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Synthesis and Testing of Antibiotic Properties of Functionalized Quinolone Analogs

Darien L. Allen
Dr. Amanda Wolfe
418: Chemical Research III

Darien Allen

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

Dr. Amanda Wolfe

Abstract

The medical field has countless issues that make it difficult to treat patients effectively, and the underlying one is antibiotic resistance. Antibiotic resistance came to be through the periodical evolution of bacteria after treatment, it makes the methods and antibiotics used to kill certain infectious bacteria less and less effective. In this work, we discuss the creation of new antibiotics and the making of new substituents for old antibiotics to make them more effective. We synthesize new molecules that resemble old antibiotics and have the capability to target and inhibit the ATP synthases of gram-negative bacteria like P. aeruginosa and A. baumannii. We have found that it is best to have primary and positive amines, as well as high overall charges. With this information, I plan to create a new quinoloine base through a Vilsmeier-Haack reaction. To create new pathways to analogs capable of both permeating and avoiding efflux. This new quinolone base will undergo various steps in a reaction process to create a breath of fresh air in the field of antibiotic synthesis.

Synthesis and Testing of Antibiotic Properties of Functionalized Quinolone Analogs

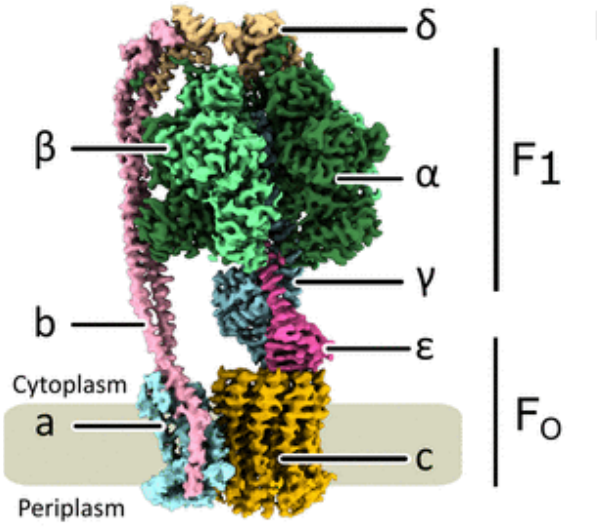
1. Introduction

Treatment for patients has become more difficult in the present day. This occurred because of antibiotic resistance, which came to be through the periodical evolution of multiple bacteria cultures within the bodies of many people that were expelled later and reintroduced to the environment.[1,2]

The Centers for Disease Control and Prevention stated that in the United States, about 48,000 people die each year from multidrug-resistant infections, and more than 3 million illnesses are caused.[2] There are also a few different ways that a bacterium can reduce the effectiveness of antibiotics. Through efflux pumps, bacteria can keep the concentrations of antibiotics within themselves low. Bacteria can also develop alterations in the components of the binding sites or changes of permeability using the membrane, degradation enzymes that break down the antibiotics, as well as changing the conformational state of the antibiotic to mollify its effects.

Currently, pathogenic gram-negative bacteria can be found anywhere. Also, they can cause the most trouble in hospitals with immune-compromised or weakened patients.[3] This is a massive problem because of the hospitals' inability to treat these infections, giving way to a high risk of morbidity and mortality.[3] These gram-negative bacteria have two membranes and the clinically observed bacteria include *Neisseria meningitidis*, *Haemophilus spp*., *Helicobacter pylori, E. coli*, *Chlamydia trachomatis, Acinetobacter baumannii* (AB), and *Pseudomonas aeruginosa* (PA). Within hospitals, there are not enough opportunities to isolate patients colonized or infected with drug-resistant strains, some are also resistant to disinfectants that are commonly used as well.[4] These bacteria can reach multiple organs; they travel in and through the digestive system, bloodstream, and nervous system. Many strains can cause multifarious diseases, such as pneumonia, urosepsis, and wound infection. Lower intestinal infections are the most difficult for doctors to treat, and they account for most

of the recorded infections. This is new treatments were discovered and researched, leading to the concepts and forms of antibiotics.



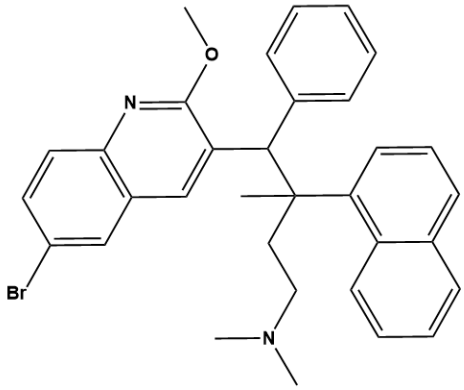


Figure. 1 *Bedaquiline (BDQ) structure*

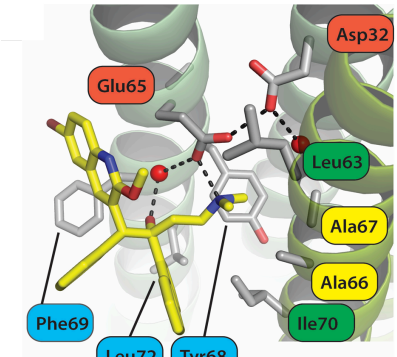
Figure 2. *A. baumanii ATP Synthase structure Cryo-EM*
Demmer et al. Science Advances ***8,*** *eabl5966 (2022)*

There have been very few discoveries of new antibiotics in the past 40 years. This has created a stagnation in the effort to prevent microbial infection.[5] Tuberculosis cases have increased substantially worldwide over the past decade, and with no newly discovered antibiotics, it has become a great example of how we can learn to analyze and destroy certain bacteria.[6] This led to the creation of diarylquinoline-based antibiotics to combat these infections. Diarylquinoline is a class of molecule that is heterocyclic because of the different elements in their rings. This class was recently approved to treat multidrug-resistant bacteria.[7] This is because it acts as a base structure that can then be edited to suit new biological purposes. One of these is the bedaquiline (BDQ) structure, which can inhibit multi-drug-resistant *Mycobacterium tuberculosis* (MT) and other antibiotic-sensitive variants; the most active compound of this experiment was the

quinoline core, a pure enantiomer with two chiral centers. (Fig. 1) This was found through pharmacodynamic studies in mice and later through human volunteers.[6] Here, it has been discovered that the use of large nonpolar sections in the molecule of choice caused mutations in the gene that encodes atpE, a part of the F0 subunit of ATP synthase (Fig. 2), where it would inhibit the proton pump within *M. tuberculosis*. This effect would terminate the function of ATP synthase.

To learn more about the capabilities of antibiotics, many research groups have begun to research the exact forms of resistance that are creating roadblocks. One such example is the MexB efflux pump found in *Pseudomonas aeruginosa.*[8] Which is an integral part of the MexAB-OprM efflux system, is from the resistance-nodulation-cell division superfamily of proteins. This is a key system that acts to pump toxins out of the bacteria, which, in their perspective, is an antibiotic. [8] The function of MexB is to be an inner membrane transporter that is responsible for the binding and recognition of various compounds. It is powered by the proton motive force of the electrochemical potential found in protons and recognizes compounds in its deep binding pocket. The two proposed mechanisms to inhibit this efflux pump are allosteric and competitive. These would hopefully stop the transition of a protomer inside the MexB pump from loose to tight, preventing it from functioning. [8] For a molecule to inhibit the MexB pump, it would need to be aromatic or hydrophobic. It was also found that positive charges increase the likelihood of antibiotic efflux. These findings could prove useful for establishing synthesis criteria.

Other challenges are the antibiotic degradation enzymes that can alter antibiotic structures and make them ineffective. For example, there is the NDM-1 enzyme that is from the metallo-β-lactamases (MBL) family, which can be transferred among different bacterial populations. The MBL superfamily can hydrolyze antibiotics, and an active site made up of two zinc ions can cleave the amide bond of the β-lactam ring of most β-lactams, thus inactivating the antibiotic.[9] This skill, combined with their wide substrate specificity, makes them a steep obstacle to surmount while keeping in line with other constraints. There have been clinically active β-lactamase inhibitors but no useful inactivators for clinical trials.[9] There are three kinds of NDM-1 inhibitors; one type acts directly on the zinc ions in the active site. The second kind acts on the amino acid residues and hinders NDM-1 and its substrate-binding capabilities. The third can act on both the zinc ions and the key amino acids that catalyze the reaction overall.[10] However, it is difficult to combat this because affecting these natural factors that can be found in the human body may lead to further damage. To survey the possible damage that these analogs could cause, we had their antibacterial activity analyzed using a standard broth microdilution MIC assay against both methicillin-susceptible PA and wild-type PA. There were tests for PA accumulation evaluation to see if the molecules that penetrated the outer membranes of bacteria could avoid the efflux protocols of bacteria. These test were used to verify the capability of molecules and generate new ideas.[11]



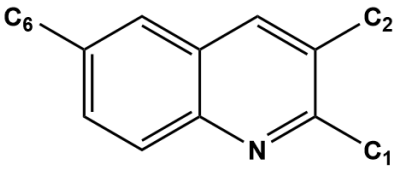


***Figure 3**. Quinoline core structure* ***Figure 4.** Bedaquiline inside Fo subunit of MT (Kumar et al., Tuber. 2017, 10)*

Past research done on MT showed BDQ as a highly potent antibiotic.[6] These bacteria have ATP synthase structure similarities to *P. aeruginosa*. Research in the Wolfe Lab began with the quinoline core, which had the best capability to create novel derivatives.[11] To create novel derivatives of BDQ, the work began with a set of 16 C1 and C2 quinoline analogs (Fig. 3).[11] Based on the binding of BDQ to the c-ring of *Mycobacterium tuberculosis* ATP synthase, it was discovered that the C1 and C2 substituents of the molecule were responsible for most interactions in the Glu61 that makes proton binding on the protein more favorable.[12] It was determined after experimentation that for the goal, stereocenters, bromine, and hydroxy groups were non-essential. The proteins that made up the pocket of PA ATP synthase were equivalent to the goal pocket (Fig. 4). This pointed to the idea that the molecule would need more non-polar subunits to be recognized. A few notable differences between PA and MT ATP were the presence of amino acids in different numbered locations, (Fig. 4.) in PA there was phenylalanine (Phe) instead of aspartate32 (Asp), tyrosine68 (Tyr), Phe69, and leucine72 (Leu), with threonine (Thr), methionine (Met), and valine (Val), respectively (Fig. 4.).[11] These discrepancies made the pocket less congested and more non-polar overall. Keeping this in mind, the similarities between PA and AB made it possible to test on both of these bacterial strains.

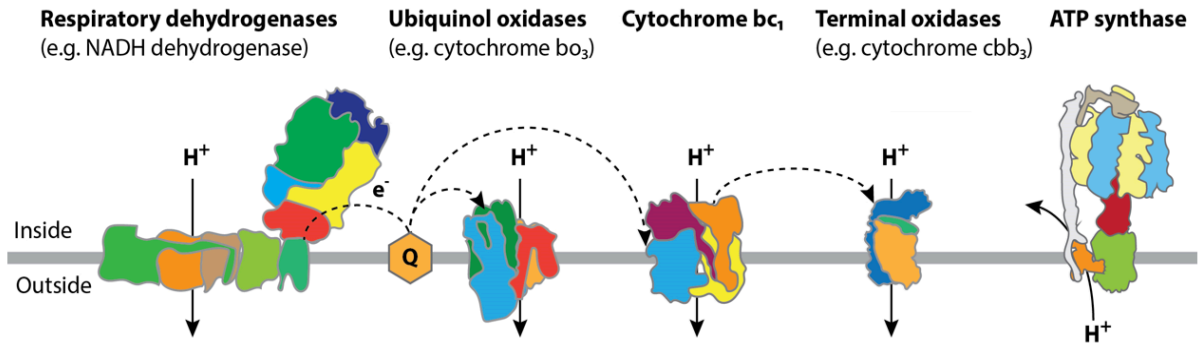


***Figure 5.** Electron transport chain (Katie Ward, Ryan Steed)*

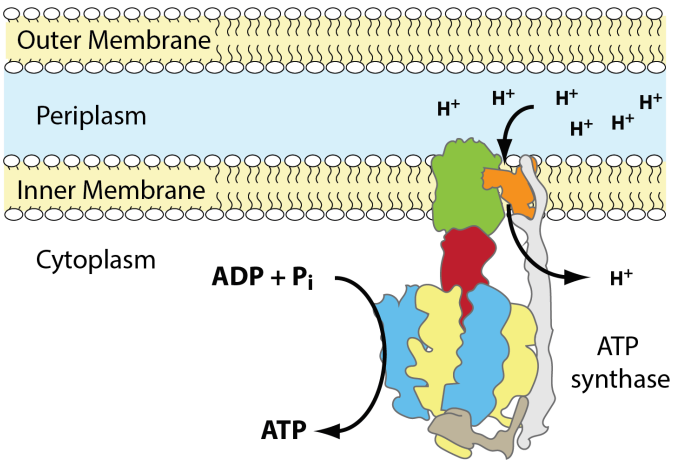


***Figure 6**. ATP synthase body in cell membrane. (Katie Ward, Ryan Steed)*

The first hypothesis was to increase π-stacking and hydrophobic surface so that binding would be promoted. At the C1 position, alkoxy groups are productive inhibitors in both ATP synthases. However, the C2 position prioritized non-polar substitutions. After the synthesis of various analogs, testing began on PA ATP synthase inhibition. In these experiments, membrane vesicles were inverted so that the target was outside of the membrane and easier to access. The ATP synthase activity was measured by the capability of the electron transport system (Fig. 5) to create a proton gradient that would drive ATP synthesis. (Fig. 6) These tests provided data proving the molecules were unsuccessful due to a lack of accumulation or penetration of the membrane.[11] This experiment concluded that six of the 16 compounds were capable of inhibiting PA ATP synthase in vitro, but only alongside a separate compound that had a methyl sulfide at C1 and tyrosine at C2. (Fig. 3) These compounds had an abundance of steric bulk at the C1 position and either methanethiol or methanol at the C2 position.[11] Unfortunately, none of the compounds displayed complete cell antibiotic activity. However, this confirmed the hypothesis that hydrophobicity, functional groups with π-stacking, and steric bulk at either the C1 or C2 position would increase activity.

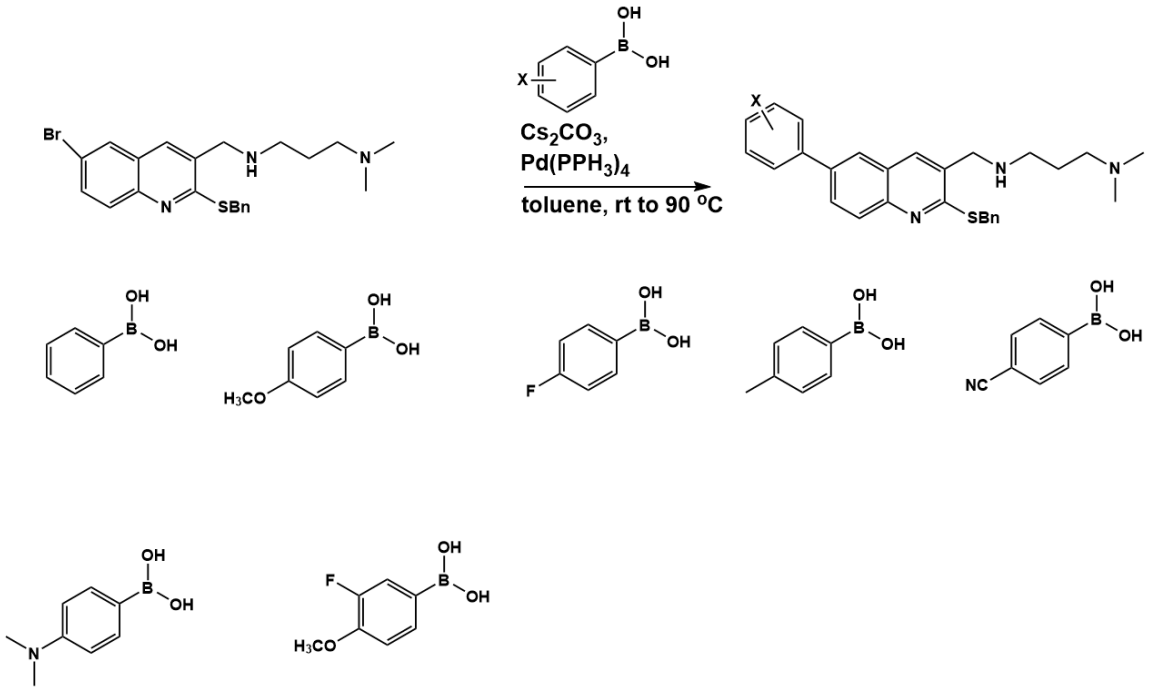


Figure 7. *Target molecules with steric bulk at the C6 position and high activity groups at the C1 and C2 positions.*

Our current goal is to create derivatives with more bulk at the C6 position while maintaining high activity at the C1 and C2 positions (Fig. 7). The first reaction scheme was a cyclization under Vilsmeier-Haack conditions.[13] The goal of this cyclization was to create a quinoline-like backbone that would have an aldehyde, a nitrogen in the added ring, and a chlorine substituent. While also containing a bromine on the sixth position of the molecule. Afterward, the objective will be to conduct a benzylation to create a sulfur-benzyl group in place of the chlorine. This will add some of the desired steric bulk and create the opportunity to perform a reductive amination. The next step in the synthesis will be a Suzuki reaction that will replace the carbon six position bromine with a boronic acid. This effort to increase steric bulk and aromaticity could be the exact thing needed to generate a lower MIC and decrease activity of ATP synthase of AB during testing.

2. Results and Discussion

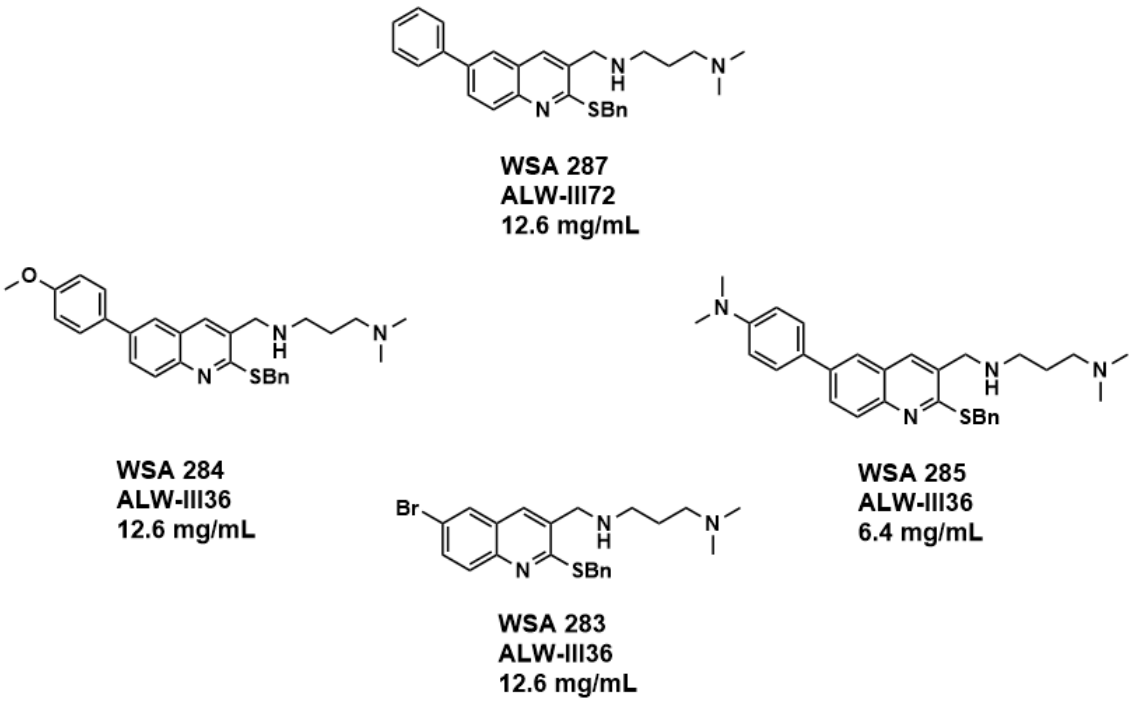
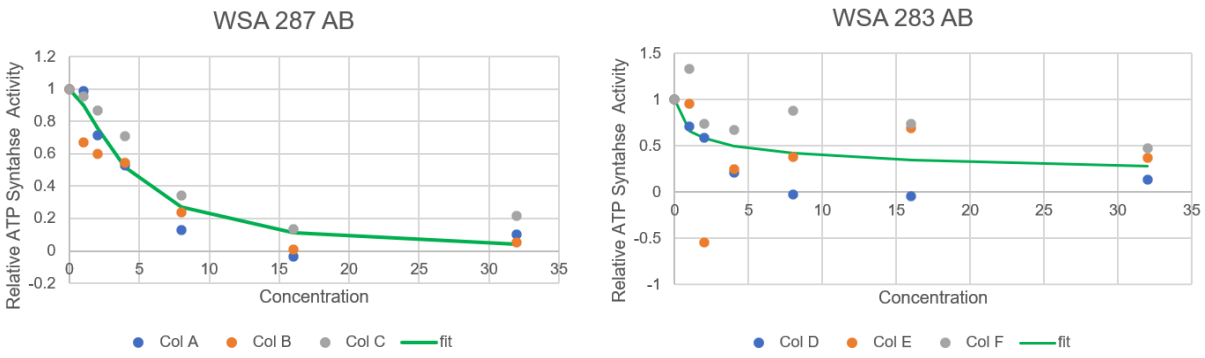


Figure. 8 *These are the compounds that were tested for ATP inhibition potential as well as the cell death.*



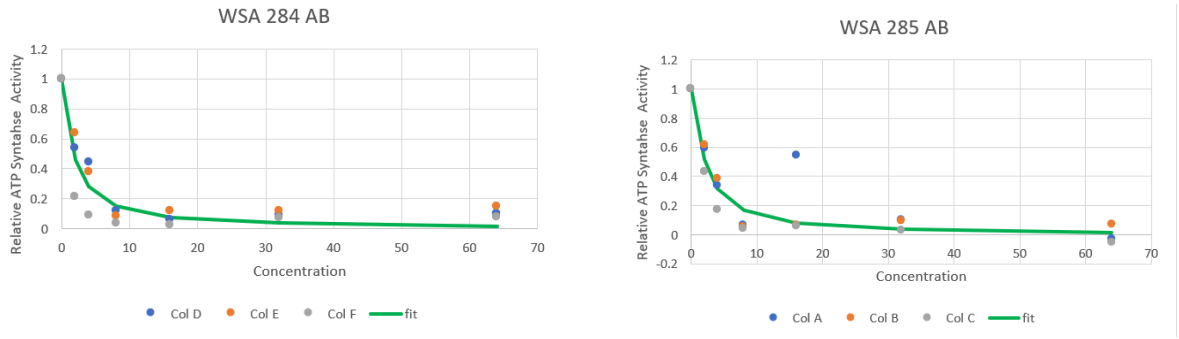


Figure. 9 *These are ATP synthase activity assays that used the compounds found in figure 9 to assess the function of ATP synthase within A. baumanii bacteria while each compound was present. WSA 287 and WSA 283 were done with a maximum 32 ug/mL concentration. WSA 284 and WSA 285 were done with a maximum 64 ug/mL concentration, all assays were performed in triplicates.*

This study aimed to generate new quinoline-based antibiotic derivatives that could reduce the activity of ATP synthase within AB. (Fig. 8) The compounds were synthesized as a library of analogs for the testing of the lab. The least functionalized compound, WSA 287, had one of the highest IC_{50} values for AB testing. The AB value was above the mean of the measured values. This, along with the inhibition tests, reveals that WSA 287 was not consistently effective in low concentrations. (Fig. 9) The data observed also demonstrates that WSA 284 has the best fit curve in terms of inhibiting ATP production. This means that there is a strong negative correlation between ATP synthase activity and inhibitor concentration. This differs from WSA 283, where certain outliers skewed the data. (Fig. 9) In this dataset the inhibition of ATP production was not consistent or prominent; however, the MIC value was lower than expected when coupled with these results. (Table 1)

	MIC (ug/mL)				
Compound	**AB 1605 (MDR)**	**AB 17978**	**PA 2108 (MDR)**	**AB IC_{50} (µg/mL)**	**PA IC_{50} (µg/mL)**
WSA 283	**8**	**8**	**8**	**4.03**	**3.39**
WSA 284	**8**	**4**	**4**	**1.71**	**2.82**
WSA 285	**32**	**16**	**8**	**2.11**	**6.53**
WSA 287	**8**	**16**	**8**	**3.88**	**4.20**

Table 1. *These are the MIC values of each compound listed in the compound library against specific strains of bacteria. MDR stands for multi-drug resistant. (Fig. 9)*

This means that this compound most likely is preventing the growth of bacterial strains in another way. This means that the compound is off-target and interferes with another organelle or process that the bacteria uses to regulate itself. With the data available and prior knowledge, the relative success of WSA 284 could possibly be attributed to the presence of the methoxy group on the para position of the C6 benzyl group. To test this theory further, computational docking and further alterations to that methoxy group could lead to new developments and elucidate the

4. Conclusion

Multidrug resistance continues to grow and outpace the research for new methods of prevention and alternative paths to combat this growing issue. The path to human trials is long and arduous, but each study narrows down future improvements and rules out certain possibilities to provide a stronger basis for the next lab to take on this surmountable challenge.In summary, this study provides new insights into potential affecting factors that affect the inhibition of ATP synthase in AB and PA. The results demonstrate that π-bonds and steric bulk are still advantageous in the production of these structures. The data provides reasoning for the improved results of WSA 284.

Highlighting the potential role of oxygen atoms in binding within the protein structure. While these findings contribute to our understanding of ATP synthase inhibitors, further investigation is needed to fully elucidate the underlying mechanisms and to explore their implications in computational settings. The next step to further investigate these phenomena is to create a checklist of criteria that can be applied to generated structures that could be tested in a computational docking program. Overall, this work lays the groundwork for future studies aimed at ATP synthase inhibition and preventing multidrug resistance from progressing and underscores the importance of continued research in this area.

Acknowledgements

I would like to thank Dr. Amanda Wolfe and Dr. Ryan Steed for this opportunity to learn from them and experiment with bacteria and organic synthesis. The Wolfe Lab team also deserves my deep gratitude due to their kindness and assistance in my efforts. I would also like to thank the University of North Carolina at Asheville Chemistry Department for funding these efforts.

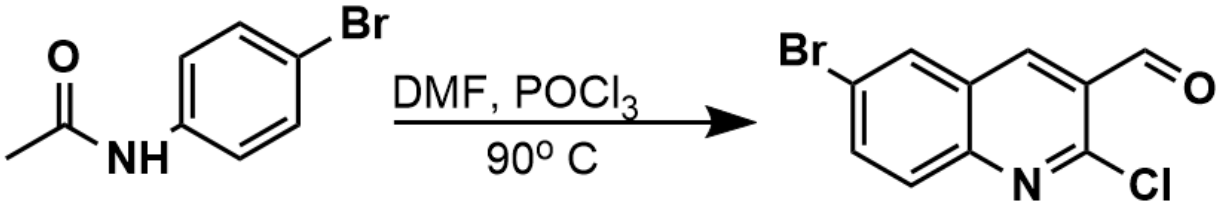
5. Materials and Methods

5.1 General Synthesis

Caution! *N-(4-bromophenyl)acetamide is very toxic orally and will cause respiratory tract irritation upon ingestion.*

Caution! *$POCl_3$ is caustic and will cause significant harm to organic materials.It should be treated as an aqueous waste during disposal, requiring rinses of all glassware.*

Caution! *Dimethyl formamide will cause damage to organs through prolonged exposure.*

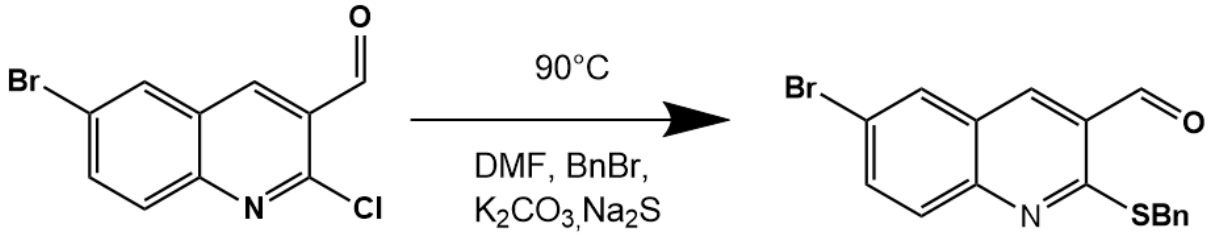


Compound was prepared via literature procedure[13], with slight modifications. In a flame dried 250 mL 2-neck round bottom flask, addition funnel, and reflux condenser added under inert conditions. The Then DMF (1.80mL, 23.25 mmol) was added directly to the round bottom after a quick removal and then replacement of the addition funnel. Then the $POCl_3$ (6.12mL, 65 mmol) was added into the addition funnel and added dropwise as slowly as possible for 10 minutes. Starting material (2.0g, 9.3 mmol, 1 eq.) was added by lifting the addition funnel and heated to 90°C oil bath. Removed the addition funnel and wrapped the septa of each neck in parafilm. The reaction ran for 15 hours and then was taken off heat to become room temperature. Quenching began with 150mL of DI H_2O stirring vigorously, the solution was added in sparingly and slowly to keep the temperature below 30°C and afford more product. After quenching filtration was done multiple times using a Buchner funnel apparatus. Recrystallization was done by heating the solids up to 120°C in 10mL of ethyl acetate. Then it was taken off heat for 15 minutes and placed in an ice bath for another 15 minutes. 1H NMR (400 MHz, CDCl3) δ 10.56 (d, J = 1.5 Hz, 1H), 8.66 (d, J = 1.8 Hz, 1H), 8.15 (dd, J = 8.3, 17.1 Hz, 1H), 7.95 (d, J = 1.2 Hz, 1H), 1.57 (d, J = 1.0 Hz, 1H)

Caution! *BnBr is a corrosive respiratory tract irritator with specific toxicity in single exposure. It is flammable if exposed to temperatures of 86°C.*

Caution! *Sodium sulfide is acutely toxic (dermal and inhalation) and will cause long-term damage to aquatic organisms.*

Caution! *Potassium carbonate is acutely toxic if inhaled and will cause respiratory tract irritation.*

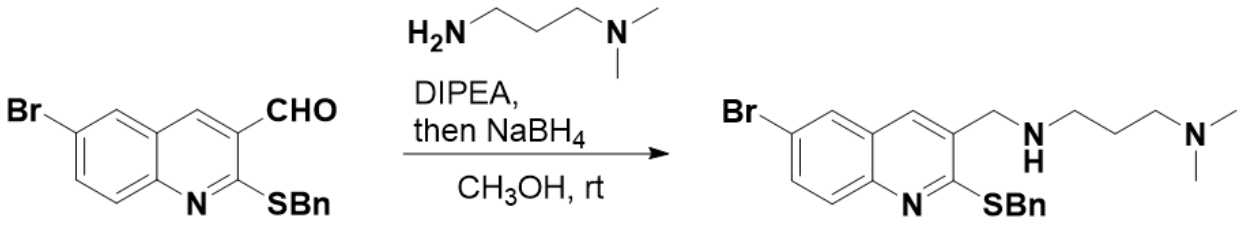


In a flame-dried 100 mL round-bottom flask under inert conditions, the previously made 6-bromo-2-chloroquinoline-3-carbaldehyde (260mg, 3.47 mmol, 1 eq) was added directly to the round. Then the DMF(0.861mL, 1 mmol) and the Na2S (18.95mg, 4.86mmol) were added and stirring began and continued for 16 hours. The,n BnBr (0.181mL, 5.2mmol) and K2CO3 (198.2mg, 5.2mmol) were added and the reaction was heated to 100°C and stirred for 4 hours. Extraction using ethyl acetate and DI H_2O afforded a crude product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.55 (s, 1H), 8.66 (s, 1H), 8.14 (s, 1H), 7.94 (d, *J* = 1.1 Hz, 1H), 7.26 (s, 1H).

Caution! *DIPEA is flammable, corrosive, and acutely toxic (ingested or inhaled) will cause respiratory tract irritation.*

Caution! *Methanol is flammable, acutely toxic (ingested, contact, or inhaled), and will cause organ damage.*

Caution! *Sodium borohydride is an irritant, corrosive, flammable, acutely toxic (ingested, contact, or inhaled) and will cause dermal irritation.*



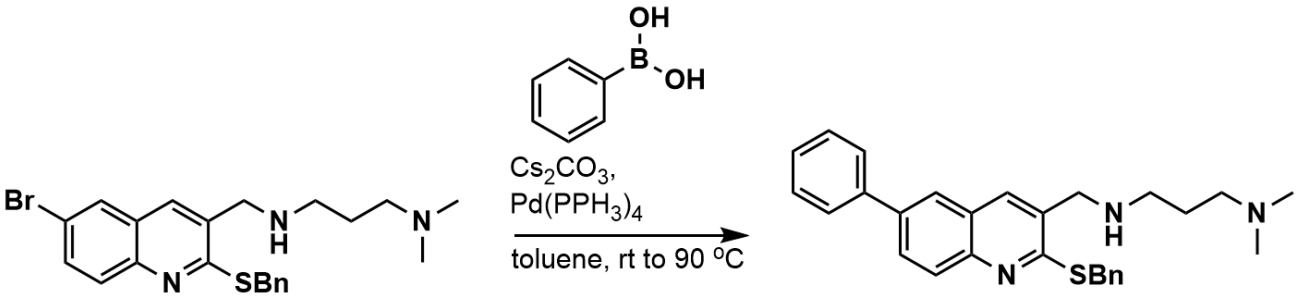
In a flame dried 100mL round bottom flask under inert conditions the benzyl sulfide quinoline (502.7mg, 1 eq) and the amine (0.218mL 1.2 eq) were dissolved in anhydrous methanol (15.62mL, 0.09 M). *N,N*-Diisopropylethylamine (0.719mL, 3 eq) was added dropwise and stirred at 23 °C for 24h. Afterwards, $NaBH_4$ (111.7mg, 2 eq) was added and the reaction stirred for 1h. Then the reaction was diluted with DI H_2O and extracted with DCM (2x) leading to the organic layers being combined and dried over Na_2SO_4. Then it was concentrated under reduced pressure in a round bottom. Flash chromatography of the crude extracts (SiO2, 3 × 10 cm, 0−100% CH3OH/CH2Cl2 gradient elution) provided the desired products. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.25 (s, 1H), 8.34 (s, 1H), 7.99 (d, *J* = 1.6 Hz, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.28 (d, *J* = 3.6 Hz, 1H), 4.61 (s, 1H).

Caution! *Toluene is an irritant, flammable, acutely toxic (ingested, contact, or inhaled) and will cause death upon ingestion.*

Caution! *Cesium carbonate is an irritant, corrosive, acutely toxic (ingested, contact, or inhaled) and will cause developmental issues.*

Caution! *Phenylboronic acid is an irritant and acutely toxic if ingested.*

Caution! *Tetrakis(triphenylphosphine)palladium acid is an irritant and acutely toxic if ingested.*



In a flame-dried round bottom, starting material brominated quinoline intermediate (32.5mg, 1 eq), phenyl boronic acid (13.6mg, 1.5 eq), Cs_2CO_3 (48mg, 2 eq), and $Pd(PPH_3)4$ (16.9mg, 0.2 eq) were all added. Then toluene (1.09mL, 0.1 M) was added, and the reaction was sparged with argon then heated to 90 ºC for 16h. The reaction was then cooled to room temperature and filtered through a pad of celite, then rinsed with DCM. The crude extracts were purified with TLC in 10% CH_3OH/CH_2Cl_2 or flash chromatography (SiO_2, 3 × 10 cm, 0−100% CH_3OH/CH_2Cl_2 gradient elution), providing the desired product. ^1H NMR (400 MHz, $CDCl_3$) δ 10.07 (s, 1H), 7.62 (d, *J* = 7.0 Hz, 1H), 7.42 (d, *J* = 12.0 Hz, 1H), 7.23 (d, *J* = 0.8 Hz, 1H), 7.03 (s, 1H), 2.07 (d, *J* = 15.0 Hz, 1H).

5.2 General Sterilization Procedure

The following are general steps unless otherwise noted. All steps were completed with aseptic techniques. All media and glassware were sterilized via autoclave at 121 °C for 60 minutes. All agitation occurred at 160 rpm in a temperature-controlled console shaker (Excella E25) at 37 °C. Full-strength tryptic soy broth (TSB) was made by dissolving 30 g BD Bacto TSB powder in 1 L deionized water. Purchased and acquired bacteria strains used were Acinetobacter baumannii (AB, ATCC 17978) and Acinetobacter baumannii 1605 (MDR) provided by Zgurskaya and co-workers at the University of Oklahoma.

5.3 Antimicrobial Susceptibility Assay Procedure

Susceptibility testing was performed in biological triplicate, using the broth microdilution method as outlined by the Clinical and Laboratory Standards Institute. Briefly, minimum inhibitory concentrations (MIC) determinations were carried out in 96-well microtiter plates with 2-fold serial dilutions of the compounds from 0 µg/mL to 256 µg/mL (final assay concentrations) in DMSO. Briefly, to each well 1 µL of compound

in DMSO, 89 µL of TSB, and 10 µL of bacterial inoculum, grown from a single colony in 10 mL of TSB for 4-6 hours, were added. After incubation for 12–15 h at 37 °C, absorbance at 590 nm was read on a Biotek Synergy HTX Multi-mode plate reader. Data was processed by background subtracting the media absorbance and then normalizing the data to full bacteria growth with only vehicle. MIC is defined as the lowest concentration of antibiotic or antibiotic/adjuvant combination that achieves ≥ 85% growth inhibition, which corresponds to no visible growth.[15]

5.4 ATP Synthesis Assay.

In a 96-well microplate, 2 µL of test compound or DMSO alone was diluted into reaction buffer, which contained 5 mM Tricine, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 3.75 mM potassium phosphate, 0.1 mM adenosine diphosphate, and 2.5 mM reduced β-D-nicotinamide adenine dinucleotide (NADH) at final dilution. The synthesis reaction was initiated by mixing in 50 µL 0.2 mg/mL vesicles (200 µL final reaction volume) and incubated for 10 min at room temperature. The reaction was stopped by diluting a 50 µL reaction sample into 1% trichloroacetic acid, 10 mM ethylenediaminetetraacetic acid, and 2 mM carbonyl cyanide 3-chlorophenylhydrazone. From the stop mix, samples were diluted 100-fold in water to dilute test compounds and stop components, and ATP concentration was determined by luminescence using a luciferin-luciferase system (ATP Determination Kit, Molecular Probes). Luminescence was measured in an opaque white microplate using a BioTek Synergy H1 microplate reader. Data were normalized to DMSO control values, and dose-response curves were fitted using GraphPad Prism 9.[15]

5.5 Determination of ATP synthesis activity.

Inhibition of ATP synthesis activity by test compounds was determined using an endpoint assay essentially as previously described.[14] (Fraunfelter page 11, 2023) In this assay, plates were activated with 25 mM NADH and 2.5 ADP. To begin the ATP synthesis reaction, vesicles were equally distributed at intervals of 30 seconds for 10 minutes. The reaction was stopped by adding 50 µL of the ATP synthase mixture to 12% trichloroacetic acid. Then the reaction was diluted by transferring 10 µL of the stopped reaction into a deep-well dilution plate with 1000 µL of dI H_2O. Afterward, 10 µL of the diluted mixture was added to each luciferase solution well, and the plates were read by a plate reader to determine the luminescence of each well. Then the Excel sheet of the data was analyzed to determine the activity of ATP synthase. Luminescence values were corrected for background by subtracting the negative control and then normalized to the positive control within the same replicate.[15]

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