Can sea cucumbers and sea anemones fight off bacterial infection?

An examination of marine natural products.

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Abstract

Natural products contribute the majority of antibiotics in clinical use today. However, since the early 1980s mortality from infectious disease has been increasing partially due to the emergence of antibiotic-resistant bacteria. The need for novel antibiotics to combat these infections has directed research towards marine natural product discovery. This study hypothesized that holothurians and actinaria—organisms bathed in a sea of bacteria but lacking well developed immune systems—produce antibacterial compounds to ward off infection and would, therefore, be excellent targets for natural product discovery. Initial antibacterial screening of crude methanolic extracts of four holothurians and one actinaria indicated that the holothurians demonstrated greater potential for antibacterial production. The holothurian extracts were fractionated by silica gel chromatography and fractions from each holothurian showed growth inhibition against *Staphylococcus aureus* with one fraction demonstrating growth inhibition against a Gram positive bacterium comparable to a commercial antibiotic. The minimum growth inhibitory concentrations from six fractions ranged from 400 μg/mL to 3200 μg/mL. Future considerations will be the purification and identification of the active compounds to categorize them as novel, variants, or known compounds; also, other biological activities of the compounds will be explored.

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Introduction

Natural products are organic chemical compounds produced by all living organisms. These compounds are often secondary metabolites (Carté, 1996). While the definition of secondary metabolites varies between sources, one definition considers secondary metatolites to be compounds that do not have a general metabolic function and are not directly related to compounds which compose the cellular material (Rose, 1979).

The biological function of secondary metabolites has been controversial. Initial theories were that secondary metabolites were purposefully produced for defense or deterrence (Faulkner, 1984; Proksch and Ebel 1998; Proksch 1999). Subsequently it was proposed that secondary metabolites were waste products of primary metabolism excreted from the cell. More recently the theory that secondary metabolites have definite biological functions has become more popular (Hartmann, 2007).

The evolution of natural products in a range of different organisms has lead to the synthesis of natural products with diverse modes of action—such as allelochemicals—and diverse structures of various compounds, including non-coded amino acids, lactones, terpenes (including sterols), phenylpropanoids, polyketides and alkaloids (Croteau *et al.*, 2000). The production of natural products by these organisms is believed to have evolved under strong selective pressure ensuring the efficiency of the compound on its target, as well as efficiency in the production of the compound (Wink, 2003). Both of these selective pressures have resulted in natural products which act at extremely low concentrations (Hartmann, 2007).

Natural products have been exploited by people for a variety of uses: food, fragrances, pigments, insecticides and medicines (Carté, 1996). Some of the first records of natural product use date back to 2600 BC, where approximately 1000 plant-derived substances were used to treat coughs and colds due to parasitic infections and inflammation; several derivatives of these substances, such as morphine and aspirin, are still in use (Newman *et al.*, 2000).

Traditionally the natural products of higher plants have been most vigorously studied but, since the discovery of penicillin, terrestrial microorganisms have been extensively exploited. These sources have been proven very profitable for the discovery of valuable drugs for the treatment of cancer and bacterial infections (Proksch *et al.*, 2002). Fungi have been crucial for the discovery of the β-lactam penicillin as well as for the production of cephalosporins; another valuable source for antibiotics is the Actinomycete genus *Streptomyces*, which is responsible for producing the aminoglycoside, tetracycline and macrolide classes of antibiotics (Newman *et al.*, 2000). However, the widespread emergence of antibiotic-resistant-bacteria has directed research to unearth new classes of antibiotics.

Over the last 100 years, North Americans and Europeans have experienced a substantial decline in mortality and an increase in life expectancy rates (Armstrong *et al.* 1999). From 1900 to 1937, there was a steady decrease in the infectious disease mortality rate in the United States by approximately 2.8% per year. This was followed by a 15-year period during which the rate fell by 8.2% per year until 1952. The decline then slowed again to 2.3% per year until 1980 (Armstrong *et al*, 1999). These trends can be attributed to

changes in sanitation and lifestyle, vaccination, and importantly, to the treatment of bacterial infections with antibiotics. The vast majority of antibacterial drugs introduced in the last 40 years have been modifications of antibiotics which were discovered and developed during the ‘‘Golden Era’’ of antibacterial discovery from the late 1930s to the 1960s (Mills, 2006). The apparent success of antimicrobial drugs lead to the misconception in the late 1960s and early 1970s that infectious diseases had been conquered (Spellberg *et al*, 2004). Since 1980 mortality from infectious diseases has increased by 4.8% per year (Armstrong *et al*, 1999). This rise in mortality rate can be attributed to the emergence of immunosuppressant viruses, such as HIV, as well as to the increase in antibiotic-resistant infections (Armstrong *et al.*, 1999). In fact, penicillin, introduced in the early 1940s, was quickly abused with use in treatment of all infections, even those non-bacterial in nature (Alanis, 2005). Predictably, within a few years penicillin-resistant *Staphylococcus aureus* strains were identified. By the late 1960s, greater than 80% of *S. aureus* isolates were penicillin resistant in United States hospitals (Mills, 2006). The emergence of multidrug resistant bacteria has created a situation in which there are few or no treatment options for infections with certain microorganisms (Spellberg *et al.*, 2004). Today, infectious disease remains the third-leading cause of death in the United States and the second-leading cause of death worldwide (Spellberg *et al*, 2004).

One may suggest that bacteria (and their potential molecular targets) are the oldest of living organisms and have been subjected to three billion years of evolution in harsh environments, and therefore have been selected to withstand chemical assault (Projan and Bradford, 2007). Due to the strong selective pressure of anti-bacterial therapies, strains

adapted to withstand their deleterious effects have emerged. For antibiotic resistance to develop, it is necessary that two key elements combine: the presence of an antibiotic capable of inhibiting the majority of bacteria present in a colony, and a heterogeneous colony of bacteria where at least one of these bacteria carries the genetic determinant capable of expressing resistance to the antibiotic (Alanis, 2005). Several mechanisms of antibiotic resistance include 1) chemical modification of the antibiotic, 2) active efflux of the antibiotic, and 3) antibiotic target modification (Alanis, 2005).

Antimicrobial drug resistance constitutes a continuing and growing problem in both developing and developed nations alike (Projan and Bradford, 2007). Rates of antimicrobial resistance among hospital and community pathogens have increased considerably during the past decade (Cosgrove, 2003). In reality, patients today are contracting multidrug-resistant infections and are suffering higher levels of morbidity and mortality than in the past. The therapeutic options for serious, life-threatening bacterial infections are becoming increasingly limited because of multidrug resistance and untreatable, pan-resistant (resistance to all antibiotics) bacterial infections do occur and are becoming increasingly common (Projan and Bradford, 2007).

Vancomycin was introduced as a first-line therapy for serious methicillin-resistant-*Staphylococcus aureus* (MRSA) infections in the 1980s. Vancomycin intermediate (VISA) and now vancomycin resistant (VRSA) MRSA are becoming more frequently documented (Mills, 2006). The emergence of vancomycin-resistant *S. aureus*, linezolid-resistant *S. aureus* and *Enterococcus*, and multidrug-resistant Gram negative bacilli may result in limited treatment options for skin, urinary tract, and systemic infections that

were formerly readily curable with commonly used antimicrobials (Spellberg *et al*, 2004). Moreover, multidrug-resistant bacteria are compromising our ability to perform what are now considered routine surgical procedures such as hip replacements or coronary artery bypass grafts (Projan and Bradford, 2007).

Several studies have demonstrated that resistance frequently leads to a delay in the administration of appropriate antibacterial therapy, which may be associated with adverse outcomes (Cosgrove *et al.*, 2003). The majority of published studies have shown an association between antibiotic resistance and adverse outcomes on the order of a 2-fold increase in mortality, morbidity, and cost for patients with resistant versus susceptible infections (Cosgrove *et al*., 2003). In order to hasten the treatment of infections, whether it is known that the infection is a resistant strain or susceptible, broader-spectrum agents are now administered. The Office of Technology Assessment estimated that the national cost of antibiotic resistance in the United States was $4 billion per year (1995 dollars); however, this assessment took into account only directly affected patients and not other ramifications of resistance, which would likely increase the estimate by several-fold (Cosgrove *et al.*, 2003).

Pharmaceutical research and development costs—estimated to be $400 to $800 million per approved agent—pose a considerable barrier to the development of new antibacterial drugs (Spellberg *et al.*, 2004). Another important reason for the dry development pipeline and decline in industrial interest is that the time it takes to develop a novel anti-infective agent from discovery to marketing has greatly increased (Projan and Bradford, 2007).

These reasons have led some large pharmaceutical companies to indicate that they are curtailing or completely abandoning anti-infective research (Spellberg *et al.*, 2004).

Between 1981 and 2006 a total of 109 antibacterial entities were discovered (Newman and Cragg, 2007). The number of newly approved antibacterial agents per 5-year-period decreased during the 20-years from 1983 to 2002 (Figure 1).

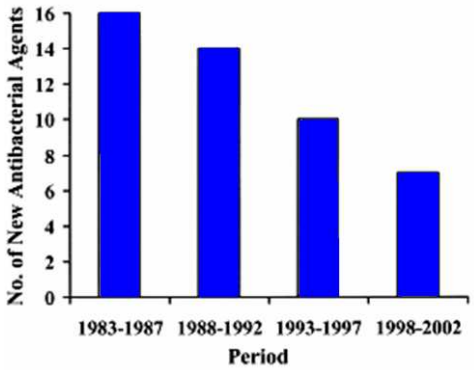

Figure 1. New antibacterial agents approved in the United States from 1983-2002, per five-year period (Newman and Cragg, 2007).

From 1998 to 2002, the Food and Drug Administration (FDA) approvals of new antibacterial agents decreased by 56%, compared with the period from 1983 to 1987. Between 1998 and 2002, only two of the approved agents, linezolid and daptomycin, had novel mechanisms of action. It is only with the development of new classes of antimicrobials with novel mechanisms of action that we can fully address the escalating drug resistance in common pathogens (Spellberg *et al*, 2004). The current method for producing the majority of new antibacterials is through the synthetic modification of existing compounds, which has been extremely inefficient for battling bacterial resistance. This practice has become archaic and bacteria are quickly evolving resistance to these therapies with redundant methods of action.

A recent report addressed the specific needs for antibacterial agents (Projan and Bradford, 2007). Among the Gram positive pathogens for which novel antibiotics are required are methicillin-resistant *Staphylococcus aureus* and *S. epidermidis*, vancomycin-resistant *Enterococcus faecium* and *E. faecalis* and the rapidly growing mycobacteria (Projan and Bradford, 2007). Among the multidrug-resistant Gram negative bacteria there is a growing unmet medical need for new antibiotics. These problematic Gram negative species include pan-resistant strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, and *Acinetobacter baumannii* (Projan and Bradford, 2007).

The technological age has seen a shift in antibacterial research towards a genomic-target-based approach as well as automated high-throughput screens (Projan and Bradford, 2007). However, it is believed that the most logical starting point is not a target-based approach but instead the tried and true method of screening for growth inhibition (Projan and Bradford, 2007). Payne *et al.* (2007) suggests that an automated, antibacterial 'high-throughput' screen is about five times less likely to yield antibiotics than for any other kind of therapeutic target (Projan and Bradford, 2007). Also, it was not until 2001 that the genomes of the important Gram positive pathogens of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *S. pyogenes* were made publicly available (Mills, 2006).

Natural products and their derivatives continue to be a vital source for antibacterial agents (Figure 2). From 1981 to 2006, 9.2% of new antibacterials were natural products, 58.7% were derivatives of natural products and 0.9% were synthetic compounds with a natural product pharmacore (Newman and Cragg, 2007). A review by Bérdy (2005)

highlights the efficiency of natural product research in comparison to synthetic methods for novel antibiotic discovery because natural products are over 100 times more likely to contain bioactivity.

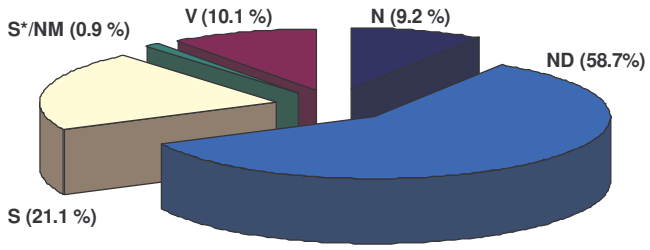

Figure 2. Source of new antibacterial entities discovered from 1981 to 2006. S (synthetic), S*/NM (created through synthesis and acts as a natural product mimic), V (vaccine), N (natural product), ND (derived from a natural product, semi-synthetic) (Newman and Cragg, 2007).

Due to the increasing demand for novel antibacterials, research has turned to the ocean for natural product discovery. The "take-off point" for marine natural product research began with the discovery of sizeable quantities of prostaglandins in the gorgonian *Plexaura homomalla* (Weinheimer and Spraggins, 1969). These compounds had just been discovered as important mediators involved in inflammatory diseases, fever and pain (Weinheimer and Spraggins, 1969). From 1969 to 1999 approximately 300 novel bioactive marine natural products were discovered. The next two years saw a dramatic increase in marine natural product research with isolated compounds exceeding 10 000 (Proksch *et al.*, 2002). A review by Faulkner (2002) reported that hundreds of these novel natural products were being isolated each year. The most intensively studied organisms are the sponges, closely followed by tunicates and coelenterates (Faulkner, 2002).

Organisms that are soft-bodied, sessile or slow-moving marine invertebrates that usually lack morphological defense structures have provided a wealth of bioactive metabolites (Proksh et a*l.*, 2002). The chemical defense through accumulation of toxic or distasteful

natural products is an effective strategy to fight off potential predators or to force back neighbours competing for space (Proksch and Ebel 1998; Proksch 1999). Therefore, organisms that thrive in spite of pronounced biotic pressures have high potential to contain metabolites that are of interest for drug prospectors searching the oceans. The flora and fauna in the marine environment are virtually bathed in a ‘‘microbial soup’’, and these organisms most likely contain a chemical means to prevent colonization by bacteria (Lippert *et al.*, 2003).

The holothurians (sea cucumbers) of the phylum Echinodermata (sub-phylum Echinozoa, class Holothuroidea) are marine invertebrates that lack physical defenses as well as an advanced immune system. Holothurians have a number of predators including crustaceans, gastropods, polychaetes and fish (Bryan *et al.*, 1997). The holothurians have been shown to produce chemical defenses in order to deter fish predators (Bryan *et al.*, 1997; McClintock, 1989). McClinktock (1989) studied three non-tropical holothurians and found one species to be highly toxic, one to be mildly toxic and the third showed no toxicity towards fish predators. Even though this study did not identify the source of the toxicity, it has been repeatedly shown that holothurians produce natural products categorized as saponins (Proksch *et al*, 2002; Faulkner, 2002). Saponins are glycosylated compounds that are also common in plants and these plant saponins have exhibited anti-fungal activity (Osbourn, 1996). The holothurian *Cucumaria japonica* produces 13 different biologically active triterpene oligoglycosides cucumariosides (Aminin *et al.*, 2006). One triterpene oligoglycoside, termed cumaside, has been shown to be biologically active with immunomodulatory action (Aminin *et al.*, 2006).

A second family of organisms that fit in this category is the actinaria (sea anemones) of the phylum Cnidaria (class Anthozoa, sub-class Hexacorallia, order Actiniaria). The phylum Cnidaria has been a very productive source of natural products; however, few studies have been undertaken into actinarian natural products (Carté, 1996). A recent paper reported the isolation of four compounds of a new class of xenicane diterpenoids isolated from an actinarian (Cao *et al*., 2005). These compounds were shown to be inhibitors of cdc25B phosphatase, revealing its potential in cancer therapy. Besides this study few actinaria natural products have been tested for biological activity.

Even though the holothurian and actinarian families produce several biologically active compounds, study of their natural products has been limited (Blunt *et al.*, 2008). Most marine natural products have been characterized by chemical structure with very little exploration into the bioactivity of these compounds; what limited bioactivity research which has been done is usually limited to anti-cancer therapy. A review by Blunt *et al.* (2008) indicated that the Cnidaria and Echinodermata are phyla with a high potential to produce bioactive natural products; however, less than 10% of the estimated number of species within each of these groups has been examined for natural product bioactivity. Also, the species that have been examined were obtained from a scuba-diving depth of approximately 40 m or less. The objective of this study was to test the hypothesis that, because of their need to defend against bacterial pathogens, some holothurians and actinaria contain antibacterial compounds. To address this hypothesis the methanol extracts from four holothurian species and one actinarian species were obtained and characterized for the presence of any antibacterial activity in the extracts.

Methods

Extraction of samples

The marine organisms that were used in this study were previously obtained by trawl of the seabed in the Dixon Inlet north of Haida Gwaii in May of 2006 (Queen Charlotte Islands, approximately 54° 30" N, 140° W) at a depth of 1000 to 1400 m. The samples were flash frozen in zip-lock freezer bags and stored at -20° C. Four holothurians that were tentatively identified by Philip Lambert (Curator of Invertebrates, Royal British Colombia Museum, Victoria, BC) as *Molpadia sp*., *Scotoplanes sp*., *Paelopadites confundens*, and *Pannychia moseleyi* and one unidentified actinarian were used in this study. Voucher specimens for each of the holothurians were fixed in 10% (v/v) formalin in sea water for 24 hours followed by preservation in 70% (v/v) methanol. All solvents used in this study were obtained from VWR (Mississauga, ON). A sample of each of the 5 organisms was lyophilized with a Thermovac (Johns Scientific Inc.; Toronto, ON). The lyophilized samples were macerated and extracted with 95% methanol 5% (v/v) water for 24 hours. Following extraction, the 95% (v/v) methanol was decanted and stored in a -4°C fridge. The extraction was repeated a further two times for a total of three 95% (v/v) methanol extractions. All three 95% (v/v) methanol extracts for each specimen were pooled in pre-weighed flasks and concentrated on an RE111 (Buchii; Flawil, Switzerland) rotary evaporator with the waterbath set to 40° C. The remaining solvent was evaporated to dryness in a fume hood. The dry extracts were re-dissolved in 95% (v/v) methanol to a concentration of 50 mg/mL and were subjected to a preliminary screening for antibacterial growth inhibition activity with a disc diffusion assay (see

'Antbacterial growth inhibition activity assay'). The extracts were then evaporated to dryness in a fume hood and then stored in the darkness at 4°C.

Fractionation of 95% (v/v) methanol extracts by liquid column chromatography

Extracts with antibacterial activity were fractionated by liquid column chromatography on silica. The extracts were absorbed onto raw polar 60Å 230-400 mesh silica beads (Whatman; Sanford, ME) in order to bind the extract to the stationary phase. This dry mixture was added to the top of a 3.75 cm diameter x 75 cm glass column (2.5 cm diameter x 75 cm for *Molpadia sp*. extract) of raw silica and hexane. A layer of silica was placed on top of the sample to prevent disturbance during the addition of solvents (Appendices 1-4). Initially the extract was eluted with either 200 or 400 mL volumes, for later separations only 100 mL volumes were used. The fractions were collected in 20 mL glass vials and placed in the fume hood to evaporate to dryness and then stored at 4°C until further use.

Thin layer chromatography (TLC) was used to determine if the eluted fractions contained the same compounds. In order to decrease the total number of fractions to be tested for antibacterial activity, the fractions that contained the same compounds were combined. A minimal amount of ethyl acetate (EtOAc) or methanol was used in order to re-dissolve the dry eluted fractions of each extract. Samples of re-dissolved fractions were spotted on plastic-backed 20x20 cm, 0.20 mm thick silica gel 60 with fluorescent indicator UV_{254} TLC plates (Machray-Nagel; Easton, PA) until visible under UV_{254} light. Based on the composition of the solvent that eluted each particular fraction from the silica gel column, a solvent system was prepared for resolving the TLC. The TLC strips were placed in the

solvent chamber and the solvent was allowed to migrate up the TLC strip until it reached a level 0.5 cm from the top. The TLC strips were then examined under a UV lamp to locate the resolved compounds and to determine the mobility of the compounds. If the compounds did not migrate as desired the solvent system was altered (Appendices 5-8). When satisfactory migration had occurred the TLC plates were visualized by spraying the TLC plates with a 10% (v/v) sulfuric acid solution, then charring them with a heat gun until the compound spots appeared. The migrations of the compounds in each fraction were compared with the neighbouring fractions. If there were compounds common to a group of fractions, then these fractions were combined into a single superfraction.

Antibacterial growth inhibition activity assay

Antibacterial growth inhibition activity is often determined by the disc diffusion assay. In this assay the extracts are loaded onto paper discs, which are then transferred to agar plate growth medium seeded with the test bacterium. Following the incubation period, growth inhibition is observed as a clear zone surrounding each disc. This method is frequently used for the rapid identification of compounds with antibacterial activity. The crude extracts and superfractions were all used in a disc diffusion assay to examine for the presence of bacterial growth inhibition. The following organisms were obtained from the American Type Culture Collection (Rockland, MD) for use in the antibacterial growth inhibition assay based on their medical importance: *Staphylococcus aureus* (ATCC 25423), *Enterococcus faecalis* (ATCC 19433), *Eschericia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). Overnight cultures of the four bacterial species were grown on Müeller-Hinton II (VWR; Mississauga, ON) agar plates at 37°C and were suspended in test tubes containing 2 mL sterile water to the same optical density as a

McFarland Standard No. 1, representing approximately 2.0 x 10^8 colony-forming units (CFU)/mL (National Committee for Clinical Laboratory Standards, 1995). Sterile cotton swabs were immersed into the inoculum and the excess fluid was removed by pressing firmly against the side of the test tube. Müeller-Hinton II agar plates were swabbed by streaking the entire surface twice with a 60° rotation between each swab (Qaiyumi, 2007). The superfractions were brought to a concentration of 50 mg/mL in either EtOAc or methanol and 5 µL (250 µg) was loaded onto sterile paper discs [6mm] (BBL; Sparks, MD). A loading control was also prepared containing 10 µL of methanol. The discs were aseptically applied to the inoculated plates. Three control plates were prepared for each of the bacteria in order to ensure consistency of the results, each containing the positive controls: ciprofloxacin [5 µg] and ceftriaxone [30 µg] (both BD BBL; Sparks, MD); as well as a loading control and a blank sterile disc. All of the plates were incubated in a 37°C incubator for 24 hours. Following the incubation the plates were visually examined for any bacterial growth inhibition and any zones of inhibition detected were measured (Diagrammed in Figure 5).

Viability testing of *P. aeruginosa* zones

Many superfractions of various extracts did not produce a zone of growth inhibition when tested against *P. aeruginosa*; however, they still appear to show some form of activity against *P. aeruginosa* as evident from the ring of morphologically different growth surrounding the disc (see Figure 4, Results). In order to determine the viability of the bacteria in these zones a subculture was obtained from around the disc and inoculated onto Müeller-Hinton agar. The subculture was obtained by scraping a sterile loop across the plate adjacent to the disc within the morphologically different region. As a control the

subculturing was also performed from beside the blank disc as well as from areas adjacent to each of the control antibiotic discs within the clear region of growth inhibition. Since ciprofloxacin and ceftriaxone are both bactericidal, there should be no viable cells within their inhibition zones. The plates were then incubated at 37°C for 24 hours and then examined for the presence of zones growth inhibition.

Identification of the compound in superfractions exhibiting antibacterial activity

The superfractions which demonstrated antibacterial activity contained a mixture of compounds based on TLC analysis. To determine which compound in the superfraction was responsible for the antibacterial activity a TLC diffusion growth inhibition assay was performed. Fifty micrograms of selected superfractions (25 µg for superfraction 16 from *Molpadia sp.*) was spotted onto duplicate TLC plates. The TLC plates were developed in the solvent system that obtained the desired resolution during the combination stage. One of the plates was visualized with 10% (v/v) sulfuric acid followed with heat; the second plate was to be used in the antibacterial growth inhibition assay. A TLC plate was also loaded with 50 µg (10 µg for superfraction 16 from *Molpadia sp.*) of each of the samples but was not subjected to a solvent system. A blank, control TLC plate was also used to determine the effects of the silica on bacterial growth. The plates were placed in sterile Petri dishes and a thin layer of sterile Müeller-Hinton agar was poured over the surface of the plates in order to allow the compounds to diffuse into the agar. A control ciprofloxacin disc was also placed in a Petri dish to have agar poured over top to determine its effects. Once the agar had sufficiently solidified, the bacterial culture (as prepared previously to a McFarland Standard 1) was swabbed over the entire surface of the agar. The plates were incubated at 37°C for 24 hours. Following incubation the plates

were stained with the red dye safranin and observed for the location of the zone of growth inhibition of the test bacterium on the agar above the TLC strip where the active compounds diffused to inhibit the growth. The location of the zone of inhibition is reflective of the mobility of the active compound. By examining the resolved duplicate TLC plate the location of the active superfraction could be determined relative to other compounds in the superfraction.

The TLC diffusion growth inhibition assay was repeated for superfraction 16 from *Molpadia sp.* with decreasing amounts loaded to determine minimal loading inhibitory amount. This superfraction had the following amounts loaded onto the TLC plate: 25 µg, 12.5 µg, 6 µg and 3 µg. The plate was developed in 65% (v/v) EtOAc in hexane and then the assay procedure above was repeated. Following the 24-hour incubation period the plate was analyzed for the inhibition of growth.

Determination of minimum inhibitory concentration (MIC) of the superfractions

The same superfractions that demonstrated antibacterial activity and that were used to locate the active compounds on TLC plates were also used to determine the MIC (growth inhibition) of the superfraction using a 96-well microtitre plate. Two-fold dilutions of the selected superfractions were performed such that the final concentration in the inoculum would be: 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.75 µg/mL, 3.38 µg/mL, 1.69 µg/mL, 0.84 µg/mL, 0.42 µg/mL. Into the first well of the microtitre plate, 100 µL of a 200 µg/mL methanol solution of the superfraction was added. Wells 2-9 were filled with 50 µl of methanol and a series of two-fold dilutions were performed by removing 50 µL of the superfraction and adding it to 50 µL of methanol in the next well. This was

continued until the lowest concentration was reached (filling nine wells). The excess 50 μL in the ninth well was discarded. The tenth well contained 50 μL of methanol to act as a solvent control. The plate was then left in the fume hood to allow the methanol to evaporate. The dried superfractions were then resuspended in 100 μL of *S. aureus* inoculum. An overnight culture of *S. aureus* grown on Müeller-Hinton II agar plates at 37°C was suspended in a test tube containing 2 mL sterile water to the same optical density as a McFarland Standard No. 0.5, representing approximately 1.0 x 10^8 CFU/mL (Qaiyumi, 2007). This suspension was then diluted 1000-fold (10^5 CFU/mL) to serve as the inoculum. The tenth well was a growth control and contained 100 μL of the inoculum and the final well was a sterility control containing 100 μL of sterile Müeller-Hinton broth. This procedure was repeated for each of the superfractions to be tested. Several control wells also served as positive growth inhibition controls where the inoculum was supplemented with 15 μg/mL tetracycline HCl (Sigma; St. Louis, MO). The plate was covered with sterile aluminum foil and placed in a zip-lock bag to be incubated at 37°C for 24-hours. Following the 24-hour incubation the plate was removed from the incubator and vigorously shaken to suspend the cells within each of the wells. The plate was visually observed and the wells where no bacterial growth was observed were noted. The MIC assay was repeated for superfractions that did not show any growth inhibition at 100 μg/mL. When repeated the highest concentration used was 12.8 mg/mL and was again decreased two-fold to fill the nine experimental wells (12.8 mg/mL, 6.4 mg/mL, 3.2 mg/mL, 1.6 mg/mL, 0.8 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL and 0.05 mg/mL).

Results

Preparation of crude extracts of marine organisms

The dry weights and the dry extract weights of each of the 5 benthic sessile marine organisms are given in Table 1.

Table 1. Summary of the starting dry weight and final extract weight of each of the five organisms with the per cent yield with the total extraction volume of methanol indicated.

Sample	Dry Weight (g)	Extract Weight (g)	% Dry Weight Yield	Volume of Methanol (mL)
Pannychia moseleyi	56.06	21.06	37.6	870
Scotoplanes sp.	71.21	14.23	20.0	660
Paelopadites confundens	30.73	12.26	39.9	1080
Molpadia sp.	64.62	8.12	12.6	900
Actinarian	77.41	12.53	16.2	900

Crude extract antibacterial growth inhibition assay

The crude extracts were initially assayed for antibacterial activity in a disc diffusion growth inhibition assay. After incubation, the plates were observed for any zones of growth inhibition against the 4 bacterial test species (Table 2).

Table 2. Antibacterial activity of crude methanol extracts of 5 deep sea benthic sessile organisms obtained from a disc diffusion assay with 250 μg of each of the 5 samples loaded onto each disc.

	Test Bacterial Species			
Sample	***S. aureus***	***E. faecalis***	***P. aeruginosa***	***E. coli***
Pannychia moseleyi	–	–	*	–
Scotoplanes sp.	–	–	*	–
Paelopadites confundens	–	–	*	–
Molpadia sp.	–	–	*	–
Actinarian	–	–	–	–

No observed growth inhibition is indicated with a (–). Ring of morphologically different growth (*)

No zones of growth inhibition were observed against *E. coli*, *E. faecalis*, *P. aeruginosa* or *S. aureus* with an assay using 250 μg of crude methanol extracts of the 5 organisms. *P. aeruginosa* characteristically shows a dull green appearance when grown on Müeller-Hinton agar. While the growth of *P. aeruginosa* maintained its green colour, all four holothurian extracts caused the growth to appear lustrous around the impregnated discs as

opposed to its dull characteristic. This change in *P. aeruginosa* morphology was not observed surrounding the disc impregnated with the actinarian extract.

Bioassay-guided fractionation of the crude extracts by silica gel chromatography

A bioassay-guided fractionation of each of the 5 crude extracts was performed by liquid column chromatography on silica gel. The liquid chromatography of the crude methanol extract of *Pannychia moseleyi* resulted in a total of 165 eluted fractions. TLC analysis of each fraction showed that the samples could be combined into 14 superfractions based on the presence of common compounds as revealed by mobility of spots on the TLC plates (Appendix 5). The total dry weights of the superfractions was 10.01 g. Comparing this weight to the dry extract weight of 21.06 g loaded onto the column shows that the per cent recovery from the column chromatography was 47.48% (Table 1).

Silica liquid chromatography separated the crude methanol extract of *Scotoplanes sp*.into 144 which were combined into 14 superfractions following TLC analysis (Appendix 6). The total weight of the compounds recovered in the superfractions was 8.51 g. Comparing this weight to the dry extract weight of 14.23 g loaded onto the column shows that the per cent recovery from the column chromatography was 59.73% (Table 1).

Silica liquid chromatography separated the crude methanol extract of *Paelopadites confundens* into 81 fractions which were later combined into 9 superfractions following TLC analysis (Appendix 7). The weight of dry extract that was loaded onto the column was 12.26 g; however, the total dry weight of the compounds recovered in the superfractions was 28.54 g (Table 1). Two of the superfractions contained a white

emulsion that trapped the solvent. These two superfractions were dried for 5 days with intermittent stirring and were still not completely dry.

Silica gel liquid chromatography resolved the crude methanol extract of *Molpadia sp*. into 85 fractions and TLC analysis led to these 85 fractions being combined into 11 superfractions (Appendix 8). The total dry weights of the superfractions was 7.59 g. Comparing this weight to the dry extract weight of 8.12 g loaded onto the column shows that the per cent recovery from the column chromatography was 93.5% (Table 1).

Antibacterial activity contained in the superfractions from each of the holothurians

None of the superfractions of the extract of *Pannychia moseleyi* demonstrated any growth inhibition activity against *P. aeruginosa*, *E. coli* or *E. faecalis*. However, five of the superfractions contained low levels of growth inhibition activity against *S. aureus* (Table 3).

All but one of the superfractions showed some type of activity against *P. aeruginosa*. A zone of growth inhibition around the discs impregnated with these superfractions was not observed; however, *P. aeruginosa* was definitely affected showing a morphologically different lustrous appearance. Wide zones of growth inhibition were observed around the ceftriaxone and ciprofloxacin discs for all 4 bacterial species. No growth inhibition activity was observed from the solvent loading control and blank discs.

Table 3. Antibacterial activity of the superfractions from the extracts of *Pannychia moseleyi*. Three plates for each organism were used as controls with each containing a ciprofloxacin, ceftriaxone, loading and blank control.

	Test Bacterial Species			
Fraction	***S. aureus***	***E.coli***	***E. faecalis***	***P. aeruginosa***
27	—	—	—	slight lustrous growth
36	—	—	—	slight lustrous growth
45	9	—	—	slight lustrous growth
50	—	—	—	slight lustrous growth
56	—	—	—	slight lustrous growth
66	—	—	—	slight lustrous growth
89	8	—	—	slight lustrous growth
100	9	—	—	slight lustrous growth
127	9	—	—	slight lustrous growth
132†	10	—	—	slight lustrous growth
138*	—	—	—	lustrous growth
149	—	—	—	slight lustrous growth
153	—	—	—	—
156	—	—	—	slight lustrous growth
Cipro	29.7 ± 1.2	42.7 ± 1.2	22.0 ± 1.0	35.7 ± 0.6
Ceftri	24.7 ± 1.2	34.3 ± 1.5	12.3 ± 0.6	21.0 ± 0.0
Loading	—	—	—	—
Blank	—	—	—	—

No growth inhibition is indicated with a (—), numerals indicate the diameter (mm) of a zone of growth inhibition. *The indicated superfraction(s) was used for further tests against *P. aeruginosa*. †The indicated superfractions were further tested on *S. aureus* for the location of the zone on TLC as well as for MIC determination.

Growth inhibition activity against *S. aureus* was observed in five of the superfractions from the *Scotoplanes sp*. extract, each with a diameter of 9 mm (Figure 3). Two concentric zones of growth inhibition were observed around the discs containing superfraction 65, the outer zone of weak growth inhibition measuring 12 mm in diameter. A yellow colour in the zone of inhibition produced around discs impregnated with superfractions 86 and 91 is most likely evidence of a compound diffusing in the agar medium. Wide zones of growth inhibition were observed around the ceftriaxone and ciprofloxacin discs for all 4 bacterial species. No growth inhibition activity was observed from the solvent loading control and blank discs.

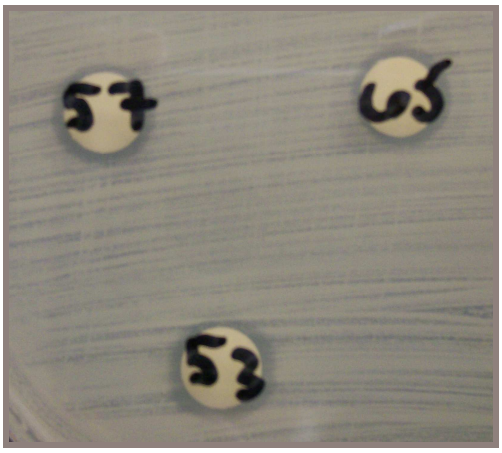

Figure 3. Demonstration of zones of growth inhibition surrounding discs impregnated with three superfractions of *Scotoplanes sp*. against *S. aureus*.

Eleven of the *Scotoplanes sp.* extract superfractions demonstrated the same zones of morphologically different lustrous growth against *P. aeruginosa* as did the *Pannychia moseleyi* superfractions. More *Scotoplanes sp.* superfractions showed a wide region of luster morphology surrounding the discs (Figure 4).

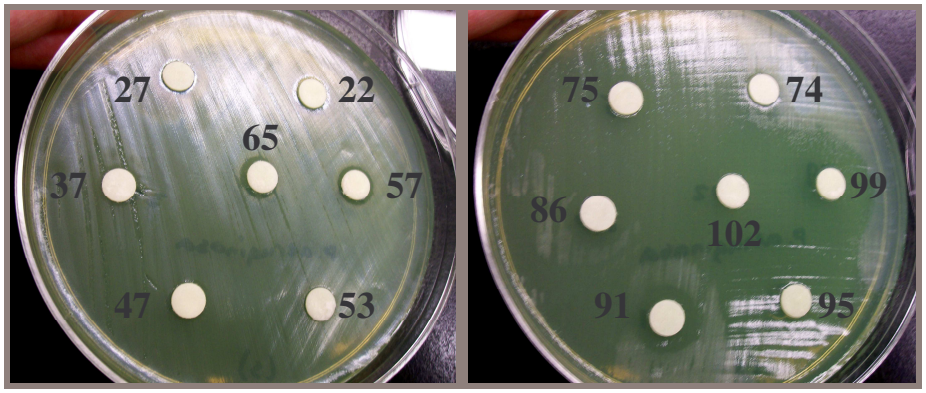

Figure 4. Zones of morphologically distinct lustrous growth of *P. aeruginosa* produced by superfractions of *Scotoplanes sp*. Numbers indicate the superfraction impregnated in each disc.

None of the superfractions from the extract of *Scotoplanes sp*. demonstrated any growth inhibition against *E. coli* or *E. faecalis* (Table 4).

Of the nine superfractions of the extract of *Paelopadites confundens*, two demonstrated weak growth inhibition activity against *S. aureus* with diameters of 8 mm each. There was no growth inhibition activity against *E. coli* or *E. faecalis* in any of the superfractions

of the *Paelopadites confundens* extract. Five of the superfractions of the *Paelopadites confundens* extract showed wide luster morphology zones against *P. aeruginosa* (Table 5).

Table 4. Antibacterial activity of the superfractions from the extracts of *Scotoplanes sp*. Three plates for each organism were used as controls with each containing a ciprofloxacin, ceftriaxone, loading and blank control.

	Test Bacterial Species			
Fraction	***S. aureus***	***E.coli***	***E. faecalis***	***P. aeruginosa***
22 *	—	—	—	lustrous growth
27	—	—	—	slight lustrous growth
37	—	—	—	—
47	—	—	—	—
53	9	—	—	—
57 † *	9	—	—	lustrous growth
65 † *	9 (weak 12)	—	—	lustrous growth
74	—	—	—	slight lustrous growth
75 *	—	—	—	lustrous growth
86 *	9 (yellow)	—	—	lustrous growth
91 *	9 (yellow)	—	—	lustrous growth
95 *	—	—	—	lustrous growth
99 *	—	—	—	lustrous growth
102 *	—	—	—	lustrous growth
Cipro	29.0 ± 0.0	43.3 ± 0.6	22.3 ± 1.2	35.7 ± 0.6
Ceftri	23.0 ± 0.0	37.7 ± 0.6	16.3 ± 0.6	21.0 ± 0.0
Loading	—	—	—	—
Blank	—	—	—	—

No growth inhibition is indicated with a (—), numerals indicate the diameter (mm) of a zone of growth inhibition. *The indicated superfraction(s) was used for further tests against *P. aeruginosa*. †The indicated superfractions were further tested on *S. aureus* for the location of the zone on TLC as well as for MIC determination.

No superfraction of the extract of *Molpadia sp*. contained any growth inhibitory activity against *E. coli* or *E. faecalis*. However, six of the superfractions demonstrated activity against *P. aeruginosa* as revealed by a wide zone of luster morphology around the discs.

Growth inhibition was observed from three of the superfractions against *S. aureus*. Two of the zones of growth inhibition were relatively small at 9 mm in diameter while superfraction 16 demonstrated considerable activity with a zone diameter of 21 mm (Table 6, Figure 5).

The zone of growth inhibition caused by superfraction 16 is comparable to the zone of inhibition produced by the ceftriaxone disc implying a potential use in antibacterial therapy.

Table 5. Antibacterial activity of the superfractions from the extracts of *Paelopadites confundens*. Three plates for each organism were used as controls with each containing a ciprofloxacin, ceftriaxone, loading and blank control.

	Test Bacterial Species			
Fraction	***S. aureus***	***E.coli***	***E. faecalis***	***P. aeruginosa***
1	—	—	—	—
9 *	—	—	—	lustrous growth
24 †	8	—	—	lustrous growth
32	8	—	—	—
60 *	—	—	—	lustrous growth
65	—	—	—	lustrous growth
68	—	—	—	lustrous growth
76	—	—	—	—
82 *	—	—	—	lustrous growth
Cipro	29.3 ± 0.6	42.7 ± 1.2	22.0 ± 1.0	38.0 ± 1.0
Ceftri	25.0 ± 1.0	34.3 ± 1.5	12.3 ± 0.6	21.0 ± 0.0
Loading	—	—	—	—
Blank	—	—	—	—

No growth inhibition is indicated with a (—), numerals indicate the diameter (mm) of a zone of growth inhibition.
*The indicated superfraction(s) was used for further tests against *P. aeruginosa*. †The indicated superfractions were further tested on *S. aureus* for the location of the zone on TLC as well as for MIC determination.

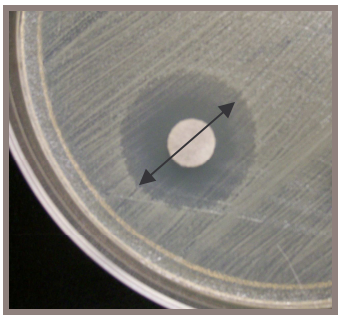

Figure 5. Representation of the large, 21 mm zone of growth inhibition against *S. aureus* produced by superfraction 16 from *Molpadia sp*. * The arrow indicates where the zone of inhibition was measured.

The solvent system compositions that were used to elute the superfractions showing antibacterial activity are summarized in Figure 6.

Table 6. Antibacterial activity of the superfractions from the extracts of *Molpadia sp*. Three plates for each organism were used as controls with each containing a ciprofloxacin, ceftriaxone, loading and blank control.

	Test Bacterial Species			
Fraction	***S. aureus***	***E.coli***	***E. faecalis***	***P. aeruginosa***
7	—	—	—	—
9	—	—	—	—
16 †	21	—	—	—
31 †	9	—	—	—
43	9	—	—	—
49 *	—	—	—	lustrous growth
58 *	—	—	—	lustrous growth
60 *	—	—	—	lustrous growth
61 *	—	—	—	lustrous growth
65 *	—	—	—	lustrous growth
70 *	—	—	—	lustrous growth
cipro	29.3 ± 0.6	42.7 ± 1.2	22.0 ± 1.0	38.0 ± 1.0
ceftri	25.0 ± 1.0	34.3 ± 1.5	12.3 ± 0.6	21.0 ± 0.0
loading	—	—	—	—
blank	—	—	—	—

No growth inhibition is indicated with a (—), numerals indicate the diameter (mm) of a zone of growth inhibition.
*The indicated superfraction(s) was used for further tests against *P. aeruginosa*. †The indicated superfractions were further tested on *S. aureus* for the location of the zone on TLC as well as for MIC determination.

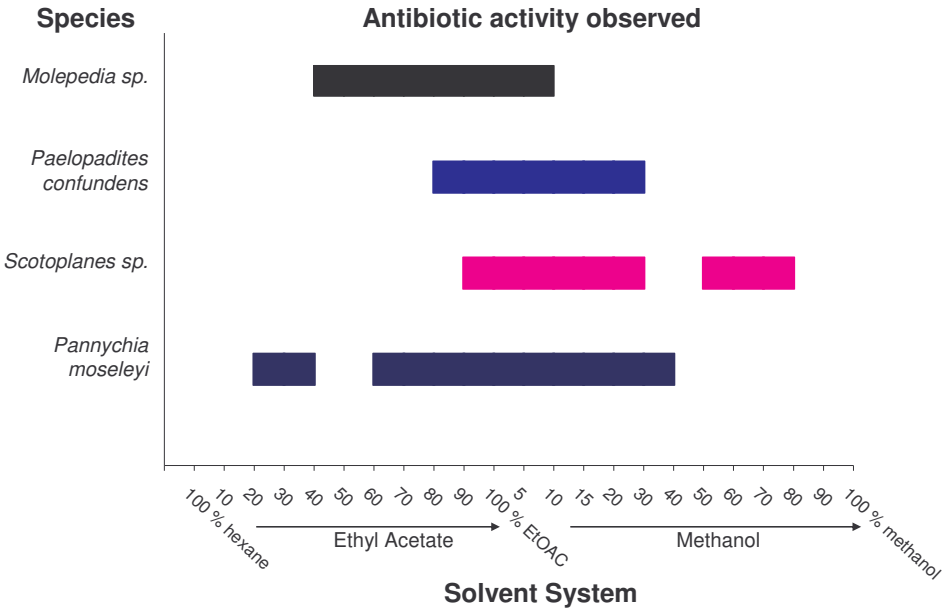

Figure 6. Representation of the solvent ranges that eluted the antibacterial superfractions for each of the four holothurian extracts. The bars correspond to the solvent composition that was used to elute each of the superfractions containing antibacterial growth inhibition activity against *S. aureus* for each of the four holothurian extracts.

Viability testing of *P. aeruginosa* in the zone of altered morphology

All of the zones of morphologically distinct growth of *P. aeruginosa* surrounding discs impregnated with various superfractions of the extracts from the four holothurians demonstrated the same lustrous appearance of *P. aeruginosa* growth (Figure 7).

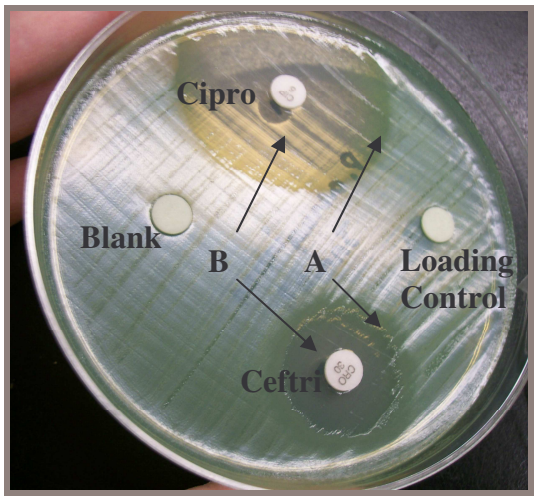

Figure 7. A picture demonstrating the lustrous growth (A) of *P. aeruginosa* at the periphery of the zone of inhibition (B) surrounding the ciprofloxaxin (cipro) and ceftriaxone (ceftri) on Müeller-Hinton media incubated at 37°C for 24 hours.

In order to determine whether the *P. aeruginosa* cells within this zone of altered growth morphology were still viable, an inoculating loop was used to transfer *P. aeruginosa* cells from an area adjacent to the discs containing the activity onto a medium which supports the growth of *P. aeruginosa*, Müeller-Hinton agar. *P. aeruginosa* cells were also transferred from the medium adjacent to the two antibiotic control discs within the clear zone of inhibition, as well as adjacent to the blank disc onto Müeller-Hinton agar. All these areas contained viable cells as shown by growth on Müeller-Hinton with exception to the areas from the zone of inhibition on the ciprofloxacin and ceftriaxone discs.

It was noted that the same lustrous appearance caused by these active superfractions of holothurian extracts was present at the edges of the zones of growth inhibition around the

antibiotic discs (Figure 7). To determine whether a greater amount of extract would cause a zone of inhibition the superfractions indicated in Table 7 were re-assayed with 500 µg and 1 mg loaded onto each disc. Following the incubation of the plates for any superfraction no zones of inhibition were observed (Table 7).

Table 7. The observed effects of increasing the amount of extract to 500 µg and 1 mg of selected superfractions from each of the four holothurians on *P. aeruginosa*. Superfractions from the different organisms are differentiated by abbreviations – PC (*Paelopadites confundens*), M (*Molpadia sp.*), S (*Scotoplanes sp*.), PM (*Pannychia moseleyi*).

	Weight of Extract Applied to Disc	
Fraction	**500 µg**	**1 mg**
138 PM 49M 57M 60M 61M 65M 70M	whole plate lustrous	whole plate lustrous
9PC	—	weak lustrous 3
60PC	lustrous, 19	strong lustrous
82PC	lustrous, 9	strong lustrous
22S	weak lustrous, 6	lustrous between 9 and 15
57S	lustrous, 1.5	lustrous, 2
65S	lustrous, 1.5	lustrous, 3
75S	lustrous, 4	
86S	lustrous, 5	whole plate lustrous with evidence of compound around the disc
91S	lustrous, 5	
95S	spotted lustrous 7	
99S	spotted lustrous ~6	
102S	spotted lustrous ~ 5	
Cipro	17.0 ± 0.6	
Ceftri	7.0 ± 0.6	
Blank Disc	—	
Loading Control	—	

No growth inhibition is indicated with a (—), numerals indicate the diameter (mm) of a zone of growth inhibition.

The lustrous appearance of *P. aeruginosa* growth over the entire surface of several of the plates was observed. Discs that had definite zones when 500 µg was applied showed zones often only slightly larger when 1 mg was applied per disc. Superfraction 9 from

Paelopadites confundens did not show any zone when 500 µg was applied but a zone of morphologically different growth was observed with the 1 mg application.

Attempt to isolate the active compound in the superfractions

A TLC diffusion growth inhibition assay against *S. aureus* was used to locate the active compound in several superfractions demonstrating antibacterial activity. Following 24-hours incubation the inoculated agar overlaying the TLC plates were observed to locate the zone of *S. aureus* growth inhibition. The growth control TLC plate had abundant *S. aureus* growth on the agar and the plate. A zone of inhibition of 32 mm was observed on the plate with the ciprofloxacin disc on top of the media; when the agar was poured over the ciprofloxacin disc the zone was increased to 39 mm. The spot control of unresolved superfraction—which tested for the presence of inhibition from the superfractions prior to separation—showed inhibition around each of the points where the superfractions were applied to the TLC plate (Figure 8).

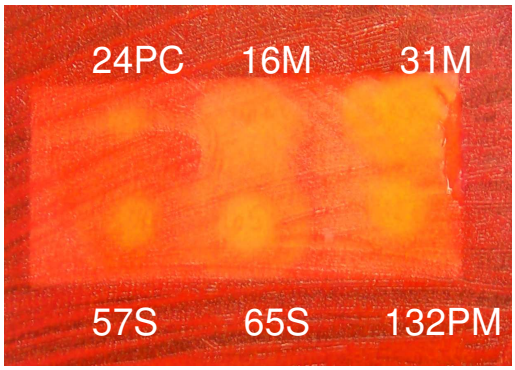

Figure 8. TLC spot no chromatography control plate with Müeller-Hinton overlay inoculated with *S. aureus* and incubated at 37°C for 24 hours. Superfractions from the different organisms are differentiated by abbreviations – PC (*Paelopadites confundens*), M (*Molpadia sp.*), S (*Scotoplanes sp.*), PM (*Pannychia moseleyi*).

Several superfractions, particularly 65 from *Scotoplanes sp*., appear to have concentric zones of inhibition surrounding the spot of application. In fact, when the compounds in

superfraction 65 from *Scotoplanes sp.* were resolved with TLC, three zones of inhibition were observed (Figure 9).

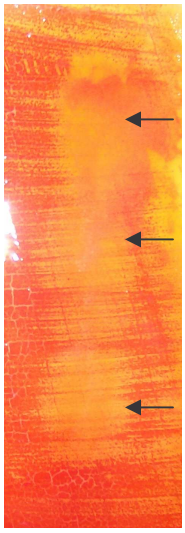

Figure 9. TLC plate of superfraction 65 from *Scotoplanes sp.* with Müeller-Hinton overlay inoculated with *S. aureus* and incubated at 37°C for 24 hours. The three regions of growth inhibition are indicated with the arrows.

The chromatographed compounds from superfractions 31 from *Molpadia sp*., 57 from *Scotoplanes sp*., 24 from *Paelopadites confundens*, and 132 from *Pannychia moseleyi* all showed growth inhibition without resolved areas of growth inhibition apparent. There appeared to be inhibition along the entire separation path of compounds in each compound on the TLC (Figure 10).

Figure 10. TLC plate of superfractions 31 from *Molpadia sp*. and 57 from *Scotoplanes sp*. with Müeller-Hinton overlay inoculated with *S. aureus* and incubated at 37°C for 24 hours. Note the inhibition of zone along the entire chromatography migration line.

Superfraction 16 from *Molpadia sp*. demonstrated the strongest antibacterial activity of all superfractions in the disc diffusion growth inhibition assay. TLC of superfraction 16 from *Molpadia sp*. demonstrated a continuous zone of growth inhibition with a prominent area of growth inhibition at the top of the migration path (Figure 11).

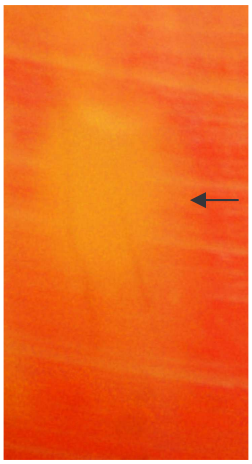

Figure 11. TLC diffusion assay of superfraction 16 from *Molpadia sp*. with Müeller-Hinton overlay inoculated with *S. aureus* and incubated at 37°C for 24 hours. Note the large zone of inhibition indicated by the arrow.

Superfraction 16 from *Molpadia sp.* was re-assayed with the TLC diffusion method with decreasing amounts of compound loaded. Following the incubation the lanes on the TLC plate that were loaded with 25 µg and 12.5 µg of sample displayed two distinct zones of inhibition (Figure 12). The zone from the more polar (lower) compound was no longer visible when 6 µg or 3 µg were applied. These two applications still demonstrated zones of inhibition, though of decreasing size, for the less polar (upper) compound.

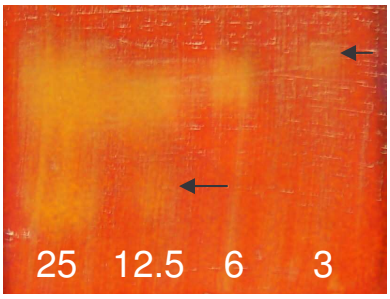

Figure 12. TLC diffusion assay for superfraction 16 from *Molpadia sp*. with Müeller-Hinton overlay inoculated with *S. aureus* and incubated at 37°C for 24 hours. The amount of compound loaded is indicated (µg). The upper arrow is indicating the small zone of inhibition for 3 µg, the lower arrow is indicating the migration of the polar compound showing inhibition.

Determination of the MIC of the various superfractions

Six superfractions were chosen based on their activity against *S. aureus*—superfractions 16 and 31 of *Molpadia sp*., superfraction 24 from *Paelopadites confundens*, superfractions 57 and 65 from *Scotoplanes sp*., and 132 from *Pannychia moseleyi* for MIC determination. Following the 24-hour incubation the microtitre plates were observed visually. No growth inhibition was observed for any concentration of any superfraction even at 100 µg/mL. The assay was repeated with the concentration of five of the superfractions increased to 12.8 mg/mL (superfraction 16 from *Molpadia sp*. was increased to 200 µg/mL due to the limited amount of sample) (Table 8).

Table 8. MIC of six selected superfractions of holothurian extracts against *S. aureus*.

Fraction	MIC (µg/mL)
16M	>200
31M	3200
24PC	3200
57S	800
65S	400
132PM	6400

Discussion

Holothurians and actinarians are prospective candidates for antibacterial chemical production; however their exploration has been extremely limited. The hypothesis of my thesis predicted the presence of antibacterial natural products from holothurians and actinaria. Their lack of a cellular immune system makes these invertebrates ideal organisms for antibacterial natural product discovery since they are bathed in a sea of bacteria. Furthermore, these compounds would most likely be novel as the holothurians and actinarian were obtained from a trawl depth of 1000 to 1400 m. The results of this research indicated that each of the holothurians studied contained several antibacterial compounds against a Gram positive bacterium.

The crude methanol extracts obtained gave relatively high per cent yields when compared to the sample dry weights. These extracts are assumed to contain sea salt, whose solubility in 95% (v/v) methanol is 1.375 (wt%) (Pinho and Macedo, 1996). It is likely that the solubility of the salt in methanol was decreased because the methanol solution contained many other natural product compounds. While the crude extracts may contain salt, this salt was most likely removed during the silica liquid chromatography of the methanol extracts.

The initial disc diffusion assay of the crude extracts showed non-growth-inhibitory activity against *P. aeruginosa* for each of the holothurian samples. The crude actinarian extract failed to show any activity against the test organisms. The lack of activity may be attributed to the minimal amount of extract loaded onto the discs. The crude extracts contain a massive quantity of natural products, diluting out the presumed antibacterial

compounds. Previous studies examining the antibacterial activity of soft corals and plants produced inhibition against *E.coli* and *S. aureus* when 1 mg and 10 mg respectively of crude extract was used to detect inhibition, four and forty times the amount loaded in this study (Kelman *et al*, 1998; Ali-Shtayeh *et al*, 1998). Based on the presence of antibacterial activity following fractionation of the holothurians, the actinarian most likely contained some antibacterial compounds that were not detectable. Moreover, studies have shown that actinaria have specialized cells that contain antibacterial activity against Gram negative bacteria, indicating their low overall concentration in the crude extract (Hutton and Smith, 1996).

The four holothurian methanol extracts were further fractionated and then re-assayed for antibacterial activity. The recovery of extract following silica liquid chromatography was relatively high. Much of the lost extract was likely the washing out of salts present in the crude methanol extract. The higher recovery percent for *Molpadia sp*. could be attributed to the smaller column diameter resulting in a narrower region for the elution of compounds.

The superfractions from the four holothurians were tested for antibacterial activity and each specimen was shown to contain compounds active against *S. aureus*. Previous studies have also shown that the largest proportion of extracts show activity against *S. aureus* (Thompson *et al.*, 1985; Ali-Shteyah *et al.*, 1998). Other researchers only used diameters of 10 mm or larger as actual zones of growth inhibition (Bergquist and Bedford, 1978); this study considered any zone around the disc as inhibition because a

critical factor in determining zone size is the ability of the compound to diffuse through the medium (Thompson *et al*., 1985).

Analysis of the overlap of the solvent systems in conjunction with the TLC analysis of the individual fractions allows the estimation of the minimum number of active compounds. *Pannychia moseleyi* had the active superfractions elute in two disjoined solvent blocks, indicating that there is a minimum of two active compounds isolated from this organism. The TLC strip used for the combination of the fractions into superfraction 45 shows the presence of a compound that was not present in either the previous superfraction or the following superfraction. The TLC strips for the fractions combined into superfraction 89 through to superfraction 132 indicates that the presence of at least two compounds in all fractions. This indicates that the same one or two compounds could be responsible for the activity in all four superfractions. The following superfraction 138 did not display any antibacterial activity; however TLC analysis showed the presence of the same two compounds as the previous superfraction. This is explained by a large quantity of other compounds also eluted from the column in these fractions and most likely diluted the active compounds below their inhibitory concentration.

The distribution of the active superfractions from *Scotoplanes sp.* indicates that there is a minimum of two antibacterial compounds in the crude methanol extract. The TLC performed on the individual *Scotoplanes sp.* fractions shows a persistent compound in the three superfractions 53, 57 and 65. The weak outer zone in superfraction 65 is most likely due to a second compound; TLC analysis shows spots that are unique in this superfraction. Superfractions 86 and 91 show a strong overlap of similar compounds

when observing the TLC strips, indicating that the antibacterial activity in each superfraction is most likely due to the same compound. These analyses indicate that the methanol extract from *Scotoplanes sp.* contains a minimum of 3 antibacterial compounds.

Two *Paelopadites confundends* superfractions contained antibacterial activity. The overlap of the solvent systems of these two superfractions as well as TLC analysis indicates that the activity is most likely due to one common compound.

Three *Molpadia sp.* superfractions contained antibacterial activity against *S. aureus*. From the TLC analysis of the individual fractions it appears that superfractions 16 and 31 contain a similar compound which most likely contains the antibacterial activity. However, as will be discussed later, it was determined that superfraction 16 contains two compounds with antibacterial activity against *S. aureus*. The TLC analysis indicates that the antibacterial compound in superfraction 43 is most likely due to a different compound not found in the other two superfractions. From this it was determined that the minimum number of antibacterial compounds from the *Molpadia sp*. methanol extract is three.

Six of the superfractions with antibacterial activity against *S. aureus* were used to determine the MIC. The lowest MIC was obtained for superfraction 65 from *Scotoplanes sp.* and the highest MIC was obtained for superfraction 132 from *Pannychia moseleyi*. These MIC values were relatively high as compared to a study examining sponge natural products (Torres *et al.*, 2002); however the MIC values obtained here are probably much higher than the actual MIC values for the pure antibacterial compounds. Since the superfractions contain many other compounds, the antibacterial compound could be present in very small relative quantities. The actual concentration of the antibacterial

compound in each extract is most likely significantly lower than the MIC determined for the crude extract or superfraction.

Several of the superfractions showing antibacterial activity against *S. aureus* were analyzed with a TLC diffusion growth inhibition assay. This method has been shown to be efficient in identifying novel growth inhibition compounds (Wedge and Nagle, 2000). The superfraction 65 from *Scotoplanes sp.* appeared to show three zones of inhibition, indicating that most likely three separate compounds in this superfraction contained antibacterial activity. As previously mentioned, superfraction 65 from *Scotoplanes sp*. had the lowest MIC, most likely due to the presence of three antibacterial growth inhibitory compounds in the superfraction. Superfraction 16 from *Molpadia sp*. showed a very pronounced zone of inhibition; and when a smaller amount was loaded onto the plate two separate antibacterial growth inhibitory compounds could be resolved from the TLC diffusion growth inhibition assay.

No inhibition was observed against *E. coli*, *P. aeruginosa* or *E. faecalis* in any of the superfractions tested. Previous studies have shown that very few natural product extracts contain compounds that are inhibitory against these three organisms (Kartal *et al*, 2003). Essawi and Srour (2002) found that, for *E. coli*, *P. aeruginosa* and *E. faecalis*, a hole-plate diffusion method was frequently more effective at identifying antibacterial compounds. This method for antibacterial testing allows for larger amounts of compounds to be tested. There are fewer antibiotics against Gram negative bacteria such as *P. aeruginosa* and *E. coli* besides the broad spectrum antibiotics (Prescott *et al.*, 2005). Being Gram negatives, *E. coli* and *P. aeruginosa* both possess outer membranes which

act as an effective permeability barrier against a large number of noxious agents (Boucabeille *et al*, 1997). The few narrow spectrum antibiotics against Gram negative bacteria, for example gentamycin and polymyxin B, are associated with occasional serious side effects such as renal damage (Prescott *et al.*, 2005). Many enterococci, such as *E. faecalis* are tolerant to the killing effects of cell-wall active agents, including ampicillin and vancomycin; recent data suggest that this property may not be inherent, but rather acquired after exposure to antibiotics (Murray, 1998). The propensity of *E. faecalis* to acquire multiple antibiotic-resistance traits may result from a variety of distinctly different mechanisms for conjugation (Murray, 1998). Moreover, antibacterial activity screening against *E. faecalis* seems unreliable and very method dependent (Naijar and Murray, 1987).

P. aeruginosa demonstrated peculiar growth surrounding discs containing several of the superfractions. It can be postulated that, while mechanisms involved in bacterial survival and growth are not inhibited, other cellular mechanisms may be affected by compounds in the superfractions. Recent studies have uncovered several instances of non-growth-inhibitory actions of compounds against bacteria (Wozniak and Keyser, 2004; Skindersoe *et al.* 2008; Vattem *et al.* 2007). This new area of research is very promising for antibacterial drug discovery. Opportunistic pathogens, such as *P. aeruginosa*, contain several non-essential virulence factors which aid in pathogenicity. One strategy to develop new mechanisms to combat bacterial infections is to develop of antipathogenic drugs that attenuate the bacterium's virulence genes (Skindersoe *et al.*, 2008). Because this can be achieved without affecting the growth of bacteria, this principle is less likely to impose a selective pressure for resistance (Skindersoe *et al.*, 2008). One system of

interest is quorum sensing pathways (Wright and Sutherland, 2007). It has been proposed that for some opportunistic pathogens, such as *P. aeruginosa*, it is essential to stay "silent" and delay production of virulence factors until the cell population has reached a level sufficient to overwhelm the host's defenses (Skindersoe *et al.*, 2008). Quorum sensing allows bacteria to determine their own density in the host and, consequently, initiate processes involved in increasing virulence, which plays an important role in the successful establishment of bacterial infections in hosts (Skindersoe *et al.* 2008; Vattem *et al.* 2007). Inhibitors of quorum sensing are ideal targets for anti-infective drug research. Since marine invertebrates rely on chemical defenses they are a logical source of quorum sensing inhibitors. Skindersoe *et al*. (2008) performed a screen of a library of marine extracts as inhibitors of two known quorum sensing mechanisms in *P. aeruginosa*. Their search revealed wide-spread production of anti-quorum sensing compounds among these organisms. A study by Zhang and Pierson (2001) showed that strains of *P. aeruginosa* quorum-sensing receptor mutants resulted in altered growth appearance. The growth of these quorum-sensing mutants was lustrous in appearance. This indicates that perhaps several of the compounds in the extracts tested that caused the peculiar growth could be interacting with cell-surface receptors involved in quorum-sensing.

This research represents a pioneer exploration of deep-sea holothurians and actinaria for antibacterial compounds. The examination of the extracts of the four holothurians uncovered multiple compounds from each specimen that demonstrated antibacterial growth inhibitory activity against a human pathogenic bacterium, *S. aureus*.

Future Work

The next phase of research for this project will be the purification of the antibacterial compounds in the active superfractions. The TLC diffusion growth inhibition method will be a very efficient means to detect the active compound separated with TLC. These pure compounds will then need to be identified to determine if they are known compounds, variants of known compounds or novel compounds. Several superfractions showing the non-growth-inhibitory activity against *P. aeruginosa* may also be purified to attempt to determine the specific cellular effects the compounds demonstrate against *P. aeruginosa.*

The remaining superfractions that showed no antibacterial activities are likely to contain different bioactivity. All of the superfractions should be screened for other activities such as for anti-cancer and anti-fungal activity.

While no antibacterial results were shown for the actinaria extract, it is most likely to contain active compounds in smaller concentrations. This extract will be fractionated and re-tested for antibacterial activity as well.

Conclusions

1. This research indicated that holothurians show an excellent potential for containing antibacterial compounds.

2. Each of the four holothurians contained multiple compounds that exhibited antibacterial activity against *S. aureus.*

3. Each of the four holothurians contained several superfractions that displayed non-inhibitory activity against *P. aeruginosa*.

Reference List

Alanis, A.J. 2005. Resistance to Antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research* **36**: 697-705.

Ali-Shtayeh, M.S., R.M. Yaghmour, Y.R. Faidi, Khalid Salem, M.A. Al-Nuri. 1998. Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. *Journal of Ethnopharmacology* **60**: 265-271.

Aminin, D.L., B.V. Pinegin, L.V. Pichugina, T.S. Zaporozhets, I.G. Agafonova, V.M. Boguslavski, A.S. Silchenko, S.A. Avilov, and V.A. Stonik. 2006. Immunomodulatory properties of Cumaside. *International Immunopharmacology* **6**(7): 1070-1082.

Armstrong, G.L., L.A. Conn, and R.W. Pinner. 1999. Trends in infectious disease mortality in the United States during the 20th century. *Journal of American Medical Association* **281**(1): 61-66.

Bérdy, J. 2005. Bioactive microbial metabolites. *Journal of Antibiotics* **58**(1): 1-26.

Bergquist, P. R. and J. J. Bedford. 1978. The incidence of antibacterial activity in marine Demospongiae; systematic and geographic considerations. *Marine Biology* 46: 215-221.

Blunt, J.W., B.R. Copp, W.-P. Hu, M.H. Munro, P.T. Northcote and M.R. Prinsep. 2008. Marine natural products. *Natural Product Reports* **25**: 35-94.

Bryan, P.J., J.B. McClintock, and T.S. Hopkins. 1997. Structural and chemical defenses of echinoderms from the northern Gulf of Mexico. *Journal of Experimental Marine Biology and Ecology* **210**(2): 173-186.

Boucabeille, C.,D. Mengin-Lecreulx, G. Henckes, J. Simonet, J. van Heijenoort. 1997. Antibacterial and hemolytic activities of linenscin OC2, a hydrophobic substance produced by *Brevibacterium linens* OC2. *FEMS Microbiology Letters* **153**(2): 295–301

Cao, S., C. Foster, J.S. Lazo and D.G.I. Kingston. 2005. Four diterpenoid inhibitors of Cdc25B phosphatase from a marine anemone. *Bioorganic & Medicinal Chemistry* **13**(20): 5830-5834.

Carté, B.K. 1996. Biomedical potential of marine natural products. *Bioscience* **46**(4):271-286.

Cosgrove, S.E. and Y. Carmeli. 2003. The impact of antimicrobial resistance on health and economic outcomes. *Clinical infectious diseases* **36**: 1433-1437.

Croteau, R., T.M. Kutchan, and N.G. Lewis. 2000. Natural products (secondary metabolites). *In* B Buchanan, W Gruissem, R Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, MD. pp 1250–1318.

Essawi, T. and M. Srour. 2002. Screening of some Palestinian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology* **70**: 343-349.

Faulkner, J.D. 1984. Marine natural products: metabolites of marine invertebrates. *Natural Product Reports* **1**(6): 551-598.

Faulkner, J.D. 2002. Marine natural products. *Natural Product Reports* **19**(1):1-48.

Girard, M., J. Bélanger, J.W. Apsimon, F.X. Garneau, C. Harvey, and J.R. Brisson. 1990. Frondoside A. A novel triterpene glycoside from the holothurian *Cucumaria frondosa*. *Canadian Journal of Chemistry* **68**(1): 11-18.

Hartmann, T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry* **68**: 2831-2846.

Hutton, D.M. and V.J. Smith. 1996. Antibacterial properties of isolated amoebocytes from the sea anemone *Actinia equine*. *The Biological Bulletin* **191**: 441-451.

Kartal, M., S. Yıldız, S. Kaya, S. Kurucu and G. Topçu. 2003. Antimicrobial activity of propolis samples from two different regions of Anatolia. *Journal of Ethnopharmacology* **86**(1): 69-73.

Kelman D, A. Kushmaro, Y. Loya, Y. Kashman, and Y. Benayahu. 1998. Antimicrobial activity of a Red Sea soft coral, *Parerythropodium fulvum fulvum*: reproductive and developmental considerations. *Marine Ecology Progress Series* **169**:87–95

Lippert, H., R. Brinkmeyer, T. Mulhaupt, and K. Iken. 2003. Antimicrobial activity in sub-Arctic marine invertebrates. *Polar Biology* **26**: 591-600.

McClintock, J.B..1989. Toxicity of shallow-water antarctic echinoderms. *Polar Biology* **9**(7): 461-465.

Mills, S.D. 2006. When will the genomics investment pay off for antibacterial discovery? *Biochemical Pharmacology* **71**: 1096-1102.

Murray, B.E.. 1998. Diversity among multidrug-resistant Enterococci. *Emerging Infectious Diseases* **4**(1): 29-36.

Naijar A and B.E. Murray. 1987. Failure to demonstrate a consistent in vitro bactericidal effect of trimethoprim-sulfamethoxazole against enterococci. *Antimicrobial Agents Chemotherapy* **31**:808-10

National Committee for Clinical Laboratory Standards. 1995. Performance standards for antimicrobial susceptibility testing. NCCLS. Villanova, PA.

Newman, D.J., G.M. Cragg, and K.M. Snader. 2000. The influence of natural products upon drug discovery. *Natural Product Reports* **17**(3): 215-234.

Newman, D.J. and G.M. Cragg. 2007. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products* **70**: 461-477.

Osbourn, A.E.. 1996. Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell* **8**(10): 1821-1831.

Payne D.J., M.N. Gwynn, D.J. Holmes, and D.L Pompliano. 2007 Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovory* **6**:29-40.

Pinho, S.B. and E.A. Macedo. 1996. Representation of salt solubility in mixed solvents: a comparison of thermodynamic models. *Fluid Phase Equilibria* **116**:209-216.

Prescott, L.M., J.P Harley and D.A. Klein, 2005. Microbiology (6th ed.). McGraw-Hill Companies Inc. New York, NY.

Projan, S.J. and P.A. Bradford. 2007. Late stage antibacterial drugs in the clinical pipeline. *Current Opinion in Microbiology* **10**: 441-446.

Proksch P. 1999. Chemical defense in marine ecosystems. In: Wink M (ed) Functions of plant secondary metabolites and their exploitation in biotechnology. Academic Press, Sheffield, pp 134–154.

Proksch P., and R.Ebel. 1998. Ecological significance of alkaloids from marine invertebrates. In: Roberts MF, Wink M (eds) Alkaloids, biochemistry, ecology and medicinal applications Plenum, New York, pp 379–394.

Proksch, P., R.A. Edrada, and R. Ebel. 2002. Drugs from the seas – current status and microbiological implications. *Applied Microbiology and Biotechnology* **59**(2-3): 125-134.

Rice LB. 2003. Do we really need new anti-infective drugs? *Current Opinion Pharmacology* **3**: 459-463.

Rose, A.H. 1979. Nature of secondary products of microbial metabolism. *Economic Microbiology*, volume 3. Academic Press Inc. New York and London: 2-3.

Qaiyumi, S. 2007. Macro- and microdilution methods of antimicrobial susceptibility testing. *Antimicrobial susceptibility testing protocols* (Schwalbe, R., L. Steele-

Moore, and A.C. Goodwin eds.). CRC Press Taylor & Francis Group. Boca Raton, FL. 75-79.

Skindersoe, M.E., P. Ettinger-Epstein, T.B. Rasmussen, T.Bjarnsholt, R. de Nys, and M. Givskov. 2008. Quorum sensing antagonism from marine organisms. *Marine Biotechnology* **10**: 56-63.

Spellberg, B., J.H. Powers, E.P. Brass, L.G. Miller, and J.E. Edwards Jr. 2004. Trends in antimicrobial drug development: implications for the future. *Clinical Infectious Diseases* **38**: 1279-1286.

Thompson, J.E., R.P. Walker, and D.J. Faulkner. 1985. Screening and bioassays for biologically-active substances from forty marine sponge species from San Diego, California, USA. *Marine Biology* **88**: 11-21.

Torres, Y.R, R.G Berlinck, G.G. Nascimento, S.C. Fortier, C. Pessoa, and M.O deMoraes. 2002. Antibacterial activity against resistant bacteria and cytotoxicity of four alkaloid toxins isolated from the marine sponge *Arenosclera brasiliensis*. *Toxicon* **40**: 885-891.

Vattem D.A., K. Mihalik, S.H. Crixell, R.J. McLean. 2007. Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia* **78**: 302-310.

Wedge, D.E. and D.G. Nagle. 2000. A new 2D-TLC bioautography method for the discovery of navel antifungal agents to control plant pathogens. *Journal of Natural Products* **63**(8):1050-1054.

Weinheimer A.J., and R.L. Spraggins. 1969. The occurrence of two new prostaglandin derivatives (15-epi-PGA2 and its acetate, methyl ester) in the gorgonian Plexaura homomalla chemistry of coelenterates. *Tetrahedron Letters* **59**: 5185-5188.

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**: 3-19.

Wright, G.D. and A.D. Sutherland. 2007. New strategies for combating multidrug-resistant bacteria. *Trends is Molecular Medicine* **13**(6): 260-267.

Wozniak, D.J. and R. Keyser. 2004. Effects of subinhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa. Chest* **125**: 62-69.

Zhang, Z. and L.S. Pierson. 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens. Applied and Environmental Microbiology* **67**(9): 4305-4315.

Appendix

Appendix 1. Solvent system and corresponding fractions obtained from silica liquid column chromatography of *Pannychia moseleyi* 95% (v/v) methanol extract.

Solvent (v/v)		Volume (mL)	Fractions
100% hexane	-	200	1-9
10% EtOAc	90% hexane	400	10-25
20% EtOAc	80% hexane	200	26-34
30% EtOAc	70% hexane	200	35-42
40% EtOAc	60% hexane	200	43-51
50% EtOAc	50% hexane	200	52-59
60% EtOAc	40% hexane	200	60-67
70% EtOAc	30% hexane	200	68-76
80% EtOAc	20% hexane	200	77-84
90% EtOAc	10% hexane	200	85-93
100% EtOAc	-	200	94-105
5% MeOH	95% EtOAc	100	106-108
10% MeOH	90% EtOAc	100	109-113
15% MeOH	85% EtOAc	100	114-117
20% MeOH	80% EtOAc	100	118-122
30% MeOH	70% EtOAc	100	123-127
40% MeOH	60% EtOAc	100	128-131
50% MeOH	50% EtOAc	100	132-135
60% MeOH	40% EtOAc	100	136-140
70% MeOH	30% EtOAc	100	141-144
80% MeOH	20% EtOAc	100	145-148
90% MeOH	10% EtOAc	100	149-152
100% MeOH	-	100	153-165

Appendix 2. Solvent system and corresponding fractions obtained from silica liquid column chromatography of *Scotoplanes sp.* 95% (v/v) methanol extract.

Solvent (v/v)		Volume (mL)	Fractions
100% hexane	-	100	1-7
10% EtOAc	90% hexane	100	8-12
20% EtOAc	80% hexane	100	13-16
30% EtOAc	70% hexane	100	19-20
40% EtOAc	60% hexane	100	21-25
50% EtOAc	50% hexane	100	26-29
60% EtOAc	40% hexane	100	30-33
70% EtOAc	30% hexane	100	34-38
80% EtOAc	20% hexane	100	39-42
90% EtOAc	10% hexane	100	43-46
100% EtOAc	-	100	47-51
5% MeOH	95% EtOAc	100	52-55
10% MeOH	90% EtOAc	100	56-59
15% MeOH	85% EtOAc	100	60-63
20% MeOH	80% EtOAc	100	64-68
30% MeOH	70% EtOAc	100	69-72
40% MeOH	60% EtOAc	100	73-76
50% MeOH	50% EtOAc	100	77-80
60% MeOH	40% EtOAc	100	81-84
70% MeOH	30% EtOAc	100	85-89
80% MeOH	20% EtOAc	100	90-93
90% MeOH	10% EtOAc	100	94-98
100% MeOH	-	100	99-144

Appendix 3. Solvent system and corresponding fractions obtained from silica liquid column chromatography of *Paelopadites confundens* 95% (v/v) methanol extract.

Solvent (v/v)		Volume (mL)	Fractions
100% hexane	-	100	1
10% EtOAc	90% hexane	100	1
20% EtOAc	80% hexane	100	1
30% EtOAc	70% hexane	100	1
40% EtOAc	60% hexane	100	1-3
50% EtOAc	50% hexane	100	4-8
60% EtOAc	40% hexane	100	9-12
70% EtOAc	30% hexane	100	13-16
80% EtOAc	20% hexane	100	17-20
90% EtOAc	10% hexane	100	21-24
100% EtOAc	-	100	25-28
5% MeOH	95% EtOAc	100	29-32
10% MeOH	90% EtOAc	100	33-36
15% MeOH	85% EtOAc	100	37-40
20% MeOH	80% EtOAc	100	41-45
30% MeOH	70% EtOAc	100	46-49
40% MeOH	60% EtOAc	100	50-53
50% MeOH	50% EtOAc	100	54-57
60% MeOH	40% EtOAc	100	58-61
70% MeOH	30% EtOAc	100	62-65
80% MeOH	20% EtOAc	100	66-70
90% MeOH	10% EtOAc	100	71-74
100% MeOH	-	100	75-81

Appendix 4. Solvent system and corresponding fractions obtained from silica liquid column chromatography of *Molpadia sp*. 95% (v/v) methanol extract.

Solvent (v/v)		Volume (mL)	Fractions
100% hexane	-	100	1
10% EtOAc	90% hexane	100	1
20% EtOAc	80% hexane	100	1
30% EtOAc	70% hexane	100	1-4
40% EtOAc	60% hexane	100	5-10
50% EtOAc	50% hexane	100	11-14
60% EtOAc	40% hexane	100	15-18
70% EtOAc	30% hexane	100	19-22
80% EtOAc	20% hexane	100	23-26
90% EtOAc	10% hexane	100	27-30
100% EtOAc	-	100	31-35
5% MeOH	95% EtOAc	100	36-39
10% MeOH	90% EtOAc	100	40-43
15% MeOH	85% EtOAc	100	44-47
20% MeOH	80% EtOAc	100	48-52
30% MeOH	70% EtOAc	100	53-56
40% MeOH	60% EtOAc	100	57-60
50% MeOH	50% EtOAc	100	61-64
60% MeOH	40% EtOAc	100	65-68
70% MeOH	30% EtOAc	100	69-72
80% MeOH	20% EtOAc	100	73-77
90% MeOH	10% EtOAc	100	78-81
100% MeOH	-	100	82-85

Appendix 5. The fractionated extract weights of *Pannychia moseleyi* crude extracts separated by silica liquid chromatography into 156 fractions. The fractions with the same constituents were combined into the indicated 14 superfractions.

Fractions Combined	Super-Fractions Renamed	TLC Solvent System	Combined Weight (mg)
1-27	27	5% EtOAc	16.3
28-36	36	15% EtOAc	10.2
37-45	45	15% EtOAc	32.4
46-50	50	15% EtOAc	28.5
51-56	56	18% EtOAc	60.3
57-66	66	18% EtOAc	193.3
67-89	89	18% EtOAc	258.2
90-100	100	100% EtOAc	61.5
101-127	127	100% EtOAc	43.7
128-132	132	5% MeOH	10.1
133-138	138	5% MeOH	439.8
139-149	149	5% MeOH	1182.6
150-153	153	30% MeOH	391.9
154-156	156	30% MeOH	7280.7

Appendix 6. The fractionated extract weights of *Scotoplanes sp.* crude extracts separated by silica liquid chromatography into 144 fractions. The fractions with the same constituents were combined into the indicated 14 superfractions.

Combined Fractions	Super-Fractions Renamed	TLC Solvent System	Combined Weight (mg)
1-22	22	5% EtOAc	19.4
23-27	27	50% EtOAc	7.6
28-37	37	50% EtOAc	101.3
38-47	47	65% EtOAc	172.4
48-53	53	65% EtOAc	119.6
54-57	57	65% EtOAc	70.5
58-65	65	100% EtOAc	46.2
66-74	74	5% MeOH	4.6
75-80	75	5% MeOH	134.8
81-86	86	5% MeOH	338.3
87-91	91	5% MeOH	163.4
92-95	95	*	441.9
96-99	99	*	509.5
100-144	102	*	6375.2

* Fractions no longer ran smoothly on the TLC and were combined based on visual determination.

Appendix 7. The fractionated extract weights of *Paelopadites confundens* crude extracts eparated by silica liquid chromatography into 81 fractions. The fractions with the same constituents were combined into the indicated 9 superfractions.

Combined Fractions	Super-Fractions Renamed	TLC Solvent System	Combined Weight (mg)
1-8	1	40% EtOAc	20.2
9-23	9	50% EtOAc	224
24-31	24	40% MeOH	63.1
32-49	32	40% MeOH	22.2
50-60	60	40% MeOH	143.3
61-65	65	50% MeOH	656.3
66-69	68	*	7275.6[†]
70-76	76	*	14715.1[†]
77-82	82	*	5418.8

[†]These fractions were unable to be completely dried. * Fractions no longer ran smoothly on the TLC and were combined based on visual determination.

Appendix 8. The fractionated extract weights of *Molpadia sp*. crude extracts separated by silica liquid chromatography into 85 fractions. The fractions with the same constituents were combined into the indicated 11 superfractions.

Combined Fractions	Super-Fractions Renamed	TLC Solvent System	Combined Weight (mg)
1-7	7	20% EtOAc	290.9
8-13	9	20% EtOAc	159.5
14-16	16	50% EtOAc	26.5
17-31	31	70% EtOAc	71.2
32-43	43	100% EtOAc	28.5
44-49	49	10% MeOH	114.7
50-57	58	30% MeOH	414.6
58-60	60	30% MeOH	438.2
61-64	61	*	3460.9
65-69	65	*	491
70-85	70	*	2096.3