SYNTHESIS OF 1-O-[hexadecyl-1',2'-^{3}H]HEXADECYL 2-ACETYL-sn-GLYCERYL 3-PHOSPHORYLCHOLINE AND 1-O-ALKYL [^{32}P]LYSOPHOSPHATIDYCHOLINE.

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SUMMARY

1-O-[hexadecyl-1',2'-^{3}H]Hexadecyl 2-acetyl-sn-glyceryl 3-phosphoryl-choline (GPC) was prepared by acetylation of 1-O-alkenyl lysophosphatidylcholine, reduction of the alkenyl lipid with tritium gas over palladium oxide, and separation of molecular species of 1-O-[^{3}H]alkyl 2-acetyl GPC by reverse phase thin-layer chromatography. 1-O-Alkyl [^{32}P]lysophosphatidylcholine was prepared by mild alkaline hydrolysis of 1-O-alkyl 2-acyl [^{32}P]phosphatidylcholine, which was enzymatically produced by treating cytidine 5'-[^{32}P]diphosphocholine with 1-O-hexadecyl 2-acetyl-sn-glycerol in the presence of rat liver microsomes. The resulting radiolabeled ether phospholipids possessed high specific radioactivity suitable for metabolic and binding studies.

Key Words: [^{32}P]Alkyl lysophosphatidylcholine, [^{3}H]Platelet Activating Factor, ether phospholipids.

INTRODUCTION

Ether phospholipids have been documented as biologically active compounds, which mediate a variety of physiological reactions such as potent platelet activation, antihypertensive activity, antitumor activity, and immunological response modulation (1). Despite the well-known biological importance of ether phospholipids, relatively little progress has been made to elucidate details of their mechanism of action and physiological functions.

To understand the basis of their physiological actions, metabolic pathways (2), physico-chemical properties, and interactions with their receptors, ether phospholipids with high specific radioactivity need to be prepared. Availability of ether phospholipids with high specific radioactivity would allow researchers to establish structure-function relationships and to design and develop new derivatives with higher biological activity and potency.

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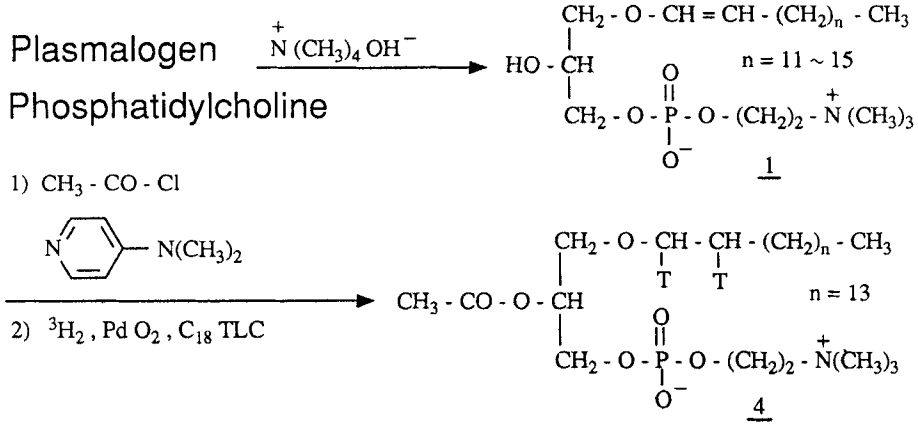
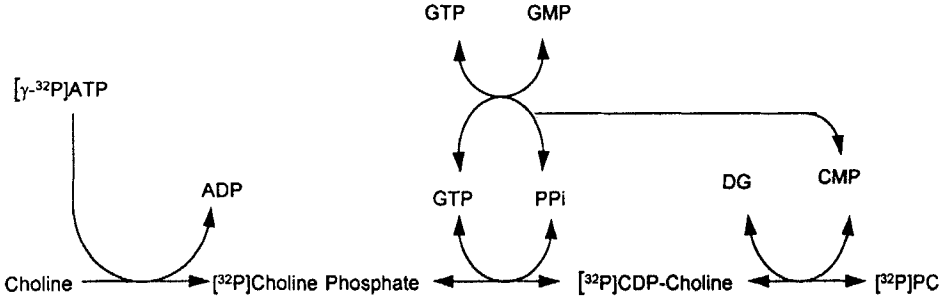
In this report, we have used a facile and efficient synthetic method to prepare 1-O-[^{3}H]hexadecyl 2-acetyl GPC from readily available bovine heart phosphatidylcholine (PC) in three steps.



We employed rat livers as sources of two enzymes, CTP: phosphocholine cytidylyltransferase (CT) and CDP-choline: Alkylacetyl glycerol cholinephosphotransferase (CACPT) for preparing 1-O-alkyl [^{32}P]Lyso PC following the Scheme 1.

SCHEME 1



RESULTS AND DISCUSSION

The preparation of 1-O-[^{3}H]alkyl 2-acetyl GPC has been reported (3). However, no attempt was made to isolate pure 1-O-[^{3}H]hexadecyl 2-acetyl GPC from the 1-O-[^{3}H]alkyl 2-acetyl GPC. A salient feature of our work is the isolation of pure 1-O-[^{3}H]hexadecyl 2-acetyl GPC by reverse phase Thin-Layer Chromatography (TLC) as described in the Experimental Section. The product was further characterized by phospholipase A_2 and C, as well as by mass spectrometry.

The presence and distribution of tritium in 1-O-[^{3}H]alkyl 2-acetyl GPC was established by ^{3}H-NMR analysis. Proton-decoupled ^{3}H-NMR spectrum in Fig. 1 showed an uneven distribution of tritium at 1' and 2' positions (42% and 58%, respectively). Closer examination of the triplet at δ = 1.48 ppm indicated that the triplet consisted of two overlapping doublets with J = 7.3 H_Z due

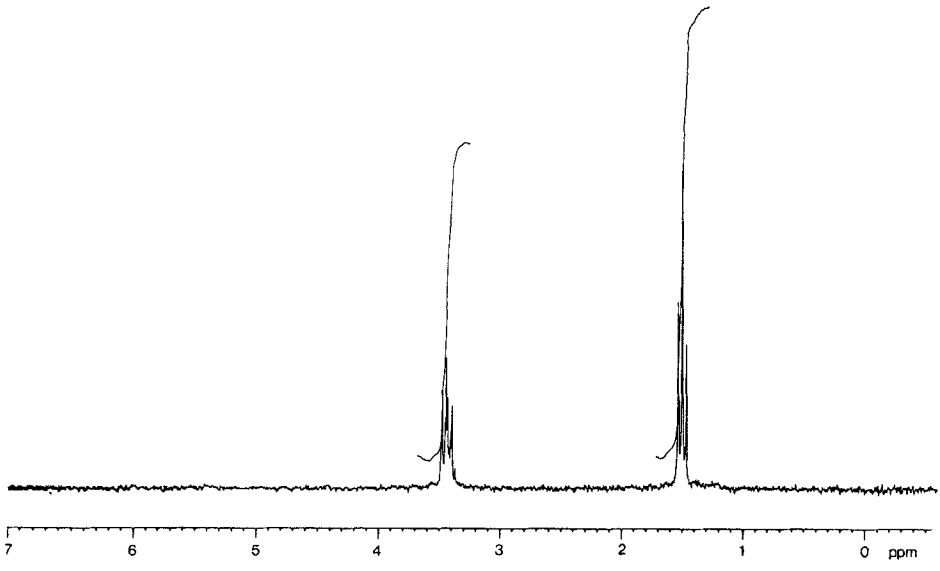


Fig.1. The ^{3}H-NMR Spectrum of 1-O-[alkyl-1',2'-^{3}H]alkyl 2-acetyl GPC in CD_3OD at 213.47 MHz

^{3}H	δ ppm	% tritium
O-CH-^{3}H	3.42 (multiplet)	42
O-CH_2-C^{3}H	1.48 (triplet)[a]	58

a The triplet consists of two overlapping doublets (J = 7.3 Hz)

to R- and S- configuration of tritium on 1' position and the inductive effect of the proton on the sn-2 position attached to the asymmetric carbon atom 2 of the glycerol backbone. The absence of any tritium resonance at δ = 1.15 ppm indicated no tritium incorporation beyond 2' position. For example, 1,2-[9,10-^{3}H]dipalmitoyl GPC has a strong multiplet tritium resonance at δ = 1.15 pm, which is indicative of tritium incorporation beyond 2' position.

To determine the composition of different [^{3}H]alkyl groups in 1-O-[^{3}H]alkyl 2-acetyl GPC, the ^{3}H-ether phospholipid was reduced with $LiAlH_4$ to 1-O-[^{3}H]alkyl glycerols, which were derivatized to the corresponding diacetates for subsequent Gas Liquid Chromatography

(GLC) analysis. Table 1 shows the distribution of different [^{3}H] alkyl moieties in 1-O-[^{3}H]alkyl 2-acetyl GPC. From this data, it is clear that hexadecyl group was the major alkyl moiety in 1-O-[^{3}H]alkyl 2-acetyl GPC. Small amounts of tetradecyl (4.5%) and octadecyl (12.0%) groups were also present.

The specific radioactivity level of 1-O-[^{3}H]hexadecyl 2-acetyl GPC, 50-65 Ci/mmol, was consistent with the expectation of an average two tritium atoms based on the incorporation of tritium on the 1' and 2' positions of hexadecyl moiety. Thus, the 3-step partial synthesis of 1-O-[^{3}H]hexadecyl 2-acetyl GPC was convenient and fast with good overall yield and high specific radioactivity.

Previously, two methods were reported for synthesis of [^{32}P]PC, using either a combination of enzymatic and chemical syntheses (4) or cell cultures (5,6). The first method required multi-step synthesis and purifications. Low recovery and potential health risk were the major concerns with this method when [^{32}P]PC of high specific radioactivity was prepared. The method using cell cultures for synthesis of [^{32}P]PC employed [^{32}P]orthophosphate as a labeling reagent. The major disadvantage of this method was the low yield due to the limited endogenous substrates. In addition, [^{32}P]PC had to be purified from many other phospholipids.

In our enzymatic method of preparing 1-O-alkyl [^{32}P]Lyso PC (Scheme 1), ^{32}P from carrier-free [γ-^{32}P]ATP was initially incorporated quantitatively into choline phosphate. The choline [^{32}P]phosphate formed in the reaction mixture was used without purification as the substrate for the succeeding reaction for [^{32}P]CDP-choline synthesis. In this rate-limiting step of

TABLE 1. Distribution of radioactivity among 1-O-[^{3}H]alkyl glycerols derived from 1-O-[alkyl-1',2'-^{3}H]alkyl 2-Acetyl GPC.

Side Chain	% Radioactivity
Tetradecyl	4.5
Hexadecyl	81.0
Octadecyl	12.0
Others	2.5
Total	100.0

Values represent means determined in triplicate observations from three separate experiments.

biosynthesis of platelet activating factor (PAF) (2), a rat liver 10,000 g supernatant was used as the source of CTP:phosphocholine cytidylyltransferase (CT).

A CTP concentration of 8 mM was tested to obtain maximum formation of [^{32}P]CDP-choline. Although 80% of the total radioactivity was incorporated into [^{32}P]CDP-choline after incubation with 8 mM CTP for 7 h (Fig. 2), the reaction mixture was not suitable for [^{32}P]PC synthesis due to gradual decomposition of [^{32}P]CDP-choline and a high concentration of CMP, which was formed from the breakdown of CTP, presumably by phosphodiesterases existing in the crude preparation of rat liver (Scheme 1). This concentration was much higher than the reported Km, 0.19 mM, of cholinephosphotransferase (CACPT) toward CMP and thus favored the reverse reaction of PC synthesis (7).

In order to decrease the final concentration of CMP in the reaction mixture of PC synthesis, 1 mM CTP was used in a test reaction. However, in this reaction the yield of

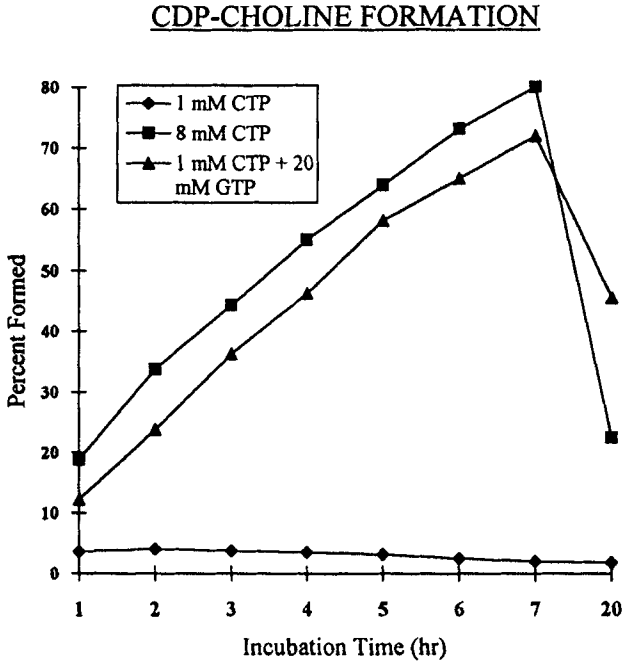


Fig. 2 Time Course of [^{32}P]CDP-Choline Formation

The incubation mixture contained 10 mCi [^{32}P]Choline phosphate, 10 mg proteins of rat liver 10,000 g supernatant, and various amounts of nucleotides as follows: 1 mM CTP, 8 mM CTP, or 1 mM CTP and 20 mM GTP.

[^{32}P]CDP-choline never exceeded 5% presumably due to the CTP breakdown and the hydrolysis of newly synthesized [^{32}P]CDP-choline by phosphodiesterases (Fig. 2).

In order to obtain a high yield of [^{32}P]CDP-choline and to maintain a low concentration of CMP, we included 20 mM GTP along with 1 mM CTP in the incubation. We intended to use a high concentration of GTP to compete with the breakdown of CTP and [^{32}P]CDP-choline. As shown in Fig. 2, [^{32}P]CDP-choline formation shows a similar time course to that of the reaction using 8 mM CTP. This result shows that a high concentration of GTP indeed protected CTP from breakdown by competing for nonspecific phosphodiesterase activity (Scheme 1). A steady concentration of CTP thus enabled CACPT reaction to continue and allowed the yield of [^{32}P]CDP-choline to reach approximately 72% after 7 h. On the other hand, we found that the breakdown product of GTP did not significantly inhibit [^{32}P]PC synthesis. Therefore, this reaction condition was employed for the routine [^{32}P]PC synthesis.

[^{32}P]PCs formed were subsequently deacetylated by methanolic NaOH and the reaction mixture was purified on a Sep-Pak column to give >99% radiochemically pure 1-O-alkyl [^{32}P]lyso PC. We were unable to determine the alkyl composition of 1-O-alkyl [^{32}P]lyso PC because of its short half life and health physics concern. However, the predominant species of alkyl group was expected to be the hexadecyl moiety because rat liver was reported to contain a very small amount of 1-O-alkyl PC (<1% total PC) and large amounts of diacyl PC (>99% total PC) (12). [^{32}P]Diacyl PC was readily hydrolyzed by methanolic NaOH to [^{32}P]GPC, which was subsequently separated from 1-O-alkyl [^{32}P]Lyso PC on the Sep-Pak column.

Overall, this method allows high conversion at each step of the enzymatic synthesis of 1-O-alkyl [^{32}P]lyso PC and eliminates extraction and purification of all intermediates. Our method has given a high yield of the final product, usually 20-30%, based on starting [γ-^{32}P]ATP. The synthesis of 1-O-alkyl [^{32}P]lyso PC by this method can be accomplished in two days. This is advantageous because of the short half-life of ^{32}P (14 days) and the risk of operator exposure to a large quantity of radioactivity.

EXPERIMENTAL

Materials: Beef heart phosphatidylcholines were obtained from Avanti Polar Lipids, Pelham, AL. Acetyl chloride, N,N-dimethylaminopyridine, lithium aluminum hydride, and 1M methanolic tetramethylammonium hydroxide were products of Aldrich, Milwaukee, WI. Palladium oxide was a product of American Platinum Works, Newark, NJ. Phospholipase A_2 from Crotalus durissus venom, phospholipase C from Clostridium perfringens, choline kinase, CTP, GTP, choline

chloride, 1-O-alkyl 2-acetyl GPC, molybdenum blue, ninhydrin and Dragendorff spray reagents were purchased from Sigma, St. Louis, MO. 1-O-hexadecyl 2-acetyl-sn-glycerol was obtained from Cayman Chemical Company, Ann Arbor, MI. [γ-^{32}P]ATP, S.A. 3,000 Ci/mmol was provided by ^{32}P-Lab, DuPont, NEN PRODUCTS, Boston, MA. Solvents were all reagent grade and, unless otherwise specified, were used as received. Precoated silica gel plates, LK5, LK5D and LKC-18, plates were purchased from Whatman.

General: ^{1}H and ^{3}H nuclear magnetic resonance (NMR) spectra were obtained on a Bruker WP 200 spectrometer in CD_3OD or $CDCl_3/CD_3OD$ (2:1) with tetramethylsilane as an internal standard. Chemical shifts are reported relative to tetramethylsilane at 0.0 ppm.

Mass spectrometry (MS) was performed on Kratos MS25 mass spectrometer using Fast Atom Bombardment mode. High performance liquid chromatography was conducted on a Waters high pressure chromatograph 201 U/6000 equipped with a UV detector and a liquid scintillation flowthrough monitor. Gas liquid chromatography was conducted on a Hewlett Packard 5710A gas chromatograph equipped with a flame ionization detector and a gas proportional monitor. A nickel column (1.8 m x 4 mm i.d.) was packed with 12% stabilized DEGS on Gas-Chrom Q (100/120 mesh). The flow rate was 40 ml/min. Temperature was programmed as follows: the initial temperature, 195°C, was maintained for two min and then increased at a rate of 8°/min until it reached 220°C, which was maintained for 16 min. The injector temperature and detector temperature were set at 250°C.

Preparation of 1-O-alkenyl lysophosphatidylcholine, 1: To 1 g of bovine heart phosphatidylcholines dissolved in 10 ml of diethyl ether was added 1 ml of 1M methanolic tetramethylammonium hydroxide solution, and the mixture was stirred at room temperature (8). After 1 h the solvent was decanted and the reaction flask was rinsed twice with 10 ml of diethyl ether to remove any residual fatty acyl groups. Yield was 550 mg (55% by weight).

Preparation of 1-O-alkenyl 2-acetyl-sn-glyceryl-3-phosphorylcholine, 2: Beef heart 1 (0.3 mmol) and 2.7 mmol of N,N-dimethylaminopyridine in a round bottom flask were dried over P_2O_5 in a vacuum dessicator for 30 min and the vacuum released with a stream of argon. The mixture was dissolved in 10 ml of dry, ethanol-free chloroform distilled over P_2O_5. To this solution was added 1.2 mmol of acetyl chloride at 0°C. The reaction mixture was allowed to reach room temperature, stirred for 2.5 h, transferred to a separatory funnel with 20 ml of chloroform and washed twice with 25 ml of methanol-water (1:1). The chloroform layer was dried in vacuo and the residue was redissolved in 10 ml of $CHCl_3$-CH_3OH (95:5) and stored at -20°C.

TLC of the product showed the presence of a single spot when the plate was visualized with molybdenum blue reagent. In order to ensure separation of close-running impurities, the following neutral, acidic acid basic solvent systems were used:

A) $CHCl_3/CH_3OH/H_2O$ (65:25:4) $R_f = 0.30$

B) $CHCl_3/CH_3OH/CH_3CO_2H/H_2O$ (100:50:14:6) $R_f = 0.40$

C) $CHCl_3/CH_3OH/29\%\ NH_4OH$ (65:35:5) $R_f = 0.30$

Preparation of 1-[^{3}H]alkyl 2-acetyl sn-glyceryl-3-phosphorylcholine, 3: A solution of 2 (15 mg) in 1 ml of dimethylformamide was reduced with tritium gas over palladium oxide for 2 h at room temperature. The crude products containing 1,263 mCi were dissolved in 80 ml of $CHCl_3/CH_3OH$ (2:1) and washed once with 20 ml of 1N HCl. The upper layer was extracted with 20 ml of chloroform. The combined chloroform layers were washed once with 50 ml of methanol/1N HCl (1:1) and then with 50 ml of methanol/water (1:1). The lower layer was dried in vacuo and then purified by preparative TLC on two PLK5 plates (20 x 20 cm) with $CHCl_3/CH_3OH/H_2O$ (65:25:4). The band corresponding to 1-O-[^{3}H]alkyl 2-acetyl GPC was scraped off and eluted with 40 ml of $CHCl_3/CH_3OH/H_2O$ (1:2:0.8) for 20 min. After the removal of silica gel by filtration, the flask and silica gel on the glass-sintered funnel were washed with 30 ml of $CHCl_3/CH_3OH$ (2:1). The filtrate was partitioned after the addition of 30 ml of chloroform and 14 ml of water. The lower layer was washed once more to remove the residual silica gel with 30 ml of CH_3OH/H_2O (1:1), evaporated to dryness, and redissolved in ethanol for storage. TLC $R_f = 0.25$ with $CHCl_3/CH_3OH/H_2O$ (65:25:4). ^{3}H-NMR (δ) 1.48 (^{3}H, t, O-CH_2-C^3H-moiety), 3.42 (^{3}H, m, O-C^3H-moiety). HPLC 10 μm Lichrosorb SI60 column (25 x 0.9 cm) was eluted with $CH_3CN/CH_3OH/H_2O$ (5:3:2). Retention time = 8 min at a flow rate of 1 ml/min.

Preparation of 1-O-[^{3}H]hexadecyl 2-acetyl GPC, 4: The molecular species of 3 (80 mCi) was fractionated by C_{18} reverse phase preparative TLC. The development solvent consisted of methanol/water/acetonitrile (91:6:3) containing 6 gm choline chloride per 100 ml. The plate was wrapped in a plastic wrap, exposed to X-ray film (DuPont, Wilmington, DE), and stored in a dry ice box. The molecular species of 1-O-[^{3}H]alkyl 2-acetyl GPC were visualized by autoradiography for 2 h. The band corresponding to 4 ($R_f = 0.27$) was eluted as described in the previous TLC purification of 3. The purified 4 had the following TLC patterns:

A) $CHCl_3/CH_3OH/H_2O$ (65:25:4) $R_f = 0.25$

B) $CHCl_3/CH_3OH/CH_3CO_2H/H_2O$ (100:50:14:6) $R_f = 0.38$

C) $CHCl_3/CH_3OH/29\%\ NH_4OH$ (65:35:5) $Rf = 0.28$

The specific radioactivity of 4 was determined by mass spectrometry. A typical result showed MH^+ peaks at 524 (unlabelled; 8.3%), 526 (1-^{3}H; 8.3%), 528 (2-^{3}H; 39.0%) and 530 (3-^{3}H; 42.9%).

Determination of Optical Purity of 3: The purified 3 (1 mCi) in 1 ml of diethyl ether was treated with 0.2 mg of phospholipase A_2 from Crotalus durissus (9) or phospholipase C from Clostridium perfringens (10) in 1 ml 0.01M borate buffer containing 0.01M $CaCl_2$, pH 7.2 for 3-6 h at room temperature. Ether was removed by a stream of nitrogen. The residue was extracted with 4 ml of $CHCl_3/CH_3OH$ (2:1). The lower layer was washed once with 1.5 ml of CH_3OH/H_2O (1:1).

An aliquot of phospholipase A_2 products was spotted on a LK5 plate and developed with $CHCl_3/CH_3OH/H_2O$ (65:25:4). From this experiment, more than 98% of 3 was found to be hydrolyzed to 1-[^{3}H]alkyl lyso PC and no radioactivity was associated with 1-O-alkyl 2-acetyl GPC.

An aliquot of phospholipase C products was co-spotted on a silica gel G plate with the authentic 1-O-hexadecyl 2-acetyl glycerol and developed with hexane/ether/acetic acid (70:30:1). More than 95% of radioactivity migrated with 1-O-hexadecyl 2-acetyl glycerol.

Preparation and acetylation of 1-O-[^{3}H]alkyl glycerols, 5: 1-O-[^{3}H]Alkyl glycerols were obtained by direct reduction of 3 with $LiAlH_4$ as described by Do and Ramachandran (11). The crude 5 were purified and converted to the corresponding diacetates as described by Do and Ramachandran (11).

Rat liver preparations: Frozen rat livers (obtained from Pel-Freez Biologicals, Roger, AR) were homogenized (1 g/1 ml) in ice cold buffer containing 0.1M K_2HPO_4, pH 7.4 and 0.25M sucrose. The homogenate was centrifuged at 10,000 g for 30 min. The supernatant was utilized as the source of CTP:phosphocholine cytidylyltransferase (CT). A portion of the supernatant was further centrifuged at 105,000 g for 1 h. The pellet was homogenized in the same buffer described above and utilized as the source of CDP-choline:alkylacetyl glycerol cholinephosphotransferase (CACPT).

Synthesis of 1-O-alkyl 2-[^{32}P]lyso PC, 6: Choline [^{32}P] phosphate was prepared by incubating 10 mM $MgCl_2$, 200 uM choline chloride, 10 mCi [γ-^{32}P]ATP, 0.5 unit choline kinase, and 100 mM tris-HCl buffer, pH 8.0. The reaction was stirred at room temperature for approximately 1 h until the radioactivity was completely incorporated into choline [^{32}P]phosphate, which was used without purification for [^{32}P]CDP-choline synthesis. A solution containing 1 mM CTP, 20 mM GTP, and 10 mg proteins of rat liver 10,000 g supernatant was added to the above reaction

mixture. The reaction was stirred at room temperature for approximately 7 h until 72% of radioactivity was incorporated into [^{32}P]CDP-choline as shown by TLC analysis. TLC plates (LK5 and LK5D) were developed in the following solvent systems: Solvent D, CH_3OH/0.9% NaCl/NH_4OH (10:10:1), was used to identify choline chloride (R_f = 0.10), choline phosphate (R_f = 0.55), and CDP-choline (R_f = 0.79). Solvent C was used to identify lyso PC and other phospholipids formed in the synthesis.

The reaction mixture was diluted with 3 ml tris-HCl buffer (100 mM, pH 8.0) followed by addition of 4 mg 1-O-hexadecyl 2-acetyl-sn-glycerol in 80 ul ethanol, 10 mM $MgCl_2$, and 10 mg rat liver microsomes. The reaction was allowed to proceed for 2 h with vigorous stirring at room temperature. The resulting 1-O-alkyl 2-acetyl [^{32}P]GPC was hydrolyzed to 6 by the addition of 4 ml of 1 M methanolic NaOH and stirring at room temperature for 1 h.

Extraction and Purification of 6: The crude 6 reaction mixture was adjusted to pH 3 with HCOOH and extracted three times with 8 ml $CHCl_3$. The extracts were applied to a silica Sep-pak (Waters). The Sep-pak was subsequently eluted with 4 x 4 ml $CH_3OH/CHCl_3$ (70:30) to remove hydrophobic impurities and with 4 x 4 ml Solvent C to elute 6. The fractions containing 6 were combined and washed twice with CH_3OH/H_2O at a ratio of $CHCl_3/CH_3OH/H_2O$ (2:1:1).

In some cases when separation with Sep-pak failed, the product was repurified by TLC on a LK5 (5 x 20 cm) plate in Solvent C. The product was located by autoradiography by exposing the plate on a X-Ray film for 5 min. The silica gel in the area containing the product was scraped and eluted with 16 ml Solvent C. The pure 6 (R_f = 0.19) co-chromatographed with 1-O-[^{3}H]alkyl lyso PC in solvent C.

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