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Metallo-β-lactamase-Catalyzed Hydrolysis of Cephalosporins: Some Mechanistic Insights into the Effect of Heterocyclic Thiones on Enzyme Activity

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The hydrolysis of β-lactam antibiotics using zinc-containing metallo-β-lactamases (mβl) is one of the major bacterial defense systems. These enzymes can catalyze the hydrolysis of a variety of antibiotics including the latest generation of cephalosporins, cephamycins, and imipenem. It is shown in this paper that the cephalosporins having heterocyclic −SR side chains are less prone to mβl-mediated hydrolysis than the antibiotics that do not have such side chains. This is partly due to the inhibition of enzyme activity by the thione moieties eliminated during hydrolysis. When the enzymatic hydrolysis of oxacillin was carried out in the presence of heterocyclic thiones such as MTT, MDT, DMETT, and MMA, the catalytic activity of the enzyme was inhibited significantly by these compounds. Although the heterocyclic −SR moieties eliminated from the β-lactams upon hydrolysis undergo a rapid tautomerism between thione and thiol forms, these compounds act as thiolate ligands toward zinc(II) ions. The structural characterization of two model tetranuclear zinc(II) thiolate complexes indicates that the −SR side chains eliminated from the antibiotics may interact with the zinc(II) metal center of mβl through their sulfur atoms.

Introduction

Antibiotics based on β-lactams such as penicillins, cephalosporins, and penems are the most commonly used drugs for bacterial infections.[1] However, the medicinal applications of most of these antibiotics are limited due to the production of β-lactamases that inactivate the antibiotics by hydrolyzing the β-lactam ring (Scheme 1). The active site of these bacterial enzymes contains either a serine residue (serine-β-lactamases) or zinc(II) ions (metallo-β-lactamases, mβl).[2,3] The metalloenzymes (mβl) represent a unique subset of zinc hydrolases that can hydrolyze a wide range of β-lactam antibiotics, including cephamycins and imipenems, both of which have relatively more stable C−N bonds as compared to the penicillin family of antibiotics.[3] These enzymes also hydrolyze serine-β-lactamase inhibitors such as clavulanic acid.[4] Therefore, the medicinal application of the most commonly used antibiotics is severely compromised in bacteria that produce the metalloenzymes.

The crystal structures of several mβl enzymes reveal a binuclear zinc center in which the two zinc ions are connected by a bridging hydroxide group. As the mβls are not susceptible to inhibition by the classic serine-β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam, considerable attention has been directed toward the design and synthesis of effective mβl inhibitors. These inhibitors include biphenyl tetrazoles, N-arylsulfonyl hydrazones, β-lactams, benzohydroxamic acid analogues, picolinic acids and its derivatives, trifluoromethyl ketones, succinic acids, and thiols such as cysteinyl peptides and thiomandelic acid.[5] Payne et al. demonstrated that mercaptoacetic acid, a product of the hydrolysis of mercaptoacetic acid thiol esters by mβl, binds irreversibly to the enzyme through the formation of a disulfide bond with the active site cysteine residue.[6] Page and co-workers reported that the degradation of cephalosporins by mβl produces thiols that can bind to the zinc center

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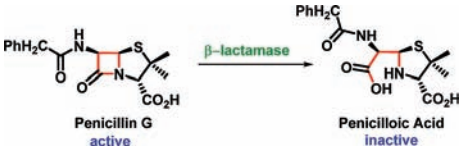
Scheme 1. Enzymatic Hydrolysis of the β-Lactam Ring in Penicillin G by β-Lactamases, Leading to the Formation of Penicilloic Acid



and inhibit the enzyme activity.[7] Kurosaki et al. have shown that the thiols capable of blocking the dinuclear zinc center can irreversibly inhibit the enzyme by forming stable thiolate-bridged complexes.[8]

Recently, we have reported that the mβl-mediated hydrolysis of antibiotics having heterocyclic thiol side chains eliminates thiones, leading to the generation of potent heme peroxidase inhibitors.[9] For example, the hydrolysis of cephalosporins having a thio-tetrazole side chain leads to the formation of 1-methyl-*1H*-tetrazole-5-thione (MTT, **1**; Scheme 2), which effectively inhibits the lactoperoxidase (LPO)-catalyzed iodination reactions. However, it is unknown whether the heterocyclic thiones eliminated from cephalosporins by enzymatic hydrolysis can inhibit the mβl activity. In this paper, we report that the heterocyclic thiones such as MTT eliminated during hydrolysis by the metalloenzyme significantly inhibit the enzyme activity. We also show that the thiones act as good ligands to produce zinc−thiolate complexes, indicating that the inhibition of mβl is due to the binding of thiones to the zinc(II) center.

Experimental Section

Chemicals. The sodium salt of penicillin G and oxacillin were obtained from Fluka. Penicillinase (β-lactamase II, BcII) from *Bacillus cereus* and deuterated solvents $CDCl_3$, D_2O, DMSO-d_6, and CD_3OD were obtained from Sigma-Aldrich. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from other commercial sources and used as received. HPLC-grade solvents for the kinetic experiments were obtained from Merck.

General Procedure. All chemical reactions were carried out in a room atmosphere, unless specifically mentioned otherwise. Buffers of desired pH were freshly prepared prior to use. The HPLC experiments were performed on an analytical HPLC system fitted with a PDA detector controlled by the EMPOWER software (Waters Corporation, Milford MA). The reaction products were isolated and purified by using reverse-phase flash chromatography (Biotage) and preparative HPLC (Waters Corporation, Milford MA) systems. The final products were obtained from the column fractions by freeze-drying the samples on a Lyophilizer (Eyela). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Bruker-Avance 400 NMR Spectrometer. Chemical shifts are cited in ppm with respect to $SiMe_4$ as an internal standard. Mass spectral studies were carried out on a Q-TOF micro mass spectrometer or with ESI-MS mode analysis. Elemental analyses were performed on a Thermo Finnigan CHN analyzer.

HPLC Studies. The kinetics of the hydrolysis of β-lactams were followed with HPLC by using a reverse-phase column. All reactions with BcII were carried out in 50 mM HEPES buffer at a pH of 7.5. The stock solutions of β-lactams and BcII were prepared in a HEPES buffer and 50 mM phosphate buffer at a pH of 7.5, respectively, and used immediately in the experiments

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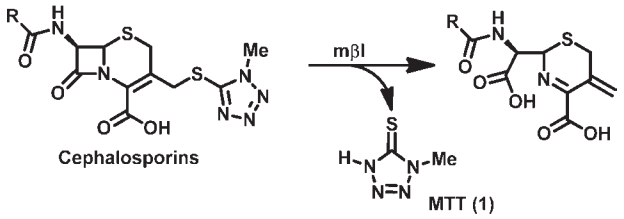
Scheme 2. Elimination of Heterocyclic Thione from the Hydrolysis of the β-Lactam Ring in Certain Cephalosporins by β-Lactamases



to avoid any possible decomposition. The concentration of the stock solution of β-lactams was fixed at 5×10^{-3} M. In a typical kinetic experiment, a sample vial containing 1.5 mL of the test samples containing an appropriate concentration of β-lactam and 0.44 nM BcII enzyme was incubated. At various time intervals, 10 μL aliquots were removed from the reaction mixture and injected directly into the HPLC column. The compounds were eluted in the linear gradient mode with a mixture of acetonitrile and 0.1% TFA (50% to 80% in the case of penicillin G, oxacillin; 30% to 80% in case of moxalactam) over 8.5 min at a flow rate of 1.0 mL/min. The chromatograms were obtained at 254 nm for penicillin G and oxacillin and at 275 nm for moxalactam. The concentration of β-lactam or the hydrolyzed product was determined for each injection from the peak area by using a calibration plot.

Synthesis of Heterocyclic Disulfides. Synthesis of MTT Disulfide (MTTox, 11). To a mixture of 1-methyl-*1H*-tetrazole-5-thione (MTT, 0.58 g, 5.0 mmol) and sodium bicarbonate (0.63 g, 7.5 mmol) was added dropwise a 40 mL solution of I_2 (0.63 g, 2.5 mmol) in a 50 mM KI solution with constant stirring. The reaction was stirred for 45 min, and the resulting mixture was extracted with chloroform. The organic layer was washed with a brine solution and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum conditions to afford a white solid. The product was recrystallized from chloroform to afford MTT disulfide as white crystals. Yield: 0.33 g (26%). ^{1}H NMR ($CDCl_3$) δ (ppm): 4.19 (s, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 35.3, 151.4. ESI-MS (*m*/*z*) calcd for $(M + Na)^+$: 253.0055. Found: 253.0059.

Synthesis of MDT Disulfide (MDTox, 12). To a mixture of 2-methyl-1,3,4-thiadiazole-2-thione (MDT, 0.68 g, 5.1 mmol) and sodium bicarbonate (0.63 g, 7.5 mmol) was added dropwise a 40 mL solution of I_2 (0.63 g, 2.5 mmol) in a 50 mM KI solution with constant stirring. The reaction mixture was stirred for 45 min, and the resulting solution was extracted two times with chloroform (2 × 50 mL). The combined organic layers was washed with brine solution and dried over anhydrous Na_2SO_4. Evaporation of the solvent afforded the product as a white solid. The desired compound was obtained in pure form by recrystallization of the crude solid from chloroform. Yield: 0.63 g (44%). ^{1}H NMR ($CDCl_3$) δ (ppm): 2.79 (s, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 16.1, 166.1, 168.9. ESI-MS (*m*/*z*) calcd for $(M + Na)^+$: 284.9373. Found: 284.9357.

Synthesis of Zinc(II)−Thiolate Complexes. Synthesis of Complex 19. The ligand 2,6-bis((dimethylamino)methyl)-4-methyl phenol (HL1; 0.22 g, 1.0 mmol) in dichloromethane (5 mL) was mixed with 60% NaH (0.04 g, 0.9 mmol) in hexane (25 mL), and the mixture was stirred at room temperature for 1 h to obtain the corresponding sodium phenolate (NaL1). Zn(OAc)$_2$·2H$_2$O (0.33 g, 1.5 mmol) and 1-(2-(dimethylamino)-ethyl)-*1H*-tetrazole-5-thione (DMETT, 0.26 g, 1.5 mmol) were then added, and the stirring was continued for an additional 3 h. This resulted in a turbid solution from which the zinc complex $[Zn_4L1_2(\mu\text{-}OH)_2(DMETT)_4]$ was filtered off as a white solid. The product was dried under vacuum conditions to afford a white crystalline solid. The solid product was dissolved in a dichloromethane/methanol mixture and was kept for

crystallization. Upon standing at room temperature, the product crystallized out as white needles suitable for single crystal X-ray diffraction analysis. Yield: 0.16 g (11%). ^{1}H NMR (CD_3OD), δ (ppm): 2.21 (s, 3H), 2.27 (s, 12H), 2.54 (s, 12H), 2.80 (t, J = 6.8 Hz, 2H), 3.30 (s, 2H), 4.38 (t, J = 6.8 Hz, 2H), 7.49 (t, J = 3.2 Hz, 1H). ^{13}C NMR (CD_3OD), δ (ppm): 19.4, 21.5, 44.5, 44.8, 57.0, 61.1, 63.2, 119.4, 124.2, 132.5, 162.8, 180.3. ESI-MS (m/z) calcd for complex **19** $(M)^+$: 1420.3. Found: 1419.8. Elem anal. calcd (%) for $C_{48}H_{84}N_{24}O_4S_4Zn_4$: C, 38.71; H, 5.93; N, 23.56. Found: C, 39.21; H, 5.88; N, 22.79.

Synthesis of Complex 20. The ligand 2,6-bis((dimethylamino)-methyl)-4-methyl phenol (HL1; 0.22 g, 1.0 mmol) in dichloromethane (5 mL) and 60% NaH (0.04 g, 0.9 mmol) in hexane (25 mL) were mixed and stirred at room temperature for 1 h to obtain the corresponding sodium phenolate (NaL1). $Zn(OAc)_2 \cdot 2H_2O$ (0.33 g, 1.5 mmol) and MDT (0.20 g, 1.5 mmol) were then added, and the stirring was continued for an additional 3 h. This resulted in a turbid solution from which the zinc complex $[Zn_4L1_2(\mu\text{-}OH)_2(MDT)_4]$ was filtered off as a white solid. The product was dried under vacuum conditions to afford a white crystalline solid. The solid product was dissolved in a dichloromethane/methanol mixture and was kept for crystallization. Upon standing at room temperature, the product crystallized out as white needles suitable for single crystal X-ray diffraction analysis. Yield: 0.19 g (15%). ^{1}H NMR (CD_3OD), δ (ppm): 2.21 (s, 3H), 2.36 (s, 3H), 2.80 (t, 2H), 3.28 (s, 12H). ^{13}C NMR (CD_3OD), δ (ppm): 16.2, 20.6, 23.3, 46.5, 60.3, 120.0, 123.1, 132.5, 161.8, 176.3. ESI-MS (m/z) calcd for complex **20** $(M + Na)^+$: 1278.9. Found: 1131.0 $([C_{35}H_{52}N_{10}O_3S_6Zn_4 + Na]^{2+}$ (one MDT and one hydroxide ligands eliminated). Elem anal. calcd (%) for $C_{38}H_{56}N_{12}O_4S_8Zn_4$: C, 36.14; H, 4.47; N, 13.31. Found: C, 35.86; H, 5.02; N, 13.05.

X-Ray Crystallography. X-ray crystallographic studies were carried out on a Bruker CCD diffractometer with graphite-monochromatized Mo Kα radiation (λ = 0.71073 Å) controlled by a pentium-based PC running the SMART software package.[10] Single crystals were mounted at room temperature on the ends of glass fiber, and data were collected at 293 K. Intensity data were measured in frames with increasing ω (width of 0.3°/frame) at a scan speed of 18 s/frame, and the SMART and SAINT software were used for data acquisition and data extraction, respectively. The structures were solved and refined using the SHELXTL software package.[11] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned at idealized locations. Empirical absorption corrections were applied to all structures using SADABS.[12] The perspective views of the complexes were obtained using PLATON[13] or ORTEP.[14]

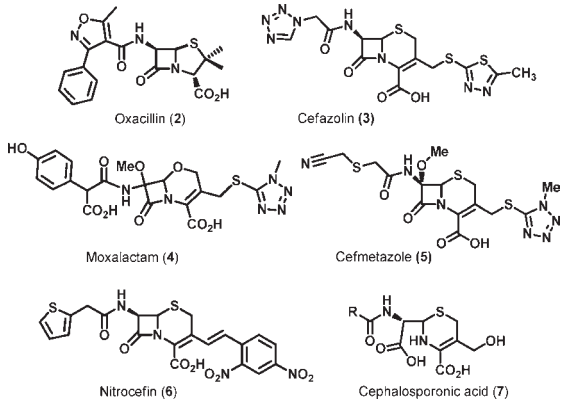


Figure 1. Chemical structures of the cephalosporins having various substitution at the 3′ position.

Computational Methods

All calculations were performed using the Gaussian 03 suite of quantum chemical programs.[15] The hybrid Becke-3-Lee−Yang−Parr (B3LYP) exchange correlation functional was applied for DFT calculations.[16] Geometries were fully optimized at the B3LYP level of theory using 6-31+G(d) basis sets. All stationary points were characterized as minima by corresponding Hessian indices. Transition states were optimized using the TS keyword. Furthermore, the transition states and the stable conformers were characterized by the presence or absence of a single imaginary frequency. IRC calculations[17] were done at the B3LYP/6-31+G(d) level on B3LYP/6-311++G(d,p) level-optimized geometries to verify a transition structure of the hydrogen transfer involved in the interconversion of different tautomeric forms. The activation energies are the difference in the electronic energy corrected for zero-point vibrational energy between the transition state and the stable conformations.

Results and Discussion

Hydrolysis of Cephalosporins by Metallo-β-lactamase. It is known that the hydrolysis of the penicillin family of antibiotics (e.g., penicillin G and oxacillin (**2**)) by β-lactamases is generally much faster than that of most of the cephalosporins (e.g., cefazolin (**3**), moxalactam (**4**), and cefmetazole (**5**)) and penem-based compounds. It has also been shown that the hydrolysis of cephalosporins by either mβls or transpeptidase leads to the elimination of the side chain at the 3′ position with the formation of cephalosporonic acid (**7**) (Figure 1).[18] Page et al. have reported an interesting observation that the hydrolysis of cephalosporins generates dihydrothiazines, which undergo isomerization at C-6 by the cleavage of the C−S bond to produce the corresponding thiol intermediates.[7] They have shown that these thiol intermediates generated during the reaction can be trapped by β-lactamase from *Bacillus cereus*, causing inhibition of the enzyme.

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Table 1. The Initial Rate (v_0) and % Conversion for the Hydrolyses of Penicillins and Cephalosporins by BcII at Room Temperature

entry	substrate	[BcII] (M)	v_0 (μM min^{-1})[a]	time (min)[b]	conversion (%)
a	penicillin G[c]	3.04×10^{-8}	4.675×10^{2}	80	100
b	oxacillin (**2**)[c]	3.04×10^{-8}	2.022	412	50
c	moxalactam (**4**)[c]	3.04×10^{-7}	0.927	487	10
d	cefmetazole (**5**)[c]	3.04×10^{-7}	0.354	666	05
e	nitrocefin (**6**)[d]	2.36×10^{-7}	0.449	50	50

[a] Calculated from the initial 5−10% of the reaction by following the decrease in the peak area due to substrates (β-lactam). [b] Time required for the % conversion of the hydrolysis of the substrate was determined in 50 mM HEPES buffer, pH 7.5, by following the decrease in the peak area due to β-lactams. [c] [β-lactam]: 1.00×10^{-3} M. [d] The hydrolysis was followed by UV−vis spectrophotometry under identical conditions. [nitrocefin]: 0.05×10^{-3} M. DMSO was used to dissolve nitrocefin, but its concentration does not exceed 2.5% in the reaction mixture.

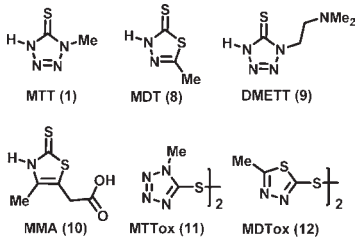

Figure 2. Thione and disulfide forms of the side chains eliminated during the hydrolysis of various cephalosporins.

Recently, we have shown that the mβl-catalyzed hydrolysis of antibiotics having heterocyclic −SR side chains eliminates the thiol moieties in addition to the C−N bond cleavage at the β-lactam ring. These thiols undergo a rapid tautomerism to produce the corresponding thiones,[9] which may bind to the enzyme active site, leading to a significant product inhibition at higher concentrations of the products. To test this hypothesis, we have studied the hydrolysis of various β-lactams by the binuclear metallo-β-lactamase from *Bacillus cereus* (BcII) using a HPLC method. The amounts of products formed at a given time were determined from the peak areas. For the hydrolysis of the chromogenic substrate nitrocefin (**6**), an UV−vis method was employed. The initial rates (v_0) for the hydrolysis were calculated by following the reactions during the first 5−10% of the conversion, where the concentrations of the products were relatively low.

As expected, penicillin G and oxacillin were hydrolyzed by mβl much faster than the cephalosporins (Table 1). The initial rate (v_0) for the hydrolysis of penicillin G was found to be almost 2 orders of magnitude higher than that of oxacillin (Table 1, entry a and b). In contrast to oxacillin and cephalosporins, a complete hydrolysis was observed when penicillin G was used as the substrate. Although the BcII enzyme was able to hydrolyze the cephalosporins at higher concentrations (3.04×10^{-7} M), the initial rates were found to be much lower than that observed for penicillin G and oxacillin. However, a significant decrease in the reaction rates was observed after ∼5−10% of the hydrolysis. For example, moxalactam (Table 1, entry c), which belongs to the latest generation of the cephalosporin family of antibiotics, was hydrolyzed much faster in the beginning of the reaction (v_0: 0.927 μM min^{-1}), but the reaction rate became very slow after ∼10% of the substrate was hydrolyzed. Similarly, the hydrolysis of cefmetazole (Table 1, entry d) was also found to be very slow, and only 5% hydrolysis was observed after 666 min. In contrast, the hydrolysis of nitrocefin (Table 1, entry e) was much faster than that of moxalactam and cefmetazole. Although moxalactam, cefmetazole, and nitrocefin belong to the family of cephalosporins, nitrocefin does not contain any heterocyclic thiols at the 3′ position. These observations indicate that the thiols/thiones eliminated during the hydrolysis may inhibit the enzyme activity by coordinating to the metal center at the active site.

Table 2. Inhibition of Metallo-β-lactamase Activity by Various Heterocyclic Thiones

		inhibition of mβl[a]	
s. no.	compound	IC_{50} (μM)[b]	K_i (μM)
1	MTT (**1**)	65.78 ± 3.55	18.33 ± 1.24
2	MDT (**8**)	47.55 ± 0.02	15.07 ± 0.48
3	DMETT (**9**)	36.64 ± 2.21	9.77 ± 0.45
4	MMA (**10**)	43.64 ± 2.89	15.57 ± 1.12
5	MTT_{ox} (**11**)	47.40 ± 4.80	17.51 ± 0.74
6	MDT_{ox} (**12**)	23.75 ± 0.16	3.73 ± 0.12
7	MAA (**13**)	0.68 ± 0.01	0.25 ± 0.02
8	MPA (**14**)	2.98 ± 0.35	0.94 ± 0.30

[a] Inhibition studies were carried out for the mβl-mediated hydrolysis of oxacillin. [BcII] = 44.0 nM; [oxacillin] = 1×10^{-3} M; 50 mM HEPES, pH = 7.5. [b] The IC_{50} values were determined from the inhibition plots obtained by plotting the percentage control activity against the concentration of inhibitors.

When the hydrolysis of oxacillin by mβl was carried out in the presence of heterocyclic thiones, the rate of hydrolysis decreased with an increase in the concentration of thiones. This indicates that the thiones inhibit the mβl activity, and the inhibition potency depends on the nature of thiones (Figure 2). The variation in the IC_{50} values for the inhibition of mβl activity by various thiones (Table 2) is probably due to the differences in the structure of the thiones. For example, MDT having a thiadiazole moiety is more efficient (IC_{50}: 47.50 ± 0.02 μM) than MTT that contains a tetrazole ring (IC_{50}: 65.78 ± 3.55 μM). Similarly, DMETT (**9**) and 2-(2-mercapto-4-methylthiazol-5-yl)acetic acid (MMA, **10**), which are present in the extended spectrum third generation antibiotics cefodizime[19] and cefotiam,[20] respectively, are more effective than MTT. Although DMETT contains a tetrazole ring similar to that of MTT, the presence of a $-CH_2CH_2-NMe_2$ group may increase the reactivity of the sulfur moiety. Furthermore, the amino group may provide a further stabilization to the enzyme−inhibitor complex if the inhibition is due to the coordination of the sulfur

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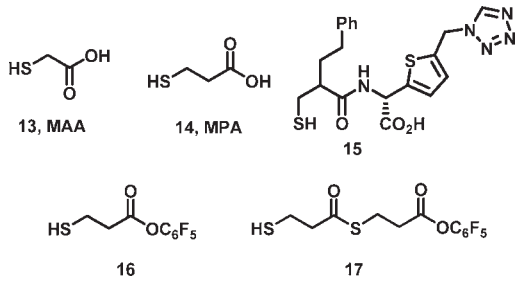

Figure 3. Chemical structures of some aliphatic thiols used as inhibitors for mβls.

moiety to the zinc(II) center. It has been previously shown that the *N*,*N*-dimethyl amino group deprotonates thiols/selenols to generate more reactive thiolates/selenolates, which can enhance the nucleophilicity of thiols/selenols.[21] The disulfides MTT_{ox} (**11**) and MDT_{ox} (**12**) also inhibited the enzyme activity, as these disulfides are converted rapidly to the corresponding thiones MTT and MDT, respectively, in the assay mixture (Figure S11, Supporting Information). As cleavage of the disulfide bond can produce two thiones, the IC_{50} values for the disulfides were found to be lower than that of the corresponding thiones.

The irreversible inhibition of mβl from *Bacillus cereus* (BcII) by mercapto acetic acid thiol ester has been ascribed to the cleavage of the thiol ester bond in this compound during the hydrolysis to produce mercapto acetic acid (**13**, MAA, Figure 3). While compound **13** has been shown to form a disulfide bond with the active site cysteine,[6] larger mercaptocarboxylates such as **15** have been shown to induce conformational changes at the active site of IMP-1 mβl from *Pseudomonos aeruginosa*.[22] This conformational change allows the thiolates to bridge between two zinc(II) ions and the carboxylates to bind to Lys224 residue at the active site.[22] Recently, Kurosaki et al. have shown that the pentafluoro phenyl-based thiols **16** and **17** inhibit the mβl from *Pseudomonoas aeruginosa* by binding to the zinc(II) sites in a bridging mode.[8] These observations lead to an assumption that MMA (**10**), which contains both thione and carboxylic acid moieties, may inhibit mβl in a similar way. However, the IC_{50} value obtained for MMA (43.64 ± 2.89 μM) indicates that this compound is a relatively poor inhibitor of mβl. This is probably due to the rigidity of the heterocyclic ring, which may not allow the sulfur moiety to act as a bridging ligand for the bimetallic center. The thiol−thione tautomerism in this compound may also reduce its possibility of acting as a thiolate bridge.

Although MTT and the related thiones inhibit mβl-mediated hydrolysis, these compounds are found to be less effective as compared to other aliphatic thiols such as mercapto acetic acid (MAA, **13**)[6] and mercapto propionic acid (MPA, **14**)[8] that have been shown to be tight-binding inhibitors for mβl. For example, the IC_{50} values observed

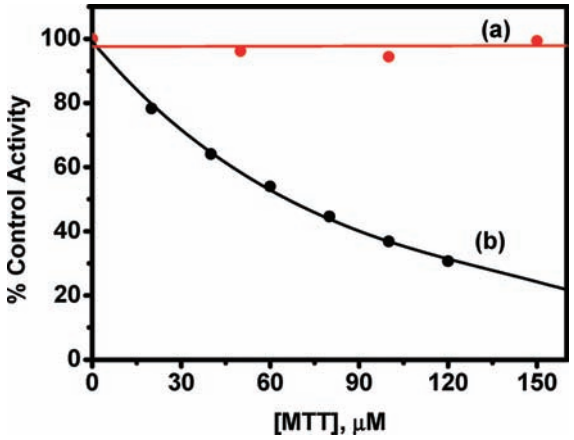

Figure 4. Inhibition of β-lactamase activity by MTT (a) for serine-β-lactamase and (b) metallo-β-lactamase. Conditions: [mβl] = 4.44×10^{-8} M; [sβl] = 1.67×10^{-7} M; [oxacillin] = 1×10^{-3} M; 50 mM HEPES, pH 7.5.

Table 3. Calculated Energy for Thiol−Water, Transition State−Water, and Thione−Water Complexes

	Δ(*E* + ZPE)		Δ(*H* + ZPE)		Δ(*G* + ZPE)	
	− H_2O	+ H_2O	− H_2O	+ H_2O	− H_2O	+ H_2O
1-thiol	0	0	0	0	0	0
1-TS	32.12	6.40	31.78	5.42	32.22	7.59
1-thione	−7.81	−8.74	−8.10	−8.86	−7.58	8.49
8-thiol	0	0	0	0	0	0
8-TS	28.75	6.92	37.90	5.88	37.97	8.33
8-thione	−9.24	−9.96	−9.52	−10.07	−8.96	−9.76
9-thiol	0	0	0	0	0	0
9-TS	31.89	6.48	31.47	5.47	32.55	7.84
9-thione	−7.54	−8.69	−7.91	−8.78	−6.69	−8.49

for MAA (0.68 ± 0.10 μM) and MPA (2.98 ± 0.35 μM) are much lower than that of MTT (65.78 ± 3.55 μM) and other heterocyclic thiones, as shown in Table 2. This indicates that the sulfur moiety in MTT and related compounds may be different from that of aliphatic thiols. As previously mentioned, the aliphatic thiols **13** and **14** have been shown to inhibit the β-lactamase activity by forming a disulfide bond with the active site cysteine residue under aerobic conditions.[6] To understand whether the heterocyclic thiones inhibit the mβl activity by a similar mechanism, we have studied the inhibition of a serine-β-lactamase, which also has cysteine residues in the active site. These experiments reveal that the heterocyclic thiones do not inhibit the serine-β-lactamase (Figure 4), indicating that the inhibition of metallo-β-lactamase by heterocyclic thiones is not due to the binding of these compounds with cysteine residues, but it is due to the coordination of the thiones to the metal center.

Theoretical Studies on Thiol−Thione Tautomerism. From Table 2, it is clear that the nature of the heterocycle plays an important role in the inhibition of mβl activity. For example, DMETT, having a *tert*-amino group, and MDT, having a thiadiazole moiety, are more efficient than MTT, having an N-methyl tetrazole moiety. In addition to the substituents, the thiol−thione tautomerism may also play an important role in the inhibition as the mβl-catalyzed hydrolysis of cephalosporins (e.g., **4**−**6**) eliminates heterocyclic thiols, which undergo a tautomerism in solution. To understand the tautomeric

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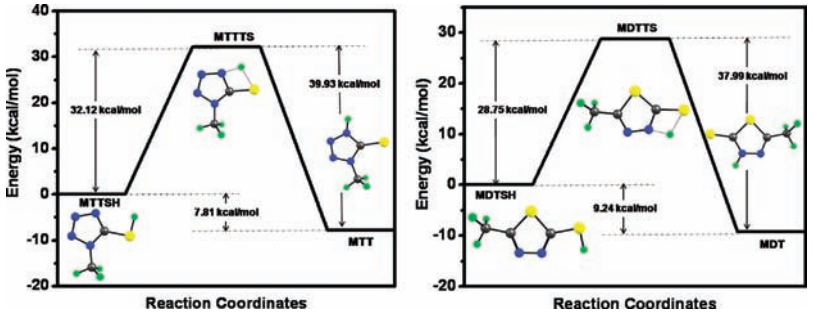

Figure 5. The potential energy profile for the conversion of MTT-thiol and MDT-thiol to MTT-thione and MDT-thione, respectively.

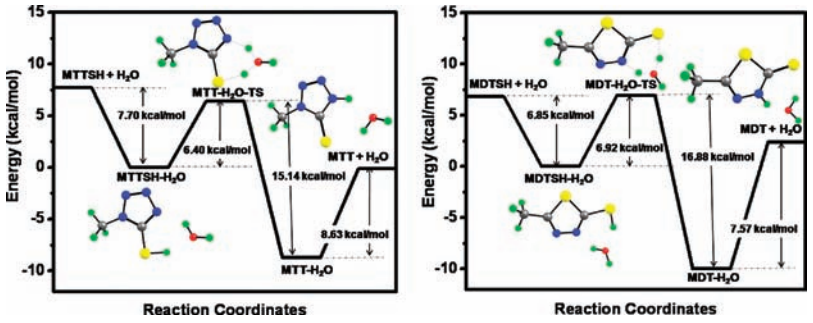

Figure 6. The potential energy profile for the water-assisted conversion of MTT-thiol and MDT-thiol to MTT-thione and MDT-thione, respectively.

behavior of MTT, MDT, and DMETT, we have performed density functional theory (DFT) calculations. The geometries of thiols, thiones, and transition states were optimized in the gas phase at the B3LYP/6-311++G(d,p) level of theory. The relative electronic energies (including the ZPVE correction) for the tautomerism are summarized in Table 3. It has been proposed that the conversion of thiols to the correponding thiones in mercapto thiadiazoles takes place in-plane *via* an intramolecular 1,3 hydrogen shift.[23] However, the activation energy barriers for the conversion of thiols to thiones are very high, although the thione tautomers are more stable than the thiols (Table 3). This indicates that the transfer of hydrogen from the exocyclic sulfur atom to the ring nitrogen *via* a 1,3 shift is not a favored process, as this process produces an unfavored, high energy transition state involving a four-membered ring, as shown in Figure 5.

Kryachko et al. have reported that the keto−enol tautomerism in uracil-based compounds can be facilitated by water molecules.[24] In this particular work, the authors have shown that water molecules transfer hydrogen between oxygen and nitrogen atoms of different uracil tautomers.[24] As the tautomerism of thiols to thiones in the heterocycles eliminated during the hydrolysis of antibiotics by mβl can also be mediated by water molecules, we have studied the effect of a water molecule on the thiol−thione tautomerism. Interestingly, the introduction of one water molecule in the calculations dramatically reduced the energy barriers for the tautomerism (Table 3). For example, the energy required for the formation of MTT (**1**)-TS from MTT (**1**)-thiol in the presence of a water molecule (6.40 kcal mol^{-1}) is almost 5 times lower than the energy required (32.12 kcal mol^{-1}) in the absence of water. Although the relative energy differences between the thiol and thione tautomers are almost identical in the presence and absence of water, the hydrogen transfer in the presence of a water molecule proceeds *via* a low energy six-membered transition state, as shown in Figure 6. This is in agreement with the report of Trujillo et al. that the presence of water molecules considerably reduces the energy barriers for the tautomerism involving selenouracils.[25] Although the relative energies of the thiones have been shown to be much lower than that of the corresponding thiols,[26] the thiones may have significant thiolate character after binding to the metal ion.

The thiol−thione tautomerism in DMETT (**9**) is particularly interesting, as the tertiary amino group attached to one of the nitrogen atoms may abstract the proton to produce a reactive thiolate anion, which could also be responsible for the inhibition. It is expected that both the thiol−thione conversion and thiolate formation lead to the generation of nucleophilic sulfur species. It has been shown previously that the deprotonation of selenols by *N*,*N*-dialkylamino substituents leads to the formation of more reactive selenolates, which significantly enhances the biological activity of selenium compounds.[21] Therefore, we have performed theoretical calculations to find

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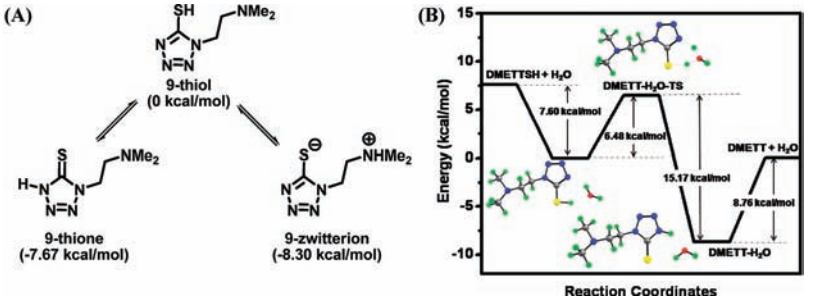

Figure 7. (A) Possible tautomeric conversions in DMETT (**9**). (B) Water-assisted thiol−thione tautomerism in DMETT.

out whether the transfer of hydrogen from the thiol moiety to ring nitrogen is more favored than the hydrogen abstraction by a basic amino group. These calculations suggest that the water-assisted tautomerism of DMETT (**9**)-thiol to DMETT (**9**)-thione (Figure 7) is more favored than the formation of DMETT (**9**)-zwitterion *via* abstraction of the S−H proton by the tertiary amino group. This is probably due to the formation of a less favored transition state involving a seven-membered ring and the comparable energies between the thione and DMETT (**9**)-zwitterion.

Synthesis and Structural Characterization of Model Zinc(II)−Thiolate Complexes. To understand the coordination properties of heterocyclic thiones toward zinc(II) ions, we have studied the interactions of MDT and DMETT with the binuclear zinc(II) complex **18** by using ESI-MS spectrometric techniques. It should be noted that complex **18** has been shown to mimic the activity of mβl by hydrolyzing a variety of β-lactam substrates.[27] When complex **18** was treated with DMETT in methanol, formation of the tetranuclear zinc(II) complex **19** was observed (Figure S12, Supporting Information). Similarly, the addition of MDT to complex **18** led to the formation of another tetranuclear zinc(II) complex **20**. However, the stability of complexes **19** and **20** was found to be poor, and ligand dissociation was observed under mass spectrometric conditions. In the presence of water, complexes **19** and **20** underwent rapid ligand exchange reactions to produce complex **21** (Figure 8). Although the mass spectral data indicated the formation of complex **21**, this complex could not be isolated.

Our attempts to crystallize the tetranuclear complexes **19** and **20** from the reactions of complex **18** with DMETT and MDT, respectively, were unsuccessful. Therefore, we have approached the synthesis of these complexes by following a different route. Treatment of the sodium salt of 2,6-bis((dimethylamino)methyl)-4-methyl phenol (HL1) with $Zn(OAc)_2 \cdot 2H_2O$ and DMETT afforded complex **19**. Crystals suitable for single-crystal X-ray diffraction studies were obtained from a methanolic solution. The synthesis of complex **19** was found to be reproducible, and the crystallization in dichloromethane/MeOH by using different concentrations of the reaction product invariably afforded complex **19**. The X-ray crystal structure of complex **19** reveals that the two identical dinuclear $Zn_2(L1)(DMETT)_2$ subunits are connected to each other *via* hydroxo bridges (Figure 9). The four zinc(II) ions are

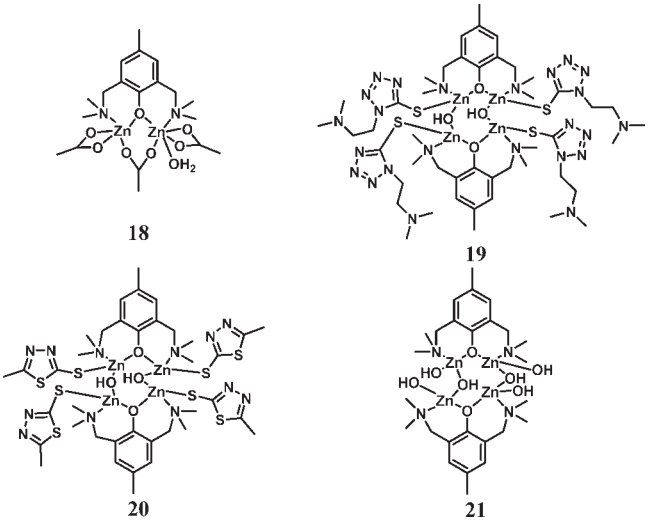

Figure 8. Chemical structures of the dinuclear complex **18**, tetranuclear zinc(II)−thiolate complexes **19** and **20**, and tetranuclear hydoxo complex **21**.

located at the corners of a nearly planar rectangle, and the two zinc ions in each subunit are bridged by two phenolate oxygen atoms. The coordination arrangement around each zinc(II) ion is tetrahedral with one amino coordination, bridging phenolate and hydroxo ligands and a terminal thiolate ligand. Interestingly, the $-NCH_2-CH_2NMe_2$ group present in the DMETT moiety is not involved in coordination to the metal ion. The Zn−O bond lengths of the phenolate oxygen atom O(1) and two zinc(II) ions are similar to that of complex **18** and are within the usual range observed for other zinc−phenolate complexes.[27,28] The N−Zn bond lengths in this complex (2.090 and 2.119 Å) are also comparable to that of complex **18** (Zn−N: 2.068 and 2.202 Å).[28] The Zn···Zn distances (3.422 and 3.603 Å) are longer than those observed for other related zinc−phenolate complexes, which normally vary from 3.1 to 3.4 Å.[28] The Zn−S bond lengths (2.292 and 2.306 Å) are comparable to those of similar zinc−thiolate complexes (2.282 and 2.314 Å) reported by Seebacher et al.[29]

Treatment of the sodium salt of HL1 with $Zn(OAc)_2 \cdot 2H_2O$ followed by the addition of MDT instead of DMETT resulted in the formation of complex **20**. Crystals suitable for X-ray diffraction studies were obtained

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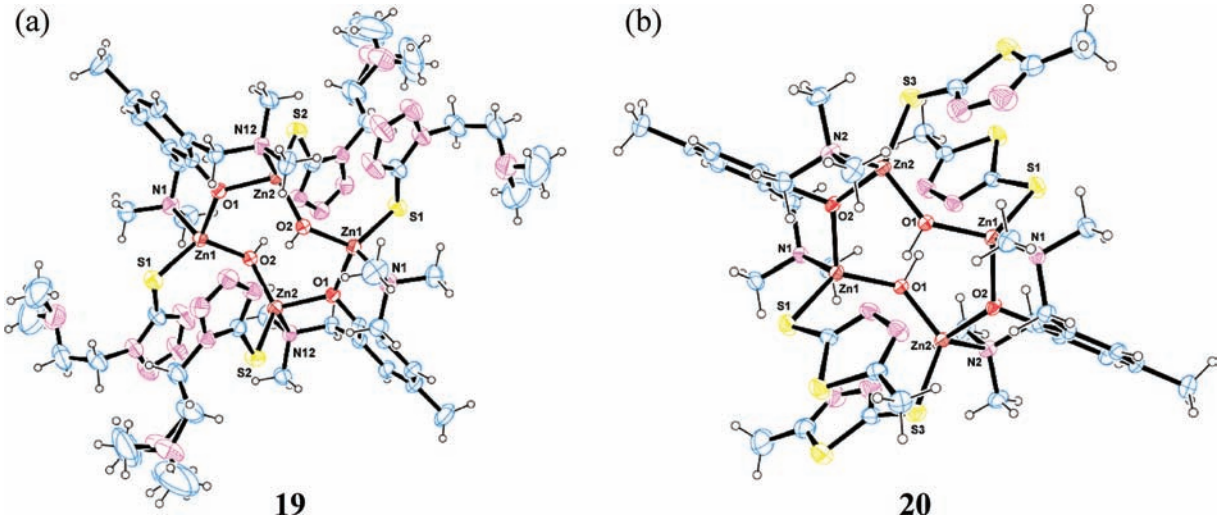

Figure 9. Crystal structures of the tetranuclear zinc complexes **19** and **20** having four DMETT and MDT ligands, respectively.

from a methanolic solution. The X-ray diffraction studies reveal that complex **20** is structurally similar to complex **19**, and each of the two zinc ions within the subunit is bridged by phenolate oxygen atoms (Figure 9). The tetrahedral arrangements of ligands around the metal center in this complex are almost identical to that of complex **19**. Although DMETT and MDT can act as N-donor or bridging ligands, no such coordination modes were observed in complexes **19** and **20**.[30] These observations indicate that the inhibition of mβl activity by the heterocyclic thiols may be due to the coordination of thiolates to the zinc(II) centers. Although the thiolate ligands in complexes **19** and **20** are very labile and can be readily replaced by other ligands, the significant inhibition of mβl activity by these thiolates indicates that some additional factors such as hydrogen bonding may influence the thiol/thione binding to the enzyme active site.[31]

Conclusions

In this paper, we have shown that the metallo-β-lactamase (mβl)-catalyzed hydrolysis of the cephalosporins having heterocyclic thiol side chains is much slower than that of the β-lactam antibiotics lacking such side chains. This is partly due to the inhibition of enzyme activity by the thiol moieties eliminated during the hydrolysis. Although the heterocyclic thiols eliminated from the β-lactams upon hydrolysis undergo a rapid tautomerism to produce the corresponding thiones, these compounds act as thiolate ligands toward zinc(II) ions. The structural characterization of two model tetranuclear zinc(II) complexes indicates that the thiolates can form reasonably stable complexes with a zinc(II) metal center. This study suggests that the introduction of $-$SR moieties in cephalosporins that can strongly inhibit the β-lactamase activity upon cleavage may help in designing new β-lactam antibiotics with enhanced resistance to β-lactamases.

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Supporting Information Available: More details on the effect of various thiones on the β-lactamase activity of metallo-β-lactamase, the HPLC chromatograms of the MTTox and MDTox at physiological pH, and the ESI-MS spectrum obtained from reaction of dinuclear zinc(II) complex **18** with DMETT in methanol. This material is available free of charge via the Internet at http://pubs.acs.org/.

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