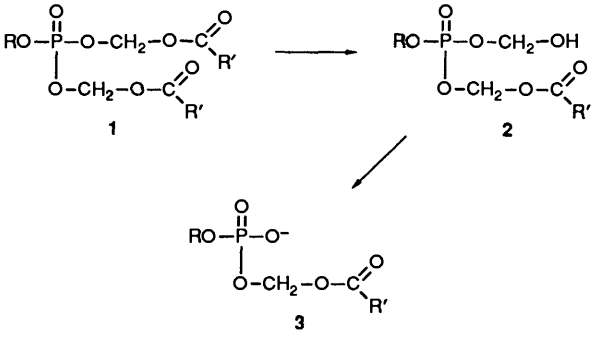
Bioreversible Protection for the Phospho Group: Bioactivation of the Di(4-acyloxybenzyl) and Mono(4-acyloxybenzyl) Phosphoesters of Methylphosphonate and Phosphonoacetate[1]

Anthony G. Mitchell, William Thomson, Dave Nicholls, William J. Irwin and Sally Freeman
Pharmaceutical Sciences Institute, Department of Pharmaceutical Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

The di(4-acetoxybenzyl) ester of methylphosphonate **4** (X = H, R = Me) and the di(4-acyloxybenzyl) esters of methoxycarbonylmethylphosphonate **4** (X = MeO_2C, R = Me, Et, Pr, Pr^i, Bu or Bu^t) were prepared from the appropriate benzyl alcohol and phosphonic dichloride. At pD 8.0 and 37 °C, both series of compounds hydrolyse with half-lives greater than 24 h to the corresponding mono(4-acyloxybenzyl) esters **5** (X = H or MeO_2C, R = Me, Et, Pr, Pr^i Bu or Bu^t) which were prepared by treatment of the di(4-acyloxybenzyl) esters **4** with sodium or lithium iodide. The mono(4-acyloxybenzyl) esters **5** (X = H, R = Me) and **5** (X = MeO_2C, R = Me, Et, Pr, Pr^i or Bu^t) undergo chemical hydrolysis to methylphosphonate **6** (X = H), and methoxycarbonylmethylphosphonate **6** (X = MeO_2C) respectively, together with 4-hydroxybenzyl alcohol and the appropriate acylate anion. The rates of hydrolysis of the mono(4-acyloxybenzyl) esters decrease as the length and steric bulk of the acyl group increases, with half-lives ranging from ~150 h for the acetyl analogues to 2240 h for the pivaloyl derivative. The hydrolyses of the di- and mono-(4-acyloxybenzyl) esters were catalysed by porcine liver carboxyesterase (PLCE), and in all cases the acylate anion was formed. The rate of enzymatic hydrolysis was most rapid for the 4-butanoyloxybenzyl and 4-isobutanoyloxybenzyl analogues. The methoxycarbonyl ester of the phosphonoacetate analogues was not cleaved by PLCE. The methylphosphonate generated from the reaction of **4** (X = H, R = Me) in the presence of esterase and $H_2{}^{18}O$, did not contain ^{18}O attached directly to phosphorus. These results suggest that both the chemical and enzymatic hydrolyses of the mono(4-acyloxybenzyl) esters and the PLCE-catalysed hydrolyses of the di(4-acyloxybenzyl) esters proceed *via* hydrolysis of the acyl group to give the acylate anion and the unstable 4-hydroxybenzyl esters. The electron-donating 4-hydroxy group facilitates the cleavage of the benzyl–oxygen bond with the formation of the 4-hydroxybenzyl carbonium ion **9**, which readily reacts either with water or the phosphate buffer. The 4-acyloxybenzyl phosphoesters provide the first example of a protecting group which will enable the bioactivation of phosphonate prodrugs at rates appropriate to biological systems.

Drugs that are charged at physiological pH often have limited cellular penetration which necessitates large intravenous doses to achieve a therapeutic effect.[2] The antiviral agent phosphonoacetic acid is partially triionic at physiological pH with p*K*a values of 2.30 (P–OH), 5.40 (CO_2H) and 8.60 (P–OH),[3] and when administered orally (20 mg kg^{-1}) to both rabbit and monkey, only 2 and 8% of the dose was absorbed, respectively.[4] The delivery of therapeutic agents is often improved by the design of prodrugs, which undergo a chemical or enzymatic transformation, within the target organ, to release the therapeutic agent.[2] One approach to prodrug design involves the conversion of an active hydrophilic drug into an inactive lipophilic molecule, thus facilitating passive diffusion through cell membranes and other physiological barriers, for example the blood–brain barrier.[5] For drugs containing the phospho group ($RPO_3{}^{2-}$), neutral lipophilic phosphoesters [RP(O)(OR′)$_2$] are prodrug candidates. With simple R′ alkyl analogues, the first R′ group may be removed readily by chemical hydrolysis under physiological conditions, however the second is usually very resistant to cleavage because the phosphorus of the anionic intermediate [RP(O)(OR′)O$^-$] is unreactive towards nucleophilic attack.[6] There is usually at least a million-fold decrease in the rate of removal of the second alkyl group, when compared with the first. To facilitate release of the phospho group, one approach would be to design R′ groups that do not require nucleophilic attack at phosphorus and subsequent P–O bond cleavage, for their removal.



Farquhar and co-workers[7,8] have examined a series of lipophilic di(acyloxymethyl) esters of benzyl and phenyl phosphate **1**, (R = Bn, Ph) as tripartite prodrug systems for the delivery of phosphates. These undergo bioactivation with esterase to give first the hydroxymethyl compounds **2** (R = Bn, Ph) which readily eliminate formaldehyde to give the diesters **3** (R = Bn, Ph). The rate of enzymatic hydrolysis can be controlled with different acyl groups, the more sterically hindered derivatives (R′ = Bu^t) undergoing only slow hydrolysis. Although the diesters **3** do degrade further to give benzyl and phenyl phosphate, this second bioactivation step is considerably slower than the first, presumably because the charged diester has a lower affinity for the esterase than the

triester. The acyloxymethyl esters of phosphonoformate have also recently been prepared.[9]

An approach to improve the monoanionic phospho intermediate as a substrate for esterases could be to distance the site of esterase attack from the anionic phospho group, by increasing the length of the linker. Instead of the acyloxymethyl group, we chose to explore the acyloxybenzyl group, which has been used for the delivery of amines as their carbamates.[10] In this study we describe the metabolic activation of the model compound di(4-acetoxybenzyl) methylphosphonate **4**, (X = H, R = Me). Here, the charge on the monoanionic phosphonate intermediate **5** (X = H, R = Me) is nine bonds removed from the site of esterase attack, an increase of some 4 Å, the length of an aromatic ring plus one single C–C bond, over the acyloxymethyl analogues. Previously we have shown that dibenzyl methoxycarbonylphosphonate, a triester of phosphonoformate was highly reactive towards chemical hydrolysis resulting in both P–O and C–P cleavage to give the diester and phosphite respectively.[11] These results were in agreement with those of Krol *et al.*[12] This instability presumably arises from the electron-withdrawing properties of the methoxycarbonyl group, suggesting that triesters of phosphonoformate might never be suitable prodrug forms. In light of these data, the di(4-acyloxybenzyl) esters of the antiviral drug phosphonoacetate **4** (X = MeO_2C), in which the phosphorus atom and the carboxy function are separated by a methylene group, were evaluated.

Results and Discussion

The di(4-acetoxybenzyl) diester **4** (X = H, R = Me) and di(4-acyloxybenzyl) triesters **4** (X = MeO_2C, R = Me, Et, Pr, Pr^i, Bu or Bu^t) were prepared by reaction of the appropriate benzyl alcohol[13] with methylphosphonic dichloride or methoxycarbonylmethylphosphonic dichloride[14] using a method similar to that previously described for the preparation of dibenzyl methoxycarbonylphosphonate.[11] The compounds were purified by flash column chromatography[15] and were fully characterised by elemental analysis and/or high resolution FAB mass spectrometry, infrared spectroscopy, and ^{1}H, ^{31}P and ^{13}C NMR spectroscopy. The ^{1}H NMR spectra in $CDCl_3$ showed that the benzylic protons were non-equivalent with the presence of 2 sets of doublets of doublets (J_{gem} ~12 Hz, J_{PH} ~9 Hz) at ~5 ppm. Interestingly, when the spectra were recorded using D_2O as solvent, the benzylic protons were equivalent giving rise to a doublet (J_{PH} ~9 Hz).

The salts of 4-acetoxybenzyl methylphosphonate **5** (X = H, R = Me) and 4-acyloxybenzyl methoxycarbonylmethylphosphonates **5** (X = MeO_2C, R = Me, Et, Pr, Pr^i, Bu or Bu^t) were prepared in yields ranging from 35–81% by the action of sodium or lithium iodide on the appropriate di(4-acyloxybenzyl) ester **4** (X = H or MeO_2C) adapting a published procedure.[16] The diesters were characterised by high resolution FAB mass spectrometry, infrared spectroscopy, and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The ^{1}H NMR (D_2O) spectra all possessed a doublet at ~5 ppm (J_{PH} ~8 Hz) for the equivalent benzylic protons.

To evaluate the stability towards chemical hydrolysis, a solution of the diester **4** (X = H, R = Me) (5 mmol dm^{-3}) in potassium phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v) was monitored by ^{31}P and ^{1}H NMR spectroscopy at 36.4 °C. The hydrolysis to potassium 4-acetoxybenzyl methylphosphonate **5** (X = H, R = Me) was slow with a half-life of 55.4 h (k = 1.25 ± 0.01 × 10^{-2} h^{-1}). The monoester decomposed further to dipotassium methylphosphonate **6** (X = H) with a half-life of 153.2 h (k = 4.52 ± 0.02 × 10^{-2} h^{-1}). The small (3.6-fold) decrease in the rate constant for the hydrolysis of the monoester when compared with the diester suggests that these compounds do not react by nucleophilic attack at phosphorus. The 4-acetoxy group should be susceptible to metabolic conversion by esterases to the electron-donating hydroxy group. In contrast to this moderate chemical stability, the addition of PLCE (50 units) to a solution of the diester **4** (X = H, R = Me) (5 mmol dm^{-3}) in phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v, 1 cm^3) at 36.4 °C resulted in the rapid decomposition of **4** to give the monoester **5** in less than 3 min, which after 2 h gave only methylphosphonate **6** (X = H). A similar reaction monitored by ^{1}H NMR spectroscopy showed that, in contrast to the chemical hydrolysis, in the presence of PLCE (100 units), the monoester **5** (X = H, R = Me) was completely metabolised within 15.5 min to the 4-hydroxybenzyl intermediate **8** (X = H), which then decomposed to methylphosphonate **6** (X = H) with a half-life of 17 min (k = 4.14 ± 0.45 × 10^{-2} min^{-1}).

The formation of potassium acetate and 4-hydroxybenzyl alcohol, and the absence of 4-acetoxybenzyl alcohol in the chemical and PLCE-catalysed reactions of **4** and **5** (X = H, R = Me) suggests that their degradation must first proceed with hydrolysis of the acetyl group to give the 4-hydroxybenzyl intermediates **7** and **8**, respectively. It is then proposed that the electron-donating 4-hydroxy group promotes cleavage of the benzyl–oxygen bond to give either monoester **5** or methylphosphonate **6** (X = H) together with the resonance-stabilised 4-hydroxybenzyl carbonium ion **9**. This proposed mechanism of hydrolysis is supported by the following evidence: (i) when the hydrolysis of diester **4** (X = H, R = Me) with PLCE (50 units) was repeated in the presence of 80% ^{18}O-enriched water, only singlets were observed by ^{31}P NMR spectroscopy for the intermediate **8** (X = H) and methylphosphonate **6** (X = H). This result confirms that (i) the ^{18}O label is not attached to phosphorus in either compound, (ii) dibenzyl methylphosphonate is completely stable under similar conditions of hydrolysis over 48 h,[11] however, at 100 °C, the reaction

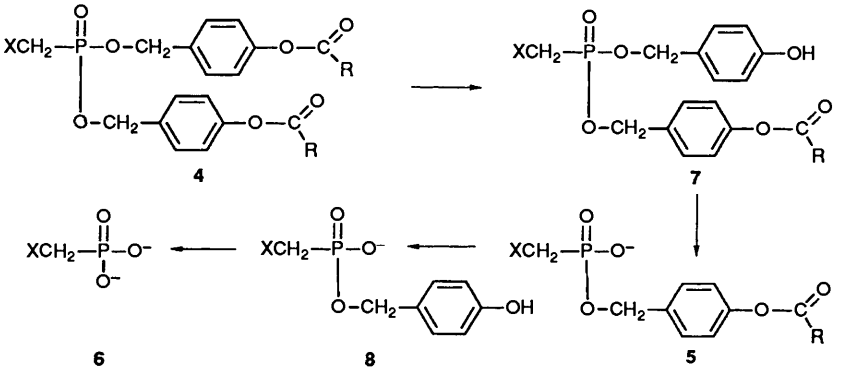
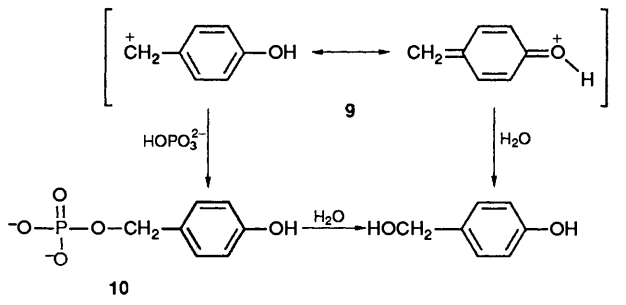


Table 1 Rate constants and half-lives ($t_{\frac{1}{2}}$) determined by ^{1}H NMR spectroscopy for the chemical hydrolysis of the phosphonoacetate diesters **5** (X = MeO_2C) (5 mmol dm^{-3}) at 37 °C in phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v)

R	$k/10^4$ h^{-1}	$t_{\frac{1}{2}}$/h
Me	45	154
Et	31	224
Pr	19	365
Pr^i	15	462
Bu^t	3.1	2240



proceeds *via* the benzyl carbonium ion with C–O cleavage,[17] (iii) after 3.3 h, 4-methoxybenzyl diethyl phosphate undergoes 88% solvolysis in methanol, whereas under identical conditions the 3-methoxy or unsubstituted benzyl analogues are completely stable,[18] and (iv) the attempted synthesis of di-(4-methoxybenzyl) methoxycarbonylmethylphosphonate from 4-methoxybenzyl alcohol and methoxycarbonylmethylphosphonic dichloride gave only di(4-methoxybenzyl) ether. A similar observation was made during the attempted synthesis of the corresponding triester of phosphonoformate.[19] This suggests that *P*-benzyl esters substituted with *para* electron-donating substituents are unstable and decompose to give a benzyl carbonium ion.

Although the products derived from the phospho group of the diester **4** (X = H, R = Me) are known, the fate of the benzyl group is more complex with only ~30% of the product derived from the proposed 4-hydroxybenzylcarbonium ion **9** being present as 4-hydroxybenzyl alcohol at early time points. It is possible that the carbonium ion may be trapped by a nucleophile other than water, and possibilities include the enzyme, products or buffer. The catalytic efficiency of the esterase was not impaired during the hydrolysis, suggesting that this intermediate does not react with enzyme. In a related reaction[20] the benzyl carbonium ion generated from the solvolysis of diphenyl benzyl phosphate in phenol is trapped by electrophilic aromatic substitution to give 2- and 4-benzylphenol. An analogous reaction of the 4-hydroxybenzyl carbonium ion with 4-hydroxybenzyl alcohol would give a trisubstituted ring, however, the ^{1}H NMR spectroscopic evidence only supports 1,4-disubstituted products. To investigate the involvement of the buffer, the reaction of **4** (X = H, R = Me) with PLCE (5 units) was performed using 0.01 mol dm^{-3} phosphate buffer. At all time points, >90% of the carbonium ion was trapped as 4-hydroxybenzyl alcohol which suggests that with the original 0.1 mol dm^{-3} buffer, inorganic phosphate can compete with water to trap the carbonium ion. Peaks in the NMR spectra of the reaction mixture with 0.1 mol dm^{-3} buffer at δ_P 3.72 and δ_H 4.64 (2 H, d, J_{PH} 5.4), 6.81 (2 H, d, J_{HH} 8.4) and 7.26 (2 H, d, J_{HH} 8.4) are for 4-hydroxybenzyl phosphate **10**, which has an approximate half-life of 1 h. The monoanion of benzyl phosphate is reported to hydrolyse with P–O cleavage, with a half-life of 86 h at 75.6 °C and pH 7.[21,22] The higher reactivity of 4-hydroxybenzyl phosphate suggests a change in mechanism, with the electron-donating hydroxy group promoting C–O cleavage.

The ready removal of both 4-acetoxybenzyl groups from **4** (X = H, R = Me) with PLCE confirms that diesters of this type are bioreversible derivatives of the phospho group. This approach was then applied to the antiviral agent phosphonoacetate. The substrate specificity of esterases is dependent on the length and steric properties of the groups on either side of the ester link.[23] The potential to control the rate of esterase-catalysed hydrolysis with the nature of the acyl group was explored with the phosphonoacetate analogues **4** and **5** (X = MeO_2C, R = Me, Et, Pr, Bu, Pr^i and Bu^t).

The chemical hydrolyses of the 4-acyloxybenzyl analogues of phosphonoacetate were examined first. A solution of the triesters **4** (X = MeO_2C) (1 mmol dm^{-3}) in potassium phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1, v/v) at 37 °C were monitored by ^{1}H NMR spectroscopy. In all cases the half-lives of the triesters were greater than 24 h. Interestingly the rates were not very sensitive to the nature of the acyl group and further experiments are required to completely elucidate their hydrolytic profile. The hydrolyses of the diesters **5** (X = MeO_2C) (5 mmol dm^{-3}) in phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v) at 37 °C followed typical first order reaction kinetics. By ^{1}H NMR spectroscopy, the diesters were shown to degrade to methoxycarbonylmethylphosphonate **6** (X = MeO_2C), 4-hydroxybenzyl alcohol and the acylate anion. The rate constants and half-lives are given in Table 1. The half-lives for the acetyl analogues of **5** (X = H and MeO_2C) are both approximately 150 h, suggesting reaction by similar mechanisms. The rate of hydrolysis decreases as the length and β-alkylation of the acyl group increases, the reactivity profile being comparable to that reported for the hydrolyses of ethyl acylates.[24] For all diesters **5** (X = MeO_2C), 4-hydroxybenzyl alcohol was formed, whereas the 4-acyloxybenzyl alcohols were not. This is consistent with hydrolysis at the acyl group to give the 4-hydroxybenzyl intermediate **8** (X = MeO_2C).

Attention was then turned to the enzymatic bioactivation of the triesters **4** and diesters **5** (R = MeO_2C). Porcine liver carboxyesterase is known to be a mixture of seven enzymes,[25] therefore a detailed study to determine K_m and V_{max} for the substrates seemed inappropriate. However, the rate of enzyme-catalysed reactions frequently follows Michaelis–Menten kinetics $\{\tau = [S_o]\cdot V_{max}/(K_m + [S_o])\}$. The integrated form of this model is shown in eqn. 1 where S_o is the initial substrate concentration, S_t is its value at time t, K_m is the Michaelis constant and V_{max} is the maximum reaction rate.

$$\mathrm{Ln}(S_t) + \frac{S_t}{K_m} = \mathrm{Ln}(S_o) + \frac{S_o}{K_m} - \frac{V_{max}}{K_m}t \qquad (1)$$

The time for 50% reaction ($t_{\frac{1}{2}}$) at various substrate concentrations (S_o') throughout the degradation profile may be calculated.[26,27] Each half-life is related to K_m and V_{max} by eqn. 2 which indicates that a plot of $t_{\frac{1}{2}}$ against S_o' should be linear.

$$t_{\frac{1}{2}} = \frac{0.6931K_m}{V_{max}} + \frac{0.5S_o'}{V_{max}} \qquad (2)$$

In the PLCE experiments, this analysis showed a generally constant value for $t_{\frac{1}{2}}$ throughout the reaction. This arises when $K_m \gg S_o'$ which approximates the model to a first order degradation when the degradation rate constant is given by $k = V_{max}/K_m$. Esterase hydrolyses have thus been analysed according to the first order kinetic model but $t_{\frac{1}{2}}$ values are quoted, rather than rate constants, to reflect this approximation.

Table 2 Half-lives ($t_{\frac{1}{2}}$) for the porcine liver carboxyesterase-catalysed hydrolyses of the phosphonoacetate triesters **4** (X = MeO_2C) (1 cm^3 of a 1 mmol dm^{-3} solution with 0.5 units of esterase for R = Me and 0.05 units for all other triesters) monitored by HPLC, and diesters **5** (X = MeO_2C) (1 cm^3 of a 5 mmol dm^{-3} solution with 10 units of esterase) monitored by ^1H NMR spectroscopy at 37 °C

	$t_{\frac{1}{2}}$/min	
R	**4**	**5**
Me	330[a]	34
Et	12	8.7
Pr	4.5	2.7
Bu	62	8.5
Pr^i	7	3.1
Bu^t	56.5	16

[a] Extrapolated to account for a 10-fold increase in esterase concentration compared to conditions for other triesters.

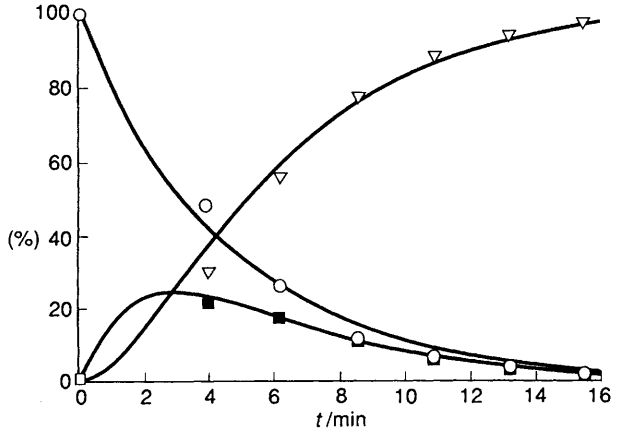


Fig 1 Reaction of lithium 4-isobutanoyloxybenzyl methoxycarbonylmethylphosphonate **5** (X=MeO_2C, R=Pr^i) (5 mmol dm^{-3}; 1 cm^3) in potassium phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1, v/v) with porcine liver carboxyesterase (10 units) at 37 °C. Monitored by ^1H NMR (250 MHz) spectroscopy using integrals of the doublets for the P-CH_2 groups to give the % of each component; ○ represents the diester **5** (X=MeO_2C, R=Pr^i), ■ the 4-hydroxybenzyl diester **8** (X=MeO_2C) and ▽ methoxycarbonylmethylphosphonate **6** (X=MeO_2C). The lines are calculated from the theoretical model

The phosphonoacetate triesters **4** (X = MeO_2C) (1 mmol dm^{-3}) were incubated with PLCE [0.5 units (R = Me), 0.05 units (all other triesters)] in potassium phosphate buffer (0.1 mol dm^{-3}; pH 7.4)–MeCN (9:1 v/v, 1 cm^3). The reactions were monitored by isocratic ion-pair reversed-phase HPLC, using acetonitrile–10 mmol dm^{-3} tetrabutylammonium hydroxide in water. The half-lives, reflecting K_m/V_{max}, are given in Table 2. The reaction of di(4-acetoxybenzyl) methoxycarbonylmethylphosphonate **4** (X = MeO_2C, R = Me) (5 mmol dm^{-3}) with PLCE (5 units) was also monitored by ^1H and ^{31}P NMR spectroscopy. The triester was initially observed to degrade to the 4-acetoxybenzyl diester **5** (X = MeO_2C, R = Me) [δ_H including 2.26 (s, CH_3CO), 3.58 (s, OCH_3), 4.86 (d, J_{PH} 7.3 Hz, CH_2OP), 7.08 (d, J_{HH} 8.4 Hz, Ar) and 7.40 (d, J_{HH} 8.4 Hz, Ar), and δ_P 15.34 (s)], 4-hydroxybenzyl alcohol [δ_H 4.45 (s, CH_2), 6.81 (d, J_{HH} 8.6 Hz, Ar) and 7.19 (d, J_{HH} 8.4 Hz, Ar)] and potassium acetate [δ_H 1.80 (s)]. In contrast to the chemical hydrolysis, in the presence of PLCE (5 units), the pivaloyl triester **4** (X = MeO_2C, R = Bu^t) (5 mmol dm^{-3}) degraded concomitantly to the diester **5** (X = MeO_2C, R = Bu^t) [δ_H including 1.27 (s) $C(CH_3)_3$] and potassium pivaloate [δ_H 1.00 (s)]. These results suggest that the PLCE-catalysed hydrolyses proceed *via* the 4-hydroxybenzyl intermediates **7** (X = MeO_2C), which then spontaneously degrade to the diesters **5** (X = MeO_2C).

The phosphonoacetate diesters **5** (X = MeO_2C) (5 mmol cm^{-3}) were incubated with PLCE (10 units) in potassium phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v, 1 cm^3). By ^1H and ^{31}P NMR spectroscopy the products were identified as dipotassium methoxycarbonylmethylphosphonate **6** (X = MeO_2C), data for which included δ_H 2.59 (d, J_{PH} 19.6 Hz, PCH_2) and 3.58 (s, OCH_3) and δ_P 11.70 (s), (t, J_{PH} 18.5 Hz, ^1H coupled)], and 4-hydroxybenzyl alcohol. In contrast to the chemical hydrolysis reactions, up to 20% of the 4-hydroxybenzyl intermediate **8** (R = MeO_2C) was detected, data of which included δ_H 2.75 (d, J_{PH} 20.5 Hz, PCH_2), 3.58 (s, OCH_3), 4.75 (d, J_{PH} 7.1 Hz, CH_2OP), 6.78 (d, J_{HH} ~8 Hz, Ar) and 7.23 (d, J_{HH} ~8 Hz, Ar) and δ_P 15.07 (s), which confirms the formation of the monoester **6** (X = MeO_2C) *via* the proposed 4-hydroxy-promoted benzyl–oxygen bond cleavage. Neither methanol [δ_H 3.25] nor phosphonoacetic acid [δ_H 2.52 (d, J_{PH} 20.1 Hz, PCH_2) and δ_P 17.41 (s)] were detected, showing that the monoester **6** (X = MeO_2C) is not a substrate for the esterase, presumably because of its dianionic nature.

The reaction profile for the PLCE-catalysed hydrolysis of **5** (X = MeO_2C, R = Pr^i) is shown in Fig. 1. This degradation follows a **5**→**8**→**6** consecutive pathway with the concentration of the individual components ($\mathbf{5}_t$, $\mathbf{8}_t$, $\mathbf{6}_t$) at time t being modelled by:

$$\mathbf{5}_t = \mathbf{5}_o \cdot \exp(-k_1 t)$$

$$\mathbf{8}_t = \frac{\mathbf{5}_o \cdot k_1}{k_2 - k_1} \cdot [\exp(-k_1 t) - \exp(-k_2 t)]$$

$$\mathbf{6}_t = \mathbf{5}_o \cdot \left[1 - \frac{k_2 \cdot \exp(-k_1 t) - k_1 \cdot \exp(-k_2 t)}{k_2 - k_1}\right]$$

Parameter estimation from these equations was undertaken by simultaneous non-linear regression of the time–concentration data collected during the degradation of **5**. The 4-hydroxybenzyl intermediate **8** gave **6** with a half-life of 1.4 min; some two-fold more reactive than the isopropyl diester **5** which had a half-life of 3.1 min. The intermediate **8** (X = MeO_2C) was also significantly less stable than the corresponding methylphosphonate analogue **8** (X = H) ($t_{\frac{1}{2}}$ 17 min), presumably by virtue of the electron-withdrawing methoxycarbonyl substituent favouring loss of the charged phosphonate.

The half-lives for the hydrolyses of the 4-acyloxybenzyl diesters **5** (X = MeO_2C) catalysed by PLCE are given in Table 2. For the straight chain acyl compounds, the acetyl analogues (R = Me) of the triesters **4** (X = MeO_2C) and diesters **5** (X = MeO_2C) are considerably less reactive than the longer chain derivatives (R = Et, Pr). This result is in agreement with those reported for the horse liver carboxyesterase-catalysed hydrolyses of ethyl acylates,[23] and the PLCE-catalysed hydrolyses of phenyl acylates.[28] The active site of PLCE appears to optimally accommodate a four carbon acyl group, with the greatest rate of hydrolysis being for the butanoyl- and isobutanoyl-oxybenzyl triesters **4** and diesters **5** (X = MeO_2C, R = Pr, Pr^i) (Table 2). Branching of the acyl chain of the ester increases the affinity ($1/K_m$) but decreases the reactivity (V_{max}) with horse liver esterase.[23] Here, similar half-lives are observed for the 4-pivaloyloxybenzyl esters, **4** and **5** (R = Bu^t) and the corresponding straight chain analogues (R = Bu), presumably resulting from a balance of these factors. The esterase-catalysed reactions of the diesters **5** (X = MeO_2C) utilised a 40-fold increase in the amount of PLCE when compared with the triesters **4** (X = MeO_2C). The diesters are much poorer substrates than the triesters, which is likely to be attributable to the anionic nature of the diester. In support, Levy and Ocken

found that ethyl potassium succinate was neither a substrate nor inhibitor of PLCE, whereas diethyl succinate is a good substrate.[29,30] They attributed this to the proposal that charged compounds are unable to form a Michaelis-complex with the enzyme, and suggested that this could be due to charge repulsion because the catalytic site can only be reached through a cluster of negatively charged groups on the enzyme.

The potential application of this bioreversible protecting group for the delivery of phosphates and phosphonates to the brain prompted us to investigate the plasma stability and bioactivation by brain S9 fraction of some of the compounds. The di(4-acyloxybenzyl) triesters **4** (X = MeO_2C, R = Me and Pr) were incubated with human plasma under physiological conditions (pH 7.4, 37 °C) and the reactions were monitored by HPLC. The triesters degraded to the diesters **5** (X = MeO_2C, R = Me and Pr) with approximate half-lives of 6.5 and 8.5 min respectively. The mono(4-acyloxybenzyl) diesters **5** (X = MeO_2C, R = Me, Pr and Bu^t) were incubated with human plasma and the reactions monitored by ^{31}P NMR spectroscopy. The acyloxybenzyl diesters **5** degrade in plasma to give methoxycarbonylmethylphosphonate **6** (X = MeO_2C). The pivaloyl analogue (R = Bu^t) shows high stability with a half-life of 154 h, whereas the acetyl (R = Me) and butanoyl (R = Pr) analogues are considerably more reactive, with half-lives of 9.2 and 8.65 h respectively. Similar to the trends observed for chemical hydrolysis (Table 1), the rates of decomposition of the triesters **4** in plasma are faster than for the diesters **5**. The rate of hydrolyses are similar for the 4-acetyl and 4-butanoyl derivatives, in contrast to the results with PLCE (Table 2) in which the 4-acetoxybenzyl diester **5** (R = Me) was much more stable than the 4-butanoyloxybenzyl derivative **5** (R = Pr). This suggests that human plasma carboxyesterases have different substrate specificities when compared to those in PLCE. Again, the methoxycarbonyl group was unaffected by human plasma, confirmed by the stability of a sample of disodium methoxycarbonylmethylphosphonate **6** (X = MeO_2C) in plasma.

Bioactivation of the 4-acyloxybenzyl diesters **5** (X = MeO_2C, R = Me, Pr and Bu^t) with the S9 fraction of porcine brain was monitored by ^{31}P NMR spectroscopy. The diesters were found to degrade to methoxycarbonylmethylphosphonate **6** (X = MeO_2C) with half-lives of 2.0, 5.3 and 48 h respectively. The porcine brain-mediated bioactivation of the diesters follow a similar trend to that observed with human plasma, the pivaloyl analogue being considerably more stable than the acetyl or butanoyl analogues.

The bioactivation of the triesters of phosphonoacetate to the methyl ester **6** (X = MeO_2C) has been demonstrated with PLCE, human plasma and porcine brain S9 fraction. Further bioactivation to the parent drug has yet to be achieved. Studies are underway to replace the methoxy group with a substituent that could be removed bioreversibly such as acyloxymethyl[2] or 4-acyloxybenzyl. A potential problem associated with the 4-acyloxybenzyl prodrug approach is the release of the highly reactive 4-hydroxybenzyl carbonium ion which may interact with cellular nucleophiles (*e.g.* DNA, glutathione) and cause toxicity.[10] Indeed, when tested for antiviral properties, the 4-acyloxybenzyl and 4-pivaloyloxybenzyl triesters, **4** (X = MeO_2C, R = Me and Bu^t) exhibited acute toxicity, possibly due to the generation of the carbonium ion.[31] For this reason methods to trap this intermediate internally are being investigated. This bioreversible protecting group could also have applications in synthesis, with the phospho moiety being liberated under very mild conditions avoiding the common methods of high pressure hydrogenation,[7,8] strong acid[32] or trimethylsilylbromide.[33] Studies are in progress to extend the 4-acyloxybenzyl prodrug system for the delivery of a range of phosphates, including the monophosphates of AZT and DDC.

Experimental

NMR spectra were recorded on a Bruker 250-AC spectrometer at ^1H (250.1 MHz), ^{31}P (101.3 MHz) and ^{13}C (62.9 MHz). ^1H and ^{13}C NMR spectra were referenced to tetramethylsilane, and ^{31}P NMR spectra were referenced to 85% H_3PO_4. All spectra are ^1H decoupled (composite pulse decoupling) unless otherwise stated: positive chemical shifts are downfield from the reference, *J* values are given in Hz. Mass spectra were recorded on a V.G. Micromass 12 instrument at 70 eV and a source temperature of 300 °C; accurate mass data were recorded on a V.G. 7070E instrument using positive ion FAB with a nitrobenzyl alcohol matrix. IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. M.p.s were measured on a Gallenkamp Electrothermal Digital apparatus and are not corrected. Flash column chromatography[15] was performed using Sorbsil C60 silica gel. TLC was performed using plastic-backed Kieselgel 60 silica gel plates containing a fluorescent indicator. Spots were visualised under 254 nm UV light or with the aid of iodine. Elemental analyses were performed by Butterworths Laboratories, Middlesex. The following solvents were dried by heating under reflux followed by distillation over the appropriate drying reagent: dichloromethane (P_2O_5), acetone (4 Å molecular sieve) and triethylamine (KOH). Chemicals were obtained from Aldrich Chemical Company and porcine liver carboxyesterase was obtained from Sigma Chemical Company. The units of enzyme added to the hydrolyses were based on the assay given by Sigma—one unit is defined as the esterase required to hydrolyse 1 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25 °C. The phosphate buffer (0.1 mol dm^{-3}; pH 7.4) was prepared by mixing aqueous solutions of dipotassium hydrogen phosphate (0.2 mol dm^{-3}; 40.5 cm^3) and potassium dihydrogen phosphate (0.2 mol dm^{-3}; 9.5 cm^3), and the volume was adjusted to 100 cm^3 with water. The D_2O phosphate buffer (0.1 mol dm^{-3}; pD 8.0) was prepared by dissolving a mixture of KH_2PO_4 (0.0572 g) and K_2HPO_4 (0.2752 g) in D_2O (20 cm^3), the pH was measured and the pD calculated.[34] High performance liquid chromatography (HPLC) was performed using a Waters 600E gradient solvent delivery system fitted with a Merck reversed-phase C-18 endcapped Lichrospher 100 column (particle size 5 μm; 250 × 4 mm), Lichrocart reversed-phase C-18 endcapped guard column and monitored by UV at λ_{max} 254 nm using a Waters 484 Tunable Absorbance Detector. Hydrolysis samples (20 mm^3) were injected directly *via* a Waters 700 Satellite WISP. Chromatograms were recorded on a Waters 745B integrator. Chromatograms were displayed and stored on an NEC Powermate SX Plus using Baseline control, collection and display software and printed on an NEC Pinwriter P5300.

The following compounds were prepared from methylphosphonic dichloride or methoxycarbonylmethylphosphonic dichloride[14] and the appropriate benzylalcohol[13] by a method similar to that previously described for dibenzyl methoxycarbonylphosphonate.[11]

Di(4-acetoxybenzyl) methylphosphonate **4** (X = H, R = Me). The title compound was isolated by flash column chromatography (EtOAc, R_f 0.29) as a colourless solid (66%); m.p. 43–47 °C; (Found: C, 58.8; H, 5.25. $C_{19}H_{21}O_7P$ requires C, 58.16; H, 5.40%); ν(thin film)/cm^{-1} 1760 (C=O) and 1220 (P=O); $\delta_H(CDCl_3)$ 1.46 (3 H, d, J_{PH} 17.6), 2.27 (6 H, s, 2 × CH_3), 4.91 (2 H, dd, J_{gem} 11.9, J_{PH} 8.5, OCH_AH), 5.01 (2 H, dd, J_{gem} 11.9, J_{PH} 9.1, $OCHH_B$), 7.05 (4 H, d, J_{HH} 8.5, Ar) and 7.33 (4 H, d, J_{HH} 8.5, Ar); δ_P 32.2 (s), (m, ^1H coupled); δ_C 11.60 (d, J_{PC} 144.3, PCH_3), 21.00 (s, 2 × CH_3), 66.34 (d, J_{PC} 6.0, 2 × OCH_2), 121.71 (s, 4 × aromatic CH), 129.02 (s, 4 × aromatic CH), 133.78 (d, J_{PC} 6.1, 2 × aromatic C), 150.56 (s, 2 × aromatic C) and 169.22 (s, C=O); *m/z* (FAB) 393 (M + H^+, 38%), 329 (34), 287 (100), 245 (43) and 201 (42). Observed accurate mass 393.1084 (M + H^+); $C_{19}H_{22}O_7P$ requires 393.1104.

Di(4-*acetoxybenzyl*) *methoxycarbonylmethylphosphonate* **4** (X = MeO_2C, R = Me). The title compound was isolated by flash column chromatography (EtOAc, R_f 0.33) as a colourless oil (15%); ν(thin film)/cm^{-1} 1740 (C=O) and 1200 (P=O); $\delta_H(CDCl_3)$ 2.29 (6 H, s, 2 × CH_3), 3.00 (2 H, d, J_{PH} 21.5, PCH_2), 3.69 (3 H, s, OCH_3), 5.02 (2 H, dd, J_{gem} 11.8, J_{PH} 9.6, OCH_AH), 5.09 (2 H, dd, J_{gem} 11.8, J_{PH} 9.6, $OCHH_B$), 7.08 (4 H, d, J_{HH} 9.2, Ar) and 7.38 (4 H, d, J_{HH} 9.2, Ar); δ_P 21.22 (s), (m, ^1H coupled); δ_C 14.08 (s, 2 × CH_3), 27.38 (d, J_{PC} 136.4, PCH_2), 45.61 (s, OCH_3), 60.42 (d, J_{PC} 6.1, 2 × CH_2O), 114.79 (s, 4 × aromatic CH), 122.21 (s, 4 × aromatic CH), 126.38 (d, J_{PC} 6.2, 2 × aromatic C), 143.73 (s, 2 × aromatic C), 162.27 (s, C=O) and 163.78 (s, C=O); *m/z* (FAB) 451 (M + H^+, 22%), 345 (35), 303 (25), 149 (54), 136 (40) and 107 (100). Observed accurate mass 451.1158 (M + H^+); $C_{21}H_{24}O_9P$ requires 451.1158.

Di(4-*propanoyloxybenzyl*) *methoxycarbonylmethylphosphonate* **4** (X = MeO_2C, R = Et). The title compound was isolated by flash column chromatography [EtOAc–Hexane (1:1), R_f 0.30] as a colourless oil (18%); (Found: C, 57.5; H, 5.75. $C_{23}H_{27}O_9P$ requires C, 57.75; H, 5.69%) ν(thin film)/cm^{-1} 1773 (C=O) and 1211 (P=O); $\delta_H(CDCl_3)$ 1.24 (6 H, t, J_{HH} 7.5, 2 × CH_3), 2.57 (4 H, q, J_{HH} 7.6, 2 × CH_2CO), 2.99 (2 H, d, J_{PH} 21.5, CH_2), 3.66 (3 H, s, OCH_3), 5.01 (2 H, dd, J_{gem} 11.8, J_{PH} 8.9, CH_AH_BO), 5.06 (2 H, dd, J_{gem} 11.8, J_{PH} 8.9, CH_AH_BO), 7.06 (4 H, d, J_{HH} 8.5, Ar) and 7.34 (4 H, d, J_{HH} 8.5, Ar); δ_P 21.19 (s), (m, ^1H coupled); δ_C 8.91 (s, 2 × CH_3), 27.59 (s, 2 × CH_2), 34.31 (d, J_{PC} 136.2, PCH_2), 52.51 (s, OCH_3), 67.37 (d, J_{PC} 6.2, 2 × CH_2), 121.70 (s, 4 × aromatic CH), 129.11 (s, 4 × aromatic CH), 133.19 (d, J_{PC} 6.2, 2 × aromatic C), 150.78 (s, 2 × aromatic C), 165.82 (d, J_{PC} 5.7, C=O), 172.65 (s, 2 × C=O); *m/z* (FAB) 479 (M + H^+, 26%), 317 (54), 163 (100) and 135 (54). Observed accurate mass 479.1471 (M + H^+); $C_{23}H_{28}O_9P$ requires 479.1471.

Di(4-*butanoyloxybenzyl*) *methoxycarbonylmethylphosphonate* **4** (X = MeO_2C, R = Pr). The title compound was isolated by flash column chromatography [EtOAc–Hexane (2:1), R_f 0.43] as a cream coloured solid (17%); (Found: C, 59.0; H, 5.95. $C_{25}H_{31}O_9P$ requires C, 59.30; H, 6.17); ν(Nujol)/cm^{-1} 1767 (C=O) and 1298 (P=O); $\delta_H(CDCl_3)$ 0.98 (6 H, t, J_{HH} 7.4, 2 × CH_3), 1.70 (4 H, tq, J_{HH} 7.3, 2 × CH_2), 2.47 (4 H, t, J_{HH} 7.3, 2 × CH_2), 2.94 (2 H, d, J_{PH} 21.5, PCH_2), 3.61 (3 H, s, OCH_3), 4.95 (2 H, dd, J_{gem} 11.8, J_{PH} 8.5, CH_AH_BO), 5.03 (2 H, dd, J_{gem} 11.8, J_{PH} 8.2, CH_AH_BO), 7.02 (4 H, d, J_{HH} 8.5, Ar) and 7.29 (4 H, d, J_{HH} 8.5, Ar); δ_P 21.20 (s), (m, ^1H coupled); δ_C 13.45 (s, 2 × CH_3), 18.22 (s, 2 × CH_2), 34.18 (d, J_{PC} 136.0, PCH_2), 35.95 (s, 2 × CH_2CO), 52.41 (s, OCH_3), 67.30 (d, J_{PC} 6.1, 2 × CH_2O), 121.68 (s, 4 × aromatic CH), 129.06 (s, 4 × aromatic CH), 133.19 (d, J_{PC} 6.1, 2 × aromatic C), 150.70 (s, 2 × aromatic C), 165.77 (d, J_{PC} 5.7, C=O) and 171.71 (s, 2 × C=O); *m/z* (FAB) 507 (M + H^+, 9%), 437 (2), 331 (35), 225 (96) and 177 (100). Observed accurate mass 507.1784 (M + H^+); $C_{25}H_{32}O_9P$ requires 507.1784.

Di(4-*isobutanoyloxybenzyl*) *methoxycarbonylmethylphosphonate* **4** (X = MeO_2C, R = Pr^i). The title compound was isolated by flash column chromatography [EtOAc–Hexane (2:1), R_f 0.29] as a cream coloured solid (23%); (Found: C, 59.2; H, 6.15. $C_{25}H_{31}O_9P$ requires C, 59.30; H, 6.17%); ν(Nujol)/cm^{-1} 1760 (C=O), 1729 (C=O) and 1267 (P=O); $\delta_H(CDCl_3)$ 1.30 [12 H, d, J_{HH} 6.9, 2 × $(CH_3)_2$], 2.70 (2 H, septet, J_{HH} 7.0, 2 × CH), 2.99 (2 H, d, J_{PH} 21.5, PCH_2), 3.67 (3 H, s, OCH_3), 5.01 (2 H, dd, J_{gem} 11.8, J_{PH} 8.6, CH_AH_BO), 5.08 (2 H, dd, J_{gem} 11.8, J_{PH} 8.6, CH_AH_BO), 7.04 (4 H, d, J_{HH} 8.5, Ar) and 7.34 (4 H, d, J_{HH} 8.5, Ar); δ_P 21.17 (s), (m, ^1H coupled); δ_C 18.78 [s, 2 × $(CH_3)_2$], 34.02 (s, 2 × CH), 34.32 (d, J_{PC} 136.2, PCH_2), 52.52 (s, OCH_3), 67.39 (d, J_{PC} 6.2, 2 × CH_2), 121.66 (s, 4 × aromatic CH), 129.11 (s, 4 × aromatic CH), 133.14 (d, J_{PC} 6.2, 2 × aromatic C), 150.90 (s, 2 × aromatic C), 165.83 (d, J_{PC} 5.7, C=O) and 175.31 (s, 2 × C=O); *m/z* (FAB) 507 (M + H^+, 11%), 435 (3), 329 (21), 225 (80), 177 (100) and 149 (78). Observed accurate mass 507.1784 (M + H^+); $C_{25}H_{32}O_9P$ requires 507.1784.

Di(4-*pivaloyloxybenzyl*) *methoxycarbonylmethylphosphonate* **4**, (X = MeO_2C, R = Bu^t). The title compound was isolated by flash column chromatography [EtOAc–Hexane (1:1), R_f 0.61] as a colourless solid which was recrystallised from toluene–hexane (1:1) (19%); m.p. 91–92 °C; (Found: C, 60.95: H, 6.4. $C_{27}H_{35}O_9P$ requires C, 60.65; H, 6.60%); ν(Nujol)/cm^{-1} 1754 (C=O), 1722 (C=O) and 1248 (P=O); $\delta_H(CDCl_3)$ 1.36 [18 H, s, 2 × $(CH_3)_3$], 3.00 (2 H, d, J_{PH} 21.5, PCH_2), 3.69 (3 H, s, OCH_3), 5.03 (2 H, dd, J_{gem} 11.8, J_{PH} 8.3, CH_AH_BO), 5.11 (2 H, dd, J_{gem} 11.7, J_{PH} 8.4, CH_AH_BO), 7.05 (4 H, d, J_{HH} 8.5, Ar) and 7.36 (4 H, d, J_{HH} 8.5, Ar); δ_P 21.18 (s), (m, ^1H coupled); δ_C 27.00 [s, 2 × $(CH_3)_3$], 34.36 (d, J_{PC} 136.2, PCH_2), 38.98 [s, 2 × $(CH_3)_3C$], 52.55 (s, OCH_3), 67.45 (d, J_{PC} 6.2, 2 × CH_2), 121.66 (s, 4 × aromatic CH), 129.11 (s, 4 × aromatic CH), 133.07 (d, J_{PC} 6.2, 2 × aromatic C), 151.15 (s, 2 × aromatic C), 165.85 (d, J_{PC} 5.8, C=O) and 176.84 (s, 2 × C=O); *m/z* (FAB) 535 (M + H^+, 6%), 343 (9), 207 (4), 191 (85), 155 (23), 107 (100), 85 (38) and 57 (100). Observed accurate mass 535.2098 (M + H^+); $C_{27}H_{36}O_9P$ requires 535.2097.

Di(4-*pentanoyloxybenzyl*) *methoxycarbonylmethylphosphonate* **4** (X = MeO_2C, R = Bu). The title compound was isolated by flash column chromatography (EtOAc, R_f 0.83) as a colourless oil (19%); (Found: C, 60.8; H, 6.45. $C_{27}H_{36}O_9P$ requires C, 60.65; H, 6.60%); $\delta_H(CDCl_3)$ 0.96 (6 H, t, J_{HH} 7.3, 2 × CH_3), 1.43 (4 H, tq, J_{HH} 7.6, 2 × CH_2), 1.73 (4 H, tt, J_{HH} 7.3, 2 × CH_2), 2.55 (4 H, t, J_{HH} 7.6, 2 × CH_2), 2.99 (2 H, d, J_{PC} 21.5, PCH_2), 3.67 (3 H, s, OCH_3), 4.98 (2 H, dd, J_{gem} 11.8, J_{PH} ~8, CH_AH_BO), 5.09 (2 H, dd, J_{gem} 11.8, J_{PH} ~8, CH_AH_BO), 7.06 (4 H, d, J_{HH} 8.5, Ar) and 7.34 (4 H, d, J_{HH} 8.5, Ar); δ_P 21.19 (s), (m, ^1H coupled); δ_C 13.61 (s, 2 × CH_3), 22.11 (s, 2 × CH_2), 26.84 (s, 2 × CH_2), 33.96 (s, 2 × CH_2CO), 34.22 (d, J_{PC} 136.8, PCH_2), 52.58 (s, OCH_3), 67.59 (d, J_{PC} 6.2, 2 × CH_2O), 121.75 (s, 4 × aromatic CH), 128.94 (s, 4 × aromatic CH), 133.06 (d, J_{PC} 6.2, 2 × aromatic C), 150.80 (s, 2 × aromatic C), 165.76 (d, J_{PC} 5.7, C=O) and 172.15 (s, 2 × C=O); *m/z* (FAB) 535 (M + H^+, 18%), 451 (8), 345 (54) and 239 (100); Observed accurate mass 535.2097 (M + H^+); $C_{27}H_{36}O_9P$ requires 535.2097.

Sodium 4-*acetoxybenzyl methylphosphonate* **5**, (X = H, R = Me).—Sodium iodide (0.15 g, 0.99 mmol) was added to a stirred solution of di(4-acetoxybenzyl) methylphosphonate (0.39 g, 0.99 mmol) in diethyl ether–acetone (1 cm^3; 3:2 v/v) in darkness in an atmosphere of argon. After 20 h, the colourless precipitate of the title compound was collected by filtration and dried under high vacuum (0.043 g, 0.16 mmol, 16%); $\delta_H(D_2O)$ 1.29 (3 H, d, J_{PH} 16.4), 2.33 (3 H, s), 4.90 (2 H, d, J_{PH} 7.3), 7.14 (2 H, d, J_{HH} 8.5) and 7.49 (2 H, d, J_{HH} 8.5); $\delta_P(D_2O)$ 27.9 (s), (qt, J_{PH} 16.4, 7.3, ^1H coupled).

Lithium 4-*acetoxybenzyl methoxycarbonylmethylphosphonate* **5**, (X = MeO_2C, R = Me).—Lithium iodide (0.054 g, 0.41 mmol) was added to a solution of di(4-acetoxybenzyl) methoxycarbonylmethylphosphonate (0.18 g, 0.41 mmol) in diethyl ether–acetone (6:4) (2.0 cm^3) and the reaction mixture stirred for 72 h at room temperature. The resulting solid was purified by dissolution in acetone and precipitation by the addition of diethyl ether to give the title compound as a colourless solid (0.10 g, 0.33 mmol, 81%); m.p. 204–206 °C; (Found: C, 45.3; H, 4.25. $C_{12}H_{14}LiO_7P$ requires C, 46.77; H, 4.58. $C_{12}H_{14}LiO_7P \cdot 0.5H_2O$ requires C, 45.44; H, 4.77%); ν(Nujol)/cm^{-1} 1754 (C=O) and 1211 (P=O); $\delta_H(D_2O)$ 2.25 (3 H, s, CH_3), 2.79 (2 H, d, J_{PH} 20.4, PCH_2), 3.58 (3 H, s, OCH_3), 4.86 (2 H, d, J_{PH} 7.3, CH_2), 7.08 (2 H, d, J_{HH} 8.5, Ar) and 7.40 (2 H, d, J_{HH} 8.5, Ar); δ_P 15.27 (s), (tt, J_{PH} 20.3, 7.3, ^1H coupled); δ_C 23.41 (s,

CH_3), 38.12 (d, J_{PC} 120.8, PCH_2), 55.58 (s, OCH_3), 69.14 (d, J_{PC} 5.3, OCH_2), 124.74 (s, 2 × aromatic CH), 132.04 (s, 2 × aromatic CH), 138.65 (d, J_{PC} 6.7, aromatic C), 152.81 (s, aromatic C), 173.87 (d, J_{PC} 6.3, C=O) and 176.50 (s, C=O); *m/z* (FAB) 315 (M + Li^+, 100%), 259 (10), 167 (50) and 136 (16). Observed accurate mass 309.0715 (M + H^+); $C_{12}H_{15}LiO_7P$ requires 309.0715.

The following compounds were prepared as colourless solids by the reaction of lithium iodide with the appropriate phosphonate triester using a method similar to that described above:

Lithium 4-propanoyloxybenzyl methoxycarbonylmethylphosphonate **5** (X = MeO_2C, R = Et). (73%); m.p. 187–188 °C; ν(Nujol)/cm^{-1} 1760 (C=O), 1704 (C=O) and 1205 (P=O); $\delta_H(D_2O)$ 1.19 (3 H, t, J_{HH} 7.5, CH_3), 2.66 (2 H, q, J_{HH} 7.5, CH_2), 2.88 (2 H, d, J_{PH} 20.4, PCH_2), 3.66 (3 H, s, OCH_3), 4.95 (2 H, d, J_{PH} 7.5, CH_2), 7.15 (2 H, d, J_{HH} 8.4, Ar) and 7.48 (2 H, d, J_{HH} 8.4, Ar); δ_P 15.58 (s), (tt, J_{PH} 20.4, 7.4, ^1H coupled); δ_C 11.17 (s, CH_3), 30.34 (s, CH_2), 38.12 (d, J_{PC} 120.8, PCH_2), 55.58 (s, OCH_3), 69.15 (d, J_{PC} 5.6, CH_2), 124.74 (s, 2 × aromatic CH), 132.04 (s, 2 × aromatic CH), 138.52 (s, aromatic C), 152.87 (s, aromatic C) and 179.89 (s, C=O), other carbonyl not detected; *m/z* (FAB) 329 (M + Li^+, 100%), 323 (M + H^+, 6), 259 (7), 167 (38) and 136 (11). Observed accurate mass 323.0872 (M + H^+); $C_{13}H_{17}LiO_7P$ requires 323.0872.

Lithium 4-butanoyloxybenzyl methoxycarbonylmethylphosphonate **5**, (X = MeO_2C, R = Pr). (68%); m.p. 189–190 °C; (Found: C, 48.75; H, 5.35. $C_{14}H_{18}LiO_7P$ requires C, 50.01; H, 5.40%. $C_{14}H_{18}LiO_7P{\cdot}0.5H_2O$ requires C, 48.71; H, 5.55%); ν(Nujol)/cm^{-1} 1760 (C=O), 1697 (C=O) and 1211 (P=O); $\delta_H(D_2O)$ 1.00 (3 H, t, J_{HH} 7.4, CH_3), 1.74 (2 H, tq, J_{HH} 7.3, CH_2), 2.63 (2 H, t, J_{HH} 7.3, CH_2), 2.89 (2 H, d, J_{PH} 20.4, PCH_2), 3.66 (3 H, s, OCH_3), 4.95 (2 H, d, J_{PH} 7.5, CH_2), 7.15 (2 H, d, J_{HH} 8.4, Ar) and 7.49 (2 H, d, J_{HH} 8.4, Ar); δ_P 15.80 (s), (tt, J_{PH} 20.4, 7.5, ^1H coupled); δ_C 15.76 (s, CH_3), 20.90 (s, CH_2), 38.11 (d, J_{PC} 120.6, PCH_2), 38.66 (s, CH_2), 55.57 (s, OCH_3), 69.13 (d, J_{PC} 5.2, CH_2), 124.74 (s, 2 × aromatic CH), 132.04 (s, 2 × aromatic CH), 138.61 (d, J_{PC} 6.3, aromatic C), 152.80 (s, aromatic C) and 179.08 (s, C=O), other carbonyl not detected; *m/z* (FAB) 343 (M + Li^+, 100%), 337 (M + H^+, 2), 309 (9), 259 (8), 167 (44) and 136 (7). Observed accurate mass 337.1028 (M + H^+); $C_{14}H_{19}LiO_7P$ requires 337.1028.

Lithium 4-isobutanoyloxybenzyl methoxycarbonylmethylphosphonate **5** (X = MeO_2C, R = Pr^i). (35%); m.p. 202–204 °C; $\delta_H(D_2O)$ 1.28 [6 H, d, J_{HH} 7.0, $(CH_3)_2$], 2.88 (1 H, sept, J_{HH} 7.0, CH), 2.88 (2 H, d, J_{PH} 20.4, PCH_2), 3.66 (3 H, s, OCH_3), 4.95 (2 H, d, J_{PH} 7.5, CH_2O), 7.14 (2 H, d, J_{HH} 8.5, Ar) and 7.48 (2 H, d, J_{HH} 8.5, Ar); δ_P 15.57 (s), (tt, J_{PH} 20.5, 7.5, ^1H coupled); δ_C 21.05 [s, $(CH_3)_2$], 36.89 (s, CH), 38.12 (d, J_{PC} 121.0, PCH_2), 55.57 (s, OCH_3), 69.14 (d, J_{PC} 5.3, CH_2O), 124.66 (s, 2 × aromatic CH), 132.05 (s, 2 × aromatic CH), 138.54 (s, aromatic C), 152.92 (s, aromatic C), 165.05 (s, C=O) and 182.61 (s, C=O); *m/z* (FAB) 343 (M + Li^+, 100%), 337 (M + H^+, 2), 259 (15) and 167 (74). Observed accurate mass 337.1028 (M + H^+); $C_{14}H_{19}LiO_7P$ requires 337.1028.

Lithium 4-pivaloyloxybenzyl methoxycarbonylmethylphosphonate **5** (X = MeO_2C, R = Bu^t). (85%); m.p. 221–222 °C; (Found: C, 50.7; H, 5.55. $C_{15}H_{20}LiO_7P$ requires C, 51.44; H, 5.76%. $C_{15}H_{20}LiO_7P{\cdot}0.25H_2O$ requires C, 50.79; H, 5.83%); ν(Nujol)/cm^{-1} 1747 (C=O), 1704 (C=O) and 1186 (P=O); $\delta_H(D_2O)$ 1.27 [9 H, s, $(CH_3)_3$], 2.81 (2 H, d, J_{PH} 20.4, PCH_2), 3.58 (3 H, s, OCH_3), 4.87 (2 H, d, J_{PH} 7.5, CH_2O), 7.06 (2 H, d, J_{HH} 8.3, Ar) and 7.41 (2 H, d, J_{HH} 8.4, Ar); δ_P 15.56 (s), (tt, J_{PH} 20.3, 7.2, ^1H coupled); δ_C 29.48 [s, $(CH_3)_3$], 38.13 (d, J_{PC} 120.8, PCH_2), 41.80 [s, $(CH_3)_3C$], 55.59 (s, OCH_3), 69.15 (d, J_{PC} 5.3, CH_2), 124.64 (s, 2 × aromatic CH), 132.09 (s, 2 × aromatic CH), 138.55 (d, J_{PC} 6.9, aromatic C), 153.20 (s, aromatic C), 173.86 (d, J_{PC} 6.3, C=O) and 184.08 (s, C=O); *m/z* (FAB) 357 (M + Li^+, 100%), 337 (5), 309 (14), 209 (8) and 149 (28). Observed accurate mass 357.1267 (M + Li^+); $C_{15}H_{20}Li_2O_7P$ requires 357.1267.

Lithium 4-pentanoyloxybenzyl methoxycarbonylmethylphosphonate **5** (X = MeO_2C, R = Bu) (46%); m.p. 177–179 °C; $\delta_H(D_2O)$ 0.92 (3 H, t, J_{HH} 7.2, CH_3), 1.39 (2 H, sextet, J_{HH} 7.4, CH_2), 1.70 (2 H, pent, J_{HH} 7.2, CH_2), 2.65 (2 H, t, J_{HH} 7.4, CH_2CO), 2.88 (2 H, d, J_{PH} 20.4, PCH_2), 3.66 (3 H, s, OCH_3), 4.95 (2 H, d, J_{PH} 7.5, CH_2O), 7.14 (2 H, d, J_{HH} 8.4, Ar) and 7.48 (2 H, d, J_{HH} 8.3, Ar); δ_P 15.57 (s), (tt, J_{PH} 20.5, 7.6 Hz, ^1H coupled); δ_C 15.94 (s, CH_3), 24.55 (s, CH_2), 29.38 (s, CH_2), 36.54 (s, CH_2), 38.12 (d, J_{PC} 121.1, PCH_2), 55.58 (s, OCH_3), 69.15 (d, J_{PC} 5.4, CH_2), 124.75 (s, 2 × aromatic CH), 132.06 (s, 2 × aromatic CH), 138.62 (d, J_{PC} 6.7 aromatic C), 152.83 (s, aromatic C), 173.92 (s, C=O) and 179.37 (s, C=O); *m/z* (FAB) 357 (M + Li^+, 100%), 309 (44), 259 (43), 167 (100) and 136 (29). Observed accurate mass 357.1332 (M + Li^+); $C_{15}H_{20}Li_2O_7P$ requires 357.1267.

Disodium Methoxycarbonylmethylphosphonate **6** (X = MeO_2C).—A solution of $NaHCO_3$ (3.02 g, 0.036 mol) in water (75 cm^3) was added dropwise to a solution of bis(trimethylsilyl) methoxycarbonylmethylphosphonate (5.45 g, 0.018 mol) in acetone (75 cm^3) and left to stir at room temperature for 48 h. The mixture was concentrated to give a cream-coloured solid, which was dissolved in water and precipitated by the addition of diethyl ether to give the title compound as a colourless solid (2.8 g, 0.014 mol, 79%); $\delta_H(CD_3CN{-}D_2O$, 1:1) 2.52 (2 H, d, J_{PH} 19.3, PCH_2) and 3.53 (3 H, s, OCH_3); δ_P 11.35 (s), (t, J_{PH} 19.3, ^1H coupled); δ_C 40.78 (d, J_{PC} 108.6, PCH_2), 55.15 (s, OCH_3) and 176.77 (d, J_{PC} 6.1, C=O).

Chemical Hydrolyses of Methylphosphonate Diester **4** (X = MeO_2C, R = Me) *and Monoester* **5**, (X = MeO_2C, R = Me).—A solution of **4** or **5** (5 mmol dm^{-3}) in potassium phosphate buffer (0.1 mol dm^{-3}; D_2O, pD 8.0)–CD_3CN (9:1 v/v, total volume 1 cm^3) was monitored by ^{31}P and ^1H NMR spectroscopy at 36.4 °C. The diester **4** gave $\delta_P(D_2O{-}CD_3CN$, 9:1) 36.5 and δ_H 1.55 (3 H, d, J_{PH} 17.5), 2.24 (6 H, s), 4.92 (4 H, d, J_{PH} 9.2), 7.05 (4 H, d, J_{HH} 8.5) and 7.31 (4 H, d, J_{HH} 8.5). The monoester **5** gave $\delta_P(D_2O{-}CD_3CN$, 9:1) 27.5 and δ_H 1.19 (3 H, d, J_{PH} 16.4), 2.25 (3 H, s), 4.81 (2 H, d, J_{PH} 7.2), 7.08 (2 H, d, J_{HH} 8.6) and 7.41 (2 H, d, J_{HH} 8.6). Dipotassium methylphosphonate **6** (X = H) gave δ_P 24.4 (s), (q, J_{PH} 16.4, ^1H coupled) and δ_H 1.15 (3 H, d, J_{PH} 16.4). Potassium acetate gave δ_H 1.80 (s) and 4-hydroxybenzyl alcohol gave δ_H 4.45 (2 H, s), 6.81 (2 H, d, J_{HH} 8.5) and 7.20 (2 H, d, J_{HH} 8.5). Potassium 4-hydroxybenzyl methylphosphonate **8** (X = H) gave δ_P 27.4 and δ_H 1.17 (3 H, d, J_{PH} 16.3), 4.71 (2 H, d, J_{PH} 7.1), 6.81 (2 H, d, J_{HH} 8.7) and 7.24 (2 H, d, J_{HH} 8.7). Dipotassium 4-hydroxybenzyl phosphate **10** gave δ_P 3.72 and δ_H 4.64 (2 H, d, J_{PH} 5.4), 6.81 (2 H, d, J_{HH} 8.4) and 7.26 (2 H, d, J_{HH} 8.4). 4-Acetoxybenzyl alcohol was not formed [authentic sample gives δ_H 2.25 (3 H, s), 4.55 (2 H, s), 7.06 (2 H, d, J_{HH} 8.5) and 7.36 (2 H, d, J_{HH} 8.5)].

Enzyme-catalysed Hydrolyses of Methylphosphonate Diester **4** (X = H, R = Me) *in* $H_2{}^{18}O$.—A solution of the diester (3.35 μmol) in CD_3CN (0.067 cm^3) was added to a solution of potassium dihydrogen phosphate (1.7 mg), dipotassium hydrogen phosphate·$3H_2O$ (10.8 mg) in 97%-enriched $H_2{}^{18}O$ (0.48 cm^3) and $H_2{}^{16}O$ (0.118 cm^3). Porcine liver carboxyesterase (50 units) was added and the reaction monitored by ^{31}P NMR spectroscopy. After 14 min, a sharp peak was observed at 27.5 ppm (peak width at half height 0.8 Hz) for the 4-hydroxybenzyl monoester **8** (X = H) without ^{18}O-label incorporation. After 12 h, 2 drops of 10 mol dm^{-3} NaOH were added and the ^{31}P spectrum gave a sharp peak at 21.4 ppm

(peak width at half height 0.8 Hz) for methylphosphonate **6** (X = H), without ^{18}O-label incorporation.

Chemical Hydrolyses of Phosphonoacetate Triesters **4** (X = MeO_2C).—A solution of the triester (1.0 mmol dm^{-3}) in potassium phosphate buffer (0.1 mol dm^{-3}, D_2O, pD 8.0)–CD_3CN (9:1 v/v, 1 cm^3) was incubated at 37 °C and monitored by ^1H NMR spectroscopy at appropriate time intervals. The P–CH_2 group could not be observed by ^1H NMR spectroscopy because the protons readily exchange with deuterium. The hydrolyses were monitored by the disappearance of the CH_2OP and aromatic peaks for the triesters.

Chemical Hydrolysis of Phosphonoacetate Diesters **5** (X = MeO_2C).—A solution of the diester (5 mmol dm^{-3}) in potassium phosphate buffer (0.1 mol dm^{-3}, D_2O, pD 8.0)–CD_3CN (9:1 v/v, 1.0 cm^3) was incubated at 37 °C and monitored by ^1H NMR spectroscopy at appropriate time intervals. The extent of hydrolysis with time was calculated using the integrals for the acyl group of the diester, data on which included δ_H 2.25 (s, Me) (R = Me); 1.12 (t, J_{HH} 7.5, CH_3) (R = Et); 0.91 (t, J_{HH} 7.4, CH_3) (R = Pr); 1.20 (d, J_{HH} 6.9, CMe_2), (R = Pr^i); 1.26 (s, CMe_3), (R = Bu^t), and the peaks for the acylate anion, data on which included δ_H 1.81 (s, Me) (R = Me); 0.945 (t, J_{HH} 7.6, CH_3), (R = Et); 0.78 (t, J_{HH} 7.4, CH_3), (R = Pr); 0.955 (d, J_{HH} 7.0, CMe_2), (R = Pr^i); 1.00 (s, CMe_3), (R = Bu^t). All of the diesters gave dipotassium methoxycarbonylmethylphosphonate **6** (X = MeO_2C), data on which included δ_H 2.59 (2 H, d, J_{PH} 19.6, PCH_2) and 3.58 (3 H, s, OCH_3); δ_P 11.70 (s), (t, J_{PH} 18.5, ^1H coupled).

Hydrolysis of Phosphonoacetate Triesters **4** (X = MeO_2C) *with Esterase.*—A solution of the triester in acetonitrile (0.10 cm^3, 1 μmol, pre-incubated at 37 °C) was added to potassium phosphate buffer (0.1 mol dm^{-3}; H_2O, pH 7.4, 0.89 cm^3, pre-incubated at 37 °C) and a sample removed for HPLC analysis (t = 0). Enzyme hydrolysis was initiated by addition of the esterase solution [0.01 cm^3, 0.5 units **4**, (R = CH_3), 0.05 units (all other triesters)] to the reaction vial at 37 °C. At the appropriate time, samples were removed from the reaction vessel for HPLC analysis. For triesters **4** (X = MeO_2C, R = Et, Pr and Pr^i) hydrolysis with enzyme was rapid and a new experiment was performed for each time point. Hydrolyses were performed in duplicate and control experiments were carried out in the absence of esterase. The samples were analysed by HPLC eluting isocratically with mixtures of 10 mmol dm^{-3} tetrabutylammonium hydroxide (TBA) in MeCN–10 mmol dm^{-3} TBA in water. The composition of the mobile phase varied according to the nature of the acyl group: 70:30 v/v MeCN–H_2O for R = Me, Et and Pr with retention times of 3.6, 5.3 and 8.8 min respectively, 80:20 v/v for R = Pr^i (R_t 5.5 min) and 85:15 v/v for R = Bu and Bu^t (R_t 6.7 and 6.5 min).

Hydrolysis of Phosphonoacetate Diesters **5** (X = MeO_2C) *with Esterase.*—A solution of the diester in potassium phosphate buffer (0.1 cm^3, 5 μmol) was added to a mixture of phosphate buffer (0.1 mol dm^{-3}; pD 8.0, D_2O, 0.78 cm^3) and CD_3CN (0.10 cm^3) incubated at 37 °C. A ^1H NMR spectrum was recorded and the enzyme hydrolysis was initiated by the addition of esterase solution (0.02 cm^3, 10 units). The reaction was monitored by ^1H or ^{31}P NMR spectroscopy every 5–10 min for several hours. For ^1H NMR, the rates of hydrolysis were determined from both the integrals of the acyl group on the diester and the acylate anion, data for which is given with the chemical hydrolyses, and from the intensities of the P–CH_2 peaks for the diesters **5** [δ_H 2.79 (d, J_{PH} 20.5)], 4-hydroxybenzyl diester **8** (X = MeO_2C) [δ_H 2.75 (d, J_{PH} 20.1)] and monoester **6** (X = MeO_2C) [δ_H 2.59 (d, J_{PH} 19.6)] at each time point. For ^{31}P NMR the rates of hydrolysis were calculated from the integrals of **5** [δ_P 15.3], **6** [δ_P 15.1] and **8** [δ_P 11.7] at each time point. To evaluate the ^{31}P and ^1H NMR response of each component type, known weights (approx. 5 μmol) of diester **5** (X = MeO_2C, R = Me) and monoester **6** (X = MeO_2C) were combined. Both ^1H (peak heights and integrals) and ^{31}P (integrals) NMR spectra confirmed the ratios from the weights.

Incubations of Phosphonoacetate Triesters **5** (X = MeO_2C, R = Me *or* Pr) *with Human Plasma.*—Human blood (30 cm^3) was centrifuged at 750 g for 10 min to give plasma as the supernatant fraction. Plasma (0.5 cm^3), pre-incubated at 37 °C was added to a solution of potassium phosphate buffer (0.1 mol dm^{-3}; pH 7.4, 0.4 cm^3) and triester (**5**, 1 μmol) in MeCN (0.1 cm^3), pre-incubated at 37 °C. Samples (200 mm^3) were removed at different time points and immediately added to centrifuge tubes containing ice-cold MeCN (200 mm^3), whereupon plasma proteins were precipitated and the reaction quenched. The samples were centrifuged at 1000 *g* for 10 min and the supernatant analysed by HPLC. Duplicate experiments were performed with a new batch of plasma.

Incubation of Phosphonoacetate Diesters **5** (X = MeO_2C, R = Me, Pr *or* Bu^t) *with Human Plasma.*—Plasma (0.5 cm^3), pre-incubated at 37 °C was added to a solution of the diester (5 μmol) in potassium phosphate buffer (0.5 cm^3, 0.1 mol dm^{-3}; pD 8.0, D_2O) pre-incubated at 37 °C. The reaction was monitored at regular time intervals by ^{31}P NMR spectroscopy.

Incubation or Phosphonoacetate Diesters **5** (X = MeO_2C, R = Me, Pr *or* Bu^t) *with* S9 *Fraction of Porcine Brain.*—A section of porcine brain (5 g) was washed in phosphate buffer (0.1 mol dm^{-3}; pH 7.4). The brain was cut into small pieces and homogenised in ice-cold buffer (20 cm^3). The homogenate was spun at 10 000 *g* for 20 min at 4 °C. The supernatant (S9 fraction) was removed and stored on ice. A solution of NADP (36 mg), glucose-6-phosphate (169.3 mg), $MgCl_2 \cdot 6H_2O$ (101.6 mg) and glucose-6-phosphate dehydrogenase (80 U) was freshly prepared in phosphate buffer (0.1 mol dm^{-3}; pH 7.4, 10 cm^3) and stored on ice. This solution (0.2 cm^3) and the S9 fraction (0.50 cm^3) were added to a solution of the diester (5 μmol) in phosphate buffer (0.1 mol dm^{-3}; pD 8.0, 0.5 cm^3) pre-incubated at 37 °C. The reaction was monitored by ^{31}P NMR spectroscopy over several hours at 36.4 °C.

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