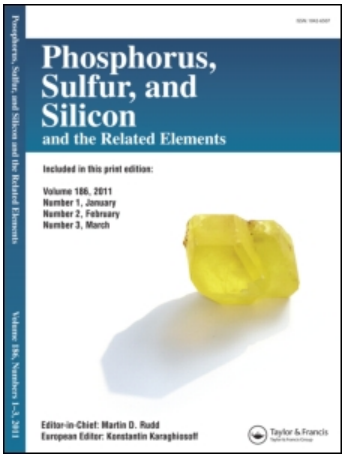

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COVALENT MODELS FOR PHOSPHOLIPID-STEROL INTERACTIONS. SYNTHESIS OF PHOSPHATIDYL-$\Delta^{5,7,9}$-CHOLESTATRIEN-3β-ol

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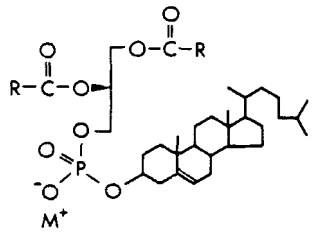
The synthesis of *O*-(1′,2′-di-*O*-palmitoyl-*sn*-glycero-3′-phosphoryl)-$\Delta^{5,7,9}$-cholestatrien-3β-ol, *O*-(1′,2′-di-*O*-myristoyl-*sn*-glycero-3′-phosphoryl)-$\Delta^{5,7,9}$-cholestatrien-3β-ol, and *O*-(1′,2′-di-*O*-palmitoyl-*sn*-glycero-3′-phosphoryl)-$\Delta^{5,7}$-cholestadien-3β-ol, as their triethylammonium and calcium salts, is described. The "double phosphorylating" reagent, 4,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphole is used to establish the phosphate bridge between the 1,2-di-*O*-acyl-*sn*-glycerol and the corresponding sterol, $\Delta^{5,7,9}$-cholestatrien-3β-ol or $\Delta^{5,7}$-cholestadien-3β-ol. The fluorescent, conjugated, polyunsaturated phosphatidylsterols are models to study noncovalent interactions between glycerophospholipids and sterols in biological membranes.

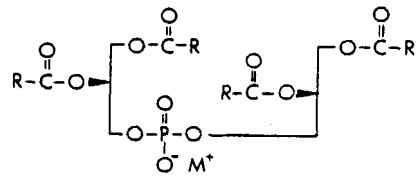
INTRODUCTION

Glycerophospholipids[1] are phosphodiesters, $(R^1O)(R^2O)P(O)OH$, derived from phosphatidic acid[2], $(R^1O)P(O)(OH)_2$, and a "second alcohol," R^2OH. Owing to their amphipathic character, i.e., to the hydrophilicity of the polar headgroup and the hydrophobicity of the nonpolar hydrocarbon region, and to certain specific packing characteristics of their molecules, the glycerophospholipids form bilayers and liposomes in aqueous dispersions under appropriate conditions.[3] Much research is being done on the specific structural features in the polar headgroups of glycerophospholipids, including the "second alcohol," R^2OH, that favor or disfavor the formation of the bilayer phase in aqueous dispersions of these amphipaths.[3] A particularly useful technique to diagnose the formation of the bilayer phase is ^{31}P-NMR spectroscopy.[4–6] However, a possible complication associated with this technique has recently emerged[7] from studies on phosphatidylcholesterol[8,9] (**1**) and phosphatidyldiacylglycerol (**2**).

It was found[7] that aqueous dispersions of these lipids, **1** and **2**, form bilayers and liposomes, as shown by various techniques: electron microscopy, diphenylhexatriene fluorescence polarization, and differential scanning calorimetry. Yet, ^{31}P NMR spectra of these dispersions exhibit characteristics previously though to be associated with the "isotropic" phase (in the case of phosphatidylcholesterol, **1**) or "hexagonal" phase (in the case of phosphatidyldiacylglycerol, **2**). It was shown[7] that differences in the shapes of the ^{31}P NMR signals can result from differences in the conformation



1a, $M^+ = (C_2H_5)_3NH^+$
1b, $M^+ = 0.5Ca^{2+}$

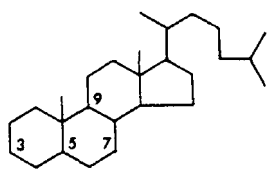


2a, $M^+ = (C_2H_5)_3NH^+$
2b, $M^+ = 0.5Ca^{2+}$

of the phosphate which forms part of the phospholipid headgroup, without significant changes in the organization and packing of the acyl chains in the bilayer phase.

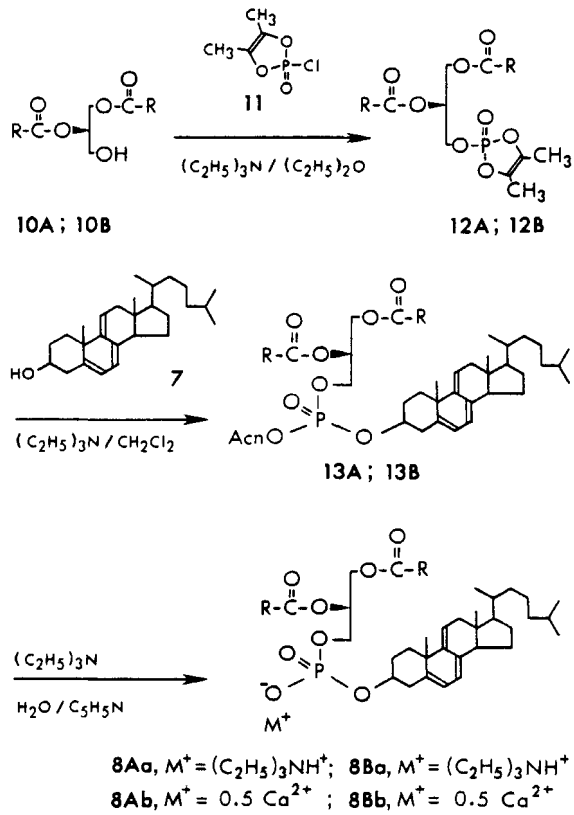
Phosphatidylcholesterol (**1**) is an unnatural phospholipid, in the sense that it is not a component of biological membranes. However, cholesterol itself (**3**) plays an important and still poorly understood role in the structure and function of membranes.[12] To probe into the nature of the *noncovalent* interactions that operate between sterols and glycerophospholipids in membranes, cholesterol and phosphatidic acid were *covalently* bonded as the phosphodiester (**1**).[8,13] From the properties of the bilayers and liposomes made from phosphatidylcholesterol, and from the effects of the charge of the cation (cf. **1a** *vs.* **1b**) on those properties, significant conclusions have been reached on the mode of organization of sterol-containing membrane models and natural membranes.[9]

In furtherance of these studies, it became necessary to synthesize phospholipids in which the "second alcohol," R^2OH, is $\Delta^{5,7}$-cholestadien-3β-ol(7-dehydrocholesterol, **4**) or the fluorescent alcohol, $\Delta^{5,7,9}$-cholestatrien-3β-ol (**7**).[14] The conjugated polyenes, in particular the triene, **7**, are considerably more labile than cholesterol.



3, Δ^5 – ene 3β – OH
4, $\Delta^{5,7}$ – diene 3β – OH
5, $\Delta^{5,7}$ – diene 3β – $OCOCH_3$
6, $\Delta^{5,7,9}$ – triene 3β – $OCOCH_3$
7, $\Delta^{5,7,9}$ – triene 3β – OH

The present paper describes the procedure by which we achieved the synthesis of the desired phosphatidylcholestatriene, **8** (see Scheme 1), and of the less labile phosphatidylcholestadiene, **9**.



SCHEME 1 Synthesis of *O*-(1′,2′-di-*O*-acyl-*sn*-glycero-3′-phosphoryl)-$\Delta^{5,7,9}$-cholestatrien-3β-ol. Compounds **A**, R = $C_{15}H_{31}$ (palmitoyl); compounds **B**, R = $C_{13}H_{27}$ (myristoyl). Acn = $CH_3CO.CH(CH_3)$ —. The synthesis of *O*-(1′,2′-di-*O*-palmitoyl-*sn*-3′-phosphoryl)-$\Delta^{5,7}$-cholestadien-3β-ol (**9A**) was carried out by the same procedure.

RESULTS AND DISCUSSION

As depicted in Scheme 1, a diacylglycerol (**10A** or **10B**) with the chirality present in the natural glycerophospholipids of biological membranes, is phosphorylated by 4,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphole[15] (**11**). Nucleophilic displacements at the phosphorus atom of the enediol cyclophosphorochloridate, **11**, proceed with complete ring-retention, probably by an addition-elimination mechanism in which the chlorotetraoxyphosphorane is an intermediate.[13] The resulting cyclic enediol phosphate ester (**12A** or **12B**) is not purified, but used directly for the phosphorylation of $\Delta^{5,7,9}$-cholestatrien-3β-ol (**7**). The nucleophilic displacement at the phosphorus atom of the cyclic enediol phosphate ester proceeds mainly, but not exclusively, with ring-opening. This step establishes the second phosphate ester bond in the acyclic triester (**13A** or **13B**), which carries also the phosphate-protective group, i.e. 3-oxo-2-butyl (or acetoinyl). Partial purification of the acyclic triester, **13**, is carried out by short-column silica gel chromatography. The final step of the synthesis is the removal of the acetoinyl protection. This step is quite mild, and is based on our

earlier finding that phosphotriesters derived from α-hydroxycarbonyl compounds undergo very rapid base-catalyzed hydrolysis with cleavage of the α-hydroxycarbonyl moiety.[16] The acyl esters of the phosphatidyl group are not affected during the hydrolytic deprotection of the phosphotriester. The fluorescent, conjugated, polyunsaturated phosphatidylsterol (**8Aa** or **8Ba**) is isolated as a triethylammonium salt, and is purified by column chromatography on silica gel. Finally, the triethylammonium salt is converted into the less hygroscopic calcium salt (**8Ab** or **8Bb**) for elemental analysis.

The calcium salt, as well as other divalent and monovalent metal ion salts that can be made from the triethylammonium salt by a similar procedure, are needed for further studies on physical properties of aqueous dispersions of natural and unnatural glycerophospholipids.[7]

Scheme 1 is also applicable to the synthesis of a phosphatidylsterol, **9**, derived from $\Delta^{5,7}$-cholestadiene-3β-ol. As expected, this diene product is less photosensitive than the corresponding triene product.

EXPERIMENTAL

All reactions involving enediol cyclophosphoryl derivatives were carried out under anhydrous conditions. Triethylamine and dichloromethane were distilled from sodium and phosphorus pentoxide, respectively. The progress of the reactions was followed by analytical T.L.C. on precoated silica gel plates (0.25 mm thick; silica gel H). (Solvent: $CHCl_3/CH_3OH$/conc. NH_4OH-30/5/1). Steroids with diene and triene functions were rendered anhydrous by storing their dichloromethane solutions over 4-A molecular sieves at 5°C for 15 h. Samples were dried for 18 h at 20°C/0.2 torr prior to microanalyses, which were carried out by Galbraith Laboratories, Knoxville, TN. All evaporations were carried out in vacuum.

$\Delta^{5,7,9}$-Cholestatrien-3β-ol (**7**). $\Delta^{5,7}$-Cholestadien-3β-ol (7-dehydrocholesterol, **4**, Sigma Chemical Co.) was converted into its acetate (**5**; m.p. 121–123°C, from ether/methanol) by treatment with acetic anhydride in pyridine (24 h at 25°). The acetate **5** was converted into $\Delta^{5,7,9}$-cholestatrien-3β-ol acetate (**6**, m.p. 91–94°C from ether/methanol; Rf 0.67, on silica gel H, hexane/ether, 1/1) by a procedure previously described.[17–19] The triene acetate, 6, was converted into $\Delta^{5,7,9}$-cholestatrien-3β-ol (**7**) by treatment with lithium aluminum hydride in ether solution, as described.[17] The triene alcohol, **7**, was protected from light and air, and was utilized soon after preparation; the sample had m.p. 106–108°C, Rf 0.20 (silica gel H, hexane/ether, 1/1), and λ_{max} 330 nm ($CHCl_3$).

O-(1',2'-Di-O-palmitoyl-sn-glycero-3'-phosphoryl)-$\Delta^{5,7,9}$-cholestatrien-3β-ol (**8A**). A diethyl ether solution (230 mL) of 1,2-di-*O*-palmitoyl-*sn*-glycerol[20] (**10A**; 2.75 g, 4.84 mmol) was added to a stirred ether solution (10 mL) of 4,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphole[14] (**11**; 0.926 g, 5.5 mmol) containing triethylamine (0.83 mL, 6 mmol) at 0°C. After 2 h at 25°C, the mixture was filtered with exclusion of moisture, and the solvent was evaporated to give the cyclic phosphate, **12A** (this compound was quite sensitive to moisture). A dichloromethane solution (30 mL) of $\Delta^{5,7,9}$-cholestatrien-3β-ol (**7**; 1.85 g, 4.84 mmol) was added to a solution containing the cyclic phosphate, **12A**, triethylamine (1.33 mL, 9.7 mmol), and dichloromethane (15 mL) at 25°C under subdued light. After 5 h at 25°C, the volume of the solution was reduced to *ca.* 10 mL. The solution was applied to a short column of silica gel (90 g, packed in ether), which was then eluted with 500 mL of ether. The ether solution was evaporated to give the partially purified phosphatidylcholestatriene triester, **13A** (4.0 g). The triester, **13A**, was mixed with pyridine (38 mL), water (38 mL) and triethylamine (2.02 mL). The mixture was protected from light, and was stirred for 48 h at 25°C. The solution was freeze-dried, and the resulting phosphate salt, **8Aa**, was purified as follows. The salt was dissolved in chloroform (5 mL), and the solution was applied to a column of silica gel (170 g, packed in ether). Elution with 600 mL of ether removed impurities. Elution with 600 mL chloroform/methanol, 8/1, did not remove material from the column. Further elution with 450 mL of the same solvent yielded the triethylammonium salt of the phosphatidylcholestatriene, **8Aa** (2.32 g; 43% overall yield based on 1,2-di-*O*-palmitoyl-*sn*-glycerol **10A**). The salt had λ_{max} 328 nm ($CHCl_3$), Rf 0.35 ($CHCl_3/CH_3OH$/conc. NH_4OH). Analytical TLC revealed the presence of traces (< 2%) of the phosphatidyldiacyl-glycerol, **2a**, which is a by-product of the synthesis.

The conversion of the triethylammonium salt **8Aa** into the calcium salt, **8Ab**, was performed as follows. A solution of the triethylammonium salt (**8Aa**, 0.159 g) in 2/1 chloroform/methanol (45 mL) was mixed with 3/48/47 chloroform/methanol/2M aqueous calcium chloride (15 mL). The upper phase was discarded, and the procedure was repeated two additional times with the lower phase. The final lower phase was washed twice with 3/48/47 chloroform/methanol/water (10 mL) and evaporated. The residue was kept 10 h (0.2 torr) to yield the calcium salt (**8Ab**, 0.140 g); Rf 0.35 ($CHCl_3/CH_3OH$/conc. NH_4OH). $C_{124}H_{216}O_{16}P_2Ca$: Calcd.: C, 72.12; H, 10.54; Ca, 1.94. Found: C, 72.31; H, 11.37; Ca, 1.69.

O-(1',2'-Di-O-myristoyl-sn-glycero-3'-phosphoryl)-$\Delta^{5,7,9}$*-cholestatrien-3β-ol* (**8B**). The triethylammonium salt, **8Ba**, was obtained from 1,2-di-*O*-myristoyl-*sn*-glycerol (**10B**) by the same procedure described above. It appeared that the condensation between $\Delta^{5,7,9}$-cholestatrien-3β-ol (**7**) and the cyclic triester, **12B**, (see Scheme 1) in the myristoyl series was significantly slower than the corresponding reaction in the palmitoyl series (i.e. **12A**). Consequently, the reaction time was extended to 25 h at 25°C in the condensation of cyclic triester **12B**. The yield of the purified salt, **8Ba**, was somewhat lower than that of the homolog, **8Aa**. Rf of **8Ba**, 0.35 ($CHCl_3/CH_3OH$/conc. NH_4OH). $C_{116}H_{200}O_{16}P_2Ca.2H_2O$: Calcd.: C, 70.05; H, 10.34; Ca, 2.02. Found: C, 69.31; H, 10.73; Ca, 1.95.

O-(1',2'-Di-O-palmitoyl-sn-glycero-3'-phosphoryl)-$\Delta^{5,7}$*-cholestadien-3β-ol* (**9A**). The triethylammonium salt (**9Aa**; overall yield: 50%) and the calcium salt (**9Ab**) were synthesized according to Scheme 1 following the procedures described above, from 7-dehydrocholesterol (**4**) and 1,2-di-*O*-palmitoyl-*sn*-glycerol (**10A**). Both salts had Rf 0.36 ($CHCl_3/CH_3OH$/conc. NH_4OH). For the calcium salt: $C_{124}H_{212}O_{16}P_2Ca.1H_2O$: Calcd.: C, 71.36; H, 10.72; Ca. 1.92. Found: C, 71.35; H, 11.1; Ca, 1.91.

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REFERENCES AND NOTES

1. G. B. Ansell, R. M. C. Dawson and J. N. Hawthorne, "Form and Function of Phospholipids," 2nd Ed., Elsevier Scientific Publishing Co., Amsterdam, 1973.
2. Phosphatidic acid = 1,2-di-*O*-acyl-*sn*-glycerol-3-phosphate, where *sn* = stereochemical numbering for chiral glycerol derivatives.
3. M. K. Jain and R. C. Wagner, "Introduction to Biological Membranes," Wiley, New York, 1980.
4. S. J. Kohler and M. P. Klein, *Biochemistry*, **16**, 519 (1977).
5. J. Seelig, *Biochim, Biophys. Acta*, **515**, 105 (1978).
6. B. De Kruijff and P. R. Cullis, *Biochim. Biophys. Acta*, **559**, 399 (1979).
7. J. H. Noggle, J. F. Marecek, S. B. Mandal, R. Van Venetie, J. Rogers, M. K. Jain and F. Ramirez, *Biochim. Biophys. Acta*, **691**, 240 (1982).
8. F. Ramirez, P. V. Ioannou and J. F. Marecek, *Synthesis*, 673 (1977).
9. M. K. Jain, F. Ramirez, T. M. McCaffrey, P. V. Ioannou, J. F. Marecek and J. Leunissen-Bijvelt, *Biochim. Biophys. Acta*, **600**, 678 (1980).
10. F. Ramirez, P. V. Ioannou, J. F. Marecek, B. T. Golding and G. H. Dodd, *Synthesis*, 769 (1976).
11. S. Rainier, M. K. Jain, F. Ramirez, P. V. Ioannou, J. F. Marecek and R. Wagner, *Biochim. Biophys. Acta*, **558**, 187 (1979).
12. M. K. Jain, *Top. Membr. Transport*, **6**, 1 (1976).
13. F. Ramirez and J. F. Marecek, *Acc. Chem. Res.*, **11**, 239 (1978).
14. J. Rogers, A. G. Lee and D. C. Wilton, *Biochim. Biophys. Acta*, **552**, 23 (1979).
15. F. Ramirez, H. Okazaki, J. F. Marecek and H. Tsuboi, *Synthesis*, 819 (1976).
16. F. Ramirez, B. Hansen and N. B. Desai, *J. Am. Chem. Soc.*, **84**, 4588 (1962).
17. F. Ramirez, J. F. Marecek and S. S. Yemul, *J. Org. Chem.*, **48**, 847 (1983).
18. A. Windaus and O. Linsert, *Justus Liebigs Ann. Chem.*, **148**, 465 (1928).
19. W. Bergman and P. B. Stevens, *J. Org. Chem.*, **13**, 10 (1948).
20. E. Baer and M. Kates, *J. Am. Chem. Soc.*, **72**, 942 (1950).