Bioreversible Protection for the Phospho Group: Chemical Stability and Bioactivation of Di(4-acetoxybenzyl) Methylphosphonate with Carboxyesterase

Sally Freeman,* William J. Irwin, Antony G. Mitchell, Dave Nicholls and William Thomson

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK

In contrast to high chemical stability ($t_{1/2}$ 55.4 h at 36.4 °C), with porcine liver carboxyesterase the title compound **1** spontaneously decomposes first to the monoester **2** then to methylphosphonate, both reactions proceeding *via* the 4-hydroxybenzyl intermediates **3** and **4**.

Several compounds containing the phospho group [-PO(OH)$_2$] are of therapeutic interest, however, at physiological pH, they are ionised and many have not achieved their therapeutic potential principally because of poor transport across cell membranes.[1] In an attempt to improve delivery, in some studies, their negative charges have been masked with the preparation of prodrug phosphoesters [-PO(OR)$_2$], whose increased lipophilicity should facilitate transport into cells by passive diffusion. The prodrug is required to liberate the parent drug and for simple R groups, the first ester could possibly be removed by hydrolysis, however, the second ester is usually very resistant to cleavage.[2] For bioactivation to be successful, the R group must be metabolically labile and towards this end the di(acyloxymethyl) esters of the phospho group [-PO(O-CH$_2$-OC(O)R′)$_2$] have been reported.[3,4] Srivastva and Farquhar have investigated the di(acyloxymethyl) derivatives of benzyl and phenyl phosphates,[3] which in the presence of porcine liver carboxyesterase readily decompose to the mono(acyloxymethyl) esters. This decomposition is thought to proceed by loss of the acyl group to give the hydroxymethyl derivative, followed by spontaneous loss of formaldehyde.[3] Although some benzyl or phenyl phosphate is released, the second acyloxymethyl group is removed only slowly by the esterase. Compounds bearing a charge are reported to be poor substrates for esterases,[5] and the slower rate for the removal of the second acyloxymethyl group is likely to be attributable to the inability of carboxyesterase to tolerate the anionic charge in close proximity to the active site. The low reactivity of the monoanion, coupled both with the chemical instability of the acyloxymethyl esters[3] and the potential problems with formaldehyde release have led us to consider alternative metabolically labile protection suitable for the phospho group.

Benzyl esters of carbamates are useful prodrugs for compounds containing the amino group.[6,7] In this report the use of carboxyesterase-susceptible dibenzyl phosphodiesters as prodrugs for the phospho group is explored. To promote the removal of the second ester, it is likely that the site of esterase attack needs to be well-separated from the monoanionic phospho group. This rationale led us to explore the 4-acetoxybenzyl derivatives, in which for the monoanions, the charge is now nine bonds removed from the site of esterase attack, an increase of ~2.7 Å over the acyloxymethyl analogue. Methylphosphonic acid [MePO(OH)$_2$] was chosen as a model compound, so that these ideas can be readily applied to the antiviral phosphonoacetate,[8] and pamidronate (APD) used in the treatment of bone metastases,[9] both of which have poor oral bioavailability.

Di(4-acetoxybenzyl) methylphosphonate **1** was prepared in 25% yield from the reaction of two equivalents of 4-acetoxybenzyl alcohol with methylphosphonic dichloride in the presence of triethylamine. The low-melting solid was purified by flash column chromatography and was fully characterised. In $CDCl_3$, the ^{1}H NMR spectrum included δ_H 5.01 (2H, dd, J_{gem} 11.9, J_{PH} 9.1 Hz) and 4.91 (2H, dd, J_{gem} 11.9, J_{PH} 8.5 Hz) for the non-equivalent protons of the POCH$_2$Ar groups.

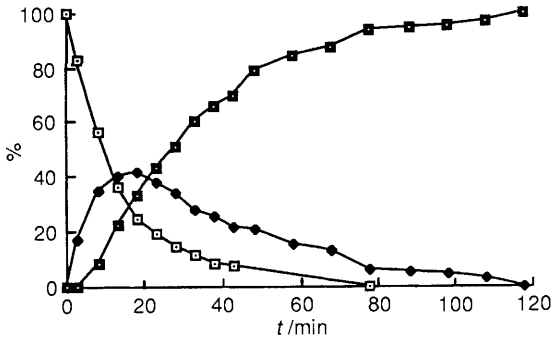
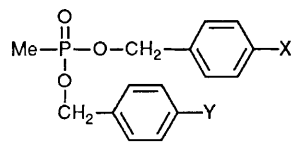


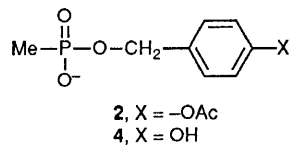
Fig. 1 Reaction of sodium 4-acetoxybenzyl methylphosphonate **2** (5 mmol dm^{-3}, 1 ml) in potassium phosphate buffer (0.1 mol dm^{-3}, D_2O, pD 8.0)–CD_3CN (9 : 1, v/v) with porcine liver carboxyesterase (50 units) at 36.4 °C. Monitored by ^1H NMR (250 MHz) spectroscopy using peak heights of P–Me doublets to give the % of each component; □ represents the 4-acetoxybenzyl monoester **2**, ◆ the 4-hydroxybenzyl monoester **4** and ■ methylphosphonate.

To test for chemical stability, a 5 mmol dm^{-3} solution of the diester **1** 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 ^1H NMR spectroscopy at 36.4 °C. The diester gave δ_P 36.5 and δ_H 7.31 (4H, d, J_{HH} 8.5), 7.05 (4H, d, J_{HH} 8.5), 4.92 (4H, d, J_{PH} 9.2), 2.24 (6H, s) and 1.55 (3H, d, J_{PH} 17.5 Hz). In contrast to the non-equivalence observed in $CDCl_3$, the methylene protons now appear equivalent. The hydrolysis of the diester was slow with a half-life of 55.4 h (k 1.251 ± 0.010 × 10^{-2} h^{-1}). The formation of 4-acetoxybenzyl methylphosphonate **2** was confirmed by the synthesis of the sodium salt from the reaction of the diester **1** with sodium iodide,[10] data on which included δ_P 27.5 and δ_H 7.41 (2H, d, J_{HH} 8.6), 7.08 (2H, d, J_{HH} 8.6 Hz), 4.81 (2H, d, J_{PH} 7.2), 2.25 (3H, s) and 1.19 (3H, d, J_{PH} 16.4 Hz). As supported by hydrolysis of this standard, **2** decomposes further to methylphosphonate, δ_P 24.4 (s, ^1H decoupled) (q, J_{PH} 16.4 Hz, ^1H coupled), and δ_H 1.15 (3H, d, J_{PH} 16.4 Hz) with a half-life of 153.2 h (k 4.525 ± 0.021 × 10^{-2} h^{-1}). Other products formed were acetate [δ_H 1.80 (3H, s)] and 4-hydroxybenzyl alcohol [δ_H 7.20 (2H, d, J_{HH} 8.5), 6.81 (2H, d, J_{HH} 8.5 Hz) and 4.45 (2H, s)]. 4-Acetoxybenzyl alcohol was not formed [authentic sample gives δ_H 7.36 (2H, d, J_{HH} 8.5 Hz), 7.06 (2H, d, J_{HH} 8.5 Hz), 4.55 (2H, s) and 2.25 (3H, s)] which suggests that the degradation of **1** and **2** must proceed with hydrolysis of the acetoxy group to give the 4-hydroxybenzyl intermediates **3** and **4**, respectively. Two studies support our view that the electron-donating 4-hydroxy group will assist in the breaking of the benzyl–oxygen bond to give either monoester **2** or methylphosphonate together with the resonance-stabilised 4-hydroxybenzyl carbonium ion. First, dibenzyl methylphosphonate **5** is completely stable under similar conditions of hydrolysis over 48 h,[11] however at 100 °C the reaction proceeds *via* the benzyl carbonium ion with C–O cleavage.[12] Secondly, after 323 min, 4-methoxybenzyl diphenyl phosphate undergoes 88% solvolysis in methanol, whereas under identical conditions the 3-methoxy or unsubstituted benzyl analogues are completely stable.[13] The slow reactivity of **1** towards chemical hydrolysis is in marked contrast to the high reactivity reported for di(acetyoxymethyl) phenyl phosphate, which has a half-life of only 193 min at 37 °C and pH 7.4.[3]

In contrast to the chemical stability, in the presence of 50 units of porcine liver carboxyesterase(SIGMA), a 5 mmol dm^{-3} solution (1 ml) of the diester **1** in phosphate buffer (0.1 mol dm^{-3}, D_2O, pD 8.0)–CD_3CN (9 : 1, v/v) at 36.4 °C rapidly decomposed in less than 3 min to give the monoester **2**, which after 2 h gave only methylphosphonate. A similar reaction with the monoester **2**, monitored by ^1H NMR spectroscopy (Fig. 1), shows that, in contrast to the chemical hydrolysis, the



1, X = Y = –OAc
3, X = –OAc, Y = OH
5, X = Y = H



2, X = –OAc
4, X = OH

4-hydroxybenzyl intermediate **4** was detected and gave δ_P 27.4 and δ_H 7.24 (2H, d, J_{HH} 8.7 Hz), 6.81 (2H, d, J_{HH} 8.7 Hz), 4.71 (2H, d, J_{PH} 7.1 Hz) and 1.17 (3H, d, J_{PH} 16.3 Hz). In the presence of 100 units of enzyme, after 15.5 min all of the monoester **2** had been metabolised to the 4-hydroxybenzyl intermediate **4**, which was shown to decompose to methylphosphonate with a half-life of 17 min (k 4.14 ± 0.45 × 10^{-2} min^{-1}).

The ready removal of the 4-acetoxybenzyl groups with carboxyesterase suggests that the 4-acyloxybenzyl diesters may be useful bioreversible derivatives of the phospho group. The lower reactivity of the monoester with carboxyesterase, when compared with the diester, could be exploited to provide a sustained release of parent drug. In theory, once inside the cell, the lipophilic diester would readily yield the anionic monoester, which being charged would be trapped and hence serve as a reservoir for the parent drug. 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,[3] strong acid[14] or trimethylsilyl bromide.[15]

Although the products derived from the phospho group of the diester **1** are known, the fate of the benzyl group is more complex with only ~30% of the product derived from the proposed carbonium ion being present as 4-hydroxybenzyl alcohol at early time points. Instead of reacting with water, the carbonium ion may be trapped by another nucleophile, and possibilities include the enzyme, products or buffer. The reaction profile for the decomposition of diester **1** with carboxyesterase is very similar to that of monoester **2** (Fig. 1). For **1**, two equivalents of the carbonium ion are generated, which does not lower catalytic efficiency, this suggests that this intermediate does not react with enzyme. In a related reaction,[16] 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 3-(4′-hydroxybenzyl)-4-hydroxybenzyl alcohol, however the ^1H NMR spectrum suggested only 1,4-disubstituted products. To investigate the involvement of the buffer, the reaction of **1** with 5 units of carboxyesterase was repeated using 0.01 mol dm^{-3} phosphate buffer. At all time points >90% of the carbonium ion was trapped as 4-hydroxybenzyl alcohol, and this result suggests that, with the original 0.1 mol dm^{-3} buffer, inorganic phosphate can compete with water to trap the carbonium ion. Although we have yet to prepare a standard, unassigned peaks in the NMR spectra of the reaction mixture with 0.1 mol dm^{-3} buffer are δ_P 3.72 and δ_H 7.26 (2H, d, J_{HH} 8.4 Hz), 6.81 (2H, d, J_{HH} 8.4 Hz) and 4.64 (2H, d, J_{PH} 5.4 Hz) consistent with 4-hydroxybenzyl phosphate, 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.[17,18] The higher reactivity of 4-hydroxybenzyl phosphate suggests a change in mechanism, with the electron-donating hydroxy group promoting C–O cleavage.

Studies are in progress to optimise the stability and bioactivation of the 4-acyloxybenzyl phosphodiesters, for both drug delivery and as a synthetic method, by altering the nature of the acyl group. The potential problems associated with the release of a highly reactive benzyl carbonium ion have been

outlined,[6] and methods to trap this intermediate internally are being investigated.

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