Title
Structure elucidation of biomedically relevant marine cyanobacterial natural products

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Structure Elucidation of Biomedically Relevant Marine Cyanobacterial Natural Products

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

in

Biomedical Sciences

by

Karla Lynn Malloy

Committee in Charge:

Professor William H. Gerwick, Chair
Professor Pieter Dorrestein
Professor William H. Fenical
Professor Lena G. Gerwick
Professor Vivian Hook
Professor Oswald Quehenberger

2011
The Dissertation of Karla Lynn Malloy is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
DEDICATION

This dissertation is dedicated to my parents for their unconditional love and support throughout the years and to all the souls lost to the ravages of human disease.
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<thead>
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<tbody>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FDVA</td>
<td>fluoro-dinitrovaline amide</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>gCOSY</td>
<td>gradient correlation spectroscopy</td>
</tr>
<tr>
<td>gHMBC</td>
<td>gradient heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>gHSQC</td>
<td>gradient heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>high resolution</td>
</tr>
<tr>
<td>IC50</td>
<td>50 % inhibitory concentration</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography / mass spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NRPS</td>
<td>non-ribosomal peptide synthetase</td>
</tr>
<tr>
<td>PKS</td>
<td>polyketide synthase</td>
</tr>
<tr>
<td>ROESY</td>
<td>rotating frame overhauser effect spectroscopy</td>
</tr>
<tr>
<td>RP</td>
<td>reversed-phase</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Si</td>
<td>silica</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TEAP</td>
<td>triethylamonium phosphate</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VLC</td>
<td>vacuum liquid chromatography</td>
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was the primary author and directed and supervised the research, which forms the basis for this chapter.

The text of III, in full, is the manuscript draft to be submitted to an academic journal as it will appear: Malloy, K. L.; Choi, H.; Fiorilla, C.; Valeriote, F.; Matainaho, T.; Gerwick, W. H. Hoiamide D, A Marine Cyanobacterial Derived Inhibitor of p53/MDM2 Interaction. The dissertation author was the primary author and directed and supervised the research which forms the basis for this chapter.

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The text of V, in part, is to be submitted to an academic journal as it will appear: Malloy, K. L.; Choi, H.; Engene, N.; Spadafora, C.; Matainaho, T.; Gerwick, W. H.; Gutierrez, M. Dudawalamides A-E, Anti-parasitic Depsipeptides from the new genus Moorea. The dissertation author was the primary author and directed and supervised the research which forms the basis for this chapter.
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PUBLICATIONS

PAPERS


**ABSTRACTS**


ABSTRACT OF THE DISSERTATION

Structure Elucidation of Biomedically Relevant Marine Cyanobacterial Natural Products

by

Karla Lynn Malloy

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2011

Professor William H. Gerwick, Chair

The use of natural products for the treatment of disease has been an age-old practice. With chemical diversity, structural complexity, and perceived ecologic roles in chemical defense, natural products, not surprisingly, continue to serve as inspiration for pharmaceutical lead compounds. In that spirit, this dissertation further delineates the novel chemistry of marine natural products by describing bioassay-guided isolation and structure elucidation of cyanobacterial secondary metabolites with efficacy against several targets of human disease.

Chapter I presents a rationale for the dissertation research by providing a general introduction of marine natural products chemistry and drug development as it relates
specifically to cyanobacteria. Chapter II describes the isolation, structure elucidation, and anti-inflammatory activity of malyngamide 2, a nitric oxide inhibitor. Chapter III explores bioassay-guided fractionation and structure elucidation of hoiamide D, a cyanobacterial derived inhibitor of p53/MDM2 interaction, an attractive target for anti-cancer drug development. Chapter IV describes the isolation and structure elucidation of the credneramides, neuromodulatory vinyl chloride-containing fatty acid amides of a new cyanobacterial species. This chapter also explores the evolution, taxonomy, and biosynthetic source of the credneramides. Chapter V expounds upon the growing class of cyanobacterial depsipeptides containing the unique DHOYA (3-hydroxy-2,2-dimethyl-7-octyenoic acid) unit by describing the isolation, structure elucidation, and anti-parasitic activity of dudawalamide E and naopopeptin, a closely related HMOYA (3-hydroxy-2-methyl-7-octyenoic acid) containing natural product. Chapter VI provides concluding thoughts with perspective on the future of marine natural products chemistry.
Chapter I.

Introduction
Overview

Because the discovery and biosynthesis of novel marine natural products (MNPs) from cyanobacteria and algae are the main foci of research in our natural products group, the rationale for this dissertation is presented with a cyanobacterial-focused approach to the discussion of marine natural products chemistry and drug development. A general discussion of cyanobacteria is followed by a chronicling of the history of marine natural products chemistry, the study of marine cyanobacterial secondary metabolites, and natural product drug development, with specific examples of cyanobacterial lead compounds given for concept illustration. Concluding thoughts detail the content of ensuing chapters.

I.1 Cyanobacteria

Having evolved more than 2 billion years ago, cyanobacteria, or blue-green algae, are one of Nature’s oldest prokaryotic organisms. This monophyletic group of bacteria is believed to be responsible for the accumulation of atmospheric oxygen and the photosynthetic capacity of plants due to evolutionary descent of chloroplasts from cyanobacteria.\(^1\)\(^-\)\(^3\) Cyanobacteria are found in a variety of habitats, including freshwater, terrestrial, and marine environments and can tolerate extremes of climate. They are morphologically quite diverse, spanning the spectrum from unicellular, photosynthetic morphotypes to multicellular, mat-forming morphotypes with terminal differentiation, branching patterns, and the capacity for nitrogen fixation.\(^1\)\(^,\)\(^4\) Schematically, cyanobacteria are categorized based on phenotype using classifications I-V. Classifications I-II constitute unicellular bacteria while classifications III-V constitute multicellular, undifferentiated or differentiated cyanobacteria.\(^1\)\(^,\)\(^4\)\(^,\)\(^5\)

As much as they are morphologically diverse, cyanobacteria are genotypically even more diverse. Many morphological species are present in several phylogenetically distinct clades, particularly those of the genus *Lyngbya*. In fact, genotypic diversity of the marine *Lyngbya* sp. lineage (Order: Oscillatoriales) outpaces phenotypic diversity by a 10:1 margin, a finding that challenges traditional morphology-based taxonomic systems and, perhaps, merits a central role of a phylogenetic-based identification system for species and sub-species resolution. Also, of interest, is that morphologically distinct cyanobacteria have been shown to have identical 16S rRNA sequences and, conversely, intragenomic 16S rRNA gene heterogeneity has resulted in variable morphologic species classification. The unreliability of 16S rRNA suggests morphologic classification systems should remain as supplemental methods for accurate and precise cyanobacterial identification.

**I.2 Marine Biodiversity and Dawn of Marine Natural Products Chemistry**

The genotypic and morphologic diversity of the marine *Lyngbya* lineage is a reflection of the extensive biodiversity of the marine environment. With $10^6$ bacterial and $10^3$ fungal cells per milliliter of seawater and $10^8$ bacterial cells per gram of ocean sediment, biodiversity of the marine environment easily surpasses that of terrestrial ecosystems. The concentration of organisms per milliliter of seawater creates an intense competition for space, resources, and energy. Marine organisms, particularly those most vulnerable to predation, have adapted defense mechanisms to provide a selective advantage for survival. These protective mechanisms not only include physical characteristics, such as spines, camouflage, and speed, but also, chemical defenses in the
form of secondary metabolites derived from metabolic routes deemed nonessential for maintenance of life. \(^9,^{10}\)

In many cases, the physiologic or ecologic roles of secondary metabolites in the native organism remain unclear; however, in some cases, these compounds are predicted to serve roles in antifeeding, antifouling, signaling, territory delineation, and mating behavior. \(^{11-14}\) Secondary metabolites have also demonstrated activity against many validated targets of human disease and have been implicated in public health issues such as harmful algal blooms and shellfish poisonings due to ciguatoxin. \(^{15}\) Evidence for the biomedical relevance of secondary metabolites, including the examples given, has emerged only through rigorous scientific study of the chemistry of marine natural products. \(^{16,17}\)

The dawn of marine natural products chemistry began in the 1970s, due in part to increasing popularity of SCUBA diving and improved analytical technology, including at the time, NMR spectrometry and more recently, 2D NMR spectroscopy. However, the field’s nascency can be traced back as early as the 1950s when Bergmann first reported the isolation of spongouridine (1) and spongothymidine (2) that then led to the development of the anti-cancer therapeutics ara-A (vidarabine) and ara-C (cytarabine) (3, 4), respectively. \(^{17-19}\) Led by pioneering work of Paul Scheuer, William Fenical, D. John Faulkner, and others, the field of marine natural products chemistry continued to explode throughout the 1980s and 1990s. Three main foci of study emerged in the field, namely, marine toxins, marine biomedicine, and marine chemical ecology; these have largely persisted, \(^20\) but the field has expanded to incorporate biochemical and genomic investigations. As a result, marine natural product biosynthesis has emerged as a
substantial sub-specialty under the umbrella of marine natural products chemistry. \textsuperscript{21} Marine biotechnology is also a growing sub-specialization, owing its exponential growth in popularity to drug discovery, use of marine pharmacologic protein probes, and algal biofuels, among other research areas.

Figure 1. Spongouridine (1) and spongothymidine (2), metabolites of the sponge \textit{Tethya crypta}, and ara-A (vidarabine) (3) and ara-C (cytarabine) (4), synthetic derivatives with anti-cancer utility.

I.3 Marine Cyanobacterial-derived Natural Products

The study of marine cyanobacterial-derived natural products was popularized by Richard E. Moore in the 1970s and accompanied by a figurative passing of the baton to William H. Gerwick in the late 1980s as Prof. Moore shifted his focus to terrestrial cyanobacteria. \textsuperscript{22} Extensive research from these laboratories has proven cyanobacteria to be prolific sources of bioactive and chemically diverse secondary metabolites. The order Oscillatoriales has produced 58\% of all reported marine cyanobacterial metabolites, due in large part to extensive study of the genus \textit{Lyngbya} which accounts for 35\% of the cyanobacterial metabolites reported. \textsuperscript{23} Within the marine \textit{Lyngbya} lineage itself, \textit{Lyngbya}
*majuscula, Lyngbya semiplena, and Lyngbya boullonii* are the most common species from which natural products have been isolated. The sheer number of natural products isolated from the *Lyngbya* genus has been historically ascribed to its perceived chemical diversity; however, recent studies indicate polyphylly of the marine *Lyngbya* lineage may also explain its status as the most prolific source of marine cyanobacterial metabolites.

Mixed Polyketide synthase-non-ribosomal synthetase (PKS-NRPS) derived compounds constitute the majority of the 678 marine cyanobacterial natural products reported in the literature, but those isolated also encompass pure polyketides, pure peptides, alkaloids, terpenes, and fatty acids. Cyclization is encountered in approximately 60% of PKS-NRPS compounds, and approximately one-third are halogenated. Nonpolar amino acids predominate in cyanobacterial peptide-derived compounds. Valine and tyrosine are the most common amino acid constituents, and if modified, the most frequently encountered amino acid modifications include *N*-methylation, *N*-*N*-dimethylation, ketide extension, and halogenation of tyrosine to a lesser extent. The molecular weight distribution of cyanobacterial metabolites is a slightly right-skewed bell-shaped curve with a median of 604 Da and a druggable mode range of 400-500 Da. Further, there is a greater degree of lipophilicity and lesser degree of charged side chains in marine cyanobacterial compounds compared to their freshwater counterparts. Therefore, many features of marine cyanobacterial compounds make them attractive drug candidates, including their molecular weight distribution, lipophilicity, and chemical diversity.
I.4 Role of Natural Products in Drug Development

For fifty years, natural products have played a major role in drug discovery programs and continue to serve as niduses for rationally designed, synthetic lead compounds (Table 1-3). Half of the molecules approved between 2000 and 2006 were natural products or derivatives thereof, and thirteen natural product–related therapeutics alone were approved between 2005 and 2007, of which five are recognized as new classes of drugs.\textsuperscript{16,25} Natural products also continue to dominate the class of approved anti-bacterials.\textsuperscript{16} Cancer therapeutics is another disease area in which natural products, particularly of marine origin, play a leading role. Approximately 160 small molecules have been approved for use in cancer therapy since the 1940s, and of those, 47% are either natural products or derivatives.\textsuperscript{16} In addition, a significant number of compounds currently or recently in clinical trials are of marine origin, including salinosporamide A (5),\textsuperscript{26} bryostatin 1 (6),\textsuperscript{27} discodermalide (7),\textsuperscript{28} trabectidin (8),\textsuperscript{28} and eribulin (9)\textsuperscript{29} (Table 3).\textsuperscript{16}
Table 1. Natural products or natural product derived therapeutics in preclinical and clinical trials. (Adapted)²⁵

<table>
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<th>Stage</th>
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<td>7</td>
<td>24</td>
<td>61</td>
<td>225</td>
</tr>
</tbody>
</table>
Table 2. Therapeutic categories of natural products or derivatives thereof in preclinical and clinical trials. (Adapted). CV= cardiovascular; GI= gastrointestinal.

<table>
<thead>
<tr>
<th>Disease Area</th>
<th>Preclinical</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
<th>Pre-regis.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>34</td>
<td>15</td>
<td>26</td>
<td>9</td>
<td>2</td>
<td>86</td>
</tr>
<tr>
<td>Infectives</td>
<td>25</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Neurological</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>CV/GI</td>
<td>9</td>
<td>0</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Inflammation</td>
<td>6</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Metabolic</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Dermatologic</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Endocrine</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Immunosupp.</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>30</td>
<td>66</td>
<td>26</td>
<td>4</td>
<td>225</td>
</tr>
</tbody>
</table>
Table 3. Selected marine natural products and their stage of clinical development. (Adapted).  

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Source Organism</th>
<th>Molecular Target</th>
<th>Development Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-C (cytarabine)</td>
<td><em>Cryptotethya crypta</em> (sponge)</td>
<td>nucleotide mimic</td>
<td>Approved; Phase I/II for other indications</td>
</tr>
<tr>
<td>Discodermalide</td>
<td><em>Discodermia dissolute</em> (sponge)</td>
<td>tubulin</td>
<td>Phase I; preclinical</td>
</tr>
<tr>
<td>Salinosporamide A</td>
<td><em>Salinospora</em> sp. (marine bacterium)</td>
<td>20S proteosome</td>
<td>Phase II</td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td><em>Bugula neritina</em> (bryozoan)</td>
<td>protein kinase C</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Eribulin</td>
<td>Synthetic analog of <em>Halichondria</em></td>
<td>microtubules</td>
<td>Approved</td>
</tr>
<tr>
<td>Trabecidin</td>
<td><em>Ecteinascidia turbinate</em> (tunicate)</td>
<td>DNA minor groove</td>
<td>Approved; Phase II/III for other indications</td>
</tr>
</tbody>
</table>
Figure 2. Salinosporamide A (5), bryostatin 1 (6), discodermalide (7), trabectidin (8), and eribulin (9), marine natural products currently or recently in clinical trials.

In contrast, only 30% of the 1184 new drugs approved for use since the 1980s are synthetic. Although combinatorial chemistry has merits as a rational approach to drug discovery, only one new chemical entity, sorafenib (10), as been approved for clinical use with this strategy. There are, however, pharmacologic classes in which synthetics continue to predominate, including diuretics, hypnotics, analgesics, antifungals, and antihistamines.
As evidenced above, natural products are and will remain a mainstay in the generation of clinically relevant therapeutics of a broad variety of pharmacologic classes. As marine natural products populate clinical trial databases in greater numbers, they are increasingly recognized as a largely untapped source for active drug leads, particularly those arising from actinomycetes and cyanobacteria. In fact, approximately one-third of the 20 marine derived anti-cancer drugs in active or recently completed clinical trials, are predicted to arise from cyanobacterial metabolism, including the dolastatins.²⁸

![Chemical structure of Sorafenib](image)

**Figure 3.** Sorafenib (10), a serine/threonine and tyrosine kinase inhibitor FDA approved in 2005 for unresectable hepatocellular carcinoma and renal cell carcinoma, and the only marketed drug to date with an origin from combinatorial chemistry.¹⁶

### I.5 Cyanobacterial-derived lead compounds

There continues to be a robust pipeline of cyanobacterial lead compounds for therapeutic development due to the diligent efforts of those natural product laboratories focusing on the isolation and structure elucidation of bioactive cyanobacterial secondary metabolites. The quantity of cyanobacterial lead compounds is too numerous to catalogue within this context; thus, representative compounds will be elaborated upon to highlight
the structural ingenuity and impressive biologic profiles of these lead compounds, as well as the geographic diversity of cyanobacteria from which these compounds arise.

**Somocystinamide**

Somocystinamide A (11) is an acid labile dimeric, disulfide-containing dienamide isolated from a Fijan mixed assemblage of *Lyngbya majuscula* and *Schizothrix* sp. Initial bioactivity data indicated neuro-2A cytotoxicity (IC$_{50}$ = 1.4 µg/ml). $^{30}$ Subsequent testing in a cancer center screening program revealed potent cytotoxic activity towards caspase 8 expressing cells. Through extensive mechanistic studies, somocystinamide A was shown to induce apoptosis via a caspase 8 dependent pathway. $^{31}$ Additionally, it inhibits endothelial tube formation at picomolar concentrations *in vitro*, and inhibits angiogenesis in zebrafish. *In vivo* inhibition of caspase 8-expressing neuroblastoma tumor growth was also demonstrated. $^{31}$ The data in totality suggest somocystinamide A is a viable lead angiogenic and caspase 8 dependent tumor inhibitor. Current barriers to progress are optimization of industrial scale synthetic routes and optimization of the nanosomal drug delivery system for somocystinamide A.

**Hoiamide A**

Hoiamide A (12) is a PKS-NRPS derived cyclic peptide with a triheterocyclic system that was isolated from a Papua New Guinea assemblage of *Lyngbya majuscula* and *Phormidium gracile*. $^{32}$ Hoiamide A features two consecutive α-methylated thiazolines and a thiazole, a modified isoleucine moiety, and a highly oxidized and methylated C15 polyketide extension. Hoiamide A inhibits [$^3$H] batrachotoxin binding to voltage-gated sodium channels (IC$_{50}$ = 92.8 nM) as a partial agonist of site 2 of voltage gated sodium channels (VGSCs). $^{32}$ Its potency far surpasses the potency of veratridine
(IC$_{50}$ = 33.0 µM), the prototypic site 2 VGSC agonist that is derived from a higher plant. Hoiamide A also activates sodium influx (EC$_{50}$ = 2.31 µM) in murine neocortical neurons. Because VGSC modifiers have the capacity to influence NMDA signaling involved in neuronal growth and plasticity, hoiamide A is a candidate lead compound for modulating neuronal growth and plasticity and harbors significant potency.  

**Coibamide A**

Coibamide A (13) is a potent, cytotoxic depsipeptide isolated from a Panamanian *Leptolyngbya sp.* collected in Coiba National Park, a Panamanian national park and World Heritage site. Its structure is purely NRPS derived with a high degree of methylated residues. Coibamide A displayed potent cytotoxicity properties against the H460 lung carcinoma and neuro-2a neuroblastoma cell lines (LC$_{50}$ < 23 nM) but was inactive in tubulin or actin assays, indicating these cytoskeletal elements were not the targets of coibamide A. Coibamide A was subsequently screened in the NCI 60 cell line cancer assay and displayed greatest potency (GI$_{50}$) in the MDA-MB-231 (2.8 nM), LOX IMVI (7.4 nM), HL-60 (TB) (7.4 nM), and SNB-75 (7.6 nM) cell lines with selectivity towards breast, CNS, colon, and ovarian cancer. Coibamide A was COMPARE negative, indicating a novel mechanism of action is in operation. Total synthesis of the compound is under way in order to attain more information regarding the scope coibamide A’s biologic activities.
Figure 4. Somocystinamide A (11), hoiamide A (12), coibamide A (13), curacin A (14), apratoxin A (15), apratoxin G (16), and largazole (17), cyanobacterial lead compounds for drug development.
**Curacin A**

Curacin A (14), a 14-carbon extended lipopeptide harboring a thiazoline and a methyl-substituted cyclopropane ring, was isolated from a Caribbean collection of *Lyngbya majuscula*. Curacin A was screened in the NCI 60 cell line assay and demonstrated potent cytotoxicity with breast, colon, and renal cell selectivity. Subsequent mechanism of action studies revealed that curacin A depolymerizes tubulin by binding to the colchicine site and inhibits cross-linking of β-tubulin. SAR studies suggested the thiazoline, olefin, and methyl group were the pertinent contributors to tubulin binding. Optimization of curacin A’s pharmacokinetic and pharmacodynamic profiles is ongoing through generation of analogs with greater bioavailability and potency.

**Apratoxins**

The apratoxins are a family of mixed PKS-NRPS depsipeptides isolated from various collections of *Lyngbya* sp. from Papua New Guinea, Guam, Palau, and Palmyra. The hallmark of this class is the presence of a tert-butyl appendage that is likely the origin of biosynthesis. These compounds harbor nanomolar cytotoxicity in several cancer cell lines; for example, apratoxin A’s (15) potency ranges from 0.36-0.52 nM in KB and LoVo cell lines, respectively. Its mechanism of action has been attributed to interaction with heat shock protein (HSP) 90 and reversible inhibition of the secretory pathway for cancer-associated receptors. Apratoxin G (16), structurally very similar to apratoxin A, incorporates an alanine residue in place of the terminal proline, as seen in all previously characterized apratoxins, and still maintains potent cytotoxicity (IC$_{50}$ = 14 nM) in the H460 cell line. Maintenance of bioactivity with this
amino acid exchange at the terminal residue suggests the terminal residue is not central to bioactivity in this class of compounds.

**Largazole**

Largazole (14) is a potent, cytotoxic depsipeptide isolated from a *Sympleoca* sp. collected in the Florida Keys. Its structure features a 4-methylthiazoline and thiazole heterocyclic system and a unique 3-hydroxy-7-mercaptohept-4-enoic acid. Initial biological screening demonstrated largazole’s potent growth inhibition and cytotoxicity in the MDA-MB-231 human mammary cell line (GI$_{50}$= 7.7 nM; LC$_{50}$= 117 nM), U2OS fibroblastic osteosarcoma cell line (GI$_{50}$= 55 nM; LC$_{50}$= 94 nM), HT29 colon cancer cell line (GI$_{50}$= 12 nM; LC$_{50}$= 22 nM), and IMR-32 neuroblastoma cell line (GI$_{50}$= 16 nM; LC$_{50}$= 22 nM). Subsequent mechanism of action studies indicate 14 induces histone hyperacetylation via HDAC inhibition. Largazole inhibits HDAC through metabolic activation to the active thiol constituent, and the mercapto group has been shown to bind Zn$^{+2}$ in the active site of HDAC. Largazole shows promising activity in HCT116 xenograft mouse models with tumor growth inhibition and induction of apoptosis. Additional studies are ongoing in other tumor types, and detailed mechanism of action studies are exploring components downstream of HDAC inhibition.

These examples discussed in detail are illustrative of the quality and quantity of pharmaceutical leads originating from cyanobacteria. These compounds showcase the structural complexity and exquisite bioactivity encountered with cyanobacterial metabolites. As biological screening is diversified and isolation techniques are varied, the number of pharmaceutical candidates is expected to multiply.
I.6 Exploring biologically relevant chemical space

Harnessing the biologic activity of marine natural products for the treatment of human disease remains an attractive concept for many natural products laboratories. As argued above, marine natural products drug discovery is a viable approach for development of therapeutic leads and subsequent approval of these compounds for clinical indications. However, the pressing question for translationally directed scientists in the field is how one can improve the efficiency of the drug discovery process and, ultimately, improve the odds for success. One can easily envision a streamlined process as a result of technological breakthroughs in molecular biological techniques, analytical technology, and dereplication methods. An additional approach to a more rational, streamlined process is to begin to systematically catalogue the biologic and chemical space of druggable compounds in order to ultimately replicate and optimize idealized chemical scaffolds with a given biological activity of interest. Drug-like compounds mapped to a certain biologic space could also inspire screening against a related target in close proximity of that space. The converse is also a possibility, in that druggable compounds mapped to a certain chemical space could inspire screening of other compounds with structural similarity for a particular biological activity.

With advances in genomic sequencing technology, an important hurdle to mapping biologic space was overcome with complete sequencing of the human genome. The human genome is approximately 3 billion base pairs in size and comprises an estimated 30,000 genes, thus defining the boundaries of biologic space. Now, with completion of human genome sequencing, pharmacologically relevant biologic space can be interrogated with greater resolution.
Contrarily, the chemical space reflecting synthetic and natural product chemodiversity is essentially infinite. If organic compounds of molecular weight constricted to 500 Da or less are considered the minimum criteria for drug candidates, an estimated $10^{60}$ compounds are plausible candidates.\textsuperscript{46} The chemical space of small molecules (CSSM) was recently defined which resulted in an estimated 1.5 million distinct scaffolds when considering topologies for up to eight rings. Only 0.6\% of these topologies representing ring structures could be mapped to chemical space attributed to known small molecules.\textsuperscript{46-48} Thus, it is imperative and most practical to limit an essentially infinite chemical space to biologically relevant chemical space when considering leads for drug development.

A large component of biologically relevant chemical space is represented by natural products, but a plethora of opportunities remain to map low density regions or regions yet to be represented by natural products. In a parallel fashion, the pharmacologic potential or biologic space of these natural products remains largely untapped with numerous disease processes and targets remaining unexplored by natural product laboratories. Historically, efforts have been focused on whole cell based screens for isolation and development of marine-derived antitumor and antibacterial agents. Recent literature indicates a shift in attention towards mechanism-based screens as well as assays devoted to understudied pathologies within the MNP community such as neurodegenerative diseases, HIV, tuberculosis, chronic inflammation, and the tropical “neglected diseases”. Opportunities abound to contribute to further mapping of the biologic space represented by natural products.
This dissertation is an attempt to incrementally expand the chemical space of natural products through systematic isolation and structure elucidation of bioactive marine cyanobacterial-derived secondary metabolites. In parallel fashion, an additional objective is to explore the frontiers of biologic space represented by these chemotypes via extensive biological assay screening of these new metabolites.

I.7 Overview of Dissertation Research

Chapter II describes the isolation, structure elucidation, and anti-inflammatory activity of malyngamide 2. Malyngamide 2 was isolated from a Papua New Guinea cf. _Lyngbya sordida_ extract using a combination of vacuum liquid chromatography (VLC) and reverse phase (RP) HPLC. Its structure features a 14-carbon PKS extended lyngbic acid that is tethered through an amide bond to a highly oxidized cyclohexanone ring. The biogenesis of the cyclohexanone ring incorporates several intriguing modifications including methylation at C-1 and hydroxylation at C-2 of acetate units. Malyngamide 2 displays nitric oxide inhibiting activity in LPS-induced RAW macrophage cells (IC$_{50}$ = 8.0 µM) with minimal cytotoxicity to a mammalian cell line.

Chapter III explores bioassay-guided fractionation and structure elucidation of hoiamide D, a cyanobacterial derived inhibitor of p53/MDM2 interaction. Hoiamide D was isolated in both its carboxylic acid and conjugate base forms from two collections of Papua New Guinea cyanobacteria _Symploca_ sp. using VLC, solid phase extraction (SPE), and RP HPLC. Its structure is PKS-NRPS derived and harbors two consecutive thiazolines and a thiazole, a modified isoleucine residue, and a highly oxidized and methylated, PKS-derived carbon chain. Hoiamide D inhibits p53/MDM2 interaction (EC$_{50}$ = 4.5 µM), an attractive target for anti-cancer drug development, and is one of the
most potent natural product inhibitors.

Chapter IV describes the isolation and structure elucidation of the credneramides A and B, neuromodulatory phenethylamine and isopentylamine vinyl chloride-containing fatty acid amides from two geographically and phylogenetically distinct species of cyanobacteria. The chapter also explores the evolution, taxonomy, and biosynthetic source of these compounds. Bioassay-guided fractionation of the parent extract using VLC and RP HPLC yielded credneramides A and B, as well as credneric acid. These compounds displayed low micromolar inhibition of calcium oscillations in murine cortical neurons (credneramide A, IC$_{50}$ = 4.0 µM; credneramide B, IC$_{50}$ = 3.8 µM).

Chapter V expounds upon the growing class of cyanobacterial depsipeptides containing the unique DHOYA (3-hydroxy-2,2-dimethyl-7-octynoic acid) unit by describing the isolation, structure elucidation, and anti-parasitic activity of dudawalamide E and naopopeptin, a closely related HMOYA (3-hydroxy-2-methyl-7-octynoic acid) containing natural product. Isolated from Lyngbya sp. using VLC and RP HPLC, these compounds are PKS-NRPS derived cyclic lipopeptides with two ester linkages. Dudawalamide E possesses broad spectrum anti-parasitic activity with minimal mammalian cell cytoxicity while naopopeptin displays selective low micromolar inhibition of Plasmodium falciparum (IC$_{50}$ = 2.7 µM) and Leishmania donovani (IC$_{50}$ = 5.8 µM), and modest inhibition of calcium oscillations in murine cortical neurons (11.7 µM). Interesting SAR features are emerging regarding this expanding class of DHOYA compounds.
Chapter VI provides concluding thoughts with perspective on the future of marine natural products chemistry.
References


Chapter II.

Malyngamide 2, an Oxidized Lipopeptide with Nitric Oxide Inhibiting Activity from a Papua New Guinea Marine Cyanobacterium
Abstract

A Papua New Guinea collection of the marine cyanobacterium cf. *Lyngbya sordida* yielded three known compounds as well as a new PKS-NRPS-derived malyngamide with anti-inflammatory and cytotoxic activity. Malyngamide 2 features an extensively oxidized cyclohexanone ring. Resolution of the ring core as a 6,8,9-triol rather than a 7,8,9-triol and relative configuration was based on chemical shift and bond geometry modeling in conjunction with homonuclear and heteronuclear coupling constants, NOE and ROE correlations, and other structural information. Malyngamide 2 exhibited anti-inflammatory activity in LPS-induced RAW macrophage cells (IC$_{50}$ = 8.0 µM) with only modest cytotoxicity to the mammalian cell line.
Introduction

Various species of the mostly marine cyanobacterial genus *Lyngbya* are renowned for their capacity to produce secondary metabolites of many different structural classes.\(^1\) One such natural product class with many members is the malyngamides, a group easily recognized by their combination of an unsaturated and methoxy-bearing fatty acid with a highly crafted and functionalized amine portion. The majority of the malyngamides isolated to date have been obtained from collections of *Lyngbya* species, although a few were isolated from the sea hares *Bursatella leachii* (malyngamides X, S) and *Stylocheilus longicauda* (malyngamides O, P).\(^2,3\) In addition, malyngamides M and N were isolated from the red alga *Gracilaria coronopifolia*.\(^4\) However, it is likely that the original natural product sources in these latter cases were also *Lyngbya* species, as these cyanobacteria are reported to be eaten by *Bursatella* and *Stylocheilus*,\(^2,3\) and form epiphytic associations with *Gracilaria*.\(^4\)

Lyngbic acid, the methoxy-containing unsaturated fatty acid portion of most malyngamides, likely derives from a PKS pathway with only a few variations having been observed, which involve its chain length (12–20 carbons).\(^5\) Thus, the principal variable domain of the malyngamides is the polar headgroup, usually a multiply functionalized cyclohexyl ring, which is attached to the lyngbic acid portion through an amide or \(N\)-methyl amide linkage. Additionally, a branching vinyl group with chlorine as a substituent, as found in the jamaicamides and recently shown to derive from a novel \(\beta\)-branch-forming reaction,\(^6\) is often present in the headgroup. The malyngamides possess a broad spectrum of biological activities, including antimicrobial, anti-inflammatory,
cytotoxicity, and antimycobacterial. Here, we report our discovery of a new member of the malyngamide family, malyngamide 2 (1), from a Papua New Guinea collection of cf. *L. sordida* (synonym: *L. polychroa*), a relatively unstudied species of *Lyngbya*. (8) Malyngamide 2 exhibits promising anti-inflammatory activity in the nitric oxide production assay (IC$_{50}$ = 8.0 µM) with only modest cytotoxic properties (IC$_{50}$ = 27.3 µM).

**Results and Discussion**

Pale red tufts of the benthic cyanobacterium cf. *Lyngbya sordida* were collected from a depth of 3 to 5 m by scuba near Dutchess Island, Papua New Guinea, in 2002. The lipophilic extract was separated by normal-phase silica vacuum liquid chromatography to generate nine subfractions. The eighth fraction harbored compounds exhibiting significant activity in a cytotoxicity assay and was subsequently subjected to RP C18 SPE cartridge purification and reversed-phase HPLC. A mixture of majusculamides A and B, wewakazole, and malyngamide 2 (1) were eluted as partially purified compounds; the two latter compounds were further purified using analytical HPLC. The known compounds were identified by comparison of their respective analytical data sets in comparison with literature values, (9, 10) whereas malyngamide 2 (1) was determined to be a new compound through dereplication using the MarinLit database program.

Malyngamide 2 (1) gave an [M + H]$^+$ m/z 488.2757, which established the molecular formula as C$_{25}$H$_{42}$ClNO$_6$ with five degrees of unsaturation (Figure 1). The $^1$H NMR spectrum was well dispersed and contained several proton resonances that are signatures for the malyngamide structure class. Specifically, a singlet proton resonance at
$\delta_H$ 6.31 ($\delta_C$ 121.1) was diagnostic of a vinyl chloride, a two-proton multiplet at $\delta_H$ 5.49 ($\delta_C$ = 128.3, 130.6) indicated a disubstituted olefin, a three-proton singlet at $\delta_H$ 3.32 ($\delta_C$ 56.4) suggested a methoxy group, and resonances at $\delta_H$ 6.54 and $\delta_C$ 173.5 indicated a secondary amide, and these were all in accord with published spectroscopic data for the malyngamide series. Ensuing 1D and 2D NMR experiments confirmed the presence of lyngbic acid, 7S-methoxytetradec-4(E)-enoic acid, as a substructure of compound 1.

Having established the lyngbic acid portion of compound 1, the polar headgroup was defined as a fragment of C$_{10}$H$_{15}$ClNO$_4$ composition and, thus, was more highly oxidized than any previously described malyngamide. The vinyl chloride appendage was connected in proximity to the amide functionality, consistent with other malyngamides, via a COSY correlation between the amide proton and the methylene protons at C-1 in combination with reciprocal H-1/C-3 and H-3/C-1 HMBC correlations. Thus, with the linear portion of the molecule accounting for three degrees of unsaturation, the remaining two degrees were assigned to a carbonyl C-5 ($\delta_C$ = 209.6) and a ring structure, again consistent with the malyngamide class. Moreover, this atom accounting required the final three oxygen atoms to be present as hydroxy groups and defined the ring as a cyclohexanone. From consideration of the unassigned remaining carbon shifts, the substituents on the ring were thus one methine, one methylene, two oxygenated methines, a quaternary oxygenated carbon, and a ketone. A final substituent that needed accommodation on this ring structure was a singlet methyl group at a chemical shift ($\delta_H$ 1.39) consistent with its attachment to an oxygenated carbon. Location of these groups about this cyclohexanone ring, especially the oxygenation pattern, was a more difficult
task than initially envisioned because of ambiguity over the number of bonds for which HMBC and COSY correlations were observed.

A unique resonance associated with this cyclohexanone ring was a fine doublet proton \((J = 1.0 \text{ Hz})\) at \(\delta 4.37\), allylically coupled with the H-3 vinyl proton and associated by HSQC spectroscopy with a carbon at 54.3 ppm. This proton was highly coupled by HMBC, showing six prominent correlations, and defined this methine as the attachment point between the cyclohexanone and linear portions of malyngamide 2. Notably H-4 was HMBC correlated to C-1, C-2, and C-3 in the linear portion of I and to the carbonyl, quaternary oxygenated carbon and singlet methyl group associated with the cyclohexanone portion, thus locating the carbonyl on one side of the attachment point and a quaternary carbon with hydroxy and methyl substituents on the other side. HMBC correlations from the quaternary methyl group \((H_3-10)\) confirmed these assignments (Table 1) and also positioned the methyl group on a carbon adjacent to oxygenated methine C-8. The proton attached to C-8 showed a 3.2 Hz coupling to one of the methylene protons, thus locating the CH\(_2\) group at C-7. H-8 was weakly coupled to the other proton at C-7, suggesting that H-8 was an equatorial proton with a nearly 90° dihedral angle to H-7\(_{ax}\). Consistent with this latter assignment, a 12 Hz coupling was observed between this H-7\(_{ax}\) proton and the last oxymethine proton at C-6, a coupling value only consistent with an axial–axial orientation. This was confirmed by observation of a 7.0 Hz coupling between H-7\(_{eq}\) and H-6\(_{ax}\). Thus, a cyclohexanone ring was defined with a ketone at C-5, hydroxy groups at C-6, C-8, and C-9, a methyl group at C-9, and a juncture to the remainder of the molecule at C-4.
However, upon completion of these assignments, a second possibility was considered that placed hydroxy groups at C-7, C-8, and C-9. Alternative assignments were reasoned as follows. The proposed methylene group at C-6 showed large (12.0 Hz) and medium (7.0 Hz) sized couplings to H-7, and thus H-7 must be axial. That H-7 and H-8 do not show any coupling could be explained by a 0 Hz axial–equatorial arrangement. The 3.2 Hz coupling between H-6$_{eq}$ and H-8$_{eq}$ could arise through $W$-type coupling about the cyclohexanone ring. Thus, this alternate arrangement was consistent, in principle, with the observed homonuclear couplings (Figure 2).

To distinguish between these two possible arrangements, data from heteronuclear couplings, ROE measurements, and conformational and chemical shift analysis were considered and led to a firm conclusion that the original formulation was correct, namely, a C-6 hydroxy rather than a C-7 hydroxy substituent was present in malyngamide 2 (1). Structure possibility 1b (Figure 2) was inconsistent with HMBC results, which measured correlations between the methylene protons and C-9 as well as from the proposed C-7 hydroxy proton to C-5, both of which would require four-bond HMBC correlations in 1b. Observation of ROE from the C-6 methylene protons to H-8 was also inconsistent with structure possibility 1b, as these protons would be predicted to be separated by greater than 4 Å. In contrast, structure possibility 1a (Figure 2), with a C-6 hydroxy group, is fully consistent with the HMBC results with all observed correlations being either two or three bonds, and ROE results are consistent with the vicinal nature of H$_2$-7 and H-8 (Figure 2). Finally, computer modeling was used to calculate the best four conformations of the 6,8,9-triol (1a) and the 7,8,9-triol (1b). Semiempirical calculation of the four best conformers for 1a and 1b, ab initio geometry optimization, and single-point energy
calculation resulted in a single best conformer at C-6/C-7 for each; the predicted coupling constants for each were compared with the observed couplings and again favored structure 1a (see Supporting Information). Finally, $^{13}C$ NMR chemical shift modeling of the two structural hypotheses was compared with the observed shifts, and interestingly, each possessed shifts at variance with the calculation; however, the 7,8,9-triol was less favored, having a greater average $\Delta\delta$ for the carbon atoms of the cyclohexanone ring as well as the single largest carbon atom shift disparity (a calculated shift for C-8 of $\delta$ 88.8; $\Delta\delta = 14.6$ from observed).

With the planar structure complete, the relative configuration of the cyclohexanone ring was assigned using 1D NOE and 2D ROESY correlations as well as homonuclear coupling constants. 1D NOEs between the H-4$_{ax}$ and H-6$_{ax}$ methines as well as a strong 2D ROESY correlation between H-4$_{ax}$ and H-10 indicated that these substituents were on the same face of the cyclohexyl ring (Figure 2). Thus, in combination with the relative relationships of H-6 and H-8 as described above, these data led to a relative configuration of 4$S^*$, 6$R^*$, 8$R^*$, 9$R^*$. Analysis of the absolute configuration of these stereocenters was precluded due to a lack of sufficient amount of compound for NMR or other methods of stereochemical analysis. Characterization of the olefin geometries and the lyngbic acid configuration was next examined. The $E$ geometry of the vinyl chloride was established from 1D NOE correlations between H-3 and H$_2$-1. The geometry of the C-4′ olefin was assigned as $E$ on the basis of the close comparison of $^{13}C$ NMR shifts of C-3′ to C-6′ with those of other malyngamides with a C-4′/C-5′ $trans$ geometry. The configuration at C-7′ is predicted to be 7′$S$ because the lyngbic acid
isolated from the same extract possessed a negative optical rotation and was consistent with the specific rotation of synthetic 7(S)-methoxytetradec-4(E)-enoic acid (lyngbic acid). 12

The biogenesis of malyngamide 2 (1) has several noteworthy features. Formation of the vinyl chloride is predicted to proceed in a similar fashion to that of the jamaicamides. 6,13 Specifically, an HMGCoA synthase is predicted to add acetate to the intermediate carbonyl formed by ketide extension of a glycyl unit attached to lyngbic acid, thereby forming a β-branch. Next, the C-2 position of the β-branch acetate unit is predicted to be halogenated via a nonheme (Fe II), α-ketoglutarate-dependent halogenase. Subsequent dehydration and decarboxylation in a regiospecific manner would then yield the vinyl chloride functionality. 6 Intriguing biosynthetic features of malyngamide 2’s PKS-derived cyclohexanone core are methylation (C-10) at a C-1-derived position (C-9) and hydroxylation at a C-2-derived position of the putative acetate subunits. Methylation at the C-1 position of acetate implies incorporation of a second HMG-CoA synthase cassette within the biosynthetic gene cluster, whereas hydroxylation at the C-2 position suggests the possible utilization of a hydroxymalonyl extender unit, as featured in the biosynthetic cluster of zwittermicin A. 14 While malyngamide 2 shares biosynthetic features with several other malyngamides reported to date, particularly with hydroxylation at the C-6 position, only malyngamide G and S share methylation at a proposed C-1 position in the cyclohexyl ring, and thus the utilization of a second HMG-CoA synthase cassette in the biosynthetic cluster of each is possible. 2b,15

Malyngamide 2 was tested in a panel of assays to explore its biological properties. In the murine RAW264.7 macrophage cell line treated with lipopolysaccharide (LPS),
malyngamide 2 (1) displayed anti-inflammatory properties by inhibiting induced nitric oxide production with an IC$_{50}$ = 8.0 µM (95% confidence interval: 4.6–13.9 µM).

Recently, several other malyngamides have been shown to possess anti-inflammatory activity in this assay, and initial SAR features are emerging, such as the requirement for a C-6 hydroxy or acetoxy group. Only modest cytotoxicity was observed for compound 1 in both the RAW macrophage cell line (94% cell survival at 21 µM) and H-460 human lung carcinoma cells (IC$_{50}$ = 27.3 µM; 95% confidence interval: 22.4–32.9 µM), indicating a reasonable therapeutic window between inhibition of nitric oxide and cytotoxicity. However, the known compounds reisolated in this study, wewakazole and a mixture of majusculamides A and B, were also tested in the H-460 cytotoxicity assay; the mixture of majusculamides A and B exhibited the strongest activity and most likely accounts for the cytotoxic activity of the parent extract. Wewakazole, originally reported without biological activity, was active in the H-460 cytotoxicity assay (IC$_{50}$ = 10.1 µM; 95% confidence interval: 8.4–12.1 µM), but was inactive in a Neuro-2A-based voltage-gated sodium channel (VGSC) assay at 17.5 µM.

**General Experimental Procedures**

Optical rotation measurements were recorded using a Jasco P1010 polarimeter, UV spectra were measured on a Beckman-Coulter DU-800 spectrophotometer, and IR spectra were obtained using a Nicolet IR-100 FT-IR spectrophotometer. NMR spectra were obtained using Varian Unity 300 and 500 MHz spectrometers. CDCl$_3$ (δ$_H$ = 7.26; δ$_C$ = 77.0) was used as an internal reference. High-resolution mass spectra were obtained
using an Agilent ESI-TOF mass spectrometer. Extracts were processed using a HPLC Waters 515 pump, Waters 996 photodiode array detector, and Millenium software for acquisition and analysis of data. All solvents were either distilled or of HPLC quality.

**Collection**

The cf. *Lyngbya sordida* sample (PNG-06/02/02-3) was collected in June 2002 at a depth of 3–5 m by scuba near Dutchess Island in Papua New Guinea with GPS coordinates of 9°57.228’ S and 150°51.054’ E. The specimens, measuring 1.5 L in total biomass, were stored in 70% EtOH at −20 °C until extraction.

**Morphological Identification**

The PNG-06/02/02-3 cyanobacterial collection was morphologically identified as *Lyngbya sordida* Gomont ex Gomont (order: Oscillatoriales). The cf. *L. sordida* collection was composed of brownish, long (>1 cm), and straight or slightly waved filaments measuring 40.5 ± 5.4 µm (n = 3) wide with distinct visible, thick, and colorless sheaths. The cells were disk-shaped, 33.0 ± 4.3 µm wide and 4.5 ± 0.4 µm long (n = 30), with distinct (7.5% ± 0.7) constrictions at the cross-walls. The terminal cells were rounded and noncapitiated and lacked calyptra (i.e., thicker outer cell walls). Taxonomic identification was performed in accordance with bacteriological systems,18 traditional as well as current phycological systems,19 and relevant taxonomic literature. Morphological characterizations were performed using an Olympus IX51 epifluorescent microscope (100× objective) equipped with an Olympus U-CMAD3 camera. Measurements were an average of 10 neighboring cells from three different filaments and calculated with standard deviations. It should be noted that the morphology of *Lyngbya* resembles that of marine *Oscillatoria* species and that these two genera cannot be distinguished without
phylogenetic analysis. Taxonomic identification of these marine cyanobacteria on the basis of morphology alone has resulted in extensive misclassification, hence the usage of the “cf” designation. Furthermore, *Lyngbya* is a polyphyletic group, and tropical marine specimens are unrelated to the genus type-strain PCC 7419$^T$. Thus, the taxonomy of tropical marine *Lyngbya* needs to be revised and separated from the genus *Lyngbya*.

**Extraction and Isolation**

The collection was extracted six times with heat using CH$_2$Cl$_2$/MeOH (2:1) and yielded an organic extract of mass 1.28 g. This extract was further processed on silica gel using vacuum liquid chromatography with a 100% hexanes–EtOAC–MeOH gradient to generate nine subfractions, A–I. Fraction H eluted with 75/25 EtOAc/MeOH and harbored the most potent cytotoxic activity. Fraction H (259 mg) was subsequently filtered using a Waters RP C18 SPE cartridge with 100% MeOH and further processed using reversed-phase HPLC. Malyngamide 2, wewakazole, and majusculamides A/B (5.0 mg) eluted with 60:40 CH$_3$CN/H$_2$O (Phenomenex Synergi Fusion 10 µm, 250 × 10 mm). Malyngamide 2 (C18 Kromasil, 5 µm, 250 × 4.6 mm; 55:45 CH$_3$CN/H$_2$O) and wewakazole (Phenomenex Synergi Fusion, 4 µm, 250 × 4.6 mm; 50:65 CH$_3$CN/H$_2$O) were further purified to yield 1.0 and 0.7 mg, respectively. Lyngbic acid was obtained by HPLC purification of fraction F of the same extract using 70:30 CH$_3$CN/H$_2$O containing 0.01% trifluoroacetic acid (Phenomenex Jupiter 10 µm, 250 × 10 mm), yielding 28.5 mg and showing \([\alpha]_D^{22.9} = -22.9 \ (c \ 2.0, \ CHCl_3)\); lit. \([\alpha]_D^{13.3} = -13.3 \ (c \ 2.5, \ CHCl_3)\). (12)
Malyngamide 2 (1): pale yellow oil; $[\alpha]_D$ 1.1 (c 2.5, CHCl$_3$); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 202 (4.34); IR (neat) $\nu_{\text{max}}$ 3300, 2927, 2855, 1720, 1641, 1546, 1458, 1372, 1251, 1128, 1063 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 500 MHz) and $^{13}$C NMR (CDCl$_3$, 75 MHz) data, see Table 1; (+)-HRESIMS [M + H]$^+$ m/z 488.2757 (calcd for C$_{25}$H$_{43}$ClNO$_6$, 488.2779).

Conformer Distributions, Ab Initio Geometry Optimization, and Single-Point Energy Calculations

Spartan 2004 software (Wavefunction, Inc.) was used to perform all molecular modeling. The best four conformers were obtained for each candidate structure using a semiempirical method (PM3), and Hartree–Fock 3-21G* ab initio calculations were used to obtain optimized geometries and single-point energies for each conformer. Dihedral angles of the optimized structures were measured, and coupling constants were subsequently calculated using the Altona$^{21}$ and Karplus$^{22}$ equations with the aid of Sweet J 2.1 software (Nucleomatica).

Biological Activity

Malyngamide 2 (1), majusculamides A/B, and wewakazole were all tested in the H-460 cytotoxicity assay and the Neuro-2A sodium channel activation and blocking assays using methods previously described.$^{17,23}$ Malyngamide 2 and wewakazole were submitted for further testing in an anti-inflammatory assay as described below. 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.$^{16}$ 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 various pure compounds (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) ethylenediamine (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).

**Supporting Information Available.** ¹H NMR, ¹³C NMR, COSY, TOCSY, HSQC, and HMBC spectra in CDCl₃ for malyngamide 2 (1). This material is available free of charge via the Internet at http://pubs.acs.org.
Figure 1. Malyngamide 2, an anti-inflammatory lipopeptide with nitric oxide inhibiting activity (IC$_{50}$ = 8.0 µM).
Figure 2. Candidate structures for the cyclohexanone ring core with key homonuclear coupling constants. **1a.** $6R^*,8R^*,9R^*$-triol; **1b.** $7R^*,8R^*,9R^*$-triol.
Table 1. $^1$H and $^{13}$C NMR assignments for malyngamide 2 (1) in CDCl$_3$.

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<th>ROE$^a$</th>
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<td>4, 8, 9, 10</td>
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References


(7) This newest representative of the malyngamide family is named “malyngamide 2” to follow a recent report of “malyngamide Z” and “malyngamide 1”. Junyang, H. Ph.D. Dissertation, National Dong Hwa University, 2008.


14349–14354.


(19) Komrek, J. and Anagnostidis, K. *Ssswasserflora von Mitteleuropa. 19/2*; Elsevier/Spektrum: Heidelberg, Germany, **2005**.


Acknowledgements

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The text of II, is published material with permission from ACS as it appears in Malloy, K. L., Villa, F. A., Engene, N., Matakaho, T., Gerwick, L., Gerwick, W. H., Malyngamide 2, an Oxidized Lipopeptide with Nitric Oxide Inhibiting Activity from a Papua New Guinea Marine Cyanobacterium, J. Nat. Prod. 2011, 74, 95-98. The dissertation author was the primary author and directed and supervised the research, which forms the basis for this chapter.
Supporting Information

**Figure 3.** $^1$H NMR spectrum of malyngamide 2 (500 MHz, CDCl$_3$)

**Figure 4.** $^{13}$C NMR spectrum of malyngamide 2 (75 MHz, CDCl$_3$)

**Figure 5.** COSY spectrum of malyngamide 2 (CDCl$_3$)

**Figure 6.** ROESY spectrum of malyngamide 2 (CDCl$_3$)

**Figure 7.** HSQC spectrum of malyngamide 2 (CDCl$_3$)

**Figure 8.** HMBC spectrum of malyngamide 2 (CDCl$_3$)

**Figure 9.** Single best conformers for the $6R^*,8R^*,9R^*$-triol and $7R^*,8R^*,9R^*$-triol with calculated and observed $^3J_{HH}$ coupling constants.

**Figure 10.** Comparison of the predicted $^{13}$C NMR shifts of two possible cyclohexanone core structures for malyngamide 2 (1) with experimentally determined $^{13}$C NMR shifts.
Figure 4. $^{13}$C NMR spectrum of malyngamide 2 (75 MHz, CDCl$_3$)
Figure 9. Single best conformers for the $6R^*,8R^*,9R^*$-triol and $7R^*,8R^*,9R^*$-triol with calculated and observed $3J_{HH}$ coupling constants.

Figure 10. Comparison of the predicted $^{13}$C NMR shifts of two possible cyclohexanone core structures. In striped bars, the 6,8,9-triol is compared with the experimentally determined $^{13}$C NMR shifts for malyngamide 2 (1) (mean deviation per carbon atom = 4.6 ± 2.8 ppm), and in solid bars, the 7,8,9-triol is compared (mean deviation per carbon atom = 6.4 ± 4.9 ppm).
Chapter III.

Hoiamide D, A Marine Cyanobacterial Derived Inhibitor of p53/MDM2 Interaction
Abstract

Bioassay-guided fractionation of two cyanobacterial extracts from Papua New Guinea has yielded hoiamide D in both its carboxylic acid and conjugate base forms. Hoiamide D is a polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS)-derived natural product that features two consecutive thiazolines and a thiazole, as well as a modified isoleucine residue. Hoiamide D displayed inhibitory activity against p53/MDM2 interaction (EC$_{50}$= 4.5 µM), an attractive target for anti-cancer drug development.
Introduction

The protein p53 is a well characterized tumor suppressor that acts as a transcription factor to regulate cell cycle dynamics, apoptosis, and DNA repair. It is encoded by the gene TP53 that is inactivated in nearly 60% of all human malignancies. Structurally, p53 harbors an NH\textsubscript{2} terminal transactivation domain, a DNA binding domain in the center, and a C-terminal locus for regulatory function and tetramerization. Its function is regulated either by mutation or deletion of the parent gene or through regulatory feedback by MDM2. MDM2, a murine ubiquitin ligase, negatively regulates p53 function by a variety of mechanisms, including degradation through proteosomal association and ubiquitylation. MDM2 also facilitates nuclear export with its export signal and binds to p53’s transactivation domain, thereby repressing transcriptional activity. Recent studies have also shown MDM2 to interact, albeit more weakly, with the C-terminal and core domains of p53. In theory, disruption of any of these regulatory functions by MDM2 is a viable strategy to reactivate p53, especially through inhibition of the p53/MDM2 binding interaction.

Based on site directed mutagenesis, the essential amino acids of the p53 N-terminal binding domain that constitute the majority of the p53/MDM2 interaction and binding energy are Phe\textsuperscript{19}-Trp\textsuperscript{23}-Leu\textsuperscript{26}, with the three terminal atoms of the phenyl ring, the indole nitrogen of tryptophan (Trp), and the isopropyl group of leucine (Leu) serving as the pertinent pharmacophore. Thus, the p53/MDM2 interaction is largely hydrophobic, and the binding interface of the two proteins is also quite small, making small, peptidic or non-peptidic molecular mimics of the p53 binding site good candidates.
for inhibitors of p53/MDM2 interaction. ³,⁶ This hydrophobic binding domain has been exploited to rationally design synthetic p53/MDM2 inhibitors with excellent affinity. Three major classes of synthetic p53/MDM2 inhibitors, the nutlins, spiro-oxindoles, and benzodiazepinediones, have taken advantage of the Phe₁⁹-Trp₂³-Leu₂⁶ binding core to displace p53 and bind MDM2 with much greater affinity and in vitro potency. However, maintaining in vivo potency has been problematic for many of these inhibitors due to their poor pharmacokinetic profiles. ⁷,⁸

Marine cyanobacteria are one of Nature’s most ancient organisms and offer a plethora of bioactive secondary metabolites, many of which are active against validated targets of human disease. Of the 678 marine cyanobacterial natural products reported in the literature, the majority is polyketide synthase-non-ribosomal peptide synthetase derived. ⁹ The molecular weight distribution forms a largely bell-shaped curve with a median of 604 Da and an attractive mode range of 400-500 Da. In addition, marine cyanobacterial compounds are generally more lipophilic than their freshwater counterparts and contain less charged side chains. There is a predominance of nonpolar amino acids in cyanobacterial peptide-derived compounds, and leucine and phenylalanine, two essential amino acids of the p53 binding pocket, are relatively common constituents in these peptidic compounds. Approximately half of all amino acids utilized in cyanobacterial natural products are modified with the most frequent modifications including N-methylation, N’-N’-dimethylation, ketide extension, and halogenation to a lesser extent. ⁹ Thus, many features of marine cyanobacterial compounds, including their molecular weight distribution, lipophilicity, and chemical diversity, lend themselves to favorable bioactivity and medicinal chemistry profiles,
particularly as it pertains to p53/MDM2 inhibition. Herein, we describe hoiamide D (1), a peptide-derived p53/MDM2 inhibitor, isolated in both its acid and carboxylate forms, from two separate collections of the Papua New Guinea cyanobacterium *Symploca* sp. Hoiamide D displays promising inhibitory activity towards p53/MDM2 interaction ($EC_{50} = 4.5 \mu M$).

**Results and Discussion**

Samples of the spongy, purple cyanobacterium *Symploca* sp. were collected by SCUBA near Kape Point, Papua New Guinea in 2006. The collection was extracted with CH$_2$Cl$_2$:MeOH (2:1) to yield a crude extract (1.37 g) that was further processed using normal phase silica vacuum liquid chromatography to generate nine subfractions. (A-I).

The ninth and most polar fraction (I) displayed inhibitory activity in the p53/MDM2 assay and was further purified using RP C18 solid phase extraction to yield an additional four relatively pure subfractions. The most polar subfraction (>95% pure) contained the new natural product, hoiamide D (23.6 mg), and harbored the greatest inhibitory activity ($EC_{50} = 4.5 \mu M$).

The HRESIMS of Hoiamide D (1), [M+H]$^+$ $m/z$ 743.3535, established its formula as C$_{35}$H$_{58}$N$_4$O$_7$S$_3$ with an isotopic pattern consistent with the presence of three sulfur atoms. The IR spectrum of 1 displayed an absorption band at 3381 cm$^{-1}$, indicating the presence of a hydroxy group while the $^1$H NMR spectrum revealed two distinctive low field shifts, a sharp singlet at $\delta_H$ 8.22 ($\delta_C$ 122.6) and a doublet at $\delta_H$ 6.85 that were indicative of a substituted alkene and an amide, respectively. A singlet at $\delta_H$ 3.23 ($\delta_C$ 56.2) revealed the presence of an O-Me substituent, and two sharp high field singlets between 1.4-1.6 ppm were suggestive of deshielded methyl groups. 2D COSY, HSQC,
TOCSY, and HMBC experiments were subsequently used to elucidate three partial structures (A-C) with key HMBC correlations used to provide attachment points and complete the planar structure, as described below.

TOCSY data afforded a two-spin system for substructure (A) with one spin system consisting of H-2/H-3/H-9 and the other spin system consisting of H4-H7. A COSY correlation between H-4 and the amide proton at $\delta_H 6.85$, as well as HMBC correlations between H-4/C-5, H3-8/C-5, H-6a/C-3, and H3-7/C-6, supported construction of a modified isoleucine with a hydroxyl at C-3 ($\delta_C 71.8, \delta_H 3.51$). The C-3 methine was coupled to the C-2 methine via a COSY correlation and through to the C-1 carbonyl with a 3-bond HMBC correlation. A methyl ($\delta_C 13.8, \delta_H 0.85$) was then assigned to C-2 via H-2/C-9 and H3-9/C-1 HMBC correlations, thus completing the planar substructure (A) or 4-amino-3-hydroxy-2,5-dimethyl-heptanoic acid (Ahdhe), a structural feature characteristic of the hoiamide class (Figure 2).

Substructure (B) featured two consecutively coupled $\alpha$-methyl thiazoline rings and a thiazole. Preliminary data analysis suggested that two methylenes with chemical shifts $\delta_H 3.59/3.13$ ($\delta_C 40.9$); $\delta_H 3.74/3.76$ ($\delta_C 42.2$) were adjacent to heteroatoms, and a low field singlet at $\delta_H 8.21$ ($\delta_C 122.6$) implied the presence an olefinic proton of a heterocycle. Atom counting and the presence of three deshielded quaternary carbons C-14/18/21 ($\delta_C 177.7, 162.5, 169.9$) also suggested that these $sp^2$ carbons were proximate to a nitrogen and a sulfur atom in each ring. HMBC correlations were then used to construct the rings of the triheterocyclic system, as follows. A correlation between the C-12 methylene ($\delta_C 40.9$) and the deshielded $sp^2$ C-14 ($\delta_C 177.7$) permitted assignment of C-12 adjacent to the sulfur atom with C-14 placed between the sulfur and nitrogen atoms. An
additional HMBC correlation between H$_2$-12 and the relatively shielded quaternary carbon at C-11 (δ$_C$ 84.4) necessitated placement of C-11 adjacent to a nitrogen atom and between the C-12 methylene and the carbonyl at C-10. Further HMBC correlations between H$_3$-13/C-10/11/12 permitted assignment of an α-methyl group on C-11 of the thiazoline ring. In a similar fashion, the second thiazoline ring was constructed with HMBC correlations between the C-16 methylene, the deshielded sp$^2$ C-18 (δ$_C$ 162.5), and the relatively shielded quaternary carbon at C-15 (δ$_C$ 158.6). Additional correlations between H$_3$-17/C-15/16 permitted assignment of an α-methyl group on C-15 of the second thiazoline ring as well. The third ring was constructed with HMBC correlations from a low field olefinic proton at δ$_H$ 8.21 (δ$_C$ 122.6) to two sp$^2$ quaternary carbons at C-19/21 which implied the presence of a thiazole ring and satisfied the remaining degrees of unsaturation for the molecule. Further correlations between H-16a/16b/C-14 and H-20/C-18 demonstrated the consecutive nature of the triheterocyclic ring system to complete substructure (B).

Substructure (C) consisted of an extended 10-carbon chain with two hydroxy and three methyl groups, and a methoxy substituent. COSY correlations were used to consecutively assign the H-22 methylene with three methines H-23/24/25. An additional COSY correlation was used to assign a methyl group at C-24 (δ$_H$ 2.08), and an HMBC correlation between C-23 and the H-35 O-methyl allowed placement of the O-methyl at this position. COSY correlations were also used to assign the H-26/27/28 methines and two methyl groups at C-26 (δ$_H$ 1.52) and C-28 (δ$_H$ 1.54), respectively. A 3-bond HMBC correlation between H$_3$-33 and C-25 allowed connection of C25/C26. Further COSY correlations were then used to place the H-29 and H-30 methylenes adjacent to the
terminal C-31 methyl group. HMBC correlations between the C-28 methine and both H-29a/H-29b protons then permitted connection of C-28/29. Accounting of the remaining atoms and chemical shift reasoning allowed hydroxy groups to be placed at C-25 ($\delta_C$ 69.4) and C-27 ($\delta_C$ 72.7) to complete substructure (C).

Finally, a key HMBC correlation between the C-10 carbonyl and the H-13 $\alpha$-methyl connected substructure (A) with substructure (B), and HMBC correlations between C21 and H22/H23 permitted connection of substructure (B) to (C), thereby completing the proposed planar structure of 1 (Figure 1).

Colon cancer selectivity-guided fractionation of a second collection of mat-forming Symplaca sp. obtained near Kolaio island, Papua New Guinea in 2003 yielded 2 with a HRESI [M+H]$^+$ m/z 743.3535 mass identical to 1. $^{12,13}$ Independent structure elucidation of 2 led to the same planar structure of 1. However, subsequent analysis of spectroscopic and analytical data revealed subtle differences between 1 and 2 that suggested the possibility of either different stereoisomers, different ionic states, or some other small difference. First, $\Delta\delta_C$ values of C-1-C-4 and C-6 and $\Delta\delta_H$ values of H-2 and H-3 are significant between 1 and 2 with the greatest $\Delta\delta_C$ nearly 3 ppm at C-1 (Figure 3a). One hypothesis accounting for the carbon shift difference was the possibility of different stereoisomers. However, coupling constant analysis did not reveal significant differences between 1 and 2. Another possibility to account for the carbon shift disparity was a difference in ionic states. The more deshielded carbon of 1 ($\delta_C$ 179.5) suggests 1 may be the conjugate base of 2. Second, 1 and 2 were obtained by different chromatographic conditions; 1 was isolated under neutral conditions while 2 was purified with an acidified buffer. A LC-ESI-MS co-injection experiment, with simultaneous
elution of 1 and 2 in various chromatographic conditions, also provided supporting evidence of constitutional equivalence but different ionic states. Third, the IR spectrum of 1 showed absorption of carbonyls at 1661 and 1580 cm\(^{-1}\), indicating the presence of an amide and carboxylate anion while the spectrum of 2 showed strong absorption of a carbonyl at 1673 cm\(^{-1}\), indicating the presence of an amide and carboxylic acid. The working hypothesis of different ionic states was finally proven by protonation of 1 with 0.1N trifluoroacetic acid, followed by comparison of spectroscopic data (supplemental info). The \(^1\)H and \(^13\)C data of protonated 1 are spectroscopically identical to those of 2, and optical rotation values are similar. In addition, protonation of 1 obliterated the CD spectroscopic differences initially observed between 1 and 2. Therefore, the proposed planar structure of 1 was amended to reflect its negatively charged state (Figure 1), and the HRESI [M+H]\(^+\) \(m/z\) 743.3535 mass of 1 is likely the result of reduction during electrospray ionization.

To ascertain the absolute stereochemistry of the Me-Cys residues, 2.1 mg of 1 was dissolved in 3 mL of CH\(_2\)Cl\(_2\) and treated with ozone at 25\(^\circ\) C for 15 min. The sample was dried and further treated with 1 mL of H\(_2\)O\(_2\)-HCOOH (1:2) at 70\(^\circ\) C for 30 min. \(L-(R)\)-2-methylcysteic acid (2.0 mg) and \(D-(S)\)-2-methylcysteic acid (2.5) mg standards were prepared and treated as above. The dry sample 1 was then hydrolyzed using 6N HCl (0.5 mL) for 2 hours at 110\(^\circ\) C and analyzed by chiral HPLC (Phenomenex Chirex 3126 (D), 4.6 x 50 mm; 2 mM CuSO\(_4\) in H\(_2\)O at 0.3 mL/min; detection at 230 nm) to yield \(D-(S)\)-2-methylcysteic acid with a retention time of 9.7 min (\(D\)-MeCysA \(t_R=10.0\) min; \(L\)-MeCysA \(t_R=12.0\) min). To determine the configuration of the remaining 10 stereocenters, semisynthetic hoiamide D, prepared by base hydrolysis of hoiamide A, was
used for comparative analysis. Semisynthetic hoiamide D was identical to 2 by LC-ESI-MS analyses (retention time, UV spectrum, and m/z value) and 1H and 13C NMR data analyses, had a similar optical rotation value and an identical CD spectrum to protonated 1 and 2 which indicated that natural hoiamide D (1 and 2) and semisynthetic hoiamide D were stereochemically identical (Figure 3b). The absolute configuration of natural hoiamide D (1 and 2) was thus determined as 2R, 3S, 4S, 5S, 11S, 15R, 23S, 24R, 25R, 26S, 27S and 28R.

The carboxylate anion (1) was screened in a panel of assays to determine its biological activity, and its greatest bioactivity was inhibition of p53/HDM2 (human homologue) interaction. A HTR- FRET-based competition assay was used to characterize antagonists of the p53/HDM2 binding interaction based on the ability of antagonist molecules to compete with biotin-labeled p53 protein (bio-QETFSDLWKLLP-Ac) binding to GST-tagged HDM2 (GST-MPRFMDYWEGLN). FRET was detected (ex 320 nm, em 615, 665 nm) when donor labeled, Europium (Mab GST-Eu cryptate, Cisbio 61GSTKLB), and acceptor labeled, XLent (streptavidin-XLent, Cisbio 611SAXLB), complexes were in close proximity due to the anti-GST/GST-HDM2 interaction and the streptavidin/ biotin-p53 interaction. The carboxylate anion (1) inhibited the p53/HDM2 protein binding (EC50= 4.5 µM; 95% confidence interval: 2.8-7.3 µM) with minimal cytotoxicity to the mammalian H460 cell line at 40 µM (16% ± 1), compared to control. Compound 2 was not tested in the p53/HDM2 assay, although the carboxyl group would be deprotonated at the pH (7.4) of the media utilized in the assay.

Chlorofusin (IC50= 4.6 µM), isolated from a marine fungus Fusarium sp., was the first natural product reported to inhibit p53/MDM2 interactions. A second natural
product inhibitor, the non-peptidic hexylitaconic acid (IC$_{50}$ = 230 µM), was isolated from a marine fungus *Arthrinium* sp. in 2006, followed by sempervirine (IC$_{50}$ = 29.4 µM). 21, 22 Hoiamide D is the second peptide-based natural product inhibitor of p53/MDM2 interaction reported, and is one of the most potent. Of note, however, there are rationally designed synthetic inhibitors, such as the spiro-oxindoles and benzodiazepinediones, with nanomolar efficacy.

Hoiamide D joins three other hoiamides isolated to date, comprising a recently described class of cyanobacterial compounds featuring a triheterocyclic system. Hoiamide A and B are cyclic whereas hoiamide C and D are linear. 23, 24 In fact, hoiamide C may be an extraction artifact of hoiamide D given utilization of ethanol in the storage conditions of the biological material. Hoiamide A and B exhibited potent inhibition of calcium oscillation in rat cortical neurons and micromolar activation of voltage gated sodium channels (VGSC) with hoiamide A reported to be a partial agonist at site 2 of the mammalian VGSC. 23 Both hoiamide C and D were inactive in these assays, suggesting a cyclic architecture is required for this potent neuromodulatory activity. 24
Supporting Information Available: $^1$H NMR, $^{13}$C NMR, COSY, TOCSY, HSQC and HMBC spectra in DMSO for hoiamide D (1 and 2).

![Chemical Structures](image)

**Figure 1.** (1). Hoiamide D, conjugate base. (2). Hoiamide D, acid form. (3). Hoiamide C.

![Partial Structures](image)

**Figure 2.** Partial structures of 1 and 2 with key COSY and HMBC correlations.
Table 1. $^1$H and $^{13}$C NMR assignments for hoiamide D (1 and 2) in DMSO-d$_6$.

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$^a$ 500 and 600 MHz for $^1$H NMR, HMBC
$^b$ 75 MHz for $^{13}$C NMR
Figure 3. (A). Carbon shift comparisons of hoiamide D. (1). (B). CD comparison of 1 (-COO$^-$ form) and 2 (-COOH form) with protonated 1 and semisynthetic 2.
**Figure 4.** Inhibition of p53/MDM2 binding by hoiamide D (1). EC\(_{50}\) = 4.5 µM; 95% confidence interval: 2.8-7.3 µM.
References and Notes


(10) Compound 1: pale green solid; [α]D -30 (c 0.05, MeCN); UV (MeCN) λmax (log ε) 252 (3.54); IR (neat) νmax 3381, 2963, 2929, 1661, 1580, 1518, 1458, 1404, 1154, 1084, 1034, 1018 cm⁻¹; 1H NMR (DMSO, 500 MHz) and 13C NMR (DMSO, 75 MHz) data, see Table 1; (+)-HRESIMS [M+H]+ m/z 743.3535 (calcd for C35H59N4O7S3, 488.3546).

(11) The collection (PNG-04-26-06-3) was extracted six times with heat using CH2Cl2:MeOH (2:1) and was further processed on silica gel using vacuum liquid chromatography with a 100% hexanes-EtOAc-MeOH gradient to generate nine subfractions A-I. Fraction I eluted with 100% MeOH and harbored the greatest p53/MDM2 inhibitory activity. Fraction I (259 mg) was subsequently filtered using a Waters RP C18 SPE cartridge with a MeOH gradient to yield four relatively pure subfractions. The most polar subfraction exhibited the greatest activity and was further examined.

(12) Compound (2): pale yellow oil; [α]D 19.6 (c 0.05, MeCN); UV (MeOH) λmax (log ε) 250 (3.49); IR (neat) νmax 3378, 2965, 2930, 1674, 1521, 1458, 1382, 1299,
1203, 1139, 1088, 1026 cm\(^{-1}\); \(^1\)H NMR (DMSO, 500 MHz) and \(^{13}\)C NMR (DMSO, 75 MHz) data, see Table 1; (+)-HRESIMS [M+H]\(^+\) \(m/z\) 743.3535 (calcd for C\(_{35}\)H\(_{59}\)N\(_{4}\)O\(_{7}\)S\(_{3}\), 488.3546).

(13) The collection (PNG-12-16-03-4) was extracted repeatedly with CH\(_2\)Cl\(_2\)-MeOH (2:1) and further fractionated by silica gel vacuum column chromatography (VLC) to produce nine fractions (A-I). Fraction I was found to possess slight colon cancer selective inhibitory activity. This fraction was subjected to RP Sep- pak followed by RP HPLC [Phenomenex Hydro RP C18, 300 Å, 10 x 250 mm, 68% CH\(_3\)CN/H\(_2\)O (0.001% trifluoroacetic acid) at 3 mL/min] to afford hoiamide D (2.4 mg).

(14) Compound 1 (3.0 mg) was treated with 0.1N trifluoroacetic acid for 1 hr and dried under N\(_2\).


(16) A semi-synthetic standard of hoiamide D was prepared according to Choi, H.; Pereira, A. R.; Cao, Z.; Shuman, C. F.; Engene, N.; Byrum, T.; Mataliho, T.; Murray, T. F.; Mangoni, A.; Gerwick, W. H. J Nat Prod. 2010, 73, 1411-21. [\(\alpha\)]\(_D\) +25.2 (c 0.05, MeCN); UV (MeOH) \(\lambda_{max}\) (log \(\varepsilon\)) 250 (3.52).

(17) The FRET buffer used for all experiments was phosphate buffered saline (pH 7.4), 100 mM KF, 0.1% bovine serum albumin, and 5 mM \(\beta\)-mercaptoethanol. Biotin-P53 (500 nM, final) was preincubated for 20 min with test compound (10-0.0781 \(\mu\)g/mL; 2-fold serial dilution), and a master mix was then added containing GST-HDM2 (2.5 nM, final), Mab GST-Eu cryptate (17 nM, final), and streptavidin XLent (9 nM, final); the solution was incubated for 1 h. Data were collected in duplicate using the PerkinElmer EnVision 2103 Multilabel Reader using excitation filter 320nm, emission filters 665nM (to detect XLent) and 615 nM (to detect Europium), respectively, and the optical module Lance Eu/APC Dual 452. Percent inhibition compared to DMSO control was calculated for each concentration of test compound.


Acknowledgements

We thank the government of Papua New Guinea for permission to make these cyanobacterial collections, A. Pereira for the synthetic standard and technical expertise, T. Suyama for helpful advice, J. Wingerd and T. Byrum for the H-460 cytotoxicity and Neuro-2A screening, and the TSRI and UCSD mass spectrometry facilities for their analytical services. This work was supported by NIH grants CA 52955, NS 053398, and NIH/NCI CA100851.

The text of III, in full, is the manuscript draft to be submitted to an academic journal as it will appear: Malloy, K. L, Choi, H., Fiorilla, C., Valeriote, F., Matainaho, T., Gerwick, W. H., Hoiamide D, A Marine Cyanobacterial Derived Inhibitor of p53/MDM2 Interaction. The dissertation author was the primary author and directed and supervised the research which forms the basis for this chapter.
Supporting Information

Figure 5. $^1$H NMR spectrum of 1 (500 MHz, DMSO)

Figure 6. $^1$H NMR spectrum of 2 (600 MHz, DMSO)

Figure 7. $^{13}$C NMR spectrum of 1 (75 MHz, DMSO)

Figure 8. $^{13}$C NMR spectrum of 2 (75 MHz, DMSO)

Figure 9. COSY spectrum of 1 (DMSO)

Figure 10. COSY spectrum of 2 (DMSO)

Figure 11. TOCSY spectrum of 1 (DMSO)

Figure 12. TOCSY spectrum of 2 (DMSO)

Figure 13. HSQC spectrum of 1 (DMSO)

Figure 14. HSQC spectrum of 2 (DMSO)

Figure 15. HMBC spectrum of 1 (DMSO)

Figure 16. HMBC spectrum of 2 (DMSO)

Figure 17. $^1$H NMR spectrum of protonated 1 (500 MHz, DMSO)

Figure 18. $^{13}$C NMR spectrum of protonated 1 (75 MHz, DMSO)

Figure 19. $^{13}$C NMR spectrum of Hoiamide A hydrolysate (75 MHz, DMSO)
Figure 14. HSQC Spectrum of 2 (600 MHz, DMSO)
Figure 16. HMBC spectrum of 2 (600 MHz, DMSO)
Chapter IV.

Credneramides A and B: Neuromodulatory Phenethylamine and Isopentylamine Derivatives of a Vinyl Chloride-Containing Fatty Acid from cf. Trichodesmium sp. nov. and Oscillatoria sp.
Abstract

Credneramides A and B, two vinyl chloride-containing metabolites, were isolated from a Papua New Guinea collection of cf. *Trichodesmium* sp. nov and expand a recently described class of vinyl chloride-containing natural products. The precursor fatty acid, credneric acid, was isolated from both the aqueous and organic fractions of the parent fraction as well as from another geographically and phylogenetically distinct cyanobacterial collection (Panama). Credneramides A and B inhibited spontaneous calcium oscillations in murine cerebrocortical neurons at low micromolar concentrations (1, IC$_{50}$= 4.0 µM; 2, IC$_{50}$= 3.8 µM).
Introduction

It has been exceptionally productive to search for structurally-novel and biologically-active natural products in marine cyanobacteria, especially among filamentous types. For example, the vinyl chloride-containing jamaicamides A-C and the structurally diverse malyngamides A-X have been found from marine cyanobacteria or opisthobranch mollusks which feed on these photosynthetic prokaryotes. However, it is important to note that field collected cyanobacteria are rich substrates for heterotrophic bacterial growth, and thus it is conceivable that these latter organisms are the source for some of these natural products. In a few cases, a microbial symbiont has been rigorously shown to be the actual producer of a compound obtained from a macroorganism. The biosynthetic machinery of the bacterial symbiont *Endobugula sertula* has been shown to produce the anticancer bryostatin natural products that were first reported from the host bryozoan *Bugula neritina*. Similarly, the unicellular symbiotic cyanobacterium *Prochloron* sp. has been shown to be the biosynthetic source of the patellamides, originally reported from the host tunicate *Lissoclinum patella*. Thus, in many cases the metabolic origin of marine secondary metabolites remains largely speculative at present.

Interestingly, the jamaicamides and malynamides have a common biosynthetic theme in that they possess a PKS-derived long lipid chain and an NRPS-derived peptidic moiety. Furthermore, all three jamaicamides and 22 of the 29 reported malyngamides contain the unusual and highly distinctive vinyl chloride functional group (Figure 2). Because the vinyl chloride unit is predicted to reside on C-1 of the glycine derived unit,
the sp² carbon bearing the chloride is predicted to derive from the C-2 carbon of another pendant acetate unit. Likewise, in the jamaicamides, the vinyl chloride carbon has been shown to be derived from C-2 of acetate via a complex biosynthetic process involving an HMGCoA synthase catalytic unit, and to be highly similar to that responsible for cyclopropyl ring formation in curacin A. 

Among cyanobacterial natural products, particularly *Lyngbya majuscula*, the phenethylamine moiety has been reported in several natural products, including grenadamide (lyngbyamide A), hermitamide A, and lyngbyamide B which harbors a p-hydroxy group on the phenethylamine group (Figure 3). Phenethylamines (PEA) are well known for their psychotropic effects and have been isolated from a variety of organisms, including octocorals, the edible mushroom *Laetiporus sulphureus*, *Streptomyces* sp., *Enterococcus* sp., and other species of cyanobacteria. In accordance with the neurotropic activity of PEAs, grenadamide exhibits cannabinoid receptor binding activity (Kᵢ = 4.7 µM) and brine shrimp toxicity (LD₅₀ = 5 µg/ml) whereas hermitamide A was cytotoxic to neuro-2A murine neuroblastoma cells (IC₅₀ = 2.2 µM) and brine shrimp (LD₅₀ = 5 µM). Lyngbyamide B was reported to be cytotoxic to brine shrimp (LD₅₀ = 6.5 ppm). For each of the cyanobacterial compounds containing a phenethylamine moiety, the “precursor” fatty acid was also isolated along with the elaborated natural product. As seen with the malyngamides, the precursor fatty acid is a chemical scaffold to which the cyanobacterium adds further functional groups, and this appears to enhance to their biological activity. This theme of chemical scaffolding to functionalize and enhance bioactivity is also observed with the credneramides. Herein, we report the isolation and structure elucidation of
credneramides A (1) and B (2) and credneric acid (3), neuromodulatory cyanobacterial compounds isolated from a Papua New Guinea collection of cf. *Trichodesmium* sp. nov. Additionally, credneric acid (3) was also isolated from a strain of cf. *Oscillatoria* sp. obtained from the Eastern Pacific (Panama). Because credneric acid was also recently reported from a field collection of the bacterium *Rhodopirellula baltica*, several interesting questions emerge as to the origin of this compound class, the phylogenetic relationships of the producing organisms, and the evolutionary history of the putative biosynthetic pathway.

**Results and Discussion**

**Natural Product Isolation and Structure Elucidation**

Credneramides A (1) and B (2) were isolated from the organic extract of the cyanobacterium cf. *Trichodesmium* sp. nov. collected near the Credner Islands, Papua New Guinea. Using silica vacuum liquid chromatography, the extract was fractionated into nine subfractions (A-I) of increasing polarity, and fraction E was further purified using reversed phase HPLC. HRESIMS data ([M+H]+ m/z 320.1779) of credneramide A (1) established the molecular formula to be C_{19}H_{26}ClNO with seven degrees of unsaturation; the presence of one chlorine atom was supported by the isotopic pattern. The ^1H NMR spectrum was well dispersed and contained two distinctive resonances similar to that of the malyngamides series that were diagnostic of a vinyl chloride functionality and a disubstituted olefin. In particular, these resonances were a sharp singlet at 5.77 ppm (δ_C 113.1 ppm) and two narrowly resolved multiplets at 5.37 and 5.45 ppm (δ_C 127.8, 131.2, respectively; Table 1). Three resonances in the aromatic region of the ^1H NMR spectrum (δ_H 7.19, δ_C 28.7; δ_H 7.24, δ_C 126.5; δ_H 7.32, δ_C 128.7) were
suggestive of the presence of a mono-substituted phenyl group, and a carbon resonance at 172.1 ppm indicated the presence of an ester or amide carbonyl.

A combination of 2D COSY, HSQC, and HMBC correlations was subsequently used to complete the planar structure of credneramide A (1)(Figure 1). COSY correlations were used to consecutively assign two methylenes (δ1.43 and 2.15) adjacent to the terminal methyl at C-10 as well as to related the C-3 and C-6 methylenes (δH 2.32 and 2.70) with each methine (δH 5.37 and 5.45) of the C-4/C-5 olefin. An HMBC correlation between H-2 and C-3 permitted connection of the C-2/C-3 methylenes, and a correlation between H-2 and C-1 allowed placement of the ester/amide carbonyl group adjacent to the C-2 methylene. Additional HMBC correlations between H-6 and C-11/C-7 permitted attachment of the vinyl chloride to this carbon chain. Integrated 1H NMR and COSY data supported the presence of a phenethyl moiety with five aromatic protons and coupling of the C-1’/2’ methylenes at 3.53 and 2.81 ppm, respectively (Figure 1). The complement of 1H NMR and 13C NMR chemical shifts for H-1’/C-1’ (δH 3.53; δC 40.5) supported its direct attachment to a nitrogen atom. HMBC correlations between H-1’ and C-2’/C-3’ and multiple correlations between the protons and carbons of the aromatic ring confirmed the presence of a phenethylamine moiety (Table 1). Lastly, a key HMBC correlation between H-1’ and C-1 permitted connection of the phenethylamine through an amide linkage to the vinyl chloride-containing fatty acid portion, thus completing the planar structure of credneramide A (1).

Assignment of the geometries of the two olefins in 1 was accomplished by 1D NOE, 2D ROE and homonuclear coupling constants. A key NOE/ROE correlation between the vinyl proton and the H-6 methylene established the C-7 olefin as E. A 3JH-H
15.3 Hz coupling constant between the vinyl protons at H-4 and H-5 protons permitted assignment of the C-4 olefin as E as well.

The second credneramide metabolite, credneramide B (2), was isolated in only about 60% of the yield of credneramide A (1), and showed by HRESIMS an M+H]⁺ peak at m/z 296.1963 for a molecular formula of C₁₆H₂₉ClNO. A comparison of the ¹H and ¹³C NMR chemical shifts of 1 and 2 revealed their close similarity with the notable absence of aromatic absorptions in the spectra for 2. Moreover, the ¹H NMR of compound 2 lacked the distinctive triplet at δ2.81 present in the spectrum of 1, and this was replaced with an additional methylene triplets at δ1.36 and a methine multiplet at δ1.60. Analysis of gHSQC data indicated the presence of two additional terminal methyl groups in the higher field region. COSY correlations defined the adjacent positions of the doublet of triplets at δ1.36 to the C1’ methylene at δ3.26 and to the C3’ methine at δ1.60. An HMBC correlation to itself (δ₉ = 0.90 ppm, δ₀ = 22.4 ppm) suggested a terminal gem-dimethyl grouping, and a correlation between the C3’ methine and the two terminal methyls allowed formation of an isopentylamine moiety. Finally, a key HMBC correlation between the C1’ methylene and the carbonyl (δ₀ = 172.0) permitted attachment of the isopentylamine to the rest of the molecule to complete the planar structure of 2. In similar fashion to credneramide A (1), a key NOE correlation between the vinyl proton and the H-6 methylene established the C-7 olefin as having E geometry in credneramide B (2), and a ³JHH 14.9 Hz coupling constant between the vinyl protons permitted assignment of the C-4 olefin as also being E.

The precursor vinyl chloride fatty acid, credneric acid (3), was isolated from both the aqueous and organic portions of the same parent extract. The initial analysis of 3 was
done by $^1$H NMR, which revealed that the compound was a short fatty acid. Analysis by COSY and HSQC revealed the presence of an $n$-propyl group. Further analysis by the same methods revealed another spin system consisting of two consecutive methylene groups, a di-substituted olefin, and another methylene group. A tri-substituted vinyl chloride functionality was postulated based on the two and three bond heteronuclear coupling information obtained by HMBC analysis. Finally, HMBC analysis also allowed connection of the three substructures to form the fatty acid portion of credneramides A and B. Again, the stereochemical assignment of the two olefins in 3 were made by the coupling constant between the two vicinal vinyl protons (5.42 ppm and 5.48 ppm; $J = 15.4$ Hz) and 1D ROESY correlations of key protons on and around the olefins, and these were in accordance with those of compounds 1 and 2. 21

Credneramides A and B, along with recently reported grenadamides B and C, constitute a new class of vinyl chloride-containing fatty acids in cyanobacteria. 23 A commonality shared among the credneramides is an apparent reductive decarboxylation of the amide head group. Reductive decarboxylation of phenylalanine (Phe) is expected to proceed via a phenylalanine decarboxylase or a tyrosine (Tyr) decarboxylase with promiscuity towards the related Phe residue. 20 Similarly, the isopentylamine functionality is predicted to arise from reductive decarboxylation of leucine by a putative leucine decarboxylase. Vinyl chloride formation in the credneramides is predicted to proceed via a $\beta$-branch acetate extension as seen with the jamaicamides and most likely occurs in malyngamide biosynthesis. 3, 12, 21 A non-heme Fe(II), $\alpha$-ketoglutarate dependent halogenase similar to the Jam halogenase is expected to halogenate C-2 of acetate during $\beta$-branch formation. Following dehydration, a decarboxylase similar to
Jam ECH$_2$ must decarboxylate this pendant group in a regiospecific manner to yield the vinyl chloride.$^3,^{12}$

**Evolution, taxonomy, and metabolic origin of the credneramides**

Morphologically, the credneramide-producing cyanobacterial specimen from Papua New Guinea (PNG-05/19/05-13; GenBank acc. Nr. 1452341) agreed well with the current definition of either *Lyngbya* or *Oscillatoria* (Figure 5, for morphological description see Supplementary Information). Marine forms of *Lyngbya* and *Oscillatoria* typically share their entire range of cellular types and are often misidentified.$^{24,25}$

Phylogenetic inferences of the SSU (16S) rRNA gene from two credneramide-producing specimens was used to determine the taxonomic identity by inferring its evolutionarily relationship to relevant reference and type-strains. The credneramide-producing strain formed a clade with other benthic tropical marine cyanobacteria with similar morphology, including the strains PAL08-3.2 (GenBank acc. Nr. HM585025) from Palmyra atoll and NAC8-50 (GenBank acc. Nr. GU724203) from the Caribbean island of Curacao. This group is evolutionarily most closely related to the planktonic genus *Trichodesmium* (reference/type-strains = *Trichodesmium erythraeum* IMS 101$^T$; p-distance 2.3%). The ecological and evolutionarily distinction suggests that the credneramide-producing strain, in fact, represents a new taxa. Technically, this new taxa could either be included in the genus *Trichodesmium*, which would require a change in the definition of this planktonic genus to also include benthic forms, or alternatively, this group needs to be erected as a separate generic entity. More careful taxonomic comparison between the two groups must be performed to firmly establish placement of this new taxa. Presently, this strain is best defined as a cf. *Trichodesmium* sp. nov.
The predicted precursor of the credneramides, credneric acid (3), was also isolated from a filamentous cyanobacterial specimen (PAC-17-FEB-10-3; GenBank acc. Nr. 1452343) obtained from the Pacific coast of Panama. Phylogenetic comparison between the strain from Papua New Guinea and the Panamanian strain revealed that these two credneric acid-producing strains were evolutionarily unrelated and belonged to two different genera (Figure 5). Strain PNG-05/19/05-13 was most closely related to the genus *Trichodesmium* whereas strain PAC-17-FEB-10-3 belonged to a clade of tropical marine *Oscillatoria*. To determine if this secondary metabolite was the result of a more ancient biosynthetic pathway, several related specimens, including the strains NAC8-50, 3LOSC (GenBank acc. Nr. EU244875), PAB-21 (EU253967), NAC8-46 (GU724197), and NAC8-5 (GU724208) were screened by ESI-LC-MS for a molecular weight corresponding to credneric acid. The fact that this secondary metabolite was not present in any other of these specimens suggests that its biosynthetic pathway has not been vertically inherited, at least within these groups of cyanobacteria.

Another possibility for this disjunct taxonomic distribution of credneric acid in these cyanobacteria is suggested by the recent isolation of credneric acid (3) from the epiphytic heterotrophic bacterium *Rhodopirellula baltica*. Since this bacterium is known to grow in association with other marine organisms, there is the possibility that credneric acid isolated from our cyanobacterial specimens is actually produced by *R. baltica* living in association with the cyanobacteria. To determine whether *R. baltica* was present in our collections and, therefore, the likely producer of credneric acid, an attempt was made to amplify the 16S rRNA gene sequence of *R. baltica* from the Panamanian and Papua New Guinean strains using specifically designed primers and to compare that
data with the 16S rRNA sequence for R. baltica (H. S Lee, personal communication).

However, sequencing of several clones from libraries generated using these specific primers resulted in gene sequences of heterotrophic bacteria other than R. baltica. Thus, because the biosynthetic machinery for production of the vinyl chloride functional group has been carefully and quite thoroughly described in other marine cyanobacteria, \(^3,12\) and the fact that another well known marine cyanobacterial metabolite (malyngic acid)\(^26\) was also isolated from the field collection of R. baltica, \(^22\) we believe that these metabolites derive from cyanobacterial metabolism. Their occurrence in the field collected R. baltica may therefore result from small filaments of Trichodesmium or Oscillatoria contaminating this environmental sample.

**Biologic Activity and Ecologic Significance of Credneramides**

Credneramides A (1) and B (2), and credneric acid (3), were subjected to a suite of bioassays to explore their biological activities. Although the parent extract possessed sodium channel blocking activity, the credneramides activity in this assay was very modest. Their most potent activity was inhibition of calcium oscillations in cerebrocortical mouse neurons (1, IC\(_{50}\) = 4.0 µM, 2, IC\(_{50}\) = 3.8 µM). Although 3 exhibited modest calcium oscillation inhibition, the Hill slope of its concentration response curve suggests nonspecific activity or 1:1 ligand-receptor binding (Figure 6). In contrast, the steep Hill slopes of the concentration curves for the 1 and 2 suggest positive cooperativity. Given that the credneric acid demonstrates a nonspecific response, and 1 and 2 confer more specific calcium inhibition, it is logical that the PEA and isopentylamine moieties are responsible for the enhanced activity of each compound. \(^21\)
The calcium oscillations observed in primary and mature cerebrocortical neurons of mice are sensitive to AMPA receptor modulation as well as group I metabotropic glutamate receptor (mgluR) agonism or antagonism. AMPA receptors regulate calcium oscillation frequency while mgluR mediate frequency and amplitude as well as release of intracellular calcium stores via phospholipase C (PLC). Thus, the bioactivity of the credneramides in this assay could be due to modulation of AMPA, mgluR, or PLC signaling or to TAAR1, dopamine, and GABA receptor activity; the latter three are known to be modulated by PEA. Unlike grenadamide A, a mixture of 1 and 2 did not possess cannabinoid receptor binding activity, and only marginal cytotoxic activity against the beet armyworm Spodoptera exigua was reported for grenadamides B and C, which are structurally most similar to the credneramides.

Fatty acids with phenethylamine have been increasingly encountered among cyanobacterial metabolites. PEA has a well recognized role in mammalian physiology as an endogenous neurotransmitter that has been shown to bind trace amine (TAAR), dopaminergic, and GABA receptors. By binding to these receptors, PEA modulates higher cognitive function via their capacity to act as hallucinogens, stimulants, and antidepressants. This class of compounds has also been implicated in the pathogenesis of several neurologic diseases including depression, attention deficit and hyperactivity disorder (ADHD), and schizophrenia. Given the substantial evidence of neuromodulation by phenethylamines, the neuromodulatory effects of credneramide A (1) are reasonable.
The ecologic and physiologic role of reductively decarboxylated fatty acid amides, particularly those containing PEA, in lower life forms is poorly understood. While they may serve in antipredatory or antifeedant roles, it is also possible that they serve a role similar to that of neurotransmitters in higher life forms, namely, to mediate intercellular signaling. Fatty acid amides with functionalized head groups similar to PEA mimic \( N \)-acyl homoserine lactones (AHL) which are quorum sensing molecules in diverse bacteria. \(^{33}\) At threshold concentrations, AHLs bind “sensor peptides” that are transcriptional regulators and activate the expression of genes of varying functions such as those involved in antibiotic synthesis and motility. \(^{33}\) Malyngamide C and \textit{epi-malyngamide C} are two other fatty acid amides isolated from cyanobacteria which have demonstrated quorum sensing properties. \(^{34}\) Thus, it is possible that the credneramides also have roles in intercellular communication and quorum sensing.

**Conclusions**

Credneramides A (1) and B (2), neuromodulatory phenethylamine and isopentylamine derivatives of a vinyl chloride-containing fatty acid, were isolated from a Papua New Guinea collection of \textit{cf. Trichodesmium} sp. nov. The precursor fatty acid, credneric acid, was also isolated from both the aqueous and organic fractions of the parent fraction, in addition, to a phylogenically distinct Panamanian collection. The true source of credneric acid production remains somewhat uncertain. However, the constellation of findings, the widespread and well characterized vinyl chloride functionality in other marine cyanobacterial metabolites, and the co-occurrence of another well known cyanobacterial metabolite in the report from the heterotrophic bacterium \textit{R. baltica}, indicates that these filamentous cyanobacteria are the likely
biosynthetic source. Credneric acid appears to serve as a template for biochemical elaboration and enhancement of bioactivity.

**General Experimental Procedures**

IR and UV spectra were obtained using a Nicolet IR-100 FT-IR and Beckman-Coulter DU800 spectrophotometer, respectively. NMR spectra were obtained using Varian Unity 300 and 500 MHz spectrometers and a Bruker 600 MHz spectrometer [CDCl$_3$ $\delta_H$ 7.26; $\delta_C$ 77.0] was used as an internal standard. High resolution mass spectra were obtained using an Agilent ESI-TOF mass spectrometer (TSRI, La Jolla, CA) and a Thermo Finnigan MAT900XL spectrometer (UCSD, La Jolla, CA). Flash chromatography was performed using EM Science silica gel (230-400 mesh). VLC was performed using TLC grade silica gel. TLC was performed using EM Science pre-coated silica gel plates (Merck 60 F$_{254}$). Extracts were processed using a HPLC Waters 515 pump, Waters 996 photodiode array detector, and Millenium software for acquisition and analysis of data.

**Collection**

The cf. *Trichodesmium* sp. nov. sample (PNG-05/19/05-13) was collected in May 2005 by snorkel at a depth of 1-2 m near the Credner Islands of Papua New Guinea (4° 14.105’ S and 152° 25.605’ E). The *Oscillatoria* sp. sample (PAC-02/15/10-3) was collected in February 2010 in shallow waters near Isla Canales de Afuera of Panama (7° 41.723’ N and 81° 38.025’ W). Both specimens were stored in 70% EtOH in a 1L container at -20°C until extraction.
DNA Extraction, Amplification, and Sequencing

Cyanobacterial biomass (~50 mg) was partly cleaned under an Olympus VMZ dissecting microscope. 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, OT106F 5’-GGACGGGTGAGTAACGCGTGA-3’ and OT1445R 5’-AGTAATGACTTCGGGCGTG-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. 1452341 and 1452343.

Phylogenetic Inference

The 16S rRNA gene sequence of PAC-17-FEB-10-2 was aligned with evolutionary informative cyanobacteria using the L-INS-I algorithm in MAFFT 6.717 and refined using the SSU secondary structures model for Escherichia coli J01695.
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 0.1.1. 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 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 (α) = 0.485, number of rate categories = 4) with 1,000 bootstrap-replicates. Bayesian inference was conducted using MrBayes 3.1 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.

**Biological Activity**

A mixture containing 1 and 2 was screened for biologic activity in an H460 cytotoxicity assay, neuro-2A sodium channel activation and blocking assays, malaria, Chagas, and Leishmania anti-parasitic assays, and a cannabinoid receptor binding assay. Compounds 1-3 were also submitted for testing in a neocortical neuron calcium oscillation assay where 1 and 2 exhibited moderately potent activity (4.0 µM, and 3.8 µM, respectively). See reported references for calcium oscillation assay, H460 cytotoxicity, neuro-2A assays, and anti-parasitic assays.

**Extraction and Isolation**

The cf. *Trichodesmium* sp. nov. collection was extracted ten times with heat using CH$_2$Cl$_2$: MeOH (2:1) to yield a crude organic extract of mass 12.2 g. Approximately 3 g
of this crude extract was further processed on silica using vacuum liquid chromatography with a 100% hexanes-EtOAc-MeOH gradient to generate nine subfractions A-I. Fraction E eluted with 60/40 EtOAc: hexanes and harbored the most potent sodium channel blocking activity in neuro-2A neuroblastoma cells. Fraction E (240.5 mg) was subsequently filtered using a Waters RP C18 SPE cartridge and was further processed using reversed phase HPLC (Phenomenex RP C18 Jupiter 250 x 10 mm, 10 µm; 50/50 CH₃CN/H₂O to 100 CH₃CN in 30 min; 100 CH₃CN 30-45 min) to yield 7.0 mg of a mixture of credneramides A (1) and B (2), and 9.6 mg of credneric acid (3). The mixture was further processed (C18 Kromasil 250 x 4.6 mm, 5 µm; 70/30 CH₃CN /H₂O isocratic) to yield 1.4 mg of pure credneramide A (1) and 0.8 mg of credneramide B (2). The remaining aqueous layers and the EtOH used for preservation of the alga were combined and the volatiles were removed under vacuum. The residues were resuspended in EtOH and the insolubles were removed by filtration. Filtration of this material over a pad of water-treated silica gel with EtOH was followed by Si gel solid phase extraction chromatography, sequentially eluting with 1:1 CH₂Cl₂/EtOAc, EtOAc, 1:9 MeOH/CH₂Cl₂, and 3:7 MeOH/CH₂Cl₂). Four fractions (4 mg, 422 mg, 1.08 g, and 1.09 g, respectively) were obtained. Based on TLC characteristics, the third SPE fraction was subjected to flash column chromatography with Si gel and a gradient mixture of MeOH and CH₂Cl₂ as the mobile phase to obtain several fractions. One of the fractions contained a partially purified compound (7 mg) and it was further purified by semi-prep reversed phase HPLC (250 x10.00 mm Synergi 4µ Hydro-RP 80A column, CH₃CN /H₂O gradient) to obtain an additional 4 mg of credneric acid (3) as a colorless oil. The Oscillatoria sp. collection was extracted five times using CH₂Cl₂: MeOH (2:1) to yield a
crude organic extract of mass 90.6 mg. This crude extract (82.9 mg) was further processed on silica using vacuum liquid chromatography as above to generate nine subfractions A-I. Fraction E eluted with 60/40 EtOAc: hexanes (5.8 mg) and was further purified using preparative TLC [hexanes: EtOAc (1:1) + 0.1% AcOH] to yield 1.9 mg of credneric acid (3).

**Credneramide A (1):** clear oil: [α]D +14 (c 0.1, MeOH); UV (MeOH) λ\text{max} (log ε) 206 (4.21), 257 (2.36); IR (neat) ν\text{max} 3289, 3068, 3028, 2959, 2930, 2869, 1644, 1551, 1453, 970, 746 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) and \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz) data, see Table 1; HRESI(+)MS obs. m/z [M+H]\textsuperscript{+} 320.1779 (calcd for C\textsubscript{19}H\textsubscript{27}ClNO, 320.1781).

**Credneramide B (2):** clear oil: [α]D -2.5 (c 0.1, MeOH); UV (MeOH) λ\text{max} (log ε) 203 (3.98); IR (neat) ν\text{max} 3305, 2958, 2926, 2857, 1709, 1644, 1554, 1461, 1376, 1172, 1071, 605 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) and \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz) data, see Table 1; HRESI(+)MS obs. m/z [M+H]\textsuperscript{+} 286.1963 (calcd for C\textsubscript{16}H\textsubscript{29}ClNO, 286.1938).

**Credneric acid (3):** clear oil: [α]D +7.4 (c 0.2, MeOH); UV (MeOH) λ\text{max} (log ε) 203 (3.77), 269 (2.81); IR (neat) ν\text{max} 3428 (br), 2965, 2930, 2873, 1711, 1632, 1431, 1410, 1284, 1252, 1212, 1158, 968, 795 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) and \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz) data, see Table 1; HREIMS m/z [M+H] 217.0995 (calcd for C\textsubscript{11}H\textsubscript{18}O\textsubscript{2}Cl, 217.0990).
**Figure 1.** Credneramide A (1), credneramide B (2), and credneric acid (3), neuromodulatory vinyl chloride-containing fatty acids.
Figure 2. Representative vinyl chloride-containing natural products from *Lyngbya* sp.
**Figure 3.** Cyanobacterial compounds containing a phenethylamine moiety.

**Table 1.** $^1$H and $^{13}$C NMR data for credneramide A (1), credneramide B (2), crednereric acid (3).

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Figure 4. Key COSY, HMBC, and ROE correlations of credneramide A (1) and credneramide B (2).
Figure 5. Evolutionary tree of the credneramide-producing strain PNG-05/19/05-13. The phylogenetic proximity with the genus *Trichodesmium* (reference/type-strains = *Trichodesmium erythraeum* IMS) suggests that this strain represents a new taxa within this genus, which would also include the related and morphologically-similar strains PAL08-3.2 (GenBank acc. Nr. HM585025) and NAC8-50 (GenBank acc. Nr. GU724203) previously putatively identified as cf. *Oscillatoria* sp. Representative reference-strains obtained from *Bergey’s Manual* are highlighted with asterisk (*) and the sensu stricto for the genera *Trichodesmium* and *Oscillatoria* are highlighted with green boxes. In addition to the strain PNG-05/19/05-13, credneric acid was also isolated from the Panamanian strain cf. *Oscillatoria* sp. PAC-17-FEB-10-3 (both credneric acid-producing strains are highlighted with red arrows). The cladogram is based on SSU (16S) rRNA gene sequences using the bayesian (MrBayes) method and the support values are indicated as posterior probability at the nodes. The specimens are indicated as species, strain, and access number in brackets. The scale bar is indicated at 0.04 expected nucleotide substitutions per site.
**Figure 6.** Calcium oscillation inhibition in cerebrocortical mouse neurons by credneramide A (IC$_{50}$ 4.0 µM), credneramide B (IC$_{50}$ 3.8 µM), and credneric acid (1550EP1, IC$_{50}$ 8.2 µM).
References


(38) Zwickl, D. J. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. Thesis, The University of Texas at Austin, Austin, TX, **2006**.


Acknowledgements

We thank the governments of Papua New Guinea and Panama for permission to make these cyanobacterial collections, J. Wingerd for the H-460 cytotoxicity and Neuro-2A sodium assay screening, C. Spadafora for the antiparasite screening, the H. S. Lee laboratory (Korea) for the R. baltica 16S RNA sequence, H. Choi for help with collection of analytical data, and The Scripps Research Institute and UCSD mass spectrometry facilities for their analytical services. This work was supported by NIH grants NS 053398 and ICBG FIC TW006634.

The text of IV, in full, is the manuscript draft to be submitted to an academic journal as it will appear: Malloy, K. L, Suyama, T. L., Engene, N., Debonsi, H., Cao, Z., Matainaho, T., Di Marzo, V., Spadafora, C., Murray, T. F., Gerwick, W. H., Credneramides A and B: Neuromodulatory Phenethylamine and Isopentylamine Derivatives of a Vinyl Chloride-Containing Fatty Acid from cf. Trichodesmium sp. nov. and Oscillatoria sp. The dissertation author was the primary author and directed and supervised the research which forms the basis for this chapter.
Supporting Information

Figure 7. $^1$H NMR spectrum of 1 (500 MHz, CDCl$_3$)
Figure 8. $^1$H NMR spectrum of 2 (600 MHz, CDCl$_3$)
Figure 9. $^1$H NMR spectrum of 3 (500 MHz, CDCl$_3$)
Figure 10. $^{13}$C NMR spectrum of 1 (75 MHz, CDCl$_3$)
Figure 11. $^{13}$C NMR spectrum of 2 (150 MHz, CDCl$_3$)
Figure 12. $^{13}$C NMR spectrum of 3 (75 MHz, CDCl$_3$)
Figure 13. COSY spectrum of 1 (CDCl$_3$)
Figure 14. COSY spectrum of 2 (CDCl$_3$)
Figure 15. COSY spectrum of 3 (CDCl$_3$)
Figure 16. HMBC spectrum of 1 (CDCl$_3$)
Figure 17. HMBC spectrum of 2 (CDCl$_3$)
Figure 18. HMBC spectrum of 3 (CDCl$_3$)
Figure 19. HSQC spectrum of 1 (CDCl$_3$)
Figure 20. HSQC spectrum of 2 (CDCl$_3$)
Figure 21. HSQC spectrum of 3 (CDCl$_3$)
Figure 22. NOESY spectrum of 1 (CDCl$_3$)
Figure 23. NOESY spectrum of 2 (CDCl$_3$)
Figure 24. ROESY spectrum of 1 (CDCl$_3$)
Figure 25. 1D NOE of 1, irr 5.77 ppm (CDCl$_3$)
Figure 26. 1D ROE of 3, irr 2.73 ppm (CDCl$_3$)
Figure 27. 1D ROE of 3, irr 5.79 ppm (CDCl₃)

Figure 28. 1D ROE of 3, irr 2.17 ppm (CDCl₃)
Figure 10. $^{13}$C NMR spectrum of 1 (300 MHz, CDCl3)
Figure 20. HSQC spectrum of 2 (600 MHz, CDCl3)
Chapter V.

Dudawalamide E and Naopopeptin, Two Anti-Parasitic Cyclic Lipopeptides from collections of the Papua New Guinea Cyanobacterium *Lyngbya majuscula*
Abstract

Dudawalamide E and naopopeptin, two new DHOYA and HMOYA containing cyclic depsipeptides, have been isolated from Papua New Guinea collections of the cyanobacterium *Lyngbya majuscula* using bioassay-guided and spectroscopic approaches. Planar structures were elucidated using standard 1D and 2D NMR experiments, and absolute stereochemistry was determined using a combination of chiral HPLC, chiral GCMS, and modified Marfey's analysis. Dudawalamide E possesses broad spectrum anti-parasitic activity with minimal mammalian cell cytotoxicity while naopopeptin displays selective low micromolar inhibition of *Plasmodium falciparum* (IC$_{50}$ = 2.7 μM) and *Leishmania donovani* (IC$_{50}$ = 5.8 μM), and modest inhibition of calcium oscillations in murine cortical neurons (11.7 μM). Comparative analysis of DHOYA-containing cyanobacterial metabolites reveals intriguing SAR features of these NRPS-PKS derived metabolites and their derivatives.
Introduction

Kulolide, a cyclic peptide isolated from the cephalaspidean mollusk *Philinopsis speciosa* by Scheuer in 1996, was the first compound reported to contain the DHOYA (3-hydroxy-2,2-dimethyl-7-octynoic acid) structural fragment. 1 Subsequent to the isolation of kulolide, this moiety and its derivatives have been identified by the Gerwick group in a number of cyanobacterial depsipeptides including yanucamides A and B, 2 mantillamide, 3 antanapeptins A-D, 4 and wewakpeptins A-D. 5 Cocosamides A and B, the most recently identified DHOYA compounds, are the first to contain only a single ester linkage. 6 Although first identified as part of a molluscan compound, the DHOYA fragment and derivatives thereof are now recognized as a unique biosynthetic hallmark of cyanobacteria. Depsipeptides containing DHOYA and HMOYA (3-hydroxy-2-methyl-7-octynoic acid) possess an array of biological activities including cytotoxicity to P388 leukemia, LoVo, and H460 cells, toxicity to brine shrimp, and anti-parasitic activity.

Reported herein are the isolation, structure elucidation, and biologic profiles of dudawalamide E (1) and naopopeptin (2), two cyclic DHOYA and HMOYA-containing lipopeptides with selective anti-parasitic activity from *Lyngbya majuscula*.

Results and Discussion

A sample of the cyanobacterium *Lyngbya majuscula* was collected by snorkel at a depth of 3 to 9 m near Dudawali Bay, Papua New Guinea, in 2006. The collection was subjected to extraction with heat CH$_2$Cl$_2$: MeOH (2:1) and yielded an organic extract of mass 1.99 g. The extract was further processed by normal phase silica vacuum liquid chromatography to generate nine subfractions (A-I) of increasing polarity. Fraction G
exhibited significant cytotoxic activity (99%; 50 µg/ml) and was subsequently subjected to RP C18 SPE cartridge filtration and reversed-phase HPLC to yield dudawalamide E (1) and the known compound compounds majusculamide A and B.

Dudawalamide E (1), with mass [M+H]⁺ m/z 794.4683, established the molecular formula as C₄₃H₆₃N₅O₉ with fifteen degrees of unsaturation (Figure 1). The ¹H NMR spectrum was well dispersed and contained several proton resonances indicative of amide bonds and an aromatic ring structure. The presence of high field methyl doublets and a methyl triplet was further suggestive of constitutive valine and isoleucine moieties.

Integrated ¹H, ¹³C, and COSY NMR data were used to initially assign six partial structures consisting of the amino acids valine, isoleucine, proline, glycine, Hiv, and N-Me-Phe (Figure 3). An additional partial structure was deduced to be DHOYA (2, 2-dimethyl-3-hydroxy-7-octynoic acid) based upon the following data. COSY correlations were used to consecutively assign the C-3 methine and C4-6 methylenes. Two and three bond HMBC correlations between the gem-dimethyl protons and C-2/C-3 placed the two methyl groups on C-2. Last, the C-7/C-8 carbon shifts (δc 83.7, 69.1), in addition to the correlation between C-7/H-8 and C-8/H-6, established the terminal acetylene of DHOYA. Further atom counting and observation of chemical shifts consistent with an amide (Table 1) established its presence and thus completed the partial structure of DHOYA. Finally, HMBC correlations were used to delineate the sequence of amino acids in 1. Correlations were observed between the Val C-12 methine and the carbonyl of DHOYA, the N-methyl of N-Me-Phe and the carbonyl of Val, the Hiv C-27 methine and the carbonyl of N-Me-Phe, the C-35 methylene of Pro and the carbonyl of Hiv, the C-37
methine of Ile and the carbonyl of Pro, and the methylene of Gly and the carbonyl of Ile, thus establishing the sequence as DHOYA, Val, N-Me-Phe, Hiv, Pro, Ile, and Gly.

Cytotoxicity guided isolation of active constituents of a *Lyngbya majuscula* sample, collected by snorkel near Naopoi Island, Papua New Guinea in 2003, yielded a second DHOYA-related derivative, naopopeptin (2). Naopopeptin (2), with mass [M+H]^+ m/z 737.4489, established the molecular formula as \( C_{41}H_{60}N_4O_8 \) with fourteen degrees of unsaturation (Figure 1). The \(^1H\) NMR spectrum was again well dispersed and contained several proton resonances indicative of valine, isoleucine, and the constitutive aromatic ring structure of phenylalanine. Integrated \(^1H\), \(^13C\), and COSY NMR data were used to initially assign five partial structures consisting of the amino acids valine, N-methyl-valine, N-methyl-isoleucine, proline, and 3-phenyllactic acid (Pla) (Figure 4). The last partial structure, HMOYA (2-dimethyl-3-hydroxy-7-octynoic acid) was elucidated in a similar fashion as above. Of note, a COSY correlation placed the single methyl group on C-2, and a C-7/H-5 HMBC correlation also confirmed the terminal acetylene placement. Likewise, atom accounting and chemical shifts consistent with an amide established the partial structure of HMOYA (Table 1). Sequencing of residues was established with HMBC correlations which demonstrated correlations between the valine C-11 methine and carbonyl of HMOYA, the N-methyl of valine and the carbonyl of valine, the C-22 methine of phenyllactic acid (Pla) and the carbonyl of N-methyl-valine, the proline C-31 methine and the carbonyl of Pla, and the N-methyl of N-methyl-isoleucine and the carbonyl of proline, thus establishing the sequence as HMOYA, Val, N-Me-Val, Pla, Pro, and N-Me-Ile.
The absolute configurations of residues in compounds 1 and 2 were determined using a combination of chiral HPLC, chiral GCMS, and modified Marfey’s analysis. The configurations of 1 were revealed as S-DHOYA, L-Val, N-Me-L-Phe, L-Hiv, L-Pro, and L-Ile, and in 2, these were revealed as L-Val, N-Me-L-Val, L-Pla, L-Pro, and N-Me-L-Ile. The HMOYA fragment of 2 was not stereochemically defined.

Dudawalamide E (1) and naopopeptin (2) likely derive from a mixed NRPS-PKS biosynthetic pathway. The biogenesis of the unique cyanobacterial DHOYA and HMOYA moieties are expected to proceed via PKS acetate extensions. SAM is likely responsible for methylation at C-2 in both of these residues, and acetylene formation is expected to proceed via oxidative dehydrogenation. 7-9

Dudawalamide E joins four other DHOYA-containing cyclic lipopeptides (dudalamides A-D (3-6)) simultaneously isolated from Lyngbya sp. in the Gerwick laboratory (Figure 2). The depsipeptides are structurally very similar to several other cyclic peptides isolated from Lyngbya, including kulolide, 1 pitipeptolides A and B, 10 antanapeptin A, 4 and mantillamide A, a low nanomolar inhibitor of Plasmodium falciparum (Table 3). 3 Genera of the order Oscillatoriales are under current revision as a result of polyphyly in the marine Lyngbya lineage 11 and if a new genus designation is approved, it is likely many of these compounds, including 1, that were initially attributed to the Lyngbya genus are actually produced by species of the new genus Moorea. Additionally, although the cyanobacterium yielding compound 2 was classified morphologically as Lyngbya majuscula, there is a distinct possibility that it phylogenetically clades as a Moorea sp. as well.
Biological Activity and SAR Features

Dudawalamide E (1) and naopopeptin (2) were extensively screened in several biological assays to ascertain the extent of their biological properties. Dudawalamide E (1) exhibited broad spectrum anti-parasitic activity against malaria, leishmaniasis, and Chagas disease, with its most potent activity directed towards the Leishmania donovani parasite (IC\_50 = 2.6 µM) (Table 2). Further, compound 1 displays a relatively large therapeutic window for Leishmania inhibition with a 5-fold difference in its cytotoxicity profile (H-460 cells, IC\_50 = 13.5 µM). Compound 1 also demonstrates modest neuro-2A sodium channel activation (IC\_50 = 13.1 µM). Naopopeptin (2) is a low micromolar inhibitor of the Plasmodium falciparum parasite (IC\_50 = 2.7 µM) and also demonstrates micromolar inhibition of Leishmania donovani (IC\_50 = 5.8 µM). Compound 2 also harbors a large therapeutic window for P. falciparum and L. donovani inhibition with a 26-fold and 12-fold difference in cytotoxicity, respectively (Vero cells, IC\_50 = 71.0 µM). Additionally, naopopeptin is a modest inhibitor of calcium oscillations in murine cortical neurons (IC\_50 = 11.7 µM). The other dudawalamides A-D exhibited similar anti-parasitic activity profiles with generous therapeutic windows as well. Dudawalamide A (3) displayed identical inhibition efficacy towards Plasmodium falciparum as dudawalamide E, and dudawalamide D (6) displayed identical inhibition efficacy of Leishmania as naopopeptin.

Intriguing SAR features are emerging as a result of comparative analysis of the DHOYA class of depsipeptides. For example, the constellation of changes between residues 1-3 of dudawalamides A (3) and D (6), L-Lac to L-Val, L-Ala to N-Me-Val, and L-N-Me-Ile to D-Hmp, results in a 10-fold difference in Leishmania activity (Figure 2,
Table 4). Because the changes in residues 1 and 2 are rather benign, it is plausible that a change in stereochemistry at residue 3 is responsible for a significant decrease in activity. In the case of wewakapeptin A and C, a change in residue 6 from L-Hiv to D-Pla results in a 20-fold decrease in activity (Table 4). However, a change in degree of unsaturation does not appear to affect bioactivity, as seen in the case of pitipeptolide A and B. Dimethylation at C-2 of the octynoic acid does appear to modestly enhance neuro-2A activation as seen in antanapeptin A and dudawalamide E. Thus, it appears that a change in stereochemistry and sequence of residues affects potency, and methylation at C-2 of DHOYA provides modest enhancement of potency. The degree of unsaturation of DHOYA has no effect in these specific examples. Extrapolation of these findings to the class in its entirety remains to be seen.

In conclusion, dudawalamamide E (1) and naopopeptin (2), two depsipeptides containing the unique cyanobacterial moieties DHOYA and HMOYA, respectively, have been isolated from Lyngbya majuscula. Isolation of these molecules again demonstrates that DHOYA and HMOYA are molecular fingerprints of cyanobacterial secondary metabolism. Naopopeptin is a low micromolar inhibitor of the Plasmodium falciparum parasite (IC$_{50}$ = 2.7 µM) and also demonstrates micromolar inhibition of Leishmania donovani (IC$_{50}$ = 5.8 µM) while dudawalamide E exhibits broad spectrum anti-parasitic activity against malaria, leishmania, and Chagas disease with its most potent activity directed towards the Leishmania donovani parasite (IC$_{50}$ = 2.6 µM). Its bioactivity is in accordance with dudawalamides A-D, and intriguing SAR features are emerging through comparative analysis of the growing class of DHOYA cyanobacterial compounds.
Experimental Section

General Experimental Procedures

Optical rotation measurements were recorded using a Jasco P1010 polarimeter. UV and IR spectra were obtained using a Beckman-Coulter DU-800 spectrophotometer and a Nicolet IR-100 FT-IR spectrophotometer, respectively. NMR spectra were obtained using Varian Unity 300, 500 and 800 MHz spectrometers. CDCl$_3$ ($\delta_H = 7.26; \delta_C = 77.0$) was used as an internal reference. High resolution mass spectra were obtained using an Agilent ESI-TOF mass spectrometer. Extracts were processed using a HPLC Waters 515 pump, Waters 996 photodiode array detector, and Millenium software for acquisition and analysis of data. All solvents used were either distilled or of HPLC quality.

Collection

The *Lyngbya majuscula* sample (PNG-04/22/06-2) yielding compound 1 was obtained in April 2006 at a depth of 3-9 m by snorkel near Dudawali Bay in Papua New Guinea (10° 17.274’ S and 151° 00.390’ E). The *Lyngbya majuscula* sample (PNG-12/16/03-8) yielding compound 2 was collected in December 2003 by snorkel at a depth of 0.5 to 3 m near Naopoi Island in Papua New Guinea (09° 14.109’ S and 150° 47.158’ E). The specimens, measuring 8 L and 7 L in total biomass, respectively, were stored in 70% EtOH at -20 °C until extraction.

Extraction and Isolation

The *Lyngbya majuscula* collection yielding (1) was extracted six times with heat using CH$_2$Cl$_2$:MeOH (2:1) and yielded an organic extract of mass 1.99 g. This extract was further processed on silica gel using vacuum liquid chromatography with a 100%
hexanes-EtOAC-MeOH gradient to generate nine subfractions A-I. Fraction G eluted with 100% EtOAc and demonstrated the most potent cytotoxic activity in the H-460 lung carcinoma cell line. Fraction G (46.8 mg) and H (177.6 mg) were subsequently filtered using a Waters RP C18 SPE cartridge with 100% MeOH and further processed using reversed-phase HPLC. Dudawalamide E (2.1 mg) and the known compounds majusculamide A and B (9.0 mg) eluted with 80:20 MeOH/H2O (Phenomenex Jupiter, 10 µm, 250 x 10 mm). Fraction H also yielded the pure known compound majusculamide D (1.3 mg) (C18 Synergi Fusion, 4 µm, 250 x 4.6 mm; 70:30 CH3CN/H2O).

The *Lyngbya* majuscula collection yielding (2) was extracted eight times with heat [CH2Cl2:MeOH (2:1)] to obtain an organic extract of mass 13.5 g. The extract (13.5 g) was further processed on silica gel using vacuum liquid chromatography in identical fashion as above to generate nine subfractions A-I. Fraction H (629 mg) eluted with 75/25 EtOAc:MeOH and harbored the most potent cytotoxic activity. This fraction was subsequently gradient filtered with 25-100% MeOH:H2O using C18 SPE to yield seven subfractions. Subfraction 4 (80% MeOH:H2O; 143 mg) was further purified (Phenomenex Hydro, 10 µm, 250 x 10 mm; 50-70% CH3CN/H2O) to yield pure naopopeptin (30.8 mg) and the known compound lyngbic acid (31.6 mg).

**Dudawalamide E (1)**: pale yellow oil; [α]D -140.5 (c 0.2, MeOH); UV (MeOH) λmax (log ε) 203 (4.44), 256 (2.89); IR (neat) νmax 3405, 2965, 2932, 2878, 1731, 1654, 1512, 1468, 1371, 1280, 1195, 1094, 1031, 752, 702 cm⁻¹; 1H NMR (CDCl3, 500 MHz) and 13C NMR (CDCl3, 75 MHz) data, see Table 1; (+)-HRESIMS [M+H]+ m/z 794.4683 (calcd for C43H64N5O9, 794.4704).
Naopopeptin (2): pale yellow oil; $[\alpha]_D$ -4 (c 0.05, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 200 (4.08); IR (neat) $\nu_{max}$ 3278, 2960, 2926, 2856, 2363, 1737, 1650, 1457, 1374, 1263, 1184, 1126, 1080, 669 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 500 MHz) and $^{13}$C NMR (CDCl$_3$, 75 MHz) data, see Table 1; (+)-HRESIMS [M+H]$^+$ m/z 737.4495 (calcd for C$_{41}$H$_{61}$N$_4$O$_8$, 737.4489).

**Hydrogenation**

0.2 mg of (1) and 0.4 mg of (2) were dissolved in 300 µl EtOH and 10% Pd/C catalyst was added in inert argon gas. Argon was evacuated, followed by addition of H$_2$ gas, and the solution was stirred for 3-4 hours at room temperature. The reaction product was filtered with a Nalgene syringe filter (2 µm, 13 mm) and dried under N$_2$ gas.

**Acid Hydrolysis and chiral HPLC**

Hydrogenated 1 and 2 were dissolved in 500 µl 6N HCl in sealed tubes. N$_2$ gas was added, and suspensions were heated at 105°C for 16 hours. The reaction products were dried under N$_2$ gas, resuspended in MeOH, and aliquoted for further analysis. The stereochemistry of the Pro residue in 1 was analyzed by chiral HPLC (Chirex 3126, 4.6 x 50 mm) using isocratic conditions [2mM CuSO$_4$: MeCN (95:5), 0.8 ml/min] and matched the retention time ($t_R$, min) of L-Pro (8.1; D-Pro, 11.8). The Hiv residue in 1 was analyzed in a similar fashion but using a different chromatographic method [2mM CuSO$_4$: MeCN (85:15), 0.8 ml/min] and yielded L-Hiv (16.6; D-Hiv, 24.2). The stereochemistry of the Pla residue in 2 was analyzed by chiral HPLC (Chirex 3126, 4.6 x 50 mm) using isocratic conditions [2mM CuSO$_4$: MeCN (85:15), 1.0 ml/min] and yielded L-Pla (17.7; D-Pla, 23.2).
Modified Marfey’s Analysis

Acid hydrolysates of 1 and 2 were derivatized with FDVA (0.1% in acetone and 0.1 M NaHCO₃) and heated at 90°C in sealed vials for 5 minutes (min). The reaction was quenched with 50 µl 1N HCl and diluted with 100 µl MeCN. Amino acid standards (1.0 mg) were derivatized in the same manner. The N-Me-Phe and Pro residues of 1 as well as the Val, N-Me-Val, and Pro residues of 2 were analyzed by RP HPLC [HP LiChrospher 100 RP-18 (5 µm, 4 x 125 mm); 0.8 ml/min, UV detection at 340 nm] using a linear gradient (30/70 to 70/30 MeCN/H₂O/0.1% HCOOH in 60 min). The retention times (tᵣ, min) of the derivatized residues in 1 matched N-Me-L-Phe (18.5; N-Me-D-Phe, 20.1) and L-Pro (8.1; D-Pro, 10.7) which confirmed the result obtained by chiral HPLC.

The derivatized residues of 2 matched the retention times of L-Val (34.4; D-Val, 51.72), N-Me-L-Val (15.73; N-Me-D-Val, 21.04), and L-Pro (8.1; D-Pro, 10.7). The stereochemistry of the N-Me-Ile residue in 2 was analyzed in a similar manner but using a different stationary phase (Symmetry C18, 4.6 mm x 250 mm; 0.8 ml/min, UV detection at 340 nm) and a different linear gradient (100% 50 mM TEAP to 50/50 TEAP: MeCN in 40 min; hold at 50/50 TEAP: MeCN for 20 min). The retention time of the N-Me-Ile residue in 2 matched N-Me-L-Ile (49.4; N-Me-D-Ile, 56.7; N-Me-D-allo-Ile, 57.2; N-Me-L-allo-Ile, 49.7).

Chiral GCMS

The dried hydrolysate of (1) was resuspended in 500 µl isopropanol and 83 µl acetyl chloride and heated in a sealed tube at 100°C for 45 min. The excess reagent was evaporated under N₂ gas. The dried products were treated with 1:1 CH₂Cl₂/(CF₃CF₂CO₂)O and heated at 100°C for 15 min. Reaction products were dried
under N\textsubscript{2} and extracted with CH\textsubscript{2}Cl\textsubscript{2}/H\textsubscript{2}O and a few drops of 1N HCl. The aqueous layer was removed and the organic layer dried under N\textsubscript{2}. Reaction products were dissolved in CH\textsubscript{2}Cl\textsubscript{2} and analyzed by chiral GCMS (Chirasil-Val, 0.25 µm, 25 m x 0.25 mm) using a step wise gradient [50°C (hold 5 min) to 110°C (2°C/min), hold 10 min]. The residues of 1 matched the retention times of L-Val (17.8; D-Val, 17.3) and L-Ile (22.0; N-Me-D-Ile, 21.3; N-Me-D-allo-Ile, 20.8; N-Me-L-allo-Ile, 21.4).

**DHOYA Stereochemical Analysis**

The hydrolysate of 1 was dissolved in MeOH to which a small volume of diazomethane (in ether) was added. The reaction mixture was dried with N\textsubscript{2} and extracted with CH\textsubscript{2}Cl\textsubscript{2}/H\textsubscript{2}O and a few drops of 1N HCl. The aqueous layer was removed and the organic layer dried under N\textsubscript{2}. Reaction products were dissolved in CH\textsubscript{2}Cl\textsubscript{2} and analyzed by chiral GCMS (J &W Scientific Cyclosil C, 0.25 µm, 30 mm x 0.250 mm) using a stepwise gradient [40°C to 100°C (20°C/min), hold 5 min, 100°C to 110°C (5°C/min)]. DHOAA synthetic standards 12 (3.0 mg) were methyl-esterified in the same manner as above. The DHOAA residue of 1 matched the retention time (t\textsubscript{R}, min) of S-DHOAA (51.5; R-DHOAA, 52.8).

**Biological Activity**

Dudawalamide E (1), naopopeptin (2), and majusculamide D were all tested in the H-460 cytotoxicity assay and the Neuro-2A sodium channel activation and blocking assays using methods previously described.13,14 Dudawalamides A-E and naopopeptin were submitted for further testing in malaria,15 leishmania,16 and Chagas17,18 anti-parasitic assays where greatest biologic activity was observed (Table 2).
Supporting Information Available: $^1$H NMR, $^{13}$C NMR, COSY, HMBC, HSQC and TOCSY spectra in CDCl$_3$ for dudawalamide E (1) and naopopeptin (2).
Figure 1. Dudawalamide A (1) and naopopeptin (2), two new cyclic depsipeptides containing DHOYA and HMOYA, respectively.
Table 1. $^1$H and $^{13}$C NMR assignments for dudawalamide E (1) and naopopeptin (2) in CDCl$_3$.

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$^a$ 500 MHz for $^1$H NMR, HMBC, and ROE

$^b$ 75 MHz for $^{13}$C NMR
Figure 2. Dudawalamides A-D, anti-parasitic depsipeptides from *Moorea* sp. Dudawalamide A (3). Dudawalamide B (4). Dudawalamide C (5). Dudawalamide D (6).
**Figure 3.** Partial structures of dudawalamide E (1) with relevant COSY and HMBC correlations.

**Figure 4.** Partial structures of naopopeptin (2) with relevant COSY and HMBC correlations.
Table 2. Anti-parasitic activity (IC$_{50}$’s) of dudawalamides A-E (1) and naopopeptin (2).

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Table 3. Sequences and biological activity of DHOYA-containing cyanobacterial metabolites or derivatives thereof. 
Hmp = 2-Hydroxy-3-Methyl-pentanoic acid (= isoleucic acid); Hiva = 2-Hydroxy-Isovaleric acid; Pla = 3-Phenyl-lactic acid; IA= inactive

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Table 4. Structure-activity relationships (SAR) of DHOYA-containing depsipeptides.

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References


Acknowledgements

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The text of V, in part, is to be submitted to an academic journal as it will appear: Malloy, K. L, Choi, H., Engene, N., Spadafora, C., Matainaho, T., Gerwick, W. H., Gutierrez, M., Dudawalamides A-E, Anti-parasitic Depsipeptides from the new genus *Moorea*. The dissertation author was the primary author and directed and supervised the research which forms the basis for this chapter.
Supporting Information

Figure 5. $^1$H NMR spectrum of 1 (500 MHz, CDCl$_3$)
Figure 6. $^1$H NMR spectrum of 2 (500 MHz, CDCl$_3$)
Figure 7. $^{13}$C NMR spectrum of 1 (75 MHz, CDCl$_3$)
Figure 8. $^{13}$C NMR spectrum of 2 (75 MHz, CDCl$_3$)
Figure 9. COSY spectrum of 1 (CDCl$_3$)
Figure 10. COSY spectrum of 2 (CDCl$_3$)
Figure 11. HMBC spectrum of 1 (CDCl$_3$)
Figure 12. HMBC spectrum of 2 (CDCl$_3$)
Figure 13. HSQC spectrum of 1 (CDCl$_3$)
Figure 14. HSQC spectrum of 2 (CDCl$_3$)
Figure 15. TOCSY spectrum of 1 (CDCl$_3$)
Figure 16. TOCSY spectrum of 2 (CDCl$_3$)
Figure 5. $^1$H NMR spectrum of 1 (500 MHz, CDCl3)
Figure 14. gHSQC spectrum of 2 (500 MHz, CDCl3)
Chapter VI.

Conclusion
This dissertation research has successfully resulted in the isolation, structure elucidation, and biological evaluation of new cyanobacterial marine natural products, as well as the re-isolation of several known natural products. The isolation and structure elucidation of malyngamide 2 (1), a linear lipopeptide with a highly oxidized cyclohexane ring, further expands the large class of vinyl chloride-containing malyngamides. Malyngamide 2’s ability to inhibit nitric oxide formation is in accordance with the reported anti-inflammatory activity of other malyngamides, including malyngamide F and malyngamide F acetate. \(^1\) Interesting SAR features are emerging in the malyngamides, such as the apparent requirement of a C-6 hydroxyl or acetoxyl group for anti-inflammatory activity. Malyngamide 2 was also screened for cytotoxicity and sodium channel blocking and activation activity; however, it showed minimal activity in those assays.

Hoiamide D (2) was also isolated and characterized in this thesis research, and is a PKS-NRPS derived lipopeptide featuring a triheterocyclic system, a modified isoleucine, and a highly oxidized and methylated PKS extension. Hoiamide D inhibits the p53/MDM2 interaction, an attractive anti-cancer target for drug development. To our knowledge, it is the second peptide-derived natural product inhibitor and one of the most potent natural product inhibitors. Hoiamide D was also screened for sodium channel blocking and activation activity as well as anti-malarial activity. It showed minimal biological activity in these latter three assays.
Figure 1. Malyngamide 2 (1), an anti-inflammatory linear lipopeptide, and hoiamide D (2), a p53/MDM2 inhibitor.

The isolation and structure elucidation of credneramides A and B (3, 4) neuromodulatory phenethylamine and isopentylamine vinyl chloride-containing fatty acids, were also a focus of this thesis research. Isolation of credneric acid (5) from the heterotrophic bacterium *R. baltica* and reisolation from collections of cf. *Trichodesmium* nov. sp. and *Oscillatoria* sp. broached provocative questions concerning the evolution and metabolic origin of this compound. The biogenesis of credneric acid is predicted to arise from cyanobacterial metabolism based upon the following reasons: 1) the requisite HMGCoA cassette needed for vinyl chloride formation has been well characterized in cyanobacteria, 2) *R. baltica* 16S rRNA could not be amplified from the environmental sample of cyanobacteria, 3) another well known cyanobacterial metabolite was isolated from the environmental *R. baltica* collection, and 4) scanning of the published genome of another strain of *R. baltica* failed to locate the genes predicted to be involved in credneric
acid biosynthesis. Credneramides A and B are low micromolar inhibitors of calcium oscillations in murine cortical neurons and may serve a physiologic role in signaling based upon precedence from other fatty acid amides. These compounds were also screened for sodium channel activation and blocking activity, cannabinoid receptor binding, and anti-parasitic activity, but were not active in any of these latter assays.

![Chemical structures of Credneramides A and B, and Credneric acid](image)

**Figure 2.** Credneramides A and B (3, 4) and credneric acid (5), neuromodulatory vinyl chloride-containing fatty acids.

Lastly, the isolation and structure elucidation of dudawalamide E (6), a low micromolar *Leishmania donovani* inhibitor and naopopeptin (7), a low micromolar inhibitor of *Plasmodium falciparum*, were detailed in this thesis. These are PKS-NRPS derived cyclic peptides featuring DHOYA and HMOYA residues, respectively. Dudawalamide E expands a growing class of cyclic peptides containing the unique DHOYA moiety, and interesting SAR features are emerging in this class as it regards their biological activity.
**Figure 3.** Dudawalamide E (6) and naopopeptin (7), two new DHOYA and HMOYA-containing cyclic lipopeptides with anti-parasitic activity.

This dissertation research has further delineated new and novel chemistry of marine cyanobacteria. Ultimately, the chemical space represented by cyanobacterial marine natural products has been expanded, and the biologic space represented by these metabolites has been preliminarily characterized. Enormous opportunities abound for further characterization of both the chemical and biological space of marine natural products as the field quickly moves to incorporate emerging technological advances.

With advances in molecular and analytical techniques, one can envision a number of breakthroughs in the near future for the various disciplines of marine natural products chemistry. Outlined below are some of my perspectives on future directions within this field.

**Natural Products Isolation and Structure Elucidation**

As it pertains to analytical instrumentation, NMR magnets of 900 MHz and greater are already in use at some research institutions. With increase of magnet strengths
to 23.5 Tesla (1000 MHz) and greater, the sample size needed to acquire adequately resolved spectra is becoming infinitesimally small. Structure elucidation on microgram amounts of material will not be the exception but the rule when these magnets are heavily utilized at academic institutions. Hybrid LC-MS-NMR and LC-MS-IR machines will also continue to be of interest because of their workflow efficiency; although, this technology remains largely cost prohibitive and, as a result, may or may not gain a foothold in academic laboratories. Mass spectrometry is expected to assume an even greater role in structure elucidation as knowledge and cataloguing of fragmentation patterns grow, and may even supplant certain NMR experiments for use in elucidation of purely NRPS-derived cyclic peptides.  

Further, increasing portability of these machines will allow researchers to take science directly into the field. The development of a handheld, lightweight, permanent magnet weighing 500 g and capable of generating high resolution spectra was recently reported and can accommodate a standard NMR sample tube.  Mass spectrometry miniaturization has been around for some time, but development of a handheld mass spectrometer with ambient pressure ionization was reported in 2007. IR technology is already portable, and portable gradient HPLC instruments have been on the market since 1998. The idealized end point with use of these combined portable technologies is a more rational, conservation directed approach to field collections in which less biomass would be harvested in exchange for increased sample turnover and increased chances of accessing novel chemistry.
Phylogenetics

Phylogenetic studies have recently demonstrated polyphyly of the marine *Lyngbya* lineage, meriting the need for genus revision. Consequently, a new genus *Moorea* has recently been proposed (N. Engene, personal communication). As other genera of the order Oscillatoriales are scrutinized, further revision is to be expected as well. The reality that genotypic diversity exceeds phenotypic diversity within the *Lyngbya* marine lineage will require a supporting or even central role for phylogenetic based identification systems for species and sub-species resolution. Further, intragenomic 16S rRNA gene heterogeneity will necessitate a more conserved gene for microscale phylogenetic resolution. The rpoC1 gene, present in single copies and shown to have five-fold greater intrastrain sequence variation than that of ribosomal genes, has been proposed as a putative replacement, and perhaps, will become the recognized standard in the future.

Natural Products Biosynthesis

As regards marine natural product biosynthesis, cutting edge molecular biology and genomic techniques are quickly advancing the frontiers of this discipline. Multiple displacement amplification (MDA) and single cell genome sequencing is currently in vogue and will become more routine. The anticipated result will be faster, more efficient sequencing of biosynthetic gene clusters. Additionally, as barriers to heterologous expression are overcome, one can envision the reality of heterologously expressing complete cyanobacterial biosynthetic gene clusters to increase yields of a given compound of interest. Heterologous expression of a cyanobacterial lyngbyatoxin tailoring gene, LtxC, was recently demonstrated in *S. coelicolor* and resulted in
production of lyngbyatoxin A (A. Jones, personal communication). As such, one can also envision genetic manipulation of a heterologously expressed gene cluster, including enzyme tailoring, deletions, additions, or even accessing the host’s enzymatic machinery, to create biosynthetic analogs for medicinal chemistry and SAR studies.

With advances in this discipline, greater scrutiny is also expected regarding the true source of biosynthetic machinery in cases of symbiotic and epiphytic associations. MDA and single cell genome sequencing coupled with MALDI-TOF-MS have provided technological means to begin shedding light on this question. 6, 10

Marine Biotechnology

Marine biotechnology is expected to continually grow as a discipline within the field of marine natural products chemistry. For example, mounting political and economic pressure has pushed energy independence and “cleaner” energy practices to the fore. The expectation is that scientists within this discipline will continue to be well funded publically and privately in order to advance technology in algal biofuels research. Further elucidation of cultivation conditions that flip the “lipid trigger switch” is foreseen, including additional insight regarding nitrogen deprivation and silicate induced stress responses. 11 Moreover, additional studies are expected to shed more light on the enzymes responsible for lipid overproduction and how gene knockdown or upregulation affects lipid yield. 11

Interdisciplinarity

The field of marine natural products is also expected to become more interdisciplinary. One can envision bioinformatics forming a niche within the field when mapping of actual and theoretical chemical and biologic space is recognized as
paramount for a streamlined drug discovery process. ChemGPS \textsuperscript{12, 13} and other bioinformatics software programs are expected to be more routinely used to provide an improved rational approach to isolation, structure elucidation, and optimization of clinically relevant natural products. In addition, a translationally-oriented niche of scientists is expected to emerge in the field that will integrate basic science with clinical trials research and clinical medicine. As metabolomics and pharmacogenetics usher in an era of personalized medicine, it is foreseen that these scientists will utilize data regarding individual genetic polymorphisms not only to inform the search and optimization of clinically relevant compounds, \textsuperscript{14-16} but also, to carefully select clinical trial subject groups, and thus effect desired clinical trial outcomes.

In summary, the field of marine natural products is expected to experience considerable growth as it continues to accommodate emerging technologies and interdisciplinarity. Natural products drug development will undoubtedly benefit as a result. One can state with great confidence that the future of marine natural products chemistry is promising indeed.
References


