papers and notes on methodology

Semi-synthetic preparation of 1-O-[1′-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) using plant cell cultures[1]

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Abstract Incubation of photomixotrophic cell suspension cultures of rape (*Brassica napus*) and heterotrophic cell suspension cultures of soya (*Glycine max*) with 1-O-[1′-^{14}C]hexadecyl-*sn*-glycerol or *rac*-1-O-[1′-^{14}C]hexadecylglycerol leads in high yield (up to 78%) to labeled 1-O-hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines. Alkaline hydrolysis of the choline glycerophospholipids yields pure 1-O-[1′-^{14}C]hexadecyl-*sn*-glycero-3-phosphocholine. 1-O-[1′-^{14}C]Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) is obtained by acetylating the lyso compound. The semi-synthetic preparation described leads to labeled platelet activating factor in an overall yield of 50–60% without loss of specific activity. — **Weber, N., and H. K. Mangold.** Semi-synthetic preparation of 1-O-[1′-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) using plant cell cultures. *J. Lipid Res.* 1985. **26:** 495–500.

Supplementary key words platelet activating factor (PAF) • lyso PAF • labeled ether glycerolipids • stereoselective preparation

Platelet activating factor (PAF) is a natural ether glycerophospholipid identified as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1, 2). It is released by cells following both immunological and non-immunological stimuli. PAF is a bio-regulator with many activities including aggregation of blood platelets, release of arachidonic acid by cells, increase in the turnover of phosphatidylinositols, and inhibition of cyclic AMP synthesis. PAF also acts as a hypotensive agent and mediates allergic responses (3, 4). Radioactively labeled PAF should be useful for studying various biological activities. We report here a procedure for the semi-synthetic preparation of 1-O-[1′-^{14}C]alkyl-2-acetyl-*sn*-glycero-3-phosphocholines using plant cell cultures. This procedure is based on earlier findings on the metabolism of 1-O-alkylglycerols by plant cell suspension cultures (5–7).

MATERIALS AND METHODS

Chemicals

rac-Isopropylideneglycerol was purchased from EGA-Chemie, Steinheim, FRG; 2,3-isopropylidene-*sn*-glycerol was a product of Calbiochem, Frankfurt/M., FRG. Phospholipase A_2 from *Crotalus durissus* and phospholipase C from *Bacillus cereus* were purchased from Boehringer-Mannheim, Mannheim, FRG. PAF derived from alkyldiacylglycerols of ratfish (*Hydrolagus colliei*) liver oil was a gift from Dr. T. Muramatsu, Tokyo, Japan.

Radioactively labeled compounds

[1-^{14}C]Palmitic acid (2.00 GBq/mmol) was purchased from Amersham Buchler, Braunschweig, FRG. Methyl [1-^{14}C]palmitate was derived from the fatty acid by the reaction with diazomethane. [1-^{14}C]Hexadecanol (0.98 GBq/mmol) was prepared from methyl [1-^{14}C]palmitate by reduction with lithium aluminum hydride (8) and purified by TLC on Silica Gel H (E. Merck, Darmstadt, FRG) with hexane–diethyl ether 80:20 (v/v). 1-O-[1′-^{14}C]Hexadecyl-*sn*-glycerol (0.98 GBq/mmol) was prepared from [1-^{14}C]hexadecanol and 2,3-isopropylidene-*sn*-glycerol, *rac*-1-O-[1′-^{14}C]hexadecylglycerol (0.98 GBq/mmol) from [1-^{14}C]hexadecanol and *rac*-isopropylidene-

Abbreviations: PAF, platelet activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines); lyso PAF, lyso platelet activating factor (1-O-alkyl-*sn*-glycero-3-phosphocholines); CGP, choline glycerophospholipids (1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholines plus 1,2-diacyl-*sn*-glycero-3-phosphocholines); TLC, thin-layer chromatography.

[1]Dedicated to Professor Walter O. Lundberg, Minneapolis, Minnesota, on the occasion of his 75th birthday.

glycerol as described earlier (9, 10). Acidic hydrolysis of 1-O-hexadecyl-2,3-isopropylideneglycerols was carried out in 10% aqueous HCl–methanol 1:6 (v/v) at room temperature for 1 hr (11). The radiochemical purity of the labeled hexadecylglycerols was better than 98%.

Cell suspension cultures

Photomixotrophic cell suspension cultures of rape were propagated in modified MS-medium (12) containing 1×10^{-6}M 2,4-dichlorophenoxyacetic acid, 1×10^{-6}M 6-benzylaminopurine, and 1×10^{-6}M gibberellic acid. Heterotrophic cell suspension cultures of soya were propagated in B_5-medium (13, 14) containing 2×10^{-6}M 2,4-dichlorophenoxyacetic acid. The photomixotrophic rape cell cultures were shaken under continuous illumination at 25°C, whereas the heterotrophic soya cell cultures were shaken in the dark at 25°C. Both the rape and the soya cells were subcultured every 10–14 days. The dry weights of the cells were determined by collecting the cells on sintered glass funnels, washing them with distilled water, and drying at 110°C for 3 hr. The rape cells, 10 g, yielded 900 mg of dry material, whereas the same amount of soya cells yielded 700 mg of dry material.

Incubation of cell suspension cultures with radioactively labeled 1-O-hexadecylglycerols

Photomixotrophic rape cells and heterotrophic soya cells, 1 g of cells/2 ml of preconditioned medium, were preincubated for 1 hr under anaerobic conditions (argon atmosphere) and then added to 37 kBq (38 nmol) of 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol or *rac*-1-O-[1'-^{14}C]hexadecylglycerol in 0.02 ml of 80% aqueous ethanol–diethyl ether 1:2 (v/v). The cells were incubated at 25°C for 1 to 8 hr under anaerobic conditions. Aliquots of about 0.2 g of plant cells each were withdrawn after 1, 2, 4, 6, and 8 hr of incubation. The cells were collected by repeated centrifugation and washing with 0.1 M sodium-potassium phosphate buffer, pH 6.0. The pellets were suspended in 2 ml of isopropanol and heated in closed tubes at 100°C for 10 min. The cells were homogenized, and the lipids were extracted according to an established procedure (15). For preparative purposes, 10 g of rape cells was incubated with 370 kBq (380 nmol) of 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol for 8 hr under the conditions described above.

Determination of radioactivity

Solutions were mixed with 'Aquasol-2' (NEN-Chemicals, Dreieich, FRG) and radioactivity was determined by liquid scintillation counting in a Tri-Carb C 2425 Instrument (Packard Instruments Company, Downers Grove, IL). The distribution of radioactive fractions on thin-layer chromatograms was determined with a Berthold TLC-Scanner LB 2760 or with a Berthold Automatic TLC-Linear Analyzer LB 2832 in combination with a data acquisition system LB 500 (BF-Vertriebsgesellschaft, Wildbad, FRG). Radio gas–liquid chromatography was carried out in a Perkin-Elmer F 22 Instrument (Perkin-Elmer Bodenseewerk, Überlingen, FRG) equipped with thermal conductivity detectors. Radioactivity in the carrier gas effluent was monitored in a Packard Gas Proportional Counter Model 894 (Packard Instruments Company) combined with a Spectra Physics Minigrator System (Spectra Physics, Darmstadt, FRG).

Isolation and analysis of radioactively labeled lipids

The total lipids extracted from the incubation mixtures were fractionated by TLC on silica gel with chloroform–methanol–water 65:25:4 (v/v/v), and the distribution of radioactivity in the various fractions was determined by scanning. The fractions of neutral lipids (R_f 0.8–1.0) and CGP (R_f 0.25) were each isolated. The neutral lipids were resolved by TLC on silica gel with hexane–diethyl ether 70:30 (v/v) twice, and the radioactivity in the fractions of 1-O-hexadecyl-2-O-acylglycerols plus 1-O-hexadecyl-3-O-acylglycerols (R_f 0.18–0.30) and 1-O-hexadecyl-2,3-diacylglycerols (R_f 0.75) was determined with the scanner. The CGP (R_f 0.32) was purified by TLC with chloroform–methanol–ammonia 65:25:4 (v/v/v). After elution from the adsorbent with chloroform–methanol–water 1:2:0.8 (v/v/v), their purity was checked by two-dimensional TLC on silica gel with chloroform–methanol–water 65:25:4 (v/v/v) in the first direction and chloroform–methanol–ammonia 65:25:4 (v/v/v) in the second. Labeled fractions were detected with the Automatic TLC-Linear Analyzer System and evaluated by using a 'perspective TLC' program.

Determination of radioactivity in acyl moieties and hexadecylglycerol moieties. An aliquot of the CGP was reduced with lithium aluminum hydride. The resulting long-chain alcohols (R_f 0.68) and hexadecylglycerol (R_f 0.20) were resolved by TLC on silica gel with hexane–diethyl ether 20:80 (v/v) and the radioactivity in the two fractions was determined by scanning. Radioactivity in the fraction of long-chain alcohols, derived from fatty acids, was taken as a measure of oxidative cleavage of the substrate. The 1-O-[1'-^{14}C]hexadecylglycerol was isolated, acetylated, and the resulting hexadecyldiacetylglycerol (R_f 0.32) was purified by TLC on silica gel with hexane–diethyl ether 70:30 (v/v). After elution from the adsorbent with water-saturated diethyl ether, the 1-O-[1'-^{14}C]hexadecyl-2,3-diacetylglycerol (R_t 23.5 min) was analyzed by gas–liquid chromatography and radio gas–liquid chromatography on a glass column, 1.8m × 4mm, packed with 3% OV-101 on Gas Chrom Q, 100–120 mesh (Applied Science Laboratories, State College, PA) at 160 to 260°C, 4°C/min, with helium as carrier gas at a flow rate of 40 ml/min.

Determination of radioactivity in phosphocholine moieties. An aliquot of labeled CGP, isolated from rape cells or soya cells, was hydrolyzed using phospholipase C (16). The

radioactivity in the resulting products, diradylglycerols and phosphocholine, was determined after TLC on silica gel with hexane–diethyl ether 70:30 (v/v) twice and after TLC on the same plate with chloroform–methanol–water 65:25:4 (v/v/v) using the TLC-Linear Analyzer System.

Determination of chiral purity. About 50 μg of CGP from rape cells, containing 3.8 nmol (3.7 kBq) of 1-O-[1'-^{14}C]hexadecyl-2-acyl-glycerophosphocholines, was dispersed by sonication in 1 ml 0.2 M Tris-HCl (pH 7.5), containing 10 mM $CaCl_2$ and 0.5 mg of bovine serum albumin. Phospholipase A_2, 0.01 ml (10 μg), was added to the dispersion and incubated for 0.5 hr at 30°C (17). Lipids were extracted according to an established procedure (18), dried, and fractionated by TLC on silica gel with chloroform–methanol–ammonia 70:35:7 (v/v/v). The distribution of radioactivity in the various fractions was determined by scanning.

Isolation of radioactive lipids from preparative experiments

The total lipids extracted from 10 g of rape cells, incubated with 370 kBq of 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol, were fractionated by TLC and isolated as described above.

Preparation of lyso PAF and PAF

A part of labeled CGP, 150 kBq, isolated from rape cells was dissolved in chloroform–methanol 2:1 (v/v) and reacted with 0.33 N methanolic potassium hydroxyde solution (19). The acidified reaction mixture was extracted with chloroform and the lipid extract was fractionated into 1-O-[1'-^{14}C]hexadecyl-*sn*-glycero-3-phosphocholine (lyso PAF; 95% yield based on the content of labeled 1-O-hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines in CGP) (R_f 0.20) and fatty acids (R_f 0.95–1.0) by TLC on silica gel with chloroform–methanol–water 65:35:7 (v/v/v); the distribution of radioactivity in the various fractions was determined by scanning. Lyso PAF was isolated, acetylated with acetic anhydride in dichloromethane in the presence of 4-(dimethylamino)pyridine (20), and the resulting 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (95% yield) (R_f 0.22) was purified by TLC on silica gel using chloroform–methanol–water–acetic acid 70:35:5:5 (v/v/v).

Characterization of 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine

The purity of the final product was checked by co-chromatography with synthetic PAF (R_f 0.24) on silica gel using chloroform–methanol–ammonia 70:35:7 (v/v/v), and radioactivity was detected by scanning. An aliquot of 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine was reduced with lithium aluminum hydride and the resulting reaction mixture was resolved by TLC as described above. The radioactivity in the various fractions was determined by scanning. Labeled hexadecylglycerol (R_f 0.20) was identified by co-chromatography with a standard, isolated, and acetylated. The resulting hexadecyldiacetylglycerol (R_f 0.32) was purified by TLC as described above and identified by co-chromatography with a standard. Radioactive purity of the derivative was checked by scanning. After elution from the adsorbent with water-saturated diethyl ether, the labeled hexadecyldiacetylglycerol was analyzed by gas–liquid chromatography and radio gas–liquid chromatography as described above. For measurements of specific radioactivity, the mass of [1'-^{14}C]hexadecyldiacetylglycerol was determined by the thermal conductivity detector using *rac*-1-O-heptadecyldiacetylglycerol as internal standard. The chiral purity of 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine was determined by enzymatic hydrolysis of 5 nmol (5.1 kBq) of the final product using phospholipase A_2, containing 50 μg of semi-synthetic PAF derived from ratfish liver oil, as described above for CGP.

RESULTS AND DISCUSSION

Both photomixotrophic rape suspension cells and heterotrophic soya suspension cells incorporated 1-O-hexadecylglycerol at a fast rate. After 2 hr of incubation, only traces of radioactivity were detected in culture media of both cell suspension cultures. **Fig. 1, a and b** shows the incorporation of radioactivity from 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol into CGP of rape cells and soya cells as well as the decrease of radioactive substrate during incubation. It is evident that both rape cells and soya cells predominantly metabolized the substrate to 1-O-hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines. After 8 hr, about 66 to 78% of radioactivity of the substrate had been incorporated into CGP of both plant cell suspension cultures. Minor amounts of radioactivity (9–16%) had been incorporated into neutral ether glycerolipids, i.e., 1-O-hexadecyl-2-acyl-glycerols and 1-O-hexadecyl-3-acylglycerols as well as 1-O-hexadecyl-2,3-diacylglycerols, of rape and soya cells after 8 hr.

The incorporation of radioactivity from *rac*-1-O-[1'-^{14}C]hexadecylglycerol into CGP of rape cells during incubation is shown in Fig. 1c. It is striking that the metabolism of the racemic substrate was practically stopped after 2–4 hr of incubation. The radioactivity incorporated into acyl moieties of CGP increased from 4% after 4 hr to 7% after 8 hr (data not shown in Fig. 1). It is conceivable that a further slow increase in radioactivity of glycerolipids was only due to incorporation of labeled fatty acids formed by oxidative cleavage of the substrate. It is obvious from these results that the 'natural' *sn*-1 isomer was by far the preferred substrate. This result is consistent with earlier findings that 1-O-alkyl-*sn*-glycerols, but not their optical antipodes, were incorporated into CGP of rape suspension cells (6, 7).

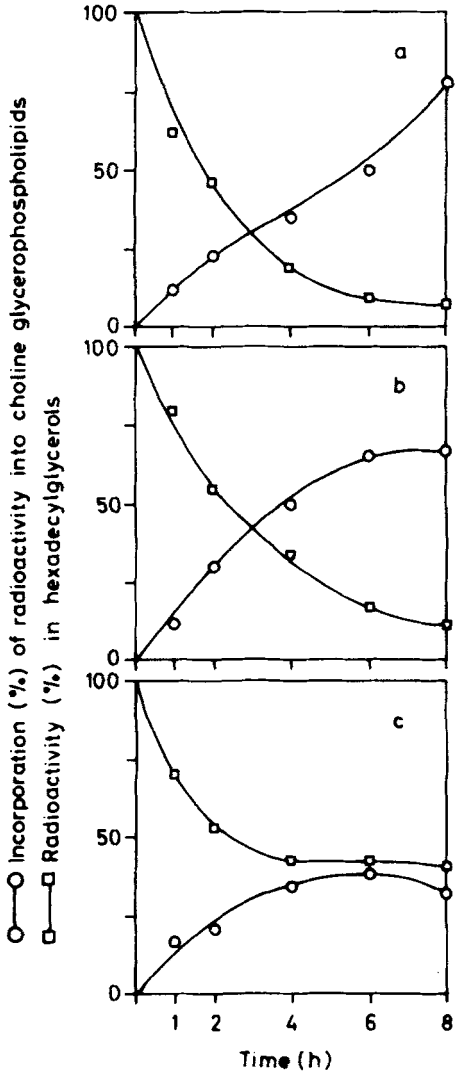


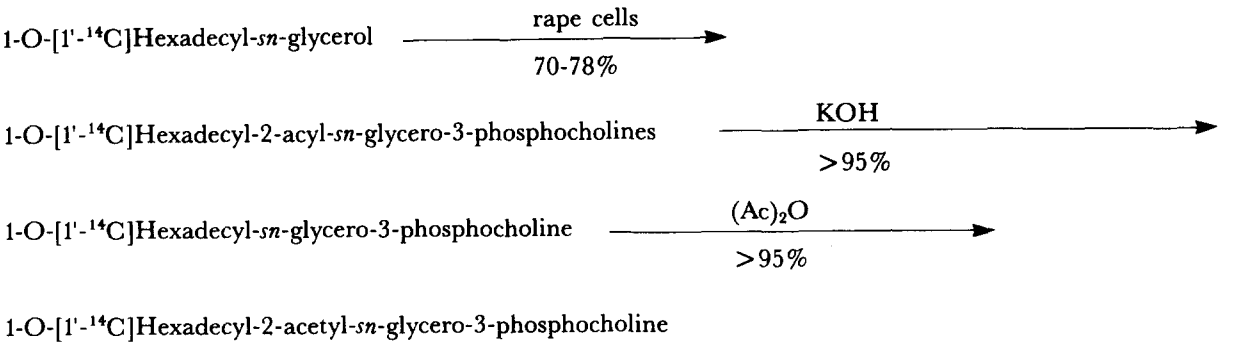
Fig. 1 Incorporation of radioactivity from 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol or *rac*-1-O-[1'-^{14}C]hexadecylglycerol into choline glycerophospholipids of rape (*Brassica napus*) cells and soya (*Glycine max*) cells in culture, as well as the decrease of radioactive substrate during incubation. a, Rape cells + 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol; b, soya cells + 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol; c, rape cells + *rac*-1-O-[1'-^{14}C]hexadecylglycerol.

The radioactivity incorporated into acyl moieties of CGP of rape cells and soya cells was investigated after incubation with labeled 1-O-hexadecylglycerols under anaerobic conditions. The lowest label in acyl moities (2% after 8 hr) was found in rape cells incubated with 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol. Higher proportions of labeled acyl moieties were detected in CGP of soya cells (9% after 8 hr), incubated with the same substrate, and CGP of rape cells incubated with the racemate (7% after 8 hr). These findings may be due to higher levels of lipoxygenase in soya cells compared to rape cells, and, in the case of racemic substrate, due to prolonged exposure of the unmetabolized *sn*-3 isomer to oxidizing enzyme systems.

Obviously, the best yield of 1-O-[1'-^{14}C]hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines was obtained by incubating rape cells with 1-O-[1'-^{14}C]hexadecyl-*sn*-glycerol. The substrate was incorporated into CGP up to 78% after 8 hr. The radiochemical purity of the fraction of CGP isolated was checked by *a*) reduction with $LiAlH_4$, *b*) enzymatic hydrolysis using phospholipase C, *c*) enzymatic hydrolysis using phospholipase A_2, and *d*) alkaline hydrolysis. After reduction of CGP with $LiAlH_4$, radioactivity was found almost exclusively in the hexadecylglycerol fraction and only trace amounts (up to 2% after 8 hr) in long-chain alcohols derived from acyl moieties. It is evident from these results that only small amounts of the substrate were metabolized by oxidative cleavage at the ether linkage. The fatty acids thus formed were predominantly incorporated into glycerophospholipids of both, rape and soya cells, as described earlier (21, 22). [1'-^{14}C]Hexadecylglycerol liberated by hydrogenolysis was acetylated. Chemical and radiochemical purity of the resulting [1'-^{14}C]hexadecyl-2,3-diacetylglycerol was determined by gas–liquid chromatography and radio gas–liquid chromatography. Alkaline hydrolysis of CGP leads to 1-O-[1'-^{14}C]hexadecyl-*sn*-glycero-3-phosphocholines and small amounts (about 2%) of radioactive fatty acids, which is consistent with the results obtained by reduction with $LiAlH_4$ described above.

No radioactivity resulting from oxidation of the substrate was detected in phosphocholine moieties of CGP liberated by enzymatic cleavage at the phosphodiester linkage with phospholipase C. The chiral purity of CGP and 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine was checked by enzymatic hydrolysis with phospholipase A_2 (17). It was found that acyl moieties at the *sn*-2 position of CGP of rape cells containing 1-O-[1'-^{14}C]hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines and acetyl moieties at the *sn*-2 position of the radioactive PAF were completely hydrolyzed by this enzyme, which indicates chiral purity of both substances. In addition, the labeled PAF was characterized by hydrogenolysis with $LiAlH_4$. The resulting radioactive 1-O-hexadecylglycerol was acetylated. Chemical and radiochemical purity of the resulting 1-O-[1'-^{14}C]hexadecyl-2,3-diacetylglycerol was determined by gas–liquid chromatography and radio gas–liquid chromatography. The ratio radioactivity/mass of hexadecyldiacetylglycerols derived from both starting material and final product was found to be unchanged. The radiochemical purity of the isolated PAF (specific activity 0.98 GBq/mmol) was better than 98%.

We have synthesized the physiologically most active PAF containing 1-O-[1'-^{14}C]hexadecyl moieties. The semisynthetic preparation shown in **Scheme 1** leads to remarkably higher yield (overall yield 65%) of 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholines than the total syntheses described recently (20, 23–26). Alkaline hydrolysis of CGP followed by acetylation of the resulting lyso PAF



Scheme 1. Schematic representation of the various steps involved in the semi-synthetic preparation of 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) using cell suspension cultures of rape.

leads to 1-O-[1'-^{14}C]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine in an overall yield of 50–60% (with respect to [1-^{14}C]palmitic acid used). The specific activity of the added precursor is not diminished during metabolism in rape cells because of the absence of ether glycerolipids in higher plants (27). The biological activity, defined as 50% of the maximum aggregation of rabbit platelets, was found to be 11.4 pg/ml (2.2×10^{-11} M) for 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine prepared by semi-synthesis. This value is considerably higher than that of synthetic preparations (28).

Much information is available on physiological activities of PAF (3, 4), yet relatively little is known about the enzymes catalyzing the biosynthesis and metabolism of this bio-regulator. Both radioactive PAF and lyso PAF containing 1-O-[1'-^{14}C]alkyl moieties should be useful in studying biosynthesis and metabolism of these biologically active ether glycerophospholipids. In addition, the lyso compound may serve as starting material for the synthesis of 1-O-[1'-^{14}C]alkyl-2-acyl-*sn*-glycero-3-phosphocholines and other ether glycerophospholipids containing defined radyl moieties in positions 1 and 2 of the glycerol backbone, which could be useful in studies relating to phospholipid exchange proteins and membrane phenomena. Furthermore, labeled lyso PAF may be of interest in studies related to food emulsification because of its surface activity. Commercially available preparations of radioactive PAF or lyso PAF usually contain ^{3}H-labeled alkyl chains or N-methyl-^{14}C groups. Preparations containing defined ^{14}C-labeled alkyl chains are not available at the present time.

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