Inhibition of the Antibacterial Target UDP-(3-*O*-acyl)-*N*-acetylglucosamine Deacetylase (LpxC): Isoxazoline Zinc Amidase Inhibitors Bearing Diverse Metal Binding Groups

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UDP-3-O-[*R*-3-hydroxymyristoyl]-GlcNAc deacetylase (LpxC) is a zinc amidase that catalyzes the second step of lipid A biosynthesis in Gram negative bacteria. Known inhibitors of this enzyme are oxazolines incorporating a hydroxamic acid at the 4-position, which is believed to coordinate to the single essential zinc ion. A new structural class of inhibitors was designed to incorporate a more stable and more synthetically versatile isoxazoline core. The synthetic versatility of the isoxazoline allowed for a broad study of metal binding groups. Nine of 17 isoxazolines, each incorporating a different potential metal binding functional group, were found to exhibit enzyme inhibitory activity, including one that is more active than the corresponding hydroxamic acid. Additionally, a designed affinity label inhibits LpxC in a time-dependent manner.

Introduction

Strains of three bacteria (*Enterococcus faecalis*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*) have recently shown resistance to every clinically available antibiotic—including vancomycin, often considered a drug of "last resort".[1] Although many groups have devised creative ways to get around resistance problems,[2,3] the most effective long-term solution is the development of drugs that act on unexploited antibacterial targets.[4] One such target in Gram negative bacteria is the biosynthesis of lipid A, a key component of the outer membrane. The minimal lipid A structure necessary for bacterial cell growth consists of a diphosphorylated tetrasaccharide acylated with six fatty acid chains on the two glucosamine subunits (Figure 1).[5] The biosynthesis of this minimal lipid A structure has been extensively studied; all of the enzymes and corresponding genes responsible for its synthesis in *Escherichia coli* have been identified.[6] Homologous genes have also been identified from the genomes of most other Gram negative bacteria.

Over the last two decades, several steps of lipid A biosynthesis have been investigated as potential antibacterial targets. Early attempts to inhibit the production of lipid A primarily focused on the biosynthesis of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO), a key sugar residue in the minimal lipid A structure (Figure 1).[7,8] Although relatively potent mechanism-based inhibitors have been found for two enzymes in this pathway, none of these inhibitors has proved therapeutically useful.[9]

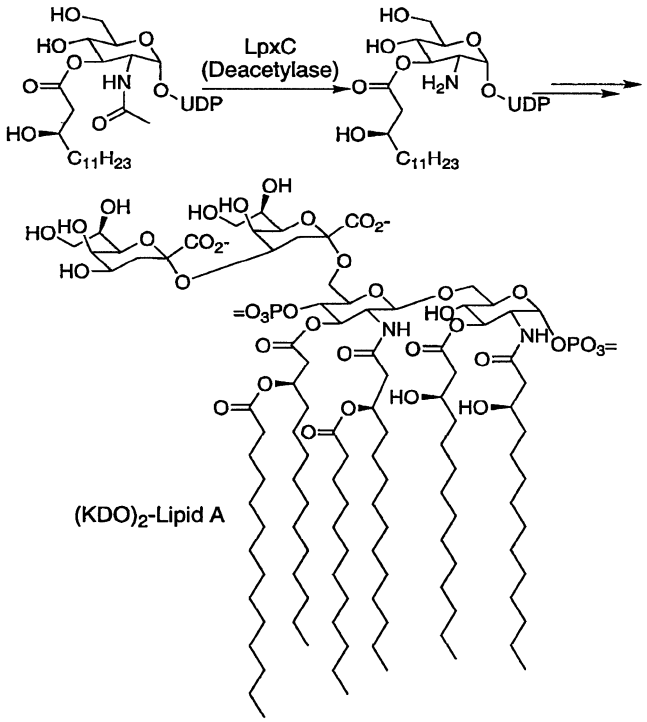

Figure 1. Biosynthesis and structure of lipid A.

Recently, a series of small heterocyclic hydroxamic acids that inhibit lipid A biosynthesis and are antibacterial against *E. coli* both in vitro and in animal models have been disclosed.[10] These compounds are aryl oxazoline hydroxamic acids (Figure 2), and the most potent compound of the series, L-161,240, has a minimal inhibitory concentration (MIC) against *E. coli* comparable to ampicillin and rifampicin (~2 μg/mL). The target of these small molecules has been shown to be

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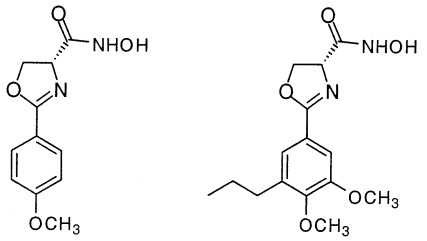


Figure 2. Structure of two of Merck's deacetylase inhibitors.

UDP-3-O-[*R*-3-hydroxymyristoyl]-GlcNAc deacetylase (LpxC), a zinc amidase encoded by the *lpxC* gene. Unfortunately, the antibacterial spectrum of this series of compounds is rather limited although a very wide variety of Gram negative bacteria have been shown to express homologous LpxCs. Although *Enterobacter cloacae* and *Klebsiella pneumoniae* are sensitive to L-161,240, the growth of *P. aeruginosa* and *Serratia marescens* is not affected.[10] This lack of activity against other Gram negative bacteria is due, at least in part, to subtle differences in enzyme structure between the various organisms.[11]

LpxC catalyzes the first committed step of lipid A biosynthesis, the removal of an *N*-acetyl group from a 3-acyl-*N*-acetyl-glucosamine moiety (Figure 1). This enzyme contains a single catalytic zinc ion that is coordinated by the nitrogen of two histidine residues, with a third coordinating group that is either a histidine nitrogen or a carboxylate of Asp or Glu.[12] The three-dimensional structure of this deacetylase is currently unknown, although efforts to determine it are underway.[13] No sequence homology with other structurally characterized zinc amidases has been identified.

One requisite feature of these LpxC inhibitors is the hydroxamic acid moiety. As shown in the earlier studies, the conversion of the hydroxamic acid to a carboxylic acid results in the loss of all inhibitory and antibacterial activity. The well-known affinity of hydroxamic acids for zinc has led to the proposal that the inhibitors act by coordinating to the single catalytic zinc ion.[14] Thus, it is thought that the inhibitors function analogously to matrix metalloproteinase (MMP) inhibitors.

A feature of MMP inhibitors that has been studied in great detail is the zinc coordinating group. Although hydroxamic acids are frequently employed as metal binding groups, they often present metabolic and pharmokinetic problems such as glucoronidation and sulfation that result in a short in vivo half-life.[15,16] Many hydroxamates are unstable in vivo, being hydrolyzed to give the toxin hydroxylamine,[17] which has fueled the search for other zinc coordinating functional groups. A variety of metal binding groups distinct from hydroxamic acids have been shown effective in in vitro and in vivo inhibition of zinc peptidases, including thiols, β-hydroxy thiols, phosphinic acids, and heterocyclic amines.[17] With these precedents in mind, we undertook a systematic study of metal binding groups for LpxC inhibitors, as it applies to both enzymatic and antibacterial activity. These data should be of value in future studies of both lipid A biosynthesis inhibitors and zinc protease inhibitors.

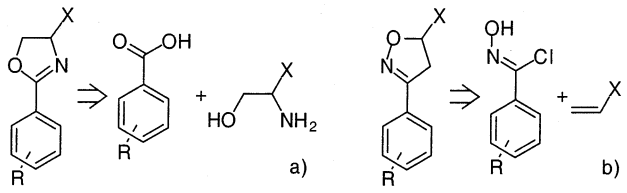
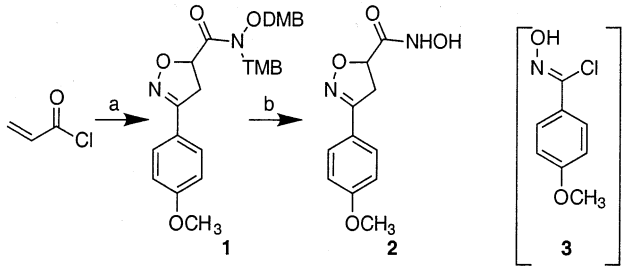


Figure 3. Retrosynthesis of oxazolines (a) and isoxazolines (b).

Scheme 1[a]



[a] Reagents: (a) (1) TMBNHODMB, *N*-methylmorpholine; (2) Compound **3**, Et_3N. (b) TFA/Et_3SiH/DCM. DMB = 2,4-Dimethoxybenzyl. TMB = 2,4,6-Trimethoxybenzyl.

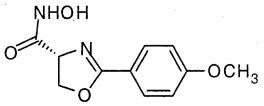
Results

One problem in targeting analogues of the original lead oxazoline hydroxamate bearing various metal binding groups is the difficulty in obtaining the appropriate serine analogues to serve as precursors (Figure 3). Although a few of these compounds have been reported, their synthesis is tedious and therefore would greatly limit the number of metal binding groups that could be studied. For instance, diethyl phosphonoserine is a known compound but is prepared in a six step synthesis from commercially available starting materials.[18] Further, oxazolines are sensitive to nucleophiles such as thiols that would be of great interest to incorporate into the inhibitors.[19]

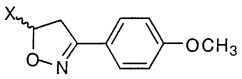
A potential solution to these problems was to change the heterocyclic scaffold to increase its stability toward nucleophiles and to allow the facile incorporation of a wide variety of groups in place of the hydroxamic acid. One class of heterocycles that fits these criteria is 4,5-dihydro-isoxazoles (Figure 3). These isoxazolines are quite stable toward nucleophiles such as thiols and are easily derived from 1,3-dipolar cycloaddition with an acrylate or a substituted ethylene.[20] Ethylenes substituted with an electron-withdrawing group generally react to give a single regioisomer (as shown in Figure 3).[21] The corresponding alkenes containing the various metal binding groups are widely available or easily synthesized.

Before large numbers of isoxazolines were made, we determined whether the change of heterocycle from an oxazoline to an isoxazoline would affect activity. A slightly simplified analogue of the most potent known inhibitor was therefore designed. Racemic hydroxamate **2** was synthesized according to Scheme 1 beginning with the previously reported doubly protected hydroxylamine shown.[22] Cycloaddition with nitrile oxide precursor **3** gave isoxazoline **1** as a single regioisomer. Acidic deprotection gave the expected hydroxamate product, which exhibited the desired biological activity (Table 1). The two enantiomers of this compound were made

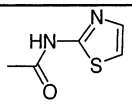
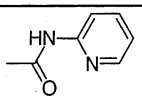
Table 1. Enzymatic and Antibacterial Activity of the Various Inhibitors



L-159,692



Compounds **2, 6 - 15, 17, 21, 22, 25, 26**

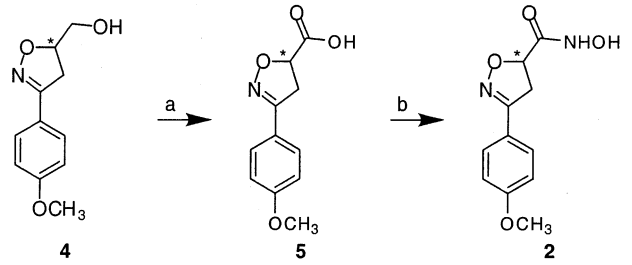
				Zone of Inhibition	
Compound #	Isomer	X	IC_{50} (μM)	*E. coli*	*EnvA1 E. coli*
L-159,692	*R*	--	3	11	24
2	*R/S*	CONHOH	13 ± 5	<6	21-23
2	*R*	CONHOH	-	<6	<6
2	*S*	CONHOH	4.37	<6	26
6	*R/S*	CO_2H	>450	<6	<6
7	*R/S*	$COCH_2SAc$	12±6[a]	<6	<6
8	*R/S*	$COCH_2SH$	27 ± 3	<6	<6
9	*R/S*	$CONHNH_2$	>425	<6	<6
10	*R/S*	CONHNHCHO	400 ± 100	<6	<6
11	*R/S*		>392	<6	<6
12	*R/S*		>336	<6	<6
13	*R/S*	SO_2NH_2	>1200	<6	<6
14	*R/S*	SO_2CH_3	>392	<6	<6
15	*R/S*	$CONHCH_2OH$	>400	<6	<6
17	*R/S*	PO_3H_2	4 ± 2	<6	<6
21	*R/S*	$PO(CH_3)OH$	170 ± 40	<6	<6
22	*R/S*	CH_2SH	22 ± 3	<6	<6
25a	*R*,R**		see text	<6	<6
25b	*R*,S**		see text	<6	<6
26a	*R*,S**	$CH(OH)CH_2SH$	>400	<6	<6
26b	*R*,R**	$CH(OH)CH_2SH$	>400	<6	<6

[a] The IC_{50} of this compound could not be determined accurately. See text.

according to Scheme 2 from the previously reported enantiomeric isoxazolines.[23,24]

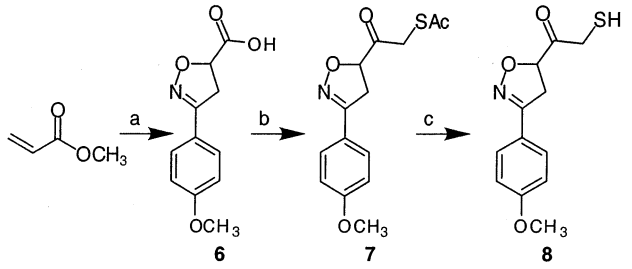
All of other compounds containing the various metal binding groups were prepared as racemates and compared to the activity of the racemic hydroxamate **2**. An α-thiol-ketone isoxazoline was synthesized from methyl acrylate according to Scheme 3. Dipolar cycloaddition followed by saponification gave carboxylic acid **6**. The carboxylic acid was converted in an Arndt-Eistert sequence to the acid chloride and then treated with diazomethane to give the corresponding α-diazoketone. Conversion to the α-chloroketone with anhydrous HCl and displacement with thioacetic acid gave thioester **7**. Removal of the acetate with hydrazine immediately gave an inseparable 1:1 mixture of the desired thiol **8** and its dimer (Figure 4). Removal of the acetate under

Scheme 2[a]



[a] Reagents: (a) Jones oxidation. (b) (1) EDC, NH_2OTr; (2) TFA/DCM.

Scheme 3[a]



[a] Reagents: (a) (1) Compound **3**, *N*-methylmorpholine; (2) NaOH. (b) (1) $SOCl_2$; (2) CH_2N_2; (3) HCl; (4) CH_3COSH, Et_3N. (c) N_2H_4.

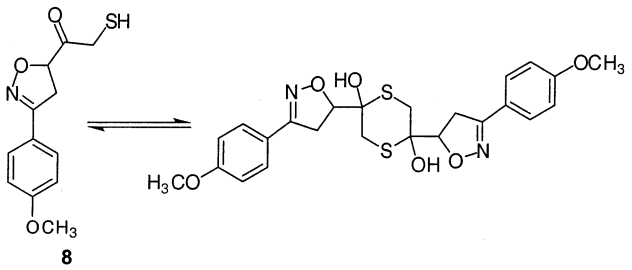


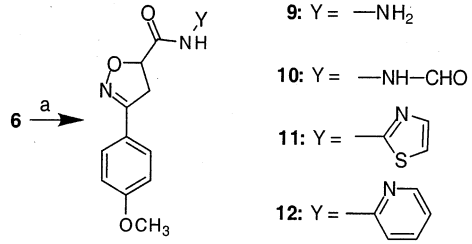
Figure 4. Compound **8** was isolated as an inseparable mixture of these two compounds.

other conditions gave little or no product. In retrospect, the formation of the dimer is not surprising given that the carbonyl is quite electrophilic (being doubly substituted with α-electron-withdrawing groups) and dimerization results in the formation of a stable six-membered ring.

Carboxylic acid **6** could easily be converted to derivatives **9**−**12** via a carbodiimide-mediated coupling reaction as shown in Scheme 4. A dipolar cycloaddition reaction with readily available alkenes gave derivatives **13**−**15** (Scheme 5). The cycloaddition reactions are quite clean and give a single regioisomer in each example. Phosphonic acid **17** was synthesized in two steps beginning with commercially available diethyl vinyl phosphonate according to Scheme 5. Again, the cycloaddition reaction gives a single regioisomer.

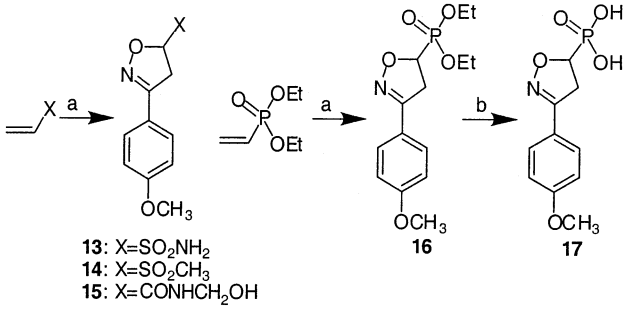
Several unsuccessful attempts were made to synthesize methyl vinyl phosphinate esters according to literature procedures. Eventually, the three step synthesis shown in Scheme 6 was adopted. Methyl dichlorophosphine was treated with 2-chloroethanol in the presence of pyridine to form the corresponding phosphine, which was concentrated and heated to 160 °C (neat). The resultant oil was heated (neat) with Et_3N to form alkene **19**, which was purified by Kugelrohr distillation. This represents a very efficient new synthesis of methyl vinyl

Scheme 4[a]



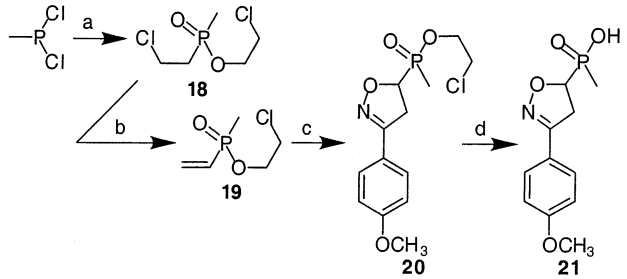
[a] Reagents: (a) EDC, Y-NH_2.

Scheme 5[a]



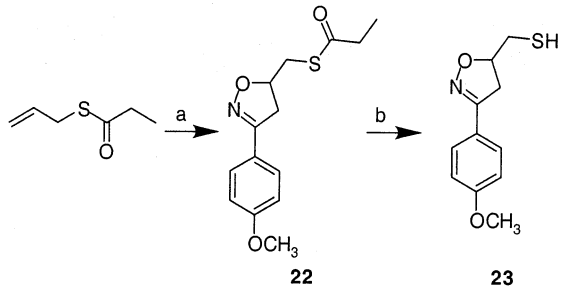
[a] Reagents: (a) Compound **3**, Et_3N. (b) Me_3SiBr.

Scheme 6[a]



[a] Reagents: (a) (1) 2-Chloroethanol, pyridine; (2) 160 °C. (b) Et_3N, 120 °C. (c) Compound **3**, Et_3N. (d) Me_3SiBr.

Scheme 7[a]

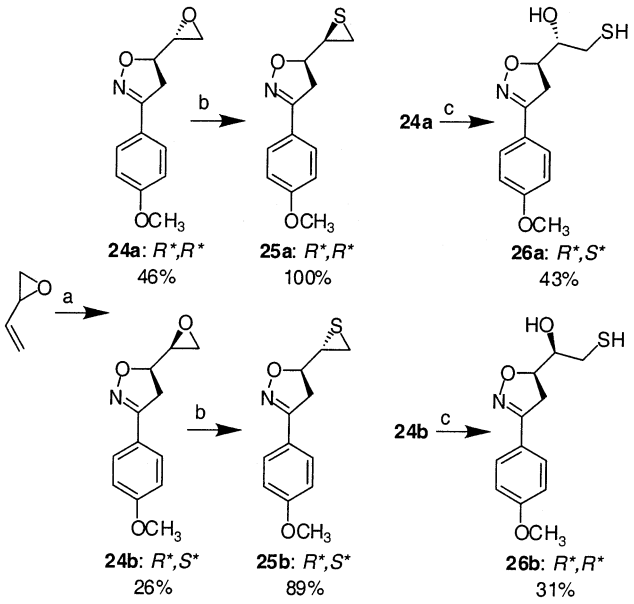


[a] Reagents: (a) Compound **3**, Et_3N. (b) NaOH.

phosphinate esters, a very useful class of compounds in natural product chemistry.[25] Dipolar cycloaddition followed by deprotection gave phosphinic acid **21**.

Thiol **22** (Scheme 7) was synthesized from the commercially available allyl thiopropionate. As may be expected, examination of the crude NMR spectrum of the cycloaddition reaction showed that a significant amount of the undesired regioisomer formed. However, the two regioisomers could be easily separated by flash chromatography. Finally, compounds **25** and **26** (Scheme 8) were synthesized from the known diastereomeric epoxides **24**, which, in turn, were derived from butadiene monoepoxide.[26] Epoxides **24** were converted to

Scheme 8[a]



[a] Reagents: (a) Compound **3**, Et_3N. (b) Thiourea. (c) $(Me_3Si)_2S$, TBAF.

thiiranes **25** with inversion of stereochemistry via a known transformation utilizing thiourea.[27] Epoxides **24** could also be opened with a hydrogen sulfide derivative[28] to give diastereomeric thiol-alcohols **26**.

Table 1 gives a complete summary of the inhibitory and antibacterial properties of the compounds prepared above. Racemic hydroxamate **2** was found to have an IC_{50} of 13 μM. Although the compound had no effect on the growth of wild type *E. coli*, it did significantly inhibit the growth of *EnvA1 E. coli*, a strain of bacteria with a mutation in lipid A biosynthesis.[10] As expected, the active enantiomer of **2** was found to have the (*S*) configuration. This corresponds to the same spatial relationship of groups as the biologically active (*R*)-oxazoline L-159,692. Interestingly, the active enantiomer of **2** has an IC_{50} very similar to that of L-159,692. Despite the equivalent inhibitory activity, the (*S*) isomer of **2** has reduced antibacterial activity as compared to that of its oxazoline counterpart. Though (*S*)-**2** has no effect on the growth of wild-type *E. coli*, the oxazoline (L-159,692) and isoxazoline (**2**) have a nearly equivalent inhibitory effect on the growth of *EnvA1 E. coli*. The MIC of (*S*)-**2** against *EnvA1 E. coli* is 3.9 μg/mL, which is comparable to the MIC of the original lead compound (3.6 μg/mL) against the same strain of bacteria.[29]

The nearly equivalent inhibitory activity of the two heterocycles (**2** and L-159,692) supports the hypothesis that the heterocycle acts as a scaffold that holds the metal binding group and the hydrophobic aryl group in a particular spatial orientation. The two heterocycles have different patterns of hydrogen bond acceptors; such properties therefore seem to be less important factors in inhibition. Overlaying the energy-minimized conformations of these two compounds shows that the positions of the hydroxamate carbons differ by only 1.1 Å (Figure 6).[30]

Several isoxazolines with metal coordinating groups other than hydroxamic acid show significant inhibition of LpxC enzymatic activity. Phosphinic acid **21** and formyl hydrazine **10** are weakly active against the

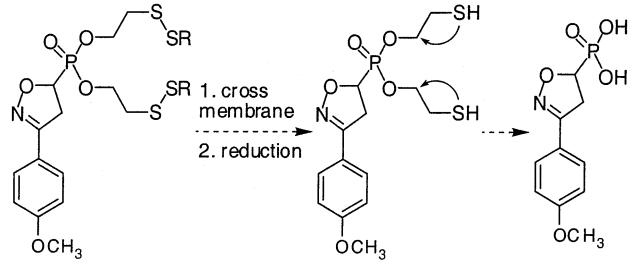


Figure 5. Pro-drug approaches for improving the antibacterial activity of compound **17** (R=CH_2CH_2OH).

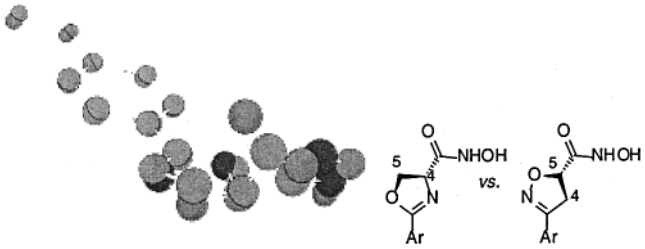


Figure 6. Structural superposition of energy-minimized structures of oxazoline and isoxazoline LpxC inhibitors.

enzyme. Even though in this case the inhibition is weak, the formyl hydrazine group present in **10** represents a previously unreported metal binding functional group. More interestingly, thiols **8** and **22** were found to have low micromolar inhibition (27 and 22 μM) of LpxC, nearly comparable to hydroxamate **2**. Their potency is predicted to be even greater at physiological pH because the thiol will be more ionized. These assays were performed at pH 6.0. It is interesting that thiol **22** is nearly as active as the hydroxamate even though it can function only as a monodentate zinc chelator.[31] This is commonly seen; it has been suggested that this observation is due to easier ionization of the thiol as compared to hydroxamates and a lower desolvation penalty.[32] Given the activity of thiol **22**, it is interesting that α-hydroxy thiols **26a**,**b** do not inhibit LpxC. α-Hydroxy thiols have made excellent zinc binding groups in various MMP inhibitors, often exhibiting equivalent or improved activity as compared to the corresponding hydroxamate.[33] Excitingly, phosphonic acid **17** has an IC_{50} of 4 μM, a considerable improvement over the corresponding hydroxamic acid. This is highly unusual: phosphonates typically inhibit zinc amidases 10−100-fold less effectively than hydroxamates. In earlier work,[11] a carbohydrate substrate analogue with a hydroxamate replacing the acetamide was about 3-fold more potent vs the *E. coli* enzyme than an analogous carbohydrate phosphinate. This compound is much more potent vs the *Aquifex aeolicus* enzyme.

Thioacetate **7** unexpectedly proved to have significant enzymatic activity, although this activity was difficult to characterize. The IC_{50} of this compound was approximately 12 μM, comparable to the corresponding hydroxamate. However, it is questionable how meaningful this value is. The activity of the enzyme dropped by 85% upon addition of 36 μM inhibitor. However, 15% residual activity remained upon addition of up to 350 μM inhibitor. Moreover, the compound appeared to show time-dependent inactivation of the enzyme. One explanation for the peculiar inhibitory activity of this compound is that it may be a substrate for the enzyme.

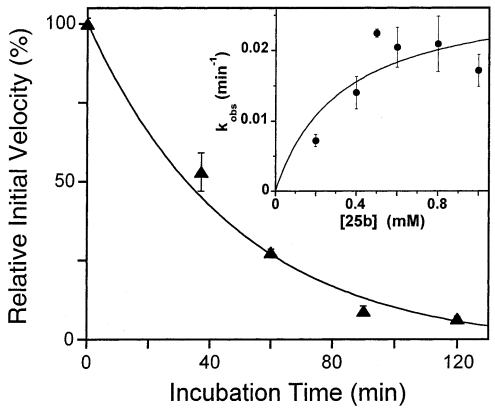


Figure 7. Time-dependent inhibition of LpxC. LpxC (100 μM) was incubated with 0.5 mM **25b** at 30 °C in 100 mM bis-Tris, pH 7, 12% DMF. At the indicated times, an aliquot was removed and diluted 10-fold into assay buffer. The initial velocity was assayed as described in the Experimental Section. The data are well-described by a single-exponential decay with an apparent first-order rate constant of 0.020 ± 0.001 min^{-1}. Inset: The observed rate constants for inhibition show a hyperbolic dependence on the inhibitor concentration. The data are well-described by eq 2 with a second-order rate constant of 90 ± 30 M^{-1} min^{-1} and K_I of 0.3 ± 0.1 mM.

Control experiments establish that **7** is stable in assay buffer alone over several hours. If the enzyme can hydrolyze **7**, the active inhibitor may be thiol **8**. However, this cannot explain the time-dependent inactivation of the enzyme. Thioesters have been used to acylate amines under physiological conditions.[34] Therefore, it may be possible that this functional group is acetylating an enzyme amine or thiol and thereby rendering the enzyme inactive. It is also conceivable that it is a slow, tight binding inhibitor, though we consider this possibility less likely. Although there have been reports of similar functional groups being used as thiol "prodrugs" in vivo, this work represents the first report of this novel zinc binding group directly having enzymatic inhibitory activity.

In addition to the competitive zinc binding groups described above, two potential affinity labels were also investigated. These inhibitors incorporate thiiranes and epoxides, functional groups that have been previously reported to covalently modify the active sites of other zinc amidases, rendering them inactive.[27,35] They are believed to act through electrophilic activation of the highly strained heterocycle by the active site zinc ion. Subsequent attack by an active site nucleophile (believed to be Glu-404 in MMPs) gives a covalently modified enzyme that is incapable of turnover.

The time-dependent inhibition of **25a**,**b** was investigated by incubating LpxC with excess inhibitor. At various times, an aliquot was removed and diluted 10-fold into assay buffer to measure the remaining enzyme activity. Compound **25b** clearly inhibits LpxC in a time-dependent manner (Figure 7). The apparent first-order rate constant for inactivation is dependent on the concentration of inhibitor, yielding a second-order rate constant (k_{inact}) of 90 ± 30 M^{-1} min^{-1} and a K_I of 0.3 ± 0.1 mM. This inactivation extrapolates to 100% inhibition at long incubation times. Furthermore, the inhibition is essentially irreversible; when the inactivated enzyme is diluted 20-fold into buffer, no increase in activity is observed over 45 h. Similar experiments demonstrated that **25a** is also a time-dependent inhibitor with a K_I of 0.8 ± 0.2 mM and k_{inact} of 4 ± 2 M^{-1} min^{-1}. While these data clearly demonstrate time-dependent inhibition, they do not demonstrate active site modification. Further studies are underway to clarify the mechanism of this inhibition. Given the dearth of structural and mechanistic knowledge of LpxC, these inhibitors may provide important clues about the active site amino acids.

Despite their similar or even improved enzymatic potency as compared to hydroxamate **2**, the compounds with the various metal binding groups described above displayed no antibacterial activity against wild-type or hypersensitive *EnvA1 E. coli* (Table 1). This was not entirely unexpected given the difficulty in finding compounds that will cross the two membranes of Gram negative bacteria. Phosphonate **17**, in particular, would be very unlikely to cross the hydrophobic barriers presented by the bacteria. Various prodrug approaches (Figure 5) were attempted in order to "mask" the polar phosphonate group as a more hydrophobic phosphonate ester that could be cleaved in vivo to form compound **17**, but these attempts were unsuccessful.[36] Phosphate prodrugs are widely utilized to deliver phosphates across mammalian cell membranes,[37] but there has been no reported prodrug system for delivering such compounds across bacterial cell membranes. Compound **7**, along with its good enzymatic activity, has the polarity and solubility profile necessary to cross the double membrane. Presumably, its lack of antibacterial activity is due to either active extrusion or fast metabolism of this compound.

Discussion

Using an isoxazoline heterocycle as a scaffold for groups that could potentially coordinate the active site zinc ion in LpxC, a number of novel inhibitors have been prepared. Our inhibition data in Table 1 suggest the following preference for zinc coordination: hydroxamate ≈ phosphonate ≈ thioacetoxyacetyl > thiolacetyl > phosphinate > carboxylate, sulfonamide, and hydrazide. This ranking is similar to that determined in a study of MMP-1 (fibroblast collagenase) inhibitors[38] where the preference for the zinc coordinating group was observed as follows: hydroxamate ≫ formyl hydroxylamine > thiol > phosphinate > α-amino acrylate > carboxylate. This study is the only reported systematic study of changes of the metal binding group in zinc proteinase inhibitors while holding the rest of the inhibitor constant. Although the rankings are similar, our studies identified two additional metal binding groups with inhibitory activity comparable to hydroxamates.

The specific ranking of the zinc binding groups presented here may not be general for this enzyme. Slight changes in the metal binding geometry are likely to translate into slight changes in the conformation of the heterocyclic scaffold or slightly alter hydrogen bond interactions with the heterocycle. This, in turn, may lead to slight changes in the way the aromatic group fits into a hydrophobic pocket of the enzyme. Therefore, a true quantitative measure of the effectiveness of the metal binding groups would require an optimization of the aromatic group and/or heterocycle for each zinc binding group tested.[17] Even if the quantitative results

presented here do not represent a general trend, they clearly represent a starting point for the design of new LpxC inhibitors with different structure/activity profiles and potentially increased or modified antibacterial activity. The four new zinc binding groups that were identified will hopefully serve as new lead structures from which more effective inhibitors can be developed. Moreover, two previously unknown zinc binding groups were identified, which may be useful in the design of inhibitors for the myriad of other therapeutically important metalloamidases.

Both oxazolines and isoxazolines tend to exist in an "envelope" conformation in which one carbon is slightly out of plane from the four remaining atoms (Figure 6). MM2 calculations and literature reports show that the C-5 position is "puckered" in both heterocycles by approximately 15°.[39,40] Importantly, the metal binding group is bound to C-5 of the isoxazoline (the puckered carbon) but to C-4 of the oxazoline (a planar carbon). Therefore, it appears that the oxazoline provides a slightly more rigid scaffold for the metal binding group than does the isoxazoline. Though this does not seem to significantly affect the IC_{50} of the isoxazoline **2**, this may have the implication of changing the structure−activity relationship (SAR) of the aromatic ring as compared to the known inhibitors when more potent compounds are tested. In fact, preliminary studies of the SAR of the isoxazoline hydroxamates as compared to the oxazoline hydroxamates indicate moderate divergences in activity when compounds in the mid-nanomolar range are tested.[41] The relative flexibility of C-5 of the isoxazoline may contribute to the wide range of metal binding groups that are effective at this position.

In summary, a comprehensive study of the utility of various zinc binding groups in LpxC inhibitors has been performed. A new class of scaffolds, the isoxazolines, was found to be effective in inhibition of this enzyme. Though the antibacterial activity of isoxazoline hydroxamate **2** was surprisingly low, additional SAR studies being performed in our labs have indicated that quite potent antibacterial compounds can be developed based on the isoxazoline scaffold given the correct choice of the aryl group at the 3-position of the ring.[42] Because we have shown that the oxazoline scaffold is not strictly required for activity, it remains to be seen the extent to which the scaffold can be varied. To this end, a systematic study of scaffolds should be undertaken.

Several zinc binding groups attached to the isoxazoline were found to have greater or nearly equal enzymatic activity as compared to the corresponding hydroxamic acid. Although the specific ranking of these inhibitors is likely of little importance, this study has identified several new lead structures from which potent inhibitors can likely be developed. Additionally, a mechanism-based inhibitor that shows time-dependent irreversible enzyme inhibition has been identified. This is the first systematic study of metal binding groups for LpxC deacetylase inhibition. This work represents the most comprehensive direct study of the zinc binding group in any family of zinc amidase inhibitors to date[43] and complements the study of Colletti et al.[35] on zinc proteases. The rankings provided should be a useful starting point for attempts to identify zinc binding groups apart from hydroxamic acids that are effective in the inhibition of a variety of zinc enzymes, though it is unlikely that the ranking of zinc binding groups presented here will apply directly to other zinc amidases.

Experimental Section

Enzyme Assays and Antibacterial Testing. Buffers and Reagents. [α-^{32}P]UTP was purchased from NEN Dupont. PEI-Cellulose thin-layer chromatography (TLC) plates were obtained from E. Merck, Darmstadt, Germany. Bis-tris buffer (Ultrapure reagent) and bovine serum albumin (BSA) were purchased from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Mallinckrodt.

LpxC Activity Assay. The *E. coli* LpxC substrate, [α-^{32}P]-UDP-3-*O*-(*R*-3-hydroxymyristoyl)GlcNAc was prepared and purified as described previously by acylation of [α-^{32}P]-UDP-GlcNAc using purified *E. coli* LpxA (provided by T. J. O. Wyckoff, Duke University). These assays were performed at 30 °C and contained 3 μM UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc, 1 mg/mL BSA in 40 mM bis-tris, pH 6.0. The activity assays were performed in plastic microcentrifuge tubes in a reaction volume of 20 μL. At each time point (chosen so that the total conversion to product was less than 10%), 5 μL portions of each reaction mixture were removed and added to 1 μL of 1.25 M NaOH to stop the reaction. The alkaline samples were incubated for an additional 10 min at 30 °C to ensure complete hydrolysis of the ester-linked acyl chains from the LpxC substrate and product and then were neutralized by the addition of 1 μL of 1.25 M acetic acid and 1 μL of 5% trichloroacetic acid. The neutralized samples were incubated on ice for 5 min and centrifuged for 2 min in a microcentrifuge. Portions of the supernatants (2 μL) were spotted onto PEI-cellulose TLC plates for separation of the remaining substrate (detected as [α-^{32}P]UDP-GlcNAc) from the product (detected as [α-^{32}P]UDP-GlcN). After they were air-dried, the plates were soaked for 10 min in methanol to improve resolution before chromatography. The plates were developed with 0.2 M aqueous guanidine-HCl as the solvent system. The radioactive spots on the plates were analyzed using a PhosphorImager equipped with ImageQuant software (Molecular Dynamics, Inc.) to determine the yields of product produced in each reaction mixture.

Inhibition of LpxC Activity. Purified *E. coli* LpxC was used to characterize inhibitors. Stock solutions (10 mg/mL) of each inhibitor were made in 100% DMSO, and any further dilutions of these compounds were also made in 100% DMSO. *E. coli* LpxC (2.5 nM) was incubated with varied concentrations (0.1−500 μg/mL) of each inhibitor in the presence of 1 mg/mL BSA for 30 min on ice prior to starting the assays. The final assay is 20% in DMSO. The LpxC/inhibitor stocks were then diluted 2.5-fold into assay mixtures containing 3 μM UDP-3-*O*-acyl-GlcNAc in 40 mM bis-tris, pH 6.0, at 30 °C, to give a final enzyme concentration of 1 nM *E. coli* LpxC. Assay tubes were supplemented with each of the inhibitors and additional DMSO, if necessary, to maintain the same concentration of the inhibitor and DMSO in the assay as was present in the preincubation with enzyme. The initial velocities were plotted as a function of inhibitor concentration for each compound. The resulting data were fit to eq 1, where v_i = the initial velocity at a given concentration of inhibitor and v_c = the initial velocity of a control reaction containing no inhibitor, to yield the IC_{50} for inhibition at 30 °C by each compound.

$$v_i/v_c = IC_{50}/([I] + IC_{50}) \quad (1)$$

Time-Dependent Inhibition of LpxC. Because high protein concentrations stabilize LpxC activity, assays were performed at concentrations >0.3 mg/mL that enabled a slow alternative substrate, UDP-GlcNAc, to be used. Stock solutions of **25a**,**b** were prepared in 100% dimethyl formamide (DMF). *E. coli* LpxC (100 μM) was incubated at 30 °C with varied concentrations (0−0.8 mM) of each inhibitor in 100 mM bis-

Tris, pH 7, 12% DMF. At various times, aliquots were diluted 10-fold into 27 mM UDP-GlcNAc in 100 mM bis-Tris, pH 7, at 30 °C in order to measure remaining enzyme activity. The initial velocity (<10% product formation) was determined from the measurement of UDP-GlcN by the following method. An aliquot (25 μL) of the assay mixture was diluted with 75 μL of 1 M borate, pH 9, and subsequently mixed with 30 μL of 10 mM fluorescamine (in acetonitrile). Fluorescamine reacts with the free amine of UDP-GlcN to form a fluorescent adduct (λ_{ex} = 395 nm, λ_{em} = 485 nm). The concentration of the product was determined from the fluorescence by comparison to a UDP-GlcN standard curve.[44] In the absence of inhibitor, enzyme activity was constant for >3 h under these conditions. The initial velocities were plotted as a function of incubation time, and the data fit to an exponential decay to determine the pseudo-first-order rate constant for inactivation. These observed rate constants were then plotted as a function of inhibitor concentration, and eq 2 was fit to these data. To examine whether this inhibition was reversible, LpxC (0.2 mM) was incubated with 0.5 mM **25b** at 30 °C for 4 h in 100 mM bis-Tris, pH 7, 12% DMF. The inactivated enzyme was then diluted 20-fold into 100 mM bis-Tris, pH 7, which decreases the inhibitor concentration to <25 μM, 10-fold below the K_I. Aliquots were removed at various times and assayed as described above. The initial velocity of both LpxC and inactivated LpxC altered by <10% over a 45 h incubation.

$$k_{obs} = k_{inact}[I]/(1 + [I]/K_I) \quad (2)$$

Disk Diffusion Test for Antibacterial Activity of LpxC Inhibitors. A 5 mL culture of *E. coli* strain R477 (wild type) was grown to stationary phase at 37 °C in LB broth containing 30 μg/mL streptomycin. This culture was diluted with LB broth to A_{600} = 0.2, and then, a sterile cotton swab was used to spread an even lawn of the diluted culture onto an LB agar plate. Filter paper disks (6 mm diameter) were saturated with 50 μg of the compound (in 100% DMSO) to be tested for antibacterial activity. The disks were placed on the lawn of freshly plated cells, and the plates were then incubated at 37 °C. Inhibition of bacterial growth was detected as a zone of clearing around each disk. The diameter of the zone of growth inhibition was measured for each compound after overnight incubation. A diameter of 6 mm indicated that there was no visible inhibition of growth beyond the edges of the disk.

MIC. Antibacterial activity of potent LpxC inhibitors (**2** and L-161,240) was determined using two strains of *E. coli*, the wild-type strain (R477), and G17S LpxC mutant strain (*EnvA*). Overnight cultures of R477 and G17S were grown at 37 °C and then diluted to OD_{660} = 0.1. The diluted culture was diluted again 1:100 into 50 μL of LB containing varied concentrations of inhibitor or DMSO as a control. A 96 well microtiter plate was used such that each well contained a different concentration of inhibitor (0.001−500 μg/mL). The cultures were allowed to grow for 7 h at 37 °C with shaking. The MIC was defined as the lowest inhibitor concentration that inhibited growth as measured by no increase in A_{660} during the time of the assay.

4-Methoxybenzohydroximinoyl Chloride (3).[45] Hydroxylamine hydrochloride (5.60 g, 80.9 mmol) was added to 2.6 mL of water. Triethylamine (11.3 mL, 80.9 mmol) was added followed by 40 mL of tetrahydrofuran (THF). The suspension was sonicated briefly. Trimethyl orthoformate (28.0 mL, 257 mmol) was added followed by *p*-anisaldehyde (8.90 mL, 73.5 mmol) and 40 mL of THF. The solution was stirred for 4 h. The solution was evaporated and partitioned between CH_2Cl_2 and water. The organic layer was dried over $MgSO_4$ and evaporated to give a white solid (11.2 g, 101%). To the white solid was added 100 mL of DMF. *N*-Chlorosuccinimide (9.80 g, 73.5 mmol) was added, and HCl gas from the headspace of a concentrated HCl bottle was bubbled into the solution until the reaction became warm. After it was stirred for 1 h at room temperature, the solution was poured over 150 mL of water and 150 mL of ether. The ether was washed 6× with water, dried over $MgSO_4$, filtered, and evaporated to give 11.1 g (81%) of a yellow oil that solidified overnight.

***N*-(2,4,6-Trimethoxybenzyl)-*O*-(2,4-dimethoxybenzyl)-3-(4-methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Hydroxyamide (24) and *N*-(2,4,6-Trimethoxybenzyl)-*O*-(2,4-dimethoxybenzyl)acrylic Hydroxamate (1).** NH(TMB)ODMB[22] (0.2 g, 0.55 mmol) and *N*-methylmorpholine (0.24 mL, 2.2 mmol) were stirred in 5 mL of CH_2Cl_2. Acryloyl chloride (0.179 mL, 2.20 mmol) was added, and the solution was stirred overnight. The reaction was quenched (carefully) with 10 mL of saturated $NaHCO_3$ and stirred for 30 min. CH_2Cl_2 (10 mL) was added, and the organic layer was removed and dried over $MgSO_4$. The mixture was filtered and evaporated to give 0.24 g (100%) of the acrylic hydroxamate as a yellow solid. A portion of solid (0.24 mmol, 0.1 g) and chlorooxime **3** (0.36 mmol, 67 mg) was stirred in 5 mL of EtOAc at room temperature. *N*-Methylmorpholine (0.36 mmol, 40 μL) was added dropwise, and the solution was stirred for 3 h. TLC indicated that the reaction was complete. The product was purified by prep TLC (3:2 EtOAc:Hex, R_f = 0.30) to give 87 mg (65%) of a clear oil. IR (thin film): 2955, 2843, 1665, 1611, 1513, 1206, 1151,1038 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.57 (2H, d, J = 8.7 Hz), 7.03 (1H, d, J = 8.7 Hz), 6.88 (2H, d, J = 8.7 Hz), 6.40−6.42 (2H, m), 6.12 (2H, s), 5.54 (1H, t, J = 9.3 Hz), 5.08 (1H, d, J = 14.1 Hz), 4.94 (1H, d, J = 14.1 Hz), 4.82 (1H, d, J = 9.9 Hz), 4.79 (1H, d, J = 9.9 Hz), 3.83 (3H, s), 3.82 (3H, s), 3.81 (3H, s), 3.77 (3H, s), 3.74 (6H, s), 3.33 (1H, d, J = 11.1 Hz), 3.32 (1H, d, J = 7.5 Hz). ^{13}C NMR: δ 169.59, 161.63, 161.04, 160.70, 159.85, 159.22, 155.12, 132.67, 128.15, 121.77, 115.07, 113.84, 104.13, 103.60, 98.29, 90.16, 76.68, 71.32, 55.61 (2C), 55.31 (3C), 39.01, 37.66. FAB MS (M + H): 567. HRMS *m*/*z* calcd for $C_{30}H_{34}N_2O_9$, 567.2342; found, 567.2329.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Hydroxyamide (2). Isoxazoline **2** (75 mg, 0.13 mmol) and 0.11 mL of triethylsilane (0.65 mmol) were stirred in 3 mL of 50% trifluoroacetic acid (TFA) in CH_2Cl_2. After 3 h, the solution was evaporated and was triturated with ether to give 22 mg (73%) of a pink solid; mp 152−153 °C (dec). IR (thin film): 3166, 2950, 2832, 1631, 1611, 1517, 1254, 828 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 10.99 (1H, bs), 9.04 (1H, bs), 7.62 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 8.4 Hz), 4.94 (1H, dd, J = 7.5, 11.1 Hz), 3.79 (3H, s), 3.59 (1H, dd, J = 11.1, 17.1 Hz), 3.52 (1H, dd, J = 7.2, 16.8 Hz). ^{13}C NMR: δ 165.64, 160.56, 155.60, 128.22, 120.93, 114.14, 77.34, 55.28, 38.33. FAB MS (M + H): 237. Anal. ($C_{11}H_{12}N_2O_4$) C, H, N.

(*R*)- and (*S*)-3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Hydroxyamide (2). (*R*)- or (*S*)-Alcohol **4**[22] (0.1 g, 0.48 mmol) was dissolved in 100 mL of acetone. Jones reagent (1.2 mmol, 2.5 equiv) was added, and the solution was stirred for 3 h. The reaction was quenched with 1 mL of MeOH and concentrated. The residue was partitioned between water and CH_2Cl_2. The organic layer was extracted with aqueous NaOH. The aqueous layer was acidified and extracted twice with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and evaporated to give 44 mg (42%) of acid **5** (see racemic **6** for characterization). Using EDC, a small sample of the acid was coupled to (*R*)-α-phenethylamine. The *de* could be measured by NMR by observing the doublets at δ 1.52 and δ 1.47. Acid **5** (0.12 g, 0.54 mmol) was dissolved in 20 mL of CH_2Cl_2. *O*-Trityl hydroxylamine (0.15 g, 0.55 mmol) was added followed by EDC (0.14 g, 0.75 mmol). The solution was stirred for 48 h after which time the solution was concentrated and partitioned between EtOAc and water. The organic layer was washed with 5% citric acid(aq), saturated aqueous $NaHCO_3$, and brine. The solution was dried over $MgSO_4$, filtered, and concentrated. The residue was treated with 30% TFA in CH_2Cl_2 (15 mL) for 20 min. MeOH (1 mL) was added, and the solution was concentrated. The residue was triturated with ether to give the desired hydroxamate **2** as a white solid (62 mg, 49% from **5**).

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid (6). *p*-Anisaldehyde oxime (0.36 g, 2.37 mmol) and *N*-chlorosuccinimide (0.32 g, 2.37 mmol) were stirred in 2 mL of DMF. A small amount of HCl gas from the headspace of a concentrated HCl bottle was bubbled into the solution until the reaction became warm. The solution was stirred for 1 h at

room temperature. CH_2Cl_2 (10 mL) was added followed by 0.45 mL (5 mmol) of methyl acrylate. *N*-Methyl morpholine (0.275 mL, 2.5 mmol) was added dropwise. After 1 h, the solution was concentrated and partitioned between H_2O and EtOAc. The organic layer was washed 4× with water and evaporated onto silica gel. The product was purified by flash chromatography (3:1 Hex:EtOAc, $R_f = 0.33$) to give 0.47 g (84%) of a white solid, which was dissolved in 20 mL of 1:1 THF:MeOH. NaOH (1 M, 2.5 mmol, 2.5 mL) was added, and the solution was stirred overnight. The solvent was evaporated, and water (10 mL) was added. The aqueous solution was washed with CH_2Cl_2 and then acidified with 3.5 mL of 1 M HCl. The mixture was extracted 2× with 15% 2-propanol in CH_2Cl_2. The solvent was dried over $MgSO_4$, filtered, and evaporated to give 0.52 g (84%) of a white solid; mp 168−170 °C. IR (thin film): 2955, 1713, 1727, 1610, 1226, 901 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.61 (2H, d, $J = 9$ Hz), 6.93 (2H, d, $J = 9$ Hz), 5.19 (1H, dd, $J = 7.2, 9.3$ Hz), 3.85 (3H, s), 3.71 (1H, d, 9.9 Hz), 3.71 (1H, d, 7.5 Hz). ^{13}C NMR: δ 175.82, 162.68, 157.26, 129.34, 122.56, 115.13, 80.06, 55.84, 40.56. FAB MS (M + H): 221. Anal. ($C_{11}H_{11}NO_4$) C, H, N.

2-Thioacetyl-1-[3-(4-methoxyphenyl)-4,5-dihydro-isoxazol-5-yl]ethanone (7). Acid **6** (1.0 g, 4.5 mmol) was stirred in 50 mL of CH_2Cl_2. $SOCl_2$ (15 mL) was added, and the solution was gently heated until it became clear. After it was stirred for 2 h, the solvent was removed and the acid chloride was dissolved in ether (25 mL). This was slowly added to a freshly prepared solution of diazomethane that was made from 4.0 g (18 mmol) of Diazald. After the mixture was allowed to stand at room temperature for 1 h, air was blown over the top to remove the solvent. The crude product was recrystallized from boiling ethanol to give 0.8 g of the α-chloroketone. A portion of this (0.4 mmol, 0.1 g) was dissolved in 7 mL of THF. Thioacetic acid (36 μL, 0.5 mmol) was added, and the solution became cloudy immediately. After 1 h, the solution was evaporated and partitioned between CH_2Cl_2 and water. The organic layer was dried over $MgSO_4$, filtered, and evaporated to give 0.12 g (69% from **6**) of the protected thiol; mp 81 °C. IR (Nujol): 1230, 1692, 1608, 1463, 1254 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.63 (2H, d, $J = 8.8$ Hz), 6.93 (2H, d, $J = 9.2$ Hz), 5.22 (1H, dd, $J = 6, 12$ Hz), 4.08 (1H, d, $J = 17.2$ Hz), 3.99 (1H, d, $J = 17.2$ Hz), 3.84 (3H, s), 3.75 (1H, dd, $J = 6, 17.2$ Hz), 3.55 (1H, dd, $J = 12, 17.2$ Hz), 2.37 (3H, s). ^{13}C NMR: δ 202.86, 194.44, 161.65, 156.68, 128.80, 121.08, 114.49, 83.93, 55.72, 38.58, 36.95, 30.44. FAB MS (M + H): 294. Anal. ($C_{14}H_{15}NO_4S$) C, H, N.

2-Mercapto-1-[3-(4-methoxyphenyl)-4,5-dihydro-isoxazol-5-yl]ethanone (8). Hydrazine (1 M, 0.18 mmol, 0.18 mL) was slowly added to protected thiol **7** (68 mg, 0.23 mmol) that was being stirred in 3 mL of THF. A new spot appeared by TLC (2.5% EtOH in CH_2Cl_2, $R_f = 0.33$) and was purified by prep TLC to give a mixture of the desired product and its dimer. ^1H NMR ($CDCl_3$): δ 7.60 (2H, d, $J = 8.7$ Hz), 6.92 (2H, d, $J = 9$ Hz), 5.25 (1H, dd, $J = 5.7, 11.7$ Hz), 3.83 (3H, s), 3.76−3.64 (3H, m), 3.55 (1H, dd, $J = 11.7, 16.8$ Hz). FAB MS (M + H and 2M + H): 252 and 503.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Hydrazide (9). Carboxylic acid **6** (0.06 g, 0.27 mmol) was stirred in 5 mL of CH_2Cl_2. EDC (0.104 g, 0.54 mmol) was added followed by 85 μL (2.7 mmol) of anhydrous hydrazine. The solution was stirred overnight and then evaporated. Water (10 mL) was added to the residue, and the suspension was sonicated briefly. The resultant white solid was filtered to give 41 mg of product (65%); mp 173−175 °C. IR (Nujol): 3297, 3245, 1689, 1609, 1460, 828 cm^{-1}. ^1H NMR (DMSO-d_6): δ 9.43 (1H, bs), 7.61 (2H, d, $J = 9$ Hz), 7.00 (2H, d, $J = 9$ Hz), 4.98 (1H, dd, $J = 7.5, 11.1$ Hz), 4.36 (1H, bs), 3.79 (3H, s), 3.60 (1H, dd, $J = 11.1, 16.8$ Hz), 3.50 (1H, dd, $J = 7.5, 17.1$ Hz). ^{13}C NMR: δ 168.79, 161.39, 156.41, 129.04, 121.80, 114.97, 78.69, 56.11, 39.30. FAB MS (M + H): 236. Acceptable combustion microanalytical data could not be obtained for this compound.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid(*N*′-formyl-hydrazide) (10). Carboxylic acid **6** (0.6 g, 0.27 mmol) was stirred in 3 mL of $CHCl_3$. EDC (61 mg, 0.32 mmol) was added followed by 18 mg (0.3 mmol) of formyl hydrazine. The solution was sonicated briefly and then allowed to stand at room temperature overnight, and a white precipitate formed. The precipitate was filtered to give 37 mg (52%) of a white solid; mp 195−200 °C (dec). IR (Nujol): 3185, 1684, 1620, 1518, 1463, 1253, 831 cm^{-1}. ^1H NMR (DMSO-d_6, 95 °C): δ 10.05 (1H, bs), 9.75 (1H, bs), 7.99 (1H, bs), 7.61 (2H, d, $J = 9$ Hz), 6.99 (2H, d, $J = 9$ Hz), 5.13 (1H, dd, $J = 7.5, 11.4$ Hz), 3.81 (3H, s), 3.66 (1H, dd, $J = 11.1, 17.1$ Hz), 3.55 (1H, dd, $J = 7.2, 17.1$ Hz). ^{13}C NMR: δ 168.84, 161.47, 159.86, 156.44, 129.13, 121.59, 115.00, 78.37, 56.12, 39.59. FAB MS (M + H): 264. HRMS *m/z* calcd for $C_{12}H_{14}N_3O_4$, 264.0984; found, 264.0974.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Thiazol-2-ylamide (11). Acid **6** (0.27 mmol, 60 mg), 2-aminothiazole (0.27 mmol, 27 mg), and EDC (0.35 mmol, 68 mg) were stirred in 5 mL of CH_2Cl_2 for 4 h. A white precipitate formed. The precipitate was filtered and washed with CH_2Cl_2 to give 32 mg (39%) of **11** as a white powder; mp 210 °C (dec). IR (Nujol): 2855, 2710, 1690, 1608, 1583, 1459, 1361, 1263 cm^{-1}. ^1H NMR ($CDCl_3$): δ 12.5 (1H, bs), 7.63 (1H, d, $J = 8.7$ Hz), 7.50 (1H, d, $J = 3.6$ Hz), 7.27 (1H, d, $J = 3.6$ Hz), 7.01 (2H, d, $J = 8.7$ Hz), 5.33 (1H, t, $J = 9$ Hz), 3.80 (3H, s), 3.69 (2H, d, 9 Hz). ^{13}C NMR: δ 167.66, 160.63, 160.60, 155.78, 137.68, 128.32, 120.75, 114.15, 114.10, 78.06, 55.28, 38.23. FAB MS (M + H): 304. Acceptable combustion microanalytical data could not be obtained for this compound. HRMS *m/z* calcd for $C_{14}H_{14}N_3O_3S$, 304.0756; found, 304.0759.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Pyridin-2-ylamide (12). Acid **6** (60 mg, 0.27 mmol), 2-aminopyridine (25 mg, 0.27 mmol), and EDC (68 mg, 0.35 mmol) were stirred in CH_2Cl_2 for 4 h. The solution was concentrated and purified by prep TLC (3:2 EtOAc:Hex, $R_f = 0.60$) to give 60 mg (75%) of compound **12**; mp 145 °C. IR (Nujol): 3184, 2855, 1702, 1610, 1583, 1435, 1299, 1257, 1177 cm^{-1}. ^1H NMR ($CDCl_3$): δ 9.12 (1H, bs), 8.30 (1H, ddd, $J = 0.6, 1.8, 4.8$ Hz), 8.18 (1H, dt, $J = 1.2, 8.4$ Hz), 7.74−7.66 (1H, m), 7.62 (2H, d, $J = 8.7$ Hz), 7.06 (1H, ddd, 0.9, $J = 4.8, 7.2$ Hz), 6.92 (2H, d, $J = 9$ Hz), 5.22 (1H, dd, $J = 6.9, 10.2$ Hz), 3.84 (3H, s), 3.75 (1H, d, $J = 6.9$ Hz), 3.75 (1H, d, $J = 10.2$ Hz). ^{13}C NMR: δ 169.78, 161.41, 156.53, 150.16, 148.00, 138.10, 128.55, 120.52, 120.26, 114.15, 113.85, 78.61, 55.37, 39.95. FAB MS (M + H): 298. Anal. ($C_{16}H_{15}N_3O_3 + H_2O$) C, H, N.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-sulfonic Acid Amide (13). Chloro-oxime **3** was generated in situ from 0.33 mmol (50 mg) of *p*-anisaldehyde oxime and *N*-chlorosuccinimide (44 mg, 0.33 mmol) as described in the synthesis of **6**. Vinyl sulfonamide[46] (43 mg, 0.4× mmol) was added as a solution in CH_2Cl_2 (10 mL). *N*-Methyl morpholine (44 μL, 0.33 mmol) was added, and the solution was stirred for 2 h. The solution was concentrated and partitioned between EtOAc and water. The organic layer was washed 3× with water, dried over $MgSO_4$, and filtered, and the solvent was evaporated. CH_2Cl_2 was added (3 mL), and a white precipitate formed. The precipitate was filtered to give 45 mg (63%) of a white solid; mp 175−176 °C. IR (Nujol): 3346, 3227, 1613, 1521, 1455,1366, 1338, 1158 cm^{-1}. ^1H NMR (acetone-d_6): δ 7.66 (2H, d, $J = 9$ Hz), 7.00 (2H, d, $J = 9$ Hz), 6.43 (2H, bs), 5.58 (1H, dd, $J = 5.1, 10.8$ Hz), 3.93 (1H, dd, $J = 10.8, 8.3$ Hz), 3.84 (3H, s), 3.82 (1H, dd, $J = 5.4, 18.6$ Hz). ^{13}C NMR: δ 162.39, 157.27, 129.44, 121.17, 115.04, 92.40, 56.04, 38.94. FAB MS (M + H): 257. Anal. ($C_{10}H_9N_2O_4S$) C, H, N.

5-Methanesulfonyl-3-(4-methoxyphenyl)-4,5-dihydro-isoxazole (14). Chloro-oxime **3** (2.74 mmol) was generated from the corresponding oxime as was described in the synthesis of **6**. CH_2Cl_2 was added followed by methyl vinyl sulfone (0.24 mL, 2.74 mmol). Triethylamine (0.38 mL, 2.74 mmol) was slowly added, and the solution was stirred for 1 h. The solution was concentrated and triturated with water. The white solid was filtered, giving 0.5 g (71%) of product; mp 167−168 °C. IR (Nujol): 3015, 1609, 1601, 1521, 1460, 1303, 1131, 1014 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.68 (2H, d, $J = 8.4$ Hz), 7.02 (2H, d, $J = 8.4$ Hz), 5.88 (1H, dd, $J = 5.1, 10.8$ Hz), 3.95 (1H,

dd, J = 10.5, 18.6 Hz), 3.87 (1H, dd, J = 4.8, 18.3 Hz), 3.80 (3H, s), 3.04 (3H, s). ^{13}C NMR: δ 161.09, 156.54, 128.75, 119.59, 114.24, 91.16, 55.36, 36.28, 35.71. FAB MS (M + H): 256. Anal. ($C_{11}H_{13}NO_4S$) C, H, N.

3-(4-Methoxyphenyl)-4,5-dihydro-isoxazole-5-carboxylic Acid Hydroxymethyl-amide (15). Chloro-oxime **23** (0.38 mmol, 70 mg) was stirred in 5 mL of THF. *N*-Hydroxymethyl acrylamide (48% solution in water, 74 μL, 0.38 mmol) was added followed by Et_3N (0.38 mmol, 53 μL). A white precipitate immediately formed. After 20 min, the filtrate was evaporated, MeOH (5 mL) was added, and the mixture was sonicated. The resultant solid was filtered giving 25 mg (26%) of nearly pure product. An analytical sample could be obtained by chromatography (3:1 EtOAc:Hex, R_f = 0.2); mp 152 °C (dec). IR (Nujol): 3353, 3300, 1663, 1611, 1518, 1462, 1254, 1050 cm^{-1}. ^{1}H NMR (acetone-d_6): δ 8.08 (1H, bs), 7.66 (2H, d, J = 9 Hz), 6.99 (2H, d, J = 9 Hz), 5.05 (1H, dd, J = 6.3, 11.7 Hz), 4.78−4.70 (2H, m), 3.84 (3H, s), 3.68 (1H, dd, J = 11.4, 16.8 Hz), 3.59 (1H, dd, J = 6.3, 16.8 Hz). ^{13}C NMR: δ 169.95, 160.61, 155.69, 128.25, 120.83, 114.14, 78.55, 62.29, 55.27, 37.95. FAB MS (M + H): 251. HRMS *m/z* calcd for $C_{12}H_{14}N_2O_4$, 250.0954; found, 250.0943.

[3-(4-Methoxyphenyl)-4,5-dihydro-isoxazol-5-yl]phosphonic Acid Diethyl Ester (16). Chloro-oxime **3** (2.74 mmol) was generated in situ as described in the synthesis of **6**. Diethyl vinylphosphonate (2.74 mmol, 0.42 mL) was added followed by slow addition of Et_3N (2.74 mmol, 0.38 mL). The solution was stirred for 1 h and then concentrated. The residue was dissolved in EtOAc (50 mL), and the mixture was washed 3× with water and once with brine. The solution was dried over $MgSO_4$, filtered, and evaporated onto silica gel. The product was purified by flash chromatography (EtOAc, R_f = 0.3) to give 0.69 g (80%) of **16** as an oil. IR (thin film): 2983, 1609, 1517, 1306, 1255, 1022, 972 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.60 (2H, d, J = 9 Hz), 6.91 (2H, d, J = 9 Hz), 4.83 (1H, ddd, J = 2.1, 10.2, 11.7), 4.28−4.18 (4H, m), 3.83 (3H, s), 3.63 (1H, dd, J = 10.2, 23.7 Hz), 3.63 (1H, dd, J = 11.7, 22.2 Hz), 1.36 (3H, t, J = 7.2 Hz), 1.32 (3H, t, J = 7.2 Hz). ^{13}C NMR: δ 161.19, 155.68 (d, J = 6 Hz), 128.38, 120.90, 114.11, 74.86 (d, J = 168 Hz), 63.57 (d, J = 6.9 Hz), 63.16 (d, J = 6.6 Hz), 55.37, 37.99, 16.59, 16.52. ^{31}P NMR: δ 19.64. FAB MS (M + H): 314. Anal. ($C_{14}H_{20}NO_5P$) C, H, N.

[3-(4-Methoxyphenyl)-4,5-dihydro-isoxazol-5-yl]phosphonic Acid (17). Phosphonate ester **16** (0.25 g, 0.80 mmol) was dissolved in 15 mL of CH_2Cl_2. Bromotrimethylsilane (0.34 mL, 6.4 mmol) was added, and the solution was stirred for 3 d at room temperature. The reaction was quenched by addition of 1 mL of water, and the mixture was stirred for 20 min. The solution was concentrated and made basic by the addition of saturated $NaHCO_3$. The solution was evaporated and dissolved in a minimal amount of water. Concentrated HCl was added to acidify the solution. The white precipitate that formed was filtered and dried to give 121 mg (59%) of **17**; mp 238−240 °C. IR (Nujol): 2775, 2356, 1610, 1516, 1459, 1376, 1180, 1040 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 8.4 (2H, bs), 7.59 (2H, d, J = 8.4 Hz), 6.98 (2H, d, J = 8.7 Hz), 4.62 (1H, t, J = 11.4 Hz), 3.78 (3H, s), 3.74−3.57 (1H, m), 3.47−3.30 (1H, m). ^{13}C NMR: δ 160.49, 155.42, 128.17, 121.17, 114.15, 75.6 (d, J = 162 Hz), 55.31, 37.31. FAB MS (M − H): 256. HRMS *m/z* calcd for $C_{10}H_{11}NO_5P$, 256.0375; found, 256.0381. Anal. ($C_{10}H_{12}NO_5P$) C, H, N.

Methyl-vinyl-phosphinic Acid 2-Chloro-ethyl Ester (19). Methyl dichlorophosphine (0.5× g, 4.3 mmol) was stirred in 25 mL of dry ether under N_2. To this solution was added 0.58 mL (8.6 mmol) of 2-chloroethanol followed by 1.7 mL (22 mmol) of pyridine. The solution was stirred overnight, filtered, and evaporated to give a clear oil. The oil was heated under N_2 in a pressure tube to 160 °C for 3 h. The reaction was allowed to cool, and 0.65 mL (4.7 mmol) of Et_3N was added. The vessel was sealed and heated to 120 °C for 2 h. The residue was purified by Kugelrohr distillation (160 °C, 2 Torr) to give 0.335 g (47%) of an oil identical to the known compound.[47]

Methyl-[3-(4-methoxyphenyl-4,5-dihydro-isoazol-5-yl)-phosphinic Acid 2-Chloro-ethyl Ester (20). Chloro-oxime **3** (0.335, 1.99 mmol) was added to a solution of vinyl phosphinate **19** (0.335 g, 1.99 mmol) in 25 mL of CH_2Cl_2. Et_3N (0.28 mL, 2 mmol) was added to the solution, and it was stirred at room temperature for 1 d. The solution was evaporated onto silica gel and purified by flash chromatography (2.5% EtOH in CH_2Cl_2, R_f = 0.1) to give 0.37 g of **20**. This was dissolved in 15 mL of CH_2Cl_2 and treated with 0.6× mL (4.7 mmol) of bromotrimethylsilane. The solution was stirred for 3 d at room temperature. Water was added (1 mL), and the solution was stirred for 3 h. The solution was evaporated, and the crude residue was recrystallized from a few milliliters of EtOH to give 0.21 g (41%) of **21**; mp 182−183 °C. IR (Nujol): 1609, 1516, 1464, 1306, 1253, 1179, 985 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 7.61 (2H, d, J = 8.4 Hz), 6.99 (2H, d, J = 8.7 Hz), 4.68 (1H, ddd, J = 4.8, 9.9, 15 Hz), 3.78 (3H, s), 3.75−3.39 (2H, m), 1.35 (3H, d, J = 14.4 Hz). ^{31}P NMR: δ 41.29. ^{13}C NMR: δ 160.55, 155.56 (d, J = 7.5 Hz), 128.25, 120.92, 114.11, 77.19 (d, J = 109 Hz), 55.27, 36.05, 12.18 (d, J = 93 Hz). FAB MS (M + H): 256. Anal. ($C_{11}H_{14}NO_4P$) C, H, N.

Thiopropionic Acid *S*-[3-(4-Methoxyphenyl)-4,5-dihydro-isoxazol-5-ylmethyl)ester (22). Chloro-oxime **3** (2.74 mmol) was generated in situ as described in the synthesis of **6**. CH_2Cl_2 (10 mL) was added followed by allyl thiopropionate (2.7 mmol, 0.37 mL). After it was stirred overnight, the solution was evaporated onto silica gel and purified by flash chromatography (3:1 Hex:EtOAc, R_f = 0.52) to give 0.487 g (64%) of a white solid; mp 62 °C. IR (thin film): 2990, 2979, 2832, 1695, 1608, 1518, 1254, 831 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.59 (2H, d, J = 9 Hz), 6.91 (2H, d, J = 9 Hz), 4.91−4.81 (1H, m), 3.83 (3H, s), 3.40 (1H, dd, J = 10.2, 16.8 Hz), 3.23 (1H, dd, J = 6, 14.1 Hz), 3.19 (1H, dd, J = 6.3, 14.1 Hz), 3.06 (1H, dd, J = 6.9, 16.5 Hz), 2.61 (q, 2H, J = 7.8 Hz), 1.18 (3H, t, J = 7.8 Hz). ^{13}C NMR: δ 199.42, 160.97, 155.83, 128.16, 121.75, 114.05, 79.17, 55.38, 39.65, 37.49, 32.63, 9.77. FAB MS (M + H): 280. Anal. ($C_{14}H_{17}NO_3S$) C, H, N.

[3-(4-Methoxyphenyl)-4,5-dihydroisoxaol-5-yl]methanethiol (23). Thioester **22** (75 mg, 0.27 mmol) was stirred in 4 mL of methanol. NaOH (1M in H_2O, 0.27 mL, 0.27 mmol) was added followed by 1 mL of water. The white precipitate that was formed after it was stirred overnight was filtered giving 19 mg (32%) of the thiol; mp 185 °C (dec). IR (Nujol): 1610, 1518, 1461, 1376, 1253, 829 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 7.59 (2H, d, J = 9 Hz), 6.98 (2H, d, J = 9 Hz), 4.96−4.85 (1H, m), 3.78 (3H, s), 3.55 (1H, dd, J = 10.5, 17.1 Hz), 3.22 (1H, dd, J = 6.9, 17.1 Hz), 3.14−2.99 (2H, m). ^{13}C NMR: δ 160.40, 155.80, 128.01, 114.05, 78.74, 55.24, 42.40. FAB MS (M + H): 224. Anal. ($C_{11}H_{13}NO_2S$) C, H, N.

(*R*,R)-3-(4-Methoxy-phenyl)-5-thiiranyl-4,5-dihydro-isoxazole (25a).** (*R*,R**)-3-(4-Methoxy-phenyl)-5-oxiranyl-4,5-dihydro-isoxazole (**24a**, 0.05 g, 0.23 mmol) was dissolved in 1 mL of MeOH. Thiourea (17 mg, 0.23 mmol) was added, and the suspension was heated to 80 °C for 5 h in a pressure tube. After it was cooled, the solution was partitioned between 10 mL each of DCM and H_2O. The organic layer was dried over $MgSO_4$ and evaporated to give 57 mg of a white solid (100%); mp 125−128 °C. IR (Nujol): 1610, 1597, 1518, 1464, 1253, 1180 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.60 (2H, d, J = 9.3 Hz), 6.92 (2H, d, J = 9.3 Hz), 4.88 (1H, ddd, J = 5.1, 6.6, 10.5 Hz), 3.84 (3H, s), 3.40 (1H, dd, J = 10.5, 16.8 Hz), 3.19 (1H, dt, J = 5.7, 6.3 Hz), 3.13 (1H, dd, J = 6.6, 16.8 Hz), 2.47 (1H, dd, J = 1.5, 6.3 Hz), 2.30 (1H, dd, J = 1.5, 5.4 Hz). ^{13}C NMR δ. FAB MS (M + H): 236. Anal. ($C_{12}H_{14}NO_2S$) C, H, N.

(*R*,S)-3-(4-Methoxy-phenyl)-5-thiiranyl-4,5-dihydro-isoxazole (25b).** (*R*,S**)-3-(4-Methoxy-phenyl)-5-oxiranyl-4,5-dihydro-isoxazole (**24b**, 82 mg, 0.37 mmol) was treated exactly as above (**25a**) to give 78 mg (100%) of a while solid; mp 127−130 °C. IR (Nujol): 1611, 1597, 1519, 1457, 1255, 1179 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.61 (2H, d, J = 8.7 Hz), 6.92 (2H, d, J = 8.7 Hz), 4.32 (1H, ddd, J = 6.9, 8.1, 10.5 Hz), 3.84 (3H, s), 3.49 (1H, dd, J = 10.2, 16.5 Hz), 3.33 (1H, dd, J = 6.9, 16.5 Hz), 3.13 (1H, ddd, J = 5.1, 6.0, 8.1 Hz), 2.62 (1H, dd, J = 1.8, 6.0 Hz), 2.37 (1H, dd, J = 1.8, 5.4 Hz). ^{13}C NMR: δ 161.32, 156.08, 128.49, 121.91, 114.38, 85.37, 55.69, 40.63, 36.24, 25.06. FAB MS (M + H): 236. Anal. ($C_{12}H_{13}NO_2S$) C, H, N.

(*R*,S)-2-Mercapto-1-[3-(4-methoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]ethanol (26a).** (*R*,R**)-3-(4-Methoxy-phenyl)-5-oxiranyl-4,5-dihydro-isoxazole (**24a**, 50 mg, 0.23 mmol) was dissolved in 4 mL of THF and 1 mL of H_2O. $(TMS)_2S$ (95 μL, 0.45 mmol, 2 equiv) was added followed quickly by TBAF (1 M in THF, 0.45 mmol, 2 equiv). After it was stirred at 0 °C for 1 h, the solution was evaporated. The residue was treated with 5 mL of boiling MeOH (the solid did not completely dissolve). After the solution was allowed to cool, the white solid was filtered to give pure product (25 mg, 43%); mp 168−170 °C. IR (Nujol): 3481, 1602, 1597, 1530, 1477, 1261 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.57 (2H, d, J = 9.0 Hz), 6.98 (2H, d, J = 9.0 Hz), 4.65−4.55 (1H, m), 4.02 (1H, bs), 3.80−3.74 (1H, m), 3.78 (3H, s), 3.50−3.29 (2H, m), 2.97 (1H, dt, J = 3.6, 13.5 Hz), 2.78 (1H, ddd, J = 8.4, 9.6, 13.2 Hz). ^{13}C NMR: δ 160.54, 155.84, 128.08, 121.89, 114.20, 82.61, 69.45, 55.29, 42.87, 35.7. FAB MS (M + H): 254. Anal. ($C_{12}H_{15}NO_3S$) C, H, N.

(*R*,R)-2-Mercapto-1-[3-(4-methoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]ethanol (26b).** (*R*,S**)-3-(4-Methoxy-phenyl)-5-oxiranyl-4,5-dihydro-isoxazole (**24b**, 50 mg, 0.23 mmol) was dissolved in 5 mL of THF. $(TMS)_2S$ (95 μL, 0.45 mmol, 2 equiv) was added followed immediately by TBAF (1 M in THF, 0.45 mmol, 2 equiv). After it was stirred for 1 h, a new spot was observed by TLC (10% EtOH in DCM, R_f = 0.65). This product was purified by flash chromatography and then recrystallized from MeOH to give 18 mg (31%) of a white solid; mp 170−174 °C. IR (Nujol): 3416, 1610, 1518, 1461, 1255 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.57 (2H, d, J = 8.7 Hz), 6.97 (2H, d, J = 8.7 Hz), 5.18 (1H, dd, J = 3.0, 6.3 Hz), 4.69 (1H, ddd, J = 3.6, 8.4, 12.0 Hz), 3.77 (3H, s), 3.66−3.58 (1H, m), 3.37 (1H, dd, J = 10.2, 16.8 Hz), 3.24 (1H, dd, J = 8.4, 16.8 Hz), 2.75−2.61 (2H, m). ^{13}C NMR: δ 160.23, 155.62, 127.83, 121.84, 114.02, 81.76, 71.24, 55.23, 36.32, 35.28. FAB MS (M + H): 254. Anal. ($C_{12}H_{15}NO_3S + H_2O$) C, H, N.

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