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A novel chemical synthesis of 1-O-hexadecyl-*rac*-[2-^{3}H]glycero-3-phosphorylethanolamine and a simple assay for plasmanyl desaturase

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Abstract A simple and efficient method for chemical synthesis of lysophosphatidylethanolamine is described. 1-O-hexadecyl diazohydroxyacetone (A. K. Hajra, T. V. Saraswathi and A. K. Das. 1983. *Chem. Phys. Lipids.* **33:** 179–193) was decomposed by benzyloxycarbonyl (CBZ) derivative of phosphorylethanolamine (I) to give 1-O-hexadecyl dihydroxyacetone-3-(N)-CBZ phosphorylethanolamine (II). Compound (II) was reduced by $NaBH_4$ and the product (III), after catalytic transfer hydrogenolysis produced the final compound, 1-O-hexadecyl-*rac*-glycero-3-phosphorylethanolamine (IV). The yield of (IV), starting from 1-O-hexadecyl diazohydroxyacetone was 53%. The identities of the compounds were verified by NMR and fast atom bombardment mass spectral (FAB–MS) analysis. 1-O-hexadecyl-*rac*-[2-^{3}H]glycero-3-phosphorylethanolamine prepared by the method above was shown to be a good in vitro substrate for plasmanyl Δ1′-desaturase (EC 1.14.99.19). Using this radioactive substrate, a simple and rapid solvent partition assay for this enzyme was developed with results comparable to those obtained by the two-dimensional thin-layer chromatographic assay method. The advantage of this rapid assay system and the applicability of the chemical synthetic method for other phosphoglycerides are discussed.—**Das, A. K., and A. K. Hajra.** A novel chemical synthesis of 1-O-hexadecyl-*rac*-[2-^{3}H]glycero-3-phosphorylethanolamine and a simple assay for plasmanyl desaturase. *J. Lipid Res.* 1996. **37:** 2706–2714.

Supplementary key words. phosphorolysis • diazoketone • lysophosphatidylethanolamine • plasmalogen biosynthesis

Glycerol ether lipids (alkyl glycerol ethers and plasmalogens) are present in most animal tissues mainly as ethanolamine phosphoglycerides (1, 2). The initial biochemical reactions for the synthesis of these lipids via the acyl dihydroxyacetone phosphate pathway occur in peroxisomes (3). The lipid intermediate, 1-O-alkyl-*sn*-glycerol-3-phosphate, is then transported to endoplasmic reticulum where it is converted to different glycerol ether lipids and plasmalogens (3–6).

In most biomembranes, ether lipids are predominantly (>90%) present as plasmalogens, i.e., 1-O-alk-1′-enyl-2-O-acyl-*sn*-glycero-3-phosphoethanolamine (1). The vinyl ether bond in plasmalogens is introduced at the terminal biosynthetic step as a dehydrogenation reaction catalyzed by plasmanyl ethanolamine desaturase (EC 1.14.99.19), a microsomal reduced pyridine nucleotide-requiring mixed function oxidase (5, 6). Though this biochemical reaction was described and extensively studied some time ago, there are still some unresolved questions about this reaction, such as the nature of the actual substrate that is desaturated and whether or not a soluble protein cofactor and ATP are required (5–7). The main reason for such slow progress in this field is the use of cumbersome two-dimensional (8, 9) or multi-developmental thin-layer chromatography (10) assay procedures. The assay procedures are also hindered by the difficulty in preparing the radioactive substrate of high specific activity either biosynthetically (8–10) or by chemical synthesis (11).

We have previously described (12, 13) the synthesis of different glycerol ether lipids via a diazoketone intermediate, and pointed out that this should be a general synthetic procedure for the easy introduction of different head groups in phosphoglycerides. We describe here the extension of this novel procedure for the synthesis of radioactively labeled (2-^{3}H) or unlabeled 1-O-hexadecyl-*rac*-glycero-3-phosphoethanolamine (Scheme 1) with high yield. The utility of the synthesized labeled lipid is demonstrated by its use as

Abbreviations: CBZ, benzyloxycarbonyl; FAB–MS, fast atom bombardment–mass spectroscopy; PAF, platelet activating factor; PMR, proton magnetic resonance; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine.

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a substrate for the biosynthesis of ethanolamine plasmalogen, and the development of a simple and rapid assay for plasmanyl Δ1′-desaturase.

EXPERIMENTAL

Materials

Carbobenzoxy chloride (benzylxycarbonyl chloride or benzyl chloroformate) and palladium (10%) on activated carbon were obtained from Aldrich Chemical Co. (Milwaukee, WI). O-phosphorylethanolamine, $NaBH_4$, phenylmethylsufonyl fluoride (PMSF), leupeptin, NADPH, primuline, and L-α-lysophosphatidylethanolamine were from Sigma Chemical Co. (St. Louis, MO). EN^{3}HANCE was purchased from DuPont (NEN Research Products, Boston, MA). Silicic acid (Unisil, 200–325 mesh) was from Clarkson Chemical Co., Inc. (Williamsport, PA). NaB^3H_4 (15 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Thin-layer chromatography (TLC) plates were from EM Science (Gibbstown, NJ). XOmat-AR film from Kodak (Rochester, New York) was used for ^{3}H-radioautography. 1-O-hexadecyloxy-3-diazoacetone was prepared according to the method described before (12).

Dioxane was distilled over KOH pellets before use. All other solvents (analytical grade) were used without further purification.

Microsomes from 13-day-old rat brains were prepared as described by Wykle and Locmiller (14), except that the homogenizing buffer used was 0.25 M sucrose containing 10 mM TES buffer (pH 7.5) and EDTA (1 mM) along with the protease inhibitors PMSF (0.2 mM) and leupeptin (1 μM). The isolated microsomes were stored in the same buffer without the protease inhibitors at −70°.

Methods

Proton magnetic resonance (PMR) spectra were taken on a 360 MHz NMR spectrophotometer (Bruker Co., Model WM-360). Positive ion fast atom bombardment mass spectra (FAB–MS) were taken on a Model 70-70E analytical mass spectrometer (VG-Analytical Co., Manchester, England). A mixture of dithiothreitol and dithioerythritol (DTT/DTE) was used as the solvent matrix (15) and the background was subtracted from the sample spectra. The TLC plates were sprayed with primuline to visualize all lipids (16) followed by ninhydrin spray (17) for the detection of free NH_2 group-containing lipids and then by molybdenum blue spray (18) to detect all phospholipids. Total phosphorus in the lipids was assayed according to Ames (19). The protein was determined by a dye-binding assay method (20) using the reagent from Pierce Chemical Co. (Rockford, IL) and bovine serum albumin as the standard.

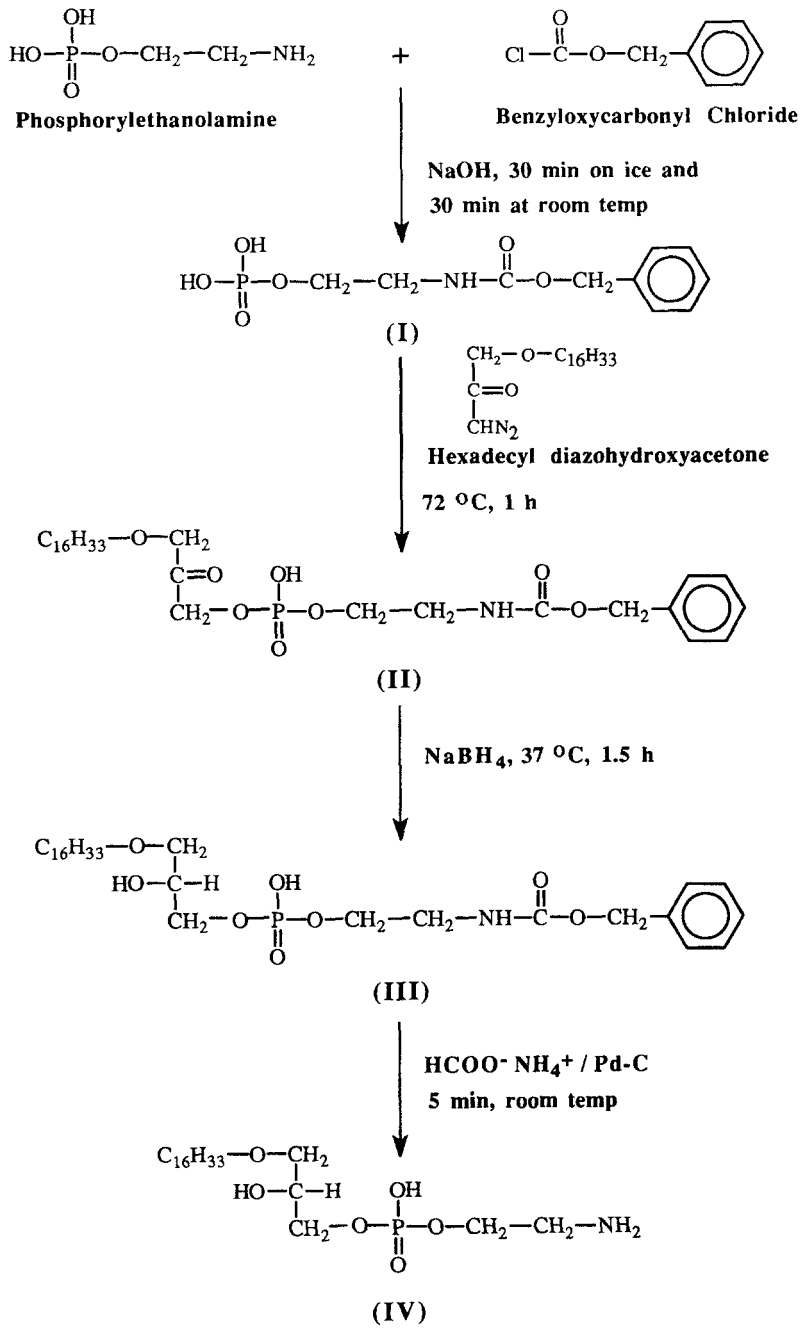
Preparation of carbobenzoxy (CBZ) derivative of phosphorylethanolamine (I. Scheme 1)

A modification of the method described previously (21) was used to prepare this compound. To a magnetically stirred solution of 0.44 g (3.2 mmol) of phosphorylethanolamine in 2 ml of 2 N NaOH cooled in an ice-water bath, a solution of carbobenzoxy chloride (3.2 mmol in 1 ml toluene) and 4 N NaOH (1 ml) was added in small aliquots over a period of 10 min. After the additions were complete, the mixture was stirred on ice for 30 min followed by 30 min at room temperature. After the reaction, the tube was centrifuged (800 *g*, 10 min) and the toluene layer was discarded. The aqueous layer was further extracted with about 3 ml of toluene to remove the unreacted carbobenzoxy chloride. The aqueous layer was acidified with 2 N HCl, and 10 ml each of water and ethyl acetate were added to it, mixed well, and centrifuged. The upper ethyl acetate layer containing the product was collected. The lower aqueous layer was re-extracted 3 times with 10 ml of ethyl acetate. TLC analysis ($CHCl_3$—CH_3OH—acetic acid–H_2O, 100:40:12:4 v/v) of the combined ethyl acetate extract showed a single spot (R_f = 0.25) that was positive towards primuline and molybdenum blue spray but negative towards ninhydrin. A trace of a ninhydrin-positive spot, probably the unreacted phosphoethanolamine, was seen at the origin.

The solvent (ethyl acetate) was removed under vacuum using a rotary evaporator and then under high vacuum in a desiccator over P_2O_5. From the dry weight, the yield was calculated to be 83.5%. This compound (I) (440 mg) was dissolved in 6 ml of a mixture of dioxane–acetonitrile 2:1 (v/v) and stored at −20°C.

Synthesis of 1-O-hexadecyldihydroxyacetone-3-(N)-CBZ phosphorylethanolamine (II)

Sixty mg (180 μmol) of 1-O-hexadecyl-3-diazohydroxyacetone was mixed with 360 μmol of compound (I) in 1.4 ml dioxane–acetonitrile 2:1 (v/v) and heated in a Reacti-Vial (Pierce Chemical Co., Rockford, IL) at 70–72°C with constant magnetic stirring. A vigorous evolution of N_2 gas was observed in a few minutes. After 1 h, the mixture was cooled and added to 6 ml of $CHCl_3$—CH_3OH 1:1. Water (2.8 ml) was added to the solution, which was then mixed and centrifuged. The upper aqueous layer was removed and the lower $CHCl_3$ layer was washed three times with 5 ml of $CHCl_3$—CH_3OH—H_2O 1:12:12 (v/v). TLC analysis ($CHCl_3$—CH_3OH–acetic acid–H_2O, 100:40:12:4, v/v) of the



Scheme 1.

washed $CHCl_3$ extract revealed the presence of one major spot (R_f = 0.8) positive to primuline and molybdenum blue spray but negative to ninhyrin. A minor phospholipid by-product was observed at R_f = 0.85 and trace amounts of polar impurities showed near the origin. From the analysis of total phosphate of the crude product (II), a yield of 80% was calculated.

Purification of compound (II) by column chromatography

The dried product (II) was dissolved in a minimum volume of $CHCl_3$ and applied to a 5-g silicic acid (Unisil) column (1 cm i.d.). The column was eluted with 80 ml each of $CHCl_3$ (fraction 1), $CHCl_3$—CH_3OH (98:2,

fraction 2), $CHCl_3$—CH_3OH (97:3, fraction 3), $CHCl_3$—CH_3OH (90:10, fraction 4), and $CHCl_3$—CH_3OH (70:30, fraction 5). TLC analysis using the same developing solvent system as above showed that the compound (II) mostly eluted out in fraction 3, while fraction 2 and fraction 4 contained a small proportion. Fraction 2 also contained some unidentified impurities (R_f = 0.85 and 0.87). Another minor and very polar phosphate positive impurity was present in fraction 5.

Fractions 3 and 4, containing the uncontaminated compound II, were combined. Yield (phosphate analysis) calculated based on the starting material, alkyl diazoketone, was about 66%. PMR (in $CDCl_3$) signals were at δ 0.87 (t, 3H, J = 7 Hz for terminal CH_3 of the aliphatic chain), 1.24 (s, sharp, 26H for next 13 long CH_2 chain), 1.52 (t, 2H for the 15th CH_2 from terminal CH_3 group), 2.32–4.13 (t, 2H for the 16th C-atom; s, 2H for O—CH_2—C═O; broad, 1H for NH; broad 4H for P—O—CH_2—CH_2—N), 4.68 (broad, 2H for CO—CH_2—O—P), 5.0 (s, 2H for CH_2—C_6H_5) and 7.25–7.29 (5H for phenyl group). Positive FAB–MS spectrum showed the presence of the molecular ion as potassium salt [m/z = 610, $(M + K)^+$] (calcd. M = 610 for $C_{29}H_{50}O_8NPK$). The other major fragments detected were at m/z 314, 205, and 162.

Reduction of (II) to 1-O-hexadecyl-*rac*-glycero-3-(N)-CBZ phosphorylethanolamine (III)

Ten mg of dry product (II) in 0.5 ml of freshly distilled (over $NaBH_4$) ethyl alcohol was treated with 0.2 ml of 0.2 M $NaBH_4$ in 0.05 M NaOH and left at 37°C for 1.5 h (22). The reaction was stopped by adding 0.5 ml of 2 N HCl. Two ml of $CHCl_3$ and 1 ml of CH_3OH were then added, mixed, and centrifuged. The lower organic phase containing the product (III) was washed twice with Bligh and Dyer (23) upper layer ($CHCl_3$—CH_3OH—H_2O, 1:12:12, v/v) and stored at −20°C. TLC analysis with the above solvent system showed complete disappearance of the keto compound (II) (R_f = 0.8) and appearance of a new phosphate positive but ninhydrin negative spot at R_f = 0.7. This product was further analyzed by TLC, using a bisulfite-containing solvent, i.e., $CHCl_3$—CH_3OH—acetic acid—10% (w/v) aqueous Na-metabisulfite (100:40:12:4, v/v) for development (22). Under these conditions, compound (III) had the same R_f (0.7) as in the regular (non-bisulfite) solvent system, whereas the mobility of the keto compound (II) was retarded (R_f = 0.5, instead of 0.8).

Hydrogenolysis of (III) to 1-O-hexadecyl-*rac*-glycero-3-phosphorylethanolamine (IV) by catalytic transfer hydrogenation

Six mg of dry compound (III) was dissolved in 0.4 ml of absolute ethanol to which 0.2 ml of 1.5 M ammonium formate and 5–6 mg of palladium on carbon (10%) were added and the mixture was stirred at room temperature for 5 min (24, 25). After the reaction, 1.5 ml CH_3OH and 1 ml $CHCl_3$ were added to the reaction mixture, mixed, and centrifuged. The extract was transferred to another tube and the residue was re-extracted with the same volume of $CHCl_3$–methanol. The combined extract was acidified with 2 N HCl followed by the addition of 2 ml of water to separate the phases for removing the excess HCl and other water-soluble materials. The purity of the hydrogenolyzed product (in the lower layer) was checked by TLC using the non-bisulfite solvent as above. The developed chromatogram showed the presence of one major spot (R_f = 0.23) with very small amounts of two minor spots above it. The major product (IV) was phosphate and ninhydrin positive and had the same R_f value as that of standard lysophosphatidylethanolamine.

Purification of (IV)

Compound (IV) was purified by chromatography on a 0.6-g silicic acid column (0.5 × 7 cm) using 5 ml each of $CHCl_3$—CH_3OH mixtures in the proportions of 65:35 (fraction 1), 60:40 (fraction 2), 50:50 (fraction 3), and 20:80 (fraction 4) as the eluting solvents. As examined by TLC, compound (IV) was mainly eluted in fraction 3 with a minor portion in fraction 2. These two fractions were combined. The impurities came out in fraction 1. The total recovery based on the starting material used for catalytic hydrogenation was 80%.

PMR (in $CDCL_3$) signals of compound (IV) appeared at δ 0.87 (t, 3H, J = 7Hz for terminal CH_3 of the aliphatic chain), 1.25 (s, sharp, 26H for next 13 long CH_2 chain), 1.54 (t, 2H for the 15th CH_2 from terminal CH_3 group), 2.32–4.15 (t, 2H for the 16th C-atom; broad, 2H for O-CH_2-CHOH, *rac*-1 glycerol; broad, 1H for HO—C—H, *rac*-2 glycerol; broad, 4H for P—O—CH_2—CH_2—N), 4.28 (broad, 2H for CHOH—CH_2—O—P, *rac*-3 glycerol) and 8.09 (broad, 2H for NH_2). Positive FAB–MS (**Fig. 1**) showed the presence of expected protonated molecular species at m/z = 440 (M^+) (calcd. M = 439 for $C_{21}H_{46}O_6NP$). The other major fragmentations were at m/z 879, 592, 397, 216.173, 142, and 99. Most of these fragments were identified and shown in the inset of Fig. 1.

Synthesis of *rac*-[2-^{3}H]compound (III) and *rac*-[2-^{3}H]compound (IV)

To 10 μmol (5.7 mg) of compound (II) 200 μl of 1 mM $NaB[^3H]_4$ (15 μCi/nmol) in 0.1 N NaOH was added and the mixture was incubated for 1 h at 37°C. To the mixture 100 μl of additional 0.1 M non-radioactive $NaBH_4$ in water was added and the incubation was continued for another 1 h to ensure complete reduction

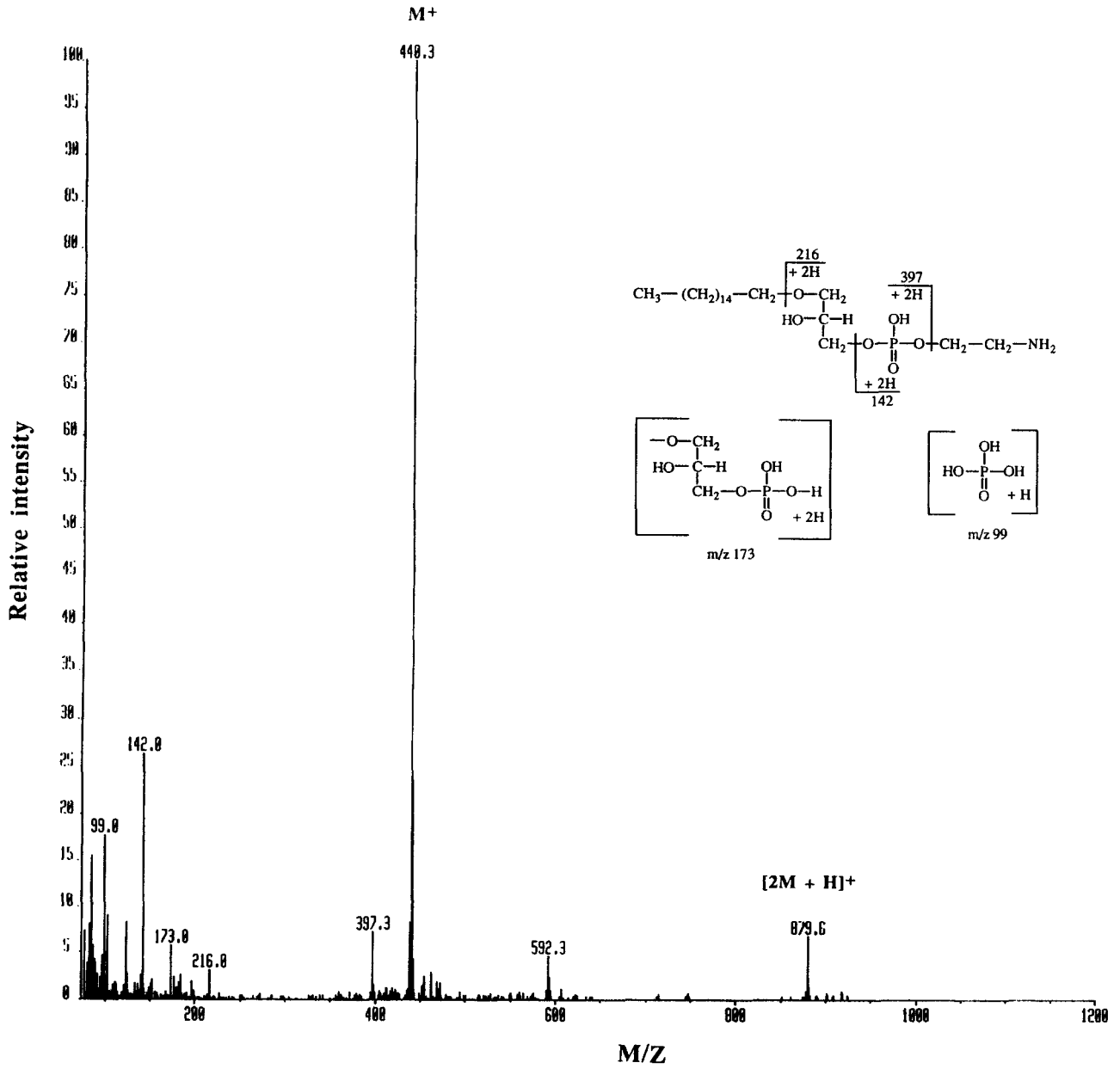


Fig. 1. Positive ion FAB–MS spectrophotometry of 1-O-hexadecyl glycerophosphorylethanolamine (IV). The *m/z* 440 represents the protonated molecular ion. The identification of major fragments is shown in the inset of the figure.

of the keto compound. The reaction method and the extraction procedure were as described above. The results as checked by TLC showed that the reduction was quantitative. The specific activity of the ^{3}H-labeled compound (III) varied from 25,000 to 30,000 cpm/nmol.

The ^{3}H-labeled compound (III) was subjected to hydrogenolysis following the protocol used for the nonradioactive compound described above. The ^{3}H-labeled compound (IV) was found to be >90% pure with a yield of 77%. This radiolabeled compound was purified by chromatography as described above, stored in $CHCl_3$–methanol 1:1 at −20°C, and used for the assay of plasmalogen biosynthesis.

Plasmalogen biosynthesis (Δ1′-desaturase) assay

Incubation. The incubation was done as described by Wykle and Lockmiller (14). A solution of 6 nmol of 1-O-hexadecyl-*rac*-[2-^{3}H]GPE (compound IV) (sp. activity, 27,000 cpm/nmol) in 10 μl of ethyl alcohol was added to the incubation mixture (0.6 ml final volume)

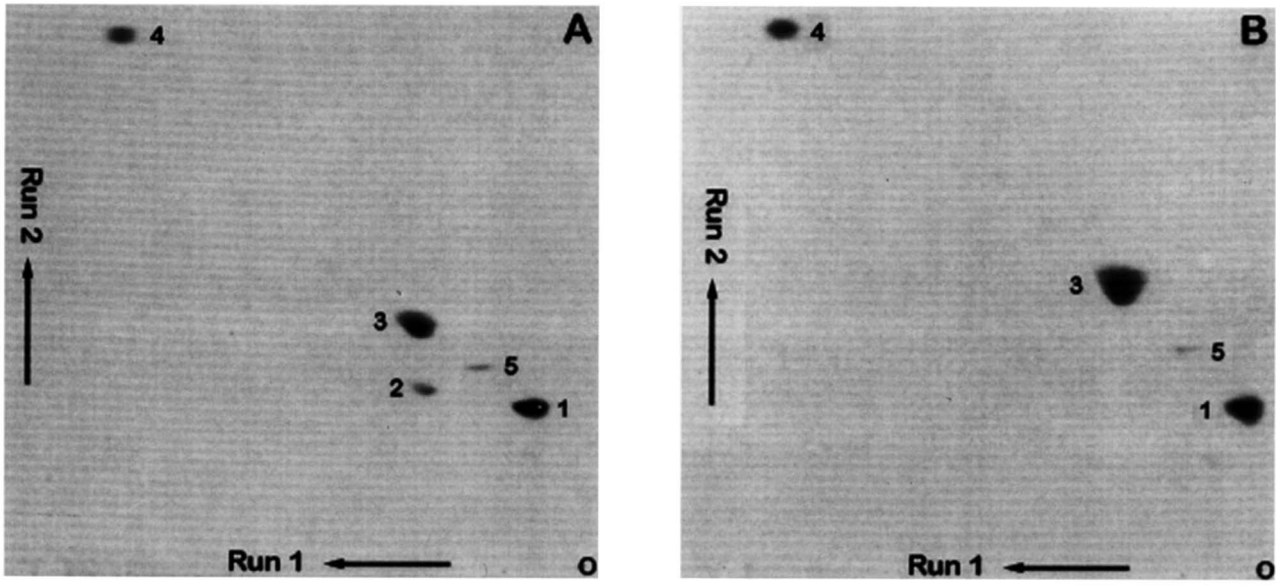


Fig. 2. Radioautogram of a 2D-TLC of the reaction products of plasmalogen biosynthesis. A: The assay mixture contained Tris-HCl buffer (0.1 M, pH 7.4), 1-O-hexadecyl-*rac*-[2-^{3}H]GPE (10 μM, 27,000 cpm/nmol), NADPH (2 mM), and brain microsomes (0.5 mg protein) in a total volume of 0.6 ml. The incubation conditions, extraction of lipids, TLC analysis, and the identification of the radioactive products were as described in the text. O, origin; 1, 1-O-hexadecyl-[2-^{3}H]GPE; 2, 2-acyl-[2-^{3}H]GPE (derived from plasmalogen); 3, 1-O-hexadecyl-2-acyl-[2-^{3}H]GPE; and 4, 1-O-hexadecyl-[2-^{3}H]glycerol. Spot no. 5 possibly represents ether analog of PtdSer formed due to base exchange reaction (see text). B: Same as A except that the experiment was performed in the absence of NADPH. GPE, glycerophosphorylethanolamine.

containing Tris-HCl (0.1 M, pH 7.4) and NADPH (2 mM). The reaction was started by adding brain microsomes (0.5 mg protein). The mixture was incubated at 37°C with vigorous shaking for 75 min and then terminated by adding 2.25 ml of $CHCl_3$—CH_3OH 1:2. A further addition of 0.75 ml of $CHCl_3$ and 0.75 ml of water was made to separate the phases. After mixing and centrifugation, the $CHCl_3$ layer containing the products was analyzed by two different assay methods.

Assay method I (*two-dimensional TLC method*). The method followed was that as described by other workers (14, 26). A known aliquot of the $CHCl_3$ solution containing ^{3}H-labeled product (see above) was applied as a single spot on a TLC plate and subjected to two-dimensional chromatography (with HCl fume treatment after first dimension) as described by Horrocks (26). After air drying, the plate was sprayed evenly with EN^{3}HANCE and radioautographed using X-ray film at −70°C overnight. The radioactive spots on the developed chromatogram were identified by comparing their R_fs with those of known standards applied on the sides of the plate.

Figure 2 shows the autoradiogram of the TLC plate and the identification of each radioactive spot. The different ^{3}H-labeled lipids on the TLC plate were extracted from the adsorbents with $CHCl_3$—CH_3OH 1:2 containing HCl (0.05 M) and the radioactivity present in the extracts was determined by liquid scintillation counting after removing the solvent. In all experiments, it was observed that about 50% of the radioactivity recovered was associated with spot #1, the starting substrate (lyso PtdEtn). This radioactivity probably represents mainly the unnatural *sn*-1 isomer, which is not enzymatically acylated. Spot 3, migrating with endogenous brain PtdEtn, represents the acylated substrate, containing 30–37% of the total recovered radioactivity. A part of the substrate is hydrolyzed to 1-O-alkyl-*sn*-glycerol (spot #4, 10–12% radioactivity), which was also observed by Wykle and Lockmiller (14). Plasmalogen is formed only when NADPH is added to the incubation mixture as evidenced by the presence of radioactive lysoPtdEtn (6–8% of total radioactivity) after HCl treatment in Fig. 2A (spot #2) but not in Fig. 2B. Small amounts (1–3%) of other radioactive lipids were also detected in the presence or absence of NADPH, which were tentatively identified as PtdSer (spot #5, Fig. 2) and PtdCho (masked by the unconverted lysoPtdEtn (spot #1), probably formed via the base exchange reactions. The amount of plasmalogen formed was then calculated from the fraction of radioactivity present in spot #2 and reported in **Table 1.**

Assay method 2 (hydrolysis followed by solvent partition). In this method, the reaction products containing the plasmalogen were sequentially subjected to alkaline methanolysis (27) to hydrolyze the *sn*-2 acyl bond followed by $HgCl_2$-catalyzed acid hydrolysis (28) to hydrolyze the *sn*-1-alk-1′-enyl bond of plasmalogens. The resulting water-soluble radioactive product, *sn*-[2-

TABLE 1. Comparison of the assay methods

Experiments	Plasmalogen Formed			
	Assay Method 1 (2D-TLC)		Assay Method 2 (Partition)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	pmol/h/mg protein			
1. Complete system	386	344	400	396
2. Complete system − NADPH	28	33	30	21
3. Complete system (0 time)	0	—	2	—

The incubation of 1-O-hexadecyl-*rac*-[2-^{3}H]glycerophosphorylethanolamine with brain microsomes was done as described in the text. The plasmalogen formed during incubation was extracted by using the Bligh and Dyer extraction method (23) and estimated by two different assay methods (see text for details).

^{3}H]glycerophosphoryl ethanolamine, was isolated by the following solvent partition method and counted.

To 1 ml of the $CHCl_3$ layer containing the ^{3}H-labeled products, 1 ml of 0.21 M NaOH in CH_3OH was added. The mixture was incubated at room temperature for 1 h. The alkaline methanolysis was stopped by adding 35 µl of 6 N HCl and then the acid hydrolysis was performed by adding 65 µl of 0.1 M $HgCl_2$ in 2 N HCl (28). After 15 min at 37°C, 125 µl of 1 M Tris-base was added to neutralize the acid. To this mixture 0.52 ml of water and 1 ml of $CHCl_3$ were added and mixed well. After centrifugation, the top aqueous phases were transferred to another set of tubes and washed with 2 ml of $CHCl_3$. The radioactivity in the washed aqueous phase was determined by liquid scintillation counting. The results are shown in Table 1 along with those from the TLC assay method.

DISCUSSION

A large number of synthetic procedures have been described for the preparation of acyl or alkyl ethanolamine phosphoglycerides (for a review see refs. 29, 30). In these methods the starting material is generally a derivative of glycerol (e.g., isopropylidine, benzidine, trityl, etc.) and different protective agents are used to selectively block specific hydroxyl groups of the glycerol moiety from acylation, alkylation, or phosphorylation. The best such procedure in terms of overall yield (~30%) is probably the one recently reported by Abdelmageed et al. (31), who prepared the ether analog of PtdEtn by using the method of Eibl (32) to introduce the Etn head group into the molecule.

We previously described a novel method of synthesizing phospholipids by phosphorolysis of acyl and alkyl derivatives of diazoacetone, followed by the reduction of the keto lipid (12, 13). We extended this method to the synthesis of a phosphodiester lipid, i.e., 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) (13). The yield of the choline phosphoglyceride, however, was comparatively low (<20%) because of the low solubility of one of the reactants, the zwitterionic phosphorylcholine, in the reaction mixture containing organic solvents. Unlike phosphocholine, phosphoethanolamine could not be directly used for the phosphorolysis step because of a side reaction of the free primary amino group of ethanolamine phosphate with the carbonyl group of the diazoketone. Using the CBZ derivative of the phosphorylethanolamine solved both the unwanted side reaction and the solubility problems, leading to a higher reaction rate and yield (80%). The resulting keto compound was reduced before removing the CBZ group, also to prevent the side reaction. Starting from hexadecyloxydiazoacetone the overall yield of the pure product (IV) was 53%. These results show that this procedure can be used as a simple method for the synthesis of not only alkyl lysoPtdEtn but also acyl lysoPtdEtn, its only drawback being that a racemic mixture instead of the naturally occurring R-(*sn*-3-phosphoethanolamine) isomer is synthesized. However, as demonstrated for PAF (13), the racemic mixture can be resolved into two isomers by chemical acylation followed by phospholipase A_2 treatment, which hydrolyzes only the natural R-isomer. For this purpose the CBZ derivative should be acylated before hydrogenolysis to prevent the acylation of the free amino group. If an unsaturated acyl group is desired at C-2, then the CBZ group should be removed by acid hydrolysis to prevent the hydrogenation of the double bonds during hydrogenolysis. Alternatively, a t-butyloxcarbonyl derivative instead of the CBZ derivative can be used, and removed under acidic conditions (11). The racemic compound (IV) can also be resolved by enzymatically acylating the 1-O-alkyl-*sn*-3-glycerophosphorylethanolamine isomer by microsomal acyl CoA: lyso PtdEtn acyltransferase (see Fig. 2).

Use of $NaBH_4$ to reduce the keto intermediate enabled us to synthesize 1-O-alkyl[2-^{3}H]glycerophosphorylethanolamine of high specific activity. This ether analog of lysoPtdEtn has been shown to be the best

precursor for the biosynthesis of plasmalogens, and such analogs isotopically (^{14}C or ^{3}H) labeled at different positions have been prepared biosynthetically (8, 10, 33) and chemically (11). As shown here, the ^{3}H-labeled compound prepared by the present method acts as a good substrate for in vitro plasmalogen biosynthesis. The plasmalogens formed in the presence of NADPH were identified and assayed by 2D-reaction TLC (26). The radioactive product (spot #2, Fig. 2A) was found to migrate with the endogenous HCl-hydrolyzed plasmalogens (i.e., 2-acyl glycerophosphorylethanolamine) present in the rat brain microsomal preparation, which was identified by spraying the plate with primuline. The identity of the product as plasmalogen was further confirmed by using a combination of alkaline and acid hydrolysis, as described by Dawson and coworkers (28), to convert the reaction product to water-soluble labeled glycerophosphorylethanolamine. We utilized this hydrolytic procedure to develop a simple assay for plasmonyl $\Delta 1'$-desaturase. As shown in Table 1, the results of this procedure agree well with the two-dimensional TLC assay procedure used by other workers (14). When the chloroform layer of the partition assay was examined by TLC, only alkyl lysoPtdEtn and 1-O-alkylglycerol (10–12% of the added substrate) were found to be present, a result similar to that seen in the TLC assay (Fig. 1). This rapid assay procedure will be helpful in answering a number of questions regarding this enzymatic reaction, such as whether or not catalase (5) or other protein cofactors (9) or ATP (5, 6, 8) or the specific reduced pyridine nucleotide (1, 5, 6) are required for the reaction, as well as helping to determine the nature of the immediate substrate for dehydrogenation (7–9).

As we have previously pointed out (12, 13) this method of synthesizing lipids by phosphorolysis of the acyl or alkyl diazoacetones can be utilized to prepare a wide variety of 2-^{3}H-labeled or unlabeled phosphoglycerides with different head groups and also with different radyl groups at the C-1 and C-2 positions. These lipids should be useful not only for studying the function of different polar groups in the lipids of biomembranes, but also for labeling specific cellular lipid pools (as has been done for PtdCho (34)), or for synthesizing head group analogs of bioactive lipid ligands (e.g., PAF) as possible agonists or antagonists (35).

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