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Immunochemical Studies of Phospholipids.[1] II. Syntheses of Cardiolipin and Its Analogues

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Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol disodium salt (I) was synthesized by the condensation of the silver salt of dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester (XI) with 1,3-diiodopropanol benzyl ether, followed by debenzylation with sodium iodide and hydrogenolysis with palladium black. Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,5-pentanediol disodium salt (III), bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,4-butanediol disodium salt (IV), bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,2-ethanediol disodium salt (VI) and bis(dipalmitoyl D,L-α-glycerylphosphoryl)methanediol disodium salt (VII) were synthesized similarly by the condensation of the silver salt (XI) with alkyl diiodide or dibromide, followed by debenzylation with sodium iodide. Bis(benzylphosphoryl)-1,3-propanediol disodium (X) was synthesized by condensation of silver dibenzyl phosphate with alkyl diiodide, followed by debenzylation with sodium iodide.

The assumption that the serological specificity of cardiolipin in reacting with Wassermann antibody is attributed mainly to the two phosphate groups and the free β-hydroxyl group of the central glycerol moiety has led the authors to synthesize several cardiolipin analogues. In a previous report,[3] the syntheses of dipalmitoyl D,L-α-glycerylphosphorylpropanol sodium salt (VIII) and bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-propanediol disodium salt (V) were reported.

This paper reports the syntheses of bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol disodium salt (synthetic cardiolipin)(I), bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,5-pentanediol disodium salt (III), bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,4-butanediol disodium salt (IV), bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,2-ethanediol disodium salt (VI), bis(dipalmitoyl D,L-α-glycerylphosphoryl)methanediol disodium salt (VII) and bis-(benzylphosphoryl)-1,3-propanediol disodium salt (X)(Fig. 1).

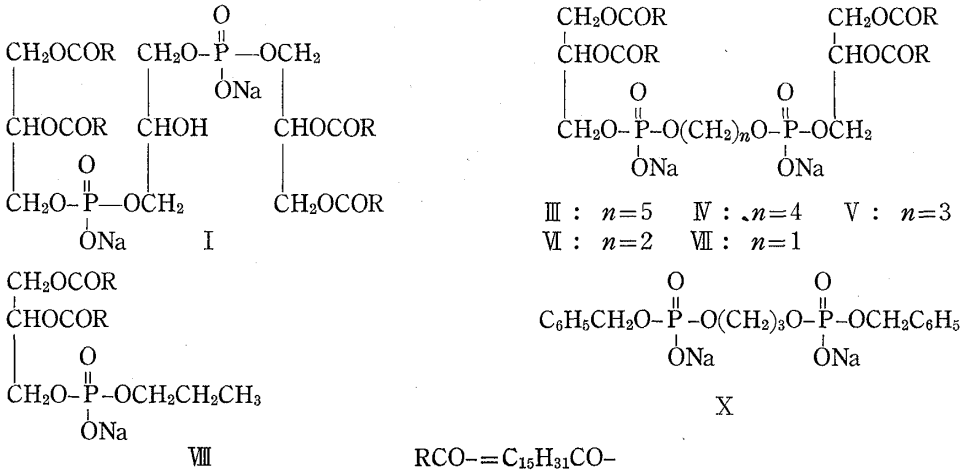
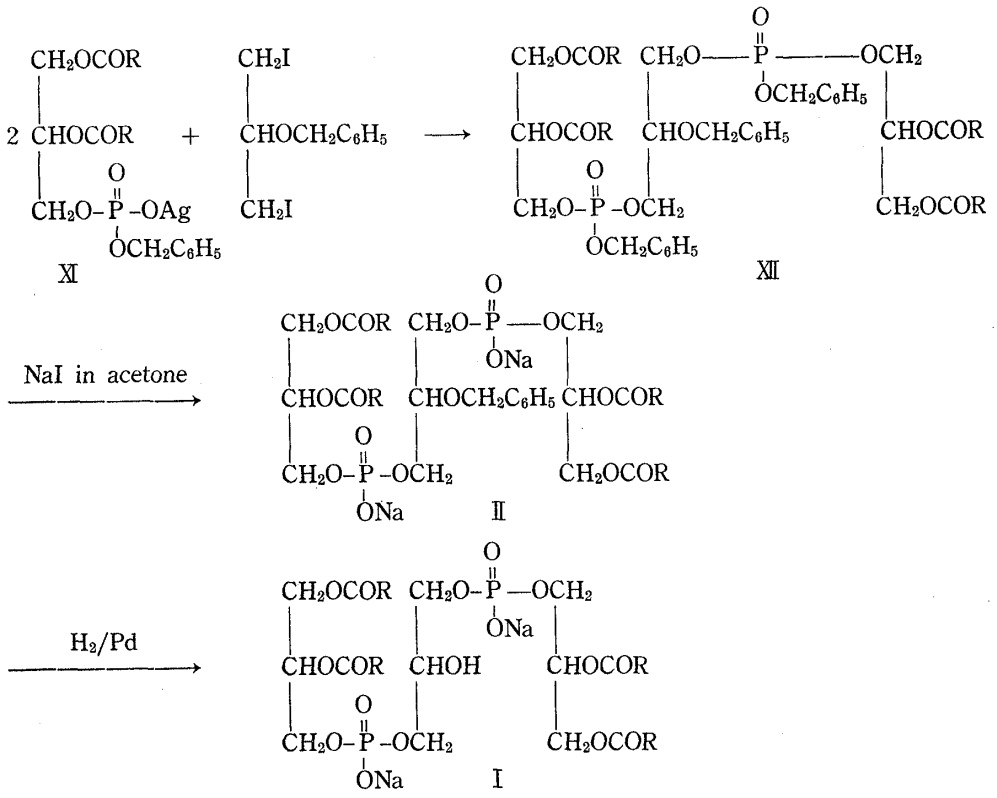


Fig. 1. Chemical Structures of Cardiolipin and Its Analogues

1) Part I: *Chem. Pharm. Bull.* (Tokyo), **11**, 1150 (1963).
2) Location: *Hongo, Tokyo.*
3) K. Inoue, Y. Suhara, and S. Nojima, *Chem. Pharm. Bull.* (Tokyo), **11**, 1150 (1963).

Although de Hass and Van Deenen[4] accomplished the total synthesis of α,γ-bis(β'-oleoyl-γ'-stearoyl-L-α'-glycerylphosphoryl)glycerol dibarium salt, the present authors had been independently trying to synthesize bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol disodium salt (I). First, attempts were made to synthesize cardiolipin by condensation of dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester silver salt (XI) with 1,3-diiodopropanol or 1,3-dibromopropanol, but this was unsuccessful, apparently due to the instability of the synthetic intermediate which is a phosphotriester with a hydroxyl function on the adjacent carbon. Next, 1,3-diiodopropanol-2-benzyl ether was used in place of 1,3-diiodopropanol. The synthetic route is shown in Scheme 1.



Scheme 1. Synthetic Pathways of Cardiolipin

After chromatography on Florisil of the condensation product between dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester silver salt (XI) and 1,3-diiodopropanol-2-benzyl ether, bis[benzyl(dipalmitoyl D,L-α-glyceryl)phosphoryl]-1,3-glycerol benzyl ether (XII) was obtained in a yield of 12%. The benzyl groups protecting the phosphate were removed by treatment with sodium iodide in dry acetone and the benzyl ether function was split off with hydrogen on palladium black. The final product (synthetic cardiolipin) showed a single spot on thin-layer chromatography (TLC) on silica gel (Fig. 2).

The synthetic cardiolipin (I) on TLC with chloroform-methanol-ammonia (75:25:4) as solvent sometimes showed slightly lower *Rf* values than that of beef heart cardiolipin, the acyl residues of which are polyunsaturated, but always had the same Rf values as cardiolipin isolated from *S. griseus*[5] or *E. coli*,[6] the acyl residues of which are saturated and monoenoic. This slight but clear difference in behavior of cardiolipins on TLC seems to be due to the difference in the fatty acid residues in the molecule. The infrared spectrum of this compound is identical with that of authentic cardiolipin (Fig. 3).

4) G.H. de Hass and L.L.M. Van Deenen, *Rec. Trav. Chim.*, **84**, 436 (1965).
5) T. Kataoka and S. Nojima, *Biochim. Biophys. Acta*, **144**, 681 (1967).
6) Y. Kanemasa, Y. Akamatsu, and S. Nojima, *Biochim. Biophys. Acta*, **144**, 382 (1967).

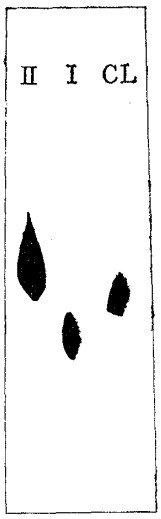


Fig. 2. Thin-layer Chromatogram of Authentic Beef Heart Cardiolipin (CL), Bis (dipalmitoyl D,L-α-glyceryl-phosphoryl)-1,3-glycerol Benzyl Ether Disodium Salt (II) and Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol Disodium Salt (I)

Solvent system : $CHCl_3$-MeOH-NH_4OH (75:25:4)

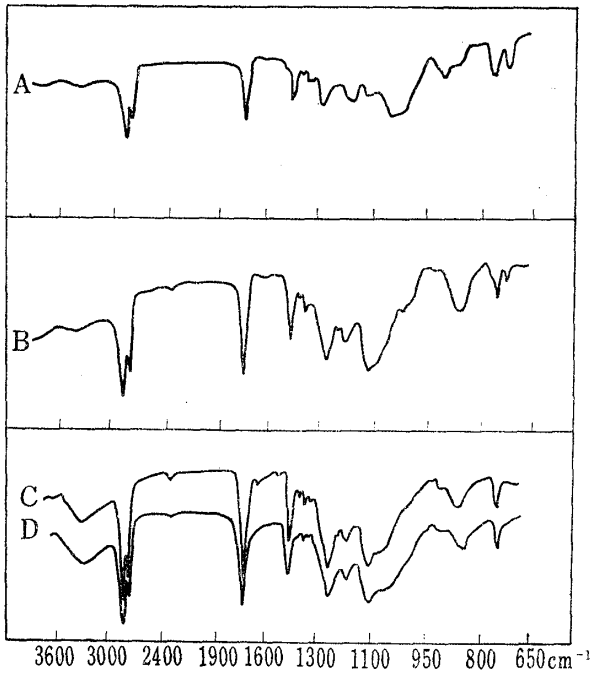


Fig. 3. Infrared Spectra of Synthetic Cardiolipin (I), Its Synthetic Intermediats ((II) and (XII)) and Natural Cardiolipin (from *Streptomyces griseus*)

A : Bis(benzyl-(dipalmitoyl D,L-α-glyceryl)phosphoryl)-1,3-glycerol benzyl ether (XII)
B : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol benzyl ether disodium salt (II)
C : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol disodium salt (I)
D : Natural cardiolipin (from *Streptomyces griseus*)
A Nihon Bunko IR-S (NaCl prism) was used. KBr disk
Ordinate : Transmission Abscissa : Wave number

This product was further characterized by its deacylated product after Dawson's mild alkaline hydrolysis.[7] The Rf value of the product was identical to that of beef heart cardiolipin on paper chromatography and paper electrophoresis. Other cardiolipin derivatives III, IV, VI and VII were synthesized similarly by condensation of dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester silver salt (XI) and 1,5-dibromopentane, 1,4-diiodobutane, 1,2-dibromoethane and diiodomethane, respectively. All of them showed a single spot, except for VII, and the same Rf value on TLC.

Compound VII, could not be isolated as a single spot on TLC even by chromatography on silicic acid treated with sodium bicarbonate.

The infrared spectra of these compounds are shown in Fig. 4. On comparison of the spectra of these synthetic phospholipids, some interesting differences are to be noted. With decrease in the distance between the two phosphate groups, the absorption peak at around 970 cm^{-1} becomes stronger. On the other hand, the spectra of the synthetic cardiolipin and natural caridolipins do not show this absorption peak at around 970 cm^{-1}. These results strongly indicate that the appearance and disappearance of the absorption peak at 970 cm^{-1} is probably due to differences in the interaction of the two phosphate groups.

The last cardiolipin derivative, bis(benzylphosphoryl)-1,3-propanediol disodium salt (X) was synthesized in the same way. It has benzyl groups as hydrophobic groups in place of the dipalmitoylglyceryl groups of V. Unlike other derivatives, this compound is insoluble in almost all organic solvents such as benzene, acetone, methanol, ethanol and even

7) R.M.C. Dawson, *Biochem. J.*, **75**, 45 (1960).

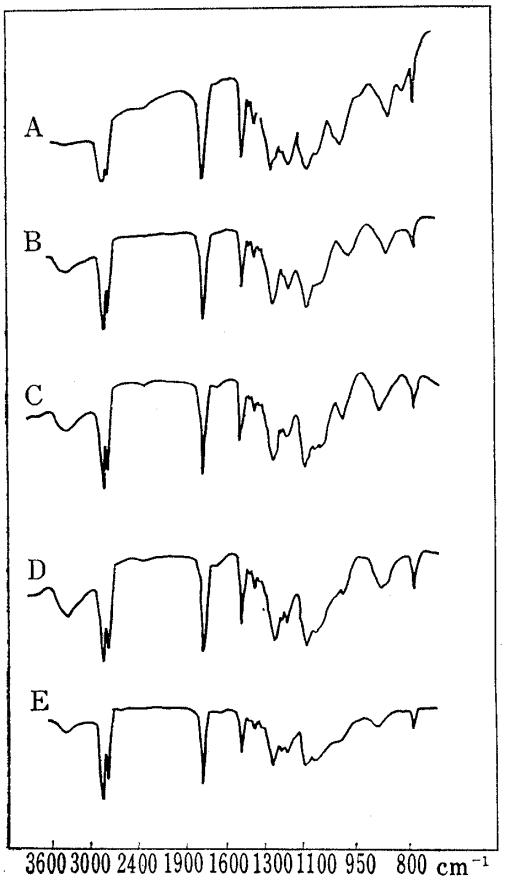


Fig. 4. Infrared Spectra of Cardiolipin Analogues

A : Bis(dipalmitoyl D,L-α-glycerylphosphoryl) methanediol disodium salt (III)
B : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,2-ethanediol disodium salt (IV)
C : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-propanediol disodium salt (V)
D : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,4-butanediol disodium salt (VI)
E : Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,5-pentanediol disodium salt (VII)
A Nihon Bunko IR-S (NaCl prism) was used. KBr disk.

tetrahydrofurane or chloroform, but freely soluble in water, water containing ethanol or water containing tetrahydrofurane.

Reactivity of these synthetic cardiolipin derivatives with Wassermann antibodies has been reported in a separate paper.[8]

Methods and Materials

The preparation of dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester silver salt (XI) was carried out according to the procedure by Stanacev and Kates.[9] Silver dibenzyl phosphate was prepared according to the method described in the previous report.[3] 2-O-benzylglycerol was prepared according to the method of Carter, *et al.*[10] mp 36—37°. 1,4-Diiodobutane was prepared according to a description in the literature.[11] bp 108—110° (10 mm Hg). 1,5-Dibromopentane, 1,2-dibromoethane and diiodomethane were commercial products (Tokyo Kasei Co., Ltd., Tokyo) and were redistilled for use. Palladium black was obtained from Nippon Engelhard, Ltd., Tokyo. Thin-layer chromatography was performed on silica gel G (Merck) with the following solvent systems: (1) $CHCl_3$–CH_3OH (100:1), (2) $CHCl_3$–CH_3OH–H_2O (65:25:4). In this report, the Rf values of the compounds found with solvents (1) and (2) are represented as Rf_1 and Rf_2, respectively. The column of silicic acid treated with sodium bicarbonate was prepared according to the method of Rathbone and Maroney.[12]

Experimental

1,3-Di-*p*-toluenesulfonyl Glycerol Benzyl Ether——To a gently agitated and cooled mixture of 2-O-benzylglycerol (2.6 g, 14.3 mmoles) and 5 ml of dry pyridine was added a solution of *p*-toluenesulfonyl chloride (6.8 g, 35.8 mmoles) in 5 ml of dry pyridine. The reaction mixture was kept in an ice-bath until the reaction had subsided.

After standing for 48 hr at 30°, a crystalline sludge had formed which was poured into 100 ml of ice-cold water with stirring. The white crystals were collected on a glass filter and washed several times with water and then with ether. After recrystallization from benzene and *n*-hexane, 3.9 g of colorless needles were obtained (55.8% of theory) mp 109.5—110.5°. *Anal.* Calcd. for $C_{24}H_{26}O_7S_2$ (mol. wt. 490.58): C, 58.75; H, 5.34. Found: C, 58.80; H, 5.58.

1,3-Diiodopropanol Benzyl Ether——A sample of 1,3-di-*p*-toluenesulfonyl glycerol benzyl ether (3.9 g, 7.9 mmoles) was refluxed in the dark with 3.5 g (23 mmoles) of sodium iodide (dried at 130°) in 30 ml of dry acetone for 10 hr. The hot reaction mixture was filtered through a glass filter to remove the precipitate of sodium *p*-toluenesulfonate and excess sodium iodide. The filtrate was evaporated to dryness under reduced pressure and the reddish brown residue was extracted with ether. The ethereal solution was washed twice with aqueous thiosulfate solution, dried with Na_2SO_4 and evaporated under reduced pressure. Then the residual colorless oil was distilled *in vacuo*, yielding 2.7 g of 1,3-diiodopropanol benzyl ether (85% of theory). bp 128—130° (10^{-3}mm Hg). *Anal.* Calcd. for $C_{10}H_{12}OI_2$ (mol. wt. 402.02): C, 29.87; H, 2.98. Found: C, 29.82; H, 2.87.

8) K. Inoue and S. Nojima, *Chemistry and Physics of Lipids*, **1**, 360 (1967).
9) N.Z. Stanacev and M. Kates, *Can. J. Biochem. Physiol.*, **38**, 297 (1960).
10) H. Hibbert and N.M. Carter, *J. Am. Chem. Soc.*, **51**, 1601 (1927).
11) *Org. Synth*, Coll. Vol., **4**, 321.
12) L. Rathbone and P.M. Marony, *Nature*, **200**, 887 (1963).

Bis[benzyl(dipalmitoyl D,L-α-glyceryl)phosphoryl]-1,3-glycerol Benzyl Ether (XII)——A solution of 3.8 g (4.5 mmoles) of dipalmitoyl D,L-α-glycerophosphoric acid benzyl ester silver salt (XI) and 720 mg (1.8 mmole) of freshly distilled 1,3-diiodopropanol benzyl ether in dry benzene was heated under reflux in the dark for 12 hr.

After cooling, the reaction mixture was centrifuged to remove the yellow precipitate of silver iodide. The resulting clear, pale yellow supernatant was tested by TLC. It showed several spots, the two main components giving Rf_1 values of 0.8 (mono-substituted product) and 0.3 (desired product). The crude product was purified on a Florisil column, eluting with chloroform-methanol mixtures and collecting the fraction (chloroform-methanol (1000:7 v/v)). This fraction was mainly composed of XII contaminated with traces of a phosphorus free compound.

After evaporation of the solvent, an analytically pure, colorless waxy mateiral was obtained. Yield 350 mg (12% of theory). *Anal.* Calcd. for $C_{94}H_{160}O_{17}P_2$ (mol. wt. 1624.18): C, 69.51; H, 9.94; P, 3.81. Found: C, 69.54; H, 10.10; P, 3.67.

This material was subjected to debenzylation without further purification.

Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol Benzyl Ether Disodium Salt (II)——A sample of 200 mg (0.12 mmole) of XII was allowed to react with 64 mg (0.4mmole) of sodium iodide by refluxing the mixture in 3 ml of dry acetone for 5 hr. After discarding the yellow supernatant, the solid material was dissolved in 2 ml of chloroform and precipitated from the solution with 10 ml of acetone. Compound II was obtained as a colorless powder, which showed a single spot (Rf_2 0.7) on TLC. Yield 134 mg (75% of theory) mp 176—179°. *Anal.* Calcd. for $C_{80}H_{146}O_{17}P_2Na_2$ (mol. wt. 1487.92): C, 64.57; H, 9.89; P, 4.16. Found: C, 64.24; H, 9.94; P, 4.01.

Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,3-glycerol Disodium Salt (I)——A sample of 100 mg (0.067 mmole) of II dissolved in 5 ml of tetrahydrofurane was hydrogenolyzed with palladium black (200 mg) for 10 hr. The progress of the reaction was checked by TLC. After 2 hr, the spot of II (Rf_2 0.7) had disappeared, and only that of the desired compound (I) (Rf_2 0.65) could be found.

After removal of the catalyst by centrifugation (3000 rpm, 5 minutes), the supernatant was concentrated to about 1 ml under reduced pressure, and five volumes of acetone were added. The white precipitate was collected on a sintered glass filter, and washed several times with acetone. Yield 73 mg (78% of theory) mp 179.5—184°. *Anal.* Calcd. for $C_{73}H_{140}O_{17}P_2Na_2$ (mol. wt. 1397.80): C, 62.72; H, 10.10; P, 4.44. Found: C, 62.94; H, 10.40; P, 4.48.

Compound I was hydrolyzed with mild alkali according to Dawson's method. A paper chromatogram of the hydrolyzate with (1) phenol-water-acetic acid-ethanol (80:20:10:12) or (2) isopropanol-ammonia-water (7:2:1) as solvent showed the same *Rf* values as those of authentic beef heart cardiolipin. This mild alkaline hydrolyzate also showed the same mobility as that of beef heart cardiolipin on paper electrophoresis.

Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,5-pentanediol Disodium Salt (III)———A sample of 2.80 g (3.2 mmoles) of the foregoing silver salt (XI) was allowed to react with 300 mg (1.3 mmole) of 1,5-dibromopentane in the dark by refluxing in 15 ml of dry benzene for 10.5 hr.

After removal of silver bromide, the clear yellow solution was passed through a Florisil column. A chromatographically and analytically pure phosphotriester intermediate, bis (dipalmitoyl D,L-α-glyceryl-phosphoryl)-1,5-pentanediol dibenzyl ester (960 mg) was obtained, mp 35—36.5°. *Anal.* Calcd for $C_{89}H_{158}O_{16}P_2$ (mol. wt. 1546.11): C, 69.13; H, 10.30: P, 4.01. Found: C, 68.89; H, 10.34; P, 3.99.

The product was converted to III by refluxing with sodium iodide in dry acetone for 5 hr. Yield 50%, mp 188—190°. *Anal.* Calcd for $C_{75}H_{144}O_{16}P_2Na_2$ (mol. wt. 1409.86): C, 63.89; H, 10.30; P, 4.39. Found: C, 63.92; H, 10.43; P, 4.14.

Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,4-butanediol Disodium Salt (IV)——The condensation of 2.36 g (2.8 mmoles) of the silver salt (XI) and 310 mg (1 mmole) of 1,4-diiodobutane was carried out for 4 hr as described above for the syntheses of I and III. A colorless waxy material, bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,4-butanediol dibenzyl ester with mp 36—37° (1.1 g) was obtained. Yield 70.4%. *Anal.* Calcd for $C_{88}H_{156}O_{16}P_2$ (mol. wt. 1532.09): C, 68.98; H, 10.24; P, 4.04. Found: C, 69.49; H, 10.37; P, 4.13.

Debenzylation of 300 mg (0.2 mmole) of this dibenzyl ester was carried out by boiling the material for 5 hr with 88 mg (0.59 mmole) of dry sodium iodide in 3 ml dry acetone, yielding 232 mg of IV (83.1% of theory) mp 182—184°. *Anal.* Calcd for $C_{74}H_{142}O_{16}P_2Na_2$ (mol. wt. 1395.83): C, 63.73; H, 10.25; P, 4.44. Found: C, 63.41; H, 10.24; P, 4.73.

Bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,2-ethanediol Disodium Salt (VI)——A sample of 2.80 g (3.21 mmoles) of the silver salt (XI) and 188 mg (1.3 mmole) of freshly distilled 1,2-dibromoethane were allowed to react in the same way for 17 hr. Even after 17 hr, a considerable amount of the mono-substituted product was found on TLC. By Florisil column chromatography, the mono-substituted product (400 mg) and the desired intermediate (635 mg) were obtained. The yield of bis(dipalmitoyl D,L-α-glycerylphosphoryl)-1,2-ethanediol dibenzyl ester was 32.4% of theory. mp 38°. *Anal.* Calcd. for $C_{86}H_{152}O_{16}P_2$ (mol. wt. 1504.03): C, 68.67; H, 10.19; P, 4.12. Found: C, 68.86; H, 10.25; P, 4.02.

Compound VI (266 mg) was obtained by debenzylation of 300 mg (0.2 mmole) of the dibenzylester with 90 mg (0.6 mmole) of dry sodium iodide (93.5% of theory, mp 197—199°). *Anal.* Calcd. for $C_{72}H_{138}O_{16}P_2Na_2$ (mol. wt. 1367.78): C, 63.22; H, 10.17; P, 4.55. Found: C, 63.11; H, 10.20; P, 4.54.

Bis(dipalmitoyl D,L-*α*-glycerylphosphoryl)methanediol Disodium Salt (VII)—The reaction of the silver salt (2.6 g, 3.1 mmoles) (XI) with diiodomethane (350 mg, 1.3 mmole) for 9.5 hr gave 535 mg of bis-(dipalmitoyl D,L-*α*-glycerylphosphoryl)methanediol dibenzyl ester, which showed a single spot on TLC (88% of theory. mp 38.5°). *Anal.* Calcd. for $C_{85}H_{150}O_{16}P_2$ (mol. wt. 1490.01): C, 68.51; H, 10.15; P, 4.16. Found: C, 67.80; H, 10.15; P, 4.14.

A sample of 300 mg (0.2 mmole) of the dibenzyl ester was allowed to react with 95 mg (0.94 mmole) of dry sodium iodide in 3 ml of boiling acetone for 1.5 hr. Unlike the other analogues described above, VII was not obtained as a single spot on TLC after reprecipitation from chloroform and acetone. Even after chromatography on silicic acid treated with sodium bicarbonate, the final product (VII) (20 mg, starting from 100 mg of material precipitated from chloroform solution with acetone) was slightly contaminated with an unknown product (mp 203—204°). *Anal.* Calcd. for $C_{71}H_{136}O_{16}P_2Na_2$ (mol. wt. 1353.75): C, 62.91; H, 10.13. Found: C, 61.89; H, 9.90.

Bis(benzylphosphoryl)-1,3-propanediol Disodium Salt (X)——A mixture of 3.16 g (8.2 mmoles) of silver dibenzylphosphate and 890 mg (3 mmoles) of 1,3-diiodopropane in 15 ml of dry benzene was heated under reflux with stirring in the dark for 11 hr. After purification on a Florisil column, 510 mg of colorless oily material was obtained, giving a single spot on TLC (Rf_1 0.45). Yield 28.4%.

This material (280 mg, 0.47 mmole) was debenzylated by refluxing with 175 mg (1.1 mmole) of sodium iodide in 3 ml of dry acetone for 2 hr. A white powder was obtained, which was insoluble in acetone, and this was washed several times with chloroform (206 mg, Yield 95.5%). This product gave a single spot on TLC (Rf_2 0.1) or paper electrophoresis. Decomposition p. 175°. *Anal.* Calcd. for $C_{17}H_{20}O_8P_2Na_2$ (mol. wt. 460.25): C, 44.36; H, 4.38; P, 13.46. Found: C, 44.35; H, 4.52; P, 13.40.

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