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Bioreversible Phosphate Protective Groups: Synthesis and Stability of Model Acyloxymethyl Phosphates

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Six bis(acyloxymethyl) esters of phenyl phosphate and benzyl phosphate and three acyloxymethylbenzylphenyl phosphates were prepared. Debenzylation of benzyl analogs gave respective free acids which were isolated as cyclohexylammonium salts. Stability of bis(acyloxymethyl)phenyl phosphates were studied in various aqueous buffers, hog liver esterase, and mice plasma.

INTRODUCTION

Organophosphate esters, particularly phosphomonoesters, play a crucial role in biological chemistry since they are involved, at one stage or other, in virtually every major metabolic pathway including the biosynthesis of nucleic acids, proteins, and carbohydrates. Despite their major role in cell metabolism, phosphomonoesters possess limited potential as thereapeutic agents. This shortcoming is due to two major factors: First, the phosphates group bears a double negative charge at physiologic pH. As a result, phosphomonoesters are highly water-soluble compounds and, generally, are unable to penetrate lipid cell membrane (*1*, *2*). Second, most phosphomonoesters are susceptible to rapid degradation by nonspecific phosphohydrolases such as alkaline and acid phosphatases which are abundant in most tissues (*3*, *4*). In an effort to restore the therapeutic potential of phosphomonoesters, we have attempted to develop biologically reversible phosphate protective groups. Our strategy was to use these protective groups to convert the ionic phosphomonoesters into neutral phosphotriesters which, conceivably, could traverse cell membranes by passive diffusion and then revert intracellularly, by enzymatic cleavage of the protective groups, to the parent phosphomonoester. From a consideration of biochemical and organic mechanistic principles, it appears that the acyloyxmethyl group might be suitable for this purpose (Scheme 1). Thus, after cell penetration, bis(acyloxymethyl) phosphotriesters should undergo cleavage of one of the acyloxy ester linkages by intracellular carboxylate esterases to yield the corresponding hydroxymethyl analogs (*5*) (**2**). These intermediates should be chemically labile and spontaneously eliminate one molecule of

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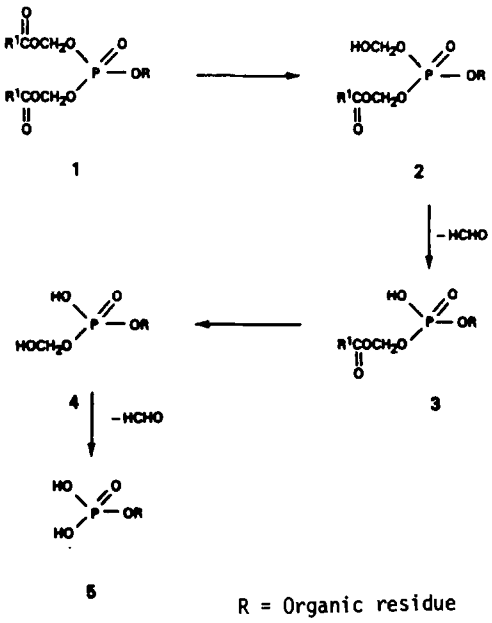
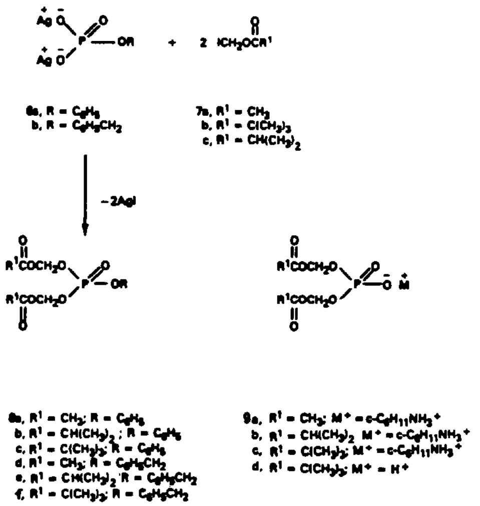


SCHEME 1

formaldehyde to yield the monoacyloxymethyl phosphodiesters (3). Repetition of this sequence with the second ester group should then regenerate the parent phosphomonoester (5). However, bis(acyloxymethyl) phosphotriesters have not been reported previously. In this manuscript, we describe the synthesis and characterization of a series of model bis(acyloxymethyl) phosphotriesters and report on their stabilities under various chemical and enzymatic conditions. A preliminary account of this work has appeared earlier (5).

EXPERIMENTAL PROCEDURES

^1H nmr were recorded at ambient temperature on a Varian Associates T-60A spectrometer in $CDCl_3$, using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnegan Model 3300 quadrapole spectrometer in the chemical ionization mode using methane as reagent gas. Elemental analyses were performed by Galbraith Laboratories Inc. All solvents were distilled immediately before use and were dried over freshly activated (300°C/4 hr) molecular sieves (type 4A) for at least 24 hr. Reactions involving silver salts were conducted in the dark under anhydrous conditions. Evaporation was carried out on a rotary evaporator under vacuum at a bath temperature of <30°C. The homogeneity of the products was determined by ascending TLC on silica gel 60 F254 (Merck), using various combinations of hexane and $CHCl_3$. Compounds were visualized under uv light (254 nm) or with iodine vapor. Analytical HPLC was carried out in the reverse phase on Bondapak C-18 columns (Waters Associates).



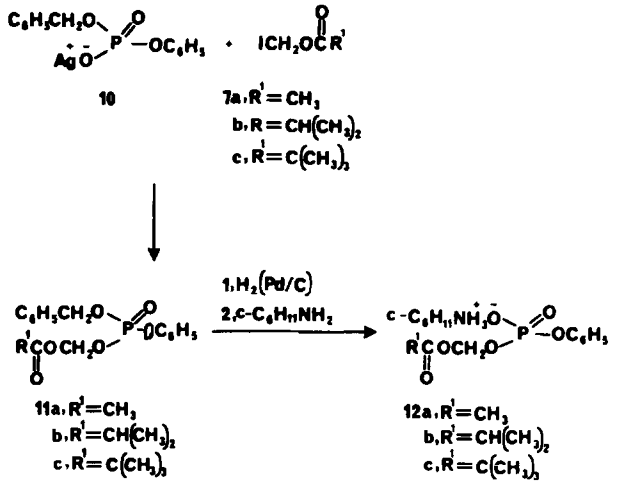
SCHEME 2

Starting Materials

The disilver phenyl phosphate **6a** (Scheme 2) (Found: Ag, 55.48; C_6H_5OPAg requires Ag, 55.67%) and disilver benzyl phosphate, **6b** (Found: Ag, 53.86; $C_7H_7O_4PAg$ requires Ag, 53.73) were prepared by treating the aqueous solution of disodium phenyl phosphate (Aldrich, St. Louis, Mo.) and disodium benzyl phosphate with aqueous silver nitrate and filtering the precipitate in the dark. The aqueous disodium benzyl phosphate (not isolated) was prepared from dicyclohexylammoniumbenzyl phosphate by passing it through AG 50W-X12 cation-exchange resin in Na^+ form. The later, dicyclohexylammoniumbenzyl phosphate (mp 243–4°C, yield 69%) (*6*), was prepared in analogy to a literature procedure given for the 4-hydroxy-but-2-enyl phosphate (*7*). The silver benzyl phenyl phosphate (Scheme 3) (**10**) (Found: Ag, 29.21; $C_{13}H_{12}O_4Ag$ requires Ag, 29.0%) (*8*) was prepared according to reported literature. Iodomethyl acylates **7a** ($R^1 = CH_3$) (*9*), (bp 61°C at 10 mm), **7b** [$R^1 = CH(CH_3)_2$] (*10*) (bp 50°C at 4 mm), and **7c** [$R^1 = C(CH_3)_3$] (*11*) (bp 68°C at 4 mm) were prepared from their respective chloromethyl acylates. The chloromethyl acetate (bp 95–100°C at 735 mm, yield 58%) (*12*) and chloromethyl isobutrate (bp 47–52°C at 25 mm, yield 73%) (*13*) were prepared as reported in the literature. An appropriate amount of crude iodomethyl acylate was dissolved in toluene (approx five times by wt), and washed with 5% aqueous sodium bisulfite (approx equal volume of toluene) and then with water (3 × 25 ml). The toluene layer after drying over anhydrous sodium sulfate was immediately used in the reaction.

Bis(acyloxymethyl) esters of phenyl and benzyl phosphate. The following general procedure was used for synthesis of these compounds (Scheme 2).

A solution of the iodomethyl acylate (6.44 mmol) in toluene (approx 5 ml) was added dropwise, with stirring, over 30 min to a suspension of disilver benzyl



Scheme 3

phosphate or disilver phenyl phosphate (2.58 mmol) in toluene (20 ml). After being stirred for a further 5 hr at room temperature, the reaction mixture was filtered to remove silver salt. The filtrate was washed successively with 5% aqueous sodium bisulfite solution (10 ml) and water (3 × 10 ml) and then dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure at room temperature. The residual oil was pure enough for further reaction. A part of the residual oil (200 mg) was taken up in acetone and purified for analytical purposes by chromatography on silica (10 g) using benzene/hexane as eluant. Fractions containing the phosphotriester as evidenced by TLC analyses were combined and evaporated. The product was dried *in vacuo* for 24 hr at room temperature.

Bis(acetoxymethyl)phenyl phosphate (**8a**). Using disilver phenyl phosphate (1.9 g, 4.89 mmol) and iodomethyl acetate (3.17 g, 15.85 mmol), **8a** was obtained as a colorless oil. It was dried under vacuum for 24 hr at room temperature. Yield, 0.82 g (53%). ^{1}H nmr ($CDCl_3$), δ7.27 (s, 5H, C_6H_5), 5.68 (d, J_{PH} = 14 Hz, 4H, OCH_2O), 2.06 (s, 6H, CH_3); MS *m/e* 319 (MH^+).

Anal. Calcd for $C_{[12}H_{15}O_8P$ $2H_2O$: C, 40.69; H, 5.40. Found: C, 40.30; H, 5.75.

Bis(isobutryloxymethyl)phenyl phosphate (**8b**). Disilver phenyl phosphate (1.0 g, 2.58 mmol) and iodomethyl isobutrate (1.5 g, 6.58 mmol) gave **8b** as a colorless, viscous oil. Yield, 0.57 g (59%). ^{1}H nmr ($CDCl_3$), δ7.17 (s, 5H, C_6H_5), 5.67 (d, J_{PH} = 14 Hz, OCH_2O), 2.17–2.84 !m, 2H, $HC(CH_3)_2$, 1.12 [d, 12H, J_{HH} = 7 Hz, $C(CH_3)_2$]; MS *m/e* 375 (MH^+).

Anal. Calcd for $C_{16}H_{23}O_8P$: C, 51.34; H, 6.19. Found: C, 51.41; H, 6.29.

Bis(pivaloyloxymethyl)phenyl phosphate (**8c**). The compound was prepared from **6a** (2.0 g, 5.16 mmol) and iodomethyl pivaloate (1.57 g, 6.49 mmol). It was isolated as a colorless, viscous oil. Yield, 1.07 (52%). ^{1}H nmr ($CDCl_3$), δ7.27 (s, 5H, C_6H_5), 5.74 (d, 4H, J_{PH} = 14 Hz, OCH_2O), 1.18 [s, 18H, $C(CH_3)_3$]; MS *m/e* 403 (MH^+).

Anal. Calcd for $C_{18}H_{27}O_8P$: C, 53.73; H, 6.77. Found: C, 53.90; H, 6.84.

Bis(acetoxymethyl)benzyl phosphate (**8d**). Disilver benzyl phosphate (7.76 g, 19.3 mmol) and iodomethyl acetate (10.10 g, 50.50 mmol) gave **8d** as a colorless, viscous oil. Yield, 3.75 g (58%). ^{1}H nmr ($CDCl_3$), δ7.33 (s, 5H, C_6H_5), 5.62 (d, J_{PH} = 14 Hz, 4H, OCH_2O), 5.13 (d, J_{PH} = 8 Hz, 2H, $PhCH_2O$), 2.06 (*s*, 6H, CH_3); MS *m/e* 333 (MH^+).

Anal. Calcd for $C_{13}H_{17}O_8P$: C, 46.99; H, 5.16. Found: C, 46.90; H, 5.30.

Bis(isobutryloxymethyl)benzyl phosphate (**8e**). This was prepared from disilver benzyl phosphate (1.0 g, 2.49 mmol) and iodomethyl isobutrate (1.5 g, 6.57 mmol) as a colorless, viscous oil. Yield, 530 mg (55%). ^{1}H nmr ($CDCl_3$), δ7.36 (s, 5H, C_6H_5), 5.66 (d, J_{PH} = 14 Hz, 4H, OCH_2O), 5.14 (d, J_{PH} = 8 Hz, 2H, $PhCH_2O$), 2.02–2.66 m, 2H, *H*$C(CH_3)_2$, 1.12 [d, J_{PH} = 7 Hz, 12H, $C(CH_3)_2$]; MS *m/e* 389 (MH^+).

Anal. Calcd for $C_{17}H_{25}O_8P$ $2H_2O$: C, 48.11, H, 6.88. Found: C, 48.11; H, 6.80.

Bis(pivaloyloxymethyl)benzyl phosphate (**8f**). The compound was prepared from disilver benzyl phosphate (51.0 g, 131 mmol) and iodomethyl pivaloate (79.5 g, 328 mmol) in dry toluene (200 ml), according to the general procedure described for **8a** and was obtained as a colorless, viscous oil. Yield, 31.7 g (58%). ^{1}H nmr ($CDCl_3$), δ7.33 (s, 5H, C_6h_5), 5.62 (d, J_{PH} = 14 Hz, 4H, OCH_2O), 5.10 (d, J_{PH} = 8 Hz, 2H, $PhCH_2O$), 1.20 [s, 18H, $C(CH_3)_3$]; MS *m/e* 417 (MH^+).

Anal. Calcd for $C_{19}H_{29}O_8P$: C, 54.81; H, 7.02. Found: C, 54.98; H, 7.00.

Bis(acetoxymethyl)phosphate, cyclohexylammonium salt (**9a**). Paladium-on-charcoal (5%, 50 mg) was added to a solution of **8d** (1.0 g, 3.01 mmol) in ethyl acetate (50 ml), and the mixture was shaken for 15 min under an atmosphere of hydrogen at 30 psi using a Parr hydrogenator apparatus. Fresh catalyst (50 mg) was added again, and the hydrogenation was continued for a further 30 min. The solution was filtered to remove the catalyst, and the filtrate was treated, dropwise, with cyclohexylamine (approx 0.3 ml) until a drop of the solution gave a pH of 7.0 when tested against wet universal pH paper. The solution was concentrated under reduced pressure at room temperature to about one-half of its original volume and then stored at 5°C for 24 hr in a closed vessel. The white, crystalline mass formed was filtered and washed with ethyl acetate/hexane (1/1, v/v). The product was recrystallized from ethyl acetate/hexane to give colorless needles. Yield, 231 mg (26%), mp 65–68°C. ^{1}H nmr ($CDCl_3$), δ7.00–7.87 (br. s, 3H, NH_3^+), 5.52 (d, J_{PH} = 13 Hz, 4H, OCH_2O), 2.60–3.27 (m, ^{1}H, *H*CNH_3^+), 2.10 (s, 6H, CH_3), 0.77–2.40 (m, 10H, *o*-, *m*-, *p*-cyclohexyl-H); MS *m/e* 342 (MH^+).

Anal. Calcd for $C_{12}H_{24}NO_8P$: C, 42.22; H, 7.08; N, 4.10. Found: C, 42.26; H, 7.07; N, 4.07.

Bis(isobutryloxymethyl)phosphate, cyclohexylammonium salt (**9b**). This compound was prepared by hydrogenolysis of **8e** (2.5 g, 6.44 mmol) in ethyl acetate (50 ml) over 5% palladium-on-charcoal (250 mg) as described for **9a**. The product was obtained as white needles after recrystallization from ethyl acetate/hexane (1/1, v/v). Yield, 1.23 g (48%); mp 59°C. ^{1}H nmr ($CDCl_3$), δ7.03–6.20 (br. s 3H, NH_3^+), 5.49 (d, J_{PH} = 13 Hz, 4H, OCH_2O), 3.20–1.33 [m, 13H, *o*-, *m*-, *p*-cyclohexyl-H, *H*CNH_3^+ and *H*$C(CH_3)_2$], 1.16 (d, J_{HH} = 7 Hz, 12H, $HC(CH_3)_2$; MS *m/e* 398 (MH^+).

Anal. Calcd for $C_{16}H_{32}NO_8P$: C, 48.35; H, 8.12; N, 3.52. Found: C, 48.59; H, 8.20; N, 3.41.

Bis(pivaloyloxymethyl)phosphate, cyclohexylammonium salt (**9d**). This compound was prepared by hydrogenolysis of **8f** (25.0 g, 60.10 mmol) in cyclohexane (400 ml) over 5% palladium-on-charcoal (2.5 g) as described for **9a**. It was recrystallized from ethyl acetate/hexane as colorless needles. Yield, 14.35 g (56%); mp 121–122°C. ^1H nmr ($CDCl_3$), δ7.60–8.27 (br. s, 3H, NH_3^+), 5.57 (d, 4H, J_{pH} = 13 Hz, OCH_2O), 2.67–3.27 (m, 1H, $HCNH_3^+$), 1.22 [s, 18H, $C(CH_3)_3$], 0.93–2.40 (m, 10H, *o*-, *m*-, *p*-cyclohexyl-H); MS *m*/*e* 426 (MH^+).

Anal. Calcd for $C_{18}H_{36}NO_8P$: C, 50.81; H, 8.53; N, 3.29. Found: C, 50.87; H, 8.61; N, 3.19.

Bis(pivaloyloxymethyl) hydrogen phosphate (**9e**). A solution of **9d**, 13.0 g in water (100 ml), on passing through a column (60 g) of Dowex 50W-X8 (H^+ form) and lyophilizing the eluant gave a white powder of **9e**, 9.9 g (99%); mp 68–9°C; ^1H nmr ($CDCl_3$), δ9.38 (br. s, 1H, P–O–H), 5.63 (d, J_{pH} = 14 Hz, 4H, OCH_2O), 1.23 [s, 18H, $C(CH_3)_3$]; MS *m*/*e* 327 (MH^+).

Anal. Calcd for $C_{12}H_{23}O_8P$: C, 44.17; H, 7.10. Found: C, 43.95; H, 7.08.

Acyloxymethylbenzylphenyl phosphate (**11a**). The following general method was used for synthesis of this class of compounds (Scheme 3).

A solution of iodomethyl acylate (3.2 mmol) in toluene (2 ml) was added, dropwise, with stirring, over 15 min to a suspension of silver phenylbenzyl phosphate, 10 (2.69 mmol) in toluene (25 ml). After being stirred for a further 5 hr at room temperature, the reaction mixture was filtered to remove silver salt. The filtrate was washed successively with 5% aqueous sodium bisulfite solution (25 ml) and water (3 × 25 ml) and then dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure at room temperature. The residual oil thus obtained was pure enough for further reaction steps. For analysis, a part of the residual oil (approx. 100 mg) was taken in acetone and purified by column chromatography on silica (10 g) using benzene/hexane as eluant. Fractions containing phosphotriester as evidenced by TLC analyses were combined and evaporated. The product was dried *in vacuo* for 24 hr at room temperature.

Acetoxymethylbenzylphenyl phosphate (**11a**). Silver salt of benzylphenyl phosphate (1.0 g, 2.69 mmol) and iodomethyl acetate (650 mg, 3.25 mmol) gave **11a**, 550 mg (61%) as viscous oil. ^1H nmr ($CDCl_3$), δ7.30 (s, 5H, $C_6H_5CH_2O$), 7.24 (s, 5H, C_6H_5O), 5.60 (d, J_{pH} = 14 Hz, 2H, OCH_2O), 5.15 (d, J_{pH} = 8 Hz, 2H, $PhCH_2O$), 1.94 (s, 3H, CH_3); MS *m*/*e* 337 (MH^+).

Anal. Calcd for $C_{16}H_{17}O_8P$: C, 57.14; H, 5.10. Found: C, 57.32; H, 5.05.

Isobutryloxymethylbenzylphenyl phosphate (**11b**). Iodomethyl isobutrate 760 mg, 3.2 mmol) and silver salt of benzylphenyl phosphate (1.0 g, 2.69 mmol) gave **11b** as colorless, viscous oil. Yield, 630 mg (64%). ^1H nmr ($CDCl_3$), δ7.27 (s, 5H, $C_6H_5CH_2O$), 7.15 (s, 5H, C_6H_5), 5.77 (d, J_{pH} = 14 Hz, 2H, OCH_2O), 5.15 (d, J_{pH} = 8 Hz, 2H, $PhCH_2O$), 1.90–2.03 [m, 1H, $HC(CH_3)_2$], 1.06 [d, J_{hH} = 7 Hz, 6H, $C(CH_3)_2$]; MS *m*/*e* 365 (MH^+).

Anal. Calcd for $C_{18}H_{21}O_6P$: C, 59.34; H, 5.81. Found: C, 59.36; H, 5.78.

Pivaloyloxymethylbenzylphenyl phosphate (**11c**). Iodomethyl pivaloate (790 mg, 3.26 mmol) and silver salt of benzylphenyl phosphate (1.0 g, 2.69 mmol) gave **11c** as colorless, viscous oil. Yield, 660 mg (65%). ^{1}H nmr ($CDCl_3$), δ7.26 (s, 5H, $C_6H_5CH_2O$), 7.16 (s, 5H, C_6H_5), 5.66 (d, J_{pH} = 14 Hz, 2H, OCH_2O), 5.16 (d, J_{PH} = 8 Hz, 2H, $PhCH_2O$), 1.16 [s, 9H, $C(CH_3)_3$]; MS *m/e* 379 (MH^+).

Anal. Calcd for $C_{19}H_{23}O_6P$: C, 60.31; H, 6.13. Found: C, 60.50, H, 6.23.

Acetoxymethylphenyl phosphate, cyclohexylammonium salt (**12a**). Palladium-on-charcoal (5%, 50 mg) was added to a solution of **11a** (500 mg, 1.49 mmol) in ethyl acetate (25 ml), and the mixture was shaken for 30 min under an atmosphere of hydrogen at 30 psi using a Parr hydrogenator apparatus. The solution was filtered to remove the catalyst, and the filtrate was treated dropwise, with stirring with cyclohexylamine (0.17 ml), until a drop of the solution gave a pH 7 when tested against wet universal pH paper. The solution was concentrated under reduced pressure at room temperature to about half of its volume, then stored at 5°C for 24 hr in a closed vessel. The white crystalline mass was filtered and washed with ethyl acetate/hexane (1/1, v/v). The product was recrystallized from ethyl acetate/hexane to give **12a** as colorless needles. Yield, 210 mg (41%), mp 75–8°C. ^{1}H nmr ($CDCl_3$), δ7.17 (s, 5H, C_6H_5), 6.66–6.18 (br. s, 3H, NH_3^+), 5.51 (d, J_{PH} = 13 Hz, 2H, OCH_2O), 3.0–2.30 (m, 1H, N^+–CH), 2.0–0.88 (m, 10H, *o*-, *m*-, *p*-cyclohexyl-H); 1.83 (s, 3H, CH_3); MS *m/e* 346 (MH^+).

Anal. Calcd for $C_{15}H_{24}NO_6P$: C, 52.17; H, 7.01; N, 4.05. Found: C, 52.40; H, 7.09, N, 4.03.

Isobutryloxymethylphenyl phosphate, cyclohexylammonium salt (**12b**). Isobutryloxymethylbenzylphenyl phosphate **11b** (400 mg, 1.10 mmol) and 5% palladium-on-charcoal (40 mg) in a manner similar to **12a** gave **12b** as needle-shaped crystals. Yield, 360 mg (88%), mp 110–11°C. ^{1}H nmr ($CDCl_3$), δ7.15 (s, 5H, C_6H_5), 6.60–6.00 (br. s, 3H, NH_3^+), 5.63–5.45 (d, J_{PH} = 14 Hz, 2H, OCH_2O), 3.01–1.00 [d, J_{HH} = 7 Hz, 6H, $HC(CH_3)_2$]; MS *m/e* 374 (MH^+).

Anal. Calcd for $C_{17}H_{28}NO_6P$: C, 54.18; H, 7.56; N, 3.75. Found: C, 54.38; H, 7.49; N, 3.78.

Pivaloyloxymethylphenyl phosphate, cyclohexylammonium salt (**12c**). Pivaloyloxymethylbenzylphenyl phosphate **11c** (400 mg, 1.06 mmol) and 5% palladium-on-charcoal (40 mg), in a manner similar to **12a**, gave **12c** as white needle crystals. Yield, 360 mg (87%), mp 129–30°C. ^{1}H nmr ($CDCl_3$), δ7.22 (s, 5H, C_6H_5), 7.90–8.06 (br. s, 3H, NH_3^+), 5.55 (d, J_{pH} = 13 Hz, OCH_2O), 2.90–2.33 (m, 1H, HC-N^+), 2.22–0.78 (m, 10H, *o*-, *m*-, *p*-cyclohexyl-H), 1.06 [s, 9H, $C(CH_3)_3$]; MS *m/e* 388 (MH^+).

Anal. Calcd for $C_{18}H_{30}NO_6P$: C, 55.80; H, 7.80; N, 3.61. Found: C, 56.04; H, 7.90; N, 3.55.

Hydrolysis Studies

Fresh methanol solutions (0.02 *M*) of each of the bis(acyloxymethyl)phenyl phosphates **8a–c** were prepared separately. An appropriate volume (10–20 μl) of this solution was mixed with aqueous buffers, hog liver carboxyl esterase (EC 3.1.1.1) (Sigma Chemical Co., St. Louis, Mo.), or mouse plasma. The initial concentrations of **8a–c** range from 1.9 to 2.0 m*M*. The final concentration of methanol in all the studies was <1% (v/v). Fresh methanol solution of bis (acyloxymethyl)phenyl phosphates **8a–c** (20 μl) was mixed in 2 ml of each buffer (*12*) [(a) 0.05 *M* ammonium phosphate buffer, pH 3; (b) 0.05 *M* sodium acetate buffer, pH 5; (c) 0.05 *M* potassium phosphate, pH 7.4, and 0.05 *M* Trisma base (Sigma Chemical Co.)–HCl buffer, pH 9]. The mixture was incubated at 37°C in a vortex shaker. Samples (100 μl) were drawn at various intervals and were analyzed by high performance liquid chromatography (HPLC) (Waters Associates Model ALC 204). The disappearance of **8a–c** was monitored by reverse-phase chromatography on a column of μBondapak-C_{18} (30 cm × 4 mm i.d., 10 μm; Waters Associates, Milford, Mass.) using solutions of 0.01 *M* potassium phosphate buffer (pH 7) methanol (various proportions, typically 25–50%) as mobile phase. The formation of **3** and **5** was monitored by ion-pair chromatography on μBondapak-C_{18} using the same buffer system as described for **8a–c** except that tetrabutylammonium hydroxide was added to a concentration of 2×10^{-3} *M* or by anion-exchange chromatography on a Partisil SAX (25 cm × 4.6 mm i.d.; 10 μm; Whatman) column using a linear gradient of 0.01–0.1 *M* potassium phosphate buffer (pH 6.5) as eluant. The flow rate ranged from 1.0 to 2.0 ml/min. The column effluents were monitored at 254 nm with a Schoeffel Model 450 uv detector, and the concentrations of **1**, **3**, and **5** were determined by comparison of the peak areas with those of reference standards. For enzyme-mediated hydrolysis studies, each incubated mixture contained hog liver esterase (60 μl, 57.6 units), triesters (10 μl of 0.02 *M* methanol solution), and 940 μl of 0.05 *M* potassium phosphate buffer (pH 7.4). The stability in plasma was studied by incubating each triester (12 μl of 0.02 *M* methanol solution), fresh mouse plasma (0.6 ml), and 0.05 *M* potassium phosphate buffer (pH 7.4) (0.6 ml) separately. An appropriate control using no phosphotriester was run simultaneously with every enzyme study and plasma-mediated hydrolysis.

RESULTS AND DISCUSSION

Phenyl phosphate was selected as a model phosphomonoester to investigate synthetic route and the chemical properties of the required bis(acyloxymethyl) phosphotriesters. However, the usual method for the synthesis of neutral esters of phenyl phosphate, namely nucleophilic displacement of chlorine from phenyl phosphorodichloridate by an appropriate alcohol, was precluded, since the required hydroxymethyl acylates are too unstable to be isolated. The synthesis of the model phosphotriesters was, therefore, attempted by nucleophilic displacement of chlorine from a series of chloromethyl acylates by phenyl phosphate. However, when chloromethyl acylate was reacted with bis(tetramethylammonium)phenyl phosphate in ethylene glycol dimethyl ether as solvent, only starting materials were recovered despite using a variety of different organophosphate salts (Na+, K+, NBu+$_4$), solvents (DMF, HMPA, $CHCl_3$, THF, CH_3CN), and temperature conditions (0–100°C) in the presence or absence of sodium iodide as catalyst. Only the intermediate mono(acetoxymethyl) phosphodiester or unchanged phenyl phosphate could be detected in the reaction mixtures. Reaction of

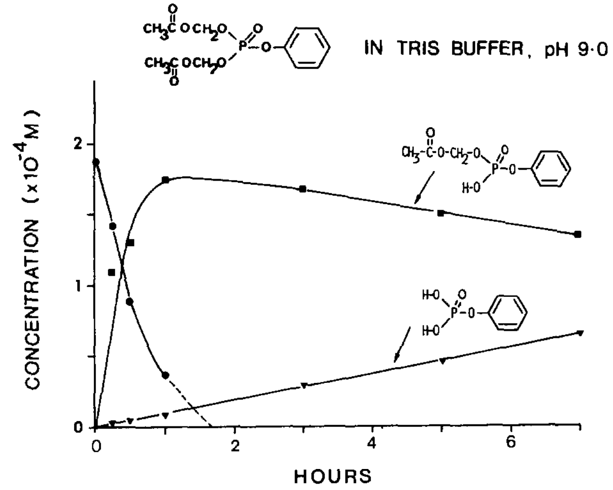


FIGURE 1

disilver aryl phosphate with a 2.5 *M* excess of iodomethyl acylate in anhydrous benzene or toluene at room temperature was the only successful method by which these compounds could be prepared.

Attempts to prepare bis(acyloxymethyl) hydrogen phosphates by catalytic hydrogenolysis of respective bis(acyloxymethyl)phenyl phosphates by Adam's catalyst (PtO_2) were unsuccessful, one of the acyloxymethyl groups being removed under the reaction conditions. However, the bis(acyloxymethyl) phosphates were readily prepared by catalytic hydrogenation of the corresponding benzyl derivatives over 5% palladium-on-charcoal.

Bis(acyloxymethyl) phosphates, although superficially simple, are, in fact, quite chemically complex, combining three different functional groups, namely a carboxylate ester, an acetal, and a phosphoester group. Naturally, these compounds are susceptible to acid as well as base hydrolysis.

Since the success of our strategy depends upon the removal of protective acyloxy groups particularly by carboxylate esterase or plasma, we studied the loss of acyloxymethyl groups in bis(acyloxymethyl)phenyl phosphate in different chemical media, hog liver esterase, and mouse plasma. Bis(acyloxymethyl)phenyl phosphates were fairly stable in neutral aprotic solvents (e.g., benzene, diethyl ether, ethyl acetate); however, in protonic solvents (e.g., ethanol, water, buffers) it was slowly converted to monoacyloxymethylphenyl phosphate. The later compounds are presumably formed by solvolysis of phosphotriesters to the hydroxymethyl intermediate (*5*) (Scheme 1), followed by spontaneous loss of formaldehyde. Repetition of this sequence with the second acyloxymethyl group regenerates the parent phenyl phosphate, the monophosphoester. Figure 1 shows the rapid degradation of bis(acetoxymethyl)phenyl phosphate in Tris buffer at pH 9. The mono(acyloxymethyl)phenyl phosphate was formed by losing one acetic acid and one formaldehyde which slowly further converted to phenyl phosphate by losing the

TABLE 1

HALF-LIVES OF **8a–c** IN DIFFERENT MEDIA

Compounds	pH 3[a]	pH 5[b]	pH 7.4[c]	pH 9[d]	Enzyme[e]	Plasma[f]
	Half-life (min)					
8a	163	278	193	24	15	<5
8b	389	521	533	93	76	27
8c	488	623	856	405	356	327

[a] 0.05 *M* ammonium phosphate buffer.
[b] 0.05 *M* sodium acetate buffer.
[c] 0.05 *M* potassium phosphate buffer.
[d] 0.05 *M* Trizma base–HCl buffer.
[e] Hog liver esterase.
[f] 50% mouse plasma in 0.05 *M* potassium phosphate buffer, pH 7.4.

second mole of acetic acid and formaldehyde. As expected, the rates of hydrolysis of these phosphotriesters **8a–c** were critically dependent on the pH of the buffer (Table 1). The parent phosphotriester was rapidly degraded to give the intermediate phosphodiester which, in turn, was degraded slowly to the parent phosphomonoester.

The mono(acyloxymethyl)phenyl analogs needed as standards for chemical and enzymatic degradation studies were used as their cyclohexylammonium salts. The cyclohexyammonium salts and the free acids showed the same retention time in the HPLC conditions used in this experiment, possibly because of the eluant

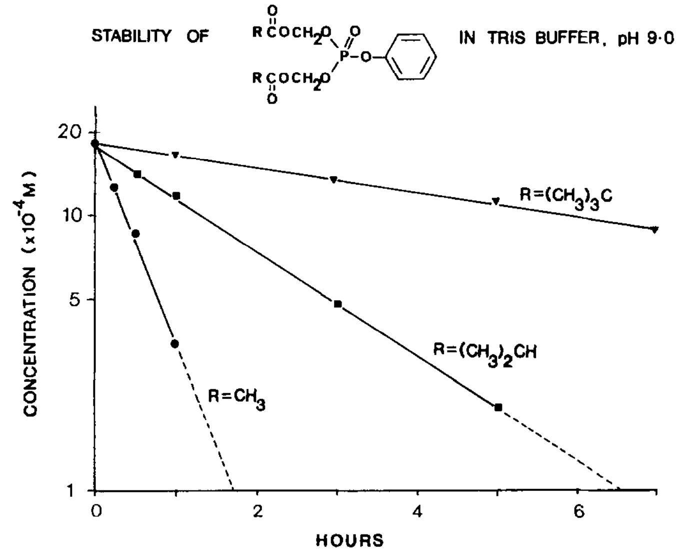


FIGURE 2

buffer in which both were ultimately converted to potassium salts. The cyclohexyammonium salts **11a–c** were prepared as indicated in Scheme 3.

The rate of hydrolysis also depends on the nature of acyl group. The importance of steric crowding adjacent to the carboxyl carbon is demonstrated by an increase in stability of phosphotriesters **8a–c,** as the bulk of alkyl substituent increases from methyl in **8a** to isopropyl in **8b** and *t*-butyl in **8c** (Fig. 2).

Enzyme-mediated hydrolysis was very similar to chemical hydrolysis (Table 1). One acetoxy group was rapidly hydrolyzed to give diester and then slowly to phenyl phosphate. The rate of hydrolysis was in the order of acetyloxy > isobutryloxy > pivaloyloxy. In the plasma-mediated hydrolysis the bis(acetoxymethyl) phenyl phosphate hydrolyzed quite fast to give di- and finally the phenyl phosphate, probably because of large amounts of nonspecific esterase present in the plasma. The rates of hydrolysis of these compounds (**8a–c**) by plasma were strongly influenced by the nature of the acyl substituent. Thus, whereas the half-life of **8a** is less than 5 min, that of **8b** is 27 min, compared with 327 min for the **8c**. By comparison, in controls in the absence of plasma, half-lives of all the three are much greater (Table 1).

The study concludes that steric crowding adjacent to the carbonyl carbon is directly related to the stability of the phosphotriesters, the rate of hydrolysis, and thus the formation of monophosphoester, and it can be used as a guideline in developing a neutral phosphotriester which conceivably could traverse cell membranes by passive diffusion and then revert biologically, possibly intracellularly, by enzymatic cleavage of the protective group to the parent phosphomonoester. Further studies using 2′-deoxy-5-fluorouridine-5′-monophosphate as monoester are in progress.

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