A Mechanistic and Structural Analysis of Novel β-lactam Antibiotics

Steven Moss
Advisor: Dr. Monika Konaklieva
Department of Chemistry
American University
Washington, DC 20016

April 30, 2012
Spring Semester 2012

Final Capstone Paper
College of Arts and Sciences – Honors College
University Honors in Biochemistry

Table of Contents

List of Figures, Tables, and Schemes

Abstract

The β-lactams are one of the most successful classes of antibiotics. However, they are rapidly losing their potency due to elaboration of β-lactamases by bacteria. Therefore, new approaches to drug development are needed to target the organisms that are resistant to β-lactam antibiotics. This can be achieved by designing antimicrobials that are resistant to the cleavage action of these enzymes. This study designed, synthesized, and tested a novel class of monocyclic β-lactams that carry an arylthio group at the C4. These lactams exhibit inhibitory and cidal activity against serine β-lactamase producing *Mycobacterium tuberculosis* wild type strain (Mtb) and multiple (n=8) β-lactamase producing *Moraxella catarrhalis* clinical isolates. Because of this, a study was performed to better understand the compounds' chemical reactivity through fragmentation analysis using electrospray ionization-mass spectrometry, and an enzyme study with penicillinase, a common β-lactamase. The main fragmentation pattern that was observed from these novel compounds was a peak produced at 177.0 m/z which correlates with the breaking of the N1-C4 and C2-C3 bonds in the β-lactam moiety. This unique fragmentation was affected by different substituents on the arylthio group. The enzyme study with penicillinase showed that the compounds did not bind, and therefore were not hydrolyzed by the enzyme. This research implicates that these novel β-lactams are both effective, and able to circumvent the main mechanism of drug resistance that microbes have against β-lactam antibiotics.

Introduction

β-lactams and Tuberculosis

Since the discovery of the first β-lactam antibiotic, penicillin (**Figure 1**), by Alexander Flemming in 1928[1], β-lactams have been commonly used to treat a wide array of diseases. β-lactams are best known for their ability to inhibit the transpeptidation of cell walls, which gives them the ability to be effective against a large array of harmful microbes including *E. coli, P. aeruginosa, S. aureus, M. tuberculosis* and many others.[1]

Structure of Penicillin

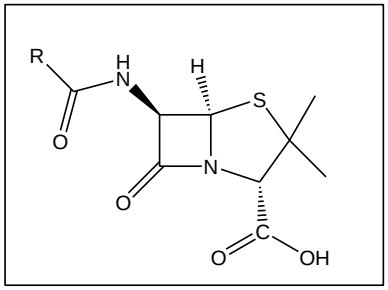


Figure 1. *Above is the general structure of the common antibiotic penicillin*

General Structure of Novel β-lactams

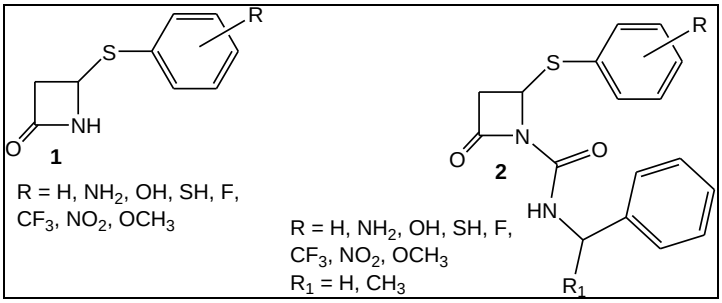


Figure 2. *The structure on the left is the arylthio attached to the β-lactam, and the structure on the right is the novel β-lactam after the addition of the carbamyl to the N1.*

The most recent project pursued by the Konaklieva lab has been the synthesis of novel monocyclic β-lactams containing an arylthio on the C4 of the β-lactam ring (**Figure 2**). . These lactams have shown inhibitory and cidal activity against *Mycobacterium tuberculosis* wild type

strain (Mtb), the microbe responsible for Tuberculosis, *Moraxella catarrhalis* clinical isolates, a microbe responsible for ear infections in children. This is shown by the low minimum inhibitory concentrations (MIC) exhibited by the compounds in relation to these two microbes (**Appedix A**). The low MICs against Mtb are particularly important because of the prevalence of TB around the globe, and the challenges this disease poses to global health.

The Importance of Overcoming TB

Tuberculosis was the number one cause of death by and infectious disease worldwide, until HIV/AIDS recently took over. While hundreds of people die from TB per day at a rate of approximately one person every 20 seconds, there are currently a limited number of methods to both diagnose and treat the disease.[3] Charles Dickens described TB in his book *Nicholas Nickleby*,[33] as:

> a disease which medicine never cured, wealth warded off, or poverty could boast exemption from – which sometimes moves in giant strides, and sometimes at a tardy sluggish pace, but, slow or quick, is ever sure and certain

With the synthesis of new potential antimicrobials, the Konaklieva lab has focused on the treatment of TB, and particularly drug-resistance in Mtb, a growing problem around the world. Drug resistant, multi-drug resistant (MDR), and even extensively/extremely-drug resistant (XDR) strands of Mtb have been found in almost all locations that exhibit clinical cases of the disease. MDR TB is defined as a strain of Mtb that is resistant to both rifampicin and isoniazid, while XDR TB has the potential to be resistant to three or more drugs.[3] A study released in 2002 stated that certain countries such as Estonia and Latvia were experiencing MDR TB rates of approximately 10% in new cases of TB,[3] a number that has risen to approximately 18% for both countries in 2012.[4] Due to the rise in observed cases, the issue of treating drug-resistance is becoming more and more eminent.

Drug-resistance in Mtb arises from cellular mechanisms in which the cell evolves to combat the normal effectiveness of a drug. One of the most prominent mechanisms of drug resistance in Mtb is the β-lactamase enzyme, which allows the microbe to be resistant to common β-lactam antibiotics.[5] Because of this, it is important to develop compounds that show antimicrobial properties while combatting resistance mechanisms such as the presence of the β-lactamase enzyme.

Drug design to fight TB is also important because there have been very few drugs released in the recent past. The last TB medication was put on the market 50 years ago,[2] and the current BCG vaccine was made available in 1921, and with almost the same original formula, is still the only vaccine available.[6] Due to the lack of both drugs and attention that TB receives from the international public health community, treating TB remains a daunting task. Drug-sensitive strains of Mtb require an intense 6 month regiment of chemotherapy, while drug-resistant forms of Mtb require up to 30 months of treatment.[2] The *TB Alliance* along with the WHO, envision a world where TB treatment regiments are only 5 to 7 days.[2] Unfortunately, this reality is far in the future, and would require the international health community to start developing and improving TB treatment. In relation to the TB vaccine, the current BCG vaccine is important to developing countries in reducing the number of cases of extra-pulmonary juvenile TB, but it is ineffective in adults, ineffective towards pulmonary TB, and not recommended for use in developed countries.[6]

The current problems with both the vaccine and chemotherapy regiments, highlight some of the divides that exist between TB in developed and developing countries. Many developed countries, such as the United States, can easily treat drug-sensitive Tuberculosis due to the wide availability of advanced diagnostic and treatment methods. However, drug-resistant TB is much

more prevalent in these countries, meaning that patients who acquire a TB infection in developed countries are usually put though one to two and a half years of chemotherapy. Because it is difficult to follow a strict drug regiment for such an extended period of time, TB re-occurrence and mortality is unnecessarily high in developed countries. The WHO reported that the treatment success rate for the USA in 2009 was down to only 60%, a number that is embarrassing for a country with plentiful medical resources.[4]

Contrary to developed countries, developing countries, especially those heavily burdened with TB, experience a prevalence of drug-sensitive TB. In order to address the TB problem, the WHO developed the directly observed treatment, short-course (DOTS) mostly through the TB work of Kyle Styblo in Tanzania in 1986.[8] In theory, DOTS is a very effective treatment program due to the fact that the core of the DOTS program is the administering of drugs directly to the patient every day of the chemotherapy regiment. DOTS was also designed with the sole intention of treating drug-sensitive TB, making it more effective in developing countries. In communities where MDR and XDR TB are not prevalent, DOTS has shown superior results.[3,7,9] However, the recent prevalence of MDR and XDR TB has caused the need for an improvement to the DOTS system. In 1998, Paul Farmer, in conjunction with the WHO and the STOP TB Partnership, developed the first "DOTS-Plus" campaign to treat MDR TB in developing countries.[9] Farmer sites several failures of DOTS, including a case in Peru where 258 patients failed to respond to DOTS, 55% of which were confirmed to have MDR TB.[9] Additionally, it was recently established that all countries surveyed for TB have reported at least one case of MDR TB.[10] DOTS-plus has been relatively successful, but its implementation has been limited due to the higher cost of drug-resistance pharmaceuticals.[9]

Despite this, drug-sensitive TB is still a much more sizable problem in developing countries because of their inability to provide sufficient diagnosis and treatment. Additionally, the population of developing countries are significantly more susceptible to experiencing complications such as HIV-TB, malnourishment, and malnutrition. These conditions can cause the body to be immunosuppressed which lead to a latent TB infection becoming and active TB infection. The higher prevalence of complications and the lack of access to health care, leads to a lethal combination that makes drug-sensitive TB mortality rates much higher in developing countries.

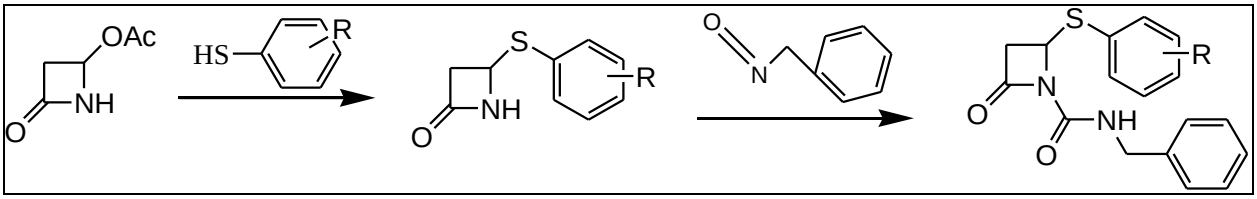
Dr. Paul Farmer wrote an editorial for *The New England Journal of Medicine*, in which he comments on the status of TB treatment, and international health care in general.[10] He claims the overlying issue of infectious disease treatment is cost, and it is responsible for a growing "outcome gap".[10] His essential argument is that because diagnosis and treatment are expensive, it is impossible to promise results in a country with a lower per capita GDP that are equivalent to the results in a developed country. Farmer ends the editorial with a call to health care officials and world leaders in saying: "excellence without equity looms as the chief human-rights dilemma of health care in the 21st century".[10]

Structural Study of Novel β-lactams Using Mass Spectrometry

Since the compounds produced by the Konaklieva lab showed antimicrobial properties towards β-lactamase producing strains of wild type Mtb, it was important to analyze their chemical reactivity, as well as develop a methodology for their target characterization. Mass Spectrometry (MS) is an important tool in both structural identification and compound characterization. MS methods were recently used by the Lee group in 2011 to perform a structural analysis of Penicillin G.[17] Other groups have done MS analyses with various β-

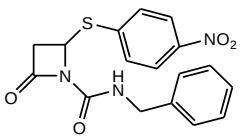
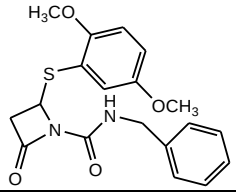
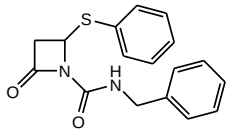
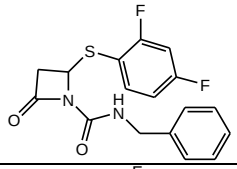
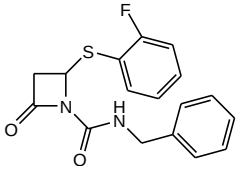
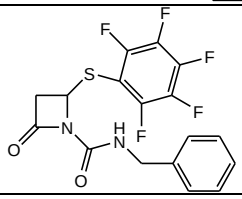
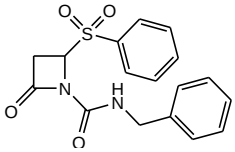
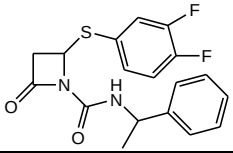
lactams and β-lactamse inhibitors, including tazobactam, cephalosporins, and clavulanic acid.[26-28] Additionally, there have been multiple groups working to establish methods of electrospray ionization (ESI) MS through the use of radical mechanisms and other common MS techniques.[11,12,15] Bruker also published the use of MS techniques (specifically Matrix Assisted Laser Desorption Ionization – Time of Flight MS) to determine β-lactam resistance in various microbes.[25]

General Synthesis of Novel β-lactam Compounds



Scheme 1. *The above scheme is the general synthesis for the novel β-lactams. The R-groups on the arylthio correspond with those in* **Figure 2**.

Compounds Observed using ESI-MS

Compound Number	Structure	Exact Weight (g/mol)	Compound Number	Structure	Exact Weight (g/mol)
1		357.08	**12**		372.11
2		312.09	**13**		348.07
3		330.08	**14**		402.05
4		344.08	**15**		362.09

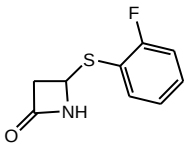
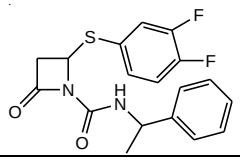
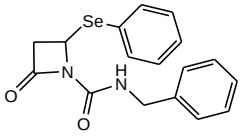
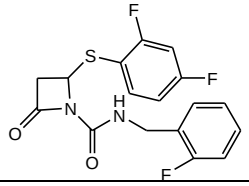
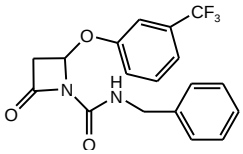
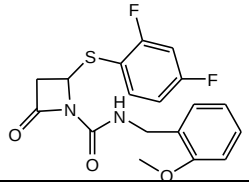
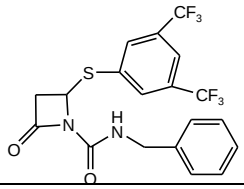
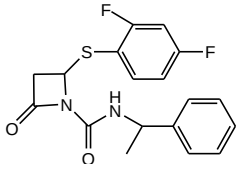
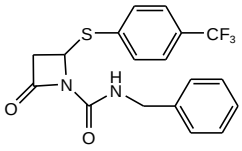
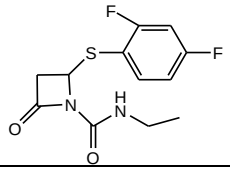
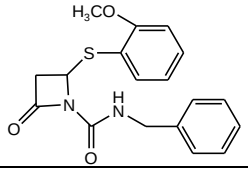
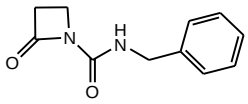
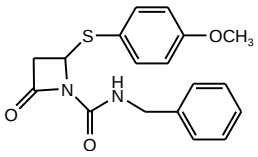
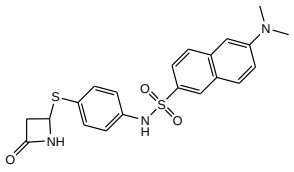
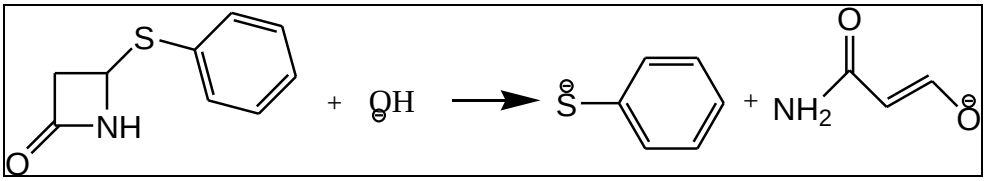
5		197.03	**16**		362.09
6		360.04	**17**		366.06
7		364.10	**18**		378.08
8		448.07	**19**		362.09
9		380.08	**20**		286.06
10		342.41	**21**		204.09
11		342.10	**22**		427.10

Table 1. *The above table shows the structures of the compounds observed using ESI-MS, as well as the exact mass of each compound.*

In this project we used ESI-MS, tandem MS, and ion trap MS to analyze the fragmentation patterns of a selection of the novel β-lactams (**Table 1**). It is important to note that the C4-arylthio contained various R groups which imparted differing levels of electron donating and withdrawing properties on the molecule (**Table 1**). Previous studies have shown that compounds with similar structures to these novel β-lactams are known to observe

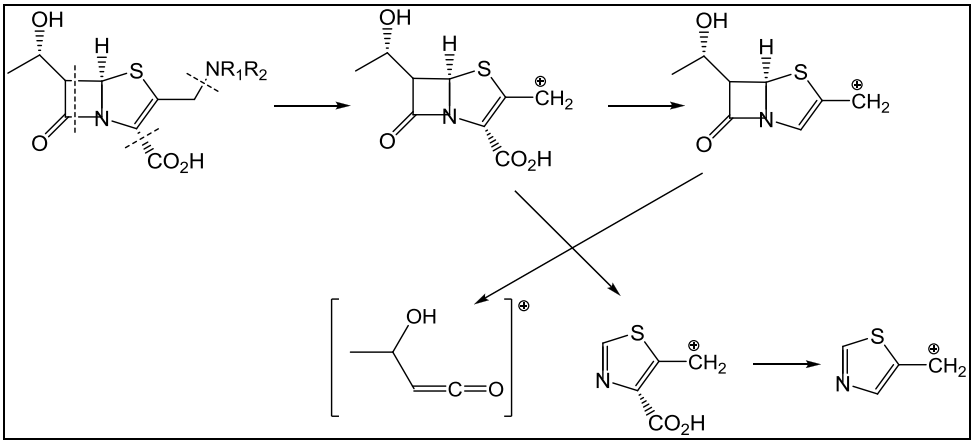
fragmentation within the β-lactam moiety with a break in both the N1-C2 bond and the C3-C4 bond under enzymatic conditions[13, 25, 32] (**Scheme 3**). The β-lactam ring has been shown to hydrolyze in a similar reaction when the compounds are put in strong basic conditions[13] (**Scheme 2**).

Reaction of β-lactam with –OH



Scheme 2. *The above scheme shows the reaction whereby the β-lactam ring is hydrolyzed by –OH and the arylthio is released.*

Previously Observed Fragmentation Mechanism of Penicillin



Scheme 3. *The above mechanism shows the various fragmentations observed by ESI-MS of penicillin. The dotted lines through the bonds in the first structure show the places of major fragmentation.*[13]

Because of this previously observed fragmentation, it was expected that a similar mechanism of fragmentation within the MS would be observed. Hard ionization techniques were initially used in this experiment and quickly dismissed due to the ease of fragmentation these β-lactams experience under harsh conditions. Soft ionization was therefore determined to be the best method for observing fragmentation patterns within the Mass Spectrometer. When the

compounds were observed with ESI-MS, the major fragmentation for most of the compounds created a strong peak at 177.0 m/z, which correlates directly with a split in the β-lactam moiety between the N1-C4 bond and the C2-C3 bond. This observed fragmentation pattern gave us interesting insights into the structure of our compounds and how they react in high energy conditions.

Enzymatic Study of Novel β-lactams with Penicillinase

Of the many mechanisms drug-resistant microbes use, one of the most common is the use of β-lactamase, an enzyme responsible for deactivating β-lactam compounds. The mechanism, as stated earlier, is effective against many common β-lactam drugs including penicillin and cephalosporin.[13, 25] The interactions that β-lactamases exhibit with various β-lactam antibiotics has been studied extensively through various enzyme studies.[19-24] Most of these studies focus on antibiotics with similar structures to penicillin, and these compounds are already known to interact with β-lactamases.[21, 23, 24] These studies also established the fact that the charged carboxylic acid group found on many common β-lactam antibiotics, including penicillin, is particularly important for its binding with β-lactamases.

Our novel β-lactams exhibit no charged moiety similar to that of penicillin, and it was therefore predicted that our compounds would not interact with beta-lactamase. This prediction was additionally based on previous MIC studies that showed good to excellent MIC values in relation to studies with β-lactamase producing Mtb, and multiple β-lactamase producing (n=8) *Moraxella catarrhalis*. In these studies, very little difference was observed in MIC values when clavulanic acid was introduced into the cells versus when it was not (**Appendix A**). Our compounds were observed interacting with penicillinase which is a class A non-metallic β-lactamase by the use of UV-Vis spectrometry, and no direct interaction was observed.

Materials and Methods

Equipment and Materials

All reactions were carried out at room temperature under normal atmospheric pressure. All common reagents and solvents were obtained from commercial suppliers and used without any further purification. Reactions were monitored by thin layer chromatography (TLC) using glass-backed analytical TLC plates coated with silica G with UV254 indicator (Sorbent Technologies Silica G TLC plates); the chromatograms were visualized under ultraviolet light and/or by staining with iodine silica chamber. Flash column chromatography was carried out on Combi flash chromatography silica gel (40 lm) or on AC- ROS aluminum oxide, activated, neutral (50–200 micron). Unless otherwise noted, the compounds were detected under UV light and iodine vapors.

Product purity was determined chromatographically and spectroscopically, unless otherwise noted. NMR spectra (25°C) were obtained at 400 MHz for proton NMR spectra and 125 MHz for carbon-13 NMR, and were recorded with the use of an internal deuterium lock at ambient temperature with a Bruker 400 spectrometer (Billerica, MA) in $CDCl_3$, unless otherwise noted. IR spectra were obtained as a thin film on NaCl plates and in solid form (KBr standard) on a Shimadzu FT-IR-8300 (Columbia, MD). Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Product names were electronically generated used ChemDraw version 12 software.

All data obtained from Mass Spectrometry studies were done in the 3200-Q-trap ESI-MS from Applied Biosystems. The formula for acidified methanol was acquired from the staff at Applied Biosystems. All UV-Vis spectrometry experiments were done with the Shimadzu-1800 UV-Vis. The penicillinase enzyme was purchased as a purified (98%) and dried sample from

Sigma-Aldrich. Additionally, all MIC tests were performed at NIH-NIAID in the TBRS research laboratory.

General Synthesis of β-lactams Containing Substituted Thiophenols

The synthetic procedure used, was adopted from Grimme et. Al.[30] and Wasserman et. al.[18] To a solution of 4-acetoxy-2-azetidinone (1 g, 8 mmol) in a 3:2 mixture acetone:water (50 mL) were added 1.05 mol eq. of the corresponding substituted thiophenol. 4 mol eq. of sodium bicarbonate were added and the mixture was stirred vigorously for 12h in a closed round bottom flask. Sodium chloride was added to the solution and the mixture was filtered out and extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate and concentrated under vacuum. The crude material was purified by flash chromatography or recrystallized from ethyl acetate/hexanes to give white crystals for all five products in quantitative yield.

General Synthesis of N-Carbamoylazetidin-2-one Derivatives

The synthetic procedure was adopted from Mulchande et al.[31] To a solution of the appropriate azetidin-2-one (1.7g, 5.4mmol) in dichloromethane (5 mL) was added to1.2mol eq. of triethylamine and 1.2 mol eq. of benzyl isocyanate. The reaction was stirred at room temperature and monitored by TLC. After completion of the reaction, the solution was evaporated under reduced pressure.

Mass Spectrometry Analysis of Compounds

ESI-MS, tandem MS, and ion-trap MS methods were used on all compounds tested. All samples were run at 10 μM using an $[M+H]^+$ method of analysis in three separate trials. The solvent system for all compounds was acidified methanol (4.0 mM ammonium formate, 27 mM formic acid). The solutions were optimized for declustering potential and entrance potential.

Analysis of compounds was done by calculating the relative intensities of the peaks as compared to the base peak of the spectra.

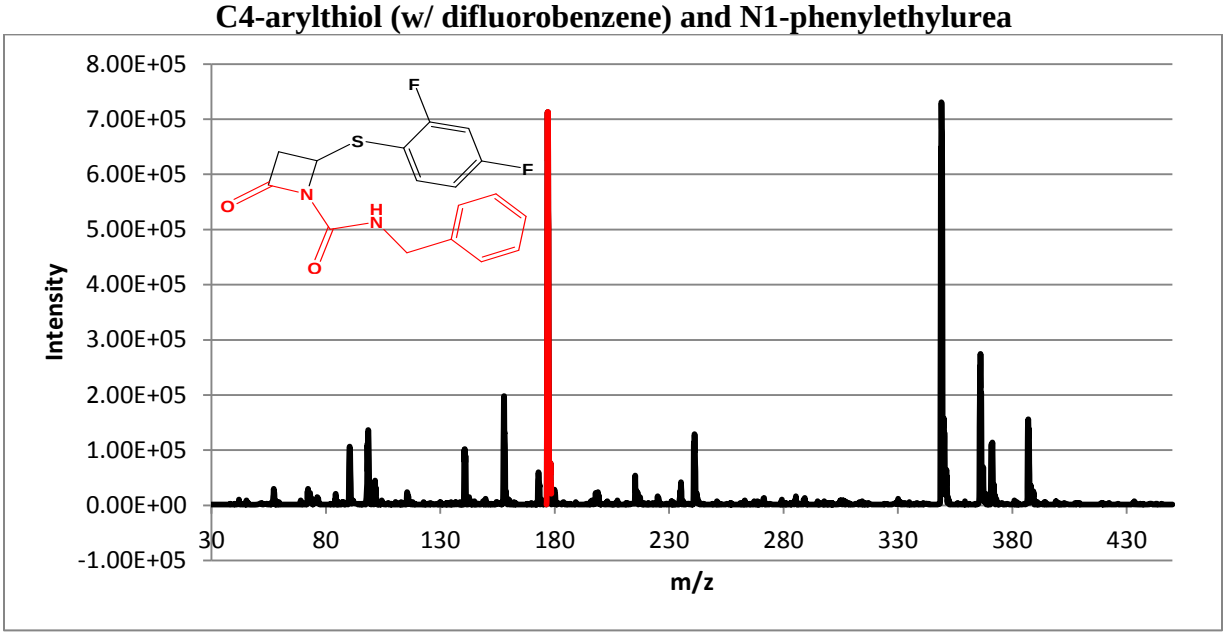
Observation of Difluoroarylthio (**13**) Interaction with Sodium Hydroxide

Compound **13** was diluted to 1.0 mM in DMSO. 3.0 mL of solution was placed in a quartz cuvette and a full range UV-Vis spectra was taken (800-200nm with 1.0 nm intervals). An initial peak was observed at λ=256nm. A 0.1 M solution of NaOH was then added dropwise. After each drop was added, the solution was mixed, allowed to incubate for 5 minutes, and then a full UV-Vis specta was observed. The products of the reaction with NaOH showed a rising peak at λ=345nm.

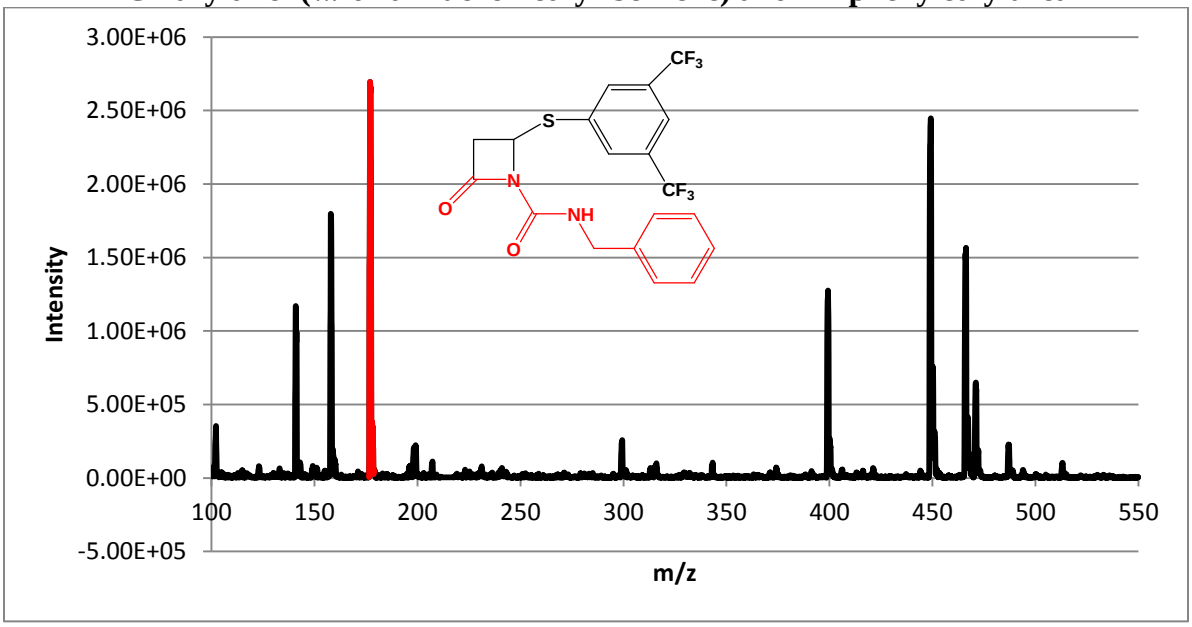
Reaction of Difluoroarylthio (**13**) with Penicillinase

Penicillinase was prepared at a 2.2μM concentration in 100 mM sodium phosphate buffer. A full range UV-Vis spectra was taken of 2.950 mL of the Penicillinase solution. Following this, 50 μL of the 1.0 mM DL02 in DMSO was added with full range spectra taken at 5 minute intervals.

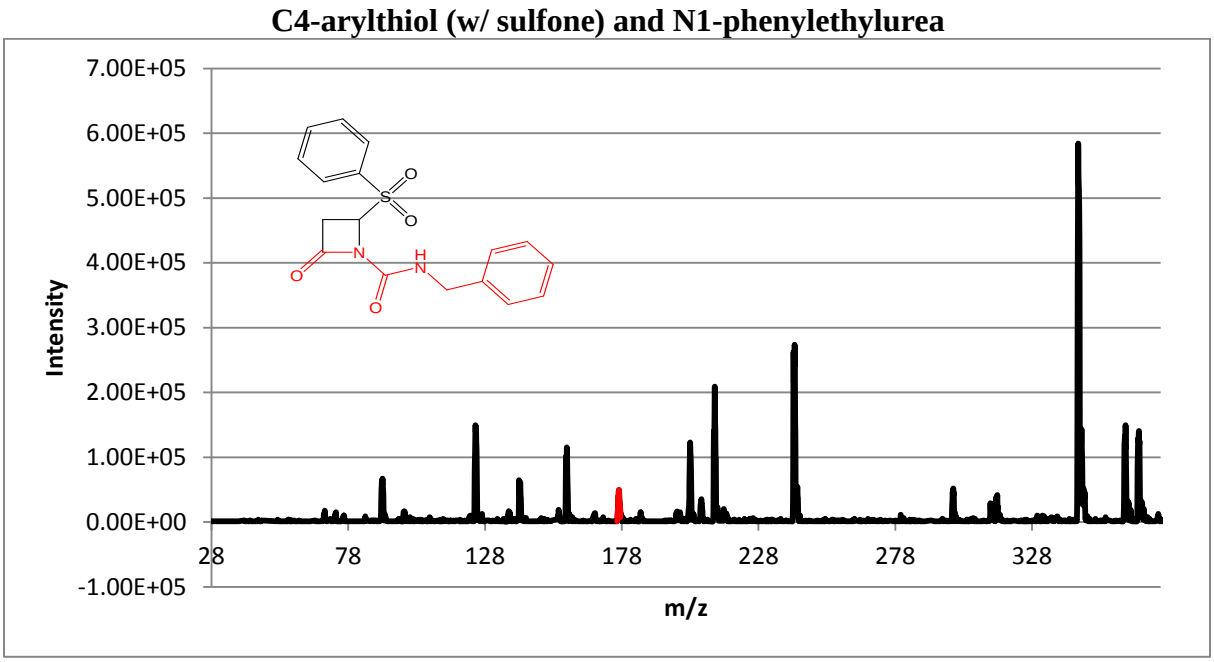
Data



Spectrum 1. *Above is the Spectra for the ESI-MS 2-(2,4-Difluoro-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide* (**13**). *The peak shown in red represents the fragmentation of the part of the compound drawn in red.*



Spectrum 2. *Above is the Spectra for the ESI-MS of the 2-(3,4-Bis-trifluoromethyl-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide compound* (**8**). *The peak shown in red represents the fragmentation of the part of the compound drawn in red.*



Spectrum 3. *Above is the Spectra for the ESI-MS of 2-Benzenesulfonyl-4-oxo-azetidine-1-carboxylic acid benzylamide* (**4**). *The peak shown in red represents the fragmentation of the part of the compound drawn in red.*

Table of Data From Fragmentation Experiments with Mass Spectrometer

Compound number	$[M+H]^+$	Important Fragmentation	$[M+H_2O]^+$	$[M+Na]^+$
1	358.0 (91)	177.0 (100) 182.2 (4)	375.0 (51)	380.2 (14)
2	313.0 (100)	177.1 (97) 137.0 (40)	330.2 (26)	335.4 (26)
3	331.1 (100)	177.1 (71) 154.8 (24)	348.2 (65)	352.9 (12)
4	345.5 (100)	177.1 (3) 168.2 (6) 212.2 (76)	362.3 (92)	367.2 (55)
5	197.8 (100)	154.8 (24) 128.5 (22)	-	-
6	361.0 (100)	177.0 (86) 185.1 (12)	377.9 (26)	383.3 (6)
7	365.1 (100)	177.0 (60) 189.1 (0)	382.0 (53)	387.1 (21)
8	449.2 (89)	177.0 (100) 273.2 (0)	466.3 (57)	471.1 (20)
9	381.3 (81)	177.1 (100) 205.3 (9)	398.4 (50)	403.4 (35)
10	343.0 (100)	177.1 (18) 166.9 (80)	360.0 (19)	365.2 (11)
11	343.3 (100)	177.1 (49) 166.9 (58)	360.2 (37)	365.0 (15)
12	373.3 (98)	177.0 (15) 197.1 (100)	390.4 (20)	395.1 (33)
13	348.9 (100)	177.0 (100) 172.9 (11)	366.3 (36)	370.9 (16)
14	403.1 (100)	177.1 (24) 227.1 (3)	420.2 (66)	425.2 (21)
15	363.2 (100)		380.2 (52)	385.3 (14)
16	363.2 (100)		380.2 (67)	385.0 (10)
17	367.2 (100)	195.1 (43) 173.2 (7)	383.9 (17)	389.0 (17)
18	379.2 (100)	207.2 (8)	395.9 (44)	401.0 (11)
19	363.0 (100)		380.2 (60)	-
20	287.0 (100)	114.6 (82) 172.9 (23)	304.0 (48)	309.1 (12)
21	205.1 (37)	148.2 (8) 77.6 (48)	222.1 (100)	226.9 (8)
22	428.1 (100)		-	450.1 (13)

Table 2. *Positive ESI-MS data for β-lactams* **1 - 22** *(in acidified Methanol). Numbers in the parenthesis show the peak ratios, 100 being the base peak in the spectrum. In "Major Splits" column, the two fragments are produced from breaking the N1 – C-4 bond on the lactam ring and the C-2 – C-3 bond on the ring.*

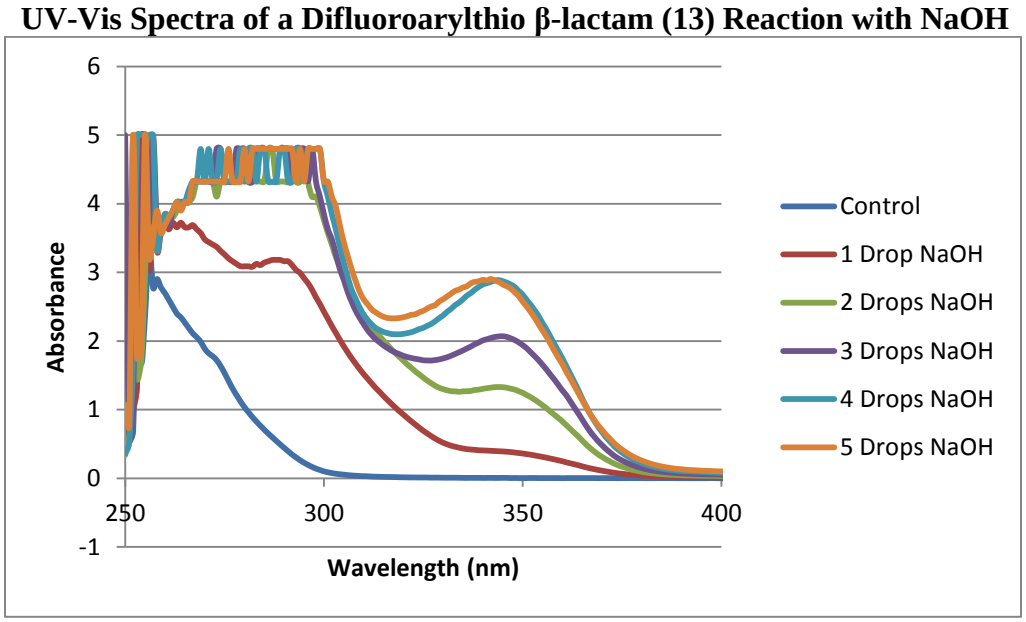


Figure 3: *Above is the UV-Vis spectra of 2-(2,4-Difluoro-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide* (**13**) *after being reacted with NaOH. The mixture was incubated for 5 minutes after each drop of NaOH was added and the rise in peak height was most prominent at λ=345nm.*

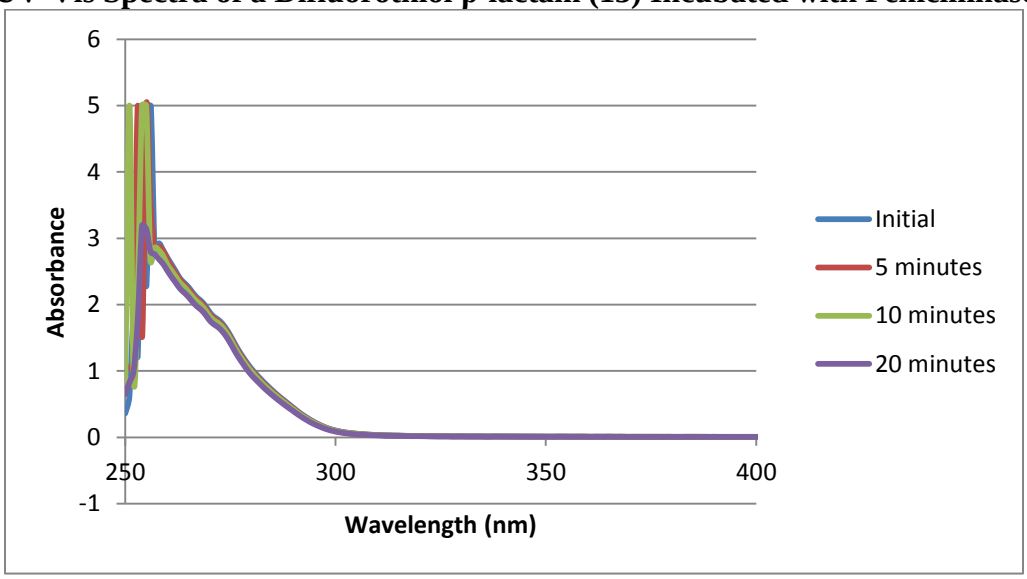


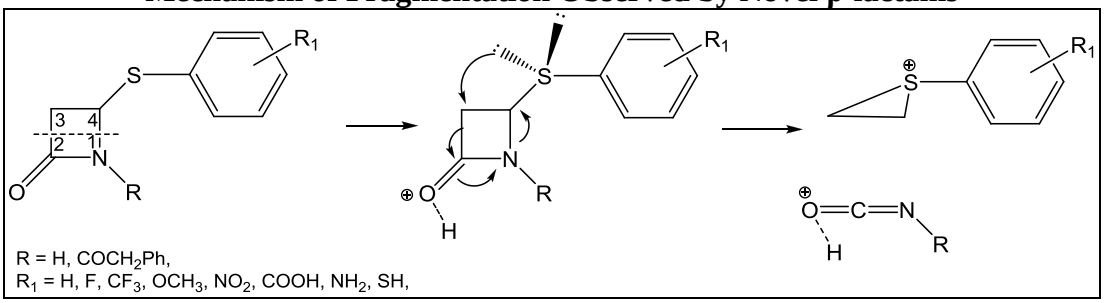
Figure 4: *Above is the UV-Vis spectra of 2-(2,4-Difluoro-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide* (**13**) *while interacting with penicillinase. The compound and enzyme were incubated for 20 minutes with spectra taken at 5 minute intervals.*

Results and Discussion

Analysis of Data from Mass Spectrometry

The most prominently observed fragmentation pattern in the positive ESI-MS data, was a break in the bonds of the β-lactam moiety between the N1-C4 bond and the C2-C3 bonds. With the addition of the carbamyl group to the N1 nitrogen that was derived from benzyl isocyanate, this fragmentation was observed with a peak at 177.0 m/z which corresponds to an exact mass of 176.0 Da. Additionally, a peak was observed that corresponded to the substituted arylthio attached to the C4 and C3 carbons of the β-lactam ring. Because of this, a mechanism was proposed that involved a nucleophilic attack of the electrons of the sulfur on the electrophilic C3 of the β-lactam ring (**Scheme 4**). This fragmentation pattern differs significantly from previously observed patterns of β-lactam structures such as penicillin as published by Cuirong et. al. (**Scheme 3**) which exhibit a [2+2] cycloreversion cleavage.[32]

Mechanism of Fragmentation Observed by Novel β-lactams



Scheme 4. *The above mechanism shows the proposed reason for the fragmentation patterns observed in the ESI-MS. The major site of fragmentation and β-lactam ring opening is between the N1-C4 and C2-C3 bonds in the β-lactam moiety.*

The proposed mechanism was further investigated by the synthesis of an arylthio sulfone which has a fully oxidized sulfur and no available electrons, and subsequently lacks the nucleophilic properties of the reduced sulfur. The resulting spectra showed a minimal fragmentation peak at 177.0 m/z, having only a 3% intensity in relation to the base peak (**Table 2**). The major fragmentation peak for the sulfone spectra was observed at 212.0 m/z, a peak

which corresponds with the mass of the full β-lactam ring attached to the sulfone arylthio substituent. This is an important result because it shows that when the sulfur is deactivated, the main fragmentation pattern ceases to exist, meaning that the sulfur plays an integral role in the N1-C4/C2-C3 fragmentation. Interestingly, the sulfone also raises the MIC of the compounds making them significantly less effective against Mtb (**Appendix A**).

Within the library of compounds, the most prominently observed difference between fragmentation patterns was based on whether the substituent on the arylthio was electron withdrawing or electron donating. The prominent electron withdrawing groups included the trifluoromethyls, the fluorines, and the nitrates. The molecules containing these groups on the arylthiol tended to exhibit a peak of higher intensity at 177.0 m/z, and in most cases, the 177.0 m/z peak was the base peak of the spectrum (**1**, **8**, **9**, **13)**. The trifluoromethyls compounds were considered to have the most electron withdrawing capability, and were carefully observed due to the fact that the $–CF_3$ unisubstituted arylthio also shows a peak at 177.0 m/z. However, since both the unisubstituted and disubstituted compounds exhibited a base peak at 177.0 m/z, their presence was considered significant.

The arylthio groups that contained the electron donating substituents, particularly the methoxy compounds, exhibited much smaller 177.0 m/z peaks ranging from 18% to 49% of the base peak (**10-12**). Additionally, these compounds all exhibited the molecular ion peak as either a very intense peak or as the base peak of the spectra. It is important to note that the compounds with electron donating substituents had considerably higher MIC values then those with electron withdrawing substituents. It was predicted that the electron withdrawing capabilities activate the compound in a variety of different settings from intracellular interactions to high energy fragmentation in the MS.

Other important observations from the ESI-MS data include the fact that the replacement of the sulfur on the arylthio with other heteroatoms such as oxygen (**7**) and selenium (**6**) still produced a peak at 177.0 m/z. However, in both cases the intensity of the peak decreased, and the molecular ion peak became the base peak of the spectra. Additionally, the compounds containing oxygen instead of sulfur had very high MIC values and were not good antimicrobials, while those containing selenium produced similar MIC values to those of the sulfur compounds (values not reported).

A variety of compounds were also tested that had different attachments to the N1 of the β-lactam moiety. The different carbamyl attachments exhibited a wide range of fragmentation patterns from no peak representing the normal fragmentation, to some with N1-C4/C2-C3 fragmentation at 82% of the base peak (**20**). Due to a small sample size within differing substituents at N1, it was difficult to draw any concrete conclusions from the data.

Analysis from UV-Vis study with Penicillinase

As can be seen by **Figure 3**, When our compounds, particularly the difluoroarylthio (**13**), were exposed to hydroxide as previously described,[13] the β-lactam ring hydrolyzed, and there was an emerging absorption at λ=345nm. Since this reaction is similar to the reaction of β-lactam with penicillinase, and no peak was observed in the UV-Vis spectra taken of the reaction with penicillinase at λ=345nm (**Figure 4**) it can be inferred that no interaction took place between the compound and the enzyme. Since these novel β-lactams contain no charged regions similar to the carboxylic acid of penicillin, it was expected that they would have no interaction with β-lactamases as was observed. Additionally, the MIC values of our compounds did not change with the introduction of clavulanic acid into the MIC experiments. This further shows that we received the expected result of β-lactamase not interacting with our compounds.

Conclusions and Future Experiments

An important step in the battle against antibiotic resistant microbes is finding compounds that are both effective antimicrobials, and have the ability to circumvent resistance mechanisms. The compounds in this study showed promise in both of these aspects due to the fact that they had good to excellent antimicrobial properties, and that they were not hydrolyzed by β-lactamase, the main cause of β-lactam antibiotic resistance in various microbes such as Mtb. It is important to continue with the synthesis of these compounds. In relation to MIC and ESI-MS data, it was established that there were four critical moieties on these compounds that enhance antimicrobial activity. Three of these are the β-lactam moiety, the sulfur between the C4 on the β-lactam ring and the aryl group, and the electron-withdrawing substituents on the arylthio group. The fourth is the carbamyl attachment to the N1 of the β-lactam ring, which appears to be necessary when no electron-withdrawing groups are present at the arylthio substituent at the C4. Without any of these substituents, there is a very apparent decrease in antimicrobial activity.

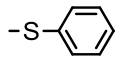
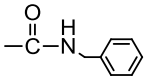
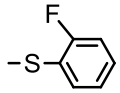
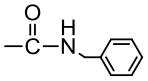
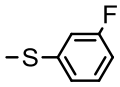
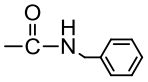
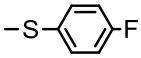
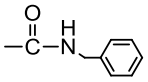
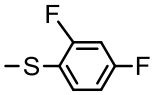
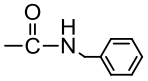
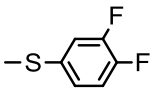
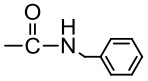
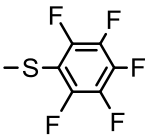
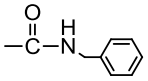
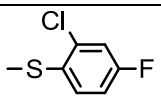
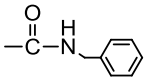
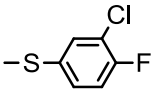
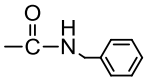
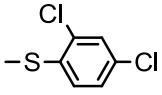
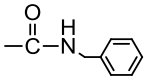
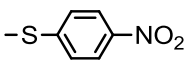
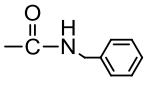
Future research will take two different paths. First, there will be a continued endeavor to synthesize compounds with lower MIC values through differing syntheses related to chirality and differing substitution. Second, there will be an effort to identify how these compounds are interacting within the cell. A method needs to be developed to identify what organelle or enzyme these compounds effect within their target microbes. With this research, a more directed synthesis can be established to focus on the most active moieties of both the compound and what the compound is targeting.

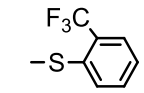
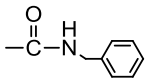
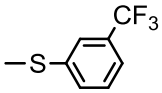
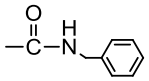
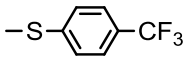
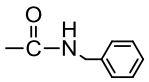
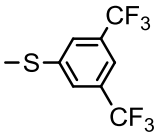
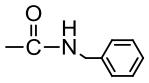
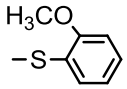
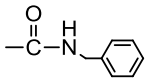
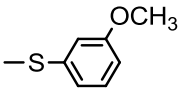
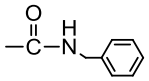
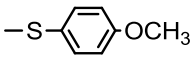
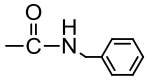
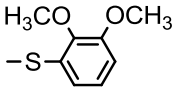
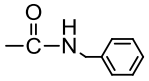
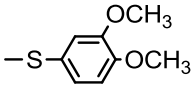
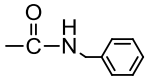
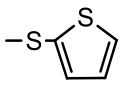
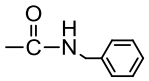
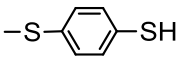
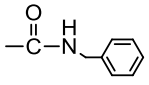
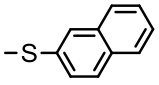
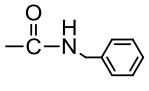
Acknowledgements

I first and foremost want to thank Dr. Monika Konaklieva who has been the best advisor, mentor, and teacher that I could have ever asked for, and who has opened up amazing opportunities for me in the future. This capstone research and my undergraduate career would not have been successful without her guiding help, kindness, and friendship. I would also like to thank all my labmates, particularly Dina Lloyd, Victor Schultz, Ross Kuskovsky, Tim Beck, Jeanette Minah, Juliana Fritz, and Susan Schultz who were responsible for the synthesis of most of the compounds used in this experiment. I additionally appreciate the support from my friends and family, particularly my mother and father who have supported me in every endeavor that I have chosen to undertake.

I want to thank the American University Honors Program for support throughout this process, both administratively and monetarily. Finally, a special thanks goes out to the AU Chemistry Department and their faculty and staff for their support of this project and my entire undergraduate career. I especially want to thank Dr. Douglas Fox, Dr. Abigail Miller, Dr. Matthew Hartings, and Arij Farran, who all played a role in ensuring my success throughout this capstone process.

Appendix A – MIC Values of Compounds

R1[b]	#	R2	M.cat.[a] MIC/MBC µg/ml	Mtb w/out and with/clav. µg/ml	#	R2	M.cat. MIC/MBC µg/ml	Mtb w/out and with/clav. µg/ml
	**2**	H	>200	25/25	**30**		12.5/12.5	6.25/6.25
	**3**	H	200	25	**31**		25/50	3.125/3.125
	**4**	H	>200	100/25	**32**		25/100	NT
	**5**	H	>200	>100/25	**33**		25/25	6.25/6.25
	**6**	H	1.5/3.1	3.125/3.125	**34**		1.625/1.625	100/12.5
	**7**	H	50/50	50/25	**35**		12.5/25	6.25/6.25
	**8**	H	1.5/1.5	>100	**36**		3.1/6.25	>100
	**9**		12.5/50	NT yet	**37**		12.5/25	NT yet
	**10**		12.5/25	Nt yet	**38**		6.25/6.25	Nt yet
	**11**		6.25/6.25	Nt yet	**39**		3.13/6.25	Nt yet
	**12**	H	6.25/12.5	25/25	**40**		3.125/25	25/25

	**13**	H	1.5/1.5	≥100	**41**		25/25	125
	**14**	H	200	6.25/12.5	**42**		3.125/6.25	25/6.25
	**15**	H	100/100	12.5/6.25	**43**		1.625/12.5	12.5/6.25
	**16**	H	12.5/100	>100	**44**		6.25/6.25	6.25/25
	**17**	H	100/>200	250/>250	**45**		200/200	125-250
	**18**	H	100/>200	>100	**46**		200/200	100
	**19**	H	200/>200	100	**47**		200/200	125
	**20**	H	100/>200	250	**48**		100/200	50
	**21**	H	200/200	250	**49**		200/>200	250
	**22**	H	25/100	6.25/12.5	**50**		50/100	6.25/6.25
	**23**	H	100/100	>100	**51**		NE	>100
	**24**	H	100/>200	>250	**52**		200/>200	>100

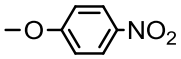
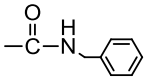
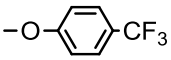
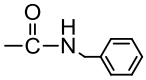
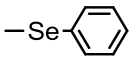
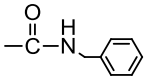
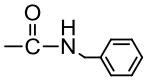
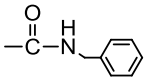
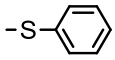
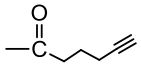
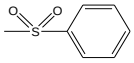
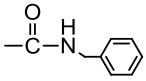
	**25**	H	50/50	25	**53**		6.25/6.25	NT- MTb is likely to respond to NO2, not tested
	**26**	H	200/200	250	**54**		50/>200	NT yet
	**27**	H	6.25/6.25	25	**55**		12.5/12.5	25
H	**28**	H	NT	NT	**56**		NT/ chemically unstable	>100
	29	H	>200	>200	**57**		NT/ chemically unstable	NT/ chemically unstable
	**2**	H	>200	25/25	**58**		100/100	45
		H	>250	>250			>250	>250

Table 1. Antimicrobial activity of C4-arylthio- and C4-aryloxy-, N1-carbamylated β-lactams against Mtb and *M.cat* (n=6).
[a]The values for M. cat are the results from the six isolates tested.
[b]All compounds are tested as racemates.

References

1. Brock, Thomas D., Katherine M. Brock, and David M. Ward. Basic Microbiology with Applications. 3rd ed. Englewood Cliffs, N.J.: *Prentice-Hall*, **1986**.

2. Cooper, C. "Experiences, successes, and challenges in developing mycobacterium tuberculosis whole cell screening hits as potential antituberculosis agents." American Chemical Society Conference in San Diego. March 25, **2012**. Room 6A – San Diego Convention Center.

3. Dye, C., B. G. Williams, M. A. Espinal, and M. C. Raviglione. "Erasing the World's Slow Stain: Strategies to Beat Muttidrug-Resistant Tuberculosis." *Science* 295, no. 5562 Mar 15 **2002**: 2042-46.

4. World Health Organization: Health Topics. "Tuberculosis." WHO. http://www.who.int/topics/tuberculosis/en/ (accessed April 19, 2012).

5. Kostova, Maya B., Carey J. Myers, Tim N. Beck, Balbina J. Plotkin, Jacalyn M. Green, Helena I. M. Boshoff, Clifton E. Barry Iii, Jeffrey R. Deschamps, and Monika I.

Konaklieva. "C4-Alkylthiols with Activity against Moraxella Catarrhalis and Mycobacterium Tuberculosis." *Bioorganic & Medicinal Chemistry* 19, no. 22. **2011**: 6842-52.

6. Kupferschmidt, K. "Taking a New Shot at a Tb Vaccine." *Science* 334, no. 6062. Dec 16 **2011**: 1488-90.

7. World Health Organization: Tuberculosis (TB). "Pursue High-Quality DOTS Expansion and Enhancement." http://www.who.int/tb/dots/en/ (accessed April 19, 2012).

8. Styblo, K. "That National Tuberculosis/Leprosy Program Tanzania." World Health Organization 88.153. Sep **1986**.

9. Farmer, P., and J. Y. Kim. "Community Based Approaches to the Control of Multidrug Resistant Tuberculosis: Introducing "Dots-Plus"." *British Medical Journal* 317, no. 7159. Sep 5 **1998**: 671-74.

10. Farmer, P. "Editorial: The Major Infectious Diseases in the World – To Treat or not to Treat?." *The New England Journal of Medicine* 345.3. Jul 19, **2001**: 208-10.

11. Osburn, S., G. Berden, J. Oomens, R. A. J. O'Hair, and V. Ryzhov. "Structure and Reactivity of the N-Acetyl-Cysteine Radical Cation and Anion: Does Radical Migration Occur?" *Journal of the American Society for Mass Spectrometry* 22, no. 10. Oct **2011**: 1794-803.

12. Kirk, B. B., D. G. Harman, and S. J. Blanksby. "Direct Observation of the Gas Phase Reaction of the Cyclohexyl Radical with Dioxygen Using a Distonic Radical Ion Approach." *Journal of Physical Chemistry* A 114, no. 3. Jan **2010**: 1446-56.

13. Gu, H. Z., and L. R. Fedor. "Base-Catalyzed Elimination of Para-Substituted Thiophenoxides from 4-(Arylthio)Azetidin-2-Ones." *Journal of Organic Chemistry* 55, no. 21. Oct **1990**: 5655-57.

14. Carr, J. A., T. F. Al-Azemi, T. E. Long, J. Y. Shim, C. M. Coates, E. Turos, and K. S. Bisht. "Lipase-Catalyzed Resolution of 4-Aryl-Substituted Beta-Lactams: Effect of Substitution on the 4-Aryl Ring." *Tetrahedron* 59, no. 46. Nov **2003**: 9147-60.

15. Brandwilliams, W., M. E. Cuvelier, and C. Berset. "Use of a Free-Radical Method to Evaluate Antioxidant Activity." *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie* 28, no. 1. **1995**: 25-30.

16. Adediran, S. A., D. Cabaret, R. R. Flavell, J. A. Sammons, M. Wakselman, and R. F. Pratt. "Synthesis and Beta-Lactamase Reactivity of Alpha-Substituted Phenaceturates." *Bioorganic & Medicinal Chemistry* 14, no. 20. Oct **2006**: 7023-33.

17. Ho, H. P., R. J. Lee, C. Y. Chen, S. R. Wang, Z. G. Li, and M. R. Lee. "Identification of New Minor Metabolites of Penicillin G in Human Serum by Multiple-Stage Tandem Mass Spectrometry." *Rapid Communications in Mass Spectrometry* 25, no. 1. Jan **2011**: 25-32.

18. Wasserman, H. H., M. D. Xia, A. J. Carr, W. T. Han, and M. G. Siegel. "Generation of Penems, Carbapenems and Aza Analogs of Cephems by the Addition of Heterocycles and Other Building Blocks to Azetinones." *Tetrahedron* 56, no. 31. Jul 28 **2000**: 5621-29.

19. Bulychev, A., M. E. Obrien, I. Massova, M. Teng, T. A. Gibson, M. J. Miller, and S. Mobashery. "Potent Mechanism-Based Inhibition of the Tem-1 Beta-Lactamase by Novel N-Sulfonyloxy Beta-Lactams." *Journal of the American Chemical Society* 117, no. 22. Jun 7 **1995**: 5938-43.

20. Achilles, K., T. Schirmeister, and H. H. Otto. "Beta-Lactam Derivatives as Enzyme Inhibitors: 1-Peptidyl Derivatives of 4-Phenylazetidin-2-One as Inhibitors of Elastase and Papain." *Archiv Der Pharmazie* 333, no. 8. Aug **2000**: 243-53.

21. Buynak, J. D., H. S. Chen, L. Vogeti, V. R. Gadhachanda, C. A. Buchanan, T. Palzkill, R. W. Shaw, J. Spencer, and T. R. Walsh. "Penicillin-Derived Inhibitors That Simultaneously Target Both Metallo- and Serine-Beta-Lactamases." *Bioorganic & Medicinal Chemistry Letters* 14, no. 5. Mar 8 **2004**: 1299-304.

22. Badarau, A., A. Llinas, A. P. Laws, C. Damblon, and M. I. Page. "Inhibitors of Metallo-Beta-Lactamase Generated from Beta-Lactam Antibiotics." *Biochemistry* 44, no. 24. Jun 21 **2005**: 8578-89.

23. Bush, K., C. Macalintal, B. A. Rasmussen, V. J. Lee, and Y. J. Yang. "Kinetic Interactions of Tazobactam with Beta-Lactamases from All Major Structural Classes." *Antimicrobial Agents and Chemotherapy* 37, no. 4. Apr **1993**: 851-58.

24. Beharry, Z., H. S. Chen, V. R. Gadhachanda, J. D. Buynak, and T. Palzkill. "Evaluation of Penicillin-Based Inhibitors of the Class a and B Beta-Lactamases from Bacillus Anthracis." *Biochemical and Biophysical Research Communications* 313, no. 3. Jan 16 **2004**: 541-45.

25. Sparbier, K., S. Schubert, U. Weller, C. Boogen, and M. Kostrzewa. "Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-Based Functional Assay for Rapid Detection of Resistance against Beta-Lactam Antibiotics." *Journal of Clinical Microbiology* 50, no. 3. Mar **2012**: 927-37.

26. Yang, Y., K. Janota, K. Tabei, N. Huang, M. M. Siegel, Y. I. Lin, B. A. Rasmussen, and D. M. Shlaes. "Mechanism of Inhibition of the Class a Beta-Lactamases Pc1 and Tem-1 by Tazobactam - Observation of Reaction Products by Electrospray Ionization Mass Spectrometry." *Journal of Biological Chemistry* 275, no. 35 Sep 1 **2000**: 26674-82.

27. Partani, P., S. Gurule, A. Khuroo, T. Monif, and S. Bhardwaj. "Liquid Chromatography/Electrospray Tandem Mass Spectrometry Method for the Determination of Cefuroxime in Human Plasma: Application to a Pharmacokinetic Study." *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 878, no. 3-4. Feb 1 **2010**: 428-34.

28. Reyns, T., S. De Boever, S. De Baere, P. De Backer, and S. Croubels. "Quantitative Analysis of Clavulanic Acid in Porcine Tissues by Liquid Chromatography Combined with Electrospray Ionization Tandem Mass Spectrometry." *Analytica Chimica Acta* 597, no. 2. Aug 10 **2007**: 282-89.

29. Janin, Y. L. "Antituberculosis Drugs: Ten Years of Research." *Bioorganic & Medicinal Chemistry* 15, no. 7. Apr 1 **2007**: 2479-513.

30. Clauss, K. G., D.; Prossel, G., *Justus Liebigs Annalen der Chemie* **1974**, (4).

31. Mulchande, J., L. Martins, R. Moreira, M. Archer, T. F. Oliveira, and J. Iley. "The Efficiency of C-4 Substituents in Activating the Beta-Lactam Scaffold Towards Serine Proteases and Hydroxide Ion." *Organic & Biomolecular Chemistry* 5, no. 16. **2007**: 2617-26.

32. Sun, C. R., J. M. Wu, and Y. J. Pan. "Characterization of Novel Hydrolysis Products of Carbapenems by Electrospray Ionization Mass Spectrometry." *Rapid Communications in Mass Spectrometry* 23, no. 19. Oct **2009**: 3205-12.

33. Dickens, Charles, and Michael Slater. Nicholas Nickleby. Penguin English Library. Harmondsworth ; New York: Penguin, 1978.