Volume 265, number 1,2, 104-106 FEBS 08477 June 1990

Biologically active ether lipids

Biotransformation of *rac*-1(3)-*O*-alkylglycerols in cell suspension cultures of rape and semisynthesis of 1-*O*-alkyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamines, potent antitumor agents

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Received 6 April 1990

Biotransformation of *rac*-1(3)-*O*-hexadecylglycerol by photomixotrophic rape (*Brassica napus*) cells in suspension culture leads to 1-*O*-hexadecyl-2-acyl-*sn*-glycero-3-phosphocholines and small proportions of other ether lipids, e.g. 1-*O*-hexadecyl-2-acyl-*sn*-glycero-3-phosphoethanolamines. Reaction of the hexadecylacyl-glycerophosphocholines with ethanolamine in the presence of phospholipase D from *Streptomyces chromofuscus* yields additional hexadecylacylglycerophosphoethanolamines. Partial hydrolysis of the combined hexadecylacylglycerophosphoethanolamines followed by reacylation of the resulting lyso compound with palmitic anhydride gives 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl) ethanolamine, a nontoxic ether glycerophospholipid with antitumor activity. The corresponding 1-*O*-tetradecyl,1-*O*-octadecyl, and 1-*O*-[(*Z*)-9'-octadecenyl] derivatives are prepared similarly.

Antitumor agent; Alkylacylglycerophospho-(*N*-acyl)ethanolamine (Plasmanyl-(*N*-acyl)ethanolamine); Ether lipid; Plant cell culture

1. INTRODUCTION

Some synthetic ether lipids, particularly ether glycerophospholipids such as *rac*-1(3)-*O*-alkyl-2-*O*-methyl-glycero-3(1)-phosphocholines exhibit antitumor activity and are being used in the therapy of cancer [1,2].

Recently, a naturally occurring class of ether glycerophospholipids, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines [plasmanyl-(*N*-acyl)ethanolamines; PNAE] has been found to exert a selective cytolytic effect on human tumor cells in vitro and in vivo [3]. Mixtures of alkylacylglycerophospho-(*N*-acyl) ethanolamines having various alkyl moieties as well as various acyl moieties can be isolated by adsorption chromatography from lipid extracts of degenerating chick embryos [3] or ischemic mammalian hearts [4].

The present communication describes the preparation of pure 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl) ethanol-amine as well as of the corresponding 1-*O*-tetradecyl, 1-*O*-octadecyl, and 1-*O*-[(*Z*)-9'-octadecenyl] derivatives by a sequence of steps outlined in Fig. 1.

Each of the final products obtained by this procedure exhibits a strong antitumor effect in cultured neoplastic cells and in mice bearing Mc 11 sarcomas (J. Kára et al., unpublished observation).

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2. MATERIALS AND METHODS

All reagents, adsorbents, and solvents were products of E. Merck, Darmstadt, FRG. Phospholipase D of *Streptomyces chromofuscus* was purchased from Boehringer Mannheim, Mannheim, FRG.

rac-1(3)-*O*-hexadecylglycerol was prepared by the reaction of hexadecyl methanesulfonate with the potassium salt of isopropylideneglycerol in toluene followed by acid-catalyzed hydrolysis of the protecting group [5].

The course of reactions and the purity of final products were assessed by thin-layer chromatography on silica gel H. Neutral lipids were fractionated with the developing solvent hexane/diethyl ether (4:1, v/v), ionic lipids with chloroform/methanol/water, (65:25:4, v/v) [6]. For the analysis of total lipid extracts of rape cells, two-dimensional thin-layer chromatography was carried out using chloroform/methanol/conc. ammonia (13:7:1, v/v) in the first direction and chloroform/acetone/methanol/acetic acid/water (6:8:2:1:1, v/v) in the second [7].

Melting points were determined on a Kofler heating block under the microscope. Optical rotations were measured on a Perkin-Elmer Polarimeter 241 MC. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Gemini 200 spectrometer.

All lipid fractions were detected by charring after spraying the plates with chromic sulfuric acid solution. Phospholipids were

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00145793/90/$3.50

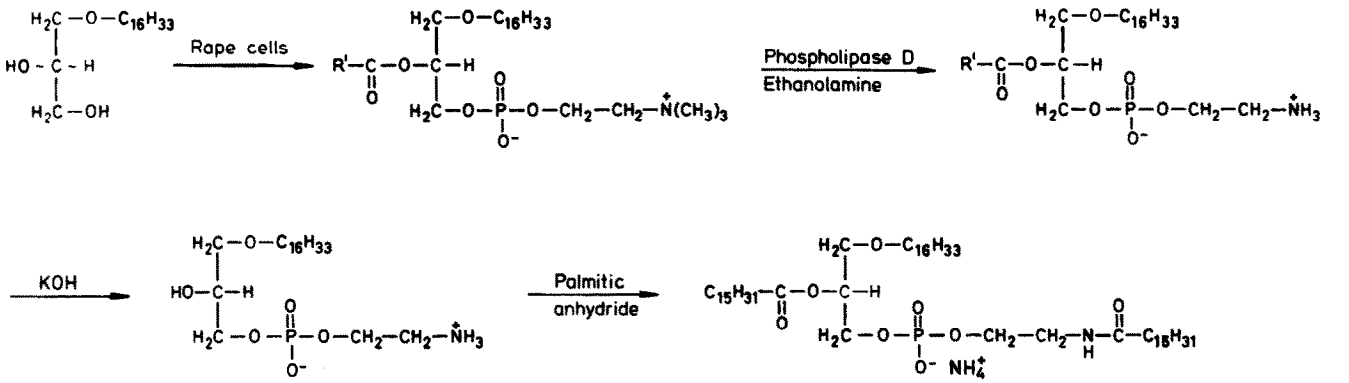

Fig. 1. Preparation of 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamine by incubating photomixotrophic cell suspension cultures of rape (*B. napus*) with *rac*-1(3)-*O*-hexadecylglycerol followed by enzymatic and chemical reactions ($C_{16}H_{33}$ = hexadecyl; $C_{15}H_{31}-CO-O-$ = palmitoyl; $R'-CO-O-$ = acyl).

detected by spraying the plates with Molybdenum blue reagent, those containing an amino group were visualized with ninhydrin and those having a choline moiety with Dragendorff reagent [8].

Photomixotrophic cell suspension cultures of rape (*B. napus*) were propagated in modified MS medium [9] containing 1×10^{-6} M 2,4-dichlorophenoxyacetic acid, 1×10^{-6} M 6-benzylaminopurine and 1×10^{-6} M giberellic acid. The cultures were shaken under continuous illumination at room temperature. A solution of 50 mg of *rac*-1(3)-*O*-alkylglycerol, e.g. *rac*-1(3)-*O*-hexadecylglycerol in 0.25 ml ethanol was added to about 50 g rape cells (wet weight) in 150 ml medium on days 7, 8, and 9, each, of growth. On day 10 the cells were collected on a sintered glass funnel and washed briefly with distilled water.

The total lipids were extracted from the cells with hot isopropanol followed by chloroform/methanol (2:1, v/v)) [10]. After evaporation of the solvents, the lipids were taken up in chloroform/methanol (2:1, v/v).

2.1. *Diradylglycerophosphocholines*

Preparative chromatography of total lipids from around 600 g rape cells (wet weight) was carried out using 110 g silica gel 60 (70–200 mesh) in a column, 30×3 cm. The lipids, ~2.5 g, were separated into 3 fractions by consecutive elution with chloroform, acetone, and methanol [11]. After evaporation of the solvent, the lipids in the third fraction, ~0.45 g, were applied as a band on layers of silica gel H, 0.5 mm thick, and developed in chloroform/methanol/water (65:25:4, v/v). The two major fractions, diradylglycerophosphocholines (~0.25 g) and diradylglycerophosphoethanolamines (~0.13 g), which were identified by cochromatography with standards were each eluted from the adsorbent with chloroform/methanol/water (1:2:0.8, v/v) [12].

2.2. *Diradylglycerophosphoethanolamines*

The mixture of diradylglycerophosphocholines, 40 mg (~0.05 mmol), which was obtained from photomixotrophic rape cells after incubation with *rac*-1(3)-*O*-hexadecylglycerol was dissolved in 1 ml of chloroform. This solution was combined with a solution of ethanolamine, 150 mg (~2.5 mmol), in 3 ml 0.1 M Tris-HCl buffer, pH 8.0, containing 40 mmol calcium chloride [13]. A solution of 100 U of phospholipase D of *S. chromofuscus* in 1 ml of Tris-HCl buffer was added, the two-phase system shaken vigorously for 30 s and then magnetically stirred at 35°C. After 2 h, the enzymatic base exchange was stopped by the addition of 2 ml 0.1 M Na_2EDTA, the mixture was diluted with 10 ml of chloroform and 7 ml of methanol, and after shaking the chloroform layer was taken off. The aqueous layer was washed twice with 10 ml of chloroform; the chloroform phases were combined, dried and concentrated. The mixture of diradylglycerophosphoethanolamines and residual diradylglycerophosphocholines was resolved by preparative thin-layer chromatography, as described above. The two fractions of phospholipids were recovered, and the unreacted diradylglycerophosphocholines of several batches were combined and subjected to the enzymatic reaction once more. Overall yields of diradylglycerophosphoethanolamines ranged from 25 to 30 mg (65–80%).

2.3. *1-O-Hexadecyl-*sn*-glycero-3-phosphoethanolamine*

The diradylglycerophosphoethanolamines, 30 mg (~0.04 mmol) dissolved in 2 ml of chloroform/methanol (2:1, v/v), were deacylated by alkaline hydrolysis with 1 ml of 0.33 N methanolic potassium hydroxide solution for 2 h at room temperature. The reaction mixture was neutralized by addition of 0.5 ml of ethyl formate, stirred for 15 min at room temperature, and diluted with ml of chloroform. After the addition of 2 ml of water, the chloroform phase was collected, the aqueous-methanolic phase was extracted with two portions of 5 ml chloroform. The combined chloroform extracts were dried, concentrated, and then fractionated by preparative thin-layer chromatography on silica gel H (0.5 mm) with chloroform/methanol/water (10:5:1, v/v). The 1-*O*-hexadecyl-*sn*-glycero-3-phosphoethanolamine (R_f 0.25) was eluted and isolated as described above; yields ranged from 6.5 to 7.5 mg (85–95%).

2.4. *1-O-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(N-palmitoyl)-ethanolamine (1-O-Hexadecyl-2-palmitoyl-PNAE)*

1-*O*-Hexadecyl-*sn*-glycero-3-phosphoethanolamine, 5 mg (~0.01 mmol), was dissolved in 5 ml of dry chloroform and 75 mg (0.15 mmol) of palmitic anhydride plus 7.5 mg of 4-(*N'*,*N'*-dimethylamino)pyridine were added. The solution was stirred for 8 h at 50°C. After reacting with 2 ml of methanol for 1 h, the reaction mixture was resolved by preparative thin-layer chromatography on silica gel H (0.5 mm) with chloroform/methanol/conc. ammonia (40:10:1, v/v), and the desired 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamine (R_f 0.4) was eluted [12] and isolated; yields ranged from 4 to 5 mg (50–65%).

3. RESULTS AND DISCUSSION

Rape cells in suspension culture are capable of incorporating 1-*O*-alkyl-*sn*-glycerols into complex ether lipids [14,15]. The enantiomeric 3-*O*-alkyl-*sn* glycerols are not utilized by the cells. Therefore, racemic mixtures of alkylglycerols which are easily prepared by chemical synthesis can be used as starting materials of biologically active ether lipids [16].

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamine (1-*O*-hexadecyl-2-palmitoyl-PNAE) is prepared in an overall yield of 2–3% on

the basis of 1-*O*-hexadecyl-*sn*-glycerol. Corresponding 1-*O*-alkyl derivatives bearing 1-*O*-tetradecyl, 1-*O*-octadecyl, and 1-*O*-[(*Z*)-9′-octadecenyl] moieties are prepared as well. Specific optical rotations of the ammonium salts were determined in chloroform/methanol (1:1, v/v; $c=1$): 1-*O*-tetradecyl-2-palmitoyl-PNAE, $[\alpha]_D$ + 1.8°; 1-*O*-hexadecyl-2-palmitoyl-PNAE, $[\alpha]_D$ +1.7°; 1-*O*-octadecyl-2-palmitoyl-PNAE, $[\alpha]_D$ + 1.5°; 1-*O*-[(*Z*)-9′-octadecenyl]-PNAE, $[\alpha]_D$ + 1.5°; melting points (NH_4-salts); mp >200°C (decomposition).

^1H and ^{13}C NMR as well as APT spectra ($CDCl_3$, TMSi as internal standard) of 1-*O*-[(*Z*)-9′-octadecenyl]-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)-ethanolamine are described below; other PNAEs show similar resonances, except for the $-C=C-$ double bond of the alkoxy chain.

^1H NMR (200 MHz), δ (ppm): 0.80 – 0.95 (9H, 3 CH_3); 1.15 – 1.40 (70H, alkyl and acyl $-CH_2-$); 1.40 – 1.65 (6H, $O-CH_2-CH_2-$ and $CO-CH_2-CH_2-$); 1.95 – 2.10 (4H, $-CH_2-CH=CH-CH_2$); 2.10 – 2.25 (2H, $-CH_2-CO-NH-$); 2.25 – 2.40 (2H, $-CH_2-COO-$); 2.60 – 3.0 (1H, $-NH-$); 3.30 – 3.60 (6H, $-CH_2-O-CH_2-$ and $-CO-NH-CH_2$); 3.80 – 4.05 (4H, $-CH_2-O-POO^--O-CH_2-$); 5.05 – 5.20 (1H, $>CH-O-CO-$); 5.25 – 5.40 (2H, $-CH=CH-$, $J,J'=5.1$ Hz).

^{13}C NMR and APT (50 MHz) δ (ppm): 14.1 (alkoxy and acyl terminal CH_3); 22.5 – 32.1 (alkoxy and acyl $-CH_2-$); 34.62, 36.60, and 40.3 ($-CH_2-CO-NH$, $-CH_2-CO-O$, and $-CH_2-NH-CO$); 64.6 and 69.48 (glycerol backbone and $PO_3-O-CH_2-CH_2-NH$); 72.09 (glyceryl $-O-CH_2-C_{17}H_{33}$); 72.3 (glycerol C – 2); 130.15 and 130.33 ($-CH=CH-$); 174.53 and 174.86 ($-CO-O$ and $-CO-NH$) [17 – 19].

The procedure described proved to be applicable to the preparation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)-ethanolamines having a saturated or an unsaturated alkyl moiety. It is particularly suitable for the preparation of radioactively labelled compounds that could be of use in studies aimed at elucidating the mechanism of their antineoplastic effects [3]. Moreover, such labelled compounds may be of use as marker substances in the diagnosis of heart stroke [4].

We are preparing a set of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines differing not only in the chain length and degree of unsaturation of their alkyl moieties, but also with regard to their *O*-acyl and *N*-acyl groups in order to assess the structural requirements for biological activity through a comprehensive pharmacological screening.

Acknowledgement: We thank Dr H.H.O. Schmid, The Hormel Institute, University of Minnesota, Austin, MN for a generous gift of diradylglycero-phospho-(*N*-acyl)ethanolamines for comparison.

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