Bounty from the Deep

An investigation of the antimicrobial properties of two species from the phylum *Porifera*

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A thesis submitted in partial fulfillment of the Honours Thesis (05.4111/6) Course

Department of Biology

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2005

Abstract

Antibiotic resistance is becoming increasingly more common in pathogenic bacteria, and could make antibiotic therapy ineffective in the future. In an effort to discover new antibiotic compounds, natural product chemists have turned their research towards the oceans. Traditionally marine invertebrates have been an excellent source of novel, bio-active compounds.

During the course of my research, I have extracted crude chemicals from two unidentified *Hexactinellida* sponges, collected from deep water, off the west coast of Vancouver Island. The crude extracts were initially screened for bio-activity against 6 strains of clinically relevant bacteria. Inhibition was observed against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus agalactiae*. The crude extract from one of the unidentified *Hexactinellida* sponges was divided by column chromatography into several fractions; each fraction was tested for bio-activity. Inhibitory activity against *Pseudomonas aeruginosa*, of varying strength, was observed in the fractions tested.

Acknowledgments

First and foremost I would like to thank my supervisor, Dr. Paul Holloway, who had the experience, knowledge and most importantly patience to guide me throughout this project. Without his most important contribution, this Honours Thesis would not have been possible. I thank my committee members: Dr. Athar Ata, who helped me every step of the way with this chemistry intensive project, with guidance, equipment and space in his laboratory and Ray Bacala, whose experience in industry, and drug development helped me avoid some potential pitfalls along the way. And finally I would like to thank the all of the individuals in the Biology and Chemistry departments, for all of their help along the way.

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Introduction

Historically, natural product chemistry has played a very important role in pharmaceutical discovery (Butler, 2004). Closely related to traditional medicine, drugs derived from natural products represent some of the most widely used and clinically important pharmaceuticals in use today, including acetylsalicylic acid (aspirin) and morphine. However, important as it was, research involving natural products remained in the background of modern medicine until the discovery of penicillin by Alexander Fleming in the late 1920's. Following the successful clinical introduction of penicillin, large pharmaceutical companies assembled and tested vast libraries of microorganisms in the hopes of mirroring the success of Fleming's penicillin, often with encouraging results. Today despite competition from other discovery methods in the last decade, natural product chemistry remains an important field in the pharmaceutical industry; its products making up 30% of the most widely used drugs on the market.

The Antibiotics

With the realization that organisms effect the growth of others through chemical interactions and that we can use these chemicals for our own purposes the age of antibiotics was born in the first half of the twentieth century. Antibiotics are compounds which stop microbes by blocking critical cell processes such as cell wall synthesis and protein synthesis. Antibiotics generally have a bactericidal or bacteriostatic effect meaning they can either kill the target cells or halt the reproduction of new cells respectively (Walsh, 2003). Antibiotics are developed through two primary processes; the

first is the design of purely synthetic compounds, obtained through careful organic synthesis while the second is through combinatorial chemistry. Traditionally, the most clinically successful antibiotics have been obtained through natural product chemistry (Butler, 2004).

The success of natural product chemistry in the development of antibiotics is evident when considering the wide range of commercially available antibiotics, developed through natural product chemistry, compared to the single class of synthetically derived antibiotics currently in clinical use, the fluoroquinolones (Walsh, 2003).

Antibiotic Resistance

After the clinical introduction of penicillin, there was a shift in the ways people thought of bacteria, from one of fear and misunderstanding to that of complacency. The common belief that microorganisms were no longer a problem created a culture where antibiotics were prescribed without proper diagnosis, and without proper instruction. In the case of penicillin it was only a short three years before infections, once easily eradicated with small doses of the drug, required higher and higher dosages of penicillin to successfully treat (Walsh, 2003). This trend continued until the dosage required to cure an infection was more than a patients body could handle. This was the first indication of the now global problem of antibiotic resistance.

With an abundant selection of antibiotics available, often, once resistance to a particular drug in a bacterial strain was observed, it was common practice to change the drug of choice to one which was still effective.

Antibiotic resistance is now considered to be inevitable, regardless of precautions and procedures (Butler, 2004). This is due for the most part to the principal of natural selection. In the most basic way, the best adapted organism will out-compete more poorly adapted individuals for space and resources; this competition is based on the ability of microorganisms to rapidly adapt to selective pressures (Townsend, 2000). When an antibiotic is introduced into the body, an artificial selective pressure is created, which will negatively effect the growth of any individuals unable to adapt to the new pressure. Ideally 100% of the pathogens should be susceptible to the drug prescribed. However in a large population of microorganisms, such as in the body of an infected person there are often resistant mutants which arise naturally.

Natural Product Chemistry

Natural product chemistry operates with the knowledge that organisms can evolve the ability to produce and release chemical molecules which serve a wide variety of functions (Proksch, 2002). When considering natural products which may negatively effect the growth and survival of microorganisms, a series of assumptions are made. The primary assumption in natural product chemistry is that organisms competing for environmental resources, such as space and organic material, generally employ mechanisms to ensure the exclusivity of these resources. Organisms which do not posses the ability to actively prevent the uptake of these resources may rely on chemical signaling molecules to give

themselves an advantage in their environmental niche. A secondary assumption is that organisms may produce chemicals which prevent colonization by other pathogenic organisms. These naturally produced chemicals would need to be powerful enough to give the organism excreting them an advantage over the target species, yet be specific enough to prevent the molecules from attacking the biological functions of the excretory individual. For example, a bacterial species is more likely to excrete a chemical affecting the synthesis of a specific protein, rather than one affecting the synthesis of cell wall components, as this would limit its own growth. These excreted chemicals are of particular interest to natural product chemists, searching for new ways to combat bacterial or fungal infection. The more specific the compound is to a particular cell function of fungi, or bacteria, the more useful it is for clinical applications, as there is always the concern about unintentional effects in clinical use.

A majority of the natural products produced by organisms are secondary metabolites. Secondary metabolites are not produced via the biosynthetic pathways necessary to sustain life, and are secondary to these processes (Nelson, 2004). The genetic pathways which produce these molecules often involve genes with promoters regulated by the health of the organism. These promoters are down regulated in times of plenty, so that metabolic pathways and resources may be directed towards critically important processes such as reproduction, however, when normal growth is affected by environmental factors, and the organism can be said to be experiencing competition, the genes which encode the biosynthesis of secondary metabolites are up-regulated, leading to the production and release of these chemicals into the environment (Walsh, 2003).

The Sponges

The sponges, phylum *Porifera,* represent a diverse and ancient group of multicellular animals, the oldest of which are thought to have developed approximately 800mya (Müller, 2003). Basic sponge body plans consist of multiple cell layers, each with individual specialization, functioning in the place of discrete organs (Thakur, 2004). As sponges do not possess vascular systems individual cells must remain close to the incoming current of water in order to ensure that nutrients and oxygen can easily diffuse into their cells. Sponges obtain nutrients and oxygen, and expel waste entirely by moving water through their body cavities. Using ciliated cells, sponges maintain a constant flow of water throughout their bodies and are able to filter up to 70 % of the suspended organic compounds and bacteria from the water. This ability to efficiently filter food from their environment allows them to inhabit nutrient poor environments, allowing them to avoid competition with other organisms. Thought to be relatively long lived, sponges spend their entire adult life attached to the substrate, where they have been reported to filter a volume of sea water equal to that of their own body every five seconds. Water is pumped in to and out of the sponge through unidirectional channels which are lined with highly specialized ciliated cells in the main cavity of the organism.

Filter feeding, while advantageous in a nutrient poor environment, also has drawbacks, such as an inability to discriminate between pathogenic and non-pathogenic organisms

which may be brought into the body. One explanation as to why sponges produce such a wide array of bio active chemicals may be to defend them selves against the effects of potentially pathogenic organisms inadvertently ingested by the sponge. Detailed microscopic studies of sponges have shown them to always be closely associated with a diverse spectrum of bacteria, however due to the feeding method of sponges it is difficult to accurately determine which bacterial species are symbiotic (Müller, 2003). It has been shown through 16S ribosomal rRNA sequencing that at least some of microflora associated with specific species of sponges is not random, but conserved among species (even those found on opposite sides of the globe) which could point to a shared natural microflora. While some experiments have demonstrated symbiotic relationships between sponges and bacteria, the details of the symbiosis is not clear. It has been suggested that the sponge's natural microflora could play roles such as aiding in the digestion of complex organic molecules to the prevention of colonization by pathogenic bacteria. These close relationships may potentially explain why sponges have been such a rich source of bioactive natural products.

One resource of particular importance to the *Hexactinellida* sponges is the availability of dissolved silica, which is used as the primary skeletal component (Whitney, 2005). During specific parts of the season, the competition for this resource becomes of primary importance to the sponge, as *Bacillariophyta* (diatom) growth blooms can use up a significant portion of dissolved silica available in the environment. Ecological observations suggest that during unusually high periods of diatom growth, sponge survival and growth (numbers) decrease drastically. Some researchers believe the rapid

evolution and subsequent population explosion of the diatoms, during the Cretaceous period, resulted in the reduction of the once global distribution of *Hexactinellida* sponges to their scattered, current distribution.

Members of the phylum *Porifera* are renowned for their medicinal applications, as important sources of chemicals used in traditional medicine. This is originally what directed natural product chemists to the sponges in search of new bioactive compounds in the early 20th century. Since then, with the exception of the *Bryozoa*, the sponges have been found to contain the highest number of biologically active natural products, among all of the *Meozoa* (Thakur, 2004). However, to date, very few of these natural products have been developed clinically due to inherent disadvantages associated with the isolation and synthesis of sponge natural products (Müller, 2003). One such disadvantage with extracting chemicals from sponges arises from the small yield of natural products obtainable from sponge extractions, as only 20% of the dry mass of a typical sponge is cellular tissue (Whitney, 2005).

Hypothesis

Sponges are the source of a remarkable number and diversity of natural products, many of which have antibiotic activity. Most sponge natural products have been isolated from shallow water tropical sponges. I hypothesize that the relatively unstudied sponges obtained from the Queen Charlotte Sound will contain novel antibiotics.

Significance

If it is shown that novel compounds exist within my samples which are active against bacteria, it is possible that this research could lead to the development of new classes of antibiotics with useful clinical applications.

Materials and Methods

All solvents used during experiment, were obtained from Anachemia (Montreal QC)

The Antibiotic disks used during this experiment were Erythromycin (15 μg/disk) and Oxytetracyclin (30 μg/disk) Sensi-Disks, obtained from BBL (Oakville ON) and Gentamicin (10 μg/disk), Vancomycin (30 μg/disk) and Streptomycin (10 μg/disk) obtained from BD (Oakville ON)

The two types of sponges used in these experiments were obtained during the first half of September 2004 in the Queen Charlotte Sound (west coast of Vancouver Island, British Columbia, Canada) at depths between 1000m and 1400m. The sponges were recovered by benthic trawls with a commercial trawl net during a Tanner Crab survey conducted by the Department of Fisheries and Oceans. Sponge materials were sorted based on appearance, packaged and frozen (-20°C) for the trip back to the University of Winnipeg. Sponge material was kept at -20°C until used.

Two morphological types of sponges were used in this study, they were labeled “A” and “B”

The “A” sponges are green in colour, and conical in shape. Extending from the apex of the cone, which points towards the substrate in nature, are a series of glass like rods which were observed via a remote camera on the trawl nets, affixing the organism to the substrate. The flesh of the sponge is soft and not obviously porous. Measured across longest the horizontal axis, the sponges range from approximately 16-20cm, and 16-21 on the vertical.

“B” sponges are dark brown, and very porous, resembling a bath sponge. The overall texture of the organism is coarse and dense. Measured across the longest axis, the sponges range from about 7-11cm, and are only 1-2 cm high.

A voucher specimen of each type of sponge was fixed by formaldehyde and preserved in 70% ethanol. The specimens were submerged in ~500 ml of 4% formaldehyde in 3.5% NaCl for 24 hours, followed by a change in formaldehyde-salt water, and an additional 24 hour exposure. Specimens were then soaked in two changes of ~500 ml 100% ethanol in 3.5% NaCl, and stored in glass jars in a solution consisting of 100% ethanol 3.5% NaCl.

Extraction of chemicals from “A” sponge

Preparation

Wet “A” type sponges were weighed and allowed to dry for 48 hours in a fume hood, in order to remove as much water as possible. The wet sponges were found to weigh 2502 g,

the final weight of the dry sponges was 644.39 g. The glasses like rods, protruding from the bottom of the sponge, were removed. The dry sponges were cut into approximately 1 cm cubes with sterile scissors.

Extraction

The dry sponge pieces were placed in a 1L flask, and immersed in solvents of varying polarity, at room temperature, such that the solvent entirely covered the sponge pieces in the flask. The extraction process began with three immersions in methanol, followed by three immersions in ethyl acetate and a final immersion in hexane. Each soak lasted for a period of 24 hours. Following each soak the sponges were allowed to dry in fume hood to remove any remaining solvent, the solvent/extract mixture from each immersion was collected and put aside.

After the solvent immersions were complete, the sponge pieces were placed in an *Imperial Osterizer Pulse-matic 16* commercial food blender with 500 ml of ethyl acetate, and blended on "high", until such time as a uniform slurry was created. This was performed to fracture remaining intact cell membranes, allowing me extract as much of the natural products as possible. The slurry was filtered through four layers of cheese cloth to remove the sponge pieces from the solvent / extract mixture. The filtrate was collected and combined with ethyl acetate extract collected from the initial extractions.

This resulted in three sets of emersion extracts one each of methanol, ethyl acetate and hexane (about 1L of crude extract / solvent mixture per immersion).

Removal of solvent from extract

Individually, the extract mixtures were transferred to a 24/40 500ml Pyrex round bottom flask, which was secured to the condenser of a rotary evaporator (Yamato re200, Orangeburg, NY) via a vacuum pump. The round bottom flask containing the extract was submerged in a 30°c water bath and rotated at the rotary evaporator's medium setting.

When a majority of the solvent had been evaporated from the individual methanol, ethyl acetate and hexane extractions they were combined in a single round bottom flask, at which point the remaining solvent was removed from the combined mixture using the rotary evaporator.

The product obtained at the end of this procedure was ~ 60 ml of an oily liquid, which consisted of a caramel colored opaque layer on top of a reddish brown translucent layer.

Extraction of chemicals from "B" sponge

Wet "B" sponge was weighed dried following the same procedure as that used for "A" sponge, resulting in wet weight of 558.5 g and a dry weight of 154.4 g.

The procedure for the extraction of chemicals from "B" sponge followed that of "A" sponge. The extract removed from the rotary evaporator consisted entirely of dry yellow crystals.

Initial antibiotic assays of crude compounds

Extract was prepared for pipetting ("A" sponge extract was mixed until the 2 layers were homogenized, "B" sponge extract was dissolved in DMSO in a ratio of 1:4 extract to DMSO). Forty micro liters of extract was pipetted, in aliquots of 10 µl, on to a sterile paper antibiotic disk, 6 mm in diameter (BBL, Oakville ON). These experimental disks were then placed on inoculated (spread or pour technique) Muller Hinton II nutrient agar (Difco, Detroit MI), prepared as per the manufacture's instructions.

Preparation of spread plates for antibiotic activity assay

Bacterial species used for antibiotic activity assay were: *Pseudomonas aeruginosa (ATCC 10145), Staphylococcus aureus (ATCC 12600), Escherichia coli (ATCC 11775), Streptococcus agalactiae (ATCC 13813), Corynebacterium xerosis (ATCC 7094)* and *Bacillus subtilis (ATCC 6051).* The cultures were maintained on Muller Hinton II agar at 4°C. Each plate was individually swabbed with sterile a cotton swab. The cotton swabs, inoculated with a species of bacteria, were immersed in a 125 ml Erlenmeyer flask containing 25 ml of sterile Muller Hinton II broth, prepared according to the manufacture's instructions. The inoculated flasks were then incubated on a shaker at 200 RPM for 24 hours at 37°C.

The Erlenmeyer flasks, containing the 25 ml of Muller Hinton II broth, now turbid with bacterial growth, were removed from the shaker immediately following the incubation.. The previously prepared Muller Hinton II nutrient media plates were inoculated by

dipping a sterile cotton swab into the broth, supporting the bacterial species, within the Erlenmeyer flasks, removing the tip of the swab from the broth, and pressing it against the inside of the flask to remove as much media as possible. The cotton swab was then streaked over the entire surface of the Petri plate; the plate was rotated 120° and streaked again, rotated a 120° and streaked a final time.

As a positive and negative control, each inoculated Muller Hinton II spread plate received a sterile, blank 6 mm paper disk (blank disk was saturated with DMSO in experiments concerning "B" sponge extract) and a disk containing a commercial antibiotic. In addition to the control disks, each plate received an experimental disk. The disks were placed on the media such that each was evenly spaced on the plate. Each plate was incubated at 37°C for 16-21 hours.

The areas of the plates around the disks were inspected after the incubation period for bioactivity. Bioactivity was classified as a change in growth from that which was observed around the blank disk. The activity was recorded.

Preparation of pour plates for antibiotic activity assay

Bacterial cultures were prepared as above.

Six 25 ml aliquots of Muller Hinton II agar were autoclaved in sealed, 200 ml media bottles. After autoclaving the individual bottles were allowed to cool to 55ºC in a water bath. Once cool, each aliquot of agar received 100 µl of an overnight bacterial culture,

and was gently mixed. The inoculated media was poured into a sterile Petri dish, and allowed to solidify at room temperature.

Once solid, control and experimental disks were placed on the media, following the procedure above, and allowed to incubate for 16-24 hours at 37°C, activity was recorded after the incubation period.

Standard eukaryotic antibiotic activity assays

Standard spread antibiotic assays were performed with *Sacchromyces cerevisiae (ATCC 9763).* Antibiotic assays were performed on YPD media (Difco, Detroit MI), made as per the manufacturer's instructions.

Fungal species used for antibiotic activity assays were: *Mucor plumbeus (ATCC 4740), Cunninghamella echinulata elegans (ATCC 9244), Cunninghamella echinulata echinulata (ATCC 9945) and Curvularia lunata (ATCC 12017).* Spores from each fungal species were aseptically removed with a sterile inoculation loop, and placed on Petri plates containing potato dextrose agar (Difco, Detroit MI), made as per the manufactures instructions. Each plate received two experimental disks, inoculated with crude extracts from "A" and "B" sponges respectively and two blank control disks. Plates were inoculated at 30°C for 16-24 hours and observed. Bioactivity was classified as a change in fungal growth around the experimental disks relative to that of the control disks.

Chromatography of "A" sponge extract

Column Chromatography

In preparation, the crude extract from "A" sponge was dissolved in a solution consisting of 125 ml of ethyl acetate and 125 ml hexane. The dissolved extract was then thoroughly mixed with an equal volume of 100-200 weight clean silica gel in a 500ml round bottom flask. The resulting slurry was placed on the rotary evaporator to remove the solvent, following the same procedures used in the extraction.

A vertical glass column (56 cm high x 5 cm in diameter) was thoroughly cleaned, and allowed to dry completely and securely clamped upright to a lab stand. Once dry, the stopcock end of the column was plugged with a wad of sterile cotton baton. The column was filled 2/3 full with clean 100-200 weight silica gel. The silica gel-extract mixture was placed on top of the clean silica gel. The extract mixture was sealed by 25 ml of clean silica gel placed on top.

The stopcock at the bottom of the column was closed completely and the column was filled with hexane, until the level of hexane was ~3 cm above the top of the clean silica gel placed on top of the extract mixture.

A 250 ml volume of hexane was added. The stopcock was opened, and 5 aliquots of 50 ml were collected in scintillation vials, each 50 ml aliquot represented one fraction. Once all 250 ml had been collected, 250 ml of a 95% hexane, 5% chloroform solution was loaded on to the column, and collected in 50ml aliquots. This elution gradient was continued in 5% increments, until a 100% chloroform solution was added on to the column. After the 100% chloroform solution was collected a chloroform / ethyl acetate elution gradient was performed, followed by an ethyl acetate / methanol gradient (see appendix 1). A total of 194 fractions were collected, sealed and put aside.

Thin Layer Chromatography

Beginning with the fraction eluted during the most polar extraction, fraction 194, and working back wards, an aliquot of each fraction was spotted onto an aluminum backed 20 cm x 20 cm, silica TLC plate with UV indicators (Whatman, Kent England), and allowed sit in a developing chamber, in 25 ml of solvent until such time as the mobile phase moved to within 1 cm of the top of the TLC plate.

TLC plates were observed under UV light following the developmental procedure; the elution profiles were observed for shape, and distance moved and compared. Fractions displaying similar profiles were pooled into groups of 3-5 fractions.

Standard spread antibacterial assays were performed on all pooled fractions.

Dry weight of combined fraction 4

One ml of combined fraction 4 was transferred to a clean, pre weighed, 20 ml scintillation vial. The vial was placed under a gentle flow of sterile air in a fume hood, until such time as all solvents had been removed from the combined fraction. The vial containing the dry fraction was weighed, and the weight of the clean vial was subtracted from this figure.

(Weight of vial containing combined fraction) – (weight of clean vial) = dry weight of combined fraction

The dry weight of 1 ml of the combined fraction was then used to quantify the dry weights of compounds added to blank 6 mm paper disks. This quantity was compared to the quantity of commercial antibiotic disks.

Results

To determine whether two types of temperate water sponges contained novel antibiotic compound, crude samples of the natural chemicals were extracted, and resolved by silica chromatography.

The crude extract from sponge "A" did posses antibiotic activity, and had an inhibitory effect on the growth of *Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus), E. coli, Streptococcus agalactiae (S. agalactiae),* and *Bacillus subtilis (B. subtilis)*. Inhibitory activity varied with species, zones of activity around each disk ranged from 7 mm to more than 21 mm in diameter (table 1, table 2). The areas of inhibition around the experimental disks, in most cases, were not circular; they were amorphic in shape (figure 1).

The zone of activity observed around disks containing crude extract from "A" sponge against *S. aureus* consisted of 2 distinct areas (figure 1). The first area was a small zone of normal growth (~12 mm in diameter) directly around the disk, surrounded by a large zone ($\geq$ 20 mm in diameter) of inhibited growth.

The crude extract from sponge "B" did posses antibiotic activity, and had an inhibitory effect on the growth of *P. aeruginosa, S. aureus, E. coli* and *B. subtilis*. Inhibitory

activity varied with species, ranging from 7 mm to more than 21 mm in diameter (table 1, table 2). The areas of inhibition around the experimental disks, in most cases, were not circular; they were amorphic in shape (figure 1).

Table 1. Bioactivity of crude extract from "A" sponge against various species of bacteria, a standard spread antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism *(bacterial species)*					
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Escherichia coli*	*Bacillus subtilis*	*Corynebacterium xerosis*
Bioactivity of crude extract	++	+++	-	+	++	-

Figure 1. Bioactivity of crude extract from sponge "A" against *S. aureus* (left) and *P.aeruginosa* (right) on 85 mm diameter Petri plates. Photographed after standard spread antibacterial assay. Disks on *S. aureus* plate, from top right, clockwise, were 10 µl, 20 µl, 30 µl and 40 µl of crude extract. Disks on *P. aeruginosa* plate, from bottom right, clockwise, were 10 µl, 20 µl, 30 µl and 40 µl of crude extract.

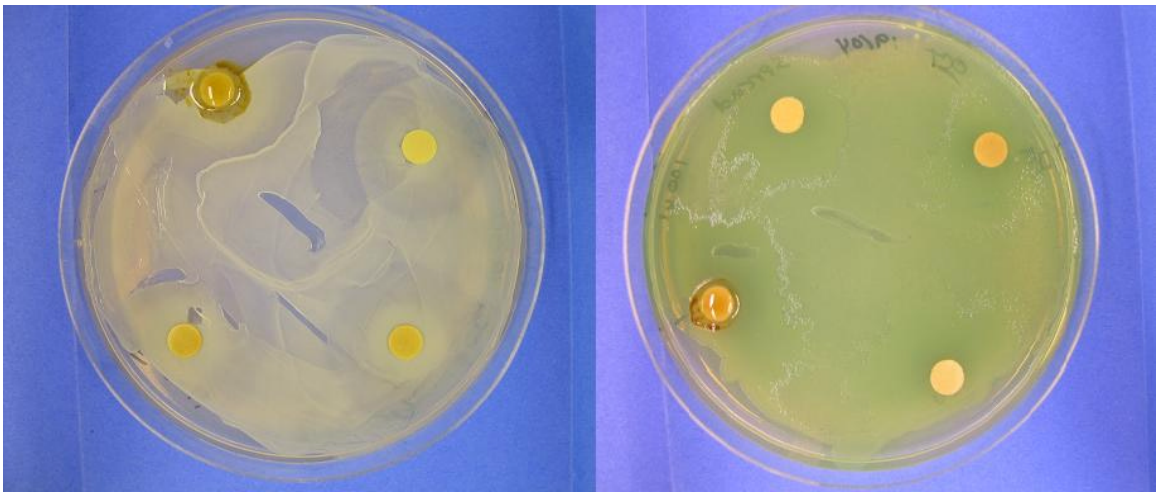


Table 2. Bioactivity of crude extract from "A" sponge against various species of bacteria, a standard pour antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism *(bacterial species)*					
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Escherichia coli*	*Bacillus subtilis*	*Corynebacterium xerosis*
Bioactivity of crude extract	+	-	+	+	++	-

Table 3. Bioactivity of crude extract from "B" sponge against various species of bacteria, a standard spread antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism *(bacterial species)*					
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Escherichia coli*	*Bacillus subtilis*	*Corynebacterium xerosis*
Bioactivity of crude extract	+	++	+	-	+++	-

Table 4. Bioactivity of crude extract from "B" sponge against various species of bacteria, a standard pour antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism *(bacterial species)*					
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Escherichia coli*	*Bacillus subtilis*	*Corynebacterium xerosis*
Bioactivity	-	-	+	+	++	-

of crude extract						

Standard antibiotic activity assays performed against *S. cerevisiae, Mucor plumbeus (M. plumbeus), Cunninghamella echinulata elegans (C. echinulata elegans), Cunninghamella echinulata echinulata (C. echinulata echinulata)* or *Curvularia lunata (C. lunata),* indicated chemicals extracted from sponge "A" or "B" had no effect on the growth of these organisms (table 5, table 6, table 7).

Table 5. Activity of "A" and "B" sponge extracts against *Sacchromyces cerevisiae*, using a standard spread antibiotic assay. Results were scored with a - (no zone of activity), + (7-14mm zone of activity), ++ (15-21mm zone of activity) and +++ (>21mm zone of activity)

	Test microorganism (yeast species)
	Sacchromyces cerevisiae
activity of "A" sponge extract	-
activity of "B" sponge extract	-

Table 6. Bioactivity of crude extract from "A" sponge against various species of fungi, a standard spread antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism (fungal species)			
	Mucor plumbeus	*Cunninghamella. echinulata elegans*	*Cunninghamella. echinulata echinulata*	*Curvularia. lunata*
Bioactivity of crude extract	-	-	-	-

Table 7. Bioactivity of crude extract from "B" sponge against various species of fungi, a standard spread antibiotic assay was used. Results were scored with - (no zone of activity), + (7-14 mm diameter zone of activity), ++ (15-21 mm diameter zone of activity) and +++ (>21 mm diameter zone of activity)

	Test microorganism (fungal species)			
	Mucor plumbeus	*Cunninghamella. echinulata elegans*	*Cunninghamella. echinulata echinulata*	*Curvularia. lunata*
Bioactivity of crude extract	-	-	-	-

Combined fractions 1, 2, 3, 4 and 5, obtained through column chromatography, of sponge "A" extract, negatively affected the growth of *P. aeruginosa,* resulting in zones of activity ranging from 7 to 21 mm in diameter. These combined fractions did not show activity against other species of bacteria tested.

Table 8. Activity of "A" extract fractions obtained through silica gel chromatography against various species of bacteria. using a standard spread antibiotic assay. Results were scored with a - (no zone of activity), + (7-14mm zone of activity), ++ (15-21mm zone of activity) and +++ (>21mm zone of activity)

combined fraction	Test microorganism *(bacterial species)*					
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Streptococcus agalactiae*	*Escherichia coli*	*Bacillus subtilis*	*Corynebacterium xerosis*
1	+	-	-	-	-	-
2	+	-	-	-	-	-
3	++	-	-	-	-	-
4	++	-	-	-	-	-
5	+	-	-	-	-	-

Figure 2. Bioactivity combined fractions 1 through against *P.aeruginosa* on 85 mm diameter Petri plates. Photographed after standard spread antibacterial assay. Disks on plate, from top left counter clockwise, contained: Blank, 40 µl combined fraction 1, 40 µl combined fraction 2, 40 µl combined fraction 3, 40 µl combined fraction 4 and a commercial antibiotic disk containing 10 µg gentamicin.



Figure 3. Activity of combined fraction number four against *P. aeruginosa. 2 ml* of combined fraction 4 obtained from "A" sponge, using a standard spread antibiotic assay. Disks on plate, from left to right, contained 10 µg gentamicin and 2ml combined fraction 4.



The dry weight of fraction four required to inhibit the growth of *P. aeruginosa* was two factors greater compared to that of commercial antibiotics.

Table 9. Estimation of potency of antibiotic compound in fraction 4 from "A" sponge. Increase in zone of activity as dry weight of compound on disk increased. Disks were loaded with 25, 50, 75, 100, 125, 150, 175 or 200 ul of combined fraction number 4, and allowed to dry, removing all traces of solvent. The disks were then aseptically transferred to Muller Hinton plates which had been previously inoculated with a 24 hours culture of *Pseudomonas aeruginosa*. The plates were incubated at 37°C for 20 hours and observed. The dry weight of 1 ml of fraction number 4, volume (ul) was converted to weight (ug), and compared with clinical antibiotics.

Compound on disk	Dry weight on disk (µg)	Size of inhibitory zone (mm)
Gentamicin	10	19
Streptomycin	10	15
Oxytetracycline	15	10
Erythromycin	30	10
Combined fraction 4	3300	8
Combined fraction 4	3960	10
Combined fraction 4	4620	20
Combined fraction 4	5280	24

Discussion

The hypothesis of this project, that the marine sponges isolated from the sea bed of the Queen Charlotte Sound at depths of 1000 to 1400 m contained substances which have growth inhibiting activity against bacteria, proved to be correct. Crude extracts obtained from both the "A" and "B" type sponges showed inhibitory activity against a wide range of Gram positive and Gram negative bacteria, often associated with infections in humans. The broad spectrum of antibacterial activity, observed in both crude extracts, may be the result of an individual compound in each extract with a wide spectrum of activity. However, in the case of the "A" sponge extract, I was able to isolate a series of fractions of the crude extracts with activity against *P. aeruginosa* but not the other five bacterial species tested (table 8 and figure 2). This may indicate that more than 1 compound in the crude extract obtained from "A" sponge is most likely responsible for the observed broad spectrum of activity.

While it is likely that this is also true of the extract obtained from the "B" sponge, only the "A" sponge extract was fractioned on a column, and I cannot make a conclusive determination at this time as to its chemical nature. However it does appear that the crude extract obtained from "B" sponge is more active against Gram positive species since it inhibited *B. subtilis, S. aureus* as well as *S. agalactiae*. More testing will need to be performed in order to make a conclusive statement.

To attempt to quantify the potency of the antibiotic compound in fraction four, from the "A" type sponge fraction four, the dry weight of compound loaded onto the blank antibiotic disk was quantified. It was found that, by weight one hundred times more sponge extract was required, when compared to a commercial antibiotic, to effect similar results. The large amount of sponge extract required to affect the growth of *Pseudomonas aeruginosa* may be due to several reasons. The chemical(s) affecting the growth are not very effective, therefore it requires large amounts of the chemical to effect the growth of the bacterium. The fractions collected after the silica gel chromatography most likely contain several different chemicals; if this is the case, and not all of the chemicals loaded onto the blank disks have inhibitory activity against *Pseudomonas aeruginosa,* the actual dry weight of active components may be significantly less than the dry weight of extract loaded onto the disk, thus the potency was underestimated.

Pseudomonas aeruginosa

The most prominent result observed during the course of this project is the strong activity of my extract against *P. aeruginosa*, seen in both the crude extract from sponge "A" and "B" as well as all fractions elucidated from the crude extract obtained from "A" sponge, tested thus far (Tables 1, 2, 3, 4 and 8). *P. aeruginosa* is a non spore-forming aerobic Gram negative rod, which prefers moist environments (Murray, 1999). It is widespread in several natural habitats such as water, soil, and on the leaves and fruit of plants. It is extremely adaptable to adverse environmental conditions such as temperature and lack of resources. *P. aeruginosa* is able to survive and grow in a wide range of curious locations. *P. aeruginosa* is commonly a member of natural micro-flora of healthy individuals,

primarily found in the gastro-intestinal tract; however it may also colonize other mucous membranes of the body. In individuals with preexisting health conditions the rates of colonization sharply increase, especially with patients who are immuno-compromised or those taking a broad spectrum antibiotics. In a high percentage of infections of individuals with compromised immune systems *P. aeruginosa* colonize the lower respiratory tract, a serious issue in cases of individuals undergoing assisted breathing in or long term care. Other associated afflictions range from the infection of cuts and scrapes to fulminant sepsis. While community acquired infections are important, the most serious infections associated with *P. aeruginosa* are those contracted by patents and workers in hospital environments, where, it is a major cause of respiratory tract infections, with a mortality rate of ~40-50%. The high rate of mortality associated with *P. aeruginosa* infections is due in part to the organism's resistance to common clinical antibiotics. The only antibiotics in clinical use, with good inhibitory activity against *P. aeruginosa* are gentamycin, tobramycin and some fluoroquinolones. Nosocomial strains tend to be resistant to these antibiotics, and often display multi-drug resistance.

If a compound does exist with in my extracts, which has strong specific activity against *P. aeruginosa,* and is able to be employed clinically, this could represent a new treatment for individuals with *Pseudomonas* infections.

Staphylococcus aureus

The initial assays performed with the crude compound obtained from the "A" and "B" sponges showed strong activity against *S. aureus* (Tables 1, 2, 3 and 4). Commonly

associated with infections of minor cuts and scrapes, *S. aureus* is a non motile, non spore forming Gram positive coccus, often observed in clusters consisting of two or more bacterium (Murray, 1999). Infections can cause serious disease, such as nosocomial pneumonia, endocarditis, septicemia, toxic shock syndrome and septic arthritis, often with high mortality and morbidity rates. In the early 1970s and 1980s strains of *S. aureus*, which displayed resistance to a wide range of antibiotics began to be described by clinical microbiologists and epidemiologists. The most serious multi drug resistant *S. aureus* strains are resistant to methicillin, a beta lactam antibiotic which is the antibiotic of choice for treating *S. aureus* infections. MRSA (methicillin resistant *S. aureus)* is currently a large challenge for health professionals, as its prevalence in nosocomial infections continues to rise. In cases of MRSA infections, vancomycin is the only treatment option, however vancomycin resistance has recently been observed in *S. aureus* strains (vancomycin resistant *S. aureus*). While the antibiotic assays of my extract have demonstrated good activity against *S. aureus,* which is sensitive to commercial antibiotics, it has not yet been tested against the methicillin resistant strains. If activity is found against these pathogens, these extracts could represent new clinical antibiotics capable of treating patients with MRSA and VRSA infections.

When the antibiotic assays of crude extract from sponge "B" was repeated a second and third time, a few weeks after the initial experiments, inhibitory activity against *S. aureus* was not observed, despite a rigid adherence to the original procedures. It may be that the chemical responsible for the initial activity degraded in the crude extract solution overtime.

Interestingly, several of the pooled fractions obtained from the fractionation of the crude extract obtained from "A" sponge increased the density growth of the *S. aureus* around the experimental disk, relative to the rest of the growth on the plate.

My hopes were that the broad spectrum of activity observed against the bacteria was a good indication that I had extracted a large number of compounds which may include some with activity against *S. cerevisiae* and fungi. Antifungal activity is important, as activity against fungi may have indicated a potential new drug to add to the very short list of clinically useful anti fungal compounds (DiDomenico, 1999). Activity against *S. cerevisiae* may have been an indication of potential anti tumor activity, as inhibition against yeast may have indicated activity against eukaryotic cell systems, which may have had an inhibitory effect on growing cancer cells. However my initial results have indicated that my compound has no effect on the growth of *S. cerevisiae* or fungi (Tables 5,7 and 6).

The lack of activity of my extracts against *S. cerevisiae* and various species of fungi is a double-edged sword. On one hand, the lack of activity initially eliminates any obvious use as an anti cancer agent. However the lack of toxicity of a compound against eukaryotes, while showing strong anti bacterial activity, may indicate a high degree of specificity towards prokaryotes with little if any activity towards eukaryotes.
This narrow specificity can be very beneficial in clinical applications, as the usefulness of many antibiotics is hampered by the negative side effects associated with their eukaryotic toxicity. If my compounds indeed show selective toxicity towards clinically important

bacterial species, while having little if any effect on a eukaryotic organism, this could represent a significant advantage inherent in these compounds and present a strong argument for future development of the active compound as an antibiotic.

Any given sponge may contain many secondary metabolites. One extraction and characterization of these metabolites would not describe the activity of all of them. Furthermore, my specific experiments focused on the antibacterial activity of sponge extracts, while no work was performed to evaluate their anti-tumor, allogesic or anti inflammatory activity, nor their anti-viral, insecticidal, anti-helminthic or other useful properties.

Location of bio-active chemicals

While I have made the statement that the sponges contained compounds which were shown to be bio-active, I was unable to determine if these were actually produced by the sponge. It is widely recognized that many species of sponges have a diverse natural bacterial microflora which live in symbiosis with the sponge (Müller, 2003). These bacteria have presented a problem for natural product chemists attempting to extract bio-active compounds from the marine sponges, as they confound the identification of the true compound producing organism. Some of the bacterial species are obligate simbionts of the sponge. This issue is of particular importance, as several studies have indicated that some symbiotic bacteria, produce a wide array of bio-active chemicals. These chemicals are thought to prevent the colonization of the sponge by foreign bacteria.

The problem presented by the natural bacterial microflora is that it is very difficult to determine if the bioactivity in the extract is caused by a chemical product of the sponge's metabolism, or that of the resident bacteria. The only way to conclusively determine this would be to isolate samples of the resident bacteria of the sponge, determine which isolates have activity, identify the chemical responsible for the activity, and compare it to the activity of the whole sponge extract.

Novelty of work

One question about the validity of my research revolves around the fact that I am working on the assumption that the sponges used in this experiment have not been characterized before. This assumption is largely based on the location and depth at which the organisms used in this experiment were obtained.

The majority of work published, that has explored the natural chemicals produced by sponges have concentrated on species found in tropical environments, at depths easily accessible by S.C.U.B.A. divers ($\leq$ 40 m). The sponges I used in this experiment were obtained from much greater depths than these (between 1000 m and 1400 m) in the temperate waters of the Queen Charlotte Sound, off of Vancouver Island. Given the location and depth, there is a high probability that the sponges I am working with have not been characterized in this manner.

At present time the compounds responsible for the antibiotic activity in both sponge extracts have not been purified, and their structures evaluated. More work will need to

further purify the extracts and identify the molecular structure of the active compounds. Once this is accomplished, we will be able to establish if this compound is novel or previously described. The lack of previous work on sponges found at the location and depth are arguments to support the assumption that these extracts do contain novel chemicals.

Future work

Given the time constraints put on this project, only a small number of fractions to be tested for specific activity, 150 remaining fractions need to be assayed for bioactivity. The fractions are probably not yet pure, and will require furthered purification by chromatography and characterization of activity before a proper molecular identification of the active compounds may be performed.

Conclusions

1. I have demonstrated that the extracts obtained from the two sponges, named "A" and "B" inhibit the growth of *P. aeruginosa, S. aureus, E. coli, S. agalactiae,* and *B. subtilis*.
2. The crude extract obtained from the sponge named "A" contained a substance with anti-*Pseudomonal* activity.
3. Neither sponge crude extract had growth inhibition towards the fungi, nor *Sacchromyces cerevisiae*.

References

Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR. 2005. Marine natural products. Natural product reports 22(1):15-61.

Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR. 2004. Marine natural products. Natural product reports 21(1):1-49.

Butler MS. 2004. The role of natural product chemistry in drug diskovery. Journal of natural products 67(12):2141-2153.

Chelossi E, Milanese M, Milano A, Pronzato R, Riccardi G. 2004. Characterization and antimicrobial activity of epibiotic bacteria from Petrosia ficiformis (Porifera, Demospongiae). Journal of experimental marine biology and ecology 309(1):21-33.

DiDomenico B. 1999. Novel antifungal drugs. Curr. Oppin. Microbiol 2 (5): 509-515.

Faulkner, DJ. 2000. Highlights of Marine Natural Products Chemistry (1972-1999). Natural Product Reports 17: 1-6.

Hornback, JM. 1998. Organic Chemistry. Pacific Grove, CA. Brooks Cole Thompson Learning.

Nelson DL and Cox MM. 2000. Lehninger Principals of Biochemistry. New York, NY. Worth Publishers.

Müller WEG, Grebenjuk VA, Thakur NL, Thakur AN, Batel R, Krasko A, Muller IM, Breter HJ. 2004. Oxygen-controlled bacterial growth in the sponge Suberites domuncula: toward a molecular understanding of the symbiotic relationships between sponge and bacteria. Applied and Environmental Microbiology 70(4):2332-2341.

Müller WEG. 2003. Sponges (Porifera). Berlin ; New York: Springer. 258 p.

Murray PR and American Society for Microbiology. 1999. Manual of clinical microbiology. 7th ed. Washington, D.C.: ASM Press.

Proksch P, Edrada RA and Ebel R. Drugs from the Sea-current status and microbiological implications. 2002. Appl Microbiol Biotechnol (59):125-134.

Takikawa H. 2004. Synthetic studies on natural products having useful bioactivity and unique structure. Nippon Nogeikagaku Kaishi-Journal of the Japan Society for Bioscience Biotechnology and Agrochemistry 78(10):944-949.

Thakur NL and Muller WEG. 2004. Biotechnological potential of marine sponges. Current science 86(11):1506-1512.

Torres YR, Berlinck RGS, Nascimento GGF, Fortier SC, Pessoa C, Moraes MO. 2002. Antibacterial activity against resistant bacteria and cytotoxicity of four alkaloid toxins isolated from the marine sponge *Arenosclera brasiliensi.* Toxicon (40): 885-891

Townsend CR, Harper JL and Begon B. 2000. Essentials of Eology. Blackwell Science, Inc.

Volk CA, Lippert H, Lichte E, Kock M. 2004. Two new haliclamines from the arctic sponge Haliclona viscosa. European Journal of Organic Chemistry (14):3154-3158.

Wagman GH and Weinstein MJ. 1973. Chromatography of antibiotics. Amsterdam ; New York: Elsevier Scientific Pub. Co. 240 p.

Walsh C. 2003. Antibiotics : actions, origins, resistance. Washington, D.C.: ASM Press.

Whitney F, Conway K, Thompson R, Vaughn B, Krautter M and Mungov G. 2004. Oceanographic habitat of sponge reefs on the Western Canadian Continental Shelf. Continental Shelf research (25): 211-226.

Appendix 1- Elution Gradient Scheme

Fraction Number	Fraction Constituents (250 ml total volume)
1	100% hexane
2	95% hexane 5% chloroform
3	90% hexane 10% chloroform
4	85% hexane 15% chloroform
5	80% hexane 20% chloroform
6	75% hexane 25% chloroform
7	70% hexane 30% chloroform
8	65% hexane 35% chloroform
9	60% hexane 40% chloroform
10	55% hexane 45% chloroform
11	50% hexane 50% chloroform
12	45% hexane 55% chloroform
13	40% hexane 60% chloroform
14	35% hexane 65% chloroform
15	30% hexane 70% chloroform
16	25% hexane 75% chloroform
17	20% hexane 80% chloroform
18	15% hexane 85% chloroform
19	10% hexane 90% chloroform

20	5% hexane 95% chloroform
21	100% chloroform
22	95% chloroform 5% ethyl acetate
23	90% chloroform 5% ethyl acetate
24	85% chloroform 5% ethyl acetate
25	80% chloroform 5% ethyl acetate
26	75% chloroform 5% ethyl acetate
27	70% chloroform 5% ethyl acetate
28	65% chloroform 5% ethyl acetate
29	60% chloroform 5% ethyl acetate
30	55% chloroform 5% ethyl acetate
31	50% chloroform 5% ethyl acetate
32	45% chloroform 5% ethyl acetate
33	40% chloroform 5% ethyl acetate
34	35% chloroform 5% ethyl acetate
35	30% chloroform 5% ethyl acetate
36	25% chloroform 5% ethyl acetate
37	20% chloroform 5% ethyl acetate
38	15% chloroform 5% ethyl acetate
39	10% chloroform 5% ethyl acetate
40	5% chloroform 5% ethyl acetate
41	100% ethyl acetate
42	95% ethyl acetate 5% methanol

43	90% ethyl acetate 10% methanol
44	85% ethyl acetate 15% methanol
45	80% ethyl acetate 20% methanol
46	75% ethyl acetate 25% methanol
47	50% ethyl acetate 50% methanol
48	25% ethyl acetate 75% methanol
49	100% methanol