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THE SYNTHESIS OF 1,2-DIOLEOYL-sn-[2-^{3}H]GLYCERO-3-PHOSPHOSERINE

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SUMMARY

Phosphatidylserine labelled in the glycerol moiety was synthesized from [2-^{3}H]glycerol through enzymatic phosphorylation of glycerol, acylation of labelled *sn*-glycerol-3-phosphate with oleic anhydride and condensation of phosphatidic acid with N-t-BOC serine benzhydryl ester. The product was chromatographically purified with an overall yield of 12%, at a molar specific activity of 25 Ci/mol.

Key-words: *tritium, glycerol, dioleoyl-glycerophosphoserine*

INTRODUCTION

The fundamental role played by phospholipids in the living matter is well known. Considerable evidence has accumulated regarding their biochemical, biophysical and pharmacological involvement in many cellular mechanisms [1]. The availability of lipid species isotopically labelled in different portions of the molecule has been essential for obtaining informations regarding such mechanisms.

Among the phospholipid classes, phosphatidylserine has been recently shown to be involved in fundamental membrane functions, such as the activation of a specific protein kinase [2]. Moreover, it has been shown that the administration of phosphatidylserine to the rat produces a series of physiological effects such as the redistribution of carbohydrate reserves [3], an increased catecholamine turnover in the hypothalamus [4], the release of acetylcholine from the brain cortex [5] and the activation of tyrosine hydroxylase metabolism [6].

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An insight into the mechanisms through which these processes take place could be obtained upon administration of labelled phosphatidylserine. Methods for synthesizing phosphatidylserine labelled in the serine moiety and in the fatty acid bound to the position 2 of the glycerol backbone have been described [7, 8]. However, the utilization of these labelled molecules cannot give complete information on the metabolism of the lipid, due to the occurrence of transacylation and base exchange reactions in animal tissues [9, 10]. Indeed, the availability of glycerol-labelled phosphatidylserine could give complementary informations regarding the fate of the glycerol backbone of the molecule. The chemical synthesis of glycerol- labelled phosphatidylserine has not been reported up to now and therefore a strategy for its preparation has been devised and the results of this study are reported in the present paper.

EXPERIMENTAL PROCEDURE

Materials

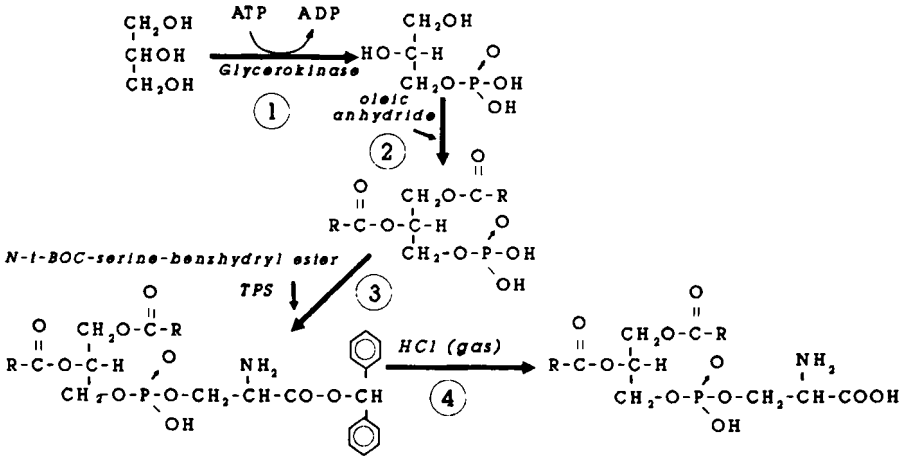
[2-^{3}H]glycerol was purchased from Amersham International plc (Buckinghamshire, England), L-glycerol-3-phosphate dicyclohexylammonium salt and glycerokinase from Bacillus stearothermophilus were from Boehringer Mannheim GmbH (West Germany). Bovine serum albumin, ATP, N-t-BOC-L-serine, Tris hydroxymethyl aminomethane and 2,4,6-triisopropyl-benzenesulfonyl chloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). . Ion exchange resins AG 1-X4 and AG 50W-X8 were from Bio-Rad (Richmond, CA, U.S.A.). Q-Sepharose was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Other chemicals were from Carlo Erba (Milano, Italy). Thin layer chromatography of phosphatidylserine and of the intermediates of its synthesis has been performed on Kieselgel 60 F254 precoated silicic acid plates (Merck, Darmstadt, West Germany).

Preparation of sn-[2-^{3}H]glycero-3-phosphoric acid

Radiolabelled glycerophosphate was synthesized enzymatically (Scheme 1, step 1) according to Chang and Kennedy [11].

A solution of 25 μmols (25 mCi) of [2-^{3}H]glycerol in 25 ml of ethanol was dried at 40°C. The residue was dissolved in 5 ml of a mixture containing 20 mM ATP (Boehringer Mannheim GmbH, West-Germany), 10 mM mercaptoethanol, 20 mM $MgCl_2$, 5 mg of bovine serum albumin (Sigma Chemical Co. St. Louis, MO, U.S.A.), 0.25 mg (40 units) of glycerokinase (Boehringer Mannheim GmbH, West-Germany) and 50 mM Tris-HCl buffer (pH 8.0). The mixture was incubated 3 h at 37°C and the enzymatic reaction was stopped by heating the incubation mixture for 5 min in boiling water. After cooling, the mixture was filtered and the filter was thoroughly washed with water. The filtrate (10 ml) was loaded on a 6 x 160 mm Ag1x4 column

Scheme 1: Synthesis of glycerol-labelled phosphatidylserine



(200-400 mesh, formate form). The column was washed with 30 ml of water to elute the unreacted glycerol and then with 10 ml of 0.1 M formic acid. Labelled sn-glycerol-3- phosphate was eluted with 50 ml of 1 M formic acid and nucleotides were eluted with 30 ml of 1 M HCl. The radioactivity and the absorbance at 260 nm of the eluates were measured on 10 μl aliquots of the fractions collected (10 ml). The fractions containing labelled sn-glycerol-3-phosphate were pooled and the resulting mixture was concentrated under vacuum to 1/5th of the original volume. After dilution with 50 ml of water, the solution was concentrated again and this procedure was repeated six times to completely remove the formic acid.

The yield of the overall procedure was 98-99 %, based on the amount of labelled sn-[2-^{3}H]glycerol-3-phosphoric acid recovered.

Preparation of oleic anhydride

Oleic anhydride was prepared according to Bergelson [12], by refluxing a solution of 10 g of oleic acid in 50 ml of acetic anhydride (1:15, by mol) for three hours. The unreacted acetic anhydride was removed by evaporation under vacuum and the oleic anhydride so obtained was utilized without further purification.

Preparation of 1,2-dioleoyl-sn-[2-^{3}H]glycerol-3-phosphate

The acylation of sn-[2-^{3}H]glycero-3-phosphate (Scheme 1, step 2) was carried out according to Lapidot et al. [13].

Unlabelled sn-glycerol-3-phosphate monocycloexylammonium salt (370.4 mg, 1 mmole) was dissolved in 5 ml of water and the solution was loaded on a 5 x 20 mm Ag50Wx 8 column (H^+ form). The column was washed with 20 ml of water to

completely elute the sn-glycerol-3-phosphate in the acid form. The sn-glycerol-3-phosphoric acid recovered was quantitated as inorganic phosphate according to van Veldhoven and Mannaerts [14]. Labelled sn-[2-^{3}H]glycerol-3-phosphoric acid was added to obtain a final specific activity of 25 Ci / mole. The sn-[2-^{3}H]glycero-3-phosphoric acid (1 mmol) was converted into its mono- tetraethylammonium salt by adding an equimolar amount of tetraethylammonium hydroxide (0.736 ml of a 20% solution). The resulting solution of sn-[2-^{3}H]glycerol-3-phosphoric acid mono-tetraethylammonium salt was freeze-dried.

Tetraethylammonium oleate was prepared adding oleic acid (2.82 g, 10 mmols) to 10 mmoles of tetraethylammonium hydroxide (7.36 ml of a 20% aqueous solution). Ethanol (3 ml) was added to the turbid suspension under vigorous stirring at 80°C, to get a homogeneous solution. The solvent was removed under vacuum and the residue was dried by lyophylization.

A solution of 301 mg (1 mmole) of sn-[2-^{3}H]glycerol-3-phosphoric acid mono-tetraethylammonium salt in 10 ml of anhydrous methanol was mixed with 30 ml of a methanol solution containing 4.11 g (10 mmoles) of tetraethylammonium oleate. The solvent was evaporated under vacuum and the residue was dried for 24 hours at 50°C under vacuum.

Oleic anhydride, 5.46 g (10 mmoles) in 30 ml of anhydrous CCl_4 was added to the residue and the mixture was refluxed for 3 h at 80°C in anhydrous conditions. The solvent was evaporated under vacuum and the residue was solubilized at 0°C, under magnetic stirring in N_2 atmosphere, with 150 ml of a $CHCl_3$/CH_3OH mixture (2:1, by vol). To this solution was added 55 ml of 0.2 N HCl and the mixture was vigorously stirred 5 min at 0°C. The aqueous phase (pH 2) was withdrawn and the lower phase was washed three times with 50 ml of a $CHCl_3$ / CH_3OH / H_2O mixture (3:48:47, by vol). The resulting organic phase was evaporated under vacuum.

Labelled phosphatidic acid was purified from the crude residue (9 g) by batch treatment with silicic acid. The residue was dissolved in 100 ml of chloroform and 35 g of silicic acid were poured into the solution under magnetic stirring. After 10 min, the chloroform was removed and the silicic acid was washed repeatedly with fresh chloroform. The chloroform phases were checked for the presence of oleic acid. After oleic acid was completely removed, the pure phosphatidic acid was desorbed from silicic acid with 600 ml of $CHCl_3$ / CH_3OH mixture (2:1, by vol). The solution of 1,2-dioleoyl-sn-[2-^{3}H]glycerol-3-phosphoric acid was evaporated under vacuum and the residue was redissolved in anhydrous pyridine. The yield of the reaction from glycerol was 56%.

Preparation of N-t-butoxycarbonyl-L-serine benzhydryl ester

The carboxylic group of N-t-BOC-serine was protected according to Hermetter and Paltauf [15]. N-t-BOC-serine, 1.15 g (5.6 mmol) was added to a solution of 1.5 g (7.74 mmol) diphenyldiazomethane [16] in 50 ml diethyl ether. After stirring over-

night at room temperature, the excess of diphenyldiazomethane was destroyed with glacial acetic acid. The solution was cooled at 0°C and 50 ml of cold water were added under stirring. The aqueous phase was neutralized with NH_4OH. The ether phase was recovered and washed three times with water, then dried over Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in 10 ml of chloroform and the chloroform solution was loaded on a 2 x 10 cm column of silicic acid packed in chloroform (approximately 10 g of silicic acid per g of residue). N-t-Butoxycarbonyl-L-serine benzhydryl ester was eluted with chloroform. Fractions (10 ml) were analyzed on silicic acid plates developed with $CHCl_3$ / CH_3OH / CH_3COOH mixture (80:10:5, by vol). The fractions containing one pure component (Rf = 0.85) were pooled and utilized for the condensation with phosphatidic acid.

Preparation of phosphatidylserine

Labelled phosphatidic acid was reacted with N-t-BOC-serine benzhydryl ester (Scheme 1, step 3) according to Hermetter and Paltauf [17]. The pyridinium salt of phosphatidic acid and 3.2 equivalents of N-t-BOC-serine benzhydryl esther (separately dried with P_2O_5 under high vacuum for 15 hours) were solubilized in 15 ml of anhydrous pyridine. The two solutions were mixed and 6.4 equivalents of 2,4,6-triisopropyl-benzenesulphonyl-chloride (TPS) were added to the pyridine solution. The reaction mixture was stirred at 30°C, under anhydrous conditions. After 24 h, few drops of water were added and the solvent was removed under reduced pressure. The residual pyridine was removed dissolving the residue in toluene and removing again the solvent under vacuum. The crude residue was dried under high vacuum and then extracted with 150 ml of anhydrous diethyl ether. The insoluble triisopropyl-benzenesulfonic acid was removed by filtration and the filter was washed with 50 ml diethyl ether. The ether solutions were combined and the solvent was removed under reduced pressure. The residue was dried overnight with P_2O_5 under high vacuum and then dissolved in 30 ml anhydrous chloroform. The solution was purged with nitrogen and saturated with anhydrous HCl for 15 min at 0°C. The solution was stirred for 2 hours at room temperature (Scheme 1, step 4). HCl was removed with a nitrogen flow, then the solvent was evaporated under reduced pressure and the residue was dried over KOH under high vacuum. Cold chloroform, 50 ml, and 50 ml of a CH_3OH / NH_4OH mixture (1:1, by vol) were added under magnetic stirring at 0°C to the residue. The aqueous phase was withdrawn and the lower phase was washed three times with 20 ml of $CHCl_3$ / CH_3OH / H_2O mixture (3:48:47, by vol). The solvent was evaporated under vacuum and the residue was dissolved in toluene under a nitrogen atmosphere.

Chromatographic purification of phosphatidylserine

Q-Sepharose (200 ml packed bed) was first washed with 500 ml of 1 M NaOH and then with doubly-distilled water, up to neutrality of the supernatant. The resin

was converted into the acetate form adding 500 ml 1 M acetic acid to the packed resin. The excess of acetic acid was drained off and completely washed out with water. After removing the water with methanol, the resin was suspended in 200 ml of $CHCl_3$ / CH_3OH mixture (3:2, by vol). A glass column (1 x 50 cm) was packed with Q-sepharose, acetate form (20 ml packed bed). The column was conditioned with 100 ml of $CHCl_3$ / CH_3OH mixture (3:2, by vol).

1,2-dioleoyl-sn[2-^{3}H]glycero-3-phosphoserine was dissolved in 10 ml of $CHCl_3$ / CH_3OH (3:2, by vol) and the solution was loaded on the column. The column was washed with 120 ml of the same solvent mixture and then with 120 ml of $CHCl_3$ / CH_3COOH mixture (9:1, by vol). Labelled phosphatidylserine was completely eluted (fig. 1) with 120 ml of a $CHCl_3$ / CH_3COOH mixture (1:6, by vol).

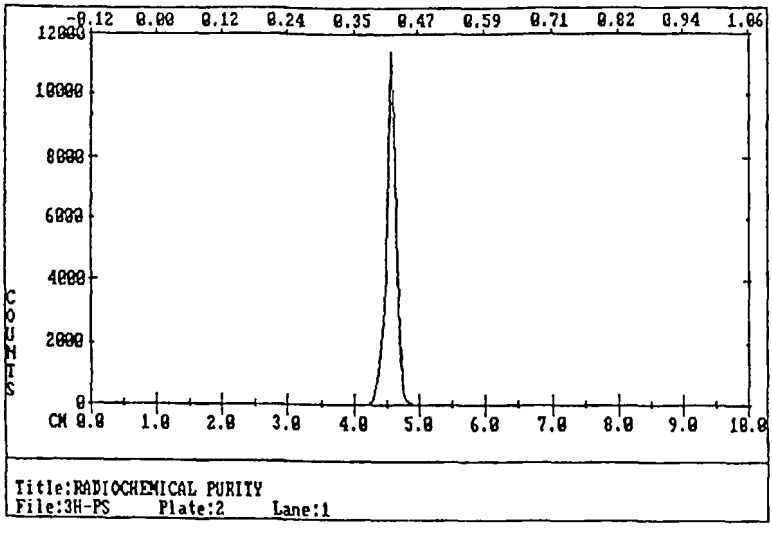


Fig. 1. Thin layer chromatographic analysis on silicic acid plates of glycerol-labelled phosphatidylserine, purified by Q-sepharose chromatography. Development: $CHCl_3$ / CH_3OH / H_2O (60:30:5, by vol). The peak of radioactivity detected by automatic radioscanning (Bioscan System 200, Canberra, Packard) contains 98% of total radioactivity loaded on the plate.

The purity of the final product (3 mCi, yield 12%) was checked by TLC on silicic acid plates (fig. 1) using as developing solvent $CHCl_3$ / CH_3OH / H_2O (60:30:4, by vol). The radiochemical purity of the product was 98% and the molar specific activity was 25 Ci/mol.

CONCLUSIONS

A laboratory procedure for synthesizing phosphatidylserine labelled in the glycerol moiety on millimolar scale is described in the present paper. Each of the steps utilized throughout the synthesis and for the purification of the intermediates have been optimized to obtain a final product of high radiochemical purity (98%), starting from a relatively low cost labelled precursor ([2-^{3}H]glycerol).

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