Amanda Wagler

The Extraction, Purification, and Characterization of a Possible Prodigiosin

Abstract

Bacteria develop resistance to drugs due to improper uses of antibiotics and mutations. This creates a need for new antibiotics. New sources of drugs can be found in nature or inspired by nature. Bacteria themselves produce secondary metabolites that ward off other bacteria, and therefore can be sources for new antibiotics. The two strains of Vibrio (MI-1 and MI-2) explored in this project have been found to produce metabolites that show antibacterial activity. Due to its characteristic pink color, it can be speculated that the secondary metabolite produced by the two Vibrio species is possibly a type of prodigiosin, a class of antibacterial compounds. Finding new types prodigiosin can lead to new drugs that can be on the market. This project aims to identify the potential prodigiosin structure. The process requires three phases: extraction, purification, and characterization. The metabolites from MI-1 and MI-2 strains were extracted and purified using chromatographic methods and solid phase extraction. Spectroscopic methods focused on only MI-1 and included NMR, mass spectrometry, Infrared Spectroscopy, and ultraviolet-visible spectroscopy to characterize the compound.

Introduction

Bacterial infections are extremely common. One of the most well-known bacterial infections is bacterial meningitis, caused by *Neisseria meningitidis*[1]. Bacterial meningitis infects over 4,000 people a year and causes 500 deaths a year in the US. Death can occur within hours if not treated properly, and permanent damage such as hearing loss, brain damage, and learning disabilities can occur after treatment[2]. Another bacterial infection that affects a wide variety of individuals is E. coli food poisoning caused by food contamination and unclean surfaces. E. coli

caused 96,000 illnesses and 3,200 hospitalizations in the US between the years 2003 and 2012[3.] Antibacterial compounds, such as prodigiosins, could be further studied to find more efficient treatments for these bacterial infections.

There has been a major shift of antimicrobial compounds used in medicine. The first antibiotic, penicillin, was discovered by Alexander Fleming in 1926[4]. Penicillins act as cell-wall inhibitors. Their beta-lactam ring interrupts the peptidoglycan cell wall synthesis, resulting in cell death. Over time, bacteria had developed a resistance against penicillin, especially *Staphylococcus aureus.* In order to combat this issue, methicillin was utilized. However, methicillin-resistant *Staphylococcus aureus* (MRSA) arose, rendering methicillin ineffective. A shift to Vancomycin then occurred, an IV-drug that also inhibits the cell wall. Certain strains of *Staphylococcus aureus* have recently become resistant to vancomycin, and carbapenems are now considered the "last-resort" antibiotic[4].

According to the World Health Organization, the four main causes of antibiotic resistance are overprescribing, incomplete treatment, overuse in livestock, lack of new antibiotics[5]. When patients are given an antibiotic that is not needed or is not taken properly, it is more likely for a bacterial strain to survive and develop resistance. The overuse in livestock causes a bacteria's exposure of an antibiotic to increase, allowing it to easily develop resistance. As more and more antibiotics are becoming ineffective, there are less options for treatment, and new antibiotics need to be available. One way to combat this ever-prevalent issue is the education of patients on how to take their antibiotic properly. Also, prescribers should be educated on when it is appropriate to prescribe an antibiotic. Another solution would be to find new antimicrobial compounds. This would involve synthesizing new structures to which drug-resistant bacteria have not developed a resistance.

Research in finding or synthesizing antibacterial compounds is extremely important due to the ever-growing antibiotic resistance that bacterial strains have built up. In this process, most of the bacteria will die off from the antibiotic, but some remaining strains will evolve to be resistant. For example, Methicillin-Resistant Staphylococcus aureus (MRSA) and *Neisseria gonorrhoeae* are resistant to penicillin.[6] In order to solve this issue, novel antibiotics need to be identified and synthesized.

Prodigiosins are secondary metabolites that have promising antibiotic activity[7]. Therefore, they can be isolated in order to identify their structures to be able to synthesize them for further studies. It is characterized by a tri-pyrrole ring, as seen in Figure 1. Typically, a prodigiosin with have an -OR group on the second ring and a long carbon chain on the third ring. It is known that different functional groups give prodigiosin novel function, making it important to continue studying these compounds. Prodigiosin is known for its red pigment, which is easily identifiable in cultured bacteria[1]. The overall goal of the project was to extract, purify, and characterize a secondary metabolite that is believed to be a prodigiosin from the bacteria named MI1 by Dr. Gasper. Metabolites usually affect the function of a bacterium, but a secondary metabolite typically does not affect the growth or reproduction[8]. A major advantage of a secondary metabolite is its toxicity toward organisms such as other surrounding bacteria. This is due to the competition for resources between bacteria. Figure 2 shows the secondary metabolite prodigiosin in several species of bacteria. Prodigiosin has shown anti-tumoral, anti-bacterial, and anti-fungal activity due to its function as a secondary metabolite[7].

Figure 1. Basic tri-pyrrole structure of a prodigiosin molecule

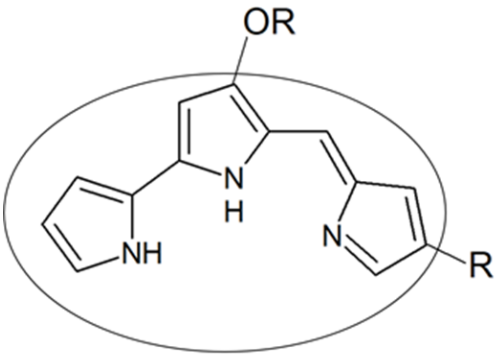
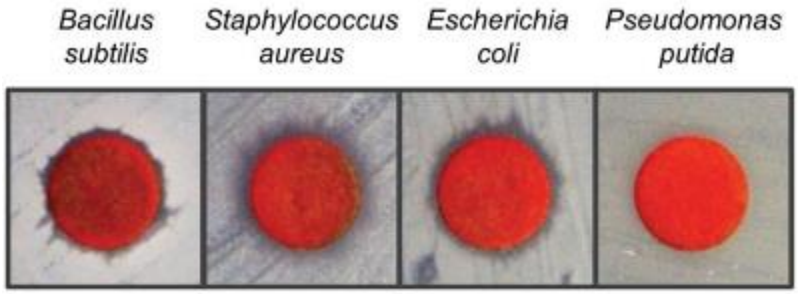


Figure 2. Prodigiosin in Several Bacterial Species[9]



Bacterial infections are not only a major target of prodigiosins. Fungal infections are common in everyday life and can be extremely dangerous if not treated. People with weak immune systems are especially susceptible to fungal infections. Fungi are present in the environment, and people breathe or come into contact with spores every day[4]. *C. neoformans* affects roughly one million individuals worldwide, and results in the death of nearly 625,000 individuals[10]. Although rare, *Mucormycosis* also causes death among its victims. Between 1992 and 1993, there were 1.7 cases per 1 million population in the US[2] Its overall mortality rate was found to be 54%. It is important to find treatment for these infections as well as novel treatments for unknown infections, and novel prodigiosins could help this cause.

Cancer affects 340,000 people per year in the U.S.[11]Prodigiosins have shown anti-tumor activity. Like most cancer-chemotherapeutic agents, prodigiosins have displayed apoptotic

effects on in-vitro specimens[11]. Prodigiosin has also showed anti-tumor activity in-vivo[11]. There have been many studies relating to the activities of compounds from the prodigiosin family.

Literature Review

Prodigiosin Activity in *Serratia marcescens*

A common marine bacterium that has shown significant prodigiosin activity is *Serratia marcescens.* One of the first prodigiosins was identified in a strain of *S. marcescens* in a study done by Robert Howard Williams[12]. In this study, a mutant strain of *S. marcescens* called H-462 was used because it was a clear, non-pigmented strain. After the strains were paired together, the pigment was extracted using centrifugation and identified using Thin Layer Chromatography (TLC), Ultraviolet-Visible Spectra, and Nuclear Magnetic Resonance spectra. When the prodigiosin was identified, a higher concentration of pigment was produced in mixed-mutant liquid broth cultures. The pigment was purified through a recrystallization using ethanol as a solvent.

Another study observed prodigiosin in *Serratia* marcescens[13].The study stated that the prodigiosin could cause apoptosis and can become a possible treatment for certain types of cancer. The study showed a detailed comparative analysis of prodigiosin and other extracellular proteins produced by *Serratia marcescens*. The prodigiosin showed extensive activity against *Botrytis cinerea*. The paper described the tri-pyrrole structure of prodigiosin and discussed how functional groups can affect function of the prodigiosin.

Prodigiosin Production in Vibrio Species

Several strains in the *Vibrio* family have been extensively studied for their production of prodigiosin. In 1974, Nancy N. Gerber and J. Y. D'Aoust found prodigiosin production in a vibrio species[14]. In this experiment, *Streptomyces longisporus ruber*, *Actinomadura madurae*,

Actinomadura pelletieri, and V. psychroerythrus were observed. Six prodigiosin-like pigments were isolated. Out of these pigments, a red pigment was found in the marine microorganism *Vibrio psychroerythrus.* The structures were compared to a prodigiosin isolated from *Serratia marcescens*. This prodigiosin of interest showed different properties than the other five, showing a positive identification of a prodigiosin.

In 2010, a *Vibrio* study was published in *Marine Drugs*[15]. Over 500 strains of bacteria were collected through a global expedition. All of the strains where known to be able to inhibit pathogens. Out of these strains, 300 were considered to be part of the *Vibrionaceae* family. Five of these strains showed significant antimicrobial activity. Liquid Chromatography Ultraviolet Mass Spectrometry (LC-UV/MS), a method that combines a separation technique with mass analysis, was used to explore genetic relationships between these five strains. *P. halotolerans* was deemed unique from any of the other strains.

In another study done in 2010, several marine bacteria were screened, and it was found that a *Vibrio* species from the coral *Sinularia polydactyla* is a unique and significant antibacterial producer[16]. The metabolites that the Vibrio produced were numerous, but seven were isolated and compared to already known metabolites. Activity against *B. subtilis* was tested with a diffusion assay, how did they test it, and the most successful metabolite was *vibrindole A*, which was found to be an adversary to cytotoxins.

Prodigiosin in *Actinomadura*

Published in the *Journal of Antibiotics*, one study discusses two prodigiosins found in Actinomadura pelletieri, a species of actinobacteria[17] Thin-Layer Chromatography, visible absorption spectroscopy, and mass spectrometry were used to identify and characterize these pigments as undecylprodiginine and methylcyclodecylprodiginine. It is still unsure which strains

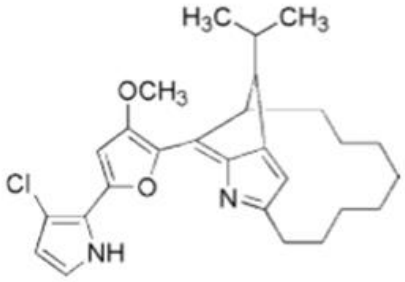
these prodigiosins are effective against, since these compounds had no effect on E. coli, P. vulgaris , C. albicans ,T. koningi , P.notatum or P. aeruginosa. Prodigiosin in this bacterium could still be explored, since this study was inconclusive.

Distinguishing Prodigiosin from Other Compounds

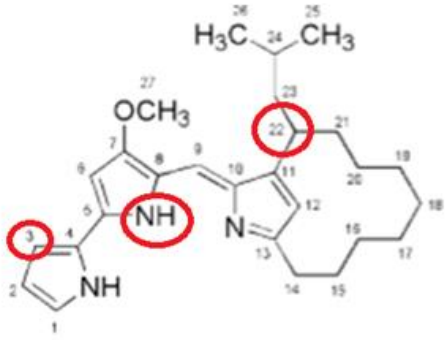
The goal of a paper published in 2013 in the *Journal of Natural Products* was to use experimental data in order to distinguish between streptorubin B and butylcycloheptylprodigiosin (BCHP). Mass spectra were run on both compounds, and BCHP was shown to have a larger mass. Since BCHP is not considered a natural product, it has been eliminated from the natural prodigiosin family. This allows for a novel hypothesis about the evolution of cyclic prodigiosins.

A paper published in 2008 in the *Journal of Natural Products* details the properties and identification of Roseophilin, which is a metabolite that is structurally related to tripyrrole antibiotics, or prodigiosins[19]. A reddish-purple pigment was isolated from Streptomyces griseoviridis. NMR, IR, and UV spectra showed that the pigment contained one furan and two pyrrole rings. Polymerase-chain reaction (PCR) products were sequenced, and homologous genes in S. griseoviridis showed that prodigiosins are also produced from the species. Figure 3 shows comparison between the structure of Roseophilin and the prodigiosin that was identified. The prodgiosin is missing the chlorine, replaced the oxygen with nitrogen to give a characteristic pyrrole ring, and lost the bicyclic ring to give three pyrrole rings.

Figure 3. Comparison between Roseophilin and Prodgiosin[19]



Roseophilin



Prodigiosin R1 (**1**)

Review of Methodology

From the literature above, there are different methods that can be used to purify and identify prodigiosins and similar anti-microbial compounds. Thin-Layer chromatography can easily analyze the separation of compounds in a sample. TLC is one of the most common and more useful forms of analysis for identifying the number of different compounds in a sample, and is used to determine a purification method. This method is the most useful when the appropriate solvent and stationary phase are used. Solvents that produce a greater minimum distance between spots are more useful. Choosing solvents according to the polarity of the compound can strength the TLC method.

Nuclear Magnetic Resonance is one of the most commonly used spectroscopies to determine structure. As seen in the study done on S. griseoviridis[19], one-dimensional NMR can be paired with two-dimensional Correlation Spectroscopy (COSY), Nuclear Overhauser effect (NOSEY), Heteronuclear Multiple Bond Correlation (HMBC) and Heteronuclear Multiple Quantum Coherence (HMQC) to give more convincing evidence of the identification of the desired compound. HMBC and HMQC can give helpful information regarding the interactions between atoms in a molecule. COSY and NOSEY NMR is able to show how individual protons

are related to each other. Each type of NMR is useful in its own way, but the evidence is stronger when these different types are paired.

Another common method of separation and identification done by previous prodigiosin studies is Gas-Chromatography Mass Spectroscopy. The spectra associated with GC-MS show peaks that correspond with the mass and fragmentation of each separated compound in a sample. High-performance liquid chromatography (HPLC) is similar, but does not require the sample to be volatized, which may be difficult for large molecules. Gas Chromatography is used to further purify the sample, while mass spectroscopy is useful for showing the mass of the compound of interest but is not sufficient on its own.

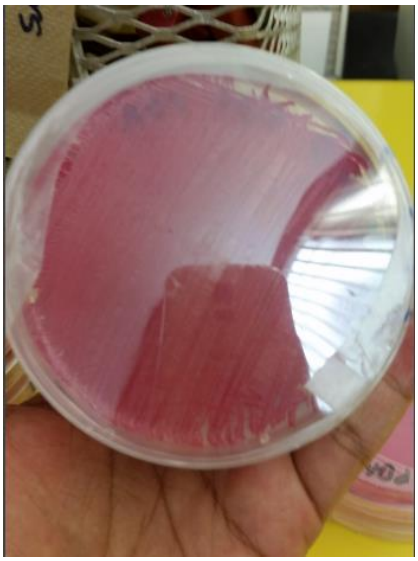
Ultraviolet-visible spectroscopy was used in past studies to show evidence of the characteristic red or pink pigmentation of prodigiosin, which appears in the 525-537 nm range. Although UV-Vis is helpful for detecting the exact wavelength associated with the color, it should be paired with other methods. UV-Vis is not able to show the structure of a compound, so its use should be paired with spectroscopy such as GC, NMR, or both. Infrared Spectroscopy(IR) is an additional characterization method that is useful in identifying the structure of a compound. It is based in absorption spectroscopy. Specific functional groups are visualized at specific wavelengths, making it possible to identify characteristic functional groups of a compound.

Current Investigation

As seen from the literature reviewed, the study of the prodigiosin family is a prevalent field with many paths to follow. My selected path was the use of prodigiosin as an antibacterial compound. My research with Dr. Bromfield Lee was in collaboration with Dr. Gasper. Dr. Gasper has cultured two bacteria isolates taken from soil in Pinellas County, FL, which she named MI1 and MI2. These isolates show a pink pigmentation, much like the expected pigment

of prodgiosin. Figure 4 shows the MI1 plated bacteria with the pigment. We hypothesized that these pigments are in fact prodigiosins and could be further isolated as an antibiotic in the distant future.

Figure 4. MI1 Plated Bacteria



The objectives of my research with Dr. Bromfield Lee were to extract the metabolite from MI1, to purify the extracted component, and to successfully characterize the metabolite as prodgiosin. Extraction was necessary in order to isolate the compound. Purification was necessary because the target prodgiosin needed to be free of any impurities before tested for characterization. The characterization of the metabolite was essential to the objective of the project, as we aimed to provide convincing data and analyses to ensure that the metabolite in question is in the prodgiosin family.

Methods

Extraction of Metabolite from MI-1 and MI-2

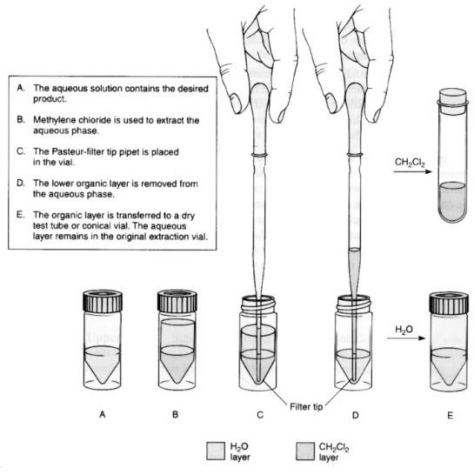
MI-1 and MI-2 were quadrant-streaked on marine agar. After growing in 30 degrees Celsius for 24 hours, pure colonies were collected, and three plates of each strain were

continuously streaked on LB agar. After five days, the plates were collected and the agar was cut into several one-centimeter cubes. The cubes were placed in separate containers. 40 mL of water was added to each, followed by 20 mL of ethyl acetate. The containers were placed in a tray containing liquid nitrogen and ethanol and were allowed to freeze for 30 minutes. This freezing lysed the bacterial cells, which allowed them to open up and release the metabolite. The containers were placed in a shaker bath at room temperature overnight.

Liquid-Liquid Extraction

The containers were collected and placed under a hood. The top, organic layer was extracted from each container using a glass pipette and pipette bulb, closely following the process found in Figure 5. The organic layers were placed in separate vials and were allowed to evaporate for two days. The remaining extract was placed in the freezer to protect the metabolite from degrading.

Figure 5. Liquid-Liquid Extraction[20]



Purification

Purification Using Thin-Layer Chromatography

Thin Layer chromatography, or TLC, was used to see the separation of compounds in the sample. It was also used to find an appropriate solvent system for column chromatography, a

purification method that was used. A study completed by Stephanie Morgan of New College of Florida suggested the use of a chloroform-water mixture, which produced promising results for her identification of prodigiosin[21]. Based on the literature above and Morgan's thesis, some solvent systems tried were chloroform mixed with water, methanol ethyl acetate, hexanes, and 1:1 ethyl acetate: hexanes. The 1:1 ethyl acetate: hexanes was the solvent that showed the greatest degree of separation.

Purification Using Prep-Thin-Layer Chromatography

A prep Thin-Layer Chromatography plate was prepared on a silica gel chromatography plate, seven centimeters wide and seven centimeters long. The extract was dotted along a line drawn 0.5 centimeters from the edge of the paper. The prep TLC was be placed in the prep column, using the 1:1 Ethyl acetate: hexanes solvent. The silica gel containing the remaining components was scraped using a small metal spatula, and the components were extracted from the gel using the 1:1 ethyl acetate: hexanes.

Purification Using Solid Phase Extraction

Another purification method that was used in the project was Solid Phase Extraction (SPE), which is a derivative of column chromatography. The purpose of the extraction was to remove nonpolar impurities to get a cleaner sample of prodigiosin. C18 cartridges were used. Sample was dissolved in minimal methanol. The sample was placed onto a pre-treated cartridge with 50% methanol. A 25% methanol solution was washed through the column, then 50%, then 75%, then 100%. Fractions were dried with a vacuum pump and evaluated using GC-MS.

Purification Using Column Chromatography

A small-scale glass column was used for column chromatography. 1:1 Ethyl Acetate: Hexanes was selected as the solvent with the best separation. MI-1 was dissolved in minimal methanol. The column was prepared with hexanes initially. The sample was added to the column and pushed through with a bulb. Hexanes was the first solvent to be used. 1 mL fractions were taken, and TLC was performed on each. Once the first spot came off of the TLC, the solvent was switched to ethyl acetate. Like-fractions were combined, and NMR and GC-MS were run.

Purification by Esterification

This method was completed so that the fatty acids from the *Vibrio* cell membrane would be purified out. The sample that was purified through column chromatography was dissolved in ethanol and then refluxed with a drop of sulfuric acid for one hour. Sodium bicarbonate was then added to neutralize the acid. The ethanol was removed by using a rotary evaporator in a 30 degrees Celsius water bath. Then, 1.5 mL water was mixed with 0.5 mL ethanol and was added to the newly dried solid. 2 mL of chloroform was placed in the vial, and it was left stirring for 15 min. The aqueous layer was taken off and saved, and the organic layer was placed in the rotary evaporator at 23 degrees Celsius. GC-MS was run on this material, but results were inconclusive.

Characterization of Metabolite

After the sample had been extracted and purified, several characterization methods were necessary to identify our sample as a prodigiosin.

UV Vis Spectroscopy

UV Vis spectroscopy does not show any structural aspects of a molecule but is a good starting point for the characterization of a pigmented molecule, such as prodigiosin. The λmax, or the maximum absorbance of light, value was taken from the prodigiosin by the spectrophotometer. Although specifics of the structure of the molecule cannot be shown, the way

that the molecule absorbs light can give hints about this structure. The sample was be tested in different pH levels in order to characterize these differences, which can ultimately tell us the planarity of the molecule.

In the current study, UV-Vs was utilized. A solid phase extraction using 100% methanol as the solvent was completed. Purified sample was placed in a cuvette, using pure methanol as the blank. Absorbance was measured in the 190-600 nm range.

Infrared Spectroscopy

IR was run on the crude MI-1 to identify any characteristic functional groups, such as a methoxy group, long carbon chain, carbon bonded to nitrogen, and carbons in the aromatic region.

Nuclear-Magnetic Resonance Spectroscopy: Proton and Carbon

NMR was used to determine the structure of the sample. A small amount of sample was placed in an NMR tube along with the NMR solvent, chloroform-D. According to the literature, this is the standard solvent and will be used initially. Carbon, Proton, and DEPT-135 NMR was run on the sample using the NMR spectrometer, and the data was collected and analyzed.

Mass Spectrometry

The purified sample was placed in a Gas Chromatography-Mass-Spectrometer. The weight of the molecule was collected from the spectra collected, along with the detection of remaining impurities in the sample through the resulting chromatogram. GC-MS was run for the samples purified by Solid Phase Extraction and column chromatography.

Results

Table 1. TLC Results of Small Scale Chromatography

Fraction Number	R_f value

1-6	0.75
7-12	0.6

UV-Vis Results

Figure 7 shows the preliminary results taken from the 100% methanol Solid Phase Extraction. Results show that the lambda max was at 253 nm, which is not within the expected range of 500-600 nm, suggesting that the metabolite is still in the cartridge and the material than was run is an impurity. More methanol washes were done in order to collect the metabolite. Figure 8 shows the second trial after a SPE column wash, and this spectrum shows a maximum peak at 534, which falls in the range of the expected literature, meaning the metabolite could be present.

Figure 6. Expected UV Vis Spectrum[22]

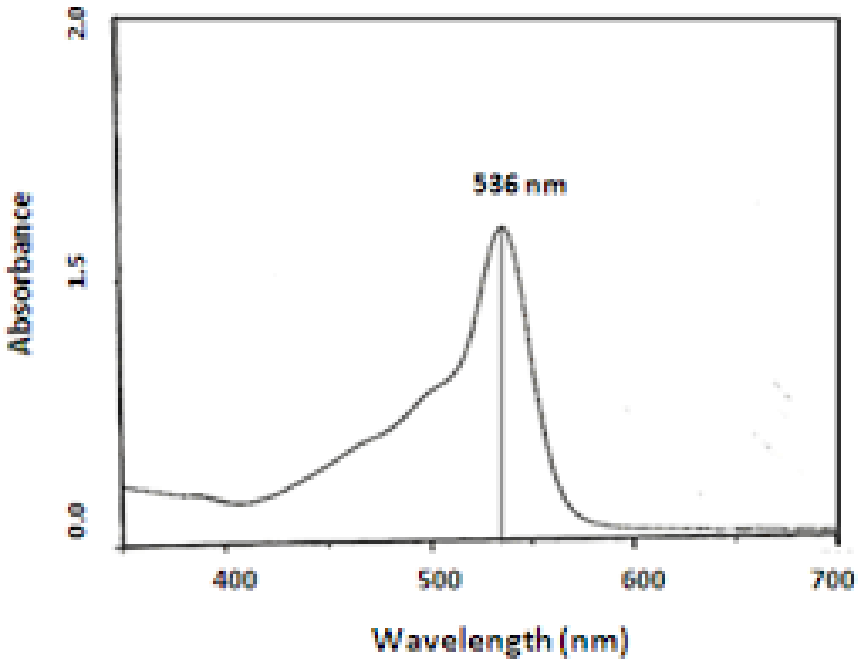


Figure 7. UV-Vis Spectrum of MI-1 Trial 1

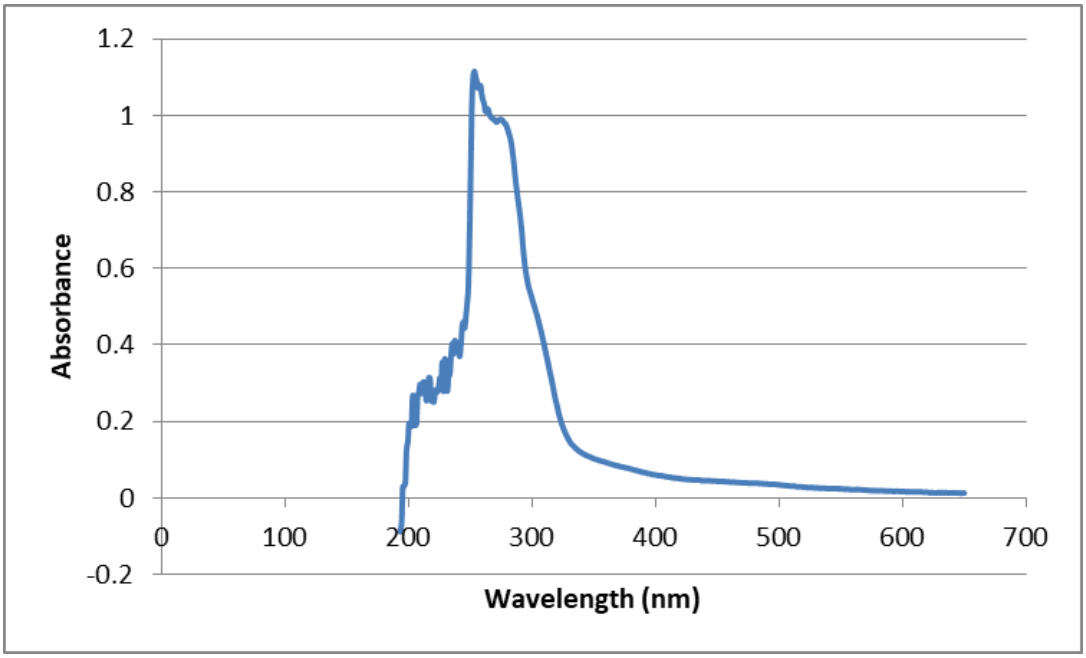
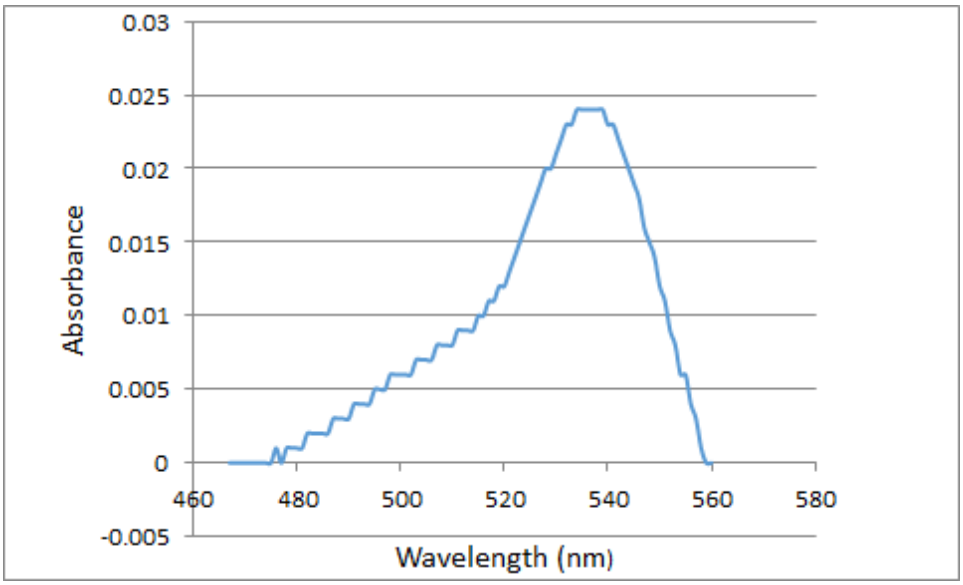


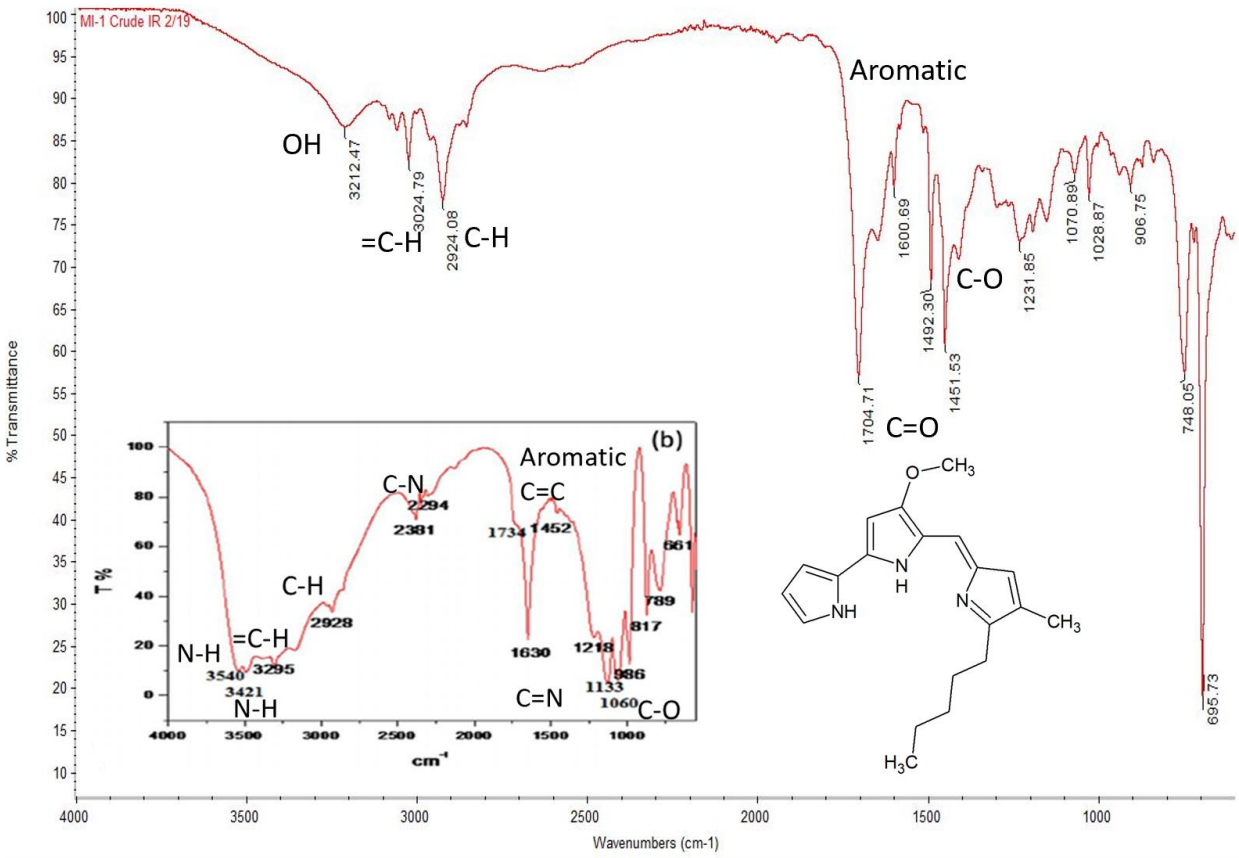
Figure 8. UV-Vis Spectrum of MI-1 Trial 2



IR Results

Figure 9 shows the IR spectra of MI-1 before it was purified. The spectra has been overlayed with an IR taken from the literature. Functional groups that were found include an alkene hydrogen bond, the alkane hydrogen bond, carbons in the aromatic range, and a methoxy group. The hydroxyl group could have been due to water in the sample, and the carbonyl could have been due to excess carbon dioxide from breathing near the machine.

Figure 9. IR Results of Crude MI-1 with Overlay from AV et al[23]



GC-MS Results

For the GC-MS, Solid Phase Extraction was performed. Methods taken from Morgenstern et. al[26]. were used for all four fractions of purified sample. Figure 10 shows the

spectra from Morgenstern et. al, while Figure 11 shows one spectra taken from the 50% methanol sample. The 393.5 peak could suggest that the possible prodigiosin has a similar structure to that of the paper, Figure 10 shows a 394 M+H peak. Another Solid phase Extraction was completed on MI-1, and the spectra in Figure 16 shows a mass of 207. This mass is two hydrogens less than the expected tri-pyrrole structure, meaning that there are two functional groups coming off in place of the hydrogens, as seen in Figure 12.

Figure 10. Mass Spectra from Morgenstern et. al[24].

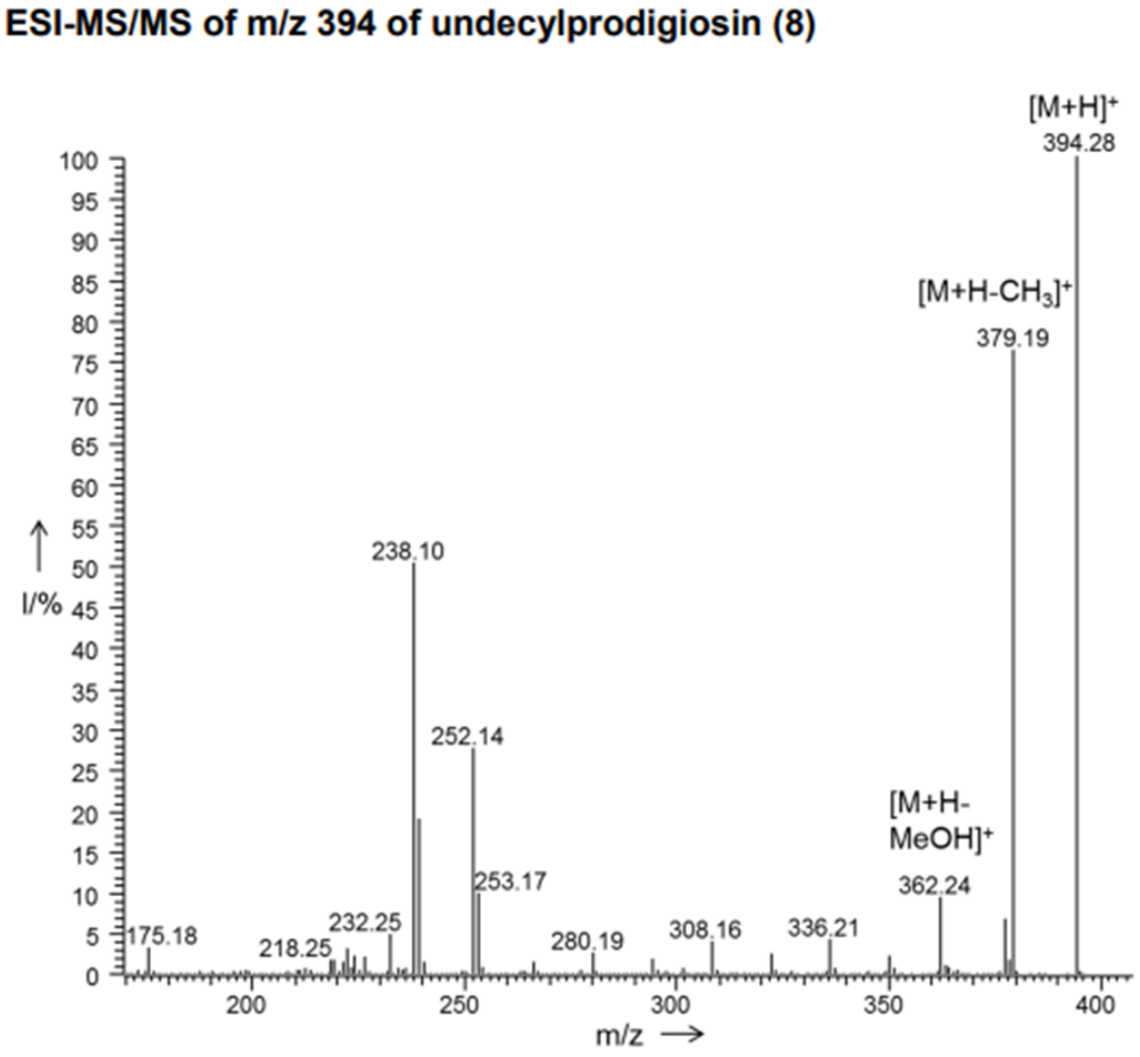


Figure 11. Mass Spectra of 50% Methanol Fraction

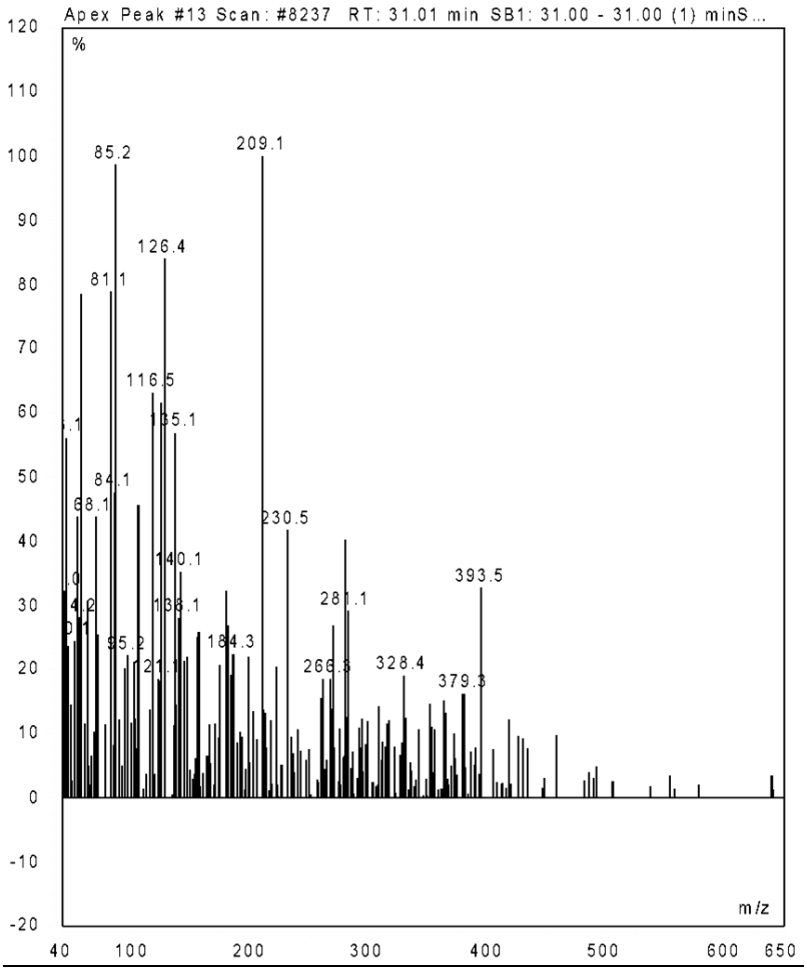
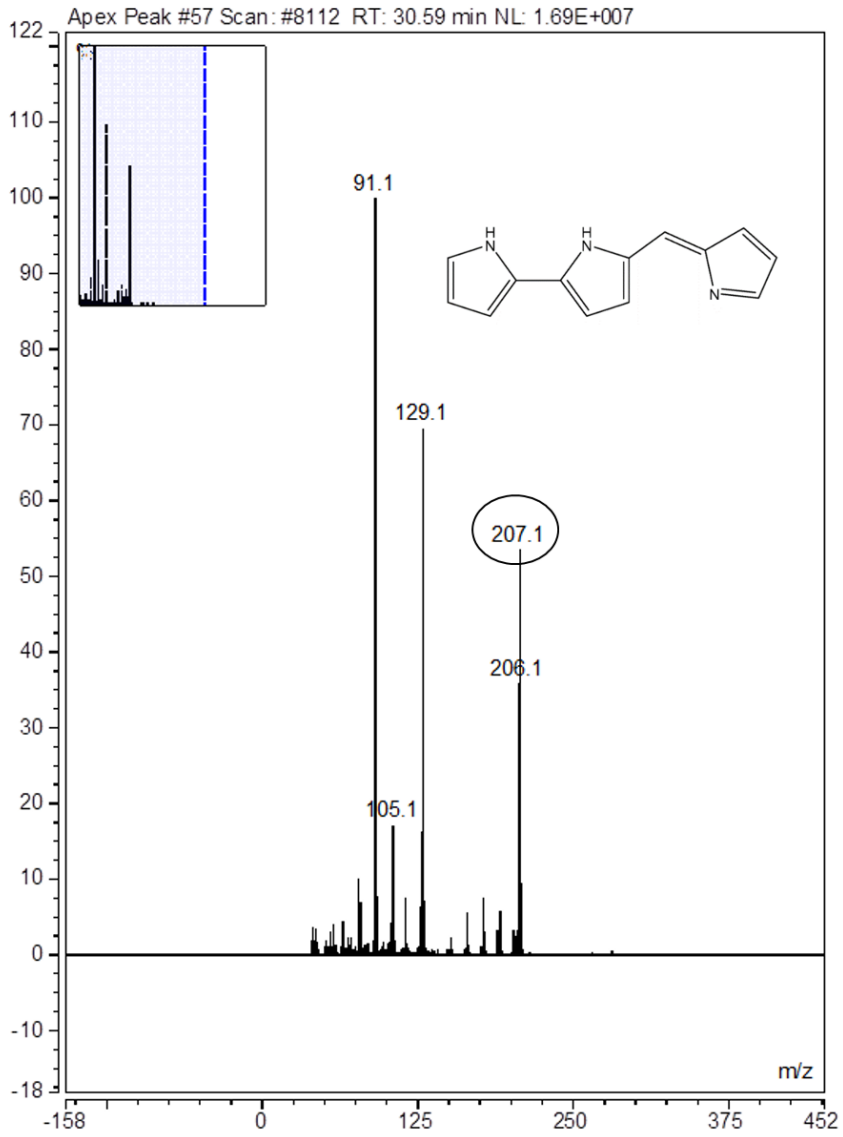


Figure 12. Mass Spectra of 100% Methanol Solid Phase Extraction



NMR Results

Figure 13 shows a predicted HNMR spectra for the tri-pyrrole structure of prodigiosin. CNMR, HNMR, and Dept-135 were run for the sample purified by column chromatography. Both the CNMR and Dept-135 spectra only showed solvent peaks. Figure 14 shows the HNMR collected from the first six fractions of the column chromatography. It was suspected that only ethyl acetate appeared in the spectra, and the sample was too dilute.

Figure 13. Expected HNMR[25]

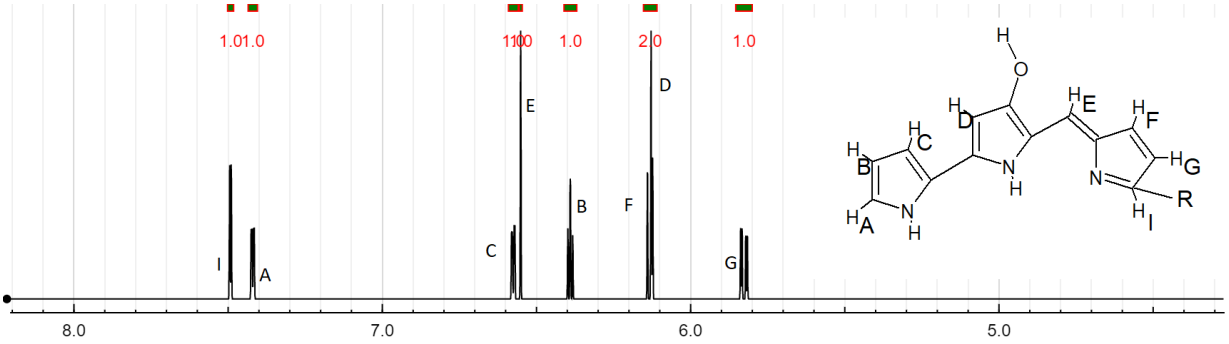
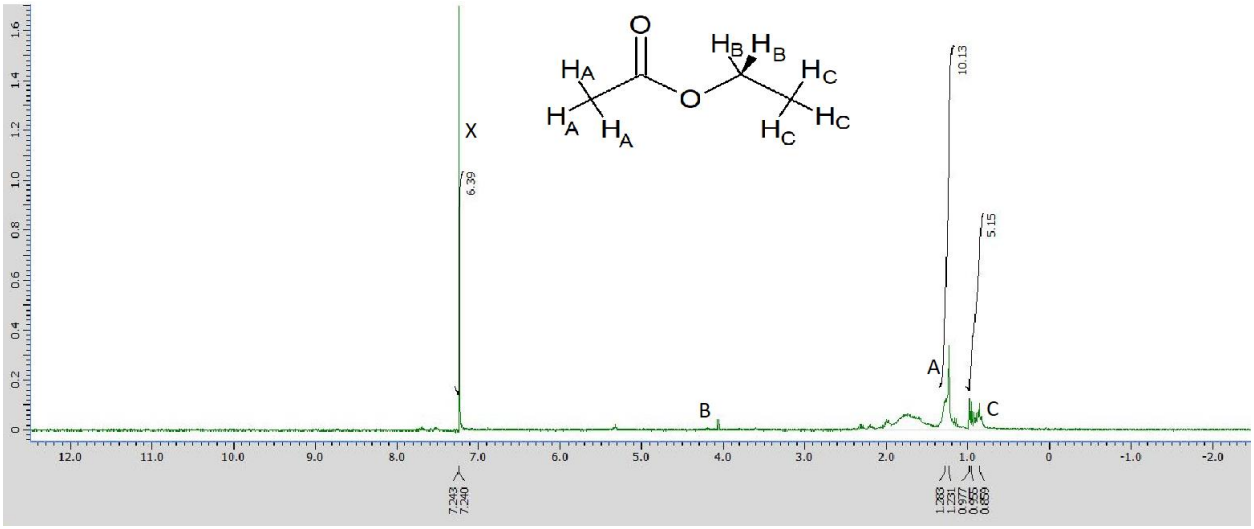


Figure 14. HNMR of Purified MI-1



Conclusions and Future Work

According to the UV-Vis and GC-MS data above, it is hopeful that the compound of interest is of the prodigiosin family. However, more characterization would have to be done in order to confidently characterize the compound and identify the entire structure. Additional NMR characterization methods COSY,NOESY, and HMBC, and HMQC could be utilized. After the prodigiosin is identified, other derivatives could be created. These derivatives could include different functional groups, such as halides, that are expected to alter the activity of the

prodgiosin. After these prodigiosin derivatives are synthesized and tested for activity, they can then be purified and characterized by the same methods outlined above. The mentioned future work could also be applied to the structure in the second *Vibrio* strain, MI-2.

References

1. Florida Hospital. Statistics of Meningitis. https://www.floridahospital.com/meningitis/statistics-meningitis (accessed Mar. 20, 2017).
2. Centers for Disease Control and Prevention. Mucormycosis Statistics. https://www.cdc.gov/fungal/diseases/mucormycosis/statistics.html (accessed Mar. 20, 2017).
3. Centers for Disease Control. Escherichia coli. https://www.cdc.gov/ecoli/ (accessed Mar. 20 2017).
4. ReAct Group. The History of Antibiotic Development. https://www.reactgroup.org/toolbox/understand/antibiotics/development-of-antibiotics-as-medicines (accessed Apr. 25, 2019).
5. World Health Organization. Antibiotic Resistance. http://www.who.int/mediacentre/events/2015/world-antibiotic-awareness-week/infographics/en/(accessed Sept 12, 2018).
6. Antibiotic resistant bacteria. Better Health, 2016 (accessed Apr. 27, 2017).
7. Darshan, N.; Manonmani, H.K. Prodigiosin and its potential applications. *J Food Sci Technol*. **2015** Sep; 52(9): 5393–5407. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4554646/ (accessed May 2, 2017).
8. Secondary Metabolite. *Biology Reference*, 2017. http://www.biologyreference.com/ (accessed May 2, 2017.).
9. Domröse, A.; Klein, A.S.; Hage-Hülsmann, J.; Thies, S.; Svensson, V.; Classen, T. Pietruszka, J.; Jaeger, J.E.; Drepper, T. ; Loeschcke, A. Efficient recombinant production

of prodigiosin in *Pseudomonas putida*. *Front. Microbiol.,* **2015**, (6):972. http://journal.frontiersin.org/article/10.3389/fmicb.2015.00972/full (accessed May 2, 2017).

10. Centers for Disease Control. Bacterial Meningitis. https://www.cdc.gov/meningitis/bacterial.html (accessed Mar. 20, 2017).
11. Pérez-Tomás, R.; Viñas, M. New insights on the antitumoral properties of prodiginines. *Curr. Med. Chem*. **2010**, 17(21), 2222-31.
12. Williams, R.H. The identification of prodigiosin and similar compounds. Iowa State University, 1965.
13. Khanafari, A.; Assadi, M.M.; Fakhr, F.A. Review of Prodigiosin, Pigmentation in Serratia marcescens. *Online Journal of Biological Sciences*. **2006**, 1, 1-13.
14. Gerber, N.N.; D'Aoust, J.Y. Isolation and Purification of Prodigiosin from Vibrio psychroerythrus. *Am. Soc. Microbiol*. **1974**, 18, 756-757.
15. Wietz, M.; Mansson ,M.; Gotfredsen , C.H.; Larsen, T.O.; Gram, L. Antibacterial Compounds from Marine Vibrionaceae Isolated on a Global Expedition. *Mar. Drugs* **2010**, 8, 2946-2960.
16. Al-Zereini, W.; Yao, C.F.; Hartmut, L.F.; Heidrun A. Aqabamycins A-G: novel nitro maleimides from a marine Vibrio species: I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot*. **2010**, 63, 297-301.
17. 17. Kumar, T.S.; Aparna, H. Anti-biofouling activity of Prodigiosin, a pigment extracted from Serratia marcescens. *Int.J.Curr.Microbiol.App.Sci* **2014**, 3, 712-725.
18. Gerber, N.N. Prodigiosin-Like Pigments from Actinomadura (Nocardia) pelletieri. *J. Antibiot*. **1971**, 24, 9, 636-640.

19. Kawasaki, T.; Sakurai, F.; Hayakawa, Y. A Prodigiosin from the Roseophilin Producer Streptomyces griseoviridis. *J. Nat. Prod*. **2008**, 71, 1265–1267

20. Extraction. The University of Arizona, n.d. http://quiz2.chem.arizona.edu/vip/extraction (accessed Apr. 27, 2017).

21. Morgan, S. Discovery of a Prodigiosin Producing *Vibrio*. New College of Florida, The Division of Natural Sciences, May 2016.

22. Casullo de Araújo, H.W.; Fukushima , K.; Takaki, G.C. Prodigiosin Production by Serratia marcescens UCP 1549 Using Renewable-Resources as a Low Cost Substrate. *Molecules* **2010**, 15, 6931-6940

23. Arivizhivendhan, K.V.; Boopathy, R.; Maharaja, P.; Mary, R.R.; Sekaran, G. Bioactive prodigiosin impregnated cellulose matrix for the removal of pathogenic bacteria from aqueous solution. RSC Advances 2015, 5(84)

24. Morgenstern, A. , Paetz, C. , Behrend, A. and Spiteller, D. (2015), Divalent Transition-Metal-Ion Stress Induces Prodigiosin Biosynthesis in Streptomyces coelicolor M145: Formation of Coeligiosins. Chem. Eur. J., 21: 6027-6032. doi:10.1002/chem.201405733

25. Institute of Chemical Sciences and Engineering. NMR Predict. http://www.nmrdb.org/new_predictor/index.shtml?v=v2.103.0 (accessed Mar 28, 2019).