Structure-Based Enhancement of Boronic Acid-Based Inhibitors of AmpC
\(\beta\)-Lactamase

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The expression of \(\beta\)-lactamase enzymes is one of the most common forms of bacterial resistance to \(\beta\)-lactam antibiotics. To combat these enzymes, agents that inhibit (e.g. clavulanic acid) or evade (e.g. aztreonam) \(\beta\)-lactamases have been developed. Both the \(\beta\)-lactamase inhibitors and the \(\beta\)-lactamase-resistant antibiotics are themselves \(\beta\)-lactams, and bacteria have responded to these compounds by expressing variant enzymes resistant to inhibition (e.g. IRT-3) or that inactivate the \(\beta\)-lactamase-resistant antibiotic (e.g. TEM-10). Moreover, these compounds have increased the frequency of bacteria with intrinsically resistant \(\beta\)-lactamases (e.g. AmpC). In an effort to identify non-\(\beta\)-lactam-based \(\beta\)-lactamase inhibitors, we used the crystallographic structure of the m-aminophenylboronic acid–Escherichia coli AmpC \(\beta\)-lactamase complex to suggest modifications that might enhance the affinity of boronic acid-based inhibitors for class C \(\beta\)-lactamases. Several types of compounds were modeled into the AmpC binding site, and a total of 37 boronic acids were ultimately tested for \(\beta\)-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 \(\beta\)-lactam antibiotics, such as amoxicillin and ceftazidime, against bacteria expressing class C \(\beta\)-lactamases. This suggests that boronic acid-based compounds may serve as leads for the development of therapeutic agents for the treatment of \(\beta\)-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 \(\beta\)-lactamase enzymes is one of the most common and well-studied forms of antibiotic resistance.7 \(\beta\)-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. \(\beta\)-lactamases protect bacteria that express them by hydrolyzing the \(\beta\)-lactam ring present in these antibiotics, inactivating them (Figure 1).8 Bacteria appear to have evolved \(\beta\)-lactamases several different times;9,10 and several different classes of these enzymes exist. The most clinically important groups are the class A \(\beta\)-lactamases, whose members include the plasmid-based TEM peni-

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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), 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, Dryjanski and Pratt reported that m-(dansylamidoaryl)-boronic acid (5) is a submicromolar inhibitor (Ki = 0.1 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 (1R)-1-aminophenylboronic acid with high affinity for this enzyme (Kii = 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 A β-lactamase.16 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 (Kii = 7.3 ± 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′-biphenylidyboronic 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 CHEMGRID29 (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

\[ \text{Enz} - \text{OH} \quad \text{boronate transition-state analog inhibitor (MAPB (7), x-ray)} \]

\[ \text{Enz} - \text{OH} \quad \text{presumed acylation transition state of β-lactam hydrolysis} \]

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.
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-nitrophosphino-boronic 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 reactions31 (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 aryboronic 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

<table>
<thead>
<tr>
<th>Boronic Acid</th>
<th>K_i (AmpC) (µM)</th>
<th>Boronic Acid</th>
<th>K_i (AmpC) (µM)</th>
<th>Boronic Acid</th>
<th>K_i (AmpC) (µM)</th>
<th>Boronic Acid</th>
<th>K_i (AmpC) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.5 ± 2.6</td>
<td>10</td>
<td>53.4 ± 6.1</td>
<td>20</td>
<td>5.4 ± 1.3</td>
<td>11</td>
<td>10.9 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>25.3 ± 4.2</td>
<td>12</td>
<td>&gt;&gt;100</td>
<td>22</td>
<td>6.4 ± 0.8</td>
<td>23</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>12.0 ± 1.0</td>
<td>13</td>
<td>5.9 ± 0.3</td>
<td>24</td>
<td>1.4 ± 0.1</td>
<td>31</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>1.1 ± 0.3</td>
<td>14</td>
<td>2.9 ± 0.3</td>
<td>25</td>
<td>15.8 ± 0.8</td>
<td>32</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>1.3 ± 0.1</td>
<td>15</td>
<td>2.9 ± 0.9</td>
<td>26</td>
<td>22.1 ± 3.5</td>
<td>33</td>
<td>8.5 ± 1.8</td>
</tr>
<tr>
<td>6</td>
<td>1.7</td>
<td>16</td>
<td>3.0 ± 0.5</td>
<td>27</td>
<td>&gt;&gt;100</td>
<td>34</td>
<td>8.5 ± 1.8</td>
</tr>
<tr>
<td>7</td>
<td>7.3 ± 0.9</td>
<td>17</td>
<td>3.6 ± 0.9</td>
<td>28</td>
<td>&gt;&gt;100</td>
<td>35</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>8</td>
<td>8.5 ± 1.8</td>
<td>18</td>
<td>4.2 ± 1.1</td>
<td>29</td>
<td>&gt;&gt;100</td>
<td>36</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>9</td>
<td>7.0 ± 1.2</td>
<td>19</td>
<td></td>
<td>30</td>
<td></td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

* a Determined from a previous Lineweaver-Burk analysis of kinetic data.  

b > = less than 50% inhibition observed at ≥ 3 times the cephalothin concentration noted.

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. 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 (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.
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).

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 $\beta$-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 $\beta$-lactamases in vitro, thus potentiating its antibiotic activity. The thiophene-2-boronic acid analogues tested showed comparable to better potency than tazobactam against bacteria expressing class C $\beta$-lactamases (Figure 8). The MIC values reported are the minimum concentrations at which no bacterial growth was observed.

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

<table>
<thead>
<tr>
<th>boronic acid</th>
<th>IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>26</td>
<td>10</td>
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<tr>
<td>33</td>
<td>&gt;200</td>
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<tr>
<td>34</td>
<td>&gt;200</td>
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<tr>
<td>35</td>
<td>&gt;200</td>
</tr>
<tr>
<td>36</td>
<td>&gt;200</td>
</tr>
<tr>
<td>37</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

\(^{a}\) CHT = $\alpha$-chymotrypsin; TRY = $\beta$-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. Structures and Inhibition Constants for Thiophene-2-boronic Acid (24) and Its Analogues

<table>
<thead>
<tr>
<th>boronic acid</th>
<th>R</th>
<th>K_i(AmpC) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>HO</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>31</td>
<td>S</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>32</td>
<td>CH3</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>33</td>
<td>Cl</td>
<td>0.41 ± 0.10</td>
</tr>
<tr>
<td>34</td>
<td>CH2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>35</td>
<td>O</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>36</td>
<td>HO</td>
<td>0.027</td>
</tr>
<tr>
<td>37</td>
<td>S</td>
<td>0.78 ± 0.08</td>
</tr>
</tbody>
</table>

\(^{a}\) The K_i value calculated from the progress curve comparison method was 0.016 ± 0.003 M.

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 $\beta$-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 $\beta$-lactamases in vitro, thus potentiating its antibiotic activity. The thiophene-2-boronic acid analogues tested showed comparable to better potency than tazobactam against bacteria expressing class C $\beta$-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 $\beta$-lactamase specificity versus $\alpha$-chymotrypsin, $\beta$-trypsin, and elastase (Table 5). The most potent of these inhibitors, benzo[b]thiophene-2-boronic acid (36), had an IC50 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 (IC50 = 5 M).
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 ($0.9 \mu$M) for AmpC, making it one of the more active boronic acid compounds previously described for this class of $\beta$-lactamase. It is considerably more active than borate, for instance, which inhibits AmpC-like enzymes only at millimolar concentrations. 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 $\beta$-lactamase active site. Boronic acids inhibit serine-type $\beta$-lactamases reversibly and rapidly, 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.

One possible explanation for the affinity of the boronic acids is that binding is driven primarily by ligand hydrophobicity. Hydrophobicity is principally a non-specific 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

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 $\beta$-lactam activity against resistant bacteria by selected compounds from the second group of boronic acids tested, using ceftazidime as the $\beta$-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

<table>
<thead>
<tr>
<th>boronic acid</th>
<th>AmpC</th>
<th>CHT*</th>
<th>TRY*</th>
<th>ELST*</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>0.15</td>
<td>5</td>
<td>&gt;200</td>
<td>200</td>
</tr>
<tr>
<td>35</td>
<td>0.6</td>
<td>14</td>
<td>&gt;200</td>
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<tr>
<td>33</td>
<td>2</td>
<td>&gt;100</td>
<td>&gt;200</td>
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</tr>
<tr>
<td>26</td>
<td>10</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>200</td>
</tr>
</tbody>
</table>

* CHT = a-chymotrypsin; TRY = $\beta$-trypsin, ELST = elastase.
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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 \(\beta\)-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 Ca–C\(_\beta\) 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 AmpC27 and a second adopted by a phosphonate inhibitor bound to the AmpC enzyme from \(\text{En. cloacae}^{30}\).

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 Asn299, 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 O\(_\beta\) of Asn343 and 3.4 Å from the N\(_\beta\) of Arg349. The heteroatom of a 4-hydroxy- or 4-aminophenylboronic acid would be positioned 2.3 Å from the N\(_\beta\) and 2.8 Å from the O\(_\alpha\)1 atom of Asn343. These residues are believed to contribute to recognition of the R2 groups of \(\beta\)-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 R3 side chains of \(\beta\)-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 \text{\mu M}), 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 \text{\mu M}) appears to fit snugly into the hydrophobic pocket created by Leu119 and Leu293, and the m-nitro group of 6 (K\(_i\) = 1.7 \text{\mu M}), adopting a slightly different “phosphonate-like” orientation, should be able to interact with Asn152. In contrast, the poor activity observed for 12 (K\(_i\) \approx 100 \text{\mu M}) 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 \text{\mu M}) 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 \text{\mu M}), formyl (16, K\(_i\) = 2.9 \pm 0.9 \text{\mu M}), trifluoromethyl (17, K\(_i\) = 3.0 \pm 0.5 \text{\mu M}), carboxyvinyl (19, K\(_i\) = 4.2 \pm 1.1 \text{\mu M}), and boronic acid (23, K\(_i\) = 3.9 \pm 0.2 \text{\mu M}) groups, with each improving affinity relative to the parent phenylboronic acid (1, K\(_i\) = 10.5 \pm 2.6 \text{\mu M}). Even larger p-substituents, such as those present in the biphenyl (14, K\(_i\) = 0.2 \pm 0.03 \text{\mu M}), 2-naphthyl (8, K\(_i\) = 8.5 \pm 1.8 \text{\mu M}), and 5-indolyl (11, K\(_i\) = 10.9 \pm 0.6 \text{\mu M}) 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 rich34 and is known to form polar transfer complexes with electropositive groups, was found to have a K\(_i\) of 2.5 \pm 0.4 \text{\mu M} 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 \pm 0.1 \text{\mu M}. 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 \pm 0.8 \text{\mu M}. The lower affinity of thiophene-3-boronic acid (27) (K\(_i\) = 22.1 \pm 3.5 \text{\mu M}) suggests that electronic effects may also play a role in the affinities of the thiophene series. The poor affinity of 2-furanyl-
boronic acid (28, \( K_i > 100 \mu M \)) 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 “MAP8-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, \( K_i = 8.5 \pm 1.8 \mu M \)), 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 \( \beta \)-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,\(^{36}\) \( \alpha \)-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\textsubscript{50} values > 200 \( \mu M \)) (Tables 3 and 5). Most inhibitors tested, except for the two 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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-lactams by \( \beta \)-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 \( \beta \)-lactamase-producing bacteria. In almost every case the boronic acids tested potentiate the effects of \( \beta \)-lactam antibiotics against resistant bacteria (Figures 6A,B, and 8). Several of the compounds tested showed protective effects in the low \( \mu g/mL \) range. These results are comparable to, or better than, those obtained for tazobactam, a clinically used \( \beta \)-lactam-based \( \beta \)-lactamase inhibitor, against strains expressing class C \( \beta \)-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 \( \beta \)-lactamase targets, perhaps owing to the barrier presented by the outer membrane of gram-negative bacteria.

**Conclusions**

An urgent need exists for novel \( \beta \)-lactamase inhibitors, not based on a \( \beta \)-lactam core structure, that are active against \( \beta \)-lactamases. Such inhibitors would not be hydrolyzable by \( \beta \)-lactamases or mutant \( \beta \)-lactamases and would not be recognized by the suite of bacterial resistance mechanisms mobilized against \( \beta \)-lactam-based \( \beta \)-lactamase inhibitors. We have applied structure-based approaches to enhance the activity of boronic acid-based inhibitors of class C \( \beta \)-lactamases. Several of these compounds are able to protect \( \beta \)-lactam antibiotics from inactivation when coadministered at low \( \mu g/mL \) concentrations against \( \beta \)-lactamase-expressing bacteria. These inhibitors demonstrate 30- to 1000-fold specificity for \( \beta \)-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, benzob[b]thiophene-2-
boronic acid (36, K(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 struc-
tural 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 Midas
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-Ci
 –
Cj
 –
O
 –
Oy
 –
Oy
 –
and Oy–B bonds and a 120° scan increment for the –C
 –
and B–OH bonds of the Ser64–boronic acid adds. An
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/ox-
yanion 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 DISTMAP28 (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
potential map lacking the O atom. The phosphonate ligand of
Lobkovsky et al.30 was subsequently cloned into pBGS18.41 Standard antibiotic
activity against AmpC was determined by measuring their
inhibition data for 24 and other boronic acids acquired in the
form of macromolecular boroxine complexes (e.g. RB(18OH)2,
where n > 1) might display variability based on the rate of
complex dissociation. However, the inhibition values for 24
and all other boronic 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 aver-
aged 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. The following strains were used:
Enterobacter cloacae cell line with derepressed β-lactamase
production (Ent-Der) and Escherichia coli RY1000 (araD139
D lacU169 rpsL D rib7 thiA gyrA recA56) cell lines, harboring
the plasmid pBSG19 (with β-lactamase) or the β-lactamase-
containing plasmids pBGAMpC (AmpC β-lactamase from E.
coli; Eco-AmpC) or pBGAMp-MHN (AmpC β-lactamase from E.
cloacae Eco-AmpCEnt). Plasmids pBGAMp-MHN and
pBGAMC were constructed by PCR amplification of the respec-
tive ampC genes from E. cloacae strain BD142 and subsequent
doping into pBSG18.31 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 concen-
trations up to a maximum of 128 μg/mL. Several ratios,
including 1:1, 2:1, 4:1, and 1:3, of β-lactam antibiotic (amox-
iciillin (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). The absence of activity 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-benzylo-L-tryptase 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-tryptase, 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 for 5 min. 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 the reaction was monitored for 100 s of each reaction were used to determine reaction rate values.

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