Synthesis of a spin-labeled phospholipid for studying membrane dynamics in intact mammalian cells

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Summary We report here the synthesis of a spin-labeled phospholipid, 1-palmitoyl-2-(4-doxylpentanoyl)glycerophosphocholine. The synthetic route for this probe involves two major steps: *1*) the synthesis of 4-doxylpentanoic acid from ethyl levulinate and 2-amino-2-methyl propanol, and *2*) the synthesis of the lipid from 4-doxylpentanoic acid and lysolecithin. The efficiency and yield of both steps have been greatly improved. This represents the first instance that the synthesis of this important spin-labeled phospholipid is described in detail. Because it mimics the native lipid molecule and can be readily incorporated into biological membranes, this probe should be extremely useful for studying lipid dynamics in the plasma membrane of intact mammalian cells using electron spin resonance (ESR) spectroscopy. —**Joseph, J., and C-S. Lai.** Synthesis of a spin-labeled phospholipid for studying membrane dynamics in intact mammalian cells. *J. Lipid Res.* 1988. **29:** 1101–1104.

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Owing to its sensitivity to a wide range of molecular motions, electron spin resonance (ESR) employing nitroxide spin probes has been frequently used for studying lipid dynamics in model and biological membranes (1). The commonly used nitroxide lipid-like spin probes can be categorized into two major classes, i.e., fatty acid spin

Abbreviations: ESR, electron spin resonance; TLC, thin-layer chromatography.

probes and phospholipid spin probes. Only the former are widely used to investigate the lipid dynamics in the membrane of the intact cell because they are highly water-soluble and readily permeate the cell membrane. However, the validity of the use of these fatty acid spin probes for studying the lipid dynamics in complex membrane systems, such as in intact cells, has been questioned because the location and distribution of these probes in the cell are not well defined (2). On the other hand, the use of phospholipid spin probes for intact cell studies is limited by their low water solubility and poor incorporation.

In 1983, Davoust and coworkers (3) reported the use of a spin-labeled phospholipid with a short β-chain (5 carbons) bearing a nitroxide group at the carbon-4 position, probe 1 (**Fig. 1**), and other similar spin-labeled phospholipids with different head groups to study lateral diffusion of lipid molecules in model membranes. In 1984, Seigneuret and Devaux (4) showed that unlike other phospholipid spin labels having two long acyl chains, which are membrane impermeable, these phospholipid spin probes possessing a short β-chain can be readily incorporated into the membrane of intact erythrocytes. Since then, the same group has published extensively on the investigations of lipid asymmetry and lipid translocation in intact erythrocytes and lymphocytes using probe 1 and other related spin-labeled phospholipids (5–8 and references therein). However, so far there has been no detailed report on the synthesis of this class of interesting spin probe. In this communication, the complete synthesis of probe 1, 1-palmitoyl-2-(4-doxylpentanoyl)glycerophosphocholine, is described in detail.

MATERIALS AND METHODS

Ethyl levulinate and 2-amino-2-methyl propanol were purchased from Fluka Chemical (Ronkonkoma, NY). Lysophosphatidylcholine was obtained from Calbiochem (San Diego, CA) and L-α-dimyristoylphosphatidylcholine was from Sigma (St. Louis, MO). HPLC grade solvents, and Analtech uniplates for TLC were obtained from Fisher Scientific (Itasca, IL). Silica gel 60 of E. Merck was purchased from American Scientific (McGaw Park, IL), and other reagent grade chemicals were from Aldrich Chemical Co. (Milwaukee, WI).

Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). All ESR spectra were recorded with a Varian Century-line spectrometer equipped with a rectangular TE_{102} cavity, operating at 9.5 GHz. The cavity temperature was regulated with a variable temperature controller and a digital thermometer. The peak-to-peak modulation amplitude was 1 G and the field sweep was 100 G.

Dimyristoylphosphatidylcholine vesicles containing spin probe 1 were prepared essentially the same as described previously for the preparations of other lipid-soluble spin probes in membranes (9).

RESULTS AND DISCUSSION

Synthesis of 4-doxylpentanoic acid

Devaux and his colleagues have cited Hubbell and McConnell's method (10) for the preparation of 4-doxylpentanoic acid. This procedure has been used for the synthesis of doxyl derivatives of fatty acids with the acyl chain length of 14–18 carbon atoms. Briefly, in the method, the acyl keto ester is refluxed with 8- to 10-fold excess of 2-amino-2- methyl propanol for 8 to 10 days in the presence of a catalytic amount of *p*-toluenesulfonic acid. When we followed this method to prepare 4-doxylpentanoic acid, we found that no oxazolidine was produced. The procedure described in the following is the modified Hubbell and McConnell method for the synthesis of 4-doxylpentanoic acid.

4-Doxylpentanoic acid was prepared by refluxing ethyl levulinate (1.44 g or 10 mmol) and 2-amino-2-methyl propanol (1.8 g or 20 mmol) in 5 ml of dry toluene for 30 hr in the presence of 25 mg of *p*-toluenesulfonic acid. Water produced in this reaction was removed continuously using a Dean-Stark water separator. The resulting liquid was diluted into 50 ml of ether at 4°C; after washing with 25 ml of saturated $NaHCO_3$ solution and 3 × 25 ml of water, the yellow product in the ether layer was dried over $MgSO_4$ and then rotary evaporated; the residual toluene was removed at 50°C on an evaporator. About 1.75 g of the orange liquid, oxazolidine, was obtained.

The liquid oxazolidine was in turn dissolved in 100 ml of dry ether and kept cooled in ice. To this was added dropwise 100 ml of ether containing 2.5 g of *m*-chloroperoxybenzoic acid. After addition of a few grams of 4-A molecular sieves, the liquid was kept at 4°C for 24 hr. The

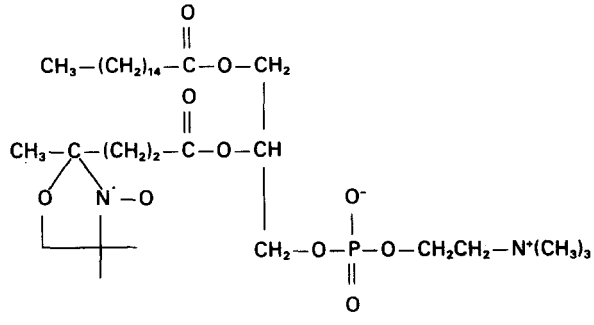

Fig. 1. The chemical structure of probe 1, 1-palmitoyl-2-(4)-doxylpentanoyl) glycerophosphocholine.

resulting deep orange ether solution was washed with 4 × 25 ml of a 4% Na_2CO_3 solution to remove all the acids. The ether layer was washed with water, dried over $MgSO_4$, and was subjected to rotary evaporation to produce an orange liquid which was further purified on a Silica Gel-60 column (80 g) with a mixture of hexane-ether (80:20) as an eluting solvent. The purity of the yellow product was examined by TLC using a mixture of hexane-ether-methanol 70:30:2 as a developing solvent. About 1.2 g of the 4-doxylpentanoic ester was yielded.

To convert the ester into 4-doxylpentanoic acid, the ester obtained was dissolved in 25 ml of methanol to which 10 ml of 4% NaOH was added. The TLC result showed that the hydrolysis was complete after refluxing for 30 min. Methanol present in the reaction mixture was subsequently removed by rotary evaporation, and the remaining aqueous solution was adjusted to pH 2-3 with a solution of 3 N HCl. The product in the acidic solution was extracted with 3 × 25 ml of chloroform and was dried over $MgSO_4$. After removing the solvent by rotary evaporation, the sample was dried over P_2O_5 in a vacuum desiccator. About 1.05 g of a bright yellow solid with a melting point of 53-54°C was produced. Anal. calcd. for $C_9H_{16}NO_4$ (4-doxylpentanoicacid): C, 53.45; H, 7.97; N, 6.93. Found: C, 53.24; H, 7.82; N, 6.88.

Thus, by shortening the reaction time (to 24-30 hr) and reducing the amount of 2-amino-2-methyl propanol (to twofold excess), 4-doxylpentanoic acid was produced with a 52% yield. Apparently, the reaction rate between the short acyl keto ester and 2-amino-2-methyl propanol is much faster than that for the long acyl keto ester and 2-amino-2-methyl propanol. Hubbell and McConnell's procedure (10) for the preparation of doxyl derivatives of long acyl chains thus has to be modified for synthesizing doxyl derivatives of shorter acyl chains such as 4-doxyl-pentanoic acid.

Synthesis of 1-palmitoyl-2-(4-doxylpentanoyl) glycerophosphocholine

In their publications, Devaux and his colleagues have also cited Hubbell and McConnell's method (10) for condensation of 4-doxylpentanoic acid and lysolecithin. The method involves the heating of the anhydride derivative of long acyl chain and lysolecithin in the presence of a catalytic amount of sodium oxide. However, we found that the yield was very poor when this procedure was used to prepare probe 1. The following is the method developed in our laboratory for condensation of 4-doxyl-pentanoic acid and 1-palmitoyllysolecithin.

To 5 ml of dry carbon tetrachloride containing 100 mg of 4-doxylpentanoic acid was added 55 mg of dicyclohexylcarbodiimide in 2 ml of dry carbon tetrachloride. The reaction mixture was kept at 22°C with vigorous stirring for 20 hr in a stoppered flask. The precipitated urea derivative was filtered off and the filtrate was dried under vacuum to obtain the anhydride. To the anhydride was added 50 mg of 1-palmitoylglycerophosphocholine (which had been dried in a vacuum desiccator over P_2O_5 for several days to remove the residual water), 3 mg of sodium oxide, and 0.25 ml of dry xylene. After purging with

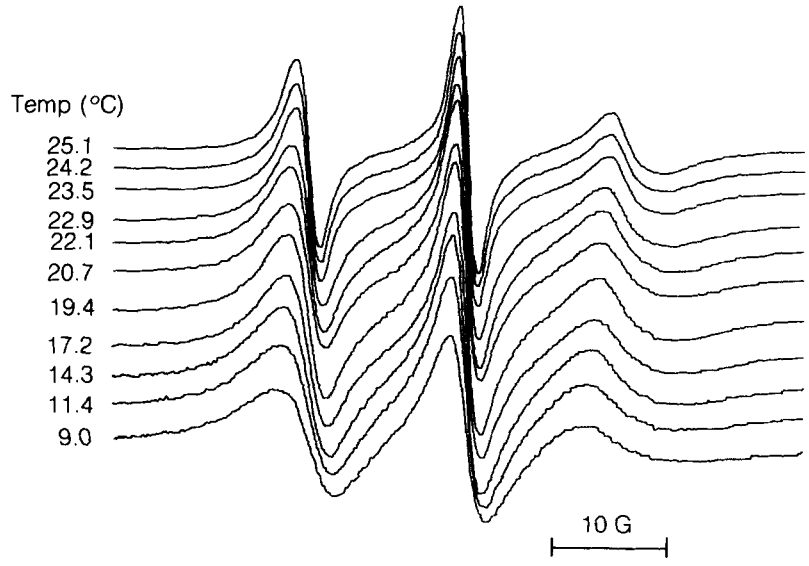

Fig. 2. Temperature-dependency of the ESR spectra of spin probe 1 in dimyristoylphosphatidylcholine multilamellar membranes in phosphate-buffered saline, pH 7.4. The membrane vesicles containing spin probe 1 were prepared as described in Methods. The host lipid concentration was 10 mM and the molar ratio of probe-to-lipid was 1-to-100.

nitrogen gas, the reaction mixture was stirred in an oil bath at 70°C for 48 hr in a sealed flask. After addition of 1 ml of chloroform, the reaction mixture was applied to a Silica Gel-60 column (with o.d. 1 cm) and eluted initially with chloroform–methanol 95:5, and then with chloroform–methanol 50:50. The purity of the yellow product was examined by using TLC. The sample was rechromatographed once to remove traces of unreacted doxylpentanoic acid and lysolecithin. The yield was about 50% based on the amount of lysolecithin used. Spin probe 1 was then introduced into dimyristoylphosphatidylcholine multilamellar membranes and changes in the ESR spectra were monitored as a function of temperature. As shown in **Fig. 2,** the spectra became broader as the temperature decreased, suggesting that the motion of spin probe 1 in dimyristoylphosphatidylcholine vesicles reflects the structural organization of the membrane as suggested previously by Devaux and coworkers using other membrane systems (3–8). Anal. calcd. for $C_{33}H_{64}N_2PO_{10}$ (1-palmitoyl-2-(4-doxylpentanoyl) glycerophosphocholine): C, 58.30; H, 9.49; N, 4.12. Found: C, 57.78; H, 9.66; N, 4.82.

During the condensation reaction, the presence of xylene may have served two purposes: *a*) to increase the miscibility of the reactants, thus enhancing the reaction rate; and *b*) the high boiling point of xylene permits the reaction to be conducted at 70°C in a nitrogen-filled and sealed flask.

Concluding remarks

In this communication, we have described in detail for the first time the synthesis of 1-palmitoyl-2-(4-doxylpentanoyl) glycerophosphocholine, a spin-labeled phospholipid potentially useful for studying lipid dynamics in the plasma membrane of intact mammalian cells (3–8). Without major modifications, our methods should be applicable for the preparation of other doxyl derivatives of short acyl chains, and for the synthesis of other similar phospholipids with different head groups.

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