Reversible Inhibitors of Penicillinases

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Reversible competitive inhibitors of a penicillinase, β -lactamase I from *Bacillus cereus*, were studied. These represent the first inhibitors of a penicillinase that lack the β -lactam ring. The products of the enzymic reaction, namely penicilloic acids, are inhibitors; their decarboxylation products, the penilloic acids, are also inhibitors, and have somewhat lower K_i values. Inhibitors have been prepared from benzylpenicillin, phenoxymethylpenicillin, methicillin (2,6-dimethoxybenzamidopenicillanic acid) and 3-hydroxy-4-nitrobenzamidopenicillanic acid. Decarboxylation of the penicilloic acids from benzylpenicillin, or from phenoxymethylpenicillin, leads to epimerization (at C-5) of the penilloic acid. Nuclear-magnetic-resonance spectroscopy at a frequency of 270 MHz can distinguish the epimers. Other competitive inhibitors studied were boric acid, benzeneboronic acid and *m*-aminobenzeneboronic acid. Boric acid itself was the best inhibitor of β -lactamase I so far found.

Reversible inhibitors have a useful part to play in the detailed study of an enzyme. Their interaction with the enzyme can be studied spectroscopically, and they may also find application in kinetic studies. Moreover, crystallographic analysis demands inhibitors to characterize the active site. Both crystallography and n.m.r. spectroscopy entail the use of high concentrations of enzyme, so that substrates that are otherwise considered unreactive are now no longer inert. β -Lactamases (penicillinases, EC 3.5.2.6), however, have hitherto lacked inert reversible inhibitors. The present paper describes several such reversible competitive inhibitors of β -lactamase I from *Bacillus cereus*; some of the results have been alluded to in the report of a meeting (Thatcher, 1975a). This enzyme has been known for some time (Kogut *et al.*, 1956), and it can be obtained in sufficient amount (Davies *et al.*, 1974) for the sequence to have been investigated (Thatcher, 1975b), and for study by n.m.r. spectroscopy and by crystallography. β -Lactamase I is related to the β -lactamases from *Bacillus licheniformis* and from *Staphylococcus aureus* (Ambler & Meadway, 1969; Ambler, 1975).

The dearth of reversible inhibitors of β -lactamases has prompted the use of poor substrates as inhibitors in kinetic experiments (e.g. Abraham & Newton, 1956; Crompton *et al.*, 1962). Indeed, hundreds of semisynthetic penicillins have been tested as inhibitors of β -lactamases from Gram-negative bacteria (Cole *et al.*, 1972). It is perhaps worth drawing attention to the meaning of the parameter obtained in these experiments. The total rate (v) of hydrolysis of a mixture of two substrates (A and B), when the method of assay measures the sum of the

two individual rates, is given by

$$v = \frac{V_A \left(\frac{a}{K_A} \right) + V_B \left(\frac{b}{K_B} \right)}{1 + \left(\frac{a}{K_A} \right) + \left(\frac{b}{K_B} \right)}$$

where V_A , V_B are the maximum velocities, K_A , K_B are the Michaelis constants, and a and b are the concentrations of A and B respectively (see, e.g. Dixon & Webb, 1958). Now if B is a poor substrate (the criterion here being the ratio of the maximum velocity to the Michaelis constant), or is present at a much lower concentration than A, then the second term in the numerator becomes much smaller than the first, and the velocity is given by

$$v = \frac{V_A a}{a + K_A \left(1 + \frac{b}{K_B} \right)}$$

which is of course the usual equation for competitive inhibition. This equation applies either when the total rate is being measured, or when the method of assay registers only the hydrolysis of A. Thus B behaves as a competitive inhibitor, but the ' K_i ' obtained is the Michaelis constant for the hydrolysis of B. It may readily be shown that this conclusion holds for a three-step mechanism in which there is a second intermediate, such as an acyl-enzyme. The physical significance of the Michaelis constant depends on the relative rate constants for the different steps (Dalziel, 1962), and its value differs from that of the dissociation constant of the enzyme-substrate complex when other intermediates are present.

Materials and Methods

Materials

Benzylpenicilloic acid (II, $R = C_6H_5CH_2$). Benzylpenicillin (7.4 g of the potassium salt) in 10 ml of water was maintained at pH 12 by the addition of 1M-NaOH; hydrolysis was complete in about 15 min. The solution was cooled and conc. HCl was added slowly to lower the pH to 2. The penicilloic acid was collected and washed with water. The n.m.r. spectrum at 270 MHz in 2H_2O (pH-meter reading 7) showed δ (p.p.m.) 1.2 and 1.4 [3 H, singlets, S-C(CH₃)₂], 3.4 (1 H, singlet, H at C-3), 3.7 (2 H, multiplet, CH₂),

4.25 and 5.1 (1 H, doublets, H at S-C and H at C-6, assignments interchangeable) 7.5 (5 H, multiplet, aromatic H) (Fig. 1).

Benzylpenicilloic acid (III, $R = C_6H_5CH_2$). Benzylpenicilloic acid (3.2 g) was suspended in 20 ml of 50% (v/v) ethanol and heated under reflux for 5 min, and then cooled to 20°C. After 5 h, the solid was collected: it was in the form of needles, and contained about 80% of the α -form (Mozingo & Folkers, 1949) as judged by n.m.r. spectroscopy, and by paper electrophoresis at pH 4.5 (Davies *et al.*, 1974) for 45 min at 75 V/cm. After being kept at 5°C, the solid that separated consisted mainly of the β -form. The nature

Fig. 1. N.m.r. spectrum of sodium benzylpenicilloate in 2H_2O
The sample was prepared by alkaline hydrolysis of benzylpenicillin as described in the text.

Fig. 2. *N.m.r. spectrum of sodium benzylpenilloate in $^2\text{H}_2\text{O}$*
The sample is mainly the α -epimer (5*R*); see the text.

of the α - and β -forms is discussed below. The n.m.r. spectrum (Fig. 2) showed two singlets near 1.2 p.p.m. (CH_3 ; the higher-field peak is from the β -epimer), two singlets near 1.6 p.p.m. (CH_3 ; the lower-field peak is from the β -epimer), two singlets near 3.5 p.p.m. (H at C-3; the higher-field peak is from the β -epimer), a multiplet 3.2–3.6 p.p.m. (CH_2 at C-6), two singlets near 3.7 p.p.m. (CH_2 ; the lower-field peak is from the β -epimer), two triplets at 4.7 p.p.m. and 4.8 p.p.m. (H at C-5; higher-field peak from the β -epimer; the other peak is often obscured by the resonance from water) and a multiplet at 7.5 p.p.m. (aromatic H). On paper electrophoresis at pH 4.5 the β -epimer travelled about 20% further than the α -epimer, i.e. about one-half of the distance of the dye marker Xylene Cyanol FF. Epimerization to an equimolar mixture occurred rapidly on heating under reflux in 50% (v/v) ethanol at pH 2; the half-life for the epimerization in neutral solution at 37°C was 10–20 h, as judged by paper electrophoresis.

Phenoxymethylpenicilloic acid (II, $R = \text{C}_6\text{H}_5\text{OCH}_2$). This was prepared by the method described for benzylpenicilloic acid.

Phenoxymethylpenilloic acid (III, $R = \text{C}_6\text{H}_5\text{OCH}_2$). This was prepared by decarboxylation of the penicilloic acid (2.5 g) in 40 ml of water and 2 ml of ethanol by heating under reflux; the solution was decanted from a small amount of oily material and freeze-dried. The product apparently consisted of an

approximately equimolar mixture of epimers. The n.m.r. spectrum showed δ (p.p.m.) 1.1 (3 H, singlet, CH_3), 1.5 (3 H, singlet, CH_3), 3.3–3.7 and 4.6–4.9 (2 H, and 1 H, multiplets, $\text{N}-\text{CH}_2$, $\text{S}-\text{CH}$), 3.4 and 3.5 (1 H each, two singlets, $\text{N}-\text{CH}$, two isomers), 4.6 (2 H, two singlets, $\text{O}-\text{CH}_2$, two isomers) and 7–7.45 (5 H, multiplet, aromatic H).

Methicillin penicilloate [II, $R = \text{C}_6\text{H}_3(\text{OCH}_3)_2$]. This was prepared from methicillin (2,6-dimethoxybenzamido penicillanic acid) by the method described for benzylpenicilloic acid (Johnson & Panetta, 1964); the n.m.r. spectrum showed δ (p.p.m.) 1.2 and 1.6 [3 H, singlets, $\text{S}-\text{C}(\text{CH}_3)_2$], 3.7 (1 H, singlet, H at C-3), 3.9 (6 H, singlet, $\text{O}-\text{CH}_3$), 4.55 and 5.15 (1 H, doublets, H at C-5 and H at C-6), 6.85 (2 H, doublet, *meta* aromatic H) and 7.6 (1 H, triplet, *para* aromatic H).

Methicillin penilloic acid [III, $R = \text{C}_6\text{H}_3(\text{OCH}_3)_2$]. The penicilloic acid (2.96 g) was heated under reflux with 12 ml of water for 10 min. The product that separated (1.6 g) apparently consisted of only one stereoisomer; it had m.p. 190–200°C [Johnson & Panetta (1964) give m.p. 195°C]. The n.m.r. spectrum showed δ (p.p.m.) 1.2 and 1.6 (3 H, singlets, $\text{S}-\text{C}-\text{CH}_3$), 3.45 (1 H, singlet, H at C-3), 3.8 (6 H, singlet, $\text{O}-\text{CH}_3$), 3.6–3.9 and 4.8 (1 H and 2 H, multiplet, partially obscured by the water resonance), 6.8 (2 H, doublet, *meta* aromatic H) and 7.5 (triplet, *para* aromatic H). This penilloic acid epimerized less readily than benzylpenilloic acid, and it was only after heating for 1 h at

100°C that an epimer was formed; it could be detected by n.m.r. spectroscopy, e.g. δ (p.p.m.) 3.6 (1 H, singlet, H at C-3).

The *N*-bromoacetyl derivative of the penilloic acid from methicillin was prepared as follows. Bromoacetyl bromide (0.24 ml) was added dropwise to a stirred solution of the methicillin penilloic acid (217 mg) in water (3.2 ml) containing NaHCO₃ (600 mg) and acetone (0.25 ml) at 0°C. The mixture was stirred for 1 h at 0°C and 0.5 h at 20°C, and acidified with 2M-HCl to pH2; the product was isolated with chloroform, the chloroform being washed with 0.2M-HCl. The residue in ether/ethanol (3:1, v/v) was converted into the potassium salt (120 mg) with potassium 2-ethylhexanoate; it had m.p. 141–143°C (Found C, 38.5; H, 4.7; N, 4.9. C₁₈H₂₂O₆N₂SBrK·3H₂O requires C, 39.3; H, 5.1; N, 5.1%).

Penilloic acid from 3-hydroxy-4-nitrobenzamido-penicillanic acid. 3-Hydroxy-4-nitrobenzoic acid (1.8 g) and thionyl chloride (10 ml) were warmed together for 40 min and then heated under reflux for 20 min. The reagent was removed by distillation; benzene was added, and distilled twice. The residue, in 30 ml of acetone, was added dropwise to a stirred, cooled solution of 6-aminopenicillanic acid (1.8 g) in 60 ml of 4% (w/v) KHCO₃. After 2.5 h, the aqueous solution was extracted with ether, cooled, covered with ether, and adjusted to pH2.5 with 10% (w/v) H₃PO₄. The dried (over Na₂SO₄) ethereal extract was treated with potassium 2-ethylhexanoate (1.5 g) in propan-2-ol (4.5 ml). The red solid that separated was hygroscopic and was used without further purification for the preparation of the penilloic acid. A portion of the potassium salt was purified by precipitation from methanol with acetone; the structure was confirmed by n.m.r. spectroscopy (in ²H₂O), by paper electrophoresis at pH4.5, by enzymic hydrolysis with β -lactamase I, and by bioassay [the activity was approx. 20 units/mg when tested with *Staphylococcus aureus* by the method of Brownlee *et al.* (1948), when the activity of benzylpenicillin was taken as 1670 units/mg].

The penilloic acid was prepared as follows. The crude potassium salt was hydrolysed at pH12 for 15 min, and the pH then lowered with 2M-HCl to 2, and 4 vol. of ethanol added. The mixture was heated under reflux for 15 min. The penilloic acid (1.1 g) crystallized (m.p. 199–202°C). A portion was recrystallized from aqueous ethanol (m.p. 203–206°C) (Found: C, 47.9; H, 5.1; N, 11.3; S, 9.3. C₁₄H₁₇O₆N₃S requires C, 47.3; H, 4.8; N, 11.8; S, 9.0%).

The i.r. spectrum of the penilloic acid showed peaks due to amide at 1655 and 1575 cm⁻¹, and carboxylate at 1825 and 1340 cm⁻¹; the u.v. spectrum in 0.1M-NaHCO₃ showed absorption maxima at 282 nm and 409 nm, the latter having ϵ 4970 litre·mol⁻¹·cm⁻¹. The n.m.r. spectrum in (C²H₅)₂SO was in accord with the structure, and at a frequency of 270 MHz in ²H₂O

there were no signs of the presence of more than one isomer.

The penilloic acid could be recovered from the solutions in which it had been used as an inhibitor as follows. The aqueous solution was concentrated by evaporation under reduced pressure about threefold and extracted at pH5 with 1 vol. of ethyl acetate. The ethyl acetate solution was then extracted with 3× $\frac{1}{6}$ vol. of saturated NaHCO₃ and the aqueous solution adjusted to pH3 with 2M-HCl; the penilloic acid separated.

Other materials. β -Lactamase I was prepared essentially as described by Davies *et al.* (1974). β -Lactamase II (Kuwabara & Abraham, 1967) and the β -lactamase from *Pseudomonas aeruginosa* (McPhail & Furth, 1973) were gifts from our colleagues Dr. G. Baldwin and Dr. McPhail-Berks respectively. Benzylpenicillin and cephalosporin 87/312 were from Glaxo Research Laboratories, Greenford, Middx., U.K., methicillin and 6-aminopenicillanic acid were from Beecham Research Laboratories, Brockham Park, Surrey, U.K., and phenoxymethylpenicillin was from Eli Lilly and Co., Indianapolis, IN, U.S.A.

Methods

Assays of β -lactamase I were carried out spectrophotometrically with benzylpenicillin in 0.05M-sodium phosphate, pH6.8, containing 0.5M-NaCl and 1 mM-EDTA, at 232 nm (Waley, 1974), or with cephalosporin 87/312 [3-(2,4-dinitrostyryl)-7-(2-thienylacetamido)ceph-3-em-4-carboxylic acid] at 500 nm (O'Callaghan *et al.*, 1972), or by the pH-stat method, in the absence of buffer (Davies *et al.*, 1974).

Fluorescence emission spectra were measured with a Farrand spectrofluorimeter mark 1 at 20°C; 5 nm slits were used, and excitation was at 285 nm.

T.l.c. was carried out in acetone/acetic acid (19:1, v/v) (Vandamme & Voets, 1972).

Proton n.m.r. spectra were observed at 270 MHz on a Bruker spectrometer with an Oxford Instrument Co. superconducting magnet. The spectra were obtained by collecting 200 transients in 4096 data points. A Fourier transformation was then performed in 8192 data points after applying a filter of line-width 1.25 Hz. The spectra were usually observed on compounds (10–20 mM) in ²H₂O at 25°C; the apparent pH was 7, and the chemical shift is given as p.p.m. downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

Results and Discussion

Preparation of penicilloates and penilloates

The product of enzymic hydrolysis by β -lactamase is penicilloic acid, and so penicilloic acids (II) were prepared for testing as inhibitors. Since the carboxylate group is larger than the carbonyl group, and the

negative charge might be unfavourable for binding to the enzyme, the decarboxylated penicilloic acids [i.e. the penilloic acids (III)] were also prepared.

The penicilloates were prepared by treatment of the corresponding penicillin with alkali, as described in the Materials and Methods section. Decarboxylation to the penilloic acids was carried out by heating under reflux in 50% (v/v) ethanol.

Stereochemistry of penicilloates and penilloates

The penicilloate from benzylpenicillin undergoes mutarotation in neutral solution (Levine, 1960), and Sabath *et al.* (1965) suggested that this was responsible for the appearance of two spots on paper electrophoresis at pH 4.5. Schneider & de Weck (1967) also noticed that the n.m.r. spectrum of the penicilloate showed 'extra' resonances, and Busson *et al.* (1976) showed that the 'natural' 5*R*,6*R*-isomer underwent isomerization into a mixture of isomers, mainly the 5*S*,6*R* one. In the present work, advantage was taken of the opportunity to obtain n.m.r. spectra in neutral aqueous solution at 270 MHz; at this frequency the upfield resonances assigned to the methyl groups as well as those assigned to protons at C-5 and C-6 can be resolved in mixtures of stereoisomers. An example of the n.m.r. spectrum at 270 MHz of the penicilloate obtained by alkaline hydrolysis of benzylpenicillin is shown in Fig. 1; one stereoisomer greatly predominates. Indistinguishable spectra were obtained when the benzylpenicillin was hydrolysed enzymically by β -lactamase I. This is the spectrum characteristic of the 'natural' (α) 5*R*,6*R*-isomer. After heating at 100°C for 30 min at pH 8 there were marked differences in the n.m.r. spectrum, which now resembled that from the 5-epipenicilloate (the β -, or 5*S*,6*R*-isomer). Schneider & de Weck (1967) pointed out that the rate of mutarotation of benzylpenicilloate did not depend on the pH (over the range pH 7.3–12.5). A possible mechanism consists of the attack of a hydroxyl ion on the nitrogen-protonated form of the thiazolidine; this would account for the lack of dependence on pH. As the half-life is about 6 h at 25°C (Schneider & de Weck, 1967), freshly prepared solutions of benzylpenicilloate were used.

Decarboxylation of a penicilloate (II) gives the penilloate (III). This change destroys the asymmetry at C-6, but may bring about epimerization at C-5. After decarboxylation of the penicilloic acid by heating under reflux in 50% (v/v) ethanol, a solid consisting largely of one stereoisomer of benzylpenilloic acid crystallizes readily at room temperature. This isomer, referred to as the α -isomer (Mozingo & Folkers, 1949), has now been shown to be the 5*R*-('natural') isomer, by crystallography (Z. Ruzic-Toros, personal communication). We have characterized this isomer in solution, both by n.m.r. spectroscopy at 270 MHz (Fig. 2) and by paper

electrophoresis at pH 4.5. If the mother liquors are kept for longer at 5°C, a second form of penilloic acid crystallizes, referred to as the β -form (Mozingo & Folkers, 1949). This material is a mixture consisting mainly of the 5*S*-stereoisomer. The resonances attributed to this isomer are distinguishable from those of the α -isomer, but in $^2\text{H}_2\text{O}$ the resonances assigned to the protons at C-5 (4.8 p.p.m. from the α -isomer, 4.7 p.p.m. from the β -isomer) were obscured by the peak corresponding to H_2O unless the samples were exceptionally free from H_2O . If the decarboxylation was carried out in $^2\text{H}_2\text{O}$, the n.m.r. spectrum was slightly simplified, and the triplets at 4.7 p.p.m. and 4.8 p.p.m. were reduced to doublets, as the proton at C-5 is now coupled to only one proton. On paper electrophoresis at pH 4.5, the mobility of the α -isomer was just less than that of the β -isomer (for conditions see the Materials and Methods section).

Various alternative conditions for the decarboxylation of benzylpenicilloic acid were investigated, but none were found in which there was decarboxylation without epimerization. Examination of the solution after decarboxylation showed that both epimers were present in equal amounts; epimerization in $^2\text{H}_2\text{O}$ did not lead to loss of the signal from the proton at C-5, and epimerization may be a consequence of transitory ring opening.

The discussion of the stereochemistry so far has been confined to benzylpenicilloic acid and benzylpenilloic acid. The penicilloic acid and penilloic acid from phenoxymethylpenicillin behaved similarly. The penilloic acid was obtained as a mixture of epimers, and was used as such. However, the penilloic acids from methicillin, and from the hydroxynitrophenylpenicillin, were obtained as one stereoisomer. It is hard to see why there should have been quantitative inversion, so these penilloic acids were probably the 5*R*-isomers.

Inhibition by penicilloates and penilloates

The penicilloates tested were competitive inhibitors, but they only bound weakly (Table 1). The 'colorimetric' substrate, cephalosporin 87/312 (O'Callaghan *et al.*, 1972), was convenient, since it can be used at low concentrations (0.05 mM), and high concentrations of penicilloates (or penilloates) do not interfere with the assay; it is for this reason that the spectrophotometric assay (Waley, 1974) cannot be used.

The penilloates were mostly rather better competitive inhibitors (Table 1). The question of the stereochemistry has to be considered. As the phenoxy-methylpenilloate was a 1:1 mixture of isomers, the K_i will be in the range 5–10 mM, depending on the relative affinity of each isomer. Two samples of benzylpenilloate, one consisting of about 80% α -isomer and the other consisting of about 60% β -

isomer, both gave a K_i of 16mM. Thus we have no evidence that the affinities of the isomers differ. The most convenient way of obtaining the value for K_i appeared to be the plot of $v_i/(v-v_i)$ against $1 + ([S]/K_m)$, where v_i and v refer to the initial rates of the inhibited and uninhibited reactions respectively, and $[S]$ is the concentration of substrate and K_m its Michaelis constant. This is a modification of the Hunter & Downs (1945) method; its advantage lies in the fact that a best (least-squares) fit of the data to a line through the origin is an efficient use of the knowledge of the theoretical form of the line. The slope of the line is $K_i/[I]$, where $[I]$ is the concentration of inhibitor.

None of these inhibitors binds particularly tightly to the enzyme. Nor do they alter the conformation of the enzyme, as judged by hydrogen exchange (Kiener & Waley, 1977). The values of K_i can only be compared with the K_m values of substrates if it is known that the latter are really dissociation constants. The arguments advanced by Cornish-Bowden (1976) suggest that the K_m for benzylpenicillin, which has a value of 0.077mM over a pH range where k_{cat} is

changing (Waley, 1975), should refer to a dissociation constant. If so, the penilloate binds about 200 times more weakly. If the K_m for methicillin is a dissociation constant, then its value of 1.2mM (Citri *et al.*, 1976) may be compared with that for penicillin, and is about 16 times as large; the K_i for methicillin penilloate (Table 1) is about 5 times that for benzylpenilloate.

The finding that penilloates are reversible inhibitors prompted the trial of alkylating agents derived from penilloates as specific irreversible inhibitors. Methicillin penilloate was converted into the *N*-bromoacetyl compound. This did not inactivate β -lactamase I at pH 6.8; in 0.1M-NaHCO₃ at pH 8.5, the bromoacetyl compound (8.8mM) brought about 80% inactivation of 10 μ M-enzyme after 40 h at 37°C. The inactivated protein was isolated by dialysis against 0.1% acetic acid and freeze-drying; amino acid analysis showed a large (about 80%) loss of both lysine and histidine. Reaction at other sites, for example carboxyl groups, is not detected by amino acid analysis of the modified protein, and may also have occurred [reagents that attack carboxyl groups have been previously found to inactivate β -lactamase I (Waley, 1975)]. Thus there was no evidence for specific inactivation of β -lactamase I, and although inactivations of β -lactamase II and the β -lactamase from *Pseudomonas aeruginosa* were somewhat faster (with half-lives of 2–3 h under the conditions described above), the reagent appears unsuitable for selective modification.

Table 1. *Inhibition of β -lactamase I by penicilloates and penilloates*

The substrate was benzylpenicillin or, more often, cephalosporin 87/312 [3-(2,4-dinitrostyryl)-7-(2-thienylacetamido)ceph-3-em-4-carboxylic acid], which was used at 30°C in 0.05M-sodium phosphate, pH 6.8, containing 0.5M-NaCl, 1mM-EDTA; the difference absorption coefficient has a maximum at 500nm.

	K_i (mM)
Penicilloates	
Benzyl	40
Phenoxymethyl	62
Penilloates	
Benzyl	16
Phenoxymethyl	9
Hydroxynitrophenyl	5
Methicillin	85

Inhibition by borate and boronates

Dobozy *et al.* (1971) reported that borate ions inhibited β -lactamase I, and that the inhibition was reversible, but that the dilution necessary for the virtually complete recovery of activity was larger than expected; this effect was said to be more marked at pH 9 than at pH 6. The experiment reported in Table 2 suggests that boric acid was behaving as a

Table 2. *Reversible inhibition of β -lactamase I by sodium borate*

The reaction mixture (volume 1 ml) contained sodium borate (0.2M) and sodium phosphate (0.1M) adjusted to pH 6 with 2M-HCl, and β -lactamase I as specified in the first column; the control lacked the sodium borate. The mixtures were kept at 30°C for 10 min, and then portions taken for assay by the spectrophotometric method (Waley, 1974). The expected inhibition was calculated assuming reversible competitive inhibition, with K_i 1.2mM.

Concn. of β -lactamase I in reaction mixture (μ g/ml)	Vol. of reaction mixture taken for assay (μ l)	Rate in assay $\Delta A_{232}/\text{min}$		Inhibition (%)	
		Reaction	Control	Found	Expected
4.1	50	0.40	0.45	11	19
8.2	25	0.37	0.43	14	11
20.5	10	0.35	0.37	7	4.5
41	5	0.35	0.36	3	2.3
82	2	0.35	0.35	0	1.2

Fig. 3. Fluorescence spectrum of β -lactamase I

(a) β -Lactamase I (60 μ g/ml) in 0.05 M-sodium phosphate buffer, pH 7, alone (—), or in the presence of 4 M-guanidinium chloride (---). (b) β -Lactamase I (55 μ g/ml) in 0.1 M-sodium phosphate, pH 6.2, alone (—), or in the presence of 20 mM-sodium borate (---). The spectra are emission spectra; excitation was at 285 nm. The fluorescence intensity is measured in arbitrary units.

reversible inhibitor at pH 6; the inhibition observed could be accounted for by the presence of boric acid (about 0.2–4 mM) in the final assay mixture. Kinetically, the inhibition was competitive, in that there was less inhibition the higher the concentration of substrate. The calculated maximum velocity was approx. 20% lower when inhibitor was present, but this difference is probably not significant. The K_i [calculated from the modified Hunter & Downs (1945) plot described above] was about 1 mM; borate is the best inhibitor of β -lactamase I so far found. Although Dobozy *et al.* (1971) suggested that the conformation of β -lactamase I was greatly affected by borate ions, we found little effect on the fluorescence spectrum

(Fig. 3). (On reversible denaturation in guanidinium chloride, the fluorescence decreases markedly, and the maximum emission shifts from 334 nm to 347 nm, the latter wavelength being close to the maximum for free tryptophan. These effects are taken to be a good indication of an extensive change in conformation.)

Benzeneboronic acid [$C_6H_5B(OH)_2$] was also an inhibitor, and had K_i 4 mM, as was the *m*-amino-benzeneboronic acid, which had K_i 2 mM; the kinetics showed similar features to those described above for sodium borate.

Benzeneboronic acid is an inhibitor of chymotrypsin (Philipp & Bender, 1971) and of subtilisin (Lindquist & Terry, 1974), and forms a covalent

adduct with subtilisin with a bond between boron and O^y of the active-site serine (Ser-221) (Matthews *et al.*, 1975). Benzeneboronic acid behaves as a reversible competitive inhibitor of subtilisin (Lindquist & Terry, 1974), in spite of the covalent bonding referred to, and Philipp & Bender (1971) have observed that reaction with chymotrypsin was fast. Whether β -lactamase I forms a covalent bond with borate or boronate ions is an open question.

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