1,2,3-Triazolylmethaneboronate: A Structure Activity Relationship Study of a Class of β-Lactamase Inhibitors against Acinetobacter baumannii Cephalosporinase

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ABSTRACT: Boronic acid transition state inhibitors (BATSIs) are known reversible covalent inhibitors of serine β-lactamases. The selectivity and high potency of specific BATSIs bearing an amide side chain mimicking the β-lactam’s amide side chain are an established and recognized synthetic strategy. Herein, we describe a new class of BATSIs where the amide group is replaced by a bioisostere triazole; these compounds were designed as molecular probes. To this end, a library of 26 α-triazolylmethaneboronic acids was synthesized and tested against the clinically concerning Acinetobacter-derived cephalosporinase, ADC-7. In steady state analyses, these compounds demonstrated \( K_i \) values ranging from 90 nM to 38 μM (±10%). Five compounds were crystallized in complex with ADC-7 β-lactamase, and all the crystal structures reveal the triazole is in the putative amide binding site, thus confirming the triazole–amide bioisosterism. The easy synthetic access of these new inhibitors as prototype scaffolds allows the insertion of a wide range of chemical groups able to explore the enzyme binding site and provides insights on the importance of specific residues in recognition and catalysis. The best inhibitor identified, compound 6q (\( K_i \) 90 nM), places a tolyl group near Arg340, making favorable cation–π interactions. Notably, the structure of 6q does not resemble the natural substrate of the β-lactamase yet displays a pronounced inhibition activity, in addition to lowering the minimum inhibitory concentration (MIC) of ceftazidime against three bacterial strains expressing class C β-lactamases. In summary, these observations validate the α-triazolylboronic acids as a promising template for further inhibitor design.

KEYWORDS: boronic acids, Acinetobacter, amide bioisostere, click chemistry, β-lactamase inhibitors

Antimicrobial resistance (AMR) is a major global health threat. Regrettably, this crisis is aggravated by the lack of new therapeutic agents in the current pharmaceutical pipeline. Economic analyses indicate that AMR increases health-care costs, the length of stay in the hospital, morbidity, and mortality.1 For these reasons, the World Health Organization (WHO) has recently designated AMR as one of the three most important problems facing human health.2 The WHO Priority List has recently assigned Acinetobacter baumannii as a critical priority pathogen due to the high prevalence of cephalosporin and carbapenem resistance and its ability to survive in adverse environmental conditions, making it one of the most threatening nosocomial pathogens.3

Common AMR mechanisms found in Acinetobacter spp. include modification of the enzymes that the antibiotic targets, decreased permeability of the outer membrane, efflux pumps, and the production of enzymes that attack and inactivate antibiotics (β-lactamases).3,4 Current antimicrobials used in the clinic to treat infections caused by multidrug resistant (MDR) or extreme drug resistant (XDR) A. baumannii are siderophore containing β-lactams (cefdicericol), polymyxins, tigecycline, and aminoglycosides. All these drugs display uncertain clinical efficacy, a high level of toxicity, and mounting resistance.4 The urgent need for new drugs active against this pathogen has recently accelerated drug development, and new therapeutic options are under study.

The attractiveness of identifying β-lactamase inhibitors effective against A. baumannii relies upon the β-lactam’s intrinsic mechanism of action. The use of combination therapy, where a
β-lactam antibiotic is combined with a β-lactamase inhibitor, is a time-honored and extremely effective approach to overcome resistance. Three new β-lactam/β-lactamase inhibitor combinations recently entered the market, namely, the diazabicyclooctane avibactam with ceftazidime (Avycaz), relebactam with imipenem/cilastatin (Recarbrio), and the boronic acid vaborbactam with Meropenem (Vabomere). While these combinations prove to be useful in the treatment of infections caused by carbapenem resistant Enterobacteriaceae and multi drug-resistant (MDR) P. aeruginosa, they are not uniformly active against A. baumannii. A. baumannii possesses many clinically diverse β-lactamases from all four classes; the most significant portion of β-lactam resistance in A. baumannii is expressed by class C Acinetobacter-derived cephalosporinases (ADCs), chromosomally encoded β-lactamases responsible for resistance to advanced generation cephalosporins.

In previous work, we systematically evaluated the activity of a series of boronic acids against ADC-7, a representative class C enzyme found in A. baumannii. A. baumannii possesses clinically diverse β-lactamases from all four classes; the most significant portion of β-lactam resistance in A. baumannii is expressed by class C Acinetobacter-derived cephalosporinases (ADCs), chromosomally encoded β-lactamases responsible for resistance to advanced generation cephalosporins. To this scaffold (A) that proved active against ADC-7 was a chiral α-acylaminoalkaneboronate (Figure 1), where the α-carbon atom was substituted by a canonical R1 amide side chain in position C6/C7, typical of penicillins/cephalosporins, and a R2 group bearing a carboxylate, which is always present in position C3/C4 of the same β-lactam antibiotics. To this scaffold belongs compound SM23, the best inhibitor of this series with a K<sub>i</sub> of 21 nM for ADC-7. A second scaffold (B) was subsequently designed that replaced the amide group with a sulfonamide. With this class of derivatives, the natural substrate mimetics of scaffold A (both the R1 and R2 inspired by the β-lactam structures) were advanced into a series of compounds that could better “fit” into the enzyme active site. Compound CR192 from series B demonstrated a K<sub>i</sub> of 0.45 nM, proving one of the most potent inhibitors of ADC-7 ever designed. Finally, in series C, the amide/sulfonamide was replaced by a triazole ring. Triazoles are nonclassical amide bioisosteres and share with the amide a wide range of properties such as planarity, size, dipole moment, and hydrogen bonding capabilities. Indeed, even though S06017 is a less potent inhibitor (K<sub>i</sub> = 6.1 μM) compared to the achiral sulfonamide CR192, the structural information from the X-ray crystal structure of the enzyme–inhibitor complex suggested that the triazole maintained two of the canonical interactions in the amide binding site, thus behaving as a good amide bioisostere.

Encouraged by the bioisosterism and the easy synthetic access of α-triazolylboronic acids C, we chose to explore the potential of this particular scaffold in the present analysis, specifically 1,2,3-triazoles 1,4-disubstituted that are easily accessible through 1–3-dipolar Cu-catalyzed azide–alkyne cycloaddition (CuAAC). Our goal was to use these compounds as molecular probes to elucidate structure activity relationships, SAR. The Cu-based process employs click chemistry, which proceeds in mild conditions, using inexpensive reagents, with high efficiency and simple product isolation. Furthermore, we have already demonstrated the tolerance of boronic esters with CuAAC. In this paper, 26 compounds were synthesized and characterized via kinetic analysis and microbiological assays. The extraordinary inhibitory activity against ADC-7 was determined (K<sub>i</sub>, values spanning from 90 nM to 33 μM) and compared with vaborbactam binding affinity of 0.72 μM (IC<sub>50</sub> 14.6 μM). Additionally, the X-ray crystal structures of ADC-7 in complex with 5 of these compounds were determined to resolutions ranging from 1.74 to 2.04 Å. Despite being different from the amide, we hypothesized that the triazole would maintain significant potency and selectivity while allowing for easy and straightforward access to a wide variety of derivatives.

### RESULTS

#### Design.
The α-triazolylmethaneboronic acid S06017 (Figure 1), described in a previous publication, displayed a lower binding affinity to ADC-7 (K<sub>i</sub> = 6.1 μM) compared to α-sulfonamido and α-acylamidomethane boronic acids. However, the crystal structure of the ADC-7/S06017 complex showed that the triazole maintained two of the three canonical interactions in the amide binding site, with two nitrogen

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**Scheme 1. Synthesis of α-Triazolylmethaneboronate 6a–q**

(i) NaN₃, CH₃CN, 85 °C; (ii) (+)-pinanediol, SiO₂, H₂O₂, overnight; (iii) a–q, CuSO₄·5H₂O, Na ascorbate, t-BuOH/H₂O, 2 h, 60 °C; (iv) isobutylboronic acid, HCl, acetonitrile, n-hexane, r.t.
atoms interacting with Gln120 and Asn152, suggesting that this heterocycle could behave as a good amide biosostere. Furthermore, the triazolylmethanoboric acid scaffold was synthetically accessible and allowed for the introduction of different groups at position 4 of the triazole (Scheme 1). For these reasons, our goals in this study were to validate whether the α-triazolylmethanoboric acid group (i) is a good scaffold for ADC-7 inhibition; (ii) serves as a template for new ADC-7 BATSIs capable of restoring antibiotic activity.

To these ends, we strategically designed four series of α-triazolylmethanoboric acids (Table 1). In Series I, five compounds (6a–e) contain a phenyl ring directly bound to the triazole with a substituent on the aromatic moiety. Series II consists of three triazoles (6f–h) bearing electron rich and electron poor heterocyclic rings replacing the phenyl, whereas Series III (compounds 6i–l) and IV (compounds 6m–q) introduce a phenoxymethyl substituent or a substituted aminomethyl bridge on the triazole in order to confer more flexibility to the structures. The substituents for each series are represented in Table 1.

### Table 1

| Series I | Series II | Series III | Series IV |
|----------|-----------|------------|-----------|
| ![Structure](image)

### Synthesis. The synthesis of α-triazolylmethanoboric was successfully performed as depicted in Scheme 1. The commercially available bromomethanetriuloroborate 1 was reacted with sodium azide in acetonitrile at 85 °C to afford the azidomethanetriuloroborate 2 in 90% yield.

Conversion of the organotrifluoroborate 2 into the (+)-pinacol-di-α-azidomethanoboric 3 was performed in degassed water in the presence of silica gel (1.5 equiv) and a stoichiometric amount of (+)-pinacol (90% yield).15–17

Compound 3 is one of the partners of CuAAC, the acetylene counterparts 4a–q were conveniently purchased or synthesized following literature procedures (see the Methods). The cyclization reactions were carried out as described.14 The expected 1,4-disubstituted triazoles 5a–q, differently substituted at the R₃ group (see Table 1), were easily isolated by extraction and used as such for the next step. Final deprotection of (+)-pinacol ester 5a–q was accomplished by trans-esterification with isobutylboronic acid (0.95 equiv) and HCl 3 M (3 equiv) in a biphasic system of acetonitrile/n-hexane, allowing one to obtain final boronic acids 6a–q.

### Inhibition Kinetics and Antibiotic Susceptibility

#### (Minimum Inhibitory Concentrations, MICs).

The binding affinities (Kᵢ) for each of the BATSIs with ADC-7 were determined using competition kinetics with nitrocefin (NCF) used as chromophore substrate. The Kᵢ values (average data from 3 experiments) for all BATSIs, corrected for the NCF affinity (Kᵢ = 20 μM), are reported in Table 2.

All compounds show inhibition of ADC-7 β-lactamase in the low micromolar range. Compounds from Series I, with an aromatic phenyl ring directly bound to the triazole, exhibit Kᵢ values spanning from 0.2 μM (compound 6e) to 1.6 μM (compound 6c). When the aromatic moiety is a heterocycle (Series II) or a substituted benzyloxy group (Series III), inhibition remains in the low micromolar range, with the thiopeptide substituent (6f) being the best from Series II (Kᵢ = 1.0 μM) and the 3-chlorophenyl (6j), from Series III (Kᵢ = 0.98 μM). Compounds from Series IV (6m–q) show the most surprising results, suggesting that the addition of moieties to the triazole to increase flexibility is not always beneficial: activity varies from 33.8 μM for compound 6p having an amide as a bridge between the triazole and the phenyl ring down to as low as 90 nM for 6q, which replaces the amide of 6p with a sulfonamide. This 300-fold difference in activity suggested a possible second round of inhibitor structure refinement. Nevertheless, we wanted to confirm 6q as the best lead compound using a microbiological profile as well.

The inhibition constant (Kᵢ) values and MIC data (Table 2) for compounds 6a–q are plotted in Chart 1. Data show a good agreement between kinetic and antimicrobial activity: the lower the MIC, the higher is the affinity of the compound. Compound 6q proved to be the best compound under both kinetic and microbiological profiles.

To further improve the structure of 6q, we designed another series of triazolyl BATSIs (Series V, Figure 2). Nine additional compounds 6r–z were synthesized with the replacement of the tollyl group of 6q with a trifluoroethyl (6r), a thiophene (6s), five different benzyl groups (6t–x), and two p-substituted phenyl groups (6y, 6z).

Synthesis of compounds 6r–z followed the same synthetic Scheme 1 (see Methods for detailed description), and their affinity toward ADC-7 (Kᵢ)’s and the enhanced activity with the antibiotic ceftazidime (CAZ) or cefepime (FEP) (MICs) are summarized in Table 3.

All compounds from Series V present nanomolar (Kᵢ = 90 nM) to low micromolar (Kᵢ = 1.46 μM) inhibitory activity against ADC-7 β-lactamase. To assess the capability of these compounds to restore β-lactam susceptibility, broth microdilution MICs were performed against three bacterial strains expressing class C β-lactamases: E. coli DH10B carrying blaCTXM-9 and P. aeruginosa 18SH strain, blaPDC) was ceftazidime (CAZ) with increasing concentrations from 0.12 to 128 μg/mL. The antibiotic partner for class A β-lactamases and one expressing a class A β-lactamase. The microdilution MICs were performed in 200 μL wells, with BATSIs concentrations maintained at 4 μg/mL. The antibiotic partner for class A β-lactamases and one expressing a class A β-lactamase. The microdilution MICs were performed in 200 μL wells, with BATSIs concentrations maintained at 4 μg/mL. The antibiotic partner for class A β-lactamases and one expressing a class A β-lactamase. The microdilution MICs were performed in 200 μL wells, with BATSIs concentrations maintained at 4 μg/mL. The antibiotic partner for class A β-lactamases and one expressing a class A β-lactamase. The microdilution MICs were performed in 200 μL wells, with BATSIs concentrations maintained at 4 μg/mL.
became susceptible to cefepime (FEP MICs decrease from 8 to 1 or 2 μg/mL). The other compounds lowered the FEP MICs by 1-fold.

Crystallographic Structures of ADC-7/Novel BATSIs Complexes. To identify the structural basis for the observed inhibition of ADC-7 by these novel triazole boronic acids as well as to confirm the triazole functionality as a bioisostere for the R1 amide group found in the natural β-lactam substrates, X-ray crystal structures of five ADC-7/BATSI complexes were determined. Two compounds from Series I (Table 1, 6d and 6e), one compound from Series II (6f), the most effective inhibitor (6q), and one from Series V (6r) were selected for crystallographic analysis.

The ADC-7/BATSI complexes were determined to resolutions ranging from 1.80 to 2.04 Å (Table 4). In summary, all complexes crystallized in the P2₁ space group with four

### Table 2. Binding Affinities ($K_i$) of Compounds 6a–q and Their Contribution to Ceftazidime (CAZ) Susceptibility (MIC)$^a$

| Compound | Structure | R         | $K_i$ (μM) ADC-7 | E. coli DH10B blaADC-7 MIC CAZ ($\mu$g/mL) |
|----------|-----------|-----------|-----------------|------------------------------------------|
| 6a       |           | -H        | 0.60 ± 0.04     | 2                                       |
| 6b       |           | -CH₃      | 0.49 ± 0.05     | 4                                       |
| 6c       |           | -SO₂NH₂   | 1.61 ± 0.2      | 4                                       |
| 6d       |           | -CO₂H     | 0.90 ± 0.12     | 2                                       |
| 6e       |           | -CONH₂    | 0.20 ± 0.03     | 2                                       |
| 6f       |           |           | 1.0 ± 0.2       | 8                                       |
| 6g       |           |           | 1.6 ± 0.2       | 4                                       |
| 6h       |           |           | 5.32 ± 0.6      | 4                                       |
| 6i       |           | -H        | 2.84 ± 0.3      | 8                                       |
| 6j       |           | -Cl       | 0.98 ± 0.1      | 4                                       |
| 6k       |           | -4-OCH₅   | 1.54 ± 0.2      | 4                                       |
| 6l       |           | -3-NHAc   | 1.52 ± 0.2      | 8                                       |
| 6m       |           |           | 8.69 ± 1        | 8                                       |
| 6n       |           |           | 14.54 ± 2       | 8                                       |
| 6o       |           |           | 3.38 ± 0.4      | 8                                       |
| 6p       |           |           | 3.38 ± 0.4      | 8                                       |
| 6q       |           |           | 0.09 ± 0.01     | 2                                       |

$^a$In contrast, the vaborbactam affinity for ADC-7 is 0.72 ± 0.1 μM.

[Crystallographic Structures of ADC-7/Novel BATSIs Complexes.](https://dx.doi.org/10.1021/acsinfecdis.0c00254)ACS Infect. Dis. 2020, 6, 1965−1975

### Chart 1. Correlation between Synergistic Activity of BATSIs in Combination with CAZ (MICs) against E. coli Expressing $bla_{ADC-7}$ and Their Binding Affinity for Purified ADC-7 Enzyme ($K_i$)

[Crystallographic Structures of ADC-7/Novel BATSIs Complexes.](https://dx.doi.org/10.1021/acsinfecdis.0c00254)ACS Infect. Dis. 2020, 6, 1965−1975

### Table 3. Binding Affinities ($K_i$ Values) of Series V BATSI Compounds against ADC-7 Enzyme and MIC Values (μg/mL) of CAZ or FEP in Combination with 4 μg/mL of Series V BATSI

| Compound | $K_i$ (μM) ADC-7 | E. coli DH10B $bla_{ADC-7}$ MIC CAZ (μg/mL) | E. coli DH10B $bla_{CMY-2}$ MIC CAZ (μg/mL) | P. aer. 18SH $bla_{PDC}$ MIC CAZ (μg/mL) | E. coli DH10B $bla_{CTX-M-9}$ MIC CAZ (μg/mL) |
|----------|-----------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| CAZ      | 16              | 128                                         | 64                                          | 64                                          | 84                                          |
| FEP      |                 |                                             |                                             |                                             |                                             |
| 6q       | 0.09 ± 0.01     | 2                                           | 16                                          | 32                                          | 1                                           |
| 6r       | 1.46 ± 0.2      | 4                                           | 32                                          | 32                                          | 4                                           |
| 6s       | 0.31 ± 0.04     | 2                                           | 8                                           | 64                                          | 2                                           |
| 6t       | 1.02 ± 0.1      | 2                                           | 32                                          | 64                                          | 4                                           |
| 6u       | 0.39 ± 0.3      | 2                                           | 64                                          | 64                                          | 8                                           |
| 6v       | 0.77 ± 0.08     | 2                                           | 32                                          | 64                                          | 4                                           |
| 6x       | 0.31 ± 0.02     | 2                                           | 32                                          | 64                                          | 2                                           |
| 6w       | 0.70 ± 0.08     | 2                                           | 32                                          | 32                                          | 4                                           |
| 6y       | 0.84 ± 0.07     | 2                                           | 32                                          | 64                                          | 4                                           |
| 6z       | 0.21 ± 0.03     | 2                                           | 64                                          | 64                                          | 4                                           |
molecules in the asymmetric unit, as previously observed for ADC-7/BATSI complexes.8,9,10,11 The quality of the final models was evaluated with the wwpdb validation service18 and showed that 96–98% of all residues were in the favorable region, with 2–4% in the allowed region, of the Ramachandran plots. The complexes with 6f, 6q, and 6r were obtained by soaking ADC-7 crystals in inhibitor solutions, and the complexes with 6d and 6e were obtained through cocrySTALLization.

In each case, the initial \(F_o - F_c\) electron density maps (contoured at 3σ) revealed unambiguous density that accounted for the presence of the inhibitor bound in the active site as well as covalent attachment to the catalytic Ser64. Inhibitors were built into the observed difference density, and the models were refined with Refmac5.19 PDB-REDO was used to analyze and improve models between rounds of manual rebuilding in Coot.20 Overall, these five structures confirm that, in ADC-7, the triazole is an effective amide bioisostere.

### Specific Characteristics of the ADC-7/Series I Complexes

In each of the monomers of the ADC-7/6d and ADC-7/6e complexes (Figure 4C,B), the inhibitor is bound in the active site in similar conformations and follows a similar trajectory. The most variability is observed at the distal end of the inhibitors. In ADC-7/6d (Figure 4C), the linear trajectory of the inhibitor orients the benzene group toward the lip of the active site, with the carboxy sensitive group making a hydrogen bond with the main chain nitrogen of Ser317, via a water molecule, whereas in the ADC-7/6e complex (Figure 4B), the inhibitor is bound in the active site in similar conformations and follows a similar trajectory. The placement of the benzamide group is seen in two distinct conformations. In one (B and C monomers), the benzamide is oriented toward Arg340, with the benzamide oxygen making a long hydrogen bond with this residue (3.2 Å). In the other (A and D monomers), the benzyl group is rotated ∼180° with the benzamide oriented away from Arg340.

### Specific Characteristics of the ADC-7/Series II Complex

In the ADC-7/6f complex (Figure 4A), the inhibitor binds in the same conformation in all active sites of the four monomers. A thiope represents the asy ring of Series I compounds at the distal end but does not make favorable interactions with the enzyme. Additionally, the shorter length of this inhibitor does not extend to the lip of the active site, where interactions with Ser317 were observed in the Series I complexes, and Arg340 is oriented toward the active site, likely due to the smaller sized inhibitor.

### Table 4. Crystallographic Summary for ADC-7/Boronic Acid Complexes

|                  | ADC-7/6d | ADC-7/6e | ADC-7/6f | ADC-7/6q | ADC-7/6r |
|------------------|----------|----------|----------|----------|----------|
| **cell constants (Å, deg)** |           |          |          |          |          |
| \(a\)             | 89.62    | 88.77    | 88.55    | 88.48    | 88.93    |
| \(b\)             | 80.78    | 81.25    | 81.46    | 80.62    | 81.10    |
| \(c\)             | 107.00   | 105.92   | 105.67   | 105.11   | 105.94   |
| \(\beta\)          | 112.47   | 112.93   | 113.10   | 113.46   | 113.06   |
| space group       | P2₁      | P2₁      | P2₁      | P2₁      | P2₁      |
| resolution (Å)    | 98.88–1.96 (1.964–1.957) | 97.55–1.82 (1.822–1.816) | 97.20–2.04 (2.042–2.033) | 50.00–1.80 (1.86–1.80) | 81.82–1.74 (1.837–1.746) |
| unique reflections | 96293 (988) | 123054 (1239) | 86979 (870) | 125707 (12526) | 116473 (5826) |
| \(R\)\(_{merge}\) (%) | 5.0 (41.5) | 5.4 (46.6) | 7.2 (64.7) | 9.0 (71.4) | 8.6 (57.1) |
| \(R\)\(_{free}\) (%) | 2.9 (23.5) | 3.1 (26.5) | 4.3 (37.8) | 4.9 (39.2) | 5.6 (39.3) |
| CC(1/2)           | 0.999 (0.929) | 0.998 (0.905) | 0.995 (0.750) | 0.940 (0.760) | 0.995 (0.647) |
| completeness (%)   | 94.6 (98.9) | 98.4 (98.8) | 98.0 (98.6) | 100.0 (100.0) | 89.1 (50.0) |
| \(〈I/σ〉\)        | 13.3 (2.0) | 11.4 (1.9) | 11.1 (2.1) | 8.48 (2.6) | 8.0 (1.5) |
| resolution range for refinement (Å) | 98.88–1.96 | 97.55–1.82 | 97.19–2.04 | 44.26–1.80 | 81.95–1.74 |
| number of protein residues | 1424 | 1422 | 1422 | 1425 | 1423 |
| number of water molecules | 330 | 488 | 236 | 694 | 871 |
| RMSD bond lengths (Å) | 0.005 | 0.005 | 0.006 | 0.008 | 0.007 |
| RMSD bond angles (deg) | 1.38 | 1.30 | 1.53 | 1.51 | 1.52 |
| \(R\)\(_{factor}\) (%) | 21.9 | 22.0 | 22.1 | 19.4 | 21.6 |
| \(R\)\(_{free}\) (%) | 25.1 | 25.6 | 25.4 | 23.8 | 27.3 |
| average B-factor, protein atoms (Å\(^2\)) | 44.77 | 47.41 | 46.86 | 35.54 | 40.68 |
| average B-factor, inhibitor atom (Å\(^2\)) | 58.36 | 66.93 | 48.98 | 58.3 | 54.82 |

Values in parentheses are for the highest resolution shell. \(^b\)\(R\)\(_{free}\) was calculated with 5% of reflections set aside randomly.
Specific Characteristics of the ADC-7/Series IV Complex. Series IV explored more flexible groups that extend from the triazole ring. The sulfonamide linker (Figure 4D) displayed the best inhibition of all molecules tested from any of the series ($K_{i}^\text{eq}$ 90 nM). Interestingly, the sulfonamide group itself does not make any favorable interactions with the enzyme. Arg340 is positioned out of the active site, with the distal tolyl group making favorable cation--π interactions with this residue. Distances from Arg340 to the centroid of the aryl ring range from 3.8 to 4.4 Å.

Specific Characteristics of the ADC-7/Series V Complex. To improve the binding affinity of 6q, Series V molecules were designed. The structure of ADC-7 in complex with 6r (Figure 4E), which replaces the tolyl group with a trifluoromethyl, was determined. The sulfonamide is oriented near Arg340 but is not within hydrogen bonding distance in monomers A and B, where Arg340 is swung out away from the active site. However, in monomers C and D, Arg340 adopts a
conformation that positions it into the active site, and in these instances, the sulfone interacts with this residue (2.5–2.9 Å). The distal trifluoromethyl substituent is bent away from Arg340 and does not favorably interact with any residues in the active site.

## DISCUSSION AND CONCLUSION

This study explores the α-triazolylmethaneboronic acid scaffold as a good template for ADC β-lactamase inhibition. Whereas boronic acids have been identified as protease inhibitors since the 1970s, only in the past decade has this class of compounds been recognized as effective “bullets” in the antimicrobial resistance arsenal.

α-Acylaminoboronic acids (Figure 1A) have been designed as a good starting point to gain the proper interactions with the enzyme. Indeed, several crystal structures of different β-lactamase/α-amidomethaneboronic acid complexes point to the presence of an amide binding site with specific enzyme residues always interacting with the amide. In previous work on ADC-7, the triazole-containing compound S06017 (Figure 1) was synthesized, tested, and co-crystallized with the enzyme.

From the crystal structure, we observed that the triazole could behave as a good amide bioisostere, with two lone pair nitrogens able to interact with the canonical R₁ amide recognition residues Asn152 and Gln120 that hydrogen bond with the two lone pairs of the amide oxygen. Given the easy and mild access to the triazole ring, with wide functional group tolerance, we wanted to prove triazole is a good amide bioisostere and to improve the activity of S06017 (Kᵢ 6.11 μM). Therefore, in this work, we designed and synthesized 26 α-triazolylmethaneboronic acids, differing the substituent at position 4 of the triazole. The Kᵢ values of these compounds vary from 90 nM to 38 μM, thus confirming a good general affinity for the enzyme and a consistent difference in activity due to insertion of varying functional groups.

Compounds with a substituted phenyl ring directly attached to the triazole (Series I, compounds 6a–e) proved to be very active with Kᵢ values spanning to 200 nM to 1.61 μM. Two compounds (6d and 6e) from this series were crystallized in complex with ADC-7: these complexes confirmed that the triazole makes two of the three canonical interactions of the β-lactam side chain, thus behaving as a good amide bioisostere. Furthermore, from the crystal structure, the benzamide carbonyl oxygen of the best inhibitor from this series, 6e (Kᵢ 200 nM), makes a hydrogen bond with Arg340 (3.2 Å), suggesting the role that interactions with Arg340 may play in increasing binding affinity for these BATSIis. With 6d, the carboxylate group of the benzoate is flipped ~180° from the benzamide, positioning the negatively charged group away from Arg340. A favorable ionic interaction might be expected between these groups in the other conformation, but rotation of the benzoate results in a steric clash between the two. Therefore, the carboxylate group is instead oriented toward the solvent.

The replacement of the substituted phenyl ring with an electron rich (i.e., the thiophene in 6f) or electron poor (the pyridine and pyrazine in 6g and 6h, respectively) heterocycle maintain a similar level of activity (Kᵢ’s from 1 to 5.3 μM). From this Series II, the structure of the enzyme in complex with compound 6f was superposed with the ADC-7/6e complex (Figure 5). The two compounds have a 5-fold difference in activity (Kᵢ of 1 μM for 6f vs 200 nM for 6e): indeed, the thienyl ring is placed in the same position as the phenyl ring from Series I and does not take advantage of any specific interaction with the enzyme. The most distinctive difference between the two structures is the positioning of Arg340, a residue that exhibits flexibility: ADC-7/6f shows Arg340 oriented toward the active site in the presence of the smaller thienyl inhibitor. In contrast, the ADC-7/6e complex (yellow) shows Arg340 oriented away from the active site to accommodate the binding of a larger inhibitor and to be positioned at a proper distance for hydrogen bonding.

In an attempt to gain interactions with Arg340, Series III and IV were synthesized to elongate the substitutent on the triazole. The addition of a substituted phenoxymethyl linker as in Series III (compounds 6i–l) did not significantly improve activity (Kᵢ’s from 0.98 to 2.84 μM), whereas the substituted aminomethyl bridge exploited the most significant differences. In Series IV, activity in fact dramatically dropped when a protonated aminomethyl (compound 6m) or acylamino side chain (compounds 6n and 6p) was introduced (Kᵢ’s from 8.7 to 33.8 μM). In contrast, compound 6q with a p-tolysulfonamido substituent displayed the best activity among the α-triazolyl BATSIis (Kᵢ 90 nM), pointing to 6q as one of the best achiral inhibitors of class C β-lactamases. The analysis of the ADC-7/6q complex revealed how the tetrahedral geometry of the sulfonamide, as in 6q, allows for cation–π interactions with Arg340 (Figure 6), which is probably not reached when a planar geometry is introduced through an amide linker as in 6p.

Notably, the structure of 6q does not resemble the natural substrate of the β-lactamase but displays a pronounced inhibition activity. In fact, when compared to α-acetylamidineboronic acids previously synthesized (Figure 7), compound 6q (Kᵢ 90 nM) is 3 times more active than the boronic acid bearing the ceftazidime side chain (Kᵢ 310 nM) and almost 9 times more active than the cephalothin analog (Kᵢ 780 nM). The activity of the α-triazolylboronic acid is significantly

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**Figure 5.** Flexibility of Arg340 in inhibitor recognition. Superposition of ADC-7 in complex with 6f (green) and 6e (yellow).
less compared to the one of the \( \alpha \)-sulfonylamidomethaneboronic acids bearing a distal tetrazole in the side chain (compound CR192). From the structural analysis of such derivatives, it became evident how the increase in activity was due to the interaction of the negatively charged tetrazole with a distal binding site formed by Asn213 and Ser317.

Given the length and trajectory of 6q, the distal functional groups of this molecule do not extend to the outer edge of the active site where Asn213 is located. However, the C\( \beta \) atom of Ser317 is within the van der Waals distance of the aryl ring of 6q (~4.3–4.5 Å), thus giving the opportunity for further optimizing the molecule. A validation of the \( \alpha \)-triazolymethaneboronic structure of 6q as a template for further derivatization was obtained through microbiological assays in \( E. \) \textit{coli} expressing ADC-7 of compounds 6a–p. All compounds lowered the MIC (16 \( \mu \)g/mL) of CAZ from 1- to 4-fold, and the MIC values were in good agreement with \( K_i \)’s (Chart 1), thus confirming a good permeability of these compounds.

In an attempt to improve 6q activity and eventually reach the distal binding site of ADC-7, we obtained an additional nine compounds (Figure 2, Series V, compounds 6r–z). Unfortunately, none of the compounds of Series V improve activity toward ADC-7 with \( K_i \)’s spanning from 0.21 to 1.46 \( \mu \)M (Table 3). Compound 6r was crystalized in complex with ADC-7. In 6r, the tolyl group of 6q is replaced with a trifluoroethyl group, which is unable to make the cation–π interaction seen in the ADC-7/6q complex: Arg340 in fact points away, likely resulting in lower binding affinity of the compound. Compounds 6t–x all contain a methylene linker that may extend the distal group away from Arg340 and prevent this interaction, resulting in lower binding affinities as well. Compounds 6y and 6z more closely resemble 6q and 6r as they lack the flexible methylene linker. Compound 6y contains a cyano group as compared to the tetrazole of 6z, which might impact the ability of the aryl rings to form cation–π interactions with Arg340. Overall, the lower binding affinities of Series V might point to the inability of reaching the distal binding site (Arg213 and Ser317) and at the same time to the loss of interaction with Arg340, which is a residue that is unique to the ADC enzymes as compared to other class C \( \beta \)-lactamases. Known to be a contributor to protein–protein and protein–ligand interactions, the cation–π interaction observed in these ADC-7/inhibitor complexes suggests that it is important for the design of future series. Arg340 may be a key residue to target as it is unique to this class of enzymes and has shown the ability to interact with a variety of different functional groups (amide, carboxylate, trifluoromethyl, phenyl) in a variety of different interactions, such as Coulombic, ionic, hydrogen bond, and cation–π. In addition, the flexibility shown by Arg340 allows ADC-7 to accommodate BATSIs with larger R1 groups that are able to reach the residues at the lip of the active site (such as Asn213 and Ser317).

In summary, when the highly efficient and versatile synthetic method known as click chemistry is employed, a new class of \( \beta \)-lactamases inhibitors has been synthesized, starting from the easily accessible pinanediol azidomethaneboronate. All 26 BATSIs displayed \( K_i \) values spanning from low micromolar to nanomolar values, with compound 6q being among the best achiral inhibitors of the class C \( \beta \)-lactamases. Five of these inhibitors were crystallized in complex with ADC-7 revealing that, besides the interaction of the boronic moiety with the catalytic serine residue, the triazole is able to maintain the typical interactions of the extensively explored and parent amidomethaneboronic inhibitors, thus acting as a good amide biosostere. Finally, this new class of inhibitors proved to be able to restore CAZ and FEP activity against class C and A \( \beta \)-lactamase strains.

### METHODS

**Synthesis.** Reactions were monitored by thin layer chromatography (TLC), which were visualized by UV fluorescence and by Hanessian’s cerium molybdate stain. Deoxygenated water was obtained through sonication. Chromatographic purification of the compounds was performed on silica gel (particle size 0.05–0.20 mm). Melting points were measured in open capillary tubes on a Stuart SMP30 Melting Point apparatus. Optical rotations were determined at 20 °C on a PerkinElmer 241 polarimeter and are expressed in 10\(^{-1}\) deg cm\(^{-1}\) g\(^{-1}\). \( ^1\)H and \( ^13\)C NMR spectra were recorded on a Bruker Avance-400 MHz spectrometer. Chemical shifts (\( \delta \)) are reported in ppm and were calibrated to the residual signals of the deuterated solvent.\(^{12} \) 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 \( ^1\)H and \( ^13\)C spectra. Particularly, in

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**Figure 6.** Cation–π interactions between Arg340 and the aryl ring of 6q, the BATSi with the highest affinity to ADC-7. Interactions, indicated by dashed yellow lines, are drawn from Arg340 to the centroid of the aryl ring, with distances ranging from 3.8 to 4.4 Å.

**Figure 7.** Structures and \( K_i \) values of previously synthesized \( \alpha \)-acylamidoboronic acids.
the $^{13}$C spectra, the signal of the boron-bearing carbon atom, which tends to be broadened, and the signal of the quaternary triazole carbon are often beyond the detection limit, but their resonances were unambiguously determined by HSQC and HMBC; melting points of free boronic acids 6b–z were not reproducible due to dehydration. 23 Mass spectra were determined on an Agilent Technologies LC-MS (n) Ion Trap 6310A (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 analytical HPLC-MS (see the Supporting Information for a detailed description). Synthesis and characterization of compounds 2, 3, 4b–e, 4g–h, 4j–w, 4y, 5b–e, 5g–h, 5j–w, 5y, 5z, 6b–e, 6g–h, and 6j–z are reported in the Supporting Information.

**Microbiology.** MICs were performed as previously described and according to Clinical and Laboratory Standards Institute (CLSI) guidelines, using a 6 × 10^6 cfu/mL inoculum. Bacterial cultures were grown overnight in Mueller-Hinton (MH) broth supplemented with 20 μg/mL chloramphenicol. We employed the E. coli construct that was previously validated as a representative of ADC-7 in a uniform genetic background (bla_{ADC-7} was directionally cloned in pBc SK (−) phagemid vector). Bacterial liquid culture was diluted using MH broth to a 6 × 10^7 cfu/mL final concentration, and the antibiotic partner, CAZ or FEP, was added at concentrations from 128 to 0.06 μg/mL. BATSIs were constant at 4 μg/mL. The plates were incubated at 37 °C overnight, and the results were recorded the next day.

**Purification and Kinetics.** ADC-7 β-lactamase was expressed as previously described and purified using cation exchange chromatography. For the purification of ADC-7, cell pellets were suspended in 25 mM 3-N-morpholino) propane-sulfonic acid (MOPS buffer), pH 6.5, with 1× HALT protease inhibitor cocktail (Sigma) and DNase I (50 Units). The solution was sonicated for 4 × 30 s intervals on ice. The lysate was centrifuged at 15 000 rpm at 4 °C for 20 min. The cell-free extract was then loaded onto a carboxymethyl-cellulose column by gravity flow at 4 °C (5 mL resin per gram of cell pellet). The column was washed with 100 mL of 25 mM MOPS, pH 6.5, at a flow rate of 0.3 mL/min followed by elution with a linear gradient of 0–0.5 M NaCl in 25 mM MOPS, pH 6.5. The fractions containing ADC-7 were collected, pooled, and then dialyzed in 2 × 5 L of 25 mM MOPS, pH 6.5 at 4 °C. The dialyzed ADC-7 was concentrated to at least 10 mg/mL using an Amicon Ultra centrifugal filter unit with Ultra-10 membrane (Millipore). The concentration of ADC-7 was determined using the A280 with an extinction coefficient of 46 300 M⁻¹ cm⁻¹, as calculated for the expressed residues D24-K383 of ADC-7 by the ProtParam tool on the ExPASy bioinformatics portal.

The inhibition constants (K_i) for each of the BATSIs with ADC-7 were determined using competition kinetics. When nitrocefin (NCF) was utilized as a colorimetric substrate of ADC-7 β-lactamase as previously described. The measurements of the initial velocities were performed with the addition of 100 μM NCF after a 5 min preincubation of the enzyme (2 nM) with increasing concentration of the inhibitor. To determine the average velocities (ν_0), data from three experiments were fit to the equation:

$$\nu_0 = \frac{\nu[I]}{IIC_{50} + [I]}$$

where ν_0 represents the NCF uninhibited velocity and IC_{50} represents the inhibitor concentration that results in a 50% reduction of ν_0. The K_i values for all 26 BATSIs were corrected for the NCF affinity (K_m = 20 μM) with the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{K_m + \frac{[NCF]}{K_m}}$$

The data analysis was performed using EnzFitter and Origin 2019b.

**Crystallography and Structure Determination.** Structures of ADC-7 in complexes with the inhibitors were obtained by both soaking and cocrystallization methods. For soaks, ADC-7 crystals were grown via hanging drop vapor diffusion at room temperature as previously described. 11 Preformed crystals were harvested using a nylon loop and soaked in crystallization buffer containing the BATSI at concentrations ranging from 2 to 16 mM for between 5 and 25 min. Co-crystals were grown in 0.1 M sucinate/phosphate/glycine (SPG buffer), pH 5.0, 25% w/v PEG-1500, with 3.5–3.75 mg/mL ADC-7 and 1 mM BATSIs in the initial crystallization buffer.

Data for each of the complexes were measured from single crystals at the Advanced Photon Source at Argonne National Laboratory (LS-CAT sector). All diffraction images were processed with XDS 25 with the exception of the ADC-7/6q data set, where HKL2000 26 was used. For the ADC-7/6r data set, additional processing of the structure factors was performed using STARANISO.27 Structures were determined by molecular replacement with Phaser, using the ADC-7/S02030 complex (PDB 4U0X), with water, ion, and inhibitor atoms removed, as the starting model. Refinement of the models was done with Refmac5 in the CCP4 suite, and model building was done with Coot.20 The coordinates and structure factors for the ADC-7/BATSI complexes were deposited in the Protein Data Bank with the following codes: 6TZF (6d), 6TZG (6e), 6TZH (6f), 6TZI (6r), and 6TJZ (6q).

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00254.

Synthesis and characterization of compounds 2, 3, 4a–z, 5b–z, and 6b–z; a statement of purity of compounds 6b–z; copies of ^1H and ^13C NMR spectra of compounds 6b–z (PDF)

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E.C., M.S., M.L.I., F.F., and F.P. synthesized and characterized all of the BATSI compounds. M.A.T. and R.A.B. performed microbiological assays and kinetics. R.A.P., B.J.W., E.R.F., and K.A.S. determined all of the crystal structures. P.N.R. contributed to the analysis of the data. E.C. wrote the first draft of the manuscript; all authors have contributed and have given approval to the final version of the manuscript.

Notes

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ABBREVIATIONS

BATSI, boronic acid transition state inhibitors; ADC, Acinetobacter derived cephalosporinase; MICs, minimum inhibitory concentrations; CAZ, ceftazidime; FEP, cefepime; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; LC/MS, liquid chromatography/mass spectrometry; CuAAC, copper-catalyzed alkyne azide cycloaddition; t-BuOH, tert-butanol

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