Secondary Metabolites from Antarctic Marine Invertebrates and their Potential as Drug Leads

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Secondary Metabolites from Antarctic Marine Invertebrates 
and their Potential as Drug Leads

by

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A thesis submitted to the Honors College of the University of South Florida 
in partial fulfillment of the requirements for the degree of 
Bachelor of Science 
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Director: Bill J. Baker, Ph.D. 
Jason Cuce 
Laurent Calcul, Ph.D.
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I would like to acknowledge Dr. Bill Baker for allowing me to participate in his research for so many years and providing me with the opportunity to learn more than I could have imagined. I would like to thank Jason Cuce for consistently going out of his way to assist me, and Garrett Craft for his HPLC expertise. Finally, I would like to thank the rest of the graduate students, post-doctoral fellows, and undergraduates for their guidance and support.
## Contents

Acknowledgements......................................................................................................................... 1
Abstract ........................................................................................................................................... 3
Introduction ..................................................................................................................................... 4
Experimental Methods .................................................................................................................... 9
Results and Discussion ................................................................................................................. 11
References ..................................................................................................................................... 12
Appendices .................................................................................................................................... 13
  Appendix A: Fractionation Schemes describe fractionation conditions ................................. 14
  Appendix B: $^1$H NMR Spectra ................................................................................................. 19
  Appendix C: Chromatograms ....................................................................................................... 23
  Appendix D: Bioassay Data ........................................................................................................... 27
Abstract

The Antarctic region is of particular interest to natural products chemists because it remains relatively unexplored and possesses a wealth of unique biodiversity. Organisms that thrive in this region must produce different chemical defenses to survive in the harsh polar environment, making their secondary metabolites original and potentially useful as drugs for human disease. These compounds often have very complex structures, making their syntheses difficult and impractical. With this knowledge, five Antarctic marine invertebrates were extracted in search of their secondary metabolites. The chemical isolation studies were performed by macroorganism solvent extraction followed by chromatographic separation and purification of metabolites guided by proton nuclear magnetic resonance spectroscopy. The isolates were screened in malaria, leishmaniasis, and cytotoxicity bioassays. Based on the bioassay data, an extract of interest was chromatographed by high performance liquid chromatography and analyzed by liquid chromatography-mass spectrometry. Potential pure compounds could not be further isolated or characterized due to an insufficient quantity.
Introduction

Pharmacognosy is a field of science devoted to the search for new drugs from natural sources, along with the study of the physical, chemical, biochemical, and biological properties of drugs, as defined by the American Society of Pharmacognosy.\textsuperscript{1} Humans have been combating disease for thousands of years. Before the days of pharmaceutical development, societies cured diseases using the elements of their natural environments. Knowledge of which plants healed specific ailments was accumulated over years by a mixture of trial and error and mere chance. Therefore, it is not surprising that many of the pharmaceutical drugs on the market today are derived from natural products, or chemicals that are produced by living organisms, including plants, animals, fungi, and bacteria. Many of these chemicals exhibit biological activity that makes them ideal candidates for drug discovery and development. In fact, over 60\% of cancer drugs and 75\% of infectious disease drugs are developed from natural products.\textsuperscript{2} The percentage is even greater for specific indications. For example, 78\% of antibacterial and 74\% of anticancer compounds originate from natural products.\textsuperscript{3} Some that are currently in medicinal use include penicillins (antibiotics), morphine (an analgesic), and paclitaxel (the anticancer drug taxol).\textsuperscript{4}

\textbf{Figure 1} Left to right: penicillin core structure, morphine, paclitaxel.
With the advent of new technologies, the focus in drug discovery has shifted toward combinatorial chemistry and virtual screening of drug-target interactions.\textsuperscript{3, 5} However, the available drug libraries are limited to known compounds and by the human imagination. There is also much greater diversity among natural products than combinatorial molecules. As a result, compounds in combinatorial libraries occupy a smaller volume of chemical space, the space spanned by all combinations of nuclei in all topology isomers, than either natural products or drugs.\textsuperscript{3, 6}

![Figure 2](image)

**Figure 2** Plots representing the volume of chemical space occupied by a) combinatorial compounds, b) natural products, and c) drugs\textsuperscript{6}. Combinatorial molecules occupy a defined region of this space, while natural products and drugs occupy this same space as well as a larger volume. There is obvious overlap between the diversity of natural products and drugs.

In traditional natural products isolation, the resulting isolate often has a complex structure that would be difficult and impractical to synthesize. Characterization of these molecules aids in the discovery of therapeutic mechanisms of action and biosynthetic pathways and also provides a
foundation for developing natural product analogs. Medicinal chemistry is generally governed by Lipinski’s Rule of Five, which is used to identify small molecules that are likely to possess desirable pharmacokinetic properties. The criteria for these molecules are that they have no more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, a molecular weight less than 500 daltons, and a log $P$ less than five. However, many important natural products violate this rule, as their molecular weights often exceed 500 daltons. One such molecule is palmerolide A, which is a macrocyclic polyketide isolated from the Antarctic tunicate *Synoicum adareanum* that exhibits activity against melanoma by inhibiting vacuolar ATPase. Another similar class of polyketides is the chrondropsins, which have been isolated from a number of marine sponges including *Ircinia ramosa* and *Chondropsis* sp. Like palmerolide A, these molecules inhibit vacuolar ATPases.

![Figure 3](image)

*Figure 3* The top is palmerolide A and the bottom is the chondropsin backbone.
The ocean comprises approximately seventy percent of the earth’s surface. Consequentially, the number of marine organisms greatly exceeds the number of terrestrial organisms. The biodiversity of the marine environment is so great that it has become one of the focuses of a number of scientific disciplines over the past few decades. The majority of the ocean’s organisms thrive on the interface between land and sea, which constitutes less than one percent of the earth’s surface.\textsuperscript{12} As a result, there is great competition among these species for food and space, especially in the case of sessile organisms such as coral, sponges, and tunicates.

In response to this, many species have developed chemical mechanisms to defend themselves.\textsuperscript{12} These chemical defenses are secondary metabolites, which are often terpenoids, alkaloids, polyketides, peptides, sugars, and steroids.\textsuperscript{12} In many cases, these molecules exhibit activity against an array of human diseases. In the past, secondary metabolites were thought to be waste products and were disregarded, but their importance in drug discovery has recently been recognized.\textsuperscript{13} So far, there have been more than 16,000 natural products isolated from marine organisms,\textsuperscript{14} which have proven to yield large quantities of chemically diverse and biologically active molecules.\textsuperscript{15} For instance, prostaglandins, which are used as non-steroidal anti-inflammatory drugs (NSAIDs), were first reported in high yields from \textit{Plexaura homomalla}, a Caribbean gorgonean.\textsuperscript{14} Another important class of compounds is the statins, which have been isolated from a number of fungi.\textsuperscript{4,15} 

\textbf{Figure 4} On the left is prostaglandin E\textsubscript{1} (PGE\textsubscript{1}); on the right is lovostatin
The Polar Regions have surprisingly great biodiversity unique to their climate, but these habitats are among the least explored due to their lack of accessibility. As a result, the ecosystems have remained relatively undisturbed and uninfluenced by humans. In addition, the Antarctic has been isolated for 25 million years by the Circumantarctic Current.\textsuperscript{16} Organisms that thrive in these harsh, cold environments are of interest because their chemical ecology may differ significantly from organisms in warmer climates. Therefore, their secondary metabolites are particularly appealing because they are exclusive to species of the Polar Regions. The benthos of Antarctica is unique in that up to 55\% of the ocean floor is covered in a variety of sponges\textsuperscript{16}, which are often the dominant organisms as a result.\textsuperscript{17} Five sessile Antarctic marine invertebrates were investigated in an attempt to identify novel secondary metabolites. Separations were performed using a variety of chromatography techniques in conjunction with proton nuclear magnetic resonance spectroscopy and bioassays to guide the isolation. Additional studies remain in order to obtain enough isolate for thorough purification and structure elucidation.
Experimental Methods

Five Antarctic marine invertebrates were extracted for analysis. An orange-top sponge with yellow interior (PSC01-53, 240 g freeze-dried), a calcaneus bryozoan (PSC03-133, 300 g freeze-dried), and a dock finger sponge (PSC08-01, 1.42 kg freeze-dried) were collected by SCUBA by the Baker research group from the waters surrounding Palmer Station, Antarctica in 2001, 2003, and 2008, respectively. *Isodictya setifera* (LMGSC10-108, 120 g freeze-dried) and *Didemnum* sp. (LMGSC10-96, 120 g freeze-dried) were collected by trawling by the Baker research group from Low Island, Antarctica in 2010. The five lyophilized samples were exhaustively extracted in 1:1 CH$_3$OH and CH$_2$Cl$_2$. The organisms were then allowed to dry for twenty-four hours before being similarly extracted in 1:1 CH$_3$OH and H$_2$O (Appendix A). Proton nuclear magnetic resonance ($^1$H NMR) spectra were obtained for each extract (Appendix B).

The lipophilic extract (50% CH$_2$Cl:CH$_3$OH) of each organism was chromatographed by normal phase medium pressure liquid chromatography (MPLC) using a solvent gradient of 100% hexane to 100% EtOAc (Appendix C). All fractions collected were then dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/mL. Aliquots of 200 µL of each fraction were transferred to a 96-well plate, which was submitted to Dr. Dennis Kyle for malaria testing with *Plasmodium falciparum* 3D7, cytotoxicity testing using the A549 cell line, and leishmaniasis testing against *Leishmania donovani*.

Based on the results of the bioassay, the malaria hit (LMGSC10-108-6-7) was dissolved in 50% CH$_3$CN:CH$_3$OH in preparation for a reverse phase MPLC wet injection. However, the sample was not completely soluble, so it was chromatographed in two separate experiments using the dry, insoluble portion of the sample and the liquid extract. Both samples were
chromatographed with a 30-g Teledyne Isco RediSep Gold High Performance C18 column using a solvent gradient of 10% H₂O:CH₃CN to 100% CH₃CN, followed by a 100% CH₃OH wash at a flow rate of 35 mL/min (Appendix C). Both injections yielded five fractions each, which were analyzed by ¹H NMR in deuterated methanol (CD₃OD).

Liquid chromatography-mass spectrometry (LC-MS) data was obtained for fraction 4 (LMGSC10-108-6-7-4b) of the CH₃CN:CH₃OH extract to determine an appropriate solvent gradient and absorption wavelengths since the compound did not absorb at 254 nm. This fraction was then chromatographed by reverse phase high performance liquid chromatography on a 4.6 x 250 mm Phenomenex Luna C18 5μ column using a solvent gradient of 10% H₂O:CH₃CN to 100% CH₃CN with 210- and 225-nm detection, yielding 13 fractions (Appendix C). ¹H NMR spectra were obtained for each fraction in deuterated dimethyl sulfoxide (DMSO-d₆).

Fraction 11 (LMGSC10-108-6-7-4b-11, 0.8 mg) was dissolved in CH₃OH and submitted for LC-MS to determine the purity of the sample, although the quantity of sample material was insufficient for additional purification (Appendix C).
Results and Discussion

Five Antarctic marine invertebrates were extracted in organic solvents. The lipophilic extract (50% CH₂Cl₂:CH₃OH) of each organism was chromatographed by normal phase MPLC and the resulting fractions were submitted for malaria, leishmaniasis, and cytotoxicity bioassays (Appendix D). Eleven fractions exhibited mild antimalarial activity with little cytotoxicity (secondary interests, partial hits) and one fraction, LMGSC10-108-6-7, exhibited moderate antimalarial activity with low cytotoxicity (primary interest, hit). None of the fractions submitted exhibited activity against leishmaniasis. Although the isolation could not be fully guided by bioassay in the interest of time, the fractions generated from the separation of the antimalarial hit will be submitted for bioassay.

The antimalarial hit, LMGSC10-108-6-7, was further chromatographed by reverse phase MPLC. A fraction of interest, identified by its ¹H NMR spectrum, was separated by reverse phase HPLC. A fraction of interest was again identified using ¹H NMR, which revealed the presence of an aromatic ring. LC-MS data was obtained, indicating that the sample is a mixture of components; however, the mass of the sample is insufficient for further purification.
References

Appendices
Appendix A: Fractionation Schemes

Orange-top sponge, yellow interior (PSC01-53) 240 g

50% CH$_3$OH:CH$_2$Cl$_2$ (-6) 16.5 g

50% H$_2$O:CH$_3$OH (-7) 45.0 g

Silica column (80 g)
100% hexane to 100% EtOAc

Fraction 1a* 18.6 mg
Fraction 1b 4.4 mg

Fraction 2a 153 mg
Fraction 2b* 642 mg

Fraction 3a 638 mg
Fraction 3b 109 mg

Fraction 4a 151 mg
Fraction 4b 243 mg

Fraction 5a* 71.6 mg
Fraction 5b 32.3 mg

Fraction 6a 24.2 mg
Fraction 6b* 9.6 mg

Fraction 7a 15.8 mg
Fraction 7b 19.2 mg

Fraction 8a 194 mg
Fraction 8b 252 mg

Fraction 9a 306 mg
Fraction 9b 2.11 g

Fraction 10a 1.23 g
Fraction 10b 947 mg

Fraction 11a 773 mg

Fraction 12a 676 mg

Fraction 13a 2.51 g

* Indicates fractions that exhibited antimalarial activity
calcaneus bryozoan
(PSC03-133)
300 g

50% CH$_3$OH:CH$_2$Cl$_2$ (-6)
5.10 g

50% H$_2$O:CH$_3$OH (-7)
8.96 g

Silica column (80 g)
100% hexane to 100% EtOAc

Fraction 1
20.2 mg

Fraction 2
26.7 mg

Fraction 3
304 mg

Fraction 4
22.6 mg

Fraction 5
49.9 mg

Fraction 6*
54.5 mg

Fraction 7*
40.7 mg

Fraction 8
22.9 mg

Fraction 9*
19.0 mg

Fraction 10*
6.1 mg

Fraction 11
4.3 mg

Fraction 12
4.7 mg

Fraction 13
9.2 mg

Fraction 14
1.5 mg

Fraction 15
1.5 mg

Fraction 16
0.4 mg

Fraction 17
13.8 mg

* Indicates fractions that exhibited antimalarial activity
Silica column (120 g)
100% hexane to 100% EtOAc

* Indicates fractions that exhibited antimalarial activity
dock finger sponge (PSC08-01) 1.42 kg

50% CH₃OH:CH₂Cl₂ (-6) 74.3 g

50% H₂O:CH₃OH (-7) 73.5 g

Vial 8 7.53 g

Silica column (80 g)
100% hexane to 100% EtOAc

Fraction 1 20.1 mg

Fraction 2 14.4 mg

Fraction 3 751 mg

Fraction 4 97.1 mg

Fraction 5 91.1 mg

Fraction 6 1.15 g

Fraction 7 1.55 g

Fraction 8 1.17 g

Fraction 9 188 mg

Fraction 10 243 mg
Isodictya setifera (LMGSC10-108)

120 g

50% CH$_3$OH:CH$_2$Cl$_2$ (-6) 7.98 g
50% H$_2$O:CH$_3$OH (-7) 12.4 g

Silica column (80 g)
100% hexane to 100% EtOAc

C18 column (30 g)
100% H$_2$O to 100% CH$_3$OH

* Indicates fractions that exhibited antimalarial activity
Appendix B: $^1$H NMR Spectra

$^1$H NMR spectrum (400 MHz, CDCl$_3$) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of the orange-top sponge with yellow interior (PSC01-53-6)

$^1$H NMR spectrum (400 MHz, CDCl$_3$) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of the calcaneus bryozoan (PSC03-133-6)
$^1$H NMR spectrum (400 MHz, CD$_3$OD) of the aqueous extract (50% CH$_3$OH:H$_2$O) of the calcaneous bryozoan (PSC03-133-7)

$^1$H NMR spectrum (400 MHz, CDCl$_3$) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of Isodictya setifera (LMGSC10-108-6)
$^1$H NMR spectrum (400 MHz, CD$_3$OD) of the malaria hit (LMGSC10-108-6-7)

$^1$H NMR spectrum (400 MHz, CD$_3$OD) of LMGSC10-108-6-7-4b
$^1$H NMR spectrum (400 MHz, DMSO-$d_6$) of LMGSC10-108-6-7-4b-11
Appendix C: Chromatograms

NP MPLC chromatogram (100% hexane to 100% EtOAc) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of *Didemnum* sp. (LMGSC10-96-6)

NP MPLC chromatogram (100% hexane to 100% EtOAc) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of dock finger sponge (PSC08-01-6)
NP MPLC chromatogram (100% hexane to 100% EtOAc) of the lipophilic extract (50% CH$_2$Cl$_2$:CH$_3$OH) of *Isodictya setifera* (LMGSC10-108-6)
RP MPLC chromatograms (100% H₂O to 100% CH₃CN) of the malaria hit, LMGSC10-108-6-7. Top: insoluble portion, Bottom: 50% CH₃CN:CH₃OH extract
RP HPLC chromatogram (10% CH₃CN:H₂O to 100% CH₃CN) of LMGSC10-108-6-7-4b

LC-MS chromatogram of pure CH₃OH

LC-MS chromatogram (10% CH₃CN:H₂O to 100% CH₃CN) of LMGSC10-108-6-7-4b-11
## Appendix D: Bioassay Data

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Malaria (*Plasmodium falciparum* 3D7) bioassay data for primary and secondary interests. Italics indicate 50-90% growth inhibition; bold face indicates >90% growth inhibition.

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Cytotoxicity (A549 cell line) bioassay data for primary and secondary interests. Italics indicate 50-90% growth inhibition; bold face indicates >90% growth inhibition.