Investigations of marine cyanobacterial secondary metabolites: isolation, structure elucidation, and synthesis

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Author
Nunnery, Joshawna Kay

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Investigations of Marine Cyanobacterial Secondary Metabolites: Isolation, Structure Elucidation, and Synthesis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Oceanography

by

Joshawna Kay Nunnery

Committee in charge:

Professor William H. Gerwick, Chair
Professor Lihini Aluwihare
Professor Michael D. Burkart
Professor Vivian Hook
Professor Bradley S. Moore
Professor Jennifer E. Smith

2012
The Dissertation of Joshawna Kay Nunnery is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012
DEDICATION

This dissertation is dedicated to my parents Ray and Wanda Nunnery. To my father who has always believed in my talents, abilities, and intellect, and who never allowed me to make excuses and to this day refuses to acknowledge my shortcomings. Thank you for believing in me and never giving up on me all of these long years. To my mother whose tireless efforts to financially support my education from primary school to undergrad and beyond have not gone unnoticed. Without your determination to help me achieve my dreams, the completion of this degree would not have been possible. Thank you for always working to support me, even if you weren’t 100% in agreement with my decisions at the time. To both of you, I love you, and I want you to know that I am especially blessed to have you as my parents.
EPIGRAPH

One should not pursue goals that are easily achieved. One must develop an instinct for what one can just barely achieve through one’s greatest efforts.

*Albert Einstein*

I am only one, but I am still one. I cannot do everything, but still I can do something; and because I cannot do everything, I will not refuse to do something that I can do.

*Helen Keller*
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LIST OF ABBREVIATIONS

CDCl$_3$- deuterated chloroform
C$_6$D$_6$- deuterated benzene
DCM- dichloromethane
DESI- desorption electrospray ionization
DNA- deoxyribose nucleic acid
DMSO-d$_6$- deuterated dimethylsulfoxide
EMEA- European Medicines Agency
ESI- electrospray ionization
Et$_2$O- diethyl ether
EtOAC- ethyl acetate
FAB- fast atom bombardment
FDA- Food and Drug Administration
FTIR- Fourier transform infrared
GCMS- gas chromatography mass spectrometry
HPLC- high performance liquid chromatography
HR- high resolution
IPA- isopropanol
LCMS- liquid chromatography mass spectrometry
LR- low resolution
MALDI- matrix-assisted laser desorption/ionization
MeCN- acetonitrile
MeOH- methanol
MS- mass spectrometry
NMR- nuclear magnetic resonance
NRPS- non-ribosomal peptide synthetase
PKS- polyketide synthase
RNA- ribonucleic acid
TLC- thin layer chromatography
TOF- time of flight
VLC- vacuum liquid chromatography
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VITA

EDUCATION AND FIELDS OF STUDY

University of California, San Diego, La Jolla, CA 2005-2012
Doctor of Philosophy in Oceanography
Advisors: William H. Gerwick and Vivian Hook

Southampton College of Long Island University, Southampton, NY 2001-2005
Bachelor of Science. Major: Marine Science, Biology Concentration

FELLOWSHIPS

NIH Neuroscience Related to Drug Abuse Training Grant 2010-2012
NIH Marine Biotechnology Training Grant 2005-2006

PUBLICATIONS


PUBLICATIONS IN PREPARATION


ABSTRACT OF THE DISSERTATION

Investigations of Marine Cyanobacterial Secondary Metabolites: Isolation, Structure Elucidation, and Synthesis

by

Joshawna Kay Nunnery

Doctor of Philosophy in Oceanography

University of California, San Diego, 2012

Professor William Gerwick, Chair

Marine cyanobacteria, especially of the genus *Lyngbya*, have been a prolific source of bioactive natural products over the past forty years. While the most prevalent biological activity reported for marine cyanobacterial secondary metabolites is
anticancer activity, the propensity of marine cyanobacteria to produce neuromodulatory active secondary metabolites has been realized in recent years. Neuromodulatory activity may have therapeutic relevance in the treatment of spinal cord injury, chronic pain, CNS disorders including stroke and epilepsy, and treatment of cardiovascular, inflammatory and neurodegenerative disorders. While a number of compounds exhibiting neuromodulatory activities have been isolated from *Lyngbya* sp., relatively few have been reported from other species belonging to the Oscillatoriaceae. The primary focus of the research herein was to evaluate the natural products richness of marine cyanobacteria belonging to the genera *Schizothrix* and *Symploca* spp. with particular emphasis on isolating compounds from extracts and fractions exhibiting neuromodulatory activity. In total, seven novel compounds from marine cyanobacteria were isolated and characterized, including four lipopeptides, janthielamide A and kimbeamides A-C, a polyketide-extended pyranone, kimbelactone A, a new iodinated and brominated diterpene, tasihalide C, and a novel lipophilic carboxylic acid, palmyric acid. Janthielamide A displayed sodium channel blocking activity in murine Neuro-2a cells with an IC$_{50}$ of 11.5 μM and antagonized veratridine-induced sodium influx in murine cerebrocortical neurons with an IC$_{50}$ of 5.2 μM. Kimbeamide A also exhibited modest sodium channel blocking activity with an IC$_{50}$ of 60 μM in murine Neuro-2a cells. Meanwhile, tasihalide C was found to possess potent anti-inflammatory activity with an IC$_{50}$ of 1.3 μM in a mouse macrophage cell line. In addition to studies regarding isolation and characterization of novel marine cyanobacterial natural products, investigations utilizing synthetic methodology to address questions regarding absolute stereochemistry of some of these compounds, as well as several depsipeptides containing an α,α-dimethyl-β-hydroxy
octynoic acid residue, were undertaken. An improved synthetic route for the generation of \( \alpha,\alpha \)-dimethyl-\( \beta \)-hydroxy octynoic acid and related residues was achieved and standards generated by this methodology have been utilized to determine the absolute configuration of the \( \beta \)-hydroxy stereocenter of this fragment in ten depsipeptides of marine cyanobacterial origins thus far.
1.0 CHAPTER 1

INTRODUCTION

1.1 History of Natural Products from Terrestrial Sources

1.1.1 History of the use of natural products in antiquity

For millennia, preparations of animals, plants and minerals in the form of ointments, powders, poultices, teas and tinctures have been used by various cultures as remedies for human ailments ranging from digestive, immune system and reproductive disorders to headaches, colds and flu. Natural products were and continue to be an integral part of traditional Chinese medicine, Ayurvedic medicine in India and the pharmacopeias of Amerindians, including indigenous peoples from North and South America, especially, the Maya civilization. Today, traditional medicine, including medicaments from natural sources such as flora and fauna, continues to be a critical component of health care, especially in rural areas. Approximately 80% of the population in some African and Asian countries rely upon traditional or alternative medicine for primary health care.

While fossil records indicate the use of plants for medicinal purposes as far back as the Middle Paleolithic Age around 60,000 years ago, the practice of utilizing natural products in the form of crude preparations for internal and external use for the treatment of a variety of human diseases and ailments for medicinal purposes dates back approximately 4,000 years to the Shang dynasty in China. Simultaneously, medicaments from plants, animals and minerals were also being implemented in ancient Egyptian, Mesopotamian and Ayurvedic medicine. The earliest recorded use of natural products
for medicinal purposes dating back to 2600 BCE was documented by the ancient Sumerians in Mesopotamia on cuneiform tablets\textsuperscript{11} and by Emperor Huang Ti in the \textit{Sheng Nung Peng Tsao}, which detailed the use of more than 10,000 medicinal substances in traditional Chinese medicine.\textsuperscript{1} Additional written records such as the Ebers papyrus,\textsuperscript{8,11} which details the use of 700 medicinal substances in Egyptian medicine circa 1500 B.C. and \textit{De Materia Medica} written by Pedanius Discorides (often called the Father of Pharmacy) in ancient Greece between 50 and 70 A.D., which identified 600 plants and listed approximately 1,000 natural products drugs, further documented the indications of plants, animals and minerals as medicaments for ancient civilizations.\textsuperscript{12} \textit{De Materia Medica}, comprised of five volumes, is considered to be the antecedent to all modern pharmacopeias and remained in use until about 1600 A.D.\textsuperscript{12}

\subsection*{1.1.2 The modern era of drug discovery from natural sources}

In the early 19\textsuperscript{th} century, various active principles were purified from terrestrial plants thereby ushering in the present era of drug discovery from natural sources and giving rise to the field of pharmacognosy. The term pharmacognosy derives from the Greek words ‘pharmakon’ or drug and ‘gnosis’ or knowledge and is described as the study of natural products with medicinal, ecological or other functional properties.\textsuperscript{13} The first discrete organic compound isolated from natural sources was the alkaloid morphine, the active constituent in opium, from the common poppy (\textit{Papaver sominiferum}) by Friedrich Wilhelm Sertürner in 1803 (Figure 1.1).\textsuperscript{14} Additional alkaloids isolated as pure compounds during this time period include emetine from ipecacuanha by Pierre-Joseph Pelletier and François Magendie in 1817,\textsuperscript{14} colchicine, from meadow saffron (\textit{Colchicum}}
autumnale), quinine, from the bark of the cinchona tree, and strychnine, from Saint Ignatius bean (seeds from the tree *Strychnos nux vomica*), all between 1817-1821 by Pelletier and Jean Bienaimé Caventou, as well as the purification of caffeine by Friedlieb Ferdinand in 1820 and atropine by Heinrich Friedrich Georg Mein in 1831. Small molecules isolated from natural sources are often referred to interchangeably as natural products or secondary metabolites. The term secondary metabolite generally refers to compounds other than amino acids and carbohydrates and those involved in growth, development and reproduction. Secondary metabolites are thought to enhance fitness and survival of the organism by playing roles in predator avoidance/deterrence, prey attraction/capture, and growth inhibition of nearby organisms.

### 1.1.3 The rise of organic synthesis and natural products chemistry

The field of organic chemistry is intricately entwined with the field of natural products chemistry. Prior to 1828, the primary distinction between organic and inorganic chemistry was the prevailing idea, known as vitalism, that organic compounds derived solely from matter that was once imbued with ‘vital force of life’ or living. However, with the synthesis of urea in 1828 by Friedrich Wöhler from the inorganic compounds cyanic acid and ammonium, the field of organic synthesis was born. Despite this experimental proof that organic compounds can be generated from inorganic substances, vitalism remained in vogue until Hermann Kolbe synthesized acetic acid from carbon disulfide in 1845, and even then renowned microbiologist and chemist Louis Pasteur remained a steadfast believer in vitalism. From these humble beginnings in the early to mid 19th century, the fields of natural products chemistry and organic synthesis have
emerged as truly fascinating areas of research, and as continued advances in technology transpire, additional compounds with novel carbon skeletons and unique structural modifications present only in miniscule quantities from natural sources will be accessible for investigation by natural products and synthetic chemists alike.

![Alkaloids](image)

**Figure 1.1.** Alkaloids isolated from terrestrial plants in the early 19th century.

Today, natural products are a critical component of Western medicine. In fact, approximately 50% of the new and approved drugs for treatment of human diseases from 1981-2006 are natural products, natural product derivatives, synthetic molecules with pharmacophores from natural products, or natural product mimics.\(^{23}\) Investigations of terrestrial plants utilized in traditional medicine has often led to the isolation of active principles which have become instrumental in modern medicine.\(^{7}\) For example, one of the medicaments described in the ancient traditional Chinese medical text *Sheng Nung Peng Tsao* is ma huang (*Ephedra sinica*) from which the ephedrine alkaloids including
ephedrine and pseudoephedrine derive (Figure 1.2).\textsuperscript{1,14} Historically, preparations from this plant were used to treat asthma, hay fever, and congestion.\textsuperscript{1} Today, pseudoephedrine remains as the active ingredient in many over the counter sinus and cold medications. In another example, the foxglove plant (Digitalis purpurea), which had historically been used to treat patients suffering from abnormal fluid build up, gave rise to digitoxin, isolated in 1841 by E. Humolle and T. Quenvenne.\textsuperscript{14} Subsequently, digoxin was isolated by Sydney Smith from D. lanata in 1929 and remains the most-widely employed cardiac glycoside for the treatment of atrial fibrillation and flutter.\textsuperscript{14,15} In the 1930s, Russell Marker discovered a compound called diosgenin from yams belonging to genus Dioscorea which had been utilized by native American women in Mexico for hundreds of years as an effective contraceptive method.\textsuperscript{14} The structure of diosgenin is similar to progesterone, a steroid hormone involved in the female menstrual cycle and reproduction, that was found to halt ovulation in rabbits.\textsuperscript{14} Unfortunately progesterone is destroyed by the digestive system in humans when ingested orally.\textsuperscript{14} In 1950, the first orally active steroidal contraceptive, an analogue of progesterone called norethindrone, was synthesized by Carl Djerassi.\textsuperscript{14} As of 2005, approximately 100 million women across the globe were using oral contraceptives.\textsuperscript{14}
1.1.4 The case for natural products in modern medicine

Several of the most important drugs in both efficacy and sales in the pharmaceutical industry today are natural products or structural analogues of natural products including various anti-tumor agents such as the vinca bis-indole alkaloids (e.g. vinblastine and vincristine),\textsuperscript{15} podophyllotoxin analogues (e.g. etoposide and teniposide),\textsuperscript{15} camptothecin analogues (e.g. irinotecan and topotecan),\textsuperscript{15} and taxanes (e.g. paclitaxel and docetaxel),\textsuperscript{15,22} as well as antibiotics including the β-lactams (e.g. penicillin

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{natural_products.png}
\caption{Natural products and derivatives from traditional medicinal sources.}
\end{figure}
and cephalosporin)\textsuperscript{15,22} and the glycopeptides (e.g. vancomycin and teicoplanin),\textsuperscript{22,24-26} as well as the cholesterol-lowering statins (e.g. atorvastatin marketed by Pfizer as Lipitor\textsuperscript{®} and lovastatin marketed by Merck as Mevacor\textsuperscript{®})\textsuperscript{14,15,22} among others (Figure 1.3). Between 1981 and 2006, approximately 54% of anticancer and 68% of antibacterial new chemical entities (NCEs) were natural products or semi-synthetic derivatives of natural products.\textsuperscript{23}

In the 1950s investigations of terrestrial plants for anti-cancer agents began in earnest with the isolation and characterization of the vinca alkaloids, from Madagascar periwinkle (\textit{Catharanthus roseus}, formerly \textit{Vinca rosea}) and the podophyllotoxins, from the American mandrake or Mayapple (\textit{Podophyllum peltatum}) and \textit{P. emodii}.\textsuperscript{27} The semi-synthetic derivatives of an epimer of podophyllotoxin, etoposide and teniposide, and the vinca alkaloids vinblastine and vincristine are still in clinical use today.\textsuperscript{27} Meanwhile, Monroe E. Wall and Mansukh C. Wani discovered camptothecin and paclitaxel from the Asian xi shu or ‘happy tree’ (\textit{Camptotheca acuminata}) and the bark of the Pacific yew tree (\textit{Taxus brevifolia}), respectively.\textsuperscript{15} Both camptothecin and paclitaxel are isolated from relatively rare trees and, especially in the case of paclitaxel, supply problems were one of the major issues that hampered its development as an anticancer agent for clinical use.\textsuperscript{15,22} At present, paclitaxel is obtained via a short synthetic sequence from 10-deacetylbaccatin III, which can be readily obtained in sustainable manner from the European yew (\textit{Taxus baccata}).\textsuperscript{22,28} Recently, one strain of the endophytic fungi \textit{Fusarium solani} has been found to produce camptothecin and two analogues,\textsuperscript{29} while several strains of endophytic fungi have been discovered to produce Taxol\textsuperscript{®} since it was first brought to market in 1993, including \textit{Taxomyces andreanae},\textsuperscript{30} as well as \textit{Pestalotiopsis microspora},\textsuperscript{31}
*Fusarium lateritium* and *Pestalotia bicilia,* among others. Optimization of compound production and growth for these endophytic fungi may result in a new source for this compound for continued use in the treatment of cancer. As of 2000, annual sales of more than $1.5 billion had been achieved for paclitaxel (Taxol®).

With regard to antibiotics, Sir Alexander Fleming first observed in 1928 that not only was the growth of bacteria inhibited, but also that otherwise healthy bacteria succumbed to cell lysis and death when they encountered the mold *Penicillium notatum.* While he was not the first to report that *Penicillium* molds inhibited bacterial growth, he was the first to identify the active constituent, a β-lactam compound, which he named penicillin, representing one of the most significant discoveries in medicine in the 20th century. Shortly thereafter, additional β-lactam antibiotics were discovered including cephalosporin C isolated from *Acremonium* sp. (formerly *Cephalosporium* sp.) by Guy Newton and Edward Abraham in 1956, clavulanic acid isolated from *Streptomyces clavuligerus* in 1970s by scientists at Beecham, and thienamycin from *Streptomyces cattleya* in 1979 by Kahan and coworkers. In addition to these natural products, semi-synthetic derivatives such as ampicillin, amoxicillin and methacillin were developed. Many of these antibiotics are still widely used today; however, abuse, misuse and overuse of these antibiotics has led to increased resistance in many microbial organisms. Since 1958, vancomycin, a glycoside antibiotic originally isolated in 1951 from *Amycolatopsis orientalis* by Edmund Kornfeld, has been the drug of last resort for the treatment of antibiotic resistant gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus.* By the late 1980s, resistance had emerged even to vancomycin. Development of new antibiotics helps to keep these potentially life-
threatening infections at bay; however acquired resistance to antibiotics in various microbes is a growing problem and there is a great need for new antibiotics with novel mechanisms of action.
Figure 1.3. Major drugs isolated from natural sources for treatment of human diseases.
Outranking cancer, heart disease is the number one cause of death in the United States.\textsuperscript{36} The development of the cholesterol lowering statins, which are 3-hydroxy-3-methylglutaryl-coenzyme A- (HMG-CoA) reductase inhibitors, represents an important development in the treatment of hypercholesterolemia and prevention of primary and secondary heart disease.\textsuperscript{14} The first anticholesterol statin, compactin, was originally discovered from \textit{Penicillium citrinum} by Akira Endo in 1973 at Sankyo Pharmaceuticals in Japan.\textsuperscript{14,37} Unfortunately, though this drug underwent some evaluation in humans, it never made it to market.\textsuperscript{14} However, a structural analogue of compactin, known as lovastatin (Mevacor\textsuperscript{®}), was isolated from the fungus \textit{Aspergillus terreus} in 1978\textsuperscript{38} and Merck began selling Mevacor\textsuperscript{®}, which was the first FDA-approved statin in 1987.\textsuperscript{39} As of 2004, cholesterol- and triglyceride-lowering drugs were the top-selling category in the world with sales of more than $30 billion, of which $10 billion was accounted for by Lipitor\textsuperscript{®}, the world’s top-selling drug overall.\textsuperscript{14}

1.2. Exploration of the Marine Environment for New Bioactive Chemical Entities

1.2.1 The case for evaluating the secondary metabolites of marine organisms

While the medicinal and biosynthetic potential of terrestrial plants and microbes is fairly well-studied, comparatively little is known regarding the chemistry and biological activity of organisms in the marine environment. Likewise, while many natural products used in ancient medicine derived from terrestrial sources, only a few were derived from marine sources. Medicaments from the marine realm used in traditional medicine in various regions of Latin America include the indication of cnidarians, such as the Portuguese-man-of-war (\textit{Physalia physalia}) to treat asthma and stony coral
(Mussismilia hartii), to treat diarrhea, as well as molluscs, including conch (Cassis tuberosa) for the treatment of asthma and oyster (Crassostrea rhizophorae) to treat cancer, pneumonia, osteoporosis and stomach ache among others. Similarly, fish were also used to treat human maladies including redband trout (Oncorhynchus mykiss) for the treatment of rheumatism and electric eel (Electrophorus electricus) in the treatment of sprains, flu, deafness, tuberculosis, osteoporosis and other ailments. Various echinoderms, including sea stars (Echinaster sp. and Luidia senegalensis) and urchins (Mellita sp. and Echinometra lucunter) were also used for the treatment of asthma and other maladies.

Figure 1.4. Hawaii centric view of Earth revealing the vastness of the world’s oceans. Adapted from Scheuer, 1991.
While the world’s oceans cover approximately 70% of Earth’s surface and possess incredible potential for the discovery of structurally-intriguing molecules with applications relevant to human disease, very few investigations of secondary metabolites from marine organisms with potential biomedical application occurred prior to the 1950s (Figure 1.4). The advent of SCUBA in the 1940s suddenly made the marine realm more accessible and avid exploration of the marine environment commenced shortly thereafter.\(^{16}\) By 1975, three sub-disciplines of marine natural products chemistry had emerged, including marine toxins, marine biomedicinals and marine chemical ecology.\(^{42}\) In the decades since, investigations of the marine environment for potential pharmaceutical agents have been quite successful, and a number of chemical entities isolated from marine organisms have proven to be exciting drug leads.\(^{42}\)

Marine organisms face many of the same challenges as their terrestrial counterparts, such as competition for space, predation avoidance, prey attraction and nutrient limitation.\(^{16}\) However, the marine realm presents an added challenge in that it is an aqueous, as opposed to gaseous, environment with high salinity and high pressure. Additionally, the world’s oceans are a rich source of halogen atoms with concentrations of 0.5 M chloride, 1 mM bromide, 1 \(\mu\)M iodide and 73 \(\mu\)M iodide.\(^{43}\) Thus, as one might expect, halogen atom incorporation into organic compounds is a phenomenon that is considerably more wide spread in the marine environment than in terrestrial environments, and in fact, as of 1997, nearly 1,000 organohalo compounds had been isolated from marine algae and cyanobacteria.\(^{44}\) Similarly, sulfur is also found in relatively high abundance in seawater with a concentration of approximately 29 mM for sulfate in open ocean waters.\(^{45}\) The high concentrations of halides and sulfur represent a
significant difference between terrestrial and marine environments that may have a substantial impact on the structural diversity of marine natural products. Increased incidence of halogen and sulfur incorporation has much relevance in the search for drug leads from nature, as both can profoundly influence the biological activity of a natural product. This unique set of conditions has resulted in the evolution of organisms that are quite different from those encountered in terrestrial biomes with 12 of 31 animal phyla relegated to the marine environment exclusively; therefore, one expects to find that marine organisms produce different natural products than terrestrial organisms.

1.2.2 Early investigations of marine natural products

Early investigations of marine organisms revealed several of the same biosynthetic pathways as those found in terrestrial organisms including, the acetate pathway, which gives rise to fatty acids and polyketides, the mevalonate pathway, which is responsible for isoprenoid/terpene biosynthesis and the shikimate pathway, which produces aromatic amino acids, as well as alkaloids derived from various routes. Interestingly, while many marine natural products appeared to derive from the same biosynthetic pathways as secondary metabolites from terrestrial organisms, they featured a myriad of unusual structural modifications in comparison. For example, not only are diterpenes more prevalent in the marine environment, whereas most terrestrial plants are rich in monoterpenes, but also the hydrogen ion mechanism of cyclization is superseded by a halogen-induced cyclization, including bromine and even sometimes chlorine, in many marine-derived terpenes.
Figure 1.5. Complex marine natural products derived from acetate pathways.

Two particularly intriguing marine natural products arising from acetate pathways are brevetoxin B and palytoxin (Figure 1.5). Brevetoxin B is a unique complicated C$_{50}$
polyether isolated from the dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve* and *Ptychodiscus brevis*), which is the causative organism of red tides occurring in coastal waters along the southeastern United States.\(^5\) Meanwhile, palytoxin is an intriguing \(\text{C}_{115}\) compound with 40 hydroxyl groups and 11 ether linkages isolated from the zoanthid *Palythoa* sp.\(^5,\)\(^5\) At the time of its characterization, palytoxin was the most poisonous nonproteinaceous compound known.\(^5\) While palytoxin was originally isolated in 1971, elucidation of the planar structure was not completed until 1981 and determination of the absolute configuration was not finished until 1982.\(^5\)\(^6\)\(^1\) Lastly, a number of nitrogen-containing metabolites with unique structural features were discovered during early investigations of marine natural products which further exemplified the diversity present in organic compounds from the marine environment. Several distinctive amino acids including \(\beta\)- and hydroxy-amino acids, as well as the fascinating aminophosphonic acid residue, have only been found in the marine environment. Additionally, halogenated amino acid derivatives, such as brominated and/or chlorinated derivatives of tyrosine are common in the marine realm. Halogenation is also a prominent feature of indole residues which derive from tryptophan.
Figure 1.6. Primary and secondary metabolites from terrestrial and marine organisms.

Primary metabolites also possess unusual structural features, such as lactone and allene functional groups in the tris-norcarotenoid peridinin from dinoflagellates\textsuperscript{62} and acetylenic and aromatic functional groups in carotenoids from sponges in the genus \textit{Reniera} (Figure 1.6).\textsuperscript{63} Even simple terpenes from the marine environment may be highly modified in comparison to similar terpenes from the terrestrial environment. For example, ochtodene from the red seaweed \textit{Ochtodes secundiramea}\textsuperscript{64} is analogous to several male boll weevil sex pheromones, including \textit{cis} and \textit{trans}-3,3-dimethyl-\(\Delta^1,\alpha\)-cyclohexaneacetaldehyde;\textsuperscript{65} however, the marine terpene ochtodene features extensive halogenation. Similarly, sterols in the marine environment feature alkylation at various positions in the side chain, including C-22 and C-23, while sterols from terrestrial plants are typically related to \(\beta\)-sitosterol with modification only at C-24 of the side chain.\textsuperscript{54} The unique structural features observed for marine natural products in comparison to
terrestrial secondary metabolites may arise from the differences in the environment (e.g. pH, pressure, temperature, ionic constitution, aquatic medium) or may be due to the fact that many taxa found in the ocean have few, if any, freshwater or terrestrial counterparts; however, it is most likely that the observed differences result from a combination of these two and other contributing factors.

1.2.3 Drugs approved for clinical use derived from or inspired by marine natural products

At present, there are nine drugs approved for use in the US or Europe that are unaltered marine natural products or chemical entities featuring significant structural homology to or bearing pharmacophores of marine-derived compounds (Figure 1.7). The drugs currently approved by either the United States Food and Drug Administration or the European Medicines Agency include vidarabine, Ara-A (Vira-A®), cytarabine, Ara-C (Cytosar-U®), zinconotide, ω-conotoxin MVIIA (Prialt®), trabectedin, ET-743 (Yondelis®), brentuximab vedotin (Adcetris®), eribulin mesylate, E7389, (Halaven®), nelarabine (Arranon®), fludarabine phosphate (Fludara®), and omega-3-acid ethyl esters (Lovaza®). Vidarabine is a synthetic purine nucleoside based on spongouridine, a nucleoside isolated from the sponge Tethya crypta (formerly Cryptotetha crypta), with antiviral activity, while cytarabine is a synthetic pyrimidine nucleoside, based on the sponge nucleoside spongothymidine, also from T. crypta, which is used as an anticancer agent. A fluorinated derivative of vidarabine known as fludarabine, has recently obtained US FDA approval for the treatment of hematological malignancies. Additionally, nelarabine, a water-soluble prodrug of another arabinose nucleoside Ara-G,
was approved by the US FDA in 2005 for treatment of T-cell acute lymphoblastic leukaemia.\textsuperscript{71}

Approved in 2004 by the FDA for the treatment of severe chronic pain in patients with AIDS or cancer, ziconotide is the synthetic equivalent of the marine natural product \(\omega\)-conotoxin MVIIA, a 25-amino acid peptide isolated from the cone snail \textit{(Conus magnus)}.\textsuperscript{66,72} Trabectedin, currently approved in the European Union for treatment of soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer, is still awaiting FDA approval in the United States.\textsuperscript{73} It is a tetrahydroisoquinone alkaloid isolated from the tunicate \textit{Ecteinascidia turbinata}.\textsuperscript{74} Eribulin mesylate is a simplified macrocyclic ketone analogue of the polyether macrolide halichondrin B, which was originally isolated from the sponge \textit{Halichondria okadai} in 1986 by Hirata and Uemura.\textsuperscript{75} Eribulin mesylate targets tubulin, which is also the molecular target for the vinca alkaloids and taxanes, however, eribulin mesylate has a different mechanism of action.\textsuperscript{66,76} Meanwhile, brentuximab vedotin is an antibody drug conjugate of auristatin E,\textsuperscript{77} a synthetic analogue of dolastatin 10,\textsuperscript{78} recently approved by the US FDA for treatment of relapsing Hodgkin’s lymphoma and anaplastic large cell lymphoma.\textsuperscript{66,79} The anti-CD30 monoclonal antibody allows the drug to travel through the bloodstream until it reaches tumors expressing CD30 where it is transported into the cell and the linker is cleaved via proteases releasing auristatin E, thus selectively targeting cancerous cells.\textsuperscript{66} Lastly, a combination of docosahexaenoic acid and eicosapentaenoic acid ethyl esters, known as Lovaza was recently approved by the US FDA for treatment of very high triglyceride levels.\textsuperscript{80}
Figure 1.7. FDA or EMEA approved drugs derived from or inspired by marine natural products.
1.2.4 Marine natural products currently in preclinical development as anti-cancer agents

One major focus in the search for drugs from the sea has been the pursuit of novel anticancer molecules. As of 2008, there were approximately 20 marine natural products and synthetic analogues of marine natural products in anticancer clinical trials with several additional compounds presently undergoing preclinical development. Intriguing marine natural products currently in phase II or phase III clinical trials include bryostatin 1, kahalide F, solibdotin (TZT-1027), and salinosporamide A (Figure 1.8). Bryostatin 1 was originally isolated from the bryozoan Bugula neritina and has been found to activate protein kinase C which induces phosphorylation of other kinases and regulatory proteins ultimately resulting in ubiquitin-mediated degradation. Kahalalide F, derived from the mollusk Elysia rufescens and the green alga Bryopsis sp., disrupts lysosome membranes resulting in apoptosis and also induces down regulation of the erbB pathway. Solibdotin (TZT-1027), a synthetic analogue of dolastatin 10 from Dolabella auricularia, has been found to target tubulin, while salinosporamide A, isolated from the marine actinomycete Salinispora sp., has been found to be a 20S proteasome inhibitor.
Figure 1.8. Marine natural products currently in Phase II/III clinical trials for cancer.

1.3 Investigations of Secondary Metabolites from Marine Cyanobacteria

1.3.1 Early discoveries regarding secondary metabolites from marine cyanobacteria

Microbial organisms in the marine environment have increasingly become one of major focal points for investigations seeking to identify new chemical entities with novel structural backbones and diverse biological activities. As many current leads from the
marine environment are likely to be produced by microbial organisms, it is worthwhile to investigate these organisms independently to evaluate their individual biosynthetic potential. In the 1970s, Professor Richard E. Moore at the University of Hawaii began exploring the chemistry of marine cyanobacteria. Early investigations of these organisms revealed that they are a prolific source of structurally intriguing bioactive secondary metabolites. In 1977 debromoaplysiatoxin was the first structurally characterized natural product to be isolated from marine cyanobacteria (Figure 1.9). Intriguingly, this compound had previously been reported from the sea hare *Stylocheilus longicauda* in the family Aplysiidae by Professor Paul Scheuer’s group, also at the University of Hawaii. Subsequent investigations have revealed that several natural products isolated from sea hares and nudibranchs including dolastatin 10, kulolide 1, aplysiatoxin, and the malyngamides, among others, are likely of cyanobacterial origin and are obtained by these opisthobranchs as a result of their dietary preference for cyanobacteria.
Figure 1.9. Marine cyanobacterial secondary metabolites from early investigations by R. E. Moore.

In addition to debromoaplysiatoxin, several other marine cyanobacterial natural products were isolated and characterized in rapid sequence, including the majusculamides A and B, lyngbyatoxin A and malyngamide A. The majusculamides A and B were reported from *Lyngbya gracilis*, however, no biological activity was reported for these compounds. Recently, a report by Tan *et al.* revealed that majusculamide A exhibits anti-fouling activities. Additional compounds in this structure class including majusculamide C, majusculamide D and deoxymajusculamide D have been found to possess cytotoxic activities. Lyngbyatoxin is an indole alkaloid isolated from *Lyngbya majuscula* that is structurally-related to teleocidin B from *Streptomyces* sp. Both
debromoaplysiatoxin and lyngbyatoxin have been implicated as the causative agents of ‘swimmer’s itch,’ a type of contact dermatitis. \(^{87,91,96}\) Similar to bryostatin 1, lyngbyatoxin has also been found to activate protein kinase C. \(^{97}\) Malyngamide A is a small amide featuring a vinyl chloride moiety isolated in 1979 from Lyngbya majuscula. \(^{92}\) To date, more than 30 malyngamides have been isolated from the marine environment. \(^{98}\) These pervasive marine cyanobacterial secondary metabolites exhibit a range of biological activities including anticancer \(^{98,99}\) and anti-inflammatory properties, \(^{100-104}\) as well as quorum sensing inhibition \(^{105}\) and ichthyotoxicity. \(^{99,106,107}\) Even these initial investigations revealed several hallmarks of marine cyanobacterial natural products including the presence of vinyl chloride moieties, interdigitation of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) pathways and extensive N-methylation. Continued investigations during the past four decades have revealed additional structural classes and features characteristic of marine cyanobacteria. The majority of secondary metabolites from marine cyanobacteria are cyclic peptides, depsipeptides or lipopeptides, \(^{108,109}\) with the latter two continuing with the theme of interdigitation of NRPS and PKS elements, previously observed in majusculamides A and B \(^{90}\) and malyngamide A. \(^{92}\) In addition to an unusually high degree of N-methylation, other distinctive features of marine cyanobacterial secondary metabolites consist of the frequent inclusion of unique β-amino, β-hydroxy, and α-hydroxy acids and the formation of heterocyclic rings from cysteine and threonine. \(^{108,110}\) Marine cyanobacterial secondary metabolites featuring amino acids, including peptides, depsipeptides and ketopeptides, are primarily composed of neutral amino acids, such as valine, alanine, proline, isoleucine, leucine, phenylalanine, and glycine, and polar non-charged amino acids, especially tyrosine and threonine. \(^{108}\) The
majority of these amino acids are incorporated as the L-form. Approximately 70% of NRPS-derived marine cyanobacterial natural products are cyclic. Marine cyanobacterial secondary metabolites often have enhanced lipophilic character due to the high proportion of polyketide elements and a greater number of hydrophobic amino acids.

1.3.2 Natural products from marine cyanobacteria with therapeutic potential in the treatment of cancer

The most prevalent therapeutically relevant biological activity reported for marine cyanobacteria is cytotoxicity. Several promising anticancer-type compounds have been isolated from these organisms during the past forty years, including apratoxin A, coibamide A, curacin A, desmethoxymajusculamide C, largazole, and somocystinamide A (Figure 1.10). Collections of cyanobacteria belonging to the genus *Lyngbya* from various localities including Guam, Palau, Papua New Guinea and Palmyra have yielded several analogues of a novel class of cyclic depsipeptides known as the apratoxins. These compounds are comprised of a tetrapeptide chain coupled to a thiazoline unit and incorporating a PKS derived 3,7-dihydroxy-2,5,8,8-tetramethylnonanoic acid chain into the cyclic carbon skeleton. The exquisite biological activity of these compounds in various in vitro cell lines has made them ideal candidates for further preclinical development, which is currently ongoing. Coibamide A is a unique lariat-type cyclic depsipeptide isolated from Panamanian collections of the cyanobacterium *Leptolyngbya* sp. This compound exemplifies the high occurrence of N-methylation often observed for marine cyanobacterial secondary peptides and
depsipeptides with 8 of 11 residues featuring N-methylation.\textsuperscript{113} Coibamide A exhibits potent cytotoxicity and appears to inhibit cancer cell proliferation through a novel mechanism.\textsuperscript{113} Curacin A, isolated from a collection of \textit{Lyngbya majuscula} obtained in Curaçao, is one of a class of four marine cyanobacterial natural products featuring a cyclopropyl and a thiazoline ring, as well as a terminal alkene functionality and exhibiting antimitotic activities.\textsuperscript{114,124,125} The biosynthesis of curacin A has been well studied and recently, the mechanisms for both cyclopropyl ring formation and terminal alkene generation have been described.\textsuperscript{126-129} Desmethoxymajusculamide C is an analogue of dolastatin 11 isolated from a Fijian collection of the cyanobacterium \textit{Lyngbya majuscula}.\textsuperscript{115} Various dolastatin analogues have been shown to exhibit potent cytotoxicity owing to the disruption of actin filaments.\textsuperscript{115,130} Particularly intriguing in the case of desmethoxymajusculamide C is the fact that both the cyclic and linear forms exhibit similar levels of cytotoxicity.\textsuperscript{115} The 16-membered depsipeptide largazole, isolated from \textit{Symploca} sp., has a number of interesting structural features including a 4-methylthiazoline linked to a thiazole, as well as a 3-hydroxy-7-mercaptohept-4-enoic acid unit, and a thioester, which had previously not been observed in marine cyanobacterial natural products.\textsuperscript{116} Largazole exhibited a variety of exciting antiproliferative activities, however, it wasn’t until total synthesis of the compound had been completed that its potent class I histone-deacetylase inhibitory activity was discovered.\textsuperscript{131} One of the most intriguing features with regard to largazole is the fact that it is a pro-drug that is activated by cleavage of the octanoyl residue from 3-hydroxy-7-mercaptohept-4-enoic acid, thereby giving rise to the active free thiol group.\textsuperscript{131} Somocystinamide A is a disulfide dimer that was originally isolated from a consortium of \textit{Lyngbya majuscula} and
Schizothrix sp. collected in Fiji.\textsuperscript{117} Though the compound initially exhibited moderate cytotoxicity, recent investigations revealed that somocystinamide A selectively induces apoptosis at nanomolar levels in cancer cell lines with caspase 8 expression.\textsuperscript{118} Additionally, somocystinamide A was found to inhibit angiogenesis in zebra fish.\textsuperscript{118} These compounds represent just a few of the numerous structural archetypes found for secondary metabolites in marine cyanobacteria. While these are exciting preclinical leads in the development of therapeutic agents for the treatment of cancer, ongoing investigations will certainly result in the isolation and characterization of new chemical entities with promising biological activities and novel structural scaffolds.
1.3.3 Secondary metabolites from marine cyanobacteria exhibiting neuropharmacological activities

Until the late 1990s, cytotoxicity was virtually the only biological activity reported for marine cyanobacteria. However, in 1999, Professor William H. Gerwick in collaboration with Professor Thomas F. Murray discovered that two compounds, previously reported as ichthyotoxins, possessed ion channel modulation properties in the form of nanomolar sodium channel activating and blocking activities for antillatoxin A
and kalkitoxin, respectively.\textsuperscript{132-134} (Figure 1.11). In the years since additional compounds deriving from marine cyanobacteria including antillatoxin B,\textsuperscript{135} jamaicamides A-C,\textsuperscript{136} hoiamides A-C\textsuperscript{137,138} and alotamide A\textsuperscript{139} have been found to exhibit neuromodulatory activities. Antillatoxins A and B, isolated from collections of \textit{Lyngbya majuscula} obtained in Curaçao and Puerto Rico, respectively, are structurally novel lipopeptides featuring extensive methylation and nanomolar level activation of sodium channels.\textsuperscript{132,134,135} Recently, antillatoxin A has been found to enhance neurite outgrowth in immature cerebrocortical neurons, which could have application in the therapeutic treatment of stroke victims.\textsuperscript{140} Meanwhile, kalkitoxin is a thiazoline-containing lipid isolated from Curaçao collections of \textit{Lyngbya majuscula} that has been found to exhibit nanomolar sodium channel blocking activity and also to cause a delayed neurotoxic response in rat cerebellar granule neurons.\textsuperscript{133-134,141}

Similarly, the jamaicamides A-C, which exhibit sodium channel blocking activities in the low \(\mu\)M range, are highly functionalized lipopeptides featuring an unprecedented terminal alkynyl bromide, as well as pendent vinyl chloride moiety, a \(\beta\)-methoxy eneone system and a pyrrolinone ring.\textsuperscript{136} These compounds, isolated from a Jamaican collection of \textit{Lyngbya majuscula}, also exhibited cytotoxicity in Neuro-2a murine neuroblastoma and \textit{H-460} human lung carcinoma cell lines, as well as ichthyotoxicity and brine shrimp toxicity.\textsuperscript{136} While the biological activities were comparable in the cellular assays, jamaicamide B, was found to be the most effective ichthyotoxin, followed by jamaicamide C; however, jamaicamide C was the only jamaicamide to exhibit any brine shrimp toxicity.\textsuperscript{136} The variability observed in these...
crude lethality assays may provide some insights into the structure-activity relationships of these compounds.\textsuperscript{136}

Recently, a new series of depsipeptides known as the hoiamides have been isolated from several independent collections of marine cyanobacteria.\textsuperscript{137,138,142} Hoiamide A, the first compound described in this class, was isolated from a consortium of \textit{Lyngbya majuscula} and \textit{Phormidium gracile} collected in Papua New Guinea.\textsuperscript{137} These unique compounds feature a triheterocyclic portion with two $\alpha$-methylated thiazolines and a thiazole, as well as an extensively oxygenated and methylated polyketide fragment.\textsuperscript{137,138,142} The cyclic depsipeptide hoiamide A was found to activate sodium influx in murine neocortical neurons at low $\mu$M levels and was also discovered to be a partial agonist on site 2 of the voltage-gated sodium channel.\textsuperscript{137} Meanwhile, hoiamide B, a similar analogue isolated from a consortium of \textit{Symploca} sp. and \textit{Oscillatoria cf.} sp. collected in Papua New Guinea, also stimulated sodium influx at low $\mu$M concentrations and suppressed spontaneous calcium oscillations at nM concentrations; however, hoiamide C, which is a linear form of these depsipeptides isolated from \textit{Symploca} sp., did not exhibit any significant activity in these assays, thus suggesting that the cyclization of the compound is required for neuromodulatory activity.\textsuperscript{138} Alotamide A represents another depsipeptide from the marine cyanobacterial genus \textit{Lyngbya} with neuromodulatory properties.\textsuperscript{139} This compound possesses an unsaturated heptaketide with highly unusual methylations and oxidations.\textsuperscript{139} Alotamide A was found to produce a concentration-dependent elevation of intracellular calcium concentration and an increase in the frequency of spontaneous calcium oscillations at low $\mu$M concentration.\textsuperscript{139} The compounds described here are representative of the neuropharmacologically active
compounds reported from marine cyanobacteria to date. While several compounds exhibiting neuromodulatory activities have now been characterized from marine cyanobacteria belonging to the genus *Lyngbya*, especially from *Lyngbya majuscula*, few investigations of neuropharmacologically active compounds from other members of the Oscillatoriaceae, such as *Schizothrix* and *Symphoca* spp., have been conducted.

![Chemical structures of marine cyanobacterial natural products](image)

**Figure 1.11.** Marine cyanobacterial natural products with neuromodulatory activities.

1.3.4 Recently discovered biological activities for marine cyanobacterial compounds

Additional biological activities continue to emerge for secondary metabolites from marine cyanobacteria as the variety of assays that these compounds are tested in is expanded. One recently observed bioactivity for compounds from marine cyanobacteria
is the inhibition of nitric oxide reported for malyngamide F acetate and malyngamide 2.\textsuperscript{102,103} In the case of malyngamide F acetate, this activity was found to be due to selective inhibition of the MyD88-dependent pathway, and as such, these compounds may have relevance in the therapeutic treatment of inflammation.\textsuperscript{102} Recently, two fatty acid amides, grenadamides B and C from a Caribbean collection of \textit{Lyngbya majuscula}, were discovered to exhibit moderate insecticidal activity against the beet armyworm (\textit{Spodoptera exigua}).\textsuperscript{143} Another intriguing bioactivity reported for secondary metabolites from marine cyanobacteria is cannabinomimetic activity.\textsuperscript{144-146} Serinolamide A, a new fatty acid amide from \textit{Lyngbya majuscula}, displayed a moderate agonist effect and selectivity for the CB\textsubscript{1} cannabinoid receptor.\textsuperscript{144} Similar activity has previously been reported for a number of marine cyanobacterial natural products including the chlorosulfolipid malhamensilipin A,\textsuperscript{145} the fatty acid grenadadiene,\textsuperscript{145} and the fatty acid amides semiplenamides A, B, and G.\textsuperscript{146} Continued investigations of marine cyanobacteria from various genera belonging to the chemically rich Oscillatoriacea including \textit{Lyngbya}, \textit{Symploca}, \textit{Leptolyngbya}, \textit{Oscillatoria}, and \textit{Phormidium}, among others, will reveal new insights into the biosynthetic capabilities of marine cyanobacteria and lead to the discovery of new biologically active chemical entities for applications in human health, agriculture and other arenas.

1.4 Dissertation Contents

The primary focus of the research described herein is the isolation and characterization of secondary metabolites from neuropharmacologically active fractions derived from the marine cyanobacterial genus \textit{Symploca}. The secondary focus is the
application of organic synthesis to address issues regarding the absolute stereochemistry of secondary metabolites from marine cyanobacteria. Chapter 2 delves into the inherent difficulties in determining the absolute stereochemistry of secondary metabolites isolated in miniscule amounts. As mentioned above, one unique feature of marine cyanobacterial metabolites is the inclusion of β-hydroxy acids in depsipeptides. A vexing problem with these acids is the determination of the absolute configuration of the β-hydroxy chiral center which forms an ester linkage in the depsipeptide. As β-hydroxy acids typically deviate from commercially available amino acid and α-hydroxy acid standards, it is often difficult to determine their absolute stereochemistry. In the case of β-hydroxy acids with quaternary α centers, even the use of chiral derivatizing agents, such as Moshers’ acid or acid chloride, on the free alcohol is often problematic due to steric congestion and limited supply of the natural product. A recently developed approach utilizing chiral GCMS to compare synthetically prepared standards is an excellent solution to this perplexing problem.147 However, the synthetic route previously reported was long and laborious.148 In the research reported here, an expedient new route to obtain α,α-dimethyl-β-hydroxy acids is reported. Synthetic standards generated using this route have been implemented to determine the absolute configuration of several marine cyanobacterial secondary metabolites including mantillamide A,150 bocapeptolide A, dudawalamides A and B, radamamide A, and palmyramide A.149 Additionally, the route was proven to be effective in producing α,α-dimethyl-β-hydroxy acids with terminal alkynes.

Chapter 3 discusses the isolation and characterization of five new vinyl-chloride containing metabolites, the lipopeptides janthielamide A and kimbeamides A-C, and the
ketide-extended pyranone kimbelactone A, which have been isolated from collections of marine cyanobacteria made in Curaçao and Papua New Guinea. Phylogenetic investigations of these cyanobacteria revealed that these organisms, while closely related to the genus *Symploca*, are evolutionarily distant enough from the type strain for *Symploca* to form a distinct clade comprised of other tropical marine strains of related cyanobacteria. With regard to biological activity, the initial fractions that ultimately gave rise to janthielamide A, kimbeamides A-C and kimbelactone A each displayed similar neuropharmacological activities including sodium channel blocking in murine Neuro-2a cells and suppression of spontaneous calcium oscillations in murine neocortical neurons. The pure compounds janthielamide A and kimbeamide A exhibited moderate sodium channel blocking activity in murine Neuro-2a cells. Additionally, janthielamide A was found to antagonize veratridine-induced sodium influx in murine cerebrocortical neurons. These lipopeptides represent the newest additions to a relatively rare family of marine cyanobacterial-derived acyl amides and a new structural class of compounds exhibiting neuromodulatory activities from marine cyanobacteria.

Chapter 4 describes the isolation, structure elucidation and biological activity of a new tasihalide analogue isolated from the same collection of *Symploca* that gave rise to janthielamide A. The initial fraction containing tasihalide C exhibited calcium ion modulation activity in murine neocortical neurons; however, it did not exhibit any activity in murine Neuro-2a cells. Thus, rather than using biological activity to guide the isolation and identification of novel compounds, an approach utilizing NMR and MS data in tandem was employed. This approach yielded the known compound tasihalide B, as well
as a new compound featuring an additional acetoxy group. Although the initial fraction displayed neuropharmacological activity, the pure compound tasihalide C was inactive in the neuropharmacological assays. As no biological activity was reported for the known compounds tasihalide A and B, tasihalide C was submitted to a variety of assays to explore its biological properties. Tasihalide C was discovered to potently inhibit nitric oxide production in murine macrophages and is undergoing further evaluation to determine the mechanism behind this observed biological activity.

Lastly, chapter 5 describes the isolation, structure elucidation and biological activity of a novel lipophilic compound named palmyric acid isolated from a Palmyra collection of *Symploca*. Similar to tasihalide C, the initial fraction only exhibited activity in the calcium ion modulation assay, and as such, an NMR/MS-guided approach was again employed. Normal phase column chromatography and HPLC gave rise to a structurally intriguing lipophilic compound unprecedented in marine cyanobacteria that contains several fascinating structural features including a methyl substituted cyclopropyl ring extended with an alkene, a thiazoline functionality and a carboxylic acid moiety. This compound was found to stimulate calcium influx in neocortical neurons, albeit only modestly in comparison to 30 μM veratridine. Additional biological properties are currently being explored.

The dissertation finishes with a conclusion chapter which summarizes the research findings of each project and elaborates upon future work for these projects. Additionally, the conclusion chapter discusses briefly the inherent difficulties in determining the ultimate producer of compounds isolated from consortia and the future of
discovery and development of marine natural products as pharmaceutical lead compounds.
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2.0 CHAPTER 2

EXPEDIENT SYNTHESIS OF $\alpha,\alpha$-DIMETHYL-$\beta$-HYDROXY CARBONYL SCAFFOLDS VIA EVANS’ ALDOL REACTION WITH A TERTIARY ENOLATE

2.0.1 Abstract

An efficient synthetic methodology for 3-hydroxy-2,2-dimethyloctynoic acid (DHOYA) and several variants, which are increasingly common fragments encountered in bioactive marine cyanobacterial metabolites, was developed. These fragments were obtained in three steps via a tertiary aldol reaction utilizing an Evans’ chiral auxiliary to afford the desired stereochemistry at the $\beta$-hydroxy carbon. Thus far, this methodology has been successfully applied for determination of the absolute stereochemistry of nine cyanobacterial natural products, including the VGSC activator palmyramide A.

2.1 Introduction

The $\alpha,\alpha$-dimethyl-$\beta$-hydroxy carbonyl functionality is a unique structural feature of a number of bioactive natural products, including the epothilones,$^1$ mycalamide A,$^2$ and peloruside A.$^3$ In 1996, a novel depsipeptide named kulolide-1 featuring an unprecedented 3-hydroxy-2,2-dimethyloctynoic acid fragment (DHOYA, 1, see Figure 2.1) was isolated from a marine cephalaspidean mollusk (Philinopsis speciosa).$^4$ Recently a number of marine cyanobacterial natural products possessing an $\alpha,\alpha$-dimethyl-$\beta$-hydroxy carbonyl functionality in the form of fragment 1, or a derivative thereof, have been described, including wewakpeptins A-D,$^5$ mantillamide A,$^6$ dudawalamide A,$^6$ guineamide G,$^7$ cocosamides A and B,$^8$ pitipeptolides C-F,$^9$ palmyramide A,$^{10}$ and
pitiprolamide,\textsuperscript{11} among others. Naturally occurring modifications of the structural motif represented by 1 include varying degrees of saturation, chain shortening, replacement of the β-hydroxy group by an amine, and mono-methylation at the α-carbon. Other derivatives of 1 feature multiple modifications; examples include antanapeptin B,\textsuperscript{12} onchidin,\textsuperscript{13} and malevamide B.\textsuperscript{14} Natural products featuring 1 or a variant on this motif display a variety of biological activities including cytotoxicity to a number of cancer cell lines,\textsuperscript{4,5,8,15} anti-microbial activity to mycobacteria,\textsuperscript{9,15} anti-parasitic activity\textsuperscript{16} and brine shrimp toxicity.\textsuperscript{7,17,18} In addition, pitipeptolide A, which exhibits both cytotoxic and antimycobacterial properties, has been discovered to have an ecological role as a feeding deterrent for both generalized and specialized grazers.\textsuperscript{19} Given the range of biological activities of these molecules and the diversity of the modifications observed for fragment 1, there is a compelling need for efficient synthetic methodologies to access this structural class as well as produce synthetic analogues for drug development purposes.
Figure 2.1. 3-hydroxy-2,2-dimethyloctynoic acid (1) and related fragments along with two representative compounds featuring \( \alpha,\alpha \)-dimethyl-\( \beta \)-hydroxy carbonyl functionalities. Fragments 2-9 are moieties related to 1 commonly observed in depsipeptides from marine cyanobacteria. Exemplary compounds featuring an \( \alpha,\alpha \)-dimethyl-\( \beta \)-hydroxy carbonyl functionality include kulolide-1 (10), isolated from a marine mollusk, and epothilone B (11), isolated from a myxobacterium.

Moreover, one of the more difficult aspects of the structure elucidation of depsipeptides containing 1 is the determination of absolute configuration of the \( \beta \)-hydroxy ester linkage of fragment 1. Chiral GCMS comparison of the hydrogenated and methyl esterified derivative of 1 (i.e. methyl 3-hydroxy-2,2-dimethyloctanoate) to synthetic standards affords a pragmatic approach for determining the absolute configuration of this stereocenter.\(^5\) However, a critical requirement for this methodology is the availability of enantiomerically pure synthetic standards.
Fragment 1 has previously been synthesized in 9 steps using an asymmetric Kiyooka-Muikaiyama aldol reaction during total synthesis of yanucamide A\textsuperscript{20} and pitipeptolide A\textsuperscript{21} and to provide standards for chiral GCMS analysis of the wewakpeptins\textsuperscript{5} (Scheme 2.1). Additional approaches for construction of the α,α-dimethyl-β-hydroxy carbonyl backbone via a tertiary enolate have been applied in partial and total syntheses of peloruside A,\textsuperscript{22,23} and in the total synthesis of pasteurestins A and B;\textsuperscript{24} in the latter case, a Reformatsky reaction employing a bromoacyl derivative of Evans’ chiral auxiliary and a TMS-protected terminal alkyne was employed.

Scheme 2.1. Synthetic route to furnish fragment 1 in the total syntheses of yanucamide A\textsuperscript{20} and pitipeptolide A.\textsuperscript{21}

However, we conceived a more efficient synthetic route in which fragment 1 could be achieved enantioselectively via a tertiary aldol reaction with an acylated Evans’ chiral auxiliary and an aldehyde possessing an unprotected terminal alkyne. As the terminal alkyne of fragment 1 is unstable to acid hydrolysis, which is usually the first step in the stereochemical determination of depsipeptides, saturated 3-hydroxy-2,2-
dimethyloctanoic acid (DHOAA, 3) is preferred for chiral GCMS analysis. Thus, our initial studies focused on the synthesis of fragment 3.

2.2 Results and Discussion

2.2.1 Initial results and validation of the proposed methodology

Since the key reaction step in this scheme requires an Evans’ chiral auxiliary that is not commercially available, the desired starting material was obtained via acylation of (R)-4-benzyl-2-oxazolidinone with isobutyryl chloride to give (R)-4-benzyl-3-isobutyryloxazolidin-2-one (12) in good yield (86%). Initially, we attempted the aldol reaction of (4′R)-12 and hexanal using LDA alone; however, the expected aldol product was not formed based on LR-ESI-LCMS and 1H NMR analyses. It was suspected that a stronger Lewis acid was required for formation and stabilization of the tertiary enolate. A titanium Lewis acid was initially selected as it was thought that the titanium enolate would best facilitate the coordination of the aldehyde via the Zimmerman-Traxler transition state (see Table 2.1). A similar strategy has been employed previously by the Kobayashi group in which LDA was used in combination with TiCl(O-i-Pr)3 to form the titanium enolate in preparation of a 1,2-diol with a quaternary chiral center.
Scheme 2.2. Optimized reaction conditions for generation of fragments 1 and 3 via tertiary aldol reaction.

Thus, we attempted the aldol addition of hexanal (1.2 equiv) and (4′R)-12 using LDA (1.5 equiv) and TiCl(O-i-Pr)₃ (4 equiv). The reaction was stirred for 1.5 h at −40°C and proceeded favorably to give (3R,4′R)-13 in 35% yield (Table 2.1, entry 1). The configuration of the β-alcohol in this intermediate was confirmed as R by Mosher’s analysis, which was consistent with the predicted Zimmerman-Traxler transition state model. The Evans’ chiral auxiliary was removed via hydrolysis to furnish (3R)-3 with an overall yield of 29%. The synthesis was also conducted starting with (S)-4-benzyl-2-oxazolidinone. The aldol reaction with (4′S)-12 gave (3S,4′S)-13 with a 32% yield (Table 2.1, entry 2), while hydrolysis afforded the final product (3S)-3 with an overall yield of 24%.

These initial results verified that this three step synthesis was indeed a viable route for the generation of (3R)-3 and (3S)-3. These products are invaluable for use as standards in the chiral GCMS analysis of the β-hydroxy stereocenter of marine natural
products featuring 1 or a saturated derivative. One such application was in the case of mantillamide A (Figure 2.2, 19) where 5.2 mg was isolated from field collections; however, only 0.2 mg of the natural product was required for chiral GCMS analysis. Comparison of the methyl esterified fragment derived from mantillamide A to methyl esterified (3R)-3 and (3S)-3 obtained using the described synthetic route revealed that mantillamide A possesses an R-DHOYA unit. The retention time for the natural product was 53.67, while the retention time for the R-enantiomer was 53.64 and the retention time for the S-enantiomer was 52.08. Co-injections of the natural product with the R- and S-enantiomers confirmed the assignment as R. In addition to mantillamide A, determination of the absolute configurations of the β-ester linkages of two novel marine cyanobacterial natural products radamamide A (20)\textsuperscript{27} and bocapeptolide A (21)\textsuperscript{28} has been completed by the author. Furthermore, the stereochemistry of this β-hydroxy chiral center has been solved for another six compounds by other investigators within the Gerwick laboratory using the standards generated herein.
2.2.2. Optimization and examination of the versatility of the tertiary aldol reaction

Table 2.1. Optimization of aldol reaction in the synthesis of 3.

<table>
<thead>
<tr>
<th>Entry</th>
<th>S.M.</th>
<th>Scale (^b)</th>
<th>Aldehyde (equiv)</th>
<th>Temperature conditions</th>
<th>Product (^c)</th>
<th>Yield (^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(4’R)-12</td>
<td>3.8</td>
<td>1.2</td>
<td>-40°C, 1.5 h</td>
<td>(3R,4’R)-13</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>(4’S)-12</td>
<td>1.7</td>
<td>1.2</td>
<td>-40°C, 1 h</td>
<td>(3S,4’S)-13</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>(4’S)-15</td>
<td>0.2</td>
<td>1.2</td>
<td>-40°C, 3 h</td>
<td>(3S,4’S)-16</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>(4’S)-15</td>
<td>0.1</td>
<td>1.2</td>
<td>-78°C→22°C, 18 h</td>
<td>(3S,4’S)-16</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>(4’S)-15</td>
<td>0.2</td>
<td>3.0</td>
<td>-40°C, 3 h</td>
<td>(3S,4’S)-16</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>(4’S)-15</td>
<td>0.2</td>
<td>3.0</td>
<td>-40°C, 3 h; 0°C, 2 h</td>
<td>(3S,4’S)-16</td>
<td>98(^d)</td>
</tr>
<tr>
<td>7</td>
<td>(4’S)-15</td>
<td>0.2</td>
<td>3.0</td>
<td>-40°C, 3 h; 0°C, 2 h</td>
<td>(3S,4’S)-16</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>(4’R)-15</td>
<td>0.2</td>
<td>3.0</td>
<td>-40°C, 3 h; 0°C, 2 h</td>
<td>(3R,4’R)-16</td>
<td>82</td>
</tr>
</tbody>
</table>

\(^{a}\)S.M.: starting material.
\(^{b}\)Scale based on mmol of starting material.
\(^{c}\)Isolated yield following flash column chromatography.
\(^{d}\)Enantiomeric ratio ≥ 50:1 according to \(^1^H\)NMR and chiral GCMS analyses.

While the route was efficient for preparation of standards for stereochemical analyses, the overall yield was quite low. Thus, optimization of the aldol reaction was undertaken to improve the overall yield making the route more attractive for utilization in total syntheses. Suspecting that an additional deprotonation may be occurring on C-5, resulting in decomposition of the starting material and, therefore, contributing to the
observed low yield, an Evans’ chiral auxiliary [(4′S)-14], featuring *gem*-dimethyl substitution at C-5, was employed as the starting material in subsequent reactions. Acylation of (4′S)-14 gave (4′S)-15 (97% yield), which was then utilized in the aldol reaction, and resulted in an increase in yield from 32% for (3S,4′S)-13 to 41% for (3S,4′S)-16 (entry 3). In subsequent reactions, 1M solutions in THF were prepared from neat TiCl(O-i-Pr)₃ due to concerns that hexanes present in the commercial 1M preparation may be adversely affecting solvation of the enolate. Additionally, the amount of aldehyde was increased from 1.2 to 3 equivalents and the reaction time was adjusted to 3 h, resulting in an increase in yield from 41% to 58% (entry 5). Next, the final temperature was increased to 0°C after 3 h and the reaction was run for an additional 2 h, resulting in an excellent yield of 98% (entry 6). While ¹H NMR analysis of the crude aldol product did not indicate the presence of diastereomers, the aldol product was hydrolyzed to give (3S)-3, which was methyl esterified and analyzed by chiral GCMS analysis. The enantiomeric ratio was determined as 50:1 for the desired enantiomer based on peak area. Repetition of these favorable aldol reaction conditions affirmed the observed result and high yield (a 91% yield was achieved, entry 7). Subsequently, the aldol reaction was conducted with (4′R)-15 and an 82% yield was obtained (entry 8, see also Scheme 2.2).
Table 2.2. Tertiary aldol reaction with additional aldehydes.

<table>
<thead>
<tr>
<th>Entry</th>
<th>S.M. a</th>
<th>Aldehyde b</th>
<th>Temperature conditions</th>
<th>Product</th>
<th>Yield c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(4'S)-15</td>
<td>Butanal</td>
<td>−40°C, 3 h</td>
<td>(3S,4'S)-17</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>(4'R)-15</td>
<td>Butanal</td>
<td>−40°C, 3 h</td>
<td>(3R,4'R)-17</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>(4'S)-15</td>
<td>5-hexynal</td>
<td>−40°C, 3 h</td>
<td>(3S,4'S)-18</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>(4'S)-15</td>
<td>5-hexynal</td>
<td>−40°C, 3 h; 0°C, 2 h</td>
<td>(3S,4'S)-18</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>(4'R)-15</td>
<td>5-hexynal</td>
<td>−40°C, 3 h; 0°C, 2 h</td>
<td>(3R,4'R)-18</td>
<td>74</td>
</tr>
</tbody>
</table>

a S.M.: starting material.
b 1.2 equivalents of aldehyde were used for entry 3, whereas 3 equivalents of aldehyde were used for all other entries.
c Isolated yield following flash column chromatography.

Additional reactions substituting butanal for hexanal were conducted to provide (S)- and (R)-3-hydroxy-2,2-dimethylhexanoic acid (DMHHA, 8) for use as standards for chiral analysis of the β-hydroxy stereocenter in palmyramid A (22), a natural product which possesses the DMHHA residue. In both of these reactions, 3 equiv of aldehyde were used and the final reaction temperature was −40°C. The S-enantiomer was obtained in 54% yield (Table 2.2, entry 1), while the R-enantiomer was obtained in 35% yield (entry 2). Comparison of the yield for the S-enantiomer from this aldol reaction to its two carbon extended counterpart reveals that the yields are not significantly different, 54%
for the former compared to 58% for the latter, suggesting that chain length does not substantially impact yield.

Having successfully developed a route for generating synthetic (3R)- and (3S)-3, as well as (3R)- and (3S)-8, as standards for stereochemical determination at the β position of fragment 1 and 8, we wanted to explore the applicability of this route for generation of chiral fragment 1 itself. Thus, the aldol reaction was conducted with (4'S)-15 utilizing 1.2 equiv of 5-hexynl-1-al (prepared from 5-hexyn-1-ol according to Böttcher and Sieber)\textsuperscript{30} with the final temperature at −40°C for 3 h, resulting in a 27% yield (Table 2.2, entry 3) as compared to a 41% yield under the same conditions using hexanal (Table 2.1, entry 3). Due to the considerable lability of the alkynyl aldehyde, in comparison with the saturated aldehyde, a lower yield was expected for this reaction; however this result revealed that the reaction conditions were amenable for use with unprotected alkynyl aldehydes. Using these improved reaction conditions with 3 equiv of aldehyde and increasing the final temperature to 0°C, the aldol reactions were conducted with 5-hexyn-1-al and both (4'S)- and (4'R)-15 to give yields of 58% and 74%, respectively (Table 2.2, entries 4 and 5).

Next, it was verified that the hydrolysis conditions were suitable for use with these various substrates to provide the desired free acids. Hydrolysis of (3R,4'R)-16 and (3S,4'S)-16 with LiOH and H\textsubscript{2}O\textsubscript{2} gave the desired products with a 68% yield for (3R)-3 and an 87% yield for (3S)-3, resulting in an overall yield of 52% for the R-enantiomer and 83% for the S-enantiomer (Scheme 2.2). This is a substantial increase in yield in comparison with the initial yields of 29% for (3R)-2 and 24% for (3S)-2. Hydrolysis of the aldol products featuring a terminal alkyne afforded (3S)-1 with a yield of 84% (47%
overall yield) and (3R)-1 with a yield of 81% (56% overall yield). This represents a significant improvement in the efficiency and yield for the synthesis of fragment 1 compared to previously published synthesis.\textsuperscript{20,21}

\textbf{Figure 2.2.} Four novel marine cyanobacterial secondary metabolites featuring fragment 1 or 8. Absolute configuration of the β-ester linkage shown as determined based on chiral GCMS analyses using standards generated by the methodology described herein.

2.3 Conclusions

In summary, a new synthetic route for the α,α-dimethyl-β-hydroxy carbonyl motif exemplified by fragment 1 has been developed. This represents a significant advance in methodology for the total synthesis of compounds featuring fragment 1 or related
residues. To date, the absolute configurations of the β-ester linkages of ten compounds isolated by various members of the Gerwick laboratory featuring either fragment 1 or 8 have been determined using standards generated from this research. Future directions of this research include exploring the utility of thiazolidinethiones and oxazolidinethiones to afford the desired (R) and (S) aldol products from a single chiral auxiliary and total synthesis of biologically active cyanobacterial secondary metabolites featuring fragment 1.
2.4 CHAPTER 2

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Author Contributions

JKN performed research. JKN, TLS, RGL, and WHG designed research and wrote the manuscript.

The authors declare no conflicts of interest.

Chapter 2, in part, includes a reprint as it appears in Tetrahedron Letters 2011, 52(23): 2929-2932. Joshawna K. Nunnery, Takashi L. Suyama, Roger G. Linington and William H. Gerwick. The dissertation author was the primary investigator and author of this paper.
2.5 CHAPTER 2

APPENDIX

General

Unless specified otherwise, all reagents and materials were purchased from commercially available sources and were used without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Diisopropylamine was distilled from CaH₂. Isobutryl chloride was distilled from quinoline (10:1). (R)-(+) and (S)-(−)-4-Benzyl-5,5-dimethyl-2-oxazolidinone (Aldrich, 98%) were recrystallized from EtOAc/hexanes prior to use. All reactions were conducted under dry argon atmosphere unless otherwise noted. Reaction temperatures reported herein are external temperatures. Flash chromatography was carried out on EMD Chemicals silica gel 60 (230-400 mesh). Thin layer chromatography (TLC) was performed on EMD Chemicals pre-coated silica gel plates (Merck 60 F₂₅₄). Optical rotations were measured on a Jasco P-2000 polarimeter. IR spectra were recorded on a Thermo Scientific Nicolet IR100 FT-IR spectrometer using a KBr disk. ¹H NMR and ¹³C NMR spectra were obtained on a 300 MHz Varian Inova spectrometer (300 MHz and 75 MHz, respectively) or a 500 MHz Varian Inova spectrometer (500 MHz and 125 MHz, respectively) in CDCl₃ using TMS (δH:0.00) or residual chloroform (δH:7.26, δC:77.16) as the internal standard (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet). High resolution mass spectra were recorded on either a Thermo Scientific LTQ-Orbitrap-XL mass spectrometer or a ThermoFinnigan MAT 900 XL mass spectrometer. HPLC was carried out using a Waters 515 pump system with a Waters 996 PDA detector. Enantiomeric ratio was determined by chiral GCMS on a Thermo Electron Trace GC Ultra DSQ mass spectrometer using a J & W Scientific Cyclosil B column (30 m × 0.25 mm).
Compound (4'R)-12: (R)-(−)-4-benzyl-oxazolidin-2-one (0.919 g, 5.19 mmol) was dissolved in dry THF (13.4 mL) and treated with n-BuLi (1.03 M in hexanes, 5.05 mL, 5.20 mmol, 1 equiv) dropwise at -78°C. After 1 hr, isobutyryl chloride (1.36 mL, 12.98 mmol, 2.5 equiv) in THF (8.32 mL) was added at -78°C. After stirring for 1 hr at -78°C, the reaction mixture was quenched with water (13.4 mL). The reaction mixture was extracted with Et₂O (3 × 45 mL), dried over MgSO₄, and concentrated in vacuo. The product was purified by flash chromatography (15% to 20% Et₂O in hexanes), yielding the title compound as a crystalline solid (0.860 g, 4.46 mmol, 86% yield): analytical TLC \( R_f = 0.36, 40\% \) Et₂O in hexanes; \([\alpha]_D^{21} = -57.4^\circ \) (c 2.0, CHCl₃); IR \( \nu_{\text{max}} \) (film): 3065-2877, 1786, 1698, 1454, 1381, 1295, 1208, 1107, 1048, 973, 736, 504 cm⁻¹; \(^1\)H NMR (300 MHz, CDCl₃) \( \delta \) 7.39-7.21 (m, 5H, H-8'-H-12'), 4.70 (m, 1H, H-4'), 4.26-4.16 (m, 2H, H-5'), 3.79 (septet, \( J = 6.8 \) Hz, 1H, H-2), 3.28 (dd, \( J = 13.3, 3.1 \) Hz, 1H, H-5'), 2.80 (dd, \( J = 8.6 \) Hz, 1H, H-6'), 1.27 (d, \( J = 6.8 \) Hz, 3H, H-3 or H-4), 1.21 (d, \( J = 6.6 \) Hz, 3H, H-3 or H-4); \(^1\)C NMR (75 MHz, CDCl₃) \( \delta \) 177.7 (C, C-1), 153.1 (C, C-2'), 135.4 (C, C-7'), 129.5 (2CH, C-8', C-12'), 129.0 (2CH, C-9', C-11'), 127.3 (CH, C-10'), 66.1 (CH₂, C-5'), 55.3 (CH, C-4'), 38.0 (CH₂, C-6'), 32.7 (CH, C-2), 19.3 (CH₃, C-3 or C-4), 18.7 (CH₃, C-3 or C-4); HR EIMS \( m/z \) 247.1200 [M⁺] (calcd. for C₁₄H₁₇NO₃, 247.1203).

Compound (4'S)-12: Oxazolidinone (4'S)-12 was prepared in the same was as oxazolidinone (4'R)-12 from the l-phenylalanine-derived (S)-(−)-4-benzyl-oxazolidin-2-one (71% yield): analytical TLC \( R_f = 0.35, 40\% \) Et₂O in hexanes; \([\alpha]_D^{21} = +57.2^\circ \) (c 1.0, CHCl₃); IR \( \nu_{\text{max}} \) (film): 3065-2880, 1785, 1700, 1456, 1381, 1296, 1210, 1111, 1049, 974, 737, 696, 505 cm⁻¹; \(^1\)H NMR (300 MHz, CDCl₃) \( \delta \) 7.38-7.21 (m, 5H, H-8'-H-12'), 4.69 (m, 1H, H-4'), 4.25-4.15 (m, 2H, H-5'), 3.78 (septet, \( J = 6.8 \) Hz, 1H, H-2), 3.28 (dd, \( J = 13.3, 3.1 \) Hz, 1H, H-6'), 2.80 (dd, \( J = 13.2, 9.4 \) Hz, 1H, H-6'), 1.26 (d, \( J = 6.8 \) Hz, 3H, H-3 or H-4), 1.21 (d, \( J = 6.6 \) Hz, 3H, H-3 or H-4); \(^1\)C NMR (75 MHz, CDCl₃) \( \delta \) 177.6 (C, C-1), 153.1 (C, C-2'), 135.4 (C, C-7'), 129.5 (2CH, C-8', C-12'), 128.9 (2CH, C-9', C-11'), 127.3 (CH, C-10'), 66.1 (CH₂, C-5'), 55.3 (CH, C-4'), 37.9 (CH₂, C-6'), 32.6 (CH, C-2), 19.3 (CH₃, C-3 or C-4), 18.7 (CH₃, C-3 or C-4); HR EIMS \( m/z \) 247.1205 [M⁺] (calcd. for C₁₄H₁₇NO₃, 247.1203).
Compound (3R,4'R)-13: Oxazolidinone (4'R)-12 (0.936 g, 3.79 mmol) was dissolved in dry THF (30 mL) and added dropwise to a solution of LDA (1.5 equiv) in dry THF (91 mL) at –78°C [prepared by addition of 1.22 M n-BuLi (4.67 mL, 5.70 mmol, 1.5 equiv) to a solution of diisopropylamine (844 µL, 6.02 mmol, 1.6 equiv) in dry THF at –78°C, the solution was warmed to 0°C for 15 min, then cooled to –78°C]. After 30 min at –78°C, chlorotriisopropoxytitanium IV (1.0 M in hexanes, 15.15 mL, 15.15 mmol, 4 equiv) was added dropwise, and the reaction mixture was warmed to –40°C. After 1.5 hr, the mixture was cooled to –78°C, hexanal (546 µL, 4.55 mmol, 1.2 equiv) was added, and the reaction mixture was warmed to –40°C. After 1 hr at –40°C, the reaction was quenched with satd NH₄Cl (90 mL) and stirred with Celite® while warming to room temperature. The filtrate was extracted with EtOAc (3 x), washed with brine, dried over MgSO₄, and concentrated in vacuo. The product was purified by flash chromatography (DCM/hexanes/MeCN, 49.5:49.5:1 to 49:49:2), yielding the title compound as a pale yellow gummy oil (0.456 g, 35% yield): analytical TLC Rf = 0.33, DCM/hexanes/MeCN (48.5:48.5:3); [α]D²¹ = -15.6° (c 1.0, CHCl₃); IR v max (film): 3534, 2930, 2863, 1779, 1685, 1458, 1386, 1349, 1258, 1191, 1108, 943, 738, 704 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.40-7.24 (m, 5H, H-8′-H-12′), 4.73 (m, 1H, H-4′), 4.28-4.14 (m, 3H, H-3, H-5′), 3.30 (dd, J = 13.2, 3.2 Hz, 1H, H-6′), 2.78 (dd, J = 13.2, 10.0, 1H, H-6′), 2.46 (bs, 1H, H-11), 1.78-1.08 (m, 8H, H-4, H-5, H-6, H-7), 1.42 (s, 3H, H-9 or H-10), 1.38 (s, 3H, H-9 or H-10), 0.93 (t, J = 6.5 Hz, 3H, H-8); ¹³C NMR (75 MHz, CDCl₃) δ 178.3 (C, C-1), 152.4 (C, C-2), 135.7 (C, C-7), 129.5 (2CH, C-8′, C-12′), 129.0 (2CH, C-9′, C-11′), 127.4 (CH, C-10′), 75.6 (CH, C-3), 66.4 (CH₂, C-5′), 57.9 (CH, C-4′), 50.3 (C, C-2), 37.9 (CH₂, C-6′), 31.9 (CH₂, C-6), 31.4 (CH₂, C-4), 26.7 (CH₂, C-5), 22.8 (CH₂, C-7), 20.5 (CH₃, C-9 or C-10), 18.8 (CH₃, C-9 or C-10), 14.2 (CH₃, C-8); HR EIMS m/z 347.2094 [M]+ (calcd. for C₂₀H₂₀NO₄, 347.2091).

Compound (3S,4'S)-13: The title compound was prepared in the same way as (3R,4'R)-13 from (4'S)-12 except that it was stirred for only 1 hr following addition of the aldehyde (32% yield): analytical TLC Rf = 0.33, DCM/hexanes/MeCN (48.5:48.5:3); [α]D²³ = +14.5° (c 1.0, CHCl₃); IR v max (film): 3536, 2929, 2862, 1779, 1686, 1459, 1386, 1349, 1258, 1191, 1108, 945, 740, 705 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.41-7.24 (m, 5H, H-8′-H12′), 4.74 (m, 1H, H-4′), 4.28-4.15 (m, 3H, H-3, H-5′), 3.31 (dd, J = 13.2, 3.2 Hz, 1H, H-6′), 2.79 (dd, J = 13.4, 9.8 Hz, 1H, H-6′), 2.37 (bs, 1H, H-11), 1.76-1.09 (m, 8H, H-4-H-7), 1.43 (s, 3H, H-9 or H-10), 1.38 (s, 3H, H-9 or H-10), 0.94 (t, J = 6.5 Hz, 3H, H-8); ¹³C NMR (75 MHz, CDCl₃) δ 178.3 (C, C-1), 152.4 (C, C-2), 135.7 (C, C-7), 129.6 (2CH, C-8′, C-12′), 129.1 (2CH, C-9′, C-11′), 127.4 (CH, C-10′), 75.7 (CH, C-3), 66.4 (CH₂, C-5′), 57.9 (CH, C-4′), 50.3 (C, C-2), 32.0 (CH₂,
C-6), 31.5 (CH2, C-4), 26.7 (CH2, C-5), 22.8 (CH2, C-7), 20.5 (CH3, C-9 or C-10), 18.8 (CH3, C-9 or C-10), 14.2 (CH3, C-8); HR EIMS m/z 347.2096 [M]+ (calcd. for C20H29NO4, 347.2091).

Compound (3R,4'R)-13 (S)-MTPA ester: To a solution of (3R,4'R)-13 (9.5 mg, 0.027 mmol) in DCM (1.8 mL) were added Et3N (19 μL, 0.137 mmol), R-MTPA chloride (25.5 μL, 0.137 mmol), and DMAP (3.6 mg, 0.030 mmol). After 16 hr stirring at room temperature, the reaction mixture was diluted with Et2O (10 mL) and 0.5 M HCl (10 mL). The solution was extracted with Et2O (3 × 10 mL), washed with H2O (10 mL), satd NaHCO3 (10 mL), brine (10 mL) and dried over Na2SO4. The product was purified by RP-HPLC to yield the S-MTPA ester (5.6 mg, 36% yield): [α]D23 = -4.0° (c 0.43, CHCl3); IR νmax (film): 2951, 2865, 1782, 1746, 1688, 1585, 1384, 1348, 1266, 1182, 1112, 1016, 721 cm⁻¹; 1H NMR (500 MHz, CDCl3) δ 7.57 (d, J = 3.9, 1.2 Hz, 2H, H-18, H-22), 7.37 (m, 3H, H-19, H-20, H-21), 7.33 (m, 2H, H-9', H-11'), 7.26 (m, 1H, H-10'), 7.21 (d, J = 7.8 Hz, 2H, H-8', H-12'), 6.20 (dd, J = 8.5, 4.2, 3.9 Hz, 1H, H-3), 4.55 (octet, J = 6.7, 3.3 Hz, 1H, H-4'), 4.17-4.11 (m, 2H, H-5'), 3.54 (s, 3H, H-7'), 2.68 (dd, J = 13.3, 10.7, 10.5 Hz, 1H, H-6'), 1.69-1.58 (m, 2H, H-4), 1.41 (s, 3H, H-9 or H-10), 1.36-1.30 (m, 2H, H-6), 1.29 (s, 3H, H-9 or H-10), 1.28-1.18 (m, 4H, H-5, H-7), 0.86 (t, J = 7.0 Hz, 3H, H-8); 13C NMR (125 MHz, CDCl3) δ 175.9 (C, C-1), 166.4 (C, C-12), 152.7 (C, C-2), 136.2 (C, C-7), 132.4 (C, C-17), 129.7 (CH, C-20), 129.5 (2CH, C-8', C-12), 129.1 (2CH, C-9', C-11'), 128.4 (2CH, C-19, C-21), 127.7 (2CH, C-18, C-22), 127.3 (CH, C-10'), 123.6 (q, J = 289.3 Hz, C, C-14), 94.6 (q, J = 27.7 Hz, C, C-13), 78.8 (CH, C-3), 66.6 (CH2, C-5'), 58.3 (CH, C-4'), 55.5 (CH3, C-16), 49.4 (C, C-2), 37.5 (CH2, C-6'), 31.6 (CH2, C-6), 29.7 (CH2, C-4), 25.9 (CH2, C-5), 22.6 (CH2, C-7), 20.7 (CH3, C-9 or C-10), 19.9 (CH3, C-9 or C-10), 14.1 (CH3, C-8); HR EIMS m/z 563.2495 [M]+ (calcd. for C30H56NO4F3, 563.2489).

Compound (3R,4'R)-13 (R)-MTPA ester: The same procedure employed for the synthesis of the S-MTPA ester of (3R,4'R)-13 was utilized with S-MTPA chloride to furnish the R-MTPA ester (47% yield): [α]D23 = +41.3° (c 0.62, CHCl3); IR νmax (film): 2952, 2863, 1782, 1743, 1686, 1456, 1384, 1348, 1266, 1182, 1112, 1016, 721 cm⁻¹; 1H NMR (500 MHz, CDCl3) δ 7.57 (d, J = 6.8 Hz, 2H, H-18, H-22), 7.34 (m, 3H, H-19, H-20, H-21), 7.31 (d, J = 7.3 Hz, 2H, H-9', H-11'), 7.26 (m, 1H, H-10'), 7.21 (d, J = 7.8 Hz,
2H, H-8′), H-12′), 6.36 (t, J = 6.3 Hz, 1H, H-3), 4.55 (m, 1H, H-4′), 4.13 (d, J = 4.4 Hz, 2H, H-5′), 3.61 (s, 3H, H-16), 3.23 (dd, J = 13.6. 2.8 Hz, 1H, H-6′), 2.64 (dd, J = 13.3, 11.4 Hz, 1H, H-6′), 1.60-1.55 (m, 2H, H-4), 1.43 (s, 3H, H-9 or H-10), 1.34 (s, 3H, H-9 or H-10), 1.24 (m, 1H, H-6), 1.16 (m, 2H, H-7), 1.13 (m, 1H, H-6), 1.06 (m, 2H, H-5), 0.81 (t, J = 6.8 Hz, 3H, H-8); ^13^C NMR (75 MHz, CDCl$_3$) $\delta$ 176.0 (C, C-1), 166.7 (C, C-12), 152.8 (C, C-2′), 136.5 (C, C-7′), 132.4 (C, C-17), 129.7 (CH, C-20), 129.5 (2CH, C-8′, C-12′), 129.0 (2CH, C-9′, C-11′), 128.3 (2CH, C-19, C-21), 127.5 (2CH, C-18, C-22), 127.2 (CH, C-10′), 125.4 (C, C-14), 84.6 (C, C-13), 78.2 (CH, C-3), 66.5 (CH$_2$, C-5′′), 58.5 (CH, C-4′), 55.9 (CH$_3$, C-16), 49.5 (C, C-2), 37.0 (CH$_2$, C-6′), 31.4 (CH$_2$, C-6), 29.0 (CH$_2$, C-4), 25.5 (CH$_2$, C-5), 22.5 (CH$_2$, C-7), 20.9 (CH$_3$, C-9 or C-10), 20.7 (CH$_3$, C-9 or C-10), 14.1 (CH$_3$, C-8); HR EIMS $m/z$ 563.2494 [M]$^+$ (calcd. for C$_{30}$H$_{36}$NO$_6$F$_3$, 563.2489).

![Chemical Structure](image)

**Compound (3R)-3:** A solution of 30% aqueous H$_2$O$_2$ (260 μL, 2.29 mmol, 3.62 equiv) was added dropwise to a solution of (3R,4′R)-13 (0.220 g, 0.63 mmol) in 4:1 THF:H$_2$O (5.5 mL) at 0°C. LiOH·H$_2$O (0.0430 g, 1.025 mmol, 1.6 equiv) in H$_2$O (1.2 mL) was added to the reaction mixture. After 1 hr, Na$_2$SO$_3$ (0.332 g, 2.63 mmol) was added and THF was removed from the slurry under vacuum. The residue was partitioned between DCM (3 × 20 mL) and H$_2$O. The aqueous layers were collected and acidified to pH 1 with 1 N HCl. The aqueous layer was extracted with Et$_2$O (3 × 20 mL), and the combined organic layers were dried over MgSO$_4$. The solvents were removed in vacuo and the residue was purified by RP-HPLC (to remove minor impurities) to yield the title compound as a colorless oil (0.108 g, 90% yield): [α]$_D$= +26.3° (c 2.14, CHCl$_3$); IR $\nu_{\text{max}}$ (film): 3415, 2939, 2866, 1704, 1466, 1401, 1270, 1170, 1117, 1073, 932 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.27 (bs, 1H, COOH), 3.67 (d, J = 8.1 Hz, 1H, H-3), 1.73-1.27 (m, 8H, H-4, H-5, H-6, H-7), 1.24 (s, 3H, H-9 or H-10), 1.21 (s, 3H, H-9 or H-10), 0.91 (t, J = 6.7 Hz, 3H, H-8); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.4 (C, C-1), 76.8 (CH, C-3), 47.2 (C, C-2), 31.9 (CH$_2$, C-4), 31.7 (CH$_2$, C-6), 26.4 (CH$_2$, C-5), 22.7 (CH$_2$, C-7), 22.5 (CH$_3$, C-9 or C-10), 20.3 (CH$_3$, C-9 or C-10), 14.1 (CH$_3$, C-8); HR EIMS $m/z$ 188.1409 [M]$^+$ (calcd. for C$_{10}$H$_{20}$O$_3$, 188.1407).

**Compound (3S)-3:** Carboxylic acid (3S)-3 was synthesized using the same procedure as (3R)-3 from (3S,4′S)-13 except that the carboxylic acid was obtained as a pure compound without further purification following extraction with Et$_2$O (98% yield): [α]$_D$= -29.0° (c 1.26, CHCl$_3$); IR $\nu_{\text{max}}$ (film): 3414, 2937, 2866, 1704, 1466, 1401, 1270, 1171, 1118, 1073, 932 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.95 (bs, 1H, COOH), 3.67 (d, J = 8.5 Hz, 1H, H-3), 1.78-1.28 (m, 8H, H-4, H-5, H-6, H-7), 1.25 (s, 3H, H-9 or H-10), 1.21 (s, 3H, H-9 or H-10), 0.92 (t, J = 6.7 Hz, 3H, H-8); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.3 (C, C-1), 76.8 (CH, C-3), 47.2 (C, C-2), 31.9 (CH$_2$, C-4), 31.7 (CH$_2$, C-
6), 26.4 (CH₂, C-5), 22.8 (CH₂, C-7), 22.5 (CH₃, C-9 or C-10), 20.3 (CH₃, C-9 or C-10), 14.2 (CH₃, C-8); HR ESIMS m/z 211.1306 [M + Na]⁺ (calcd. for C₁₀H₁₈O₃Na, 211.1305).

**Compound (4'R)-15:** (R)-(+)‐4‐benzyl‐5,5‐dimethyl‐2‐oxazolidinone (0.299 g, 1.46 mmol) was dissolved in dry THF (3.7 mL) and treated with n‐BuLi (1.45 M in hexanes, 1.01 mL, 1.46 mmol, 1 equiv) dropwise at -78°C. After 1 hr, isobutyryl chloride (382 μL, 3.65 mmol, 2.5 equiv) in THF (2.4 mL) was added at -78°C. After stirring for 1 hr at -78°C, the reaction mixture was quenched with water (6.1 mL). The reaction mixture was extracted with Et₂O (4 × 10 mL), dried over MgSO₄, and concentrated in vacuo. The product was purified by flash chromatography (5% to 7% Et₂O in hexanes), yielding the title compound as a nearly colorless oil (0.371 g, 1.35 mmol, 94% yield): analytical TLC Rf = 0.60, 40% Et₂O in hexanes; [α]D²² = +38.2° (c 1.0, CHCl₃); IR νmax (film): 2976, 2935, 1775, 1700, 1508, 1458, 1356, 1279, 1240, 1160, 1089, 992 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.35 (m, 5H, H-8'-H-12'), 7.36 (m, 5H, H-8'-H-12'), 6.8 (m, 1H, H-1'H), 4.51 (dd, J = 9.5, 3.9 Hz, 1H, H-4'), 3.76 (septet, J = 6.8 Hz, 1H, H-2'), 3.09 (dd, J = 14.3, 3.9, 3.7 Hz, 1H, H-6'), 2.89 (dd, J = 14.2, 9.5 Hz, 1H, H-6'), 1.38 (s, 3H, H-13' or H-14'), 1.36 (s, 3H, H-13' or H-14'), 1.17 (s, 3H, H-3 or H-4), 1.15 (s, 3H, H-3 or H-4); ¹³C NMR (75 MHz, CDCl₃) δ 177.9 (C, C-1), 152.4 (C, C-2'), 137.0 (C, C-7'), 129.2 (2CH, C-8', C-12'), 128.7 (2CH, C-9', C-11'), 126.9 (CH, C-10'), 82.1 (C, C-5'), 63.7 (CH, C-4'), 35.5 (CH₂, C-6'), 32.8 (CH, C-5'), 28.6 (CH₃, C-13' or C-14'), 22.4 (CH₃, C-13' or C-14'), 19.3 (CH₃, C-3 or C-4), 18.8 (CH₃, C-3 or C-4); HR EIMS m/z 275.1517 [M]+ (calcd. for C₁₆H₂₁NO₃, 275.1516).

**Compound (4'S)-15:** Oxazolidinone (4'S)-15 was prepared in the same way as oxazolidinone (4'R)-15 from the l-phenylalanine‐derivived (S)(−)-4‐benzyl‐5,5‐dimethyl‐2‐oxazolidinone (97% yield): analytical TLC Rf = 0.59, 40% Et₂O in hexanes; [α]D²² = -31.7° (c 2.0, CHCl₃); IR νmax (film): 2977, 2935, 1775, 1699, 1458, 1356, 1280, 1240, 1160, 1089, 992 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.36–7.17 (m, 5H, H-8'-H-12'), 4.51 (dd, J = 9.4, 4.2, 3.9 Hz, 1H, H-4'), 3.76 (septet, J = 6.8 Hz, 1H, H-2'), 3.10 (dd, J = 14.4, 3.9 Hz, 1H, H-6'), 2.89 (dd, J = 14.2, 9.3 Hz, 1H, H-6'), 1.36 (s, 3H, H-13' or H-14'), 1.35 (s, 3H, H-13' or H-14'), 1.17 (s, 3H, H-3 or H-4), 1.15 (s, 3H, H-3 or H-4); ¹³C NMR (75 MHz, CDCl₃) δ 177.9 (C, C-1), 152.4 (C, C-2'), 137.0 (C, C-7'), 129.2 (2CH, C-8', C-12'), 128.7 (2CH, C-9', C-11'), 126.9 (CH, C-10'), 82.0 (C, C-5'), 63.7 (CH, C-4'), 35.5 (CH₂, C-6'), 32.8 (CH, C-5'), 28.6 (CH₃, C-13' or C-14'), 22.4 (CH₃, C-13' or C-14'), 19.3 (CH₃, C-3 or C-4), 18.7 (CH₃, C-3 or C-4); HR EIMS m/z 275.1516 [M]+ (calcd. for C₁₆H₂₁NO₃, 275.1516).
(3R,4'R)-16: Oxazolidinone (4'R)-15 (50.7 mg, 0.184 mmol) was dissolved in dry THF (1.8 mL) and added dropwise to a solution of LDA (1.5 equiv) in dry THF (2 mL) at –78°C [prepared by addition of 1.5 M n-BuLi (184 μL, 0.276 mmol, 1.5 equiv) to a solution of diisopropylamine (38.7 μL, 0.276 mmol, 1.5 equiv) in dry THF at –78°C, the solution was warmed to 0°C for 15 min, then cooled to –78°C]. The flask containing oxazolidinone (4'R)-6 was rinsed with THF (0.5 mL). After 30 min at –78°C, chlorotriisopropoxytitanium IV (1.0 M in THF, prepared immediately before use from neat chlorotriisopropoxytitanium IV, 736.5 μL, 0.737 mmol, 4 equiv) was added dropwise, and the reaction mixture was warmed to –40°C. After 1 hr, the mixture was cooled to –78°C, hexanal (68.0 μL, 0.552 mmol, 3 equiv) in THF (1.8 mL) was added, and the reaction mixture was warmed to –40°C. After 3 hr at –40°C, the reaction mixture was warmed to 0°C. After 2 hr at 0°C, the reaction was quenched with satd NH₄Cl (5 mL) and stirred with Celite® for 30 min while warming to room temperature. The filtrate was extracted with EtOAc (3 × 15 mL), washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The product was purified by flash chromatography (DCM/hexanes/MeCN, 49.5:49.5:1 to 48.5:48.5:3), yielding the title compound as a pale yellow gummy oil (56.7 mg, 82% yield): analytical TLC Rₐ= 0.32, DCM/hexane/MeCN (48.5:48.5:3). [α]D²¹ = +40.0° (c 1.0, CHCl₃); IR νmax (film): 3529, 2931, 2859, 1775, 1686, 1458, 1352, 1279, 1178, 1100, 732, 701 cm⁻¹; ¹H NMR (500MHz, CDCl₃) δ 7.34–7.19 (m, 5H, H₈′–H₁₂′), 4.56 (dd, J = 9.5, 3.7 Hz, 1H, H₄′), 4.10 (d, J = 8.1 Hz, 1H, H₃), 3.10 (dd, J = 14.4, 3.7 Hz, 1H, H₆′), 2.89 (dd, J = 14.3, 9.8, 9.5 Hz, 1H, H₆′), 2.39 (bs, 1H, OH), 1.67–1.54 (m, 1H, H₄), 1.50–1.11 (m, 7H, H₄, H₅, H₆, H₇), 1.37 (s, 3H, H₁₃′ or H₁₄′), 1.35 (s, 3H, H₉–H₁₀), 1.34 (s, 3H, H₉ or H₁₀), 1.33 (s, 3H, H₁₃′ or H₁₄′), 0.89 (t, J = 6.8 Hz, 3H, H₈), ¹³C NMR (75 MHz, CDCl₃) δ 178.9 (C, C₁), 151.9 (C, C₂′), 137.1 (C, C₇), 129.2 (2CH, C₈′–C₁₂′), 128.8 (2CH, C₉′–C₁₁′), 126.9 (CH, C₁₀′), 82.1 (C, C₅′), 75.8 (CH, C₃), 65.9 (CH, C₄′), 50.3 (C, C₂), 35.3 (CH₂, C₆′), 32.0 (CH₂, C₆), 31.6 (CH₂, C₄), 28.3 (CH₃, C₁₃′ or C₁₄′), 26.7 (CH₂, C₅), 22.8 (CH₂, C₇), 22.3 (CH₃, C₁₃′ or C₁₄′), 20.6 (CH₃, C₉ or C₁₀), 19.0 (CH₃, C₉ or C₁₀), 14.2 (CH₃, C₈); HR EIMS m/z 375.2400 [M]+ (calcld. for C₂₂H₃₃NO₄, 375.2404).

(3S,4'S)-16: The title compound was prepared in the same way as (3R,4'R)-16 from oxazolidinone (4'S)-15 (91% yield): analytical TLC Rf = 0.32, DCM/hexanes/MeCN (48.5:48.5:3); [α]D²² = -34.8° (c 1.0, CHCl₃); IR νmax (film): 3448, 2932, 2861, 1776, 1686, 1459, 1351, 1280, 1178, 1101, 945, 733, 700 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.35–7.17 (m, 5H, H₈′–H₁₂′), 4.56 (dd, J = 9.5, 3.7 Hz, 1H, H₄′), 4.11 (d, J = 6.8 Hz, 1H, H₃), 3.11 (dd, J = 14.2, 3.7 Hz, 1H, H₆′), 2.89 (dd, J = 14.4,
9.5, 1H, H-6'), 2.40 (bs, 1H, OH), 1.73-0.97 (m, 8H, H-4, H-5, H-6, H-7), 1.37 (s, 3H, H-13' or H-14'), 1.35 (s, 3H, H-9 or H-10), 1.34 (s, 3H, H-9 or H-10), 1.33 (s, 3H, H-13' or H-14'), 0.89 (t, J = 6.5 Hz, 3H, H-8); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.9 (C, C-1), 151.9 (C, C-2'), 137.1 (C, C-7'), 129.2 (2CH, C-8', C-12'), 128.8 (2CH, C-9', C-11'), 126.9 (CH, C-10'), 82.1 (C, C-5'), 75.8 (CH, C-3), 65.9 (CH, C-4'), 50.4 (C, C-2'), 35.3 (CH$_2$, C-6'), 32.0 (CH$_2$, C-6), 31.6 (CH$_2$, C-4), 28.3 (CH$_3$, C-13' or C-14'), 26.7 (CH$_2$, C-5), 22.8 (CH$_2$, C-7), 22.3 (CH$_3$, C-13' or C-14), 20.6 (CH$_3$, C-9 or C-10), 19.0 (CH$_3$, C-9 or C-10), 14.2 (CH$_3$, C-8); HR EIMS $m/z$ 375.2401 [M]$^+$ (calcd. for C$_{22}$H$_{33}$NO$_4$, 375.2404).

![Structure of Compound (3R,4'R)-17](image)

**Compound (3R,4'R)-17:** Oxazolidinone (4'R)-15 (43.7 mg, 0.159 mmol) was dissolved in dry THF (2 mL) and added dropwise to a solution of LDA (1.5 equiv) in dry THF (1.8 mL) at –78°C [prepared by addition of 1.35 M n-BuLi (176 µL, 0.238 mmol, 1.5 equiv) to a solution of diisopropylamine (35.6 µL, 0.254 mmol, 1.6 equiv) in dry THF] at –78°C. The reaction mixture was warmed to 0°C for 15 min, then cooled to –78°C. After 30 min at –78°C, chlorotriisopropoxytitanium IV (1.0 M in THF, prepared immediately before use from neat chlorotrisopropoxytitanium IV, 635 µL, 0.635 mmol, 4 equiv) was added dropwise, and the reaction mixture was warmed to –40°C. After 1 hr, the mixture was cooled to –78°C, butyraldehyde (42.6 µL, 0.476 mmol, 3 equiv) was added, and the reaction mixture was warmed to –40°C. After 3 hr at –40°C, the reaction was quenched with satd NH$_4$Cl (5 mL) and stirred with Celite® while warming to room temperature. The filtrate was extracted with EtOAc (4 × 4 mL), washed with brine (4 mL), dried over Na$_2$SO$_4$, and concentrated in vacuo. The product was purified by flash chromatography (DCM/hexanes/McCN, 49.5:49.5:1), yielding the title compound as a pale yellow gummy oil (19.4 mg, 35% yield): analytical TLC $R_f$ = 0.30, DCM/hexanes/McCN (48.5:48.5:3); $[\alpha]_D^{21}$ = +50.1° (c 0.42, CHCl$_3$); IR $\nu_{\text{max}}$ (film): 3511, 2959, 2872, 1775, 1685, 1459, 1351, 1280, 1179, 1102, 971, 731, 700 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.37-7.20 (m, 5H, H-8'-H-12'), 4.56 (dd, $J = 9.5, 3.9$ Hz, 1H, H-4'), 4.11 (m, 1H, H-5'), 3.92 (dd, $J = 14.4, 9.5$ Hz, 1H, H-6'), 2.90 (dd, $J = 14.4, 9.5$ Hz, 1H, H-6'), 2.39 (bs, 1H, OH), 1.68-0.76 (m, 4H, H-4, H-5), 1.37 (s, 3H, H-13' or H-14'), 1.35 (s, 3H, H-7 or H-8), 1.33 (s, 3H, H-13' or H-14'), 0.94 (t, $J = 7.1$ Hz, 3H, H-6); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.9 (C, C-1), 151.9 (C, C-2'), 137.1 (C, C-7'), 129.3 (2CH, C-8', C-12'), 128.8 (2CH, C-9', C-11'), 126.9 (CH, C-10'), 82.1 (C, C-5'), 75.6 (CH, C-3), 65.9 (CH, C-4'), 50.3 (C, C-2), 35.3 (CH$_2$, C-6'), 33.8 (CH$_2$, C-4), 28.3 (CH$_3$, C-13' or C-14'), 22.4 (CH$_3$, C-13' or C-14'), 20.6 (CH$_3$, C-7 or C-8), 20.1 (CH$_2$, C-5), 18.9 (CH$_3$, C-7 or C-8), 14.2 (CH$_3$, C-6); HR ESI MS $m/z$ 370.1991 [M + Na]$^+$ (calcd. for C$_{29}$H$_{29}$NO$_4$Na, 370.1989).
Compound (3S,4′S)-17: The title compound was prepared in the same way as (3R,4′R)-17 from oxazolidinone (4′S)-15 (54% yield): analytical TLC $R_f = 0.29$, DCM/hexanes/MeCN (48.5:48.5:3); $[\alpha]_D^{21} = -45.2^\circ$ (c 0.83, CHCl$_3$); IR $\nu_{\max}$ (film): 3504, 2959, 2874, 1775, 1685, 1458, 1352, 1280, 1156, 1102, 972, 846, 734, 700 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.34-7.20 (m, 5H, H-8′-H-12′), 4.56 (dd, $J = 9.5$, 3.7 Hz, 1H, H-4′), 4.11 (m, 1H, H-3), 3.10 (dd, $J = 14.4$, 3.9 Hz, 1H, H-6′), 2.90 (dd, $J = 14.4$, 9.5 Hz, 1H, H-6′), 2.39 (bs, 1H, OH), 1.67-0.77 (m, 4H, H-4, H-5), 1.37 (s, 3H, H-13′ or H-14′), 1.35 (s, 3H, H-7 or H-8), 1.34 (s, 3H, H-7 or H-8), 1.33 (s, 3H, H-13′ or H-14′), 0.94 (t, $J = 7.1$, 3H, H-6); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.9 (C, C-1), 151.9 (C, C-2), 137.1 (C, C-7), 129.2 (2CH, C-8′, C-12′), 128.8 (2CH, C-9′, C-11′), 126.9 (CH, C-10′), 82.1 (C, C-5′), 75.9 (CH, C-3), 65.6 (CH, C-4′), 50.3 (C, C-2), 35.3 (CH$_2$, C-6′), 33.7 (CH$_2$, C-4′), 28.3 (CH$_3$, C-13′ or C-14′), 22.3 (CH$_3$, C-13′ or C-14′), 20.6 (CH$_3$, C-7 or C-8), 20.1 (CH$_2$, C-5), 18.9 (CH$_3$, C-7 or C-8), 14.2 (CH$_3$, C-6); HR ESIMS $m/z$ 370.1992 [M + Na]$^+$ (calcd. for C$_{20}$H$_{29}$NO$_4$Na, 370.1989).

![Chemical Structure](image_url)

Compound (3R)-8: A solution of 30% aqueous H$_2$O$_2$ (16.2 μL, 0.143 mmol, 3.62 equiv) was added dropwise to a solution of (3R,4′R)-17 (13.6 mg, 0.039 mmol) in 4:1 THF:H$_2$O (340 μL) at 0ºC. LiOH·H$_2$O (2.6 mg, 0.063 mmol, 1.6 equiv) in H$_2$O (75 μL) was added to the solution. After 1 hr, Na$_2$SO$_4$ (20.6 mg, 0.163 mmol) was added and THF was removed from the slurry under vacuum. The residue was partitioned between DCM (3 × 1 mL) and H$_2$O. The aqueous layers were collected and acidified to pH 1 with 1 N HCl. The aqueous layer was extracted with Et$_2$O (3 × 4 mL), and the combined organic layers were dried over MgSO$_4$, filtered and concentrated to yield the title compound as a colorless oil (4.3 mg, 69% yield); $[\alpha]_D^{22} = +26.5^\circ$ (c 0.28, CHCl$_3$); IR $\nu_{\max}$ (film): 3385, 2962, 2876, 1704, 1468, 1398, 1261, 1151, 1115, 1073, 982, 869, 658 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.66 (m, 1H, H-3), 1.69-1.56 (m, 1H, H-5), 1.53-1.42 (m, 1H, H-4), 1.41-1.29 (m, 2H, H-4, H-5), 1.25 (s, 3H, H-7 or H-8), 1.20 (s, 3H, H-7 or H-8), 0.95 (t, $J = 7.1$ Hz, 3H, H-6); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 182.1 (C, C-1), 76.6 (CH, C-3), 47.1 (C, C-2), 33.9 (CH$_2$, C-4), 22.7 (CH$_3$, C-7 or C-8), 20.4 (CH$_3$, C-7 or C-8), 19.9 (CH$_2$, C-5), 14.1 (CH$_3$, C-6); HR ESIMS $m/z$ 183.0994 [M + Na]$^+$ (calcd. for C$_8$H$_{16}$O$_3$Na, 183.0992).

Compound (3S)-8: Carboxylic acid (3S)-8 was synthesized using the same procedure as (3R)-8 from (3S,4′S)-17 (75% yield): $[\alpha]_D^{22} = -35.5^\circ$ (c 0.24, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.66 (m, 1H, H-3), 1.68-1.56 (m, 1H, H-5), 1.52-1.42 (m, 1H, H-4), 1.42-1.30 (m, 2H, H-4, H-5), 1.25 (s, 3H, H-7 or H-8), 1.20 (s, 3H, H-7 or H-8), 0.95 (t, $J = 7.1$ Hz, 3H, H-6); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 182.2 (C, C-1), 76.6 (CH, C-3), 47.1 (C, C-2), 33.9 (CH$_2$, C-4), 22.7 (CH$_3$, C-7 or C-8), 20.4 (CH$_3$, C-7 or C-8), 19.9 (CH$_2$, C-5), 14.1 (CH$_3$, C-6); HR ESIMS $m/z$ 183.0995 [M + Na]$^+$ (calcd. for C$_8$H$_{16}$O$_3$Na, 183.0992).
Compound (3R,4′R)-18: Oxazolidinone (4′R)-15 (51.3 mg, 0.186 mmol) was dissolved in dry THF (1.9 mL) and added dropwise to a solution of LDA (1.5 equiv) in dry THF (2 mL) at –78°C [prepared by addition of 1.45 M n-BuLi (193 μL, 0.279 mmol, 1.5 equiv) to a solution of diisopropylamine (39.2 μL, 0.279 mmol, 1.5 equiv) in dry THF at –78°C, the solution was warmed to 0°C for 15 min, then cooled to –78°C]. The flask containing oxazolidinone (4′R)-15 was rinsed with THF (0.5 mL). After 30 min at –78°C, chlorotriisopropoxytitanium IV (1.0 M in THF, prepared immediately before use from neat chlorotriisopropoxytitanium IV, 745 μL, 0.745 mmol, 4 equiv) was added dropwise, and the reaction mixture was warmed to –40°C. After 1 hr, the mixture was cooled to –40°C, hexynal (53.7 mg, 0.559 mmol, 3 equiv, prepared from 5-hexyn-1-ol according to Böttcher and Sieber in THF (1.8 mL) was added, and the reaction mixture was warmed to –40°C. After 3 hr at –40°C, the reaction mixture was warmed to 0°C. After 2 hr at 0°C, the reaction was quenched with satd NH₄Cl (5 mL) and stirred with Celite® for 30 min while warming to room temperature. The filtrate was extracted with EtOAc (3 × 15 mL), washed with brine (15 mL), and concentrated in vacuo. The product was purified by flash chromatography (DCM/hexanes/MeCN, 48.5:48.5:3), yielding the title compound as a pale yellow oil (51.3 mg, 74% yield): analytical TLC \( R_f = 0.22 \), DCM/hexanes/MeCN (48.5:48.5:3); \([\alpha]_D^{21} = +36.4^\circ \) (c 1.0, CHCl₃); IR \( \nu_{\text{max}} \) (film): 3532, 3291, 2937, 1773, 1686, 1352, 1280, 1162, 1101, 953 cm⁻¹; \( ^1H \) NMR (500 MHz, CDCl₃) \( \delta \) 7.33-7.20 (m, 5H, H-8′-12′), 5.30 (residual DCM), 4.56 (dd, \( J = 9.4, 4.1, 3.9 \) Hz, 1H, H-4′), 4.06 (dd, \( J = 10.0, 5.1 \) Hz, 1H, H-3), 3.10 (dd, \( J = 14.3, 3.9, 3.7 \) Hz, 1H, H-6′), 2.89 (dd, \( J = 14.3, 9.5 \) Hz, 1H, H-6′), 2.50 (d, \( J = 5.9 \) Hz, 1H, OH), 2.25 (m, 2H, H-6), 1.93 (t, \( J = 2.7 \) Hz, 1H, H-8), 1.89-1.76 (m, 1H, H-5), 1.66-1.54 (m, 2H, H-4, H-5), 1.53-1.39 (m, 1H, H-4), 1.37 (s, 3H, H-13′ or H-14′), 1.37 (s, 3H, H-9 or H-10), 1.35 (s, 3H, H-9 or H-10), 1.33 (s, 3H, H-13′ or H-14′); \( ^{13}C \) NMR (75 MHz, CDCl₃) \( \delta \) 178.9 (C, C-1), 151.9 (C, C-2′), 137.0 (C, C-7′), 129.2 (2CH, C-8′, C-12′), 128.8 (2CH, C-9′, C-11′), 127.0 (CH, C-10′), 84.5 (C, C-7), 82.2 (C, C-5′), 75.6 (CH, C-3), 68.7 (CH, C-8), 65.8 (CH, C-4′), 50.3 (C, C-2), 35.3 (CH₂, C-6′), 30.4 (CH₃, C-4), 28.3 (CH₃, C-13′ or C-14′), 25.8 (CH₂, C-5), 22.3 (CH₃, C-13′ or C-14′), 20.5 (CH₃, C-9 or C-10), 18.7 (CH₃, C-9 or C-10), 18.4 (CH₂, C-6); HR ESIMS \( m/z \) 394.1990 [M + Na]^+ (calcd. for C₂₂H₂₉NO₄Na, 394.1989).

Compound (3S,4′S)-18: The title compound was prepared in the same way as (3R,4′R)-18 from oxazolidinone (4′S)-15 (58% yield): TLC \( R_f = 0.19 \), DCM/hexanes/MeCN (48.5:48.5:3); \([\alpha]_D^{21} = -43.8^\circ \) (c 1.0, CHCl₃); IR \( \nu_{\text{max}} \) (film): 3532, 3291, 2931, 1773, 1686, 1352, 1280, 1162, 1101, 953 cm⁻¹; \( ^1H \) NMR (500 MHz, CDCl₃)
Compound (3R)-1: A solution of 30% aqueous H$_2$O$_2$ (42.8 μL, 0.377 mmol, 3.66 equiv) was added dropwise to a solution of (3R,4'R)-18 (38.3 mg, 0.103 mmol) in 4:1 THF:H$_2$O (854 μL) at 0°C. LiOH·H$_2$O (7.0 mg, 0.167 mmol, 1.6 equiv) in H$_2$O (198 μL) was added to the solution. After 1 hr, Na$_2$SO$_4$ (53.8 mg, 0.427 mmol) was added and THF was removed from the slurry under vacuum. The residue was partitioned between DCM (3 × 1 mL) and H$_2$O. The aqeous layers were collected and acidified to pH 1 with 1 N HCl. The aqueous layers were extracted with Et$_2$O (3 × 1 mL), and the combined organic layers were dried over MgSO$_4$, filtered and concentrated to yield the title compound as a colorless oil (15.4 mg, 81% yield): [α]$^21_D = +25.3^\circ$ (c 1.0, CHCl$_3$); IR $\nu_{max}$ (film): 3298, 2948, 1704, 1471, 1399, 1268, 1166, 1074, 972, 641 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.53 (bs, 1H, H-3), 3.68 (d, $J = 10.7$ Hz, 1H, H-3), 2.26 (m, 2H, H-6), 1.96 (q, $J = 4.8$, 2.4, 2.2 Hz, 1H, H-8), 1.89-1.77 (m, 1H, H-5), 1.72-1.64 (m, 1H, H-4), 1.64-1.55 (m, 1H, H-5), 1.48-1.39 (m, 1H, H-4), 1.25 (s, 3H, H-9 or H-10), 1.21 (s, 3H, H-9 or H-10); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.5 (C, C-1), 84.3 (C, C-7), 76.2 (CH, C-3), 68.9 (CH, C-8), 47.2 (C, C-2), 30.5 (CH$_2$, C-4), 25.5 (CH$_2$, C-5), 22.7 (CH$_3$, C-9 or C-10), 20.1 (CH$_3$, C-9 or C-10), 18.3 (CH$_2$, C-6); HR EIMS $m/z$ 207.0991 [M + Na]$^+$ (calcd. for C$_{10}$H$_{16}$O$_3$Na, 207.0992).

Compound (3S)-1: Carboxylic acid (3S)-1 was synthesized using the same procedure as (3R)-1 from (3S,4'S)-18 (84% yield): [α]$^21_D = -26.3^\circ$ (c 1.0, CHCl$_3$); IR $\nu_{max}$ (film): 3297, 2944, 1703, 1470, 1398, 1265, 1168, 1074, 971, 640 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.42 (bs, 1H, COOH), 3.68 (d, $J = 10.9$ Hz, 1H, H-3), 2.26 (m, 2H, H-6), 1.97 (t, $J = 2.6$ Hz, 1H, H-8), 1.89-1.78 (m, 1H, H-5), 1.71-1.64 (m, 1H, H-4), 1.64-1.55 (m, 1H, H-5), 1.50-1.36 (m, 1H, H-4), 1.25 (s, 3H, H-9 or H-10), 1.21 (s, 3H, H-9 or H-10); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.4 (C, C-1), 84.3 (C, C-7), 76.2 (CH, C-3), 68.8 (CH, C-8), 47.2 (C, C-2), 30.5 (CH$_2$, C-4), 25.5 (CH$_2$, C-5), 22.7 (CH$_3$, C-9 or C-10), 20.1 (CH$_3$, C-9 or C-10), 18.3 (CH$_2$, C-6); HR EIMS $m/z$ 207.0991 [M + Na]$^+$ (calcd. for C$_{10}$H$_{16}$O$_3$Na, 207.0992).
Figure 2.5.1. $^1$H NMR spectrum of compound (4'R)-12 (CDCl$_3$, 300 MHz).
Figure 2.5.2. $^{13}$C NMR spectrum of compound (4'R)-12 (CDCl$_3$, 75 MHz).
Figure 2.5.3. $^1$H NMR spectrum of compound (4'S)-12 (CDCl$_3$, 300 MHz).
Figure 2.5.4. $^{13}$C NMR spectrum of compound (4'S)-12 (CDCl$_3$, 75 MHz).
Figure 2.5.5. $^1$H NMR spectrum of compound (3R,4'R)-13 (CDCl$_3$, 300 MHz).
Figure 2.5.6. $^{13}$C NMR spectrum of compound (3R,4'R)-13 (CDCl$_3$, 75 MHz).
Figure 2.5.7. $^1$H NMR spectrum of compound (3S,4'S)-13 (CDCl$_3$, 300 MHz).
Figure 2.5.8. $^{13}$C NMR spectrum of compound (3S,4'S)-13 (CDCl$_3$, 75 MHz).
Figure 2.5.9. $^1$H NMR spectrum of (S)-MTPA ester of compound (3R,4'R)-13 (CDCl$_3$, 500 MHz).
Figure 2.5.10. $^{13}$C NMR spectrum of (S)-MTPA ester of compound (3R,4'R)-13 (CDCl$_3$, 125 MHz).
Figure 2.5.11. $^1$H NMR spectrum of (R)-MTPA ester of compound (3R,4'R)-13 (CDCl$_3$, 500 MHz).
Figure 2.5.12. $^{13}$C NMR spectrum of (R)-MTPA ester of compound (3R,4'R)-13 (CDCl$_3$, 75 MHz).
Figure 2.5.13. $^1$H NMR spectrum of compound (3R)-3 (CDCl$_3$, 300 MHz).
Figure 2.5.14. $^{13}$C NMR spectrum of compound (3R)-3 (CDCl$_3$, 75 MHz).
Figure 2.5.15. $^1$H NMR spectrum of compound (3S)-3 (CDCl$_3$, 300 MHz).
Figure 2.5.16. $^{13}$C NMR spectrum of compound (3S)-3 (CDCl$_3$, 75 MHz).
Figure 2.5.17. $^1$H NMR spectrum of compound (4'R)-15 (CDCl$_3$, 300 MHz).
Figure 2.5.18. $^{13}$C NMR spectrum of compound (4'R)-15 (CDCl$_3$, 75 MHz).
Figure 2.5.19. $^1$H NMR spectrum of compound (4'S)-15 (CDCl₃, 300 MHz).
Figure 2.5.20. $^{13}$C NMR spectrum of compound (4′S)-15 (CDCl$_3$, 75 MHz).
Figure 2.5.21. $^1$H NMR spectrum of compound (3S,4'S)-16 (CDCl$_3$, 300 MHz).
Figure 2.5.22. $^{13}$C NMR spectrum of compound (3S,4'S)-16 (CDCl$_3$, 75 MHz).
Figure 2.5.23. $^1$H NMR spectrum of compound (3$R$,4$'R$)-17 (CDCl$_3$, 500 MHz).
Figure 2.5.24. $^{13}$C NMR spectrum of compound (3R,4'R)-17 (CDCl$_3$, 75 MHz).
Figure 2.5.25. $^1$H NMR spectrum of compound (3S,4'S)-17 (CDCl$_3$, 500 MHz).
Figure 2.5.26. $^{13}$C NMR spectrum of compound (35,4'S)-17 (CDCl$_3$, 75 MHz).
Figure 2.5.27. $^1$H NMR spectrum of (3R)-8 (CDCl$_3$, 500 MHz).
Figure 2.5.28. $^{13}$C NMR spectrum of compound (3R)-8 (CDCl$_3$, 75 MHz).
Figure 2.5.29. $^1$H NMR spectrum of compound (3S)-8 (CDCl$_3$, 500 MHz).
Figure 2.5.30. $^{13}$C NMR spectrum of compound (3S)-8 (CDCl$_3$, 75 MHz).
Figure 2.5.31. $^1$H NMR spectrum of compound (3R,4'R)-18 (CDCl$_3$, 500 MHz).
Figure 2.5.32. $^{13}$C NMR spectrum of compound (3$R$,4$R'$)-18 (CDCl$_3$, 75 MHz).
Figure 2.5.33. $^1$H NMR spectrum of compound (3S,4'S)-18 (CDCl$_3$, 500 MHz).
Figure 2.5.34. $^{13}$C NMR spectrum of compound (3S,4'S)-18 (CDCl$_3$, 75 MHz).
Figure 2.5.35. $^1$H NMR spectrum of compound (3R)-1 (CDCl$_3$, 500 MHz).
Figure 2.5.36. $^{13}$C NMR spectrum of compound (3R)-1 (CDCl$_3$, 75 MHz).
Figure 2.5.37. $^1$H NMR spectrum of compound (3S)-1 (CDCl$_3$, 500 MHz).
Figure 2.5.38. $^{13}$C NMR spectrum of compound (3S)-1 (CDCl$_3$, 75 MHz).
REFERENCES


(28) Balunas, M. J., unpublished results.


3.0 CHAPTER 3
BIOSYNTHETICALLY-INTRIGUING CHLORINATED LIPOPEPTIDES AND A
PYRANONE FROM GEOGRAPHICALLY DISTANT TROPICAL MARINE
CYANOBACTERIA

3.0.1 Abstract

Five new vinyl-chlorine containing metabolites, the lipopeptides janthielamide A and kimbeamides A-C, and the ketide-extended pyranone kimbelactone A, have been isolated from collections of marine cyanobacteria made in Curaçao and Papua New Guinea. Both janthielamide A and kimbeamide A exhibited moderate sodium channel blocking activity in murine Neuro-2a cells. Consistent with this activity, janthielamide A was also found to antagonize veratridine-induced sodium influx in murine cerebrocortical neurons. These lipopeptides represent the newest additions to a relatively rare family of marine cyanobacterial-derived acyl amides and a new structural class of compounds exhibiting neuromodulatory activities from marine cyanobacteria.

3.1 Introduction

Marine cyanobacteria are prolific producers of diverse bioactive secondary metabolites with many exhibiting anticancer activity largely owing to interference with tubulin (e.g. dolastatin 10, curacin A, and desmethoxymajusculamide C) or actin (e.g. hectochlorin, lyngbyabellins A and E) formation and function. While freshwater and terrestrial cyanobacteria have long been recognized as producers of potent neurotoxins, such as anatoxin-α, β-methylaminoalanine and saxitoxin, marine cyanobacteria have only recently garnered attention for their potential to produce neuromodulatory
Metabolites exhibiting such activity, including voltage-gated sodium channel (VGSC) activation or blocking effects, and/or intracellular calcium ion modulation, have potential therapeutic utility in the treatment of spinal cord injury, chronic pain, CNS disorders including stroke and epilepsy, and treatment of cardiovascular, inflammatory and neurodegenerative disorders. With the discovery of potent VGSC activation for antillatoxin A and nanomolar VGSC blocking activity for kalkitoxin, two metabolites obtained from collections of marine cyanobacteria, the capacity of these oceanic prokaryotes to produce neuroactive compounds was realized.

Halogen atom incorporation is common in natural products from the marine realm in part owing to the relatively high concentrations of bromide, chloride and iodide present in seawater. Chloride, the most common halogen atom incorporated into marine cyanobacterial natural products, is present in several linear lipophilic metabolites which feature terminal or pendant vinyl chloride functionalities. These vinyl chloride-containing cyanobacterial compounds exhibit a variety of biological activities, such as VGSC blocking activity, cytotoxicity, toxicity, and insecticidal activity.

In the present work, two independent collections of cyanobacteria from Caribbean and Indo-Pacific locations yielded chromatographic fractions that had similar $^1$H NMR profiles, molecular weights, isotopic patterns and exhibited identical neuromodulatory activity profiles. Subsequently, assay-guided isolation of an extract derived from the Curaçao collection yielded a new lipopeptide, janthielamide A (1), whereas the Papua New Guinea collections of electric orange “puffballs” yielded the novel lipopeptide kimbeamide A (2) and two geometrical isomers, kimbeamides B (3) and C (4), as well as the polyketide kimbelactone A (5). Despite geographical distance and morphological
differences, both collections were found to contain evolutionarily closely-related cyanobacteria based on phylogenetic analyses. Herein, we report the isolation, structure elucidation and bioactivity of these metabolites, and a thorough characterization of the putative marine cyanobacterial producers.

3.2 Results and Discussion

3.2.1 Collection, isolation and structure elucidation

A green-pigmented filamentous cyanobacterial mat was collected by hand from Jan Thiel Bay in Curaçao. The cyanobacterial tissue was extracted repeatedly with DCM/MeOH (2:1) and then fractioned by silica gel vacuum column chromatography to produce nine subfractions (A-I). Bioassay-guided fractionation of the main bioactive fraction (D) utilizing normal phase column chromatography led to the isolation of janthielamide A (1) as a dark yellow, optically active amorphous solid {34.2 mg, 1.54%, [α]D 10.2 (c 0.60, CHCl3)}. The LR-ESI-MS spectrum of 1 revealed a 3:1 ratio at m/z 310/312 for the [M+H]+ pseudo-molecular ion, consistent with the presence of a single chlorine atom. HR-ESI-TOFMS established the molecular formula as C18H28NOCl, revealing that 1 contained five double bond equivalents. The IR spectrum displayed absorptions characteristic for NH or OH protons (3297 cm⁻¹) and an amide carbonyl group (1732 cm⁻¹), while UV showed a maximum at 224 nm (log ε = 4.4) consistent with the presence of at least one conjugated diene or enone. The ¹H NMR spectrum of 1 (Table 3.1) contained two doublet methyls (δH 0.98 and 1.02), two singlet methyls (δH 1.82 and 2.14), a diastereotopic deshielded methylene (δH 3.19 and 3.27), two upfield shifted methines (δH 2.14 and 2.29), three resonances at 1-2 ppm accounting for two
methylenes, one NH proton at $\delta_H$ 5.35, and seven olefinic resonances between 5-6 ppm. The $^{13}$C NMR spectrum of 1 included two quaternary ($\delta_C$ 167.0 and 150.5), seven methine ($\delta_C$ 144.1, 137.5, 130.1, 127.2, 122.3, 118.8 and 116.3), five methylene ($\delta_C$ 39.8, 37.7, 37.4, 36.9 and 35.2) and four methyl carbons ($\delta_C$ 27.2, 21.2, 19.9 and 19.8), accounting for all 18 carbon atoms in the molecular formula. The quaternary and methine carbons were further assigned as four double bonds and one carbonyl moiety according to their chemical shifts.

Two $^1$H spin systems were assembled by COSY (Figure 3.1); fragment 1a featured an interesting vinylic gem-dimethyl functionality while fragment 1b contained a conjugated diene, two methyl substituted methine carbons, a third olefin group, and two adjacent methylene groups. The lone carbonyl carbon observed in the $^{13}$C NMR spectrum was assigned to fragment 1a based on an HMBC correlation from H2' to C1'. Subsequently, spin systems 1a and 1b were connected through an amide bond based on a correlation from the amide proton to C2' in the HMBC spectrum. Lastly, the chlorine atom was attached to the highly deshielded C1 position ($\delta_C$ 116.3) on the basis of chemical shift arguments and comparison with literature values for similar systems.$^{22-24}$
The configurations of the double bonds were determined via coupling constant data. The vicinal coupling constant for H1/H2 was 7.0 Hz, a value consistent with a polarized Z double bond. The C3-C4 and C7-C8 olefins were both assigned as $E$ based on vicinal coupling constants of 15.4 Hz and 15.3 Hz for H3/H4 and H7/H8, respectively.
Table 3.1. NMR spectroscopic data for janthielamide A (1) in CDCl$_3$ ($^1$H at 500 MHz, $^{13}$C at 75 MHz).

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta_c$ $^{a,d}$</th>
<th>$\delta_h$ mult ($J$ in Hz)$^{c}$</th>
<th>COSY</th>
<th>HMBC</th>
</tr>
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<tr>
<td>1</td>
<td>116.3 CH</td>
<td>5.88 d (7.0)</td>
<td>2</td>
<td>2, 3</td>
</tr>
<tr>
<td>2</td>
<td>130.1 CH</td>
<td>6.25 dd (10.4, 7.0)</td>
<td>1, 3</td>
<td>1, 4</td>
</tr>
<tr>
<td>3</td>
<td>122.3 CH</td>
<td>6.39 dd (15.4, 10.3)</td>
<td>2, 4</td>
<td>1, 2, 5</td>
</tr>
<tr>
<td>4</td>
<td>144.1 CH</td>
<td>5.76 dd (15.4, 7.6)</td>
<td>3, 5</td>
<td>2, 3, 12</td>
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<tr>
<td>5</td>
<td>37.4 CH</td>
<td>2.29 m</td>
<td>4, 6, 12</td>
<td>3, 4, 6, 7, 12</td>
</tr>
<tr>
<td>6</td>
<td>39.8 CH$_2$</td>
<td>2.03 m</td>
<td>5, 7</td>
<td>4, 5, 7, 8, 9, 12</td>
</tr>
<tr>
<td>7</td>
<td>127.2 CH</td>
<td>5.33 dt (15.3, 6.7)</td>
<td>6, 8, 11a/b</td>
<td>5, 6, 8</td>
</tr>
<tr>
<td>8</td>
<td>137.5 CH</td>
<td>5.25 dd (15.3, 7.9)</td>
<td>7, 9</td>
<td>5, 6, 7, 13</td>
</tr>
<tr>
<td>9</td>
<td>19.9 CH</td>
<td>2.14 m</td>
<td>8, 10a/b, 13</td>
<td>7, 8, 10, 13</td>
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<tr>
<td>10a</td>
<td>36.9 CH$_2$</td>
<td>1.53 m</td>
<td>9, 10b, 11a, 12</td>
<td>8, 11, 13</td>
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<tr>
<td>10b</td>
<td></td>
<td>1.40 m</td>
<td>9, 10a, 11b</td>
<td>8, 11, 13</td>
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<tr>
<td>11a</td>
<td>37.7 CH$_2$</td>
<td>3.27 m</td>
<td>7, 10a</td>
<td>10, 1'</td>
</tr>
<tr>
<td>11b</td>
<td></td>
<td>3.19 m</td>
<td>7, 10b</td>
<td>10, 1'</td>
</tr>
<tr>
<td>12</td>
<td>19.8 CH$_3$</td>
<td>1.02 d (6.7)</td>
<td>5</td>
<td>4, 5, 6</td>
</tr>
<tr>
<td>13</td>
<td>21.2 CH$_3$</td>
<td>0.98 d (6.7)</td>
<td>9</td>
<td>8, 10</td>
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<tr>
<td>NH</td>
<td></td>
<td>5.35</td>
<td></td>
<td>2', 4'</td>
</tr>
<tr>
<td>1'</td>
<td>167.0 qC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>118.8 CH</td>
<td>5.51 s</td>
<td>4', 5'</td>
<td>12, 1', 3', 5'</td>
</tr>
<tr>
<td>3'</td>
<td>150.5 qC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>35.2 CH$_3$</td>
<td>2.14 s</td>
<td>2'</td>
<td>1', 2', 3', 4', 5'</td>
</tr>
<tr>
<td>5'</td>
<td>27.2 CH$_3$</td>
<td>1.82 s</td>
<td>2'</td>
<td>1', 2', 3', 12</td>
</tr>
</tbody>
</table>

$^a$ Recorded at 75 MHz. $^b$ Multiplicity deduced from HSQC. $^c$ Recorded at 500 MHz.
Regarding the absolute configuration of 1, we envisioned that hydrolysis and ozonolysis followed by oxidative workup would yield 2-methylsuccinic acid (6) and 2-methyl-γ-aminobutyric acid (GABA, 7) as key degradation products containing the stereocenters in question (C5 and C9, Scheme 3.1). Enantiomerically pure (R)- and (S)-6 are commercially available and thus, were initially derivatized to afford methyl or isopropyl esters; however, these proved inseparable on chiral GCMS. Ultimately, esterification with (S)-2-(+)-octanol\textsuperscript{26} afforded di(octan-2-yl) 2-methylsuccinate diastereomers (8) that separated well on achiral GCMS. The products of hydrolysis and ozonolysis of janthielamide A (1) were similarly esterified with (S)-2-(+)-octanol. Comparison of the retention times for (R)- and (S)-8 with janthielamide A-derived 8 allowed for assignment of the absolute configuration at C5 as R.

Unfortunately, in the case of 2-methyl-GABA, enantiomerically pure standards were not commercially available. This analogue of the neurotransmitter GABA has previously been synthesized;\textsuperscript{27} however, the starting material is a controlled substance and the synthetic route is complex. We envisioned a more facile synthetic route whereby the desired standards could be afforded via oxidation of the corresponding amino alcohol. The standards were prepared as follows: the (S)-2 methyl-GABA standard (7a) was obtained from commercially available (R)-(−)-3-bromo-2-methyl-1-propanol (12) via an S\textsubscript{N}2 substitution of bromine with cyanide as described by Evans and Morken\textsuperscript{28}, followed by reduction with LiAlH\textsubscript{4} as described by Zoidis et al.\textsuperscript{29} to give (S)-4-amino-2-methyl-1-butanol (14a). This was N-protected with an acetyl group (16a) and oxidized to the carboxylic acid (7a) using Jones’ reagent. To obtain the (R)-2-methyl-GABA (7b) standard, commercially available (R)-4-amino-2-methyl-1-butanol (14b) was oxidized as
described above for 7a. Both 7a and 7b were then esterified with (S)-2-(-)-octanol. These diastereomers (9a, 9b) were successfully resolved utilizing achiral reverse phase LCMS, and comparison of their retention times with that of the corresponding janthielamide A (1) derivative led us to assign the absolute configuration at C9 as \( R \), completing the structure elucidation of compound 1.

Scheme 3.1. Methodology for determining the absolute configuration at C5 and C9 of janthielamide A (1) and at C1’ of kimbeamide A (2). (A) Fragmentation strategy for 1 and 2. (B) Preparation of enantiomerically pure 2-methyl-\( \gamma \)-aminobutyric acid standards for determining the absolute configuration of 1.

In parallel with the above study, two extracts from Papua New Guinea collections of orange-colored cyanobacterial consortia were combined (See Experimental details) and subjected to a similar bioassay-guided protocol employing normal phase column
chromatography and HPLC to yield kimbeamide A (2) as an optically active pale yellow oil {1.6 mg, 0.10%, $[\alpha]_D$ +44 (c 0.05, MeOH)}. The LR-ESI-MS of 2 revealed an isotopic pattern for the [M+H]$^+$ pseudo-molecular ion cluster consistent with the presence of two chlorine atoms $[328/330/332 = 1:0.5:0.15]$. The HR-ESI-TOFMS of 2 gave an [M+H]$^+$ at $m/z$ 328.1234 (calcd for $C_{17}H_{24}NOCl_2$, 328.1229) in agreement with a molecular formula of $C_{17}H_{23}NOCl_2$, therefore requiring 6 degrees of unsaturation. The IR spectrum featured absorptions for NH or OH protons (3290 cm$^{-1}$) and an amide carbonyl group (1718 cm$^{-1}$), while UV showed a maximum at 250 nm (log $\varepsilon$ = 4.01) suggesting the presence of a conjugated dienone system.

The $^1$H NMR spectrum of compound 2 (Table 3.2) had resonances for a methyl doublet at $\delta_H$ 1.01, a methyl singlet at $\delta_H$ 1.54, a doublet at $\delta_H$ 2.31 integrating for two protons and indicative of a bis-allylic methylene, four resonances between 1-2 ppm accounting for two diastereotopic methylenes, an NH proton at $\delta_H$ 4.70, nine resonances from 5-6 ppm characteristic of olefinic protons, and a doublet of doublets at $\delta_H$ 7.44 for the $\beta$ proton in a conjugated dienone system. Analysis of the $^{13}$C NMR and HSQC spectra revealed two quaternary ($\delta_C$ 164.1 and 136.4), ten methine ($\delta_C$ 140.5, 137.5, 133.3, 132.6, 130.6, 130.3, 124, 117.8, 114.2 and 42.4), three methylene ($\delta_C$ 40.1, 30.8 and 27.1) and two methyl ($\delta_C$ 21.7 and 16.6) carbons, accounting for all 17 carbon atoms required by the molecular formula. Two spin systems were assembled by COSY (Figure 3.1); fragment 2a featured a conjugated diene whereas fragment 2b featured two isolated alkene functionalities separated by two methylene groups. The lone carbonyl carbon was assigned to fragment 2a at position C1 based on correlations in the HMBC from H2 to C1 and H3 to C1, respectively. Spin systems 2a and 2b were connected through an amide
bond based on HMBC data including correlations from NH to C1 and C1′ as well as from H1′ to C1. HMBC correlations from H8 to C6, C7 and C9 allowed for placement of a methyl substituted alkene on the distal side of fragment 2a. Additionally, correlations from H7′ to C6′ and C5′, as well as from H5′ and H6′ to C7′, allowed for assignment of the remaining proton of the terminating alkene of fragment 2b. As with janthielamide A (1), the relatively shielded chemical shifts exhibited by C8 (δC 114.2) and C7′ (δC 117.8) indicated that chlorine atoms were appended to these vinylic carbons.

The configurations of the double bonds were determined based on JHH vicinal coupling constants and NOE correlations. The C2-C3, C4-C5, and C6′-C7′ olefins were assigned as E according to J values of 14.9 Hz, 15.0 Hz and 13.4 Hz for H2/H3, H4/H5, and H6′/H7′, respectively. The C7-C8 olefin, in turn, was also assigned as E based on an NOE correlation between H8 and H6. Lastly, the C2′-C3′ olefin was determined to be Z given a JH2′-H3′ value of 10.5 Hz.

The absolute configuration of kimbeamide (2) at C1′ was determined by cleaving the C2′-C3′ olefin and the amide bond via ozonolysis followed by oxidative workup and hydrolysis to produce alanine as a degradation product (Scheme 3.1). This residue, as well as enantiomerically pure L- and D-alanine standards, were esterified with isopropanol and acylated with trifluoroacetic anhydride to furnish volatile derivatives for chiral GCMS analysis.30,31 By comparison of retention times of the cleaved product and standards, metabolite 2 was found to possess the S configuration at C1′, thus completing the structure elucidation of kimbeamide A (2).
Table 3.2. NMR spectroscopic data for kimbeamide A (2) in C$_6$D$_6$ ($^1$H at 600 MHz, $^{13}$C at 125 MHz).

<table>
<thead>
<tr>
<th>No.</th>
<th>δc$^{a,b}$</th>
<th>δH ($^1$H in Hz)$^c$</th>
<th>COSY</th>
<th>HMBC</th>
<th>NOESY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>164.1 qC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>124.0 CH</td>
<td>5.32 d (14.9)</td>
<td>3</td>
<td>1, 3, 4, 5</td>
<td>4, NH</td>
</tr>
<tr>
<td>3</td>
<td>140.5 CH</td>
<td>7.45 dd (14.9, 11.2)</td>
<td>2, 4</td>
<td>1, 2, 4, 5</td>
<td>5</td>
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<td>5.53 bs</td>
<td></td>
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<td>16.6 CH$_3$</td>
<td>1.54 s</td>
<td></td>
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<td>3', 4'a</td>
<td>3', 5', 6'</td>
<td>1'</td>
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<td>5'a</td>
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<td>1.76 m</td>
<td>4'b, 6'</td>
<td>3', 4', 6', 7'</td>
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<tr>
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<td></td>
<td>1.71 m</td>
<td>4'b, 6'</td>
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<td>21.7 CH$_3$</td>
<td>1.01 d (6.6)</td>
<td>1'</td>
<td>1', 2'</td>
<td>NH</td>
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</table>

$^a$ Recorded at 125 MHz. $^b$ Multiplicity deduced from HSQC. $^c$ Recorded at 600 MHz.

Two geometrical isomers of 2 were also isolated from the orange-colored cyanobacterial collections from Kimbe Bay. Kimbeamide B (3) differed from 2 in the
geometry of the C4-C5 olefin (Z, $^3J_{H4-H5} = 10.9$ Hz), while kimbeamide C (4) differed from 3 in the geometry of the C2'-C3' olefin ($E$, $^3J_{H2'-H3'} = 15.4$ Hz). Unfortunately, because compounds 3 and 4 were isolated in very low yields and were unstable, we were unable to complete the analysis of their stereochemistry. Nevertheless, we hypothesize that C1' of compounds 3 and 4 is S based on their similarity to and co-occurrence with kimbeamide A (1).

Kimbelactone A (5) was isolated as an optically active pale yellow oil {3.3 mg, 0.20%, $[\alpha]_D -164.7$ ($c$ 0.17, DCM)} from a slightly more polar chromatographic fraction of the orange Papua New Guinea collections. The LR-ESI-MS spectrum of 5 revealed a 3:1 ratio at $m/z$ 359/361 for the [M+Na]$^+$ pseudo-molecular ion, consistent with one chlorine atom. The HR-ESI-TOFMS established the molecular formula as C$_{19}$H$_{25}$O$_3$Cl revealing that 5 contained 7 double bond equivalents. A UV maximum of 229 nm suggested the presence of one or more conjugated diene or enone systems.

Examination of the $^1$H NMR spectrum of 5 (Table 3.3) revealed a methyl doublet at $\delta_H 1.02$, a methyl singlet at $\delta_H 1.76$, an unusual O-methyl at $\delta_H 2.91$, five resonances between 1-2 ppm accounting for six methylene protons, two midfield shifted methine protons at $\delta_H 3.32$ and $\delta_H 3.85$, as well as eight resonances between 5-6 ppm characteristic of olefinic protons. The $^{13}$C NMR and HSQC spectra revealed three quaternary ($\delta_C$ 171.7, 165.8, and 137.2), ten methine ($\delta_C$ 134.5, 133.9, 133.8, 128.4, 128.2, 127.2, 118.5, 91.2, 74.2 and 35.6), three methylene ($\delta_C$ 34.4, 33.0 and 28.5), one O-methyl ($\delta_C$ 55.1) and two aliphatic methyl ($\delta_C$ 21.2 and 12.9) carbons accounting for all 19 carbons required by the molecular formula.
One large $^1$H NMR spin system (fragment 5b) featuring a conjugated diene, a methyl substituted methine, an oxygen substituted methine (based on a chemical shift of $\delta_C$ 74.2) and an additional alkene moiety was assembled by COSY data (Figure 3.1). A second fragment, 5a, contained a methyl substituted vinyl chloride functionality, and was assembled based on HMBC correlations from H19 to C15 and H16 to C19. The chemical shift of C16 at $\delta_C$ 118.5 allowed for placement of the chlorine atom at this position. Fragment 5c possessed an enone with O-Me substitution at the $\beta$ carbon as revealed by HMBC correlations from H2 to C1 and C3 and H17 to C2 and C3. Fragment 5a was connected to the distal side of fragment 5b based on HMBC correlations from H14 to C15, C16, and C19 and from H16 to C14. Reciprocal HMBC correlations from H19 to C14 and C16 further corroborated this connection. Fragment 5c was connected to the proximal side of fragment 5b forming a $\beta$-methoxy-substituted pyranone ring based on HMBC correlations from H5 to C3 and from H4 to C1, C2 and C3, as well as HMBC correlations from H17 to C4 and from H2 to C5.

As before, the geometries of the double bonds were determined based on $^3J_{HH}$ coupling constants and NOE correlations. The C8-C9 and C13-C14 olefins were found to be $E$ on the basis of large $J$ values (14.8 Hz and 15.6 Hz, respectively), whereas the C10-C11 olefin was determined to be $Z$ ($^3J_{H10-11} = 10.5$ Hz). Lastly, the configuration of the C15-C16 olefin was determined to be $E$ based on an NOE correlation between H19 and H13. The absolute configurations at C5 and C12 of kimbelactone A (5) remain unknown as the compound decomposed before such studies could be undertaken.
Table 3.3. NMR spectroscopic data for kimbealactone A (5) in C$_6$D$_6$ ($^1$H at 600 MHz, $^{13}$C at 125 MHz).

<table>
<thead>
<tr>
<th>N$^a$</th>
<th>$\delta$$_C$$^{a,b}$</th>
<th>$\delta$$_H$ ($J$ in Hz)$^c$</th>
<th>COSY</th>
<th>HMBC</th>
<th>NOESY</th>
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<td>2</td>
<td>91.2 CH</td>
<td>4.97 s</td>
<td></td>
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<td>6a/b</td>
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<tr>
<td>3</td>
<td>171.7 qC</td>
<td></td>
<td>4b, 5</td>
<td></td>
<td>6b</td>
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<td>1.84 dd (16.6, 12.1)</td>
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<td>1, 2, 3, 5, 6</td>
<td>6a/b</td>
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<td>1.61 dd (16.8, 3.4)</td>
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<td>6b</td>
</tr>
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<td>5</td>
<td>74.2 CH</td>
<td>3.85 dddd (16.0, 11.9, 8.0, 4.0)</td>
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<tr>
<td>6a</td>
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<td>1.51 m</td>
<td></td>
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<td>4a</td>
</tr>
<tr>
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<td></td>
<td>1.14 m</td>
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<td>9, 11, 12</td>
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<td>13</td>
<td>134.5 CH</td>
<td>5.51 dd (15.7, 6.6)</td>
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<td>1.76 s</td>
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<td>14, 15, 16</td>
<td>13</td>
</tr>
</tbody>
</table>

$^a$ Recorded at 125 MHz. $^b$ Multiplicity deduced from HSQC. $^c$ Recorded at 600 MHz.
3.2.2 Biological activity

Biological evaluation of fractions arising from extracts of these two Curaçao and Papua New Guinea collections of tropical marine cyanobacteria revealed similar neuromodulatory activity profiles. The most potent bioactive fractions from both collections exhibited sodium channel blocking activity in murine Neuro-2a cells at 20 μg/mL and suppressed spontaneous calcium oscillations in murine neocortical neurons at 5 μg/mL. The lipopeptide janthielamide A (1), obtained as the main bioactive constituent of the Curaçao collection, exhibited sodium channel blocking activity in murine Neuro-2a cells with an IC\textsubscript{50} value of 11.5 μM. Janthielamide A was also found to antagonize veratridine-induced sodium influx in cerebrocortical neurons with an IC\textsubscript{50} value of 5.2 μM. From the Papua New Guinea collections, pure kimbeamide A (2) was found to exhibit sodium channel blocking activity in murine Neuro-2a cells at 20 μg/mL however, further biological evaluation of these latter lipopeptides was prevented by the rapid decomposition of 2-5 under either dry or organic solvent storage. Decomposition due to autoxidation is common in polyunsaturated compounds such as fatty acids, and thus, it is not surprising that these lipopeptides readily decomposed despite efforts to prevent autoxidation by cold storage in benzene.\textsuperscript{32,33}

3.2.3 Characterization of the cyanobacterial collections

The similar neuromodulatory activity profiles and related natural product skeletal types from these two collections suggested that the producing cyanobacteria might be closely related, despite their geographic distance and macroscopic morphological differences. Based on their growth morphologies as well as their filament and cell
morphologies, the kimbeamide- and the janthielamide-producing strains best fell under the taxonomic definition of the genus *Symploca*. However, phylogenetic inferences of the 16S rRNA gene sequenced from each collection revealed that these two natural product-producing strains form a separate clade that is evolutionarily distant (p-distance $= \sim 5\%$ gene sequence divergence) from the genus *Symploca* (reference strain: PCC 8002$^T$, GenBank acc. nr. AB03902). This clade of “tropical marine *Symploca*” have yielded several important natural products, including dolastatin 10 (e.g. strain VP377; GenBank acc. nr. AF306497),$^{34}$ symplostatin 1 (e.g. strain VP642b; GenBank acc. nr. AY032933),$^{34}$ and the hoiamides (strain PNG06-65.1; GenBank acc. nr. HM072001) (Figure 3.2).$^{35}$ Although strains of this group have been published as *Symploca*, the evolutionary divergence together with the distinct ecological habitats of these two groups suggests that this clade of “tropical marine *Symploca*” needs to be described as a new genus.

In addition, microscopic morphological and phylogenetic analyses indicated that the cyanobacterial collections from Kimbe Bay, Papua New Guinea consisted of a consortium of *Moorea producta*$^{36}$ (formerly *Lyngbya majuscula*) and “tropical marine *Symploca*”. However, because the biomass was primarily composed of the finer “*Symploca*” filaments, and the fact that the *M. producta* was only found in one of the kimbeamide-producing specimens, it is likely that these are metabolites of the “tropical marine *Symploca*” clade.
Figure 3.2. Molecular-phylogenetic inference of the kimbeamide-producing strains PNG 07/14/07-6.1 (JQ388599) and PNG 07/18/07-3 (JQ396912) from Papua New Guinea as well as the janthielamide-producing strain NAC 12/21/08-3 (JQ388601). The clade that includes the NP-producing strains is highlighted with a red box. The closest related group is the genus Symphocia (reference strain: PCC 8002T, GenBank acc. nr. AB039021) with a p-distance of 4.9% based on the SSU rRNA gene sequence divergence (highlighted with a gray box). The cladogram is based on SSU (16S) rRNA gene sequences using the bayesian (MrBayes) and maximum likelihood (PhyML) methods, and the support values are indicated as posterior probability at the nodes. The specimens are indicated as species, strain and access number in brackets. Specimens designated with (T) represent type-strains obtained from Bergey’s Manual. The scale bar is indicated at 0.03 expected nucleotide substitutions per site, corrected using the General Time Reversal (GTR) model.
3.2.4 Predicted biogenesis

It is tempting to speculate on the biogenesis of the compounds described here. Presumably, janthielamide A (1) and the kimbeamides A-C (2-4) arise via interdigitation of polyketide synthase and non-ribosomal synthetase modules, with each featuring a single probable amino acid residue, glycine in the case of janthielamide A and alanine in the case of kimbeamides A-C, flanked by acetate extensions on both sides; however, kimbelactone A appears to derive solely from polyketide synthase modules (Figure 3.3). The C5 and C9 methyl substituents of janthielamide A and the C12 and O-methyl groups of kimbelactone A appear to arise from S-adenosyl-L-methionine mediated enzymatic methylation, while the methyl substituent at C3’ in janthielamide A appears to arise from a 3-hydroxy-3-methylglutaryl-coenzyme A-like methyl addition.

**Figure 3.3.** Expected acetate and/or amino acid incorporation patterns for janthielamide A, kimbeamide A and kimbelactone A.
Analyses of the vinyl chloride termini reveal that two different types of vinyl chloride moieties are featured in janthielamide A and kimbeamides A-C. With regard to kimbeamides A-C (2-4) and kimbelactone A (5), which possess a methyl-substituted vinyl chloride moiety, and grenadamides B^{22} and C^{22}, which feature a propyl-substituted vinyl chloride, it is hypothesized that this functionality arises in a similar fashion to the pendant vinyl chloride moiety present in the jamaicamides A-C.^{20,38} This is likely occurring through a hydroxymethylglutaryl CoA synthase (HMGCS)-like addition of acetate to a β-keto-thioester intermediate, followed by chlorination, dehydration and decarboxylation. As for the terminal vinyl chloride functionality present in janthielamide A (1), kimbeamides A-C (2-4), grenadamide C^{22} and pitiamide A^{23}, it is conceivable that this moiety arises first via radical chlorination at the penultimate carbon of a β-hydroxy ACP thioester, followed by sulfation of the β-hydroxy group (Figure 3.4). This functionally dense system is then predicted to undergo a concerted thioester-mediated hydrolysis, decarboxylation and sulfate elimination similar to the mechanism shown for terminal alkene formation in curacin A.^{39,40}
3.3 Conclusions

The lipopeptides janthielamide A and kimbeamides A-C, as well as the pyranone kimbelactone A, were isolated from independent collections of marine cyanobacteria from Curaçao and Papua New Guinea. All of these new cyanobacterial natural products are characterized by multiple unsaturations and an intriguing terminal vinyl-chloride moiety. Both janthielamide A (1) and kimbeamide A (2) exhibited modest sodium channel blocking activity in murine Neuro-2a cells. Additionally, compound 1 antagonized veratridine-induced sodium influx in murine cerebrocortical neurons with an IC$_{50}$ value of 5.2 μM. Thus, these metabolites constitute new lead molecules in the development of potential neuromodulatory agents.

Similar compounds in this structure class, such as pitiamide A, grenadamide B and grenadamide C (Figure 3.5), have been reported without characterization of their absolute configurations, likely due to the difficulties in determining the chirality of
remote methyl substituents and the small quantities isolated. Following the initial isolation of pitiamide A, the absolute stereochemistry of the compound was determined using optical rotation calculations in concert with comparisons of the measured optical rotation and NMR data for the natural product with the data from synthetically prepared (7S,10R) and (7R,10R)-isomers of pitiamide A. The determination of absolute configuration of the lipopeptides presented here was accomplished using a variety of approaches including fragmentation and derivatization followed by chiral or achiral GCMS or achiral LCMS analyses. In the case of janthielamide A (1), hydrolysis and ozonolysis afforded two fragments, 2-methyl-succinic acid and 2-methyl-γ-aminobutyric acid, for which standards could be obtained commercially or via synthesis. Similarly, ozonolysis and hydrolysis of kimbeamide A (2) yielded alanine, which was readily analyzed and compared with amino acid standards. The generation of diastereomers via esterification of carboxylic acids with (S)-2-(-)-octanol, as previously explored by our group, has proven to be a robust method for determining the absolute configurations of small chiral fragments of natural products. Implementation of this methodology was key in the assignment of the chiral centers at C5 and C9 in 1, which had proven difficult to solve using standard chiral GCMS and LCMS methodologies. Additionally, the synthetic route utilized to generate the 2-methyl-γ-aminobutyric acid here could be further explored and optimized to provide a faster and more facile generation of this fragment than current methodologies, which are cumbersome and require the use of controlled substances.
Despite macroscopic morphological differences, the putative producing organisms were revealed by microscopic and phylogenetic analyses to be closely related, belonging to an as yet undescribed genus of tropical marine cyanobacteria closest related to *Symplaca*. The similarities in the biological activity profiles, secondary metabolite chemical structure classes, and microscopic morphological differences all suggested that these organisms may be evolutionarily closely related. Ongoing investigation of the chemistry of morphologically similar and phylogenetically related organisms will certainly continue to reveal intriguing chemotaxonomic relationships in the marine cyanobacteria.

Figure 3.5. Janthielamide A, kimbeamides A-C, kimbelactone A and structurally similar known compounds. Janthielamide A and kimbeamides A-C are comparable to pitiamide A and grenadamides A and B, which are also secondary metabolites from marine cyanobacteria. Meanwhile, kimbelactone A is structurally most analogous to fuligoic acid a chlorinated polyene-pyrone acid from a myxomycete.
Janthielamide A (1) and kimbeamides A-C (2-4), presumably of a mixed PKS/NRPS biosynthetic origin, further expand the halogenated acyl amide chemotype previously found in marine cyanobacteria, which includes pitiamide A$^{23}$ and grenadamides B$^{22}$ and C$^{22}$ (Figure 3.5). On the other hand, kimbelactone A (5) is most closely structurally-related to fuligoic acid, a chlorinated polyene-pyrone from a myxomycete.$^{43}$

3.4 Experimental Details

3.4.1 General experimental procedures

Optical rotations were measured with a Jasco P-2000 polarimeter. CD spectra were obtained on a Jasco J-815 CD spectrometer. UV spectra were obtained on a Beckman Coulter DU-800 spectrophotometer, and FT-IR spectra were recorded on a Nicolet IR 100 FT-IR spectrophotometer. NMR spectra were recorded on a Varian Inova 300 MHz or 500 MHz with a 5 mm probe, a Bruker Avance III DRX600 spectrometer equipped with a 1.7 mm MicroCryoProbe, or a Varian VX500 equipped with an XSens cold probe for direct observe $^{13}$C. NMR spectra were referenced to residual solvent $^1$H and $^{13}$C signals ($\delta$$_H$ 7.26, $\delta$$_C$ 77.16 for CDCl$_3$ and $\delta$$_H$ 7.16, $\delta$$_C$ 128.62 for C$_6$D$_6$). Low-resolution ESIMS spectra were acquired on a Finnigan LCQ Advantage Max mass spectrometer, while high-resolution ESIMS spectra were obtained by the UCSD Chemistry and Biochemistry Molecular MS Facility on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. HPLC separation was completed using a Waters HPLC system equipped with a Waters 515 binary pump, a Waters 996 PDA detector, and a
Phenomenex Maxil Silica column (10 μm, 10 × 500 mm). GCMS analysis was accomplished using a Thermo Electron Corporation Trace GC Ultra gas chromatograph with a Thermo Electron Corporation DSQ single quadrupole mass spectrometer, and either a chiral Cyclocil B Alltech capillary column (25 m × 0.25 mm) or an achiral DB-5 Agilent capillary column (30 m × 0.25 mm). LCMS analysis was accomplished using a Finnigan Surveyor LC Pump Plus, Autosampler Plus and PDA Plus Detector with an LCQ Advantage Max Mass Spectrometer and a Phenomenex Luna 5μ C18(2) (250 × 4.60 mm). Ozonolysis was conducted using a Yanco Industries LTD Ozone Services Ozone Lab OL80F/Basic Ozone Generator.

3.4.2 Biological material collection and identification

For the Curaçao collection, approximately 3 L of a lime green filamentous cyanobacterial mat was collected by hand at 1 m depth from a sandy reef substrate in Jan Thiel Bay on the leeward side of Curaçao (N 12°07.634´, W 68°88.088´). Following collection the cyanobacterium was stored in 1:1 EtOH:seawater and stored at −20°C; prior to shipping, the samples were thawed and the supernatant was discarded. For longer storage, EtOH was added and the samples were stored at −20°C until workup. A voucher specimen (NAC 12/21/08-3) is maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA.

For the Papua New Guinea collections, approximately 1.5 L of bright orange cyanobacterial puffballs (PNG 07/14/07-6) were collected by hand at 20 m depth from at least eight sites located throughout Kimbe Bay off the North coast of New Britain, Papua New Guinea (S 5°26.192´, E 150°40.813´). Field notes identified the puffballs as a
consortium of *Schizothrix* sp. with a minor amount of *Lyngbya* sp. present. A separate collection of 250 mL of the cyanobacterium (PNG 07/18/07-3) was made at 25 m depth in Kimbe Bay (S 5°19.588’, E 150°18.034’). The collected samples were soaked in 1:1 EtOH:seawater in the field; the supernatant was decanted and discarded before shipment. For longer storage, EtOH was added and the samples were stored at –20°C until workup. Voucher specimens [collection numbers PNG 07/14/07-6 (1.5 L collection) and PNG 07/18/07-3 (250 mL collection)] are maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA.

3.4.3 Morphological characterization

Morphological characterization was performed using an Olympus IX51 epifluorescent microscope (1000X) equipped with an Olympus U-CMAD3 camera. Morphological comparison and putative taxonomic identification of the cyanobacterial specimen was performed in accordance with modern classification systems.

3.4.4 DNA extraction, PCR amplification, and cloning

Algal biomass (~50 mg) was partly cleaned under an Olympus VMZ dissecting microscope. The biomass was pretreated using TE (10 mM Tris; 0.1M EDTA; 0.5 % SDS; 20 µg/mL RNase)/lysozyme (1 mg/mL) at 37°C for 30 min followed by incubation with proteinase K (0.5 mg/mL) at 50°C for 1 h. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer’s specifications. DNA concentration and purity was measured on a DU® 800 spectrophotometer (Beckman Coulter). The 16S rRNA genes were PCR-amplified from
isolated DNA using the modified lineage-specific primers, CYA106F 5’-CGGACGGGTGAGTAACGCCTGA -3’ and CSL1445R 5’-GGTAACGACTTCGCGGCTG -3’. The PCR reaction volumes were 25 µL containing 0.5 µL (~50 ng) of DNA, 2.5 µL of 10 × PfuUltra IV reaction buffer, 0.5 µL (25 mM) of dNTP mix, 0.5 µL of each primer (10 µM), 0.5 µL of PfuUltra IV fusion HS DNA polymerase and 20.5 µL dH₂O. The PCR reactions were performed in an Eppendorf® Mastercycler® gradient as follows: initial denaturation for 2 min at 95°C, 25 cycles of amplification, followed by 20 sec at 95°C, 20 sec at 55°C and 1.5 min at 72°C, and final elongation for 3 min at 72°C. PCR products were purified using a MinElute® PCR Purification Kit (Qiagen) before subcloning using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) following the manufacturer’s specifications. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and sequenced with M13 primers. The 16S rRNA gene sequences are available in the DDBJ/EMBL/GenBank databases under acc. No. JQ388601 (NAC 12/21/08-3), JQ396912 (PNG 07/18/07-3), JQ388599 (PNG 07/14/07-6.1), and JQ388600 (PNG 07/14/07-6.2).

3.4.5. Phylogenetic inference

The 16S rRNA gene sequences were aligned with evolutionary informative cyanobacteria using the L-INS-I algorithm in MAFFT 6.7174 and refined using the SSU secondary structures model for *Escherichia coli* J016955 without data exclusion. The best-fitting nucleotide substitution model optimized by maximum likelihood was selected using corrected Akaike/Bayesian Information Criterion (AICc/BIC) in jModeltest 0.1.146. The evolutionary histories of the cyanobacterial genes were inferred using Maximum
likelihood (ML) and Bayesian inference algorithms. The ML inference was performed using GARLI 1.0\textsuperscript{47} for the GTR+I+G model assuming a heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.494, shape parameter ($\alpha$) = 0.485, number of rate categories = 4) with 1,000 bootstrap-replicates. Bayesian inference was conducted using MrBayes 3.1\textsuperscript{48} with four Metropolis-coupled MCMC chains (one cold and three heated) ran for 3,000,000 generations. The first 25% were discarded as burn-in and the following data set were sampled with a frequency of every 100 generations. The MCMC convergence was detected by AWTY.

3.4.6 Extraction and isolation

Two of the three liters of EtOH-preserved biomass (884.6 g, dry weight) of the Curaçao collection of “tropical marine Symplaca” (NAC 12/21/08-3) were extracted with 2:1 DCM/MeOH eight times to give 2.77 g of crude extract (extract #1869). A portion of the crude extract (2.22 g) was fractionated using vacuum liquid chromatography (VLC) on Silica gel (Type H, 10-40 µM, Sigma-Aldrich) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A-I). Fraction D, eluted with 40% EtOAc in hexanes (307 mg), was subjected to two iterations of normal-phase chromatography using Si Varian Bond Elut Sep-Paks. During the first NP-SPE cartridge chromatography step, the initial condition of 2.5% EtOAc in hexanes eluted 185 mg of material. A second NP-SPE cartridge chromatography, employing a stepwise gradient of 100% hexanes to 100% EtOAc, a fraction (D1F) eluting with 5% EtOAc in hexanes gave compound 1 (34.2 mg, 1.5% of extract). Surrounding sub-fractions D1E (22.4 mg) and
D1G (6.2 mg) eluted with 2% and 10% EtOAc in hexanes, respectively, also contained 1, however these fractions contained additional impurities.

An EtOH-preserved consortium of “tropical marine Symploca sp.” and Moorea producta (PNG 07/14/07-6, 101.7 g dry weight) was extracted with 2:1 DCM/MeOH six times to give 1.82 g of crude extract (extract #1707), while an additional sample of “tropical marine Symploca sp.” (PNG 7/18/07-3, 5.97 g dry weight) was extracted with 2:1 DCM/MeOH five times to give 0.151 g of crude extract (extract #1708). Based on preliminary 1H NMR and LCMS analyses indicating that the extracts were virtually identical, the crude extracts were combined and fractionated using vacuum liquid chromatography (VLC) on Silica gel (Type H, 10-40 µM, Sigma-Aldrich) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A-I). Fraction D, eluted with 40% EtOAc in hexanes (159 mg) was subjected to two iterations of normal-phase chromatography using Si Varian Bond Elut Sep-Paks. In the first round of NP-SPE column chromatography, the majority of the material (127 mg) eluted under the loading condition of 30% EtOAc in hexanes. The second round of NP-SPE cartridge chromatography utilizing a stepwise gradient of 100% hexanes to 100% EtOAc was more successful. Sub-fraction D1E (24.7 mg), eluted with 8% EtOAc in hexanes, was then fractionated using normal-phase HPLC with an isocratic condition of 18.5% EtOAc in hexanes at 6 mL/min on a Phenomenex Maxil 10 Silica (500 × 10.0 mm, 10 µ) column to yield 2 (peak centered at 19.5 min, 1.6 mg, 0.08% of extract), 3 (peak centered at 15.5 min, 1.4 mg, 0.07% of extract) 4 (peak centered at 21 min, 0.8 mg, 0.04% of extract) and 5 (peak centered at 33 min, 1.0 mg, 0.05% of extract. Sub-fraction D1F (14.4 mg), eluted with 11% EtOAc in hexanes, was then fractionated using normal-phase HPLC with an
isocratic condition of 30% EtOAc in hexanes at 6 mL/min on a Phenomenex Maxil 10 Silica (500 × 10.0 mm, 10 µ) column to yield 5 (peak centered at 17.5 min, 2.6 mg, 0.13% of extract).

3.4.7 Characterization of purified compounds

Janthielamide A (1): 34.2 mg (1.54%) dark yellow amorphous solid \([\alpha]^{24}_D +10.2\) (c 0.60, CHCl₃), UV (MeCN) \(\lambda_{\text{max}} (\log \varepsilon) 224 (4.4) \text{ nm};\) IR \(\nu_{\text{max}} (\text{film}) 3297, 2961, 2925, 1732, 1667, 1540, 1452, 1376, 1259, 1180, 973, 851, 755, 716 \text{ cm}^{-1};\) \(^1\)H and \(^{13}\)C NMR data, see Table 1; ESIMS \(m/z 310 (100\% \text{ rel. abund.}) [M+H]^+;\) HRESI-TOFMS \(m/z [M+H]^+ 310.1934\) (calcd for C₁₈H₂₉NOCl, 310.1932).

Kimbeamide A (2): 1.6 mg (0.10%) pale yellow oil \([\alpha]^{23}_D +44.0\) (c 0.05, MeOH); UV (MeOH) \(\lambda_{\text{max}} (\log \varepsilon) 250 (4.0) \text{ nm};\) IR \(\nu_{\text{max}} (\text{film}) 3290, 2926, 2855, 1718, 1660, 1541, 1449, 1373, 1255, 1140, 1115, 1078, 1001, 936 \text{ cm}^{-1};\) \(^1\)H and \(^{13}\)C NMR data, see Table 2; ESIMS \(m/z 328 (28\% \text{ rel. abund.}) [M+H]^+, 373 (100\% \text{ rel. abund.}) [M+2 Na]^+, 655 (5\% \text{ rel. abund.}) [2 M+H]^+, 677 (14\% \text{ rel. abund.}) [2 M+Na]^+, 700 (6\% \text{ rel. abund.}) [2 M+2 Na]^+;\) HRESI-TOFMS \(m/z [M+H]^+ 328.1234\) (calcd for C₁₇H₂₄NOCl₂, 328.1229).

Kimbeamide B (3): 1.4 mg (0.09%) pale yellow oil \([\alpha]^{23}_D +33.4\) (c 0.17, DCM); \(^1\)H NMR data see Supporting Information; ESIMS \(m/z 328 (13\% \text{ rel. abund.}) [M+H]^+, 373 (100\% \text{ rel. abund.}) [M+2Na]^+\).

Kimbeamide C (4): 0.8 mg (0.05%) pale yellow oil; \(^1\)H and \(^{13}\)C NMR data, see Table S2 in Supporting Information; ESIMS \(m/z 328 (23\% \text{ rel. abund.}) [M+H]^+, 350 (11\% \text{ rel. abund.}) [M+Na]^+, 373 (100\% \text{ rel. abund.}) [M+2 Na]^+, 677 (47\% \text{ rel. abund.}) [2 M+Na]^+\).
Kimbelactone A (5): 3.3 mg (0.20%) pale yellow oil \([\alpha]^{23}_D -164.7\) \((c 0.17, DCM)\); UV (MeCN) \(\lambda_{\text{max}} (\log \varepsilon) 227 (4.2)\) nm; \(^1\)H and \(^{13}\)C NMR data, see Table 3; ESIMS \(m/z\) 301 (2% rel. abund.) \([M-\text{Cl}]^+, 337 (\text{<1\% rel. abund.}) [M+H]^+, 359 (9\% rel. abund.) [M+Na]^+, 695 (100\% rel. abund.) [2 M+Na]^+\); HRESI-TOFMS \(m/z\) \([M+Na]^+\) 359.1383 (calcd for \(C_{19}H_{25}O_3\text{ClNa, 359.1384}\)).

3.4.8 Stereoanalysis of C5 in janthielamide A (1)

Janthielamide A (1) was hydrolyzed (2.8 mg of 1 in 1 mL of 6\(N\) HCl, 110\(^\circ\)C, 15 h), dried under a stream of \(N_2\) gas and then ozonized (1.5 mL DCM, 20 min, \(-78^\circ\)C) with oxidative workup (5 drops 30\% \(H_2O_2\)). The product was dried and then divided into two parts. The ozonized hydrolysate (0.8 mg) was treated with 300 \(\mu\)L \((S)-(+)-2\)-octanol and 150 \(\mu\)L of acetyl chloride, and heated at 110\(^\circ\)C for 4 h to generate the di(octan-2-yl) \(2\)-methylsuccinate diastereomer. The excess reagent was evaporated under a constant stream of \(N_2\) gas, and the dried residue was resuspended in DCM. Both \((R)-(+)\)-methyl succinic acid (2.1 mg) and \((S)-(-)\)-methyl succinic acid (1.3 mg) were derivatized using the same conditions as described for the reaction product of 1 to furnish the standards. All samples were analyzed by GCMS under identical conditions; the initial oven temperature of \(40^\circ\)C was held for 1 min, then the temperature was increased to \(200^\circ\)C at a rate of 4.0\(^\circ\)C/min and held for 20 min. Co-injection of the derivatized \((R)\)-methyl succinic acid standard with the derivatized fragment from 1 gave one peak at 46.06 min, while co-injection of the \((S)\)-methyl succinic acid standard with the derivatized fragment from 1 gave two peaks at 44.93 min \((S)\) and 46.05 min (janthielamide A). Thus, the absolute configuration at C5 was assigned as \(R\).
3.4.9 Stereoanalysis of C9 in janthielamide A (1)

The desired fragment for stereoanalysis of C9, N-acetyl protected and (S)-2-octanoyl-esterified 2-methyl-γ-aminobutyric acid (GABA), was obtained in addition to the derivatized 2-methyl-succinic acid under the conditions employed for stereoanalysis of C5 from the initial aliquot of 1 {HR-ESI-TOFMS m/z 294.2035 [M+Na]^+ (calcd C_{15}H_{29}NO_{3}Na, 294.2040)}. The (R)-2-methyl-GABA standard was obtained via oxidation of N-acetyl protected (R)-4-amino-2-methyl-1-butanol. To (R)-4-amino-2-methyl-1-butanol (100 mg, 106.4 μL) was added 4-dimethylaminopyridine (11.8 mg, 0.097 mmol, 0.1 eq), pyridine (3.34 mL) and acetic anhydride (3.34 mL). The reaction mixture was stirred 15 h at rt, then dried under a stream of N₂ gas affording the diprotected (amine and alcohol) fragment. The residue was resuspended in 2:1 1,4-dioxane:H₂O (8.66 mL) and LiOH·H₂O (407 mg, 9.7 mmol, 10 eq) was added. After 1.5 h, solvents were removed under vacuum and the residue was resuspended in H₂O (2 mL), acidified to pH 1 with 6N HCl and extracted with EtOAc (3 ×3 mL). The combined organic layer was dried over Na₂SO₄ and evaporated to dryness under a stream of nitrogen to give the N-acetyl protected fragment. To N-acetyl protected (R)-4-amino-2-methyl-1-butanol in acetone (1 mL) at 0°C was added dropwise Jones’ reagent (400 μL, prepared by dissolving 2.7 g CrO₃ in 2.3 mL of conc. H₂SO₄ and diluting to 10 mL at 0°C). After 1 h at 0°C, isopropanol (800 μL) was added and the mixture was stirred for 15 min. A small volume of H₂O was added and the organic solvents were removed via a stream of nitrogen. A small volume of acetonitrile was added and the solution was vortexed for 5 sec, then the organic layer was removed and the process was repeated two
additional times until the chromium residue appeared dry. The combined acetonitrile layers were dried under N\textsubscript{2} gas. (S)-2-octanol (200 \mu L) and acetyl chloride (100 \mu L) were added to the N-protected GABA and the reaction was sealed and warmed to 110\textdegree C. After 4 h, the reaction was cooled and the dried under N\textsubscript{2} gas, thus affording the N-acetyl (S)-2-octanol esterified (R)-2-methyl-GABA standard \{HR-ESI-TOFMS \textit{m/z} 294.2039 \textit{[M+Na]}\textsuperscript{+} (calcd for C\textsubscript{15}H\textsubscript{29}NO\textsubscript{3}Na, 294.2040)\}.

(S)-4-amino-2-methyl-1-butanol was obtained from (R)-(\textsuperscript{-})-3-bromo-2-methyl-1-propanol via an \textit{S\textsubscript{2}N}2 reaction in which the bromine was replace with cyanide, followed by reduction with LiAlH\textsubscript{4}. To sodium cyanide (0.076 g, 1.55 mmol, 1.1 eq) was added DMSO (2.24 mL) and (R)-(\textsuperscript{-})-3-bromo-2-methyl-1-propanol (148 \mu L, 1.41 mmol). After 24 h, the reaction was quenched with H\textsubscript{2}O (2.2 mL) and extracted with EtOAc (3 \times 3 mL). The aqueous layer was acidified to pH 3 with 10\% H\textsubscript{2}SO\textsubscript{4} and extracted with EtOAc (3 \times 3 mL). The organic layers were combined, washed with brine, dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated to dryness under N\textsubscript{2} gas. The residue was resuspended in THF (3.5 mL) and added dropwise to a solution of LiAlH\textsubscript{4} (0.271 g, 7.40 mmol, 5.25 eq) in THF (3.7 mL) at 0\textdegree C. The reaction was allowed to warm to rt and stirred 19 h. The reaction was cooled to 0\textdegree C and quenched with H\textsubscript{2}O (3 mL) and a few drops of 10\% NaOH. After stirring 30 min, a small amount of Na\textsubscript{2}SO\textsubscript{3} was added and the mixture was stirred for 10 min and then filtered over qualitative paper \#1. The filtrate was dried under N\textsubscript{2} to yield (S)-4-amino-2-methyl-1-butanol \{HR-ESI-TOFMS \textit{m/z} 104.1072 \textit{[M+H]}\textsuperscript{+} (calcd for C\textsubscript{5}H\textsubscript{14}NO, 104.1070)\}. The N-acetyl (S)-2-octanol esterified (S)-2-methyl-GABA standard \{HR-ESI-TOFMS \textit{m/z} 294.2042 \textit{[M+Na]}\textsuperscript{+} (calcd for C\textsubscript{15}H\textsubscript{29}NO\textsubscript{3}Na, 294.2040)\} was obtained as described above for the (R)-2-methyl-GABA standard from
(S)-4-amino-2-methyl-1-butanol. The standards and natural product derived fragment were resuspended in MeCN at a concentration of 1 mg/mL and subjected to analysis by LCMS. At a 0.4 ml/min flow rate, the run conditions were as follows: 60% acidified water (0.1% formic acid in 99.9% H₂O):40% MeCN held for 15 min, then ramped to 35% acidified water:65% MeCN over 90 min, then ramped to 100% MeCN over 10 min and held for 15 min, then ramped back to 60% acidified water:40% MeCN over 10 min and held 10 min. Co-injection of the derivatized (R)-2-methyl-GABA standard with the derivatized fragment from 1 gave one peak at 71.47 min, while co-injection of the (S)-2-methyl-GABA standard with the derivatized fragment from 1 gave two peaks at 70.47 min (S) and 71.51 min (janthielamide A). Thus, the absolute configuration at C9 was assigned as R.

3.4.10 Stereoanalysis of C1’ in kimbeamide A (2)

Kimbeamide A (2) was ozonized (0.2 mg of 2 in 400 μL DCM, 5 min, –78°C) with oxidative workup, dried under a stream of N₂ gas, and hydrolyzed (1 mL of 6 N HCl, 110°C, 16 h). The hydrolysate was dried under a constant stream of N₂ gas and treated with 150 μL of acetyl chloride and 500 μL of IPA and heated at 110°C for 1 h to generate the isopropyl ester of alanine. The excess reagent was evaporated under a constant stream of N₂ gas, and the dried residue was derivatized with trifluoroacetic anhydride (400 μL) in DCM (400 μL) at 110°C for 15 min. The N-(trifluoroaceto)isopropyl ester of the alanine residue was solubilized in EtOAc. Both L- and D-alanine (0.5 mg each) were derivatized using the same conditions as described for the ozonized hydrolysate of 2 to furnish standards. All samples were analyzed by
chiral GCMS under identical conditions; the initial oven temperature of 50°C was held for 3 min, then increased to 90°C at a rate of 1°C/min and held for 10 min. Co-injection of the derivatized L-alanine standard with the derivatized fragment from 2 gave one peak at 18.82 min, while co-injection of the D-alanine standard with the derivatized fragment from 2 gave two peaks at 16.87 min (D-alanine) and 18.82 min (kimbeamide A).

3.4.11 Sodium channel activation and blocking assay

Neuro-2a cells were added to 96-well plates at 3.0 x 10^5 cells/mL of RPMI 1640 medium with 10% FBS and 1% Penicillin/Streptomycin. The cells, in a volume of 200 μL per well, were incubated (37°C, 5% CO₂) overnight to allow recovery before treatment with compounds. Compounds were dissolved in DMSO to a stock concentration of 10mg/mL. Working solutions of the compounds were made in RPMI 1640 medium without FBS, with a volume of 10 μL added to each well to give a final compound concentration of 20 μg/mL. An equal volume of RPMI 1640 medium without FBS was added to 16 wells designated as negative controls for each plate. Brevetoxin-2, at a final concentration of 0.435 μg/mL, was used as the positive control for the sodium channel activating assay and tetrodotoxin, at a final concentration of 0.0435 μg/mL, was the positive control for the blocking assay. Eight wells were used for each treatment. A mixture of ouabain, veratridine and HCl/PBS was applied to the bottom half of each plate to cause sodium overload to varying degrees for the blocking and activating assays. For the blocking assay, a solution of 5 mM ouabain/0.35 mM veratridine/0.75 mM HCl/PBS was added and for the activating assay a solution of 5 mM ouabain/0.15 mM
veratridine/1.75 mM HCl/PBS was added. A solution of PBS/5 mM HCl without ouabain or veratridine was added to the top half of each plate to give the general toxicity of the test compounds unrelated to sodium channel regulation. The final volume for each well was 230 μL. Plates were incubated for approximately 16 h before staining with MTT for the activating assay. For the blocking assay, plates were incubated for approximately 24 h before MTT staining. Using a ThermoElectron Multiskan Ascent plate reader, plates were read at 570 and 630 nm. Concentration-response graphs were generated using GraphPad Prism.

3.4.12 Neocortical neuron culture

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice as described elsewhere. Briefly, pregnant mice were euthanized by CO₂ asphyxiation, and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle’s minimal essential medium with Earle’s salt (MEM) and supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin, and 0.10 mg/mL streptomycin (pH 7.4). Cells were plated onto poly-L-lysine-coated 96-well (9 mm), clear-bottomed, black-well culture plates (Costar) at a density of 1.5 × 10⁵ cells/well. Cells were then incubated at 37°C in a 5% CO₂ and 95% humidity atmosphere. Cytosine arabinoside (10 mM) was added to the culture medium on day 2 after plating to prevent proliferation of
nonneuronal cells. The culture media was changed every other day, starting from day 5 in vitro, using a serumfree growth medium containing Neurobasal Medium supplemented with B-27, 100 IU/mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM l-glutamine. Neocortical cultures were used in experiments between 8–13 days in vitro (DIV). All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.

3.4.13 Intracellular sodium concentration measurement

$[\text{Na}^+]_i$ measurement and full in situ calibration of sodium-binding benzofuran isophthalate (SBFI) fluorescence ratio were performed as described previously.49 Cells grown in 96-well plates were washed four times with Locke's buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM MgCl$_2$, 2.3 mM CaCl$_2$, 0.1 mM glycine, pH 7.4) using an automated microplate washer (BioTek Instruments, Winooski, VT). After measuring the background fluorescence of each well, cells were incubated for 1 h at 37°C with dye-loading buffer (100 μL/well) containing 10 μM SBFI-AM (Invitrogen) and 0.02% Pluronic F-127 (Invitrogen). Cells were then washed five times with Locke's buffer, leaving a final volume of 150 μL in each well. The plate was then transferred back to the incubator for 15 min to allow the cells to equilibrate after washing and then placed in a FlexStation II (Molecular Devices, Sunnyvale, CA) chamber to detect Na$^+$-bound SBFI emission at 505 nm (cells were excited at 340 and 380 nm). Fluorescence readings were taken once every 5 s for 60 s to establish the baseline, and then 50 μL of drug was added to each well from the compound plate at a rate of 26 μL/s, yielding a final volume of 200 μL/well. After correcting for background fluorescence,
SBFI fluorescence ratios (340/380) versus time were analyzed, and time- or concentration-response graphs were generated using GraphPad Prism (GraphPad Software Inc., San Diego, CA).
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Author Contributions

JKN and NE performed research. TB, ZC, and SVJ conducted bioassays. JKN, NE, ARP, TFM, and WHG designed research and wrote the manuscript.

The authors declare no conflicts of interest.

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### Table 3.6.1. $^1$H NMR data for kimbeamide A (2), kimbeamide B (3) and kimbeamide C (4) at 600 MHz in C$_6$D$_6$.

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Table 3.6.2. NMR spectroscopic data for kimbeamide B (3) in C$_6$D$_6$ ($^1$H at 600 MHz, $^{13}$C at 125 MHz).

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<th>N$^a$</th>
<th>$\delta_c$$^{a,b}$</th>
<th>$\delta_h$ ($J$ in Hz)$^c$</th>
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<th>HMBC</th>
<th>NOESY</th>
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<td>1', 2'</td>
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$^a$ Recorded at 125 MHz. $^b$ Multiplicity deduced from HSQC. $^c$ Recorded at 600 MHz.
Figure 3.6.1. $^1$H NMR spectrum of janthielamide A (1), recorded in CDCl$_3$ at 500 MHz.
Figure 3.6. $^{13}$C NMR spectrum of janthielamide A (1), recorded in CDCl$_3$ at 75 MHz.
Figure 3.6.3. HMBC spectrum of janthielamide A (1), recorded in CDCl$_3$ at 500 MHz.
Figure 3.6.4. HSQC spectrum of janthielamide A (1), recorded in CDCl$_3$ at 500 MHz.
Figure 3.6.5. COSY spectrum of janthielamide A (1), recorded in CDCl$_3$ at 500 MHz.
Figure 3.6. $^1$H NMR of kimbeamide A (2), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.7. $^{13}$C NMR spectrum of kimbeamide A (2), recorded in C$_6$D$_6$ at 125 MHz.
Figure 3.6.8. HMBC spectrum of kimbeamide A (2), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.9. HSQC spectrum of kimbeamide A (2), recorded in C₆D₆ at 600 MHz.
Figure 3.6.10. COSY spectrum of kimbeamide A (2), recorded in C₆D₆ at 600 MHz.
Figure 3.6.11. NOESY spectrum of kimbeamide A (2), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.12. $^1$H NMR spectrum of kimbeamide B (3), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.13. $^{13}$C NMR spectrum of kimbeamide B (3), recorded in C$_6$D$_6$ at 125 MHz.
Figure 3.6.14. HMBC spectrum of kimbeamide B (3), recorded in C₆D₆ at 600 MHz.
Figure 3.6.15. HSQC spectrum of kimbeamide B (3), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.16. COSY spectrum of kimbeamide B (3), recorded in C₆D₆ at 600 MHz.
Figure 3.6.17. NOESY spectrum of kimbeamide B (3), recorded in C₆D₆ at 600 MHz.
Figure 3.6.18. $^1$H NMR spectrum of kimbeamide C (4), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.19. $^1$H NMR spectrum of kimbelactone A (5), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.20. $^{13}$C NMR spectrum of kimbelactone A (5), recorded in C$_6$D$_6$ at 125 MHz.
Figure 3.6.21. HMBC spectrum of kimbelactone A (5), recorded in C₆D₆ at 600 MHz.
Figure 3.6.22. HSQC spectrum of kimbelactone A (5), recorded in C$_6$D$_6$ at 600 MHz.
Figure 3.6.23. COSY spectrum of kimbelactone A (5), recorded in C₆D₆ at 600 MHz.
Figure 3.6.24. NOESY spectrum of kimbelactone A(5), recorded in C6D6 at 600 MHz.
Figure 3.6.25. (A) Achiral GCMS total ion chromatograms for the derivatized methylsuccinic acid fragment from janthielamide A (I) in comparison with standards. (B) Mass spectrum of janthielamide A derivatized methylsuccinic acid fragment (RT:46.05 min).
Figure 3.6.26. (A) Achiral LCMS selective ion scan (m/z 271-273) chromatograms for the derivatized 2-methyl-γ-aminobutyric acid (GABA) fragment from janthielamide A (1) in comparison with standards. (B) Mass spectrum of janthielamide A derivatized (GABA) fragment (RT:71.47 min).
Figure 3.6.27. (A) Chiral GCMS selective ion scan (m/z 139-141) chromatograms for the derivatized alanine residue from kimbeamide A (2) in comparison with standards. (B) Mass spectrum of kimbeamide A derivatized alanine fragment (RT:18.82 min).
Figure 3.6.28. CD spectrum for kimbelactone A (5) in MeCN.

Figure 3.6.29. Chem 3D modeling of kimbelactone A (5). The circular dichroism spectrum of compound 5 revealed a bisignate couplet interpreted to result from interactions between the two conjugated diene systems across C12 in which the cotton effect (CE) at longer wavelengths was negative, while the CE at shorter wavelengths was positive, thus suggesting negative chirality. Molecular modeling studies in Chem3D taking into account the dihedral torsion angles between H11 and H12 as well as H12 and H13 allowed for determination of the lowest energy conformation for both the (12R)- and (12S)-isomers of 5. The dihedral torsion angles were determined by measuring the $^3J_{HH}$ coupling constants and comparing the values with the Karplus curve. Analysis of the relative positions of the dienes to one another allowed for the tentative assignment of the absolute configuration of the methyl substituent as $S$. Confirmation of this assignment was impeded due to decomposition of compound 5, thus the absolute configuration of C12 and C5 remain unknown.
Figure 3.6.30. Macroscopic photographs of PNG 07/14/07-6.

Figure 3.6.31. Microscopic photographs of filaments from cyanobacterial collections from Curaçao (NAC 12/21/08-3) and Papua New Guinea (PNG 07/18/07-3, PNG 07/14/07-6.2, 07/14/07-6.1). In PNG 07/14/07-6.2, arrows indicate filaments of *Moorea producta* interspersed with the *Symploca*-like filaments.
3.7 CHAPTER 3
REFERENCES


(9) Alam, M.; Shimizu, Y.; Ikawa, M.; Sasner, J. J. Reinvestigation of the toxins from the blue-green alga, Aphanizomenon flos-aquae, by a high performance


(19) A MarinLit search of chlorine-containing compounds revealed 166 unique compounds, while a search for bromine-containing compounds yielded 58 distinct compounds.


4.0 CHAPTER 4

ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL ACTIVITY OF TASIHALIDE C FROM A CURAÇAO COLLECTION OF CYANOBACTERIA

4.01 Abstract

A new member of the tasihalide family of brominated and iodinated diterpenes, tasihalide C, is reported here from a collection of marine cyanobacteria obtained in Curaçao that previously gave rise to the novel lipopeptide, janthielamide A. This collection of cyanobacteria has also been found to contain the known compounds tasihalide B and dolastatin 10, the latter being a potent antitumor agent. Previously, no biological activity has been reported for the tasihalides. Herein, we report modest anti-inflammatory activity in LPS-induced murine macrophage cells RAW264.7 for tasihalide C with an IC\textsubscript{50} of 1.3 μM.

4.1 Introduction

Terpenes and sterols, organic small molecules comprised of five carbon isoprene units typically arranged in a head to tail fashion, represent one of the most prevalent structure classes observed from marine organisms. In 1904, the first sterol obtained from the marine environment was isolated from the sponge *Suberites domuncula* by Martin Henze.\textsuperscript{1} However, structure elucidation for this compound, known as spongosterol, was not completed until 1945 when Werner Bergmann and co-workers re-isolated the compound from *Suberites compacta*. Comparison of the properties of the newly isolated compound with those of spongosterol, revealed that the compound was actually a mixture
of cholestanol and neospongosterol\(^2\). The pioneering investigations of marine sponge\(^3\) and mollusk\(^4\) sterols by Bergmann in the 1930s, and his student Leon Ciereszko’s later explorations of terpenoids from gorgonians\(^5\) in the 1960’s, incited the field of marine isoprenoid chemistry. Since then, scores of novel terpenoids have been isolated from sponges, gorgonians, soft corals, algae, and opisthobranch mollusks, among others.\(^6\)-\(^14\)

While some terpenes from terrestrial environs are found unaltered in the marine realm, many others are present as optical enantiomers of their terrestrial counterparts\(^6\) or feature unique modifications, including chlorination,\(^6\),\(^12\) bromination\(^6\),\(^12\) and bromonium ion-induced cyclization,\(^15\),\(^16\) or unusual functional groups such as isonitriles, isocyanates isothiocyanates, and dichloroimines.\(^13\),\(^14\),\(^17\),\(^18\) Additionally, carbon skeletons including the sesquiterpene chamigrenes and the diterpene amphilectanes and cembranes that are atypical in terrestrial environs are prevalent in the marine realm.\(^13\)

A number of terpenes from marine macroorganisms have been found to exhibit ecologically relevant activities with roles in predation deterrence, competition, anti-fouling and reproduction (Figure 4.1).\(^19\),\(^20\) For example, flexibilide and dihydroflexibilide from the soft coral \textit{Sinularia flexibilis}\(^21\) are thought to play a role in chemical defense as these compounds have been found to have ichthyotoxic activities, as well as necrotizing effects on scleractinian coral tissue and lethality to fertilized eggs of hard corals.\(^19\),\(^22\),\(^23\) The sesquiterpene 7-deacetoxy-olepupuane from the marine sponge \textit{Dysidea} sp.\(^24\) exhibits duality as well playing a role in both spatial competition and predation deterrence causing tissue necrosis in \textit{Cacospongia} sp. and ichthyotoxicity, respectively.\(^25\) Meanwhile, a chlorinated monoterpenic, chloromertensene from the red alga \textit{Plocamium}
*hamatum* is one of the causative agents of tissue necrosis in the soft coral *Sinularia cruciata*.\(^{26,27}\)

**Figure 4.1.** Marine-derived terpenes with known ecological roles.

In addition to ecologically important activities, many marine-derived terpenes possess potent pharmacologically relevant properties, including antibacterial, anti-inflammatory, anti-oxidant, antiparasitic and cytotoxic activities (Figure 4.2).\(^{13,28-31}\) A number of marine-derived terpenes, including laurinterol and debromolaurinterol, phenolic sesquiterpenes from various species of the red algal genus *Laurencia*,\(^{32,33}\) exhibit antibiotic activities, while other terpenes, such as haliconadin C, a sesquiterpene from the sponge *Halichondria* sp.\(^{34}\), and bromosphaerone, a diterpene from the red alga *Sphaerococcus coronopifolius*,\(^{35}\) display potent antibacterial activities. Additionally, several compounds including the pseudopterosins,\(^{36-39}\) which are diterpene glycosides with an amphilectane skeleton from the gorgonian coral *Pseudopterogorgia* sp., and manoalide, which is a sesterterpene from the sponge *Luffariella variabilis*,\(^{40}\) exhibit anti-inflammatory activity.\(^{41,42}\) Similarly, the sesquiterpene axisonitrile-3\(^{43,44}\) and the diterpene kalihinananes from sponges belonging to the genus *Acanthella* sp.,\(^{45,46}\) as well as diisocyanoadociane, a diterpene from the sponge *Cymbastela hooperi*,\(^{47,48}\) each feature at
least one isocyanate and display anti-parasitic activity against *Plasmodium* sp. Lastly, eleutherobin, from the soft coral *Eleutherobia* sp.\(^49\) and sarcodictyin from the stoloniferan coral *Sarcodictyon roseum*,\(^{50,51}\) both diterpene glycosides with mechanisms of action similar to paclitaxel (Taxol\(^\text{®}\)), are just two of many examples of terpenes with potent antitumor activities.\(^{13,31}\)
Herein, a new member of the brominated and iodinated diterpene family of compounds known as the tasihalides\textsuperscript{52} is reported. NMR and MS guided fractionation of
a chromatographic fraction from an extract of a Curaçao collection of cyanobacteria that previously yielded janthielamide A\textsuperscript{53} gave rise to the novel compound tasihalide C (I). This compound features an additional acetoxy functional group in comparison to the known compound tasihalide B, which was also isolated as a minor constituent. Tasihalide C exhibits modest anti-inflammatory activity in LPS-induced murine macrophage RAW cells with an IC\textsubscript{50} of 1.3 μM.

4.2 Results and Discussion

4.2.1 Collection, isolation and structure elucidation

A green filamentous cyanobacterial mat was collected by hand at a depth of 1 m in Jan Thiel Bay, Curaçao in 2008. Repetitive extraction of the biological material with 2:1 DCM:MeOH resulted in a crude lipophilic extract which was subjected to vacuum liquid chromatography on silica gel using a stepwise gradient from non-polar to polar to obtain nine fractions. The fraction that eluted with 60% EtOAc in hexanes was found to increase calcium oscillations in murine neocortical neurons at 5 μg/mL. Subsequent NMR- and MS-guided fractionation employing normal phase Silica SPE cartridges and HPLC led to the isolation of the new compound tasihalide C as an optically active amorphous pale yellow solid {13.1 mg, 0.6%, [α]_D -26.9 (c 0.60, MeOH)}, and the known compound tasihalide B.

The LR-ESI-MS spectrum of I revealed a 1:1 ratio at m/z 807/809 for the [M+Na]\textsuperscript{+} pseudo-molecular ion consistent with the presence of a single bromine atom. The \textsuperscript{1}H NMR spectrum of I (Table 4.1) contained 1 doublet methyl (δ\textsubscript{H} 1.04), 8 singlet methyls (δ\textsubscript{H} 1.93, 1.86, 1.70, 1.59, 1.541, 1.536, 1.15 and 0.97), two diastereotopic
deshielded methylenes ($\delta_H$ 4.03 and 3.11; 2.49 and 2.32), six downfield shifted methines ($\delta_H$ 6.00, 5.58, 5.56, 5.26, 4.97 and 4.85) and five midfield methylenes ($\delta_H$ 3.15, 3.06, 2.29, 1.98 and 1.96). The $^{13}$C NMR spectrum of 1 included 8 quaternary ($\delta_C$ 170.2, 169.1, 168.8, 168.5, 168.2, 77.1, 75.3 and 46.1), 11 methine ($\delta_C$ 79.2, 74.2, 74.1, 73.7, 70.1, 53.4, 50.8, 46.0, 38.6, 33.4 and 31.2), 2 methylene ($\delta_C$ 39.6 and 15.1) and nine methyl ($\delta_C$ 26.9, 21.8, 20.8, 20.7, 20.22, 20.18, 18.6, 18.4 and 18.1) carbons. The presence of five acetoxy moieties was suggested based on $^1$H and $^{13}$C NMR data as described above, including five sharp methyl singlets (1.5-2 ppm), five oxygenated methine (70-80 ppm) and five ester carbonyls (168-170 ppm).

Meanwhile, the upfield shifted methylene at $\delta_C$ 15.1 suggested the presence of an iodine atom, which was further supported by the observed loss of 128 in the MS²/MS³ of compound 1 indicative of an [M-HI]$^+$ adduct. The HR-ESI-TOFMS of 1 gave an [M+Na]$^+$ adduct at $m/z$ 807.0847 (calcd for C$_{30}$H$_{42}$O$_{11}$BrINa, 807.0847) establishing the molecular formula as C$_{30}$H$_{42}$O$_{11}$BrI and revealing that 1 contained nine double bond equivalents. The inclusion of iodine and bromine together with the presence of multiple acetoxy groups is characteristic of only one family of known compounds from marine cyanobacteria, the tasihalides, which were first described by Williams et al. in 2003 from a consortium of Symploca sp. with trace amounts of red algae. Comparison of the $^1$H and $^{13}$C NMR chemical shift data and molecular weight of our compound with the previously described tasihalide B, suggested that our compound featured an additional acetoxy group.
Comprehensive analyses of the COSY and HMBC data led to the elucidation of the planar structure of tasihalide C (Figure 4.3). COSY correlations revealed large portions of the diterpene core structure, while HMBC correlations allowed for placement of the methyl and acetoxy substituents and closure of the ring systems. In particular, HMBC correlations from H21 to C4, C5, C6 and C10 provided for the assignment of the C21 methyl as a substituent on C5 and closure of the decalin ring system. Similarly, HMBC correlations from H26 to C8, C13 and C14 allowed for placement of the C26 methyl as a substituent on C14 and closure of the six-membered ring. A COSY correlation from H17 to H16 a/b combined with HMBC correlations from H17 to C11 and C15, as well as HMBC correlations from H16b to C11 and from H16a/b to C15 and C17 allowed for closure of the bicyclic ether bridge and placement of the C17 methyl and the C16 iodinated methylene as substituents of C15. Meanwhile, HMBC correlations
from H2 to C19, H6 to C22, H7 to C24, H12 to C29 and H13 to C27 allowed for assignment of the acetoxy groups at C2, C6, C7, C12 and C13, respectively. Thus, tasihalide C features an additional acetoxy group at position C13 in comparison to tasihalide B.

**Figure 4.4.** Key ROESY correlations observed for tasihalide C (1). Dashed double headed arrow indicates key ROESY correlations for the acetoxy group at the new stereocenter at C13.

Careful analysis of the coupling constants and ROE correlations allowed for determination of the relative configuration for tasihalide C (Figure 4.4). ROESY correlations between H10 and H21 suggested a cis ring fusion at this juncture, while a large coupling constant of 11.0 Hz between H8 and H9 suggested a trans fusion between
these two six-membered rings. A sharp singlet for H13 suggests a coupling constant near zero indicating that the dihedral angle between H13 and H12 must be nearly 90°; thus the acetoxy group at C13 must be axial. This is further supported by the observation of ROESY correlations between H13 and H8, as well as H28 of the acetoxy methyl group and H16a of the iodinated methylene. Meanwhile, coupling constants and ROESY correlations for the remaining protons revealed that with the exception of the new acetoxy group, the relative configuration of tasihalide C was the same as tasihalide B.
Table 4.1. NMR spectroscopic data for tasihalide C (1) in CDCl$_3$ ($^1$H at 500 MHz, $^{13}$C at 75 MHz).

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<td>75.3 qC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>77.1 qC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16a</td>
<td>15.1 CH$_2$</td>
<td>4.03 d (10.4)</td>
<td>16b, 17</td>
<td>15, 17</td>
<td>12, 16b</td>
</tr>
</tbody>
</table>

$a$ : signals assigned by $^{13}$C resonance.
$^{b}$ : signals assigned by $^1$H resonance.
$^c$ : $^1$H chemical shifts are in ppm (referenced to TMS) and $^1$C chemical shifts are in ppm (referenced to CDCl$_3$).
4.2.2 Examination of the localization of marine cyanobacterial secondary metabolites utilizing mass spectrometry approaches

The discovery of tasihalide C represents the second incidence of the isolation of this family of compounds from a collection of "tropical marine Symploca sp." cyanobacteria; however, the previous cyanobacterial collection was found to contain...
trace amounts of a red alga. As the compound structure class is characteristic of red algae, but rare in marine cyanobacteria,\textsuperscript{54} and as iodination is present, though rare, in red algae,\textsuperscript{55} but virtually absent in cyanobacteria, it was suggested that the red alga is likely the producing organism. This was further supported by the fact that approximately 0.04% of the dry extract was comprised of tasihalides A and B,\textsuperscript{52} individually, while the peptides tasiamide\textsuperscript{56} and tasiamide B\textsuperscript{57} and the depsipeptides tasipeptin A and B,\textsuperscript{58} consistent with known marine cyanobacterial secondary metabolites, averaged approximately 0.16% of the dry extract per compound. In our collections, no noticeable amounts of eukaryotic algae were observed macroscopically, however, small microscopic fragments of a branched red alga, an unbranched red alga and a green branched alga, as well as, filaments of another cyanobacterium were teased out of the RNA later specimen preserved for phylogenetic analyses.

While the amount of biological material was too small for 18S rRNA analyses thus preventing taxonomic characterization of the contaminating organisms beyond the division level, enough material was available to attempt identification of the putative producing organism using matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) and nano-desorption electrospray ionization (nano-DESI).\textsuperscript{59,60} Filaments from the “tropical marine \textit{Symphloca} sp.” cyanobacterium, as well as the assorted eukaryotic algal fragments and the additional cyanobacterium were subjected to matrix-assisted laser desorption/ionization imaging mass spectrometry. Initially, negative ionization mode was employed with proton-sponge (\textit{N,N,N′,N′}-tetramethyl-1,8-naphthalenediamine) as the matrix. Localization of janthielamide A (exact mass 309.1856, [M+Na]\textsuperscript{+} exact mass 332.1757),\textsuperscript{53} previously isolated from this cyanobacterial
collection, and dolastatin 10 (exact mass 784.4913, [M+Na]$^+$ 807.4819), a potent anticancer marine natural product originally isolated from the sea hare Dolabella auricularia$^{61}$ and later identified in cyanobacterial species of the genus Symploca sp.,$^{62}$ was observed in the “tropical marine Symploca sp.” filaments (see Chapter 4 Appendix). Unfortunately, tasihalide C was not observed under these conditions. It is interesting to note that the exact mass of dolastatin 10 is quite similar to that of the target compound tasihalide C (exact mass 784.0949). Fortunately though, tasihalide C is brominated, while dolastatin 10 is non-halogenated, and thus the isotope patterns are quite distinct. During this initial experiment, a pure compound spot of tasihalide C mixed with Universal MALDI matrix (1:1 mixture of 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxy-cinnamic acid) was found to ionize well in positive ion mode. Thus, a 96-well MALDI plate containing a representative fragment or filament from each of the contaminating organisms, as well as filaments from the “tropical marine Symploca sp.” cyanobacterium, was prepared by coating the dried specimens with Universal MALDI matrix. During subsequent analysis using MALDI-IMS, only dolastatin-10, a known cyanobacterial metabolite present in the collection of cyanobacteria, was identified in the “tropical marine Symploca sp.” fragments.

Subsequent analyses of the cyanobacterial and algal fragments employing high resolution nano-DESI mass spectrometry revealed the presence of tasihalide C superficially on all specimens, as well as in an aliquot of the RNAlater® solution in which the specimens had been stored since the initial collection. It is worth noting that the amount of tasihalide C, as evidenced by total ion count (TIC), was considerably higher for the “tropical marine Symploca sp.” filaments than for all other samples (TIC 32,400
for “tropical marine Symploca sp.”, 1,090 for red unbranched alga, and 2,520 for RNALater® aliquot). However, this may be due to the fact that a larger sample size was used for the “tropical marine Symploca sp.” filaments than for all other specimens. Unfortunately, these investigations were not conclusive on the algal versus cyanobacterial source of tasihalide C.

4.2.3 Biological activity of tasihalide C (1)

While fraction 1869 E from VLC of the extract from this assemblage produced an increase in calcium oscillations at 5 μg/mL, the fraction from which tasihalide C was derived (1869 E8I) was inactive in this bioassay. Subsequently, the pure compound was submitted to a variety of bioassays, including assays assessing cathepsin L and V modulation, voltage-gated sodium channel blocking or activating activity, cytotoxicity, and nitric oxide inhibition. Tasihalide C was virtually inactive in all assays, except for the nitric oxide inhibition assay which is employed as a proxy for immunomodulatory activities, including anti-inflammation. RAW264.7 murine macrophage cells stimulated with LPS were utilized to assess the inhibition of nitric oxide production due to concentration-dependent addition of tasihalide C. The IC_{50} for tasihalide C (1) was 1.3 μM indicating that compound 1 is a modest anti-inflammatory agent. Tasihalide C appears to exhibit anti-inflammatory activity that is slightly more potent than that observed for other marine cyanobacterial natural products, including malyngamide F (IC_{50} = 5.4 μM),\textsuperscript{63} malyngamide F acetate (IC_{50} = 7.1 μM),\textsuperscript{63} malyngamide J (IC_{50} = 7.7 μM),\textsuperscript{63} and malyngamide 2 (IC_{50} = 8.0 μM).\textsuperscript{64}
While relatively few investigations regarding anti-inflammatory properties of secondary metabolites from marine cyanobacteria have been conducted to date, extensive investigations of terpenes exhibiting anti-inflammatory activities have been conducted. The pseudopterosins and manoalide are examples of marine-derived terpenes that exhibit potent anti-inflammatory activities. The pseudopterosins possess anti-inflammatory properties that are superior to current drugs, such as indomethacin, and appear to interfere in the arachidonic cascade and inhibit the release of eicosanoids, though their exact mechanism of action is not known. Meanwhile, manoalide inhibits phospholipase A\(_2\) (PLA\(_2\)) via irreversible binding of the masked aldehyde groups to several lysine residues on the interfacial binding site of PLA\(_2\). The novel carbon skeleton of the tasihalides, as well as the unusual presence of iodine in a terpenoid compound, indicate that these compounds may have unique pharmacological activities in comparison to other marine-derived terpenoids; therefore, further investigations of the observed anti-inflammatory activity of tasihalide C may reveal a novel mechanism for these terpenoid compounds.
4.3 Conclusions

Tasihalide C is a new member of the tasihalide family of compounds, which have been isolated only one time prior to this investigation (Figure 4.5). These compounds feature a unique carbon skeleton for terpenoid compounds and both iodination and bromination. Nearly ten years after their initial isolation, these compounds are still an enigma to the marine natural products community due to the difficulties in definitively identifying the producing organism, the relatively unusual incorporation of iodine on an isoprene tail, the unexplored biological properties, and the rarity of encountering iodinated diterpenes. Since tasihalides A and B were first described, only one other family of iodinated diterpenes has been described from the marine environment. The dichotellides are chlorinated and iodinated briarane diterpenoids from the gorgonian *Dichotella gemmacea*. While iodination is considerably less common than chlorination
and bromination, it seems that iodination of terpenes is exceedingly rare. In addition to
the tasihalides and the dichotellides, the only other known iodinated terpenes are two
iodobromo aromatic sesquiterpenes, 10-bromo-7-hydroxy-11-iodolaurene and a related
ether, isolated in 1979 from *Laurencia nana* Howe, 67 and 8-iodo-laurinterol, isolated in
2007 from *Laurencia microcladia*. 68 It is intriguing that the tasihalides have now been
isolated twice from marine cyanobacterial consortia containing trace amounts of
eukaryotic algae, but they have still not been found in assemblages of macro-algae.
Future investigations of these unique secondary metabolites may resolve some of the
remaining questions regarding these compounds, including determination of their
absolute configuration, identification of the producing organism, and the therapeutic
relevance of these compounds as potential anti-inflammatory agents.

4.4 Experimental Details

4.4.1 General experimental procedures

Optical rotation was measured with a Jasco P-2000 polarimeter. CD spectrum was
obtained on a Jasco J-815 CD spectrometer. UV spectrum was obtained on a Beckman
Coulter DU-800 spectrophotometer, and FT-IR spectrum was recorded on a Nicolet IR
100 FT-IR spectrophotometer. NMR spectra were recorded on a Varian Inova 300 MHz
or 500 MHz with a 5 mm probe and were referenced to residual solvent 1H and 13C
signals (δH 7.26, δC 77.16 for CDCl3 and δH 7.16, δC 128.62 for C6D6). Low-resolution
ESIMS spectra were acquired on a Finnigan LCQ Advantage Max mass spectrometer,
while high-resolution ESIMS spectra were obtained by the UCSD Chemistry and
Biochemistry Molecular MS Facility on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. HPLC separation was completed using a Waters HPLC system equipped with a Waters 515 binary pump and a Waters 996 PDA detector.

4.4.2. Biological material collection and identification

Approximately 3 L of a lime green filamentous cyanobacterial mat was collected by hand at 1 m depth from a sandy reef substrate in Jan Thiel Bay on the leeward side of Curaçao (N 12°07.634’, W 68°88.088’). Following collection, the cyanobacterium was stored in 1:1 EtOH:seawater and stored at −20°C; prior to shipping, the samples were thawed and the supernatant was discarded. For longer storage, EtOH was added and the samples were stored at −20°C until workup. A voucher specimen (NAC 12/21/08-3) is maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA. Morphological characterization and phylogenetic analyses of this specimen have been described previously (see Chapter 3 or Nunnery et al., 2012).

4.4.3 Extraction and isolation

Two of the three liters of EtOH-preserved biomass (884.6 g, dry weight) of “tropical marine Symploca” (NAC 12/21/08-3) were extracted with 2:1 DCM/MeOH eight times to give 2.77 g of crude extract (extract #1869). A portion of the crude extract (2.22 g) was fractionated using vacuum liquid chromatography (VLC) on Silica gel (Type H, 10-40 µM, Sigma-Aldrich) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A-I). Fraction E, eluted with 60% EtOAc in
hexanes (210 mg), was subjected to two iterations of normal-phase chromatography using Si Varian Bond Elut Sep-Paks. During the first NP-SPE cartridge chromatography step, a stepwise gradient from 100% hexanes to 100% EtOAc was employed. The majority of the material eluted off of the column at 20% EtOAc (fraction 8, 150 mg). NP-SPE cartridge chromatography of fraction 8, increasing from 0% hexanes to 30% EtOAc in increments of 2.5% resulted in four major fractions at 15% (E8G, 19.9 mg), 17.5% (E8H, 47.8 mg), 20% (1869 E8I, 29.9 mg) 22.5% (1869 E8J, 11.2 mg) EtOAc and several minor fractions. Normal phase HPLC of fraction 1869 E8I on a Phenomenex Luna 5 μ Silica (2) (100 Å, 250 × 10.0 mm) column under an isocratic condition of 3 mL/min 5% isopropyl alcohol in hexanes gave 1 (fraction 9, peak centered at 34.5 min, 8.5 mg, 0.4% of extract). Fractions 1869 E8G and E8H also contained compound 1 based on LCMS analyses. Subsequent purification of 1869 E8G gave an additional 4.6 mg of 1 (fraction 6), increasing the percentage of extract to 0.6%. Purification of tasihalides B and C from E8H is ongoing; however, as E8H was the largest fraction deriving from the E fraction, it is likely that it will yield a substantial amount of tasihalide C and thus increase the percent yield significantly. A total of 2.7 mg of tasihalide B was also isolated to date from fraction E8 (1.5 mg from E8G and 1.2 mg from E8I).

4.4.4 Characterization of purified tasihalide C (1)

Tasihalide C (1): pale yellow amorphous solid [α]$_{25}$D -26.9 (c 0.60, MeOH), UV (MeOH) $\lambda_{\text{max}}$ (log ε) 202 (3.6), 252 (2.8) nm; IR $\nu_{\text{max}}$ (film) 1749, 1373, 1233, 1032 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; ESIMS $m/z$ (relative intensity) [M+Na]$^+$ 807 (1:1).
HRESI-TOFMS $m/z [M+Na]^+$ 807.0847 (calcd for $C_{30}H_{42}O_{11}BrNa$, 807.0847, 0.04 mmu error).

4.4.5 Neocortical neuron culture

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice as described elsewhere. Briefly, pregnant mice were euthanized by CO$_2$ asphyxiation, and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle’s minimal essential medium with Earle’s salt (MEM) and supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin, and 0.10 mg/mL streptomycin (pH 7.4). Cells were plated onto poly-L-lysine-coated 96-well (9 mm), clear-bottomed, black-well culture plates (Costar) at a density of $1.5 \times 10^5$ cells/well. Cells were then incubated at 37°C in a 5% CO$_2$ and 95% humidity atmosphere. Cytosine arabinoside (10 mM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed every other day, starting from day 5 in vitro, using a serum-free growth medium containing Neurobasal Medium supplemented with B-27, 100 IU/mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine. Neocortical cultures were used in experiments between 8–13 days in vitro (DIV). All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.
4.4.6 Nitric oxide inhibition

Anti-inflammatory activity was evaluated using the mouse macrophage cell line RAW 264.7 (ATCC) cultured in DMEM with 4 mM l-glutamine and 4.5 g/L glucose. Media was further supplemented with 10% FBS, penicillin, and streptomycin. RAW 264.7 cells were seeded in 96-well plates (5 × 10^4 cells/well) and after one day were stimulated in triplicate with 3 μg/mL LPS in the absence or presence of pure compound (1 to 30 μg/mL) for 24 h at 37°C with 5% CO₂. The generation of NO was assessed in the supernatant of cell cultures by quantification of nitrite using the Griess reaction. In brief, 50 μL of each supernatant was added to 96-well plates together with 50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% N-(1-naphthyl)ethylendiamine (NED) in H₂O. All assays were run in triplicate with lipopolysaccharide (LPS) as the positive control (assigned as 100%) and cells alone as the negative control (average = 2.5 ± 0.3%). Optical density was measured at 570 nm. IC₅₀ values, the sample concentrations that resulted in 50% inhibition of NO production, were determined using nonlinear regression analysis (percent nitrite versus concentration).
I thank the government of Curaçao and the CARMABI research station for permission to collect the cyanobacterial specimen. I thank Dr. Y. Su (UCSD Chemistry and Biochemistry Mass Spectrometry Facility) for acquiring HRMS and MS" data. Support was provided by a graduate fellowship to JKN from the National Institutes of Health Neuroscience Related to Drug Abuse Training Grant (T32DA007315). The statements, findings, conclusions, and recommendations provided here are those of the authors and do not necessarily reflect the views of the National Institutes of Health.

Author Contributions

JKN, JH, SM, JW and NE performed research. SM, SVJ conducted bioassays. JKN, SM, JW, TFM, PCD and WHG designed research and wrote the manuscript.

The authors declare no conflicts of interest.

Chapter 4, in essence, is currently being prepared for submission in 2012. Joshawna K. Nunnery, Jennifer Hao, Samantha Mascuch, Jeramie Watrous, Niclas Engene, Sairam V. Jabba, Thomas F. Murray, Pieter C. Dorrestein, and William H. Gerwick. The dissertation author was the primary investigator and author of this material.
Figure 4.6.1. MALDI-IMS of “tropical marine Symploca sp.” cyanobacterial filaments at m/z 784 (red) and 332 (green).
Table 4.6.1. Tabulated nano-DESI data obtained for cyanobacterial filaments and eukaryotic algal fragments in “tropical marine *Symploca* sp.” collection.

<table>
<thead>
<tr>
<th></th>
<th>Tropical Marine <em>Symploca</em></th>
<th>Branched red alga</th>
<th>Unbranched red alga</th>
<th>Large filamentous cyanobacterium</th>
<th>RNAlater</th>
</tr>
</thead>
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<tr>
<td><strong>Dolastatin 10</strong></td>
<td>785.5213 (12100)</td>
<td>785.5083 (205)</td>
<td>785.5040 (2790)</td>
<td>785.5043 (244)</td>
<td>785.5264 (2520)</td>
</tr>
<tr>
<td>[M+H]+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dolastatin 10</strong></td>
<td>807.5015 (525)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[M+Na]+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tasihalide C</strong></td>
<td>807.1060 (32400)</td>
<td>807.0959 (10)</td>
<td>807.0897 (1090)</td>
<td>807.0934 (8)</td>
<td>785.0573 (2520)</td>
</tr>
<tr>
<td>[M+Na]+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tasihalide B</strong></td>
<td>744.1411 (754)</td>
<td>-</td>
<td>744.1296 (90)</td>
<td>-</td>
<td>744.1616 (550)</td>
</tr>
<tr>
<td>[M+NH₄]+</td>
<td></td>
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<tr>
<td><strong>Tasihalide B</strong></td>
<td>749.0990 (327)</td>
<td>-</td>
<td>749.0839 (180)</td>
<td>-</td>
<td>749.1023 (552)</td>
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<td>[M+Na]+</td>
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</table>
Figure 4.6.2. $^1$H NMR spectrum of tasihalide C (I), recorded in C$_6$D$_6$ at 500 MHz.
Figure 4.6.3. $^{13}$C NMR spectrum of tasihalide C (1), recorded in $C_6D_6$ at 75 MHz.
Figure 4.6.4. HMBC spectrum of tashihalide C (1), recorded in C₆D₆ at 500 MHz.
Figure 4.6.5. HSQC spectrum of tasihalide C (1), recorded in C₆D₆ at 500 MHz.
Figure 4.6.6. COSY spectrum of tasihalide C (1), recorded in C₆D₆ at 500 MHz.
Figure 4.6.7. ROESY spectrum of tasihalide C (1), recorded in C₆D₆ at 500 MHz.
Figure 4.6.8. Microscopic photographs of filaments from cyanobacterial collection from Curaçao (NAC 12/21/08-3).
REFERENCES


(55) Based on a MarinLit search for compounds containing iodine there are 37 iodine-containing compounds from the division Rhodophyta, however, there are only 2 compounds, tasihalides A and B, from the division Cyanophyta.


5.0 CHAPTER 5

PALMYRIC ACID, AN INTRIGUING LIPI D METABOLITE FROM A PALMYRA ATOLL MARINE CYANOBACTERIUM

5.0.1 Abstract

An intriguing lipophilic carboxylic acid, palmyric acid (1), was isolated from a collection of marine cyanobacteria obtained near Barren Island in the Palmyra Atoll. The planar structure of compound 1 was determined based on interpretation of 1D and 2D homonuclear and inverse heteronuclear NMR experiments, as well as analyses of HR-ESI-MS and MS^n data. Palmyric acid features a methyl-substituted cyclopropyl ring, a thiazoline ring, and an extended lipophilic chain terminating with a carboxylic acid, thus yielding an novel carbon skeleton arising from a proposed PKS-NRPS hybrid pathway. Based on structural similarity, palmyric acid may be derived from a pathway related to the curacin A marine cyanobacterial secondary metabolite gene cluster; however, palmyric acid appears to show alternative PKS processing and a normal hydrolytic off-loading versus the decarboxylative mechanism shown for curacin A.

5.1 Introduction

Secondary metabolites from marine cyanobacteria often include diverse functionalities with unique modifications, including the incorporation of intriguing β-amino and hydroxy acids, heterocyclization of cysteine, as well as, chlorination, bromination, and N- and O-methylation.\textsuperscript{1-3} Curacin A, which features cyclopropyl and thiazoline rings, as well as O-methylation and a terminal alkene, possesses potent cancer
cell cytotoxicity due to its antimitotic properties (Figure 5.1). Another marine cyanobacterial metabolite, kalkitoxin, also possesses a thiazoline ring, as well as N-methylation and a terminal alkene, but exhibits potent neuromodulatory activities. A further 26 unique natural products featuring thiazoline rings have been discovered from marine cyanobacteria, including curacins B-D, apratoxins A-G and grassypeptolides A-G, as well as bisebromoamide and alotamide A among others. Additionally, at least 31 distinct marine cyanobacterial secondary metabolites containing one or more thiazole rings have been isolated, including lyngbyabellins A-J, ulongamides A-F, guineamides A and B, and venturamides A and B. Interestingly, a few marine cyanobacterial natural products, including largazole, kororamide, lyngbyabellin B and the hoiamides A-D possess both thiazoline and thiazole rings.

![Figure 5.1. Sulfur-containing metabolites from marine cyanobacteria.](image-url)
A number of additional marine cyanobacterial metabolites contain sulfur in other functional groups, such as disulfides (e.g. somocystinamide A), sulfate groups (e.g. lyngbyastatin 4, crossbyanols B-D), methionine sulfoxides (e.g. pompanopeptin, tiglicamides A-C) and thioesters (e.g. largazole, thiopalmyrone). The disulfide dimer somocystinamide A possesses exceptional cytotoxic properties and subsequently was shown to trigger apoptosis via a caspase 8 dependent mechanism. Sulfate-containing lyngbyastatin 4 was found to inhibit elastase and chymotrypsin selectively over other serine proteases, while the disulfated compound crossbyanol B exhibits antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and brine shrimp toxicity. Similar to lyngbyastatin 4, methionine sulfoxide-containing pompanopeptin A displayed inhibitory activity against serine proteases, with selective activity against trypsin, while tiglicamides A-C modestly inhibited porcine pancreatic elastase. One of the more intriguing marine cyanobacterial compounds isolated to date with regard to sulfur atom incorporation is largazole, a potent anticancer agent that features not only thiazoline and thiazole rings, but also a thioester functionality. The only other thioester-containing marine cyanobacterial metabolite isolated to date is thiopalmyrone, a molluscicidal compound recently described from a Palmyra Atoll collection of *Oscillatoria* and *Hormoscilla* spp. that features a unique six-membered ring incorporating an α,β-unsaturated thioester. As evident from this discussion, sulfur-containing marine cyanobacterial metabolites may be of considerable therapeutic relevance due to their varied pharmacological properties.

In the present work, red filamentous tufts of a cyanobacterium from Palmyra Atoll in the Pacific Ocean yielded a chromatographic fraction that produced an influx of
calcium at 50 μg/mL, while suppressing spontaneous calcium oscillations at 5 μg/mL, in murine neocortical neurons. NMR- and MS-guided fractionation of this active fraction led to the isolation of a novel lipophilic acid, palmyric acid (1), as the major constituent. Herein, we report the isolation, structure elucidation and bioactivity of this intriguing metabolite as well as a phylogenetic analysis of the putative marine cyanobacterial producer.

5.2 Results and Discussion

5.2.1 Collection, isolation and structure elucidation

Red filamentous cyanobacterial tufts were collected by hand in a shallow lagoon north of Barren Island on Palmyra Atoll. The resultant biomass was subjected to repeated extraction with DCM/MeOH (2:1) and then fractioned by silica gel vacuum column chromatography using a step-wise gradient from hexanes to EtOAc to MeOH to produce nine subfractions (A-I). Fraction E displayed neuromodulatory activity and was thus fractionated by normal phase column chromatography and HPLC to provide pure palmyric acid (1) as a nearly colorless, optically active amorphous solid {4.4 mg, 0.68%, [α]D +39.8 (c 1.00, CHCl3)}. The LR-ESI-MS of 1 revealed an m/z 352 for the [M+H]+ adduct, while in negative ion mode, an m/z of 350 for the [M-H]- adduct was observed, indicating a mass of 351 daltons, and thus the presence of an odd number of nitrogen atoms. HR-ESI-TOFMS established the molecular formula as C20H33NO2S, revealing that 1 contained a sulfur atom as well as five degrees of unsaturation. The 1H NMR spectrum of 1 (Table 5.1) revealed a highly shielded methylene (δH 0.69 and 0.78) and methine (δH 0.98), as well as a methyl doublet (δH 1.10), a large methylene envelope (δH 1.29) two
diastereotopic methylenes ($\delta_H$ 1.57 and 1.79; 2.92 and 3.32), three additional methylenes ($\delta_H$ 1.43, 1.64 and 2.34), a deshielded methine ($\delta_H$ 4.48) and two olefinic protons at $\delta_H$ 5.87 (dd) and 6.45 (d). The $^{13}$C NMR spectrum of 1 included two quaternary ($\delta_C$ 177.6 and 167.1), five methine ($\delta_C$ 149.8, 121.7, 76.4, 23.7 and 17.4), twelve methylene ($\delta_C$ 37.2, 34.9, 34.3, 29.9, 29.0, 28.9, 28.76, 28.75, 28.67, 26.3, 24.9 and 17.1) and one methyl carbon ($\delta_C$ 18.5), accounting for all 20 carbon atoms in the molecular formula. An HSQC correlation from $\delta_H$ 1.29 to $\delta_C$ 23.7 suggested that a single methine proton overlapped with the methylene envelope at $\delta_H$ 1.29.

![Figure 5.2](image.png)

**Figure 5.2** Partial structures a-c and planar structure for palmyric acid (1).

COSY correlations readily revealed three partial structures (see Figure 5.2). Fragment a consisted of an extended lipophilic chain with a deshielded quaternary carbon at one terminus. Fragment b featured a heteroatom substituted methine bordered by two distinct methylene branches. Lastly, fragment c was comprised of a methyl-substituted cyclopropyl ring, which was adjacent to a disubstituted alkene moiety. The connections between these three partial structures proved challenging due to unusual chemical shifts
for some of these functional groups. For example, the chemical shift of $\delta_C$ 76.4 suggested that C2 may be oxygenated, while the distinctive chemical shifts of the olefin from C2'-C3' of $\delta_C$ 121.7 and 149.8, respectively, suggested that an amide or ester functionality was present at C1'. However, tentative initial structures based on these predicted functional groups did not correlate well with the overall $^1$H and $^{13}$C NMR data. Resolution of this dilemma was provided by recognition of the presence of sulfur in 1 in combination with proton and carbon chemical shifts from the literature, suggesting that palmyric acid likely contained a thiazoline ring. Specifically, the $^1$H and $^{13}$C NMR shifts of $\delta_H$ 4.48 and $\delta_C$ 76.4, respectively, for the C2 methine, in combination with the diastereotopic methylene shifts of $\delta_H$ 3.32 and 2.92 and $\delta_C$ 76.4 for C1 and the $\delta_C$ of 167.1 for the quaternary carbon C1', correlated well with the chemical shifts for curacin A (C$_6$D$_6$)$_4$ and largazole (CDCl$_3$)$_3$.

HMBC correlations from H2 to C1, C3 and C4 and from H3 to C1, C2, C4 and C5, as well as from H1 to C2 and C3, allowed for connection of the lipophilic chain to the methine of the thiazoline ring. Likewise, HMBC correlations from H1 to C1' and from H2' to C1', as well as a correlation from H3' to C1' revealed the connection between the alkene-extended and methyl-substituted cyclopropyl ring and the quaternary carbon of the thiazoline. Assembly of these partial structures accounted for all of the carbon, nitrogen and sulfur atoms of compound 1; however, one hydrogen and two oxygen atoms remained unaccounted. As the terminus of partial fragment a required two heteroatoms based on the chemical shift of $\delta_C$ 177.6, it was apparent that a carboxylic acid was likely installed at this position. A carboxylic acid at this position correlated well with the $^1$H and $^{13}$C NMR chemical shift data obtained for 1 in comparison with literature data for
malygic acid, which also features an extended lipophilic tail that terminates in a carboxylic acid.\textsuperscript{43}

![Diagram of Palmyric Acid and Methyl Ester](image)

**Palmyric acid:** $R = \text{H}$, $[\text{M+H}]^+ = 352.2302$, molecular formula = $\text{C}_{20}\text{H}_{33}\text{NO}_2\text{S}$

1: $m/z$ 334.2198, consistent with $\text{C}_{20}\text{H}_{32}\text{NOS}^-$ (calc'd for 334.2205)  
2: $m/z$ 227.1463, consistent with $\text{C}_{13}\text{H}_{23}\text{OS}^-$ (calc'd for 227.1470)  
3: $m/z$ 310.1835, consistent with $\text{C}_{17}\text{H}_{26}\text{NO}_2\text{S}^-$ (calc'd for 310.1841)

**Palmyric acid methyl ester:** $R = \text{CH}_3$, $[\text{M+H}]^+ = 366.2468$, molecular formula = $\text{C}_{21}\text{H}_{36}\text{NO}_2\text{S}$

1: $m/z$ 334.2203, consistent with $\text{C}_{20}\text{H}_{35}\text{NOS}^-$ (calc'd for 334.2205)  
2: $m/z$ 227.1466, consistent with $\text{C}_{13}\text{H}_{25}\text{OS}^-$ (calc'd for 227.1470)  
3: $m/z$ 324.1996, consistent with $\text{C}_{18}\text{H}_{36}\text{NO}_2\text{S}^-$ (calc'd for 324.1997)

**Figure 5.3.** MS/MS fragmentation patterns for palmyric acid (1) and the methyl ester of 1.

To confirm the carboxylic acid in 1, palmyric acid was methyl esterified using diazomethane and subjected to HR-MS and high resolution MS\textsuperscript{2} analyses. Methyl esterified 1 revealed an $[\text{M+H}]^+$ adduct at $m/z$ of 366.2468 consistent with a molecular formula of $\text{C}_{21}\text{H}_{35}\text{NO}_2\text{S}$ (calc'd for $\text{C}_{21}\text{H}_{36}\text{NO}_2\text{S}$, 366.2467), verifying the addition of one methyl group to compound 1. MS/MS analyses of the parent ions $m/z$ 352.23 for 1 and $m/z$ 366.25 for methyl esterified 1 revealed fragments of $m/z$ 334.22 in each, suggesting the loss of OH or OMe, respectively (Figure 5.3). Similarly, the MS\textsuperscript{2} spectra of both compounds revealed peaks with $m/z$ values of 227.15, consistent with both loss of the hydroxyl or methoxy substituent on the carboxy terminus for compound 1 or methyl esterified 1, respectively, and opening of the thiazoline ring. Furthermore, the MS\textsuperscript{2}
spectrum of compound 1 featured a fragment at \( m/z \) of 310.18 consistent with opening of the cyclopropyl ring and loss of C5′-C7′, while in the MS\(^2\) of methyl esterified 1, a corresponding fragment was observed at \( m/z \) 324.20. Finally, a fragment with an \( m/z \) value of 306.24 in the MS\(^2\) spectrum of 1 was consistent with opening of the thiazoline ring and loss of C1 and S; however, in the MS\(^2\) spectrum of methyl esterified 1, this mass was absent, but a mass of \( m/z \) 318.94, consistent with the corresponding fragment for the methyl ester derivative was observed. These analyses further confirmed the planar structure of palmyric acid as shown in Figure 5.2.

**Figure 5.4.** Relative configuration of the methyl-substituted cyclopropyl ring of palmyric acid (1). A) Key ROESY correlations observed for compound 1. B) Vicinal coupling constants in Hertz (Hz) for H4′ with its four adjacent protons. C) Geminal and vicinal coupling constants for H5′a, H5′b and H6′ of compound 1 in Hertz (Hz). D) Structure of 1 with relative configuration of methyl-susbstituted cyclopropyl ring.

The geometry of the C2′-C3′ olefin was assigned as \( E \) based on a vicinal coupling constant of 15.5 Hz. The relative configuration of the cyclopropyl ring was assigned
based on ROESY correlations and confirmed based on coupling constant analyses (see Table 5.6.1 in Chapter 5 Appendix for 2-D NMR data acquired in C₆D₆). ROESY correlations from H4′ to H2′ and H7′, as well as from H7′ to H5′b and from H5′a to H3′ revealed that the methyl-substituted cyclopropyl ring has a \textit{trans} configuration (Figure 5.4). Coupling constant analyses of H3′, H4′, H5′a and H5′b further supported this assignment.⁴⁴ The coupling constants for H3′ were deduced as 15.6 Hz and 9.4 Hz for H2′-H3′ and H3′-H4′, respectively. The remaining J values for H4′ were determined as a large \textit{cis} coupling of 8.1 Hz with H5′b, and two \textit{trans} couplings of 4.5 Hz with H6′ and 4.5 Hz with H5′a. For H5′a, the \textit{cis} coupling constant with H6′ was deduced as 8.5 Hz, while the geminal coupling constant with H5′b was determined to be 4.5 Hz. Lastly, the remaining \textit{trans} coupling constant for H5′b was deduced to be 5.7 Hz with H6′. Unfortunately, compound 1 degraded during lyophilization to remove DMSO, thus prohibiting determination of the relative configuration at C2 and the overall absolute configuration of palmyric acid. However, based on the precedent with curacin A, which has a similar overall architecture and thiazoline ring, it can be predicted that C2 might have an \textit{R} absolute configuration due to its probable origin from L-cysteine.⁴⁵
Table 5.1. NMR spectroscopic data for palmyric acid (1) in CDCl$_3$ ($^1$H at 500 MHz, $^{13}$C at 75 MHz).

<table>
<thead>
<tr>
<th>N$^a$</th>
<th>$\delta_C^{a,b}$</th>
<th>$\delta_H$ mult (J in Hz)$^c$</th>
<th>COSY</th>
<th>HMBC</th>
</tr>
</thead>
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<tr>
<td>1a</td>
<td>37.2 CH$_2$</td>
<td>3.32 dd (10.6, 8.3)</td>
<td>1b, 2</td>
<td>2, 3, 1$'$</td>
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<tr>
<td>1b</td>
<td></td>
<td>2.92 dd (10.6, 7.9)</td>
<td>1a, 2</td>
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<tr>
<td>2</td>
<td>76.4 CH</td>
<td>4.48 m</td>
<td>1a, 1b, 3a, 3b</td>
<td>3w, 4w, 1$'$w</td>
</tr>
<tr>
<td>3a</td>
<td>34.9 CH$_2$</td>
<td>1.79 m</td>
<td>2, 3b, 4</td>
<td>1, 2, 4, 5w</td>
</tr>
<tr>
<td>3b</td>
<td></td>
<td>1.57 m</td>
<td>2, 3a, 4</td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>1.43 m</td>
<td>3a, 4, 5</td>
<td>2w, 3w, 5w</td>
</tr>
<tr>
<td>5</td>
<td>29.9 CH$_2$</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29.0 CH$_2$</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>28.9 CH$_2$</td>
<td>1.29</td>
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<td>8</td>
<td>28.76 CH$_2$</td>
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<td>28.67 CH$_2$</td>
<td>1.29</td>
<td></td>
<td>11w, 12w</td>
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<tr>
<td>11</td>
<td>24.9 CH$_2$</td>
<td>1.64 m</td>
<td>10, 12</td>
<td>10, 12, 13</td>
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<tr>
<td>12</td>
<td>34.3 CH$_2$</td>
<td>2.34 m</td>
<td>11</td>
<td>10, 11, 13</td>
</tr>
<tr>
<td>13</td>
<td>177.6 qC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1$'$</td>
<td>167.1 qC</td>
<td></td>
<td></td>
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<tr>
<td>2$'$</td>
<td>121.7 CH</td>
<td>6.45 d (15.5)</td>
<td>3$'$</td>
<td>1$'$, 4w, 5$'$</td>
</tr>
<tr>
<td>3$'$</td>
<td>149.8 CH</td>
<td>5.87 dd (15.5, 9.4)</td>
<td>2$'$, 4$'$</td>
<td>1$'$, 4$, 5w, 6w</td>
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<td>4$'$</td>
<td>23.7 CH</td>
<td>1.29</td>
<td>3$'$, 5$'$a, 5$'$b</td>
<td>2w, 6w</td>
</tr>
<tr>
<td>5$'$a</td>
<td>17.1 CH$_2$</td>
<td>0.78 ddd (8.3, 4.7, 4.4)</td>
<td>4$'$, 5$'$b, 6$'$</td>
<td>3$'$, 4$'$, 7$'$</td>
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<tr>
<td>5$'$b</td>
<td></td>
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<td></td>
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<td>6$'$</td>
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<td>0.98 m</td>
<td>4$'$, 5$'$a, 5$'$b, 7$'$</td>
<td>3$'$, 4w, 7$'$</td>
</tr>
<tr>
<td>7$'$</td>
<td>18.5 CH$_3$</td>
<td>1.10 d (5.9)</td>
<td>6$'$</td>
<td>4$'$, 6$'$</td>
</tr>
</tbody>
</table>

$^a$ Recorded at 75 MHz. $^b$ Multiplicity deduced from HSQC. $^c$ Recorded at 500 MHz.
5.2.2 Biological activity

The initial bioactive fraction (E, 60% EtOAc in hexanes) obtained from VLC of the crude extract displayed neuromodulatory activity in murine neocortical neurons, as evidenced by an influx of calcium at 50 μg/mL and suppression of spontaneous calcium oscillations at 5 μg/mL. Additionally, this fraction exhibited marginal cytotoxicity in a human large-cell lung carcinoma H460 cell line (63% inhibition at 30 μg/mL, 0% at 3 μg/mL). Similarly, modest activity was observed for three of twelve cell lines in disk diffusion assays, including murine granulocyte/macrophage colony-forming units (CFU-GM), human colon carcinoma (H116) and human breast adenocarcinoma (MCF-7); however, the fraction was neither potent, nor selective. Evaluation of the effect of the lipophilic carboxylic acid, palmyric acid (1), on calcium ion modulation in murine neocortical neurons, revealed that 1 produced a very modest calcium influx when compared to 30 μM veratridine, however, even this marginal activity rapidly diminished over time. It is suspected that this loss of activity is due to stability and/or solubility issues. Additional investigation of the biological properties of palmyric acid will be conducted in due course if additional material is obtained by reisolation or chemical synthesis.

5.2.3. Characterization of the cyanobacterium

Similar to previous investigations of cyanobacteria identified morphologically as either Schizothrix or Symploca spp., phylogenetic analyses of the 16S rRNA gene sequence obtained from PAL 08/22/08-1 (indicated by a blue box in Figure 5.5) revealed that this organism claded with other tropical marine specimens of “Symploca sp.” The
most closely related strains appear to be *Symploca* sp. VP642a-c,⁴⁶ which are recollections of a strain known to produce dolastatin 10 from Palau and *Symploca* sp. VP377,⁴⁶ which is a symplostatin 1-producing strain originally identified as *Symploca hydnoides* from Guam. As observed previously, this clade of “tropical marine *Symploca* sp.” appears to be distinct from the type strain for the genus *Symploca* (PCC 8002, GenBank acc. nr. AB03902, indicated by a red box in Figure 5.5).⁴⁷
Figure 5.5 Molecular-phylogenetic inference of the palmyric acid producing strain of marine cyanobacterium from Palmyra Atoll. The specimen PAL 08/22/08-1 is indicated with a blue box, while the closest type strain as designated in Bergey's Manual is indicated with a red box. The clade includes two other secondary metabolite producing tropical marine cyanobacterial strains, including Symploca sp. VP642a-c and VP377. The cladogram is based on SSU (16S) rRNA gene sequences using the bayesian (MrBayes) and maximum likelihood (PhyML) methods. The specimens are indicated as genus/species/strain with the accession number in parentheses. The scale bar is indicated at 0.03 expected nucleotide substitutions per site, corrected using the General Time Reversal (GTR) model.
5.2.4 Proposed biogenesis of palmyric acid (1)

![Chemical structures of palmyric acid and curacin A](image)

**Figure 5.6.** Predicted acetate labeling pattern for palmyric acid as compared with the acetate labeling pattern observed for curacin A.

Previously, a biosynthetic gene cluster has been identified for curacin A from a Curaçao strain of *Moorea producta* (formerly *Lyngbya majuscula*). The structural similarity between curacin A and palmyric acid suggests that parallels might exist between the biosynthetic gene cluster for curacin A and the hypothetical biogenesis of palmyric acid. Feeding studies with *Moorea producta* revealed the incorporation of eight pairs of carbons deriving from acetate in curacin A, as well as two lone C2 carbons, one in the methyl-substituted cyclopropyl ring at the methylene position and one at the terminal alkene (Figure 5.6). The lipophilic chain is comprised of six intact acetate units, while the methyl-substituted cyclopropyl moiety and the quaternary carbon of the thiazoline ring are composed of two intact acetate units. By comparison, it is suspected
that the lipophilic chain of palmyric acid is comprised of five acetate extensions, while
the alkene-extended and methyl-substituted cyclopropyl ring is composed of three intact
acetates (Figure 5.6). Furthermore, it appears that a full complement of ketoreductase,
dehydratase and enoyl reductase domains is present for each acetate extension in the
lipophilic chain of palmyric acid, while several enoyl reductases, and at least one full
complement of tailoring domains are absent from the curacin A biogenetic pathway by
analogy\textsuperscript{50,51} (Figure 5.7). Lastly, while curacin A has an unusual terminal module
whereby β-hydroxy acyl ACP is sulfonated by a sulfotransferase, before undergoing
thioesterase hydrolysis, decarboxylation and sulfate elimination to form a terminal alkene
moiety, palmyric acid seems to be offloaded by a typical thioesterase to give a carboxylic
acid terminus.\textsuperscript{52,53}

\textbf{Figure 5.7.} Hypothetical biogenetic pathway for palmyric acid based on the probable
curacin A biosynthetic gene cluster (adapted from Chang \textit{et al.}, 2004 and Gu \textit{et al.},
2009).\textsuperscript{50,51}

Investigations regarding the ECH domains of curacin A revealed a high degree of
sequence identity to the ECH domains present in the jamaicamide pathway.\textsuperscript{54}
Subsequently, these domains have been discovered to catalyze the formation of a vinyl
chloride moiety in the jamaicamide pathway; however, in the curacin pathway, they catalyze the formation of a cyclopropyl ring functionality.\textsuperscript{51} Identification of the biosynthetic gene cluster responsible for palmyric acid production may reveal new insights into the biosynthesis of these unique molecules.

5.3 Conclusions

Intriguingly, palmyric acid possesses features characteristic of the curacin A class of natural products including the presence of a thiazoline and a methyl-substituted cyclopropane ring; however, the carbon skeleton of palmyric acid has an additional acetate extension between the cyclopropyl ring functionality and the putative cysteine residue. Also of note is that the lipophilic chain of palmyric acid appears to have undergone a complete complement of ketoreduction, dehydration and enoylreduction for each acetate extension whereas the lipophilic chain in the curacins A-D features several olefins and one methylated hydroxyl group, the result of a lack of dehydratase and enoylreduction domains, as shown by a detailed biosynthetic gene cluster analysis.\textsuperscript{50} It is also interesting that the lipophilic chain in palmyric acid terminates in a carboxylic acid as opposed to an alkene as observed in the curacins. To our knowledge, this is the first report of both a curacin A-like compound and a methyl- or alkyl-substituted cyclopropyl ring-containing compound from an organism other than \textit{Moorea producta}.

Many thiazoline-containing marine cyanobacterial secondary metabolites exhibit potent biological activities. A majority of these compounds are principally peptidic in nature; however, an increasing number feature an extensive PKS-derived fragment, such
as largazole,\textsuperscript{30} alotamide \textsuperscript{A,19} kalkitoxin\textsuperscript{6} and the curacins.\textsuperscript{4,7,8} Both alotamide \textsuperscript{A,19} and kalkitoxin\textsuperscript{5,6} have been found to possess neuromodulatory activities. Alotamide \textsuperscript{A} produces an unusual calcium influx activation profile in murine neocortical neurons,\textsuperscript{19} while kalkitoxin inhibits the increase in intracellular calcium concentrations due to exposure of cerebellar granule neurons to veratridine.\textsuperscript{54} Meanwhile, both largazole and the curacins are lead compounds for the development as anticancer therapeutics. Largazole exhibits selective and potent antiproliferative activities against transformed cancer cell lines over nontransformed cells,\textsuperscript{30} while the curacins display potent antimitotic activities.\textsuperscript{4,7,8} Thus far, palmyric acid (1) appears to only possess neuromodulatory activity. Interestingly, palmyric acid exerts an effect on intracellular calcium concentrations, but does not appear to have any effect on the VGSC as determined by a lack of significant activity of parent fraction 1882 E4 in both sodium channel activating and blocking assays in murine Neuro-2a cells.

Future investigations exploring the biosynthetic pathway for this unusual secondary metabolite will help to determine if this molecule is produced via a modified version of the curacin A pathway or if it is generated through a novel biosynthetic route.\textsuperscript{53} Additionally, based on its biological profile, further examination of the ion modulation effects displayed by this compound may reveal a novel target. Nevertheless, it is clear that a much broader evaluation of the biological properties of palmyric acid is warranted.
5.4 Experimental Details

5.4.1 General experimental procedures

Optical rotation was measured with a Jasco P-2000 polarimeter. CD spectrum was obtained on a Jasco J-815 CD spectrometer. UV spectrum was obtained on a Beckman Coulter DU-800 spectrophotometer, and FT-IR spectrum was recorded on a Nicolet IR 100 FT-IR spectrophotometer. NMR spectra were recorded on a Varian Inova 300 MHz or 500 MHz with a 5 mm probe and were referenced to residual solvent $^1$H and $^{13}$C signals ($\delta^H 7.26, \delta^C 77.16$ for CDCl$_3$ and $\delta^H 7.16, \delta^C 128.62$ for C$_6$D$_6$). Low-resolution ESIMS spectra were acquired on a Finnigan LCQ Advantage Max mass spectrometer, while high-resolution ESIMS spectra were obtained by the UCSD Chemistry and Biochemistry Molecular MS Facility on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. HPLC separation was completed using a Waters HPLC system equipped with a Waters 515 binary pump and a Waters 996 PDA detector.

5.4.2 Biological material collection and identification

Approximately 300 mL of red filamentous cyanobacterial tufts were collected by hand from a depth of 1-3 m in a shallow lagoon north of Barren Island in the Palmyra Atoll. Following collection the cyanobacterium was stored in 1:1 EtOH:seawater at $-20^\circ$C; prior to shipping, the samples were thawed and the supernatant was discarded. For longer storage, EtOH was added and the samples were stored at $-20^\circ$C until workup. A voucher specimen (PAL 08/22/08-1) is maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA.
5.4.3 Polymerase chain reaction (PCR) and cloning

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Inc., Madison, WI, USA) following the manufacturer’s specifications. DNA concentration and purity were measured on a DU® 800 spectrophotometer (Beckman Coulter). The 16S rRNA genes were PCR-amplified from isolated DNA using the cyanobacterial specific primers 27F 5’-AGAGTTTGATCCTGCTGAGCAG-3’ and 809R 5’-GCTTCGGCACGGCTCGGGTCAGA-3’. The PCR reaction contained 1.0 µL (~100 ng) of DNA, 2.5 µL of 10 × PfuUltra IV reaction buffer, 1.0 µL (10 mM) of dNTP mix, 1.0 µL of each primer (10 µM), 1.0 µL of PfuUltra IV fusion HS DNA polymerase and 17.5 µL H2O for a total volume of 25 µL. The PCR reactions were performed in an Eppendorf® Mastercycler® gradient as follows: initial denaturation for 4 min at 95°C, amplification by 30 cycles of 30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C, and final elongation for 7 min at 72°C. PCR products were purified using a MinElute® PCR Purification Kit (Qiagen) before subcloning with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) following the manufacturer’s specifications. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and sequenced with M13 primers.

5.4.4. Phylogenetic inferences

All gene sequences were analyzed using Geneious Pro v.5.5.4.55 The 16S rRNA gene sequences were aligned using the L-INS-I algorithm in MAFFT v6.814b.56 Best-fitting nucleotide substitution models optimized by maximum likelihood were selected using corrected Akaike/Bayesian Information Criterion (AIC/BIC) in jModelTest
The evolutionary histories of the cyanobacterial genes were inferred using Maximum Likelihood (ML) and Bayesian inference algorithms. The Maximum Likelihood (ML) inference was performed using PhyML in Geneious Pro v5.5.4. The analysis was run using the GTR+I+G model (selected by AIC and BIC criteria) assuming heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.042, shape parameter (α) = 0.182, number of rate categories = 4). Bootstrap resampling was performed on 1,000 replicates. Bayesian analysis was conducted using MrBayes in Geneious Pro v5.5.4 with four Metropolis-coupled MCMC chains (one cold and three heated) run for 3,000,000 generations. The first 25% were discarded as burn-in and data set was sampled with a frequency of every 200 generations.

5.4.5 Extraction and isolation

All 300 mL of EtOH-preserved biomass (81.4 g, dry weight) of the filamentous red cyanobacterial tufts (PAL 08/22/08-1) were extracted with 2:1 CH2Cl2/MeOH six times to give 0.833 g of crude extract (extract #1882). A portion of the crude extract (0.644 g) was fractionated using vacuum liquid chromatography (VLC) on Silica gel (Type H, 10-40 μM, Sigma-Aldrich) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A-I). Fraction E, eluted with 60% EtOAc in hexanes (69 mg), was subjected to normal-phase chromatography using a Phenomenex Strata SI-1 Silica (55 mm, 70Å) 1000 mg/6 mL Sep-Pak with a stepwise gradient from 0% EtOAc in hexanes to 100% EtOAc. The largest fraction (E1) containing 16.6 mg eluted with 100% hexanes and appeared to be largely comprised of standard fatty acids based on the presence of a large bis-allylic methylene envelope at 2.8 ppm and a broad
olefinic envelope at 5.35 ppm in the ¹H NMR spectrum. However, the ¹H NMR of the second largest fraction (E4) containing 7.5 mg eluted with 12.5% EtOAc in hexanes featured several interesting resonances, including a doublet at 6.4 ppm, a doublet of doublets at 5.95 ppm, a multiplet at 4.45 ppm, as well as two additional doublet of doublets at 3.35 and 2.95, respectively, and resonances characteristic of a cyclopropane between 0.5 and 1 ppm, among others. LR-ESI-MS in positive and negative mode revealed an [M+H]^+ adduct of 352 and an [M-H]^− adduct of 350, respectively, suggesting a mass of approximately 351, which was dereplicated against the MarinLit database. MarinLit gave 31 hits for a mass range of 351 to 352, 14 of which were brominated and/or chlorinated; however, as the isotopic pattern for the major compound in fraction E4 did not feature a significant M+2 peak, the compound was presumed not to be halogenated, thus these compounds were eliminated. Of the remaining compounds, only one, laingolide, had been isolated from marine cyanobacterial sources. As laingolide features five methyl groups and one N-Me, but ¹H NMR for E4 revealed only a single methyl resonance, it was presumed that the major component of E4 was a novel compound. Thus, fraction E4 was subjected to normal phase HPLC on a Phenomenex Luna 5μ Silica (2) 100Å column (5 μm, 10 × 250 mm) with a gradient as follows: 2.5% IPA in hexanes at 3mL/min from 0-18 min, then ramped from 2.5% to 20% IPA in hexanes from 18-23 min, held at 20% IPA in hexanes from 23-40 min, then returned to initial condition from 40-45 min and held for 15 min. Compound 1 eluted over 8 min beginning at 16 min, and was collected in several fractions designated at B-E. Fractions B-E all appeared to be pure based on NMR and LCMS analyses. Fraction 1882 E4B
contained 2.8 mg, while C contained 0.9 mg, D contained 0.4 mg and E contained 0.3 mg, thus compound 1 comprised 0.68% of the extract.

5.4.6. Characterization of purified palmyric acid (1)

Palmyric Acid (1): 4.4 mg (0.68%) white amorphous solid $[\alpha]^{24}_D +39.8$ (c 1.0, CHCl$_3$), UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 247 (4.2) nm; IR $\nu_{\text{max}}$ (film) 2999, 2924, 2853, 1713, 1641, 1578, 1457, 1207, 1098, 1072, 1030, 961, 869, 572 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 5.1; ESIMS positive mode $m/z$ 352 (100% rel. abund.) [M+H]$^+$; ESIMS negative mode $m/z$ 350 [M-H]; HRESI-TOFMS $m/z$ [M+H]$^+$ 352.2299 (calcd for C$_{20}$H$_{34}$NO$_2$S, 352.2305).

5.4.7. Neocortical neuron culture

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice as described elsewhere. Briefly, pregnant mice were euthanized by CO$_2$ asphyxiation, and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle’s minimal essential medium with Earle’s salt (MEM) and supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin, and 0.10 mg/mL streptomycin (pH 7.4). Cells were plated onto poly-L-lysine-coated 96-well (9 mm), clear-bottomed, black-well culture plates (Costar) at a density of $1.5 \times 10^5$ cells/well. Cells were then incubated at
37°C in a 5% CO₂ and 95% humidity atmosphere. Cytosine arabinoside (10 mM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed every other day, starting from day 5 in vitro, using a serum-free growth medium containing Neurobasal Medium supplemented with B-27, 100 IU/mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM l-glutamine. Neocortical cultures were used in experiments between 8–13 days in vitro (DIV). All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.
I thank the National Wildlife Service for permission to collect the cyanobacterial specimen. I thank Dr. Y. Su (UCSD Chemistry and Biochemistry Mass Spectrometry Facility) for acquiring HRMS and MS\textsuperscript{n} data. Support was provided by a graduate fellowship to JKN from the National Institutes of Health Neuroscience Related to Drug Abuse Training Grant (T32DA007315). The statements, findings, conclusions, and recommendations provided here are those of the authors and do not necessarily reflect the views of the National Institutes of Health.

Author Contributions

JKN TB, and TTT performed research. TB, SVJ conducted bioassays. JKN, TB, ARP, TFM and WHG designed research and wrote the manuscript.

The authors declare no conflicts of interest.

Chapter 5, in essence, is currently being prepared for submission in 2012. Joshawna K. Nunnery, Tamara T. Tran, Tara Byrum, Sairam V. Jabba, Alban R. Pereira, Thomas F. Murray, and William H. Gerwick. The dissertation author was the primary investigator and author of this material.
Figure 5.6.1. High-resolution electrospray ionization Fourier transform mass spectrum of MS² analysis of m/z 352 from 1 (peak at m/z 183.3 is from FT-MS background).
Figure 5.6.2. High-resolution electrospray ionization Fourier transform mass spectrum of MS$^3$ analysis of $m/z$ 227 from 1 (peak at $m/z$ 183.3 is from FT-MS background).
Figure 5.6.3. High-resolution electrospray ionization Fourier transform mass spectrum of MS² analysis of m/z 366 from methyl esterified 1.
Figure 5.6. High-resolution electrospray ionization Fourier transform mass spectrum of MS$^3$ analysis of $m/z$ 227 from methyl esterified 1.
Figure 5.6.5. $^1$H NMR spectrum of palmyric acid (1) in CDCl$_3$ at 500 MHz.
Figure 5.6.6. $^{13}$C NMR spectrum of palmyric acid (1), recorded in CDCl$_3$ at 75 MHz.
Figure 5.6.7. HMBC spectrum of palmyric acid (1), recorded in CDCl$_3$ at 500 MHz.
Figure 5.6.8. HSQC spectrum of palmyric acid (1), recorded in CDCl₃ at 500 MHz.
Figure 5.6.9. COSY spectrum of palmyric acid (1), recorded in CDCl₃ at 500 MHz.
Table 5.6.1. NMR spectroscopic data for palmyric acid (1) in C₆D₆.

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<th>δC&lt;sup&gt;a,b&lt;/sup&gt;</th>
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<td>0.74 d (6.0)</td>
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<sup>a</sup> Recorded at 75 MHz. <sup>b</sup> Multiplicity deduced from HSQC. <sup>c</sup> Recorded at 500 MHz.
Figure 5.6.10. $^1$H NMR spectrum of palmyric acid (1) in C$_6$D$_6$ at 500 MHz.
Figure 5.6.11. $^{13}$C NMR spectrum of palmyric acid (1), recorded in C$_6$D$_6$ at 75 MHz.
Figure 5.6.12. HMBC spectrum of palmyric acid (1), recorded in C\textsubscript{6}D\textsubscript{6} at 500 MHz.
Figure 5.6.13. HSQC spectrum of palmyric acid (1), recorded in C₆D₆ at 500 MHz.
Figure 5.6.14. COSY spectrum of palmyric acid (1), recorded in C₆D₆ at 500 MHz.
Figure 5.6.15. ROESY spectrum of palmyric acid (1), recorded in C₆D₆ at 500 MHz.


(43) Cardellina, J. H., II; Moore, R. E. Mallyngic acid, a new fatty acid from Lyngbya majuscula. Tetrahedron 1980, 36, 993-996.


6.0 CHAPTER 6
CONCLUSIONS & FUTURE WORK

6.1 Marine Cyanobacteria as Prolific Producers of Bioactive Natural Products

Over the past three decades, marine cyanobacteria have emerged as a prolific source of natural products with biological activities relevant for the treatment of human diseases. To date, much of the emphasis regarding drug discovery from marine cyanobacteria has been placed on the isolation and characterization of marine cyanobacterial secondary metabolites with anticancer activities.\(^1\) However, several therapeutically relevant properties have been discovered recently for marine cyanobacterial compounds, including neuromodulatory (e.g. antillatoxins A,\(^2\) kalkitoxin,\(^2\) jamaicamide A,\(^3\) hoiamide A\(^4\))\(^5,6\) anti-inflammatory (e.g. scytonemin,\(^7\) malyngamide F acetate,\(^8\) malyngamide 2\(^9\)), and antimalarial (e.g. venturamides A and B,\(^10\) carmbin A,\(^11\) lagunamides A-C,\(^12,13\) gallinamide A\(^14\)) activities. These recent revelations regarding the varied biological activities of marine cyanobacterial secondary metabolites indicate that broader evaluation of the biological properties of marine cyanobacterial compounds should be conducted. The order Oscillatoriales, comprised of approximately 30 genera, includes several that significantly contribute to the overall production of secondary metabolites in marine cyanobacteria.\(^15,16\) These genera, including *Lyngbya*, *Oscillatoria*, *Schizothrix*, *Symploca*, *Phormidium*, and *Leptolyngbya*, account for approximately 58% of the secondary metabolites isolated from marine cyanobacteria.\(^16\)

As of 2010, a total of 240 compounds accounting for 35% of all cyanobacterial metabolites had been reported from the genus *Lyngbya*, with *Lyngbya majuscula*
attributed as the source of 76% of these compounds.\(^{16}\) Recently, genome sequencing of one strain of *Lyngbya majuscula* from Curaçao revealed only 126 genes predicted to play a role in secondary metabolite biosynthesis.\(^{17}\) Furthermore, while eight gene clusters potentially involved in natural product biosynthesis were identified, only two of the clusters had been previously described and only one additional cluster appeared to encode a known marine cyanobacterial natural product, thus indicating that secondary metabolite production in marine cyanobacteria may be strain specific.\(^{17}\) Recently, this strain of *Lyngbya* was described as a new genus of cyanobacteria and is now identified as *Moorea producta*.\(^{18}\)

6.2 Summary of the Research Presented in the Dissertation and Future Work

The major objective of the research presented herein was to explore the chemical diversity of compounds from extracts or fractions displaying neuromodulatory activities derived from tropical marine collections of cyanobacteria. In particular, the focus of these investigations was to evaluate the chemical richness of marine cyanobacteria identified as belonging to either the genus *Schizothrix* or *Symploca* spp. Previously, the genera *Schizothrix* and *Symploca* have been found to collectively account for approximately 9% (61 compounds) of cyanobacterial secondary metabolites.\(^{16}\) As chirality can significantly impact biological activity, determination of absolute stereochemistry is a critical facet of the development of marine natural products as pharmaceuticals. Therefore, one of the minor objectives was to utilize synthetic methodologies to provide standards for stereochemical analyses. Chapter 2 addressed this secondary objective by describing synthetic efforts to obtain the \(R\)- and \(S\)-enantiomers of three fragments featuring an \(\alpha,\alpha\)-
dimethyl-\(\beta\)-hydroxy carbonyl scaffold for stereochemical analyses and total syntheses applications and in this context, explored the utility of tertiary aldol reactions in organic synthesis. While chapter 3 largely focused on the primary objective presenting the isolation, structure elucidation, and biological activity evaluation of janthielamide A and kimbeamide A, which are lipopeptides exhibiting modest neuromodulatory activity, it also addressed the secondary objective in the absolute configuration determination of janthielamide A. Furthermore, chapter 3 recounted the isolation and characterization of the planar structures of two geometrical isomers of kimbeamide A, designated as kimbeamides B and C, and a ketide-extended pyranone. Chapter 4 discussed the isolation, structure elucidation and biological activity of a new member of the tasihalide family, tasihalide C, which features an additional acetoxy moiety in comparison to tasihalide B, and exhibits immunomodulatory activity. Lastly, chapter 5 chronicled the isolation, structure elucidation and preliminary biological evaluation of palmyric acid, a novel marine cyanobacterial natural product with structural homology to the curacin family of compounds. A brief summary of each research chapter and future work for each project are described below.

Stereochemical analyses of non standard amino acid residues, as well as \(\beta\)-hydroxy acid moieties and other fragments containing chiral centers are often challenging. As demonstrated in chapter 2 with the synthesis of \(\alpha,\alpha\)-dimethyl-\(\beta\)-hydroxy octanoic acid and \(\alpha,\alpha\)-dimethyl-\(\beta\)-hydroxy hexanoic acid and in chapter 3 with the generation of 2-methyl-\(\gamma\)-aminobutyric acid, synthetic methodologies may provide facile access to non-commercially available standards for comparative analyses with derivatized degradation products of secondary metabolites for the determination of
absolute configuration. Desaturated analogues of α,α-dimethyl-β-hydroxy octanoic acid including fragments featuring either a terminal alkene or alkyne moiety are often encountered in natural products from marine cyanobacteria, including yanucamides A and B,\textsuperscript{22} georgamide,\textsuperscript{23} pitipeptolides A-F\textsuperscript{24,25} and wewakpeptins A-D.\textsuperscript{26} Recently, several secondary metabolites possessing an α,α-dimethyl-β-hydroxy octynoic acid fragment have been isolated in the Gerwick laboratory. While this fragment has previously been synthesized by Xu \textit{et al.},\textsuperscript{27} the synthetic route was nine steps long and involved several deprotection/protection steps to mask the alkyne functionality. However, the saturated fragment is preferred for stereochemical analyses as the alkyne has been proven to be unstable to the acidic conditions employed for hydrolysis of the natural product to yield the desired fragment.\textsuperscript{28}

Thus, an improved synthesis was devised and optimized to provide α,α-dimethyl-β-hydroxy octanoic acid in three steps with overall yields of 52\% and 83\% for the \(R\) and \(S\)-enantiomers, respectively. The absolute configurations of the β-ester linkages of eight marine cyanobacterial secondary metabolites possessing an α,α-dimethyl-β-hydroxy octynoic acid fragment, including mantillamide A,\textsuperscript{29} bocapeptolide,\textsuperscript{30} radamamide A\textsuperscript{31} and the dudawalamides A-E\textsuperscript{32} have been determined using the standards afforded via the synthetic route described in chapter 2. Furthermore, the versatility of this synthetic route was explored by substituting hexanal with either hexynal or butanal to provide α,α-dimethyl-β-hydroxy octynoic acid and α,α-dimethyl-β-hydroxy hexanoic acid, respectively. Overall yields of 56\% and 47\% for the \(R\)- and \(S\)-enantiomers, respectively, were achieved for α,α-dimethyl-β-hydroxy octynoic acid, representing a significant improvement in yield in comparison to the synthesis of α,α-dimethyl-β-hydroxy octynoic acid.
acid by Xu et al. for which an overall yield of 21% was obtained. Lastly, the α,α-dimethyl-β-hydroxy hexanoic acid standards were employed in the stereochemical analysis of palmyramide A, which was found to have the \( R \) configuration for the β-ester linkage.

Future directions of this research may include the generation of both enantiomers of α,α-dimethyl-β-hydroxy hexanoic acid under the optimized conditions for the 3° aldol reaction to evaluate overall yield. Also, additional experiments to evaluate the utility of various Lewis acids in the 3° aldol reaction could be conducted to determine if a higher yield might be obtained in a shorter timeframe or under less stringent temperature requirements. Further, implementation of thiazolidinethiones and oxazolidinethiones in place of the oxazolidinones employed here could be explored. Research by Crimmins et al. regarding secondary aldol reactions with sulfur-containing Evans’-type auxiliaries has shown that the \( \text{syn} \) or \( \text{anti} \) product can be obtained with high diastereoselectivity from the same starting material with the addition of different equivalents \((-\text{-sparteine and the Lewis acid TiCl}_4\). Therefore, one may expect that a more economical route could be achieved utilizing a thiazolidinethione or an oxazolidinethione starting material. Lastly, the products afforded by this route, or a modified route employing a different Lewis acid or Evans’-type auxiliary or both, may be utilized in the total syntheses of secondary metabolites featuring these fragments that possess potent biological activities.

The synthetic route described in chapter 2 will allow other researchers to obtain the requisite standards for stereochemical analyses of natural products featuring α,α-dimethyl-β-hydroxy octynoic acid or a related fragment in an expedient manner. The development of facile routes to obtain standards for comparison with derivatized
degradation products from natural products more readily allows for complete characterization of compounds with multiple stereocenters. In addition, exploration of new routes for the production of standards for stereochemical analyses may lead to improved routes to obtain fragments for implementation in total syntheses efforts.

In chapter 3, five new vinyl-chloride-containing secondary metabolites were described from marine cyanobacteria collected in Curaçao and Papua New Guinea. The lipopeptides, janthielamide A and kimbeamides A-C possess novel backbones, but display structural similarity to other marine cyanobacterial secondary metabolites, including pitiamide A \(^{35}\) and grenadamides B and C.\(^{36}\) Meanwhile, kimbelactone A exhibits structural similarity to fuligoic acid, a chlorinated polyene-pyrone from the myxomycete *Fuligoseptica f. flava*.\(^{37}\) The planar structures of these compounds were largely assembled based on homonuclear and heteronuclear 2-D NMR experimental data including COSY, HMBC, and multiplicity-edited HSQC. For all compounds, the positions of the chlorine atoms were assigned based on chemical shift arguments and comparison with published compounds featuring similar functionalities. The geometries of the double bond configurations for each compound were established based on \(^3J_{HH}\) vicinal coupling constants or NOE correlations.

The absolute configuration of the methyl group of kimbeamide A, presumed to derive from alanine, was determined by ozonolyzing the natural product followed by an oxidative workup and subsequent acidic hydrolysis to obtain an alanine residue. This residue was then esterified with isopropanol and acylated with trifluoroacetic anhydride to furnish a derivative for comparison by chiral GCMS with similarly derivatized L-
D-alanine standards. Ultimately, it was revealed that the absolute configuration of C1’ on kimbeamide A was $S$.

Determination of the absolute configuration of janthielamide A proved to be more challenging. Acidic hydrolysis followed by ozonolysis with oxidative workup readily provided two fragments with each containing one of the desired chiral centers. Standards for the 2-methylsuccinic acid fragment were commercially available and derivatization with the chiral alcohol (S)-2- (+)-octanol\(^{38}\) afforded diastereomers which resolved well under achiral GCMS conditions. Thus, the absolute configuration at C5 was determined to be $R$. Though 2-methyl-$\gamma$-aminobutyric acid (2-methyl-GABA) was readily obtained from the natural product following the degradation pathway described above, standards were not commercially available despite the fact that this fragment is an analogue of the extensively studied neurotransmitter GABA.\(^{39}\) Ultimately, the requisite standards ($R$)- and (S)-2-methyl-GABA were obtained from commercially available ($R$)-4-amino-2-methyl-1-butanol and ($R$)-(−)-3-bromo-2-methyl-1-propanol, respectively.\(^{40,41}\) Derivatization with (S)-2- (+)-octanol\(^{38}\) and subsequent analysis by achiral LCMS revealed that the absolute configuration of C9 was $R$.

Janthielamide A exhibited sodium channel blocking activity in murine Neuro-2a cells with an $IC_{50}$ of 11.5 $\mu$M and antagonized veratridine-induced sodium influx in murine cerebrocortical neurons with an $IC_{50}$ of 5.2 $\mu$M. Kimbeamide A exhibited sodium channel blocking activity 60 $\mu$M; however, an $IC_{50}$ value was unable to be obtained for this compound due to degradation and solubility issues. Unfortunately, the kimbeamides B and C, as well as kimbelactone A, also readily decomposed, thus prohibiting determination of their absolute configurations and evaluation of their biological activities.
Morphological analyses of the source marine cyanobacteria from both the Curaçao and Papua New Guinea collections revealed that the major cyanobacterial organism present in each collection fell under the taxonomic classification of *Symploca*. However, 16S rRNA phylogenetic analyses revealed that while these organisms are related to *Symploca*, there is approximately 5% gene sequence divergence for the “tropical marine *Symploca*” clade in comparison with the type strain for *Symploca* (PCC 8002). The “tropical marine *Symploca*” clade contains additional strains identified as *Symploca* from tropical marine collections, including VP377 (symplostatin 1 putative producer), VP642 (dolastatin 10 putative producer) and PNG 06-65.1 (hoiamides A and B putative producer).

Future research regarding this project might include recollection of these marine cyanobacteria for culture and additional phylogenetic analyses, including formal description of the “tropical marine *Symploca*” genus. Recollection and reisolation of the secondary metabolites or total syntheses of these compounds would provide additional material for biological testing, as well as further investigations of solubility and stability issues. Kimbeamides B and C, which are geometrical isomers of kimbeamide A, may have been generated due to exposure of kimbeamide A to either light or heat; re-isolation of these compounds would allow for investigations into whether or not the isomers are natural products or artifacts. In the case of kimbelactone A, re-isolation or total synthesis would be beneficial to allow for elucidation of the absolute configuration of the two chiral centers present in the molecule and evaluation of its biological properties. It would also be worthwhile to obtain samples of grenadamides B and C, as well as pitiamide
A,\textsuperscript{35} for comparison in the sodium channel blocking assay in murine Neuro-2a cells to begin to evaluate structure activity relationships.

In chapter 4, the isolation and biological activity evaluation of a new analogue in the tasihalide family of compounds, previously described Williams et al.,\textsuperscript{46} was presented. These intriguing diterpenes feature both bromination and iodination and were isolated from collections of marine cyanobacteria related to Symploca sp. which were found to contain trace amounts of eukaryotic algae.\textsuperscript{46} The planar structure of tasihalide C was assembled based on COSY, HMBC and multiplicity-edited HSQC. The presence of bromine was deduced from the 1:1 isotopic ratio observed for the [M+Na]\textsuperscript{+} adduct present in the LCMS spectrum, while the presence of iodine was suspected due unique chemical shifts of $\delta_C$ 15.1 for C15 and $\delta_H$ 4.03 and 3.11 for the associated protons. Comparison with tasihalide B revealed that tasihalide C featured an acetoxy on C13 not present in tasihalide A or B. Analysis of the ROESY data and coupling constants for tasihalide C indicated that the relative stereochemistry of tasihalide C is identical to that of tasihalides A and B with the exception of the new stereocenter at C13.\textsuperscript{46}

Evaluation of the biological activity of the initial fractions obtained from the crude extract of the cyanobacterium revealed three fractions that increased calcium oscillations at 5 $\mu$g/mL in murine cerebrocortical neurons. The fraction investigated here was selected as it contained significantly more material than the other two fractions. Interestingly, the fraction preceding the purification of tasihalide C did not exhibit any effect on calcium in murine cerebrocortical neurons. As a result, tasihalide C was submitted to a variety of bioassays and was found to exhibit modest activity in an immunomodulatory assay. Tasihalide C exhibited a decrease in the nitric oxide
production in LPS-induced murine macrophage cells RAW264.7 with an IC$_{50}$ of 1.3 $\mu$M suggesting that tasihalide C may have anti-inflammatory activity. As anti-oxidant activity can skew the data in this anti-inflammatory assay, tasihalide C was also evaluated for anti-oxidant activity and found to be inactive.

Future work for tasihalide C will include additional evaluation of the observed biological activity and determination of its absolute stereochemistry if acceptable crystals can be obtained. Further investigations regarding the potent anti-inflammatory activity observed for tasihalide C may include evaluation of the compound using a mouse ear edema model by assessing the potential decrease of the edema of the PMA painted ear, as well as the completion of an ELISA assay of the ear tissue to assess cytokine expression with emphasis on IL-6 and TNF-$\alpha$. Attempts to grow suitable crystals for X-ray crystallography are currently underway using a variety of conditions. It is suspected that tasihalide C should readily crystallize as the cage structure seems to be tightly constrained and as the compound features two heavy halide atoms. Once acceptable crystals are obtained, they will be submitted for X-ray crystallography. With regard to the ultimate producer of the tasihalides, it would be beneficial to recollect the cyanobacterium and to subject live filaments to analysis using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) and/or nano-desorption electrospray ionization (nano-DESI) to determine if the compound is localized to the cyanobacterial filaments or to the eukaryotic algal fragments. While the chemistry is more consistent with red algal secondary metabolite chemistry than marine cyanobacterial chemistry, the metabolites have not yet been isolated from red algae, nor
have other brominated and iodinated diterpenes been reported, therefore, although it is unexpected, it is possible that these natural products are of cyanobacterial origin.

Finally, chapter 5 described the isolation, structure elucidation and preliminary biological evaluation of a novel compound, palmyric acid, with structural similarity to the curacin A family of marine cyanobacterial secondary metabolites.\textsuperscript{19-21} The planar structure of palmyric acid was determined based on analysis of 1-D and 2-D NMR experimental data, including COSY, HMBC and multiplicity edited HSQC. Chemical shift data, including shifts at $\delta_C$ 177.6, consistent with an ester, and $\delta_C$ 76.4, consistent with an oxygenated carbon, were initially interpreted as suggesting the presence of at least three oxygen atoms in the molecule; however, high-resolution mass spectrometric analysis indicated the presence of a sulfur atom and only two oxygen atoms as opposed to three. Ultimately, palmyric acid was discovered to have a thiazoline functionality and a carboxylic acid moiety, accounting for the chemical shifts of $\delta_C$ 177.6 and $\delta_C$ 76.4, as well as a methyl-substituted cyclopropane. Overall, palmyric acid displays structural homology to the curacins with a few key structural differences including an additional ketide extension on the cyclopropyl ring-containing portion of the compound and a carboxylic acid terminus in place of a terminal alkene on the lipophilic chain.\textsuperscript{19-21}

Fractionation of the crude extract and subsequent bioassay of the preliminary fractions identified a fraction that produced an influx of calcium in murine cerebrocortical neurons at 50 $\mu$g/mL, but suppressed spontaneous calcium oscillations at 5 $\mu$g/mL. Purification of the major non-fatty acid component of this fraction led to the isolation of palmyric acid, which was discovered to have very modest effects in the calcium influx assay. It is suspected that palmyric acid readily decomposes as new peaks
were observed in both the $^1$H NMR spectrum and the LCMS spectrum following exposure to and removal of DMSO. The susceptibility of this compound to degradation may impede further evaluation of its biological activity and stereochemical analyses.

Future research concerning palmyric acid might include additional evaluation of the biological activity of the compound in other biological assays and under different solvent conditions, as well as determination of the absolute stereochemistry of the molecule. Previously, other marine cyanobacterial metabolites featuring cyclopropyl rings and carboxylic acids have shown intriguing biological activities, including majusculoic acid,\textsuperscript{48} which was found to exhibit antifungal activity against \textit{Candida albicans} ATCC 14503 with an MIC of 8 $\mu$M, and lyngbyoic acid,\textsuperscript{49} which was discovered to disrupt quorum sensing in \textit{Pseudomonas aeruginosa}. Based on the presence of similar functional groups in palmyric acid, it might be worthwhile to evaluate this compound in additional bioassays, especially antifungal, antibacterial and quorum sensing assays. As palmyric acid appears to be unstable in DMSO, it might be useful to investigate the biological activity of the compound under different solvent conditions.

As far as the absolute configuration of palmyric acid is concerned, it can be envisioned that degradation of the compound via ozonolysis with an oxidative workup might afford 2-methylcyclopropanecarboxylic acid. This fragment could be derivatized with a chiral ester or alcohol and compared with synthetically prepared standards to determine the absolute configuration of the two chiral centers on the cyclopropyl ring. Meanwhile, the absolute stereochemistry of C2 could conceivably be determined by one of two ways: 1) palmyric acid could be hydrolyzed and ozonolyzed to give 12-amino-13-sulfotridecanoic acid, which could then be purified and compared with model compounds
to assess the absolute configuration based on optical rotation or 2) a naphthylene or other functionality with a strong chromophore could be appended to the carboxylic acid and the absolute configuration could be determined using circular dichroism spectroscopy. Unfortunately, only a very small amount of palmyric acid was isolated and due to stability issues, it might be quite difficult to determine the stereochemistry using the miniscule amount of remaining material. Total synthesis of this compound may be the most straightforward approach for determining the absolute stereochemistry.

Figure 6.1. New secondary metabolites discovered from collections of tropical marine Symploca sp.

In total, the discovery and characterization of seven new marine natural products were presented in this dissertation (Figure 6.1). The marine natural products described here further support the commonly observed trend of NRPS-PKS interdigitation for marine cyanobacterial secondary metabolites as five of the seven compounds feature at least one amino acid residue. Of the remaining two compounds, one is derived purely
from PKS machinery, while the other is derived from isoprene condensation and suspected to be of red algal origin.\textsuperscript{46} Additionally, six of the seven compounds featured at least one chlorine or bromine atom consistent with the high occurrence of halogenation typically observed for marine natural products, especially those of cyanobacteria and red algae.\textsuperscript{50} Janthielamide A, kimbeamide A and palmyric acid expand our knowledge of compounds possessing neuromodulatory activity from marine cyanobacteria, while the discovery of immunomodulatory activity for tasihalide C, which belongs to a class of compounds for which no bioactivity had previously been reported, may provide a new lead compound for development as an anti-inflammatory agent.

### 6.3 Expected Future Developments in Marine Natural Products Chemistry

As the case with the tasihalides reveals, it can often be difficult to determine which organism is ultimately responsible for the production of bioactive compounds when consortia are intentionally or inadvertently investigated for novel chemistry. Bacteria and cyanobacteria are increasingly being implicated as the ultimate source of many potently bioactive lead compounds from the sea. A number of marine natural products which have recently been approved or are currently undergoing phase I-III clinical trials were initially isolated from invertebrates, but are suspected to be of bacterial or cyanobacterial origins.\textsuperscript{51} For example, the tetrahydroisoquinoline chemotherapeutic agent trabectedin (ET-743) approved by the European Medicines Agency for the treatment of ovarian cancer and advanced soft-tissue sarcoma, was originally described in 1990 by Rinehart \textit{et al.} from the tunicate \textit{Ecteinascidia turbinata}.\textsuperscript{52,53} However, it has long been suspected to be of microbial origins based on
structural similarity to prokaryotic secondary metabolites,\textsuperscript{54} including saframycin A,\textsuperscript{55} saframycin Mxl,\textsuperscript{56} and safracin B.\textsuperscript{57} Recently, employing metagenomic and metaproteomic approaches in tandem, Rath et al. revealed that the unculturable symbiont \textit{Candidatus Endoecteinascidia frumentensis} is the ultimate producer of trabectedin.\textsuperscript{58}

Similarly, bryostatin 1, an anticancer agent in phase II clinical trials in the US\textsuperscript{59} discovered by Pettit \textit{et al.} in 1982 from the bryozoan \textit{Bugula neritina},\textsuperscript{60} is now believed to be biosynthesized by the uncultivated symbiont “\textit{Candidatus Endobugula sertula}.”\textsuperscript{61,62}

Meanwhile, solibdotin (TZT-1027), currently in phase III trials,\textsuperscript{59,63} is a synthetic analogue of a natural product originally identified from the sea hare \textit{Dolabella auricularia} known as dolastatin 10.\textsuperscript{64} Subsequently, dolastatin 10 and an analogue named symplostatin 1 were isolated from the cyanobacterium \textit{Symploca} sp. VP642,\textsuperscript{44} and therefore, it is presumed that this herbivorous sea hare acquires these compounds from dietary sources.\textsuperscript{65} An assessment by Simmons and Gerwick in 2008 of 20 anticancer agents derived from marine organisms presently or previously in clinical trials suggested that while approximately 60\% of the compounds were isolated from marine macro-organisms and only 5\% and 15\% were isolated from bacteria and cyanobacteria, respectively, up to 75\% of the compounds were likely produced by bacteria and cyanobacteria (Figure 6.2).\textsuperscript{51}
One of the most challenging obstacles in the advancement of marine natural products to the clinic is the necessity of obtaining a sustainable supply of these potent biologically-active molecules which are often rare, structurally complex compounds.
isolated in extremely small yields from the natural environment. An increasingly large number of compounds with varied therapeutically-relevant activities have been isolated from marine invertebrates, including sponges, tunicates, mollusks and corals, among others. However, as shown above, many of these natural products are suspected to be of bacterial or cyanobacterial origins based on structural homology to known compound classes from prokaryotic organisms and circumstantial evidence regarding dietary sources and/or symbiotic relationships. New developments in instrumentation and techniques for molecular biology and mass spectrometry are providing novel ways to address questions regarding the ultimate producers of rare compounds with potential for clinical development. By identifying the ultimate producing organism, new routes for the production of these compounds may be realized, including scaleable culture of the producing organism, heterologous expression of the relevant pathway in robust prokaryotic organisms, such as *Escherichia coli* or *Pseudomonas aeruginosa*, or semi-synthesis of complex natural products from simplified scaffolds obtained from prokaryotes possessing similar biosynthetic pathways.

To date, there are approximately 250,000 species known from the marine realm; however, it is suspected that the world's oceans contain nearly 2.2 million eukaryotic species with many phyla unique to the marine environment.\textsuperscript{66,67} Furthermore, microbes are expected to comprise up to 90\% of the overall weight of marine life with 10 to the 29\textsuperscript{th} power cells believed to be present in the water column.\textsuperscript{66} Currently, it is estimated that more than 38,000 kinds of bacteria may be found in a liter of seawater, while between 5,000 and 19,000 kinds may be encountered in a gram of sand.\textsuperscript{66} At present, a meager 9\% of species in the ocean have even been described and by comparison, only a
handful of marine species has been explored regarding the production of secondary metabolites. Based on the present knowledge concerning the structural diversity and therapeutic potential of marine natural products from known species, it is certain that continued explorations of organisms from the marine environment will reveal novel chemical entities with relevance in the treatment of human disease.


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