A facile route to semi-synthesis of acetyl glycerylether phosphoethanolamine and its choline analogue

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Abstract A facile route to the semi-synthesis of acetyl glycerylether phosphoethanolamine and, subsequently, its choline analogue (platelet-activating factor) has been developed. In essence, this technique takes advantage of the fact that the phosphatidylethanolamine fraction of bovine erythrocytes contains 75–80% of a 1-*O*-alkyl-2 fatty acyl derivative. Isolation of the latter by silicic acid chromatography followed by base-catalyzed methanolysis allowed good recovery (60–70%) of the 1-*O*-alkyl-(lyso)-*sn*-glyceryl-3-phosphoethanolamine, which contained a mixture of long chain alkyl ethers. This compound was treated with acetic anhydride in the presence of trace amounts of perchloric acid for 45 sec to give, in excellent yield, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphoethanolamine (AGEPE). This procedure gave a 70% yield of purified AGEPE, based on the starting component, 1-*O*-alkyl-(lyso)-*sn*-glyceryl-3-phosphoethanolamine. Separation of AGEPE into fractions individually enriched in the 16:0, 18:0, and 18:1 alkylether substituents was accomplished by silica gel G combined with silver nitrate-impregnated silica gel H thin-layer chromatography. The AGEPE or its individual molecular species can be converted in high yields to the corresponding 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (AGEPC) analogues by reaction with methyl iodide in the presence of a crown ether. Characterization of the derivatives was achieved through thin-layer chromatography, infrared spectroscopy, gas–liquid chromatography, and combined gas–liquid chromatography–mass spectrometry. The ability of these analogues to induce irreversible aggregation and secretion of serotonin from washed rabbit platelets was evaluated.—**Kumar, R., S. T. Weintraub, L. M. McManus, R. N. Pinckard, and D. J. Hanahan.** A facile route to semi-synthesis of acetyl glycerylether phosphoethanolamine and its choline analogue. *J. Lipid Res.* 1984. **25:** 198–208.

Supplementary key words choline • GLC–mass spectrometry • platelet activity

The recent discovery that a unique glycerophospholipid, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (AGEPC), possessed potent biological activity which was comparable to that exhibited by a naturally produced platelet-activating factor (1) has prompted considerable interest in the biochemical and physiological behavior and role of this molecule. A challenging component to any study of the metabolic behavior of this biologically active glycerophospholipid is its very high potency, e.g., AGEPC can elicit 50% secretion of serotonin from rabbit platelets in 60 sec at a concentration of 1×10^{-10} M (1). Thus, in order to investigate the biochemical characteristics of this compound and related derivatives at a physiologically relevant concentration, it is important that chemically pure material, which can be adapted to radioactive labeling, is available. To date, attention has been centered on the total chemical or semi-synthesis of AGEPC using starting materials such as diacetone mannitol (2), the vinyl ether choline plasmalogens from bovine heart muscle (3), the glyceryl ethers from hog leucocytes (4), and from ratfish liver (5). In order to have other derivatives analogous to AGEPC available for study, an attractive approach was to use the alkyl ether-rich phosphatidylethanolamine fraction of bovine erythrocytes as the starting material for the synthesis of 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphoethanolamine, which could then be separated into molecular species by argentation thin-layer chromatography. These latter preparations can then serve as the starting material for the synthesis of other derivatives such as the choline-containing analogues.

This communication, for the first time, describes a route by which AGEPE can be synthesized without protection of the amino group and by which various ho-

Abbreviations: AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine; AGEPE, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphoethanolamine; lyso-GEPE, 1-*O*-alkyl-(lyso)-*sn*-glyceryl-3-phosphoethanolamine; PE, phosphatidylethanolamine; AGEPMME, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phospho-N-monomethylethanolamine; AGEPDME, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phospho-N,N-dimethylethanolamine; TMP, trimethylphosphite; Vitride, [$NaAlH_2$ ($OCH_2CH_2OCH_3)_2$]; TNS, 6-p-toluidine-2-naphthalene sulfonic acid; P, phosphorus; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; LDH, lactate dehydrogenase; BSA, bovine serum albumin.

mologues of AGEPE and AGEPC can be prepared. The biological activity of these derivatives with respect to aggregation and serotonin release from washed rabbit platelets is also discussed.

EXPERIMENTAL PROCEDURE

Materials

1-*O*-Hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (16:0 AGEPC) and 1-*O*-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (18:0 AGEPC) were obtained from Bachem, Switzerland. Monoglyceryl ethers were purchased from Serdary Research Laboratories, London, Ontario. PE, phosphatidylethanolamine, was purchased from Sigma Chemical Company, St. Louis, MO. Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] was a product of Alfa Products, Danvers, MA. 18-Crown-6-ether, (1,4,7,10,13,16 hexacyclooctadecane) was obtained from Aldrich, Milwaukee, WI. 2-7-Dichlorofluorescein was a product of Sigma Chemical Company. Platinum oxide was purchased from Engelhard Co., Newark, NJ. TNS (6-*p*-toluidine-2-napthalene sulfonic acid), ninhydrin, and molybdate reagents were prepared in the laboratory as previously described (6). Silver nitrate (analytical grade) and SilicAR CC-7-size mesh was purchased from Mallinckrodt, St. Louis, MO. Silica gel H was obtained from Brinkmann Instrument Co., New York. BSA was a product of Miles Laboratories, Kankakee, IL. Thin-layer chromatography plates precoated with silica gel G, 250 and 500 μm, were products of Analtech, Newark, NJ, and were prewashed with the appropriate developing solvent system and then activated by heating for 30 min at 140°C immediately before use. All solvents were of reagent grade. The 10% SP 2330 on 100/120 mesh Chromosorb WAW was obtained from Supelco, Bellefonte, PA. Unless otherwise stated, all solvent mixtures for extraction and thin-layer chromatography were prepared on a v/v basis.

METHODS

Thin-layer chromatography

Prewashed plates were dried in an oven at 140°C for 30 min prior to sample application; they were developed in a tank containing a wick saturated with the required solvent. Compounds were located by sequential spraying with TNS, ninhydrin, and molybdate reagents, and finally charring with sulfuric acid at 500°C. For preparative TLC, the sample was applied as a streak across the plate as well as in reference lanes marked about 2 cm from the edge of the plate. The desired phospholipid fractions were scraped and extracted three times with chloroform–methanol–water 1:2:0.8 and the lipid products were recovered in chloroform phase after addition of 1 vol of chloroform and 1 vol of water.

Extraction of total lipids from bovine erythrocytes

A modification of the procedure of Hanahan, Watts, and Pappajohn (7) for the isolation and extraction of total lipids from the bovine erythrocyte was used.

Bovine blood (4 l) was collected into six 1-liter plastic bottles containing sodium citrate (2 g), glucose (20 g), and deionized water (10 ml). The mixture was centrifuged at 1033 *g* at 4°C for 20 min. The supernatant and the buffy coat fractions were separated from the red cell pellet by gentle aspiration and the cells were resuspended to the original volume in normal saline (0.9%, pH 7.4). This mixture was recentrifuged and the cells were washed one additional time. The lipids were extracted from the washed cells by the method of Bligh and Dyer (8). Chloroform–methanol–water 1:2:0.8 was added to the packed cells and the mixture was stirred. After stirring for 2 hr at room temperature, the mixture was filtered through Whatman #1 filter paper. The residue was resuspended in the same solvent mixture and again filtered. The combined filtrates were mixed with 1 vol of chloroform and 1 vol of water. The chloroform-rich lower phase was evaporated to dryness under vacuum at 30–35°C in a rotary evaporator and redissolved in a mixture of chloroform–methanol 1:1, analyzed for total phosphorus content, and stored at −20°C until needed.

Preparation of 1-*O*-alkyl-(lyso)-*sn*-glyceryl-3-phosphoethanolamine (lyso-GEPE)

An excellent separation of phosphatidylethanolamine (PE) from the total lipid extract was achieved by a modification of the procedure of Hanahan and Watts (9). The total lipid extract (103 mg of P) in 110 ml of chloroform was loaded on to 200 g of SilicAR CC-7, in a glass column measuring 4 cm × 30 cm, and sequentially eluted with chloroform, 200 ml; chloroform–methanol 20:1 (v/v), 300 ml; chloroform–methanol 6:1 (v/v), 1000 ml; and finally with methanol, 300 ml. Pure PE was recovered in the chloroform–methanol 6:1 fraction and gave a total phosphorus value of 28 mg (yield 27%, based on total added P). The purity of the eluate was checked on TLC using two different solvent systems, chloroform–methanol–water 65:35:6 and chloroform–methanol–ammonium hydroxide 70:30:5. A single sharp band corresponding to a PE standard was detected in each system. The infrared spectrum gave the expected absorption peaks (10). A strong band characteristic of primary amine was observed at 1076 cm^{-1}.

A sample containing 12.0 mg of P of the purified PE in 1 ml of chloroform was mixed with 10 ml of 0.5 N methanolic sodium hydroxide and stirred at room tem-

perature for 45 min. The reaction was neutralized with 1 N HCl and mixed by vortexing with chloroform–water 10:9 to make a final ratio of chloroform–methanol–water 1:1:0.9. The lower chloroform-rich layer containing primarily lyso-GEPE and the methyl esters of long chain fatty acids was subjected to preparative TLC. The desired product, lyso-GEPE, was recovered by extracting the appropriate portion of the plate by the procedure of Bligh and Dyer (8).

Lyso-GEPE migrated as a single band, R_f 0.34, on TLC in a chloroform–methanol–water 65:35:6 system and gave a total phosphorus value of 7.5 mg (yield, 62.5% based on purified PE). The infrared spectrum showed all the expected bands. A strong absorption band, characteristic of a free amine, was observed at 1076 cm^{-1}; CH_3-CH_2, 2920 cm^{-1}; P—O—C, 1060 cm^{-1}; C—O—C, 1110 cm^{-1}. No fatty acyl ester (C=O) band at 1760 cm^{-1} was detected. Measurement of optical activity gave a value $[\alpha]_D^{23} = -1.47°$ (543 μg of P/ml in chloroform–methanol 1:1 (v/v)).

1-*O*-Alkyl-2-acetyl-*sn*-glyceryl-3-phosphoethanolamine (AGEPE)

Lyso-GEPE, 990 μg of P, was dissolved in 0.5 ml of chloroform and treated with 0.1 ml of acetic anhydride. This mixture was stirred for 30 sec. To the cloudy suspension, 0.05 ml of 12 N perchloric acid was carefully added and the mixture was stirred again for 5 to 10 sec. There was an almost immediate reaction and a clear solution resulted. The reaction was immediately stopped by cooling the tube in ice and adding 1.8 ml of cold water, 1.95 ml of chloroform, and 2 ml of methanol. The organic phase was separated after centrifugation, washed twice with methanol–water 10:9, and the AGEPE was purified by preparative TLC on 500 μm silica gel G plates using a solvent system of chloroform–methanol–ammonium hydroxide 70:30:5. In this system, AGEPE migrates with an R_f value of 0.31. The purified compound also migrated as a single spot in a neutral TLC system and gave a positive reaction both with ninhydrin and phosphate spray. Total recovery of phosphorus was 708 μg, representing a yield of 72% based on the starting P value (lyso-GEPE). The infrared spectrum exhibited all the expected bands plus an ester band at 1769 cm^{-1}. Measurement of optical activity gave a value of $[\alpha]_D^{23} = +2.11°$ (340 mg of P/ml in chloroform–methanol 1:1 (v/v)).

1-*O*-Alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (AGEPC)

This compound was synthesized essentially by the method of Patel, Morrisett, and Sparrow (11). To 2 ml of benzene, dried over P_2O_5, was added 2.84 mg of anhydrous potassium carbonate and 148 mg of 18-crown-6-ether; the mixture was stirred for 5 min at 23°C. A sample of AGEPE containing 476 μg of P, dissolved in 3 ml of anhydrous benzene was added to the above mixture. Methyl iodide (8.3 ml) was then introduced into the reaction tube which was sealed under nitrogen and stirred at 37°C. The progress of the reaction was monitored by silica gel G TLC using chloroform–methanol–water 65:35:6. The reaction was usually complete by the end of 3 hr, at which time the solvent was evaporated under nitrogen and the residue was treated with chloroform–methanol–water 1:1:0.9. The desired derivative was recovered by preparative TLC using a solvent system of chloroform–methanol–water 65:35:6. The yield was 332 μg of P, (70% based on P value). The infrared spectrum showed the following prominent bands: CH_3-CH_2 (2920 cm^{-1}, 2850 cm^{-1}); P=O, 1220 cm^{-1}; P—O, 1087 cm^{-1}; P—O—C (1052, 962 cm^{-1}), bound water (3370 cm^{-1}). The optical activity was measured and a value, $[\alpha]_D^{23} = -2.85°$ (526 μg of P/ml in chloroform–methanol 1:1 (v/v)) was obtained for this AGEPC.

Separation of AGEPE into hexadecyl (16:0)-, octadecyl (18:0)-, and octadecenyl (18:1)-rich species

The separation of the AGEPE into various alkylether chain species was achieved by TLC in two steps. The hexadecyl (16:0) plus octadecenyl (18:1) fraction was separated from the octadecyl species by using silica gel G plates, developed in the methanol–water 2:1 system. Subsequently, separation of the hexadecyl from octadecenyl derivative was achieved on 30% $AgNO_3$-impregnated plates. In a typical experiment, a sample of AGEPE (200 μg of P) was applied to a preparative 500 μm, 20 × 20 cm plate as described in the TLC section and developed in methanol–water 2:1. Reference lanes were sprayed with TNS reagent, and the area corresponding to the reference bands was scraped and extracted by the procedure of Bligh and Dyer (8). The combined recovery of the lower and upper bands was in the range of 80–85%. A small portion of each fraction was subjected to acetolysis and the products were analyzed by GLC. The upper band (A) showed the following mol % composition: 16:0, 54%; 18:1, 43%, plus two additional small peaks representing about 3% of the total amount; however, no attempt was made to purify these fractions further. The lower band (B) was rich in 18:0 (84 mol %), but was contaminated with the 16:0 species. A rerun of the lower band (B) in the same TLC system yielded a pure octadecyl fraction.

The upper band (A), isolated from the above described TLC plate, was further separated into 16:0- and 18:1-rich species on $AgNO_3$-impregnated silica gel H plates (500 μm, 20 × 20 cm) that had been activated at 170°C for 7 hr and then cooled in a desiccator containing silica gel (12). A maximum amount of 100 μg of P was applied as a band and developed in chloroform–methanol–water 55:35:7. After development, plates were sprayed with a

0.2% solution of dichlorofluorescein in ethanol and the two fractions, an upper band of 16:0 (R_f value 0.70) and the lower band rich in 18:1 (R_f value 0.63) were located under ultraviolet light. The bands were removed by scraping and extracted as described earlier. The upper aqueous phase containing the dye and the silver ions was discarded and the chloroform phase was washed three times with methanol–water 10:9. The samples were purified further by TLC using methanol–water 2:1. Analysis of the upper and lower bands indicated that each substance was between 94 and 97% pure. The recovery of compounds based on the phosphate (P) values ranged between 80 to 82% (upper band P = 54 μg, lower band P = 26 μg).

1-*O*-Alkyl-2-acetyl-*sn*-glyceryl-3-phospho-N-acetyl ethanolamine (AGEPE amide)

AGEPE, (150 μg of P) was dissolved in a mixture of 0.5 ml of dry chloroform and 0.5 ml of pyridine. Acetic anhydride, 20 μl, was added to the reaction mixture which was then stirred for 30 min at room temperature. After the reaction period, the mixture was washed successively with 0.5 N HCl (2 × 1 ml) and cold water (2 × 1 ml). The organic layer was dried over anhydrous Na_2SO_4 and the desired compound was purified by preparative TLC in the chloroform–methanol–ammonium hydroxide system and gave a negative reaction to ninhydrin. Yield, 135 μg of P; 90% based on starting P. The infrared spectrum showed all the expected bands as shown by AGEPE plus an additional strong amide band at 1660 cm^{-1}.

Lipid phosphorus

This was determined by the method of Bartlett (13) subsequent to perchloric acid digestion of the lipid.

Chemical analysis

The identification of the alkylether residue in the phospholipids was accomplished by subjecting a sample containing 10–15 μg of P to (*a*) an acetolysis procedure by using acetic anhydride and glacial acetic acid (0.5 ml) in a ratio of 2:3 (v/v) at 150°C for 5 hr (14) or (*b*) reduction with Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] by adding 0.5 ml of the latter reagent to a sample dissolved in diethyl ether and heating in a closed tube at 37°C for 30 min (15). The resulting dephosphorylated mixture obtained in procedure (*b*) was acetylated by using acetic anhydride and perchloric acid as described above. Alternatively, the isopropylidine derivative of the dephosphorylated preparation was prepared by reaction with acetone in the presence of perchloric acid (16). Subsequently, these latter derivatives were purified on TLC and examined by gas–liquid chromatography (GLC) and/or gas–liquid chromatography–mass spectrometry (GLC–MS). A Varian 3700 unit, equipped with a flame ionization detector, and a 6 ft × 2 mm ID glass column, packed with 10% SP 2330 on 100–120 Chrombsorb WAW was utilized. The column was operated isothermally at 210°C with detector and injector temperatures maintained at 230°C. Various mixtures of standard glycerylethers, as the diacetyl or the isopropylidine derivatives, were run and the unknown glycerylethers were tentatively identified by comparing the retention times. The areas of the peaks corresponding to the glycerylethers were obtained directly by use of a programmable computer (Varian CDS III) attached to the GLC unit. A Finnigan MAT Model 212 mass spectrometer in combination with a Varian Model 3700 gas chromatograph and an INCOS 2200 data system was also utilized for structural analysis of the derivatized samples. Gas–liquid chromatographic separation of acetylated compounds was accomplished with a 20-meter SE 54 glass capillary column, using helium as the carrier gas with a column head pressure of 10 p.s.i. The ion source temperature was 250°C, the accelerating voltage was 3 kV, the electron energy was 70 eV, and the cathode current was 1 mA. Samples were introduced into the mass spectrometer by means of an open split separator and platinum capillary interface which was maintained at 240°C. Positive ion electron impact mass spectra were obtained from full mass scans which were recorded and stored continuously during the GLC–MS run. Selected ion retrieval traces were acquired by computer evaluation of the date.

Infrared spectroscopy

Infrared spectra were recorded on a Perkin Elmer 283 spectrometer using IRTran-2 cells with 1 mm optical path. Samples were dissolved in pure chloroform and usually contained a minimum of 100 μg of phosphorus/ml.

NMR spectroscopy

^{31}P NMR spectra were obtained on a Nicollet-200 superconducting Fourier transform NMR spectrometer at the University of Texas in Austin. Samples containing 15 mg of material were dissolved in deuterated organic solvents (chloroform and methanol) and the spectra were run at ambient temperature (22°C) using an external reference standard of trimethylphosphite (TMP).

Optical rotation

Optical activity was determined on a Perkin Elmer Model 241 automatic polarimeter using 1 dm tube. All samples were dissolved in chloroform–methanol 1:1 (v/v).

Platelet-stimulating activity of AGEPE and analogues

The biological activity of these analogues were assessed by previously described techniques (1, 17). Briefly, 200

μl of prewarmed washed rabbit platelets ($1.25 \times 10^6/\mu l$) labeled with [^{3}H]serotonin was added to 4 μl of various concentrations of AGEPE or analogues dissolved in pyrogen-free 0.15 M NaCl containing 2.5 mg/ml of bovine serum albumin. Sixty seconds later, 20 μl of cold (0°C) 1.5 M formaldehyde was added to stop the reaction; the tubes were cooled immediately to 0°C and centrifuged at 2,200 *g* for 10 min. The supernates then were assayed by beta-scintillation spectrometry for the percent of [^{3}H]serotonin release relative to 100% control prepared by the addition of 4 μl of 10% Triton X-100 to 200 μl of unstimulated rabbit platelets. The percentage serotonin released by various concentrations of AGEPC or AGEPE and its analogues was plotted linearly; one unit of activity was defined as the final molar concentration of the particular compound required to effect the release of 50% [^{3}H]serotonin under standardized conditions.

RESULTS AND DISCUSSION

General

The results presented here show that acetyl glycerylether phosphorylethanolamine can be easily prepared in good yields from the glycerylether phosphatidylethanolamine-rich fraction of bovine erythrocytes. The data indicate quite clearly that the lyso-PE used as a starting material is not altered in its glycerylether composition or other physical-chemical characteristics by conversion to the acetyl derivative. The resulting acetyl GEPE, containing a mixture of alkylether residues, could be separated in good yields into individual molecular species by a combined silica gel and argentation thin-layer chromatography. In addition, either the mixed chain or the individual molecular species of AGEPE could be converted very smoothly to the choline analogue by methylation with methyl iodide. Other derivatives such as the monomethyl (AGEPMME) or dimethyl (AGEPDME) can be prepared from the starting AGEPE but these were not investigated in this study.

If a single molecular species of AGEPC is the primary goal, then the preferred avenue to its preparation is through use of the same molecular counterpart of AGEPE. As had been noted earlier (18) and observed in this study, a mixed alkyl ether containing AGEPE is much more sensitive to separation on silica gel G and argentation thin-layer chromatography into individual molecular species than is the mixed alkylether containing AGEPC.

Further characteristics of these compounds and reaction procedures are described in the following sections.

Acetylation

The acetylation of lyso-GEPE, at the free hydroxyl and not the free amino group, with acetic acid anhydride in the presence of a trace amount of perchloric acid proceeded smoothly within a few seconds giving recoveries in the range of 85 to 90% based on the starting lyso-GEPE. Further purification on TLC in a basic system (chloroform–methanol–ammonium hydroxide) gave an overall yield of pure AGEPE in the range of 70 to 75%. Of interest, acetylation of the amino group also occurred as the consequence of neutralization of the reaction mixture with sodium bicarbonate. This problem can be circumvented by washing the reaction mixture three times with methanol–water 10:9 instead of using sodium bicarbonate.

Isolation of molecular species of AGEPE

Separation of individual species of AGEPE, e.g., the 18:0 species from the 16:0 plus 18:1 species in methanol–water and the 16:0 species from the 18:1 species on $AgNO_3$-impregnated plates in a chloroform–methanol–water system, were reproducible and allowed a good recovery (80 to 85%) of the desired products. It can be seen from **Table 1** that the purity of the individual molecular species varied from 95 to 98% and in addition to the indicated chain lengths, trace amounts of 14:0, 15:0, 17:1 were also present.

Thin-layer chromatographic behavior

The starting material (purified bovine PE) and all the semi-synthetic compounds were subjected to TLC in three different solvent systems. The R_f values of these compounds are shown in **Table 2.** It can be seen that in the neutral system AGEPE has an R_f value of 0.52, slightly less than that of the amide with an R_f of 0.54. Thus, purification on a preparative scale in this system was not feasible if the possibility of amide formation was considered. Consequently, AGEPE was purified in the basic

TABLE 1. Alkylether composition of specific phospholipids[a]

Compounds	Chain Length in Mole %			
	16:0	17:0	18:0	18:1
Lyso-GEPE	39	2	28	29
AGEPE				
Mixed	38	3	29	27
Fractioned				
16:0	95	3	1	ND[b]
18:0	0.5	1	98	ND[b]
18:1	2	1	ND[b]	97
AGEPC				
Mixed	39	3	29	28

[a] Phospholipids were subjected to acetolysis procedure and the percentage composition of the diacetyl derivatives was calculated from the gas chromatograms obtained from a Varian 3700 Gas Chromatograph with a flame ionization detector. Column 10% SP-2330, temperature 210°C; injector and detector, 230°C.

[b] ND, not detectable.

TABLE 2. R_f values of synthetic glycerylether phosphoglycerides

Compounds	Acidic[a]	Neutral[b]	Basic[c]
Bovine PE[d]	0.66	0.60	0.48
Lyso-GEPE	0.44	0.34	0.21
AGEPE	0.52	0.46	0.31
AGEPE amide	0.54	0.50	0.60
AGEPC	0.37	0.26	0.15

[a] Chloroform–methanol–acetic acid–water 60:35:1:8 (v/v).
[b] Chloroform–methanol–water 65:35:6 (v/v).
[c] Chloroform–methanol–28% ammonium hydroxide 70:30:5 (v/v).
[d] Mixed PE purified by silicic acid column chromatography (see Methods).

system where marked differences in the R_f values of these two compounds were noticed. A single concise spot in each of three systems was taken as one criteria of purity in studying biological activity for the present report.

Characterization of the alkyl glycerylether residue

The characterization of the alkylether residue in the individual phospholipids was carried out by subjecting these compounds to acetolysis, as described in the Experimental section, and analyzing the resulting 1-*O*-alkyl-2,3-diacetyl glycerol derivatives on GLC–MS. Typical mass spectra of the diacetyl glycerylethers derived from AGEPE are shown in **Fig. 1, Fig. 2,** and **Fig. 3.** In each instance, a peak at m/z 43, attributable mainly to the acetyl ion, was observed. For elucidation of the alkyl chain length of saturated glycerylethers, a peak resulting from the loss of an acetyl group in addition to a molecule of acetic acid [M − (43 + 60)] was observed at m/z 297 (16:0) or m/z 297 (16:0) or m/z 325 (18:0). Of further use in characterizing the chain length of the alkyl group were the ions that were derived from cleavage of the bond between carbon 1 and 2 of the glycerol backbone (m/z 255, 16:0; m/z 283, 18:0). For unsaturated diacetate derivatives, a slightly different fragmentation pattern was observed. The peaks important for structural evaluation were found at [M − 60], m/z 366, 18:0, and m/z 159 [M − O-alkyl]. For further comparison, diacetate derivatives of commercially available 1-mono oleyl glycerol and AGEPC containing only the 16:0 or 18:0 alkyl chain length were analyzed. The GLC–MS results agreed with those obtained for the diacetate derivatives of the semi-synthetic AGEPE or AGEPC. From the GLC–MS analysis

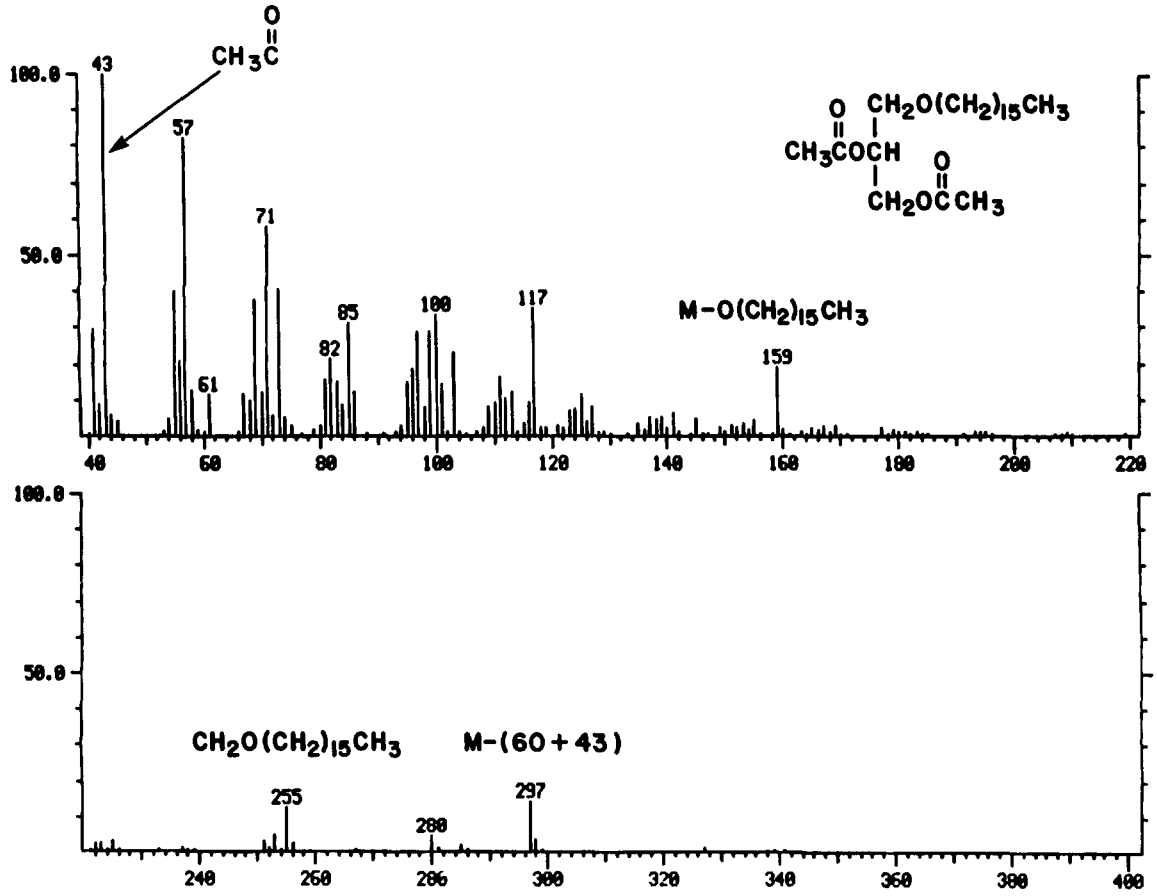

Fig. 1. Electron impact mass spectrum (70 EV) of 1-*O*-hexadecyl-2,3-diacetyl-glycerol.

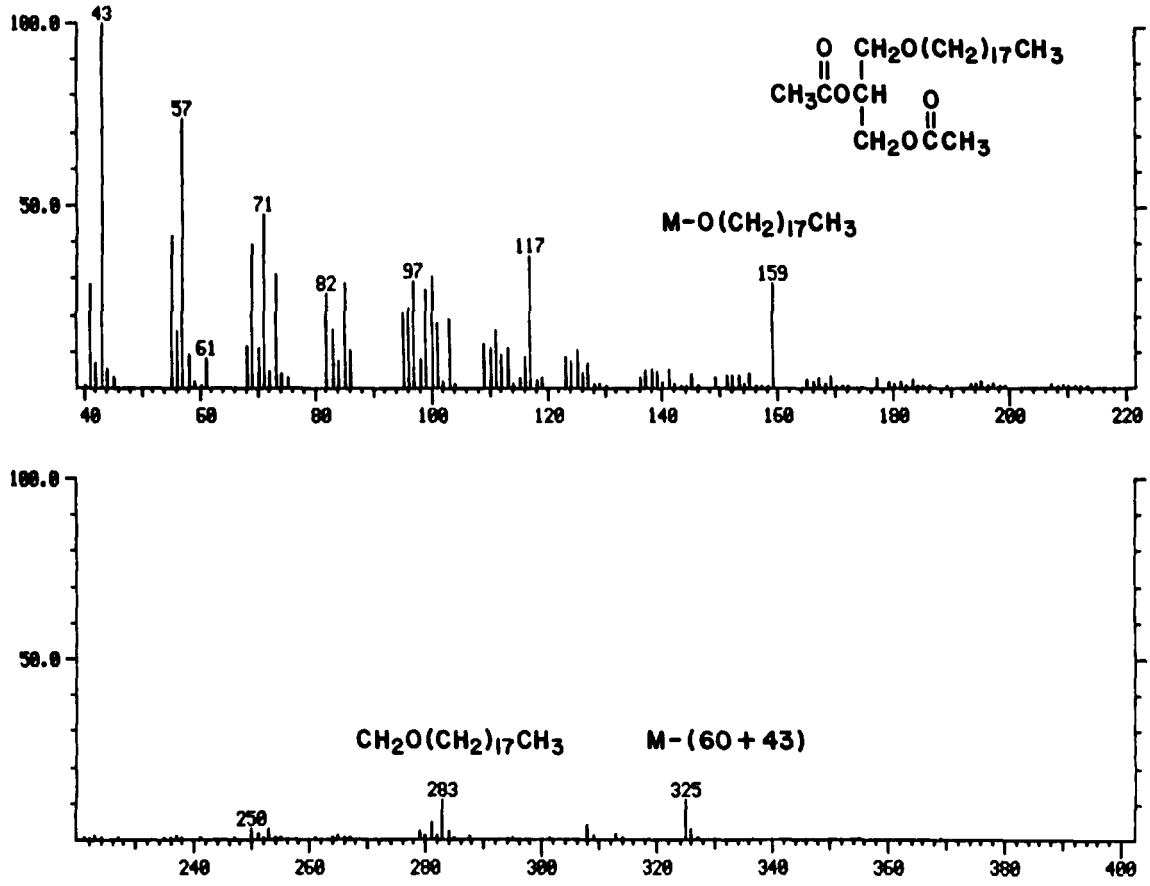

Fig. 2. Electron impact mass spectrum (70 EV) of 1-*O*-octadecyl-2,3-diacetyl-glycerol.

of the semi-synthetic AGEPE, as exemplified by the reconstructed ion chromatograms shown in **Fig. 4,** it can be seen that along with the 16:0, 18:0, and 18:1 constituents, glycerylether diacetates containing 14:0, 15:0, 17:0, and 17:1 were also present, albeit in minor amounts. In addition to the peak with a GLC retention time of 3.4 min representing the straight chain 17:0 glycerylether diacetate, a second peak with a similar mass spectrum was observed at 3.0 min, which may represent a 17:0 branched chain alkylether. This latter peak was not characterized further. It can also be seen from Fig. 4 that there was no appreciable difference in the percentage composition of alkyl ether residue of lyso-GEPE, AGEPE, and AGEPC. The presence of an 18:1 species was further substantiated by catalytic hydrogenation of the desired AGEPE and then analysis of the derivatized product by GLC–MS (Fig. 4D).

^{31}P NMR spectroscopy

Although the infrared spectrum gave some supporting proof of the structure of the synthetic compounds, additional insight into the structural configuration was gained by the use of ^{31}P NMR. The chemical shifts relative to the internal standard (TMP) were found to be 0.83 PPM for lyso-GEPE, 0.38 PPM for AGEPE, and −0.90 PPM for AGEPC. Isomeric phosphatidylcholines showing two distinct chemical shifts at 0.86 PPM for the *sn*-3 form and 1.45 PPM for the *sn*-2 form have been reported (19). A mixture of the *sn*-2 and *sn*-3 isomers of AGEPC exhibits two clearly separated peaks (20).[1] Inasmuch as the spectrum for AGEPC derived from AGEPE subsequent to the acetylation of lyso-GEPE was identical to synthetic *sn*-3 AGEPC, and as no detectable signal corresponding to *sn*-2 isomer was observed, it is reasonable to conclude that semi-synthetic AGEPE and AGEPC have retained the *sn*-3 configuration.

Optical rotation

The pure mixed ether AGEPE, which showed an optical reaction $[\alpha]^{23} = +2.11°$, was base-treated with methanolic sodium hydroxide (0.5 N) at room temperature for 45 min. The resulting lyso-GEPE gave the same optical rotation as that obtained with pure starting lyso-GEPE

[1] Kumar, R., and W. Kramp. Unpublished observations.

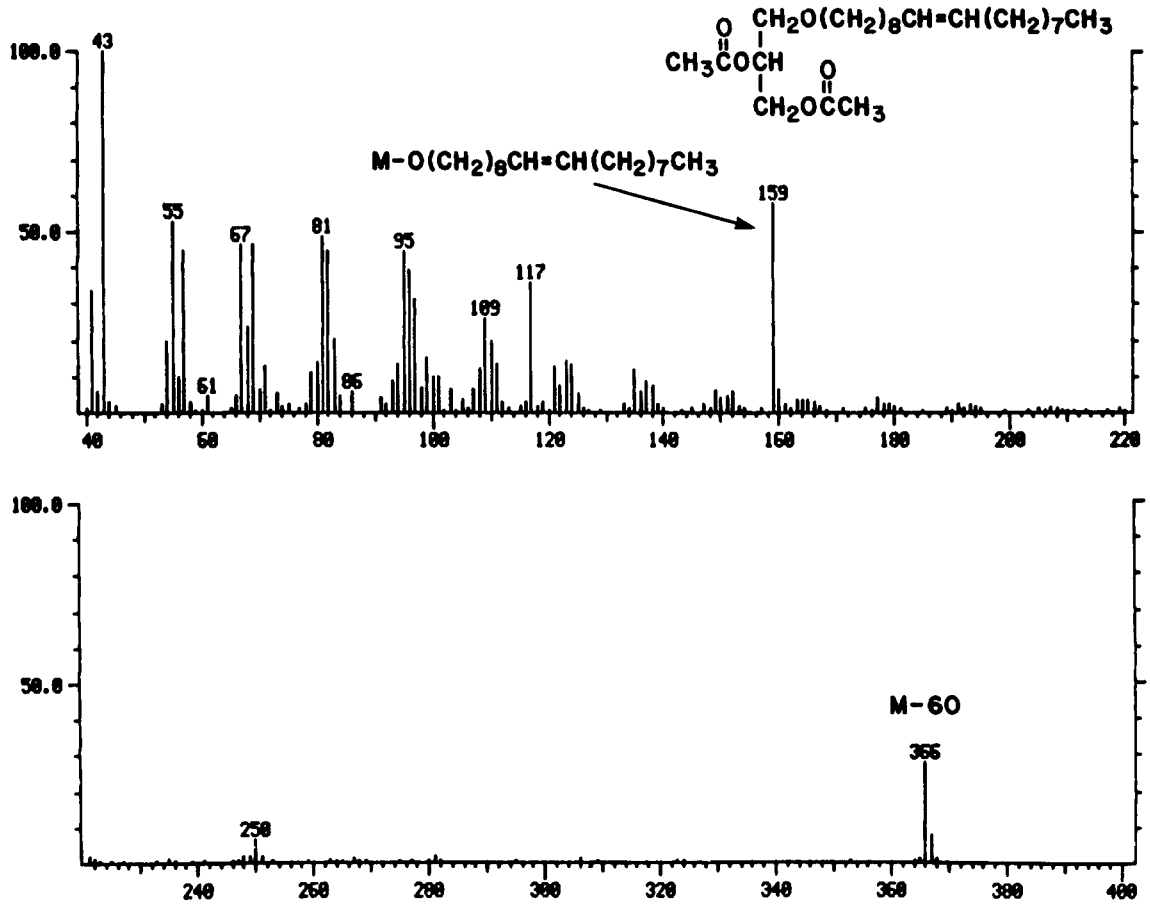

Fig. 3. Electron impact mass spectrum (70 EV) of 1-*O*-octadecenyl-2,3-diacetyl-glycerol.

$[\alpha]_D^{23} = -1.46°$ (see above). This supported the contention that no change in stereochemical configuration of the glycerol backbone occurred during acetylation of lyso-GEPE.

Platelet activity

The ability of the various species of AGEPC and AGEPE to induce 50% serotonin secretion from rabbit platelets is shown in **Table 3.** It is quite evident that the hexadecyl-rich species of AGEPE is significantly more potent than the corresponding octadecyl- and octadecenyl-rich species. As had been noted in other studies (3) and is obvious from the data in Table 3, the AGEPE samples were nearly 5000-fold less active than the corresponding AGEPC preparation in stimulating secretion of serotonin from washed rabbit platelets.

CONCLUSION

The major accomplishment of this study was the development of a facile, high-yield semi-synthetic procedure for the preparation of 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphoethanolamine, AGEPE. There was a threefold purpose in choosing this approach, the first being that AGEPE could be separated surprisingly well by a two-step thin-layer chromatographic procedure into individual molecular species, e.g., the 16:0, 18:0, or 18:1 types; and a second being that AGEPE, which has low platelet-activating activity, can be converted very smoothly into the very potent AGEPC. This synthetic technique definitely avoids the problem attendant upon the use of phospholipase D to convert AGEPC, for example, into other derivatives by the transphosphatidylation reaction. In the latter case enormous care and attention must be focused on the removal of any trace amounts of the starting AGEPC. The third advantage was to provide a starting material which could be easily labeled with radioisotopes for in vitro as well as in vivo metabolic studies. For example, radio-labeled methyl iodide can be employed to tag the amino group in AGEPE, and tritiation of the 18:1 molecular species of any of the derivatives can provide another site for labeling. However, it should be noted that labeling of these compounds at a specific activity

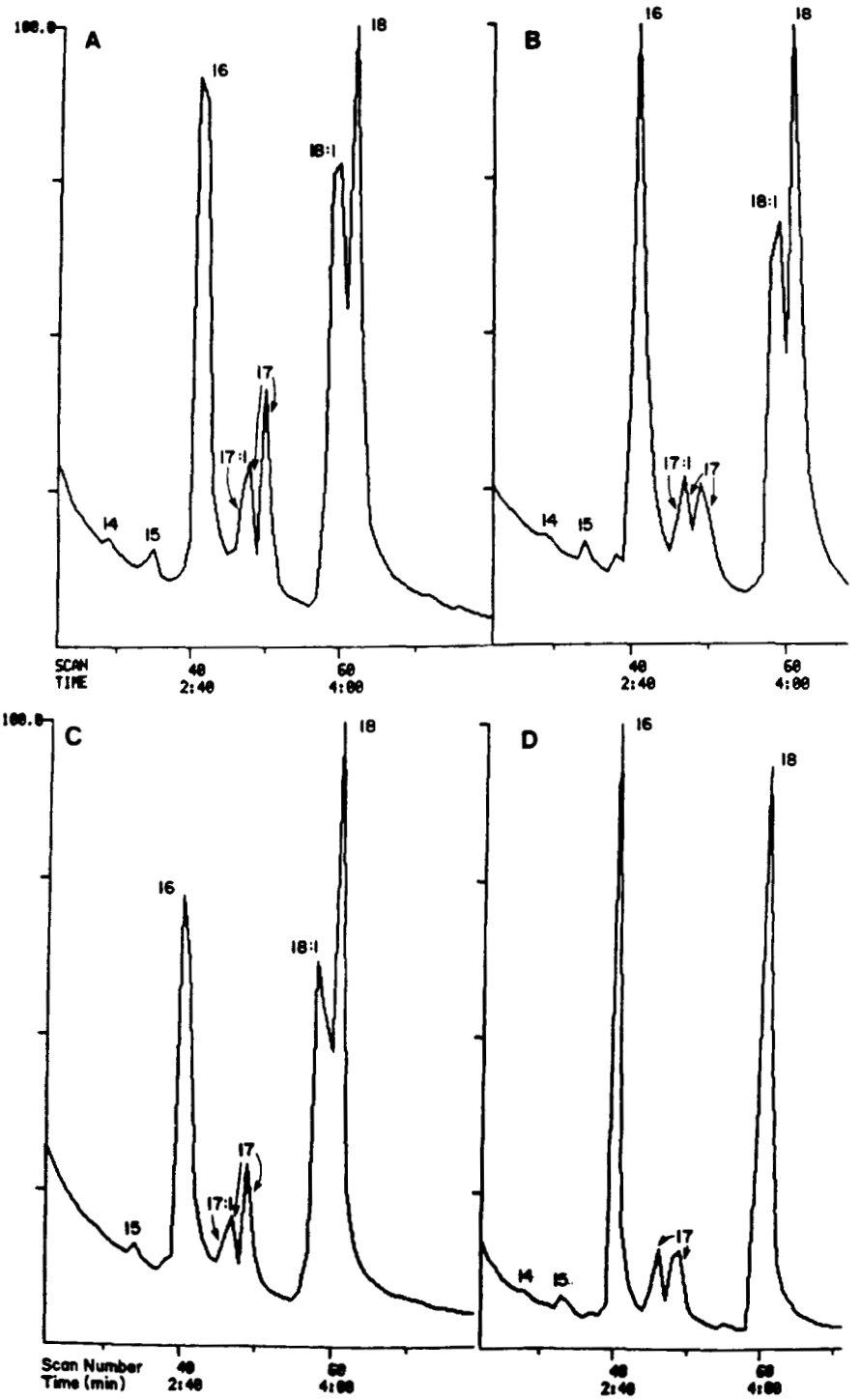

Fig. 4. Reconstructed ion chromatogram of acetoylsis products of: A, lyso-GEPE; B, AGEPE; C, AGEPC; D, hydrogenated AGEPE. Separation of these 1-*O*-alkyl-2,3-diacetyl-glycerols was accomplished with a 20-meter Se 54 glass capillary column at 240°C, using helium as the carrier gas with a column head pressure of 10 PSI.

sufficiently high to allow use at levels near the biological activity of AGEPC, i.e., 10^{-10} M, does pose certain limitations. At present, tritiated derivatives provide the best route to high specific activity substrates. In any event, bovine erythrocytes provide an excellent source of ethanolamine glycerophospholipids with a high content of

TABLE 3. Specific glycerylether phospholipid stimulation of serotonin release from rabbit platelets

Compounds	Biological Activity[a]
AGEPE, 16:0	$1.02 \pm 0.16 \times 10^{-6}$[b] (N = 6)
AGEPE, 18:0	$9.94 \pm 1.45 \times 10^{-6}$[b] (N = 5)
AGEPE, 18:1	$1.07 \pm 0.08 \times 10^{-5}$[b] (N = 4)
AGEPE, 16:0 + 18:0 + 18:1	$1.75 \pm 0.51 \times 10^{-6}$[b] (N = 6)
Lyso-GEPE, 16:0 + 18:0 + 18:1	Inactive[c]
AGEPC, 16:0 + 18:0 + 18:1	$3.51 \pm 0.90 \times 10^{-10}$[b] (N = 6)
AGEPC, 16:0[d]	$1.38 \pm 0.35 \times 10^{-10}$[b] (N = 6)

[a] Biological activity expressed as the final concentration (molar) of indicated lipid that was required to induce the release of 50% [^{3}H]serotonin from washed rabbit platelets within 60 sec at 37°C. Under these conditions, no platelet lactate dehydrogenase (LDH) was released as assessed in a spectrophotometric assay with a sensitivity of 1% platelet LDH.

[b] Mean ± one standard deviation (N, number of different washed rabbit platelet preparations).

[c] This derivative was without any platelet-stimulating activity (both aggregation and secretion) when assessed at a final concentration of 10^{-5} M.

[d] Although the 18:1 species of AGEPC derived from AGEPE has not been tested for biological activity, an 18:1 AGEPC prepared by a total organic chemical synthetic route showed an activity only slightly less than that of the 16:0 derivative.

glycerylethers with the desired *sn*-3-stereochemical configuration.

One important component of this synthetic route was the acetylation reaction in which the catalyst used, i.e., perchloric acid, as suggested by Eibl (21), allowed complete acetylation of the free hydroxyl of the starting substrate, lyso-PE, within 30 sec. Apparently the lack of reactivity of the acetic anhydride with the free amino was due to formation of a perchlorate salt. That this was presumably the case was illustrated by the fact that neutralization of the acetylation mixture with $NaHCO_3$ at the end of the reaction time led to formation of the amide in addition to the expected acetylation at the 2-hydroxyl. This problem could be circumvented by omitting the neutralization with sodium bicarbonate or any base and simply washing the chloroform-soluble reaction mixture several times with methanol–water mixtures. A detailed examination of the physical and chemical characteristics of the derivatives formed by this acetylation procedure (and also the argentation TLC) demonstrated that there had been no attack on the olefinic double bonds in the alkyl side chains and as a consequence no change in the composition or chemical nature of the glyceryl ethers.

An evaluation of the biological activity of the AGEPE series showed that the hexadecyl-rich sample exhibited an activity which was six to eight times greater than the octadecyl-rich derivative. It was noteworthy that there was no appreciable difference between the biological activity of the octadecyl- and the octadecenyl-rich species. It is also important to note that the major molecular-enriched species of AGEPE contained small but detectable levels of pentadecyl, heptadecyl, heptadecenyl, and some branched chain alkylethers. At this point, however, it is not possible to define their exact structure or possible effects of these minor components on the biological activity.

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