Structure-Based Enhancement of Boronic Acid-Based Inhibitors of AmpC β-Lactamase

G. Scott Weston,[†] Jesús Blázquez,[‡] Fernando Baquero,[‡] and Brian K. Shoichet*,[†]

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611, and Servicio de Microbiología, Hospital Ramón y Cajal, National Institute of Health (INSALUD), Madrid, Spain

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The expression of β-lactamases is the most common form of bacterial resistance to β-lactam antibiotics. To combat these enzymes, agents that inhibit (e.g. clavulanic acid) or evade (e.g. aztreonam) β-lactamases have been developed. Both the β-lactamase inhibitors and the β-lactamase-resistant antibiotics are themselves β-lactams, and bacteria have responded to these compounds by expressing variant enzymes resistant to inhibition (e.g. IRT-3) or that inactivate the β-lactamase-resistant antibiotic (e.g. TEM-10). Moreover, these compounds have increased the frequency of bacteria with intrinsically resistant β-lactamases (e.g. AmpC). In an effort to identify non-β-lactam-based β-lactamase inhibitors, we used the crystallographic structure of the *m*-aminophenylboronic acid−*Escherichia coli* AmpC β-lactamase complex to suggest modifications that might enhance the affinity of boronic acid-based inhibitors for class C β-lactamases. Several types of compounds were modeled into the AmpC binding site, and a total of 37 boronic acids were ultimately tested for β-lactamase inhibition. The most potent of these compounds, benzo[*b*]thiophene-2-boronic acid (**36**), has an affinity for *E. coli* AmpC of 27 nM. The wide range of functionality represented by these compounds allows for the steric and chemical "mapping" of the AmpC active site in the region of the catalytic Ser64 residue, which may be useful in subsequent inhibitor discovery efforts. Also, the new boronic acid-based inhibitors were found to potentiate the activity of β-lactam antibiotics, such as amoxicillin and ceftazidime, against bacteria expressing class C β-lactamases. This suggests that boronic acid-based compounds may serve as leads for the development of therapeutic agents for the treatment of β-lactam-resistant infections.

Introduction

Microbial resistance to current antibiotics is one of the most serious problems facing health care systems today.[1−4] The overuse and misuse of broad-spectrum antibiotics and the capability of microbes to exchange resistance genes has accelerated the development of this public health problem. The explosive increase in antibiotic resistance has led some to suggest that we are now entering a "post-antibiotic" era in which we will no longer be able to rely on traditional antibiotics to cure common infections.[5,6]

The expression of β-lactamase enzymes is one of the most common and well-studied forms of antibiotic resistance.[7] β-lactam antibiotics exert their therapeutic effects by inhibiting penicillin-binding proteins, enzymes that cross-link muramyl peptides of the bacterial cell wall. This disrupts microbial cell wall biosynthesis, leading to lysis and bacterial cell death. β-lactamases protect bacteria that express them by hydrolyzing the β-lactam ring present in these antibiotics, inactivating them (Figure 1).[8] Bacteria appear to have evolved β-lactamases several different times,[9,10] and several different classes of these enzymes exist. The most clinically important groups are the class A β-lactamases, whose members include the plasmid-based TEM penicillinases, and the class C β-lactamases, represented by cephalosporinases such as AmpC.[11,12]

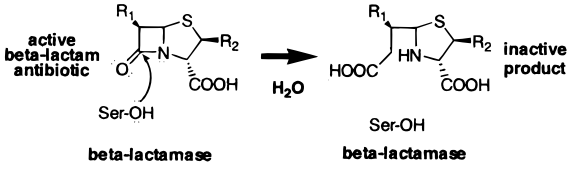

Figure 1. General mechanism of hydrolysis of a β-lactam antibiotic by a serine β-lactamase.

To overcome the actions of these enzymes, medicinal chemists have introduced β-lactam molecules that inhibit (e.g. clavulanic acid) or evade (e.g. aztreonam) β-lactamases. Each of the three clinically used β-lactamase inhibitors[11] shares the same β-lactam core found in β-lactam antibiotics (Figure 2). This is also true, of course, of "β-lactamase-resistant" antibiotics such as aztreonam. This reliance upon a common β-lactam core has made it easier for bacteria to respond to these molecules. Bacterial mechanisms designed to evade β-lactam-based antibiotics have adapted to evade β-lactam-based lactamase inhibitors and to hydrolyze "β-lactamase-resistant" antibiotics.[13] For example, sensor proteins that up-regulate lactamase production in the presence of β-lactams may also be activated by the currently available β-lactamase inhibitors.[14] Other bacterial adaptations, including mutations in porin channels,[15] the recruitment of active efflux pumps,[16] and

* To whom correspondence should be addressed.
[†] Northwestern University Medical School.
[‡] INSALUD.

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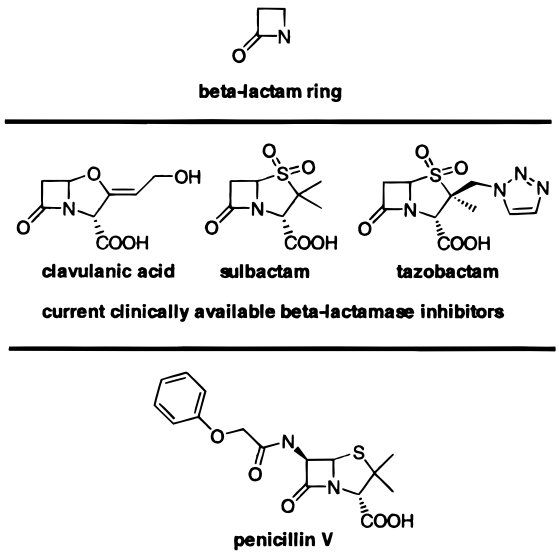


Figure 2. Chemical structures of a β-lactam ring, the currently available β-lactamase inhibitors, and a typical β-lactam-based antibiotic.

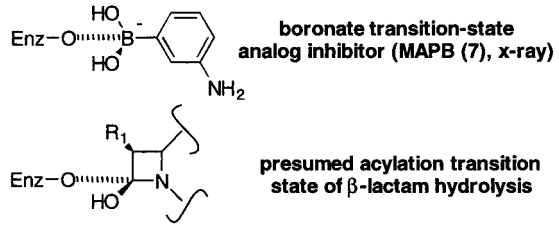


Figure 3. Comparison of a boronic acid-based transition-state analogue inhibitor (**7**, top) and a typical β-lactam antibiotic (bottom) in complex with a β-lactamase.

the expression of mutant β-lactamases,[17] have led to increasing reports of bacterial resistance to current β-lactamase inhibitors and "β-lactamase-resistant" antibiotics. Perhaps the most serious adaptations have arisen in the β-lactamases themselves. Mutant variants of the TEM-1 class A β-lactamase, such as IRT-1, IRT-2, and IRT-3, have arisen that can evade β-lactam-based β-lactamase inhibitors such as clavulanic acid.[13,17] Enzymes that are naturally resistant to current β-lactamase inhibitors, including the class C β-lactamases such as AmpC, have become increasingly prominent.[11] There is a pressing need for novel β-lactamase inhibitors, not based on a β-lactam core structure. Such inhibitors would not be hydrolyzable by β-lactamases or mutant β-lactamases and would not be recognized by the suite of bacterial resistance mechanisms mobilized against β-lactam-based β-lactamase inhibitors.

An intriguing class of compounds long known to inhibit β-lactamases is the boronic acids. These compounds act as transition-state analogue inhibitors of lactamases and other amidases (Figure 3).[18−20] Approximately two decades ago, Kiener and Waley found that boric acid, phenylboronic acid (**1**), and *m*-aminophenylboronic acid (**7**, MAPB) weakly inhibit a class A β-lactamase from *Bacillus cereus* (K_i range = 1.2−4.0 mM).[21] No bacteriological data was reported for any of these compounds. Later, Beesley et al. identified 12 substituted phenylboronic acids (including **1**, **2**, **7**, **16**, and **20**) that act as weak to moderate covalent, reversible inhibitors of class C β-lactamases from *Pseudomonas aeruginosa* and *Escherichia coli* (K_i range = 2.4−920.0 μM).[22] One of the more potent compounds reported, 3-iodoacetamidophenylboronic acid (K_i (*P. aeruginosa* β-lactamase) = 2.4 μM, K_i (*E. coli* AmpC) = 23 μM), was synergistic with cephalosporin C against *P. aeruginosa*, albeit at high (=1.64 mM; =0.5 mg/mL) concentrations. Also, Amicosante et al. found that boric acid, phenylboronic acid (**1**), *m*-aminophenylboronic acid (**7**), and tetraphenylboronic acid potentiate the activity of cephalosporins against β-lactamase-expressing strains of *C. diversus* and *P. aeruginosa*;[23] here too, the activity was weak, with the compounds only showing activity at millimolar concentrations. More recently, Dryjankski and Pratt reported that *m*-(dansylamidophenyl)-boronic acid (**5**) is a submicromolar inhibitor (K_i = 0.6 μM) of the *Enterobacter cloacae* P99 β-lactamase (a class C β-lactamase).[24] No bacteriological data were reported for this inhibitor. In addition, Strynadka and colleagues used the crystallographic structure of a mutant TEM-1 (a class A β-lactamase) enzyme−penicillin G complex to design a novel alkylboronic acid inhibitor ((1*R*)-1-acetamido-2-(3-carboxyphenyl)ethane boronic acid) with high affinity for this enzyme (K_i (TEM-1) = 110 nM).[25]

Our goal was to use structure-assisted methods to identify new boronic acid-based agents with improved affinity for class C β-lactamases. Inhibitors with improved affinities might serve as lead "antiresistance" compounds suitable for possible synthetic elaboration, crystallographic studies, and bacteriological testing. In addition, we wanted to "map" the binding site of a class C (*E. coli* AmpC[26]) β-lactamase for further inhibitor discovery. Boronic acids are useful agents in this regard, as they possess a wide range of structural functionality and inhibit β-lactamases reversibly and competitively. We began our efforts with the crystallographic structure of the MAPB (**7**)−*E. coli* AmpC complex.[27] Although MAPB inhibits the enzyme with only moderate affinity ($K_i = 7.3 \pm 0.9$ μM), the structure of this complex revealed several possible interactions that might be improved by analogues with altered functionality. Using a cycle of structural modeling, enzymatic testing, and antibacterial evaluation, we attempted to discover compounds with improved affinity for AmpC and the ability to potentiate the effect of β-lactam antibiotics against resistant bacteria.

Results

Modeling of Selected Boronic Acids into the AmpC Active Site. We selected 2-phenylboronic acid (**1**), *m*-aminophenylboronic acid (**7**), thiophene-2-boronic acid (**26**), *m*-nitrophenylboronic acid (**6**), and 4,4′-biphenyldiboronic acid (**14**) as a representative sample of available boronic acids for modeling into the AmpC active site. Structures and conformational libraries for each compound were created using the Sybyl molecular modeling suite (Figure 4). Conformer interactions with AmpC were checked for steric conflicts with AmpC using DISTMAP.[28] Conformers which passed this steric filter were then scored based on complementarity to the enzyme as judged by the AMBER potential function as implemented in CHEMGRID[29] (Table 1). The energies reported did not consider a number of factors, including ligand and receptor desolvation and receptor flexibility, and thus should not be expected to correlate with

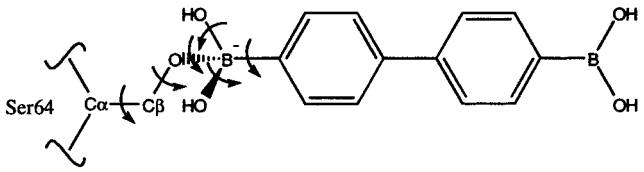


Figure 4. Diagram of torsional angles explored in modeling selected boronic acids into the AmpC active site. 4,4′-biphenyldiboronic acid (**14**) is shown as an example. The same torsions were explored for the other inhibitors noted.

Table 1. Results of Ligand Conformer Analysis[a]

	orientation		
ligand	"MAPB-like" best force field score (kcal/mol)	"phosphonate-like" best force field score (kcal/mol)	K_i AmpC (μM)
Selected Compounds from Initial Group of Boronic Acids			
1	−22.9	−20.6	10.5 ± 2.6
7	−18.3	−18.8	7.3 ± 0.9
26	−22.4	−20.2	2.5 ± 0.4
6	−15.4	−18.3	1.7
14	29.4	−22.3	0.20 ± 0.03
Selected Thiophene-2-boronic Acid (**26**) Analogues			
31	−24.8	−22.0	3.8 ± 1.4
34	−13.9	−18.0	1.6 ± 0.4
33	−19.6	−16.2	0.4 ± 0.1
36	−5.4	−17.8	0.027

[a] Of the 746 496 total conformations possible for each boronic acid, approximately 5000 conformers passed the distance constraints used and tens to hundreds passed the steric filter employed. For purposes of comparison, the phosphonate ligand of Lobkovsky et al.,[30] the canonical "phosphonate-like" ligand, fit into the A monomer of *E. coli* AmpC gets scores of −2.639 (monomer A, no "bumps") and −13.545 (monomer B, 1 "bump").

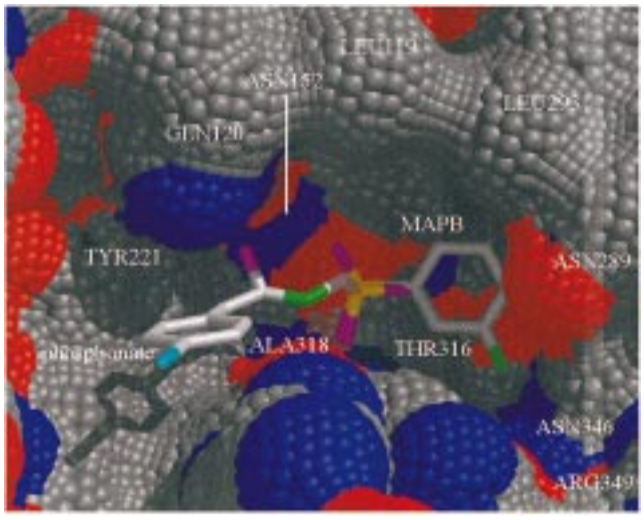


Figure 5. Two major families of possible boronic acid ligand orientations as illustrated by the crystallographic orientations of MAPB (**7**)[27] and a phosphonate inhibitor of AmpC.[30] For the surface, carbon atoms are gray, oxygens are red, and nitrogens are blue. For the ligands, carbon atoms are white, boron/phosphorus atoms are yellow, nitrogen atoms are green, iodine atoms are cyan, and oxygen atoms are magenta.

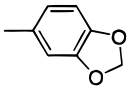
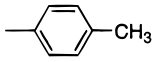
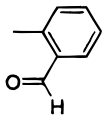
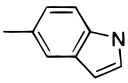
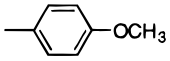
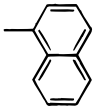
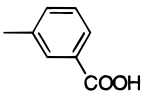
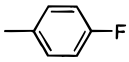
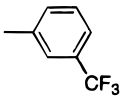
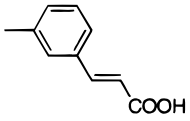
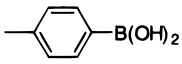
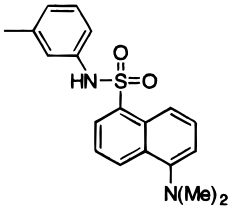
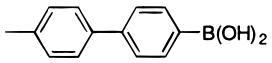
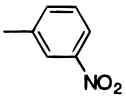
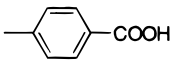
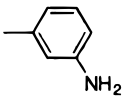
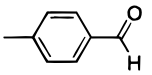
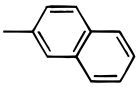
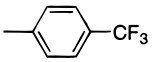
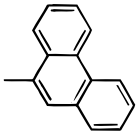
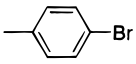
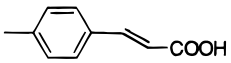
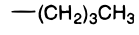
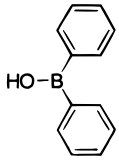
binding energies. However, these calculated energies may be used as a guide to possible ligand conformations in the binding site. Two major families of ligand orientations were identified (Figure 5): a "MAPB-like" mode in which the boronic acid ligand is oriented similarly to the inhibitor in the MAPB (**7**)−AmpC structure (near residues Thr316, Asn346, and Asn289), and a "phosphonate-like" mode in which the boronic acid ligand is oriented similarly to the phosphonate ligand in an AmpC−inhibitor complex determined by Lobkovsky et al.[30] (close to residues Asn152 and Gln120). The distribution of orientations in the two modes is partially correlated with the size of the boronic acid ligand, with some of the larger ligands favoring the "phosphonate-like" mode because of steric clashes with the receptor in the "MAPB-like" mode. For example, the energies of interaction calculated for the relatively large biphenyl derivative **14** seem to favor a "phosphonate-like" binding mode. Conversely, the much smaller thiophene-2-boronic acid (**26**) appears to fit better in the MAPB-like binding mode. It should be noted that we cannot distinguish the crystallographic binding mode for **7** from the alternative "phosphonate-like" binding mode; the two binding modes have equal energies of interaction in these calculations. These calculations are clearly approximate and can only suggest possibilities.

Enzymatic Testing of the Initial Group of Boronic Acids against *E. coli* AmpC. We identified an initial group of 30 boronic acids that allowed us to test the impact of a wide variety of functionality on the affinity of boronic acid-based lactamase inhibitors (Table 2). For one boronic acid compound (3-nitrophenylboronic acid, **6**), we had previously determined a K_i value for AmpC using Lineweaver−Burk/double-reciprocal plots of kinetic data.[27] We compared the K_i value for **6** determined in this manner (1.7 μM) to that calculated by a much faster method using the progress curves of uninhibited and inhibited enzymatic reactions[31] (1.2 ± 0.1 μM). The inhibition constants determined by these two methods were within the error of our measurement. We thus decided to use the progress curve comparison method to determine the inhibition constants for the rest of the boronic acids that were tested against the AmpC enzyme (Table 2). Many of the arylboronic acids, as well as some of the heterocyclic alkyl- and arylboronic acids, tested showed activity against AmpC at low micromolar concentrations.

Bacteriological Susceptibility Testing of the Most Active β-Lactamase Inhibitors from the Initial Group of Boronic Acids. Several of the more potent compounds from this initial series of boronic acids were selected for assays against class C β-lactamase-expressing bacteria in combination with the cephalosporin ceftazidime and the penicillin amoxicillin. The goal of the assays was to determine if the boronic acids protected the two β-lactam antibiotics from inactivation by β-lactamases. Several of the compounds showed protective effects comparable to that of tazobactam, a clinically used β-lactam-based β-lactamase inhibitor, against strains expressing class C β-lactamases (Figure 6A,B). The MIC values reported are the minimum concentrations at which no bacterial growth was observed.

Specificity Testing of the Most Active Inhibitors from the Initial Group of Boronic Acids. Several of the more potent compounds from this initial series of boronic acids were also tested against three serine proteases—α-chymotrypsin, β-trypsin, and elastase—to determine their specificity for β-lactamases (Table 3). A few of the boronic acids (**5**, **35**, **36**) showed some activity against the proteases used, but at concentra-

Table 2. Chemical Structures and Inhibition Constants for the First Group of Boronic Acids Tested

boronic acid	R	K_i(AmpC) (μM)[b]	boronic acid	R	K_i(AmpC) (μM)[b]	boronic acid	R	K_i(AmpC) (μM)[b]
1		10.5 ± 2.6	10		53.4 ± 6.1	20		5.4 ± 1.3
2		25.3 ± 4.2	11		10.9 ± 0.6	21		5.9 ± 0.8
3		12.0 ± 1.0	12		>>100	22		6.4 ± 0.8
4		1.1 ± 0.3	13		5.9 ± 0.3	23		3.9 ± 0.2
5		1.3 ± 0.1	14		0.20 ± 0.03	24		1.4 ± 0.1
6		1.7[a]	15		2.9 ± 0.3	25		15.8 ± 0.8
7		7.3 ± 0.9	16		2.9 ± 0.9	26		2.5 ± 0.4
8		8.5 ± 1.8	17		3.0 ± 0.5	27		22.1 ± 3.5
9		7.0 ± 1.2	18		3.6 ± 0.9	28		>>100
			19		4.2 ± 1.1	29		>>100
						30		>>100

[a] Determined from a previous Lineweaver−Burk analysis of kinetic data.[27] [b] ≫ = less than 50% inhibition observed at ≥3 times the cephalothin concentration noted.

tions 10−40 times greater than those required for AmpC inhibition. Most boronic acids had no significant affinity for the proteases.

Modeling of Thiophene-2-boronic Acid (26) Analogues into the AmpC Active Site. On the basis of the success of thiophene-2-boronic acid (**26**) in the enzymatic and bacterial assays, we investigated the complementarity to the AmpC active site for several analogues of this compound. As a small representative set of the thiophene-2-boronic acid derivatives available, structures and conformer libraries for 3-formylthiophene-2-boronic acid (**31**), 5-acetylthiophene-2-boronic acid (**34**), 5-chlorothiophene-2-boronic acid (**33**), and benzo-[*b*]thiophene-2-boronic acid (**36**) were generated and scored for interaction with AmpC. Conformer interactions with AmpC were checked for steric conflicts with AmpC using DISTMAP.[28] Conformers which passed this steric filter were then scored based on complementarity to the enzyme as judged by the AMBER potential function as implemented in CHEMGRID[29] (Table 1). The energies reported did not consider a number of factors, including ligand and receptor desolvation and receptor flexibility, and thus should not be expected to correlate with binding energies. However, these calculated energies may be used as a guide to possible ligand conformations in the binding site. As with the initial group of boronic acids, two major families of ligand conformations were also identified for the thiophene-2-boronic acid analogues.

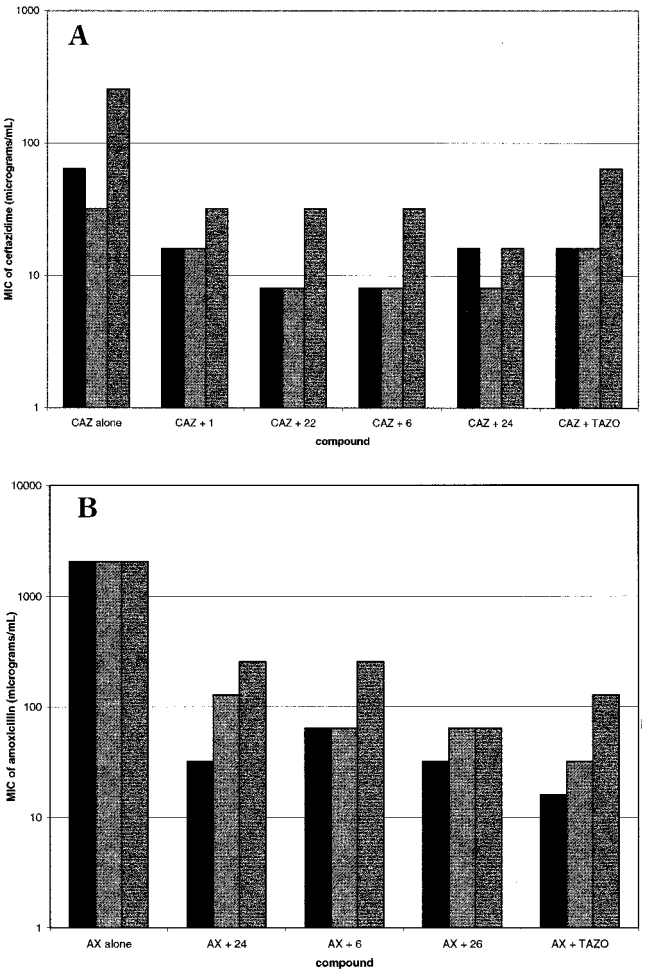


Figure 6. Potentiation of β-lactam activity against resistant bacteria by selected compounds from the first group of boronic acids tested, using (A) ceftazidime as the β-lactam (ceftazidime-to-inhibitor ratio of 2:1) and (B) amoxicillin as the β-lactam (amoxicillin-to-inhibitor ratio of 2:1). The bars represent, from left to right, Eco-AmpCEnt, Eco-AmpC, and Ent-Der. Data are considered accurate and reproducible to a factor of 2. Abbreviations used for compounds: CAZ = ceftazidime, TAZO = tazobactam, AX = amoxicillin. For bacterial strain nomenclature, see Experimental section.

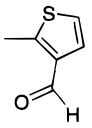
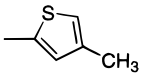
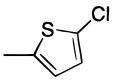
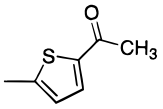
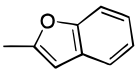
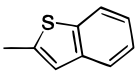
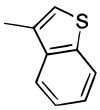
Table 3. Specificity of Selected Members of the First Group of Boronic Acids Tested

	IC_{50} (μM)			
boronic acid	AmpC	CHT[a]	TRY[a]	ELST[a]
24	5	>200	>200	>200
4	5	>200	>200	>200
5	5	>100	>100	50
6	5	>200	>200	>200
26	10	>200	>200	200

[a] CHT = α-chymotrypsin; TRY = β-trypsin; ELST = elastase.

Enzymatic Testing of Thiophene-2-boronic Acid (26) Analogues. We obtained seven analogues of thiophene-2-boronic acid (**26**) for a second round of testing against *E. coli* AmpC (Table 4). As with the initial group of boronic acids, inhibition constants for this series were determined using the progress curve comparison method.[31] For the most potent member of this series, benzo[*b*]thiophene-2-boronic acid (**36**), a more rigorous determination of binding affinity was performed using a Lineweaver−Burk/double-reciprocal analysis of kinetic data (Figure 7).

Table 4. Strucutres and Inhibition Constants for Thiophene-2-boronic Acid (**24**) and Its Analogues

boronic acid	R	K_i(AmpC) (μM)
26		2.5 ± 0.4
31		3.8 ± 1.4
32		0.50 ± 0.05
33		0.41 ± 0.10
34		1.6 ± 0.4
35		0.22 ± 0.05
36		0.027[a]
37		0.78 ± 0.08

[a] The K_i value calculated from the progress curve comparison method was 0.016 ± 0.003 μM.

Bacteriological Susceptibility Testing of Thiophene-2-boronic Acid (26) Analogues. We selected three of the more potent compounds from this series (**33**, **35**, **36**) for testing against β-lactam-resistant bacteria in combination with the cephalosporin ceftazidime. The goal of the assays was to determine if the boronic acids protected ceftazidime from inactivation by class C β-lactamases in vitro, thus potentiating its antibiotic activitity. The thiophene-2-boronic acid analogues tested showed comparable to better potency than tazobactam against bacteria expressing class C β-lactamases (Figure 8). The MIC values reported are the minimum concentrations at which no bacterial growth was observed.

Specificity Testing of the Most Active Thiophene-2-boronic Acid (26) Analogues. The three thiophene-2-boronic acid analogues selected for bacterial assays were also tested for β-lactamase specificity versus α-chymotrypsin, β-trypsin, and elastase (Table 5). The most potent of these inhibitors, benzo[*b*]thiophene-2-boronic acid (**36**), had an IC_{50} of 150 nM for AmpC but showed little inhibition of trypsin or elastase at concentrations up to 1000-fold higher. However, **36** displays significant affinity for chymotrypsin (IC_{50} = 5 μM).

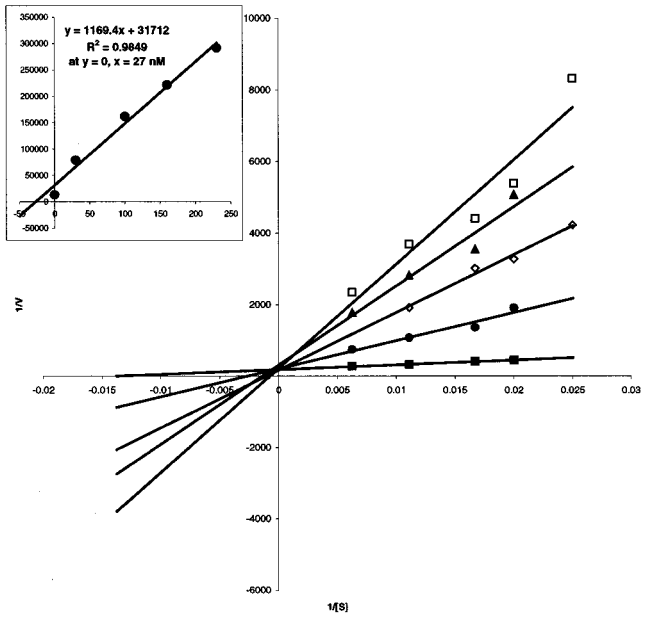


Figure 7. K_i calculation for **36** using Lineweaver−Burk analysis (■ no **36**, ● 30 nM **36**, ◇ 100 nM **36**, ▲ 160 nM **36**, □ 230 nM **36**). Replot of slopes from the **34**−AmpC Lineweaver−Burk plot to determine K_i value is shown in the inset.

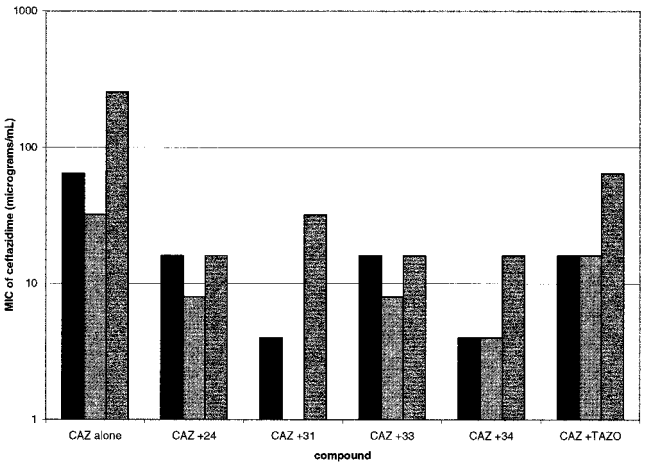


Figure 8. Potentiation of β-lactam activity against resistant bacteria by selected compounds from the second group of boronic acids tested, using ceftazidime as the β-lactam (ceftazidime-to-inhibitor ratio of 2:1). The bars represent, from left to right, Eco-AmpCEnt, Eco-AmpC, and Ent-Der. Data are considered accurate and reproducible to a factor of 2. Abbreviations used for compounds: CAZ = ceftazidime, TAZO = tazobactam. For bacterial strain nomenclature, see Experimental Section. Note: data for **31** + CAZ was not collected for Eco-AmpC.

Table 5. Specificity of Thiophene-2-boronic Acid (**26**) and Selected Analogues

	IC_{50} (μM)			
boronic acid	AmpC	CHT[a]	TRY[a]	ELST[a]
36	0.15	5	>200	200
35	0.6	14	>200	>200
33	2	>100	>200	200
26	10	>200	>200	200

[a] CHT = α-chymotrypsin; TRY = β-trypsin, ELST = elastase.

Discussion

Probing the Structural Determinants of Binding to AmpC. An intriguing feature of the MAPB (**7**)−AmpC complex is how few obviously favorable interactions are observed between the aryl group of the inhibitor and the enzyme (Figures 5 and 9). Still, **7** has a K_i of 7.3 ± 0.9 μM for AmpC, making it one of the more active boronic acid compounds previously described for this class of β-lactamase. It is considerably more active than borate, for instance, which inhibits AmpC-like enzymes only at millimolar concentrations.[22] We thus were interested to know what structural features determine the affinity for this class of inhibitor, and whether inhibition could be improved upon by taking advantage of potential interactions in the β-lactamase active site. Boronic acids inhibit serine-type β-lactamases reversibly and rapidly,[21−25] allowing for simple kinetic analyses of inhibition patterns (Figure 7). In this class of compounds, changes in observed inhibition can be easily converted to changes in binding affinity.

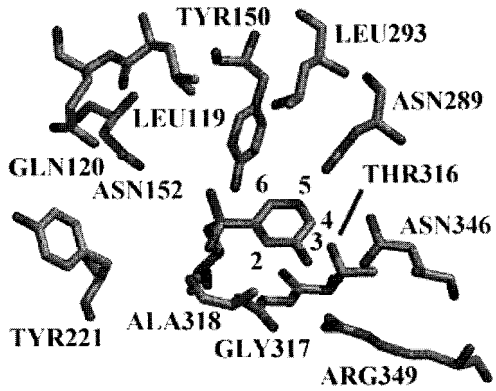


Figure 9. View of the *E. coli* AmpC active site from the crystallographic complex with *m*-aminophenylboronic acid (MAPB, **7**) bound; all ring positions are labeled.

One possible explanation for the affinity of the boronic acids is that binding is driven primarily by ligand hydrophobicity. Hydrophobicity is principally a nonspecific unfavorable interaction with solvent. The 1-naphthyl- (**3**), the 9-phenanthrene- (**9**), and 2-naphthyl- (**8**) boronic acids are among the most hydrophobic members of this series and also among the weaker inhibitors identified. Although poor steric complementarity to the target cannot be ruled out for these inhibitors, the smaller and more flexible *n*-butylboronic acid (**29**) should have no problems with steric hindrance and yet it also is a poor inhibitor of AmpC. Taken together, these results suggest that boronic acids must have the correct stereochemical arrangement of functionality; hydrophobicity alone is not sufficient to explain affinity.

The differential affinities of the boronic acids for AmpC might owe to activation of the boronic acid group as an electrophile by substituents on the aryl ring. We compared the affinities of 2-formyl- (**2**) and 4-formyl- (**16**) phenylboronic acids, and those of 3-trifluoro- (**4**) and 4-trifluoro- (**17**) phenylboronic acids (Table 2). If affinity is modulated mostly by effects on electrophilicity of the boronic acid group, one might expect electron-withdrawing groups at the 2 and 4 positions to be about equal activators but both to be better than similar groups at the 3 position. Instead, we found that derivatives at the 3 position are more active than ones at 4 and that groups at 2 are much less active than derivatives either

at 3 or 4. We also found that the (*S*) and (*R*) stereoisomers of 3-tetrahydrofuranyl boronic acid (**24**, **25**) differ in affinity for AmpC by an order of magnitude. The electronic effects on the boron atom should be identical for stereoisomers. This suggests that electrophilic activation is certainly not the only factor underlying the AmpC binding affinity of boronic acids.

In an effort to understand which residues might be interacting with the different inhibitors, we first fit the analogues onto the conformation of **7** found in the crystallographic complex with AmpC.[27] Transition-state analogues such as boronic acids are known to adopt different conformations in β-lactamases, depending upon which microscopic transition state they are mimicking.[25,27,30,32,33] It therefore seemed prudent to also consider other conformations of the arylboronic acids in the site. This was accomplished by rotating the boronic acid adducts about several of their single bonds, beginning with the Cα−Cβ bond of Ser64 (Figure 4). These conformations were evaluated for steric and electrostatic complementarity to the enzyme. Most conformations clustered into two families—one that resembled that of **7** in its adduct with AmpC[27] and a second adopted by a phosphonate inhibitor bound to the AmpC enzyme from *En. cloacae*.[30]

We begin by discussing general features of the two conformational families and continue with an analysis of specific interactions observed for specific compounds. In the case of the "MAPB-like" conformation, hydrogen-bonding groups from Asn289, Asn346, and Arg349 are in position to make polar contacts with substituents at the 3- and 4-positions of an arylboronic acid. For example, the nitrogen of MAPB (**7**) lies 2.8 Å from the Oδ of Asn343 and 3.4 Å from the Nη of Arg349. The heteroatom of a 4-hydroxy- or 4-aminophenylboronic acid would be positioned 2.3 Å from the Nδ2 and 2.8 Å from the Oδ1 atom of Asn343. These residues are believed to contribute to recognition of the R_2 groups of β-lactam antibiotics.[30] Alternatively, if the boronic acid ligand binds in a "phosphonate-like" orientation, hydrogen-bonding groups from Gln120, Asn152, Tyr221, and Ala318 lie nearby. These residues are thought to be involved in binding the R_1 side chains of β-lactam antibiotics.[30] Consistent with the greater openness of the site in the area of residues Gln120 and Asn152 (Figure 5), the larger boronic acid ligands often prefer the "phosphonate-like" conformation while the smaller ligands may fit in either conformation. We note that the energies used to evaluate the conformations can only be used as a guide and for distinguishing among different conformations of the same ligand. No effort has been made to consider solvation effects, and thus the rankings between ligands has little meaning. In the discussion that follows, we will restrict ourselves to discussing the predominant binding mode, as judged by the number of conformations found and their energies, always allowing that the other conformation might also be sampled.

The largest 3-derivative tested was the dansyl analogue **5** ($K_i = 1.3 \pm 0.1$ μM), and based on its size, it seems likely to bind in a "phosphonate-like" manner. Several smaller 3-derivatives were also best accommodated in this binding orientation. For instance, MAPB (**7**) analogues such as *m*-nitrophenylboronic acid (**6**), *m*-trifluoromethylphenylboronic acid (**4**), *m*-carboxyvinylphenylboronic acid (**13**), and *m*-carboxyphenylboronic acid (**12**) seem unlikely to bind to AmpC in a "MAPB-like" manner due to steric conflicts with the enzyme. However, each should be able to bind in a "phosphonate-like" manner and this binding mode appears to be consistent with the structure−activity relationships observed for these compounds. Thus, the *m*-trifluoromethyl substituent of **4** ($K_i = 1.1 \pm 0.3$ μM) appears to fit snugly into the hydrophobic pocket created by Leu119 and Leu293, and the *m*-nitro group of **6** ($K_i = 1.7$ μM), adopting a slightly different "phosphonate-like" orientation, should be able to interact with Asn152. In contrast, the poor activity observed for **12** ($K_i \gg 100$ μM) may be due to its poor interaction with the Leu119−Leu293 hydrophobic pocket and the burial of its charged carboxylate moiety. The better affinity of the *m*-carboxyvinyl derivative **13** ($K_i = 5.9 \pm 0.3$ μM) relative to its smaller *m*-carboxy analogue **12** may be due to the ability of the larger compound to form complementary interactions with more distant residues, such as Gln120, not accessible to **12**.

Assumption of a "phosphonate-like" binding mode would also help explain the activity of many of the *p*-substituted arylboronic acids studied. The region of AmpC which includes residues Ala318, Tyr221, Gln120, and Asn152 is much less sterically restricted than the MAPB (**7**) binding site and has a variety of functional groups present for potential ligand−receptor interactions. This may explain why, in general, *p*-substituted arylboronic acids are effective AmpC inhibitors. A variety of *p*-substituents are tolerated by AmpC, including carboxy (**15**, $K_i = 2.9 \pm 0.3$ μM), formyl (**16**, $K_i = 2.9 \pm 0.9$ μM), trifluoromethyl (**17**, $K_i = 3.0 \pm 0.5$ μM), carboxyvinyl (**19**, $K_i = 4.2 \pm 1.1$ μM), and boronic acid (**23**, $K_i = 3.9 \pm 0.2$ μM) groups, with each improving affinity relative to the parent phenylboronic acid (**1**, $K_i = 10.5 \pm 2.6$ μM). Even larger *p*-substituents, such as those present in the biphenyl (**14**, $K_i = 0.2 \pm 0.03$ μM), 2-naphthyl (**8**, $K_i = 8.5 \pm 1.8$ μM), and 5-indolyl (**11**, $K_i = 10.9 \pm 0.6$ μM) compounds, are tolerated in this binding mode.

In contrast to the bulkier members of this series, smaller boronic acids should be able to assume either a "MAPB-like" or "phosphonate-like" binding orientation. The proximity of the hydroxyl groups of Tyr150 and Thr316 to ring atoms of MAPB (**7**) in the crystallographic complex suggested that polar or polarizable groups might better complement the enzyme than the phenyl ring of **7** (Figure 9). Consistent with this view, thiophene-2-boronic acid (**26**), whose ring should be electron rich[34] and is known to form polar transfer complexes with electropositive groups, was found to have a K_i of 2.5 ± 0.4 μM against AmpC. (*R*)-3-tetrahydrofuranyl-boronic acid (**24**), which should be able to interact with Tyr150 or Thr316 or both through its ring oxygen, was found to have a K_i of 1.4 ± 0.1 μM. The (*S*)-3-tetrahydrofuranylboronic acid (**25**), the heteroatom of which, in a "MAPB-like" binding orientation, should be unable to interact with Thr316 or Tyr150, has a K_i of 15.8 ± 0.8 μM. The lower affinity of thiophene-3-boronic acid (**27**) ($K_i = 22.1 \pm 3.5$ μM) suggests that electronic effects may also play a role in the affinities of the thiophene series. The poor affinity of 2-furanyl-

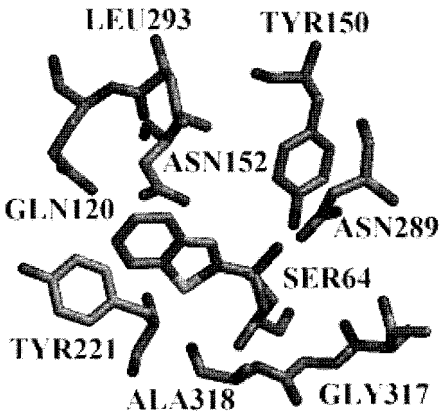


Figure 10. One possible conformation for **36** from the modeling results.

boronic acid (**28**, $K_i \gg 100$ μM) may be due to its reduced ability to form quadrupole interactions versus its sulfur analogue[35] and perhaps also to electronic effects. We note that the putative dipole−quadrupole discrimination between thiophene-2-boronic acid (**26**) and furan-2-boronic acid (**28**) would also be consistent with a "phosphonate-like" binding orientation in which the Asn152 side chain would provide the electropositive group with which the thiophene ring might interact.

We also considered the possibility that substitutions, including the presence of larger heteroaryl groups, might improve upon the potency of **26**. Allowing for the assumptions noted earlier, modeling suggested that although ligands containing larger systems such as benzo[*b*]heteroarylboronic acids probably would not fit into the AmpC site in a "MAPB-like" binding mode, these compounds should still be able to bind to the enzyme in other productive orientations. Several derivatives of **26** were tested (Table 4), the most potent of which was benzo[*b*]thiophene-2-boronic acid (**36**). This compound has a K_i of 27 nM for AmpC.

Benzo[*b*]thiophene-2-boronic acid (**36**) is approximately 100-fold more active than thiophene-2-boronic acid (**26**), suggesting that interactions with the second aryl ring contribute considerably to affinity. Modeling results suggest that dipole−quadrupole interactions between Asn152 and **36** and quadrupole−quadrupole interactions between Tyr221 and **36** may be responsible for the contribution of the second aryl ring to affinity (Figure 10). The importance of the second aryl ring is also indicated by the activity of benzo[*b*]furan-2-boronic acid (**35**), which is about 1000-fold more active than the furan-2-boronic acid parent (Tables 2 and 4). At the same time, a comparison of the activity of **36** with 2-naphthylboronic acid (**8**, $K_i = 8.5 \pm 1.8$ μM), which should place its distal aryl ring in approximately the same area as the benzo[*b*]thiophene derivative, confirms the importance of the thiophene ring for affinity.

Specificity for β-Lactamase. Boronic acids form complexes with catalytic serines that resemble the geometry of high-energy intermediates along the reaction pathway of peptidases (Figure 3). Peptidic boronic acids have been widely investigated as inhibitors of serine proteases such as trypsin,[36] α-lytic protease,[19] chymotrypsin,[37] and elastase.[37] To further investigate the specificity of the boronic acids, it seemed prudent to investigate the affinity of the aryl and heterocyclic boronic acids against representative proteases.

For trypsin and elastase, most inhibitors, with the exception of the dansyl-based **5**, have no measurable activity (IC_{50} values >200 μM) (Tables 3 and 5). Most inhibitors tested, except for the two larger bicyclic compounds **35** and **36**, also have little or no measurable activity against chymotrypsin. The differences in chymotrypsin inhibition observed for the two "benzo[*b*]" compounds (**35**, **36**) compared to that for their smaller monocyclic analogues (**28**, **26**) suggest that the basis for this affinity may lie in the similarity of the structure of the larger compounds to the aromatic amino acids present in substrates preferred by chymotrypsin. Overall, the boronic acids presented here have little measurable activity against the proteases, showing specificities for the β-lactamases that are often as high as 1000-fold or better. Even the most active protease inhibitor, **36**, is at least 30-fold more active against AmpC than it was against chymotrypsin. Consistent with the structure−activity data in this series, these results suggest that the aryl- and heterocyclic boronic acids presented here bind to AmpC β-lactamase with some specificity.

In Vitro Activity against Resistant Bacteria. Previous investigations have suggested that arylboronic acids have only modest ability to overcome resistance to β-lactams by β-lactamase-producing organisms.[22,23] The increased potency of these new compounds against AmpC led us to wonder if they might also have improved activity against β-lactamase-producing bacteria. In almost every case the boronic acids tested potentiate the effects of β-lactam antibiotics against resistant bacteria (Figures 6A,B, and 8). Several of the compounds tested showed protective effects in the low μg/mL range. These results are comparable to, or better than, those obtained for tazobactam, a clinically used β-lactam-based β-lactamase inhibitor, against strains expressing class C β-lactamases. However, trends in enzyme inhibition are not always fully reflected in the bacterial testing data. For example, although the benzo[*b*]thiophene **36** has a K_i value that is 100-fold lower than that of the related thiophene **26**, it is at best only 4-fold more active than **26** in the bacterial assays. This suggests that some of the more potent AmpC inhibitors, such as **36**, may not have full access to β-lactamase targets, perhaps owing to the barrier presented by the outer membrane of gram-negative bacteria.

Conclusions

An urgent need exists for novel β-lactamase inhibitors, not based on a β-lactam core structure, that are active against β-lactamases. Such inhibitors would not be hydrolyzable by β-lactamases or mutant β-lactamases and would not be recognized by the suite of bacterial resistance mechanisms mobilized against β-lactam-based β-lactamase inhibitors. We have applied structure-based approaches to enhance the activity of boronic acid-based inhibitors of class C β-lactamases. Several of these compounds are able to protect β-lactam antibiotics from inactivation when coadministered at low μg/mL concentrations against β-lactamase-expressing bacteria. These inhibitors demonstrate 30- to 1000-fold specificity for β-lactamases over representative serine proteases. The wide variety of chemical functionality present in

the boronic acid compounds tested has also allowed us to map the AmpC binding site and suggest modifications to improve the potency of the agents tested. We also report the identification of a potent non-β-lactam-based class C β-lactamase inhibitor, benzo[*b*]thiophene-2-boronic acid (**36**, K_i (*E. coli* AmpC) = 27 nM). Modeling of these inhibitors suggests that they may be interacting with the enzyme in manners unanticipated by earlier classes of inhibitors. At the same time, it must be admitted that such modeling carries with it some ambiguity, and key questions regarding the structural bases for activity remain unanswered. Further structural and chemical investigations in this series seem warranted.

Experimental Section

The MAPB (**7**)−*E. coli* AmpC complex structure used for analysis was previously determined to 2.3 Å resolution with an *R*-factor of 15.6%.[27] Crystallographically determined structures were examined for potential interactions using the Sybyl (version 6.4, Tripos Associates, St. Louis, MO) and MidasPlus[38] interactive molecular graphics programs. The numbering scheme used to refer to *E. coli* AmpC residues is that of Galleni et al.[39]

Boronic acid compounds were constructed from the Sybyl fragment library and assigned Gasteiger−Huckel electrostatic charges, and conformer generation was performed using systematic search within Sybyl (version 6.4, Tripos Associates, St. Louis, MO) using a 15° scan increment for the Cα−Cβ, Cβ−Oγ, and Oγ−B bonds and a 120° scan increment for the B−C and B−OH bonds of the Ser64−boronic acid adducts. No internal energy evaluation was performed, and all van der Waals radii scaling factors were set to 0.5. Only conformers that placed one of the two boronic acid oxygen atoms near (2.4−3.4 Å range) the nitrogens of the putative hydroxyl/oxyanion hole (backbone N atoms of Ser64 and Ala318) were scored. Two separate systematic searches, one for each boronic acid oxygen atom involved in the distance constraints, were performed for each compound. Initial scoring was performed using the DOCK accessory program SCOREOPT2 based on steric criteria from DISTMAP[28] (values of 2.3 and 2.6 Å used for receptor polar and apolar close contact limits, respectively) for monomer A of the AmpC unit cell. Conformers lacking steric clashes with the binding site were then scored using a potential map composed of van der Waals and electrostatic terms for the AMBER potential function as calculated by the chemgrid[29] program. A potential map lacking the Oγ and Cβ of Ser64 was used for chemgrid scoring to allow corrections of the boronic acid ligands without steric conflicts with the receptor. The phosphonate ligand of Lobkovsky et al.[30] was scored in a similar manner, using only the crystallographic conformations as input to chemgrid-based scoring.

9-Phenanthreneboronic acid (**9**) was obtained from TCI America, Portland, OR. *m*-Aminophenylboronic acid (**7**) and phenylboronic acid (**1**) were obtained from Sigma Chemical, St. Louis, MO. Butylboronic acid (**29**), 4-bromophenylboronic acid (**18**), 3-nitrophenylboronic acid (**6**), and both enantiomers of diethanolamine-tetrahydrofuranylboronic acid (**24**, **25**) were obtained from Aldrich Chemical, Milwaukee, WI. 3-dihydroxyborane-benzoic acid (**12**), 4-methylthiophene-2-boronic acid (**32**), and benzothiophene-3-boronic acid (**37**), and 5-indolylboronic acid (**11**) were supplied by Frontier Scientific, Logan, UT. The remaining compounds tested for β-lactamase inhibition were obtained from Lancaster Synthesis, Windham, NH. The identity and purity of thiophene-2-boronic acid (**26**), furan-2-boronic acid (**27**), benzo[*b*]thiophene-2-boronic acid (**36**), and benzo[*b*]furan-2-boronic acid (**35**) were verified by HPLC and FAB-MS. All other compounds were used as provided by the manufacturers without additional verification.

Boronic acid compounds were initially dissolved in DMSO at concentrations of 1−100 mM. Solubility and absorbance profiles were determined by incremental addition of small volumes of DMSO compound stocks to assay buffer at 25 °C using an HP8543 UV/visible spectrophotometer with multicell transport running HP ChemStation software (version 2.5). Compounds were delivered from the same DMSO stocks for assays against the AmpC enzyme from *E. coli*. Assay conditions for AmpC were as follows: pH 7.0, 100 μM cephalothin (sodium salt, Sigma) as substrate, reaction monitored at 265 nm, 25 °C, 50 mM phosphate buffer, pH 7.0. Reactions were initiated with addition of 1.5 nM AmpC. The background rate of cephalothin hydrolysis was found to be negligible under these conditions (approximately 1%). Because the boronic acids were delivered from DMSO stock solutions, appropriate volumes of DMSO were added to enzyme control reactions in all cases. Standard 1 cm path length quartz spectrophotometric cells (Hellma Cells, Inc., Jamaica, NY) were used in the assays. All assays were performed on the HP8543 spectrophotometer described earlier. We were concerned that the inhibition data for **24** and other boronic acids acquired in the form of macromolecular boroxine complexes (e.g. $RB_n(OH)_{2n}$ where $n > 1$) might display variability based on the rate of complex dissociation. However, the inhibition values for **24** and all other boroxine compounds tested showed no significant differences regardless of the preincubation time allowed.

For the Lineweaver−Burk analysis of **36**, reaction rates for each spectrophotometric cell were calculated from quadratic fits to the absorbance data for the full time course of each reaction. This typically resulted in a standard deviation that was 2−3 orders of magnitude less than the reaction rate value. At least 3 reaction rate values for each substrate−inhibitor combination were determined. These values were then averaged together to generate each data point plotted on the Lineweaver−Burk plot. Data for some of the lowest substrate values used (40 μM cephalothin) were inconsistent with the rest of the data, and these outliers were not included in the plot.

Bacterial cell culture testing was performed and interpreted following the guidelines of the National Committee for Clinical Laboratory Standards.[40] The following strains were used: *Enterobacter cloacae* cell line with derepressed β-lactamase production (Ent-Der) and *Escherichia coli* RYC1000 (*ara*D139 D *lac*U169 *rps*L D *rib*7 *thi*A *gyr*A *rec*A56) cell lines, harboring the plasmid pBGS19 (with no β-lactamase) or the β-lactamase-containing plasmids pBGAmpC (AmpC β-lactamase from *E. coli*; Eco-AmpC) or pBGAmpC-MHN (AmpC β-lactamase from *En. cloacae*; Eco-AmpCEnt). Plasmids pBGAmp-MHN and pBGAmpC were constructed by PCR amplification of the respective *En. cloacae* and *E. coli* chromosomal *ampC* genes and subsequent cloning into pBGS18.[41] Standard antibiotic powders were kindly provided by pharmaceutical companies as follows: amoxicillin (SmithKline Beecham Laboratories), ceftazidime (Glaxo), tazobactam (Cyanamid-Lederle).

Boronic acid inhibitors were tested over a range of concentrations up to a maximum of 128 μg/mL. Several ratios, including 1:1, 2:1, 4:1, and 1:3, of β-lactam antibiotic (amoxicillin (AX) or ceftazidime (CAZ)) to boronic acid compound were used in the assays. Some of the boronic acids tested, including **6** and **26**, showed weak antibiotic activity (MIC values of 64−128 μg/mL) in the absence of a β-lactam antibiotic against some bacteria. Tazobactam (TAZO), a clinically used β-lactamase inhibitor, was used as a positive control.

The specificity of several of the most potent boronic acid inhibitors for AmpC was determined by measuring their activity against α-chymotrypsin (bovine pancreatic), β-trypsin (bovine pancreatic), and elastase (porcine pancreatic). Substrates for α-chymotrypsin (*N*-benzoyl-L-arginine ethyl ester, BAEE) and β-trypsin (*N*-benzoyl-L-tyrosine ethyl ester, BTEE) were purchased from Sigma Chemical, St. Louis, MO. The elastase substrate used (elastase substrate 1, MeOSuc-Ala-Ala-Pro-Val-pNA) was purchased from Calbiochem, San Diego, CA. All enzymes used for specificity testing were purchased from Sigma Chemical, St. Louis, MO. For α-chymotrypsin, 3 μL of a 0.2 mg/mL enzyme stock (50 mM phosphate buffer, pH 7) was incubated with the boronic acid being tested for 5 min, and then the reaction was initialized by addition of 200

μM BTEE from a DMSO stock. The reaction was performed at 25 °C and monitored at 260 nm. For β-trypsin, 5 μL of a 0.2 mg/mL enzyme stock (50 mM phosphate buffer, pH 7) was incubated with the boronic acid being tested for 5 min, and then the reaction was initialized by addition of 90 μM BAEE from a DMSO stock. The reaction was performed at 25 °C and monitored at 260 nm. For elastase, 30 μL of a 0.2 mg/mL enzyme stock (50 mM phosphate buffer, pH 7) was incubated with the boronic acid being tested for 5 min, and then the reaction was initialized by addition of 640 μM elastase substrate 1 from a DMSO stock. The reaction was performed at 25 °C and monitored at 385 nm. Compound testing was typically initiated at concentrations of 200 μM and titrated downward as needed. Linear fits to the absorbance data for the first 100 s of each reaction were used to determine reaction rate values.

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