A simple chemical synthesis of the ether analog of lysophosphatidylcholine and platelet-activating factor

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Summary A simple chemical procedure for synthesis of 1-O-alkyl(\(\text{rac}\) or \(\text{sn}\))glycerol-3-phosphocholine (alkyl analog of lysophosphatidylcholine, II) and platelet activating factor (PAF), 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine (III) has been described. The key step of the method is the decomposition of 1-O-hexadecyl-3-diahydroxyacetone (A. K. Hajra, T. V. Saraswathi and A. K. Das, 1983. Chem. Phys. Lipids. 33: 179–193) with phospholipase to synthesize 1-O-hexadecyl dihydroxyacetone-3-phosphocholine (I). Conditions for this phospholysis were studied with respect to the reaction medium, temperature, and optimum proportion of the reactants. Compound (I) was quantitatively reduced with \(\text{NaBH}_4\) to synthesize (II) which was acetylated to prepare compound (III). Phospholipase \(A_2\) hydrolysis of compound (III) followed by separation of the products afforded the unreacted sn-3-hexadecyl isomer (III) and sn-1-hexadecyl isomer (II) which was acetylated to PAF. The structures of the compounds were verified by NMR and FAB-MS spectra, and their biological activities were determined by measuring the release of serotonin from rabbit blood platelets in response to different doses of these compounds. The suitability of the method as a general technique for synthesis of different ether phosphoglycerides is discussed.—Das, A. K., and A. K. Hajra. A simple chemical synthesis of the ether analog of lysophosphatidylcholine and platelet-activating factor. J. Lipid Res. 1995. 36: 2459–2468.

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Platelet-activating factor (PAF), an ether phospholipid, has potent biological activity in a variety of cellular phenomena such as platelet aggregation, chemotaxis, hypotension, bronchoconstriction, vascular permeability regulation, inflammation, glyco- genolysis, and in regulating cellular breakdown of phosphatidylinositol (1–3). The naturally occurring substance is secreted from antigen-stimulated IgE-sensitized basophils and also from other activated cells (1, 2). The structure of PAF has been established as 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine (1–3). Molecules containing alkyl chain lengths of both C16 and C18 have been shown to exhibit PAF activity (1–3). A number of convenient semi-synthetic methods have been described (4–7) for preparation of this bioactive molecule. However, because the semi-synthetic procedures start from naturally occurring glycerol ether lipids (choline plasmalogen from beef heart or alkylacyl glycerol from shark liver oil) such syntheses (4–7) lead to the production of PAF with a mixture of alkyl chains. A number of chemical synthetic procedures using D-mannitol or D- or L-tartrate or epoxides as the starting material have been described to prepare this compound with specific alkyl chain length composition and steric configuration (8–12). However, these methods are lengthy, complicated multistep procedures. A number of workers introduced a shorter route for chemical synthesis of PAF and lysoPAF starting from synthetic 1-O-alkyl sn-glycerol derivatives (13–15). The overall yield, however, in these methods is reported to be relatively low (<10%).

Previously, we have described methods of preparing 1-O-acyl and 1-O-alkyl dihydroxyacetone-3-phosphate in high yield by decomposing the corresponding 1-O-acetyl and 1-O-alkyl-3-diahydroxyacetone with phosphoric acid (16). These keto lipids could be chemically or enzymatically reduced to form the corresponding 1-rac- or sn-1-glycero-3-phosphate (17). It has been indicated that the intermediate alkyl or acyl diahydroxyacetone could also be decomposed by different phosphorylated compounds to introduce different head groups to the glycerol moiety (16). We describe here such a novel procedure for the preparation of 1-O-hexadecyl dihydroxyacetone-3-phosphocholine (I), which was chemically reduced to 1-O-hexadecyl-rac-glycero-3-phosphocholine, the ether analog of lysophosphatidylcholine (lysoPtdCho) or lysoPAF (II), and subsequently acetylated to form 1-O-hexadecyl-2-acetyl-rac-glycero-3-phosphocholine, the PAF (III) (Scheme 1). The natural form of PAF (III) was made by hydrolyzing rac-PAF with phospholipase \(A_2\) followed by re-acetylation of the 1-alkyl sn-glycerol derivative. The preparation of \(^3\)H- and \(^14\)C-labeled derivatives of PAF and the possible application of the method for the synthesis of similar compounds are also described in this paper.

EXPERIMENTAL PROCEDURES

Materials

The Ca-salt of phosphocholine chloride, \(\text{NaBH}_4\), 4-pyrrolidinopyridine (4PP), acetic anhydride, lysoPtdCho, 1-O-hexadecyl-2-acetyl-rac-glycero-3-phosphocholine (PAF) and phospholipase \(A_2\) (lyophilized

Abbreviations: PtdCho, phosphatidylcholine; PEtn, phosphorylethanolamine; PtdIns, phosphatidylinositol; PAF, platelet-activating factor; PMR, proton magnetic resonance; 4PP, 4-pyrrolidinopyridine; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography.

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powder from *Naja naja* venom) were purchased from Sigma Chemical Co. (St. Louis, MO). The cationic exchange resin AG 50W-X4, (H+ form, 200-400 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA). E. Merck silica gel 60 plates (EM Science, Gibbstown, NJ) were used for thin-layer chromatography (TLC). [Methyl-$^{14}$C]phosphorylcholine (55 mCi/mmol), [2-$^{14}$C]serotonin (40 mCi/mmol), and NaB$_3$H$_4$ (15 Ci/mmol in 0.1 N NaOH) were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively. 1-O-hexadecyl-3-diazo-hydroxyacetone was prepared as described previously (16). All solvents were of analytical grade. Chloroform was distilled over P$_2$O$_5$ and dioxane from KOH pellets.
before use. All other solvents obtained as analytical reagents from Aldrich Chemical Co. (Milwaukee, WI) were used without any further purification.

Methods

All melting points were taken in a Thomas Hoover capillary melting point apparatus and are reported uncorrected. Proton magnetic resonance (PMR) spectra were taken on a 360 MHz NMR spectrophotometer from Bruker Co., Model WM-360. The TLC plate was sprayed with primuline to visualize all the lipids (18) followed by molybdenum blue spray (19) to detect phospholipids. The total lipid phosphorus was assayed according to Ames (20). Positive ion fast atom bombardment mass spectra (FAB-MS) were taken on a Model 70-70E analytical mass spectrometer (VG-Analytical Co., Manchester, England). Nitrobenzyl alcohol was used as the solvent matrix and the solvent background was subtracted from the spectra obtained.

Preparation of Ca\(^{2+}\)-free phosphorylcholine

One hundred fifty mg (500 pmol) of the Ca-salt of phosphocholine chloride was dissolved in about 1 ml H\(_2\)O and the solution was passed through a small AG 50W-X4 (H\(^+\)-form) column (0.6 × 4 cm). The column was further washed with 8 ml H\(_2\)O and the eluates were combined. The combined acidic (pH ~ 1.85) eluate was shown to be Ca\(^{2+}\)-free, as indicated by the non-precipitation of calcium oxalate when potassium oxalate was added to the neutralized eluate. The Ca\(^{2+}\)-free aqueous solution of phosphocholine was lyophilized overnight in a Speed Vac concentrator. The solid glossy mass was dissolved in 1 ml of dimethyl sulfoxide (DMSO) by sonication for 3–5 min to obtain a clear solution. When not used immediately, this solution was stored at 4°C.

Synthesis of 1-O-hexadecyl-dihydroxyacetone-3-phosphocholine (I)

The optimum reaction conditions, such as solvent composition of the reaction medium, reaction time, temperature, and the molar ratio of the substrate concentrations, were studied for the decomposition of 1-O-hexadecyl-3-diazohydroxyacetone with phosphocholine. Different solvent mixtures were tried to make a homogenous reaction medium with the reactants, and a mixture of dioxane-dimethyl formamide (DMF)-DMSO 1:2:4 (by volume) was found to be most suitable (see Discussion). The optimum concentrations and ratio of the reactants, the optimum temperature for the reaction, and isolation of the products were studied by using hexadecyl diazohydroxyacetone and phospho[methyl-\(^{14}\)C]choline as shown in Fig. 1. Maximum yield was obtained when the molar ratio of the phosphocholine to the diazoketone was about 3.0 (Fig. 1A).

![Fig. 1. Optimum substrate concentrations, molar ratio of the reactants, and temperature of the reaction for the phosphorolysis of 1-O-hexadecyl-3-diazohydroxyacetone by phosphocholine.](image-url)

A: The reaction mixtures contained 3 pmol of 1-O-hexadecyl-3-diazohydroxyacetone and increasing concentrations (1.5–30 pmol) of phospho[methyl-\(^{14}\)C]choline (sp. act. = 40,000 cpm/pmol) in 0.14 ml of dioxane-DMSO-DMF 1:2:4 mixture. The mixtures were magnetically stirred for 24 h at 72°C and the radioactive lipid was isolated by CHCl\(_3\)-methanol extraction as described in the text. The radioactivity in the washed CHCl\(_3\) layer was determined to calculate the amount of 1-O-hexadecyl-dihydroxyacetone-3-phospho[methyl-\(^{14}\)C]choline formed from the diazoketone.

B: The reaction mixture contained 3 pmol of the diazoketone, 9 pmol of phospho[methyl-\(^{14}\)C]choline in 0.14 ml dioxane-DMSO-DMF 1:2:4 as described above. The magnetically stirred mixtures were heated at three different temperatures (72°C, 80°C, and 90°C) for different periods of time as indicated. The amount of the radioactive lipid product formed was determined as described in A.

Under such conditions, increasing yield (up to 25%) with shorter reaction time was obtained with increasing reac-
tion temperature (Fig. 1B). However, decomposition of the product was also seen with the increase in temperature and the colored impurities formed were difficult to separate from the desired compound. Therefore, an optimum reaction temperature between 72° and 75°C was used. The details of the preparative method are described below.

Fifty mg (150 μmol) of 1-O-hexadecyl-3-diazohydroxyacetone was dissolved in 0.75 ml distilled dioxane. To this was added 450 μmol of Ca²⁺-free phosphocholine in 0.9 ml of DMSO followed immediately by the addition of 1.5 ml of DMF and 2.1 ml of DMSO. The whole turbid mixture (5.25 ml) was then sonicated for 3–5 min and heated on a Reacti-Therm (Pierce Chemical Co., Rockford, IL) at 72–75°C with continuous magnetic stirring. The solution became clear within a few minutes (if not, a small portion of DMSO was added till the solution was visibly clear). After a period of 24 h, the reaction was stopped by cooling the mixture to room temperature and dissolving in 26 ml of CHCl₃-methanol 1:1. To the solution, 6.5 ml H₂O was added, mixed well, and centrifuged to separate the phases. The lower layer was saved and the upper layer was re-extracted two times, each with about 5 ml CHCl₃. Chloroform from the combined extracts was removed by vacuum evaporation in a rotary evaporator. The oily residue, which contained the reaction product and a portion of the high boiling DMSO and DMF from the reaction mixture, was dissolved in a few ml of CHCl₃ and loaded onto a silicic acid (Unisil) column (1 x 12 cm). CHCl₃ (100 ml) was first used as the eluant which eluted out all of the DMSO and DMF (fraction 1). The column was subsequently eluted with 100 ml of CHCl₃–methanol 7:3 (fraction 2), 150 ml of CHCl₃–methanol 1:4 (fraction 3) and 100 ml of methanol (fraction 4). When examined by thin-layer chromatography (CHCl₃–methanol–acetic acid–10% (w/v) aqueous sodium bisulfite 60:40:12:8), fraction 3 was found to contain most of the desired phosphorylated product, i.e., 1-O-hexadecyl-dihydroxyacetone-3-phosphocholine (Rᵣ = 0.11) (molybdenum blue spray) uncontaminated by any other lipid. Unidentified byproducts (primuline-positive but molybdenum blue-negative) of the reaction were eluted out in fractions 1 and 2. The solvent from fraction 3 was removed in a rotary evaporator and finally dried under vacuum. From the phospho-

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2 The bisulfite was included in the solvent to separate the keto-lipid (which forms a bisulfite-addition compound) from the corresponding hydroxy lipid, i.e., lysoPAF (see later). Without bisulfite, the keto and the hydroxy compounds migrate on TLC at the same rate (17).
Fig. 3. Positive ion FAB mass spectrum of compound I. The m/z 480 is the protonated molecular ion. The identification of major fragments is shown in the inset (see text for details).

The PMR (in CDCl₃) data (Fig. 2) showed peaks at 8.07 (t, 3 H, J = 7 Hz, for terminal CH₃ of the long hydrophobic chain denoted by a), 1.24 (s, sharp, 26 H for the next 13 long chain CH₂ denoted by b), 1.56 (t, 2 H for CH₂ of the 15th C-atom from the terminal CH₃, denoted by c), 3.37 (s, sharp, 9 H for N(CH₃)₃ denoted by d), 3.43 (t, 2 H for CH₂ of the 16th C-atom from terminal CH₃, denoted by e), 3.85 (s, broad, 2 H for CH₂, denoted by f), 4.15 (s, sharp, 2 H for CH₂ denoted by g), 4.36 (s, broad, 2 H for CH₂ denoted by h), and 4.68 (d, 2 H for the CH₂ attached to phosphodiester bond, denoted by H₂ and H₃).

The FAB-MS of the compound (Fig. 3) showed the expected protonated molecular species (m/z = 480). The major fragments, i.e., m/z 254, 224, 184, 166, 104, and 86, are identified as shown in the legend of Fig. 3. The fragmentation pattern was similar to that of lysoPAF (15); however, for the keto compound, an additional fragment of significant intensity at m/z 254 was seen. This was probably due to the McLafferty rearrangement.
of the carbonyl group containing fragment as indicated in Fig. 3.

A $^{14}$C-labeled compound (I) was also made by phosphorolysis of 4 mg (12 pmol) of 1-O-hexadecyl-3-diazohydroxyacetone with 36 pmol of [methyl-$^{14}$C]phosphocholine (40,000 cpm/µmol) following the above reaction method. Determination of radioactivity of the extracted $^{14}$C-labeled compound (I) showed a conversion of 18.3% of the diazo compound to (I). On TLC, using the above bisulfite solvent system, the product showed a major (95%) $^{14}$C spot with $R_f = 0.11$, the same as the purified non-radioactive compound (I).

**Reduction of (I) with NaBH$_4$ to 1-O-hexadecyl-rac-glycero-3-phosphocholine (II)**

Ten mg of dry 1-O-hexadecyl dihydroxyacetone-3-phosphocholine (I) was dissolved in 0.2 ml of freshly distilled (over NaBH$_4$) ethyl alcohol by sonication. To the solution, 0.1 ml of 0.3 M Tris-HCl (pH 7.6) and 0.2 ml of NaBH$_4$ (1 M in 0.025 M NaOH) were added and the mixture was kept at 37°C for 2 h (17). The reaction was terminated by adding 3 ml of CHCl$_3$-methanol 2:1 followed by 0.5 ml of 1 N HCl to decompose the excess NaBH$_4$. The aqueous and organic phases were separated out by mixing and centrifuging. The upper layer was removed and the lower layer was washed with 1.5 ml of CHCl$_3$-methanol–H$_2$O 3:48:47. TLC of the product using the above bisulfite-containing solvent showed complete disappearance of the keto compound ($R_f = 0.11$) and appearance of a new spot corresponding to the $R_f$ of standard lysoPtdCho ($R_f = 0.20$), indicating that the reduction was complete.

The reduction was also performed by using either NaB$_3$H$_4$ or 1-hexadecyl-dihydroxyacetone-3-phospho[methyl-$^{14}$C]choline, as prepared above. With NaB$_3$H$_4$ (15 µCi/nmol) the $^3$H-labeled lipid formed had a TLC migration rate the same as that of the lysoPtdCho ($R_f = 0.20$) and no other $^3$H-labeled lipid was detected in the chromatogram. With the $^{14}$C-labeled keto compound, the NaBH$_4$ reduced product also yielded a single $^{14}$C-labeled compound with a TLC migration rate the same as that of lysoPtdCho ($R_f = 0.20$). These BH$_4$ reduced $^3$H- and $^{14}$C-labeled lipids and the lipid synthesized as above (compound II), also migrated together on TLC at the same rate as lyso PAF in different other solvent systems. (CHCl$_3$–methanol–acetic acid–H$_2$O 60:40:12:8, $R_f = 0.20$; CHCl$_3$–methanol–8 M NH$_4$OH 60:40:5, $R_f = 0.1$).

**Acetylation of (II) to 1-O-hexadecyl-2-acetyl-rac-glycero-3-phosphocholine (III)**

Solvent-free 1-O-hexadecyl-rac-glycero-3-phosphocholine (II) (5 mg) was dissolved in 0.5 ml of dry CHCl$_3$ containing 4PP (1–2 mg) in a 1 ml Reacti-Vial. To this solution, 0.05 ml of acetic anhydride was added. The Reacti-Vial was closed under N$_2$ gas and stirred with a small magnet at room temperature for 2 h. After the reaction, 0.5 ml more of CHCl$_3$, 1 ml methanol, and 1 ml H$_2$O were added, the mixture was vortexed and
centrifuged. The CHCl₃ layer was washed two times with 2 ml of methanol–0.9% (w/v) NaCl mixture 1:1 and subjected to TLC analysis. Using the same solvent system as described above, the chromatogram showed the presence of only a single phospholipid-positive (19) spot with the 

result of TLC analysis revealed a major (95% of the radioactivity) ¹⁴C-labeled phospholipid spot with Rₜ (0.29) the same as that of compound (III). However, some minor ¹⁴C-labeled impurities were also detected (but not identified) above and below the major radioactive product on TLC.

The compound (III) was purified by loading the sample onto a 0.75 g silicic acid column (0.6 cm i.d.) and eluting successively with 25 ml each of CHCl₃ (fraction 1), CHCl₃–methanol 4:1 (fraction 2), CHCl₃–methanol
1:1 (fraction 3), and CHCl₃–methanol 1:9 (fraction 4). TLC analysis of these fractions showed the presence of PAF only in fraction 4 and some very minor impurities in the other three fractions. The recovery of the material after complete drying of fraction 4 was close to 99% as calculated based upon the starting material, hexadecyl-rac-glycerophosphocholine. The m.p. of the acetylated compound was 218°C (with decomposition). PMR (in CDCls) data (Fig. 4) showed peaks at 80.87 (t, 3 H, J = 7 Hz for CH₃ denoted by a), 1.12 (s, sharp, 28 H for 14 long chain C-atom denoted by b), 1.52 (t, 2 H for CH₂ denoted by c), 2.07 (s, sharp, 3 H for CH₃-CO-O- denoted by d), 3.97 (s, sharp, 9 H for N(CH₃)₃ denoted by e), 3.54 (triplet-like, 2 H for CH₂ denoted by f), 3.82 (s, broad, 2 H for CH₂ denoted by g), 3.94 (multiplet, 2 H for CH₂ denoted by h), 4.30 (s, broad, 2 H for CH₂ denoted by i) and 5.12 (quintuplet, 1 H for -C-H denoted by j). These data were consistent with the structure of the molecule and in good agreement with the values reported by other workers (9, 15). The FAB-MS of the compound (Fig. 5) showed the expected protonated molecular ion (m/z = 524). The identified major fragments (Fig. 5) m/z 224, 184, 166, 104 and 86 are similar to those described for the deuterated PAF (15).

**Phospholipase A₂ hydrolysis of PAF and separation of the optically active lyso PAF by TLC**

The stereoisomers of rac PAF were resolved from each other by phospholipase A₂ hydrolysis followed by racetylation of the hydrolysis product. Phospholipase A₂ has been shown to stereospecifically hydrolyze the 2-acetyl group (R-isomer) of PAF (21, 22). The racemic PAF (7 µmol), prepared as above, was dissolved in 0.5 ml of a mixture of diethyl ether–methanol–CHCl₃ 85:10:5 (23). The solution, 15 µl of Naja naja venom (12 mg/ml of 10 mM CaCl₂) was added and the reaction mixture was magnetically stirred in a Reacti-Vial at room temperature. The reaction was allowed to continue for 2 h with the periodic addition of 15 µl of the enzyme preparation at 20-min intervals throughout the course of the reaction (23). After 2 h most of the solvents were removed in a stream of N₂. The products were extracted with CHCl₃ and an aliquot was applied as a band on a TLC plate that was developed in CHCl₃–methanol–acetic acid–H₂O 60:40:12:8. Two bands corresponding to the Rs of lyso PAF and PAF were located. The bands were scraped out and the powders were extracted with CHCl₃–methanol 1:2 containing 0.1 N HCl, followed by an acid wash. The amount of phospholipid present was estimated by phosphorus determination (20). The upper band on the TLC plate corresponding to unreacted PAF (Rₛ = 0.29) was found to contain 1.5 µmol of phospholipid phosphorus and the lower band corre-
phosphorolysis of the diazo compound may also be used for introducing different headgroups, such as PEs or PIns, to synthesize different glycerophospholipids. As shown above, the reactions described here are also applicable to the easy preparation of radioactive glycerophospholipids labeled with $^3$H at the 2-position of the glycerol moiety or with other isotopes in the head groups. We are presently exploring the possibility of using this diazoketone intermediate in a general synthetic method for the preparation of different labeled and unlabeled glycerophospholipids.

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