4-6-2018

Synthesis of Oxygenated Boronic Acid Substituted a-Cyanostilbenes For Use as Antibacterial

Yewon Kim
Andrews University, yewon@andrews.edu

J. N. Andrews Honors Program
Andrews University

HONS 497
Honors Thesis

Synthesis of Oxygenated Boronic Acid Substituted α-Cyanostilbenes for use as Antibacterial

Yewon Kim

April 6, 2018

Advisor: Dr. Desmond Murray

Primary Advisor Signature: Desmond Murray

Department of Chemistry and Biochemistry

Abstract

Stilbenes are naturally occurring compounds that have exhibited antibacterial activity, although the biological effect of various stilbenes differs for Gram-positive and Gram-negative strains of bacteria. In prior research, cyanostilbenes have shown "slight" antibacterial activity (Brownlee, *et al.* 1943). This project aims to explore whether hybrid oxygenated α-cyanostilbenes possessing a boronic acid pharmacophore exhibits significant antibacterial activity (Das *et al.* 2013). The biological activity against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) was tested using the Kirby-Bauer test. No inhibition of *E. coli* growth was shown while *S. aureus* was partially inhibited.

Introduction

A 2014 study by the Center for Disease Control showed that for every 1,000 people, 836 prescriptions had been written for antibiotics. Within this large amount of antibacterial use, an alarming 30% of written prescriptions in US ambulatory care patients were prescribed inappropriately (Fleming-Dutra, *et. al*). Examples of inappropriate antibiotic prescriptions include the use of antibiotics for the common cold, bronchitis, or other viral infections that are not bacterial infections. As antibiotics are increasingly used inappropriately, antibiotic-resistant microbes remain while other "good" bacteria are killed, therefore allowing the antibiotic-resistant microbes to take over. By perpetuating this pattern of misuse in the United States' healthcare system, antibiotic resistance continues to grow and thus looms as one of the most prevalent issues in healthcare today.

In order to combat antibiotic resistance, healthcare providers and the public alike must be educated on the proper use of antibiotics. However, in the meantime, new modes of antibiotic

treatment must be developed. Often times, creating a hybrid drug may be the answer to drug resistance. Pokrovskaya and Baasov (2010) conclude that the benefit to synthesizing hybrid drugs include "i) activity against drug-resistant bacteria, ii) expanded spectrum of activity and iii) reduced potential for generating bacterial resistance".

In this study, a family of 15 boronic-acid substituted α-cyanostilbenes were synthesized and tested for antibacterial activity against the Gram-negative *E. coli* and the Gram-positive *S. aureus*. A study by Trippier *et. al* demonstrated that boronic acid compounds exhibit inhibitory activity against both Gram-positive and Gram-negative bacteria. On the other hand, there is not much prior research done on the antibacterial effect of α-cyanostilbenes. The little research that does exist suggests that there is "slight" antibacterial activity against only Gram-positive bacteria (Brownlee, *et. al*). As suggested by Pokrovskaya and Baasov, creating this hybrid drug would not only hold the potential for an increased, synergistic antibacterial effect by the hybrid drug, but also increase the potential of resisting the onset of antibiotic resistance. During this study, the antibacterial effect of the newly synthesized hybrid drugs will be tested in order to first determine whether synthesizing a boronic-acid containing hybrid drug will have increased the antibacterial effect of the cyanostilbene.

Methodology

Five arylacetonitriles and three boronic acids were used to synthesize a family of 15 boronic acid substituted α-cyanostilbenes (Figure 1).

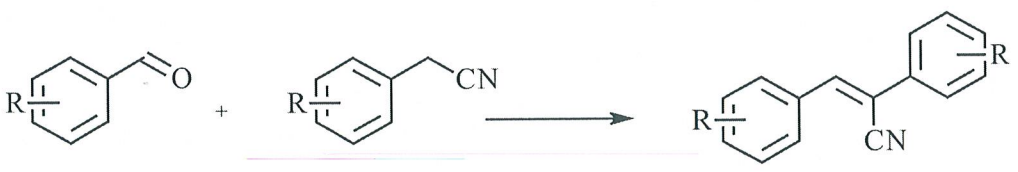


Figure 1. Proposed reaction for the synthesis of an α-cyanostilbenes.

The arylacetonitriles (Figure 3) used were 4-methoxyphenyl acetonitrile (4MAN), 2-thiopheneacetonitrile (2TAN), 2-pyridinylacetonitrile (2PAN), 3,5-dimethoxyphenylacetonitrile (35DM), and 3,5-difluorophenylacetonitrile (35DF). The three boronic acids (Figure 2) used were 2-formylphenylboronic acid (2FPBA), 3-formylphenylboronic acid (3FPBA), and 4-formylphenylboronic acid (4FPBA). A set of 5 non-boronic acid stilbenes were also synthesized using *p*-tolualdehyde and the same 5 arylacetonitriles.

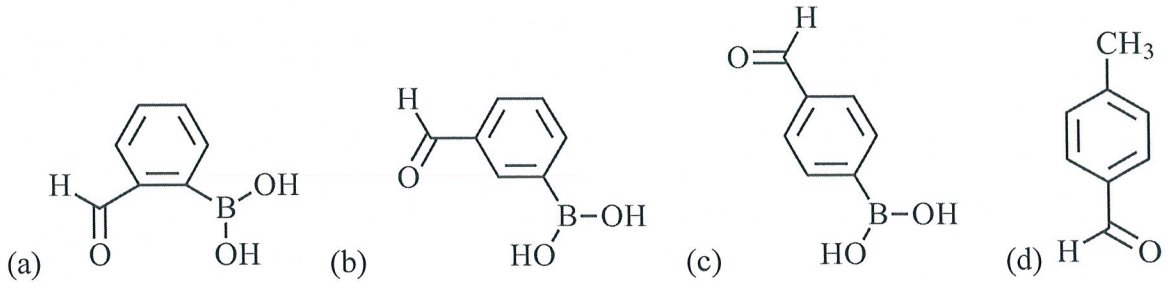


Figure 2. The three boronic acids used (a) 2-formylphenylboronic acid, (b) 3-formylphenylboronic acid, (c) 4-formylphenylboronic acid and (d) *p*-tolualdehyde used to synthesize the control antibiotics.

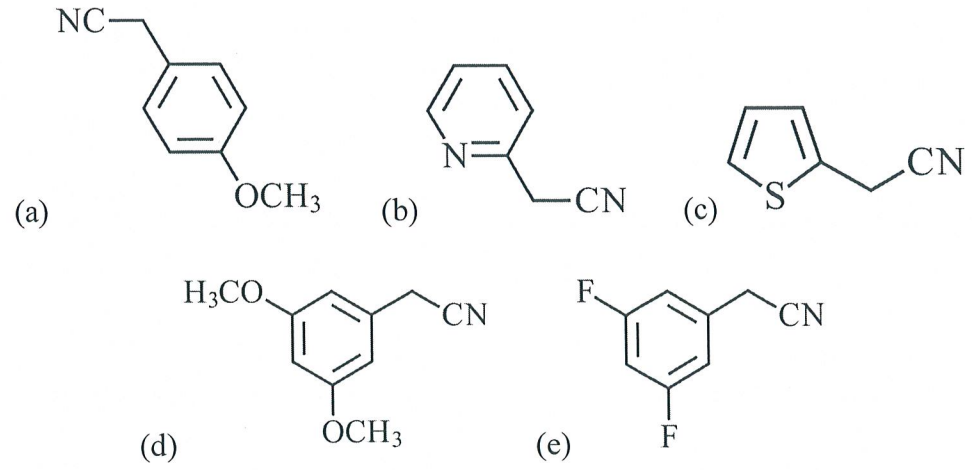


Figure 3. The five arylacetonitriles used for synthesis. (a) 4-methoxyphenylacetonitrile, (b) 2-pyridinylacetonitrile, (c) 2-thiopheneacetonitrile, (d) 3,5-dimethoxyphenylacetonitrile, and (e) 3,5-difluorophenylacetonitrile

For each of the synthesized products, the method of synthesis used was a three-hour reflux. To begin preparing the reflux for each compound, a clean 50 mL round-bottom flask was clamped into place above a hot plate and magnetic stirrer. In the round-bottom flask, a clean

magnetic stirrer was placed. 16 mL of deionized (DI) water and 4 mL of ethanol were measured into the round-bottom flask. Then, in order, the necessary arylacetonitrile, boronic acid, and then calcium oxide were added to the round bottom flask. The amounts needed for each reagent is listed below in Table 1. The mixture was stirred and a condenser, connected to a water source and proper drainage, was placed on the clamped round bottom flask.

Reagent	**Molar weight (g/mol)**	**Density (g/mL)**	**Moles**	**g or mL needed**
Water (H_2O)	18.02	1.00	0.89	16 mL
Ethanol	46.07	0.789	0.069	4 mL
Boronic Acid (2FPB, 3FPB, 4FPB)	149.94	--	0.0025	0.3745 g
Calcium Oxide	56.08	--	0.010	0.561 g
4MAN	147.17	1.085	0.0025	0.3391 mL
2TAN	123.18	1.157	0.0025	0.3662 mL
2PAN	118.14	1.059	0.0025	0.279 mL
35DM	177.20	--	0.0025	0.443 g
35DF	153.1	--	0.0025	0.3828 g

Table 1. The reagents used in the reactions, properties of the reagents, and the amounts of each reagent needed for the reactions.

The reaction was refluxed for three hours, and then removed from the heat source. The mixture was poured over ice and neutralized using 30 mL of saturated ammonium chloride (NH_4Cl). Using a pH strip, the mixture was checked for neutralization. This mixture was then vacuum filtered for approximately one hour (or until dry) using a clamped side-arm flask with a Buchner funnel, equipped with an appropriately sized Whatman® filter paper. The dried product was then weighed, stored in labeled scintillation vials, and the percent yield was calculated for each product.

The collected products were analyzed by nuclear magnetic resonance (NMR). The solvent chosen for NMR was methanol-d4 (solvent peak at 4.9 ppm), as it produced the smallest amount of "noise", which, when present, often obscures necessary peaks and makes the spectra difficult to read. Based on the results of the NMR and the best percent yields, seven of the synthesized products were chosen for antibacterial testing using the Kirby-Bauer test. The seven products chosen are listed below in Table 2, along with the disk number.

Disk number	Product
1	4-formylphenylboronic acid + 3,5-difluorophenylacetonitrile
2	2-thiopheneacetonitrile + *p*-tolualdehyde
3	2-pyridylacetonitirle + *p*-tolualdehyde
4	2-formylphenylboronic acid + 2-thiopheneacetonitrile
5	4-formylphenylboronic acid + 4-methoxyphenylacetonitrile
6	3-formylphenylboronic acid + 3,5-dimethoxyphenylacetonitrile
7	2-formylphenylboronic acid + 2-pyridylacentonitrile.

Table 2. Seven of the twenty synthesized compounds were tested for antibacterial activity using the Kirby Bauer test. This table lists the seven compounds along with the assigned disk number of each.

Two dilutions of each of these seven products were prepared using serial dilutions. First, a stock solution of each product was created by dissolving 0.1 g product in 1 mL of dimethylsulfoxide (DMSO) to obtain a concentration of 100,000 μgram/mL. This stock solution was then diluted to 1000 μgram/mL and then subsequently 500 μgram/mL using DMSO and the stock solution.

Three disks were prepared for each of the two dilutions of each product. Each of the three diffusion disks was soaked with 20 microliters of solution. Two petri dishes were labeled on the bottom as "1000" and "500" for the two dilutions, and the perimeter of the dish was split into seven regions, numbered 1-7. Each of the disks was placed in its appropriate area and left to dry overnight.

In order to prepare the plates for antibacterial activity, one American Type Culture Collection (ATCC) incubated plate of *E. coli* (ATCC# 25922) and one plate of *S. aureus* (ATCC# 29213) were obtained. Two plates for each type of bacteria were prepared- one was labeled on the bottom "1000" and the other labeled "500". Each plate was split into seven regions, numbered 1-7, as shown below in Figure 4.

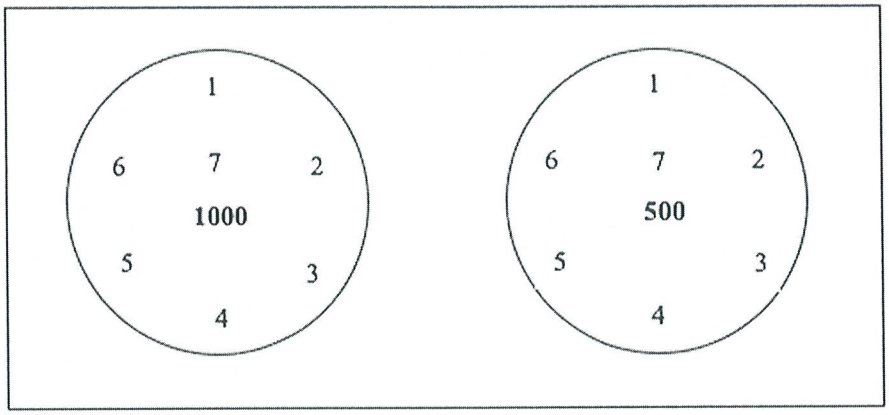


Figure 4. This figure shows the labeling scheme of each disk and the relative location of each disk. One 1000 μgram/mL and one 500 μgram/mL test was performed for each of the seven chosen compounds with both E. coli and *S. aureus.*

Two inoculants were prepared: one with *E. coli* and one with *S. aureus.* A sterile swab was used to pick up a single colony of each bacteria, and the swabs were swirled in separate test tubes containing sterile saline. The swabs were swirled in the saline until enough bacteria had been deposited for the inoculants' turbidity matched that of a 0.5 McFarland standard, which was used to obtain a standardized amount of bacteria in each inoculant. The 0.5 McFarland standard was chosen because this amount has been successful in previous experiments. Another sterile swab was dipped into the prepared inoculant, and the plates were streaked using the lawn streak method, in which the entire surface of the plate is evenly coated to ensure even growth of bacteria. Once the plate was coated, the dry disk was placed in its appropriate place on the disk and lightly pressed onto the agar. The plates were covered and incubated upside down at 37 ± 2

°C in ambient air for 18-24 hours. At the end of the incubation period, the plates were examined for zones of inhibition and the disks were identified as partial, complete, or no inhibition.

Results:

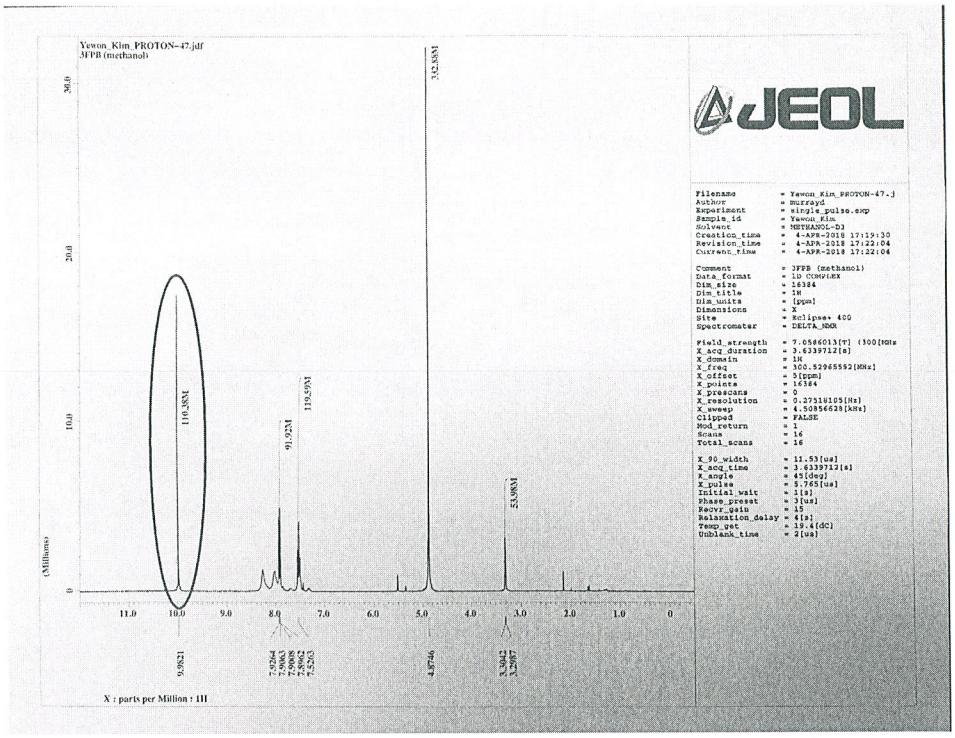
The percent yield for each synthesized compound is listed below in Table 3.

Nitrile	Aldehyde	% Yield	Nitrile	Aldehyde	% Yield
4MAN	2FPBA	46.9	35DF	3FPBA	138
	3FPBA	113		4FPBA	114
	4FPBA	57.2	35DM	2FPBA	43.2
2PAN	2FPBA	109		3FPBA	119
	3FPBA	125		4FPBA	119
	4FPBA	129	4MAN	*p*-TA	54.5
2TAN	2FPBA	70.1	2PAN		98.1
	3FPBA	255	2TAN		104
	4FPBA	144	35DF		105
35DF	2FPBA	47.3	35DM		132.2

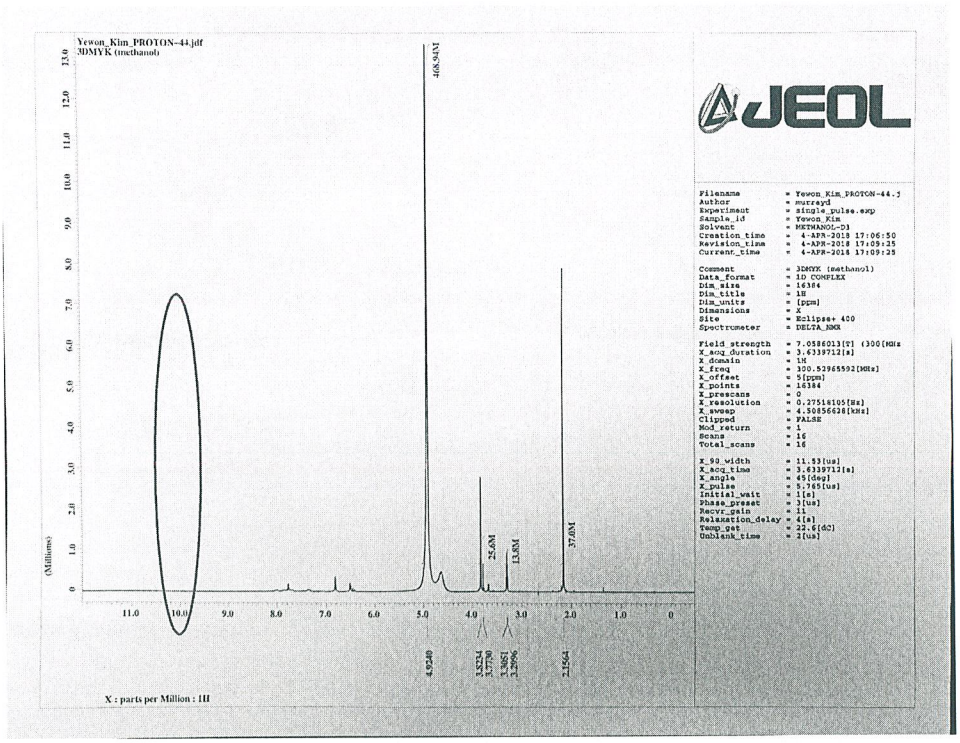
Table 3. Percent yield for each of the twenty synthesized compounds.

Each of the twenty compounds was analyzed by proton (H^1) NMR in order to confirm that the aldehyde peak (~9-10 ppm) had disappeared. Initially, the aldehyde peak (called a "diagnostic peak" for the purpose of this experiment) would have been present in the structure for the 2, 3, or 4-formylphenylboronic acid (Figure 5). If the synthesis proceeds in the expected manner, the diagnostic peak would disappear, as the aldehyde would no longer be present.

During the condensation reaction that occurs, the aldehyde in the boronic acid compound and the carbon, on which the cyano- group attaches on the acetonitrile, condense in order to form a double bond, resulting in the loss of the hydrogen attached to the aldehyde. Therefore, the loss of the aldehyde peak is a good indicator that the reaction has most likely proceeded as expected.



(a)



(b)

Figure 5. NMR spectra of 3-formylphenylboronic acid. In each of the 2, 3, and 4-formylphenylboronic acids, there is an aldehyde present in the compound structure. The H^1 NMR peak for each aldehyde (a) is found around 9-10. The circled peak is the readily identifiable aldehyde peak, which is called the "diagnostic peak". However, when the reaction proceeds as expected (b), the aldehyde will disappear from the structure, as will the peak for the hydrogen attached to the NMR.

The proton NMR of all twenty compounds was obtained. For each of the compounds, the aldehyde proton had disappeared, indicating that the expected synthesis reaction had most likely occurred. Because all 20 NMR spectra indicated successful syntheses, the percent yield of the reactions was used as a secondary basis for selecting the reactions that were most likely to be successful. The selected synthesized products had percent yields closest to 100%-- if the yield was much higher than 100%, the purity of the product would most likely be low and therefore would not be an ideal antibacterial. In each reaction, the products synthesized using 3-formylphenylboronic acid yielded >100% yields and typically had the highest yields.

Because of the extremely high percent yields from the 3-formylphenylboronic acid products, it was hypothesized that these products would not be ideal for use as an antibacterial. However, in the synthesis of products with 3,5-dimethoxyphenyl acetonitrile, the yields for the 3-formylphenylboronic acid and 4-formylphenylboronic acids were the same while the percent yield for the 2-formylphenylboronic acid compound was rather low. In order to have some representation for a product using 3-formylphenylboronic acid, the product of 3FPBA+35DM was used in the Kirby Bauer test. Two controls (non-boronic acid products), synthesized using *p*-tolualdehyde, were chosen as well as five boronic-acid containing products, one from each acetonitrile, for a total of seven products. Table 4 below shows the assigned disk number and the type of bacterial inhibition shown from the Kirby Bauer test for both *S. aureus* and *E. coli*.

Disk	Compounds Used	Bacteria	Inhibition
1	4-formylphenylboronic acid + 3,5-difluorophenylacetonitrile	*S. aureus*	Partial
		E. coli	None
2	2-thiopheneacetonitrile + *p*-tolualdehyde	*S. aureus*	None
		E. coli	None
3	2-pyridinylacetonitrile + *p*-tolualdehyde	*S. aureus*	None
		E. coli	None

4	2-formylphenylboronic acid + 2-thiopheneacetonitrile	*S. aureus*	Partial
		E. coli	None
5	4-formylphenylboronic acid + 4-methoxyphenylacetonitrile	*S. aureus*	Partial
		E. coli	None
6	3-formylphenylboronic acid + 3,5-dimethoxyphenylacetonitrile	*S. aureus*	Complete
		E. coli	None
7	2-formylphenylboronic acid + 2-pyridylacentonitrile.	*S. aureus*	Partial
		E. coli	None

Table 4. Out of the twenty synthesized products, seven were tested for antibacterial activity with *S. aureus* and *E. coli*. There was no activity shown against *E. coli* and partial inhibition against *S. aureus*.

There was no inhibition for either *S. aureus* or *E. coli* for the controls (compounds 2, 3). None of the synthesized compounds were able to inhibit the Gram-negative *E. coli*. All of the boronic acid substituted compounds had inhibitory effects on *S. aureus*. Initially, it was hypothesized that the products of 3-formylphenylboronic acid would not be very effective because of its high impurity (as seen by >100% yields), but compound 6, which was synthesized using 3-formylphenylboronic acid, showed complete inhibition while the other products only showed partial or no inhibition.

Discussion:

Based on the results of NMR and the disappearance of the diagnostic peaks, it can be assumed that the desired products have been synthesized. However, a deeper structural confirmation is necessary in order to truly confirm the formation of the desired compounds. In the future, in order to truly be able to compare the antibacterial activity of each of the compounds, each of the products should be purified. Currently, the Kirby-Bauer test used the synthesized product without any mode of purification, which means that a mixture of active antibacterial and byproducts were present in the tested solutions. The test would be more

effective and more meaningful if the products were as pure as possible, which would allow the same amount of antibacterial to be used in each of the solutions. Further, studies on drug concentration and antibacterial activity can be performed in the future if the products can be purified.

According to the Brownlee et al study, a non-boronic acid substituted cyanostilbene showed only "slight" antibacterial activity. For this study, the two controls tested (disks 2 and 3), which lacked boronic acid, showed no antibacterial activity for both Gram-positive and Gram negative bacteria, although their boronic acid containing counterparts proved to have antibacterial activity. Overall, no activity was shown against the Gram-negative *E. coli* while the Gram-positive *S. aureus* was more readily inhibited by the synthesized compounds. An explanation for this observed effect could have been due to the difference in cell wall structure for Gram-negative and Gram-positive bacteria. Although the walls of Gram-negative bacteria are significantly thinner than those of Gram-positive bacteria, Gram-negative bacteria tend to be more resistant to antibiotics, as they have an extra, relatively impermeable outer membrane.

It is uncertain why specific products showed antibacterial activity against *S. aureus* and others didn't, though there are several factors that could have led to this activity. The cell walls of Gram-positive bacteria also have pores which allow for the passage of specific molecules, assuming that they are small enough. The differing polarities and hydrophobicity of the synthesized compounds could also have played a role in the activity of the various antibiotics.

Because of time and resource constraints, only 7 of 20 compounds were tested for antibacterial activity. If this project is ever to be replicated, it would be recommended that each of the compounds are tested for antibacterial activity in order to obtain a clearer, more complete picture of the behavior of each of the compounds.

References

Brownlee, G., Copp, F. C., Duffin, W. M., Tonkin, I. M. 1943. *The Antibacterial action of some stilbene derivatives.* Vol. 37(5). Biochemical Journal.

Brownlee et al. in their paper, *The antibacterial action of some stilbene derivatives*, tested seven categories of stilbenes and stilbene derivatives on staphylococcus and streptococcus. For the preparation of the stilbene-containing solutions, the research team created 10% solutions in 90% ethanol. In total, 34 compounds were tested. One of the compounds tested was a cyano-derivative of a stilbene. However, the results were poor—the bactericidal activity against staphylococcus was reported as "inactive" and against streptococcus, "slight activity" was reported. This finding is important to my study because it has prompted me to investigate a method of creating a cyanostilbene that will have considerable antibacterial activity.

Das, B. C., Thapa, P., Karki, R., Schinke, C., Das, S., Kambhampati, S., Banerjee, S. K., Veldhuizen, P. V., Verma, A., Weiss, L. M., Evans, T. 2013. *Boron chemicals in diagnosis and therapeutics.* Vol. 5(6). Future Med Chem.

This paper by Das et al. investigates the medicinal uses of boron-containing compounds. The authors of this paper argue that boron, a metalloid, has versatile uses. Because boronic acid has a high pKa of 9-10 and therefore remains protonated under most physiological conditions, it may be able to form hydrogen bonds with target proteins and act with high specificity and produce strong biological activity, whether it may be antifungal, antibacterial, etc. Further, the authors of this study found that boron-containing compounds acted highly specifically against Gram-negative bacteria, leading me to believe that the compounds I am synthesizing will be able to target Gram-negative bacteria due to the presence of the substituted boronic acid.

Docherty, J. J., McEwen, H. A., Sweet, T. J., Bailey, E., Booth, T. D. 2007. *Resveratrol inhibition of Propionibacterium acnes.* Vol. 59. Journal of Antimicrobial Chemotherapy.

In this study conducted by Docherty et al., several concentrations of resveratrol were tested against *Propionibacterium acnes*, a type of Gram-positive bacteria that is found on human skin and is closely associated with human acne. The purpose of the study was to test the inhibitory effects of resveratrol based on the concentrations of resveratrol present. 24-hour old plates of *P. acnes* were incubated in broths of various concentrations of resveratrol (0-200 mg/L) and were incubated between 12-24 hours, then plated on agar plates. Following another 48 hours, the colonies of *P. acnes* were counted. The results of the study showed that resveratrol was bacteriostatic at lower concentrations and only at the highest concentration tested (200 mg/L). This study is useful for my

research in order to understand the antibacterial behavior of stilbene compounds. I plan on modeling my antibacterial portion of my study on the methods presented in this paper.

Fleming-Dutra, K.E., Hersh, A. L., Shapiro, D. J.. 2016. *Prevalence of Inappropriate Antibiotic Prescriptions Among US Ambulatory Care Visits, 2010-2011.* Vol 315. Journal of the American Medical Association.

An investigation into the antibiotic prescriptions among ambulatory care patients revealed that an overwhelming 30% of prescriptions were for inappropriate causes. The reasons for these prescriptions range from over-diagnosis to incorrect diagnosis. This study is helpful to establish a basis for the necessity of projects like mine- as antibiotics are overused, antibiotic resistance grows, creating a need for the development of hybrid drugs.

Paulo, L., Ferreira, S., Gallardo, E., Quieroz, J. A., Dominguez, F.. 2010. *Antimacrobial activity and effects of resveratrol on human pathogenic bacteria.* Vol. 26. World J. Microbiol Biotechnol.

Paulo et al. conducted a study on six Gram-positive bacteria strains and seven Gram-negative bacteria strains, using the disc diffusion method for testing the bactericidal effect of resveratrol. The research group tested resveratrol concentrations ranging from 3.125 to 400 μg/mL in order to identify the minimum inhibitory concentration necessary for each strain of bacteria tested in the study. The study found that resveratrol exhibited antibacterial behavior for each of the Gram-positive bacteria tested, but only five of the seven Gram-negative bacteria were acted on antibacterially. Additionally, the results showed that while the Gram-negative bacteria decreased their growth when exposed to resveratrol, the Gram-positive bacteria stopped growing altogether. This is essential for my research project, as it shows what type of bacteria will be most effectively targeted when I test the products of synthesis.

Trippier, P. C., McGuigan, C. 2010. *Boronic acids in medicinal chemistry: anticancer, antibacterial and antiviral applications.* Vol. 1. Med Chem Comm.

This paper by Trippier and McGuigan discusses the anticancer, antiviral, and antibacterial applications of boronic acid. The section on antibacterial application discusses the inhibitory mechanisms of boronic acid, specifically in the case of bacteria that have developed resistance to penicillin and cephalosporins. These bacteria achieve this resistance by the production of β-lactamases, which hydrolyze the β-lactam rings of penicillin and cephalosporin, thus inactivating the drug. The way in which a boronic acid compound behaves as an antibacterial is by acting as a transition state inhibitor. Trippier and McGuigan discovered that the closer the boronic acid mimicked the structure of the

natural compound, the more potent the inhibitory action was. This study also investigated the ortho-, meta-, para- directed analogues of the boronic acid compounds and found that the para-directing boronic acids were the most potent as an antibacterial. The research presented in this paper is important because it confirms my choice of boronic acid-containing reactant. The reactant being used in my project, if synthesis goes as proposed, will result in a para-directing product.