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Inhibition of *Acinetobacter*-Derived Cephalosporinase: Exploring the Carboxylate Recognition Site Using Novel β -Lactamase Inhibitors

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Abstract

Boronic acids are attracting a lot of attention as β -lactamase inhibitors, and in particular, compound **S02030** ($K_i = 44$ nM) proved to be a good lead compound against ADC-7 (*Acinetobacter*-derived cephalosporinase), one of the most significant resistance determinants in *A. baumannii*. The atomic structure of the ADC-7/**S02030** complex highlighted the importance of critical structural determinants for recognition of the boronic acids. Herein, to elucidate the role in recognition of the R2-carboxylate, which mimics the C₃/C₄ found in β -lactams, we designed, synthesized, and characterized six derivatives of **S02030** (**3a**). Out of the six compounds, the best inhibitors proved to be those with an explicit negative charge (compounds **3a–c**, **3h**, and **3j**, $K_i = 44–115$ nM), which is in contrast to the derivatives where the negative charge is omitted, such as the amide derivative **3d** ($K_i = 224$ nM) and the hydroxyamide derivative **3e** ($K_i = 155$ nM). To

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Author Contributions

E.C., C.R., F.F., and F.P. synthesized all of the BATSI compounds. M.A.T. and R.A.B. performed microbiological assays and kinetics. R.A.P., B.J.W., A.A.B., H.C.S., and K.A.S. performed kinetics and determined all of the crystal structures. All authors have contributed to the manuscript and have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

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¹HNMR and ¹³CNMR spectra of compounds **3b**, **3d**, **3e**, **3h**, and **3j** (PDF)

develop a structural characterization of inhibitor binding in the active site, the X-ray crystal structures of ADC-7 in a complex with compounds **3c**, **SM23**, and **EC04** were determined. All three compounds share the same structural features as in **S02030** but only differ in the carboxy-R2 side chain, thereby providing the opportunity of exploring the distinct binding mode of the negatively charged R2 side chain. This cephalosporinase demonstrates a high degree of versatility in recognition, employing different residues to directly interact with the carboxylate, thus suggesting the existence of a “carboxylate binding region” rather than a binding site in ADC enzymes. Furthermore, this class of compounds was tested against resistant clinical strains of *A. baumannii* and are effective at inhibiting bacterial growth in conjunction with a β -lactam antibiotic.

Graphical Abstract

Keywords

β -lactamase; boronic acid; structure activity relationship; click chemistry; carboxylate; structure activity relationship study; enzyme plasticity; *Acinetobacter*

Bacterial production of β -lactamases represents the most clinically challenging mechanism of resistance to β -lactam antibiotics in Gram-negative bacteria. Among these multidrug resistant bacteria, *Acinetobacter baumannii* is one of the greatest clinical threats due to its acquisition of numerous genetic determinants conferring resistance to all antibiotics.¹ *A. baumannii* harbors multiple antibiotic resistance determinants, such as class C, D, and B β -lactamases, multidrug resistance efflux pumps, and a modified outer membrane that decreases permeability to antimicrobials. Furthermore, *A. baumannii* possesses the ability to acquire new resistance determinants by genetic exchange, natural transformation, and outer membrane vesicles. Preserving and restoring the efficacy of β -lactam antibiotics, which are still the major class of drugs for the treatment of bacterial infections, is of critical importance as a strategy to overcome and circumvent resistance mechanisms against this challenging pathogen.

The *Acinetobacter*-derived cephalosporinases (ADCs) are chromosomally encoded class C β -lactamases found in *A. baumannii* and other *Acinetobacter* spp. and are responsible for resistance to penicillins, cephalosporins, and β -lactam/ β -lactamase inhibitor combinations. Since clavulanic acid, sulbactam, and tazobactam, the commercially available β -lactamase inhibitors bearing a β -lactam ring as their core structure, are not effective against ADC,² the

urgency of discovering new active inhibitors is pressing. The bridged diazabicyclo [3.2.1]-octanone avibactam entered the market in combination with ceftazidime and demonstrated potent inhibitory activity against most *Enterobacteriaceae* that produce class A and class C β -lactamases, with some inhibitory capabilities against organisms producing class D enzymes. Notably, this combination is not targeted for *A. baumannii*,³ and other solutions are urgently needed to treat infections caused by *A. baumannii*.

A class of non- β -lactam β -lactamase inhibitors that is recently attracting considerable attention for their activity and pharmacological profile are boronic acids transition state inhibitors (BATSIs).^{4–7} Mechanistically, the electrophilic boron reacts with the nucleophilic catalytic serine leading to the formation of a tetrahedral adduct that mimics the high-energy intermediate of the deacylation reaction with β -lactams (Scheme 1). Previous studies showed that the insertion of different chemical groups on the carbon bearing the boronic moiety results in specific interactions with different β -lactamase enzymes, allowing for a gain in inhibitory function and selectivity tuned for a specific class of β -lactamases. Boronic acids have been widely used for inhibition of class C β -lactamases, such as AmpC, and recently, they have been studied primarily for inhibition of the cephalosporinase of *Acinetobacter* spp. (ADC).⁸ Of the reported BATSIs, **S02030** (compound **3a**) is comprised with a set of features that makes it a promising lead compound against ADC, both for its structure and activity ($K_i = 44$ nM), as well as its efficient synthetic pathway.⁷

The chemical structure of **S02030** was specifically designed to mimic the structure of the natural substrate cephalothin in the deacylation high energy intermediate with the β -lactamase (Figure 1). The structural features important for enzyme recognition and inhibition are depicted in Figure 1: an R1 amide side chain bearing a 2-thienylmethyl group, an oxyanion group represented by one boronic hydroxyl, and a R2 cyclic side chain bearing a carboxylate meant to mimic the C3/C4 carboxylate found in β -lactams. All these groups are spatially oriented as in the natural substrate, in order to gain the proper interactions with the enzyme.

The crystal structure of ADC-7 in complex with **S02030** demonstrated that the inhibitor is interacting with highly conserved residues in the catalytic pocket.⁷ In particular, the R1 amide side chain is positioned in a region analogous to where β -lactam substrates bind in other class C β -lactamases, interacting with highly conserved Asn152 and Gln120. Also, one hydroxyl group of the boronic acid is positioned in the oxy-anion hole and made hydrogen bonds with the main chain nitrogens of Ser64 and Ser315 as well as the main chain carbonyl oxygen of Ser315. Interestingly, the position and role of the R2 side chain, and specifically the carboxylate, is not similar to other class C structures. In general, the C3/C4 carboxylate recognition site in class C enzymes is reported to be comprised of Asn346 and Arg349 (AmpC numbering), but while these residues were conserved in ADC-7 (Asn343 and Arg346), the carboxylate of **S02030** interacted with Arg340, a residue that distinguishes ADC-7 from other AmpCs. In class A β -lactamases, the carboxylate binding pocket and the role of the carboxylate itself is well established;^{9–11} in class C β -lactamases, we confirmed that neither the residues involved in recognition nor the role of the carboxylate are sufficiently known.

To elucidate the residues that take part in carboxylate recognition in ADC-7 and its importance for β -lactamase inhibition, we report the synthesis and characterization of six **S02030** derivatives (**3b–e**, **3h**, **3j**) specifically designed to insert unique modifications localized on the R2 triazole substituent. Specific localized modifications were easily introduced using the flexible and efficient synthesis previously described which relies on “click chemistry”, specifically, the well-known copper-catalyzed azide–alkyne cycloaddition (CuAAC).¹² According to this procedure, the substituted triazole moiety is formed by reaction of the chiral boronic scaffold **1**, bearing both the R1 thienylacetamido side chain and an azido group, with the proper mono-substituted alkyne (Scheme 2). These alkynes are specifically chosen to introduce rational variation of the carboxylate.

All synthesized BATSIs were tested against ADC-7 and confirmed to be potent inhibitors. Since the best inhibition values were obtained for compounds with an explicit negative charge, two additional boronic acids (**SM23**¹³ and **EC014**¹⁴) were tested and confirmed to be among the best inhibitors. To elucidate the specific residues that interact with the carboxylate, we determined the crystal structures of ADC-7 in complex with the boronic acids **3c**, **SM23**, and **EC04**. Comparison of these structures reveals critical differences in binding of the carboxylate. The variability in the active site interactions with the R2-carboxylate suggests that class C β -lactamases display a high degree of plasticity and flexibility for recognition, pointing to the existence of a carboxylate “binding region” rather than a “binding site” in ADC-7. The synergistic effect of these inhibitors paired with β -lactams to reverse antibiotic resistance in whole cell assays against *Escherichia coli* and *Acinetobacter baumannii* strains expressing ADC-7 was also explored.

RESULTS

Design

In previous studies, we demonstrated that boronic acids of general structure **3** were excellent inhibitors against class C β -lactamases,¹² and in particular, **3a** was a 44 nM inhibitor of ADC-7 cephalosporinase.⁷ In this study, our goal was to elucidate the role of the carboxylate in enzyme–inhibitor recognition. To achieve this end, we introduced specific modifications near this R2-carboxylate group. Specifically, the carboxylate of **3a** is elongated with addition of a methylene bridge (**3b**), inserted on the *meta*-position of a phenyl ring (**3c**), modified to an amide group (**3d**) and to an hydroxamate (**3e**), and finally replaced by the bioisostere tetrazole, either directly bonded to the triazole (**3h**) or as a *meta*-substituent on the phenyl ring (**3j**). Compounds **3b**, **3c**, **3h**, and **3j** are explicitly designed to introduce distance modifications between the negative charge of the carboxylate and the electrophilic boron atom, whereas the amide **3d** and the hydroxamate **3e** change the protonation profile of the substituent while still maintaining heteroatoms able to form hydrogen bonds with the enzyme residues.

Synthesis of α -2-Thienylacetamido- β -Triazolylethane-boronic Acids

The synthesis of compounds **3b–e**, **3h**, and **3j** is depicted in Scheme 3.

(+)-Pinanediol (1*R*)-2-azido-1-[(2-thienylacetyl)amino]-ethaneboronate **1** was obtained as previously described.¹² Compound **1** was then reacted with six terminal alkynes in the copper catalyzed azide–alkyne cycloaddition (CuAAC). Among the six chosen alkynes, 3-butyric acid ($R = -CH_2COOH$, for the synthesis of compound **2b**) and 3-carboxyphenylacetylene ($R = -m-C_6H_5COOH$, for compound **2c**) are commercially available, whereas the other four alkynes, propiolamide¹⁵ ($R = -CONH_2$, for compound **2d**), *N*-hydroxypropiolamide ($R = -CONHOH$, for compound **2e**),¹⁶ propiolonitrile (**2f**),¹⁷ and 3-ethynylbenzonitrile (**2g**)¹⁸ were prepared following established procedures.

As already described for compound **3a**, formation of the triazole was conducted in very mild conditions, in water/*tert*-butyl alcohol as solvent, generating the Cu(I) catalyst *in situ* from $CuSO_4$ in the presence of an excess of ascorbate as reducing agent (5% catalyst, 20% ascorbate) and at 60 °C for approximately 2 h. The expected triazole **2b–g** were recovered in good-excellent yields (72–99%) by extraction with ethyl acetate and crystallization of the crude (Scheme 3). The formation of the triazole was confirmed by the presence in the ¹H NMR spectra of a singlet downfield at 8.0–8.7 ppm which correlates (heteronuclear single quantum coherence spectroscopy (HSQC) experiments) with a signal at 118–131 ppm of the ¹³C NMR spectra, as expected for a triazolic CH. Moreover, the 1,4 substitution was confirmed by the long-range correlation (heteronuclear multiple bond correlation (HMBC) experiment). Triazoles **2h** and **2j** were obtained by treatment of the cyano precursors with tetrabutylammonium fluoride trihydrate in trimethylsililazide as solvent. Final deprotection of (+)-pinanediol esters was accomplished by transesterification with phenylboronic acid (isobutylboronic acid in the case of **3h** and **3j**) in a biphasic system of acetonitrile/*n*-hexane, to afford the final BATSIs **3b–e**, **3h**, and **3j** in 81–96% yield.

Inhibition Kinetics

The binding affinities for each of the BATSIs with ADC-7 were determined using competition kinetics. Utilizing nitrocefin, NCF, as a colorimetric substrate of ADC-7, boronic acids **3a–e**, **3h**, and **3j** were tested as inhibitors of ADC-7 β -lactamase. Additionally, three reference BATSIs were tested: compound **s08127**,¹² which lacks the substituent on the triazole ring, compound **EC04**,¹⁴ which replaces the triazole with a phenyl group, and compound **SM23**¹³ where the phenyl aromatic moiety is directly connected to the boron bearing carbon atom. The K_i values were calculated for all ten BATSIs and reported in Table 1.

All BATSIs were shown to be very active ADC-7 inhibitors with K_i values in the nanomolar range. We observed that affinity is diminished when the inhibitor lacks the carboxylate group, such as for **s08127** ($K_i = 658$ nM), and is less significant when the negative charge is not present, as for the amide and hydroxyamate derivatives **3d** and **3e** ($K_i = 224$ and 155 nM, respectively). Compound **3a** proved to be a potent inhibitor of ADC-7, with a K_i of 44 nM. The boronic acid **3b**, which exhibits an additional methylene linker between the carboxylate and the triazole, demonstrates less activity by 3-fold ($K_i = 115$ nM), whereas **3c** ($K_i = 46$ nM), bearing a phenyl linker, exhibits an equivalent activity to **3a**. Inhibition is diminished when the carboxylate negative charge is replaced by an amide derivative **3d** ($K_i = 224$ nM) or a hydroxyamide group **3e** ($K_i = 155$ nM). We synthesized two additional boronates where

the negatively charged carboxylate is substituted with its bioisostere tetrazole, a group with a similar pK_a value, but a wider distribution of the negative charge. Indeed, the presence of a net negative charge as for compound **3h** ($K_i = 76$ nM) and for the phenyltetrazole **3j** ($K_i = 99$ nM) improves activity with respect to inhibitors lacking this negative charge. The binding affinity of all the other BATSIs spans from 22 nM (for **SM23**) to 115 nM (**3b**), thus confirming the importance of a negative charge in the R2 side chain for recognition and simultaneously suggesting the presence of a “binding region” for this carboxylate rather than of a “binding site” (Table 1).

X-ray Crystal Structures of ADC-7 in Complex with **SM23**, **EC04**, and **3c** BATSIs

To further define the role of the carboxylate group for ADC-7 inhibition, the complexes of ADC-7 with **SM23**, **EC04**, and **3c** were determined by X-ray crystallography to resolutions ranging from 1.88 to 1.95 Å (Table 2, Figure 1). Each of the three inhibitors bears an unmasked carboxylate at varying distances from the electrophilic boron. Complexes were obtained by soaking the inhibitors into pre-formed ADC-7 crystals. The complexes crystallized in the same space group ($P2_1$) with similar cell parameters. Inspection of the initial $F_o - F_c$ maps countered at 3σ showed unambiguous electron density for the inhibitors bound in the active sites of each structure. The density was contiguous with the $O\gamma$ atom of Ser64, suggesting that the inhibitors were covalently attached to the catalytic serine residue, Ser64. Refinement of the inhibitors modeled into the density revealed a tetrahedral geometry around the boron atom as expected. For compounds **EC04** and **3c**, the position of the carboxylate was unambiguous, but electron density for the **SM23** carboxylate suggests two distinct conformations for the negative charge.

The structures of the three BATSIs bound in the active site reconfirm the features essential for enzyme–inhibitor recognition of both the R1 and R2 groups (Figure 2), initially described in the ADC-7/**S02030** complex. Briefly, the inhibitor R1 side chain is bound in the highly conserved amide binding site, where the amide oxygen hydrogen bonds with the side chain nitrogen atoms of Gln120 and Asn152 and the main chain amide nitrogen and oxygen atoms of Ser315, reminiscent of β -lactamase recognition of the R1 amide group of β -lactam substrates. The inhibitor O1 hydroxyl groups are positioned in the oxyanion hole, hydrogen bonding with the main chain nitrogens of Ser315 and Ser64 and also with the main chain oxygen of Ser315. The inhibitor O2 hydroxyl groups make hydrogen bonds with the side chain hydroxyl of Tyr150, as well as water molecule observed in most class C β -lactamase complexes. The distal thiophene ring common to the inhibitors is positioned near the lip of the active site in all three structures, extending over Tyr222 (Tyr221 in AmpC numbering). The thiophene ring of **EC04** and **SM23** is observed to adopt two distinct conformations that exhibit an approximate 180° rotation of the thiophene ring; in **3c**, only one conformer is observed.

The R2 side chains display more variability in their binding modes than the R1 groups. The carboxylate-bearing R2 side chain is structurally different in the three BATSIs, and the negatively charged group is observed to bind in two regions of the ADC-7 active site. The chemical structures of **SM23** and **EC04** are relatively similar, differing from each other by a single carbon spacer between the boron-bearing carbon atom and the aromatic ring of **EC04**.

The carboxylate groups are bound in essentially the same region, a site comprised of residues Ser315, Arg340, and Asn343. In the **EC04** complex, the inhibitor carboxylate hydrogen bonds with the amide nitrogen of Asn343 (3.2 Å) and interacts with Arg340 via a water-mediated bridge (Wat412). This water molecule is not observed in the ADC-7/**SM23** complex, where the carboxylate of **SM23** occupies that space and adopts two conformations. In the first conformation, the carboxylate hydrogen bonds directly to Arg340 (2.6 Å) and Asn343N82 (3.2 Å) and, in the second, with the side chain hydroxyl of Ser315 (3.0 Å) and a water molecule (Wat227; 2.9 Å). Compound **3c** varies more from the chemical structures of **SM23** and **EC04** in that **3c** is more extended due to the addition of a triazole linker between the boron and the benzoic acid functionality. This linker allows the R2 group of **3c** to extend over the R1 group and places the carboxylate group toward an area near the edge of the active site. In this location, the carboxylate is observed in a hydrogen bond with the side chain of Ser317 and near the bulk solvent. Additionally, in this extended conformation, the aryl ring of **3c** is positioned near Arg340 (C ζ -centroid of aryl ring = 4.0 Å), making a potential cation– π interaction with this conserved residue in the ADC enzymes.

Antimicrobial Activity

Disk susceptibility assays (DSAs) were performed using an *E. coli* carrying the phagemid pBC SK expressing *bla*_{ADC-7} construct and in an *A. baumannii* clinical strain. In both cases, significant inhibition of ADC-7 β -lactamase by the BATSI is observed at a concentration of 10 μ g/mL when partnered with the β -lactam ceftazidime (CAZ) for the *E. coli* strain and sulbactam (SUL) for the *A. baumannii* isolate tested (Table 3). The DSA zone disk increased from 12 mm for CAZ alone to up to 26 mm for the best inhibitor **3a**. The results were very encouraging as the boronate compounds show cell penetration against clinical isolates of *A. baumannii* when paired with sulbactam with the best ones, **3a** and **3c** increasing the disk size from 6 mm to 16 and 15 mm, respectively. The broth minimum inhibitory concentrations (MICs) results show that the addition of the BAI decreases the CAZ MIC from 32 to 1–2 μ g/mL for **3a**, **SM23**, **3b**, **3c**, **3e**, **3h**, and **EC04**. The results from DSA and broth MIC reveal that most of the compounds perform similarly under the agar or liquid condition. The slight difference is for s08127, which performs relatively better in liquid than in agar media, which can be attributed to lower diffusion properties in agar. The highest MIC (8 μ g/mL) for compound **3j** (despite a very good affinity K_i = 99 nM) can be due to cell penetration.

DISCUSSION AND CONCLUSIONS

Although boronic acids are a class of compounds known since the 1980s to act as β -lactamase inhibitors,^{19,20} a significant amount of effort is presently focused on the discovery of potent and selective boronic inhibitors to be administered together with a β -lactam. Currently, a boronic acid, RPX7009 (vaborbactam), is commercially available in combination with meropenem to treat carbapenem resistant *Enterobacteriaceae* infections.²¹ Unfortunately, this combination displays only a little enhancement of meropenem activity when tested against carbapenem-resistant *A. baumannii* or *P. aeruginosa*.²²

Thus far, several different classes of boronic acid inhibitors have been discovered. These compounds display different profiles of activity, in terms of both microbiological and

enzymatic potency. In this study, we report our attempt to identify specific molecular determinants for the recognition of BATSIs by a representative class C β -lactamase expressed in *A. baumannii*, ADC-7. We conducted a structure activity relationship (SAR) study on the BATSI's R2-carboxylate which is designed to mimic the C₃/C₄ carboxylate found in β -lactams, starting from the lead compound **S02030** (compound **3a**). The high degree of flexibility of the synthesis developed for this lead compound that bears a simple carboxylate and the triazole substituent allows easy access of a focused small library of compounds bearing different substituents that aim to (1) distance the carboxylate from the electrophilic center, (2) substitute the carboxylate with the bioisostere tetrazole, or (3) replace the negative charge with an amide or a hydroxamide.

The data reported herein suggest that ADC-7 β -lactamase recognizes a negative charge in this region, probably employing different residues in recognition. Indeed, compound **s08127**, which lacks the substituent on the triazole ring, shows a significantly less potent K_i of 658 nM, whereas the other two BATSI, bearing a *m*-carboxyphenyl (**SM23**, K_i = 22 nM) or a *m*-carboxybenzyl (**EC04**, K_i = 56 nM) side chain, restore a comparable activity to the β -triazole derivatives.

To better understand the role of the carboxylate in this class of inhibitors, we determined crystallographic complexes of ADC-7 in a complex with three BATSI that all have a carboxylate in the R2 side chain: **SM23**, **EC04**, and **3c**. The carboxylate is positioned on a phenyl ring in both **SM23** and **EC04**, with the only difference being that **EC04** contains an additional methylene bridge between the aromatic moiety and the α -carbon. Compound **3c** is structurally different, since it keeps the additional methylene linker but replaces the phenyl with a triazole that bears a *meta*-carboxyphenyl substituent at position 4. The presence of two aromatic rings between the boronic group and the carboxylate not only alters the distance of the negative charge to the electrophilic center but also adds hydrophobic character to the inhibitor.

A superposition of the ADC-7 active site in complex with the three R2-containing BATSI highlights that the R1 side chain of all three structures maintain a similar conformation and therefore the same interactions with active site residues. However, the R2-carboxylate is located in distinct binding regions (Figure 3).

In compounds **SM23** and **EC04**, the carboxylate is pointing toward a binding region previously identified in the crystal structure of ADC-7 in complex with the lead compound **S02030**. This region is positively charged due to the presence of Arg340, which is interacting either directly with the carboxylate (**SM23**) or through a water molecule (**EC04**) making it an attractive binding site for a negatively charged carboxylate group in ADC-7 and possibly other ADCs that contain this conserved residue. However, Arg340 is not ubiquitous among all class C β -lactamases. For example, the crystal structure of *E. coli* AmpC in complex with **SM23**¹³ shows a direct hydrogen bond between the inhibitor carboxylate and Asn289; a residue that is not conserved in related class C lactamases, such as *E. cloacae* P99, where this residue is a serine. Interestingly in ADC-7, this interaction is not present due to an Asp at the analogous position, as this negatively charged residue is flipped away from the inhibitor R2-carboxylate group. The presence of Arg340 seems crucial for inhibitor

recognition in ADC-7: compound **3c** has an extended R2 side chain due to the presence of two aromatic rings; therefore, its carboxylate is placed in a different region of the enzyme, interacting with the side chain of Ser317, whereas Arg340 is in the position for a cation- π interaction with the inhibitor aryl ring. Together, these two interactions seem to contribute to reach the same level of inhibition as for compounds **SM23** and **EC04** (Figure 2 and Table 1). Cation- π interactions have been shown to contribute to protein-protein and protein-ligand interactions across a broad range of the biological realm,²³ and arginine residues have been proposed to play a role in the structural stability of numerous β -lactamases.²⁴ The Arg340 residue therefore seems to attract a negative charge in this region but also demonstrates a high degree of versatility in recognition.

On the other hand, data previously obtained on compounds **SM23** and **EC04** against *E. coli* AmpC (K_i values of 1 nM)¹³ and *Pseudomonas* PDC-3 (K_i values of 4 and 220 nM, respectively),¹⁴ two class C enzymes lacking Arg340, point to the high plasticity of this class of enzymes. This plasticity allows the class C β -lactamases to take advantage of different functional groups that are not conserved with respect to position in primary sequence but which can mediate the same role in recognition of inhibitors and possibly β -lactam substrates.

Microbiological data suggest the importance of the carboxylate, not only for recognition but also for cell activity. Our data suggest that cell penetration is enhanced by compounds possessing a carboxylate functional group, followed by ones with hydroxamide functionality. These data also show that cell penetration correlates well with affinity. The only compound with good binding affinity but poor microbiological activity is compound **3j**. For this compound, the R2 chain comprises three aromatic rings with the triazole and the phenyl ring that are likely to conserve the binding interaction observed with **3c** (cation- π) with Arg340 and the third tetrazole ring placing the negative charge toward an area near the distal site (Ser317-Asn213) and the bulk solvent. However, the dispersion of the negative charge on an aromatic moiety is not beneficial for the cell penetration.

In conclusion, this work describes a SAR study on the role of the carboxylate R2 group of a previously identified β -lactamase BATSI, compound **S02030 (3a)**, which proved to be an excellent inhibitor of ADC-7. The synthetic approach to **3a** and its derivatives, which relies on the robust CuAAC click reaction, gives easy access to specifically designed modifications of the carboxylate position. This study demonstrates that insertion of a negative charge in this position improves binding affinity and cell penetration; however, due to the high degree of plasticity of ADC, a wider choice of R2 negatively charged groups could be explored in future inhibitor design.

METHODS

Protein Preparation and Purification

A version of ADC-7 beginning at amino acid D24 and ending at K383 was cloned into a pET28a(+) plasmid (no His-tag), expressed in the competent *E. coli* strain, BL21 (DE3), and affinity purified by a *m*-aminophenyl boronic acid column as previously described.⁷ Using the A_{280} with an extinction coefficient of 46 300 M⁻¹, the concentration of ADC-7 was

determined. The extinction coefficient was calculated for the expressed residues in the ADC-7 construct by the ProtParam tool on the ExPASy bioinformatics portal.

ADC-7 X-ray Structure Determination

The ADC-7 crystals were grown by hanging drop vapor diffusion at room temperature. The crystallization conditions consisted of 3 mg/mL ADC-7 in 25% w/v polyethylene glycol 1500 and 0.1 M succinate/phosphate/glycine buffer at pH 5.0 (SPG buffer, Molecular Dimensions). The crystals were harvested from the drop using a nylon loop, soaked in crystallization buffer containing 1 mM BATSI for 5–10 min, and subsequently cryocooled in liquid nitrogen. Data were measured for each ADC-7/BATSI complex from an individual crystal at LS-CAT sector (21-ID-G Beamline) at the Advanced Photon Source at Argonne National Laboratory (Argonne, IL). The images were indexed, integrated, and scaled with XDS.²⁵ Molecular replacement was carried out with Phaser²⁶ using the ADC-7 apo structure (PDB 4U0T).⁷ Refmac5 in the CCP4 program suite was utilized to perform refinements.^{27,28} Repeated rounds of model building optimization were completed using Coot.²⁹ The coordinates and structure parameters for the ADC-7/BATSI complexes are stored with the Protein Data Bank as 5W12 (**EC04**), 5W13 (**SM23**), and 5W14 (**3c**).

Steady State Kinetics

Using an Agilent 8453 diode array spectrophotometer at room temperature, competition kinetics were conducted in 10 mM phosphate-buffered saline, pH 7.4. The β -lactam substrate and colorimetric indicator, nitrocefin (NCF, $\epsilon_{482} = 17400 \text{ M}^{-1} \text{ cm}^{-1}$), was utilized. In each assay, the measurements were taken after a 5 min preincubation of enzyme with BATSI. The average velocities (v_0) were then fitted to eq 1,

$$v_0 = v_u - \frac{v_u[I]}{K_{i(\text{observed})} + [I]} \quad (1)$$

where v_u represents the uninhibited turnover of NCF, $K_{i(\text{observed})}$ is the concentration of inhibitor that results in a 50% reduction of v_u , and $[I]$ is the concentration of inhibitor in the experiment. K_i values were also corrected for NCF affinity ($K_m = 21.2 \mu\text{M}$) according to eq 2,

$$K_{i(\text{corrected})} = \frac{K_{i(\text{observed})}}{1 + \frac{[\text{NCF}]}{K_{m\text{NCF}}}} \quad (2)$$

Disk Susceptibility Assays (DSAs) and Minimum Inhibitory Concentrations (MICs)

DSAs and MICs broth microdilutions were performed as previously described⁷ and according to Clinical and Laboratory Standards Institute (CLSI) guidelines and methods.³⁰ Bacterial cultures were grown overnight in Mueller-Hinton (MH) broth supplemented with

20 $\mu\text{g/mL}$ of chloramphenicol to ensure maintenance of the β -lactamase plasmid in pBC SK (–) containing *Escherichia coli* DH10B strains. Liquid cultures were then diluted using MH broth to a McFarland Standard ($\text{OD}_{600} = 0.224$). For the DSA assay, bacteria were plated on MH agar and a disk containing 10 μg of boronic acid compounds and 10 μg of ceftazidime or sulbactam was added. After an overnight incubation at 37 °C, zone sizes were measured and reported. For the MIC broth microdilution determinations, 200 μL of MH broth containing 10 $\mu\text{g/mL}$ BAI and increasing concentration of CAZ (from 0 to 128 $\mu\text{g/mL}$) was inoculated with the overnight culture. After overnight incubation, MICs were determined.

Synthesis

All reactions were performed under argon using oven-dried glassware and dry solvents. Dry tetrahydrofuran (THF) and diethyl ether were obtained by standard methods and freshly distilled under argon from sodium benzophenone ketyl prior to use. The $-100\text{ }^{\circ}\text{C}$ bath was prepared by addition of liquid nitrogen to a precooled ($-78\text{ }^{\circ}\text{C}$) mixture of ethanol/methanol (1:1). Preloaded (0.25 mm) glass supported silica gel plates (Kieselgel 60, Merck) were used for TLC analysis, and compounds were visualized by exposure to UV light and by dipping the plates in 1% $\text{Ce}(\text{SO}_4)\cdot 4\text{H}_2\text{O}$ and 2.5% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ in 10% sulfuric acid followed by heating on a hot plate. Chromatographic purification of the compounds was performed on silica gel (particle size of 0.05–0.20 mm). Melting points were measured in open capillary tubes on a Stuart SMP30 Melting Point apparatus and are uncorrected. Optical rotations were determined at $+20\text{ }^{\circ}\text{C}$ on a PerkinElmer 241 polarimeter and are expressed in $10^{-1}\text{ deg cm}^2\text{ g}^{-1}$. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance-400 MHz spectrometer. Chemical shifts (δ) are reported in ppm and were calibrated to the residual signals of the deuterated solvent.^{27,28} Multiplicity is given as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad signal; coupling constants (J) are given in Hz. Two-dimensional NMR techniques (COSY, HMBC, HSQC) were used to aid in the assignment of signals in ^1H and ^{13}C spectra. Particularly, in the ^{13}C spectra, the signal of the boron-bearing carbon atom tends to be broadened, often beyond the detection limit; however, its resonance was unambiguously determined by HSQC. Mass spectra were determined on an Agilent Technologies LC-MS (n) Ion Trap 6310A (electrospray ionization (ESI), 70 eV). High-resolution mass spectra were recorded on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS.

The purity of all tested compounds was above 95%, determined by elemental analysis performed on a Carlo Erba elemental analyzer 1110; results from elemental analyses for the compounds were within 0.3% of the theoretical values. Combustion analysis and mass spectrometry of free boronic acids provided inaccurate results because of the formation of dehydration products.³¹ Nevertheless, these boronic acids could be converted into analytically pure pinanediol esters by exposure to an equimolar amount of pinanediol in anhydrous THF. (+)-Pinanediol (1*R*)-2-azido-1-(2-thienylacetylaminio)ethaneboronate (**1**), (1*R*)-2-[4-carboxy-[1,2,3]triazol-1-yl]-1-(2-thienylacetylaminio)ethaneboronic acid (**3a**), and (1*R*)-2-[4-(3-carboxyphenyl)-[1,2,3]triazol-1-yl]-1-(2-thienylacetylaminio)ethaneboronic acid (**3c**) were synthesized according to the literature.¹²

General Procedure for Cu/C-Catalyzed “Click” Reaction

In a glass vial, (+)-pinanediol (1*R*)-2-azido-1-(2-thienyla-cetyl-amino)-ethaneboronate **1** (0.4 mmol) and the proper alkyne (0.4 mmol) were dissolved in *tert*-BuOH (3 mL) and CuSO₄ (0.04 mmol); sodium ascorbate (0.08 mmol) and water (3 mL) were added. The vessel was sealed, and the temperature was raised to 60 °C. The reaction was monitored through TLC until disappearance of the azidoboronate. After the proper reaction time, that spanned from 0.5 h for more activated alkynes to 2 h for less activated alkynes, the reaction mixture was partitioned between EtOAc (30 mL), H₂O (12 mL), and saturated NaCl (7 mL). The aqueous phase was extracted with EtOAc (2 × 20 mL), and the combined organic phases were washed with saturated NaCl, dried over Na₂SO₄, and concentrated *under vacuum* to afford the crude product which was thereafter purified as described.

(+)-Pinanediol (1*R*)-2-(4-carboxymethyl-[1,2,3]-triazol-1-yl)-1-(2-thienylacetyl-amino)-ethaneboronate (**2b**)

The reaction was terminated after 1 h. The crude residue was triturated from diethyl ether to afford **2b** as a white solid (117 mg, 80% yield), mp 180–182 °C. [α]_D – 94.5 (*c* 0.9, methanol (MeOH)). ¹H NMR (400 MHz, dimethyl sulfoxide (DMSO)): δ 0.81 (3H, s, pinanyl CH₃), 1.22 (3H, s, pinanyl CH₃), 1.24 (3H, s, pinanyl CH₃), 1.36 (1H, d, *J* = 10.0, pinanyl H_{endo}), 1.64–2.33 (5H, m, pinanyl protons), 2.98–3.02 (1H, m, BCHCH₂), 3.66 (2H, s, CH₂COOH), 3.90 (2H, s, CH₂CONH), 4.05 (1H, d, *J* = 6.7, CHOB), 4.22–4.39 (2H, m, BCHCH₂), 6.95–6.99 (2H, m, H_{ar}), 7.42–7.44 (1H, m, H_{ar}), 8.00 (1H, s, CH_{triazole}), 9.75 (1H, s, CH₂COOH). ¹³C NMR (100 MHz, DMSO): δ 24.4, 26.7, 27.7, 29.6, 32.1, 37.0, 38.1, 43.1 (br, CB), 52.4, 76.0, 83.0, 124.2 (CH_{triazole}), 126.2, 127.3, 127.4, 135.2, 140.6, 171.9, 175.4. MS (ESI, Ion Trap): 473 [M + H]⁺, MS/MS 473, *m/z* (%): 346 (50), 321 (100), 168 (8). Anal. Calcd for C₂₂H₂₉BN₄O₅S: C, 55.94; H, 6.19; N, 11.86; S, 6.79. Found: C, 55.69; H, 6.21; N, 11.70; S, 6.93

(+)-Pinanediol (1*R*)-2-(4-carboxamido-[1,2,3]triazol-1-yl)-1-(2-thienylacetyl-amino)ethaneboronate (**2d**)

Propiolamide was prepared from ethylpropiolate according to the literature procedure.¹⁵ The reaction was terminated after 30 min. The crude residue was crystallized from EtOAc and *n*-hexane to afford **2d** as a beige solid (162 mg, 89% yield), mp 144–146 °C. [α]_D – 50.9 (*c* 0.9, MeOH). ¹H NMR (400 MHz, MeOD): δ 0.91 (3H, s, pinanyl CH₃), 1.33 (3H, s, pinanyl CH₃), 1.38 (3H, s, pinanyl CH₃), 1.43 (1H, d, *J* = 9.4, pinanyl H_{endo}), 1.80–2.40 (5H, m, pinanyl protons), 3.20 (1H, dd, *J* = 3.9, 10.6, BCHCH₂), 3.98 (2H, s, CH₂CONH), 4.22 (1H, d, *J* = 6.9, CHOB), 4.43 (1H, dd, *J* = 10.6, 14.5, BCHCH₂), 4.60 (1H, dd, *J* = 4.0, 14.5, BCHCH₂), 6.97–7.00 (2H, m, H_{ar}), 7.34 (1H, dd, *J* = 1.5, 5.0, H_{ar}), 8.45 (1H, s, CH_{triazole}). ¹³C NMR (100 MHz, MeOD): δ 23.2, 26.2, 26.4, 28.3, 31.0, 36.3, 37.7, 40.0, 44.4 (br, CB), 52.2, 52.3, 76.1, 83.2, 125.4, 126.7 (CH_{triazole}), 126.8, 127.3, 133.2, 142.3, 163.4, 177.8. MS (ESI, Ion Trap): 458 [M + H]⁺, MS/MS 458, *m/z* (%): 387 (3), 346 (22), 306 (100), 168 (4). Anal. Calcd for C₂₁H₂₈BN₅O₄S: C, 55.15; H, 6.17; N, 15.31, S, 7.01. Found: C, 54.88; H, 6.01; N, 15.25; S, 6.88.

(+)-Pinanediol (1*R*)-2-(4-idroxy-carbamoyl-[1,2,3]triazol-1-yl)-1-(2-thienylacetyl-amino)ethaneboronate (**2e**)

N-Hydroxypropiolamide was prepared from propiolic acid according to the literature procedure.¹⁶ The reaction was terminated

after 30 min. The crude residue was crystallized from EtOAc and *n*-hexane to afford **2e** as a beige solid (155 mg, 82% yield), mp 105 °C (dec). $[\alpha]_D - 64.3$ (*c* 0.6, CHCl₃). ¹H NMR (400 MHz, MeOD): δ 0.90 (3H, s, pinanyl CH₃), 1.31 (3H, s, pinanyl CH₃), 1.38 (3H, s, pinanyl CH₃), 1.43 (1H, d, *J* = 9.4, pinanyl *H*_{endo}), 1.79–2.40 (5H, m, pinanyl protons), 3.20 (1H, dd, *J* = 3.9, 10.3, BCHCH₂), 3.98 (2H, s, CH₂CONH), 4.22 (1H, d, *J* = 8.5, CHOB), 4.40–4.62 (2H, m, BCHCH₂), 6.95–7.00 (2H, m, *H*_{ar}), 7.34 (1H, dd, *J* = 1.5, 5.0, *H*_{ar}), 8.44 (1H, s, CH_{triazole}). ¹³C NMR (100 MHz, MeOD): δ 24.6, 27.6, 27.8, 29.7, 32.4, 37.7, 39.2, 41.4, 45.7 (br, CB), 53.6, 53.7, 77.5, 84.6, 126.8, 127.8 (CH_{triazole}), 128.1, 128.7, 134.6, 139.8, 160.6, 178.6. MS (ESI, Ion Trap): 474 [M + H]⁺, MS/MS 474, *m/z* (%): 346 (18), 322 (100), 168 (4). Anal. Calcd for C₂₁H₂₈BN₅O₅S: C, 53.28; H, 5.96; N, 14.80, S, 6.77. Found: C, 53.01; H, 6.10; N, 14.66; S, 6.55.

(+)-Pinanediol (1R)-2-[4-cyano-[1,2,3]triazol-1-yl]-1-(2-thienylacetyl amino)ethaneboronate (2f)—Propiolonitrile was prepared according to the literature procedure.¹⁷ The reaction was terminated after 2 h. The crude residue triturated from Et₂O to afford **2f** as a beige solid (174 mg, 99% yield), mp 73 °C (dec). $[\alpha]_D - 60.0$ (*c* 1.0, MeOH). ¹H NMR (400 MHz, MeOD): δ 0.90 (3H, s, pinanyl CH₃), 1.31 (3H, s, pinanyl CH₃), 1.38 (3H, s, pinanyl CH₃), 1.43 (1H, d, *J* = 10.4, pinanyl *H*_{endo}), 1.79–2.40 (5H, m, pinanyl protons), 3.19 (1H, dd, *J* = 3.9, 10.2, BCHCH₂), 3.97 (2H, s, CH₂CONH), 4.22 (1H, d, *J* = 6.8, CHOB), 4.47 (1H, dd, *J* = 10.2, 14.6, BCHCH₂), 4.62 (1H, dd, *J* = 3.9, 14.6, BCHCH₂), 6.98–7.00 (2H, m, *H*_{ar}), 7.35 (1H, d, *J* = 4.8, *H*_{ar}), 8.73 (1H, s, CH_{triazole}). ¹³C NMR (100 MHz, MeOD): δ 22.3, 26.2, 26.4, 28.3, 31.0, 36.3, 37.8, 40.0, 44.0 (br, CB), 52.2, 52.7, 76.2, 83.3, 111.4, 120.2, 125.4, 126.7, 127.3, 131.3 (CH_{triazole}), 133.1, 177.3. MS (ESI, Ion Trap): 440 [M + H]⁺, MS/MS 440, *m/z* (%): 346 (26), 288 (100), 212 (8), 194 (6), 150 (5). Anal. Calcd for C₂₁H₂₆BN₅O₃S: C, 57.41; H, 5.96; N, 15.94, S, 7.30. Found: C, 57.45; H, 6.11; N, 15.79; S, 7.08.

(+)-Pinanediol (1R)-2-[4-(3-cyanophenyl)-[1,2,3]triazol-1-yl]-1-(2-thienylacetyl amino)ethaneboronate (2g)—3-Ethynyl-benzonitrile was prepared according to the literature procedure.¹⁸ The reaction was terminated after 2 h. The crude residue triturated from Et₂O to afford **2g** as a beige solid (148 mg, 72% yield), mp 78 °C (dec). $[\alpha]_D - 64.8$ (*c* 1.2, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 0.87 (3H, s, pinanyl CH₃), 1.29 (3H, s, pinanyl CH₃), 1.41 (1H, d, *J* = 13.6, pinanyl *H*_{endo}), 1.42 (3H, s, pinanyl CH₃), 1.82–2.40 (5H, m, pinanyl protons), 3.32 (1H, b, BCHCH₂), 3.91 (2H, s, CH₂CONH), 4.30 (1H, d, *J* = 7.5, CHOB), 4.50–4.60 (2H, m, BCHCH₂), 6.94–6.98 (2H, m, *H*_{ar}), 7.23 (1H, d, *J* = 5.0, *H*_{ar}), 7.26 (1H, b, NHCO), 7.52–7.61 (3H, m, *H*_{ar}), 7.85 (1H, s, *H*_{ar}), 8.01 (1H, s, CH_{triazole}). ¹³C NMR (100 MHz, CDCl₃): δ 24.3, 26.8, 27.4, 29.1, 34.0, 36.6, 38.4, 40.1, 42.3 (br, CB), 52.2, 52.5, 77.3, 84.8, 113.3, 118.6 (CH_{triazole}), 126.4, 127.8, 128.3, 129.2, 129.87, 129.90, 131.6, 131.9, 133.1, 175. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₂₇H₃₀BN₅O₃S: 516.223; Found: 516.225. Anal. Calcd for C₂₇H₃₀BN₅O₃S: C, 62.92; H, 5.87; N, 13.59, S, 6.22. Found: C, 63.05; H, 5.70; N, 13.49; S, 6.00.

General Procedure for Tetrazole Formation

In a microwave glass vial, the cyano derivatives **2f** and **2g** (0.4 mmol) and tetrabutylammonium fluoride trihydrate (0.4) were dissolved in trimethylsililazide (6.0

mmol) under Argon. The vessel was sealed and heated under microwave irradiation at 110 °C for 30 min. The reaction mixture was diluted with EtOAc (30 mL) and washed with HCl (1N, 3 × 10 mL), dried over Na₂SO₄, and concentrated *under vacuum* to afford the crude product which was thereafter purified as described.

(+)-Pinanediol (1R)-2-[4-(2H-tetrazol-5-yl)-[1,2,3]triazol-1-yl]-1-(2-thienylacetylamino)ethaneboronate (2h)—The crude residue was triturated from Et₂O to afford **2h** as a beige solid (153 mg, 79% yield), mp 165–168 °C (dec). [α]_D – 80.2 (*c* 1.1, MeOH). ¹H NMR (400 MHz, MeOH): δ 0.88 (3H, s, pinanyl CH₃), 1.29 (3H, s, pinanyl CH₃), 1.37 (3H, s, pinanyl CH₃), 1.43 (1H, d, *J* = 10.4, pinanyl H_{endo}), 1.78–2.38 (5H, m, pinanyl protons), 3.24 (1H, dd, *J* = 4.2, 10.2, BCHCH₂), 3.96 (2H, s, CH₂CONH), 4.22 (1H, dd, *J* = 2.1, 8.8, CHOB), 4.50 (1H, dd, *J* = 10.2, 14.5, BCHCH₂), 4.67 (1H, dd, *J* = 4.2, 14.5, BCHCH₂), 6.91–6.98 (2H, m, H_{ar}), 7.28 (1H, dd, *J* = 1.2, 5.2, H_{ar}), 8.71 (1H, s, CH_{triazole}). ¹³C NMR (100 MHz, CDCl₃): δ 24.5, 27.6, 27.8, 29.7, 32.4, 37.7, 39.2, 41.4, 45.8 (br, CB), 53.6, 53.8, 77.5, 84.6, 126.6 (CH_{triazole}), 126.7, 128.0, 128.6, 134.5, 134.9, 150.54, 178.7. HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd for C₂₁H₂₈BN₈O₃S: 483.209; Found: 483.209. Anal. Calcd for C₂₁H₂₇BN₈O₃S: C, 52.29; H, 5.64; N, 23.23, S, 6.65. Found: C, 52.10; H, 5.55; N, 23.18; S, 6.59.

(+)-Pinanediol (1R)-2-[4-(3-(2H-tetrazol-5-yl)phenyl)-[1,2,3]triazol-1-yl]-1-(2-thienylacetylamino)ethaneboronate (2j)—The crude residue was triturated from Et₂O to afford **2j** as a beige solid (205 mg, 92% yield), mp 140–142 °C (dec). [α]_D – 70.3 (*c* 1.3, MeOH). ¹H NMR (400 MHz, MeOH): δ 0.88 (3H, s, pinanyl CH₃), 1.28 (3H, s, pinanyl CH₃), 1.38 (3H, s, pinanyl CH₃), 1.43 (1H, d, *J* = 10.3, pinanyl H_{endo}), 1.78–2.38 (5H, m, pinanyl protons), 3.26 (1H, dd, *J* = 4.1, 10.5, BCHCH₂), 3.98 (2H, s, CH₂CONH), 4.22 (1H, dd, *J* = 2.1, 8.5, CHOB), 4.57 (1H, dd, *J* = 10.5, 14.5, BCHCH₂), 4.60 (1H, dd, *J* = 4.1, 14.5, BCHCH₂), 6.94 (1H, dd, *J* = 3.4, 5.2, H_{ar}), 6.99 (1H, dd, *J* = 1.1, 3.4, H_{ar}), 7.30 (1H, dd, *J* = 1.1, 5.2, H_{ar}), 7.64 (1H, t, *J* = 7.9, H_{ar}), 7.98 (1H, dt, *J* = 1.4, 7.9, H_{ar}), 8.46 (1H, s, CH_{triazole} and H_{ar}). ¹³C NMR (100 MHz, CDCl₃): δ 24.5, 27.6, 27.8, 29.7, 32.5, 37.7, 39.1, 41.4, 45.7 (br, CB), 53.6, 53.7, 77.5, 84.6, 123.3, 125.2, 126.4 (CH_{triazole}), 126.8, 127.7, 128.0, 128.7, 129.4, 131.1, 133.3, 134.6, 147.6, 157.7, 178.6. HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd for C₂₇H₃₂BN₈O₃S: 559.2411; Found: 559.243. Anal. Calcd for C₂₇H₃₁BN₈O₃S: C, 58.07; H, 5.60; N, 20.06, S, 5.74. Found: C, 57.81; H, 5.66; N, 19.89; S, 5.70.

General Procedure for Pinanediol Removal

The pinanediol esters **2b–e**, **2h**, and **2j** (0.2 mmol) were dissolved in CH₃CN (3 mL) and HCl (0.6 mmol of a 1 M solution in degassed H₂O), phenylboronic acid (isobutylboronic acid in the case of **3h** and **3j**) (0.18 mmol) and *n*-hexane (2 mL) were sequentially added and the resulting biphasic solution was vigorously stirred. After 20 min, the *n*-hexane solution (containing the pinanediol phenylboronate or isobutylboronate) was removed and fresh *n*-hexane (3 mL) added. This last procedure was repeated several times until a TLC analysis of the *n*-hexane layer did not reveal any more phenylboronate or isobutylboronate production (total reaction time of 3 h). The acetonitrile phase was concentrated, and the crude purified as reported, affording the desired compounds **3b–e**, **3h**, and **3j**. Copper(II) concentration in these products was found below 0.5 ppm through plasma analysis on a ICP-MS instrument.

The enantiomeric purity of chiral boronic acids was checked by reversion into their pinanediol esters. In particular, final compounds **3b**, **3d**, **3e**, **3h**, and **3j** were allowed to react with an equimolar amount of (+)-pinanediol in anhydrous THF: the NMR spectra of the crude products displayed the presence of a single diastereoisomer, proving that no racemization occurred during transesterification.

(1R)-2-(4-carboxymethyl-[1,2,3]triazol-1-yl)-(2-thienylace-tylamino)ethaneboronic acid (3b)—

The crude residue was triturated from Et₂O to afford **3b** as beige solid (62 mg, 92% yield), mp 84 °C (dec). [α]_D = −75.0 (*c* 1.0, MeOH). ¹H NMR (400 MHz, MeOD): δ 3.25 (1H, dd, *J* = 4.5, 9.7, BCH), 4.03 (4H, s, CH₂CO and CH₂COOH), 4.52 (1H, dd, *J* = 9.7, 14.4, BCHCH₂), 4.66 (1H, dd, *J* = 4.5, 14.4, BCHCH₂), 6.99 (1H, dd, *J* 3.5, 5.0, H_{ar}), 7.03–7.15 (1H, m, H_{ar}), 7.36 (1H, dd, *J* = 1.4, 5.0, H_{ar}), 8.47 (1H, s, CH_{triaz}). ¹³C NMR (100 MHz, MeOD): δ 30.2, 32.3, 46.3 (br, CB, not seen), 56.2, 127.0, 128.2, 129.0, 134.5 (CH_{triazole}), 170.1, 179.4. MS (ESI, Ion Trap): 339 [M + H]⁺, MS/MS 339, *m/z* (%): EI-MS and elemental analysis results were not obtainable,²⁴ but exposure of **3b** to an equimolar amount of (+)-pinanediol in anhydrous THF afforded compound **2b** in quantitative yield and satisfactory elemental analysis results. Anal. Calcd for C₂₂H₂₉BN₄O₅S: C, 55.94; H, 6.19; N, 11.86; S, 6.79. Found: C, 56.18; H, 6.33; N, 11.68; S, 6.60.

(1R)-2-(4-carboxamido-[1,2,3]triazol-1-yl)-1-(2-thienylacetylaminio)ethaneboronate (3d)—

The crude residue was triturated from Et₂O to afford **3d** as beige solid (62 mg, 96% yield), mp 90–92 °C. [α]_D = −107.9 (*c* 0.8, MeOH). ¹H NMR (400 MHz, MeOD): δ 3.21 (1H, dd, *J* = 3.8, 10.2, BCH), 4.02 (2H, s, CH₂CO), 4.22 (1H, dd, *J* = 10.2, 14.4, BCHCH₂), 4.55 (1H, dd, *J* = 3.8, 14.4, BCHCH₂), 6.99–7.04 (2H, m, H_{ar}), 7.34–7.37 (1H, m, H_{ar}), 8.46 (1H, s, CH_{triaz}). ¹³C NMR (100 MHz, MeOD): δ ¹³C NMR (101 MHz, CDCl₃) δ 32.0, 47.2 (br, CB), 53.7, 126.8, 128.1, 128.2, 128.5, 128.3, 131.0, 134.5, 134.7, 179.1. EI-MS and elemental analysis results were not obtainable,²⁴ but exposure of **3d** to an equimolar amount of (+)-pinanediol in anhydrous THF afforded compound **2d** in quantitative yield and satisfactory elemental analysis results. Anal. Calcd for C₂₁H₂₈BN₅O₄S: C, 55.15; H, 6.17; N, 15.31, S, 7.01. Found: C, 55.36; H, 6.10; N, 15.09; S, 6.84.

(1R)-2-(4-idroxicarbamoil-[1,2,3]triazol-1-yl)-1-(2-thienylacetylaminio)ethaneboronic acid (3e)—

The crude residue was triturated from Et₂O to afford **3e** as beige solid (62 mg, 91% yield), mp 241–243 °C. [α]_D = −80.9 (*c* 0.9, MeOH). ¹H NMR (400 MHz, MeOD): δ 3.19 (1H, dd, *J* = 3.5, 10.2, BCH), 4.00 (2H, s, CH₂CO), 4.41 (1H, dd, *J* = 10.2, 14.2, BCHCH₂), 4.52 (1H, dd, *J* = 3.5, 14.2, BCHCH₂), 6.95–7.04 (2H, m, H_{ar}), 7.29–7.41 (1H, m, H_{ar}), 8.41 (1H, s, CH_{triaz}). ¹³C NMR (100 MHz, MeOD): δ 32.0, 47.3 (br, CB), 53.5, 126.9, 128.1, 128.5, 128.9, 134.4 (CH_{triazole}), 134.7, 179.3. EI-MS and elemental analysis results were not obtainable,²⁴ but exposure of **3e** to an equimolar amount of (+)-pinanediol in anhydrous THF afforded compound **2e** in quantitative yield and satisfactory elemental analysis results. Anal. Calcd for C₂₁H₂₈BN₅O₅S: C, 53.28; H, 5.96; N, 14.80, S, 6.77. Found: C, 52.90; H, 6.17; N, 14.96; S, 6.60.

(1R)-2-[4-(2H-tetrazol-5-yl)-[1,2,3]triazol-1-yl]-1-(2-

thienylacetylaminio)ethaneboronic acid (3h)—The crude residue was triturated from acetonitrile to afford **3h** as yellowish solid (56 mg, 81% yield), mp 221 °C. $[\alpha]_D = -96.5$ (*c* 0.5, MeOH). ^1H NMR (400 MHz, MeOD): δ 3.25 (1H, dd, $J = 4.8, 10.1$ BCH), 4.00 (2H, s, CH_2CO), 4.48 (1H, dd, $J = 10.1, 14.5$, BCHCH₂), 4.61 (1H, dd, $J = 4.2, 14.5$, BCHCH₂), 6.96 (1H, dd, $J = 3.5, 5.1$, H_{ar}), 7.01 (1H, dd, $J = 1.3, 3.5$, H_{ar}), 7.31 (1H, dd, $J = 1.3, 5.1$, H_{ar}), 8.66 (1H, s, CH_{triaz}). ^{13}C NMR (100 MHz, MeOD): δ 32.1, 47.2 (br, CB), 53.7, 126.5 ($\text{CH}_{\text{triazole}}$), 126.8, 128.1, 128.8, 134.5, 134.9, 150.6, 179.2. EI-MS and elemental analysis results were not obtainable,²⁴ but exposure of **3h** to an equimolar amount of (+)-pinanediol in anhydrous THF afforded compound **2h** in quantitative yield and satisfactory elemental analysis results. Anal. Calcd for C₂₁H₂₇BN₈O₃S: C, 52.29; H, 5.64; N, 23.23; S, 6.65. Found: C, 52.07; H, 5.59; N, 23.18; S, 6.57.

(1R)-2-[4-(3-(2H-tetrazol-5-yl)phenyl)-[1,2,3]triazol-1-yl]-1-(2-

thienylacetylaminio)ethaneboronate (3j)—The crude residue was triturated from acetonitrile to afford **3j** as beige solid (81 mg, 96% yield), mp 189–191 °C. $[\alpha]_D = -81.9$ (*c* 0.4, MeOH). ^1H NMR (400 MHz, MeOD): δ 3.28 (1H, d, $J = 4.0$ BCH signal under MeOH), 4.03 (2H, s, CH_2CO), 4.49 (1H, dd, $J = 10.5, 14.5$, BCHCH₂), 4.62 (1H, dd, $J = 4.0, 14.5$, BCHCH₂), 6.97 (1H, dd, $J = 3.5, 5.3$, H_{ar}), 7.03 (1H, dd, $J = 1.2, 3.5$, H_{ar}), 7.33 (1H, dd, $J = 1.2, 5.3$, H_{ar}), 7.71 (1H, dt, $J = 0.4, 2.0$, H_{ar}), 8.02–8.07 (2H, m, H_{ar}), 8.52 (1H, dt, 0.4, 1.7, H_{ar}), 8.63 (1H, s, CH_{triaz}). ^{13}C NMR (100 MHz, MeOD): δ 32.1, 47.0 (br, CB), 54.8, 124.8 ($\text{CH}_{\text{triazole}}$), 125.6, 126.88, 126.92, 128.2, 128.7, 128.9, 129.8, 130.9, 131.4, 134.5, 146.3, 157.8, 179.3. EI-MS and elemental analysis results were not obtainable,³¹ but exposure of **3j** to an equimolar amount of (+)-pinanediol in anhydrous THF afforded compound **2j** in quantitative yield and satisfactory elemental analysis results. Anal. Calcd for C₂₇H₃₁BN₈O₃S: C, 58.07; H, 5.60; N, 20.06; S, 5.74. Found: C, 57.83; H, 5.73; N, 19.99; S, 5.64.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ADC	acinetobacter derived cephalosporinases
BATSIs	boronic acids transition state inhibitors

CuAAC	copper-catalyzed azide–alkyne cycloaddition
BL	β -lactamase
MICs	minimum inhibitory concentrations
SAR	structure activity relationship
HSQC	heteronuclear single quantum coherence spectroscopy
HMBC	heteronuclear multiple bond correlation
NCF	nitrocefin
CAZ	ceftazidime
SUL	sulbactam
THF	tetrahydrofuran
LC/MS	liquid chromatography/mass spectrometry
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
MeOH	methanol

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Figure 1.

Stereoview of the **SM23**, **EC04**, and **3c** BATSIs bound to the catalytic residue, Ser64 of ADC-7. $F_o - F_c$ omit maps are contoured at 2.5σ . Atom coloring is as follows: oxygen atoms, red; nitrogens, blue; sulfurs, yellow; borons, pink; carbon atoms of **SM23**, white; carbons atoms of **EC04**, cyan; carbons atoms of **3c**, green. This figure was generated with PyMOL [Schrodinger].

Figure 2. **SM23** (white), **EC04** (cyan), **3c** (lime green). Stereoview of the ADC-7 active site in complex with three, R2-containing BATSIs. Hydrogen bonds observed are shown as yellow, dashed lines. Cation– π interactions are shown as magenta dashed lines. Water molecules are shown as red spheres. The colors are as follows: oxygens, red; nitrogens, blue; boron, pink; sulfur, yellow. The carbon atoms of the active site residues and BATSIs are colored by the various inhibitors in complex with ADC-7.

Figure 3. Stereoview superposition of the ADC-7 active site in complex with the R2-containing BATSIs; **3c** (green), **EC04** (cyan), and **SM23** (white).

Scheme 1.

(a) Cephalothin- β -Lactamase (BL) Deacylation High Energy Intermediate; (b) S02030 in Complex with a Serine β -Lactamase

Scheme 2.

Scheme 3. General Synthesis of α -2-Thienylacetamido- β -Triazolylethaneboronic Acids 3

Table 1 K_i Values of Compound 3 Derivatives with ADC-7

Cmp	Structure	R	K_i [nM]
3a			44 ± 2
3b			115 ± 2
3c			46 ± 3
3d			224 ± 2
3e			155 ± 3
3h			76 ± 2
3j			99 ± 2
s08127			658 ± 6
EC04		—	53 ± 4
SM23		—	22 ± 2

Table 2

Crystallographic Summary for the ADC-7/BATSI Complexes

	ADC-7/3c	ADC-7/EC04	ADC-7/SM23
cell constants (Å; deg)	<i>a</i> = 89.01 <i>b</i> = 81.30 <i>c</i> = 106.44 β = 112.70	<i>a</i> = 89.53 <i>b</i> = 81.30 <i>c</i> = 106.69 β = 112.89	<i>a</i> = 88.71 <i>b</i> = 81.65 <i>c</i> = 106.42 β = 112.84
space group	P2 ₁	P2 ₁	P2 ₁
resolution (Å)	1.88 (1.89–1.88) ^a	1.88 (1.89–1.88) ^a	1.95 (1.96–1.95) ^a
unique reflections	113 977	113 662	101 377
total observations	475 114	477 304	418 250
<i>R</i> _{merge} (%)	6.7 (62.4)	5.9 (65.3)	9.0 (70.6)
completeness (%) ^b	99.8 (99.8)	99.0 (98.1)	99.6 (99.6)
$\langle I/\sigma_I \rangle$	13.5 (2.2)	15.7 (2.2)	11.9 (2.3)
resolution range for refinement (Å)	0.998 (0.732)	0.996 (0.734)	0.997 (0.719)
number of protein residues	50.00–1.88	98.28–1.88	98.07–1.95
number of water molecules	1424	1423	1426
RMSD bond lengths (Å)	589	516	628
RMSD bond angles (deg)	0.018	0.018	0.018
<i>R</i> -factor (%)	1.896	1.865	1.915
<i>R</i> _{free} (%) ^c	20.2	20.1	18.7
average B-factor, protein atoms (Å ²)	25.6	25.5	24.6
average B-factor, water molecules (Å ²)	37.0	40.5	37.0
average B-factor, inhibitor atoms (Å ²)	36.7	38.4	37.1

^aValues in parentheses are for the highest resolution shell.^bFraction of theoretically possible reflections observed.^c*R*_{free} was calculated with 5% of reflections set aside randomly.

Table 3

MICs Were Determined Using the Broth Microdilution Method with 10 $\mu\text{g/mL}$ Compound and Varying Amounts of CAZ (from 0 to 128 $\mu\text{g/mL}$)^a

cmpd.	<i>E. coli</i> carrying <i>bla</i> _{ADC37} encoded in pBC SK (–)		AB0057 <i>A. baumannii</i> spp. clinical strain
	MIC broth dilution [$\mu\text{g/mL}$]	disk assay [mm]	disk assay [mm]
none	32	CAZ 12 mm	SUL 6 mm
3a	1	26	16
3b	2	18	10
3c	2	24	15
3d	4	18	10
3e	2	16	8
3h	2	23	10
3j	8	15	6
s08127	4	12	6
EC04	2	21	11
SM23	1	25	12

^aThe DSAs were performed against *E. coli* DH10B using ceftazidime as the BATSI antibiotic partner and sulbactam for the AB0057 clinical strain (10 μg of BAI and 10 μg of antibiotic partner).