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Unsymmetric aryl–alkyl disulfide growth inhibitors of methicillin-resistant *Staphylococcus aureus* and *Bacillus anthracis*

Edward Turos [a,*], Kevin D. Revell [b], Praveen Ramaraju [a], Danielle A. Gergeres [a], Kerriann Greenhalgh [a], Ashley Young [a], Nalini Sathyanarayan [b], Sonja Dickey [c], Daniel Lim [c], Mamoun M. Alhamadsheh [d], Kevin Reynolds [d]

[a] *Center for Molecular Diversity in Drug Design, Discovery, and Delivery, Department of Chemistry, 4202 East Fowler Avenue, CHE 205, University of South Florida, Tampa, FL 33620, USA*
[b] *Department of Chemistry, Murray State University, 456 Blackburn, Murray, KY 42071, USA*
[c] *Department of Biology, 4202 East Fowler Avenue, IDRB 404, University of South Florida, Tampa, FL 33620, USA*
[d] *Department of Chemistry, Portland State University, PO Box 751, Portland, OR 97207, USA*

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ABSTRACT

This study describes the antibacterial properties of synthetically produced mixed aryl–alkyl disulfide compounds as a means to control the growth of *Staphylococcus aureus* and *Bacillus anthracis*. Some of these compounds exerted strong in vitro bioactivity. Our results indicate that among the 12 different aryl substituents examined, nitrophenyl derivatives provide the strongest antibiotic activities. This may be the result of electronic activation of the arylthio moiety as a leaving group for nucleophilic attack on the disulfide bond. Small alkyl residues on the other sulfur provide the best activity as well, which for different bacteria appears to be somewhat dependent on the nature of the alkyl moiety. The mechanism of action of these lipophilic disulfides is likely similar to that of previously reported *N*-thiolated β-lactams, which have been shown to produce alkyl-CoA disulfides through a thiol-disulfide exchange within the cytoplasm, ultimately inhibiting type II fatty acid synthesis. However, the mixed alkyl-CoA disulfides themselves show no antibacterial activity, presumably due to the inability of the highly polar compounds to cross the bacterial cell membrane. These structurally simple disulfides have been found to inhibit β-ketoacyl-acyl carrier protein synthase III, or FabH, a key enzyme in type II fatty acid biosynthesis, and thus may serve as new leads to the development of effective antibacterials for MRSA and anthrax infections.

1. Introduction

The design of new antimicrobial agents has reached unprecedented urgency as drug resistance in certain medically relevant bacteria continues to climb. Our laboratory has recently been investigating the microbiological properties of *N*-alkylthio β-lactams[1–9] and *N*-alkylthio-2-oxazolidinones[10] (Fig. 1) as potential antibiotics for treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus anthracis* (the causative agent of anthrax).

These compounds exert selective bacteriostatic properties against *Staphylococcus* and *Bacillus* microbes over most other common bacterial genera, and demonstrate unique structure–activity profiles never seen before for β-lactam and 2-oxazolidinone antibiotics. One of the structural requirements of these compounds is that the residues on the heterocyclic ring and sulfur side chain must be highly lipophilic in order to bestow the most potent antibacterial activities. Most recently our studies have determined that *N*-methylthio β-lactams block type II fatty acid biosynthesis in *S. aureus* through the initial transfer of the *N*-alkylthio moiety from the ring nitrogen onto a cellular target.[9] We have since identified this target as being coenzyme A (CoASH), which is present in large quantities in the cytoplasm and comprises the redox buffer system of these bacteria. It is postulated that sulfenylation of coenzyme A produces an alkyl-CoA disulfide (CoASSR) responsible for inhibiting lipid biosynthesis (Scheme 1).

***N*-thiolated β-lactams** ***N*-thiolated oxazolidinones**

Figure 1. Antibacterially active sulfenylating agents.

* Corresponding author. Tel.: +1 813 974 7312; fax: +1 813 974 1733.
E-mail address: eturos@cas.usf.edu (E. Turos).

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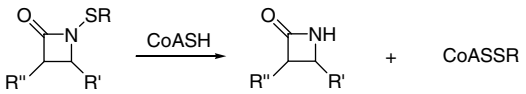


Scheme 1. Sulfenylation of coenzyme A by *N*-thiolated β-lactams.

Recent work in the Reynolds laboratory has demonstrated that these mixed alkyl-CoA disulfides, CoASSR, covalently modify the fatty acid biosynthesis protein, FabH, and that the inhibitory effects are highly dependent on the nature of the alkyl side chain R.[11] In our initial investigations, we examined the possibility that the CoASSR disulfides could directly inhibit microbial growth if fed to bacteria growing in culture media. However, incubation of *S. aureus* cells with CoASSMe does not lead to growth inhibition, as judged by both Kirby–Bauer well diffusion studies and minimum inhibitory concentration assays in broth. Although this seemed surprising at first, given that this compound is likely responsible for bioactivity in the cell, this suggests that the multiply charged CoA moiety may not easily traverse the cell membrane to be able to exert an inhibitory effect within the cytoplasm.[12] This led to the realization that the *N*-thiolated β-lactam, and presumably the *N*-thiolated oxazolidinone as well, may have the requisite cell permeability and sulfenylation capabilities for antibacterial activity, and could generate the CoASSR adduct intracellularly. Since it was apparent that the lactam or the oxazolidinone rings did not have any special structural features needed for activity, other than being relatively lipophilic and able to serve as a leaving group during nucleophilic attack on sulfur, we thought that we could utilize lipophilic disulfides in place of the *N*-thiolated β-lactams as a means to deliver electrophilic sulfur species more effectively into the bacterial cell. In fact, nature has long used disulfides and trisulfides as natural sulfenylation agents, as evidenced by the large number of naturally occurring compounds that have anti-infective properties.[13] Highly notable examples include diallyl disulfide, diallyl trisulfide, ajoene, *S*-allylmercaptocysteine, and allicin, found only in freshly crushed garlic (Fig. 2).[14–17] The biological targets and mode of action of most of these compounds are not precisely known, but in the case of allicin, there are distinct similarities in the antibiotic properties to the *N*-thiolated β-lactams (and *N*-thiolated oxazolidinones). In addition to exhibiting activity against a wide range of bacteria,[18–21] allicin possesses antifungal,[22–24] antiviral,[25,26] antiparasitic,[27,28] and anticancer properties. Both the lactams and allicin strongly inhibit fatty acid synthesis,[29–32] but also partially inhibit protein and nucleic acid biosynthesis.[33] Both compounds exert their strongest antibacterial activities against microbes such as *S. aureus* and *B. anthracis* that express unusually low levels of glutathione and relatively high levels of coenzyme A. Allicin reacts with sulfhydryl residues of various proteins such as RNA polymerase, thioredoxin reductase, and alcohol dehydrogenase, and reversibly inhibits acetyl-CoA synthetases.[34,35] This certainly suggests similar modes of action in causing intracellular sulfenylation of their biological targets. A survey of the patent literature also uncovered two recent US patent applications describing the effects of mixed alkyl disulfides as inhibitors of redox buffers in mammalian cells as a means to restore normal cellular function, a feature attributed to their high reactivity toward thioredoxin.[36,37]

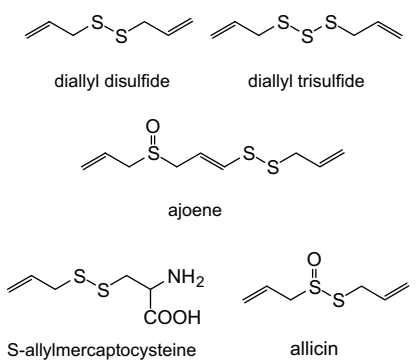
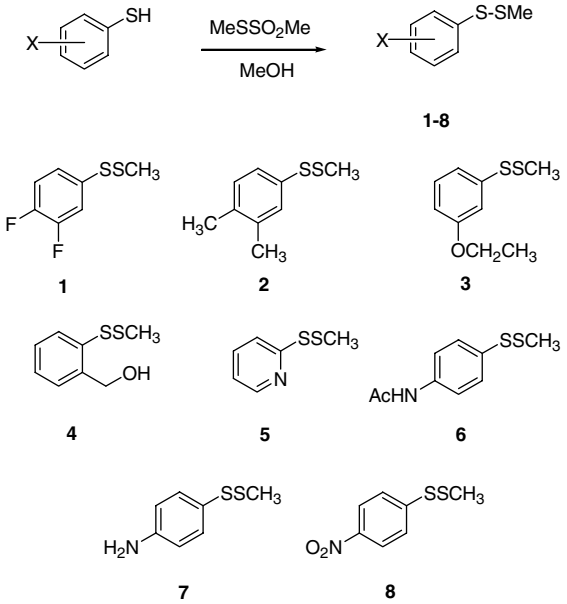


Figure 2. Naturally occurring disulfide and trisulfide antibacterials from garlic.

2. Results and discussion

To initiate these investigations, a selection of unsymmetrical aryl methyl disulfides was synthesized from commercially available arylthiols as shown in Scheme 2. We selected a group of electronically diverse arylthiols as coupling partners in this reaction to obtain aryl methyl disulfides **1**–**8** for evaluation. The synthesis was done simply by combining a methanolic solution of the arylthiol (100 ml of 1.0 M) with a slight excess of the alkyl methanethiolsulfonate (105 ml of 1.0 M methanolic solution) and stirring under an inert atmosphere for 1 h. The solutions were then treated with a small amount (about 0.3 equiv) of cysteine hydrochloride to consume the excess methanethiolsulfonate. The solution was concentrated and taken up in dichloromethane (1 ml), filtered to remove any cysteine products, treated with Amberlyst 21 resin (weakly basic), then flushed through a small silica plug to remove impurities. In most instances, this yielded products >90% pure by ^{1}H NMR. Yields for these reactions were in most cases high and the disulfides were used in microbiological screening assays without further purification. We carried out initial antibacterial assays on these eight aryl methyl disulfides against methicillin-susceptible *S. aureus* by Kirby–Bauer disk diffusion on agar plates, according to National Committee for Clinical Laboratory Standards guidelines.[38] In each case, 20 μg of the test compound in DMSO was applied to 6-mm wide wells bored directly into the agar, prior to inoculation and incubation. The average zones of growth inhibition (from three trials) produced by the compounds after 24 h of incubation at 37 °C are presented in Table 1. The Kirby–Bauer data indicate that most of the disulfides have weak or no antimicrobial activity, which suggests that the disulfide bond itself is not enough of a prerequisite to induce antibacterial activity. Of these eight compounds, the *p*-nitrophenyl analogue **8** showed the strongest



Scheme 2. Synthesis of aryl methyl disulfides **1**–**8**.

Table 1
Zones of bacterial growth inhibition data from Kirby–Bauer testing of disulfides **1–8** against *S. aureus* (ATCC 25923) on agar plates

	1	2	3	4	5	6	7	8
Diameter of zone (mm)	11	10	9	14	23	20	0	35

in vitro activity against *S. aureus*, with zones sizes comparable to some of our previously reported *N*-thiolated β-lactam compounds.[3–8]

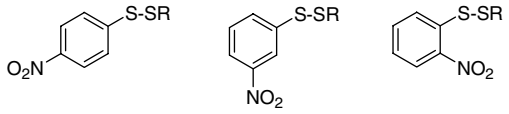
We then expanded on the structure–activity properties of these compounds by making structural modifications to the most potent one, aryl disulfide **8**. These include changing the location of the electron-withdrawing nitro group on the phenyl ring, as well as the length and branching of the *S*-alkyl substituent. The same procedure shown in Scheme 1 was used to prepare *m*-nitrophenyl disulfides **9** and *o*-nitrophenyl disulfides **10**, employing the corresponding alkylthio sulfonates as sulfenylating agents.

Antimicrobial screening was done by Kirby–Bauer testing as well as by determining the minimum inhibitory concentration (MIC) values of compounds **8**–**10** by agar serial dilution. The data shown in Table 2 indicate that all three nitrophenyl disulfide systems were active against *S. aureus* and *B. anthracis*, but it appeared that *p*-nitrophenyl disulfides **8** and *m*-nitro isomers **9** were both considerably more active than the *o*-nitro analogues **10**. The enhanced bioactivity of these nitrophenyl disulfides compared to other aryl-substituted analogues suggests that nucleophilic scission of the disulfide linkage is enhanced by strong electron deficiency on the sulfur centers.

These data revealed that increasing the alkyl chain length of the aryl–alkyl disulfide from methyl to ethyl to propyl to butyl increased bioactivity somewhat, but not as much as introducing branching in the chain (Table 2). Thus, the isopropyl disulfides and *sec*-butyl, **8c** and **8d**, respectively, were considerably more active than the unbranched side-chain analogues. Additionally, all of the compounds appeared to be somewhat more active against *S. aureus* than MRSA, which is opposite to what we observed previously for the *N*-thiolated β-lactams.[7] We do not believe that this difference has anything to do with penicillinase production in MRSA, since repeating the testing of *S. aureus* in the presence of penicillinase protein does not cause a reduction in bioactivity.

The most active series of compounds, *p*-nitrophenyl disulfides **8a**–**f**, was also tested against *Escherichia coli* (K12 strain, ATCC 23590) (Table 3). With the lowest MICs being around 16 μg/ml, none of these derivatives were appreciably active against *E. coli*. However, it is interesting that the methyl and ethyl disulfides possess the strongest activity, which drops off dramatically as the alkyl chain is elongated or branched. This is opposite to that observed for *S. aureus* and *B. anthracis*.

Table 2
Zones of inhibition (on agar plates) and MIC values (in broth) for aryl disulfides **8a–f**, **9a–d**, and **10a–d** against *S. aureus*, MRSA, and *B. anthracis*



8a-f **9a-d** **10a-d**

Compound	Alkyl R	*S. aureus* (ATCC 25923)	MRSA (ATCC 43300)	*B. anthracis*
8a	Methyl	35 mm	23 mm	54 mm
		16 μg/ml	32 μg/ml	1 μg/ml
8b	Ethyl	54 mm	38 mm	67 mm
		1 μg/ml	16 μg/ml	0.25 μg/ml
8c	Isopropyl	85 mm	75 mm	71 mm
		<0.125 μg/ml	<0.125 μg/ml	0.125 μg/ml
8d	*sec*-Butyl	68 mm	54 mm	62 mm
		0.8 μg/ml	1.0 μg/ml	0.5 μg/ml
8e	*n*-Propyl	43 mm	38 mm	57 mm
		16 μg/ml	16 μg/ml	1 μg/ml
8f	*n*-Butyl	53 mm	28 mm	52 mm
		1 μg/ml	32 μg/ml	1 μg/ml
9a	Methyl	36 mm	15 mm	43 mm
		16 μg/ml	64 μg/ml	8 μg/ml
9b	Ethyl	45 mm	12 mm	40 mm
		8 μg/ml	64 μg/ml	8 μg/ml
9c	Isopropyl	85 mm	38 mm	54 mm
		<0.125 μg/ml	16 μg/ml	1 μg/ml
9d	*sec*-Butyl	45 mm	22 mm	41 mm
		8 μg/ml	32 μg/ml	8 μg/ml
10a	Methyl	37 mm	26 mm	31 mm
		16 μg/ml	32 μg/ml	16 μg/ml
10b	Ethyl	40 mm	28 mm	35 mm
		16 μg/ml	32 μg/ml	16 μg/ml
10c	Isopropyl	31 mm	20 mm	27 mm
		16 μg/ml	32 μg/ml	32 μg/ml
10d	*sec*-Butyl	24 mm	20 mm	23 mm
		32 μg/ml	32 μg/ml	32 μg/ml
Penicillin G		33 mm	14 mm	nt
		0.03 μg/ml	64 μg/ml	nt
Vancomycin		27 mm	22 mm	nt
		0.25 μg/ml	0.5 μg/ml	nt

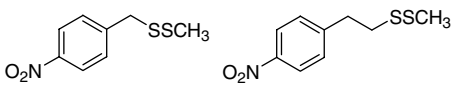
The top value for each compound and microbe is the average diameter of growth inhibition (3 trials) produced after 24 h of incubation using 20 μg of test compound (in DMSO). The lower value is the minimum inhibitory concentration (MIC) of drug (in μg/ml), the lowest concentration of compound in the agar where bacterial growth is completely inhibited. MIC values were determined by serial dilution in 24-well plates according to NCCLS protocols.[38] nt = not tested.

Table 3
Comparison of the minimum inhibitory concentration (MIC) values of *p*-nitrophenyl alkyl disulfides **8a–f** against *E. coli* in broth

Compound	*E. coli* (ATCC 23590)
8a	16 μg/ml
8b	16 μg/ml
8c	96 μg/ml
8d	128 μg/ml
8e	64 μg/ml
8f	96 μg/ml

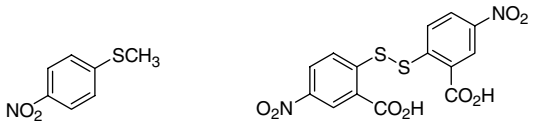
The need for an aryl moiety directly on one of the sulfurs was made apparent when we tested *p*-nitrobenzyl and *p*-nitrophenethyl disulfides, **11** and **12** (Fig. 3). Neither of these compounds showed antibacterial activity against *S. aureus*, but had very weak activities against *B. anthracis* (MICs of 32 and 64 μg/ml, respectively). Thus, insertion of a methylene or ethylene group between the aryl ring and sulfur center dramatically reduces antibacterial activity, probably due to significantly lower reactivity of the disulfide toward nucleophilic displacement.

Likewise, the corresponding sulfide **13** and Ellman's reagent (**14**) were both found to be effectively inactive against *S. aureus*,



11 **12**

Figure 3. *p*-Nitrobenzyl and *p*-nitrophenethyl disulfides **11** and **12**.



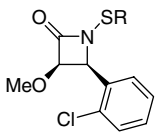
13 **14**

Figure 4. Methyl *p*-nitrophenyl sulfide (**13**) and Ellman's reagent (**14**).

Table 4
Percent inhibition[a] of FabH derived from *E. coli* by disulfides **8a–f**

Compound	*ec*FabH (%)
8a	94
8b	98
8c	98.5
8d	96
8e	91
8f	93

[a] Measured as the percent decrease in activity of FabH in the presence of 5 μM of disulfides **8a–f**, as compared to the same enzyme in the absence of the disulfide.



15a: R = Me
15b: R = *s*-Bu

Figure 5. *N*-Alkylthio β-lactams (**15a** and **15b**).

illustrating the need for both a disulfide linkage (in the case of inactive compound **13**) and high lipophilicity (in the case of inactive disulfide **14**) for antimicrobial activity (Fig. 4).

In addition to the in vitro properties, we were interested to determine if the antibacterial activity of the disulfides could be due to inhibition of a key enzymatic step in lipid biosynthesis in bacteria. Given our recent finding that *N*-thiolated lactams react with coenzyme A to create a mixed CoA disulfide and inhibit fatty acid biosynthesis, we decided to investigate whether the disulfides could directly inhibit β-ketoacyl-acyl carrier protein synthase III, or FabH, a key enzyme in type II fatty acid biosynthesis. Thus, additional experiments using compounds **8a–f** to determine inhibition capabilities against purified *E. coli* FabH verify that the disulfides are highly active against this enzyme. This was carried out as described previously.[39] Each compound in this series showed >90% inhibition of *E. coli* FabH at a disulfide concentration of 5 μM (Table 4).

For comparison, *N*-alkylthio β-lactams **15a** and **15b** were also tested, and both of these thiolating lactams exhibited inhibitory activity against purified *E. coli* FabH (Fig. 5). At 1 μM, methylthio lactam **15a** inhibits FabH catalytic activity by 89%, while *sec*-butylthio lactam **15b** induces a 30% decrease in activity. These assays were done in the absence of coenzyme A, thus showing that the compounds directly act on FabH protein.

These results provide the first evidence that the disulfides and the *N*-alkylthio β-lactams can inhibit bacterial growth in the same manner, directly through FabH inactivation. However, once inside the bacterial cell, the disulfides and *N*-alkylthio β-lactams can *indirectly* inhibit FabH catalysis by readily forming alkyl-CoA mixed disulfide adducts, which in turn can impede FabH function. Indeed, Reynolds has previously reported that methyl-CoA mixed disulfide effectively inhibits *E. coli*-expressed FabH (IC_{50} = 57 μM) (Fig. 6).[11] Data is not yet available for inhibition studies on FabH from *S. aureus*.

It is noteworthy that the trends in activity observed for the nitrophenyl disulfides **8–10** coincide with previous observations on the relative dimensions of the active site in each of the microbial species studied. The active site of the *E. coli* FabH has been reported to preferentially bind *S*-acetyl-CoA over larger or branched *S*-acyl-CoA analogues.[40] By contrast, the FabH from both *Staphylococcus*[41] and *Bacillus*[42] have larger active-site pockets, which favor reactions with branched *S*-acyl-CoA species such as *S*-isobutyryl-CoA.[43] These trends are followed precisely in the bioactivities of the disulfide series, providing strong indirect evidence that these compounds may selectively bind to FabH in a mechanism-based manner. One possibility is that the aryl–alkyl disulfides may inhibit FabH by reversibly 'capping' the active-site cysteine through a thiol-disulfide exchange. The reported X-ray crystal structures[44,45] of FabH from *S. aureus* indicate a fairly deep, lipophilic 'pocket' into which *S*-acyl-CoA or *S*-acyl-ACP are able to stretch their lipophilic arm during binding. A proximal cysteine residue is located within the active site, and it is this available thiol that we speculate may be sulfenylated by entry of the aryl–alkyl disulfides into the en-

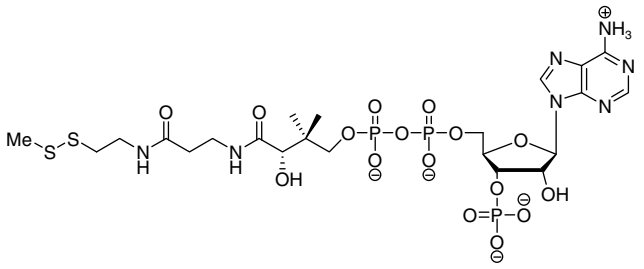
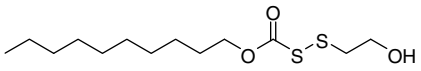


Figure 6. Methyl-coenzyme A mixed disulfide.



16

Figure 7. FabH inhibitor disulfide **16**.

zyme. Reynolds has recently reported that the asymmetric disulfide **16** inhibits the activity of *Mycobacterium tuberculosis* and *E. coli* FabH enzymes, by capping an active-site cysteine thiol by sulfenylation, and that this capping destroys catalytic functioning of the enzyme (Fig. 7). We presume that a similar event could be occurring intracellularly in the FabH protein of *S. aureus* in the presence of the disulfides.[46]

3. Conclusions

The aryl–alkyl disulfides examined in this initial study possess strong in vitro antimicrobial properties against *S. aureus* (including MRSA) and *B. anthracis*, and only weak activity against *E. coli*. Of the structural variants included in our study, the nitrophenyl alkyl disulfides exhibit the greatest in vitro potency. Perhaps even more interestingly, it seems that the efficacy of these compounds may be 'tuned' to select for a particular bacterial species: for *E. coli*, the methyl aryl disulfides are optimal, while for *Staphylococcus* and *Bacillus*, the isopropyl- and *sec*-butyl aryl disulfides appear to be more efficacious. The fact that the in vitro microbiological properties of these compounds, determined from agar diffusion and agar MIC measurements, coincide with the FabH inhibition capabilities certainly points to the fact that this key enzyme is associated with the biological properties of the compounds. These results also highlight the fact that the *N*-thiolated β-lactams (and presumably *N*-thiolated oxazolidinones), reported previously as selective bacteriostatic agents for *Staphylococcus*and *Bacillus*, may act similarly by directly inhibiting FabH protein in MRSA and *B. anthracis*.

The structural effects on bioactivity of these compounds raise several key questions regarding the role of the aryl nitro substituent, whether it can be satisfactorily substituted for other functionalities (such as water solubilizing groups), and if the aryl ring substituent in some way aids in specific binding of the molecule to the FabH active site prior to sulfenylation. Understanding the ability of alkyl-CoA mixed disulfides to covalently deactivate bacterial fatty acid synthesis represents a major advance in the quest for novel antibacterials. However, due to the demonstrated inability of CoA mixed disulfides to traverse the cell membrane, the alkyl-CoA disulfides are not directly useful as therapeutics. This research shows that prodrugs such as the described disulfides and the previously reported *N*-alkylthio β-lactams can be used to produce the CoA mixed disulfides within the bacterial target.

The activity of methyl-CoA disulfide against *ec*FabH provides strong evidence that in prokaryotic cells which contain CoA, the alkyl–aryl disulfides and *N*-alkylthio β-lactams may function as prodrugs to produce alkyl-CoA disulfides, and that these mixed disulfides can then inhibit the fatty acid cycle through inhibition of FabH. This mechanism is unique in that it involves a thiol-disulfide exchange from the alkyl-CoA disulfide to the active-site cysteine of FabH, a suggestion that is supported by protein crystallographic studies. Reynolds reported an X-ray crystal structure of *E. coli* FabH showing the methylthio group from methyl-CoA disulfide being covalently bound to the active-site cysteine (^{112}Cys). While the catalytic activity of the methylthiolated (deactivated) FabH protein could not be regenerated simply by dialysis, treatment with dithiothreitol (DTT) quickly restores full catalytic function. This suggests that formation of the cysteine-alkyl FabH disulfide (either via the alkyl-CoA disulfide, the alkyl–aryl disulfide, or *N*-alkylthio β-lactam) is irreversible under buffered aqueous conditions, but that addition of a thiol can regenerate the catalytic form through thiol-disulfide exchange.

The finding that these simple alkyl–aryl disulfides are generally less active against *E. coli* than *S. aureus* or *B. anthracis* may also reflect differences in the thiol-redox buffers of these three microbes.[13,47] *Staphylococcus* and *Bacillus* utilize a coenzyme A based thiol-redox buffer, while *E. coli* uses a glutathione buffer. Consequently, the effects of the aryl–alkyl disulfides in glutathione-based eukaryotic cells would likely also be dampened, which is a question we are now exploring.

For the past decade, there had been considerable interest in the development of FabH inhibitors as potential antibacterial agents.[48,49] The key role of FabH in Type II fatty acid synthesis, as well as the unique differences between bacterial and mammalian FAS pathways, makes this protein a desirable target for the selective inhibition of bacterial growth. We are exploring potential applications of these disulfide inhibitors of FabH as antibacterial agents for life-threatening bacterial infections.

4. Experimental

4.1. General methods

Reagents were purchased from Sigma–Aldrich Chemical Company or Acros Chemical Company. Methanethiolsulfonates were purchased from Toronto Research Chemicals. Reagents were used without further purification. Solvents were obtained from Fischer Scientific Company. Thin-layer chromatography (TLC) was carried out using EM Reagent plates with fluorescence indicator (SiO_2-60, F-254). Products were purified by flash chromatography using J.T. Baker flash chromatography silica gel (40 μm). NMR spectra were recorded in $CDCl_3$ unless otherwise noted. ^{13}C NMR spectra were proton broad-band decoupled. Methylene chloride and THF were distilled prior to use. Prior to the preparation of the disulfides, methanol was purged of oxygen by bubbling nitrogen an inert gas through it for several minutes.

4.2. *m*-Nitrobenzenethiol[50]

To a solution of *m*-nitrobenzene disulfide (300 mg, 0.97 mmol) in anhydrous THF (2 ml) was added solid $NaBH_4$ (140 mg, 3.4 mmol) in small portions. The resulting mixture was stirred at rt under an inert atmosphere. After 2 h, the reaction mixture was cooled in an ice bath, then about 5 ml of icewater was added to the mixture. The resulting mixture was acidified with HCl (1 M), then extracted with CH_2Cl_2 (10 ml). The organic layer was then washed with water (10 ml), then brine (10 ml), dried over $MgSO_4$, and concentrated in vacuo to give the thiol (248 mg, 83%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.10 (d, J = 2.0 Hz, 1H); 7.97 (dd, J = 8.0, 2.0 Hz, 1H); 7.54 (d, J = 8.0 Hz, 1H); 7.38 (t, J = 8.0 Hz, 1H); 3.68 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 134.9, 130.0, 123.9, 120.7.

4.3. *o*-Nitrobenzenethiol

Prepared exactly as described above, except that the reaction was allowed to run for 3 h, and the products required chromatography (silica with hexanes:CH_2Cl_2) to give the thiol (160 mg, 61%) as a pale yellow flocculent solid. Mp: 44–45 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.24 (d, J = 8.0 Hz, 1H); 7.42 (d, J = 4.0 Hz, 1H); 7.29–7.24 (m, 1H); 4.00 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 133.9, 132.2, 126.5, 126.0.

4.4. *p*-Nitrobenzyl thiol

Thioacetic acid (386 μl, 5.40 mmol) was added to a mixture of 4-nitrobenzyl bromide (600 mg, 2.79 mmol) and anhydrous K_2CO_3 (380 mg, 2.75 mmol) in acetone (3 ml), and the resulting mixture was stirred at rt for 2 h under an inert atmosphere. The mix was then partitioned between CH_2Cl_2 (10 ml) and water (10 ml). The organic layer was separated, then washed quickly with saturated aqueous $NaHCO_3$ (10 ml) and brine (10 ml). The organic solution was concentrated in vacuo, then taken up in methanol (5 ml). Aqueous H_2SO_4 (50%, 1 ml) was added, and the solution was heated to reflux for 3 h, under N_2. The solution was partitioned between ether (30 ml) and water (30 ml). The ether layer was washed with water (2× 30 ml), then brine (10 ml). The solution was dried over Na_2SO_4, then concentrated in vacuo to give the thiol (317 mg, 67%) as a yellow solid. Mp: 49–52 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.19 (d, J = 8.8 Hz, 2H); 7.02 (d, J = 8.6 Hz, 2H); 3.82 (d, J = 7.8 Hz, 2H); 1.84 (t, J = 8.1 Hz, 1H).

4.5. *p*-Nitrophenethyl thiol

Prepared according to the general procedure described above, except that the reaction time was 45 min. Isolated 170 mg (69%) as a yellow liquid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.17 (d, J = 8.8 Hz, 2H); 7.35 (d, J = 8.8 Hz, 2H); 3.04 (t, J = 7.7 Hz, 2H); 2.84 (dt, J = 5.9 Hz, 7.7 Hz, 2H); 1.38 (t, J = 5.9 Hz, 1H).

4.6. Synthesis of alkyl–aryl disulfides: general procedure

To a 1.0 M solution of arylthiol in MeOH (100 μl) was added a solution of alkyl methanethiolsulfonate in MeOH (105 μl of a 1.0 M solution, 1.05 equiv). After stirring at rt under an inert atmosphere for 1 h, the solutions were treated with a small amount (about 0.3 equiv) of cysteine hydrochloride in order to consume the excess thiosulfonate. The mixture was then concentrated under reduced pressure. The solid was taken up in CH_2Cl_2 (1 ml), and the insoluble material was filtered off. The solution was stirred with Amberlyst-21 resin (weakly basic), (approx. 0.2 equiv, pre-swelled in CH_2Cl_2) for 3 min. The solution was drawn off of the resin, quickly passed through a small silica plug using dichloromethane, and concentrated in vacuo to give the desired product.

4.6.1. 3,4-Difluorophenyl methyl disulfide (1)

9.6 mg (50%) as a colorless oil. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.37–7.29 (m, 1H); 7.18–7.00 (m, 2H); 2.34 (s, 3H).

4.6.2. 3,4-Dimethylphenyl methyl disulfide (2)

Isolated 9.7 mg (53%) as a colorless oil. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.22 (d, J = 8.4 Hz, 1H); 7.18 (s, 1H); 7.02 (d, J = 7.7 Hz, 1H); 2.70 (s, 3H); 2.19 (s, 3H); 2.18 (s, 3H).

4.6.3. *m*-Ethoxyphenyl methyl disulfide (3)

Isolated 5.6 mg (28%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.21 (t, J = 8.0 Hz, 1H); 7.09–7.05 (m, 2H); 6.74 (dd, J = 8.0, 1.2 Hz, 1H); 4.04 (q, J = 7.1 Hz, 2H); 2.43 (s, 3H); 1.41 (t, J = 7.4 Hz, 3H).

4.6.4. *o*-(Hydroxymethyl)phenyl methyl disulfide (4)

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, J = 8.8 Hz, 1H); 7.44 (dd, J = 6.6, 2.2 Hz, 1H); 7.32–7.24 (m, 2H); 4.83 (d, J = 5.2 Hz, 2H); 2.42 (s, 3H); 2.02 (br t, J = 6.0 Hz, 1H).

4.6.5. Methyl 2-pyridinyl disulfide (5)

^{1}H NMR indicates that about 10% of material is unreacted thiol. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.46 (d, J = 4.4 Hz, 1H); 7.68–7.61 (m, 2H); 7.07 (t, J = 6.6 Hz, 1H); 2.49 (s, 3H).

4.6.6. *p*-Acetamidophenyl methyl disulfide (6)

To a solution of *p*-acetamidobenzenethiol (50 mg, 0.3 mmol) in dry CH_2Cl_2 (3 ml) was added triethylamine (127 μl, 0.9 mmol, 3 equiv), followed by acetyl chloride (32 μl, 0.45 mmol). The reaction mixture was stirred at rt for 1 h, then it was diluted with dichloromethane, washed with water, then 5% aq HCl, then 5% aq $NaHCO_3$, dried over $MgSO_4$, then concentrated in vacuo to give a yellow oil which solidified on standing. Chromotography (1:1 petroleum ether:ethyl acetate) gave 18 mg (29%) as a light brown solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (s, 4H); 2.41 (s, 3H); 2.16 (s, 3H).

4.6.7. *p*-Aminophenyl methyl disulfide (7)

To a solution of *p*-aminobenzenethiol (125 mg, 1 mmol) in methanol (1 ml) was added methylthio methanesulfonate (94 μl, 1 mmol) in a single portion. The reaction mixture was stirred at rt under N_2. After 2 h, the solution was loaded onto an SCX column and eluted with methanol, followed by 1 M ammonia in methanol. The basic eluents were concentrated in vacuo to a yellow oil. Chromatography (1:1 hexanes/CH_2Cl_2 with 1% isopropyl amine) yielded **8** (140 mg, 82%) as a pale yellow oil. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.28 (d, J = 8.6 Hz, 2H); 6.54 (d, J = 8.6 Hz, 2H); 3.60 (br s, 2H); 2.34 (s, 3H). Treatment with HCl/ether gave 5 mg of the HCl salt. Biological testing was performed on the acid salt. ^{1}H NMR (400 MHz, d_6-DMSO): δ 7.46 (br s, 2H); 7.05 (br s, 2H); 2.47 (s, 3H); 2.43 (s, 3H).

4.6.8. *p*-Nitrophenyl methyl disulfide (8a)

Isolated 15.3 mg (76%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, J = 8.8 Hz, 2H); 7.63 (d, J = 8.8 Hz, 2H); 2.46 (s, 3H). Resynthesis using 10× scale yielded 184 mg (92%) as a slightly oily, bright yellow solid. Mp: 29–32 °C. ^{13}C NMR (100 MHz, $CDCl_3$): δ 126.6, 126.0, 124.7, 124.4, 22.9.

4.6.9. Ethyl *p*-nitrophenyl disulfide (8b)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H); 7.64 (d, J = 8.8 Hz, 2H); 2.77 (q, J = 7.0 Hz, 2H); 1.31 (t, J = 7.2 Hz, 3H).

4.6.10. Isopropyl *p*-nitrophenyl disulfide (8c)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H); 7.64 (d, J = 8.8 Hz, 2H); 3.10 (septuplet, J = 6.5 Hz, 1H); 1.30 (d, J = 6.8 Hz, 6H).

4.6.11. *sec*-Butyl *p*-nitrophenyl disulfide (8d)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H); 7.64 (d, J = 8.8 Hz, 2H); 2.88–2.83 (m, 1H); 1.73–1.64 (m, 1H); 1.57–1.50 (m, 1H); 1.27 (d, J = 6.8 Hz, 3H); 0.97 (t, J = 7.2 Hz, 3H).

4.6.12. *p*-Nitrophenyl *n*-propyl disulfide (8e)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H); 7.63 (d, J = 8.8 Hz, 2H); 2.73 (t, J = 7.2 Hz, 2H); 1.68 (tq, J = 7.2, 7.6 Hz, 2H); 0.98 (t, J = 7.6 Hz, 3H).

4.6.13. *n*-Butyl *p*-nitrophenyl disulfide (8f)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15 (d, J = 8.0 Hz, 2H); δ 7.64 (d, J = 8.8 Hz, 2H); δ 2.95 (t, J = 7.4 Hz, 2H); *δ 1.65–1.61 (m, 2H); δ 1.42–1.37 (m, 2H);* δ 0.88 (t, J = 7.2 Hz, 3H).

4.6.14. Methyl *o*-nitrophenyl disulfide (9a)

Isolated 13.9 mg (69%) as an oily yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.27–8.23 (m, 2H); 7.69 (t, J = 7.4 Hz, 1H); 7.35 (d, J = 7.2 Hz, 1H); 2.41 (s, 3H).

4.6.15. Ethyl *o*-nitrophenyl disulfide (9b)

Isolated 13.2 mg (62%) as an oily yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, J = 8.0 Hz, 1H); 8.24 (d, J = 8.0 Hz,

1H); 7.66 (t, J = 7.4 Hz, 1H); 7.33 (t, J = 7.6 Hz, 1H); 2.74 (q, J = 7.3 Hz, 2H); 1.31 (t, J = 7.2 Hz, 3H).

4.6.16. Isopropyl *o*-nitrophenyl disulfide (9c)

Chromatographed on silica with 3% dichloromethane in hexanes as eluent. Isolated 13.7 mg (60%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, J = 8.8 Hz, 1H); 8.23 (d, J = 8.8 Hz, 1H); 7.64 (t, J = 7.9 Hz, 1H); 7.31 (t, J = 7.6 Hz, 1H); 3.05 (s, J = 6.8 Hz, 1H); 1.31 (d, J = 6.8 Hz, 6H).

4.6.17. Butyl *o*-nitrophenyl disulfide (9d)

Chromatographed on silica with 3% dichloromethane in hexanes as eluent. Isolated 12.9 mg (54%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (d, J = 8.0 Hz, 1H); 8.22 (d, J = 8.8 Hz, 1H); 7.64 (t, J = 7.8 Hz, 1H); 7.31 (t, J = 8.0 Hz, 1H); 2.86–2.79 (m, 1H); 1.75–1.60 (m, 1H); 1.58–1.50 (m, 1H); 1.28 (d, J = 7.2 Hz, 3H); 0.98 (t, J = 7.8 Hz, 3H).

4.6.18. Methyl *m*-nitrophenyl disulfide (10a)

Isolated 7.6 mg (38%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H); 8.04 (d, J = 6.4Hz, 1H); 7.79 (d, J = 8.0 Hz, 1H); 7.49 (t, J = 8.0 Hz, 1H); 2.47 (s, 3H).

4.6.19. Ethyl *m*-nitrophenyl disulfide (10b)

Isolated 7.7 mg (36%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.41 (s, 1H); 8.02 (d, J = 7.2 Hz, 1H); 7.80 (d, J = 7.2 Hz, 1H); 7.47 (t, J = 7.2 Hz, 1H); 2.78 (q, J = 7.3 Hz, 2H); 1.32 (t, J = 7.2 Hz, 3H).

4.6.20. Isopropyl *m*-nitrophenyl disulfide (10c)

Isolated 17.0 mg (75%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.41 (s, 1H); 8.01 (d, J = 8.0 Hz, 1H); 7.80 (d, J = 7.2 Hz, 1H); 7.46 (t, J = 7.2 Hz, 1H); 3.09 (septet, J = 6.8 Hz, 1H); 1.30 (d, J = 6.8 Hz, 6H).

4.6.21. Butyl *m*-nitrophenyl disulfide (10d)

Isolated 12.6 mg (52%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H) 8.00 (d, J = 8.0 Hz, 1H); 7.80 (d, J = 8.0 Hz, 1H); 7.45 (t, J = 8.0 Hz, 1H); 2.88–2.83 (m, 1H); 1.73–1.68 (m, 1H); 1.57–1.50 (m, 1H); 1.28 (d, J = 7.2 Hz, 3H); 0.97 (t, J = 7.2 Hz, 3H).

4.6.22. Methyl 4-nitrobenzyl disulfide (11)

Prepared according to the general procedure outlined above for the aryl–alkyl disulfides. Following chromatography (3:1 hexanes/CH_2Cl_2), 3.1 mg (5%) was obtained as a pale yellow solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.20 (d, J = 8.7 Hz, 2H); 7.51 (d, J = 8.6 Hz, 2H); 3.94 (s, 2H); 2.15 (s, 3H).

4.6.23. Methyl 2-(*p*-nitrophenethyl) disulfide (12)

Obtained 9.7 mg (15%) as a pale yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.17 (d, J = 8.0 Hz, 2H); 7.37 (d, J = 8.0 Hz, 2H); 3.19–2.85 (m, 4H); 2.43 (s, 3H). MS (EI) m/z: 229 (M^+).

4.7. General microbiological methods

Staphylococcus aureus (ATCC 25923) and MRSA (ATCC 43300) were purchased from ATCC sources. *Bacillus* bacteria from Sterne spore vaccine was purchased from Colorado Serum Co., Denver, CO.

4.7.1. 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.

4.7.2. Antimicrobial testing: Kirby–Bauer method

Sterile saline (5 ml) was inoculated with a swab of bacteria, then the concentration was adjusted to 0.5 McFarland standard. Bacterial solution was then streaked across a TSA plate to give an even lawn of bacteria. Sterile pipet tips (1–30 μl) 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 inoculated overnight at 37 °C.

4.7.3. Agar dilution minimal inhibitory concentration (MIC) assay

A stock solution of 1 mg/ml of each disulfide was prepared in DMSO. Freshly prepared Mueller-Hinton agar (1.5 ml) was added to each well of a 24-well plate, and to each well was then added a specified amount of the disulfide test solution in order to a give a final drug concentration of 256 mg/ml down to 0.125 μg/ml. One well within each series received 0.1 ml of DMSO as a blank. The contents of each well were thoroughly stirred to evenly distribute the antimicrobial solution within the agar. Once the agar completely solidified, 10 μl of Mueller-Hinton broth saline containing 0.5 McFarland standard of the test bacteria was pipetted on top of each well and the plates were then incubated for 24 h at 37 °C. Bacterial growth was assessed by visual observation of growth.

4.7.4. FabH enzyme assay

FabH assays were carried out using a standard coupled trichloroacetic acid precipitation assay which determines the rate of formation of radiolabeled 3-ketoacyl ACP from malonyl ACP and radiolabeled acetyl-CoA. In this coupled assay *Streptomyces glauscescens* FabD is used to generate the malonyl ACP substrate from malonyl-CoA and *Streptomyces glauscescens* ACP. For inhibition studies, A standard 20 μl reaction mixture of *ec*FabH (2 pmol of monomer, 100 nM) and test compound (0.02 nmol, 1 μM for compounds **15a** and **15b**; 0.1 nmol, 5 μM for compounds **8a–f**) in 50 mM sodium phosphate buffer (pH 7.4) was incubated at room temperature for 15 min prior to addition to a solution of [1-^{14}C]acetyl-CoA (0.8 nmol, 40 μM) and MACP (0.2 nmol, 10 μM). The inhibition was measured using standard FabH assay.[51] The % inhibition was determined by measuring FabH activity in the presence of test compound relative to a negative control that has no inhibitor (100% activity). The assay was run in duplicate.

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