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The Relative Catalytic Efficiency of β-Lactamase Catalyzed Acyl and Phosphyl Transfer

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Phosphonamidates which bear a simple resemblance to penicillin type structures have been synthesised as potential inhibitors of β-lactamases: -ethyl *N*-(benzyloxycarbonyl) amidomethyl phosphonyl amides, $PhCH_2OCONHCH_2P(O)(OEt)NR_2$, the amines HNR_2 being L-proline, D-proline, L-thiazolidine, and *o*-anthranilic acid. The proline derivatives completely and irreversibly inactivated the class C β-lactamase from *Enterobacter cloacae* P99, in a time-dependent manner, indicative of covalent inhibition. The inactivation was found to be exclusive to the class C enzyme and no significant inhibition was observed with any other class of β-lactamase. The anthranilic acid derivative exhibited no appreciable inactivation of the β-lactamases. The phosphonyl proline and phosphonyl thioproline derivatives were separated into their diastereoisomers and their individual second order rate constants for inhibition were found to be 7.72 ± 0.37 and $8.3 \times 10^{-2} \pm 0.004$ M^{-1} s^{-1} for the L-proline derivatives, at pH 7.0. The products of the inhibition reaction of each individual diastereoisomer, analyzed by electrospray mass spectroscopy, indicate that the more reactive diastereoisomers phosphonylate the enzyme by P-N bond fission with the elimination of proline. Conversely, gas chromatographic detection of ethanol release by the less reactive proline diastereoisomer suggests phosphonylation occurs by P-O bond fission. The enzyme enhances the rate of phosphonylation with P-N fission by at least 10^6 compared with that effected by hydroxide-ion. The pH dependence of the rate of inhibition of the β-lactamase by the more reactive diasteroisomer is consistent with the reaction of the diprotonated form of the enzyme, EH_2, with the inhibitor, I (or its kinetic equivalents EH with IH). This pH dependence and the rate enhancement indicate that the enzyme appears to use the same catalytic apparatus for phosphonylation as that used for hydrolysis of β-lactams. The stereochemical consequences of nucleophilic displacement at the phosphonyl centre are discussed.

It is generally accepted that nucleophilic substitution at acyl centres proceeds through the formation of an unstable tetrahedral intermediate (TI) (*1*). The reaction pathway thus involves a change in geometry and the conversion of the carbonyl carbon from three to four coordination. Furthermore, it is assumed that there is some preferential direction of nucleophilic attack such that the incoming nucleophile approaches at approximately the tetrahedral angle to the carbonyl group (*2*).

By contrast, the associative mechanism for phosphyl group transfer involves a

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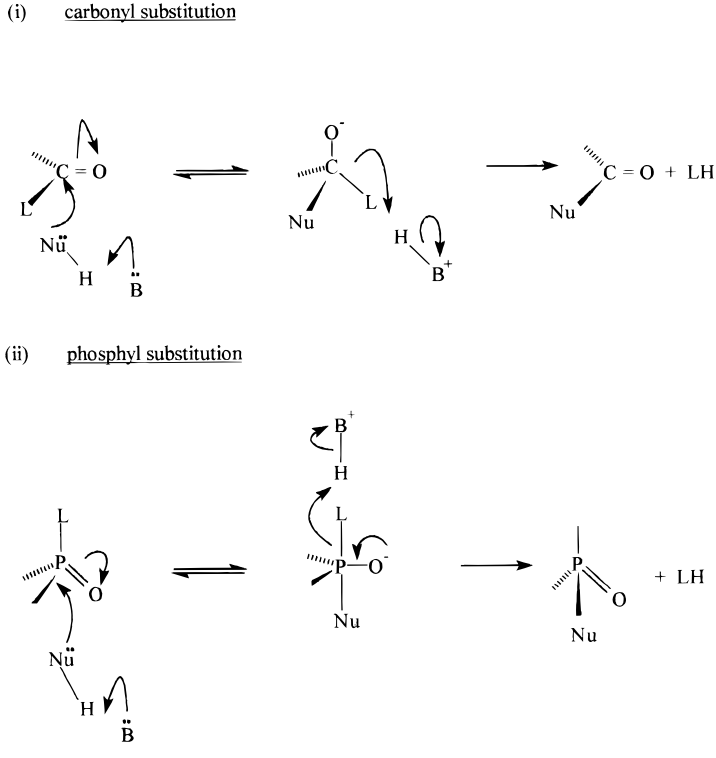
pentacoordinate intermediate with trigonal bipyramidal geometry (*1,3*). Here an initially four coordinate and tetrahedral phosphorus centre is converted to a five coordinate one and, in general, it is assumed that the preferential pathway involves the nucleophile taking up the apical position and the leaving group departing from an apical position of the trigonal bipyramidal intermediate (TBPI) (*1,3*).

Nucleophilic attack at both carbonyl and phosphyl centres is often facilitated by general base catalysis when covalent bond formation to the incoming nucleophile generates an acidic center in the intermediate. Similarly if the leaving group is very basic, bond fission and expulsion of the leaving group may be assisted by general acid catalysis. Given the preferential geometrical requirements for incoming nucleophiles and departing leaving groups, there should be a favoured relative positioning of the general base and general acid catalysts in an enzyme catalyzed reaction (Scheme 1). In principle, the general base, which accepts a proton from the attacking nucleophile, is not necessarily the same residue, which then acts as a general acid to donate a proton to the leaving group. Conceptually this is neater and appears to be the case for some serine proteases such as α-chymotrypsin. However, this may not always be the situation.

It is often assumed, but with little actual supporting evidence, that enzymes catalyze



SCHEME 1.

reactions by an exquisite positioning of the catalytic groups (*4*). If this were the case then it is doubtful if an enzyme with a primary function, say, as a catalyst for acyl transfer could be an effective catalyst for phosphyl transfer because of the geometrical differences just described.

Herein, we report that a serine enzyme—β-lactamase class C—is, in fact, also an extremely efficient catalyst for phosphyl transfer. The pH dependence of the kinetic parameters indicate that similar catalytic machinery is used for both types of reactions. A preliminary description of the inactivation of the β-lactamase by phosphonylation has been reported (*5*).

MATERIALS AND METHODS

Enzymes. The class C β-lactamase of *Enterobacter cloacae* P99 was obtained from the Centre of Applied Microbiology and Research (Porton Down, UK). The class A and B β-lactamases from *Bacillus cereus* were gifts from M. Galleni (University of Liege, Belgium).

Starting compounds were purchased from Aldrich/Sigma Chemical Co. All solvents, unless stated otherwise, were used as purchased without purification. Ethanol free chloroform was prepared by shaking with 5% (v/v) conc H_2SO_4 and then washing with distilled water. The chloroform layer was then separated, dried ($CaCl_2$) and then distilled with exclusion of moisture from P_2O_5. Triethylamine was usually distilled over KOH. Thionyl chloride was distilled under reduced pressure with exclusion of moisture, at least twice, before use.

Benzyl penicillin was kindly donated by Glaxo Wellcome and cephaloridine was kindly donated by SmithKline Beecham.

HPLC. All HPLC analyses were performed using a Lichrosorb 7 micron reverse phase RP 18 column (250 × 4 mm). A standard gradient elution programme was used involving elution from 100% aq. ammonium acetate (0.1% w/v) to 100% acetonitrile over a 30 min period.

Diethyl N-(phenylacetyl) amidomethyl phosphonate (1). This compound was synthesized from diethyl *N*-(phthalimido)-methylphosphonate (20 g, 67 mM) using the method of Bartlett (*6*). Initial N-deprotection with hydrazine monohydrate (3.78 ml, 71 mM) gave the crude amine (diethyl aminomethylphosphonate, 11.5 g), which was condensed with phenylacetyl chloride (11.11 ml, 84 mM) to give the product (3) in 55% yield (10.6 g) as a yellow oil. HPLC (220 nm): 16.1 min. TLC (Rf in EtOAc): 0.23 ^{1}H ($CDCl_3$) δ 1.26 (t, *J* 7.1 Hz, 6H, CH_3CH_2OP), 3.60 (s, 2H CH_2Ph), 3.68 (dd, $J_{P\text{-}CH}$ 11.96 Hz, $J_{NH\text{-}CH}$ 6.00 Hz, 2H, CH_2P), 4.06 (quin., $J_{P\text{-}OCH}$ 7.1 Hz, J_{CH_3} 7.1 Hz, 4H, CH_3CH_2OP), 6.0 (broad s, 1H, NH), 7.30 (m, 5H, ArH).

Diethyl N-(benzyloxycarbonyl) amidomethyl phosphonate (2). This compound was synthesized as described above except that, in this case, the amine (diethyl aminomethylphosphonate, 11.25 g) was condensed with benzyl chloroformate (12 ml, 84 mM), to give the product (4), in 60% yield (12.2 g) as an oil. HPLC (257 nm): 17.3 min, TLC (Rf in EtOAc): 0.26. ^{1}H ($CDCl_3$) δ 1.29 (t, *J* 7.2 Hz, 6H, CH_3CH_2OP), 3.61 (dd, $J_{P\text{-}CH}$ 11.24 Hz, $J_{NH\text{-}CH}$ 6.04 Hz, 2H CH_2P), 4.10 (quin., $J_{P\text{-}OCH}$ 7.2 Hz, *J* 7.34 Hz, 4H, CH_3CH_2OP), 5.11 (s, 2H OCH_2Ph), 5.30 (broad s, 1H, NH), 7.35 (m, 5H, ArH). IR (liquid film): 3266 cm^{-1}(NH), 1719 cm^{-1} (CO).

Ethyl N-(benzyloxycarbonyl)amidomethyl phosphonate (3). Diethyl (*N*-benzyloxycarbonyl) amidomethyl phosphonate (9.43 g, 31 mM) was dissolved in 62 ml of dioxane 3 equivalents of sodium hydroxide were added (23 ml of a 4 M solution) and the resulting emulsion was left to stir overnight at room temperature. The reaction mixture was diluted with 23 ml of distilled water and then washed with two 56-ml portions of chloroform to remove unreacted starting material. A further 112 ml of chloroform were added to the aq. layer before acidification with 51 ml of H_2SO_4 (1M). The organic layer was separated and the aq. layer extracted with two 140-ml portions of chloroform. The combined portions of chloroform were dried (anhyd. $MgSO_4$) and rotary evaporated under reduced pressure to yield an oil, which solidified on standing to give product as a white powder in 93.5% yield. Mpt 98.7–101.3°C (lit. 106–106.5[6]). HPLC (257 nm): 9.2 min. TLC (Rf in EtOAc): 0.4. 1H ($CDCl_3$) δ 1.30 (t, *J* 7.1 Hz, 3H, CH_3CH_2OP), 3.62 (d, $J_{P\text{-}CH}$ 11.48 Hz, 2H, CH_2P), 4.11 (quin. $J_{P\text{-}OCH}$ 7.26 Hz, J_{CH_3} 7.1 Hz, 2H, CH_2OP), 5.12 (s, 2H, CH_2Ph), 5.61 (broad s, 2H, NH/POH), 7.33 (m, 5H, ArH).

Ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl L-proline (benzyl ester) (4). Benzyl L-proline was obtained by adding satd. sodium bicarbonate to a solution of the hydrochloride salt (3 g in 37 ml water/50 ml EtOAc) at 0°C. The aq. layer was quickly extracted with three 75-ml portions of ethyl acetate, which were then combined, dried ($MgSO_4$), and rotary evaporated to give the desired amine (2.4 g) as a liquid. The phosphonate monoester (*3*) was converted to the corresponding phosphonyl chloride by the treatment of a solution of the monoester (1 g, 3.7 mM in 7 ml $CHCl_3$) with thionyl chloride (0.26 ml, 3.7 mM). After the removal of solvent, residual HCl and SO_2, the resulting oil was dissolved in chloroform (7 ml), and then condensed with benzyl proline (0.9 g in 7 ml $CHCl_3$) at 0°C, using triethylamine (0.93 ml) as a base. The product was obtained as a mixture of two diastereoisomers in the form of a thick yellow oil (1.4 g, 83%). Yields were typically in excess of 70% (1.2 g) after purification by column chromatography on silica 60 gel. HPLC (220nm): 22.85 and 23.13 min. (2\3 ratio of two diastereoisomers). TLC (Rf in EtOAc): 0.26 and 0.33. ^{13}C ($CDCl_3$) ppm 15.90, 16.00 (CH_3CH_2OP), 24.78, 24.88 (Pro γ CH_2), 30.74, 30.84 (Pro β CH_2), 36.0 (d, $J_{C\text{-}P}$ 148.9 Hz, CH_2P), 45.72, 45.80 (Pro δ CH_2N), 59.20, 60.00 (Pro α CH), 60.42, 60.52 (CH_3CH_2OP), 66.41 (CH_2 Bzl), 66.66 (CH_2 Cbz), 127.57–128.27 (ArCH × 10), 135.22, 135.45 (quat. Cbz), 136.40 (quat. Bzl), 156.31 (CO Cbz), 173.94, 174.06 (CO Bzl). IR (liquid film): 3235 cm^{-1} (NH), 1727, 1716 cm^{-1-} (CO). GCMS: *m/z* 461 (M +1), 325 (M -Z), 256 (M - Pro(OBzl)), 204 (Pro(OBzl)). The two diastereoisomers were separated by column chromatography on silica 60 gel using gradient elution (from 100% diethyl ether to100% ethyl acetate). The less polar diastereoisomer, major component (4a) was eluted first. 1H ($CDCl_3$) δ 1.24 (t, *J* 6.96 Hz, 3H, CH_3CH_2OP), 1.87 (m, 3H, Pro β CH + γ CH_2), 2.13 (m, 1H, Pro β CH), 3.18 (m, 1H, Pro δ CH), 3.35 (m, 1H, Pro δ CH), 3.62 (ABCX, *J* 6.06 Hz, J 11.35 Hz, $J_{P\text{-}CH}$ 6.8 Hz, 1H, CH_AP), 3.80 (ABCX, *J* 8.0 Hz, *J* 11.35 Hz, $J_{P\text{-}CH}$ 15.8 Hz, 1H, CH_BP), 4.10 (m, 2H, CH_2OP), 4.27 (dt, *J* 3.35 Hz, $J_{P\text{-}CH}$ 7.64 Hz, 1H, Pro α CH), 5.06 (AB, *J* 12.4 Hz, 1H, Cbz CH), 5.09 (AB, *J* 12.4 Hz, 1H, OBzl CH), 5.12 (AB, *J* 12.4 Hz, 1H, cbz CH), 5.13 (AB, J 12.4 Hz, 1H, OBzl CH), 5.55 (m, 1H, NH), 7.32 (m, 10H, ArH). The more polar, minor component (4b) was eluted second. 1H ($CDCl_3$) δ1.2 (t, *J* 6.98 Hz, 3H, CH_3CH_2OP),

1.82 (quin., *J* 7.4 Hz, 2H, Pro γ CH_2), 1.97 (ABC_2X, *J* 4.49 Hz, *J* 6.70 Hz, J 11 Hz, 1H, Pro β CH), 2.15 (ABC_2X, *J* 8.28 Hz, *J* 8.28 Hz, *J* 11 Hz, 1H, Pro β CH), 3.23 (m, 2H, Pro δ CH_2), 3.60 (ABCX, *J* 5.68 Hz, $J_{P\text{-}CH}$ 12.57 Hz, *J* 15.4 Hz, 1H, CHP), 3.73 (ABCX, *J* 7.57 Hz, $J_{P\text{-}CH}$ 10.58 Hz, *J*, 15.4 Hz, 1H, CHP), 3.91 (ABC_3X, *J* 7.39 Hz, $J_{P\text{-}OCH}$ 7.4 Hz, *J* 10.08 Hz, 1H, CHOP), 4.08 (ABC_3X, *J* 7.26 Hz, $J_{P\text{-}OCH}$ 7.4 Hz, *J* 10.08 Hz, 1H, CHOP), 4.45 (dt, *J* 3.52 Hz, $J_{P\text{-}CH}$ 8.59 Hz, 1H, Pro α CH), 5.09 (AB, *J* 12.28 Hz, 1H, OBzl CH), 5.12 (s, 2H, cbz CH_2), 5.18 (AB, *J* 12.28 Hz, 1H, OBzl CH), 6.0 (m, 1H, NH), 7.31 (m, 10H, ArH).

Ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl D*-proline (methyl ester) (5).* Methyl D-prolate was obtained by treating a suspension of D-proline (590 mg) in ethanol (10 ml) at 0°C with ethereal diazomethane. Rotary evaporation of the solvent under reduced pressure gave the desired amine as a colourless liquid (471.5 mg) in 71.2% yield. The ester amine (471.5 mg) was condensed with the phosphonyl chloride (obtained from 0.52 g, 1.9 mM, of the monoester 3, as before), using the method described previously, to give a yellow oil (620 mg) in 84% yield. A mixture of the two diastereoisomers was obtained (560 mg) in 76.5% yield (after purification by column chromatography on silica 60 gel, as described previously). The ratio of the less polar isomer (5a) relative to the more polar isomer (5b) was found to be 2\1 by ^{1}H nmr. HPLC (220nm): 17.4 min. TLC (Rf in 10% EtOH/EtOAc): 0.31 and 0.37. ^{13}C ($CDCl_3$) ppm 16.24, 16.33 (CH_3CH_2OP), 25.08, 25.33 (Pro γ CH_2), 31.03, 31.13 (Pro β CH_2), 37.40, 37.76 (doublet × 2 , $J_{C\text{-}P}$ 148.7, 158.00 Hz, CH_2P), 46.00, 46.07 (Pro δ CH_2N), 52.08, 52.34 (Pro OCH_3), 60.09, 60.16 (Pro α CH), 60.36, 60.74 (CH_2OP), 66.78, 67.04 (CH_2 Cbz), 127.91−128.45 (ArCH × 3), 136.36, 136.66 (quat. Cbz), 156.47, 156.55 (CO Cbz), 174.92, 175.05 (CO OCH_3) 5b. ^{1}H ($CDCl_3$) δ 1.3 (t, *J* 7.1 Hz, 3H, CH_3CH_2OP), 1.86 (m, 4H, Pro β CH_2 + γ CH_2), 3.18 (m, 1H, Pro δ CH), 3.32 (m, 1H, Pro δ CH), 3.61 (ABCX, *J* 6.08 Hz, *J* 11.32 Hz, $J_{P\text{-}CH}$ 6.8 Hz, 1H, CH_AP), 3.67 (s, 3H, OCH_3), 3.82 (ABCX, *J* 8.10 Hz, *J* 11.32 Hz, $J_{P\text{-}CH}$ 16.88 Hz, 1H, CH_BP), 4.13 (m, 2H, CH_2OP), 4.21 (dt, *J* 4.05 Hz, $J_{P\text{-}CH}$ 6.80 Hz, 1H, Pro α CH), 5.05 (AB, *J* 12.33 Hz, 1H, Cbz CH), 5.13 (AB, *J* 12.33 Hz, 1H, Cbz CH), 6.06 (m, 1H, NH), 7.32 (m, 5H, ArH) 5a. ^{1}H ($CDCl_3$) δ 1.25 (t, *J* 8.3 Hz, 3H, CH_3 CH_2OP), 1.83 (quin., *J* 7.0 Hz, 2H, Pro γ CH_2), 1.95 (ABC_2 X, *J* 4.5 Hz, J 6.75 Hz, *J* 12.5 Hz, 1H, Pro β CH), 2.38 (ABC_2X, *J* 8.1 Hz, *J* 8.1 Hz, *J* 12.5 Hz, 1H, Pro β CH), 3.01 (m, 2H, Pro δ CH_2), 3.69 (s, 3H, OCH_3), 3.93 (m, 2H, CH_2P), 4.11 (m, 2H, CH_2OP), 4.39 (dt, *J* 4.05 Hz, $J_{P\text{-}CH}$ 8.1 Hz, 1H, Pro α CH), 5.10 (s, 2H, cbz CH_2), 6.4 (m, 1H, NH), 7.33 (m, 5H, ArH).

Ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl L*-thioproline (methyl ester) (6).* L-Thioproline methyl ester (1.14 g) was obtained from 1 g of the corresponding acid. The amine (1 g in 10 ml $CHCl_3$) was condensed with the phosphonyl chloride (obtained from 1.55 g of the monoester (*3*). An unresolved mixture of the pure diastereoisomers (1:1 ratio) was obtained in 30% yield (300 mg), after separation by column chromatography on silica 60 gel. HPLC (220 nm): 18.20 min. TLC (Rf in 10% EtOH/EtOAc): 0.39 and 0.48. ^{1}H ($CDCl_3$) δ 1.26 (t, *J* 7.4 Hz, 3H, *CH_3*CH_2OP, 6a), 1.27 (t, *J* 7.4 Hz, 3H, *CH_3*CH_2OP, 6b), 2.96 (ABX, *J* 6.75 Hz, *J* 10.8 Hz, 1H, β CHS, 6b), 3.16 (ABX, *J* 6.8 Hz, *J* 10.8 Hz, 1H, β CHS, 6b), 3.18 (ABX, *J* 6.8 Hz, *J* 10.8 Hz, 1H, β CHS, 6a), 3.28 (ABX, *J* 2.2 Hz, *J* 10.8 Hz, 1H, β CHS, 6a), 3.48 (ABCX, *J* 4.7 Hz, $J_{P\text{-}CH}$ 10.8 Hz, *J* 15.5 Hz, 1H, CHP), 3.69 (s, 3H, CH_3OP,

6b), 3.7 (s, 3H, *CH*$_3$OP, 6a), 3.80 (m, 3H, CHP + CH_2P), 4.19 (m, 6H, CH_2OP + α CH), 3.38 (ABX, *J* 5.4 Hz, *J* 8.1 Hz, 1H, δCH,6b), 4.49 (ABX, *J* 5.4 Hz, *J* 8.1 Hz, 1H, δCH, 6a), 4.84 (ABX, *J* 5.4 Hz, *J* 8.1 Hz, 1H, δCH, 6b), 4.95 (ABX, *J* 5.4 Hz, *J* 8.1 Hz, 1H, δCH, 6a), 5.06 (AB, *J* 12.5 Hz, 1H, cbz CH, 6b), 5.10 (s, 2H, Cbz CH_2, 6a), 5.13 (AB, *J* 12.5 Hz, 1H, Cbz CH, 6b), 6.2 (m, 1H, NH 6b), 6.35 (m, 1H, NH, 6a), 7.34 (m, 10H, ArH). ^{13}C ($CDCl_3$) ppm 16.09, 16.19 (CH_3CH_2OP), 34.66, 34.73 (Thiopro NCH_2S), 37.66, (Thiopro β CH_2), 37.40, 37.72 (doublet $\times$ 2 , $J_{C\text{-}P}$ 153.92,154.60 Hz, CH_2P), 48.06, 48.13 (Thiopro CH_2N), 52.58, 52.75 (Thiopro OCH_3), 61.35, 61.45 (CH_2OP), 62.90, 62.99 (Thiopro CH), 69.42 (CH_2 Cbz), 128.03–128.44 (ArCH $\times$ 4), 136.45, 136.53 (quat. Cbz), 156.49, 156.57 (CO Cbz), 171.66, 171.70 (CO OCH_3).

Ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl anthranilate (methyl ester) (7). This compound was synthesised from methyl anthranilate (0.56 ml) and the phosphonyl chloride (obtained from 1g of the monoester (*3*)) as described previously. The product was obtained as a white solid in 68% yield, after purification by column chromatography on silica 60 gel Mpt. 91–92°C. HPLC (220 nm): 21.34 min. TLC (R_f in EtOAc): 0.29. ^{13}C ($CDCl_3$) ppm 16.19, 16.28 (*CH*$_3$$CH_2OP$), 38.43 (d, $J_{C\text{-}P}$ 143.66 Hz, *CH*$_2$P), 52.04 (OCH_3), 61.67, 61.77 (CH_2OP), 67.00 (*CH*$_2$Cbz), 114.32, 114.44, 117.70, 117.75 (ArCH Anth. C4), 120.54 (ArCH Anth C3), 127.90–128.35 (ArCH Cbz), 131.23 (ArCH Anth. C2), 134.56 (ArCH Anth. C5), 136.17 (ipso Cbz), 143.94 (C CO), 156.04, 156.13 (CO Cbz), 168.68 (CO Cbz). ^{1}H ($CDCl_3$) δ 1.37 (t, *J* 7.09 Hz, 3H, *CH*$_3$$CH_2OP$), 3.76 (ABX, $J_{P\text{-}CH}$ 10.37 Hz, $J_{NH\text{-}CH}$ 6.02 Hz, 2H, CH_2P), 3.90 (s, 3H, OCH_3), 4.18 (m, 2H, CH_2OP), 5.00, (AB, *J* 14.8 Hz, 1H, CH_2Ph), 5.05 (AB, *J* 14.8 Hz, 1H, CH_2Ph), 5.15 (broad s, 1H, NH), 6.95 (t, *J* 7.35 Hz, 1H, (C4)-ArH), 7.33 (m, 5H, Cbz ArH), 7.41 (t, *J* 7.82 Hz, 1H, (C3)-ArH), 7.60 (d, *J* 8.33 Hz, 1H, (C2)-ArH), 7.97 (d, *J* 8.00 Hz, 1H, (C5)-ArH), 9.20 (d, $J_{P\text{-}NH}$ 8.00 Hz, 1H, PNH). IR (liquid film): 3226.4 cm^{-1} (NH CO), 1717.2 cm^{-1} (CO Cbz), 1682.6 cm^{-1} (CO ester). Elemtl. anal.: expect -C(51.93), H(5.05), N(3.19), found -C(51.68), H(5.08), N(3.09).

Ammonium salt of ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl L-proline (8). This compound was synthesized from the diester (*4*) by hydrolysis of a 0.1M solution in a mixture of 50% acetonitrile/water (v/v), with 3 equiv. of lithium hydroxide. Benzyl alcohol was removed by extraction with diethyl ether followed by freeze-drying to give the lithium salts. HPLC (220 nm): 10.62 and 11.24 min (in the ratio 3\2). ^{13}C (D_2O) ppm 14.87, 14.95 (*CH*$_3$$CH_2OP$), 24.38, 24.49 (Pro γ CH_2), 31.08, 31.21 (Pro β CH_2), 35.81 (doublet, $J_{C\text{-}P}$ 147.67 Hz, CH_2P), 46.27, 46.35 (Pro δ CH_2N), 61.02, 61.11 (Pro α CH), 61.34, 61.48 (CH_2OP), 66.45, 66.56 (*CH*$_2$Cbz), 127.03–128.13 (ArCH $\times$ 3), 135.77 (ipso. Cbz), 157.50 (CO Cbz), 181.39 (CO pro). ^{1}H (D2O) δ 1.24 (t, *J* 7.0 Hz, 3H, *CH*$_3$$CH_2OP$, 8b), 1.30 (t, *J* 7.0 Hz, 3H, *CH*$_3$$CH_2OP$, 8a), 1.83 (m, 6H, Pro γ CH_2, and β CH), 2.13 (m, 2H, Pro β CH), 3.26 (m, 4H, Pro δ CH2), 3.62 (d, $J_{P\text{-}CH}$ 10.2 Hz, 2H, CH_2 P, 8a), 3.67 (d, $J_{P\text{-}CH}$ 10.2 Hz, 2H, CH_2P, 8b), 3.99 (dt, $J_{P\text{-}NCH}$ 13.5 Hz, 2H, Pro α CH), 4.11 (quin., *J* 7.0 Hz, $J_{P\text{-}OCH}$ 7.0 Hz, 4H, CH_2OP), 5.15 (s, 4H, cbz CH_2), 7.42 (m, 10H, ArH). The two diastereoisomers were separated by HPLC using a Dynamax 60 A reverse phase C 18 column (25 $\times$ 2.14 cm, flow rate 12 ml/min) and eluting with 13% acetonitrile–87% (v/v) of an aqueous ammonium acetate solution (1% w/v). The ammonium acetate solutions

containing the pure diastereoisomers were freeze-dried and then chromatographed a second time, eluting with 10% acetonitrile–90% (v/v) distilled water, to remove ammonium acetate. The resulting fractions were freeze dried to yield white hygroscopic powders (*8a*) and (*8b*) as their ammonium salts. HPLC at 220nm confirmed the presence of the two diastereoisomers without contamination by either the other isomer or any other impurity. FABMS (*8a*): *m\z* 393, 20 (MNa^+), 372, 3 (MH^+), 256,10 (M − Pro), 116, 14 ($ProH^+$), 91, 100 ($PhCH_2^+$). ESMS (*8a*): *mz* 415, 10 (MH^+, Na_2^+), 409, 5 (MK^+), 393, 100 (MNa^+), 371, 5 (MH^+). ESMS (*8b*): *m/z* 409, 100 (MK^+), 393, 25 (MNa^+), 371, 20 (MH^+), 256, 12 (M − Pro), 116, 22 ($ProH^+$).

Ammonium salt of ethyl N-(benzyloxycarbonyl)amidomethylphosphonyl L*-thioproline (9).* The lithium salt of this compound was synthesized from (*6*) as previously described. HPLC (220 nm): 11.3 and 11.64 min (in the ratio 1.1:1, (*9a*,*9b*)). The two diastereoisomers were separated by HPLC and the products were obtained as hygroscopic powders as their ammonium salts, which showed no contamination from either the other isomer or any other impurity. ESMS (9a): *m/z* 427, 90 (MK^+), 389, 15 (MH^+), 256, 65 (M − thiopro), 145, 100. ESMS (9b): *m/z* 427, 100 (MK^+), 411, 40 (MNa^+), 389, 20 (MH^+), 256, 85 (M − thiopro), 145, 30.

Lithium salt of ethyl N-(benzyloxycarbonyl) amidomethylphosphonyl anthranilate (10). The lithium salt of this compound was synthesized from (*7*) as previously described. HPLC (220 nm): 11.79 min. ^1H (D_2O) δ 1.34 (t, *J* 7.0 Hz, 3H, CH_3CH_2OP), 3.81 (d, $J_{P\text{-}CH}$ 9.4 Hz, 2H, CH_2P), 4.21 (ABX *J* 7.0 Hz, $J_{P\text{-}OCH}$ 7.0 Hz, 2H, CH_2OP), 5.00 (s, 2H, cbz CH_2), 7.04 (t, *J* 7.4 Hz, 1H, (C4)-ArH), 7.41 (m, 7H, Anth. (C2-C3) and Cbz ArH), 7.92 (d, *J* 8.03 Hz, 1H, (C5) ArH).

Kinetics. Some of the phosphonamidate salts were hygroscopic and determination of their concentration by weighing was not possible. An HPLC calibration curve for an authentic sample of the phosphonate monoester (*3*) was obtained using the conditions described previously. The concentration of the phosphonamidate salt stock solution was then determined from its complete hydrolysis in HCl and measuring the concentration of (*3*) produced.

β-Lactamase activity was determined against benzylpenicillin by a spectrophotometric method (*7*). Concentrations of the *Ent. cloacae* P99 β-lactamase were also determined spectrophotometrically, using an extinction coefficient at 280 nm, $7.1 \times 10^4\ M^{-1}\ cm^{-1}$ (*8*). Concentrated stock solutions of the phosphonamidates were prepared in 20 mM MOPS buffer at pH 7.5.

For the kinetic measurements required for the pH-rate profiles the buffer systems were HCl, formate, acetate, Mes, Mops, Hepes, Tapso, Ches, and NaOH. In all cases, an ionic strength of 1.0 M (KCl), and 30°C was used. Rates of enzyme inactivation were determined from measurements of enzyme activity against benzylpenicillin as a function of time. In the general procedure, enzyme (ca. 5 μM) and the inhibitor (20 μM to 20 mM) were incubated together in buffer at the required pH. Aliquots of the reaction mixture were withdrawn at relevant time intervals and immediately assayed for enzyme activity. The activity of samples of enzyme without inhibitor was also routinely monitored as a control. Pseudo-first order rate constants of inactivation were determined from the Enzfitter programme and second-order rate constants were then obtained from the slopes of plots of the first-order rate constants against inhibitor concentration.

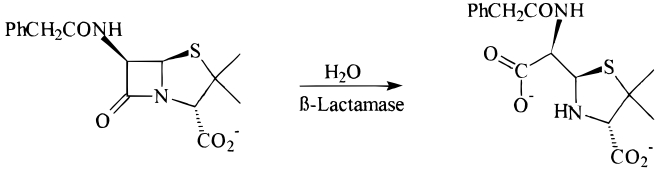
RESULTS AND DISCUSSION

The major cause of the resistance of some bacteria to the normally lethal action of β-lactam antibiotics is the ability of the bacteria to produce β-lactamase enzymes, which catalyze the hydrolysis of the β-lactam of penicillins **(11)** (Scheme 2) and cephalosporins. The most prevalent and clinically important β-lactamases are the class A and C type, which are both serine enzymes and act by a mechanism involving the formation of a relatively unstable acyl enzyme (EA) intermediate, Eq. [1] (*9*).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P \qquad [1]$$

Although there have been several crystal structures of β-lactamases and their derivatives reported (*10–12*) and although the nature and degree of the conservation of the amino acid residues in and near the active site are known (*13*) there is little detailed evidence of the groups presumed to be involved with the necessary proton transfer steps (*14,15*). Comparison with serine proteases would indicate the need for general acid–base catalysis (*16*). The main contenders for these roles are, in class A β-lactamase, the carboxyl group of Glu-166 and the amino group of Lys-73 (*9*). In class C β-lactamase there is no equivalent glutamate residue but tyrosine-150 may take its role and some unusual solvent kinetic isotope effect studies indicate that the tyrosine may have a low pK_a of *ca.* 6 (*17*). It is assumed that this pK_a corresponds to that of the general base catalyst required for proton removal from serine 64 and used in the formation of the tetrahedral intermediate leading to acylation (*10,17,18*). Site-directed mutagenesis of Tyr-150 has not led to firm conclusions although it appears to be essential for hydrolysis of the best substrates (*19*).

We have previously demonstrated that the class C β-lactamase from *Enterobacter cloacae* P99 is inactivated by the phosphonamidate **(13)** (R = $PhCH_2O$) (*5*). Our preliminary report indicated that the enzyme was phosphonylated in a process analogous to the enzyme catalysed acylation by the penicillins (Scheme 3). We are interested in the detailed mechanism of this inactivation and the geometrical differences in the catalytic machinery required for acyl and phosphyl transfer. It is necessary to demonstrate that the enzyme *increases the rate* of phosphonylation reaction. It is well known that enzymes can catalyse the same reaction of a variety of substrates and even different



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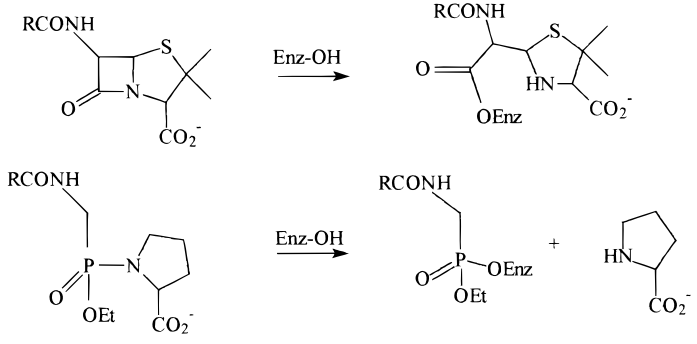
SCHEME 2.

reactions with alternative substrates (*20*). However, demonstrating the efficiency of the catalysis when the substrate is modified is not straight-forward. Modification of the substrate structure can affect the free energies of both the initial reactant state and the transition state, whereas the observed *differences* in rate constants for the enzyme catalysed reaction only reflect the difference in energies between these two states. Importantly, changes in the activity of an enzyme catalysed reaction can result from differences in intrinsic "chemical" effects within the substrate as well as from differences in binding interactions between the substrate and enzyme (*20*). Different chemical structures can affect the ease of bond-making and -breaking by classical electronic factors such as inductive, resonance and steric effects. However, the free energy of activation of an enzyme-catalyzed reaction is also affected by the favourable binding energies between the protein and substrate substituents not directly involved with the reaction site. It is therefore important to separate these two effects before conclusions about the efficiency of enzyme catalysis can be made. We have suggested (*21*) that an "enzyme rate-enhancement factor" (EREF) can be evaluated by dividing the second-order rate constant for the enzyme catalysed reaction, k_{cat}/K_m, by that for hydrolysis of the same substrate catalysed by hydroxide ion, k_{OH}. To demonstrate enzyme "catalyzed" phosphonylation, it is therefore necessary to compare the relative "intrinsic" reactivities of the two "substrates"—the penicillin **(11)** and the phosphonamidate **(13)** toward hydrolysis.

The phosphonamidate **(13)** has two asymmetric centres and exists as four diastereoisomers. Reactions of these diastereoisomers at the chiral active site of the enzyme would be expected to show differences in reactivity and perhaps even reaction type such as P-O or P-N bond fission. Any such observed discrimination would also be indicative of the enzyme using its catalytic machinery for phosphonylation.

(i) Hydrolysis of the phosphonamidate **(8).** The pH rate profile for the hydrolysis of the phosphonamidate **(8)** is shown in Fig. 1. There are two acid catalyzed reactions corresponding to the reaction of the phosphonamidate with an undissociated and dissociated carboxylic acid. The inflection point corresponds to the expected pK_a of the carboxylic acid, 3.86 ± 0.1. At pHs above this pK_a, there is a pH independent



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SCHEME 3.

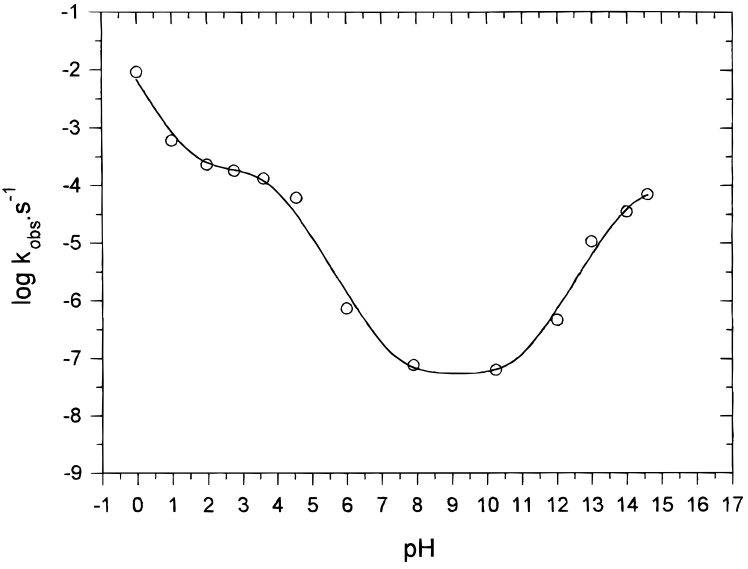


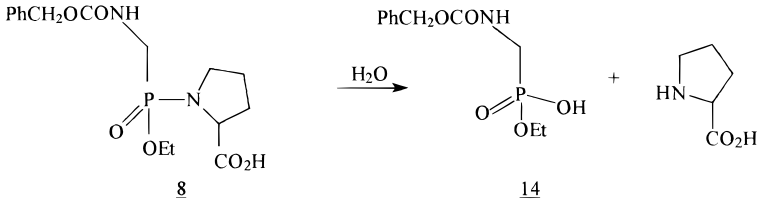
FIG. 1. A plot of the first order rate constant, k_{obs}/s^{-1}, for the hydrolysis of the phosphonamidate (8) as a function of pH at 30°C and I = 1.0M (KCl).

and a hydroxide ion dependent reaction of the phosphonamidate with the carboxylic acid residue ionized. At very high pH, the rate again starts to fall off which is presumably the result of ionisation of the side chain amido group. The overall rate of hydrolysis is therefore given by Eq. [2], where k_{OH} is the second order rate constant for the hydroxide ion catalyzed hydrolysis and k_o is the pH-independent first-order rate constant for hydrolysis of **(8)** with the carboxylic acid ionised; k_H and k_H^H, are the second-order rate constants for the acid catalysed hydrolysis for the phosphonamidate **(8)** with a dissociated and undissociated carboxyl group, respectively; k_{a1} is the dissociation constant for the carboxylic acid and K_{a2} is the dissociation constant of the amido side chain.

$$k_{obs} = k_{OH}(OH^-)\left(\frac{(H^+)}{K_{a2} + (H^+)}\right) + k_O + k_H(H^+)\left(\frac{K_{a1}}{K_{a1} + H^+}\right) + k_H^H(H^+)\left[\frac{H^+}{K_{a1} + H^+}\right] \quad [2]$$

Below pH 11 the reaction occurs with P-N fission and the hydrolysis products are the monoester **(14)** and proline (Scheme 4) as expected from previous studies of the hydrolysis of phosphonamidates (*3*).

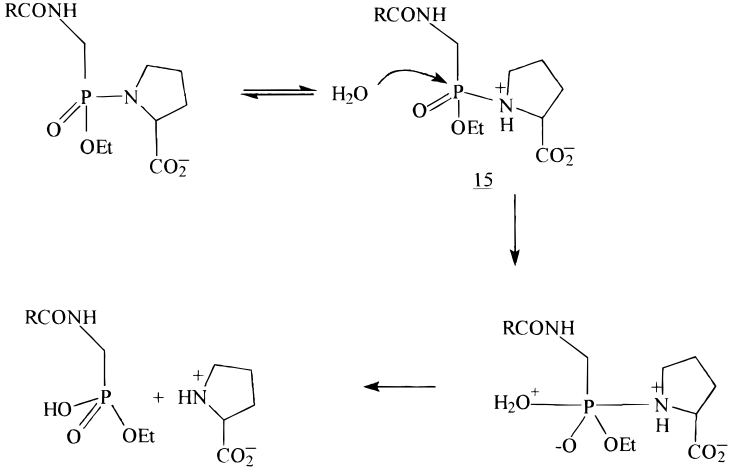
The value of k_H, the acid-catalyzed hydrolysis of the phosphonamidate with a dissociated carboxylic acid, is 1.36 $dm^3\ mol^{-1}\ s^{-1}$, which is 200-fold greater than k_H^H ($6.59 \times 10^{-3}\ dm^3\ mol^{-1}\ s^{-1}$)—the acid-catalyzed hydrolysis of the same compound



SCHEME 4.

with an undissociated carboxylic acid. It is possible that this rate difference indicates neighboring group participation by the carboxylate anion or the kinetically equivalent intramolecular general acid catalysed mechanism of water attack on the phosphonamidate with an undissociated carboxylic acid (Scheme 8). The rate of the acid catalyzed hydrolysis of the phosphonamidate with the carboxyl group esterified as the benzyl ester is also about 100-fold less than that for the carboxylate anion derivative and is similar to that for the phosphonamidate with an undissociated carboxylic acid. However, the simplest explanation for these rate differences is that the acid catalyzed hydrolysis of the phosphonamidate occurs by initial *N*-protonation followed by attack of water on the phosphonyl centre (*3,22*) (Scheme 5). The N-protonated phosphonamidate **(15)** is stabilized by the negatively charged carboxylate anion relative to the undissociated carboxylic acid, giving rise to a higher concentration of the conjugate acid and thus an overall faster rate of hydrolysis. The proline nitrogen is about 2 pK_a units less basic with an undissociated or esterified carboxylic acid. The rate difference of 200 between the two species gives a crude estimate of a Bronsted β_{1g} of *ca.*1.0 which is indicative of a positively charged nitrogen in the transition state.

The pH-independent hydrolysis occurs with predominant P-N fission and is very



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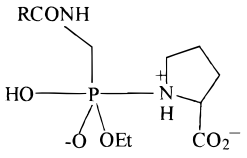

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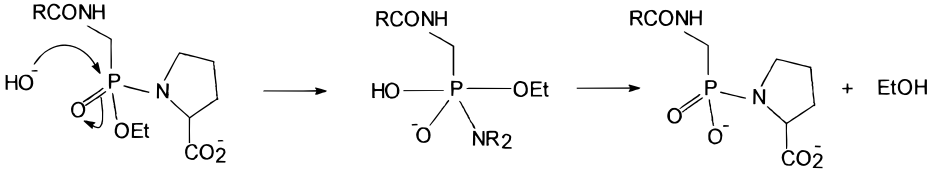
slow ($k_o = 5.29 \times 10^{-8}$ s^{-1}). This pathway probably involves rate-limiting breakdown of the intermediate (16). Above pH 11 the rate of hydrolysis of the phosphonamidate is first order in hydroxide ion (Fig. 1) and the second-order rate constant for the hydroxide ion catalysed hydrolysis, k_{OH}, is 6.79×10^{-5} dm^3 mol^{-1} s^{-1}. In this region P-O fission occurs to expel ethanol—again as expected from previous studies of phosphonamidates (*3*) (Scheme 6). The basic limb of the pH-rate profile (Fig. 1) indicates a slight decrease in the dependence upon hydroxide ion at very high pH which is compatible with a reduced rate of hydrolysis of the phosphonamidate with its amide side chain ionised ($pK_{a2} = 14.1$).

In summary, the phosphonamidate **(8)** is a relatively stable derivative with a reactivity similar to that predicted from previous studies (*3*), and any reaction of it with enzymes is likely to require the protein to lower the activation energy.

(ii) Enzyme inactivation. Both diastereoisomers of the phosphonamidate of L-proline **(8)** completely and irreversibly inactivate the class C β-lactamase from *Enterobacter cloacae* (P99) in a time-dependent manner. There is an exponential decrease in enzyme activity, measured by its hydrolysis of benzylpenicillin, as a function of time, from which an apparent first order rate constant, k_{obs}, for inactivation could be obtained. The experiments were conducted with excess phosphonamidate relative to enzyme concentration and there was no evidence of a zero order decline in enzyme activity, which would be indicative of saturation of the enzyme by the phosphonamidate **(8).** The pseudo first-order rate constants for inactivation show a first order dependence on the concentration of the phosphonamidate **(8)** (Fig. 2). The slopes of these graphs give the second-order rate constants, k_i, for inactivation. Although there is no discernible difference in the chemical reactivity of the two diastereoisomers towards alkaline hydrolysis, for the enzyme reaction they do show different rates of inactivation, the two values of k_i being 7.72 dm^3 mol^{-1} s^{-1} and 8.3×10^{-2} dm^3 mol^{-1} s^{-1} at pH 7.0



SCHEME 6.

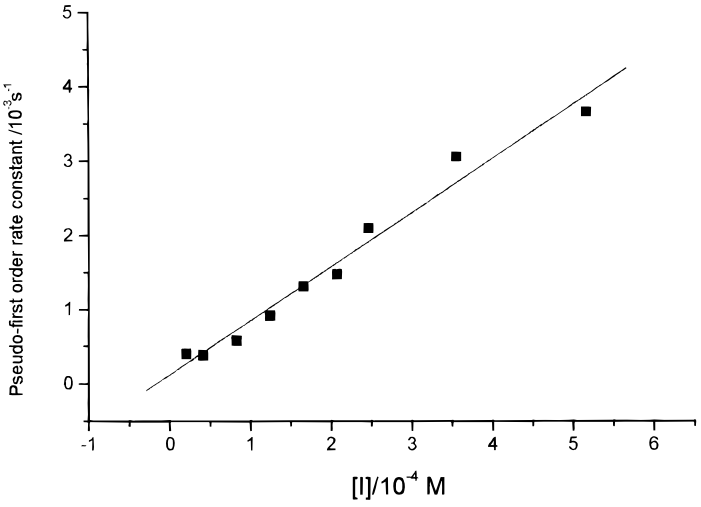


FIG. 2. A plot of the observed first order rate constant for the inactivation of the class C *E.cloacae* β-lactamase by the phosphonamidate (8) at pH 7 as a function of the concentration of the inactivator.

and 30°C. The 93-fold difference in reactivity is therefore good evidence of selectivity in the reaction of the enzyme with the two phosphonamidates **(8)** and indicative of specific interactions between the inactivators and the protein. Further evidence of selectivity is seen from the fact that the phosphonamidate **(8)** is not a significant inhibitor of either the class A β-lactamase or the class B zinc dependent β-lactamase from *B.cereus.*

The time-dependent inactivation is indicative of covalent bond formation between the enzyme and the phosphonamidate. For the most active diastereoisomer this was confirmed by electrospray mass spectrometry (ESMS). The positive ion ESMS mass transformed spectrum of the native P99 β-lactamase gave a molecular mass M_r of 39,202 ± 5.3. After incubation of the enzyme with the more reactive phosphonamidate **(8)** the molecular mass M_r increased to 39,442 ± 3.1, a mass shift of + 240, which suggests that the enzyme had been phosphonylated by displacing proline from the phosphonamidate. The calculated mass for a phosphonylated enzyme formed by displacing proline would be 39,459; that for an enzyme-phosphonamidate complex would be 39,573 and for that formed by displacing ethanol it would be 39,527. The discrepancy of 17 in the mass could be experimental error or indicative of the accompanying loss of, say, ammonia from glutamine. It seems clear that inactivation of the enzyme occurs by formation of a 1:1 covalently bound enzyme-inactivator complex in which a proline residue has been displaced by a nucleophilic group on the enzyme—presumably the active site serine. There was no evidence from gas chromatography that any ethanol was displaced as a result of the reaction between β-lactamase and the more reactive phosphonamidate.

Conversely, the less reactive diastereoisomer reacts with β-lactamase by displacing ethanol, which could be detected by gas chromatography. The observation that the enzyme reacts with one diastereoisomer by displacing proline and with another by

displacing ethanol is again indicative of a stereoselective reaction occurring at the active site.

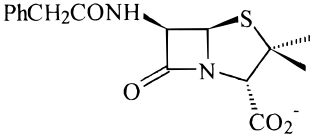
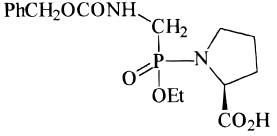
(iii) Enzyme promoted phosphonylation. It is often assumed that the phosphonylation of serine enzymes can only occur with organophosphorus compounds with good leaving groups because these do not require proton transfer from general acid catalysts to aid departure of the leaving group (*23*). However, the class C β-lactamase is obviously capable of displacing the proline residue from the phosphonamidate **(8)** (Scheme 3) despite the poor leaving group and the presumed need for protonation of the amine nitrogen.

The phosphonylation of the enzyme displays signs that the reaction takes place at the active site—other β-lactamases are not inactivated by the phosphonamidate and differential P-N and P-O bond fission occurs with the two diastereoisomers. Phosphonamidates are relatively chemically stable and the fast reaction with the β-lactamase indicates that the catalytic machinery of the enzyme used for hydrolysis is also used for phosphonylation. This is confirmed by the enzyme rate enhancement factors (EREF). The P99 β-lactamase catalyses the hydrolysis of benzylpenicillin with an EREF of 2.6×10^7 (Table 1). The hydroxide ion catalysed hydrolysis of the phosphonamidate **(8)** occurs with P-O fission and displacement of ethanol as does the reaction of the less reactive diastereoisomer with the P99 β-lactamase. The EREF value for enzyme catalysed phosphonylation by P-O fission is obtained from k_i/k_{OH} to give 1.2×10^3. However, phosphonylation of β-lactamase by the most reactive diastereoisomer of the phosphonamidate **(8)** occurs by P-N fission and displacement of proline. Because the base hydrolysis of the phosphonamidate **(8)** occurs with P-O fission, and as no proline could be detected in the alkaline hydrolysis of the phosphonamidate **(8),** the rate constant for hydrolysis by P-N fission must be at least 30 times smaller than the observed value, i.e., $k_{OH} < 2 \times 10^{-6}$ dm^3 mol^{-1} s^{-1}. The calculated EREF value for phosphonylation of β-lactamase is therefore $> 3 \times 10^6$. Clearly, the enzyme is facilitating P-N fission almost as effectively as it does C-N fission in β-lactams. Despite the differences in geometrical requirements for substitution at acyl and phosphyl centers and the enormous differences in intrinsic chemical reactivities between the β-lactam in penicillin and the phosphonamidate, the β-lactamase enzyme is able to significantly lower the activation energy for reactions of both compounds.

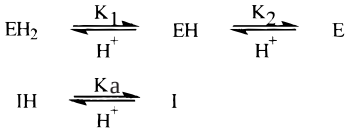
Further evidence that the phosphonylation process is "catalyzed" by the enzyme comes from the pH dependence of the rate of inactivation. The dependence of the rate constant, k_{cat}/K_m, for the P99 class C β-lactamase catalysed hydrolysis of benzylpenicillin on pH (Fig. 3) indicates that there are two ionisations which control hydrolytic catalytic activity corresponding to groups of pK_a *ca* 6.1 and 10.1, representing the two protonic forms of the enzyme, EH_2 and EH, respectively (*17*). The catalytically important form of the enzyme for hydrolysis is thus EH. The pH-dependence of the second order rate constant, k_i, for the inactivation of β-lactamase by the phosphonamidate**(8)** (Fig. 3) shows that it also depends on a catalytic group of pK_a 6.2, which suggests that the catalytic machinery used for the hydrolysis of β-lactams is also used for the phosphonylation reaction. However, the pH-rate profile for inactivation indicates an additional proton is required, i.e., whereas hydrolysis apparently requires the group of pK_a 6 to be in its deprotonated, basic form, phosphonylation apparently requires this group to be in its protonated, acidic form, although the difference may

TABLE 1

Enzyme Rate Enhancement Factors (EREF) for the P99 Class C β-Lactamse Catalyzed Hydrolysis of Benzylpenicillin (11) and the Enzyme Promoted Phosphonylation of the Phosphonamidate (8)

	k_{OH}/dm^3 mol^{-1} s^{-1}	k_{cat}/K_m/dm^3 mol^{-1} s^{-1}	EREF
 11	0.15 ± .01	3.97 ± .04 × 10^6	2.6 × 10^7
 8	6.79 ± .08 × 10^{-5} (P-O) fission) <2 × 10^{-6} (P-N fission)	k_i/dm^3 mol^{-1} s^{-1} 7.72 ± .15 (P-N fission)	>3 × 10^6 (P-N fission)

Note. k_{OH} is the second order rate constant for the hydroxide ion catalysed hydrolysis, k_{cat}/k_m and k_i the second-order rate constants for enzyme catalyzed hydrolysis and phosphonylation, respectively, at pH 7.0 and 30°C.



SCHEME 7.

be the result of kinetic ambiguity. The rate of hydrolysis of the substrate *S* is proportional to (EH) (*S*), where (EH) is the protonic form of the enzyme with the groups of pK_a 6 and 10 in their basic and acidic forms, respectively (Scheme 7). The observed pH-rate dependence (Fig. 3) for inactivation by the inhibitor, *I*, indicates a rate proportional to (EH_2) (*I*), which, for a process of phosphonylation of the active site serine (Scheme 3) is difficult to interpret mechanistically. Of course, the rate law can have various kinetic equivalents (Eq. [3]), where k_i is the observed second order rate constant for inactivation, K_a and K_1 are the acid dissociation constants for the phosphonamidate and the enzyme EH_2, respectively.

$$\text{Rate} = k_i(EH_2)(I) = \frac{k_i}{K_1}(EH)(I)(H^+) = \frac{k_i K_a}{K_1}(EH)(IH) \qquad [3]$$

Phosphonylation of the enzyme could occur through the protonic form EH with the group of pK_a 6 acting as a general base for serine attack, as is thought to occur in the hydrolytic reaction (Scheme 8). Breakdown of trigonal bipyramidal intermediate,

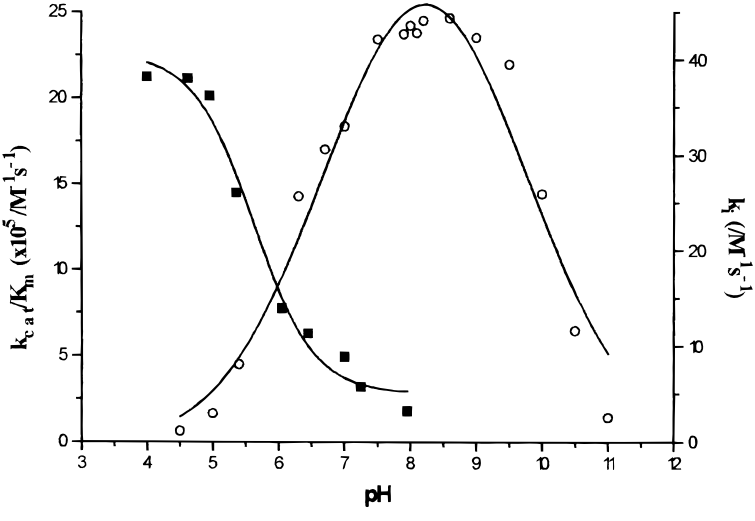


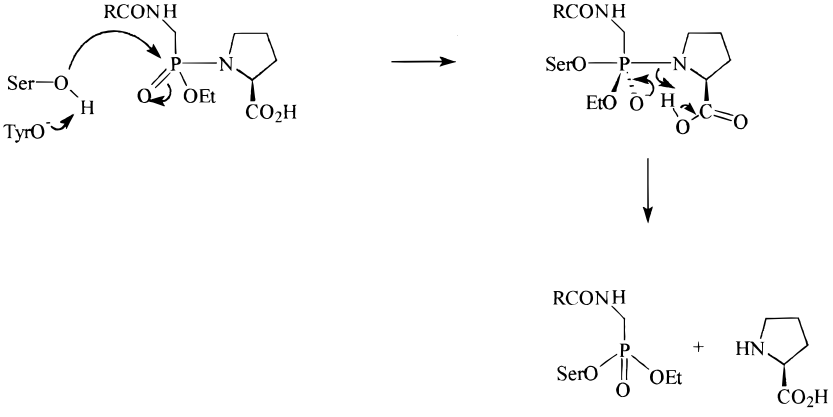
FIG. 3. A plot of k_{cat}/K_m (left hand axis and open circles O) for the class C *E.cloacae* β-lactamase catalyzed hydrolysis of benzylpenicillin against pH and k_i (right-hand axis and closed squares ■) against pH for the inactivation of the enzyme by the phosphonamidate (8).

however, could occur by proton donation to the proline nitrogen from the solvent hydronium ion and not from the protein. Only the L-proline phosphonamidate **(8)** is an effective inhibitor and, surprisingly, the D-proline isomer is a much weaker inactivator by a factor of at least 20-fold. This may reflect the different geometrical binding requirements of the normal substrate and the phosphonamidate or the possibility of an inactivator intramolecular catalyzed reaction discussed later. The corresponding carboxylic acid methyl ester is completely ineffective. which could reflect the normal requirement of a carboxylate anion for molecular recognition. It could, however, indicate that the reaction occurs through EH and IH (Eq. [3]), where the latter is the conjugate acid of the phosphonamidate **(8)**, *i.e.*, with the carboxylic acid group undissociated. The pK_a of this carboxylic acid is 3.86, so, over the pH range studied for inactivation, the major species present is the carboxylate anion of the phosphonamidate. The pH dependence for inactivation (Fig. 3) would then be given by Eq. [4]. The true second-order rate constant for phosphonylation

$$k_{obs}^{inact} = k_i \cdot \frac{K_1}{K_a} \cdot \frac{H^+}{K_1 + H^+} \qquad [4]$$

would then be given by $k_i \cdot K_1/K_a$, which is calculated to be 6.43×10^3 dm^3 mol^{-1} s^{-1}, which in turn would give an EREF of $> 3 \times 10^9$. The class C β-lactamase would then indeed be very effective at enhancing the rate of phosphonylation. If the mechanism does involve the undissociated carboxylic acid of the phosphonamidate as "substrate" then this is probably the result of substrate-assisted catalysis with the carboxylic acid acting as an intramolecular general acid catalyst facilitating P-N fission from the trigonal bipyramidal intermediate (Scheme 8).

The fact that the enzyme can lower the activation energy for the phosphonylation reaction as well as the acyl transfer reaction of the hydrolysis of the "normal" β-lactam substrates indicates a reasonable degree of flexibilty in the active site. In



SCHEME 8.

aqueous solution, the generation of a formal negative charge on oxygen in the tetrahedral intermediate is accompanied by a large change in the basicity of the oxygen—a change in pK_a of the corresponding conjugate acids of at least 12 pK_a units (Scheme 1). The alkoxide anion must be strongly solvated by H-bonding and many enzymes, which catalyse acyl transfer reactions, have an "oxyanion hole," which stabilizes the presumed tetrahedral intermediate by H-bonding from adjacent peptide links. The *difference* in the interaction between the protein and carbonyl oxygen in the initial state and the oxygen in the tetrahedral intermediate, as a result of the change in oxygen basicity, makes a major contribution to the lowering of the activation energy compared with the nonenzyme catalyzed reaction (*24*). Such an "oxyanion hole" exists for the serine β-lactamases (*25*) and, in common with serine proteases, one of the H-bond donors is the peptide N-H of serine residue Ser64 in the class C enzymes. This 'intramolecular' H-bond with the active site serine peptide NH presents a fairly well defined "cyclic" geometry and may be expected to hinder conformational changes which would be unfavorable for reaction. In the equivalent pentavalent phosphonyl enzyme intermediate (Scheme 8) the negative charge on the phosphonyl oxygen presumably takes up an equatorial position so that it is *ca.* 90° to the newly formed serine O-P bond compared with the approximately tetrahedral angel formed with the β-lactam substrate (Scheme 1). Yet despite all these differences, the enzyme is capable of catalysing the phosphonylation with extreme efficiency, indicative of a flexible active site, which remains capable of stabilizing all the necessary transition states in the reaction.

ACKNOWLEDGMENTS

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