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CHEMICAL SYNTHESIS OF 1-*O*-ALKYL AND 1-*O*-ACYL DIHYDROXYACETONE-3-PHOSPHATE

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A method for the chemical synthesis of 1-*O*-hexadecyl dihydroxyacetone-3-phosphate is described. The synthesis was started with the preparation of *O*-hexadecyl glycolic acid by condensing 1-iodohexadecane with ethyl glycolate in the presence of silver oxide, followed by saponification and free acid liberation with HCl. *O*-Hexadecyl glycolic acid was converted to the acid chloride (with oxalyl chloride) which was condensed with diazomethane in diethyl ether to form hexadecyloxy diazoacetone. The diazoketone was decomposed by H_3PO_4 in dioxane to give the desired product, 1-*O*-hexadecyl dihydroxyacetone-3-phosphate. The product was purified by chromatography on silicic acid column followed by an acid wash. The final yield was 50% starting from *O*-hexadecyl glycolic acid. Analytical, spectral (IR, NMR) and chromatographic properties of 1-*O*-hexadecyl dihydroxyacetone-3-phosphate are described. The method described here may be used to prepare different acyl and alkyl derivatives of dihydroxyacetone phosphate in good yield as illustrated by describing the procedure for the synthesis of 1-*O*-palmitoyl dihydroxyacetone-3-phosphate, 1-*O*-hexadecyl dihydroxyacetone-3-[^{32}P] phosphate and the dimethyl ketal of 1-*O*-palmitoyl [2-^{14}C]dihydroxyacetone phosphate.

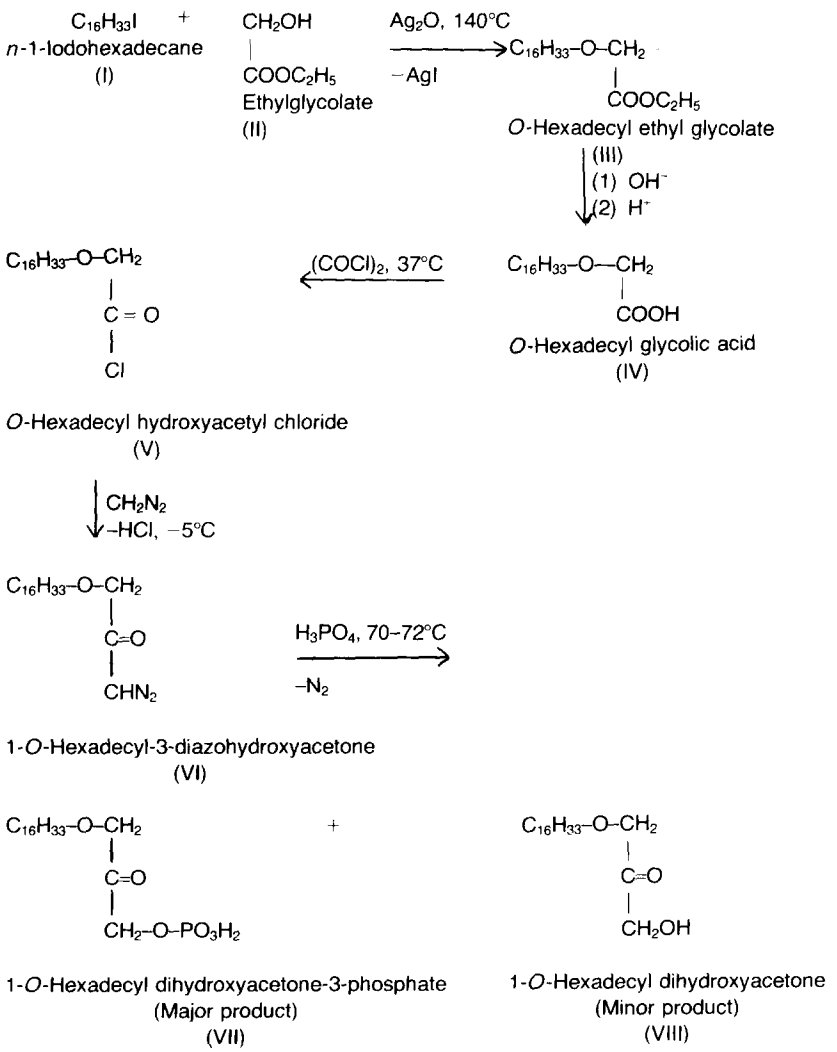
Keywords: *O*-alkyldihydroxyacetone phosphate; acyl dihydroxyacetone phosphate; diazoketones; phosphate esters.

Introduction

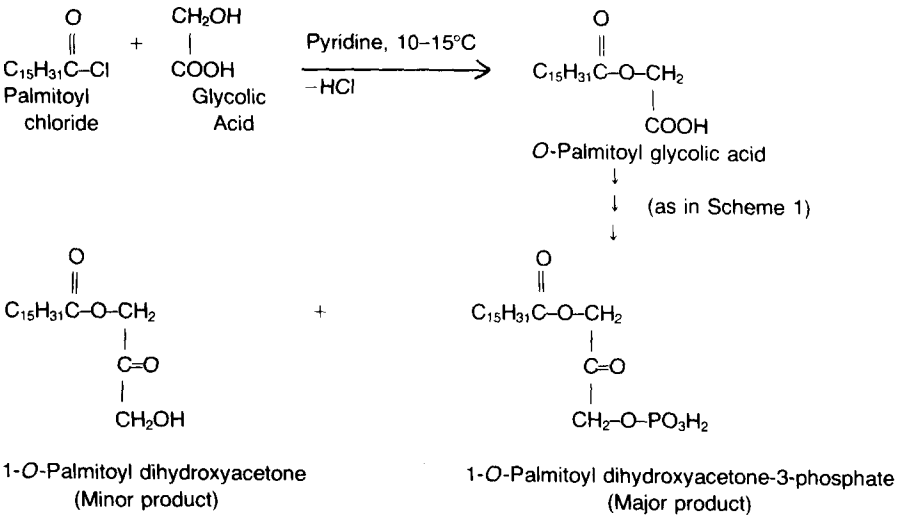
1-*O*-Alkyl and 1-*O*-acyl dihydroxyacetone-3-phosphate have been shown to be important intermediates in the biosynthesis of glycerolipids containing both ester and ether bonds [1–4]. A brief description of the chemical synthesis of acyl dihydroxyacetone phosphate and the outlines for the chemical synthesis of alkyl dihydroxyacetone phosphate via diazoketone intermediates have been published from this laboratory [5–7]. Following the method of Hartman [8], Piantadosi and coworkers [9,10] also described the synthesis of alkyl and acyl dihydroxyacetone phosphate. The detailed methods for the preparation of the long chain ester and ether derivatives of dihydroxyacetone phosphate are described here.

The main principles involved in these methods are the preparations of *O*-alkyl or *O*-acyl derivatives of hydroxyacetyl chloride, condensation of

these compounds with diazomethane to form the corresponding diazoketones and subsequent phosphorolysis to form the phosphate esters. The outline for the whole synthesis is shown in Schemes 1 and 2. We also describe methods for the synthesis of ^{32}P-labeled *O*-hexadecyl dihydroxyacetone phosphate and the dimethyl ketal of palmitoyl [2-^{14}C]dihydroxyacetone phosphate.



Scheme 1. *O*-Hexadecyl dihydroxyacetone-3-phosphate.



Scheme 2. Palmitoyl dihydroxyacetone phosphate.

Experimental

Materials

Silver oxide (Ag_2O), purified powder, was obtained from General Chemical (Division of Allied Chemical Corporation), 1-iodohexadecane, ethyl glycolate and tri-methylorthoformate were obtained from Eastman Kodak Company, (Rochester, NY). Oxalyl chloride, palmitoyl chloride, dibenzyl phosphoric acid and palladium on charcoal were purchased from Aldrich Chemical Co. $H_3{}^{32}PO_4$ and [1-^{14}C]glycolic acid were obtained from Amersham. Purity of 1-*O*-iodohexadecane and palmitoyl chloride were checked by gas-liquid chromatography and were found to be 98% and 94% respectively, the remainders being some homologous (14:0? 18:0) compounds. The gas chromatography (Varian Aerograph Model 920 with thermal conductivity detector) was done in a 5′ × 1/4″ OV-101 column on chromosorb 9 (1.5%) at 185°C with helium gas flowing at 60 ml/min. The iodohexadecane was analyzed directly and the palmitoyl chloride was converted to methyl ester with methyl alcohol and pyridine [11].

All solvents were of analytical grade; dry chloroform was prepared by distilling over P_2O_5 (Baker Chemical Co.), benzene and pyridine were dried over CaH_2 (Aldrich Chemical Co.), xylene and dioxane were freshly distilled over KOH pellets before use. Anhydrous diethyl ether from E. Merck (D-6100 Darmstadt) was used without any other prior treatment.

Methods

All melting points were uncorrected and were taken in a Thomas Hoover capillary melting point apparatus. The elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). The infrared (IR) spectra were taken on a Perkin-Elmer Model 283 spectrophotometer and proton magnetic resonance (PMR) spectra were taken on a 360 MKz NMR spectrophotometer of Bruker Co., Model WM-360. The total lipid phosphorus was determined by the method of Ames and Dubin [12].

O-Hexadecyl ethyl glycolate (III)

A mixture of 3.13 ml (10 mmol) of 1-iodohexadecane (I), 2.5 ml (25 mmol) of ethyl glycolate (II), 3.46 g (15 mmol) of Ag_2O and 10 ml of xylene in a 50-ml round-bottomed flask fitted with a refluxing condenser was refluxed for 20 h on a heating mantle. The reaction mixture was cooled, filtered from inorganic residue using a sintered-glass funnel and the xylene was removed at 40–45°C by blowing a stream of nitrogen.

Saponification of III to give IV was carried out by dissolving the above product in 35 ml of ethanol, 10 ml of water and 10 ml of 6N KOH and refluxing for 1.5 h. The reaction mixture was extracted repeatedly (5–6 times) with hexane until no more non-saponifiables were extracted (checked by thin-layer chromatography (TLC), see below). Hexadecyl alcohol was found to be the main side product extracted by hexane (about 25–30%) as seen by TLC using a solvent mixture of petroleum ether/ether/acetic acid (50:50:2, $R_f = 0.45$). Approximately 12 ml of 6N HCl was added to the alkaline-aqueous layer to generate the free acid which was subsequently extracted with diethyl ether. While extracting with ether, continuous shaking of the mixture with occasional addition of some more acid (6 N) helped to dissolve the sparingly soluble *O*-hexadecyl glycolic acid in the ethereal layer. After complete extraction with ether, the ether layer was washed with water several times to remove inorganic acid and then dried over anhydrous Na_2SO_4. Evaporation of ether and recrystallization twice from hexane gave rise to 1.8 g (60% of theoretical) of pure *O*-hexadecyl glycolic acid (IV), m.p. 62–64°C. TLC in petroleum ether/ether/acetic acid (50:50:2) gave a single spot having R_f value of 0.37. Analysis: calc. for $C_{18}H_{36}O_3$: C 72.00, H 12.07; found: C 72.12, H 12.16.

The IR spectrum (in KBr) showed the presence of expected C–O–C band (1140 cm^{-1}), C=O absorption band of the carboxylic acid (1710 cm^{-1}), OH (broad, 3040–3200 cm^{-1}), sharp CH_2 and CH_3 (2960, 2930, 2862, 1475 cm^{-1}) and CH_2 (rocking, 715 cm^{-1}). PMR ($CDCl_3$) data showed the peaks at δ 0.85 (t, 3H, J = 7 cps, for terminal CH_3 of long chain C-atom), 1.24 (s, sharp, 26 H for next 13 long chain CH_2), 1.60 (t, 2H for CH_2 of the 15th C-atom from terminal CH_3), 3.55 (t, 2H for the last CH_2 of the C-chain attached to ether

bond), 4.1 (s, sharp, 2H for CH_2 adjacent to C=O) and 14.36 (s, broad, for acidic OH).

O-Hexadecyl hydroxyacetyl chloride (V)

To 1.2 g (4 mmol) of compound IV in 4 ml of dry benzene, 4 ml of oxalyl chloride (a large excess) were added and incubated at 37°C for 15 h. Benzene and excess oxalyl chloride was then removed by blowing a gentle stream of nitrogen in a water bath at 35–40°C. Three 3-ml portions of dry benzene were added and the solvents was evaporated again under nitrogen to completely eliminate the excess oxalyl chloride. The process was repeated once more. The acid chloride was immediately converted to compound VI by condensing with freshly prepared diazomethane as follows.

1-O-Hexadecyl-3-diazohydroxyacetone (VI)

Ethereal solution of distilled diazomethane was prepared from Diazald (Aldrich Chemical Co.) according to the procedure of de Boer and Backer [13]. The above material (compound V) was dissolved in 10 ml of anhydrous ether and added in 10 min with stirring to 30 mmol of diazomethane (freshly prepared in about 100 ml of ether) which was cooled to −5°C in an ice-salt bath. The reaction mixture was stirred by a magnetic stirrer for an additional 0.5 h at −5°C and at room temperature for 0.5 h. The excess of diazomethane was removed by blowing N_2 at room temperature. The ether solution was concentrated to about one-third of the original volume and then cooled at −20°C for 0.5 h. The pale yellow crystals obtained were filtered under suction through a sintered-glass funnel precooled at −20°C. The crystals obtained were redissolved in a minimum volume of ether, filtered quickly and the filtrate was cooled for 20–30 min at −20°C. The recrystallized very pale yellow silky product obtained was filtered as above, dried in a vacuum dessicator over P_2O_5. The yield was 1.05 g (81%), m.p. 60°C.

The main byproduct formed during the reaction was checked before crystallization of the crude diazo-compound and was found to be the chloro compound ($C_{16}H_{33}OCH_2COCH_2Cl$) in trace quantity (<5%). This was identified by TLC using the solvent system, petroleum ether/ether/acetic acid (50:50:2) where the chloro-compound had an R_f value of 0.68. Authentic standard chloro-compound was prepared by decomposing the diazo compound with HCl. The R_f value of the diazo compound itself was 0.60. The recrystallized 1-*O*-hexadecyl-3-diazohydroxyacetone showed the following analytical results. Analysis: calc. for $C_{19}H_{36}N_2O_2$: C 70.32, H 11.18, N 8.63; found: C 70.37, H 11.29, N 8.58.

The IR spectrum (in KBr) showed the absorption bands for C–O–C (1130–1140 cm^{-1}), C=O (1665 cm^{-1}), CHN_2 (3155, 2120 cm^{-1}), CH_2 and CH_3

(2965, 2930, 2860, 1475, 1365 cm^{-1}) and CH_2 (rocking, 715 cm^{-1}). PMR ($CDCl_3$) data showed the peaks at δ0.85 (t, 3H, J = 7 cps, for terminal CH_3 of long chain C-atom), 1.24 (s, sharp, 26H for next 13 long chain CH_2), 1.57 (t, 2H for CH_2 of the 15th C-atom from terminal CH_3), 3.45 (t, 2H for the last CH_2 of C-chain attached to ether bond) 4.01 (s, sharp 2H for CH_2 adjacent to C=O), 5.73 (s, sharp, 1H for methine of CHN_2).

1-O-Hexadecyl dihydroxyacetone-3-phosphate (VII)

About 50 mg (154 μmol) of the diazo compound (VI) was dissolved in about 0.5 ml of dioxane which was then added to 0.5 ml of a mixture of dioxane and H_3PO_4 (1.2 mmol) (1 ml of dioxane +0.2 ml of 85% H_3PO_4) at 70–72°C. The whole reaction was carried out in a 2-ml Reactivial (Pierce Chemical Co., Rockford, IL) containing a small triangular magnetic stirring bar and the vial was heated at 70–72°C with constant stirring for 1 h. The solution was cooled, transferred to a tube with 2 ml of diethyl ether and 2 ml of water were added, mixed well, centrifuged and the ether layer was collected. Ether extractions were performed three more times, each time adding 2-ml portions of ether. The combined ether layer was washed twice with water, then the solvents were completely removed by blowing a stream of N_2 gas. Traces of water was removed by adding benzene and re-evaporation under N_2. The crude product was subjected to column chromatography on silicic acid (Unisil) for purification.

Purification by column chromatography

The dried phospho-compound (VII) was applied in $CHCl_3$ to a 5 g silicic acid (Unisil) column (1 cm i.d.) and the column was eluted with three different solvent mixtures, viz. 80 ml of chloroform (Fraction 1), 80 ml of chloroform/methanol (7:3) (Fraction 2) and 80 ml of chloroform/methanol (1:2) (Fraction 3). Solvents from these fractions were removed in a rotary evaporator at 40°C and finally dried completely by blowing a stream of N_2 gas at room temperature. TLC of the product from different fractions using a solvent system of $CHCl_3$/methanol/acetic acid/water (100:40:12:4) showed the presence of 1-*O*-hexacecyl dihydroxyacetone-3-phosphate mainly in Fraction 2 (R_f value 0.35) with a negligible amount in Fraction 3. Besides the desired product, some 1-*O*-hexadecyl dihydroxyacetone was found as a main byproduct and eluted in Fraction 1 (R_f value 0.90 on TLC).

The residue from Fraction 2 was dissolved in 10 ml of chloroform/methanol (2:1) to which 0.5 ml of 2 N HCl and 2 ml of water were added, mixed well and centrifuged. The upper aqueous layer was removed and the chloroform extract was washed twice with a mixture of $CHCl_3$/methanol/water (1:12:12). The lower $CHCl_3$ layer was finally taken out, dried by blowing N_2 and suspended in cyclohexane and lyophilized. The solid material was dried again under vacuum in a desiccator over KOH. The

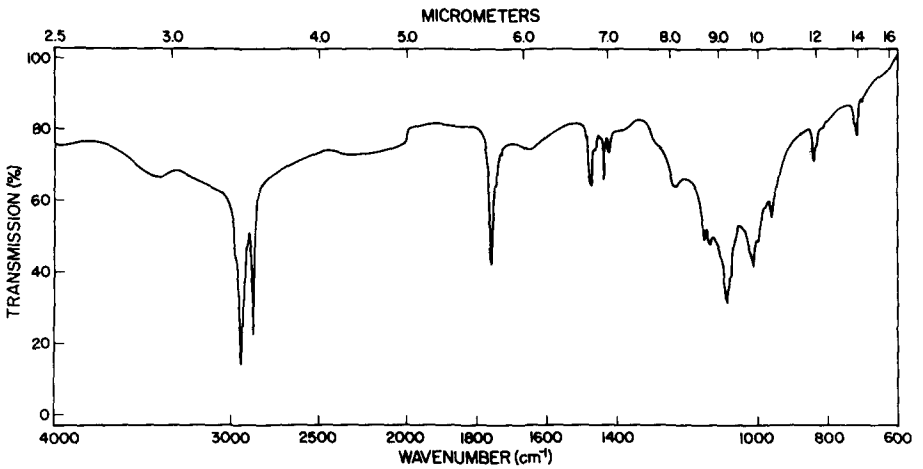


Fig. 1. IR spectrum of 1-*O*-hexadecyl dihydroxyacetone-3-phosphate.

yield was 37 mg (60%) and m.p. 85–86°C. Analysis: calc. for $C_{19}H_{39}O_6P$: C 57.85, H 9.96, P 7.85; found C 57.71, H 9.74, P 7.6.

The IR spectrum (in KBr) is shown in Fig. 1. Characteristic bands were C–O–C (1085 cm^{-1}), C=O (1757 cm^{-1}), CH_2 and CH_3 (2930, 2865,1475, 1435 and 1380 cm^{-1}), CH_2 (rocking, 720 cm^{-1}). and OH of $-PO_3H_2$ (broad at 1640 and 1015 cm^{-1}). The PMR spectroscopy (Fig. 2) of VII was performed using 2–3 mg of the sample dissolved in a solvent of 0.5 ml of $CDCl_3$ which contained a drop of $(CD_3)_2SO$. The peaks were at δ0.75 (t, 3H, J = 7 cps, for CH_3 denoted by a), 1.15 (s, sharp, 26 H for CH_2 denoted by b) 1.45 (t, 2H for

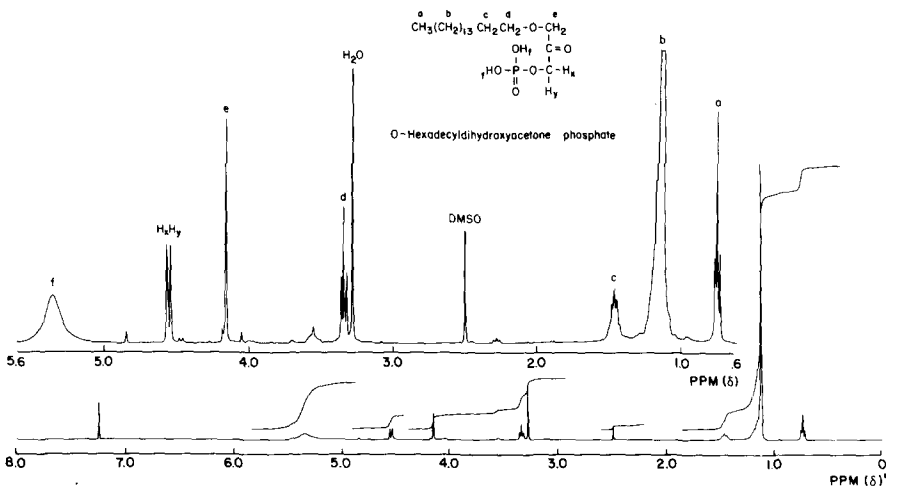


Fig. 2. PMR spectrum of 1-*O*-hexadecyl dihydroxyacetone-3-phosphate.

CH_2 denoted by c), 3.36 (t, 2H for CH_2 attached to glycerol via ether bond, denoted by d) 4.15 (s, sharp, 2 H for CH_2 denoted by e) 4.55 (d, 2H of the CH_2 attached to phosphoester bond, denoted by H_x and H_y), 5.34 (s, broad, OH attached to phosphorus and denoted by f). The peaks at δ 2.49 and 7.26 were due to the presence of protons in DMSO-d_6 and $CDCl_3$ used as solvents and that at δ 3.28 was due to H_2O possibly absorbed during operation. Irradiation corresponding to the H-nucleus having peaks at δ 1.45 (peak no. c, Fig. 2) and 3.36 (peak no. d, Fig. 2) and the results of respective effect due to decoupling from the irradiated nucleus clearly confirmed the presence of $-CH_2-CH_2-O$-moiety.

1-O-hexadecyl-dihydroxyacetone-3-[^{32}P]phosphate

The method described above was used to prepare different radioactively-labeled derivatives of alkyl dihydroxyacetone phosphate. An example is given here, where the purification method was modified to remove a major phosphodioester byproduct which was inevitably formed when a stoichiometric amount, instead of an excess, of $H_3\,^{32}PO_4$ was used to decompose the intermediate diazo derivative. Five milliCuries of carrier free $H_3\,^{32}PO_4$ in 1 ml of 0.01 N HCl was dried down in a 3 ml Reactivial (Pierce Chemical Co.) kept at 50°C in a water bath by blowing a stream of N_2 gas. Last traces of water were removed by adding 1 ml dry benzene to the residue and drying under N_2. The process was repeated twice. Non-radioactive carrier H_3PO_4 (25 μmol) in 0.5 ml dioxane was added and the mixture was dried again by blowing N_2. After the water was removed by drying in the presence of benzene as described above, the mixture was dissolved in 0.5 ml dioxane. The vial has heated at 70°C with constant stirring using a triangular magnetic bar and 0.1 ml of dioxane containing 25 μmol of VI was added into the mixture. The mixture was stirred constantly for 2 h at 70°C, then cooled to room temperature and transferred to a screw-topped test tube with 5 ml diethyl ether. The ether layer was washed 2–3 times with a solution of KCl (2 M)–H_3PO_4 (0.02 M) and the formation of alkyl dihydroxyacetone-3-[^{32}P]phosphate was checked by TLC ($CHCl_3$/methanol/acetic acid/water, 25:10:3:1) and autoradiography. The expected product was seen on the radioautogram (R_f = 0.35) along with a faster-moving spot (R_f = 0.65). The latter byproduct was identified as the phosphodiester (i.e. bis(1-*O*-alkyl dihydroxyacetone)phosphate) derivative formed as a byproduct due to use of equivalent amounts of H_3PO_4 and the diazo-compound for the reaction. A solvent partition method [14] was used to purify the product. The crude mixture was dissolved in 2.4 ml of 0.1 M triethanolamine buffer (pH 8.0) to which 9 ml $CHCl_3$/methanol (1:2) was added and mixed well. To this one phase mixture, 3 ml $CHCl_3$ and 3 ml water were added. After mixing, the two layers were separated from each other by centrifugation. The upper layer containing most of the alkyl dihydroxyacetone [^{32}P]phosphate was

transferred to another tube. The lower layer containing the phosphodiester impurity was extracted with 5 ml of a mixture of $CHCl_3$/methanol/water 1:12:12). The combined upper layer was acidified with 1 N HCl and extracted twice with 4 ml $CHCl_3$. The $CHCl_3$ layer contained the alkyl dihydroxyacetone-3-[^{32}P]phosphate free from any other radioactive impurities. The yield of alkyl dihydroxyacetone-3-[^{32}P]phosphate from $^{32}P_i$ was 10%. The labeled compound was found to be enzymatically as active towards alkyl dihydroxyacetone phosphate: NADPH oxidoreductase as other chemically or biochemically synthesized alkyl dihydroxyacetone phosphate. After reduction with $NaBH_4$ the product was 1-alkyl-*rac*-glycerol-3-[^{32}P]phosphate which was used as a substrate either for lipid phosphomonoesterase [7] or for the alkyl glycerophosphate:acyl CoA acyltransferase [15].

Preparation of 1-O-palmitoyl dihydroxyacetone phosphate

1-*O*-Palmitoyl dihydroxyacetone phosphate was prepared in a manner essentially similar to that of 1-*O*-hexadecyl dihydroxyacetone phosphate. The method had already been reported briefly in a previous work of Hajra and Agranoff [5]. The detailed procedure for the synthesis of palmitoyl glycolic acid is described below. Two grams of glycolic acid was taken in a stoppered ice-cooled 50-ml round bottomed flask containing a magnetic bar and the flask was fitted with a dropping funnel and a $CaCl_2$ guard tube. To this was added 6 ml of anhydrous pyridine and 2.5 ml of alcohol free dry $CHCl_3$ and the mixture was kept in a water bath at 10–15°C. The reaction was started with very slow addition of 6.56 g of palmitoyl chloride in 12.5 ml of dry chloroform from the dropping funnel. The addition was completed within 40–45 min, and the mixture was stirred continuously through the course of the reaction. Stirring was continued for an additional period of 1–1.5 h at 10–15°C and 1 h at room temperature. After the reaction was over, most of the solvent was removed by evaporation under N_2.

About 200 ml of each of ether and water were added and the whole mixture was shaken in a separatory funnel. The aqueous layer was removed and the ether layer was washed twice with methanol/0.2 N HCl (1:1) to remove pyridine and then with water to remove inorganic acid. The ether extract containing the product *O*-palmitoyl glycolic acid was dried over anhydrous Na_2SO_4. The solvents were evaporated in a rotary evaporator and the residue was crystallized from *n*-heptane. The crystals were filtered, washed with a little *n*-heptane and dried under vacuum. The yield was 5.54 g (73.6%) and m.p. 83–85°C. The rest of the procedures for preparation of acid chloride, diazoketone and that of 1-*O*-palmitoyl dihydroxyacetone phosphate were the same as described under 1-*O*-hexadecyl dihydroxyacetone phosphate. Final yield of the purified palmitoyl dihydroxyacetone phosphate was found to be 68.2% having m.p. 80–81°C.

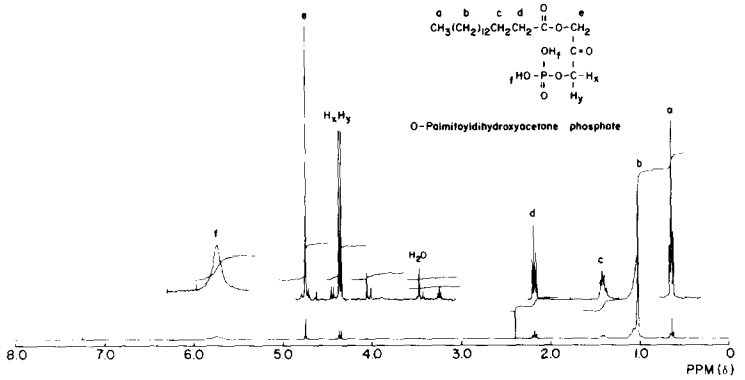


Fig. 3. PMR spectrum of 1-*O*-palmitoyl dihydroxyacetone-3-phosphate.

Analysis: Calc. for $C_{19}H_{37}O_7P$: C 55.85, H 9.13, P 7.57; found: C 55.69, H 9.10, P 7.3.

The structure of 1-*O*-palmitoyl dihydroxyacetone phosphate was further confirmed by IR [16] and PMR spectra (Fig. 3) as before.

1-O-hexadecanoyl-[2-^{14}C]dihydroxyacetone-3-phosphate, dimethyl ketal

The above method can be modified to prepare stable derivatives of acyl and alkyl dihydroxyacetone phosphate. This is illustrated below by describing the preparation of the ^{14}C-labeled derivative of the dimethyl ketal of palmitoyl dihydroxyacetone phosphate and its hydrolysis to the free ketone.

[1-^{14}C]Glycolic acid along with carrier glycolic acid (final spec. act. 5 μCi/μmol, 20 μmol total) was dissolved in 10 ml cyclohexane in a 25-ml round bottom flask and then lyophilized to dryness. The residue was dissolved in 0.5 ml dry pyridine (cooled in an ice-water bath) to which 25 μmol of palmitoyl chloride in 1 ml dry $CHCl_3$ was slowly added (15 min). The mixture, protected from moisture by a $CaCl_2$ dry tube, was continuously stirred with a Teflon-covered magnetic bar for 1 h at 4°C and another hour at room temperature. The reaction was stopped by adding a few drops of water and most of the solvents were removed by blowing a stream of N_2 while the flask was kept in a water bath at 35°C. The residue was dissolved in ether and the ether extract was washed twice with 0.1 N HCl and once with water. The total radioactivity recovered in the ether layer was 83% of the starting amount. On silica gel TLC (petroleum ether/ether/acetic acid, 50:50:2) only one radioactive spot corresponding to authentic palmitoyl glycolic acid (R_f = 0.36) was found.

The rest of the ether from the extract was removed by blowing a stream of nitrogen and the residue was dried twice after adding 2 ml of dry benzene each time to remove traces of water. The dry residue was then dissolved in

0.5 ml dry benzene to which 0.5 ml oxalyl chloride was added. The stoppered flask was incubated at 37°C overnight. At the end of incubation the oxalyl chloride and benzene was removed by blowing N_2 at 40°C. The residue was dried twice under N_2 after adding 1 ml of dry benzene each time. It was then dissolved in 1 ml of dry diethyl ether and the solution was then slowly added to a freshly distilled solution of diazomethane (1.5 mmol) in 3 ml of ether at −5°C. After the addition was complete (15 min), the mixture was stirred for 1 h at −5°C and then an additional 30 min at room temperature. The ether was removed by blowing a stream of nitrogen, the residue was dissolved in 0.5 ml dioxane and then slowly added to a solution of 40 μmol of dibenzyl phosphoric acid in 0.5 ml dioxane contained in a Reactivial at 70°C as described above. The mixture was stirred at 70°C for 2 h, cooled to room temperature and the content was transferred to a screw-topped test tube with 5 ml ether. The ether solution was washed 5 times with 3 ml of 0.1 M $KHCO_3$ and twice with water to remove the excess dibenzyl phosphoric acid. The total recovery of the radioactivity in the washed ether extract, starting from the palmitoyl [^{14}C]glycolic acid, was 85%. On TLC on silica gel plate (hexane/ether/acetic acid, 50:50:2) the main radioactive spot (R_f = 0.41) had >90% of the radioactivity and had the same migration rate as the authentic hexadecanoyl dihydroxyacetone dibenzyl phosphate (The authentic non-radioactive compound was prepared by the same way, purified by chromatography and analyzed (IR, NMR, elemental analysis) to establish the assigned structure.). The dimethyl ketal derivative of the compound was prepared by dissolving it in 1 ml of a mixture of trimethylorthoformate, methanol and conc. H_2SO_4 (100:50:0.6) and incubating at room temperature for 5 h [8]. The reaction was stopped by adding 0.1 g $KHCO_3$ followed by 5 ml water. To the mixture 5 ml $CHCl_3$ was added, mixed well and centrifuged. The upper layer was removed and the lower $CHCl_3$ layer was washed successively with 2 × 5 ml methanol/0.1 M $KHCO_3$ (1:1) and 2 × 5 ml methanol/water (1:1). On TLC ($CHCl_3$/methanol, 96:4), the major radioactive spot (~90% radioactivity) had an R_f = 0.72 with the rest of the radioactivity being in a spot having R_f = 0.25. This latter spot was identified to be dimethyl ketal of dihydroxyacetone dibenzyl phosphate, a hydrolytic byproduct formed during the ketalization step. The main compound was purified by a silicic acid column (0.5 cm × 10 cm) chromatography from which it was eluted out with 20 ml $CHCl_3$, leaving behind the impurities in the column. The solvent was removed from the column fraction in a rotary evaporator and the product was debenzoylated by hydrogenolysis as described below. The residue was dissolved in 1 ml ethanol to which 5 mg of palladium on charcoal was added. Ethanol saturated H_2 gas was bubbled through the mixture for 30 min. The mixture was centrifuged and the clear supernatant was transferred to another tube. The residue was washed twice with 1 ml ethanol. The ethanolic solution was neutralized with cyclohexyl-

amine and the product was purified, after drying, on another silicic acid column (0.5 cm × 10 cm). The column was first eluted with 20 ml $CHCl_3$, followed by 20 ml $CHCl_3$/methanol (8:2). All the radioactivity was eluted in Fraction 2. When checked by TLC ($CHCl_3$/methanol/acetic acid/water, 25:10:3:1) Fraction 2 was found to contain a single radioactive spot (R_f = 0.75) the same as that of authentic palmitoyl dihydroxyacetone phosphate dimethyl ketal. The solvent was removed from Fraction 2 in a rotary evaporator, the residue was dissolved in methanol and the solution was passed through a Dowex 50-X8, cyclohexylammonium form column (0.5 cm × 5 cm) and washed out with 10 ml methanol. The eluate was dried by blowing a stream of N_2, suspended in cyclohexane and lyophilized to dryness. The fluffy white powder, palmitoyl [2-^{14}C]dihydroxyacetone phosphate, dimethyl ketal, dicyclohexylammonium salt, was found to be stable (no other radioactive spots on TLC) on storage at −20°C over Drierite. The total yield was 55% (55 μCi) starting from the [1-^{14}C]glycolic acid. The free ketone was regenerated from the dimethyl ketal by the following way. The dimethyl ketal (10 μCi, 2 μmol) was dissolved in 1 ml methanol/water (1:1) and then passed through a Dowex 50-X4 H^+ column (0.5 cm × 4 cm). The column was washed with an additional 2 ml of methanol/water. The eluate was incubated at 37°C for 4 h, evaporated to dryness and dissolved in $CHCl_3$/methanol (2:1). On TLC, a complete conversion of the dimethyl ketal (R_f = 0.75) to the free ketone form (R_f = 0.4) was observed. The radioactive material was also found to be an active substrate for the acyl dihydroxyacetone phosphate: NADPH oxidoreductase and also for the alkyl dihydroxyacetone phosphate synthase [1,6]. In the dry state (free ketone) the material was not very stable on storage at −20°C (the decomposition products could be seen on TLC after 2 weeks) but is somewhat more stable (for 1–2 months) in $CHCl_3$/methanol (1:1) at −20°C.

Discussion

Using the methods described above the long chain ester and ether derivatives of dihydroxyacetone phosphate can be chemically synthesized in a few steps at high yields. In contrast, the procedures described by Piantadosi and coworkers [9,10] for the preparation of these compounds consisted of a number of steps with the resultant low yields of the final compounds. For example, starting from chimyl alcohol these authors described the synthesis of alkyl dihydroxyacetone phosphate in nine steps with a final yield of less than 8% [9]. Whereas, as described above, in the present method starting from hexadecyl glycolic acid, the hexadecyl dihydroxyacetone phosphate is synthesized in three steps with a final yield of about 50%. We also encountered problems in reproducing some of the procedures

described by Piantadosi et al.; for example, the methods for the hydrolysis of dimethyl ketal either by using 0.1 N HCl [9] or anhydrous HCl gas [17], resulted in incomplete hydrolysis and also decomposition of the acyl and alkyl dihydroxyacetone phosphate. The use of aqueous Dowex $50H^+$ resin [9] was also not feasible as the compound (VII, Scheme 1)) was completely insoluble in water at low pH. The use of methanol/water and Dowex $50H^+$ resin, as described above, turned out to be the most suitable method in our view for the regeneration of the ketone from the ketal.

The long-chain ether intermediate, i.e. *O*-alkyl glycolic acid is prepared by a new method with a moderate yield. This compound could be purified easily by saponification as described above. The *O*-hexadecyl glycolic acid can also be prepared by the oxidation of chimyl alcohol with a mixture of $NaIO_4$ and $KMnO_4$ [6]. The synthesis of short chain *O*-alkyl derivatives of glycolic acid has been described previously [18]; however, that method was found not to be suitable for the synthesis of the long chain alkyl ethers of glycolic acid.

The aliphatic diazo intermediates have been used for a long time for the synthesis of different organic compounds. Nierenstein and coworkers were the first to show the formation of chloro-ketones from acid chlorides and diazomethane [19,20]. Robinson and coworkers then demonstrated the formation of diazoketone as an intermediate in such reactions [21,22]. Arndt and Eistert in their well-known synthetic procedure described the conversion of an acid to its next higher homolog via the diazoketone intermediate [23]. Wolfram and coworkers used the diazoketone intermediates for the synthesis of a number of monosaccharides [24]. The diazoketones have also been used for the synthesis of sphingolipids and glycerolipids (see the review by Mangold [25]). Schlenk et al. [26] first described the use of the diazoketone intermediates for the chemical synthesis of glycerolipids. These authors synthesized acyl dihydroxyacetone by decomposing the acyloxydiazocetone with $HClO_4$ [26]. We showed that the same intermediate could be decomposed with H_3PO_4 to form acyl dihydroxyacetone phosphate [5]. We also improved the yield of the diazoketone by making a reverse addition, i.e. adding the acid chloride to excess diazomethane, from that described by Schlenk et al. This reverse addition prevented the formation of the chloroketone by immediately removing the HCl as CH_3Cl by reaction with the excess diazomethane. Here, we extend the procedure described for the synthesis of acyl dihydroxyacetone phosphate to synthesize the alkyl derivatives of dihydroxyacetone phosphate. After we described this method of producing phosphate esters [5], we discovered that almost 40 years ago Reichstein and Schindler used a similar phosphorolysis of diazoketone to synthesized deoxycorticosterone phosphate [27].

Use of a large excess of H_3PO_4 for the decomposition of the diazoketones minimized the formation of phosphodiesters. However, when the phos-

phodiesters are formed, these can be easily removed from the desired products by solvent partitions as described for the synthesis of alkyl dihydroxyacetone [^{32}P]phosphate. The other byproducts (mainly acyl or alkyl dihydroxyacetone) are easily separated by chromatography on silicic acid. It was found that the phosphorylated compounds are co-eluted in Fraction 2 with some impurities from silicic acid (probably Na^+ ions) which made these phosphate esters sparingly soluble in $CHCl_3$. Erratic analytical values were also obtained with these phosphate esters recovered directly from the silicic acid columns. A simple acid wash, as described above, removed these impurities so that there was no problem in dissolving these phosphate esters in $CHCl_3$ or in obtaining good analytical results after this acid treatment.

The synthesized acyl and alkyl dihydroxyacetone phosphate are found to be stable to a brief exposure (10–15 min) to 1 N HCl at room temperature. As described previously [5], the acyl dihydroxyacetone phosphate is extremely labile towards mild alkaline treatment. Alkyl dihydroxyacetone phosphate is found to be stable towards alkali (1 N NaOH) at room temperature for a short period of time (10–15 min). However, alkyl dihydroxyacetone phosphate was found to be somewhat labile towards acid and alkali at elevated temperature. About 20% of hexadecyl dihydroxyacetone phosphate was found to be decomposed to hexadecanol and water soluble phosphate derivatives by heating at 100°C in either 1 N HCl or 1 N NaOH. Both acyl and alkyl dihydroxyacetone phosphate are found to be stable on storage in $CHCl_3$ at −20°C for up to 1 month. Prolonged storage (>6 months) under such conditions did result in some decomposition as indicated by the presence of a number of spots, other than acyl or alkyl dihydroxyacetone phosphate, on thin-layer chromatograms. The diazoketones are fairly stable when stored over desiccants at −20°C. We found that it is preferable to store the diazoketone intermediate for a long period of time and prepare from time to time as needed the acyl or alkyl dihydroxyacetone phosphate from this diazoketone intermediate.

The method described here may be further extended to synthesize a number of other phospholipids. We have previously described that lysophosphatidic acid (*rac*) and its ether analog can be synthesized by the reduction of acyl and alkyl dihydroxyacetone phosphate with $NaBH_4$ [7,15]. In preliminary experiments we found that the hexadecyloxy diazo acetone (Compound VI, Scheme 1) could be decomposed with phosphatidic acid (free acid form) to form 1-*O*-alkyl-3-phosphatidyl-dihydroxyacetone in moderate (20–30%) yield. Work in our laboratory is presently directed towards the use of other phosphate esters such as inositol phosphate or (N-protected) ethanolamine phosphate etc., for the chemical synthesis of different phosphate esters from these diazoketone intermediates.

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