papers and notes on methodology

A novel method for the analysis of platelet-activating factor: direct derivatization of glycerophospholipids

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Abstract A novel, facile, and sensitive method for the quantitative and complete structure-proof analysis of platelet-activating factor (PAF) and other glycerophospholipids is described. 1-*O*-Alkyl/acyl-2-acyl-3-glycerophospholipids were treated with heptafluorobutyric anhydride in a one-step reaction to yield 1-*O*-alkyl/acyl-2-acyl-3-heptafluorobutyroyl-*sn*-glycerols as gas–liquid chromatography (GLC)-compatible derivatives. Furthermore, the components of the polar head group were also analyzed from the aqueous extract of the same reaction mixture as t-butyldimethylsilyl derivatives. Thus, this new method eliminates the need for phospholipase C treatment and subsequent purification procedures. Moreover, the direct derivatization of PAF homologs and analogs with heptafluorobutyric anhydride does not result in positional isomerization of the product, providing increased specificity for gas–liquid chromatography–mass spectrometric (MS) analysis. It has also been shown that the heptafluorobutyroyl (HFB) derivative can easily be converted to the respective t-butyldimethylsilyl analog in a one-step reaction using t-butyldimethylsilyl chloride/imidazole reagent. Analogous to the formation of heptafluorobutyroyl derivatives, PAF also was reacted with pentafluorobenzoyl chloride to generate the pentafluorobenzoyl derivative. Therefore, this method has wide applicability for the formation of GLC-compatible derivatives of various glycerophospholipids. Our successful HFB derivatization and GLC–MS detection of subnanogram quantities of PAF indicate that this analytical procedure will greatly facilitate complete and quantitative identification of each of the molecular species of biologically derived PAF.—**Satsangi, R. K., J. C. Ludwig, S. T. Weintraub, and R. N. Pinckard.** A novel method for the analysis of platelet-activating factor: direct derivatization of glycerophospholipids. *J. Lipid Res.* 1989. **30:** 929–937.

Supplementary key words 1,2-disubstituted-3-heptafluorobutyroyl-*sn*-glycerol • gas–liquid chromatography–mass spectrometry • polar head group • acetyl glyceryl ether phosphocholine

Platelet-activating factor (PAF) is comprised of a family of closely related acetylated glycerophospholipids that have been found to mediate many important physiological and pathological responses (1–3). Various types of inflammatory cells synthesize and release this heterogeneous group of compounds in nanogram quantities (c.f. 1). PAF derived from stimulated PMN has been documented to be comprised of saturated and unsaturated alkyl chain homologs of AGEPC (4–6), 1-O-acyl analogs (6, 7), and 1-O-alkyl and alk-1-enyl ethanolamine analogs (8, 9), all of which have differing biological potencies (8, 10, 11). As a result of the relatively small amounts of each PAF molecule synthesized by various cells, the assessment of PAF production has been difficult. The levels of PAF have been determined by measuring their ability to activate platelets (4, 5, 12) or by evaluation of [^{3}H]acetate incorporation into the PAF molecule (13). However, these procedures do not provide definitive information about the molar amounts of each molecular species of PAF produced by cells. Physicochemical methods such as fast atom bombardment (FAB) mass spectrometry (14, 15) and gas–liquid chromatography–mass spectrometry (GLC–MS) with selected ion monitoring (16, 17) have been used successfully for structure-proof and quantitation of phospholipids. However, the analysis of these molecules by GLC–MS requires several enzymatic and/or chemical reactions to derivatize these compounds into less polar and more volatile GLC-compatible forms. To achieve this goal, the glycerophospholipids have most commonly been hydrolyzed by phospholipase C, and derivatives such as the t-butyldimethylsilyl (TBDMS) (17, 18), trimethylsilyl (TMS) (19), and pentafluorobenzoyl (PFB) (20) have been made. Phospholipase C treatment of glycerophospholipids generates two isomers of

Abbreviations: PAF, platelet-activating factor; PMN, neutrophilic polymorphonuclear leukocyte; AGEPC, acetyl glyceryl ether phosphocholine; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; HFB, heptafluorobutyroyl; PFB, pentafluorobenzoyl; TBDMS, *t*-butyldimethylsilyl; TMS, trimethylsilyl; FAB, fast atom bombardment; GLC–MS, gas–liquid chromatography–mass spectrometry; EI, electron impact; CI, chemical ionization; EC, electron capture.

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the hydrolyzed product (21) and consequently, results in formation of two isomers of the volatile derivative. This significantly complicates interpretation of subsequent GLC or GLC–MS analysis and can cause reduced sensitivity, because each molecular species of PAF can generate two GLC peaks.

In addition to the problems described above for analysis of the glyceride portion of compounds such as PAF, structural elucidation of the polar head group is exceedingly difficult following phospholipase C treatment, mainly due to difficulties in purification. The polar head group is isolated in an aqueous extract along with copious amounts of salts. This complicates the isolation procedure and greatly interferes with FAB–MS analysis.

In view of the above, we have developed a facile, quantitative, one-step procedure for structure-proof analysis and quantitation of glycerophospholipids. Treatment of AGEPC with heptafluorobutyric anhydride followed by extraction into hexane generates the 3-heptofluorobutyroyl (HFB) derivative of the respective glycerophospholipid as a clean, single isomer, with virtually quantitative yield. By an analogous procedure, direct derivatization of AGEPC with pentafluorobenzoyl chloride results in a volatile product, as does treatment of the HFB derivative of AGEPC with TBDMS/imidazole reagent. Thus, complete and quantitative chemical identification of all of the molecular species of biologically derived PAF is now possible.

MATERIALS AND METHODS

Materials

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0-AGEPC) was purchased from Bachem Fine Chemicals (Torrance, CA). 1-*O*-Octadec-9-enyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:1-AGEPC), 1,2-dioctadecanoyl-*sn*- glycero-3-phosphoethanolamine, 1-*O*-hexadecyl-2-methoxy-*sn*-glycero-3-phosphocholine, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphatidic acid, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoserine, 1,2-diacyl-*sn*-glycero-3-phosphoinositol (linoleoyl and palmitoyl), and 1-*O*-hexadecyl-*sn*-glycerol were obtained from Sigma Chemical Co. (St. Louis, MO), as was phospholipase C (*B. cereus*). Tritium-labeled C16:0-AGEPC was purified as described (22) from beef heart-derived semisynthetic 1-*O*-[alkyl-1',2'-^{3}H]-2-acetyl-*sn*-glycero-3-phosphocholine (New England Nuclear, Boston, MA, 30–60 Ci/mmol). C16:0-AGEPC labeled with ^{3}H in the acetyl residue was also bought from New England Nuclear (10 Ci/mmol). N-Methyl-N-(t-butyl)dimethylsilyl-trifluoroacetamide and dimethylformamide (silylation grade) were purchased from Pierce Chemical Co. (Rockford, IL). t-Butyldimethylsilyl chloride/imidazole reagent was obtained from Applied Science Labs (Deerfield, IL). Pentafluorobenzoyl chloride and heptafluorobutyric anhydride were bought from Aldrich Chemical Co. (Milwaukee, WS). Acetic anhydride was purchased from Fisher Scientific Co. (Fairlawn, NJ). Pyridine was dried by refluxing over barium oxide, and after distillation it was stored over 3-Å molecular sieves. All other solvents used were HPLC grade. Thin-layer chromatographic (TLC) analyses were performed on 250-μm precoated silica gel G plates (Analtech Inc., Newark, DE). Six different solvent systems were used in the analysis of the various compounds: system A, chloroform–methanol–water 65:35:6 (v/v/v); system B, hexane–diethyl ether 70:30 (v/v); system C, hexane–diethyl ether 90:10 (v/v); system D, silica gel G plates saturated with 4% aqueous boric acid and dried, chloroform–methanol 98:1.5 (v/v); system E, silica gel G plates saturated with 4% aqueous boric acid and dried, hexane–diethyl ether 70:30 (v/v); system F, hexane–diethyl ether 80:20 (v/v). Samples were visualized by charring with 50% H_2SO_4 in water. When using radiolabeled compounds, 5-mm increments of the gel were scraped into scintillation vials and radioactivity was assessed by liquid scintillation spectroscopy.

Nuclear magnetic resonance (NMR) spectroscopy

Proton NMR studies were performed on a JEOL FX-90Q FT NMR spectrometer at 90 MHz using tetramethylsilane as an internal reference. The samples were dissolved in $CDCl_3$ (Aldrich Chemical Co.).

Mass spectrometry (MS)

Fast atom bombardment (FAB) and positive ion electron impact (EI) GLC–MS analyses were performed on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS data system. For FAB, xenon was used with an Ion Tech saddle field atom gun operating at 8 kV. The accelerating voltage in the mass spectrometer was 3 kV, and the ion source temperature was 70°C. Sample spectra were evaluated after subtraction of contributions from the thioglycerol matrix. GLC separation of volatile compounds was accomplished in a Varian model 3700 gas chromatograph. The 12 m × 0.32 mm i.d. BP-1 fused silica column (Scientific Glass Engineering, Austin, TX) used for these purposes was connected directly to the mass spectrometer through a transfer region that was maintained at 250°C. Helium was used at a linear velocity of approximately 80 cm/sec. The injector temperature was 250°C, and the column temperature was adjusted as needed. The accelerating voltage in the mass spectrometer was 3 kV, and the ion source temperature was 250°C. For solids probe and chemical ionization (CI) GLC–MS analyses, a Finnigan-MAT model 4615 mass spectrometer in combination with an INCOS data system was utilized. For CI–GLC–MS, the ion source temperature was 100°C, and the methane pressure was 0.5 Torr. GLC separation was accomplished by means of a 12 m × 0.32 mm BP-1 fused silica column (Scientific Glass Engineering, Inc.) which was connected directly to the mass

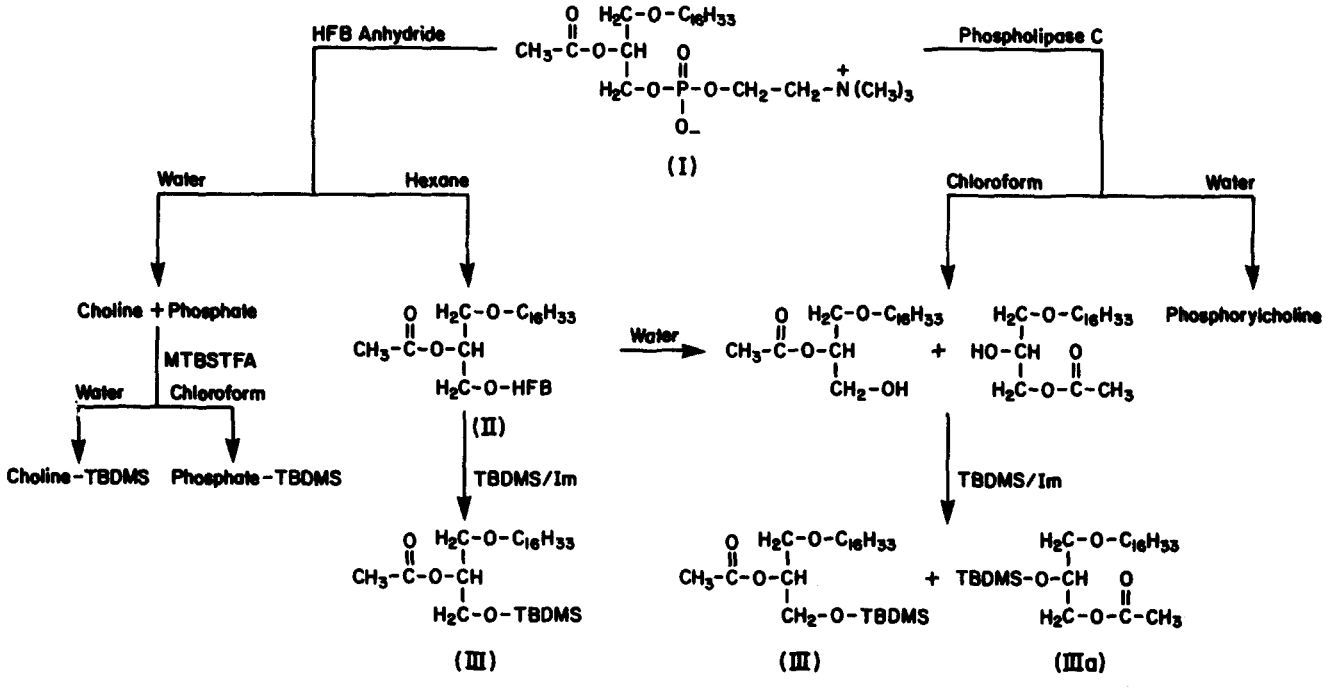


Fig. 1. Derivatization procedures for analysis of AGEPC.

spectrometer via a short length of 0.25-mm deactivated fused silica tubing. The linear velocity of the helium carrier gas was approximately 80 cm/sec. The injector temperature was 250°C; a splitless mode of injection was used. The column temperature was initially maintained for 1 min at 180°C, and was then increased to 225°C at a rate of 30°C/min.

Synthesis of heptafluorobutyroyl (HFB) derivative (II) of C16:0-AGEPC

In a 10-ml screw-cap tube, 12.5 μg of C16:0-AGEPC (compound I in **Fig. 1**) in $CHCl_3$ was taken to dryness, and heptafluorobutyric (HFB) anhydride (100 μl) was added. The atmosphere above the reaction mixture was purged with N_2, and the tube was sealed with a Teflon-lined screw-cap. Then the reaction mixture was heated at 35°C, with continuous stirring. The progress of the reaction was checked hourly by TLC using solvent systems A and B separately. The reaction was approximately 90% complete after 4 h, and attained nearly 100% conversion after incubation at room temperature overnight. The sample was dried under a stream of N_2 at room temperature, and the residue was partitioned between 500 μl hexane and 500 μl H_2O. The aqueous layer was washed twice with 250 μl hexane. The combined hexane layers were washed twice with 250 μl H_2O. The organic phase was dried under a stream of N_2 and stored in hexane.

In order to prepare large quantities of the HFB derivative of C16:0-AGEPC, 14.7 mg (28 μmol) of C16:0-AGEPC was treated with HFB anhydride (200 μl) as described above. The excess HFB anhydride was removed under a stream of N_2 at room temperature, and the residue was partitioned between hexane (1 ml) and H_2O (1 ml). The H_2O layer was washed twice with 1 ml hexane. The combined hexane layer was washed with H_2O (500 μl), dried over anhydrous sodium sulfate, and passed over a column of activated charcoal (0.6 × 2 cm). The column was washed with 5 ml $CHCl_3$. After evaporating the $CHCl_3$ under a stream of N_2, the residue was dried under vacuum. The overall yield of the product was 15.5 mg (96.8% of theory). The R_f of compound II was 0.14, with streaking, in TLC system B and 0.90 as a single spot in TLC system E.

^1H NMR ($CDCl_3$): δ 0.87–1.54 (31H, complex m, C(1)-O-CH_2-$C_{15}\underline{H}_{31}$); 2.06 (3H, s, C(2)-O-CO-$C\underline{H}_3$); 3.43 (2H, t(J6.2), C(1)-O-$C\underline{H}_2$-$C_{15}H_{31}$); 3.54 (2H, d(J5.7), C(1)$\underline{H}_2$-O-CH_2-$C_{15}H_{31}$); 4.58 (2H, distorted t, C(3)$\underline{H}_2$-O-HFB); 5.20 (1H, m, C(2)$\underline{H}$-O-CO-CH_3). Positive ion electron impact GLC-MS analysis was performed as described above. At a column temperature of 200°C, the retention time was 2.5 min. The EI mass spectrum typified this class of compounds, with predominant hydrocarbon fragments and low intensity ions with pertinent structural information. No $[M]^+$ was observed. The fragments of interest were: *m/z* 494, [M-acetic acid]$^+$; *m/z* 341, [M-HFBO]$^+$; *m/z* 313, [M-$C_{16}H_{33}O$]$^+$; *m/z* 271, [M-($C_{16}H_{33}O$ + $COCH_2$)]$^+$.

HFB derivatives of other phosphoglycerides were prepared as described above.

^{1}H NMR of HFB derivative of 1,2-distearoyl-glycerophospholipids ($CDCl_3$): δ 0.87–1.67 (66H, complex m, C(1,2)-O-CO-CH_2-$C_{16}\underline{H}_{33}$); 2.32 (4H, distorted t, C(1,2)-O-CO-$C\underline{H}_2$-$C_{16}H_{33}$); 4.23 (2H, distorted dd, C(1)$\underline{H}_2$-O-CO-CH_2-$C_{16}H_{33}$); 4.54 (2H, distorted t, C(3)$\underline{H}_2$-O-HFB); 5.33 (1H, m, C(2)$\underline{H}$-O-CO-CH_2-$C_{16}H_{33}$).

^{1}H NMR of HFB derivatives of other 1,2-diacyl-glycerophospholipids were similar to those described above and confirmed their proposed structures.

Analysis of polar head group

After synthesizing the HFB derivative of 12.5 μg of C16:0-AGEPC, as described above, the aqueous layer remaining after removal of the glyceryl moiety was dried under a stream of N_2 at room temperature in a screw-cap tube. Dimethylformamide (100 μl) and N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (25 μl) were added to the residue. The atmosphere in the reaction tube was purged with N_2, and the tube was sealed with a Teflon-lined screw-cap. The reaction mixture was heated at 60°C for 3 h, with continuous stirring. After drying the mixture under a stream of N_2 at 37°C, the residue was extracted by the method of Bligh and Dyer (23). The upper and lower phases were separated and dried under a stream of N_2. The residues were separately dissolved in 100 μl acetonitrile and were individually analyzed by positive ion FAB-MS. In the material remaining from the organic phase, the major ions were *m/z* 441 and *m/z* 327, consistent with $[HPO_4TBDMS_3]^+$, and $[H_2PO_4TBDMS_2]^+$, respectively. In the residue of the aqueous phase, the predominant ion was observed at *m/z* 218, interpreted as [cholineTBDMS]$^+$.

Synthesis of 1-*O*-hexadecyl-2-acetyl-3-TBDMS-*sn*-glycerol (III) directly from (II)

1-*O*-Hexadecyl-2-acetyl-3-heptafluorobutyroyl-*sn*-glycerol (compound II, 50 μg) was taken to dryness. t-Butyldimethylsilyl chloride/imidazole (100 μl) was added, and the atmosphere in the tube was purged with N_2. The tube was sealed with a Teflon-lined screw-cap. The reaction mixture was heated at 65–70°C for 3 h with continuous stirring, and then the excess TBDMS chloride was evaporated under N_2 at 50°C for 1 h. The residue was partitioned between $CHCl_3$ (500 μl) and H_2O (500 μl). The $CHCl_3$ layer was washed twice with 500-μl portions of H_2O, and the $CHCl_3$ phase was dried under a stream of N_2. The sample was dissolved in hexane and analyzed by GLC-MS.

Using a glass falling-needle injector (R. H. Allen Co., Boulder, CO) with a pressure of 4 psi and a column temperature of 250°C, the product eluted as a single peak with a retention time of 2.68 min. The EI mass spectrum was typical for TBDMS derivatives, with no [M]$^+$ being observed. Characteristic ions were: *m/z* 415, [M-t-butyl]$^+$; *m/z* 355, [M-(t-butyl + acetic acid)]$^+$; *m/z* 131, [TBDMSO]$^+$; *m/z* 117, $[CH_3COSi(CH_3)_2]^+$. The TLC R_f was 0.68 using TLC system C. Using previously published procedures (20, 21), formation of the TBDMS derivative after phospholipase C treatment of C16:0-AGEPC or after aqueous hydrolysis of the HFB analog resulted in two GLC peaks with retention times of 2.68 min and 2.80 min, respectively, which exhibited mass spectra similar to that described above.

Synthesis of pentafluorobenzoyl (PFB) derivative (IV) of C16:0-AGEPC

C16:0-AGEPC (125 μg) in $CHCl_3$ was added to a screw-cap tube and dried under a stream of N_2. Pentafluorobenzoyl chloride (100 μl) was added, and the reaction mixture was heated at 120°C, with stirring. The reaction was monitored periodically with TLC system A and was found to be complete after 18 h. The excess pentafluorobenzoyl chloride was removed by a stream of N_2 at 60°C. Chloroform (500 μl) was added to the residue, and the sample was dried by a stream of N_2 at 60°C. Chloroform (500 μl) was again added, and the sample was dried under N_2 in order to aid in removal of any remaining PFB chloride. The residue was then partitioned between H_2O (1 ml) and hexane (1 ml). The aqueous phase was washed twice with hexane (500 μl each), and the combined organic layer was dried under a stream of N_2. The sample was dissolved in hexane and analyzed by GLC-MS. When a column temperature of 230°C was used, the product eluted at 3.87 min. No [M]$^+$ was found in the EI mass spectrum. Ions useful for structural elucidation were: *m/z* 311, $[M-C_{16}H_{33}O]^+$; *m/z* 269, $[M-(C_{16}H_{33}O + CH_2CO)]^+$; *m/z* 195, $[C_6F_5CO]^+$. Using TLC system F, the R_f was 0.68. The GLC-MS and TLC characteristics of this product were the same as those obtained for 1-*O*-hexadecyl-2-acetyl-3-pentafluorobenzoyl-*sn*-glycerol prepared by the method of Ramesha and Pickett (20), using phospholipase C.

Reaction of heptafluorobutyroyl (HFB) derivative of C16:0-AGEPC with H_2O: formation of 1-*O*-hexadacyl-2-acetyl-*sn*-glycerol

1-*O*-Hexadecyl-2-acetyl-3-heptafluorobutyroyl-*sn*-glycerol (compound II, 50 μg) was taken to dryness in a screw-cap tube. Water (500 μl) was added, and the mixture was heated at 35°C overnight, with stirring. Chloroform (500 μl) was then added to the reaction mixture. The organic phase was removed and dried under a stream of N_2. The products were analyzed by TLC system D and compared to the products isolated from the phospholipase C treatment of C16:0-AGEPC (20, 21).

Synthesis of 1-*O*-hexadecyl-2,3-diheptafluorobutyroyl-*sn*-glycerol (V)

1-*O*-Hexadecyl-glycerol (10 mg) was placed in a screw-cap tube, and HFB anhydride (150 μl) was added. The atmosphere in the tube was purged with N_2 for 1 min, and

the tube was sealed with a Teflon-lined screw-cap. The reaction mixture was heated at 50°C for 3 h and then stirred at room temperature overnight. The solvent was removed under a stream of N_2 at room temperature. The remaining residue was partitioned between H_2O (1 ml) and $CHCl_3$ (1 ml). The aqueous layer was washed twice with $CHCl_3$ (1 ml), and the combined $CHCl_3$ extract was washed twice with H_2O (1 ml). The organic extract was dried under a stream of N_2 and then under vacuum. The overall yield of the product was 20 mg (89% of theory). Pyridine was not used for this acylation procedure because it was anticipated that the HFB derivative would not be stable in base.

The R_f of compound V using TLC system B was 0.24. ^{1}H NMR ($CDCl_3$): δ 0.87–1.54 (31H, complex m, C(1)-O-CH_2-$C_{15}\underline{H}_{31}$); 3.45 (2H, distorted t, C(1)-O-$C\underline{H}_2$-$C_{15}H_{31}$); 3.64 (2H, d(J5.7), C(1)$\underline{H}_2$-O-CH_2-$C_{15}H_{31}$); 4.64 (2H, distorted dd, C(3)$\underline{H}_2$-O-HFB); 5.43 (1H, m, C(2)$\underline{H}$-O-HFB).

Synthesis of 1-*O*-hexadecyl-2,3-diacetyl-*sn*-glycerol (VI)

1-*O*-Hexadecyl-glycerol (8 mg) was placed in a screw-cap tube, and acetic anhydride (200 μl) was added. The mixture was cooled on ice for 15 min, and then dry pyridine (50 μl) was added. The tube was sealed with a Teflon-lined screw-cap, and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was lyophilized, and the resulting residue was partitioned between 5% HCl (1 ml) and $CHCl_3$ (1 ml). The aqueous phase was extracted twice with $CHCl_3$ (1 ml each). The combined organic phase was extracted twice with H_2O (1 ml each). The $CHCl_3$ layer was evaporated to dryness under a stream of N_2 and further dried under vacuum. The overall yield of the product was 9.5 mg (95% of theory). Using TLC system B the R_f was 0.49; the product comigrated with the acetolysis product of AGEPC (24, 25).

^{1}H NMR ($CDCl_3$): δ 0.87–1.57 (31H, complex m, C(1)-O-CH_2-$C_{15}\underline{H}_{31}$); 2.06 and 2.08 (3H + 3H, 2xs, C(2)-O-CO-$C\underline{H}_3$, C(3)-O-CO-$C\underline{H}_3$); 3.43 (2H, t(J6.2), C(1)-O-$C\underline{H}_2$-$C_{15}H_{31}$); 3.53 (2H, d(J5.3), C(1)$\underline{H}_2$-O-CH_2-$C_{15}H_{31}$); 4.23 (2H, dd(J6.1), C(3)$\underline{H}_2$-O-CO-CH_3); 5.20 (1H, m, C(2)$\underline{H}$-O-CO-CH_3).

RESULTS

In initial experiments, the synthesis of heptafluorobutyroyl (HFB) derivatives was attempted by reacting C16:0-AGEPC with HFB anhydride and heptafluorobutyric acid at 150°C, a procedure analogous to that of acetolysis of glycerophospholipids (24, 25). However, this procedure resulted in a brown mixture of unidentified decomposition products. Modifying the reaction conditions, such as lowering the temperature and using milder acids, did not yield the required product. It was not until HFB anhydride alone was reacted with C16:0-AGEPC at 35°C for 4 h that the polar head group was effectively replaced by HFB.

To verify the structure of the HFB derivative of C16:0-AGEPC (compound II in Fig. 1), large amounts were prepared for analysis by NMR spectroscopy. The peaks at chemical shifts of δ 3.54, 4.58, and 5.20 were appropriate for the methylene and methine protons on the glycerol backbone. Furthermore, the integration and coupling patterns of each signal were compatible with the proposed structure. Confirmatory assignment of each signal to its respective protons was accomplished by comparing the NMR spectrum of II (**Fig. 2**) to that of 1-*O*-hexadecyl-2,3-diheptafluorobutyroyl-*sn*-glycerol (**Fig. 3**) and 1-*O*-hexadecyl-2,3-diacetyl-*sn*-glycerol (**Fig. 4**). NMR spectroscopy revealed that the methylene and methine protons of the glycerol backbone in the diacetate derivative (compound VI) resonated at δ 3.53, 4.23, and 5.20, while those in the diHFB derivative (compound V) were seen at δ 3.64, 4.64, and 5.43. From these results it can be deduced that the methylene protons attached to C(1) of the glycerol backbone (substituted with the least electronegative group) should be assigned to the highest field signal (i.e., δ 3.64 in compound V and δ 3.53 in compound VI). Further, a comparison of methylene and methine protons in diacetate and diHFB derivatives indicated that the methine proton adjacent to the acyl group resonated further downfield than the C(3) methylene protons. Moreover, HFB derivatization imparted a stronger downfield chemical shift as compared to acetyl derivatization. Thus, the C(2) methine proton adjacent to the acetyl group in compound VI resonated at δ 5.20, while the corresponding proton on C(2) in the diHFB derivative (compound V) resonated at δ 5.43. Similarly, the methylene protons C(3) in compound VI resonated at δ 4.23, and those adjacent to the HFB group in compound V resonated at δ 4.64. After comparing these chemical shifts with those observed for compound II, it was

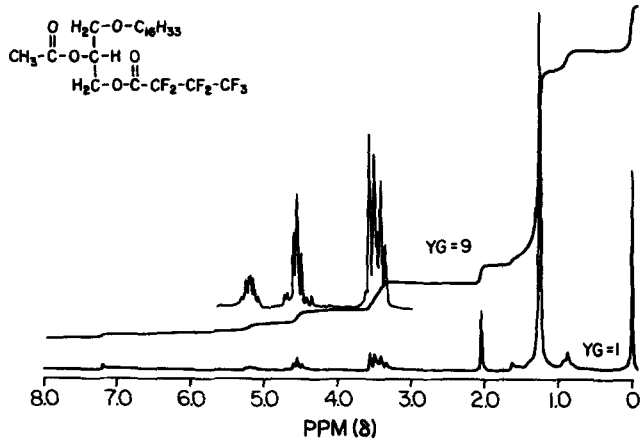


Fig. 2. ^{1}H NMR of 1-*O*-hexadecyl-2-acetyl-3-heptafluorobutyroyl-*sn*-glycerol (II).

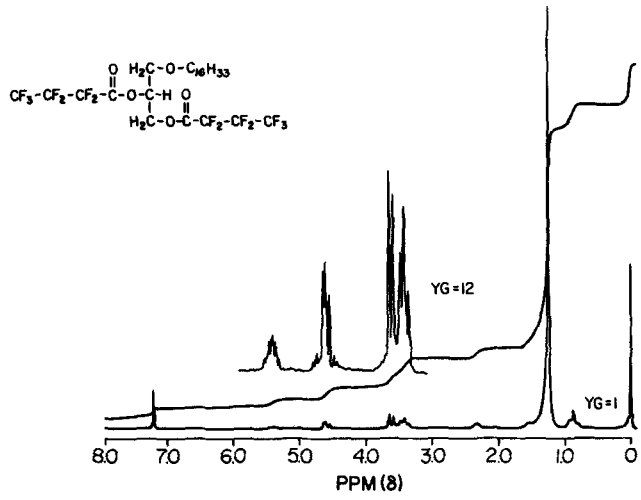


Fig. 3. ^{1}H NMR of 1-*O*-hexadecyl-2,3-diheptafluorobutyroyl-*sn*-glycerol (V).

clear that in compound II the HFB was attached to C(3) of the glycerol backbone, since the methylene protons at this position resonated at δ 4.58; moreover, C(2) was proved to be attached to an acetyl group, because the methine proton at this position resonated at δ 5.20. Therefore, the structure of the HFB derivative of C16:0-AGEPC was confirmed to be 1-*O*-hexadecyl-2-acetyl-3-HFB-*sn*-glycerol, as represented by compound II in Fig. 1.

It was anticipated that the treatment of C16:0-AGEPC with HFB anhydride would liberate the polar head group into the aqueous extract of the reaction mixture. The dried residue obtained from the aqueous extract of this reaction was analyzed by FAB-MS; however, only sodium ions were detected. It was found that since HFB anhydride is sufficiently corrosive, it removes sodium ions from glass reaction vessels. This prevented observation of the desired FAB mass spectrum of the polar head group fraction. To overcome this problem, we derivatized the polar head group in such a way that it could be extracted into organic solvents while leaving behind the salts. Thus, the aqueous phase from the HFB derivatization procedure was dried, and the residue was treated with N-methyl-N-(t-butyldimethylsilyl)trifluoro-acetamide. FAB-MS of the Bligh-Dyer extract of the TBDMS derivative showed that the HFB treatment had split the polar head group into choline and phosphate. The FAB mass spectrum of the residue from the organic phase revealed the presence of an ion at *m/z* 441 which corresponds to $[HPO_4TBDMS_3]^+$ and another ion at *m/z* 327 from $[H_2PO_4TBDMS_2]^+$. The aqueous phase from this Bligh-Dyer extraction was dried under N_2, and the residue was dissolved in acetonitrile, leaving behind the insoluble salts. FAB-MS of the solution showed that the major component exhibited an ion at *m/z* 218 which is representative of [cholineTBDMS]$^+$.

Further evaluation of the HFB derivative of C16:0-AGEPC (compound II) revealed that treatment of this product with water resulted in a mixture of two compounds, analogous to those obtained following the action of phospholipase C on C16:0-AGEPC (21). GLC-MS analysis of the TBDMS derivatives of these hydroxy analogs indicated that two isomers, presumably 1-*O*-hexadecyl-2-acetyl-3-TBDMS-*sn*-glycerol and 1-*O*-hexadecyl-2-TBDMS-3-acetyl-*sn*-glycerol (compounds III and IIIa) were present. The fact that the HFB moiety is a good leaving group was exploited as a means to ensure production of a single TBDMS derivative. The direct replacement of the HFB in compound II by a TBDMS group was accomplished by treating compound II with TBDMS-chloride/imidazole. The derivative obtained by this method (compound III) developed as a single spot by analytical TLC (system C). Furthermore, GLC-MS analysis revealed a single peak with a retention time of 2.68 min, which gave the characteristic $[M-57]^+$ ion at *m/z* 415. Thus, direct treatment of the HFB derivative with TBDMS/imidazole reagent yielded only one isomeric form, as represented by compound III in Fig. 1.

Based upon the observation that derivatization of AGEPC with HFB anhydride resulted in the formation of only one isomer of the HFB derivative of AGEPC (compound II), C16:0-AGEPC was directly treated with pentafluorobenzoyl chloride at 120°C, and the resulting 1-*O*-hexadecyl-2-acetyl-3-pentafluorobenzoyl-*sn*-glycerol (compound IV, **Fig. 5**) was obtained in almost quantitative yield. The analysis of this product by GLC-MS revealed that it exhibited the expected mass spectral pattern.

In order to test the applicability of the HFB derivatization procedure to other glycerophospholipids, the reaction of various molecules with HFB anhydride was investigated. The results are shown in **Table 1**. Out of all the compounds

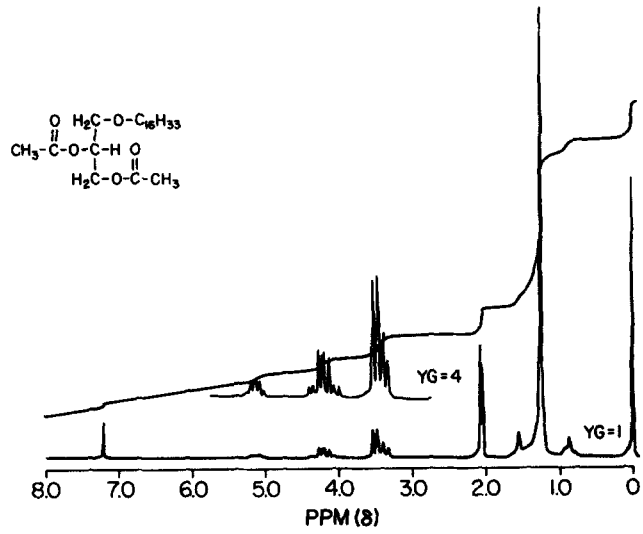
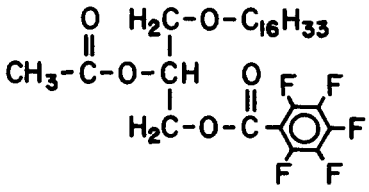


Fig. 4. ^{1}H NMR of 1-*O*-hexadecyl-2,3-diacetyl-*sn*-glycerol (VI).



(IV)

Fig. 5. Structure of 1-*O*-hexadecyl-2-acetyl-3-pentafluorobenzoyl-*sn*-glycerol (IV).

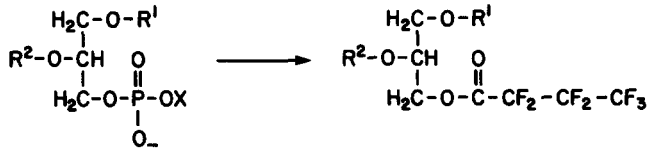
studied, only the di-*O*-alkyl-glycerophospholipid was resistant to replacement of the polar head group by HFB. The structure of each of these HFB analogs was confirmed by mass spectrometry and NMR spectroscopy. The HFB derivatives of the compounds containing two long chain fatty acyl groups were found to decompose during GLC–MS analysis and, therefore, were characterized after introduction into the mass spectrometer by means of a solids probe.

Finally, a series of experiments was conducted to determine the applicability of direct derivatization of PAF with HFB for detection of physiologically relevant amounts of the autacoid. In initial studies, when nanogram quantities of C16:0-AGEPC were analyzed, the recovery of the HFB derivative was only 50% as compared to the quantitative yield obtained when microgram amounts of AGEPC were used. Subsequently, however, we found that identical high yields of HFB derivatives could consistently be obtained from amounts of AGEPC ranging from 10 pg to 10 μg, providing that the derivatization procedure was conducted in siliconized vessels. To evaluate detection by GLC–MS, 50 ng, 5 ng, and 0.5 ng of C16:0-AGEPC were separately reacted with HFB, resulting in quantitative recovery (as assessed by parallel studies with radiolabeled AGEPC). After dissolving each sample in 10 μl of hexane, a 1-μl aliquot was subjected to positive ion methane chemical ionization GLC–MS analysis. Each HFB derivative eluted as a single peak with a retention time of 3.0 min, using a 12 m × 0.32 mm BP-1 column under the conditions described in Methods. By monitoring the $[MH]^+$ ion at *m/z* 555, each sample produced a readily distinguishable peak, with a > 10:1 signal-to-noise ratio being observed from the aliquot from the 0.5 ng derivative (i.e., 50 pg).

DISCUSSION

Characterization of each of the molecular species of PAF is important to our understanding of the role of this class of autacoid in modulating inflammatory and physiological processes. The present method is a novel, complete, and easy analytical procedure for the identification of PAF molecules as well as other types of glycerophospholipids. Using only one chemical reaction, the separate isolation of the glyceryl backbone and the constituents of the polar head group can be accomplished. The reagent, HFB anhydride or PFB, cleaves the phosphodiester bond of the glycerophospholipid, but leaves the ether and carboxyester linkages intact. Thus, this analytical method has widespread applicability towards the identification and quantitation of small amounts of glycerophospholipids, including the various molecular species of PAF. With respect to HFB, derivatization from 10 pg to 10 mg of C16:0-AGEPC resulted in quantitative recovery of the HFB derivative. Furthermore, 50 pg of the C16:0-AGEPC HFB derivative could

TABLE 1. Yield of 1,2-diradyl-3-heptafluorobutyroyl-*sn*-glycerols from the reaction of respective phosphoglyceride with heptafluorobutyric anhydride



R¹	R²	X	Yield (%)	TLC[a] R_{fB}	TLC[a] R_{fE}
Hexadecyl	Acetyl	Choline	96.8	0.14	0.90
Octadecenyl ($\Delta^{9,10}$)	Acetyl	Choline	nd[b]	0.14	0.90
Hexadecyl	Methyl	Choline	0		
Stearoyl	Stearoyl	Ethanolamine	92.6	0.20	0.90
Stearoyl	Stearoyl	H	96.7	0.20	0.90
Palmitoyl	Palmitoyl	Serine	87.9	0.20	0.85
Linoleoyl and	Palmitoyl	Inositol	92.3	0.20	0.90

[a]TLC: R_{fB}, R_f in TLC system "B"; R_{fE}, R_f in TLC system "E."
[b]Not determined.

easily be detected by positive ion CI-GLC-MS; this level of detection is comparable to that reported in a study which utilized GLC and electron capture (EC) detection (26). In the case of the PFB derivative, even greater sensitivity would be expected since amounts as low as 100 fg have been detected by CI-GLC-MS analysis using negative ion detection (20).

The present work with C16:0-AGEPC and various other glycerophospholipids indicates that the procedure works equally well with saturated or unsaturated alkyl or fatty acyl linkages at position-1 and acyl substituents at position-2 of glycerophospholipids. Relative to plasmalogens, HFB would most likely cause acid-catalyzed hydrolysis of the alk-1-enyl linkage, resulting in HFB derivatization at position-1, and cleavage of the phosphodiester bond and HFB derivatization at position-3. While the product 1,3-diheptafluorobutyroyl-2-acyl-*sn*-glycerol could easily be quantitated by GLC-MS or EC-GLC, no structural information would be gained about the chain length of the alk-1-enyl group at position-1. Such information could, however, be obtained by catalytic reduction of the plasmalogen species prior to HFB derivatization. Finally, we have shown that HFB derivatization at position-3 is similarly effective when the polar head group is either phosphocholine, phosphoethanolamine, phosphoserine, phosphoinositol, or unesterified phosphate.

In other methods used for the analysis of glycerophospholipids (17-20), the polar head group is generally removed by means of phospholipase C. Subsequent derivatization of the neutral glyceryl backbone portion of the molecule is then used to obtain a product suitable for GLC-MS analysis. There are several drawbacks to this protocol. First, the phospholipase C procedure requires significant sample purification prior to qualitative or quantitative measurements. Second, the use of phospholipase C could pose problems in quantitative measurements, since the various classes of phospholipids exhibit different relative susceptibilities to the action of the enzyme (27). Third, phospholipase C treatment generates two positional isomers of the hydroxy product (i.e., 1-*O*-alkyl-2-acetyl-*sn*-glycerol and 1-*O*-alkyl-3-acetyl-*sn*-glycerol) (21), hampering interpretation of the analytical results. In our laboratory we have observed the formation of these isomers and have separated their TBDMS derivatives by GLC and TLC. However, the current treatment of glycerophospholipids with HFB anhydride generates only one isomer of the GLC-compatible HFB derivative, in a single step, without the need for chromatographic purification. Furthermore, we have demonstrated that direct treatment of the HFB derivative of C16:0-AGEPC with TBDMS/imidazole reagent also produces only one TBDMS isomer. In addition, the reaction of C16:0-AGEPC with pentafluorobenzoyl chloride was found to proceed in a similar manner. Thus, this method allows flexibility in the choice of derivatives for analysis of glycerophospholipids.

Chemical identification of the polar head group of small amounts of biologically derived glycerophospholipids has been difficult, mainly due to problems in isolation and purification after enzymatic hydrolysis. The treatment of C16:0-AGEPC with HFB anhydride, as described in this report, splits the polar head group into water-soluble phosphate and choline. TBDMS derivatization of these components shifts their solubility into organic solvents which facilitates their purification and identification.

In summary, the current HFB derivatization of biologically relevant glycerophospholipids will facilitate the complete and quantitative chemical identification of these molecules, which are produced in very small amounts. As a result of this procedure, the chemical structures of large numbers of biologically derived PAF molecules will be able to be rapidly determined. Thus, studies using this technique on biological samples currently are being conducted and should provide valuable information towards understanding PAF-mediated tissue dysfunction and injury.

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REFERENCES

1. Pinckard, R. N., J. C. Ludwig, and L. M. McManus. 1988. Platelet-activating factors. *In* Inflammation: Basic Principles and Clinical Correlates. I. M. Goldstein and R. Snyderman, editors. Raven Press, New York. 139-167.
2. Lee, T. C., and F. Snyder. 1985. Function, metabolism and regulation of platelet activating factor and related ether lipids. *Med. Res. Rev.* **5:** 107-140.
3. Braquet, P., L. Touqui, T. Y. Shen, and B. B. Vargaftig. 1987. Perspectives in platelet-activating factor research. *Pharmacol. Rev.* **39:** 97-145.
4. Pinckard, R. N., E. Jackson, C. Hoppens, S. T. Weintraub, J. C. Ludwig, L. M. McManus, and G. E. Mott. 1984. Molecular heterogeneity of platelet-activating factor produced by stimulated human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **122:** 325-332.
5. Weintraub, S. T., J. C. Ludwig, G. E. Mott, L. M. McManus, C. Lear, and R. N. Pinckard. 1985. Fast atom bombardment-mass spectrometric identification of molecular species of platelet-activating factor produced by stimulated human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **129:** 868-876.
6. Mueller, H. W., J. T. O'Flaherty, and R. L. Wykle. 1984. The molecular species distribution of platelet-activating factor synthesized by rabbit and human neutrophils. *J. Biol. Chem.* **259:** 14554-14559.
7. Satouchi, K., M. Oda, K. Yasunaga, and K. Saito. 1985. Evidence for the production of 1-acyl-2-acetyl-*sn*-glycerol-3-phosphorylcholine concomitantly with platelet-activating factor. *Biochem. Biophys. Res. Commun.* **128:** 1409-1417.
8. Ludwig, J. C., and R. N. Pinckard. 1987. Diversity in the chemical structures of neutrophil-derived platelet-activating factors. *In* New Horizons in Platelet Activating Factor Research. C. Winslow and M. Lee, editors. John Wiley & Sons, New York. 59-71.
9. Tessner, T. G., and R. L. Wykle. 1987. Stimulated neutrophils produce an ethanolamine plasmalogen analog of platelet-activating factor. *J. Biol. Chem.* **262:** 12660-12664.

10. Humphrey, D. M., L. M. McManus, K. Satouchi, D. J. Hanahan, and R. N. Pinckard. 1982. Vasoactive properties of acetyl glyceryl ether phosphorylcholine (AGEPC) and AGEPC analogues. *Lab. Invest.* **46:** 422–427.
11. Surles, J. R., R. L. Wykle, J. T. O'Flaherty, W. L. Salzer, M. J. Thomas, F. Snyder, and C. Piantadosi. 1985. Facile synthesis of platelet-activating factor and racemic analogues containing unsaturation in the *sn*-1 alkyl chain. *J. Med. Chem.* **28:** 73–78.
12. Ludwig, J. C., C. L. Hoppens, L. M. McManus, G. E. Mott, and R. N. Pinckard. 1985. Modulation of platelet-activating factor (PAF) synthesis and release from human polymorphonuclear leukocytes (PMN): role of extracellular albumin. *Arch. Biochem. Biophys.* **241:** 337–347.
13. Mueller, H. W., J. T. O'Flaherty, and R. L. Wykle. 1983. Biosynthesis of platelet-activating factor in rabbit polymorphonuclear neutrophils. *J. Biol. Chem.* **258:** 6213–6218.
14. Clay, K. L., D. O. Stene, and R. C. Murphy. 1984. Quantitative analysis of platelet activating factor (AGEPC) by fast atom bombardment mass spectrometry. *Biomed. Mass Spectrom.* **11:** 47–49.
15. Varenne, P., B. C. Das, J. Polonsky, and M. Tence. 1985. Chemical ionization and fast atom bombardment mass spectrometry of platelet activating factor (PAF-acether) and related phospholipids. *Biomed. Mass Spectrom.* **12:** 6–10.
16. Satouchi, K., M. Oda, K. Yasunaga, and K. Saito. 1983. Application of selected ion monitoring to determination of platelet-activating factor. *J. Biochem.* **94:** 2067–2070.
17. Oda, M., K. Satouchi, K. Yasunaga, and K. Saito. 1985. Molecular species of platelet-activating factor generated by human neutrophils challenged with ionophore A23187. *J. Immunol.* **134:** 1090–1093.
18. Satouchi, K., and K. Saito. 1979. Use of *t*-butyl dimethyl chlorosilane/imidazole reagent for identification of molecular species of phospholipids by gas–liquid chromatography–mass spectrometry. *Biomed. Mass Spectrom.* **6:** 396–402.
19. Satouchi, K., and K. Saito. 1976. Studies on trimethylsilyl derivatives of 1-alkyl-2-acylglycerols by gas–liquid chromatography–mass spectrometry. *Biomed. Mass Spectrom.* **3:** 122–126.
20. Ramesha, C. S., and W. C. Pickett. 1986. Measurement of sub picogram quantities of platelet activating factor (AGEPC) by gas chromatography/negative ion chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* **13:** 107–111.
21. Satouchi, K., R. N. Pinckard, L. M. McManus, and D. J. Hanahan. 1981. Modification of the polar head group of acetyl glyceryl ether phosphorylcholine and its ethanolamine analog on biological activity towards rabbit platelets. *Arch. Biochem. Biophys.* **211:** 683–688.
22. Jackson, E. M., G. E. Mott, C. Hoppens, L. M. McManus, S. T. Weintraub, J. C. Ludwig, and R. N. Pinckard. 1984. High performance liquid chromatography of platelet-activating factor. *J. Lipid Res.* **25:** 753–757.
23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
24. Renkonen, O. 1965. Individual molecular species of different phospholipid classes. *J. Am. Oil. Chem. Soc.* **42:** 298–304.
25. Kumar, R., S. T. Weintraub, and D. J. Hanahan. 1983. Differential susceptibility of mono- and di-O-alkyl ether phosphoglycerides to acetolysis. *J. Lipid Res.* **24:** 930–937.
26. Bossant, M. J., R. Farinotti, J. M. Mencia-Huerta, and J. Benveniste. 1987. Characterization and quantification of PAF-acether (platelet-activating factor) as a heptafluorobutyrate derivative of 1-*O*-alkyl-2-acetyl-*sn*-glycerol by capillary column gas chromatography with electron-capture detection. *J. Chromatogr.* **423:** 23–31.
27. Waku, K., and Y. Nakazawa. 1972. Hydrolysis of 1-alkyl-, 1-*O*-alkenyl-, and 1-acyl-2[1-^{14}C]linoleoyl-glycero-3-phosphorylcholine by various phospholipases. *J. Biochem.* **72:** 149–155.