MULTI-FUNCTION ANTIBIOTICS: β-LACTAMS AS NEUROPROTECTIVE AND ANTIMICROBIAL AGENTS

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ABSTRACT

Glutamate is the principal excitatory neurotransmitter in the nervous system. Although crucial for normal neuronal excitability, high doses of glutamate in the synapse, often induced by stress, can lead to nervous cell damage and cell death – glutamate excitotoxicity. Neuronal damage due to excess of glutamate is associated with diseases such as Parkinson's, Alzheimer's, ALS, and stroke. A library of β-lactams was created using a combination of novel research and previous literature of natural antibiotic compounds as potential neuroprotective agents. These β-lactams differ from the ones reported in the literature by being monocyclic, which allows for more synthetic flexibility, and in general being more lipophilic, which allows for crossing the blood-brain barrier. These β-lactams have also been tested for antimicrobial effectiveness, particularly as anti-tuberculosis and anti-*Moraxella* agents.

1. INTRODUCTION

The discovery and production of antibiotics is considered to be one of the greatest advancements of modern medicine. Early antibiotics were compounds extracted from microorganisms that demonstrated a protective potential against invasion by other microorganisms. Thanks to Alexander Fleming in 1929, one of the earliest antibiotics, penicillin, was extracted from the mold *Penicillium notatum* (Shetty, Tang, & Andrews, 2009; Xing, Rao, & Rongrong, 2008). In particular, the β-lactam class of antibiotics has included some of the most clinically important antibacterial agents. With the synthetic development of new broad-spectrum cephalosporin β-lactams, both gram-negative and gram-positive bacterial infections have been successfully treated (Bush & Mobashery, 1998).

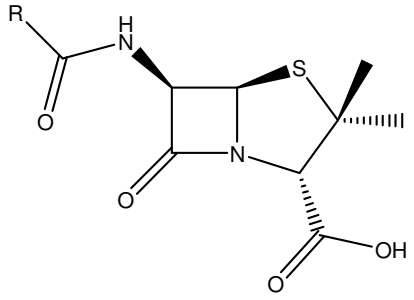


Figure 1. Penicillin core structure.

β-lactams antibiotics fight bacterial growth by inhibition of penicillin-binding proteins (PBPs), proteins necessary for the cross-linking process of bacterial cell wall synthesis. PBPs mistake β-lactam antibiotics for the C-terminal D-Ala-D-Ala end of newly formed peptidoglycan (a bacterial cell wall component). The active site serine on the electrophilic β-lactam ring initiates a nucleophilic attack on the PBP enzymes, forming an inert acyl-enzyme intermediate (Buynak, 2007). With the enzymes unable to function, cell wall synthesis is halted, leading to cell death via hydrolysis of existing peptidoglycan (Staub & Sieber, 2008).

With the widespread use of these “wonder drugs”, pathogens require a more sophisticated method to evade eradication. Some bacterial pathogens have developed enzymes, β-lactamases, which are effective at destabilizing the β-lactam ring, destroying the drug before it can operate on the bacterium (Bush & Sykes, 1986). Two pathogens in particular, *Moraxella catarrhalis* and *Mycobacterium tuberculosis*, have uniquely similar methods of rendering the β-lactam class of antibiotics largely ineffective.

M. catarrhalis, responsible for upper respiratory tract infections in children and the elderly, has an almost universal β-lactamase mediated resistance to penicillins, but remains sensitive to most antibiotics (Murphy, 1996). Despite its current sensitivity, it must be taken into account that prior to 1970, no *M. catarrhalis* isolate had been observed to produce β-lactamase. However, in 1980, 75% of United States isolates showed β-lactamase production, and in 1990, 80-90% of all strains (US and UK), demonstrated this adaptation (Wallace, Nash, & Steingrube, 1990). Currently, there is a trend toward reduced susceptibility to four β-lactam antibiotics: penicillin G, ceftriaxone, amoxicillin-clavulanic acid, and imipenem, indicative of genetic selection for β-lactamase strains, and in particular, more efficient β-lactamases (Livermore, 1995). These developments pose not only a concern for fighting *M. catarrhalis*, but is also a significant issue when treating other, more serious concomitant infections; for β-lactamase from *M. catarrhalis* protects not only its producer bacteria but also inactivates the therapy in place for other more serious concomitant airway infections, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Tan, Morgelin, Forsgren, & Riesbeck, 2007; Verduin, Hol, Fleer, van Dijk, & van Belkum, 2002).

M. tuberculosis has an intrinsic resistance to β-lactams that stems from its genetically expressed extended spectrum class A β-lactamase, BlaC. This enzyme catalyzes the hydrolysis of the β-lactam ring, releasing the nucleophilic "trap" of the ring before it can attack PBPs on the bacterial cell wall. Because of this pre-made resistance, β-lactams have been in the past avoided when treating tuberculosis infections; however, with the emerging development of multidrug-resistant and extremely drug-resistant strains of TB, there is a pressing need to discover novel treatments and drugs to counter the infection (Tremblay, Hugonnet, & Blanchard, 2008).

Though developed by nature for that purpose, β-lactams can be used in other ways than as antibiotics. Recent studies have demonstrated that β-lactams are also capable of increasing the expression and activity of the glutamate transporter subtype 1 (GLT-1), which is responsible for 90% of glutamate uptake in the central nervous system (CNS) (Rawls, Tallarida, Robinson, & Amin, 2007). Glutamate is the principal excitatory neurotransmitter in the brain, and is known to play a key role in memory consolidation and retrieval (long term potentiation) (Fedulov et. al 2007; Wang et al., 2006). Normally, glutamate is released by glutaminergic neurons into the synapse, binding to its ionotropic and metabotropic receptors, and resulting in the formation of strengthened signaling patterns – long term potentiation – that is vital for memory formation and learning. Reuptake of excess glutamate into the glia, or accessory neural cells, occurs via the glial glutamate transporter (GLT). The recovered glutamate is converted to glutamine and returned to the pre-synaptic neuron for reconversion into glutamate, restarting the process (Beart & O'Shea, 2007). In conditions of neuronal stress, glutamate mediated calcium influx (from excitation of the receptor neurons) triggers oxidative stress and apoptosis leading to neuronal degeneration, a symptom of many chronic illnesses such as Alzheimer's Disease (Hota, Barhwal, Ray, Singh, & Ilavazhagan, 2008; Scott, Gebhardt, Mitrovic, Vandenberg, & Dodd, 2010).

Ceftriaxone is a third-generation cephalosporin β-lactam antibiotic used for the clearance of various bacterial infections, including bacterial meningitis (Tunkel, 2006). Like other β-lactam antibiotics, its mechanism of action in antibacterial processes is in destruction of cell wall synthesis (Shetty, Tang, & Andrews, 2009).However, as proven in recent studies, when delivered to animals, ceftriaxone increases both brain expression of the glutamate transporter protein (GLT1) and its biochemical and functional activity. Furthermore, it is neuroprotective *in vitro* when used to prevent motor neuron degradation and ischemic injury, both which are conditions that can be partly attributed to glutamate toxicity. In murine models, ceftriaxone was able to delay neuronal and muscle loss due to ALS, increasing mouse survival (Rothstein, et al., 2005). When testing for neuroprotection against HIV-associated dementia, ceftriaxone was also protective against Tat- and gp120-induced cell death – both proteins which are known to down-regulate the GLT1 transporter, causing glutamate toxicity in HIVD (Rumbaugh, et al., 2007).

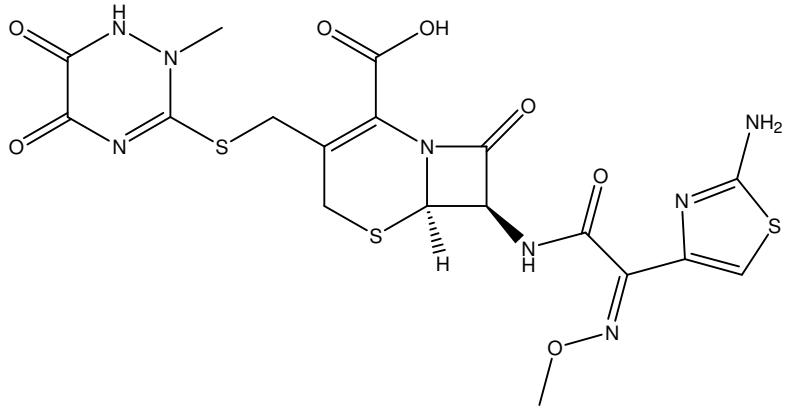


Figure 2. Ceftriaxone

Notably ceftriaxone and related β-lactam antibiotics have no substantial toxic CNS actions at normal antibacterial dosages; however there are negative aspects to using ceftriaxone for neuroprotection (Rothstein, et al., 2005). Primarily, ceftriaxone is only available in intravenous and intramuscular forms. Also, continuous treatment with ceftriaxone, a potent

antibiotic, may lead to unintended antimicrobial resistance. Overall, the neuroprotective property of these drugs appears to be a result of activation of the genetic promoter for GLT1 (Rothstein, et al., 2005). What is more uncertain is the neuromolecular pathway for this activation and the specific properties of the β-lactam chemical structure that allow for its effectiveness.

This investigation combines the traditional use of β-lactams as antibiotics with its novel application as neuroprotective agents to present a library of compounds that may be effective in one or both areas of application. The new compounds are *N*-alkylthio β-lactams (monobactams with sulfur side chains), which demonstrate unique structure activities that could provide significant antimicrobial and neuroprotective benefits.

Studies for the neuroprotective properties of β-lactams have only examined ceftriaxone and other related bicyclic compounds (Rothstein, et al., 2005). This new study presents a new compound which may have a stronger neuroprotective effect than ceftriaxone, while limiting the possibility of further antibiotic resistance. This new compound is more lipophilic, making for a more efficient uptake across the blood-brain barrier *in vivo*. It is also intended for the new compounds to be administered as an oral agent, making it more accessible than the previously utilized ceftriaxone.

2. RESULTS AND DISCUSSION

The synthesis of these novel β-lactams was intended be performed in several phases over the course of multiple generations of testing. In the initial phase, five compounds were synthesized based upon the efficacy of *N*-thiolated β-lactams as antimicrobials (Turos, et al., 2008). Fluorothiophenol groups appear to possess a greater ability against multiple types of β-lactamase than hydroxyl groups, making these compounds of interest to exploit (Buynak, et al., 2004). In addition, the sulfone group is an extremely powerful activator of the β-lactam ring toward the Ser group in PBPs (Mulchande, Martins, Moreira, Archer, Oliveira, & Hey, 2007). These minimalistic β-lactams may also aid in isolating the mechanism of action for β-lactams as neuroprotective agents, although that was not the focus of this investigation.

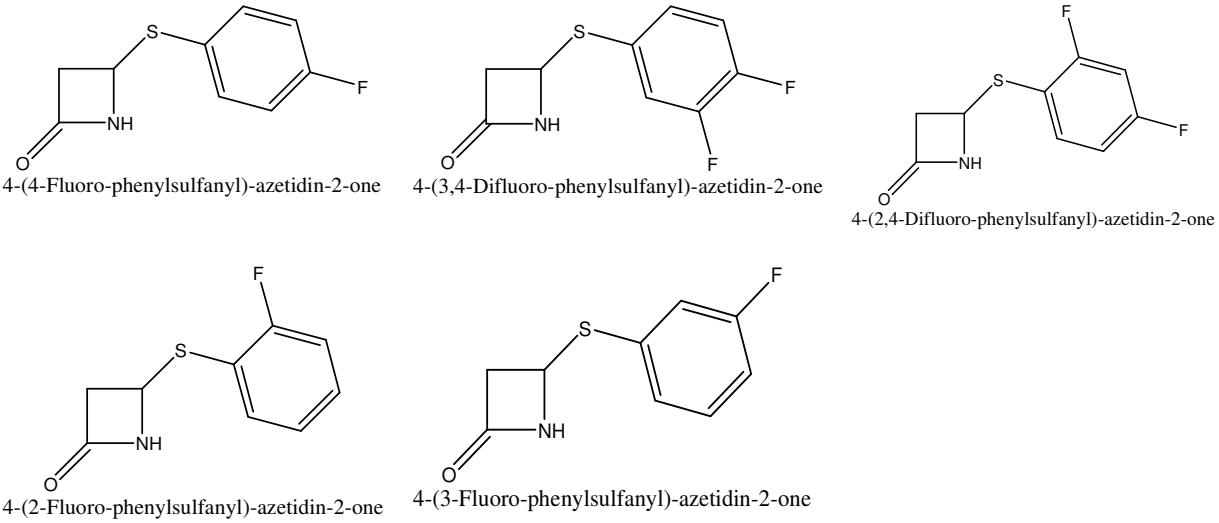
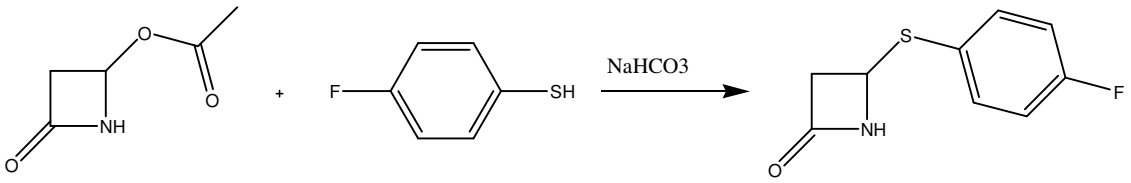


Figure 3. 1st phase compounds.

All initial phase compounds were created following the methodology utilized by Gu and Fedor and developed by Clauss et. al (Gu & Fedor, 1990; Clauss, Grimm, & Prossel, 1974). A fluorothiophenol was combined with 4-acetoxy-2-azetidinone, a C4 substituted monobactam, to produce the 1st phase compound.



Scheme 1. General synthesis of 1st phase compounds.

The moderately activating acetoxy group of 4-acetoxy-2-azetidinone is replaced in a substitution reaction by a much stronger activating fluorothiophenol. These initial phase β-lactams were designed to maximize the electrophilic nature of the β-lactam ring, the center of attack against PBPs, by modification of the C4 position. The C4 position was chosen for modification for its more effective electronics and lesser steric effects (than C3 derivatives) ((Rao & O'Ferrall, 1989). When comparing leaving groups for the C4 position, it was previously demonstrated that fluoro derivatives offer a safer alternative *in vitro* than other more electron-withdrawing groups (e.g. nitro groups) (Murugesan, Palaniappan, Perumal, Arnab, Valakunja, & Sriram, 2008). The single fluoro addition to the thiophenol group was compared to difluorophenol to determine whether the quantity of fluorines added to the phenol group affected the electronics and overall effectiveness of the β-lactam. Due to external circumstances, only one of the compounds was able to be tested for neuroprotection; however, two 1st phase compounds were analyzed for efficacy against TB and all were examined for *Moraxella* .

Biological testing for neuroprotection was performed using primary neuronal cell cultures prepared from cerebellar tissues removed from prenatal day 15 Sprague–Dawley rat fetuses. Cells were dissociated by mechanical titration in neuronal culture medium (NCM). Collected cells were centrifuged at 1000 rpm for 5 min. at 4°C. Supernatant was carefully removed and the cellular pellet was re-suspended in 10 ml NCM. Cell counts were made by combining an aliquot of the re-suspended cells with an equal volume of trypan blue and counting the number of

unstained cells on a hemacytometer. Cells were diluted to a final concentration of 1 x 106 cells/ml in serum-containing medium and plated at density of 500,000/well onto 48-well plates pre-treated with poly-L-lysine. Following plating, cultures were maintained in an incubator (5% CO2, 37°C) until they were used in experiments at 7-10 days in vitro. The compound was dissolved in DMSO to a final concentration of 20mM. 1mM concentrations were prepared by mixing 250µl of 20mM stock with 750µl Locke's. 500µM concentrations were prepared by mixing 500µl from 1mM solution with 500µl Locke's. 250µM concentrations were prepared by mixing 500µl from 500mM solution with 500µl Locke's (Herbert, Richards, Girard, & Konaklieva, 2009).

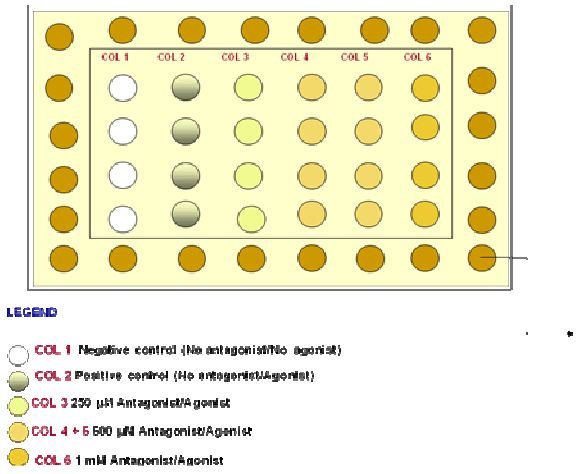


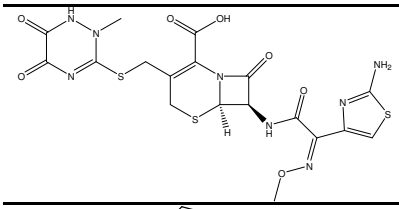
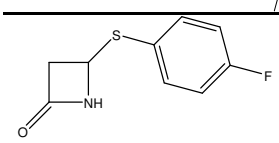
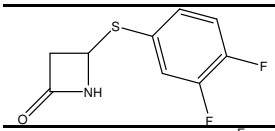
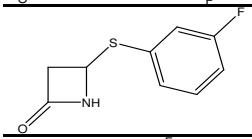
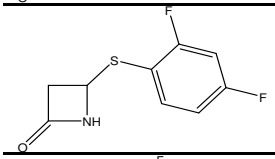
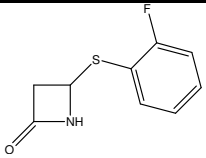
Figure 4. Plate layout for neuroprotective assay.

Neurons were exposed to the potential antagonist/neuroprotectant for 5 minutes followed by glutamate (agonist) for 30 minutes. Wells were then emptied followed by the addition of 450 µl MEM/well. Plates were then returned back to the incubator and stored overnight. Toxicity and protection were assessed by using the MTT assay the next morning after which viability relative to appropriate controls is determined. The antagonist was considered neuroprotective if cell

viability is statistically greater than the agonist treatment alone (Herbert, Richards, Girard, & Konaklieva, 2009).

For antibiotic biological testing, samples were sent to external laboratories for testing. Compounds tested for efficacy against *M. tuberculosis* were also measured for any improvement in effect when clavulanic acid, a known inhibitor of *M. tuberculosis*'s β-lactamase, was added to the plate. Compounds tested for efficacy against *M. catarrhalis* were measured against multiple strains of the bacterium, with varying results for each strain.

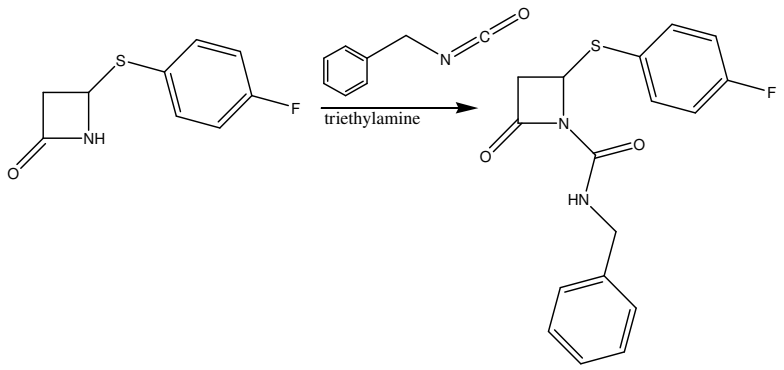
Table 1. Summary of 1st phase neuroprotection and antimicrobial biological testing.

Compound	**Neuroprotection**	***M. tuberculosis***	***M. tuberculosis* + clavulanic acid**	***M. catarrhalis***
	55% cell survival at 250 μM 50% cell survival at 500 μM 50% cell survival at 1000 μM			
	85% cell survival at 250 μM 95% cell survival at 500 μM 97% cell survival at 1000 μM	MIC >100 μg/mL	MIC >100 μg/mL	No
		MIC at 100 μg/mL	MIC at 100 μg/mL	MIC at 200 μg/mL
				MIC at 200 μg/mL
				Not effective
				MIC at 100, 200 μg/mL, NE (strains vary)

These compounds, while showing some antimicrobial activity with both *M. tuberculosis* and *M. catarrhalis*, failed to provide the significant antimicrobial effectiveness necessary for an effective drug treatment. The minimum inhibitory concentration (MIC) of all compounds was too high to be utilized *in vivo*, requiring a large dose of the drug to be effective at clearing bacterial growth. Surprisingly, 4-(4-Fluoro-phenylsulfanyl)-azetidin-2-one, the only compound tested for neuroprotection, was shown to be significantly more effective at neuroprotection than ceftriaxone, the previously published neuroprotective β-lactam. At medium to high concentrations of antibiotic, cell survival was nearly 100%.

From an antimicrobial perspective: although the mechanism for action for these compounds is not precisely known, it can be surmised by these results that these compounds could not effectively inhibit bacterial growth. This can be either due to (1) weakness in lipophilicity, (2) unsuccessful mimicking of the β-lactamase or PBP substrate, (3) slowness of action compared to bacterial β-lactamase, or (4) weakness of the ring to β-lactamase or cellular environmental conditions. As there was some antimicrobial effect, the first three options may be the more likely.

The second phase of modification involved addition of an *N*-linked tail to the initial phase compound. Methodology for synthesis was adapted from Mulchande et. al (Mulchande, Martins, Moreira, Archer, Oliveira, & Hey, 2007). Benzyl isocyanate was added to the 1st phase compound in a triethylamine / methylene chloride solution and stirred for 18h before purification and collection.

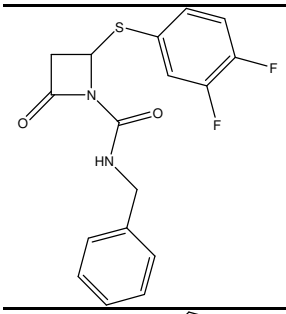
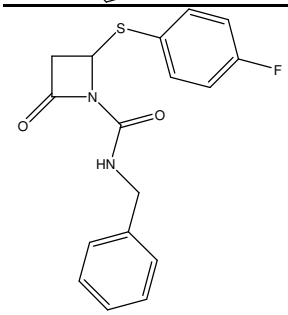


Scheme 2. General Synthesis for 2nd phase compounds.

In this nucleophilic acyl addition, the nitride from the β-lactam ring itself attacks the highly electron deficient carbonyl carbon. The resultant products are the 2nd phase benzylamides.

Similar type compounds have previously been determined to be effective against β-lactamase-like enzymes (Mulchande, Martins, Moreira, Archer, Oliveira, & Hey, 2007). Two of these compounds were tested for antimicrobial effectiveness, with improved results from the initial phase compounds.

Table 2. Summary of 2nd phase antimicrobial biological testing.

Compound	***M. tuberculosis***	***M. tuberculosis* + clavulanic acid**	***M. catarrhalis***
	MIC at 50 µg/mL	MIC at 50 µg/mL	MIC at 25-100 µg/mL, depending on strain
	MIC at 50 µg/mL	MIC at 25-50 µg/mL	MIC at 25-50 µg/mL, depending on strain

The 2nd phase compounds tested showed bacterial clearance at low MICs, indicative of an effective treatment. MICs varied by strain, but were overall far more effective than their phase 1 counterparts. Neuroprotection was not examined for these compounds. The evidence of success in these compounds indicates that lipophilicity may play a role in the effectiveness of these compounds, or that the new tail group provides a conformation that is closer to the β-lactamase/PBP substrate in the bacteria tested.

Future Direction

The future direction for this investigation is to continue neuroprotection tests on all synthesized compounds, from all phases. The 2nd phase should also be rounded out with tail additions of each of the 1st phase compounds. A 3rd phase has been postulated, and would involve oxidation of the sulfur located on the C4 group of the β-lactam ring. This is thought to provide additional activation of the β-lactam ring, making for a more powerful antibiotic. The 2nd phase and 3rd phase would be altered interchangeably, with production of the oxidized sulfur prior to addition of the *N* tail and vice versa, determining the more effective of the two potential antibiotics.

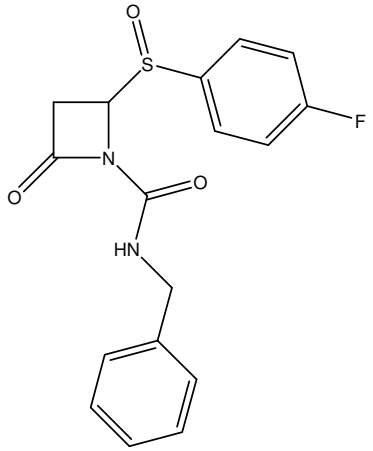


Figure 5. Potential 3rd Phase Compound, 2-(4-Fluoro-benzenesulfinyl)-4-oxo-azetidine-1-carboxylic acid benzylamide.

3. CONCLUSIONS

Seven new compounds were synthesized and tested for neuroprotective and/or antimicrobial activity. One compound was proven to be effective in neuroprotective testing alone, while others, untested for neuroprotection varied in success against *M. catarrhalis*. All 1st phase compounds synthesized and tested for TB activity were largely ineffective against *M. tuberculosis*; however, given that 2nd phase compounds tested better across the board for both TB and *M. catarrhalis,* the likely cause of 1st phase's impotency is lipophilicity of the compound.

Based on the results, further synthesis will be made of monocyclic *N*-thiolated β-lactams, varying the electronics of the β-lactam ring via the thio group and comparing C4 versus *N*-linked substitutions. The structure-activity relationship of these new compounds will then be analyzed for improved efficacy both as antimicrobials and neuroprotectives.

4. EXPERIMENTAL

4.1. Equipment and Materials

All moisture-sensitive reactions were conducted under argon. All solvents – acetone, hexanes, triethylamine, methanol (CH_3OH), dicloromethane (CH_2Cl_2), and ethyl acetate – were purchased from Fisher (HPLC grade) or Spectrum (HPLC grade) and used without distillation. EM reagent plates with a fluorescence indicator (SiO_2-60, F-254) were used for thin-layer chromatography (TLC). All other reagents were obtained from Sigma-Aldrich and Matrix Scientific. Solid-state column chromatography was performed using JT Baker Flash Chromatography Silica Gel (40 μm). ^1H spectra were obtained using a Bruker 400MHz instrument at 400 MHz with $CDCl_3$ as solvent. Chemical shifts are referenced to that of TMS (0 ppm) as per usual for measurements in organic solvents.

4.2. 4-(4-Fluoro-phenylsulfanyl)-azetidin-2-one, 4-(3-Fluoro-phenylsulfanyl)-azetidin-2-one, and 4-(2-Fluoro-phenylsulfanyl)-azetidin-2-one

4-acetoxy-2-azetidinone (2.000 g, 15.49 mmol) was added to a solution of 4-fluorothiophenol, 3-fluorothiophenol, or 2-fluorothiophenol (2.045g, 15.49 mmol), sodium bicarbonate (5.200 g, 61.90 mmol) acetone (50 mL), and distilled water (30 mL). The resulting mixture was sealed and stirred at room temperature. After 18 h, a spoonful of NaCl was added to the reaction mixture and the resultant solid layer removed by filtration. The aqueous layer was thrice extracted with ethyl acetate (50 mL), isolating the compound of interest in the organic layer. This layer was subsequently dried over $MgSO_4$, and concentrated in vacuo to give the β-lactam (2.34g, 77%) as a white crystalline solid. ^1H NMR ($CDCl_3$) 400MHz: δ 3.4 (d, 2H), 4.5 (t, 1H), 6.9 (d, 1H, J=8), 7.2 (d, 1H, J=8).

4.3 4-(3,4-Difluoro-phenylsulfanyl)-azetidin-2-one and 4-(2,4-Difluoro-phenylsulfanyl)-azetidin-2-one

4-acetoxy-2-azetidinone (1.000 g, 7.745 mmol) was added to a solution of 3,4-difluorothiophenol or 2,4-difluorothiophenol (1.166g, 7.745 mmol), sodium bicarbonate (2.6g, 30.95 mmol) acetone (50 mL), and distilled water (30 mL). The resulting mixture was sealed and stirred at room temperature. After 18 h, a spoonful of NaCl was added to the reaction mixture and the resultant solid layer removed by filtration. The aqueous layer was thrice extracted with ethyl acetate (50 mL), isolating the compound of interest in the organic layer. This layer was subsequently dried over $MgSO_4$, and concentrated in vacuo to give the β-lactam (1.22g, 73%) as a white crystalline solid. 4-(3,4-Difluoro-phenylsulfanyl)-azetidin-2-one: ^1H NMR ($CDCl_3$) 400MHz: δ 3.4 (d, 2H), 4.5 (t, 1H), 6.9 (d, 1H, J=7.3), 6.9 (d, 1H). 4-(2,4-Difluoro-phenylsulfanyl)-azetidin-2-one: ^1H NMR ($CDCl_3$) 400MHz: δ 3.4 (d, 2H), 4.5 (t, 1H), 6.5 (s, 1H), 6.6 (d, 1H), 7.1 (d, 1H).

4.4 2-(4-Fluoro-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide

4-(4-Fluoro-phenylsulfanyl)-azetidin-2-one (1.000 g, 5.070 mmol) was added to a solution of benzyl isocyanate (0.8101 g, 6.084 mmol), triethylamine (0.6157 g, 6.084 mmol), and CH_2Cl_2 (40 mL). The resulting mixture was sealed and stirred at room temperature. After 18 h, the mixture was concentrated in vacuo, and required chromatography (silica with hexanes:CH_2Cl_2, CH_2Cl_2:ethyl acetate) to yield the benzylamide as a white crystalline solid. ^1H NMR ($CDCl_3$) 400MHz: δ 3.4 (d, 2H), 4.4 (s, 2H), 5.1(t, 1H), 6 (s, 1H), 6.9 (d, 1H, J=8), 7.1 (d, 1, J=3.2), 7.2 (d, 1H, J=8).

4.5 2-(3,4-difluoro-phenylsulfanyl)-4-oxo-azetidine-1-carboxylic acid benzylamide

4-(3,4-Difluoro-phenylsulfanyl)-azetidin-2-one (1.000 g, 4.646 mmol) was added to a solution of benzyl isocyanate (0.742 g, 5.576 mmol), triethylamine (0.564 g, 6.084 mmol), and CH_2Cl_2 (40 mL). The resulting mixture was sealed and stirred at room temperature. After 18 h, the mixture was concentrated in vacuo, and required chromatography (silica with hexanes:CH_2Cl_2, CH_2Cl_2:ethyl acetate) to yield the benzylamide as a white crystalline solid. ^1H NMR ($CDCl_3$) 400MHz: δ 3.4 (d, 2H), 4.4 (s, 2H), 5.1(t, 1H), 6 (s, 1H), 6.9 (d, 1H, J=4.2), 7.1 (d, 1, J=3.2).

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