6 β -Bromopenicillanic Acid Inactivates β -Lactamase I

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The inactivation of β -lactamase I by preparations of 6 α -bromopenicillanic acid showed unexpected kinetic features that indicated that the active species was the 6 β -epimer. Samples containing 6 β -bromopenicillanic acid have been synthesized and shown to inactivate the enzyme in a rapid stoichiometric reaction.

β -Lactamases (penicillinases, EC 3.5.2.6) are efficient enzymes whose mechanism of action is unknown. They are important clinically as some pathogenic bacteria owe their resistance to β -lactam antibiotics to the production of β -lactamases. β -Lactamase I from *Bacillus cereus* can be prepared efficiently (Davies *et al.*, 1974) and is related to the enzymes from *Staphylococcus aureus* and *Escherichia coli* (R factor TEM) (Thatcher, 1975; Ambler, 1975; R. P. Ambler, personal communication). Crystallographic studies have been initiated on β -lactamase I (Aschaffenburg *et al.*, 1978) and therefore there is a need, for several reasons, for specific inhibitors of the enzyme that label the active site. The results described in the present paper concern a reagent that promises to fulfil this need. During the course of this work we learnt that Pratt & Loosemore (1978) had independently discovered the usefulness of 6 β -bromopenicillanic acid.

Materials and Methods

Materials

6 α -Bromopenicillanic acid. This was prepared by the method of Cignarella *et al.* (1962) by the diazotization of 6 β -aminopenicillanic acid. It was utilized as its freshly prepared, dried potassium salt. The dibenzylethylenediamine salt had m.p. 163–165°C [Cignarella *et al.* (1962) give a value of 164–165°C].

³H-labelled 6 α -bromopenicillanic acid. 6 β -Aminopenicillanic acid labelled with ³H in its 2 β -methyl group (Usher *et al.*, 1975) was similarly diazotized in the presence of NaBr by the method of Cignarella *et al.* (1962) and utilized as its potassium salt. This compound ran as one spot on t.l.c. with a trace of material remaining at the baseline. Radiochemically the major spot contained 93% of the total radioactivity. The material had a sp. radioactivity of 22.9 μ Ci/ μ mol.

6 β -Bromopenicillanic acid. Treatment of the

6 α -epimer (100mg) in 10ml of buffer at pH9.2 (0.025M-Na₂B₄O₇·10H₂O adjusted to pH9.2 with 0.1M-NaOH) at 25°C for 89h afforded an epimeric mixture of the 6 α - and 6 β -isomers. A similar procedure was used by Pratt & Loosemore (1978). ¹H n.m.r. analysis of the mixture indicated 5% of the β -isomer [δ (p.p.m.)] ([²H]chloroform) 1.57 (2 α -CH₃), 1.71 (2 β -CH₃), 4.58 (3 H), 5.36 (6 H, J 4Hz), 5.60 (5 H, J 4Hz).

The epimeric mixture was purified by acid/base extraction and isolated as the potassium salt. Purer samples of the 6 β -bromopenicillanic acid have been prepared by selective reduction of the 6,6-dibromo acid and by the epimerization of esters before release of the free acid, by the method of Roets *et al.* (1976).

Other materials. β -Lactamase I was prepared as previously described (Davies *et al.*, 1974; Kiener, 1976); other materials were from sources given by Kiener & Waley (1978). The pH7 buffer was 0.1M-NaH₂PO₄/0.1M-K₂HPO₄. The pH9.2 buffer was prepared from 0.2M-KHCO₃ and 0.2M-Na₂CO₃ in suitable proportions in 0.5M-NaCl/1mM-EDTA, the proportions being such that the pH was 9.2 at 20°C.

Methods

Assays of β -lactamase I were carried out as described by Kiener & Waley (1978), as were observations of ¹H n.m.r. spectra on 6 α -bromopenicillanic acid in ²H₂O. ³H radioactivity was determined as described by Kiener & Waley (1977). The solvent for t.l.c. was toluene/acetone/acetic acid (12:7:1, by vol.), and compounds were detected by spraying with 0.5% potassium permanganate.

Results and Discussion

Kinetics of inactivation by preparations of 6 α -bromopenicillanic acid

Inactivation of β -lactamase I by 6 α -bromopenicillanate was progressive at pH9.2. Unexpectedly, the

Table 1. *Inactivation of β -lactamase I by 6 α -bromopenicillanic acid at pH 9.2*

The enzyme and the reagent were kept in pH 9.2 buffer (see under 'Materials'); periodically portions were withdrawn for assay by the spectrophotometric method (Waley, 1974). The values given in parentheses show the initial rate ($\mu\text{M} \cdot \text{min}^{-1}$) of inactivation.

[Potassium 6-bromo- penicillanate] (mM)	Temperature (°C)	β -Lactamase concn. (μM) ...	Half-life of inactivation (min)		
			0.7	3.1	8.3
0.25	30		6.1		
0.5	30		3.25		
2.5	30				24
10	12				5
25	12			2.7 (0.79)	7.3 (0.79)
				0.8 (2.7)	2.2 (2.6)

Table 2. *Partial inactivation of β -lactamase I by 'pure' 6 α -bromopenicillanic acid at pH 7*

Successive additions of reagent (50 mM-potassium 6-bromopenicillanate) or enzyme (400 μg of β -lactamase I/ml) were made to β -lactamase I (16 μg) in 0.5 ml of the pH 7 buffer (see under 'Materials') at 20°C; 5 μl portions were taken for assay. The activities (units/ml) are given for samples taken about 10 min after the addition; there was little change between 5 and 10 min. One unit of activity is the amount of enzyme required to convert 1 μmol of substrate/min. The expected gain in activity brought about by the second addition was 195 units/ml.

Addition	Activity of solution (units/ml)		
	Before addition	After addition	Change
First; reagent (250 nmol)	390	110	-280
Second; enzyme (8 μg)	110	300	+190
Third; reagent (100 nmol)	300	150	-150
Fourth; reagent (100 nmol)	150	30	-120

rate of inactivation was independent of the concentration of enzyme and, correspondingly, the half-life for inactivation was directly proportional to the concentration of enzyme (Table 1). Usually the rate of inactivation is proportional to, and the half-life independent of, the concentration of enzyme. The results can be explained if the rate-determining step is the conversion of the 6 α -bromopenicillanate (A) into a more reactive form (B), which then inactivates the enzyme in a relatively rapid step. A kinetic scheme that incorporated these assumptions gave a half-life of the order of 20 h for the conversion of A into B at pH 9.2 and 30°C. Small amounts of conversion could be detected because the concentration of the reagent was so much greater than that of the enzyme.

The conversion of A into B did not occur significantly at pH 7 and the extent of inactivation at this pH was limited by the amount of B initially present. With the 'pure' 6 α -penicillanate (A), inactivation was partial (Table 2). Addition of fresh enzyme enhanced the activity, which was then stable; thus the inhibitor was no longer present. Addition of fresh reagent brought about a further drop in enzyme activity. Partial, rather than complete, inactivation thus appears to be due to consumption of the inhibitor. Analysis (by t.l.c. and by n.m.r. spectroscopy)

of such solutions, however, showed only the 6 α -epimer. This raised the possibility that inactivation of the enzyme might be caused by a minor component in the reagent.

Use of 6 β -bromopenicillanic acid

The results described above suggested that the reactive species B might be the 6 β -bromopenicillanic acid. For this reason synthetic approaches to the β -epimer have been developed (see under 'Materials'). The present results have been obtained with material that contained only 5% of the β -isomer: they confirm the hypothesis. Inactivation at pH 7 was no longer limited and only small amounts of reagent were needed. Thus, one molar proportion of β -epimer brought about 50% inactivation and two molar proportions brought about 86% inactivation of 1 μM - β -lactamase I at pH 7 within 15 min at 30°C. When the concentration of β -epimer was 1 μM and the concentration of the enzyme was 0.1 μM the half-life for inactivation was of the order of 1 min: this corresponds to a rate constant of the order of $10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$. The fact that the rate of inactivation decreased more rapidly than expected for a second-order reaction suggests that hydrolysis of the β -bromopenicillanic acid may compete with its inactivation of the enzyme. The rates of both processes

appear to be comparable. For comparison, the value of $k_{\text{cat.}}/K_m$ for 6 β -aminopenicillanate is in the order of $10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Waley, 1974).

Use of radioactive 6 α -bromopenicillanic acid

The reagent, labelled in the 2 β -methyl group (Usher *et al.*, 1975), was prepared as described above. β -Lactamase I (3 mg/ml) in pH 9.2 buffer (see under 'Materials') was treated with 15 mM-potassium 6 α -bromo[^3H]penicillanate (3.44 mCi; sp. radioactivity 1.5×10^7 c.p.m./ μmol) for 15 min at 30°C; over 90% of the activity was lost. The solution (5 ml) was fractionated on a column (34 cm $\times$ 2.6 cm) of Sephadex G-25 with pH 7 buffer (see under 'Materials') diluted with an equal volume of water. There were two well separated peaks of radioactivity in the elution profile. The six tubes that comprised the high-molecular-weight peak had a sp. radioactivity of $(15.5 \pm 4) \times 10^6$ c.p.m./ μmol (mean $\pm$ s.d.) [taking the mol.wt. as 28000 (Davies *et al.*, 1974)] corresponding to incorporation of 1 mol of ^3H -labelled penicillanic acid/mol of enzyme.

When a portion (about 0.4 mg) of the labelled enzyme was kept in 0.5 ml of 0.02 M-NaOH for 1 h at 30°C and the solution then fractionated as described above, 80% of the radioactivity in the eluate was in the low-molecular-weight compounds and 20% in the protein peak; when 0.2 M-NaOH for 2 h at 37°C was used, virtually all the radioactivity was in low-molecular-weight compounds. These results suggest the possibility of an ester, or ether, linkage formed by reaction of a carboxy (cf. Waley, 1975) or hydroxy group at C-6 of the reagent with displacement of the bromine atom. The rate constant of the order of $10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ given above for the formation

of this linkage is of the order of 10^9 -fold greater than that for the corresponding reactions of low-molecular-weight compounds. This is a marked acceleration. Moreover, it encourages hope that the inactivation is due to reaction at the active site of the enzyme.

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