</td>
<td>-</td>
<td>Y5/Y7</td>
<td>Y5/Y7, Y6</td>
</tr>
<tr>
<td>NH</td>
<td>7.99 d (7.7)</td>
<td>-</td>
<td>Y1</td>
<td>Y1, Y2, Y2', Y4/Y8, OA2</td>
<td>Y1, Y2, OA1</td>
</tr>
</tbody>
</table>

7-methyloctanoamide, OA

| 1  | 172.4   | -       | -      | -      | -               | -         |
| 2  | 35.1    | 2.00 m, 2H | OA3  | OA3, Y-NH | OA1, OA3, OA4 |
| 3  | 25.2    | 1.35 m, 2H | OA2, OA4 | OA2  | OA1 |
| 4  | 38.3    | 1.08 m, 2H | -      | -      | -               | -         |
| 5  | 1.14 m, 2H | -      | -      | -      | -               | -         |
| 6  | 38.3    | 1.08 m, 2H | -      | -      | -               | -         |
| 7  | 27.4    | 1.45 m | OA6, OA8, OA8' | OA8, OA8' | OA6, OA8, OA8' |
| 8  | 22.5    | 0.83 d (6.7), 3H | OA7  | OA7  | OA6, OA7 |
| 8' | 22.5    | 0.83 d (6.7), 3H | OA7  | OA7  | OA6, OA7 |
The data collected was completely consistent with the formation of the ethyl ester during isolation. The ethyl ester was readily evident by a $^1\text{H}$ signal at $\delta$ 1.18 (EE1) which showed a COSY correlation to a proton resonance at $\delta$ 4.09 (EE2). An HMBC correlation from a proton resonance at $\delta$ 4.09 (EE2) to a carbon resonance at $\delta_c$ 172.0 (R-CO) established the arginine residue at the C-terminus of the peptide. An HMBC correlation from $\delta$ 8.43 (R-NH) to a carbon resonance at $\delta_c$ 172.0 (R-CO) supports this assignment. Multiple ROESY and HMBC correlations were present which confirmed the peptide sequence as shown in Figure 2.29. It follows that the full constitution of tauramamide ethyl ester is 2.15.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Structure of tauramamide ethyl ester (2.15) showing selected inter-residue correlations}
\end{figure}
2.3.3 Comments on Tauramamide Ethyl Ester

The isolation of the ethyl ester from the EtOH extract, coupled with the absence of the methyl ester indicates that the ester is formed during the isolation steps. It is thus likely that the free acid is the true natural product, and the esters are simply formed as a result of repeated exposure to alcohol during extraction and chromatography. This metabolite was reproducibly synthesized by PNG-276.

2.4 Configurational Information

Marfey’s analysis\textsuperscript{130} of the 6M HCl hydrolysate of tauramamide methyl ester was performed to determine the configuration of the amino acids. A series of standards (both L- and D,L-mixtures) were reacted with Na\textsubscript{\((2,4\text{-dinitro-5-fluorophenyl})\text{-L-alaninamide (FDAA), and the derivatised standards analyzed by gradient HPLC to determine their relative retention times.\textsuperscript{131} Injections of the derivatised acid hydrolysate, as well as coinjections with standards confirmed four of the five amino acid configurations as outlined in Table 2.3. No peak was evident for tryptophan in the original hydrolysate, so a second hydrolysis was performed on fresh tauramamide methyl ester, this time under milder (1/16\textsuperscript{th} duration) hydrolysis conditions. Injections of the second hydrolysate, complete with coinjection of standards, provided the data necessary to determine the
configuration of the tryptophan residue. In all cases the FDAA-L conjugate eluted before the FDAA-D conjugate.

Table 2.3 Retention times (minutes) of FDAA-modified standards and derivatised tauramamide methyl ester acid hydrolysate from first hydrolysis

<table>
<thead>
<tr>
<th>FDAA-Amino Acid</th>
<th>L-FDAA</th>
<th>D-FDAA</th>
<th>Acid Hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>31.3</td>
<td>32.3</td>
<td>31.0 (L)</td>
</tr>
<tr>
<td>Leucine</td>
<td>55.9</td>
<td>62.8</td>
<td>61.6 (D)</td>
</tr>
<tr>
<td>Serine</td>
<td>35.1</td>
<td>36.9</td>
<td>35.4 (L)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>47.2</td>
<td>50.5</td>
<td>50.5 (D)</td>
</tr>
</tbody>
</table>

Table 2.4 Retention times (minutes) of FDAA-modified standards and derivatised tauramamide methyl ester acid hydrolysate from second hydrolysis

<table>
<thead>
<tr>
<th>FDAA-Amino Acid</th>
<th>L-FDAA</th>
<th>D-FDAA</th>
<th>Acid Hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>55.9</td>
<td>59.2</td>
<td>55.3 (L)</td>
</tr>
</tbody>
</table>

Marfey's analysis of tauramamide revealed the presence of two \( D \) amino acids (Leu and Tyr, Figure 2.30), further supporting the proposal that the compound is of nonribosomal origin. Certainly, the presence of non protein amino acids is not unusual for this organism, although it certainly adds to the novelty of tauramamide. The loloatins, bogorols and tupuseleiamides all feature \( D \) amino acids. It has been observed that \( D \) amino acids in oligopeptides give increased resistance to enzymatic proteolysis.\(^{123,132}\) Although no studies in our lab have focussed on this issue to date, it would be interesting to assemble a combinatorial library of the various configurational isomers of tauramamide, and potentially the other PNG-276 compounds to examine the relationship between their structure and antibacterial activity.
2.5 Synthesis of Tauramamide Ethyl Ester (2.15)

A complete synthesis of tauramamide ethyl ester (2.15) was performed by Alban Pereira, a fellow graduate student in the Andersen Lab, in order to confirm the stereochemical assignment of tauramamide as well as to investigate the importance of the C-terminal free carboxylic acid to antibacterial activity. The synthetic scheme followed is outlined in Figures 2.31 and 2.32. The details of this synthesis will be reported elsewhere.
Figure 2.31 Synthetic scheme for tauramamide: synthesis of the first subunit
Figure 2.32 Synthetic scheme for tauramamide: synthesis of the second subunit, final assembly and deprotection
Using the synthetic schemes outlined in Figures 2.31 and 2.32, samples of both the free acid (2.13) and ethyl ester (2.15) were prepared. They were then subjected to standard characterization techniques (MS, NMR, OR) and the resulting data compared with that of natural tauramamide ethyl ester (2.15).

The natural and synthetic ethyl esters of tauramamide were identical by both $^1$H NMR spectroscopy (Figure 2.33) and mass spectrometry comparison. The total synthesis of 2.15 confirms the structure proposed from analysis of the spectroscopic data of the isolated compound. The optical rotation data corroborates this assessment ($[\alpha]^\circ_{25} -31.9$ (c = 0.8 MeOH) for the natural product, $[\alpha]^\circ_{25} -23.4$ (c = 0.7 MeOH) for the synthetic) and confirms the assignment of tauramamide as containing $D$-leucine and tyrosine, and $L$-arginine, serine and tryptophan.
Figure 2.33. Comparison of synthetic and natural tauramamide ethyl ester. 

Synthetic

Natural

\(^1\)H NMR spectra (600 MHz, recorded in DMSO-\(d_6\))
2.6 Biological Activity of Tauramamide

Tauramamide was found to be antibacterial against MRSA, *C. albicans*, and *Enterococcus* sp. A number of bioassays were employed on both 2.15 and 2.13, the results of two of which are summarized in Table 2.5. The initial assay used to follow the activity during the isolation was the Alamar Blue™ assay, which uses a dye to measure the presence of proliferating pathogen cells.\(^{133}\) Dilutions of test compounds are placed into 96-well plates, and pathogen in growth medium is added. Examination of plates following an incubation period shows the presence of viable cells with a blue colour, and inhibition of growth with a pink colour. Compound 2.15 showed reasonable activity against MRSA using this method (MIC = 25 \(\mu\)g/mL), and slight inhibition of *C. albicans* at 200 \(\mu\)g/mL. Compound 2.13 showed no inhibition of these two pathogens, although it was observed to precipitate out of solution in the wells. Both were active against *Enterococcus* sp. at 0.1 \(\mu\)g/mL.
Table 2.5  Bioassay data (MIC) from both tauramamide (2.13) and tauramamide ethyl ester (2.15)

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Pathogen</th>
<th>Vancomycin µg/mL</th>
<th>Amphotericin B µg/mL</th>
<th>2.13 (µg/mL)</th>
<th>2.15 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar Inclusion</td>
<td>MRSA</td>
<td>0.625</td>
<td>-</td>
<td>200</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>C. albicans</td>
<td>-</td>
<td>No endpoint</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>Alamar Blue</td>
<td>C. albicans</td>
<td>-</td>
<td>-</td>
<td>ppt</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>MRSA</td>
<td>-</td>
<td>-</td>
<td>ppt</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Enterococcus sp.</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

More interesting was the data obtained from the agar inclusion bioassay. In this assay, small agar plates are made containing growth medium and pathogen of a standard concentration, and test compounds are diluted into multiple plates. Following incubation, plates are inspected for pathogen growth. The plate with the lowest concentration of compound that shows lack of growth gives the minimum inhibitory concentration. Standard antibiotics were also introduced as positive controls. Compound 2.15 was found to inhibit MRSA at a MIC of 9.4 µg/mL, which, while ten-fold less active than vancomycin (0.625 µg/mL), is still of interest, and considerably more active than 2.13 (200 µg/mL). However, 2.13 was more active than 2.15 (50 µg/mL vs. 75 µg/mL) against C. albicans. In addition, making the sample up in solid phase medium seems to remove the problems associated with precipitation of 2.13 in the Alamar Blue™ assay. The high activity against Enterococcus sp. (0.1 µg/mL) is uniform for both 2.13 and 2.15. This is indicative of even higher potential activity, given the
precipitation of sample which occurs in the Alamar Blue™ assay. However, neither 2.13 and 2.15 were tested in the agar inclusion assay against Enterococcus sp.

2.7 Discussion and Conclusions

The isolation of tauramamide esters from cultures of PNG-276 provides yet another example of a novel antimicrobial product from this organism. The isolation of both the methyl (2.14) and ethyl (2.15) esters of tauramamide from MeOH and EtOH extracts of PNG-276, respectively, indicates that the ester functionality is an artifact of the isolation, and thus the natural product is likely the free acid (2.13). Marfey's analysis of the amino acids indicated that two (leucine and tyrosine) of the five amino acids possess the D configuration. This, coupled with mixed biogenesis, indicates a non-ribosomal origin for the compounds. Independent synthesis of the free acid allowed for further biological testing to be done, resulting in the observation that the free acid is more active against C. albicans (MIC 50 μg/mL), whereas the ethyl ester is more active against MRSA (MIC 9.38 μg/mL).

The different bioassays employed help to explain the results observed during the isolation. The initial focus of this project was upon finding the known antifungal basiliskamides (2.9 and 2.10) from PNG-276 cultures. Upon treatment with MeOH in the extraction process and chromatography, however, the C. albicans activity seemed to weaken although the MRSA activity remained strong.
This is consistent with the conversion of 2.13 to 2.14 during the procedure. As for a putative structure activity relationship shown by the relative activities of 2.13 and 2.15, it is difficult to say which functionalities are required without further study. It may be that the change in charge due to esterification allows a different interaction with a specific target, or perhaps the cell membrane as a whole. With the ester in place, perhaps 2.15 interacts with the cell membrane in a different way, due to the net positive charge of the compound, and either is able to be transported across the membrane or not, depending on the nature of the membrane. Given the differences between eukaryotic and prokaryotic membranes, the difference in the effect of esterification of 2.13 on the C. albicans and MRSA activities is not unusual.

It is interesting to compare the structure of 2.13 with other metabolites obtained from the same organism. The lipid tail of 2.13 is identical to that present in tupuseleiamide A (2.11), although in that compound both the tyrosine and the serine are in the D configuration, and in the inverse order when compared to 2.13. The bogorol class of compounds possess D-leucine, and the loloatins have been found to contain D-tyrosine. All of these features, put together, provide the necessary building blocks to make the novel aspects of 2.13. The rest could be incorporated via normal metabolic pathways. It could be said that the species, in its ongoing process of experimentation and genetic manipulation, has come upon a particularly fruitful subset of molecular tools that can be used to produce a number of useful compounds. The discovery of
tauramamide is yet another testament to the versatility of these tools. It possesses features found in the bogorols, tupuseleiamides and loloatins.

Given the highly modular nature of non-ribosomal and polyketide biosynthesis, it is entirely consistent to see classes of compounds which contain substructures from several disparate classes of compounds. In fact, this modularity of polyketide and NRPS is currently being used to create new natural products.\textsuperscript{126,134} As discussed earlier the NRPS can contain any number of domains, each of which will have an impact on the end product produced by the enzyme. Including or excluding a given domain will form a different product.

In a sense, this is evidence of the wide array of biosynthetic pathways available to an organism. A mix and match method (like a biological version of combinatorial chemistry) is ideal for creating a large number of possible compounds. Over time, organisms which are successful at this might survive, while others may not. PNG-276 has certainly been shown to be one of these biosynthetically gifted organisms. It is also testament to the possibilities which exist in the realm of marine drug discovery. While much of the research on terrestrial microbes seems to be slowing, studies into marine microorganisms show great promise.
2.8 Experimental

2.8.1 General Experimental Procedures

2D-NMR spectra were recorded on either Bruker AMX-500 or AV-600 spectrometers. The AV-600 is equipped with a cryoprobe. $^{13}$C NMR data for tauramamide methyl ester (2.14) was recorded on a Bruker AM400 spectrometer while all other $^{13}$C NMR data was recorded on the AV-600 system. $^1$H NMR chemical shifts were referenced to the residual DMSO-$d_6$ signal (δ 2.50 ppm), and $^{13}$C NMR chemical shifts were referenced to the DMSO-$d_6$ solvent peak (δ 39.51 ppm). All NMR solvents were obtained from Cambridge Isotope Laboratories. All NMR data was processed using Bruker WINNMR® software.

All chromatography was performed using HPLC grade solvents from Fisher Scientific with no further purification. Water was purified using a Millipore MQ filter system. Reversed-phase chromatography was performed using 10, 5 or 2 gram Waters C-18 Sep-Paks. HPLC isolation was performed using a Waters Breeze HPLC system consisting of a Waters 1525 Binary HPLC Pump and Waters 2487 Dual Wavelength Absorbance Detector interfaced to a PC, whereas the Marfey’s analysis was performed using a Waters 600E system controller liquid chromatograph interfaced to a Waters 486 tunable absorbance detector and a Waters 994 programmable photodiode array detector. Optical rotations were measured with a JASCO J-1010 spectrophotometer using a 10 mm cell with a sodium light (589 nm). High-resolution electrospray mass spectra were collected on a Micromass LCT mass spectrometer, whereas low-resolution
mass spectra were collected on a Bruker Esquire LC mass spectrometer. Liquid secondary ion mass spectrometry (LSIMS) was performed on a Kratos Concept IIHQ mass spectrometer using a thioglycerol matrix. Amino acid standards were obtained from Sigma-Aldrich, as was the FDAA (Na-(2,4-dinitro-5-fluorophenyl)-L-alaninamide, D7906). PNG 276 was identified as *Brevibacillus laterosporus* by 16S RNA analysis.

### 2.8.2 Culture Conditions for PNG 276

PNG-276 was cultured on 21 pans of solid tryptic soy agar supplemented with NaCl to a final concentration of 1%. Each pan was 24 cm X 37 cm X 0.5 cm deep, and the cultures were grown at room temperature for five days. Live cells were then scraped from the solid agar and lyophilized.

### 2.8.3 Isolation of Tauramamide Methyl Ester (2.14)

For the general isolation scheme see section 2.2.1. Lyophilized cells (~30 g) were exhaustively extracted with MeOH (3 X 250 mL). The combined methanol extract was concentrated *in vacuo* to yield a brown gum (3.8 g) and then partitioned between H$_2$O and EtOAc, both of which were concentrated *in vacuo*. The organic partition (1.0 g) showed activity against *C. albicans*. Initial lipophilic size exclusion chromatography (Sephadex LH-20®) in MeOH was followed by partitioning size exclusion chromatography (Sephadex LH-20®) in
20:5:2 EtOAc/MeOH/H$_2$O yielding a number of fractions which were pooled based upon bioassay information, NMR and TLC characteristics ($R_f = 0.7$ on normal phase silica (4:1:1 n-BuOH:MeOH:H$_2$O, visualized with ceric sulfate/phosphomolybdic acid dip)).

The most active fraction (140.9 mg) was then subjected to reversed-phase C-18 chromatography (Waters 2 g Sep-Pak$^\circledR$) as follows. Sample was dissolved in MeOH, and adsorbed in vacuo onto reversed phase C-18 silica. A step gradient of 10 mL volumes of 10% increments of H$_2$O:MeOH (ie. 10 mL of 100% H$_2$O, then 10 mL of 90 % H$_2$O:MeOH, etc.) was performed, with an additional 10 mL steps of 50% MeOH:DCM and 100% DCM used to flush the column, resulting in 36 fractions. From this, TLC characteristics were again used to pool the fractions of interest.

This sample (92.8 mg) was then separated by repeated injections on isocratic reversed-phase HPLC (40% MeCN/H$_2$O + 0.1 % TFA, CSC-Inertsil$^\circledR$ ODS2 column, 2 mL/min flow rate, dual wavelength (210 nm and 254 nm) uv detection) yielding tauramamide methyl ester (2.14, 7.4 mg, 0.02% dry cell weight).

### 2.8.4 Tauramamide Methyl Ester (2.14) Physical Data

Clear, pale yellow glass (7.4 mg); $[\alpha]_D^{25} -14.6$ (c=0.6 MeOH). For 1D and 2D NMR data see Table 2.1. HRESIMS: [M+H]$^+$ $m/z = 878.5181$ (calcd for $C_{45}H_{67}N_9O_9$, 878.5185).
2.8.5 Acid Hydrolysis of Tauramamide Methyl Ester (2.14)

Purified 2.14 (0.8 mg, 0.9 μmol) was hydrolyzed in 0.5 mL of 6 N HCl, and sealed in a screw top vial. The mixture was heated to 108 °C with stirring for 16 hr. Upon cooling, the mixture was evaporated under N₂, and repeatedly under same from H₂O (3 x 0.5 mL) to removed traces of HCl.

A second hydrolysis was performed using purified 2.14 (0.2 mg, 0.2 μmol) under identical conditions, but heated for 1 hr instead of 16 hrs.

2.8.6 Preparation of FDAA Derivative Standards

Amino acid standards (2.0 μmol) were dissolved in H₂O (40 μL). To this was added Marfey's reagent (Na-(2,4-dinitro-5-fluorophenyl)-L-alaninamide, 62.5 mM in Me₂CO, 60μL), followed by NaHCO₃ (1 M, 20 μL). The mixture was heated for 1 hr at 43 °C, after which HCl (2 N, 10 μL) was added. An additional aliquot of Me₂CO (50 μL) was added to solubilize the samples. The acid hydrolysate of 2.14 was treated in the same fashion as the amino acid standards.
2.8.7 Marfey's Analysis of Tauramamide Methyl Ester (2.14)

Repeated HPLC injections were performed using an Alltech Econosil C18 5μm HPLC column, and run using the following solutions. Solution A was prepared by adding Et₃N (28 mL, 0.2 mol) to H₂O (3.572 L) while stirring. To this mixture, H₃PO₄ was added drop-wise until the mixture was pH 3.0. MeCN (400 mL) was then added, resulting in a mixture of 9:1 triethylammonium phosphate (50 mM, ph 3.0)/MeCN. Solution B was HPLC grade MeCN. The HPLC conditions used were a linear gradient from 100 % Solution A to 60 % Solution A/40 % Solution B over 40 minutes at a flow rate of 1 mL/min, with uv detection at 210 nm.

2.8.8 Isolation of Tauramamide Ethyl Ester (2.15)

Cells were cultured as before (Section 2.8.2). Cells were collected by scraping the agar surface, and lyophilized (~28g) prior to exhaustive EtOH extraction (3 X 250 mL). The ethanolic extract was dried in vacuo to yield a brown gum, and then partitioned between H₂O and EtOAc. The EtOAc soluble material was concentrated in vacuo, then dissolved in EtOH (5mL) and separated by lipophilic size exclusion chromatography (Sephadex LH-20®) in EtOH and fractions were pooled based upon ¹H NMR and TLC characteristics (Rf = 0.63 on reversed-phase C-18 silica, 100 % MeOH solvent, visualized with vanillin acid spray). The ethyl ester-containing fraction (230.4 mg) was then
subjected to reversed-phase C-18 HPLC (50% MeCN/H2O + 0.1% TFA linear gradient to 70% MeCN/H2O + 0.1% TFA over 40 minutes, CSC-Inertsil® ODS2 column, 2 mL/min flow rate, dual wavelength (210 nm and 254 nm) uv detection) yielding tauramamide A ethyl ester (2.15, 4.1 mg, 0.015% dry cell weight).

2.8.9 Tauramamide Ethyl Ester (2.15) Physical Data

Clear, pale yellow glass (2.1 mg); [α]D25 -31.9 (c = 0.8 MeOH). For 1D and 2D NMR data see Table 2.2. HRESIMS: [M+H]⁺ m/z = 892.5294 (calcd for C₄₆H₇₀N₉O₉, 892.5297).

2.8.10 Synthetic Tauramamide (2.13) Physical Data

Clear, pale yellow glass (5.8 mg); [α]D25 -51.8 (c = 0.9 MeOH). HRESIMS: [M+H]⁺ m/z = 864.4982 (calcd for C₄₄H₆₆N₉O₉, 864.4984).

2.8.8 Synthetic Tauramamide Ethyl Ester (2.15) Physical Data

Clear, pale yellow glass (3.7 mg); [α]D25 -23.4 (c = 0.7 MeOH). HRESIMS: [M+H]⁺ m/z = 892.5291 (calcd for C₄₆H₇₀N₉O₉, 892.5297).
Chapter 3: Methylspirastrellolide C, an Antimitotic Macrolide Isolated from the Marine Sponge Spirastrella coccinea

3.1 Introduction

Mitosis is the step in the eukaryotic cell cycle where replicated chromosomes are distributed into two daughter cells. It is an attractive target for cancer therapy, and is the focus for a number of classes of cancer chemotherapeutics.\textsuperscript{135,136} Although mitotic cancer cells can be targeted in a number of different ways, one of the classic methods is to target microtubule function. Microtubules are important structures in a mitotic cell that are vital for the proper separation and integrity of the DNA.\textsuperscript{137} The majority of antimitotic drugs are compounds which target microtubule function.

![Figure 3.1 The eukaryotic cell cycle](image-url)
The taxanes are a very well studied class of microtubule binding drugs, isolated initially from the bark of *Taxus brevifolia*.\textsuperscript{138} The natural product paclitaxel (Taxol\textsuperscript{®}, 1.4), along with the related synthetic analogue docetaxel (Taxotere\textsuperscript{®}, 3.1) are the two commercially available taxanes employed in the treatment of ovarian, lung, and metastatic breast cancers, as well as the management of early breast cancer.\textsuperscript{139} One of the many activities of the taxanes is to promote the assembly of microtubule polymers and stabilize the polymerized structure, thereby inhibiting the normal progression of mitosis, leading to apoptosis and cell death.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{docetaxel_paclitaxel.png}
\caption{Docetaxel (3.1) and paclitaxel (1.4)}
\end{figure}

This is in contrast to the function of the vinca alkaloids, such as vincristine (3.2) and vinblastine (3.3), which were isolated from the leaf extract of *Vinca rosea* by two groups independently in the late 1950s,\textsuperscript{140,141} and were initially thought to act by inhibiting microtubule formation by binding specifically in the *Vinca* domain of tubulin.\textsuperscript{142} It is now known, however, that they possess multiple types of activity, depending upon the drug concentration. At high concentrations
they indeed act as a microtubule depolymerizer, however, at low concentrations they stabilize microtubules.\textsuperscript{143} As is common with many biological systems, the seemingly straightforward activity of a compound is in reality much more complicated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.3}
\caption{Vincristine (3.2) and vinblastine (3.3)}
\end{figure}

Although the vinca alkaloids and the taxanes are some of the most prominent antimitotic agents currently known, a host of others have been discovered. In contrast to the plant compounds mentioned above, a large number of promising antimitotic agents have been isolated from marine sources. These will be discussed below, and grouped based upon their mode of action.

Discodermolide (1.27) is a polyketide isolated from the marine sponge \textit{Discodermia dissoluta} collected off Grand Bahama Island, and reported in 1990 as possessing both immunosuppressive and cytotoxic properties.\textsuperscript{144} Subsequent studies indicated that 1.27 halts cells in the G2 or M phase of the cell cycle at low concentrations (IC\textsubscript{50} 3-80 nM), and have also established the absolute configuration of 1.27 through synthesis.\textsuperscript{145} Compound 1.27 was found to bind on or near the same site on microtubules as paclitaxel, although with higher affinity,
and is thus thought of as a possible alternative or supplement to paclitaxel therapy. A number of syntheses have been performed to make 1.27, its enantiomer, and structural analogues. Novartis is currently conducting Phase I clinical trials with discodermolide.

![Figure 3.4 Discodermolide (1.27)](image)

**Figure 3.4 Discodermolide (1.27)**

Eleutherobin (3.4) is a diterpene glycoside originally isolated from *Eleutherobia* sp., a soft coral, and subsequently isolated from the soft coral *Erythropodium caribaeorum* along with related diterpene glycosides. Compound 3.4 was found to possess significant cytotoxicity against a range of cancer cell lines (IC$_{50}$ 10-15 nM) and it binds tubulin in a similar fashion to paclitaxel and discodermolide. Together with the development of paclitaxel analogues, eleutherobin has been used to help establish a common pharmacophore for tubulin stabilizing agents. Clinical studies of 3.4 have been hampered by inadequate supply.
A second class of antimitotic agents are exemplified by dolastatin 10 (3.5) and hemiasterlin (3.6). Compound 3.5 is one of a number of interesting bioactive compounds isolated from the sea hare *Dolabella auricularia* found to be extremely cytotoxic against P388 leukemia cells (ED$_{50}$ 0.1 ng/mL). Hemiasterlin was originally reported in 1994 from the sponge *Hemiasterella minor* collected in South Africa. Subsequently, analogues hemiasterlin A (3.7) and B (3.8) were isolated, and the synthetic analogue HTI-286 (3.9) developed. All were found to be potent cytotoxins active against a range of cancer cell lines. Both the hemiasterlins and dolastatin 10 are thought to act by binding tubulin in the vicinity of the *Vinca* binding site, and have been shown to competitively inhibit each other. Dolastatin 10 has been evaluated in a number of clinical trials, but did not pass Phase II due to a low therapeutic index. HTI-286 has passed Phase I clinical trials, and is in the process of being licensed for further studies.
Spongistatin 1 (3.10) is the first of a group of nine related compounds isolated from the sponges *Spongia* sp. and *Spirastrella spinispirulifera* by the Pettit group in the early 1990s, and reported as exhibiting growth inhibition against a number of chemoresistant cell types in the US National Cancer Institute’s panel of 60 human cancer cell lines.\(^{165}\) It was simultaneously reported by Kobayashi and co-workers from the Okinawan sponge *Hyrtios altum*.\(^{166}\) Subsequent studies showed that 3.10 bound to tubulin in the *Vinca* domain, and inhibited tubulin assembly, thus manifesting antimitotic activity.\(^{167}\) It was found that this binding is unlike both dolastatin 10 (3.5) and the vinca alkaloids, in that
a tubulin polymer is not formed upon treatment of cells with 3.10. It is not surprising that 3.10 interacts differently than 3.5, considering the marked difference in chemical structure between the two. Further analysis indicated that the unique activity of 3.10 may be attributed to the two spiroketal pyran moieties. A synthetic compound, SPIKET-P (3.11), exhibited similar activity to 3.10, and is currently under further investigation.168

Figure 3.7 Spongistatin (3.10) and SPIKET-P (3.11)

Microtubule function is not the only process that, when targeted for chemotherapy, can lead to cancer cell death via mitotic interference. Okadaic acid (3.12) was originally isolated from the Japanese sponge Halichondria okadai and the Caribbean sponge Halichondria melanodocia as a cytotoxin, but was subsequently identified as antimitotic agent acting through inhibition of serine/threonine protein phosphatase activity.169-171 It has subsequently been
shown that okadaic acid (3.12) and structurally similar compounds are produced by the marine dinoflagellates *Prorocentrum* and *Dinophysis* sp.\textsuperscript{172,173}

![Okadaic acid (3.12)](image)

**Figure 3.8** Okadaic acid (3.12)

Serine/threonine protein phosphatases are enzymes that catalyze the removal of phosphate from serine and threonine residues on phosphoproteins.\textsuperscript{174} Reversible phosphorylation is an important process used by the cell to modify the biological activity of a number of structural and regulatory proteins.\textsuperscript{175} Because of this important function, a number of studies now focus on finding inhibitors of protein kinases, enzymes which are responsible for adding phosphates to serine and threonine residues. Kinases outnumber the protein phosphatases by approximately 15:1 and were originally thought to be the main players in phosphorylation pathways, although it has been recently shown that both processes are important to cellular function.\textsuperscript{175} The protein phosphatases can be grouped genetically into two families: the PPP family (PP1, PP2A and PP2B), and the PPM family (PP2C).\textsuperscript{174} Okadaic acid was found to inhibit the activity of PP2A most strongly, and PP2C not at all.\textsuperscript{171} As PP2A has been
shown to play a central role in the cell cycle, it is thought that protein phosphatase inhibitors such as okadaic acid may become clinically useful.\textsuperscript{176,177}

Figure 3.9 Proposed role of protein phosphatase 2A (PP2A) in control of mitosis (adapted from Wera and Hemmings).\textsuperscript{178} PP2A controls activation of cyclin-dependant kinase 1 (cdk1) via inhibition of cdk1 activating kinase (CAK). Cdk1 activation is central in the cellular entry into mitosis. PP2A also is implicated in controlling levels of cdc25, a protein phosphatase that, among other functions, can itself activate cdk1. Inhibition of PP2A results in the loss of these and other controls, and results in uncontrolled entry into mitosis.

The structure of spirastrellolide A (3.13) was initially reported by our research group in 2003 as an antimitotic compound from the marine sponge \textit{Spirastrella coccinea}, and was amended in 2004 following further mass spectral and configurational analysis.\textsuperscript{179,180} Interestingly, although it is structurally similar to the spongistatins, it does not appear to interact with purified tubulin. In fact, 3.13 drove cells directly from S phase into mitosis, similar to okadaic acid and
other serine/threonine phosphatase inhibitors.\textsuperscript{176} It inhibited protein phosphatase 2A (PP2A) strongly (IC\textsubscript{50} = 1 nM) and protein phosphatase 1 (PP1) less effectively (IC\textsubscript{50} = 50 nM).\textsuperscript{180} No inhibition was observed for protein phosphatase 2C (PP2C).

![Figure 3.10 Spirastrellolide A (3.13)](image)

**Figure 3.10** Spirastrellolide A (3.13)

As well as 3.13, up to 15 other related macrolides were observed in the extract of *S. coccinea*. Unfortunately, limited supply and instability of the components initially prohibited analysis of the minor spirastrellolides. A recent recollection of *S. coccinea* permitted the analysis of these side fractions. In the following chapter the isolation and structural elucidation of methylspirastrellolide C (3.14), a novel antimitotic analogue of spirastrellolide A, is reported.
Compound 3.14 differs from 3.13 in three areas. It lacks the C28 chlorine, the C15-C16 alkene, and is hydroxylated at C8. While interesting due to its unique composition, the elucidation of 3.14 also contributed a valuable piece of information to the study of the relative configuration of spirastrellolide A. Prior to this study, only the relative configuration of three segments of 3.13 were known (Figure 3.12). This has been the focus of a great deal of study, with several synthetic groups attempting to synthesize the possible combinations of these subunits in the pursuit of the complete relative configuration of 3.13.\textsuperscript{181-186}
The relative configuration of these three areas of 3.13 was established using ROESY and ¹H-¹H coupling constant data. To establish the relative configuration of C₉-C₁₃, as well as that of C₂₁-C₂₃, acetonides were formed and analyzed.¹⁸⁰ The presence of the C₈ alcohol in 3.14, compared to 3.13, allowed the complete assignment of the relative configuration of the C₃⁻C₂₄ segment of the molecule using a similar analysis, without the formation of the acetonides. This is thus the first assignment of the C₃-C₂₁ relative configuration of the spirastrellolide core. It has been subsequently verified through the use of X-ray crystallographic techniques on a modified fragment of methylspirastrellolide B (3.15).¹⁸⁷ Using this recent information, the complete absolute configuration of 3.14 is proposed, with the exception of that of the C₄₆ alcohol, which remains unknown.

Figure 3.13 Structure of methylspirastrellolide B (3.15)
3.2 Isolation and Characterization of Methylspirastrellolide C

3.2.1 Isolation of Methylspirastrellolide C

*Spirastrella coccinea* was collected from reef walls at depth of 2-5 m near Capucin, Dominica. The sponge was extracted repeatedly with MeOH and resulting extracts were dried *in vacuo*. The resulting reddish gum was subjected to a number of solvent-solvent partitions (see Experimental), culminating in a CH$_2$Cl$_2$ extract which was evaporated to dryness to yield a potently antimitotic amorphous red solid (2.21 g).

At this point, the CH$_2$Cl$_2$ extract was methylated to simplify the isolation process. The sample was treated with diazomethane generated *in situ* by addition of trimethylsilyldiazomethane in hexanes to anhydrous MeOH in C$_6$D$_6$, and left stirring at room temperature for 16 hours. All reagents were evaporated, and the sample fractionated using normal phase step gradient silica gel chromatography (from 19:1 hexanes/EtOAc to MeOH). Two fractions, eluting with EtOAc (363.7 mg) and 1:9 MeOH/EtOAc (110.6 mg) were biologically active, and further separated using size exclusion chromatography (Sephadex LH-20, 4:1 MeOH/CH$_2$Cl$_2$). From these two separations were obtained potently antimitotic fractions (135.1 and 39.2 mg, respectively), both of which contained a complex mixture of methylspirastrellolides. Methylspirastrellolide A and a number of analogues were obtained from the first fraction using a range of reversed-phase HPLC conditions. The second fraction yielded impure

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$^1$ Initial isolation work performed by Dr. D. E. Williams, Andersen Lab, University of British Columbia.
methylspirastrellolide C using reversed-phase HPLC (3:1 MeOH/H$_2$O), as well as trace quantities of other analogues. This sample was subjected to further reversed-phase HPLC (gradient MeCN/H$_2$O) to yield pure methylspirastrellolide C (3.14, 1.8 mg).

Initial inspection of the proton NMR spectrum of 3.14 was promising, although subsequent collection of 1D and 2D data showed that the sample resonances changed over a period of weeks when stored in an NMR tube in C$_6$D$_6$ at -20 °C (Figure 3.14). This is likely due to small changes in the concentration of the sample, as well as absorption of small amounts of water from the surroundings. This complicated the analysis of data sets, as the same sample would yield slightly different 2D data over a period of several weeks due to minute changes in sample composition. Fortunately, more sponge had been collected and extracted for studies on other analogues, allowing for the isolation of fresh 3.14 in greater quantities than before.
3.2.2 Subsequent Isolation of Methylspirastrellolide C

*Spirastrella coccinea* was collected from reef walls at depths of 2-5 m near Capucin, Dominica. Sponge (22 kg) was extracted repeatedly with MeOH, and the resulting extracts dried *in vacuo*. The resulting reddish gum was

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\(^1\) Sponge workup prior to HPLC performed by Dr. K. Warabi, Andersen Lab, University of British Columbia.
partitioned between H₂O and EtOAc. The two layers were treated as follows (Figure 3.15).

Figure 3.15 Isolation scheme for methylspirastrellolide C (3.14)
The H$_2$O layer was extracted with $n$-BuOH, and the organic layer concentrated *in vacuo* prior to partition between hexanes and 4:1 MeOH/H$_2$O. The methanolic layer was adjusted to 4:2 MeOH/H$_2$O and extracted with CH$_2$Cl$_2$. The original EtOAc layer was dried *in vacuo* and partitioned between 4:1 MeOH/H$_2$O and hexanes. The methanolic layer was adjusted to 4:2 MeOH/H$_2$O, and extracted with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ layers from both original phases were combined and concentrated to yield a deep red oil (7.4 g). This was then methylated with trimethylsilyldiazomethane in anhydrous MeOH and toluene and stirred at room temperature for 18 hours. The reaction was quenched with acetic acid (6 mL, 0 °C), diluted with toluene, and evaporated to dryness. Methylation conditions are described in the Experimental at 3.5.4.

The resulting mixture was subjected to stepwise normal-phase silica gel chromatography (hexanes to MeOH), from which fractions were pooled according to TLC characteristics (0.66 g). This mixture was then separated by size exclusion chromatography (LH-20, 4:1 MeOH/CH$_2$Cl$_2$) to yield a mixture of methylspirastrellolides (0.13 g). Reversed-phase HPLC (3:1 MeOH/H$_2$O) produced pure methylspirastrellolide C (3.14, 4.4 mg), and related analogues.

Due to the relative instability of 3.14 all 1D and 2D NMR data needed to be collected within a short period of time. A sample of 3.14 (1.4 mg) was isolated and all data was collected immediately, over a 72-hour period on a Bruker AV-600 with cryoprobe.
3.2.3 Structure Elucidation of Methylspirastrellolide C

High-resolution electrospray mass spectrometric (HRESIMS) analysis of 3.14 indicated a molecular formula of C_{53}H_{86}O_{18}, indicating 11 degrees of unsaturation. Low-resolution ESIMS in CD_{3}OD yielded a pseudo-molecular ion of \( m/z \) 1040, whereas the corresponding measurement in CH_{3}OH yielded a pseudo-molecular ion of \( m/z \) 1034, indicating the presence of 6 exchangeable protons. Inspection of the 1D and 2D NMR data obtained for 3.14 (Figures 3.16-3.23) allowed the elucidation of its structure as follows. All one bond C-H correlations were established by analysis of the HSQC spectrum, and TOCSY and HSQC-TOCSY data were used minimally due to the large number of correlations and presence of superfluous data, and are thus reported only when utilized. A complete assignment of the \(^1\)H, \(^{13}\)C, COSY, HSQC, HMBC and ROESY spectra can be found in Table 3.1, as well as selected correlations from the HSQC-TOCSY and TOCSY. The numbering scheme for 3.14 is found in Figures 3.11, 3.16 and 3.17.
Figure 3.17 150 MHz $^{13}$C NMR spectrum of methylspirastrellolide C (3.14) recorded in C$_6$D$_6$
Figure 3.18 600 MHz COSY NMR spectrum of methylspirastrellolide C (3.14) recorded in C\textsubscript{6}D\textsubscript{6}
Figure 3.19 600 MHz HSQC NMR spectrum of methylspirastrellolide C (3.14) recorded in C$_6$D$_6$.
Figure 3.20 600 MHz HMBC NMR spectrum of methylspirastrellolide C (3.14) recorded in $C_6D_6$
Figure 3.21 600 MHz ROESY NMR spectrum of methylspirastrellolide C (3.14) recorded in C₆D₆
Figure 3.22 600 MHz TOCSY NMR spectrum of methylspirastrellolide C (3.14) recorded in C$_6$D$_6$
Figure 3.23 600 MHz HSQC-TOCSY NMR spectrum of methylspirastrellolide C (3.14) recorded in C₆D₆
Table 3.1 1D and 2D NMR data for methylspirastrellolide C (3.14) recorded at 600 MHz (1H) and 150 MHz (13C) in C6D6

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<th>1H (J)</th>
<th>COSY/TOSY</th>
<th>ROESY</th>
<th>HMBC (1H to 13C)</th>
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<td>1</td>
<td>169.9</td>
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<td>H45 2.35</td>
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The structure elucidation can be broken down into four sections of the molecule based upon contiguous $^1$H NMR spin systems, and will be discussed as such. The first segment, from C₁-C₁₇ (Figure 3.24), initiates at an ester carbonyl at δc 169.9 (C1), which correlates in the HMBC spectrum to two protons at δ 2.20 (H2) and δ 2.58 (H2'). From here, correlations in the COSY spectrum between δ 2.58 (H2') and δ 3.82 (H3) establish the adjacent proton. It is then possible in the COSY spectrum to work around the tetrahydropyran ring from H₄ to H₆ (Figure 3.25). The protons at δ 1.63 and δ 1.47 (H₆) correlate in the COSY to a broad doublet δ 3.97 (H₇). An HMBC correlation from δ 3.97 (H₇) to δc 74.4 (C₃) connects C₃ and C₇ via an ether oxygen, establishing the tetrahydropyran ring.
Figure 3.24 C$_1$-C$_{17}$ fragment of methylspirastrellolide C (3.14) with key correlations, $^1$H and $^{13}$C NMR assignments shown.

Figure 3.25 Expansion of COSY NMR spectrum, showing some correlations from C$_1$-C$_7$ in methylspirastrellolide C (3.14)
A COSY correlation from δ 3.97 (H7) to δ 3.54 (H8) establishes the adjacent carbinol methine. From here COSY correlations can be observed from δ 3.54 (H8) to δ 4.22 (H9), and onward to δ 1.77 (H10) and δ 2.37 (H10'). The methylene protons at δ 2.37 (H10') and δ 1.77 (H10) correlate in the COSY spectrum into an additional carbinol methine at δ 4.69 (H11), which correlates into the adjacent methylene protons at δ 2.02 and δ 1.52 (H12). All carbinol methines in the C₆-C₁₂ chain showed COSY correlations to their respective alcohol protons (Figure 3.26). HSQC information completes the assignment of the corresponding carbon atoms, and HMBC evidence corroborates this data (Table 3.1).

**Figure 3.26** Expansion of COSY NMR spectrum, showing correlations from C₇-C₁₂ in methylspirastrellolide C (3.14)
From the methylene protons at δ 2.02 and δ 1.52 (H12) a COSY correlation is observed into δ 3.74 (H13). This proton correlates into a quaternary carbon at δc 96.0 (C17) in the HMBC spectrum, suggesting the presence of a spiroketal moiety. A weak correlation in the COSY spectrum connects the resonance at δ 3.74 to δ 1.23, necessitating the analysis of TOCSY data to confirm this connection (Figure 3.27). Correlations in the TOCSY spectrum complete the spin system, connecting H13 to a tertiary methine at δ 1.23 (H14), which in turn correlates to a methyl at δ 0.80 (H48) and a methylene at δ 1.52 and δ 1.38 (H15). This final methylene correlates to δ 1.50 and δ 1.40 (H16) in the COSY spectrum, and all protons on C15 and C16 correlate in the HMBC spectrum into the C17 quaternary centre, completing the ring and the first substructure (Figure 3.24).

**Figure 3.27** Expansion of TOCSY NMR spectrum of methylspirastrellolide C (3.14)
Five additional protons correlate in the HMBC spectrum to δc 96.0, linking to the second fragment of methylspirastrrellolide C (Figure 3.28). The proton at δ 4.27 (H21) is one of these, and also shows a COSY correlation to the proton at δ 3.29 (H20). This proton further correlates into protons at δ 1.97 and δ 1.69 (H19), which subsequently correlate into protons at δ 1.23 and δ 1.61 (H18). All of the protons on C18 and C19 correlate in the HMBC spectrum into δc 96.0 (C17), completing what is now clearly a spiroketal ring system. A second COSY correlation from δ 4.27 (H21) to δ 4.20 (H22) provides the link to the adjacent carbinol methine, which in turn correlates to a second carbinol methine at δ 3.78 (H23). A COSY correlation from δ 3.78 (H23) to an aliphatic methine at δ 2.28 (H29) links to the adjacent atom, which correlates to a broad peak consisting of both the methyl at δ 1.30 (H50) and a methylene proton at δ 1.29 (H25). The lack of dispersion in the proton spectrum in this region required that HMBC data be used to verify these assignments.
Correlations in the HMBC spectrum from δ 3.78 (H23) to δc 20.0 (C50), δc 27.7 (C25) and δc 34.4 (C24) were observed (Figures 3.28 and 3.29). HSQC
information was used to identify the chemical shifts of the protons on these nuclei as δ 1.30 (H50), δ 2.27 (H25') and δ 1.29 (H25), and δ 2.28 (H24), respectively. With this information in hand, COSY correlations from δ 2.27 (H25') and δ 1.29 (H25) to δ 1.63 (H26) connect to the adjacent methylene in the chain. This in turn correlates to a methine at δ 3.69 (H27). COSY correlations from δ 3.69 (H27) to δ 1.97 (H28') and δ 1.18 (H28), and on to δ 3.75 (H29) and δ 2.16 (H30') and δ 1.40 (H30) complete the proton spin system (Figure 3.30). HMBC correlations from δ 2.16 (H30') and δ 1.40 (H30) and δ 3.69 (H27) to a quaternary carbon at δC 98.8 (C31) suggest a second spiroketal system, and complete the C17-C31 substructure (Figure 3.29). Alcohol protons on C22 and C23 were assigned through COSY correlations to H22 and H23, respectively.

Figure 3.30 Expansion of COSY NMR spectrum, showing correlations from C25-C30 in methylspirastrellolide C (3.14)
The third (C₃₁-C₃₅) spin system was elucidated as follows. Two methylene protons at δ 1.81 (H₃₂') and δ 1.44 (H₃₂) show HMBC correlations into both δc 98.8 (C₃₁) and δc 44.7 (C₃₀), placing them nearest the C₃₁ spiroketal. One set of methylene protons at δ 2.07 and δ 1.24 (H₃₃) show HMBC correlations to both δc 98.8 (C₃₁) and δc 109.0 (C₃₅), placing them next along the chain. A final methine proton at δ 1.51 (H₃₄) correlates in the HMBC to δc 109.0 (C₃₅), linking to the adjacent spiroketal. A COSY correlation from δ 1.51 (H₃₄) to δ 1.09 (H₅₂) establishes a methyl on C₃₄, and completes the C₃₁-C₃₅ substructure (Figure 3.31).

![COSY/TOCSY](image1.png) ![HMBC (¹H to ¹³C)](image2.png)

**Figure 3.31** C₃₁-C₃₅ fragment of methylspirastrellolide C (3.14) with key correlations, ¹H and ¹³C NMR assignments shown

The final substructure originates at both the C₃₅ spiroketal and the C₁ carbonyl. An HMBC correlation from δ 5.70 (H₃₇) to δc 169.9 (C₁) links this fragment to the C₁-C₁₇ fragment via an ester linkage. The proton at δ 5.70 (H₃₇) shows COSY correlations to δ 2.35 and δ 2.01 (H₃₆) (Figure 3.33), which along with δ 5.70 (H₃₇) correlate in the HMBC into δc 109.0 (C₃₅), thus linking to the
C_{17}-C_{31} fragment. An additional COSY correlation from δ 5.70 (H37) to an oxymethine at δ 4.29 (H38) establishes the adjacent atom, and places C36 next to the C35 spiroketal. The presence of the final ring is inferred both by the requirement of a ketal at C35 (δ_c 109.0), the necessity of oxygen substitution at C38 (δ_c 83.6). The proton at δ 4.29 (H38) correlates to a pair of methylene protons at δ 3.09 and δ 2.65 (H39), which in turn correlate to an alkenyl methine at δ 5.68 (H40). COSY correlations are evident down the side chain to δ 5.97 (H41), δ 2.79 and δ 2.69 (H42), δ 5.72 (H43), δ 5.44 (H44), δ 2.49 and δ 2.35 (H45), and δ 4.16 (H46) (Figure 3.33). An HMBC correlation from δ 4.16 (H46) to a carbonyl at δ_c 175.1 (C47) completes the side chain, yielding the C_{35}-C_{37} fragment (Figure 3.32).

**Figure 3.32** C_{35}-C_{47} fragment of methylspirastrellolide C (3.14) with key correlations, ^1H and ^13C assignments shown
Figure 3.33 Expansion of COSY NMR spectrum, showing correlations from $C_{36}^-$
$C_{46}$ in methylspirastrellolide C (3.14)
With the majority of the connectivity of methylspirastrellolide established, only a few more connections need to be made to complete the flat structure of 3.14. Protons from two methyl ethers are observed at δ 3.13 (H49) and δ 3.25 (H51), and correlate in the HMBC spectrum to δc 75.3 (C20) and δc 73.9 (C29), respectively. Protons at δ 3.31 (H53) are likewise established as a methyl ester by the presence of an HMBC correlation to δc 175.1 (C47). Two carbonyls, two double bonds, and the presence of a total of seven rings result in eleven degrees of unsaturation, as required by the high-resolution mass spectrometry measurements. A summary of key COSY and HMBC correlations can be found in Figure 3.34.

Figure 3.34 Complete structure of methylspirastrellolide C (3.14) with key correlations shown
3.2.4 Relative Configuration of Methylspirastrellolide C

The relative configuration of spirastrellolide A has been the focus of much study (vide supra).\textsuperscript{181-186} Previous data established the relative configurations in subunits of the molecule, however, gaps remained. Analysis of the ROESY spectrum of 3.14 in concert with $^3J$ coupling constant data allowed the connection of two of these subunits (C$_3$-C$_7$ and C$_9$-C$_{24}$). The dihedral angle between two vicinal protons can be inferred from the magnitude of their $^3J$ coupling constant.\textsuperscript{188} The orientation of the other substituents is then inferred by the presence of correlations in the ROESY spectrum. As the conformation of the C$_7$-C$_{13}$ chain is relatively rigid, the relative configurations of the two can be connected using this $^3J$ and ROESY information. For the purpose of clarity, unless otherwise noted all correlations are from the ROESY spectrum.

The C$_3$-C$_7$ tetrahydropyran ring is established in a chair conformation by axial ROESY correlations between $\delta$ 3.97 (H7), $\delta$ 3.82 (H3) and $\delta$ 1.30 (H5), as well as axial correlations between $\delta$ 1.63 (H6') and $\delta$ 1.04 (H4). A weak W (four bond) coupling in the COSY spectrum between $\delta$ 1.47 (H6) and $\delta$ 1.04 (H4) supports their placement in equatorial orientations. H3 and H7 are established as cis in the ring (Figure 3.35) due to their diaxial relationship. H7 is observed in the $^1$H NMR spectrum as a broad doublet ($^3J = 11.6$ Hz), trans to H6' ($\delta$ 1.63). The small coupling constant between H7 and H8 places the angle between them close to 90°. H8 is a broad doublet of doublets ($^3J = 8.6$ and 7.7 Hz), coupled both to C8-OH (7.7 Hz), and H9 (8.6 Hz), which must be anti due to the large
coupling constant. A ROESY correlation between δ 1.47 (H6) and δ 3.54 (H8), as well as between δ 4.69 (H11) and δ 3.54 (H8) fixes the C8-C9 bond in such a way that the C8-OH is anti to H7, and anti to the C9-OH (Figure 3.36). A weak ROESY correlation between δ 3.97 (H7) and δ 4.13 (C9-OH) further establishes C8-OH anti to H7. A consideration of the alternative configuration at C8 would require a ROESY correlation between H11 and H8 that would not be possible given the extended chain between the two centres.
Figure 3.35 A) ROESY information, B) proton assignments and coupling constant data for the C₃-C₁₃ segment of methylspirastrellolide C (3.14). C) An alternate configuration at C8 requires a ROESY coupling between very distant protons, thus this C8 configuration is not likely
Figure 3.36 Expansions of the ROESY NMR spectrum, showing key correlations from C₃-C₁₃ in methylspirastrellolide C (3.14)
The relative configurations established above are consistent with previous investigations of the relative configuration of spirastrellolide A (3.13), which is thought to possess the same relative configuration throughout as 3.14. Multiple ROESY correlations are observed which support the staggered structure of the C7-C13 chain (see Figures 3.35, 3.36 and Table 3.1), which is proposed for spirastrellolide A.180 In particular, the extensive ROESY correlations between the protons on C10 and the protons on C12 confirm their close proximity in space. A correlation between δ 4.13 (C9-OH) and δ 4.57 (C11-OH) conforms with their axial positions on the C7-C13 chain (Figure 3.35), as observed from the acetonide of spirastrellolide A (3.13). A correlation in the ROESY spectrum between δ 3.54 (H8) and δ 4.69 (H11), as mentioned previously, establishes close proximity between H8 and H11. A further ROESY correlation between δ 3.74 (H13) and δ 4.57 (C11-OH) establishes H13 in an axial conformation as shown in Figure 3.35. Further support for placing H13 axial in the C13-C21 segment is found below. Finally, ROESY correlations between δ 0.80 (H48) and δ 2.02 (H12'), as well as between δ 1.23 (H14) and δ 1.52 (H12) confirm the equatorial position of the C12-C13 bond in relation to the C13-C21 structure.

Analysis of ROESY data for the C13-C21 segment is consistent with that of 3.13.180 Diaxial ROESY correlations between δ 1.23 (H14) and δ 1.50 (H16'), as well as diaxial ROESY correlations between δ 1.38 (H15) and δ 3.74 (H13) place the ring in a chair conformation, with the C48 methyl and C12 alkyl chain trans and equatorial (Figure 3.37). Additional ROESY correlations between δ 3.74 (H13) and δ 4.27 (H21) as well as a weak correlation between δ 1.40 (H16) and
δ 1.61 (H18') establish the relative configuration of the spiroketal. Further diaxial ROESY correlations between δ 1.97 (H19') and δ 4.27 (H21), as well as between δ 1.23 (H18) and δ 3.29 (H20) set the second ring in a chair conformation, with the C21 alkyl chain and C49 methoxy group equatorial (Figure 3.38).

![Diagram of molecular structure with ROESY correlations](image)

**Figure 3.37** Summary of ROESY data for C13-C21 segment of methylspirastrellolide C (3.14)
Figure 3.38 ROESY NMR spectrum, showing key correlations from C$_{13}$-C$_{21}$ in methylspirastrellolide C (3.14)

The C$_{21}$-C$_{24}$ segment of the molecule can be established as follows. H$_{21}$ had previously been established as axial, and is observed as a broad doublet (9.8 Hz), \textit{anti} to H$_{20}$. The small coupling to $\delta$ 4.27 (H$_{21}$) places it \textit{gauche} to $\delta$ 4.20 (H$_{22}$). The proton signal at $\delta$ 4.20 (H$_{22}$) is observed as a doublet of doublets, coupled to $\delta$ 1.68 (C$_{22}$-OH) and $\delta$ 3.78 (H$_{23}$), placing H$_{23}$ \textit{anti} to H$_{22}$. Finally, ROESY correlations between $\delta$ 2.28 (H$_{24}$) and $\delta$ 4.20 (H$_{22}$), as well as between $\delta$ 3.13 (H$_{49}$) and $\delta$ 1.30 (H$_{50}$) orient the chain in such a way that the relative configurations are as shown in Figure 3.39. These are in accordance with data obtained from the acetonide of 3.13.
The key ROESY correlation that establishes the relative configuration of the C$_{27}$-C$_{39}$ subunit is observed between $\delta$ 3.69 (H27) and $\delta$ 4.29 (H38) (Figure 3.40). This places C30 and C36 cis on the C$_{31}$-C$_{35}$ ring, with H27 and H39 both facing into the bowl-like structure (Figure 3.41). Diaxial ROESY correlations between $\delta$ 1.40 (H30) and $\delta$ 1.18 (H28) place the C$_{27}$-C$_{31}$ ring in a chair conformation. Strong correlations between $\delta$ 3.69 (H27) and $\delta$ 3.75 (H29) to equatorial $\delta$ 1.97 (H28') support a cis orientation between C28 and C29. The C$_{31}$-C$_{35}$ ring is likewise in a chair conformation, as shown the diaxial ROESY correlation between $\delta$ 1.44 (H32) and $\delta$ 1.51 (H34). The axial proton $\delta$ 1.44 (H32) and the equatorial proton $\delta$ 2.16 (H30') also show a correlation in the ROESY, further fixing the relative configuration of the C31 spiro centre. Likewise a strong ROESY correlation between pseudo-equatorial $\delta$ 2.01 (H36) and axial $\delta$ 1.51 (H34) fix the configuration of the C35 spiro centre. Protons resonating at $\delta$ 4.29 (H38) and $\delta$ 5.70 (H37) are shown to be cis by a correlation in the ROESY.
spectrum, as is the pseudo-axial δ 2.35 (H36'). This analysis is consistent with that of 3.13, and is summarized in Figure 3.41.

Figure 3.40 ROESY NMR spectrum, showing key correlations from C_{27}-C_{38} in methylspirastrellolide C (3.14)
Figure 3.41 Summary of ROESY NMR data for C\textsubscript{27}-C\textsubscript{38} segment of methylspirastrellolide C (3.14)

The remaining relative configuration questions relate to the C\textsubscript{38}-C\textsubscript{47} side chain of 3.14. The C43-C44 double bond was established as cis due to the existence of a ROESY correlation between δ 5.72 (H43) and δ 5.44 (H44). A similar correlation appears to exist in the ROESY spectrum between δ 5.68 (H40) and δ 5.97 (H41), although the difference in phase to other ROESY correlations proves it to be due to a TOCSY correlation. The large coupling constant in δ 5.97 (H41) ($^3J = 15.3 \text{ Hz}$) is attributed to a trans coupling to δ 5.68 (H40). The configuration at the final chiral centre, at δ 4.16 (H46), is unassigned at this point.

By connecting the relative configuration of C\textsubscript{3}-C\textsubscript{8} to the C\textsubscript{9}-C\textsubscript{24} segment, a large portion of the overall relative configuration is in hand. As mentioned
previously, this is in full agreement with the relative configurations determined for methylspirastrellolide A,\textsuperscript{180} and those recently determined by X-ray crystallography of methylspirastrellolide B (3.15).\textsuperscript{187} The optical rotation of methylspirastrellolide C (3.14, $[\alpha]_D^{25} +17.6$ (c=0.9 CH$_2$Cl$_2$)) is also comparable to that obtained for methylspirastrellolide A (3.13, $[\alpha]_D^{25} +27$ (c=0.16 CH$_2$Cl$_2$)) and methylspirastrellolide B (3.15, $[\alpha]_D^{25} +44.7$ (c=0.5 MeOH)). Given the similarities of both ROESY and optical rotation data, the absolute configuration of methylspirastrellolide C is assigned as 3R, 7S, 8S, 9S, 11S, 13R, 14S, 17S, 20S, 21S, 22S, 23S, 24S, 27R, 29S, 31R, 34S, 35R, 37S, 38S.

3.3 Biological Activity of Methylspirastrellolide C

Methylspirastrellolide C (3.14) was tested against protein phosphatase 2A (PP2A), and found to be a potent ($IC_{50} \approx 1nM$) inhibitor, similar to methylspirastrellolide A.\textsuperscript{180} Further structure-activity relationship (SAR) studies are underway to look at the effect of structural changes on the observed biological activity of the compounds, however extremely limited supply currently hampers these efforts.
3.4 Discussion and Conclusions

With the isolation and elucidation of methylspirastrellolide C (3.14) complete, a third member is added to the number of known spirastrellolides. The elucidation is complicated by the complex nature of the NMR spectra observed, as well as the relative instability of the compounds upon isolation. The structural types thus elucidated present a common core, with typical polyketide variations on that core (hydroxylation and dehydration) as well as the less typical chlorination found in spirastrellolide A (3.13).

The sole barrier to establishing the absolute configuration of the methyspirastrellolides is the configuration at C46. Although a number of techniques exist which would allow the determination of the stereochemistry of this center in the natural product, this has not been possible as of yet due to the extremely limited supply of compound. More likely, the synthetic groups currently studying the compound will synthesize the appropriate core, and append side chains with different stereochemistry. Detailed analysis of the resulting products and comparison with the natural products may reveal the correct configuration. Alternatively, the center may be important for the biological activity of the compound, and thus the two resultant diastereomers may exhibit different biological activities. Regardless, this center will likely be assigned in the near future.

In addition to the three spirastrellolides already isolated, it appears from HPLC analysis that a number of other analogues are present in the
spirastrellolide-enriched fractions. The elucidation of another analogue simplifies the analysis of the remaining compounds, which in turn may shed some light upon SAR as their structures are elucidated and biological activities tested. There is much more to do with these fascinating compounds.

Indeed the spirastrellolides are prime examples of a typical marine natural products research project. The study of a sponge which showed anti-mitotic activity led to the discovery of an interesting and highly active compound, spirastrellolide A (3.13). While biologists studied the compound and established its mode of action, our laboratory studied the structure with the aim of establishing the absolute configuration of the compound. Other research labs began to synthesize increasingly complex portions of the molecule, and comparing the synthesized products with data obtained from the natural product to see what fit and what did not. After a re-collection of the same sponge, a number of analogues of methylspirastrellolide A were isolated. Analysis of one (methylspirastrellolide C (3.14)) revealed the small, but important inclusion of a C8 alcohol, which allowed the relative configuration of the C3-C23 segment to be established. Degradation and derivatization of a third analogue (methylspirastrellolide B (3.15)) allowed for the growth of crystals and the determination of the absolute configuration of the macrolide core. Both methylspirastrellolides B (3.15) and C (3.14) are highly active against protein phosphatase 2A, as is spirastrellolide A (3.13).
3.5 Experimental

3.5.1 General Experimental Procedures

All 1D and 2D NMR data was collected on a Bruker AV-600 spectrometer equipped with a cryoprobe (\(^1\)H at 600 MHz, \(^{13}\)C at 150 MHz). \(^1\)H chemical shifts were referenced to the residual \(\text{C}_6\text{D}_5\text{H}\) signal (\(\delta 7.16\) ppm), and \(^{13}\)C chemical shifts were referenced to the \(\text{C}_6\text{D}_6\) solvent peak (\(\delta_c 128.39\) ppm). All NMR solvents were obtained from Cambridge Isotope Laboratories.

All chromatography was performed using HPLC grade solvents from Fisher Scientific with no further purification. Water was purified using a Millipore reverse osmosis filter system. Normal phase chromatography was performed using Silicycle Ultra Pure silica gel (230-400 mesh) under compressed air. HPLC isolation was performed using a Waters 600E system controller liquid chromatograph interfaced to a Waters 486 tunable absorbance detector and a Waters 994 programmable photodiode array detector. Optical rotations were measured with a JASCO J-1010 polarimeter using a 10 mm cell. High-resolution mass spectra were collected on a Micromass LCT mass spectrometer, whereas low-resolution mass spectra were collected on a Bruker Esquire LC mass spectrometer. (Trimethylsilyl)diazomethane was obtained from Sigma-Aldrich (362832-5mL).
3.5.2 Initial Isolation of Methylspirastrellolide C

*Spirastrella coccinea* was collected from reef walls at depth of 2-5 m near Capucin, Dominica. The sponge (19 kg) was extracted repeatedly with MeOH (3 X 20 L), and resulting extracts dried in vacuo. The resulting reddish gum was partitioned between H$_2$O (6 L) and EtOAc (5 X 1 L), and the EtOAc partition evaporated to yield a red oil (42 g). This was partitioned between hexanes (4 X 300 mL) and 4:1 MeOH/H$_2$O (1000 mL). The methanolic layer was adjusted to 2:1 MeOH/H$_2$O (to a total of 1200 mL) and extracted with CH$_2$Cl$_2$ (4 X 200 mL). The CH$_2$Cl$_2$ extracts were evaporated to dryness to yield a potently antimitotic amorphous red solid (2.21 g).

At this point, the CH$_2$Cl$_2$ extract was methylated to simplify the isolation (see Experimental 3.5.4, *vide infra*). All reagents were evaporated under a stream of N$_2$, and the sample fractionated using normal phase silica gel chromatography (step gradient from 19:1 hexanes/EtOAc to MeOH). Two fractions, eluting with EtOAc (363.7 mg) and 1:9 MeOH/EtOAc (110.6 mg) were both biologically active, and further separated using size exclusion chromatography (Sephadex LH-20, 4:1 MeOH/CH$_2$Cl$_2$). From these two separations were obtained potently antimitotic fractions (135.1 and 39.2 mg, respectively) both of which consisted of a complex mixture of methylspirastrellolides as viewed by NMR. Methylspirastrellolid A and a number of analogues were obtained from the first fraction using a range of reversed-phase HPLC conditions. The second fraction yielded impure
methylspirastrellolide C using reversed-phase HPLC (3:1 MeOH/H₂O), as well as trace quantities of other analogues. This sample was subjected to further reversed-phase HPLC (gradient of 44% MeCN/H₂O to 50% MeCN/H₂O over 60 minutes) to yield pure methylspirastrellolide C (3.14, 1.8 mg). Small sample volume coupled with sample instability necessitated further sponge collection and extraction.

3.5.3 Second Isolation of Methylspirastrellolide C

*Spirastrella coccinea* was collected from reef walls at depth of 2-5 m near Capucin, Dominica. Sponge (22 kg) was extracted repeatedly with MeOH (4 X 22 L), and resulting extracts dried *in vacuo*. The resulting reddish gum was partitioned between H₂O (3.8 L) and EtOAc (9.3 L). The two layers were treated as outlined in Figure 3.15. The H₂O layer was extracted with n-BuOH (2.9 L), and the organic layer concentrated *in vacuo* prior to partition between hexanes (450 mL) and 4:1 MeOH/H₂O (500 mL). The methanolic layer was adjusted to 4:2 MeOH/H₂O (600 mL), and extracted with CH₂Cl₂ (400 mL). The original EtOAc layer was dried *in vacuo* and partitioned between 4:1 MeOH/H₂O (1000 mL) and hexanes (900 mL). The methanolic layer was adjusted to 4:2 MeOH/H₂O (1200 mL), and extracted with CH₂Cl₂ (800 mL). The CH₂Cl₂ layers from both original phases were combined and concentrated to yield a deep red oil (7.4 g). This mixture was then methylated (*vide infra*).
3.5.4 Methylation of Spirastrellolide Mixture

The deep red oil (7.4 g) was dissolved in anhydrous MeOH (40 mL) and toluene (160 mL), to which was added 30 mL of trimethylsilyldiazomethane (2.0 M in hexanes, 0 °C), and stirred at room temperature for 18 hours. The reaction was quenched with acetic acid (6 mL, 0 °C), diluted with toluene, and evaporated to dryness under a stream of N₂ followed by lyophilization to remove trace solvents.

3.5.5 Chromatography of Spirastrellolides

The resulting mixture was subjected to stepwise normal-phase silica gel chromatography (hexanes to MeOH), from which fractions were pooled according to TLC characteristics (0.66 g). This mixture was then separated by size exclusion chromatography (LH-20, 4:1 MeOH/CH₂Cl₂) to yield a mixture of methylspirastrellolides (0.13 g). Reversed-phase HPLC (Inertsil C-28 column, 3:1 MeOH/H₂O, 2 mL/min flow, uv detected at 203 nm) produced pure methylspirastrellolide C (3.14, 4.4 mg), and related analogues.

Due to the relative instability of 3.14 all 1D and 2D NMR data was collected immediately. A sample of 3.14 (1.4 mg) was isolated and all data was collected over a 72 hour period on a Bruker AV-600 with cryoprobe.
3.5.6 Methylspirastrellolide C Physical Data

Clear, pale yellow glass (1.8 mg); $[\alpha]_D^{25} + 17.6$ (c=0.9 CHCl₃). For 1D and 2D NMR data see Table 3.1. HRESIMS: [M+Na]$^+$ m/z = 1033.5721 (calcd for C₅₃H₈₆O₁₉Na, 1033.5712).
Chapter 4: Cannabinoid Receptor Studies

4.1 Cannabinoids and Cannabinoid Receptors

The cannabinoids are a group of naturally occurring compounds isolated from the *Cannabis sativa* L. plant, along with related terpenoids and flavonoids. *C. sativa* had been used medicinally for upwards of 5000 years, and was studied in great detail during a flurry of plant natural product research during the late 1800s and early 1900s.\(^{189-191}\) The first natural cannabinoid isolated was cannabinol (4.1) in 1899.\(^{192}\) It was acetylated and subjected to chemical degradation studies, although it was not tested for biological activity. Later studies resulted in the isolation of (-)-cannabidiol (CBD, 4.2), a cannabinoid now known to be a minor constituent of fresh *C. sativa* extracts.\(^{193}\) The structure of 4.2 was determined much later using early NMR techniques.\(^{194}\) The first report of a biologically active *C. sativa* compound was that of Δ⁹-tetrahydrocannabinol (Δ⁹-THC, 1.39) in 1964.\(^{195}\) A host of related compounds were subsequently isolated and characterized, on which a number of excellent reviews have been written.\(^{196-198}\)

![Chemical structures](image)

**Figure 4.1** Some compounds isolated from *C. sativa*
The increase in the number of studies on *C. sativa* during the mid-1960s and 70s can be attributed, in part, to the increase in its use as a recreational drug during that era. Studies focused on the psychoactive properties of *C. sativa*, and specifically, to the detriment of the study of the other pharmacological effects of the plant.\(^{190}\) However, a number of potential roles have been proposed for \(1.39\) including appetite stimulation, nausea control and pain relief.\(^{199}\) Compound \(4.2\) has been investigated for the management of cancer, acute schizophrenia, nausea, anxiety, epilepsy, glaucoma, inflammatory disorders, and for neuroprotection.\(^{200}\) Of particular interest is the fact that \(4.2\) seems to not carry the psychotropic affects associated with \(1.39\).

With the study of the plant cannabinoids (phytocannabinoids) and their effects on animal physiology came the natural questions regarding their mode of action. The search for receptors of the phytocannabinoid agonists produced a number of studies which established a dose-dependant, stereoselective response to phytocannabinoids, and eventually, cloning of the receptors themselves.\(^{201,202}\) There are two primary receptor subtypes, \(\text{CB}_1\) and \(\text{CB}_2\), located in the neuronal tissue of the nervous system and immune tissue, respectively.\(^{203}\) Both possess 7-transmembrane spanning segments, similar to rhodopsin, and bind their agonists at the central core of the subunits.\(^{204}\) Their mode of action is to inhibit adenylyl cyclase via associated G proteins, as well as modulating the function of potassium and calcium channels.\(^{201,202,205,206}\)
Several other structural classes of compounds have been observed to be active on the CB receptors. All told, there are currently thought to be four classes of CB agonists. The first type of agonist is the classical phytocannabinoid, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, 1.39), consisting generally of a dihydrobenzopyran-type structure, with an alkyl group at the C-3 aromatic position, and a hydroxyl group at the C-1 aromatic position (Figure 4.1). The second group is typically called the non-classical cannabinoids, as evidenced by CP 55,940 (4.3) and other synthetic analogues. These are typically more water-soluble and potent than classical cannabinoids, and thus have served as important tools in discovering and exploring the CB receptors. The third class of CB agonists is the aminoalkylindoles, such as WIN 55,212-2 (4.4). These are structurally different from the classical and non-classical cannabinoids, and are thought to bind to a slightly different site on the receptor. The fourth class of known CB agonists encompasses the eicosanoids, of which anandamide (1.38) was the first isolated from mammalian brain tissue. This was a particularly interesting discovery, as it was the first of the five known endogenous CB agonists to be discovered.
The topic of CB receptor antagonists is slightly more complicated due to the complexity of the receptors themselves. SR141716A (4.5) potently binds CB₁ receptors,²¹⁰ whereas SR144528 (4.6) strongly binds CB₂ receptors.²¹¹ Compound 4.5 has been observed to display both antagonist and inverse agonist activities, which is thought to be due to binding to two different sites on the CB₁ receptor.²¹² This inverse agonism arises from the interaction between constitutively activated CB receptor and 4.5. Compounds 4.7 (6-bromopraivadoline) and 4.8 (LY320135) also exhibit this mixed antagonist/inverse agonist activity, and are structurally similar to the aminoalkylindole agonists such as 4.4.²¹³,²¹⁴ Finally, O-1184 (4.9) acts on CB₁ as low efficacy agonist, with little of the inverse agonist activity associated with the other CB antagonists.²¹⁵ A great deal of work has gone into looking at these interactions, but for the purpose of this study it is sufficient to note that there are a host of possible interactions between ligands and CB receptors, including both

Figure 4.2 Structures of some CB agonists. Shown are CP 55,940 (4.3), WIN 55,212-2 (4.4), and anandamide (1.38)
antagonism and potential allosteric interactions. A number of excellent reviews explore this subject in greater detail.

![Structures of some CB antagonists](image)

**Figure 4.3** Structures of some CB antagonists. Shown are SR141716A (4.5), SR144528 (4.6), WIN54461 (4.7), LY320135 (4.8) and O-1184 (4.9)

Given the interesting activity shown in the endogenous cannabinoids, phytocannabinoids and synthetic compounds, it became clear that a wealth of information about these receptors could be gathered from the study of compounds which interact with them. The lab of Tom Grigliatti at UBC has studied the molecular biology of insects, and recently published a technique to study human G-protein-coupled receptors (GPCRs) in insect cell lines. To
advantages are noted for the use of insect cell lines: the cells do not possess the endogenous GPCRs found in mammalian cells (thus avoiding false positive results); and the GPCRs are properly expressed and modified post-translationally unlike those found in yeast cell lines (thus avoiding false negative results). The assay utilizes a light producing reaction to follow the interaction of an agonist or antagonist with the GPCR in question (Figure 4.4).

**Figure 4.4.** Schematic for a general GPCR bioassay (from Knight et al.)\textsuperscript{221} Human GPCR, Gα human G protein subunit and jellyfish aequorin reporter are expressed in an insect cell line. Binding of agonist to GPCR produces changes in GTP-bound Gα, which is linked via phospholipase Cβ (PLCβ) pathway to cellular Ca\textsuperscript{2+} levels. The aequorin photoprotein reports this change in Ca\textsuperscript{2+} levels with the creation of light and conversion of cellular O\textsubscript{2} to CO\textsubscript{2}.
A range of marine sponge extracts were tested in cells expressing a host of human GPCRs, including those expressing CB₁ and CB₂. Several extracts from our library were identified as having activity in this CB bioassay. The first extract was 03-039, subsequently identified as derived from *Stylissa massa* (Carter, 1881). The crude methanolic extract was found to be active primarily as a CB₂ antagonist with lesser agonism and antagonism of both CB₁ and CB₂. Bioassay guided fractionation of this extract yielded a complex mixture of A-nor steroids. Derivatization with *m*-bromophenyl isocyanate was performed on this mixture both to provide a chromophore for HPLC and to attempt recrystallization for X-ray analysis. After numerous unsuccessful attempts to grow crystals, repeated HPLC separation resulted in the purification and characterization of the 3-bromophenyl isocyanate derivative of 3β-hydroxymethyl-A-nor-5α-cholesta-22E-ene (4.10, hereinafter *A-nor*-C₂₇Δ²² derivative). The second was extract 03-253, identified as from *Hemiasterella aff. affinis*. The crude methanolic extract was found to be a CB₁ antagonist. Bioassay guided fractionation of this extract yielded a mixture of bengamides, primarily bengamide A (4.11). Both purified compounds failed to show activity in the CB assay.
4.2 A-nor-steroids

4.2.1 Isolation of A-nor-steroids from Stylissa massa (Carter, 1881)

The orange cake sponge *Stylissa massa* (Carter, 1881) was collected at a depth of 10 m near Kavieng, Papua New Guinea (2° 36.71' S, 150° 42.56' E) on Sept. 11, 2003 (Figure 4.6). During an assay of our library of marine extracts it was identified as possessing a number of activities in the CB bioassay, including both CB₁ and CB₂ agonism and antagonism.
A sample of the sponge was extracted repeatedly with MeOH. The organic extract was dried in vacuo to yield an orange paste (109.6 mg). A portion of this paste was then partitioned between H$_2$O and EtOAc, each of which was dried in vacuo. The organic fraction was subjected to normal phase silica gel chromatography (Hexanes/EtOac). TLC analysis resulted in the pooling of a steroid enriched fraction, which was then derivatized with m-bromophenyl isocyanate to both attempt crystallization for X-ray analysis and to provide a uv chromophore for HPLC analysis. Repeated HPLC separation of the resulting sample yielded increasingly pure steroid samples under carefully moderated conditions (MeOH/H$_2$O, C18) giving 0.6 mg of partially pure steroid derivative, followed by a second separation (propanol/H$_2$O, ODS2), ultimately providing a purified steroid derivative (0.1 mg). NMR and MS studies identified this sample as the A-nor-$\Delta^{22}$ steroid derivative (4.10).
4.2.2 Structure Elucidation of A-nor-Δ^{22} Steroid Derivative

Standard 1D and 2D NMR data sets were collected in C\textsubscript{6}D\textsubscript{6} on a Bruker 600 MHz spectrometer with a TXI probe. Spectra are shown in Figures 4.7-4.11. High-resolution mass spectrometry data indicated a molecular formula of C\textsubscript{34}H\textsubscript{50}NO\textsubscript{2}Br, requiring 10 degrees of unsaturation. A summary of the NMR data is found in Table 4.1. Only clearly discernable ROESY and TOCSY correlations are tabulated. Due to a paucity of material, \textsuperscript{13}C resonances were determined from HSQC and HMBC correlations.
Figure 4.7 600 MHz $^1$H NMR spectrum of 4.10 recorded in $C_6D_6$. 
Figure 4.8 600 MHz COSY NMR spectrum of 4.10 recorded in C₆D₆
Figure 4.9 600 MHz TOCSY NMR spectrum of 4.10 recorded in C₆D₆
Figure 4.10 600 MHz HSQC NMR spectrum of 4.10 recorded in C₆D₆
Figure 4.11 600 MHz HMBC NMR spectrum of 4.10 recorded in C₆D₆
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<td></td>
</tr>
<tr>
<td>34</td>
<td>122.0</td>
<td>7.23 m</td>
<td>H33</td>
<td>-NH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-NH</td>
<td></td>
<td>5.86 s</td>
<td></td>
<td></td>
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</table>
The structure elucidation of 4.10 is both complicated and simplified by the lack of functionality on the steroid nucleus. The lack of double bonds within the ring structure means that nearly all of the structure is a continuous spin system, allowing great use of extended 2D correlations. However, this lack of functionality also results in very little dispersion in the $^1$H spectrum, resulting in few unique resonances from which one can access the steroid core. A total of two spin systems are present, with one encompassing the whole of the sterol subunit and the second in the aromatic derivatized alcohol side chain. The TOCSY spectrum reveals valuable information on the relative connectivity in the aliphatic spin system, allowing a total assignment of the structure.

The primary carbinol at C4 is observed by a characteristic pair of multiplets at $\delta$ 4.07 (H4) and $\delta$ 4.29 (H4') which correlate in the HSQC into $\delta_c$ 69.4 (C4). The protons at $\delta$ 4.07 (H4) and $\delta$ 4.29 (H4') correlate in the COSY to a multiplet at $\delta$ 2.36 (H3), which correlates in the HSQC to $\delta_c$ 39.3 (C3). Correlations between $\delta$ 2.36 (H3) and all of $\delta$ 1.50 (H2), $\delta$ 1.96 (H2') and $\delta$ 1.34 (H5) establish the protons on the adjacent centres, with HSQC correlations establishing their respective carbon signals at $\delta_c$ 27.7 (C2) and $\delta_c$ 30.3 (C5). COSY correlations from $\delta$ 1.50 (H2) and $\delta$ 1.96 (H2') to $\delta$ 1.01 (H1) and $\delta$ 1.54 (H1') establish the adjacent centre. Both $\delta$ 1.01 (H1) and $\delta$ 1.54 (H1') correlate in the HSQC spectrum to a signal at $\delta_c$ 39.5 (C1), completing all protonated centres on the A ring (Figure 4.12). At this point COSY correlations became difficult to distinguish due to the lack of dispersion, thus COSY data was used in concert with TOCSY and HMBC data to work through the rest of the steroid.
A correlation in the COSY spectrum from $\delta$ 1.31 (H5) to both $\delta$ 1.25 (H6) and $\delta$ 1.56 (H6') established the adjacent centre in the B ring of the nucleus. Both $\delta$ 1.25 (H6) and $\delta$ 1.56 (H6') correlate in the COSY to $\delta$ 0.79 (H7) and $\delta$ 1.70 (H7') on the subsequent methylene, which in turn correlate in the COSY spectrum to a methine at $\delta$ 1.22 (H8). The observation of a correlation from $\delta$ 1.22 (H8) to at methine at $\delta$ 0.66 (H9) completes the protonated portion of the B ring. All protonated carbons were assigned from the HSQC spectrum as: $\delta_c$ 23.0 (C6), $\delta_c$ 33.1 (C7), $\delta_c$ 35.8 (C8) and $\delta_c$ 35.8 (C9), respectively. The remaining centre at C10 is the site of the C19 methyl group, and thus assigned by observation of HMBC correlations from the methyl singlet at $\delta$ 0.69 (H19) to $\delta_c$ 45.0 (C10). HMBC correlations were also observed from $\delta$ 0.69 (H19) to $\delta_c$ 39.5
(C1), $\delta_c$ 52.6 (C5) and $\delta_c$ 55.8 (C9), which allows the assembly of the completed A-B ring bicycle (Figure 4.13).

Figure 4.13 A) A-B ring structure showing COSY and HMBC correlations, B) $^1H$ assignments, and C) $^{13}C$ assignments

A correlation in the COSY spectrum from $\delta$ 1.22 (H8) to $\delta$ 0.96 (H14) establishes the adjacent methine in the structure. Correlations from $\delta$ 0.66 (H9) to both $\delta$ 1.33 (H11) and $\delta$ 1.42 (H11’) work around the other side of the C ring to establish the next centre. Both $\delta$ 1.33 (H11) and $\delta$ 1.42 (H11’) correlate to the adjacent methylene at $\delta$ 1.11 (H12) and $\delta$ 1.94 (H12’), which completes the assignment of the protonated carbons in the C ring. HSQC correlations are again used to establish the protonated carbons as $\delta_c$ 23.7 (C11), $\delta_c$ 42.5 (C12) and $\delta_c$ 56.7 (C14).
Correlations from $\delta$ 0.96 (H14) to $\delta$ 1.07 (H15) and $\delta$ 1.59 (H15') in the COSY spectrum establish the adjacent methylene in the D ring. Both $\delta$ 1.07 (H15) and $\delta$ 1.81 (H15') in turn correlate in the COSY to $\delta$ 1.37 (H16) and $\delta$ 1.81 (H16'), establishing the next methylene. A subsequent correlation from $\delta$ 1.37 (H16) and $\delta$ 1.81 (H16') to $\delta$ 1.17 (H17) in the COSY spectrum completes the protonated centres on the D ring. The remaining quaternary centre at C 13 is again assigned by a correlation in the HMBC spectrum from the attached C18 methyl group. In this case, an HMBC correlation from $\delta$ 0.70 (H18) to $\delta_c$ 40.5 (C13) assigns the centre, while additional HMBC correlations from $\delta$ 0.70 (H18) to $\delta_c$ 42.5 (C12), $\delta_c$ 56.7 (C14) and $\delta_c$ 56.5 (C17) establish the adjacent centres. HSQC information is used to assign all protonated carbons (Figure 4.14).

![Figure 4.14](image)

**Figure 4.14** A) C-D ring structure showing key COSY and HMBC correlations, B) $^1$H assignments, and C) $^{13}$C assignments.
A COSY correlation from $\delta$ 1.17 (H17) to $\delta$ 2.12 (H20) establishes the link from the core steroid to the side chain. The C21 methyl group is established by the presence of a COSY correlation from $\delta$ 2.12 (H20) to $\delta$ 1.14 (H21). A site of unsaturation is present on the side chain, as observed by a COSY correlation from $\delta$ 2.12 (H20) to $\delta$ 5.35 (H22), and onwards by a COSY correlation from $\delta$ 5.35 (H22) to $\delta$ 5.38 (H23). The $^3J$ coupling constant between H22 and H23 is 15.2 Hz, as measured from the $^1$H spectrum, indicating a trans relationship between H22 and H23. From $\delta$ 5.38 (H23) additional COSY correlations establish the neighboring protons as $\delta$ 1.17 (H24) and $\delta$ 1.97 (H24'), which in turn correlate into a methine at $\delta$ 1.61 (H25). The side chain terminates in a geminal dimethyl as shown by two signals at $\delta$ 0.93 (H26) and $\delta$ 0.95 (H27), each of which integrates for 3 protons. These correlate in the COSY spectrum from $\delta$ 0.93 (H26) and $\delta$ 0.95 (H27) to $\delta$ 1.61 (H25), establishing the final connection in the steroid side chain. HSQC correlations were used to assign the protonated carbons as $\delta_c$ 40.8 (C20), $\delta_c$ 21.4 (C21), $\delta_c$ 138.7 (C22), $\delta_c$ 126.7 (C23), $\delta_c$ 40.3 (C24), $\delta_c$ 29.0 (C25) and $\delta_c$ 22.6 (C26 and C27), respectively.
The aromatic residue is connected to the steroid core by observation of HMBC correlations from $\delta$ 4.07 (H4) and $\delta$ 4.29 (H4') to an amide carbonyl at $\delta_c$ 153.1 (C28). The amide NH is observed by a similar HMBC correlation from $\delta$ 5.86 (NH) to $\delta_c$ 153.1 (C28). The presence of ROESY correlations from $\delta$ 5.86 (NH) to $\delta$ 7.27 (H30) and $\delta$ 7.23 (H34) place these aromatic protons closest to the amide bond. Correlations in the COSY between $\delta$ 7.23 (H34) and $\delta$ 6.69 (H33), which itself correlates to $\delta$ 6.95 (H32) completes the assignment of protonated centres in the aromatic ring. HSQC correlations establish the carbon centres at $\delta_c$ 116.5 (C30), $\delta_c$ 126.0 (C32), $\delta_c$ 130.5 (C33) and $\delta_c$ 122.0 (C34),
respectively. The final centres were assigned by the observation of HMBC correlations from δ 7.23 (H34) to δc 123.0 (C29) and from δ 6.69 (H33) to δc 140.3 (C31). This places the bromine on C31 (Figure 4.16).

Figure 4.16 A) Key COSY, HMBC and ROESY NMR correlations in the side chain derivative, B) ¹H NMR assignments, and C) ¹³C NMR assignments

All of the above COSY correlations were supported by excellent data in the TOCSY spectrum, as listed in Table 4.1. This proved invaluable during the initial structural elucidation. Less data was available in the ROESY spectrum, although 1,3-diaxial correlations support the placement of C18 and C19 in the axial position, with a chair-chair relative configuration of the B-C rings. A ROESY correlation between δ 0.70 (H18) and δ 2.12 (H20) supports the
placement of the side chain in the pseudoaxial position. Likewise, the observation of a ROESY correlation between δ 2.36 (H3) and δ 1.31 (H5) indicates that H3 and H5 are cis, and since H5 is axial, C4 should be pseudoequatorial. A summary of the ROESY information can be found in Figure 4.17. Due to the extremely small sample size no optical rotation measurements were made.

![ROESY diagram]

**Figure 4.17** Relative configuration of 4.10, with key ROESY NMR correlations

### 4.2.3 Biological Activity of A-nor-Steroid

Initial bioassay results showed the full spectrum of CB activities in the 03-39 extract. However, as purification proceeded biological activity diminished in the resulting fractions. Due to small sample size the A-nor-C_{27}Δ^{22} derivative (4.10) was not bioassayed, however precursor fractions from which 4.10 was isolated, including one containing a mixture of non-derivatized A-nor-sterols showed no activity at relatively high concentrations (16.5 mM). This leads to one of two possible conclusions: either the biologically active compound was lost somewhere during the isolation, or the observed biological activity was a result of
a number of different compounds acting in concert on the CB receptor. A review of the literature shows some reference to membranolytic activity in sponge extracts. This is proposed to be due to $\Delta^7$-sterols present in a similarly complex mixture to that found in this sample of *Stylissa massa*. Given the membrane-spanning properties of the CB receptors, it is possible to imagine this as a more general membrane receptor interaction. At the least, the A-nor-sterols are identified as potential nuisance compounds in this assay.

This group of compounds has been the subject of some study. A great deal of work was performed on these and other related minor sterols from marine sponges by the laboratory of Carl Djerassi, particularly in the early 1980s. It has been shown that these compounds are produced in the sponge by contraction of the A ring of exogenous steroids. While they have been observed in a number of sponges in the order Axinellida, this is to the author’s knowledge the first report of A-nor-steroids from *Stylissa massa*, a sponge from the order Halichondria.

4.3 Bengamide A

4.3.1 Isolation and Characterization of Bengamide A from *Hemiasterella aff. affinis*

The orange fan sponge *Hemiasterella aff. affinis* was collected from a vertical wall at a depth of 15 m in Milne Bay, Papua New Guinea (10° 32.02' S, 150° 39.07' E) on September 17, 2003 (Figure 4.18). During an assay of our
library of marine extracts, it was identified as possessing CB₁ antagonism in the CB bioassay.

A sample of sponge was extracted repeatedly with MeOH. The methanolic extract was dried *in vacuo* to yield an orange paste. A sample of this paste (1.4 g) was then partitioned between H₂O and EtOAc, each of which was dried *in vacuo*. The EtOAc fraction was subjected to lipophilic size-exclusion chromatography (Sephadex LH-20®) in MeOH. The resulting fractions were submitted for bioassay, and one of the active fractions (81.7 mg) subjected to both normal and reversed-phase column chromatography with little success in purification as seen by TLC analysis. The resulting sample (34.8 mg) was

![Image](image.png)

Figure 4.18 *Hemiasterella aff. affinis* sponge, collected in Milne Bay, Papua New Guinea

A sample of sponge was extracted repeatedly with MeOH. The methanolic extract was dried *in vacuo* to yield an orange paste. A sample of this paste (1.4 g) was then partitioned between H₂O and EtOAc, each of which was dried *in vacuo*. The EtOAc fraction was subjected to lipophilic size-exclusion chromatography (Sephadex LH-20®) in MeOH. The resulting fractions were submitted for bioassay, and one of the active fractions (81.7 mg) subjected to both normal and reversed-phase column chromatography with little success in purification as seen by TLC analysis. The resulting sample (34.8 mg) was
separated by reversed-phase C-18 HPLC (MeOH/H$_2$O, ODS2 column) to yield a series of bengamide containing fractions. The main fraction (5.9 mg) was found to be active as a CB$_1$ agonist. Further HPLC purification was performed on this sample, yielding (2.7 mg) of relatively pure sample, as observed by TLC, NMR and MS analysis. NMR and MS studies identified this sample as bengamide A (4.11).

![Structure of bengamide A (4.11)](image)

**Figure 4.19** Structure of bengamide A (4.11)

### 4.3.2 Structure Elucidation of Bengamide A

Standard 1D and 2D NMR data sets were collected on a sample of 4.11 in CDCl$_3$ on a Bruker 600 MHz spectrometer with a TXI probe. Spectra are found in Figures 4.20-4.24. High-resolution mass spectrometry data indicated a molecular formula of C$_{31}$H$_{56}$N$_2$O$_8$, requiring 5 degrees of unsaturation. A summary of NMR data is found in Table 4.2.
Figure 4.20 600 MHz $^1$H NMR spectrum of bengamide A (4.11) recorded in CDCl$_3$. 

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Figure 4.21 150 MHz $^{13}$C NMR spectrum of bengamide A (4.11) recorded in CDCl$_3$.
Figure 4.22 600 MHz COSY NMR spectrum of bengamide A (4.11) recorded in CDCl₃
Figure 4.23 600 MHz HSQC NMR spectrum of bengamide A (4.11) recorded in CDCl₃
Figure 4.24 600 MHz HMBC NMR spectrum of bengamide A (4.11) recorded in CDCl$_3$
<table>
<thead>
<tr>
<th>C#</th>
<th>$^1$H ($J$)</th>
<th>COSY</th>
<th>HMBC</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>22.4 0.98 d (3.3)</td>
<td>H2</td>
<td>C3</td>
</tr>
<tr>
<td>2</td>
<td>31.0 2.30 m (6.5)</td>
<td>H1, H3</td>
<td>C1, C3, C4, C15</td>
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<tr>
<td>3</td>
<td>142.2 5.77 dd (6.5, 15.4)</td>
<td>H2, H4</td>
<td>C1, C2, C5, C15</td>
</tr>
<tr>
<td>4</td>
<td>125.6 5.43 dd (6.8, 15.4)</td>
<td>H3, H5</td>
<td>C2, C5, C6</td>
</tr>
<tr>
<td>5</td>
<td>74.5 4.21 m</td>
<td>H4, H6</td>
<td>C3, C4, C6</td>
</tr>
<tr>
<td>6</td>
<td>72.5 3.59 bs</td>
<td>H5</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>73.0 3.81 bd (7.0)</td>
<td>H8</td>
<td>C5, C8, C9</td>
</tr>
<tr>
<td>8</td>
<td>81.1 3.77 bd (7.0)</td>
<td>H7</td>
<td>C6, C7, C31</td>
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<tr>
<td>9</td>
<td>172.6 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>51.7 4.57 dd (6.1, 10.4)</td>
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<td>C9, C11, C12, C16</td>
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<tr>
<td>11</td>
<td>29.1 1.72 ddd (2.5, 13.3, 24)</td>
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<td>C10, C13, C16</td>
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<tr>
<td>12</td>
<td>33.2 1.94 ddd (3.7, 13.5, 24.5)</td>
<td>H11, H11', H13</td>
<td>C10, C13, C14</td>
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<tr>
<td>13</td>
<td>71.0 4.63 m (10.5, 3.3)</td>
<td>H12, H12', H14, H14'</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>45.3 3.29 bdd (8.0, 14.5)</td>
<td>H13, N_b</td>
<td>C12, C13, C16</td>
</tr>
<tr>
<td>15</td>
<td>22.3 0.97 d (3.3)</td>
<td>H2</td>
<td>C3</td>
</tr>
<tr>
<td>16</td>
<td>174.2 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>173.1 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>34.6 2.28 t (7.6)</td>
<td>H19</td>
<td>C17, C19</td>
</tr>
<tr>
<td>19</td>
<td>25.1 1.58 m (7.1)</td>
<td>H18, H20</td>
<td>C17, C18, C21-28</td>
</tr>
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<td>20</td>
<td>22.9 1.28 m</td>
<td>H19, H21-28</td>
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</tr>
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<td>21-28</td>
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<td>Aliphatic envelope</td>
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<td>29</td>
<td>32.1 1.24 m</td>
<td>H21-28, H30</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>14.3 0.86 t (6.9)</td>
<td>H29</td>
<td>C29</td>
</tr>
<tr>
<td>31</td>
<td>60.3 3.53 s</td>
<td>C8</td>
<td>-</td>
</tr>
<tr>
<td>N_a</td>
<td>- 7.98 d (6.1)</td>
<td>H10</td>
<td>C9, C10, C16</td>
</tr>
<tr>
<td>N_b</td>
<td>- 5.95 bs</td>
<td>H14, H14'</td>
<td>C10, C16</td>
</tr>
</tbody>
</table>

Compound 4.11 consists of three separate proton NMR spin systems. Each of these can be readily assembled using COSY data, and the three connected using HMBC correlations. Six methyl protons at δ 0.98 (H1) and δ 0.97 (H15) correlate in the COSY spectrum to a tertiary methine at δ 2.30 (H2), which then correlates into an alkenyl methine at δ 5.77 (H3). This correlates in the COSY to another alkenyl methine at δ 5.43 (H4), and the olefin configuration is established as *trans* to H3 due to the large $^3$J coupling constant (15.4 Hz) between them. A COSY cross peak between δ 5.43 (H4) and δ 4.21 (H5) establishes the next proton in the chain. A COSY correlation between δ 4.21 (H5) and δ 3.59 (H6) establishes the subsequent centre, which weakly correlates in the COSY spectrum to the proton at δ 3.81 (H7). A final COSY correlation...
from δ 3.81 (H7) to δ 3.77 (H8) completes the proton spin system (Figure 4.25).
The HSQC spectrum was used to assign protonated carbons, and HMBC correlations used to confirm COSY information. HMBC correlations from both δ 3.81 (H7) and δ 3.77 (H8) to a carbonyl at δC 172.6 (C9) establish the adjacent centre in the chain. Three methoxy protons at δ 3.53 (H31) are placed on C8, as a strong HMBC correlation to δC 81.1 (C8) is observed. The chemical shifts of C5-7 establish them as being hydroxylated.

Figure 4.25 A) Fragment of bengamide A (4.11) showing key COSY and HMBC NMR correlations, B) ¹H NMR assignments, and C) ¹³C NMR assignments

The second spin system begins at a methine at δ 4.57 (H10), which correlated in the COSY spectrum into an adjacent methylene at δ 1.72 (H11) and δ 2.14 (H11'). Both δ 1.72 (H11) and δ 2.14 (H11') correlate in the COSY spectrum to the adjacent methylene at δ 1.94 (H12) and δ 2.19 (H12'), which in turn correlated in the COSY spectrum to an oxymethine at δ 4.63 (H13). A
correlation from $\delta$ 4.63 (H13) to a methylene at $\delta$ 3.29 (H14) and $\delta$ 3.36 (H14') established the adjacent methylene. A final correlation in the COSY spectrum between $\delta$ 3.29 (H14) and $\delta$ 5.95 (N_b) established a link to the amide bond. All of $\delta$ 4.57 (H10), $\delta$ 3.29 (H14) and 3.36 (H14') show strong HMBC correlations to a carbonyl at $\delta_c$ 174.2 (C16), which places the amide between C10 and C6. HSQC data was used to establish the carbon resonances of this fragment as $\delta_c$ 51.7 (C10), $\delta_c$ 29.1 (C11), $\delta_c$ 33.2 (C12), $\delta_c$ 71.0 (C13), $\delta_c$ 45.3 (C14) and $\delta_c$ 174.2 (C16), respectively. HMBC data was consistent with these assignments.

Figure 4.26 A) Fragment of bengamide A (4.11) showing COSY and HMBC NMR correlations, B) $^1$H NMR assignments, and C) $^{13}$C NMR assignments

The final fragment of bengamide A (4.11) was established by an HMBC correlation from $\delta$ 4.63 (H13) to a carbonyl at $\delta_c$ 173.1 (C17). Two methylene protons at $\delta$ 2.28 (H18) also correlated in the HMBC spectrum to $\delta_c$ 173.1 (C17), which places an ester between C13 and C17. COSY correlations from $\delta$ 2.28
(H18) proceed down the alkyl chain into an aliphatic envelope at δ 1.18-1.32. The chain terminates at δ 0.86 (H30) in a methyl triplet.

![Figure 4.27 A) Fragment of bengamide A (4.11) showing COSY and HMBC correlations, B) $^1$H assignments, and C) $^{13}$C assignments](image)

Connecting the three fragments was relatively straightforward, yielding the planar structure of 4.11. Given that the optical rotation data is relatively close to the literature value ([α]$_D^{25}$ + 51.3 (c =0.9 MeOH) measured, [α]$_D^{25}$ + 30.3 (c =0.081 MeOH) literature value), it was reasonable to propose the same absolute configuration as the previously isolated natural product (Figure 4.19).

### 4.3.3 Biological Activity of Bengamide A

Throughout the bioassay-guided fractionation, samples were assayed against both CB$_1$ and CB$_2$, and tested for both agonism and antagonism, using the Grigliatti assay.† The initial extract of 03-253 was active as a CB$_1$ antagonist,

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† All CB bioassays conducted by Tom Pfeifer in the laboratory of Tom Grigliatti.
however subsequent fractions were active as CB$_1$ agonists throughout the isolation, although at relatively weak concentrations (2.5 mM). In comparison, the natural agonist, anandamide, is used as a positive control at 0.3 mM. This CB$_1$ agonism was focussed in the bengamide containing fractions, although exhaustive purification of the major bengamide (bengamide A, 4.1) showed no response in the CB bioassay at high (16.5 mM) concentration. This is suggestive of non-specific interaction with the receptors, particularly in the presence of several different bengamides, or perhaps that the active compound is one of the minor bengamides.

4.4 Future Directions with the CB Bioassay

While both compounds isolated in this project were known compounds, neither of the sponges that they were isolated from have been identified as sources of these compounds to date. The A-nor sterols, as exemplified by 4.10, are relatively rare steroids, and to date (to this author’s knowledge) this is the first report of A-nor-sterols from Stylissa massa.$^+$ Although they have been isolated from several sponges in the family Axinellidae, this seems to be the first report from the family Dictyonellidae.$^{223,224}$ In fact, the only compounds reported in the marine natural products literature from this sponge are oroidin alkaloids such as massadine (4.12), which are reported to have activity against geranylgeranyl transferase and some protein kinases.$^{229}$

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$^+$ Search run on MarinLit Marine Literature Database, Version pc13.4.
With regards to the isolation of bengamide A (4.11) from *Hemiasterella aff. affinis*, this is also the first reported isolation of a bengamide from this genus of sponge. In fact, all other isolations of bengamide A were from the *Jaspis* species of sponge. The bengamides were initially identified as antihelmenthic compounds, and subsequently identified as methionine aminopeptidase inhibitors, although in general they are found to be inhibitory to tumor growth. An anti-cancer Phase 1 trial was begun with a synthetic analogue of bengamide A, LAF-389 (4.13), although it was shortly withdrawn due to issues with toxicity and uncertainty of the molecular target. Although purified bengamide A (4.11) was not found to be active in the CB assay against either CB₁ or CB₂, it is possible that a component in the fractions from which compound 4.11 was isolated will be a related bengamide and found to possess the observed activity. Further studies of these minor components will need to be undertaken.

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† Search run on MarinLit Marine Literature Database, Version pc13.4.
4.5 Experimental

4.5.1 General Experimental Procedures

All 1D and 2D were recorded on a Bruker AV-600 spectrometer equipped with a cryoprobe or TXI probe. $^1$H chemical shifts were referenced to the residual C$_6$D$_6$ or CDCl$_3$ solvent peak ($\delta$ 7.16 and $\delta$ 7.24 ppm, respectively), and $^{13}$C chemical shifts were referenced to the C$_6$D$_6$ or CDCl$_3$ solvent peak ($\delta_c$ 128.39 and $\delta_c$ 77.23 ppm, respectively). All NMR solvents were obtained from Cambridge Isotope Laboratories.

All chromatography was performed using HPLC grade solvents from Fisher Scientific with no further purification. Water was purified using a Millipore reverse osmosis filter system. Reversed-phase chromatography was performed using 10, 5 or 2 gram Waters C-18 Sep-Paks$^\circledR$. Normal phase chromatography was performed using Silicycle Ultra Pure silica gel (230-400 mesh) under N$_2$ pressure. HPLC isolation was performed using a Waters 600E system controller liquid chromatograph interfaced to a Waters 486 tunable absorbance detector. Optical rotations were measured with a JASCO J-1010 spectrophotometer using a 10 mm cell. Reagents for derivatization were obtained from Sigma-Aldrich.
4.5.2 Isolation of A-nor-steroid

A sample of sponge (~ 100 g wet weight) was extracted repeatedly with MeOH (3 X 100 mL). The organic extract was dried in vacuo to yield an orange paste (109.6 mg). A portion of this paste was then partitioned between H₂O (75 mL) and EtOAc (3 X 100 mL), each of which was dried in vacuo. The EtOAc fraction was subjected to normal phase silica gel chromatography (100% Hexane to 100% EtOAc step gradient, washed with 10% MeOH/DCM and 100% MeOH) yielding 81 fractions. TLC analysis resulted in the pooling of a steroid enriched fraction (3.7 mg). This mixture of sterols was then derivatized with m-bromophenyl isocyanate in CCl₄ and monitored by TLC (Experimental 4.5.3). Repeated HPLC separation of the resulting sample first yielded increasingly pure steroid samples under carefully moderated conditions (97% MeOH/H₂O on a Phenomnex Gemini® 5μ C18 110A column) giving 0.6 mg of partially pure steroid, followed by a second separation (30% propanol/70% H₂O to 100% propanol over 70 minutes, CSC-Inertsil® ODS2 column), ultimately providing a purified steroid derivative (0.1 mg). NMR and MS studies identified this sample as the A-nor-C₂₇Δ¹² derivative (4.10).

4.5.3 Derivatization of A-nor-steroid

A mixture of A-nor-sterols (1.7 mg) was dissolved in CCl₄ (1 mL) in a screw-top vial. To this was added 3-bromophenyl isocyanate (10.0 mg, µmol).
The reaction was sealed, and stirred at 65 °C for 3 hours. TLC analysis showed complete reaction, and CCl₄ was removed under a stream of N₂. The residue was lyophilized overnight prior to NP column chromatography.

4.5.4 A-nor-steroid Physical Data

Clear, colorless glass (0.1 mg). For 1D and 2D NMR data see Table 4.1. HRESIMS: [M + Na]+ m/z = 606.2900 (calcd for C₃₄H₅₀NO₂⁷⁹BrNa, 606.2923).

4.5.5 Isolation of Bengamide A

A sample of sponge (~30 g wet weight) was extracted repeatedly with MeOH (3 X 100 mL). The methanolic extract was dried in vacuo to yield an orange paste. A sample of this paste (1.4 g) was then partitioned between H₂O (75 mL) and EtOAc (3 X 100 mL), each of which was dried in vacuo. The EtOAc fraction was subjected to lipophilic size-exclusion chromatography (Sephadex LH-20®) in MeOH. The resulting fractions were submitted for bioassay, and one of the active fractions (81.7 mg) subjected to both normal (CH₂Cl₂ to 50% MeOH in CH₂Cl₂ on SiO₂) and reversed-phase (50% MeOH/H₂O to MeOH on Waters 2g Sep-Pak®) column chromatography with little success in purification as seen by TLC analysis. The resulting pooled fractions (34.8 mg) were separated by reversed-phase C-18 HPLC (gradient from 80% MeOH/H₂O to 100% MeOH over 40 minutes, CSC-Inertsil® ODS2 column) to yield a series of bengamide
containing fractions. The main fraction (5.9 mg) was found to be active as a CB₁ agonist. Further HPLC purification (85 % MeOH/H₂O isocratic, CSC-Inertsil® ODS2 column) was performed on this sample, yielding (2.7 mg) of extremely pure sample. NMR and MS studies identified this sample as bengamide A (4.11).

4.5.6 Bengamide A Physical Data

Clear, colorless glass (2.7 mg); [α]₀^{25} + 51.3 (c =0.9 MeOH). For 1D and 2D NMR data see Table 4.2. HRESIMS: [M+H]⁺ m/z = 607.3951 (calcd for C₃₁H₅₆N₂O₈Na, 607.3934).
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Appendix I

ROESY spectrum of tauramamide methyl ester (2.14), recorded at 500 MHz in DMSO-$d_6$, showing inter-residue correlations
Appendix II

LSIMS spectrum of tauramamide methyl ester (2:14)