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-(*rac* or *sn*)-glycero-3-phosphocholine (alkyl analog of lysophosphatidylcholine, **II**) and platelet activating factor (PAF), 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (**III**) has been described. The key step of the method is the decomposition of 1-O-hexadecyl-3-diazohydroxyacetone (A. K. Hajra, T. V. Saraswathi and A. K. Das, 1983. *Chem. Phys. Lipids.* **33:** 179–193) with phosphocholine to synthesize 1-O-hexadecyl dihydroxyacetone-3-phosphocholine (**I**). Conditions for this phosphorolysis were studied with respect to the reaction medium, temperature, and optimum proportion of the reactants. Compound (**I**) was quantitatively reduced with $NaBH_4$ 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.

Supplementary key words phosphorolysis • diazoketone • 1-O-hexadecyl dihydroxyacetone-3-phosphocholine

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, glycogenolysis, 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*-glycero-3-phosphocholine (1–3). Molecules containing alkyl chain lengths of both C_{16} and C_{18} 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 multi-step 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-acyl and 1-O-alkyl-3-diazohydroxyacetone with phosphoric acid (16). These keto lipids could be chemically or enzymatically reduced to form the corresponding 1-radyl-(*rac* or *sn*)-glycerol-3-phosphate (17). It has been indicated that the intermediate alkyl or acyl diazohydroxyacetone 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 ^3H- 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, $NaBH_4$, 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; **PIns**, phosphorylinositol; **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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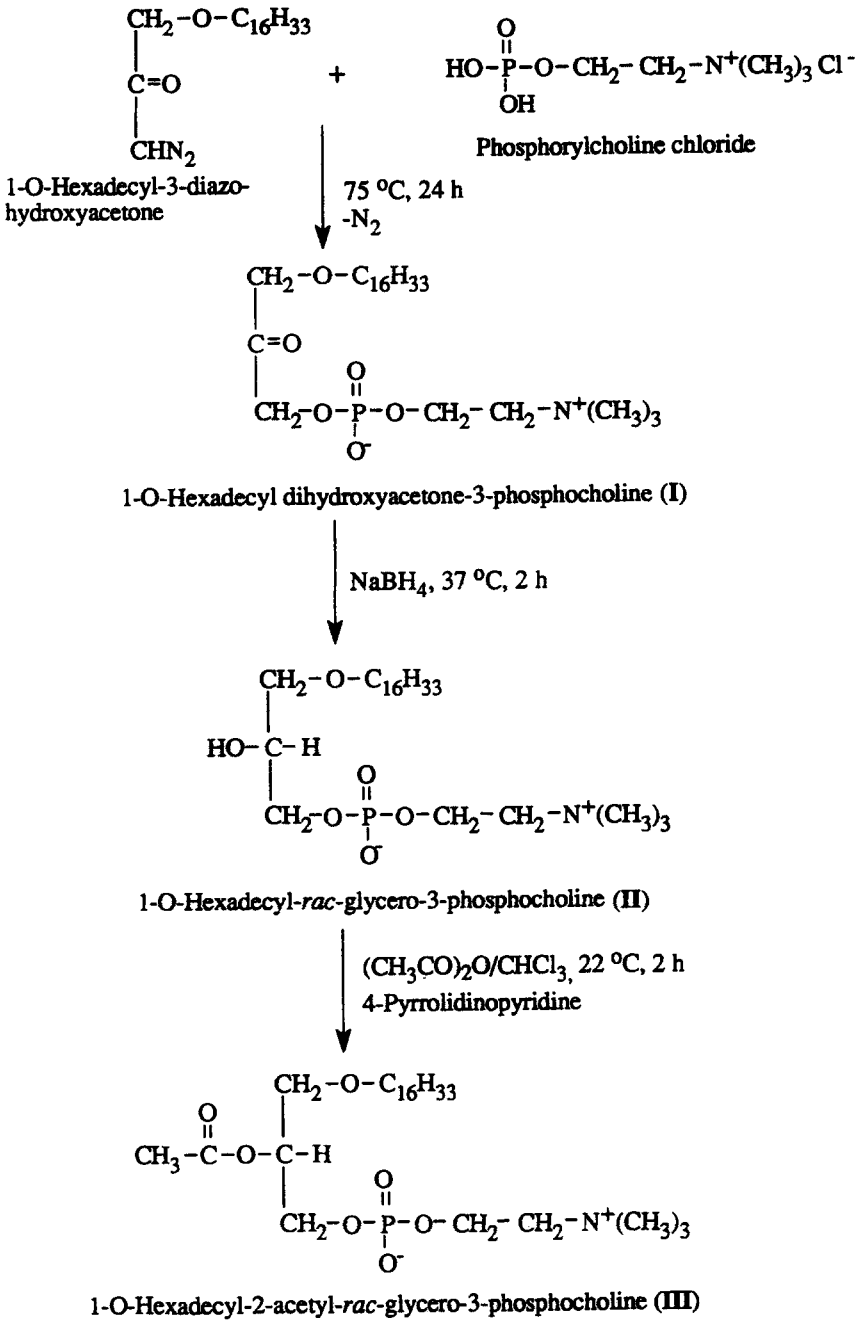

Scheme 1.

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^3H_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-diazohydroxyacetone was prepared as described previously (16). All solvents were of analytical grade. Chloroform was distilled over P_2O_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 μmol) of the Ca-salt of phosphocholine chloride was dissolved in about 1 ml H_2O 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_2O 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).

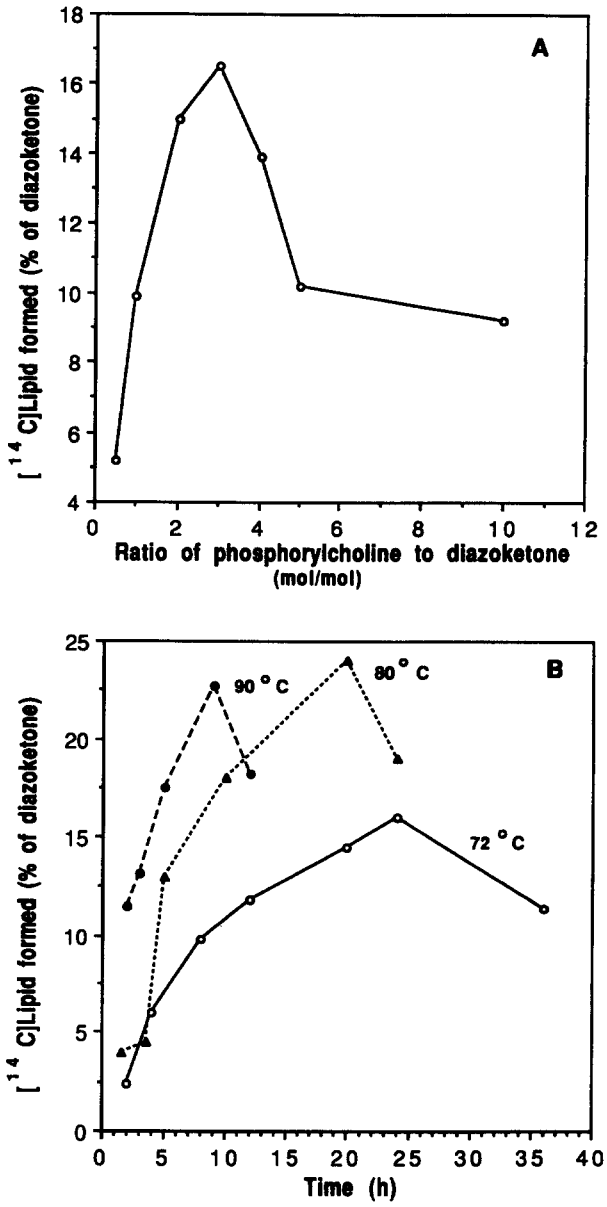

Fig. 1. Optimum substrate concentrations, molar ratio of the reactants, and temperature of the reaction for the phosphorolysis of 1-O-hexadecyl-3-diazohydroxyacetone by phosphocholine. A: The reaction mixtures contained 3 μmol of 1-O-hexadecyl-3-diazohydroxyacetone and increasing concentrations (1.5–30 μmol) of phospho[methyl-^{14}C]choline (sp. act. = 40,000 cpm/μmol) 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 μmol of the diazoketone, 9 μmol 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-

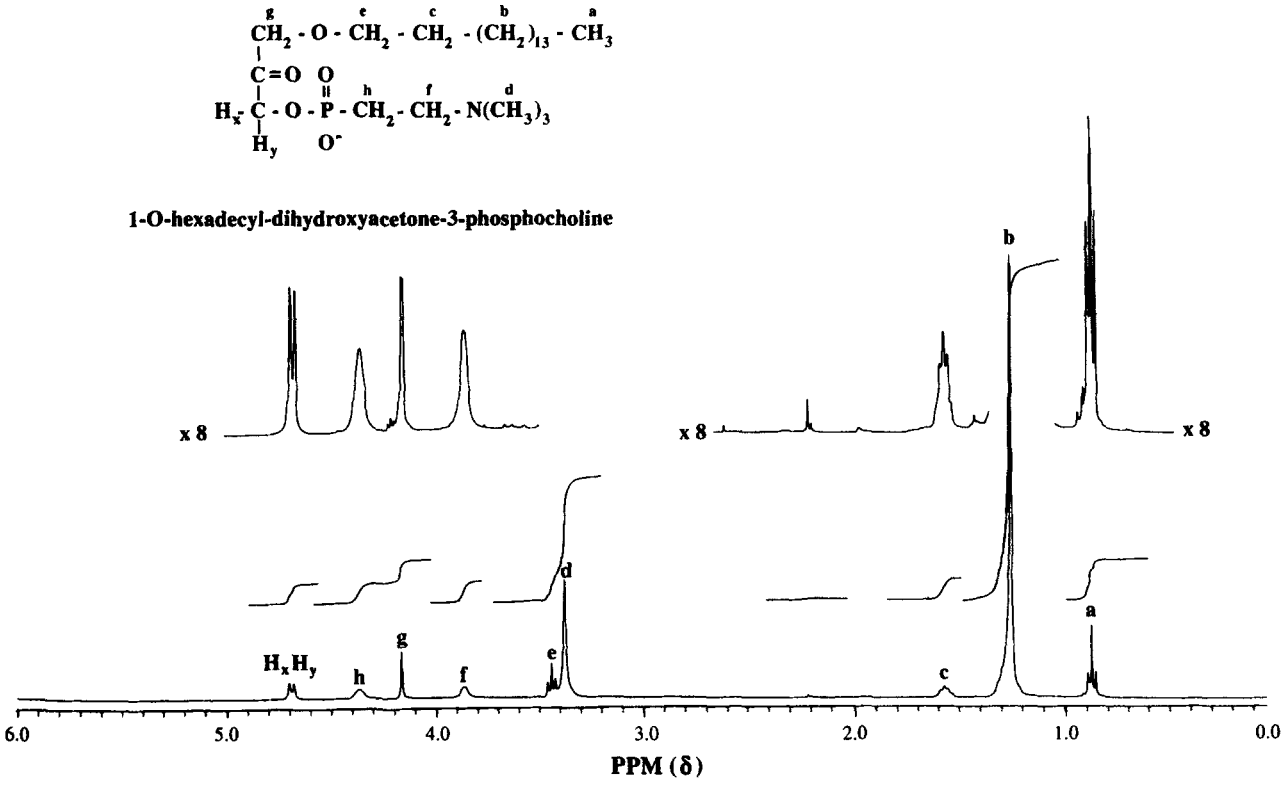

Fig. 2. PMR spectrum of 1-O-hexacecyl dihydroxyacetone-3-phosphocholine (**I**). The signals corresponding to the specific groups are denoted by small alphabets in the inset (see text for details).

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 Ca^{2+}-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 $CHCl_3$–methanol 1:1. To the solution, 6.5 ml H_2O 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 $CHCl_3$. 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 $CHCl_3$ and loaded onto a silicic acid (Unisil) column (1 × 12 cm). $CHCl_3$ (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 $CHCl_3$–methanol 7:3 (fraction 2), 150 ml of $CHCl_3$–methanol 1:4 (fraction 3) and 100 ml of methanol (fraction 4). When examined by thin-layer chromatography ($CHCl_3$–methanol–acetic acid–10% (w/v) aqueous sodium bisulfite 60:40:12:8),[2] fraction 3 was found to contain most of the desired phosphorylated product, i.e., 1-O-hexadecyl-dihydroxyacetone-3-phosphocholine (R_f = 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-

[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).

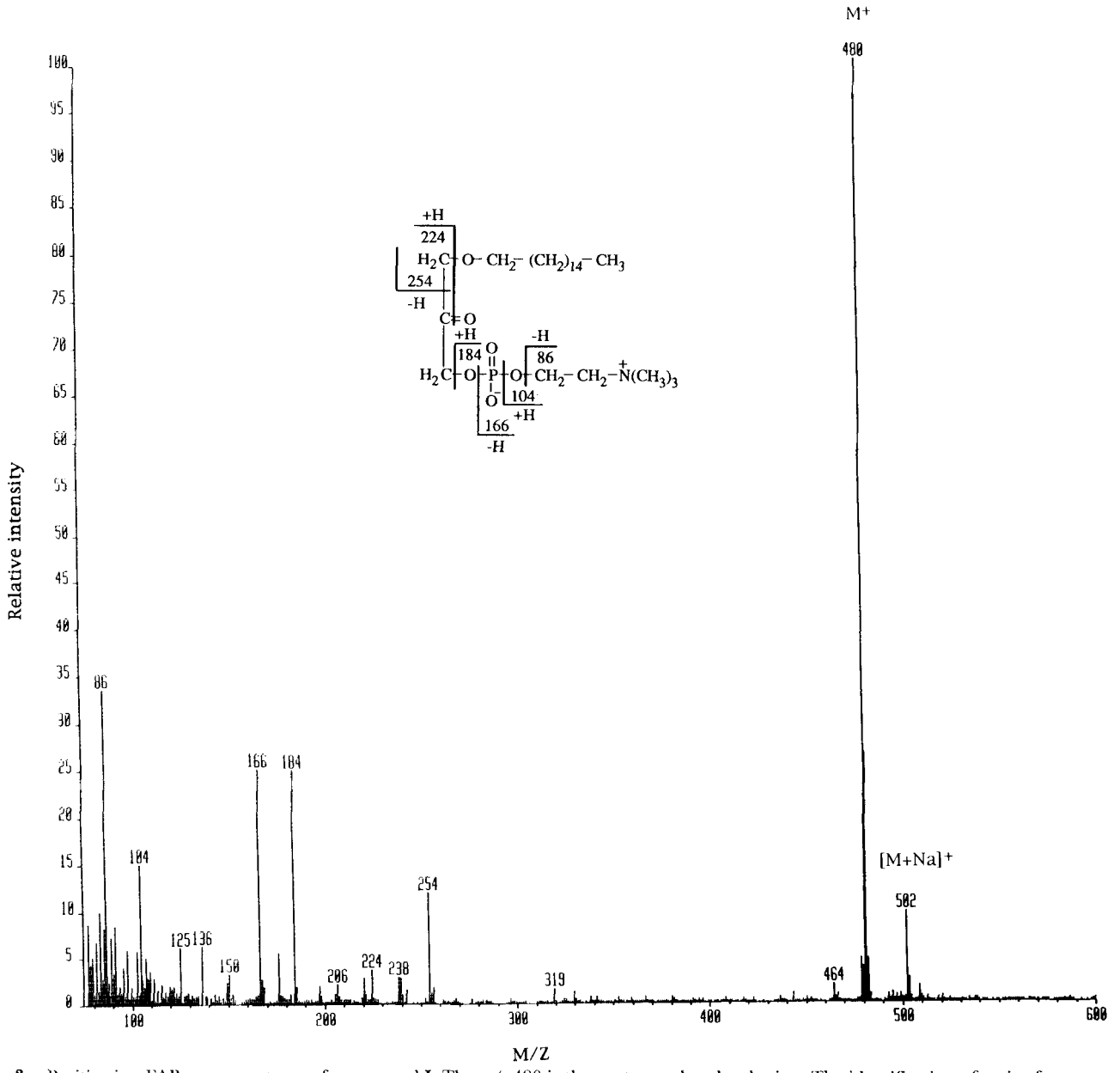

Fig. 3. Positive ion FAB mass spectrum of compound **I.** The *m/z* 480 is the protonated molecular ion. The identification of major fragments is shown in the inset (see text for details).

rus content of the purified lipid, the amount of the product formed was calculated to be 26.5 μmol, indicating a yield of about 18% of the starting diazo compound. The m.p. was 145–150°C (with decomposition). The sample was stored in $CHCl_3$. PMR (in $CDCl_3$) data (**Fig. 2**) showed peaks at δ0.87 (t, 3 H, J = 7 *Hz*, for terminal CH_3 of the long hydrophobic chain denoted by a), 1.24 (s, sharp, 26 H for the next 13 long chain CH_2 denoted by b), 1.56 (t, 2 H for CH_2 of the 15th C-atom from the terminal CH_3, denoted by c), 3.37 (s, sharp, 9 H for $N(CH_3)_3$ denoted by d), 3.43 (t, 2 H for CH_2 of the 16th C-atom from terminal CH_3, denoted by e), 3.85 (s, broad, 2 H for CH_2, denoted by f), 4.15 (s, sharp, 2 H for CH_2 denoted by g), 4.36 (s, broad, 2 H for CH_2 denoted by h), and 4.68 (d, 2 H for the CH_2 attached to phosphodiester bond, denoted by H_x and H_y).

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

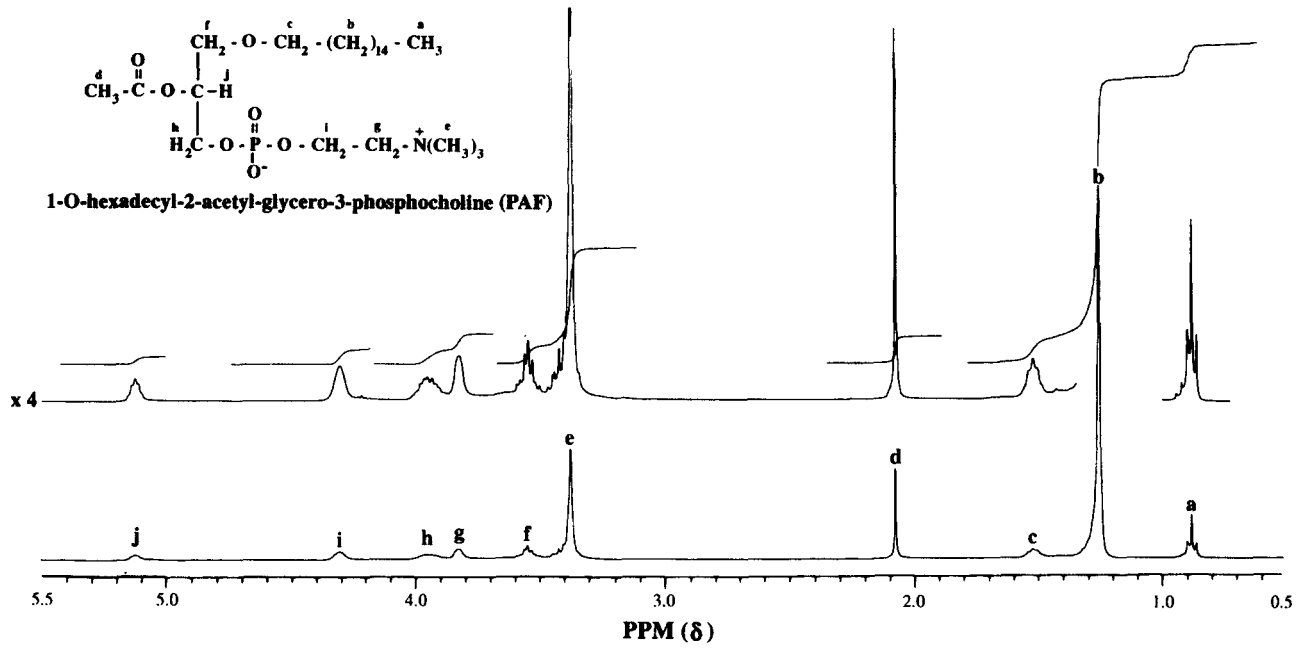

Fig. 4. PMR spectrum of 1-O-hexadecyl-2-acetyl-glycero-3-phosphocholine (PAF, **III**). The correspondence of signals with the structure is denoted by small alphabets in the inset (see text for details).

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 μmol) of 1-O-hexadecyl-3-diazohydroxyacetone with 36 μmol 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-hexadexyl 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_2O 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^3H_4 or 1-hexadecyl-dihydroxyacetone-3-phospho [methyl-^{14}C]choline, as prepared above. With NaB^3H_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_2O 60:40:12:8, R_f = 0.20; $CHCl_3$–methanol–8 M NH_4OH 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_2O were added, the mixture was vortexed and

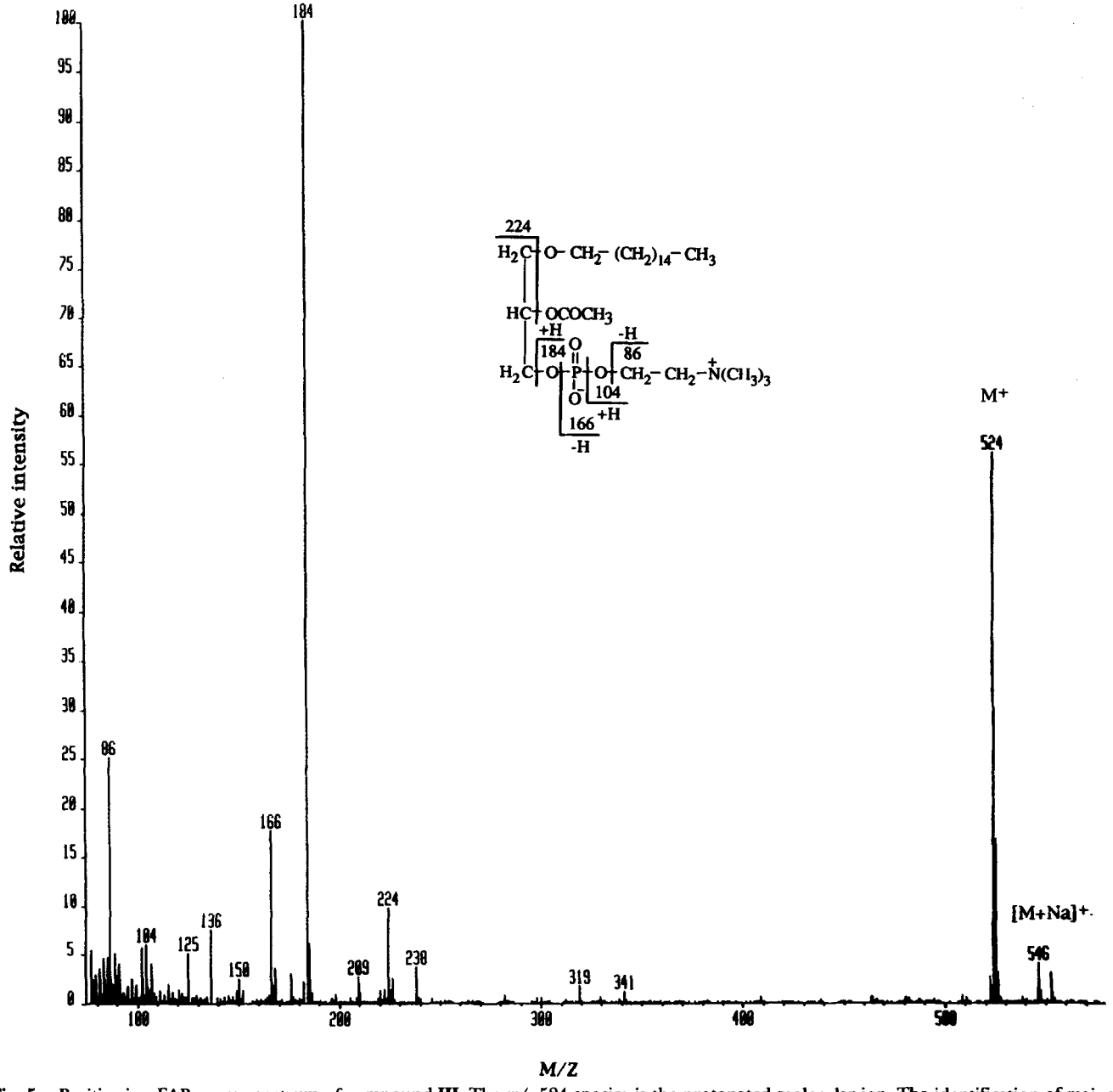

Fig. 5. Positive ion FAB-mass spectrum of compound **III**. The *m/z* 524 species is the protonated molecular ion. The identification of major fragments is shown in the inset (see text for details).

centrifuged. The $CHCl_3$ 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 R_f the same as that of authentic PAF (R_f = 0.29), confirming that the acetylation was complete in 2 h (quantitative yield). The experimental condition for acetylation was verified with about 1 mg of ^{14}C-labeled compound (**II**) (1.2×10^5 cpm) obtained above. The result of TLC analysis revealed a major (95% of the radioactivity) ^{14}C-labeled phospholipid spot with R_f (0.29) the same as that of compound (**III**). However, some minor ^{14}C-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 $CHCl_3$ (fraction 1), $CHCl_3$–methanol 4:1 (fraction 2), $CHCl_3$–methanol

1:1 (fraction 3), and $CHCl_3$–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 $CDCl_3$) data (**Fig. 4**) showed peaks at δ0.87 (t, 3 H, J = 7 Hz for CH_3 denoted by a), 1.12 (s, sharp, 28 H for 14 long chain C-atom denoted by b), 1.52 (t, 2 H for CH_2 denoted by c), 2.07 (s, sharp, 3 H for CH_3–CO–O– denoted by d), 3.37 (s, sharp, 9 H for $N(CH_3)_3$ denoted by e), 3.54 (triplet-like, 2 H for CH_2 denoted by f), 3.82 (s, broad, 2 H for CH_2 denoted by g), 3.94 (multiplet, 2 H for CH_2 denoted by h), 4.30 (s, broad, 2 H for CH_2 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 A_2 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 A_2 hydrolysis followed by reacetylation of the hydrolysis product. Phospholipase A_2 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–$CHCl_3$ 85:10:5 (23). To the solution, 15 μl of *Naja naja* venom (12 mg/ml of 10 mM $CaCl_2$) 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 N_2. The products were extracted with $CHCl_3$ and an aliquot was applied as a band on a TLC plate that was developed in $CHCl_3$–methanol–acetic acid–H_2O 60:40:12:8. Two bands corresponding to the R_fs of lyso PAF and PAF were located. The bands were scraped out and the powders were extracted with $CHCl_3$–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 (R_f = 0.29) was found to contain 1.5 μmol of phospholipid phosphorus and the lower band corresponding to optically active (R-form) lyso PAF (R_f = 0.20) contained 1.3 μmol of phospholipid phosphorus. The ratio between these two compounds corresponded closely to the theoretical value, indicating that almost all of the natural (R or *sn*-1-O-alkyl) isomer of PAF present in the racemic mixture was deacetylated under the experimental conditions used. The isolated lyso PAF was re-acetylated as described above to obtain the optically active form of PAF.

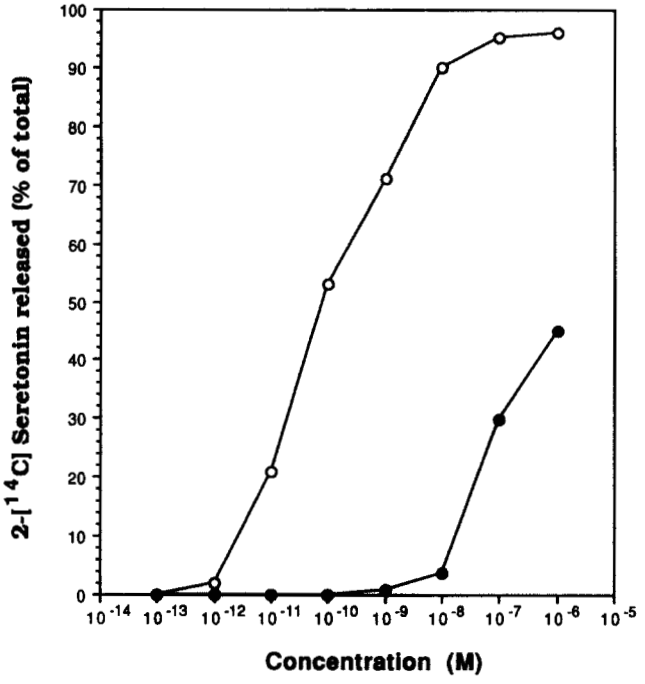

Fig. 6. Release of serotonin from rabbit platelets by compounds I and III. Platelets preloaded with [2-^{14}C]serotonin (see text) were suspended in Tyrode buffer with Ca^{2+} (4 mM) at a concentration of 2.5 × 10^8 cells/ml as described (4). To the suspension (200 μl), 10 μl of the compounds at different concentrations dissolved in bovine serum albumin (2.5 mg/ml)-containing normal saline were added and incubated at 37°C for 1.0 min. The reactions were stopped by adding 20 μl of formaldehyde (1.5 M), cooled on ice, and centrifuged at 2,000 *g* for 10 min. Serotonin released was determined by measuring the radioactivity in the supernatants. Total release of labeled serotonin was determined by adding 10 μl of Triton X-100 to 200 μl of the suspension and measuring the radioactivity in the supernatant after centrifugation. The data are expressed as the percent of total serotonin release by compound **I** (●-●) and compound **III** (○-○) at the indicated concentrations.

Release of serotonin by the synthetic compounds from rabbit blood platelets

The biological activities of the synthesized compounds were determined by studying the release of serotonin from platelets in response to these compounds. Rabbit blood platelets were loaded with radioactive serotonin by incubating platelet-rich plasma (2,000 *g*-min supernatant of blood) with [2-^{14}C]sero-

tonin (6 μCi) for 20 min at 37°C. The platelets were isolated from the incubation mixture by centrifugation (8,000 *g*-min) and washing in Tyrode Ca^{2+}-free buffer solution as described (4, 13). The prelabeled platelets were used to assay the release of the radioactive serotonin in response to the compounds **I, II** and **III** at concentrations ranging from 10^{-6} M to 10^{-13} M following published methods (4, 13) as summarized in the legend of **Fig. 6.** Compound **III** is especially potent in causing this release of 50% of serotonin at a concentration of 10^{-10} M (Fig. 6). This activity was found to be the same when compared with commercial *rac*-PAF (Sigma) and similar to the activity reported by other authors (13, 22). The keto compound (compound **I**) had some serotonin release activity (Fig. 6) but was almost three orders of magnitude less potent than compound **III.** Compound **II** (lysoPAF) did not have any biological activity at the concentrations used.

DISCUSSION

A chemical synthesis of 1-O-hexadecyl-*rac*-glycero-3-phosphocholine (alkyl lyso phosphatidylcholine or lyso PAF) and *rac*-PAF is described here. The method involves three sequential steps (Scheme 1), i.e., phosphorolysis of an alkyl diazoketone, $NaBH_4$ reduction of the alkyl keto phospholipid (compound **I**), and acetylation of the reduced product (compound **II**). The last two steps follow well-established quantitative methods (17, 23). The novel step in the whole procedure is the decomposition of 1-O-hexadecyl diazohydroxyacetone with phosphocholine to form the 2-keto analog of lysoPtdCho. The mechanism of this phosphorolysis reaction is the initial protonation of the diazo compound by the acid form of choline phosphate (PK_1 ~2.0 as determined by titration) to a diazonium intermediate which is attacked by the nucleophilic phosphate anion with the release of N_2 (24). The main problem we encountered in the phosphorolysis step was selecting a proper solvent medium to dissolve both the hydrophobic hexadecyl diazohydroxyacetone and the hydrophilic phosphocholine in the same mixture. In our previous study, for the preparation of 1-O-alkyl (or acyl) dihydroxyacetone phosphate, both the reactants, alkyl diazoketone and orthophosphoric acid, were freely soluble in dioxane (16) and a high yield (70%) was obtained. In the present case, while phosphocholine was insoluble in dioxane but soluble in DMSO, hexadecyl diazohydroxyacetone behaved in the opposite manner. Consequently, both phosphocholine and hexadecyl diazohydroxyacetone precipitated out when their individual solutions in DMSO and dioxane, respectively, were mixed. Addition of a detergent (Triton-X-100) or a putative phase-transfer catalyst (tetraphenylboron) did not facilitate the reaction. Addition of a third solvent of intermediate polarity was found to be necessary to keep the reactants in solution. Among various solvents investigated (acetonitrile, tetrahydrofuran, DMF, tetramethylsulfone), a mixture of DMF and DMSO along with dioxane was found to be most effective. However, because of the large volume of solvents needed to be used for the reaction, thus lowering the concentration of the reactants, the rate of phosphorolysis was slow and so the yield was comparatively low.

Despite such problems of low reaction rate and yield, our synthetic strategy provides a useful technique for quick preparation of 1-O-alkyl glycero-3-phosphocholine or PAF in small or large scale quantities. The procedure does not require prior protection of the different functional groups, thus minimizing the number of steps necessary to synthesize the glycerol derivatives. Starting from commercially available hexadecyl iodide and ethyl glycolate (16), the whole synthesis was accomplished in six reaction steps. The overall yield (~9% for *rac* PAF) is comparable to most other chemical synthetic methods described for PAF (9–15). The intermediate keto compound was found to be stable for months when stored in $CHCl_3$ solution at -20°C under N_2.

Diazoketones have been used for a long time as a reactive intermediate for the synthesis of a variety of organic compounds (24) and also lipids (16, 25). By extending our previous method (16), we show here that lipids containing phosphodiester bond can also be easily synthesized from diazoketone. The phosphorolytic product, i.e., the keto intermediate, may be utilized to form analogs of lysoPtdCho or PAF, such as ketalization to form dimethyl, diethyl ketals (A. K. Das, and A. K. Hajra, unpublished results) or reductive amination to form the 2-amino or acetamido derivatives, which have been shown to be potent agonist or antagonist of PAF (26). The phosphorolysis of the diazo compound may also be used for introducing different headgroups, such as PEtn 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 ^3H 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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REFERENCES

1. Benveniste, J., and B. B. Vargaftig. 1983. Platelet-activating factor: an ether lipid with biological activity. *In* Ether Lipids: Biochemical and Biomedical Aspects. H. K. Mangold and F. Paltauf, editors. Academic Press, New York. 355–376.
2. Hanahan, D. J. 1986. Platelet-activating factor: a biologically active phosphoglyceride. *Annu. Rev. Biochem.* **55:** 483–509.
3. Snyder, F. 1985. Chemical and biochemical aspects of platelet-activating factor: a novel class of acetylated ether-linked choline-phospholipids. *Med. Res. Rev.* **5:** 107–140.
4. Demopoulos, C. A., R. N. Pinckard, and D. J. Hanahan. 1979. Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* **254:** 9355–9358.
5. Blank, M. L., F. Snyder, L. W. Byers, B. Brooks, and E. E. Muirhead. 1979. Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. *Biochem. Biophys. Res. Commun.* **90:** 1194–1200.
6. Muramatsu, T., N. Totani, and H. K. Mangold. 1981. A facile method for the preparation of 1-O-alkyl-2-O-acetoyl-*sn*-glycero-3-phosphocholines (platelet activating factor). *Chem. Phys. Lipids.* **29:** 121–127.
7. Lekka, M. E., D. C. Tsoukatos, A. D. Tselepis, and V. M. Kapoulas. 1988. Semisynthetic preparation of 1-O-hexadecyl-2-acetyl-*sn*-glyceryl -3-phosphorylcholine (platelet activating factor). *Z. Naturforsch [C]* **43:** 665–670.
8. Godfroid, J-J., F. Heymans, E. Michel, C. Redeuilh, E. Steiner, and J. Benveniste. 1980. Platelet activating factor (PAF-acether): total synthesis of 1-O-octadecyl-2-O-acetyl *sn*-glycero-3-phosphorylcholine. *FEBS Lett.* **116:** 161–164.
9. Heymans, F., E. Michel, M-C. Borrel, B. Wichrowski, J-J. Godfroid, O. Convert, E. Coeffier, M. Tence, and J. Benveniste. 1981. New total synthesis and high resolution ^{1}H NMR spectrum of platelet activating factor, its enantiomer and racemic mixtures. *Biochim. Biophys. Acta.* **666:** 230–237.
10. Fujita, K., H. Nakai, S. Kobayashi, K. Inoue, S. Nojima, and M. Ohno. 1982. An efficient stereoselective synthesis of platelet activating factors and the enantiomers from D- and L-tartaric acids. *Tetrahedron Lett.* **34:** 3507–3510.
11. Mangold, H. K. 1979. Synthesis and biosynthesis of alkoxylipids. *Angew. Chem. Intl. Ed. Engl.* **18:** 493.
12. Guivisdalsky, P. N., and R. Bittman. 1989. An efficient stereocontrolled route to both enantiomers of platelet activating factor and analogues with long-chain esters at C2: saturated and unsaturated ether glycerolipids by opening of glycidyl arenesulfonates. *J. Org. Chem.* **54:** 4643–4648.
13. Surles, J. R., R. L. Wykle, J. T. O'Flaherty, W. L. Salzer, M. J. Thomas, F. Snyder, and C. Piantadosi. 1985. Facile synthesis of platelet-activating factor and racemic analogues containing unsaturation in the *sn*-1-alkyl chain. *J. Med. Chem.* **28:** 73–78.
14. Murari, M. P., R. Murari, S. Parthasarathy, C. A. Guy, V. V. Kumar, B. Malewicz, and W. J. Baumann. 1990. Lyso platelet activating factor (LysoPAF) and its enantiomer. Total synthesis and carbon-13 NMR spectroscopy. *Lipids.* **25:** 606–612.
15. Prakash, C., S. Saleh, F. T. Douglass, and I. A. Blair. 1989. Synthesis of trideuterated O-alkyl platelet activating factor and lyso derivatives. *Lipids.* **24:** 786–792.
16. Hajra, A. K., T. V. Saraswathi, and A. K. Das. 1983. Chemical synthesis of 1-O-alkyl and 1-O-acyl dihydroxyacetone-3-phosphate. *Chem. Phys. Lipids.* **33:** 179–193.
17. Das, A. K., and A. K. Hajra. 1984. Estimation of acyldihydroxyacetone phosphate and lysophosphatidate in animal tissues. *Biochim. Biophys. Acta.* **796:** 178–189.
18. Wright, R. S. 1971. A reagent for the non-destructive location of steroids and some other lipophilic materials on silica gel thin-layer chromatograms. *J. Chromatogr.* **59:** 220–221.
19. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126–127.
20. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8:** 115–118.
21. VanDeenen, L. L. M., and G. H. DeHaas. 1964. The synthesis of phosphoglycerides and some biochemical applications. *Adv. Lipid Res.* **2:** 167–234.
22. Wykle, R. L., C. H. Miller, J. C. Lewis, J. D. Schmitt, J. A. Smith, J. R. Surles, C. Piantadosi, and J. T. O'Flaherty. 1981. Stereospecific activity of 1-O-alkyl-2-O-acetyl-*sn*-glycero-3-phosphocholine and comparison of analogs in the degranulation of platelets and neutrophils. *Biochem. Biophys. Res. Commun.* **100:** 1651–1658.
23. Mason, J. T., B. V. Anthony, and C-H. Huang. 1981. A method for the synthesis of isomerically pure saturated mixed-chain phosphatidylcholines. *Anal. Biochem.* **113:** 96–101.
24. Smith, A. B., and R. K. Dieter. 1981. The acid promoted decomposition of a-diazoketones. *Tetrahedron.* **37:** 2407–2439.
25. Mangold, H. K. 1973. Reactions of aliphatic diazoketones and haloketones. *Chem. Phys. Lipids.* **11:** 244–253.
26. Chandrakumar, N. S., and J. Hajdu. 1983. Stereospecific synthesis of ether phospholipids. Preparation of 1-alkyl-2-(acylamino)-2-deoxyglycero-phosphorylcholines. *J. Org. Chem.* **48:** 1197–1202.