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Geographic and Host-Microbe Symbiotic Influence on Secondary Metabolism

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GEOGRAPHIC AND HOST-MICROBE SYMBIOTIC INFLUENCE ON SECONDARY METABOLISM

A Thesis
presented in partial fulfillment of requirements
for the degree of Master of Science
in the Department of Pharmacognosy
The University of Mississippi

By
SAMUEL H. ABBAS
August 2012
This thesis is composed of studies that regard the advancement to the Arctic region and the importance of host-microbe interactions in natural product discovery. Bioactive metabolites have been reported from a myriad of marine and terrestrial organisms around the world including plants, insects, sponges, tunicates, bacteria, and fungi among others. Many macroorganisms, of which the metabolites are found, depend on the symbiotic relationship of microorganisms for metabolite production. The scope of this work is to investigate secondary metabolism of both marine and terrestrial organisms from different areas of the world as well as search for the importance of host-microbe symbiosis on a chemical level.

Chapter 1 comprises a review of bioactive sponge secondary metabolites reported from Arctic sponge species. Although more tropical and easily accessible waters have been investigated, other regions including the more dark, cold polar regions, specifically the Arctic, represent a less explored frontier for secondary metabolite discovery. Additionally, a survey of a 2010 sponge collection in the Aleutian Islands, AK, is displayed and assesses the chemical potential of sponge species in the area. The description of two small molecule aldehydes with reported broad spectrum bioactivity from a new species of *Guitarra* obtained through this collection is also given.

Chapter 2 discusses the chemical investigation of a new sponge species of the genus *Monanchora* from the Aleutian Islands. Different species of *Monanchora*, including *M. pulchra*, have been classified within the region. Through de-replication, members of the class of potent cytotoxic, antimicrobial compounds known as the monanchocidins were identified from the
sponge. Further investigation of the metabolome and development of bioactivity will continue to be investigated in the future.

Studies have suggested that the chemical complexity of sponges is dependent upon not only the sponge itself, but rather an intricate associated microbial community. Chapter 3 presents the results of fermentations of sponge-associated *Micromonospora* sp. M42, yielding seven small molecule secondary metabolites, with four belonging to a class of broad spectrum bioactive molecules known as diketopiperazines. Previous research suggests that these compounds possess bioactivities that can benefit the sponge in a symbiotic relationship, including growth promoter and antifouling properties. Screening of the crude extracts revealed the presence of the diketopiperazines in the sponge. Additionally, a genomic evaluation of the biosynthetic machinery of M42 was performed. This data was generated in supplement of previously done work regarding the entire sponge-associated microbiome and previous confirmation of manzamine production by M42 as well as a series of biotranformation studies that add insight to the generation of the array of manzamine derivatives found in the sponge.

Chapter 4 discusses the metabolite nicotianamine, a compound found in all higher plant species that possesses antioxidant and metal-binding properties that can be helpful in use as a food preservative in replacement of EDTA. Preliminary data into the optimization of the isolation and quantification of nicotianamine on an analytical scale is presented.
DEDICATION

This work is dedicated to my family.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my family: my parents, brother, and sisters, for their continual support throughout my education and particularly my graduate tenure. I also want to acknowledge my advisor, Professor Mark Hamann, for his support and guidance during my masters research. Kraft Foods Global deserves appreciation and acknowledgment for their funding of my graduate education. Additionally, thanks are given to the members of my committee, Dr. Daneel Ferreira and Dr. Jordan Zjawiony. Special thanks are given to John Bowling for teaching me most laboratory techniques as well as providing guidance on my projects.

I give a special thanks to Dr. Michelle Kelly for contributing taxonomic information for the Alaskan sponge species discussed in this thesis. Dr. James Sims deserves many thanks for performing the sponge collection. Amanda Waters, Kamilla Alves, Serena Ellison, and Alexis Fullmer deserve recognition for their aid in the large scale screening of sponge extracts. I want to thank Joonseok Oh for his collaboration in developing the monanchocidin project.

I would like to thank Dr. Noer Kasanah for her work in the isolation of manzamine from Micromonospora sp. M42 as well as the manzamine biotransformation studies. I also want to thank Dr. Olivier Peraud for his work in assessing the microbial community of Acanthostrongylophora sp. and constructing the phylogenetic analysis. Thanks are given to Dr. Russell Hill and Dr. Matthew Anderson for their contribution to the project and provision of cultures of M42. I want to express my appreciation for Dr. Jon Clardy and co-workers for the
gene sequencing of M42. I am grateful for the aid of Dr. Sims in developing the genomic evaluation of M42.

Special thanks are given to members of our group for their contribution to the nicotainamine project. Yike Zou deserves credit for the nicotianamine modeling work regarding the chelating complex with iron. I am grateful for the help of Bin Wang in the production of the calibration curve and other analytical work regarding nicotianamine. Also, thanks are given to Chris James for his work in the isolation of nicotianamine from soy flour and research towards the introduction of nicotianamine.

I would like to thank the rest of the Hamann research group that has worked with me and helped further my knowledge and education, in particular John Bowling, Joonseok Oh, Amanda Waters, and Bin Wang. I would also like to express my appreciation for Casey Stauber for her user-friendly assistance whenever I had any technical issues to address. I want to give appreciation to Dr. Melissa Jacob for performing the opportunistic infection assays in my work and the National Cancer Institute for their cancer assay data. Finally, I give thanks to my friends and fellow colleagues in the School of Pharmacy that made my graduate tenure enjoyable.
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Marine sponges have provided a vast resource in the search for bioactive secondary metabolites and potential drug leads. With most research in the area of bioactive sponge metabolites being conducted in temperate and tropical areas and the strong emergence of a myriad of disease resistance, it is becoming increasingly important to survey the fauna of remote regions of the globe for new and replacement drug therapies or compounds with other useful bioactivities. Porifera of cold water environments, including the deep sea and northern polar regions in particular, are marginally known in terms of their faunas, and as such are a relatively untapped resource for scientific discovery. In fact, of all marine natural products described, less than 3% originate from organisms in polar environments. Among the research conducted in
polar environments, the majority of compounds isolated and characterized have come from the Antarctic region.\textsuperscript{1,2} This article discusses bioactive molecules that have been discovered in the southern reaches of the Arctic Circle and the particular significance of a recent sponge collection conducted in the Aleutian Islands, Alaska. The handful of studies that have been completed in this area are primarily represented by sponges collected in more easily accessible coastal waters of Alaska and off the coast of Sweden and Norway. A potentially fruitful opportunity for new bioactive marine secondary metabolites lies within the cold water regions of the earth, where little, but promising, research has been completed.

Articles addressing cold water bioactive metabolites and the chemical ecology of cold water sponges, particularly the Antarctic region, have been published by our group and others.\textsuperscript{1-4} These articles cover some of the compounds listed here and should be consulted for a recording of cold water marine metabolites from various organisms.\textsuperscript{4} This work focuses primarily on bioactive sponge metabolites from Alaska and other more northern Arctic cold water regions.

SECONDARY METABOLITES ISOLATED FROM ARCTIC SPONGES

There are many reasons for the lack of exploration of the metabolites of polar sponges, but the most obvious are the difficulties associated with the access to polar regions and the harsh working environments. Several decades ago the misconception was held that, due to the harshness of the general environment, sponge biodiversity in polar regions would be poor, and competitive pressure to develop chemical defenses were low or non-existent.\textsuperscript{1} However, today we know from work in the Antarctic and revised opinions of Arctic benthos that polar regions are rich in sponge diversity\textsuperscript{5-7} with comparable species numbers to southern cold-temperate regions\textsuperscript{8} and that polar marine invertebrates, including sponges, possess a high number of
chemical defenses. Despite the suggestions of high polar sponge biodiversity and thus a potential source for marine drug discovery, less than 100 metabolites from cold water sponges have been described as of 2010, as compared to thousands from temperate and tropical regions. Consequently, extensive sponge surveys in the polar environments for the discovery of new bioactive molecules have merit. The following information reviews the bioactive compounds that have been discovered from marine sponges collected in the southern-most limits of the Arctic Ocean.

**Discorhabdin Alkaloids**

One of the major groups of compounds that have been found in polar sponges of the genus *Latrunculia* (Class Demospongiae, Order Poecilosclerida, Family Latrunculiidae) are the discorhabdin alkaloids. Many different derivatives of discorhabdins were originally isolated from Antarctic and New Zealand species of *Latrunculia* yielding an impressive array of activities. Discorhabdin C (1) (Figure I.1) was first described in 1986 as isolated from the New Zealand species *L. cf bocagei* Ridley and Dendy, 1886, and exhibited potent antitumor bioactivity against L1210 tumor cells with an ED$_{50}$ of less than 100 ng/mL. Discorhabdin F$^{12}$ was subsequently isolated in 1990 from the Antarctic species *L. biformis* Kirkpatrick, 1907 followed by discorhabdin G from *L. apicalis* Ridley and Dendy, 1886.$^{13, 14}$ Discorhabdin R (2) (Figure I.1) was isolated in 2000 from an unidentified species of *Latrunculia* collected from Prydz Bay, Antarctica, exhibiting activity similar to other discorhabdins encompassing Gram positive and Gram negative species of bacteria among others.$^{15}$
Discorhabdin C (1) and discorhabdin R (2); members of the discorhabdin alkaloids, exhibiting a spectrum of bioactivities, isolated in 1986 and 2000, respectively, from New Zealand and Antarctic sponge species of the genus *Latrunculia*.\textsuperscript{11,15}

In 2009, eight members of the same class of discorhabdin alkaloids, including the new dihydrodiscorhabdin B (3) and discorhabdin Y (4), were isolated from members of *Latrunculia* in the Arctic region off the coast of Alaska in the Aleutian Islands (Figure I.2).\textsuperscript{4} This was the first report of bioactive compounds from sponges collected in the Alaskan region. The compounds isolated from this new, undescribed species of *Latrunculia*, demonstrated anti-HCV, antimalarial, and antibacterial activities along with two previously described discorhabdins, dihydrodiscorhabdin C (5) and discorhabdin A (6), showing selective anti/protozoal activity in vitro.\textsuperscript{4} From this report, compounds 5 and 6, along with compound 1, had reported IC\textsubscript{50} values of 170, 53, and 2800 nM against chloroquine-susceptible *Plasmodium falciparum* and 130, 53, and 2000 nM against chloroquine-resistant *P. falciparum*, respectively. Compounds 5 and 6 were tested in vivo using a murine model for antimalarial activity, however, high levels of toxicity were observed including weight loss, movement reduction, and dehydration. Regardless of these in vivo results, the first report of bioactive compounds from an Alaskan sponge being used in an animal model provides promise for future bioactive molecules from the region. Reports suggest that the discorhabdin alkaloids have potential due to their varied biological activities, and new
information on the group and the species they have been isolated from\textsuperscript{16-19} have been discovered during the past 25 years.

![Compounds isolated from a new, undescribed species of Latrunculia from the Aleutian Islands.](image)

**Figure I.2.** Compounds isolated from a new, undescribed species of *Latrunculia* from the Aleutian Islands. The compounds listed are dihydrodiscorhabdin B (configuration unassigned) (3), discorhabdin Y (4), dihydrodiscorhabdin C (5), discorhabdin A (6), discorhabdin E (7), discorhabdin L (8), and also discorhabdin C (1, Figure I.1).\textsuperscript{4}

**Monanchocidins**

A group of new polycyclic guanidine containing alkaloids were discovered from *Monanchora pulchra* in 2010 and 2011\textsuperscript{20,21} near Urup Island in the southern Sea of Okhotsk at a similar latitude to the discorhabdins in Figure I.2. Monanchocidins A–E (9–13) (Figure I.3) demonstrated apoptosis-inducing activity against HL-60 human leukemia cells at 540, 200, 110, 830, and 650 nM respectively.\textsuperscript{21}
3-Alkylpyridinium Alkaloids

A series of 3-alkylpyridinium alkaloids have been isolated from the Arctic sponge

*Haliclona (Rhizoniera) viscosa* (Topsent, 1888) (Class Demospongiae, Order Haplosclerida, Family Chalinidae) with interesting bioactivity including antibacterial, antifungal, cytotoxic, and feeding deterrent effects. Until 2004 *Haliclona* spp. and related genera had only been described from more tropical and temperate environments where they are more abundant and diverse in terms of species. Specimens of *H. viscosa* were collected in Kongsfjorden, an inlet on the west coast of Spitsbergen, an island which is part of the Svalbard Archipelago in the Arctic Ocean. The specimens yielded two compounds elucidated as viscosamine (14) and viscosaline (15) (Figure I.4), the first acyclic dimeric 3-alkylpyridinium alkaloid isolated from nature. Both compounds exhibit antibacterial activity while viscosaline has been reported as a feeding deterrent against the amphipod *Anonyx nugax* and starfish.
Figure I.4. Bioactive compounds viscosamine (14) and viscosaline (15) isolated from the Arctic sponge *Haliclona viscosa*.  

Diketopiperazines

Two diketopiperazines, barettin (16) (Figure I.5) and 8,9-dihydrobarettin were isolated from the sponge *Geodia barretti* Bowerbank, 1858 (Class Demospongiae, Order Astrophorida, Family Geodiidae) in the North Sea off the coast of Sweden. These compounds exhibited extremely interesting non-toxic bioactivity as an antifouling agent, inhibiting the settlement of larvae of the barnacle *Balanus improvisus* and the blue mussel *Mytilus edulis* when mixed with surface coatings.

Figure I.5. Barettin (16), isolated from the sponge *Geodia barretti* collected in the North Sea.
Polymastiamides

Polymastiamide A (17) (Figure I.6), the first reported marine natural product derived from a steroid and α-amino acid component, was isolated from the Norwegian sponge Polymastia boletiformis (Lamarck, 1815) (Class Demospongiae, Order Hadromerida, Family Polymastiidae), and showed in vitro activity against the microorganisms Staph. aureus, Candida albicans, and Pythium ultimum. Conjugates with similar steroid/amino acid constitutions were subsequently isolated from the same sponge species to give polymastiamides B (18), C (20), D (21), E (19), and F (22) (Figure I.6).

Figure I.6. Polymastiamide A, B, E (17, 18, 19) and C, D, F (20, 21, 22) isolated from the Norwegian sponge Polymastia boletiformis.

Cyclic Peroxides

Another Norwegian sponge, Plakortis simplex Schulze, 1880 (Class Demospongiae, Order Homosclerophorida, Family Plakiniidae), yielded two new cyclic peroxides (23, 24) (Figure I.7) with 24 showing in vitro IC₅₀ values between 7 and 15 µg/mL for a number of different solid human tumor cell lines.
Two recent collection trips were performed in collaboration with the NOAA/AFSC annual groundfish survey. Collection sites in the summer of 2010 were in the western Aleutian Islands between Adak Island and Stalemate Bank. The depths of these collections ranged 60–400 m. Specimens were collected by fishing trawl and were sorted by morphology and immediately frozen at −20 °C. Following the survey samples were shipped frozen for extraction and chemical analysis.

**Taxonomic Overview of the Region**

Our understanding of the sponge fauna of the Aleutian Islands and the Alaskan Arctic is aided by taxonomic literature based on collections principally from the Chukchi and East Siberian Seas, the Aleutian Island chain and Alaskan coastline to the north, Kamchatka Peninsula, Kurile Islands, the Sea of Okhotsk to the west, and the Bering Sea to the north. However, unlike the Antarctic region, few large scale collections and taxonomic studies of the overall sponge fauna of this region have been produced, and many reports are old and in need of revision. A full review of the species known from the region has not been available, but we
know from recent studies\textsuperscript{29,30} and collections that the sponge/demosponge fauna, at least, is
diverse and dominated by poecilosclerid taxa.

General literature for the Bering Sea and Alaskan Arctic includes references\textsuperscript{31-34} for the
southwestern Sea of Okhotsk and reference\textsuperscript{35} for the northwest Pacific in general. More recently,
Lehnert \textit{et al.} \textsuperscript{28} (and in previous reports), commenced the documentation of a number of deep
and shallow water sponges from the region, many of which were found to be new species.

Taxonomic literature from the Gulf of Alaska and British Columbia to the southeast of the
Aleutians, and to a lesser extent, the coasts of Washington, Oregon, and northern California, are
important resources for any work in the Aleutians and Alaskan Arctic. Lambe,\textsuperscript{36-38} more recently
Austin,\textsuperscript{29} and Austin and Ott\textsuperscript{30} provide the best starting points for any taxonomic work in the
region. Lee \textit{et al.} \textsuperscript{39} on the sponges of California is also particularly relevant.

\textbf{New Species Found from the Collection}

Of the 93 sponge specimens collected during the 2010 voyage, six have been formally
identified and two are new, undescribed species. Five out of the six specimens are in the Order
Poecilosclerida, the most diverse order of demosponges, and typically the most common type of
demosponge found in polar regions.\textsuperscript{6} The sixth specimen is from the Order Astrophorida, an
order of Demospongiae much less common in polar regions than in temperate regions such as
around New Zealand.\textsuperscript{6,8}

Of the five poecilosclerid sponges, two were species of \textit{Latrunculia} (Family
Latrunculiidae); \textit{L. oparinae} (Samaai and Krasokhin, 2002) and the new species detailed in
reference 4. This genus has presented a major group of compounds, the discorhabdin alkaloids,
with a striking array of activities as detailed above. Research on this important group has focused
on Antarctic and New Zealand species, but these current explorations are documenting their existence in the North Pacific and southern Arctic regions. Prior to this work, *L. oparinae* was known only from the Russian Kurile Islands, in the Sea of Okhotsk, between depths of 127 and 238 m. In the Aleutian Islands, the species is quite common where it is found between depths of 81 and 288 m. The species has a globular shape with tall cylindrical oscules (exhalent structures), and is light olive to khaki green in life. The new, and as yet, undescribed species detailed in reference 4 is similar to *L. oparinae* but differentiated by color in life. It is dark purple brown, possesses the short flat oscules, and the perfectly hemispherical shape. *L. austini* (Samaai, Gibbons and Kelly, 2006) known from the Vancouver Coast of British Columbia, Canada, and further south, is a relatively shallow water species (20–50 m), grayish brown in life, spherical in shape, and with broad crater-like areolate porefields that dominate the sponge surface. *Latrunculia velera* (Lehnert, Stone and Heimler, 2006) from the Aleutian Islands, is a dark brown elongate to subglobose sponge with short tiny oscules. The primary feature that differentiates each of these species is the shape and ornamentation of the family-specific microsclere, the anisodiscorhabd (Figure I.8). *L. occulta* (Lehnert, Stone and Heimler, 2006) also from the Aleutian Islands, is now considered to be a species of the genus *Chondrocladia* (*Meliiderma*) (Order Poecilosclerida, Family Cladorhizidae). While these species produce an array of different compounds, they are difficult to differentiate at the species level other than by the shape and ornamentation of the family-specific microsclere, the anisodiscorhabd (Figure I.8).
Of the five poecilosclerid sponges, two were species of *Monanchora* (Family Crambeidae); *M. pulchra* (Lambe, 1895), first described from the Aleutian Islands, and a new, undescribed species, *M. n. sp. 1* (yellow fan). There are two additional species of this genus known from the Aleutian Islands, Eastern Bering Sea, and the Gulf of Alaska; *M. alaskensis* (Lambe, 1895) and *M. laminachela* (Lehnert, Stone, and Heimler, 2006). The key differences between these four species are in the gross morphology of the sponge, the coloration life, the length of the skeleton-forming megascleres, and the length and shape of the microscleres (Table I.1).
Table I.1. Key differences between species of *Monanchora* (Order Poecilosclerida, Family Crambeidae) in the Aleutians-Arctic region.

<table>
<thead>
<tr>
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<th><em>Monanchora</em></th>
<th><em>Monanchora</em> n. sp.</th>
<th><em>Monanchora</em></th>
<th><em>Monanchora</em> laminachela</th>
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<tr>
<td><em>Monanchora</em> pulchra</td>
<td>ramose fan</td>
<td>fan</td>
<td>short flabby</td>
<td>subglobular</td>
</tr>
<tr>
<td><em>Monanchora</em> alaskensis</td>
<td>fan</td>
<td>yellow</td>
<td>brown</td>
<td>yellow</td>
</tr>
<tr>
<td>shape</td>
<td>1 (yellow fan)</td>
<td>1 (yellow fan)</td>
<td>1 (yellow fan)</td>
<td>1 (yellow fan)</td>
</tr>
<tr>
<td>color in life</td>
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<td>yellow</td>
<td>brown</td>
<td>yellow</td>
</tr>
<tr>
<td>styles (interior) (µm)</td>
<td>1100</td>
<td>480–510</td>
<td>262</td>
<td>840–1170</td>
</tr>
<tr>
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<td>200–250</td>
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<td>350–395</td>
</tr>
<tr>
<td>microscleres 1 (µm)</td>
<td>19</td>
<td>30–35</td>
<td>91</td>
<td>22–25</td>
</tr>
<tr>
<td>microscleres 2 (µm)</td>
<td>13</td>
<td>20</td>
<td>32</td>
<td>19–23</td>
</tr>
</tbody>
</table>

The single astrophorid species is *Poecillastra rickettsi* de Laubenfels, 1930 (Family Pachastrellidae), first described from the northern Californian coast.

**PROCESSING OF COLLECTED ARCTIC SPONGE SAMPLES**

Each of the 93 sponge samples were initially processed by extracting 50 g portions (wet) with ethanol. The resulting extract (5 mg of each sample) was submitted for opportunistic infections, antimalarial, and anti-hepatitis C virus assays. For more rapid results, each extract was screened using a disc diffusion assay against *Bacillus cereus*. 
**Disc Diffusion Assay**

Extracts were tested with kanamycin used as a positive control. Extracts were applied to the disc using methanol to give a total of 1 mg of extract on each disc. A total of 100 µg of kanamycin was applied to the control disc. A bacterial lawn of *Bacillus cereus* was created by spreading 250 µL of water containing live cells. The discs were then placed upon the media and allowed to incubate for 24 hours before examining for zones of inhibition.

**In vitro Bioactivity**

An initial *in vitro* screen of 93 crude sponge extracts revealed bioactivity against many microorganisms associated with opportunistic infections (Figure I.9), indicating a broader than expected activity against a small sample set of different microorganisms. The extracts were also screened against *Plasmodium falciparum* (malaria) and HCV resulting in the identification of several samples with significant activity.

![Graph showing bioactivity](image)

Figure I.9. The percent of crude extracts of 93 Alaskan sponge samples that yielded significant activity (greater than 50% inhibition in vitro) against different opportunistic infections (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*), malaria, and hepatitis C virus (HCV)(methods described in reference 4).
The IC\textsubscript{50} values for active sponge extracts ranged from 200 to 5000 ng/mL against the different opportunistic infections (ciprofloxacin and amphotericin B as controls) and antimalarial IC\textsubscript{50} ranged between 500 and 2200 ng/mL (chloroquine and artemisinin as controls); anti-HCV IC\textsubscript{50} values were not determined. Many of the active samples are currently being examined for their active components and will be reported in due time. The initial screen for activity combined with the evidence of interesting taxonomic classification highlights the potential of the polar region for new bioactive compounds.

**CONCLUSIONS**

With roughly 70% of the earth covered with ocean, marine sources provide an enormous source for scientific research and discovery. Numerous collections conducted for over half a century to collect sponges in particular for their bioactive secondary metabolites have been carried out in many cold temperate, temperate, tropical, and subtropical locations across the Atlantic and Pacific Oceans and in other parts of the globe. These efforts and their results are described in a series of reviews.\textsuperscript{41} By contrast, dedicated collections of sponges to specifically isolate and characterize metabolites from sponges in the Arctic and Alaskan regions have been few and far between as illustrated in Figure I.10. Yet, the biodiversity of the Gulf of Alaska and British Columbia in particular, is known to be considerable. The discovery of new species with diverse bioactivity as indicated by the initial screen of the 2010 collection of Aleutian sponges and previous collections made by our group in the general area bodes well for the future of new and valuable bioactive compounds.
ISOLATION EFFORTS FOR NEW SPECIES OF *GUITARRA*

**Taxonomic Overview**

A new undescribed species of *Guitarra* (Order Poecilosclerida, Family Guitarridae) was amongst the six poecilosclerid sponges identified and was further investigated for bioactive components. The specimen was dredged from a depth of 94 m from the Aleutian Islands, where it was moderately common. In life the sponge forms a massive encrustation with deep cracks outlining polygonal “plates”. The texture is tough, the color in life is peach to orange-yellow. A photograph of the sponge outside of water can be seen in Figure I.11. The sponge is characterized by long megascleres (oxeas 580–600 µm long) and three forms of microscleres:

- **A. Discorhabdins (3-8)**; Aleutian Islands collection
- **B. Monanchidins (9-13)**
- **C. Pyridinium alkaloids (14,15)**
- **D. Diketopiperazines (16)**
- **E. Polymastiamides (17-22)**
- **F. Cyclic peroxides (23,24)**
(biplochelae, c. 47 µm long, in addition to the usual placochelae, c. 47 µm long, and small, spiny bipocillae and anisobipocillae, c. 15 µm long). The most closely comparable species to *Guitarra* n. sp. is *G. abbotti* Lee, 1987, from the Cordell Bank, northern California. *Guitarra abbotti* also has the unusual biplochelae in addition to the usual oxeas, placochelae, and bipocillae microscleres, but these differ considerably in length from those in *Guitarra* n. sp. The oxeas of *G. abbotti* are half the length (330 µm) of those in *Guitarra* n. sp., and there are two sizes of placochelae in the former species, the larger of which is twice the length of those in *Guitarra* n. sp. (83 µm long) with the smaller size being 37 µm long. The biplochelae of *G. abbotti* are slightly smaller (36 µm) than those in *Guitarra* n. sp, and the bipocillae are half the length (7 µm) of those in the new species.

Figure I.11. An out-of-water photograph of the new species of the genus *Guitarra*.
Isolation Methods

The new species of Guitarra was examined because of initial activity from the disc diffusion assay against *B. cereus*. The complete 1 kg (wet wt.) specimen was extracted with ethyl acetate to yield 11 g of crude material. The resulting extract was subject to a silica flash column and the active material eluted from a fraction of 1:1 hexane and ethyl acetate. The resulting fraction (483 mg) was subject to separation using a 3 cm diameter LH20 column and a 1:1 mixture of dichloromethane and methanol. The activity was traced to a group of fractions that were combined and subjected to further separation on a Waters Delta HPLC (λ 280 nm) using a 4.6 × 150 mm Phenomenex Luna Silica column employing a linear gradient from 100% pentane to 100% dichloromethane over 75 min. The HPLC separation yielded sub-miligram amounts of two pure compounds that were identified as 4-hydroxybenzaldehyde (25) and indole-3-carboxaldehyde (26) (Figure I.12) based on comparison of standards with $^1$H-NMR and GC-MS results. Spectral information can be seen in Figures I.13-I.16.

![Figure I.12. 4-Hydroxybenzaldehyde (25) and indole-3-carboxaldehyde (26) isolated from a new species of Guitarra.](image-url)
Figure I.13. $^1$H NMR spectrum of 25 in CDCl$_3$.

Figure I.14. GC-MS analysis of 25.
Figure I.15. $^1$H NMR spectrum of 26 in CDCl$_3$.

Figure I.16. GC-MS analysis of 26.
Discussion

The antibacterial activity of a crude extract against *Bacillus cereus* guided the isolation and identification of two known aldehydes previously identified from the new sponge species of the genus *Guitarra*. Indole-3-carboxaldehyde was isolated previously from marine *Pseudomonas* species.\(^{42}\) Using purchased standards of these molecules, the original bioactivity of the crude extract was not reproduced. In spite of this result, 4-hydroxybenzaldehyde has been reported as active against some bacterial and yeast species including *Staph. aureus*, *E. coli*, and *P. oryzae*, and also against some tumor cell lines.\(^{43, 44}\) Interestingly, it has also been isolated from a perennial saprophytic herb *Gastrodia elata* and demonstrated unique bioactivity. Assays performed *in vivo* using rats showed that 4-hydroxybenzaldehyde has anticonvulsive and antiepileptic properties.\(^{45}\)
CHAPTER 2

MONANCHOCIDINS WITH BROAD SPECTRUM BIOACTIVITY FROM A NEW SPECIES
OF MONANCHORA

INTRODUCTION

The Aleutian Islands Archipelago is a chain of 14 major islands and roughly 55 smaller islands that extend 1100 miles from Alaska westward towards Russia to the final island Attu Island, AK. The Aleutian Islands, formed by years of volcanic activity, separate the Bering Sea to the North from the Pacific Ocean. Water temperatures around the Aleutian Islands range from 8°C at the surface to around 3°C between depths of 200 and 400 m.46 The cold and dark environment provides an unexplored and rich diversity of undiscovered marine fauna. The initial sponge collection in the Aleutian Islands provided 28 new species from a total of 102 collected, and now an aggregate of 125 species have been characterized from the region.47 A preliminary taxonomic break down of the species from the phylum Porifera of the Aleutian Islands comprises 10 calcareous, 20 hexactinelli, and 95 demosponge species, yet there is still estimated to be hundreds of species from Porifera yet to be discovered from the Aleutian Islands region.47
Among the 95 species of demosponge native to the Aleutian Islands are three members of the genus *Monanchora* belonging to the family Crambeidae. *M. alaskensis*, *M. pulchra*, and *M. laminochela* have all been characterized from the region.

*M. alaskensis* (Figure. II.1) is uncommon to the Pacific Ocean but can be found in the bedrock and cobble of parts of the Aleutian Islands and Bering sea where it is seen at depths between roughly 150 and 350 meters and water temperatures between 1.4 and 6.5 °C.

![Figure II.1. M. alaskensis collected from the Aleutian Islands, AK.](image)

*M. pulchra* is found in great numbers in the Aleutian Islands, as well as the Canadian Pacific coastline, and other archipelago in the region. Found in more shallow regions than *M. alaskensis*, *M. pulchra* is typically located attached to bedrock and boulders between depths of 80 and 330 meters. *M. pulchra* is orange-colored in life but can be seen in varying shades. Figure II.2 displays photographs of both *M. pulchra* in natural habitat as well as out of the water.
Figure II.2. Photographs of *M. pulchra*: the same specimen out of water (A) and in life (B); another photograph of *M. pulchra* in life (C).\(^{47}\)

*M. laminachela* is reported as common in the Aleutian Islands but has not been reported anywhere else worldwide. Found at greater depths than both *M. alaskensis* and *M. pulchra*, *M. laminachela* grows attached to boulders and cobbles between roughly 200 and 500 meters.\(^{47}\) Photographs of *M. laminachela* both out of water and in life can be seen in Figure II.3.
Members of *Monanchora* have been the source of bioactive secondary metabolites such as the cytotoxic monanchocidins isolated for *M. pulchra* presented in Chapter 1, Figure I.3. Here we present a new species of *Monanchora* collected in Aleutian Islands, AK, which produces known and new analogues of bioactive secondary metabolites previously isolated from *M. pulchra*.

**Taxonomic Overview**

The specimen has been compared to the three known species of *Monanchora* in the Aleutian Islands area: *M. alaskensis* (Lambe, 1894), *M. pulchra* (Lambe, 1894) (2010-AK-48)
and *M. laminochela* Lehnert et al. 2006. It is neither of these three, presenting much smaller megascleres and microscleres overall. The specimen is a new and undescribed species of *Monanchora*. A post-collection photograph is shown in Figure II.4.

![Figure II.4. A post-collection photograph of a new Monanchora sp. collected in the Aleutian Islands, AK (51.6415, -177.45301)](image)

**Bioactivity**

The crude extract of the new *Monanchora* sp. was active in a variety of assays against opportunistic infectious organisms, *Plasmodium falciparum* (malaria), hepatitis C virus, and the disc diffusion assay mentioned in Chapter 1.
Table II.1. IC\textsubscript{50} values in µg/mL for the ethanol crude extract of the new species of \textit{Monanchora} collected in the Aleutian Islands, AK. (\textit{C. albicans}, \textit{C. glabrata}, \textit{C. krusei}, \textit{A. fumigatus}, \textit{C. neoformans}, \textit{Staph. aureus}, Methicillin-resistant \textit{Staph.}, \textit{E. coli}) *Amphotericin B and ciprofloxacin as a control

<table>
<thead>
<tr>
<th></th>
<th>\textit{C. albicans}</th>
<th>\textit{C. glabrata}</th>
<th>\textit{C. krusei}</th>
<th>\textit{A. fumigatus}</th>
<th>\textit{C. neoformans}</th>
<th>\textit{Staph. aureus}</th>
<th>\textit{MRS}</th>
<th>\textit{E. coli}</th>
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<tbody>
<tr>
<td>Crude extract</td>
<td>23.07</td>
<td>4.51</td>
<td>3.42</td>
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<td>2.77</td>
<td>6.03</td>
<td>5.49</td>
<td>17.75</td>
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<tr>
<td>Amphotericin B</td>
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<td>1.04</td>
<td>1.599</td>
<td>0.293</td>
<td>0.695</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.082</td>
<td>0.091</td>
</tr>
</tbody>
</table>

\textbf{Isolation Methods}

The specimen was lyophilized and the subsequent 60 g dry weight sample was extracted with ethyl acetate followed by ethanol. The resulting ethanol extract (14.35 g wet) was subjected to a liquid-liquid partition. The extract was partitioned between 200 mL of water and 200 mL of ethyl acetate three times. The resulting precipitate formed from the partition was removed to yield 174.3 mg of white powder, and the organic layer was separated and dried to yield 38.1 mg of dark green oil. The resulting aqueous layer was subject to partition with n-butanol three times. These layers were separated and the water fraction yielded 3.9 g of white powder while the butanol fraction gave 1.5 g of a viscous orange liquid. The four fractions resulting from the liquid-liquid partition were submitted for bioactivity with the results shown in Figure II.2.
Table II.2. *Monanchora* sp. bioactivity results (IC\textsubscript{50} values, µg/mL) against a number of opportunistic infections for the fractions resulting from the ethanol extract undergoing liquid-liquid partition.

<table>
<thead>
<tr>
<th></th>
<th><em>C. albicans</em></th>
<th><em>C. glabrata</em></th>
<th><em>C. krusei</em></th>
<th><em>A. fumigatus</em></th>
<th><em>C. neoformans</em></th>
<th><em>Staph. aureus</em></th>
<th>MRS</th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>23.07</td>
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<td>3.42</td>
<td>21.06</td>
<td>2.77</td>
<td>6.03</td>
<td>5.49</td>
<td>17.75</td>
</tr>
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<td>n-butanol</td>
<td>2.51</td>
<td>1.63</td>
<td>1.96</td>
<td>9.06</td>
<td>&lt;0.8</td>
<td>1.52</td>
<td>1.40</td>
<td>7.08</td>
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<td>EtOAc</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Precip.</td>
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<td>-</td>
<td>14.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

With all bioactivity retained in the n-butanol fraction, this material was subjected to separation using a 6 cm Sephadex LH20 with a 1:1 mixture of dichloromethane and methanol. A flow rate of 2 mL/min was employed with fractions collected every 30 minutes to give 12 fractions. All fractions were screened for bioactivity using the disc diffusion assay against *B. cereus*. Fractions 6-9 proved to possess bioactivity (Figure II.5).

![Figure II.5. Zones of inhibition of crude fractions 6-8 from the Sephadex LH20 separation in comparison to the kanamycin control in the middle of the plate. Fraction 9 also retained bioactivity but was tested on a different plate.](image)
Fractions 6 (229 mg), 7 (430 mg), 8 (111 mg), and 9 (20 mg) were combined to conserve material before purification. The mixture was subjected to a reversed phase C18 cartridge (Phenomenex Strata C18-E, 55µm, 70A) to give four fractions as follows: 100% water (22 mg), 1:1 water:methanol (104 mg), 100% methanol (245 mg), and 100% chloroform (42 mg). These fractions were tested for bioactivity using the disc diffusion assay against *B. cereus*. The results are shown in Figure II.6.

![Figure II.6. Zone of inhibition for fractions 2 (1:1 water:methanol) and 3 (100% methanol) in relation to the kanamycin (K).](image)

Using reversed phase C8 HPLC, Phenomenex (5 micron, 250 x 21.2 mm) employing a gradient of 1:9 (acetonitrile:water) to 100% acetonitrile over 60 minutes (monitored at λ 245 nm, 10 mL/min), 40 mg of active fraction 3 from reversed phase VLC yielded a purified sample of monanchocidin A (Figure II.12) was obtained by splitting the UV signal into multiple fractions.
RESULTS AND DISCUSSION

Fractions 2 and 3 contained mixtures of closely related metabolites that are not easily separated. The $^1$H-NMR (Figure II.7), $^{13}$C-NMR, and mass profiles of these fractions reveal that they contain a group of molecules known as monanchocidins (Figure II.8) that have been recently published.$^{20,21}$ The group of compounds is highlighted in Chapter 1 as compounds isolated from sponges in the Arctic region.

Figure II.7. $^1$H-NMR spectrum in CD$_3$OD of fraction 3 which contains monanchocidin A and derivatives from a new Monanchora sp.
The structural difference between the known monanchocidins is minimal, which provides difficulties in the purification of mixtures. The two distinct differences reside in the guanidine region of the molecule in which two of the described compounds contain a 7-membered oxygen heterocyclic ring (moiety A) and three of the compounds contain a 5-membered oxygen heterocycle (moiety B). The two distinct reported moieties are displayed in Figure II.9. This particular difference is not so easily distinguishable in that the molecular weight of the compounds remains identical. The other difference among the known monanchocidins is the length of the alkyl chain that connects the guanidine portion to the other heterocyclic region, which is ubiquitous among all analogues. These slight differences provided barriers for purification and characterization of potentially new compounds.
Because of the difficulties of separating these compounds, the components of the mixture were classified through LCTOF-MS. Using a Bruker Daltonics LCTOF-MS system, monanchocidins with different alkyl chain lengths were identified by using mass ($m/z$) extraction of predicted molecular weights from the total ion chromatogram for both bioactive fractions 2 and 3. The results and predicted compounds are displayed in Figures II.10 and II.11 with unreported molecular weights indicated with a star.

Figure II.10. Extracted ion traces of various monanchocidins in bioactive fraction 2 (Total Ion Chromatogram on top). Mass values present at significant intensities that have not been reported are indicated by a star.
Figure II.11. Extracted ion traces of various monanchocidins in bioactive fraction 3 (Total Ion Chromatogram on top). Mass values present at significant intensities that have not been reported are indicated by a star.

Monanchocidin A, the analogue in series, has a molecular weight of 859 Da. All predictions were based off of varying numbers of methylene units composing the alkyl linker chain in the molecule. As indicated by the TIC in Figures II.10 and II.11, the potentially new monanchocidins cannot be easily purified from the mixture. Some of the extracted ion chromatograms reveal two distinct mass signals with probable indication of the two distinct guanidine moiety portions of the molecule seen in Figure II.9.
The utility of the bioactivity of these compounds will be further investigated using the acquired pure sample of monanchocidin A. The $^1$H-NMR, $^{13}$C-NMR, MS, and comparison with published $^{13}$C-NMR data can be seen in Figures II.12, II.13, II.14, and Table II.3.

![Figure II.12. $^1$H-NMR spectrum of monanchocidin A dissolved in CD$_3$OD.](image-url)
Figure II.13. $^{13}$C-NMR spectrum of monanchocidin A in CD$_3$OD.

Figure II.14. Mass spectrum analysis of monanchocidin A.
Table II.3. $^{13}$C-NMR chemical shift comparison between experimental data and reported monanchocidin A in CD$_3$OD. Carbons indicated with (-) were reported as overlapped.

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CHAPTER 3

MICROBIOME AND METABOLOME STUDIES OF THE SPONGE

ACANTHOSTRONGYLOPHORA SP. AND THE POTENTIAL ROLE OF THE SPONGE-
ASSOCIATED BACTERIA MICROMONOSPORA SP. M42 IN THE HOST METABOLOME

The interaction between the microbiome and the host organism plays a crucial role in the
development of host-microbe ecosystems. Nearly all organisms exist in coordination with a
number of bacterial and fungal species that compromise their particular microbiome. These floral
communities typically live amongst their host in one of three types of symbiotic relationship
including commensalism, mutualism, and parasitism. Symbiotic microorganisms have been
shown to play a number of beneficial roles for the host organism including aid in primary
metabolism through nitrogen fixation and transformations in marine invertebrates, growth
promotion in plants, and provision of critical chemical defense systems for marine
invertebrates, plants, and humans among other organisms. The specific roles of microbes in
the host life cycle can be greatly dictated and more precisely defined by understanding its small
molecule secondary metabolism. Thousands of secondary metabolites have been
discovered from microorganisms with an array of different bioactivities that can significantly alter the dynamics of open biosystems upon production.

The investigation of microbial symbiosis with larger host organisms provides an interesting frontier for natural product discovery. Understanding the intended purpose of microbial secondary metabolism to host specific responses can help evaluate the potential therapeutic use or other real world application of newly discovered metabolites. Specific examples of host-microbe interactions have been reported in a variety of different species types including insects, plants, humans and other mammals, other microorganisms, and most relevant to this work, marine invertebrates. Actinobacteria are hypothesized to provide antibiotic defense against pathogenic microbes in wasp species with more than 200 strains isolated and many producing a myriad of bioactive secondary metabolites. In a more complex biosystem ant species that depend on the growth of certain fungi for nutrition possess an associated actinobacteria of the genus *Pseudonocardia* that retains antibiotic activity towards parasites that attack the ant food source. In plants, microbial symbionts have shown to have a number of positive and negative effects on host organisms. Associated microbes can produce antibiotic metabolites that aid in host defense mechanisms or can also induce host susceptibility to other infections of herbivores. Alternatively, *Pseudomonas* species have been demonstrated to produce small diketopiperazines that influence root growth and architecture of its host plant. An increased complexity of plant-microbe interactions has been presented with the report of plant-pathogenic fungi, *Rhizopus* sp., that rely on harbored symbiotic bacterial species for toxin production. In addition, the enormous biosynthetic capabilities of some plant-associated bacteria has been illustrated through recent research, including the discovery of 65 total biosynthetic gene clusters in three different strains of *Frankia* sp. derived from actinorhizal
The proficiency of microbial secondary metabolism in association with plants suggests a number of symbiotic roles, many of which remain to be described. The normal flora associated with the human body has yielded important benefits for the human host. These include providing physical and chemical defenses on the epidermal layer as well as within the gastrointestinal lining and contributing enzymes for digestion and metabolism all represent valuable benefits from human associated microorganisms. The abundance and diversity of the human microbiome indicates an articulate relationship between microorganisms and humans. As of 2011, the Human Microbiome Project established by the NIH has deposited over 600 referenced genomes with up to 60 million predicted gene products from normal human flora with anticipation of understanding human-microbe interactions for therapeutic value.

Host-microbe interactions in marine invertebrates share common characteristics with terrestrial models in that growing evidence reveals microorganisms are responsible for chemical defenses through secondary metabolite production. However, the diversity and physiology of the microbiome are more difficult to understand because of its unique environment and struggle to successfully culture these microbes in the laboratory. Microbial species have been identified and studied for their symbiotic relationships in many marine microorganisms, namely tunicates and sponges. Special symbiosis between the cyanobacteria Prochloron didemni and the tunicate Lissoclinum patella has been studied for its extremely large yield of bioactive patellamide compounds. Although the patellamides could not be detected when the bacterium was separated from the host, the biosynthetic gene clusters were definitively identified in P. didemni, suggesting the host-microbe interactions play a crucial role in patellamide biosynthesis. In addition to the varying production of promising bioactive metabolites isolated from L. patella, other studies of this symbiosis explain the importance of P. didemni in aiding the tunicate in both
primary and secondary metabolism to ensure its survival,\textsuperscript{67} providing further motivation for researching host-microbe symbiosis for the benefit of drug discovery. Furthermore, tunicate-derived $\alpha$-proteobacteria have been characterized as a producer of didemnin B, which was the first marine drug to be tested in humans.\textsuperscript{68} Similarly in sponge species, early studies regarding their microbial communities showed the microbiome to be somewhat uniform and phylogenetically distinct from microbes characterized from other marine sources.\textsuperscript{69} Other studies of the sponge microbial community provided evidence towards the vertical transmission of the microbiome in the sponge \textit{Corticium} sp.,\textsuperscript{70} suggesting the importance of the microbial community in proper sponge development. Early work in sponge host-microbe research suggested that two different types of metabolites were produced by two distinct microbial inhabitants of the sponge \textit{Theonella swinhoei} by analyzing distinct cell types using transmission electron microscopy.\textsuperscript{71} This data provided both evidence for a possible microbial origin and cell specific sequestration of secondary metabolites as well as the complexity of the sponge microbiome.

In this research effort, we present a thoroughly interrogated microbiome and metabolome of the sponge \textit{Acanthostrongylophora} sp. (Fig. III.1). From the complex microbiome, a small subset of 12 bacteria could be successfully cultured in the laboratory thus far. One bacterium in particular, identified as \textit{Micromonospora} sp. M42 (Fig. III.1), appears to play a critical role in defensive secondary metabolism and potentially growth of the sponge. Using 16s rRNA gene sequence analysis, the sponge-associated microbial community was assessed and microbial isolates were obtained. This study revealed that \textit{Micromonospora} sp. M42 to be capable of biosynthesis of manzamine A, a metabolite found in high yield in all \textit{Acanthostrongylophora} sp.
as well as the producer of a series of diketopiperazines which possess a variety of bioactivities and may play a role in sponge growth and integrity.

![Image of sponge and bacterial colony](image)

Figure III.1. *Acanthostrongylophora* sp., a common Indo-Pacific manzamine producing sponge with the image of a colony of *Micromonospora* sp. strain M42 with orange mycelial growth and black spores. Manzamine A, B, and 8-hydroxymanzamine A.

**Results**

**Microbiome Analysis of the Sponge *Acanthostrongylophora* sp.**

The 16S rRNA gene sequence analysis of the sponge-associated bacterial community on a library of 117 clones showed three dominating groups, deltaproteobacteria (30%, n=36), chloroflexi (19%, n=23), and actinobacteria (16%, n=19), the remainder of the community includes acidobacteria, gammaproteobacteria, alphaproteobacteria, spirochaetes, and CFB related bacteria (Fig. III.2). The *Acanthostrongylophora* sp. bacterial community is similar to previously reported sponge-associated bacterial communities in being highly diverse with many of the clones having sponge-associated bacteria as their closest relative. Interestingly, a cluster of 23 deltaproteobacteria sequences were identified, which are closely related to the potentially new genus *Plakortis* sp. sponge clone PK003. Actinobacteria are one of the major components of the bacterial community in *Acanthostrongylophora* sp., as seen in previously reported sponge community analysis.
Figure III.2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the bacterial diversity of *Acanthostrongylophora* sp.
Denaturing gradient gel electrophoresis (DGGE) comparison of the sponge bacterial community to the surrounding water column (Fig. III.3) showed that the sponge bacterial community was both distinct and more diverse than the water column. Numerous high GC bacteria (possibly actinobacteria) were undetectable in the water column, but clearly visible in the sponge. Visualization of actinobacteria within *Acanthostrongylophora* sp. tissue by fluorescence in-situ hybridization (FISH) using a high-GC probe specific for actinobacteria revealed distribution throughout the sponge. Clusters of 20 to 50 high-GC bacteria were randomly distributed throughout the mesohyl (Fig. III.4).

Figure III.3. DGGE analysis of a PCR negative control (C), seawater (W), and *Acanthostrongylophora* sp. (S). The increase in bands in the sponge sample indicates a higher diversity in the sponge than in the surrounding water column.
Isolation of sponge-associated heterotrophic bacteria was performed. Standard marine agar 2216, actinomycete media ISP2, and starch casein agar were modified by the addition of NaCl for sponge derived actinomycete isolation and yielded 11 pure cultures, one gammaproteobacteria (M37), two alphaproteobacteria (M31, M36), five firmicutes (M28, M29, M39, M34, M40) and three actinomycetes (M41, M42, M62). The two alphaproteobacteria isolated belong to a group of common sponge symbionts that are vertically transmitted via sponge larvae.\textsuperscript{74}

Multi-spectrum bioactive manzamine alkaloids have been isolated in large quantity and variety from \textit{Acanthostrongylophora} sp.\textsuperscript{75} and a myriad of other species,\textsuperscript{76} suggesting the potential importance of a sponge-associated microbe in symbiosis through manzamine metabolite production. Thus, each \textit{Acanthostrongylophora} sp. isolate was tested for the presence of manzamines by TLC with \textit{Acanthostrongylophora} sp. isolated MA and 8OHMA standards and detected by the alkaloid specific Dragendorff solution (Fig. III.5, left inset). LC-TOF-MS
analysis of isolates testing positive by TLC quickly identified a single bacterial strain (M42) producing a compound with identical retention time, mass and $^1$H-NMR of sponge derived MA, thus confirming the identity of the metabolite (Fig. III.5). This strain was identified as a *Micromonospora* sp., closely related to *Micromonospora chalcea* (X92594) through phylogenetic analysis, and thus named *Micromonospora* sp. M42 (Fig. III.6).

Figure III.5. Chemical confirmation of MA production. Left insert) TLC of alkaloids produced by M42 in either ISP2 or YM, A and B, respectively, compared to media and standards. Right insert) $^1$H-NMR analysis comparison of sponge derived MA. Main image) $^1$H-NMR of bacterial derived MA.
Figure III.6. Phylogenetic analysis based on 16S rRNA gene sequence of cultured bacteria isolated from sponge *Acanthostrongylophora* sp. noted in bold.

**Genome Sequencing and Metabolomic Analysis of Micromonospora sp. M42**

A great portion of the genome of *Micromonospora* sp. M42 has been sequenced by Clardy and coworkers and deposited to the Broad Institute Streptomyces database. The annotation of the M42 genome was retrieved for analysis from this database. The sequencing revealed 14 super contiguous (supercontigs) segments of DNA with an estimation of 6.78 Mbp in total. Of the 6.78 Mbp, nearly 6.7 Mbp are found on one supercontig. The entire annotation contains multiple small gaps of unidentified sequence.

The entire available genome annotation of *Micromonopsora* sp. M42 was screened using Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH). The antiSMASH program
screened the inputted sequence against a database containing identified secondary metabolite and antibiotic gene clusters including polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS), terpene biosynthetic enzymes, hybrid clusters, and others. The screening revealed at least 17 biosynthetic gene clusters in M42. Although we were unable to specifically designate the products of these clusters because of gaps in the sequence and a small degree of relation between manzamine A to known biosynthetic genes, enough sequence was available to identify biosynthetic clusters by family. Among the 17 identified secondary metabolite gene clusters a total of four PKS, one NRPS, six hybrids, five terpenes, and one lantibiotic were discovered. The gene cluster descriptions are presented in Table III.1. The analysis of the genome of M42 reveals a great number of genes that code for machinery necessary to produce an array of secondary metabolites that have yet to be described from the species that potentially play roles in sponge symbiosis. A circular mapping of the genome of M42 containing the discovered secondary metabolite biosynthetic gene clusters was also performed (Fig. III.7).

Table III.1. Assignment and associated genomic data for each of the 17 secondary metabolite gene clusters of *Micromonospora* sp. M42.

<table>
<thead>
<tr>
<th>Cluster Assignment</th>
<th>Predicted Product</th>
<th>Type</th>
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<th>Size, kb</th>
<th>Cluster Location</th>
<th>Promoter</th>
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<tr>
<td>pks1</td>
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<td>62</td>
<td>136 - 61803</td>
<td>LuxRx2</td>
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<td>89</td>
<td>174331 - 263486</td>
<td>LuxRx3 TetR</td>
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<tr>
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<td>62</td>
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<td>LuxR, LacL SARP</td>
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<tr>
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<td>665765 - 699240</td>
<td>SARP LuxR</td>
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<td>unknown</td>
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<tr>
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<td>Type I PKS</td>
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<td>70.2</td>
<td>56</td>
<td>2268788 - 2324751</td>
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<tr>
<td>lan1</td>
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<td>Lantibiotic</td>
<td>69.4</td>
<td>26</td>
<td>2487725 - 2513477</td>
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<td>Terpene</td>
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<td>4018209 - 4040214</td>
<td>MarR AsnC</td>
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<tr>
<td>terp3</td>
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<td>Terpene</td>
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<td>TetR MarR</td>
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<td>TetR</td>
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<td>PKS-NRPS</td>
<td>74.7</td>
<td>115</td>
<td>6587812 - 6702591</td>
<td>LacI</td>
</tr>
</tbody>
</table>
Figure III.7. A circular mapping of the available genome of Micromonospora sp. M42. The outside ring presents the biosynthetic gene clusters in their position along the genome. The middle ring illustrates normalized GC content while the inner ring shows a normalized plot of GC skew.

Metabolites Identified from Micromonospora sp. M42 and Potential Role in Acanthostrongylophora sp. Symbiosis

Fermentation studies of M42 were performed to confirm the production of manzamine and also assess other secondary metabolites to further understand the role of M42 in Acanthostrongylophora sp. symbiosis. Manzamine alkaloids were purified (Fig. III.5) from the media by extraction into chloroform and the extracted material subjected to two HPLC purification steps, first reverse phase C8 followed by normal phase silica both monitored at 350 nm. Analysis of manzamine A (MA) production over 16 days was performed in triplicate and
revealed that MA is initially produced in a linear fashion and then metabolized by the organisms resulting in undetectable levels of MA (Fig. III.8A-C). The peak of production occurred after 4-7 days of incubation, apparently corresponding to log phase growth (Fig. III.8D), and by day 10 no MA was detected, after the log phase has ended. This is intriguing as the molecule is extraordinarily stable in all solvents as well as during in vitro and in vivo evaluations. Further stability studies revealed that degradation of the metabolite is not caused by solvent; however, we cannot rule out external enzymatic degradation or sequestration into cellular components. While these experiments show that M42 is capable of producing MA, they also introduce complications of further metabolism of the molecule. Further support of the biogenesis of MA by M42 was obtained through biosynthetic studies. In the first biosynthetic scheme for MA proposed by Baldwin and Whitehead,\(^{79, 80}\) a pyridinium dimer undergoes a Diels-Alder reaction followed by hydrolysis to generate an aldehyde similar to ircinal A. This aldehyde can further react with tryptamine through a Pictet-Spengler reaction, producing manzamine D in a similar biochemical mechanism as plant strictosidine synthase.\(^{81}\) Further oxidation would yield the aromatic β-carboline ring system. Based on this proposed biosynthetic route tryptamine and ircinal A were added to M42 cultures in ISP2 media and resulted in an approximate 5-fold increase in MA production demonstrating the ability of M42 to convert precursors to MA.
Figure III.8. Production curve of MA from M42 in ISP2. A-C are production charts normalized to mg MA/ g cell mass. Production increases in the first 7 days and dramatically drops afterwards. D is a growth curve of the bacteria based on OD 600 measurements. This data suggests that MA is best produced during logarithmic growth.

Interestingly, additional fermentations yielded a series of seven additional small-molecule compounds that display a broad range of bioactivities including tryptophol (1), N-acetyltryptamine (2), phenethylacetamide (3), brevianamide F (cyclo-(L-Pro-L-Trp) (4), cyclo-(L-Pro-L-Phe) (5), cyclo-(L-Pro-L-Leu) (6), and cyclo-(L-Pro-L-Val) (7) (Fig. III.9). Structural information can be seen in supplementary Figures III.13-III.30. These compounds were purified from extraction using methanol-chloroform, followed by a reversed phase C18 flash column and subsequent C18 then NH2 HPLC. In the fermentations that yielded compounds 1-7, no manzamine related molecules were detected. It is possible that the biosynthetic machinery necessary for manzamine production is plasmid derived and can be easily lost or acquired through horizontal gene transfer. The wide distribution of manzamine alkaloids in different sponge species suggest that the machinery is able to be acquired by multiple microorganisms, most suitably in plasmid form. Other research suggests that secondary metabolite production can decrease and even become non-existent after multiple generations of subcultures. The production of camptothecin by the endophytic fungi *Fusarium solani* in axenic culture initially
decreased and then was eliminated after multiple subcultures. It was also reported that reinoculation into host plant species did not restore metabolite production, indicating that biosynthetic machinery can be quickly mutated in axenic culture. These data reveal the difficulties of producing large scale drug candidates in laboratory settings from host-associated microbes, but offers insight towards the reliance on host and other microbe mutualism in the quality production of some compounds, as appears to be the case for *Micromonospora* sp. M42.

Figure III.9. Tryptophol (1), N-acetyltryptamine (2), phenethylacetamide (3), and four diketopiperazines composed of cyclo(L-Trp, L-Pro) (brevianamide F) (4), cyclo(L-Phe, L-Pro) (5), and cyclo(L-Pro, L-Leu) (6), cyclo(L-Pro, L-Val) (7) isolated from *Micromonospora* sp. M42.

The significance of the production of the manzamine alkaloids and compounds 1-7 by M42 for the development and protection of the host *Acanthostrongylophora* sp. can be predicted from examination of what is known of the properties of the metabolites. Manzamine A was originally described as a possible cancer lead, but since then has shown significant activity against *Plasmodium falciparum* including single dose clearance and a 2-7 fold greater activity compared to artemisinin in vitro. Manzamine A was originally described as a possible cancer lead, but since then has shown significant activity against *Plasmodium falciparum* including single dose clearance and a 2-7 fold greater activity compared to artemisinin in vitro. Mycobacterium tuberculosis, and serine/threonine kinases, such as GKS3-β and CDK5, are also inhibited by MA. Treatment with MA impedes metastasis in cancer cell lines and sensitizes them to TRAIL-induced apoptosis. The broad spectrum antibiotic activity of MA appears to provide an extensive chemical defense system for
the host sponge. We have shown that MA has an inhibitory effect on the growth of M42 at concentrations exceeding 12µg/mL (Figure III.10) which is much lower than the concentration found in the sponge supporting the sequestration of the metabolite by the host. These data indicate that M42 could provide chemical defense for *Acanthostrongylephyora* sp. in exchange for habitat and the ability to sequester large amounts of manzamine alkaloids in a manner that is not lethal to the bacteria. As for compounds 1-7, all products have been reported from an assortment of marine and terrestrial organisms and display a number of bioactivities including antimicrobial, antitumor, and cytoprotective properties. These small molecules could also benefit the sponge with chemical defenses. In addition to antibiotic activity, compounds 5-7 are reported as antifouling agents that inhibit the attachment of barnacle larva. These compounds may help maintain the integrity and proper development of *Acanthostrongylephyora* sp. environment by not allowing other organisms to inhabit areas directly on or around the sponge. Some of these metabolites may aid in the development and growth of sponge species. Three diketopiperazines, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr), were shown to be produced from plant-associated *Pseudomonas aeruginosa* and promote growth and alter root architecture in plants by mimicking the hormone auxin. Two of the three active components (4, 7) were isolated from M42 and may play a role in sponge growth, although a lengthy study is necessary to identify any correlation. Furthermore, the four diketopiperazines 4-7 were identified in *Acanthostrongylephyora* sp. crude extract using standards and LC-MS (Figure III.11). Compounds 1-3 were not detected in the sponge extract, and 4-7 appeared to be in low amounts compared to the laboratory fermentation production which could likely be due to their rapid metabolism in the marine biosystem by the host or other associated microbes.
Figure III.10. Inhibition of M42 growth by MA. Increasing MA concentration inhibits the growth of M42 in a dose dependent fashion.
Figure III.11. LC-MS traces of crude sponge extracts, standard compounds, and the co-injection of both showing the extracted ion (m/z) for the compound of interest in each chromatogram. A) cyclo(L-Pro, L-Leu) (6); B) cyclo(L-Pro, L-Val) (7); C) cyclo(L-Phe, L-Pro) (5); D) cyclo(L-Trp, L-Pro) (Brevianamide F) (4). Standard LC-MS profiles correspond for 2µg on the column. Elution was performed on reversed phase HPLC using a gradient of 15:85 MeOH:H2O to 100% MeOH over 40 minutes.

Proposed biosynthetic origin and route for manzamine alkaloids

Using the antiSMASH software as well as NCBI genomic databases, a number of potentially important biosynthetic enzymes in manzamine biogenesis were identified. The hyb6 cluster, identified through antiSMASH, contains the majority of the enzymes predicted in the
biosynthesis of manzamine (mnz cluster). The biosynthesis initiates with formation of a pyrrolidinium monomer formed by the condensation of acetate units to nicotinic acid. A constant supply of nicotinic acid can be maintained by the expression of MnzA, an amino hydroxyl benzoic acid (AHBA) homologue. MnzA is predicted to convert a nicotinic acid degradation product to nicotinic acid in a similar dehydration mechanism seen in the formation of AHBA synthesis.\textsuperscript{93} The nicotinic acid unit is proposed to be activated by the adenylation domain found in MnzB. This can subsequently be elongated using the PKS machinery in MnzE and MnzF. The dimerization would proceed through an uncharacterized mechanism by an unknown enzyme. The enzyme catalyzed Diels-Alder reaction is also catalyzed by an unidentified enzyme with no clear relation to an identified natural Diels-Alderase.\textsuperscript{94} Following the Diels-Alder reaction the ring opens to produce the carbon skeleton of ircinal A. MnzG is a putative Pictet-Spengler catalyzing protein that can add tryptamine to the aliphatic chain previously produced. Currently, it is unclear if this is clustered with hyb6; however, it is possible as hyb6 is located at the end of a 7Mbp contig separate of the MnzG containing contig. These two contigs could be adjacent to each other, forming a complete Mnz cluster. The P450 homolog MnzD would form an epoxide from the product of MnzG, producing manzamine B. Opening of the epoxide through proton rearrangement, which may or may not be enzyme catalyzed, leads to manzamine A formation. A reductive domain can remove or close the bottom ring of the structure. A putative biosynthetic mechanism as well as a rough annotation of hyb6 is presented in Figure III.12. It is important to note that the last 30kb of hyb6 is unpredictable because 30\% (~10kb) of the sequence is undefined.
Figure III.12. Proposed biosynthetic scheme for manzamine alkaloids with genomic representation of proposed machinery located in and adjacent to the hyb6 gene cluster.

*Micromonospora* sp. M42 Metabolome with respect to the entire *Acanthostrongylophora* sp.

**Metabolome**

With greater evidence for the biosynthetic capabilities of M42, its fingerprint and role within the entire sponge microbiome and resultant metabolome became a focus. There have been
over 35 manzamine derivatives isolated from *Acanthostrongylophora* sp. to date. The genesis for this diverse array of metabolites is likely the ability of the entire microbiome to carry out biotransformation of the prototype manzamine core structure. The conversion of manzamine alkaloids to a series of derivatives provides the symbiotic environment with varying chemical properties that can be suitable for adaptation to current stressors. As a proof of principle experiment, a series of biotransformation experiments have been conducted.\textsuperscript{95-97} MA was incubated with terrestrial microbes and one isolated from the same sponge as M42, *Bacillus* sp. VAN35 (M28). Under laboratory conditions, M28 converted MA into ircinal A. Terrestrial bacteria and fungi were used as models for other biotransformation pathways for MA including various *Fusarium* sp., *Streptomyces* sp., and *Nocaridia* sp. It was found that through incubation of both marine and terrestrial microbes, MA could be transformed to related molecules originally reported from the sponge, thereby providing another route through which the suite of metabolites found in the sponge may be generated. An illustration providing routes to the manzamine-related molecules from *Acanthostrongylophora* sp. from proven microbial biotransformations, tryptamine-ircinal precursor feeding experiments, as well as plausible routes can be seen in Figure III.13. The results of biotranformation studies show that microbial species are capable of performing a series of oxidations, dehydrations, rearrangements, and other modifications to manzamines that can logically explain the presence of most manzamine derivatives isolated from the sponge. These studies offer further clarity on the effects of symbiosis between M42 and the entire sponge microbiome on a chemical level.
Figure III.13. Potential routes for Acanthostrongylus sp. derived metabolites. Biotransformation results using manzamine standards are displayed as well as plausible explanation for related metabolites. Microorganisms: (1). Fusarium solani F0007, (2). Micromonospora M42, (3). Fusarium oxysporum f.sp. gladioli ATCC 1113713, (4) Bacillus sp. VAN35 (M28) (5). Streptomyces seokies, (6). Fusarium oxysporium ATCC 760114 (7). Nocardia sp ATCC 2114514 were capable of transforming MA into a related metabolite. [(Ref.) = 98, Plausible biosynthetic route for manadomanzamines in this reference]
Conclusion

The complexity of host-microbe relationships drives the growth and development of ecological systems of all environments, including the oceans. Here, the rich diversity of the microbiome present in *Acanthostrongylophora* sp. in conjunction with the myriad of bioactive secondary metabolites isolated from the sponge, delivers evidence of the significance and complexity of the symbiosis in the overall metabolome. Additionally, the biosynthetic capabilities assessed in the sponge-associated *Micromonospora* sp. M42 genome displays the ability of the bacteria to produce a number of compounds that would influence the success of the host. Chemically, the secondary metabolites isolated have displayed a variety of activities that can logically benefit the host, while the sponge provides a unique habitat for microorganisms that have been proven exceedingly difficult to reproduce in laboratory environments. The enzymatic capabilities of other microbes to biotranform manzamine alkaloids to other related molecules that have been isolated from *Acanthostrongylophora* sp. illustrate the potential activity of the entire sponge microbiome in the generation of the complex sponge metabolome. Understanding the remarkable potential of the sponge microbiome and the influence it has on secondary metabolism provides interesting insight into how this host-microbial relationship ultimately impacts the diversity and final products that compose the complex metabolome of the sponge.

Materials and Methods

Sponge Sampling. Specimens of *Acanthostrongylophora* sp. were collected by SCUBA diving in Manado Bay, Indonesia at depths between 6 and 33 m. Sponges were transferred directly to plastic bags containing seawater. Sponge tissue was stored frozen at -80°C until used. The sponge tissue was lyophilized prior to analysis.
Total DNA Extraction from the Sponge *Acanthostrongylophora* sp. DNA was extracted from lyophilized sponge tissue using a bead-beater method adapted from Pitcher et al.\textsuperscript{99} Dried tissue (4 g) was ground with a mortar and pestle and resuspended in 16 mL of TE buffer and 4 mL of isoamyl alcohol. The solution was transferred to a 50 ml bead-beater chamber (Biospec Products, Inc.). Zirconia/Silica beads (0.1 mm and 1 mm) were added to 1/4 volume of the chamber and homogenized four times in 1 min via the bead-beater. The solution was transferred to a 50 ml tube, 10 ml of guanidium thiocyanate buffer was added, mixed gently, and transferred to ice. Ammonium acetate (10 M) was added to a 2.5 M final concentration. Standard phenol/chloroform extraction and chloroform/isoamyl alcohol extraction followed. DNA was precipitated with cold isopropanol, cleaned with 70% (v/v) ethanol, and resuspended in TE buffer (pH 8). DNA was quantified using a UV/Vis spectrophotometer.

**Indonesian Sponge *Acanthostrongylophora* sp. Bacterial Clone Library.** Polymerase Chain Reaction (PCR) was performed using 100 ng of DNA with universal 16S rRNA gene primers 8-27\textsuperscript{f} and 1492r\textsuperscript{r} using Hi-Fi Platinum *Taq* (Invitrogen). Cycling conditions were as follow: initial denaturation at 94°C for 5 min, 20 cycles of 94°C for 30 sec, 48°C for 2 min, 72°C for 1.5 min, and a final extension of 5 min at 72°C in a PTC-200 MJ-research thermal cycler (Bio-Rad). PCR products were purified by electrophoresis in a 1% (wt/vol) agarose gel and bands of approximately 1500 bp were excised and recovered using a gel extraction kit (Qiagen, Inc.). Purified PCR products were cloned with a TOPO-XL cloning kit (Invitrogen) according to the manufacturer’s instructions. The sequence of 108 clones (>500 bp) were manually aligned with Phydit software.\textsuperscript{102} The tree was generated by the neighbor-joining algorithm\textsuperscript{103} implemented in Phydit. The robustness of inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values >50 % were shown.
Isolation and Identification of Sponge-Associated Bacteria. Sponge extract was obtained by grinding 1 cm³ of sponge tissue in sterile artificial seawater using a mortar and pestle. The extract was plated in a serial dilution (10⁰ to 10⁻⁴) on three different media types and incubated at 30°C for up to 3 weeks. Marine Agar 2216 (Difco), a non-selective medium, ISP2 (Difco) and starch casein agar, both improve the growth of Actinobacteria and were supplemented with a final concentration of nalidixic acid (10 μg/ml), cycloheximide (10 μg/ml), nystatin (25 μg/ml) and 2% (wt/vol) NaCl. One morphotype of each bacterial colony was selected and subcultured until pure. Isolates were identified by 16S rRNA gene sequence analysis. Bacterial isolates were grown at 30°C, in 50 ml broth of the medium from which they were originally isolated. After 5 days of growth, DNA was extracted from cell pellets using the Ultra-Clean Microbial DNA Isolation kit (Mo Bio Laboratories Inc.). The 16S rRNA gene fragments were amplified by PCR using Platinum Taq DNA polymerase High Fidelity, primers 8-27f, 1001492 r and sequenced. Phylogenetic analysis of isolates sequence was carried as described above.

Culture Methods for Micromonospora sp. M42. Micromonospora sp. strain M42 was grown in shake flasks and a 20 L fermenter in ISP2 liquid medium with vigorous aeration at 30°C. Ethyl acetate extracts from these cultures were screened by thin layer chromatography followed by LC TOFMS for manzamine production. A similar method was used for production of 1-7 with 10 individual 1L cultures were prepared.

Extraction and Isolation. M42 was extracted with ethyl acetate at room temperature. The extract was dried and subjected to silica gel vacuum-liquid chromatography and eluted beginning with hexanes (100%), hexanes–acetone (9:1, 3:1, 1:1), acetone (100%), chloroform–methanol (1:1) and finally with methanol (100%). Fraction 3, eluted with a gradient system of hexane–acetone (3:1) was subjected to HPLC chromatography (Phenomenex Luna 5 μM 250 x 10.0 mm
column, flow rate 3 mL/min, λ 254, 360 nm) using a gradient solvent system of acetonitrile and water both with 0.1% TFA to obtain MA and MB. For purification of compounds 1-7, each 1 L culture of M42 was extracted on day four of fermentation by initially adding 75 mL of MeOH followed by 3x partition with 150 ml chloroform. The organic layer was removed and dried to afford 511 mg of a brown solid material. The material was subjected to a C18 flash cartridge give four fractions eluting, respectively, with water (100%), water:methanol (1:1), methanol (100%), and chloroform (100%). The water:methanol (1:1) fraction (67 mg of green/brown solid) was then fractionated using HPLC employing a Phenomenex 250 x 10 mm Ultracarb column with the following gradient: 1:4 acetonitrile:water (5 min isocratic) to 100% acetonitrile (55 min linear gradient, 3 mL/min, λ 260 nm) to give 22 fractions. Fractions containing aromatic 1H-NMR signals were further separated using HPLC with a Phenomenex 10 mm Luna amino column employing a gradient of 100% hexane to 100% dichloromethane (45 min linear gradient, 3 mL/min, λ 260 nm) to give compounds 1-7.

**Precursor Directed Biosynthetic Experiments.** Commercially available tryptamine and ircinal A isolated from the sponge were fed to M42. A 1L culture of M42 in ISP2 media supplemented with 2% (wt/vol) NaCl was shaken at 28°C and 150 rpm for two days followed by addition of tryptamine and ircinal A (each 25 mg). Cultures were further incubated for three days. Cells and supernatant were separated by centrifugation. The supernatant was extracted with chloroform and evaporated under vacuum. The crude extract was passed though SPE C8 column before analysis using HPLC, LC TOF and NMR. These experiments were repeated four times yielding a 5-fold increase in manzamine production.
Figure III.14. $^1$H-NMR spectrum of 1 in CDCl$_3$.

Figure III.15. $^{13}$C-NMR spectrum of 1 in CDCl$_3$. 
Figure III.16. High resolution mass spectrum profile of 1 with the generated molecular formula. The mass was measured using a Bruker Daltonics micrOTOF system.

Figure III.17. $^1$H-NMR spectrum of 2 in CDCl$_3$. 
Figure III.18. $^{13}$C-NMR spectrum of 2 in CDCl$_3$.

Figure III.19. High resolution mass spectrum profile of 2 with the generated molecular formula. The mass was measured using a Bruker Daltonics micrOTOF system.
Figure III.20. $^1$H-NMR spectrum of 3 in CDCl$_3$.

Figure III.21. $^{13}$C-NMR spectrum of 3 in CDCl$_3$.
Figure III.22. High resolution mass spectrum profile of 3 with the generated molecular formula. The mass was measured using a Bruker Daltonics micrOTOF system.

Figure III.23. $^1$H-NMR spectrum of 4 in CDCl$_3$. 
Figure III.24. $^{13}$C-NMR spectrum of 4 in CDCl$_3$. 
Figure III.25. High resolution mass spectrum profile of 4 with the generated molecular formula (3rd formula). The mass was measured using a Bruker Daltonics micrOTOF system.

Figure III.26. $^1$H-NMR spectrum of 5 in CDCl$_3$. 
Figure III.27. High resolution mass spectrum profile of 5 with the generated molecular formula. The mass was measured using a Bruker Daltonics micrOTOF system.

Figure III.28. $^1$H-NMR spectrum of 6 in CDCl$_3$. 
Figure III.29. High resolution mass spectrum of 6 generated using Bruker Daltonics micrOTOF system. The spectrum shows the dimer of 6 plus sodium ion. The formula for the molecular weight of the dimer is shown.

Figure III.30. $^1$H-NMR spectrum of 7 in CDCl$_3$. 
Figure III.31. Mass spectrum of 7 generated using Bruker Daltonics micrOTOF system.
CHAPTER 4

EXPLORING SOURCES AND OPTIMIZING ISOLATION OF NICOTIANAMINE

INTRODUCTION

Nicotianamine (NA) is a metabolite found in all higher plants functioning as a metal chelating compound in plant tissues, binding metal ions so they can be transferred throughout the plant. Another possible role includes protecting plants from oxidative stress. Recent studies have shown that NA possesses anti-hypertensive effects which are related to its ability to inhibit the angiotensin I-converting enzyme (ACE) in the renin-angiotensin system. Pharmacological ACE inhibitors are widely used for the control of high blood pressure, as well as in the treatment of diabetic neuropathy and hypertensive related congestive heart failure. The ACE enzyme must bind zinc in each present active site for catalytic activity, leading researchers to speculate that NA inhibits ACE activity via zinc chelation. However, recent studies have shown that NA demonstrates preferential inhibition towards ACE. NA has a similar inhibition rate as the known metal chelator, EDTA, for the zinc-containing enzyme carboxypeptidase A. Interestingly, NA has a 15 times higher inhibition rate for ACE than EDTA. This preferential mixed inhibition indicates that NA could possibly have medicinal utility as an ACE inhibitor.
NA has also displayed potential as a replacement for EDTA as a food additive to prevent spoilage. Metal ions can act as catalysts for oxidative reactions that can cause spoilage in food products. EDTA is a synthetic chelator that is currently added to many food products to sequester metal ions and prevent oxidative reactions. Utilizing NA as a natural metal chelator could allow companies to label food products as “all-natural” while still controlling spoilage.

NA functions mainly as an iron chelator in plants, and previous reports have demonstrated how NA possesses the optimal molecular structure for Fe(II) complex formation (Figure IV.1). The particular location of the oxygen atoms on one side of the complex and the methylene groups and/or the azetidine ring system on the other might play a significant role in the biological function of the complex.

![Figure IV.1. Model of the Fe(II)-NA complex.](image)

Although all higher plants contain NA, graminaceous and non-graminaceous plants can be differentiated based on NA use. Graminaceous plants use NA as a precursor in the biosynthetic production of the mugineic acid family of phytosiderophores, while non-
graminaceous plants do not utilize this pathway. The biosynthetic pathway that is utilized for the production of NA is identical for both sets of plants and is shown in Figure IV.2. NA is formed by the trimerization of three molecules of S-adenosyl methionine. Graminaceous plants that thrive in iron deficient conditions show a higher degree of plasticity in regards to the NA synthase gene. Another important distinction between plants with respect to NA production relates to plants involved in metal hyperaccumulation. *Thlaspi caerulescens* can thrive in soil with concentrations of nickel that would be toxic to other plants. More NA is produced and resistance to high nickel soil concentrations is conferred when the NA synthase gene from *T. caerulescens* is spliced into *Arabidopsis thaliana*.

Figure IV.2. Biosynthetic pathway of nicotianamine

All of these considerations can be taken into account when deciding on source material for NA extraction. Iron deficient conditions would only need to be utilized if the plant species is both graminaceous and adaptive to iron deficient conditions. For example, if rice were chosen, it would not be cost effective to transport plants to iron deficient soils for a certain time period.
before starting the extraction process. However, if barley were considered, this could potentially be a vital step in increasing NA production.

**Nicotianamine Sources**

Because NA is found in many different commonly found plant species, an abundance of available resources can be analyzed for the best possible NA yield. The goal is to use analytical methods to efficiently screen a high number of different samples for NA content and further be able to isolate and purify gram scale quantities. Soybean and soy products are likely to be a reasonable starting point for the NA analysis based on patented processes. A number of commercially available soy products as well as different hybrids of unprocessed soybean material can be readily obtained from agricultural services in the greater Mississippi area such as Monsanto and the USDA. Further accessible sources that have been cited for NA production include tobacco, tomato, and beechnuts. A listing with reported yields of NA in different plant species is presented in Table IV.1. Bioengineering approaches could possibly utilize NA synthase genes from plants involved with metal hyperaccumulation where they are constitutively expressed.
Table IV.1. Reported sources and yields of NA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reported Yield of NA (μmol/g fr. Wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Datura metel</em> (perennial herb)</td>
<td>0.12</td>
</tr>
<tr>
<td><em>Lycium chinense</em> (Wolfberry)</td>
<td>0.69</td>
</tr>
<tr>
<td><em>Solanum melongena</em> (eggplant)</td>
<td>0.05</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>0.05</td>
</tr>
<tr>
<td><em>(tomato)</em></td>
<td></td>
</tr>
<tr>
<td><em>Nicotiana tabacum B. Y.</em></td>
<td>0.05</td>
</tr>
<tr>
<td><em>(tobacco)</em></td>
<td></td>
</tr>
<tr>
<td><em>N. glutinosa</em> (tobacco)</td>
<td>0.07</td>
</tr>
<tr>
<td><em>N. rustica</em> (tobacco)</td>
<td>0.19</td>
</tr>
<tr>
<td><em>N. arentsii</em> (tobacco)</td>
<td>0.12</td>
</tr>
<tr>
<td><em>N. alata</em> (tobacco)</td>
<td>0.03</td>
</tr>
<tr>
<td><em>N. debneyi</em> (tobacco)</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Zea mays</em> (corn)</td>
<td>0.02</td>
</tr>
<tr>
<td><em>Rohdea japonica</em> (flower)</td>
<td>trace</td>
</tr>
<tr>
<td><em>Fagus silvatica</em> (beechnuts)</td>
<td>Not reported</td>
</tr>
<tr>
<td>Soybeans</td>
<td>0.3 % aqueous extract of soybeans</td>
</tr>
</tbody>
</table>

From Table IV.1, it is apparent that a multitude of common agricultural products contain NA. *Lycium chinense*, commonly known as the Chinese Wolfberry plant, is notably richer in NA content. The berries of the Wolfberry plant are commercially available at extremely affordable prices although the reported yield resides in the Wolfberry leaves which can also be purchased. Aside from the most easily acquired soy sources, tobacco species stand as a strong and reasonable source of NA. The reported yields from Table IV.1 in regards to the tobacco species are derived from the leaves of the plant as well. Tobacco leaves can also be purchased commercially and analyzed using our analytical methods.

**DETECTION LIMITS OF NA**

**Quantification Optimization**

Since NA is amphiprotic (Figure IV.3), it could be relatively unreliable to quantify under neutral pH and standard conditions of MS. Figure IV.3 illustrates the amount of ionic species of
NA present at varying pH. From the figure, we can make predictions on the intensity of the ionization detection based on pH. Using these calculated values, methods using volatile buffers can be employed for the best quantification and detection of NA. The quantity of compounds can be determined by our LC-TOF system in both positive and negative ion settings for optimal measurements.

<table>
<thead>
<tr>
<th>pH</th>
<th>%-3</th>
<th>%-2</th>
<th>%-1</th>
<th>%neutral</th>
<th>%+1</th>
<th>%+2</th>
<th>%+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.42</td>
<td>7.89</td>
<td>91.69</td>
</tr>
<tr>
<td>1.20</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.87</td>
<td>32.25</td>
<td>38.59</td>
<td>28.29</td>
</tr>
<tr>
<td>2.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>12.32</td>
<td>72.36</td>
<td>13.72</td>
<td>1.59</td>
</tr>
<tr>
<td>5.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.43</td>
<td>98.99</td>
<td>0.58</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>8.00</td>
<td>0.03</td>
<td>17.69</td>
<td>66.83</td>
<td>15.46</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>9.60</td>
<td>5.71</td>
<td>86.07</td>
<td>8.17</td>
<td>0.05</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>14.00</td>
<td>99.94</td>
<td>0.06</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Figure IV.3. Percent of ionic states present at different pH values for NA (MarvinSketch 5.6.0.0)
Testing Quantification with Buffer Solutions

To determine methods for the best quantification of NA on our TOF system and to also find the best conditions to generate a calibration curve, standard solutions of different concentration of buffer were created and analyzed. Equal amounts of NA standard were dissolved in water, 0.05% HCOOH, 0.05% HCOOH with 5mM HCO$_2$NH$_4^+$, 0.05% HCOOH with 10 mM HCO$_2$NH$_4^+$, 5 mM HCO$_2$NH$_4^+$, 10 mM HCO$_2$NH$_4^+$, respectively. Samples were analyzed on MS by flow injection (FIA) to determine the best condition for quantification. Both positive and negative ion modes were considered. As a result, 10mM ammonium formate solution gave the best intensity using negative mode (Fig. IV.4) (extracted ion at $m/z$ 302, [M-H]$^+$), while H$_2$O gave the best intensity on positive mode (Fig. IV.5) (extracted ion at $m/z$ 304, [M+H]$^+$), while 0.05% formic acid gave significant intensity as well. When comparing the two detection modes, the positive mode provides a higher response (Fig. IV.6).

![Figure IV.4. FIA analysis of NA in negative mode.](image-url)
Figure IV.5. FIA analysis of NA in positive mode.

Figure IV.6. Comparison of positive and negative modes.
Sample Preparation

Commercially available NA was used to determine the detection limits. NA was dissolved in water to create a stock solution of 1 mg/mL. A 10 fold serial dilution of the stock solution was performed to create multiple data points until the limit of detection was reached.

LCMS Analysis

All data were generated using LCMS with our Bruker Daltonics micrOTOF instrument in conjunction with Agilent 1100 series HPLC. A calibration curve (Fig. IV.7) was generated for nicotianamine detection. The retention time for NA was determined as 2.75 min on a C8 column (4.6 mm×150 mm, 5 μm, Phenomenex Luna) using an isocratic system with water-methanol (98:2).

The limit of detection (counted as S/N = 10) of NA on our instrument was 4.2 ng and can be seen in Figure IV.7. The standard curve was $y = 10.06x - 287.97$ ($R^2 = 0.9966$, $n = 3$), which was linear over the range of 0.21-4.2 μg/mL (Figure 4), the precision was calculated as shown in Table IV.2. The exact mass of NA (304 $m/z$, [M+H]$^+$) was extracted from the chromatogram for precise quantification.
Figure IV.7. LC trace of each data point and calibration curve of NA

Table IV.2. Calibration of NA using LC-MS (n=3).

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Area</th>
<th>Average</th>
<th>*RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>1350</td>
<td>1224</td>
<td>9.34</td>
</tr>
<tr>
<td></td>
<td>1195</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>420</td>
<td>3579</td>
<td>3679</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>3933</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3524</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1050</td>
<td>9600</td>
<td>10248</td>
<td>5.47</td>
</tr>
<tr>
<td></td>
<td>10565</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10578</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2100</td>
<td>22021</td>
<td>22486</td>
<td>6.63</td>
</tr>
<tr>
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<td>24154</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21284</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4200</td>
<td>39385</td>
<td>41203</td>
<td>5.59</td>
</tr>
<tr>
<td></td>
<td>40431</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>43794</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RSD (%)=((std dev*100)/mean)

**EXPLORING SOURCES**

Because NA is ubiquitous among plant species, options for finding a viable source are broad. After searching what is known about NA production and yields, soybeans and soybean...
products were chosen for the initial attempt to isolate significant amounts. The samples that have been retrieved for analysis include whole commercially produced soybeans from five different hybrid species of whole soybeans and soy flour. Using techniques mentioned previously,\textsuperscript{110} method development was completed for efficient isolation and quantification of NA.

**Whole Soybean Analysis**

A total of 1.785 kg of the commercially available Asgrow 4531 strain soybeans from Monsanto-Asgrow brand markets in Leland, MS, were ground and prepared for extraction to validate the method. The ground material was soaked in 8 L warm water (40°C) for roughly 20 hours and subjected to sonication. The aqueous extract (6 L) was removed from the soybean material by use of mesh wiring filtration. Using a 1M HCl solution, the pH of the resulting aqueous extract was adjusted to 4.5 to precipitate proteins. Using centrifugation, the proteins from the pellet were removed and the aqueous extract retained. A total of 5.5 L of supernatant was recovered and subsequently concentrated to 2.25 L using rotary evaporation. Ethanol (2.25 L) was added to the concentrated aqueous extract in order to precipitate the NA-containing material. The mixture was stirred and placed under refrigeration overnight to maximize precipitation. The resulting mixture was centrifuged to separate the precipitate from the aqueous extract. The precipitate was lyophilized and yielded 15 g of dry material. NA was detected in the material by LCTOF-MS using the same method (1 mL/min 98:2 water-methanol isocratic) used for the standard curve (Figure IV.8).
Soy Flour Analysis

Soy flour was obtained from a 1.5 lb (680 g) bag of Hodgson Mill soy flour expiration date 11/10/12, barcode: 0 71518 05039 9. Water (2.5 L) was added to each of two 2.8 L flasks containing 200 g of soy flour and pH was adjusted to 9 using sodium hydroxide and set to stir magnetically at 25°C. The flour was then filtered using wire mesh filtration, and the pH was adjusted to 4.5. The aqueous extract was centrifuged, and the protein-containing pellet was removed. Ethanol was added to the supernatant at a concentration of 80% of total volume. The resulting solution was centrifuged, and the NA-containing pellet was removed. The material was lyophilized to yield 12.4 g of dry material. NA was detected by LCTOF-MS (Figure IV.8).

Figure IV.8. LCTOF MS traces of the whole soybean precipitate, soy flour precipitate, and NA standard. Each trace represents the extracted ion for 304.2 (m/z) of NA.
Quantification

Using a fresh standard of NA, the relative amounts of NA in the two sources (whole soy and soy flour) was determined in the precipitate mixtures. Solutions (1 mg/mL) of the precipitated mixtures were prepared and 20 µL were injected into the LC-MS system for a total of 20 µg of material on the column. NA standard solution (5 µL of a 10 µg/mL stock) was injected for a total of 50 ng of pure NA on the column. The determination was calculated based on the one spot calibration (y = 60.11x). The ionization intensity of the extracted m/z 304.2 ion for NA is shown in Figure IV.8 for the whole soy precipitate, soy flour precipitate, and the NA standard obtained from Kraft Foods Global. The integration intensity of extracted m/z 304.2 ion for 20 µg soy flour precipitate, 20 µg whole soy precipitate, and 50 ng of pure NA standard was 621051, 228178, and 603387, respectively. The following equations give the percent composition of NA in the precipitate mixtures:

\[
\frac{(50 \text{ ng pure NA} \times 621051)}{(603387)} \div (20000 \text{ ng crude precipitate}) \times 100\% = 0.26\% \text{ NA in soy flour precipitate}
\]

\[
\frac{(50 \text{ ng pure NA} \times 228178)}{(603387)} \div (20000 \text{ ng crude precipitate}) \times 100\% = 0.1\% \text{ NA in whole soy precipitate}
\]

The percent of NA in precipitated mixtures from this process from the reported patent range from 0.3-0.5%, making soy flour near to the reported range. Using the percent mass composition found, the amount of NA in the 12.4g of soy flour precipitate is 31.93 mg. The percent yield from a total of 400 g soy flour was determined to be 0.008%. For the whole soy
bean precipitate, calculation determined that the 15 g of precipitate mixture contains 14.18 mg. The percent yield from a total of 1.785 kg was determined to be 0.0007%. Comparing the yields and convenience in handling necessary to produce the NA containing mixture from the two sources, soy flour is easily a more efficient choice for retrieving respectable quantities of NA. A comparison of LC traces of 1 mg/mL solutions of both whole soybean and soy flour precipitate against a 50 ng standard of NA is shown in Figure IV.10. Based on the quantification of 31.9 mg of NA per 400 g of soy flour, 62.7 kg are necessary to recover 5 g of NA according to the following equation:

\[
(5 \text{ g NA})(400 \text{ g soy flour}) / (0.0319 \text{ g NA}) = 62.7 \text{ kg soy flour}
\]

Figure IV.10. LCTOF MS traces for 1 mg/mL solutions of whole soy precipitate (green), soy flour precipitate (blue), and 50 ng NA standard (red).


32. Fristedt, K., Sponges from the Atlantic and Arctic Oceans and the Behring Sea. *Iakttagelser (Nordenskiöld)* 1887, 4, 401-471.


77. Broad Institute. [http://www.broadinstitute.org/annotation/genome/streptomyces_collab.1/Contact.html](http://www.broadinstitute.org/annotation/genome/streptomyces_collab.1/Contact.html)


VITA

Samuel Abbas was born in Minneapolis, Minnesota on August 5, 1988. He graduated with honors with a Bachelor of Science degree in microbiology from Mississippi State University in 2010. Following completion of his undergraduate degree, Samuel began his graduate studies in Pharmacognosy under the supervision of Dr. Mark Hamann at the University of Mississippi in the fall of 2010.