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Synthesis and Antimicrobial Activities of S,S'-Heterosubstituted Disulfides

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Synthesis and Antimicrobial Activities of S,S’-heterosubstituted Disulfides

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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LIST OF ABBREVIATIONS

ATP = adenosine triphosphate
ATPase = adenosine triphosphate synthase
Bn = benzyl
°C = degrees Celsius
CoA = coenzyme A
CoADR = coenzyme A disulfide reductase
Cys = cysteine
δ = delta or chemical shift (NMR)
DBE = disulfide bridge forming enzyme
DID = 5,5’-diphenyl-3,3’-diisothiazole disulfide
dNTP = deoxyribonucleoside triphosphate
DMSO: Dimethylsulfoxide
EC = effective concentration
Et3N = triethylamine
ETP = epipoly(thiodioxopiperazine)
GDR: glutathione disulfide reductase
GSH: glutathione
1H NMR= proton nuclear magnetic resonance
HIV = human immunodeficiency virus
HIV-RT = human immunodeficiency virus reverse transcription
Hz = hertz
J = coupling constant (NMR)
JUNV = Junin (agent of Argentine hemorrhagic fever)
MEA = 2-mercaptoethylamine (cysteamine)
MIC = minimum inhibitory concentration
MSH: Mycothiol
µg = micrograms
µM = micromolar
mM = millimolar
MRSA = methicillin-resistant \textit{Staphylococcus aureus}
MRSE = methicillin-resistant \textit{Staphylococcus epidermidis}
NAC = N-acetyl-L-cysteine
NCCLS: National Committee for Clinical Laboratory Standards
ng = nanogram
PenG: Penicillin G
Ph = phenyl
ppm = parts per million
$S_8$ = elemental sulfur
TCBZ = triclabendazole
TLC = thin layer chromatography
TSA: Trypticase®, Soy Agar
trx = thioredoxin
Antibiotic resistance is a particularly critical health concern and has increased dramatically over the past two decades. For over a decade the Turos laboratory has been designing small molecules to target pathogenic microbes such as *Staphylococcus aureus* and the resistant variants like methicillin-resistant *Staphylococcus aureus* (MRSA). Previously, N-thiolated β-lactams, N-thiolated 2-oxazolidinones and aromatic disulfides that were synthesized in Dr. Turos’ lab have shown strong activity against these bacteria. The present work describes the synthesis and antimicrobial activities of a related structural class called S,S’-heterosubstituted disulfides. For ages, sulfur (elemental) has been used as an antibacterial for controlling infestation and bacterial diseases. This is the starting point of this thesis. Chapter 1 discusses the various sulfur-containing antibiotic compounds and the importance of sulfur compounds to exhibit inhibitory activity against disease-causing pathogens. Also in this introductory chapter, the different sulfur functionalities and their respective modes of action were presented. The synthesis and the antimicrobial activities of the title compounds are described in chapter 2. S,S’-heterosubstituted disulfides were found to possess inhibitory activities against *Staphylococcus aureus*, methicillin-
resistant *Staphylococcus aureus* (MRSA), *Francisella tulerensis* and the fungi *Candida albicans*. From the bacterial viability assay and the trypan staining assay, these compounds were found to be bacteriostatic and fungistatic, and these structurally-simple disulfides may serve as new leads to the development of effective antibacterials for drug-resistant staph infections.
1.1 Introduction

The use of organosulfur compounds to control infectious diseases has its roots dating back to early times, when ancient Egyptians recognized the potent medicinal effects of naturally occurring organosulfur substances from leeks. In 1824 powered sulfur was shown to treat peach mildew\(^1\) and over the centuries, it has successfully been applied as a fungicide to protect a wide variety of plants against fungal infestation\(^1\). Although the mode of antifungal action of elemental sulfur is not fully defined, there is no doubt of its dependency on the reaction of sulfur with a biological target.

1.2 Biological Targets of Organosulfur Drugs

Due to the exposure of living organisms to various infection-causing pathogens, over time they developed defenses against harmful biological oxidants and disinfectants. The thiol-disulfide equilibrium in cells plays a vital role, creating a natural defense system that helps rid the cell of potentially damaging chemicals and metabolites that cause oxidative degradation. Nature protects various bacteria from oxidative stress by maintaining high thiol:disulfide ratios in the cytosol, 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 biological processes where an antioxidant is required in the cell.

1.2.1 Glutathione-based Systems

For many years, glutathione (1) (γ-L-glutamyl-L-cysteinylglycine), a tripeptide, serves as the active component of the most common thiol/disulfide redox system in many cells. Glutathione is produced biologically via the glutaredoxin enzymatic pathway (Fig 1.1). Biosynthesis of GSH occurs in all cell types via two reactions catalyzed by γ-glutamyl cysteine synthetase (γ-GCS) and GSH synthetase (GS). γ-GCS catalyzes the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine. Glutathione synthetase forms the peptide bond between the α-carboxyl of cysteine in γ-glutamyl cysteine and the α-amino group of glycine. The equilibrium between free thiol and disulfide, at stasis, is typically maintained at a cytoplasmic thiol concentration of around 90%. Cells with high intracellular glutathione levels are generally less susceptible to damage by organosulfur drugs. 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 cause any 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 endiyne 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 mixed glutathione drug disulfides\textsuperscript{4}. Reduction of glutathione disulfide (GSSG) and mixed disulfides to GSH is catalyzed by glutathione reductase, which seems to play a crucial role in evolutionary adaptation of organisms to atmospheric oxygen. Glutathione reductase is widespread among bacteria, fungi, plants, protozoa, and animals. All glutathione reductases isolated from different sources are highly homologous, showing high evolutionary conservativeness of this protein.\textsuperscript{3}

![Biosynthetic pathway of Glutathione]

\textbf{Figure} (1.1) Biosynthetic pathway of Glutathione

\textbf{1.2.2 Coenzyme A-based Systems}

For many years, glutathione was assumed to be present in all cells. However, it is not entirely ubiquitous. Some bacteria are totally devoid of glutathione, but in its place have
some other thiol/disulfide based redox system. *S. aureus* does not utilize glutathione at all, but rather produces millimolar levels of the nucleosidic thiol, coenzyme A (2) (CoA). At stasis, the bacterium maintains a ratio of thiol:disulfide of about 95:5, via coenzyme A disulfide reductase (CoADR) (Fig 1.2). This redox system is very selective 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. The fate of these mixed disulfides is not clearly known. Therefore, anti-infective compounds that can form CoADR resistant mixed disulfides with CoA can offer an effective mode of inhibition interfering with the redox buffer, providing an opportunity to enhance the susceptibility of the bacterial cell to oxidative damage. Indeed, glutathione is an inhibitor of the redox cycle of CoA, as it forms mixed CoA-glutathione disulfides that cannot be reductively cleaved, and is detrimental to the growth of this bacterium.

![Figure 1.2 Coenzyme A redox system](image)

**Figure (1.2)** Coenzyme A redox system

### 1.2.3 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 (DBE’s), which
span through the bacterial membrane connecting the cytoplasm with the periplasm (Fig 1.3). 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 DBE’s, could intervene. Formation of mixed disulfides between the trx’s and an organothio compound can potentially inhibit enzymatic reduction by disulfide reductases and thus can perturb proper cellular function.

**Figure (1.3) Regular thiol and disulfide interactions with Thioredoxins**

DBE’s, for the most part, inhabit the cytoplasmic membrane with exposed (Cys-X-X-Cys) functionalities on both the cytoplasmic and periplasmic surfaces. DBE’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 DBE’s electron transport abilities, affecting a host of cellular processes such as respiration and cytochrome syntheses. Although there is evidence that DBE repair enzymes exist\textsuperscript{7,8}, their effectiveness and versatility against non-native thiols or sulfides has not been reported.

\textbf{Figure (1.4)} Thiol and disulfide bearing drug interactions with Thioredoxins

So, these extraordinarily reactive, native groups represent significant potential as drug targets.
1.2.4 Mycothiol-based Systems

The redox buffer in case of Actinomycetes is that of mycothiol (3), which acts as the 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. Mycothiol undergoes metal-catalyzed autoxidation at a slower rate than GSH and is maintained in the reduced state by mycothiol disulfide reductase.

![Mycothiol](image)

**Figure (1.5)** Mycothiol

1.2.5 Bacillithiol-based Systems

Bacillithiol (4) is the α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid and most probably functions as an antioxidant. Bacillithiol, like the structurally similar mycothiol, may serve as a substitute for glutathione. Bacillithiol is used as a buffer system in *Bacillus* species, *Staphylococcus aureus* and *Deinococcus radiodurans*.

![Bacillithiol](image)

**Figure (1.6)** Bacillithiol
1.2.6 Other Cellular Targets of Organosulfur Antiinfectives

Another potential intracellular thiol target is adenosine triphosphate synthase (ATPase). ATPase is responsible for regeneration of the energy source of cells, adenosine triphosphate (ATP) (5), from adenosine diphosphate and inorganic phosphate (Fig 1.7). ATPase, which sits on the membrane of mitochondria, contains a reactive, free thiol that is vital to activity. Inhibition of ATPase, therefore, can be achieved by formation of a mixed disulfide.

![Figure (1.7)](image)

**Figure (1.7)** Synthesis of Adenosine Triphosphate (5)

1.3 Classes of Biologically-Active Organosulfur Compounds

Organic compounds that contain sulfur exhibit 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 be a non-participant in a side chain residue or an innocuous constituency of the molecular framework. Important examples of this are the bicyclic β-lactams, including penicillins (6), cephalosporins (7), and penems (8), and the sulfa drugs (9). Most of the β-lactam drugs act by inhibiting the transpeptidation reaction responsible for the synthesis of peptidoglycan, a key component of the bacterial cell wall that provides rigidity. 13, 14 Sulfa drugs are known to inhibit the
synthesis of folic acid in bacteria by competing with para-aminobenzoic acid. The anti-infective activity of these drugs does not appear to be directly related to a sulfur-centered event nor its presence in the molecule.

![Chemical Structures](image)

**Figure (1.8)** Bicyclic β-Lactams and Sulfa Drugs.

In case of thiosugars, the sulfur atom exerts a more definitive, yet subtle, effect on its biological activity. These sulfur analogs of the natural sugars and nucleosides act as inhibitors of glycosidases and reverse transcriptases, respectively\(^{15-18}\). 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 play a central role in the reaction of the molecule with a biological entity.
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 (Fig 9). Here, the sulfur atom is responsible for activating the compound toward nucleophilic attack by displacing a chlorine atom and forming an episulfonium ion \((\text{10})\). Thus formed, this cationic electrophile is prone to attack by a biological nucleophile, and can lead to crosslinking of duplex DNA.

Thiol-bearing enzymes often lose some or all of their activity in the presence of non-native thiols like allyl mercaptan due to the formation of mixed disulfide. Thus, thiol-bearing therapeutics are often effective at inhibiting enzymatic pathways regulated by these proteins. Although there are a large number of small, natural product thiols known
to possess antibiotic activities, there are only a few synthetically-derived thiols that have been shown to be antibacterial. A trio of thiodiazole aromatics (11) is reported to have minimum inhibitory concentrations (MICs) against *Staphylococcus aureus* in the 31-62 µg/ml range, but impose no effect on *E. coli* (Fig 1.11).

\[
\text{Figure (1.11) } S. \text{ aureus-Active Thiodiazoles.}
\]

A similar effect was observed in case of 1,3,4-thiadiazolium-5-thiolates\(^\text{20}\) (Fig 1.12), whose MIC’s against *Staphylococcus aureus* range from 1-8 µg/ml, and which also exhibit moderate activities against *E.coli*. The mechanism of action of these compounds is yet to be studied.

\[
\text{Figure (1.12) Substituted 1,3,4-thiadiazolium-5-thiolates}
\]

### 1.3.1.2 Antifungal 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. Most of the previously discussed antibacterial thiols have been observed to possess some antifungal
activity. Closely related derivatives of the antibacterial mercaptotriazoles (12) inhibit *Candida albicans* and *Saccharomyces cerevisiae*, with minimum fungicidal concentration (MFC) values in the range of 12.5 to 61 µg/ml\(^2\). Both the thiol and amine groups are believed to be required for antifungal activity, since the thiodiazole aromatics (which lack the amino moiety) and their precursors have no antifungal properties.

![Figure (1.13) Mercaptotriazole antifungals](image)

A new class of 1,3,4-oxadiazole compounds (13) have reportedly shown growth inhibition zones of 20-25 mm at 100 µg, equivalent to the control antifungal compound griseofulvin.

![Figure (1.14) Antifungal 1,3,4-oxadiazoles](image)
1.3.1.3 Antiviral Thiols

Intracellular redox activity controls replication and virulence of viruses\(^{22}\). The cellular thiol, glutathione, itself has purported \textit{in vitro} and \textit{in vivo} anti-influenza activity. As levels of glutathione are depleted in mucosal cells lining the oral, nasal and upper airways, susceptibility to viral infection is enhanced. Decreased intracellular glutathione levels are also implicated in Human Immunodeficiency Virus (HIV) infections, and methods to increase glutathione production, in human monocyte derived macrophages and lymphocytes, have been proposed as a means to stop these infections\(^ {23-25}\). \textit{N}-Acetyl-L-cysteine (NAC) and 2-mercaptoethylamine (MEA) have been shown to strongly increase glutathione levels in various cell lines\(^ {26}\). Several new \textit{N}-((\textit{N}-acetyl-L-cysteinyl})-\textit{S}-acetylcysteamine derivatives (Fig 1.15) have also been reported to actively release NAC and MEA intracellularly, which in turn improve glutathione levels.

![Chemical structures](image)

\textbf{Figure (1.15)} S-Acetyl-cysteamine derivatives

These compounds display an EC90 (effective concentration for 90% inhibition of virus yields) ranging from 80 to 380 \(\mu\text{M}\) against HIV in human monocyte-derived macrophages (MDM). 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. Examples of this include a group of thiocyanatopyrimidine nucleosides (14), which are reported to display reasonable activity (EC75 = 100 µM) against vaccinia virus replication in HeLa cells.

![Thiocyanatopyrimidine nucleoside (R = ribofuranosyl nucleoside).](image)

**Figure (1.16)** Thiocyanatopyrimidine nucleoside (R = ribofuranosyl nucleoside).

### 1.3.1.4 Antiparasitic Thiols

A novel sesquiterpene named T-cadinthiol (15) shows significant antiparasitic properties. This terpenoid metabolite, with four fixed stereocenters, displays activity towards cultured *Plasmodium falciparum*, a species of malaria, with an IC50 of 3.6 µg/ml. The mode of action of this agent is still unexplored, however, and a number of derivatives of 15, including the corresponding alcohol analog, were tested and shown to have absolutely no biological activity. The thiol group appears to be essential for antiparasitic effects.

![Antiprotazoaal agent T-Cadinthiol.](image)

**Figure (1.17)** Antiprotazoaal agent T-Cadinthiol.
African trypanosomiasis caused by *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* was treated with melaminophenylarsine melarsoprol (MelB), introduced in 1947\(^3\). MelB is insoluble in water and is administered by intravenous injection as a propylene glycol solution. This arsenical agent is frequently associated with a number of serious side effects and the solvent is an irritant that often causes thrombophlebitis. Water-soluble arsenical agents such as melarsonyl potassium (MelW) showed little improvement over MelB. Later a new, water-soluble trivalent arsenical agent, melarsamine hydrochloride (MelCy) (trade name: Cymelarsan), was shown to be very effective against *T. brucei brucei*, *T. evansi*, and *T. equiperdum* \(^3\), \(^3\). 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\(^3\). The most active thiol system is the propane-1,3-dithiol (2-melarsenyl).

![Trypanocidal agents](image)

**Figure (1.18a)** Trypanocidal agents
In fact, 2-melarsenyl is twice as potent as Cymelasan (MelCy) 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 thiol groups and thus the activity\textsuperscript{33}.

![Equilibrium between Cymelarsan and melarsen oxide in water](image)

**Figure (1.18b)** Equilibrium between Cymelarsan and melarsen oxide in water

### 1.3.2 Sulfide Reagents

Penicillin (6), cephalosporins (7) and penems (8), as discussed earlier in this chapter, are some of the most potent sulfide anti-infectives. Diallyl sulfide (Fig 1.19), isolated from
garlic, is another natural antimicrobial compound which has shown inhibitory activity against MRSA in mice.\(^{34}\)

\[
\begin{align*}
\text{S} & \quad \text{S}
\end{align*}
\]

**Figure (1.19)** Diallyl sulfide, isolated from garlic extract

### 1.3.2.1 Antifungal Sulfides

Six newly reported sulfide-bearing 1,4-naphthoquinones (16-21) have been found to display good to potent activities against *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Microsporum cannis*, with MFCs ranging from less than 0.78 to 50 \(\mu\)g/ml, a marked improvement over their respective oxygen counterparts\(^ {35}\). 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 and not to the presence of the sulfur moieties.

**Figure (1.20)** Antifungal Naphthoquinone Sulfides
1.3.2.2 Antiparasitic 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. A new bioactive derivative of TCBZ, 5-chloro-2-methylthio-6-(1-naphthyloxy)-1*H*-benzimidazole (22) has been recently discovered. While this analog has an effective dose of 15 mg/kg, the marketed TCBZ displays a 5 to 10 mg/kg effective dose against *Fasciola hepatica*. The mechanism of action of this analog has not been elucidated, and the role of the sulfide moiety is not known.

![Methylthiobenzimidazole fasciolicide (22)](image)

**Figure (1.21)** Methylthiobenzimidazole fasciolicide (22)

1.3.3 Disulfide Reagents

1.3.3.1 Antibacterial Disulfides

As therapeutic agents, disulfides usually serve as inactive structural components of biomolecules or 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 (23), create the same type of mixed disulfide products with native thiols that are seen with the corresponding thiol drugs.
When various concentrations of cysteine were added to ajoene-containing media, the ajoene concentrations were decreased. Similar effects were also observed with dithiothreitol instead of cysteine. The sulfhydryl groups in cysteine and dithiothreitol are highly reactive to disulfides and tend to form mixed disulfides, which are biologically inactive. This likely explains the decrease in concentration of ajoene and inhibitory activity towards various pathogens.\(^{38}\)

\[
\text{HO-S-S-S-HO} \\
\text{SH} \\
\text{S-allylmethymercaptocysteine} \\
\text{SH} \\
\text{S-allyldithiothreitol}
\]

**Figure (1.22)** Formation of mixed disulfides of Ajoene.

Epipolythiodioxopiperezines (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 are likely related to their outer membrane permeability, and are prone to nucleophilic attack on their electrophilic disulfide bridge. Gliotoxin (24) 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) sulffenylation of native thiols of certain proteins to make catalytically defunct mixed disulfides\textsuperscript{39, 40}. Which sulfur center in gliotoxin is the site of enzymatic attack is still unclear but it is likely that both may be involved.

\textbf{Figure (1.23)} Gliotoxin, an Epipolythiodioxopiperazine (ETP) antibacterial reacts with thiols.

Isolated from both a species of \textit{Psammaplysilla} and \textit{Thorectopsamma xana}, a trio of closely related disulfides was discovered with antibacterial activity\textsuperscript{41}. Psammaplin A (25) and the dimer, Bisaprasin (26), along with Psammaplin D (27) all inhibit growth of \textit{Staphylococcus aureus} and \textit{Bacillus subtilis}. Psammaplin D also shows activity against the Gram-negative bacterium \textit{Trichophyton mentagrophytes}\textsuperscript{42}.

\textbf{Figure (1.24)} Psammaplin A

\begin{center}
\includegraphics[width=0.8\textwidth]{gliotoxin.png}
\end{center}
Isolated from a tunicate, *Polycitorella mariae*, a novel disulfide termed Citorellamine (28), has been reported to have significant antimicrobial activity. Originally assigned the structure of a sulfide, the disulfide Citorellamine demonstrates potent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilus* and *Escherichia coli* as well as cytotoxicity in some cancer cell lines.

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 due to the presence of disulfide bonds\textsuperscript{45}. It was found to be cationic, yet hydrophobic, with a molecular mass of about 11.5kDa.

\begin{center}
\begin{tikzpicture}
  \node (a) at (0,0) {\includegraphics[width=0.5\textwidth]{citorellamine.png}};
\end{tikzpicture}
\end{center}

**Figure (1.27) Citorellamine**

A series of simple aryl–alkyl disulfides have also been looked at previously in the Turos lab. These aryl–alkyl disulfides\textsuperscript{46} demonstrated strong in vitro antimicrobial properties against \textit{S. aureus}, MRSA and \textit{B. anthracis}. The nitrophenyl alkyl disulfides exhibited the most in vitro potency, and bioactivity seems to require that the aryl substituent (X) be electron-withdrawing.

\begin{center}
\begin{tikzpicture}
  \node (a) at (0,0) {\includegraphics[width=0.5\textwidth]{arylalkyldisulfides.png}};
\end{tikzpicture}
\end{center}

**Figure (1.28) Aryl-alkyl Disulfides synthesized in Dr.Turos’ lab.**

In vitro microbiological properties of these compounds, determined from agar diffusion and agar MIC measurements, coincides with the FabH inhibition capabilities, which points to the likelihood that this key bacterial enzyme is associated with the biological properties of the disulfide compounds.
1.3.3.2 Antifungal Disulfides

Although the mechanism of action of the organodisulfide, Ajoene (23) 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. 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 (29), 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.

![Core Structure of the Thiarubine Group of Antifungals.](image)

The tunicate-generated disulfide, Citorellamine (28), not only possesses strong antibacterial activity, but is also very active against Saccharomyces cerevisiae and mildly active towards Pseudomonas aeruginosa. The other group of disulfides, the 1,2-dithiole-3-thiones (30), 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, Sacharomyces cerevisiae, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger,
Microsporum canis, Microsporis gypseum, Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentagrophytes. The main activity is due to the disulfide-thione functionality. Although the pendant sulfide (SR) is not strictly required for activity\textsuperscript{51}, its presence, as compared to alkyl or aryl groups, increases activity greatly. After lengthening the sulfur side chain beyond ethyl, an inverse relationship between chain length and antifungal activity begins to develop. However, against all of the fungi tested the thiobenzyl analog maintained the greatest potency, with MFC’s ranging from 0.7 to 6.25 µg/ml.

\[
\text{R} = \text{ethyl, propyl, butyl, hexyl, decyl, dodecyl, phenyl, benzyl, cyclopentyl}
\]

**Figure (1.30)** Dithiole-Thione antifungals.

1.3.3.3 Antiviral Disulfides

As discussed in relation to antibacterials, organodisulfides can act as \textit{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 have been reported with potent antiviral activities against the arena viruses Junin (JUNV), the causative agent of Argentine hemorrhagic fever, and Tacaribe (TCRV)\textsuperscript{52}. The disulfides and thiosulfonate (31-34) displayed 50% effective
concentration (EC50) values, the concentration at which 50% of the viral 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.

\[
\text{Cl}_2\text{PhHCOCNHN} - \text{NHCOCHPhCl}_2
\]

31

\[
\text{HN(H}_2\text{N)HCHN} - \text{NHCH(NH}_2\text{)NH}
\]

32

\[
\text{N} - \text{S} - \text{N} - \text{S}
\]

33

\[
\text{O} - \text{O}
\]

34

**Figure (1.31)** Antiarenavirus disulfides.

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 (35), 7-methyl-6,7,8,9-tetrahydrodibenzon [c,k] [1,2,6,9]-dithiadiazacyclododecine-5,10-dione\(^{53}\). This compound displays an EC50 of 0.05 µg/ml against HIV-infected macrophages, compared to the current standard AZT (3’-azido-3’-deoxythymidine) that has an EC50 of 0.004 µg/ml.
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 combination.

![Anti-HIV macrocyclic disulfide](image)

**Figure (1.32) Anti-HIV macrocyclic disulfide.**

Another disulfide with promising antiviral properties is 5,5'-diphenyl-3,3'-diisothiazole disulfide (36a) (DID). DID induces potent inhibition of plaque-infected cells derived from invasion of poliovirus type 1, with an IC$_{50}$ 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$_{50}$), the concentrations where normal human cell proliferation is inhibited by 50%, were more than 200 times higher than the IC$_{50}$'s, illustrating the exquisite selectivity this compound has for the viral infected cells. The reduced form, thiol 36b, has almost the same activity and selectivity as 36a (Table 1.1).

![5,5'-Diphenyl-3,3'-diisothiazole disulfide and thiol antipoliovirus agents](image)

**Figure (1.33) 5,5'-Diphenyl-3,3'-diisothiazole disulfide and thiol antipoliovirus agents.**
Table 1.1 Isothiazole anti-poliovirus activities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Selectivity CC&lt;sub&gt;50&lt;/sub&gt;/ IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>36a</td>
<td>0.35</td>
<td>89.28</td>
<td>255</td>
</tr>
<tr>
<td>36b</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<sup>56</sup>. A number of sulfur-bearing nucleotide HIV-RT inhibitors, which have similar effects to AZT, include 3’-mercapto-2’,3’-dideoxynucleotides (37) and 2’-deoxy-2’-mercaptopuridine-5’-diphosphate (38)<sup>57, 58</sup>. These nucleosides serve to transfer a radical to a thiol of the transcriptase as shown in Fig 1.33.

![Figure 1.33](image)

X = Adenine, cytosine, guanine, thymine

**Figure (1.34)** Dideoxynucleotides thiol radical-based reverse transcriptase inhibitors.
Figure (1.35) Postulated mechanism of reduction of natural ribonucleoside 5’-diphosphates.

A new pyrimidine nucleoside disulfide (39) has been synthesized and shown to inhibit both HIV-RT and dNTP. The disulfide also has an EC50 of 10 µM and an IC50 of 25 µM against proliferation of human T-lymphocyte cells. Very interestingly, the corresponding thiol derivative had no activity at all.

\[ Y = \text{SH, SSC}_3\text{H}_7 \]

Figure (1.36) Mercaptouridine Thiyl Radical-based Reverse Transcriptase Inhibitors.
Figure (1.37) Nucleosidic disulfide anti-HIV agent.

1.3.4 Trisulfide Reagents

1.3.4.1 Antibacterial Trisulfides

Trisulfides found in garlic, such as diallyl trisulfide and allyl methyl trisulfide (Fig 1.38), can act as antibacterial agents in the same way as disulfides, but with greater efficacy.

![Diallyl trisulfide](image1)

![Allyl methyl trisulfide](image2)

Figure (1.38) Anti-infectives from garlic

The antibacterial activity of some cyclic polysulfides like tetrathiepanes can be attributed to a trisulfide bridge, as much as previous examples owe their activity to a disulfide bridge. 4-Dioxo-1,2,4,6-tetrathiepane (40), an extract from the red alga Chondria californica, has potent antibacterial activity against Vibrio anguillarum, the causative agent of a tropical fish disease.
Also part of the trisulfide family of antibiotics are the enediyne trisulfide antitumor antibiotics, calichaemycin (41a), a natural product of *Micromonospora echinospora*, Namenamicin (41b), and esperamicins, natural products of *Actinmadura verrucosospora*. Calichaemycin is at least a thousand times more potent than penicillin G against *S. aureus*. Although these antibiotics are not currently used to treat infection because of their elevated toxicity, in anticancer studies these compounds have been shown to undergo an intricate cascade of intramolecular reactions initiated by glutathione attack, resulting in an intensely reactive radical species.
Figure (1.40) Enediyne Trisulfides: Calichaemycin (41a), Esperamicin A₁ (41b) and Namenamicin (41c).

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 orbital 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, it is likely that the trisulfide moiety is involved as an electrophilic reactant with cellular thiols (Table 1.2).
**Figure (1.41) Bioactivation of Enediynes**

**Table 1.2 Enediyne Antibacterial Activities**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC(µg/ml)</th>
<th>Calichaemycin</th>
<th>Namenamicin</th>
<th>Penicillin G</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td>0.00005</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>0.000001</td>
<td>0.001</td>
<td>0.015</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td></td>
<td>0.00012</td>
<td>0.03</td>
<td>128</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>0.12</td>
<td>0.12</td>
<td>32</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td>0.25</td>
<td>0.06</td>
<td>128</td>
</tr>
</tbody>
</table>

1.3.4.2 Antifungal 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\(^6\). 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 cellular thiophile.
Table 1.3 Enediyne Antifungal Activities

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Calichaemycin (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>

1.3.5 Polysulfide Reagents

1.3.5.1 Antibacterial Polysulfides

Within the Didemnidae or tunicate family, *Lissoclinum* species are rich sources of organosulfur antibiotics. One such isolate, Lissoclinotoxin A (42), 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*, *Actinetobacter*, and *Proteus* species.62

![Lissoclinotoxin A](image)

**Figure (1.42)** Lissoclinotoxin A.

1.3.5.2 Antifungal Polysulfides

Lissoclinotoxins A (42) and D (43) (disulfide analog), 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.

![Figure](image.png)

**Figure (1.43)** Lissoclinotoxin D.

### 1.3.5.3 Antiparasitic Polysulfides

Another anti-malarial agent, Lissoclinotoxin A (42) has also demonstrated potent activity towards a resistant strain of the parasite *Plasmodium falciparum*\(^6\). Lissoclinotoxin A is intermediate in activity compared to the usual antimalarials quinine, mefloquine, halofantrine and chloroquine, with an IC\(_{50}\) of 296 nM (Table 1.4). Although not yet defined conclusively, its mechanism is likely similar to that described for di- and trisulfide antibacterials.

**Table 1.4** 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 (42)</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>
1.3.6 Other Organosulfur agents

1.3.6.1 N-Thiolated Antibacterials

N-Sulfenylated monocyclic β-lactams (44) 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. Regardless of 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.

![Figure (1.44) N-Sulfenylated-β-Lactam Antibacterials](image)

It is believed that an intracellular thiol in susceptible bacteria, coenzyme A, attacks the sulfur atom to form a mixed disulfide which in turn causes inhibition of bacterial growth by blocking lipid biosynthesis. These thiolated lactams show a narrow range of antibacterial properties, including *Staphylococcus* strains such as MRSA and *S. epidermidis*, with MIC’s as low as 0.125 µg/ml.
Another sulfenamide, 1,2-benzoisothiazolin-3-one (BIT) (45), has shown weak activity against *Staphylococcus aureus*, with an MIC around 100 µg/ml [57].

**Figure (1.46) BIT.**

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 (46), a drug designed as a gastric acid pump inhibitor, has been shown to rearrange in the acidic environment of the stomach to a sulfenamide (47), which is an inhibitor of *Helicobacter pylori* [65]. *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 sulfinamide affords fast action with an MIC value of 10 µg/ml.
1.4 Conclusions

This chapter discusses the various classes of organosulfur compounds which have shown efficient antimicrobial activities against bacteria, viruses, fungi and various parasites. Most of these compounds are either made by Nature or inspired from them. The mode of action of the majority of these organosulfur compounds has not been defined and requires further investigation. One of the rich sources of natural organosulfur compounds is *Allium sativum* (garlic), which has potent antibacterial and antifungal activity. A major inevitable problem with bacteria is the development of antibiotic resistance. The scope of these organosulfurs as clinical anti-infectives could possibly be improved if they could be functionalized innovatively to circumvent the resistance mechanisms or act in multi-modal ways.

Figure (1.47) Lansoprazole and *in vivo* Sulfenamide.
CHAPTER TWO

SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF S, S’-HETEROSUBSTITUTED DISULFIDES

2.1 Introduction

The concern over multidrug-resistant bacteria and the need for more effective antibacterials has reached a critical level, given that successful treatment of common bacterial infections can no longer be taken for granted. Staphylococcus aureus (Staph), a gram-positive facultative anaerobe, resides in the nostrils and on the skin of human beings. S. aureus starts to cause infection once it enters the bloodstream. Antibiotic-resistant variants of S. aureus, mainly methicillin-resistant Staphylococcus aureus (MRSA), is the single most important pathogen causing infections. The mutants from the community, referred to as community-acquired MRSA (CA-MRSA), are known to be more lethal and complex in nature than the variants originating in hospitals, which are referred to as hospital-acquired MRSA (HA-MRSA). MRSA is known to cause infections of skin and soft tissue, as well as pulmonary, osteoarticular, hemorrhagic adrenal gland (Waterhouse-Friderichsen syndrome) and opthalmic infections. According to the United States Centers for Disease Control and Prevention (CDC), MRSA accounted for more than 94,000 life-threatening infections and nearly 19,000 deaths in the United
States alone in 2005\textsuperscript{71}. CDC also emphasizes that the prevalence of multi-drug resistant bacteria is rising at an alarming rate.

The Turos laboratory has been studying the effects of a variety of synthetic antibacterial agents against common microbes. Previously we have investigated N-thiolated β-lactams\textsuperscript{64, 72-77}, N-thiolated 2-oxazolidinones\textsuperscript{78} and recently, a novel family of aryl-alkyl disulfides,\textsuperscript{46} are which all effective growth inhibitors of MRSA. The mode of action and structure–activity profiles of all these substances differ dramatically from those of other analogs of β-lactams, oxazolidinones, and disulfides. Investigations in our laboratory have shown that these compounds can each carry a wide range of substituents, and are reactive towards thiophilic agents under certain conditions (such as in bacterial cytoplasm).

![Various antibacterials synthesized in the Turos lab.](image)

**Figure (2.1)** Various antibacterials synthesized in the Turos lab.

The aryl–alkyl disulfides were looked at previously in the Turos lab by Dr. Kevin Revell, and found to have strong in vitro antimicrobial properties against *S. aureus*, MRSA and *B. anthracis*. The nitrophenyl alkyl disulfides exhibited the most in vitro potency.
Figure (2.2) Various aryl-alkyl disulfides synthesized in the Turos lab.

The fact that the in vitro microbiological properties of these disulfides, determined from agar diffusion and agar MIC measurements, coincides with the FabH inhibition capabilities points to the likelihood that this key bacterial enzyme is associated with the biological properties of the compounds. More detailed studies into this are now being done in collaboration with Dr. Lindsey Shaw’s laboratory in the USF Biology department.

This chapter describes experiments on a series of new sulfur-containing antibacterials, S,S’-heterosubstituted disulfides. In this study, we investigated the synthesis and antibacterial and antifungal properties of these structurally intriguing compounds.

2.2 Synthesis

These investigations were initiated by the synthesis of a group of S,S’-heterosubstituted disulfides shown in figure 2.2. These compounds were chosen as a means to assess the electronic effects of different heteroatoms on bioactivity. These derivatives were prepared by treating an alcohol, amine or thiol with sulfur monochloride at -20 °C in the
presence of triethylamine as a Lewis base (Fig 2.3). The desired disulfide products were obtained in 70-90% yields after column chromatography, and characterized by $^1$H NMR spectroscopy.

![Figure (2.3)](image-url) Synthesis of S,S'-heterosubstituted disulfides.
2.3 Elucidation of $^1$H NMR spectrum of di sec-butoxy disulfide (1d):

The structure of the disulfide products 1-4 were determined by $^1$H NMR spectroscopy. The $^1$H NMR spectrum of di-sec-butoxy disulfide (1d) is illustrated below as a representative example (Fig 2.5). Apart from the obvious lack of the alcohol O-H peak in $^1$H NMR spectrum (from the starting alcohol), the chemical shifts of the disulfide protons

Figure (2.4) Synthesized S,S'-heterosubstituted disulfides.
are slightly downfield as compared to those of sec-butanol. This can be attributed to the presence of the oxygen-sulfur bond which deshields the neighbouring protons more than in the case of the alcohol, due to the electronegativity of the O-S bond.

**Figure (2.5)** $^1$HNMR spectra of sec-butanol and di sec-butoxy disulfide (1d).

The protons of carbon-4 of butanol (marked in red) have a chemical shift of 0.70-0.76 ppm and appear as a clearly-defined triplet whereas in the disulfide they appear as an
unresolved multiplet at 0.83-0.91 ppm. The C-1 methyl protons appear as a doublet at 1.17-1.23 ppm in case of the disulfide and at 0.97-0.99 ppm for the alcohol. The methylene protons show up at 1.46-1.61 ppm as a multiplet for the disulfide and at 1.22-1.31 ppm in case of the alcohol. And finally methine (–CH) proton appears at 3.77-3.86 ppm for the disulfide and at 3.49-3.56 ppm in the alcohol. Similar trend was observed in rest of the disulfides.

2.4. Reactivity of S,S’-heterosubstituted disulfides

The reactivity of S,S’-heterosubstituted disulfides has been previously explored by Motoki et al. Dialkoxy disulfides reportedly react with mercaptans or secondary amines (Fig 2.6) at reflux conditions in carbon tetrachloride to give alkoxy alkyl trisulfides (2) or alkoxy amino disulfides (3) with elimination of alcohol. The yields are typically below 50% for reactions with thiols, and 22-74% for amines.

\[
\begin{align*}
\text{ROSSSR'} & \overset{\text{R'SH} \quad 50^\circ \text{C}}{\longrightarrow} \overset{\text{CCl}_4 \quad 3\text{hrs}}{\text{ROSSOR}} \quad \overset{\text{HN}_1^\text{R''} \quad \text{R''}}{\longrightarrow} \overset{\text{CCl}_4 \quad \text{4hrs \ reflux}}{\text{R''O-S-S-N-R''}}
\end{align*}
\]

Figure (2.6) Reactivity of dialkoxy disulfides with thiols and secondary amines.

These alkoxy alkyl trisulfides (2) further react with mercaptans or secondary amines at refluxing conditions in carbon tetrachloride to give unsymmetrical dialkyl tetrasulfides (4) and alkylamino trisulfides (5) in varying yields.
Figure (2.7) Reactivity of alkoxy trisulfides (2) with thiols and secondary amines.

The alkoxy diamino disulfides (3) also react with thiols and amines in the similar fashion (Fig 2.8) to yield alkylamino trisulfides (5) and unsymmetrical diamino disulfide (6), respectively.

Figure (2.8) Reactivity of alkoxy amino disulfides (3) with thiols and secondary amines.
Motoki found that when diethoxy disulfide was reacted with 1,1-disubstituted thioureas, thiadiazoles (7) were obtained, whereas 1,3-disubstituted thioureas yielded carbodiimides (9) in moderate yields.\textsuperscript{80}

\[
\begin{align*}
\text{R}_2\text{N}=\text{C}=\text{N}\text{R}_1 & \quad \xrightarrow{\text{CH}_2\text{Cl}_2 \ 2 - 23 \text{ hrs}} \quad \text{ROSSOR} \\
\text{R}_1\text{N} & \quad \xrightarrow{\text{CCl}_4 \ 6 - 11 \text{ hrs}} \quad \text{ROSSOR}
\end{align*}
\]

\[7 \quad \xrightarrow{\text{Reflux}} \quad 80 - 66\% \]

\[8 \quad \xrightarrow{\text{Reflux}} \quad 19 - 66\% \]

**Figure (2.9)** Reactivity of dialkoxy disulfides (1) with 1,1 and 1,3-disubstituted thioureas.

Motoki et al. also studied the reactions of diethoxy disulfide with various arylhydrazines (Fig 2.10). Diethoxy disulfide on refluxing with arylhydrazines in benzene loses ethanol and yields aryl ethoxy tetrasulfides (10), aryl benzenes and diaryl sulfides.\textsuperscript{81}

\[
\begin{align*}
\text{ROSSOR} & \quad \xrightarrow{\text{PhH} \ 10 - 30 \text{ hrs}} \quad \text{Ar}\text{NHNH}_2 \\
\text{R}_1\text{N} & \quad \xrightarrow{\text{PhH} \ 10 - 30 \text{ hrs}} \quad \text{Ar}\text{NHNH}_2
\end{align*}
\]

**Figure (2.10)** Reactivity of dialkoxy disulfide (1) with monosubstituted hydrazine.

All these compounds were purified either by recrystallization or column chromatography and characterized by elemental analysis and $^1$H NMR spectroscopy. A detailed look at the various reaction conditions can be seen in table 2.1.
Table (2.1) Reaction of diethoxy disulfide with monosubstituted hydrazine.

<table>
<thead>
<tr>
<th>Hydrazine (ArNHNH₂)</th>
<th>Benzene refluxing time (hrs)</th>
<th>Products- Isolated yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar</td>
<td></td>
<td>Ar-C₆H₅</td>
</tr>
<tr>
<td>4-NO₂C₆H₄</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td>2-ClC₆H₄</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>4-BrC₆H₄</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>4-CH₃C₆H₅</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>2-C₁₀H₇</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

2.5. Antimicrobial activity

The antimicrobial activity of these S,S’-heterosubstituted disulfide compounds 1-5 (Fig 2.2) was evaluated against a selection of available common bacteria by determination of the minimum inhibitory concentration (MIC), Kirby-Bauer diffusion assay and growth viability assay. Minimum fungicidal concentration (MFC) and viability assays were also performed against the fungus, Candida albicans.

2.5.1. Determination of the minimum inhibitory concentrations of disulfides against bacteria

According to the National Committee for Clinical Laboratory Standards (NCCLS), the MIC is the lowest concentration of antimicrobial agent that completely inhibits visible growth of the organism as detected by the unaided eye. The minimum
inhibitory concentrations of the S,S’-heterosubstituted disulfides were evaluated against *Staphylococcus aureus* (S.A - ATCC 25923) and a methicillin-resistant strain of *Staphylococcus aureus* (MRSA – ATCC 43300) by Danielle Gergeres in Dr. Turos’ lab by agar dilution using a 24-well plate. This was done by allowing the bacteria to grow for 24 hours in presence of varying concentrations of antibiotic. All the antimicrobial assays were performed in triplicate. Penicillin G was used as a positive control and DMSO as negative control in all these assays. The averaged MIC values of the S,S’-dialkoxy disulfides are shown in table 2.2. For this series, antibacterial activity improved with the increase in the chain length, with exception for the sec-butoxy derivative. The most active of the five analogs is the isopropoxy compound (1b).

**Table (2.2)** MIC’s of S,S’-dialkoxy substituted disulfides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>S.A (µg/mL)</th>
<th>MRSA (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>propyl</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>1b</td>
<td>isopropyl</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>1c</td>
<td>butyl</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>1d</td>
<td>s-buty</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>1e</td>
<td>phenyl</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

The activity among the thiol substituted disulfides (Table 2.3) decreases with increase in the alkyl chain branching. Surprisingly these compounds were not as active even with the
presence of four sulfur atoms, indicating that the number of sulfur atoms is not directly related to the antibacterial activity. The phenyl substituent (2e) was found to provide the greatest potency among the five R substituents examined. In general, the activity decreased with the increase in the hydrophobic nature of the chain. In case of the S,S’-diamino disulfides (3a-e), those made from primary amines (Table 2.4) have weaker activity than those of secondary amines (Table 2.5).

**Table (2.3) MIC’s of S,S’-dithio substituted disulfides.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>S.A (µg/mL)</th>
<th>MRSA (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>propyl</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>2b</td>
<td>isopropyl</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>2c</td>
<td>butyl</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>2d</td>
<td>s-butyl</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>2e</td>
<td>phenyl</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
Table (2.4) MIC’s of S,S’-diamino substituted disulfides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>S.A (µg/mL)</th>
<th>MRSA (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>propyl</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>3b</td>
<td>isopropyl</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>3c</td>
<td>butyl</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>3d</td>
<td>s-butyl</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>3e</td>
<td>phenyl</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

For the S,S’-diamino substituted disulfides also, the most active compound was the phenyl substituted one, followed by the propyl. Compound 4a, synthesized from the dimethylamine, showed potent activity among the secondary amine disulfides. Increase in the chain length and branching only resulted in the loss of activity in this case. In comparison the S,S’-diamino disubstituted disulfides had a smaller range of MIC values than the other analogues.
Apart from varying the alkyl chains on the heteroatoms, we also wanted to observe the effects of chirality on the bioactivity. To accomplish this, various chiral alcohols and amines were used as substrates for the reaction with sulfur monochloride. It was observed that the activities of the chiral disulfide analogs 5 & 6 (Table 2.6) were not much different from their racemic counterparts. This was observed in case of sec-butyl and menthyl disulfide analogs 5a-g. Starting materials for compounds 5c, 5d and 5g were racemic mixture of diastereomers, while the rest were optically pure.
Chiral analogs:

![Chiral analogs](image)

**Figure (2.11)** Synthesized chiral S, S'-diheterosubstituted disulfides.

**Table (2.6) MIC's of Chiral disulfides 5&6.**

<table>
<thead>
<tr>
<th>Chiral Substrates</th>
<th>Compound</th>
<th>R</th>
<th>S.A</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>(S) - s-butyl</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>(R) - s-butyl</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td>1-phenylpropyl</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>2-phenylpropyl</td>
<td>16 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td>(1S, 2R, 5S)-(+)-menthyl</td>
<td>8 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>----------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5f</td>
<td></td>
<td>L(-)-menthyl</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>5g</td>
<td></td>
<td>(±)-menthyl</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

### Amines

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>(R)-benzyl-(1-phenylethyl)</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>6b</td>
<td>(S)-benzyl-(1-phenylethyl)</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

#### 2.5.2 Bacterial Viability Assay

A bacterial viability assay was carried out by Sonja Dickey in Dr. Daniel Lim’s lab in the Department of Biology, University of South Florida, to further assess the antibacterial activity of the most active compound, diisopropoxy disulfide (1b). This assay was conducted by counting the viable bacterial cells before and after incubation. The initial plate count in all tubes was $10^5$ cfu/ml. *Staphylococcus aureus* 25923 and MRSA 43300 used as test microbes showed a 3-log increase in the number of bacteria in the absence of the disulfide. Diisopropoxy disulfide (1b) was tested against *S. aureus* 25923 and MRSA 43300 in concentrations of 2 and 4 µg/ml. In the tubes with 2 µg/ml disulfide, *S. aureus* 25923 was decreased in number of cells by one log, but still not all were killed. In the MRSA 43300 2 µg/ml tube, it appears that the cell number increased by one log. In fact there were too many cells (>1200 cfu/ml) to count on those plates; and since plate counts are considered accurate only when there are 30 – 300 cells on the plate, this cannot be considered an accurate count. In the tubes with 4 µg/ml of disulfide, the number of cells decreased by 2 logs in both *S. aureus* 25923 and MRSA 43300. While there was a significant decrease in the number of cells, not all were killed. Even with the growth in
the 2 µg/ml MRSA tube, it is still less by 2 logs than the control. Also, the minimum inhibitory concentration of diisopropoxy disulfide (1b) is 2 µg/ml since there was no visible growth of bacteria in the broth until the concentration of disulfide was lowered to 1 µg/ml. These factors point towards a bacteriostatic nature of the compound.

Table (2.7) Bacterial viability assay.

<table>
<thead>
<tr>
<th></th>
<th>Drug Concentration (µg/mL)</th>
<th>Before Incubation (cfu/mL)</th>
<th>After Incubation (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S.aureus 25923</strong></td>
<td>Control (no drug)</td>
<td>2.11 x 10⁵</td>
<td>7.6 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.22 x 10³</td>
<td>2.04 x 10³</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1 x 10⁴</td>
<td>3.86 x 10⁴</td>
</tr>
<tr>
<td><strong>MRSA 43300</strong></td>
<td>Control (no drug)</td>
<td>3.09 x 10⁵</td>
<td>4.02 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.96 x 10³</td>
<td>2.88 x 10³</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1 x 10⁴</td>
<td>&gt;1.2 x 10⁶</td>
</tr>
</tbody>
</table>

2.5.3 Anti-staphylococcal assay by Kirby-Bauer testing on agar plates

In addition to evaluating the biological activity by Kirby-Bauer diffusion assay, the effect of various additives on the bioactivity of the dialkoxy disulfides was also investigated. The intent was to select one of the most bioactive S,S’-heterosubstituted disulfide compound, diisopropoxy disulfide, as a representative for all the testing. Two more groups of antibacterials, N-thiolated β-lactams and alkyl-aryl disulfides (Figure 2.12) previously synthesized in Turos’ lab, were also tested simultaneously. All the
experiments were performed against MRSA (ATCC 43300) in Mueller-Hinton agar in duplicates. 10µg of 1-isopropyldisulfanyl-4-nitrobenzene, 20µg of acrylic acid 2-(2-chlorophenyl)-1-methylsulfanyl-4-oxo-azetidin-3-yl ester, 50µg of diisopropoxydisulfide (1b) and 10µg of penicillin G were used for the assay. All the assays were performed by agar diffusion with 1mg of additive in the centre well. These amounts were considered after optimization to obtain comparable zones of growth inhibition for easier comparison.

![Diisopropoxy disulfide](image)

![1-isopropyldisulfanyl-4-nitrobenzene](image)

![Acrylic acid 2-(2-chlorophenyl)-1-methyl sulfanyl-4-oxo-azetidin-3-yl ester](image)

![Penicillin G potassium salt](image)

**Figure (2.12)** Compounds tested by Kirby-Bauer diffusion assay.
The anti-MRSA activity of diisopropoxy disulfide was neutralized by the presence of a free thiol diffusing away from the centre well. This cancelling effect was also observed in case of the aryl disulfide and beta-lactam. However penicillin G salt had clear zones with no inhibition observed. This inhibition was further confirmed by the absence of zones around the wells containing both disulfide and glutathione (GSH) or coenzyme A (CoA). Since all the compounds were tested as a solution in DMSO, another assay was performed to make sure the solvent did not show any inhibitory activity by itself. As expected, DMSO did not induce, nor diminish, activity of the compounds as it diffuses outward from the center well (Figure 2.13).

In assays with compounds containing a free thiol group such as coenzyme A (CoA) glutathione (GSH) and cysteine (Cys) the antibacterial activity of the compounds was completely cancelled (Figure 2.14). This was evident from the indented regions of growth inhibition after 24 hours of incubation.

Figure (2.13) Kirby-Bauer Assay showing no growth inhibition by DMSO.
Figure (2.14) Kirby-Bauer assay showing the ability of CoA, GSH and cysteine to inhibit antimicrobial effects of the test compounds on MRSA.

Figure (2.15) Structures of cysteine, coenzyme A and glutathione.
Other additives that were tested as controls included glycine, lysine, valine, isopropyl disulfide and triphenylphosphine. There was no inhibition observed in any of these cases. This can be attributed to the absence of free thiol (Figures 2.16 & 2.17). In case of triphenylphosphine a small zone was observed around its well indicating antimicrobial activity. The activity may be due to its ability to reduce disulfides responsible for the redox equilibrium in the bacteria.

\[\text{Figure (2.16) Kirby-Bauer assay showing no cancellation effect of diisopropyl disulfide by isopropyl disulfide or triphenylphosphine.}\]
Figure (2.17) Kirby-Bauer Assay showing no inhibition effect from the amino acids valine, lysine and glycine.

2.6 Studies of the mode of action of S,S’-heterosubstituted disulfides

It can be inferred from the Kirby-Bauer experiments that the interaction between the free thiol group of the additives and the S,S’-heterosubstituted disulfides is responsible for the inhibition of the biological activity.

In order to determine the exact concentrations of the coenzyme A and glutathione at which they can completely inhibit the activity of S,S’-heterosubstituted disulfides,
another Kirby-Bauer assay was performed. In this assay both the disulfide and the additive (coenzyme A or glutathione) were added in the same well, using varying amounts of the additive, while maintaining a constant initial amount of disulfide.

Thus, disulfide 1b was added along with 0.25, 0.5, 0.75 and 1 molar equivalent of glutathione into different wells cut into the agar in a Petri plate with MRSA (Fig 2.18) and incubated for 24 hours at 37 °C. A parallel assay was also done using coenzyme A in place of glutathione. There were no zones observed around any of the wells, indicating that the concentration of glutathione and coenzyme A required to inhibit the activity of compound 1b was less than 0.25 molar equivalents.

![Figure (2.18)](image)

**Figure (2.18)** Kirby-Bauer assay with compound 1b and glutathione in different molar ratios in the same well.

To explore more on the type of conjugates formed, compound 1b was reacted directly with coenzyme A in phosphate buffer solution (pH = ~ 7) at 37 °C. HPLC traces did not
show any evidence of formation of a new adduct even after 24 hours. A similar reaction was also monitored in an NMR tube. Compound 1b was co-mixed with equimolar amount of propane-2-thiol at room temperature and heated to 50 °C for 24 hours. This experiment also did not show any change in the starting materials. This finding indicates something interesting, in that although the mixture was heated to 50 °C, there was no apparent reaction between the disulfide and the thiol; however, there must be a reaction occurring between the disulfide and thiol additive in the well (or agar) of the Petri plates at 37 °C, since the disulfide completely loses its bioactivity. This suggests the possibility of bacterial enzymes influencing the formation of the biologically inactive conjugates, perhaps via a thiol transfer process.

**Figure (2.19)** $^1$HNMR tube experiment of diisopropoxy disulfide and propane-2-thiol in CDCl$_3$. 
In order to explore the possible mode of action, an experiment was conducted to check if the disulfides were interacting with the formation of the bacterial cell wall. The MRSA cells were observed under scanning electron microscope (SEM) before and after the treatment with diisopropoxy disulfide. It was observed that there was no change in their external morphology. The cell wall looked intact, indicating that the compound did not disrupt or interact with bacterial transpeptidases or any cell wall-related proteins. If the compounds were to interact with these components, the spherical structure of the cells would most likely have been disrupted as what happens in the case of penicillin G.

![Scanning electron microscopy pictures of MRSA cells before and after treatment with diisopropoxy disulfide.](image)

**Figure (2.20)** Scanning electron microscopy pictures of MRSA cells before and after treatment with diisopropoxy disulfide.

### 2.7 Activity of S,S’-heterosubstituted disulfides against other microbes

To assess the range of antimicrobial activity, S,S’-heterosubstituted disulfides were also evaluated against various other bacteria (*Francisella tulerensis, Bartonella sp., Escherichia coli*) and fungus (*Candida albicans*). The testing of *Francisella* and *Bartonella* was carried out by John Thomas in the biosafety level (BSL) 3 labs of Dr. Burt Anderson at the College of Medicine, University of South Florida.
2.8 Anti-Francisella activity

*Francisella tularensis* is a pathogenic species of gram-negative bacteria and the causative agent of tularemia, also known as rabbit fever or “cat scratch fever”. Symptoms of tularemia include fever, lethargy, anorexia and signs of septicemia, and in extreme cases can cause death. As low as 10-50 cfu\(^82\) (colony-forming units) are sufficient to cause an infection. Due to its high pathogenicity and ease of spread by aerosol they were and still are considered for usage in biological warfare\(^83,84\). Hence *F.tularensis* is classified as a Class A agent by the U.S. government. Class A agents have a moderate to high likelihood for large-scale dissemination or a heightened general awareness that could cause mass fear and civil disruption. *F. tularensis* is susceptible to carbapenems, ceftriazone, ceftazidime, rifampin and certain macrolides, but a lack of clinical data to recommend any of these compounds for clinical use\(^85\). Moreover, there is broad resistance to erythromycin among strains of *F. tularensis* subspecies *holarctica*\(^86\). Ciprofloxacin is the only drug that is considered ideal for the treatment of tularemia and it may not be long before antibiotic resistance mechanisms initiate. In addition, very little is known about the molecular basis of this pathogen\(^82\). The availability of the genome-sequence will help better understand this organism. So there is a need for new antibacterial agents and protocols for treating these infections.

The minimum inhibitory concentrations (MIC) of the 29 S,S`-heterosubstituted disulfides 1-6 were evaluated against a live vaccine strain (LVS) of *Francisella tulerensis* (F.T) by broth dilution (Table 2.8).
Table (2.8) MIC’s of S,S’-heterosubstituted disulfides against *Francisella tularensis*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Compound</th>
<th>R</th>
<th><em>Francisella tularensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>S,S’- dialkoxy substituted disulfides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>propyl</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>isopropyl</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>butyl</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>s-butyl</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>1e</td>
<td>phenyl</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S,S’- dithio substituted disulfides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>propyl</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>isopropyl</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>butyl</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>s-butyl</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>2e</td>
<td>phenyl</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S,S’- diamino substituted disulfides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>propyl</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>isopropyl</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3c</td>
<td>butyl</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>s-butyl</td>
<td>8</td>
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</tr>
<tr>
<td>3e</td>
<td>phenyl</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>S,S’- diamino disubstituted disulfides</td>
<td></td>
<td></td>
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<tr>
<td>4a</td>
<td>methyl</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>ethyl</td>
<td>16</td>
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</tr>
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</table>
There was no obvious structure-activity relationship among these 29 analogues, but some of the compounds showed good activities with MIC’s as low as 0.5 µg/ml. Bisdiaminophenyl disulfide (3e) was observed to be the most active compound.

### 2.9 Anti-Bartonella activity:

Similarly, a selection of disulfides were tested against various species of Bartonella, namely *B. henselae (Houston-1)*, *B. quintana (U-mass)*, *B. henselae Marseille* and *B.
*henselae (SA-1)* by disk diffusion assay. Rifampicin was used as a positive control along with DMSO, which was used to solubulize the compounds.

**Table (2.9)** Zone of inhibition data of S,S’-heterosubstituted disulfides against *Bartonella* sp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>B. henselae Houston-1</th>
<th>B. henselae U-mass</th>
<th>B. henselae Marseille</th>
<th>B. henselae SA-1</th>
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</thead>
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<tr>
<td>Zone of inhibition (mm)</td>
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<tr>
<td><strong>S,S’- dialkoxy substituted disulfides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>12</td>
<td>25</td>
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<td>11</td>
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<td>1c</td>
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<td>0</td>
</tr>
<tr>
<td>1e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>S,S’- dithio substituted disulfides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>0</td>
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<td>17</td>
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<tr>
<td><strong>Rifampin</strong></td>
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<td>22</td>
<td>68</td>
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<tr>
<td><strong>DMSO</strong></td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Surprisingly, most of the disulfides were completely inactive (Table 2.9). Only compound **1b**, diisoproxy disulfide, which was the most active compound against
Staphylococcus aureus, showed a small zone of inhibition. S,S’-heterosubstituted disulfides were also tested against Escherichia coli and were found to be inactive. MIC’s of all the disulfides were greater than 128 µg/ml.

2.10 Anti-fungal activity

Given that, previously, N-thiolated β-lactams were found to have antifungal properties, we decided to examine the disulfides against Candida albicans, one of the most common human commensal organisms that lives in the mouth and gastrointestinal tract. It is present in about 80% of healthy individuals. Entry of these fungal cells in the bloodstream causes candidiasis, infections which vary from superficial to systemic, and are often life threatening. Invasive candidiasis is very prevalent in intensive care units and is a major cause of mortality in 33-47% of immunocompromised individuals in hospitals. Reduced susceptibility of Candida species towards the commonly used antifungal agents has raised concerns over a need for effective antifungal agents.

Fungal susceptibility testing of diisopropoxy disulfide (1b) against C. albicans was done, by Sonja Dickey in Dr. Daniel Lim’s lab in the USF Biology department, according to NCCLS document M27-A2 in Yeast Nitrogen Broth (YNB) using 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, and 0.125 µg/ml concentrations of diisopropoxy disulfide (1b). Trypan blue was used for staining of non-viable cells. Two controls were used as well, a viable control using only C. albicans in YNB and a dead control using C. albicans in phosphate buffered saline (PBS) that was heat killed (boiled for 5 minutes). The MIC of diisopropoxy disulfide (1b) towards C. albicans was found to be 0.5 µg/ml.
2.10.1 Trypan Blue Staining:

To further assess the antifungal activity of the most active disulfide compound, diisoproxy disulfide (1b), a cell staining assay was carried out by Sonja Dickey in Dr. Daniel Lim’s lab in the Department of Biology, University of South Florida. This staining procedure is based on the observation that viable fungal cells do not take up the trypan blue dye while the non-viable cells do, and indicate cell viability in the presence of the drug.

![Trypan Blue](image)

**Figure (2.21) Trypan Blue**

Trypan staining assay images (Figures 2.22 & 2.23) of *C.albicans* treated with diisoproxy disulfide show that only the cells in the heat-killed control absorb the dye. None of the cells from the susceptibility test or the viable control from the yeast nutrient broth (YNB) stained blue, indicating that diisoproxy disulfide is fungistatic and not fungicidal.
Figure (2.22) Trypan Blue staining showing live fungal cells.

Figure (2.23) Trypan Blue staining of heat killed control showing dead fungal cells.
2.11 Efforts towards prodrug/dual action drug synthesis

The advent of multi-drug resistant microbes has made it very difficult to treat the diseases caused by them. A way to circumvent this would be by means of dual-action drug approach. In this approach two drugs with different modes of action are connected via a cleavable covalent linkage and introduced into the microbial cell. Upon entering the cell the linkage is broken enzymatically and releases both the drugs. These drugs target different biological pathways and so have a better chance to inhibit the microbe than in case of a drug with a single target.

In our efforts to make disulfide-based dual-action antibiotics, we unsuccessfully tried to couple disulfide 1b with 4-(2-chlorophenyl)-3-methoxyazetidin-2-one, ciprofloxacin and penicillin G (Fig 2.24). All these reactions were tried varying the solvents (dichloromethane, benzene), bases (triethylamine, diisopropylamine and pyridine) and temperatures (0 – 50 °C). Ciprofloxacin and penicillin G were used from commercially available sources. 4-(2-chlorophenyl)-3-methoxyazetidin-2-one was synthesized according to the previously published protocol in Dr. Turos’ lab.
Apart from these, the synthesis of disulfide linked ciprofloxacin and penicillin dimers prepared from sulfur monochloride were also attempted (Fig 2.25). These results also were not encouraging. The reactions did not yield any new products at ambient temperature and decomposed at elevated temperatures (50 °C).
Figure (2.25) Attempted synthesis of ciprofloxacin and penicillin disulfides.

The only reaction that worked well was the synthesis of a t-butyl carbamate analog of ciprofloxacin. This ciprofloxacin prodrug exhibited potent activity, having an MIC < 0.125 µg/ml against *Staphylococcus aureus* and MRSA, on par with ciprofloxacin.

Figure (2.26) Synthesis of N-Boc ciprofloxacin.

2.12 Conclusions and future directions
S,S’-Heterosubstituted disulfides were synthesized in good yields from sulfur monochloride, and characterized by $^1$H NMR. Most of these compounds showed promising growth inhibition activities against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. Diisopropoxy disulfide (1b) and bisdimethylamino disulfide (4a) were found to be the most potent among the set of compounds. The data from the Kirby-Bauer assay concluded that the activity diminished in presence of additives that contain a free sulfhydryl group. It is likely that this might be due to the cleavage of the S-heteroatom or disulfide bonds, and the formation of a biologically inactive mixed disulfide. As of yet, we have not been able to confirm this in solution, or to obtain isolable mixed disulfide adducts. From the bacterial viability assay these disulfides were found to be bacteriostatic in nature. These compounds have also shown decent activities against *Francisella tulerensis*, the lowest MIC was observed for diphenyl diamino disulfide (3e).

![Sulfhydryl groups](image)

**Figure (2.27)** Most active disulfide analogs 1b and 3e

From the various antimicrobial tests and assays performed, S,S’-heterosubstituted disulfides were observed to be bacteriostatic inhibiting gram-positive bacteria such as *Staphylococcus aureus* and its multi-drug resistant variant.
The SEM images (Fig 2.20) of MRSA treated with bis-isopropoxy disulfide (1b) also showed similarity to those of N-thiolated β-lactams. Neither affects the integrity of the bacterial cell wall, nor alters cell morphology.

These compounds were tested alongside N-thiolated β-lactams and aryl-alkyl disulfides synthesized previously in Dr. Turos’ lab and have shown comparable zones of inhibition against MRSA. In addition, it has been also shown that their bioactivity can be cancelled out in the presence of thiophilic additives, such as cysteine, glutathione and coenzyme A (Figures 2.14, 2.16 & 2.17).

It can also be observed that the structural similarity in having a cleavable S-heteroatom bond in all the three mentioned antibacterials (a sulfur-nitrogen bond in N-thiolated β-lactams, sulfur-sulfur bond in aryl-alkyl disulfides and three cleavable S-heteroatom bonds in S,S’-heterosubstituted disulfides) gives an understanding of the possible active component and consequently the similarity in the target moiety.

It was established that N-thiolated β-lactams target coenzyme A and, subsequently the FabH enzyme regulating bacterial fatty acid pathway. It can now be hypothesized there might be a parallel in the mode of action between the N-thiolated β-lactams and the S,S’-heterosubstituted disulfides.

The next logical step would be to confirm the hypothesized mode of action of these compounds. With the aid of tools like proteomics, one can get an understanding as to which bacterial pathway, if any, is being inhibited by the drug. Proteomics is the branch of genetics that deals with the full set of proteins encoded by the genome. A basic experiment would include incubating the bacteria with a drug and analyzing the bacterial
contents for any over or under expressed proteins with the help of a mass spectrometer. This would specify the protein and in turn the pathway that is being affected by the drug.

Apart from being antibacterials themselves, the presence of cleavable S-heteroatom bonds in their structure gives S,S’-heterosubstituted disulfides a potential to be modified as prodrugs or dual-action drugs. The prodrug nature facilitates the drug to be masked from the various bacterial metabolic enzymes and gives the drug extra time to show its inhibitory activity. This helps in lowering the dose of the drug that needs to be administered. The advantage with dual-action drugs is the presence of two antibacterial agents with different modes of action. Once these drugs enter the bacterial cell, they are prone to enzymatic cleavage and can simultaneously target different bacterial metabolic pathways. This can possibly be a means of overcoming bacterial resistance mechanisms.

Due to the advent of so many different bacterial resistance mechanisms there is always a need for effective antimicrobial agents with novel modes of action. The present work shows S,S’-heterosubstituted disulfides as antimicrobial agents against various bacteria including Staphylococcus aureus, MRSA, Francisella tulerensis and the fungus Candida albicans. These structurally-simple disulfides may serve as new leads to the development of effective antibacterials for drug-resistant microbial infections.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Antimicrobial Testing to Determine Minimum Inhibitory Concentration

All the testing was performed according to NCCLS guidelines. [NCCLS (National Committee for Clinical Laboratory Standards) Methods for Dilution of Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. NCCLS Document M7-A5, Vol. 17, No. 2, 1997.]

3.1.1 Inoculum Preparation

10 ml of Mueller Hinton broth was added to a sterile test tube. Using a sterile cotton swab, the broth-containing test tube was inoculated with 3-5 colonies from the appropriate overnight culture. The test tube was placed in an incubator for approximately 2 hours at 35 to 37°C, or until the culture reached an optical density of 0.08-0.10 at 625 nm wavelength. This is equal to 0.5 McFarland standard [1.5X10^8 CFU (colony forming units) /ml]. The culture as needed was adjusted to obtain the appropriate optical density. If the bacterial suspension is too turbid, it can be diluted with more diluent. If the suspension is not turbid enough, more bacteria can be added. Once the appropriate optical
density was obtained, a 1:1000 dilution of the above culture in a sterile media storage bottle was prepared.

### 3.1.2 Preparation of Drug-Containing Mueller Hinton Agar in 24-well Plates

Using a 1 mg/mL stock solution of the test drug in DMSO, the following volumes were added in each of the 12 wells: 256 µl, 128 µl, 64 µl, 32 µl, 16 µl, 8 µl, 4 µl, 2 µl, 1 µl, 0.5 µl, 0.25 µl, 0.125 µl, respectively. The following volume of molten Mueller Hinton Agar was then added into each of the 12 wells containing the above volume of test drug: 744 µl, 872 µl, 936 µl, 968 µl, 984 µl, 992 µl, 996 µl, 998 µl, 999 µl, 999.50 µl, 999.75 µl, 999.875 µl, respectively. Each well was mixed using a pipette and allowed the drug-containing agar to solidify at room temperature before inoculation. This gives the following order of drug concentrations in the wells: 256 µg/ml, 128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml and 0.125 µg/ml.

### 3.1.3 Inoculation of Drug-Containing Mueller Hinton Agar

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension and preparing a 1000-fold dilution, 1 µl of the appropriate inoculum was added to each well of the 24-well plate, containing solidified agar/test drug. The plates were then placed into the incubator at 35 to 37 °C.
3.1.4 Reading the Plates and Interpreting the Results

After 16 to 18 hours of incubation, each plate was examined for bacterial growth. The drug concentration in the well showing no visible bacterial growth as detected with the unaided eye is considered the MIC value. These values are recorded in table 3.1.

3.2 Kirby-Bauer Diffusion Assay

3.2.1 Culture preparation

From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20 % glycerol, a culture of each microorganism was transferred with a sterile Dacron swab to Trypticase® Soy Agar (TSA) plates (Becton Dickinson Laboratories, Cockeysville, MD), streaked for isolation, and incubated at 37 °C for 24 h. A $10^8$ standardized cell count suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates.

3.2.2 Testing procedure

Sterile saline (5 mL) was inoculated with a swab of bacteria with a sterile cotton plug. The concentration was then adjusted to 0.5 McFarland standard as explained earlier. This bacterial solution was then streaked across a TSA plate to a give an even lawn of bacteria. Sterile pipet tips were used to drill 6 mm wells into the agar plate, then 20 µL of 1 mg/mL drug in DMSO was added to the well. Plates were incubated overnight at 37 °C.
3.3 Anti-Francisella testing:

All 29 disulfides were tested against a live vaccine strain (LVS) of *Francisella tularensis* by the broth dilution technique as described below. This was carried out in a biosafety level 3 facility by John Thomas in Dr. Burt Anderson’s laboratory at USF College of Medicine.

3.3.1 Culture preparation

In a 50 ml conical tube, 320 µg of the test disulfide was added to 10 ml of Mueller-Hinton (MH) broth at room temperature to give a concentration of 32 µg/ml. 5 mL of this broth was added and mixed into another conical tube containing 5 mL of MH broth to afford a final disulfide concentration of 16 µg/ml. These steps were repeated to obtain various concentrations. A 5 mL aliquot was removed from the final tube having a disulfide concentration of 0.25µg/mL.

3.3.2 Addition of LVS

Two full cotton swabs worth of LVS growth culture was suspended into 10 mL of MH broth at room temperature. From this stock, 25 µl was added into each of the dilution tubes prepared above. These tubes were incubated at 36 °C in an incubator with 0% CO₂.

3.3.3 Controls

Three control tubes were also prepared, containing the following:

1. 25 µl LVS in 5 mL of MH broth in a 50 mL conical tube.

2. 25 µl LVS in 5 mL of MH broth in a 50 mL conical tube with dilutions of DMSO added that match those of the above drug dilutions.
3. 5 mL of MH broth in a 50 mL conical tube.

After 48 hours of incubation, all of the tubes were examined visually to define the concentration of disulfide that inhibits LVS growth. These values are recorded in table 3.1.

**Table 3.1** Antimicrobial activities of S,S’-heterosubstituted disulfides 1-6.

<table>
<thead>
<tr>
<th>Disulfide</th>
<th>Compound number</th>
<th>alkyl</th>
<th>Minimum Inhibitory Concentration (µg/mL)</th>
<th>F.T</th>
<th>S.A</th>
<th>MRSA</th>
</tr>
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<tbody>
<tr>
<td>(RO-S)₂</td>
<td></td>
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<tr>
<td>1a</td>
<td></td>
<td>propyl</td>
<td>16</td>
<td>32</td>
<td>32</td>
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<td>1</td>
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Control

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<td>Penicillin-G</td>
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F.T: Francisella tularensis (Live Vaccine Strain)

S.A: Staphylococcus aureus (ATCC-25923)

MRSA: Methicillin-resistant Staphylococcus aureus (ATCC-43300)

3.4 In vitro Anti-Bartonella activity by disk diffusion method

B. henselae (hoston-1), B. quintana (U-mass), B. henselae Marseille and B. henselae (SA-1) were inoculated onto chocolate agar plates to create a confluent lawn of growth. A 6 mm diameter paper disk was then placed in the center of the plate before incubation. Plates were incubated at 37 °C for one week and the diameter of growth inhibition was then measured and recorded.

3.4.1 Preparation of antibiotic-containing paper disks

The preparation of disks was done in an identical fashion each time, as it is important to standardize the procedure as much as possible. All antibiotics were predissolved in DMSO to a final concentration of 1 mg/ml, and added to the paper disks as a 1 mg/ml solution using a volume of 20 µl. This volume readily adsorbed into the paper disks quickly, and gave a total standard amount of 20 µg of antibiotic per disk. To load the antibiotics, 6 mm paper disks were placed on a sheet of aluminum foil in the biological safety cabinet (BSC). Disks can be easily handled without damage using fine-point forceps. To each disk was added 20 µl of antibiotic solution at a concentration of 1 mg/ml
dissolved in DMSO. A negative control consisting of 20 µl DMSO/disk and a positive control consisting of 20 µl of rifampicin at 0.1mg/ml in DMSO were also prepared. Each disk contained 20 µg of the antibiotic being tested while the positive control disk contained 2.0 µg of rifampicin. The disks were allowed to dry in the BSC for at least 20 minutes, then placed by forceps in a sealable bag with desiccant and stored sealed in the refrigerator.

3.4.2 Inoculation of agar plates for disk diffusion testing

Bacteria from 4-5 day old plates was harvested and resuspended in 1.0 ml sterile HIB. Turbidity was adjusted to about McFarland 2.0 by inspection. The suspension was spread over the surface of a labeled chocolate agar plate using a swab and allowed to dry into the agar in the BSC for 10-15 minutes. Forceps were used to place the disk in the center of the plate. The disk was tapped gently to make sure it adhered to the plate. The plates were then inverted and kept at 35 °C in a CO₂ incubator for one week. The zones of inhibition were then measured and recorded to the nearest mm for each plate.

3.5 Fungal viability assay

Diisopropoxy disulfide was tested for fungal viability by a Trypan Blue staining protocol. 50 µl of a Candida albicans culture and 50 µl of a 0.4% Trypan Blue solution in water (w/v) were mixed in a 2 ml plastic microcentrifuge tube. After 5 minutes, 20 µl of the mix was loaded into a disposable cell-counting chamber (Cellometer (Registered), Nexcelom Bioscience, Lawrence, MA) and viewed microscopically (Olympus BX60, Olympus, Center Valley, PA) at 40X magnification. Pictures were taken using a Spot Flex Camera (Diagnostic Instruments, Inc., Sterling Heights, MI).
3.6 Cytotoxicity testing

Primary bovine primary aortic endothelial cells and EBM-2 (endothelial basal medium) cell culture medium were from Lonza Walkersville, Inc. Cells were maintained in culture medium supplemented with 10% FBS (fetal bovine serum) and 100 IU penicillin/100 µg/ml streptomycin sulfate in a humidified incubator containing 5% CO₂ at 37 °C. For cytotoxicity testing, cells were trypsinized, counted, centrifuged at 120 x g, and resuspended in fresh culture medium. Cells were plated in a volume of 100 µl (2.5 x 10⁴ cells/well) in 96-well flat-bottom culture dishes. After 16-18 h of incubation, 100 µl of medium or medium containing test samples was added to each well. Tests were performed in triplicate. After 48 h, 20 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (1.25 - 4 mg/ml in DPBS) was added to each well, and the cells were further incubated for 2 - 3.5 h. The medium was aspirated, and the formazan product generated in each well was dissolved in 100 µl DMSO. Plates were read in a BioTek Synergy 2 SLFA plate reader set at 540 nm (background subtract at 660 nm). Cell viability was calculated as percent of control (sample absorbance/control medium absorbance x 100).
Table 3.2 Cytotoxicity data of diisopropoxy disulfide (1b)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>% control</th>
<th>Appearance after 48hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>0.3125</td>
<td>0.248</td>
<td>0.006</td>
<td>90.6</td>
<td>ok</td>
</tr>
<tr>
<td>1b</td>
<td>1.25</td>
<td>0.271</td>
<td>0.017</td>
<td>98.9</td>
<td>ok</td>
</tr>
<tr>
<td>1b</td>
<td>5</td>
<td>0.299</td>
<td>0.021</td>
<td>109.4</td>
<td>ok</td>
</tr>
<tr>
<td>1b</td>
<td>20</td>
<td>0.252</td>
<td>0.010</td>
<td>92.0</td>
<td>ok</td>
</tr>
</tbody>
</table>

Diisopropoxy disulfide was checked for cytotoxicity by MTT assay as explained above. It was observed that this compound did not show any cytotoxic activity against bovine aortic endothelial cells up to the concentration of 20 µg/ml.

3.7 Synthetic procedures

All the chemicals used for the synthesis of the disulfide compounds were purchased from Aldrich Chemical Company and used without further purification. Sulfur monochloride was purified by distilling from sulfur and charcoal (100:4:1 S2Cl2/sulfur/charcoal) (Fieser, L. F.; Fieser, M. Reagents for Organic Synthesis; John Wiley and Sons: New York, 1967; Vol. 1). Thin layer chromatography was performed using Silica Gel 60 F254 purchased from EMD Chemicals. A UVG-11 Minera light lamp was used to visualize the TLC plates. The NMR spectra were recorded in deuterated chloroform on a Bruker 250 MHz instrument. High performance liquid chromatography (HPLC) was done on a Shimadzu Prominence system using a reverse-phase Shimadzu column (C18, 0.46×5 cm). Samples were eluted using a gradient from 100% of a 10 mM PBS solution (pH 7.4).
to 100% of acetonitrile in 22 min at a flow rate of 1 ml/min. The detection was performed using a Shimadzu SPD-20A UV–visible detector at 254 nm.

3.7.1 General procedure for the synthesis of S,S’-heterosubstituted disulfide 1-6.

To a stirred solution of equimolar concentrations (1 mmol) of the selected alcohol, amine, or thiol and triethylamine in dry dichloromethane at -20 °C was added dropwise a solution of sulfur monochloride (0.5 mmol) in dichloromethane. The reaction was brought to room temperature after the addition was completed and stirring was continued for about 1 hr. The reaction was worked up by the addition of about 100 mL of ice-cold water. This was stirred for a few minutes, transferred to a separatory funnel, and the aqueous phase was removed by draining the organic layer. The organic layer was further washed twice with 50 mL of ice-cold water to remove the triethylamine hydrochloride, and washed with 50 mL of brine to dry the organic layer. The dichloromethane layer was further dried by the addition of anhydrous magnesium sulfate, filtered and concentrated in vacuo. The product obtained was purified by silica gel chromatography and characterized by 1H NMR. Due to the absence of any ionizable functionalities in the S,S’-heterosubstituted disulfides, mass spectroscopy was not helpful because no parent ion could be identified for any of the compounds.

Synthesis of S,S’-dipropoxy disulfide (1a)

To a stirred solution of n-propanol (5 mL, 1mmol) and triethylamine (9.36 mL, 1 mmol) in 20 mL of anhydrous dichloromethane at -20 °C was added dropwise a solution of sulfur monochloride (2.68 mL, 0.5mmol) in 10 mL of dichloromethane. The reaction was brought to room temperature after the addition was completed and stirring was continued
for about 1 hr. The reaction was worked up as described in the general procedure. All the yields mentioned below are after column chromatography.

**1a**: Isolated 77.3 mg (85%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 3.38-3.43 (t, 2H, $J$= 6.8 Hz), 1.82-1.96 (m, 2H), 1.01-1.07 (t, 3H, $J$= 7.3 Hz).

**1b**: Isolated 79.2 mg (87%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 3.95-4.15 (m, 1H), 1.24-1.32 (m, 6H).

**1c**: Isolated 87.2 mg (83%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 3.4-3.46 (t, 2H, $J$= 6.8 Hz), 1.8-1.91 (m, 2H), 1.4-1.55 (m, 2H), 0.91-0.97 (t, 3H, $J$= 7.4 Hz).

**1d**: Isolated 85 mg (81%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 3.67-3.93 (m, 1H), 1.38-1.68 (m, 2H), 1.08-1.28 (d, 3H, $J$= 7.5 Hz), 0.75-0.96 (t, 3H, $J$= 6.3 Hz).

**1e**: Isolated 95 mg (76%) as a dark green oil $^1$H NMR (250 MHz, CDCl$_3$): δ 7.27-7.40 (5H).

**2a**: Isolated 92.1 mg (86%) as a yellow oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 2.91-2.97 (t, 2H, $J$= 7.1 Hz), 1.73-1.76 (m, 2H), 1.06 (t, 3H, $J$= 7.3 Hz).

**2b**: Isolated 90.2 mg (85%) as a yellow oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 3.19-3.37 (m, 1H), 1.38-1.41 (d, 6H, $J$= 6.8 Hz).

**2c**: Isolated 108.9 mg (90%) as a yellow oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 2.68-3.01 (m, 2H), 1.71-1.80 (m, 2H), 1.41-1.51 (m, 2H), 0.92-0.99 (t, 3H, $J$= 7.7 Hz).

**2d**: Isolated 100.44 mg (83%) as a yellow oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 2.95-3.12 (m, 1H), 1.58-1.83 (m, 2H), 1.36-1.41 (d, 3H, $J$= 6.2 Hz), 0.97-1.05 (t, 3H, $J$= 7.4 Hz).
2e: Isolated 90.2 mg (85%) as pale yellow crystals; mp 31-33 °C; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.51-7.55 (m, 2H), 7.25-7.37 (m, 3H).

3a: Isolated 58.5 mg (65%) as a reddish oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 3.11-3.17 (t, 2H, $J$= 7.9 Hz), 1.55-1.65 (m, 2H), 1.46-1.52 (bs, 1H), 0.84-0.89 (t, 3H, $J$= 7.3 Hz).

3b: Isolated 58.5 mg (65%) as a reddish oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 3.79-3.95 (m, 1H), 1.28-1.3 (d, 6H, $J$= 6.5 Hz).

3c: Isolated 62.43 mg (60%) as a reddish oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 3.63-3.71 (m, 2H), 1.8-1.89 (m, 2H), 1.34-1.48 (m, 2H), 0.93-0.99 (t, 3H, $J$= 7.3 Hz).

3d: Isolated 65.5 mg (63%) as a reddish oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 2.95-3.12 (m, 1H), 1.58-1.83 (m, 2H), 1.36-1.41 (m, 3H), 0.97-1.05 (m, 3H).

3e: Isolated 80.6 mg (65%) as a reddish oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 7.14-7.27 (m, 2H), 6.68-6.81 (m, 3H), 3.65 (bs, 1H).

4a: Isolated 55.5 mg (73%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 2.52 (s, 3H).

4b: Isolated 78.1 mg (75%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 3.40-3.48 (q, 2H, $J$= 7.4 Hz), 1.65-1.71 (t, 3H, $J$= 7.3 Hz).

4c: Isolated 99.1 mg (75%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 4.05-4.15 (m, 1H), 1.27-1.33 (t, 6H, $J$= 7 Hz).

4d: Isolated 89.6 mg (70%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 5.73-5.9 (m, 4H), 5.07-5.16 (m, 8H), 3.34-3.37 (d, 8H, $J$= 6.3 Hz).
4e: Isolated 113.6 mg (71%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 2.64-2.7 (bs, 1H), 2.42-2.45 (d, 1H, $J=7.1$ Hz), 1.93-2.13 (m, 1H), 0.89-0.92 (d, 6H, $J=6.6$ Hz).

5a: Isolated 81.9 mg (78%) as a colorless oil; [α]$^{25}_D$ –10.1° (c 0.29, CH$_2$Cl$_2$); $^1$H NMR (250 MHz, CDCl$_3$): δ 3.85-3.91 (m, 1H), 1.53-1.69 (m, 2H), 1.26-1.31 (m, 3H), 0.91-0.97 (m, 3H).

5b: Isolated 79.8 mg (76%) as a colorless oil; [α]$^{25}_D$ +9.2° (c 0.25, CH$_2$Cl$_2$); $^1$H NMR (250 MHz, CDCl$_3$): δ 3.86-3.91 (m, 1H), 1.55-1.67 (m, 2H), 1.26-1.31 (m, 3H), 0.91-0.97 (m, 3H).

5c: Isolated 58.45 mg (70%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 7.28-7.39 (m, 5H), 4.59-4.65 (m, 1H), 1.77-1.87 (m, 2H), 0.92-0.98 (t, 3H, $J=7.4$ Hz).

5d: Isolated 60.95 mg (73%) as a colorless oil; $^1$H NMR (250 MHz, CDCl$_3$): δ 7.03-7.18 (m, 5H), 3.50-3.53 (d, 2H, $J=6.8$ Hz), 2.69-2.83 (m, 1H), 1.07-1.1 (d, 3H, $J=6.7$ Hz).

3.7.2 Synthesis of S,S'-heterosubstituted disulfides of menthol:

To an ice-cooled suspension of sodium hydride (oil removed from hexanes) (1.5 mmol) in anhydrous tetrahydrofuran (THF), a solution of menthol (1 mmol) in THF was added dropwise and was stirred until the evolution of hydrogen stopped. This was followed by the addition of a solution of sulfur monochloride (0.5 mmol) in THF dropwise under argon atmosphere. The reaction was stirred until all the starting material was consumed, monitoring by TLC. The reaction mixture was then concentrated in vacuo and purified by silica gel chromatography.
5e: Isolated 56.1 mg (60%) as a colorless oil; \([\alpha]^25_D +32.1^\circ \) (c 0.35, CH₂Cl₂); \(^1\)H NMR (250 MHz, CDCl₃): δ 3.38-3.46 (m, 1H), 2.15-2.21 (m, 1H), 1.95-2 (m, 1H), 1.59-1.70 (m, 2H), 1.42-1.45 (m, 1H), 1.25-1.27 (m, 1H), 0.98-1.17 (m, 3H), 0.91-0.95 (m, 6H), 0.81-0.84 (m, 3H).

5f: Isolated 58 mg (62%) as a colorless oil; \([\alpha]^25_D –28.7^\circ \) (c 0.15, CH₂Cl₂); \(^1\)H NMR (250 MHz, CDCl₃): δ 3.37-3.47 (m, 1H), 2.15-2.21 (m, 1H), 1.93-2 (m, 1H), 1.59-1.70 (m, 2H), 1.26-1.39 (m, 3H), 1.01-1.13 (m, 2H), 0.94-0.95 (m, 3H), 0.91-0.93 (m, 3H), 0.81-0.84 (m, 3H).

5g: Isolated 60.7 mg (65%) as a colorless oil; \(^1\)H NMR (250 MHz, CDCl₃): δ 3.37-3.47 (m, 1H), 2.15-2.21 (m, 1H), 1.93-2 (m, 1H), 1.59-1.70 (m, 2H), 1.26-1.39 (m, 3H), 1.01-1.13 (m, 2H), 0.94 (d, 3H, \(J= 4.3 \) Hz), 0.92 (d, 3H, \(J= 3.8 \) Hz), 0.81-0.84 (d, 3H, \(J= 6.8 \) Hz).

The compounds 6a and 6b were synthesized according to the general procedure explained in section 3.6.1.

6a: Isolated 67.7 mg (56%) as a colorless oil; \([\alpha]^25_D +23^\circ \) (c 0.59, CH₂Cl₂); \(^1\)H NMR (250 MHz, CDCl₃): δ 7.13-7.29 (m, 10H), 3.86-3.88 (m, 3H), 1.42-1.46 (m, 3H).

6b: Isolated 70.2 mg (58%) as a colorless oil; \([\alpha]^25_D –25^\circ \) (c 0.45, CH₂Cl₂); \(^1\)H NMR (250 MHz, CDCl₃): δ 7.17-7.27 (m, 10H), 3.82-4.3 (m, 3H), 1.44 (d, 3H, \(J= 6.6 \) Hz).

3.7.3 Synthesis of 7-(4-tert-Butoxycarbonyl-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid: To a stirred solution of ciprofloxacin (390
mg, 1.0 mmol) and 1M sodium hydroxide solution (3 ml, 2.5 mmol) in 20mL of tetrahydrofuran was added di-tert-butyl dicarbonate (0.20 ml, 1.1 mmol). The reaction was stirred until all the starting material was consumed by monitoring with TLC. The reaction mixture was then concentrated in vacuo and purified by silica gel chromatography to give 339.4 mg (67%) of the title compound as a white solid. $^1$H NMR (250 MHz, CDCl$_3$): 14.95 (bs, 1H), 8.79 (s, 1H), 8.06 (d, 1H, $J= 13.0$ Hz), 7.36-7.39 (d, 1H, $J= 7.3$ Hz), 3.66-3.70 (m, 4H), 3.50-3.60 (m, 1H), 3.25-3.35 (m, 4H), 1.51 (s, 9H), 1.40-1.43 (m, 2H), 1.20-1.23 (m, 2H).
Spectrum 4.1: $^1\text{H}$ NMR (250 MHz, CDCl$_3$) of compound 1a:
Spectrum 4.2: $^1$H NMR (250 MHz, CDCl$_3$) of compound 1c:

Spectrum 4.3: $^1$H NMR (250 MHz, CDCl$_3$) of compound 1d:
**Spectrum 4.4:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 1b:

![Spectrum 4.4](image1.png)

**Spectrum 4.5:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 1e:

![Spectrum 4.5](image2.png)
**Spectrum 4.6:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 2b:

![NMR Spectrum of Compound 2b]

**Spectrum 4.7:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 2c:

![NMR Spectrum of Compound 2c]
Spectrum 4.8: $^1$H NMR (250 MHz, CDCl$_3$) of compound 2e:

Spectrum 4.9: $^1$H NMR (250 MHz, CDCl$_3$) of compound 2d:
Spectrum 4.10: $^1$H NMR (250 MHz, CDCl$_3$) of compound 2a:

Spectrum 4.11: $^1$H NMR (250 MHz, CDCl$_3$) of compound 3b:
Spectrum 4.12: \(^1\)H NMR (250 MHz, CDCl\(_3\)) of compound 3d:

Spectrum 4.13: \(^1\)H NMR (250 MHz, CDCl\(_3\)) of compound 3e:
Spectrum 4.14: $^1$H NMR (250 MHz, CDCl$_3$) of compound 3a:

Spectrum 4.15: $^1$H NMR (250 MHz, CDCl$_3$) of compound 4e:
**Spectrum 4.16:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 4a:

![Spectrum 4.16](image)

**Spectrum 4.17:** $^1$H NMR (250 MHz, CDCl$_3$) of compound 4c:

![Spectrum 4.17](image)
**Spectrum 4.18**: $^1$H NMR (250 MHz, CDCl$_3$) of compound 4b:

![NMR Spectrum of 4b](AmDS.009.esp)

**Spectrum 4.19**: $^1$H NMR (250 MHz, CDCl$_3$) of compound 4d:

![NMR Spectrum of 4d](Desktop.002.esp)
Spectrum 4.20: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5c:

Spectrum 4.21: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5g:
Spectrum 4.22: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5d:

![Spectrum 4.22](image)

Spectrum 4.23: $^1$H NMR (250 MHz, CDCl$_3$) of compound 6b:

![Spectrum 4.23](image)
Spectrum 4.24: $^1$H NMR (250 MHz, CDCl$_3$) of compound 6a:

Spectrum 4.25: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5a:
Spectrum 4.26: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5e:

Spectrum 4.27: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5b:
Spectrum 4.28: $^1$H NMR (250 MHz, CDCl$_3$) of compound 5f:

Spectrum 4.29: $^1$H NMR (250 MHz, CDCl$_3$) of compound 7:
Spectrum 4.30: HPLC trace of (1b) and CoA after 30 min in phosphate buffer solution:

CoA + Diisopropoxy disulfide (1b) in PBS after 30min at 254nm

DMSO

CoA

1b

Minutes
Spectrum 4.31: HPLC trace of 1b and CoA after 24 hrs in phosphate buffer solution:

CoA + Diisopropoxy disulfide (1b) in PBS after 24 hrs at 254nm

Minutes
**Spectrum 4.32:** HPLC trace of CoA in phosphate buffer solution:

CoA in PBS after 24 hrs at 254nm
REFERENCES


