Synthesis and biological properties of the fluorescent ether lipid precursor 1-O-[9′-(1″-pyrenyl)]nonyl-sn-glycerol

Hongying Zheng,[1],* Richard I. Duclos, Jr.,[2],* Conor C. Smith,[†] Harrison W. Farber,[†] and Raphael A. Zoeller[3],*

Department of Physiology and Biophysics* and Pulmonary Center,[†] Boston University School of Medicine, Boston, MA 02118-2526

Abstract The synthesis of an ω-pyrene-labeled 1-O-alkyl-sn-glycerol was performed using a chirospecific method starting from R-(−)-2,3-O-isopropylidene-sn-glycerol. The product, 1-O-[9′-(1″-pyrenyl)]nonyl-sn-glycerol (pAG), is a fluorescent ether lipid that has a pyrene moiety covalently attached at the alkyl chain terminus. pAG was taken into CHO-K1 cells and a plasmalogen-deficient variant of CHO-K1, NRel-4. This variant is defective in dihydroxyacetonephosphate acyltransferase, which catalyzes the first step in plasmenylethanolamine (PlsEtn) biosynthesis. pAG was incorporated primarily into ethanolamine and choline phospholipids as well as a neutral lipid fraction tentatively identified as alkyldiacylglycerol. NRel-4 accumulated more fluorescence in the phospholipid fraction than CHO-K1, specifically in the ethanolamine phospholipids. Analysis of the fluorescent lipids showed that 93% of the pAG was incorporated into glycerolipids with the ether bond intact. Although the addition of 20 μM 1-O-hexadecyl-sn-glycerol to the medium fully restored PlsEtn biosynthesis in NRel-4 cells, pAG only partially restored PlsEtn synthesis. Incubation of cells with pAG followed by irradiation with long-wavelength (>300 nm) ultraviolet light resulted in cytotoxicity. NRel-4 cells displayed an increased sensitivity to this treatment compared with CHO-K1 cells.■ This photodynamic cytotoxicity approach could be used to select for mutants that are defective in downstream steps in ether lipid biosynthesis.—Zheng, H., R. I. Duclos, Jr., C. C. Smith, H. W. Farber, and R. A. Zoeller. Synthesis and biological properties of the fluorescent ether lipid precursor 1-O-[9′-(1″-pyrenyl)]nonyl-sn-glycerol. *J. Lipid Res.* 2006. **47:** 633–642.

Supplementary key words pyrene • fluorescence • chemical synthesis • ether lipid • plasmalogen • alkylglycerol

Mammalian cell membranes contain phospholipids in which the sn-1 and sn-2 positions of the glycerol backbone are occupied by fatty acids attached through ester linkages. They can also contain significant quantities of ether-linked phospholipids, in which the sn-1 position is occupied by an ether- or vinyl ether-linked alkyl chain (1), the latter species being called plasmalogens. Plasmalogens are the most abundant of the ether phospholipids, making up 18% of the total phospholipid mass in humans (2). Plasmenylethanolamine (PlsEtn; 1-O-alk-1′-enyl-2-O-acyl-sn-glycero-3-phosphoethanolamine) and plasmenylcholine (PlsCho; 1-O-alk-1′-enyl-2-O-acyl-sn-glycero-3-phosphocholine) are the two major plasmalogen species found in mammalian cell membranes. In different tissues and cell types, the content of plasmalogens varies widely; however, in most tissues and cultured cells, PlsEtn is the dominant plasmalogen species (2).

A number of questions remain concerning ether lipids, including their topology, function, and biosynthesis. One of the major questions with respect to plasmalogens (and other ether lipids) is what determines their levels in the different cell types. **Figure 1** summarizes the steps involved in the synthesis of PlsEtn in animal cells. Control of a biosynthetic pathway often involves the earliest steps. However, previous studies have suggested that the control of plasmalogen biosynthesis is exerted in steps distal to the first three steps (3–5). There are questions concerning whether some of the later steps in the pathway, which are analogous to those used in the synthesis of nonether glycerolipids, are catalyzed by the same enzymes (1). Also, the exact mechanism by which PlsCho is synthesized is still unclear, although PlsEtn appears to be a precursor (6, 7).

Labeled precursors that can enter the biosynthetic pathway to produce labeled ether lipids would be valuable

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Fig. 1. The biosynthesis of plasmenylethanolamine (PlsEtn) in animal cells. Step 1, dihydroxyacetonephosphate acyltransferase (DHAPAT); step 2, alkyl-dihydroxyacetonephosphate (alkyl-DHAP) synthase (the fatty acid is replaced by a fatty alcohol); step 3, acyl/alkyl-DHAP reductase; step 4, lysophosphatidate acyltransferase; step 5, phosphatidate phosphohydrolase; step 6, CDP-ethanolamine:diacylglycerol ethanolamine-phosphotransferase; step 7, PlsEtn desaturase; step 8, alkylglycerol phosphotransferase. The exact pathway for plasmenylcholine has not been elucidated. CDP-Etn, CDP-ethanolamine.

tools. Fatty alcohols, which enter the pathway at the second step of ether lipid biosynthesis, have been used to label ether lipids, but they are also readily converted to fatty acids once in the cells. The fatty acids can then be incorporated into nonether lipids (3, 8). Alkylglycerols can enter the plasmalogen biosynthetic pathway after the third step in the pathway (Fig. 1). Alkylglycerols such as 1-O-hexadecyl-sn-glycerol (HG) can rapidly restore plasmalogen biosynthesis and plasmalogen levels in variant cell lines defective in the first three steps in their synthesis (9). Labeled alkylglycerols might be better candidates as precursors in that they should specifically label ether lipids, including plasmalogens, assuming there is not significant cleavage of the ether bond.

Pyrene-labeled lipids have been used in a number of ways to examine membrane dynamics, lipid movement, and lipid/protein interactions (10). Pyrene, although bulky, has the advantage of being hydrophobic and is readily incorporated into membranes. Labeling cells with pyrene-labeled lipids also avoids the difficulty associated with the use and disposal of radioactive compounds. We have successfully used a pyrene-labeled fatty alcohol [9-(1′-pyrenyl)-1-nonanol; P9OH] for the selection of mutants deficient in ether lipid biosynthesis through ultraviolet (UV) light-mediated cytotoxicity (9, 11, 12). However, these mutants have been defective in early steps in the pathway. A pyrene-labeled ether lipid precursor that enters the pathway distal to these first three steps could possibly be used to isolate mutants defective in later steps in the pathway. We performed the chemical synthesis of a pyrene-labeled alkylglycerol, 1-O-[9′-(1″-pyrenyl)]nonyl-sn-glycerol (pAG; **Fig. 2**) and examined its distribution into complex cellular lipids and the metabolic stability of the ether bond in an effort to assess its usefulness as a probe to investigate ether lipid biosynthesis and as a tool for mutant isolation.
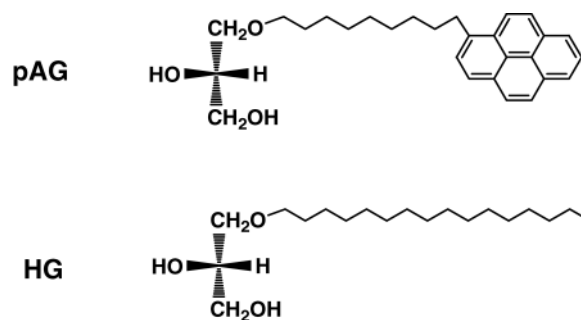
Fig. 2. Structural comparison of 1-O-hexadecyl-sn-glycerol (HG) and 1-O-[9′-(1″-pyrenyl)]nonyl-sn-glycerol (pAG).

MATERIALS AND METHODS

Materials

[1-^{3}H]ethanol-2-amine hydrochloride (27 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). [^{32}P]inorganic phosphate (^{32}P$_i$; 9,000 Ci/mmol) and EN^{3}HANCE spray were obtained from Perkin-Elmer/New England Nuclear (Boston, MA). Ham's F12 medium was purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Ecoscint A liquid scintillation fluid was obtained from National Diagnostics (Atlanta, GA). Scintillation vials, silica gel 60, and silica gel G TLC plates (Merck) were obtained from VWR Scientific (Boston, MA). Phosphatidylethanolamine (PtdEtn) standard (from bovine brain) and HG were purchased from Doosan/Serdary Chemicals (Englewood Cliffs, NJ). Total bovine heart lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl 1-pyrene-dodecanoate and P9OH were purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Chemical synthesis of pAG

General. The scheme for the synthesis of pAG is shown in **Fig. 3**. Reagents CH$_2$Cl$_2$ (from P$_2$O$_5$), triethylamine (TEA; from CaH$_2$), and toluene (from Na/benzophenone) were dried by distillation. Reactions were carried out under argon with magnetic stirring at the reported bath temperature (bT). Solvent removal used rotary evaporation under reduced pressure. All solvent ratios were by volume. Silica gel 60 (35–70 μm, irregular particle) was activated at 120°C for 12 h before use. Acid-sensitive compounds were often protected during silica gel chromatography by the use of TEA in the elution mixture. All reaction products were purified to homogeneity as judged by TLC on silica gel plates, which were visualized by the reported (13) charring method. Product solutions were filtered through 0.5 μm Teflon membranes. NMR used tetramethylsilane as an internal reference. Chemical shifts were often assigned with the assistance of ^{1}H-^{1}H correlation spectroscopy and ^{1}H-^{13}C heteronuclear multiple-quantum coherence spectroscopy. Electron-impact (70 eV) mass spectrometric analysis was used, and the molecular ion and major peaks have been reported.

9-(1′-Pyrenyl)nonyl methanesulfonate [2]. A magnetically stirred solution of 9-(1′-pyrenyl)-1-nonanol [1] (125 mg, 0.363 mmol, 100 mol%) and TEA (184 mg, 1.82 mmol, 500 mol%) in 5 ml of CH$_2$Cl$_2$ was protected from light and cooled to −20°C (bT; CCl$_4$/N$_2$). A solution of mesyl chloride (93.5 mg, 0.816 mmol, 225 mol%) in 1 ml of CH$_2$Cl$_2$ was added drop-wise over 2 min and was stirred for an additional 10 min, during which the reaction mixture became cloudy. The reaction mixture was then warmed to 0°C (bT) for an additional 45 min, during which the reaction mixture became nearly homogeneous. The disappearance of starting material [1] [relative mobility (R$_f$) = 0.26] and conversion to mesylate [2] (R$_f$ = 0.60) were demonstrated by TLC CH$_2$Cl$_2$/ethyl acetate/TEA (95:5:0.5). The reaction mixture was concentrated and filtered through a short column CH$_2$Cl$_2$/ethyl acetate/TEA (95:5:0.5). The crude product [2] was carefully re-chromatographed to give the homogeneous mesylate [2] (146 mg, 0.345 mmol, 95%), which was a crystalline white solid: TLC CH$_2$Cl$_2$/ethyl acetate/TEA (95:5:0.5) R$_f$ = 0.60; mp 81–82°C; ^{1}H NMR (CDCl$_3$): δ 2.96 (s, 3 H, Me), 4.19 (t, 2 H, J = 6.6 Hz, C1H$_2$), 1.71 (appar quint, 2 H, C2H$_2$), 1.25–1.40 (m, 8 H, C3H$_2$-C6H$_2$), 1.45–1.51 (m, 2 H, C7H$_2$), 1.86 (appar quint, 2 H, C8H$_2$), 3.34 (t, 2 H, J = 7.8 Hz, C9H$_2$), 7.87 (d, 1 H, J = 7.7 Hz, Ar), 7.99 (dd, 1 H, J = 7.5, 7.5 Hz, Ar), 8.01 (d, 1 H, J = 8.9 Hz, Ar), 8.04 (d, 1 H, J = 8.9 Hz, Ar), 8.10 (d, 1 H, J = 9.2 Hz, Ar), 8.11 (d, 1 H, J = 7.7 Hz, Ar), 8.15 (d, 1 H, J = 7.5 Hz, Ar), 8.17 (d, 1 H, J = 7.5 Hz, Ar), 8.28 (d, 1 H, J = 9.2 Hz, Ar); ^{13}C NMR (CDCl$_3$): δ 70.12 (C1), 28.91–29.63 (C2, C4–C7), 25.30 (C3), 31.81 (C8), 33.50 (C9); aromatics 123.43, 124.55, 124.71 (2 × C), 124.98, 125.00, 125.70, 126.41, 127.02, 127.17, 127.46, 128.52, 129.62, 130.86, 131.37, and 137.17.

1-O-[9′-(1″-Pyrenyl)]nonyl-2,3-O-isopropylidene-sn-glycerol [4]. A stirred mixture of KOH (21.3 mg, 0.380 mmol, 110 mol%) and
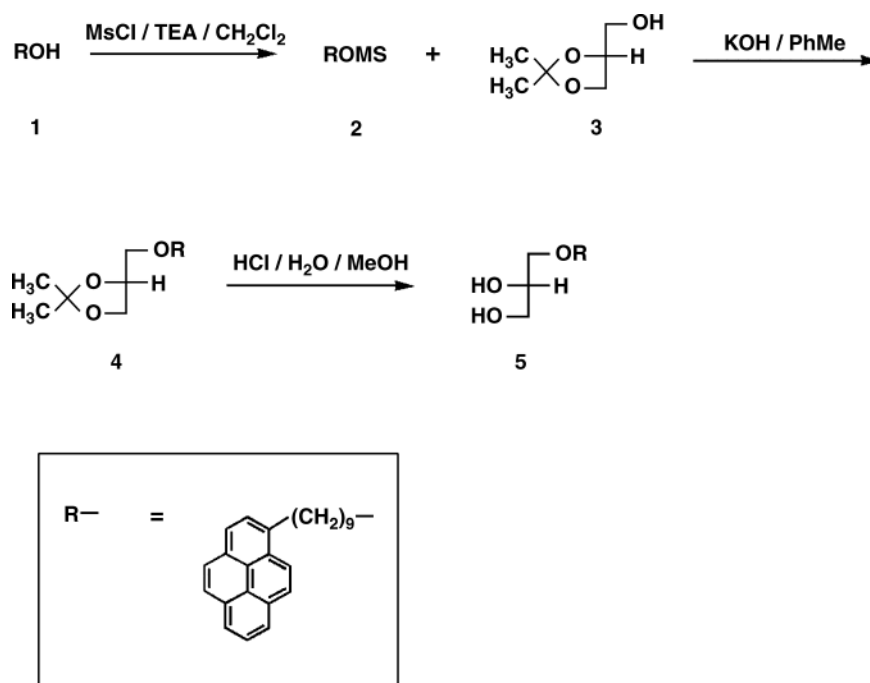

Fig. 3. Chemical synthesis of pAG. MeOH, methanol; TEA, triethylamine.

R-(−)-2,3-*O*-isopropylidene-*sn*-glycerol **[3]** (50.1 mg, 0.379 mmol, 110 mol%) in 40 ml of toluene was heated to reflux, and water was removed with a Dean Stark trap. After 15 min, the reaction mixture was cooled slightly and protected from light, and a solution of mesylate **[2]** (0.146 mg, 0.345 mmol, 100 mol%) in 5 ml of dry toluene was added rapidly drop-wise. The reaction mixture was maintained at a gentle reflux, and the conversion of mesylate **[2]** (R_f = 0.66) to product **[4]** (R_f = 0.52) was followed by TLC CH_2Cl_2/ethyl acetate/TEA (95:5:0.5). After 3 h, the reaction mixture was cooled to ambient temperature, concentrated, and partitioned between 20 ml of CH_2Cl_2 and 10 ml of saturated aqueous NaCl. The organic phase was dried over Na_2SO_4, concentrated, and filtered through a short column CH_2Cl_2/ethyl acetate/TEA (95:5:0.5). The crude product **[4]** was carefully rechromatographed to give the homogeneous 1-*O*-alkyl-*sn*-glycerol derivative **[4]** (141 mg, 0.307 mmol, 89%), which was a crystalline white solid: TLC CH_2Cl_2/EtOAc/TEA (95:5:0.5) R_f = 0.52; mp 56–57°C; ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 3 H, Me), 1.46 (s, 3 H, Me), 3.42 (dd, 1 H, *J* = 9.9, 5.7 Hz, C1Ha), 3.52 (dd 1 H, *J* = 9.9, 5.7 Hz, C1Hb), 4.28 (appar quint, 1 H, C2H), 3.75 (dd, 1 H, *J* = 8.1, 6.5 Hz, C3Ha), 4.07 (dd, 1 H, *J* = 8.1, 6.5 Hz, C3Hb), 3.43–3.49 (m, 2 H, C1′H$_2$), 1.56–1.62 (m, 2 H, C2′H$_2$), 1.32 (br s, 6 H, C3′H$_2$–C5′H$_2$), 1.35–1.42 (m, 2 H, C6′H$_2$), 1.45–1.52 (m, 2 H, C7′H$_2$), 1.84–1.90 (m, 2 H, C8′H$_2$), 3.34 (t, 2 H, *J* = 7.8 Hz, C9′H$_2$), 7.87 (d, 1 H, *J* = 7.7 Hz, Ar), 7.99 (dd, 1 H, *J* = 7.5, 7.5 Hz, Ar), 8.02 (d, 1 H, *J* = 8.9 Hz, Ar), 8.04 (d, 1 H, *J* = 8.9 Hz, Ar), 8.11 (d, 1 H, *J* = 9.2 Hz, Ar), 8.12 (d, 1 H, *J* = 7.7 Hz, Ar), 8.16 (d, 1 H, *J* = 7.5 Hz, Ar), 8.17 (d, 1 H, *J* = 7.5 Hz, Ar), 8.29 (d, 1 H, *J* = 9.2 Hz, Ar); ^{13}C NMR ($CDCl_3$): δ 26.74 (Me), 25.38 (Me), 109.28 (CMe$_2$), 71.75 and 71.78 (C1 and C1′), 74.70 (C2), 66.86 (C3), 29.39–29.73 (C2′, C4′–C7′), 25.94 (C3′), 31.87 (C8′), 33.54 (C9′); aromatics 123.44, 124.53, 124.69 (2 × C), 125.01 (2 × C), 125.67, 126.39, 127.00, 127.15, 127.46, 128.52, 129.61, 130.87, 131.38, and 137.23; mass spectrum *m/z* 458 (M$^+$), 215; [α]$_D^{24}$ −6.0° (c 3.0, CH_2Cl_2).

*p*AG **[5]**. To a stirred solution of the isopropylidene derivative **[4]** (105 mg, 0.229 mmol, 100 mol%) in 5 ml of warm methanol was added 0.15 ml of 12 N aqueous HCl, and the reaction mixture was warmed to 45°C (bT) for 1.5 h. The conversion of the isopropylidene derivative **[4]** (R_f = 0.74) to product pAG **[5]** (R_f = 0.13) was followed by TLC CH_2Cl_2/ethyl acetate (65:35). The reaction mixture was cooled to ambient temperature and partitioned between 30 ml of CH_2Cl_2 and 10 ml of water. The organic phase was washed with 10 ml of half-saturated aqueous $NaHCO_3$ and 10 ml of water, dried over Na_2SO_4, and concentrated. Chromatography CH_2Cl_2/ethyl acetate (65:35) gave homogeneous pAG **[5]** (92 mg, 0.22 mmol, 96%), which was crystallized from pure ethyl acetate to give short fine white needles: TLC (ethyl acetate) R_f = 0.30; mp 81–82°C; ^{1}H NMR ($CDCl_3$): δ 3.46 (dd, 1 H, *J* = 9.7, 3.6 Hz, C1Ha), 3.50 (dd 1 H, *J* = 9.7, 4.2 Hz, C1Hb), 3.84–3.88 (m, 1 H, C2H), 3.63 (dd, 1 H, *J* = 11.4, 5.4 Hz, C3Ha), 3.71 (dd, 1 H, *J* = 11.4, 3.7 Hz, C3Hb), 3.40–3.45 (m, 2 H, C1′H$_2$), 1.54–1.58 (m, 2 H, C2′H$_2$), 1.31 (br s, 6 H, C3′H$_2$–C5′H$_2$), 1.32–1.45 (m, 2 H, C6′H$_2$), 1.45–1.52 (m, 2 H, C7′H$_2$), 1.83–1.89 (m, 2 H, C8′H$_2$), 3.33 (t, 2 H, *J* = 7.8 Hz, C9′H$_2$), 7.86 (d, 1 H, *J* = 7.7 Hz, Ar), 7.99 (dd, 1 H, *J* = 7.5, 7.4 Hz, Ar), 8.01 (d, 1 H, *J* = 8.9 Hz, Ar), 8.03 (d, 1 H, *J* = 8.9 Hz, Ar), 8.09 (d, 1 H, *J* = 9.2 Hz, Ar), 8.10 (d, 1 H, *J* = 7.7 Hz, Ar), 8.15 (d, 1 H, *J* = 7.4 Hz, Ar), 8.16 (d, 1 H, *J* = 7.5 Hz, Ar), 8.28 (d, 1 H, *J* = 9.2 Hz, Ar); ^{13}C NMR ($CDCl_3$): δ 72.34 (C1), 70.45 (C2), 64.16 (C3), 71.72 (C1′), 29.36–29.71 (C2′, C4′–C7′), 25.97 (C3′), 31.84 (C8′), 33.52 (C9′); aromatics 123.43, 124.53, 124.69 (2 × C), 124.99 (2 × C), 125.67, 126.39, 127.00, 127.15, 127.46, 128.52, 129.60, 130.86, 131.37, and 137.21; mass spectrum *m/z* 418 (M$^+$), 215; [α]$_D^{24}$ +2° (c 3.0, CH_2Cl_2).

Cells and culture conditions

CHO-K1 (wild-type) cells were obtained from the American Type Culture Collection (Rockville, MD). NRel-4 is a plasmalogen-deficient CHO-K1 variant cell line that is defective in dihydroxyacetonephosphate acyltransferase (DHAPAT), the first enzyme in plasmalogen biosynthesis (9). "CHO cells" refers to NRel-4 and CHO-K1 cells. Both cell lines were routinely grown at 37°C in Ham's F12 nutrient mix supplemented with 10% fetal bovine serum, glutamine (1 mM), penicillin G (100 U/ml), and streptomycin (75 U/ml). This growth medium is designated F12c throughout the text.

Labeling cellular lipids using [1-^{3}H]ethanolamine and $^{32}P_i$

For labeling cellular lipids with [^{3}H]ethanolamine, cells (1.5×10^5) were plated into sterile glass scintillation vials in 1 ml of F12c medium. After attachment overnight at 37°C, medium was replaced with 1 ml of F12c medium containing [^{3}H]ethanolamine (2 μCi/ml) and different concentrations of HG or pAG. Cells were incubated at 37°C. After 3 h, medium was removed, the cells were washed once with 2 ml of PBS, and the lipids were extracted with 3.8 ml of chloroform/methanol/PBS (1:2:0.8) containing 200 μg of carrier lipid (bovine brain PtdEtn). After transferring the extract to test tubes, 1 ml of chloroform and 1 ml of PBS were added to form a two-phase Bligh and Dyer system (14), and the lower (organic) phase was collected after centrifugation at 600 *g* for 5 min. The aqueous phase was reextracted using 2 ml of chloroform, and the extracts were combined. Solvent was removed with a stream of nitrogen, and labeled lipids were resuspended in chloroform, spotted onto 20 cm × 20 cm silica gel 60 TLC plates, and developed to 10 cm using chloroform-methanol-acetic acid-water (25:15:3:1.5). This separated intact ethanolamine phospholipids from the small amount of lysoethanolamine phospholipids. The plates were then sprayed with 10 mM $HgCl_2$ in acetic acid to cleave the vinyl ether double bond associated with plasmalogens (15), and after 45 min of drying in a fume hood, the samples were redeveloped in the same solvent system to the top of the plate. This double development resulted in the separation of lysoPtdEtn, PlsEtn, and PtdEtn (16). The plates were sprayed with EN[3]HANCE (Perkin-Elmer/New England Nuclear) and exposed to X-ray film at −80°C to locate the labeled species. The labeled bands were scraped into scintillation vials and quantitated by liquid scintillation counting. It should be noted that PtdEtn and the saturated ether phospholipid, 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmanylethanolamine), could not be separated using this technique, and the band containing PtdEtn may contain significant levels of the plasmanyl phospholipid.

The labeling and extraction of cellular lipids with $^{32}P_i$ (20 μCi/ml) was performed similarly to the labeling with [^{3}H]ethanolamine, although the phospholipids were separated using two-dimensional TLC (17). Each sample was spotted onto silica gel 60 TLC plates and developed in chloroform-methanol-acetic acid-water (25:15:3:1.5). After drying, the plate was sprayed with 10 mM $HgCl_2$ in acetic acid as described above, and after 45 min of drying in a fume hood, the samples were developed in the second dimension using chloroform-methanol-formic acid (65:25:10). The radioactive phospholipids were located by exposure of the plate to X-ray film at −80°C, and the radioactivity was quantitated as described above.

Cell growth measurements

Cells were plated at low density (5×10^3 cells/well) on a 24-well plate. The next day, different concentrations of HG or pAG were added to each well, and the cells were allowed to grow

for 3 days at 37°C. Cell monolayers were washed with 1.5 ml of PBS, and cellular protein was determined. Protein was determined either by the Lowry method (18) after solubilization of cellular proteins in 0.5 N NaOH or by Coomassie staining; both methods yielded similar results. For Coomassie staining, PBS-washed monolayers were stained with 0.5 ml of 0.1% Coomassie blue in methanol-water-acetic acid (45:45:10) for 30 min at room temperature. The staining solution was removed, and cells were washed with 2 ml of destaining solution [methanol-water-acetic acid (45:45:10)] three times. After drying the plates, 1.0 ml of 0.5 N NaOH was added to each well, and the plates were incubated at 37°C for 1 h to solubilize the stain. The plates were placed in a tissue culture incubator adjusted to 5% CO_2 for 3 h for development of a stable blue color. Absorbance was read at 595 nm using a spectrophotometer. Care was taken to ensure that the cells had not reached confluence in the control (untreated) wells.

Uptake and distribution of pAG into cellular lipids

Cells (5×10^6) were plated into 100 mm diameter tissue culture dishes and allowed to attach overnight at 37°C. The cells were then incubated in F12c medium containing 10 μM pAG for 6 h at 37°C. Medium was removed, and cells were incubated for an additional 1 h in F12c without pAG to allow unmetabolized pAG to be removed from the cells. Cells were harvested with trypsin, pelleted by centrifugation at 600 g for 7 min, and resuspended in 0.8 ml of PBS, and the cell suspension was transferred to a test tube containing 1 ml of chloroform, 2 ml of methanol, and 200 μg of carrier lipids (total lipid from bovine heart). The lipids were extracted as described above. Individual lipid species were separated on 20 cm × 20 cm silica gel G TLC plates. The plates were first developed in chloroform-methanol-acetic acid-water (25:15:3:1.5) approximately half the distance of the plate (10 cm) to separate phospholipids. After drying, they were developed in *n*-hexane-diethyl ether-acetic acid (70:30:1) to the top to separate the neutral lipids. The bands with fluorescence were localized by irradiating the plate with UV light (Black-Ray UV lamp model XX-15L; UVP, Inc., San Gabriel, CA). The bands were scraped from the plate, and the lipids were eluted using chloroform-methanol (2:1). The solvent was evaporated under a stream of nitrogen, and the lipids were resuspended in chloroform and quantitated using a FluoroMax-2 spectrofluorometer (HORIBA Jobin Yvon, Inc., Edison, NJ) with an excitation wavelength of 341 nm and an emission wavelength of 378 nm. To separate PlsEtn from the PtdEtn form, the ethanolamine phospholipids that had been collected from the TLC plates were rechromatographed using treatment with $HgCl_2$ as described above. In this case, two fluorescent bands were observed. The first comigrated with PtdEtn and the second traveled with fatty aldehyde (a result of the cleavage of PlsEtn's vinyl ether double bond). The bands were recovered and quantitated as described above. As before, the PtdEtn band may contain significant amounts of the plasmanyl form.

Vitride reduction of cellular lipids

Cells (5×10^6) were labeled for 6 h with 10 μM pAG, and cellular lipids were extracted as described above. Extracted lipids were blown to dryness with a stream of nitrogen and dissolved in 0.75 ml of dry benzene-diethyl ether (1:4), and 0.15 ml of Vitride reagent [bis(2-methoxyethoxy)aluminum hydride] was added (19). The tubes were tightly capped, vortexed, and incubated at 37°C for 1 h. The excess Vitride was destroyed by drop-wise addition of 3 ml of ice-cold 20% ethanol, and the lipids were extracted three times using 3 ml of hexane. Samples were blown to dryness using nitrogen and resuspended in chloroform. The

lipids were spotted and separated on silica gel G TLC plates using hexane-ethyl ether-acetic acid (20:80:1). The pyrene-labeled fatty alcohol, P9OH, and the pyrene-labeled alkylglycerol, pAG, were used as standards to identify the fluorescent Vitride-generated products. The fluorescent bands were localized by brief UV light exposure and recovered from the plates using chloroform-methanol (2:1). Solvent was evaporated by nitrogen, the samples were resuspended in chloroform, and fluorescence was determined as described above.

Sensitivity to pAG/UV treatment

Cells (5×10^3/well) were plated on a 24-well plate. After attachment overnight at 37°C, the cells were incubated in F12c medium containing 2.5 μM pAG for 9 h at 37°C. The medium was removed, and the cells were incubated in F12c medium without pAG for an additional 1 h. The cells were then placed on a glass plate suspended over a UV source (same lamp as described above) and exposed from underneath to long-wavelength (>300 nm) UV light for different time intervals. A piece of aluminum foil was placed between the plate and the glass to block UV light for wells being exposed for less time. The intensity at the surface of the glass plate was 2,000 μW/cm^2 as measured using a Black-Ray long-wavelength UV light meter. Surviving cells were allowed to grow and fill in the wells for 3 days at 37°C before staining and quantitation of proteins as described above. Care was taken so that the untreated (no UV light exposure) wells did not reach confluence.

Statistical analysis

Each value in cell-related experiments represents the mean ± SD of triplicate samples. Similar results were obtained in multiple independent experiments. P values were calculated according to Student's two-tailed t-test. The differences were considered significant at $P < 0.05$.

RESULTS

pAG synthesis and characterization

The synthesis and chemical characterization of pAG was thoroughly described in Materials and Methods. Briefly, the 9-(1′-pyrenyl)nonyl methanesulfonate [2] was prepared in a 95% yield, according to a variation of known procedures (20–22) to prepare alkyl methanesulfonates. Commercial 2,3-*O*-isopropylidene-*sn*-glycerol [3] was available in nearly optically pure form and was used as the starting material to prepare the 1-*O*-alkyl-*sn*-glycerol derivatives by a variation of the previously reported methodology (20, 21, 23). The alkylation of [3] gave the 1-*O*-alkyl-2,3-*O*-isopropylidene-*sn*-glycerol derivative [4] in an 89% yield. The removal of the isopropylidene protecting group gave the desired fluorescently labeled 1-*O*-alkyl-*sn*-glycerol pAG [5] in a 96% yield. Structural analysis revealed that pAG is an analog of natural alkylglycerols such as HG, with a hydrocarbon chain of approximately the same length. Both have the ether linkage at the *sn*-1 position of glycerol.

Effects of pAG and HG on cell growth

The effects of both HG and pAG on cell growth were determined using CHO-K1 cells (**Fig. 4**). HG, when present in the medium at levels up to 15 μM, had a limited effect on cell growth, at 20 μM growth was reduced by
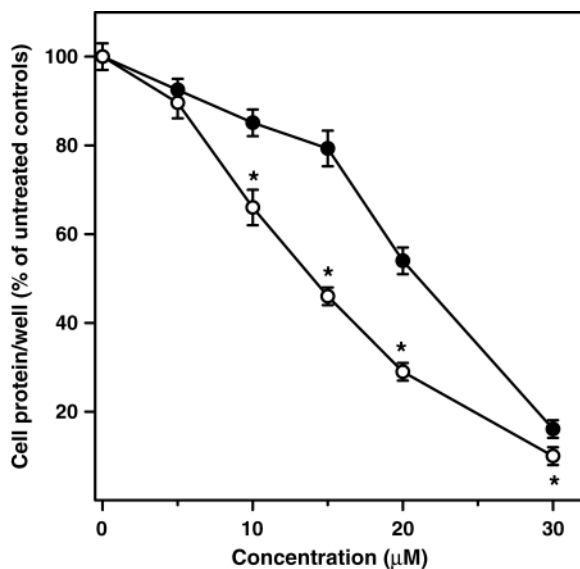
Fig. 4. Effects of HG and pAG on cell growth. CHO-K1 cells were plated onto 24-well dishes and allowed to grow for 3 days in the presence of various levels of either HG (closed circles) or pAG (open circles). Cellular protein in each well was then determined as described in Materials and Methods, and values are expressed as the amount present relative to untreated controls. All values represent means ± SD of three samples. Values for pAG-treated cells that were significantly different ($P < 0.05$) from those for HG-treated cells are identified by asterisks. Similar results were obtained using NRel-4 cells (not shown).

45%, and growth was severely reduced at higher concentrations (30 μM). There was no evidence of cytotoxicity at 30 μM HG over 72 h as judged by trypan blue (data not shown). pAG also inhibited cell growth, but lower concentrations were sufficient. At 10 μM, pAG inhibited cell growth by ∼30%, and 20 μM pAG resulted in 70% inhibition. There was no evidence of cytotoxicity within these concentrations over a period of 24 h, although there was some small amount of cytotoxicity evident within 72 h at 20 μM. At 30 μM pAG, there was severe growth inhibition, with evidence of significant cytotoxicity within 24 h and complete cytotoxicity within 72 h. NRel-4 cells displayed almost identical sensitivities to both HG and pAG (data not shown).
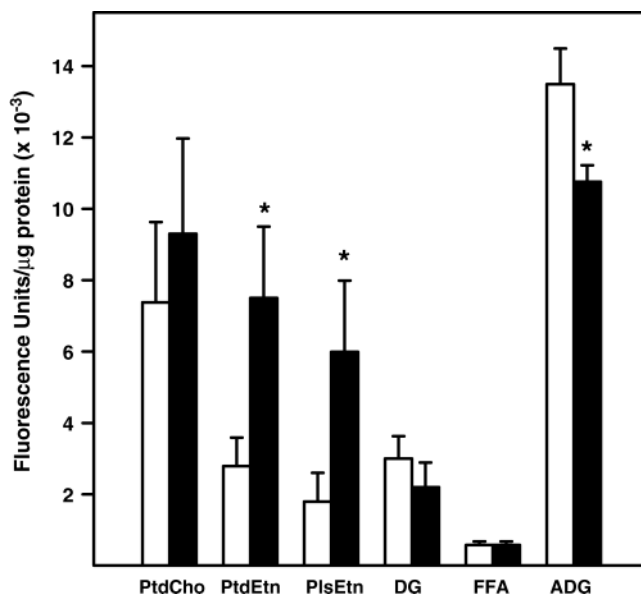
Distribution of pAG into cellular glycerolipids

The wild-type CHO-K1 and the plasmalogen-deficient variant strain, NRel-4, were labeled for 6 h using 10 μM pAG, and the distribution of the fluorescence among the various lipid classes was measured. In both cell lines, fluorescence was found primarily associated with the ethanolamine and choline phospholipids and with a neutral lipid fraction that migrated just below triglycerides on TLC ($R_f = 0.50$ vs. $R_f = 0.56$). We tentatively identified this neutral lipid species as alkyldiacylglycerol (ADG) (**Fig. 5**). Normally, ADGs travel slightly higher than triacylglycerols using this development system (24); however, the addition of a pyrene significantly alters the migration of neutral lipids. As an example, cholesteryl ester bearing a pyrene-labeled fatty acid (cholesteryl 1-pyrenedodecanoate) trav-

eled with a significantly lower R_f than cholesteryl oleate using this system ($R_f = 0.59$ vs. $R_f = 0.70$; data not shown). We would expect a similar pattern from pyrene-labeled ADG. Also, this putative ADG comigrated with a fluorescent lipid formed in CHO-K1 cells that were labeled with a pyrene-tagged fatty alcohol (P9OH). This compound was not present in similarly labeled, ether lipid-deficient NRel-4 cells (data not shown). There was also a small amount of fluorescence associated with diradylglycerols and free fatty acids.

There was a marked increase in fluorescence associated with the ethanolamine phospholipids in NRel-4. When the choline and ethanolamine phospholipids were treated with $HgCl_2$ to cleave the vinyl ether bond of the plasmalogen form (15), only the ethanolamine phospholipids displayed the generation of fluorescent fatty aldehyde (the product of $HgCl_2$-mediated cleavage). The fact that pyrene label was not detected in PlsCho is consistent with the fact that CHO cells contain very little of this phospholipid (9).

Fluorescence from pAG remains associated with ether lipids

The majority of the fluorescence associated with the cellular lipids was not in plasmalogens. This could result from the incorporation of intact pAG into ether lipids that remain in either the alkylacyl or plasmanyl form. Alter-


Fig. 5. Distribution of pAG into complex cellular lipids. Cells were labeled using 10 μM pAG for 6 h at 37°C followed by a 1 h incubation in unlabeled medium to remove unmetabolized pAG. Labeled lipids were extracted and separated, and the fluorescence was quantitated as described in Materials and Methods. CHO-K1 (open bars), NRel-4 (closed bars). All values represent averages ± SD of three samples. NRel-4 values that were significantly different ($P < 0.05$) from those obtained from CHO-K1 are identified by asterisks. ADG, alkyldiacylglycerol; DG, diradylglycerol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine. The phosphatidyl (diacyl) and the plasmanyl (alkylacyl) forms of the ethanolamine and choline phospholipids could not be separated using this analysis.

natively, the ether bond could be cleaved by monooxygenases, resulting in pyrene-labeled fatty aldehyde (25). This labeled fatty aldehyde could then be converted to fatty acid (8) and incorporated into pyrene-labeled nonether lipids through acyl linkages. To determine how much of the pAG that enters cells remains associated with an ether or vinyl ether bond, we treated the lipids of pAG-labeled cells with Vitride reagent. This cleaves all ester-linked fatty acids, resulting in the generation of fatty alcohols. This also cleaves the phosphodiester bonds but leaves ether and vinyl ether linkages intact (19). Total lipids extracted from pAG-labeled cells were reduced by Vitride reagent and the products separated by TLC. If the pyrene is still associated with the *sn*-1 ether linkage, Vitride cleavage should result in pyrene-labeled alkylglycerol or alkenylglycerol. If the pyrene is associated with ester-linked fatty acids, Vitride reduction should result in the generation of a pyrene-labeled fatty alcohol. Two fluorescent species were generated from Vitride reduction. More than 90% of the fluorescence comigrated with standard pAG (alkylglycerol and alkenylglycerol could not be separated using the TLC system), whereas <10% appeared as pyrene-labeled fatty alcohol (**Fig. 6**).

Effects of pAG and HG on phospholipid biosynthesis

NRel-4 is a plasmalogen-deficient variant of the CHO-K1 cell line with a deficiency in DHAPAT activity, which catalyzes the first step in PlsEtn biosynthesis (Fig. 1). Addition of HG to the medium bypasses the lesion and restores plasmalogen biosynthesis to this cell line (9). We measured the ability of both HG and pAG to influence the biosynthesis of ethanolamine phospholipids using [³H]ethanolamine
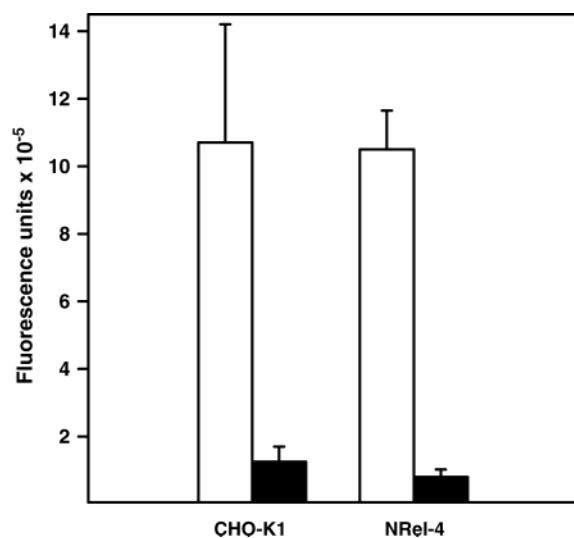

Fig. 6. Distribution of Vitride reduction products from pAG-labeled cellular lipids. Cells were labeled for 6 h with pAG as described for Fig. 5. The extracted lipids were reduced using Vitride reagent, and the products were extracted. The products, alkylglycerol (open bars) and fatty alcohol (closed bars), were separated on TLC, and fluorescence was quantitated as described in Materials and Methods. The values for CHO-K1 and NRel-4 were obtained in separate experiments. All values represent averages ± SD of three samples.

labeling (**Fig. 7**). Both compounds caused the reduction of PtdEtn labeling, inhibiting PtdEtn biosynthesis to similar degrees at both 10 and 20 μM. However, HG was very effective at stimulating PlsEtn biosynthesis, whereas the pyrene-labeled analog, pAG, was less effective, stimulating labeling of this phospholipid species by only 2.5-fold at 10 μM; at 20 μM pAG, this stimulation was not increased.

The cellular phospholipids were also labeled using ³²P$_i$ to assess the effects of these compounds on the synthesis of other phospholipids, including the ethanolamine phospholipids (**Fig. 8**). As with ethanolamine labeling, both compounds caused a reduction in PtdEtn labeling and an increase in PlsEtn labeling, with pAG being less effective in the latter. Phosphatidylcholine and sphingomyelin labeling was reduced slightly, although less so by pAG. Phosphatidylinositol labeling, unaffected by HG, was increased slightly in the presence of pAG.

pAG treatment results in sensitivity of cells to long-wavelength UV irradiation

A cell that takes up pyrene-containing compounds becomes sensitive to long-wavelength UV (>300 nm) irra-
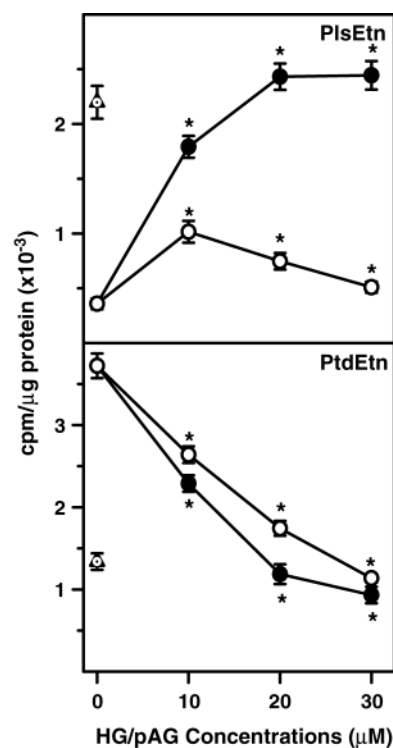
Fig. 7. Effects of HG and pAG on ethanolamine phospholipid biosynthesis. NRel-4 cells were labeled for 3 h at 37°C using [³H]ethanolamine in the presence of different concentrations of HG (closed circles) or pAG (open circles). Triangles represent values obtained from unsupplemented CHO-K1 cells. Cellular lipids were extracted and separated, and the radioactivity was quantitated as described in Materials and Methods. All values represent averages ± SD of three samples. Values that were significantly different from those of the untreated (no HG or pAG) NRel-4 controls are identified by asterisks. The phosphatidyl (diacyl) and the plasmanyl (alkylacyl) forms of the ethanolamine phospholipids could not be separated using this analysis.
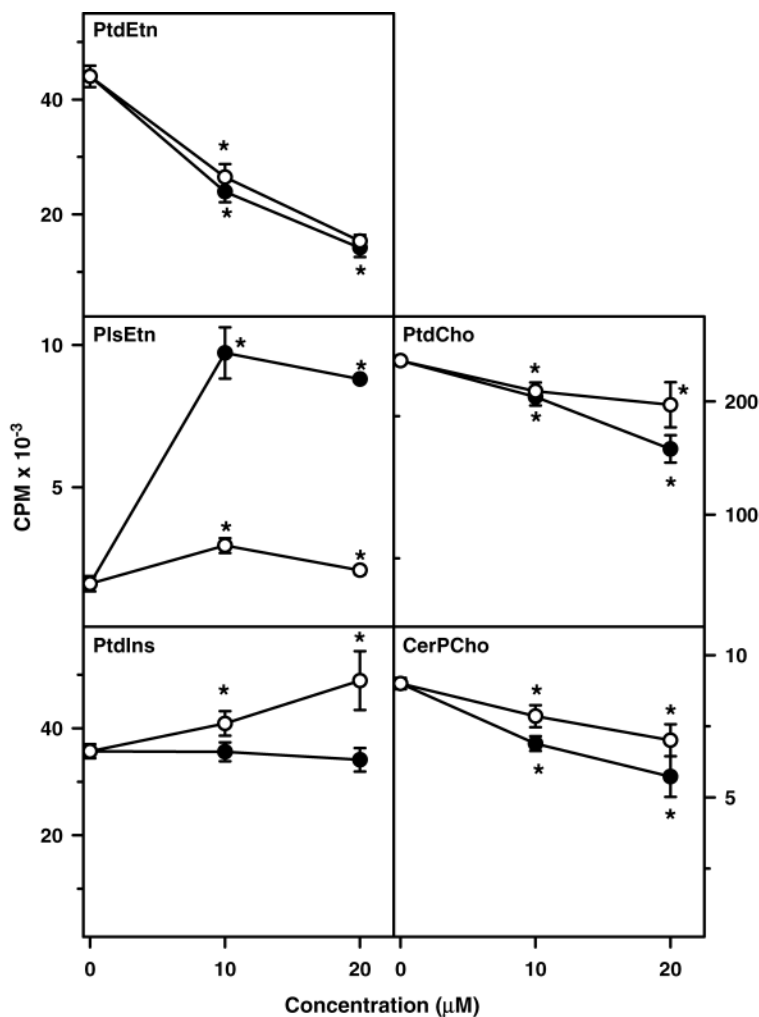
Fig. 8. Effects of HG and pAG on the biosynthesis of various phospholipid head group classes. NRel-4 cells were labeled for 3 h at 37°C using [^{32}P]inorganic phosphate and different concentrations of HG (closed circles) or pAG (open circles). Cellular lipids were extracted and separated, and the radioactivity was quantitated as described in Materials and Methods. All values represent averages ± SD of three samples. Values that were significantly different from those of the untreated controls are identified by asterisks. CerPCho, sphingomyelin; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine. The phosphatidyl (diacyl) and the plasmanyl (alkylacyl) forms of the ethanolamine and choline phospholipids could not be separated using this analysis.

diation. This is attributable to the generation of radical oxygen species after the excitation of pyrene within the cells. The toxicity is directly related to the amount of pyrene in the cells as well as to the duration and intensity of the UV light exposure. Therefore, cells that accumulate more pAG in membrane lipids should be more sensitive to long-wavelength UV irradiation. This was found to be the case, in that NRel-4 cells incorporated significantly more pAG into phospholipids than CHO-K1 cells (Fig. 5) and were more sensitive to UV irradiation (**Fig. 9**).

DISCUSSION

We have described the synthesis of a fluorescent alkylglycerol, pAG, and compared its biochemical properties with those of a natural ether lipid precursor, HG. This

fluorescent analog demonstrated properties that make it useful for studies concerning ether lipids. First, an important requirement for using pAG as a labeling probe is that it must be incorporated, with the ether bond intact, into complex lipids. One of our concerns was that cleavage of the ether bond by a monooxygenase and rapid turnover of the fluorescent ether lipids would result in significant labeling of nonether lipids. In vitro, HG is a substrate for O-alkylglycerol monooxygenase (25). Our data showed that after 6 h only 7% of the fluorescence from cell-associated pAG was recovered as fluorescent fatty alcohol (the predicted product of an esterified fatty acid) after Vitride reduction of the lipids from pAG-labeled cells. The remainder of the fluorescence was recovered as either alkylglycerol or alkenylglycerol. Therefore, over a significant period of time (6 h), the fluorescence is ether lipid-specific. This is consistent with the report that a number of
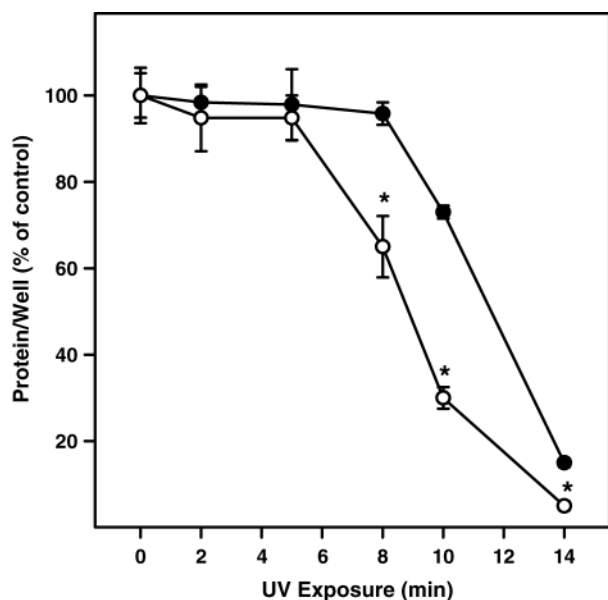
Fig. 9. Killing of CHO-K1 and NRel-4 cells using pAG and ultraviolet (UV) irradiation. CHO-K1 (closed circles) and NRel-4 (open circles) cells were plated on a 24-well plate at 5×10^3/well and incubated in F12c medium containing 2.5 μM pAG for 9 h at 37°C followed by a 1 h incubation in F12c medium without pAG. The cells were then irradiated by a long-wavelength UV light source from underneath for different time intervals as described in Materials and Methods. Surviving cells were allowed to grow and fill in the wells for 3 days at 37°C before staining and quantitation of cellular protein per well as described in Materials and Methods. Values are expressed as the amount of protein present relative to nonirradiated controls. All values represent averages ± SD of three samples. Values from NRel-4 cells that were significantly different from those of CHO-K1 cells ($P < 0.05$) are identified by asterisks.

transplantable tumor cell lines have very low levels of the monooxygenase (26).

Both pAG and HG inhibited cell growth. The pyrene analog had a small but significant effect on cell growth even at 5 μM. The mechanism behind the growth inhibition by pAG as well as for HG is not clear. Previous studies have linked growth quiescence to increases in cellular alkylglycerol levels (27). This may be linked to the inhibition of protein kinase C (28). Also, alkylglycerol treatment resulted in decreased cancer cell reproduction and a reduced ability of the cancer cells to metastasize (29). It is not clear whether HG or a metabolite (acylated or phosphorylated) is responsible for these effects. The inhibition of cell growth by pAG is not likely to be related to the decrease in PtdEtn biosynthesis. HG, which also inhibits cell growth, does not severely inhibit total ethanolamine phospholipid biosynthesis; the decrease in PtdEtn synthesis is compensated for by an increase in PlsEtn synthesis. Regardless of the mechanism, our data show that studies using pAG should use levels ≤15 μM.

PlsEtn is the only significant plasmalogen species found in CHO-K1 cells, and a significant portion of the pyrene label was found associated with this lipid. Twice as much fluorescence was found associated with this phospholipid in the NRel-4 cells, demonstrating that pAG could be suc-

cessfully converted into plasmalogens. However, pAG was not as effective as HG at restoring PlsEtn biosynthesis in the plasmalogen-deficient cell line NRel-4. Although the addition of 20 μM HG was capable of restoring PlsEtn biosynthesis to wild-type rates, pAG was only capable of restoring this rate to 40% of the wild-type rate at 10 μM. Concentrations of 20 μM and greater were no more effective. It is possible that the bulky pyrene group interferes with the Δ1′-desaturase, which catalyzes the final step in PlsEtn biosynthesis, inserting the vinyl ether double bonds into the alkyl chain (Fig. 1). Pyrene-labeled alkylglycerols with different alkyl chain lengths may result in more effective substrates for the complete conversion to PlsEtn. Although the majority of the label was found associated with lipid classes other than PlsEtn, these were primarily ether-linked lipids (1-*O*-alkyl-2-acyl-*sn*-glycerolipids), because Vitride reduction demonstrated that the ether bond remained intact.

Cells treated with pAG became sensitive to UV light exposure as a result of the generation of reactive oxygen species such as singlet oxygen (30). The NRel-4 cell line was more sensitive to UV light killing than the parent strain under the same conditions. This was likely attributable to the fact that NRel-4 cells accumulated more fluorescence in the phospholipid pools and to the fact that plasmalogen levels are reduced in this cell line; plasmalogens have been shown to have antioxidant capabilities (31–33).

We previously used a combination of pyrene-labeled plasmalogen precursor, the pyrene-labeled fatty alcohol P9OH, and UV light exposure to isolate variant cell lines, including NRel-4, that are deficient in ether lipid biosynthesis (9, 11, 12). The fatty alcohol, which enters the biosynthetic pathway in the second step, catalyzed by alkyldihydroxyacetonephosphate synthase (Fig. 1), was incorporated into pyrene-labeled ether lipids. Cells that did not synthesize ether lipids incorporated less pyrene-labeled fatty alcohol and were less sensitive to UV irradiation. The variant cell lines that have emerged from this protocol have, with only one exception (11), been defective in one of the first three steps in the biosynthetic pathway. The fact that pAG would be incorporated into ether lipids downstream of the first three steps should allow for the selection of ether lipid-deficient cell lines that are defective in downstream steps in the biosynthetic pathway. In a manner similar to that used to isolate the mutants mentioned above (9, 11, 12), a population of cells could be repeatedly subjected to pAG followed by UV light exposure under conditions that resulted in ≥90% cell death. This should result in a population of cells less capable of taking up or using pAG for ether lipid biosynthesis. Clonal cell lines generated from these surviving populations would be useful in answering questions concerning ether lipid biosynthesis and function. For instance, the pathway for the assimilation of HG into ether lipids is not certain. Although it is typically assumed that phosphorylation of HG is the first step in this process, there are few data to support this. Variants unable to catalyze the first step would be revealing. They may also

shed light on the mechanism behind the biological activities of alkylglycerols.

In summary, we have described the chemical synthesis of a fluorescent alkylglycerol analog that will be useful in studying ether lipid biosynthesis and alkylglycerol metabolism. This compound may also be a useful tool in the isolation of novel mutants in downstream steps of ether lipid biosynthesis.▓

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