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N-thiolated β-lactams: Studies on the mode of action and identification of a primary cellular target in *Staphylococcus aureus*

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Abstract—This study focuses on the mechanism of action of *N*-alkylthio β-lactams, a new family of antibacterial compounds that show promising activity against *Staphylococcus* and *Bacillus* microbes. Previous investigations have determined that these compounds are highly selective towards these bacteria, and possess completely unprecedented structure–activity profiles for a β-lactam antibiotic. Unlike penicillin, which inhibits cell wall crosslinking proteins and affords a broad spectrum of bacteriocidal activity, these N-thiolated lactams are bacteriostatic in their behavior and act through a different mechanistic mode. Our current findings indicate that the compounds react rapidly within the bacterial cell with coenzyme A (CoA) through in vivo transfer of the *N*-thio group to produce an alkyl-CoA mixed disulfide species, which then interferes with fatty acid biosynthesis. Our studies on coenzyme A disulfide reductase show that the CoA thiol-redox buffer is not perturbed by these compounds; however, the lactams appear to act as prodrugs. The experimental evidence that these β-lactams inhibit fatty acid biosynthesis in bacteria, and the elucidation of coenzyme A as a primary cellular target, offers opportunities for the discovery of other small organic compounds that can be developed as therapeutics for MRSA and anthrax infections.

1. Introduction

N-Alkylthio β-lactams represent a recently discovered family of antibacterial compounds that selectively inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA)[1–6] and *Bacillus anthracis*[7] (Fig. 1). Despite their apparent structural similarities to the penicillins and other members of the β-lactam family of antibiotics, these N-thiolated β-lactam compounds behave, in many ways, differently to all other known antibacterial β-lactams. First, these N-thiolated compounds only target a few genera of bacteria such as *Staphylococcus* and *Bacillus*, yet show anticancer properties,[8–12] and are non-lethal to healthy human cells (fibroblasts).[2] Second, the bioactivity of these N-thiolated lactams is largely insensitive to changes in structure of the ring substituents at the C3 and C4 centers of the lactam ring, strongly suggesting that the compounds exert their antibacterial effects on a completely different cellular target and through a different mode of action than previously studied β-lactam antibacterials. Unfortunately, neither the target nor the mode of action of these new antibacterial agents is presently known.

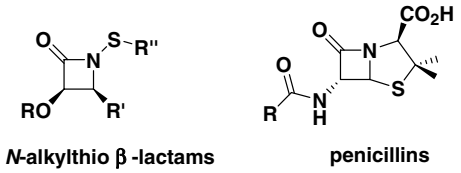

Figure 1. Structures of *N*-alkylthio β-lactams and penicillins, two different families of β-lactam antibiotics.

2. Results and discussion

2.1. Structure–activity studies

Ongoing experiments in our laboratory on the N-thiolated systems have explored the effect of the substituents around the β-lactam ring on antibacterial activity.[1–7] Our results so far indicate that the structure–bioactivity patterns for these N-thiolated lactams are completely different to those of other β-lactam antibacterial drugs. In fact, these are the first β-lactam compounds which

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possess antibiotic properties despite the absence of any ionizable ring functionality (typically a carboxylic acid) within its core structure. A wide variety of substituents at the C3 and C4 positions can be tolerated without significant effects on biological activity, and stereochemistry at these centers is also irrelevant. However, the *N*-alkylthio moiety is absolutely essential.[6] Finding that thiolation of the nitrogen is a prerequisite for activity, we then further investigated the effect of various organothio moieties. While arylthio substituents are tolerated on the nitrogen, small alkylthio substituents (four carbons or less) provide for the strongest bioactivity. Further, alkylthio groups bearing a single branched site (i.e., isopropyl and *sec*-butyl) produce better activities against *S. aureus* than do the straight chain *n*-alkylthio analogs, while a high degree of branching (i.e., *t*-butylthio) results in a significant decrease in activity. The oxidation state of the sulfur also plays a substantial role in the activity of the compounds: *N*-sulfenyl and *N*-sulfinyl groups provide for much more elevated anti-MRSA activities than *N*-sulfonyl or *N*-sulfonic acid functionalities.[6]

The clear dependency of substituents at the lactam nitrogen, as compared to the C3 and C4 ring centers, suggests that this organothio moiety is central to the compound's biological mechanism. By contrast, the broad tolerance of substituents at the other two positions suggests that these groups may not play a direct role in the binding or reactivity of the drug with a biological target, but, more likely, may influence bacterial cell uptake and membrane permeability. Further, it was recently shown that structurally related *N*-alkylthio-2-oxazolidinones (Fig. 2) closely mirror these same trends, demonstrating that the four-membered β-lactam ring itself is not even essential for bioactivity.[13]

2.2. Genera-selective antibacterials

Antimicrobial screening of the various lactam derivatives outlined above included a fairly wide range of Gram-positive and Gram-negative bacteria. Approximately 65 microbes of different genera or species were examined.[14] Although there were fluctuations in the levels of bioactivity seen among the different lactam variants against any particular bacterium, the data were consistent in terms of which microbes were most susceptible. This indicates that these N-thiolated lactams function as narrow-spectrum antibiotics for *Staphylococcus*, *Micrococcus*, and *Bacillus* bacteria, although they also are somewhat less powerful inhibitors of *Bacteroides, Streptococcus, Neisserria gonorrhoeae*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Mycobacterium tuberculosis*. On the other hand, these compounds were found to be consistently ineffective against

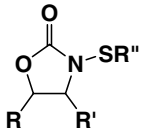
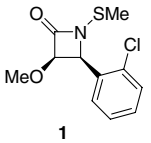


Figure 2. Structure of *N*-alkylthio 2-oxazolidinone antibiotics.

Enterobactor cloacae, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, and *Proteus mirabilis*. Thus, activity does not appear to be related to whether the microbe is Gram-positive or Gram-negative, unlike that seen for many other antibacterial drugs. Peculiarly, the most sensitive microbes were those from four distinct taxonomic orders defined by their genetic, morphological, and metabolic traits. Although nine genera in total were found to be susceptible to the lactams, few taxonomic relationships could be used to ascertain the spectrum of activity. The class of bacteria most sensitive to the *N*-thiolated β-lactams was the 'Bacilli', which consists of two orders: *Bacillales* and *Lactobacillales*. Susceptible members of this taxonomic class included species of *Bacillus*, *Staphylococcus*, and *Streptococcus*. *Enterococcus*, *Lactococcus*, and *Listeria* which are also affiliated with the 'Bacilli' were mostly insensitive to the drugs. This sporadic but narrow spectrum of activity supports the conclusions of the above-described structure–activity data, that the compounds are acting in a different manner from traditional broad-spectrum β-lactam antibiotics.



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Effects of the lactam on cell growth were studied by measuring cell survival for methicillin-susceptible *S. aureus* (MSSA) and MRSA, respectively, when cultured in the absence or presence of lactam **1** over a 2 h time frame. In the absence of lactam **1**, MSSA and MRSA grew logarithmically; in the presence of **1**, bacterial growth was immediately halted. While reproduction ceased at the MIC level of the lactam as well as at 10× MIC, the number of viable cells remained constant throughout the duration of the experiment. Consequently, bacterial growth was clearly inhibited by lactam **1**, but little to no decrease in cell population was observed. These data provide substantial evidence that even at high drug concentrations, N-methylthio β-lactams are bacteriostatic agents toward staphylococci, and do not reduce cell counts that would be expected if the effects were bacteriocidal.[14]

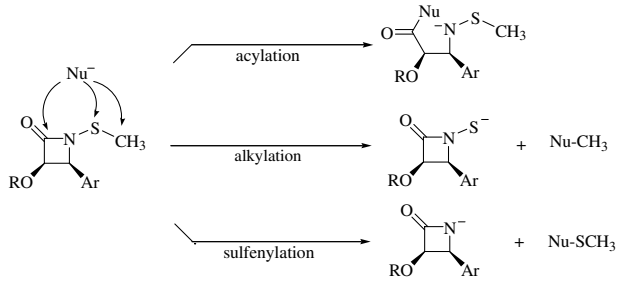
Thus, the finding that N-thiolated β-lactams are bacteriostatic agents possessing a narrow range of activity strongly implies that the biological target is internalized within the cell. Preliminary information on the cellular target of a new antibiotic can be extracted from the intrinsic nature of other known antibacterial drugs: those antibiotics that inhibit intracellular processes such as protein synthesis (chloramphenicol) or secondary metabolites (sulfa drugs) are generally bacteriostatic agents, whereas those that disrupt the outer periphery of cells such as the bacterial membrane (polymyxins, bacitracin, cationic lipoproteins), the cell wall (penicillin, vancomycin) or directly damage DNA (metronidazole) are bacteriocidal. Thus, our first efforts to determine mechanism of action were directed at identi-

fying the chemical pathway by which the lactams exert their effects, and which cellular process or processes are most greatly affected by the lactams.

2.3. Considerations regarding the mode of action

To account for the antibiotic properties of these compounds, at least three types of reactions between *N*-methylthio β-lactams and a biological nucleophile (Nu^-) can be envisioned: acylation (ring opening), alkylation, and sulfenylation (Scheme 1). For the β-lactam class of molecules, it is naturally assumed that nucleophilic addition occurs on the carbonyl center of the reactive four-membered ring. As in the case of penicillins and cephalosporins, the four-membered lactam ring is susceptible to this type of acylation mechanism through recognition by and reaction with an active site serine of cell wall transpeptidases.[15,16] It is through this mechanism, the covalent interaction with the penicillin-binding proteins (PBPs), that β-lactam antibiotics are known to eradicate bacteria. During this process, a serine residue in the active site of the PBPs adds to the carbonyl center of penicillin to create a stable ester linkage. The addition reaction occurring on the hydroxyl of serine is enhanced by ring strain and a carboxylic acid moiety located in proximity to the β-lactam carbonyl. Repositioning or eliminating the carboxylate negates the ability of the antibiotics to irreversibly bind to the PBPs. Therefore, the lack of an acidic functionality in the *N*-methylthio β-lactams suggests that binding within the PBP active site, and subsequent nucleophilic attack on the carbonyl carbon, would be extremely unlikely. Moreover, chemical studies of N-thiolated β-lactams verified the ring's stability. Compound **1** is stable to cleavage in water when exposed to equimolar amounts of potassium hydroxide and serine. In addition, **1** is completely unreactive to commercial β-lactamases even after several days in pH 7.4 buffer. Moreover, lactam **1** does not inhibit penicillinase in catalyzing the hydrolysis of penicillin G.

Further evidence of the stability of N-thiolated lactams to cleavage by bacterial penicillinases is shown in Figure 3. Two experiments are shown: on the left (Fig. 3a), 20 μg of lactam **1** was added to one well (left side well) bored into the agar, and 20 μg of penicillin G was added to the other well (right side), and the plate was inoculated with a penicillin-susceptible form of *S. aureus*. After 24 h of incubation, large growth inhibition zones were observed for both antibiotics. This experiment was then repeated (Fig. 3b), but this time 20 μg of penicillinase enzyme was mixed into the agar prior to incubation with MRSA. The consequences of this are clear: penicillin G (right well) loses all of its antibiotic activity, while lactam **1** retains all of its anti-MRSA activity.



Scheme 1. Probable reaction pathways of N-thiolated β-lactams with nucleophiles.

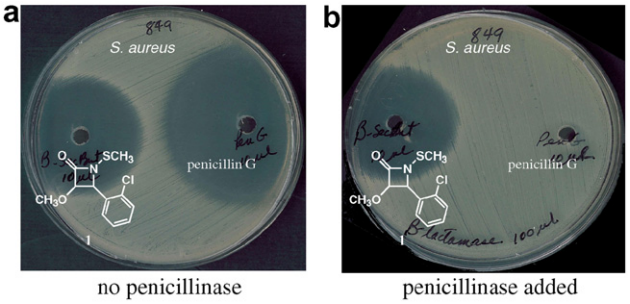


Figure 3. Comparison of bioactivities of lactam **1** and penicillin G toward MRSA in the absence (a) versus presence (b) of bacterial penicillinase. In (a), when no penicillinase is present, lactam **1** and penicillin each produce large zones of growth inhibition. However, when penicillinase is present in the growth media (b), penicillin G is completely devoid of activity while lactam **1** retains its full activity.

However, to prove unequivocally that the N-thiolated β-lactam was not inhibiting the PBPs, light microscopy and electron microscopy were used to probe for damage or thinning of the cell wall in *S. aureus* treated with high concentrations of lactam **1**.

Bacteria exposed to antibiotics that disrupt the cell wall (i.e., β-lactams) or cytoplasmic membrane (i.e., polymyxins) can be observed by scanning electron microscopy (SEM) for physiological damage elicited by the drugs. Cultures of *S. aureus* inoculated with either lactam **1** or penicillin G were inspected by SEM for changes in cell size and appearance in comparison to a culture with no antibiotic (Fig. 4).[14] The SEM samples were prepared from Kirby–Bauer agar diffusion plates by incision of the agar along the outermost regions of cleared zones (see Fig. 3a) where sub-lethal doses of the drugs are present and bacterial growth is only partially inhibited.[17] The first image in Figure 4a portrays the appearance of *S. aureus* grown with no antibiotic present. In its natural state, staphylococci grow to about 1 μm in diameter as grape-like clusters of spherical-shaped cells. Cultures with lactam **1** (Fig. 4b) also show the presence of surviving colonies of bacteria which resemble *S. aureus* in its natural state, appearing spherical and uniform with no apparent deformities. This provided the first substantial evidence that N-thiolated β-lactams do not inhibit bacterial transpeptidases or cause rupturing of the cytoplasmic membrane in staphylococcus in the manner of penicillin, which produces cocci that are wrinkled, concaved, and sheared due to deterioration of the bacterial cell wall (Fig. 4c).

The inhibition of cell wall biosynthesis in Gram-positive bacteria can also be detected by light microscopy upon Gram-staining of the cells. This allows bacteria to be

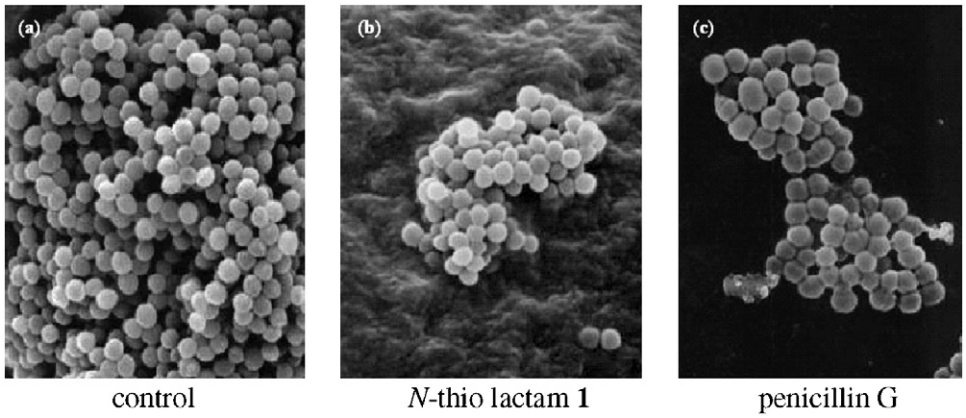


Figure 4. Scanning electron microscope images of *Staphylococcus aureus* cultured on agar in the presence of (a) no antibiotic, (b) lactam **1**, and (c) penicillin G. These images show that cells which survive exposure to N-thiolated lactam **1** (b) look normal in morphology and clustering behavior, while those treated with penicillin (c) suffer severe damage indicative of inhibition of cell wall biosynthesis.

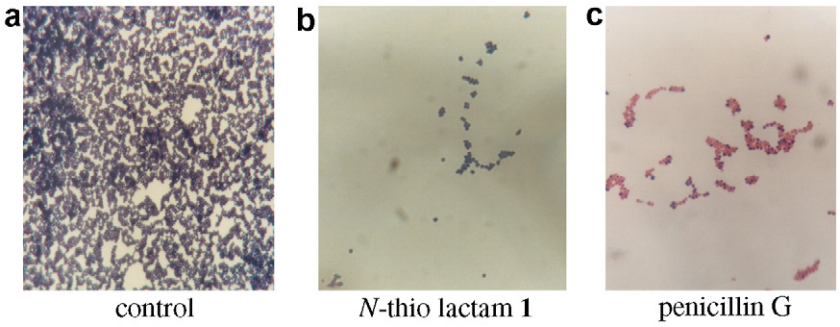


Figure 5. Light microscopy of *Staphylococcus aureus* cells exposed to (a) no antibiotic, (b) lactam **1**, and (c) penicillin G. Staphylococci which appear purple after Gram-staining (a, b) have intact cell walls; those which stain pink (c) have deformed cell walls.

readily distinguished by the thickness and structural integrity of their cell walls. Figure 5 shows the results of these experiments, and the appearances of *S. aureus* cells exposed to (a) no antibiotic, (b) lactam **1**, and (c) penicillin G after staining.[14] Untreated *S. aureus* possesses a well-developed, thick cell wall which retains the crystal-violet stain upon decolorization (Fig. 5a). Cells treated with lactam **1** likewise stain uniformly purple, indicating a fully intact and mature cell wall (Fig. 5b). However, bacteria treated with penicillin G stain mostly pink, indicative of a weakened or sheared cell wall (Fig. 5c). This confirms the results of the SEM experiments, which indicate that the N-thiolated lactams inhibit *S. aureus* through a pathway other than interfering with cell wall biosynthesis.

Scheme 2. Proposed nucleophilic attack on the alkyl residue of the *N*-alkylthio side chain.

Next, we considered the possibility that the compounds could serve as alkylating agents toward cellular nucleophiles, as depicted hypothetically in Scheme 2. Initial structure–activity studies, however, did not support this. It could be predicted that nucleophilic attack on the carbon of the alkylthio moiety could be deterred by placement of bulkier, branched alkyl groups on the sulfur center. In fact, what we observed was completely opposite to this prediction, that the *N*-ethylthio, *N*-isopropylthio, and *N*-*sec*-butylthio lactams were increasingly *more active*, not less active, than the *N*-methylthio analog **1**.[6] But we decided to look into this further by studying the ability of lactam **1** to serve as a DNA alkylating agent in vitro. Most known nucleic acid alkylating drugs react with nucleotides to inhibit DNA replication or compromise the integrity of the super helices. Examples of DNA alkylating agents include leinamycin[19] and mitomycin,[20] which exhibit bactericidal behavior. The *N*-methylthio lactam compounds, however, are bacteriostatic and thus seem unlikely to function as alkylating agents. Nevertheless, experiments were conducted to confirm this.

First, studies were run to determine if N-thiolated β-lactams could directly cause strand breakage of supercoiled

DNA.[14] Plasmid pBR322 was incubated in the presence of varying amounts of lactam **1** (5–100 μM) at 37 °C in sodium phosphate buffer (50 mM, pH 7.4) for 24 h, and double strand (fragmentation) and single strand (linearization) breakage was then analyzed by agarose gel electrophoresis containing 1% ethidium bromide (Fig. 6).

The results show that in lanes 2 and 3, two bands were observed for the plasmid in the absence of lactam **1**. With the supercoiled pBR322 giving rise to the denser band, a weak band of relaxed or 'nicked' DNA was formed by the electrical current and passage through the gel. Lanes 3–8 contained plasmid samples treated with various concentrations of lactam **1**. It was evident that the compound did not cause fragmentation or relaxation of the superhelix. The bands were equivalent in location and illumination to those observed for the untreated plasmids (lanes 2 and 3) thus indicating that chemical modification of the DNA did not occur in the presence of 5–100 μM of lactam **1**.

Recognizing that DNA alkylators are often in a prodrug form, requiring chemical activation before lethal effects on the nucleotide can occur, we carried out some additional experiments. Leinamycin, for example, is transformed into a potent alkylating antibiotic by a thiol-mediated reaction with cysteine or glutathione.[21] Cells susceptible to leinamycin possess thiol-rich intracellular environments which we considered may also be needed for N-thiolated β-lactams to be able to alkylate their cellular target. A second study was therefore performed to examine the stability of plasmid pBR322

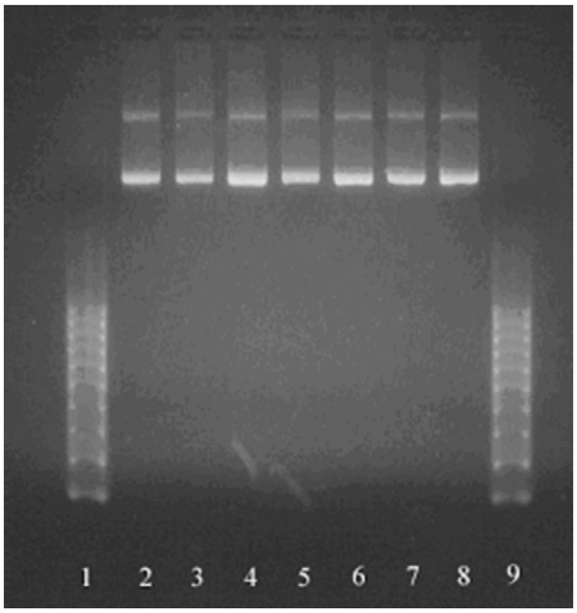


Figure 6. Supercoiled DNA treated with lactam **1** at 5–100 μM. Plasmid pBR322 (0.5 μg) was incubated with lactam **1** at 37 °C in sodium phosphate buffer (50 mM, pH 7.4) for 24 h and analyzed by agarose gel electrophoresis (ethidium bromide staining). Lane 1: marker, lane 2: pBR322, lane 3: pBR322 + DMSO, lane 4: pBR322 + 5 μM **1**, lane 5: pBR322 + 10 μM **1**, lane 6: pBR322 + 25 μM **1**, lane 7: pBR322 + 50 μM **1**, lane 8: pBR322 + 100 μM **1**, lane 9: marker.

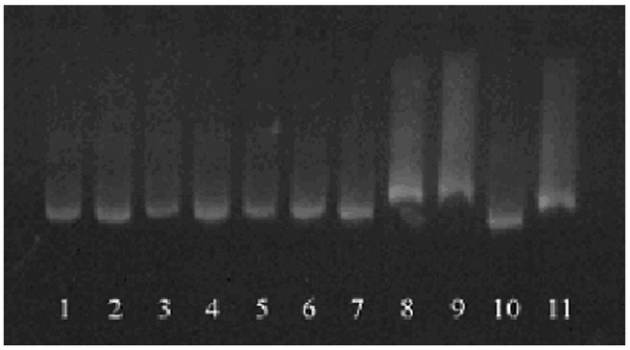


Figure 7. Gel electrophoresis analysis of super-coiled DNA treated with N-thiolated lactam **1** in the presence of thiols. Plasmid pBR322 (0.5 μg) was incubated with lactam **1** at 37 °C in sodium phosphate buffer (50 mM, pH 7.2) for 24 h and analyzed by agarose gel electrophoresis (ethidium bromide staining). Restriction digests conducted with *Eco*R1 were performed at 37 °C for 1 h. Lane 1: pBR322 + 100 μM **1**, lane 2: pBR322 + 100 μM glutathione, lane 3: pBR322 + 100 μM glutathione + 100 μM **1**, lane 4: pBR322 + 100 μM DTT, lane 5: pBR322 + 100 μM DTT + 100 μM **1**, lane 6: pBR322 + 100 μM 2-mercaptoethanol, lane 7: pBR322 + 100 μM 2-mercaptoethanol + 100 μM **1**, lane 8: linearized pBR322 (*Eco*R1 digest), lane 9: pBR322 + *Eco*R1 + 100 μM **1**, lane 10: pBR322 + DMSO, lane 11: pBR322 + *Eco*R1 + DMSO.

(5 μM) to treatment with a large amount of lactam **1** (100 μM) in the presence of various thiols (100 μM) (Fig. 7). In lane 1, the plasmid was treated with the lactam for 24 h.

As expected, the supercoil remained completely intact with only the antibiotic present. DNA samples loaded in lanes 2–7 were incubated with glutathione (lanes 2 and 3), dithiothreitol (DTT; lanes 4 and 5), or β-mercaptoethanol (lanes 6 and 7). Samples which also contained the lactam are located in lanes 3, 5, and 7. To verify the cleavability of the plasmid, pBR322 was also linearized with the endonuclease, *Eco*R1. Samples containing the digested DNA are located in lanes 8 and 11. A loading comprised of lactam **1** and *Eco*R1 was applied to lane 9 to establish if the compound could function as an endonuclease inhibitor. After 24 h, the superhelix of the DNA appeared completely unaltered by the lactam either in the presence, or absence, of glutathione, DTT, or β-mercaptoethanol. The bands of lanes 1–7 were comparable to that of the antibiotic-free plasmid loading (lane 10), indicating no DNA cleavage by the N-thiolated lactam.

Disruption of the superhelix by chemical modifications of nucleotide bases can cause the arrest of DNA replication. To further substantiate that N-thiolated β-lactams are not alkylating native nucleic acids or affecting nucleotide assimilation into double-stranded DNA, pulse-labeling experiments with ^{3}H-thymidine were next performed to monitor DNA replication in living bacteria, in the presence versus in the absence of lactam **1** and controls.[14] The results of the radio-uptake experiments are shown in Figure 8. Thymidine incorporation was measured as a percent of the DMSO control. As expected, ciprofloxacin was potent, inhibiting 65% of thymidine utilization after 30 min. Conversely, with

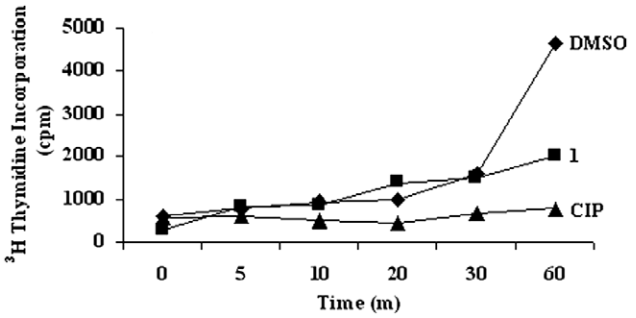


Figure 8. The rate of radiolabeled thymidine incorporation into growing *Staphylococcus aureus* cells (relative to an untreated control) for ciprofloxacin (CIP) and lactam **1**.

lactam **1** present, more than 90% of the nucleotide was converted into DNA. Lactam **1** appeared not to affect thymidine incorporation for at least the first 30 min. After that, however, the rate of thymidine incorporation leveled off.

Gene expression is a biological process targeted by many families of antibiotics including tetracyclines, macrolides, aminoglycosides, streptogramins, and oxazolidinones. In most cases, drugs that affect transcription or translation are bacteriostatic, suggesting that one of these processes may be inhibited by N-thiolated β-lactams. To test this hypothesis, incorporation of ^{3}H-uridine and ^{3}H-isoleucine into RNA and proteins, respectively, was measured in a broth culture of *S. aureus* treated with lactam **1**. RNA-labeling studies[14] with ^{3}H-uridine were conducted to assess the influence of lactam **1** on transcription in *S. aureus*. Cultures were grown to early logarithmic phase and inoculated with ^{3}H-uridine and lactam **1**, rifampicin (an inhibitor of RNA synthesis), or DMSO (Fig. 9). As expected, rifampicin immediately blocked the transcription of RNA, with nucleotide uptake being less than 20% of that for the DMSO control after 20 min. Lactam **1** was also found to modulate uridine incorporation, though certainly not to the extent or rapidity of rifampicin. After 20 min, the nucleotide uptake in the presence of **1** remained nearly 80% of the untreated (DMSO) control, suggesting that transcription cannot be the primary process targeted by the lactam.

To determine if the primary role of growth inhibition by N-thiolated β-lactams could be as a translation inhibitor, the rate of protein synthesis was monitored by the uptake of ^{3}H-isoleucine.[14] An overnight culture of *S. aureus* was grown to early logarithmic phase and inoculated with ^{3}H-isoleucine (5 μCi/ml). The cultures were then treated with either lactam **1** or chloramphenicol (a translation inhibitor), or DMSO as a control (Fig. 10).

For the initial 10 min, a linear progression of isoleucine incorporation was observed for the culture containing lactam **1**, but beyond that, effects on protein synthesis became more pronounced.[22] While these studies indicate that the lactams have a delayed effect on protein synthesis in bacteria, we decided to then investigate the effect of the lactams on fatty acid biosynthesis in *S. aureus*.[22–25] In this case, these experiments demonstrated that treatment of *S. aureus* cells with lactam **1** either at the MIC (10 μg/mL) or twice the MIC (20 μg/mL) had a striking effect on the rate of uptake of radiolabeled acetate compared to the controls, DMSO and penicillin G (Fig. 11). This indicates that a primary mode of action for the compounds in *S. aureus* is inhibition of fatty acid biosynthesis.

2.4. N-thiolated β-lactams as thiolating agents

N-thiolated β-lactams have similar sulfenylating properties to the sulfur transfer reagents used for their preparation (Fig. 12). Previous chemical studies of *N*-methylthio lactams revealed that the N–S bond is resistant to cleavage by most types of nucleophiles, but is susceptible to thiophiles.[27,28]

Further analysis of the cultures of *S. aureus* treated with sub-MIC amounts of *N*-alkylthio β-lactam **1** under steady-state growth conditions showed the formation of the dethiolated β-lactam in the lysate, which could easily be isolated from the growth media by extraction with ethyl acetate. The ^{1}H NMR spectrum of the crude isolate revealed the clean formation of the *N*-protio β-lactam as represented by the broad singlet positioned at 6.37 ppm (Fig. 13).[14] The loss of the *N*-methylthio substituent was also confirmed by the absence of the

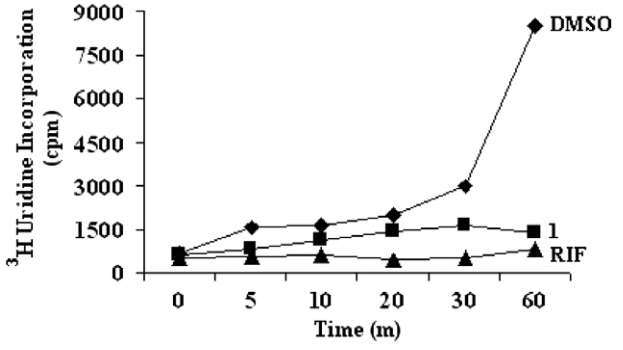


Figure 9. The rate of radiolabeled uridine incorporation into growing *Staphylococcus aureus* cells (relative to an untreated control) in the presence of rifampicin (RIF) and lactam **1**.

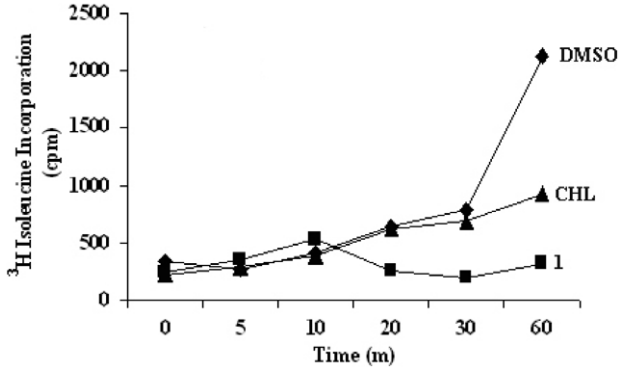


Figure 10. The rate of radiolabeled isoleucine incorporation into growing *Staphylococcus aureus* cells (relative to an untreated control) in the presence of chloramphenicol (CHL), lactam **1**, and DMSO.

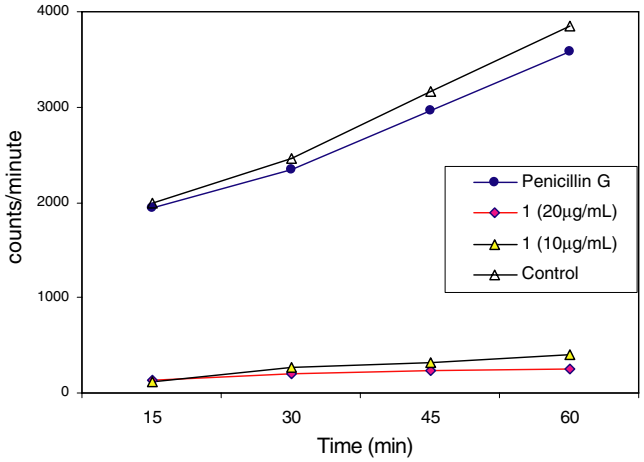
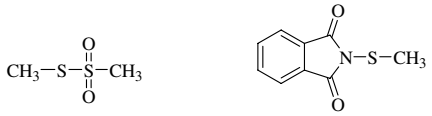


Figure 11. Effect of *N*-alkylthio β-lactam **1** on fatty acid synthesis. Measurement of uptake of radiolabeled acetate. Incidence of radiation versus time for *Staphylococcus aureus* treated with: penicillin G (2 μg/mL, 2× MIC), compound **1** (20 μg/mL, 2× MIC), compound **1** (10 μg/mL, 1× MIC), DMSO control in the presence of ^{3}H acetate.



methylthio methanesulfonate **N-methylthio phthalimide**

Figure 12. Electrophilic sulfur-transfer reagents.

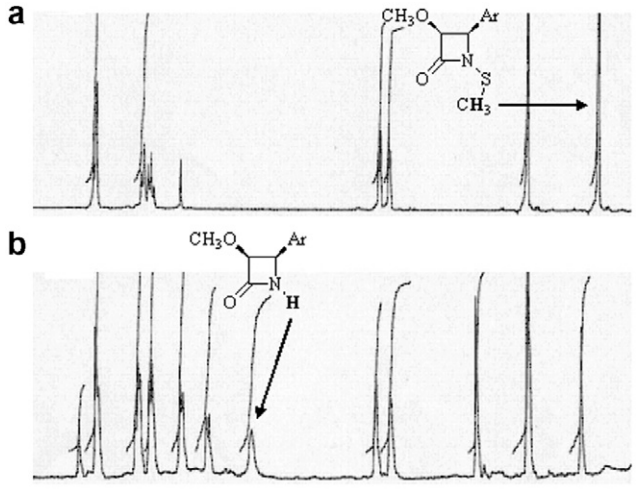
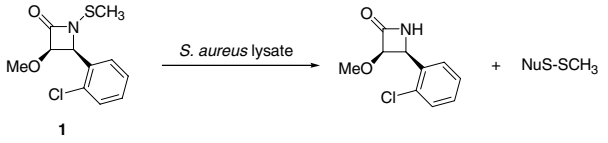


Figure 13. ^{1}H NMR spectra of (a) lactam **1** prior to inoculation of *Staphylococcus aureus* and (b) lactam **1** following incubation with *Staphylococcus aureus* for 24 h. The N–H signal noted in (b) is indicative of a thio-transfer reaction.

methylthio protons' signal at 2.41 ppm. The evidence from this and prior experiments suggests that the methylthio moiety is cleaved off by a protein or metabolite following entry of the lactam into the cytoplasm. This provides clear evidence that a primary metabolic pathway for the N-thiolated lactams proceeds through a thiol-transfer process (Scheme 3). In light of these findings, we turned to consider the likelihood that an intracellular



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Scheme 3. Thio-transfer from the lactam **1** to a nucleophilic reactant present in the lysate of cultured *Staphylococcus aureus.*

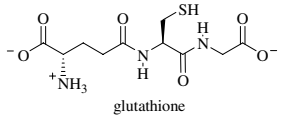
thiol was the recipient of this transfer to produce a mixed disulfide within the cell.[9]

We initially considered the likelihood that N-thiolated β-lactams may inhibit bacterial growth by transfer of the alkylthio moiety onto a sulfhydryl-containing target such as a cysteine residue in a protein. A survey conducted in our laboratory using the 20 essential L-amino acids showed that cysteine was the only one that reacted directly with lactam **1** under physiological conditions (pH 7.4), affording the N-dethiolated β-lactam. Similar results were obtained with glutathione, a tripeptide that is found in the cytoplasm of most bacteria and in human cells (Fig. 14).

To evaluate whether glutathione could affect the anti-staphylococcal properties of N-thiolated β-lactams, a Kirby–Bauer diffusion experiment was run.[22] Equimolar amounts of glutathione and lactam **1** were added to the same well while only the lactam was added to another well. Following overnight incubation, the plate inoculated with *S. aureus* was examined for growth inhibition. As expected, a large zone of inhibition was found around the well containing the lactam **1**, but no zone was found around the well containing lactam **1** that had been comixed with glutathione. It was subsequently observed by varying the ratio of lactam-to-glutathione that the antimicrobial activity of *N*-methylthio lactam **1** against *S. aureus* can be partially or completely reduced, depending on the quantity of glutathione present in the media.

To further substantiate the neutralizing effect of glutathione on the lactams, an agar plate was prepared with 1 mg of glutathione (in pH 7.4 buffer) added into a well located at the center of the plate, and 20 μg of the *N*-methylthio β-lactams **1**–**3** were each loaded into three surrounding wells (Fig. 15). The ability of glutathione to protect the bacteria from the anti-proliferation effects of these antibiotics was again clearly evident by the presence of concaved zones between the wells containing glutathione (in the center) and the three lactams (on the outside).

We then followed this experiment up with an additional assay to evaluate the stability of other *N*-alkylthio



glutathione

Figure 14. Structure of glutathione.

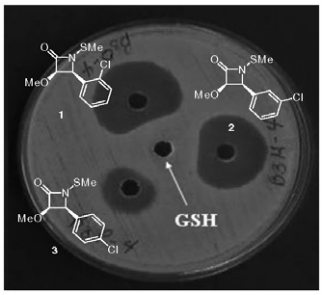
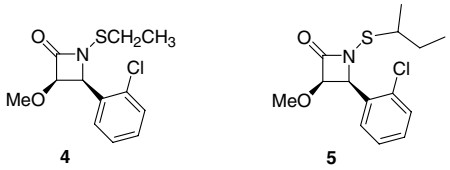


Figure 15. Effect of glutathione (GSH) on the antimicrobial properties of N-thiolated β-lactams **1**–**3** against *Staphylococcus aureus*. Each 6-mm well contains 20 μg of the indicated β-lactam, and the center well contains 1 mg of glutathione. The neutralizing effect of glutathione on the lactams is apparent from the indented regions of growth inhibition after 24 h of incubation.



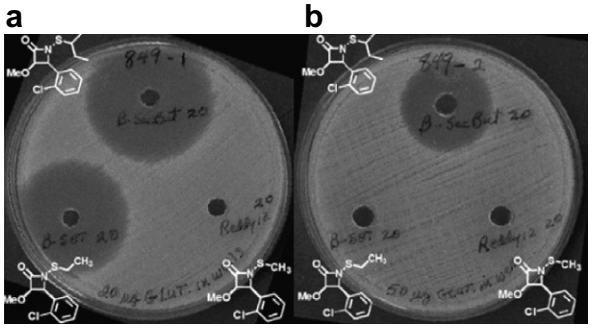


Figure 16. Comparison of bioactivities of *N*-methylthio lactam **1**, *N*-ethylthio lactam **4**, and *N*-*sec*-butylthio lactam **5** against *Staphylococcus aureus* in the presence of glutathione. In (a), each well contains 20 μg of the lactam and 20 μg of glutathione. In (b) 50 μg of glutathione is added to each well containing 20 μg of the lactam. Growth inhibition was checked after 24 h of incubation.

lactam derivatives to glutathione. Figure 16 compares the relative antibacterial activities of three *N*-alkylthio-substituted lactams, *N*-methylthio lactam **1**, *N*-ethylthio lactam **4**, and *N*-*sec*-butylthio lactam **5**, against *S. aureus* in the presence of different amounts of glutathione. For the plate shown in Figure 16a, 20 μg glutathione was added to the wells containing 20 μg of the three lactams, and for the plate shown in Figure 16b, 50 μg of glutathione was added to the three wells. As observed in Figure 16a, *N*-ethylthio lactam **4** and *N*-*sec*-butylthio lactam **5** both possess full activity against *S. aureus* in the presence of 20 μg of glutathione, while *N*-methylthio lactam **1** is completely inactivated. At the higher amount of glutathione (50 μg) shown in Figure 16b, the *N*-ethylthio lactam also loses all of its bioactivity, while the *N*-*sec*-butylthio compound is still strongly active. Thus, these studies show that the differences in biological activity of the different *N*-alkylthio lactam derivatives seem to be due to the different reactivities of those lactams toward thiophilic nucleophiles.

From these experiments, we surmised that the protective role of glutathione likely explains the selectivity that is observed among the different bacterial genera, such that those microbes having the highest levels of glutathione in their cytoplasm are more resistant to the antibacterial effects of these lactams. Closer inspection of the list of bacteria that we tested indicates that the microbes having the greatest susceptibilities to the lactams also are those that reportedly contain low cytoplasmic levels of glutathione.[29] In addition, *E. coli* and *Pseudomonas aeruginosa*, which show no susceptibility to these drugs, have high levels of glutathione. The correlations between reported thiol levels and the observed bioactivities of the *N*-alkylthio β-lactams are shown in Table 1.

Once the role of glutathione in protecting bacteria from the inhibitory effects of the N-thiolated lactams was established, it was important to identify the cellular target in S. *aureus*. In order to trap the active species, resin-bound β-lactam **6** was prepared. The resin was stirred for 24 h with homogenized S. *aureus* lysate, then washed with hot water and ethanol to remove non-bound components. The resin was then reacted with diisobutylaluminum hydride (DIBAL) in anhydrous methylene chloride to cleave off any covalently bound constituents (Scheme 4). Upon purification and analysis by HPLC and NMR, the product was identified as coenzyme A (CoA).

These results are consistent with a previous report by Fahey (Table 1) that CoA is the predominant small thiol (and thus the major thiophilic nucleophile) in *S. aureus*,[29] and our own observation that bacteria with the highest reported levels of CoA (*Staphylococcus*, *Bacillus*, and *Micrococcus*) are most susceptible to the *N*-alkylthio β-lactams.

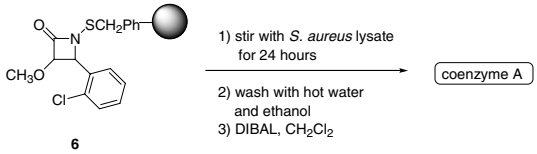
Table 1. Correlation between reported[30] percentages of intracellular glutathione (GSH) and CoA and the observed susceptibility of selected microbes to *N*-alkylthio β-lactams (by agar diffusion)

Species	% GSH[a]	% CoA	Susceptibility
Staphylococcus aureus	<1	67	High[b]
Bacillus subtilis	<2	21	High
Micrococcus luteus	<0.2	17	High
Mycobacterium tuberculosis	<0.2	14	Medium[c]
Pseudomonas aeruginosa	70	15	None
Escherichia coli	80	11	None

[a] Percentages of GSH and CoA (relative to total thiol content) derived from data.[30]

[b] High: average zones of inhibition > 20 mm.

[c] *M. tuberculosis* showed no activity in the Kirby–Bauer assays, but showed activity in MIC assays.

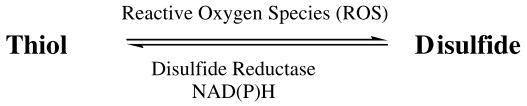


Scheme 4. Identification of primary cellular target in *Staphylococcus aureus* using resin-bound lactam **6**.

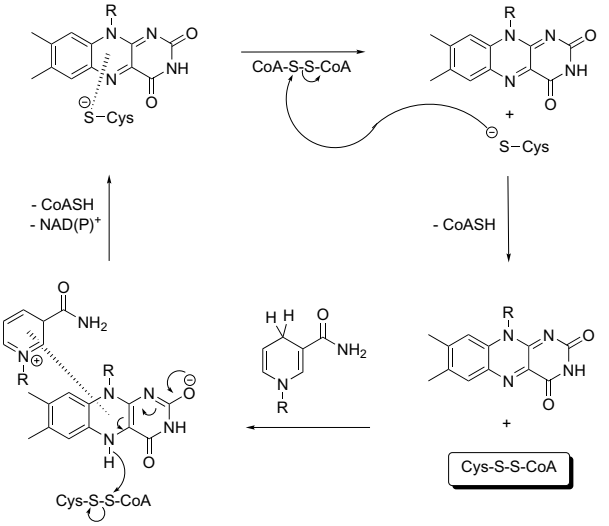
2.5. The role of coenzyme A disulfide reductase in bacterial cells

The unusually high concentrations of CoA in some bacteria, particularly *Staphylococcus* and *Bacillus*, stem from the role of CoA within the thiol-redox buffer (Scheme 5). Thiol-redox buffer systems are present in all cells, and consist of free cytosolic thiols which are present in high (often millimolar) concentrations. An NAD(P)H-dependent disulfide reductase[30–32] regulates the balance of this equilibrium in bacterial cells by converting the disulfide to the reductively active thiol form. Eukaryotes and many prokaryotes utilize glutathione in their thiol-redox buffers. Indeed, glutathione was long thought to be ubiquitous.[29] However, recent findings have shown that some prokaryotes, including those in the *Staphylococcus* and *Bacillus* genera, do not produce glutathione, and instead rely upon CoA or mycothiol in their thiol-redox buffer. This buffer consists of millimolar levels of free CoA and a CoA disulfide reductase, an NADPH-dependent enzyme in the flavoprotein disulfide reductase family. Originally isolated from *S. aureus* by delCardayre and coworkers in the late 1990s, CoA disulfide reductase has been thoroughly characterized.[30,31] A member of the flavoprotein disulfide reductase family, CoA disulfide reductase, contains a single cysteine residue and an integral but non-covalently bound flavin molecule.[32] Reduction of CoA disulfide occurs through two steps: thiol-disulfide exchange with the active site cysteine, followed by flavin-mediated hydride transfer from NADPH to reduce the cysteine-CoA disulfide bond and regenerate the active site (Scheme 6).

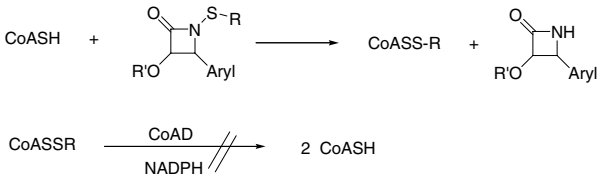
The correlation between the antibacterial activity of the *N*-alkylthio β-lactams and the CoA-based thiol-redox buffer system led us to consider whether CoA disulfide reductase might be a key target in the mode of action of these compounds. Despite a coarse resemblance, glutathione disulfide reductase and coenzyme A disulfide reductase are substantially different in mechanism and substrate selectivity, and so it was postulated that the activity of the *N*-alkylthio β-lactams against CoA-rich bacteria might be a function of these differences. In particular, we hypothesized that the mixed CoA-alkyl disulfide produced by the alkylthio transfer from the β-lactam might inhibit the CoA-dependent pathway in the manner shown in Scheme 7. Although this might take place through a non-covalent inhibition, the asymmetric CoA disulfide might also transfer the alkylthio portion to the active site cysteine for covalent modification.



Scheme 5. A generalized thiol-redox buffer. The thiol-disulfide equilibrium is responsible for absorbing reactive oxygen species (ROS) generated in the cell by oxidative stress, and is closely maintained by an enzyme, disulfide reductase.



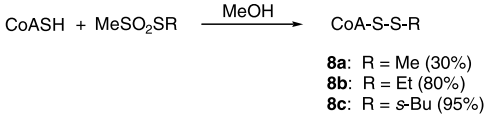
Scheme 6. Mode of action of CoA disulfide reductase.



Scheme 7. A possible result of transferring the alkylthio residue from the lactam to CoA, leading to the disruption of the CoA disulfide reductase.

To test these hypotheses, asymmetric CoA disulfides **8a–c** were prepared[33] from free CoA (trilithium salt) and the corresponding alkyl methanethiolsulfonates (Scheme 8).

Rates of reduction of the CoA mixed disulfides **8a–c** versus the native CoA disulfide by CoA disulfide reductase were determined spectrophotometrically and the values of V_{max}, K_m, and k_{cat} were found through best fit analysis to the Michaelis–Menten equation (Table 2).[34,35] In each case, the rates of catalysis of the mixed disulfides were found to be within an order of magnitude of those of the native substrate (CoA disulfide). The most drastic difference in rate of reduction was observed for the *sec*-butyl CoA mixed disulfide (**8c**), whose V_{max} and k_{cat} were approximately 30% of CoA disulfide. This steady drop in both the V_{max} and K_m upon incrementally increasing the bulk of the disulfide alkyl residue from methyl to ethyl to *sec*-butyl reflects diminishing reducibility and increasing stability of the disulfides by the reductase. Despite the retardation of reductase activity,



Scheme 8. Formation of mixed CoA disulfides **8a–c**.

Table 2. Kinetic data for the reduction of CoA disulfides by CoA disulfide reductase[36]

Compound	V_{max} (μM/min)	K_m (μM)	k_{cat} (min^{-1})
CoA-disulfide	1.9	6	190
CoA-methyl disulfide (**8a**)	2.6	8	260
CoA-ethyl disulfide (**8b**)	1.6	5	160
CoA-*sec*-butyl disulfide (**8c**)	0.57	4	57

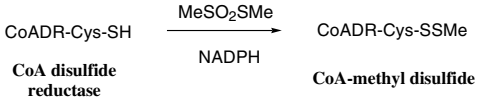
Kinetic data were determined by spectrophotometric measurement (340 nm, 25 °C) of the rate of NADPH oxidation in the presence of CoA disulfide reductase (10 nM). Concentrations of disulfides were varied from 2 to 200 μM.

the mixed CoA disulfides do not substantially inhibit CoA disulfide reductase.

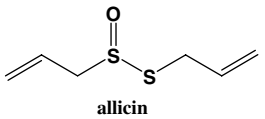
Although from these studies we conclude that CoA disulfide reductase is not the primary enzymatic target of the *N*-alkylthio β-lactams, the capping of the active cysteine in CoA disulfide reductase through formation of an unreactive disulfide remained an intriguing possibility. If mixed CoA disulfides interact with CoA disulfide reductase in such a way that the alkyl thiol is preferentially expelled instead of transferred, then the cysteine-CoA disulfide bond is selectively formed over the cysteine–alkyl disulfide bond. This postulate is supported by the report of delCardayre et al., who noted that in the reaction of CoA-glutathione mixed disulfide with CoA disulfide reductase, the CoA selectively formed the disulfide bond with the enzyme, while free glutathione was released.[31] To explore this latter possibility, the rate of catalysis of CoA disulfide reductase in the presence of methylthio methanesulfonate was measured (Scheme 9). This thio-transfer compound was indeed found to be a viable substrate for CoA disulfide reductase, having a V_{max} of 2.3 μM/min and a K_m of 172 μM. The fact that the V_{max} is nearly identical to that of the other CoA disulfides (Table 2) indicates that there is no difference in the rate of cleavage between a cysteine-CoA disulfide and a cysteine–methyl disulfide.

Since the thiol-redox buffer is not itself affected by the *N*-alkylthio β-lactams, we are now looking into the possibility that the mixed CoA-alkyl disulfides might directly inhibit one or more CoA-dependent enzymes, such as those involved in fatty acid biosynthesis. This coincided with our findings that lactam **1** inhibits the uptake of acetate, and thus fatty acid biosynthesis, in *S. aureus*.

The demonstrated ability of CoA-mixed disulfides to inhibit bacterial fatty acid synthesis represents a significant advance in the quest for novel antibacterials. However, due to the inability of CoA mixed disulfides to traverse the cell membrane, these CoA disulfides are not directly useful as therapeutics. This research shows that *N*-alkylthio β-lactams can serve as a prodrug to



Scheme 9. Disulfide capping of the active site cysteine in CoA disulfide reductase.



allicin

Figure 17. Structure of allicin, the active constituent from crushed garlic.

produce the CoA mixed disulfides within the bacterial target, and that it is through the formation of these mixed disulfides that inhibition of bacterial fatty acid synthesis can be effected.

Finally, it might be informative to compare the properties of these N-thiolated lactams to those of allicin, a natural product found in garlic (Fig. 17).[35–38] In addition to exhibiting activity against a wide range of bacteria,[39–42] allicin also shows antifungal,[44,45] antiviral,[47–49] antiparaisitic,[49,50] and anticancer properties. Like the N-thiolated lactams, allicin shows the strongest activity against those bacteria that express low levels of glutathione, and blocks fatty acid biosynthesis.[49–52] Additionally, both compounds partially inhibit protein and nucleic acid synthesis.[55] Allicin is reported to be a specific inhibitor of acetyl-CoA synthetases through reversible and non-covalent binding.[56] On the other hand, allicin reacts directly with sulfhydryl residues of various proteins such as alcohol dehydrogenases, thioredoxin reductase, and RNA polymerase, and it is believed that through this covalent modification it may also inhibit RNA polymerase.[57] The similarities in the reactivities and biological properties of allicin (and related compounds such as ajoene,[58,59] thiolactomycin, and cerulenin) with those of N-thiolated lactams do strongly suggest common biochemical targets and mechanisms of action.

3. Conclusions

Over the past several years, substantial progress has been made toward understanding the structural and mechanistic basis of the observed antibacterial properties of *N*-alkylthio β-lactams. As described, the metabolism of these compounds proceeds through a thiol-transfer pathway to produce alkyl-CoA mixed disulfides within the cell. Further, it was demonstrated that the bacteria most susceptible (*Staphylococcus* and *Bacillus*) are those which utilize CoA in their thiol-redox buffers. Although the high levels of coenzyme A required for the activity of these compounds arise from the role of CoA within the thiol-redox buffer, kinetic studies on the CoA disulfide reductase enzyme provided strong evidence that the CoA thiol-redox buffer itself is not appreciably affected by the *N*-alkylthio β-lactams. The finding that bacterial fatty acid synthesis is strongly inhibited by the *N*-alkylthio β-lactams has led to the hypothesis that CoA-dependent enzymes within this pathway may be inhibited by alkyl-CoA mixed disulfides. In vitro experiments in MRSA show that alkyl-CoA mixed disulfides, the ones thought to form from the interaction of CoA and the lactams inside the cell, are in fact biologically inactive against these microbes in culture. This is presumably due to the inability of the disulfides to cross

the cell membrane into the cytoplasm. This finding highlights the utility of the *N*-alkylthio β-lactams as prodrugs to produce alkyl CoA mixed disulfides within the bacterial cells. Furthermore, it was demonstrated that covalent attachment of an alkylthio group to the active site cysteine of CoA disulfide reductase is reversible, due to an NADPH-mediated reduction of the disulfide bond to regenerate the active sulfhydryl form of the enzyme.

While there is strong likelihood that these thiolating compounds produce multiple inhibitory effects on thiol-dependent enzymatic pathways, the finding that coenzyme A is a primary target of the N-thiolated lactams brings further attention to CoA and, specifically, CoA-dependent processes, as a viable target for the development of antibacterial drugs. Within this context, these studies may prove informative, particularly in light of the increasing incidences of bacterial drug resistance and the growing need for new antibiotics with alternative mechanisms of action. Although this current study has ruled out CoA disulfide reductase as the primary enzyme affected, other CoA-dependent enzymes which utilize a thiol in its active site may be involved. Our laboratory is now examining this in more detail.

4. Experimental

4.1. General procedures

Commercially available reagents and solvents were used without further purification. Lactams **1**–**5** were prepared and purified as previously reported.[1,6]

4.2. Antimicrobial susceptibility testing of lactams 1–5

4.2.1. Culture preparation. From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20% glycerol, a culture of each microorganism was grown on tryptic soy agar (TSA) plates (Becton–Dickinson Laboratories, Cockeysville, MD) at 37 °C for 24 h. A 10^8 suspension was then made in sterile phosphate-buffered saline (pH 7.4) and swabbed across fresh TSA plates.

4.2.2. Kirby–Bauer agar diffusion assays. A 10^8-standardized cell count suspension was made in sterile phosphate-buffered saline (pH 7.4) and swabbed across fresh TSA plates. Circular wells (6 mm in diameter) were cut into the inoculated plates and 20 μL of a 1 mg/mL stock solution of the test lactam in dimethylsulfoxide (DMSO) was pipeted into the wells. The plates were incubated for 24 h at 37 °C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each well.

4.3. Minimum inhibitory concentration (MIC) assays

4.3.1. Media preparation. The minimum inhibitory concentrations were determined by agar dilution. The test media were prepared in 24-well plates (Costar 3524, Cambridge, MA) by adding a known concentration of the test drug in DMSO together with a solution of Mueller–Hinton II agar (Becton–Dickinson Laboratories, Cockeysville, MD) for a total volume of 1 mL in each well. Calculations of the overall concentration of antibiotic in the wells were standardized by measuring from a 1 mg/mL stock solution of the test drug. At this concentration, the microliter volume is equivalent to the micrograms in solution. The amount of agar solution added to the wells was determined by subtracting 1000 μL from the quantity of test drug in each well to give a combined volume of 1 mL. Following preparation of the well plates, the media were allowed to solidify at room temperature for 24 h before inoculation.

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

4.3.3. Growth studies. Overnight cultures of the test strains were grown to logarithmic phase in Mueller–Hinton Broth (MHB). An inoculum of 10^6 cfu/mL was added to freshly prepared MHB and grown for 1 h at 35 °C while shaking. The test compounds diluted to 1, 5, or 10 times the MICs in DMSO were applied to each tube. Viable cell counts were determined by plating adequate dilutions of each culture. The plates were incubated and colony counts were taken after 24 h. Turbidity measurements were determined by transferring 0.2 mL aliquots of culture to a 48-well plate (Costar 3524, Cambridge, MA) and optical density readings taken at 630 nm with a Bio-Tek EL800 plate reader. Metabolism studies. To a fresh 10^6 cfu/ml suspension of *S. aureus* (ATCC 25923) in 9 mL of sterile saline was added 1 mL of a 400 μM solution of lactam 1 in DMSO. After 1 h, 10 mL of deionized H_2O was added and the solution was extracted three times with 5 mL of ethyl acetate. The organic layers were combined, dried with magnesium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in 500 μL $CDCl_3$ and the chemical structure was elucidated by ^1H NMR.

4.4. Studies on the inhibition of CoA disulfide reductase by disulfides and thiosulfonates

A sample of CoA disulfide reductase was provided by Professor Al Claiborne (Wake Forest University). Coenzyme A (hexahydrate, trilithium salt), coenzyme A disulfide, and S-methyl methanethiolsulfonate were obtained from Sigma–Aldrich Co. The remaining thiosulfonate reagents and disulfides were synthesized as described below.

4.5. S-*sec*-Butyl methanethiosulfonate

To a mixture of sodium methanethiolsulfonate (88 mg, 0.86 mmol, 3.2 equiv) and *sec*-butyl disulfide (50 μL, 0.27 mmol, 1 equiv) in dry CH_2Cl_2 (0.5 mL) was added

I_2 (137 mg, 0.54 mmol, 2 equiv). The mixture was allowed to stir at rt under an inert atmosphere. After 10 h, TLC indicated no disulfide remaining. The reaction mixture was diluted to 25 mL with CH_2Cl_2, then 10 mL $Na_2S_2O_3$ (1 M) was added with stirring until the reddish color disappeared (about 10 min). The CH_2Cl_2 layer was separated, washed with water (2×20 mL), dried over $MgSO_4$, and then concentrated in vacuo to give 63 mg (70%) of a pale yellow oil. ^{1}H NMR (250 MHz, $CDCl_3$) δ 3.50–3.42 (m, 1H); 3.27 (s, 3H); 1.75–1.64 (m, 2H); 1.43 (d, $J = 7.0$ Hz, 3H); 0.98 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 51.4, 49.9, 29.9, 21.8, 11.2.

4.6. S-Ethyl methanethiosulfonate

Prepared according to the procedure described above, except the reaction time was 16 h. Obtained 45 mg (60%) as a pale yellow oil. ^{1}H NMR (250 MHz, $CDCl_3$) δ 3.267 (s, 3H); 3.14 (q, $J = 7.6$ Hz, 2H); 1.39 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 50.8, 30.9, 15.0.

4.7. Methyl-CoA disulfide (8a)

To a solution of coenzyme A (5 mg of the trilithium salt, 6.37 μmol) in methanol (300 μL) was added S-methyl methanethiosulfonate (106 μL of a 10 mg/mL methanolic solution, 1.1 mg, 6.4 μmol, 1 equiv). The resulting solution was stirred at rt under N_2 for 30 min. The solvent was evaporated under a stream of nitrogen, and the residual solid was taken up in water (1 mL), analyzed by HPLC, and lyophilized. Compound **8a** (1.6 mg, 30%) was isolated as a flocculent white solid. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 8.36 (s, 1H); 8.16 (d, $J = 5.4$ Hz, 1H); 8.12 (s, 1H); 7.69 (t, $J = 6.0$ Hz, 1H); 7.23 (br s, 2H); 5.87 (d, $J = 6.4$ Hz, 2H); 4.64 (t, $J = 5.2$ Hz, 1H); 4.21 (br s, 1H); 3.94 (br s, 2H); 3.78 (m, 4H); 2.74 (t, $J = 7.0$ Hz, 2H); 2.38 (s, 2H); 2.37 (s, 3H); 2.56–2.23 (m, 2H); 0.92 (s, 3H); 0.63 (s, 3H). MS (MALDI): *m/z* 814.550 (M^+, all sites protonated).

4.8. Ethyl-CoA disulfide (8b)

Prepared as described for **8a**. 4.0 mg (80%) was obtained as a flocculent white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (s, 1H); 8.15 (t, $J = 5.6$ Hz, 1H); 8.11 (s, 1H); 7.69 (t, $J = 5.8$ Hz, 1H); 7.22 (br s, 2H); 5.87 (d, $J = 6.4$ Hz, 1H); 4.77 (br s, 1H); 4.62 (t, $J = 5.6$ Hz, 1H); 4.19 (br s, 1H); 3.93 (br s, 2H); 3.77 (br s, 4H); 2.72–2.64 (m, 4H); 2.35 (s, 2H); 2.23 (t, $J = 7.2$ Hz, 2H); 1.20 (t, $J = 7.2$ Hz, 3H); 0.92 (s, 3H); 0.62 (s, 3H). MS (MALDI): *m/z* 828.619 (M^+, all sites protonated).

4.9. *sec*-Butyl-CoA disulfide (8c)

Prepared as described for **8a**. 4.9 mg (95%) was isolated as a flocculent white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (s, 1H); 8.14 (t, $J = 5.6$ Hz, 1H); 8.12 (s, 1H); 7.68 (t, $J = 5.8$ Hz, 1H); 7.23 (br s, 2H); 5.87 (d, $J = 6.4$ Hz, 1H); 4.79 (br s, 1H); 4.64 (t, $J = 5.4$ Hz, 1H); 4.21 (br s, 1H); 3.95 (br s, 2H); 3.78 (br s, 4H); 2.90–2.74 (m, 1H); 2.69 (t, $J = 7.0$ Hz, 2H); 2.37 (s, 2H); 2.29–2.23 (m, 2H); 1.60–1.53 (m, 1H); 1.48–1.40 (m, 1H); 1.19 (d, $J = 6.8$ Hz, 3H); 0.92 (s, 3H); 0.88 (t, $J = 7.4$ Hz, 3H); 0.63 (s, 3H). MS (MALDI): *m/z* 856.592 (M^+, all sites protonated).

4.10. CoA disulfide reductase inhibition assays

Spectrophotometric assays were done using a Perkins-Elmer spectrophotometer equipped with a 6-cuvette changer. Measurements were made at 340 nm and 25 °C. Absorbances were measured every 5–10 s for 3 min versus a blank consisting of the buffer solution. Using Tris–HCl (50 mM, pH 7.80), stock solutions of CoA disulfide reductase (500 nM), NADPH (100 μM), CoA disulfide (1.0 mM), and the mixed disulfides (1.0 mM) were prepared. CoA disulfide reductase was kept cold during the experiments; all other solutions were allowed to warm to rt.

4.10.1. Determination of CoA disulfide reductase inhibition for mixed disulfides 8a–c and CoA disulfide. To each 1-mL cuvette were added NADPH, CoA disulfide reductase, and pH 7.4 phosphate buffer solution. Addition of the disulfide initiated the kinetic run. Amounts were added volumetrically from stock solutions (described above) such that the following final concentrations were reached: buffer, 50 mM; CoA disulfide reductase, 10 nM; NADPH, 50 μM. Disulfide concentrations were varied as follows:

CoA disulfide = 20, 40, 80, 100 μM (Trial 1); 5, 10, 20, 40, 60, 80, 100 μM (Trial 2);

[**8a**] = 5, 10, 20, 40, 60, 100, 150 μM;

[**8b**] = 2, 10, 20, 40, 60, 100, 150, 200 μM;

[**8c**] = 20, 40, 60, 100, 150 μM.

Kinetic parameters were determined by finding the best hyperbolic fit to the Michaelis–Menten equation using Kaleidagraph® software, version 3.5.1.[34]

4.10.2. Measurement of inhibition effects for β-Lactams 1 and 2. To each 1-mL cuvette were added NADPH, CoAD, and lactams **1** or **2** to produce the following final concentrations: buffer, 50 mM; NADPH, 50 μM; CoA disulfide, 20 μM; CoA disulfide reductase, 20 nM. Concentrations of *N*-alkylthio β-lactam were varied as follows:

[**1**] = 0, 5, 10, 15, 20, 25, 50, 70 μM;

[**2**] = 0, 10, 15, 20, 25, 50, 70 μM.

Addition of the enzyme was done last, to mark the start of the run.

4.10.3. Determination of inhibition of CoA disulfide reductase by methylthio methanesulfonate. To each 1-mL cuvette were added NADPH, CoA disulfide reductase, and pH 7.4 phosphate buffer solution. Addition

of the methylthio methanesulfonate initiated the kinetic run. Amounts were added volumetrically from stock solutions (described above) such that the following final concentrations were used: buffer: 50 μM; CoA disulfide reductase: 10 nM; NADPH: 50 μM; Methylthio methanesulfonate concentrations were varied as follows: 20, 40, 60, 200, 400, 600, 1000, 2000, 3000 μM.

4.11. Scanning electron microscopy (SEM) experiments

Samples were prepared from sections of agar taken from the disk-diffusion experiment. Sterile 6-mm diameter paper disks (Becton–Dickinson Laboratories, Cockeysville, MD) were impregnated with 20 μg of penicillin G potassium salt (Sigma Chemical Co., St. Louis, MO) and lactam **1** from their 1 mg/mL stock solutions in DMSO. From a 24-h culture of *S. aureus* (ATCC 25923) grown on TSA plates (Becton–Dickinson Laboratories, Cockeysville, MD), a 10^8 suspension was made in sterile phosphate-buffered saline (pH 7.4) and swabbed across two separate plates. The disks were placed on the inoculated plates and incubated for 24 h at 37 °C. After 24 h, sections of agar in areas containing the division between the zones of inhibition and lawn of bacteria were cut out and placed into Petri dishes for the SEM preparation. The agar sections were flooded with 10 mL of a pre-made glutaraldehyde-osmium fixative. After 1 h, the sections were removed and washed three times with 0.1 M sodium cacodylate. The samples were then sequentially submerged in 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol for periods of 5 min. Following alcohol treatment, the sections were placed into hexamethyldisilazane (HMDS) for chemical drying. The samples were next mounted onto stubs and coated with gold/palladium by a Pelco Model 3 Sputter Coater. The morphology of the cells was examined with a Novascan 30 scanning electron microscope.

4.12. Light microscopy experiments

Sterile 6-mm paper disks (Becton–Dickinson Laboratories, Cockeysville, MD) containing 20 μg of lactam 1 and penicillin G potassium salt (Sigma Chemical Co., St. Louis, MO) were placed on separate TSA plates inoculated by *S. aureus* (ATCC 25923) from an overnight culture. The plates were incubated at 37 °C for 4–5 h or until the zones of inhibition were visible. Glass 24 × 60-mm coverslips (Corning Glass works, Corning, NY) were then gently pressed across the zones to adhere the bacteria. Following brief heat fixing over a Bunsen burner flame, the coverslips were flooded by Gram crystal-violet stain (Becton–Dickinson Laboratories, Cockeysville, MD) for one minute. The coverslips were rinsed with water and flooded by Gram iodine (Difco Laboratories, Detroit, MI) for one minute then decolorized by adding 95% ethanol dropwise until the crystal-violet no longer flowed off the coverslips. The coverslips were rinsed again with water and counterstained with Gram safranin (Difco Laboratories, Detroit, MI) for one minute. They were then thoroughly rinsed with water, blotted dry, and mounted on glass microscope slides (Fisher Scientific, Pittsburgh, PA). The slides were viewed with a Nikon bright-field light microscope.

4.13. DNA cleavage experiments

To 17 μL of sodium phosphate buffer (50 mM, pH 7.4) was added 0.5 μg of pBR322 (ICN Biomedicals Inc., Aurora, OH) in 200 μL microfuge tubes. Two microliter of lactam **1** at 5, 10, 25, 50, and 100 μM concentrations in DMSO was added and the samples were vortexed then incubated at 37 °C. After 24 h, 2 μL of Blue/Orange 6× Loading Dye (Promega Corp., Madison, WI) was added to 8 μL aliquots of the DNA mixtures. The samples were loaded on a 1.2% agarose gel (1.8 g medium EEO agarose; 150 mL TAE; 1 μg/mL ethidium bromide) and horizontal electrophoresis was performed at 80 V/10 cm for 2.5 h in TAE (40 mM Tris–acetate, pH 7.8, 1 mM EDTA). The agarose gel was visualized and photographed under UV transillumination. The same procedure was used in experiments conducted with glutathione, dithiothreitol, and 2-mercaptoethanol. For the enzyme digest samples, 20 μL of sterile deionized H_2O, 2 μL of 100 μM **1**, 2 μL of restriction enzyme buffer H (90 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5), 1 μg of pBR322, and 2 μL of *Eco*R1 were added sequentially to a 1.5 mL microfuge tube and incubated in a 37 °C water bath for 1 h.

4.14. Radioisotope-uptake experiments

The effects of lactam **1** on DNA, RNA, and protein biosynthesis in *S. aureus* ATCC 25923 were determined by measuring the respective incorporations of [methyl-^{3}H]thymidine, [5-^{3}H]uridine, or L-[4,5-^{3}H]isoleucine (Amersham Life Sciences). Ciprofloxacin, rifampicin, and chloramphenicol were used as controls for the inhibition of DNA, RNA, and protein synthesis, respectively. Radioactive precursors (3 μCi of [methyl-^{3}H]thymidine, 3 μCi of [5-^{3}H]uridine, or 5 μCi of L-[4,5-^{3}H]isoleucine) in Luria Broth in the presence or absence of an antibiotic at twice the MIC value. To assess the effect on DNA, RNA, and protein synthesis, 50 μL samples were removed from each reaction tube at designated time intervals (5, 10, 20, 30, 45, and 60 min) and precipitated in 1 mL of ice-cold 10% trichloroacetic acid. After 1 h, the samples were filtered through glass fiber filters (GF/A; Whatman), washed with 2 mL of ice-cold 5% trichloroacetic acid and 2 mL of ice-cold 95% ethanol, and dried at room temperature overnight. The dried filters were placed in 10 mL vials containing 7 mL of counting fluid (Cytoscint, ICN International). Radioactivity was measured by liquid scintillation (Beckman Instruments).

4.15. ^{3}H-Acetate-uptake studies

A culture of *S. aureus* (strain RN4220 in 1% Luria Broth) was used as 1% inoculum in freshly prepared broth (20 mL) and cells were grown at 37 °C until the A_{600} reached 0.11 (1.3×10^7 cfu/mL). The culture was then divided into four test tubes (5 mL each) and kept in the water bath at 37 °C to let the bacteria grow for 5 min, and then sufficient drug was added such that the final concentration of DMSO in each sample was 2% by volume, and each sample contained one of the following: (a) penicillin G (2 μg/mL, 2 × MIC); (b)

lactam **1** (10 μg/mL, 1 × MIC); (c) lactam **1** (20 μg/mL, 2 × MIC); (d) no drug added. Each tube was incubated for 5 min. at 37 °C, then 2 μL of ^{3}H-acetate (10 μCi/uL in ethanol solution, sodium salt) was added to each tube (4 μCi/mL radioactivity per tube). At intervals of 15, 30, 45, and 60 min, 0.8 mL aliquots were taken from each tube, homogenized, and diluted with chloroform (1 mL) and methanol (2 mL). An additional 1 mL of chloroform and 1 mL of distilled water were added, and the solution was mixed. The organic solution was then washed with distilled water (1 mL), then 2 M KCl (3× 1 mL), and then 0.1 M sodium acetate (3× 1 mL). The radioactivity in the organic phase was then analyzed by scintillation counting.

4.16. Preparation of resin-bound β-lactam 6

A mixture of Merrifield's resin, thioacetic acid and triethylamine was stirred at room temperature, after which the resin was filtered. The resin was then reacted with sulfuryl chloride in carbon tetrachloride to produce the resin-bound sulfenyl chloride in situ. To this mixture was added a solution of phthalimide and Hunig's base in benzene. The mixture was allowed to stir at rt, then filtered and washed to give resin-bound phthalimide, The resin was then combined with N–H β-lactam precursor, and heated to reflux in benzene for 24 h, under nitrogen. The mixture was cooled, and the resin filtered and washed. The presence of the resin-bound lactam was verified by reaction of a small quantity of the resin with DIBAL, and subsequent analysis by ^{1}H NMR and HPLC.

4.17. Procedure for the isolation of CoA from *S. aureus* lysate using Lactam-bound resin 6

A quantity of 1 liter of *S. aureus* (ATCC 25923) in broth was cultured at 37 °C for 24 h. The culture was then centrifuged and washed. The resultant pellet was washed with phosphate buffer and then resuspended in 5 mL of phosphate buffer. The cells were then sonicated in an ice bath for 30 min, stopping every 5 min to avoid overheating. The lysed cells were then centrifuged and the lysate was centrifuged again, and the resulting lysate was separated off from the solids. Filtering of the lysate yielded a slightly opaque yellow solution. 0.252 g of resin **6** was swelled in 0.5 mL DMSO and added to the lysate solution. This mixture was then centrifuged at 200 rpm for 24 h. Next, the lysate was filtered and the solid was repeatedly washed with boiling water and boiling ethanol. The solid was dried and 196 mg of material was collected. The material was dissolved in 5 mL of dry CH_2Cl_2, and 1 mL of diisobutylaluminum hydride (1.0 M in hexanes) was added at 0 °C. The reaction was worked up with 0.1 M HCl followed by freeze-drying. The crude extract was analyzed by high-performance liquid chromatography using a Shimadzu LC-8A HPLC on an analytical reverse phase column. A 9:1 mixture of acetonitrile:water at a 2 mL/min flow rate was used, and detection was done using a Shimadzu SPD-10A UV–vis detector. Identification of CoA was achieved by comparison of the retention time to a commercial sample.

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References and notes

1. Turos, E.; Konaklieva, M. I.; Ren, R. X.-F.; Shi, H.; Gonzalez, J.; Dickey, S.; Lim, D. *Tetrahedron* **2000**, *56*, 8193.
2. Turos, E.; Long, T. E.; Konaklieva, M. I.; Coates, C.; Shim, J.-Y.; Dickey, S.; Lim, D. V.; Cannons, A. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2229.
3. Coates, C.; Long, T. E.; Turos, E.; Dickey, S.; Lim, D. V. *Bioorg. Med. Chem. Lett.* **2003**, *11*, 193.
4. Long, T. E.; Turos, E.; Konaklieva, M. I.; Blum, A. L.; Amry, A.; Baker, E. A.; Suwandi, L. S.; McCain, M. D.; Rahman, M. F.; Dickey, S.; Lim, D. V. *Bioorg. Med. Chem.* **2003**, *11*, 1859.
5. Turos, E.; Coates, C.; Shim, J.-Y.; Wang, Y.; Roettgers, J. M.; Long, T. E.; Reddy, G. S. K.; Ortiz, A.; Culbreath, M.; Dickey, S.; Lim, D. V.; Alonso, E.; Gonzalez, J. *Bioorg. Med. Chem.* **2005**, *13*, 6289.
6. Heldreth, B.; Long, T. E.; Jang, S.; Guntireddygari, S. K. R.; Turos, E.; Dickey, S.; Lim, D. V. *Bioorg. Med. Chem.* **2006**, *14*, 3775.
7. Turos, E.; Long, T. E.; Heldreth, B.; Leslie, J. M.; Guntireddygari, S. K. R.; Wang, Y.; Coates, C.; Konaklieva, M.; Dickey, S.; Lim, D. V.; Alonso, E.; Gonzalez, J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2084.
8. Smith, D. M.; Kazi, A.; Smith, L.; Long, T. E.; Heldreth, B.; Turos, E.; Dou, Q. P. *Mol. Pharm.* **2002**, *61*, 1348.
9. Kazi, A.; Hill, R.; Long, T. E.; Kuhn, D. J.; Turos, E.; Dou, Q. P. *Biochem. Pharm.* **2004**, *67*, 365.
10. Kuhn, D.; Coates, C.; Daniel, K.; Chen, D.; Bhuiyan, M.; Turos, E.; Dou, Q. P. *Front. Biosci.* **2004**, *9*, 2605.
11. Kuhn, D.; Wang, Y.; Minic, V.; Coates, C.; Reddy, G. S. K.; Daniel, K. G.; Shim, J. Y.; Chen, D.; Landis-Piwowar, K. R.; Miller, F. R.; Turos, E.; Dou, Q. P. *Front. Biosci.* **2005**, *10*, 1183.
12. Private communication, Q. Ping Dou, Wayne State University.
13. Mishra, R. K.; Revell, K. D.; Coates, C. M.; Dickey, S.; Lim, D. V. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2081.
14. Timothy Long, Ph.D. dissertation, University of South Florida, **2003**.
15. Tipper, D. J.; Strominger, J. L. *Proc. Natl. Acad. Soc. U.S.A.* **1965**, *54*, 1133.
16. Waxman, D. J.; Strominger, J. L.. In *Chemistry and Biology of β-Lactam Antibiotics*; Morin, R. B., Gorman, M., Eds.; Academic Press: New York, 1982; Vol. 3, p 209.
17. The advantage of preparing the samples from agar cultures as opposed to broth is that a concentration gradient is produced as the antibiotics diffuse through the solid media, allowing microscopic examination among a range of exposure amounts.
19. Hara, M.; Takahashi, I.; Yoshida, M.; Asano, K.; Kawamoto, I.; Morimoto, M.; Nakano, H. *J. Antibiot.* **1989**, *42*, 333.
20. Hata, T.; Hoshi, T.; Kanamori, K.; Matsumae, A.; Sano, Y.; Shima, T.; Sugawara, R. *J. Antibiot.* **1956**, *9*, 141.

21. Hara, M.; Saitoh, Y.; Nakano, H. *Biochemistry* **1990**, *29*, 5676.
22. In addition to potential inhibitory effects on protein and nucleic acid biosynthesis, we also considered the possibility that the lactams may alter bacterial respiration by inhibiting the cellular uptake and phosphorylation of glucose. Thus, we examined the propensity of lactam **1** to induce metabolic starvation in *S. aureus* by measuring uptake of radiolabeled glucose in the presence versus the absence of lactam **1**. It was found that sugar uptake was not inhibited to any detectable extent at the MIC of lactam **1**. Thus, metabolic respiration does not seem to be at all perturbed by these N-thiolated lactams. For more details, see Bart Heldreth, Ph.D. dissertation, University of South Florida, **2004**.
23. Leonardi, R.; Chohnan, S.; Zhang, Y.-M.; Virga, K. G.; Lee, R. L.; Rock, C. O.; Jackowski, S. *J. Biol. Chem.* **2005**, *280*, 3314.
24. Slater-Radosti, C.; Van Aller, G.; Greenwood, R.; Nicholar, R.; Keller, P. M.; DeWolf, W. E., Jr.; Fan, F.; Payne, D. J.; Jaworski, D. D. *J. Antimicrob. Chemother.* **2001**, *48*, 1.
25. Higgins, D. L.; Chang, R.; Debabov, D. V.; Leung, J.; Wu, T.; Krause, K. M.; Sandvik, E.; Hubard, J. M.; Kaniga, K.; Schmidt, D. E., Jr.; Gao, Q.; Cass, R. T.; Karr, D. E.; Benton, B. M.; Humphrey, P. P. *Antimicrob. Agents Chemother.* **2005**, *49*, 1127.
26. Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Phys.* **1959**, *37*, 911.
27. Shah, N. V.; Cama, L. D. *Heterocycles* **1987**, *25*, 221.
28. Woulfe, S. R.; Iwagami, H.; Miller, M. J. *Tetrahedron Lett.* **1985**, *26*, 3891.
29. Newton, G. L.; Arnold, K.; Price, M. S.; Sherrill, C.; delCardayre, S. B.; Aharonowitz, Y.; Cohen, G.; Daview, J.; Fahey, R. C.; Davis, C. *J. Bacteriol.* **1996**, *178*, 1990.
30. delCardayre, S. B.; Stock, K. P.; Newton, G. L.; Fahey, R. C.; Davies, J. E. *J. Biol. Chem.* **1998**, *273*, 5744.
31. delCardayre, S. B.; Davies, J. E. *J. Biol. Chem.* **1998**, *273*, 5752.
32. Argyrou, A.; Blanchard, J. S. *Prog. Nucleic Acid Res. Mol. Biol.* **2004**, *78*, 89.
33. Fujiki, K.; Tanifuji, N.; Sasaki, Y.; Yokoyama, T. *Synthesis* **2002**, *3*, 343.
34. Analysis done using Kaleidagraph software, version 3.5.1, available from Synergy Software.
35. Purified CoADR was provided by Professor Al Claiborne (Wake Forest Medical School). For more details, see Kevin Revell, Ph.D. dissertation, University of South Florida, **2006**.
36. Cavallito, C.; Bailey, J. H. *J. Am. Chem. Soc.* **1944**, *66*, 1944.
37. Ankri, S.; Mirelman, D. *Microbes Infect.* **1999**, *2*, 125.
38. Block, E. *Scient. Am.* **1985**, *252*, 94.
39. Koch, H. P.; Lawson, L. D. *Garlic: The Science and Therapeutic Application of Allium sativum L.*; Williams and Wilkins: Baltimore, 1996, pp. 1–233.
40. Uchida, Y.; Takahashi, T.; Sato, N. *Jpn. J. Antibiotics* **1975**, *28*, 638.
41. Cellini, L.; Campli, E.; Masulli, M.; Di Bartolomeo, S.; Allocati, N. *FEMS Immunol. Med. Microbiol.* **1996**, *13*, 273.
42. Gonzales-Fandos, E.; Garcia-Lopez, M. L.; Sierrra, M. L.; Otera, A. *J. Appl. Bacteriol.* **1994**, *77*, 549.
43. Gimenez, M. A.; Solanes, R. E.; Gimenez, D. F. *Rev. Argent. Microbiol.* **1988**, *20*, 17.
44. Davis, L. E.; Shen, J.; Royer, R. E. *Planta Med.* **1994**, *60*, 546.
45. Hughes, B. G.; Lawson, L. D. *Photother. Res.* **1991**, *5*, 154.
47. Tsai, Y.; Cole, L. L.; Dav Lockwood, S. J.; Simmons, V.; Wild, G. C. *Planta Med.* **1985**, *5*, 460.
48. Tatarintsev, A. V.; Vrzhets, P. V.; Ershov, D. E.; Turgiev, A. S.; Karamov, E. V.; Kornilaeva, G. V.; Makarova, T. V.; Federov, N. A.; Varfolomeev, S. D. *Vestn. Ross. Akad. Med. Nauk.* **1992**, *11*, 6.
49. Mirelman, D.; Monheit, D.; Varon, S. *J. Infect. Dis.* **1987**, *156*, 243.
50. Ankri, S.; Miron, T.; Rabinkov, A.; Wilchek, M.; Mirelman, D. *Antimicrob. Agents Chemother.* **1997**, *10*, 2286.
51. Adetumbi, M.; Javor, G. T.; Lau, B. H. *Antimicrob. Agents Chemother.* **1986**, *30*, 499.
52. Ghannoum, M. A. *J. Gen. Microbiol.* **1988**, *134*, 2917.
53. Neuwirth, Z.; Sundstrom, D. C.; Thompson, N. H. *Antimicrob. Agents Chemother.* **1988**, *32*, 1763.
54. Augusti, K. T.; Mathew, P. T. *Experentia* **1974**, *30*, 468.
55. Feldberg, R. S.; Chang, S. C.; Kotik, A. N.; Nadler, M.; Neuwirth, Z.; Sundstrom, D. C.; Thompson, N. H. *Antimicrob. Agents Chemother.* **1988**, *32*, 1763.
56. Focke, M.; Feld, A.; Lichtenthaler, H. K. *FEBS Lett.* **1990**, *261*, 106.
57. Ozolin, O. N.; Uteshev, T. A.; Kim, I. A.; Deev, A. A.; Kamzolova, S. G. *Mol. Biol. (Mosk.)* **1990**, *24*, 1057.
58. Urbina, J. A.; Marchan, E.; Lazardi, K.; Visbal, G.; Apitz-Castro, R.; Gil, F.; Aguirre, T.; Piras, M. M.; Piras, R. *Biochem. Pharm.* **1993**, *45*, 2381.
59. San-Blas, G.; Urbina, J. A.; Marchan, E.; Contreras, L. M.; Sorais, F.; San-Blas, F. *Microbiology* **1997**, *143*, 1583.