Biochem. J. (1983) **209**, 229–233
Printed in Great Britain

The inhibition of class C β-lactamases by boronic acids

Tim BEESLEY,* Norman GASCOYNE,* Vroni KNOTT-HUNZIKER,* Sigthor PETURSSON,* Stephen G. WALEY,* Bengtake JAURIN† and Thomas GRUNDSTRÖM†
**Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K., and †Department of Microbiology, University of Umea, S-901 87 Umea, Sweden*

(*Received 9 August 1982/Accepted 12 October 1982*)

Aromatic boronic acids are reversible inhibitors of the recently classified class C β-lactamases. The boronic acids studied include *ortho*-, *meta*- and *para*-methyl-, -hydroxymethyl- and -formyl-phenylboronic acid. The β-lactamases were chromosomally-encoded enzymes, one from *Pseudomonas aeruginosa*, and the other specified by the *ampC* gene of *Escherichia coli*. The inhibition may be correlated with our finding that these β-lactamases are serine enzymes, i.e. their function entails the hydroxy group of a serine residue acting as a nucleophile.

Inert reversible inhibitors of β-lactamases are hard to come by. Although some (e.g. izumenolide) have been reported recently (Bush *et al.*, 1980), the commonest inhibitors of β-lactamases are β-lactams, and they are seldom inert when high concentrations of enzyme are used. This is an important point for those interested in the mechanism of β-lactamase action because truly inert inhibitors are required for the most informative techniques (crystallography, nuclear magnetic resonance). The molecular classification of β-lactamases now recognizes three classes: A, B and C (Ambler, 1980; Jaurin & Grundström, 1981). Recently, boric acid and phenylboronic acid were found to be inhibitors of β-lactamase I from *Bacillus cereus* (Kiener & Waley, 1978), a class A β-lactamase that we have also shown to be a serine enzyme (Knott-Hunziker *et al.*, 1979, 1980*a*). This conclusion has been confirmed both for this and for other class A β-lactamases (Cohen & Pratt, 1980; Fisher *et al.*, 1981). It is now clear that class C β-lactamases are also serine enzymes (Knott-Hunziker *et al.*, 1980*b*, 1982*a*,*b*), although they are otherwise unrelated to the class A enzymes (Jaurin & Grundström, 1981). This raised the question whether class C β-lactamases are inhibited by boric acid or boronic acids. The present paper answers this question. The β-lactamases studied were from a mutant of *Pseudomonas aeruginosa* 18S that produces the enzyme constitutively (Flett *et al.*, 1976; Berks *et al.*, 1982) and from *Escherichia coli* K12 (the *ampC* β-lactamase) (Lindström *et al.*, 1970).

Inhibitors of class A β-lactamases are in clinical use, but effective inhibitors of the class C β-lactamases are lacking. The boronic acid with the highest affinity for the *Pseudomonas* β-lactamase in fact showed a modest degree of synergy when tested with cephalosporin C against the strain of *Pseudomonas aeruginosa* from which the β-lactamase was obtained. The compounds used in the present study are shown below. The synergistic action of penicillins and cephalosporins had previously been demonstrated with the strain of *Pseudomonas aeruginosa* containing the inducible β-lactamase (Sabath & Abraham, 1964).

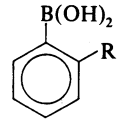
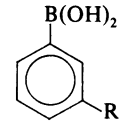
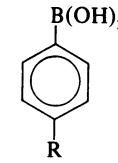
Experimental

^{1}H n.m.r. spectra were recorded on Bruker WH90 and Bruker WH300 instruments with tetramethylsilane as an internal reference. The ^{13}C n.m.r. spectrum was measured at a frequency of 22.63 MHz with a Bruker WH90 instrument. The chemical shifts are relative to tetramethylsilane, the solvent ([^{2}H]methanol) carbon peaks at δ 49.0 p.p.m. being a secondary reference.

3-Hydroxyphenylboronic acid

This was prepared from 3-aminophenylboronic acid by diazotization (Bean & Johnson, 1932) followed by hydrolysis. The diazonium salt solution was poured into an equal volume of water at 50°C, and the solution was then stirred at room temperature for 1 h. The product was extracted into diethyl ether, which was dried ($MgSO_4$) and evaporated. After purification on a column of silica gel (elution was with diethyl ether at 4°C), 3-hydroxyphenylboronic acid (R_F 0.29, on silica gel

0306-3275/83/010229-05$02.00

(1)
(a) R = CH_3
(b) R = CH_2OH
(c) R = CHO

(2)
(a) R = CH_3
(b) R = CH_2OH
(c) R = CHO
(d) R = OH
(e) R = $NHCOCH_3$
(f) R = $NHCOCH_2I$

(3)
(a) R = CH_3
(b) R = CH_2OH
(c) R = CHO

t.l.c., elution being with ether) was isolated in 46% yield and crystallized from dry 1,2-dichloroethane [m.p. 220–223°C; cf. m.p. value of 219–225°C recorded by Bean & Johnson (1932)].

3-(Iodoacetamido)phenylboronic acid

This was prepared by reaction of iodoacetyl chloride (Abderhalden & Guggenheim, 1908) with 3-aminophenylboronic acid hemisulphate in 50% acetone containing 2.4 molar equivalents of $NaHCO_3$. On evaporation of the acetone the crude product precipitated. This was decolourized in warm methanol with animal charcoal. After filtration and evaporation of the solvent, the residue was crystallized from boiling water. Yield, 51%; m.p. (anhydride), 232°C (decomposition). ^1H n.m.r. ([^2H]-methanol): δ (p.p.m.) 3.83 (2H, s, $-CH_2-$), 7.2–7.8 (4H, m, aromatic protons). (Found: C, 31.19; H, 2.96; N, 4.47. Calc. for $C_8H_9BINO_3$: C, 31.52; H, 2.97; N, 4.60%).

3-Acetamidophenylboronic acid

This was prepared by reaction of acetic anhydride with 3-aminophenylboronic acid, as described above. The crude product was purified on a column of silica gel [elution was with chloroform/methanol (9:1, v/v)] before crystallization from water. M.p. 255°C (decomposition) [cf. m.p. value of 250°C [recorded by Torsell (1957*a*)]. ^{13}C n.m.r. ([^2H]-methanol) (multiplicity of each signal was determined by single frequency off-resonance decoupling, and this is given in parentheses after the chemical shifts): δ (p.p.m.) 23.7 (quartet, $-CH_3$), 123.3 (d), 126.7 (d), 128.9 (d and s), 130.5 (d), 139.0 (s, aromatic carbons), 171.6 (s, carbonyl carbon).

Tolylboronic acids

The 2-, 3- and 4-tolylboronic acids were prepared by published methods (Bean & Johnson, 1932; Torssell, 1954).

2-, 3- and 4-hydroxymethyl- and -formylphenylboronic acids

These were prepared by the method of Torssell (1957*b*), who reported that the 3- and 4-hydroxymethyl compounds were not crystalline and therefore did not characterize them. They were therefore purified in the following manner (described for the 3-hydroxymethyl compound). After hydrolysis of the 2-bromo-3-tolylboronic acid in boiling water, an equal volume of 1 M-HCl was added and the product was extracted into ethyl acetate. After drying ($MgSO_4$) and evaporation of the solvent, the hydroxymethyl compound [R_F 0.35 on silica gel t.l.c. (chloroform/methanol (37:3, v/v)] was purified on a column of silica gel with chloroform/methanol (37:3, v/v) as eluent. After evaporation of the solvent the residue was dissolved in a small amount of water and freeze-dried. For elemental analysis the compound was converted into the anhydride by drying under high vacuum at 110°C for 6 h. N.m.r. ([^2H]dimethyl sulphoxide) (solvent and water peaks at δ 2.5 and 3.3 are not listed) of 3-hydroxymethylphenylboronic acid: δ (p.p.m.) 4.5 (2H, d, J_{CH_2-OH} 5HZ, $-CH_2-$), 5.08 (1H, t, J_{OH-CH_2} 5Hz, CH_2-OH), 7.25 (1H, apparent t, $J_{H\text{-}5—H\text{-}4 \text{ and } \text{-}6}$ 7Hz, H-5), 7.34 (1H, d, $J_{H\text{-}4—H\text{-}5}$ 7Hz, H-4), 7.66 (1H, d, $J_{H\text{-}6—H\text{-}5}$ 7Hz, H-6), 7.74 (1H, s, H-2) and 7.92 [2H, s, $-B(OH)_2$]. After ^2H_2O shake, the doublet at δ 4.5 goes to a 2H singlet and –OH peaks at δ 5.08 and 7.92 disappear. N.m.r. for 4-hydroxymethylphenylboronic acid: δ (p.p.m.) 4.5 (2H, d, J_{CH_2-OH} 5Hz, $-CH_2-$), 5.18 (1H, t, J_{OH-CH_2} 5Hz, $-CH_2-OH$), 7.28 (2H, d, $J_{m\text{-}H—o\text{-}H}$ 7.5 Hz, *m*-H), 7.75 (2H, d, $J_{o\text{-}H—m\text{-}H}$, *o*-H) and 7.97 [2H, s, $-B(OH)_2$]. After ^2H_2O shake, the doublet at δ 4.5 goes to a 2H singlet and –OH peaks at δ 5.18 and 7.95 disappear.

For 3-hydroxymethylphenylboronic acid, found: C, 62.50; H, 5.29. Calc. for $C_7H_7BO_2$: C, 62.76; H, 5.27%. For 4-hydroxymethylphenylboronic acid, found: C, 62.24; H, 5.30. Calc. for $C_7H_7BO_2$: C, 62.76; H, 5.27%.

Enzymic methods

The β-lactamases from *E. coli* and *Pseudomonas aeruginosa* were prepared as described previously (Knott-Hunziker *et al.*, 1980*b*; Jaurin & Grundström, 1981). The K_i values were obtained by comparison of progress curves carried out in the presence and absence of inhibitor, with cephalosporin C (0.1 or 0.2mM) as substrate, in 100mM-triethanolamine hydrochloride buffer, pH8.0, at 21°C. The hydrolysis was followed by the change in $A_{259.7}$ (path-length = 10mm), the concentration of β-lactamase being around 3nM. About seven readings were taken from each progress curve, and K_i was obtained by the novel, quick method described previously (Waley, 1982). The mean value of the quotient, S.E.M./mean was 7% (Table 1).

Irreversible inhibition was tested as follows. The formylphenylboronic acid (*o*-, *m*- or *p*-) (1mM) and the *Pseudomonas* β-lactamase (3μM) were incubated together in 100mM-triethanolamine hydrochloride buffer, pH8, at 30°C. At times of up to 20h, portions (2μl) of the incubation mixture were withdrawn for assay against 3mM-cephalosporin C as substrate. This assay was carried out in 0.5M-NaCl in the pH-stat at pH8 and 30°C. There was no significant loss of enzyme activity brought about by any of the formylphenylboronic acids. Similarly, there was no loss in activity when the *Pseudomonas* β-lactamase (10nM) was incubated for 48h with 10mM-3-iodoacetamidophenylboronic acid (2f) at 37°C and pH7 (0.1M-phosphate), pH8 (0.1M-triethanolamine hydrochloride) or pH9 (0.1M-bicarbonate/carbonate) (S. J. Cartwright, personal communication).

Microbiological methods

The standard hole-plate assay failed to show any synergy when 3-iodoacetamidophenylboronic acid (2f) and cephalosporin C were tested against *Pseudomonas aeruginosa* 1822S/H [this strain produces the *Pseudomonas* β-lactamase constitutively (Flett *et al.*, 1976; Berks *et al.*, 1982)]. Instead, minimum inhibitory concentrations were determined as follows. An overnight culture in nutrient broth at 37°C provided the inoculum; portions were diluted 10-fold and incubated with a range of concentrations of cephalosporin C, 3-iodoacetamidophenylboronic acid or both, for 16h at 37°C. Absence of any visible turbidity on shaking was regarded as evidence of inhibition of growth.

Table 1. *Inhibition constants for boric acid and boronic acid*

The constants were obtained by comparison of progress curves, carried out in the presence and absence of inhibitor (Waley, 1982), of hydrolyses of cephalosporin C (0.1 or 0.2mM) in 0.1M-triethanolamine hydrochloride, pH8, at 30°C. The change in $A_{259.7}$ was measured in 1cm path-length cells. The value of K_i was obtained as outlined in the Experimental section. The value of K_i given is the mean ± S.E.M., for the number of values shown in parentheses.

Compound	K_i (μM) *Pseudomonas* β-lactamase	K_i (μM) *ampC* β-lactamase
Boric acid	630 ± 75 (4)	920 ± 120 (6)
Phenylboronic acids		
o-Methyl	15 ± 1 (5)	
m-Methyl	4.5 ± 0.5 (7)	7.5 ± 0.2 (6)
p-Methyl	6 ± 0.2 (5)	
o-Hydroxymethyl	29 ± 2 (6)	
m-Hydroxymethyl	11 ± 1.5 (6)	21 ± 0.8 (6)
p-Hydroxymethyl	16 ± 1.5 (6)	
o-Formyl	98 ± 4 (6)	82 ± 5 (6)
m-Formyl	6.1 ± 0.3 (6)	18 ± 1 (6)
p-Formyl	9.5 ± 0.4 (8)	
3-Hydroxy	9.8 ± 0.4 (6)	
3-Acetamido	7.6 ± 0.3 (6)	
3-Iodoacetamido	2.4 ± 0.1 (5)	23 ± 2 (7)

Results and discussion

Boronic acids as reversible inhibitors

Boric acid and the boronic acids behaved as reversible competitive inhibitors of these class C β-lactamases. Some of the compounds contained potentially reactive functional groups. We had hoped that one of the aldehydes (1c, 2c or 3c) might behave as an irreversible inhibitor by virtue of combination with a nearby amino group in the enzyme (if such exists). Irreversible inhibition, however, could not be detected. Nor did the iodo-compound, compound (2f), a potential alkylating agent, behave as an irreversible inhibitor. This compound exhibited synergy when tested with cephalosporin C, the minimum inhibitory concentrations of cephalosporin C and the compound being lowered to one-quarter and one-half respectively, when both were present (Table 2). However, the concentration

Table 2. *Synergy between 3-iodoacetamidophenylboronic acid and cephalosporin C against Pseudomonas aeruginosa 1822/H*

Growth (+) or absence of growth (−) are shown; details are given in the Experimental section.

Concn. of cephalosporin C (mg/ml)	Concn. of the boronic acid (mg/ml): 0	0.25	0.5	1
1	+	+	+	+
2	+	+	+	+
4	+	+	+	−
8	+	+	−	−

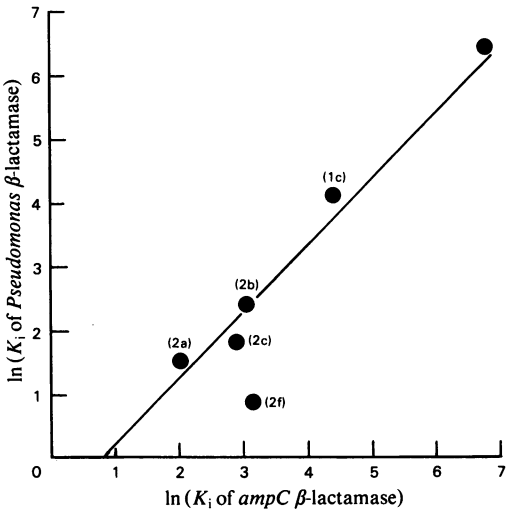


Fig. 1. *Structure–activity relationships in the inhibition of class C β-lactamases by boronic acids*

The values of $\ln K_i$ for the *Pseudomonas* β-lactamase are plotted on the ordinate against those of $\ln K_i$ for the *ampC* β-lactamase on the abscissa. The structures of the compounds are given in the text (the top point is boric acid); the line is drawn by the 'median' method (Atkins & Nimmo, 1980) in which each point is connected pair-wise with all the other points, the slopes of the lines ranked and the median taken.

of compound was then around 4 mM, whereas the K_i was 2.4 μM. Thus, although it seems reasonable to ascribe the synergy to inhibition of the β-lactamase, the weakness of the effect suggests that the boronic acid may penetrate poorly. 3-Iodoacetamidophenylboronic acid is a hydrophobic substance. The outer membrane of most Gram-negative bacteria is known to be a very effective barrier against such compounds (Nikaido, 1976).

Relative affinities: comparison of compounds

The dissociation constants in Table 1 cover a fairly wide range of values, from about 2 μM [for 3-iodoacetamidophenylboronic acid (2f)] to around 1 mM for boric acid. When translated into free energies of binding, the range is from about 18 kJ/mol to about 32 kJ/mol. Thus even for compound (2f), which binds most avidly, about half the free-energy change on binding can probably be assigned to interaction with the boronic acid group. The compounds containing methyl groups (1a–3a) bind somewhat more tightly than those of compounds (1b)–(3b) or (1c)–(3c), which have more polar substituents, and so the other half of the free-energy change can be regarded as resulting from non-polar (hydrophobic) interactions.

Within each of the three series (a, b and c), the affinities fall in the order *meta*>*para*>*ortho*; the differences between the *meta*- and *para*-compounds are barely significant, but the *ortho*-compounds clearly bind the most weakly, presumably owing to an unfavourable steric interaction.

Relative affinities: comparison of enzymes

There is a clear correlation between the affinities of the two enzymes for the boron-containing inhibitors. This holds both for boric acid and for the boronic acids (Fig. 1). The points are fitted by a line with slope 1.0, showing that changes in structure generally have comparable effects on the affinities for the two enzymes. Our previous work has shown that, although they are qualitatively similar, there are some appreciable quantitative differences between the kinetic properties of these class C β-lactamases (Knott-Hunziker *et al.*, 1982*b*). The most noticeable exception to the correlation in Fig. 1 is the iodoacetamido compound (2f), which may be regarded as binding exceptionally weakly to the *ampC* β-lactamase or exceptionally tightly to the *Pseudomonas* enzyme.

Mode of interaction between boronic acids and β-lactamases

The phenylboronic acids may interact with class C β-lactamases, which are serine enzymes (Knott-Hunziker *et al.*, 1980*b*, 1982*a*,*b*), in the same way as they do with the proteolytic enzyme, subtilisin. In that case, there is crystallographic evidence that the active-site serine forms a covalent bond with the boronic acid (Matthews *et al.*, 1975), the latter being regarded as a transition-state analogue. Whether the same applies to β-lactamases is an interesting, and unanswered, question.

The support of the Medical Research Council is gratefully acknowledged. This work was also supported by grants from the Swedish Natural Science Research Council (Dnr 3373) and from the Swedish Medical Research Council (Dnr 5428). We thank Glaxo Research Laboratories for gifts, and Lady Richards and the Dyson Perrins Laboratory for performing the n.m.r. spectra; elemental analyses were carried out by Dr. F. B. Strauss.

References

Abderhalden, E. & Guggenheim, M. (1908) *Ber. Dtsch. Chem. Ges.* **41**, 2852–2857

Ambler, R. P. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, 321–331

Atkins, G. L. & Nimmo, I. A. (1980) *Anal. Biochem.* **104**, 1–9

Bean, F. R. & Johnson, J. R. (1932) *J. Am. Chem. Soc.* **54**, 4415–4425

Berks, M., Redhead, K. & Abraham, E. P. (1982) *J. Gen. Microbiol.* **128**, 155–159

Bush, K., Bonner, D. P. & Sykes, R. B. (1980) *J. Antibiot.* **33**, 1262–1269
Cohen, S. A. & Pratt, R. F. (1980) *Biochemistry* **19**, 3996–4003
Fisher, J., Charnas, R. L., Bradley, S. M. & Knowles, J. R. (1981) *Biochemistry* **20**, 2726–2731
Flett, F., Curtis, N. A. C. & Richmond, M. H. (1976) *J. Bacteriol.* **127**, 1585–1586
Jaurin, B. & Grundström, T. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4897–4901
Kiener, P. A. & Waley, S. G. (1978) *Biochem. J.* **169**, 197–204
Knott-Hunziker, V., Waley, S. G., Orlek, B. S. & Sammes, P. G. (1979) *FEBS Lett.* **99**, 59–61
Knott-Hunziker, V., Orlek, B. S., Sammes, P. G. & Waley, S. G. (1980*a*) *Biochem. J.* **187**, 797–802
Knott-Hunziker, V., Redhead, K., Petursson, S. & Waley, S. G. (1980*b*) *FEBS Lett.* **121**, 8–10
Knott-Hunziker, V., Petursson, S., Jayatilake, G. S., Waley, S. G., Jaurin, B. & Grundström, T. (1982*a*) *Biochem. J.* **201**, 621–627
Knott-Hunziker, V., Petursson, S., Waley, S. G., Jaurin, B. & Grundström, T. (1982*b*) *Biochem. J.* **207**, 315–322
Lindström, E. B., Boman, H. G. & Steele, B. B. (1970) *J. Bacteriol.* **101**, 218–231
Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T. & Kraut, J. (1975) *J. Biol. Chem.* **250**, 7120–7126
Nikaido, H. (1976) *Angew. Chem. Int. Ed. Engl.* **18**, 337–350
Sabath, L. D. & Abraham, E. P. (1964) *Nature* (*London*) **204**, 1066–1069
Torssell, K. (1954) *Acta Chem. Scand.* **8**, 1779–1786
Torssell, K. (1957*a*) *Ark. Kemi* **10**, 513–521
Torssell, K. (1957*b*) *Ark. Kemi* **10**, 507–511
Waley, S. G. (1982) *Biochem. J.* **205**, 631–633