Prodrugs of Phosphonoformate: Products, Kinetics and Mechanisms of Hydrolysis of Dibenzyl (Methoxycarbonyl)phosphonate†

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Dibenzyl (methoxycarbonyl)phosphonate (**1**) has been prepared by the reaction of benzyl alcohol with (methoxycarbonyl)phosphonic dichloride. The hydrolysis of **1** proceeded rapidly, with a half-life of 60 min at 36.4 °C and pH 7.4, by two main pathways. The dominant pathway (k_1, 6.56×10^{-3} min^{-1}) yielded the diester, benzyl (methoxycarbonyl)phosphonate (**2**) and benzyl alcohol (**3**), with P–O cleavage. The second (k_2, 3.55×10^{-3} min^{-1}) gave dibenzyl phosphite (**4**), possibly arising from hydrolysis of the carboxyl ester followed by decarboxylation. Benzyl phosphite (**5**) was also observed, which arises from the hydrolysis of **4** with P–O cleavage (k_5, 9.04×10^{-4} min^{-1}). Other products formed in small amounts were, benzyl (benzyloxycarbonyl)phosphonate (**6**) (k_3, 3.59×10^{-4} min^{-1}) and dibenzyl phosphate (**7**) (k_4, 4.24×10^{-4} min^{-1}). The rapid and complicated hydrolysis of **1** involving four competitive reactions, two of which involve C–P bond cleavage, suggests that triesters of phosphonoformate are unlikely to be suitable prodrug forms.

Phosphonoformate (FOSCARNET) is a useful antiviral agent, showing activity against most retro-viruses.[1] Phosphonoformate cannot readily diffuse through cell membranes,[1,2] presumably because it is triionic at physiological pH (pK_a 0.49, 3.41 and 7.27), and in the clinic high dose infusions of phosphonoformate are required to achieve therapeutic effects.[3] Autoradiographic studies have shown that phosphonoformate cannot traverse the blood–cerebral spinal fluid barrier[1] and thus cannot be effective against viral infections within the brain.[4] In an attempt to improve the delivery of phosphonoformate, a range of *P,P*-di(alkyl) triesters have been prepared as lipophilic prodrugs,[5] and more recently the *P,P*-di(acyloxymethyl) esters have been reported.[6] None of these compounds have shown antiviral activity greater than that of phosphonoformate,[5,6] and 'hydrolytic behaviour' was given as the reason for the unsuitability of the acyloxymethyl derivatives,[6] although details were not presented.

Benzyl esters of carbamates are useful prodrugs for compounds containing the amino group,[7] and it was hoped that these ideas could be extended to the delivery of phosphonoformate, with the design of metabolically unstable benzyl esters of the phosphonate group. Here the synthesis of dibenzyl (methoxycarbonyl)phosphonate (**1**) is presented, and its fate upon hydrolysis is elucidated.

Results and Discussion

Dibenzyl (methoxycarbonyl)phosphonate (**1**) was readily prepared in 64% yield from the reaction of two equivalents of benzyl alcohol with (methoxycarbonyl)phosphonic dichloride[8] in the presence of triethylamine. The triester was purified by flash column chromatography,[9] and was fully characterised spectroscopically. The ^1H NMR spectrum showed the presence of a doublet at 5.18 ppm, integrating for 4 H, with a J_{PH} coupling constant of 8.3 Hz confirming that the benzyloxy groups are attached to phosphorus. We anticipated that benzyl (methoxycarbonyl)phosphonate (**2**) and benzyl alcohol (**3**) would be formed on hydrolysis and an authentic sample of this diester **2** was prepared by treatment of a solution of **1** in acetone with sodium iodide.[10]

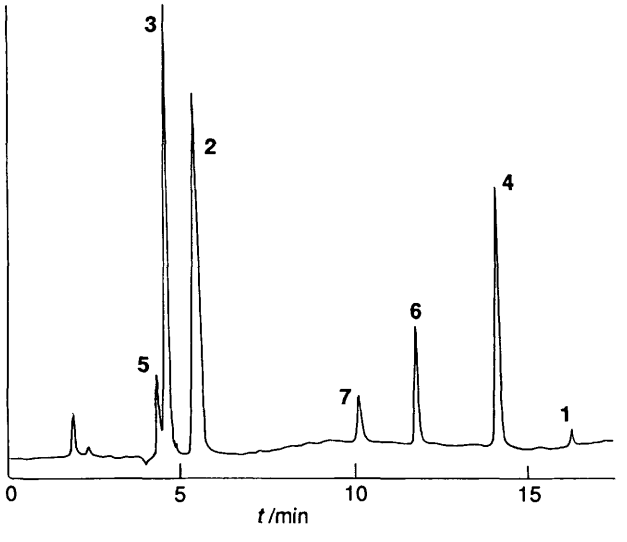


Fig. 1 HPLC chromatogram showing the hydrolysis of **1** in phosphate buffer (pH 7.4, 0.1 mol dm^{-3})–acetonitrile mixture (1:1 v/v) at 37 °C after 210 min. Peak **1** elutes with dibenzyl (methoxycarbonyl)-phosphonate, **2** with benzyl (methoxycarbonyl)phosphonate, **3** with benzyl alcohol, **4** with dibenzyl phosphite, **5** with benzyl phosphite, **6** with benzyl (benzyloxycarbonyl)phosphonate and **7** with dibenzyl phosphate.

The triester **1** was subjected to hydrolysis in a phosphate buffer (pH 7.4, 0.1 mol dm^{-3})–acetonitrile mixture (1:1 v/v) at 36.4 °C and the reaction monitored by ion-pair reversed-phase HPLC. In a typical HPLC chromatogram (Fig. 1) benzyl alcohol (**3**) eluted with a retention time of 4.6 minutes, the diester (**2**) at 5.4 minutes and the lipophilic triester (**1**) after 16.2 minutes. The diester and benzyl alcohol were the major products, however there were 4 unexpected peaks **4**, **5**, **6** and **7** having retention times of 14.1, 4.3, 11.75 and 10.1 min respectively.

In an attempt to characterise these products, the reaction was monitored by ^{31}P NMR (^1H decoupled) spectroscopy and after 90 min, there were three main peaks corresponding to the triester **1** (δ_P −3.70), the diester **2** (δ_P −4.71) and an

† Preliminary accounts of this work were presented at the MRC AIDS-directed programme workshop, Sheffield, September 1989, and as an abstract at the Sixth International Conference on AIDS, San Francisco, June 1990.

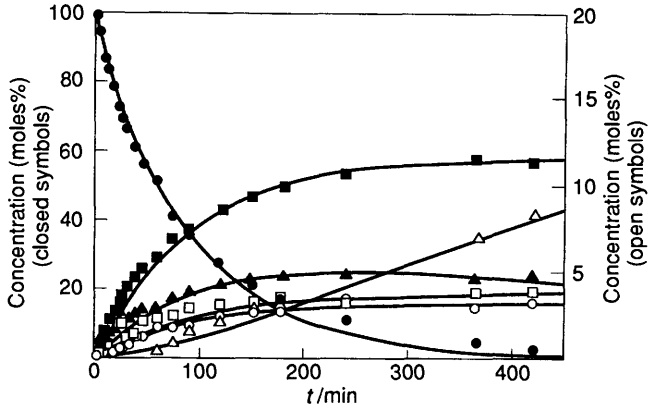


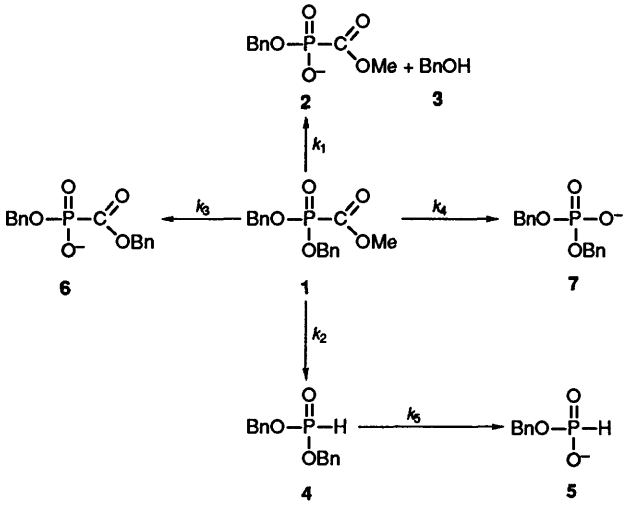
Fig. 2 Time–concentration profile for the degradation of dibenzyl (methoxycarbonyl)phosphonate in aqueous acetonitrile (50%) at 36.4 °C and pH 7.4 determined by ^{31}P NMR. The symbols represent the experimental points and the lines are calculated from eqns. (7)–(12) using k_1, 6.56 × 10^{-3}; k_2, 3.55 × 10^{-3}; k_3, 3.59 × 10^{-4}; k_4, 4.24 × 10^{-4}; k_5, 9.04 × 10^{-4} min^{-1}. {●, [1]$_t$, dibenzyl (methoxycarbonyl)phosphonate; ■, [2]$_t$, benzyl (methoxycarbonyl)phosphonate; ▲, [4]$_t$, dibenzyl phosphite; ○, [6]$_t$, benzyl (benzyloxycarbonyl)phosphonate; □, [7]$_t$, dibenzyl phosphate; △, [5]$_t$, benzyl phosphite} The left-hand axis refers to species designated by closed symbols and the right-hand axis refers to species designated by open symbols.

unknown compound at δ_P 10.82 ppm. The ^{31}P NMR spectrum was recorded with ^{1}H coupling and the peak at δ_P 10.82 gave a doublet of quintets with coupling constants of 719 and 9.6 Hz. The very large coupling to phosphorus is consistent only with a P–H group, suggesting the formation of dibenzyl phosphite (**4**). This compound was isolated on a solid phase extraction column, and the ^{31}P NMR spectrum and HPLC retention time were identical with a commercially available sample of dibenzyl phosphite. Phosphite formation from the hydrolysis of trialkyl esters of phosphonoformate has been observed,[5] and under acid catalysis the decarboxylation of phosphonoformate occurs[11] to give phosphorous acid. The hydrolysis was left for a further 2 weeks, after which time the triester (**1**) and dibenzyl phosphite (**4**) had degraded and a new peak at δ_P 5.90 ppm was observed, which when ^{1}H coupled gave a doublet of triplets, with coupling constants of 625 and 8.3 Hz. This was readily assigned as benzyl phosphite (**5**) and its identity was confirmed by comparison with the retention time on HPLC (4.3 min) and ^{31}P NMR spectrum of an authentic sample prepared from the reaction of dibenzyl phosphite with NaI.

The minor products detected by HPLC at retention times of 11.75 min (**6**) and 10.1 min (**7**), proved more difficult to characterise. To establish whether these compounds were anionic, the HPLC analysis was repeated in the absence of the ion-pairing agent. The triester **1**, dibenzyl phosphite (**4**) and benzyl alcohol (**3**) eluted at their usual retention times, whereas the anionic diester **2**, together with the peaks for **6** and **7**, now eluted at the solvent front. This indicated that the unknowns were charged, and from their chromatographic characteristics probably monoanionic. The ^{31}P NMR (^{1}H decoupled) spectrum gave minor unassigned peaks at −4.97 and 1.07 ppm, which when ^{1}H coupled gave a triplet (J_{PH} 6.65 Hz) and a pentet (J_{PH} 6.7 Hz) respectively. The unknown **6**, was shown to elute at the same retention time (11.75 min) as sodium benzyl (benzyloxycarbonyl)phosphonate which was prepared from the reaction of dibenzyl (benzyloxycarbonyl)phosphonate with sodium iodide. Purification of **6** from the reaction mixture by solid phase extraction, followed by ^{31}P NMR (^{1}H coupled) spectroscopy gave a triplet at −4.79 ppm with J_{PH} 7.3 Hz.

Interestingly, the minor product **7**, with a retention time of 10.1 min by HPLC, coeluted with dibenzyl phosphate. This product was partially purified by solid phase extraction, which by ^{31}P NMR (^{1}H coupled) spectroscopy gave a quintet at 1.06 ppm with J_{PH} 7.2 Hz.

Having established the identity of the products, the reaction was monitored over 6 h by ^{31}P NMR spectroscopy. The time course from peak areas, corrected for the differing responses of the components, for the disappearance of triester and appearance of products is shown in Fig. 2. The degradation is represented by Scheme 1, where [1]–[7] are the concentrations



Scheme 1

of compounds **1**–**7** and k_1–k_5 are the first-order rate constants. The kinetic equations which describe this scheme are eqns. (1)–(6).

$$\frac{d[1]}{dt} = -(k_1 + k_2 + k_3 + k_4)\,[1] \quad (1)$$

$$\frac{d[2]}{dt} = k_1\,[1] \quad (2)$$

$$\frac{d[4]}{dt} = k_2\,[1] - k_5\,[4] \quad (3)$$

$$\frac{d[5]}{dt} = k_5\,[4] \quad (4)$$

$$\frac{d[6]}{dt} = k_3\,[1] \quad (5)$$

$$\frac{d[7]}{dt} = k_4\,[1] \quad (6)$$

Integration of these expressions between initial (t = 0) and current (t = t) time limits provides equations which allow the concentration of the individual species ([1]$_t$, [2]$_t$, [4]$_t$, [5]$_t$, [6]$_t$, [7]$_t$) to be evaluated at any time t [eqns. (7)–(12)].

$$[1]_t = [1]_0 \cdot \exp(-k \cdot t) \quad (7)$$

$$[2]_t = \frac{[1]_0 \cdot k_1}{k} \cdot [1 - \exp(-k \cdot t)] \quad (8)$$

$$[4]_t = \frac{[1]_0 \cdot k_2}{k - k_5} \cdot [\exp(-k_5 \cdot t) - \exp(-k \cdot t)] \quad (9)$$

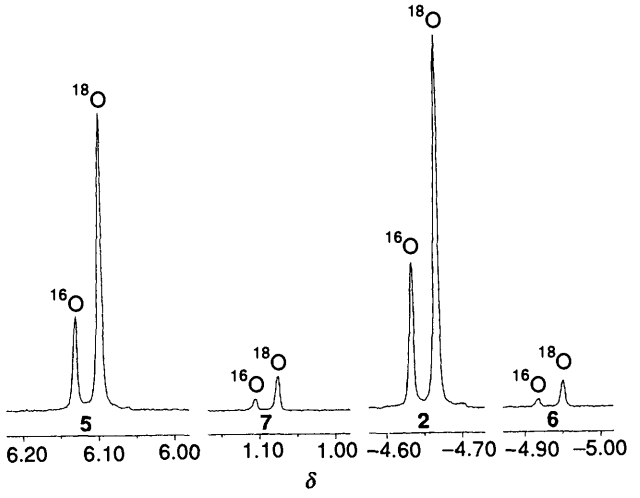


Fig. 3 ^{31}P NMR spectrum of the product from the hydrolysis of dibenzyl (methoxycarbonyl)phosphonate with $H_2{}^{18}O$. Peaks at −4.635 (27.5%) and −4.668 (72.5%) ppm are for ^{16}O- and ^{18}O-labelled benzyl (methoxycarbonyl)phosphonate (**2**). Peaks at −4.919 (23.5%) and −4.951 (76.5%) are for ^{16}O- and ^{18}O-labelled benzyl (benzyloxycarbonyl)phosphonate (**6**). Peaks at 6.130 (23.5%) and 6.097 (76.5%) ppm are for ^{16}O- and ^{18}O-labelled benzyl phosphite (**5**). Peaks at 1.105 (25%) and 1.075 (75%) ppm are for ^{16}O- and ^{18}O-labelled dibenzyl phosphate (**7**).

$$[\mathbf{5}]_t = [\mathbf{1}]_0 \cdot k_2 \cdot k_5 \cdot \left(\frac{1}{k_5 \cdot k} - \frac{\exp(-k_5 \cdot t)}{k_5 \cdot (k - k_5)} - \frac{\exp(-k \cdot t)}{k \cdot (k_5 - k)} \right) \quad (10)$$

$$[\mathbf{6}]_t = \frac{[\mathbf{1}]_0 \cdot k_3}{k} \cdot [1 - \exp(-k \cdot t)] \quad (11)$$

$$[\mathbf{7}]_t = \frac{[\mathbf{1}]_0 \cdot k_4}{k} \cdot [1 - \exp(-k \cdot t)] \quad (12)$$

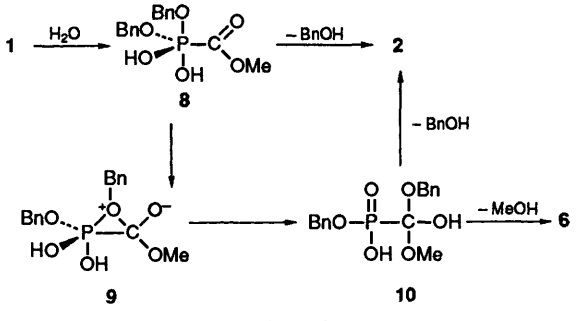
where $k = k_1 + k_2 + k_3 + k_4$.

The time-concentration data were fitted to eqns. (7)–(12) by means of non-linear least squares regression using program NONREG[12] which was extended to deal with the six simultaneous functions required in this analysis. Iteration converged rapidly to provide estimates for each of the five rate constants with standard deviations of k_1, 6.56 ± 0.07 × 10^{-3}; k_2, 3.55 ± 0.06 × 10^{-3}; k_3, 3.59 ± 0.45 × 10^{-4}; k_4, 4.24 ± 0.45 × 10^{-4}; k_5, 9.04 ± 0.29 × 10^{-4} min^{-1}. The consistency of the rate constants is indicated by, for example, the half-life of the triester which may be calculated from $t_{\frac{1}{2}} = 0.6932/(k_1 + k_2 + k_3 + k_4)$ to be 64 min. This is in agreement with the value of 60 min measured experimentally. The reasonable degree of correspondence between experimental data and theoretical profiles calculated from these rate constants using eqns. (7)–(12) is shown in Fig. 2. The correlation coefficients, r, for the fit were 0.9974 for the degradation of **1**, 0.9966 for the appearance of **2**, 0.9948 for **4**, 0.9907 for **5**, 0.9445 for **6** and 0.9743 for **7**. The rate constants confirm that the major reactions involve hydrolysis, to benzyl (methoxycarbonyl)phosphonate (**2**, k_1) and dibenzyl phosphite (**4**, k_2) and that competing rearrangements, to benzyl (benzyloxycarbonyl)-phosphonate (**6**, k_3) and dibenzyl phosphate (**7**, k_4), are an order of magnitude slower. Additionally, hydrolysis of dibenzyl phosphite (**4**) occurs at about one-fifth of the rate of its formation.

The triester **1**, with a half-life of 60 min, was considerably more reactive towards chemical hydrolysis than expected. Under identical reaction conditions, dibenzyl (methoxycarbonylmethyl)phosphonate and dibenzyl methylphosphonate were completely stable over 48 h. From the literature, the half-lives of dibenzyl methylphosphonate in water[13] and tetrabenzyl pyrophosphate in propan-1-ol[14] are 11 min at 100 °C and 4.1 h at 50 °C respectively.

To gain information on the mechanisms of decomposition of the triester **1**, the hydrolysis was repeated in an 80% enriched ^{18}O-labelled water–acetonitrile mixture (1:1) at pH 7.4. After 24 h, the reaction mixture was examined by ^{31}P NMR spectroscopy (Fig. 3), a technique which has been elegantly used to probe for the presence of ^{18}O-label attached directly to phosphorus, and which relies upon a small, but measurable, up-field shift in the resonance caused by ^{18}O.[15] The diester **2** gave peaks at −4.635 (^{16}O, 27.5%) and −4.668 (^{18}O, 72.5%) ppm showing that it contains ^{18}O label attached to phosphorus. The reaction mixture was further examined by ^{13}C NMR spectroscopy and the benzyl alcohol was shown not to contain ^{18}O, as the methylene group at 64.4 ppm gave only a singlet. We were also interested in establishing whether the carbonyl of the diester **2** contained ^{18}O label, however the carbonyl could not be detected by ^{13}C NMR. In a similar experiment, the reaction mixture was extracted with dichloromethane, and concentration of the aqueous layer gave the diesters **2** and **6**. This mixture was analysed by fast atom bombardment (FAB) mass spectrometry for the incorporation of ^{18}O label. By comparison with the unlabelled material, the mass spectrum showed peaks at 275 [^{16}O, 30%] and 277 [^{18}O, 70%] for an ion corresponding to sodium benzyl (methoxycarbonyl)phosphonate (**2**) plus a sodium cation. There was no evidence for the incorporation of two ^{18}O labels, which rules out exchange into the carbonyl group.

These results require that the hydrolysis of the triester **1** to give **2** proceeds by P–O bond cleavage with nucleophilic attack of water at phosphorus, most likely *via* the pentacoordinate intermediate **8** (Scheme 2). In contrast, studies of dibenzyl



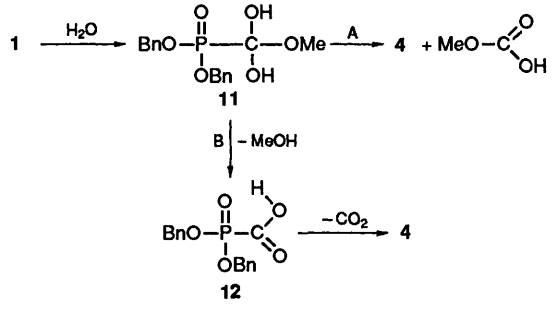
Scheme 2

methylphosphonate[13] and tetrabenzyl pyrophosphate[14] have shown that these compounds lose a benzyl group with C–O cleavage. The high reactivity and change in mechanism for the phosphonoformate triester must be attributed to the electron-withdrawing effect of the methoxycarbonyl group making the phosphorus sensitive to nucleophilic attack.

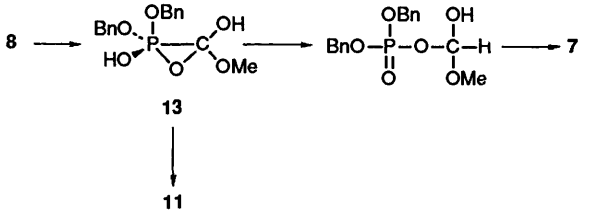
The minor diester **6**, also contained one ^{18}O attached directly to phosphorus as shown by ^{31}P NMR (^{18}O, 76.5%, Fig. 3) and FAB mass spectrometry with peaks at 350 [^{16}O, 30%] and 352 [^{18}O, 70%] consistent with the molecular ion of sodium benzyl (benzyloxycarbonyl)phosphonate (**6**) plus sodium. At first, it was considered that it may arise from the hydrolysis of the triester, dibenzyl (benzyloxycarbonyl)phosphonate, which could be formed from the transesterification of **1** with benzyl alcohol. However, the tribenzyl triester could not be detected by HPLC (standard elutes at 16.7 min using convex gradient 5 with which **1** elutes at 13.6 min) during the course of the reaction, even in the presence of ten equivalents of benzyl alcohol. Moreover, the ratio of **2**:**6** was constant at

17 ± 2 throughout the reaction, which suggests that benzyl alcohol is not utilised in the formation of **6**. A possible route to **6** involves the pentacoordinate intermediate **8**, which instead of losing benzyl alcohol, could react by a 1,2-shift of the benzyloxy group from phosphorus to carbon to give **10**, proceeding *via* the intermediate **9** (Scheme 2). Loss of methanol from **10** would give **6**, whereas loss of benzyl alcohol from **10** would give **2**.

Parallel to the mechanism proposed for the hydrolyses of dialkyl acylphosphonates, which undergo ready C–P bond cleavage with the formation of the dialkyl phosphite and the carboxylic acid,[18] the hydrolysis of **1** to give dibenzyl phosphite **4** could proceed with nucleophilic attack at the carbonyl to give the hydrate **11** (Scheme 3). Alternatively **11** could be



Scheme 3



Scheme 4

formed from **8** by a hydroxy group migration proceeding *via* the epoxide **13** (Scheme 4). Lack of incorporation of ^{18}O label from $H_2{}^{18}O$ into the carbonyls of the diesters **2** and **6**, suggests that the formation of the hydrate **11** cannot be reversible. This step should be base-catalysed, and in support of this proposal, the hydrolysis of the triester **1** is very rapid at pH 9.0 with complete reaction after 35 min, whereas at pH 4 the triester is much more stable with a half-life of *ca.* 90 h at 37 °C. The C–P bond of **11** may cleave to give the phosphite **4** and methyl carbonate, which could undergo decarboxylation to methanol and CO_2 (Scheme 3, pathway A). Alternatively methanol could be lost from **11** to give the carboxylic acid **12** which, by an intramolecular five-membered-ring proton transfer, can decarboxylate to give the phosphite **4** (Scheme 3, pathway B). A related mechanism to pathway B has been proposed for the reaction of $Ph_2P(O)$–$C(O)OCH_2Ph$ with iodide anions.[17]

In an attempt to distinguish between mechanisms A and B, two experiments were performed. In the first experiment, **1** was incubated with porcine liver carboxyesterase at pH 5.0 and 37 °C. HPLC analysis showed that after 10 min **1** could not be detected and that the only product was dibenzyl phosphite (**4**). Although this result could be consistent with either pathway, catalysis of P–C bond cleavage by an esterase is without precedent and the initial formation of the carboxylic acid **12** is therefore considered to be more likely under these conditions. In the second experiment, the hydrolysis of dimethyl (phenoxycarbonyl)phosphonate,[5] bearing a better ester leaving group, was monitored by ^{31}P NMR spectroscopy. This substrate was hydrolysed instantly and the only product detected was dimethyl phosphite [δ_P(^1H coupled) 14.6 ppm, d × sept, J_{PH} 718 Hz, J_{PH} 12 Hz], which was slowly hydrolysed to methyl phosphite [δ_P(^1H coupled) 8.2 ppm, d × q, J_{PH} 626 Hz, J_{PH} 12 Hz]. The enhanced leaving ability of the phenoxy group is compatible with the reaction proceeding *via* the carboxylic acid **12**, in agreement with a recent study on the hydrolysis of phosphonoformate esters.[18]

Benzyl phosphite (**5**) was formed from the hydrolysis of dibenzyl phosphite (**4**). The reaction in $H_2{}^{18}O$ showed that the hydrolysis proceeds with P–O cleavage with the ^{18}O label attached directly to phosphorus (^{18}O, 76.5%, Fig. 3), as observed for dimethyl phosphite.[19]

It was possible that dibenzyl phosphate (**7**) could simply be formed from the oxidation of dibenzyl phosphite (**4**), however three observations suggest that this cannot be the case. First, from the time course, the formation of **7** did not increase with the degradation of **4**. Second, when **4** was incubated under the reaction conditions for 24 h, **7** could not be detected by ^{31}P NMR spectroscopy. Third, from the experiment with $H_2{}^{18}O$, the label becomes attached to phosphorus in **7** (^{18}O, 75%, Fig. 3). Dibenzyl phosphate (**7**) is a primary product, and the mechanism must involve nucleophilic attack of water at phosphorus. A possible route, again involving a rearrangement of the pentacoordinate intermediate **8**, *via* the epoxide **13** is shown in Scheme 4.

In support of this study, Thatcher *et al.* have recently reported that phosphonoformate triesters are hydrolysed to mixtures of hydrogen phosphonate and phosphonoformate esters.[18]

The hydrolysis of dibenzyl (methoxycarbonyl)phosphonate (**1**) was more rapid and considerably more complicated than expected. Because of competing pathways of hydrolysis, involving nucleophilic attack both at phosphorus and at the carbonyl group, the preparation of stable triester analogues of phosphonoformate will be difficult. As the hydrolysis, in part, proceeds with C–P bond cleavage, triesters will be unsuitable as prodrug forms of phosphonoformate.

Experimental

^1H (300 or 250 MHz), ^{31}P (121.5 or 101.3 MHz) and ^{13}C (75.5 or 62.9 MHz) NMR were recorded on Bruker AC spectrometers. The spectra were referenced to tetramethylsilane (^1H and ^{13}C) and 85% H_3PO_4 (^{31}P): positive chemical shifts are downfield from the reference. ^{31}P and ^{13}C spectra were ^1H decoupled (composite pulse decoupling), unless otherwise stated. Coupling constants are 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 obtained on a V.G. 7070E instrument under FAB (glycerol, thioglycerol or nitrobenzyl alcohol matrix). IR spectra were recorded on a Perkin-Elmer 1310 Spectrophotometer. Melting points were measured on an Electrothermal Digital apparatus and are not corrected. Flash column chromatography[9] was performed using Sorbsil C60 silica gel. TLC was performed using 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. All chemicals were obtained from Aldrich Chemical Company. Enzyme was obtained from Sigma Chemical Company. The following solvents were dried by refluxing and distillation over the appropriate drying reagent: dichloromethane (P_2O_5), acetone (4Å molecular sieve), toluene (Na) and triethylamine (KOH). The phosphate buffer (0.1 mol dm^{-3}, pH 7.4) was prepared by mixing aqueous solutions of disodium hydrogen phosphate (0.2 mol dm^{-3}, 40.5 cm^3) and sodium dihydrogen phosphate (0.2 mol dm^{-3}, 9.5 cm^3), and then the volume was adjusted to 100 cm^3 with water.

Dibenzyl (methoxycarbonyl)phosphonate (**1**).—A solution of (methoxycarbonyl)phosphonic dichloride[8] (2.00 g, 11.2 mmol) in dichloromethane (10 cm^3) was added dropwise over 20 min to a stirred solution of benzyl alcohol (2.43 g, 22.5 mmol) and triethylamine (2.22 g, 22.5 mmol) in dichloromethane (30 cm^3) at 0 °C under argon. The mixture was stirred for 1 h at room temperature. The triethylammonium hydrochloride was removed by filtration through Celite. Concentration and purification by flash column chromatography[9] [EtOAc–hexane (1:1), R_f 0.39] gave **1** as a colourless oil (2.30 g, 7.17 mmol, 64%) (Found: C, 60.21; H, 5.53. $C_{16}H_{17}O_5P$ requires C, 60.00; H, 5.35); ν/cm^{-1} (thin film) 1710 (C=O) and 1280 (P=O); $\delta_H(CDCl_3)$ 3.77 (3 H, d, J_{PH} 1.1, OCH_3), 5.18 (4 H, d, J_{PH} 8.3, 2 × CH_2) and 7.33 (10 H, s, 2 × C_6H_5); $\delta_P(CD_3CN)$ −3.75 (s), (quint. q, J_{PH} 8.5, 1.0, ^1H coupled); $\delta_C(CDCl_3)$ 52.53 (d, J_{PC} 5.5, OCH_3), 69.66 (d, J_{PC} 6.3, 2 × CH_2), 128.20 (s, 4 × aromatic CH), 128.63 (s, 4 × aromatic CH), 128.80 (s, 2 × aromatic CH), 135.05 (d, J_{PC} 6.2, 2 × aromatic C) and 166.88 (d, J_{PC} 272.1, C=O); *m/z* (FAB, thioglycerol matrix) 321 (M + H, 57%), 229 (10), 214 (13), 181 (100), 141 (17) and 91 (87); observed accurate FAB *m/z* on M + H 321.0892. $C_{16}H_{18}O_5P$ requires 321.0892.

Dibenzyl (benzyloxycarbonyl)phosphonate was prepared in a similar way to **1** from (benzyloxycarbonyl)phosphonic dichloride and benzyl alcohol. Purification by flash column chromatography[9] [EtOAc–hexane (1:1), R_f 0.81] gave the tribenzyl ester as a yellow oil, (34%) (Found: C, 65.28; H, 5.35. $C_{22}H_{21}O_5P$ requires C, 66.66; H, 5.34); ν/cm^{-1} (thin film) 1720 (C=O) and 1270 (P=O); $\delta_H(CDCl_3)$ 5.16 (4 H, d, J_{PH} 7.9, 2 × CH_2OP), 5.21 (2 H, s, CH_2OC) and 7.32 (15 H, m, 3 × Ph); δ_P −5.08 ppm (s), (quint., J_{PH} 8.0, ^1H coupled); δ_C 67.65 (d, J_{PC} 4.1, $COCH_2$), 69.72 (d, J_{PC} 5.8, 2 × CH_2OP), 128.17 (s, aromatic CH), 128.60 (s, aromatic CH), 128.69 (s, aromatic CH), 128.75 (s, aromatic CH), 134.33 (s, aromatic C) and 135.05 (d, J_{PC} 6.8, 2 × aromatic C), 2 aromatic CH overlapping and carbonyl not detected; *m/z* (FAB, nitrobenzyl alcohol matrix) 397 (M + H, 23%), 181 (63), 107 (17), 91 (100) and 77 (24); observed accurate FAB *m/z* on M + H 397.1205. $C_{22}H_{22}O_5P$ requires 397.1205.

Dibenzyl (methoxycarbonylmethyl)phosphonate was prepared in the same way as **1** from (methoxycarbonylmethyl)phosphonic dichloride[20] and benzyl alcohol. Flash column chromatography[9] [EtOAc–hexane (2:1), R_f 0.2] gave the triester as a yellow oil (34%) (Found: C, 58.70; H, 5.71. $C_{17}H_{19}O_5P$ requires C, 61.08; H, 5.73); ν/cm^{-1} (thin film) 1740 (C=O) and 1275 (P=O); $\delta_H(CDCl_3)$ 2.97 (2 H, d, J_{PH} 21.5, CH_2), 3.66 (3 H, s, OCH_3), 5.03 (2 H, dd, J_{gem} 12, J_{PH} 8.4, CH_2), 5.10 (2 H, dd, J_{gem} 12, J_{PH} 9.4, CH_2) and 7.33 (10 H, s, 2 × Ph); δ_P 20.36 (s); δ_C 34.11 (d, J_{PC} 135.8, PCH_2), 52.25 (s, OCH_3), 67.76 (d, J_{PC} 6.0, 2 × CH_2O), 127.66 (s, aromatic CH), 128.27 (s, aromatic CH), 128.21 (s, aromatic CH) and 135.56 (d, J_{PC} 6.0, aromatic C), 165.69 (d, J_{PC} 6.0, C=O); *m/z* (FAB, nitrobenzyl alcohol) 669 (2 M + H, 12.4%), 335 (M + H, 68), 181 (24) and 91 (100); observed accurate FAB *m/z* on M + H 335.111. $C_{17}H_{20}O_5P$ requires 335.105.

Dibenzyl methylphosphonate[13] was prepared in the same way as **1** from methylphosphonic dichloride and benzyl alcohol. Flash column chromatography[9] (EtOAc, R_f 0.33) gave dibenzyl methylphosphonate as a brown oil (40%); ν/cm^{-1} (thin film) 1240 (P=O); $\delta_H(CDCl_3)$ 1.50 (3 H, d, J_{PH} 17, CH_3), 5.01 (4 H, d, J_{PH} 8.2, 2 × CH_2) and 7.35 (10 H, s, Ph); δ_P 31.38 (s).

Sodium benzyl (methoxycarbonyl)phosphonate (**2**).—Using a method similar to that described for other diesters of phosphonoformate[10] a solution of sodium iodide (1.03 g, 6.88 mmol) in acetone (15 cm^3) was added to a solution of **1** (2.00 g, 6.25 mmol) in acetone (20 cm^3). The reaction mixture was stirred and heated under reflux for 1 h. The diester **2** was precipitated as a colourless powder. After filtration the sample was dissolved in water and precipitated by the addition of acetone (0.30 g, 1.06 mmol, 17%), m.p. >210 °C (Found: C, 42.52; H, 3.78. $C_9H_{10}O_5PNa$ requires C, 42.87; H, 4.00); ν/cm^{-1} (Nujol) 1690 (C=O) and 1265 (P=O); $\delta_H(D_2O)$ 3.48 (3 H, s, OCH_3), 4.80 (2 H, d, J_{PH} 7.8, CH_2) and 7.21 (5 H, s, C_6H_5); δ_P −2.65 (s); *m/z* (FAB, glycerol matrix) 275 (M + Na, 100%), 253 (M + H, 71), 185 (22), 177 (2), 115 (61) and 91 (55).

Sodium benzyl (benzyloxycarbonyl)phosphonate was prepared in a similar way to **2** from dibenzyl (benzyloxycarbonyl)-phosphonate with sodium iodide. The dibenzyl diester was precipitated as a colourless powder (64%), m.p. >210 °C; ν/cm^{-1} (Nujol) 1680 (C=O) and 1260 (P=O); $\delta_H(D_2O)$ 4.70 (2 H, d, J_{PH} 7.7, CH_2OP), 4.88 (2 H, s, $COCH_2$) and 7.20–7.03 (10 H, m, 2 × Ph); δ_P −3.04 (s); δ_C 69.42 (d, J_{PH} 3.2, $COCH_2$), 70.95 (d, J_{PH} 5.2, CH_2OP), 130.54 (s, aromatic CH), 131.03 (s, aromatic CH), 131.25 (s, aromatic CH), 131.33 (s, aromatic CH), 131.44 (s, aromatic CH), 137.91 (s, aromatic C), 139.34 (d, J_{PC} 6.0, aromatic C) and 175.45 (d, J_{PC} 241.9, C=O), one aromatic CH not observed; *m/z* (FAB, nitrobenzyl alcohol matrix) 351 (M + Na, 58%), 329 (M + H, 50), 176 (100), 91 (33) and 77 (12). Observed accurate FAB *m/z* on (M + Na) gives 351.0374. $C_{15}H_{14}O_5Na_2P$ requires 351.0374.

Benzyl Phosphite.—A solution of sodium iodide (2.81 g, 19.0 mmol) in acetone (10 cm^3) was added slowly to a solution of dibenzyl phosphite (4.5 g, 17.0 mmol) in acetone (10 cm^3). The mixture was heated at reflux for 3 h. On cooling, sodium benzyl phosphite precipitated as a colourless solid (2.30 g, 71%), ν/cm^{-1} (Nujol) 1205 (P=O); $\delta_H(D_2O)$ 4.86 (2 H, d, J_{PH} 8.0, CH_2OP), 7.37 (5 H, s, Ph) and 6.75 (1 H, d, J_{PH} 632); δ_P 6.91 ppm (s), (dt, J_{PH} 637, J_{PH} 8.7, ^1H coupled).

Reaction of **1** *with* $H_2{}^{18}O$.—A solution of **1** (17 mg, 0.053 mmol) in dry acetonitrile (1.00 cm^3) was added to $H_2{}^{18}O$–$H_2{}^{16}O$ (4:1 v/v, 1.00 cm^3) buffered with NaH_2PO_4/Na_2HPO_4 (pH 7.4) and left to stand for 24 h at room temperature. The reaction mixture was analysed by ^{31}P NMR (101.3 MHz) spectroscopy with a sweep width of 1309 Hz, a data block size of 32K, an acquisition time of 12.5 s, 3720 scans and 12.5 data points per Hz. The FID was transformed with a line broadening of 0.1 Hz (Fig. 3). The reaction mixture was analysed by ^{13}C NMR (62.90 MHz) spectroscopy which included δ 68.46 (d, J_{PC} 5.5, CH_2) and 52.07 (d, J_{PC} 4.7, OMe) for diester **2**, 66.13 (d, J_{PC} 3.9, CH_2) for benzyl phosphite (**5**) and 64.46 (s, CH_2) for benzyl alcohol (**3**). There was no evidence for the incorporation of ^{18}O into benzyl alcohol. In a similar experiment, the reaction mixture was concentrated, and the organic components were extracted into CH_2Cl_2 (3 cm^3) and the diesters **2** and **6** into water. The aqueous fraction was concentrated on the freeze-drier to give the diesters which were characterised by FAB mass spectrometry using a nitrobenzyl alcohol matrix.

Hydrolysis of Phosphonates Monitored by HPLC.—High performance liquid chromatography 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. Hydrolysis samples (20 mm^3) were injected directly and eluted with a linear or convex (Waters pre-programmed gradient curve 5) gradient of acetonitrile–10 mmol dm^{-3} tetrabutylammonium hydroxide in water, initial conditions, 35:65 v/v; final conditions, 90:10 v/v; gradient time 20 min. Chromatograms were recorded on a Waters 745B integrator.

Hydrolyses were performed in glass screw-capped vials

using a total volume of 1 cm^3 and a final concentration of triester of 0.1 mg cm^{-3} or 0.4 mg cm^{-3}. A mixture of acetonitrile and the appropriate 0.1 mol dm^{-3} phosphate buffer (pH 4, 7.4, 9) (1:1 v/v) was pre-incubated at 37 °C for 10 min. Hydrolysis was initiated by the addition of an acetonitrile solution of the triester **1**, dibenzyl (methoxycarbonylmethyl)phosphonate or dibenzyl methylphosphonate (50 mm^3) to give the required final concentration and then incubated at 37 °C on a shaking water-bath. At the appropriate time the incubation was removed from the water-bath and the solution used directly for HPLC analysis. Hydrolyses were monitored for approximately 2–3 half-lives.

Hydrolysis of **1** *with Esterase.*—Incubations were performed at 37 °C in glass screw-capped vials using a total incubation volume of 1 cm^3 containing 15% MeCN and a final concentration of triester **1** of 1 mg cm^{-3}. Prior to incubation, a solution of porcine liver carboxyesterase was prepared in 0.1 mol dm^{-3} phosphate buffer (pH 8.0) containing 0.4–0.5 units/mm^3. Phosphate buffer (0.1 mol dm^{-3}, pH 5.0, 0.80 cm^3), MeCN (0.10 cm^3) and esterase solution (50 mm^3) were pre-incubated for 10 min at 37 °C and the reaction initiated by the dropwise addition of an acetonitrile solution of triester (50 mm^3). Control incubations were performed in the absence of enzyme. The reactions were monitored by HPLC.

Hydrolysis of **1** *Monitored by* ^{31}P *NMR Spectroscopy.*—A solution of **1** (6.0 mg, 18.75 μmoles) was dissolved in CD_3CN (0.5 cm^3). With the NMR probe warmed to 36.4 °C, the ^{31}P NMR spectrum of this mixture was recorded [δ_P(101.3 MHz) −3.75 ppm (s)]. Phosphate buffer (0.1 mol dm^{-3}, pH 7.4, 0.5 cm^3, pre-equilibrated at 37 °C) was added to initiate the reaction, and the ^{31}P NMR spectrum was recorded at 4.25 min intervals for the first 45 min, then at t = 1, 1.25, 1.5, 2, 2.5, 3, 4, 5 and 6 h. A ^1H coupled spectrum was recorded at 6.5 h. The reaction mixture was left for 2 weeks at room temperature, after which time the ^{31}P NMR spectrum was recorded with and without ^1H decoupling. To confirm the identity of the products, authentic samples of **2**, **4**, **5**, **6** and **7** were added to the reaction mixture and in each case the appropriate peak increased in size. Each FID was transformed using a line broadening of 1 Hz and the spectrum plotted on a wide expansion. The area of each peak was determined from its width and height. To evaluate the ^{31}P NMR response of each component type, known weights (*ca.* 10 μmol) of diester **2**, benzyl phosphite (**5**) and dibenzyl phosphate (**7**) were combined. ^1H NMR spectroscopy confirmed the ratios from the weights, however, under identical spectrometer conditions to the kinetics experiment, by ^{31}P NMR **5** had an area response 1.55 × greater than **2**, and **7** had an area response 1.1 × greater than **2**. These factors were applied to the data, using the assumption that **4** responds identically to **5** and that **1** and **6** respond identically to **2**. The % of the components present at each time point were calculated and the time course is shown in Fig. 2.

After complete reaction, the pH of the mixture was 7.20. During the course of the reaction, there was some change in pH as the chemical shift of the phosphate buffer drifted from 2.50 to 1.63 ppm. The ^{31}P chemical shifts of the products were constant throughout the experiment: −3.70 (s), (quint. q, J_{PH} 8.5, J_{PH} 1.0, ^1H coupled) (**1**); −4.71 (s), (t q, J_{PH} 7.2, J_{PH} 0.9, ^1H coupled) (**2**); 10.82 (s), (d, quint., J_{PH} 719, J_{PH} 9.6, ^1H coupled) (**4**); 5.90 (s), (d t, J_{PH} 625, J_{PH} 8.3, ^1H coupled) (**5**); −4.97 (s), (t, J_{PH} 6.65, ^1H coupled) (**6**); 1.07 ppm (s), (quint., J_{PH} 6.7, ^1H coupled) (**7**).

Hydrolysis of Dimethyl (Phenoxycarbonyl)phosphonate Monitored by ^{31}P *NMR Spectroscopy.*—A solution of the triester[5] (80 mg, 0.35 mmol) in acetonitrile (4 cm^3) was added to a 10 mm ^{31}P NMR tube, containing a 5 mm D_2O inner lock. After the ^{31}P NMR spectrum was recorded (−1.9 ppm), 4 cm^3 of phosphate buffer (pH 7.4, 0.1 mol dm^{-3}) were added to initiate the hydrolysis. After *ca.* 5 min the mixture was monitored by ^{31}P NMR which showed only dimethyl phosphite (δ_P 14.6 ppm (s) (d sept, J_{PH} 718, J_{PH} 12, ^1H coupled), which was slowly hydrolysed to methyl phosphite (δ_P 8.2 ppm (s) (d q, J_{PH} 626, J_{PH} 12, ^1H coupled). To confirm the identity of the product, dimethyl phosphite was added to the sample and the ^{31}P NMR spectrum was repeated and the peak for this compound increased in intensity.

Control Reaction between Benzyl Alcohol and **1**.—A solution of **1** (0.4 mg, 1.25 μmol) in acetonitrile (50 mm^3) was added to a solution of benzyl alcohol (1.35 mg, 1.25 μmol, 10 equiv.) in acetonitrile–0.1 mol dm^{-3} phosphate buffer pH 7.4 (1:1, v/v, 950 mm^3) at 37 °C. The reaction mixture was monitored by HPLC every 30 min for a total of 4 h.

Hydrolysis of Dibenzyl Phosphite (**4**).—Dibenzyl phosphite (4.0 mg, 15.0 μmol) was added to a solution of CD_3CN–0.1 mol dm^3 phosphate buffer (pH 7.4) (1 cm^3, 1:1, v/v). The solution was analysed by ^{31}P NMR spectroscopy, δ 10.92 (s). The mixture was incubated at 37 °C for 24 h, after which time the only product observed by ^{31}P NMR was benzyl phosphite, δ 6.08 (s). Dibenzyl phosphate was not detected.

Large Scale Hydrolysis of Dibenzyl (Methoxycarbonyl)phosphonate: Isolation of Dibenzyl Phosphite, Benzyl (Benzyloxycarbonyl)phosphonate and Dibenzyl Phosphate.—A solution of dibenzyl (methoxycarbonyl)phosphonate (300 mg) in acetonitrile (10 cm^3) was added dropwise to a mixture of acetonitrile–phosphate buffer (0.1 mol dm^{-3}, pH 7.4) (150 cm^3, 1:1, v/v) at 37 °C on a shaking water-bath. The mixture was monitored by HPLC, and after complete hydrolysis of the triester, the volume was reduced to *ca.* 100 cm^3 (contains *ca.* 30% MeCN). A 30 cm^3 portion was applied to an activated 1 g C-18 Bond-Elut solid phase extraction column. The column was washed with water (3 × 3 cm^3). Elution with acetonitrile (2 × 2 cm^3), gave dibenzyl phosphite, which was characterised by ^{31}P NMR spectroscopy, δ_P 11.15 ppm, (d pent, J_{PH} 721, J_{PH} 9.6, ^1H coupled). By HPLC, this coeluted with peak **4**.

All of the other components had eluted, either in the load or water eluates. These were pooled (35 cm^3) and diluted with water (105 cm^3) to lower the concentration of the MeCN to *ca.* 7%, thereby facilitating the retention of the more polar components. This solution was applied to a new 1 g C-18 Bond-Elut solid phase extraction column, which was then washed successively with water (3 × 3 cm^3), 15% acetonitrile in water (2 × 2 cm^3) and 25% acetonitrile in water (2 × 2 cm^3). Each wash was analysed by HPLC and ^{31}P NMR spectroscopy. The second 15% acetonitrile wash contained dibenzyl phosphate (25%), δ_P 1.06 ppm (s), (quint., J_{PH} 7.2, ^1H coupled) and benzyl (benzyloxycarbonyl)phosphonate (75%), δ_P −4.68 ppm (s), (t, J_{PH} 7.8, ^1H coupled). Addition of authentic samples of these compounds enhanced the intensity of the appropriate peaks in ^{31}P NMR and the sample gave peaks coeluting with **7** and **6** by HPLC. The first 25% acetonitrile wash contained dibenzyl phosphate (10%) and benzyl (benzyloxycarbonyl)phosphonate (90%). The second 25% acetonitrile wash contained only benzyl (benzyloxycarbonyl)phosphonate, δ_P −4.79 ppm (2), (t, J_{PH} 7.3, ^1H coupled).

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