Unnatural Dipeptide Synthesis with Bioactivity Against *P. aeruginosa*

K. Geren, A. Lamb

Mentor: K. Mudrack

Introduction

Previous research on dipeptide antibiotics

The complexity of the immune response to pathogens led early researchers to discover the concept of innate immunity, which is predetermined by genetics and does not rely on previous exposure to the pathogen. Natural antibiotic peptides, or antimicrobial peptides (AMPs), found in humans, mainly in the skin, are essential to the start of the innate immune response against viruses, fungi, gram-negative, and gram-positive bacteria. Cathelicidins are cationic AMPs with variation at the C-terminus and are among the first AMPs; they were discovered when analyzing wound fluid from the skin and were found to have increased activity against *Staphylococcus aureus*.[1]

The two major classes of peptide antibiotics are non-ribosomally synthesized peptides and ribosomally synthesized (natural) peptides. Non-ribosomally synthesized peptides are not synthesized as proteins on ribosomes but are instead made on multi-enzyme complexes. An application of this includes the antibiotic colimycin, a derivative of a lipopeptide called colistin used in an aerosol formula against *Pseudomonas aeruginosa*. The natural form of the lipopeptide colistin is largely toxic to humans, so chemically modifying it to create colimycin is extremely beneficial. Ribosomally synthesized peptides may originate from plants, mammals, amphibians,

bacteria, and viruses. These peptides may have a beta structure with disulfide bonds or an alpha helix.

AMPs from bacteria can come from either gram-positive or gram-negative bacteria and are known as bacteriocins, which can inhibit protein synthesis within the bacterium. Unnatural amino acid combinations have also been found effective against *S. aureus*, which is antibiotic resistant but can be inhibited as a result of side benefits from these peptides including neutralizing endotoxins.[2] Many biologically active natural drugs contain amino acids and their derivatives.

Previous dipeptide antibiotics have been derived from natural strains of bacteria, commonly from the genus *Streptomyces.* Specifically, *Streptomyces* contains Gram-positive bacteria that have the ability to produce metabolites such as "antifungals, antivirals, antitumorals, anti-hypertensives, immunosuppressants, and especially antibiotics".[3] Examples of these natural dipeptide antibiotics from different strains of *Streptomyces* include alahopcin, which contains L-alanine and was found to be active against the antibiotic resistant *S. aureus* by inhibiting the synthesis of the bacterial cell wall.[4] Cyclic dipeptides containing various amino acids such as L-leucyl, L-prolyl, and L-phenylanyl also have antibiotic properties and have proven to be effective against *S. aureus, Escherichia coli,* and *Candida albicans.*[5]

Why This Is Important

The need for new antibiotics that can be developed efficiently and inexpensively is clearly evidenced when reviewing how bacteria can quickly become resistant, diseases in the developing world are overlooked, and economic pressures affect health-care decisions. According to the

U.S. Centers for Disease Control and Prevention, antibiotic-resistant pathogens cause 2 million illnesses and 23,000 deaths per year in the United States.[6]

Some of the most well-known bacteria, typically Gram-negative, that pose a serious threat are known as "ESKAPE" pathogens and include drug-resistant forms of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter (E. coli)* species.[6] When pathogens such as these develop resistance to an antibiotic in a certain class, the rest of the antibiotics in the class tend to follow soon after which eliminates a whole class of antibiotics, such as tetracyclines, against that pathogen. It is extremely difficult for small companies to fund their antibiotic research, and investors are not particularly interested in antibiotic-resistance investments because of the significant challenges and costs.

Part of the reason why antibiotics become resistant so quickly is contributed to doctors overprescribing antibiotics for both inpatient and outpatient use. To combat this, doctors may hold new antibiotics back and use them only when extremely necessary; however, investors view this as detrimental to business because of the lack of total revenue. Doctors also may opt to purchase the generic version of a drug since it is cheaper than the new brand name.[6] Despite some of these economic challenges in the area of antibiotic-resistance, there are significantly more incentives that drive investors to fund equipment and procedures in the pharmaceutical industry. However, these incentives are essentially absent in the developing world which leads to unnecessary diseases and deaths.

Cystic Fibrosis

Cystic Fibrosis (CF) is the most common life-threatening disease in Caucasians.[7] It is a progressive genetic disorder caused by the recessive CFTR gene. The lungs and the pancreas are the main two organs it impacts. The buildup of thick mucus in the pancreas makes it difficult for the release of digestive enzymes. Because of this, it is challenging for CF patients to get the nutrients they need. In the lungs, thick mucus makes it difficult to breathe and leads to an increased risk of infection.

For more than 80% of patients, respiratory failure is brought about by bacterial infections.[7] Treatments try to break up the mucus and clear the airway. Inflatable vests that vibrate at high frequency to clear the airway are the most common. Inhaled medicines are also available help clear the airways. Enzymatic supplements are used to help with the absorption of nutrients. A number of new therapies are being researched and clinical trials are taking place. However, no lifelong cure is currently available.

Pseudomonas aeruginosa

CF patients are likely to be affected by the bacteria *Pseudomonas aeruginosa*. By the age of 20, around 80% of patients are colonized by *P. aeruginosa*.[7] *P. aeruginosa* is a gram-negative bacterium that infects the airway. The accumulation of mucus makes it difficult to clear bacteria and allows for colonization of the bacteria in the lungs.[7] *P. aeruginosa* is exposed to the immune response of the person, to the antibiotics taken, and to the reduced oxygen environment inside the lungs. Because of these factors, *P. aeruginosa* can rapidly adapt and become resistant.[8] It is challenging to treat with antibiotics because of the protective biofilm that forms around the

bacteria. It is a lifelong infection for patients with CF due to these characteristics. The rate of resistance to the antibiotics to treat *P. aeruginosa* is on the rise.[7]

Biofilms

Gram negative bacteria like *P. aeruginosa* forms a capsule surrounding it for protection. Bacteria attaches to the surface of the area it is infecting. Eventually, the bacteria will produce a layer known as the extracellular polymeric matrix (EPS).[9] The EPS is a slime like layer made up of different components such as glycoproteins, polysaccharides, and proteins. The biofilm can thicken with time which allows more bacteria to reproduce and the colony to expand.[10] *P. aeruginosa* uses cell-cell communication known as N-acylhomoserine lactone (AHL). AHL uses molecular signals to regulate the formation of the biofilm.[9] The bacteria within the is altered by the biofilm formation and it is less susceptible to outside agents. It is defended from threats such as antibiotics and UV light.[9] To effectively treat *P. aeruginosa*, the biofilm must be removed. Some strategies proposed to overcome biofilm formation include the following: preventing attachment to the surface the bacteria is infecting, interfering with development of the biofilm, and disrupting the biofilm if it has already formed.[10]

Antibiotics

Antibiotics are medicines that are used to halt the spread of an infection by killing the bacteria responsible. They are active against both Gram-positive and Gram-negative bacteria. Most antibiotic-resistant bacteria are Gram-negative, meaning they have a very large cell wall that is hard to penetrate. Different classes of antibiotics include beta-lactams, aminoglycosides, quinolones, lipopeptides, streptogramins, ansamycins, glycopeptides, chloramphenicol, sulfonamides, tetracyclines, and macrolides. Penicillin was the first antibiotic to be discovered,

done by Alexander Fleming in 1928; it is of the beta-lactam class. Each class of antibiotics is effective against bacteria in different ways, whether it is inhibiting bacterial protein synthesis, inhibiting bacterial DNA replication, or destroying the cell wall.[11]

Materials and Methods

Synthesis of Unnatural Dipeptides

Attaching the R1 group to the resin

Each of the 6 vessels in the Bill-Board, as seen in *Figure 1*, will contain 50 mmol of resin, which is an amino acid protected by an imine, connected to a polystyrene bead by a Wang linker, and held in an isopycnic (neutral buoyancy) suspension. The resin in each vessel should be washed 3 times using a 1.0 mL calibrated disposable pipette and 3 mL quantities of NMP. Use the air "push" apparatus and make sure solvent and resin is not drawn back up into the apparatus; keep going until all of the solvent has been pushed through. Once each vessel has been washed and wiped of residual NMP, catalyze the alkylation reaction by adding base, which consists of 0.5 mL of 0.20 M BTPP in NMP to each vessel. Color change of the resin should be visible. Obtain a few disposable pipettes and add 0.5 mL of 0.20 M ${}^{1}R^{1}$-X solution in NMP to the resin in vessels across Row A, including positions A1, A2, and A3. Next, add 0.5 mL of 0.20 M ${}^{2}R^{1}$-X solution in NMP to the resin in vessels across row B, including positions B1, B2, and B3. Cap each vessel, wipe the Bill-Board dry, and invert it a couple times to mix the solution, let the Bill-Board stand, and allow the reaction to proceed for 7 days at room temperature. Record the start time. Clean glassware with acetone and dispose of washes in designated waste container.

Protecting of acylating agent

Record the end time. Utilizing the uncapping procedure listed above, take off the caps. After consulting the general washing procedure, use 3mL of THF clean and filter the alkylated resin product. Cap the bottom of the vessels with clean caps. Add 2.5 mL of 1N aqueous HCl-THF mixture (1:2) to the six vessels, capping each afterwards. To mix the solution, invert the Bill-Board. Leave the Bill-Board to react for 20 minutes. Once the time is up, use the general uncapping procedure previously mentioned. Using 3 mL of THF, wash and filter product 3 once. Wash twice with 2.5mL for 5 minutes using .20 M DIEA in NMP, and then wash twice with 2.5mL of NMP.

Deprotecting the acylating agent

Cap the bottom of the reaction vessels with clean caps. To the deprotected resin 3 down column 1 (A1 and B1), add 1.0 mL of the standard acylating agent (.25 M R-COOH in .25M HOBt in NMP). To column 2(A2 and B2), add 1.0 mL of the second acylating agent (.25M R-COOH in .25M HOBT in NMP). To column 3 (A3 and B3), add the third acylating agent (.25 M R-COOH in .25M HOBt in NMP). To all six vessels, add .5 mL of .50M "DIC" in NMP. Cap and dry the Bill-Boards. Mix the solvents by inverting the Bill-Boards, and then leave for 5 days. Record the ending time, and then follow the uncapping procedure. Carefully and thoroughly wash the acylated product twice using 3 mL NMP, twice using 3 mL of THF, and three times using 3 mL of CH2Cl2. Wash the sides of the vessels to ensure all impurities and contaminants are properly removed. Follow the general washing procedure previously stated.

Cleave dipeptide off of resin

Using a permanent marker, label six tool-necked vials with the correct label corresponding to each vessel. With the caps off, tare the empty vials to 0.0001 g and record the mass of each; place them in the collection tray in the designated position. Remember which balance was used. The chemicals used in the following steps are hazardous and extremely volatile so it will be of utmost importance to do the transfers with disposable pipettes without chemical dripping from the end. In order to achieve this, fill the dead space in the pipette with solvent vapors instead of air by immersing the pipette end fully in the liquid and pulling up the liquid 3 different times, allowing it to push out each time. This equilibrates the vapors of each respective chemical, dichloromethane and trifluoroacetic acid, into the bulb of the pipette which allows easier withdrawal of the liquid and transfer to the vessels without spurting. Place the previously drained Bill-Board rack over the collection rack, aligning the vials with the corresponding Bill-Board position. Add 2 mL of TFA-CH_2Cl_2-H_2O to each vessel. Do not agitate the Bill-Board; allow it to stand for 30 minutes. The product will cleave from the resin as the solution passes through; the product will drain into the collection vials. Allow 30 minutes to pass and rinse each resin once with 2 more mL of TFA-CH_2Cl_2-H_2O and 2 mL of CH_2Cl_2. Collect ALL of these rinses into each respective vial. Fully drain each vessel using the air push apparatus. Keep these filtrates because they contain the product. When extracting the Bill-Board from the collection vials, make sure the vials do not stick because this would cause them to spill. Using separate 300 microliter pipettes for each product, stir the solution to make the cleavage solution and rinses homogenous. Transfer 100 microliters (0.1 mL) of each product to the correct HPLC autosampler vial with the matching label. Leave the vials uncapped in the order A1, A2, A3, B1, B2, B3 in the collection rack to allow the cleavage solution to evaporate, leaving the remaining crude product. Place the

rack in the hood, and wash materials with acetone, disposing of washes in the designated waste container.

Thin Layer Chromatography

Find a TLC plate and lightly trace 6 pencil marks about 0.5 cm from the bottom of the plate. Mark each pencil mark A1, A2, A3, B1, B2, B3, respectively. Spot each sample on its corresponding pencil mark with a microcapillary tube. Allow to dry and place the plate into the TLC chamber which contains 9:2:1 isopropanol:methanol:NH_4OH (Solvent C). Once the solvent front is ¾ up the plate, mark the solvent front, and remove it from the chamber. View the TLC plate under UV light and circle each spot with a pencil. Using forceps, dip the plate in ninhydrin up to the solvent front. Continue holding the plate with forceps and allow it to dry; heat the plate until colored spots are visible. Photograph the TLC plate, and mark "N" next to the ninhydrin stain.

Purification

Load only half of the headspace with Solvent B. Collect .5 mL from the Fraction 1 vial using the air push. Repeat this step using the Fraction 2 vial. For fractions 3-10, use the same process to collect 1.5 mL fractions.

Microbiology Assay

Orient a sterile 24 well plate with A1 in the upper left-hand corner (rows A-D, columns 1-6) Place 490 microliters of PA14 in M63 media to every well plate except for C6 and D6. Use sterile pipettes for each new compound in the following procedure. For columns 1-6, since there are 6 new test compounds, add 10 microliters of the respective new test compound down one

column, for both rows A and B. To column 1, row C and D, add 10 microliters of a purified control compound, 4-fluorophenylalanine or 2-fluorophenylalanine. To column 2, row C and D, add 10 microliters of unpurified sample of the control compound in column 1. To column 3, row C and D, add 10 microliters of gentamycin which will result in no biofilm by killing the bacteria. 10 microliters of tobramycin, an antibiotic typically used to treat infections caused by P. Aeruginosa, should be added to column 4, row C and D. In column 5, row C and D, place 10 microliters of 10% DMSO. To column 6, row C and D, add 490 microliters of M63 media and 10 microliters of 10% DMSO. When returning the next day, remove the contents of the 24 well plate with a vacuum aspirator. Do not touch the side of the well so that the biofilm is not disturbed. Pipette 1 mL of distilled water into each to rinse them and aspirate to remove the water; rinse one more time. To each well, add 750 microliters of 0.1% crystal violet stain in deionized water. Incubate for 10 minutes and empty the contents into the sink. Run water over the wells gently and then shake it off; leave the plate inverted.

Figure 1: Bill-board set up for synthesizing dipeptide antibiotics

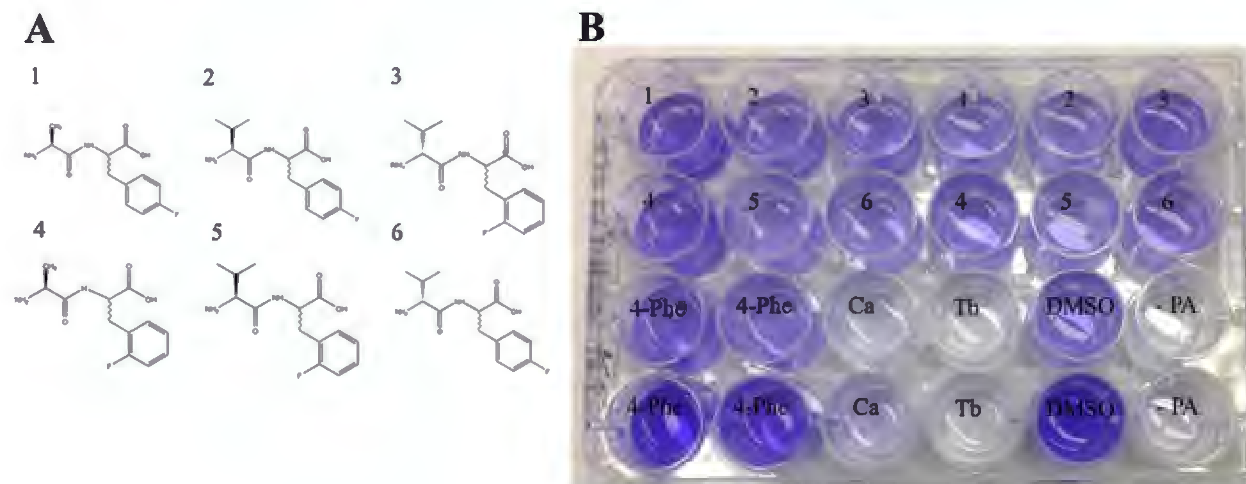
Results

Percent yields were calculated for each compound, which can be seen in Table 1. Compound A2, 4-flurophenylalanine with the amino acid valine, had the highest percent yield at 92.18%. Compound B9, 2-flurophenylalanine with methionine, and compound B10, 2-flurophenylalanine with D-methionine, had the lowest percent yields at 30.92%.

Microbiology assay was run on each compound, which can be seen in Figure 2B, Figure 3B, and Figure 4B. In Figure 2B, 2-flurophenylalanine and the amino acid valine is noted to have a lighter purple color in wells 2/5. The darkest purple wells are noted to be 4-flurophenylalanine along with 4-flurophenylalanine with D-valine. In Figure 3B, 2-flurophenylalanine and the amino acid alanine and D-methionine had the lightest purple color in the plate. Those wells using 4-flurophenylalanine were noted to have the darkest purple color. In Figure 4B, 2-flurophenylalanine along with the amino acids D-methionine and D-alanine exhibited the lightest purple color in the plate. 4-flurophenylalanine was observed to have the darkest purple tint. Wells containing Cayston and Tobramycin were used due to their known activity against *P. aeruginosa*; the structures of each antibiotic can be seen in Figure 5. Wells with Cayston are noted to have a slightly more of a purple tint than the wells with Tobramycin which are clear. Wells containing 4-phenylalanine and DMSO with and without *P. aeruginosa* were used as controls. These were consistently clear or very light purple in each of the plates. The wells without *P. aeruginosa* are clear whereas the wells with *P. aeruginosa* are a darker purple.

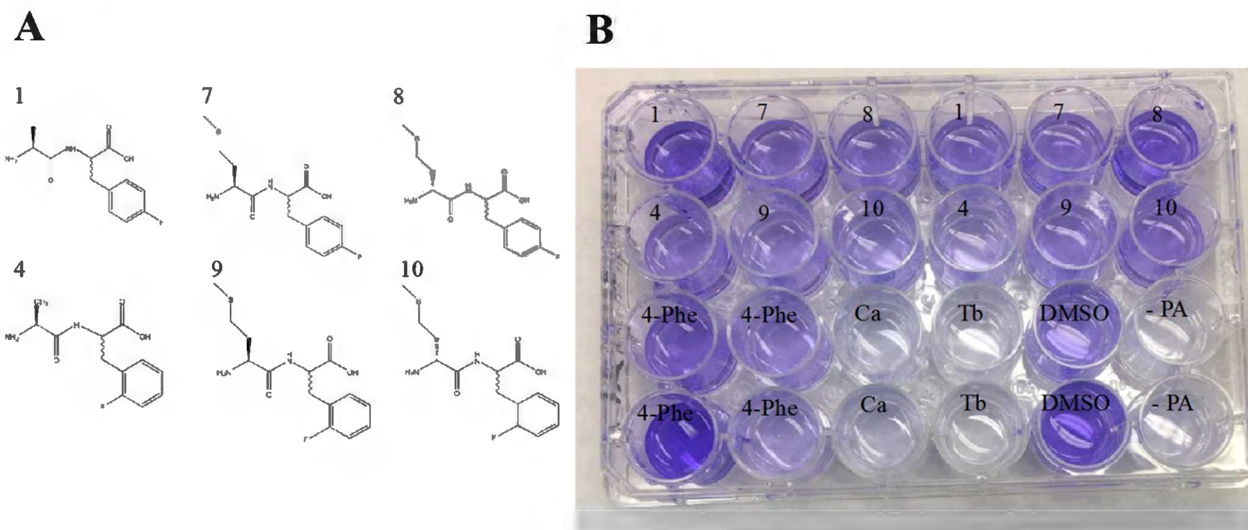
Compound	Percent yield
A1	76.71%
A2	92.18%
A3	75.67%
B4	43.84%
B5	49.35%
B6	65.80%
Compound	**Percent yield**
A1	65.75%
A7	56.22%
A8	44.98%
B4	32.88%
B9	30.92%
B10	30.92%
Compound	**Percent yield**
A1	76.71%
A11	73.06%
A8	53.41%
B4	76.71%
A13	80.36%
A10	42.16%

<u>Table 1:</u> Percent yields of all compounds. Corresponding compound numbers can be found in 2A, 3A, and 4A.

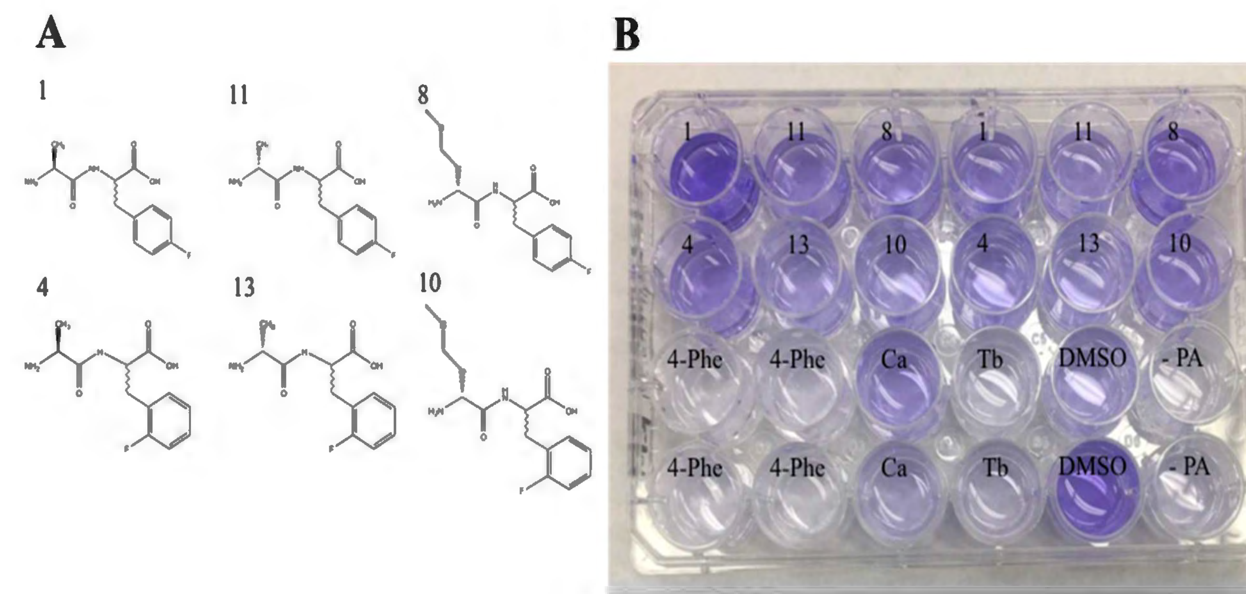


<u>Figure 2:</u> (A) 4-fluorophenylalanine is used across row A. 2-fluorophenylalanine is used across row B. A1/B4 uses alanine, A2/B5 valine, and A3/B6 D-valine. (B) Synthesized antibiotics were used in the labeled wells to test effectiveness against *P. aeruginosa*. 4-fluorophenylalanine and DMSO are used as controls in the labeled wells. *P. aeruginosa* is not present in wells C6/D6 as labeled. Antibiotics Cayston (C3/D3) and Tobramycin

(C4/D4) are also used as controls as labeled. Corresponding structures 1-6 are on the left in Figure 1A.



<u>Figure 3:</u> (A) 4-fluorophenylalanine is used across row A. 2-fluorophenylalanine is used across row B. A1/B4 uses alanine, A7/B9 methionine, and A8/B10 D-methionine. (B) Synthesized antibiotics were used in the labeled wells to test effectiveness against *P. aeruginosa.* 4-fluorophenylalanine and DMSO are used as controls. *P. aeruginosa* is not present in wells C6/D6. Antibiotics Cayston (C3/D3) and Tobramycin (C4/D4) are also used as controls. Numbered structures are on the left in Figure 2A.



<u>Figure 4:</u> **(A) 4-fluorophenylalanine is used across row A. 2-fluorophenylalanine is used across row B. A1/B4 is uses alanine, A11/A13 D-alanine, and A8/B10 D-methionine. (B) Synthesized antibiotics were used in the labeled wells to test effectiveness against *P. aeruginosa.* 4-fluorophenylalanine and DMSO are used as controls. *P. aeruginosa* is not present in wells C6/D6. Antibiotics Cayston (C3/D3) and Tobramycin (C4/D4) are also used as controls. Numbered structures are on the left in Figure 3A.**

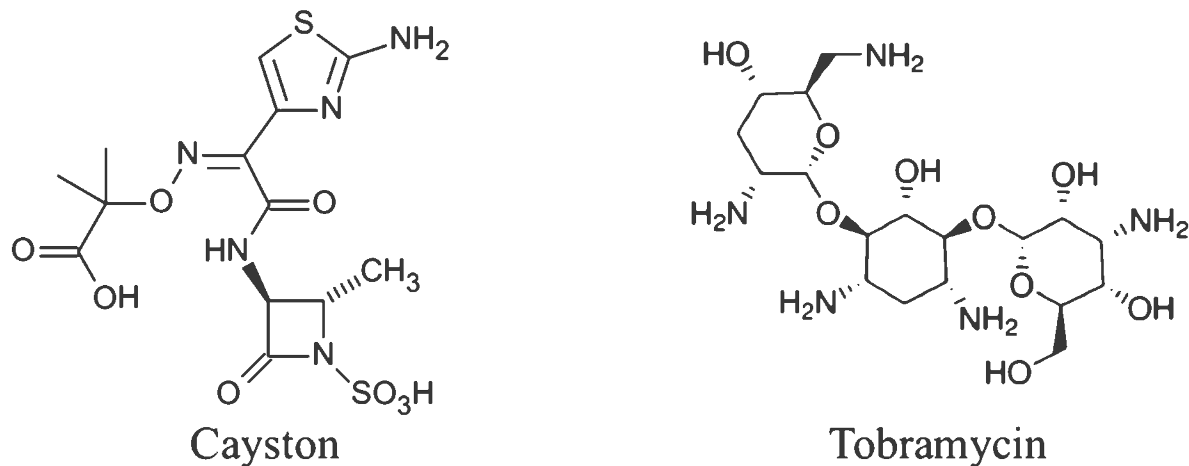


Figure 5: Shown are the structures of the antibiotics Cayston and Tobramycin currently used against *P. aeruginosa*. Cayston is used in wells C3/D3. Tobramycin is used in wells C4/D4.

Discussion

Bioactivity against *P. aeruginosa* is indicated by a light purple color or no color at all in each well. Poor activity or no activity is indicated by a dark purple color. Alanine was used in each Bill-Board in wells labeled A1/B4 and had the worst activity of the synthesized dipeptides against *P. aeruginosa* in each figure when alkylated with 4-fluorophenylalanine. 4-fluorophenylalanine was also used as a control and did not have significant activity as shown by the dark purple color in Figures 2B and 3B. However, 4-fluorophenylalanine did have some activity in Figure 4B. Compounds synthesized with 2-fluorophenylalanine were more effective than those alkylated with 4-fluorophenylalanine. DMSO had poor to no activity in Figure 2B, 3B, and 4B which indicates that the solvent is not active against *P. aeruginosa*.

In Figure 2B, wells labeled 2/5 seemed to have the best activity shown by the lighter purple color; valine was the amino acid used. D-valine also had some activity, which is more than alanine, but it did not have as much as valine. Compounds using 4-flurophenylalanine along with the amino acids alanine and valine were observed to have the darkest purple color, which indicates these compounds showed less activity. Cayston and Tobramycin both had complete activity indicated by no purple color.

In Figure 3B, wells labeled 4/10, which are those alkylated with 2-fluorophenylalanine, and using alanine and D-methionine, respectively, were the most effective against *P. aeruginosa* shown by the light purple color. Wells labeled 1/8, which are those alkylated with 4-fluorophenylalanine, using alanine and D-methionine, respectively, were the least effective against *P. aeruginosa* shown by the dark purple color. Cayston is noted to have a slight purple tint whereas Tobramycin was completely clear. This is evidence that Tobramycin is more effective than Cayston.

The Bill-Board synthesized in Figure 4 had the best activity overall. Although alanine was not effective, D-alanine and D-methionine did exhibit activity. D-alanine was the most effective, indicated by the very light purple color of wells labeled 7/9 in Figure 4B. 4-flurophenylalanine along with the amino acid alanine are noted to have the darkest purple tint, which is evidence that this compound is less effective. Cayston, which had a slight purple tint, was less successful than Tobramycin, which was clear.

Conclusion

Compounds synthesized using 4-fluorophenylalanine as the alkylating agent were less effective against *P. aeruginosa.* The combination of alanine and 4-fluorophenylalanine was the least

effective overall. Compounds synthesized using 2-fluorophenlyalanine were shown to be more effective against *P. aeruginosa.* Specifically, the combination of 2-flurophenylalanine and D-methionine were consistently more successful. Valine combined with 2-fluorophenylalanine was also successful, as well as D-alanine. Some compounds had lower yields or lower purities which may have impacted the results. A higher yield corresponded with increased bioactivity.

In future experiments, 2-fluorophenylalanine could be used to synthesize other compounds. The amino acids alanine, D-methionine, and valine could be utilized in further experiments. 3-fluorophenylalanine could be tested to see if it would be active against *P. aeruginosa*.

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