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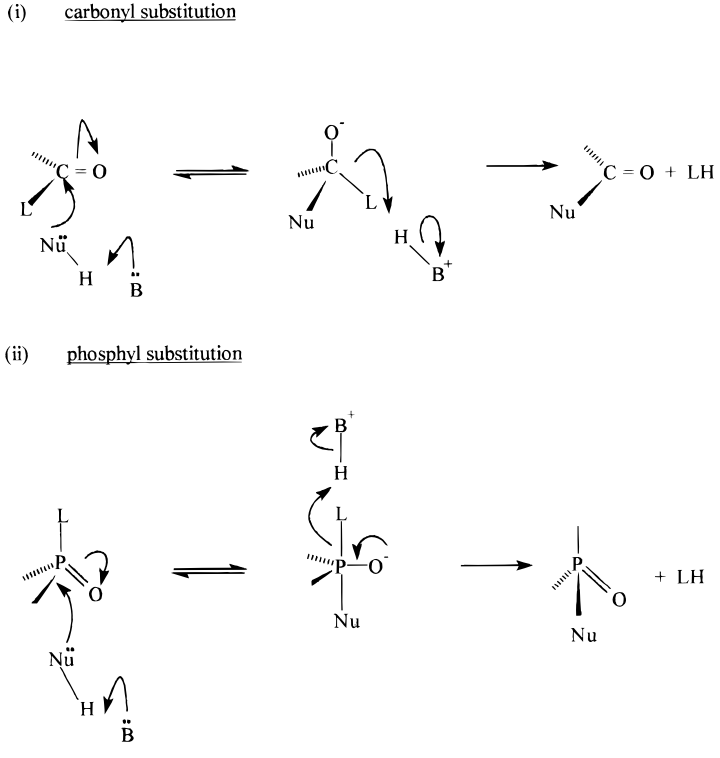
pentacoordinate intermediate with trigonal bipyramidal geometry (*1,3*). Here an initially four coordinate and tetrahedral phosphorus centre is converted to a five coordinate one and, in general, it is assumed that the preferential pathway involves the nucleophile taking up the apical position and the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

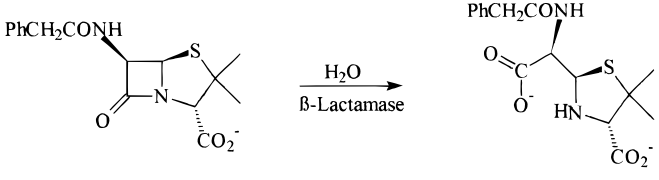
RESULTS AND DISCUSSION

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*).

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

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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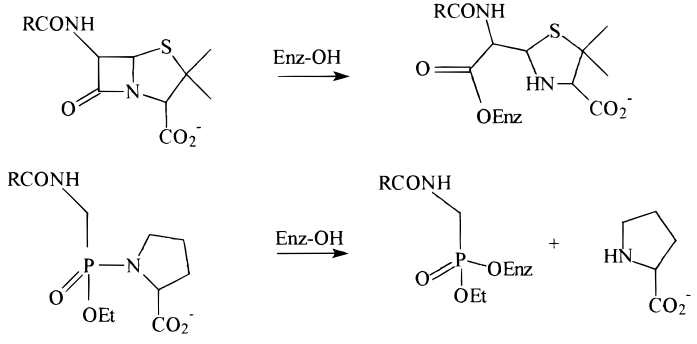
SCHEME 2.

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.