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Interactions of "Bora-Penicilloates" with Serine β-Lactamases and DD-Peptidases

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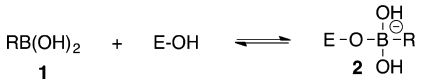
Supporting Information

ABSTRACT: Specific boronic acids are generally powerful tetrahedral intermediate/transition state analogue inhibitors of serine amidohydrolases. This group of enzymes includes bacterial β-lactamases and DD-peptidases where there has been considerable development of boronic acid inhibitors. This paper describes the synthesis, determination of the inhibitory activity, and analysis of the results from two α-(2-thiazolidinyl) boronic acids that are closer analogues of particular tetrahedral intermediates involved in β-lactamase and DD-peptidase catalysis than those previously described. One of them, 2-[1-(dihydroxyboranyl)(2-phenylacetamido)methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid, is a direct analogue of the deacylation tetrahedral intermediates of these enzymes. These compounds are micromolar inhibitors of class C β-lactamases but, very unexpectedly, not inhibitors of class A β-lactamases. We rationalize the latter result on the basis of a new mechanism of boronic acid inhibition of the class A enzymes. A stable inhibitory complex is not accessible because of the instability of an intermediate on its pathway of formation. The new boronic acids also do not inhibit bacterial DD-peptidases (penicillin-binding proteins). This result strongly supports a central feature of a previously proposed mechanism of action of β-lactam antibiotics, where deacylation of β-lactam-derived acyl-enzymes is not possible because of unfavorable steric interactions.

Enzyme inhibitors remain important as drug leads.[1] Boronic acids, **1**, have for quite some time now been designed and used as sources of active site-specific, anionic, tetrahedral transition state analogue complexes, **2**, of serine amidohydrolases (Scheme 1). They are thus very effective inhibitors of

Scheme 1

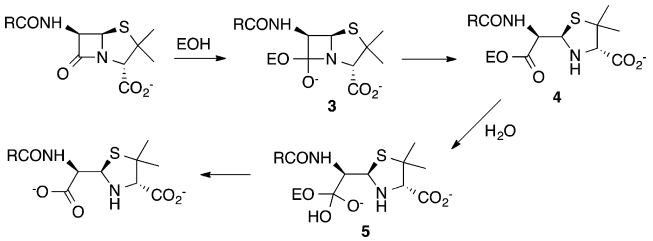


1 2

these enzymes and potential drug candidates.[2−4] Among the enzymes that are inhibited by these compounds are the β-lactam-recognizing enzymes, the serine β-lactamases and DD-peptidases. Boronic acid inhibition of serine β-lactamases has been recognized for many years,[5,6] but only more recently have such inhibitors of DD-peptidases been identified.[7,8] The time gap between these developments may reflect the increasing awareness of the evolutionary relationship between DD-peptidases and β-lactamases and thus their close structural and functional similarity.[9−11]

β-Lactamases catalyze the hydrolysis of β-lactam antibiotics and are thus an important source of bacterial resistance to these molecules.[12] The reaction (Scheme 2; shown with a penicillin) proceeds by way of a covalent acyl enzyme intermediate **4** and, therefore, through tetrahedral intermediates **3** and **5**. Acyl-enzymes, analogous to **4**, are formed on reaction of DD-peptidases with β-lactams but in this case hydrolyze very slowly leading to effective inhibition of these enzymes and thus interruption of bacterial cell wall synthesis. One would expect that the closest boronate analogue to a β-lactamase deacylation

Scheme 2



tetrahedral intermediate/transition state **5** would be **6**, arising from reaction between the enzyme and boronic acid **7**. A number of approximations to the structure **7** have been described, for example, initially, amidoalkyl boronic acids such as **8**.[13,14] Subsequently, closer analogues, such as **9** and **10**, were found to be very powerful β-lactamase inhibitors.[15,16] Crystal structures showed them to form the anticipated tetrahedral adducts **2** at the β-lactamase active site. To complement these developments, we describe here the syntheses of the boronic acids **11** and **12**. We follow this with a description and analysis of their inhibitory activity against representative serine β-lactamases and DD-peptidases.

MATERIALS AND METHODS

The boronic acids **11** and **12** were synthesized as described in detail in Supporting Information. The *Actinomadura* R39 and

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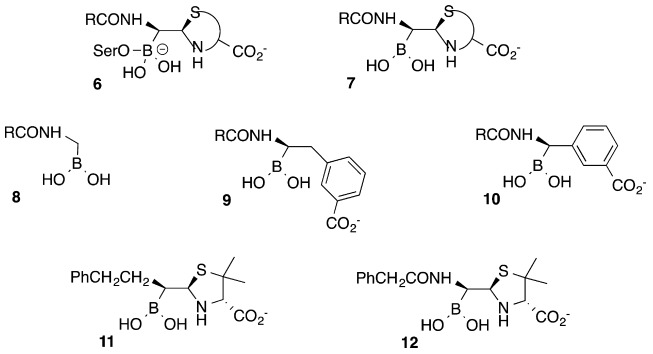


Streptomyces R61 DD-peptidases, and *Bacillus subtilis* PBP4a, were generous gifts from Dr. J.-M. Frère and Dr. P. Charlier of the University of Liège, Liège, Belgium. The *Escherichia coli* PBP5 DD-peptidase was a generous gift from Dr. R. A. Nicholas of the University of North Carolina, Chapel Hill, NC. The AmpC β-lactamase was provided by Dr. B. K. Shoichet of the University of California at San Francisco, San Francisco, CA. The class C P99 β-lactamase from *Enterobacter cloacae*, the class A TEM-2 β-lactamase from *E. coli* W3310, and the class A *Staphylococcus aureus* PC1 β-lactamase were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, UK). The class A SHV-1 enzyme was a gift from Dr. Michiyoshi Nukaga of Jyosai International University, Japan.

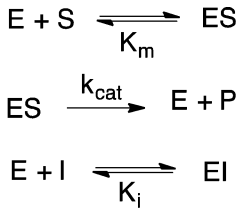
Enzyme Kinetics Studies. *DD-Peptidase Inhibition. a. In Solution.* Experiments designed to obtain equilibrium constants of inhibition of the *Actinomadura* R39 DD-peptidase, *B. subtilis* PBP4a, and *E. coli* PBP5 in solution by compounds **11** and **12** were performed as described previously[17] from steady-state competition experiments where *N*-(phenylacetyl)glycyl-D-thiolactic acid was employed as a spectrophotometric (245 nm, $\Delta\varepsilon$ = 2500 cm^{-1} M^{-1}) substrate (0.5 mM). Enzyme concentrations were between 0.1 and 0.2 μM. Initial rates of substrate hydrolysis in the presence of a range of concentrations of **11** and **12** (0−1.0 mM) were obtained.

b. Membrane-Bound Enzymes. Equilibrium constants for inhibition of *E. coli* DD-peptidases (PBPs) in membranes were obtained as described previously, employing Bocillin Fl as a fluorescent competitive β-lactam.[18] Compounds **11** (0−1.0 mM) and **12** (0−100 μM) were incubated with *E. coli* membrane preparations for 1 h prior to addition of Bocillin Fl (20 μM).

β-Lactamase Inhibition. Equilibrium constants of inhibition of the P99 and AmpC β-lactamases by compounds **11** and **12** (0−100 μM) were obtained from steady-state competition experiments where cephalothin was employed as a spectrophotometric (262 nm, $\Delta\varepsilon$ = 7660 cm^{-1} M^{-1}) substrate (0.2 mM). The reaction conditions were 20 mM MOPS buffer, pH 7.50, 25 °C, and enzyme concentrations of 2 nM, stabilized by 0.1% bovine serum albumin in solution. Under these conditions, the K_m value of the substrate was 9.0 μM for the P99 enzyme[19] and 13.8 μM for AmpC. No time-dependence of inhibition was observed in the manual mixing time frame in these experiments. Measurements of the initial rates, v, of cephalothin hydrolysis catalyzed by the β-lactamase in the absence and presence of **11** or **12** were fitted to Scheme 3 by means of eq 1 (least-squares), where v_0 is the initial rate in the absence of inhibitor, to obtain the K_i values. Attempts to obtain K_i values of **11** and **12** for the class A TEM-2, SHV-1, and PC1 β-lactamases were conducted similarly.

Molecular Modeling. Simulations were performed on a SGI workstation running the program Insight II, essentially as

Scheme 3



$$v/v_0 = (K_m + s)/[K_m(1 + i/K_i) + s] \quad (1)$$

previously described.[21,22] The crystal structure of the ampC β-lactamase, inhibited by **15** [PDB entry 1MXO[23]], including the crystallographic water molecules, was modified to construct the adduct of **12** with the active site Ser 64. The pH was set to 7.0, and the total charge on the complex was zero. At this pH, the side chain of Tyr 150 was neutral, and those of Lys 67 and 315 were cationic. The partial charges of the enzyme were assigned by Insight II. Partial charges (MNDO) of the inhibitor in the complex were calculated from a model adduct with serine. Boron is not parametrized in Insight II so fixed standard crystallographic B−C and B−O bond distances and a rigid tetrahedral geometry at boron were assumed. The active site was hydrated with a 15 Å sphere of water centered on Oγ of the nucleophilic serine 64. The models were then subjected to 100 steps of steepest descent energy minimization followed by short molecular dynamics runs (40 ps) to relax the active site side chains and inhibitor. A typical snapshot from the molecular dynamics runs was selected and subjected to 1000 steps of steepest descent energy minimization, followed by 2000 steps of conjugate gradients.

A similar procedure was followed to obtain a model of the deacylation analogue adduct of **12** with the TEM-1 β-lactamase. This was derived from the crystal structure of the complex of **15** with this enzyme [PDP entry 1NXY[16]]. Note, however, that the thiophene ring of **15** has been retained in this model rather than changed to phenyl. In the TEM active site, Glu166 was neutral and Lys73 and Lys234 cationic. A generic model of a tetrahedral acylation adduct of **12** with the TEM-1 enzyme was derived directly from the crystal structure of its complex with **27** [PDB entry 1NY0[16]]. Models of benzylpenicillin at the CTX-M-9 and *E. coli* PBP4 active sites were built directly from the published crystal structures [PDB entries 3HUO[24] and 2EX8,[25] respectively]. In each case, the acyl forms were converted to tetrahedral intermediates by Insight modeling.

RESULTS AND DISCUSSION

The syntheses of the boronic acids **11** and **12** are outlined in Schemes 4 and 5, respectively. In these syntheses, we made use of the recent discovery that stable α-boryl aldehydes can be prepared when *N*-methyl or pinene iminodiacetic acid (MIDA or PIDA) boronic acid protecting groups are employed.[26,27] Our use of MIDA thus allowed synthesis of aldehydes **13** and **14** from which the thiazolidine rings of **11** and **12** could readily be constructed. We also found that the MIDA protecting group was conveniently removed when treated with penicillamine in methanol during the last step of each synthesis, yielding **11** and **12** in unprotected form. High resolution mass spectral characterization of **11** and **12** presented difficulties since free boronic acids are generally not volatile or stable enough under the conditions required.[28] The mass spectrum of **11** as

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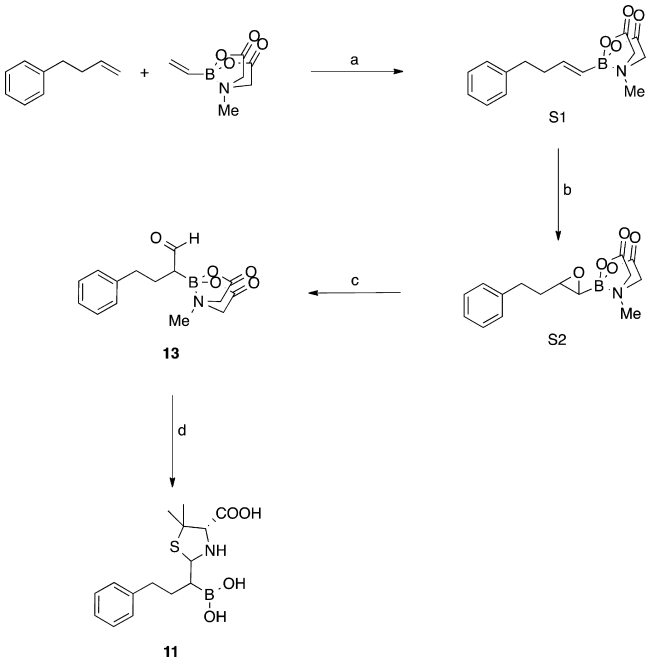
Scheme 4. Synthesis of 11[a]

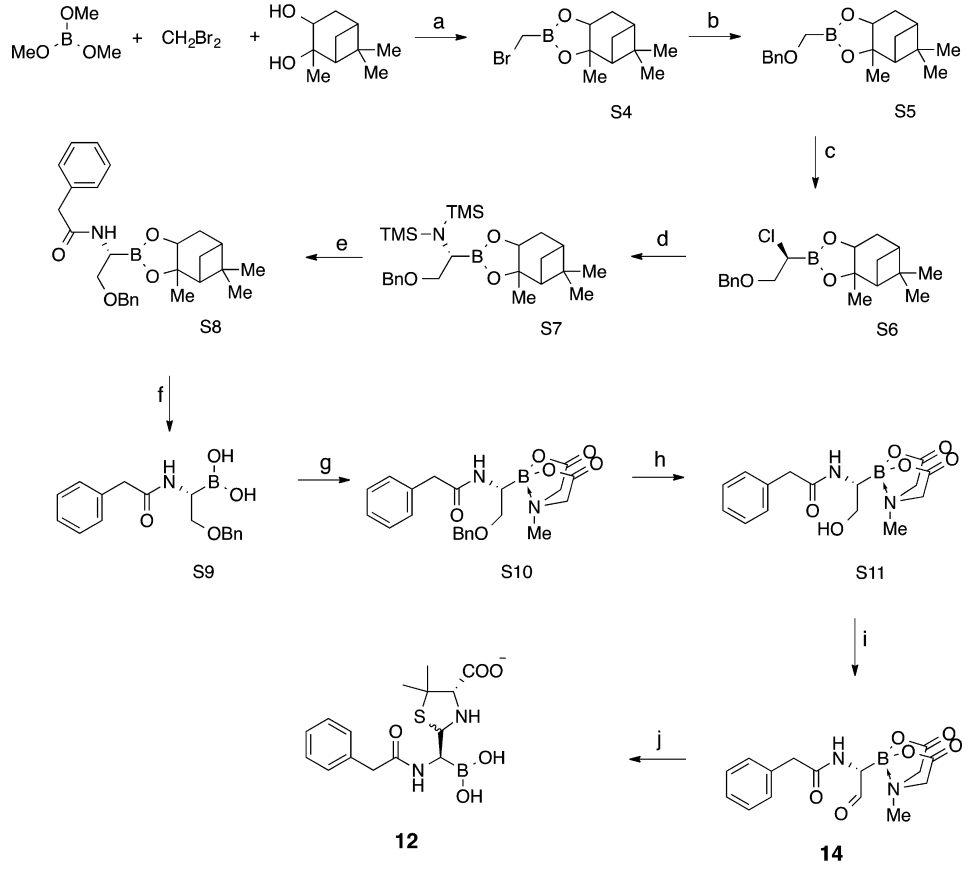


[a]Stereochemistry is discussed in the text. Reagents and conditions: (a) Grubb's II catalyst, CH_2Cl_2, reflux; (b) mCPBA, CH_2Cl_2, 0 °C; (c) $BF_3.Et_2O$, CH_2Cl_2, −30 to 0 °C; (d) D-penicillamine, MeOH-H_2O, rt.

the pinacol ester, however, displayed a small peak at the appropriate mass (m/z = 406.3), but even this represented a metastable ion and was thus not suitable for accurate mass measurement. For both **11** and **12** we were able to obtain accurate mass measurements for M-BO_2 cations from these pinacol esters (see Supporting Information). The presence of the boronic acid moiety in the parent boronic acids was indicated in their ^{1}H NMR spectra by the absence of a methylene hydrogen resonance in the spectrum and the characteristic upfield shift of the hydrogen α to the boryl group.[29]

These syntheses yielded, as expected, mixtures of diastereoisomers that we did not attempt to separate since we could get a good estimate of the activity of the likely most active diastereoisomer from the activity of the mixture. The preparation of **12** yielded a mixture of two diasteroisomers in an essentially 1:1 ratio as indicated by the ^{1}H NMR spectrum. Presumably these would have structures **12a** and **12b** (Scheme 6), formed in the last step (j) of the synthesis. All precedent with β-lactam-recognizing enzymes would suggest that **12a**, which has the stereochemistry of bicyclic β-lactams, would be the active inhibitor. The situation with **11** was more complicated. Epoxidation and rearrangement of the intermediate alkenes (Scheme 4) led to a mixture of two enantiomers with a chiral carbon α to the boronic acid group (Scheme 6, a/c and b/d) in a 4:1 ratio, where the stereochemistry of (a), α to the boryl group, is likely to be present in the major component.[27] Subsequent formation of the thiazolidine ring (step d) would

Scheme 5. Synthesis of 12[a]



[a]Stereochemistry is discussed in the text. Reagents and conditions: (a) nBuLi, THF, −78 °C; (b) BnOH, nBuLi, THF, −78 °C; (c) CH_2Cl_2, nBuLi, THF, −100 °C; (d) LHMDS, THF, −100 °C; (e) $PhCH_2COCl$, CH_2Cl_2, −78 °C; (f) 1-methylpropylboronic acid, MeOH − H_2O/hexane, 6 h; (g) MIDA, DMF, 80 °C, 12 h; (h) H_2, Pd on carbon, MeOH, 40 psi, 12 h; (i) DMP, AcOH − CH_3CN; (j) D-penicillamine, MeOH.

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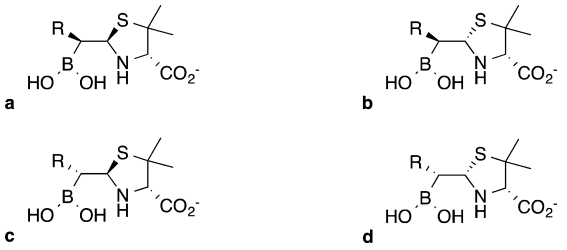
Scheme 6. Boronic Acid Stereochemistry



presumably, as with **12**, subdivide these equally into a final 4:4:1:1 mixture, as observed by ^{1}H NMR (Supporting Information), where the likely most active **11a** would represent 40% of the total. A minor component **11c** might also have some activity since this stereochemistry is found in carbapenem antibiotics.[30]

The first important result was that neither **11** nor **12** inhibited representative DD-peptidases. In solution, no inhibition of the low molecular mass DD-peptidases of *Streptomyces* R61 and *Actinomadura* R39, or of *B. subtilis* PBP4a was observed. Gel experiments also demonstrated that these compounds did not inhibit *E. coli* PBP1a/1b, 2, 3, 4, and 5. These results concretely support a current general proposal for the mechanism of inhibition of DD-peptidases by β-lactams. Figure 1 shows the

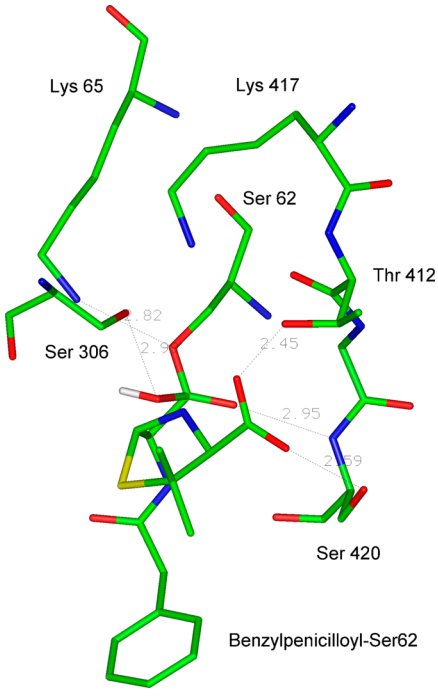


Figure 1. Active site of the *E. coli* PBP4 DD-peptidase with benzylpenicillin bound as a deacylation tetrahedral intermediate. Modeled from the crystal structure of the acyl-enzyme.[25]

structure of a model of the putative tetrahedral intermediate for deacylation of the covalent acyl-enzyme formed on reaction of benzylpenicillin with *E. coli* PBP4. This structure was generated directly from the acyl-enzyme structure.[25] This structure is not stable (and, consequently, deacylation is very slow) because of unfavorable steric interactions between the hydroxyl group of the water nucleophile and the adjacent carbon and nitrogen atoms of the thiazolidine ring. These interactions are nicely seen in a space-filling model (Supporting Information, Figure S1). If this rationale for the slow deacylation of β-lactams from DD-peptidases were true, the tetrahedral boronate adduct **6** would also be unstable, and thus **11** and **12** would not be DD-peptidase inhibitors. Therefore, our results with these inhibitors support the mechanism of action of β-lactams described above. It is also reported that **15** does not inhibit *Streptococcus pneumoniae* PBP1b.[31] As would be anticipated from the above discussion, analogues of **16**, unsubstituted α to the boronic acid, are more likely to be DD-peptidase inhibitors.[32−36]

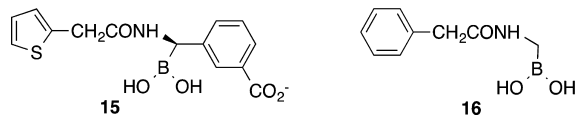
The results of Table 1 show that the new boronic acids **11** and **12** at micromolar concentrations do inhibit class C

Table 1. Enzyme Inhibition by Boronic Acids 11 and 12

	K_i (μM)[a]	
enzyme	**11**	**12**
P99 β-lactamase	0.62 ± 0.15	0.95 ± 0.11
AmpC β-lactamase	0.38 ± 0.12	1.3 ± 0.4
TEM-2 β-lactamase	NI[b]	NI[b]
PCI β-lactamase	NI[b]	NI[b]
R39 DD-peptidase	NI[c]	NI[c]

[a]The K_i values above are not corrected for the presence of stereoisomers of **11** and **12** (see text). If it were assumed that the most likely stereoisomer in each case was the only one with activity, the values for **11** reported above would be multiplied by 0.4 and those for **12** by 0.5 (see text) to obtain the K_i values of the active isomers. [b]NI, no inhibition observed at the concentration 1.0 mM. [c]NI, no inhibition observed at the concentration 0.10 mM.

β-lactamases, the P99 and AmpC enzymes. Notably, **11** is comparably effective to **12**, despite the absence in the former of the amido side chain which is present in good substrates and generally thought to be important for active site recognition through hydrogen bonding.[37] Apparently the hydrophobic side chain of **11** is just as effective for inhibition as the amido side chain of **12**, which we can assume binds in the usual site between the backbone carbonyl oxygen of residue 318 and the amide side chain of the conserved Asn152.



Boronic acid **12** is similar in structure to **15**, an inhibitor described by Morandi et al.[23] The latter compound is a 1 nM inhibitor of the AmpC β-lactamase. A crystal structure of the inhibitory complex has been published,[20] from which the active site diagram of Figure 2A has been taken. This shows the amide side chain of **15** firmly hydrogen-bonded to the protein as described above, and the boronate present as a tetrahedral anion covalently bound to the nucleophilic serine of the active site. One boronate oxygen is in the oxyanion hole (hydrogen-bonded to backbone NH groups of Ser64 and Ala318), and the other takes up the position of a leaving group or of the deacylating water molecule, depending on whether the complex is seen as an analogue of an acylation or deacylation tetrahedral intermediate (see below).

Finally, the carboxyphenyl group is oriented with its plane perpendicular to the chain formed by the amido side group, the alkyl boronate, and the side chain of Ser64. In this position, the carboxylate is directed above the β_2-strand where it forms a hydrogen bond with the amido side chain of Asn 289. This is

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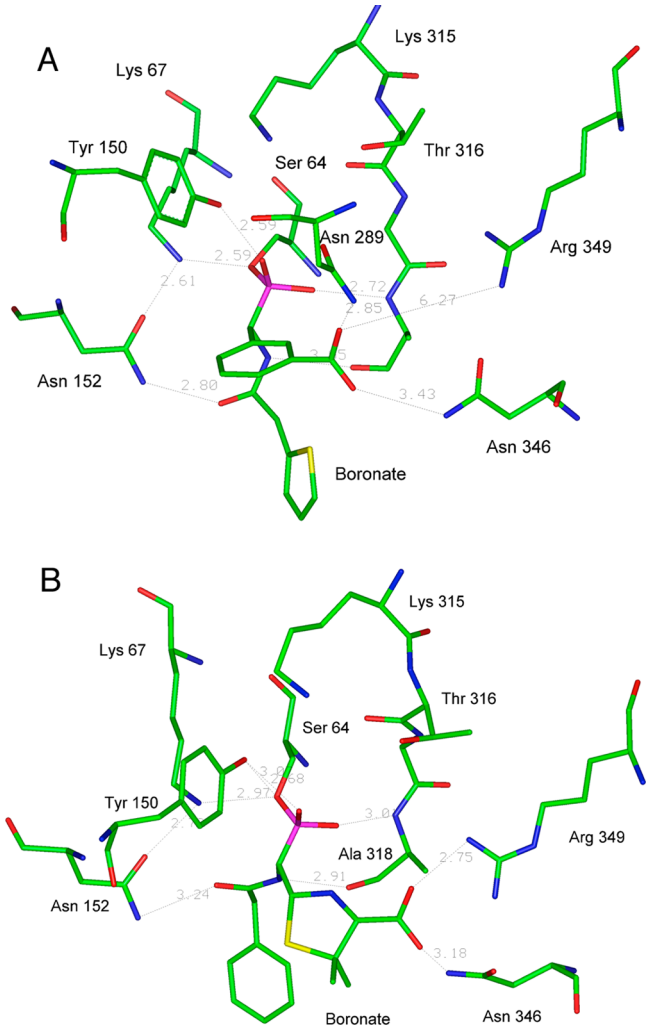
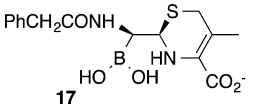


Figure 2. (A) Active site of the AmpC β-lactamase with the boronic acid **15** bound, from the crystal structure.[23] (B) An energy-minimized model of **12** bound to the AmpC active site, derived directly from the structure in A.

apparently an effective arrangement since the boronic acid **16**, lacking the carboxyphenyl group, has a K_i value of 0.57 μM.[14]

A model of the complex between **12** and the AmpC β-lactamase was constructed based on the crystal structure with **15** as described in Materials and Methods. The initial structure was subjected to a short molecular dynamics simulation to relax active site interactions and a typical snapshot energy-minimized (see Materials and Methods). This procedure led to the structure of Figure 2B. This resembles the structure of the complex of **15** described above but differs in the positioning of the thiazolidine carboxylate vs the phenyl carboxylate of **15**, due to the nonplanar nature of the five- (vs six-) membered thiazolidine ring and thus to the positioning of its substituents. The short MD run suggested that the thiazolidine carboxylate may prefer interaction with Arg349 over that with Asn 289. A similar dynamics run on the complex with **15** suggested that it too may be mobile in solution, with carboxylate access even to the Arg204 side chain. At any event, it appeared that the carboxylates of **12** and **15** may prefer somewhat different environments with some complementary adjustment of the active site structure. These effects presumably lead to the weaker binding of **12** than **15** to the enzyme. It is possible that a cephalosporin analog of **12**, e.g., **17**, which more closely resembles **15**, may be a better inhibitor of class C β-lactamases: k_{cat} values (deacylation rate constants) of cephalothin and cephalexin are larger than that of benzylpenicillin for the P99 enzyme,[28,29] suggesting that deacylation transition states of the former may be better stabilized by the enzyme.



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It might be noticed in passing that the interactions of the inhibitor carboxylate group with the enzyme described above hold the thiazolidine ring of **12** from sterically impeding nucleophilic attack by water on the acyl-enzyme intermediate; the thiazolidine nitrogen is held well away from both boronate hydroxyl groups. This situation is in clear contrast to that seen in DD-peptidases[11] (see above) and presumably allows facile hydrolysis of the acyl-enzyme by β-lactamases.

It might also be noted that Gln289 is not strictly conserved in class C β-lactamases but is replaced by serine in *Enterobacter* homologues such as the P99 β-lactamase. The hydroxyl of serine in this position may also be able to form a hydrogen bond with the carboxylate of a penicillin substrate and of an inhibitor such as **15**. Certainly **12** inhibits the P99 and AmpC β-lactamases to very similar extents (Table 1).

Perhaps more striking than the results with class C enzymes are those with class A. Neither the TEM-1, SHV-1, nor PCI β-lactamases were inhibited by **11** or **12**, even at 1 mM concentrations. Given the fact that **12** should be the source of a precise transition state analogue structure by reaction with these enzymes (Schemes 1 and 2), much as with the class C β-lactamases, the result is quite astonishing. In stark contrast, **15** is a powerful inhibitor of the TEM-2 β-lactamase; K_i = 64 nM.[16] This profound and unexpected difference between **12** and **15** is discussed below.

First, it is necessary to recall that the geometry of the acylation and deacylation transition states (and tetrahedral intermediates, Scheme 2) is different in class A β-lactamases, unlike the situation with the class C enzymes where the acylation and deacylation tetrahedral intermediates have similar structure (see above). This situation arises because, although the hydrolytic water molecule is believed to attack the acyl-enzyme intermediate from the solution side (Re face) in class C β-lactamases, this attack comes from the protein side (Si face), catalyzed by Glu 166, in class A β-lactamases.[40,41] During acylation, the leaving group is thought to depart from the Re face of the acyl-enzyme in each case. These details are shown diagrammatically in Scheme 7.

Thus, although one would expect all boronic acid complexes of class C enzymes to resemble **18** and **20** (i.e., have structure **21**, the two forms of which are interconvertible by a small single bond rotation), two possibilities, **24** and **26**, exist for class A β-lactamases. Indeed, examples of each kind have been observed with class A β-lactamases in crystal structures of boronate complexes.[16] Note that **22** and **23** (or **24** and **26**) are not interconvertible by single bond rotations.

The crystal structure of the strongly inhibitory (64 nM) complex between **15** and the TEM-1 β-lactamase represents a mimic of a deacylation tetrahedral intermediate (Figure 3A), where one boronate hydroxyl group is in hydrogen bond contact with the deacylation catalyst Glu166.[16] In this structure,

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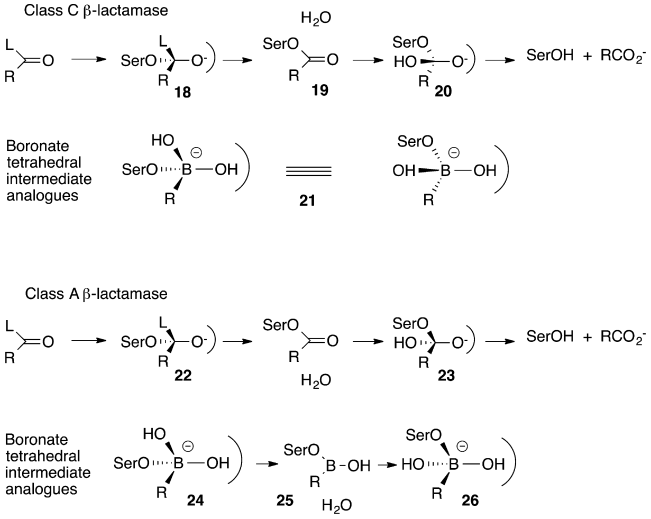
Scheme 7. Mechanism of Formation of Tetrahedral Intermediates and Their Boronate Analogues[a]



[a]In this diagram, **18** and **20** represent the acylation and deacylation tetrahedral intermediates in class C β-lactamase catalysis, and **22** and **23**, respectively, represent these species for a class A enzyme. The boronate analogues **21**, **24**, and **26** are shown below their respective intermediates, and **19** is the central acyl-enzyme. The arc adjacent to one oxygen ligand in each case represents the oxyanion hole, the presence of which serves to distinguish the two boronate hydroxyl groups.

the carboxylate group of the inhibitor forms a strong hydrogen bond with the Thr235 hydroxyl group and, probably, with the Arg244 side chain via a water molecule. An energy-minimized model of a complex of **12** with this enzyme is shown in Figure 3B. In this structure, the interactions with the active site are the same as that of the complex with **15**. Both structures seemed stable during short MD runs. The experimental result, that **12** (or **11**) is not an inhibitor, therefore remains a puzzle.

A possible way out of this dilemma may be found when the mechanism of formation of deacylation analogue boronate complexes is considered. Direct formation of these complexes from the free boronic acids and enzyme might be difficult since it would require prior or concerted displacement of the deacylating water molecule from Glu166. An alternative, perhaps more facile, mechanism would proceed by way of initial direct formation of an acylation tetrahedral intermediate analogue, **24**. In an analogous fashion to the acylation/deacylation sequence of a substrate (Scheme 7), consecutively a neutral trigonal boronic acid intermediate **25** may be formed from **24**, followed by its attack by the hydrolytic water molecule, presumably catalyzed by Glu166, to form the deacylation tetrahedral intermediate analogue **26**.

The crystal structure of the complex between the class A TEM-1 β-lactamase with the boronic acid **27** resembles the acylation tetrahedral intermediate **22** (i.e., it has the structure **24**) while, as noted above, that with **15** resembles the deacylation intermediate **23** (i.e., it has the structure **26**). Wang et al. have suggested that the preference for the deacylation analogue structure **26** by **15** derives from the presence of the pendant carboxylate group which interacts with Arg244 in the complex, as seen in the crystal structure[16] (Figure 3A). One might expect that the boronic acids, **11** and **12**, bearing the thiazolidine carboxylate, would also form stable complexes analogous to **26**. Ke et al. have also discussed the

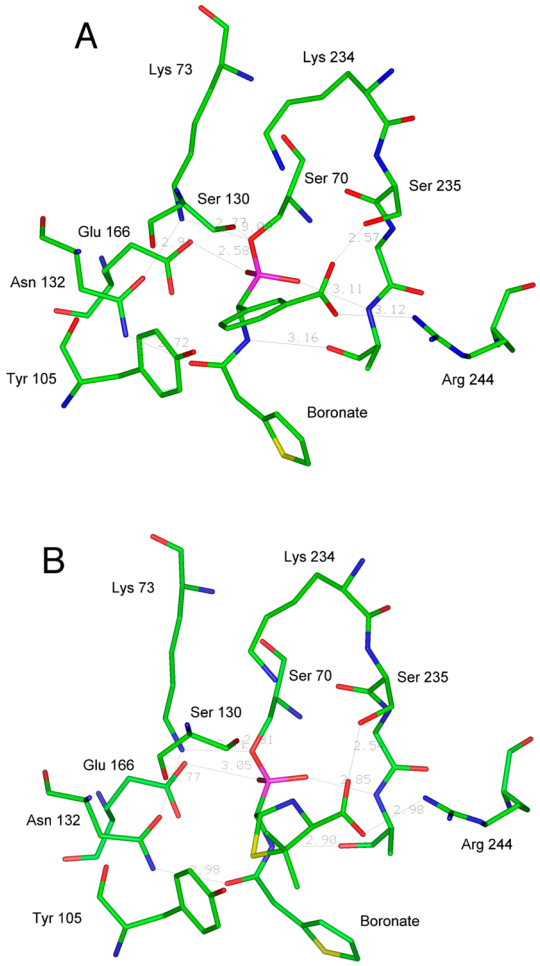
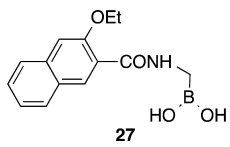


Figure 3. (A) Active site of the TEM-1 β-lactamase with the boronic acid **15** bound, from the crystal structure.[16] (B) An energy-minimized model of **12** bound to the TEM-1 active site, derived directly from the structure in A. Note, however, that the thiophene ring of **15** has been retained in this model rather than changed to phenyl.

relative merits of acylation vs deacylation complexes in various specific instances.[42]



We approached a structural model of acylation tetrahedral intermediate complexes by way of the crystal structure of the complex of **27** with the TEM-1 enzyme.[16] An immediate problem was that a bulky substituent α to the boronic acid, as present in **11**, **12**, and **15**, sterically interacts unfavorably with Tyr105 (Figure 4), raising the likelihood of significant conformational adjustment (Figure 4). Models of both **12** and **15** were unstable, both to energy minimization and to MD simulation. In both cases, particularly that of **12**, expansion of the lower part of the active site, comprising Tyr105, Asn132, and Glu166, occurred. In particular, Tyr 105 was "pushed away" with motion of the Asp101 − Leu108 loop. This distortion was more extreme with the bulkier (nonplanar) thiazolidine of **12**. Acylation tetrahedral intermediates thus appeared likely to be less stable than the deacylation species described above. This is certainly a reasonable explanation for

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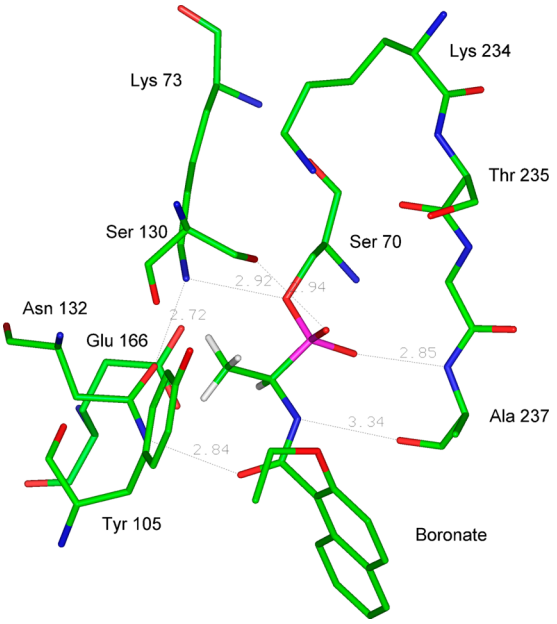


Figure 4. Active site of the TEM-1 β-lactamase with the boronic acid **27** bound, from the crystal structure.[16] Also shown, in the form of an added methyl group, is the general orientation of an L-α substituent, as present in **11** and **12**.

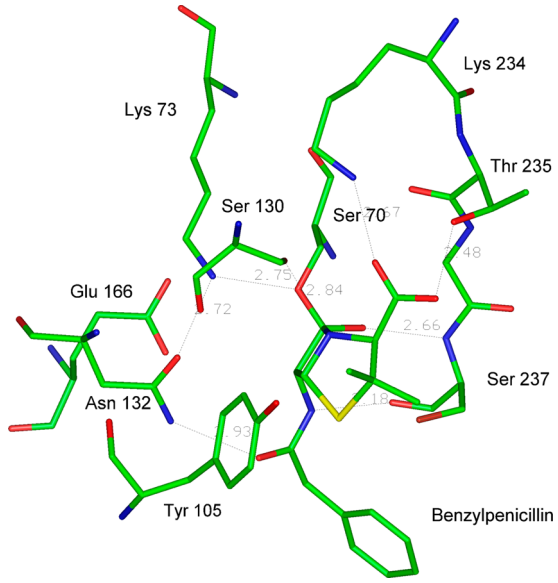


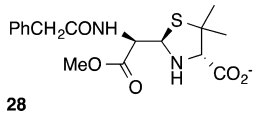
Figure 5. Active site of the class A CTX-M-9 β-lactamase with benzylpenicillin bound as an acylation tetrahedral intermediate. Modeled from the crystal structure of the noncovalent complex of penicillin with the Ser70Gly mutant.[24]

the two types of boronate structures obtained, the nature of the complex depending on whether a bulky substituent α to the boronic acid is present.

This problem of the instability of the intermediate **22** (and thus the analogue **24**) might also be sufficient to explain the lack of inhibition by **11** and **12**. The ability of **15** to act as inhibitor but not **11** and **12** can be supposed to arise from the greater bulk of the thiazolidine substituent of the latter than the phenyl of the former (Figure S2, Supporting Information) and thus the greater difficulty of the latter to achieve the acylation analogue structure **24** required (Scheme 7) as a precursor of the likely stable deacylation analogue structure **26**. This is an explanation in terms of unfavorable kinetics, but it should be noted that no inhibition of the TEM-2 enzyme was observed in 24 h.

In support of the rationalization above, it should be pointed out here that acylation tetrahedral intermediate analogues generated from acyclic boronic acids such as **11**, **12**, and **15** are not likely to closely resemble the structure of a "real" acylation tetrahedral intermediate **3** (Figure 5). This structure was generated from the crystal structure of a noncovalent complex between benzylpenicillin and a Ser70Ala mutant of the class A CTX-M-9 β-lactamase.[21] In this structure, the azetidine ring is still intact, with the scissile C−N bond eclipsed by the C−C bond on the opposite side of the ring. Such eclipsing will be lost for steric reasons when the four-membered ring is opened, as seen in the structures of Figure 3. In Figure 5, the carboxylate substituent of the thiazolidine ring forms hydrogen bonds with the side chains of Lys234 and Thr235. After rotation away of the thiazolidine ring with opening of the four-membered ring, this hydrogen-bond pattern is lost and replaced by the interactions seen in Figure 3. Thus, acyclic boronates cannot generate close acylation transition state analogue structures, at least of bicyclic β-lactam substrates, hence the instability of the boronate acylation complexes **24** of class A β-lactamases. On the other hand, acyclic boronic acids can form direct analogues of deacylation tetrahedral species, **23** (Scheme 7). It is possible that recently discovered cyclic borinates[43,44] may better approximate acylation complexes.

These considerations lead to another point and one that provides direct evidence for the instability of acyclic boronate adducts **24**. Methyl penicilloate, **28**, is known to be a substrate of a *Pseudomonas* class C β-lactamase,[45] and we have extended this result to the P99 β-lactamase (see Supporting Information). This compound is, however, neither a substrate nor a covalent inhibitor of the class A BCI β-lactamase,[46] and we have extended this point by observations with the TEM-2 enzyme (Supporting Information). These observations prove that while the class C β-lactamase active site can significantly stabilize the acylation tetrahedral intermediate **29** (a direct analogue of **18**), the class A active site cannot (stabilize the analogue of **22**), presumably for the reasons discussed above.



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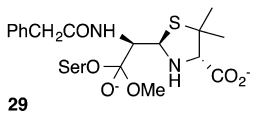

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SUMMARY AND CONCLUSIONS

Neither **11** nor **12** [or, most likely, **15**[31]] inhibit DD-peptidases, even at 0.1 mM concentrations, probably because of unfavorable steric interactions at the active site of these enzymes (Figure 1). Deacylation tetrahedral intermediates of DD-peptidase catalysis are thought to be destabilized in the same way.[9,11] The results with **11** and **12** from experiments in solution therefore strongly support the steric mechanism of inhibition of DD-peptidases by β-lactams and thus the mechanism of antibiotic action by these compounds. Previous evidence for this mechanism largely rested on inspection of crystal structures of inert complexes.

The boronic acids **11** and **12** inhibit class C β-lactamases, at micromolar concentrations, presumably by formation of covalent tetrahedral adducts (Figure 2B) that resemble the

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high energy tetrahedral intermediates of penicillin turnover. Compounds **11** and **12** are not, however, as effective as inhibitors as the phenyl analogue **15**.[23] It is possible that **17**, a cephalosporin analogue, closer in structure to **15**, may be more effective than **12**. The new compounds do not inhibit class A β-lactamases even at millimolar concentrations, in strong contrast to **15**, which, at nanomolar concentrations, forms **26**, a structural analogue of the deacylation tetrahedral intermediate **23**[16] (Scheme 7). A rationale for this surprising result is offered in terms of a mechanism of formation of **26** (Scheme 7), which requires the initial formation of an acylation tetrahedral analogue **24**, followed by that of a neutral trigonal boronic acid intermediate **25**, analogous to an acyl-enzyme, and finally formation of **26** by intramolecular water attack on **25**, presumably catalyzed by Glu166. A model of the acylation tetrahedral intermediate analogue from **12** suggests that this species may be unstable on steric grounds, precluding progress of **11** and **12** toward the deacylation analogue **26**. An effective transition state analogue inhibitor requires an energetically accessible path to the inhibitory complex as well as transition state mimicry in that complex.[47]

■ ASSOCIATED CONTENT

Supporting Information

Synthetic details for the preparation of compounds **11** and **12**. Figures S1and S2 illustrate steric effects of the thiazolidine ring. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DMP, Dess−Martin periodinane; ESMS, electrospray ionization mass spectroscopy; LHMDS, lithium hexamethyldisilazane; mCPBA, *m*-chloroperbenzoic acid; MIDA, methyl iminodiacetic acid; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; PBP, penicillin-binding protein; THF, tetrahydofuran; DMF, dimethylformamide; DMSO, dimethyl sulfoxide

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