Thiomandelic Acid, a Broad Spectrum Inhibitor of Zinc β-Lactamases

KINETIC AND SPECTROSCOPIC STUDIES*§

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Resistance to β-lactam antibiotics mediated by metallo-β-lactamases is an increasingly worrying clinical problem. Candidate inhibitors include mercapto-carboxylic acids, and we report studies of a simple such compound, thiomandelic acid. A series of 35 analogues were synthesized and examined as metallo-β-lactamase inhibitors. The Ki values (Bacillus cereus enzyme) are 0.09 μM for R-thiomandelic acid and 1.28 μM for the S-isomer. Structure-activity relationships show that the thiol is essential for activity and the carboxylate increases potency; the affinity is greatest when these groups are close together. Thioesters of thiomandelic acid are substrates for the enzyme, liberating thiomandelic acid, suggesting a starting point for the design of “pro-drugs.” Importantly, thiomandelic acid is a broad spectrum inhibitor of metallo-β-lactamases, with a submicromolar Ki value for all nine enzymes tested, except the Aeromonas hydrophila enzyme; such a wide spectrum of activity is unprecedented. The binding of thiomandelic acid to the B. cereus enzyme was studied by NMR; the results are consistent with the idea that the inhibitor thiol binds to both zinc ions, while its carboxylate binds to Arg91. Amide chemical shift perturbations for residues 30–40 (the β3-β2 loop) suggest that this small inhibitor induces a movement of this loop of the kind seen for other larger inhibitors.

The β-lactam antibiotics are among the most useful antibacterial chemotherapeutic agents, but their efficiency is being continuously challenged by the emergence of resistant strains of pathogenic bacteria. β-Lactamases, which inactivate these antibiotics by hydrolyzing their endocyclic amide bond, play a major role in this resistance (1). β-Lactamases have been divided into four classes on the basis of their amino acid sequences and catalytic mechanisms (2). The mechanisms of class A, C, and D enzymes, which contain a nucleophilic serine side chain as a key component of their active site, have been extensively studied, due to their established clinical importance. Class B enzymes are metalloproteins that require one or two zinc ion(s) for their activity (3). The first metallo-β-lactamase (MBL)1 to be discovered was produced by an innocuous strain of Bacillus cereus (4), but in the last 20 years, MBL-mediated resistance has appeared in several pathogenic strains including Bacteroides fragilis, Aeromonas hydrophila, Stenotrophomonas maltophilia, and Serratia marcescens (5). Even more threatening is the rapid dissemination of some metallo-β-lactamase genes by horizontal transfer, involving both plasmid- and integron-borne genetic elements. For instance, the IMP enzymes that were first isolated in clinical isolates of S. marcescens and Pseudomonas aeruginosa have also been found in Klebsiella, Alcaligenes, Acinetobacter, and Shigella strains (6), and up to four variants of IMP-1 have been described so far (7–10).

The MBLs with known sequences share a small number of conserved motifs, but otherwise they show significant sequence diversity and have thus been classified into three subclasses: subclass B1 includes BeII from B. cereus (11, 12), CfA (also called CcrA) from B. fragilis (13, 14), IMP-1 and VIM-1 from P. aeruginosa (6, 15), and BLaB from Chrysobacterium meningosepticum (16); subclass B2 essentially consists of enzymes from Aeromonas strains (e.g. CpaA from A. hydrophila (17); and subclass B3 includes the L1 enzyme from Stenotrophomonas maltophilia (18, 19), along with FEZ-1 from Legionella gormanii (20) and GOb-1 from C. meningosepticum (21). Crystal structures have been determined for the B. cereus (11, 22), B. fragilis (13, 23, 24), and IMP-1 (25) enzymes, as well as for the subclass B3 enzyme from S. maltophilia (26). These reveal a unique αβ/βα “sandwich” topology characteristic of this family of enzymes, distinct from other zinc enzymes such as thermolysin and carboxypeptidase A.

Two zinc cations are present in the active site of the four MBLs for which crystal structures are available (although in the case of the B. cereus enzyme not in all reported structures); the zinc ligands are not fully conserved between the different

1 The abbreviations used are: MBL, metallo-β-lactamase; BeII, the zinc metallo-β-lactamase from B. cereus; HSQC, heteronuclear single quantum coherence; HMQC, heteronuclear multiple quantum coherence; MES, 2-[N-morpholino]ethanesulfonic acid.
subclasses, perhaps contributing to some of the observed differences in substrate profiles and zinc affinities. Considering only the subclass B1 enzymes, in site I the zinc is coordinated by the imidazole rings of three histidine residues (His$^{36}$, His$^{88}$, and His$^{149}$ in the B. cereus enzyme; His$^{116}$, His$^{118}$, and His$^{196}$ according to the recently proposed standard numbering of MBLs (27)) and one water molecule. In the structures with two zinc, this water (or hydroxide) bridges to the zinc in site II, which is also coordinated by a histidine (His$^{110}$ in the B. cereus enzyme), a cysteine, an aspartate, and a second water (or a carbonate ion). The bridging water molecule is believed to be the nucleophile responsible for lactam cleavage, but the precise role of the two metals in catalysis is still unclear; mechanisms have been proposed in which only site I plays a direct role in catalysis (28), or in which the two zinc ions are both involved, as a binuclear center (3, 26, 29). Despite the conservation of all zinc ligands in this subclass, some enzymes (e.g. BcII (12, 30, 31)) seem to bind zinc rather loosely, whereas in other cases zinc is more difficult to remove and reincorporate (14). Interestingly, whenever they have been prepared, mononuclear forms of subclass B1 enzymes are active, and addition of the second zinc ion usually results in only rather moderate increases of activity (12, 14).

Since MBLs are resistant to inactivators, such as clavulanic acid, of the serine enzymes, the spread of MBL-mediated bacterial resistance to β-lactams (including carbapenems) is a matter of real concern. No satisfactory inhibitors of MBLs are yet available for clinical use, and the range of active site architectures for the MBLs makes the discovery of useful broad spectrum inhibitors a challenging task. Work to date allows the identification of at least five major groups of potential MBL inhibitors. 1) Biphenyl tetrazoles are potent inhibitors of the B. fragilis MBL, although they are much less active toward other important MBLs such as IMP-1 (32); the crystal structure of a complex between B. fragilis MBL and a biphenyl tetrazole shows that the inhibitor binds to the zinc in site II, with the displacement of a water molecule (32). 2) Some carbapenem derivatives are potent inhibitors of several β-lactamases including class B enzymes (33), but they behave as poor substrates of MBLs and may be too rapidly hydrolyzed by some of them to be effective therapeutic agents. 3) Inactivators of the A. hydrophila enzyme such as trifluoromethyl alcohols and ketones (34) or hydroxamates (35) have been described, but the mechanism of inhibition is still unclear, and their activity against other MBLs is much lower. 4) Very recently, succinic acid derivatives with aromatic substituents have been shown to be potent inhibitors of the IMP-1 enzyme (36). 5) Thiol compounds as simple as mercaptoacetic acid (37) can be potent inhibitors of MBLs, either as free thiols or in the protected form of a thioester (38). A number of thiols (24, 28, 39, 40) and thioesters (41–43) have been studied as inhibitors, but again, they often show activity only against a restricted panel of MBLs. Crystal structures are available for the complex of the IMP-1 enzyme with a potent mercaptoacrylate substrate analogue inhibitor (25), and NMR data are available for the binding of other mercaptoacrylate inhibitors to the MBLs from B. fragilis (40, 44) and B. cereus.3

We now report kinetic studies of the inhibition of MBLs by one of the simplest potent mercaptoacrylate inhibitors, thiomandelic acid (Fig. 1; a-mercaptophenylacetic acid), and a number of analogues, together with NMR studies of thiomandelic acid binding to the B. cereus MBL, BcII, as a starting point for the design of broad spectrum MBL inhibitors.

2 C. Moali, unpublished results obtained with IMP-1.
3 C. F. Dambion, I. G. Barsukov, C. Molland, and G. C. K. Roberts, manuscript in preparation.
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Thiomandelic acid itself and the two para-substituted analogues (4a, b, d), with $K_I$ values of 0.2–0.5 $\mu M$ as the racemates. The nature of the para substituent had little effect on the inhibition constant, suggesting that this group does not make specific interactions with the active site. Comparison of the relative potencies of compounds 4a, 8, and 9 shows that the affinity for Bcll is greatest when the thiol and carboxylate groups are in close spatial proximity. Of these two groups, the thiol is clearly the major driving force for binding, since its replacement even by a hydroxyl group completely abolishes activity (6a, Fig. 2), whereas removal of the carboxylate group increases $K_I$ by only ~30-fold (4a versus 11, Table I).

The $K_I$ values of the disulfide (5a) and thioesters (3aa-ab) of thiomandelic acid are 100-fold higher than those of the thiol compound, and we cannot rule out the possibility that the inhibitory activity observed in these cases is due to the generation of small amounts of thiols. The possible generation of thiomandelic acid by enzyme-catalyzed hydrolysis of the thioesters was investigated by using 4,4′-dithiodypipyridine (50) to measure thiol formation. This possibility is also of interest in considering the potential of thioesters as "pro-drugs" for in vivo use. The increase in absorbance at 324 nm characteristic of the reaction of 4,4′-dithiodypipyridine with thiols was found to be time-dependent, substrate-dependent, and enzyme-dependent for both 3aa and 3ab; subtraction of the nonenzymatic rate revealed clear catalysis of the hydrolysis of these thioesters by Bcll. An attempt was made to determine the $K_m$ and $V_{max}$ values for this reaction, but product inhibition (presumably by thiomandelic acid) was too great to reach substrate saturation. At 1 mM substrate concentrations, the specific activity of Bcll was found to be 424 ± 60 nmol/min/mg (0.18 ± 0.02 s⁻¹) versus 3aa and 460 ± 60 nmol/min/mg (0.18 ± 0.02 s⁻¹) versus 3ab.

In view of the evidence, discussed above, that simple thiol compounds and mercaptocetic acid thio esters are inhibitors of MBLs and that the most effective compounds contain both thiol and carboxylate groups, we have investigated the inhibition of MBLs by thiomandelic acid (α-mercaptophenylacetic acid) and a number of structurally related compounds. Thiomandelic acid and a series of para-substituted analogues were synthesized in racemic form by a modified version of the method described by Bonner (46), and the individual optical isomers, R- and S-thiomandelic acids (15a and 15b), were prepared by the procedure of Strijtveen and Kellogg (48).

Screening for MBL Inhibition—Racemic thiomandelic acid (4a) and a number of analogues and synthetic intermediates (Fig. 1) were initially screened for inhibitory activity against B. cereus MBL (Bcll) by measuring the residual activity against nitrocefin in the presence of each compound at a concentration of 1 mM. The results are presented in Fig. 2 and show that 10 of the 35 compounds tested inhibited Bcll by >50% under these conditions. Comparison of the residual activity in the presence of compounds 4a-b, d (thiomandelic acid and para-substituted analogues) with that for compounds 1aa-d (lacking the SH) and 11 (lacking the COOH) shows that the thiol group is essential for inhibition but the carboxylate group is not, although it leads to a significant increase in potency. Replacement of the thiol by a hydroxyl (6a-b, d-f), bromo (2a-d), or amidoxime (7a-c, g-h) function effectively abolishes inhibitory activity. Amidoximes 7a-c, g-h were also inactive toward CphA, despite their resemblance to previously described hydroxamate inhibitors of this enzyme (35). Protection of the thiol as thioncarboethoxy (3ac-d), thioester (3aa-ab) or disulfide (5aa-b, d) derivatives is detrimental, but some inhibitory activity is retained for compounds 3c, 3aa-ab and 5aa-b, d. Altogether, these preliminary results clearly demonstrate that the thiol group plays a major role in the interaction between MBLs and derivatives of mandelic acid.

Inhibition constants were determined for those compounds exhibiting more than 50% inhibition at 1 mM (Fig. 2), and these are presented in Table I. The most potent inhibitors are thiomandelic acid itself and the two para-substituted analogues (4a, b, d), with $K_I$ values of 0.2–0.5 $\mu M$ as the racemates.
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This activity is low compared with the usual activities of MBLs against β-lactams, and the classical competitive inhibition model used here should not be perturbed at the thioester and enzyme concentrations required to measure the \( K_i \) values in Table I. Thus, the amount of thiomandelic acid generated during the incubation will be much too low to account for the observed inhibition, which must be due to the thioesters themselves. In such conditions, to a good approximation, \( K_i = K_e \) for 3aa and 3ab, but the corresponding \( k_{cat} \) cannot be determined.

**Thiomandelic Acid Is a Broad Spectrum MBL Inhibitor**—In light of these observations, thiomandelic acid was chosen for more detailed investigation. We determined the inhibitory activity of the racemic compound against most of the MBLs currently available (Table II). Thiomandelic acid was found to be a reasonably potent (submicromolar) inhibitor for all of the MBLs tested, except for the subclass B2 enzyme from *A. hydrophila* (CphA). Such a wide spectrum of activity against MBLs of subclasses B1 and B3 is unprecedented in previously published data concerning MBL inhibitors. For instance, thiomandelic acid is only about 25-fold less potent as an inhibitor of the CfIA (CcrA) enzyme from *B. fragilis* than of the IMP-1 enzyme from *P. aeruginosa*. This is in marked contrast to the reported thioester MBL inhibitors, which are very much poorer inhibitors of the *B. fragilis* enzyme (38, 41, 43).

**Characteristics of Inhibition by Thiomandelic Acid**—Synthesis and analysis of the individual stereoisomers showed that the stereochemistry at the α-carbon has a modest effect on the binding of thiomandelic acid to the BcII enzyme. Pre-incubation of the enzyme with thiomandelic acid for 30 min at 30 °C did not modify the degree of inhibition. Although the time dependence of the activity was not linear over the first few minutes (suggesting that a two-step process may occur), the inhibition was fully and quickly reversible upon dilution or upon the addition of excess zinc (which binds free thiomandelic acid). Mass spectrometry of the enzyme after incubation with thiomandelic acid gave no evidence for a covalent adduct.

To investigate the possibility that thiomandelic acid might form a disulfide bridge with the cysteine involved in zinc binding (the only cysteine residue conserved in all of the zinc β-lactamases), as demonstrated earlier for mercuriaconetic acid thioesters (38), we examined thiomandelic acid inhibition of the C15SA mutant of IMP-1. The \( K_i \) value of thiomandelic acid for this mutant was found to be 1.83 μM, 63-fold greater than that for the wild-type enzyme but still indicating significant inhibition. This higher \( K_i \) may reflect the much weaker binding of the second zinc to this mutant. A much greater loss of potency would have been expected if cysteine 158 had been directly involved in the inhibitory process. It is possible that the disulfide bond formation previously observed (38) results from slow adventitious oxidation, which is not relevant on the time scale of our kinetic analyses.

**NMR Spectroscopy**—To obtain direct information on the binding of thiomandelic acid to the BcII enzyme, its effects on the backbone and imidazole NH resonances of the enzyme were investigated.

**Effect of Inhibitors on the Imidazole Resonances of the Metal Ligands**—The imidazole resonances of the metal-binding histidine residues (His36, His88, and His149 in site I, His210 in site II), which have been assigned to individual residues (49), provide valuable probes of the effects of inhibitors on the active site. The imidazole NH resonances appear between 12 and 15 ppm (Fig. 3). In the absence of inhibitors at pH 6.4, 288 K, relatively sharp resonances are observed for His36 and His88 and a broad resonance for His210, but no signal is apparent for His149. At lower pH or temperature, the His210 resonance sharpens, and a signal for His149 appears. These differences in line width reflect the differences in accessibility of the histidine imidazole NHs for exchange with the solvent. The gradual addition of either R- or S-thiomandelic acid to the enzyme resulted in a progressive decrease in the intensity of the imidazole NH signals from the free enzyme and a progressive increase in a new set of signals attributable to the enzyme-inhibitor complex. The changes were complete at a 1:1 ratio of inhibitor to enzyme. The spectra of the BcII- R-thiomandelic acid and BcII-S-thiomandelic acid complexes are shown in Fig. 3. The imidazole NH resonances of these complexes were assigned by using \(^{1}H\)\(^{15}N\) HMQC spectra optimized for observa-
tion of the long range $^1H-^{15}N$ couplings in the imidazole ring (49) (see “Supplemental Material”). These spectra allow one to connect the NH resonances to those of the two nitrogens and the two CH protons in each imidazole. Whereas there are changes in $^{15}N$ and CH chemical shift on inhibitor binding, the pattern of connectivities in the HMQC spectrum permits the assignment of the imidazole resonances in the spectrum of the $R$- and $S$-thiomandelic acid complexes by comparison with the spectrum of the free enzyme, and the chemical shifts are summarized in Table III.

It is clear that the imidazoles in both metal binding sites are markedly affected by inhibitor binding. In site I, both His$^{86}$ and His$^{88}$ show large (>0.5 ppm) changes in NH chemical shift on inhibitor binding, upfield for His$^{88}$ and downfield for His$^{86}$, the shifts being somewhat greater for the $S$- than the $R$-isomer. The CεH proton resonance of His$^{86}$ also shows a striking downfield shift of >2 ppm on inhibitor binding. The resonances of the unprotonated imidazole nitrogen (the zinc ligand; Ne for His$^{86}$ and His$^{149}$, Nò for His$^{88}$) of the histidines in site I show large downfield shifts (up to ∼9 ppm); for His$^{88}$ and His$^{149}$, but not His$^{86}$, these are larger with the $S$-isomer than with the $R$-isomer. In site II, by contrast, the Ne (zinc ligand) resonance of His$^{210}$ shows a large downfield shift on binding $R$-thiomandelic acid but a large upfield shift on binding the $S$-isomer. In the case of the cadmium-substituted BcII enzyme, $^{113}$Cd NMR has provided strong evidence for the binding of the sulfur atom of $R$-thiomandelic acid to both the metal ions in the active site, presumably displacing the “bridging” water molecule, as seen for a much more complex thiocarbonate inhibitor bound to the IMP-1 enzyme (25). The present data for the zinc BcII enzyme are entirely consistent with this mode of binding of thiomandelic acid. The different shifts seen for the resonances of the histidines in sites I and II for the $R$- and $S$-isomers of the inhibitor, which presumably reflect differences in electron distribution on the metal ligands, may represent a slightly different position of the sulfur atom between the two zinc atoms imposed by the interactions of the rest of the molecule with the enzyme.

A second clear difference between the complexes of the enzyme with the two isomers of thiomandelic acid is in the line width of the imidazole NH resonance of His$^{149}$. Inhibitor binding clearly tends to decrease the rate of exchange with water of the imidazole NHs of the metal-binding histidines, thus sharpening their resonances, but the magnitude of this effect varies significantly from one residue to another. For example, the resonances of His$^{86}$ and His$^{88}$ are reasonably sharp in the spectrum of the free enzyme and their line widths are little affected by inhibitor binding, whereas the resonance of His$^{210}$ is broad in the spectrum of the free enzyme and their line widths are little affected by inhibitor binding, whereas the resonance of His$^{210}$ is broad in the spectrum of the free enzyme and is markedly sharpened, to approximately the same extent, by the binding of either $R$- or $S$-thiomandelic acid. The NH resonance of His$^{149}$, on the other hand, is too broad to see in the spectrum of the free enzyme and also in that of the $S$-thiomandelic acid complex; only the binding of the $R$-isomer of the inhibitor decreases the imidazole NH exchange rate of this residue sufficiently to yield an observable resonance, in fact one as sharp as those of the other three active site histidines in this complex (Fig. 3).

**Effect of Inhibitor Binding on the Backbone Amide Resonances**—A two-dimensional proton-nitrogen correlation spectrum ($^1H-^{15}N$ HSQC) of $^{15}N$-labeled BcII allows the rapid observation of most of the backbone amide $^1H$ and $^{15}N$ resonances. These resonances have been assigned to individual residues in the free protein, and monitoring changes in these resonances thus provides a convenient method for identifying the regions of the enzyme affected by inhibitor binding.

Fig. 4 shows a comparison of the $^1H-^{15}N$ HSQC spectra of the free enzyme and of the enzyme in the presence of $S$-thiomandelic acid. Most of the spectrum remains essentially unaltered, but some significant changes are observed, generally involving residues that are close to the zinc binding sites. Only very few residues in the secondary structure elements of the core of the protein seemed to be affected by the inhibitor; some 90 residues

### Table II

| Enzyme          | B. cereus | B. fragilis | S. maltophilia | P. aeruginosa | A. baumanii | P. aeruginosa | C. meningosepticum | L. gormanii | A. hydrophila |
|-----------------|-----------|-------------|----------------|---------------|-------------|---------------|---------------------|-------------|---------------|
| $K_i$ (µM)      | 0.34      | 0.80        | 0.081          | 0.029         | 0.059       | 0.23          | 0.56                | 0.27        | 144           |

$K_i$ values are calculated assuming a competitive pattern of inhibition. Mean values of at least two separate experiments are shown (S.D. < 20%).
in this part of the structure that give resolved resonance signals were unaffected by the addition of inhibitors. Whereas the resonance assignment for the free enzyme is complete, the resonances in the complex are not yet assigned, so that the magnitude of the shifts of individual resonances cannot be accurately determined. We have thus used the “minimum chemical shift” approach (52, 55, 56), in which the chemical shift difference from a given cross-peak in the free protein to the closest cross-peak in the complex is calculated, as described under “Experimental Procedures.” Whereas this method can of course lead to underestimation of chemical shift changes, particularly in crowded regions of the spectrum, it can provide a reliable identification of the interaction site(s) (56, 57). The “minimum chemical shift index” (Ref. 52; see “Experimental Procedures”) is plotted as a function of residue number for both the R- and S-thiomandelic acid complexes in Fig. 5, and the residues showing the greatest shifts are mapped on to the structure of the enzyme in Fig. 6. A notable feature of the chemical shift changes on complex formation is the strong qualitative similarity in the effects of R- and S-thiomandelic acid; it is clear from Fig. 5 that, with few exceptions, the same residues are affected by the binding of both isomers. The amide resonances of the zinc binding residues of the enzyme in the presence and absence of R- and S-thiomandelic acid are listed in Table III. The cross-peaks are shown in black for the spectrum of the enzyme alone and in red for that of the S-thiomandelic acid complex. Resonance assignments are indicated for a number of the better resolved signals that are affected by inhibitor binding.

| Residue | Nucleus | Chemical shift (ppm) |
|---------|---------|----------------------|
|         |         | Enzyme alone | R-Thiomandelic acid complex | S-Thiomandelic acid complex |
| His86   | Nδ      | 175.61       | 176.98                 | 176.18                 |
|         | Nε      | 175.61       | 176.98                 | 176.18                 |
|         | CεH     | 6.26         | 6.14                   | 6.08                   |
|         | CεH     | 7.66         | 10.36                  | 9.89                   |
|         | NδH     | 12.03        | 12.87                  | 12.98                  |
|         | NεH     | 14.19        | 13.66                  | 13.37                  |
| His88   | Nδ      | 205.26       | 208.22                 | 208.27                 |
|         | Nε      | 173.14       | 170.53                 | 169.41                 |
|         | CεH     | 7.33         | 7.32                   | 7.77                   |
|         | CεH     | 7.66         | 7.76                   | 8.30                   |
|         | NδH     | 11.49        | 13.66                  | 13.37                  |
|         | NεH     | 201.87       | 203.62                 | 208.27                 |
|         | CεH     | 5.18         | 4.95                   | 4.96                   |
|         | CεH     | 8.43         | 8.40                   | 8.30                   |
|         | NδH     | 14.19        | 13.66                  | 13.37                  |
|         | NεH     | 170.10       | 168.58                 | 169.41                 |
| His149  | Nδ      | 217.65       | 224.44                 | 210.43                 |
|         | Nε      | 6.93         | 7.00                   | 7.25                   |
|         | CεH     | 7.23         | 7.40                   | 7.48                   |
|         | CεH     | 7.23         | 7.40                   | 7.48                   |
|         | NδH     | 13.14        | 13.19                  | 12.66                  |

FIG. 4. 1H-15N HSQC spectra of BcII in the presence and absence of S-thiomandelic acid, showing the backbone and side chain amide and tryptophan indole NH resonances. The cross-peaks are shown in black for the spectrum of the enzyme alone and in red for that of the S-thiomandelic acid complex. Resonance assignments are indicated for a number of the better resolved signals that are affected by inhibitor binding.

TABLE III

1H and 15N chemical shifts of the imidazole rings of the metal-binding histidine residues of BcII in the presence and absence of R- and S-thiomandelic acid

| Residue | Nucleus | Chemical shift (ppm) |
|---------|---------|----------------------|
|         |         | Enzyme alone | R-Thiomandelic acid complex | S-Thiomandelic acid complex |
| His86   | Nδ      | 175.61       | 176.98                 | 176.18                 |
|         | Nε      | 175.61       | 176.98                 | 176.18                 |
|         | CεH     | 6.26         | 6.14                   | 6.08                   |
|         | CεH     | 7.66         | 10.36                  | 9.89                   |
|         | NδH     | 12.03        | 12.87                  | 12.98                  |
|         | NεH     | 14.19        | 13.66                  | 13.37                  |
| His88   | Nδ      | 205.26       | 208.22                 | 208.27                 |
|         | Nε      | 173.14       | 170.53                 | 169.41                 |
|         | CεH     | 7.33         | 7.32                   | 7.77                   |
|         | CεH     | 7.66         | 7.76                   | 8.30                   |
|         | NδH     | 11.49        | 13.66                  | 13.37                  |
|         | NεH     | 201.87       | 203.62                 | 208.27                 |
|         | CεH     | 5.18         | 4.95                   | 4.96                   |
|         | CεH     | 8.43         | 8.40                   | 8.30                   |
|         | NδH     | 14.19        | 13.66                  | 13.37                  |
|         | NεH     | 170.10       | 168.58                 | 169.41                 |
| His149  | Nδ      | 217.65       | 224.44                 | 210.43                 |
|         | Nε      | 6.93         | 7.00                   | 7.25                   |
|         | CεH     | 7.23         | 7.40                   | 7.48                   |
|         | CεH     | 7.23         | 7.40                   | 7.48                   |
|         | NδH     | 13.14        | 13.19                  | 12.66                  |
dues Asp90, His88, His149, and Cys168 are all clearly affected; that of His210 is in a crowded region of the spectrum and cannot be followed with certainty. For several of the residues in the active site, including the zinc ligands His88 and His149, as well as the nearby residues Ile84, Asp152, and Gly167, the changes in the shift index are greater in the S- than in the R-thiomandelic acid complex; the only exception is Gly211, which is affected more by the R-isomer.

The largest change in amide chemical shift on inhibitor binding is seen for Arg91, which is affected to a very similar extent by both isomers of thiomandelic acid. This suggests the possibility that the guanidino group of this residue might be responsible for interacting with the carboxylate group of the inhibitor. This residue does not correspond to the residue Lys161, which binds the inhibitor carboxylate in the complex of the IMP-1 enzyme with a mercaptocarboxylate (25), but the relationship between the thiol and the carboxylate of this inhibitor is quite different from that in thiomandelic acid. It must be noted, however, that the guanidino group of Arg91 in BcII is close to the two zinc binding sites and hydrogen-bonds to the zinc ligand Asp90; we cannot, therefore, exclude the possibility that the effects of thiomandelic acid binding on this residue are secondary to the thiol binding to the metal ions nor indeed that the carboxylate also interacts with one of the zincs, as seen in the recent structures of substituted succinic acid derivatives with the IMP-1 enzyme (36).

Substantial amide chemical shift perturbations are also observed for residues in the “flexible flap” region (the β3-β4 loop, residues 30–40), which in the absence of inhibitor is some distance from the active site (Fig. 6). Shift changes are seen for Glu30, Leu31, Phe34, and Val39. A significant chemical shift perturbation was also observed for Trp59, which is located at the beginning of a loop on the top of the cavity; since the side
chain of Trp\(^9\) extends toward the \(\beta_2-\beta_4\) loop (see Fig. 6), this could result from a movement of the latter. Chemical shift effects on residues in the corresponding loop or \(\beta\)-hairpin were noted by Scrofani \textit{et al.} (40) in their studies of a tightly binding inhibitor of the \(B.\) \textit{fragilis} MBL. Crystallographic studies of inhibitor binding to the IMP-1 MBL (25) clearly show that, upon the binding of a rather large mercapto-carboxylate inhibitor, the corresponding loop or \(\beta\)-hairpin folds over the active site, making important contacts with the bound inhibitor. The evidence described here on the \(B.\) \textit{cereus} enzyme, together with the chemical shift and relaxation studies of inhibitor binding to the \(B.\) \textit{fragilis} enzyme (40, 44), strongly suggests that a movement of this loop is a general and important feature of inhibitor binding to MBLs. It is particularly interesting that the loop movement apparently takes place even on the binding of an inhibitor as small as thiomandelic acid, suggesting the possibility that the trigger for this movement is conformational, rather than simply arising from direct interactions of loop residues with the inhibitor. The linkage of the relatively distant loop movement with inhibitor binding may have mechanistic relevance. Thus, the loop may be involved in substrate capture and help to direct the \(\beta\)-lactam of the substrate toward the metal center; in turn, the hydrolytic chemistry at the metal center may be coordinated with product release through movement of this loop.

Conclusions—The data presented here demonstrate that the simple molecule thiomandelic acid is a reasonably potent and broad spectrum inhibitor of metallo-\(\beta\)-lactamases. This report is the first demonstration that broad spectrum inhibition of MBLs is feasible. The data are consistent with the idea that both \(R\) and \(S\)-thiomandelic acid bind in such a way that the thiol group of the inhibitor binds to the two zinc atoms in the active site. The chemical shift changes suggest that the carboxylate group of the inhibitor may bind to Arg\(^{31}\) in a well conserved residue in subclass B1 enzymes, and this appears to be the case for both optical isomers of the inhibitor, since both produce similar effects on the amide resonance of this residue. The structure-activity relationship among the thiomandelic acid isomers binds in a different position in the active site. The modest observed difference in \(K_i\) would then indicate that this phenyl ring does not contribute to binding in a major way, again consistent with the structure-activity relationships. The different positions of the phenyl rings of \(R\) and \(S\)-thiomandelic acid in the active site are likely to contribute to the different effects of the two compounds on the chemical shifts of residues in the active site, both directly, through the magnetic anisotropy of the phenyl ring itself, and indirectly, through its interactions with the protein, which may lead to slight differences in position of the thiol and carboxylate groups of the inhibitor. The \(\beta_2-\beta_4\) loop, or "flap," is clearly affected even by the binding of this small inhibitor, confirming its role as an important determinant of ligand binding in subclass B1 enzymes. Finally, the observation that thioesters of thiomandelic acid, themselves rather weak inhibitors, are hydrolyzed by the enzyme to yield the much more inhibitory thiomandelic acid suggests that these compounds should be valuable alternatives for \textit{in vivo} use and deserve further study.

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