Design, Synthesis, Crystal Structures, and Antimicrobial Activity of Sulfonamide Boronic Acids as β-Lactamase Inhibitors

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We investigated a series of sulfonamide boronic acids that resulted from the merging of two unrelated AmpC β-lactamase inhibitor series. The new boronic acids differed in the replacement of the canonical carboxamide, found in all penicillin and cephalosporin antibiotics, with a sulfonamide. Surprisingly, these sulfonamides had a highly distinct structure−activity relationship from the previously explored carboxamides, high ligand efficiencies (up to 0.91), and Ki values down to 25 nM and up to 23 times better for smaller analogues. Conversely, Ki values were 10−20 times worse for larger molecules than in the carboxamide congener series. X-ray crystal structures (1.6−1.8 A) of AmpC with three of the new sulfonamides suggest that this altered structure−activity relationship results from the different geometry and polarity of the sulfonamide versus the carboxamide. The most potent inhibitor reversed β-lactamase-mediated resistance to third generation cephalosporins, lowering their minimum inhibitory concentrations up to 32-fold in cell culture.

Introduction

The expression of β-lactamases is the most common cause of resistance to β-lactam antibiotics, such as penicillins and cephalosporins.1−3 β-Lactamases catalyze the hydrolysis of the critical β-lactam ring in β-lactam antibiotics, thereby inactivating them.4 To overcome this problem, β-lactamase inhibitors, such as clavulanic acid or sulbactam, are coadministered with the β-lactam (Figure 1).5−7 However, the active core of these inhibitors remains a β-lactam ring, enabling the rapid development of resistance.8 For example, AmpC, a class C β-lactamase expressed by many nosocomial pathogens, is not inhibited by clavulanic acid or sulbactam at clinically relevant concentrations, leading to substantial problems in the clinic.9,10

The broad activity of class C β-lactamases has motivated a search for novel inhibitors. New departures based on the β-lactam core have shown substantial, typically mechanism-based activity in vitro and in vivo as inhibitors.10−13 There is also a motivation to discover inhibitors structurally unrelated to β-lactams, which might evade pre-evolved bacterial resistance mechanisms.14−16 Lacking the β-lactam core, these inhibitors should not be hydrolyzed by mutant enzymes that arise in response to new β-lactams, should not be recognized by β-lactam signaling receptors, and might be unaffected by porin channel mutations that decrease permeability.2,17

1The coordinates and structure factors have been deposited in the Protein Data Bank with the accession codes 3O86, 3O87, and 3O88 for AmpC in complex with 4, 9, and 17, respectively.
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Boronic acids are transition-state analogues that lack the β-lactam recognition motif and are distinct enough chemically to evade pre-evolved resistance mechanisms.17−19 They form dative, rapidly reversible covalent bonds with the active site serine, forming a tetrahedral adduct that mimics the high-energy intermediate of β-lactams along the β-lactamase reaction coordinate (Figure 2).20

We found previously that acylglycyl boronic acids bearing side chains characteristic of penicillins and cephalosporins (R4 group in Figure 2A) have Ki values as low as 20 nM against AmpC.19 Using a structure-based drug design approach, we designed m-carboxyphenylboronic acids having a negatively charged group in a position corresponding to the C4 of cephalosporins, such as cephalothin (Figure 1).21,22 Compound 18c has a Ki value of 1 nM, improving potency up to 300-fold compared to the corresponding acylglycylboronic acid 10c (Table 1).

In the crystal structure of the AmpC/18c complex, the oxygen atom of the carboxamide forms a hydrogen bond with Asn152, a conserved residue in the AmpC active site, while the carboxamide nitrogen hydrogen-bonds with the backbone carbonyl of Ala318, a residue that contributes to the enzyme’s oxyanion hole (Figure 3A). In an unrelated series of non-covalent β-lactamase inhibitors, discovered by molecular docking, a sulfonamide group is similarly positioned and makes the same interactions (Figure 3B).23

It therefore seemed interesting to replace the ubiquitous carboxamide, present in the R4 side chain of both β-lactams and their boronic acid mimics, by a sulfonamide (Scheme 1). Whereas sulfonamide replacements for carboxamides date
back to the seminal synthetic work of Sheehan in the 1950s, these derivatives were never used clinically and, as far as we know, have never been explored among β-lactamase inhibitors previous to this year. Very recent work by Tan et al. at Merck revealed two arylsulfonamide boronic acids with activity in the low micromolar range (IC50 between 0.57 and 5.6 μM) against five different β-lactamases.

We synthesized 10 sulfonamide boronic acids and tested their inhibition of AmpC β-lactamase. Among them are seven different R1 groups including the penicillin G and nafcillin side chains. We found that the simplest of these new sulfonamides were unexpectedly the most potent (Ki values in the 25 nM range), over 23 times more than their carboxamide analogues, and that the SAR of this series differed completely from what was observed in the carboxamide series. To understand these differences, we determined X-ray structures of three inhibitors in complex with AmpC. Finally, we investigated the efficacy of the most potent inhibitors to reverse antibiotic resistance in bacterial cell cultures.

Results

Design. The geometry and electronic properties of a sulfonamide are quite different from those of a carboxamide, and we were interested in how this substitution would affect affinity (Scheme 1). First, we explored the sulfonamide substitution with a methyl alone as the R group. Since this compound was unexpectedly potent against AmpC, we then synthesized and tested sulfonamide boronic acids with seven more elaborate R groups (Table 1). Finally, we synthesized molecules with a benzyl and m-carboxybenzyl groups as R2 substituents.

Synthesis. The sulfonamidomethaneboronic acids 3–9 and 11 were synthesized through sulfonylation of the amonomethaneboronate 2 (Scheme 2). The key intermediate 2 was obtained by treating the chloromethylboronic acid pinacol ester 1 with lithium bis(trimethylsilyl)amide. In situ deprotection of 2 with equimolar methanol, followed by condensation with suitable sulfonyl chlorides, afforded the sulfonamidomethaneboronic acids 3–9 and 11.

Compound 10 (Table 1), bearing the cephalothin side chain, could not be obtained because of instability of the

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**Table 1.** Ki Values (μM) of Sulfonamide and Carboxamide Boronic Acids

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<thead>
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<th>Sulfonamide boronic acids</th>
<th>Carboxamide boronic acids</th>
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*(a) Abbreviations: LE, ligand efficiency (= (ΔG_binding (kcal/mol)) / (number of non-hydrogen atoms)); PDB, Protein Data Bank; IC50, half-maximal inhibitory concentration; SAR, structure–activity relationship; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; CAZ, ceftazidime; FOX, cefoxitin; THF, tetrahydrofuran; TLC, thin layer chromatography.
suitable sulfonyl chloride. In fact, several attempts to obtain the (thiophene-2-yl)methanesulfonyl chloride led to the corresponding 2-chloromethylthiophene instead, as reported for other electron rich phenylmethanesulfonyl chlorides.26

According to recent literature reports, related sulfonylaminomethaneboronate compounds27,28 can also be prepared by alkylation of suitable N-tert-butylsulfonamides with pinacol chloromethaneboronate 1, followed by acidic tert-butyl removal. However, this reaction sequence proved to be less efficient, and the method was therefore abandoned.

Following our previous approach with carboxamides,21 we attempted to synthesize compound 18 (Table 1), but the synthesis failed because protodeboronation during sulfonylation reaction occurred. The loss of the boronic moiety may occur because of stabilization of the intermediate carb-anion formed when a base coordinates to boron.29 Because the presence of the m-carboxyphenyl ring in the present case can account for this stabilization, we decided to add an extra carbon between the boron atom and the phenyl ring (Scheme 3). Therefore, to build the more elaborated inhibitors 16 and 17, boronates 12a,b were first homologated with chloromethyl-lithium.30 Subsequent Matteson stereoselective homologation with dichloromethyl-lithium,29,31-33 followed by substitution with bis(trimethylsilyl)amide and sulfonylation afforded compounds 15a,b. The use of (+)-pinanediol as chiral auxiliary allowed the formation of the final R configuration, which mimics the stereochemistry of carbon atom C6/7 of penicillin/cephalosporin. The conversion of pinanediol esters 15a,b to free boronic acids 16 and 17 was achieved through transesterification with phenylboronic acid in a biphasic acetonitrile/water system.34

Enzymology and Binding Affinities. Except for those binding in the low nanomolar range, all previously studied boronic acids inhibitors of AmpC β-lactamase have been reversible, competitive inhibitors with fast-on, fast-off rates.17,18 The same was found for the new sulfonamide boronic acid series, as no incubation effect was detected for any of the compounds.

To investigate the effect of the carboxamide replacement with a sulfonamide, we first measured the potency of methanesulfonamide boronic acid 3 (Table 1). Compound 3 inhibits AmpC with a $K_i$ of 789 nM, a 23-fold improvement of potency compared to the carboxamide analogue 3c ($K_i = 18.5 \mu M$). The high affinity of this relatively small molecule prompted us to study larger derivatives. Compound 4, bearing the penicillin G side chain has a $K_i$ of 70 nM, a 10-fold improvement over the initial sulfonamide 3 and an 8-fold improvement compared to its exact carboxamide analogue 4c ($K_i = 570 \mu M$) (Table 1).19 Because compound 5, isomer of compound 4, has a $K_i$ of 210 nM, we decided to focus on benzylic derivatives and synthesized compounds 6–9, for which required sulfonylchlorides were commercially available or the final products easily accessible. Although most of those derivatives have a lower potency than 4, compound 9

![Figure 3. Inspiration for sulfonamide boronic acids. (A) The carboxamide of boronic acid 18c hydrogen-bonds with Asn152 and Ala318 of AmpC β-lactamase (PDB code 1MXO). Carbon atoms of inhibitor are colored green, oxygens red, nitrogens blue, sulfur yellow, boron atom amaranth pink. (B) The sulfonamide of a noncovalent β-lactamase inhibitor hydrogen-bonds with Asn152, Ala318, Ser64, and Lys67 (PDB code 1L2S). Carbon atoms of inhibitor are colored cyan, oxygens red, nitrogens blue, sulfur yellow, chloride green.](image-url)

**Scheme 1.** Conversion of Carboxamide Boronic Acids into Sulfonamide Boronic Acids

![Scheme 1](image-url)

**Scheme 2.** General Scheme of the Synthesis of Sulfonamidomethaneboronic Acids

![Scheme 2](image-url)

**Scheme 3.** Asymmetric Synthesis of 1-Sulfonamido-2-phenylethaneboronic Acids 16 and 17

![Scheme 3](image-url)
has a $K_i$ of 25 nM, a 3-fold improvement versus compound 4. We tried to synthesize compound 10, the exact analogue of the carboxamide 10c, but found that the required (thiophene-2-yl)methanesulfonylchloride was unstable. Unexpectedly, compound 11, bearing the nafcilin side chain, inhibits AmpC with a $K_i$ of 670 nM, 20-fold weaker than the carboxamide analogue 11c ($K_i = 33$ nM). This was another indicator that the SAR changes substantially in the sulfonamide series.

In the carboxamide series, addition of a $m$-carboxybenzyl group to 10c had resulted in a 7-fold improvement of potency for compound 17c. We decided to investigate the same modification for the sulfonamide 4 and synthesized the chiral compound 17. This latter has a $K_i$ of 430 nM; thus, rather than a 7-fold improvement, adding a $m$-carboxybenzyl to compound 4 led to a 6-fold decrease in inhibition. Compound 16, only with a benzyl as R$_2$ group, has an even worse $K_i$ of 3.7 μM. The weaker potency of compounds 16 and 17 further demonstrate the changed SAR resulting from the carboxamide to sulfonamide conversion.

**Structural Analysis.** To investigate the structural basis of the carboxamide to sulfonamide conversion, we determined co-crystal structures of AmpC in complex with 4, 9, and 17 (Table 2). All complexes were obtained from the same crystallization condition yielding centered monoclinic C2 crystals with two AmpC complexes in the asymmetric unit. The positions of the inhibitors in the active site were unambiguously identified in both chains in the initial $F_o - F_c$ difference density maps (Figure 4). With the exception of the complex with compound 4, the conformations of the inhibitors were very similar in both monomers.

For all three inhibitors, electron density connected the $O_T$ of the catalytic Ser64 to the boron atoms of the inhibitors. The boron geometry was tetrahedral, as expected, and key hydrogen bond interactions in the active site closely resembled those typically observed in $β$-lactamase structures with transition-state analogues and with $β$-lactams (Figure 5A–C).14,19,21,22 One oxygen atom of the boronic acid is placed in the “oxyanion hole” formed by the backbone amide groups of Ser64 and Ala318. The other oxygen atom of the boronic acid interacts with the conserved Tyr150, which contributes to activating the deacylating water Wat402 for attack on the acyl intermediate.12,35–39

Both oxygen atoms are also within hydrogen bonding distance to the conserved water molecule Wat402.

The crystal structure of compound 4 ($K_i = 70$ nM) in complex with AmpC was determined at 1.60 Å (Figure 5A). Whereas there were several differences between the two monomers in the asymmetric unit, such as the rotation of the benzyl ring to stack with Tyr221 in either a parallel or herringbone conformation, we will focus on the interaction in chain A. Here, one oxygen atom of the sulfonamide hydrogen-bonds to Asn152 (nitrogen-π distance: 2.6 Å). The other sulfonamide oxygen atom hydrogen-bonds with a water molecule bridging to Gln120. The benzyl group makes a T-shaped π–π interaction (90° angle) with Tyr221. The sulfonamide nitrogen interacts neither with the protein nor with a well-ordered water molecule.

To understand the influence of the $p$-carboxylate group, responsible for a 3-fold increase affinity of compound 9 ($K_i = 25$ nM) compared to compound 4, we determined the crystal structure of the AmpC/9 complex to 1.78 Å resolution (Figure 5B). The conformation of 9 is similar in both monomers and resembles the conformation of 4 observed in chain A except that the plane of its phenyl ring is slightly offset relative to that of 4 (Figure 5D). The slightly altered conformation allows the $p$-carboxylate group to hydrogen-bond with the side chain hydroxyl and amide.

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**Table 2. Data Collection and Refinement Statistics**

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$^a$ Values in parentheses represent values for the highest resolution shells. $^b$ Calculated for both molecules in asymmetric unit.
nitrogen of Ser212. The other carboxylate oxygen atom hydrogen-bonds with an ordered water molecule.

To understand the reduced activity of 17 (K\textsubscript{i} = 430 nM) against AmpC relative to compounds 4 and 9, we determined its crystal structure in complex with AmpC at 1.64 Å resolution (Figure 5C). In this complex, the boron—carbon bond is rotated by 90° compared to the conformation adopted by compound 4. This is necessary to accommodate the additional m-carboxybenzyl R\textsubscript{2} group of 17. Consequently, the sulfonamide is reoriented in the active site, placing the benzylsulfonamide in a totally different location relative to compounds 4 and 9 (Figure 5E). One sulfonamide oxygen of compound 17 makes stretched hydrogen bonds with Asn152 and Lys67 (3.2 and 3.1 Å, respectively), and the nitrogen atom of the sulfonamide hydrogen-bonds with the backbone amide of Ala318 (3.1 Å), an interaction missed in the other two structures. Conversely, the second oxygen atom now abuts the backbone carbonyl of Ala318 (2.8 Å) and the quality of the other sulfonamide hydrogen bonds, as judged by distance and angle, has deteriorated substantially. The R phenyl group makes a T-shaped π—π interaction with Tyr221 but in a different conformation than observed in the AmpC/4 and AmpC/9 complex structures.

**Antimicrobial Activity.** The antiresistance activity of inhibitors 4 and 9 was investigated against clinical bacteria exhibiting high level of resistance against third-generation cephalosporins, such as ceftazidime, via expression of class A or class C β-lactamases. When the compounds were administered alone in a disk diffusion assay, they had little or no detectable activity on bacteria growth, as expected. However, in combination with ceftazidime the compounds produced a large inhibition halo surrounding the disk (Figure 6A). This inhibition of the bacterial growth revealed clear synergy between these compounds. The size of the inhibition zone was similar for both compounds and showed improved inhibition of bacterial growth compared to ceftazidime alone.

Antimicrobial activity was investigated quantitatively to determine the minimum inhibitory concentrations (MICs) of the β-lactam/inhibitor combination necessary to inhibit the bacterial growth (Table 3). The MICs of β-lactams alone against the strains corresponded to a high level of resistance according to Clinical and Laboratory Standards Institute...
The compounds had no measurable antibiotic activity when used alone. In combination with β-lactams, the compounds decreased MIC values by 8-fold on average. Interestingly, even larger relative decreases in MIC values were observed for an E. coli strain producing the plasmid-mediated class A β-lactamase CTX-M-14 (16- to 32-fold). This offers preliminary evidence that the sulfonamide boronic acids may inhibit both class C β-lactamases and class A enzymes, consistent with studies revealing activity against this class of enzymes in the low micromolar range described in this study (Table 4 and below) and results from Tan and colleagues. More detailed studies will be necessary before inhibition of the class A β-lactamases can be shown to be broad or robust.

A traditional β-lactam resistance mechanism is the up-regulation of β-lactamase-encoding gene transcription caused by β-lactams such as cefoxitin and the β-lactamase inhibitor clavulanate. To test the role of compounds on the induction of AmpC expression, we investigated their ability to potentiate the action of the β-lactam ceftazidime. We compared the potentiation effect of compounds with that of cefoxitin (Figure 6B and Figure 6C). Agar plates were inoculated with K. pneumoniae and E. cloacae strains in which AmpC expression is inducible. A ceftazidime-containing disk and a disk containing the inhibitors (cefoxitin or compounds 4 and 9) were placed on each plate. As ceftazidime diffuses from disks into the agar, a clear zone is created around the ceftazidime-containing disk, indicating where bacteria are unable to grow. The shape of this halo in the vicinity of the inhibitor-containing disk indicated the effect of these compounds on the induction of AmpC. The inhibition halos normally surrounding ceftazidime were substantially diminished in the regions nearest the cefoxitin disk. This well-known antagonist picture is characteristic of the ampC gene induction by cefoxitin. The antagonist picture was replaced by a synergic picture with compounds 4 and 9, the inhibition halos of ceftazidime were dramatically increased in the region near the compound disks. This result indicates that the compounds inhibit AmpC and do not induce significant up-regulation of AmpC-encoding genes, in contrast to β-lactams such as cefoxitin.

Selectivity. To investigate the selectivity of sulfonamide boronic acids, compounds 4 and 9 were tested against the serine protease α-chymotrypsin, the cysteine protease cruzain, and the class A β-lactamase CTX-M-9, which is structurally different from AmpC (Table 4). Compounds 4 and 9 showed no measurable inhibition at 100 μM against cruzain and α-chymotrypsin. Interestingly, the compounds are active against CTX-M-9 with IC50 values in the low micromolar range. The Ki values corresponding to the IC50 values are 2.529 and 0.552 μM, respectively, indicating 36-fold and 22-fold selectivity toward AmpC versus CTX-M-9 β-lactamase.

Discussion

Three key observations emerge from this study. First, the new sulfonamide boronic acids derived from the conversion of the canonical R1 carboxamide retain substantial inhibition activity against β-lactamases. Second, they rescue antibiotic resistance when used in combination with third generation antibiotics in bacterial cell cultures. Third, this superficially modest substitution changes the geometry of the inhibitors enough to scramble the SAR observed in the analogue carboxamides.

A favorable change in the SAR was observed for the smaller compounds of this study. The most substantial change in activity was observed for compound 3, which has a Ki of 789 nM against AmpC. Its exact carboxamide analogue 3c is 23-fold less potent (18.5 μM). The high affinity of 3 corresponds...
to a ligand efficiency of 0.91, placing it among the most efficient of enzyme inhibitors. Given the simplicity of this inhibitor, it seems clear that its improved affinity can be laid at the door of the advantages of the sulfonamide versus the carboxamide group in the AmpC site; this in turn may reflect the improved hydrogen bonds in this site owing to differences in polarity and geometry. This advantage was maintained during the SAR development among the smaller sulfonamide analogues, with potency improving 11-fold in compound 4 ($K_i = 70 \text{ nM}$) and by another 3-fold in compound 9 ($K_i = 25 \text{ nM}$). The X-ray complexes of these inhibitors underscore the favorable hydrogen bonds made between them and canonical recognition residues.

Conversely, in larger sulfonamides the SAR is inverted with affinities generally getting worse and relative to the carboxamides getting much worse. An example for the inverted SAR is the low affinity of the sulfonamide with the nafcillin side chain. Compound 11 is a 670 nM inhibitor compared to the carboxamide analogue 11c with a $K_i$ of 33 nM. This result suggests that adding a bulky side chain to the sulfonamide weakens, if not completely disrupts, the hydrogen bonds with residues Asn152 and Gln120 observed in the crystal structures of compounds 4 and 9. Favorable stacking and van der Waals interaction of the elaborated aryl side chain may also be affected negatively.

We observed another change in the SAR when we tested compounds 16 and 17, having a benzyl and m-carboxybenzyl as R2 group, respectively. In the carboxamide boronic acids, adding a m-carboxybenzyl improved affinity 8-fold from compound 10c to 17c. In our sulfonamide series, the same modification (compound 4 to 17) leads to a 6-fold drop in potency. In the structure of the AmpC/17 complex, the sulfonamide hydrogen bonds have been completely disrupted and rearranged versus those made by 4 and 9 (Figure 5E). To understand the flip of the sulfonamide, we modeled compound 17 in the conformation observed for compound 4 (Figure 5F). The protein—inhibitor interactions in this model looked quite reasonable, but the ligand internal interactions were strained, with the aliphatic chain in the m-carboxybenzyl group very close to one of the sulfonamide oxygens (3.0 Å). We reasoned that internal 1,5 repulsion between those two atoms could force the reorientation of the sulfonamide. We therefore calculated internal energies for both the model and the crystal structure of compound 17 using the semiempirical QM method AMSOL. The internal energy of the ligand alone in the crystal structure conformation (~90.89 kcal/mol) was substantially lower than the internal energy of the modeled ligand (~55.08 kcal/mol), consistent with the internal strain hypothesis.

The unexpected SAR of these inhibitors suggests that we cannot simply adopt lessons learned from earlier series. Perhaps the most encouraging observation to emerge is that sulfonamide boronic acids with small R1 side chains are very active against AmpC and can substantially reverse antibiotic resistance in bacterial cell culture. In addition, their activity against other β-lactamases classes, as shown here and by Tan et al., supports further studies on this new class of β-lactamase inhibitors.

Table 3. Minimum Inhibitory Concentrations of Third-Generation Cephalosporins Alone and in Association with Compound 4 or 9 (Ratio 1:1) for Clinical Bacteria Exhibiting a High Level of Resistance

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<tr>
<th></th>
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<sup>a</sup>Bacteria overproducing chromosomally mediated class C β-lactamase.<br><sup>b</sup>K. pneumoniae producing the plasmid-mediated class C β-lactamase DHA-1.<br><sup>c</sup>E. coli producing plasmid-mediated class A β-lactamase CTX-M-14.
Thereafter, the reaction mixture was cooled again to
expected sulfonamides.

rated in vacuo and the residue purified by chromatography
a solution of the selected sulfonyl chloride (1.10 mmol) in THF
57.3, 84.7, 128.6, 128.7, 129.6, 130.6. EI-MS, m/z: 311 (M+1, 2%), 156 (18), 120 (5),
106 (8), 94 (17), 92 (93), 80 (117), 65 (11), 59 (16). Anal.
Calcd for C14H21BNO4S: C, 54.03; H, 7.13; N, 4.50; S, 10.30.
Found: C, 54.32; H, 7.30; N, 4.29; S, 10.55.

Pinacol (4-Methylbenzenesulfonylimino)methaneboronate (5).
White solid (84% yield). Mp 103–105 °C. 1H NMR (200 MHz,
DMSO): δ 1.17 (12H, s, pinacol protons), 2.25 (2H, d, J = 5.5, BCH2),
2.38 (3H, s, CH3Ph), 7.06 (1H, t, J = 5.5, SO2NH), 7.38
(2H, d, J = 8.2, H3, H5), 7.65 (2H, d, J = 8.2, H2, H6). 13C NMR
(50 MHz, CDCl3): δ 21.4, 24.7, 27.6 (CB), 84.6, 127.5, 129.6,
135.8, 143.2. EI-MS, m/z: 296 (M+1, 15%), 282 (55), 253 (31),
212 (19), 180 (78), 166 (26), 150 (100), 152 (39), 151 (46), 143 (20),
140 (30), 139 (89), 137 (23), 91 (80), 85 (19), 83 (25), 74 (98),
73 (30), 65 (31), 59 (20), 55 (23). Anal. Calcld for C14H21BNO4S:
C, 54.03; H, 7.13; N, 4.50; S, 10.30. Found: C, 54.26; H, 7.24; N,
4.32; S, 10.38.

Pinacol (3-Nitrophenylmethanesulfonylimino)methaneboronate (6).
Yellowish viscous oil (84% yield). 1H NMR (400 MHz,
CDCl3): δ 1.29 (12H, s, pinacol protons), 2.80 (2H, d, J = 4.4, BCH2),
4.39 (2H, s, PhCH2), 4.44 (1H, t, J = 4.4, SO2NH), 7.59
(1H, J = 8.0, H3, H5), 7.80 (1H, d, J = 8.0, H2, H6), 8.23 (1H, d, J = 8.0, Ha), 8.29 (2H, s, H2). 13C NMR
(100 MHz, CDCl3): δ 24.8, 28.3 (CB), 56.6, 84.9, 123.5, 125.5, 129.8,
131.8, 136.9, 148.3. EI-MS, m/z: 341 (M+1, 2%), 327 (2), 240 (8), 165 (8), 156
(24), 137 (11), 136 (100), 129 (8), 104 (54), 103 (14), 90 (32), 89
(32), 83 (36), 74 (34), 59 (38), 43 (15), 41 (19). Anal. Calcld for
C15H13B2NO5S: C, 47.21; H, 5.94; N, 7.86; S, 9.00. Found: C, 47.35; H, 6.05; N, 7.73; S, 8.74.

Pinacol (3-Aminophenylmethanesulfonylimino)methaneboronate
Hydrochloride (7). Palladium 10 wt % on activated carbon (51 mg,
40% w/w) was added to a solution of 6 (128 mg, 0.36 mmol)
in ethyl acetate (4 mL) and allowed to react under hydrogen atmosphere for 16 h. The catalyst was filtered off, and the solvent was evaporated in vacuo. The crude residue was
solved in HCl (0.1 M solution in MeOH, 3.6 mL, 0.36 mmol).
After 45 min the mixture was concentrated under reduced pressure
and the residue repeatedly washed with diethyl ether, affording
7 as a yellowish solid (118 mg, 90% yield). Mp 193–196 °C.

1H NMR (400 MHz, DMSO): δ 0.123 (12H, s, pinacol protons),
2.59 (2H, d, J = 3.82, BCh2), 4.37 (2H, s, PhCH2), 6.76 (1H, br,
SO2NH), 7.30–7.36 (3H, m, H1Ar), 7.52–7.55 (2H, m, H2Ar),
7.58 (1H, d, J = 8.5, H3, H5), 7.60 (2H, d, J = 8.5, H2, H6), 8.00
(2H, s, COOH), 8.17 (2H, t, J = 5.5, BCh2), 8.48, 106, 100, 95
(100), 74 (17), 65 (11), 59 (16). Anal. Calcld for C14H13B2ClNO4S:
δ 25.0, 27.5 (CB), 55.9, 84.2, 122.8, 125.3, 130.1, 130.5,
132.7, 133.0. EI-MS, m/z: 326 (M+1, 5%), 156 (8), 107 (64), 106
(100), 104 (15), 77 (11), 59 (11), 41 (8). Anal. Calcld for
C14H13B2ClNO4S: C, 46.63; H, 6.67; N, 7.72; S, 8.84. Found: C, 46.41; H,
6.50; N, 7.55; S, 8.68.

Pinacol (4-[Methoxycarbonyl]phenylmethanesulfonylimino)-
methaneboronate (8). According to the general procedure described
above, the compound was recovered as a white solid
(84% yield). Mp 104–107 °C. 1H NMR (400 MHz, CDCl3): δ 1.27 (12H,
s, pinacol protons), 2.73 (2H, d, J = 4.1, BCh2), 3.93
(3H, s, COOCH3), 4.33 (2H, s, PhCH2), 4.37 (1H, br, SO2NH),
(100 MHz, CDCl3): δ 24.8, 28.4 (CB), 52.2, 57.2, 64.8,
129.9, 130.3, 130.7, 134.6, 166.6. EI-MS, m/z: 354 (M+1, 2%),
338 (2), 311(2), 269 (3), 156 (25), 150 (24), 149 (100),
121 (14), 118 (3), 104 (19), 90 (19), 74 (26), 59 (13), 41 (9). Anal.
Calcld for C14H14NO4S: C, 52.04; H, 6.55; N, 3.79; S, 8.68.
Found: C, 51.88; H, 6.74; N, 3.56; S, 8.51.

[4-Carboxyphenyl]methanesulfonylimino)methaneboronic Acid
(9). A mixture of 8 (67 mg, 0.18 mmol) and HCl 3 N degassed
(3 mL) was allowed to react at reflux for 1 h 30 min. After
cooling, the reaction mixture was diluted with water (15 mL) and
washed twice with diethyl ether (2 x 20 mL). The aqueous phase
was concentrated in vacuo, affording 9 as a yellow solid
(42 mg, 86% yield). Mp 118–122 °C (dec). 1H NMR (400 MHz,
DMSO): δ 2.5 (2H, d, J = 4.9, BCh2), 4.42 (2H, s, PhCH2), 6.22
The combined organic phases were washed with brine, dried, and finally, (over 15 min, during which the solution turned cherry red. After chromatography (light petroleum to light petroleum/ethyl ether (2:40 mL). The organic phases were dried, filtered, and concentrated, affording 11 as white solid free from bromine (80% yield). H NMR (200 MHz, MeOD): δ = 1.46 (3H, t, J = 6.9, OCH2CH3), 1.94 (2H, q, J = 6.9, OCH2CH3), 2.74 (1H, dd, J = 9.0, 2.0, H395<x>); 7.29 (1H, s, H492); 7.70–7.91 (3H, m, H arom); 8.32 (1H, s, H arom). 13C NMR (50 MHz, MeOD): δ = 13.6, 20.7 (CB), 63.3, 106.2, 120.1, 123.2, 127.3, 127.4, 127.9, 130.1, 133.8, 136.4, 159.0. EI-MS and elemental analysis were not obtained, but exposure of 11 to an equimolar amount of pinacol in anhydrous THF afforded the corresponding pinacol boronate in quantitative yield and satisfactory elemental analysis results. Anal. Caled for C19H27BNO5S: C, 50.72; H, 6.24; N, 3.94; S, 9.03. Found: C, 50.98; H, 6.41; N, 3.80; S, 8.77.

(2-Ethynaphthalene-1-sulfonylamino)methaneboronic Acid (11). According to the general procedure described above, reaction of 2 with 2-ethynaphthalene-1-sulfonyl chloride, obtained from 2-ethynaphthalene, afforded 11 as white solid free from boronic acid (80% yield). H NMR (200 MHz, MeOD): δ = 1.46 (3H, t, J = 6.9, OCH2CH3), 1.94 (2H, q, J = 6.9, OCH2CH3), 2.74 (1H, dd, J = 9.0, 2.0, H arom); 7.29 (1H, s, H arom); 7.70–7.91 (3H, m, H arom); 8.32 (1H, s, H arom). 13C NMR (50 MHz, MeOD): δ = 13.6, 20.7 (CB), 63.3, 106.2, 120.1, 123.2, 127.3, 127.4, 127.9, 130.1, 133.8, 136.4, 159.0. EI-MS and elemental analysis were not obtained, but exposure of 11 to an equimolar amount of pinacol in anhydrous THF afforded the corresponding pinacol boronate in quantitative yield and satisfactory elemental analysis results. Anal. Caled for C19H27BNO5S: C, 50.72; H, 6.24; N, 3.94; S, 9.03. Found: C, 50.98; H, 6.41; N, 3.80; S, 8.77.

(+-)Pinanediol 3-[3-(tert-Butyloxy)phenyl]benzenecarboxylate (12b). A solution of tert-butyl 3-bromobenzonate (1.70 g, 6.61 mmol), obtained from 3-bromobenzoic acid, and freshly distilled triisopropyl borate (1.53 mL, 6.61 mmol) in THF (17 mL) was cooled to −100 °C and treated with 1.0 M solution of diborane (0.50 mL). The white inorganic precipitate was recovered from the reaction mixture by filtration, and the filtrate was concentrated in vacuo to give a residue which was subjected to column chromatography (light petroleum/ethyl ether/triethylamine 98:2:5) recovering (38), 119 (32), 118 (26), 117 (28), 109 (24), 93 (44), 91 (74), 83 (75), 82 (32), 81 (34), 79 (24), 77 (26), 66 (67), 65 (27), 55 (47), 53 (29). Anal. Caled for C17H22O3: C, 75.57; H, 8.58. Found: C, 75.39; H, 8.44.

(+-)Pinanediol 3-[3-(tert-Butyloxy)phenyl]benzenecarboxylate (13b). Following the procedure described for the synthesis of 13a, compound 13b was recovered from 12b as a yellow oil (89% yield): [α]D + 8.7 (c = 1.4, CHCl3). H NMR (400 MHz, CDCl3): δ = 0.87 (3H, s, pinanyl CH3), 1.11 (1H, d, J = 11.0, pinanyl H9); 3.29 (3H, s, pinanyl CH3), 1.43 (3H, s, pinanyl CH3), 1.63 (9H, s, -Bu), 1.84–2.39 (5H, m, pinanyl protons), 2.42 (2H, s, BCH3), 4.32 (1H, dd, J = 8.8, 2.0, CHOB), 7.33 (1H, t, J = 7.7, H3), 7.40 (1H, d, J = 7.7, H3), 7.80 (1H, d, J = 7.7, H3), 7.81 (1H, s, H3). 13C NMR (100 MHz, CDCl3): δ = 19.3 (CB), 24.0, 26.5, 27.1, 28.2, 28.6, 35.4, 38.2, 39.5, 51.3, 78.0, 80.7, 86.0, 126.2, 128.1, 129.2, 132.0, 132.2, 139.0, 166.0. EI-MS: m/z = 370 (M+), 184, 314 (29), 279 (59), 135 (55), 57 (100). Anal. Caled for C17H22O3: C, 71.36; H, 8.44. Found: C, 71.60; H, 8.27.

(+-)Pinanediol (1R)-1-(N-Bis(trimethylsilyl)amino)-2-phenylethanol (14a). A solution of diborane (1.00 g, 3.70 mmol) in THF (8 mL) was slowly added at the same temperature. The white precipitate disappeared, and the mixture was allowed to gradually reach room temperature overnight. The resulting solution was concentrated under reduced pressure. The crude was treated with petroleum ether (50 mL), and the resulting white inorganic precipitate was filtered off and washed with abundant petroleum ether. The solvent was evaporated in vacuo afforded the pinanediol (1S)-1-chloro-2-phenylethanol, used as such for the subsequent reaction. 1H NMR (400 MHz, CDCl3): δ = 0.12 (18H, s, 2Si(CH3)3), 0.87 (3H, s, pinanyl CH3), 0.99 (1H, d, J = 10.8, pinanyl Hendo), 1.31 (3H, s, pinanyl CH3), 1.41 (3H, s, pinanyl CH3), 2.71 (1H, dd, J = 13.1, 7.6, CH3), 2.87 (1H, t, J = 7.4, BCH3), 3.05 (1H, dd, J = 13.1, 7.3, CH2CH3), 4.31 (1H, dd, J = 8.7, 2.0, CH2O), 7.17–7.31 (5H, m, H arom). 13C NMR (100 MHz, CDCl3): δ = 30.0, 24.0, 26.0, 27.1, 28.4, 35.2, 38.1, 39.5, 42.0, 45.0 (CB), 51.4, 78.2, 85.6, 125.7, 127.8, 129.7, 141.7. Anal. Caled for C24H22BNO3Si: C, 69.48; H, 9.54; N, 3.16. Found: C, 65.19; H, 9.46; N, 2.95.

(+-)Pinanediol (1R)-2-(3-(tert-Butyloxy)phenyl)-1-(N-bis(trimethylsilyl)amino)ethaneboronate (14b). Following the procedure described for the synthesis of 14a, compound 14b was recovered as a colorless oil (28% yield): [α]D + 12.6 (c = 3.9, CHCl3). H NMR (200 MHz, CDCl3): δ = 0.10 (18H, s, 2Si(CH3)3), 0.83 (3H, s, pinanyl CH3), 0.94 (1H, d, J = 11.1,
pinanyl $H_{\text{endo}}$, 1.28 (3H, s, pinanyl $CH_2$), 1.38 (3H, s, pinanyl $CH_2$), 1.61 (9H, s, $t$-Bu), 1.78–2.35 (5H, m, pinanyl protons), 2.66–2.87 (2H, m, $BCH$, $CHCCH$), 3.06–3.15 (1H, m, $CHCCH$), 4.28 (2H, $t$, J = 7.1 Hz, $CHOB$). 7.27–7.43 (2H, m, $H_\text{endo}$), 7.82–7.91 (2H, m, $H_\text{exo}$). $^{13}$C NMR (50 MHz, CDCl$_3$): δ 30.0, 23.9, 26.2, 27.0, 28.2, 28.4, 35.1, 38.1, 39.5, 41.8, 44.8 (CB), 51.4, 78.3, 80.5, 85.6, 126.9, 127.7, 130.8, 131.5, 133.8, 141.7, 166.0. Anal. Calcld for C$_{34}$H$_{50}$BNO$_4$Si$_2$: C, 64.06; H, 9.27; N, 2.58.

(1R)-1-(3-Carbonylphenyl)-1-(phenylmethane)sulfonylaminoo)ethaneboronic Acid (17). A solution of 15b (90 mg, 0.16 mmol) in dichloromethane (2 mL) was cooled to −10 °C and treated with an excess of trifluoroacetic acid (10% v/v solution in dichloromethane, 2 mL). After 10 min at −10 °C, the mixture was allowed to react at room temperature for 5 h. The solution was concentrated in vacuo and the residue crystallized from diethyl ether, affording (1R)-1-(3-carbonylphenyl)-1-(phenylmethanesulfonylaminoo)ethaneboronic acid (79 mg, 100% yield) as a cream colored solid. Mp 155–158 °C. [α]$_D$ +25.9 (c 0.6, CHCl$_3$). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.87 (3H, s, pinanyl $CH_3$), 1.06 (1H, d, J = 10.8, pinanyl $H_{\text{endo}}$), 1.31 (3H, s, pinanyl $CH_3$), 1.33 (3H, s, pinanyl $CH_3$), 1.88–2.38 (5H, m, pinanyl protons), 2.99 (1H, dd, J = 13.7, 6.0, $CHCCH$), 3.15 (1H, dd, J = 13.7, 5.6, $CHCCH$), 3.37 (1H, br, $BCH$), 4.31 (2H, s, $CH_2SO_2$), 4.40 (1H, d, J = 7.7, $CHOB$), 4.51 (1H, br, $SO_2NH$), 7.30–7.58 (7H, m, $H_{\text{Arom}}$). 7.97–8.01 (2H, m, $H_{\text{Arom}}$), 10.2 (1H, br, COOH). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 24.0, 26.3, 27.0, 28.3, 35.0, 38.2, 38.5, 39.4, 42.4 (CB), 51.1, 59.1, 78.8, 87.3, 128.5, 128.7, 127.2, 129.3, 129.4, 130.8, 131.2, 135.6, 139.0, 171.8. Anal. Calcld for C$_{35}$H$_{52}$BNO$_4$S$_2$: C, 67.28; H, 6.48; N, 2.82; S 6.45. Found: C, 62.61; H, 6.72; N, 2.64; S, 6.21.

This compound was then treated according to the procedure described for 16 to recover 17 as a yellowish solid (54 mg, 92% yield). Mp 118–122 °C (dec). [α]$_D$ +136.0 (c 0.5, MeOH). $^1$H NMR (400 MHz, MeOD): δ 2.87 (2H, d, J = 7.0, $CHCCH$), 3.05 (1H, d, J = 7.0, $BCH$), 4.24 (2H, s, $CH_2SO_2$), 7.15–7.62 (10H, m, $H_{\text{Arom}}$). $^1$C NMR (100 MHz, MeOD): δ 38.4, 42.9 (CB), 58.8, 127.5, 128.0, 128.1, 128.2, 130.1, 130.2, 130.6, 133.8, 139.1, 168.4. EI-MS and elemental analysis were not obtained, but exposure of 11 to an equimolar amount of (1R)-pinanediol in anhydrous THF afforded the corresponding pinanediol boronate in quantitative yield and satisfactory elemental analysis. Anal. Calcld for C$_{34}$H$_{52}$BNO$_4$S$_2$: C, 67.28; H, 6.48; N, 2.82; S 6.45. Found: C, 62.56; H, 6.71; N, 2.59; S, 6.28.

Enzymology. Sulfonamidemethaneboronic acids were dissolved in 100% DMSO at 100 mM. More diluted solutions were prepared as necessary. AmpC activity was determined by monitoring the change of absorbance at 405 nm due to hydrolyzed sulfonyl-penicillin, and the reaction mixture was prepared as necessary. AmpC activity was determined by monitoring the change of absorbance at 405 nm due to hydrolyzed sulfonyl-penicillin. Kinetic measurements were run at room temperature in 50 mM sodium cacodylate, pH 6.5, in the presence of 0.01% Triton-X to prevent nonspecific inhibition via compound aggregation. Kinetic measurements were run at room temperature in 50 mM sodium cacodylate, pH 6.5, in the presence of 0.01% Triton-X to prevent nonspecific inhibition via compound aggregation. Kinetic measurements were run at room temperature in 50 mM sodium cacodylate, pH 6.5, in the presence of 0.01% Triton-X to prevent nonspecific inhibition via compound aggregation.
α-Chymotrypsin assays were performed in 50 mM Tris buffer, pH 7.5, with 0.01% Triton-X. Inhibitor and 0.001 mg/mL enzyme were incubated at their final concentration for 10 min before the reaction was initiated by the addition of 200 μM N-succ-Ala-Ala-Pro-Phe p-nitroanilide and monitored at 410 nm.

Cruzain assays were performed in 100 mM sodium acetate, pH 5.5, with 0.01% Triton-X and 5 mM diithiothreitol. Cruzain activity was measured in a 96-well format fluorimetric assay, monitoring the cleavage of the substrate Z-Phe-Arg-aminoethylcoumarin (Z-FR-AMC), in a SpectraMax M5 Molecular Devices spectrofluorimeter. Inhibitor and 0.4 nM enzyme were incubated at their final concentration for 10 min before the reactions were initiated by the addition of 2.5 μM Z-FR-AMC (Km = 2 μM) and monitored for 5 min, determining activity based on initial rates.

Modeling. Compound 17 was modeled in complex with AmpC by superposing 17 on the crystal structure of compound 4 in chain A. To remove clashes of the additional m-carboxybenzyl group with the protein, we minimized 17 in the AmpC active site by an all-atom energy minimization using PLOP. The oxygen atom of Ser64 was allowed to move while the rest of the enzyme was kept rigid during the minimization. This resulted in a model with the phenylmethanesulfonylamide conformation resembling the conformation observed for compounds 4 and 9, in which the sulfonylamide oxygen atoms pointed in the direction of the m-carboxybenzyl group.

Crystal Growth and Structure Determination. Crystals of AmpC in complex with compound 4, 9, and 17 were grown by vapor diffusion in hanging drop vapor diffusion experiments equilibrated over 1.7 M potassium phosphate buffer (pH 8.7–8.9) using microseeding technique. The initial concentration of the protein was 3.9 mg/mL. The concentrations of the compounds were 625 μM, and the DMSO concentration was 1.25%. Crystals appeared 4–7 days after equilibration at 20 °C. Before data collection, crystals were immersed in a cryoprotectant solution of 25% sucrose, 1.7 M potassium phosphate, pH 8.7, for about 30 s and were flash-cooled in liquid nitrogen.

Diffraction data were measured at beamline 8.3.1 of the Advanced Light Source (ALS, Lawrence Berkeley Lab, CA). Reflections were indexed, integrated, and scaled using the XDS package. The space group was C2 with two molecules in the asymmetric unit. The initial phasing model was an apo AmpC model (PDB entry 1KE4), with water molecules and ions removed. The model was positioned initially by rigid body refinement and further refined with L-BFGS minimization, simulated annealing, individual B-factor refinement, and water picking using PHENIX. Coot was used for model building, and the PRODRG server was used to generate lipid restraints. Ligand restraints were modified manually because the PRODRG server does not support boron atoms.

Microbiology. Susceptibility testing was performed and interpreted following the guidelines of CLSI. The compounds were dissolved in 50% DMSO, and dilutions were performed using Muller–Hinton medium. An adequate final concentration was obtained to determine the inhibitory minimum concentrations (MICs). The concentration of DMSO was maintained below 5%. The inhibitors were tested for synergy with the third-generation β-lactams ceftazidime and cefotaxime against several clinical bacteria. The ratio of β-lactams to inhibitors was 1:1. Each value reported reflects the average of three independent experiments. The bacteria exhibited a high level of resistance to β-lactams because of the expression of class C or class A β-lactamases. One Escherichia coli strain showed an AmpC-overproduced phenotype, and the second one produced the plasmid-mediated class A β-lactamase CTX-M-14. Citrobacter freundii, Enterobacter cloacae, and Pseudomonas aeruginosa strains showed AmpC-deregulated phenotype. Klebsiella pneumoniae isolate produced the inducible plasmid-mediated class C enzyme DHA-1.

For disk diffusion plate assays, bacterial strains were diluted in sterile water to a turbidity equivalent to 0.5 McFarland turbidity standards. After a subsequent 10-fold dilution, the bacterial suspensions were inoculated on Mueller–Hinton agar. The plates were dried for 10 min before applying the disks containing cefotaxime or ceftazidime antibiotics (30 μg) or the inhibitor (30 μg) or both. After overnight incubation at 37 °C, the zones of bacterial-growth inhibition were measured.

For the β-lactamase induction experiments, plates of Mueller–Hinton agar were inoculated with two clinical strains (E. cloacae and K. pneumoniae) in which production of AmpC-type enzyme is inducible by β-lactam antibiotics. Inhibitors were added to blank disks, and the final content of inhibitor per disk was 64 μg of compounds and 32 μg cefoxitin. Disks of ceftazidime contained 30 μg of antibiotic.

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