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Synthesis and evaluation of boronic acids as inhibitors of Penicillin Binding Proteins of classes A, B and C

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ABSTRACT

In response to the widespread use of β-lactam antibiotics bacteria have evolved drug resistance mechanisms that include the production of resistant Penicillin Binding Proteins (PBPs). Boronic acids are potent β-lactamase inhibitors and have been shown to display some specificity for soluble transpeptidases and PBPs, but their potential as inhibitors of the latter enzymes is yet to be widely explored. Recently, a (2,6-dimethoxybenzamido)methylboronic acid was identified as being a potent inhibitor of *Actinomadura* sp. R39 transpeptidase (IC_{50}: 1.3 μM). In this work, we synthesized and studied the potential of a number of acylaminomethylboronic acids as inhibitors of PBPs from different classes. Several derivatives inhibited PBPs of classes A, B and C from penicillin sensitive strains. The (2-nitrobenzamido)methylboronic acid was identified as a good inhibitor of a class A PBP (PBP1b from *Streptococcus pneumoniae*, IC_{50} = 26 μM), a class B PBP (PBP2xR6 from *Streptococcus pneumoniae*, IC_{50} = 138 μM) and a class C PBP (R39 from *Actinomadura* sp., IC_{50} = 0.6 μM). This work opens new avenues towards the development of molecules that inhibit PBPs, and eventually display bactericidal effects, on distinct bacterial species.

1. Introduction

The widespread use of β-lactam antibiotics resulted in the worldwide appearance of drug-resistant strains. Bacteria have developed resistance to β-lactams by three main mechanisms: the production of β-lactamases that catalyze the hydrolysis of β-lactams, the production of low-affinity, drug resistant Penicillin Binding Proteins (PBPs) and the overexpression of resistant PBPs. PBPs are generally membrane-associated proteins. The PBP family is divided in three classes.[1,2] The high-molecular mass PBPs of classes A and B contain two catalytic domains. Class A PBPs such as PBP1b from *Streptococcus pneumoniae* catalyze the last two steps of the biosynthesis of peptidoglycan (PG): the glycosyltransfer (GT, the formation of glycan chain (GlcNAc-MurNAc-peptide-)$_n$) and the transpeptidation (TP, cross-linking of stem peptides).[3] The N-terminal domains of class B PBPs such as PBP2xR6 from *Streptococcus pneumoniae* and PBP2x5204 from a penicillin resistant pneumococcal strain are believed to play a role in cell morphogenesis, while their C-terminal domains exhibit a TP activity as do those of class A PBPs.[4] Class C PBPs are low-molecular mass PBPs. R39, a soluble D,D-peptidase from *Actinomadura*, is a class C type-4 PBP[2,5] with an endopeptidase activity. The TP domains mostly catalyze the cross-linking of stem peptides via an acyl-enzyme intermediate formed by nucleophilic attack of a serine residue on the amide carbonyl group of the penultimate D-Ala of the stem peptide. The TP domains of PBPs are the main targets of β-lactam antibiotics, due to the structural similarity between penicillin and the stem-peptide. β-Lactams are suicide substrates of the TP domains of PBPs forming a stable, covalent adduct with the serine residue of the active site (Fig. 1).

Partly in response to the emergence and spread of β-lactamases, many β-lactam derivatives have been developed. For example libraries of penams, which preserve the β-lactam core of the drug but explore diverse R functionalities on the carboxyamido side chain at the C6 position of the penicillin ring, have been synthesized (Fig. 2). Different R side chains confer different antibacterial activities, levels of resistance to β-lactamases and kinetics of PBP inhibition[3,6–10] (Fig. 2, Table 1). Another approach has been the development of non-β-lactam inhibitors, with the objective of attempting to stall the development of β-lactam resistance. Various non-β-lactam inhibitors of PBPs are described in the literature.[3,11–13]

Abbreviations: GT, glycosyltransfer; TP, transpeptidation; PG, peptide glycan; PBP, Penicillin Binding Proteins; RA, residual activity.

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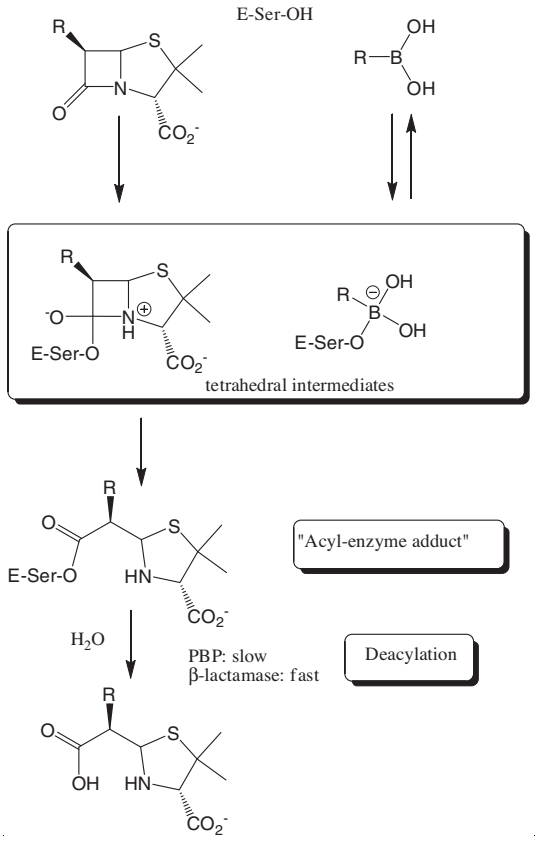


Figure 1. β-Lactams and boronic acids form tetrahedral intermediates with the serine residue of the active site of PBPs and β-lactamases (E).

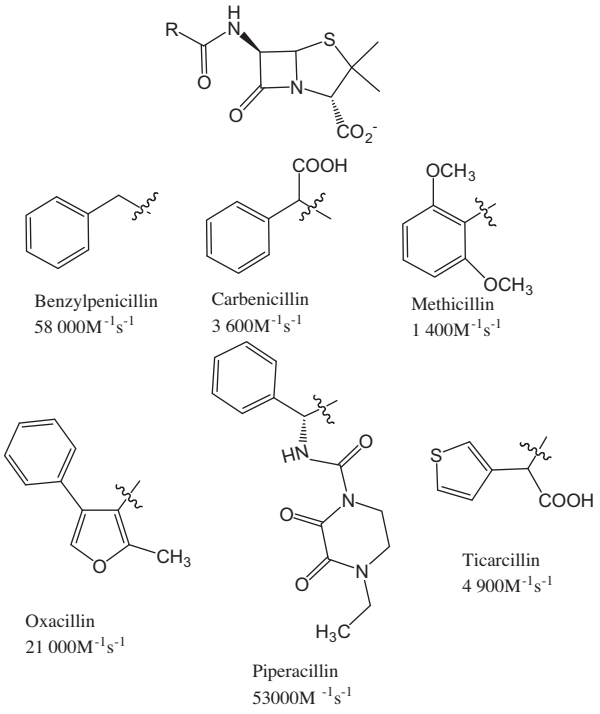


Figure 2. Acylation rate constants k_2/K for commonly used penams of PBP2xR6.[8]

Boronic acids have been shown to be potent and specific inhibitors of serine proteases and of other enzymes.[14] The dipeptidyl boronic acid derivative Bortezomib, a proteasome inhibitor, is currently in clinical use.[15] Furthermore, boronic acids have been shown to inhibit β-lactamases like AmpC and TEM[16–19] as well as PBPs.[20–25] Boronic acids form reversible covalent adducts, which mimic the tetrahedral intermediates in the PBP-catalyzed acylation reactions[22] (Fig. 1) and are thus interesting starting points for the development of non-β-lactam inhibitors of PBPs. Crystal structures of adducts formed by boronic acid analogs and R39 described here (**4**, **11**, **14**, **19**, Table 2) have already been solved and an unexpected tricovalent binding mode of these molecules within the active site of R39 was observed.[25] In addition, crystal structures of the pneumococcal PBP1b complexed to different alkyl and aryl boronic acid analogs (**4**, **7**, **11**, **13**, **14**, Table 2) have been used in combination with other structures of ethylboronic acids in the structure-guided design of PBP inhibitors that overcome β-lactam resistance in *Staphylococcus aureus* (MRSA).[20] (2,6-Dimethoxybenzamido)methylboronic acid is an inhibitor of β-lactamases from *Bacillus cereus* and *Pseudomonas aeruginosa*.[26] This boronic acid was identified as a potent inhibitor of R39 and was used in this work as a lead-structure for the synthesis of various acylaminomethylboronic acids. We show that a number of methylboronic acid analogs inhibit PBPs from all three classes and thus constitute promising leads for the development of new inhibitors of peptidoglycan biosynthesis.

Table 1
Acylation rate constants k_2/K of different PBPs by penicillin G as found in the literature[3,6–10]

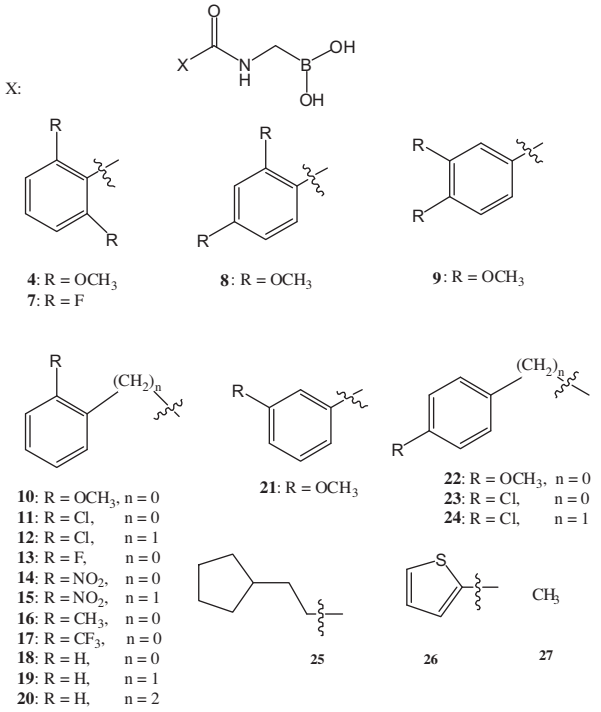
PBP	Benzylpenicillin
PBP1b	20,000[6]
PBP2xR6	110.000,[3] 58 000[8]
R39	300.000[7]
PBP2x5204	104[3]
PBP2a	17[9]
PBP5fm	20[10]

2. Results and discussion

2.1. Identification of acylaminomethylboronic acids as R39 inhibitors

The overall fold of the transpeptidase domains of all PBPs is very similar to that of class A β-lactamases.[1,2] The active site is at the interface of two subdomains and is defined by three motifs common to all PBPs and serine-β-lactamases as shown by Sauvage[5] for the R39 D,D-peptidase and the class A β-lactamase from *Bacillus licheniformis*. PBPs form long-lived acylenzymes with β-lactams. A loop in the active site of class A β-lactamases bearing an asparagine and a glutamic acid is responsible for the rapid deacylation of most β-lactam antibiotics. Because of the structural similarity with β-lactamases it is not surprising that some powerful β-lactamase inhibitors like 6-β-iodopenicillanate[5] have been shown to inactivate PBPs however rather poorly. (2,6-Dimethoxybenzamido)-methylboronic acid **4** was identified as a good inhibitor of R39 (IC_{50}: 1.3 μM, Table 2) by an initial screening of a library containing several β-lactamase inhibitors. Two fragments of molecule **4**, the aminomethylboronic acid **5** and 2-(2,6-dimethoxybenzamido)acetic acid **6**, were not or only poor inhibitors at a concentration of 1 mM (Fig. 3) underlining the importance of the presence of the 2,6-dimethoxybenzoyl residue, the side chain of methicillin (Fig. 2), and of the aminomethylboronic acid function of **4** for the inhibition of R39. Powerful β-lactamase inhibitors have been synthesized by introducing the R side chains of different penams (Fig. 2) in acylaminomethylboronic acids.[16–19] Here, in addition to the R side chains of methicillin **4** and benzylpenicillin **19** new side chains were introduced and some potent inhibitors were found.

Table 2
Screening of lead-structure **4** analogs performed after a 1 h preincubation



Compound	R39 % residual activity	PBP2xR6 % residual activity	PBP1b % residual activity
4	0 (IC_{50}: 1.3 ± 0.05)	26 ± 2 (IC_{50}: 278 ± 17)	67 ± 3[b]
7	6 ± 6 (IC_{50}: 35 ± 7)	40 ± 1 (IC_{50}: 906 ± 47)	7 ± 3(100 μM) (IC_{50}: 7 ± 0.1)[cb]
8	69 ± 6[c]	69 ± 1[c]	18 [ca]; 76 ± 5 (100 μM)[cb]
9	81 ± 2	82 ± 3	90 ±17[ca]
10	43 ± 1	86 ± 1	1[cb]; 67 ± 8 (100 μM)[cb]
11	15 ± 1[c] (IC_{50}: 85 ± 25)	31 ± 0[c] (IC_{50}: 262 ± 25)	20 ± 1(100 μM)[cb] (IC_{50}: 27 ± 1)[cb]
12	57 ± 3	26 ± 1 (IC_{50}: 649 ± 30)	24[ca]; 96 ± 7 (100 μM)[cb]
13	30 ± 1 (IC_{50}: 505)	56 ± 1	14 ± 4 (100 μM)[cb] (IC_{50}: 16 ± 1)[cb]
14	0[c] (IC_{50}: 0.6 ± 0.07)	21 ± 1[c] (IC_{50}: 138 ± 13)	24 ± 1(100 μM)[cb] (IC_{50}: 26 ± 3)[cb]
15	52 ± 9	31 ± 2 (IC_{50}: 339 ± 45)	52 ± 7 (100 μM)[cb]
16	45 ± 1	70 ± 4	27 ± 1 (100 μM)[ca] (IC_{50}: 18 ± 1)[cb]
17	5 ± 1 (IC_{50}: 15 ± 2)	44 ± 2 (IC_{50}: 481 ± 29)	69[ca]
18	80 ± 1[c]	78 ± 3	56 ± 7[ca]
19	29 ± 1 (IC_{50}: 320)	57 ± 2	56 ± 3[cb]
20	51 ± 0	71 ± 2	0[ca]; 95 ± 4 (100 μM)[ca]
21	50 ± 2	55 ± 2	23 ± 2[ca] (IC_{50}: 79 ± 16)[ca]
22	50 ± 2	81 ± 4	38 ± 2[ca]; 82 ± 1 (100 μM)[cb]
23	73 ± 9	89 ± 2	29 ± 3[ca]; 70 ± 1 (100 μM)[cb]
24	71 ± 3	32 ± 2 (IC_{50}: 376 ± 61)	20 ± 1[ca]; 69 ± 1 (100 μM)[cb]
25	71 ± 4	79 ± 7	59 ± 2[ca]
26	73 ± 1[c]	66 ± 4[c]	nd
27	74 ± 5	100 ± 1	87 ± 3[cb]

The concentration of acylaminomethylboronic acid derivatives, if not specifically mentioned, was 1 mM. IC_{50} values are given in μM.
[a] Assay A.
[b] Assay B.
[c] 0.01% Triton-X-100.

2.2. Synthesis of acylaminomethylboronic acids

The synthesis described in Scheme 1 was used for the preparation of acylaminomethylboronic acid derivatives using methods described in the literature. The diisopropyl-*N*,*N*-bis(trimethylsilyl)aminomethyl boronate **3** was synthesized in two steps from dibromomethane.[27,28] Compound **3** and acylchlorides were used for the synthesis of various acylaminomethylboronic acids.[26] Low chemical yields reflected the reactivity of different acylchlorides and the synthesis has yet to be optimized.

2.3. Activity of acylaminomethylboronic acids

All compounds were tested for inhibition of PBP1b, a class A PBP, two class B PBPs, PBP2xR6 (penicillin sensitive) and PBP2x5204 (penicillin resistant, see Supplementary data) all from *Streptococcus pneumoniae*, the penicillin resistant PBP2a from *Staphylococcus aureus* and PBP5fm from *Enterococcus faecium* as well as R39 from *Actinomadura* sp. strain, a class C PBP. Thioesters were used as reporter substrates in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for the determination of the

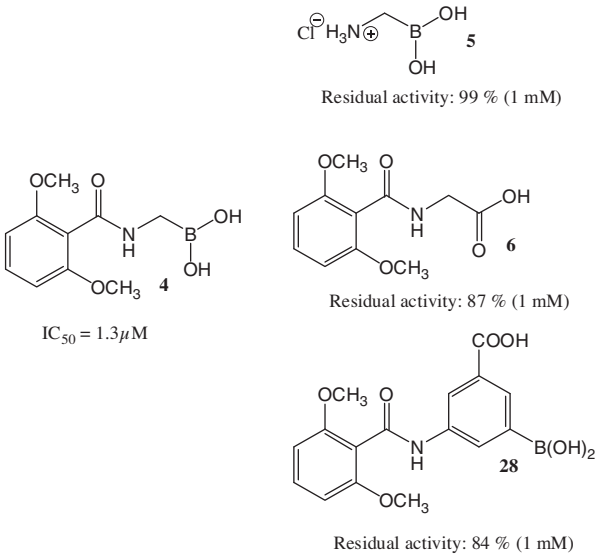
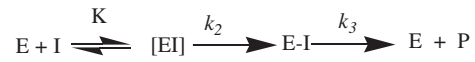


Figure 3. (2,6-Dimethoxybenzamido)methylboronic acid **4** was identified as a good inhibitor of R39 (IC_{50}: 1.3 μM). Two fragments of **4**: the aminomethylboronic acid **5** and 2-(2,6-dimethoxybenzamido)acetic acid **6**, and 3-(dihydroxyboryl)-benzoic acid **28** described by Inglis[21] were not or only poor inhibitors at a concentration of 1 mM.

residual activity of PBP1b, PBP2x and R39. PBP2a and PBP5fm from penicillin resistant bacterial strains do not hydrolyze the thioesters 2-(2-benzamidopropanoylthio)acetic acid (S2d) and 2-(2-(2-phenylacetamido)propanoylthio)propanoic acid (PATP) (see Supplementary data). Thus counter labeling of active PBPs with



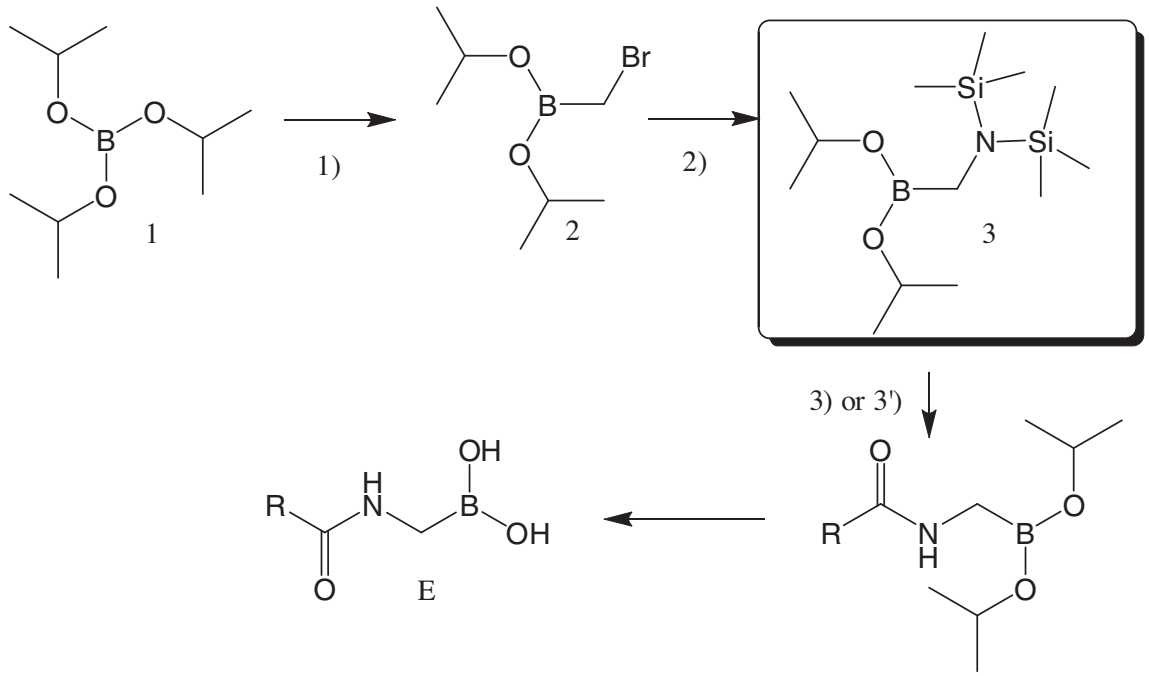
Scheme 2. Model of the inactivation of PBPs by penams. The deacylation rate constant k_3 is very small.

fluoresceyl ampicillin was used to determine the residual activity. Inhibition of PBPs by boronic acids is time dependent (data not shown).[25] Residual activities were measured after pre-incubations of PBPs with acylaminomethylboronic acid for 1 h (PBPs from penicillin sensitive bacteria strains) or 4 h (PBPs from penicillin resistant bacteria strains, see Supplementary data). Inhibition was monitored in the presence of 0.01% Triton-X-100.

Several acylaminomethylboronic acids were inhibitors of PBPs (Table 2). The acylaminomethylboronic acid derivative **14** with a 2-nitrobenzoyl side chain was a potent inhibitor of all PBPs of classes A (PBP1b: IC_{50} = 26 μM), B (PBP2xR6: IC_{50} = 138 μM) and C (R39: IC_{50} = 0.6 μM) from penicillin sensitive strains. Some of the boronic acids also displayed low activities against class B PBPs from penicillin resistant strains (PBP2x5204, PBP2a and PBP5fm) (Table 1, Supplementary data).

The results summarized in Table 2 show that the nature of the X side chain significantly influences the inhibitory ability of acylaminomethylboronic acids. This observation is similar to the results obtained by kinetic studies with penams bearing different side chains[8] (Fig. 2). The second order rate constant k_2/K, which characterizes the formation of the E-I acyl-enzyme (Scheme 2), depends on the nature of the side chain R (Fig. 2).

Compound **4** was a good inhibitor of R39 (IC_{50} = 1.3 μM), a mediocre inhibitor of PBP2xR6 (IC_{50} = 278 μM) and a poor inhibitor of PBP1b (IC_{50} >1000 μM) (Table 2). Similar observations can be made by comparing the results of inhibition experiments



1) n-BuLi in hexane, CH_2Br_2, THF, -78°C, 90 min, 79 %
2) n-BuLi, HMDS, THF, -78°C, 30 min, then to rt overnight, 58 %
3) 5 equiv. RCOCl, CH_2Cl_2, -78°C → rt, 120 min; MeOH, -78°C → rt, 30 min, H_2O (method A)
3') 1 equiv MeOH, -78°C → rt, 2 h ; 1 equiv RCOCl, CH_2Cl_2, -78°C → rt, 17 h; H_2O (method B)

Scheme 1. Synthesis of acylaminomethylboronic acids.

performed with compounds **7** and **13**. In contrast to compound **7**, which carries a 2,6-difluorobenzoyl-side chain, compound **13**, with a single fluoro substituent, was a poor inhibitor of R39 (**13**: IC_{50} = 505 μM, **7**: IC_{50} = 35 μM) while both were good inhibitors of PBP1b (**7** and **13**: IC_{50} <20 μM). These results reflect the structural differences between the active sites of PBPs as do the very different k_2/K second order rate constants observed for penicillin G with various PBPs (Table 1).

The nature of the substituent on the boron atom (aryl or alky) also influences the inhibition. A compound with a 2,6-dimethoxybenzoylamino side chain in the meta position of 3-(dihydroxyboryl) benzoic acid (such as **28**[21] (Fig. 3)) was a poor inhibitor of R39 while **4**, with a aminomethylboronic acid group, was a potent inhibitor. 3-(Dihydroxyboryl)benzoic acid derivatives with a 2-methoxybenzoylamino, a 2-phenyacetylamino or a benzoylamino side chains were described as good inhibitors of R39 (IC_{50} <100 μM)[21] while the corresponding aminomethylboronic acid derivatives **10** (RA = 43% at 1 mM), **19** (IC_{50}: 320 μM) and **18** (RA = 80% at 1 mM) were less potent.

Crystal structures of adducts of R39 with **4**, **11**, **14** and **19**[25] and PBP1b with **4**, **7**, **11**, **13**, and **14**[20] were solved and allowed an interpretation of the results. No crystal structure of a complex of PBP2xR6 with a boronic acid has been described yet.

An unexpected tricovalent-binding mode was observed in the active site of R39 with **4**, **11**, **14** and **19** (Fig. 4). In the tricovalent adduct specific interactions of *ortho* substituents on the phenyl group of the aminomethylboronic acids within the active site of R39 explain the decrease of K_i[25] and IC_{50}-values of **4**, **7**, **11**, **14** and **17** when compared with compounds without *ortho* substituents like **19** (IC_{50} = 320 μM, K_i = 125 μM[25]). The improvement was weak in the case of **7** (fluoro, IC_{50} = 35 μM), **11** (chloro, IC_{50} = 85 μM, K_i = 42 μM[25]) and **17** (trifluoromethyl, IC_{50} = 15 μM) but strong with **4** (methoxy, IC_{50} = 1.3 μM, K_i = 1.5 μM[25]) and **14** (nitro, IC_{50} = 0.6 μM, K_i = 0.36 μM[25]). Furthermore the introduction of a methylene group between the 2-chlorophenyl or the 2-nitrophenyl-side chains and the aminomethylboronic acid (**12** and **15**, respectively), presumably reducing the prevalence of the tricovalent adduct in the inhibition of R39 led to an important increase of the IC_{50}-value (IC_{50} >1000 μM) while the IC_{50}-values of **11** and **14** were 85 and 0.6 μM, respectively. **20**, with a 3-phenylpropanoyl side chain (RA: 51% at 1 mM), was also less active than **19**, with a 2-phenylacetyl side chain (IC_{50} = 320 μM). In the tricovalent adduct formed between R39 and **4**, one methoxy group occupies the hydrophobic pocket of PBPs that is thought to accommodate the methyl group of the penultimate D-alanine of the natural substrate (a pentapeptide ending with D-Ala-D-Ala). A methoxy group in position 3 (**21**, **9**) or 4 (**22**, **9**) of the phenyl group would certainly not allow such hydrophobic interactions and could clash with amino acids side chains in the R39 active site (Y147, L349) disfavoring the formation of the tricovalent adduct that is probably the species responsible for the strong inhibition of R39 by aminomethylboronic acids. Thus **8** with methoxy groups in positions 2 and 4 was a poor inhibitor. Compound **10** was expected to be a good inhibitor but its methoxy group might be oriented to the exterior of the active site. A similar observation was made with **13** (IC_{50} = 505 μM), which was also less potent than **7** (IC_{50} = 35 μM), which bears a 2,6-difluorobenzoyl side chain.

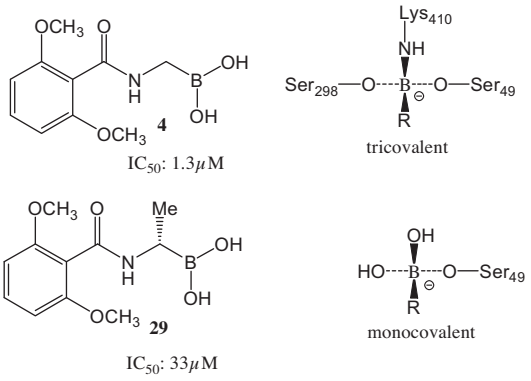


Figure 4. Crystal structures of adducts of R39 with **4** and **29**.[24,25] An unexpected tricovalent binding mode was observed with glycine analog **4** while the ethylboronic acid **29**[24] that mimics the D-alanine residue formed a monocovalent complex.

In the presence of ethylboronic acids, which mimic the D-alanine residue, the natural amino acid that acylates the active serine of PBPs, only monocovalent complexes (Fig. 4) were observed with R39.[24,25] The IC_{50}-value of **29** (Fig. 4), bearing a 2,6-dimethoxybenzoyl side chain, was increased (IC_{50} = 33 μM[24], IC_{50} (**4**) = 1.3 μM), while an IC_{50}-value of 3.3 μM[24] was observed for the ethylboronic acid analogs of **7** (IC_{50} = 35 μM) indicating that the 2,6-difluorobenzoyl side chain probably fits better in the monocovalent complex than the 2,6-dimethoxybenzoyl group.

The co-crystal structures of the monocovalent complexes of PBP1b were classified into two 'families' depending on the occupation of the pockets by the aryl groups.[20] The aryl groups of glycine analogs (**7**, **11**, **13** and **14**) and the *R*-methyl-substituted analog of **29** occupied pocket 1 while the side chains of the glycine analog **4** and of the *S*-methyl substituted analogs were found in pocket 2 indicating that the C-α chirality may direct the side chain to a specific pocket. The C-6/C-7 amide side chains of cephalosporins and penicillins, including methicillin, which has a 2,6-dimethoxybenzoyl side chain (Fig. 2) also fill pocket 2.[20] Compound **4** was a poor inhibitor of PBP1b (IC_{50} >1000 μM) indicating that its side chain does not fit very well in pocket 2. *S*-Methyl substituted analogs with a hydrophobic group in the *ortho* position of the aryl side chains such as the 2-fluoro-6-phenylbenzoyl group (E10[20]), fit better and display good inhibitory activity against PBP1b (IC_{50} of E10 = 20 μM).

Compounds **7**, **11**, **13**, **14** and **16** are good inhibitors of PBP1b (IC_{50}: 7–27 μM). No co-crystal structure with **16** has been solved but results of inhibition studies indicate that this boronic acid probably occupies pocket 1. The introduction of a methylene group between the 2-chlorophenyl or the 2-nitrophenyl side chains and the aminomethylboronic acid (**12** and **15**, respectively), increased the IC_{50}-values (**12**: RA = 97% at 100 μM and 24% at 1 mM, **15**: RA = 52% at 100 μM) while the IC_{50}-values of **11** and **14** were 27 and 26 μM, respectively. An increase of IC_{50}-values could reflect that molecules fit poorly in pocket 1 and that they could occupy pocket 2 like compound **4**.

In conclusion, glycine analogs can form unexpected complexes with PBPs (R39: tricovalent complex,[25] PBP1b: **7**, **11**, **13** and **14** in pocket 1[20]) while crystal structures with D-alanine analogs (*S*-methyl-substituted boronic acids) were more similar to those formed with β-lactams (R39: monocovalent[24]; PBP1b: pocket 2[20]).

Compounds **4**, **7**, **11**, **13** and **14** showed no antibacterial activity when tested on a broad range of bacterial strains (data not shown). The (*S*)-1-(2-fluoro-6-phenylbenzamido)ethaneboronic acid E10 described before was active against pathogens including a methicillin-resistant *S. aureus* (MRSA).[20] Compounds **4** and **19** are serine-β-lactamase inhibitors.[16,26] Inhibition studies of β-lactamases with the acylaminomethylboronic acids presented in this study are underway. The utilization of acylaminomethylboronic acids in combination with a β-lactam can potentially broaden the spectrum of antibacterial activity.[16]

3. Conclusion

After the identification of (2,6-dimethoxybenzamido)methylboronic acid **4** as a potent R39 inhibitor (IC_{50} = 1.3 μM) analogs of **4** were prepared. Inhibition studies with PBPs of classes A, B and C

from penicillin sensitive strains showed the importance of the nature of the side chain for the inhibition properties. The (2-nitrobenzamido)methylboronic acid **14** was a potent inhibitor of all studied PBPs from penicillin sensitive strains.

4. Experimental

All chemicals and reagents were either purchased p.A. from commercial suppliers. All solvents used were HPLC grade. The HPLC chain consists of a pump (Waters 600) and an UV detector (PDA Waters 996), (200–400 nm). Analytical HPLC analysis was performed on a XTerra RP18 (150 × 4.6 mm, 3.5 μm) column. The following protocol was used: solvent A: water (MilliporeQ) containing 0.1% TFA v/v and solvent B: acetonitrile. Flow: 0.7 mL/min, gradient: 0–30 min: 0–100% B, 30–35 min: 100% B, 35–36 min: 0–100% A, 36–56 min: 100% A. The injection volumes were 20 μL.

Manual chromatography was performed with Sep-Pak C18 Cartridges from Waters. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on either Bruker 250 or 400 MHz. Chemical shifts δ are given in ppm. The employed abbreviations for the multiplicities are the following ones: s = singlet, d = doublet, t = triplet, q = quadruplet, p = quintet, m = multiplet, br = broad. The coupling constant *J* is given in Hz. Spectra were recorded as solutions in d_6-DMSO (δ_H at 2.5 ppm, δ_C at 39.5 ppm) or D_2O (δ_H at 4.79 ppm) which were used as internal references. No ^{11}B NMR spectra were recorded because α-amido boronic acids have different chemical shifts at different pH values corresponding to different possible conformations.[29] TMSP (trimethylsilyl propionate) was used as external reference for ^{13}C NMR spectra done with D_2O. MS analysis was made on a device TSQ 7000 Thermoquest Finnigan equipped with an electrospray source. The co-solvent (injected in 200 μL/min) is a mixture 50/50: H_2O/acetonitrile containing 0.1% acetic acid v/v. FT-MS were done on a device 9,4 Tesla Apex-QeFTICR (Bruker Daltonics, Billerica, MA) with an electrospray source using a ESI-Positive mode. The conditions of injection were: solvent is a mixture 50/50: H_2O/acetonitrile containing 0.1% formic acid v/v containing ~25 μM of test compound. FT-MS* were done on a SYNAPT HDMS (Waters) with an internal calibration (72 *m*/*z* to 1285 *m*/*z*, error <2 ppm) using a nano ESI-Positive mode. Test compounds were injected at a concentration of ±10 μM in a solution of 50/50: H_2O/acetonitrile. Determination of an exact melting point of boronic acids was not possible.

4.1. Preparation of diisopropyl-*N*,*N*-bis(trimethylsilyl)-aminomethylboronate 3

17.5 g Diisopropyl (bromomethyl) boronate **2** were prepared from dibromomethane as described in literature (**2**, 17.5 g, 79%).[28] For the second step, an identical protocol as described by Martichonok[27] was used. After the synthesis of lithiohexamethyldisilazane ($LiN(TMS)_2$) from hexamethyldisilane (16.5 mL, 79 mmol) in 100 mL THF and *n*-BuLi (49 mL of 1.6 M solution in hexane, 78 mmol)[27], the resulting solution was cooled (−78 °C) and diisopropyl (bromomethyl) boronate **2** (17.5 g, 78 mmol) in THF (100 mL) was slowly added. The reaction mixture was then allowed to warm to 20 °C and stirred overnight. THF was removed under vacuum and the residue Kugelrohr distilled to give diisopropyl-*N*,*N*-bis(trimethylsilyl)-aminomethylboronate **3** (14.5 g, 46 mmol, 59%). ^{1}H NMR-data were in agreement with the literature.[30]

4.2. General procedure for the preparation of a solution of acid chlorides in CH_2Cl_2

To a solution of carboxylic acids (1.7 mmol) in dry DMF (100 μL) and dry toluene (15 mL) under nitrogen atmosphere, freshly distillated thionyl chloride (3.4 mmol) was added and the solution was stirred at room temperature overnight. The solvent and thionyl chloride were removed under vacuum. The acid chloride was dissolved with dichloromethane (15 mL) under nitrogen atmosphere and directly used for the synthesis of the boronic acid.

4.3. General procedure for the preparation of boronic acids (method A)

For the preparation of boronic acids a protocol described by Crompton[26] was used. The previously prepared solution of acid chloride (11.6 mmol) in dichloromethane (15 mL) under nitrogen atmosphere (see above) was slowly added to diisopropyl-*N*,*N*-bis(trimethylsilyl)aminomethylboronate (**3**, 5 mL, 2.2 mmol) at −78 °C. After stirring for 2 h at room temperature the solution was cooled to −78 °C and methanol (2.5 mL) was added. After stirring for 30 min at room temperature, water (10 mL) was added and the solution was stirred for 1 h. Organic solvents were removed and the product was suspended in water (15 mL). After extraction with ethyl acetate (3 × 15 mL) the aqueous layer was lyophilized and the products were collected. Further purification was performed by chromatography on Sep-Pak C18 Cartridges. Crude products were suspended in water, put on the top of a Sep-Pak column and the column was washed with water containing 0.1% TFA. The products were eluted using water with 0.1% TFA/acetonitrile 50/50 v/v. After the removing of acetonitrile under vacuum, aqueous solution was lyophilized.

4.4. General procedure for the preparation of boronic acids (method B)

To a solution of diisopropyl-*N*,*N*-bis(trimethylsilyl)aminomethylboronate (**3**, 1.7 mmol) in dichloromethane (15 mL) under nitrogen atmosphere 2 mL of a dichloromethane solution containing 0.068 mL (1.7 mmol) methanol at −78 °C were added. After stirring for 2 h at room temperature, the solution was cooled to −78 °C and a solution (2 mL) of acid chloride (1.7 mmol) in dichloromethane was added. After stirring overnight at room temperature the solvent was removed under vacuum and the product was resolved in water (15 mL) and upload to a Sep-Pak as described earlier in method A.

4.5. Preparation of various molecules

4.5.1. (2,6-Dimethoxybenzamido)methylboronic acid 4 (method A)

Three hundred and twenty milligrams (1.3 mmol) of a white solid (yield: 88%) were obtained from 2,6-dimethoxybenzoyl chloride and **3** (1.5 mmol). NMR-data were in agreement with Crompton.[26] HPLC: Rt = 12.6 min (210 nm). MS (ESI, positive ion) *m*/*z:* [221 (24%), 222 (100%), 223 (12%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 222.0932, observed: 222.0932.

4.5.2. Aminomethylboronic acid 5

A solution of **3** (5 mL) was mixed with a solution of methanol/HCl 6 N v/v (20 mL) at 0 °C for 1 h. After another hour at room temperature 10 mL of water was added. After washing with diethyl ether (3 × 20 mL) the water phase was lyophilized. Eight hundred and fifty milligrams of aminomethylboronic acid hydrochloride were obtained. ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.56 (s, CH_2). MS (ESI, positive ion) *m*/*z:* [76 (100%), 75 (25%)] (M−H_2O+H).

4.5.3. 2-(2,6-Dimethoxybenzamido)acetic acid 6

2,6-Dimethoxybenzoic acid (1.83 g, 10 mmol) in 20 mL DMF was mixed with the glycine methyl ester (HCl, 1.39 g, 11 mmol). Under nitrogen atmosphere the solution was cooled at 0 °C.

2.6 mL Diphenylphosphoryl azide (DPPA) (10 mmol) and 3.75 mL di-isopropyl ethylamine (0.02 mol) were slowly added. After 4 h at 0 °C the solution was stirred overnight at room temperature. The reaction was followed by TLC (silica gel 60, ethyl acetate, RF (product): 0.62). After the addition of 100 mL dichloromethane the solution was washed first with 2 N HCl and then with $NaHCO_3$ (5% w/v) and dried over Na_2SO_4. After filtration and the solvents removal the product was solubilized in a small amount of dichloromethane and purified by filtration through silica gel using ethyl acetate/petroleum ether 40–60, 4:1. After the elimination of the solvent under vacuum a white solid (1.65 g, 0.0065 mol, yield: 65%) was obtained. The product was mixed with 2 equiv LiOH solved in THF/water 1:1 v/v (20 mL). The pH of the solution was adjusted to 1 with 6 N HCl. After the extraction of the product with ethyl acetate the solution was washed with water and dried over Na_2SO_4. After filtration the solvent was removed under vacuum and a white solid (1.52 g, 6.4 mmol, yield: 98%) was obtained.

Mp: 110 °C. HPLC: Rt: 11.6 min (210 nm). ^{1}H NMR was in agreement with Chavez.[31] ^{13}C NMR (100 MHz, DMSO): δ (ppm) = 171.1 (COOH), 164.9 (CO), [156.9, 130.2, 116.4, 104.4 (Ar)], 55.8 (OCH_3), 40.9 (CH_2). MS (ESI, positive ion): *m*/*z*: [240 (100%), 241 (12%), 242 (2%)], (M+1). FT-MS: calculated (M+Na): 262.0691, observed: 262.0691.

4.5.4. (2,6-Difluorobenzamido)methylboronic acid 7 (method A)

One hundred and sixty-two milligrams (0.75 mmol) of a white solid (yield: 34%) were obtained from 2,6-difluorobenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR was in agreement with Inglis.[32] ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 31.6 (br, *C*H_2-B), 113.9 (t, $^2J_{CF}$ = 18.6 Hz, C-1), 114.8 (dd, $^4J_{CF}$ = 3.2 Hz, $^2J_{CF}$ = 22 Hz, C-3 and C-5), 136.0 (t, $^3J_{CF}$ = 10.7 Hz, C-4), 162.2 (dd, $^1J_{FC}$ = 247 Hz, $^3J_{CF}$ = 6.6 Hz, C-2 and C-6), 166.9 (*C*=O). HPLC: Rt = 9.9 min (210 nm). MS (ESI, positive ion) *m*/*z:* [197 (25%), 198 (100%), 199 (10%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 198.0532, observed: 198.0532.

4.5.5. (2,4-Dimethoxybenzamido)methylboronic acid 8 (method B)

Forty two milligrams (0.18 mmol) of a white solid (yield: 10%) were obtained from 2,4-dimethoxybenzoyl chloride and **3** (1.7 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.52 (s, 2H, C*H*$_2$-B), 3.88 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.6 (s, 1H, Ar-H), 6.68 (dd, ^3J = 8.8 Hz, ^4J = 2.3 Hz, 1H, Ar-H), 7.91 (d, ^3J = 9 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 39.0 (br, *C*H_2-B), 58.5 (OCH_3), [100.7, 108.2, 109.4, 136.1, 164.0, 168.2 (Ar)], 172.2 (*C*=O). HPLC: Rt = 18.5 min (210 nm). MS (ESI, positive ion) *m*/*z:* [221 (24%), 222 (100%), 223 (11%)] (M−H_2O+H). FT-MS*: calculated (M+Na): 262.0868, observed: 262.0864.

4.5.6. (3,4-Dimethoxybenzamido)methylboronic acid 9 (method A)

Two hundred and eighty-three milligrams (1.2 mmol) of a white solid (yield: 54%) were obtained from 3,4-dimethoxybenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.54 (s, 2H, C*H*$_2$-B), 3.85 (s, 3H, OC*H*$_3$), 3.88 (s, 3H, OC*H*$_3$), 7.05 (d, ^3J = 8.4 Hz, 1H, Ar-H), 7.37 (s, 1H, Ar-H), 7.51 (d, ^3J = 8.4 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 37.8 (br, *C*H_2-B), 58.5 (OCH_3), 58.6 (OCH_3), [113.3, 114.1, 121.9, 125.4, 150.8, 155.6 (Ar)], 174 (*C*=O). HPLC: Rt = 13.2 min (210 nm). MS (ESI, positive ion) *m*/*z*: [221 (23%), 222 (100%), 223 (17%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 222.0932, observed: 222.0932.

4.5.7. (2-Methoxybenzamido)methylboronic acid 10 (method A)

One hundred and sixty-one milligrams (0.77 mmol) of a white solid (yield: 35%) were obtained from 2-methoxybenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.56 (s, 2H, C*H*$_2$-B), 4.01 (s, 3H, OC*H*$_3$), 7.18 (t, ^3J = 7.6 Hz, 1H, Ar-H), 7.24 (d, ^3J = 7.6 Hz, 1H, Ar-H), 7.69 (t, ^3J = 7.2 Hz, 1H, Ar-H), 8.01 (d, ^3J = 7.6 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 28.8 (br, *C*H_2-B), 58.7 (OCH_3), [114.9, 115.2, 123.7, 134.2, 139.1, 162.1 (Ar)], 172.7 (*C*=O). HPLC: Rt = 13.5 min (210 nm). MS (ESI, positive ion) *m*/*z:* [191 (25%), 192 (100%), 193 (10%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 192.0827, observed: 192.0826.

4.5.8. (2-Chlorobenzamido)methylboronic acid 11 (method A)

Two hundred and six milligrams (0.97 mmol) of a white solid (yield: 44%) were obtained from 2-chlorobenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.67 (s, 2H, C*H*$_2$-B), 7.36–7.52 (m, 4H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 33.5 (br, *C*H_2-B), [130.1, 132.1, 133.0, 133.6, 134.2, 135.3 (Ar)], 173.8 (*C*=O). HPLC: Rt = 12 min (210 nm). MS (ESI, positive ion) *m*/*z*: [195 (24%), 196 (100%), 197 (20%), 198 (34%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 196.0331, observed: 196.0331.

4.5.9. (2-(2-Chlorophenyl)acetamido)methylboronic acid 12 (method B)

One hundred milligrams (0.44 mmol) of a white solid (yield: 26%) were obtained from 2-(2-chlorophenyl)acetyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.44 (s, 2H, C*H*$_2$-B), 3.88 (s, 2H, Ar-C*H*$_2$), 7.35 (m, 3H, Ar-H), 7.48 (m, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 34.8 (br, *C*H_2-B), 37.3 (Ar-*C*H_2), [130.3, 132.4, 134.0, 134.8, 137.0 (Ar)], 178.3 (*C*=O). HPLC: Rt = 14.5 min (210 nm). MS (ESI, positive ion) *m*/*z* = [209, 210 (64%), 211, 212] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 210.0488, observed: 210.0488.

4.5.10. (2-Fluorobenzylamido)methylboronic acid 13 (method B)

One hundred and eighty milligrams (0.91 mmol) of a white solid (yield: 54%) were obtained from 2-fluorobenzoyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.66 (s, 2H, C*H*$_2$-B), 7.29 (dd, $^3J_{HH}$ = 8.6 Hz, $^3J_{HF}$ = 11.8 Hz 1H, Ar-H), 7.35 (t, $^3J_{HH}$ = 7.6 Hz, 1H, Ar-H), 7.66 (m, 1H, Ar), 7.87 (td, $^3J_{HH}$ = 7.6 Hz, $^4J_{HF}$ = 1.6 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 36.1 (br, *C*H_2-B), 119.2 (d, $^2J_{CF}$ = 12 Hz, Ar), 119.3 (d, $^2J_{CF}$ = 21.7 Hz, Ar), 127.7 (d, $^4J_{CF}$ = 3.2 Hz, Ar), 133.4 (d, $^3J_{CF}$ = 1.8 Hz, Ar), 138.1 (d, $^3J_{Cf}$ = 9.3 Hz, Ar), 163.4(d, $^1J_{CF}$ = 249 Hz, Ar), 170.9 (d, $^3J_{CF}$ = 2.2 Hz, *C*=O). HPLC: Rt = 12.0 min (210 nm). MS (ESI, positive ion) *m*/*z:* [179 (25%), 180 (100%), 181 (14%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+1): 180.0627, observed: 180.0627.

4.5.11. (2-Nitrobenzamido)methylboronic acid 14 (method A)

Two hundred and twenty-one milligrams (0.099 mmol) of a white solid (yield: 45%) were obtained from 2-nitrobenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.77 (s, 2H, C*H*$_2$-B), 7.52 (m, 1H, Ar-H), 7.62 (m, 1H, Ar-H), 7.72 (m, 1H, Ar-H), 8.06 (m, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 32.4 (br, *C*H_2-B), [127.6, 131.7, 132.4, 134.3, 137.4, 148.3 (Ar)], 172.9 (*C*=O). HPLC: Rt = 9.4 min (210 nm). MS (ESI, positive ion) *m*/*z:* [206 (25%), 207 (100%), 208 (17%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 207.0571, observed: 207.0571.

4.5.12. (2-(2-Nitrophenyl)acetamido)methylboronic acid 15 (method B)

One hundred and seventy-five milligrams (0.74 mmol) of a white solid (yield: 43%) were obtained from 2-(2-nitrophenyl)acetyl chloride and **3** (1.7 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.52 (s, 2H, C*H*$_2$-B), 4.09 (s, 2H, Ar-C*H*$_2$), 7.55 (dd, ^3J = 7.5 Hz, ^4J = 1.5 Hz, 1H, Ar-H), 7.63 (td, ^3J = 7.5 Hz, ^4J = 1.5 Hz, 1H, Ar-H), 7.77 (td, ^3J = 7.5 Hz, ^4J = 1.5 Hz, 1H, Ar-H), 8.2 (dd, ^3J = 8.0 Hz, ^4J = 1.5 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ

(ppm) = 34.3 (br, CH_2-B), 40.4 (Ar-CH_2), [128.3, 131.5, 132.2, 136.7, 137.5, 150.9 (Ar)], 177.7 (C=O). HPLC: Rt = 11.6 min (210 nm). MS (ESI, positive ion) *m/z:* [220 (24%), 221 (100%), 222 (16%)] (M−H_2O+H). FT-MS*: calculated (M+Na): 261.0656, observed: 261.0657.

4.5.13. (2-Methylbenzamido)methylboronic acid 16 (method A)

Three hundred and seventy-seven milligrams (1.95 mmol) of a white solid (yield: 89%) were obtained from 2-methylbenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.15 (s, 3H, Ar-CH_3), 2.44 (s, 2H, CH_2-B), 7.03 (m, 2H, Ar-H), 7.25 (m, 2H, Ar-H).. ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 22.1 (Ar-CH_3), 37.4 (br, CH_2-B), [128.7, 129.2, 131.1, 134.1, 135.4, 140.6 (Ar)], 177.3 (C=O). HPLC: Rt = 12.4 min (210 nm). MS (ESI, positive ion) *m/z:* [175 (25%), 176 (100%), 177 (10%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 176.0877, observed: 176.0877.

4.5.14. (2-(Trifluoromethyl)benzamido)methylboronic acid 17 (method B)

One hundred and seventeen milligrams (0.47 mmol) of a white solid (yield: 28%) were obtained from 2-(trifluoromethyl)benzoyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.83 (s, 2H, CH_2-B), 7.59 (d, ^3J = 6.8 Hz, 1H, Ar-H), 7.71 (m, 2H, Ar-H), 7.84 (d, ^3J = 7.2 Hz, 1H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 32.8 (CH_2-B(OH)$_2$), 126.3 (q, $^1J_{CF}$ = 269 Hz, CF_3) 129.4 (q, $^3J_{CF}$ = 4 Hz, Ar), 129.35 (q, $^2J_{CF}$ = 32 Hz, Ar), 131.1, 133.5(Ar), 135.0 (m, Ar), 135.2 (m, Ar), 174.4 (C=O). HPLC: Rt = 15.5 min (210 nm). MS (ESI, positive ion) *m/z:* [229 (28%), 230 (100%), 231 (8%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 230.0595, observed: 230.0595.

4.5.15. Benzamidomethylboronic acid 18 (method B)

Two hundred and eleven milligrams (1.2 mmol) of a white solid (yield: 70%) were obtained from benzoylchloride and **3** (1.7 mmol). NMR-data were in agreement with Lai.[29] HPLC: Rt = 11.3 min (210 nm). MS (ESI, positive ion) *m/z:* [161 (24%), 162 (100%), 163 (13%)] (M−H_2O+H). FT-MS*: calculated (M+Na): 202.0651, observed: 202.0651.

4.5.16. (2-Phenylacetamido)methylboronic acid 19 (method B)

One hundred and twenty-eight milligrams (0.66 mmol) of a white solid (yield: 39%) were obtained from 2-phenylacetyl chloride and **3** (1.7 mmol). NMR-data were in agreement with Crompton.[26] HPLC: Rt = 15.1 min (210 nm). MS (ESI, positive ion) *m/z:* [175 (28%), 176 (100%), 177 (14%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 176.0877, observed: 176.0877.

4.5.17. (3-Phenylpropanamido)methylboronic acid 20 (method B)

Two hundred and eighteen milligrams (1.1 mmol) of a white solid (yield: 64%) were obtained from 3-phenylpropanoyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.30 (s, 2H, CH_2-B), 2.71 (t, ^3J = 7.2 Hz, 2H, CH_2–C=O), 2.97 (t, ^3J = 7.2 Hz, 2H, Ar-CH_2), 7.28-7.40 (m, 5H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 33.3 (Ar-CH_2), 35.7 (CH_2–C=O), 36.0 (br, CH_2-B), [129.4, 131.2, 131.6, 142.7 (Ar)], 180.6 (C=O). HPLC: Rt = 21.2 min (210 nm). MS (ESI, positive ion) *m/z:* [189 (25%), 190 (100%), 191 (10%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 190.1034, observed: 190.1034.

4.5.18. (3-Methoxybenzamido)methylboronic acid 21 (method A)

One hundred and sixty-seven milligrams (0.8 mmol) of a white solid (yield: 36%) were obtained from 3-methoxybenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.62 (s, 2H, CH_2-B(OH)$_2$), 3.89 (s, 3H, OCH_3), 7.29 (m, 1H, Ar-H), 7.44 (m, 1H, Ar-H), 7.51 (m, 2H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 58.5 (OCH_3), [115.9, 122.5, 123.4, 131.9, 133.3, 162.0 (Ar)], 174.5 (C=O). HPLC: Rt = 13.6 min (210 nm). MS (ESI, positive ion) *m/z:* [191 (25%), 192 (100%), 193 (10%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 192.0827, observed: 192.0826.

4.5.19. (4-Methoxybenzamido)methylboronic acid 22 (method A)

One hundred and eighty-seven milligrams (0.89 mmol) of a white solid (yield: 40%) were obtained from 4-methoxybenzoyl chloride and **3** (2.2 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.52 (s, 2H, CH_2-B), 3.88 (s, 3H, OCH_3), 7.08 (d, ^3J = 8.4 Hz, 2H, Ar-H), 7.86 (d, ^3J = 8.4 Hz, 2H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 35.4 (br, CH_2-B), 56.3 (OCH_3), [115.1, 118.9, 131.0,, 164.3 (Ar)], 172.2 (C=O). HPLC: Rt = 13.4 min (210 nm). MS (ESI, positive ion) *m/z:* [191 (25%), 192 (100%), 193 (10%)] (M−H_2O+H). FT-MS* calculated (M−H_2O+H): 192.0827, observed: 192.0833.

4.5.20. (4-Chlorobenzamido)methylboronic acid 23 (method A)

One hundred and eleven milligrams (0.51 mmol) of a white solid (yield: 39%) were obtained from 4-chlorobenzoyl chloride and **3** (1.3 mmol). ^{1}H NMR (250 MHz, D_2O): δ (ppm) = 2.61 (s, 2H, CH_2-B), 7.53 (d, ^3J = 8.5 Hz, 2H, Ar-H), 7.81 (d, ^3J = 8.5 Hz, 2H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 27.0 (br, CH_2-B), [126.4, 129.8, 130.0, 140.0 (Ar)], 171.6 (C=O). HPLC: Rt = 15.8 min (210 nm). MS (ESI, positive ion) *m/z:* [195 (24%), 196 (100%), 197 (19%), 198 (34%)] (M−H_2O+H). FT-MS*: calculated (M−H_2O+H): 196.0337, observed: 196.0336.

4.5.21. (2-(4-Chlorophenyl)acetamido)methylboronic acid 24 (method B)

One hundred and twenty-five milligrams (0.55 mmol) of a white solid (yield: 32%) were obtained from 2-(4-chlorophenyl)acetyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.38 (s, 2H, CH_2-B), 3.69 (s, 2H, Ar-CH_2), 7.25 (d, ^3J = 8.8 Hz, 2H, Ar-H), 7.38 (d, ^3J = 8.5 Hz, 2H, Ar-H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 37.5 (Ar-CH_2), [129.9, 131.9, 133.3, 134.6 (Ar)], 178.7 (C=O). HPLC: Rt = 17.7 min (210 nm). MS (ESI, positive ion) *m/z:* [209 (25%), 210 (100%), 211 (19%), 212 (34%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 210.0488, observed: 210.0488.

4.5.22. (3-Cyclopentylpropanamido)methylboronic acid 25 (method B)

One hundred and ninety-seven milligrams (1 mmol) of a white solid (yield: 58%) were obtained from cyclopentanepropionyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 1.09 (m, 2H, cyclopentyle-CH_2), 1.63 (m, 6H, cyclopentyle-CH_2), 1.75 (m, 3H, cyclopentyle-CH_2), 2.34 (s, 2H, CH_2-B), 2.42 (t, ^3J = 7.2 Hz, 2H, CH_2–C=O). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = [27.5, 32.8, 33.5, 34.6, 41.7 (cyclopentyle-CH_2–CH_2)], 37.2 (br, CH_2-B), 182.4 (C=O). MS (ESI, positive ion) *m/z:* [181 (25%), 182 (100%), 183 (12%)] (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 182.1347, observed: 182.1347.

4.5.23. (Thiophene-2-carboxamido)methylboronic acid 26 (method B)

One hundred and forty-five milligrams (0.78 mmol) of a white solid (yield: 46%) were obtained from thiophenyl-2-carbonyl chlorid and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.63 (s, 2H, CH_2-B), 7.22 (t, ^3J = 4.4 Hz, 1H), 7.80 (m, 2H). ^{13}C NMR (62 MHz, D_2O): δ (ppm) = 35.2 (br, CH_2-B), [131.3, 134.4, 134.5, 135.9 (Ar)], 168.7 (C=O). HPLC: Rt = 11.3 min (210 nm). MS (ESI, positive ion) *m/z*: 168 (M−H_2O+H). FT-MS: calculated (M−H_2O+H): 168.0285, observed: 168.0285.

4.5.24. Acetamidomethylboronic acid 27 (method A)

One hundred and fifty-nine milligrams (1.36 mmol) of a white solid (yield: 80%) were obtained from acetyl chloride and **3** (1.7 mmol). ^{1}H NMR (400 MHz, D_2O): δ (ppm) = 2.06 (s, 3H, CH_3) 2.32 (s, 2H, CH_2-B), ^{13}C NMR (100 MHz, D_2O): δ (ppm) = 19.2, 37.8 (br, CH_2-B), 179.7 (C=O). MS (ES+): *m/z*: [100 (100%), 101 (10%), 102 (5%)] ($M-H_2O+H$). FT-MS*: calculated ($M-H_2O+H$): 100.0570, observed: 100.0569.

4.6. Biological assays

R39 from *Actinomadura* was prepared and purified as described by Granier.[33] PBP2x-5204 and, PBP2x-R6 from *Streptococcus pneumoniae* were prepared as described by Carapito[4] while PBP1b from the same organism was purified as described by Di Guilmi.[6] Fluorescein labeled ampicillin was prepared as described by Lakaye.[34] The thioester 2-(2-benzamidopropanylthio)acetic acid S2d was prepared as described by Adam[35] and Schwyzer.[36] The preparation of 2-(2-(2-phenylacetamido)propanoylthio)propanoic acid PATP will be described elsewhere.

4.6.1. Inhibition tests of R39 and PBP2xR6

Inhibition experiments with R39 and PBP2xR6 were performed by monitoring the degree of hydrolysis of the substrate S2d using microtiter 96 well plates and a Power Wave microtiter plate reader (Bio-Tek Instruments). Enzyme residual activity (RA) was determined after pre-incubation of the PBPs in the presence of potential inhibitors. The initial rate of hydrolysis of S2d and the rate of spontaneous hydrolysis of S2d in the presence of the inhibitors was determined in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, $\varepsilon_{412\ nm}$ = 14,150 $M^{-1}\ s^{-1}$). All experiments were performed in triplicate. Activity of PBPs in absence of inhibitors (100% RA) was measured with six replicates on each plate. Potential inhibitors were solubilized in DMF. The final concentration of DMF in the assays was 1%. Potential inhibitors were incubated with 3.5 nM R39 in 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. In the case of PBP2xR6, 0.09 μM enzyme was incubated in the presence of potential inhibitors in 10 mM sodium phosphate buffer (pH 7.0) and 0.01 mg/mL BSA for 60 min at 25 °C. After the pre-incubation RAs were determined by adding S2d (1 mM) and DTNB (1 mM) and measuring the initial rate of hydrolysis of S2d at 412 nm (total test volume: 150 μL).

4.6.2. Inhibition tests of PBP1b

4.6.2.1. Assay A. PBP1b (0.7 μM) was incubated with potential inhibitors (1 mM) in 10 mM sodium phosphate (pH 7.0) for 60 min at 25 °C. Active PBP1b was then counter labeled with fluoresceyl-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.

4.6.2.2. Assay B. Inhibition experiments with PBP1b were also performed by monitoring the degree of hydrolysis of the PATP thioester as described before. Enzyme residual activity (RA) was calculated from initial rates after pre-incubation of PBP1b (0.2 μM) in the presence of potential inhibitors in 10 mM sodium phosphate buffer (pH 7.0), 100 mM D-alanine and 0.01 mg/mL BSA for 60 min at 25 °C. Initial rates of hydrolysis of PATP (5 mM) were determined in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 1.0 mM) as described (total test volume: 150 μL).

4.6.3. Screening of 'false positives' and determination of IC_{50} values

False positives (promiscuous inhibitors) were detected by performing assays under the same conditions but in the presence of 0.01% Triton-X-100 v/v. As described in the literature promiscuous inhibitors are slow binding, non-competitive inhibitors. In order to avoid a detailed kinetic study[13], it is possible to identify such compounds by performing tests in the presence of Triton-X-100.[37,38] Promiscuous inhibitors show no inhibition in the presence of Triton-X-100. IC_{50}-values of R39, PBP2xR6 and PBP1b were determined in the presence of 0.01% Triton-X-100 v/v. RA was measured over a range of concentrations from which IC_{50} values were determined by performing a non-linear regression analysis using Sigma Plot (Systat software) and fitting the data to the equation[3] $y = y_0 + (a \times b)/(b + x)$.

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Supplementary data

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