University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2017

Use Of Secondary Metabolites For Novel Antibacterial Compound Discovery Produced By *Chromobacterium* And *Pseudomonas*

Delaney Beals
Chemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Amanda Wolfe

Abstract

Multidrug resistant bacterial infections, which arise due to misuse and overuse of antibiotics, are responsible for many nosocomial infections and are a threat to human health. Derivatization of known antibiotic compounds via total or semisynthesis can be time consuming and ineffective at targeting specific bacteria. This investigation focuses on bacteria found in the phytotelmata of *Sarracenia* pitcher plants and the natural antibiotic compounds they secrete under varying conditions. The aim of the project is to find single-producer and co-culture producing bacteria that secrete secondary metabolites effective against broad spectrum of Gram-positive and -negative pathogens. Two different species of *Pseudomonas* (CMCP E3, SS 827) and a *Chromobacterium* (CP SSIV) isolated in this study have been found to be effective against Gram-positive bacteria *Staphylococcus aureus* and the fungi *Fusarium solani*. The bacteria strains were cultured in minimal media containing either succinate or citrate that showed the densest growth after 72 hours. A 6 L citrate culture of the CMCP E3 bacterium yielded on average 32 mg of crude product and 9 mg of antibacterial compound. CPSS IV yielded an average of 16.8 mg crude natural product per 6 L culture. Characterization of these compounds has not been completed.

1. Introduction

Bacteria resistant to a variety of drugs are responsible for many nosocomial infections and are a threat to human health. The development of novel antibiotics for each new strain of infection-causing bacteria that circulate throughout the population often do not have high profit margins for the companies developing them or may not be the most effective antibiotic against certain strains. Resistance by bacteria, noncompliance of patients, and inappropriate antibiotic prescriptions exacerbates the chance of creating antibiotic resistant bacterial strains, making it more difficult to find novel drugs that are potent and not extensively time consuming to develop.[1] New methods of developing therapeutic antibiotics that can be translated to a large scale are of increasing importance due to the rising trend of antibiotic resistance in the population.[2]

The beginning of the modern age of antibiotics is often cited at the discovery of penicillin in 1929. Since then, natural products such as plants and microbes have been the source of new antibacterials, and their analogs have been responsible for the creation of two-thirds of clinically-useful antibiotic therapies.[3] This route of production, which utilizes natural sources for novel drugs, has likely been the most successful in that the mechanisms behind the antibacterials have been evolutionarily selected for over generations of bacterial growth.[4] More recently, the industrial rates of antibiotic production have decreased due to a perceived oversaturation of similar antimicrobial drugs and the fact that finding new targets and the drugs to reach them is becoming increasingly more difficult.[5] However, the challenging method of seeking out highly specific targets and attempting to synthetically develop or alter pre-existing drugs may be overhauled by reverting back to natural sources.[6]

As previously mentioned, the success of developing antibiotics derived from natural sources stems from the evolutionary traits that many bacteria have gained.[4] One of these traits is the release of bioactive compounds or antibiotics from bacteria themselves, which may be used for signaling, regulation, or as weapons against competing

microbes.[8] In general, these bioactive compounds are found in bacteria populations as a way for conspecific bacteria to regulate biological processes such as developmental changes that increase survival and can be virulent, or disease-causing, towards plants.[4] Creating conditions to encourage the release of these bioactive compounds and isolating them may be the key to solving the antibiotic development issue. By manipulating the types of bacteria and the growth conditions they are in, a potential for different genes within the bacteria to be expressed (and thus new compounds) can be created.[9]

The goal or this research was to use bacteria that produce their own bioactive compounds as a natural source for novel antibiotics. There are many situations in which bacteria are stimulated to produce antibiotics, one of which is due to quorum sensing (QS).[10] This process regulates gene expression in bacteria as a response to changes in the population density. The change in gene expression of a given bacterium may produce a bioactive compound, which could then be isolated and characterized to determine whether it can be used for therapeutic purposes.[11] The process behind unsilencing specific gene expression can be done using QS due to small molecules, known as pheromones or autoinducers, that can signal to other bacteria either within the same species or across a diverse population.[12] The genes that become expressed as a result of QS can factor into the virulence factor of bacteria, their biofilm formation capabilities, and any resistance towards antibiotics. [13]

Another useful trait of bacteria is the social interactions between two different strains that may produce antibiotics. Co-cultivation, or growing two types of different microbes together, can produce or increase production of many bioactives or antibiotics depending on the growth conditions. These testing conditions may include simply exposing two types of bacteria to one another, known as asocial growth, or by adding a third type to the environment or culture and observing how each strain responds, or social growth.[14] By using different combinations of bacteria, new bioactive compounds can be found *in vitro*. One such study by Marmann and coworkers found that several bacteria strains (MH1, MH2, MH3) showed increased antibiotic activity when exposed to another strain known as *B. subtilis.*[15] This bacterial strain is found in human gastrointestinal tracts, and the antibiotic found that works against it may be modified to use against similar, more harmful bacteria. These two methods of cocultivation and quorum sensing have been the main methods of using bacteria to produce novel antimicrobial compounds.

Because the role of gene expression is such a large part of finding and developing novel antibiotics based off naturally occurring compounds produced by bacteria, the classification and screening of these genes is highly useful and important. A common method of this is to categorize and find clusters of genes that affect bioactives production in the most common antibiotics. From there, scientists can find trends among the actual mechanisms that the genes influence, which can help to find new targets for antibiotics. One example of this type of screening is the BioMAP system that can show characteristics of different gene clusters found in bacteria strains.[16] In addition to being useful for finding new places to look for antibiotics, the BioMAP system can help classify new antibiotics and bioactive compounds that may not be identified fully. Using this method allows for a direct comparison between a new compound's gene cluster and any of the gene clusters already derived from the 72 commercially available antibiotics whose mechanism of action are already known.

The previous work done in our research group in collaboration with Dr. Sarah Seaton involved the isolation of bacteria from soil and the phytotelmata of pitcher plants, which act as a marine source. The goal of this project was to find different strains of bacteria, both Gram-positive and Gram-negative, that produced antibiotics or bioactive compounds without the introduction of other bacteria or microbes. Previous colleagues assayed over 400 different soil bacteria samples for antibiotic activity against Gram-positive *Staphylococcus aureus* both in single- and co-culture production. The assay was performed using 10% Tryptic Soy agar plates that had a thin overlay of *S. aureus* dried at 23°C.

To induce co-culture antibiotic production, randomly selected bacteria strains that were not determined to be single producers were grown in the same media and assayed to determine whether they produced any bioactive compounds. However, no samples studied were viable co-cultures in producing discernible antibiotic compounds.[15] Samples that produced a zone of inhibition as single producers were selected for future research, including the bacteria of this specific study, which are *Pseudomonas* labelled as CMCP E3 and SS 827 as well as a *Chromobacterium* labelled as CPSS IV.

There are several promising routes of finding and developing new antibiotics that will ideally outpace the rate of resistance on both a microscopic and macroscopic scale in the human population. Multidrug resistance is a complex issue, and no single method of drug development will be able to solve it. However, by referring back to the mechanisms of nature and bacteria themselves, these efforts will be more successful in finding new targets and decreasing the time required to develop a drug against them. In this research, the naturally-occurring, dynamic characteristics of bacteria that produce their own bioactive compounds will be studied, and in turn be used to increase the efficacy and availability of therapeutic antibiotics. Understanding and optimizing how bacteria produce bioactive compounds is an essential step in the characterization and manipulation of its chemical structure, and thus its mechanism and function. In this research, the naturally-occurring, dynamic characteristics of bacteria that produce their own bioactive compounds are studied, and may be used to increase the efficacy and availability of therapeutic antibiotics.

2. Experimental Procedures

2.1 Sterile Technique

All methods were carried out under sterile conditions which included sterilization of counter tops and gloves using ethanol and bleach. Work was done under a flame, and containers, pipette tips, inoculating loops, and all other materials were autoclaved before and after use (121℃, 20 min., 15 psi).

2.2 Agar Streaking Technique

All bacteria were streaked using the same method on 10% Tryptic Soy agar (Difco™ Tryptic Soy Agar Soybean-Casein Digest Agar) plates (TSA; 3g per one liter of water with 2% agar). A toothpick was used to pick up one colony off a given bacteria plate and then streaked in quadrants on the new plate, flipping the toothpick halfway through. This ensured that the bacteria would be distributed sparsely enough for individual colonies to be visualized after 48 hours of growth at 23 ℃ for soil bacteria or 37 °C for *S. aureus* and *E. coli*.

2.3 Culture Conditions

Bacteria strains were grown in citrate (CMCP E3, SS 827) and succinate (CPSS IV) carbon sources after determination of the most prolific source via monitoring changes in absorbance over time. A salt minimal medium containing one of each carbon source was used to grow the bacteria. Per one liter, the media contained 100 mL of 10x minimal media salt [0.35M $K_2HPO_4 \cdot 3H_2O$, 0.22M KH_2PO_4, 0.08M $(NH_4)_2SO_4$)], 1M $MgSO_4$, and 1 M of the specific carbon source. The bacteria were inoculated under sterile conditions by scraping one colony of cells with a pipette tip and placing into an aliquot (approximately 10 mL) of minimal media, which could later be added to a large sterile amount of media to continue growing. After 48 hours of growth, the absorbance was measured of the bacteria in minimal media using 1 mL of sample in a plastic cuvette in a spectrophotometer at 600 nm.

2.4 Simple Antibiotic Assay Method

To detect the antibiotic activity of the bacteria strains, a culture of *S. aureus* was prepared by inoculating 10 mL full strength tryptic soy broth (Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium) (TSB) under sterile conditions. After 24 hours at 37 °C, 200 µL was evenly spread on an agar plate made with 10% TSA. After drying, 3 µL of the selected minimal media culture was pipetted in the center of the sectioned plate. After 24-48 hours at 23 ℃, a zone of inhibition around each 3 µL spot was visualized.

2.5 Time trial

To determine at what time after inoculation bacterial growth was most dense, and thus at what time it produced the maximum amount of antibacterial compound, media was inoculated at time 0 hours. At each interval (hours 0, 2, 4, 8, 16, 24, 48, and 72), 3 mL of culture was removed from the flask after filtration using a syringe attachment. The filtrate was placed in a test tube to be used for a well assay. Another 1 mL was taken from the large culture without filtration and placed into a quartz cuvette and the absorbance read.

2.6 Extraction

Large scale cultures were used to extract antibiotic compounds from the media and bacterial cells. A small culture of each bacteria (10 mL of minimal media inoculated with bacteria) grown in the most prolific carbon source was added to 1 L of the respective minimal media and allowed to grow while shaking at 25 °C for 48 hours. The cultures were then centrifuged at 5000 RPM for ten minutes. The supernatant was then separated from the cell pellet and was extracted in a 4 L separatory funnel. The extraction was carried out three times using ethyl acetate and saturated aqueous NaCl was added prior to separation. The organic layers were collected, washed with saturated aqueous NaCl, dried over Na_2SO_4 and concentrated under reduced pressure.

An alternative extraction method was also used for CPSS IV. Per each 3L portion, the supernatant was washed once with hexane, diethyl ether, and ethyl acetate successively to further separate compounds by polarity. Each crude product from the different washes were collected and saved for testing.

2.7 Column chromatography

To further isolate compounds within the crude product, a solvent system of hexane and ethyl acetate was determined by selecting the ratio that displayed the most amount of compound separation on a TLC (thin layer chromatography) plate. This solvent system was then used to run a dry-packed SiO_2 column for each crude product. Columns were wet with a solvent more non-polar than the optimal system, then increased in polarity until ideal ratio was achieved. Columns were flushed with ethyl acetate, and all fractions were monitored by TLC. Similar fractions were collected, evaporated, and massed.

2.8 Well Assay Method

Each dried sample from CMCP E3, SS 827, and CPSS IV was dissolved in dimethyl sulfoxide (DMSO). The dissolved compound and DMSO was diluted with full strength TSB to 100x concentration, and a negative control was made using the same concentration. Wells were created in a 10% TSB agar plate and 45 µL of each sample and control each were added to one well. The plates remained at 23 ℃ for 48 hours and the zones of inhibition visualized and measured.

2.8 Microplate Assay Method

Compounds to be tested for antibiotic properties were pipetted into a 96-well microplate and read with a BioTek Synergy HTX Multimode Reader spectrophotometer. To each experimental well, 89 µL full strength TSB, 10 µL *S. aureus* liquid full strength TSB culture, and 1 µL of sample dissolved in DMSO were added. Positive control for the study included a known antibiotic, chloramphenicol, to S. aureus-containing wells, as well as a negative control containing only 1 µL of DMSO with no antibacterial compound. Once prepared, the microplate was left in a 25℃-shaker overnight before being read.

3. Results and Discussion

3.1 Optimization of culture conditions

Once single-producer strains of bacteria were identified from Dr. Seaton's laboratory, small cultures of five different carbon source-containing minimal media were grown. After 24 hours, the absorbances were read to determine the carbon source that produced the densest and ideally most antibiotic producing bacteria (Table 1). The highest absorbance for CMCP E3 was observed using the citrate media, and the second highest absorbance was succinate for CPSS IV. Glucose-containing minimal media was known to produce the densest culture of CPSS IV, but was not chosen due to difficulties in isolation as identified by previous co-workers within the Wolfe group. SS 827 was determined to be most prolific in citrate minimal media. Small cultures (10 mL) of all types of minimal media were each inoculated with CMCP E3 and left to grow in a shaker (Eppendorf New Brunswick Excella 25 Inc Shaker) above 90 RPM for 24 hours. A simple antibiotic assay against *S. aureus* was performed to ensure that a CMCP E3 citrate culture allowed for antibiotic production, with zone of inhibition of 15 mm.

A time trial from 0 to 72 hours was run using CMCP E3 to determine at what point after inoculation was the growth of bacteria the densest and produced the most antibiotic compound. Results were read using a spectrophotometer and an assay of samples from each time period was performed. The results showed that growth approached a maximum after 24 hours, and began to decrease at 72 hours (Table 2). Zones of inhibition from different times were inconsistent, but antibiotic production was clearly present at 48 hours (Table 2). These results provided a guideline for future culture growth times for CMCP E3. The time trial was not performed for CPSS IV or SS 827 due to time constraints.

Table 1. Relative densities of bacteria grown in liquid media containing one of five potential carbon sources. The corresponding carbon source to the densest growth was chosen for future cultures for each bacterial strain.

CMCP E3	carbon source	CPSS IV
0.337	glucose	0.325
0.013	acetate	0.038
0.339	succinate	**0.255**
0.002	salicylate	0.003
0.384	citrate	0.000

Table 2. Time trial results that show the increase in growth density with increasing time. The results of a well assay are present to test for antibiotic presence at various times after inoculation.

Time after inoculation (h)	Absorbance	Zone of inhibition (cm)
t=1	0.000	N/A
t=2	0.000	1.0
t=4	0.001	N/A
t=8	0.009	1.2
t=16	0.302	N/A
t=24	0.366	N/A
t=48	0.268	0.7
t=72	0.325	N/A

3.2 Isolation of antibiotic compounds

Using the determined carbon source for each bacteria strain, large scale (1 L to 6 L) cultures were made. After 24-48 hours of growth, the cultures became cloudy as compared to the control media, indicating bacterial growth. These cultures were centrifuged to remove cell debris, and the supernatant was extracted using ethyl acetate and saturated aqueous NaCl. After evaporation under reduced pressure, crude product masses were obtained (table 3). A well assay against *S. aureus* was run using the crude product of a 1 L CMCP E3 culture, giving a zone of inhibition of 15 mm. This assay showed that the antibiotic compound of CMCP E3 was found in the organic layer of the extraction and was still a viable antibacterial after the extraction process.

The crude products of CMCP E3, CPSS IV, and SS 827 were individually separated using column chromatography. Small fractions (1-2 mL) were collected and qualitatively analyzed using TLC. Upon completion of the column and elution of all compounds via flushing with ethyl acetate, the like fractions were collected, evaporated, and massed (table 4). A well assay was performed testing each fraction from both bacteria. The zones of inhibition were observed. In both strains of bacteria, one respective fraction was clearly identified as containing the antibiotic compound, which could be isolated further and characterized. The fraction 3 from CMCP E3 showed a zone of inhibition of 0.9 cm, and fraction 6 from CPSS IV showed a zone of 2.0 cm (Figure 1a-b).

Antibiotic results of CPSS IV fractions were reconfirmed using a microplate assay. The assay compared the antibiotic effects of CPSS IV fractions to a known antibiotic chloramphenicol against *S. aureus*, which served as a positive control. The assay showed again that fraction 6 had the most antibacterial effect in comparison to the other fractions, which showed an absorbance similar to the negative control containing no antibiotic (Table 5a-b). Several further efforts to isolate the antibiotic compound located at Rf= 0.38 produced by CPSS IV. This included an alternative extraction scheme as mentioned in section 2.6, as well as secondary columns on fractions that contained the desired compound in addition to others, according to TLC. Isolation has proven to be especially difficult, due to the high number of compounds within the crude product as well as the low concentration of the antibacterial

compound. Separation methods used in this study have been designed to reduce confounding factors such as these, but a completely optimized separation scheme has yet to be identified and performed.

Table 3. Average masses of crude products per size of liquid culture for all strains.

Bacterial strain	Culture size	Average mass of crude product
CMCP E3	7 L	60.8 mg
CPSS IV	6 L	25.2 mg
SS 827	6 L	113 mg

Table 4. Mass of compounds and associated TLC Rf values obtained from most productive column of CPSS IV crude product.

CPSS IV fraction	Mass product	Rf value
A	1.5 mg	0.48
B	2.5 mg	0.40
C	2.7 mg	0.38
D	1.6 mg	0.38 & 0.32
E	0.9 mg	0.23

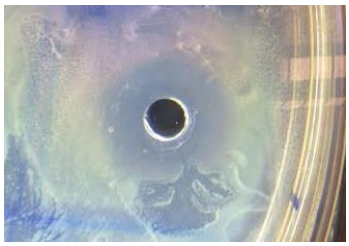


Figure 1a. Characteristic well assay of CPSS IV fraction 6. Dissolved compounds were pipetted into wells created from *S. aureus*-streaked agar.

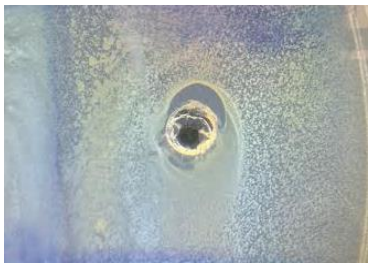
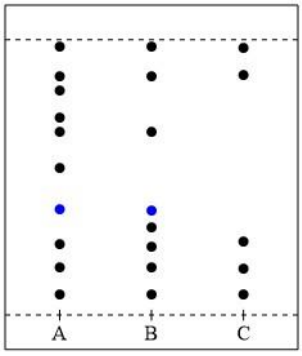
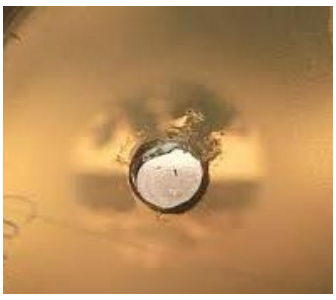


Figure 1b. Control well containing DMSO and TSB pipetted into S. aureus-streaked agar.



2a



2b

2c

Figure 2. TLC of alternative extraction of CPSS IV using hexane (C), diethyl ether (B), and ethyl acetate (A) to wash each layer of supernatant (2a).

Antibiotic compound as identified from previous columns has Rf= 0.38, and is present in the ethyl acetate and diethyl ether lanes, indicated by a blue spot. These results are consistent with the well assay against S. aureus showing zones of inhibition around product obtained from diethyl ether (left well) and ethyl acetate (right well) extraction layers (2c). Zones show comparable size to positive control well with antibiotic chloramphenicol (2b).

Table 5. Raw data of microplate assay measured in duplicate. Absorbances were read at 590 nm.

a

	1	2
A	0.093	0.504
B	0.116	0.431
C	0.116	0.386
D	0.118	0.287
E	0.456	0.367
F	0.424	0.386
G	0.417	0.414
H	0.393	0.441

b

	1	2
A	0.135	0.438
B	0.139	0.418
C	0.145	0.401
D	0.143	0.321
E	0.439	0.295
F	0.446	0.305
G	0.429	0.348
H	0.379	0.412

Low values indicate relatively high antibiotic activity as compared to the known positive control wells containing chloramphenicol (Column 1, Rows A, B, C, D). The negative controls (Column 1, Rows E, F, G, H) indicate no inhibition of *S. aureus* growth. Several different compounds isolated from an ethyl acetate extraction of CPSS IV were diluted 100-fold (as described in section 2.8) and added to wells (Column 2, Rows A, B, C, D, E). The well containing antibacterial compound on each plate (Table 5a: Column 2, Row D; Table 5b: Column 2, Row E), showed a decrease in absorbance as compared to other isolated fractions.

3.3 Characterization of compounds

Once the antibiotic compounds were identified from the crude product of CMCP E3, mass spectroscopy and an ^{1}H NMR using $CDCl_3$ were run. Data from both characterization methods were inconclusive, with results suggesting that the antibiotic product contained mixture of compounds. Further attempts to isolate the single compounds were performed for CMCP E3, including column chromatography of the fraction with a wider solvent system range. This technique did not yield greater separation of the compounds within the original fraction. Characterization of the antibiotic fraction of CMCP E3 and CPSS IV is ongoing at the time of writing this report.

4. Conclusion

The process of optimization and isolation of compounds is moderately successful, allowing for the identification of column fractions that contain an antibiotic compound. The method yields a small amount of crude product for the amount of liquid media culture, but this result is comparable to other methods performed using *Chromobacterium*.[15] The gradient extraction that used increasingly polar solvents proved to be the most effective method for obtaining both a high yield of crude product and good separation of the antibiotic compound in CPSS IV. This extraction method

will be utilized for any further isolation attempts. Several antibiotic assays have shown both through large zones of inhibition and low absorbance values that CPSS IV secretes a compound with strong inhibitory activity towards *S. aureus*.

Future directions for this project involve elucidating the chemical structure of antibiotic metabolites from both CMCP E3 and CPSS IV using NMR and mass spectrometry. Further analysis includes determination of minimum inhibitory concentration (MIC) and IC50 values of the antibiotic compound. Once characterized, derivatives of the structure can be synthesized to potentially create more novel antibiotic candidates and manipulate functional groups through a structure-activity relationship (SAR) analysis, to better understand the compound's mode of action. However, further optimization of the extraction process may be needed to obtain more pure samples of the antibiotic compound from CMCP E3 in order to characterize it accurately. The results of this research will contribute to the overall project of reliably and rapidly identifying and analyzing novel antibiotics from natural bacterial sources.

5. Acknowledgements

The author wishes to express her appreciation to Dr. Amanda Wolfe, Dr. Sarah C. Seaton, UNC Asheville's Department of Chemistry, Maddy Thurman, Leah Bouthillette, and Tess Handy.

6. References

1. Bologna CG, Ursu O, Oprea TI, Melançon III CE, Tegos GP. Emerging trends in the discovery of natural product antibacterials. *Current Opinion in Pharmacology* **2013,** *13*, 678-687.

2. Butler CC and Nagel J. A Review of Quality Measures for Assessing the Impact of Antimicrobial Stewardship Programs in Hospitals. *Antibiotics* **2016**, *5*(1), 5.

3. Lucas X, et al. StreptomeDB: a resource for natural compounds isolated from *Streptomyces* species. *Nucleic Acids Research* **2013**, *41*, 1130-1136.

4. McKellar MR and Fendrick AM. *Clinical Infectious Diseases* **2015**, *61*(11), 1695-1702

5. Molony MG. Natural Products as a Source for Novel Antibiotics. *Trends in Pharmacological Sciences* **2016**, *37*(8), 689-701.

6. Aussel L, Beuzon CR, Cascales E. Adaptation and communication of bacterial pathogens. *Virulence* **2016**, *7*(4).

7. de Lima Procopío RD, et al. Antibiotics produced by *Streptomyces*. *The Brazilian Journal of Infectious Diseases* **2012**, *16*(5), 466-471.

8. Hassan, et al. Antibiotics as Microbial Secondary Metabolites: Production and Application. *Jurnal Teknologi (Science and Engineering)* **2012**, *59*, 101-111.

9. Liu Z, et al. Antibiotics at subinhibitory concentration improve the quorum sensing behavior of *Chromobacterium violaceum*. *FEMS Microbiology Letters* **2013**, *341*, 37-44.

10.Chifiriuc MC, Grumezescu AM, Lazar V. Quorum Sensing Inhibitors from the Sea: Lessons from Marine Symbiotic Relationships. *Current Organic Chemistry* **2014**, *18*, 823-839.

11. Deryabin DG and Tolmacheva AA. Antibacterial and Anti-Quorum Sensing Molecular Composition Derived from *Quercus cortex* (Oak bark) Extract. *Molecules* **2015**, *20*(9), 17093-17108.

12. Abrudan MI, Smakman F, Grimbergen AJ, Westhoff S, Miller EL, van Wezel GP, Rozen DE. Socially mediated induction and suppression of antibiosis during bacterial coexistence. *PNAS* **2015**, *112*, 11054-11059.

13. Marmann A, Aly AH, Lin W, Wang B, Proksch P. Co-Cultivation—A Powerful Emerging Tool for Enhancing the Chemical Diversity of Microorganisms. *Marine Drugs* **2014**, *12*, 1043-1065.

14. Wong, Weng, Oliver, Allen, Linington, Roger. Development of Antibiotic Activity Profile Screening for the Classification and Discovery of Natural Product Antibiotics. *Chemistry & Biology* **2012**, *19*, 1438-1495.

15. Parker, et al. *Aerocyanidin*, a New Antibiotic Produced by *Chromobacterium violaceum. Journal of Antibiotics* **1988**, *41*, 454-460.

16. Darcey C. Isolation and Characterization of Natural, Antibiotic-Producing Bacteria in Pure-and Co-Culture. Journal on Undergraduate Research. Published May 2016.