Pharmaceutical Research, Vol. 6, No. 3, 1989

Research Article

Synthesis and Biological Evaluation of Ether-Linked Derivatives of Phosphatidylinositol

Khalid S. Ishaq,[1,2] Maria Capobianco,[1] Claude Piantadosi,[1] Alessandro Noseda,[3,4] Larry W. Daniel,[3] and Edward J. Modest[3]

Received April 21, 1988; accepted October 17, 1988

The synthesis of two novel glycero-3-phosphoinositol ether lipid analogues, *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phospho-myo-inositol **6** (an ether lipid analogue of *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine; ET-18-OMe) and *rac*-1-*O*-octadecyl-2-*O*-acetylglycero-3-phospho-myo-inositol **11** (an ether lipid analogue of platelet-activating factor), is described. The two target compounds and the synthetic intermediates were evaluated for inhibition of HL60, BG1, and BG3 human malignant cells *in vitro* and inhibition of protein kinase C. Tumor inhibitory activity was found for compounds **6** and **11** in all systems but not for their synthetic intermediates. However, compounds **6** and **11** as well as the synthetic intermediates **5** and **13**, but not **9**, exhibited protein kinase C inhibitory activity.

KEY WORDS: synthesis; inositol phospholipids; malignant cells; ether lipid analogue; protein kinase C; enzyme inhibitors.

INTRODUCTION

Ether lipid analogues of platelet-activating factor (PAF; 1-*O*-octadecyl-2-*O*-acetylglycero-3-phosphocholine) (1) have recently attracted considerable attention since they are potent inhibitors with a broad spectrum of biological properties including antitumor activity (2–4). The range of biological and pharmacological effects includes neutrophil activation (5), macrophage activation (6), protein kinase C (PKC) inhibition (7–9), alteration of phospholipid metabolism (8–11), malignant-cell differentiation (12), membrane interactions (13–17), and cytotoxicity and direct tumor-cell destruction (2,3,18). The most often studied compound of this type is *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine (ET-18-OMe), first reported by Munder *et al.* (2,3).

The ability of these analogues to inhibit neoplastic cell growth both *in vitro* and *in vivo* is of particular interest (2–4,18), and compounds of this class appear to be promising antineoplastic agents. The biochemical and biological properties enumerated could account for the antineoplastic activity, but further research on the mechanism of antitumor action is required.

In a collaborative program, we have designed, synthesized, and evaluated novel alkyl ether phospholipid analogues (4,19–21) as improved antineoplastic agents, with the emphasis on glycero-3-phosphocholine analogues. We have now extended this research program on the structure–activity relationships of ether lipid analogues to include glycero-3-phosphoinositol ether lipids, in order to examine their biochemical and biological properties and their potential as antitumor agents.

Considerable emphasis has recently been devoted to the possible regulatory role of the breakdown products of phosphoinositol 4,5-biphosphate, diacylglycerols, and inositol 1,4,5-triphosphate, through stimulation of protein kinase C and regulation of intracellular calcium (22,23). We believe that effective inhibitors of transmembrane signaling via the phosphatidyl inositol (PI) cascade may be important pharmacological agents in the regulation of neoplastic growth, as expressed in an earlier publication (4).

Accordingly, we have synthesized two unique compounds, *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phospho-myo-inositol **6** and *rac*-1-*O*-octadecyl-2-*O*-acetylglycero-3-phospho-myo-inositol **11** (Fig. 1), the latter being the PI-containing counterpart of PAF. This is the first report that describes the synthetic approaches utilized for the preparation of these compounds.

The PI analogues and their intermediates were evaluated *in vitro* against the HL60 human leukemia and the BG1 and BG3 human ovarian adenocarcinoma cell lines, using a cytotoxicity test (trypan blue dye exclusion) and an antiproliferative assay (semisoft agarose clonogenic assay), respectively. Preliminary data concerning the antineoplastic activity of the two target compounds have been reported (21). In this communication we report more extensive studies on the

[1] School of Pharmacy, Division of Medicinal Chemistry and Natural Products, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599.

[2] To whom correspondence should be addressed.

[3] Department of Biochemistry and Cancer Center, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103.

[4] Present address: "Mario Negri" Institute for Pharmacological Research, via Eritrea 62, 20157 Milan, Italy.

0724-8741/89/0300-0216$06.00/0

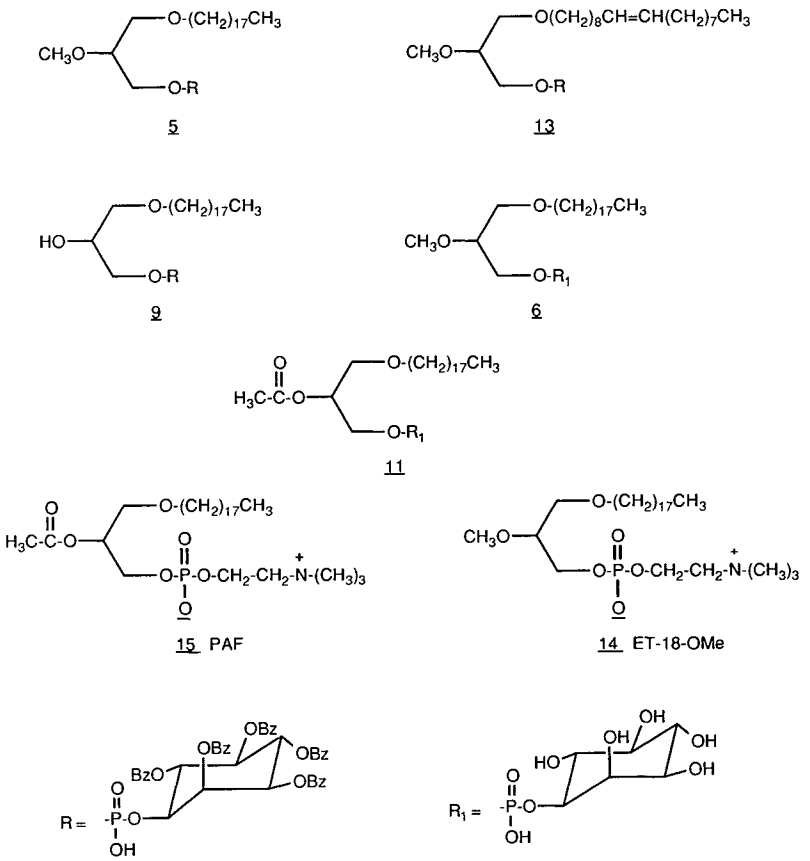


Fig. 1. Structural formulas of analogues of inositol phospholipids.

inhibition of human malignant cells and the activity of the target analogues 6 and 11 against HL60-derived protein kinase C (PKC).

MATERIALS AND METHODS

Materials

Melting points were determined on a Thomas–Hoover capillary apparatus and are uncorrected. Routine proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL-JNM-Fx60 spectrometer (59.75 MHz). Proton NMR spectra of the target compounds were obtained from a Bruker Wm-250 spectrospin spectrophotometer (250.137 MHz). Chemical shifts are reported as δ (ppm) units relative to the internal reference tetramethylsilane. Thin-layer chromatography (TLC) plates (1 × 3 in.) precoated with silica gel MK6F (layer thickness, 200 μm) from Whatman were used. Compounds were visualized with iodine vapors, 15% sulfuric acid, or short-wave UV light (Chromato-Vue Model CC-20). The preparative thin-layer chromatography plates used in the purification of the final products were obtained from Analtech Uniplates. These were precoated with silica gel GF (20 × 20 cm; layer thickness, 1000 μm). Column chromatography was performed on silica gel 60 (70- to 230- and 230- to 400-mesh ASTM) from E. M. Science. Dimethyl sulfoxide, tetrahydrofuran, and methylene chloride were dried over molecular sieves. Benzene and toluene were dried over sodium metal. Pyridine was dried and stored over potassium hydroxide pellets. Ethanol-free chloroform was prepared by shaking with concentrated sulfuric acid, washing several times with water, drying over calcium chloride, and finally, distilling. Triisopropylbenzenesulfonyl chloride (TPS) was recrystallized from *n*-pentane before use. Reagent-grade solvents and reactants were used as obtained from suppliers unless stated otherwise. Mass spectra (FAB-MS) were obtained on VG7070EQ.

Chemistry

The myo-inositol phospholipid analogues shown in Fig. 1 were synthesized according to Figs. 2–5.

The problem of protecting the 2,3,4,5,6-hydroxy groups of myo-inositol was resolved by following the procedure described by Gigg and Warren (24) for the synthesis of *rac*-2,3,4,5,6-penta-*O*-benzyl-myo-inositol-1-phosphate, compound 1. This procedure involves the preparation of 1,2-*O*-isopropylidene-myo-inositol followed by benzylation to protect the remaining hydroxyl groups of myo-inositol. Acid hydrolysis of 3,4,5,6-tetra-*O*-benzyl-1,2-*O*-isopropyl idene-myo-inositol gave the desired 3,4,5,6-tetra-*O*-benzyl-myo-inositol. Treatment of the latter with allyl bromide gave predominantly the monoallyl ether due to preferential allylation of the equatorial hydroxyl group at the 1 position. Purification and benzylation of the resultant compound produced the 1-*O*-allyl-2,3,4,5,6-penta-*O*-benzyl-myo-inositol, which was in turn isomerized to the 1-prop-1-enyl ether derivative to ensure the facile removal of this protecting group under

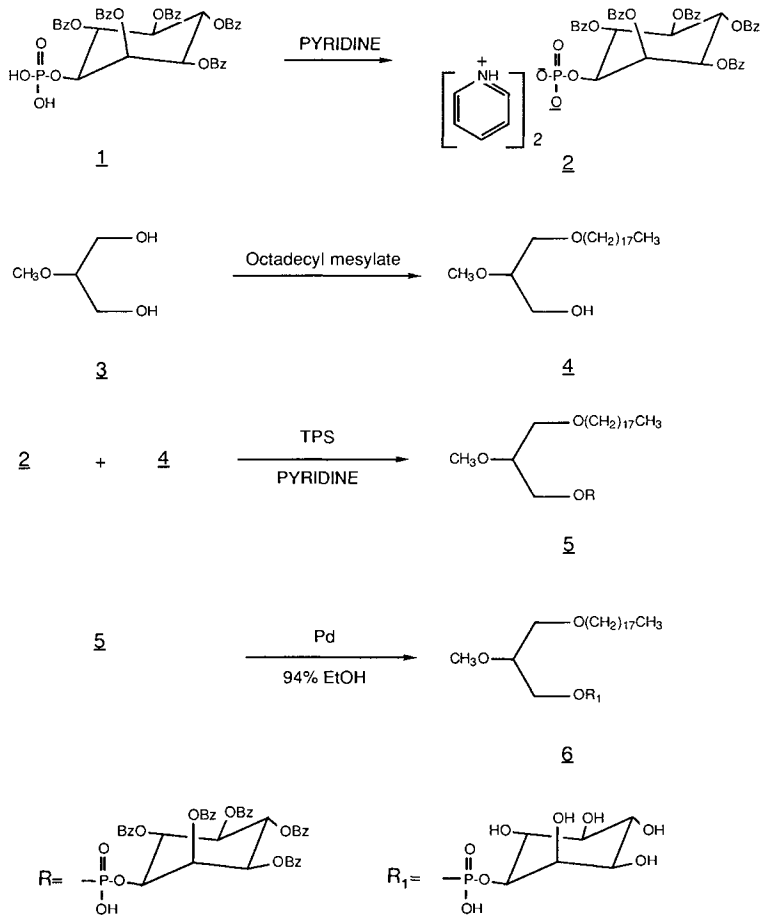


Fig. 2. Synthesis of *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phospho-myo-inositol: compound 6. TPS, 2,4,6-triisopropylbenzenesulfonyl chloride.

acidic conditions. Acid hydrolysis of the 1-prop-1-enyl-2,3,4,5,6-penta-*O*-benzyl-myo-inositol followed by phosphorylation gave compound 1. Attempts to crystallize this compound were unsuccessful. Accordingly, it was purified by washing it thoroughly with hexane and oiling it out from diethyl ether–petroleum ether in order to remove any residual impurity. This resulted in a clear oil whose IR spectrum was consistent with the proposed structure. This 1-phosphate derivative was then converted to the pyridinium salt, compound 2 (Fig. 2), which served as the common intermediate for the synthesis of the desired analogues, compounds 6, 11, and 13 (Fig. 1).

The preparation of *rac*-1-*O*-octadecyl-2-*O*-methylglycerol, compound 4, was accomplished as follows. Benzylideneglycerol was converted to the methyl ether derivative and the protecting benzylidene moiety removed under acidic conditions to give the 1,3-dihydroxy-2-methoxypropane, compound 3. This dihydroxy compound was then reacted

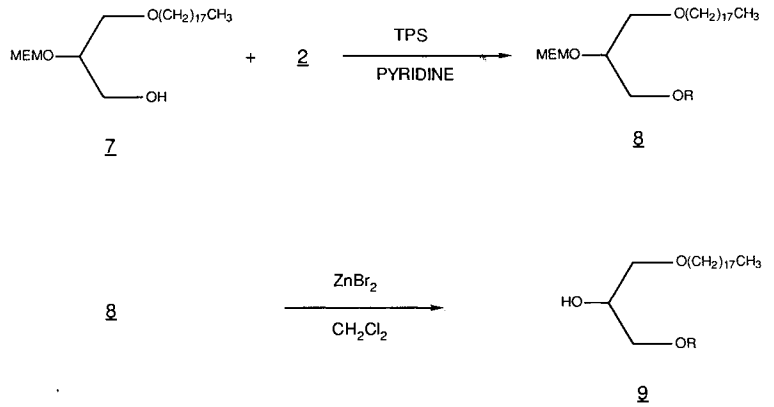


Fig. 3. Synthesis of *rac*-1-*O*-octadecylglycero-3-phospho-penta-*O*-benzyl-myo-inositol: compound 9. MEM, methoxyethoxymethyl.

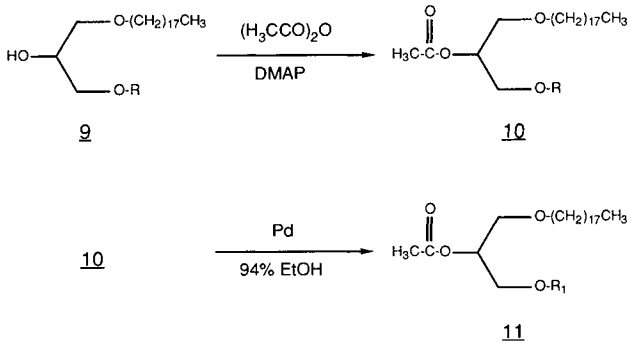


Fig. 4. Synthesis of *rac*-1-*O*-octadecyl-2-*O*-acetylglycero-3-phospho-myo-inositol: compound 11. DMAP, (*N*,*N*-dimethylamino)pyridine. R and R_1 are identical to those in Fig. 2.

with octadecyl mesylate to afford the *rac*-1-*O*-octadecyl-2-*O*-methylglycerol.

The condensation of the pyridinium salt of *rac*-2,3,4,5,6-penta-*O*-benzyl-myo-inositol-1-phosphate, compound 2, with *rac*-1-*O*-octadecyl-2-*O*-methylglycerol, compound 4, in the presence of freshly recrystallized 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) resulted in the formation of *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phospho-penta-*O*-benzyl-myo-inositol, compound 5 (Fig. 2) (25). The reaction was carried out in a desiccator over Drierite. In addition, all reactants were dried under vacuum for 48 hr. The protecting benzyl groups were then removed by hydrogenolysis in the presence of larger than normal amounts of palladium black in aqueous ethanol to ensure complete debenzylation to the desired product *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phospho-myo-inositol, compound 6 (26) (Fig. 2). Further, the percentage of water in ethanol is determined by the extent of the solubility of the benzylated and the debenzylated product in aqueous ethanol. It was necessary at times to recycle the unreacted pentabenzyl derivative and subject it again to hydrogenolysis in order to ensure the complete removal of the protecting groups. Our attempts to debenzylate compound 5 by using 10% Pd/C in absolute ethanol and by catalytic transfer hydrogenation were unsuccessful.

The synthesis of *rac*-1-*O*-octadecyl-2-*O*-[(methoxyethoxy)methyl]glycerol, compound 7, was accomplished by following the procedure of Surles *et al.* (27). Condensing this compound with the pyridinium salt of *rac*-2,3,4,5,6-penta-*O*-benzyl-myo-inositol, compound 2, in presence of TPS as described for compound 5, gave *rac*-1-*O*-octadecyl-2-*O*-[(methoxyethoxy)methyl]glycero-3-phospho-penta-*O*-benzyl-myo-inositol, compound 8. The methoxyethoxymethyl (MEM) group was cleaved by the Lewis acid zinc bromide selectively to give compound 9 (Fig. 3). The generated hydroxyl group was then acetylated to give compound 10 (Fig. 4). The resultant acetylated compound was subjected to hydrogenolysis in the same manner as described for compound 5, yielding *rac*-1-*O*-octadecyl-2-*O*-acetylglycero-3-phospho-myo-inositol, compound 11 (Fig. 4).

The synthetic pathway followed for the preparation of the oleyl derivative, compound 13, the analogue of compound 5, is depicted in Fig. 5. It is synthesized as a precursor for *rac*-1-*O*-[9,10-$^3H_2$-octadecyl]-2-*O*-methylglycero-3-phospho-myo-inositol.

The purity of the target compounds, 6 and 11, was ascertained by thin-layer chromatography; the NMR and mass spectra (MS) were consistent with the proposed structures of these compounds.

Experimental

rac-*2,3,4,5,6-Penta*-O-*benzyl-myo-inositol-1-phosphate (Compound 1)*

The procedure of Gigg and Warren (24) was followed to synthesize this compound.

Pyridinium Salt of rac-*2,3,4,5,6-penta*-O-*benzyl-myo-inositol-1-phosphate (Compound 2)*

The pyridinium salt was synthesized according to the procedure of Hermetter *et al.* (25).

2-O-Methylglycerol (Compound 3)

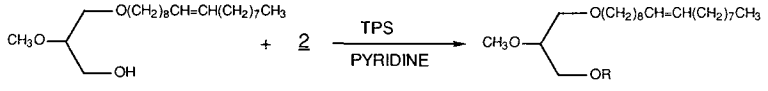
To pure 2-*O*-methyl-1-benzylideneglycerol (28) (25 g, 129 mmol) was added 112.8 ml of a solution of concentrated sulfuric acid, water, and ethyl alcohol (1:7.5:5, respectively). The reaction mixture was then magnetically stirred and refluxed gently for 7 hr (29), after which it was cooled to room temperature and 100 ml of H_2O was added. The ethyl alcohol was removed *in vacuo* and the aqueous layer was extracted with ether to remove the benzaldehyde formed during the reaction. The reaction mixture was then neutralized with potassium carbonate and the H_2O was removed *in vacuo*. The residue was dissolved in 300 ml of ethyl acetate, filtered to remove the potassium salts, and dried over anhydrous magnesium sulfate. After removal of the ethyl acetate, the oily residue weighed 7.8 g. Yield, 57.4%.

^{1}H-NMR (δ, $CDCl_3$): 3.15 (m, 1H, CH_2CHCH_2); 3.5 (s, 3H, OCH_3); 3.75 (m, 6H, CH_2CHCH_2 and 2 × OH).

rac-*1*-O-*Octadecyl-2*-O-methylglycerol *(Compound 4)*

This intermediate was synthesized according to the procedure of Surles *et al.* (27).

^{1}H-NMR (δ, $CDCl_3$): 0.87 (t, 3H, CH_3); 1.25 (m, 3OH, $C_{15}H_{30}$); 1.5 (m, 2H, $OCH_2CH_2C_{16}H_{33}$); 2.3 (s, 1H, OH); 3.35 (m, 1H, CH_2CHCH_2); 3.4 (s, 3H, OCH_3); 3.6 (m, 6H, 3 × CH_2O).



12 13

Fig. 5. Synthesis of *rac*-1-*O*-oleyl-2-*O*-methylglycero-3-phospho-penta-*O*-benzyl-myo-inositol: compound 13. R is identical to that in Fig. 2.

rac-*1*-O-*Octadecyl-2*-O-*methylglycero-3-phospho-penta*-O-*benzyl-myo-inositol (Compound 5)*

The procedure of Hermetter *et al.* (25) was slightly modified. To a magnetically stirred solution of the pyridinium salt of the *rac*-2,3,4,5,6-penta-*O*-benzyl-myo-inositol-1-phosphate (2.5 g, 2.9 mmol) and 1-*O*-octadecyl-2-*O*-methylglycerol (3.5 g, 10 mmol) in 20 ml of dry pyridine was added 2,4,6-triisopropylbenzenesulfonyl chloride (6.1 g, 21.8 mmol). The stirring was continued at room temperature under anhydrous conditions for 24 hr. The reaction mixture was then immersed in ice, 0.5 ml of water was added, and stirring was continued for another 10 min. The pyridine was evaporated without heating *in vacuo* and 50 ml of anhydrous diethyl ether was added to the residue.

The precipitated triisopropylbenzenesulfonic acid pyridinium salt was filtered off and washed thoroughly with diethyl ether. The ether was removed *in vacuo* and the residue weighed 7 g. Purification of the crude product was accomplished by column chromatography on silica gel with a discontinuous gradient of $CHCl_3$, followed by 95/5 and 8/2 (v/v) $CHCl_3$/MeOH, resulting in 2.8 g of a viscous clear oil.

Further purification on silica gel preparative TLC plates using 170/30/3 (v/v/v) $CHCl_3$/MeOH/NH_4OH as eluent gave 2.33 g of the pure clear semisolid product. Yield, 83% (based on the pyridinium salt).

^{1}H-NMR (δ, $CDCl_3$): 0.87 (t, 3H, CH_3); 1.26 (m, 3OH, $C_{15}H_{30}$); 1.38 (m, 2H, $OCH_2CH_2C_{16}H_{33}$); 3.13 (m, 2H, $OCH_2C_{17}H_{35}$); 3.23 (s, 3H, OCH_3); 3.57 (m, 2H, $CH_2OC_{18}H_{37}$); 4.03 (d, 2H, CH_2OP); 3.65–4.20 (m, 5H, 5 × CH-ring); 4.5–4.95 (m, 11H, 5 × $CH_2C_6H_5$ and 1 × CH-ring); 4.98 (m, 1H, CH_2CHCH_2); 7.22 (s, 25H, 5 × C_6H_5).

rac-*1*-O-*Octadecyl-2*-O-*methylglycero-3-phospho-myo-inositol (Compound 6)*

A solution of compound 5 (0.5 g, 0.48 mmol) in 145 ml of 94% aqueous EtOH was added to 0.75 g of palladium black suspended in 10 ml of 94% aqueous EtOH (26). The flask was connected to an atmospheric hydrogenation apparatus and the reaction mixture was magnetically stirred overnight at room temperature. After filtration and solvent removal *in vacuo*, 0.27 g of a white solid was obtained. The crude product was purified by column chromatography on silica gel with a discontinuous gradient of 65/35/0.5, 65/35/1, 65/35/2 (v/v/v) $CHCl_3$/CH_3OH/NH_4OH to give 243 mg of pure product as a white solid. Yield, 84%. m.p., 185–190°C.

^{1}H-NMR (δ, $CDCl_3$–D_3COD): 0.816 (t, 3H, CH_3); 1.18 (m, 3OH, $C_{15}H_{30}$); 1.48 (m, 2H, $OCH_2CH_2C_{16}H_{33}$); 2.83 (s, 6H, 6 × OH); 3.33 (m, 2H, $OCH_2C_{17}H_{35}$); 3.4 (s, 3H, OCH_3); 3.5 (m, 2H, $CH_2OC_{17}H_{35}$); 3.58 (d, 2H, CH_2OP); 3.7–4.0 (m, 5H, 5 × CH-ring); 4.17 (m, 2H, CH_2CHCH_2 and 1 × CH-ring). MS [(M + 1)thioglycerol, MW = 601 *m/z*].

rac-*1*-O-*Octadecyl-2*-O-*[(methoxyethoxy)methyl]glycerol (Compound 7)*

This compound was prepared according to the method described by Surles *et al.* (27).

^{1}H-NMR (δ, $CDCl_3$): 0.85 (t, 3H, CH_3); 1.25 (m, 32H, $C_{16}H_{32}$); 3.38 (s, 3H, OCH_3); 3.42–3.9 (m, 12H, OH, OCH_2CH_2O, CH_2CHCH_2 and $OCH_2C_{17}H_{35}$); 4.8 (s, 2H, OCH_2O).

rac-*1*-O-*Octadecyl-2*-O-*[(methoxyethoxy)methyl]glycero-3-phospho-penta*-O-*benzyl-myo-inositol (Compound 8)*

To a magnetically stirred solution of compound 7 (6.5 g, 14.8 mmol) and the pyridinium salt of *rac*-2,3,4,5,6-penta-*O*-benzyl-myo-inositol-1-phosphate (4 g, 4.6 mmol) in 80 ml of dry pyridine was added 2,4,6-triisopropylbenzenesulfonyl chloride (9.1 g, 30 mmol). The procedure described for the synthesis of compound 5 was then followed to obtain 3 g of the desired product. Yield, 57%.

^{1}H-NMR (δ, $CDCl_3$): 0.88 (t, 3H, CH_3); 1.25 (m, 32H, $C_{16}H_{32}$); 3.22 (s, 3H, OCH_3); 3.3–3.7 (m, 8H, OCH_2CH_2O, $CH_2OCH_2C_{17}H_{35}$); 3.7–4.3 (m, 7H, 5 × CH-ring and CH_2OP); 4.3–5.1 (m, 14H, 1 × CH-ring, 5 × $OCH_2C_6H_5$, CH_2CHCH_2 and OCH_2O); 7.22 (s, 25H, 5 × C_6H_5).

rac-*1*-O-*Octadecylglycero-3-phospho-penta*-O-*benzyl-myo-inositol (Compound 9)*

To a solution of compound 8 (0.8 g, 0.7 mmol) in 10 ml of dry CH_2Cl_2, anhydrous zinc bromide (0.79 g, 3.5 mmol) was added and the reaction mixture was magnetically stirred for 24 hr at room temperature (30), after which the reaction mixture was extracted with 3 × 15 ml of H_2O and the organic layer was dried over anhydrous sodium sulfate. The drying agent was suction filtered, and the solvent removed *in vacuo* to give 0.8 g of the crude product. This was chromatographed on silica gel with a discontinuous gradient of 170/3/3 (v/v/v) $CHCl_3$/CH_3OH/NH_4OH to give 0.5 g of the pure lyso product as a slightly yellow oil. Yield, 68%.

^{1}H-NMR (δ, $CDCl_3$): 0.78 (t, 3H, CH_3); 1.15 (m, 32H, $C_{16}H_{32}$); 2.75–3.2 (m, 4H, $CH_2OCH_2C_{17}H_{35}$); 3.2–4.2 (m, 7H, 5 × CH-ring and CH_2OP); 4.2–5.1 (m, 12H, 5 × $CH_2C_6H_5$, 1 × CH-ring and CH_2CHCH_2); 7.12 (s, 25H, 5 × C_6H_5).

rac-*1*-O-*Octadecyl-2*-O-*acetylglycero-3-phospho-penta*-O-*benzyl-myo-inositol (Compound 10)*

Compound 9 (0.5 g, 0.5 mmol) and 4-dimethylaminopyridine (0.061 g, 0.5 mmol) were dissolved in a mixture of 2.4 ml of ethanol-free $CHCl_3$ and 0.6 ml of dry pyridine. To this solution 5.6 ml of freshly distilled acetic anhydride was added, the flask stoppered under a nitrogen atmosphere, and the mixture magnetically stirred for 24 hr (27).

The volatile material was removed by a stream of nitrogen and the product was extracted with $CHCl_3$ and washed with 5 × 50 ml of H_2O using methanol to break the emulsions formed during the extraction procedure. The $CHCl_3$ layer was dried over anhydrous sodium sulfate, the drying agent suction filtered, and the solvent removed *in vacuo* to give 0.4 g of a semisolid crude product. This was purified on silica gel preparative TLC plates using 170/30/3 (v/v/v) $CHCl_3$/MeOH/NH_4OH as eluent to give 0.25 g of a yellow semisolid product. Yield, 48%.

^{1}H-NMR (δ, $CDCl_3$): 0.87 (t, 3H, CH_3); 1.25 (m, 3OH, $C_{15}H_{30}$); 1.57 (m, 2H, $OCH_2CH_2C_{16}H_{33}$); 1.86 (s, 3H, H_3CCOO); 3.23 (m, 2H, $OCH_2C_{17}H_{35}$); 3.36 (m, 2H,

$CH_2OC_{18}H_{37}$); 3.5–4.4 (m, 7H, 5 × CH-ring and CH_2OP); 4.5–5.03 (m, 11H, 5 × $OCH_2C_6H_5$, and 1 × CH-ring); 5.17 (m, 1H, CH_2CHCH_2); 7.2 (s, 25H, 5 × C_6H_5).

rac-1-O-Octadecyl-2-O-acetylglycero-3-phospho-myo-inositol (Compound 11)

A solution of compound **10** (0.3 g, 323.4 mmol) in 50 ml of 94.5% aqueous EtOH was added to 0.45 g of palladium black suspended in 10 ml of 94.5% aqueous EtOH. The flask was connected to an atmospheric hydrogenation apparatus and the reaction mixture was magnetically stirred overnight at room temperature.

After filtration and solvent removal *in vacuo*, 0.11 g of crude product was obtained. This was chromatographed on silica gel using a discontinuous gradient of 65/35/0.5, 65/35/1, and 65/35/2 (v/v/v) $CHCl_3$/MeOH/NH_4OH to give 40 mg of pure product as a white solid. Yield, 39.3%. m.p., 190°C with decomposition.

^{1}H-NMR (δ, $CDCl_3$–CD_3OD): 0.8 (t, 3H, CH_3); 1.18 (m, 30H, $C_{15}H_{30}$); 1.45 (m, 2H, $OCH_2CH_2C_{16}H_{33}$); 2.12 (s, 3H, H_3CCOO); 3.03 (m, 8H, 6 × OH and $OCH_2C_{17}H_{35}$); 3.3–4.2 (m, 9H, 5 × CH-ring and CH_2CHCH_2); 5.10 (m, 2H, CH_2CHCH_2 and 1 × CH-ring). MS [(M + 1) = 629 *m/z*].

rac-*1*-O-*Oleyl-2*-O-*methylglycerol (Compound 12)*

The procedure of Baumann and Mangold (31) was followed to prepare this compound.

^{1}H-NMR (δ, $CDCl_3$): 0.88 (t, 3H, CH_3); 1.25 (m, 24H, 2 × $(CH_2)_6$); 2.05 (m, 4H, 2 × $CH_2CH=CH$); 3.45 (s, 3H, OCH_3); 3.3–3.8 (m, 7H, CH_2CHCH_2 and $OCH_2C_{17}H_{33}$); 5.35 (m, 2H, CH=CH).

rac-*1*-O-*Oleyl-2*-O-*methylglycero-3-phospho-penta*-O-*benzyl-myo-inositol (Compound 13)*

This compound was prepared according to the procedure described for the synthesis of compound 5.

^{1}H-NMR (δ, $CDCl_3$): 0.83 (t, 3H, CH_3); 1.1–1.4 [m, 24H, 2 × $(CH_2)_6$]; 2.0 (m, 4H, 2 × $CH_2CH=CH$); 3.11 (m, 2H, $OCH_2C_{17}H_{33}$); 3.23 (s, 3H, OCH_3); 3.33 (m, 2H, $CH_2OC_{18}H_{35}$); 3.48 (d, 2H, CH_2OP); 3.85–4.05 (m, 5 × CH-ring); 4.39 (m, 2H, CH=CH); 4.5–4.95 (m, 12H, 5 × $CH_2C_6H_5$, 1 × CH-ring and CH_2CHCH_2); 7.2 (s, 25H, 5 × C_6H_5).

Biological Evaluation

Growth Inhibition of Malignant Cells

Cell lines of HL60 human leukemia (American Type Culture Collection, Bethesda, Maryland) were cultured in RMPI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin (all from GIBCO, Grand Island, New York). Human ovarian carcinoma cell lines BG1 and BG3 (32) were propagated as monolayers in McCoy's 5A culture medium (GIBCO) with 10% fetal calf serum, 0.1% penicillin, and 100 μg/ml streptomycin. Serial passage following mild trypsinization was done weekly in culture flasks (Costar, Cambridge, Massachusetts).

Two techniques were used to evaluate the biological activity of the compounds. The cytotoxic potential against HL60 was measured by trypan blue dye exclusion at different concentrations of compound incubated with 5 × 10^5 cells/ml in 24-well culture plates. Following incubation for 48 hr at 37°C in an atmosphere of 5% CO_2 and 95% humidified air, viable cells were enumerated by hemocytometer as those that retained a cell membrane permeability barrier against trypan blue dye intrusion. Changes in the clonogenicity of the BG1 and BG3 tumor cells as a result of treatment with the test compounds were determined with a modified two-layer semisoft agarose assay as described previously (32,33). *rac*-1-*O*-Octadecyl-2-*O*-methylglycero-3-phosphocholine 14 (ET-18-OMe) was the kind gift of Dr. Wolfgang Berdel, Technical University, Munich, Federal Republic of Germany, and served as a reference compound in all experiments.

Preparation of Protein Kinase C

HL60 cells were grown in 750-ml flasks, harvested, and washed with ice-cold normal saline. After centrifugation (600*g*, 5 min, 4°C) the cell pellet was resuspended (20 m*M* Tris–HCl, pH 7.5, 2 m*M* EDTA, 10 m*M* EGTA, 50 m*M* mercaptoethanol, 2 m*M* phenylmethylsulfonyl fluoride) and sonicated for 20 sec with a stepped microprobe sonicator. Unbroken cells were removed by centrifugation as above and the supernatant centrifuged (120,000*g*, 90 min, 4°C). The supernatant from this step (cytosol) was then fractionated on a 1 × 8-cm DEAE-Sephacel column after the addition of sucrose to a final concentration of 10%. After equilibrating the column (20 m*M* Tris, pH 7.5, 0.2 m*M* EDTA, 0.2 m*M* EGTA, 50 m*M* mercaptoethanol, 10% sucrose), the sample was loaded and unbound material was washed through with 40 ml of the equilibration buffer. Then protein kinase C was eluted by a gradient from 0 to 0.5 *M* NaCl in the buffer. Fractions of 1 ml were collected at 25 ml/hr, and 0.05-ml aliquots assayed for protein kinase C as below. The fractions with the highest activity were pooled and used in further experiments.

Assays of Protein Kinase C Activity

The assays were done at pH 7.5 in a total volume of 0.25 ml and all tubes contained 25 m*M* Tris, 10 m*M* $MgCl_2$, 40 μg histone, 10 μ*M* ATP (including 1 μCi of [γ-^{32}P]ATP), 0.1 m*M* $CaCl_2$, 20 μg ml phosphatidylserine, and 2.5 μ*M* *rac-*

Table I. Cytotoxic Effect of Analogues of Inositol Phospholipids Against HL60 Human Leukemia Cells (Cell Survival)[a]

Compound	Dose (μ*M*)			
	10	20	40	80
5	121 ± 16	126 ± 14	125 ± 11	121 ± 11
13	125 ± 25	137 ± 17	109 ± 14	125 ± 27
9	96 ± 2	85 ± 14	55 ± 16	61 ± 1
6	87 ± 6	94 ± 6	65 ± 10	38 ± 5
11	110 ± 10	140 ± 7	79 ± 4	13 ± 4

[a] Values are expressed as percentage of control and each represents the mean ± SD of three determinations of the same assay. The corresponding control value is 1.044 ± 0.128 × 10^6 cells/ml. ID_{50} for compound 14 (ET-18-OMe) is 2.5 μ*M*.

Table II. Effect of Analogues of Inositol Phospholipids on Colony Growth of BG3 Ovarian Adenocarcinoma Cells[a]

Compound	Dose (μM)					
	1.25	2.5	5	10	20	40
14	66 ± 3	6 ± 3	1 ± 0.5	1 ± 1	1 ± 0.3	1 ± .03
5	90 ± 1	83 ± 1	88 ± 7	NA	101 ± 8	103[b]
13	102 ± 8	102 ± 7	78 ± 6	82 ± 8	69 ± 6	76 ± 2
9	78 ± 8	85 ± 15	68 ± 6	78 ± 2	67 ± 5	63 ± 2
6	91 ± 2	90 ± 9	113 ± 1	93 ± 8	64 ± 2	47 ± 4
11	120 ± 3	109 ± 11	94 ± 8	105 ± 3	107 ± 8	49 ± 3

[a] Values are expressed as percentage of control growth and each represents the mean ± SD of three determinations of the same assay. The corresponding control value is 1965 ± 236 (N = 18) colonies/well. NA, not available.
[b] N = 2.

1-oleoyl-2-acetylglycerol (OAG) plus 0.05 ml protein kinase C. Enzymatic activity was determined as the incorporation of ^{32}P from [γ-^{32}P]ATP into histone in the presence of Ca^{2+}, phosphatidylserine, and OAG minus the incorporation in the absence of OAG. Reactions were initiated by the addition of protein kinase C and halted after 20 min at 30°C by the addition of 0.05 ml bovine serum albumin (10 mg/ml) and 1 ml 25% ice-cold trichloroacetic acid. The tubes were kept on ice and then filtered in a Millipore vacuum box using Millipore HA filters and washed with 25% trichloroacetic acid. The radioactivity bound to the filters was determined by scintillation counting in 5 ml Budget Solve. The amount of enzyme used was shown to result in linear activity for at least 20 min and the assay was linearly dependent on the amount of enzyme used.

rac-1-*O*-Octadecyl-2-*O*-methylglycero-3-phosphocholine (ET-18-OMe) or the analogue to be tested was added directly to the reaction mixture before the addition of protein kinase C. As a control for the small amount of ethanol from the stock solution of ET-18-OMe, 0.1% ethanol was included in the samples with the enzyme and without ET-18-OMe.

RESULTS AND DISCUSSION

The data obtained from the viability test with HL60 human leukemic cells are shown in Table I. Compounds 5 and 13 are not cytotoxic in this system at any of the concentrations tested. Compound 9 is poorly active, with a cell kill of 40% at 80 μ*M*, while compounds 6 and 11 are active at high concentrations (ID_{50} values of 62 and 57 μ*M*, respectively). The dose–response curve of ET-18-OMe, compound 14, in this assay revealed an ID_{50} concentration corresponding to 2.35 ± 0.47 μ*M*, consistent with previously reported data (2,21).

The clonogenic assay results obtained with the ovarian adenocarcinoma BG3 are shown in Table II, where these cells were insensitive to compound 5 and moderately sensitive to compounds 13 and 9, while compounds 6 and 11 showed ID_{50} values of 37 and 40 μ*M*, respectively. The ID_{50} of ET-18-OMe in this assay corresponds to 1.45 ± 0.4 μ*M*, as previously determined. Against the BG1 cell line (Table III), of the compounds assayed, only compound 11 showed inhibitory activity at the concentration tested, with an ID_{50} of 25 μ*M*.

In a previous article (21) a summary of *in vitro* inhibitory data for the target compounds 6 and 11 has been given. In this paper, we report and compare complete data, including those obtained testing the synthetic intermediates. Interestingly, only the final compounds 6 and 11 are active against all the experimental tumors tested. Compound 9 is slightly active against BG3 and HL60. These data suggest that the removal of the benzyl groups from the inositol ring during the synthetic procedure corresponds to the appearance of activity against neoplastic cells.

Helfman *et al.* (7) have shown that ET-18-OMe inhibits PKC and suggested that this enzyme system may be the site of action of the alkylphospholipids. In addition, *rac*-1-*O*-alkyl-2-*O*-methoxyglycerol, a cellular metabolite of ET-

Table III. Effect of Analogues of Inositol Phospholipids on Colony Growth of BG1 Ovarian Adenocarcinoma Cells

Compound	Dose (μM)					
	1.25	2.5	5	10	20	40
14	59 ± 2	9 ± 1	3 ± 3	3 ± 0.2	3 ± 1	1 ± 0.3
5	105 ± 12	95 ± 12	95 ± 14	103 ± 9	88 ± 3	92 ± 10
13	101 ± 9	91 ± 7	96 ± 9	100 ± 9	81 ± 4	98 ± 15
11	86 ± 9	83 ± 9	51 ± 4	54 ± 6	59 ± 1	19 ± 5

[a] Values are expressed as percentage of control colony growth and each represents the mean ± SD of three different assays. The corresponding control value is 1210 ± 111 (N = 12) colonies/well.

18-OMe (34), has recently been shown to inhibit PKC activity (35,36). We have recently reported that 1-amido analogues of glycero-3-phosphocholine inhibit PKC (20). Therefore, PKC inhibition appears to be a common feature among the ether lipid analogues and may be required for activity. In order to extend the studies to determine the structural requirements for PKC inhibition, we tested compounds 6 and 11 in an *in vitro* PKC assay system. Neither compound affected the basal protein kinase activity in the presence of Ca^{2+} and phosphatidylserine (Fig. 6). However, when PKC activity was stimulated by diacylglycerol, in the form of OAG, as described previously (36), the addition of either compound 6 or compound 11 caused a dose-dependent inhibition of PKC activity (Fig. 6). In the presence of 2.5 μM OAG, compound 6 exhibited 64% inhibitory activity at a 10 μM concentration, while compound 11 exhibited 60% inhibitory activity at the same concentration. At the same concentration, compounds 5 and 13 exhibited PKC inhibitory activity of 43 and 63%, respectively. However, the lyso derivative compound 9 had no PKC inhibitory activity. Taken together these studies indicate that the choline moiety is not required for PKC inhibition and that the 1-alkyl (or amido) group is required. Future studies will include the effects of compounds 6 and 11 on phosphatidylinositol turnover and inhibition of other enzymes in this transmembrane signaling pathway (4).

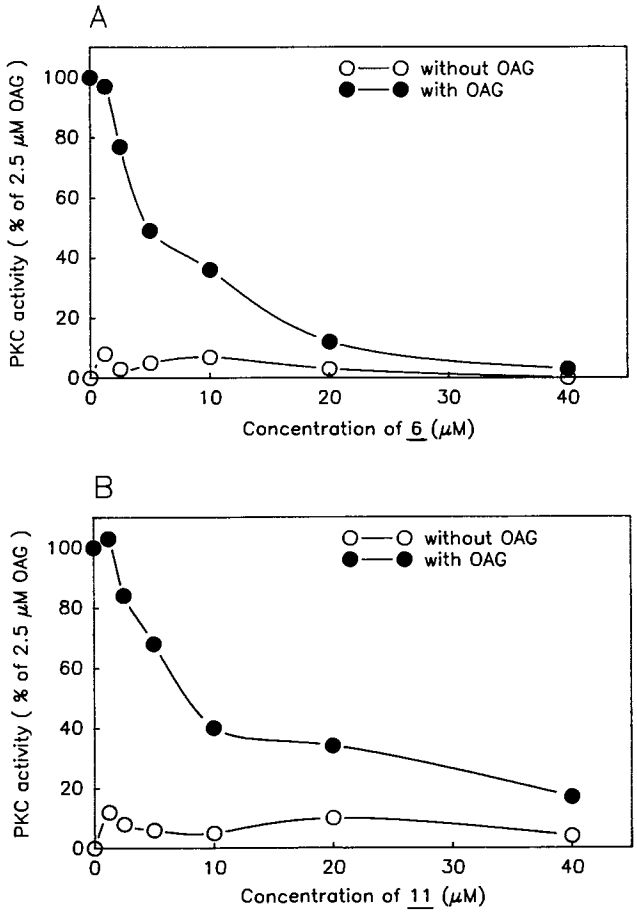


Fig. 6. Effect of compounds 6 and 11 on PKC activity. PKC activity was measured using a partially purified preparation from HL-60 cells. PKC activity is defined as the total activity minus the basal activity in the abscence of OAG. The data are presented as the average of duplicate determinations from one of two similar experiments. (A) Compound 6; (B) compound 11.

ACKNOWLEDGMENTS

This work was supported in part by National Cancer Institute Research Grants CA 41314 (EJM), CA 43297 (LWD), and CA 42216 (CP) and American Cancer Society Research Grant RD-262 (CP).

Dr. Noseda was supported by a fellowship kindly provided by Dr. Luciano Berti, Champion Industries, Winston-Salem, N.C. 27115.

The authors are grateful to J. Gaines White, Patrick L. Godwin, and George W. Small for their skillful technical assistance.

REFERENCES

1. T.-C. Lee, and F. Snyder. In J. F. Kuo (ed.), *Phospholipids and Cellular Regulation,* CRC Press, Boca Raton, Fla., 1985, Vol. II, pp. 2–39.
2. W. E. Berdel, R. Andreesen, and P. G. Munder. In J. F. Kuo (ed.) *Phospholipids and Cellular Regulation,* CRC Press, Boca Raton, Fla., 1985, Vol. II, pp. 41–73.
3. W. E. Berdel and P. G. Munder. Antineoplastic actions of ether lipids related to platelet-activating factor. In F. Snyder (ed.), *Platelet-Activiting Factor and Related Lipid Mediators,* Plenum Press, New York, 1987, pp. 449–467.
4. E. J. Modest, L. W. Daniel, R. L. Wykle, M. E. Berens, C. Piantadosi, J. R. Surles, and S. Morris-Natschke. In K. Harrap and T. Connors (eds.), *New Avenues in Developmental Cancer Chemotherapy,* Bristol-Myers Cancer Symposia, Academic Press, New York, 1987, Vol. 8, pp. 387–400.
5. J. T. O'Flaherty, R. L. Wykle, C. H. Miller, J. C. Lewis, M. Waite, D. A. Bass, C. E. McCall, and L. R. DeChatelet. *Am. J. Pathol.* 103:70–78 (1981).
6. P. G. Munder, M. Modolell, W. Bausert, H. F. Oettgen, and O. Westphal. In E. M. Hersh, M. A. Chirigos, and M. J. Mastrangelo (eds.), *Augmenting Agents in Cancer Therapy,* Raven Press, New York, 1981, pp. 441–458.
7. D. N. Helfman, K. C. Baines, J. M. Kinkade, W. R. Vogler, M. Shoji, and J. F. Kuo. *Cancer Res.* 43:2955–2961 (1983).
8. J. Parker, L. W. Daniel, and M. Waite. *J. Biol. Chem.* 262: 5385–5393 (1987).
9. L. W. Daniel, L. A. Etkin, B. T. Morrison, J. Parker, S. Morris-Natschke, J. R. Surles, and C. Piantadosi. *Lipids* 22:851–855 (1987).
10. M. Modolell, R. Andreesen, W. Pahlke, U. Brugger, and P. G. Munder. *Cancer Res.* 39:4681–4686 (1979).
11. D. B. J. Herrmann. *J. Natl. Cancer Inst.* 75:423–430 (1985).
12. Y. Honma, T. Kasukabe, M. Hozumi, S. Tsushima, and H. Nomura. *Cancer Res.* 41:3211–3216 (1981).
13. G. Storme, W. E. Berdel, W. J. van Blitterswijk, E. A. Bruyneel, G. K. DeBruyne, and M. M. Mareel. *Cancer Res.* 45:351–357 (1985).
14. W. J. van Blitterswijk, H. Hilkmann, and G. A. Storme. *Lipids* 22:820–823 (1987).
15. A. Noseda, J. G. White, and E. J. Modest. *Proc. Am. Assoc. Cancer Res.* 28:13 (1987).
16. A. Noseda, P. L. Godwin, and E. J. Modest. *Biochim. Biophys. Acta* 945:92–100 (1988).
17. A. Noseda, J. G. White, P. L. Godwin, W. G. Jerome, and E. J. Modest. *Exp. Mol. Pathol.* (in press).
18. A. Noseda, P. L. Godwin, and E. J. Modest. *Proc. Am. Assoc. Cancer Res.* 29:321 (1988).
19. S. Morris-Natschke, J. R. Surles, L. W. Daniel, M. E. Berens, E. J. Modest, and C. Piantadosi. *J. Med. Chem.* 29:2114–2117 (1986).

20. M. H. Marx, C. Piantadosi, A. Noseda, L. W. Daniel, and E. J. Modest. *J. Med. Chem.* **31**:858–863 (1988).
21. A. Noseda, M. E. Berens, C. Piantadosi, and E. J. Modest. *Lipids* **22**:878–883 (1987).
22. M. J. Berridge. *Annu. Rev. Biochem.* **56**:159–193 (1987).
23. A. Kishimoto, Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka. *J. Biol. Chem.* **255**:2273–2276 (1980).
24. R. Gigg and C. D. Warren. *J. Chem. Soc. C* 2367–2371 (1969).
25. A. Hermetter, F. Paltauf, and H. Hauser. *Chem. Phys. Lipids* **30**:35–45 (1982).
26. S. S. Yang, T. R. Beattie, and T. Y. Shen. *Tetrahedron Lett.* **23**:5517–5520 (1982).
27. J. R. Surles, R. L. Wykle, J. T. O'Flaherty, W. L. Salzer, F. Snyder, and C. Piantadosi. *J. Med. Chem.* **38**:73–78 (1985).
28. B. A. Stoochnoff and N. L. Benoiton. *Tetrahedron Lett.* **1**:21–24 (1973).
29. A. J. E. Porck and B. M. Craig. *Can. J. Chem.* **33**:1286–1289 (1955).
30. E. J. Corey, J. L. Gras, and P. Ulrich. *Tetrahedron Lett.* **11**: 809–812 (1976).
31. W. J. Baumann and H. K. Mangold. *J. Org. Chem.* **29**:3055–3057 (1967).
32. C. E. Welander, T. M. Morgan, H. D. Homesley, P. P. Trotta, and R. J. Spiegel. *Int. J. Cancer* **35**:721–729 (1985).
33. A. W. Hamburger and S. E. Salmon. *Science* **197**:461–463 (1977).
34. R. W. Wilcox, R. L. Wykle, J. D. Schmitt, and L. W. Daniel. *Lipids* **22**:800–807 (1987).
35. W. J. van Blitterswijk, R. L. van der Bend, I. J. M. Kramer, A. J. Verhoeven, H. Hilkmann, and J. de Witt. *Lipids* **22**:842–846 (1987).
36. L. W. Daniel, G. W. Small, J. D. Schmitt, C. J. Marasco, K. S. Ishaq, and C. Piantadosi. *Biochem. Biophys. Res. Commun.* **151**:291–297 (1988).