A Reversible Inhibitor of β-Lactamase I from *Bacillus cereus*

Mark Jones,[a] Stephen C. Buckwell,[a] Michael I. Page,*[a] and Roger Wrigglesworth[b]

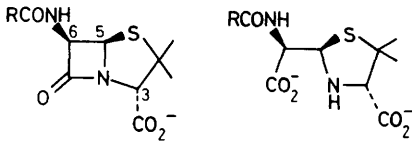
[a] *Department of Chemical and Physical Sciences, The Polytechnic, Queensgate, Huddersfield HD1 3DH, U.K.*
[b] *Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN, U.K.*

α-Methyl (3*S*,5*R*,6*R*)benzylpenicilloate is a reversible competitive inhibitor of β-lactamase I from *Bacillus cereus*; the inhibition constant K_i is 6.38×10^{-4} M and is independent of pH between pH 5 and 8 which is compatible with binding of the free base form of the thiazolidine residue to the enzyme.

The major cause of bacterial resistance to β-lactam antibiotics is the ability of bacteria to produce a β-lactamase enzyme which catalyses the hydrolysis of the β-lactam to antibiotically inactive products. For example, β-lactamase I is a very efficient catalyst for the hydrolysis of penicillins (**1**) to penicilloic acids (**2**).

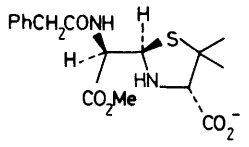
The inhibition of β-lactamases is therefore of interest and there have been several reports of good inactivators of these enzymes.[1] However, there are no efficient reversible non-covalent inhibitors known which would be useful for developing the recently reported *X*-ray structure of a β-lactamase.[2] We report herein that the methyl ester of (3*S*,5*R*,6*R*)-benzylpenicilloate (**3**) is a competitive reversible inhibitor of β-lactamase I from *Bacillus cereus* (strain 569/H/9 obtained from Porton Products). It is not an inhibitor of the zinc enzyme, β-lactamase II.

The ester (**3**) is readily prepared from the methanolysis of benzylpenicillin and the 3*S*,5*R*,6*R* epimer is readily characterised by its ^{1}H n.m.r. spectrum (δ 1.22, s, 2α-CH_3; δ 1.52, s, 2β-CH_3; δ 3.43, s, H-3; δ 3.69, s, PhCH_2; δ 3.74, s, CH_3O; δ 4.55, d, H-6; δ 5.12, s, 2β-CH_3; δ 3.43, s, H-3; δ 3.69, s, PhCH_2; δ 3.74, s, CH_3O; δ 4.55, d, H-6; δ 5.12, d, H-5; δ 7.38, m, Ph). Although epimerisation of the ester (**3**) does occur at C(6), particularly at lower pH, this is of little significance

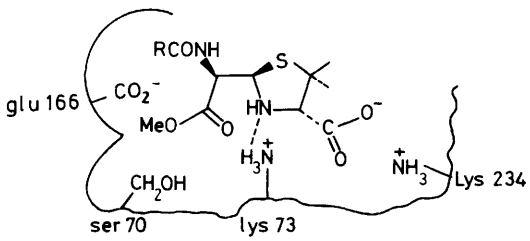


(1)

(2)



(3)



Scheme 1

during the time period required for the inhibition studies. β-Lactamase does not catalyse either hydrolysis or epimerisation of the ester (**3**).

The hydrolysis of β-lactam antibiotics is readily monitored spectrophotometrically.[4,5] Kinetic parameters for the enzyme catalysed reaction were obtained by both the method of initial rates and using the integrated rate equation.[5] The inhibition studies were analysed using Lineweaver–Burke, Eadie–Hofstee, Hanes plots, and the newer method of Waley[6] (Figure 1) to determine the inhibition constant for the β-lactamase catalysed hydrolysis of cephaloridine at different concentrations of the inhibitor, (**3**). Lineweaver–Burke analysis indicates that the inhibition is competitive and the inhibitor (**3**) is recovered unchanged after 24 h, *i.e.* there is no enzyme catalysed hydrolysis of the ester. At 30 °C the inhibition constant K_i was determined to be $6.70 \pm 1.08 \times 10^{-4}$ and $6.06 \pm 0.79 \times 10^{-4}$ M using cephaloridine and benzylpenicillin as the substrates, respectively.

The pK_a of the thiazolidine nitrogen conjugate acid, IH, of the methyl ester (**3**) was obtained from pH measurements at partial neutralisation as 3.80 ± 0.10. β-Lactamase shows two ionisable groups, EH and EH_2 of apparent pK_a 5.60 and 8.64, obtained from the pH-dependence of k_{cat}/K_m for the hydrolysis of both penicillins and cephalosporins.[5] The catalytically active form of the enzyme is EH. There are thus several possibilities for the protonated nature of binding the inhibitor to enzyme. The inhibition constant, K_i, is apparently pH independent between pH 5 and 8. Over this pH range the inhibitor exists predominantly in its free base form, although the concentration of its conjugate acid changes by a factor of 10^3. The simplest interpretation of the pH independence of K_i is that the inhibitor binds in its free base form, (**3**), to the active form of the enzyme, EH. Other binding modes would be possible if the pK_as of the enzyme or the inhibitor are severely modified upon binding compared with the aqueous solution pK_as.

Unlike the methyl ester, (**3**), the corresponding trifluorethyl ester is not an inhibitor of β-lactamase, which suggests that in this case there is an unfavourable steric interaction upon binding.

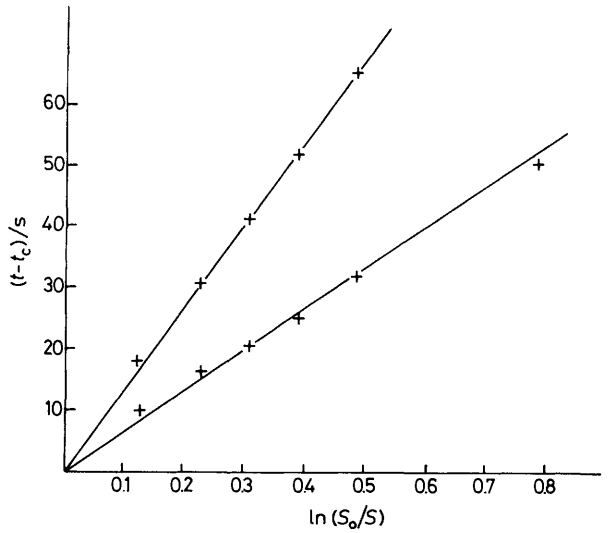


Figure 1. A plot of $(t - t_c)$ (the time difference required to give equal values of substrate S concentration with and without inhibitor) against $\ln(S_0/S)$ where S_0 is the initial substrate concentration. The data are for the *B. cereus* β-lactamase I (1×10^{-7} M) catalysed hydrolysis of cephaloridine at 30 °C, pH 7.0, in the presence of inhibitor (**3**) at different concentrations.

The hydrolysis product of benzylpenicillin, benzylpenicilloate (**2**; R = $PhCH_2$), is a very weak inhibitor of β-lactamase and it is difficult to measure K_i accurately. Using cephaloridine as a substrate the inhibition constant was found to be $>5 \times 10^{-2}$ M.

The increased binding of the ester (**3**), relative to the carboxylate (**2**; R = $PhCH_2$) suggests that the C(6) carboxylate residue in (**2**) may be near an anionic site on the enzyme. The recent *X*-ray crystallographic study[2] of β-lactamase from *Staphyllococus aureus* suggests that the active site contains, in addition to the catalytically important serine 70 residue, lysine and glutamate residues (Scheme 1). The mechanism of the reaction requires proton removal from the serine hydroxyl and protonation of the thiazolidine but unlike the serine proteases it is not known which, if any, residues act as general acid base catalysts.[4] Because the free base form rather than the protonated form of the inhibitor binds to the enzyme this may be indicative that the thiazolidine nitrogen acts as a proton acceptor rather than donor with respect to the protein. This suggests hydrogen bonding to 73-Lys-NH_3^+ (Scheme 1) which would also be compatible with this group acting as a general acid catalyst in the protonation of the β-lactam nitrogen facilitating C–N bond cleavage.[5]

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