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Synthesis and Evaluation of 3-(Dihydroxyboryl)benzoic Acids as D,D-Carboxypeptidase R39 Inhibitors

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Penicillin binding proteins (PBPs) catalyze steps in the biosynthesis of bacterial cell walls and are the targets for the β-lactam antibiotics. Non-β-lactam based antibiotics that target PBPs are of interest because bacteria have evolved resistance to the β-lactam antibiotics. Boronic acids have been developed as inhibitors of the mechanistically related serine β-lactamases and serine proteases; however, they have not been explored extensively as PBP inhibitors. Here we report aromatic boronic acid inhibitors of the D,D-carboxypeptidase R39 from *Actinomadura* sp. strain. Analogues of an initially identified inhibitor [3-(dihydroxyboryl)benzoic acid **1**, IC_{50} 400 μM] were prepared via routes involving pinacol boronate esters, which were deprotected via a two-stage procedure involving intermediate trifluorborate salts that were hydrolyzed to provide the free boronic acids. 3-(Dihydroxyboryl)benzoic acid analogues containing an amide substituent in the meta, but not ortho position were up to 17-fold more potent inhibitors of the R39 PBP and displayed some activity against other PBPs. These compounds may be useful for the development of even more potent boronic acid based PBP inhibitors with a broad spectrum of antibacterial activity.

Introduction

The bacterial cell wall consists of a complex network of sugars connected to peptides that are cross-linked by the transpeptidase activity of penicillin binding proteins (PBPs[a]) (reviewed in Macheboeuf et al.[1] and Sauvage et al.[2]). PBP catalysis proceeds via acyl-enzyme intermediates formed by nucleophilic reaction of a serine residue onto the amide carbonyl group of a C-terminal D-ala-D-ala dipeptide fragment of a stem peptide (Figure 1A) with the concomitant loss of the terminal D-ala fragment. The acyl-enzyme intermediate then undergoes reaction with a nucleophilic side chain, often lysine (in Gram-positive bacteria or diaminopimelic acid (DAP) Gram-negative bacteria), from a neighboring stem peptide to form a cross-linked peptide with regeneration of the free PBP.

The PBPs can be classified into high molecular weight and low molecular weight enzymes. Within the high molecular weight class, there are two subclasses: the bifunctional class A and monofunctional class B (which may also be bifunctional). The low molecular weight PBPs are sometimes referred to as class C PBPs.[1] The high molecular weight proteins are generally multifunctional, and their inhibition leads to cell death. Inhibition of the low molecular weight proteins is generally nonlethal but leads to a change in the cell wall cross-linking pattern.[3] β-Lactam antibiotics (e.g., penicillins) react irreversibly with the nucleophilic serinyl residue of the PBP, causing irreversible inhibition (Figure 1B). Bacteria have developed resistance to β-lactams by mechanisms including the production of β-lactamases that catalyze β-lactam hydrolysis (Figure 1B), and production of PBPs that no longer recognize, or have reduced affinity for, β-lactams. While attempts have been made to prepare alternative lactam structures that also lead to a stable acyl-enzyme complex (including both synthetic compounds[4,5] and derivatives of the natural product γ-lactam, lactivicin[6−9]), these compounds are also likely to be prone to hydrolysis by β-lactamases. Thus, there is a need to develop transpeptidase inhibitors that are not acylating agents.

Boronic acids have received attention as inhibitors of enzymes employing nucleophilic catalysis, in particular as inhibitors of serine and other proteases (reviewed by Yang et al.[10]). Inhibition of nucleophilic enzymes by boronic acids employs the electron deficient nature of the boron atom in its trivalent form which enables it to form covalent, "tetrahedral" adducts with nucleophilic residues (Figure 1C). Boronic acid inhibitors of both the AmpC[11−14] and TEM β-lactamases[15−17] have been reported. However, boronic acids have received relatively little attention as PBP inhibitors. The kinetics of peptide based boronic acid inhibitors of PBP3, PBP4, and PBP5 from *Neisseria gonorrhoeae* have been studied[18] and a crystal structure of a peptide boronic acid in complex with PBP5 has been reported.[19] Phenyl- and methylboronic acids have been tested against these same enzymes, and modest activity was observed for phenylboronic acid against PBP3.[20]

Here we describe the identification and development 3-(dihydroxyboryl)benzoic acid derivatives as inhibitors of the D,D-carboxypeptidase R39 from *Actinomadura* sp. strain (R39). R39 is a low molecular weight PBP that is related to

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[a] Abbreviations: PBP, penicillin binding protein; RA, residual activity.

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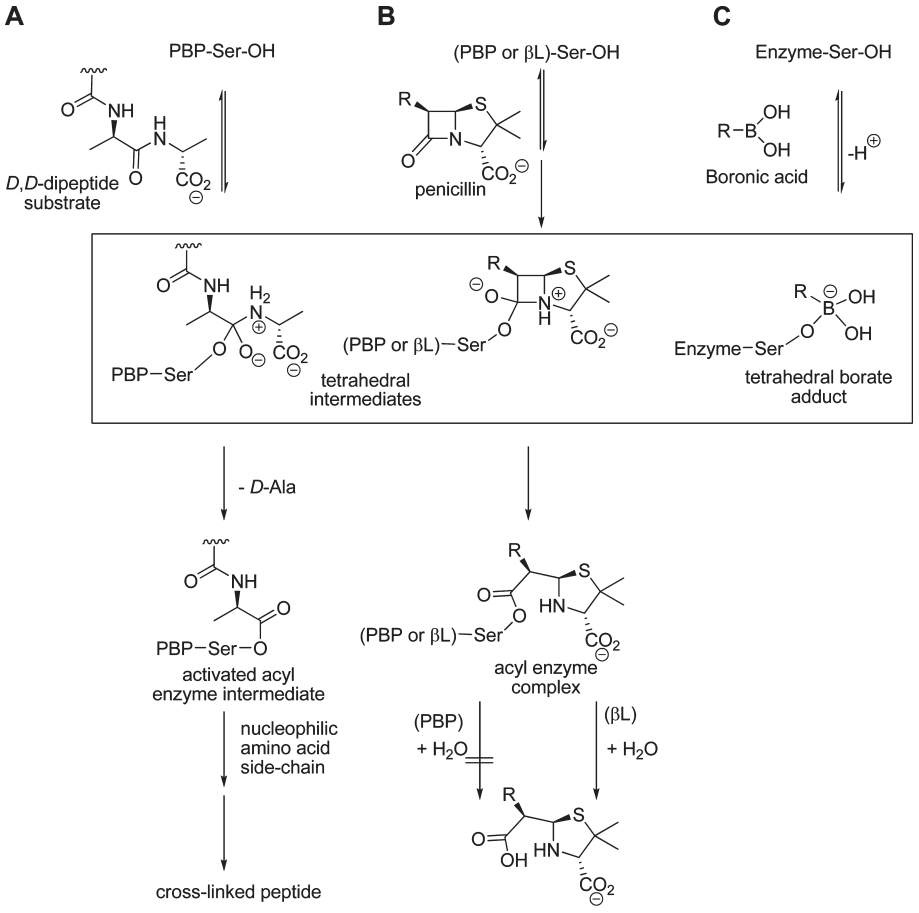


Figure 1. Reactions of nucleophilic serine enzymes: (A) transpeptidase activity, (B) differential reactivities of PBPs and serine β-lactamases (βL) with a β-lactam antibiotic, and (C) reaction with a boronic acid inhibitor.

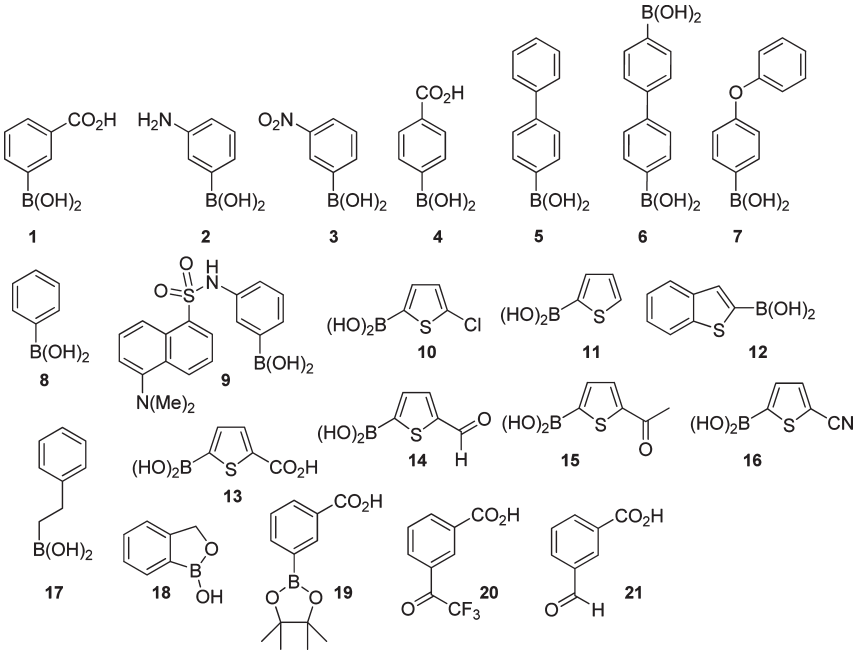
PBP4 from *Escherichia coli*,[21] PBP4a from *Bacillus subtilis*,[22] and PBP3 from *Neisseria gonorrheae*.[23] With a view to development of potent inhibitors of more clinically important high molecular weight PBPs (e.g., PBP1b, PBP2xR6, and the penicillin resistant strain PBP2x5204), we used R39 as a model system with established kinetic assays for the initial development stages. We identified lead compounds from a set of commercially available boronic acids tested against three different PBPs. We found that 3-(dihydroxyboryl)benzoic acid **1** was a modest inhibitor of R39. The design and synthesis of analogues of **1** are described, from which we developed inhibitors with improved potency for R39.

Identification of Arylboronic Acids as R39 Inhibitors

Initially, a set of commercially available boronic acids including phenylboronic acids (**1**−**9**), thiophenyl boronic acids (**10**−**16**), an alkylboronic acid **17**, a bicyclic benzoxaborole **18**, and a boronic acid pinacol ester **19** were tested for inhibition of the R39 D,D-peptidase from *Actinomadura* sp. strain and of PBP2xR6 and PBP2x5204 (penicillin resistant) from *Streptococcus pneumoniae*. The assays involved hydrolysis of a reporter substrate (*R*)-[2-(benzoylamino)propionylsulfanyl]acetic acid[24] in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (monitoring at 412 nm) in a procedure similar to that reported.[7] Four compounds (**1**, **8**, **18**, and **19**) displayed at least 50% inhibition of the R39 enzyme at the initially tested concentrations (1 mM for **1** and **18**; 0.5 mM for compounds **8** and **19**, which were not fully soluble under the assay conditions at 1 mM) (Table 1). The most potent inhibitor identified in this initial screen was **1** [residual activity (RA) of 20% at 1 mM], in both the absence and presence of Triton-X detergent, which is used to test for the possibility of nonspecific interactions/aggregation.[25,26] The IC_{50} of **1** was determined as 400 ± 19 μM; the corresponding pinacol ester **19** showed similar activity (RA = 38% at 500 μM), suggesting that pinacol esters are hydrolyzed under the assay conditions. The thiophene boronic acid **13**, analogous with **1**, displayed less inhibition (57% at 1 mM), while the remaining thiophene analogues **10**, **11**, and **14**−**16** and the known β-lactamase inhibitor **12**[13] were even less active. The derivatives with bulky substituents (**5** and **6**) were poorly soluble under the assay conditions and could only be tested at a lower concentration of 100 μM, at which they showed negligible inhibition. The trifluoromethyl and formyl analogues (**20** and **21**) of **1** were also tested because reactive carbonyl compounds are used as inhibitors of nucleophilic enzymes.[27−29] These compounds displayed some inhibition; however, they were less active than the boronic acid **1**. This observation is consistent with work on proteases where boronic acid inhibition was more potent than with an analogous aldehyde[30] or fluorinated ketone.[31] None of the set showed any significant inhibition against the PBP2xR6 and PBP2x5204 penicillin resistant strains (Supporting Information).

These initial tests suggested that phenylboronic acids with a carboxylic acid at the meta- position (as in **1**) can inhibit R39. In the case where the carboxylate group was at the para-position (as in **4**), no inhibition was observed. Similarly, when the carboxylate group was absent as in **8**, reduced inhibition

Table 1. Inhibition Data for the Initial Set of Compounds Tested as R39 Inhibitors



compd	concn (μM)	residual activity (%)[a]	compd	concn (μM)	residual activity (%)[a]
1	1000	20 ± 5 ($IC_{50} = 400 \pm 19$)	**12**	1000	86 ± 5
2	1000	82 ± 3	**13**	1000	57 ± 6
3	1000	85 ± 3	**14**	1000	99 ± 2
4	1000	84 ± 4	**15**	1000	88 ± 3
5	100	104 ± 5	**16**	1000	95 ± 4
6	100	97 ± 5	**17**	1000	91 ± 5
7	1000	98 ± 2	**18**	1000	45 ± 5
8	500	50 ± 3	**19**	500	38 ± 4
9	500	60 ± 8	**20**	1000	60 ± 2
10	1000	91 ± 5	**21**	1000	77 ± 3
11	1000	88 ± 6			

[a] Quoted values are mean $\pm$ standard deviation over three replicate experiments.

was observed. In cases where alternative substituents were present (as in **2** and **3**), only modest inhibition was observed. Compound **9**, a known inhibitor of the serine protease elastase,[13] also displayed activity against R39, suggesting that compounds with a *m*-(sulfon)amido side chain may be accommodated at the active site. These results suggested that a combination of meta-arrangements of both the boronic acid and the carboxylate (as in **1**) and of the boronic acid and an amido group (as in **9**) may give rise to more potent inhibitors.

Docking studies (Figure 2) based on a crystal structure for the acyl-enzyme complex formed between the β-lactam nitrocefin and R39[32] were then performed in an attempt to identify improved boronic acid inhibitors. These studies assumed that the boronic acid reacted with the nucleophilic serine (ser 49) to form a covalent tetrahedral complex. The results suggested that the carboxylate group of **1** binds in a similar way to the carboxylate of nitrocefin (Figure 2A) and that **1** analogues with an *o*- or *m*-acetamido group (**22a** and **23**) would bind with the acetamido side chain interacting in a way similar to that of nitrocefin (Figure 2B).

Synthesis of Derivatives of 1

The synthesis of derivatives of **1** with acetamido side chains at the ortho-position relative to the boronic acid was then explored with the phenylacetamido derivative being chosen for method development (Scheme 1). The boronic acid was introduced as its pinacol ester protected form, by reaction of **24** with bis(pinacolato)diboron and catalysis using [1,1′-bis(diphenylphosphino)ferrocene]palladium(II) chloride ($PdCl_2 \cdot$dppf) in the presence of potassium acetate using a modified version of a reported procedure[35−37] to give **25**. As observed with analogous palladium catalyzed couplings,[35] a low yield of the reduced coproduct **26** was consistently isolated.

Prior to establishing a reliable method for the deprotection of pinacol boronate esters, hydrolysis of the methyl ester to yield the carboxylic acid **27** was attempted, in part because we had previously shown that the pinacol ester **19** of the lead **1** had similar inhibitory activity to the free boronic acid. Furthermore, pinacol boronate esters have previously been assumed to hydrolyze under the aqueous conditions used for biological studies.[11,30] The carboxylic acid **27** was prepared by treatment of **25** with lithium hydroxide in a mixture of THF and water. After workup, the NMR of the crude material in acetone-d_6 indicated that **27** was the major species present, with no evidence for hydrolysis of the pinacol ester.

In order to reliably obtain boronic acids free of pinacol for the inhibition assay, there was a need to synthesize the free

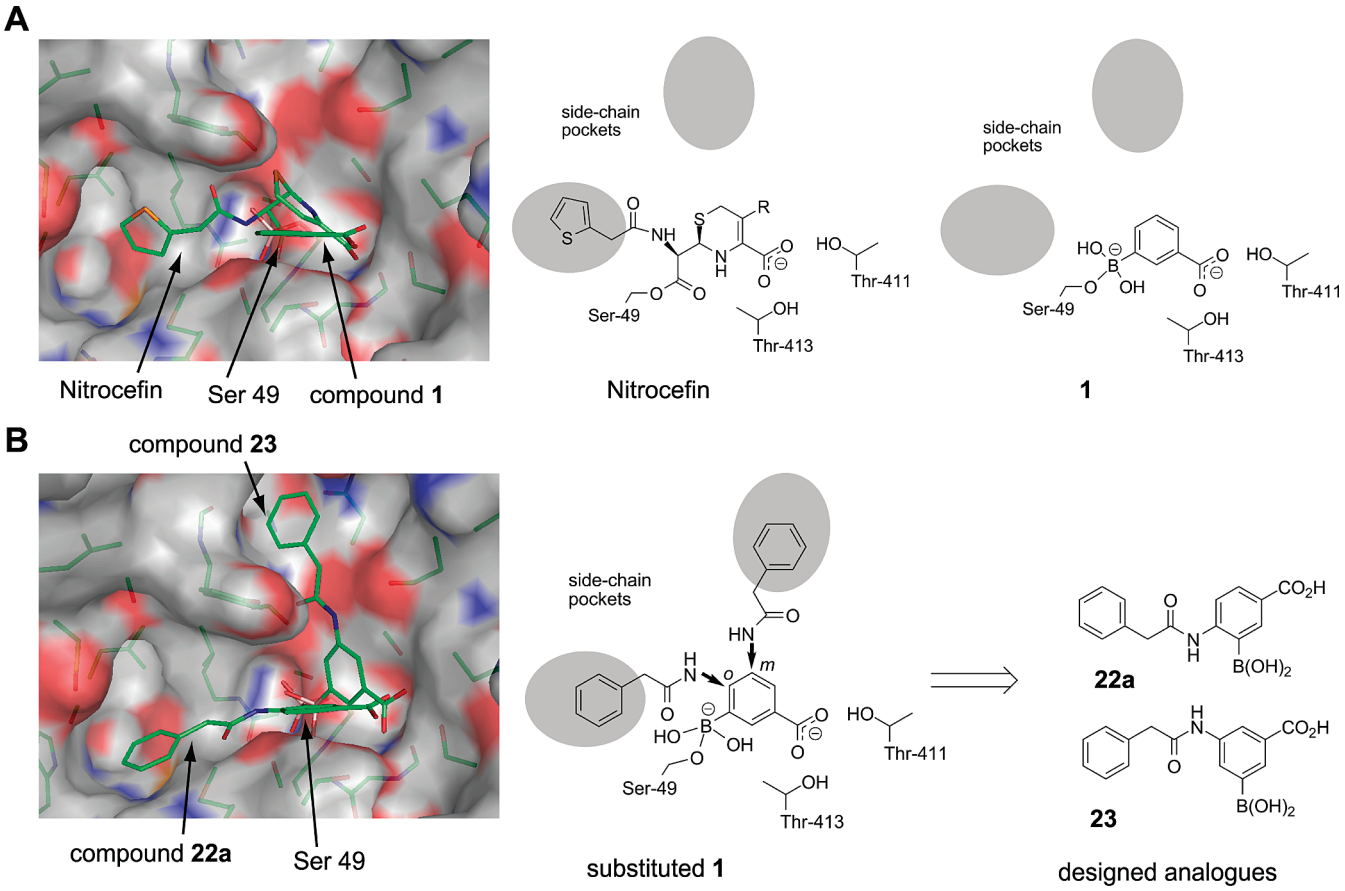
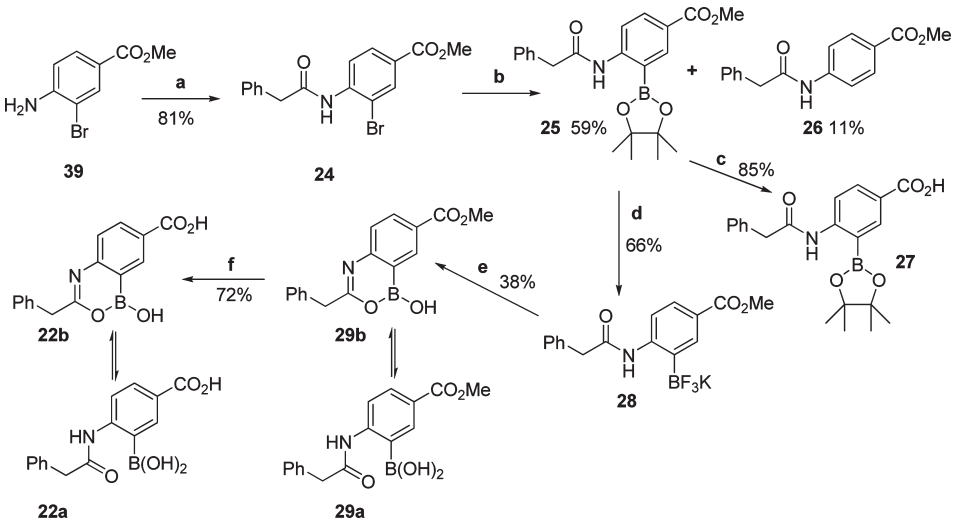


Figure 2. Rationale for derivatives of **1**. (A) Docking of **1** using GOLD[33,34] and comparison with a nitrocefin/R39 complex crystal structure (PDB code 1W8Y).[32] Note the C-3 side chain of nitrocefin was not observed in this structure. (B) Docking of proposed derivatives of **1**, **22a**, and **23**.

Scheme 1. Synthesis of *o*-Phenylacetamido Derivative of **1**[a]



[a] Reagents and conditions: (a) $PhCH_2COCl$/*N*,*N*-dimethylacetamide, room temp overnight; (b) bis(pinacolato)diboron, $PdCl_2$·dppf, AcOK, toluene, 90 °C overnight; (c) $LiOH·H_2O$/THF/H_2O, room temp overnight; (d) $KHF_{2(aq)}$/MeOH, room temp 1 h; (e) TMSCl/CH_3CN/H_2O, room temp 1 h; (f) $LiOH·H_2O$/dioxane/water, 60 °C 1 h, then room temp overnight. In solution, **29b** and **22b** may be in equilibrium with ring opened forms **29a** and **22a**, respectively.

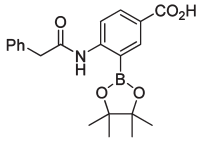
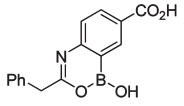
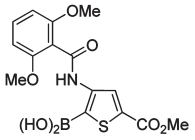
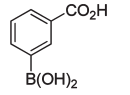
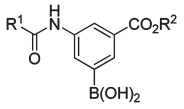
boronic acid **22a**. A commonly used method for deprotection of pinacol boronate esters is treatment with sodium periodate under acidic conditions.[38,39] When applied to **25**, a low recovery of a mixture of products was isolated. We then explored the method of Yuen and Hutton[40] for the deprotection of pinacol esters via the formation of intermediate trifluoroborate salts (Scheme 1). Thus, intermediate **28** was obtained after treatment of **25** with aqueous potassium hydrogen difluoride in methanol. The salt **28** was then treated with trimethylsilyl chloride in aqueous acetonitrile to give ester **29b**.

The structure of **29b** was assigned, at least in the solid state, as the bicyclic benzoxazaborinine on the basis of elemental analysis and mass spectrometric data. The formation of such a bicyclic boron containing heterocycle, instead of the free boronic acid form **29a**, has been observed previously in attempts to prepare *o*-amidophenylboronic acids.[41,42] It has been suggested that such compounds exist in equilibrium with the monocyclic boronic acid form in solution.[43] An analogous ortho-substituted thiophenylboronic acid **30** (Table 2 below), derived from **13**, was also prepared (Supporting Information).

Benzoxazaborinine **29b** was then hydrolyzed with lithium hydroxide to give carboxylic acid **22b**. **29b** and **22b** have very similar NMR spectra (aside from the anticipated loss of the ester CH_3 peak and the appearance of a CO_2H peak), suggesting that the form of the product obtained in the NMR solution was unchanged following exposure to the basic and then acidic deprotection conditions (i.e., the product is predominantly in the bicyclic form **22b**). NMR analysis under aqueous conditions indicated that pinacol ester **27** undergoes rapid hydrolysis to give **22b** (Supporting Information). However, we found that the pinacol ester **27** re-forms during acidic workup if the pinacol is not removed.

Meta-substituted boronic acids (**23** and **31**−**37**) were then prepared by a route analogous to that used for the ortho-derivative **22b** (Scheme 2). In contrast to the comparable reaction with the ortho-derivative, no reduced byproduct analogous with **26** (Scheme 1) was obtained during the palladium catalyzed preparation of the *m*-pinacol esters leading to higher yields in most cases. There was no evidence for intramolecular interactions between the *m*-amido group and the boronic acid for this series.

Table 2. Inhibitory Activity of Ayrlboronic Acids against R39[a]

N°	Structure	Residual Activity (1mM) (%)*	IC_{50} (μM)†
27		81 ± 13	nd§
22b		80 ± 3	nd§
30		98 ± 2	nd§
1		20 ± 5	400 ± 19
			

	R^1	R^2		
23	$PhCH_2$	H	3 ± 0	88 ± 2.6
31	Ph	H	9 ± 31	34 ± 0.3
32		H	10 ± 19	23 ± 1
33		H	84 ± 2	nd§
34		H	18 ± 8	32 ± 2.2
35		H	5 ± 2	78 ± 5.5
36	$PhOCH_2$	H	2 ± 10	28 ± 0.6
37	CH_3	H	50 ± 4	nd§
38	$PhCH_2$	CH_3	100 ± 3	nd§

[a] Symbols in the table have the following meaning: (∗) mean values ± standard deviation over three replicate experiments; (†) values calculated based on three replicate experiments and adjusted to account for standard error of regression of about 50% inhibition; (§) not determined.

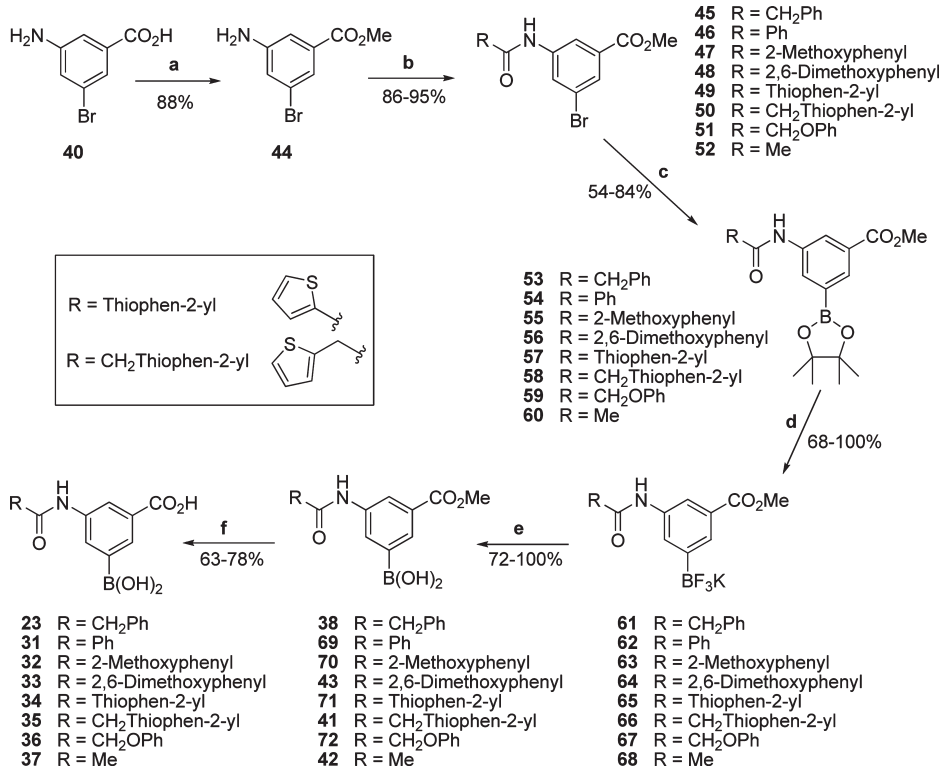
Activity of Boronic Acid Analogues against PBPs

The two ortho-substituted **1** derivatives **27** and **22b** and the analogous thiophene methyl ester **30**, derived from **13**, were poor inhibitors of R39 (RA = 80−100% at 1 mM, Table 2), displaying a reduction in potency compared with their respective leads **1** and **13**. This lack of activity may be a consequence of the tendency of these compounds to exist in the bicyclic benzoxazaborinine form of structure **22b** (Scheme 1). However, it is notable that the bicyclic benzoxaborole **18** (Table 1) shows a better level of inhibition than **22b**, suggesting that the position of the substituents on the structure **22b** may be responsible for its poor activity.

In contrast to the ortho-compounds, nearly all of the meta-substituted **1** derivatives (**23**, **31**, **32** and **34**−**36**) displayed improved inhibition against R39 relative to **1**, with residual activities in the range 2−18% at 1 mM (Table 2). Inhibition for selected compounds was also observed in the presence of 0.01% Triton-X (data not shown). The most active compounds had IC_{50} values in the range 20−90 μM. The 2-methoxybenzoyl derivative **32** was the most potent inhibitor (IC_{50} = 23 μM), a significant improvement from the parent compound **1** (IC_{50} = 400 μM).

The results indicate that the number of atoms between the aromatic side chain and carbonyl group is important; the two arylacetamido derivatives **23** and **35** were less potent ($IC_{50} \approx$ 80−90 μM) than the derivatives, which have the aromatic group attached directly to the carbonyl group (as in **31**, **32**, and **34**, $IC_{50} \approx$ 20−30 μM) or the phenoxyacetamido derivative **36** (IC_{50} = 28 μM). The observation that the derivative containing the 2,6-dimethoxybenzoyl side chain **33** was a poor inhibitor indicates that this side chain is too bulky to be accommodated into the adjacent pocket in the active site. This result is of interest given that the analogue with a single methoxy substituent (**32**) was the most potent inhibitor, demonstrating that one, but not two, methoxy group can be accommodated. In the case where the *m*-amido group is less bulky and lacked an aromatic group as in acetamide **37**, a reduction in inhibition was observed (RA = 50% at 1 mM) relative to **1**, suggesting that the aromatic group is important and that a lipophilic contact is likely to provide the enhanced inhibition. The importance of the carboxylate group on the **1** derivatives was shown by testing the methyl ester of **23**, as in **38**, which displayed no inhibition. Some of the

Scheme 2. Synthesis of *m*-Amido Derivatives of **1**[a]



[a] Reagents and conditions: (a) $SOCl_2$/MeOH, Δ, 2 h; (b) RCOCl/*N*,*N*-dimethylacetamide, room temp overnight; R = Me, Ac_2O/THF, room temp overnight; (c) bis(pinacolato)diboron, $PdCl_2$·dppf, AcOK, toluene, 90 °C overnight; (d) $KHF_{2(aq)}$/MeOH/THF, room temp 1 h; (e) TMSCl/CH_3CN/H_2O, room temp 1 h; (f) LiOH·H_2O/THF/H_2O, room temp overnight.

meta-substituted **1** derivatives also displayed activity against PBP2xR6, PBP2x5204 (penicillin resistant), and PBP1b, from *Streptococcus pneumoniae* (Supporting Information).

Conclusions

We have used the low molecular weight PBP, R39, from *Actinomadura* sp. strain as a model system for the development of inhibitors against higher molecular weight, more clinically important and penicillin resistant PBPs. Following the identification of 3-(dihydroxyboryl)benzoic acid **1** as an R39 inhibitor (IC_{50} = 400 μM), derivatives of **1** with substitution in both the ortho- and meta-positions were prepared. Ortho-substituted derivatives were poor inhibitors; however, compounds with up to 17-fold improved potency were identified from the meta-substituted series [e.g., 3-(dihydroxyboryl)-5-(2-methoxybenzamido)benzoic acid **32**, IC_{50} = 23 μM]. Some of the more potent derivatives displayed activity against higher molecular weight PBPs. These compounds may be useful for the development of even more potent boronic acid based PBP-inhibitors with a broad spectrum of antibacterial activity. Given the potential of boronic acids to inhibit serine β-lactamases,[11−17] it is also possible that they could be used in conjunction with a β-lactam both for protection from β-lactamases and to extend the spectrum of antibacterial activity.

Experimental Section

Biological Reagents. R39 D,D-peptidase from *Actinomadura* was prepared and purified as described by Granier et al.[44] PBP2x-5204, PBP2x-R6, and PBP1b from *Streptococcus pneumoniae* were prepared as described by Carapito et al.[45] Fluorescein-labeled ampicillin was prepared as described by Lakaye et al.[46] (*R*)-[2-(Benzoylamino)propionylsulfanyl]acetic acid **S2d** was prepared as described by Adam et al.[24] and Schwyzer and Hurlimann.[47]

Testing of Compounds for Inhibition of R39, PBP2xR6, and PBP2x5204. Inhibition experiments with R39, PBP2xR6, and PBP2x5204 were performed by monitoring the degree of hydrolysis of the substrate **S2d** in microtiter 96-well plates using a Power Wave microtiter plate reader, using procedures previously described.[7] Enzyme residual activity (RA) was determined after preincubation of the PBPs in the presence of potential inhibitors. The initial rate of hydrolysis of **S2d** (1 mM) was determined in the presence of 5,5′-dithiobis(2-nitrobenzoic acid), $\varepsilon[\Delta\varepsilon]$ = 13 600 M^{-1} s^{-1} monitoring at 412 nm. The rate of spontaneous hydrolysis of **S2d** in the presence of the inhibitors was also determined in absence of the enzymes. All experiments were performed in triplicate. Activity of PBPs in the absence of inhibitors (RA=100%) was measured with six replicates on each plate. Compounds were initially prepared as 100 mM solutions in DMF and diluted to 10 mM in sodium phosphate buffer (pH 7.0). In cases where compounds were insoluble under these conditions, solutions of compound were prepared at 1 mM in 10% DMF. For initial tests, potential inhibitors were incubated with the enzymes at a concentration of either 1.18 mM or 118 μM (depending on the solubility) in the presence of 3.5 nM R39, 10 mM sodium phosphate buffer (pH 7.2) with 100 mM NaCl, 100 mM D-alanine, and 0.01 mg/mL BSA for 60 min at 25 °C (total volume = 127 μL). After incubation, the substrate mixture [**S2d** and 5,5′-dithiobis(2-nitrobenzoic acid)] was added (23 μL) to give the final solution (150 μL) [final concentration is 1 mM for **S2d**, 0.5 mM for 5,5′-dithiobis(2-nitrobenzoic acid), 1 mM for potential inhibitor (or 100 μM for low solubility compounds), and 1% v/v DMF], and the RA was then measured at 412 nm. In the case of PBP2xR6, 0.09 μM enzyme was incubated in the presence of potential inhibitors (1.18 mM or 118 μM) in 10 mM sodium phosphate

buffer (pH 7.0) and 0.01 mg/mL BSA for 60 min at 25 °C, and for PBP2x5204, 0.6 μM enzyme was incubated in the presence of potential inhibitors (1.18 mM or 118 μM) in 10 mM sodium phosphate buffer (pH 7.0), 70 mM D-alanine, and 0.01 mg/mL BSA for 4 h at 25 °C, prior to the addition of the substrate mixtures and measurement of the RA. When RA was $<80\%$, the RA measurement was repeated in the presence of 0.01% Triton-X-100 v/v to identify possible false positive results. If the RA for compounds was $<50\%$ at 1 mM (or 100 μM), the RA was measured over a range of concentrations from which IC_{50} values were determined by performing a nonlinear regression analysis using Sigma Plot (Systat software) and fitting the data to the equation[7] $y = y_0 + (ab)/(b + x)$ where y_0 is the activity with high concentration of inhibitor ($y_0 \approx 0$), $y_0 + a$ is the activity in the absence of the inhibitor ($x = 0$), and $b = IC_{50}(a - y_0)/(a + y_0)$; so for $y_0 \approx 0$, $b \approx IC_{50}$. The IC_{50} values are adjusted to account for standard error of regression of about 50% inhibition.

Testing of Compounds for PBP1b Inhibition. PBP1b (0.7 μM) was incubated with inhibitors (1 mM) in 10 mM sodium phosphate (pH 7.0) for 60 min at 30 °C. Active PBP was then counterlabeled with fluorescein-labeled ampicillin (10 μM) for 20 min. The reaction was stopped and analyzed by SDS−PAGE followed by fluorescence visualization using a Molecular Imager FX (Bio-Rad) and the program Quantity One (Bio-Rad). Background fluorescence was subtracted.

Docking Studies. Docking of boronic acids was performed using the R39/nitrocefin structure (PDB code 1W8Y). Monomer A was selected from an ensemble of four structures, and the nitrocefin molecule was removed from the active site. The PDB file was prepared for the docking experiment using WHAT IF[48] online resource (http://swift.cmbi.ru.nl/servers/html/index.html) using the "prepare PDB file for docking programs" feature. Ligand files were prepared using Marvin Sketch/View, version 4.1.7.[49] Boronic acids were prepared in tetrahedral form by incorporation of an additional oxygen atom bonded to the boron to facilitate covalent linkage. Explicit hydrogen atoms were added before optimization of structures and writing output files as MDL mol files. Docking experiments were performed using GOLD,[33,34] version 3.2. The ligand binding site was centered about the nucleophilic serine residue O-γ (serine 49) atom with a radius of 15 Å. Atom-to-atom covalent linkage was used with connection of the serine 49 O-γ atom with the added oxygen atom from the ligands (see above). Flip ring corners, flip amide bonds, flip all planar R-NR1R2 (including ring NH-R, including ring NR1NR2) were used under the fitness and searching options. Otherwise all settings were as per default. Orientations of docked ligands were inspected using Pymol, version 0.99.[50]

Sources and Purities of Compounds for Inhibition Studies. Boronic acids **2**, **5**, **6**, **8**, **9**, and **19** were from Aldrich, and their stated purities were all >95%. **10**, **12**, **14**−**16**, and **18** were from Alfa Aesar with stated purities of >95%. Boronic acid **13** was from Maybridge with stated purity of 95%. Boronic acids **3**, **4**, **7**, **11**, and **17** were from Aldrich and were specified to contain varying amounts of anhydrides and were tested for inhibition without purification. Aldehyde **21** was from Aldrich, and its purity was 97%. Compound **1** was from Aldrich and was analytically pure as determined by C and H elemental analysis. Trifluoromethylketone **20** and thiophenylboronic acid analogue **30** were synthesized as described in the Supporting Information and were >95% pure (HPLC). All other boronic acids used for inhibition assays were prepared as described in the example procedures below or in the Supporting Information and were analytically pure as determined by C, H, and N elemental analysis (Table S-1), with the exception of **22b** which was 90% pure (HPLC).

General Synthetic Considerations. Compounds **39** and **40** were from Aldrich and used without further purification. Bis(pinacolato)diboron and the complex of [1,1′-bis(diphenylphosphino)ferrocene]palladium(II) chloride with dichloromethane (1:1) ($PdCl_2 \cdot$dppf) were from Alfa Aesar. All solvents used were HPLC grade. Where possible, reactions were monitored by TLC, which was performed on precoated aluminum-backed plates (Merck, silica 60 F_{254}). Spots were visualized using UV light (λ = 254 or 221 nm) and/or by staining with a potassium permanganate/ethanol solution. Chromatography was performed using a Biotage SP4 chromatography system, using prepacked Biotage columns, or by filtration through silica purchased from VWR (40−63 μm particle size). NMR spectra were recorded on a Bruker DPX250 NMR spectrometer fitted with a 5 mm broadband heteronuclear probe (^{1}H, 250.13 MHz), a Bruker AV400 spectrometer fitted with a 5 mm z-gradient ^{1}H/^{13}C/^{19}F/^{31}P quad-nucleus probe (^{1}H, 400.133; ^{13}C, 100.630 MHz; ^{19}F, 376.507 MHz) or a Bruker AVII 500 spectrometer fitted with a 5 mm ^{13}C(^{1}H) dual cryoprobe optimized for ^{13}C detection (^{1}H, 500.303 MHz; ^{13}C, 125.813 MHz). Chemical shifts (δ) are given in ppm, and the multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br). Coupling constants J are given in Hz to the nearest 0.1 Hz. Spectra were recorded as solutions in $CDCl_3$ (δ_H at 7.26 ppm, δ_C at 77.0 ppm), acetone-d_6 (δ_H at 2.05 ppm, δ_C at 29.9 ppm), or DMSO-d_6 (δ_H at 2.50 ppm, δ_C at 39.5 ppm), used as internal references. Low resolution mass spectra were recorded on a LCT Premier, operating in electrospray ionization (ESI) mode with medium-high (7000) resolution running OpenLynx. Samples were introduced by a PAL autosampler and were prepared as methanol solutions, unless otherwise stated. Field ionization (FI) mass spectra were recorded on a Micromass GCT reflectron time-of-flight spectrometer in FI mode with a temperature programmed solids probe inlet system (resolution = 5000 fwhm). High resolution mass spectra (HRMS) were recorded under ESI conditions on a Bruker MicroTOF (resolution = 10 000 fwhm). Unless otherwise stated, infrared spectra were recorded from Nujol mulls between sodium chloride plates, using a Bruker Tensor 27 FT-IR spectrometer. Elemental analyses were performed at London Metropolitan University, London, U.K. The results of elemental analysis are given in Table S-1. Melting points were determined using a Leica Galen III hot-stage melting point apparatus and microscope.

The ^{13}C NMR spectra of some of the compounds contained some resonances that were observed as two peaks, likely due to conformational isomers due to hindered rotation about amide groups. This feature was observed for methyl esters **38** and **41**−**43**, where the carbons adjacent to the amide functionality were observed as two signals separated by ~0.1 ppm in ratios of ~3:1; in these cases only the major peaks have been reported. For **43**, a 1:1 ratio was observed and both sets of peaks are reported. Similarly, small additional peaks were observed for the same carbon atoms of carboxylic acids **23** and **31**−**37** for which only the major species is reported. The signals for the quaternary carbons directly attached to the boron atom of all the boronic acid derivatives were usually observed as small broad humps in the baseline. The signal could be enhanced by increasing the line broadening to ~20 Hz in the exponential multiplication window function, and this has been used to identify and assign this signal where possible.

General Procedure for the Synthesis of Anilides. The aniline **39** or **44** (4.37 mmol) was stirred in *N*,*N*-dimethylacetamide (10 mL) under an atmosphere of nitrogen and ice cooling before addition of the appropriate acid chloride (8.73 mmol) dropwise over 20 min. The mixture was stirred on ice for 1 h and then at room temperature overnight. The solution was diluted with ethyl acetate (70 mL), washed with 1 M HCl (3 × 40 mL), 0.2 M NaOH (3 × 40 mL), brine (40 mL), and dried (Na_2SO_4), and the solvent was removed to afford the products which were used as indicated.

Methyl 3-Bromo-5-(2-phenylacetamido)benzoate 45. Methyl 3-bromo-5-aminobenzoate **44** (1.00 g, 4.37 mmol) was treated with phenylacetyl chloride (1.15 mL, 1.35 g, 8.73 mmol) in

N,*N*-dimethylacetamide (10 mL) as described in the general procedure. After workup, the residue was purified by filtration through silica gel using 10% ethyl acetate/dichloromethane as eluant to afford the title compound (1.35 g, 89%) as a white waxy solid, mp 86−90 °C. Anal. ($C_{16}H_{14}BrNO_3$) C, H, N. IR (nujol) ν/cm^{-1}: 3284 (NH), 3246 (NH), 3184 (NH), 3092 (NH), 1726 (CO_2), 1655 (CON). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.76 (2H, s, CH_2), 3.89 (3H, s, CH_3), 7.30 (1H, br s, NH), 7.32−7.43 (5H, m, H2′−H6′), 7.77 (1H, br t, J = 1.5 Hz, H6), 7.88 (1H, br t, J = 1.5 Hz, H2), 8.11 (1H, m, H4). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 44.7 (CH_2), 52.2 (CH_3), 119.1 (C6), 122.8 (C3), 126.8 (C4), 128.0 (C4′), 128.2 (C2), 129.4 ([C2′ and C6′] or [C3′ and C5′]), 129.5 ([C3′ and C5′] or [C2′ and C6′]), 132.1 (C1), 133.7 (C1′), 138.9 (C5), 165.4 (CO_2), 169.3 (CON). m/z (ESI, negative ion) 697 ({2M} − H, 85%,), 695 ({2M} − H, 100), 693 ({2M} − H, 80), 349 (30), 348 (M − H[^{81}Br], 85), 347 (30), 346 (M − H[^{79}Br], 85).

General Procedure for the Preparation of Pinacol Boronate Esters from Aryl Bromides. The aryl bromide (3.24 mmol) was treated with bis(pinacolato)diboron (3.57 mmol), [1,1′-bis-(diphenylphosphino)ferrocene]palladium(II) chloride, complex with dichloromethane (1:1), (hereafter referred to as $PdCl_2$·dppf) (0.1 mmol) and potassium acetate (9.70 mmol) in dry degassed toluene (32 mL) with stirring at 95 °C overnight under an atmosphere of nitrogen. After cooling, the mixture was concentrated, and the residue was resuspended in ethyl acetate (70 mL), washed with water (1 × 40 mL) and brine (1 × 40 mL), and dried (Na_2SO_4), and the solvent was removed. The residue (a dark-brown solid) was purified by chromatography on silica gel as indicated to afford the pure product.

Methyl 3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-5-(2-phenylacetamido)benzoate 53. Methyl 3-bromo-5-(2-phenylacetamido)benzoate **45** (0.430 g, 1.24 mmol) was treated with bis(pinacolato)diboron (0.347 g, 1.36 mmol), $PdCl_2$·dppf (0.031 g, 0.037 mmol), and potassium acetate (0.364 g, 3.71 mmol) in toluene (13 mL) as described in the general procedure. After workup the residue was purified by filtration through silica gel using 20% ethyl acetate/dichloromethane as eluant to afford the title compound (0.330 g, 67%) as an off-white solid, mp 132−137 °C. Anal. ($C_{22}H_{26}BNO_5$) C, H, N. IR (nujol) ν/cm^{-1}: 3300 (NH), 1724 (CO_2), 1662 (CON). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.33 (12H, s, 4 × CH_3[pinacolyl]), 3.76 (2H, s, CH_2), 3.89 (3H, s, CH_3O), 7.18 (1H, br s, NH), 7.33−7.43 (5H, m, H2′-H6′), 7.87 (1H, br d, J = 1.5 Hz, H4), 8.20 (1H, bs s, H2), 8.31 (1H, br s, H6). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 24.8 (4 × CH_3[pinacolyl]), 44.8 (CH_2), 52.1 (CH_3), 84.2 (C4″ and C5″), 123.7 (C6), 127.8 (C4′), 129.4 ([C2′ and C6′] or [C3′ and C5′]), 129.6 ([C3′ and C5′] or [C2′ and C6′]), 130.1 (C4), 130.4 (v br, C3), 130.5 (C1′), 131.9 (C2), 134.1 (C1′), 137.3 (C5), 166.7 (CO_2), 169.1 (CON). m/z (ESI, negative ion) 822 ({2M} + MeO, 75%), 821 ({2M} + MeO, 100), 820 ({2M} + MeO, 70), 427 (30), 426 (M + MeO[^{11}B], 90), 425 (M + MeO[^{10}B], 40), 395 (15), 394 (M − H[^{11}B], 50), 393 (M − H[^{10}B], 12).

General Procedure for Conversion of Pinacol Esters to Trifluoroborate Salts. The pinacol ester (2.32 mmol) is dissolved in a mixture of methanol (15 mL) and THF (10 mL) with stirring before addition of 4.5 M $KHF_{2(aq)}$ (20.7 mmol). The resulting mixture was stirred for 1 h before concentrating to dryness. The residue, a white solid, was collected by filtration, washed several times with hot diethyl ether (total of ∼100 mL) followed by water (∼50 mL), and dried to afford the trifluoroborate salt.

Potassium Trifluoro{3-(methoxycarbonyl)-5-[2-phenylacetamido]phenyl}borate 61. A solution of methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5-(2-phenylacetamido)benzoate **53** (0.400 g, 1.01 mmol) in methanol (6 mL) and THF (2 mL) was treated with 4.5 M $KHF_{2(aq)}$ (2.0 mL, 9.00 mmol) as described in the general procedure. After workup, the title compound (0.292 g, 77%) was isolated as a white solid, mp 278−284 °C. Anal. ($C_{16}H_{14}BF_3KNO_3$) H, N. C: calcd 51.22; found, 50.38. HRMS (ESI, negative ion) $C_{16}H_{14}{}^{11}BF_3NO_3^-$ (M − K$^+$) requires 336.1024. Found 336.1016. IR (Nujol) ν/cm^{-1}: 3356 (NH), 1703 (CO_2), 1698 (CON). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.62 (2H, s, CH_2), 3.80 (3H, s, CH_3), 7.22−7.26 (1H, m, H4′), 7.31−7.36 (4H, m, H2′, H3′, H5′, H6′), 7.60 (1H, br d, J = 2.0 Hz, H6), 7.69 (1H, br s, H2), 8.19 (1H, t, J = 2.0 Hz, H4), 10.06 (1H, br s, NH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.4 (CH_2), 51.6 (CH_3), 117.0 (C4), 126.4 (C4′), 127.3 (C6), 127.7 (C2), 127.8 (C3), 128.3 ([C2′ and C6′] or [C3′ and C5′]), 129.0 ([C3′ and C5′] or [C2′ and C6′]), 136.2 (C1′), 137.7 (C5), 167.3 (CO_2), 168.8 (CON), C1 not observed. ^{19}F NMR (DMSO-d_6, 366 MHz) δ: −139.8 (3F, br s, BF_3). m/z (ESI, negative ion) 337 (60%), 336 (M − K[^{11}B]), 100), 335 (M − K[^{10}B], 80).

General Procedure for the Preparation of Boronic Acids from Trifluoroborate Salts. A stirred solution of the trifluoroborate salt (1.90 mmol) in acetonitrile and water as indicated (total volume of ∼35 mL) was treated with TMSCl (7.25 mmol) for 1 h. The resulting mixture was treated with saturated $NaHCO_3$ (4 mL) before drying (Na_2SO_4) and concentrating to afford the product.

Methyl 3-(Dihydroxyboryl)-5-(2-phenylacetamido)benzoate 38. A solution of potassium trifluoro{3-(methoxycarbonyl)-5-[2-phenylacetamido]phenyl}borate **61** (0.440 g, 1.17 mmol) in acetonitrile (18 mL) and water (5 mL) was treated with TMSCl (0.560 mL, 0.480 g, 4.44 mmol) for 1 h, followed by addition of $NaHCO_3$ solution (3.5 mL), as described in the general procedure. After workup the title compound (0.269 g, 73%) was obtained as a white solid, mp 273−276 °C. Anal. ($C_{16}H_{16}BNO_5$) C, H, N. IR (Nujol) ν/cm^{-1}: 3404 (NH or OH), 3340 (NH or OH), 3262 (NH or OH), 1712 (CO_2), 1652 (CON). ^{1}H NMR (500 MHz, acetone-d_6) δ: 3.76 (2H, s, CH_2), 3.88 (3H, s, CH_3), 7.25−7.28 (1H, m, H4′), 7.33−7.36 (2H, m, [H2′ and H6′] or [H3′ and H5′]), 7.42−7.43 (2H, m, [H3′ and H5′] or [H2′ and H6′]), 7.45 (2H, s, 2 × BOH), 8.23 (1H, br s, H2), 8.25 (1H, br s, H4), 8.47 (1H, br s, H6), 9.44 (1H, br s, NH). ^{13}C NMR (125 MHz, acetone-d_6) δ: 44.8 (CH_2), 52.3 (CH_3), 122.8 (C6), 127.5 (C4′), 129.2 ([C2′ and C6′] or [C3′ and C5′]), 130.2 ([C3′ and C5′] or [C2′ and C6′]), 130.3 (C4), 130.9 (C2), 131.0 (C1), 135.6 (v br, C3), 136.8 (C1′), 140.0 (C5), 167.4 (CO_2), 170.0 (CON). m/z (ESI, negative ion) 326 (90%), 313 (20), 312 (M − H[^{11}B], 100), 311 (M − H[^{10}B], 50).

General Procedure for Hydrolysis of Methyl Esters to Carboxylic Acids. A mixture of the methyl ester (0.82 mmol) and LiOH·H_2O (2.86 mmol) was stirred overnight in THF (10 mL) and water (7.5 mL). The mixture was concentrated to ∼2 mL and the resulting solution diluted with additional water (1 mL). This mixture was extracted with ethyl acetate (2 × 2 mL), and the aqueous layer was acidified with 1 M HCl until substantial precipitation was observed, with assistance by scratching if necessary. The precipitate was collected and dried to afford the carboxylic acids. The crude products were accompanied by small amounts of apparent impurities thought to be corresponding anhydrides or semianhydrides. In all cases, the analytically pure free boronic acids were obtained after recrystallization from water/methanol mixtures as indicated.

3-(Dihydroxyboryl)-5-(2-phenylacetamido)benzoic Acid 23. Methyl 3-(dihydroxyboryl)-5-(2-phenylacetamido)benzoate **38** (0.210 g, 0.67 mmol) was treated with LiOH·H_2O (0.099 g, 2.35 mmol) in THF (9 mL) and water (7 mL) as described in the general procedure. After workup the title compound (0.147 g, 74%) was obtained as a white solid. A portion was recrystallized from 30% methanol/water solution (3×) to obtain an analytical sample as a white fluffy solid, mp 200−210 °C. Anal. ($C_{15}H_{14}BNO_5$) C, H, N. IR (Nujol) ν/cm^{-1}: 3273 (NH and OH), 1698 (CO_2), 1631 (CON). ^{1}H NMR (500 MHz, acetone-d_6) δ: 3.75 (2H, s, CH_2), 7.25−7.28 (1H, m, H4′), 7.33−7.36 (2H, m, [H2′ and H6′] or [H3′ and H5′]), 7.40−7.44 (4H, m, [H3′ and H5′] or [H2′ and H6′] and 2 × BOH), 8.26 (2H, br s, H2 and H4), 8.48 (1H, t, J = 2.0 Hz, H6), 9.42 (1H, br s, NH), 11.20 (1H, br s,

CO_2H). ^{13}C NMR (125 MHz, acetone-d_6) δ: 44.8 (CH_2), 123.2 (C6), 127.5 (C4′), 129.2 ([C2′ and C6′] or [C3′ and C5′]), 130.1 ([C3′ and C5′] or [C2′ and C6′]), 130.3 (C4), 131.2 (C2), 131.3 (C1), 135.7 (v br, C3), 136.9 (C1′), 139.9 (C5), 167.8 (CO_2), 170.0 (CON). *m*/*z* (ESI, negative ion) 313 (20%), 312 (M + MeO − OH − H[^{11}B], 60), 311 (M + MeO − OH − H[^{10}B], 25), 299 (40), 298 (M − H[^{11}B], 100), 297 (M − H[^{10}B], 50).

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Supporting Information Available: Additional synthetic procedures, elemental analysis results for compounds, NMR spectra of the solution studies of aromatic boronic acids, and inhibition data from testing compounds against other PBPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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