N-Thiolated β-Lactams: Chemistry, SAR and Intracellular Target of a Novel Class of Antimicrobial and Anticancer Agents

by

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ABBREVIATIONS

α = alpha
ATP = adenosine triphosphate
ATPase = adenosine triphosphate synthase
β = beta
BisBAL = bismuth-2,3-dimercaptopropanol
BisEDT = bismuth-1,2-ethanedithiol
BisPYR = bismuth-pyrithione
BisTOL = bismuth-3,4-dimercaptotoluene
BisβME = bismuth-2-mercaptoethanol
Bn = benzyl
BTs = bismuth thiol
°C = degrees Celsius
¹³C = carbon 13
CAN = ceric ammonium nitrate
CMV = cytomegalovirus
CoA = coenzyme A
CoADR = coenzyme A disulfide reductase
Cys = cysteine
δ = delta or chemical shift
DIAD = diisopropyl azodicarboxylate
DIBAL = diisobutylaluminum hydride
DID = 5,5′-diphenyl-3,3′-diisothiazole disulfide
dNTP = deoxyribonucleoside triphosphate
Dsb = disulfide bridge forming enzyme
EC = effective concentration
Et₃N = triethylamine
ETP = epipoly(thiodioxopiperazine)
FRET = Forster Resonance Energy Transfer
¹H = proton
¹H NMR = proton nuclear magnetic resonance
HIV = human immunodeficiency virus
HIV-RT = human immunodeficiency virus reverse transcription
HPLC = high pressure liquid chromatography
Hz = hertz
IR = infrared
J = coupling constant
JUNV = Junin (agent of Argentine hemorrhagic fever)
LAH = lithium aluminum hydride
mCPBA = meta-chloroperoxybenzoic acid
MDM = monocyte-derived macrophages
MEA = 2-mercaptoethylamine (cysteamine)
MHz = megahertz
MIC = minimum inhibitory concentration
µg = micrograms
µM = micromolar
mM = millimolar
MRSA = methicillin-resistant Staphylococcus aureus
MRSE = methicillin-resistant Staphylococcus epidermidis
NAC = N-acetyl-L-cysteine
ng = nanogram
Ph = phenyl
ppm = parts per million
S₈ = elemental sulfur
TCBZ = triclabendazole
TCRV = Tacaribe
TLC = thin layer chromatography
trx = thioredoxin
VRE = Vancomycin Resistant enterococci
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N-Thiolated β-Lactams: Chemistry, SAR and Intracellular Target of a Novel Class of Antimicrobial and Anticancer Agents

Bart Allan Heldreth

ABSTRACT

N-Thiolated β-lactams (1) represent a promising new group of compounds with potent inhibition effects on bacteria, like Bacillus anthracis and methicillin resistant Staphylococcus aureus, and onco-systems, like breast cancer and leukemia. Originally developed as part of a synthetic pathway to bicyclic lactams, N-thiolated β-lactams have been shown in this laboratory to possess intriguing biological activities. The antibacterial activities of this new class of agents rely on novel structural features unlike those of any existing family of β-lactam drugs. The lactams seem to exert their effects intracellularly, requiring passage of the bioactive species through the cellular membrane, rather than acting extracellularly on cell wall components in the manner of penicillin and related antibiotics. The lipophilic nature of these molecules, which lack the polar side chain functionality of all other microbially-active β-lactams, suggests the compounds do not target the penicillin binding proteins within bacterial membranes but instead pass through these membranes. The biological target of these compounds has been investigated. The most active members of this β-lactam class appear to be those bearing a small branched alkyl chain on the sulfur atom. The effects of stereochemistry, branching and chain length of the sulfur group on bioactivities were studied. This dissertation is divided into six chapters. A review of organosulfur anti-infectives is discussed in Chapter 1. The types of existing antibiotics and their modes of action will be discussed in Chapter 2. The
synthesis of these novel agents is discussed in Chapter 3. A structure-activity relationship of these lactam analogues is discussed in Chapter 4. And Chapters 5 and 6 demonstrate a novel mode of action and biological target for these drugs using techniques which include target identification, metabolic effects, and reactivity kinetics.

![Chemical Structure](image)

(1)
CHAPTER ONE
INVESTIGATIONS IN ORGANOSULFUR ANTI-INFECTIVES

An all too often overlooked group of anti-infective compounds are those whose biological activity is based on sulfur functionalities. As a prelude to my research on \( N \)-thiolated \( \beta \)-lactams, this chapter provides a review of organosulfur anti-infectives.

1.1.1 General Introduction
As far back as 1000 B.C. elemental sulfur (\( S_8 \)) (1) was used, at the very least, as a pesticide. In 1824 \( S_8 \) was shown to treat peach mildew\[1\] and over the centuries, \( S_8 \) has successfully been applied as a fungicide to protect a wide variety of plants against fungal infestation. The current rationale for these antifungal properties is that \( S_8 \) is absorbed by the spores of the fungi, and converted within the fungi to toxic hydrogen sulfide, a compound which could have deleterious effects on mitochondrial respiration.\[1\] Although the mode of \( S_8 \)’s antifungal action is not fully defined, there is no doubt of its dependency on the reaction of sulfur with a biological target.

![Figure (1.1). Elemental Sulfur \( S_8 \)](image)

The use of organosulfur compounds to control the onset or progression of infectious diseases in humans and animals also has its roots dating back to early times, when ancient Egyptians recognized the potent medicinal effects of naturally occurring

\[1\]
organosulfur substances from leeks. In this report we summarize the types of molecules and reaction mechanisms associated with the anti-infective properties of various organosulfur substances.

1.1.2 Classes of Biologically-Active Organosulfur Compounds

Organic compounds that contain sulfur cover an extraordinary range of chemical structures and reactivities. Many of these have biological activity. In the simplest case, the presence of one or more sulfur atoms in a biologically active molecule may not actually give the compound its biological effects, but rather may act as a non-participant in a side chain residue or an innocuous constituency of the molecular framework.

A few prime examples are the bicyclic beta-lactams, including penicillins (2), cephalosporins (3), and penems (4), and the sulfa drugs (5), whose anti-infective activity is not related to sulfur-centered events (Figure 1.2).

![Bicyclic beta-lactams and sulfa drugs](image)

Figure (1.2). Bicyclic β-Lactams and Sulfa Drugs.

Alternatively, the presence of a sulfur atom in a bioactive molecule may exert a more definitive, yet subtle, effect on its biological activity, such as the case of the
thiosugars (6a-c) and thionucleosides (7). These sulfur analogues of the natural sugars and nucleosides act as inhibitors of glycosidases and reverse transcriptases, respectively.\textsuperscript{[2-8]} It has been documented that these properties are due to the conformational and stereoelectronic changes brought about by the replacement of oxygen with sulfur in the heterocyclic ring. However, even in these molecules, the sulfur atom does not, per se, play a central role in the reaction of the molecule with a biological entity.

\[ \text{S} \quad \text{OH} \quad \text{HO} \quad \text{HO} \quad \text{N} \quad \text{NH} \quad \text{R} \quad \text{O} \quad \text{O} \]

\[ \text{S} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \]

\[ \text{D-5-Thioglucose} \quad \text{L-5-Thiofucose} \quad \text{D-5-Thiomannose} \]

(6a) (6b) (6c)

7

**Figure (1.3). Thiosugars and Thionucleosides.**

In many other cases, however, the bioactivity of an organosulfur compound may be *directly* attributable to the reactivity of the sulfur center, which is certainly the case for the sulfur mustards (8). Here, the sulfur atom is responsible for activating the compound toward nucleophilic attack by displacing a chlorine atom. Once activated, the sulfur atom is then a potent electrophile for attack by a biological nucleophile (Figure 1.4).
Figure (1.4). Sulfur Mustard.

In many instances, it is the sulfur center itself which is the site of chemical attachment of the biomolecules to its target. What follows in this review is a discussion of the antibacterial, antifungal, antiviral, and antiparasitic activities of organosulfur compounds whose mode of action depends on the reactivity of one or more sulfur atoms in that molecule with a biological target.

1.1.3 Relationship Between Reactivity of Organosulfur Compounds and Microbiological Activity.

Biologically active organosulfur compounds can interfere with the processes associated with human infections and disease through a number of common pathways, depending on the type of sulfur functionality that is present and the nature of its reactivity. For instance, thiols are relatively reactive groups which can act as either powerful nucleophiles or reducing agents, depending on the nature of the electrophile (E$^+$), the thiol substituent (R), and the local environment in which the reaction occurs (equations 1 and 2).
Disulfides, on the other hand, behave as electrophilic reactants in the presence of various thiophilic nucleophiles, resulting in the heterolytic cleavage of the sulfur-sulfur bond (equation 3). Intracellular nucleophiles such as glutathione (9) and thioredoxins (trx’s), for example, can react rapidly with di- and trisulfide compounds to disrupt cellular stasis (Figure 1.6).\(^{[9,10]}\)

Alternatively, disulfides can act as electrophilic oxidants (equation 4), which can lead to the generation of superoxide and hydrogen peroxide within the cell via redox pathways, thereby affecting oxidative conditions in and around the cell (Figure 1.8). These two pathways, represented by equations 3 and 4, are generally competing within the cell, and may both lead to cell death.
A number of other sulfur functionalities act in similar fashion to either thiols or disulfides and thus serve as masked versions of these reactive groups. Bismuth thiols (Figure 1.21), for instance, have greater potency, increased selectivity, reduced toxicity, and better stability than the free thiol, while trisulfides, such as that found in calichemicin (10), and certain sulfenamides, behave mechanistically similarly to disulfides. Some trisulfide compounds may also result in the generation of a destructive radical species, such as the case of calichemicin (10) and esperamicins (11).[11-16]

![Figure (1.7). Calichemicin, an Enediyne Trisulfide.](image)

\[
\text{R-S-Nu} + \overset{\ominus}{\text{SR}} \quad (3)
\]

\[
\text{R-S-S-R} + \overset{\ominus}{\text{Nu}} \quad \overset{\ominus}{\text{R-S-Nu}} + \overset{\ominus}{\text{SR}} \quad (4)
\]

![Figure (1.8). Reactions of Disulfide Compounds with Nucleophiles.](image)
Figure (1.9). Namenamicin, a Novel Enediyne.

The focus of this review centers on the structures and modes of action of those agents whose anti-infective properties have been proven, or postulated, to be dependent on the sulfur atom as the primary site of biochemical reactivity. This list includes those already mentioned, thiols, disulfides, trisulfides, as well as related moieties such as sulfenamides, thiosulfinates and thiosulfonates. Compounds that contain groups such as sulfenic acids, sulfoxides and more highly oxidized compounds like sulfinic acids, sulfones, sulfonic acids, sulfamates and sulfate esters are not discussed here since their activities are rarely directly dependent on the sulfur atoms. Those compounds that are only dependent on sulfur for such properties as architecture, polarity, stability or solubility are also not included here.

1.2.1 The Key Biological Targets of Organosulfur Drugs

Over the course of time, living cells have developed defenses against the harmful effect of biological oxidants, or disinfectants. The thiol-disulfide redox equilibrium in cells is central to this natural defense mechanism, and thus serves as a potentially valuable target of sulfur-based antibiotics. Nature protects microbes from oxidative stress
by maintaining high thiol:disulfide ratios, typically 19:1 or higher. Disruption of this redox system can alter many vital cellular activities such as regulation of protein activity, regeneration of enzymatic cofactors and reductases, like ribonucleotide reductase, and a host of other processes where an antioxidant is required.$^{[17]}$

### 1.2.2 Glutathione-based Systems

One of the most important thiol/disulfide dyads found in many cell types is glutathione (9) (γ-L-glutamyl-L-cysteinylglycine), a tripeptide assembled exclusively in the cell. Glutathione is produced biologically via the glutaredoxin enzymatic pathway in two steps: 1) formation of the glutamyl-cysteine adduct and 2) subsequent attachment addition of glycine to the c-terminus. The equilibrium between free thiol and disulfide, at stasis, is typically maintained at a cytoplasmic thiol concentration of around 90%. Although intracellular glutathione concentrations are notoriously difficult to measure, and can vary throughout the life cycle of a cell, cell lines with high intracellular glutathione levels are generally less susceptible to damage by organosulfur drugs.$^{[18]}$ Serving a primary role as an antioxidant, glutathione is extremely effective in scavenging reactive free radicals, electrophiles and other destructive oxidants in the cytoplasm. Glutathione can react directly with various drugs to deactivate them before they are able to inflict irreversible damage, while also serving to reactivate enzymes that have been inhibited as mixed disulfides formed between a drug and enzyme. In response to this protective mechanism afforded by glutathione, nature has cleverly designed prodrug molecules such as the enediyne trisulfides and anthracyclines which can interact directly with glutathione as a way to be biochemically activated within the target cell. Inhibition of glutathione’s
antioxidant abilities can be induced by formation of glutathione-S-S-drug mixed disulfides. The rate of thiol cleavage of the sulfur-sulfur bond of the glutathione disulfide is directly proportional to twice the protic acidity of the free thiol.\cite{19}

1.2.3 Coenzyme A-based Systems

In the past it was commonplace to assume the presence of glutathione in all organisms. Glutathione, however, is not entirely ubiquitous. Some bacteria are totally devoid of glutathione, but in its place have some other thiol/disulfide-based redox system. \textit{S. aureus}, for example, does not generate or utilize glutathione at all, but rather produces millimolar levels of the nucleosidic entity, coenzyme A (12) (CoA).\cite{20}

At stasis, the bacterium maintains a ratio of thiol:disulfide of about 95:5, via coenzyme A disulfide reductase (CoADR) (Figure 1.10). This is extraordinarily specific in its ability to reduce CoA disulfide. Mixed disulfides formed between CoA and glutathione, or other thiophilic agents, are typically unable to be reduced by CoADR.\cite{20,21} Therefore, anti-infective compounds that can form CoADR resistant mixed disulfides with CoA can offer an effective mode of inhibition against \textit{S. aureus}. Indeed, glutathione is an inhibitor of the redox cycle of CoA and is detrimental to the growth of this bacterium.
Figure (1.10). Coenzyme A Redox System.

1.2.4 Thioredoxin-based Systems

Another very important group of native cellular thiols / disulfides are the thioredoxins (trx’s) and a related subfamily, the disulfide bridge forming enzymes (Dsb’s), which span through the bacterial membrane connecting the cytoplasm with the periplasm. The characteristic Cys-X-X-Cys motif in the structures of the trx’s is highly conserved in many bacteria. These dithiols undergo reversible oxidation and can quickly react with non-native thiols or disulfides. Before a drug even passes through the membrane of a bacterium however, a subfamily of thioredoxins, the Dsb’s, could potentially intervene. Formation of mixed disulfides between the trx’s and the organothio compound can thereby inhibit enzymatic reduction and thus can shut down cellular function. Figure 11 illustrates the cellular function of trx’s and Dsb’s.

Dsb’s, for the most part, inhabit the cytoplasmic membrane with exposed (Cys-X-X-X-Cys) functionalities on both the cytoplasmic and periplasmic surfaces. Dsb’s are involved in electron transport across the membrane and serve as a signaling mechanism,
communicating the oxidation state of the cytoplasmic trx’s with the oxidation state on the periplasmic side. Disulfide formation involving trx motifs on either side of the membrane can cause disruption of the Dsb’s electron transport abilities, affecting a host of cellular processes such as respiration and cytochrome syntheses. Although there is evidence that Dsb repair enzymes exist\(^{[9,10]}\), their effectiveness and versatility against non-native thiols or sulfides has not been charted. So, these extraordinarily reactive, native groups represent significant potential as drug targets. Especially of interest is that human thioredoxin has a greater distance between cysteine residues, Cys-X-X-X-X-Cys, which could allow for development of inactivators which are specific for the bacterial thioredoxins.\(^{[22]}\)
Figure (1.11). Potential Drug Interactions with Thioredoxins.
(a) Normal function of disulfide bridge forming enzymes (Dsb’s), allowing electron flow between Dsb’s and thioredoxins (trx’s). (b) Drug molecules with thiol or disulfide functionalities can bind irreversibly with the Dsb, on either the cytoplasmic or periplasmic side, and thereby inhibit communication across the bacterial membrane. (c) As well, direct, irreversible, binding of an organosulfur molecule to free thioredoxins (trx) can also shut down these important redox mechanisms.

1.2.5 Mycothiol-based Systems
Actinomycetes also do not produce glutathione, but generate mycothiol (13) instead as their primary antioxidant. First discovered in a species of Streptomyces, and then identified in Mycobacterium bovis, mycothiol has since been found to be prevalent only amongst actinomycetes and is produced in high levels by mycobacteria.
Consequently, these cellular antioxidants have great potential as targets for anti-tuberculosis agents.

![Mycothiol (13)](image)

**Figure (1.12). Mycothiol (13).**

### 1.2.6 Other Cellular Targets

Another potential intracellular thiol target is adenosine triphosphate synthase (ATPase). ATPase is the enzyme responsible for regeneration of the all-important energy source of cells, adenosine triphosphate (ATP) (14). ATPase, which sits on the mitochondria, contains a reactive, free thiol which is paramount to activity. Inhibition of ATPase, therefore, can be achieved by formation of a mixed disulfide.

One final target of note is the phosphoenol pyruvate phosphotransferase system which is involved in the sugar uptake of some bacteria. This system employs an enzyme whose structure is moderately conserved between species, carries a molecular weight of approximately 70,000, and is extremely vulnerable to attack by free thiols. [24]
1.3.0 Antibacterial Organosulfur Compounds

1.3.1 Introduction

Bacterial cells differ greatly from human and animal cells, thus providing a number of targets that are specific to bacterial infection, but not healthy tissues. A major focus to antibacterial development has been on finding compounds that can interrupt bacterial cell wall biosynthesis without inducing harm to human cells. In the case of Gram-negative bacteria there is also an additional cell membrane on the exterior of the cell wall which can block the entry of some polar antibacterial agents. The remaining classical targets of antibiotics are tetrahydrofolate, DNA, RNA and protein synthesis. However, the search is on for novel bacterial targets that can damage bacteria through non-classical pathways.

For thousands of years homeopathic medicine extracts, and oils from leeks such as garlic, onions, shallots, chives and scallions, have been successfully used to treat bacterial infections. Early experimentation found that thoroughly dried formulations were totally ineffective, thus pointing to volatile reagents as the active anti-infective
agents. We now know that the responsible compounds in these remedies are low molecular weight organosulfur compounds.\textsuperscript{[24,25]}

Today, extracts are still used abundantly. In China, Dasuansu (diallyl trisulfide) is a popular commercial product for the treatment of bacterial, fungal and parasitic infections. Garlic-based remedies spawned the initial interest of medicinal sulfur chemistry and continue to be actively investigated.\textsuperscript{[26]}

In 1944 a rather smelly extract was determined to be a primary agent responsible for garlic’s wide range of antibacterial properties.\textsuperscript{[21]} Three years later the first structural proof of a sulfur containing garlic extract was determined, by chemical synthesis, to be a thiosulfinate. This component was named Allicin (\textsuperscript{15}) (originating from \textit{Allium sativum}, the Latin name for garlic).

Allicin has been shown to have potent \textit{in vitro} antibiotic activity against \textit{Escherichia coli}, \textit{Staphylococcus aureus}, \textit{Streptococcus pyogenes}, \textit{Proteus mirabilis}, \textit{Pseudomonas aeruginosa}, \textit{Acinetobacter baumanii}, and \textit{Klebsiella pneumoniae} (Table 1.1).\textsuperscript{[27,28]} The likely mode of action of Allicin involves reaction of the thiosulfinate with a cellular thiol to produce a mixed disulfide.\textsuperscript{[29]} The identity of the native thiol has not been definitively proven, but is likely to be a thioredoxin or glutaredoxin.
Although Allicin was the first sulfur-containing extract identified from garlic, it is certainly not the only one: other isolates with in vitro antibacterial activities include diallyl disulfide (16), diallyl trisulfide (17), Ajoene (18), diallyl sulfide (19), S-allylmercaptocysteine (20), and S-allyl-L-cysteine (21).[25,27-31]
Figure (1.15). Natural Antibiotics from Garlic.

Many of these substances have also been tested for \textit{in vivo} activities, including the diallyl sulfide and diallyl disulfide, which have potent activity against murine methicillin-resistant \textit{Staphylococcus aureus} (MRSA) infections\cite{32}. The mechanism by which they exert their bacteriostatic effects has not been fully defined but undoubtedly relies on the sulfur functionalities.

1.3.2 Thiols

Thiol-bearing enzymes, such as glutaredoxin and thioredoxin, often lose some or all of their activity in the presence of non-native (to the cell) thiols like allyl mercaptan. To that effect, thiol-bearing therapeutics are often effective at shutting down enzymatic pathways regulated by these proteins. Although there are a large number of small, natural product thiols now known to possess antibiotic activities, there are only a few synthetically-derived thiols that have been shown to have antibacterial properties. A trio of thiodiazole aromatics, \textbf{22a-c}, are reported to have minimum inhibitory concentrations
(MICs) against *Staphylococcus aureus* in the 31-62 µg/ml range, but impose no effect on *E. coli.*[^33] The reasons for this are not known.

Compounds 23a-c, synthetic precursors to 22a-c, possess much lower antibiotic activities, with MIC’s above 125 µg/ml for *Staphylococcus aureus*.

![Figure (1.16). *S. aureus*-Active Thiodiazoles.](image1)

![Figure (1.17). Mercaptotriazoles, Precursors to the Thiodiazoles.](image2)

Some mercaptopyrimidines have also been shown to have antibiotic activities.[^34] Biological evaluation of 5-mercapto-2’-deoxyuridine (24b) in comparison to a known antibiotic, 5-fluoro analogue (24a), has been reported. Although weaker than the 5-fluoro analogue, the mercapto analogue shows selectivity to *Lactobacillus leichmannii* over *Lactobacillus arabinosus* and *Streptococcus faecalis*, which is not observed for the fluoro analogue (Table 1.2). As well, there is a synergistic effect when 5-mercapto-2’-deoxyuridine is used in tandem with the fluoro analogue, indicating that the two compounds exert different modes of action in their inhibition of *Lactobacillus leichmannii*. The potency of the drug is displayed by an IC$_{50}$ of 0.06 µM. Interestingly, for the 5-mercaptouracil, this selectivity seems to be reversed, with *L. faecalis* being the most greatly affected. Similar properties are seen with uracil analogues 25.
Figure (1.18). 5-Fluoro-2'-deoxyuridine (24a) and 5-Thio Analogue (24b).

Figure (1.19). 5-Fluorouracil (25a) and 5-Thio Analogue (25b).

Figure (1.20). Example of a Boron-Complexed Benzothiazoline Antibacterial.

Nisin Z is an antimicrobial peptide (lantibiotic), comprised of 34 amino acids with 5 free thiols, that effectively inhibits growth of various gram-positive bacteria. It exerts its antimicrobial activity by permeabilizing the cytoplasmic membrane of target bacteria. This leads to the release of small cytoplasmic compounds, depolarization of the membrane potential, and ultimately cell death. The free thiols along the backbone of this peptide are required for the antibiotic properties, since blockage or removal of these thiols completely destroys activity. The reasons for this are not yet fully understood.
Table 1.2. IC$_{50}$ Values (µM) of Fluoro- and Mercapto-Substituted Pyrimidines (24a,b and 25a,b).[34]

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. leichmannii</th>
<th>L. arabinosus</th>
<th>L. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-fluorodeoxyuridine (24a)</td>
<td>0.03</td>
<td>0.001</td>
<td>0.00001</td>
</tr>
<tr>
<td>5-mercaptodeoxyuridine (24b)</td>
<td>0.06</td>
<td>4</td>
<td>0.1</td>
</tr>
<tr>
<td>5-fluorouracil (25a)</td>
<td>0.04</td>
<td>0.2</td>
<td>0.00005</td>
</tr>
<tr>
<td>5-mercaptouracil (25b)</td>
<td>30</td>
<td>700</td>
<td>3</td>
</tr>
</tbody>
</table>

As mentioned, certain groups of enzymes within bacteria cells require free thiols for their activity, which can in some cases be inhibited by non-native thiols. These proteins are especially susceptible to inactivation by metal thiol complexes, such as boron-complexed benzothiazolines (26), which form mixed disulfides with thiol-containing proteins to block the metabolic pathway.[35] Compounds 26 have shown promising in vitro activities against Escherichia coli, Staphylococcus aureus, Klebsiella aerogenous and Pseudomonas cepacicola. The levels of biocidal activity such complexes have against different microorganisms depend primarily on the permeability of the cells.

Bearing the idea that free thiols can be responsible for biological activity, it is important to realize the potential for these reagents to dimerize and thus lower, or even abolish, their in vivo activity. Metal-thiol coordinations can prevent these dimerizations without destroying activity of the thiol.

To prove this, one report compared the antibacterial effects of the sodium thiolate, free thiol, and tin thiolate of 5,7-dichloroquinone (27a-c).[37,38] Presumably, disulfide formation would be more likely for the sodium thiolate than free thiol, and more for the free thiol than for the tin thiolate. The three compounds were evaluated against four bacteria: Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Bacillus
cereus (Table 1.3). The sodium thiolate displayed no antibacterial activity, while the free thiol showed MICs in the 100 to 200 µg/ml range and the tin thiolate had MICs in the 37.5 to 50 µg/ml range. This suggests that spontaneous dimerization of thiol antibacterials may be a limiting effect which can be overcome by metal coordination.

\[
\begin{align*}
27a &= \text{Na} \\
27b &= \text{H} \\
27c &= \text{Sn}
\end{align*}
\]

Figure (1.21). 5,7-Dichloroquinoline Thiol and Metallated Derivatives.

It seems that the tin entity is not responsible for this activity, but merely preserves the thiolate from disulfide formation. This has been demonstrated conclusively in the use of tin thiol derivatives for antifungal treatment (vide infra).

<table>
<thead>
<tr>
<th>Compound</th>
<th>S.aureus</th>
<th>E.coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>27a</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>27b</td>
<td>103</td>
<td>102.5</td>
</tr>
<tr>
<td>27c</td>
<td>50</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Table 1.3. MIC’s of Quinoline Derivatives 27a-c.

Similarly, some simple bismuth-coordinated thiols (BTs) have been reported to possess potent antibacterial properties.\[^{39}\] Bismuth-2,3-mercaptopropanol (BisBAL) (28), bismuth-3,4-dimercaptotoluene (BisTOL) (29), bismuth-1,2-ethanedithiol (BisEDT) (30), and bismuth-pyrithione (BisPYR) (31) have been shown to effectively inhibit the growth of MRSA and methicillin-resistant Staphylococcus epidermidis (MRSE), with MIC’s in the single digits and low MBC’s (Table 1.4).
Table 1.4. Bioactivities of Bismuth-Coordinated Thiols Against Methicillin-resistant *S. aureus*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisBAL (28)</td>
<td>8.3</td>
<td>18</td>
</tr>
<tr>
<td>BisTOL (29)</td>
<td>4.4</td>
<td>9</td>
</tr>
<tr>
<td>BisEDT (30)</td>
<td>2.4</td>
<td>73</td>
</tr>
<tr>
<td>BisPyr (31)</td>
<td>6.7</td>
<td>14</td>
</tr>
</tbody>
</table>

As well, these bismuth thiols prevent growth of bacterial biofilms on coated indwelling medical devices, such as catheters and intravascular lines. Synthesis of BTs is straightforward, simply requiring the addition of bismuth nitrate to the appropriate thiol.\[40\]

### 1.3.3 Disulfides

As therapeutic agents, disulfides usually serve one of two purposes: 1) as inactive structural components of biomolecules, or 2) as biological oxidants. In the latter role, disulfides are particularly prone to react with thiols to give biologically-inert mixed disulfide adducts.
Many of the leek extracts, like Ajoene (18), create the same type of mixed disulfide products with native thiols that are seen with the corresponding thiol drugs.\[31\] This leads to effective antibacterial activities such as those shown in Table 1.5.

Table 1.5. Antibacterial Activities of Ajoene (18).

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Mycobacterium phlei</em></td>
<td>14</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>136</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>19</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>56</td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>116</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>152</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>Xanthomonas maltophilia</em></td>
<td>118</td>
</tr>
</tbody>
</table>

Epipoly-thiodioxopiperazines (ETP’s) are a class of fungal metabolites of *Candida, Thermoascus* and *Penicillium*, to name a few, that possess characteristic bridged disulfide piperazinedione six-membered rings. These antibiotics only inhibit Gram-negative bacteria, which is likely related to their outer membrane permeability, and are prone to nucleophilic attack on their electrophilic disulfide bridge. Gliotoxin (32) and related ETP’s are reported to act as oxidants by at least two different pathways: 1) generation of superoxide and hydrogen peroxide via glutathione redox cycling, and 2) sulfenylation of native thiols of certain proteins to make catalytically defunct mixed
disulfides.\textsuperscript{[41,42]} Which sulfur center in gliotoxin is the site of enzymatic attack is still unclear but it is likely that both may be involved.

![Figure (1.23). Gliotoxin, an Epipolythiodioxopiperazine (ETP) Antibacterial.](image)

Although somewhat rare in nature, some cyclic polysulfides (33-35) occur in a few fungi and aquatic organic organisms.\textsuperscript{[43,44]} The intermittent carbon linkages in these molecules distinguish these analogues from S\textsubscript{8} and enhance solubility in non-aqueous environments.

![Figure (1.24). Cyclic Polysulfide Natural Products.](image)

These cyclic systems contain reactive disulfide bridges that most likely behave like those of other disulfide antibiotics. These compounds have been found to inhibit \textit{Staphylococcus aureus, Streptococcus faecium, Escherichia coli, Klebsiella sp., Proteus mirabilis} and \textit{Pseudomonas aeruginosa}.

24
Similarly, the natural product Leinamycin (36) has potent inhibition activity, MIC = 0.03 µg/ml, against *Bacillus subtilis*.\textsuperscript{[45]} Leinamycin has an intriguing mechanism of action. It is believed that Leinamycin reacts with cellular thiols to form, in a few additional intramolecular steps, an episulfenium cation which can, in turn, alkylate DNA.\textsuperscript{[46-48]} As a DNA alkylater, Leinamycin blocks cellular replication in bacteria.

![Figure (1.25) Leinamycin’s Mode of Action](Image)

**Figure (1.25) Leinamycin’s Mode of Action**

Isolated from both a species of *Psammaplysilla* and *Thorectopsamma xana*, a trio of closely related disulfides were discovered with noteworthy antibacterial activity.\textsuperscript{[49]} Psammaplin A (37) and the dimer, Bisaprasin (38), along with Psammaplin D (39) all demonstrated growth inhibition of *Staphylococcus aureus* and *Bacillus subtilis*. Psammaplin D, interestingly, also shows activity against the Gram-negative bacterium *Trichophyton mentagrophytes*.\textsuperscript{[50]}
Isolated from a tunicate, *Polycitorella mariae*, a novel disulfide termed Citorellamine (40) has been reported to have significant antimicrobial activity.\[^{[51]}\] Originally assigned the structure of a sulfide, the disulfide Citorellamine demonstrates potent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* as well as cytotoxicity in some cancer cell lines.\[^{[52]}\]

Disulfides with interesting antibacterial activities also occur in proteins specifically synthesized by life forms for defense. These antibiotics are found most commonly in marine sources, and usually contain two neighboring cysteine residues. One such antibacterial protein, from a marine decapod, displayed potent inhibition of *Planococcus citreus*, *Planococcus kocurii*, *Aerococcus viridans*, and *Micrococcus luteus* and an extraordinarily strong resistance to heat damage.\[^{[53]}\]

![Psammaplin A (37)](image1)

Figure (1.26). Psammaplin A (37).

![Bisaprasin (38)](image2)

Figure (27). Bisaprasin (38)
1.3.4 Trisulfides

Trisulfides found in garlic, such as diallyl trisulfide and allyl methyl trisulfide, can act as antibacterial agents in the same way as disulfides, but with greater efficacy. The antibacterial activity of some cyclic polysulfides can be attributed to a trisulfide bridge, as much as previous examples owe their activity to a disulfide bridge.\textsuperscript{[43,44]} 4-Dioxo-1,2,4,6-tetrathiepane (41), an extract from the red alga \textit{Chondria californica}, has potent antibacterial activity against \textit{Vibrio anguillarium}, the causative agent of a tropical fish disease.
Enantiomeric marine natural products are fairly uncommon, especially those with two chiral centers. However, a pair of Enantiomeric trisulfide alkaloids (42) from the New Zealand ascidian *Hypistylozoa fischeriana* have been identified and shown to possess weak (4 mm zone of inhibition by a 120 µg/disk), but identical, activities against *Bacillus subtilis* and weak (10% inhibition at 12.5µg/ml) activities against *Mycobacterium tuberculosis*.[54] Similar Ascidian natural products without the trisulfide functionality (43), but with a 1,3-dithiane instead, are completely devoid of antimicrobial activity.

![Figure (1.31). Ascidian Trisulfide and Dithiane.](image)

Also part of the trisulfide family of antibiotics are the enediyne trisulfide antitumor antibiotics, Calichemicin (10), natural product of *Micromonospora echinospora*, and Namenamicin (11), Esperamicins natural product of *Actinmadura verrucosospora*, Although these antibiotics are not currently used to treat infection, in anticancer systems these compounds have been shown to undergo an intricate cascade of intramolecular reactions initiated by glutathione attack, resulting in an intensely reactive radical species. In the first step of this process, reaction of the trisulfide group with glutathione generates a free thiolate anion which in turn undergoes a Michael addition
across the α,β-saturated ketone. This subtle change in hybridization of the carbons allows the enediyne to undergo Bergman cycloaromatization, producing the phenylene diradical. These diradicals are believed to cleave DNA by sequentially stripping off hydrogen atoms along the minor groove of the double helix. Even though their mechanism of antibacterial action has not been proven to be the same as that of the anticancer mechanism (Figure 36), it is likely that the trisulfide moiety is involved as an electrophilic reactant with cellular thiols (Table 1.6).

![Diagram](image)

**Figure (1.32). Proposed Mechanism of Enediyne Bioactivation.**

**Table 1.6. Antibacterial Activity of Representative Enediynes, 10 and 11, versus Penicillin G.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (µg/ml) Calicheamicin</th>
<th>MIC (µg/ml) Namenamicin</th>
<th>MIC (µg/ml) Penicillin G</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.00005</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0.00001</td>
<td>0.001</td>
<td>0.015</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>0.00012</td>
<td>0.03</td>
<td>128</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.12</td>
<td>0.12</td>
<td>32</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>0.25</td>
<td>0.06</td>
<td>128</td>
</tr>
</tbody>
</table>
1.3.5 Pentasulfides

Within the Didemnidae or tunicate family, *Lissoclinum* species are rich sources of organosulfur antibiotics. One such isolate Lissoclinotoxin A (44), demonstrated potent growth inhibition of a number of bacteria, including *S. aureus*, *Streptococcus faecalis*, *Cirrobacter* species, *Klebsiella* species, *E. coli*, *Enterobacter* species, *Serratia* species, *Salmonella* species, *Pseudomonas aeruginosa*, *Acinetobacter*, and *Proteus* species (Table 1.7).[55]

![Chemical Structure of Lissoclinotoxin A](image)

**Figure (1.33). Lissoclinotoxin A**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (µg/ml)</th>
<th>Cefotaxim (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>0.08 - 0.15</td>
<td>1.2 - 10</td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td>0.3 - 0.6</td>
<td>0.6 - 2.5</td>
</tr>
<tr>
<td><em>Cirrobacter</em> spp.</td>
<td>0.3 - 0.6</td>
<td>0.08 - 10</td>
</tr>
<tr>
<td><em>Klesiella</em> spp.</td>
<td>0.3 - 0.6</td>
<td>0.01 - 0.15</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.15 - 0.6</td>
<td>0.005 - 5</td>
</tr>
<tr>
<td><em>Enterobacter</em> spp.</td>
<td>0.3 - 0.6</td>
<td>0.08 - 40</td>
</tr>
<tr>
<td><em>Serratia</em> spp.</td>
<td>0.3 - 0.6</td>
<td>0.08 - 2.5</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>0.3 - 0.6</td>
<td>0.08 - 3</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>2.5 - 10</td>
<td>2.5 - 40</td>
</tr>
<tr>
<td><em>Acinetobacter</em></td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td><em>Proteus</em> spp.</td>
<td>0.15 - 0.6</td>
<td>0.005 - 0.3</td>
</tr>
</tbody>
</table>
1.3.6 *N*-Thiolated Compounds

*N*-Sulfenylated monocyclic β-lactams (45) are another class of sulfur-containing antibacterial compounds recently discovered to have an unusual mode of action, where the N-S functionality may interact with cellular thiols in the same fashion as a disulfide.\[^{56}\]

Despite the presence of a β-lactam ring, the mode of action of these *N*-thiolated compounds is totally different to that of the penicillins and other beta-lactam drugs which act as cell wall biosynthesis inhibitors. It is believed that an intracellular thiol attacks the sulfur atom to form a mixed disulfide which in turn causes inhibition of bacterial growth. These thiolated lactams show a narrow range of antibacterial properties, including *Staphylococcus* strains such as MRSA and *S. epidermidis*. Full details on the synthesis and biological activities is covered in the following chapters.

![Figure (1.34). *N*-Sulfenylated-β-Lactam](image)

Another sulfenamide, 1,2-benzoisothiazolin-3-one (BIT) (46), has shown weak activity against *Staphylococcus aureus*, with an MIC around 100 µg/ml.\[^{57}\]

![Figure (1.35). BIT](image)
BIT has been shown to inhibit the action of a number of intracellular thiols such as glutathione and ATPase. The mode of action has been linked to an inhibitory effect on cellular respiration upon metabolic uptake.

Lansoprazole (47), a drug originally designed as a gastric acid pump inhibitor, has been shown to rearrange in the acidic environment of the stomach to a sulfenamide (48), which is an inhibitor of Helicobacter pylori.[58,59] H. pylori is considered to be a main culprit in the cause of gastric ulcers and therefore lansoprazole serves double duty as an antibiotic and an acid production reducer. Even though an intermediate sulfenic acid derivative of lansoprazole also displays anti-pylori activity, the sulfenamide affords fast action with an MIC value of 10 µg/ml.

![Figure (1.36). Lansoprazole and in vivo Sulfenamide.](image)

1.4.0 Antifungal Organosulfur Compounds

1.4.1 Introduction

In addition to their antibacterial properties many organosulfur compounds have fungistatic or fungicidal activity. Being eukaryotes, fungi comprise a separate group of
microorganisms, having a membrane bound nucleus, a more extensive endoplasmic reticulum and mitochondria. Compared to bacteria, fungi have a very different cell wall which is built from a complex set of constituents including chitin (aminoglucans), polyuronids, galactosamine, melanin and various lipids. Fungal DNA is found isolated within the nucleus instead of dispersed throughout the cytoplasm, as in bacteria. Fungal infections are usually isolated to the dermis or mucosal membranes (superficial mycosis). However, with the increasing prevalence of immune deficiency diseases, like HIV-AIDS, development of treatments for internal and systemic fungal infections (deep mycosis) are now of significant concern.

Although antifungal activity attributed to garlic extracts dates back to 1936, the first garlic isolate to display antifungal activity, Ajoene (Fig. (18)) against *Aspergillus*, was not reported until 1987.\textsuperscript{[60]} Since then a number of other antifungal activities have been found for Ajoene, such as *Candida* and *Paracoccidioides* (Table 1.8).\textsuperscript{[31]}

Table 1.8. Antifungal Activities of Ajoene (18).

<table>
<thead>
<tr>
<th>Fungal Strain</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>13</td>
</tr>
<tr>
<td><em>Hanseniaspora valbyensis</em></td>
<td>11</td>
</tr>
<tr>
<td><em>Pichia anomala</em></td>
<td>11</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>5.5</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>12</td>
</tr>
</tbody>
</table>

Other garlic-derived natural products that exhibit potent antifungal activities include diallyl trisulfide (against *Cryptococcal meningitis*) and Allicin (against species of
Candida, Cryptococcus, Trichophyton, Epidermophyton, and Microsporum, with MICs as low as 1.57 µg/ml)(Table 1.9).[28,32] The mode of action of these antifungal agents has yet to be fully elucidated, however, they are believed to function as sulfenylating agents, as they do in bacteria.

Table 1.9. Antifungal Activities of Allicin (15).

<table>
<thead>
<tr>
<th>Fungal Strain</th>
<th>MIC(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>0.3</td>
</tr>
<tr>
<td>C. albicans (clinical isolate)</td>
<td>0.8</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>0.3</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>0.15</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>0.3</td>
</tr>
<tr>
<td>C. krusei</td>
<td>0.3</td>
</tr>
<tr>
<td>Torulopsis glabrata</td>
<td>0.3</td>
</tr>
<tr>
<td>T. glabrata (clinical isolate)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

1.4.2 Thiols

Due to the proliferation of thiol-bearing enzymes in a large majority of life forms, it is to be expected that anti-infective thiol compounds could be found in nature that can successfully inhibit the growth of fungi by formation of mixed disulfides. Consequently, some of the thiols examined earlier in this report for antibacterial activity are also effective antifungal reagents.

Closely related derivatives (49a-c) of the antibacterial mercaptotriazoles inhibit Candida albicans and Saccharomyces cerevisiae, with MIC values in the range of 12.5 to 61 µg/ml.[34] Both the thiol and amine groups are believed to be required for antifungal activity, since the thiodiazole aromatics 22a-c (which lack the amino moiety) and their precursors 23a-c have no antifungal properties.
The unsymmetrical boron-complexed benzothiazolines, BisBAL, BisTOL, BisEDT, BisPYR and BisβME, whose antibacterial properties were previously discussed (Table 1.4), all display weak antifungal activities, with MICs just above 200 µg/ml against *Macrophomina phaseolina, Fusarium oxysporum* and *Aspergillus niger*.\[31\] Comparatively, the marketed non-sulfur-containing drug, Bavistin, exhibits MIC values of approximately 100 µg/ml each.

Likewise, 5,7-dichloroquinoline-8-thiol and its sodium and tin thiolates (Figure 1.21) have been examined against fungi.\[28\] As in the case of the antibacterials, the stannous thiolate showed the best antifungal activities, with MICs against *Candida albicans* and *Saccharomyces cerevisiae* of 50 µg/ml, while the free thiol and sodium thiolate displayed MICs of 128 and greater, respectively. To substantiate the claim that this is an antifungal effect of the thiol and not strictly a function of tin toxicity, another lab compared a relatively inactive control group of tin-2-thionaphthalenes to their free thiols and discovered almost equal antifungal activities. As well, the tin-thiolated compound was shown to be no more toxic (lethal dose = LD$_{50}$ >100 µg/ml) than the free thiol itself.\[29\]

### 1.4.3 Sulfides

Although the mechanism of action of the Ajoene organosulfide, 18, is still under active investigation, it is likely that antifungal activity is the result of induced cell wall
damage, since morphological changes in fungal cells treated with these compounds have been observed via scanning and transmission electron microscopy.$^{[54]}$

Six newly reported sulfide-bearing 1,4-naphthoquinones (50-55) have been found to display good to potent activities against *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Microsporum canis*, with MICs ranging from less than 0.78 to 50 µg/ml, a marked improvement over their respective oxygen counterparts.$^{[60]}$ As of yet, the mechanism of their activity is not understood, but may in fact be related to the oxidative potential of the naphthoquinone ring system.

![Figure (1.38) Naphthoquinone Sulfides Antifungal Compounds.](image-url)
1.4.4 Disulfides

Disulfides are commonly found as toxins produced by fungi, however, two groups of disulfides having antifungal activity have been reported. The first group of compounds contains some of the most potent antifungal agents ever known, the thiarubines (56), with MIC’s in the ng/ml range. Isolated as natural products from the Compositae (Asteraceae) family of plants, the thiarubines display activities against Cryptococcus neoformans, Aspergillus fumigatus, Candida albicans and other species of Candida.

\[
\begin{array}{c}
R \equiv S \equiv S \equiv R' \\
\end{array}
\]

**Figure (1.39). Core Structure of the Thiarubine Group of Antifungals.**

The tunicate-generated disulfide, Citorellamine, not only possesses strong antibacterial activity, but is also very active against Saccharomyces cerevisiae and mildly active towards Pseudomonas aeruginosa (Figure 1.31). The other group of disulfides, the 1,2-dithiole-3-thiones (57a-j), is very interesting because it consists of compounds that could possibly behave as a disulfide, sulfide, or thiol in its activity. The compounds are fungicidal against Candida albicans, Candida tropicalis, Cryptococcus neoformans, Saccharomyces cerevisiae, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Microsporum cannis, Microsporis gypseum, Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentagrophytes. The main activity is due to the disulfide-thione functionality. Although, the pendant sulfide (thioether) is not strictly required for activity, its presence, as compared to alkyl or aryl groups,
increases activity greatly. After lengthening the sulfur sidechain beyond ethyl, an inverse relationship between chain length and antifungal activity begins to develop. However, against all of the fungi tested the benzylthio analogue \((57i)\) maintained the greatest potency, with MIC’s ranging from 0.7 to 6.25 µg/ml (Table 1.10).

![Figure (1.40). Core Structure of the Dithiole-Thione Group of Antifungals.](image)

### Table 1.10. Antifungal Activities of Thione Derivatives 57.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>57a</th>
<th>57b</th>
<th>57c</th>
<th>57d</th>
<th>57e</th>
<th>57f</th>
<th>57g</th>
<th>57h</th>
<th>57i</th>
<th>57j</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>50</td>
<td>25</td>
<td>125</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>50</td>
<td>6.25</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>50</td>
<td>25</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>C. neofomans</td>
<td>62.5</td>
<td>12.5</td>
<td>50</td>
<td>62.5</td>
<td>125</td>
<td>125</td>
<td>250</td>
<td>6.25</td>
<td>6.25</td>
<td>50</td>
</tr>
<tr>
<td>S. cerevisiae</td>
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<td>25</td>
<td>&gt;250</td>
<td>&gt;250</td>
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<td>&gt;250</td>
<td>12.5</td>
<td>6.25</td>
<td>&gt;250</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>50</td>
<td>6.25</td>
<td>50</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>6.25</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>125</td>
<td>25</td>
<td>125</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>25</td>
<td>6.25</td>
<td>250</td>
</tr>
<tr>
<td>A. niger</td>
<td>125</td>
<td>12.5</td>
<td>125</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>25</td>
<td>3.12</td>
<td>250</td>
</tr>
<tr>
<td>M. Canis</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>3.12</td>
<td>0.7</td>
<td>3.12</td>
</tr>
<tr>
<td>M. gypseum</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>0.7</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>E. floccosum</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>50</td>
<td>&gt;250</td>
<td>250</td>
<td>250</td>
<td>6.25</td>
<td>0.7</td>
<td>6.25</td>
</tr>
<tr>
<td>T. rubrum</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>50</td>
<td>&gt;250</td>
<td>250</td>
<td>250</td>
<td>6.25</td>
<td>0.7</td>
<td>6.25</td>
</tr>
<tr>
<td>T. mentagrop.</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>250</td>
<td>&gt;250</td>
<td>250</td>
<td>250</td>
<td>3.12</td>
<td>12.5</td>
<td></td>
</tr>
</tbody>
</table>

### 1.4.5 Trisulfides

Although not currently used in clinical settings, enediyne antitumor antibiotics also display potent antifungal activities. *Candida albicans, Ustilago maydis,*
Saccharomyces cerevisiae, and Neurospora crassa are all inhibited by Calicheamicin and Namenamicin with MIC’s below 1 µg/ml (Table 1.1).[65] Their mechanism of action is presumably similar to that of their antibacterial and anticancer properties as DNA cleaving agents triggered by S-thiolation of glutathione or a related thiophile.

Table 1.11. Enediyne Antifungal Activities

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Calicheamicin (µg/ml)</th>
<th>Namenamicin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>U. maydis</td>
<td>0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.008</td>
<td>0.06</td>
</tr>
<tr>
<td>N. crassa</td>
<td>0.06</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Isolated from an ascidian, trans-5-hydroxy-4-(4’-hydroxy-3’-methoxyphenyl)-4-(2”-imidazolyl)-1,2,3- trisulfide, 42, also displays moderate antifungal activity against Candida albicans.[48]

1.4.6 Polysulfides

Lissoclinotoxins A (44) (Figure 1.34) and D (58), pyridoacridine alkaloids from ascidians, both display potent antifungal activities against Candida albicans and Trichosporon mentagrophytes, with cell-mediated immunity levels (CMI) of 40 and 20 µg/ml, respectively. More moderate activities (CMI’s greater than 40 µg/ml) were observed against several other fungi.[66] Pyridoacridines have been shown to intercalate DNA and in some cases inhibit topoisomerase II.[66]
1.5.0 Antiviral Organosulfur Compounds

1.5.1 Introduction

For many years the use of antiviral agents in the clinical setting was a dream yet to be realized. Now there are a plethora of researchers studying viral pathways, discovering new drugs, and delineating novel modes of action. Viruses differ immensely from all other classes of infective agents. With no real cell to speak of, a virus consists of simply a “shell” or hard protein “coating” that encapsulates viral DNA or RNA. “Classical” modes of anti-viral action include the incorporation of “false” DNA building blocks, which leads to a blockage of replication, inhibition of the virally-induced DNA polymerase (which can be done with some selectivity in relation to the endogenous enzyme) inhibition of reverse transcriptase (a virus specific enzyme), inhibition of viral protein synthesis, and interference with the “uncoating” process, by which viruses release their genetic material. Within this context, organosulfur compounds may play an increasingly important role. A recent report shows conclusively that cytomegalovirus (CMV), a member of the herpes virus family which can induce mononucleosis-like
symptoms in immunocompromised patients, contains free thiol groups which are paramount to infectivity. It has been found that if these groups are blocked as disulfides the virus is unable to infect, while de-blocking the disulfide back to the free thiol returns full infectivity. Potentially other viruses may contain similar thiols important to their ability to infect, and therein lies the possibility to design novel organosulfur compounds for use as antiviral agents. In the search for novel nucleosides with AZT (3’-azido-3’-deoxythymidine) like activities, a variety of thionucleosides have been examined. Here, a sulfur atom occupies the site of the ribose ring oxygen, allowing the molecules to be introduced during reverse transcription into viral DNA. A good review of the synthesis and biological evaluation of antiviral thionucleoside analogues has been published by Wnuk.

Although the antiviral properties of garlic natural products are not as highly appreciated as their antibacterial effects, Ajoene, Allicin, allyl methythiosulfinate, and methyl allylthiosulfinate (Figure 1.16 and Figure 1.18) have all been reported to have antiviral activity. Specifically, these organosulfur compounds show detectable inhibitory activity against herpes simplex virus type 1, herpes simplex virus type 2, parainfluenza virus type 3, vaccinia virus, vesicular stomatitis virus, and human rhinovirus type 2. Ajoene and allyldisulfide are also active against human immunodeficiency virus (HIV), apparently by inhibiting integrin-dependent processes. Integrins are a family of cytokines that provide costimulatory signals to T cells and protect them from abnormal cell death. In AIDS patients the action of integrins is
impaired by the viral consumption of these T cell protectors. It is interesting to note, however, that alliin and S-allyl cysteine have no antiviral activity.

1.5.2 Thiols

Intracellular redox activity plays an integral part in regulating replication and infectivity of viruses.\textsuperscript{71} The cellular thiol, glutathione, itself has purported \textit{in vitro} and \textit{in vivo} anti-influenza activity. As levels of glutathione are depleted in the oral, nasal and upper airways, susceptibility to viral infection is enhanced. Decreased intracellular glutathione levels are also implicated in HIV, and methods to increase glutathione production have been proposed as a means to stem these infections.\textsuperscript{72} \textit{N}-Acetyl-L-cysteine (NAC) and 2-mercaptoethylamine (MEA) have been shown to strongly increase glutathione levels in various cell lines.\textsuperscript{73} Several new \textit{N}-(\textit{N}-acetyl–L-cysteinyl)-\textit{S}-acetylcysteamine derivatives (59-61) have also been reported to actively release NAC and MEA, which in turn strongly bolsters glutathione levels.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{59_60_61.png}
\caption{S-Acetylcysteamines Derivatives}
\end{figure}

These compounds display \textit{EC}_{90} (effective concentration for 90\% inhibition of virus yields) ranging from 80 to 380 \textmu M against HIV in human monocyte-derived macrophages (MDM). Organosulfur compounds 60 and 61 with small chain thioester moieties are the most active analogues. \textit{S}-Acylated derivatives are believed to have
increased activity by 1) having a protected thiol and 2) increasing lipophilicity relative to
the free thiol.

Another way reported to protect a free thiol is by use of a thiocyanate, which is
likely reduced in vivo to the thiol through an equilibrium exchange with a native thiol
such as glutathione.\[74\] Examples of this include a group of thiocyanatopyrimidine
nucleosides (62), which are reported to display reasonable activity (EC\textsubscript{75} = 100 µM)
against vaccinia virus replication in HeLa cells.

\[62\]

Figure (1.43). Thiocyanatopyrimidine Nucleoside (R = Ribofuranosyl Nucleoside.)

1.5.3 Disulfides

As discussed in relation to antibacterials, organodisulfides can act as in vivo
oxidants of thiols. It is likely that disulfides, and their related thiosulfonates, behave in
the same manner in terms of their activity in viral systems. A group of aromatic disulfides
and a thiosulfonate (63-68) have been reported with potent antiviral activities against the
arenaviruses Junin (JUNV), agent of Argentine hemorrhagic fever, and Tacaribe
(TCRV).\[75\] The disulfides and thiosulfonate displayed 50% effective concentration
(EC\textsubscript{50}) values, the concentration where 50% of the virus yield is eliminated, ranging from
3.6 to 100 µM towards these microbes. This is at least ten times lower than the
concentrations needed to induce cytotoxic effects.
A large focus of preclinical and clinical development of anti-HIV drugs is in protease inhibition. However, other processes are certainly important to viral infectivity and replication. Metabolic pathways of infected cells, such as precursor protein processing, have been shown to be inhibited by a macrocyclic disulfide, 7-methyl-6,7,8,9-tetrahydrodibenzo[c,k][1,2,6,9]-dithiadiazacyclodecine-5,10-dione (69).[76]

This compound displays an EC₅₀ of 0.05 µg/ml against HIV-infected macrophages. Compared to the current standard AZT that has an EC₅₀ of 0.004. With a different mode of action from AZT however, the disulfide acts synergistically with AZT when tested in vitro, and could potentially be used in tandem.
Another disulfide with promising antiviral properties is 5,5’-diphenyl-3,3’-diisothiazole disulfide (DID) (70a).\textsuperscript{[77]} DID induces potent inhibition of plaque-infected cells derived from invasion of poliovirus type 1, with an IC\textsubscript{50} of 0.35 µM. Cytotoxicity to healthy human cells was also examined, and no adverse effects were observed with uninfected cell cultures at 50 µM concentration of DID. This agent is believed to inhibit an enzyme associated with RNA synthesis.

50% cytotoxic concentrations (CC\textsubscript{50}), the concentrations where normal cell proliferation is inhibited by 50%, were more that 200 times higher that the IC\textsubscript{50}’s, illustrating the exquisite selectivity this compound has for the viral infected cells.\textsuperscript{[77]} The reduced form, thiol 70b, has almost the same activity and selectivity as 70a (Table 1.12).

![Figure (1.46). 5,5’-Diphenyl-3,3’-diisothiazole Disulfide and Thiol Antipoliovirus Agents.](image)

Table 1.12. Isothiazole Anti-Poliovirus Activities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} µM</th>
<th>CC\textsubscript{50} µM</th>
<th>Selectivity CC\textsubscript{50}/IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>70a</td>
<td>0.35</td>
<td>89.28</td>
<td>255</td>
</tr>
<tr>
<td>70b</td>
<td>0.42</td>
<td>90.75</td>
<td>216</td>
</tr>
</tbody>
</table>
HIV reverse transcription (HIV-RT) and deoxyribonucleoside triphosphate (dNTP) synthesis are paramount to viral replication, and thus are prime inhibition targets for anti-HIV therapy. A number of sulfur bearing nucleotide HIV-RT inhibitors, which have similar effects to AZT, include 3’-mercapto-2’,3’-dideoxynucleotides (71) and 2’-deoxy-2’-mercaptopuridine-5’-diphosphate (72). These nucleosides serve to transfer a radical to a thiol of the transcriptase as shown in Figure 1.50. A new pyrimidine nucleoside disulfide (73) has been synthesized and shown to inhibit both HIV-RT and dNTP. The disulfide also has an EC$_{50}$ of 10 µM and an IC$_{50}$ of 25 µM against proliferation of human T-lymphocyte cells. Very interestingly, the corresponding thiol derivative had no activity at all. The mechanism of 73 is thought to involve release of the thiryl radical, RSS•, as the primary active species (Figure 1.50).

![Figure 1.47. Dideoxynucleotides Thiryl Radical-based Reverse Transcriptase Inhibitors.](image)

![Figure 1.48. Mercaptouridine Thiryl Radical-based Reverse Transcriptase Inhibitors.](image)
Figure (1.49). Nucleosidic Disulfide anti-HIV Agent.

Figure (1.50). Proposed Mechanism of Sulfur-bearing Nucleotide-based Reverse Transcriptase Inhibition

Gliotoxin (Figure 1.23), although a somewhat non-specific toxin, is a potent inhibitor of poliovirus.\textsuperscript{[41]} Although Gliotoxin has a cytotoxicity which is too high to be a useful clinical agent, the compounds antiviral effects warrant further investigation in an effort to find an analogue with lower toxicity.

1.6.0 Antiparasitic Organosulfur Compounds

1.6.1 Introduction

As with all three of the previously discussed types of infections, bacterial, fungal and viral, garlic extracts have been known the longest to exhibit antiparasitic properties. Generally, the most serious of these infections involve invasion of the intestinal tract or other internal organs. Since these microbes generally have low intracellular glutathione or
trypanothione concentrations they are proposed to have a higher sensitivity toward sulfur anthelmintics compared to mammalian cells.

Allicin (Figure 1.16) has been tested against a number of protozoan parasites, *Giardia lamblia, Leishmania major, Leptomonas colosoma* and *Crithidia fasciculata*, and determined to have MIC<sub>50</sub> values equal to about 30 µg/ml. Toxicity levels toward tissue-cultured mammalian cells of Allicin is above 100 µM.[69] Allicin has also been reported to display potent inhibition of *Entamoeba histolytica*, with complete inhibition at 30 µg/ml and an IC<sub>90</sub> of only 5 µg/ml.[82, 83]

Diallyl trisulfide, 17, which Allicin has been shown to degrade to *in situ*, also shows potent inhibition of *Entamoeba histolytica* with an IC<sub>50</sub> equal to 14 µg/ml.[27] Diallyl trisulfide displays the same MIC’s against *Giardia lamblia* and *Trypanosoma* species (Table 1.13).[84]

**Table 1.13. Growth Inhibition (IC<sub>50</sub>) Activities of Dasuansu (Diallyl Trisulfide (17)) Against Parasites.**[26]

<table>
<thead>
<tr>
<th>Parasite</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma brucei brucei</em></td>
<td>2.5</td>
</tr>
<tr>
<td><em>T. b. gambiense</em></td>
<td>1.8</td>
</tr>
<tr>
<td><em>T. b. rhodesiense</em></td>
<td>2.9</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>0.8</td>
</tr>
<tr>
<td><em>T. congolense</em></td>
<td>5.5</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>1.2</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>59</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>14</td>
</tr>
</tbody>
</table>
Ajoene (Figure 1.18) inhibits the proliferation of both epimastigotes and amastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease.$^{[84]}$ Ajoene alters the composition of phospholipids, most likely through a sulfenylation that inhibits the phosphatidylcholine biosynthesis and cell proliferation. A 40 µM concentration of Ajoene is all that is required to eradicate the parasite, in amastigote form, from host cells in 96 hours, while an 80 µM concentration is enough to immediately inhibit growth in the epimastigote form. Ajoene is also cytocidal to epimastigotes at 100 µM within 24 hours.

As well, an entire host of garlic extracts, methyl propyl sulfide, allyl methylsulfide, diallyl sulfide, dimethyl sulfide, diallyl disulfide, dimethyl disulfide, methyl propyl disulfide, allyl mercaptan and dipropyl disulfide (some in Figure 1.16), have been tested separately against *Giardia intestinalis*, displaying IC$_{50}$ values ranging from 100 to 1300 µg/ml (Table 1.14).$^{[85]}$

### Table 1.14. Antiprotazoal Activity of Garlic Extracts.

<table>
<thead>
<tr>
<th>Structure</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>250</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>550</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>1300</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>1300</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>100</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>200</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>300</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>37</td>
</tr>
<tr>
<td>$\text{S} \ \text{S}$</td>
<td>450</td>
</tr>
</tbody>
</table>
1.6.2 Thiols

A novel sesquiterpene named T-cadinthiol (74), also shows significant antiparasitic properties. This terpenoid metabolite, with four fixed stereocenters, displays activity towards cultured *Plasmodium falciparum*, a species of malaria, with an IC\textsubscript{50} of 3.6 µg/ml. The mode of action of this agent is still unexplored, however, and a number of derivatives of 74, including the corresponding alcohol analogue, were tested and shown to have absolutely no biological activity. The thiol group appears to be essential for anti-parasitic effects.

![Figure (1.51). Antiprotazoal Agent T-Cadinthiol.](image)

Until the availability of Cymelarsan (75) (melarsamine hydrochloride), treatment of African trypanosomiasis had relied heavily on antiviral agents which have a number of strong side effects. A successful attempt to increase the activity of this drug, by derivatizing the thiol groups to get the level of affinity between arsenic and sulfur atoms optimal for biological activity, has been reported. The most active thiol system is the propane-1,3-dithiol (76) (2-merlarsenyl). In fact, 2-merlarsenyl is twice as potent as Cymelasan against *Trypanosoma brucei brucei* strains (0.025 versus 0.05 µM concentration to terminate all growth in 1 hour). It is believed that in aqueous solution Cymelasan is in equilibrium with the hydrolyzed oxide form (melarsen oxide), which has lost one thiol group and thus half the activity.
1.6.3 Sulfides

Fasciolosis (Fasciola hepatica) is a serious parasitic disease in humans and livestock. Few new anti-fasciolitic compounds have been marketed since triclabendazole (TCBZ), a benzimidazole used routinely in veterinary medicine since 1983 and for human use, in some regions, since 1989, was patented in 1978.\textsuperscript{[90]} A new bioactive derivative of TCBZ, 5-chloro-2-methylthio-6-(1-naphthyloxy)-1\textsubscript{H}-benzimidazole (77) has been recently discovered.\textsuperscript{[91]} While this analogue has an effective dose of 15 mg/kg with 100\% effectivity, the marketed TCBZ displays a 5 to 10 mg/kg effective dose against Fasciola hepatica. The mechanism of action of this analogue has not been elucidated, and the role of the sulfide moiety is not known.

Figure (1.53). A Methylthiobenzimidazole Fasciolicide
1.6.4 Pentasulfide

Another anti-malarial agent, Lissoclinotoxin A (45), has demonstrated potent activity towards a resistant strain of the parasite *Plasmodium falciparum.*[^55] Lissoclinotoxin A is intermediate in activity compared to the usual antimalarials quinine, mefloquine, halofantrine and chloroquine, with an IC$_{50}$ of 296 nM (Table 1.15). Although not yet defined conclusively, its mechanism is likely similar to that described for di- and trisulfide antibacterials.

Table 1.15. Anti-Malarial Activities of Lissoclinotoxin A and a Few Clinical Standards.

<table>
<thead>
<tr>
<th>Anti-Malaria Agent</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lissoclinotoxin A (45)</td>
<td>296</td>
</tr>
<tr>
<td>Quinine</td>
<td>350</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>40</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>2</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>580</td>
</tr>
</tbody>
</table>

1.7.1 Conclusions & Future Prospects

Garlic formulations have been used for thousands of years for the treatment of many types of infections. We now know that the attributable anti-infective effects are caused by the active organosulfur species within. The failure to commercialization these compounds most likely stems from their volatility and instability. However, other active sulfur reagents have been discovered, or synthesized, which have similar or better biological activities with good stabilities and very low vapor pressures. Numerous sulfur compounds are yet to be evaluated for biological activity. The precise targets and modes
of action of many sulfur reagents are still unexamined. It has been proposed that the thiol-disulfide redox metabolisms of infectious organisms might serve as a potentially valuable target for development of new anti-infectives.\textsuperscript{[11]} For decades, no new targets have been discovered and brought to bear in clinical usage, even though these systems are essential for pathogenic growth and viability. The rapid procession of resistance to available antimicrobials, which target essentially three types of cell processes (cell wall synthesis, protein synthesis, and DNA synthesis), is depleting the current arsenal of antibiotics that remain effective. Often, these redox systems are extraordinarily divergent from mammalian physiology and therefore provide targets where selectivity should be very easy to come by. Stripping these infectious agents of their natural antioxidants will leave them wide open to the stresses of the external environment, or cause them to invest significant resources to develop resistance mechanisms for survival. Therefore compounds that recognize these systems should be excellent drug candidates. The genes for these systems, in a number of microbes, have been cloned and sequenced. Imaginative, novel designs and development of new structural classes of anti-infective agents, with the intention of affecting these thiol-disulfide redox systems, is a rational but nearly unexplored avenue of drug discovery and is the focus of this dissertation.

An introduction of classical antibiotics follows in Chapter 2. The detailed organic synthetic methodology used to generate this new class of $\beta$-lactams is provided in Chapter 3. An explanation of how $N$-thiolated $\beta$-lactams have been found to be active organosulfur agents against bacteria, such as \textit{Staphylococcus} and \textit{Bacillus}, fungi such as
*Candida*, and even neoplastic systems, such as those related to leukemia and cancerous tumors, then follows in Chapters 4 through 6.
2.1.1 Introduction

*Staphylococcus aureus*, a species of bacteria which is often referred to as "staph", can live harmlessly on many skin surfaces, especially around portals such as the nose, mouth, genitals, and rectum. But when the skin is punctured or broken for any reason, *S. aureus* can enter the wound and cause an infection. *S. aureus* can cause folliculitis, boils, scalded skin syndrome, impetigo, toxic shock syndrome, cellulitis, and other types of infections. After the discovery of penicillin (78) by Alexander Fleming in 1928, the elucidation of a crystal structure by Hodgekins and Rodgers-Low, and the scale-up production processes of Flory and Chain, *S. aureus* infections became very treatable by penicillin, and eventually by a variety of penicillin analogues 78-82.

![Penicillin analogues](image)

Figure (2.1) Penicillin analogues.
During the 1960’s and 70’s a number of other β-lactam antibiotic classes, with the ability to inhibit growth of \textit{S. aureus} as well as other bacteria, were either discovered from natural sources or generated via synthetic manipulations \((2-4,83-86)\).

\begin{align*}
\text{penicillins} & \quad 2 \\
\text{cephalosporins} & \quad 3 \\
\text{penams} & \quad 4 \\
\text{carbapenams} & \quad 83 \\
\text{clavulanic acids} & \quad 84 \\
\text{nocardicins} & \quad 85 \\
\text{monobactams} & \quad 86
\end{align*}

\textbf{Figure (2.2) Common Classes of β-Lactam Antibiotics.}

Unfortunately, the efficacy of these drugs has steadily declined since their initial conception, due to the ability of \textit{S. aureus} to acquire resistance mechanisms. Methicillin, a standard lactam for measuring resistance, resistant \textit{Staphylococcus aureus} (MRSA), is
a multi-drug resistant bacteria whose immunity to antibiotics was first discovered in 1961 (only one year after clinical introduction of methicillin). The percentage of *S. aureus* strains with resistance to standard β-lactam antibiotics has doubled over the past fourteen years, from 30% to 60% as seen in Figure 2.3.

![Proportion of *S. aureus* Nosocomial Infections Resistant to Oxacillin (MRSA) Among Intensive Care Unit Patients, 1989-2003](image)

**Figure (2.3) Increase of *S. aureus* Resistance, as Reported by the Centers for Disease Control (CDC).**

The classical method of MRSA infection is acquisition during unrelated treatments in a hospital setting. For example, a patient may visit a hospital for a puncture wound and become infected with MRSA. With low drug efficacies against these infections, it often becomes quite difficult to treat MRSA. Even other types of antibiotics, such as Vancomycin, considered the last line of defense against MRSA, are now exhibiting lower efficacy levels due to acquired resistance.
Recent headlines are providing additional urgency to develop new antibiotics for the treatment of resistant infections, as reports have come out that MRSA is no longer constrained to the hospital setting. This year a large number of the MRSA infections reported included infections acquired in the community. A group of novel drugs, whose mode of action are not affected by current resistance pathways, are therefore in high demand as this scourge is becoming untreatable.

*Bacillus anthracis* (Anthrax) has also received much attention, in recent years, as a matter of national security. Currently the most effective antibiotic for this infection is Ciprofloxacin (Cipro). Generating more, equally potent, defenses against this potential terrorist weapon is certainly an appropriate goal in light of the evolution of drug resistance seen in other bacterial systems. This chapter will focus on the development of antibiotic agents for MRSA and Anthrax, and on investigations into their modes of action.

2.2.1 β-Lactam Antibiotics: Anti-MRSA

Penicillins and Cephalosporins have disputably been the most clinically important group of compounds discovered to date. Extensive investigations have led to a clear picture of biosynthesis and antibacterial modes of action.

The biosynthetic pathway of penicillin begins with the condensation of L-aminoacidipic acid (87), L-cysteine (88) and L-valine (89) via δ-(L-α-aminoacidipyl)-L-cysteinyld-valine (ACV) synthetase to form a tripeptide intermediate (89) with epimerization at the α-carbon of the valine residue.
Figure (2.4) Open ring Penicillin Biosynthetic Intermediate.

Next, is the annulation of the bicyclic core skeleton to isopenicillin N (IPN) (91), by what else but IPN synthase, followed by epimerization of the α-aminoadipoyl α-carbon, by IPN epimerase, to provide penicillin N.

Figure (2.5) Annulation and Epimerization of Open ring Lactam.

Need a cephalosporin? Ring expansion and hydroxylation of penicillin N, by deacetoxycephalosporin C/deacetyl cephalosporin C (DAOC/DAC) synthase(s), effectually produces deacetyl cephalosporin C (92) from penicillin N.
Penicillins are well known for there mode of action involving the coupling with penicillin binding proteins (PBP’s). In this manner, penicillin antibiotics kill bacteria by disrupting cell wall synthesis and as effected cells attempt to expand they become deformed or even lysed. Simply speaking, penicillin binds to a serine residue of the transpeptidase enzyme (the primary enzyme of cell wall crosslinking), irreversibly, and inhibits that enzyme from being involved in cell wall crosslinking. Like a broken link in a chain, this results in a malformed peptidoglycan network and thus deformed or lysed cells. Without this strong crosslinked network, ordinary osmotic pressure within the cells can cause them to rupture.

Due to widespread over usage, β-lactam antibiotics continue to succumb to developed resistance. Researchers have had great success synthesizing new lactam...
derivatives to combat resistance. However, none of these is immune to the cell defense enzyme β-lactamase, except the N-thiolated β-lactams (93) (Chapters 3 through 6).

\[
\text{Figure 2.8 } N\text{-Thiolated β-Lactam Antibiotics.}
\]

Despite the continuous increase in resistance to multiple analogs of cephalosporins and penicillins, these drugs remain the most abundantly prescribed class of antibiotics. Primary resistance to these antibiotics is related to cleavage of the β-lactam ring by a specialized bacterial defense enzyme that is released extracellularly, β-lactamase. β-Lactamase interacts with the lactam ring in much the same way as PBP’s do, by breaking open the four membered ring. This ring opened form does not inhibit cell wall synthesis or disrupt any other cell function. So, in effect, the drug is deactivated by formation of the lactam-lactamase adduct, before it can reach the transpeptidase target. Except in the case of N-thiolated β-lactams, this weakness is ubiquitous amongst β-lactam antibiotics due to the extreme lability of this strained ring system. Again, the N-thiolated β-lactams are unaffected by β-lactamase and are discussed in greater detail in the following chapters.

\[
\text{Figure (2.9) β-Lactamase Ring Opening of Penicillin.}
\]
2.3.1 Vancomycin and the Peptide Antibiotics

Vancomycin (94) is the most successful clinical treatment for MRSA infections thus far. Like the standard β-lactam family, this antibiotic acts by interrupting cell wall synthesis, albeit in a different way. Part of a family of peptide antibiotics, the glycopeptides, Vancomycin is a potent antibacterial agent which, unlike ordinary β-lactams, is impervious to β-lactamase defenses. However, bacteria with specific defenses that can deactivate Vancomycin have developed over the course of time. The first of these was from a strain of enterococci, now termed Vancomycin resistant enterococci (VRE). The resistance has since jumped, via a plasmid, to S. aureus, alerting a need for new antibiotics to fight this cross-resistance. Synthetic modifications have successfully defeated VRE and Vancomycin resistant strains of S. aureus (VRSA), however development of strains with resistance to these analogues is bound to occur. Other peptide antibiotics, depsipeptides and lipopeptides, are reported to display potent activities against VRSA, but are, as of yet, not clinically available.
Figure (2.10) Vancomycin.

Figure (2.11) Teicoplanin, an Example of a Semi-synthetic Analogue of Vancomycin.
2.4.1 DNA and Protein Synthesis Inhibiting Antibiotics

Although DNA synthesis inhibition or the cleavage of fully formed DNA are accepted targets of antibacterial exploitation, there currently are no clinically available analogues. One group of promising DNA synthesis inhibitors are the coumarins. These type II topoisomerase inhibitors have shown great selectivity in targeting DNA gyrase\(^96\). X-ray crystallographic analysis of a 24-kDa N-terminal fragment of DNA gyrase with bound novobiocin (96), a typical coumarin, reveals that the antibiotic binds competitively at the ATP site and that the aminocoumarin bicyclic ring is the scaffold for presenting the L-noviosyl sugar moiety to interact with the gyrase.\(^97\)

![Figure (2.12) Novobiocin.](image)

Linezolid (97, trade name Zyvox), an oxazolidinone, is a fairly recent addition to the clinically available arsenal of antibiotics known to affect \(S. \text{ aureus}\). Most, oxazolidinones are protein synthesis inhibitors by irreversible binding to ribosomes. Their bacteriostatic activities are most pronounced amongst Gram-positive bacteria.
2.5.1 Anthrax

*Bacillus anthracis*, Anthrax, was once a common problem amongst people and cattle. With the advent of antibiotics like penicillin, doxycycline, and amoxicillin this scourge was all but wiped out. Unfortunately, Anthrax has now become a weapon which is popular amongst terrorists. While, these antibiotics can be very effective against susceptible strains, a better defense has been developed, Ciprofilaxin (98). Ciprofilaxin retains potent activity against most strains of *anthracis* and even acts effectively as a prophylactic treatment for suspected exposures. However, resistant strains are emerging. A nalidixic acid-resistant strain of *anthracis* has already been discovered with a 10-fold lower susceptibility to Ciprofilaxin than other strains. Therefore, it is equally important to find antibiotics with a novel mode of action which impedes induction of resistance amongst these bacteria.

Figure (2.13) Linezolid.
Figure (2.14) Ciprofloxacin.

2.6.1 Conclusions

For a number of years, pharmaceutical companies all but abandoned the search for new antibiotics, out of complacency. Now, as the advent of resistant bacteria is on a steady climb, researchers, industrial and academic, are coming back into this area of research. While new derivatives of currently available drugs seem to be a quick-fix, agents with entirely novel modes of action must be discovered to establish a more permanent solution to the ever-present problem of antibiotic resistance.
CHAPTER THREE
N-ORGANOTHIOLATED β-LACTAMS: SYNTHESIS

3.1.1 A Brief History

Since Alexander Fleming’s discovery of penicillin in 1928, β-lactam antibiotics have developed into the most profound medical discovery to date. Though, it was not until 1940 that Florey and Chain showed penicillin’s value as a potent systemic antibacterial agent. Fortunately, this discovery came just in time to be of use during World War II, thus saving many lives. Although bacterial resistance was already rearing its ugly head just a year after penicillin’s introduction, it was not until the 1960’s that a multitude of β-lactam analogues, to defeat these resistant microbes, were either discovered from natural products or developed synthetically, as discussed in Chapter 2. Most structurally related to the N-thiolated β-lactam project is the first monocyclic class of β-lactam antibiotics, the monobactams (discussed in chapter 2). Mechanistically however, these agents are drastically different from N-thiolated β-lactams. The N-thiolated β-lactams are truly a novel class of antibiotics, separate from penicillins, cephalosporins, carbapenams, penams and monobactams, not only by structural differences but also in their mechanisms of antibacterial activity (as will be shown in chapter 5).

In earlier studies an N-sulfenylated β-lactam (99) was isolated on synthetic route to the generation of novel isopenems (100). Testing this intermediate against a panel of
microbes revealed potent inhibition of an array of bacteria, including MRSA. This sparked our laboratory’s interest in studying \( N \)-thiolated monocyclic \( \beta \)-lactams.

![Chemical structure](image)

Figure (3.1) Discovery of Novel \( N \)-Substituted Monocyclic \( \beta \)-lactams.

3.2.0 Synthesis Towards an SAR

3.2.1 Introduction

With five possible sites of substitution on the lactam ring, two at \( C_3 \) (\( R_2 \) & \( R_3 \)), two at \( C_4 \) (\( R_4 \) & \( R_5 \)) and one at \( N_1 \) (\( R_1 \)), a large variety of variations could be explored as long as they are synthetically feasible (Figure 3.2).

![Chemical structure](image)

Figure (3.2) \( \beta \)-Lactam Substitutions

Initial structure-activity studies focused on determining the importance of the \( C_4 \) substituent.\(^{[100]}\) Although monocyclic \( \beta \)-lactams have antimicrobial activity with their monocyclic form, certain amido functional groups are required for their activity. However, substitutions at either \( C_3 \) or \( C_4 \) on these \( N \)-thiolated monocyclic \( \beta \)-lactams demonstrated no prominent effect on antimicrobial activity.\(^{[100]}\) Activities were different
for various substitutions at these loci, but the absence or presence of saturation levels or functional groups did not turn activity on or off like a switch. So, for all intense purposes, variation of substituents at sites $R_2$ through $R_5$ does not radically affect antibacterial activity. For example, changing the substitution of $R_2$ from a methoxy group to an acetoxy group has almost no effect. As long as there is some electronegative group at $R_2$ or $R_3$, activity does not seem to change drastically. In fact, with no substituent at $C_3$, antibacterial activity still persists. As well, a variety of different halo-substituted aromatic functionalities $R_5$ affords very little flux in antibacterial activity. As long as an unsaturated group or electronegative element exists at $C_4$ antibacterial activity changes very little. Lipophilicity can assist $N$-thiolated $\beta$-lactams though, as demonstrated by better bioactivities for derivatives having longer $C_3$ side chains. However, total absence of this side chain results in a compound which still maintains potent biological activities.

This led me to believe that activity had a greater dependence on some other structural motif than these side chains. Substitution at $R_1$, indeed, is paramount to activity (Table 3.1). Without an appropriate functional group at this position biological activity is non-existent. The focus of this chapter will be the synthesis of varied $N$-substituted $\beta$-lactams for the SAR which follows in the next chapter.
Table 3.1 Effect of β-Lactam Substitutions on Antibacterial Activities.

3.2.2 Synthetic Strategy

The synthesis of these $N$-thiolated β-lactams, like that in Figure 3.3, is comprised of two major steps. First, the annulation of the β-lactam ring from an acid chloride and imine. Secondly, $N$-thiolation (or sulfenylation) of the β-lactam nitrogen.

![Retrosynthetic Analysis](image)

**Figure (3.3) Retrosynthetic Analysis**

Closer inspection of the standard synthetic steps towards $N$-thiolated β-lactam syntheses reveals a few additional nuances (Figure 3.4). First is the generation of an appropriate acid chloride. Variation of the acid sidechain will directly translate to a
variation in the identity of the functional groups at C$_3$. Next, a simple synthesis of an imine is performed by the addition of para-anisidine with an aldehyde of choice. Here, as well, choice of the starting material translates directly into an β-lactam substitution pattern, for whatever side groups are attached to the chosen imine will become the substituents at C$_4$. With these coupling agents in hand, the aforementioned annulation occurs by a Staudinger type coupling to generated an $N$-protected β-lactam. Deprotection (or dearylation) is induced to generate the $N$-protio β-lactam. Finally, $N$-thiolation occurs readily with an appropriate transfer reagent. Greater detail follows in the subsequent sections.

**Figure (3.4) Standard Synthetic Overview**
3.2.2 Acid Chloride Synthesis

Although some of the necessary acid chlorides were available for purchase, the low purity and moderately high expense made the synthesis of these reagents more attractive. The synthesis of these acid chlorides is simple and straightforward. Since the free acids were inexpensive and of relatively high purity upon purchase, they were used without further purification. The activating agent, thionyl chloride, was always racked with impurities such as sulfates and sulfoxides. A two step distillation process, with the use of linseed oil, proceeded to produce fairly pure thionyl chloride. This was then immediately used after purification. In most cases the resulting acid chloride required purification via one last fractional distillation.

![Figure (3.5) Acid Chlorination](image)

3.2.3 Imine Synthesis

\textit{N-}(2-Chlorobenzylidene)-4-methoxybenzenamine (105) was easily prepared via the condensation of 2-chlorobenzaldehyde (103) and 4-anisidine (104). Both reagents, however, required purification before use, even when newly purchased. The amine (105) was recrystallized from water and dried under reduced pressure. Aldehyde (103) was purified via atmospheric distillation. Originally the reaction was commenced by dissolution of 4-anisidine in freshly distilled methylene chloride, followed by pipette
addition of 2-chlorobenzaldehyde. It was later discovered that the presence of the solvent was completely unnecessary. Later reactions were performed by simple addition of (6) to (5). In the solvent free methodology a significant portion of released thermal energy could be observed. Camphorsulfonic was added in a few instances to trigger the condensation, however this was usually unnecessary. In either case, long, sharp, bright yellow crystals of product were formed along with an equivalent amount of water. A spot to spot conversion could also be followed by thin layer chromatography (TLC), but reaction completion was rarely of doubt. Proton nuclear magnetic resonance (¹HNMR) spectroscopy was used to confirm complete formation of the imine.

![Imine Formation](image.png)

**Figure (3.6) Imine Formation**

### 3.2.4 Staudinger Coupling

The Staudinger coupling is a formal [2+2] cycloaddition process of an acid chloride (102) and a Schiff base, imine (105). The Staudinger coupling is the most prevalent method of monocyclic β-lactam formation and it is successfully used to prepare N-protected lactam (106). In this case, a ketene-mediated cycloaddition is believed to be one of the possible mechanisms of annulation, versus a direct acylation of the imine. Ketene formation is initiated via deprotonation by a Bronsted-Lowry base, triethylamine or diisopropylethylamine (Hunig’s base). This solution is normally refluxed in freshly
distilled methylene chloride for 12 to 24 hours and closely monitored by TLC. The product obtained always demonstrated the cis relative stereochemistry, although this is not the case under modified conditions. In the case of Figure 4.3 the imine used is always of the E-configuration which ultimately leads to the cis formation if the cyclization is truly [2+2]. It is possible, for the cases where a trans substituted lactam is formed via a Staudinger coupling, that either a Z-imine was used or a potent nucleophile can interrupt the cyclization and allow free rotation of the imine unsaturation. All of these steps are part of an equilibrium, therefore it is also possible that the lactam ring may reopen and interconvert between forms. Regardless of the mechanistic pathway, this cyclization always provided a racemic mixture of cis diastereomers. Exploration of the properties of each separate diastereomer is detailed in later sections.
3.2.5 Dearylation

A procedure was reported in the literature which effectively deprotects lactam (106) by oxidative cleavage of the N-methoxyphenyl carbon-nitrogen bond. In this case, ceric ammonium nitrate (CAN) is used as the oxidant in an aqueous acetonitrile
solution. CAN initiates a radical mechanism which results in a di-hydroxylated, pre-quinone form. Washing with a mild reagent, such as sodium bisulfite, drives the reaction to completion with generation of the protonated lactam (107) and one equivalent of benzoquinone (108). In small scale reactions, less than 2 grams, dearylation with three equivalents of CAN proceeded, at zero degrees Celsius, in moderate yields within 10 minutes. Larger scale reactions also proceeded within 10 minutes, however yields suffered significantly at these larger quantities. Once the reaction was determined to be complete, by TLC, the reaction mixture was poured into water, extracted with ethyl acetate, washed with sodium bisulfite and sodium bicarbonate, dried over magnesium sulfate and concentrated via rotary evaporation. Attempts to increase yields with extended reaction times, variations in temperatures or different stiochiometric ratios failed to provide any benefit.

\[
\begin{align*}
&\text{106} \quad \text{CAN} \quad \text{CH}_3\text{CN-H}_2\text{O} \\
\rightarrow &\quad \text{107} \quad \text{108}
\end{align*}
\]

**Figure (3.8) Dearylation.**

### 3.2.6 \(N\)-Substitution: Basis for the Phthalimide Transfer Reagent

A series of sulfenyl, sulfinyl, and sulfonyl analogues was synthesized using \(N\)-thiolating reagents and some direct modifications. Extremely important to the thiolation of the monocyclic \(\beta\)-lactams is an organothio-transfer reagent, the \(N\)-thiolated phthalimide. More direct methods failed to succeed (Figure 3.10). What seemed like the
most direct method was to first generate the sulfenyl chloride which could then be attacked by the deprotonated β-lactam nitrogen. However, this resulted in opening of the lactam ring, as crude $^1$HNR spectra show absolutely no lactam ring protons. The most likely culprit for opening the ring in this case is the ejected chloride anion. Once the sulfur is installed, it is very plausible that the chloride anion could act as a nucleophile, attacking the carbonyl of the ring and forcing the ring to cleave between the carbonyl-carbon and nitrogen bond, although this was never isolated. Regardless of conditions, this pathway yielded no product, yet consumed all of the $N$-protio lactam starting material.

A gentler method was then attempted where the sulfenyl chloride was first reacted with deprotonated succinimide to generate the $N$-thiolated succinimide. This reagent transfers the organothiol group to the lactam with extremely poor yields, below usable limits. However, no lysis of the lactam ring occurred, making this pathway somewhat better than direct introduction using sulfenyl chlorides.

Figure (3.9) Failed Direct $N$-Organothiolation Method.
Figure (3.10) Failed Transfer $N$-Organothiolation Method.

The best method for sulfonylating the lactam-nitrogen was via another transfer reagent 111 made from phthalimide. A number of organothiol groups could be transferred to the lactam with this type of reagent, whether by using a tertiary amine or carbonate base.

Figure (3.11) $N$-Organothiolation Methods.

Synthetic pathways where a sulfur functionality is already installed on the imine prior to annulation may be plausible for some trityl systems, however this method was
Although often problematic, thio-transfer seems to be the best method, as shown in Figure 3.11.

Most thiol reactions can be chlorinated in a straightforward manner by dissolution in a benzene solution which contains an equimolar quantity of dissolved chlorine gas. This sulfenyl chloride solution can then be added, in situ, to a preformed slurry of Hunig’s base and phthalimide in ice cold benzene. As the reaction proceeds and the thiolated phthalimide 111 is formed, the mixture dissolves into a clear solution. Unreacted phthalimide 112 remains as a solid and can be easily filtered off. After aqueous workup with solutions of bicarbonate, bisulfite and saturated salt, the dried and concentrated solid can be easily purified via recrystallization from ethanol. For cases which did not proceed smoothly via this route, greater success could be found via reaction of the thiol with N-bromo phthalimide reagent 113. This method brought higher yields to reactions with longer chain thiols.

![Phthalimide Synthesis](image)

**Figure (3.12) Phthalimide Synthesis.**

The yields for the synthesis of N-thiolated phthalimide reagents bearing large aryl substituents were increased by using potassium carbonate and sonication rather than Hunig’s base or sodium hydride. Likewise, some N-thio phthalimides could be formed by first oxidizing the thiol to the thiosulfone via mCBPA, which is more easily attacked by phthalimide. Alkyl and aryl substituents were attainable through these routes, however,
great resistance was confronted with all attempts to install sulfurs of different oxidation states or hetero-groups on the thiol side chain.

![Chemical structures](image)

**Figure (3.13) Alternative Phthalimide Synthesis Routes.**

Synthetically, the *N*-octylthiolated 109e lactam was extraordinarily challenging at the phthalimide transfer reagent synthesis step. More than 10 sets of conditions were attempted before any *N*-octylthiophthalimide was observed, and even in this one case the yield was extremely poor, as the equilibrium between octylthiol and 1,2-dioctyl disulfide heavily favored the disulfide. The yield was improved by *m*CPBA oxidation of the disulfide to the thiosulfonate, making the chlorination and addition to phthalimide easier.

The *N*-butylthio lactam 109d was generated quite simply using *N*-bromophthalimide with n-butylthiol and triethylamine, where the other three analogues were synthesized in the highest yields from the chlorinated thiol via addition of chlorine gas to the thiols, followed by addition of phthalimide and a tertiary amine base.

Also, transfer of organothio-groups from the phthalimide to the lactam was seriously hindered by steric bulk. Although phthalimide transfer reagent synthesis was achieved for highly hindered organothio systems, anything larger or more branched
proved difficult to transfer to the lactam by this method. sec-Butylthio and tert-butylthio groups were successfully installed, but with difficulty. Phthalimide reagents which were either unsuccessfully prepared, as with the branched alkyl chains, or were prepared but unsuccessfully used to make the corresponding β-lactam derivatives, as with the heteroatom containing sidechains, are shown in Figure 3.14.

Figure (3.14) Unsuccessful Phthalimide-based Transfers.
As described, most of the \(N\)-organothio phthalimides were prepared via chlorination of the corresponding thiol or disulfide to generate the sulfonyl chloride in situ and then added dropwise into a chilled flask containing phthalimide, and triethylamine or Hunig’s base. After purification by flash chromatography the phthalimide reagents (111) were then added to an equimolar quantities of \(\text{\textit{N}}\)-protio \(\beta\)-lactam (108) and an amine base, usually Hunig’s base or triethylamine, and refluxed in dry methylene chloride or benzene for 1 to 10 days. After aqueous workup with solutions of bicarbonate, bisulfite and saturated salt, the resulting phthalimide was removed via trituration with chloroform. Residual organothiolated phthalimide was removed, painstakingly, via column chromatography.

### 3.3.1 Asymmetric Synthesis

Of course not all of the desired thiols or disulfides needed for these studies were commercially available. For example, when this project was initiated enantiomerically pure thiols were unavailable and enantiomerically pure alcohol precursors were cost prohibitive. So, the first method attempted to generate enantiomerically pure thiols involved enzymatic resolution of esters 114 via Lipase PS-30, a generous gift from the Amano enzyme company. In a slightly less than pH 7 buffered solution, complete 50:50 resolution of racemic esters could be accomplished in about a week. After filtration through Celite, the alcohol 115 and unaffected ester 116 were separated via column chromatography. Mitsunobu coupling of 115 and acetylated 116, however, to afford 117 and 118 did not proceed so smoothly. Steric hindrance was a serious problem for these secondary centers, forcing the crude yield to be quite low. Though a similar literature
procedure exists for the thioester formation starting from 2-nonanol, the shorter chain analogues proved to be much more difficult to purify due to their volatility. Reduction of the thioester to the free thiol (119 and 120) also proved problematic. While some conversion occurred, the volatility of the thiol product made purification impossible (Figure 3.16).

Figure (3.15) Initial Enzymatic Scheme Towards Enantiomerically Pure Thiols.

Next, a pathway was tried which involved a chirality inducing, nicotine based rearrangement process.[95] The first step of this process involved the hydride assisted attack of racemic 2-butanol 121 on carbon disulfide, which in turn activated attack on methyl iodide to form dithioate 122. Rearrangement of this sec-butyloxy-dithioate 122 to the sec-butythio-dithioate 125 proceeded via a dioxy-(S)-(−)-nicotine reagent 124 (from oxidation of 123 via mCPBA). Unfortunately, the following reduction step suffered
similar problems of low yields. Purification was again made impossible by the extreme volatility of the product thiol 119.

![Scheme Diagram](image)

**Figure (3.16) Nicotine Based Scheme Towards Enantiomerically Pure Thiols.**

At this point, attempts to obtain enantiomerically pure sec-butylthiol were abandoned in favor of a less volatile system such as 2-phenylethylthiol. Beginning with the acetic ester 126, Lipase PS-30 was used again for enzymatic resolution. This worked very well. After filtration and concentration, purification simply involved the separation of the two layers, alcohol 127 and unaffected acetic ester 128. Unfortunately, this is as far as this pathway would proceed, for the subsequent Mitsunobu substitution on the alcohol would not take place at any appreciable level to generate enough product (129 and 130) for purification.

84
Figure (3.17) Second Enzymatic Scheme Towards Enantiomerically Pure Thiols.

The ability to generate the enantiomerically pure alcohol 127 suggested that it may provide a way to produce the enantiomerically pure thiol. This pathway was first tested with a racemic mixture of 2-phenylethanol (131), which is easier to handle than the more volatile, more soluble 2-butanol (Figure 3.18). Chloride substitution of the alcohol with thionyl chloride was straightforward and proceeded smoothly. This reaction was assumed to go through an $S_{N}2$ process to afford the stereochemically-inverted chloride 132. Thioesterification also proceeded smoothly by a second nucleophilic substitution with thiolacetic acid to generate thioester 133. Next, a one-pot, two-step procedure produced the $N$-thiolated phthalimide 134, without any free thiol intermediate.[84] The first step involves the sulfuryl chloride-induced oxidative cleavage of 133 to generate the sulfenyl chloride. In step two, this sulfinyl chloride solution is added to a preformed slurry of phthalimide and Hunig’s base to generate the $N$-thiolated phthalimide 134. With
this transfer reagent in hand, production of the \(N\)-(1-phenylethyl)thiolated \(\beta\)-lactam 135 proceeded smoothly by addition of one equivalent of the \(N\)-protio \(\beta\)-lactam 108 and three equivalents of triethylamine in a benzene reflux. Adduct 135 was obtained as an equimolar mixture of racemic diastereomers.

### Figure (3.18) Successful Synthesis of an \(N\)-Thiolated \(\beta\)-Lactam from an Alcohol.

With the absence of a free thiol in this synthesis, this appeared to be an effective method for generation of the enantiomerically pure sec-butylthio \(\beta\)-lactams. At about this time, the R and S sec-butanols became available at a reduced cost and were therefore purchased, instead of being independently synthesized. Alcohols in hand, each one was chlorinated, thioesterified, and eventually converted to the \(N\)-thio lactams via the route developed in Figure 3.19 for the R enantiomer (136-140). The process was repeated to generate the S enantiomer (141-145).
Figure (3.19) Enantiospecific Synthesis of Enantiomerically Pure N-sec-Butylthio β-Lactams from Optically-pure Alcohols.

To prepare enantiomerically and diastereomerically pure lactams for more detailed bioassays it was necessary to couple an enantiomerically pure version of lactam 108 and 139. Fortunately, this proceeded smoothly utilizing the same enzymatic resolution scheme described above.\footnote{103} To provide a handle for resolution, the C₃ acetylated β-lactam 146 was synthesized via the standard procedures previously described, simply starting with acetoxyacetyl chloride instead of methoxyacetyl chloride. As developed earlier through a collaboration with Dr. Bisht (University of South Florida), Lipase PS-30 efficiently hydrolyzed only one enantiomer 147 from the racemic mixture of acetoxy-lactams, leaving the other enantiomer 148 unaffected (with greater than 97% ee’s). These two products were then separated via column chromatography. The
hydrolyzed enantiomer, 147, was re-acetylated by acetic anhydride in pyridine to give (+) 149.\textsuperscript{[104]}

Figure (3.20) Successful Synthesis of Enantiomerically Pure β-Lactams.

With both lactam enantiomers 148 and 149 prepared, what followed was the \textit{N}-dearylation with ceric ammonium nitrate to produce enantiomerically pure \textit{N}-protio β-lactams 150 and 151, respectively.
Figure (3.21) Dearylation of Enantiomeric β-Lactams 150 and 151.

By applying both R and S sec-butylthio phthalimde transfer reagents 142 and 143 to each of these β-lactam enantiomers 50 and 51, four separate stereoisomers were produced (152-155). Lactams 152 and 155 are enantiomeric with each other, as are compounds 153 and 154. These four compounds were tested independently for antibacterial activities.
3.4.1 Oxidation

For comparison of the N-sulfenyl lactams just described, more highly oxidized sulfur side chain analogues were also needed for biological screening. The structures of these compounds are shown below (Figures 3.25 and 3.26). Unfortunately, the preparation of these derivatives by thiolation of the N-protio lactam was made difficult by the inability to synthesize the necessary N-thio phthalimide transfer reagents, such as 156.

Figure (3.23) Attempted Preparation of N-Sulfinyl Phthalimide

Oxidation of the phthalimide sulfenamide was successful, but transfer of the sulfinyl group to the lactam did not work under the usual conditions (refluxing dichloromethane, Hunig’s base and N-protio β-lactam) (Figure 3.24).
Figure (3.24) Second Ineffectual Method of Sulfoxyl and Sulfonyl Transfer

The $N$-sulfinyl and $N$-sulfonyl $\beta$-lactams were instead prepared from the $N$-sulfenyl lactams via oxidation (Figure 3.25). There were two sets of oxidation conditions that were successful for this procedure, depending on the organothio substituent. One is by $m$CPBA in ether, and the other is by 30% hydrogen peroxide in acetic acid. In the case of the $N$-cyclohexylthio lactam $109i$, only the peroxide method worked to give the $N$-sulfinyl product $157b$, whereas $m$CPBA cleaved the sulfur-nitrogen bond. Re-subjection of this $N$-sulfinyl compound to the hydrogen peroxide conditions afforded $N$-sulfonyl product $158b$ in a nearly quantitative yield. This pathway also worked well for the generation of $N$-phenylsulfinyl $157a$ and $N$-phenylsulfonyl $158a$ $\beta$-lactams from the $N$-phenylthio $\beta$-lactam $109j$. In the other case examined, where the sulfur sidechain is the sec-butyl in $109g$, peroxide was completely ineffectual. Here, $m$CPBA allowed for the conversion of the sulfenamide to the $N$-sulfinyl lactam $157c$; however further oxidation to
the sulfonamide 158c with additional mCPBA could not be accomplished. Instead, cleavage of the nitrogen-sulfur bond was again observed.

\[
\begin{align*}
\text{H}_3\text{C} & \text{-} \text{O} \quad \text{H}_3\text{C} & \text{-} \text{O} \quad \text{H}_3\text{C} & \text{-} \text{O} \\
\text{N} & \text{O} \quad \text{N} & \text{O} \quad \text{N} & \text{O} \\
\text{SO}_2 & \text{-} \text{Cl} & \text{SO}_2 & \text{-} \text{Cl} & \text{SO}_2 & \text{-} \text{Cl}
\end{align*}
\]

Figure (3.25) Oxidative Routes to N-Sulfinyl and N-Sulfonyl Lactams.

N-Sulfonic acids and sulfonate salts were formed in a completely different manner than above. This turned out to be the only direct method of installing a sulfur sidechain on the lactam nitrogen. A sulfur trioxide / pyridine / DMF transfer solution was used\(^{[105]}\). This pathway directly provides the potassium N-sulfonate salt which can be converted by ion exchange to the tetrabutylammonium salt 159. Eluting the tetrabutylammonium salt 159 down a standard silica gel chromatography column gave the sulfonic acid derivative 160 in good overall yield.

\[
\begin{align*}
\text{H}_3\text{C} & \text{-} \text{O} \quad \text{H}_3\text{C} & \text{-} \text{O} \quad \text{H}_3\text{C} & \text{-} \text{O} \\
\text{N} & \text{O} \quad \text{N} & \text{O} \quad \text{N} & \text{O} \\
\text{SO}_3 & \text{-} \text{Cl} & \text{SO}_3 & \text{-} \text{Cl} & \text{SO}_3 & \text{-} \text{Cl}
\end{align*}
\]

Figure (3.26) Route to N-Sulfonate and N-Sulfonic Acid Lactams.
3.5.1 An N-Resinthiolated β-Lactam

For the purpose of trying to identify the intracellular targets of these novel antibacterial agents, as to be discussed in chapter 5, a lactam-bound polymer resin was synthesized. The hypothesis is that an intracellular target could be physically captured by the appropriate resin. The first of two methods examined in this study
involved Merrifield’s resin (161) as a solid support on which the lactam could be attached through an N-thiolation process. This chloro benzyl material was subjected to thiolation via sodium hydrosulfide in water with cetyltriethylammonium bromide. Since this commercially available resin, and the expected product, is 2% crosslinked with divinylbenzene, it is completely insoluble in the available solvents, thus making NMR verification of the product difficult without a solid phase NMR probe. However, a standard chemical test for the presence of free thiols, sodium nitroprusside, demonstrated the total absence of the expected thiolation product (162). Infrared spectroscopy also indicated the absence of the S-H stretch that would be expected around 2500 cm\(^{-1}\) for 162. Potentially, 162 could dimerize to form a disulfide but no disulfide S-S stretching peaks were observed in the expected region around 500 cm\(^{-1}\).

\[
\begin{align*}
\text{Cl} & \quad \text{NaSH} \\
\text{161} & \quad \text{SH} \\
\text{162}
\end{align*}
\]

**Figure (3.28) Attempted Direct Thiolation of Merrifield’s Resin**

Fortunately, another, although less direct, method of resin thiolation was successful. With the insolubility of this resin, workups at each stage of the synthesis were simple and consisted of a set of filtrations with boiling solvent washings. The first step of the procedure was to thioesterify Merrifield’s resin (161) with thiolacetic acid and triethylamine, a method similar to that previously used for the synthesis of chiral thiolated lactams.\(^\text{102}\) This provided thioester 163, which was confirmed by a single
carbonyl stretch which was apparent in the infrared spectrum. Next, a two-step, one-pot procedure converted thioester 163 to the N-thiolated phthalimide transfer reagent 164. This analogue was also confirmed by infrared spectroscopy through the appearance of two carbonyl stretching peaks representing the phthalimide carbonyls. The first step involved the addition of sulfuryl chloride in carbon tetrachloride to the thioester 163 to generate the in situ the sulfenyl chloride. This solution was then added to a benzene solution of phthalimide and Hunig’s base. Hunig’s base proved to be the highest yielding choice of amine bases, over triethylamine and pyridine, for this deprotonation. With the synthesis of this N-resinthiolated phthalimide transfer reagent 164 complete, the production of the N-resinthiolated β-lactam 165 followed through the standard transfer conditions of mixing 164 with N-protio β-lactam 107 in the presence of Hunig’s base. The reaction was done in benzene and refluxed for 24 hours. After completion, thorough washing of the resulting resin commenced with a myriad of solvents until no solutes were detectable by TLC or NMR. However, exposure of the resin to diisobutyl aluminum hydride (DIBAL) resulted in the release of a nearly quantitative yield of the N-protio β-lactam 107, which was observed in the 1H NMR spectrum of the post-reaction washings. As well, infrared examination of resin 165 demonstrated only one carbonyl stretching indicating the absence of the phthalimide and providing evidence to formation of the resin-lactam adduct.
Figure (3.29) Successful Synthesis of an N-Resinthiolated β-Lactam.

3.6.1 A Fluorescence System

Another synthetic target, proposed for experiments to probe the basis for biological activity of these lactams, consisted of a Forster Resonance Energy Transfer (FRET) quenching pair, to be discussed in further detail in chapter 5. The model system involved the placement of N-naphthylthio and C$_3$-dansyl groups onto the β-lactam ring, where dansyl is a fluorescence quenching accepter for naphthalene. The first attempt to synthesize this compound, 77, involved deacetylation of β-lactam 166 with potassium hydroxide in methanol to give alcohol 167, which was then deprotonated with sodium hydride and coupled with dansyl chloride. This product, 168, was isolated but could not
be further derivatized to the \(N\)-naphthylthiolated \(\beta\)-lactam. Instead, all attempts to install this thiol group with phthalimide transfer reagent 169 resulted in lactam ring cleavage. Therefore, another route was proposed in which the thiol moiety would be installed first, producing lactam 170. The dansyl substituent would then be introduced to produce FRET product 171. However, while the naphthylthiol substituent was installed smoothly, the addition of dansyl chloride resulted, again, in lactam ring cleavage. Thus, 171 could not be prepared via either route shown in Figure 3.30.

![Figure (3.30) Attempted Synthesis of a Model FRET System.](image)

Another FRET paired system was then targeted with the fluorescein analogue 172 and 4-\(N,N\)-dimethylaminoazobenzene-4’-sulfonyl (dabsyl), donor-acceptor pair. This first step of this process involved the esterification of this carboxylic acid and etherification of the alcohol with methyl iodide in an acetonitrile slurry of potassium carbonate, which proceeded but with a very small yield.
Figure (3.31) Methylation of Fluorescein.

From 173, two routes were attempted to yield the free thiol 175. The first route was meant to utilize the successful processes of past experiments by first generating the thioacetate 174, which would then be saponified to the thiol. However, thioesterification of the chloro precursor 173 completely failed. Secondly, a very direct method of arylthiolation through the use of sodium hydrosulfide in water, with the phase transfer catalyst cetyltriethylammonium bromide (CTAB), was carried out, but did not generate product.\[106\] Finally, a literature procedure for thiolating aryl halides via sodium thiophosphate dodecahydrate directly from 173 delivered about a 50% yield of 176.\[107\] Selectivity was not as high as hoped though, and a mixture of thiolation products 176a and 176b from substitution of either of the two chloride groups and 176c, from displacement of both chlorides, was obtained. This mixture appeared as one spot by thin layer chromatography and was therefore used as a mixture without attempts at separation.
This mixture was then subjected to the standard procedures of chlorination, followed by reaction of the in situ generated sulfonyl chlorides with phthalimide, to give N-thio phthalimides in a about a 40% yield of 177a-c. This mixture was then subjected directly to reaction with the N-protio β-lactam 107 in the presence of triethylamine in refluxing benzene. Visible in the crude $^1$H NMR spectrum were two doublets ($J = 5.0$ Hz) for the two β-lactam protons. As well, these doublets were significantly shifted downfield from the doublet and triplet positions of the N-protio β-lactam 107. Unfortunately, this product could not be purified. The only recoverable lactam following column 99
chromatography was the starting material, 107. Without the ability to cleanly transfer the N-fluoresceinylthio group to the β-lactam, there was no point in pursuing the C₃ dabsylated lactam.

![Chemical Structures and Reactions]

Figure (3.33) Fluoresceinylthiolation of the Phthalimide Reagent.

Figure (3.34) Fluoresceinylthiolation of the β-Lactam.

Future work should probe the viability of a coumarin/dinitrophenyl FRET paired β-lactam, which may encounter fewer synthetic problems.

Figure (3.35) Coumarin / Dinitrophenyl FRET Paired β-Lactam.
CHAPTER FOUR
N-ORGANOTHIOLATED β-LACTAMS: STRUCTURE ACTIVITY RELATIONSHIPS

4.1.1 Introduction: Finding Structure Activity Relationships

One of the original goals of this project, in essence, was to understand the minimum structural requirements of N-thiolated β-lactam antibiotics to sustain antimicrobial activity. In this chapter, the testing of compounds prepared in chapter 3, and the effects that sulfur side chains have on activity of the lactams is presented.

4.1.2 Initial Antibacterial Screening

The initial method for antibacterial screening was executed by the Kirby-Bauer disk diffusion method. In this procedure, a cultured bacterium such as Staphylococcus aureus was streaked out (inoculated) on an agar filled Petri dish to encourage a homogeneous coat of growth across the surface of the plate. Next, 6 mm blank paper disks impregnated with 20 µg of the chosen β-lactam analogue (or control agent such as penicillin G or Vancomycin) were arranged on the streaked plate, spaced with an appropriate distance as to not interfere with each other. These plates were then covered and incubated at 37°C for 24 hours. The expected result for antibacterial agents was to see what is called a halo effect, or zone of growth inhibition, around the impregnated disk. This halo is due to the inhibition of bacterial growth elicited by the antibacterial agent. Demonstrated in Figure 4.1 is such an assay comparing the effects of penicillin G, (to the left), Vancomycin (at the top), an inactive analogue (to the right) and a very active
$N$-thiolated $\beta$-lactam (at the bottom). The larger these halos, or zones of inhibition, the more potent the antibacterial activity. In this example, the bacterium was a methicillin-resistant strain of *Staphylococcus aureus*, MRSA. It is quite clear from this test that vancomycin is more potent than penicillin, but the $N$-thiolated $\beta$-lactams are stronger still.

Figure (4.1) Antibacterial Testing by Kirby-Bauer Disk Diffusion.

4.2.1 MRSA Activities

As mentioned, substitution on the nitrogen center of the $\beta$-lactam ring is key to activity. Early in this program the importance of a sulfur group directly bonded to nitrogen quickly became quite evident. $N$-Protio and $N$-para-methoxyphenyl lactams 179 and 180, as well as others having an $N$-phenylselenyl, $N$-chloro, and $N$-benzyl groups, were chosen as a first round of $N$-substituents (Figure 4.2). These analogues proved (179-
to have no activity against a panel of bacterial species. However, an additional sulfenyl analogue, the \(N\)-phenylthiolated \(\beta\)-lactam 184, demonstrated potent activity against a variety of bacteria.

![Chemical structures](image_url)

**Figure (4.2) Different \(N\)-Substituted \(\beta\)-Lactam Analogues Tested.**

Although activity persisted with variation in the identity of the sulfur sidechain from S-alkyl to S-aryl, changes in the oxidation state of the sulfur center was extraordinarily detrimental to activity (Figure 4.3). Thus, disulfide 185 and methylsulfonyl lactam 186 were devoid of activity. \(N\)-Benzylthio and \(N\)-methylthio lactams, 187 and 188, were about equally as potent. These early studies led us to believe that any increase in the oxidation state of the sulfur substituents on these \(N\)-thiolated \(\beta\)-
lactams completely obliterates antibacterial activity. However, later results with different types of sulfur-substituted derivatives showed that some S-oxidized forms are tolerated.

Figure (4.3) Initial Variations in the Sulfur Sidechain Oxidation State.

To further evaluate the influence of the $N$-thio moiety on antibacterial activity an SAR study of alkyl-thiolated $\beta$-lactams was conducted, the synthesis of which is described in chapter 3. The $N$-methythio $\beta$-lactam 109a was shown to be a potent inhibitor of MRSA strains, with an average zone of inhibition of about 28 mm (Table 4.1). Comparatively, penicillin G demonstrates a zone of less than 15 mm against the same strains. And Vancomycin (94), the most potent clinical antibiotic for MRSA, only ranks a zone of about 18 mm. The next step was to extend the chain of methylthio compound 109a by one methylene unit to the $N$-ethylthio $\beta$-lactam 109b. This change raised the anti-MRSA activity to 31 mm. Further elongation of the straight chain, however, only demonstrated a steady decline in zone of inhibition sizes. As can be seen in Table 4.1, $S$-propyl- 109c, $S$-butyl- 109d, and $S$-octyl- 109e moieties had diminished activities compared to that of the $N$-ethylthiolated $\beta$-lactam 109b.
Examining these bioactivity trends, the notion may arise that this diminishing effect on antibacterial activity as a function of S-alkyl chain length may simply be proportional to the analogues ability (or inability) to diffuse across the agar surface. Increasing S-alkyl chain length could certainly inhibit migration from the disk, leading to decreasing zone sizes as an artifact of the experiment.

This exact problem arose upon the search for an SAR between different alkyl chain lengths at C$_3$.[87] In that case longer chain lengths also show decreased antibacterial activities via Kirby-Bauer screening. However, upon further investigation by methods
which are independent of diffusability requirements, the trend in bioactivities was actually shown to be reversed to that of Kirby-Bauer testing. In other words, the longer C₃ sidechain analogues were actually more active than their shorter chain counterparts, and the trend in diminishing activities as measured by zone of inhibition testing was indeed an artifact of the method (Figure 4.4).

Figure (4.4) Relationship of C₃ Side Length to Antibacterial Activities.\[87\]

The first step taken to determine if a similar scenario is being created with the sulfur sidechain was to repeat the Kirby-Bauer disk diffusion experiments by a similar method, well diffusion. This methodology involves burrowing a 6 mm cylindrical well into the agar after streaking out the bacteria, and adding 20µl of a 1µg/µl solution of the analogues into the wells. The plates were then incubated as usual for 24 hours. The reason for doing the agar diffusion assays this way was to see if there was a problem of
the lactams not being able to diffuse off of the cellulose disk. By adding the lactam solution to the wells, there could be no interference due to physical adsorption onto the surface of the disks. These results, however, were essentially the same. Overall, the zone of inhibition values were slightly larger than with disk diffusion, but the relative trends in bioactivity were unchanged.

Believing that the sudden drop in activity for the long chain lactam analogues could still be an artifact of agar plate testing, a different type of experiment was performed to measure minimum inhibitory concentration, or MIC, values in broth media. MIC determination is an accepted means for assessing antimicrobial activity of compounds. This method is an aqueous phase technique, where bacteria, nutrients (like agar, called Mueller-Hinton broth), and antibiotic candidates are all suspended in solution together. The minimum amount of compound required to completely inhibit visible bacterial growth (100% growth inhibition) is the MIC value for that compound. The lower the MIC is, the stronger is the drug’s bioactivity. This method is without concerns of diffusion since the drug is dispersed homogeneously throughout the experimental vessel. Bacterial growth is examined optically for changes in opacity due to increasing cell counts, and is considered to be very accurate (done in triplicate). Indeed, these experiments further substantiated that the increase in sulfur chain length leads to a steady increase in MIC values, and thus to a drop in bioactivity. For example, the $N$-ethylthio β-lactam 109b has an MIC value against MRSA of only 8 µg/ml, while $N$-octylthio β-lactam 109e has an MIC of 64 µg/ml (Table 4.2). So, a shorter sulfur sidechain must either react better with the cellular target or positively influence delivery to the target.
The differences in reactivity between analogues of different bioactivity are explored more deeply in chapter 5.

Along with the MIC testing, it is quite convenient to do another test to determine the minimum bactericidal concentration (MBC). MBC screening quickly determines if a compound is bacteriostatic or bactericidal. Classical β-lactam antibiotics are bactericidal, however, of the N-thiolated β-lactams which were tested for an MBC all were shown to be bacteriostatic. Each MIC sample that displayed 100% inhibition of bacterial growth was streaked out onto an agar plate, allowed to incubate for an additional 24 hours, and yet displayed a positive growth of bacteria. This MBC evidence again points to a mode of action for these compounds differing from that of penicillin.

Table 4.2

<table>
<thead>
<tr>
<th>N-Alkylthiolated β–Lactams Vs Standard Antibiotics</th>
<th></th>
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<tbody>
<tr>
<td>[Chemical Structure]</td>
<td>[Chemical Structure]</td>
<td>[MIC = 16µg/ml]</td>
<td>[MIC = 8µg/ml]</td>
<td>[MIC = 16µg/ml]</td>
<td>[MIC = 64µg/ml]</td>
</tr>
<tr>
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<td>MSSA</td>
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<tr>
<td>Gray bars = MSSA, Black bars = MRSA strains</td>
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Of course, linear alkyl chains are not the only possibility for the sulfur substituents. Investigating the effects of branching with these chains, or aromaticity, should provide some evidence for, or against, a steric effect on bioactivity. The first study involves a simple variation of the ethylthio substituent with a longer, branched N-isopropyl group \textbf{109f}. This change in structure led to an increase in the anti-MRSA activity, from 31 mm for ethyl to 33 mm (Table 4.3). Steric hindrance around sulfur seems to be ineffectual, or to even enhance growth inhibition. Further lengthening of the alkyl chain, to \textit{sec}-butyl \textbf{109g}, further increased potency to 40 mm. This is greater than twice the zone size of the same quantity (20 µg) of vancomycin (94)! MIC testing concurred with the potency of this compound, yielding a value of less than 0.5 µg/ml for 100% growth inhibition (Figure 4.5).

A alternative way to compare the relative potencies of the \textit{N-sec}-butylthiol analogue \textbf{109g} to that of other compounds is by Kirby-Bauer testing against MRSA, as shown in Fig. 4.5. This plate contains four disks loaded with different test compounds. To the right of the plate is a disk containing 20 µg of an inactive compound To the left is a disk impregnated with 20 µg of penicillin G (78), which produces a very small zone of inhibition. At the top of the plate is another disk impregnated with 20 µg of vancomycin (94), a non-β-lactam antibiotic considered the last line of defense against MRSA. Lastly, at the bottom of the plate is a disk containing \textit{N-sec}-butylthio β-lactam \textbf{109g}. The differences in bioactivities are clearly visible.
The next step was to further hinder the sulfur center of these lactams by synthesizing and testing the $N$-tert-butylthio analogue 109h. The synthesis of this compound was described in chapter 3, and turned out to be a difficult task. Fortunately, a small amount was synthesized for testing. Surprisingly, this exchange of $N$-sec-butyl to $N$-tert-butyl nearly obliterated anti-MRSA activity, with a zone size being reduced down to 10 mm. The reason for this appreciable drop in bioactivity in going from $N$-sec-butylthio to $N$-tert-butylthio is not entirely clear. Sterically, this change certainly creates greater bulk directly around the sulfur center, but electronically there is also a greater induction of electrons into the sulfur atom. Both factors may decrease electrophilicity toward cellular thiophiles. However, if steric bulk alone was influencing the lability of
the N-S bond, then one must wonder why the thiol transfer process occurred from phthalimide to lactam occurred so readily.

Another series of N-thiolated lactams to examine was that of cyclic and aromatic systems. To this end, N-cyclohexyl, N-phenyl and N-benzyl β-lactams 109i-k, synthesized as shown in chapter 3, proved valuable. Table 4.3 compares the bioactivity of these three compounds to the branched lactams 109f-h. All three of the cyclic variants showed lower activity that the sec-butyl and isopropyl analogues, and were roughly equal to each other in activity. Thus, the presence of cyclic side chains on sulfur diminished anti-MRSA activity to some degree.

Table 4.3.

![Effects of Chain Branching and Cyclic Substituents](image)

Gray bars = MSSA, Black bars = MRSA strains
Attempts to synthesize more complicated branched sidechain derivatives for further studies were unsuccessful, all examples of which are shown in Figure 3.29. In these cases, even the phthalimide transfer reagents were difficult to synthesize. This, of course, is likely due to major steric hindrances afforded by bulky constituents.

The next objective was to study some N-thiolated lactams bearing heteroatom-containing side chains on sulfur. A series of these were attempted without success as shown in Figure 3.27. Attempts to prepare these compounds failed. In most cases the synthesis of the requisite phthalimide transfer reagent occurred without any problems, such as the case of a N-p-methoxyphenylthio analogue. However, transfer of the thio moiety onto the lactam never occurred in a useful yield after purification. Other methods of N-thiolation also failed. Direct N-thiolation with a sulfenyl chloride always resulted in either recovery of the starting N-proto lactam 107 or in cleavage of the lactam ring as shown in Figure 3.9. Installing the thiol groups onto the imine prior to the Staudinger cyclization, as suggested in the literature[101], also proved ineffective for the synthesis of these compounds (Figure 4.6).

![Figure (4.6) Attempt to Prepare N-Tritylthio Lactams from N-Tritylthioimines.](image-url)
Next, compounds having additional heteroatoms (oxygen) directly on the sulfur center were examined. As shown in Figure 4.3, initial results demonstrated no activity for lactams bearing a sulfur at a greater oxidation state than the sulfenyl systems discussed thus far. Further investigation with some additional analogues, however, seemed warranted. Thus, the synthesis of \(N\)-cyclohexyl lactams 109i, 157b, and 158b and \(N\)-sulfonic acid analogue 160 described in Chapter 3 enabled a comparison of their bioactivities. For these four compounds, the \(N\)-sulfenyl and \(N\)-sulfinyl lactams exhibited similar bioactivities, while the \(N\)-sulfonyl lactam was appreciably less active, and the \(N\)-sulfonic acid was devoid of bioactivity. This differs from the previous observations that \(S\)-oxidation destroys activity. Logically, the most active analogue, \(N\)-sec-butylthiol \(\beta\)-lactam 109g, should be the analogue to oxidize and test comparatively for antibacterial effects. However, oxidation of 109g with hydrogen peroxide (Chapter 3) led to cleavage of the nitrogen-sulfur bond. Fortunately though, oxidation of 109g to \(N\)-sec-butylsulfinyl 157c analogue could be achieved with slightly less than one equivalent of \textit{meta}-chloroperoxybenzoic acid (\textit{mCPBA}). Further oxidation with another equivalent of \textit{mCPBA} did not facilitate the synthesis \(N\)-sec-butylsulfonyl analogue however. Instead, the lactam ring was deannulated and the same results occurred with the use of other oxidants under a variety of conditions. Unlike \(N\)-cyclohexylsulfinyl analogue 157b, this \(N\)-sec-butylsulfinyl compound 157c retained only weak antibiotic activity as evidenced by a zone size \(\sim 25\) percent the diameter of that produced by the \(N\)-sulfenyl lactam.
Thus far, all of the β-lactam antibacterial activities discussed have been prepared and tested as racemic mixtures. The bioactivity of many drugs depend heavily on stereochemistry. So, it seemed very important to examine these chiral N-thiolated β-lactams more closely with respect to their relative and absolute stereochemistry. Even though no cytotoxic effects have been demonstrated for these antibiotics\textsuperscript{[113]}, perhaps one or more analogues might be more potent than the others.

To examine these possible stereochemical effects the $N$-sec-butylthio β-lactam 109g was chosen as the test candidate for its three chiral centers, and potency. Unremarkably, there was very little difference between each of the four stereoisomers
However, what is curious and potentially meaningful is that cis “up” stereoisomers 152-153 have equal potencies, which are greater than that of their β-lactam enantiomers cis “down” stereoisomers 154-155. Also of note is that variations in β-lactam ring stereochemistry have no effect on bioactivity when the N-organothio substituent is not chiral, such as N-methylthio.\textsuperscript{100}

Figure (4.7) Dependency of Anti-MRSA Activity on Lactam Stereochemistry.

So it seems that the overall configuration of N-thiolated β-lactams is important to bioactivity, perhaps for delivery to a target or to avoid aggregation of the drug, but stereochemistry of the most important substituent, the N-organothio group, is unimportant.
4.3.1 Anthrax

In addition to *Staphylococcus* species, the *N*-thiolated β-lactams have potent antibacterial effects on *Bacillus* species. Initial experiments with *Bacillus anthracis* showed that the same SAR as that for MRSA seems to hold up for *B. anthracis*. The analogues with the most potent activities against MRSA, such as *N*-sec-butylthio β-lactam 109g, are still these most potent against *B. anthracis*. So, it seems very likely that whatever is causing activity of the lactams against MRSA is the same for *B. anthracis*. This will be further explored and discussed in chapter 5.

Figure (4.8) Kirby-Bauer Screening of *N*-Thiolated β-Lactams Against Anthrax.

Table 4.5 shows a sampling of structures and respective activities of *N*-thiolated β-lactams against *Bacillus anthracis*, the causative agent of anthrax. The standard clinical antibiotic of choice to treat anthrax infections, Ciprofloxacin, has a zone of inhibition of
40 mm by agar disk diffusion. This is slightly less than that of the sec-butyl analogue 109g.

Table 4.5

Ciprofloxacin, the current drug of choice to treat *Bacillus anthracis*, demonstrates a zone of inhibition of 40mm.

Anthrax was not the only *Bacillus* species tested, however. *B. cereus*, *B. coagulans*, *B. globigii*, *B. megaterium*, *B. subtilis*, and *B. thuringenesis* were also inhibited by *N*-sec-butylthiol β-lactam 109g, with zones of comparable size to those produced by Ciprofloxacin (Table 4.6). As well, the same trends seen for *anthracis* hold true for the remainder of these six species of *Bacilli*. These shared trends seem to point to a common mode of antibacterial action of the *N*-thiolated β-lactams in *Staphylococcus* and *Bacillus*, which will be explored further in chapter 5.
Table 4.6

*N-*sec-Butylthiolated β-Lactam vs. Cipro Against *Bacillus* Species

![Bar chart showing zone of inhibition (mm) for *Bacillus* species comparing *N*-sec-butylthiolated β-lactam and Cipro.]

- *anthracis*
- *cereus*
- *coli*
- *globigii*
- *megaterium*
- *subtilis*
- *lanceolatus*

*Legend:*
- ■ *N*-secbutylthiolated β-lactam
- □ Cipro
5.1.1 Introduction

As discussed in chapters 1 and 2, classical β-lactam antibiotics inhibit bacterial growth by interrupting cell wall crosslinking. Studies completed in this lab have shown that cell wall crosslinking inhibition is not the mode of action of N-thiolated β-lactams.\[18\] Evidence to this fact includes no changes in cellular morphologies, via examination by scanning electron microscopy of cells treated with N-thiolated β-lactams, and no change in cell wall density as determined by Gram-staining. Drastic alterations in morphology and cell wall thickness occur for the same cells treated with penicillins. This means that for the first time a β-lactam antibiotic has a mode of action not directly related to cell wall synthesis. The purpose of the work discussed in this chapter was to find out where in the cell these drugs are going, what are they interacting with and how do these interactions produce inhibitive effects.

5.2.1 Chemical Interaction

Since it seems apparent from the SAR studies that the sulfur sidechain of these N-thiolated β-lactams is paramount to antibacterial activity, and that other evidence from our laboratories suggests that these lactams are impervious to radicals, there are really only three logical reaction mechanisms to consider in terms of the compounds biological mode of action. The first pathway is that some bio-nucleophile attacks the carbonyl carbon, \( C_2 \) of the lactam, thus opening the ring (Figure 5.1). This pathway is identical to
the fate of all other \( \beta \)-lactam antibiotics whose mode of action involves attack at that very same site by a serine residue of the transpeptidase enzyme. However, logic would dictate that if this was the site of attack, stronger electron-withdrawing groups on the lactam nitrogen would be expected to increase the electrophilicity, and thus reactivity, of the lactam ring. Thus, although increased reactivity does not always mean increased activity an \( N \)-sulfonyl lactam should be more active than an \( N \)-sulfinyl, which should, in turn, be more active than the \( N \)-sulfenyl derivatives. In actuality though, the precise reverse trend in bioactivities is seen. If this was not enough to rule out this pathway, it is important to point out that the \( N \)-protio \( \beta \)-lactam can be isolated un-cleaved after treatment with bacterial culture media.

![Chemical Structure](image)

*Activity should decrease for \( N \)-substituent = \( \text{SO}_3\text{H} \) > \( \text{SO}_2\text{R} \) > \( \text{SOR} \) > \( \text{SR} \)
Reverse is observed \( \text{SR} \) > \( \text{SOR} \) > \( \text{SO}_2\text{R} \) > \( \text{SO}_3\text{H} \)*

**Figure (5.1) Nucleophilic Attack at \( C_2 \).**

The second potential site of attack on the lactam by a biological nucleophile is the first carbon of the sulfur sidechain (Figure 5.2). If this site was the point of nucleophilic attack, then it would be expected that less hindered groups like methyl, as compared to ethyl or even isopropyl, on sulfur would be the most reactive. However, the exact reverse trend in bioactivity is seen. The isopropyl-substituted system was one of the most active analogues, certainly more active than the ethyl and methyl analogues.
Activity should decrease for $R = \text{CH}_3 > \text{CH}_2\text{CH}_3 > \text{CH}$(CH$_3$)$_2$

We observe the reverse $R = \text{CH}_3 < \text{CH}_2\text{CH}_3 < \text{CH}$(CH$_3$)$_2$

**Figure (5.2) Nucleophilic Attack at the Alpha-carbon of Sulfur Sidechain.**

The third, and final, site of attack to consider is the sulfur center itself (Figure 5.3), which seems very likely. First of all, the sulfur atom has proven to be absolutely required for antibacterial activity of these compounds and therefore attack at that site is possible. Secondly, attack at the sulfur center, and thus ejection of the lactam ring intact, fits well with the finding that the intact $N$-protio lactam is recovered cleanly from culture media. Shorter sulfur sidechains mean lower lipophilicity and therefore a better chance for the lactam molecule to pass completely through the cellular membrane into the cytoplasm to interact with some intracellular constituent.

**Figure (5.3) Nucleophilic Attack at Sulfur.**

Also shown previously in this lab and in others (Shah/ Cama), is the extreme reactivity of these $N$-thiolated $\beta$-lactams toward free thiols, such as 2-mercaptopyridine (Figure 5.4). This shows a selectivity of thiophilic nucleophiles for this electrophilic
site, and points to the likelihood of attack here by some intracellular thiol nucleophile. N-
Thiolated β-lactams are otherwise quite stable towards radicals, acidic conditions, and
weakly basic conditions.

\[
\begin{align*}
&\text{HS} \quad \text{N} \\
&\text{O} \quad \text{MeO} \\
&\text{MeO} \quad \text{Cl} \\
&\text{N} \quad \text{HS} \\
&\text{S} \quad \text{R} \\
&\text{O} \quad \text{MeO} \\
&\text{MeO} \quad \text{Cl} \\
&\text{N} \quad \text{HS} \\
&\text{S} \quad \text{R}
\end{align*}
\]

Figure (5.4) Chemical Reactivity of N-Thiolated β-Lactams to Thiophilic Reagents.

So the questions that remain are: where does the biological action occur, what is
the intracellular target, and how does this interaction confer antibacterial activity?
Attempts to answer these questions are what follows in this chapter.

5.3.1 Loci

The first experiments done in this lab by Dr. Timothy Long to determine where
these drugs may end up in a bacterial cell involved radiolabeling.\textsuperscript{108} For the lowest cost
and smallest number of “hot” synthetic steps, the N-methylthio β-lactam was synthesized
with all three methyl hydrogens swapped for tritium labels. Unfortunately, the results
from this experiment did not show where the drug went, only where it did not go. These
lactams were not detected in any appreciable levels in cellular fractions containing DNA,
RNA, or proteins. At the time, low molecular weight targets were not considered and
quite possibly the radio-tag was thrown out with the cellular bath water. Experiments
were underway, at the time of this dissertation’s completion, to use radiolabeling to detect small molecule targets in aqueous fractions.

A different method to follow the drug’s pathway into the cell was considered to measure fluorescence uptake. The first idea was to attach a fluorophore as part of the sulfur sidechain, to trace the path of this fluorophore through the bacterial cell membrane and into the cell. To see precisely the location within the cell where the lactam may interact with its target, a Forster Resonance Energy Transfer (FRET) pair was built into the N-thiolated β-lactam framework. The principle with FRET is that two functional moieties are installed in the framework where the fluorescence wavelength of one, the donor, would overlap with the absorption wavelength of the other, the acceptor. In this way the fluorescence of the donor would be quenched by the acceptor. Also of note is that this FRET phenomenon is greatly distance-dependent. Therefore, as the donor and acceptor are separated, the quenching effect exponentially decreases and the donor “lights up” as its fluorescence is released. If a donor, like a naphthyl group, is incorporated in the sulfur sidechain and the acceptor is attached to the lactam moiety, like a dansyl group, perhaps at C₃ or C₄, then once the sulfur-nitrogen bond is cleaved fluorescence would be emitted (Figure 5.5) as it moved away from the lactam. So the model system was a naphthyl-dansyl FRET pair 171.
Probing the Mechanism of Action via Forster Resonance Energy Transfer

Potential Action of N-Thiolated β-Lactams

Figure (5.5) FRET Pair Concept.

In practice, however, there were significant problems. Unforeseen synthetic challenges thwarted efforts to synthesize this FRET pair 171 impossible. Even after

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changing the order of steps for introducing the two fluorescent side chains, lactam ring decomposition still occurred, preventing formation achievement of the final FRET product. As well, this was only, at best, a model system, as the donor fluorophore (naphthyl) is simply not red-shifted enough to be of use for cell structures. Naphthalene’s fluorescence wavelength is fairly blue ($\lambda_{\text{emission}} = 380 \text{ nm}$), as is the auto-fluorescence of bacteria. So, the small, faint blue light of this fluorophore would be difficult to observe amongst the large intense blue background of the cell. The compound and the background could, theoretically, be differentiated based on fluorescence lifetimes, but no real-time imaging would be possible.

Thus, another FRET candidate, with the appropriate fluorescence wavelength, was selected for synthesis, a fluoresceinyl / dabsyl paired compound 178. However, initial attempts tp prepare this compound were also unsuccessful for reasons noted above for 178. In the future, it may be possible to overcome the difficulties of the synthesis, and perhaps use a similar procedure to make coumarin / dinitrophenyl FRET paired analogues.

5.4.1 Sugar Uptake

Another sulfenamide, 1,2-benzoisothiazolin-3-one (BIT) (46), has shown weak activity against Staphylococcus aureus, with an MIC around 100 µg/ml[57], as discussed in Chapter 1. It has been suggested to interact with thiols of biological importance as part of its mode of action, such as glutthione. Particularly, however, the molecule has been shown to inhibit the glucose uptake pathway of staph. This pathway, called the phosphoenol pyruvate phosphotransferase system is responsible for the uptake and
phosphorylation of sugars, such as glucose, for metabolism. Inhibition of this pathway starves the cell from nutrient absorption. Importantly, the important phosphotransferase is highly thiophilic and sharply inhibited by free thiols. Indeed, 46 is known to form an adduct with this enzyme an inhibit the glucose uptake of *Staphylococcus aureus* by 96%. It is thus evident that this molecule ring opens across the nitrogen-sulfur bond to reveal a free thiol for bioactivity. Since *N*-thiolated β-lactams have been shown to interact with thiols there was reasonable suspicion that they may interact with this sugar uptake system in a similar pattern.

Using a fluorescence based monitoring kit, glucose uptake of *Staphylococcus aureus* cultures were monitor with and without the presence of *N*-sec-butylthio β-lactam 109g, as well as a series of positive an negative controls. As seen in Table 5.1, *N*-thiolated β-lactams do not appear to inhibit this pathway, in light of the marginal decrease in sugar uptake. Apparently, these drugs are effecting a different system.

Table 5.1

![Lactam Effect on Glucose Uptake](image)

5.5.1 Identity of the Intracellular Target

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Since radiolabeling and fluorescence tagging methods has thus far failed to produce insightful data on the N-thiolated β-lactam’s mode of action, another novel method of for identifying the intracellular target(s) was investigated. In this experiment the goal was to synthesize a polymer resin onto which is covalently bonded an N-thiolated β-lactam through the sulfur moiety. In this way, it may be possible to use this lactam-conjugated resin to capture the cellular target. The plan was to expose the contents of bacterial cells to the lactam-conjugated resin, and upon reaction of the cellular target with the N-thio moiety, the nucleophile would then be covalently bonded to the resin as glutathione is shown to do in Figure 5.6. Once the nucleophile is bonded to the resin, all impurities could be washed away. The nucleophile, or intracellular target, could then be chemically cleaved from the resin, further purified if necessary, and characterized.

This indeed proved to be possible. As described in chapter 3, the synthesis of N-thiolated lactam resin 165 proceeded smoothly to generate a crosslinked N-polystyrenylthio β-lactam resin (Figure 5.6). To test this resin’s abilities to capture thiophilic nucleophiles, it was first subjected to glutathione 9, a common intracellular thiol, under pseudo-physiological conditions. The washings of this resin were examine by HPLC and $^1$H NMR for organic compounds, and the N-protio β-lactam 108 was thus observed. This experiment successfully produced a resin-glutathione adduct 192 which could be chemically cleaved by a number of conditions, like triphenyl phosphine / water, or DIBAL / ether, $^1$H NMR confirmed the recovery of the glutathione 9 after cleavage from the resin.
Figure (5.6) Testing of the Lactam-thiolated Resin.

The resin was also tested in an aqueous solution containing equimolar amounts of 18 different amino acids including glutathione and cysteine, all at 2 millimolar concentrations, with this DMSO swelled resin. After thorough washings, this adduct was then cleaved with DIBAL in ether to afford glutathione only.

Table 5.2

<table>
<thead>
<tr>
<th>Amino Acids Stirred with N-Thiolated Resin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine</td>
<td>glutathione</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>L-methionine</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>L-histidine</td>
<td>L-threonine</td>
</tr>
<tr>
<td>D-threonine</td>
<td>L-valine</td>
</tr>
<tr>
<td>L-serine</td>
<td>DL-phenylalanine</td>
</tr>
<tr>
<td>DL-β-3,4-dihydroxyphenylalanine</td>
<td></td>
</tr>
</tbody>
</table>

The real goal of these experiments was, of course, to see if the resin could extract targets from the intracellular contents of cells. This was completed by first cultivating a large number of *Staphylococcus aureus* cells, then concentrated them from their growth media before washing and lysing them via sonication. After centrifugation, the lysate was
filtered to obtain only the soluble contents of the cells. These lysates were then shaken with a significant portion of the resin for 24 hours at 37°C. The resin was then thoroughly washed and chemically cleaved with DIBAL to obtain the putative target species. One and only one compound was captured by this method, Coenzyme A (CoA) (12).

A simple high pressure liquid chromatography (HPLC) experiment (traces shown as spectra 8.51-8.59 on pages 213-221) was performed to demonstrate the formation of an organothio-coenzyme A adduct 193. This was quite simple, once the proper eluent was discovered. There was an immediate generation of two new, less polar peaks which were representative of the adduct 193 and the resultant N-protio β-lactam.

![Figure (5.7) Thiol-CoA Adduct 193](image)

These experiments supported the claim that exposure of the *S. aureus* lysate to the *N*-thiolated β-lactam would generate the same type of disulfide adduct. This was also proven to be so. A relationship between the rate of adduct formation and the antibacterial potency of various *N*-thiolated β-lactams was explored. However, either there is no relationship between these properties, or the rate of thiol reaction between coenzyme A and the lactam is very fast. Different *N*-organothio β-lactams, ethyl, phenyl and sec-butyl, were separately combined with coenzyme A all at 10 µM in a solution of aqueous, pH 7 phosphate buffer and DMSO, and immediately injected into the HPLC. For each
sample, it appears that the adducts is formed instantaneously under these experimental conditions, with no observable differences in the reactivity of the different \(N\)-thiolated lactams with coenzyme A. Several questions thus remain. Do different \(N\)-thiolated \(\beta\)-lactams react more readily with the target? Is coenzyme A the only target? Does the organosulfur substituent server to enhance activity of the lactam by promoting its stability, reactivity, or delivery? Are the disulfide adducts formed between the lactam and different thiols stable to reductases? Do the lactams of different bioactivities have different stabilities to non-target thiophiles?

5.6.1 CoA-Antibacterial Effects

To counteract the effects of oxidative stress, cells have developed an important defense mechanism: a thiol / disulfide redox buffer consisting of small molecules and proteins with redox-active thiol moieties, disulfides and disulfide reductases (also called thiol-disulfide oxidoreductases). Through redox regulation of different target proteins and small molecules, disulfide reductases control diverse cellular functions including apoptosis, cell proliferation, protein folding, oxidative stress and signal transduction, by constantly keeping a fresh supply of free thiols. These antioxidants are particularly susceptible to attack by foreign thiophilic agents. At stasis, the reductases keep the equilibrium between thiol and disulfide at over 90% thiol. The most well known of these is the glutathione / glutathione reductase system. Classically, glutathione was thought to be the ubiquitous thiol involved in the thiol / disulfide redox metabolism of all life and thus was the thiol of choice for the model experiments with the \(N\)-resinthiolated \(\beta\)-lactam experiments.
So, how does this tie in with Coenzyme A (9)? CoA is a fairly ubiquitous chaperone enzyme used, by definition, to assist in enzymatic processes such as re-naturing and folding. CoA’s role in *Staphylococcus aureus*, however, has recently been found to be much more profound.\(^{[109]}\) The exciting part of this discovery is that *S. aureus* has been found to neither produce nor utilize glutathione. Instead, *S. aureus* utilizes CoA, which it generates in millimolar quantities, and coenzyme A reductase (CoADR) as its primary thiol / disulfide redox metabolism. Therefore, *S. aureus* is highly dependant on this system to prevent oxidation of important cellular systems, and any disruption, say by *N*-thiolated β-lactams, of this metabolism could result in significant detrimental effects for this bacterium.

Of equal significance, it is important to realize that CoADR is extraordinarily selective for CoA-CoA disulfides.\(^{[110]}\) It has been shown that CoADR is incapable of reducing a mixed disulfide between CoA and glutathione. It is therefore likely that CoADR is incapable at reducing a mixed disulfide formed between CoA and the organothiolate procured from an *N*-thiolated β-lactam. In this way, *N*-thiolated β-lactams can possibly inhibit this redox cycle and, eventually downstream, inhibit cell growth. It is also possible that the enzyme, CoADR, itself forms an adduct with the thiol from these lactams and is thereby irreversibly deactivated. Since the enzyme only bears one thiol, on a cysteine residue (Cys43), which is needed for enzymatic activity\(^{[110]}\), blockage of this site could quickly shut down the entire redox metabolism.

There is additional evidence to support this mode of action. The bacteria that are affected by *N*-thiolated β-lactams, such as multiple species of *Staphylococcus* and
Bacillus, each use CoA for their primary redox metabolism. Bacteria which are resistant to N-thiolated β-lactams, such as Mycobacterium spp., Streptococcus spp., Enterococcus spp., and Escherichia coli, are known to utilize other thiols like mycothiol or glutathione for their redox cycling. The reductases for these systems are not as selective for their homo-disulfide and therefore may simply reduce any mixed disulfides that are formed. Human cells also use glutathione and this may be why, amongst other reasons, these compounds are non-cytotoxic to healthy tissue.

These lactams indeed react well with other thiols such as glutathione. This was shown in the case of the lactam-bound solid phase. As well, thin layer chromatography experiments show immediate formation of the N-protio β-lactam, when any organothiolated β-lactam is mixed in solution with the free glutathione thiol at μM concentrations, roughly equal to that known to be present in cytoplasmic fluid. Regardless of the identity of the organothio-substituent, the reaction is instantaneous. So it seems that the trends in biological activity can be based on neither the reactivity of the thiolate with the target, CoA, or the ability to not react with other antioxidants such as glutathione.

Given glutathione’s reactivity toward different N-thiolated β-lactams seems to be the equivalent, the question arises as to what effect glutathione levels in a cell correspond to sensitivity of those cells to N-thiolated lactams. Glutathione demonstrates a greater ability to inhibit the potency of less active N-thiolated analogues as shown in Figure 5.7. As can be seen, three lactams were exposed to zone of inhibition testing, which resulted in the less active analogues, 109a and 109b, having greatly diminished zones, while a
more active analogue, like 109g, was less affected. So although, all tested N-thiolated analogues have a very high rate of reaction with free thiols, like glutathione, biological interaction is not a question of rate. Certain structural difference, the same ones that make an N-thiolated β-lactam more bioactive, bestow a level of defense against free media thiols such as the glutathione distributed in these experiments.

Figure (5.8) Effect of Glutathione on Growth Inhibition by N-Thiolated β-Lactams

Picture A) Each well (small dark circles) was filled with 20 µg of the shown drugs plus 20 µg of glutathione.
Picture B) Each well was filled with 20 µg of the shown drugs plus 50 µg of glutathione.
In both cases, incubation was for 24 hours.
CHAPTER SIX
BEYOND BACTERIA: OTHER BIOLOGICAL ACTIVITIES AND CONCLUSIONS

6.1.1 Fungi

As mentioned in chapter 1, fungi, which are eukaryotes, comprise a separate group of microorganisms, having a membrane bound nucleus, a more extensive endoplasmic reticulum and mitochondria. Compared to bacteria, fungi have a mostly chitin-based cell wall, instead of a peptidoglycan like that in Gram-positive bacteria. This is why fungi are completely unaffected by traditional β-lactam drugs like penicillin and other peptidoglycan cell wall crosslinking inhibitors like Vancomycin. As well, fungal DNA is found isolated within the nucleus instead of dispersed throughout the cytoplasm as in bacteria. Fungal infections are usually constrained to only the dermis or mucosal membranes (superficial mycosis). However, just as resistant bacterial infections, like MRSA, are becoming a problem with the increasing prevalence of immune deficiency diseases like HIV-AIDS, development of treatments for internal and systemic fungal infections (deep mycosis) are of great importance.

As discussed in chapter 5, N-thiolated β-lactams have a completely different mode of action from that of traditional β-lactam antibiotics, which likely involves the inhibition of a cellular redox metabolism. Fungi, are also known to rely on thiol / disulfide redox metabolisms to defend themselves from over-oxidation and therefore are potentially susceptible to drugs that inhibit these systems.
To determine if these \(N\)-thiolated \(\beta\)-lactams can serve as antifungal agents, Dr. Timothy Long and Marci Culbreath tested \(N\)-methylthio lactam 109a against a panel of eight different species of *Candida*, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis* and *C. utilis*. The antifungal screening method closely mimicked the Kirby-Bauer method used for antibacterial screenings. Compared to a standard clinical antifungal agent, Clotrimazole, the \(N\)-thiolated \(\beta\)-lactams, specifically 109a, faired quite well. For some species Clotrimazole was more potent, but in others the \(N\)-thiolated \(\beta\)-lactam was more potent (Fig. (6.1)).

**Table 6.1**

![Graph: N-Methylthio Lactams vs Candida Species](image)
This is certainly a departure from standard β-lactam antibiotics. To verify this data MIC testing was also performed (Table 6.2). MIC values obtained after 24 hours were in the 10 to 15 µg/ml range, with some even lower.

**Table 6.2 MIC’s of Lactam 109a Against Candida Species.**

<table>
<thead>
<tr>
<th>Candida sp.</th>
<th>MIC 24 hrs.</th>
<th>MIC 48 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>&lt;5 µg/ml</td>
<td>&lt;5 µg/ml</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>10-15 µg/ml</td>
<td>30-35 µg/ml</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>10-15 µg/ml</td>
<td>10-15 µg/ml</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>10-15 µg/ml</td>
<td>35-40 µg/ml</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>10-15 µg/ml</td>
<td>15-20 µg/ml</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>&lt;5 µg/ml</td>
<td>&lt;5 µg/ml</td>
</tr>
<tr>
<td>C. utilis</td>
<td>10-15 µg/ml</td>
<td>15-20 µg/ml</td>
</tr>
</tbody>
</table>

This verifies, yet again, that the mode of action of these N-thiolated β-lactams is completely novel. The identities of the thiol / disulfide redox metabolisms of these fungi have thus far been assumed to be glutathione based. In light of these results, it would be very interesting to conclusively determine the true redox metabolism of those fungi which are inhibited by N-thiolated β-lactams versus those fungi which are not. If there is a strong line of difference between inhibited fungi thiols and uninhibited fungi thiols, a potentially important avenue to the design of new antifungal agents would be identified.

**6.2.1 Neoplastic Systems**

As remarkable as it is that these N-thiolated β-lactam antibiotics are antifungal agents, it is perhaps even more intriguing that they are also anti-cancer agents. This is the first time that a β-lactam antibiotic has been shown to possess any anti-cancer properties. In leukemic Jurkat T-cells, N-thiolated β-lactams have demonstrated the ability to induce DNA damage and inhibit DNA replication. Eventually, downstream this
cascades to p38 mitogen-activated protein kinase activation, S-phase arrest, and apoptotic cell death. This apoptotic program was also induced in human leukemia, breast, prostate and head-and-neck cancer cell lines.

Figure (6.1) N-Thiolated β-Lactam Anti-Cancer Mode of Action.¹¹⁰

Still unknown is how do N-thiolated β-lactams induce DNA damage in tumor cells? Is there a direct interaction between these compounds and DNA? Our labs have
shown that these compounds are non-cytotoxic at five times the concentration needed to induce DNA damage and inhibit DNA replication in leukemic Jurkat T cells within 2 hours\textsuperscript{110}. If the lactams damage DNA directly then why do they not affect the DNA of healthy human cells? What difference between healthy cells and cancer cells can these lactams differentiate between? A major difference between these two types of cells is intracellular glutathione concentration. It has been shown that tumor cells generate much higher concentrations of glutathione that do non-cancerous cells. Much like an infection, tumor cells are constantly fighting off oxidation, and by evidence of these increased glutathione levels, appear to be doing so more than non-cancerous cells. So, in essence, tumor cell redox metabolisms may be more susceptible to inhibition by \textit{N}-thiolated \textit{β}-lactams. As well, the influx roads into tumor cells are known to be quite different from other types of cells. Much is yet to be learned about these lactam’s mode of action, however the exciting results of induced apoptosis (Figure 6.2) are not diminished.
In cancer cell lines, there is also a pattern of activity seen amongst sulfur sidechain analogues that is similar to that previously described in Chapter 4 for anti-MRSA activity. In the case of these cancer cell lines, longer chain lactams have lower potencies, as shown with compounds 109a, 109b and 109d in Figure 6.3. (This is demonstrated by %Caspase 3 activity values of 100%, 40% and 30% respectively.) However, the more potent antibacterial lactams, like 109g, are yet to be tested against these cell lines, and lactams with an oxidized sulfur, like $N$-cyclohexylsulfinyl $\beta$-lactam 109i, have recently shown decreased in vitro growth of breast cancer cell lines by more than 50%.

Figure (6.2) Apoptotic Effects of $N$-Thiolated $\beta$-Lactams.$^{[110]}$
Increased Chain Length

Decreased Anticancer Activities

Figure (6.3) Trend of Anticancer Activity.

6.3 Conclusions and Future Directions

The rapid procession of resistance to available antimicrobials is depleting the current arsenal of antibiotics that remain effective against infections. For decades, no new antibacterial targets have been discovered and brought to bear in clinical usage. Numerous sulfur compounds are yet to be evaluated for biological activity, and precise targets and modes of action of many sulfur reagents are still unexamined. It has been proposed that the thiol-disulfide redox metabolisms of infectious organisms might serve as a potentially valuable target for development of new anti-infectives. Often, these redox systems are extraordinarily divergent from healthy mammalian physiology and therefore provide targets where selectivity could be very high. Therefore, N-thiolated β-lactams, with their novel mode of action, are excellent drug candidates, as shown here, for the treatment of cancer, as well as drug-resistant bacterial and fungal infections. Further analogue synthesis does not appear to be warranted, however, with the potential of a novel target across a number of systems, further biochemical and in vivo investigations are definitely worth exploring. It is the hope of this author that this work may contribute to the development of the thiol-disulfide redox as a target of rational drug design.
CHAPTER SEVEN
MATERIALS AND METHODS

7.1 Synthetic Procedures

All chemicals required for the synthesis of $N$-thiolated $\beta$-lactams were purchased from one of the following sources: Sigma Aldrich, Fisher Scientific, Acros Organics, TCI Organic Chemicals, or Lancaster Research Chemicals. Most were used without further purification. Solvents were obtained from Fisher Scientific. Products were purified by flash chromatography were done with either J.T.Baker or Whatman flash chromatography silica gels (40 $\mu$m). NMR spectra were recorded in either CDCl$_3$ or D$_2$O as indicated.

7.1.2 Preparation of Imines

$N$-Anisylimine (105): $p$-Anisidine, regardless of the source company, was always impure upon receipt and therefore required purification by recrystallization from water. (1eq.) 11.20g (0.0797 moles) of $o$-chlorobenzaldehyde (103) and 11.00g (0.0894 moles) of recrystallized $p$-anisidine (104) were stirred together neat, at room temperature, open to the atmosphere, for a couple of minutes. The solid product was then dissolved in dry dichloromethane, dried over magnesium sulfate, and concentrated via rotary evaporation. The imine product was quite pure at this stage, but was further purified by recrystallization from methanol prior to further use.
(E)-N-(2-Chlorobenzylidene)-4-methoxybenzenimine (105): yellow solid; mp 51-52°C; $^1$H NMR (250 MHz, CDCl, CDCl$_3$): $\delta$ 8.94 (s, 1H), 7.4-6.9 (m, 8H), 3.85 (s, 3H).

7.1.3 Preparation of Acid Chlorides

Methoxyacetyl chloride (102): Although this acid chloride was available for purchase, the price was moderately high and the purity was very poor. Thus, it was preferable to synthesized it fresh. Purification of the thionyl is achieved through distillations with quinoline and then linseed oil as directed in the literature procedure: Rigby, Chem. Ind., 1969, 1508. To a dry round bottom flask 30.00g (0.335 moles) of methoxyacetic acid (3) was added, followed by dropwise addition of 39.58g (0.335 moles) of thionyl chloride at 0°C. The mixture was allowed to stir and warm to room temperature overnight. The solution was then distilled, open to the atmosphere from 111 to 114°C (literature value = 112-113°C), to obtain pure product.
7.1.4 Preparation of \(N\)-4-Anisyl Azetidin-2-ones (\(\beta\)-Lactams)

\(N\)-4-Anisyl azetidin-2-ones (106): In a round bottom flask, 6.00g (0.0245 moles) of \(N\)-Anisylimine 7 and 7.5g (0.0742 moles) of triethyl amine were dissolved in minimal amount of dry dichloromethane and cooled to 0\(^{\circ}\)C. A solution of 2.81g (0.0260 moles) of methoxyacetyl chloride in an equal volume of dry dichloromethane was added dropwise with stirring. The solution was then heated to reflux, which was maintained overnight. After cooling, the solution was poured into an equal volume of water, extracted thrice with the dichloromethane, dried over magnesium sulfate and concentrated via rotary evaporation. Column chromatography was used for purification, eluting with dichloromethane or a mixture of hexanes and ethyl acetate.

\[
\begin{align*}
\text{Cl} & \quad \text{N} & \quad \text{O} & \quad \text{CH}_3 \\
\text{105} & \quad \text{Cl} & \quad \text{O} & \quad \text{R} \\
\text{102} & \quad \text{N} & \quad \text{O} & \quad \text{R} & \quad \text{Cl} \\
\text{106} & \quad \text{H}_3\text{C} & \quad \text{O} & \quad \text{R} & \quad \text{O} & \quad \text{Cl} & \quad \text{phenyl} \\
\end{align*}
\]

\((\pm)-(3S,4R)-4-(2\text{-chlorophenyl})-3\text{-methoxy}-1-(4\text{-methoxyphenyl})\text{azetidin-2-one (106): white solid; mp 183-184}^{\circ}\text{C} \ \text{\textsuperscript{1}H NMR (250 MHz, CDCl}_3): \ \delta 7.43 \ (d, 1H, J = 7.4 \text{ Hz}), 7.29-7.19 \ (m, 5H), 6.80 \ (d, 2H, J = 9.0 \text{ Hz}), 5.61 \ (d, 1H, J = 4.8 \text{ Hz}), 4.89 \ (d, 1H, J = 4.8 \text{ Hz}), 3.73 \ (s, 3H), 3.27 \ (s, 3H).}

7.1.5 Dearylation of \(N\)-Anisyl Azetidin-2-ones

\(N\)-Protio Azetidin-2-ones (107a): In a round bottom flask 1.00g (0.0035 moles) of \(N\)-anisyl azetidin-2-one 106 was dissolved in a minimal amount of acetonitrile at 0\(^{\circ}\)C. A solution of 5.77g (0.01053 moles) of ceric ammonium nitrate, dissolved in a minimal
amount of water, was added dropwise via addition funnel. The reaction was monitored
via thin layer chromatography (TLC) and was usually complete within 10 minutes. The
reaction mixture was then poured into three volume equivalents of water and extracted
thrice with ethyl acetate. The organic extracts were washed sequentially with a 5%
sodium bicarbonate solution, a 1% sodium bisulfate solution, and a saturated sodium
chloride solution, then dried over magnesium sulfate and concentrated via rotary
evaporation. The β-lactam product was purified via column chromatography with a
mixture of ethyl acetate and hexanes.

(±)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxyazetidin-2-one (107a): white solid; mp 93-95°C; ¹H NMR
(250 MHz, CDCl₃): δ 7.34 (m, 4H), 6.55 (bs, 1H), 5.27 (d, 1H, J = 4.6 Hz), 4.85 (d, 1H, J = 4.6 Hz), 3.29 (s, 3H).

(±)-(3S,4R)-3-Methoxy-4-phenylazetidin-2-one (107b): white solid; mp 130-131°C; ¹H NMR (250 MHz,
CDCl₃): δ 7.35 (m, 5H), 6.24 (bs, 1H), 5.30 (d, 1H, J = 4.6 Hz), 4.87 (dd, 1H, J = 3.4, 1.2 Hz), 3.30 (s, 3H).

(±)-(3S,4R)-3-Acetoxy-4-(2-chlorophenyl)azetidin-2-one (107c): white solid; mp 100-101°C; ¹H NMR
(250 MHz, CDCl₃): δ 7.37 (m, 4H), 5.30 (d, 1H, J = 4.6 Hz), 4.87 (t, 1H, J = 3.8 Hz), 3.29 (s, 3H), 1.59 (s, 3H).
7.1.6 Preparation of Phthalimide-based Sulfur Transfer Reagents (111)

**Method 1:** In dry benzene, chlorine gas was bubbled until the increase in weight equaled 0.93g (0.01313 moles). This was then added to a round bottom flask containing 1.00g (0.01313 moles) 2-propanethiol in a minimal quantity of benzene and then sealed at 0°C with stirring for 1 hour. The solution was then added dropwise to a second round bottom flask containing a slurry of 1.93g (0.01313 moles) phthalimide (110) and 2.89g (0.02232 moles) of Hunig’s base and allowed to reach room temperature over 2 hours with stirring. The solution was then poured into water, extracted thrice with benzene, dried over magnesium sulfate and concentrated via rotary evaporation. This often generated a pure product. When unreacted phthalimide remained, tritration with chloroform dissolved the product nicely, leaving the starting material to be filtered off. When present, other impurities were removed by recrystallization with methanol or column chromatography.

![Reaction scheme](image)

2-(Methylthio)isoindoline-1,3-dione (111a): white solid; ^1^H NMR (250 MHz, CDCl₃): mp 178-180°C; δ 7.82 (m, 4H), 3.77 (s, 3H).

2-(Ethylthio)isoindoline-1,3-dione (111b): white solid; mp 158-160°C; ^1^H NMR (250 MHz, CDCl₃): δ 7.86 (m, 4H), 2.92 (q, 2H, J = 7.34 Hz), 1.56 (t, 3H, J = 8.1 Hz).

2-(Propylthio)isoindoline-1,3-dione (111c): white solid; 77-78°C; ^1^H NMR (250 MHz, CDCl₃): δ 7.87 (m, 4H), 2.86 (t, 2H, J = 7.3 Hz), 1.62 (m, 2H), 1.04 (t, 3H, J = 8.1 Hz).

2-(Butylthio)isoindoline-1,3-dione (111d): white solid; mp 189-190°C; ^1^H NMR (250 MHz, CDCl₃): δ 7.8 (m, 4H), 2.85 (t, 2H, J = 7.4 Hz), 1.61 (m, 2H), 1.42 (m, 2H), 0.91 (t, 3H, J = 5.0Hz).
2-(Isopropylthio)isoindoline-1,3-dione (111f): white solid; mp 61-62°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.8-7.5 (m, 4H), 4.9 (m, 1H), 2.17 (d, 6H, $J = 6.9$ Hz).

(+)-2-(sec-Butylthio)isoindoline-1,3-dione (111g): white solid; mp 43-45°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.83 (m, 4H), 3.21 (m, 1H), 1.55 (m, 2H), 1.24 (d, 3H, $J = 6.8$ Hz), 1.04 (t, 3H, $J = 7.3$ Hz).

2-(Cyclohexylthio)isoindoline-1,3-dione (111i): white solid; mp 92-94°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.80 (m, 4H), 3.05 (m, 1H), 1.90-1.25 (m, 11H).

2-(Phenylthio)isoindoline-1,3-dione (111j): white solid; mp 149-155°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 8.0-6.8 (m, 9H).

2-(Benzylthio)isoindoline-1,3-dione (111k): white solid; mp 165-168°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.9-7.5 (m, 4H), 7.31 (m, 5H), 1.75 (s, 2H).

(+)-2-(1-Phenylethylthio)isoindoline-1,3-dione (111l): white solid; mp 100-104°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.20 (m, 9H), 3.67 (q, 1H, $J = 6.2$), 1.76 (d, 3H, $J = 5.0$).

2-(2-Napthylthio)isoindoline-1,3-dione (111m): white solid; mp 120-128°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.65 (m, 11H).

**Method 2:** In dry benzene 0.1g (0.0004424 moles) of $N$-bromophthalimide was dissolved in a minimal quantity of benzene and stoppered at 0°C with stirring. The solution was then added dropwise to a second round bottom flask containing a solution of 0.034g (0.0004424 moles) of 1-butnaethiol and 0.06g (0.0004424 moles) of triethyl amine. The mixture was allowed to achieve room temperature over 2 hours with stirring. The solution was then poured into an equal volume of water, extracted thrice with equal volumes of benzene, dried over magnesium sulfate and concentrated via rotary evaporation. This often generated a fairly pure product. Unreacted phthalimide was removed via triteration with chloroform which dissolved the product nicely, leaving the starting material to be
filtered off. Also present, were other impurities which were removed via recrystallization with methanol and column chromatography.

2-(Octylthio)isoindoline-1,3-dione (111e): white solid; mp 55-58°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.88 (m, 4H), 2.91 (t, 2H, $J = 7.3$ Hz), 1.76-1.28 (m, 12H), 0.89 (t, 3H, $J = 6.4$ Hz).

2-(tert-Butylthio)isoindoline-1,3-dione (111h): white solid; mp 130-133°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.87 (m, 4H), 1.36 (s, 9H).

Method 3: In a round bottom flask was added 0.0061g (0.0000272 moles) of (anthracen-10-yl)methanethiol, 0.0035g (0.0000354 moles) potassium carbonate, and 0.0040g (0.0000272 moles) phthalimide in 5ml of reagent grade acetone. The vessel was sealed and the liquid meniscus was submerged below an active sonication bath for 24 hours. The solution was concentrated via rotary evaporation and re-dissolved in 10ml of dichloromethane. The solution was washed with and equal volume of water, dried over magnesium sulfate and concentrated via rotary evaporation. The product yields were very low and purification was not attempted for loss of all product.

2-((Anthracen-10-yl)methylthio)isoindoline-1,3-dione (111n): white solid; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 8.38-7.17 (m, 13H), 3.12 (s, 2H).

Method 4: In a round bottom flask was added the thioester dissolved in a minimal quantity of carbon tetrachloride and cooled to 0°C with stirring. A solution of sulfuryl chloride in an equal volume of carbon tetrachloride was then added dropwise and the solution was allowed to come to room temperature with stirring over 30 minutes. The solution was then added dropwise into a slurry of phthalimide and Hunig’s base in an
equal volume of carbon tetrachloride cooled to 0°C with stirring. After the mixture was allowed to warm to room temperature over 3 hours with stirring, 40 ml of water was then added and the mixture was then allowed to stir at high speed for 20 min. The mixture was filtered and the solid washed with 40 ml more of water. The solid was then triturated with chloroform and the dissolved product was dried over magnesium sulfate and concentrated via rotary evaporation.

2-(7-(Methyl2-(2-chloro-6-methoxy-3-oxo-3H-xanthen-9-yl)benzoate)thio)isoindoline-1,3-dione (111o): white solid; mp 140-146°C; ¹H NMR (250 MHz, CDCl₃): δ 9.2-7.3 (m, 9H), 6.05 (s, 1H), 5.35 (s, 1H), 3.8 (s, 3H), 3.05 (s, 3H).

2-(Polystyrenylthio)isoindoline-1,3-dione (164): light brown powder; IR 3015 (aromatic C-H stretch), 2980 (aliphatic C-H stretch), 1490, 1450 (C=O stretches).

7.1.7 Preparation of N-Thiolated Azetidin-2-ones

N-Thiolated azetidin-2-ones (109j): In a round bottom flask was added 0.005g (0.0000236 moles) N-protio β-lactam 107, 0.0067g (0.000236 moles) of N-phenylthiolated phthalimide and 0.0119g (0.000118 moles) of triethylamine, in a minimal quantity of dichloromethane. The solution was refluxed and followed via TLC. Reaction was complete after 12 hours. After cooling, the solution was poured into an equal volume of water, washed with aqueous solutions of 5% sodium bicarbonate, 1% sodium bisulfate, and saturated sodium chloride. The extracts were then dried over magnesium sulfate and concentrated via rotary evaporation. Phthalimide was removed via tritration with chloroform and the remaining impurities were removed via column chromatography, eluting with either dichloromethane.
(±)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-N-(methylthio)azetidin-2-one (109a) white crystal; mp 71-73 °C; $^1$H NMR (250 MHz, CDCl3): $\delta$ 7.35 (d, 1H, $J$ = 7.4 Hz), 7.24 (m, 3H), 5.29 (d, 1H, $J$ = 4.9 Hz), 4.80 (d, 1H, $J$ = 4.9 Hz), 3.16 (s, 3H), 2.40 (s, 3H); $^{13}$C NMR (63 MHz, CDCl3): $\delta$ 170.4, 133.8, 131.4, 129.6, 128.9, 126.8, 86.7, 62.7, 58.9, 21.8.

(+)-(3S,4R)-4-(2-Chlorophenyl)-N-ethylthio-3-methoxyazetidin-2-one (109b): white solid; mp 68-70 °C; $^1$H NMR (250 MHz, CDCl3) $\delta$ 7.34 (4H, m), 5.33 (1H, d, $J$ = 5.0 Hz), 4.88 (1H, d, $J$ = 5.0 Hz), 3.20 (3H, s), 1.35 (2H, q, $J$ = 10.0 Hz), 0.92 (3H, t, $J$ = 6.8 Hz); $^{13}$C NMR (63 MHz, CDCl3): $\delta$ 172.5, 142.1, 137.5, 130.8, 128.4, 70.5, 46.7, 34.4, 22.0.

(±)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-N-(propylthio)azetidin-2-one (109c) colorless oil; $^1$H NMR (250 MHz, CDCl3): $\delta$ 7.6-7.2 (m, 4H), 5.45 (d, 1H, $J$ = 4.9 Hz), 5.09 (d, 1H, $J$ = 4.9 Hz), 3.21 (s, 3H), 2.49 (m, 2H), 1.55 (m, 2H), 0.82 (t, 3H, $J$ = 6.9 Hz); $^{13}$C NMR (63 MHz, CDCl3): $\delta$ 171.0, 133.6, 128.3, 128.3, 86.3, 66.9, 58.3, 38.2, 30.8, 21.5, 13.6.

(+)-(3S,4R)-N-Butylthio-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (109d): light yellow, viscous oil, $^1$H NMR (250 MHz, CDCl3): $\delta$ 7.30 (4H, m), 5.35 (1H, d, $J$ = 4.9 Hz), 4.89 (1H, d, $J$ = 4.9 Hz), 3.23 (3H, s), 2.85 (2H, t, $J$ = 7.0), 1.60 (4H, m), 0.91 (3H, t, $J$ = 7.3 Hz); $^{13}$C NMR (63 MHz, CDCl3): $\delta$ 176.5, 134.0, 132.1, 129.7, 129.0, 126.9, 86.8, 64.8, 58.1, 48.0, 12.3.

(+)-(3S,4R)-4-(2-Chlorophenyl)-N-octylthio-3-methoxyazetidin-2-one (109e): white solid, $^1$H NMR (250 MHz, CDCl3) $\delta$ 7.35 (4H, m), 5.35 (1H, d, $J$ = 4.9 Hz), 4.89 (1H, d, $J$ = 4.9 Hz), 3.23 (1H, m), 2.77 (2H m), 1.64-1.26 (12H, m), 0.88 (t, 3H, $J$ = 6.2 Hz); $^{13}$C NMR (63 MHz, CDCl3): $\delta$ 167.8, 132.5, 131.1, 127.5, 127.0, 80.3, 52.7, 43.7, 28.0, 26.0, 24.5, 22.3, 18.9.
(+)-(3S,4R)-4-(2-Chlorophenyl)-N-isopropylthio-3-methoxyazetidin-2-one (109f): light yellow paste, mp 30-38°C; $^1$H NMR (250MHz, CDCl$_3$) δ 7.34 (4H, m), 5.39 (1H, d, $J = 4.9$ Hz), 4.95 (1H, d, $J = 4.9$ Hz), 3.27 (1H, m), 3.25 (3H, s), 1.27 (6H, d, $J = 5.7$Hz); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 171.3, 133.2, 128.9, 127.0, 87.1, 65.2, 59.7, 49.4, 34.0, 30.1, 28.5, 25.8, 21.5.

(+)-(3S,4R)-N-sec-Butylthio-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (109g): light yellow, viscous oil, $\pm$ cis mixture. $^1$H NMR (250MHz, CDCl$_3$) δ 7.4 (1H, d, $J = 7.4$ Hz), 7.3 (3H, m), 5.3 (1H, d, $J = 4.7$ Hz), 4.9 (1H, d, $J = 4.8$ Hz), 3.2 (3H, s), 0.94 (3H, q, $J = 6.0$ Hz); $^{13}$C NMR (63 MHz) δ 171.0, 133.8, 131.4, 129.5, 128.9, 126.8, 86.3, 64.1, 58.8, 48.1, 28.1, 19.0, 18.6, 11.1.

(+)-(3S,4R)-N-tert-Butylthio-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (109h): white solid, $^1$H NMR (250MHz, CDCl$_3$) δ 7.34 (4H, m), 5.50 (1H, d, $J = 4.8$ Hz), 4.99 (1H, d, $J = 4.8$ Hz), 3.25 (3H, s), 1.35 (9H, s); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 166.0, 133.0, 129.0, 88.0, 65.0, 60.0, 44.0, 34.0, 31.0, 27.0, 19.0.

(+)-(3S,4R)-4-(2-Chlorophenyl)-N-cyclohexylthio-3-methoxyazetidin-2-one (109i): brown solid; mp 78-79°C; $^1$H NMR (250MHz, CDCl$_3$) δ 7.34 (4H, m), 5.35 (1H, d, $J = 4.9$ Hz), 4.93 (1H, d, $J = 4.9$ Hz), 3.24 (3H, s), 3.05 (1H, m), 2.01-1.41 (10H, m); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 171.3, 133.5, 128.9, 128.7, 128.3, 85.2, 67.6, 58.3, 49.5, 32.2, 30.9, 25.6, 25.4.

(+)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-N-phenylthioazetidin-2-one (109j): light yellow, oily solid, mp 60-62°C $^1$H NMR (250MHz, CDCl$_3$) δ 7.34 (9H, m), 5.35 (1H, d, $J = 5.0$Hz), 4.91 (1H, d, $J = 5.0$Hz), 3.25 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 172.0, 137.0, 135.0, 134.0, 132.0, 130.0, 129.0, 128.0, 127.0, 125.0, 82.0, 59.0, 48.0.

(+)-(3S,4R)-N-Benzylthio-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (109k): light yellow, oily solid; mp 68-70°C; $^1$H NMR (250MHz, CDCl$_3$) δ 7.22 (9H, m), 5.30 (1H, d, $J = 4.7$ Hz), 4.55 (1H, d, $J = 4.8$ Hz), 3.16 (3H, s), 1.25 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 179.0, 142.0, 138.0, 136.0, 135.0, 134.0, 132.0, 130.0, 128.0, 126.0, 88.0, 59.0, 48.0, 40.0.
(\(+\)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-N-(1-phenylethylthio)-azetidin-2-one (109i): white solid; mp 65-67\(^\circ\)C; \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.56 (9H, m), 5.25 (1H, d, \(J = 4.2\) Hz), 4.84 (1H, d, \(J = 4.2\) Hz), 3.27 (3H, s), 3.01 (2H, m), 0.93 (3H, d, \(J = 7.1\) Hz); \(^13\)C NMR (63 MHz, CDCl3): \(\delta\) 168.9, 164.1, 139.6, 138.3, 134.5, 133.5, 118.5, 79.9, 51.4, 43.7, 40.8, 29.3.

(\(+\)-(3S,4R)-4-(2-Chlorophenyl)-3-hydroxy-N-naphthylthioazetidin-2-one (109m1): white solid; \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.9-7.2 (11H, m), 5.3 (2H, m); \(^13\)C NMR (63 MHz, CDCl3): \(\delta\) 172.0, 142.0, 135.0, 133.0, 132.0, 131.0, 129.0, 128.0, 127.0, 126.0, 125.0, 124.0, 123.0, 81.0, 58.0.

(\(+\)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-N-naphthylthioazetidin-2-one (109m): yellow solid, \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 8.13-7.19 (11H, m), 5.35 (1H, d, \(J = 5.0\) Hz), 4.91 (1H, d, \(J = 5.0\) Hz), 3.24 (3H, s); \(^13\)C NMR (63 MHz, CDCl3): \(\delta\) 170.0, 142.0, 130.0, 134.0, 132.0, 131.0, 130.0, 129.0, 128.0, 127.0, 126.0, 125.0, 123.0, 121.0, 82.0, 58.0, 55.0.

(\(+\)-(3S,4R)-N-(Anthracen-10-yl)methylthio)-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (109n): pink solid, \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.48-7.19 (13H, m), 5.25 (1H, d, \(J = 4.8\) Hz), 4.81 (1H, d, \(J = 4.8\) Hz), 4.078 (2H, s), 3.25 (3H, s).

(\(+\)-(3S,4R)-N-(R)(sec-Butylthio)-3-methoxy-4-phenylazetidin-2-one (152): white oil; \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.29 (m, 5H), 5.94 (d, 1H, \(J = 4.9\) Hz), 5.01 (d, 1H, \(J = 4.9\) Hz), 2.99 (m, 1H), 1.47 (m, 2H), 1.28 (d, 3H, \(J = 6.8\) Hz), 0.94 (t, 3H, \(J = 7.4\) Hz).

(\(-\)-(3S,4R)-N-(R)(sec-Butylthio)-3-methoxy-4-phenylazetidin-2-one (153): white oil; \(^1\)H NMR (500MHz, CDCl3) \(\delta\) 7.30 (m, 5H), 5.87 (d, 1H, \(J = 3.5\) Hz), 4.94 (d, 1H, \(J = 4.0\) Hz), 2.90 (m, 1H), 1.62-1.40 (m, 2H), 1.12 (d, 3H, \(J = 6.5\) Hz), 0.90 (t, 3H, \(J = 8.0\) Hz).

(\(+\)-(3S,4S)-N-(S)(sec-Butylthio)-3-methoxy-4-phenylazetidin-2-one (154): white oil; \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.32 (m, 5H), 5.94 (d, 1H, \(J = 4.9\) Hz), 5.01 (d, 1H, \(J = 4.9\) Hz), 2.97 (m, 1H), 1.56 (m, 2H), 1.18 (d, 3H, \(J = 6.9\) Hz), 0.97 (t, 3H, \(J = 7.4\) Hz).

(\(-\)-(3S,4S)-N-(S)(sec-Butylthio)-3-methoxy-4-phenylazetidin-2-one (155): white oil; \(^1\)H NMR (250MHz, CDCl3) \(\delta\) 7.29 (m, 5H), 5.94 (d, 1H, \(J = 4.9\) Hz), 5.01 (d, 1H, \(J = 4.9\) Hz), 2.99 (m, 1H), 1.51 (m, 2H), 1.28 (d, 3H, \(J = 6.75\) Hz), 0.94 (t, 3H, 7.4 Hz).
7.1.8 Preparation of Thioesters

**Method 1 (Mitsunobu Reaction):** In a round bottom flask was added 9ml (0.04506 moles) of diisopropyl azodicarboxylate (DIAD) and 12.16g (0.04506 moles) of triphenylphosphine were combined in THF at 0°C, under nitrogen with stirring. This solution was allow to stir for 30 min, or until a white precipitate formed. Then a solution of 3.43g (0.04506 moles) thiolacetic acid and 3.25g (0.0225 moles) of 2-nonanol in a minimal quantity of THF was added carefully, dropwise. After work-up it was very difficult to remove all of the triphenylphosphine as it shared a close R_f value with the desired products.

**Method 2 (Substitution):** In a round bottom flask was added (1 eq.) an alkyl halide in a minimal amount of benzene under nitrogen, at 0°C with stirring. To this, a solution, of (1eq.) triethylamine and (1 eq.) thiolacetic acid in enough benzene, was added dropwise. The solution was allowed to come to room temperature over 3 hours with stirring. The reaction mixture was then poured into an equivalent portion of water, extracted with benzene, dried over magnesium sulfate and concentrated via rotary evaporator. Continued exposure to a stream of nitrogen successfully removed any remaining triethylamine.

7.1.9 Preparation of N-Sulfoxylated β-Lactams

**Method 1 H_2O_2 (157b):** Inspired via Prinzbach and Netscher, *Synthesis, 1987*, 683-688. In a round bottom flask was added 0.0135g (0.0000415 moles) an N-cyclohexylthiolated β-lactam in a minimal amount of glacial acetic acid at 0°C with stirring. To this, a solution, of 0.008g (0.0000415 moles) of 30% hydrogen peroxide in few drops of glacial
acetic acid, was added dropwise. The solution was allowed to come to room temperature over 3 hours with stirring. The reaction mixture was then poured into an equivalent portion of water, extracted with three equivalent volumes of benzene, washed with water until the washings were at neutral pH, as observed via pH paper, dried over magnesium sulfate and concentrated via rotary evaporation.

(+)-(3S,4R)-N-Cyclohexylsulfinyl-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (157b): white oil, $^1$H NMR (250MHz, CDCl$_3$) $\delta$ 7.41 (8H, m), 5.85 (1H, d, $J = 6.0$Hz), 5.71 (1H, d, $J = 5.6$), 4.94 (1H, d, $J = 6.0$Hz), 4.91 (1H, d $J = 6.0$Hz), 3.75 (2H, m), 3.29 (3H, s), 3.23 (3H, s), 2.72 (2H, m), 2.2-0.80 (20H, m); $^{13}$C NMR (63 MHz, CDCl$_3$): $\delta$ 171.4, 133.6, 130.9, 130.8, 129.3, 123.5, 83.4, 61.6, 53.2, 50.5, 37.4, 29.9, 25.6.

(+)-(3S,4R)-N-Phenylsulfinyl-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (157a): white oil, $^1$H NMR (250MHz, CDCl$_3$) $\delta$ 7.30 (m, 9H), 5.18 (bs, 1H), 4.80 (bs, 1H), 3.25 (bs, 1H); $^{13}$C NMR (63 MHz, CDCl$_3$): $\delta$ 168.9, 164.1, 131.4, 128.1, 131.5, 118.0, 113.9, 108.8, 79.7, 54.7, 43.9.

**Method 2 mCPBA:** In a round bottom flask was added 0.20 g (0.00085 moles) an $N$-sec-butylthiolated $\beta$-lactam in a minimal amount of diethyl ether at 0$^\circ$C with stirring. To this, a solution, of 0.14 g (0.00080 moles) meta-chloroperoxybenzoic acid in a minimal amount of diethyl ether, was added dropwise. The solution was monitored via thin layer chromatography. The reaction mixture was then poured into an equivalent portion of water, extracted thrice with equivalent volumes of benzene, washed twice with water, dried over magnesium sulfate and concentrated via rotary evaporation.
(+)-(3S,4R)-N-sec-Butylsulfinyl-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (157c): dark brown paste, mp 35-43°C; $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.32 (4H, m), 5.38 (1H, d, $J$ = 4.9 Hz), 4.94 (1H, d, $J$ = 4.9 Hz), 3.11 (2H, m), 2.11 (3H, s), 1.75-0.96 (8H, m); $^{13}$C NMR (63 MHz, CDCl$_3$): $\delta$ 171.2, 149.8, 131.4, 129.5, 129.0, 126.8, 86.5, 74.1, 58.8, 48.1, 28.1, 19.0, 14.6, 11.2.

7.1.10 Preparation of N-Sulfonylated \(\beta\)-Lactams

**Method:** Identical to 7.1.9 Method 1.

(+)-(3S,4R)-N-Cyclohexylsulfonyl-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (158b): white, oily solid; mp 152-158°C; $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.47 (m, 4H), 5.79 (d, 1H, $J$ = 5.0 Hz), 4.97 (d, 1H, $J$ = 4.6 Hz), 3.29 (s, 3H), 3.12 (m, 1H), 2.20-0.86 (m, 10H); $^{13}$C NMR (63 MHz, CDCl$_3$): $\delta$ 174.9, 143.8, 127.9, 125.8, 120.1, 118.5, 84.6, 61.9, 52.0, 45.2, 40.1, 34.9, 33.6.

(+)-(3S,4R)-N-Phenylsulfonyl-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (158a): white oil, $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.96-7.16 (9H, m), 5.70 (1H, d, $J$ = 5.6 Hz), 4.82 (1H, d, $J$ = 5.6 Hz), 3.35 (3H, s) $^{13}$C NMR (63 MHz, CDCl$_3$): $\delta$ 168.9, 164.1, 144.4, 139.5, 134.2, 128.0, 125.7, 119.0, 79.9, 54.5, 43.8.

7.1.11 Preparation of a \(\beta\)-Lactam Sulfonic Acid

**Method (160):** Literature Procedure Cimarusti, C. *Tetrahedron*, 1983, 39, 2577. In a round bottom flask a solution 0.01g (0.472 mmol) of N-protio \(\beta\)-lactam in 2 ml of freshly distilled dichloromethane and 2 ml of dry DMF was stirred with 0.014g (0.00944 moles) of 50% sulfur trioxide-pyridine under nitrogen at room temperature for 2 hours. The solution was concentrated via rotary evaporator. The resultant salt was ion exchanged for a potassium salt. Application of this salt to column chromatography afforded the necessary protonation to afford the sulfonic acid in a pure form.
(+)-(3S,4R)-2-(2-Chlorophenyl)-3-methoxy-4-oxoazetidine-1-sulfonic acid (160): dark brown oil, $^1$H NMR (250 MHz, CDCl₃) $\delta$ 7.32 (4H, m), 5.69 (1H, d, $J = 4.9$ Hz), 4.98 (1H, d, $J = 4.9$ Hz), 3.32 (2H, m), 0.89 (1H, s); $^{13}$C NMR (63 MHz, CDCl₃): $\delta$ 179.9, 145.2, 137.7, 131.0, 122.7, 83.4. 55.2. 43.3.

7.1.12 De-Acetylation of a C₃ Acetoxy Substituted β-Lactam

**Method:** To a round bottom flask 0.184g (0.0329 moles) of potassium hydroxide was dissolved in a minimal quantity of methanol. To this solution was added a solution of 0.787g (0.0329 moles) of (+)-(3S,4R)-4-(2-chlorophenyl)-3-acetoxy-1-azetidin-2-one in enough 0°C. The solution was allowed to warm to room temperature over night with stirring. The solution was then concentrated via rotary evaporation, re-dissolved in methylene chloride, washed with water until the washing were neutral pH via pH paper, dried over magnesium sulfate, and concentrated via rotary evaporation.

(+)-(3S,4R)-4-(2-Chlorophenyl)-3-hydroxy-N-azetidin-2-one (108OH): white solid; mp 204-205°C; $^1$H NMR (250 MHz, CDCl₃): $\delta$ 7.30 (m, 4H), 6.82 (s, 1H), 5.64 (d, 1H, $J = 5.2$ Hz), 5.34 (d, 1H, $J = 4.9$ Hz), 3.79 (s, 3H).


**Method:** To a round bottom flask was added 10.0ml (excess) of pyridine and 0.119ml (0.0001283 moles) of acetic anhydride and chilled to 0°C. To this solution was added 0.0345g (0.0001283 moles) of (-)-(3S,4R)-4-(2-Chlorophenyl)-3-hydroxy-$N$-(4-methoxyphenyl)azetidin-2-one. The resultant solution was stirred for 1 hour at 0°C and was allowed to stir overnight at room temperature, then poured into ice water. The mixture was filtered to yield a pure white solid.
(-)-(3S,4R)-3-Acetoxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (p152): white solid; mp 165-167°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.30 (m, 8H), 5.94 (d, 1H, $J$ = 4.9 Hz), 5.34 (d, 1H, $J$ = 4.9 Hz), 3.76 (s, 3H), 1.68 (s, 3H).

7.1.14 Dansylation

Method: To a round bottom flask was added 0.005 g (0.0261 mmol) to a minimal quantity of tetrahydrofuran for dissolution. The flask was then chilled to 0°C with stirring. To this was added 0.002 (0.0522 mmol) of sodium hydride, pre-dissolved in a minimal quantity of tetrahydrofuran. This solution was allowed to stir for 30 minutes. This solution was then added to 0.007 (0.0261 mmol) of dansyl chloride, pre-dissolved in a minimal quantity of tetrahydrofuran. This mixture was allowed to stir for an additional 30 minutes. The remaining sodium hydride was quenched with slightly wet methanol. The reaction mixture was then poured into an equal volume of water and extracted thrice with equal portions of tetrahydrofuran. The organic layers were combined and washed with an equal volume of water. The organic extracts were then dried over magnesium sulfate and concentrated via rotary evaporation.

2-(2-Chlorophenyl)-4-oxoazetidin-3-yl 5-(dimethylamino)naphthalene-1-sulfonate: yellow solid; mp 135-140°C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 8.48 (b, 1H), 8.25 (b, 1H), 8.05 (b, 1H), 7.52 (b, 1H), 7.40 (b, 1H), 7.30 (b, 2H), 6.90 (b, 1H), 6.75 (b, 1H), 6.60 (b, 1H), 5.25 (b, 1H), 4.80 (b, 1H), 2.85 (s, 6H).

7.2 Microbiological Test Procedures

The following bacteria were used for the antimicrobial evaluation of $N$-thiolated $\beta$-lactams: Bacillus anthracis (Sterne strain), Bacillus cereus (ATCC 14579), Bacillus coagulans (USF 546), Bacillus globigii (Department of Defense Reagents Program), 156
Bacillus megaterium (ATCC 14581), Bacillus subtilis (19569), Bacillus thuringensis (ATCC 10792), Bacteroides fragilis (obtained from Smith-Kline Laboratory), Candida albicans (clinical isolate), Candida tropicalis (clinical isolate), Enterobacter cloace (environmental isolate, USF510), Enterococcus gallinarium (ATCC 49573), Enterococcus faecalis (ATCC 19433), Enterococcus casseliflavus (ATCC 700327), Enterococcus durans (ATCC 6056), Enterococcus avirum (ATCC 14025), Enterococcus saccharolyticus (ATCC 43076), Escherichia coli (ATCC 23590), Haemophilus influenzae (USF 561), Klebsiella pneumoniae (USF 512), Lactococcus lactis (ATCC 11454), Listeria monocytogenes (ATCC 19115), Micrococcus luteus (environmental isolate, USF681), Niesseria gonnorheae (obtained from the Tampa Branch State Laboratory, β-lactamase positive, USF 662), Pseudomonas aeruginosa (ATCC 15442), Salmonella typhimurium (obtained from University of South Florida Medical Clinic, USF 515), Serratia marcescens (ATCC 29634), Staphylococcus aureus USF525 (ATCC 25923) Staphylococcus aureus USF652-658 (obtained from Lakeland Regional Medical Center, β-lactamase positive), Staphylococcus epidermidis (environmental isolate, USF528), Staphylococcus saprophyticus (ATCC 35552), Staphylococcus simulans (ATCC 11631), Staphylococcus capitis (ATCC 35661), Staphylococcus cohnii (ATCC 35662), Staphylococcus lentus (ATCC 700403), Staphylococcus lugdunensis (ATCC 700328), Staphylococcus xylosus (ATCC 29971), Streptococcus pyogenes, Streptococcus agalactiae, Vibrio cholerae (biotype El Tor Ogawa, cholera toxin positive, CDC E5906),
7.2.1 Antimicrobial Susceptibility Testing

**Culture preparation:** From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20% glycerol, a culture of each organism was grown on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD) at 37°C for 24 hours. A 10^8 suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates.

**Disc method:** From each 1mg/ml stock solution in dimethyl sulfoxide (DMSO), sterile 6mm paper discs (Becton-Dickinson Laboratories, Cockeysville, MD) were impregnated with 20 µl of the test compounds. At this concentration, the microliter quantity is equivalent to the micrograms in solution. The discs were allowed to dry in a biohazard safety hood then placed onto the inoculated TSA plates. The plates were incubated for 24 hours at 37°C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each disc.

**Well method:** A 10^8 standardized cell count suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates. Circular wells (6 mm in diameter) were cut into the inoculated plates and 20 µL of a 1 mg/ml stock solution of the test lactam in dimethylsulfoxide (DMSO) was pipetted into the wells. The plates were incubated for 24 hours at 37°C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each well.

7.2.2 MIC Calculations

**Media preparation:** The minimum inhibitory concentrations were determined by the agar plate dilution (need reference). The test media were prepared in 24 well plates
(Costar 3524, Cambridge, MA) by adding a known concentration of the test drug in DMSO together with a solution of Mueller-Hinton II agar (Becton-Dickinson Laboratories, Cockeysville, MD) for a total volume of 1 ml in each well. Calculations of the overall concentration of antibiotic in the wells were standardized by measuring from a 1mg/ml stock solution of the test drug. At this concentration, the microliter quantity is equivalent to the micrograms in solution. The amount of agar solution added to the wells was determined by adding to the quantity of test drug in each well to give a combined volume of 1 ml. Following preparation of the well plates, the media were allowed to solidify at room temperature for 24 hours before inoculation.

**Inoculation:** From an 24 hour culture of each organism on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD), the *Staphylococcal* strains were grown overnight in 5 ml of tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C. One microliter of each culture was then applied to the appropriate well of agar and incubated at 37°C overnight. After 24 hr, the MICs were determined by examining the wells for growth.

**7.2.4 Glucose Uptake / Respiration Study**

A fresh 200 ml culture of an 106 cfu/ml suspension of *S. aureus* (ATCC 25923) was precipitated via centrifugation. The solid contents were separated from the supernatant and washed with a phosphate buffer solution (PBS). The mixture was then precipitated again and the supernatant removed. The cells were resuspended in 10 ml of PBS to an approximate concentration of 5 X 10⁹ cells/ml. This is the bacteria stock
The solutions of 1 µM horseradish peroxidase (HRP), and glucose oxidase were prepared. 4mL of 5X buffer was added to 16 mL of DI water to generate a 1X buffer. Then, 1ml of 1X buffer was used to dissolve 5.9 mg of glucose. Then 50 µL of this solution was diluted in 3950µL of 1X buffer. This is the glucose stock solution.

Next, 1mg of lactam was dissolved in 10mL of DMSO/water. This is the lactam stock solution. 1.2 mg of glutathione was added to 10 mL of water. This is the glutathione stock solution. Then 1.2 mg of erythromycin was dissolved in 10 ml DMSO/water. This is the erythromycin stock solution. Finally, 1.2 mg of dithiothritol (DTT) was dissolved in 10ml DMSO/water. This is the DTT stock solution. The experimental tubes were then set up as follows.

**Experiment tubes:**

1) **Tube 1. Maximum Glucose Uptake.**
   Add 1mL of Bacteria Stock Solution
   Add 303µL of Glucose Stock Solution
   Add 3.99mL of buffer.

2) **Tube 2. Just Sugar.**
   Add 303µL of Glucose Stock Solution
   Add 4.99mL of buffer.

3) **Tube 3. Sugar with Lactam.**
   Add 303µL of Glucose Stock Solution
   Add 1mL of Lactam Stock Solution
   Add 3.99mL of buffer.

4) **Tube 4. Test 0.5 MIC (0.25µg/mL)**
   Add 303µL of Glucose Stock Solution
   Add 0.250mL of Lactam Stock Solution
   Add 1mL of Bacteria Stock Solution
   Add 3.74mL of buffer.
5) Tube 5. Test 1.0 MIC (0.5µg/mL)
Add 303µL of Glucose Stock Solution
Add 0.50mL of Lactam Stock Solution
Add 1mL of Bacteria Stock Solution
Add 3.49mL of buffer.

6) Tube 6. Test 2.0 MIC (1.0µg/mL)
Add 303µL of Glucose Stock Solution
Add 1.0mL of Lactam Stock Solution
Add 1mL of Bacteria Stock Solution
Add 2.74mL of buffer.

7) Tube 7. Test 4.0 MIC (2.0µg/mL)
Add 303µL of Glucose Stock Solution
Add 2.0mL of Lactam Stock Solution
Add 1mL of Bacteria Stock Solution
Add 1.74mL of buffer.

Add 5mL of buffer.

9) Tube 9. Glutathione
Add 303µL of Glucose Stock Solution
Add 1mL of Bacteria Stock Solution
Add 1mL of Glutathione Stock Solution
Add 2.74mL of buffer.

10) Tube 10. Erythromycin
Add 303µL of Glucose Stock Solution
Add 1mL of Bacteria Stock Solution
Add 1mL of Erythromycin Stock Solution
Add 2.74mL of buffer.

11) Tube 11. DTT
Add 303µL of Glucose Stock Solution
Add 1mL of Bacteria Stock Solution
Add 1mL of DTT Stock Solution
Add 2.74mL of buffer.

12) Tube 12. Erythromycin Blank
Add 303µL of Glucose Stock Solution
Add 1mL of Doxycyclin Stock Solution
Add 3.74mL of buffer.
Once these were prepared, all 12 tubes were incubated with shaking at 37°C for 30 minutes. A standard curve was prepared as at the concentrations shown in table 7.1.

**Table 7.1 Standard Curve**

<table>
<thead>
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<th>Well</th>
<th>Final Concentrations of Sugar (micromolar)</th>
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Each tube was then precipitated via centrifugation and the supernatant was filtered with 0.2µm cellulose nitrate membrane. 50µL of each supernatant was pipetted into three wells each. The Amplex Red stock was prepared by dissolving the contents of the vial of Amplex Red reagent in 60 µL of DMSO. A Working Solution of fluorophore was prepared by mixing 1) 50µL of Amplex Red Stock, 2) 100µL of HRP, 3) 100µL of Glucose Oxidase, and 4) 4.75mL 1X Buffer. Then 50µL of this Working Solution was added to each well. The entire plate of wells was then incubated in a drawer for 30 minutes. Fluorescence was then measured via a fluorometer.
7.2.5 Resin / Lysate Exposure

1 liter of *Staphylococcus aureus* USF849 was cultured at 37°C for 24 hours. The culture was then centrifuged and washed. The resultant pellet was washed with PBS buffer and then resuspended in 5 ml of PBS buffer. The cells were then sonicated in an icebath for 30 minutes total, stopping every 5 minutes to check for overheating. The lysed cells were then centrifuged and the lysate extracted. The lysate was centrifuged again and the lysate extracted from the solids. Filtering the lysate yielded a slightly opaque yellow solution. 0.25226g of Lactam Thiolated Resin (12) was swelled in 0.5ml of DMSO and added the lysate solution. This mixture was then setup for 200 rpm shaking for 24 hours. Next, the lysate was filtered and repeatedly washed solid with boiling water and boiling ethanol. The solid was dried and 196mg of post lysate exposed resin material was collected which was a light brown mixture of amorphous and crystalline solid (looked like sand). After DIBAL cleavage, the solid was repeatedly freeze-dried with 100% deuterium oxide. Spectrum 8.49 was observed bearing stark similarity to Conezyme A.

7.2.6 HPLC Experiments

All HPLC experiments (Spectra 8.51 through 8.59) were done using a Shimadzu LC-8A HPLC through a analytical reverse phase column to a Shimadzu SPD-10A UV-VIS Detector at a 2ml/min flow rate. 10 µM solutions of standards: coenzyme A, *N*-protio β-lactam, *N*-ethylthiolated β-lactam, *N*-sec-butylthiolated β-lactam, phenylthiolated β-lactam and the ethylthio-coenzyme A disulfide adduct were prepared. As well, mixtures of each thiolated β-lactam with coenzyme A were also produced. A cell lysate of *S. aureus*, prepared as above, was prepared and mixed with a equal volume of a
20 µM solution of N-thiolated β-lactam. Each mixture of coenzyme A and lactam provided two new peaks, apparently the adduct and the resultant N-protio β-lactam. The mixture of lysate and lactam appeared to present the same adduct and N-protio β-lactam peaks. A reverse phase column was used with a eluent equal to 90% acetonitrile 10% water.
CHAPTER EIGHT

SPECTRA

Spectrum 8.01: $^1$H NMR (250 MHz, CDCl$_3$) of imine 105:
Spectrum 8.02: $^1$H NMR (250 MHz, CDCl$_3$) of $\beta$-lactam 106:
Spectrum 8.03: $^1$H NMR (250 MHz, CDCl$_3$) of β-lactam 107:
Spectrum 8.04: $^1$H NMR (250 MHz, CDCl$_3$) of β-lactam 107b:
Spectrum 8.05: $^1$H NMR (250 MHz, CDCl$_3$) of phthalamide 111a:
Spectrum 8.06: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111b:
Spectrum 8.07: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111c:
Spectrum 8.08: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111d:
**Spectrum 8.09:** $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111e:
**Spectrum 8.10:** $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111f.
**Spectrum 8.11**: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111g:
Spectrum 8.12: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111h:
Spectrum 8.13: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111i:
Spectrum 8.14: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111j:


**Spectrum 8.15:** $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111k:

![NMR Spectrum]

111k

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Spectrum 8.16: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 1111:
Spectrum 8.17: $^1$H NMR (250 MHz, CDCl₃) of phthalimide 111m:
Spectrum 8.18: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111n:
Spectrum 8.19: $^1$H NMR (250 MHz, CDCl$_3$) of phthalimide 111o:
Spectrum 8.20: FTIR of phthalimide resin 164:
Spectrum 8.21: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109a:
Spectrum 8.22: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109b:
Spectrum 8.23: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109c:
Spectrum 8.24: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109d:
Spectrum 8.25: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109e:
Spectrum 8.26: $^1$H NMR (250 MHz, CDCl$_3$) of lactam $^{109f}$.
Spectrum 8.27: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109g:

![Chemical Structure]

109g

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Spectrum 8.28: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109h:
Spectrum 8.29: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109i:
Spectrum 8.30: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109j:
Spectrum 8.31: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109k:
**Spectrum 8.32:** $^1$H NMR (250 MHz, CDCl$_3$) of lactam **109l**:
Spectrum 8.33: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109m1:
Spectrum 8.34: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109m:
Spectrum 8.35: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 109n:
Spectrum 8.36: $^1$H NMR (250 MHz, CDCl$_3$) of (R)(+) lactam 152:
Spectrum 8.37: $^1$H NMR (500 MHz, CDCl$_3$) of (R)-lactam 153:
Spectrum 8.38: $^1$H NMR (250 MHz, CDCl$_3$) of (S)(+) lactam 154:
Spectrum 8.39 $^1$H NMR (250 MHz, CDCl$_3$) of (S)-lactam 155:
Spectrum 8.40: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 157b:
Spectrum 8.41: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 157a:
Spectrum 8.42: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 157c:
Spectrum 8.43: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 158b:
**Spectrum 8.44:** $^1$H NMR (250 MHz, CDCl$_3$) of lactam 158a:
Spectrum 8.45: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 160:
Spectrum 8.46: FTIR of lactam resin 165:
**Spectrum 8.47:** $^1$H NMR (250 MHz, CDCl$_3$) of lactam 108OH:

![Chemical Structure Diagram]

**Graphical Representation:**

- Peaks at various ppm values.
- Integration peaks at 1.0, 2.0, 3.0, 4.0, 5.0, 7.0.

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Spectrum 8.48: $^1$H NMR (250 MHz, CDCl$_3$) of lactam p152:
Spectrum 8.49: $^1$H NMR (250 MHz, CDCl$_3$) of lactam 168:
Spectrum 8.50: $^1$H NMR (250 MHz, CDCl₃) of Coenzyme A Standard 9 and Resin-Lysate Extract 193:
**Spectrum 8.51: HPLC of Coenzyme A**

- Coenzyme A
- Coenzyme A Disulfide
Spectrum 8.52: HPLC N-Protio β-Lactam 107:
Spectrum 8.53: HPLC of ethylthiolated β-lactam 109b:
Spectrum 8.54: HPLC Purified Thioethyl Adduct:
Spectrum 8.55: HPLC Immediately after mixture of Ethylthiolated β-Lactam and Coenzyme A:
Spectrum 8.56: HPLC Immediately after mixture of sec-Butylthiolated β-Lactam and Coenzyme A:
Spectrum 8.57: HPLC Immediately after mixture of Phenylthiolated β-Lactam and Coenzyme A:
Spectrum 8.58: HPLC Immediately after mixture of ethylthiolated β-Lactam and *S. aureus* lysate:
Spectrum 8.59: HPLC *S. aureus* lysate:
REFERENCES


50) “Isolation of Psammaplin A 11'-Sulfate and Bisaprasin 11'-Sulfate From the Marine Sponge *Aplysinella rhax*,” Pham, N.; Butler, M.; Quinn, R. *Journal of Natural Products*, 2000, 63, 393-395.


ABOUT THE AUTHOR

Bart Heldreth received his bachelor’s degree in chemistry at Kent State University, Kent, Ohio. After marrying Amy, he pursued a doctorate in chemistry in the synthetic laboratory of Professor Edward Turos. Bart continues to follow his interest in organic synthetic chemistry, as well as chemical biology.