Isolation and Structure Elucidation of Bioactive Marine Natural Products

by

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Abstract

Clionamines A-D (2.6-2.9) are new aminosteroids isolated from South African specimens of the sponge *Cliona celata*. All four compounds (2.6-2.9) are activators of autophagy in MCF-7 cells. Autophagy is a catabolic process that plays an important role in maintaining cellular homeostasis. Autophagy is also directly involved in the removal of bacterial and viral antigens and in the development of cancerous tumors.

The novel sesterterpenoid ansellone A (3.4) was isolated from the nudibranch *Cadlina luteomarginata* and was later found to have been sequestered by the nudibranch from the sponge *Phorbas* sp. Ansellone A (3.4) is an activator of the cAMP signalling pathway. Following the isolation of 3.4, the novel sesterterpenoids ansellones B-D (4.3-4.5) as well as alotaketal E (4.6) were isolated from the sponge *Phorbas* sp. and were found to also be activators of the cAMP signalling pathway.

Several bacterial isolates were obtained from the sponge *Phorbas* sp. in order to investigate the possibility that the ansellones and the alotaketals isolated from this sponge were biosynthesized by a bacterial symbiont. Since these sesterterpenoids were activators of the cAMP signalling pathway, the investigation was conducted using bioassay guided fractionation of the bacterial isolates. The new meroterpenoid phorbasolic acid (5.1) was isolated, but no sesterterpenoids were found in the bacterial isolates.

In an effort to identify molecules with antibiotic properties, a biological assay was designed to screen for inhibitors of the citrate synthase type II enzyme. One aspect of this enzyme that is of therapeutic interest is that Gram-negative bacteria possess a very different isoform of the enzyme than Gram-positive bacteria and eukaryotes. Therefore, an antibiotic specific to type II citrate synthase would target Gram-negative bacteria selectively. An extract from a culture of *Bacillus pumillus* inhibited
the enzyme in the assay. Although the molecule responsible for this effect has yet to be identified, the new aliphatic amide 12-methyl tridecanamide (6.1) was isolated.
Preface

For chapter 2, my contributions include the isolation and structure elucidation of clionamine C (2.8) and the deprotection of clionamines B-D (2.7-2.9) and the spectroscopy and confirmation of the structure of the deprotected clionamines B-D (2.7-2.9). I also re-isolated clionamine A (2.6) to acquire enough material for biological assay and I prepared the steroidal derivatives used in the bioassay. The isolation of clionamine A (2.6) and of the Fmoc protected clionamines B (2.7) and D (2.9) was done by Dr. Robert Keyzers. The biological assay was conducted by Aruna Balgi from the Roberge laboratory in the department of Biochemistry and Cellular Biology at the University of British Columbia. My contributions to the writing of the manuscript include part of experimental section and the supporting information. The body of the manuscript was written by my supervisor. Although, chapter 2 is based on a published article, I am responsible for the redaction of the text presented in this thesis.

My contributions to chapter 3 include the collection of the animal material with the help of Michael Leblanc. Dr. Angelo Fontana and I isolated and elucidated the structure of ansellone A (3.4). The biological assay was conducted by Catherine Merchant from the Kieffer laboratory at the department of Cellular and Physiological Sciences and Surgery at the University of British Columbia. I contributed to the writing of the experimental section and the supporting information for the publication on ansellone A (3.4), while my supervisor contributed to the body of the manuscript. I am also responsible for the redaction of chapter 3, although some of the material presented in this chapter is based on a published article.
For chapter 4, I collected the sponge material with the help of Michael Leblanc. I conducted the isolation and structure elucidation of ansellones B to D (4.3-4.5) and of alotaketal E (4.6). Again, Catherine Merchant from the Kieffer laboratory carried out the biological testing.

My contributions for chapter 5 include the isolation and the structure elucidation of the meroterpenoid (5.1) from the culture of Enterococcus phorbasii and the fractionation and preparation of various bacterial extracts for the chemical prospecting aspect of this chapter. I assisted Derek Smith, from the mass spectrometry facility of our chemistry department with the LC-MS experiment conducted on the bacterial and the sponge extracts. The bacterial cell cultures were grown by Dr. Doralyn Dalisay from our laboratory.

For chapter 6, my contributions include the isolation and structure elucidation of 12-methyl tridecanamide (6.1). I carried out some of the bacterial culturing, while most of the bacterial cultures were grown by Dr. Doralyn Dalisay. The biological assay was performed by Aruna Balgi of the Roberge laboratory.

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<table>
<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>1D</td>
<td>one dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>ribosomal ribonucleic acid with a sedimentation factor of 16 Svedberg units</td>
</tr>
<tr>
<td>[α]_D^{20}</td>
<td>specific rotation at sodium D-line (589 nm) recorded at 20°C</td>
</tr>
<tr>
<td>atg</td>
<td>autophagy related gene</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>13C</td>
<td>carbon 13</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C₆D₆</td>
<td>deuterated benzene</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CD₃OD</td>
<td>deuterated methanol</td>
</tr>
<tr>
<td>CCl₄</td>
<td>carbon tetrafluoride</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic adenosine monophosphate response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate response element binding</td>
</tr>
<tr>
<td>CREB-P</td>
<td>cyclic adenosine monophosphate response element binding protein</td>
</tr>
<tr>
<td>δH</td>
<td>¹H NMR chemical shift in parts per million from tetramethyl silane</td>
</tr>
<tr>
<td>δC</td>
<td>¹³C NMR chemical shift in parts per million from tetramethyl silane</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO-d₆</td>
<td>deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dt</td>
<td>doublet of triplets</td>
</tr>
<tr>
<td>ε</td>
<td>molar extinction coefficient (M⁻¹ cm⁻¹)</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>concentration giving 50% of the maximum effectiveness</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESIMS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>Et₂O</td>
<td>diethyl ether</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-Cl</td>
<td>fluorenylmethyloxycarbonyl chloride</td>
</tr>
</tbody>
</table>
g  - gram (s)
GDP  - guanidine diphosphate
GFP  - green fluorescent protein
GIP  - gastric inhibitory polypeptide
GLP-1  - glucagon like peptide
GPCR  - G-protein coupled receptor
GTP  - guanidine triphosphate
h  - hour (s)
HCl  - hydrochloric acid
HEK293  - human embryonic kidney cells
HMBC  - heteronuclear multiple bond coherence
HPLC  - high performance liquid chromatography
HRESIMS  - high resolution electron spray ionization mass spectrometry
HSQC  - heteronuclear single bond coherence
Hz  - hertz
J  - coupling constant (in hertz)
λ  - wavelength
λ_max  - wavelength, giving a local maximum absorbance
L  - litre (s)
LC3  - light chain 3
LRESIMS  - low resolution electrospray ionization mass spectrometry
μg  - microgram (s)
μM  - micromolar
m  - multiplet
MCF-7  - Michigan cancer foundation 7
MeCN  - acetonitrile
MeOH  - methanol
mg  - milligram (s)
MHz  - megahertz
min  - minute (s)
ml  - millilitre (s)
MS  - mass spectrometry
mTORC1  - mammalian target of rapamycin complex 1
m/z  - mass-to-charge ratio
Na_2CO_3  - sodium carbonate
Na^+  - sodium ion
NaBH_3CN  - sodium cyanoborohydride
NaCl  - sodium chloride
NADH  - nicotinamide adenine dinucleotide
NH_3  - ammonia
NMR  - nuclear magnetic resonance
NOESY  - nuclear Overhauser effect spectroscopy
NP HPLC - normal phase high performance liquid chromatography
PDA - photodiode array
PKA - protein kinase A
pH - \(-\log_{10}[H^+]\)
pHTS-CRE - luciferase reporter vector
PKS - polyketide synthase
ppm - parts per million
RP HPLC - reverse phase high performance liquid chromatography
ROESY - rotating frame nuclear Overhauser effect spectroscopy
r.t. - room temperature
s - singlet
SAR - structure activity relationship
SCUBA - self-contained underwater breathing apparatus
sp. - species
t - triplet
td - triplet of doublets
tt - triplet of triplets
TFA - trifluoroacetic acid
THF - tetrahydrofuran
TLC - thin layer chromatography
UV - ultraviolet
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“We must not cease from exploration. And the end of all our exploring will be to arrive where we began and to know the place for the first time.” T.S. Eliot
1. Introduction

1.1 Natural Products

The vast array of organic molecules found in living organisms can be classified into two main categories; primary and secondary metabolites. Primary metabolites include nucleic acids, fats, proteins, and carbohydrates and they are considered to be essential for the survival of an organism. The mechanisms involved in the biosynthesis of primary metabolites are generally highly conserved across all living organisms. On the other hand, secondary metabolites are as structurally diverse as the species that produce them. Although secondary metabolites are not considered essential for the survival of an organism, they are thought to confer a selective advantage to the producing organism such as defence against predators or protection against fouling organisms. Secondary metabolites are divided into several classes of molecules such as the terpenoids, the polyketides, the alkaloids, the glycosides, the phenyl propanoids, and the non-ribosomal peptides. The boundaries between primary and secondary metabolites can often be blurry, certain fatty acids and carbohydrates can be very specific to a particular genus, while some terpenes are highly conserved in a wide range of organisms. In plants, many terpenoids are considered primary metabolites as they play functional roles as photosynthetic pigments, plant hormones, and membrane structural components. Nevertheless, secondary metabolites are, generally speaking, species-specific, whereas primary metabolites represent the building blocks of all living creatures.

Natural products, a term commonly used to describe secondary metabolites, have been used as medicines for several thousand years and continue to be an important source of pharmaceuticals today. The latest National Cancer Institute review on natural products as a source of pharmaceuticals revealed
that 57% of the new chemical entities introduced in clinical use in the past 25 years were derived from natural products. Secondary metabolites isolated from plants, fungi, bacteria and some venoms have all served to inspire drugs currently on the market. Over the past half century, marine natural products have emerged as a rich source of novel pharmacophores and represent a new frontier for the expansion of the chemical space as we know it.

1.2 Terpenoids

Most of the molecules described in the following chapters are terpenoids and this warrants a more in depth survey of the biosynthesis of this class of metabolites. The terpenoids are biosynthesized from C5 isoprene units derived either from dimethylallyl pyrophosphate (1.1) or from isopentenyl pyrophosphate (1.2). The term terpene is often used to describe terpenoids, although it specifically refers to hydrocarbons built from isoprene units, while terpenoids may contain other atoms such as oxygen. The number of isoprene units present in a specific terpenoid dictates its classification, as listed in Table 1-1.

Table 1-1 Terpenoid classification

<table>
<thead>
<tr>
<th>Number of isoprene units</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemiterpenes</td>
<td>1</td>
</tr>
<tr>
<td>Monoterpene</td>
<td>2</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td>3</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>4</td>
</tr>
<tr>
<td>Sesterterpenes</td>
<td>5</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>6</td>
</tr>
<tr>
<td>Tetraterpenes</td>
<td>8</td>
</tr>
</tbody>
</table>

Generally, isoprene units are joined together in a head to tail fashion. Figure 1.1 illustrates the enzyme-catalyzed reaction between dimethylallyl pyrophosphate (1.1) and isopentenyl pyrophosphate.
(1.2) to form geranyl diphosphate (1.3), a monoterpenoid. Additional isoprene units are added in the same manner to form sesquiterpenes, diterpenes and sesterterpenes. The terpenoids described in chapters 3 and 4 are sesterterpenoids.

Sponges appear to be the principal source of marine sesterterpenes while triterpenes, represented mainly by steroids, are distributed across a wide range of species.

![Figure 1.1 Formation of geranyl diphosphate.](image)

Triterpenes are produced through the tail to tail addition of two molecules of farnesyl pyrophosphate (1.4) to form squalene (1.5). The first step in the biosynthesis of squalene results in the formation of a tertiary carbocation (Figure 1.2). Loss of a proton leads to the formation of a cyclopropane ring. After the loss of the remaining diphosphate, the cyclopropane ring rearranges into a cyclobutane ring. The opening of this cyclobutane ring results in the formation of an allylic cation that is subsequently quenched to yield squalene (1.5).
Figure 1.2 Biosynthesis of squalene (1.5).

Squalene (1.5) is the basic carbon skeleton from which steroids arise. The cyclization of the triterpene skeleton occurs through 2,3-oxidosqualene as shown in Figure 1.3. The protonation of epoxide I will trigger a series of stepwise cyclizations towards the formation of the cyclic triterpene. Each step creates a tertiary carbocation that undergoes an electrophilic addition to the nearest double bond. The diversity observed in naturally occurring steroidal skeletons arises through this polycyclization but also through a series of Wagner-Meerwein 1,2 alkyl and hydride shifts that occur after the
cyclization. The skeleton of the aminosteroids described in chapter 2 is derived from lanosterol (1.6). In lanosterol (1.6), a tertiary carbocation (II) is formed after the cyclization of the first three rings (Figure 1.3). The opening of the five-membered ring to form a six-membered ring yields a secondary carbocation (III) that is then attacked by the adjacent double bond to form a five-membered ring (IV). A series of Wagner-Meerwein rearrangements occur thereafter and set the configuration of the lanosterol skeleton.

Figure 1.3 Formation of lanosterol (1.6).
Tetraterpenes are synthesized from two molecules of geranylgeranyl diphosphate joined in a similar manner as the two molecules of farnesyl pyrophosphate involved in the biosynthesis of squalene. The tetraterpenes are represented by the carotenoids and they are found in plants, fungi, bacteria, and animals.

1.3 Marine Natural Products Chemistry

The field of marine natural products chemistry spans multiple disciplines. Research programs in this field can include aspects of bioprospecting, chemical ecology, biosynthesis, organic synthesis or structure-activity relationship (SAR) studies. The focus of these research programs can, therefore, be based on ecological interests, or on expanding the known chemical space, or it can be based on the discovery of commercially relevant molecules. Nonetheless, all marine natural products studies must begin by the isolation and structure elucidation of secondary metabolites.

Organisms found in the marine environment have proven to be rich in structurally diverse secondary metabolites. Unique scaffolds and rare functionalities have been found in marine natural products and their discovery continues to motivate natural products chemists to search for novel molecules despite the intricate nature of their task. Indeed, the isolation of marine metabolites is complicated by the difficulty in acquiring sufficient material for characterization and biological studies since these metabolites are often extracted from new or rare invertebrates. These organisms are collected by hand using SCUBA diving in areas that are often secluded, hard to access, and where the sample collection can easily be complicated by weather or in some cases political unrest. These conditions make additional collections of a specific organism difficult, if not impossible. Thus, the sample handling during the transport of the biological material and during the isolation process must be done very cautiously in order to obtain sufficient material for spectroscopic characterization of the secondary metabolites and for any other studies as the case may be. Thankfully, continued improvements in
Nuclear Magnetic Resonance (NMR) spectroscopy have lowered the amount of material necessary for a full characterization of the constitution and relative configuration of a molecule to sub-milligram amounts. The advancements in NMR technology have also encouraged natural products chemists to revisit the isolation of very minor metabolites from species that were studied in years past.

As mentioned above, the isolation of marine natural products can lead to multiple scientific endeavours. Some scientists choose to study the ecological role of secondary metabolites, but overwhelmingly natural products chemists choose to isolate molecules that may serve to improve our lives through medicine or agriculture. Therefore, most natural products discovery programs begin by the screening of crude extract libraries in biological assays. Once a molecule is isolated and characterized, its biological properties are explored. These studies are often supported by the work of synthetic chemists who seek to provide a stable supply of the biologically active molecule, although many are also motivated by the intellectual challenge that these complete syntheses represent. Additionally, synthetic chemists participate in SAR studies that can lead to more cost effective solutions to the development of a marine derived product.

Several marine natural products and marine natural products derivatives are now being sold as treatments for a variety of diseases. Cytarabine (1.7), a drug inspired by a nucleoside found in a marine sponge, was approved for the treatment of leukemia in the 1970’s and was the first marine derived drug to enter the market. Recently, trabectedin (1.8), isolated from the tunicate Ecteinascidia turbinata, was approved in Europe and in Canada for the treatment of soft tissue sarcoma and ovarian cancer. Eribulin mesylate (1.9) was derived from halichondrin B, a sponge metabolite, and has been approved for clinical use as a tubulin destabilizing agent for the treatment of cancer. In addition, a number of promising marine derived compounds are now in clinical or preclinical trials. The synthetic compound E7974 (1.10), an analog of the hemiasterlins, is currently in phase I clinical trials. Hemiasterlin was
isolated from the sponge *Hemiasterella vasiformis* while hemiasterlin A and B were isolated from *Cymbastella* sp. by our laboratory a few years later.\textsuperscript{7,8} These molecules are antimitotic agents that lead to tumor cell apoptosis through their binding to α-tubulin.\textsuperscript{9}

The drug candidates mentioned above represent a fraction of the marine natural products and their derivatives in clinical and preclinical trials, but they serve to illustrate the potential of these molecules as therapeutic entities.

![Chemical structures](1.7, 1.8, 1.9, 1.10)

**Figure 1.4 Pharmaceuticals derived from marine natural products.**

The largest hurdle for getting natural products through clinical trials has been the supply issue. Providing the necessary quantity of a molecule to undergo clinical trials can be very challenging. Furthermore, when a drug is approved to enter the market the supply issue becomes an even greater
problem. The natural product halichondrin B was originally pulled out of clinical trials as an anti-cancer drug due to supply issues and was later replaced by the synthetic analog eribulin mesylate (1.9). Had it made it to market, the annual need for halichondrin B would have hovered around 1.5 kg, corresponding to several thousand metric tonnes of sponge per year. Such a quantity of sponge tissue could not have been obtained without causing serious environmental damage. While the supply of natural products isolated from invertebrates can be limiting, the same is not true of the secondary metabolites produced by microorganisms. Natural products derived from bacteria and algae can be generated through large scale cultures in biofermentors. Marine microorganisms, therefore, represent an attractive source of potential pharmaceuticals.

1.4 Bacterial Marine Natural Products

The focus of many marine natural products isolation groups has now shifted to marine bacteria for the discovery of novel molecules. This shift has been motivated by both the difficulties involved in collecting marine invertebrates and by the wealth of molecules that have been isolated from terrestrial bacteria.

The study of bacterial natural products began with soil bacteria, most specifically actinobacteria, which have proven to be a major source of clinically useful molecules such as vancomycin and erythromycin. Consequently, the focus of microbial natural product chemistry has been invested on these terrestrial sources. Until recently, it was believed that actinobacteria existed only on land and were not adapted to living in the ocean. It has now been shown that certain actinobacteria are metabolically active in the marine environment. However, accessing the natural products produced by marine actinobacteria has proven challenging since relatively few bacteria can be grown in the laboratory due to poorly optimized culture conditions. It is estimated that less than 1% of the bacteria in the ocean can be cultured with standard growth conditions. Nevertheless, new methods are becoming
available to optimize the culture of marine bacteria and many new microbial species are described every year. Also, culture independent techniques such as genomics can be used to guide cultivation strategies.\(^\text{18}\) Gaining access to the chemical diversity held by marine bacteria through newly developed growth conditions represents a great opportunity for natural products chemists to uncover novel molecules.

Recently, several marine obligate bacteria, bacteria that are specific to the marine environment, have been grown in the laboratory and novel molecules have been isolated from their cultures. One of the first marine obligate actinobacteria species to be cultured was *Salinispora tropica* and it has since become a valuable source of novel metabolites.\(^\text{15}\) Salinisporamide A (1.11) and salinisporamide B (1.12) were isolated from *S. tropica*. Salinisporamide A (1.11) is structurally related to omurolide (1.13) and, like omurolide, it is a 20S protease inhibitor. Salinisporamide A (1.11) is now in phase I clinical trial for the treatment of multiple myeloma.\(^\text{19}\) The novel polycyclic macrolides sporolide A (1.14) and sporolide B (1.15) were also isolated from *S. tropica*. Moreover, other species belonging to the genus *Salinispora* have yielded interesting chemistry. Two polyketides with an unusual bicyclic ketal portion, saliniketal A (1.16) and saliniketal B (1.17), were isolated from *Salinispora arenicola*.\(^\text{20}\) Investigation of the metabolites produced by *S. pacifica* revealed the presence of cyanosporaside A (1.18) and cyanosporaside B (1.19). Macrolides, such as marinomycin A (1.20), were isolated from a marine actinobacteria of the tentative genus *Marinispora*.\(^\text{21}\)
Figure 1.5 Natural products isolated from obligate marine actinobacteria

Marine microorganisms have been collected from the water column and from sediments, but increasingly they have been isolated from marine invertebrates. Many have suggested that secondary metabolites isolated from sponges could in fact be produced by microorganisms living within the sponge. This theory has been difficult to prove due to the difficulties surrounding the culture of marine microorganisms in the laboratory. Nonetheless, several natural products isolated from sponges were later found in microorganisms that were not sponge symbionts. This was the case for okadaic acid, a cytotoxic polyether that was originally isolated from the sponges *Halichondria okadai* and *Halichondria melanodocia*, but was later found in a species of free-living dinoflagellate of the genus *Prorocentrum*.
Perhaps a more convincing piece of evidence for the symbiotic origin of natural products comes from the work by Faulkner’s laboratory on the two marine sponges *Dysidea herbacea* and *Theonella swinhoei* and their cyanobacterial symbionts. Faulkner’s group was able to separate the sponge cells from the cyanobacterial cells using a cell sorter that separated cells based on the fluorescence of the chlorophyll in the cyanobacterial cells. The amino-acid derivative 13-demethylisodysidenin was isolated from the cyanobacterial cells whereas the sponge cells contained spirodysin.\(^{23}\) Both of these compounds had previously been isolated from the sponge and Faulkner’s work confirmed the hypothesis that 13-demethylisodysidenin was of cyanobacterial origin.

More recently, many researchers have turned to metagenomic techniques to isolate the genes responsible for the production of the secondary metabolites isolated from sponges. A number of studies of the polyketide synthase (PKS) genes present in sponges have uncovered unusual PKS types that were shown to be of bacterial origin.\(^{24}\) Despite the substantial amount of research that has been done to establish that bacterial symbionts are responsible for the production of some of the secondary metabolites isolated from sponges, there have been no examples of the isolation of a molecule originally found in a sponge and later found in bacteria cultured from the same sponge.

### 1.5 Research Goals

The work presented in this manuscript was conducted in an effort to isolate novel natural products with therapeutic properties from marine bacteria and invertebrates. Another goal of this work was to explore the possibility that sponge symbionts were responsible for the metabolites isolated from their host.
2. The Clionamines

2.1 Natural Products Reported from Sponges Belonging to the Family Clionaidae

Sponges in the taxonomic family Clionaidae are known as the “boring sponges” since they bore holes in the substrates they grow on. The Clionaidae are commonly found growing on rocks, mollusc shells, and corals. Members of the Clionaidae family are strong competitors for space on coral reefs, where they can dramatically undermine the viability of the coral tissue with the excavating filaments they use to attach themselves to the coral reef. *Cliona tenuis*, a Caribbean species of Clionaidae is one of the fastest growing fouling organisms threatening coral reefs.\(^2^5\) It also produces clionapyrrolidine (2.1), a molecule with potent negative allelopathic effects on corals (Figure 2.1).\(^2^6\) Clionapyrrolidine was shown to be lethal when administered to the corals *Madracis mirabilis*, *Montrastea cavernosa*, and *Siderastrea siderea*.\(^2^6\)

![Clionapyrrolidine](image)

**Figure 2.1 Clionapyrrolidine isolated from *C. tenuis***.

In addition to being a source of concern to the health of coral reefs, the Clionaidae are also a threat to various types of aquaculture. Another member of the Clionaidae family that has attracted

scientific attention is *Cliona celata*. *C. celata* is a cosmopolitan species that causes considerable economic losses to the oyster aquaculture industry.\(^2\)\(^7\) The shell of cultured oysters can become heavily encrusted with the sponge. *C. celata* overgrowth significantly decreases the market value of the oysters by making their shells more fragile. Additionally, the sponge gives off a strong sulphurous odour that can discourage potential buyers. Similarly, *C. celata* is a significant cause of concern for the pearl industry as it bores into the shell of pearl oysters and damages their nacre. The infected oysters must then divert the nacre they produce to repair the shell instead of producing pearls, which inevitably results in slower pearl growth rates.\(^2\)\(^8\) Due to the economic significance of the damage caused by *C. celata*, many studies have focused on the mechanical and chemical boring mechanisms used by the sponge.\(^2\)\(^9\) In addition, the widespread geographical distribution of *C. celata* has made it the subject of many natural products investigations.

Several compounds have been isolated from *C. celata* to date. In fact, Bergmann, a pioneer of marine natural products chemistry, isolated clianosterol (2.2) from *C. celata* in 1941 (Figure 2.2).\(^3\)\(^0\) In the late 1970’s our group studied specimens of *C. celata* collected in Bamfield, on the west coast of Vancouver Island. From these specimens, the indole alkaloid clionamide (2.3) was isolated, as well as celenamides A (2.4)-D, a group of linear peptide alkaloids.\(^3\)\(^1\)\(^,\)\(^3\)\(^2\) In 1996, Lenis and co-workers reported the isolation of the alkaloid acetylhomoagmatine (2.5) from *C. celata* collected in Spain.\(^3\)\(^3\) Finally, in 2008, our research group reported the isolation of the steroids clionamines A through D (2.6-2.9) from a sample of *C. celata* collected in South Africa (Figure 2.3).
2.2 Steroids from Marine Sponges

The marine environment has been a source of structurally unusual steroids over the last 40 years. Prior to the isolation of a C\textsubscript{26} steroid from the mollusc *Placopeden magellanicus*, steroids had been known to contain between 27 and 30 carbon atoms.\textsuperscript{34} However, since then a multitude of steroids with unusual side chains, ring sizes, and substitution patterns have been isolated. Among the unusual sterols isolated from the marine environment are clionamines A-D (2.6-2.9) described in this chapter (Figure 2.3).
The clionamines each possess a γ-lactone attached to ring D of their steroidal core at C-16 and C-17. To the best of our knowledge, the presence of a γ-lactone at C-16/C-17 in the clionamines is unique amongst naturally occurring steroids. Frondoside A (2.10), a saponin isolated from a holothuroid also has a γ-lactone ring fused to C-13 and C-17 in ring D of the cyclopentanoperhydrophenanthrene steroid core (Figure 2.4).\textsuperscript{35} It should be noted that C-16/C-17 γ-lactone E rings like those found in the clionamines have been reported in degradation products of terrestrial steroidal sapogenins.\textsuperscript{36,37} Nevertheless, the most noteworthy structural feature of the clionamines is the spiro bislactone moiety found in clionamine D (2.9), which is unprecedented both in synthetic and naturally occurring steroids.
Nitrogen containing steroids are a rare group of marine natural products. Previously isolated examples include ritterazine A (2.11), a heterodimeric aminosteroid, cortistatin A (2.12), an abeo-androstane type aminosteroid, and ergosterimide (2.13), a Diels-Alder adduct of an ergosteroid with maleimide (Figure 2.5).
There are even fewer examples of marine aminosteroids with a primary amine functionality at C-3. One such example is plakinamine A (2.14) isolated from the sponge *Plakina* sp., a member of the Plakinidae family (Figure 2.5).\(^{41}\) Lokysterolamine A and B (2.15-2.16) are also examples of 3-aminosteroids that are related to plakinamine A. They were isolated from the Plakinidae sponge *Corticum* sp.\(^ {42}\) Unlike the clionamines, the lokysterolamines do not have a primary amine at C-3, instead the amino group at C-3 is dimethylated in lokysterolamine A (2.15) and acetylated in lokysterolamine B (2.16).
In summary, the clionamines are a new family of aminosteroids that add to the diversity of molecules isolated from C. celata. The clionamines were isolated due to their biological activity in a chemical genetics assay designed to uncover modulators of autophagy.

2.3 Chemical Genetics and Autophagy

Traditionally, biological pathways have been studied using mutagenetics methods where a gene is knocked out to uncover its function. More recently, small molecules and, in particular, natural products have been used to better understand biochemical pathways in the context of chemical genetics. In chemical genetics, small molecules are used to mimic expressional changes in the host organism by acting upon a gene product (a protein) thereby producing a specific phenotype. There are several advantages to using chemical genetics over traditional mutagenetics methods for the identification of new drug targets. With gene knock-out, the changes observed in the cell are irreversible. On the other hand, in chemical genetics studies, the effects of small molecules are often reversible and concentration dependent. In addition, small molecules used in chemical genetics studies can serve to study essential genes, interfere with protein-protein interactions and disrupt a single function of a multifunctional protein, all of which are not possible using mutagenetics methods.43

Chemical genetics is used to gain biological insight and discover new molecular probes. It is used in this study with the encompassing goal of uncovering new natural products. Natural products and derivatives are the most important source of clinically relevant molecules and they represent a rich source of chemical genetics tools.44,45 Natural products are relevant tools for chemical genetics studies due to their structural diversity and inherent fit to cellular targets.
Chemical genetics was also used in the work presented in this chapter in order to gain biological insight into the mechanisms regulating autophagy and describe new molecular probes of autophagy. Chemical genetics is an appropriate tool for studying autophagy since this cellular mechanism is controlled by various complex and interconnected molecular pathways. In such a system, standard knockout methods create permanent changes in the signalling pathways that prevent a clear understanding of the changes caused by the knockout. Furthermore, there is a therapeutic need for autophagy regulating molecules that could impact biochemical pathways that are specific to autophagy.

2.4 Autophagy as a Therapeutic Target

The viability of a cell largely depends on cellular homeostasis for maintaining a constant internal environment. When cellular homeostasis is disturbed, undesired material such as misfolded proteins and damaged organelles accumulate in the cytoplasm. This accumulation is a hallmark of many neurodegenerative diseases as well as many types of cancer. Autophagy is a degradation process that plays an important role in maintaining cellular homeostasis by sequestering superfluous or damaged intracellular material in a double membrane vesicle called autophagosome and delivering it to the lysosomic machinery for degradation.

In healthy cells, autophagy serves a protective role in stressful cellular environments such as nutrient depletion by recycling cellular material. Autophagy also plays a protective role by removing bacterial and viral antigens and has recently been identified as a potential therapeutic target for the treatment of infections by Mycobacterium tuberculosis. The treatment of tuberculosis is complicated by the many mechanisms used by the microorganism to evade antimicrobial defences. Mycobacteria appear to accumulate in macrophages and halt their delivery to the lysosomes, therefore, preventing the processing of antigens against mycobacteria. This is detrimental to both the normal
immunity against tuberculosis and the tuberculosis vaccine efficiency.\textsuperscript{50} Stimulating autophagy has been shown to improve the delivery of the mycobacteria filled macrophages to the lysosomes, and molecules that can activate autophagy could improve the efficiency of a tuberculosis vaccine.\textsuperscript{50}

In neurodegenerative disorders such as Parkinson’s disease and Huntington’s disease, autophagy is downregulated and the cell is overwhelmed with aberrant and misfolded proteins.\textsuperscript{51} In cancer cells, autophagy may be upregulated to protect the cell against metabolic stress, thereby promoting tumorigenesis.\textsuperscript{52} Autophagy can also protect cancer cells against radiation therapy by discarding materials damaged by the radiation.\textsuperscript{53} Conversely, certain upregulators of autophagy are involved in tumor suppression.\textsuperscript{54, 55} Autophagy is also thought to be linked to the programmed cell death of oncogenic cells.\textsuperscript{56} These findings underline the need for a better understanding of the processes involved in both the upregulation and the downregulation of autophagy for therapeutic purposes.

In the early 1990s, a group of researchers identified 14 autophagy-related genes (atg) in yeast.\textsuperscript{57} These genes were conserved in humans and provided a stepping stone for understanding the mechanisms regulating autophagy.\textsuperscript{58} These findings have led to the elucidation of pathways upstream of autophagy that can play a regulatory role in the formation of autophagosomes.\textsuperscript{59} One such pathway relies on the deactivation of mTORC1 kinase, a protein involved in nutrient sensing. When the nutrients in the cell are depleted the mTORC1 kinase is inhibited, which allows for the formation of autophagosomes. After a period of nutrient depletion, the degradation products of the autophagosome trigger the activation of the mTORC1 kinase once again.\textsuperscript{60} Since mTORC1 kinase is of great importance in the control of autophagic processes, many of the inhibitors or activators of autophagy identified thus far target the mTORC1 kinase. A well known inhibitor of the mTORC1 kinase is rapamycin (2.17), a macrolide that prevents the formation of the mTORC1 kinase complex leading to the upregulation of autophagy (Figure 2.6).\textsuperscript{59, 60}
Autophagy is also regulated by myo-inositol-3, 4, 5-triphosphate (IP₃) cellular levels. IP₃ plays a role in the control of the Ca²⁺ channels in the cell membrane. The Ca²⁺ channels appear to be essential to the formation of autophagosomes although the precise mechanism by which they regulate their formation is not well understood. Lithium, carbamazepine (2.18) and sodium valproate (2.19) are proven inhibitors of autophagy that reduce the intracellular levels of IP₃ (Figure 2.6). Although these drugs are promising leads, they do not directly target atg gene products and, therefore, their effects are not exclusive to autophagy pathways. Interestingly, lithium, carbamazepine (2.18) and sodium valproate (2.19) are all mood stabilizing drugs.

Chloroquine (2.20) is another inhibitor of autophagy and it acts by preventing the lysosomal degradation of the material delivered by the autophagosome. Chloroquine is a weak base and appears to affect the pH inside the lysosome and in turn causes the release of important proteases from the lysosome. Similarly, bafilomycin A₁ (2.21), a macrolide isolated from *Streptomyces griseus*, was found to inhibit autophagy by altering the pH in the autophagosome and preventing its fusion with the lysosome (Figure 2.6). Bafilomycin A₁ is a specific inhibitor of vacuolar type H⁺ ATPase, a regulator of the proton pumps on the cell membrane. By inhibiting the H⁺ ATPase of the autophagosomes, bafilomycin A₁ prevents their acidification and their fusion to the lysosomes and ultimately inhibits autophagy.

Although several inhibitors of autophagy are known, few activators have been identified and most of them are mTOR inhibitors. Since mTOR encoding genes control cellular pathways other than autophagy, there is a need to identify novel activators of autophagy that act upon other atg genes.
Our collaborator, Dr. Michel Roberge has developed an assay using automated cell imaging to identify cellular phenotypes indicative of autophagy activation. The assay uses a marker made from Light Chain 3 (LC3) that was modified with Green Fluorescent Protein (GFP-LC3) to visualize autophagosome formation through microscopy in MCF-7 cells (human breast adenocarcinoma cell line). When autophagy is induced in the cell, LC3 accumulates on the membrane of the forming autophagosome. In the assay, increased autophagic activity translates into conversion of GFP-LC3 from its cytosolic form to its lipidated form and its subsequent attachment to the autophagosome.

The recruitment of GFP-LC3 to the autophagosome results in GFP-LC3 fluorescent puncta that can be detected through microscopy whereas the cytosolic GFP-LC3 is dispersed across the cell and is detected as diffuse light. Figure 2.7 shows the accumulation of these GFP-LC3 fluorescent puncta in MCF-7 cells after treatment with rapamycin (2.17), a stimulator of autophagy and after nutrient deprivation, a condition that stimulates the formation of autophagosomes.
The cells treated with rapamycin and the nutrient deprived cells display a greater number of GFP-LC3 puncta compared to the untreated cells due to the presence of a greater number of autophagosomes. This method, therefore, allows for the identification of molecules that can upregulate autophagy in a whole cell system. Consequently, this assay makes it possible to simultaneously measure the cellular toxicity of small molecules.

Using this biological assay, we aimed to identify marine natural products that could act as modulators of autophagy.

2.5 Isolation and Structure Elucidation of the Clionamines

Our laboratory has an extensive library of extracts from marine invertebrates and algae. In an effort to find small molecules involved in the regulation of autophagy, our extract library was tested in the GFP-LC3 autophagy assay developed by the Roberge laboratory. One of the most promising extracts
from this large screening program was the methanolic extract of a South African sample of the sponge *C. celata*.

*C. celata* was collected using SCUBA at a depth of 20 metres off Coffee Bay, on the Wild Coast of South Africa. The sponge tissue was deep frozen for transport and storage. The isolation procedure is summarized in Figure 2.8. Clionamine A (2.6) was the only pure aminosteroid to be isolated by Reversed Phase HPLC (RP-HPLC).

![Isolation scheme of the clionamines](image)

Figure 2.8 Isolation scheme of the clionamines

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2 The sponge samples were collected as part of a collaboration between Dr. M. Davies-Coleman, Dr. D. J. Faulkner and Pfizer. The sponge samples were later donated to the laboratory of Dr. Andersen at UBC when Pfizer research laboratories in St Louis terminated their natural products program.
Additional aminosteroids were isolated as a mixture that also contained clionamine A (2.6). Separation of the clionamines proved challenging as they do not have a strong chromophore and are, consequently, difficult to detect using UV/Vis or photodiode array HPLC detectors. Also, the free amine functionality makes their separation difficult. In order to circumvent these problems, the mixture of aminosteroids was treated with 9-fluorenylmethyl carbamate chloride (Fmoc-Cl) and Na$_2$CO$_3$ in 9:1 MeCN: H$_2$O (Scheme 2.1). The protected aminosteroids could then be separated by NP-HPLC to yield the Fmoc derivatives of clionamines B (2.22), C (2.23) and D (2.24).

Scheme 2.1 Protection of clionamine B (2.7).

Clionamine A (2.6) was isolated as an optically active white amorphous solid that gave a [M+H]$^+$ ion at $m/z$ 444.3465 in the HRESIMS (calculated for C$_{28}$H$_{46}$NO$_3$, 444.3478) allowing us to assign a molecular formula of C$_{28}$H$_{45}$NO$_3$, requiring seven degrees of unsaturation. The $^{13}$C/DEPT spectra showed 27 carbons and 42 hydrogens attached to carbons of which five were methyls, eight were methylenes, eleven were methines and three were quaternary carbons (Figure 2.9, Figure 2.10). The remaining carbon resonance was seen in the $^{13}$C NMR spectrum as a weak resonance at $\delta$ 76.6. A [M+D]$^+$ ion at $m/z$ 448 in the LRESIMS of clionamine A (2.6) recorded in CD$_3$OD confirmed the presence of three exchangeable protons. The $^1$H NMR spectrum is displayed in Figure 2.11.
Figure 2.9 $^{13}$C NMR spectrum of clionamine A (2.6) recorded in CD$_3$OD at 150 MHz.
Figure 2.10 DEPT spectrum of clionamine A (2.6) recorded in CD$_3$OD at 150 MHz.
Figure 2.11 $^1$H NMR spectrum of clionamine A (2.6) recorded in CD$_3$OD at 600 MHz.
Table 2-1 NMR assignments for clionamine A (2.6) at 600 MHz.

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The $^{1}$H NMR spectrum displayed several resonances that are characteristic of steroids derived from the ergostane skeleton (2.25) (Figure 2.12). The two methyl singlets at $\delta$ 0.81 and $\delta$ 0.87 were characteristic of Me-18 and Me-19 of a typical ergostane steroidal core. In addition, the overlapping resonances between $\delta$ 1.0 and $\delta$ 2.3 could be attributed to the methylenes and methine of the steroidal core. Finally, the methyl doublets at $\delta$ 0.91, 0.91, and 1.02 were characteristic of methyls 26, 27 and 28 of the ergostane side chain. Detailed analysis of the COSY, TOCSY, ROESY and HMBC data for clionamine A (2.6) confirmed the presence of a steroidal ABCD ring system and of an unsaturated side chain. The ergostane skeleton numbering scheme is provided in Figure 2.12 to assist with the analysis of the structure elucidation of clionamine A (2.6).

From the two methyl singlets at $\delta$ 0.81 (Me-18) and $\delta$ 0.87 (Me-19), it was possible to assign the protonated carbon resonances in fragment A shown in Figure 2.13. From the methyl singlet at $\delta$ 0.87 (Me-19), HMBC correlations were seen to one quaternary carbon resonance at $\delta$ 36.5 (C-10), to two methine resonances at $\delta$ 46.0 (C-5) and at $\delta$ 55.0 (C-9), and to one methylene resonance at $\delta$ 37.6 (C-1) (Figure 2.14). Using the HSQC data it was possible to assign a pair of geminal protons at $\delta$ 1.10 (H-1$\beta$) and at $\delta$ 1.82 (H-1$\alpha$) to the methine carbon at $\delta$ 37.6 (C-1), while the proton at $\delta$ 0.76 (H-9) was assigned to the methine carbon at $\delta$ 55.0 (C-9).
Figure 2.13 Selected COSY and HMBC correlations for fragments A and B of clionamine A (2.6) in CD$_3$OD.
A linear COSY spin system, described in detail in the following paragraphs, could be traced from the pair of geminal protons at $\delta$ 1.82 (H-1$\alpha$) and at $\delta$ 1.10 (H-1$\beta$) around a decalin moiety to the methine proton resonance at $\delta$ 0.76 (H-9). The geminal proton at $\delta$ 1.82 (H-1$\alpha$) was correlated in the COSY spectrum to a proton at $\delta$ 1.53 (H-2$\beta$) (Figure 2.15). The proton at $\delta$ 1.53 (H-2$\beta$) had a COSY correlation to $\delta$ 1.85 (H-2$\alpha$) and both protons were correlated to the carbon resonance at $\delta$ 27.5 in the HSQC data.
and were, therefore, geminal methylene protons. Both the proton resonances at $\delta$ 1.53 (H-2$\beta$) and $\delta$ 1.85 (H-2$\alpha$) had COSY correlations to the proton resonance at $\delta$ 3.08 (H-3, HSQC to $\delta$ 51.7). The proton resonance at $\delta$ 3.08 (H-3) integrated for one hydrogen atom and was correlated in the COSY spectrum to the protons at $\delta$ 1.60 (H-4$\alpha$, HSQC to $\delta$ 34.0) and $\delta$ 1.41 (H-4$\beta$, HSQC to $\delta$ 34.0), which were in turn correlated to each other (Figure 2.16). The proton resonance at $\delta$ 1.41 (H-4$\beta$) was correlated to a proton at $\delta$ 1.23 (H-5, HSQC to $\delta$ 46.0). The carbon resonance at $\delta$ 46.0 was mentioned previously as the methine carbon resonance that was correlated to the methyl resonance at $\delta$ 0.87 (Me-19) in the HMBC data. Consequently, the data was consistent with the presence of a cyclohexane ring starting with the carbon at $\delta$ 37.6 (C-1) and ending with the quaternary carbon at $\delta$ 36.5 (C-10).
Figure 2.15 Expanded COSY spectrum of 2.6 recorded in CD$_3$OD at 600 MHz showing the correlations for ring A and B.

An additional COSY correlation was seen from the proton resonance at $\delta$ 1.23 (H-5) to the resonance at $\delta$ 1.35 (H-6$\alpha$) (Figure 2.15). This latter proton resonance was also correlated in the COSY
spectrum to the resonance at \( \delta 1.10 \) (H-6\( \beta \)), and both the resonances at \( \delta 1.35 \) (H-6\( \alpha \)) and at \( \delta 1.10 \) (H-6\( \beta \)) were correlated to the carbon at \( \delta 29.4 \) (C-6) in the HSQC spectrum and were, consequently, geminal protons. Another COSY correlation was seen from the proton resonance at \( \delta 1.35 \) (H-6\( \alpha \)) to the proton resonance at \( \delta 1.74 \) (H-7\( \beta \)) which was also correlated to the resonance at \( \delta 1.00 \) (H-7\( \alpha \)) in the COSY (Figure 2.16). The two proton resonances at \( \delta 1.74 \) (H-7\( \beta \)) and at \( \delta 1.00 \) (H-7\( \alpha \)) were correlated to \( \delta 32.8 \) (C-7) in the HSQC. The proton resonance at \( \delta 1.74 \) (H-7\( \beta \)) also had a COSY correlation to the methine proton resonance at \( \delta 1.56 \) (H-8, HSQC to \( \delta 35.8 \)). The methine proton at \( \delta 1.56 \) (H-8) had a COSY correlation to \( \delta 0.76 \) (H-9), the methine correlated to the methyl at \( \delta 0.87 \) (Me-19) in the HMBC. The HMBC correlations from Me-19 and the large COSY spin system just described served as evidence to support the presence of a decalin system methylated on the junction between the A ring and the B ring of the steroidal core (Figure 2.13).

From the other methyl singlet resonance at \( \delta 0.81 \) (Me-18) present in the \( ^1H \) NMR data of clionamine A (2.6), HMBC correlations were seen to four carbons. From the \( ^{13}C \) NMR data and the HSQC data, it was possible to determine that one was a quaternary carbon resonance at \( \delta 41.7 \) (C-13), two were methine resonances at \( \delta 56.5 \) (C-14, HSQC with \( \delta 1.17 \)) and at \( \delta 65.8 \) (C-17, HSQC with \( \delta 2.14 \)) and one was a methylene resonance at \( \delta 39.8 \) (C-12, HSQC with \( \delta 1.21 \) and \( \delta 1.92 \)) (Figure 2.14). The proton resonances from this last methylene were part of the COSY spin system described in the previous paragraphs and they were assigned to the C ring of the steroidal core. The proton resonance at \( \delta 1.92 \) (H-12\( \beta \)) was correlated to a proton at \( \delta 1.55 \) (H-11\( \alpha \)) and this latter proton resonance had a COSY correlation to the resonance at \( \delta 1.33 \) (H-11\( \beta \)) (Figure 2.16). Both proton resonances at \( \delta 1.55 \) (H-11\( \alpha \)) and \( \delta 1.33 \) (H-11\( \beta \)) were correlated to the same carbon resonance at \( \delta 21.2 \) in the HSQC data. The proton resonance at \( \delta 1.33 \) (H-11\( \beta \)) had a COSY correlation to the proton signal at \( \delta 0.76 \) assigned to H-9 (Figure 2.16). As mentioned in the previous paragraph, the proton assigned as H-9 was correlated to the
methine proton resonance at δ 1.56 (H-8) in the COSY spectrum, but this methine proton signal also had a COSY correlation to the methine resonance (δ 1.17, H-14) correlated to Me-18 in the HMBC spectrum. This confirmed the presence of a six-membered C ring in the steroidal core of clionamine A (2.6) (Figure 2.13).

As mentioned above, the proton resonance for the methyl singlet at δ 0.81 (Me-18) had HMBC correlations with the carbon resonances at δ 41.7 (C-13), δ 56.5 (C-14) and δ 65.8 (C-17). In order to confirm the presence of a standard steroidal five-membered D ring in clionamine A (2.6), the COSY data was analysed to uncover a COSY spin system joining the proton resonances associated with the carbons at δ 56.5 (C-14) and at δ 65.8 (C-17), since both these carbons were correlated with Me-18 in the HMBC spectrum. A COSY correlation was seen between the proton resonance at δ 1.17 (H-14) and proton resonances at δ 2.25 (H-15α, HSQC to δ 32.7), which was also correlated to the resonance at δ 1.48 (H-15β, HSQC to δ 32.7) (Figure 2.16). Both of the resonances at δ 2.25 (H-15α) and δ 1.48 (H-15β) had a COSY correlation with the downfield proton resonance at δ 5.07 (H-16, HSQC to δ 84.0), which was in turn correlated to the resonance at δ 2.14 (H-17, HSQC to δ 65.8), confirming the presence of a five carbon D ring in clionamine A (2.6) (Figure 2.13).
Figure 2.16 Expanded COSY spectrum of 2.6 recorded in CD$_3$OD at 600 MHz showing the correlations for ring C, D, and fragment B.

COSY and HMBC correlations for the three doublet methyl signals seen in the $^1$H NMR of clionamine A (2.6) and the olefinic resonances at $\delta$ 5.75 (H-22) and $\delta$ 6.13 (H-23) were consistent with fragment B, a seven carbon C-24 methylated unsaturated steroidal side chain shown in Figure 2.13. The HMBC resonances for the doublet integrating for six protons at $\delta$ 0.91 (Me-26 and Me-27) revealed the presence of a geminal methyls on the carbon resonance at $\delta$ 34.3 (C-25) (Figure 2.17). This carbon was correlated to the proton resonance at $\delta$ 1.61 (H-25) in the HSQC and from this proton a COSY correlation
was seen to a signal at δ 2.09 (H-24, HSQC to δ 44.6) (Figure 2.16). An HMBC correlation between the methyl doublet signal at δ 1.02 (Me-28) and the carbon resonance at δ 44.6 (C-24) indicated that this carbon was methylated (Figure 2.17). In the COSY spectrum, the proton resonance at δ 2.09 (H-24) was also correlated to the olefinic resonances at δ 5.75 (H-22, HSQC to δ 126.4) and δ 6.13 (H-23, HSQC to δ 140.6) (Figure 2.16). The two olefinic protons shared a 15.8 Hz coupling but the proton signal at δ 6.13 (H-23) was a doublet of doublets suggesting that it may be vicinal to the methine assigned to C-24, whereas the proton signal at δ 5.75 (H-22) was a doublet and could be vicinal to a quaternary carbon. The position of the proton assigned as H-23 next to the methylated C-24 methine was supported by an HMBC correlation between the the proton at δ 6.13 (H-23) and both the methyl carbon assigned as Me-28 and methine carbon δ 34.3 (C-25) (Figure 2.18).
Figure 2.17 Expanded HMBC spectrum of clionamine A (2.6) recorded in CD$_3$OD at 600 MHz.
The olefinic methine signal at \( \delta \) 5.75 (H-22) was correlated to an ester carbonyl signal at \( \delta \) 178.7 (C-21) (Figure 2.18). Both the olefinic methine signals at \( \delta \) 5.75 (H-22) and \( \delta \) 6.13 (H-23), and the methine signal at \( \delta \) 2.14 (H-17) were correlated to an oxygenated tertiary carbon at \( \delta \) 76.6 (C-20) in the HMBC spectrum. To determine if this oxygenated tertiary carbon was hydroxylated, a complete NMR data set for clionamine A (2.6) was collected in DMSO-\( d_6 \), since no exchangeable proton had been
detected in C₆D₆. The HMBC spectrum in DMSO-d₆ revealed a correlation between an oxygenated tertiary carbon resonance at δ 74.7 (C-20) and a proton resonance at δ 6.07 (Figure 2.19). The proton resonance δ 6.07 was assigned to a hydroxyl group since it lacked a HSQC correlation with a carbon. In the HMBC spectrum, the resonance at δ 6.07 (OH-20) was also correlated to a methine carbon resonance at δ 64.0 (C-17) and to an ester carbonyl signal at δ 176.1 (C-21) (Figure 2.20).

Figure 2.19 Expanded HMBC spectrum of clionamine A (2.6) recorded in DMSO-d₆ at 600 MHz.

In the HMBC data collected in DMSO-d₆, the two olefinic methine resonances at δ 5.67 (H-22) and δ 5.98 (H-23) were also correlated to the oxygenated tertiary carbon resonance at δ 74.7 (C-20) and
the signal at δ 5.67 (H-22) was correlated to the ester carbonyl resonance at δ 176.1 (C-21). The proton resonance for δ 2.08 (H-17) was correlated to the tertiary carbon resonance at δ 74.7 (C-20) and to the ester carbonyl at δ 176.1 (C-21). This established that the ester carbonyl (C-21), the olefinic methine (C-22), and the methine at δ 64.0 (C-17) were all substituents on the tertiary carbon at δ 74.7 (C-20) (Figure 2.20).

One more ring had to be present in clionamine A (2.6) in order to satisfy the molecular formula obtained from the HRESIMS data. The deshielded chemical shift of the proton signal at δ 4.98 (H-16, in DMSO-d$_6$) and of the carbon signal at δ 81.7 (C-16, in DMSO-d$_6$) indicated that the alkoxyl group of the ester carbonyl was attached there. Thus, a γ-lactone ring was formed by C-16, C-17, C-20 and the ester carbonyl C-21.

The chemical shift of the proton and carbon resonance for C-3 (δ $^1$H 2.96, δ $^{13}$C 49.4 in DMSO-d$_6$) suggested that it could be the site of attachment of a primary amine instead of the secondary alcohol usually found at this position in steroids. This assignment was also supported by the even molecular mass [M+H]$^+$ obtained in the HRESIMS that indicated the presence of a nitrogen atom in clionamine A (2.6). The primary amine proton resonance was detected when the data was collected in DMSO-d$_6$ at δ 7.71 and it integrated for two protons.
The ROESY data obtained in CD$_3$OD was used to determine that the amino group was equatorial based on the correlation between the methine proton resonance at $\delta$ 3.08 (H-3), and both of the proton resonances at $\delta$ 1.82 (H-1$\alpha$) and $\delta$ 1.23 (H-5) (Figure 2.21).

Figure 2.21 Expanded ROESY spectrum of clionamine A (2.6) recorded in MeOD at 600 MHz.
Figure 2.22 Expanded ROESY spectrum of clionamine A (2.6) recorded in MeOD at 600 MHz.

The ROESY data was also used to establish the trans, anti, trans, anti, trans relative configuration in the ABCD ring system shown in Figure 2.23. The resonance for Me-19 (δ 0.87) was correlated to H-2β (δ 1.53) and to H-8 (δ 1.56), which was also correlated to Me-18 (δ 0.81) (Figure 2.22). The resonance for Me-18 (δ 0.81) was correlated to H-22 (δ 5.75), indicating that C-20 was a β substituent on C-17 and that C-22 was a β substituent on C-20 (Figure 2.21). The correlations from H-16
(δ 5.07) to H-17 (δ 2.14) and H-14 (δ 1.17) indicated that these protons were α substituents on their respective carbons (Figure 2.21).

Figure 2.23 Selected ROESY correlations for clionamine A (2.6) in MeOD.

Detailed analysis of the 1D and 2D NMR data obtained for clionamine A (2.6) served to elucidate its structure and relative configuration. Clionamine A (2.6) is a novel ergostane-derived aminosteroid for which the side chain was arranged into a γ-lactone.

Clionamines B (2.7), C (2.8), and D (2.9) were isolated as their Fmoc derivatives along with the Fmoc derivative of clionamine A (2.6). The 1D and 2D NMR data of the Fmoc- clionamines B (2.22), C (2.23), and D (2.24) in C6D6 were compared to the NMR data obtained for Fmoc-clionamine A and the steroidal nuclei were found to be virtually identical in all four Fmoc protected clionamines. The γ-lactone ring was also present in all the clionamines, but it was clear that they differed in the structures of their side chains.

The Fmoc derivative of clionamine B (2.22) was isolated as a white amorphous solid that gave a [M+Na]+ ion at m/z 676.3975 in the HRESIMS corresponding to a molecular formula of C42H55NO5 (calculated for C42H53NO5Na, 676.3978). After the subtraction of the atoms present in the Fmoc
protecting group (C$_{13}$H$_{20}$O$_{2}$), we found the molecular formula for clionamine B (2.7) to be C$_{27}$H$_{45}$NO$_{3}$, which required six degrees of unsaturation. The $^{13}$C NMR and $^{1}$H NMR spectra are shown in Figure 2.24-2.25, respectively.

Figure 2.24 $^{13}$C NMR spectrum of Fmoc-clionamine B (2.22) recorded in C$_{6}$D$_{6}$ at 150 MHz.
Figure 2.25 $^1$H NMR spectrum of Fmoc-clionamine B (2.22) recorded in $CD_6$ at 600 MHz.
Table 2-2 NMR assignments for Fmoc-clionamine B (2.22) in C₆D₆ at 600 MHz.

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The $^{13}$C NMR spectrum (Figure 2.24) and the HSQC data showed that clionamine B (2.22) had 27 carbons, of which four were methyls, eleven were methylenes, eight were methines, and four were quaternary carbons. The $^1$H NMR and the HSQC data revealed the absence of the olefinic methines at C-22 and C-23 that were seen in clionamine A (2.6). The absence of the olefin signals along with the fact that clionamine B (2.7) only had 6 degrees of unsaturation compared to seven in clionamine A (2.6) suggested that the side chain of clionamine B (2.7) was fully saturated.
Figure 2.26. Selected HMBC and COSY correlation for the side chain of Fmoc-clionamine B (2.22) in C<sub>6</sub>D<sub>6</sub>. 

The structure of the saturated side chain of 2.22 was derived through the information obtained in the COSY, HSQC and HMBC spectra (Figure 2.26). A proton at δ 1.95 (H-22a) showed an HMBC correlation to an oxygenated tertiary carbon at δ 77.6 (C-20) and to a methine at δ 62.1 (C-17) indicating that the carbon at δ 33.0 (C-22) was attached to the lactone moiety (Figure 2.27). As with clionamine A (2.6), Fmoc-clionamine B (2.22) was found to be hydroxylated on C-20, based on an HMBC correlation between the oxygenated tertiary carbon at δ 77.6 (C-20) and a proton resonance at δ 1.98 that had no correlation to any of the carbons in the HSQC spectrum in C<sub>6</sub>D<sub>6</sub>. 

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Figure 2.26: Diagram showing the HMBC and COSY correlations for the side chain of Fmoc-clionamine B (2.22) in C<sub>6</sub>D<sub>6</sub>.
Figure 2.27 Expanded HMBC spectrum of Fmoc-clionamine B (2.22) recorded in C₆D₆ at 600 MHz.

The proton signal at δ 1.95 (H-22a) had a COSY correlation to a signal at δ 1.66 (H-22b) and the two protons both had an HSQC correlation to the carbon at δ 33.0 (C-22) (Figure 2.28). The proton resonance at δ 1.95 (H-22a) was also correlated to proton resonance at δ 1.29 (H-23b) in the COSY spectrum (Figure 2.28). The latter resonance was correlated to the proton at δ 1.66 (H-23a), and the two were found to be a pair of geminal protons based on their HSQC correlation to the same carbon resonance at δ 22.0 (C-23). The proton resonance at δ 1.66 (H-23a) was correlated to another pair of
geminal protons in the COSY data (δ 1.21, H-24a; δ 1.15, H-24b, HSQC to δ 40.1) which were in turn correlated to a methine (δ 1.55, H-25, HSQC to 28.9). The resonance at δ 1.55 (H-25) was correlated to two methyl doublet resonances at δ 0.95 (Me-26) and δ 0.93 (Me-27) in the COSY spectrum. Only two methyl doublet signals were present in 2.22, therefore, the side chain lacked the methyl attached to carbon 24 found in clionamine A (2.6).
Figure 2.28 Expanded COSY spectrum of Fmoc-clionamine B (2.22) recorded in C₆D₆ at 600 MHz.

The ROESY data confirmed that as in clionamine A (2.6), the nitrogen atom was in the equatorial position on carbon 3 of Fmoc-clionamine B (2.22) (Figure 2.29). The ROESY data also confirmed the *trans, anti, trans, anti, trans* relative configuration in the ABCD ring system. Thus Me-18 (δ 0.70) showed a ROESY correlation to H-22b (δ 1.66), indicating that C-20 was a β substituent on C-17 and that C-22
was a \( \beta \) substituent on C-20. In addition, the ROESY correlation from both H-17 (\( \delta \) 1.82) and H-16 (\( \delta \) 4.91) to OH-20 (\( \delta \) 1.98) indicated that these protons were \( \alpha \) substituents on their respective carbons (Figure 2.30).

![Figure 2.29 Selected ROESY correlations for Fmoc-cionamine B (2.22) in C$_6$D$_6$.](image-url)
Figure 2.30 Expanded ROESY spectrum of Fmoc-clionamine B (2.22) recorded in C₆D₆ at 600 MHz.

The Fmoc protected clionamine C (2.23) was isolated as a colorless glass that gave a [M+Na]⁺ ion at m/z 688.3961 in the HRESIMS corresponding to a molecular formula of C₄₃H₅₅NO₅ (calculated for C₄₃H₅₅NO₅Na, 688.3978). After subtracting the atoms of the Fmoc protecting group, the molecular
formula was \( \text{C}_{28}\text{H}_{45}\text{NO}_3 \). This molecular formula was identical to the molecular formula of clionamine A (2.6) and required seven degrees of unsaturation.

Figure 2.31 \( ^{13}\text{C} \) NMR spectrum of Fmoc-clionamine C (2.23) recorded in \( \text{C}_6\text{D}_6 \) at 150 MHz.
Figure 2.32 $^1$H NMR spectrum of Fmoc-clionamine C (2.23) recorded in CD$_6$ at 600 MHz.
Table 2-3 NMR assignments for Fmoc-clionamine C (2.23) in C_{6}D_{6} at 600 MHz.

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The $^{13}$C NMR and the $^1$H NMR data indicated the presence of an olefin in Fmoc-clionamine C (2.23) (Figure 2.31-2.32). The HSQC data revealed that the singlet proton resonances at δ 4.84 (H-26a) and δ 4.86 (H-26b) were correlated to the same carbon resonance at δ 110.0 (C-26), and these chemical shifts were diagnostic of a terminal alkene. Unlike clionamine A (2.6), Fmoc-clionamine C (2.23) appeared to be saturated at C-22 since the olefinic protons resonances at δ 4.84 (H-26a) and δ 4.86 (H-26b) did not have HMBC correlations to any of the lactone carbon signals. Instead, a methylene proton resonance at δ 1.96 (H-22b, HSQC to δ 30.5), was correlated to the ester carbonyl signal at δ 176.5 (C-
21) of the lactone ring in the HMBC spectrum, confirming that Fmoc-clionamine C (2.23) was saturated at C-22 (Figure 2.33).

![Expanded HMBC spectrum of 2.23 recorded in C₆D₆ at 600 MHz showing the correlations to C-21.](image)

A hydroxyl proton resonance at δ 1.98 (OH-20) was seen in the NMR spectrum of Fmoc-clionamine C (2.23) and it was useful in confirming the presence of the γ-lactone moiety. HMBC correlations were seen from the hydroxyl proton resonance at δ 1.98 (OH-20) to the tertiary carbon at δ 76.0 (C-20), to the methine carbon at δ 62.4 (C-17), to the methylene carbon signal at δ 28.8 (C-23) (Figure 2.34), and finally to the ester carbonyl signal at δ 176.5 (C-21) (Figure 2.34).
The proton at resonance at $\delta$ 1.96 (H-22b) was coupled to the proton resonance at $\delta$ 1.34 (H-22b) in the COSY data (Figure 2.35). From the two proton resonances assigned to H-22a and H-22b, COSY correlations were observed to $\delta$ 1.26 (H-23a) and $\delta$ 1.89 (H-23b), a pair of protons correlated to the carbon at $\delta$ 28.8 (C-23) in the HSQC spectrum. From both of these geminal methylene protons, a COSY correlation was seen to a methine proton signal at $\delta$ 2.07 (H-24, HSQC to $\delta$ 42.5) (Figure 2.35).
Figure 2.35 Expanded COSY spectrum of Fmoc-clionamine C (2.23) recorded in $\text{C}_6\text{D}_6$ at 600 MHz.

In the HMBC spectrum, the olefinic protons $\delta$ 4.84 (H-26a) and $\delta$ 4.86 (H-26b) were correlated to the methine carbon signal at $\delta$ 42.5 (C-24), to a quaternary carbon resonance at $\delta$ 150.0 (C-25) as well as to the methyl singlet signal at $\delta$ 19.2 (Me-27) (Figure 2.36). Only one methyl doublet signal at $\delta$ 1.03 (Me-28) was present in the $^1\text{H}$ NMR data for Fmoc-clionamine C (2.23) and it was correlated to the methine carbon signal at $\delta$ 42.5 (C-24) in the HMBC spectrum indicating that Fmoc-clionamine C (2.23) was methylated on C-24 as was clionamine A (2.6).
Figure 2.36 Expanded HMBC spectrum of 2.23 recorded in C₆D₆ at 600 MHz showing the correlations to H-26a & H-26b.

The methyl singlet at δ 1.66 (Me-27) had HMBC correlations into the quaternary carbon resonance at δ 150.0 (C-25), to the terminal olefin carbon signal at δ 110.0 (C-26), and to the methine carbon signal at δ 42.5 (C-24) (Figure 2.37).
Figure 2.37 Expanded HMBC spectrum of 2.23 recorded in C₆D₆ at 600 MHz showing the correlations to Me-27.

Therefore Fmoc-clionamine C (2.23) was methylated on C-24 and C-25 and had a Δ²⁵,²⁶ olefin in accordance with the sub-structure shown in Figure 2.38.
The ROESY data confirmed that as in clionamine A (2.6) and clionamine B (2.7), the amino group was in the equatorial position on carbon 3 of the Fmoc derivative of clionamine C (2.8) (Figure 2.39). The ROESY data also confirmed the trans, anti, trans, anti, trans relative configuration in the ABCD ring system. Again, the resonance for Me-18 (\(\delta 0.71\)) was correlated to H-22b (\(\delta 1.96\)) in the ROESY spectrum, indicating that C-20 was a \(\beta\) substituent on C-17 and that C-22 was a \(\beta\) substituent on C-20. In addition, the ROESY correlation from both H-17 (\(\delta 1.79\)) and H-16 (\(\delta 4.90\)) to OH-20 (\(\delta 1.98\)) indicated that these protons were \(\alpha\) substituents on their respective carbons (Figure 2.40).
The Fmoc protected clionamine D (2.24) was isolated as a yellow solid that gave a [M+Na]^+ ion at m/z 646.3130 in the HRESIMS corresponding to a molecular formula of C_{39}H_{45}NO_{6} (calculated for C_{39}H_{45}NO_{6}Na, 646.3145). Once the atoms for the Fmoc protecting group were subtracted, the molecular formula for clionamine D (2.9) was found to be C_{24}H_{35}NO_{4}, which required eight degrees of unsaturation. The $^{13}$C NMR and the $^1$H NMR spectra are displayed in Figure 2.41 and 2.42 respectively.
Figure 2.41 $^{13}$C NMR spectrum of Fmoc-clionamine D (2.24) recorded in $\text{C}_6\text{D}_6$ at 150 MHz.
Figure 2.42 $^1$H NMR spectrum of Fmoc-clionamine D (2.24) recorded in CD$_6$ at 600 MHz.
Table 2-4 NMR assignments for Fmoc-clionamine D (2.24) in C_6D_6 at 600 MHz.

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Detailed analysis of the NMR data revealed that the steroidal core and the γ-lactone ring found in clionamines A (2.6), B (2.7), and C (2.8) were present in Fmoc-clionamine D (2.24), although the carbon signal for C-20 in the γ-lactone ring seen in 2.6-2.8 at δ 76.0 was not present in Fmoc-clionamine D (2.24). Instead a more downfield signal was seen at δ 86.0. An HMBC correlation from the proton resonance at δ 1.69, assigned to H-17, was seen to the carbon signal at δ 86.0 and suggested that this oxygenated tertiary carbon resonance was part of the γ-lactone ring (Figure 2.43). The NMR data showed that the side chain present in Fmoc-clionamine D (2.24) was highly modified when compared with clionamines A-C (2.6-2.8), offering an explanation for the deshielded chemical shift of the
oxygenated tertiary carbon resonance. The $^{13}$C NMR data for Fmoc-clionamine D (2.24) only presented three carbons that did not belong to the steroidal core and the lactone ring found in clionamines A-C (2.6-2.8) (Figure 2.41). One was an ester carbonyl at $\delta$ 173.9 (C-24) and the other two were carbon resonances at $\delta$ 25.0 (C-22) and $\delta$ 28.3 (C-23) assigned as methylenes using the HSQC data. The proton signals for these methylenes [(H-22a); $\delta$ 1.60 (H-22b); $\delta$ 2.58 (H-23a); $\delta$ 1.91 (H-23b)] formed an isolated COSY spin system. The HMBC data revealed that all four methylene protons resonances were correlated to the ester carbonyl signal at $\delta$ 173.9 (C-24) and to the quaternary carbon at $\delta$ 86.0, assigned as C-20 (Figure 2.43). In addition, the proton signal at $\delta$ 1.60 (H-22b) was correlated to the ester carbonyl signal of the $\gamma$-lactone ring at $\delta$ 174.5 (C-21), as well as to the methine carbon resonance at $\delta$ 60.5 (C-17). Based on the molecular formula and on the analysis of the DEPT data, only one exchangeable proton was present in Fmoc-protected clionamine D (2.24) and it was associated with the amino group at C-3. As a result, Fmoc-protected clionamine D (2.24) did not have a hydroxyl group on C-20. This piece of evidence coupled with the HMBC correlations mentioned above for the vicinal methylene signals at $\delta$ 2.13 (H-22a), $\delta$ 1.60 (H-22b), $\delta$ 2.58 (H-23a) and $\delta$ 1.91 (H-23b) pointed to the presence of a second lactone ring forming an unprecedented spiro bislactone side chain on clionamine D (2.9). The proposed structure, shown in Figure 2.44, also accounts for the downfield carbon chemical shift of C-20.
Figure 2.43 Expanded HMBC spectrum for Fmoc-clionamine D (2.24) in CD$_2$D$_6$ at 600 MHz.
Figure 2.44 Selected HMBC and COSY correlation for the side chain of Fmoc-clionamine D (2.24) in C₆D₆.

The ROESY data for cliona mine D (2.9) revealed that Me-18 (δ 0.43) was correlated to H-22a (δ 2.13) and H-22b (δ 1.60) (Figure 2.45). This was consistent with C-20 being β on C-17 and with C-22 being β on C-20 (Figure 2.46). As in clionamine A (2.6), a ROESY correlation was seen between H-16 (δ 4.58) and H-17 (δ 1.69), confirming that the two protons were cis to each other. The configuration around the rest of the molecule was consistent with what was found for clionamine A (2.6).
Figure 2.45 Expanded ROESY spectrum for Fmoc-clionamine D (2.24) in C₆D₆ at 600 MHz.
The deprotection of Fmoc-clionamine B (2.22), C (2.23) and D (2.24) was performed using standard conditions; 2 mg portions of each of the protected clionamines were stirred in 90:10 DMF:piperidine solution (10 mL) at room temperature for one hour (Scheme 2.2). The DMF and piperidine were then removed by vacuum distillation and the resulting residues were titurated in ether and 0.1% trifluoroacetic acid. Considering the amount of material available, filtration would have resulted in a considerable loss of material. Therefore, the precipitate was centrifuged and the supernatant removed. The deprotection of Fmoc-clionamine B (2.22), C (2.23) and D (2.24) was successful in yielding clionamine B (2.7) (≈ 0.5 mg), clionamine C (2.8) (≈ 0.5 mg) and clionamine D (2.9) (1 mg).
Clionamine B (2.7) was isolated as white solid that gave a [M+H]^+ ion at m/z 432.3474 in the HRESIMS corresponding to a molecular formula of C_{27}H_{45}NO_{3} (calculated for C_{27}H_{46}NO_{3}, 432.3478). The $^1$H NMR spectrum for clionamine B (2.7) recorded in CD$_3$OD is shown in Figure 2.47 and the NMR assignments for 2.7 are listed in Table 2-5.

![Figure 2.47 $^1$H NMR spectrum for clionamine B (2.7) in CD$_3$OD at 600 MHz.](image-url)
Table 2-5 NMR assignments for clionamine B (2.7) in CD$_3$OD at 600 MHz.

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Clionamine C (2.8) was isolated as a white solid that gave a [M+H]$^+$ ion at m/z 444.3462 in the HRESIMS corresponding to a molecular formula of C$_{28}$H$_{45}$NO$_3$ (calculated for C$_{28}$H$_{46}$NO$_3$, 444.3478). The $^1$H NMR spectrum for clionamine C (2.8) recorded in DMSO- $d_6$ is shown in Figure 2.48 and the NMR assignments are listed in Table 2-6.
Figure 2.48 $^1$H NMR spectrum for clonamine C (2.8) in DMSO-$d_6$ at 600 MHz.
Clionamine D (2.9) was isolated as a yellow solid that gave a [M+H]$^+$ ion at m/z 402.2632 in the HRESIMS corresponding to a molecular formula of C$_{24}$H$_{35}$NO$_4$ (calculated for C$_{24}$H$_{36}$NO$_4$, 402.2644). The $^1$H NMR spectrum for clionamine D (2.9) recorded in DMSO- $d_6$ is shown in Figure 2.49 and the NMR assignments for 2.9 are listed in Table 2-7.
Figure 2.49 $^1$H NMR spectrum for clionamine D (2.9) in DMSO- $d_6$ at 600 MHz.
Table 2-7 NMR assignments for clionamine D (2.9) in DMSO- d$_6$ at 600 MHz.

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<th>$\delta_H$</th>
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</table>

It was necessary to obtain the deprotected clionamines (2.7-2.9) in order to establish their biological activity in the autophagy assay. The results of the assay are presented in the following section.

2.6 The Clionamines as Autophagy Activators

The phenotypic response of MCF-7 cells to clionamine A (2.6) was investigated and the phenotypes obtained upon treatment with various concentrations of clionamine A (2.6) and chloroquine (2.20) (100 μM) are shown in Figure 2.50. In the first column of Figure 2.50, the images obtained using the Hoechst channel of a Cellomics™ microscope show the number of cells present in each well of a plate and allows the measurement of the cytotoxicity of the drug administered to the cell. The GFP-LC3 proteins are detected in the second channel as they aggregate on the forming autophagosomes. The composite image, shown in the third column of Figure 2.50 is used to visualize the presence of
autophagosomes in the cells by detecting the GFP-LC3 that has concentrated on the surface of the autophagosomes. In the control cells (first row of Figure 2.50), the composite image displays a diffuse blue color across all the cells. In these cells, autophagy is at a basal level and most of the GFP-LC3 is spread across the cytosol and a blue diffuse light is observed across the cell. On the other hand, the results shown in the second row of Figure 2.50 illustrate that in the cells treated with clionamine A (2.6), at a concentration of 10 μg/mL, the GFP-LC3 is concentrated on the autophagosomes and small blue dots are detected within each cell. However, 2.6 appeared to be toxic at a concentration of 10 μg/mL based on the low cell count at this concentration. The cells treated with a concentration of 3 μg/mL of clionamine A (2.6) showed an increase in the number of autophagosomes formed compared with the control cells and 2.6 was not cytotoxic at that concentration. At a concentration of 1 μg/mL, clionamine A (2.6) did not cause an increase in the number of autophagosomes compared with the control cells. The phenotypes obtained after treatment with chloroquine (2.20) are shown in the last row of Figure 2.50. Chloroquine (2.20) is used as a positive control in this assay since it inhibits the last step of autophagy by preventing the delivery of the autophagosomes to the lysosomes. The composite image of the cells treated with chloroquine shows the blue dots corresponding to the aggregation of GFP-LC3 on the autophagosomes, but since the autophagosomes of cells under treatment with chloroquine cannot be degraded by the lysosomes, the process of autophagy is stopped and the autophagosomes accumulate in the cells, filling the cytosol with blue dots.
Figure 2.50 MCF-7 GFP-LC3 cells incubated for 4 h with various concentrations of clionamine A (2.6) and chloroquine.

The deprotected clionamines B-D (2.7-2.9) were also tested in the GFP-LC3 biological assay and were shown to be autophagy activators. The clionamines (2.6-2.9) were compared in terms of the average minimum concentration needed to induce a 20 fold increase in the number of autophagosomes in the MCF-7 cell line. Each clionamine was tested in triplicates at 8 different concentrations. The results are summarised in Table 2-8.
Table 2-8 Average minimum concentration of clionamine A (2.6), B (2.7), C (2.8) and D (2.9) needed to obtain a 20-fold increase in the number of autophagosomes detected in the MCF-7 GFP-LC3 cells.

<table>
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<th>Minimum Concentration (ug/mL)</th>
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<tr>
<td>clionamine A (2.6)</td>
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<td>Clionamine B (2.7)</td>
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<tr>
<td>Clionamine C (2.8)</td>
</tr>
<tr>
<td>Clionamine D (2.9)</td>
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</table>

Clionamine A (2.6) and C (2.8) appear to be the most active compounds present in the original extract. Clionamine A (2.6) differs from clionamine C (2.8) only by the position of the olefin on the side chain. On the other hand, unlike clionamines A (2.6) and C (2.8), clionamine B (2.7) is not methylated at carbon 24 and its side chain is saturated. These structural differences in the side chain may account for the concentration of clionamine B (2.7) needed to induce autophagy. Similarly, clionamine D (2.9) has a highly modified side chain arranged into a spiro bislactone. Again, the structure of the side chain appears to decrease the biological activity of this clionamine. Since we had significantly more clionamine A (2.6) than clionamine C (2.8) available, we used clionamine A for most of the biological testing.

Clionamine A (2.6) was tested in MCF-7 cells under nutrient poor and nutrient rich conditions. Under nutrient poor condition, the formation of autophagosomes in the cell is normally upregulated in order to recycle superfluous cellular material and maintain essential cell functions. Accordingly, the activating effect of clionamine A (2.6) was enhanced in cells lacking amino acids and serum in their media compared with cells under normal growth conditions (Figure 2.51a). This induction of autophagy under nutrient poor conditions was not seen with rapamycin (2.17). This suggests that clionamine A (2.6) does not act on the protein kinase mTOR, the target of most modulators of autophagy such as rapamycin (2.17). The exact molecular target of clionamine A (2.6) is currently being investigated.
To verify that the autophagosomes formed under treatment with clionamine A (2.6) were in fact delivered to the lysosome for degradation, the levels of free GFP in the cells treated with 2.6 were measured. The rationale behind measuring the levels of GFP is as follows; in the cells used in the assay, the autophagosomes with the GFP-LC3 on their surface are delivered to the lysosomes but since GFP is not a human protein, it is degraded very slowly by the human lysosomes while LC3 and the rest of the autophagosomes are readily degraded. Immunoblotting using an anti-GFP antibody demonstrated that the levels of free GFP were increased under treatment with clionamine A (2.6) (Figure 2.51b). These results indicate that LC3 is being degraded along with the autophagosome, presumably by the lysosomal machinery, and that clionamine A (2.6) does stimulate the entire autophagy process and not only the formation of autophagosomes.

Figure 2.51 Effects of clionamine A (2.6) on the accumulation of autophagosomes under nutrient rich and nutrient poor conditions. Induction of autophagosome accumulation for different concentrations of clionamine A (2.6) over a period of 4 hours (A). Electrophoresis gels showing the relative amount of GFP-LC3 and of GFP present in the cells for various concentrations of clionamine A (2.6) (B).

The presence of the amine on the clionamines raised concerns that it may affect the pH of the lysosome like chloroquine and that therein lays their biological activity. To answer this concern, the amine functionality of clionamine A (2.6) was acetylated using acetic anhydride in pyridine. The resulting acetylated aminosteroid (2.25) did not induce the formation of autophagosomes in the biological assay.
Scheme 2.3 Acetylation of clionamine A (2.6).

To verify that the biological activity was not only due to the presence of an amine on a steroidal core, a reductive amination of 5α-cholestan-3-one (2.26) was performed with sodium cyanoborohydride and ammonium acetate in MeOH (Scheme 2.4).\textsuperscript{74}

Scheme 2.4 Reductive amination of 5α-cholestan-3-one (2.26).

The aminosteroid 2.27 did not induce the formation of autophagosomes in the biological assay, indicating that the γ-lactone ring functionality may also play an important role in the binding of clionamines A-D (2.6-2.9) to their target.

### 2.7 Conclusion

The biological assay developed by the Roberge laboratory was successful in guiding the isolation of autophagy modulators. The assay allowed for the isolation of clionamines A through D (2.6-2.9). The clionamines are novel aminosteroids and have several unprecedented structural features. Firstly, as 3-
aminosteroids, they are amongst a small group of nitrogen containing steroids isolated from natural sources. Another notable feature of the clionamines is the presence of a C-16/C-17 fused γ-lactone E ring. This structural feature is common as a degradation product of terrestrial steroidal saponins, but the clionamines represent the first marine examples of molecules with such functionality. Clionamine D (2.9) has the most unique features of the clionamines. Its highly modified side chain forms a spiro bislactone, a moiety that is novel to both synthetic and natural steroids isolated to date. The clionamines also add to the diversity of metabolites known to be produced by *C. celata*.

Clionamines A-D (2.6-2.9) were found to be activators of autophagy as they induce the formation of autophagosomes in the MCF-7 cell line, with clionamines A (2.6) and C (2.8) inducing autophagy at lower concentration than clionamines B (2.7) and D (2.9). The amine functionality of the clionamines appeared to be necessary to their biological activity as the acetylated clionamine A (2.6) was not found to be an inducer of autophagy. The γ-lactone ring may also play a role in the mechanism of action of the clionamines on their target since 5α-cholestan-3-amine (2.27) did not induce autophagy.

Clionamine A (2.6) was shown to induce autophagy under normal growth conditions but its effect was increased for cells grown in nutrient-poor conditions. In addition, the autophagosomes formed in the cells treated with clionamine A (2.6) were shown to be delivered to the lysosomes for degradation. Therefore, clionamine A (2.6) induces autophagy in normal growth conditions, but especially under starvation conditions, an effect not seen in other activators of autophagy.

Finally, clionamine A (2.6) is currently being studied for its antibiotic effects on *Mycobacterium tuberculosis* since it can assist the cells infected by *M. tuberculosis* to deliver the bacteria to the lysosomes by stimulating autophagy.
3. **Ansellone A**

3.1 **Overview of the Metabolites Isolated from *Cadlina luteomarginata***

Nudibranchs, unlike most of their molluscan relatives, lack the hard shell that could serve to protect them against potential predators. Despite this obvious lack of protection, nudibranchs are usually very colourful and they are slow moving. One would assume that such a seemingly defenceless creature would be preyed upon by many predators; however adult nudibranchs have virtually no predators in the wild.

Chemical investigations conducted on nudibranchs have demonstrated that they use chemical defences to fend off potential predators. Most nudibranchs concentrate secondary metabolites in the skin on their dorsum and on colourful appendages called ceratas. Some of the metabolites found in nudibranchs are sequestered from their diet while others are made *de novo* by the animal.

Due to the vast number of secondary metabolites that they contain and the relative ease in isolating these molecules from their skin, nudibranchs have been the darlings of marine natural products chemists. One local nudibranch species has been particularly prolific at producing and sequestering novel molecules; nearly forty novel terpenoids have been isolated from the dorid nudibranch *Cadlina luteomarginata*. These compounds comprise 21 terpenoid skeletons including monoterpenoids, diterpenoids, sesterterpenoids, degraded diterpenoids and degraded sesterterpenoids. *C. luteomarginata* is a common nudibranch species in the rocky intertidal and in the shallow subtidal zones of the west coast of North America. These molluscs mostly feed on sponges from which they sequester a

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large number of the molecules found in their skin.\textsuperscript{78} As a result, the chemical make-up of the skin extracts of specimens of \textit{C. luteomarginata} varies depending on its geographical location.\textsuperscript{80} Nevertheless, \textit{C. luteomarginata} has been shown to also synthesise terpenoids \textit{de novo}. The terpenoids albicanyl acetate (3.1), cadlinaldehyde (3.2), and luteone (3.3) were found to be synthesized by the nudibranch using \textsuperscript{13}C labelling experiments.\textsuperscript{79}

![Chemical structures of terpenoids](image)

**Figure 3.1** Terpenoids biosynthesized by \textit{C. luteomarginata}.

Recently, we isolated the novel sesterterpenoid ansellone A (3.4) from \textit{C. luteomarginata} and we later established that ansellone A (3.4) was derived from the diet of the nudibranch. This finding further underlies the incredible diversity of metabolites present in this dorid nudibranch.
Sesterterpenes

Terpenes are the largest class of natural products and they have been isolated from virtually all living organisms. However, sesterterpenes, a class of terpenes made from five isoprene units, are the rarest group of terpenes. Despite their small number, they present an impressive structural diversity ranging from regular acyclic skeletons to highly modified cyclic skeletons. Sesterterpenes were first isolated from terrestrial fungi. Ophiobolin A (3.5) was the first known sesterterpenoid and it was isolated from the terrestrial fungus *Ophiobolus miyabeanus* and related ophiobolins were later found in both terrestrial and marine fungi (Figure 3.3). The linear sesterterpenoid geranylenoridol (3.6) was isolated from a phytopathogenic fungus and from the insect *Cerosplaste albolineatus* and it represented the first example of a linear sesterterpene. The cyclic sesterterpenoid ceroestrol (3.7) was isolated from the insect *Cerosplaste albolineatus* and together with geranylenoridol (3.6) represents one of the few examples of this class of terpenoids to be isolated from insects.
Figure 3.3 Examples of sesterterpenoids isolated from fungi and an insects.

Most sesterterpenoids are of marine origin and have been isolated from sponges or from nudibranchs. Manoalide (3.8) is a well known sponge sesterterpenoid due to its potent inhibitory effect on phospholipase A$_2$, an important enzyme of the inflammatory response system in mammals (Figure 3.4).

Figure 3.4 Manoalide

Like manoalide, many sponge sesterterpenoids such as luffolide (3.9) and inorolide A (3.10) have a butenolide moiety (Figure 3.5). Luffolide (3.9) and inorolide A (3.10) are cheilanthane sesterterpenoids, a group of terpenoids which have a 25 carbon skeleton consisting of three carbocyclic rings fused in a perhydrophenanthrene fashion and a five carbon side chain methylated on C-17 (3.11). The first cheilanthane terpenoid to be described, cheilanthatriol (3.12), was isolated from the fern *Cheilanthes farinose*, while most other cheilanthane terpenoids were isolated from marine sponges. The side chain of the cheilanthane terpenoids is often highly functionalized as in petrosaspongiolide C.
(3.13), where a hemiacetal is present on both carbon 24 and 25, thus forming an oxecane ring as well as a dioxolane ring.92

![Chemical structures](image)

**Figure 3.5 Cheilanthane sesterterpenoids.**

The suberitanes are class of sesterterpenes that are characteristic of sponges from the genus *Suberites*. Similar to the cheilanthanes, their skeleton contains three carbocyclic rings fused in a perhydrophenanthrene fashion. However, their side-chain is modified into a cyclohexenone moiety. The sesterterpene 3.14 isolated from the Antarctic sponge *Suberites caminatus* displays this moiety (Figure 3.6).93 The cyclohexenone moiety is not unique to the suberitanes and has been found in other sesterterpenoids such as sarcodictyenone (3.15), a linear polyprenylquinone.94
More recently, a number of novel sesterterpenoids with a cyclohexenone moiety have been isolated from sponges. They included the alotaketals (3.16, 3.17), the phorbaketals (3.19-3.21), the phorbasones (3.22-3.23) and ansellone A (3.4) (Figure 3.7-3.9).

3.3 The Alotane Skeleton

In 2009, our laboratory isolated the sesterterpenoids alotaketals A (3.16) and B (3.17) from the sponge *Hamigera* sp. collected in Papua New Guinea (Figure 3.7). The two molecules represent the first examples of the alotane sesterterpenoid skeleton (3.18), a monocyclic regular terpene skeleton containing 25 carbons. The alotaketals also contain an unusual spiroketal moiety and a cyclohexenone moiety.
Figure 3.7 Alotane sesterterpenoids

Shortly after the isolation of the alotaketals by our group, Rho and co-workers published the structures of three other alotane sesterterpenoids, named phorbaketals A-C (3.19-3.21) (Figure 3.8).\(^{96}\) They differ from the alotaketals due to the oxidation on the C ring and the substitution on C-11. The ketone functionality of the alotanes was reduced to a secondary alcohol in phorbaketals B and C. A year later, Rho’s group reported the isolation of yet another group of sesterterpenoids from the sponge *Phorbas sp.* that they named phorbasones A (3.22) and B (3.23) (Figure 3.8).\(^{97}\)
Figure 3.8 Sesterterpenoids isolated from a sample of Phorbas sp. collected off the coast of Korea.

Ansellone A (3.4) was isolated from a sample of Phorbas sp. collected in British Columbia and represents yet another example of the rich diversity of secondary metabolites found in the genus Phorbas (Figure 3.9). Ansellone A (3.4) is the first example of the ansellane sesterterpenoid skeleton (3.24) but it shares many structural features with the alotaketals (3.16-3.17) and also shares their biological properties as an activator of the cAMP signalling pathway.

Figure 3.9 Ansellone A (3.4) and the ansellane skeleton (3.24).
3.4 The Incretin Receptors as Therapeutic Targets

Diabetes is a disease that occurs when cells inadequately uptake glucose, thereby causing high levels of glucose in the bloodstream, a condition called hyperglycemia. Type 1 diabetes is caused by a failure to produce insulin and is treated by administering insulin. Insulin stimulates cells from all tissues to take up glucose and also prevents glycogen (a form of sugar stored in the liver), fatty acids, and amino acids from being converted to sugar while the levels of sugars in the blood are high. In type 2 diabetes, insulin is produced but there is a loss of sensitivity to the hormone and therefore, insulin administration alone cannot alleviate the symptoms of type 2 diabetes. A number of anti-hyperglycemic drugs are used today to treat patients suffering from type 2 diabetes, but many fail to bring the blood sugar levels down to normal levels. Consequently, there is a need for an efficient treatment of type 2 diabetes, especially when considering that an estimated 242 million adults worldwide suffer from this condition. Recently, new treatments have emerged in the form of agonists of the incretin receptors. These G protein coupled receptors (GPCR) are activated by hormones secreted in the gut and they regulate the signalling cascade leading to the release and uptake of insulin. The GPCR receptors are activated by a number of hormones involved in the release of insulin such as the hormone Glucagon like Peptide-1. Currently, many GLP-1 agonists are being developed as potential treatments for diabetes. However, all GLP-1 agonists found to date have very short biological half lives since they are degraded rapidly by enzymes naturally targeting GLP-1 in the body. This means that patients being treated with GLP-1 agonists must take their medication multiple times a day. Consequently, there is a need to find modulators of the incretin receptors that have a longer half life in the body.

Our collaborators from the Kieffer Laboratory in the Department of Cellular and Physiological Sciences and Surgery at UBC have been dedicated to finding modulators of the incretin receptors and have developed a cell-based assay to screen for potential modulators. Alotaketals A (3.17) and B
(3.18) were isolated from the extract of the sponge *Hamigera* sp. guided by their biological assay. The assay used HEK293 cells transfected with a GLP-1 plasmid and a pHTS-CRE plasmid responsible for the production of luciferase. When a small molecule activates the G coupled protein receptor the entire signalling pathway is activated and light is produced (Figure 3.10).

![Schematic of the enzymatic pathway downstream of the gastric protein receptors (GPCR) in cells transfected with a GPCR plasmid and a luciferase plasmid.](image)

Figure 3.10 Schematic of the enzymatic pathway downstream of the gastric protein receptors (GPCR) in cells transfected with a GPCR plasmid and a luciferase plasmid. The G-proteins coupled receptor (GPCR) is activated and guanidine diphosphate (GDP) is phosphorylated to form guanidine triphosphate (GTP). GTP activates adenyl cyclase which catalyzes the conversion of ATP to cAMP. cAMP then activates protein kinase A (PKA) to enter the nucleus where it causes the cAMP response element-binding (CRE) protein to bind to the CREB binding protein (CREB-P) allowing CREB to bind to the cAMP response element (CRE) region of the DNA strand where it can turn genes on or off. In this case, the luciferase genes are expressed and light is produced. (Source: Catherine Merchant)

Upon further investigation, it was found that the alotaketals (3.17-3.18) were not modulators of the incretin receptors, but rather activators of the secondary messenger cyclic AMP (cAMP) signalling pathway. The alotaketals were tested in HEK293 cells transfected with the pHTS-CRE plasmid but lacking the GLP-1 receptor. In these cells, the signalling pathway was activated without any hormone binding event on the GLP-1 receptor. A series of enzyme-linked immunosorbent assay (ELISA) experiments were conducted in order to narrow down the possible target of the alotaketals (3.17-3.18). The ELISA
experiments showed that the concentration of cAMP in the cells was significantly increased upon treatment with either alotaketals A or B (3.17-3.18).

Adenylyl cyclase is the enzyme responsible for breaking down ATP into cAMP. Once formed the cAMP binds to a cAMP-dependent protein kinase (PKA) and activates the kinase to catalyze the phosphorylation of proteins involved in a number of cellular functions such as the expression of specific genes or control of the ion channels of the cell. Since cAMP signalling plays a central role in cell signalling, small molecules that can modulate its effects are useful as molecular tools. The terpenoid forskolin (3.25) is an activator of adenylyl cyclase and is used today as a cell biology tool. Alotaketals A and B (3.17-3.18) were shown to be more potent than forskolin at activating the cyclic AMP signalling pathway in HEK293 cells transfected with the pHTS-CRE plasmid in the absence of hormone binding event on the GPC receptor. When ansellone A (3.4) was isolated, it was tested in the same cell assay and was also found to be an activator of the cAMP signalling pathway.

![Forskolin](image)

Figure 3.11 Forskolin

### 3.5 Isolation and Structure Elucidation of Ansellone A (3.4)

A specimen of *Cadlina luteomarginata* was collected using SCUBA on the rocky walls off of Ansell Place in Howe Sound, British Columbia. The freshly collected specimen of *C. luteomarginata* was immersed in MeOH and subsequently extracted twice in fresh MeOH (2 x 5 mL). The methanolic extracts
were combined and concentrated in vacuo to afford 3 mg of orange oil. The extract was fractionated between H₂O (1 mL) and Et₂O (2 x 2 mL). The Et₂O partition was evaporated under reduced pressure and chromatographed on a silica gel pencil column using a gradient from 100% hexanes to 3:7 hexanes: EtOAc. Fractions A to G were obtained, with 3.4 present in fraction E (0.6 mg).
Ansellone A (3.4) was isolated as an orange oil that gave a [M+Na]\(^+\) ion at m/z 465.2621 in the HRESIMS consistent with a molecular formula of C\(_{27}\)H\(_{38}\)O\(_5\) (calculated for C\(_{27}\)H\(_{38}\)O\(_5\)Na, 465.2617), requiring nine degrees of unsaturation. All 27 carbon resonances were seen in the \(^{13}\)C NMR spectrum of anellone A (3.4) recorded in C\(_6\)D\(_6\) and they are tabulated in Table 3-1. The \(^1\)H NMR and the HSQC spectra revealed the presence of six methyl groups, five methylenes, and nine methines for a total of 37 hydrogen atoms attached to carbons (Figure 3.12). A low resolution ESIMS recorded in CD\(_3\)OD gave a [M + Na]\(^+\) ion at m/z 466 indicating the presence of one exchangeable hydrogen in 3.4. This hydrogen atom along with the hydrogen atoms shown to be attached to carbons by the HSQC data accounted for the 38 hydrogen atoms required by the molecular formula derived from the HRESIMS data.

A total of six alkene carbons (δ 128.0, C-13; δ 131.4, C-7; δ 134.7, C-8; δ 135.7, C-12; δ 138.6, C-3; δ 139.4, C-2), one α,β-unsaturated ketone (δ 197.7, C-4), and one ester carbonyl (δ 170.2, C-26) were...
present in the $^{13}$C NMR spectrum of anellone A (3.4), accounting for five degrees of unsaturation (Figure 3.13). Since the HRESIMS data indicated that 3.4 had nine degrees of unsaturation, four rings had to be present.

![Figure 3.13 $^{13}$C NMR spectrum of anellone A (3.4) recorded in C$_6$D$_6$ at 150 MHz.](image)
100

Table 3-1 NMR assignments for ansellone A (3.4) recorded in C₆D₆ at 600 MHz.

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<td>6.14 (1H, d, J= 4.8Hz)</td>
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The analysis of the COSY and HMBC data for ansellone A (3.4) allowed us to identify the fragments A, B, and C shown in Figure 3.14. In fragment A, a linear COSY spin system started from the two proton resonance at δ 2.67 (H₅-5) and continued in sequence to the methine at δ 2.10 (H-6) and then to the oxymethine at δ 3.44 (H-1) and from there to the olefinic methine at δ 6.14 (H-2) and finally
to the olefinic methyl at δ 1.69 (Me-21) (Figure 3.15). In the HMBC data, the α,β-unsaturated ketone carbon resonance at δ 197.7 (C-4) was correlated to both the olefinic methine at δ 6.14 (H-2) and the olefinic methyl resonance at δ 1.69 (Me-21), as well as to a two proton doublet at δ 2.67 (H₂-5) suggesting that the COSY spin system described above together with the ketone at C-4 formed a cyclohexenone ring (Figure 3.16). Additional HMBC and HSQC correlations served to assign the carbons around the cyclohexenone ring and confirmed the presence of such a moiety in ansellone A (3.4). Firstly, the olefinic proton resonance at δ 6.14 (H-2) was correlated to the carbon resonance at δ 139.4 (C-2) in the HSQC spectrum. This carbon had an HMBC correlation to the proton resonance at δ 1.69 (Me-21, HSQC with 16.3) and this set of correlations confirmed the presence of a methylated olefin in the cyclohexenone ring of ansellone A (3.4). Also, the methylene proton at δ 2.67 (H₂-5, HSQC to δ 38.6) showed an HMBC correlation to the oxymethine carbon at δ 70.9 (C-1) found to be correlated to the proton signal at δ 3.44 (H-1) in the HSQC.
Figure 3.14 Selected HMBC and COSY correlations for fragments of ansellone A (3.4).
Figure 3.15 Expanded COSY spectrum of 3.4 recorded in C₆D₆ at 600 MHz showing the correlations of fragment A.
Figure 3.16 Expanded HMBC spectrum of 3.4 recorded in C$_6$D$_6$ at 600 MHz showing the correlations of fragment A.
In fragment B, a linear COSY spin system started from the pair of geminal oxymethylene protons at δ 4.27 (H-22a) and δ 4.42 (H-22b) and went to the olefinic methine resonance at δ 5.70 (H-8), to an oxymethine at δ 4.77 (H-9) and ended at a methine at δ 1.46 (H-10) (Figure 3.17). The pair of geminal protons [δ 4.27 (H-22a), δ 4.42 (H-22b), HSQC to δ 66.1] were determined to be allylic to the olefinic methine at δ 5.70 (H-8, HSQC to δ 134.7) based on the small coupling constant ($J < 1$ Hz) that they shared with H-8. This arrangement was supported by the HMBC data where the olefinic carbon at δ 134.7 (C-8) was correlated to the oxymethylene resonances at δ 4.27 (H-22a) and δ 4.42 (H-22b) (Figure 3.18). A homoallylic coupling between the oxymethylene protons [δ 4.27 (H-22a), δ 4.42 (H-22b)] and the oxymethine at δ 4.77 (H-9) was also observed in the COSY spectrum. Both the oxymethylene resonances δ 4.27 (H-22a) and δ 4.42 (H-22b) and a methyl resonances at δ 1.67 (Me-27) had HMBC correlations to the carbonyl at δ 170.2 (C-26) and demonstrated that the oxymethylene carbon (δ 66.1; C-22) was acetylated (Figure 3.18).
Figure 3.17 Expanded COSY spectrum of 3.4 recorded in C₆D₆ at 600 MHz showing the correlations of fragment B.
Figure 3.18 Expanded HMBC spectrum of 3.4 recorded in C$_6$D$_6$ at 600 MHz showing the correlations of fragment B.
Fragment C included another linear COSY spin system incorporating three contiguous methylene resonances starting from δ 1.02 and δ 1.82 (H-16a and H-16b, HSQC to δ 38.9) to δ 1.33 and δ 1.53 (H-17a and H-17b, HSQC to δ 18.9), and ending with δ 1.04 and δ 1.29 (H-18a and H-18b, HSQC to δ 41.7) (Figure 3.19). The methylene resonance at δ 1.04 (H-18a) had an HMBC correlation to a quaternary carbon resonance at δ 33.1 (C-19) (Figure 3.20). The quaternary carbon resonance at δ 33.1 (C-19) was in turn correlated to a pair of methyl resonances at δ 0.78 (Me-25) and δ 0.86 (Me-20) in the HMBC spectrum suggesting that the methylene spin system ended in a quaternary carbon bound to a pair of geminal methyls as shown in Figure 3.14. A different methyl resonance at δ 1.20 (Me-24) had an HMBC correlation to a quaternary carbon at δ 42.1 (C-15) and to one of the methylene carbons at δ 38.6 (C-16) (Figure 3.20). Therefore the three contiguous methylenes were attached to quaternary carbons on both ends.

The methyl proton resonance at δ 1.20 (Me-24) also had an HMBC correlation into the methine carbon signal at δ 57.0 (C-14, HSQC with δ 1.17) (Figure 3.20). The proton resonance for one of the geminal methyls (Me-20, δ 0.86) was also correlated to the methine carbon resonance at δ 57.0 (C-14) in the HMBC. In addition, a correlation between the methine proton resonance at δ 1.71 (H-14) and the quaternary carbon at δ 42.1 (C-15) was seen in the HMBC and this suggested the presence of a cyclohexane ring containing the three contiguous methylenes, the quaternary carbon substituted with geminal methyls (δ 33.1, C-19), the methylated quaternary carbon at δ 42.1 (C-15) and the methine at δ 57.0 (C-14).
Figure 3.19 Expanded COSY spectrum of 3.4 recorded in CD$_6$ at 600 MHz showing the correlations of fragment C.
Figure 3.20 Expanded HMBC spectrum of 3.4 recorded in C₆D₆ at 600 MHz showing the correlations of fragment C.
The proton resonance at δ 1.71 (H-14) was part of the other COSY spin system shown in fragment C that went to the olefinic methine proton resonance at δ 5.59 (H-13, HSQC to δ 128.0) and from there to another olefinic methine proton resonance at δ 5.52 (H-12, HSQC to δ 135.7) (Figure 3.19). The olefinic methine proton at δ 5.59 (H-13) was correlated to an oxygenated tertiary carbon resonance at δ 73.1 (C-11) in the HMBC spectrum (Figure 3.20). A methyl proton resonance at δ 1.50 (Me-23) and the methine proton resonance at δ 1.46 (H-10, HSQC to δ 63.8) also had an HMBC correlation to the oxygenated tertiary carbon resonance at δ 73.1 (C-11). Since the methine resonance at δ 1.46 (H-10) had an HMBC correlation to the quaternary carbon signal at δ 42.1 (C-15), it was concluded that a cyclohexene ring was formed with the olefinic carbons at δ 128.0 (C-13) and δ 135.7 (C-12), the oxygenated tertiary carbon at δ 73.1 (C-11), the methine at δ 63.8 (C-10), the quaternary carbon at δ 42.1 (C-15) and the methine at δ 57.0 (C-14). Fragments B and C were, therefore, linked through the methine at δ 63.8 (C-10) (Figure 3.21).

![Figure 3.21 Selected COSY and HMBC correlations for anellone A (3.4) in C₆D₆.](image)

It was possible to put together fragment A and B based on HMBC correlations from the methine at δ 2.10 (H-6, HSQC to δ 34.8) to the quaternary carbon at δ 131.4 (C-7) and from the oxygenated methylene at δ 4.27 and δ 4.27 (H-22α & β) to the methine carbon at δ 34.8 (C-6) (Figure 3.20).
The $^{13}$C NMR data indicated the presence of four oxygenated carbons in ansellone A. At this stage, the nature of the oxygen on the carbon at $\delta$ 66.1 (C-22) was the only one that had been resolved out of the four oxygenated carbons. The LRESIMS in CD$_3$OD had indicated that one of the oxygen was a hydroxyl group and the molecular formula required nine degrees of unsaturation, eight of which had been satisfied by the structural fragments resolved so far. Therefore, another ring had to be present and it needed to be an ether linked ring. To determine which carbon was hydroxylated, the NMR data for ansellone A (3.4) was collected in DMSO-$d_6$ (Figure 3.22). A proton resonance at $\delta$ 4.32, which was not correlated to any carbons in the HSQC, was correlated to an oxygenated tertiary carbon at $\delta$ 70.1 (C-11), to a tertiary carbon at $\delta$ 61.6 (C-10) and to a methyl at $\delta$ 25.2 (C-23) in the HMBC spectrum in DMSO-$d_6$. The proton resonance for the methyl at $\delta$ 1.25 (Me-23, HSQC to 25.2) also had an HMBC correlation to the oxygenated tertiary carbon resonance at $\delta$ 70.1 (C-11) and to the tertiary carbon at $\delta$ 61.6 (C-10) as well as an HMBC correlation to an olefinic methine carbon signal at $\delta$ 135.5 (C-12). Based on these HMBC correlations, the oxygenated tertiary carbon resonance at $\delta$ 70.1 (C-11) was assigned as C-11 and was determined to be the hydroxylated carbon (Figure 3.22).

Therefore, the two oxygenated carbons still to be assigned were C-1 and C-9. Since only one oxygen atom had not been accounted for, the two carbons at C-1 and C-9 had to be linked through an ether bond, although no HMBC correlation was seen between them in either solvent.
Figure 3.22 HMBC correlations for the hydroxyl resonance (OH-13) of ansellone A (3.4) in DMSO-d$_6$.

A 1D NOE correlation in C$_6$D$_6$ between the proton resonances at δ 3.44 (H-1) and at δ 4.77 (H-9) confirmed the presence of an ether bond between C-1 and C-9 and it also established that the two protons were cis to each other (Figure 3.23, Figure 3.24). Another strong NOE correlation was present between H-1 (δ 3.44) and H-6 (δ 2.10). This correlation indicated that the cyclohexenone and the dihydropyran ring were cis fused (Figure 3.23). The ROESY correlations between Me-24 (δ 1.20) and both Me-23 (δ 1.50) and Me-25 (δ 0.78) established that all three methyls were axial and that the C-10 to C-19 decalin ring system was trans fused (Figure 3.25). The presence of a NOE correlation between H-9 (δ 4.77) and Me-23 (δ 1.50) required that the dihydroxypyrany ring was equatorial on C-10. The
resonance for H-9 was also correlated to H-10 (δ 1.46) in the 1D NOE spectra and H-10 was in turn correlated to H-8 (δ 5.70). To illustrate this assignment a Newman projection of C-10 is given in Figure 3.23. Based on the NOE data, the relative configuration between the two rings was defined as C-9R*/C-10S*.

Figure 3.23 Selected NOE correlations for ansellone A (3.4) in CD₆₃.
Figure 3.24 1D NOE spectra for ansellone A (3.4) in C₆D₆ at 600 MHz.
Figure 3.25 ROESY spectrum for ansellone A (3.4) in C₆D₆ at 600 MHz showing the correlations to Me-24.

The absolute configuration of ansellone A (3.4) now had to be determined but the minute amount of 3.4 available (0.6mg) and its oily nature made it difficult to obtain a suitable crystal for X-ray diffraction studies. As a result, the determination of the absolute configuration was put on hold until additional material was isolated.
Ansellone A (3.4) had never been isolated from *Cadlina luteomarginata* in previous collections and since an extensive review of the metabolites found in *C. luteomarginata* had been done in the past, we began to suspect that ansellone A (3.4) came from the diet of the nudibranch. Nevertheless, doubts arose about the identity of the original specimen of *C. luteomarginata* from which 3.4 was isolated. Another collection of *C. luteomarginata* specimens from Ansell Place was done to acquire more material and ascertain that 3.4 did in fact come from *C. luteomarginata*. However, the organic extract of the newly collected specimens did not appear to contain 3.4. The nudibranch specimen from the original collection trip was taken to Sandra Millen, a nudibranch taxonomist at the University of British Columbia. She identified the specimen as *Cadlina luteomarginata* based on the anatomy of its rhinophores, the sensory appendages of nudibranchs, and on its radula, a grazing structure used by the nudibranchs to feed. Therefore, ansellone A (3.4) must have come from the diet of this particular nudibranch specimen. Upon dissection of the digestive gland of the original nudibranch specimen, orange sponge tissue was uncovered. Another sample collection was organized, but this time, small samples of multiple sponge species (~ 5 gr wet weight) were collected from Ansell Place in order to uncover the source of ansellone A (3.4).

Sponges can be difficult to identify based on their morphology and they are often identified based on the structure of their spicules, the structural elements made of silica found in the tissue of sponges. One of the sponges collected at Ansell Place had spicules that matched the spicules found in the sponge tissue from the stomach of the dissected *C. luteomarginata* specimen. The sponge was initially identified as *Hamigera* sp., but was later revised to *Phorbas* sp. by Dr. Nicole J. de Voogd, a sponge taxonomist at the Netherlands Centre for Biodiversity Naturalis.

A TLC (Thin Layer Chromatography) comparison of the contents of the organic extracts of both the original *C. luteomarginata* specimen and the sample of Phorbas sp. was found to be comparable.
and, more importantly, ansellone A (3.4) appeared to be present in both. More sponge tissue was collected in order to isolate 3.4 and other sesterterpenoids that appeared to be present in the sponge tissue based on the TLC and $^1$H NMR spectrum of the crude sponge extract.

A 400g (wet weight) sample of Phorbas sp. was collected using SCUBA off Ansell Place. The freshly collected sponge was immediately extracted with MeOH (2 x 100 mL). The methanolic extracts were combined and concentrated in vacuo to afford 400 mg of an orange gum. A portion of the gum was partitioned between H$_2$O (50 mL) and EtOAc (3 x 10 mL). The EtOAc partition was evaporated under reduced pressure and chromatographed on a silica gel column using a solvent gradient from 100% hexanes to 3:7 hexanes: EtOAc. Fractions A to L were obtained with ansellone A (3.4) present in fraction H (8 mg) as indicated by TLC analysis.

Enough material for the crystallization of ansellone A (3.4) was now available. The acetyl functionality of 3.4 was hydrolysed using LiOH/H$_2$O in a 1:1 mixture of THF and MeOH. The deacetylated product (3.26) was then recrystallized in CCl$_4$ and crystals suitable for X-ray diffraction analysis were obtained. The ORTEP diagram shown below confirmed the relative configuration that was assigned to ansellone A (3.4) by analysis of the NMR data (Figure 3.26). The X-ray structure indicated that the absolute configuration of the deacetylated ansellone A (3.26) was 1S, 6R, 9R, 10S, 11R, 14S, 15S. The X-ray structure was obtained by differences in anomalous dispersion using Cu radiation. The refined Flack parameter for this measurement was -0.01(16).
As mentioned in section 3.3, a number of molecules that are biogenetically related to ansellone A (3.4) were recently isolated. The alotaketals (3.16, 3.17) and the phorbaketals (3.19-3.21) are among them and although they share a very similar skeleton, their absolute configuration around carbon 1 and 6 was reported as opposite. The absolute configuration of the alotaketals could not be determined by Mosher ester hydrolysis methodology since not enough material was available. Therefore, the absolute configuration was determined from the CD spectrum of alotaketal A using Snatzke’s rules in order to predict the sign of the Cotton effect from the enone n→π* transition. Using this method the absolute configuration was determined to be 1R, 6S, whereas the absolute configuration of phorbaketal A was determined to be 1S, 6R using Mosher ester methodology. The phorbaketals had the same absolute configuration as ansellone A (3.4). A CD spectrum of 3.4 was then taken for comparison with the CD spectrum of alotaketal A (3.16) and the two spectra showed a positive Cotton effect for the n→π* transition at λ=330 nm (Figure 3.27). Thus, their dihydrochromenone ring systems have the same absolute configuration.
Figure 3.27 CD spectra for ansellone A (3.4) and alotaketal A (3.16) recorded in MeOH.
3.6 Biological Activity

The similarities in structures of alotaketals A and B (3.16, 3.17) and ansellone A (3.4) suggested that they may share similar biological properties. Alotaketals A and B (3.16, 3.17) are activators of the cAMP signalling pathway in HEK293 cells that have been transfected with a pHTS-CRE plasmid in the absence of hormone binding on the GLP-1 receptor (Figure 3.28). Alotaketal A (3.16) had an EC₅₀ of 18 nM and is much more potent than forskolin (3.25) (EC₅₀ 3 μM) the current molecular probe used for the cAMP signalling pathway.

![Dose response curves of alotaketal A (3.16) and alotaketal B (3.17) in HEK-pHTS-CRE cells.](image)

Ansellone A (3.4) was also tested under the same conditions and it had an EC₅₀ of 14 μM (Figure 3.29) and was, therefore, not as potent as alotaketals A (3.16) and B (3.17) but was comparable in potency with forskolin (3.24).
Figure 3.29 Ansellone A (3.4) dose response curve in HEK-pHTS-CRE cells.

3.7 Conclusion

Ansellone A (3.4) is a novel sesterterpenoid isolated from the nudibranch Cadlina luteomarginata. This molecule is the first example of a terpenoid with the ansellane skeleton (3.24). The isolation of ansellone A (3.4) from C. luteomarginata further confirmed that this species of nudibranch will remain of great interest to natural products chemists as it concentrates a wide array of terpenoids that it sequesters from its diet. In a sense, it can serve as a biological indicator of the terpenoid content of the sponges in its habitat. In this study, we were able to identify the sponge species from which the nudibranch had isolated ansellone A (3.4). The sponge species that produced 3.4 was originally called Hamigera sp. but the species was revised as Phorbas sp. based on the specimen collected for this study.

The presence of a \( 4\alpha, 5\)-dihydro-2H chromen-6(8\(\alpha\)H)-one moiety in ansellone A (3.4) and in alotaketel A (3.16) suggested that they may share similar biological properties. Alotaketel A (3.16) is an
activator of the cAMP signalling pathway and ansellone A (3.4) was also found to be an activator of the same pathway, albeit 3.4 was less potent than alotaketal A (3.16).

The isolation of the new sesterterpenoid ansellone A (3.4) from Phorbas sp. motivated the search for other related sesterterpenoids in the extract of this sponge and this will be the subject of the following chapter.
4. **Sesterterpenoids from *Phorbas* sp.**

4.1 **Metabolites Produced by Sponges of the Genus *Phorbas***

The genus *Phorbas* has been shown to produce several classes of natural products. Many oxazole containing macrolides such as hemiphorboxazole A (4.1) have been isolated from sponges of the genus *Phorbas*. The diterpenoid, phorbasin A (4.2) was also isolated from this genus. Recently, a number of sesterterpenoids such as phorbaketal A (3.19), phorbasones A (3.22) and ansellone A (3.4) have been reported from the sponges of the genus *Phorbas* (Figure 4.1).

![Figure 4.1 Metabolites isolated from sponges of the genus *Phorbas.*](image-url)
The isolation of ansellone A (3.4) from our sample of \textit{Phorbas} sp. motivated further investigations into the sesterterpenoids that could be isolated from this sponge. The search for novel sesterterpenoids in \textit{Phorbas} sp. led to the isolation of ansellones B-D (4.3-4.5) and alotaketal E (4.6) (Figure 4.2).

Figure 4.2. Sesterterpenoids isolated from a sample of \textit{Phorbas} sp. collected in B.C.

4.2 Isolation and Structure Elucidation of Ansellones B-D and Alotaketal E

The method used for the isolation of ansellones B-D (4.3-4.5) and alotaketal E (4.6) is summarized in Figure 4.3. The methanolic extract from the sample of \textit{Phorbas} sp. collected for the
isolation of ansellone A (3.4) was partitioned between H₂O and EtOAc, and the EtOAc partition was concentrated *in vacuo* and then chromatographed on a silica gel column using a solvent gradient from 100% hexanes to 4:6 hexanes: EtOAc. A mixture of ansellones B (4.3), C (4.4) and D (4.5) eluted at 8:2 hexanes/ EtOAc. The fraction that eluted at 7:3 hexanes:EtOAc contained pure ansellone A (3.4) (8.0 mg) and the fraction that eluted at 1:1 hexanes: EtOAc contained alotaketal E (4.6). The fraction containing 4.3, 4.4, and 4.5 was further separated using normal phase HPLC and pure 4.3 (3.4 mg), 4.4 (2.3 mg) and 4.5 (1.4 mg) were eluted using the solvent mixture 7:3 hexanes:EtOAc. The fraction containing alotaketal E (4.6) was separated on a HPLC equipped with a C8 column using a 6:4 mixture of MeCN: H₂O as eluent and pure 4.6 (1.2 mg) was obtained.

Figure 4.3 Isolation scheme for ansellone A to D (4.3-4.5) and alotaketal E (4.6).
Ansellone B (4.3) was isolated as a yellow oil that gave an [M+Na]^+ ion at m/z 467.2783 (calculated for C_{27}H_{40}O_{5}Na, 467.2773) in the HRESIMS consistent with a molecular formula of C_{27}H_{40}O_{5}, requiring eight degrees of unsaturation. The $^{13}$C NMR spectrum recorded for ansellone B (4.3) in C_{6}D_{6} showed 27 well-resolved resonances in agreement with the HRESIMS (Figure 4.4). The $^1$H NMR spectrum (Figure 4.5) and HSQC spectra identified 39 hydrogen atoms attached to carbon (6 x CH$_3$, 7 x CH$_2$, 7 x CH) the remaining hydrogen was an exchangeable hydrogen identified through low resolution ESIMS using CD$_3$OD as the eluent ([M + Na]^+ ion at m/z 468). The $^{13}$C and $^1$H NMR spectra for ansellone B (4.3) are shown in Figure 4.4-4.5, respectively.

Figure 4.4 $^{13}$C NMR spectrum of ansellone B (4.3) recorded in C_{6}D_{6} at 150 MHz.
Figure 4.5. $^1$H NMR spectrum of ansellone B (4.3) recorded in C$_6$D$_6$ at 600 MHz.
Table 4-1. NMR assignments for ansellone B (4.3) recorded in C$_6$D$_6$ at 600 MHz.

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The downfield resonances in the $^{13}$C NMR spectrum were assigned to four olefinic carbons [$\delta$
116.7 (C-22), 136.4 (C-3), 141.9 (C-2), and 146.0 (C-7)], one $\alpha,\beta$-unsaturated ketone carbonyl ($\delta$ 198.4, C-
4) and an ester or carboxylic acid carbonyl (δ 169.7, C-26), which together accounted for four degrees of unsaturation. The absence of 13C NMR evidence for additional unsaturated functionality indicated that ansellone B (4.3) was tetracyclic.

Detailed analysis of the COSY and HMBC data collected for ansellone B (4.3) served to identify the fragments A, B and C shown in Figure 4.6. As in ansellone A (3.4), the presence of a cyclohexenone ring (fragment A) was evident in the COSY and HMBC data. The COSY correlations for the cyclohexenone ring define a linear 1H spin system starting with a pair of geminal protons at δ 2.23 (H-5a) and δ 2.94 (H-5b) continuing in sequence to a methine at δ 2.37 (H-6), an oxymethine at δ 3.72 (H-1), an olefinic methine at δ 6.03 (H-2), and ending with an olefinic methyl at δ 1.81 (Me-21) (Figure 4.7). HMBC correlations observed from each of the olefinic methyl (δ 1.81, Me-21), the olefinic methine (δ 6.03, H-2) and the methylene (δ 2.23, H-5a; δ 2.94, H-5b) proton resonances to the ketone carbonyl resonance at δ 198.4 (C-4) revealed that the linear 1H spin system and the conjugated ketone were part of a cyclohexenone ring (Figure 4.8).
Figure 4.6 Selected COSY and HMBC correlations for fragments A, B and C of ansellone B (4.3) in C$_6$D$_6$. 
Figure 4.7 Expanded COSY NMR spectrum of 4.3 recorded in C$_6$D$_6$ at 600 MHz showing the correlations for fragment A.
Figure 4.8 Expanded HMBC NMR spectrum of 4.3 recorded in C₆D₆ at 600 MHz showing the correlations for fragment A.

The COSY data revealed the presence of another spin system that included a pair of resonances at δ 4.70 (H-22a) and 4.90 (H-22b), assigned to geminal olefinic protons based on their HSQC correlation to a downfield olefinic carbon resonance at δ 116.7 (C-22) and based on the COSY correlation that the two protons had with each other. The geminal olefinic protons were correlated to the geminal methylene protons at δ 2.46 (H-8a) and 2.83 (H-8b) which were in turn correlated to the oxymethine resonance at δ 5.53 (H-9, HSQC to δ 75.2) as shown in fragment B and in Figure 4.9. The proton resonance at δ 5.53 (H-9) was also correlated to a methine at δ 1.39 (H-10). The HMBC data showed that
the geminal olefinic proton at δ 4.90 (H-22b) was correlated to a quaternary carbon resonance at δ 146.0 (C-7) and that both olefinic protons (H-22a & b) were correlated to the methylene carbon resonance at δ 34.2 (C-8, HSQC with δ 2.46, 2.83) suggesting that the COSY correlation between the methylene (H-8a, H-8b) and the geminal olefinic protons (H-22a, H-22b) was allylic (Figure 4.10). The HMBC data also showed correlations between an ester resonance at δ 169.7 (C-26) and the oxymethine proton at δ 5.53 (H-9) demonstrating that C-9 was acylated. An HMBC correlation between the methyl singlet at δ 1.67 (Me-27) and the ester resonance at δ 169.7 (C-26) showed that the acyl group on C-9 was in fact an acetyl group.

![Figure 4.9 Expanded COSY NMR spectrum of 4.3 recorded in C6D6 at 600 MHz showing the correlations for fragment B.](image)
A third linear COSY spin system contained three contiguous methylenes (C-16: $\delta^{1}H$ 0.92, 1.87, $^{13}C$ 41.6, C-17: $\delta^{1}H$ 1.33, 1.49, $^{13}C$ 18.6, C-18: $\delta^{1}H$ 1.11, 1.31, $^{13}C$ 43.9) (Figure 4.11). As with ansellone A (3.4), the HMBC data for ansellone B (4.3) showed a pair of aliphatic methyl proton resonances (Me-25: $\delta^{1}H$ 1.03, $^{13}C$ 22.9 and Me-20: $\delta^{1}H$ 1.34, $^{13}C$ 37.6) correlating to a quaternary carbon resonance (C-19: $\delta^{13}C$ 34.5) and to a downfield methine carbon at $\delta$ 61.5 (C-14, HSQC with $\delta$ 0.80), revealing that the methyl resonances were geminal on a quaternary carbon that joined the methylene spin system and the downfield aliphatic methine carbon. A methyl resonance at $\delta$ 0.97 (Me-24, $^{13}C$ 18.9) had HMBC
correlations with both the methine carbon signals at δ 51.1 (C-10) and at δ 61.5 (C-14) and with the quaternary carbon resonance at δ 39.5 (C-15) supporting the presence of a cyclohexane ring between C-14 and C-19 in agreement with the proposed structure of fragment C (Figure 4.12).

Figure 4.11 Expanded COSY NMR spectrum of 4.3 recorded in C6D6 at 600 MHz showing the correlations for fragment C.
A fourth COSY spin system started at a methylene proton resonance (C-12: δ\(^1\)H \(=\) 1.50, δ\(^{13}\)C 51.1) and proceeded to an oxymethine resonance (C-13: δ\(^1\)H 3.54, δ\(^{13}\)C 68.4) and ended with an aliphatic methine (C-14: δ\(^1\)H 0.80, δ\(^{13}\)C 61.5) (Figure 4.11). A COSY correlation between the proton resonance at δ 3.54 (H-13) and an exchangeable \(^1\)H resonance at δ 0.48 (OH-13) suggested the presence of a secondary alcohol at C-13.
This was confirmed by an HMBC correlation from the proton resonance at δ 0.48 (OH-13) to the carbon resonance at δ 68.4 (C-13) (Figure 4.12). HMBC correlations from a methyl proton resonance at δ 1.55 (Me-23) to the quaternary carbon at δ 79.8 (C-11), and to the methylene carbon signal at δ 51.1 (C-12) were detected. These HMBC correlations to Me-23 along with the HMBC correlations from the methine proton at δ 1.39 (H-10) to the oxygenated tertiary carbon at δ 79.8 (C-11) were helpful in determining the attachment of fragment C to fragment B through the oxygenated tertiary carbon at δ 79.8 (C-11) (Figure 4.13). This data was consistent with the structure of ansellone B (4.3) shown in Figure 4.14. An additional HMBC correlation from the methine proton at δ 1.39 (H-10) to the quaternary carbon at δ 39.5 (C-15) supported this assignment. The oxymethine resonance at δ 3.72 (H-1) had an HMBC correlation into the oxygenated tertiary carbon at δ 79.8 (C-11), thus revealing the presence of an oxocane functionality in ansellone B (4.3). Additional HMBC correlations from δ 1.39 (H-10) and δ 1.50 (H-12) into the oxygenated tertiary carbon at δ 79.8 (C-11) confirmed the attachment of the oxocane moiety to the decalin portion of the molecule. Finally, the HMBC correlations between the geminal olefinic protons at δ 4.70 (H-22a) and δ 4.90 (H-22b) and δ 46.3 (C-6) confirmed the attachment of the oxocane moiety to the cyclohexenone ring (Figure 4.13).
Figure 4.13 Expanded HMBC NMR spectrum of ansellone B (4.3) recorded in C₆D₆ at 600 MHz.

Figure 4.14 Selected COSY and HMBC correlations for ansellone B (4.3) in C₆D₆.
The relative configuration of ansellone B (4.3) was determined using 2D ROESY experiments and the key correlations are highlighted in Figure 4.15. The 2D ROESY data showed a NOE correlation between H-1 (δ 3.72) and H-6 (δ 2.37) revealing that the cyclohexenone and the oxocane were cis fused (Figure 4.16). A NOE correlation between H-1 and H-10 (δ 1.39) demonstrated these two protons were cis to each other. The resonance for H-10 was also correlated with H-14 (δ 0.8). Adding to this, correlations between Me-24 (δ 0.97) and Me-23 (δ 1.55), Me-25 (δ 1.03) and H-13 (δ 3.54) suggested that the decalin system was trans fused.

Figure 4.15 Selected 2D ROESY correlations for ansellone B (4.3) in C6D6.
The relative configuration around C-13 was deduced from the $^1$H NMR and the 2D ROESY NMR data. The resonance for H-13 ($\delta$ 3.54) and H-14 ($\delta$ 0.8) had a coupling constant of 10.5Hz which suggested that the 2 protons were anti-periplanar to each other. In the 2D ROESY data, H-13 was correlated to three methyl resonances; Me-20, Me-23, and Me-24. Based on these pieces of evidence the relative configuration at C-13 was assigned as C-135*. The relative configuration around C-9 was established to be C-9S* based on the ROESY correlation between H-9 ($\delta$ 5.53) and H-10 ($\delta$ 1.39) as well as the correlation between Me-24 ($\delta$ 0.97) and Me-27 ($\delta$ 1.67).
The CD spectrum obtained for ansellone B (4.3) was similar to the one obtained for ansellone A (3.4). They both had a positive Cotton effect for the n→π* transition at about λ ≈ 330 nm (Figure 4.17). Therefore, the absolute configuration of ansellone B (4.3) was assigned as 1S, 6R, 9S, 10S, 11R, 13S, 14S, 15S.

![Ansellone A (3.4)](image1.png) ![Ansellone B (4.3)](image2.png)

Figure 4.17 CD spectra for ansellones A (3.4) and B (4.3) collected in MeOH.

Ansellone C (4.4) was also isolated as a white residue that gave an [M+Na]⁺ ion at m/z 467.2764 in the HRESIMS consistent with a molecular formula of C₂₇H₄₀O₅ (calculated for C₂₇H₄₀O₅Na, 467.2773), requiring eight degrees of unsaturation. The ¹³C NMR spectrum recorded for ansellone C (4.4) in C₆D₆ showed 27 well resolved resonances in agreement with the HRESIMS, and the HSQC data identified 39 hydrogen atoms attached to carbon (6 x CH₃, 7 x CH₂, 7 x CH). Again, the missing hydrogen from the HSQC data was identified as an exchangeable hydrogen through low resolution ESIMS using CD₃OD as the solvent ([M + Na]⁺ ion at m/z 468).

The ¹³C NMR spectra of ansellones B (4.3) and C (4.4) were virtually identical. In contrast, the ¹H NMR data of the two molecules displayed subtle differences. The oxymethine signal at δ 3.54 associated
with C-13 in ansellone B (4.3) was absent in ansellone C (4.4) (Figure 4.5). Instead a broad signal at $\delta$ 4.16 was present as highlighted in Figure 4.18.

Figure 4.18 $^1$H NMR spectrum of ansellone C (4.4) recorded in C$_6$D$_6$ at 600 MHz.
Table 4-2 NMR assignments for anellone C (4.4) recorded in C_6D_6 at 600 MHz.

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<tr>
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<td>27</td>
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The HSQC data revealed that in ansellone C (4.4), the oxygenated carbon C-13 was attached to the broad singlet at δ 4.16 (H-13). In the COSY data, this signal was coupled to an exchangeable resonance at δ 0.45 (13-OH), a broad singlet at δ 0.64 (H-14), and a methylene resonance at δ 1.45 (H-12b) (Figure 4.19).

Since the substituents around C-13 seemed to be the same in ansellones B (4.3) and C (4.4), it was hypothesized that the differences in coupling constants and chemical shifts were due to the configuration around C-13. This hypothesis was confirmed by the 2D ROESY data and the coupling constant between H-13 (δ 4.16) and H-14 (δ 0.64). In ansellone B (4.3), H-13 and H-14 had a coupling constant of 10.5 Hz whereas in ansellone C (4.4) their coupling constant was less than 2Hz suggesting that H-13 was equatorial and H-14 axial on the trans decalin system. The ROESY correlations that
confirmed this arrangement are highlighted in Figure 4.20. ROESY correlations between H-13 (δ 4.16) and H-14 (δ 0.64) and Me-20 (δ 0.94) supported the assignment of the relative configuration at carbon 13 as C-13R* (Figure 4.21). This assignment was further confirmed by the ROESY correlations between the hydroxyl proton at δ 0.45 (OH-13) and Me-23 (δ 1.85), Me-24 (δ 1.46) and Me-25 (δ 1.26).

Figure 4.20 ROESY NMR spectrum of ansellone C (4.4) recorded in C₆D₆ at 600 MHz.
Ansellone D (4.5) was isolated as a yellow oil that gave an [M+Na]$^+$ ion at m/z 465.2620 in the HRESIMS consistent with a molecular formula of C$_{27}$H$_{40}$O$_5$ (calculated for C$_{27}$H$_{38}$O$_5$Na, 465.2617), requiring nine degrees of unsaturation. The $^1$H NMR spectrum (Figure 4.18) HSQC data identified 37 hydrogen atoms attached to carbon (6 x CH$_3$, 5 x CH$_2$, 9 x CH). The missing hydrogen from the HSQC data was identified as an exchangeable hydrogen through low resolution ESIMS using CD$_3$OD as the solvent ([M + Na]$^+$ ion at m/z 466).

As with the previously isolated anellone A (3.4), downfield resonances in the $^{13}$C NMR spectrum were assigned to six olefinic carbons [$\delta$ 126.6 (C-12), 134.7 (C-11), 129.2 (C-8), 132.4 (C-7), 130.1 (C-3) and 138.1 (C-2)], one $\alpha,\beta$-unsaturated ketone carbonyl ($\delta$ 198.2, C-4), and an ester or a carboxylic acid carbonyl (δ 169.3, C-26), which together accounted for six degrees of unsaturation. The absence of $^{13}$C NMR evidence for additional unsaturated functionality indicated that anellone D (4.5) was tetracyclic.
Figure 4.22 $^1$H NMR spectrum of ansellone D (4.5) recorded in C$_6$D$_6$ at 600 MHz.
Table 4-3 NMR assignments for ansellone D (4.5) recorded in C$_6$D$_6$ at 600 MHz.

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<td>3</td>
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<td>4</td>
<td>198.2</td>
<td>—</td>
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Upon inspection of the $^1H$ and $^{13}C$ NMR data, ansellone D (4.5) appeared to bear many of the structural characteristics of ansellone A (3.4), although the resonances associated with ring C appeared to differ with respect to the position of the hydroxyl group and of the olefin. Indeed, an additional
oxymethine ($\delta^1H 5.73, \delta^{13}C 71.6$) was present in the HSQC of ansellone D (4.5), while the signal for the hydroxylated tertiary carbon C-11 of ansellone A (3.4) was absent (Figure 4.23).

![Diagram](image)

**Figure 4.23 Selected COSY and HMBC correlations for rings C and D of ansellone D (4.5).**

Furthermore, the HMBC data revealed that the olefin in ring C was tri-substituted since the methyl signal at $\delta 1.72$ (Me-23) was correlated to both the olefinic carbons at $\delta 126.6$ (C-12) and $\delta 134.7$ (C-11) and to the methine carbon at $\delta 57.9$ (C-10) (Figure 4.24). The HMBC correlation between Me-23 and C-10 confirmed the arrangement showed in Figure 4.25.
Figure 4.24 Expanded HMBC spectrum of ansellone D (4.5) recorded in C₆D₆ at 600 MHz.

Figure 4.25 Selected COSY and HMBC correlations for ansellone D (4.5) in C₆D₆.
The COSY data revealed a large spin system that could be traced sequentially from the methine at δ 1.48 (H-14) to the olefinic methine at δ 5.65 (H-8) (Figure 4.25). The methine at δ 1.48 (H-14) was correlated to the oxymethine at δ 5.73 (H-13) which was correlated to the olefinic proton at δ 5.50 (H-12). The spin system continued from the olefinic methine at δ 5.50 (H-12) to the allylic methyl signal at δ 1.72 (Me-23) and to the methine at δ 2.28 (H-10). In addition, an allylic COSY coupling was seen between the hydroxylated proton resonance at δ 5.73 (H-13) and both the methine proton at δ 2.28 (H-10) and the allylic methyl at δ 1.72 (Me-23) (Figure 4.26). The COSY spin system continued from the methine proton at δ 2.28 (H-10) to the oxymethine at δ 4.23 (H-9) and from the oxymethine to the olefinic methine at δ 5.65 (H-8).
Figure 4.26 Expanded COSY spectrum of ansellone D (4.5) recorded in C₆D₆ at 600 MHz.

This COSY spin system just described was determined to be attached on one end to a quaternary carbon at δ 39.0 (C-15) based on an HMBC correlation from the methine at δ 1.48 (H-14, HSQC to δ 53.0) to the carbon at δ 39.0 (C-15) (Figure 4.27). Both of C-14 and C-15 were also correlated to a methyl group at δ 0.83 (Me-24, HSQC to δ 15.9) in the HMBC. The same methyl group (Me-24) shared a correlation with the methine carbon at δ 57.9 (C-10), thus indicating the presence of cyclohexenone ring in ansellone D (4.5) extending from carbon 10 to carbon 15.
Figure 4.27 Expanded HMBC spectrum of ansellone D (4.5) recorded in C$_6$D$_6$ at 600 MHz.

The relative configuration of ansellone D (4.5) was determined through a 2D ROESY experiment (Figure 4.28). The relative configuration around the cyclohexenone (ring A) and the dihydropyran ring (ring B) was the same in ansellones A (3.4) and D (4.5). ROESY correlations between H-13 (δ 5.73) and both Me-24 (δ 0.83) and Me-25 (δ 0.98) suggested that H-13 was in an axial position on C-13 and that the relative configuration around C-13 was C-13S* (Figure 4.28).
Figure 4.28 Expanded 2D ROESY spectrum of ansellone D (4.5) recorded in C₆D₆ at 600 MHz.

The latter correlations and a ROESY correlation between H-14 (δ 1.48) and H-10 (δ 2.28) revealed that the decalin ring system from C-10 to C-19 was trans fused. This was also supported by the presence of a ROESY correlation between Me-23 (δ 1.72) and H-9 (δ 4.23), that required that H-10 was axial as in ansellone A (3.4). The ROESY correlations between H-9 and both Me-23 and H-10 could only be accommodated by the C-9R*/C-10S* relative configuration as it was the case with ansellone A (3.4).
The CD spectra of ansellone D (4.5) was also similar to the one obtained for ansellone A (3.4) and displayed a positive Cotton effect for the n→π* transition at λ = 330nm. The absolute configuration of ansellone D (4.5) was, therefore, assigned as 1S, 6R, 9R, 10S, 11R, 13S, 14S, 15S.

Alotaketal E (4.6) was isolated as a yellow oil that gave an [M+Na]+ ion at m/z 481.2555 in the HRESIMS consistent with a molecular formula of C_{27}H_{38}O_{6} (calculated for C_{27}H_{38}O_{6}Na, 481.2668), requiring nine degrees of unsaturation. The $^{13}$C NMR spectrum for alotaketal E (4.6) recorded in C_{6}D_{6} showed 27 well resolved resonances in agreement with the HRESIMS, and the HSQC data identified 37 hydrogen atoms attached to carbon (6 x CH_{3}, 6 x CH_{2}, 7 x CH). The $^{13}$C NMR and the $^{1}$H NMR spectra are displayed in Figure 4.30 and 4.31, respectively.
Figure 4.30 $^{13}\text{C}$ NMR spectrum of alotaketal E (4.6) recorded in $\text{C}_6\text{D}_6$ at 150 MHz.
Figure 4.3. $^1$H NMR spectrum of alotaketal E (4.6) recorded in C$_6$D$_6$ at 600 MHz.
Table 4-4 NMR assignments for alotaketal E (4.6) recorded in C₆D₆ at 600 MHz.

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The presence of 27 carbons in alotaketal E (4.6) along with the presence of a proton singlet resonance at δ 6.27 (H-2) and of six methyl singlets in the ¹H NMR suggested that it was another sesterterpenoid related to the ansellones. Despite the structural differences of ansellones A-D (3.4, 4.3-4.5), they all shared very similar chemical shifts for the signals associated with the atoms in the
cyclohexenone ring. A particularly diagnostic resonance for the presence of the cyclohexenone ring in the sesterterpenoids of the ansellane and alotane families had been the $^1$H NMR resonance around $\delta$ 6.20 integrating for one proton, which was characteristic of the olefinic methine signal in the cyclohexenone. The COSY and HMBC data for alotaketal E (4.6) confirmed the presence of the cyclohexenone moiety as shown in Figure 4.32. A COSY spin system was seen starting from the methylene signal at $\delta$ 2.39 (H$_2$-5) and ending at the olefinic methyl at $\delta$ 1.77 (Me-21), as in other sesterterpenoids with this functionality (Figure 4.33). Both the methylene signal at $\delta$ 2.39 (H$_2$-5) and the olefinic methyl at $\delta$ 1.77 (Me-21) had an HMBC correlation to the carbonyl carbon resonance at $\delta$ 197.7 (C-4,) while the methyl at $\delta$ 1.77 (Me-21) also had a correlation to the carbon at $\delta$ 139.1 (C-2), and confirmed the presence of a cyclohexenone ring in alotaketal E (4.6) (Figure 4.34).

![Figure 4.32 Selected COSY and HMBC correlations for fragment A of alotaketal E (4.6).](image)

Figure 4.32 Selected COSY and HMBC correlations for fragment A of alotaketal E (4.6).
Figure 4.33 Expanded COSY NMR spectrum of 4.6 recorded in C$_6$D$_6$ at 600 MHz showing the correlations for fragment A.
Figure 4.34 Expanded HMBC NMR spectrum of 4.6 recorded in C₆D₆ at 600 MHz showing the correlations for fragment A.

The remainder of the molecule is illustrated as fragment B in Figure 4.35. From fragment A, the methine resonance at δ 1.99 (H-6) was correlated to a quaternary carbon at δ 142.4 (C-7) in the HMBC spectrum (Figure 4.36). An oxygenated methylene resonance at δ 3.51 (H₂-22) and an olefinic methine resonance at δ 5.44 (H-8) were also correlated to the same quaternary carbon at δ 142.4 (C-7) in the HMBC spectrum. The two proton resonances just mentioned (H-8 and H₂-22) were correlated to each
other in the COSY spectrum (Figure 4.37). The olefinic methine at δ 5.44 (H-8) also had an HMBC
correlations to the quaternary carbon at δ 96.8 (C-9), a chemical shift that is diagnostic for ketals. The
presence of a ketal and of a cyclohexenone ring suggested that this sesterterpenoid may be related to
alotaketals A (3.16) and B (3.17) (Figure 3.7).

Figure 4.35 Selected COSY and HMBC correlations for fragment B of alotaketal E (4.6) in C6D6.
Figure 4.36 Expanded HMBC for 4.6 recorded in C₆D₆ at 600 MHz showing selected correlations of fragment B.
Figure 4.37 COSY NMR spectrum of 4.6 recorded in C₆D₆ at 600 MHz showing correlations for fragment B.

The oxygenated methine of the cyclohexenone ring at δ 4.27 (H-1) and a pair of geminal methylene protons at δ 3.11 (H-10a) and δ 1.19 (H-10b) were also correlated to the ketal resonance at δ 96.8 (C-9) in the HMBC (Figure 4.36). These correlations suggested that like in alotaketals A and B (3.16-3.17) the ketal was connected to C-8 (δ 125.2) and C-10 (δ 39.9) and that there was an ether bond between C-1 (δ 63.2) and C-9 (δ 96.8) (Figure 4.35).

The geminal methylene protons (H-10a and H-10b) were correlated to another oxygenated tertiary carbon at δ 77.4 (C-11) in the HMBC spectrum (Figure 4.38). The oxygenated tertiary carbon at δ 77.4 (C-11) was found to be methylated based on an HMBC correlation to a methyl singlet at δ 1.45 (Me-23). Carbon 11 was also found to be acetylated based on the HMBC correlation from δ 1.19 (H-10b) to an ester carbonyl at δ 170.4 (C-26) and the HMBC correlation from a methyl resonance at δ 1.77 (Me-27) to the same ester carbonyl (C-26) (Figure 4.38).
Figure 4.38 Expanded HMBC for 4.6 recorded in C₆D₆ at 600 MHz showing selected correlations of fragment B.

A COSY system spin system went from a pair of geminal methylene protons at δ 1.19 (H-12a) and δ 1.81 (H-12b) to an oxygenated methine at δ 5.13 (H-13) and from there to the olefinic methine at δ 5.46 (H-14) (Figure 4.37). The olefinic methine at δ 5.46 (H-14) had a COSY correlation to one of the methyl signals at δ 1.77 (Me-24). This methyl resonance (Me-24) had an HMBC correlation to the
quaternary carbon at δ 139.4 (C-15) (Figure 4.38), indicating that the COSY correlation it shared with the olefinic methine at δ 5.46 (H-14) was allylic and that the methyl (Me-24) was a substituent on the quaternary carbon. Based on the HMBC data, the other substituent on the quaternary carbon appeared to be the methylene at δ 2.11 (H2-16). From this methylene, a linear spin system could be traced sequentially in the COSY spectrum all the way to the two allylic methyl groups at δ 1.67 (Me-25) and δ 1.55 (Me-20). The methylene resonance at δ 2.11 (H2-16) was correlated to another methylene resonance at δ 2.21 (H2-17), which was correlated to the olefinic proton resonance at δ 5.23 (H-18, HSQC to δ 124.7). The two methyl groups at δ 1.67 (Me-25) and δ 1.55 (Me-20) were shown to have an allylic coupling with the olefinic proton at δ 5.23 (H-18) since they were both correlated to the olefinic methine in the COSY spectrum and also had an HMBC correlation to the quaternary carbon at δ 132.0 (C-19) in the HMBC spectrum (Figure 4.37, Figure 4.38).

The ROESY data allowed us to confirm the presence of a pyran ring between C-9 and C-13 due to the presence of a correlation between H-1 (δ 4.27) and H-13 (δ 5.13) (Figure 4.39). Consequently, the seiterterpenoid skeleton of alotaketal E (4.6) was consistent with alotaketal A (3.16) and B (3.17).
Figure 4.39 Expanded 2D ROESY for Alotaketal E (4.6) recorded in C$_6$D$_6$ at 600 MHz.

The ROESY data was used to establish the relative configuration of alotaketal E (4.6). As with the other alotaketals and the ansellones, a ROESY correlation between H-1 (δ 4.27) and H-6 (δ 1.99) established that the cyclohexenone and the dihydropyran ring were cis fused. The correlation between H-1 (δ 4.27) and H-13 (δ 5.13) required that the oxygen of the pyran ring be cis to H-1 and that the oxygen of the dihydropyran ring was cis to H-13 as shown in Figure 4.40. A large coupling constant ($J= 14$ Hz) was observed between H-13 and H-12b (δ 1.81) and was consistent with H-13 having a pseudoaxial
orientation on the pyran ring. This would also place H-13 in close proximity to H-1 and explain the ROESY correlation between the two protons. A ROESY correlation between H-12b (δ 1.81) and Me-23 (δ 1.45) and Me-23 and H-10a (δ 3.11) indicated that they were arranged in pseudoaxial orientation and that the relative configuration at carbon 11 was C-11S*(Figure 4.39). The proton assigned to H-13 (δ 5.13) also had a correlation to Me-24 (δ 1.77) and H-14 (δ 5.46) had a ROESY correlation to H-16 (δ 2.11) demonstrating that the Δ14,15 olefin had an E configuration.

![Molecule structure](image)

**Figure 4.40** Selected 2D ROESY correlations for alotaketal E (4.6) in C6D6.

The CD spectrum for alotaketal E (4.6) was similar to the one obtained for ansellone A (3.4) and displayed a positive Cotton effect for the n→π* transition at λ ≈ 330nm. The absolute configuration of alotaketal E was, therefore, established as 1S, 6R, 9S, 11S, 13S, 14E.

Alotaketal E (4.6) had a very similar structure to alotaketal B (3.17). The two molecules differ in their substituent at carbon 11 and in the position of the double bond on carbon 19 as seen in Figure 4.41. Alotaketal E (4.6) is substituted with an acetyl group at carbon 11 whereas alotaketal B (3.17) is substituted with an isovalerate. The terminal olefin present in alotaketal B (3.17) is replaced by a dimethyl substituted double bond in alotaketal E (4.6).
4.3 Biological Activity

Alotaketals A (3.19) and B (3.20) and ansellone A (3.4) are activators of the cAMP signalling pathway in HEK293 cells that have been transfected with a pHTS-CRE plasmid in the absence of hormone binding on the GLP-1 receptor (Section 3.6). Consequently, ansellones B to D (4.3-4.5) were also tested in the assay but ansellones B and C (4.3-4.4) were cytotoxic in the assay and ansellone D (4.5) was inactive in the assay (Figure 4.42).
Alotaketal E (4.6) was also tested in the assay and was less active than alotaketals A (3.16) and B (3.17) with an EC$_{50}$ of 6.54 μM, but it was more potent than ansellone A (3.4) and comparable with forskolin (3.25), the current molecular probe used to study the cAMP signalling pathway. Investigations into the specific target of the alotaketals and of the ansellones are currently ongoing and may help to explain the difference in potency between these two families of sesterterpenoids.

Figure 4.43 Dose response curves of ansellone A (3.4) and alotaketal E (4.6) in HEK-pHTS-CRE cells.

4.4 Proposed Biogenesis

The alotaketals (3.16, 3.17, 4.6), the phorbaketals (3.19-3.21), the suberitanes (3.14), the ansellones (3.4, 4.3-4.5), and the phorbasones (3.22, 3.23) are all sesterterpenoids and appear to be derived from geranylgeranyl pyrophosphate. The initial cyclization of geranylgeranyl pyrophosphate to form the alotane skeleton most likely occurs through the formation of an allylic cation created by the loss of the pyrophosphate and the subsequent electrophilic addition to the cation by the nearest double bond (Figure 4.44). The protonation of the dimethylated alkene may trigger the stepwise cyclization of the alotane skeleton that produces a carbocation which is quenched by water to form the suberitane skeleton. The sesterterpenoid 19-episuberitone (3.14) is formed from the suberitane skeleton after a
series of oxidation steps. Alotaketal E (4.6) presumably arises from the alotane skeleton after being oxidized to form intermediate I, which then undergoes two intramolecular nucleophilic attacks to create the spiro-ketal moiety (Figure 4.44).

![Diagram showing the biogenesis of the suberitones and the alotaketals.]

Figure 4.44 Proposed biogenesis of the suberitones and the alotaketals.

The ansellones (3.4, 4.3-4.5) most likely arise from the alotane skeleton. The protonation of the dimethylated double bond could trigger a series of electrophilic additions that could result in the cyclization of the farnesyl portion of the alotane skeleton to produce the ansellane skeleton (Figure 4.45). The oxidation of intermediate II would lead to the formation of ansellone A (3.4) and ansellone B (4.3). The formation of the oxocane moiety of ansellone B (4.3) likely takes place through the protonation of the cyclohexene followed by the addition of the hydroxyl group to the newly formed cation. Intermediate III is most likely formed from intermediate II after an oxidation step and a
nucleophilic attack on the newly oxidized carbon by the oxygen attached to carbon 1. Intermediate III probably gives rise to ansellone A (3.22) after a series of oxidations.

The phorbasones (3.22-3.23) are likely formed from the ansellane skeleton after a series of rearrangements. A cationic cyclization of the ansellane skeleton generates the bicyclic intermediate IV (Figure 4.45). The intermediate V is then formed through a series of proton shifts followed by a ring cleavage in intermediate IV. The sesterterpenoid phorbasone A (3.22) arises from intermediate IV after a series of oxidation steps.

Figure 4.45 Proposed biogenesis of the ansellones and the phorbasones.
4.5 Conclusion

Ansellones B (4.3) and C (4.4) are among the sesterterpenoids that have been isolated from our sample of *Phorbas* sp. They are unusual sesterterpenoids that have an ansellane carbon skeleton arranged into an oxocane moiety. Ansellone D (4.5) also has the ansellane skeleton and differs from ansellone A (3.4) based on the oxidation pattern on ring C. Alotaketal E (4.6) was also isolated from our sample of *Phorbas* sp. and shares many similarities with the phorbaketals (3.19-3.21) and the alotaketals (3.16-3.17) isolated from a sample of *Phorbas* sp. collected in Korea and a sample of *Hamigera* sp. collected in Papua New Guinea, respectively. Alotaketal E (4.6) is also an activator cAMP signalling pathway in HEK293 cells that have been transfected with a pHTS-CRE plasmid in the absence of hormone binding on the GLP-1 receptor.

Some preliminary results obtained by the laboratory of Dr. Rho at the Kunsan National University in Korea, that isolated the phorbaketals (3.19-3.21), suggested that these sesterterpenoids may be produced by a bacterial symbiont. We decided to investigate this possibility for the production of the ansellanes and the alotanes isolated in *Phorbas* sp. from British Columbia. Our work on bacterial isolates found in *Phorbas* sp. is the subject of the next chapter.
5. Chemical Investigations of Bacterial Isolates from *Phorbas* sp.

5.1 Bacterial Symbionts in Sponges

Since the dawn of marine natural products exploration, the phylum Porifera has been a prolific source of novel molecules. The marine sponges making up this phylum are under a variety of environmental stresses that may explain the wide array of secondary metabolites they produce. As sessile organisms, sponges need to prevent competitors from overtaking their substrate since space is a finite resource on the marine benthos. Many studies have shown that sponges can be incited to produce allelopathic chemicals when under competitive pressure for space.\(^\text{106, 107}\) Sponges are also under pressure to repel fouling organisms from overgrowing them.\(^\text{108}\) Fending off predators is also accomplished through chemical defence.\(^\text{109}\)

Perhaps a more important explanation for the richness of sponges as a source of natural products is the bacterial flora that inhabits them. It is becoming increasingly obvious that many bioactive compounds isolated from marine invertebrates are in fact produced by bacterial symbionts.\(^\text{11}\) As filter feeders, sponges can pump thousands of litres of seawater through their tissues each day allowing for the presence of microbes and bacteria.\(^\text{110}\) Microorganisms that can resist the sponge digestive and immune system have the potential of becoming symbionts. A large portion of the microfauna present in a sponge at any one time is made up of microorganisms that are found in the water column around the sponge and that are simply “pumped” in the sponge. However, some microorganisms are present within the sponge tissue and some symbionts are even intracellular. These intracellular symbionts can be transmitted through the gametes of the sponge and be found in the sponge larvae.\(^\text{111}\) Regardless of their origin, microorganisms are concentrated by the sponge, and their
number in the sponge tissue can be several times greater than the number of bacteria found in the surrounding sea water, as exemplified by studies done on the sponge *Aplysina Aerophoba*.\textsuperscript{112}

Since two groups of closely related sesterterpenoids, the alotaketals (3.16, 3.17, 4.6) and the ansellones (3.4, 4.3-4.5), were isolated from distinct species of sponges, we decided to undertake a chemical investigation of the metabolites produced by the bacterial cultures isolated from our sample of *Phorbas* sp. We wanted to explore the possibility that a bacterial symbiont present in both sponges was responsible for the biosynthesis of the sesterterpenoids our group had isolated. This hypothesis was particularly intriguing since Dr. Rho’s group had reported that they had isolated the sesterterpenoid phorbaketal A (3.19) from *Phorbas* sp. and from mixed microbial cultures obtained from the homogenized sponge tissue.\textsuperscript{96} However, Dr. Rho did not publish clear evidence that his group had in fact isolated the sesterterpenoids from the cultures themselves and not from the sponge tissue present in the media.

Our approach to the isolation of sesterterpenoids from the bacterial isolates obtained from *Phorbas* sp. was twofold. First, we tested crude extracts of cultures of each bacterial strain isolated from the sponge in the biological assay that led our group to the isolation of alotaketals A and B (3.16, 3.17). Second, we screened the bacterial extract using LC-MS and compared their LC-MS traces to the LC-MS trace of the sponge extract.

### 5.2 Bacterial Symbionts Isolated from *Phorbas* sp.

A total of fourteen distinct bacterial colonies were isolated from the tissue of *Phorbas* sp. and grown on marine media. Unlike Dr. Rho’s group, we did not include sponge tissue homogenate in the marine media so that we would not contaminate the bacterial cultures with sesterterpenoids from the sponge tissue. The cultures of the fourteen isolates were then screened for their biological effects on
the cAMP signalling pathway. Three of the isolates showed moderate biological activity in the assay. Following these results the 16S rRNA of the same three isolates was sent for sequencing and all three were determined to be the same novel species of bacteria with a 99.0% match with *Erythrobacter* sp. PaD3.33a. The species was named *Erythrobacter phorbasii* (GenBank Accession # JN368468) after the sponge from which it had been isolated. Most *Erythrobacter* species are marine species and have been isolated from sea water and invertebrates.113,114 Bacteria from the genus *Erythrobacter* are photoheterotrophs and are known to contain bacteriophyll (similar to chlorophyll in eukaryotes) to conduct photosynthesis. They also contain a number of carotenoids, which give them a red colour. Most of the past chemical investigations on *Erythrobacter* species were focused on identifying their pigments.115,116

The presence of sesterterpenoids in the organic extract of the culture of *Erythrobacter phorbasii* was suggested by a chromatogram obtained using LC-MS and the TLC chromatogram of the bacterial extract. Although several peaks were seen between m/z 450 and 500 (the mass range of the anseellones and alotaketals) in the LC-MS chromatogram, only one exact match to a known sesterterpenoid was found. A peak at m/z 481 in the bacterial extract had nearly the same elution time and molecular mass as alotaketal E (4.6). Unfortunately, when alotaketal E (4.6) was co-injected with the bacterial extract on LC-MS, the elution time of the two peaks at 481 differed slightly. Therefore, alotaketal E (4.6) was not present in the bacterial culture extract. Further, comparison between the LC-MS trace of the bacterial extract with the sponge extract was conducted but no peaks with identical molecular mass and elution time were found. The complexity of the metabolite mixture in the extracts and the low concentration of the terpenoids may have been to blame for the negative result obtained.

Since the bacterial extract was active in the same biological assay as the alotaketals and the anseellones, we decided to pursue the isolation of the biologically active component in the culture of
*Erythrobacter phorbasii*. Bioassay-guided fractionation of the bacterial extract led to the isolation of phorbasolic acid (5.1) (Figure 5.1).

![Figure 5.1. Phorbasolic acid](image)

### 5.3 Meroterpenoids

Meroterpenoids are natural products derived from the terpene biosynthetic pathway and an additional biosynthetic pathway, generally the polyketide synthase pathway. Meroterpenoids have been isolated mostly from fungi and marine invertebrates but they have also been found in plants. Bakuchiol (5.2) is an example of a meroterpenoid isolated from a plant (Figure 5.2).\(^{117}\) A large number of meroterpenoids have been isolated from both marine and terrestrial species of fungi from the genus *Aspergillus*. Tropolactone A (5.3) was isolated from a species of marine fungi of this genus.\(^ {118}\) Few examples of bacterial meroterpenoids exist. The napyradiomycins, a family of halogenated meroterpenoids, are amongst a handful of bacterial meroterpenoids to have been described to date. Like the other bacterial meroterpenoids, they were isolated from terrestrial species of *Streptomyces* or in the case of 5.4 from a marine *Streptomyces*.\(^ {119}\) To the best of our knowledge, phorbasolic acid (5.1) is the first example of a bacterial meroterpenoid isolated outside the genus *Streptomyces*. 
5.4 A Meroterpenoid Isolated from *Erythrobacter Phorbasii* Cultures

The isolation protocol that led to the discovery of phorbasolic acid is summarized in Figure 5.3. The protocol had to be repeated multiple times in order to isolate a spectroscopically detectable amount of the biologically active component. *Erythrobacter phorbasii* was grown on solid agar for fourteen days, and the bacterial cells and the agar were extracted twice in EtOAc (2 x 15 L). The solvent was evaporated *in vacuo* and the extract was dissolved in 1.5 L of EtOAc and back extracted with water (3 x 500 mL). The organic layer was dried *in vacuo* and separated with a Sephadex LH-20 column eluted with MeOH. The resulting fractions were submitted for biological testing in the assay described in section 3.4. The active fractions were then combined and separated on a normal phase column eluting with 85:15 hexanes:EtOAc to EtOAc. Again the fractions were tested and the active fractions were combined and separated on normal phase HPLC. The HPLC separation was performed using a 60:40
hexanes:EtOAc mixture. Although this step provided a significant purification of the active component, the resolution of the biologically active peaks on the HPLC chromatogram was poor. Therefore, the next step of purification was done using a C8 column and a mixture of 60:40 MeCN:H2O as the eluent. The biologically active fraction from this step of purification contained 1.1 mg of the meroterpenoid phorbasolic acid (5.1).

**Figure 5.3. Isolation scheme for phorbasolic acid (5.1).**
Table 5-1. NMR assignments for phorbasolic acid (5.1) recorded in C₆D₆ at 600 MHz.

<table>
<thead>
<tr>
<th>Position #</th>
<th>δ¹³C</th>
<th>δ¹H (J/Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>182.8</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>38.7</td>
<td>2.34 (1H, m, J = 6.8 Hz)</td>
</tr>
<tr>
<td>3a</td>
<td>32.7</td>
<td>1.26 (1H, m)</td>
</tr>
<tr>
<td>3b</td>
<td></td>
<td>1.70 (1H, m)</td>
</tr>
<tr>
<td>4</td>
<td>25.1</td>
<td>1.39 (2H, m)</td>
</tr>
<tr>
<td>5a</td>
<td>36.7</td>
<td>1.88 (1H, m)</td>
</tr>
<tr>
<td>5b</td>
<td></td>
<td>2.34 (1H, m)</td>
</tr>
<tr>
<td>6</td>
<td>137.2</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>121.3</td>
<td>5.28 (1H, t, J = 7.2 Hz)</td>
</tr>
<tr>
<td>8</td>
<td>27.2</td>
<td>3.22 (2H, d, J = 7.2 Hz)</td>
</tr>
<tr>
<td>9</td>
<td>127.5</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>159.1</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>114.5</td>
<td>6.10 (1H, d, J = 8.3 Hz)</td>
</tr>
<tr>
<td>12</td>
<td>129.8</td>
<td>7.91 (1H, d, J = 8.3 Hz)</td>
</tr>
<tr>
<td>13</td>
<td>122.1</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>132.8</td>
<td>8.08 (1H, s)</td>
</tr>
<tr>
<td>15</td>
<td>16.6</td>
<td>1.07 (3H, d, J = 6.8 Hz)</td>
</tr>
<tr>
<td>16</td>
<td>15.8</td>
<td>1.48 (3H, s)</td>
</tr>
<tr>
<td>17</td>
<td>171.8</td>
<td>—</td>
</tr>
</tbody>
</table>

Phorbasolic acid (5.1) was isolated as a white solid that gave a [M-H]⁻ ion at m/z 305.1383 in the HRESIMS consistent with a molecular formula of C₁₇H₂₂O₅ (calculated for C₁₇H₂₁O₅, 305.1389), requiring seven degrees of unsaturation. The HSQC data showed that phorbasolic acid (5.1) had five methines, 4 methylenes, and two methyls accounting for 19 hydrogen atoms attached to carbons as listed in Table 5-1. Three hydrogen atoms were unaccounted for based on the HRESIMS data. The ¹³C NMR spectrum revealed the presence of 2 carboxylic acid or ester carbonyls [δ 182.8 (C-1), 171.8 (C-17)]. These functional groups accounted for 4 of the 5 oxygen atoms present in the molecule. If the two functional groups were carboxylic acids, they would also account for two of the three remaining hydrogen atoms. A quaternary carbon resonance at δ 159.1 that was correlated to aromatic proton resonances in the HMBC data was characteristic of a phenol carbon resonance. The oxygen and the
hydrogen atoms of the phenol, together with the oxygen and hydrogen atoms of the two carboxylic acids and the 19 hydrogens attached to carbons, could account for the five oxygens and 22 hydrogens indicated by the HRESIMS measurement.

The COSY data identified three linear spin systems. One of the COSY spin systems went from the methyl doublet resonance at δ 1.07 (Me-15) to the methine at δ 2.34 (H-2) and from there to three contiguous sets of methylene resonances [δ 1.26 (H-3a), 1.70 (H-3b), 1.39 (H-4), 1.88 (H-5a), 2.34 (H-5b)] (Figure 5.5). An HMBC correlation was seen from both the proton resonance at δ 2.34 (H-2) and at δ 1.07 (Me-15) to the carbonyl at δ 182.8 (C-1) indicating that this first COSY spin system had a carbonyl on one end (Figure 5.6).

Figure 5.4. ¹H NMR spectrum at 600 MHz of phorbasolic acid (5.1) recorded in C₆D₆
Figure 5.5 Expanded COSY spectrum of phorbasolic acid recorded (5.1) in C$_6$D$_6$ at 600 MHz.
The next COSY spin system could be followed from the methyl resonance at δ 1.48 (Me-16) to the olefinic methine at δ 5.28 (H-7) to the methylene at δ 3.22 (H$_2$-8) (Figure 5.7). Based on an HMBC correlation between the methylene proton resonance at δ 1.88 (H-5a, HSQC to δ 36.7) and the quaternary olefinic carbon at δ 137.2 (C-6), it was concluded that the first spin system was connected to the second spin system through a bond between the methylene carbon at δ 36.7 (C-5) and the quaternary olefinic carbon (C-6) (Figure 5.8). An HMBC correlation from both the proton resonances at δ 3.22 (H$_2$-8) and the methyl proton signal at δ 1.48 (Me-16) to the quaternary carbon at δ 137.2 (C-6) confirmed the proposed arrangement around the alkene in 5.1 (Figure 5.6).
The third COSY spin system included only two aromatic proton resonances at δ 6.10 (H-11, HSQC to 114.5) and δ 7.91 (H-12, HSQC to δ 129.8) (Figure 5.7). HMBC correlations were seen from the aromatic proton at δ 6.10 (H-11) to the aromatic carbons at δ 127.5 (C-9), at δ 159.1 (C-10) and at δ 122.1 (C-13) (Figure 5.6). The proton resonance for δ 7.91 (H-12) was correlated to the aromatic carbon at δ 159.1 (C-10) as well as to the aromatic methine at δ 132.8 (C-14) and to the carbonyl resonance at δ 171.8 (C-17). These correlations confirmed the presence of an aromatic ring including carbons 9 to 14. Additional HMBC correlations served to assign the arrangement of the substituent on the aromatic ring. First, the methylene resonance at δ 3.22 (H$_2$-8) was correlated into three aromatic carbons at δ 127.5 (C-
9), at δ 159.1 (C-10) and δ 132.8 (C-14) (Figure 5.6). It was concluded that the aromatic ring was attached to the terpene fragment (C-1 to C-8) through the quaternary aromatic carbon at δ 127.5 (C-9), since the carbon at δ 132.8 (C-14, HSQC with δ 8.08) was protonated and the carbon at δ 159.1 (C-10) was thought to be substituted with a hydroxyl group, based on its chemical shift. The HMBC correlation between the proton at δ 6.10 (H-11) and the phenol carbon at δ 159.1 (C-10) suggested that the two centers were adjacent. Both the aromatic protons at δ 7.91 (H-12) and at δ 8.08 (H-14) had an HMBC correlation to the carbonyl at δ 171.8 (C-17) (Figure 5.6). Only one quaternary aromatic carbon at δ 122.1 (C-13) remained unassigned and it was hypothesised to be between C-12 and C-14 in the aromatic ring and substituted with the carbonyl C-17. This was supported by an HMBC correlation between H-11 and C-13.

![Diagram of phorbasolic acid](image.png)

**Figure 5.8.** Selected COSY and HMBC correlations for phorbasolic acid (5.1) in C6D6.

No carbons were left that could be assigned to an ester resonance, therefore, the two carbonyls of phorbasolic acid had to be carboxylic acids. This was consistent with the molecular formula obtained for phorbasolic acid from the HRESIMS data.
5.5 Biological Activity

The isolation of phorbasolic acid (5.1) was guided by the biological assay that allowed our group to isolate alotaketals A (3.16) and B (3.17). Like the alotaketals, phorbasolic acid (5.1) (30 μM) was found to be an activator of the cAMP signalling pathway in HEK293 cells that have been transfected with a pHTS-CRE plasmid in the absence of hormone binding event on the GLP-1 receptor (See section 3.4). The exact EC_{50} value of Phorbasolic acid (5.1) is currently being determined.

5.6 Conclusion

Following the isolation of closely related sesterterpenoids from the sponge *Phorbas* sp. and the sponge *Hamigera* sp., we investigated the possibility that these sesterterpenoids were produced by a microorganism living in both species of sponges. It has been suggested that many molecules isolated from sponges are in fact synthesized by microorganisms ingested or living in these sponges. However, most marine bacteria are difficult to grow in the laboratory and, so far, no one has succeeded in isolating a molecule from both a sponge and the culture of one of its microbial symbionts.

As part of this investigation, various bacterial and fungal isolates were cultured from our sample of *Phorbas* sp. The isolates were screened using the biological assay that served to isolate the sesterterpenoids, alotaketals A (3.16) and B (3.17), from the sponge *Hamigera* sp. The assay was used as a screen for identifying activators of the cAMP signalling pathway in HEK293 cells that have been transfected with a pHTS-CRE plasmid in the absence of hormone binding event on the GLP-1 receptor (See section 3.4). One of the bacterial isolates was found to activate the cAMP signalling pathway in this assay. This bacterial isolate was a new species of *Erythrobacter* and was named *Erythrobacter phorbasi*. Bacteria from the genus *Erythrobacter* are mostly found in the marine environment and many species of *Erythrobacter* have been isolated from marine invertebrates. The bioassay guided fractionation of the
extract of *E. phorbasii* led to the isolation of phorbasolic acid (5.1), a novel meroterpenoid, built from a hydroxybenzoic acid moiety and a monoterpenoid fragment. Meroterpenoids are not common bacterial metabolites and to the best of our knowledge they have never been isolated from a species of *Erythrobacter*. 
6. **Type II Citrate Synthase Inhibitors**

6.1 **Microbial Natural Products as a Source of Novel Antibiotics**

Microbial natural products have been a major source of antibiotics over the last century, starting from the discovery of penicillin to the more recent isolation of daptomycin. Microbial metabolites have also proven to have therapeutic uses reaching beyond antibiotics. For example, salinisporamide A and cryptomycin, both isolated from marine bacteria, have now entered clinical trial as anticancer agents.\(^{19, 120, 121}\) The efforts to uncover new molecules from bacteria have highlighted the vast chemical diversity of nature. Microorganisms have evolved to produce chemicals in order to interact with other life beings, and this long legacy of biological interactions represents a unique opportunity to encounter molecules that are therapeutically relevant.\(^{122, 123}\)

The need for new antibiotics is pressing when considering the emergence of microbial resistance to currently available antibiotics. The Canadian Nosocomial Infection Surveillance Program reported that the occurrence of methicillin resistant *Staphylococcus aureus* in Canadian hospitals has increased from 1 case per 1000 patients in 1995 to 9 cases per 1000 patients in 2007.\(^{124}\) Furthermore, the Infectious Disease Society of America estimates that 70% of bacterial infections in hospitals are resistant to at least one antibiotic.\(^{125}\) This is especially worrisome since hospitals are using the last line of antibiotic defence available. Contrasting with the increase in bacterial resistance, are the dwindling numbers of new antibiotics entering clinical trials.\(^{126, 127}\) Moreover, many of the new antibiotics coming out of the drug pipeline are variations on antibiotics already on the market. Only two new classes of antibiotics have been approved since the 1960's, neither of which is active against Gram-negative bacteria.\(^{126, 128}\) Gram-negative bacteria include some of the multidrug resistant bacteria placed in the ESKAPE group, named for their resistance to most available antibiotics. The ESKAPE group includes *Enterococcus*
faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter sp. Multidrug resistant strains of all of the species from the ESKAPE group have been found in Canadian hospitals. Therefore, novel antibiotics are needed and history has shown that they can be found in living organisms or at least be inspired by molecules from nature. Under these circumstances, marine microorganisms should be considered as a rich potential source of new antibiotics. The investigation described in this chapter focused on the isolation of novel antibiotics from marine microbes.

6.2 The Citrate Synthase Enzyme

The antibiotic target used in this study is the citrate synthase enzyme which is involved in the citric acid cycle and is, therefore, necessary for the bioenergetics of all living organisms. The citrate synthase enzyme catalyses the first step of the citric acid cycle and is necessary for the production of adenosine triphosphate (ATP) and nicotinamide adenine nucleotide (NADH) in all living organisms.

One aspect of the citrate synthase enzyme that is of therapeutic interest is that Gram-negative bacteria possess a very different isoform of the enzyme than Gram-positive bacteria and eukaryotes. The isoform present in archa, Gram-positive bacteria and in eukaryotes is named type I citrate synthase. It is a dimeric enzyme, and it is not allosterically controlled. On the other hand, type II citrate synthase, found only in Gram-negative bacteria, is allosterically controlled by NADH and its structure is hexameric. The differences between these two types of enzymes are being used in this project to search for an antibiotic specific to type II citrate synthase and, therefore, specific to Gram-negative bacteria. To date, no antibiotic targeting citrate synthase type II has been described.
6.3 Biological Assay

Our collaborators in the Brayer and Roberge laboratories at the University of British Columbia have expressed the citrate synthase type II enzyme in a yeast model in order to design an efficient screening program to isolate microbial natural products that inhibit the enzyme. The citrate synthase assay falls in line with previous growth restoration assays using yeast developed by the Roberge laboratory. In the yeast strain used for this particular assay, the overexpression of a protein of interest can lead to the accumulation of that protein in the cytoplasm, thereby inhibiting the growth of the yeast culture, while the inhibition of the overexpressed protein can lead to the restoration of the growth. Furthermore, the yeast cells used for the assay do not have efflux pumps and consequently, they have a greater sensitivity to chemicals as they cannot remove them from the cytoplasm. To screen for inhibitors of a specific protein, the yeast cells are infected with an expression plasmid that will allow the gene for the target protein to be induced. Once the expression of the target protein is induced, the cells are exposed to a potential inhibitor of the target protein. If, after a treatment with a potential inhibitor, the growth of the yeast cells is restored, the molecule is tested in a secondary assay to verify its selectivity for the target protein.

In the present study, the citrate type II enzyme was overexpressed in the yeast assay and inhibitors of the enzyme would restore the growth of the yeast cell. The yeast cells themselves have the type I citrate synthase enzyme therefore an inhibitor of the type I isoform would restrict the growth of the yeast cell. Consequently, the selectivity of a particular inhibitor to the type II citrate synthase could be tested in one assay.
6.4 Isolation of Citrate Synthase Inhibitors

Our extensive bacterial extract library was screened using the citrate synthase type II growth restoration assay. An extract of *Bacillus pumilus* caused a 60% growth restoration in the yeast model. This level of restoration was higher than what was previously seen with the commercially available chemical library that had been tested. Based on these encouraging results, a large scale culture of *B. pumilus* was undertaken, and the isolation of the biologically active component(s) was done as summarized in Figure 6.1.
**Figure 6.1. Isolation scheme for extract of B. pumillus**

*B. pumillus* was grown on solid agar in 160 pans measuring 50 cm x 30 cm. The bacterial cultures and the agar were extracted twice with EtOAc (2 x 80 L). The extracts were combined and dried *in vacuo* before being re-dissolved in 2.5 L of EtOAc and back extracted three times with 600 mL of water. The organic and aqueous fractions were then dried *in vacuo* and tested in the growth restoration assay. The organic fraction caused a 61% growth restoration in the assay while the aqueous fraction did not restore the growth of the yeast cultures.

The organic fraction (2.1 g) was separated on a LH-20 column using 100% MeOH as eluent. The fractions collected from the LH-20 column were combined based on their TLC patterns and dried *in vacuo* and submitted for biological testing. The biologically active fraction was crystalline and contained a large amount of steroids. The steroids were recrystallized and removed via filtration to give a filtrate largely free of steroids. The filtrate was dried *in vacuo*, and the crystals and the filtrate were submitted for biological assay. The filtrate caused a 60% growth restoration in the yeast assay whereas the crystals did not cause growth restoration. The filtrate contained 200 mg of material and was fractionated using a chromatotron.

The chromatotron is a circular preparative thin layer chromatography instrument. The sample and the solvent are applied near the centre of the rotating plate and the sample elutes with the solvent in circular bands which can be visualised with a UV lamp. The bands are eventually spun off the plate and are collected in test tubes. The chromatotron is a convenient preparative technique as the bands can be visualised before they elute from the silica plate and the elution conditions can be adjusted during the separation. Unlike preparative TLC, the chromatotron allows the sample to be conveniently eluted off the plate into test tubes and there is no need to separate it from the silica as it is the case.
with preparative TLC. In addition, the chromatotron is much faster than preparative TLC or flash chromatography columns as it is accelerated by centrifugal force and requires very little preparation.

For the reasons stated above, a chromatotron equipped with a normal phase plate was used to fractionate the 200 mg of fraction containing the biologically active component. The sample was eluted using 62% chloroform and 38% MeOH. The fractions were pooled based on their UV absorption during elution and based on their TLC pattern when stained with p-anisaldehyde. The fifteen pooled fractions were dried *in vacuo* and submitted for biological testing.

Three contiguous fractions were active in the biological assay and they were pooled to afford 8 mg of material. The material was partitioned further on normal phase HPLC using an isocratic mixture of 60% hexanes and 40% acetone. The seven fractions collected from this separation step were tested for their biological activity. Only one fraction was biologically active and it contained 1.2 mg of material. This fraction contained a mixture of molecules based on its TLC trace and $^1$H NMR spectrum.

The next fractionation step was performed using a C8 column on reverse phase HPLC using 80% MeCN and 20% H$_2$O as eluent. The five fractions collected were tested in the biological assay and only one caused growth restoration in the yeast cultures. The active fraction contained 0.5 mg of the amide 12-methyl tridecanamide shown in Figure 6.2. This fraction caused a 63% growth restoration response at a concentration of 0.1 μg/mL and an 84% growth restoration response at 0.3 μg/mL.

![6.1](Image)

*Figure 6.2. Novel aliphatic amide (6.1) isolated from B. pumillus.*
6.5 Structure Elucidation of 12-Methyl Tridecanamide (6.1)

The aliphatic amide 12-methyl tridecanamide (6.1) was isolated as a white powder that gave a [M+H]$^+$ ion of 228.2336 (228.2327, calculated for C$_{14}$H$_{30}$NO) in the HRESIMS. The molecular formula accounted for 1 degree of unsaturation. Based on the HSQC, the $^1$H NMR (Figure 5.4) and the $^{13}$C NMR data, the molecule contained two methyl groups, 10 methylenes, one methine and one quaternary carbon that was later assigned as an amide (δ 174.15).

![Structure of 12-Methyl Tridecanamide (6.1)](image)

**Figure 6.3.** $^1$H NMR spectrum of 12-methyl tridecanamide (6.1) recorded DMSO- $d_6$ 600 MHz.

The presence of an amide was determined based partly on the $^{15}$N HSQC data where the two proton resonances at δ 6.6 and δ 7.2 were both correlated to the same nitrogen resonance at δ 271. Furthermore, the absence of correlations between the proton resonances at δ 6.6 and δ 7.2 and the
other carbon and proton resonances from rest of the molecule indicated that the NH$_2$ was isolated.

Consequently, the NH$_2$, was assumed to be part of amide functionality. The methyl doublet integrating for six protons at $\delta$ 0.85 (Me-13 and Me-14) indicated the presence of an iso-propyl group in the molecule. The methyl doublet at $\delta$ 0.85 was correlated to the methine resonance at $\delta$ 1.48 (H-12) in the COSY spectrum. From the methine resonance at $\delta$ 1.48 (H-12), a linear spin system could be followed sequentially to the methylene at $\delta$ 2.01 (H$_2$-2) via COSY correlations. The proton resonance at $\delta$ 2.01 (H$_2$-2) was correlated to the amide carbonyl (C-1) in the HMBC data.

To the best of our knowledge, 12-methyl tridecanamide (6.1) has never been isolated from a natural source, although it has been synthesized from its corresponding acid.

Table 6-1. NMR assignments for 12-methyl tridecanamide (6.1) in DMSO-$d_6$

<table>
<thead>
<tr>
<th>Position #</th>
<th>$\delta^{13}$C/$^{15}$N</th>
<th>$\delta^1$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>174.15</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>34.93</td>
<td>2.01 (2H, t, J = 7.1 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>24.96</td>
<td>1.46 (2H, m.)</td>
</tr>
<tr>
<td>4-11</td>
<td>28.6</td>
<td>1.25 (8H, br.s)</td>
</tr>
<tr>
<td>12</td>
<td>26.64</td>
<td>1.48 (1H, sept., J = 6.65 Hz)</td>
</tr>
<tr>
<td>13-14</td>
<td>22.39</td>
<td>0.85 (6H, d, J = 6.65 Hz)</td>
</tr>
<tr>
<td>NH-1</td>
<td>271</td>
<td>6.6 (1H, br. s.)</td>
</tr>
<tr>
<td>NH-1</td>
<td>271</td>
<td>7.2 (1H, br. s.)</td>
</tr>
</tbody>
</table>

Although the amide 6.1 isolated from B. pumillus has not been isolated from a natural source before, several closely related molecules have been described. Molecules related to the amide isolated from B. pumillus include 12-methyl tridecanoic acid (6.2) a common fatty acid found mostly in plants and bacteria. In addition, an unbranched analog (6.3) of the amide has been described and was found to have deer repellent properties. The corresponding acetamide, capsiamide (6.4), was isolated from the hot pepper Capsicum annuum and has analgesic properties.
Figure 6.4. Molecules structurally related to 12-methyl tridecanamide (6.1).

6.6 Preparation of 12-Methyl Tridecanamide (6.1)

The amide (6.1) isolated from the B. pumillus extract was synthesized in order to verify that it was responsible for the biological activity observed in the yeast assay (Scheme 6.1). 12-methyltridecanol acid (6.1) was dissolved in a solution of methanolic HCl to afford the corresponding ester (6.5). The ester was then dissolved in a solution of methanolic NH₃ to yield the target amide.

The synthetic amide was tested in the biological assay and did not cause any growth restoration in the yeast cultures. Therefore, the biological activity of the fraction that contained the amide must have been caused by another molecule that co-eluted with the amide. A 300 scan $^1$H NMR spectrum of the biologically active fraction was taken on a 600 MHz spectrometer to look for the presence of the minor active component. Several low intensity signals were detected as seen in Figure 6.5. The molecule that caused the growth restoration response in the biological assay appears to make up no more than 5% of the 0.5 mg of sample shown in Figure 6.5. This sample caused a 63% growth restoration at a concentration of 0.1 μg/mL, therefore, the active molecule must have a potency in the ng/mL range. No other extracts from our library or from the library of commercially available chemicals that was tested in this assay had a comparable potency.

Since only 0.5 mg of material were available for further fractionation after several scale-ups of the bacterial culture, the optimization of the culture conditions and large scale cultures will be necessary to lead to the successful isolation of the target molecule.
6.7 Conclusion

Due to the emergence of microbial resistance to currently available antibiotics, there is a pressing need to find novel antibiotics. In an effort to find a selective antibiotic against Gram-negative bacteria, a yeast growth restoration assay was developed. This assay serves as a screen for inhibitors of citrate synthase type II, an enzyme that catalyses the first step of the citric acid cycle and that is essential for survival. The type II isoform of citrate synthase is specific to Gram-negative bacteria and would, therefore, present a selective antibiotic target.
Using the growth restoration assay to screen our microbial extract library, an extract of *B. pumillus* was found to restore the growth of the yeast cells that were overexpressing citrate synthase type II. Using the assay to guide the fractionation of the bacterial extract, 12-methyl tridecanamide was isolated. This aliphatic amide was synthesized and tested in the growth restoration assay. The synthetic amide did not restore the growth of the yeast cells. Trace amounts of another compound were detected in the bacterial extract fraction containing the aliphatic amide and we estimate that this trace component has a potency in the ng/mL range. However, the amount of material available did not allow for further purification of this fraction. A larger culture volume of *B. pumillus* will be necessary to achieve a successful isolation of the molecule(s) responsible for the growth restoration observed in the biological assay.
7. Conclusion

The work presented in this thesis was conducted in an effort to isolate novel marine natural products with potential therapeutic properties from marine bacteria and invertebrates. These organisms are a relevant source of therapeutic molecules as exemplified by several marine natural products currently on the market and in clinical trials for the treatment of various ailments. Even if not directly used as drugs, marine natural products have served as molecular tools for studying biological processes. The isolation and structure elucidation of secondary metabolites is the first step in the chain of discoveries that ultimately leads to the production of a pharmaceutical or the development of a molecular tool. Several novel marine natural products were isolated and their structures were elucidated as part of the work presented here. These molecules not only serve as novel molecular tools and potential lead compounds but also serve to expand the known chemical space.

Two novel families of terpenoids were isolated from marine sponges; the clionamines and the ansellones. Clionamines A-D (2.6-2.9), a group of aminosteroids, were isolated from a specimen of Cliona celata and were found to be activators of autophagy. Few activators of autophagy are known, and clionamine A (2.6) is currently being used as a molecular tool to further our understanding of the processes controlling autophagy. Clionamine A (2.6) induces autophagy in normal growth conditions but especially under starvation conditions, an effect not seen in other activators of autophagy such as rapamycin. Many activators of autophagy act upon the mTOR encoding genes which control cellular pathways other than autophagy. Clionamine A does not appear to act upon the mTOR genes and could have a much more specific effect when administered in vivo. In addition, clionamine A (2.6) is currently being studied for its antibiotic effects on Mycobacterium tuberculosis.

Sesterterpenoids are the rarest class of terpenoids and are most commonly found in marine sponges. The novel sesterterpenoid ansellone A (3.4) was isolated from the nudibranch Cadlina...
luteomarginata and later found in a local specimen of the sponge Phorbas sp. along with ansellones B-D (4.3-4.5) and alotaketal E (4.6). The ansellones are the first examples of sesterterpenoids with the ansellane carbon skeleton, and they add to the structural diversity of this rare class of terpenoids. Ansellone A (3.4) and alotaketal E (4.6) are both activators of the cAMP signalling pathway.

Another goal of this work was to explore the possibility that bacteria present in the sponge Phorbas sp. were responsible for the metabolites isolated from their host. No conclusive evidence was found to support this hypothesis, but the novel meroterpenoid phorbasolic acid (5.1) was isolated from one of the bacterial isolates of Phorbas sp. based on its biological activity as an activator of the cAMP signalling pathway. Phorbasolic acid (5.1) was isolated from a new species of bacteria named Erythrobacter phorbasii, and it represents the first example of a meroterpenoid isolated from a bacterium outside of the genus Streptomyces.

In an effort to uncover a novel antibiotic to treat infections by Gram-negative bacteria, an assay was developed that targets the isoform of the citrate synthase enzyme specific to this type of bacteria. Our effort to uncover an inhibitor of the citrate synthase enzyme led to the extract of a culture of Bacillus pumillus which had an unprecedented level of potency in the biological assay. Although the molecule responsible for this effect has yet to be identified, the aliphatic amide 6.1 was isolated.

In conclusion, novel molecules with therapeutic properties were isolated as a result of this work. Several of these molecules now serve as molecular tools to study biological processes relevant to the treatment of diseases.
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Appendix A. Chapter 2 Experimental

A 1. General Experimental Procedure

Solvents for chromatography were HPLC grade obtained from Fisher Scientific Inc., Canada, and were used without further purification. Milli-Q H$_2$O was used for all chromatography. Analytical thin layer chromatography was performed on silica gel 60 F$_{254}$ pre-coated aluminum plates (Merck). Size-exclusion separations were achieved using Sephadex LH-20$^{TM}$ (Sigma). RP-HPLC separations were performed using either a Waters Breeze 600E binary pumping system equipped with a Waters 486 tunable UV detector or a Waters Breeze 1525 binary pumping system equipped with a Waters 2487 Dual λ UV detector, using a CSC-Inertsil 150A/ODS-2 semi-preparative column (9.4 x 250 mm, 5 μm) eluting at 2.5 mL/min. Normal-phase HPLC was carried out using a Waters 515 isocratic pump with a Waters 2487 Dual λ UV spectrophotometer coupled to an Alltech Apollo silica semi-preparative column (10 x 250 mm, 5 μm) eluting at 4.5 mL/min. NMR spectra were recorded using a Brüker Avance 600 MHz NMR system equipped with a CPTCI cryoprobe. All $^{13}$C and $^1$H NMR spectra were internally referenced to the residual solvent signal (DMSO- $d_6$: $\delta_H$ 2.50, $\delta_C$ 39.5; CD$_3$OD: $\delta_H$ 3.31, $\delta_C$ 49.0,C$_6$D$_6$: $\delta_H$ 7.16, $\delta_C$ 128.4; CD$_2$Cl$_2$: $\delta_H$ 5.32, $\delta_C$ 54.0) while $^{15}$N NMR spectra were externally calibrated against CH$_3$NO$_2$ (0 ppm) and are accurate to ±1 ppm. All NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Low and high resolution ESI mass spectra were obtained using a Micromass LCT-TOF spectrometer. UV spectra were recorded using a Waters 2487 Dual λ UV spectrophotometer while specific rotations were obtained with a Jasco P-1010 polarimeter at the sodium D line (589 nm).
A 2. Isolation of Clionamines A to D (2.6-2.9)

Specimens of *Cliona celata* were collected by hand using SCUBA at a depth of 20 m off Coffee Bay, a resort on the warm temperate Wild Coast of South Africa in April, 2002. Freshly collected sponge was immediately frozen for storage until needed. A voucher sample (ZMAPOR19970) has been deposited at the University of Amsterdam.

Freeze dried *Cliona celata* (~10 g dry weight) was extracted with MeOH (2 x 20 mL) at room temperature after which the solvent was removed *in vacuo*. The resulting brown gum was partitioned between H₂O (15 mL) and EtOAc (3 x 5 mL). The EtOAc partition was evaporated under reduced pressure and the resulting solid was chromatogrammed on a Sephadex LH-20™ column (2.8 x 140 cm) eluting with MeOH and the fractions combined on the basis of TLC signatures. The most biologically active fraction was further purified by semi-preparative RP-HPLC using 35% ACN/0.1% TFA to yield 2.6 (1.4 mg).

Freeze dried *C. celata* (56.3 g dry weight) was cut into small pieces that were extracted exhaustively with 95% MeOH/5% H₂O (500 mL) and with MeOH (2 x 350 mL) at room temperature. The combined methanolic extracts were evaporated *in vacuo* with the resulting brown gum (3.2 g) being partitioned between H₂O (150 mL) and EtOAc (150 mL). The EtOAc partition was evaporated under reduced pressure and the resulting solid was further partitioned between 90% MeOH/10% H₂O (225 mL) and hexanes (225 mL). A 453 mg portion of the dried methanolic partition (1.106 g) was chromatographed on a Sephadex LH-20™ column (2.8 x 140 cm) eluting with MeOH. Fractions collected were combined on the basis of their biological activity profiles to give an aminosteroid fraction (58 mg) which was re-chromatographed on an LH-20™ column (2.8 x 105 cm) using 40:5:4 EtOAc:MeOH:H₂O as eluent. Again, fractions were combined on the basis of their bioactivity profile to give a further amino steroid enriched fraction (24.3 mg). The enriched fraction was divided in two and each portion Fmoc
protected by stirring in 9:1 ACN:H2O (1.35 mL) with Na2CO3 (10.0 mg) and Fmoc-Cl (9.0 mg) for 1 hr. The reaction mixtures were dried in vacuo and partitioned between EtOAc (3 x 20 mL) and H2O (3 x 20 mL). The dried organic layers were repeatedly purified by NP-HPLC (15% EtOAc/85% Hexanes) to yield Fmoc-clionamine B (2.22)(1.5 mg), Fmoc-clionamine C (2.23)(1.0 mg) and Fmoc-clionamine D (2.24)(8.7 mg).


Deprotection was performed using standard conditions. 2 mg portions of the Fmoc protected clionamines were stirred in 90:10 DMF:piperidine (10 mL) solution at room temperature for one hour. The DMF and piperidine were removed by vacuum distillation. The residues were then titrated in ether and 0.1% trifluoroacetic acid. The suspensions were centrifuged and the supernatant removed to give pure clionamines B to D (2.7-2.9) as white precipitates.

A 4. Clionamine A (2.6) Physical Data

White amorphous solid; [α]D22 –9.7 (c., 0.36, MeOH); UV/Vis (MeOH) λmax (α) 201 (9160), 276 (870); 1H and 13C NMR, see Table 2-1.; LRESIMS [M+H]+ m/z 444.3; HRESIMS [M+H]+ m/z 444.3465 (calculated for C28H46NO3, 444.3478).

A 5. Clionamine B (2.7) Physical Data

White solid; [α]D22 –15.3 (c., 0.40, MeOH); UV/Vis (MeOH) λmax (c) 201 (9160), 276 (870); 1H and 13C NMR see Table 2-5, HRESIMS [M+H]+ m/z 432.3474 (calculated for C27H46NO3, 432.3478).

A 6. Clionamine C (2.8) Physical Data

White solid, [α]D22 –5.0 (c., 0.32, MeOH); 1H and 13C NMR see Table 2-6; HRESIMS [M+H]+ m/z 444.3462 (calculated for C28H46NO3, 444.3478).
A 7. Clionamine D (2.9) Physical Data

Yellow solid, $[\alpha]_D^{22} -33.3$ (c., 0.45, MeOH); $^1$H and $^{13}$C NMR, see Table 2-7; LRESIMS [M+H]$^+$ m/z 402.4; HRESIMS [M+H]$^+$ m/z 402.2632 (calculated for C$_{24}$H$_{36}$NO$_4$, 402.2644).

A 8. Fmoc-clionamine B (2.22) Physical Data

White amorphous solid; $[\alpha]_D^{22} -11.5$ (c., 0.75, CH$_2$Cl$_2$); UV/Vis (CH$_2$Cl$_2$) $\lambda_{max}$ ($\varepsilon$) 235 (14215), 267 (12520), 301 (3910); $^1$H and $^{13}$C NMR, see Table 2-2.; LRESIMS [M+Na]$^+$ m/z 676.6; HRESIMS [M+Na]$^+$ m/z 676.3975 (calculated for C$_{42}$H$_{55}$NO$_5$Na, 676.3978).

A 9. Fmoc-clionamine C (2.23) Physical Data

White amorphous solid; $[\alpha]_D^{22} -11.5$ (c., 0.40, CH$_2$Cl$_2$); UV/Vis (CH$_2$Cl$_2$) $\lambda_{max}$ ($\varepsilon$) 226(2460), 265 (6285), 289 (1930), 30 (1910); $^1$H and $^{13}$C NMR, see Table 2.3; LRESIMS [M+Na]$^+$ m/z 688.6; HRESIMS [M+Na]$^+$ m/z 688.3955 (calculated for C$_{42}$H$_{55}$NO$_5$Na, 676.3978).

A 10. Fmoc-clionamine D (2.24) Physical Data

White amorphous solid; $[\alpha]_D^{22} -34.5$ (c., 1.68, CH$_2$Cl$_2$); UV/Vis (CH$_2$Cl$_2$) $\lambda_{max}$ ($\varepsilon$) 241 (15100), 267 (25900), 290 (7540), 301 (7920); $^1$H and $^{13}$C NMR, see Table 2.3.; LRESIMS [M+Na]$^+$ m/z 646.1; HRESIMS [M+Na]$^+$ m/z 646.3130 (calculated for C$_{39}$H$_{45}$NO$_6$Na, 646.3145).

A 11. Acetylation of Clionamine A (2.6)

To 1.3 mg of 2.6 (0.0029mmol) in pyridine (1.5mL) was added 27.4µl of acetic anhydride (0.058mmol). The reaction was stirred under argon at room temperature overnight. The resulting acetylated clionamine was purified using silica flash chromatography (pencil column) eluted with 70% hexanes and 30% EtOAc pure 0.9mg of acetylated clionamine A.
A 12. Acetylated Clionamine A (2.25) Physical Data

¹H NMR (600 MHz, DMSO-\textit{d}_6) δ 5.98 (1H, dd, J = 15.5, 7.8 Hz, H.22), δ 5.67 (1H, d, J = 15.5Hz, H.13), δ 4.98 (1H, m, H.16), δ 3.54 (1H, m, H.3), δ 2.15 (1H, m, H.15α), δ 2.08 (1H, m, H.17), δ 2.05 (1H, m, H.24), δ 1.79 (3H, s, Me.31), δ 1.77 (1H, m, H.12β), δ 1.70 (1H, m, H.2α), δ 1.66 (1H, m, H.1β), δ 1.60 (1H, m, H.7β), δ 1.55 (1H, m, H.26), δ 1.43 (1H, m, H.6α), δ 1.42 (1H, m, H.4α), δ 1.42 (1H, m, H.8), δ 1.38 (1H, m, H.2β), δ 1.35 (1H, m, H.15β), δ 1.42 (1H, m, H.11β), δ 1.22 (1H, m, H.4β), δ 1.21 (1H, m, H.11β), δ 1.20 (1H, m, H.6β), δ 1.15 (1H, m, H.12α), δ 1.11 (1H, m, H.5), δ 1.09 (1H, m, H.14), δ 0.95 (3H, d, J = 6.7Hz, H.29), δ 0.87 (1H, m, H.7α), δ 0.85 (3H, d, J = 6.7Hz, Me.27), δ 0.85 (3H, d, J = 6.7Hz, Me.28), δ 0.74 (3H, m, Me.19), δ 0.70 (3H, m, Me.18), δ 0.64 (1H, m, H.9); ¹³C NMR (600 MHz, CDCl₃) δ 176.1 (C.21), δ 170.4 (C.30), δ 137.9 (C.23), δ 125.5 (C.22), δ 81.7 (C.16), δ 74.7 (C.20), δ 64.0 (C.17), δ 54.6 (C.14), δ 52.9 (C.9), δ 52.3 (C.3), δ 43.9 (C.5), δ 42.2 (C.24), δ 39.5 (C.13), δ 37.9 (C.12), δ 36.0 (C.1), δ 35.0 (C.10), δ 34.0 (C.8), δ 33.3 (C.4), δ 32.4 (C.26), δ 31.3 (C.15), δ 31.3 (C.7), δ 27.8 (C.6), δ 25.9 (C.2), δ 23.1 (C.31), δ 19.7 (C.11), δ 19.5 (C.27), δ 19.5 (C.28), δ 16.6 (C.29), δ 13.5 (C.18), δ 11.8 (C.19); HRESI-MS [M+Na]^+ m/z 508.7042 (calculated for C₄₂H₅₅NO₅Na).

A 13. Reductive Amination of 5α-Cholestan-3-one (2.26)

A solution of 5α-cholestan-3-one (2.26) (0.3g, 0.77mmol) was stirred in dry MeOH under argon at room temperature. To this solution was added ammonium acetate (0.59g, 7.7mmol) and sodium cyanoborohydride (0.034g, 0.54mmol). The reaction was stirred overnight and was quenched using HCl to pH 2. The reaction mixture was partitioned between water and EtOAc. The aqueous layer was basified using KOH to pH 10 and NaCl was added. The aqueous layer was partitioned with EtOAc and the organic layer was dried in vacuo. The amine was purified on silica flash chromatography (90% CH₂Cl₂: 10% MeOH) and the pure amine 2.27 was obtained (60mg).
A 14. Physical Properties of 5α-Cholestan-3-amine (2.27)

$^1$H NMR (300 MHz, CD$_2$Cl$_2$) $\delta$ 5.31 (2H, s, NH$_2$-3) $\delta$ 3.1 (1H, br. s, H-3), $\delta$ 2.45 (1H, m), $\delta$ 1.96 (2H, br.d, $J$ = 12Hz), $\delta$ 1.80 (2H, m), $\delta$ 0.96 (3H, s, Me-18), $\delta$ 0.94 (3H, s, Me-19), $\delta$ 0.91 (3H, s, Me-27), $\delta$ 0.85 (3H, d, $J$ = 3Hz, Me-25), $\delta$ 0.84 (3H, d, $J$ = 3Hz, Me-26). HRESIMS [M+H]$^+$ m/z 388.6812.

A 15. 2D NMR Data for Clionamine A (2.6)

Figure A-1 COSY spectrum of clionamine A (2.6) recorded in CD$_2$OD at 600 MHz.
Figure A-2 HSQC spectrum of clionamine A (2.6) recorded in CD$_3$OD at 600 MHz.
Figure A-3 HMBC spectrum of clionamine A (2.6) recorded in CD$_2$OD at 600 MHz.
Figure A-4 ROESY spectrum of clionamine A (2.6) recorded in CD$_3$OD at 600 MHz.
A 16. 2D NMR Data for Fmoc-clionamine B (2.22)

Figure A-5 HSQC spectrum (600 MHz) of Fmoc-clionamine B (2.22) recorded in C6D6.

Figure A-6 COSY spectrum (600 MHz) of Fmoc-clionamine B (2.22) recorded in C₆D₆.
Figure A-7 HMBC spectrum (600 MHz) of Fmoc-clionamine B (2.22) recorded in $C_6D_6$. 
Figure A-8 COSY spectrum (600 MHz) of Fmoc-clionamine C (2.23) recorded in C₆D₆.
Figure A-9 HSQC spectrum (600 MHz) of Fmoc-clionamine C (2.23) recorded in C$_6$D$_6$. 
Figure A-10 HMBC spectrum (600 MHz) of Fmoc-clionamine C (2.23) recorded in C$_6$D$_6$. 
A 18. 2D NMR Data for Fmoc-clionamine D (2.24)

Figure A-11 COSY NMR spectrum (600 MHz) of Fmoc-clionamine D (2.24) recorded in C$_6$D$_6$. 
Figure A-12 HSQC NMR spectrum (600 MHz) of Fmoc-clionamine D (2.24) recorded in C$_6$D$_6$. 
Figure A-13  HMBC NMR spectrum (600 MHz) of Fmoc-clionamine D (2.24) recorded in C₆D₆.
Figure A-14 ROESY spectrum (600 MHz) of Fmoc-clionamine D (2.24) recorded in C$_6$D$_6$. 
Appendix B. Chapter 3 Experimental

B 1. General Experimental Procedures

Optical rotations were measured using a Jasco P-1010 spectrophotometer. The CD spectrum was collected using a Jasco J-730 (NIR) spectropolarimeter using MeOH as the solvent. The $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe. All $^{13}$C and $^1$H NMR spectra were internally referenced to the residual solvent signal (DMSO- $d_6$: $\delta^H$ 2.50, $\delta^C$ 39.5; $C_6D_6$: $\delta^H$ 7.16 ppm, $\delta^C$ 128.39 ppm). All NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Low resolutions ESI +/- were recorded on Bruker Esquire LC ion trap mass spectrometer equipped with an electrospray ion source. The solvent for ESI-MS experiments was MeOH. The sample solution concentration was 100μM. It was infused into the ion source by a syringe pump at flow rate of 200 μL/min. High resolution ESI+ were recorded on a Micromass LCT time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source. The samples were dissolved in MeOH. Solvents for chromatography were HPLC grade and were used without further purification. Merck Type 5554 silica gel plates were used for analytical thin layer chromatography. Normal phase chromatography was achieved using silica Flash F60. Solvents used for HPLC were HPLC grade and were filtered through a 0.45 μM filter (Osmotics, Inc.) prior to use. HPLC separation was performed using a Waters 600 pump system and a Waters PDA 900 detector equipped with an Alltech Apollo silica column or a Phenomenex Luna C8 column.


A specimen of Cadlina luteomarginata was collected by hand using SCUBA at a depth of 10m off Ansell Place, in Howe Sound, British Columbia, Canada in November 2009. The specimen was maintained live and frozen on dry ice before being dissected. A 400g sample of Phorbas sp. was
collected by hand using SCUBA at a depth of 10m off Ansell Place, in Howe Sound, British Columbia, Canada in February 2010. The freshly collected sponge tissue was immediately extracted in MeOH. A voucher sample (RMNH POR. 5227) has been deposited at the University of Amsterdam.

### B 3. Extraction of the Nudibranch

A specimen of *Cadlina luteomarginata* was extracted in MeOH (2 x 5mL). The methanolic extracts were combined and concentrated *in vacuo* to afford 3 mg of an orange oil. The extract was fractionated between H₂O (1mL) and Et₂O (2 x 2mL). The Et₂O partition was evaporated under reduced pressure and chromatographed on a silica gel column using a gradient from 100% hexanes to 3:7 hexanes: EtOAc. Fractions A to G were obtained with ansellone A (3.4) present in fraction E (0.6mg).

### B 4. Extraction of the Sponge

A fresh sample of *Phorbas* sp. (400g) was extracted in MeOH (2 x 100mL). The methanolic extracts were combined and concentrated *in vacuo* to afford 400mg of an orange gum. The extract was fractionated between H₂O (50mL) and EtOAc (3x 10mL). The EtOAc partition was evaporated under reduced pressure and 100 mg of an orange residue was obtained. The residue was chromatographed on a silica gel column using a gradient from 100% hexanes to 3:7 hexanes: ethyl acetate to yield pure ansellone A (3.4) (8.0 mg).

### B 5. Ansellone A (3.4) Physical Data

Isolated as an orange oil; [α]_{D}^{25} = -15.4 (c 0.05, MeOH); $^{1}$H NMR and $^{13}$C NMR, see Table 3.1; positive ion HRESIMS [M+Na]$^{+}$ m/z 465.2621 (calculated for C$_{27}$H$_{38}$O$_{5}$Na).

### B 6. Hydrolysis and Crystallization of Ansellone A (3.4)

To a solution of ansellone A (3mg, 0.067mmol) stirred at room temperature in a 1:1 mixture of THF:MeOH (2 mL) was added LiOH/H$_{2}$O (0.56mg, 0.014mmol). After 6 hours the solution was dried in
vacuo and the residue was extracted with EtOAc (3x 1 mL) and H₂O (3x 1 mL). The organic layers were combined and evaporated to dryness. The residue was dissolved in CCl₄, the solvent was then evaporated overnight and crystals were formed.

B 7. 2D NMR Data for Ansellone A (3.4)

![2D NMR spectrum](image)

Figure B-1 HSQC spectrum (600 MHz) of ansellone A (3.4) recorded in C₆D₆.
Figure B-2 HMBC spectrum (600 MHz) of ansellone A (3.4) recorded in C6D6.
Figure B-3 ROESY spectrum (600 MHz) of anellone A (3.4) recorded in C$_6$D$_6$. 
Appendix C. Chapter 4 Experimental

C 1. General Experimental Procedures

Optical rotations were measured using a Jasco P-1010 spectrophotometer. The CD spectra were collected using a Jasco J-730 (NIR) spectropolarimeter using MeOH as the solvent. The $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe. All $^{13}$C and $^1$H NMR spectra were internally referenced to the residual solvent signal (DMSO- $d_6$: $\delta_H$ 2.50, $\delta_C$ 39.5; C$_6$D$_6$: $\delta_H$ 7.16 ppm, $\delta_C$ 128.39 ppm). All NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Low resolutions ESI +/- were recorded on Bruker Esquire LC ion trap mass spectrometer equipped with an electrospray ion source. The solvent for ESI-MS experiments was MeOH. The sample solution concentration was 100μM. It was infused into the ion source by a syringe pump at flow rate of 200 μL/min. High resolution ESI+ were recorded on a Micromass LCT time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source. The samples were dissolved in MeOH. Solvents for chromatography were HPLC grade and were used without further purification. Merck Type 5554 silica gel plates were used for analytical thin layer chromatography. Normal phase chromatography was achieved using silica Flash F60. Solvents used for HPLC were HPLC grade and were filtered through a 0.45 μM filter (Osmotics, Inc.) prior to use. HPLC separation was performed using a Waters 600 pump system and a Waters PDA 900 detector equipped with an Alltech Apollo silica column or a Phenomenex Luna C8 column.

C 2. Animal Material

A 400g sample of Phorbas sp. was collected by hand using SCUBA at a depth of 10m off Ansell Place, in Howe Sound, British Columbia, Canada in February 2010. The freshly collected sponge tissue
was immediately extracted in MeOH. A voucher sample (RMNH POR. 5227) has been deposited at the University of Amsterdam.

C 3. **Extraction of the Sponge**

A fresh sample of *Phorbas sp.* (400g wet weight) was extracted in MeOH (2 x 100 mL). The methanolic extracts were combined and concentrated *in vacuo* to afford approx. 400mg of an orange gum. The extract was fractionated between H₂O (50 mL) and EtOAc (3x 10 mL). The EtOAc partition was evaporated under reduced pressure and 75 mg of an orange residue was obtained. The residue was chromatographed on a silica gel column using a gradient from 100% hexanes to 4:6 hexanes: ethyl acetate. An orange oily residue (18 mg) containing a mixture of ansellone B (4.3), C (4.4) and D (4.5) eluted at 8:2 hexanes/ethyl acetate. The fraction that eluted at 7:3 hexanes: ethyl acetate contained pure ansellone A (3.4) (8.0 mg) and the fraction that eluted at 1:1 hexanes: ethyl acetate contained alotaketal E (4.6) and other metabolites. The fraction containing ansellone B, C and D was placed on normal phase HPLC and pure 4.3 (3.4 mg), 4.4 (2.3 mg) and 4.5 (1.4 mg) were eluted using a mixture of 7:3 hexanes: ethyl acetate with a flow rate of 2ml/min. The fraction containing 4.6 was placed a HPLC equipped with a C8 column using a 6:4 mixture of MeCN: H₂O as eluent with a flow rate of 2ml/min. Pure 4.6 (1.2 mg) was obtained.

C 4. **Ansellone B (4.3) Physical Data**

Isolated as white residue; [α]²⁵₀-12.5 = (c 0.05, MeOH); ¹H NMR and ¹³C NMR, see Table 4-1; positive ion HRESIMS [M+Na]⁺ m/z 467.2783 (calculated for C₂₇H₄₀O₅Na).

C 5. **Ansellone C (4.4) Physical Data**

Isolated as a white residue; [α]²⁵₀-8.2 = (c 0.05, MeOH); ¹H NMR and ¹³C NMR, see Table 4-2; positive ion HRESIMS [M+Na]⁺ m/z 467.2764 (calculated for C₂₇H₄₀O₅Na).
C 6. **Ansellone D (4.5) Physical Data**

Isolated as a yellow oil; \([\alpha]^{25}_{D} -32.0 = (c 0.05, \text{MeOH})\); \(^1\text{H} \text{NMR and } ^{13}\text{C} \text{NMR, see Table 4-3;}

positive ion HRESIMS [M+Na]^+ m/z at m/z 465.2620 (calculated for C\(_{27}\)H\(_{38}\)O\(_{5}\)Na).

C 7. **Alotaketal E (4.6) Physical Data**

Isolated as an orange oil; \([\alpha]^{25}_{D} -8.05 = (c 0.05, \text{MeOH})\); \(^1\text{H} \text{NMR and } ^{13}\text{C} \text{NMR, see Table 4-4;}

positive ion HRESIMS [M+Na]^+ m/z 481.2555 (calculated for C\(_{27}\)H\(_{38}\)O\(_{6}\)Na).

C 8. **CD Spectra**

![CD spectrum](image)

*Figure C-1 CD spectrum for ansellone C (4.4) recorded in MeOH.*
Figure C-2 CD spectrum for ansellone D (4.5) recorded in MeOH.

Figure C-3 CD spectrum for Alotaketal E (4.6) recorded in MeOH.
C 9. 2D NMR Data for Ansellone B (4.3)

Figure C-4 HSQC NMR spectrum (600 MHz) of ansellone B (4.3) recorded in C₆D₆.

Figure C-5 COSY NMR spectrum of ansellone B (4.3) recorded in C₆D₆ at 600 MHz.
Figure C-6 HMBC NMR spectrum of ansellone B (4.3) recorded in C$_6$D$_6$ at 600 MHz.

Figure C-7 2D ROESY NMR spectrum of ansellone B (4.3) recorded in C$_6$D$_6$ at 600 MHz.
C 10. 2D NMR Data for Ansellone C (4.4)

Figure C-8 HSQC NMR spectrum (600 MHz) of anellone C (4.4) recorded in C$_6$D$_6$.

Figure C-9 HMBC NMR spectrum (600 MHz) of anellone C (4.4) recorded in C$_6$D$_6$. 
Figure C.10 COSY NMR spectrum (600 MHz) of ansellone C (4.4) recorded in C$_6$D$_6$. 
Figure C-11 ROESY NMR spectrum (600 MHz) of ansellone C (4.4) recorded in C$_6$D$_6$. 
C 11. NMR Data for Ansellone D (4.5)

Figure C-12 $^{13}$C NMR spectrum (600 MHz) of ansellone D (4.5) recorded in C$_6$D$_6$.

Figure C-13 HMBC NMR spectrum (600 MHz) of ansellone D (4.5) recorded in C$_6$D$_6$. 

Figure C-14 COSY NMR spectrum (600 MHz) of ansellone D (4.5) recorded in C₆D₆.

Figure C-15 HSQC NMR spectrum (600 MHz) of ansellone D (4.5) recorded in C₆D₆.
Figure C-16 2D ROESY NMR spectrum (600 MHz) of ansellone D (4,5) recorded in C$_6$D$_6$. 
C 12. 2D NMR Data for Alotaketal E (4.6)

Figure C-17 COSY NMR spectrum (600 MHz) of alotaketal E (4.6) recorded in C₆D₆.

Figure C-18 HSQC NMR spectrum (600 MHz) of alotaketal E (4.6) recorded in C₆D₆.
Figure C-19 HMBC NMR spectrum (600 MHz) of alotaketal E (4.6) recorded in C$_6$D$_6$.

Figure C-20 2D ROESY NMR spectrum (600 MHz) of alotaketal E (4.6) recorded in C$_6$D$_6$. 
Appendix D. Chapter 5 Experimental

D 1. General Experimental Procedures

The $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe. All $^{13}$C and $^1$H NMR spectra were internally referenced to the residual solvent signal (DMSO-$d_6$: $\delta_H$ 2.50, $\delta_C$ 39.5; C$_6$D$_6$: $\delta_H$ 7.16 ppm, $\delta_C$ 128.39 ppm). All NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Low resolutions ESI +/- were recorded on Bruker Esquire LC ion trap mass spectrometer equipped with an electrospray ion source. High resolution ESI+ were recorded on a Micromass LCT time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source. The samples were dissolved in MeOH. The LC-MS analysis was performed using a Bruker Esquire-LC equipped with a electrospray ion source and an Agilent HPLC. Solvents for column chromatography were HPLC grade and were used without further purification. Solvents used for HPLC were HPLC grade and were filtered through a 0.45 μM filter (Osmotics, Inc.) prior to use. HPLC separation was performed using a Waters 600 pump system and a Waters PDA 900 detector equipped with an Alltech Apollo silica column or a Phenomenex Luna C8 column. Merck Type 5554 silica gel plates were used for analytical thin layer chromatography. Normal phase chromatography was performed using silicaFlash F60. Size exclusion chromatography was done using sephadex$^{\text{TM}}$ LH-20.

D 2. Culture Conditions of Bacterial Cells

_Erythrobacter phorbasii_ (GenBank Accession # JN368468) was isolated on Marine Medium 49 (0.5 g mannitol, 0.1 g tryptose, 0.01 g FeSO$_4$$\cdot$7H$_2$O, 0.01 g of NH$_4$Cl, 0.10g petone, 10 g phytage, 0.1 L nine salt solution and 0.9 L sea water) from the sponge _Phorbas sp._ collected at Ansell Place, B.C.. The strain was cultures on production medium equivalent to 56 L of the Marine Medium 1 (10.0 g of soluble starch, 4.0 g of yeast extract, 2.0 g of peptone, 0.001 g of FeSO$_4$$\cdot$7H$_2$O, 0.001 g of KBr, 18.0 g agar, 1 L sea
water) at room temperature for 14 days in pans measuring 50 cm x 30 cm. The mature cells and the agar were soaked in EtOAc for extraction.

**D 3. Isolation of Phorbasolic Acid (5.1)**

The bacterial cells and the agar were extracted twice in EtOAc (2 x 15 L). The solvent was evaporated *in vacuo* and the extract was dissolved in 1.5 litres of EtOAc and back extracted with water (3 x 500 mL). The organic layer was dried *in vacuo* and 1.2 g of a red oily residue was obtained. The residue was separated with a Sephadex LH-20 (8 x 120 cm) column eluted with MeOH. The resulting fractions were submitted for biological testing. The active fractions were then combined to obtain 0.42 g of a red residue and separated on a normal phase column eluting with 85:15 hexanes:EtOAc to EtOAc. The active fractions eluted from the column with a solvent mixture of 55:45 hexanes:EtOAc. After establishing their biological activity, the active fractions were combined to afford 0.011 g of a yellow residue, which was then separated on normal phase HPLC. The HPLC separation was performed using a 60:40 hexanes:EtOAc mixture at a flow rate of 2 ml/min. The active fraction purified using normal phase HPLC eluted at 46 minutes and weighed approximately 1.8 mg. The next step of purification was done using HPLC equipped with a C8 column and a mixture of 60:40 MeCN:H2O as the eluent with a flow rate of 2 ml/min. The biologically active fraction from this step of purification contained 1.1 mg of the meroterpenoid phorbasolic acid (5.1).

**D 4. Screening with LC-MS**

A 0.3 g portion of the dried organic layer obtained from the solvent partition of the crude extract of the culture of *Erythrobacter phorbasii* described above was chromatographed on a silica gel column using a gradient from 100% hexanes to 4:6 hexanes:EtOAc as it was done for the sesterterpenoids (4.3-4.6). The fractions that eluted at 1:1 hexanes:EtOAc contained a group of molecules with similar Rf to alotaketal E (4.6) when chromatographed on TLC with a solvent mixture of
6:4 hexanes: EtOAc. The fractions containing these molecules were combined to afford 0.050g of an orange residue and the residue chromatographed using LC-MS to investigate if any known sesterterpenoids were present. The LC-MS was equipped with a reversed-phase column and elute with a gradient of 60:40 MeOH:H₂O → 90:10 MeOH: H₂O for 35 minutes. Several peaks were obtained with m/z ion between 450 and 500. A peak at 481.3 was obtained, which matches the mass of alotaketal E (4.6). Alotaketal E was co-injected with the bacterial fraction but two peaks were obtained with a mass of 481.3 (Figure D-1).

Figure D-1 LC-MS chromatograph of a fraction of the extract of E. phorbasii and alotaketal E (4.6).
D 5.  **Phorbasolic Acid (5.1) Physical Data**

Isolated as a white solid; $^1$H NMR, see Table 5-1; $^{13}$C NMR, see Table 5-1; negative ion HRESIMS $[\text{M-H}]^+ m/z$ 305.1383 (calculated for $\text{C}_{17}\text{H}_{21}\text{O}_5$, 305.1389).

D 6.  **2D NMR Spectra of Phorbasolic Acid (5.1)**

![Figure D-2 HSQC spectrum of phorbasolic acid (5.1) recorded in C$_6$D$_6$ at 600 MHz.](image)

Figure D-2 HSQC spectrum of phorbasolic acid (5.1) recorded in C$_6$D$_6$ at 600 MHz.
Figure D-3 HMBC spectrum of phorbasolic acid (S.1) recorded in C<sub>6</sub>D<sub>6</sub> at 600 MHz.
Appendix E. Chapter 6 Experimental

E 1. General Experimental Procedure

The $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe. All $^{13}$C and $^1$H NMR spectra were internally referenced to the residual solvent signal (DMSO-$d_6$: $\delta$H 2.50 ppm, $\delta$C 39.51 ppm). All NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Low resolutions ESI +/- were recorded on Bruker Esquire LC ion trap mass spectrometer equipped with an electrospray ion source. High resolution ESI+ were recorded on a Micromass LCT time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source. The samples were dissolved in MeOH. Solvents for column chromatography were HPLC grade and were used without further purification. Solvents used for HPLC were HPLC grade and were filtered through a 0.45 μM filter (Osmotics, Inc.) prior to use. HPLC separation was performed using a Waters 600 pump system and a Waters PDA 900 detector equipped with an Alltech Apollo silica column or a Phenomenex Luna C8 column. Merck Type 5554 silica gel plates were used for analytical thin layer chromatography. Normal phase chromatography was achieved using silica Flash F60. Size exclusion chromatography was done using sephadex™ LH-20. The chromatotron by Harrison Research Inc. (Model 7924) was equipped with a silica plate.

E 2. Bacterial Culture Conditions

*Bacillus pumillus* strain RJA 1515 was isolated on Mueller Hinton Agar medium from a marine sediment sample collected in Bamfield, British Columbia at a depth of 84 m. The strain was cultured on Mueller Hinton Agar at room temperature for 14 days in 160 pans measuring 50 cm x 30cm. The mature cells and the agar were soaked in EtOAc for extraction.
**E 3. Isolation of Citrate Synthase Inhibitor(s)**

The bacterial cultures and the agar were extracted twice with EtOAc (2 x 80 L). The extracts were combined and dried *in vacuo* before being re-dissolved in 2.5 L of EtOAc and back extracted three times with 600 mL of water. The organic and aqueous fractions were then dried *in vacuo* and tested in the growth restoration assay. The organic fraction (2.10 g) was active in the assay and it was separated on a LH-20 (6 x 140 cm) column using 100% MeOH as eluent. The fractions collected from the LH-20 column were combined based on their TLC patterns and dried *in vacuo* and submitted for biological testing. The biologically active fraction (0.406 g) was crystalline and contained a large amount of steroids. The steroids were recrystallized in benzene and removed via filtration to give a filtrate largely free of steroids. The filtrate was dried *in vacuo* and the crystals and the filtrate (0.201 g) were submitted for biological assay. The filtrate was fractionated using a chromatotron equipped with a normal phase plate. The sample was eluted using 62% chloroform and 38% MeOH. The fractions were pooled based on their UV absorption during elution as well as their TLC pattern when stained with p-anisaldehyde. The fifteen pooled fractions were dried *in vacuo* and submitted for biological testing. Three contiguous fractions were active in the biological assay and they were pooled to afford 0.008 g of material. The procedure described above had to be repeated multiple to afford enough material (0.008 g) to proceed to the following steps.

The material was partitioned on normal phase HPLC using an isocratic mixture of 60% hexanes and 40% acetone. The seven fractions collected from this separation step were tested for their biological activity. Only one fraction was biologically active and it contained 0.001 mg of material. The active fraction was separated on a C₈ column on reverse phase HPLC using 80% MeCN and 20% H₂O as eluent. The five fractions collected were tested in the biological assay. The active fraction contained approximately 0.5 mg of material and one of the molecules it contained was the amide 12-methyl tridecanamide.
E 4.  NMR Data for 12-Methyl Tridecanamide (6.1)

Figure E-1 HMBC NMR spectrum (600 MHz) of 12-methyl tridecanamide (6.1) recorded in DMSO- $d_6$.

Figure E-2 Expanded COSY NMR spectrum (600 MHz) of 12-methyl tridecanamide (6.1) recorded in DMSO- $d_6$. 
Figure E-3 Expanded COSY NMR spectrum (600 MHz) of 12-methyl tridecanamide (6.1) recorded in DMSO- $d_6$.

Figure E-4 HSQC NMR spectrum (600 MHz) of 12-methyl tridecanamide (6.1) recorded in DMSO- $d_6$. 
E 5. **Synthesis of Ester 6.5**

12-methyltridecanoic acid (6.2) (10mg, 0.0039mmol) was dissolved in a solution of HCl (0.006mol) in MeOH to afford the corresponding ester as white crystals. 

$^1$H NMR (600 MHz, DMSO- $d_6$) δ 3.57 (3H, s, Me-14), δ 2.2 (2H, t, $J =$ 7.1 Hz, H$_2$-2), δ 1.50 (1H, m, H-12), 1.50 (2H, m, H$_2$-3), 1.24 (8H, br.s, H$_2$-4 to H$_2$-11), 0.85 (6H, d, $J =$ 6.65 Hz, Me-13 and Me-15). ESIMS [M+H]$^+$ 242.02 calculated for C$_{15}$H$_{30}$O$_2$.

E 6. **Synthesis of Amide 6.1**

The ester 6.5 was dissolved in a solution containing NH$_3$ in MeOH (1.7μl, 0.0117mmol) in 2 mL of MeOH. The reaction was stirred for 5 hours at 50˚C and was then dried in vacuo to yield a white solid. 

$^1$H NMR (600 MHz, DMSO- $d_6$) δ 7.3 (1H, s, NH-1), δ 6.6 (1H, s, NH-2), 2.0 (2H, t, $J =$ 7.1 Hz, H$_2$-2), 1.48 (1H, sept, $J =$ 6.65 Hz, H-12), 1.46 (2H, m, H$_2$-3), 1.247 (8H, br.s, H$_2$-4 to H$_2$-11), 0.85 (6H, d, $J =$ 6.65 Hz, Me-13 and M-14). ESIMS [M+H]$^+$ 227.04 calculated for C$_{14}$H$_{29}$NO.