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

KINETIC AND SPECTROSCOPIC STUDIES*[S]

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Resistance to β-lactam antibiotics mediated by metallo-β-lactamases is an increasingly worrying clinical problem. Candidate inhibitors include mercaptocarboxylic 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 K_i 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 K_i 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 Arg^{91}. Amide chemical shift perturbations for residues 30–40 (the β_3-β_4 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*, *Alcalinogenes*, *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 BcII from *B. cereus* (11, 12), CfiA (also called CcrA) from *B. fragilis* (13, 14), IMP-1 and VIM-1 from *P. aeruginosa* (6, 15), and BlaB from *Chryseobacterium meningosepticum* (16); subclass B2 essentially consists of enzymes from *Aeromonas* strains (*e.g.* CphA 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

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[S] The on-line version of this article (available at http://www.jbc.org) contains two schemes and one figure.

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[1] The abbreviations used are: MBL, metallo-β–lactamase; BcII, the zinc metallo-β-lactamase from *B. cereus*; HSQC, heteronuclear single quantum coherence; HMQC, heteronuclear multiple quantum coherence; MES, 2-[*N*-morpholino]ethanesulfonic acid.

Supplemental Material can be found at:
http://www.jbc.org/cgi/content/full/276/48/45015/DC1

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^{86}, 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 zincs, this water (or hydroxide) bridges to the zinc in site II, which is also coordinated by a histidine (His^{210} 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).[2] 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 mercaptocarboxylate substrate analogue inhibitor (25), and NMR data are available for the binding of other mercaptocarboxylate 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 mercaptocarboxylate inhibitors, thiomandelic acid (Fig. 1; α-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. Damblon, I. G. Barsukov, C. Mollard, and G. C. K. Roberts, manuscript in preparation.

EXPERIMENTAL PROCEDURES

Chemicals and Syntheses—4-Methoxyphenylacetic acid (**1b**), 4-nitrophenylacetic acid (**1c**), α-bromophenylacetic acid (**2a**), 4-mercaptobenzoic acid (**9**), and 2-thiophenecarboxylic acid (**10**) were purchased from Aldrich. 4-Fluorophenylacetic acid (**1d**) and thiosalicylic acid (**8**) were from Acros Organics, and benzylmercaptan (**11**) was from Fluka. Compounds **7a–c** and **g-h** (Fig. 1) were generous gifts from Jean-Luc Boucher (CNRS, University Paris V) and were synthesized according to Eloy and Lenaers (45). Thiomandelic acid and a series of *para*-substituted analogues were synthesized in racemic form by a modified version of the procedure described by Bonner (46). Starting from the readily accessible appropriate α-bromobenzeneacetic acids **2b–d** and commercially available **2a**, compounds **3a–d** were synthesized by reaction with potassium *o*-ethyldithiocarbonate, and **3aa-ab** were synthesized with the corresponding cesium salt. The *o*-ethyldithiocarbonate group was then converted into a thiol moiety via basic hydrolysis using concentrated ammonia in ethyl alcohol. The α-mercaptophenylacetic acids **4a-b, d** were obtained as a mixture with the corresponding disulfide derivatives **5a-b, d**, whereas the nitro derivative **3c** gave only undesired side products. The oxidation of the thiol groups by using catalytic ferric chloride and sodium iodide (47) led to pure (>95% by ^{1}H NMR analysis) disulfides **5a-b, d**. The disulfide moiety is often a convenient protecting group, and thiols are easily obtained by the reduction of disulfides. Here, compounds **5a-b**, **d** were reduced with sodium borohydride to give pure (>95% by ^{1}H NMR analysis) thiols **4a-b, d**. The individual optical isomers, *R*- and *S*-thiomandelic acids (**15a** and **15b**), were synthesized by the procedure of Strijtveen and Kellog (48). The ethyl esters **12a** and **12b** were prepared from the commercially available *S*- and *R*-mandelic acids. Methanesulfonates **13a** and **13b** were then obtained by reacting methanesulfonyl chloride with **12a** and **12b**, respectively. Reaction of these mesylate derivatives with the cesium salt of thioacetic acid led to the thioesters **14a** and **14b**, which were hydrolyzed. However, after 4 days in concentrated hydrochloric acid at room temperature, only starting material was recovered. Hydrolysis was thus carried out at 50 °C for 12 h to give the desired compounds **15a** and **15b** in an optical purity of 73–74%. Details of the synthesis and characterization of individual compounds are given in the Supplemental Material.

Enzyme Preparation—All the MBLs used here were purified as described in the following references: BcII (12), CfiA (23), L1 (26), IMP-1 (6), IMP-2 (7), VIM-1 (15), BlaB (16), FEZ-1 (20), and CphA (17). ^{2}H,^{15}N-Labeled BcII for NMR spectroscopy was prepared as described previously (49). For the cysteine 158 to alanine mutant of IMP-1 (C158A), the pET9a construct bearing the entire bla_{IMP} gene (6) was used as a template for PCR amplification with the following overlapping primers: 5′-GTACGGTTTAATAAATGCACCACCGAATAATATTTTC-C-3′ and 5′-GGAAAATATTATTCGGTGGTGCATTTATTAAACCGTA-C-3′. The C158A enzyme was produced and purified as for the wild-type enzyme (6).

Enzyme Kinetics—Determinations of catalytic activity were performed in 10 mM HEPES, pH 7.5, at 30 °C using a Uvikon XL (Bio-tek instruments) spectrophotometer equipped with thermostatically controlled cells. Substrate hydrolysis was followed by monitoring the change in absorbance at the appropriate wavelength (nitrocefin for BcII, CfiA, L1, IMP-1, and FEZ-1, $\Delta\epsilon^{482} = 17{,}500\ M^{-1}\ cm^{-1}$; imipenem for IMP-2, BlaB, and CphA, $\Delta\epsilon^{300} = -9000\ M^{-1}\ cm^{-1}$; meropenem for VIM-1, $\Delta\epsilon^{297} = -6500\ M^{-1}\ cm^{-1}$), and the substrate concentrations used were chosen to be close to the K_m value. For the screening of potential inhibitors, nitrocefin (20 μM) was used as a substrate, and the experiments were carried out at a final enzyme (BcII) concentration of 4.8 nM. The compounds to be tested were dissolved in ethanol at a concentration of 0.1 M and then diluted to 1 mM in the enzyme assay. The presence of 1% ethanol proved to be without effect on the BcII activity (data not shown). Quoted K_i values are derived assuming a competitive pattern of inhibition and plotting $v_0/v_i = f([I])$, where v_0 is the initial rate in the absence of inhibitor, and v_i is the initial rate in the presence of inhibitor. The slope of the resulting straight line is then given by $K_m/((K_m + [S])\cdot K_i)$. The specific activity toward the thioesters **3aa** and **3ab** was also determined spectrophotometrically in the presence of 4,4′-dithiodipyridine (Sigma), a sensitive thiol-specific reagent that has an absorption maximum at 324 nm ($\epsilon = 19{,}800\ M^{-1}\ cm^{-1}$) after reaction with two equivalents of thiol (50). Incubations contained 1 mM thioester, 2.5 mM 4,4′-dithiodipyridine (0.1 M stock solutions of both compounds prepared in ethanol) and 0.6 μM BcII in 500 μl of 10 mM HEPES, pH 7.5. Subtraction of the side reaction due to the nonenzymatic release of thiol from the thioester in the presence of 4,4′-dithiodipyridine was performed by using a double beam spectrophotometer in

which the reference cuvette contained thioester and 4,4′-dithiodipyridine at the same concentration as in the sample cuvette but no enzyme. Control experiments with nitrocefin as substrate demonstrated that the presence of 4,4′-dithiodipyridine at the concentration used did not inhibit the catalytic activity of the enzyme.

Protein NMR Spectroscopy—Samples of the inhibitor complexes of the BCII enzyme for NMR spectroscopy were prepared by the addition of microliter volumes of stock inhibitor solution (50 mM in 0.2 M MES buffer, pH 6.4) to a solution of 1.0 mM ^{15}N-labeled enzyme in the same buffer. Spectra were acquired at 298 K using Bruker DMX 500-MHz or DRX 600-MHz spectrometers equipped with 5-mm inverse detection triple resonance probes with *z* axis gradient. Backbone amide NH resonances were observed using the ^{1}H-^{15}N HSQC experiment with gradient coherence selection and sensitivity enhancement (51). The assignment of these resonances by means of triple resonance experiments using ^{2}H,^{13}C,^{15}N-labeled enzyme is described elsewhere.[3] The effects of inhibitor binding on the amide resonances were analyzed as follows. For each cross-peak in the ^{1}H-^{15}N HSQC spectrum of the free protein, the nearest cross-peak (in terms of ^{1}H and ^{15}N chemical shifts) in the spectrum of the inhibitor complex was identified. The ^{1}H and ^{15}N chemical shift differences, ΔH and ΔN, between each such pair of cross-peaks were measured and used to calculate a "minimum shift index" (52).

$$\sqrt{(\Delta H/0.03)^2 + (\Delta N/0.3)^2} \qquad \text{(Eq. 1)}$$

One-dimensional ^{1}H spectra of histidine imidazole (N)H protons were obtained by using the Watergate experiment with water flipback pulse (53, 54); ^{1}H-^{15}N correlation spectra of the histidine imidazole resonances were obtained using ^{1}H-^{15}N HMQC as previously described (49). Spectra were processed using UXNMR (Bruker) software.

RESULTS AND DISCUSSION

In view of the evidence, discussed above, that simple thiol compounds and mercaptoacetic acid thiol 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 Kellog (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 (BcII) 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 inhibit BcII 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 **1a–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 (**3a–d**), thioester (**3aa-ab**) or disulfide (**5a-b, d**) derivatives is detrimental, but some inhibitory activity is retained for compounds **3c, 3aa-ab** and **5a-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 μM 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 BcII 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).

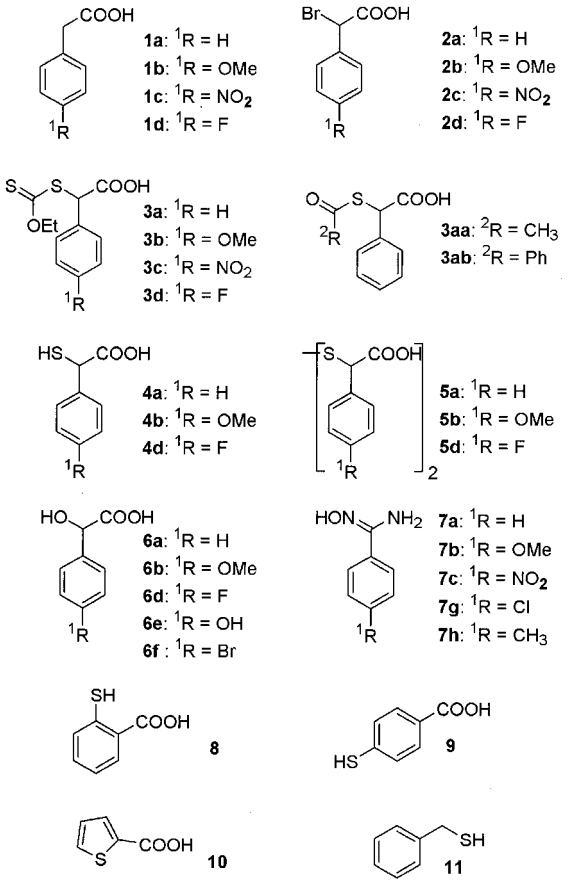

FIG. 1. **Structures of thiomandelic acid and related compounds studied as inhibitors of metallo-β-lactamases.**

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′-dithiodipyridine (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′-dithiodipyridine 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 BcII. 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 BcII was found to be 424 ± 55 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**.

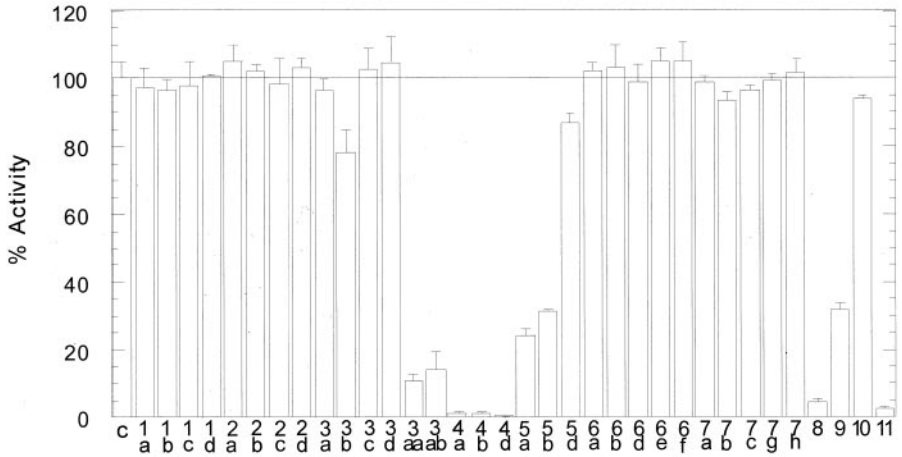

FIG. 2. **Comparison of the activity of the BcII enzyme in the absence (control) or presence of 1 mM concentrations of the compounds shown in Fig. 1.** Activity was measured with nitrocefin as substrate, as described under "Experimental Procedures," and corresponds to 400 nmol/s/mg in the absence of inhibitor (*C*).

TABLE I

Inhibition constants of some thiomandelic acid derivatives for the B. cereus metallo-β-lactamase (BcII)

K_i values are calculated assuming a competitive pattern of inhibition. Mean values of at least two separate experiments are shown (S.D. < 20%).

Inhibitor	**3aa**	**3ab**	Thiomandelic acid			**4b**	**4d**	**5a**	**5b**	**8**	**9**	**11**
			RS **(4a)**	*R* **(15a)**	*S* **(15b)**							
K_i (μM)	36	37	0.34	0.09	1.28	0.21	0.46	33	237	29	346	9.2

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, $K_m = K_i$ 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, the K_i of *R*-thiomandelic acid (**15a**) being 14-fold less than that of the *S*-isomer (**15b**) (Table I).

The kinetic data for thiomandelic acid fitted a competitive pattern of inhibition, and no evidence was obtained for irreversible binding of thiomandelic acid to the BcII enzyme. Preincubation 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 mercaptoacetic acid thioesters (38), we examined thiomandelic acid inhibition of the C158A 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 (His^{86}, His^{88}, and His^{149} in site I, His^{210} in site II), which have been assigned to individual residues (49),[3] 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, 298 K, relatively sharp resonances are observed for His^{86} and His^{88} and a broad resonance for His^{210}, but no signal is apparent for His^{149}. At lower pH or temperature, the His^{210} resonance sharpens, and a signal for His^{149} 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 ^1H-^{15}N HMQC spectra optimized for observa-

TABLE II
Inhibition constants of racemic thiomandelic acid for metallo-β-lactamases

Enzyme	*B. cereus* BcII	*B. fragilis* CfiA	*S. maltophilia* L1	*P. aeruginosa* IMP-1	*A. baumanii* IMP-2	*P. aeruginosa* VIM-1	*C. meningosepticum* BlaB	*L. gormanii* FEZ-1	*A. hydrophila* CPhA
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%).

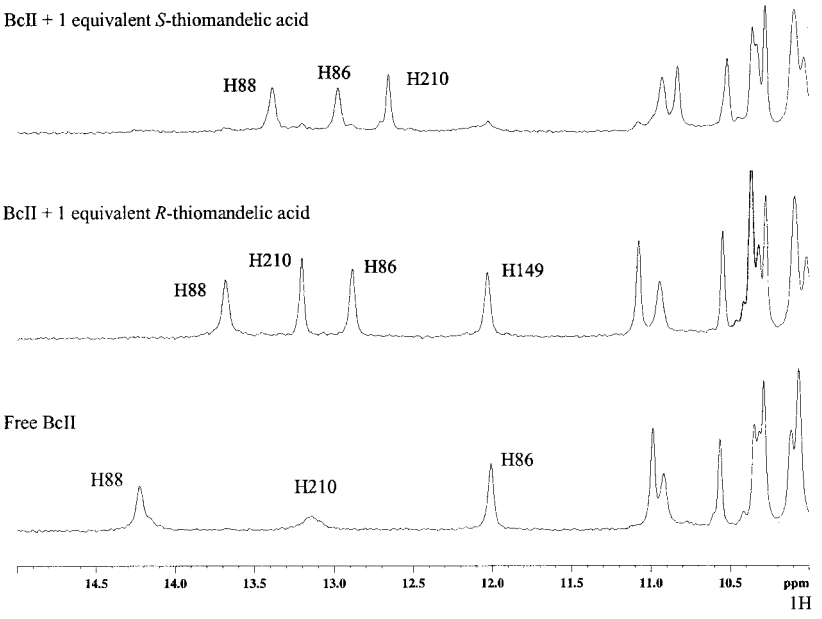

FIG. 3. **Part of the ^{1}H NMR spectrum of BcII in the presence and absence of *R*- and *S*-thiomandelic acid, showing the imidazole NH resonances of the metal-binding histidine residues.**

tion of the long range ^{1}H-^{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; Nϵ for His^{86} and His^{149}, Nδ 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 Nϵ (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,[3] presumably displacing the "bridging" water molecule, as seen for a much more complex thiocarboxylate 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 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 (^{1}H-^{15}N HSQC) of ^{15}N-labeled BcII allows the rapid observation of most of the backbone amide ^{1}H and ^{15}N resonances. These resonances have been assigned to individual residues in the free protein,[3] 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 ^{1}H-^{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 unaffected, 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 III
^{1}H and ^{15}N 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		
		Enzyme alone	*R*-Thiomandelic acid complex	S-Thiomandelic acid complex
			ppm	
His^{86}	Nδ	175.61	176.98	176.18
	Nε	207.83	207.83	217.07
	CδH	6.26	6.14	6.08
	CεH	7.66	10.36	9.89
	NδH	12.03	12.87	12.98
His^{88}	Nδ	205.26	208.22	208.27
	Nε	173.14	170.53	169.41
	CδH	7.33	7.32	7.77
	CεH	7.66	7.76	8.30
	NεH	14.19	13.66	13.37
His^{149}	Nδ	169.98	169.13	169.27
	Nε	201.87	203.62	208.27
	CδH	5.18	4.95	4.96
	CεH	8.43	8.40	8.30
	NδH		12.03	
His^{210}	Nδ	170.10	168.58	169.41
	Nε	217.65	224.44	210.43
	CδH	6.93	7.00	7.25
	CεH	7.23	7.40	7.48
	NδH	13.14	13.19	12.66

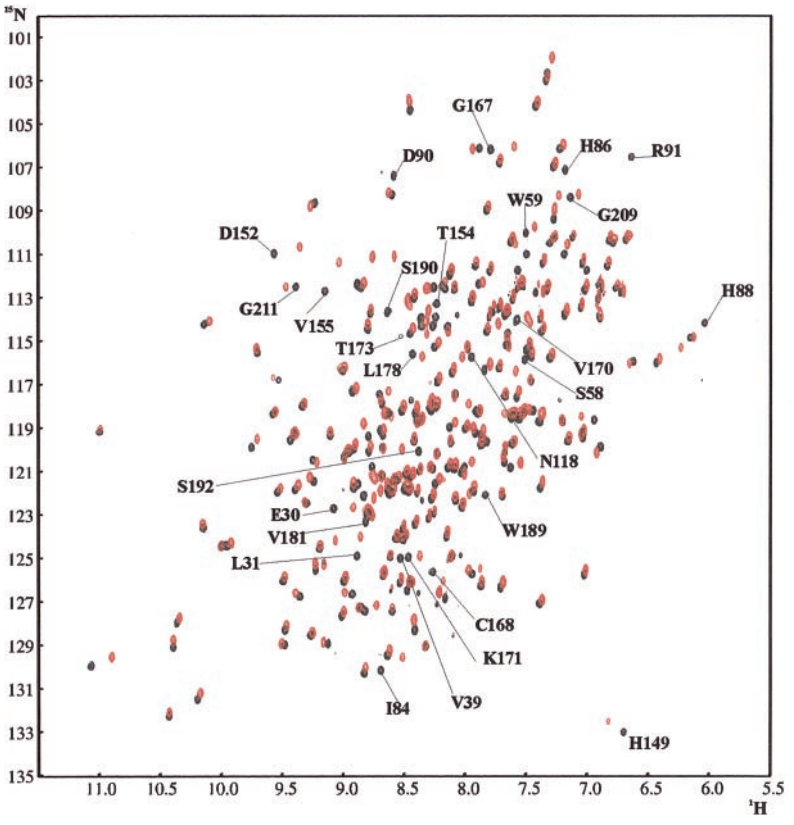

FIG. 4. **^{1}H-^{15}N HSQC spectra of BcII in the presence and absence of *S*-thiomandelic acid, showing the backbone and side chain amide and tryptophan indole NH resonances.** The cross-peaks are shown in *black* for the spectrum of the enzyme alone and in *red* for that of the *S*-thiomandelic acid complex. Resonance assignments[3] are indicated for a number of the better resolved signals that are affected by inhibitor binding.

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 resi-

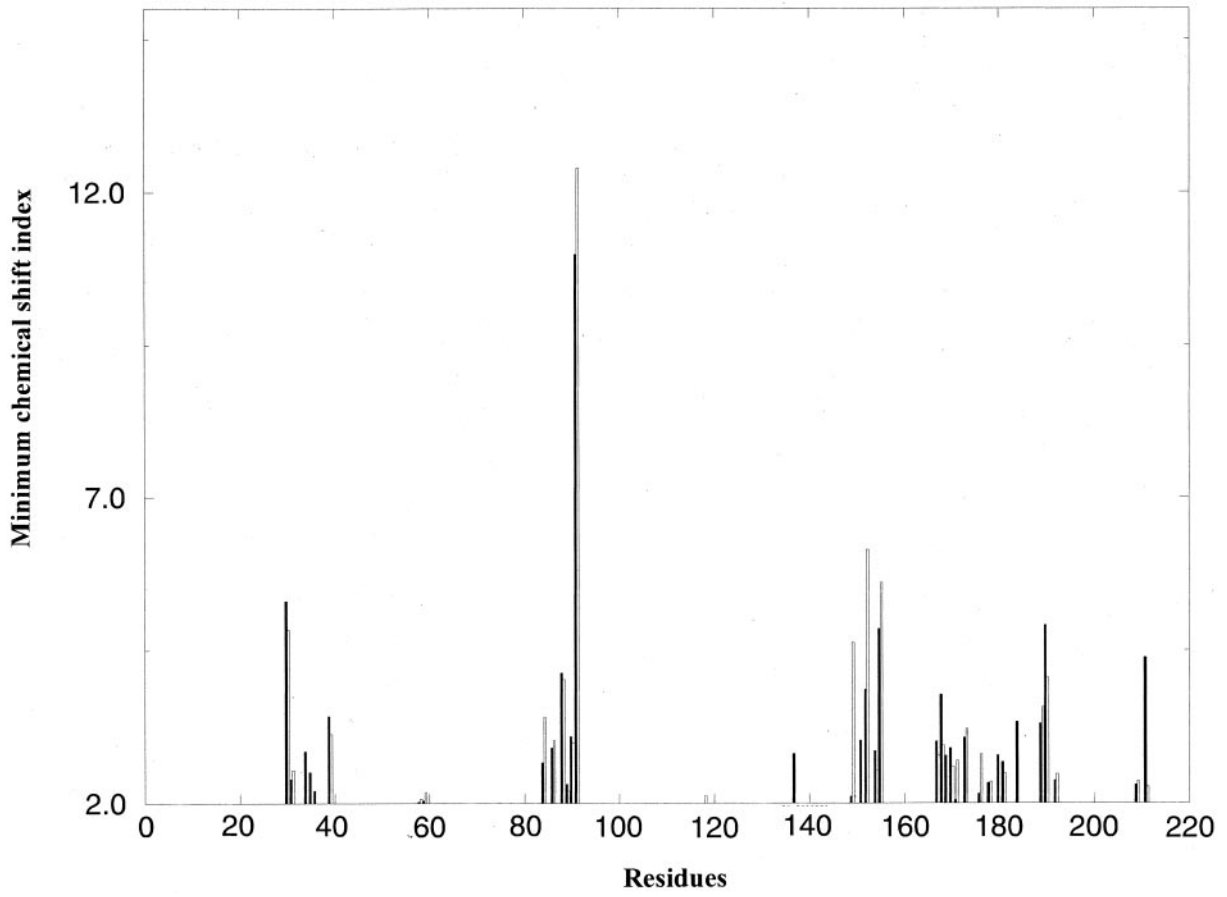

FIG. 5. **The changes in chemical shift of the amide resonances of BcII on the binding of *R*- thiomandelic acid (*solid bars*) or *S*-thiomandelic acid (*open bars*).** As discussed under "Results and Discussion," the changes are expressed as the "minimum chemical shift index," calculated as described under "Experimental Procedures."

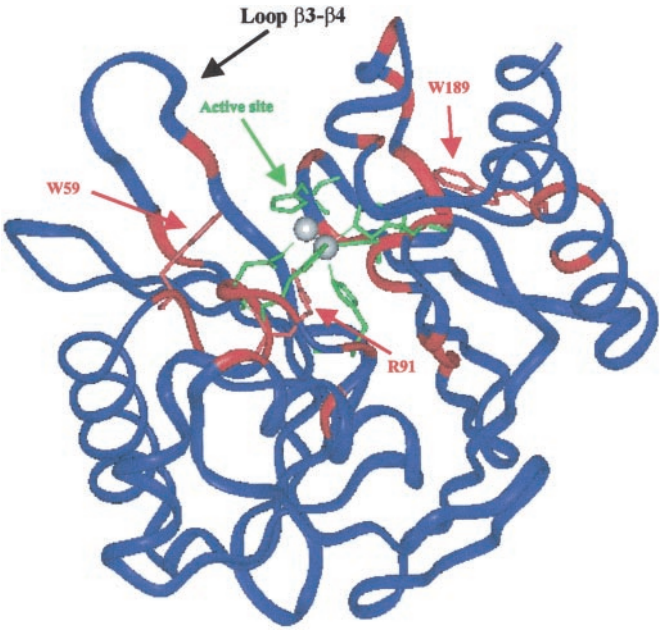

FIG. 6. **Ribbon diagram of the structure of BcII.** The residues whose amide resonances are most affected by the binding of *S*-thiomandelic acid are indicated in *red* on the backbone ribbon; side chains are shown for the zinc ligands and some other residues mentioned under "Results and Discussion."

dues Asp[90], His[88], His[86], His[149], and Cys[168] are all clearly affected; that of His[210] 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 His[88] and His[149], as well as the nearby residues Ile[84], Asp[152], and Gly[167], the changes in the shift index are greater in the *S*- than in the *R*-thiomandelic acid complex; the only exception is Gly[211], which is affected more by the *R*-isomer.

The largest change in amide chemical shift on inhibitor binding is seen for Arg[91], 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 Lys[161], 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 Arg[91] in BcII is close to the two zinc binding sites and hydrogen-bonds to the zinc ligand Asp[90]; 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 β_3-β_4 loop, residues 30–40), which in the absence of inhibitor is some distance from the active site (Fig. 6). Shift changes are seen for Glu[30], Leu[31], Phe[34], and Val[39]. (A significant chemical shift perturbation was also observed for Trp[59], which is located at the beginning of a loop on the top of the cavity; since the side

chain of Trp^{59} extends toward the β_3-β_4 loop (see Fig. 6), this could result from a movement of the latter). Chemical shift effects on residues in the corresponding loop or β-hairpin were noted by Scrofani *et al.* (40) in their studies of a tightly binding inhibitor of the *B. fragilis* MBL. Crystallographic studies of inhibitor binding to the IMP-1 MBL (25) clearly show that, upon the binding of a rather large mercaptocarboxylate inhibitor, the corresponding loop or β-hairpin folds over the active site, making important contacts with the bound inhibitor. The evidence described here on the *B. cereus* enzyme, together with the chemical shift and relaxation studies of inhibitor binding to the *B. 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 β-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-β-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^{91}, 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 analogues studied here indicates that the carboxylate and, particularly, the thiol groups are most important for binding, and the conclusion that these two groups bind similarly to the enzyme in both *R*- and *S*-thiomandelic acid is consistent with the finding that there is a relatively modest difference in K_i between the two isomers. Similar binding of the thiol and carboxylate groups would imply that the phenyl ring of the two 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 β_3-β_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 *in vivo* use and deserve further study.

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REFERENCES

1. Frère, J. M. (1995) *Mol. Microbiol.* **16,** 385–395
2. Matagne, A., Dubus, A., Galleni, M., and Frère, J. M. (1999) *Nat. Prod. Rep.* **16,** 1–19
3. Wang, Z., Fast, W., Valentine, A. M., and Benkovic, S. J. (1999) *Curr. Opin. Chem. Biol.* **3,** 614–622
4. Kuwabara, S., and Abraham, E. P. (1967) *Biochem. J.* **103,** 27C–30C
5. Payne, D. J. (1993) *J. Med. Microbiol.* **39,** 93–99
6. Laraki, N., Franceschini, N., Rossolini, G. M., Santucci, P., Meunier, C., de Pauw, E., Amicosante, G., Frère, J. M., and Galleni, M. (1999) *Antimicrob. Agents Chemother.* **43,** 902–906
7. Riccio, M. L., Franceschini, N., Boschi, L., Caravelli, B., Cornaglia, G., Fontana, R., Amicosante, G., and Rossolini, G. M. (2000) *Antimicrob. Agents Chemother.* **44,** 1229–1235
8. Iyobe, S., Kusadokoro, H., Ozaki, J., Matsumura, N., Minami, S., Haruta, S., Sawai, T., and O'Hara, K. (2000) *Antimicrob. Agents Chemother.* **44,** 2023–2027
9. Chu, Y. W., Afzal-Shah, M., Houang, E. T., Palepou, M. F., Lyon, D. J., Woodford, N., and Livermore, D. M. (2001) *Antimicrob. Agents Chemother.* **45,** 710–714
10. Yano, H., Kuga, A., Okamoto, R., Kitasato, H., Kobayashi, T., and Inoue, M. (2001) *Antimicrob. Agents Chemother.* **45,** 1343–1348
11. Fabiane, S. M., Sohi, M. K., Wan, T., Payne, D. J., Bateson, J. H., Mitchell, T., and Sutton, B. J. (1998) *Biochemistry* **37,** 12404–12411
12. Paul-Soto, R., Bauer, R., Frère, J. M., Galleni, M., Meyer-Klaucke, W., Nolting, H., Rossolini, G. M., de Seny, D., Hernandez-Valladares, M., Zeppezauer, M., and Adolph, H. W. (1999) *J. Biol. Chem.* **274,** 13242–13249
13. Concha, N. O., Rasmussen, B. A., Bush, K., and Herzberg, O. (1996) *Structure* **4,** 823–836
14. Paul-Soto, R., Hernadez-Valladares, M., Galleni, M., Bauer, R., Zeppezauer, M., Frère, J. M., and Adolph, H. W. (1998) *FEBS Lett.* **438,** 137–140
15. Franceschini, N., Caravelli, B., Docquier, J. D., Galleni, M., Frère, J. M., Amicosante, G., and Rossolini, G. M. (2000) *Antimicrob. Agents Chemother.* **44,** 3003–3007
16. Rossolini, G. M., Franceschini, N., Riccio, M. L., Mercuri, P. S., Perilli, M., Galleni, M., Frère, J. M., and Amicosante, G. (1998) *Biochem. J.* **332,** 145–152
17. Hernandez Valladares, M., Felici, A., Weber, G., Adolph, H. W., Zeppezauer, M., Rossolini, G. M., Amicosante, G., Frère, J. M., and Galleni, M. (1997) *Biochemistry* **36,** 11534–11541
18. Walsh, T. R., Hall, L., Assinder, S. J., Nichols, W. W., Cartwright, S. J., MacGowan, A. P., and Bennett, P. M. (1994) *Biochim. Biophys. Acta* **1218,** 199–201
19. Crowder, M. W., Walsh, T. R., Banovic, L., Pettit, M., and Spencer, J. (1998) *Antimicrob. Agents Chemother.* **42,** 921–926
20. Mercuri, P. S., Bouillenne, F., Boschi, L., Lamotte-Brasseur, J., Amicosante, G., Devreese, B., Van Beeumen, J., Frère, J. M., Rossolini, G. M., and Galleni, M. (2001) *Antimicrob. Agents Chemother.* **45,** 921–926
21. Bellais, S., Aubert, D., Naas, T., and Nordmann, P. (2000) *Antimicrob. Agents Chemother.* **44,** 1878–1886
22. Carfi, A., Duée, E., Galleni, M., Frère, J. M., and Dideberg, O. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54,** 313–323
23. Carfi, A., Duée, E., Paul-Soto, R., Galleni, M., Frere, J. M., and Dideberg, O. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54,** 45–57
24. Fitzgerald, P. M., Wu, J. K., and Toney, J. H. (1998) *Biochemistry* **37,** 6791–6800
25. Concha, N. O., Janson, C. A., Rowling, P., Pearson, S., Cheever, C. A., Clarke, B. P., Lewis, C., Galleni, M., Frère, J. M., Payne, D. J., Bateson, J. H., and Abdel-Meguid, S. S. (2000) *Biochemistry* **39,** 4288–4298
26. Ullah, J. H., Walsh, T. R., Taylor, I. A., Emery, D. C., Verma, C. S., Gamblin, S. J., and Spencer, J. (1998) *J. Mol. Biol.* **284,** 125–136
27. Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O., and Frère, J. M. (2001) *Antimicrob. Agents Chemother.* **45,** 660–663
28. Bounaga, S., Laws, A. P., Galleni, M., and Page, M. I. (1998) *Biochem. J.* **331,** 703–711
29. Wang, Z. G., Fast, W., and Benkovic, S. J. (1999) *Biochemistry* **38,** 10013–10023
30. Baldwin, G. S., Galdes, A., Hill, H. A., Waley, S. G., and Abraham, E. P. (1980) *J. Inorg. Biochem.* **13,** 189–204
31. Orellano, E. G., Girardini, J. E., Cricco, J. A., Ceccarelli, E. A., and Vila, A. J. (1998) *Biochemistry* **37,** 10173–10180
32. Toney, J. H., Fitzgerald, P. M., Grover-Sharma, N., Olson, S. H., May, W. J., Sundelof, J. G., Vanderwall, D. E., Cleary, K. A., Grant, S. K., Wu, J. K., Kozarich, J. W., Pompliano, D. L., and Hammond, G. G. (1998) *Chem. Biol.* **5,** 185–196
33. Nagano, R., Adachi, Y., Imamura, H., Yamada, K., Hashizume, T., and Morishima, H. (1999) *Antimicrob. Agents Chemother.* **43,** 2497–2503
34. Walter, M. W., Felici, A., Galleni, M., Paul-Soto, R., Adlington, R. M., Baldwin, J. E., Frère, J. M., Gololobov, M., and Schofield, C. J. (1996) *Bioorg. Med. Chem. Lett.* **6,** 2455–2458
35. Walter, M. W., Hernandez Valladres, M., Adlington, R. M., Amicosante, G., Baldwin, J. E., Frère, J. M., Galleni, M., Rossolini, G. M., and Schofield, C. J. (1999) *Bioorg. Chem.* **27,** 35–40
36. Toney, J. H., Hammond, G. G., Fitzgerald, P. M. D., Sharma, N., Balkovec, J. M., Rouen, G. P., Olson, S. H., Hammond, M. L., Greenlee, M. L., and Gao, Y.-D. (2001) *J. Biol. Chem.* **276,** 31913–31918
37. Goto, M., Takahashi, T., Yamashita, F., Koreeda, A., Mori, H., Ohta, M., and Arakawa, Y. (1997) *Biol. Pharm. Bull.* **20,** 1136–1140
38. Payne, D. J., Bateson, J. H., Gasson, B. C., Proctor, D., Khushi, T., Farmer, T. H., Tolson, D. A., Bell, D., Skett, P. W., Marshall, A. C., Reid, R., Ghosez, L., Combret, Y., and Marchand-Brynaert, J. (1997) *Antimicrob. Agents Chemother.* **41,** 135–140
39. Page, M. I., and Laws, A. P. (1998) *Chem. Commun.* 1609–1617

40. Scrofani, S. D., Chung, J., Huntley, J. J., Benkovic, S. J., Wright, P. E., and Dyson, H. J. (1999) *Biochemistry* **38,** 14507–14514
41. Payne, D. J., Bateson, J. H., Gasson, B. C., Khushi, T., Proctor, D., Pearson, S. C., and Reid, R. (1997) *FEMS Microbiol. Lett.* **157,** 171–175
42. Greenlee, M. L., Laub, J. B., Balkovec, J. M., Hammond, M. L., Hammond, G. G., Pompliano, D. L., and Epstein-Toney, J. H. (1999) *Bioorg. Med. Chem. Lett.* **9,** 2549–2554
43. Hammond, G. G., Huber, J. L., Greenlee, M. L., Laub, J. B., Young, K., Silver, L. L., Balkovec, J. M., Pryor, K. D., Wu, J. K., Leiting, B., Pompliano, D. L., and Toney, J. H. (1999) *FEMS Microbiol. Lett.* **179,** 289–296
44. Huntley, J. J. A., Scrofani, S. D. B., Osborne, M. J., Wright, P. E., and Dyson, H. J. (2000) *Biochemistry* **39,** 13356–13364
45. Eloy, F., and Lenaers, R. (1962) *Chem. Rev.* **62,** 155–183
46. Bonner, W. A. (1968) *J. Org. Chem.* **33,** 1831–1836
47. Iranpoor, N., and Zeynizadeh, B. (1999) *Synthesis* **1,** 49–50
48. Strijtveen, B., and Kellog, R. M. (1986) *J. Org. Chem.* **51,** 3664–3671
49. Damblon, C., Prosperi, C., Lian, L. Y., Barsukov, I., Paul-Soto, R., Galleni, M., Frère, J. M., and Roberts, G. C. K. (1999) *J. Am. Chem. Soc.* **121,** 11575–11576
50. Grassetti, D. R., and Murray, J. F. (1967) *Arch. Biochem. Biophys.* **119,** 41–49
51. Kay, L. E., Keifer, P., and Saarinen, T. (1992) *J. Am. Chem. Soc.* **114,** 10663–10665
52. Lian, L.-Y., Barsukov, I., Golovanov, A., Hawkins, D., Sze, K.-H., Badii, R., Keep, N. H., and Roberts, G. C. K. (2000) *Structure* **8,** 47–55
53. Piotto, M., Saudek, V., and Sklenar, V. (1992) *J. Biomol. NMR* **2,** 661–665
54. Kuboniwa, H., Grzesiek, S., Delaglio, F., and Bax, A. (1994) *J. Biomol. NMR* **4,** 871–878
55. Farmer, B. T. (1996) *Nat. Struct. Biol.* **3,** 995–997
56. Williamson, R. A., Carr, M. D., Frenkiel, T. A., Feeney, J., and Freedman, R. B. (1997) *Biochemistry* **36,** 13882–13889
57. Muskett, F. W., Frenkiel, T. A. Feeney, J., Freedman, R. B., Carr, M. D., and Williamson, R. A. (1998) *J. Biol. Chem.* **273,** 21736–21743