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Biochemistry

Glycerophospholipid synthesis: Improved general method and new analogs containing photoactivable groups

(*p*-dimethylaminopyridine/membrane bilayer/crosslinking/lipid–lipid interactions/phospholipid–protein interactions)

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ABSTRACT **Current methods for phospholipid synthesis involving acylation of *sn*-glycero-3-phosphorylcholine, lysolecithins, and related glycerophosphate esters are not satisfactory. With *N,N*-dimethyl-4-aminopyridine as a catalyst and moderate amounts of fatty acid anhydrides (1.2–1.5 mol equiv per OH group), diacyl or 1,2-mixed diacylphosphatidylcholines, *N*-protected phosphatidylethanolamines, and phosphatidic acids now can be conveniently prepared in high yields (75–90%). New phospholipids containing photoactivable groups, such as trifluorodiazopropionyl, diazirinophenoxy, 2-nitro-4-azidophenoxy, *m*-azidophenoxy, and α,β-unsaturated keto groups, in the fatty acyl chains have been prepared. These phospholipids are of interest in studies of lipid–lipid and lipid–protein interactions in biological membranes.**

Biochemical studies of phospholipid–phospholipid and phospholipid–protein interactions are of interest in obtaining further insight into the functions of biological membranes. A general chemical approach to this problem which is being investigated in this laboratory starts with the preparation of fatty acid analogs that carry suitable photoactivable groups (1) and aims at bringing about crosslinking between the phospholipids carrying these groups and the neighboring phospholipids or proteins. For studies *in vivo*, the synthetic fatty acids may be incorporated into the membrane phospholipids, and this has already been demonstrated for an *Escherichia coli* unsaturated fatty acid auxotroph (2, 3). For *in vitro* studies, the above fatty acid analogs are first incorporated synthetically into phospholipids, and the phospholipids thus obtained can then be used in various defined systems involving phospholipid–phospholipid and phospholipid–protein interactions. In the latter studies, mild and efficient methods for the chemical synthesis of 1,2-diacyl and mixed diacyl glycerophospholipids are clearly desirable because the synthetic photoreactive fatty acids are relatively inaccessible and, in addition, may be sensitive to harsh conditions. The present communication describes a new and efficient procedure for phospholipid synthesis that has a marked advantage over the procedures currently available (4–8). The new method has been applied to the synthesis of various 1,2-mixed diacyl phospholipids containing photoactivable groups in the fatty acyl moieties.

Of the different approaches to the synthesis of glycerophospholipids (4–8), the one involving acylation(s) of a preformed phospholipid backbone, such as *sn*-glycero-3-phosphorylcholine (I) (GPC) is particularly attractive. The diacyl product may, when necessary, be converted to the 2-lysophospholipid by treatment with phospholipase A_2, and subsequent acylation of the 2-OH group (Fig. 1) then yields the mixed diacyl phospholipid. Commonly used reagents for acylation of GPC and of lysophospholipids (III) are fatty acyl chlorides (9), anhydrides (10, 11), or mixed anhydrides (7, 12). The reagents are usually used in large excess (5- to 10-fold), especially because the acylation of the 2-OH group is particularly sluggish. Further, the use of fatty acyl chlorides may be accompanied by the formation of significant amounts of several side products (13); acylation with anhydrides requires relatively vigorous conditions—e.g., 48 hr at 80° (10, 11). Finally, the yields most often are unsatisfactory. In the present work, it has been found that *N,N*-dimethyl-4-aminopyridine, which has been shown to be a powerful catalyst in many acylation reactions in organic synthesis (14, 15), is also very efficient in phospholipid synthesis. Acylations of GPC, lysolecithins, *N*-protected lysophosphatidylethanolamines, and *sn*-glycero-3-phosphate (Fig. 1) all could be accomplished in 75–90% yields at room temperature with moderate amounts (1.2–1.5 mol equiv per OH group) of fatty acyl anhydrides. From the standpoint of phospholipid–phospholipid and phospholipid–protein interaction studies, different photoactivable groups, which have been successfully prepared, are shown in Fig. 2.

MATERIALS AND METHODS

Materials. DL-α-Glycerol phosphate (disodium salt), *sn*-glycero-3-phosphate (dicyclohexylammonium salt), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine were purchased from Sigma Chemical Co. The purity of these materials was checked by thin-layer chromatography on silica gel plates and by paper chromatography using the solvent systems described below. 11-Bromo-undecanoic acid (recrystallized from diethyl ether/petroleum ether, mp 50–51°), *N,N*-dimethyl-4-aminopyridine (recrystallized from chloroform/diethyl ether, mp 112°), *m*-aminophenol (recrystallized from toluene, mp 122–123°), and 4-amino-2-nitrophenol (reagent grade, >99% pure) were obtained from Aldrich Chemical Co. Ricinoleic acid (12-hydroxyoleic acid) was purchased from NuChek Preparations, Inc., MN. 1-[^{14}C]Palmitic acid was purchased from New England Nuclear. Crude rattlesnake venom (*Crotalus adamanteus*) (Ross Allen Reptile Farm, FL) was used as the source of phospholipase A_2.

Silica gel-60 (70–230 mesh), Sephadex LH-20 (25–100-μm beads), and Rexyn I-300 mixed resin were bought from E. M. Laboratories, Inc., Pharmacia Fine Chemicals, and Fisher Scientific Co., respectively.

Methods. GPC as the $CdCl_2$ complex was prepared from purified soya lecithin essentially by the procedure of Chadha (16). 1-Palmitoyl- and 1-stearoyl-GPC were prepared from 1,2-dipalmitoyl- or 1,2-distearoyl-GPC, respectively, by phospholipase A_2 treatment (1). 1,2-Dipalmitoyl-*sn*-glycero-

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Abbreviations: GPC, *sn*-glycero-3-phosphorylcholine (IUPAC-IUB recommended name, glycerophosphocholine); IR, infrared; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance (peaks shown as s = singlet, d = doublet, t = triplet, and m = multiplet).

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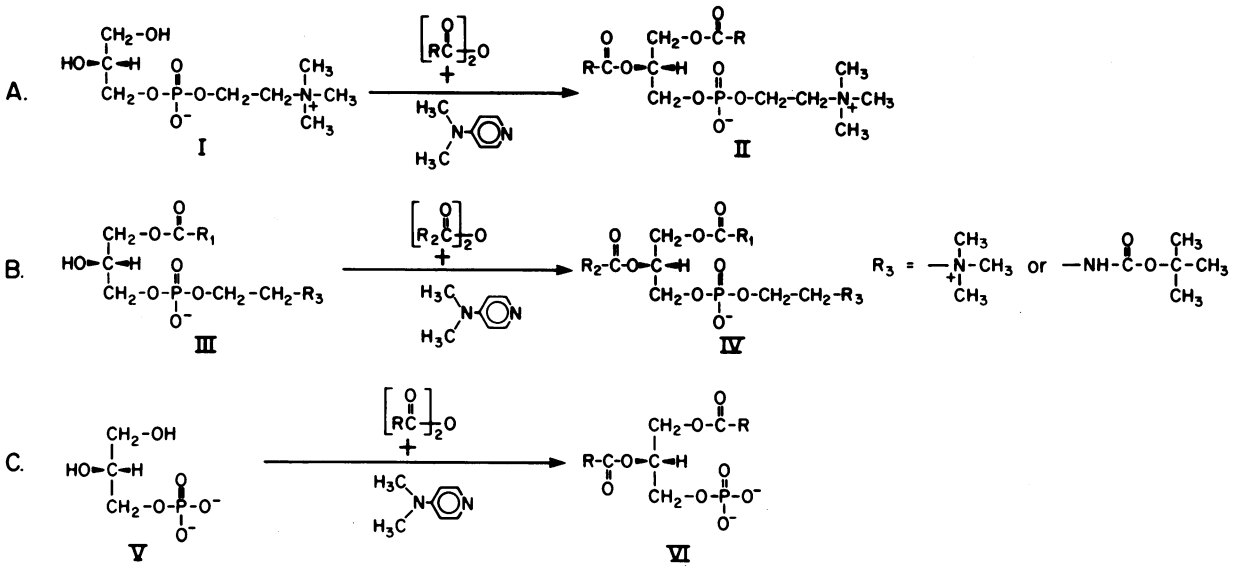


FIG. 1. *N,N*-Dimethyl-4-aminopyridine-catalyzed acylation reactions in the synthesis of phospholipids. (*A*) Acylations of *sn*-glycero-3-phosphorylcholine (GPC). (*B*) Acylation of 1-fatty acyl-*sn*-glycero-3-phosphorylcholines and *N*-protected ethanolamines. (*C*) Synthesis of phosphatidic acids by acylation of *sn*-glycero-3-phosphate.

3-phosphorylethanolamine was converted to its *N-tert*-butoxycarbonyl derivative according to the procedure of Chakrabarti and Khorana (1). Treatment of the protected phosphatidylethanolamine with phospholipase A_2 gave the Ca^{2+} salt of 1-palmitoyl-*sn*-glycero-3-(*N-tert*-butoxycarbonyl)aminoethyl phosphate. To avoid possible acyl group migration from C-1 to C-2, the lysolecithins used in these preparations were not subjected to silica gel column chromatography; instead they were obtained in reasonably pure state simply by washing the insoluble Ca^{2+} salts several times with anhydrous ether.

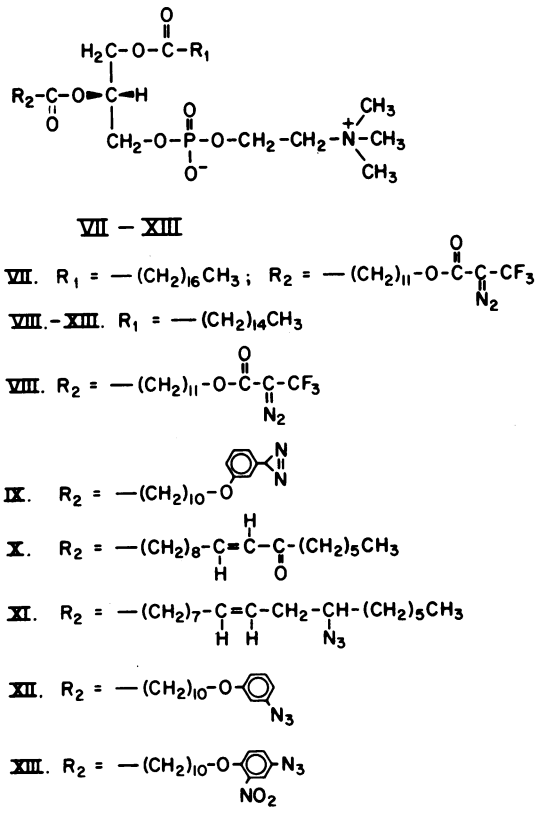


FIG. 2. Synthetic 1,2-diacyl-*sn*-glycero-3-phosphorylcholines. The phospholipids **VII–XIII** were prepared from 1-acyl-*sn*-glycero-3-phosphorylcholines by acylation with anhydrides containing photoactivable groups in the fatty acyl chains.

Fatty acid anhydrides were made by the reaction of fatty acids with 0.55 mol equiv of dicyclohexylcarbodiimide in dry carbon tetrachloride or methylene chloride (17). The anhydrides were characterized by infrared (IR) spectra.

Dry benzene was prepared by distillation over sodium hydride. Dry chloroform was prepared by distillation of the reagent grade solvent over phosphorus pentoxide just before use.

12-Azidooleic acid was prepared from ricinoleic acid according to the described procedure (1). 3,3,3-Trifluoro-2-diazopropionyl chloride was synthesized according to the method of Chowdhry *et al.* (18). *m*-Azidophenol and 4-azido-2-nitrophenol were prepared as described (19). Dl-α-Glycerol 1,2-cyclic phosphate was prepared according to the method of Lapidot *et al.* (10). Methyl 11-iodo-undecanoate was prepared by treatment of 11-bromo-undecanoic acid with potassium iodide in refluxing ethyl methyl ketone followed by esterification of the iodo acid with methanol in the presence of catalytic amounts of *p*-toluenesulfonic acid.

Purity of the various compounds was routinely checked by thin-layer chromatography (TLC) using Merck silica gel plates, or by paper chromatography using Whatman no. 1 paper. The solvents used were: A, chloroform/methanol/water, 65:25:4 (vol/vol), and B, 2-propanol/concentrated ammonia/water, 7:1:2 (vol/vol). The phospholipids were visualized by the molybdenum blue spray (20); the non-fatty-acid-containing phosphate compounds were visualized by the phosphorus spray (21, 22).

For column chromatography, 20–25 g of silica gel was used per mmol scale reaction. Fatty acids were removed by washing the column with $CHCl_3$ (about 500 ml/mmol scale reaction); the phospholipid could be eluted with $CHCl_3$/MeOH, 1:1 (vol/vol). Sephadex LH-20 (25–100-μm beads) chromatography was performed on a 2.5 × 100 cm column with $CHCl_3$/MeOH, 1:1 (vol/vol) as the eluant. Diacylphosphatidylcholines were eluted faster than monoacylphosphatidylcholines and the fatty acids. The separation between lecithins and lysolecithins was marginal but the fatty acids separated quite well from the phospholipids. Reverse-phase high-pressure liquid chromatography (HPLC), as described elsewhere (23), was also used

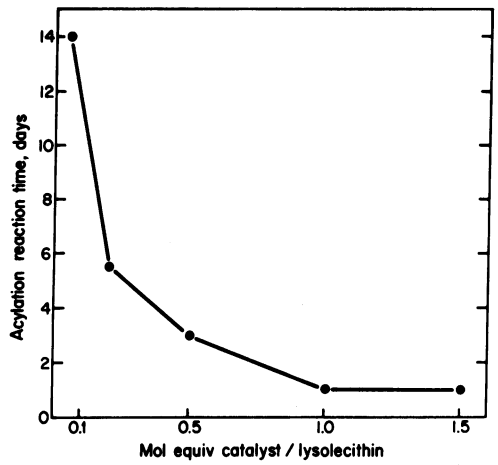


FIG. 3. Time required for the completion of the acylation of 2-hydroxyl group in 1-palmitoyl-GPC as a function of the concentration of *N,N*-dimethyl-4-aminopyridine. In the complete absence of the latter, the extent of acylation did not exceed 10% in 20 days.

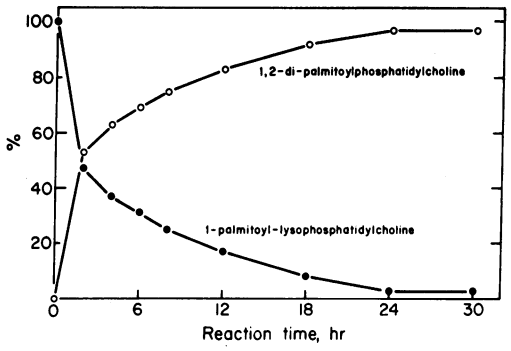


FIG. 4. Rate of acylation of 2-OH group in 1-[^{14}C]palmitoyl-GPC with palmitic anhydride using 1.0 mol equiv of *N,N*-dimethyl-4-aminopyridine. From a vigorously stirred reaction mixture, prepared as described in *text*, aliquots were removed at different time intervals and treated with an equal volume of methanol. After some minutes, they were applied to TLC plates that were developed in solvent A. The products were visualized by autoradiography. The spots corresponding to the monoacyl and diacyl products were assayed for radioactivity.

for the separation of lecithins. The elution solvent was methanol/chloroform/water, 90:10:4 (vol/vol). The rate of elution was 2 ml/min. Total phosphorus was determined by the method of Ames and Dubin (24).

All the compounds were characterized by ultraviolet (UV), IR, and nuclear magnetic resonance (NMR) spectra. The UV spectra were recorded in a Cary-15 UV spectrophotometer; IR spectra were recorded in a Beckman IR 4-210 spectrophotometer; NMR spectra were recorded in a Varian (T-60) spectrometer. Resonance(s) (δ) in the NMR spectra is given in parts per million down field from tetramethylsilane.

The assay of radioactive isotopes was carried out in a Beckman LS-250 liquid scintillation spectrometer with 2,5-diphenyloxazole (15.3 g) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.38 g) in toluene (1.0 gallon; 3.78 liters) as the scintillator.

RESULTS AND DISCUSSION

Catalysis of Acylation Reaction by *N,N*-Dimethyl-4-aminopyridine. Because acylation of the 2-OH group in lysolecithins is particularly sluggish, this reaction was chosen for systematic study.

To an anhydrous suspension of 1-palmitoyl-GPC (0.1 mmol) in 2.5 ml of anhydrous chloroform/pyridine, 4:1 (vol/vol), was added stearic anhydride (0.15 mmol). After being flushed with dry N_2, the sealed reaction mixture was stirred with a magnetic stirrer at 25° for different lengths of time. A parallel series of reaction mixtures was set up containing, in addition, varying amounts of *N,N*-dimethyl-4-aminopyridine. The reaction mixtures were all worked up by evaporation of the solvent followed by chromatography on silica gel or gel filtration on Sephadex LH-20. The times required for completion of the acylation reaction using different amounts of the catalyst are shown in Fig. 3. With chloroform/pyridine alone, the extent of acylation was less than 10% in 20 days. With progressively increasing amounts of dimethylaminopyridine, the time required for completion (>95%) of the reaction continued to decrease. The use of 1.0 mol equiv relative to the hydroxyl group gave optimal results.

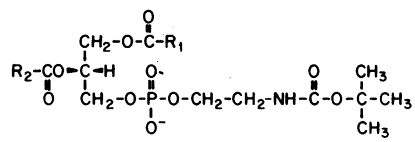
Kinetics of the 2-OH Acylation of 1-[^{14}C]Palmitoyl-GPC with Stearic Anhydride. The results of a second experiment in which the kinetics of acylation of 1-[^{14}C]palmitoyl-GPC, were followed in the presence of dimethylaminopyridine are shown in Fig. 4. The final yield in the acylation was high.

An important consideration for the use of the ^{14}C-labeled palmitoyl group in the present experiment was to determine if any acyl group migration, from C-1 to C-2, occurred during the dimethylaminopyridine-catalyzed acylation of the 2-OH group. After acylation of 1-[^{14}C]palmitoyl-GPC, any migration of the 1-[^{14}C]palmitoyl group would be readily detected by the presence of radioactivity in the fatty acid formed on treatment of the diacyl product with phospholipase A_2. Therefore, in the above experiment, the product, 1-[^{14}C]palmitoyl-2-stearoyl-GPC, was purified by using a Sephadex LH-20 column. Treatment of a portion with phospholipase A_2 was followed by a work-up involving removal of the ether and the buffer under a stream of N_2 and dissolving the residue in methanol/chloroform/water, 5:4:1 (vol/vol). The solution was subjected to TLC on a silica gel plate along with appropriate markers ([^{14}C]palmitic acid, 1-[^{14}C]palmitoyl-GPC, and the corresponding 2-stearoyl derivative). Of the total radioactivity, lysolecithin contained 6500 cpm (95%) and the fatty acid spot contained 350 cpm (5%).

General Procedure for the 1,2-Diacylation of GPC (as $CdCl_2$ Complex). The GPC–$CdCl_2$ complex (1.0 mmol) was rendered anhydrous by repeated evaporation of added dry benzene. The residue was suspended in 25 ml of freshly distilled anhydrous chloroform, the fatty acid anhydride (2.5 mmol) and *N,N*-dimethyl-4-aminopyridine (2.0 mmol) were added, and the reaction flask was tightly sealed after being flushed with dry N_2. The mixture was stirred with a magnetic stirrer at room temperature (about 25°) under protection from direct laboratory light for 30 hr. The solvent was then removed under reduced pressure and the residue was treated with 20 ml of methanol/chloroform/water, 5:4:1 (vol/vol). After the insoluble suspension was removed by filtration, the clear solution was passed through a column of Rexyn I-300 resin† (50 ml). The resin was washed with two bed volumes of the same solvent and, after removal of the solvents, the product in the total effluent, was purifed either by silica gel or Sephadex LH-20 column chromatography. Yields of the products exceeded 80%.

Preparation of 1,2-Mixed Diacyl-*sn*-glycero-3-phosphoryl(*N*-*tert*-butoxycarbonyl)ethanolamines (Fig. 5). Typically, the calcium salt of 1-palmitoyl-*sn*-glycero-3-phosphoryl(*N*-*tert*-butoxycarbonyl)ethanolamine (0.1 mmol) was stirred with

† In addition to removing $CdCl_2$ and the aminopyridine, the resin was found to remove some of the fatty acid, but no loss of the phospholipid was observed.



XIV. $R_1, R_2 = -(CH_2)_{14}CH_3$

XV. $R_1 = -(CH_2)_{14}CH_3$; $R_2 = -(CH_2)_{16}CH_3$

FIG. 5. Synthetic 1,2-mixed diacyl-*sn*-glycero-3-phosphoryl(*N*-*tert*-butoxycarbonyl)ethanolamines prepared by acylation of the 2-OH group in 1-acyl-*sn*-glycero-3-phosphoryl(*N*-*tert*-butoxycarbonyl)ethanolamines as shown in Fig. 1*B*.

stearic anhydride (0.15 mmol) and *N,N*-dimethyl-4-aminopyridine (0.15 mmol) in chloroform (2.5 ml) for 24 hr. The required product was isolated in 85–90% yield after silicic acid chromatography as described above.

1-Palmitoyl-2-ω-(3,3,3-trifluoro-2-diazopropionyl)lauryl-GPC (VIII). (*a*) *12-Hydroxylauric acid.* 11-Bromo-undecanoic acid was converted to the corresponding acid chloride by treatment with thionyl chloride in benzene. Reduction of the resulting acid chloride in ether with lithium aluminum hydride at −78° gave 11-bromo-undecanol. The latter, on treatment with potassium cyanide (5 mol equiv) under reflux in aqueous ethanol (50%) followed by alkaline hydrolysis of the resulting cyano intermediate, gave 12-hydroxylauric acid in better than 60% overall yield. It was crystallized from diethyl ether; mp 82–83°; reported mp 84° (25). Treatment of the acid with methanol under reflux in the presence of *p*-toluenesulfonic acid gave the corresponding methyl ester; mp 33–34° (petroleum ether); reported mp 34.5° (25). This preparation showed a single peak on HPLC with acetonitrile/water/acetic acid, 60:40:20 (vol/vol), as the eluant (retention time, 4.3 min). The spectral characteristics were: IR (Nujol)—3400 (broad band, OH) and 1750 cm^{-1} (C=O); NMR ($CDCl_3$) δ 3.6 (s, 3H), 3.59 (t, 2H, J = 7 Hz), 2.26 (t, 2H, J = 7 Hz), 0.9–2.0 (m, 18H).

(*b*) *12-(3,3,3-Trifluoro-2-diazopropionyl)lauric acid anhydride.* 12-Hydroxylauric acid was converted to the corresponding anhydride as in *Materials and Methods*. To an ice-cold solution (0.5 M) of the anhydride in dry methylene chloride (10 ml) were added 1.5 mol equiv of 3,3,3-trifluoro-2-diazopropionyl chloride and dry pyridine (3.0 mol equiv). The reaction mixture was slowly allowed to attain room temperature and was stirred at this temperature for 12–14 hr. After evaporation of the solvent, the residue was dried thoroughly under reduced pressure. This was extracted with petroleum ether and the insoluble material was removed by filtration through a bed of Celite. Evaporation of the filtrate gave the required 12-trifluorodiazopropionyllauric acid anhydride as a light yellow syrup. The IR spectrum (liquid film) showed peaks at 2140 (N_2), 1825, and 1740 cm^{-1} (C=O).

(*c*) *The phospholipid* (VIII). Acylation of 1-palmitoyl- or 1-stearoyl-GPC by the above anhydride was carried out under standard acylation conditions. Purification was by silica gel preparative TLC followed by flow through a Sephadex LH-20 column or by HPLC. Retention time on the μC_{18} reversed-phase HPLC column (250-μg injection) was 3.6 min for the 1-palmitoyl- and 4.1 min for the 1-stearoyl-2-ω-(trifluorodiazopropionyl)lauroyl-*sn*-glycero-3-phosphorylcholine. The IR spectrum (Nujol) showed a sharp peak at 2240 cm^{-1} (N_2) and a broad band at 1735 cm^{-1} (C=O); NMR ($CDCl_3$) showed δ 5.2 (m, 1H), 3.4 (s, 9H). Phosphorus content was 3.73%; expected for 1-palmitoyl-2-ω-(trifluorodiazopropionyl)lauroyl-lecithin ($C_{39}H_{71}F_3N_3O_{10}P$; 829.95) is 3.82%.

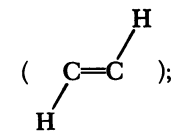
1-Palmitoyl-2-ω-(*m*-3H-diazirinophenoxy)undecanoyl-GPC (IX). (*a*) *Methyl 11-(m-diazirinophenoxy)undecanoate. m*-Methoxymethyleneoxyphenyl-3H-diazirine was prepared from *m*-methoxymethylenoxybenzaldehyde by the procedure of Smith and Knowles (26). Treatment of the diazirine with glacial acetic acid containing a catalytic amount of 1 M HCl gave *m*-hydroxyphenyl-3H-diazirine. The following UV, IR, and NMR characteristics were consistent with the structure: IR (Nujol): 1580 cm^{-1} (N=N); UV (cyclohexane) λmax: 358 nm (ε 265); NMR ($CDCl_3$): δ 5.6–7.2 (m, 5H; one of which disappears after D_2O shake), 2.4 (s, 1H).

The phenolic diazirine (3 mmol) was converted to the sodium salt and later, after complete removal of methanol, was mixed with methyl 11-iodo-undecanoate (980 mg; 3 mmol) in dry dimethyl formamide (5.0 ml). The mixture was stirred at room temperature (22°–24°) for 2 hr. The reaction mixture was partitioned between water and ether (100 ml each). The organic phase was washed three times with cold water (50 ml each time) and dried over anhydrous Na_2SO_4. Removal of ether followed by silica gel preparative TLC of the residue gave methyl 11-(*m*-diazirinophenoxy)undecanoate as a colorless syrup. This product was pure as judged by HPLC (monitored by UV absorption). The μPorasil column was 0.4 × 30 cm; ligroin/benzene, 60:40 (vol/vol), was the eluant; rate of elution was 2 ml/min. The retention time was 9.0 min. IR (liquid film): 1750 (C=O) and 1590 cm^{-1} (N=N); UV (cyclohexane) λmax: 358 nm (ε 286); NMR ($CDCl_3$): δ 6.3–7.2 (m, 3H), 5.65 (d, 1H, J = 2 Hz), 3.5 (s, 3H), 3.8 (t, 2H, J = 7 Hz). Saponification of this ester with 5% potassium hydroxide in 90% aqueous ethanol at room temperature gave the acid as a gum, which crystallized from diethyl ether/petroleum ether (mp 73–75°). IR, UV, and NMR spectra of this compound were in agreement with the structure.

(*b*) *The phospholipid* (IX). The above acid was converted to the corresponding anhydride as described in *Materials and Methods*. The anhydride was directly used to acylate 1-palmitoyl-GPC by the general method. The phospholipid was purified by silica gel preparative TLC followed by HPLC. The IR spectrum (AgCl) showed peaks at 1740 (C=O) and 1570 cm^{-1} (N=N); UV ($CHCl_3$) λmax: 340 (ε 250) and 288 nm (ε 1850); NMR ($CDCl_3$): δ 6.3–7.2 (m, 3H), 5.8 (d, 1H, J = 2 Hz), 5.2 (m, 1H), 3.4 (s, 9H). The retention time on the μC_{18} reversed-phase HPLC column (250-μg injection) was 4.2 min.

Phosphorus content was 3.84%; expected for structure IX ($C_{42}H_{74}N_3O_9P$; 796.01) is 3.89%.

1-Palmitoyl-2-(12-oxo-*trans*-10-octadecenoyl)-GPC (X). 12-Oxo-*trans*-10-octadecenoic acid was prepared from ricinoleic acid by the procedure of Ellis (27). This was converted to its anhydride which was directly used to acylate 1-palmitoyl-GPC. The product was purified by preparative TLC. IR (Nujol) showed peaks at 1740 (C=O) and 1630 cm^{-1}



UV ($CHCl_3$) λmax: 293 nm (ε 50.2); NMR ($CDCl_3$): δ 6.65 (m, 1H), 6.0 (d, 1H, J = 14 Hz), 5.2 (m, 1H), 3.4 (s, 9H). Retention time on the standard reversed-phase HPLC column [methanol/chloroform/water, 80:20:4 (vol/vol); 250-μg injection] was 3.7 min. The phosphorus content was 4.10%; expected for 1-palmitoyl-2-(12-oxo-*trans*-10-octadecenoyl)lecithin ($C_{42}H_{80}NO_9P$; 774.08) is 4.00%.

11-(*m*-Azidophenoxy)undecanoic acid and 11-(4-azido-2-nitrophenoxy)undecanoic acid were prepared by reaction of the sodium salts of *m*-azidophenol and 4-azido-2-nitrophenol with methyl 11-iodo-undecanoate in dimethyl formamide. The esters were saponified to the corresponding acids with 5% KOH

in 90% ethanol. The acids were then converted to anhydrides and used to acylate 1-palmitoyl-GPC, to give **XII** and **XIII**.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphate. An aqueous solution of dicyclohexylammonium *sn*-glycero-3-phosphate (30 mg; 0.074 mmol) was converted to the pyridinium salt by passage through pyridinium Dowex-50 ion exchange resin. The aqueous pyridine solution was evaporated under reduced pressure and the gummy residue of the pyridinium salt was rendered anhydrous by repeated evaporation of added dry pyridine. The dry residue was resuspended in anhydrous chloroform (5 ml) and treated with *N,N*-dimethyl-4-aminopyridine (36.1 mg; 4 equiv) and palmitic anhydride (110 mg; 3 equiv). After being flushed with N_2, the reaction vessel was sealed and the mixture was stirred at room temperature; a practically clear solution resulted in 2 days. After 4 days, the reaction mixture was evaporated and the residue was dissolved in a 5 ml of methanol/chloroform/pyridine/water, 2:1:1:1 (vol/vol), and the solution was kept overnight. A fast-travelling product, presumably palmitoyl phosphate anhydride, disappeared and only a single product with mobility identical to that of dipalmitoylphosphatidic acid was present. The solution was evaporated and the residue, as a solution in chloroform, was passed through a column of silicic acid. Elution with chloroform removed the fatty acid and subsequent elution with methanol/chloroform, 1:1, gave the required phosphatidic acid. This was converted to the pyridinium salt by passage through pyridinium Dowex-50 resin. The yield was 52.0 mg (87%).

Absence of 1,2-Cyclic Phosphate Formation in the Synthesis of Phosphatidic Acid. To show the absence of 1,2-cyclic phosphate formation, dipyridinium dipalmitoylphosphatidic acid (30 mg) as prepared above was dissolved in 0.5 ml of chloroform and the solution was treated with 1 ml of 50% methanolic ammonium hydroxide (methanol/conc NH_4OH, 1:1). The stirred reaction mixture was examined by TLC (solvent A) and by paper chromatography (solvent B). Formation of glycerol phosphate was complete after 2 days. Glycerol 1,2-cyclic phosphate was subjected to the identical ammoniacal conditions. The R_Fs of *sn*-glycero-3-phosphate and of glycerol 1,2-cyclic phosphate in solvent B were 0.25 and 0.55, respectively. Only the product with the lower R_F was obtained from the synthetic phosphatidic acid, glycerol cyclic phosphate being stable to the ammoniacal conditions.

CONCLUDING REMARKS

Various methods have previously been introduced for the acylation of GPC, lysolecithins, and *sn*-glycero-3-phosphate but, in our hands, none of these proved to be satisfactory and, especially in the acylation of lysolecithins, only very modest yields were obtained. The method described is uniformly efficient in the acylation of glycerophosphoryl compounds, and it should find wide application in the phospholipid field.

During acylation of *sn*-glycero-3-phosphate, there exists the possibility of 2,3-cyclic phosphate formation, and special conditions were used to avoid this reaction (10, 11). In the present work, no cyclic phosphate formation was observed under the standard acylation conditions, and excellent yields of the expected phosphatidic acids were obtained by using economical amounts of the acyl anhydrides. Because *sn*-glycero-3-phosphate can be prepared by phosphorylation of glycerol with the readily available glycerokinase (28), the general approach to the synthesis of phospholipids using phosphatidic acids as the intermediates becomes more attractive.

Finally, acyl group migration in mono- and diglycerides has previously been studied by a number of investigators (ref. 29 and references cited therein). Migration, in general, is rapid and favors predominantly the 1-position. In the present work, a small (5%) extent of migration was detected from the 1- to the 2-OH group during acylation of 2-lysolecithins. The finding, which is not inconsistent with the previous results, probably will not cause any serious complication in the membrane studies contemplated.

Note Added in Proof: T. G. Warner and A. A. Benson [*J. Lipid Res.* **18**, 548 (1977)] have recently reported on a new method for the preparation of symmetrical unsaturated phosphatidylcholines.

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1. Chakrabarti, P. & Khorana, H. G. (1975) *Biochemistry* **14**, 5021–5033.
2. Greenberg, G. R., Chakrabarti, P. & Khorana, H. G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 86–90.
3. Olsen, W. L., Gupta, C. M., Mann, C., Radhakrishnan, R., Schaechter, M. & Khorana, H. G. (1977) "Incorporation of Photoactivable Fatty Acids Into *Escherichia coli* Phospholipids," American Society for Microbiology Annual Meeting (Abstract).
4. Slotboom, A. J., Verheij, H. M. & deHaas, G. H. (1973) *Chem. Phys. Lipids* **11**, 295–317.
5. Rosenthal, A. R. (1975) in *Methods in Enzymology*, ed. Lowenstein, J. M. (Academic Press, New York), Vol. 35, part B, pp. 429–528.
6. Kates, M. (1977) in *Methods in Membrane Biology*, ed. Korn, E. D. (Plenum Press, New York), Vol. 8, pp. 219–290.
7. Keana, J. F. W. & Ertle, A. R. (1976) *Chem. Phys. Lipids* **17**, 402–406.
8. Boss, W. F., Kelley, C. J. & Landsberger, F. R. (1975) *Anal. Biochem.* **64**, 289–292.
9. Baer, E. & Buchnea, D. (1959) *Can. J. Biochem. Physiol.* **37**, 953–959.
10. Lapidot, Y., Barzilay, I. & Hajdu, J. (1969) *Chem. Phys. Lipids* **3**, 125–134.
11. Robles, E. C. & van DenBerg, D. (1969) *Biochim. Biophys. Acta* **187**, 520–526.
12. Pugh, E. & Kates, M. (1975) *J. Lipid Res.* **16**, 392–394.
13. Aneja, R. & Chadha, J. S. (1971) *Biochim. Biophys. Acta* **239**, 84–91.
14. Steglich, W. & Höfle, G. (1969) *Angew. Chem., Int. Ed. Engl.* **8**, 981.
15. Reese, C. B. (1973) in *Protective Groups in Organic Chemistry*, ed. McOmie, J. F. W. (Plenum Press, New York), pp. 95–143.
16. Chadha, J. S. (1970) *Chem. Phys. Lipids* **4**, 104–108.
17. Salinger, Z. & Lapidot, Y. (1966) *J. Lipid Res.* **7**, 174–175.
18. Chowdhry, V., Vaughan, R. & Westheimer, F. H. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1406–1408.
19. Forster, M. O. & Fierz, H. E. (1907) *J. Chem. Soc.* **91**, 859–867.
20. Goswami, S. K. & Frey, C. F. (1971) *J. Lipid Res.* **12**, 509–510.
21. Hanes, C. S. & Isherwood, F. A. (1949) *Nature* **164**, 1107–1112.
22. Bandurski, R. S. & Axelrod, B. (1951) *J. Biol. Chem.* **193**, 405–410.
23. Fritz, H.-J., Belagaje, R., Brown, E. L., Fritz, R. H., Lees, R. G. & Khorana, H. G. (1977) "High pressure liquid chromatography in polynucleotide synthesis," *Biochemistry*, in press.
24. Ames, B. N. & Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769–775.
25. Tong, S. T. (1954) *Soap, Perfum. Cosmet.* **27**, 58–61; cf. (1955) *Chem. Abstr.* **49**, 2339^b.
26. Smith, R. A. G. & Knowles, J. R. (1975) *J. Chem. Soc. Perkin Trans. 2*, 686–694.
27. Ellis, G. W. (1950) *J. Chem. Soc.*, 9–12.
28. Chang, Y. & Kennedy, E. P. (1967) *J. Lipid Res.* **8**, 447–455.
29. Wolfenden, R., Rammler, D. H. & Lipmann, F. (1964) *Biochemistry* **3**, 329–338.