Spring 5-9-2014

Chemical Profiling and Biological Activity of Two Tunicate-Associated Marine Bacteria

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Date Submitted: 5/9/2014

List of Abbreviations

HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry

NMR: Nuclear Magnetic Resonance

BioMAP: Antibiotic Mode of Action Profile

MSSA: Methicillin-sensitive *Staphylococcus aureus*

MRSA: Methicillin-resistant *Staphylococcus aureus*

MIC: Minimum Inhibitory Concentration

IO: Instant Ocean

DI: Deionized

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

SPE: Solid Phase Extraction

ISP4: Inorganic Salts-Starch Agar

$CDCl_3$: Deuterated Chloroform

^1H NMR: Proton Nuclear Magnetic Resonance

CHX: cycloheximide

NDA: nalidixic acid

MeOH: methanol

Abstract

Marine natural products have recently been an increasingly abundant source of novel antibiotics. Given that there is an increasing resistance to current drug therapies, finding new sources such as marine natural products is essential. Tunicate-associated marine bacteria can be a significant source of antibacterial compounds. Two tunicates of the species *Eudistoma* were collected from Portobelo National Park on the Salmedina Reef of Panama in the Caribbean Sea. Bacteria associated with the tunicate were isolated, cultured, extracted, and fractionated. Fractions were tested against an array of clinically relevant bacterial pathogens in the BioMAP assay. Two fractions MB0086E and MB0088E demonstrated activity and specificity against *Staphylococcus aureus*, *Enterococcus faecium*, and *Listeria ivanovii*. This study focuses on developing the chemical profiles of the fractions using High Performance Liquid Chromatography and Nuclear Magnetic Resonance as well as further examining their biological activity using various assays.

Introduction

Although natural products from land species have been extensively studied, many more remain undiscovered in aquatic life. Natural compounds can provide starting structures for synthetic chemists to modify in order to make them less cytotoxic or more potent (Genilloud et al. 2011). Most microorganisms produce secondary metabolites, organic compounds that are not directly involved in the main functions of an organism's survival. Instead, these metabolites often assist in the increase of an organism's lifespan, ability to survive, and overall success in their environment. Marine microorganisms may provide biologically active compounds distinct from those discovered in terrestrial microorganisms, because their environmental conditions are substantially different (Subramani and Aalbersberg 2011).

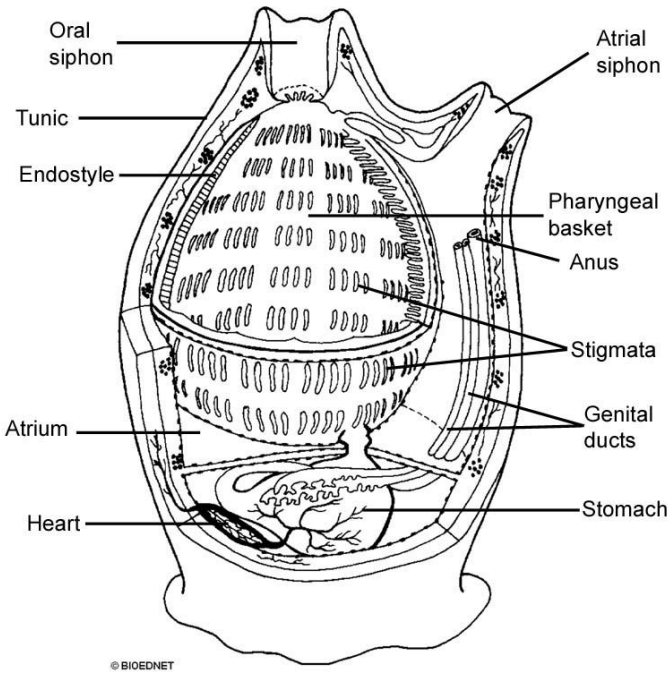


Figure 1. Diagram of a tunicate (figure from Hosie 2014).

Tunicates, also known as ascidians, are marine organisms that contain a significant amount of microbes. Tunicates get their name from the tunic-like sac (Figure

1) that surrounds them (Davidson 1993). They are also sometimes referred to as sea squirts because of the water they eject. These organisms are vulnerable to predation since they are often bright, attractive, and stationary. Since they have no escape mechanism, they must rely on producing chemical toxins to ward off predators (Flam 1994). Some of these toxins are produced by bacterial flora associated with the tunicates.

Many antibacterial compounds have previously been isolated from tunicates or tunicate-associated marine bacteria. Figure 2 displays antibacterial compounds isolated from tunicates or tunicate-associated marine bacteria discovered between 2010 and the present. Antibacterial activity was observed in brominated rubrolides **1-4** which were isolated from an African ascidian *Synoicum globosum* (Sikorska et al. 2013). Peptidolipins **5-9**, lipopeptide compounds produced by the *Nocardia* sp. bacteria from the Florida Keys tunicate *Tridemnum orbiculatum*, showed activity against methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Wyche et al. 2012). N-deacetylshermilamine B **10**, cystodimine A **11**, and cystodimine B **12** are three pyridoacridine alkaloid compounds isolated from the Mediterranean ascidian *Cystodytes dellechiajei*, which are active against *Escherichia coli* and *Micrococcus luteus* (Bontemps et al. 2010). Synoxazolidinone A **13**, an alkaloid isolated from Norwegian ascidian *Synoicum pulmonaria*, is active against *Corynebacterium glutamicum* (Tadesse 2010).

BioMAP (antibiotic mode of action profile), an assay developed by the Linington lab at the University of California in Santa Cruz, is an efficient method for screening extracts against multiple bacterial pathogens. The assay assigns biological “fingerprints” to both known antibiotics and extracts. These “fingerprints” indicate against which of the

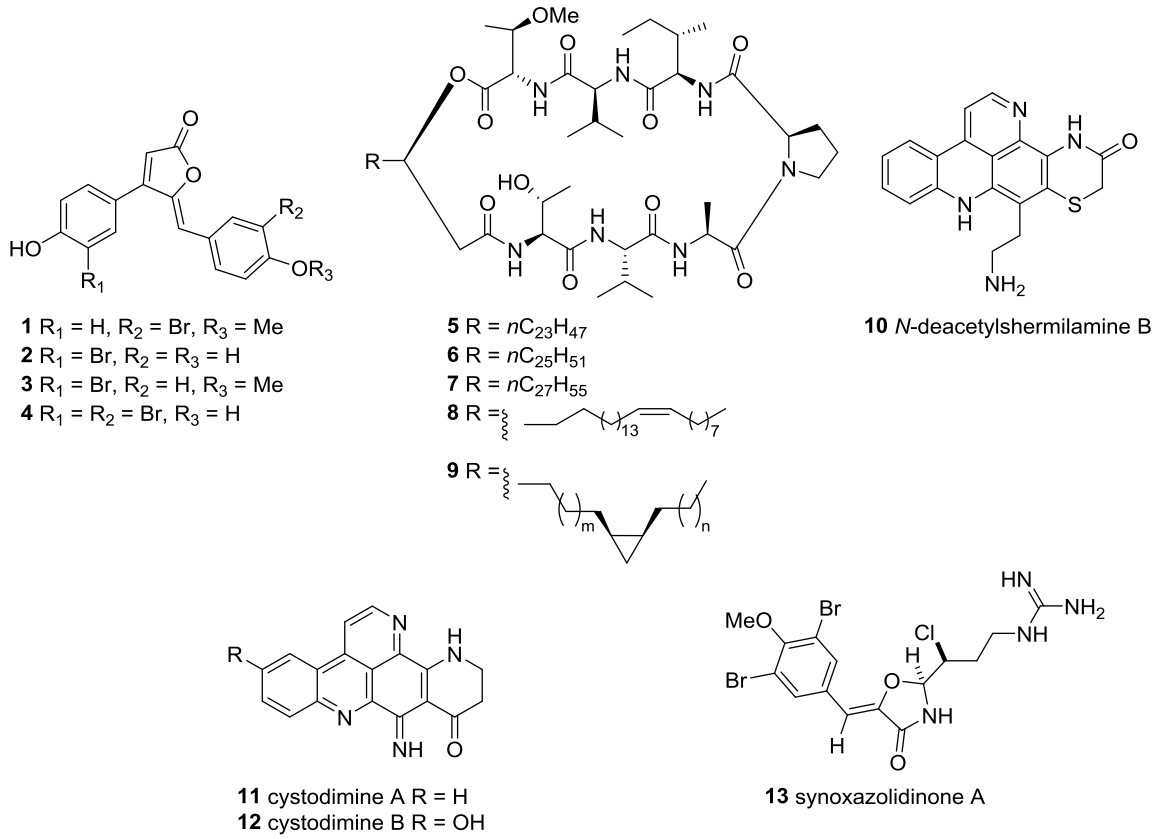


Figure 2. Recent antibacterial compounds isolated from tunicates (ascidians) or tunicate-associated bacteria.

clinically relevant bacterial pathogens the antibiotics show activity. Both Gram-positive and Gram-negative bacteria are included in the assay. An example of BioMAP shown in Figure 3 displays the varying levels of activity of various compounds against the bacterial pathogens. The figure indicates activity based on a range of shades from black (inactive) to red (highly active). BioMAP also clusters the known antibiotics and extracts by structural class. The structural class can often be determined by the biological activity, since antibiotics from the same structural class usually implement similar mechanisms

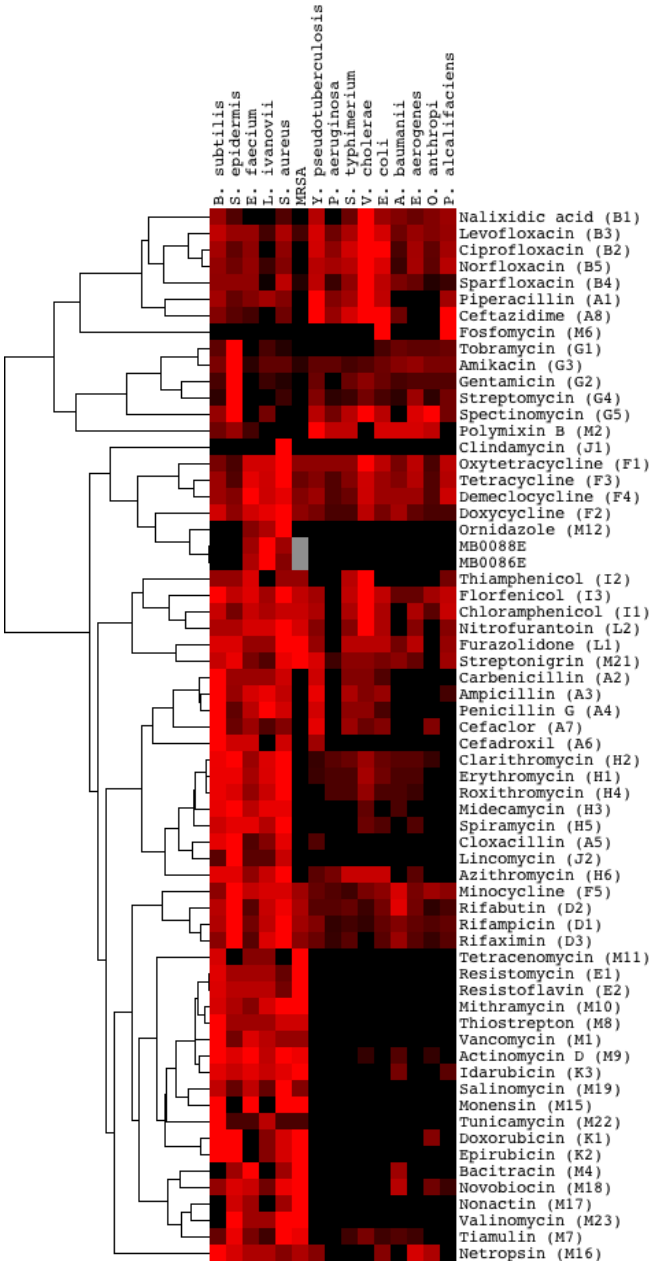


Figure 3. BioMap. Extracts MB0088E and MB0086E were tested against 14 organisms (listed on map) and placed next to compounds of similar antimicrobial activity. In this cluster heat map, the shade of red depicts degree of antimicrobial activity (brighter shades show stronger activity). MB0088E and MB0086E show possible activity against *Staphylococcus aureus, Listeria ivanovii,* and *Enterococcus faecium* as well as similar activity to the compound ornidazole (courtesy of Linington lab).

for inhibiting bacterial growth. This assay is informative for two reasons: discovering the activity of extracts against clinically relevant bacterial pathogens and determining their possible structural classes (Wong et al. 2012).

Recently, our lab tested extracts and fractions from tunicate-associated bacteria in the BioMAP assay and found two fractions with interesting activity. The sources of these fractions were two Panamanian tunicates of the species *Eudistoma* (Figure 4), collected from Portobelo National Park on the Salmedina Reef in the Caribbean Sea at a depth of 50-60 feet. The bacteria associated with the tunicates were isolated and grown in liquid culture. The liquid cultures were then extracted and fractionated to be prepared for biological testing. The extracts and fractions were sent to the Linington lab at the University of California in Santa Cruz where they were tested in BioMAP (Figure 3). Our active fractions, labeled MB0086E and MB0088E, are grouped on the right side of the cluster heat map with ornidazole, due to their similar activity. Ornidazole is used to treat protozoan diseases such as giardiasis, trichomoniasis, and bacterial vaginosis (Saracoglu et al. 1998; Ozbilgin et al. 2004; Inceboz et al. 2004).

Figure 4. A representative *Eudistoma* sp. ("strawberry tunicate") collected from Portobelo National Park on the Salmedina Reef in Panama (photo Marcy Balunas).

In the BioMAP assay, these fractions showed potent activity against *Staphylococcus aureus, Enterococcus faecium,* and *Listeria ivanovii* (Figures 3 and 5). *S. aureus* can cause severe skin infections (Lowy 1998); *E. faecium* causes urinary tract infections (Huycke et al. 1998); and *L. ivanovii* is a ruminant pathogen (Fentahun and Fresebehat 2012). Notably, the fractions were not active against the entire panel of bacterial pathogens in the BioMAP assay. This indicates that the active compounds in the fractions are selective. This is beneficial because compounds intended for an antibiotic which are specific to only a few types of bacteria are more desirable. Specificity is crucial since antibacterial compounds should not disrupt or kill host cells or non-pathogenic bacterial strains. Non-specific, or broad-spectrum antibiotics, can contribute to drug resistance by allowing the growth and spread of bacteria which are not susceptible to that antibiotic (Huycke et al. 1998).

The activity shows potential for a possible new antibiotic for humans and/or cattle. *S. aureus* is a common Gram-positive coccal bacterium in skin infections that causes sores. *S. aureus* can also cause the sometimes fatal toxic shock syndrome, endocarditis, and sepsis (Lowy 1998). In addition, once a human is infected with the bacteria, they become more susceptible to future infections. Even though antibiotics for *S. aureus* already exist, new antibiotics are always desired that are selective and help the issue of growing resistance to current antibiotics (Giedraitiene et al. 2011).

The other human pathogen that the fractions show activity against, *E. faecium,* is one of the primary bacteria causing urinary tract infections. Other complications these Gram-positive bacteria may cause are bacteremia and surgical wound infections. Infections from strains that originate in the body's normal flora are usually easily treated;

however, strains originating from nosocomial infections are likely to be antibiotic resistant and therefore more difficult to treat (Huycke et al. 1998). The antibiotic resistant strains make the search for novel antibiotic compounds crucial to treating future infections.

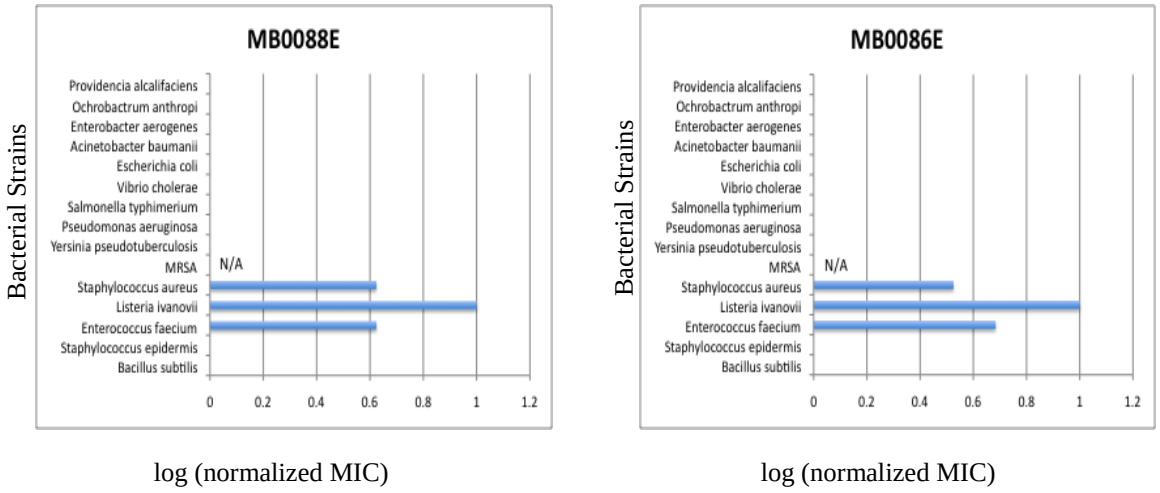


Figure 5. BioMap profile of MB0088E and MB0086E with normalized MIC values (courtesy of Linington lab).

L. ivanovii, the third bacteria which the fractions are active against, mainly affects ruminants. *L. ivanovii* are Gram-positive bacilli. Infection by *L. ivanovii* in ruminants may cause abortions (Fentahun and Fresebehat 2012). This pathogen rarely affects humans; however, recent cases of *L. ivanovii* causing gastroenteritis and bacteremia have been reported (Guillet et al. 2010). Another species in the same genus, *Listeria monocytogenes*, can cause listeriosis in humans. *L. monocytogenes* is a food-borne pathogen that could be fatal. This species may also cause abortions in pregnant women

(Lamont et al. 2011). If the active fractions target a mechanism implemented by all bacteria of the *Listeria* genus, then they may inhibit *L. monocytogenes* as well.

The bacterium from which MB0086E was extracted is an Actinomycete. MB0088E may be an Actinomycete as well, although this is not as clear from the bacteria's morphology. Actinomycetes are an especially significant source of biologically active compounds. There is great diversity amongst these microorganisms and they have been previously shown to produce antibiotic compounds (Subramani and Aalbersberg 2011). More than 22,000 secondary metabolites from microbes have been studied and 70% of these come from Actinomycetes (Subramani and Aalbersberg 2011).

The activity of fractions MB0086E and MB0088E against two human pathogens could be significant in the search for novel antibiotics. Drug resistant strains of human pathogenic bacteria are becoming an increasing threat, making these two fractions of valuable interest. *E. faecium* is intrinsically resistant to some antibiotics since the resistance is not acquired, but encoded in the bacterial genome. The surface penicillin binding proteins of *E. faecium* do not bind β-lactam antibiotics with high affinity (Giedriatiene et al. 2011). There are also vancomycin-resistant strains of *E. faecium* due to inducible genes which change the cell wall synthesis. These bacteria can become even more drug resistant through conjugation or plasmid transfer from other bacteria (Huycke et al. 1998). *S. aureus* can acquire resistance through similar methods. Drug resistant strains also contain new penicillin binding proteins that prevent β-lactam antibiotics from working. Vancomycin-resistant *S. aureus* strains are also on the rise (Lowy 1998). Currently, a solution to this problem is treating patients with multiple antibiotics simultaneously; however, this method has not been proven to be fully successful (Huycke

et al. 1998). Novel antibiotic compounds with specificity, such as the compounds present in MB0086E and MB0088E could be a superior solution.

The goal is to further the research and data on the two bacteria, the compounds they produce, and their biological activity. The two bacteria must be regrown, extracted, and fractionated. The extracts and fractions will be tested again for biological activity against *S. aureus*. If the activity is found again in the new fractions, then the active compounds contained in the fractions will be isolated. Then we must determine if the two compounds are the same and whether or not they are analogs of ornidazole. Further research will focus on modifying the compound's structure to achieve the optimal activity for use as an antibiotic against *S. aureus*, *E. faecium*, and *L. ivanovii*.

Materials and Methods

General Experimental Procedures

Each extract and fraction was prepared at 1 mg/mL in either HPLC grade MeOH or acetonitrile for HPLC-MS or HPLC analysis. Samples were run on an Agilent ESI single quadrupole mass spectrometer connected to an Agilent HPLC system containing a G1311 quaternary pump, G1322 degasser, and a G1315 diode array detector with an Eclipse XDB-C18 reverse phase-HPLC column. Fractions were dissolved in $CDCl_3$ for ^1H NMR analysis. ^1H NMR analysis was conducted on a Brüker Avance 500 MHz spectrometer which gives chemical shifts in ppm downfield from TMS.

Tunicate Collection, Plating, and Bacterial Isolation

Tunicates were collected from Portobelo Park in Salmedina, Panama from the Caribbean

Sea (N 9 33.746 W 79 41.617) at a depth of 50-60 feet. Tunicates were rinsed with sterile sea water and cut into small pieces. Tunicates were homogenized by crushing with a mortar and pestle in 1-2 mL sterile sea water. A sterile cotton swab was dipped in homogenate and spread onto ISP4 agar (soluble starch 10 g, K_2HPO_4 1 g, $MgSO_4$ USP 1 g, NaCl 1 g, $(NH_4)_2SO_4$ 2 g, $CaCO_3$ 2g, $FeSO_4$ 1 mg, $MnCl_2$ 1 mg, $ZnSO_4$ 1 mg, agar 20 g, 1 L water) with cycloheximide (CHX) and nalidixic acid (NDA). Bacteria PTY138G1 (Actinomycete) and PTY139L1 were selected from the ISP4 plates and further isolated on marine agar. Bacterial isolates were stored in liquid nitrogen.

Regrowing of Bacteria

PTY138G1 and PTY139L1 were removed from liquid nitrogen and streaked onto agar plates of various media in order to determine ideal growth conditions. PTY138G1 was streaked onto ISP4 agar (with DI, CHX, and NDA), ISP4 agar (with IO, CHX, and NDA), and marine agar (peptone 5 g, yeast extract 1 g, ferric citrate 0.1 g, NaCl 19.45 g, $MgCl_2$ 8.8 g, Na_2SO_4 3.24 g, $CaCl_2$ 1.8 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, KBr 0.08 g, $SrCl_2$ 34 mg, H_3BO_3 22 mg, $Na_2(SiO_2)_nO$ 4 mg, NaF 2.4 mg, NH_4NO_3 1.6 mg, Na_2HPO_4 8 mg, agar 15 g, 1 L DI water). PTY139L1 was also streaked onto ISP4 agar (with IO, CHX, and NDA), ISP4 agar (with DI, CHX, and NDA) and marine agar. Plates were incubated for 1-2 weeks at 30° C.

Bacterial growth in liquid culture

Liquid media was prepared according to method and ingredients mentioned above, but without agar. ISP4 + DI water (1000 mL) and marine broth (1000 mL) were each

inoculated with single isolated colonies from the PTY138G1 plates. Marine broth (1000 mL) was inoculated with single isolated colonies from the PTY139L1 plates. The flasks were incubated at 30° C with shaking, for two weeks. After removal from the incubator, each flask was streaked onto plates to check for purity of cultures.

Extraction

Diaion HP-20 beads (200 g) were first prepared by washing them in a frit filter with methanol and then water. The beads were then transferred to a 1 L flask where the liquid bacterial culture, PTY183G1 grown in marine broth or ISP4 broth, was also added. The flask was covered and shaken for 24 hours at 135 rpm. After 18-24 hours, the flask was removed from the shaker and prepared for extraction. The contents of the flask were poured into a frit filter set up on a vacuum flask. The water was removed by vacuum and the beads were washed two times with 600 mL of DI water, after allowing the water to sit in the filter for five minutes. The water layers were discarded. The beads were then washed with 600 mL of 100% MeOH, 600 mL of 100% DCM, and 600 mL of 100% acetone. The solvents were evaporated. The PTY138G1 extract grown in marine broth was named LY0086-MB and the PTY138G1 extract grown in ISP4 broth was named LY0086-ISP4.

Fractionation

The extracts LY0086-MB and LY0086-ISP4 were subject to fractionation using a Discovery DSC-18 reverse phase-solid phase extraction cartridge with a MeOH— H_2O

solvent gradient (1:4, 1:1, 4:1, 100% MeOH, 100% Ace). The fractions were named LY0086-MB (A-E) and LY0086-ISP4 (A-E).

Extraction/Fractionation

A separate set of flasks of PTY138G1 and PTY139L1 liquid cultures (both grown in marine agar with the same method mentioned above) underwent a combined step of extraction and fractionation instead of sequential extraction followed by fractionation. Diaion HP-20 beads were first prepared by washing them in a frit filter with methanol and then water. The beads were then transferred to a 1 L flask where the liquid bacterial culture, either PTY183G1 (grown in marine broth) or PTY139L1, was also added. The flask was shaken for 24 hours at 135 rpm. After 18-24 hours, the flask was removed from the shaker and prepared for extraction. The contents of the flask were poured into a 600 mL frit filter set up on a vacuum flask. The water was removed by vacuum and the beads were washed two times with 600 mL of DI water, after allowing the water to sit in the filter for five minutes. The water layers were discarded. The beads were then subject to an extraction/fractionation with a MeOH—H_2O solvent gradient (1:4, 1:1, 4:1, 100% MeOH, 100% Ace), each with a volume of 600 mL. Solvents were allowed to sit in the frit filter with the beads for five minutes before vacuum was applied. The fractions from PTY138G1 were named LY0086 (A-E) and the fractions from PTY139L1 were named LY0088 (A-E).

Bioassay procedure

The 96 well plate bioassay procedure was modified from Zgoda and Porter (2001).

Day 1:

5 mL of tryptic soy broth (Bacto™ Tryptone [Pancreatic Digest of Casein] 17 g, Bacto Soytone [Peptic Digest of Soybean Meal] 3 g, glucose 2.5 g, NaCl 5 g, K_2HPO_4 2.5 g, 1 L DI water) were placed in two 15 mL Falcon tubes. One tube was inoculated with 1 colony of methicillin-sensitive *Staphylococcus aureus* (MSSA) and the other tube was left to serve as a blank. The tubes were incubated for 18 hours at 37° C.

Day 2:

The blank was checked for absence of growth. Bacteria were diluted 1:1,000 with sterile water. A mix was then created with 2 mL inoculum, 8 mL tryptic soy broth, and 9.5 mL sterile water. Each well in a 96 well plate was filled with 195 μL of this mix (except for sterility control and color control wells). The assay was conducted at 500 μg/mL so extracts were prepared at 20 mg/mL with DMSO and 5 μL of the extract was added to the assay wells. Negative control wells contained 195 μL of the mix and 5 μL sterile DMSO. Positive control wells contained 195 μL mix and 5 μL of vancomycin at 200 μg/mL. Sterility control wells contained 80 μL broth, 115 μL sterile water, and 5 μL DMSO. Color control wells contained 80 μL broth, 115 μL sterile water, and 5 μL of the extract (Table 1). The negative, positive, and sterility controls had 8 wells each. The assay wells with the extract were performed in triplicate and the color control wells were performed in duplicate. The 96 well plate was then incubated for 24 hours at 37° C.

Day 3:

The 96 well plate was run on a Gen51.11 Plate reader. The plate was read at an absorbance of 750 nm.

Control	Components
Negative control	195 μL mix (inoculum, water, and broth) 5 μL sterile DMSO
Positive control	195 μL mix (inoculum, water, and broth) 5 μL vancomycin (at 200 μg/mL)
Sterility control	80 μL broth 115 μL sterile water 5 μL sterile DMSO
Color control	80 μL broth 115 μL sterile water 5 μL extract

Table 1. Components of 96 well plate bioassay controls.

Disc Diffusion Assay

The disc diffusion assay procedure was modified from Bauer et al. (1966).

Day 1:

A liquid *S. aureus* culture (100 μL) of optical density .0132 was plated uniformly on tryptic soy agar (Bacto™ Tryptone [Pancreatic Digest of Casein] 15 g, Bacto Soytone [Papaic Digest of Soybean Meal] 5 g, NaCl 5 g, agar 15 g, 1 L DI water). Fractions were prepared at 2 mg/mL and 5 mg/mL in DMSO. Discs were soaked in 10 μL of the prepared fractions and placed on *S. aureus* plates. Each fraction was tested in triplicate. Each plate contained one disc with 10 μL DMSO and one disc with 10 μL vancomycin to serve as controls. Plates were incubated for 18 hours at 37° C.

Day 2:

Plates were checked for zones of inhibition around discs. Diameters of zones of inhibition were measured in mm if present.

Results and Discussion

Bacteria were grown on various media to observe differences in growth and to determine the optimal growth conditions. PTY138G1 was grown on ISP4 + DI and ISP4 + IO plates. The bacteria were able to grow on both types of agar, but at different rates. The bacteria took one week to grow on ISP4 + DI, but two weeks to grow on ISP4 + IO. PTY138G1 was also grown on marine agar and compared to its growth on ISP4 + DI. The bacterial growth looked different on these two types of agar. On ISP4 + DI the colonies were clearly Actinomycetes with large gray centers and white outlines (Figure 6A). The classification of PTY138G1 as an Actinomycete was not as clear on marine agar, and the colonies were small and black (Figure 6B).

A) 

B) 

Figure 6. PTY138G1 growth on agar (2 week incubation period at 30° C): (A) ISP4 agar with deionized water, and (B) marine agar with deionized water.

The differences in growth amongst media were even more apparent when the bacteria were grown in liquid media. In liquid ISP4 + DI media, the surrounding media was opaque with floating white solid colonies (Figure 7A). In liquid marine broth, the

surrounding media was dark black with floating grey solid colonies (Figure 7B). The floating colonies were similar in size.

A) 

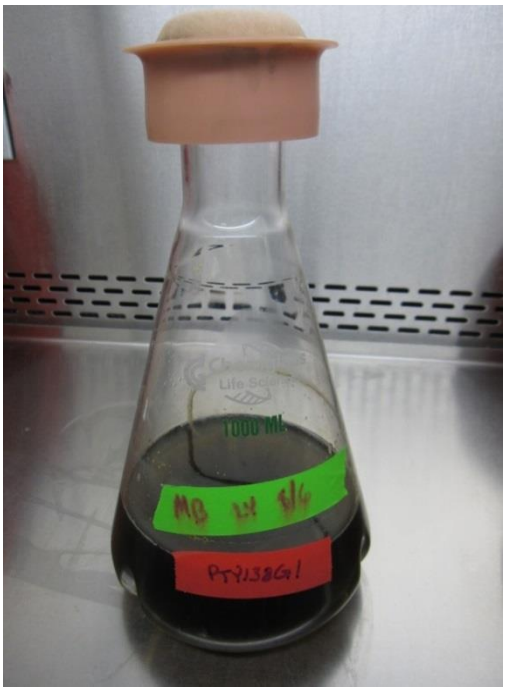
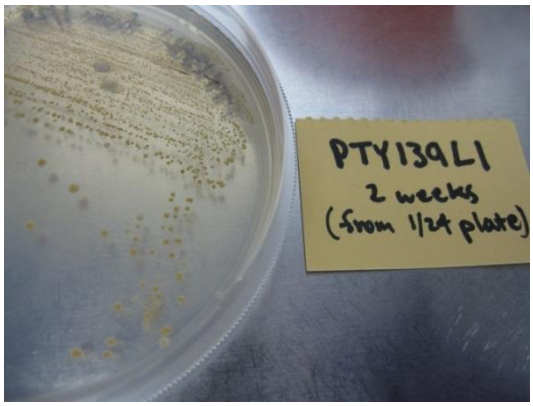
B) 

Figure 7. PTY138G1 growth in liquid media (2 week incubation period at 30°C): (A) ISP4 media with deionized water, and (B) marine broth with deionized water.

PTY139L1 was also grown on both ISP4 agar and marine agar. There was no growth on ISP4 agar with either DI or IO. The bacteria grew on marine agar and showed a color change after many weeks of incubation. After two weeks of growth, the bacteria consisted of small, dull orange colonies with an umbonate elevation (Figure 8A). This may be a sign that PTY139L1 is an Actinomycete as well. After more than four weeks of incubation, the colonies underwent a color change to black (Figure 8B). The color change was not clearly the result of the same compound that caused the black color in PTY138G1 in marine broth.

A) 

B) 

Figure 8. PTY139L1 growth on marine agar: A) 2 week incubation period at 30° C., and B) >4 weeks incubation at 30° C.

All samples were run on HPLC-MS or HPLC to compare metabolite profiles. The UV spectra of MB0086E (Figure 9) and MB0088E (Figure 10) (the original extracts used in BioMAP) were different from LY0086E (Figure 11) and LY0088E (Figure 12) (the new large scale extracts). Peaks of interest in MB0086E appear between minutes 50-56, 69-71, and 22-24. Peaks of interest in MB0088E appear at minutes 23, 29, 41, 55, 69, 75. LY0086E and LY0088E both have interesting peaks at minutes 70, 74, and especially at 64. Based on these peaks, there is a possibility that LY0086E and LY0088E contain the same compounds.

MB0086E, MB0088E, LY0086E, and LY0088E were also all examined via ^{1}H NMR in $CDCl_{3.}$ LY0086E and LY0088E both show possible aromatic rings between 7 and 7.5 ppm (Figures 13 and 14). Comparison of the two ^{1}H NMR spectra indicates that the two extracts are similar. However, LY0086E and LY0088E are not pure compounds, making this difficult to determine. The ^{1}H NMR of MB0086E and MB0088E are difficult to evaluate, because the samples were small, giving an unclear baseline. However, the

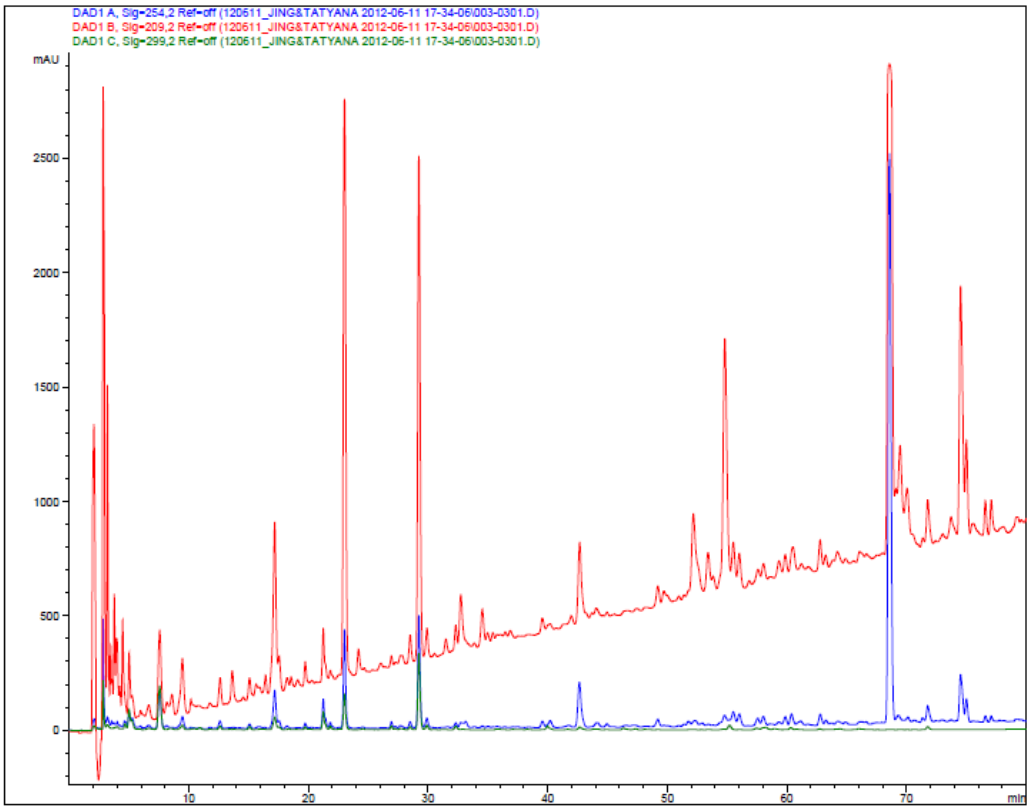


Figure 9. UV chromatogram from HPLC-MS of original fraction MB0086E.

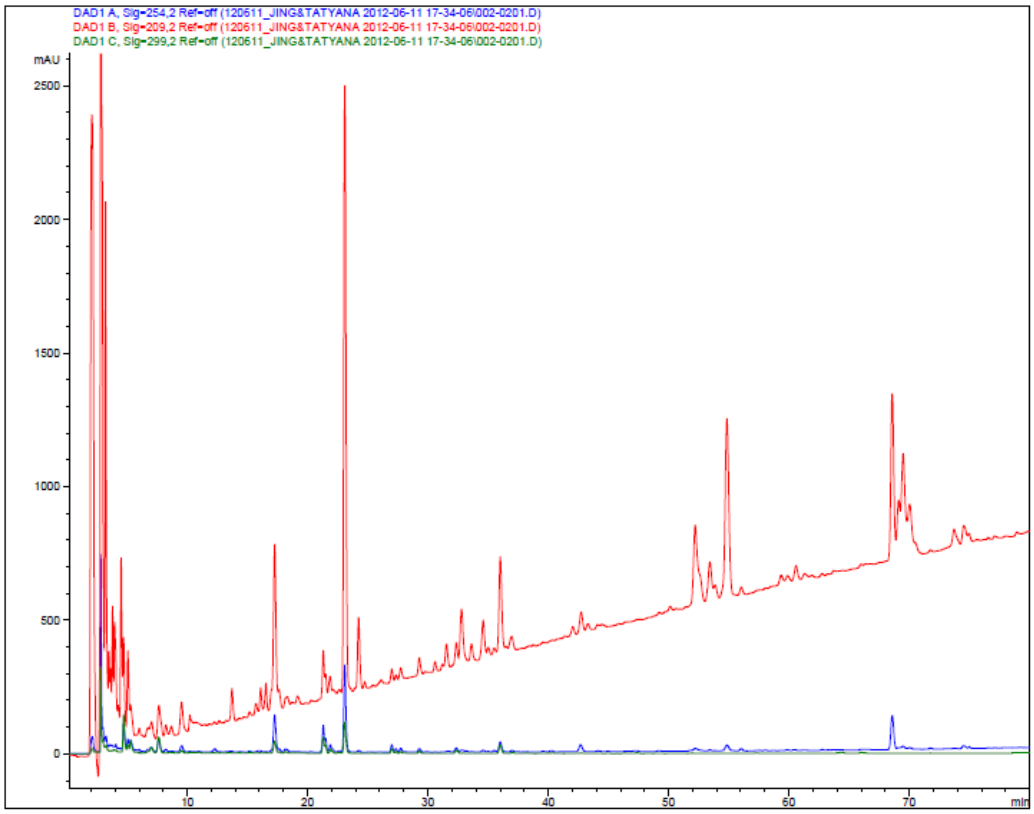


Figure 10. UV chromatogram from HPLC-MS of original fraction MB0088E.

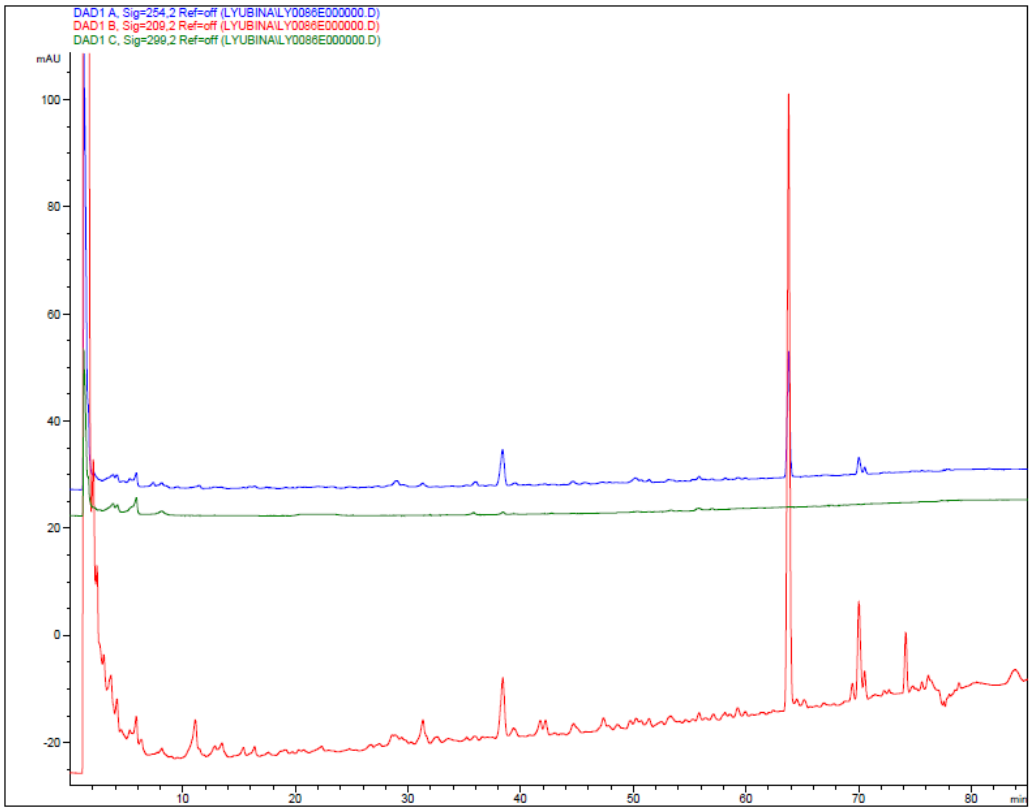


Figure 11. UV chromatogram from HPLC of new fraction LY0086E.

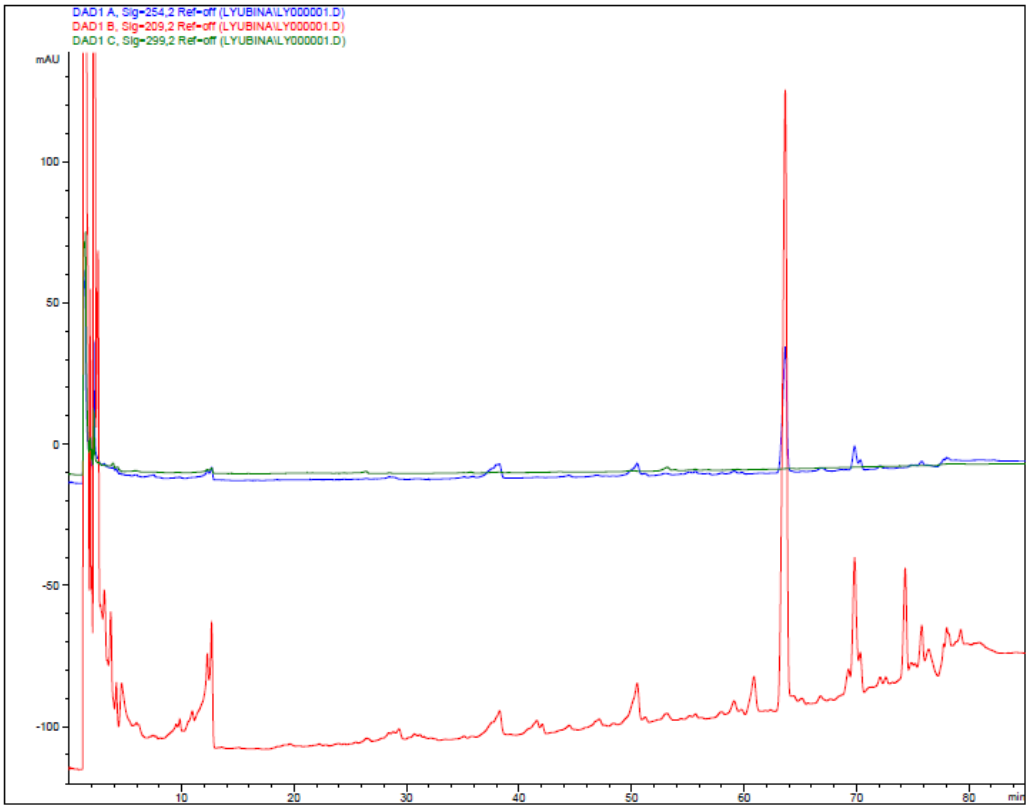


Figure 12. UV chromatogram from HPLC of new fraction LY0088E.

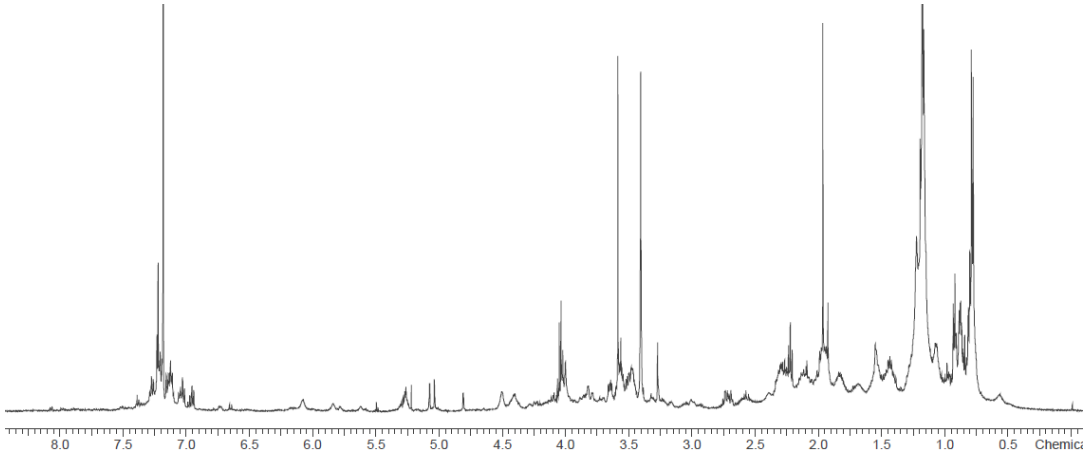


Figure 13. ^{1}H NMR of new fraction LY0086E in $CDCl_3$.

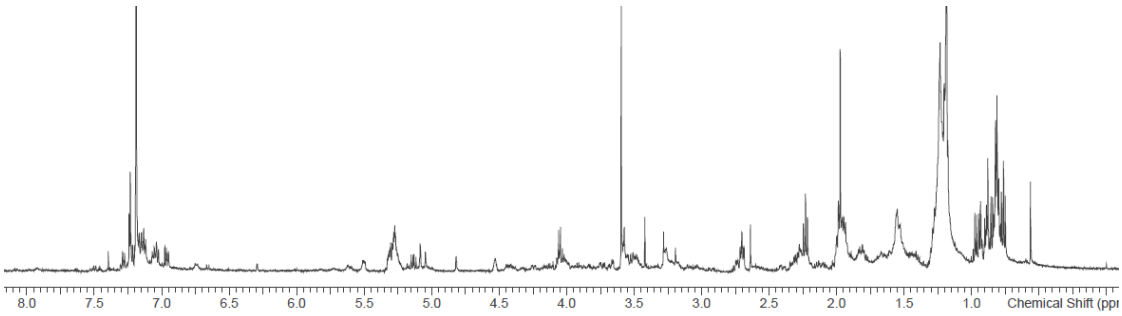


Figure 14. ^{1}H NMR of new fraction LY0088E in $CDCl_3$.

two have similar spectra, indicating that the compounds may be the same or analogs. Based on the NMR, MB0086E and MB0088E are likely pure compounds. The two spectra also show similarities when compared to ornidazole's NMR (Figure 17) predicted with ChemBioDraw Ultra 13.0 (PerkinElmer, Inc., Cambridge, Massachusetts, USA). The two compounds may be analogs of ornidazole, the structure of which is shown in Figure 18.

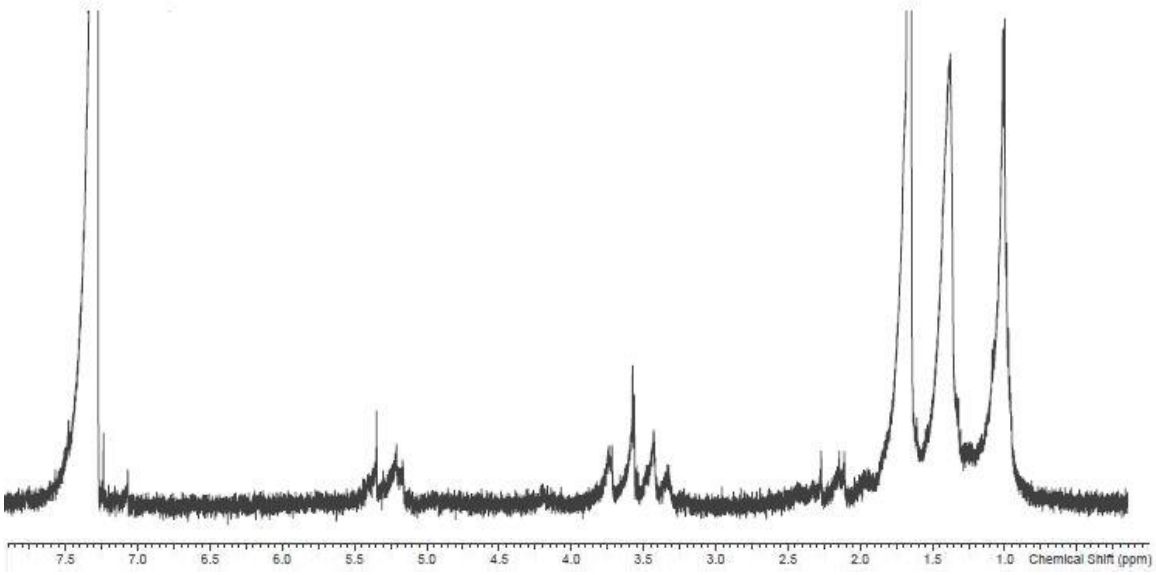


Figure 15. ^{1}H NMR of original fraction MB0086E in $CDCl_{3.}$

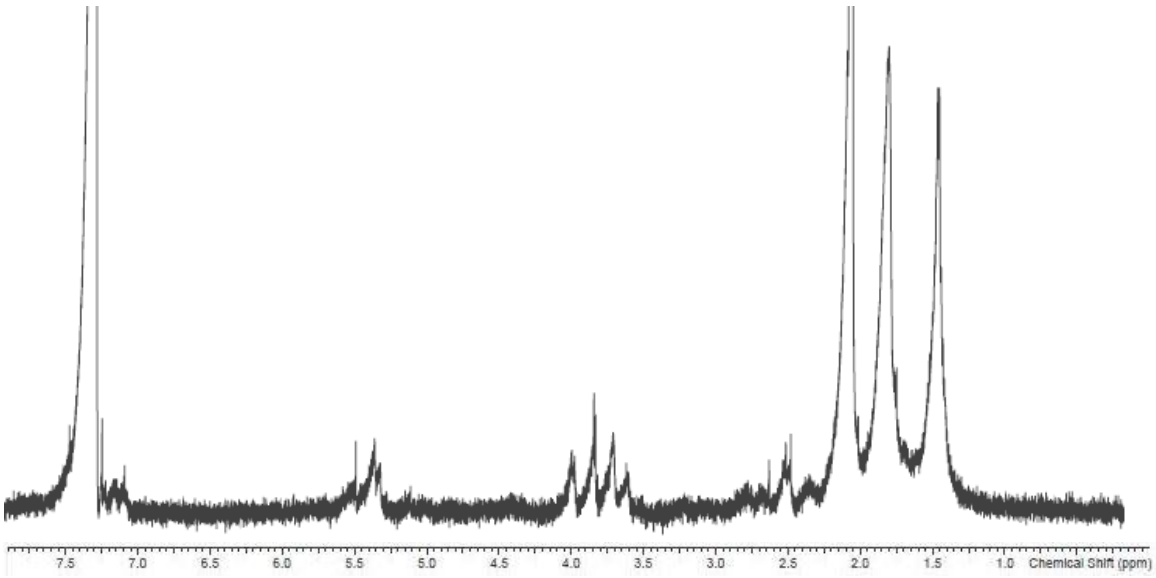


Figure 16. ^{1}H NMR of original fraction MB0088E in $CDCl_{3.}$

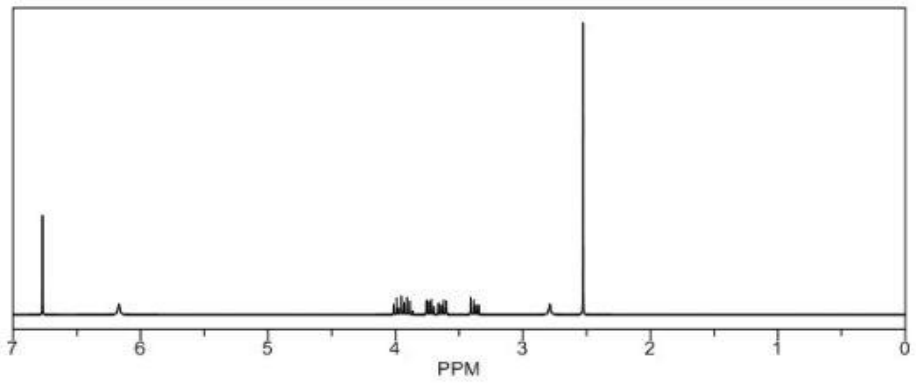


Figure 17. Predicted ^{1}H NMR of ornidazole.

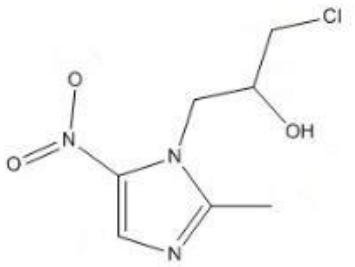


Figure 18. Structure of ornidazole.

Bioassays were conducted in order to determine if the newly extracted fractions (LY0086E and LY0088E) had the same activity as (MB0086E and MB0088E). The bioassay did not show activity against *S. aureus* compared to vancomycin. The bioassay often had high absorbance values in the color controls. This indicates that there was something in the extracts causing this absorbance. Growth was even sometimes observed in the color controls, even though there was no growth in the sterility controls. The high absorbance in the color controls could have been affecting the values for the wells in which the extracts were tested against *S. aureus.* After many attempts in which the color controls showed high absorbance values, the extracts were sterile filtered before the assay was performed. However, even after filtration, the extracts did not show activity against *S. aureus* (Figures 19 and 20).

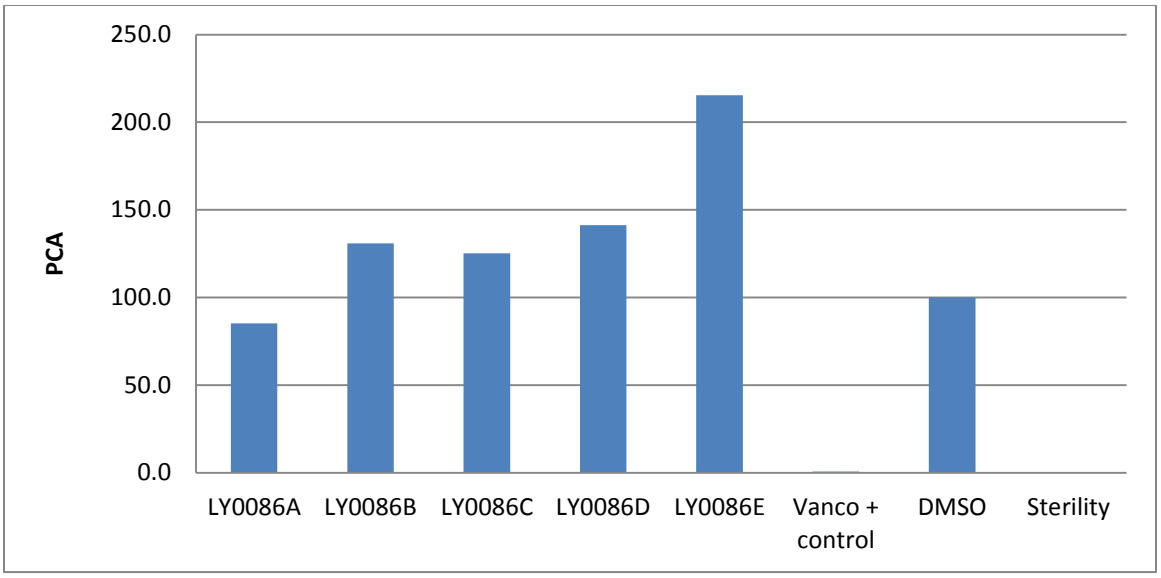


Figure 19. Bioassay of fractions LY0086A, LY0086B, LY0086C, LY0086D, LY0086E against methicillin-sensitive *Staphylococcus aureus* (MSSA). In a 96 well assay, MSSA was incubated for 24 hours with the five fractions, vancomycin as the positive control, and DMSO as the negative control. No inhibition of MSSA growth occurred.

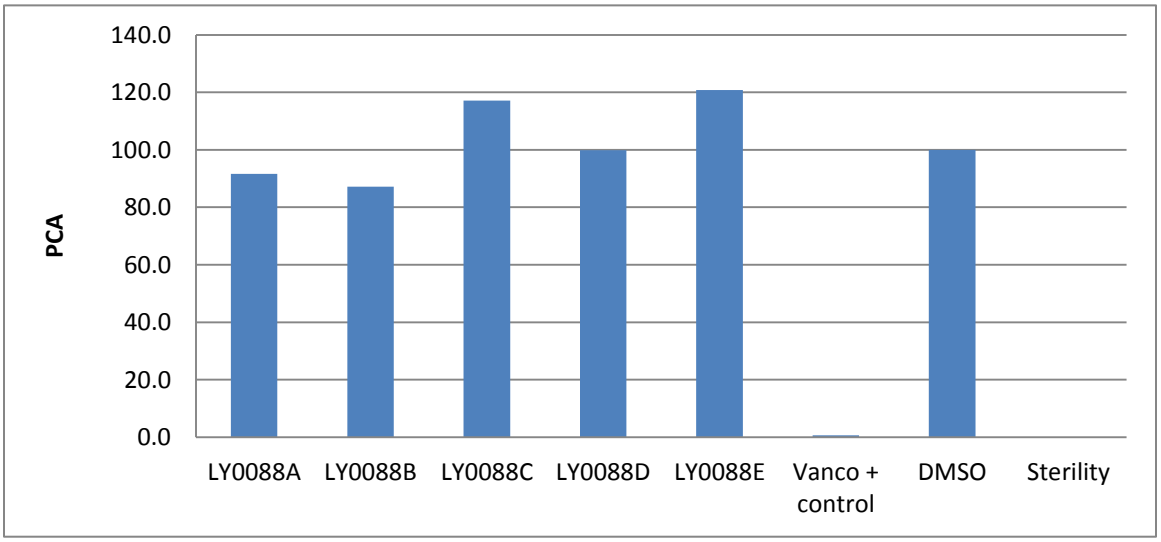


Figure 20. Bioassay of fractions LY0088A, LY0088B, LY0088C, LY0088D, LY0088E against methicillin-sensitive *Staphylococcus aureus* (MSSA). In a 96 well assay, MSSA was incubated for 24 hours with the five fractions, vancomycin as the positive control, and DMSO as the negative control. No inhibition of MSSA growth occurred.

After no activity was found, fractions LY0086E and LY0088E were sent to collaborators at the University of California, Santa Cruz in order to be tested in the assay in which MB0086E and MB0088E were originally active. The fractions still showed no activity against *S. aureus*, *E. faecium*, or *L. ivanovii*. In addition, the fractions LY0086A-E, LY0088A-E, LY0086A-E (SPE fractions from bacteria grown in ISP4 media), and LY0086A-E (SPE fractions from bacteria grown in marine broth) were not found to be active against *S. aureus* in the disk diffusion assay at 2 mg/mL or 5 mg/mL. LY0086E and LY0088E were also not active in the disk diffusion assay at 10 mg/mL.

An obvious obstacle in this experiment was the inability to repeat the activity previously shown in MB0086E and MB0088E. Many reasons could explain this inactivity. In the original experiment, fractions MB0086E and MB0088E could have been contaminated with something active that showed a false result in the BioMAP assay. The active compound might not have been produced in the new experiment. In the original experiment the bacteria were grown in 125 mL of media, which could have provided a better environment for the bacteria to produce these active fractions, whereas in the new one they were grown in 500 mL of media. The problem could also have been in the extraction of the bacteria. The active compounds may have remained on the HP-20 Diaion beads. Also, the incubation period before extraction may not have been sufficient to allow production of the active compounds.

Conclusion

Additional research must be conducted on the two bacteria and their extracts in order to determine their level of biological activity. HPLC and NMR indicate possibly interesting compounds that are worth pursuing. Bacteria should be regrown in 125 mL

cultures and incubated for two weeks to completely replicate the method used to grow the bacteria with the original active fractions. There is a possibility that the bacteria did not produce the active compounds in the larger volume of media. Another explanation is that the larger volume needed a longer incubation period in order to produce the compound. In addition to the 125 mL cultures, the bacteria can be grown again in 500 mL cultures with a longer incubation period such as three or four weeks. The bacteria should then undergo the combined extraction/fractionation step described in the methods section. Special attention should be given to dissolving the fractions and whether or not there are solubility issues. The extracts and fractions should be tested in the disc diffusion assay, the 96 well bioassay, and BioMAP. Testing them against parasites would be of interest, because of their probable structural similarity to ornidazole, an anti-parasitic drug.

If any fractions demonstrate activity against bacteria, or parasites, compound isolation should follow. Active fractions should be isolated using HPLC. Isolated compounds should be run on NMR, IR and HPLC-MS to determine their structures. These spectra will aid in determining whether or not the two active compounds are the same, and if they are analogs of ornidazole.

In order to make the study more thorough, the genomes of the two bacteria can be sequenced. This could determine the genera of the bacteria and possibly the species if they are previously discovered bacteria. Research looking into the mechanism used by the antibiotic compound can also be conducted. Eventually, the compounds can be synthetically altered to determine if there is a more potent structure. If the strength of the compound is comparable to known antibiotics, the antibacterial may move on to clinical trials.

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