Biochem. J. (1985) **225**, 435–439
Printed in Great Britain

Inhibition of class C β-lactamases by (1′*R*,6*R*)-6-(1′-hydroxy)benzylpenicillanic acid *SS*-dioxide

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(*Received 23 July 1984/Accepted 24 September 1984*)

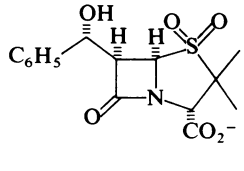
β-Lactamases, enzymes that catalyse the hydrolysis of the β-lactam ring in β-lactam antibiotics, are divided into three classes, A, B and C, on the basis of the structures so far determined. There are relatively few effective inhibitors of class C β-lactamases. A β-lactam sulphone with a hydroxybenzyl side chain, namely (1′*R*,6*R*)-6-(1′-hydroxy)benzylpenicillanic acid *SS*-dioxide (I), has now been studied. The sulphone is a good mechanism-based inhibitor of class C β-lactamases. At pH 8, the inhibition of a *Pseudomonas* β-lactamase is irreversible, and proceeds at a rate that is about one-tenth the rate of concurrent hydrolysis. The labelled enzyme has enhanced u.v. absorption and is probably an enamine. At a lower pH, however, inhibition is transitory.

β-Lactamases are clinically important and mechanistically interesting enzymes, notable for their prevalence and diversity. No three-dimensional structures are yet known, but several primary structures are. These have permitted division into three classes, A, B and C (Ambler, 1980; Jaurin & Grundström, 1981). Members of the different classes have differing susceptibilities to inhibitors (Cartwright & Waley, 1983), class C β-lactamases being inhibited but poorly by several widely used inhibitors. Penam sulphones are effective inhibitors of class A β-lactamases (English *et al.*, 1978; Fisher *et al.*, 1981), and the introduction of a hydroxyalkyl group into the side chain, rather as in some naturally occurring carbapenems (Brown, 1981), seemed attractive. As part of a study on the effect of side-chain structure on activity (cf. Foulds *et al.*, 1984*a*), a series of aromatic substituted hydroxyalkyl derivatives has been prepared. The present paper describes studies on reactions of one of these derivatives, the hydroxybenzyl sulphone (I), with β-lactamases. The compound, although ineffective against class A and class B β-lactamases, turned out to be a potent inhibitor of class C β-lactamases. The reaction, at pH 8, leads to the formation of a covalently labelled enzyme with a changed isoelectric point and u.v.-absorption spectrum. Thus the hydroxybenzyl sulphone (I) has the characteristics of a 'branched-pathway inhibitor' (Cartwright & Waley, 1983). This selective reaction is assumed to involve the active-site serine residue of the enzyme (Knott-Hunziker *et al.*, 1982*a*,*b*). At lower pH values, the inhibition is but transitory, and the time course shows the loss and then regain of activity so characteristic of the reactions of β-lactamases (Fisher *et al.*, 1978; Pain & Virden, 1979). The enzyme kinetics of transient inhibition are considered elsewhere (Bicknell *et al.*, 1984).

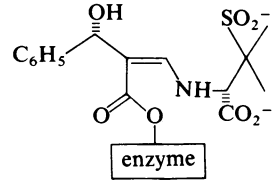
Materials and methods

Materials

The β-lactamase from a mutant of *Pseudomonas aeruginosa* 18S (Sabath *et al.*, 1965; Flett *et al.*, 1976; Berks *et al.*, 1982) was purified by affinity chromatography (Cartwright & Waley, 1984). The *amp*C β-lactamase was from *Escherichia coli* (Jaurin & Grundström, 1981). β-Lactamase I and β-lactamase II from *Bacillus cereus* 569/H/9 were prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980).



(I)



(II)

The hydroxybenzyl sulphone (I) was synthesized as described elsewhere (Foulds *et al.*, 1984*b*). The material referred to as hydroxybenzyl sulphone here was the crystalline potassium salt of (1′*R*,6*R*)-6-(1′-hydroxy)benzylpenicillanic acid *SS*-dioxide.

Methods

The activity of the *Pseudomonas* β-lactamase was measured, in the pH-stat, in 0.5M-NaCl, pH8, at 30°C, in a volume of 2ml, with cephalosporin C (5mM) as substrate. When the hydroxybenzyl sulphone was also present, its concentration was 20–150μM, the concentration of cephalosporin C was 10mM or 20mM (the K_m being 0.07mM), and the concentration of *Pseudomonas* β-lactamase (added last) was 8.65nM. A high concentration of cephalosporin C was used to maintain zero-order kinetics.

Isoelectric focusing of the reaction mixture could be carried out directly when sufficiently dilute buffer was used. *Pseudomonas* β-lactamase (21.9μM) in 0.5ml of 2mM-triethanolammonium chloride, pH8, was treated with 62μl of 4mM-hydroxybenzyl sulphone for 15min at 30°C; this brought about 96% inactivation. Isoelectric focusing was carried out on LKB PAG plates, pH range 3.5–9.5, in an LKB Multiphor apparatus, with proteins for calibration from Pharmacia (Milton Keynes, U.K.).

Labelled *Pseudomonas* β-lactamase was isolated as follows. The enzyme (0.5ml of 0.148mM) was treated with 0.2ml of 8mM-hydroxybenzyl sulphone for 10min at 30°C and then freed from low-M_r material by gel filtration on Sephadex G-25 in 20mM-triethanolammonium chloride, pH8. A control sample of enzyme was treated similarly, but with buffer lacking sulphone.

Hydrolysis of hydroxybenzyl sulphone was carried out by adding 0.05ml of β-lactamase II (1mg/ml in ^2H_2O) to 0.5ml of 4mM-hydroxybenzyl sulphone in 50mM-sodium phosphate, pD7, in ^2H_2O, and keeping at 30°C for 30min. The n.m.r. spectrum of the mixture was complex, and breakdown to a mixture of products had occurred.

Transitory inhibition at pH5.3 was carried out by keeping *Pseudomonas* β-lactamase (9.2μM) and the hydroxybenzyl sulphone (92μM) in 0.2M-sodium acetate buffer pH5.3, at 30°C.

Results and discussion

Mechanism of inhibition (kinetic aspects)

When the *Pseudomonas* β-lactamase was incubated with various amounts of the hydroxybenzyl sulphone at pH8, the fractional activity remaining was proportional to the molar ratio of sulphone to enzyme (Fig. 1). By extrapolation, 10 molecules of sulphone were required to inactivate one molecule of enzyme. The fractional activity remaining was not appreciably different when the times of incubation were varied from a few minutes to a few hours. Thus the reaction leading to incomplete (but apparently permanent) inhibition had come to a halt. These kinetics can be interpreted by a branched pathway in which an intermediate may give either active enzyme and product, or inactive enzyme (see Scheme 1). When less than 10 molar proportions of sulphone were used the reaction ceased because all the sulphone had been consumed. The kinetics predicted by Scheme 1 (Waley, 1980; Tatsunami *et al.*, 1981) give the slope of the line in Fig. 1 as $(1+k_{+3}/k_{+4})$;

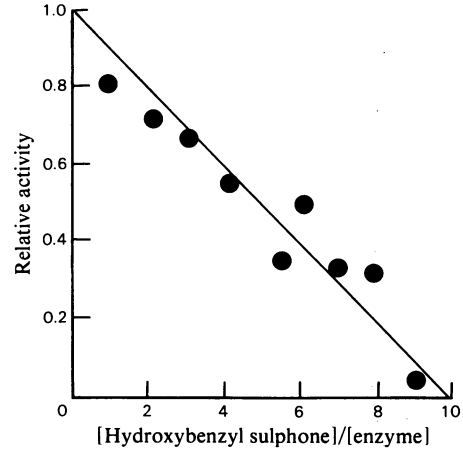
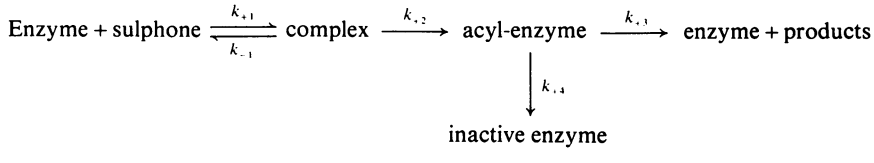


Fig. 1. *'Titration' of Pseudomonas β-lactamase with the hydroxybenzyl sulphone*

The enzyme (1.7μM) and the hydroxybenzyl sulphone (2–20μM) in 20mM-triethanolamine hydrochloride, pH8, were incubated at 30°C, and portions were withdrawn for assay.



Scheme 1

thus $k_{+3}/k_{+4} = 9$ here. The hydrolysis of β-lactams by this *Pseudomonas* enzyme is known to proceed by an acyl-enzyme mechanism, and the acyl-enzyme accumulates in certain instances (Knott-Hunziker *et al.*, 1982*a*,*b*).

The kinetics of inhibition at pH 8 were most conveniently studied by measuring the hydrolysis of substrate (cephalosporin C) in the presence of the hydroxybenzyl sulphone (Fig. 2). The concentration of sulphone was much greater than the concentration of enzyme. The exponential decrease in the rate of hydrolysis of cephalosporin C gave a first-order rate constant, $k_{obs.}$. The variation of $k_{obs.}$ with the concentrations of the hydroxybenzyl sulphone (H) and cephalosporin C (C) are given by eqn. (1) (Knott-Hunziker *et al.*, 1980; Frère *et al.*, 1982):

$$k_{obs.} = \frac{k_i[H]}{[H] + K_H\left(1 + \frac{[C]}{K_C}\right)} \quad (1)$$

In eqn. (1), k_i is the first-order rate constant for inhibition, and K_C and K_H are the Michaelis constants for cephalosporin C and the hydroxybenzyl sulphone respectively. When eqn. (1) is written as:

$$\frac{[H]}{k_{obs.}} = \frac{[H]}{k_i} + \frac{K_H}{k_i}\left(1 + \frac{[C]}{K_C}\right) \quad (2)$$

it is clear that a plot of $[H]/k_{obs.}$ against [H] will be linear, as was found (Fig. 3), and that k_i and K_H can be found from the intercept and slope. Further discussion is in terms of k_i and K_H because the individual rate constants of Scheme 1 cannot be evaluated from the present results. The value of k_i was not high (Table 1), but the low value of K_H meant that the second-order rate constant (k_i/K_H) was quite large, comparable with the value for 6β-bromopenicillanic acid and β-lactamase I (Knott-Hunziker *et al.*, 1980). Thus the sulphone is, roughly speaking, a 'low-reactivity, high-affinity' inhibitor of the class C β-lactamase, whereas 6β-bromopenicillanic acid, by contrast, is a 'high-reactivity, low-affinity' inhibitor of the class A β-lactamase, β-lactamase I. The *amp*C β-lactamase (Jaurin & Grundström, 1981) gave results with the hydroxybenzyl sulphone virtually indistinguishable from those obtained with the *Pseudomonas* β-lactamase (results not shown).

The experiments described so far had been carried out at pH 8, and little activity was regained on prolonged incubation. At a lower pH, inhibition was transient (Fig. 4). Curves of this shape are frequently encountered when β-lactamases are incubated with branched-pathway inhibitors. A quantitative treatment of the loss and regain of enzymic activity in transient inhibition can account for the characteristic features observed

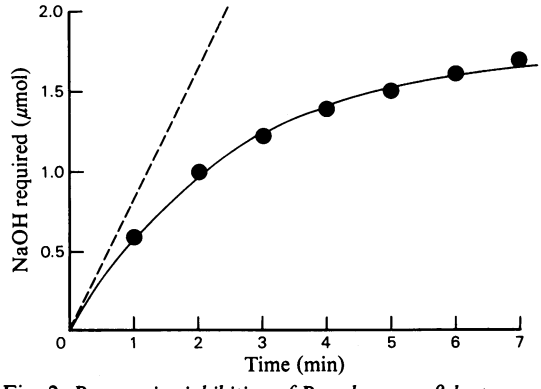


Fig. 2. *Progressive inhibition of Pseudomonas β-lactamase by the hydroxybenzyl sulphone*

The hydrolysis of cephalosporin C (10 mM) by the β-lactamase (8.65 nM) was monitored in the presence (●) and in the absence (-----) of the hydroxybenzyl sulphone (40 μM). The rate was measured in the pH-stat at pH 8 at 30°C.

Table 1. *Kinetic parameters for progressive inhibition of Pseudomonas β-lactamase by the hydroxybenzyl sulphone at pH 8*

β-Lactamase was added to cephalosporin C (10 mM or 20 mM) and the sulphone (0.02–0.15 mM) in 0.5 M-NaCl. The hydrolysis of cephalosporin C was measured at pH 8 at 30°C in the pH-stat. The rate of reaction declined exponentially, and the first-order rate constant for inhibition depended hyperbolically on the concentration of sulphone. The results are given as means ± S.E.M. (no. of experiments). The values for 6β-bromopenicillanic acid are from unpublished experiments (S. G. Waley), and those for benzylpenicillin from Knott-Hunziker *et al.* (1982*b*) and Bicknell *et al.* (1983).

β-Lactam	First-order rate constant for inhibition (k_i) (min^{-1})	Apparent dissociation constant (K_H) (μM)	Second-order rate constant (k_i/K_H) ($\mu M^{-1} \cdot min^{-1}$)
Hydroxybenzyl sulphone (I)	1.9 ± 0.01 (6)	1.1 ± 0.2 (6)	1.8 ± 0.3 (6)
6β-Bromopenicillanic acid	0.5	40	0.013
Benzylpenicillin*	4700	2.8	1680

* Benzylpenicillin is a substrate, not an inhibitor, and the values in the three columns refer to $k_{cat.}$, K_m and $k_{cat.}/K_m$ respectively.

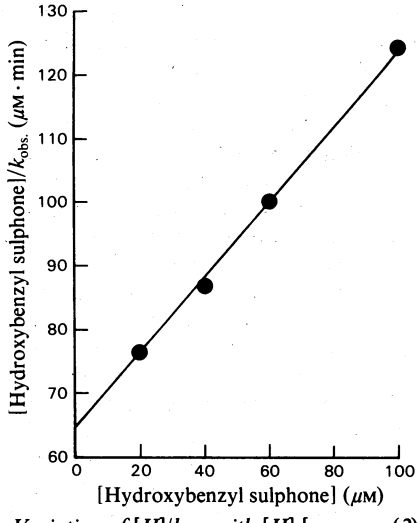


Fig. 3. *Variation of* $[H]/k_{obs.}$ *with* $[H]$ *[see eqn. (2) in the text], where* $[H]$ *is the concentration of the hydroxybenzyl sulphone and* $k_{obs.}$ *is the rate constant for inactivation during the hydrolysis of cephalosporin C (10mM) by the Pseudomonas β-lactamase (8.65nM) at pH8 at 30°C*

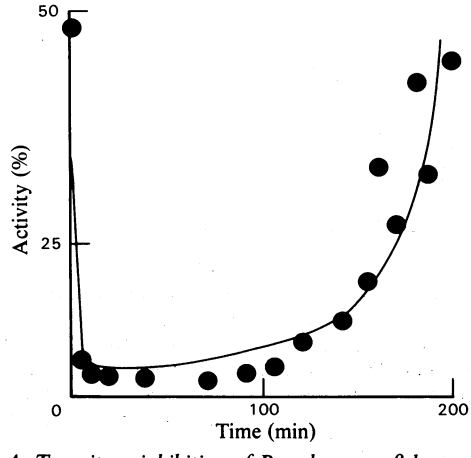


Fig. 4. *Transitory inhibition of Pseudomonas β-lactamase by the hydroxybenzyl sulphone at pH5.3*
The β-lactamase (9.2μM) and the hydroxybenzyl sulphone (92μM) were incubated in 0.2M-sodium acetate buffer, pH5.3, at 30°C. Portions were withdrawn and the enzymic activity was measured in the pH-stat, with cephalosporin C as substrate. The continuous curve is calculated as described by Bicknell *et al.* (1984). The initial fall from 100% activity was too rapid to follow.

(Bicknell *et al.*, 1984). That the transient inhibition is due to covalent modification is shown by the increase in the rate of regain of activity in the presence of hydroxylamine (Table 2). This supports the view that material accumulates in some form of acyl-enzyme. Whether the structure of the modified enzyme is the same at both pH values is unclear.

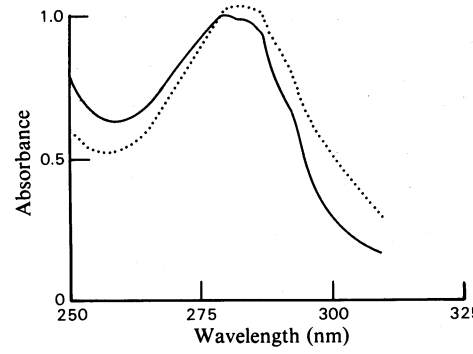


Fig. 5. *Absorption spectrum of Pseudomonas β-lactamase after treatment with the hydroxybenzyl sulphone*
The enzyme was treated with the hydroxybenzyl sulphone as described in the Materials and methods section, and the high-M_r fraction was isolated by gel filtration. The spectra of untreated (——) and treated (·····) samples are shown.

Table 2. *Effect of hydroxylamine on rate of regain of enzymic activity by Pseudomonas β-lactamase*
Expt. 1: *Pseudomonas* β-lactamase was treated with 12 molar proportions of the hydroxybenzyl sulphone in 10mM-triethanolammonium chloride, pH8, for 10min at 30°C, and then diluted with an equal volume of 0.1M-sodium acetate buffer, pH5.3, and incubated at 30°C; portions were withdrawn for assay of enzymic activity. Expt. 2: *Pseudomonas* β-lactamase was treated with 20 molar proportions of the sulphone at pH8, and the inactivated enzyme was then isolated by gel filtration on Sephadex G-25 and treated with 0.1M-hydroxylamine hydrochloride at pH5.2 at 30°C. The regain of enzymic activity was first-order in both experiments.

Expt. no.	Concn. of hydroxylamine (M)	Rate constant for regain of enzymic activity (min^{-1})
1	0	0.0083
2	0.1	0.055

Mechanism of inhibition (structural aspects)

Investigation of the properties of the inactivated enzyme gave direct information about the course of the reaction at pH 8 leading to inactivation. Isoelectric focusing is particularly useful because of its high resolving power. The *Pseudomonas* β-lactamase was treated with about 20 molar proportions of the hydroxybenzyl sulphone at pH8, which brought about 96% inactivation. The pattern of bands on isoelectric focusing was altered, suggesting a covalent modification of the

enzyme. One band predominated, suggesting that mainly one product was formed. The untreated enzyme had an apparent isoelectric point of pH8.7, whereas the treated enzyme had an apparent isoelectric point of 8.15. This change points to a structure in the modified enzyme that has gained 1 or 2 units of negative charge. Further information was provided by the u.v.-absorption spectrum of the modified enzyme after separation from low-M_r material by gel filtration (Fig. 5). The appearance of a new chromophore ($\lambda_{max.}$ about 293nm, ε about $16000 M^{-1} \cdot cm^{-1}$) suggested the formation of an enamine. The initially formed thiazolidine sulphones would not be expected to be stable (Peck & Folkers, 1949), and moreover the product from a β-lactam sulphone and the TEM-2 β-lactamase also had an intense chromophore of $\lambda_{max.}$ 289nm (Fisher *et al.*, 1981), and was believed to be an enamine (Brenner & Knowles, 1981; Kemal & Knowles, 1981). Hence, by analogy, it is probable that the modified *Pseudomonas* β-lactamase contained an enamine moiety (II), as is consistent with its isoelectric point.

The complexity of the reaction of β-lactam sulphones and other branched-pathway inhibitors with β-lactamases (Kemal & Knowles, 1981; Cartwright & Waley, 1983) makes any simple relationship between structure and inhibitory capacity unlikely. The hydroxybenzyl sulphone is perhaps the first effective synthetic inhibitor of class C β-lactamases; it owes much of its effectiveness against the *Pseudomonas* enzyme to the low apparent dissociation constant (Table 1). This constant has a higher value for 6β-bromopenicillanic acid (Table 1), an effective inhibitor of class A β-lactamases but a relatively poor inhibitor of class C β-lactamases. It seems unlikely that understanding of these differences will be attained until the three-dimensional structures of class A and class C β-lactamases are known.

The support of the Medical Research Council and the British Technology Group is gratefully acknowledged; we thank Glaxo Research Laboratories for gifts, and Professor P. G. Sammes both for compounds and for stimulating discussions.

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