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Inhibition of Metallo-β-Lactamases by a Series of Mercaptoacetic Acid Thiol Ester Derivatives

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A series of mercaptoacetic acid thiol esters have been identified as metallo-β-lactamase inhibitors. Electrospray mass spectrometry (ESMS) has shown that irreversible inhibition of the *Bacillus cereus* II metallo-β-lactamase by SB214751, SB214752, and SB213079 was concomitant with a 90-Da increase in mass of the enzyme. Tryptic digestion of the *B. cereus* II inhibited with SB214751 illustrated that the peptide fragment, containing the only cysteine of the enzyme, had undergone a mass increment of 90 Da. It was further demonstrated that *B. cereus* II hydrolyzed this type of compound across the thiol ester bond to yield mercaptoacetic acid. Mercaptoacetic acid is the only molecular fragment common to SB214751, SB214752, and SB213079, and free mercaptoacetic acid does not bind covalently to *B. cereus* II. Therefore, it is concluded that these compounds inhibit *B. cereus* II by the mechanism-based delivery of mercaptoacetic acid, forming a disulfide linkage with the active site cysteine (predicted mass shift = +90 Da) under the aerobic conditions of the assay. The different thiol esters examined had a broad range of potencies against the metallo-β-lactamases tested. For example SB214751, SB214752, and SB213079 all had 50% inhibitory concentrations of <10 and >1,000 μM for the *Stenotrophomonas maltophilia* L-1 and *Bacteroides fragilis* CfiA enzymes, respectively. SB216968 was particularly active against the *Aeromonas hydrophila* CphA metallo-β-lactamase and was found to be an uncompetitive inhibitor of this enzyme (K_i = 3.9 μM), whereas it exhibited irreversible inhibition of the L-1 enzyme. These observations with this series of compounds have revealed subtle differences between the active sites of different metallo-β-lactamases. Finally, a novel application for isothermal titration calorimetry for assessing the zinc chelating activity of candidate inhibitors is also presented.

Carbapenems have a broad spectrum of antibacterial activity, and they are resistant to hydrolysis by the majority of serine-based β-lactamases. For these reasons the medical community will become increasingly dependent on this type of antibiotic and its usage will increase. However, most metallo-β-lactamases hydrolyze the majority of commercial β-lactam antibiotics, including carbapenems. As plasmid-encoded metallo-β-lactamases are now produced by common clinical pathogens (e.g., *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Bacteroides fragilis* [12, 17, 23]) and the selective pressure for these enzymes is increasing, it is probable that the prevalence of metallo-β-lactamases will increase.

The metallo-β-lactamases produced by these different organisms have homologous active sites. The earliest structural studies with the prototypic *Bacillus cereus* II enzyme concluded that the active site Zn^{2+} cation was coordinated by histidine residues and (distantly) by the only cysteine residue of the enzyme, Cys168 (21). Early biochemical work with this enzyme has shown that this cysteine residue is accessible to thiol reagents, such as Ellman's reagent, and also to iodoacetic acid, only in the absence of Zn^{2+}, suggesting that the reactivity of the cysteine thiol group is downmodulated in the presence of the metal cation (20). More recently, an X-ray crystallographic study of *B. cereus* II at 2.5 A refined the structure as a mononuclear zinc hydrolase resembling the metallo zinc carboxypeptidases (4). However, the most recent, high-resolution, synchrotron radiation studies have revealed that the complete structures, both of this enzyme at 1.9 A (8) and of the *B. fragilis* CcrA metallo β-lactamase (CcrA has an amino acid sequence identical to that of CfiA) (5), possess a dinuclear zinc active site. In these structures, the cysteine is seen at last to fulfill a satisfactory role as a ligand to the second zinc atom.

One approach to overcoming the threat of metallo-β-lactamase would be the discovery or design of inhibitors of these enzymes to be used in combination with β-lactam antibiotics. We report the discovery of a series of thiol ester (thiadepsipeptide) metallo-β-lactamase inhibitors. Figure 1 shows the structural relationship of these inhibitors compared with the generalized structure of metallo-β-lactamase substrates. Representatives of this class of thiol esters have been shown to be substrates for serine and metallo-β-lactamases (25, 26) and D,D-peptidases (6, 11, 13) and are slowly hydrolyzed by the *Streptomyces albus* G D,D-metalloprotease (1, 6). The study investigating the interaction of thiol esters with *Streptomyces* K15 active site serine D,D-transpeptidase deployed SB214751 in its racemic form (11). The present study describes the interaction of the resolved enantiomeric forms of this compound and other related compounds with metallo-β-lactamases. Data are presented on the interesting inhibition of the *Aeromonas hydrophila* CphA and *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) L-1 metallo-β-lactamases. However, our detailed mechanistic studies have focused on the inhibition of the *B. cereus* II enzyme, as purified quantities of this enzyme were readily available.

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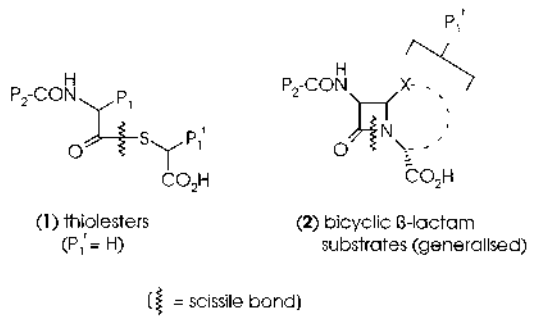


FIG. 1. Relationship between the thiol ester metallo-β-lactamase inhibitors (1) and bicyclic β-lactam substrates (2) of the metallo-β-lactamases. P_2, P_1, and P_1', terminology for specificity of subsites as developed for proteases and their substrates after the recommendations of Berger and Schechter (3).

MATERIALS AND METHODS

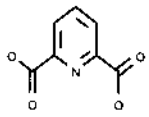
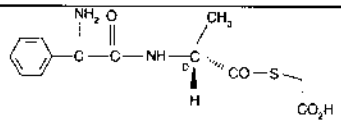
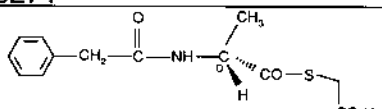
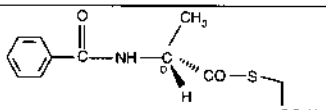
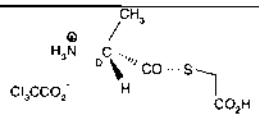
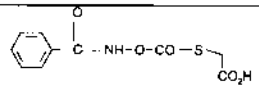
Bacterial strains and enzymes. *B. cereus* II metallo-β-lactamase was purchased from Porton Products (Porton Products Ltd., Maidenhead, Berkshire, United Kingdom), and the L-1 and CphA metallo-β-lactamases were partially purified as described previously from *S. maltophilia* 511 (10) and *A. hydrophila* AE036 (16), respectively. The CfiA-type enzyme was obtained from *B. fragilis* 262; this was used as a crude preparation, as no other β-lactamases were produced by the strain (14).

The mercaptoacetic acid thiol ester inhibitors. SB216271, SB214751, and SB214752 are *N*-acylated variants of the thiol ester SB216968, 2-(D-alanylthio) acetic acid, trichloroacetate salt. SB216271 and SB214751 contain P_2-type recognition features associated, respectively, with the ampicillin and benzyl penicillin substrates of metallo-β-lactamases. SB213079 contains an *O*-acylated hydroxamic acid functionality (Table 1).

The thiol esters in Table 1 were prepared by methods similar to those previously described (6). The following synthesis of the *N*-phenylacetyl D-alanyl analog SB214751, the principal compound of the present investigation, is typical of the procedure. Phenylacetyl D-alanine (1 mol equivalent) was added to ethyl chloroformate (1 mol equivalent) and triethylamine (2 mol equivalent) in dimethylformamide-ethyl acetate (1:1), with stirring, at −5°C. Mercaptoacetic acid (1 mol equivalent) and triethylamine (1 mol equivalent) were added and stirring was continued, first at room temperature and then at 50°C. The product was converted to the sodium salt (sodium hydrogencarbonate, 1 mol equivalent) and purified by HP20SS chromatography, affording SB 214751 as its sodium salt.

Determination of IC_{50}s. The 50% inhibitory concentrations (IC_{50}s) were determined by previously described methodology (18). IC_{50}s for *B. cereus* II, *S. maltophilia* L-1, and *B. fragilis* CfiA β-lactamases were determined with 40 μM nitrocefin as reporter substrate, using 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer at 1 μM and 100 μM Zn^{2+} (pH 7.0). All IC_{50}s were measured following a 5-min incubation of enzyme and inhibitor. IC_{50}s for the *A. hydrophila* CphA enzyme were determined in the presence of 1 μM Zn^{2+}. Imipenem (100 μM) was used as the reporter substrate, since nitrocefin is a very poor substrate for this enzyme (10).

TABLE 1. Inhibitory activity of thiolesters[a]

	IC50 (μM)						
	B.cereus II		*B.fragilis* CfiA		*X.maltophilia* L-1		*A.hydrophila* CphA
Concentration of Zn^{2+}:	1	100	1	100	1	100	1
 Dipicolinic acid	28	871	40	437	66	309	NT
 SB216271	38	479	-	-	3	5	3.5
 SB214751	479	-	-	-	8	7	N T
 SB214752	645	-	-	-	2	3	NT
 SB216968	-	631	-	-	186	115	0.55
 SB213079	288	-	-	-	10	15	30

[a] Thiol esters were mercaptoacetic acid derivatives. $IC_{50} > 1{,}000$ μM.

ESMS and preparation of samples. Electrospray mass spectroscopy (ESMS) has been utilized previously to characterize inhibitors covalently bound to serine β-lactamases (2, 9, 19). Although the substrate hydrolysis of metallo-β-lactamases is not expressed by means of covalent intermediates, the presence of a cysteine residue close to the active site of metallo-β-lactamases affords the prospect of covalent intervention at this residue in achieving inhibition.

As 25 mM Tris HCl (pH 7.0) buffer is an appropriate medium for ESMS sampling, this buffer was used throughout the ESMS and tryptic digest experiments. A few ESMS experiments were also performed with 25 mM PIPES (pH 7.0) buffer to confirm that the observed effects were not buffer artifacts. Inhibition of *B. cereus* II by SB214751, SB214752, SB213079, and mercaptoacetic acid was studied by ESMS. One hundred fifty microliters of a 16 mM solution of each inhibitor was added to 150 μl of 80 μM *B. cereus* II (final Zn^{2+} concentration = 300 μM), with mixing, to provide a 200:1 molar ratio of inhibitor-*B. cereus* II. A *B. cereus* II control solution, containing 40 μM enzyme, was prepared at the same time. After 1.5 h at 37°C a 5-μl aliquot was removed from each of the samples and the control and then diluted 10,000 times into 100 μM nitrocefin. The activity of the enzyme was then measured and compared to that of the untreated control.

The mixture (20 μl) was then examined by electrospray liquid chromatography (LC)-MS using a gradient system in order to prevent possible interference from excess inhibitor. The chromatography was performed on a Hewlett-Packard 1090 liquid chromatograph with an Applied Biosystems Aquapore RP-300 column (100 by 2.1 mm) with a linear gradient of 0.1% TFA in water to 0.085% TFA in 7:3 acetonitrile-water (15 min, 100 μl/min, 214 nm).

The eluent from the column was fed directly into the electrospray interface of a Finnigan MAT TSQ 700 mass spectrometer. Spectra were averaged over the peak of interest and deconvoluted by using ICIS V1.00 software. Controls containing the same concentrations of Zn^{2+}, SO_4^{2-}, and dimethyl sulfoxide (DMSO) as the samples treated with inhibitor were also assessed.

A separate series of experiments was performed directly to ascertain which active site residue was bound to the inhibitor. Here, iodoacetic acid was added (final concentration, 1 mM) to 150 μl of a *B. cereus* II control and also to a sample containing a 200:1 mixture of SB214751-*B. cereus* II, prepared as described above. The samples were incubated for 90 min.

Tryptic digests of *B. cereus* II. A *B. cereus* II control and the sample containing a 200:1 ratio of SB214751-*B. cereus* II were each subjected to tryptic digestion. Excess of inhibitor was removed from the sample by using a Bio-Spin 30 chromatography column (Bio-Rad Laboratories) as directed by the manufacturers. A 5.4-μl volume of a 0.5-μg/ml solution of trypsin was then added to 135 μl of *B. cereus* II (final concentration, 40 μM) inhibited with SB214751. The mixture was incubated at 37°C for 8 h and then a 20-μl aliquot was analyzed by LC-MS. The high-performance liquid chromatography (HPLC) conditions were those used above except that a 220-by-2.1-mm column was used and the solvent gradient was applied over a 70-min period.

Inhibition of *B. cereus* II by SB214751. Assays were carried out at 37°C in PIPES (pH 7.0) plus or minus 300 μM zinc sulfate. The parameters k_{inact}, K_i, and k_{inact}/K_i were determined by monitoring the progressive inactivation of the enzyme (15). This was done discontinuously, by measuring residual enzyme activity in the presence of 100 μM nitrocefin against time for a range of inhibitor concentrations. Comparison of the initial reaction rates in the presence of the inhibitor with those of the control reaction enabled the rate of inactivation to be monitored as a function of time. The first-order rate constants were determined from plots of ln (percentage of enzyme activity remaining) against time. These values were then replotted as reciprocals against the reciprocal of the inhibitor concentration in order to calculate the inactivation rate constants, k_{inact}, K_i, and k_{inact}/K_i.

Detection of the hydrolysis products of thiol esters by *B. cereus* II by HPLC. It was important to determine if mercaptoacetic acid was a product of the metallo-β-lactamase hydrolysis reaction of the thiol esters and therefore released in the active site rather than from simple chemical hydrolysis. HPLC was carried out using a Waters 600E pump and a Waters 990 diode-array detector (Waters, Milford, Mass.) scanned from 210 to 400 nm at 5-nm intervals. The column was a 150-by-4.6-mm Ultrasphere ODS (5 μm). The following linear gradient was applied: 0.1% TFA in water to 0.085% TFA in 4:1 acetonitrile-water (15 min, 1 ml/min, 230 nm).

The weak UV chromophore of SB214751 rendered the compound unsuitable for this analysis. The structurally similar SB214752 was therefore chosen for these investigations, since it provided a conjugated chromophore within its benzoylalanine component, thereby increasing the sensitivity of detection. One hundred fifty microliters of 648 μM SB214752 and 150 μl of 65.4 μM *B. cereus* II (final Zn^{2+} concentration, 6.7 μM) were mixed to provide a 10:1 molar ratio of SB214752-*B. cereus* II. A *B. cereus* II control, containing a final concentration of 32.7 μM enzyme, was prepared at the same time. Samples were incubated at 37°C and aliquots were removed and analyzed by HPLC.

The use of ITC to estimate the affinity of inhibitors for Zn^{2+}. To facilitate the mechanistic studies it was necessary to determine the affinity of the inhibitors for Zn^{2+}. Such data provide an indication of whether compounds could be inhibiting by a sequestration process resulting in depletion of the active site Zn^{2+} via an equilibrium process involving chelation of extraneous zinc. Isothermal titration calorimetry (ITC) was used to estimate the zinc affinity of the thiol esters. *K* represents the equilibrium constant for the dissociation of the Zn-inhibitor complex: $[ZnI]^{2+} \leftrightarrow Zn^{2+} + I$, where I represents the inhibitor and ZnI represents the complex. Therefore, *K* is given by the concentration ratio:

$$K = \frac{[Zn^{2+}]\,[I]}{[ZnI]^{2+}}$$

Titrations to determine *K* were performed with an OMEGA titration calorimeter (supplied by Microcal Inc., Northampton, Mass.). All titrations were run at 28°C in 25 mM PIPES (pH 7.0). The cell was filled with 1 mM zinc sulfate in buffer, and the titrant was 50 mM inhibitor also dissolved in buffer. Data were collected for 25 5-μl injections. The data were processed by using the ORIGIN software supplied by Microcal (22). Dipicolinic acid and EDTA were positive controls.

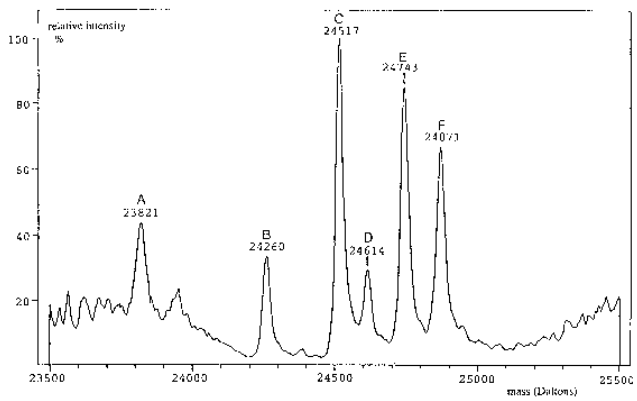


FIG. 2. ESMS of native *B. cereus* II enzyme. Peaks A through F represent ragged ends of *B. cereus* II. Peak A, loss of SQKVEKTVIK; peak B, loss of SQKVEK; peak C, loss of SQKV; peak D, loss of SQK; peak E, loss of SQ; peak F, loss of S.

RESULTS

Activities of compounds. SB214751, SB214752, SB213079, and SB216271 all showed their greatest inhibitory activities against the L-1 metalloenzyme. None of the compounds had detectable activity against the CfiA enzyme, and with the exception of SB216968, activity against *B. cereus* II appeared to be greater at 1 than at 100 μM Zn^{2+}. This effect may be rationalized in terms of the proposed mechanism and the dinuclear active site (see Discussion). SB216968 was exceptionally active against the CphA metallo-β-lactamase (Table 1).

ESMS. ESMS of the *B. cereus* II enzyme gave 6 peaks, A through F (Fig. 2), each of which corresponded to ragged ends comprising the loss of N-terminal amino acid residues (19). Following a 1:10,000 dilution, *B. cereus* II enzyme was inhibited 95% by each of the inhibitors, SB214751, SB214752, or SB213079. However, under the same conditions, no inhibition by mercaptoacetic acid was observed. ESMS indicated a time-dependent mass increment of 88 to 92 Da for *B. cereus* II enzyme solutions incubated with SB214751, each peak increased by 88 to 92 Da (Fig. 3). An identical adduct was observed when the reactions were performed in PIPES buffer. SB213079 and SB214752 also gave rise to a mass increment of approximately 90 Da for the enzyme. The increment must therefore arise from these compounds as a consequence of adduct formation with their common $-SCH_2CO_2H$ component. No adduct was observed with *B. cereus* II incubated with mercaptoacetic acid. Although the theoretical mass increments for the following would fall outside the observed range of 88 to 92 Da, control experiments have shown that this increment was not caused by adducts arising from the presence of DMSO, Zn^{2+}, or SO_4^{2-} species.

Kinetics of inhibition of metallo-β-lactamases by SB216968 and SB214751. The inhibition of *B. cereus* II by SB214751 and

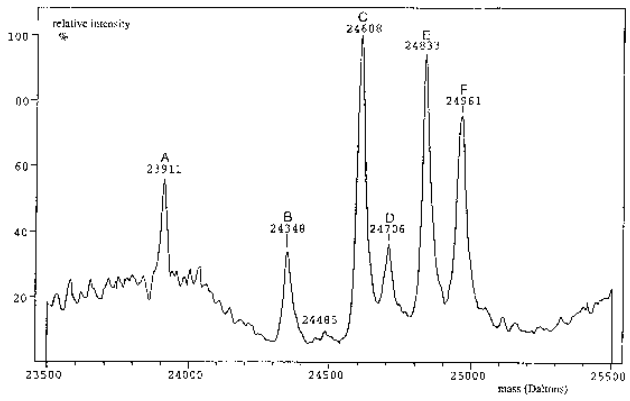


FIG. 3. ESMS of *B. cereus* II incubated with 1:200 molar ratio of SB214751 for 90 min. Peaks A to F represent ragged ends of *B. cereus* II plus 88 to 92 Da. Peak A, +90; peak B, +88; peak C, +91; peak D, +92; peak E, +90; peak F, +90.

mercaptoacetic acid and inhibition of CphA by SB216968 were chosen for more detailed studies.

In the absence of 300 μM zinc sulfate the inactivation of *B. cereus* II by SB214751 was found to be nonsaturating; it was not therefore possible to calculate either the inhibitor constant K_i or the maximum rate of inactivation k_{inact}. However, by the method of Kitz and Wilson (15) the second-order constant of inactivation was calculated as 0.0714 mM^{-1} min^{-1}. In contrast, when 300 μM zinc sulfate was added, inactivation was found to be saturated, giving a value of 0.2 mM for K_i and 2.21×10^{-3} min^{-1} for the maximum rate of inactivation and a value of 0.0108 mM^{-1} min^{-1} for the corresponding second-order rate of inactivation (half-life [$t_{1/2}$] = 314 min). The stability of the inhibited enzyme complex was measured by allowing the enzyme to react with SB214751 to give 100% inhibition. Subsequently samples were removed and diluted into excess substrate and the activity was compared to that of an untreated control. It was found that over a 24-h period no recovery of enzyme activity occurred. Mercaptoacetic acid, the thiol component of SB214751, exhibited no progressive inhibition of *B. cereus* II, and a K_i of 27.9 μM was determined in the presence of 100 μM zinc sulfate.

Inhibition of *A. hydrophila* CphA metallo-β-lactamase by SB216968 was studied with imipenem as the reference substrate, and the inhibition was not reduced by increasing the substrate concentration. Analysis of initial rate data (i.e., the first 30 s of the reactions) using 1/v against [I] and [S]/v against [I] plots for different imipenem concentrations showed uncompetitive inhibition, with a K_i of 3.9 μM. However, preincubation (5 min) of enzyme with inhibitor reduced the IC_{50} from 1.8 to 0.55 μM, indicating a time-dependent component to the inhibition. (Extended incubation times were not possible because of enzyme instability.) Furthermore, incubating the metallo-β-lactamase with concentrations of SB216968 shown previously to be inhibitory (0.78 to 6.25 μM), then diluting into excess imipenem and assaying for activity, gave essentially no inhibition of the enzyme. Increasing the inhibitor concentration to 50 μM, then diluting, gave only the degree of inhibition expected from carryover. Inhibition was unaffected by increasing the Zn^{2+} concentration from 1 to 10 μM.

ITC. As expected, the ITC data for EDTA showed very high affinity for Zn^{2+}; the operational dissociation constant K was outside the range of the instrument, giving a K of $\leq$ 1 nM. The shape of the titration curve for dipicolinic acid (control) and stoichiometry of the endpoint was more complex than a simple 1:1 interaction. The data fit illustrated a stepwise binding of two molecules of dipicolinic acid by Zn^{2+} with a K of ~1 μM for each step. The results for SB214751, SB214752, and SB216968 indicated that their dissociation constants were also very high ($K > 1$ mM), corresponding to very low affinity of the compounds for Zn^{2+}.

TABLE 2. Summary of LC-MS data from the tryptic digestion of *B. cereus* II treated with SB214751

HPLC peak no.	Mol mass (Da)	*B. cereus* II amino acid sequence assignment
1	ND[a]	NA[b]
2	ND	NA
3	ND	NA
4	1,348	NA
5	1,504	206–216
6	1,447	79–91
7	1,212	105–116
8	1,403	11–23
9	989	66–73
10	1,332	51–62
11	2,018	117–134
12	1,890	118–134
13	2,870	24–50
14	ND	NA
15	ND	NA
16	1,234	217–227
17	ND	NA
18	**2,758**	**148–171[c] + 90 Da**
19	2,436	177–198
20	ND	NA
21	24,259	*B. cereus* II[d]

[a] ND, not detectable.
[b] NA, cannot be assigned.
[c] Peptide containing the active site cysteine.
[d] Mass of *B. cereus* II minus N-terminal residues 1 to 6.

Hydrolysis of SB214752 by *B. cereus* II. HPLC analysis of the *B. cereus* II-treated samples illustrated that the metallo-β-lactamase caused a reduction in the peak which corresponded to SB214752 and an increase in a peak which corresponded to *N*-benzoyl alanine. The hydrolysis of the thiol ester by *B. cereus* II was three times greater than the background rate of the control sample. Subtracting the background rate, under these conditions SB214752 was hydrolyzed at a rate of 10.4 to 11.8 nM/min/μM enzyme (k_{cat} = 0.01 min^{-1}; $t_{1/2}$ = 63 min). These data were determined from the decrease in substrate peak and increase in product peak, respectively. It is inferred that the second product from the enzyme hydrolysis is, therefore, mercaptoacetic acid.

Identification of the binding site of the +90 fragment. The purpose of this work was to identify the amino acid residue in *B. cereus* II which had bound the 90-Da fragment from SB214751. Following the tryptic digestion of *B. cereus* II the mass of 14 peptides was determined (Table 2) and assigned to a specific part of the *B. cereus* II sequence. The peptide which contained the only cysteine residue of the enzyme (peptide 18) was not observed in tryptic digests of native *B. cereus* II. This may be a result of the high reactivity of fragments containing the free thiol. However, peptide 18 of the enzyme treated with SB214751 had undergone a mass increment of 90 Da (Table 2). The identity of this modified cysteine peptide was confirmed by collecting the HPLC fractions followed by manual Edman sequencing of the first 20 amino acids.

ESMS illustrated that the mass of *B. cereus* II treated with iodoacetic acid increased by 116 Da. This indicates that two molecules of iodoacetic acid bind to the enzyme. One of these

binding sites is putatively the active site cysteine (20). Only one molecule of iodoacetic acid bound to *B. cereus* II enzyme inhibited with SB214751 (mass increment of 58 Da). This illustrates that only one derivatizable group was now available, suggesting that the 90-Da fragment from SB214751 was probably bound to the active site cysteine.

DISCUSSION

The results above have shown that these mercaptoacetic acid thiol ester compounds exhibit a variety of potencies against the different metallo-β-lactamases tested. The most significant observation was the irreversible inhibition of *B. cereus* II, and this merited in-depth evaluation. Overall, the L-1 enzyme appears to be the most susceptible to inhibition by these compounds, although SB216968 shows very good potency against the *A. hydrophila* CphA enzyme. Interestingly, no inhibition of the CfiA enzyme was detected and therefore this series of inhibitors has identified differences in the active site of the CfiA and those of the *B. cereus* II, CphA, and L-1 enzymes.

The ITC results for the thiol esters show that their affinity for Zn^{2+} under the conditions used was very much lower than the affinity of the *B. cereus* II enzyme for Zn^{2+} ($K = 1$ μM [7]). This implies that the inhibition exhibited by our compounds was not simply due to chelation of Zn^{2+} released from the active site. This was illustrated by the fact that the inhibition of L-1 metallo-β-lactamase by the compounds was essentially unaffected by the concentration of Zn^{2+} present during testing. In contrast, dipicolinic acid, a metallo-β-lactamase inhibitor and Zn^{2+} chelator (24), was five times more potent against this enzyme at 1 μM Zn^{2+} than at 100 μM Zn^{2+}. Inhibition of *B. cereus* II by the thiol esters was affected by the concentration of Zn^{2+}. As stated previously, this is unlikely to be caused by the sequestration of Zn^{2+} by these compounds, and an alternative hypothesis is offered below. The use of ITC to determine the potential chelating activity of compounds has provided a novel approach for the evaluation of candidate metalloenzyme inhibitors.

The compounds also had different mechanisms of inhibition for the different enzymes: the ESMS experiments and kinetic evaluation clearly illustrated that inhibition of *B. cereus* II by SB214751 was progressive and irreversible. In marked contrast, initial rate data (imipenem as reference substrate) indicated that SB216968 was an uncompetitive inhibitor of CphA metallo-β-lactamase. This observation would suggest that the inhibitor binds to the ES complex, as in scheme 1 below:

$$\begin{array}{ccccc} E + S & \leftrightarrow & ES & \rightarrow & E + P \\ & & +I & & \\ & & \updownarrow & & \\ & & ESI & & \end{array}$$

where S is the substrate (imipenem) and I is the inhibitor (SB216968). Such a situation is necessarily very different from that exhibited in the interaction of SB217451 with *B. cereus* II. Binding of substrate to CphA may give rise to a conformational change and render the complex vulnerable to attack by SB216968 at a site as yet unspecified. Some support for this is provided by the observation that bovine serum albumin (50 μg ml^{-1}) afforded protection against inhibition. However, the improved inhibition on preincubation, followed by recovery of activity on dilution, shows that this inhibition of CphA is more complicated than the classical reversible type. This behavior is consistent with relatively slow binding of SB216968 to the enzyme, with release of inhibitor and recovery of free enzyme when its concentration is decreased by dilution. There may be more than one inhibited species, one being formed slowly from the other.

The irreversible inhibition of *B. cereus* II was studied in more detail. HPLC analysis showed that the interaction of *B. cereus* II with these compounds achieves a hydrolytic scission of the thiol ester bond to release mercaptoacetic acid. ESMS studies proved the irreversible inhibition of *B. cereus* II by SB213079, SB214751, and SB241752 to be associated with the covalent binding of a 90-Da fragment to the enzyme. Mercaptoacetic acid has a mass of 92 Da, and if this fragment formed a mixed disulfide covalent linkage with the *B. cereus* II enzyme under the aerobic conditions employed, it would cause a mass increment of exactly 90 Da. As this is the only molecular fragment, of mass of 90 Da which is common to all three thiol esters tested, we propose that this part of the molecule, mercaptoacetic acid, binds to the enzyme in the manner stated. Tryptic digests of *B. cereus* II treated with SB214751 and iodoacetic acid provided substantial evidence that the mercaptoacetic acid fragment binds to the active site cysteine of the enzyme.

No irreversible inhibition of *B. cereus* II by free mercaptoacetic acid (at high or low concentrations of Zn^{2+}) could be detected by kinetic or ESMS analysis, in agreement with the observation that it is a competitive inhibitor of *B. cereus* II. Therefore, these results suggest that mechanism-based delivery of mercaptoacetic acid, via the thiol ester, facilitates the binding of the resulting thiol fragment (i.e., the hydrolysis product) to the active site cysteine of the enzyme. Knowledge of the dinuclear active site structure of the enzyme now permits a greater understanding of these phenomena, which differ only in the genesis of the mercaptoacetic acid associated with them: the lack of covalent inhibition arising from free (i.e., extraneous) mercaptoacetic acid may result from the binding of its thiol function to Zn^{2+} 1. Accordingly, it may never become orientated toward the Cys168 thiol, which is ligated at Zn^{2+} 2. The mechanism-based release of mercaptoacetic acid from the thiol ester can, however, deliver the molecule in a favorable alignment toward the Cys thiol at Zn^{2+} 2 for the disulfide formation to occur after interaction with an oxidant (e.g., O_2), thereby establishing the covalency. The latter oxidative step will be rate determining in the inhibition process.

Furthermore, our mechanism permits an explanation of the observation of reduced inhibition at higher zinc concentrations: the final step of the mechanism may require the departure of the second, more weakly bound zinc (7), in order for the sulfur atoms to become proximate and for the oxidation step to occur (i.e., Zn^{2+} 2 must depart prior to formation of the mixed disulfide). In *B. cereus* II greater occupancy of this second site will be promoted at higher concentrations of extraneous zinc. The high-equilibrium Zn^{2+} 2 occupancy will therefore suppress the final process of reaction of the released mercaptoacetic acid with the cysteine thiol, the latter remaining coordinated to Zn^{2+} 2.

It is also of interest to note that the L-alanyl enantiomer of SB214751 did not exhibit any time-dependent inhibition of *B. cereus* II. This may be a consequence of substrate stereoselectivity by the enzyme, in favor of hydrolysis only of the D-alanyl enantiomers. (A similar specificity has been observed in the hydrolysis of such molecules by some class A and class D serine β-lactamases [6].)

In conclusion, we propose that the mercaptoacetic acid thiol ester derivatives are hydrolyzed by *B. cereus* II, releasing the mercaptoacetic acid component, which then in a process of lower efficiency irreversibly binds to the enzyme, forming a disulfide with the active site cysteine under the aerobic conditions of the assay. Free mercaptoacetic acid functions solely as a competitive inhibitor, and therefore these results suggest that

it is able to bind to the enzyme only when it is delivered via the thiol ester. Accordingly, this is the first class of mechanism-based inhibitors of metallo-β-lactamases and is illustrative of non-β-lactam substrates for the enzyme.

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