The Synthesis of Unnatural Dipeptides Against *Pseudomonas Aeruginosa*

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Abstract

Pseudomonas aeruginosa is a bacteria prominent in the lungs of patients with Cystic Fibrosis. The current antibiotics against it are becoming less effective due to the rise in antibiotic resistant strains of *P. aeruginosa*. As a result, there has been an urgent need to revise the production of antibiotics against this bacteria, and this improvement has come with the production of unnatural dipeptides. In this project, the amino acids used were either alanine, valine, or D-valine with 2 or 3 -fluorophenylalanine as the second amino acid group. The biofilm assay results indicated that 3-fluorophenylalanine with D-valine was the most effective combination against *P. aeruginosa*, which adds to the results of previous researchers, Andi Lamb and Kellee Geren. Continued research is needed in the future to ensure significance.

Introduction

Pseudomonas Aeruginosa is an opportunistic pathogen, which means it affects immunocompromised patients, especially people with Cystic Fibrosis (CF).[1] More than 30,000 people in the United States have CF, and about 1,000 new cases occur each year.[2] This disease is an autosomal recessive genetic disorder that causes a mutation on the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene on chromosome 7. This gene codes for the CFTR channel protein, which allows for the transport of chloride ions in and out of cells.[3] When this pathway becomes dysfunctional, a lot of problems ensue. There are common misconceptions that CF is only a lung disease; however, it is a whole body disease. It affects the pancreas by mucus blocking the pancreatic duct. This leads to problems with the release and production of digestive enzymes involving fats and proteins. Insulin production can also be damaged in this process, and CF-related diabetes can happen as a result.[4] Other symptoms include salty skin and infertility.[2] The main organ in focus, however, is the lungs. In a normal airway, the mucus is thin and free-flowing due to the negative charge on the chloride ions causing water to move along with it into the airway. In patients with CF, the chloride ions cannot go to the cell surface due to the dysfunction of the protein channel. Since the chloride ions can't leave the cell, the water stays and does not go to the airway. This is what causes the mucus to become thick, sticky, and dehydrated.[5] This creates an optimal environment for bacteria like *Pseudomonas Aeruginosa (P. aeruginosa)* to reside and grow.[2] The problem that *P. aeruginosa* presents is that this bacteria has built resistance against most current antibiotics including beta lactam drugs, aminoglycosides, and fluoroquinolones.[6]

Resistance occurs when a bacteria is exposed to a certain antibiotic, and the pathogenic cells that aren't killed start to develop ways to prevent this harm from happening again. The

susceptible bacteria still left will still be killed by the antibiotic, but the amount of resistant bacteria grows by either spontaneous mutations or gene transfer from one bacteria to another.[7] Therefore, after some time, the medication is no longer efficient enough to fight the growing population of resistant bacteria. Cayston and Tobramycin are specific examples of antibiotics that fight against *P. aeruginosa*; however, this bacteria has developed distinctive resistance mechanisms that shut down these drugs. Cayston, also called Aztreonam, is in the beta-lactam drug class like penicillin, and Tobramycin is in the aminoglycoside drug class, which targets ribosomes.[8] A resistance mechanism *P. aeruginosa* has developed against Cayston is a deactivating enzyme called beta-lactamase that breaks apart the lactam on the structure, which renders the drug useless. This is because the lactam provides the therapeutic effect, and when it is broken, it no longer works.[6] *P. aeruginosa* also develops deactivating enzymes against Tobramycin, which attach acetyl, amino, and hydroxy substituents to the antibiotic. This lowers the affinity of the antibiotic to the ribosome of the bacteria, and the drug can no longer destroy protein synthesis.[9] All of these mechanisms are defeating prominent antibiotics, and it is leaving medical professionals with no other options but to create new antibiotics against the resistant *P. aeruginosa* strains.

This is why antibiotic research is vital to future human existence. According to *The Centers for Disease Control and Prevention* (CDC), there are 23,000 deaths and 2 million illnesses each year in the U.S. due to drug-resistant bacteria. Antibiotic resistance endangers the ability to treat bacterial infections, which is needed to carry out chemotherapy, surgery, organ transplantation, and other medical procedures.[10] In March 2015, the Obama Administration acknowledged this problem, and they released a national action plan to combat antibiotic resistant bacteria.This plan included a 1.2 billion dollar budget with several goals in mind. These goals listed slowing the high rates of resistant bacteria, improving diagnostic tests for identifying resistant bacteria, and fast-tracking research for the development of new antibiotics.[10] This problem is urgent, and it needs to be addressed now rather than later.

The present research seeks to create a series of antimicrobial dipeptide antibiotics via solid phase peptide synthesis. This type of antibiotic has a positive charge on the peptides, which allows the peptides to be attracted to the negative charge on the cell wall of the gram negative *P. aeruginosa* biofilms. The antibiotic also has a hydrophobic component that allows for entry of the peptide through the lipid bilayer of the bacterial cells. Due to the disturbance of the cell membrane and the change in the cells' ion gradient, it can no longer function appropriately and will eventually lyse.[11] These dipeptides are also effective in breaking down biofilms. A biofilm is a group of bacteria which first attach to a surface and exchange genetic material, then they colonize or multiply, and finally, they develop into mature bacteria. Biofilms help bacteria to have a defense mechanism and a favorable environment to live in.[12]

The drug production process of these dipeptides starts with discovering a compound that will kill the bacteria. Sometimes, researchers can start with lead compounds that other researchers have developed, and they can modify the known compound based on previous knowledge of

the lead molecule. Therefore, each research group has the same pharmacophore or “active backbone,” and then molecules differ in regards to additional functional groups.[13] This project involves the latter with collaboration with other institutions.

The schools participating in this project are Milligan College, Goshen College, Colorado College, IUPUI, and a college in Cuba. They all have contributed to this diverse project with the common goal to modify and improve the antimicrobial drugs that combat *P. aeruginosa*. At Milligan College, Andi Lamb, Kellee Geren, and Dr. Kristen Mudrack, PhD, have worked together in order to obtain information about various drugs that can help attack *P. aeruginosa* with a specific concentration on CF.

Research previously conducted by Geren and Lamb has shown that the 4-fluorophenylalanine substituent was unsuccessful against *P. aeruginosa.* In contrast, the 2-fluorophenylalanine substituent with valine was the most successful. Their results exhibited a higher efficacy with the 2-fluorophenylalanine substituent than the 4-fluorophenylalanine substituent. This might be due to the substituent position on the ring being the pharmacophore. Their research also indicated that the D-stereoisomers were generally more effective than the L-stereoisomers. Geren and Lamb suggested future experiments that would explore the difference in efficiency further. This included analyzing the effect of 2-fluorophenylalanine on various other compounds. Another idea included examining the effects of other alkylating agents like 3-fluorophenylalanine on *P. aeruginosa*, which is part of the present experiments.[14]

Figure 1: SPPS Vessels with Billboard. Containers used for the solid phase peptide synthesis in the billboard with a drain.

Materials and Methods

Preparation of the Resin

A SPPS vessel shown in Figure 1 was obtained, and the researchers added FMOC-Gly-Wang resin. The resins were then allowed to swell in 20% piperidine in NMP for 20 minutes. Afterward, the vessel was drained, and then the resin was washed 5 more times with 20% piperidine in NMP for 2 minutes for each wash. They were drained with each subsequent wash. Then benzophenone imine in NMP was added followed by the addition of acetic acid.[15]

Washing, Capping, and Uncapping

A beaker was put underneath the spigot of each drain. Washing solvents are added with the bottom of the vessels open for draining. After 30 seconds of letting wash solvents drain, an air

push was used. Before capping vessels, excess residue was wiped away with a Kimwipe. Each reaction vessel was then capped, and inverted. Then, the bottom caps were removed, and the vessels were placed right-side up in the drain. Finally, the top caps were also removed to add new reagents.[15]

Alkylation

The resins were washed 3 times with NMP with the washing and uncapping procedure as mentioned above. Next, .2M BTTP in NMP was added to each vessel followed by the addition of 2-fluorobenzyl bromide in NMP across row A and the addition of 3-fluorobenzyl bromide in NMP across row B. Then, the vessels were capped on the top and bottom, wiped, inverted, and then left alone to sit for 7 days at room temperature.[15]

Hydrolysis

Once 7 days passed, the uncapping procedure was used as mentioned above, and the vessels were allowed to drain. Then the vessels were washed with THF with the washing procedure mentioned above. Clean caps were obtained. Next, 1 M aqueous HCL-THF was added to each vessel, inverted a few times, and left to sit for 20 minutes. Then the uncapping procedure was used again as mentioned above. The product was then filtered and washed with THF, then washed with 0.2 M of DIEA in NMP twice, sat for 5 minutes for each wash, and then washed twice with NMP with the washing, capping, and uncapping procedure mentioned above.[15]

Figure 2: Tool Neck Vials. The containers used to put the crude products in.

Acylation

The bottom of each reaction vessel was capped. Then, 0.25 M of alanine in 0.25 M HOBt in NMP was added to both A1 and B1. 0.25 M of valine in 0.25 M HOBt in NMP was added to both A2 and B2. 0.25 M of D-valine in 0.25 M HOBt in NMP was added to A3 and B3. Then, .50 M diisopropylcarbodiimide in NMP was added to all vessels. The top of the vessels were capped, inverted, and left to sit for 5 days.[15]

Removal of Resin

After 5 days, follow the uncapping procedure as mentioned above. The vessels were washed twice with 3 mL of NMP, then washed twice with THF, and then washed 3 times with CH_2Cl_2. With each wash, the washing, capping, and uncapping procedure were used as mentioned above. 6 tool neck vials were labeled and weighed. The mass was recorded.

The drained Bill-Board was placed over the collection rack so that the vessels align with the correct tool neck vials. The bill-board set still for 30 minutes, was rinsed with 35:60:5 TFA-CH_2Cl_2-H_2O, and then rinsed with CH_2Cl_2. Each vessel was then drained with an airpush, and the drainage with the products was collected. Each product solution was stirred and transferred to the appropriate tool neck vial.[15]

TLC Visualization of Crude Products

A TLC plate was obtained. 10 mg/mL product solutions in 18:21:1 isopropanol:methanol:NH_4OH (Solvent B) were made, and they were used to spot the TLC plate. A 9:2:1 isopropanol:methanol:NH_4OH solution (Solvent C) was made and used as the mobile phase in the TLC chamber. A .3% ninhydrin solution in 3% acetic acid in n-butanol (Solvent A) was made, and the developed TLC plate was dipped in Solvent A, set out to dry, and heated over a hot plate to visualize the plate.[15]

Purification of Crude Products

A 500 mg Hyper-Sep SI cartridge column was obtained and mounted on a clamp. Solvent B was used to wet the column followed by the addition of one of the crude products. Solvent B was added until 14 fractions were obtained. Repeated with all products, and each fraction for each product was developed on a TLC plate using the same TLC visualization procedure as mentioned above. All the fractions with the pure product were pooled into one tool neck vial.[15]

Biofilm

All crude and purified samples were made into 20 ug/mL solutions in .2% DMSO. Tobramycin was made into a 0.5 mg/mL in 10% DMSO, and Aztreonam was made into a 10 mg/mL solution in 10% DMSO. A M64 media was made with KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, H_2O, 20% arginine, and 1 M $MgSO_4$. All these solutions were autoclaved at 121℃ for 20 minutes. A small colony of *P. aeruginosa* was transferred to a test tube with autoclaved nutrient broth, and it was incubated for 24 hours at 37℃. Once the 24 hours passed, the *P. aeruginosa* was diluted in complete M63 media with a dilution of 1:100. The diluted bacterial solution was added to all wells except C1 shown in Figure 4. All contents of each well are also in Figure 4, and the bacteria was grown overnight at 37℃. After 24 hours, contents from the wells were aspirated without contacting the bottom of the well. Then the wells were rinsed by pipetting water into each well. The water was aspirated, and rinsing repeated. Then .1% crystal violet was added to each well, and the plate was incubated for 10 minutes at 37℃. After 10 minutes, the crystal violet was discarded, the wells were rinsed with water, and the plate was inverted. 750 uL of acetic acid was then added to visualize the color.[12]

Crystal Violet UV-Vis Assay

UV-Vis Spectrophotometry was used to measure the concentration of crystal violet in each well. This was done by using the absorbance measured and Beer's Law to get the concentration. Beer's Law is $A = \varepsilon l c$ where ε is the molar absorptivity constant of 87,000 $M^{-1}cm^{-1}$, l is length of 1 cm, and c is the concentration (M). A blank was created using 750 µL of Acetic acid and 2250 µL of water. For each sample, 750 µL of acetic acid from each well was added to a clean cuvette with 2250 µL of water as well in order to read the absorbance of crystal violet at 590 nm.

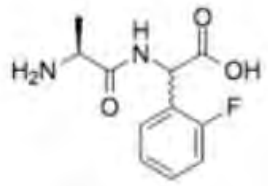
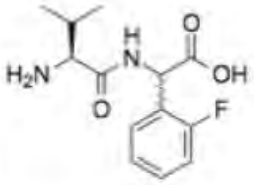
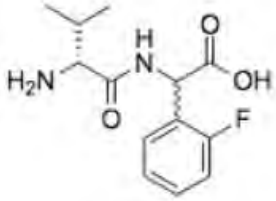
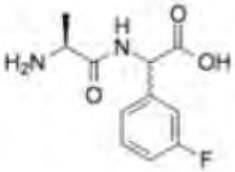
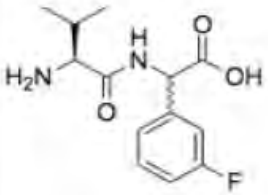
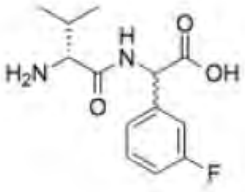
	1: Alanine	2: Valine	3: D-Valine
A: 2-fluoro			
B: 3-fluoro			

Figure 3: Hannah's Billboard. Row A: 2-fluorophenylalanine, Row B: 3-fluorophenylalanine, Column 1: Alanine, Column 2: Valine, and Column 3: D-Valine.

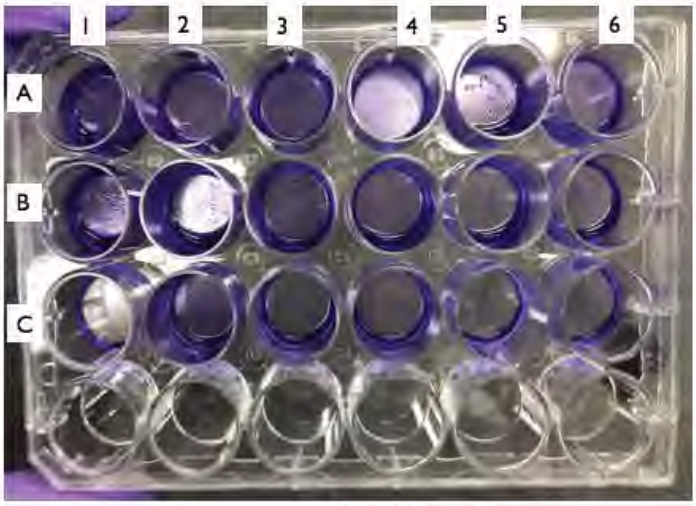


Figure 4: Biofilm Assay. Row A: 2 fluorophenylalanine, Row B: 3 fluorophenylalanine, Row C: Controls (From left to right: Just Media, 10% DMSO, Tobramycin, Aztreonam, 4-fluorobenzyl bromide, 4-fluorobenzyl bromide). Only in Row A and B: Column 1: Alanine crude, Column 2: Alanine pure, Column 3: Valine pure, Column 5: D-Valine crude, Column 6: D-Valine pure. All have *P. Aeruginosa* except C1.

Results/Discussion

Compounds were successfully created using solid phase peptide synthesis on the Billboard apparatus. Purity of the compounds will be assessed upon collection of spectra from a liquid chromatography mass spectrometer. This data is currently unavailable due to the COVID-19 shutdown of nonessential businesses.

Crude and pure compounds were tested in the biofilm assay to determine if the purification scheme was effective in making the antibiotic more effective. In addition, controls were used to compare the efficacy of the novel antibiotics with those currently on the market, Tobramycin and Cayston.

According to Figure 5, one can see that Cayston is a less effective antibiotic than Tobramycin, likely because of dosing. Cayston is given to patients at 75 mg doses (nebulized) three times

daily (Minimum Inhibitory Concentration, MIC, is the lowest concentration that inhibits the growth of a microorganism in patients. MIC 0.016 mg/mL), while Tobramycin is given to patients at 300 mg (nebulized) twice daily (MIC 0.004 mg/mL). The concentrations used in the biofilm assay were 0.5 mg/mL.

As one can see in Figure 5, without antibiotics (10%DMSO), the concentration of crystal violet is high, indicating a large amount of growth of *P. aeruginosa*. In general, all pure antibiotic

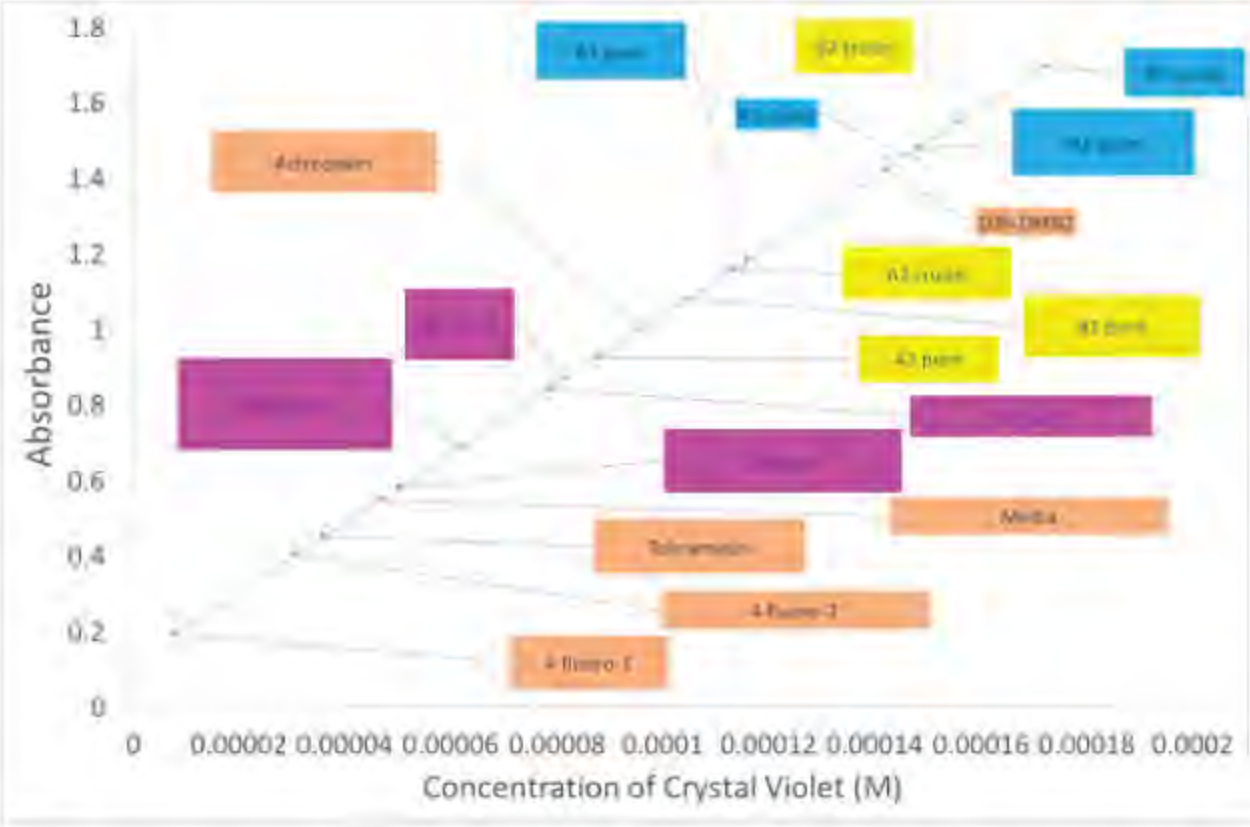


Figure 5: UV Vis Graph. Concentration of Crystal Violet (M) vs. Absorbance. A1: 2-fluorophenylalanine and alanine, B1: 3-fluorophenylalanine and alanine, A2: 2-fluorophenylalanine and valine, B2: 3-fluorophenylalanine and valine, A3: 2-fluorophenylalanine and D-valine, B3: 3-fluorophenylalanine and D-valine. Orange represents the controls.

compounds were more effective at killing the *P. aeruginosa* biofilms than their crude counterparts, as expected, though to varying degrees. The antibiotic compounds that lie above the 10% DMSO control in crystal violet concentration did not inhibit growth of *P. aeruginosa* at all, and those below the 10% DMSO control did inhibit growth to varying extent. The goal of this project is to create antibiotics that are as effective or more effective than the two most common antibiotics prescribed today, Aztreonam and Tobramycin. Though Aztreonman is less effective than Tobramycin, this is likely due to MIC and dosing considerations, as mentioned above.

According to both Figure 5 and Table 1, 3-fluorophenylalanine with D-valine was the most effective against *P. aeruginosa* whereas alanine with either 2 or 3-fluorophenylalanine was the least effective. This is seen in Figure 5 with 3-fluorophenylalanine with D-valine (B3) having the lowest concentration of crystal violet, and 2-fluorophenylalanine with alanine (A1) and 3-fluorophenylalanine with alanine (B1) having the highest crystal violet concentrations. This is also seen in Table 1 with 3-fluorophenylalanine with alanine (B1) and 3-fluorophenylalanine with D-valine (B3) having the most significant difference with the highest T-value and lowest p-value in the table. There was a lower significant difference between 3-fluorophenylalanine with valine (B2) and 3-fluorophenylalanine with D-valine (B3); however, there wasn't a significant difference between 2-fluorophenylalanine with valine (A2) and 2-fluorophenylalanine with D-valine (A3) . This shows that 3-fluorophenylalanine is more efficient than the 2-fluorophenylalanine position.This point is also seen with 2-fluorophenylalanine with alanine (A1) and 2-fluorophenylalanine with D-valine (A3) having less of a significant difference than B1 and B3 in Table 1. Also, in Table 1, there is a significant difference between A3 and B3, which shows again that the 3-fluoro position is more efficient in the case of D-valine. These results added on to the results of Geren and Lamb's results. Their results indicated that the 2-fluorophenylalanine position was more efficient than the 4-fluorophenylalanine position,[14] and the results of this research showed that the 3 position was more efficient than the 2 position. Also, their results indicated that the D-stereoisomers were more efficient against *P. aeruginosa* than the L-stereoisomers.[14] The results of this research reiterate this hypothesis with D-valine being more effective against *P. aeruginosa* than valine when paired with 3-fluorophenylalanine.

Table 1: Significant Differences for Crystal Violet Concentrations. A One-Tailed T-test for 2 Independent Means ($p<.05$)[16]

Dipeptides	Well Number	T- value	P-Value
Alanine and D-Valine	A1 and A3	3.39623	0.038419
	B1 and B3	7.8629	0.007896
3-fluorophenylalanine Valine and D-Valine	B2 and B3	2.84576	0.052242
2-fluorophenylanine D-valine and 3-fluorophenylalanine D-valine	A3 and B3	4.01312	0.028424

The researcher was able to use 4-fluorobenzyl bromide (similar to 4-fluorophenylalanine) as a control in this experiment, but was unable to use 2-fluorobenzyl bromide or 3-fluorobenzyl bromide alone in the biofilm assay. As a result, the researcher is unsure at the present time if the effectiveness of the 3-fluorophenylalanine + D-valine peptide is due to the efficacy of

3-fluorophenylalanine or the fact that it is combined with D-valine. More controls would have been good to use in this experiment, however, COVID-19 again prohibited these experiments.

Further research is to be conducted by Blanton, Mudrack, and other students will endeavor to build upon the work presented here, including a large range of controls in the biofilm assay and the creation of novel antibiotic compounds built off of this research.

Conclusion

This research has shown that 3-fluorophenylalanine with D-valine was effective against P. aeruginosa whereas alanine with either 2 or 3-fluorophenylalanine was the least effective. This project has given this research group more information moving forward. Future researchers can look further into the significance of the 3-fluorophenylalanine position with D-valine more by looking at the effects of 3-fluorobenzyl bromide with *P. aeruginosa* as a control instead of 4-fluorobenzyl bromide. They also can use all three fluoro positions with various other amino acid combinations. In addition, they can look into other amino acids and the effects of D vs L forms to see if the D stereoisomer is more efficient against *P. aeruginosa* like it was in this project. Furthermore, they can redo Lamb's and Geren's antibiotics and test them with UV Vis since it wasn't used in their project. They can also continue the purification process and send current and future antibiotics to be screened through a liquid chromatography mass spectrometer. This project is a step forward, and other researchers can add onto it in semesters to come.

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