Chapter 2

Chemical synthesis of phospholipids
This chapter describes the synthesis, purification and characterization of NBD-labeled and non-labeled glycerophospholipids and their corresponding 1,3,4-butanetriol-containing analogs. The synthesis of the butanetriol-containing phosphatidylycerolines has been described earlier by Arora and Gupta (1997a). However, the synthesis of the corresponding NBD-labeled phosphatidylycerolines, -ethanolamines and -serines has been carried out for the first time.

2.1 MATERIALS

All the reagents and the solvents used in the synthesis were of the highest purity available. rac-Butanetriol was purchased from Aldrich Chemical company. N,N-dimethyl-4-aminopyridine (DMAP), Palmitic acid, diisopropylethylamine, trimethylammonium chloride, sn-glycero-3-phosphocholine, dicyclohexylcarbodiimide (DCC), Phospholipase A$_2$ from Naja naja snake venom, NBD-C1 (6-N-(7-nitrobenz-2-oxa-1,3-diazo-4-yl) chloride), 6-aminohexanoic acid, 12-aminododecanoic acid, Triton X-100, 4-chloro-7-nitrobenz-2-oxa-1,3-diazol, 1,1'-carbonyldimidazole, L-serine, calcium chloride, ethanolamine, Sephadex LH-20, EGTA, EDTA and octylglucoside were bought from Sigma Chemical Company. Benzylchloride, 10% Pd-C, and silica gel (60-120 mesh) were from Sisco Research laboratories. Boron trifluoride-etherate and triphenylmethylchloride were from Spectrochem Ltd., Bombay and Fluka A. G., Switzerland, respectively.
2.2 GENERAL METHODS

Most of the reagents were purified prior to their use in the chemical synthesis. Palmitic acid recrystallized from dry hexane. DMAP was recrystallized from a hot solution of dry hexane-benzene (3:2 v/v). rac-1,2,4-Butanetriol, DCC, diisopropylethylamine and benzylchloride were purified by distillation under vacuo. Phosphorustrichloride and boron trifluoride-etherate were purified by simple distillation. The solvents used in recrystallization and chemical reactions were dried exhaustively using the published procedures.

Palmitic acid anhydride was prepared by reaction of palmitic acid with DCC (Salinger and Lapidot, 1966). 2-Chloro-2-oxo-1,3,2-dioxaphospholane was prepared by reacting ethyleneglycol with phosphorustrichloride (Lucas et al., 1950) followed by oxidation of the reaction product (Edmundson, 1962). N-Triphenylmethyl-N',N'-dimethyl-4-aminopyridinium chloride was prepared by reacting triphenylmethylchloride with DMAP in dichloromethane (Hernandez, et al., 1981). 6-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoic acid and 12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoic acid were prepared from 6-aminohexanoic acid and 12-aminododecanoic acid respectively, by using standard procedures. The lysophospholipids were prepared by treatment of diacylglycerophospholipids with *Naja naja* phospholipase A₂ in the presence of calcium chloride (Dennis, 1983). These methods are briefly described in the miscellaneous section.

Purity of various reaction intermediates was checked by TLC on silica gel G-60 plates. Homogeneity of phospholipids was established by both TLC and HPLC. TLC
plates were developed in chloroform/methanol/water (65:25:4, v/v/v) mixture and the phospholipid spots visualized by staining the plate with iodine vapor followed by molybdenum-blue spray (Goswami and Frey, 1971). HPLC was performed using Waters HPLC pump (Model 510) along with Waters R 401 differential refractometer or Waters fluorescence detector (Model 420-AC) interfaced with Waters System Interface Module. Chromatogrammes were analyzed by using baseline 810 software. Analytical HPLC was performed on an Altex Ultrasphere C18 reverse-phase column (4.6 x 250 mm, particle size 5 μm) using methanol/chloroform/water (87:13:6, v/v/v) containing 176 mM choline chloride (Krisovitch and Regen, 1992) at a flow rate of 1.0 ml/min as the elution system, whereas preparative HPLC was carried out on an LKB TSK-ODS-120T semiprep column (7.8 x 300 mm, particle size 10 μm) using methanol/chloroform/water (90:10:4, v/v/v) at a flow rate of 2 ml/min as the eluant (Gupta et al., 1977).

Phospholipids were purified by silica gel column chromatography, Sephadex LH-20 column (2.5 x 100 cm) chromatography, and preparative HPLC. The Sephadex LH-20 column was eluted with chloroform/methanol (1:1, v/v) mixture at a flow rate of about 60 ml/h. All the phospholipids thus-purified exhibited single spots on TLC and single peaks on analytical HPLC, and were characterized by NMR and mass spectrometry analysis. 1H-NMR spectra were recorded on a Bruker ACP 300 FT-NMR spectrometer, and the chemical shifts have been expressed in parts per million down-field from tetramethylsilane. Positive ion FAB mass spectrometry was performed
on a Jeol JMS-SX 102 FAB mass spectrometer equipped with JMA-DA 6000 data system.

2.3 Chemical Synthesis of Phospholipids

1,2-Dihexadecanoyl-sn-glycero-3-phosphocholine (I) (Fig. 17) was prepared by acylation of sn-glycero-3-phosphocholine with palmitic acid anhydride in the presence of DMAP, followed by removal of cadmium chloride over mixed-bed resin, and purification over Sephadex LH-20 (Gupta et al., 1977).

1,3-Dihexadecanoyl-rac-but-4-yl-[2-(triethylammonium) ethyl] phosphate was prepared from rac-1,2,4-butanetriol in several steps (Arora and Gupta, 1997a), which are described below.

2.3.1 Preparation of 1,3-dihexadecanoyl-rac-but-4-yl-[2-(triethylammonium) ethyl] phosphate (II)

The synthesis was carried out according to the Scheme 1.

4-hydroxymethyl-1,3-(2-phenyl)dioxane (III): Distilled rac-1,2,4-butanetriol (10.6 g, 0.1 mole) and benzaldehyde (25.4 ml, 0.25 mole) were treated with 10% HCl in dry methanol (100 μl). The heterogeneous mixture was stirred vigorously for 1h at room temperature and then at 45 - 50°C for 1h under vacuo (4 mm pf Hg). The reaction mixture was cooled, and to it was added 100 ml of chloroform. The resulting solution was washed with 5% sodium bicarbonate (2 × 50 ml) followed with water (3 × 50 ml). The organic layer was dried over anhydrous sodium sulfate. The solvent was removed.
and the residue subjected to fractional distillation under vacuo (b.p. 126°C at 0.8 mm of Hg) to obtain 4-hydroxymethyl-1,3-(2-phenyl)dioxane (III). It was further purified by column chromatography over silica gel (1.8 × 100 cm) using benzene/ethylacetate mixture as the eluant. The pure III was eluted in benzene containing 3% ethylacetate. The purity was checked by TLC using benzene/chloroform/ethylacetate/methanol (60:20:4:1 v/v/v/v) as the solvent system. Yield: 11.2-12 g (57-60%). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.50-7.30 (m, 5H), 5.47 (s, 1H), 4.25-4.20 (m, 1H), 3.98-3.85 (m, 2H), 3.65-3.55 (m, 2H), 1.88-1.74 (m, 1H), 1.38-1.32 (m, 1H).

4-benzyloxyethyl-1,3-(2-phenyl)dioxane (IV) : A solution of III (20 g, 103 mmole) in benzene, freshly distilled benzylchloride (13.05 ml, 0.1 mole), powdered potassium hydroxide (87 g, 1.55 mole) and DMAP (100 mg) were taken in dry benzene (400 ml) in a 500 ml round-bottomed flask fitted with a Dean-Stark apparatus for the removal of water formed in the reaction. It was stirred at 100-200°C (bath temperature) for 12 h (Hübner et. al., 1994). The progress of the reaction was monitored by TLC using benzene/chloroform/ethylacetate/methanol (60:20:4:1 v/v/v/v) as the solvent system. After completion of the reaction, the benzene layer was collected in a separating funnel and washed with 1% aqueous hydrochloric acid (150 ml \(\times\) 3) followed by saturated saline (150 ml \(\times\) 6). The washed organic phase was dried over anhydrous sodium sulfate. The solvent was removed and the residue was fractionally distilled under vacuo to get 4-benzyloxyethyl-1,3-(2-phenyl)dioxane (IV). Yield: 20 g (83%); b. p. 173 °C at 0.8 mm, \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 7.60-7.20 (m, 10H), 5.53 (s, 1H), 4.64-4.55 (m, 2H), 4.31-4.25 (m, 1H), 4.18-4.16 (m, 1H), 4.02-3.93 (dt, \(J=\) 11.90 Hz and 2.60 Hz, 1H),
3.69-3.64 (dd, J=10.20 Hz and 6.00 Hz, 1H), 3.55-3.50 (dd, J=10.20 Hz and 4.80 Hz, 1H), 1.95-1.80 (m, 1H), 1.62-1.54 (m, 1H).

4-benzyloxy-1,3-dihydroxy-rac-butane (V) : Compound IV (5 g, 176 mmole) was treated with 0.01 N aqueous hydrochloric acid (150 ml) at 120-130°C for 6 h. The reaction mixture after cooling, was neutralized with 5% sodium carbonate solution saturated with sodium chloride. It was extracted several times with chloroform (50 ml x 6). The organic phase was dried over anhydrous sodium sulphate and the solvent was removed. The residue thus-obtained was chromatographed over silica gel column using chloroform/benzene mixture as the eluant. 4-Benzyloxy-1,3-dihydroxy-rac-butane (V) was eluted in chloroform containing 30% benzene (v/v). Yield: 2.3 g (70 %); m.p. 74-77 °C; H-NMR (CDCl₃) δ 7.45-7.25 (m, 5H), 4.54 (s, 2H), 4.12-4.04 (m, 1H), 3.85-3.76 (t, J=5.50 Hz, 2H), 3.55-3.48 (dd, J=9.40 Hz and 3.50 Hz, 1H), 3.44-3.36 (dd, J=9.40 and 7.50 Hz, 1H), 1.88-1.65 (m, 2H).

1,3-Dihexadecanoyloxy-4-benzyloxy-rac-butane (VI) : A solution of V (1.0 g, 5 mmole), DMAP (1.25 g, 10 mmole) and palmitic anhydride (5.6 g, 11 mmole) in dry chloroform (100 ml) was stirred under nitrogen for 60-70 h in dark (Gupta et. al., 1977). The progress of the reaction was monitored by TLC using hexane/ethylacetate (95:5, v/v) as the solvent system. After completion of the reaction, chloroform was evaporated under reduced pressure. The residue was dissolved in n-hexane and cooled. The palmitic acid thus-crystallized was removed by filtration and the filtrate was washed with 1% aqueous hydrochloric acid (20 ml x 2) followed by water (20 ml x 4). It was dried over anhydrous sodium sulphate. The solvent was removed and the residue
chromatographed first over silica gel (60-120 mesh), using ethylacetate/n-hexane as the eluant, and then over Sephadex LH-20 to give 1,3-dihexadecanoyloxy-4-benzyloxy-rac-butane (VI). Yield: 2.8 g (80%); m.p. 38-40°C; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$ 1760 (C=O); $^1$H-NMR (CDCl$_3$) $\delta$ 7.45-7.30 (m, 5H), 5.34-5.26 (m, 1H), 4.64-4.50 (m, 2H), 4.20-4.10 (m, 2H), 3.60-3.52 (m, 2H).

1,3-Dihexadecanoyloxy-4-hydroxy-rac-butane (VII): A solution of VI (2 g, 3 mmole) in ethanol/ethylacetate (85:15, v/v) mixture (100 ml) was hydrogenated over 10% palladium on charcoal (700 mg) at room temperature at 60 psi hydrogen pressure for 12 h. The progress of the reaction was monitored by TLC using chloroform/acetone (95:5, v/v) as a solvent system. The catalyst was removed by filtration through a celite bed and the solvent evaporated off under vacuo. The residue was purified by chromatography over Sephadex LH 20 column to give 1,3-dihexadecanoyloxy-4-hydroxy-rac-butane (VII). Yield: 1.6 g (90%); m.p. 73-75°C; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3300 (OH) and 1740 (C=O); $^1$H-NMR (CDCl$_3$) $\delta$ 5.1-5.0 (m, 1H), 4.24-4.10 (m, 2H), 3.80-3.60 (m, 2H).

1,3-Dihexadecanoyloxy-rac-but-4-yl-[2-(trimethylammonium) ethyl] phosphate (II): A solution of 2-chloro-2-oxo-1,3,2-dioxaphospholane (25 mg, 0.175 mmole) in dry benzene (2 ml) was cooled to 4°C under stirring. To this was added a solution of VII (100 mg, 0.170 mmole), triethylamine (23 µl, 0.258 mmole) and 5 mg of DMAP in dry benzene (3 ml) during 15-20 min. After the addition was complete, stirring was continued further for 40-50 min at 4°C and then at room temperature for 24 h (Chandrakumar and Hajdu, 1982; Arora and Gupta, 1997a). The progress of the
reaction was monitored by TLC using chloroform/acetone (95:5, v/v) as a solvent system. The insoluble material was removed by filtration, and the solvent from the filtrate was removed under reduced pressure. The residue was dried in vacuo for 3 h at 40-50°C. It was dissolved in 2 ml of dry acetonitrile and transferred to a reaction bomb vessel to which was added a 2 ml solution of anhydrous triethylamine in acetonitrile (32 g/100 ml). After sealing, the vessel was heated at 60-70°C for 40 h. It was cooled and the solvent was removed in vacuo. The residue was purified first over silica gel and then over Sephadex LH-20 column to get pure 1,3-dihexadecanoyloxy-rac-but-4-yl-[2-(trimethylammonium) ethyl] phosphate (II). Yield: 93 mg (65%); IR (KBr) νmax cm⁻¹: 3430 (OH), 1735 (O=O), 1240 (P=O), 1090 (P=O); FAB mass 749 (MH⁺); 1H-NMR (CDCl₃) δ 5.06-5.05 (m, 1H, -CH=O-C≡O-CH₂-), 4.45-4.35 (b, 2H, -P-O-CH₂-), 4.15-4.05 (m, 2H, -CH₂-OC(=O)-CH₂-), 3.95-3.85 (m, 2H, -OCH₂-C(CH₃)₃), 3.85-3.75 (b, 2H, -CH₂-N(CH₃)₂), 3.40-3.30 (s, 9H, -N(CH₃)₃), 2.45-2.35 (m, 4H, -CH₂-C(=O)O-), 2.05-1.85 (m, 2H, -CH₂-CH₂-CH₂-), 1.70-1.55 (m, 4H, -CH₂-CH₂-CO(=O)-).

2.3.2 Preparation of 1,3-dihexadecanoyl-(3R)-but-4-yl-[2-(trimethylammonium) ethyl] phosphate (DPBPC)

DPBPC was prepared from 1,3-dihexadecanoyloxy-rac-but-4-yl-[2-(trimethylammonium)ethyl] phosphate according to the scheme II. The details of the steps involved are given below.
Figure 17. Molecular structures of phosphatidylcholine analogs wherein glycerol moiety have been replaced by the butanetriol residue.
1-Hexadecanoyloxy-3-hydroxy-(3R)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate (XVIII)

A thin film of II (100 mg, 0.134 mmole) was treated under shaking with CaCl₂·2H₂O (64 mg, 0.435 mmole), Naja naja snake venom (3.0 mg, 0.23 μmole), 2% methanol in dry diethyl ether (20 ml) and 10 ml of Tris buffer (20 mM, pH 8.8) in a 125 ml reagent bottle for 12 h at 37°C. Progress of the reaction was monitored by TLC using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. The organic solvents from the reaction mixture were evaporated at 40°C under a jet of N₂. The remaining reaction mixture was carefully transferred to a separatory funnel. The phospholipids were extracted by Bligh Dyer extraction procedure (Bligh and Dyer, 1959) using chloroform/methanol (1:2, v/v). The extracts were concentrated under reduced pressure at <40°C and the residue dried under vacuo for 1-2 h. The solid so-obtained was dissolved in chloroform/methanol (1:1, v/v). The insoluble impurities at this stage were removed by filtration. The solvents were removed from the filtrate and the residue was chromatographed over Sephadex LH-20 column to give 1-hexadecanoyloxy-3-hydroxy-(3R)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate. Yield: 28.5 mg (35%).

1,3-Dihexadecanoyloxy-(3R)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate (DPBPC)

DPBPC was prepared according to the procedure of Gupta et al. (1977). A mixture of XVIII (28 mg, 0.0374 mmole), palmitic anhydride (46.26 mg, 0.0935...
SCHEME II

Phospholipase A₂

Tris buffer; methanol/ether

C₁₉H₅₆C-0-CH₂

HO-C-H-O

CH₂-O-P-O-CH₂-CH₂-N-C₆H₅

O⁻

XVIII

Sephadex LH-20 chromatography

Pure XVIII

DMAP

(C₁₄H₂₇-C-₁)₂O

C₁₉H₅₆C-0-CH₂

O

C₁₉H₅₆C-H

CH₂-O-P-O-CH₂-CH₂-N-C₆H₅

O⁻

DPBPC

unhydrolysed (3S)-isomer
mmole), DMAP (4 mg, 0.050 mmole) in dry chloroform (100 ml) was stirred under N₂ atmosphere in dark. Course of the reaction was monitored by TLC. Chloroform/Methanol/water (65:25/4, v/v/v) was used as the solvent system for TLC. After completion of the reaction (48-72 hr), methanol (100 ml) was added and the insoluble material was removed by filtration through a sintered funnel. The solvent was evaporated from the filtrate at <40°C, and the residue dried in vacuo for 30 min. It was then dissolved in a minimum quantity of chloroform/methanol/water (4:5:1, v/v/v; 15 ml) and stirred with mixed bed resin (10 g) on a shaker for 2 h. The resin was removed by filtration. The solvents were evaporated from the filtrate and the residue dried in vacuo for 3-4 h. DPBPC was isolated from the residue by sephadex LH-20 chromatography using chloroform/methanol (1:1, v/v) as the eluant. Yield: 1.8-2.0 g (59-65%).

2.3.3 Preparation of fluorescent-labeled phospholipid analogs

NBD-labeled phosphatidylcholine analogs were prepared according to the Scheme III and Scheme IV. The details of the steps involved are described below.

6-Aminohexananoic acid-methyl ester hydrochloride (Xa)

Thionyl chloride (3.00 ml, 4.92 g, 41.35 mmole) was added dropwise to an ice chilled methanol (30 ml) with stirring and the reaction continued overnight at room temperature. The solvent and other volatile materials were evaporated off under vacuo. The residue was left overnight in vaccum in the presence of sodium hydroxide to
remove the absorbed hydrogen chloride. The material (Xa) thus-obtained was, without any further purification, yield: 2.76 g (~ 100%).

**Methyl-6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoate (Xla)**

A solution of Xa (2.76 g), DMAP (5.4 g, 44.2 mmole), NBD-Cl (969 mg, 4.85 mmole) and triethyl amine in dry chloroform was stirred at room temperature for 24 h, in dark. The progress of the reaction was monitored by TLC, using chloroform/methanol (95:5, v/v) as the solvent system (Rf 0.9) The solvent was evaporated in vacuo. The residue was purified over silica gel using chloroform as the eluant to give Xla. Yield : 1.15 g (42%); $^1$H-NMR (CDCl$_3$) δ 8.5 (d, J=9 Hz, 2H), 6.45-6.35 (t, 1H), 6.19 (d, J=9 Hz, 1H), 3.80-3.60 (s, 3H), 3.59-3.45 (m, 2H), 2.45-2.32 (t, 2H), 1.91-1.73 (m, 2H), 1.80-1.64 (m, 2H), 1.60-1.49 (m, 2H).

**6-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoic acid (XIIIa)**

Compound XI (5.75 mg, 1.86 mmole) was dissolved in dry tetrahydrofuran (10 ml) under stirring at 4°C. To this was added aqueous solution of sodium hydroxide (72 mg in 1 ml water) dropwise, under constant stirring. The reaction was completed in 45 min. The solvent was evaporated off in vacuo. The aqueous phase was acidified with citric acid to a pH 5.0. The compound was extracted from the aqueous phase with ethyl acetate (100 x 5 ml). The combined ethyl acetate extracts were washed several times with equal volumes of saline. The washed organic phase was dried over anhydrous sodium sulphate. The solvent was removed. The residue was purified over silica gel using chloroform/methanol as the eluant. 6-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoic acid (XIIIa) was eluted in chloroform containing 10% methanol. Yield
SCHEME III

NH₂(CH₂)ₙ-COOH  \( \xrightarrow{\text{CH₃OH}} \)  HCl-NH₂(CH₂)ₙ-COOCH₃

(X)

(i) NBD-Cl  
(ii) DMAP  
(iii) Et₃N  
(iv) CHCl₃ (dry)

NH-(CH₂)ₙ-COOCH₃

(XI)

(i) NaOH  
(ii) THF (dry)

NH-(CH₂)ₙ-COOH  \( \xrightarrow{\text{Citric Acid}} \)  NH-(CH₂)ₙ-COONa

(XII)

a, n=5  
b, n=11
520 mg (95%). 1H-NMR (CDCl3 / CH3OH) δ 12.25-11.15 (b, 1H), 8.5 (d, J=9 Hz, 1H), 6.45-6.34 (t, 1H), 6.19 (d, J=9 Hz, 1H), 3.57-3.44 (m, 2H), 2.45-2.31 (t, 2H), 1.90-1.72 (m, 2H), 1.80-1.65 (m, 2H), 1.60-1.50 (m, 2H).

12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoic acid (XIIIb)

It was prepared following similar steps as described for the synthesis of 6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoic acid (XIIIa).

2.3.3.1 1-Hexadecanoyloxy-3-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-(3R)-but-4-yl-[(2-trimethylammonium) ethyl] phosphate (XVIa, C6-NBD-bPC)

A solution of XIIIa (112 mg, 0.495 mmole) and 1,1'-carbonyldiimidazole (80.28 mg, 0.495 mmole) in dry THF (2 ml) was stirred at room temperature in a sealed round bottom flask (Yu and Dennis, 1992). Since the reaction generates carbon dioxide, pressure builds up inside the vial, and therefore, the pressure was released three to four times. The reaction was allowed to continue until no further pressure build up was observed (about 1 h). This mixture was then added to the mixture of 1-hexadecanoyloxy-3-hydroxy-(3R)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate (81 mg, 0.495 mmole), triethylamine (50 mg, 0.495 mmole) and DMAP (60.47 mg, 0.495 mmole) in 1.0 ml of dry chloroform, and the resulting mixture was stirred at room temperature for 48 h. The reaction was monitored by TLC using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. Without further
treatment, the reaction mixture was applied directly to a preparative TLC and chromatographed, using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. It was further purified by chromatography followed by purification over Sephadex LH-20, using chloroform/methanol (1:1, v/v) as the eluant. Yield: 45-50 mg (35-40%); FAB mass 772 (M+); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.49 (d, J=9 Hz, 1H), 6.23 (d, J=9 Hz, 1H), 5.28-5.21 (m, 1H), 4.37-4.29 (b, 2H), 4.17-4.08 (m, 2H), 4.03-3.97 (m, 2H), 3.85 - 3.75 (b, 2H), 3.30 (s, 9H).

Similarly, 1-hexadecanoyloxy-3-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyloxy]-3R)-but-4-yl-[(2-trimethylammonium) ethyl] phosphate (XVIb, C$_{12}$-NBD-bPC), 1-hexadecanoyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexyl]-sn-glycero-3-phosphocholine (XVII, C$_6$-NBD-PC) and 1-hexadecanoyl-2-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-sn-glycero-3-phosphocholine (XVII,C$_{12}$-NBD-PC) were synthesized. The characterization of these compounds is given below.

**C$_6$-NBD-PC:** FAB mass 772 (M$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.49 (d, J=9 Hz, 1H), 6.23 (d, J=9 Hz, 1H), 5.28-5.21 (m, 1H), 4.37-4.29 (b, 2H), 4.17-4.08 (m, 2H), 4.03-3.97 (m, 2H), 3.85 - 3.75 (b, 2H), 3.30 (s, 9H).

**C$_{12}$-NBD-bPC:** FAB mass 856 (M$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.44 (d, J=9 Hz, 1H), 6.19 (d, J=9 Hz, 1H), 5.27-5.20 (m, 1H), 4.30-4.20 (b, 2H), 4.18-4.10 (m, 2H), 4.02-3.97 (m, 2H), 3.63-3.56 (b, 2H), 3.22 (s, 9H).

**C$_6$-NBD-bPC:** FAB mass 786 (M$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.44 (d, J=9 Hz, 1H), 6.14 (d, J=9 Hz, 1H), 5.08-5.01 (m, 1H),
4.27-4.21 (b, 2H), 4.14 - 4.04 (b, 2H), 4.02-3.99 (m, 2H), 3.58-3.49 (b, 2H), 3.14 (s, 9H), 2.03-1.82 (m, 2H). C_{12}-NBD-bPC: FAB mass 870 (MH⁺); ¹H-NMR (CDCl₃/CD₃OD) δ 8.43 (d, J=9 Hz, 1H), 6.14 (d, J= 9 Hz, 1H), 5.06-5.00 (m, 1H), 4.29-4.21 (b, 2H), 4.16-4.05 (b, 2H), 4.03-4.01 (m, 2H), 3.56-3.46 (b, 2H), 3.14 (s, 9H), 2.04-1.82 (m, 2H).

2.3.3.2 1-Hexadecanoyloxy-3-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-{(3R)-but-4-yl-phosphoserine (C₆-NBD-bPS), 1-hexadecanoyloxy-3-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyloxy]-{(3R)-but-4-yl-phosphoserine (C₁₂-NBD-bPS), 1-hexadecanoyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohecanoyl]-sn-glycerol-3-phosphoserine (C₆-NBD-PS) and 1-hexadecanoyl-2-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminodocecanoyl]-sn-glycerol-3-phosphoserine (C₁₂-NBD-PS) (fig. 18, 19)

The NBD-labeled PS analogs were prepared by transesterification of their corresponding NBD-bPC and NBD-PC analogs (Comfurius et. al., 1990). NBD-PC (or NBD-bPC) in chloroform/methanol (1:1, v/v) was dried in a glass tube under a slow jet of nitrogen gas. The lipid was solubilised again in pure chloroform and dried under a slow jet of nitrogen, and the procedure was repeated three times to remove traces of methanol or ethanol. A solution of 45% (w/v) serine, 0.1 M CaCl₂ and 4% (w/v) n-octyl-β-D-gluco side in 0.1 M acetate buffer was prepared at 45°C and pH adjusted to 5.6. Ten times (w/v) of this solution was added to the dried NBD-bPC (or NBD-PC).
Figure 18. Molecular structures of butanetriol containing NBD-labeled phospholipid analogs.

C_{15}H_{31}C—O—CH_{2}

NH—(CH_{2})_{n}C—O—C—H

NO

CH_{2}O—P—O—CH_{2}X

XVI

C_{6}-NBD-bPC: n=5; X=—CH—N

C_{6}-NBD-bPE: n=5; X=—CH—NH_{3}

C_{6}-NBD-bPS: n=5; X=—CH—NH_{3}

C_{12}-NBD-bPC: n=11; X=—CH—N

C_{12}-NBD-bPE: n=11; X=—CH—NH_{3}

C_{12}-NBD-bPS: n=11; X=—CH—NH_{3}

COOH
The transesterification reaction was initiated by adding phospholipase D from Streptomyces species to a final concentration of 5 μg/ml. The reaction mixture was incubated at 45°C for 2 h under shaking. The reaction was stopped by adding two volumes of EDTA solution (100 mM in water). To it was added ten volumes of chloroform/methanol (1:1, v/v), the mixture was stirred for 10 min and then centrifuged to remove the insoluble material. The supernatant was collected, evaporated and then dried by azeotroping with dry benzene containing a few drops of absolute ethanol. Finally the lipid was extracted from the dried material with pure chloroform and purified by preparative TLC, Sephadex LH-20 column chromatography and HPLC. 

$C_{0\text{-NBD-PS}}$: FAB mass 774 (MH$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.47 (d, J=8.7 Hz, 1H), 6.16 (d, J= 8.7 Hz, 1H), 5.38-5.26 (m, 1H), 4.36-4.24 (b, 2H), 4.20-4.13 (b, 2H), 4.04-3.95 (m, 2H), 3.65 - 3.58 (b, 1H). 

$C_{0\text{-NBD-PS}}$: FAB mass 788 (MH$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.44 (d, J=9 Hz, 1H), 6.11 (d, J= 9 Hz, 1H), 5.32-5.24 (m, 1H), 4.37-4.23 (b, 2H), 4.20-4.12 (b, 2H), 4.03-3.97 (m, 2H), 3.65-3.59 (b, 1H), 2.33 - 2.10 (m, 2H). 

$C_{12\text{-NBD-PS}}$: FAB mass 858 (MH$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.48 (d, J=8.7 Hz, 1H), 6.21 (d, J= 8.7 Hz, 1H), 5.22-5.16 (m, 1H), 4.34-4.26 (b, 2H), 4.20-4.08 (b, 2H), 4.07-4.01 (m, 2H), 3.62-3.53 (b, 1H). 

$C_{12\text{-NBD-PS}}$: FAB mass 872 (MH$^+$); $^1$H-NMR (CDCl$_3$/CD$_3$OD) $\delta$ 8.37 (d, J=9 Hz, 1H), 6.09 (d, J= 9 Hz, 1H), 5.07-5.01 (m, 1H), 4.29-4.21 (b, 2H), 4.14-4.04 (b, 2H), 4.03-3.97 (m, 2H), 3.59-3.46 (b, 1H), 1.98-1.82 (m, 2H).
Figure 19. Molecular structures of NBD-labeled glycerophospholipid analogs.
2.3.3.3 1-Hexadecanoyloxy-3-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-(3R)-but-4-yl-phosphoethanolamine (C₆-NBD-bPE), 1-hexadecanoyloxy-3-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyloxy]-(3R)-but-4-yl-phosphoethanolamine (C₁₂-NBD-bPE), 1-hexadecanoyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-sn-glycero-3-phosphoethanolamine (C₆-NBD-PE) and 1-hexadecanoyl-2-[12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-sn-glycero-3-phosphoethanolamine (C₁₂-NBD-PE) (fig. 18, 19)

The NBD-labeled PE analogs were prepared by transesterification of their corresponding NBD-bPC and NBD-PC analogs (Comfurius et al., 1990). NBD-PC (or NBD-bPC) was dried in a glass tube under a slow jet of nitrogen gas. The lipid was solubilised again in pure chloroform and dried under a slow jet of nitrogen, and the procedure was repeated three times to remove traces of methanol or ethanol. Ethanolamine (4%, w/v) (10 times of the weight of lipid), 0.1 M CaCl₂ and 4% (w/v) n-octyl-β-D-glucoside in 0.1 M acetate buffer (pH 5.6) was added to the dried NBD-PC (or NBD-bPC). The transesterification reaction was initiated by adding phospholipase D from Streptomyces species to a final concentration of 5 μg/ml. The reaction mixture was incubated at 35°C for 2 h under shaking. The reaction was stopped by adding two volumes of EDTA solution (100 mM in water). To it was added ten volumes of chloroform/methanol (1:1, v/v), the mixture was stirred for 10 min and then centrifuged to remove the insoluble material. The supernatant was collected, evaporated and then dried by azeotroping with dry benzene containing a few drops of
absolute ethanol. Finally, the lipid was extracted from the dried material with pure chloroform and purified by preparative TLC, Sephadex LH-20 column chromatography and HPLC. C\textsubscript{6}-NBD-PE: FAB mass 730 (MH\textsuperscript{+}); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}/CD\textsubscript{3}OD) \(\delta\) 8.43 (d, J=8.7 Hz, 1H), 6.13 (d, J= 8.7 Hz, 1H), 5.22-5.15 (m, 1H), 4.35-4.24 (b, 2H), 4.22-4.10 (b, 2H), 4.05-3.98 (m, 2H), 3.61-3.47 (b, 2H). C\textsubscript{6}-NBD-bPE: FAB mass 744 (MH\textsuperscript{+}); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}/CD\textsubscript{3}OD) \(\delta\) 8.46 (d, J=9 Hz, 1H), 6.16 (d, J= 9 Hz, 1H), 5.29-5.21 (m, 1H), 4.38-4.26 (b, 2H), 4.24-4.13 (b, 2H), 4.06-3.99 (m, 2H), 3.64-3.51 (b, 2H), 2.31-2.09 (m, 2H). C\textsubscript{12}-NBD-PE: FAB mass 814 (MH\textsuperscript{+}); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}/CD\textsubscript{3}OD) \(\delta\) 8.44 (d, J=8.7 Hz, 1H), 6.12 (d, J= 8.7 Hz, 1H), 5.16-5.07 (m, 1H), 4.38-4.25 (b, 2H), 4.20-4.11 (b, 2H), 4.10 - 4.03 (m, 2H), 3.67-3.49 (b, 2H). C\textsubscript{12}-NBD-bPE: FAB mass 828 (MH\textsuperscript{+}); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}/CD\textsubscript{3}OD) \(\delta\) 8.45 (d, J=9 Hz, 1H), 6.13 (d, J= 9 Hz, 1H), 5.13-5.06 (m, 1H), 4.36-4.24 (b, 2H), 4.19-4.09 (b, 2H), 4.06-3.99 (m, 2H), 3.67-3.49 (b, 2H), 2.02-1.81 (m, 2H).

2.3.4 MISCELLANEOUS

Palmitic Anhydride : Recrystallised palmitic acid (10 g, 39 mmoles) was dissolved in dry carbontetrachloride (50 ml) and to it was added a solution of DCC (4.43 g, 21 mmoles) in dry carbontetrachloride (10 ml) (Salinger and Lapidot, 1966). After stirring at room temperature for 3h, the precipitated dicyclohexylurea was removed by filtration through celite. Solvent was evaporated and residue dried and recrystallized from dry hexane to give pure palmitic acid anhydride. Yield : 7.2 g (75%); m. p. 62-64\textdegree C; IR (KBr) \(\nu_{\text{max}}\) cm\textsuperscript{-1} 1740 and 1810 (C=O).
1-Triphenylmethyl-N',N'-dimethyl-4-aminopyridinium chloride: Freshly crystallized triphenylmethyl chloride (3.06 g, 11 mmole) and 4-dimethylaminopyridine (1.22 g, 10 mmole) in dry dichloromethane (20 ml) were stirred for 1 h under nitrogen at room temperature (Hernandez et al., 1981). Addition of 100 ml of absolute diethyl ether precipitated 1-triphenylmethyl-N',N'-dimethyl-4-aminopyridinium chloride, which was filtered and washed with ether, and dried. Yield: 3.90 g (95%); m. p. 126-128°C.

2-Chloro-2-oxo-1,3,2-dioxaphospholane: Freshly distilled phosphorus trichloride (22 ml, 0.25 mole) in dry dichloromethane (50 ml) was taken in a two-necked flask fitted with a reflux condenser and a dropping funnel. To it was added distilledethylene glycol (13.9 ml, 0.25 mole), dropwise and under constant stirring, at such a rate that the heat generated was sufficient to keep the mixture under gentle reflux (Lucas et al., 1950). After completion, the flask was adapted for distillation and most of the dichloromethane was distilled off. The remainder, distilled under vacuo, afforded pure 2-chloro-1,3,2-dioxaphospholane. Yield: 18 g (80%); b. p. 60°C at 17 mm of Hg.

2-Chloro-1,3,2-dioxaphospholane (18 g, 0.20 mole) was dissolved in dry benzene (30 ml). Dry oxygen gas was slowly bubbled through the solution, under stirring for 16 h (Edmundson, 1962). After completion, the solvent was removed and pure 2-chloro-2-oxa-1,3,2-dioxaphospholane was obtained by subjecting the residue to fractional distillation under vacuo. Yield: 12.7 g (60%); b. p. 90°C at 0.8 mm of Hg.
2.3.5 Isolation and Purification of egg PC

Egg PC was isolated according to the procedure of Singleton et. al (1965) after slight modifications. Briefly, egg yolk of two dozen eggs was taken out carefully and washed with acetone (100 ml) in a mixer for 5-6 min and filtered. The residue was resuspended in acetone (100 ml). This process was repeated 5-6 times to get rid of colored impurities, until the filtrate was colorless. The resulting white solid was powdered and dried in vacuo for 1h to remove traces of the solvent. The extraction was carried out by stirring the solid mass with 1L of absolute ethanol. It was then filtered and the residue was washed with absolute ethanol (200 ml). The filtrates were mixed and evaporated off at 40-45°C. The sticky mass thus obtained was dried under high vacuum to remove the traces of solvent, dissolved in a minimum amount of petroleum ether (b. p. 60-80°C) and poured onto chilled acetone to obtain a white sticky precipitate. The solvents was decanted off. The process was repeated 2-3 times to obtain crude PC. It was then chromatographed on a neutral alumina (7-8% hydrated). The elution was performed using increasing amounts of methanol in chloroform. Pure egg PC got eluted at 5-7 % methanol in chloroform. It was further purified by Sephadex LH-20 chromatography using chloroform/methanol (1:1, v/v). The purity was ascertained on silica gel G-60 plates, using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system and staining with iodine vapors followed by molybdenum-blue spray (Goswami et. al., 1971).
2.3.6 Preparation of 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (I) (DPPC)

DPPC was prepared essentially by the procedure of Gupta et. al. (1977). A mixture of sn-glycero-3-phosphorylcholine CdCl₂ complex (1.83 g, 4.16 mmole), palmitic anhydride (2.57 g, 5.2 mmole), DMAP (0.2 g, 1.66 mmole) in dry chloroform (100 ml) was stirred under N₂ atmosphere in dark. Course of the reaction was monitored by TLC. Chloroform/Methanol/water (65:25/4, v/v/v) was used as the solvent system for TLC. After completion of the reaction (48-72 hr), methanol (100 ml) was added and the insoluble material was removed by filtration through a sintered funnel. The solvent was evaporated from the filtrate at <40°C, and the residue dried in vacuo for 30 min. It was then dissolved in a minimum quantity of chloroform/methanol/water (4:5:1, v/v/v; 15 ml) and stirred with mixed bed resin (10 g) on a shaker for 2 h. The resin was removed by filtration. The solvents were evaporated from the filtrate and the residue dried in vacuo for 3-4 h. 1,2-Dipalmitoyl-sn-glycero-3-phosphorylcholine was isolated from the residue by Sephadex LH-20 chromatography using chloroform/methanol (1:1, v/v) as the eluant. Yield: 1.8-2.0 g (59-65%).

2.3.7 Preparation of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphorylcholine

A mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (0.8 g, 1.09 mmole), CaCl₂:2H₂O (547.9 mg, 3.72 mmole), Naja naja snake venom (10.7 mg, 81.0 μmole), 2% methanol in dry diethyl ether (160 ml) and 80 ml of Tris buffer (20 mM, pH 8.8) in a 500 ml reagent bottle was stirred at 30°C for 3-4 h. Progress of the...
reaction was monitored by TLC using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. The organic solvents from the reaction mixture were evaporated at 40°C under a jet of N₂. The remaining reaction mixture was carefully transferred to a separatory funnel. The phospholipids were extracted by Bligh Dyer extraction procedure (Bligh and Dyer, 1959) using chloroform/methanol (1:2, v/v). The extracts were concentrated under reduced pressure at <40°C and residue was dried under vacuo for 1-2 h. The solid so-obtained was dissolved in chloroform/methanol (1:1, v/v). The insoluble impurities at this stage were removed by filtration. The solvents were removed from the filtrate and the residue chromatographed over Sephadex LH-20 column to give 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphorylcholine. Yield: 0.35-0.40 g (65-75%).

2.4 RESULTS AND DISCUSSION

The modification in the interface region of the phospholipids was introduced by changing the glycerol backbone with butanetriol-moiety. For this a multistep synthetic strategy was employed starting with rac-1,2,4-butanetriol (Scheme I). The design of the modified phospholipids was based on the consideration that the additional methylene residue between the C1 and C2 carbon atoms in the glycerol backbone would lead to an altered phospholipid conformation, such that the C1 methylen would serve as the proximal beginning of the primary acyl chain (Arora and Gupta, 1997a). This would help in analyzing the role of the glycerol backbone or the ester moiety in determining the interaction of the phospholipids with various proteins involved in
regulating the phospholipid metabolism, such as, phospholipase A_2, aminophospholipid translocase and human apolipoprotein A-II.

DPPC and egg PC were prepared following the standard procedures (Gupta et al., 1977, Singleton et. al., 1965) and the corresponding rac-butaneetriol analog (II) was synthesized according to the procedure of Arora and Gupta (1997a) without much modifications. These phospholipids were subsequently purified by silica-gel and Sephadex LH-20 column chromatography and finally by HPLC and characterized by \textsuperscript{1}H-NMR and FAB mass spectroscopy. The \(\text{L}(-)\) isomer of DPBPC was prepared by first treating rac-butaneetriol analog (II) with phospholipase \(\text{A}_2\) and then reacylating the lyso compound (Scheme II).

DPPC and DPBPC were fluorescently labeled with NBD by acylation of the corresponding lyso-phosphatidylcholine analogs with NBD-labeled amino hexanoic and amino dodecanoic acids. The procedure used for acylation (Scheme IV) was the modified version of that used by Yu and Dennis (1992) for the incorporation of fatty acid into the amide analogue of phosphatidylcholines. This procedure was adopted in the present work to acylate lyso-PC with NBD-labeled fatty acids. Since the NBD-labeled \(\text{C}_6\) and \(\text{C}_{12}\) fatty acids were only partially soluble in chloroform, dry THF was used in the reaction and the reaction was increased to 48 h. The purification of the NBD-labeled phosphatidylcholines was carried out by preparative-TLC followed by sephadex LH-20 and HPLC, as described in 'methods'. The final yield after purification was about 40%.
The enzymatic synthesis of NBD-labeled PS and PE analogs was carried out by transesterification of phosphatidylcholines using phospholipase D. The yield of NBD-PS and NBD-PE was significantly increased by replacing phospholipase D from cabbage (~5 % yield) by the enzyme from *Streptomyces species* (Comfurius et. al., 1990). A modification in phospholipase D-catalyzed synthesis of PS and PE was introduced by using one-phase system, that is, using octylglucoside to disperse the lipid instead of the ether-water system, for the enzymatic conversion (Comfurius et. al., 1990). When exactly the same conditions as reported by Comfurius et. al. (1990) were used, the yield was ~40%. Upon increasing the amount of detergent upto 4% (w/v) instead of 2% (w/v), the yield increased upto 60%. Moreover, the reaction time was also reduced from 3 h to 2h. The purification method of NBD-PE and NBD-PS analogs was also changed, that is, instead of purifying by CM-cellulose ion-exchange chromatography, where it was found that even a slight variation in the working procedure results in a major loss of the NBD-labeled PS and PE analogs, the purification was carried out by two-dimensional preparative-thin layer chromatography, using a solvent system chloroform/methanol/ammonia (65:25:5, v/v/v) in the first dimension and chloroform/methanol/acetone/acetic acid/water (40:8:16:9:4, v/v/v/v/v) in the second dimension.