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SYNTHESIS OF CHIRAL DIESTER- AND ESTER/ETHER-PHOSPHATIDYLCHOLINES. CALORIMETRIC STUDIES OF ASYMMETRIC-CHAIN PHOSPHATIDYLCHOLINES IN LIPOSOMES

by

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Abstract

Synthesis of Chiral Diester- and Ester/ether-Phosphatidylcholines. Calorimetric Studies of Asymmetric-Chain Phosphatidylcholines in Liposomes

by

Shaukat Ali

Advisor: **Professor Robert Bittman**

Diester- and ester/ether-phosphatidylcholines (PCs) have been prepared from (*R*)-glycidyl tosylate in good yields. The key step in the synthesis of 1,2-distearoyl-*sn*-glycero-3-PC was the ring opening of the epoxide with stearic anhydride (1.5 equiv) and $BF_3 \cdot Et_2O$ as a catalyst. The resulting 1,2-diacyl-*sn*-glycerol 3-tosylate was obtained in a one-pot synthetic procedure in good yield (76%). The tosylate group was converted into the phosphocholine moiety by using standard procedures. The key step in the synthesis of ester/ether-PC was the $BF_3 \cdot Et_2O$-mediated ring opening of glycidyl tosylate with benzyl alcohol as a nucleophile to give the ring-opened product in good yield (84%) and in high optical purity (~96% enantiomeric excess). Catalytic hydrogenolysis gave the *sn*-1 hydroxyl group, which was acylated by using known procedures. Etherification of the *sn*-2 hydroxyl group with alkyl triflate using 2,6-di-*tert*-butyl-4-methylpyridine as a catalyst, followed by conversion of the tosylate group to the phosphocholine moiety, gave ester/ether-PC. Both of the commercially available enantiomers of glycidyl tosylate can be used in these syntheses, giving PCs with either the natural or unnatural configuration.

The $CdCl_2$-complex of glycero-*sn*-3-phosphocholine has been used to prepare a 1,2-di-[5'*R*/*S*,9'*R*,13'*R*)-5',9',13',17'-tetramethyloctadecanoyl]-*sn*-glycero-3-PC (di-C_{22}-chain PC). The C_{22}-chain was prepared in many steps from commercially available phytol, which was oxidized and then extended by Emmons-Wittig-Horner type reaction. High-resolution differential scanning calorimetric (DSC) studies of the di-C_{22}-chain PC and a highly asymmetric-chain PC having a di-C_{22}-chain at the *sn*-1 position and a C_{10}-chain at the *sn*-2 position did not show any phase transition temperature between -28 ° and 68 °C, suggesting that both the PCs are most probably in the liquid-crystalline states within the perturbed bilayers. The phosphatidic acid monomethyl ester of di-C_{22}-chain PC prepared by the transesterification in the presence of excess of methanol and phospholipase D from *Streptomyces chromofuscus* was a better substrate of pancreatic phospholipase A_2 than the corresponding di-C_{22}-chain PC.

Asymmetric-chain PCs [1-octadecanoyl-2-decanoyl-*sn*-3-glycerophosphocholines (C18:C10PC)] modified in the choline moiety have been prepared in four steps by using a semisynthetic approach. Acyl migration during the acylation procedure of 1-stearoyl-2-lyso-PC with fatty acid anhydride using 4-pyrrolidinopyridine was assayed by gas chromatography of fatty acids released by phospholipase A_2 digestion. Under the reactions employed, asymmetric-chain PCs were obtained with <1% acyl migration. The mixed-chain PC was hydrolyzed with phospholipase D (*Streptomyces chromofuscus*) to give the corresponding phosphatidic acid, which was coupled with different aminoalkanols by using known procedures, giving the asymmetric-chain phospholipids modified in the polar head group.

Liposomes were prepared from mixtures of C18:C10PC and asymmetric-chain PCs having ω-CH_2=CH, ω-CH_3CO_2, and ω-CH_3CO

moieties. DSC results suggested that an asymmetric-chain PC with a ω-CH_2=CH moiety is miscible at all proportions in the bilayers of C18:C10PC and C18:C11PC, indicating an ideal mixing of C18:C(11:1Δ^{10})PC in C18:C10PC and C18:C11PC. The phase diagrams constructed based on the thermograms from different mol % of C18:C(11:1Δ^{10})PC in C18:C10PC and C18:C11PC suggested that the ω-CH_2=CH moiety in the *sn*-2 acyl chain has virtually no perturbing effect on the packing of the hydrocarbon chains at temperatures below and above the phase transition temperatures; thus, a near ideal mixing behavior is observed for both of these binary systems. Since the mixing is an ideal, it is assumed that lipid-lipid lateral interactions in C18:C(11:1Δ^{10})PC are the same as in C18:C10PC; therefore, C18:C(11:1Δ^{10})PC is considered to form mixed interdigitated bilayers at $T < T_m$, and partially interdigitated bilayers at $T > T_m$.

The effects of the ω-CH_3CO_2 and ω-CH_3CO groups of C18:C(10:ω-CH_3CO_2)- and C18:C(10:ω-CH_3CO)PCs on the mixing behavior in the liposomes of C18:C10PC were studied by DSC. Phase diagrams for the binary systems C18:C10PC/C18:C(10:ω-CH_3CO_2)PC and C18:C10PC/C18:C(10:ω-CH_3CO)PC were constructed based on the calorimetric data. DSC results suggested that C18:C10PC liposomes enriched in PCs with ω-CH_3CO_2 and ω-CH_3CO moieties show gel-phase partial immiscibility, but miscibliIty in the liquid-crystalline state. Although PCs with ω-CH_3CO_2 and ω-CH_3CO moieties show almost a similar trend on the gel phase immiscibility at low temperatures, the effect is more pronounced in the PC having a ω-CH_3CO moiety. The mixing is near-ideal for both the binary systems in the liquid-crystalline state.

Dedicated to my dear wife and to our newly born daughter Farah; to my parents, and all my younger brothers

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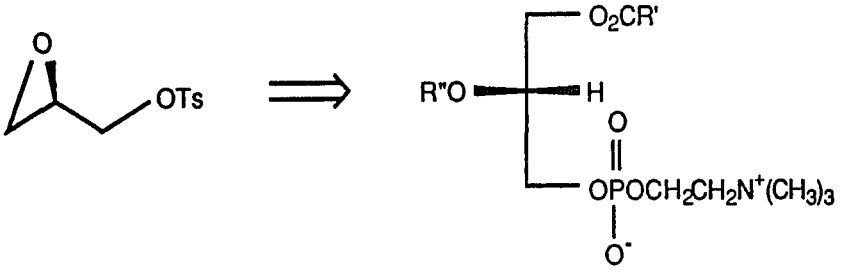
Chapter 1

General introduction

Acylalkyl, alkylacyl, and alkenylacyl analogs of phospholipids, together with diacyl phospholipids, are present in significant amounts in many biological membranes.[1] To assess the role of these species in membranes isomerically and optically pure materials are required. Their syntheses require considerable expertise in synthetic lipid chemistry. Equations 1-4 outline the preparations of a number of phosphatidylcholines (PCs) that are described in detail in chapters 2-5 and 7.



(eq. 1)

1 R' = $C_{17}H_{35}$; R"O = $C_{17}H_{35}CO_2$ (symmetric-chain diacyl-PC)

2 R' = $C_{15}H_{31}$; R"O = $C_{16}H_{33}O$ (acylalkyl-PC)

Synthesis of diacyl- and acylalkyl phospholipids. The synthesis of symmetric-chain diacyl-PCs such as **1** (eq. 1) from the reaction of *sn*-glycero-3-phosphocholine (GPC) or its $CdCl_2$ salt with fatty acid anhydrides has been reported.[2] Because of the poor solubility of GPC or its $CdCl_2$ salt in aprotic solvents, this method often requires long reaction times and high temperatures. The 1,2-diacyl-*sn*-glycero-3-phosphocholine product is contaminated considerably with 1,3-diacyl-*sn*-glycero-2-PC which arises by phosphoryl and acyl migration.[2h-j] Mixed-chain 1,2-diacyl-PCs have been synthesized by the reaction of 1-acyl-2-lyso-PC with fatty acid anhydrides having a different acyl chain than that in lyso-PC. This method requires efficient

catalysts such as 4-(dimethylamino)pyridine and 4-pyrrolidinopyridine;[2h-j,3c] in some instances heating at ~ 35 °C or higher temperatures is required to bring about reaction, which results in the contamination of the desired 1,2-diacyl-*sn*-glycero-3-PC with 1,3-diacyl-*sn*-glycero-2-PC and mixed 1,2-diacyl-*sn*-glycero-3-PC by acyl and phosphoryl migration.[2f,h,i,3a] The latter are difficult to separate from the desired 1,2-diacyl-*sn*-glycero-3-PC isomer. Another disadvantage of the above procedures is that they require a large amount of fatty acid anhydride. To avoid using a large excess of fatty acid anhydrides, the acylation procedure has been carried out with acid chlorides, fatty acid imidazolides, and other activated acid derivatives as the acylating agents.[2b,d,f,g,3b]

D-Mannitol has been used as the precursor to prepare optically active 1,2-diacyl-*sn*-glycerols or phospholipids via 1,2-isopropylidene-*sn*-3-glycerol or 2,3-isopropylidene-*sn*-1-glycerol. This procedure involves a number of protection and deprotection steps.[4] Moreover, the optical purity of the product obtained is dependent on the substrate (D-mannitol or glycerol derivative) used as the precursor. Epoxides have been used frequently as synthons of various natural products because of their extensive reactivity, ease of preparation, and availability in optically active form. Recently, epoxides have been found to be valuable intermediates in the synthesis of many racemic and optically active glycerophospholipids and other natural products.[5] For example, *rac*-glycidol has been used to synthesize *rac*-mono-acyl and 1,2-diacyl-*rac*-3-glycerol,[6] and 1,2 diacyl-*rac*-3-glycerophospholipids.[7] *rac*-Glycidyl ester has also been used to synthesize *rac*-1,3-diacylglycerols.[8]

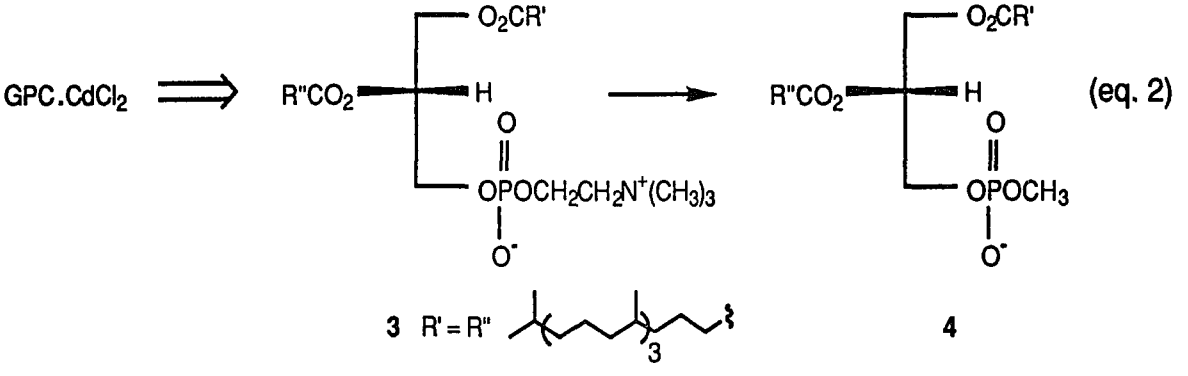
In our laboratory, however, it is shown that use of BF_3 etherate as a Lewis acid catalyst for the opening of (*R*)-glycidyl tosylate in the presence of stearic anhydride gives the diacylation product in one step in good yield

(76%).[9a] In subsequent steps, the tosylate group is converted into phosphocholine by using known procedures.[10] While our work was in progress the titanium(IV) isopropoxide-assisted nucleophilic epoxide opening of (*S*)-glycidol with stearic acid was reported to give 1-stearoyl-*sn*-glycerol in low (~ 25%) yield.[9b]

Another application of glycidyl tosylate as a precursor in phospholipid synthesis is in the preparation of 1-acyl-2-*O*-alkyl-*sn*-3-glycero-PC (eq. 1, **2**). This synthesis requires only five steps and gives the product in high optical purity. The key step is the regio- and stereospecific ring opening of glycidyl tosylate with 1.5 equiv of benzyl alcohol and a catalytic amount of BF_3 etherate, giving 1-*O*-benzyl-*sn*-3-glycerol tosylate. The latter is alkylated under mildy basic reaction conditions using alkyl triflates in the presence of 2,6-di-*tert*-butyl-4-methylpyridine as a catalyst.[9c]

In this dissertation the synthetic usefulness of the tosylate derivative of glycidol is demonstrated by (i) facile diacylation in the presence of BF_3 etherate, (ii) regioselectivity, with attack of benzyl alcohol as the nucleophile exclusively at C-3 in the presence of BF_3 etherate,[9d] and (iii) stereospecific opening of the epoxide (established by ^1H NMR of the Mosher esters of both of the enantiomers), and (iv) conversion of the tosyl group into the phosphocholine moiety, with retention of configuration at the C-2 position of the glycerol derivative. Since allyl alcohol is readily converted to either (*R*)- or (*S*)-glycidyl tosylate by asymmetric epoxidation and in-situ derivatization,[11] the procedures can be used to prepare the phospholipids with the *sn*-1 configuration. Since the configuration at the C-2 position is important with respect to the packing organization in the subgel phase formed by diacyl PCs, the acyl/alkyl or alkyl/acyl PCs of *sn*-1 and *sn*-3 configurations might have an influence on the structural arrangements in the gel phases.[12]

The effects of acyl chain length and position of methyl branching on the thermotropic properties and molecular areas of PCs have been investigated.[13] The synthesis of diester and diether PCs bearing 20-carbon atoms including four methyl branches in each chain have been reported. The synthesis of the diester-PC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, for instance, used phytanic acid as the hydrocarbon-chain precursor, which was obtained from naturally occurring dihydrophytol.[14] The synthesis of the isopranyl glyceryl diether phospholipid, 2,3-di-*O*-phytanyl-*sn*-glycerol-1-phosphocholine, on the other hand, has been reported in a semisynthetic way by phosphorylation of natural 2,3-di-*O*-phytanyl-*sn*-glycerol, which is derived from *Halobacterium halobium*.[15] To study the effect of methyl branching on the packing of acyl chain(s), we have synthesized a diester-PC which has a C_{22} chain bearing four methyl branches at both the *sn*-1 and *sn*-2 positions (eq. 2, **3**).



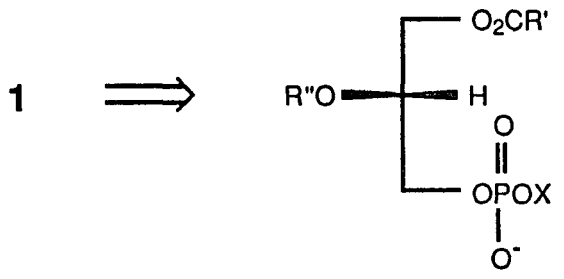
(eq. 2)

The methyl ester **4**, which was prepared from **3** by transesterification reaction with phospholipase D from *Streptomyces chromofuscus* in excess of methanol at room temperature, has been used to probe the kinetic properties of phospholipase A_2. It has been found that methyl ester **4** is a better substrate for phospholipase A_2 than that PC **3**, suggesting that head group modification has a pronounced effect on the lipid-protein interaction. It has also been observed that phospholipase A_2 hydrolyses sluggishly the di-C_{22} symmetric-chain PC to form the C_{22}-lyso PC. No phase transition temperature

of PC **3** has been detected when scanned in a high-resolution calorimeter from -28 ° to 68 °C.

Synthesis of head-group analogs of phospholipids. Although efficient procedures for the synthesis of phospholipid head group analogs have been reported,[16a,b] the range of acyl groups reported is limited to symmetric chains (in synthetic phospholipids) and to chains of similar lengths (as in naturally occurring PCs). These phospholipids have been used to study the effects of phospholipid head groups on various physical properties. These studies include the role of head group structure on the phase behavior and relative stabilities of lamellar and non-lamellar phases,[17] on the interaction of phospholipids with phospholipid-requiring enzymes[18] or with cholesterol,[19] and on the distribution of phospholipids between the leaflets of the bilayer of small unilamellar vesicles.[20] To assess the role of bulky head groups on the packing of the terminal methyl group (ω-CH_3) of the longer hydrocarbon chain in the interdigitated gel state bilayer, it is required to synthesize the analogs of asymmetric-chain PCs modified in polar head groups.

For the synthesis of asymmetric PCs with bulky head groups (eq. 3, **5a-e**), we found that many preparations of phospholipase D from peanut or cabbage failed to hydrolyze mixed-chain PCs having widely different chain lengths at the *sn*-1 and *sn*-2 positions. We also found that phospholipase D preparation from *Streptomyces chromofuscus* did catalyze the hydrolysis of mixed-chain PCs to the corresponding phosphatidic acids (PAs), but did not catalyze the transesterification reaction with bulky head group analogs.



(eq. 3)

R' = $C_{17}H_{35}$, R"O = $C_9H_{19}CO_2$

X = **5a** $CH_2CH_2CH_2N^+(CH_3)_3$
b $CH_2CH_2CH_2N^+(CH_3)_2C_2H_5$
c $CH_2CH_2CH_2N^+(C_2H_5)_3$
d $CH(CH_3)CH_2CH_2N^+(CH_3)_3$
e $CH_2CH_2CH_2CH_2CH_2N^+(CH_3)_3$

We have prepared mixed-chains PCs (**5**) modified in the choline moiety by a semisynthetic route. The first step is the acylation of 1-stearoyl-2-lysoPC with a 10-fold excess of fatty acid anhydride at room temperature in 6 h using 1.2 equiv of 4-pyrrolidinopyridine as a catalyst. The mixed-chain PCs are formed in high yield with a very small amount (< 1%) of contamination arising from acyl migration during the reaction.[16c] The resulting mixed-chain PC is hydrolyzed with phospholipase D (*Streptomyces chromofuscus*) to give PA, which is then coupled with the bulky, *N,N,N*-trialkylammonium alcohols (as the *p*-toluenesulfonate or tetraphenylborate salt) by using a previously reported procedure.[18] Compounds **5a-e** had broad $\Delta T_{1/2}$ values associated with the phase transition; therefore, calorimetric studies of binary mixtures consisting of these analogs and other PC could not be carried out. The mixing behavior of these PCs analogs with other phospholipids will be studied by using Raman spectroscopy in Dr. I. W. Levin's laboratory in the near future. The peak intensity ratios of Raman signals (I_{2834}/I_{2882} and I_{2935}/I_{2882}) arise from the symmetric and asymmetric mode sof C-H stretching will be taken as the Raman indices to study the gel state packings of hydrocarbon chains.[21a,b]

Phase behavior of phospholipids in bilayer assemblies. In recent

years there has been considerable interest in determining the phase behavior of a single lipid and lipid mixtures in order to obtain information about the mixing of lipid components in membrane bilayers.[21] It is possible that biological membranes contain domains that are enriched in certain lipid components. The existence of laterally separated domains within the plane of bilayers might play an important role in membrane function. For instance, a change in membrane morphology from the lamellar to hexagonal (H_{II}) phase results in membranes that are more prone to diffusion of metabolites,[22] and to activation of protein kinase C by 1,2-diacylglycerols and phosphatidylethanolamines, which are involved in signal transduction across the bilayer.[23]

A number of instrumental techniques have been used to study the phase behavior of phospholipids in model and biological membranes. However, none of the techniques used gives in-depth information about the structural organization of phospholipids within the plane of bilayers. High-resolution differential scanning calorimetry (DSC), along with X-ray, NMR, and Raman spectroscopy have frequently been used to study the structure of natural and synthetic phospholipids in bilayer assemblies.[21a,b]

Interdigitation: Based on calorimetric data and spectroscopic techniques, it has been established that the hydrocarbon chains of saturated PCs at low temperatures can adopt four types of gel-state packings: non-interdigitated, partially interdigitated, mixed-interdigitated, and fully interdigitated bilayers (Fig. 1a-d).[21a] The parameter $\Delta C/CL$ is helpful in identifying a particular packing mode in the gel state.[21a,b] ΔC is the absolute difference in chain length of the two hydrocarbon chains in C-C bond lengths, and is given by $\Delta C = |n_1 - n_2 + 1.5|$ where n_1 and n_2 are the number of carbons in the *sn*-1 and *sn*-2 acyl chains, respectively. CL is the length of the longer of the two hydrocarbon

chains in C-C bonds. The value of 1.5 C-C bond lengths or ~1.8 Å length inequivalence is due to the abrupt *sn*-2 bending at the C(2) atom near the glycerol backbone.[24] Thus, there is an inherent asymmetry between two hydrocarbon chains of the same number of carbon atoms at the *sn*-1 and *sn*-2 positions.

Phospholipids such as C14:C16PC and C16:C18PC with ΔC = 0.5 C-C bond length (or $\Delta C/CL$ = 0.035) adopt a noninterdigitated packing arrangement in which the methyl termini of the acyl cahins are accommodated in the center of the bilayer (Fig. 1a). Phospholipid molecules with ΔC values larger than 0.5 but smaller than 6 C-C bond lengths ($6 > \Delta C > 0.5$) adopt a partially interdigitated packing in the gel state, in which the longer chain of the lipid on one side of the bilayer packs end-to-end with the shorter chain of another lipid molecule in the opposing leaflet (Fig. 1b). With a further increase in chain-length difference, where $\Delta C/CL \sim 0.5$, as in phospholipids such as C18:C12PC, C18:C11PC, and C18:C10PC, a mixed interdigitated packing mode is formed. In this arrangement, the methyl terminus of the shorter chain is packed end-to-end with the methyl terminus of the shorter chain from another lipid molecule in the opposing bilayer leaflet, while the longer chain from the two leaflets span the entire hydrocarbon width of the bilayer (Fig. 1c). If the value of $\Delta C/CL$ approaches unity (~ 1.1), fully interdigitated bilayers are formed, as with highly asymmetric phospholipids such as C18:C0PC and C16:C0PC, in which each long acyl chain spans the entire hydrocarbon width of the bilayers and interacts laterally with the long acyl chain of lipid molecules from the opposing leaflet (Fig. 1d). The mixed interdigitated bilayers are thus characterized by having the area per molecule at the lipid/water interface encompass three hydrocarbon chains per head group, in contrast to the two hydrocarbon chains per head group for PCs in the noninterdigitated, partially

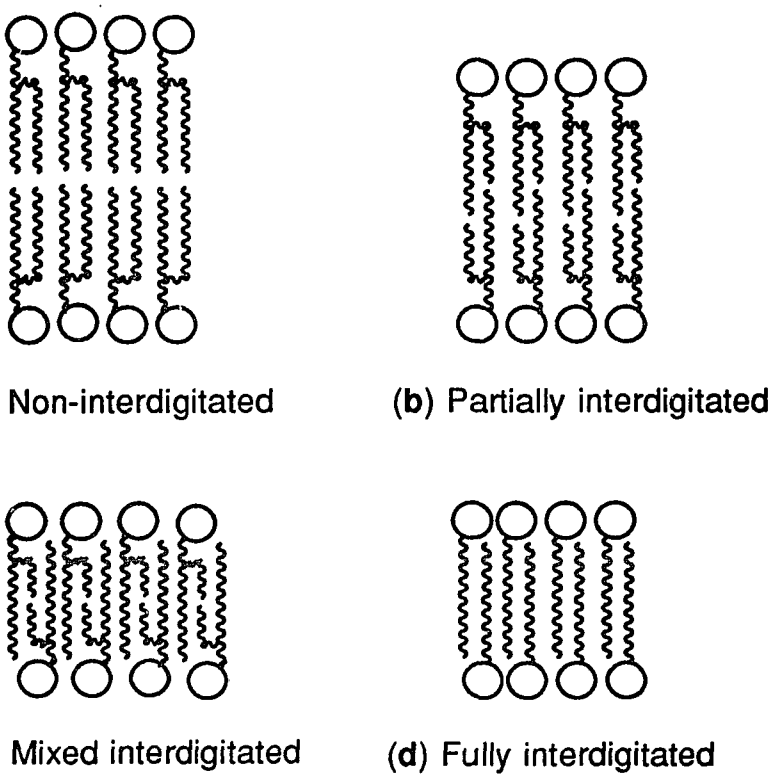
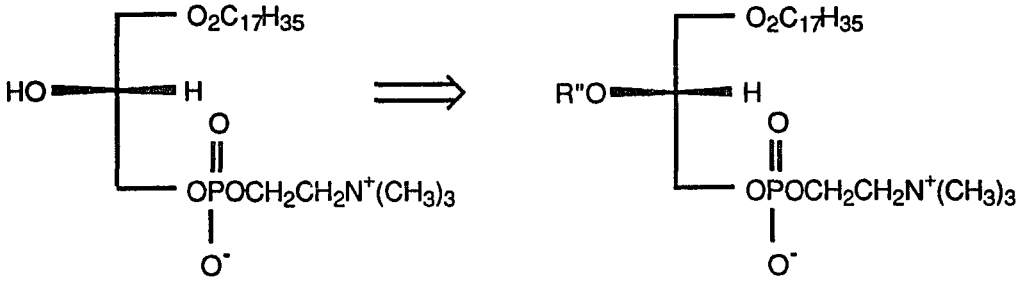


Fig. 1 Gel state packing of hydrocarbon chains: the circles represent the polar head groups and the wiggly lines represent the acyl chains.

interdigitated, and fully interdigitated bilayers.

Naturally occurring phospholipids containing a long chain at the *sn*-1 position and a short chain the *sn*-2 position may be produced in nature by lipid peroxidation of the *cis*-double bond(s) at the *sn*-2 acyl chain of long-chain phospholipids (such as 1-palmitoyl-2-oleoyl-PC). This reaction would result in the presence of an oxygen atom at the end of the short chain. Since asymmetric-chain phospholipids with $\Delta C/CL \sim 0.5$ are known to self-assemble in excess water to form mixed interdigitated bilayers at low temperatures,[21a,b] it would be interesting to see whether an oxygen atom has a perturbing effect on the packing behavior of the hydrocarbon chains in the gel state and in the

liquid-crystalline state. The ideal candidate to study this effect would be an asymmetric-chain PC having an aldehyde moiety at the the end of the short chain at the *sn*-2 position. Since the aldehyde is unstable and may undergo oxidation during the slow heating-cooling cycles used in calorimetric studies, we have synthesized structurally similar asymmetric-chain PCs terminating in an ester and a ketone moiety (eq. 4, **6**, **7**).



(eq. 4)

6 R"O = $MeO_2C(CH_2)_8CO_2$

7 R"O = $MeCO(CH_2)_7CO_2$

The mixing properties of two of the similar asymmetric-chain phospholipids (such as C18:C(11:1Δ^{10})PC/C18:C11PC, C18:C(11:1Δ^{10})PC/ C18:C11PC, an ester-PC/C18:C10PC, and a methyl ketone-PC/C18:C10PC as binary mixtures are carried out by using DSC.

DSC studies of phospholipid bilayers. DSC is a powerful, non-perturbing thermodynamic technique for characterizing the thermotropic phase behavior associated with the transition between the gel and liquid-crystalline states of lipids in model and biological membranes. It has been used to study the phase behavior of binary and ternary mixtures of lipids. The effects of cholesterol, drugs, and proteins on the phase transitions of phospholipids exhibited by single or two components have extensively been studied by DSC.[25]

In DSC, an aqueous dispersion of a lipid sample and an inert reference

material (buffer) are heated simultaneously in ampoules at the same rate. If the sample undergoes a thermally induced endothermic or exothermic process, a portion of the power is absorbed or liberated by the sample, respectively. The detector senses the temperature difference between the sample and the reference cells and provides more (or less) heat to the sample cell in order to maintain the temperature of the sample cell equal to that of the reference cell. The heat then is recorded with respect to the reference cell as the difference in heat or the excess heat as a function of sample temperature.

Fig. 2 shows a typical DSC thermogram showing the excess specific heat with temperature for a simple two-state, first-order endothermic transition such as the gel to liquid-crystalline phase transition. The area under the curve is proportional to the amount of heat absorbed (ΔH) during the phase transition. The phase transition temperature, T_m, is the temperature of the hydrocarbon-chain melting at which the excess heat absorbed by the system reaches a maximum. For a symmetric thermogram, T_m is the temperature at which both the gel and liquid-crystalline phases are in equilibrium. The sharpness of the phase transition is often expressed as the temperature width at half-height, $\Delta T_{1/2}$, or as the temperature difference between the onset (T_o) and completion (T_c) temperatures of a gel to liquid-crystalline phase transition. Table I shows T_m, ΔH, and $\Delta T_{1/2}$ values for some synthetic PCs.

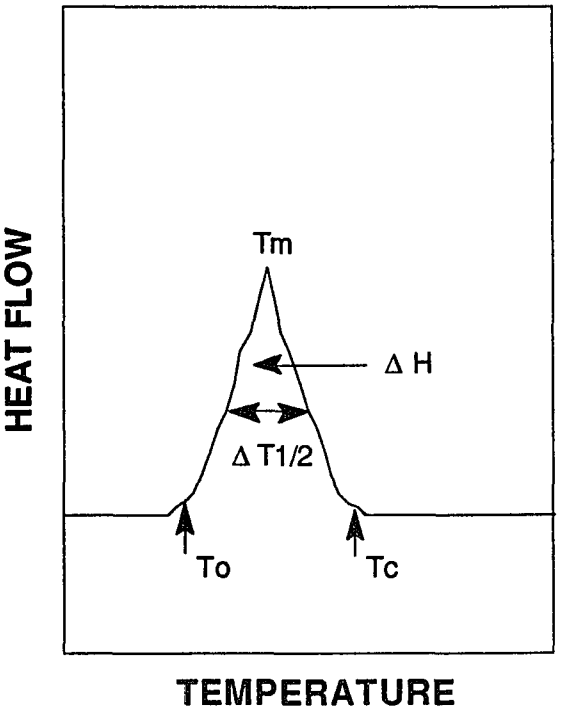


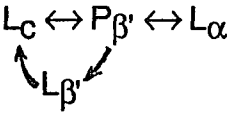
Fig. 2 A typical DSC thermogram

Table I: Thermodynamic Parameters of some of the synthetic PCs

PC	T_m (°C)	ΔH (kcal/mol)	$\Delta T_{1/2}$ (°C)	Ref.
C18:C18PC	54.9	10.6	0.51	33
C18:C0PC	26.2	7.0	0.51	34
C18:C2PC	19.8	1.8	0.83	34
C18:C8PC	3.1	13.7	2.3	21a
C18:C11PC	21.4	9.9	0.4	21a
C18:C14PC	29.8	5.6	1.7	35
C14:C18PC	38.5	6.9	0.16	36
C16:C18PC	48.9	8.3	0.14	36
C16:C(18:1)PC	-0.8	5.4	2.4	37
C(18:1):C16PC	-7.9	4.6	2.3	37

The transition from the gel to liquid-crystalline phase arises from the cooperative melting of the hydrocarbon chains of the phospholipid molecules, and is fully reversible. The hydrocarbon chains are converted from a relatively rigid, ordered, extended, and largely all-*trans* conformation in the gel phase to a disordered liquid-crystalline state which is characterized by *gauche* conformations with increased rates of intra- and intermolecular motions.[26] The hydrocarbon chain melting results in a lateral chain expansion and in a decrease in the thickness of the bilayer. The phase transition temperature of a phospholipid depends markedly on the length of the hydrocarbon chains, the structure of the hydrocarbon chains, and the structure of the polar head group. The gel to liquid-crystalline phase transition of a pure single lipid is sharp and symmetric, and is a rapid process. However, in biological membranes the gel to liquid-crystalline phase transition is usually broad and asymmetric because of heterogeneity in the hydrocarbon chains and the head groups of the constituent phospholipids. In addition to the gel to liquid-crystalline phase transition, other transitions can occur in model membranes prepared with pure phospholipids. For instance, dipalmitoylphosphatidylcholine (DPPC) undergoes two thermally induced lamellar gel-state transitions. These transitions occur usually between 11-18 °C and are less cooperative than the main transition; they arise from tilted hydrocarbon chains in the $L_{\beta'}$ phase where the chains are still fully extended, rotationally disordered and packed less, probably in a disordered orthorhombic lattice.[27] At the more cooperative and less energetic pretransition that occurs at 35 °C, the $L_{\beta'}$ phase is converted into the "rippled" gel state ($P_{\beta'}$ phase), in which the hydrocarbon chains remain largely extended and tilted. At 41 °C, the $P_{\beta'}$ phase is finally converted into the liquid-crystalline phase (L_{α}), a more highly hydrated phase in which the hydrocarbon chains are highly disordered.

The phase behavior of saturated 1,2-diacylphospholipids can be outlined as:



where $P_{\beta'} \rightarrow L_{\alpha}$ is the main transition (gel to liquid), $P_{\beta'} \rightarrow L_{\beta'}$ is cooling transition indicating that the planar $L_{\beta'}$ phase actually converts to the crystalline L_c phase. $L_c \rightarrow P_{\beta'}$ is the low temperature transition corresponding to the crystalline to gel transformation.[28]

Biological membranes have a heterogeneous distribution of chain lengths. For example, lysophospholipids (which comprise < 1% of the total PCs in biological membranes) are highly asymmetric in chain length, having a hydroxy group at the *sn*-2 position. Naturally occurring lipids such as platelet activating factor, sphingosines, cerebrosides, gangliosides and sulfatides of ceramide have considerable amounts of unsaturation and asymmetry in their hydrocarbon moieties. This chain-length asymmetry plays an important role in lipid distribution in the plane of bilayer, since these lipids form compositional domains within the plane.[29] The domains formed might influence membrane functions under certain physiological conditions. It has been demonstrated that gangliosides can form clustered domains in dimyristoyl-PC bilayers, which serve as identifying surface structures involved in cellular recognition or cell-cell interactions.[29] There is evidence that suggests that cholesterol may interact with asymmetric-chain phospholipids under certain conditions. For instance, it has been found that the cholesterol strongly interacts with sphingomyelin, a highly asymmetric-chain phospholipid with sphingosine backbone, found in mammalian cells.[30]

Mixed-chain phospholipids, like the saturated and symmetric-chain lipids, undergo thermotropic phase transitions. Most mixed-chain

phospholipids exhibit only the gel to liquid-crystalline phase transition. The transition temperature and the enthalpy of mixed acyl phospholipid bilayers are found to lie between the values for the corresponding symmetric-chain phospholipids.[31] For pairs of positional isomers C18:C16PC and C16:C18PC, for instance, the PC with the longer acyl chain at the *sn*-2 position shows a higher transition temperature and heat of enthalpy. The physical parameters for a particular transition are dependent on the packing behavior of hydrocarbon chains within the plane of bilayers. In order to compensate for the perturbing effects of hydrocarbon chains arising from the chain inequivalence, the acyl chains undergo a conformational change with respect to the normal bilayers.

Unlike saturated asymmetric chain length phospholipids, the thermotropic phase behavior of unsaturated and other structurally modified symmetric and asymmetric chain length phospholipids are quite different from saturated 1,2-diacyl-PCs, and even sometimes difficult to study calorimetrically. The thermal transitions observed for these kinds of lipids are usually broad and less cooperative, presumably due to the disruptive effect of the double bonds or other groups. Bilayers formed from *trans*-unsaturated lipids exhibit lower phase transition temperatures than their saturated anologs, indicating that the hydrocarbon chains are more loosely packed; thus, they show better miscibility in the gel phase than do saturated PCs. *cis*-Unsaturated PCs, on the other hand, can adopt a gel phase whose structure differs from that of the $L_{\beta'}$ phase formed by saturated phospholipids at lower temperatures. Thus, extensive gel phase immiscibility is observed in mixtures of both *cis*-unsaturated and saturated PCs.

DSC studies of synthetic PCs prepared in this laboratory. Work reported in this dissertation is described on the synthesis of analogs of

glycerophospholipids modified in the acyl chains and in the polar head group. The mixing behavior of highly asymmetric phospholipids dispersed in aqueous solutions is reported based on the DSC observations. It is found by phase diagrams constructed based on the calorimetric data of binary mixtures of PCs that an asymmetric-chain PC having a terminal double bond in the acyl chain at the *sn*-2 position (C18:C(11:1Δ^{10})PC) is miscible at all proportions with C18:C10PC and C18:C11PC in the gel state and in the liquid-crystalline state, and forms a mixed interdigitated bilayer at $T < T_m$ and a partially interdigitated bilayer at $T > T_m$.[32] On the other hand, asymmetric-chain PCs having an ester or a ketone moiety at the terminal position of the *sn*-2 (**6** and **7**, respectively) exhibit a solid phase immiscibility with C18:C10PC over a wide composition range (chapter 7).

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Chapter 2

Facile diacylation of glycidyl tosylate: chiral synthesis of symmetric-chain glycerophospholipids

Abstract: Optically active symmetric-chain 1,2-diacyl-*sn*-3-glycero-phosphocholine was prepared from the derivatized epoxy alcohol, (*R*)-glycidyl tosylate. It is shown that the same procedure can be used to prepare the enantiomer, 2,3-diacyl-*sn*-1-glycerophosphocholine, from (*S*)-glycidyl tosylate. The key step in the synthetic scheme is the stereoselective ring opening of the chiral epoxide with 1.5 equivalents of fatty acid anhydride in the presence of a catalytic amount of boron trifluoride etherate in dichloromethane over a 2-h period. The tosyl group was converted to the iodide, which was then converted to the phosphatidic acid and phosphocholine using standard methods. Since no protecting groups are used to generate the glyceryl esters, a substantial reduction in time is realized compared with published methods.

Introduction: The chemical synthesis of optically active phospholipids involves the extensive use of protecting groups and requires considerable expertise in synthetic lipid chemistry. For example, the synthesis of enantiomerically pure mixed-chain glycerophospholipids from 1,2-isopropylidene-*sn*-3-glycerol or 2,3-isopropylidene-*sn*-1-glycerol entails the use of three protecting groups.[1] Since chiral epoxides have been found to be valuable intermediates in the synthesis of many optically active natural products, we have sought to prepare the natural 1,2-diacyl-*sn*-3-glycerophosphocholines by Lewis acid catalyzed ring opening of chiral epoxides. Conversion of racemic glycidol to racemic ester-linked glycerols has been reported,[2] and optically active glycidol was used as the precursor of triacylglycerols.[3] The preparation of a monoacylglycerol from

optically active glycidol in the presence of titanium (IV) isopropoxide was reported during the course of our investigations; titanium-assisted nucleophilic epoxide opening with stearic acid gave glycidyl stearate in low yield.[4]

We report here an efficient enantiospecific synthesis of 1,2-diacyl-*sn*-3-glycerophosphocholines from (*R*)-(-)-glycidyl tosylate (**1**). The synthetic usefulness of the tosyl derivative of glycidol is demonstrated by (a) facile diacylation in the presence of BF_3 etherate, and (b) conversion of the 3-tosyl group into the 3-phosphocholine moiety, with retention of configuration at C-2 in both steps. Since allyl alcohol is readily converted to either (*R*)- or (*S*)-glycidyl tosylate by asymmetric epoxidation and in situ derivatization,[5] the procedures described here are also applicable to the preparation of phospholipids with the *sn*-1 configuration.

The attachment of two identical fatty acid ester linkages simultaneously to *sn*-glycero-3-phosphocholine or its $CdCl_2$ complex, to give symmetric-chain diacylphosphocholines, has been achieved by well-known methods.[6] In the absence of efficient catalysts, these methods suffer from the need to use severe reaction conditions such as high temperature and long times to obtain a homogeneous mixture of glycerophosphocholine in the aprotic solvent used for the acylation reaction, sometimes resulting in extensive acyl and phosphoryl migration.[6h,j] In contrast, the procedure described here takes place relatively rapidly and under mild conditions, and is not limited to the stereochemistry available in a naturally occurring precursor such as glycerophosphocholine.

In our attempts to use glycidyl derivatives as C_3-synthons for the convenient preparation of glycerophospholipids, we found that exposure of *rac*-epibromohydrin to BF_3 etherate catalyst and 1.5 equivalents of stearic, oleic, and capric anhydrides afforded the corresponding diacylglycerol bromohydrins in yields of 64-84%. We therefore subjected optically active

(*R*)-glycidyl tosylate (-)-**1**, and its enantiomer (+)-**1**, to similar reaction conditions. Scheme I summarizes the synthesis of optically active diacylglycerophosphocholines from glycidyl tosylate. The key feature is the ring opening with 1.5 equivalents of fatty acid anhydride. This is accomplished in 76% yield by stirring with a catalytic amount of BF_3 etherate in dichloromethane over 2 h. The observation that the product **6** has a specific rotation at least as high as that reported in the literature[6a,6i,7] for the optically pure sample indicates that transformation of **(-)-1** into **(+)-2** occurred with complete retention of configuration at C-2. The corresponding enantiomer, **(-)-2**, was prepared analogously from (+)-**1** in 77% yield. Scheme II outlines a mechanism which may explain the stereochemistry observed. The conversion of **1** to **2** is postulated to proceed via initial attack of the anhydride carboxylic oxygen on the primary carbon of **1**, affording intermediate **7** which undergoes acyl migration to give optically active tosylate **2**.

Direct conversion of tosylate **2** with silver diphenyl phosphate to ester **4** in 77% isolated yield was carried out in refluxing xylenes, since lower temperatures (refluxing benzene and toluene) were insufficient to effect the conversion. Unfortunately, the optical purity of the resulting phosphocholine **6** was only about 35%, suggesting that partial racemization took place at refluxing xylene temperature (eq 1). We therefore converted tosylate **2** to iodide **3**; the latter reacted with silver diphenyl phosphate in refluxing benzene, giving ester **4** in 59% yield. Phosphate ester **4** was further converted to diacylphosphocholine **6** by standard procedures.[8]

In summary, we have presented an efficient synthesis of enantiomerically pure diacylglycerophosphocholines from the derivatized epoxy alcohols **(*R*)-(-)-1** and **(*S*)-(+)-1**. The synthetic scheme does not involve the use of protecting groups in the generation of the glyceryl esters, thus making possible

a substantial reduction in time compared with published methods. The procedures are suitable for large-scale preparation of phospholipids because of the relatively low costs of the commercially available (*R*)- and (*S*)-glycidyl tosylate and the long-chain fatty acid anhydrides.

Experimental Section

General. The solvents used were dried and/or distilled as follows: THF, from sodium benzophenone ketyl; pyridine, CH_2Cl_2, benzene, toluene, and xylenes, from calcium hydride (then stored over type 4A molecular sieves); acetone, dried over $CaSO_4$. Boron trifluoride etherate was freshly distilled. Silver diphenyl phosphate was prepared as described previously.[9] Choline tosylate was prepared by the reaction of *N*,*N*-dimethylethanolamine and methyl tosylate in dry THF.[10] ^1H NMR spectra were recorded on a GE Model QE spectrometer (300.5 MHz). Chemical shifts are given in parts per million from tetramethylsilane as internal standard. Optical rotations were measured on a JASCO Model DIP-140 digital polarimeter. Elemental analyses were performed by Desert Analytics, Tucson, AZ (C, H, and N) and by Schwarzkopf Microanalytical Laboratory, Woodside, NY (P). Silica gel G TLC plates of 0.25-mm thickness from Analtech, Newark, DE, were used to monitor reactions, and E. Merck silica gel 60 (230-400 ASTM mesh) was used for flash chromatography. Phospholipids were detected on TLC plates by spraying as described previously.[8b]

1,2-Distearoyl-*sn*-glycero-3-*p*-toluenesulfonate ((+)-2). In a dry 2-necked 100-mL flask fitted with a condenser were placed 1.5 eq of stearic anhydride (413.2 mg, 0.75 mmol) and 114.1 mg (0.5 mmol) of (*R*)-(-)-glycidyl tosylate (**1**) in 20 mL of CH_2Cl_2. Boron trifluoride etherate (4-5 drops, about 0.4

mmol)[11] was added, and the mixture was stirred and refluxed for 2 h under nitrogen. After the reaction mixture had cooled to room temperature, 30 mL of anhydrous ether was added. The organic layer was washed with 5% aqueous sodium bicarbonate (10 mL) and brine (10 mL), dried over sodium sulfate, filtered, and evaporated under reduced pressure. The residue (580 mg) was purified by flash chromatography on silica gel, eluting with 10% ethyl acetate-hexanes, to yield 295 mg (76%) of **(+)-2**; mp 73-75°C; lit.[12] mp 75.4°C; $[\alpha]_D^{25}$ +5.50° (*c* 0.545, $CHCl_3$-CH_3OH, 1:1). ^1H NMR ($CDCl_3$) δ 7.8-7.37 (dd, J = 8.2, 8.3 Hz, 4H, C_6H_4), 5.18-5.13 (m, 1H, $CH_2C\underline{H}CH_2$), 4.29-4.05 (m, 4H, $C\underline{H}_2CHC\underline{H}_2$), 2.46 (s, 3H, CH_3), 2.38-2.20 (t, J = 7.6 Hz, 4H, $COCH_2$), 1.67-1.25 (m, 60H, $(CH_2)_{15}$), 0.90-0.84 (t, J = 6.3 Hz, 6H, ω-CH_3). Anal. Calcd for $C_{46}H_{82}O_7S$: C, 70.90; H, 10.60; S, 4.11. Found: C, 70.66; H, 10.82; S, 4.15.

2,3-Distearoyl-*sn*-glycero-1-*p*-toluenesulfonate ((-)-2). The above procedure was repeated using (*S*)-(+)-glycidyl tosylate, giving the enantiomer of **2** as a white solid in 77% yield; mp 68-70 °C; $[\alpha]_D^{25}$ -5.13° (*c* 0.545, $CHCl_3$-CH_3OH, 1:1).

(*R*)-1,2-Distearoyl-3-iodopropane ((+)-3). To a solution of 200 mg (0.26 mmol) of **(+)-2** in 10 mL of dry acetone was added 250 mg (1.5 mmol) of sodium iodide. After the mixture was refluxed overnight under nitrogen, the solvent was removed under reduced pressure. Anhydrous ether (25 mL) was added to the residue, and the mixture was filtered through Celite to give a yellow residue on removal of the solvent. TLC analysis (elution with hexanes-ethyl acetate 9:1) showed product **3** (R_f 0.60) and a trace of **2** (R_f 0.25). Recrystallization from methanol-petroleum ether (9:1) gave 120 mg (63%) of **(+)-3**, mp 54-55 °C; lit.[13] mp 53-54 °C; $[\alpha]_D^{25}$ +1.47° (*c* 3.185, $CHCl_3$); lit.[13] $[\alpha]_D^{20}$ +2.5° (*c* 10, $CHCl_3$).

Diphenyl 1,2-distearoyl-*sn*-glycero-3-phosphate ((+)-4). The iodide

3 (90 mg, 0.12 mmol) was dried over P_2O_5 in a desiccator and dissolved in 10 mL of dry refluxing benzene in a flask protected from light with aluminum foil. Silver diphenyl phosphate (175 mg, 0.35 mmol) was added and the reaction mixture was refluxed for 4 h, after which time TLC analysis (hexanes-ethyl acetate 4:1) showed complete conversion of **3** into the desired diphenyl phosphate ester **4** (R_f 0.50); traces of tosylate **2** present in **3** remained unreacted. The mixture was cooled to room temperature, filtered through a sintered glass funnel packed with Celite, and washed with chloroform (3 x 50 mL). Removal of solvent left a white solid that was dissolved in hexanes-ethyl acetate (95:5) and purified by flash chromatography on silica gel in the same solvent system, yielding 60 mg (59%) of product **4**; $[\alpha]_D^{25}$ +1.55° (*c* 1.42, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 7.4-7.2 (m, 10H, C_6H_5), 5.28-5.19 (m, 1H, $CH_2C\underline{H}CH_2$), 4.42-4.08 (m, 4H, $C\underline{H}_2CHC\underline{H}_2$), 2.38-2.15 (m, 4H, $COCH_2$), 1.7-1.1 (m, 60H, $(CH_2)_{15}$), 0.92-0.80 (t, J = 7.3 Hz, 6H, ω-CH_3). Anal. Calcd for $C_{51}H_{85}O_8P$: C, 71.46; H, 9.99; P, 3.61. Found: C, 71.86; H, 10.19; P, 3.40.

(+)-**4** was also prepared in 77% yield directly from tosylate (+)-**2** by refluxing for 6 h in dry xylenes with 2.5 eq of silver diphenyl phosphate. Purification by flash chromatography (elution with hexanes-ethyl acetate 95:5) gave a white solid, mp 55-56 °C; lit.[14a] mp 58-59 °C, [14b] 54.5-55°C; *rac*-**4** lit. [14c,d] mp 58-59°C; $[\alpha]_D^{25}$ +0.52° (*c* 5.07, $CHCl_3$) (34% optical purity).

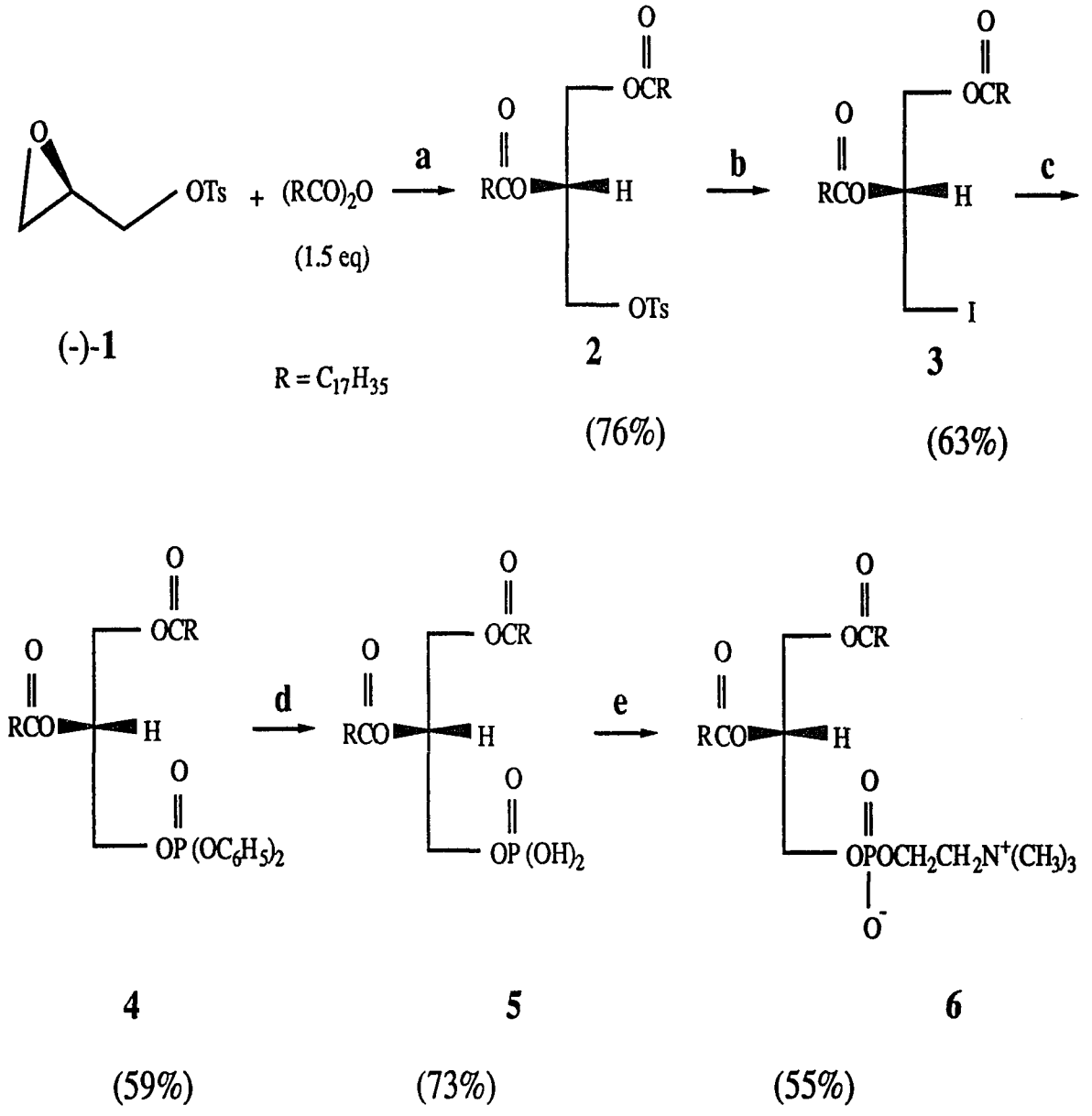
1,2-Distearoyl-*sn*-glycero-3-phosphatidic acid (5). Adams catalyst (80 mg) was suspended in 10 mL of glacial acetic acid and reduced with hydrogen for about 1 h until black granules of platinum black appeared. A solution of 60 mg (0.070 mmol) of diphenyl phosphate **4** in 10 mL of cyclohexane-glacial acetic acid (1:1) was injected into the flask through a rubber septum. The mixture was stirred vigorously at room temperature for 3 h, filtered through a sintered glass funnel packed with Celite, and washed with $CHCl_3$ (3 x 10 mL).

The filtrate was concentrated and the product was obtained by precipitation using 10 mL of cold (-20 °C) acetonitrile. After two precipitations, phosphatidic acid **5** (R_f ~ 0.5 in $CHCl_3$-CH_3OH-HCO_2H, 80:15:5) was obtained as a hygroscopic white solid (36 mg, 73%), which was used in the next step without further purification.

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (6). Phosphatidic acid **5** (36 mg, 0.050 mmol) was dissolved by heating (oil bath) in dry pyridine (10 mL) at 50±5 °C for 30 min. Choline tosylate (140 mg, 0.50 mmol), freshly dried over P_2O_5, and trichloroacetonitrile (2 mL) were added, and the reaction mixture was stirred for 48 h at 50±5 °C. The solvent was removed under reduced pressure; to ensure complete removal of pyridine, the residue was dissolved three times successively in 25 mL of $CHCl_3$-CH_3OH (1:1) and the solvents were evaporated each time under vacuum. The residue was dissolved in THF-water (9:1) and purified by column chromatography on Amberlite MB-3 (20 g; THF-water, 9:1) to give (+)-**6** as a tan solid. Chromatography on silica gel, eluting with $CHCl_3$-CH_3OH (first 9:1, then 3:2), gave 32 mg (55%) of the desired phosphocholine **6** (R_f 0.37 in $CHCl_3$-CH_3OH-H_2O, 65:25:4). The suspended silica gel was removed by filtering a chloroform solution of **6** through a 0.45-mm Metricel filter; (+)-**6**: $[\alpha]_D^{25}$ +6.95° (*c* 0.097, $CHCl_3$-CH_3OH 1:1); an authentic sample purchased from Sigma Chemical Co. had $[\alpha]_D^{25}$ +6.80° ($CHCl_3$-CH_3OH, 1:1); lit.[6a] $[\alpha]_D^{29}$ +6.2° ($CHCl_3$-CH_3OH, 1:1); lit.[6i] $[\alpha]_D^{25}$ +6.4° ($CHCl_3$-CH_3OH, 1:1); lit.[7a] $[\alpha]_D^{25}$ +6.1° ($CHCl_3$-CH_3OH, 1:1); lit.[7b] $[\alpha]_D^{20}$ +6.95° ($CHCl_3$-CH_3OH, 1:1); ^1H NMR ($CDCl_3$) δ 5.30-5.18 (m, 1H, $CH_2C\underline{H}CH_2$), 4.62-4.42 (m, 2H, CH_2OP), 4.40-4.25 (m, 2H, CH_2N), 4.18-3.98 (m, 4H, $C\underline{H}_2CHC\underline{H}_2$), 3.4 (s, 9H, $N(CH_3)_3$), 2.74 (broad s, H_2O), 2.38-2.20 (m, 4H, $COCH_2$), 1.65-1.50 (m, 4H, $COCH_2C\underline{H}_2$), 1.38-1.0 (m, 56H, $(CH_2)_{14}$), 0.92-0.80 (t, J = 7.8 Hz, 6H, ω-CH_3). Anal. Calcd for $C_{44}H_{88}O_8NP{\cdot}3H_2O$: C,

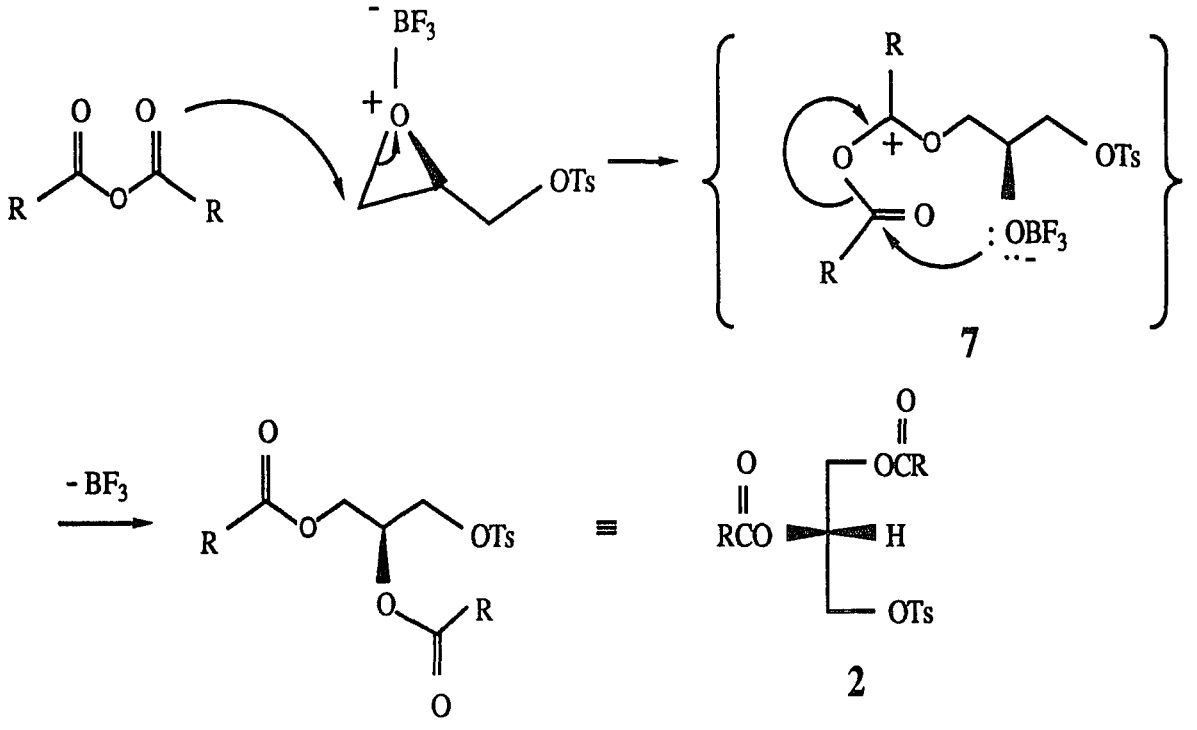
62.60; H, 11.22; N, 1.65; P, 3.67. Found: C, 62.52; H, 11.31; N, 1.44; P, 3.53.

1-Stearoyl-2-lyso-*sn*-glycero-3-phosphocholine. The optical purity of phosphocholine (+)-**6** was examined by treatment with phospholipase A_2 (*Naja naja*, Sigma Chemical Co.) in pH 7.4 buffer for 1 h at 38 °C as described previously.[15] TLC analysis ($CHCl_3$-CH_3OH-H_2O, 65:25:4) showed complete hydrolysis of **6** (R_f 0.37) to 1-stearoyl-2-lysophosphatidylcholine (R_f 0.12), and stearic acid (R_f 0.85), confirming retention of the natural *sn*-3 configuration in 1,2-distearoylphosphatidylcholine (**6**).

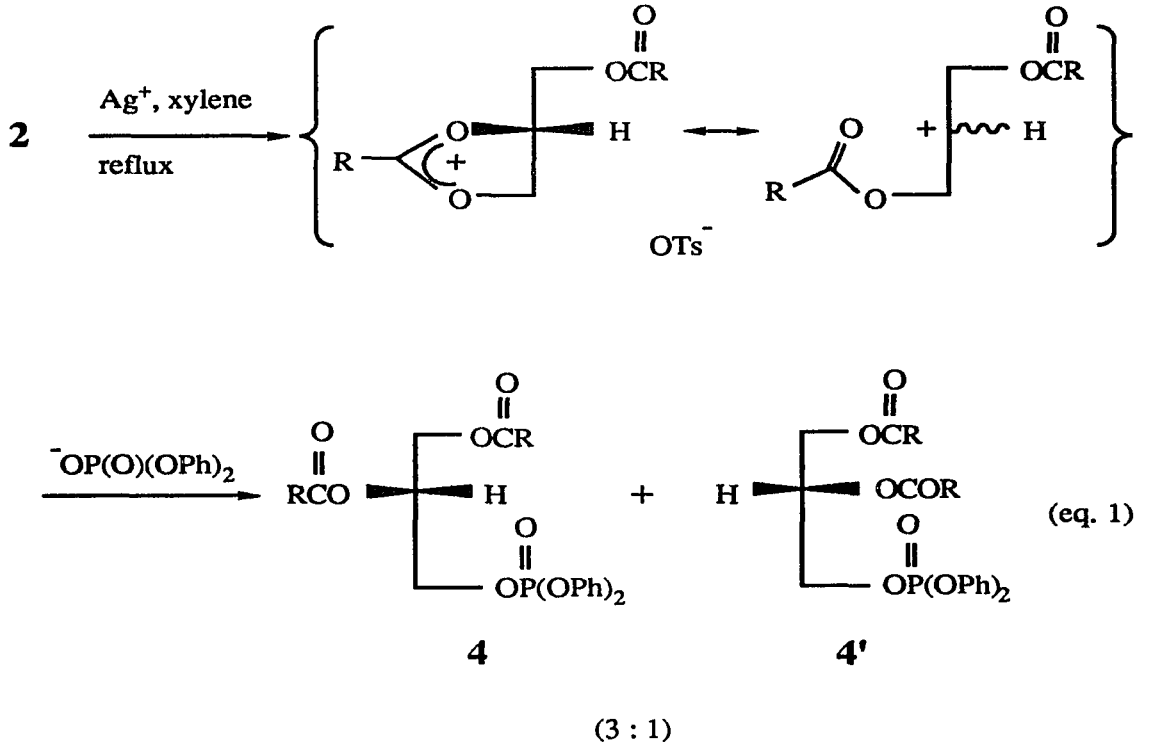


Scheme I: Synthesis of 1,2-distearoyl PC (**6**)

Reagents: (**a**) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 40 °C, 2 h (**b**) NaI (excess), acetone, reflux, 24 h (**c**) $AgOP(O)(OPh)_2$, benzene, 80 °C, 4 h (**d**) H_2/PtO_2, cyclohexane-HOAc, 1:1, 4 h (**e**) $HOCH_2CH_2N^+(CH_3)_3$ OTs^-, Cl_3CCN, Py, 50 °C, 48 h.



Scheme II: A possible mechanism of diacylation of (-)-**1**



Eq. 1: Conversion of tosylate **2** into diphenyl phosphate ester **4** with partial racemization at C-2.

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Chapter 3

Synthesis of optically active 1-acyl-2-O-alkyl-sn-glycero-3-PC 1 via 1-O-benzyl-sn-glycero-3-arenesulfonate

Abstract: A stereocontrolled route to 1-palmitoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphocholine from (*R*)-glycidyl tosylate is described. This method gives very high enantioselectivity (93-96% enantiomeric excess) and can be used to prepare 3-acyl-2-*O*-alkyl-*sn*-glycero-1-phosphocholines from (*S*)-glycidyl tosylate. The key step is the preparation of 1-*O*-benzyl-*sn*-glycerol 3-tosylate by the boron trifluoride etherate catalyzed regio- and stereospecific opening of the epoxide ring with excess benzyl alcohol. The alkyl group is introduced using alkyl trifluoromethanesulfonate in the presence of excess 2,6-di-*tert*-butyl-4-methylpyridine. Debenzylation gives 2-*O*-alkyl-*sn*-glycerol 3-arenesulfonate, which is acylated and then converted into the phosphocholine. The use of chiral glycidyl derivatives as starting materials for the synthesis of glycerophospholipids is discussed.

Introduction: Although saturated and unsaturated mixed-acid PCs are the major components of many biological membranes, alkylacyl and alkenylacyl analogues are also present in significant amounts and acylalkyl analogues are present in small amounts (Horrocks 1972). In order to compare the behavior of the different molecular species of phospholipids, isomerically and optically pure synthetic materials are required. We reported previously that small but significant differences exist in the degree of interaction of cholesterol with 1-acyl-2-*O*-alkyl-PC compared with 1-*O*-alkyl-2-acyl-PC (Bittman *et al.* 1984). Previous papers have described the syntheses of alkylacyl and alkenylacyl phospholipids (e.g., Paltauf 1983). We report here the preparation of chiral

acylalkyl-PC **1**. Epoxides have attracted considerable popularity in organic synthesis because of their high reactivity, ease of preparation, and availability in optically active form; for some recent applications of glycidol and related C_3-synthons and their ring-opened derivatives as chiral building blocks in enantioselective syntheses of a variety of natural products, see references cited in Burgos, Ayer, and Johnson 1987; Klunder, Onami, and Sharpless 1989; Guivisdalsky and Bittman 1989a,b; Byun and Bittman 1989. (*R*)-(-)-Glycidyl arenesulfonates (**2**), which are prepared from allyl alcohol by asymmetric epoxidation followed by sulfonation (Gao *et al.* 1987), are used as the starting materials in this communication for the synthesis of 1-acyl-2-*O*-alkyl-*sn*-glycero-3-phosphocholine. The key step is the BF_3 etherate-catalyzed regio- and stereospecific opening of the epoxide at C_3 using benzyl alcohol as the nucleophile, giving ring-opened intermediate **3** in high yield. In addition, we have developed a very mild procedure for the etherification of **3** that avoids epoxide formation. We have recently reported that **2** is useful for the preparation of PCs with acyl chains at the *sn*-1 and *sn*-2 positions (Ali and Bittman 1988) and with a saturated or unsaturated alkyl chain at the *sn*-1 position (Guivisdalsky and Bittman 1989b,c). The present report extends the utility of arenesulfonates derivatives of glycidol as valuable precursors of 1,2-diradylglycerophosphocholines.

Experimental

Our general analytical and chromatographic methods have been described previously (Ali and Bittman 1988). Reagents were purchased from Aldrich Chemical Co. unless otherwise noted. (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)phenyl acetic acid (MTPA), which was used to prepare the "Mosher ester" for determination of optical purity (Dale et al. 1969), was

obtained from Fluka and Aldrich. Silica gel 60 (230-400 ASTM mesh) was used for flash chromatography. Optical rotations were measured on a JASCO Model DIP-140 digital polarimeter using a 1-dm cell. Most ^1H NMR spectra were recorded at 200 MHz on an IBM-Bruker spectrometer; however, some spectra (indicated below) were recorded at 400 MHz and 500 MHz on JEOL and Varian spectrometers, respectively. Melting points are uncorrected. The solvents used were distilled and dried as follows: dichloromethane, chloroform, and dimethylformamide (DMF) were distilled from calcium hydride and stored over type 3A molecular sieves; benzene was distilled and stored over sodium. Boron trifluoride etherate was distilled before use.

Syntheses

1-*O*-Benzyl-*sn*-glycerol 3-*O*-*p*-Toluenesulfonate (3). To a solution of 1.0 g (4.38 mmol) of (*R*)-(-)-**2** [$[\alpha]^{25}_D$ - 17.0° (*c* 2.75, $CHCl_3$)] in 40 mL of dry CH_2Cl_2 was added 0.95 g (8.76 mmol) of benzyl alcohol and a catalytic amount (4 drops, ~0.40 mmol) of BF_3-etherate. The reaction mixture was stirred at room temperature under nitrogen atmosphere for 2 h. Anhydrous ether (50 mL) was added and the organic phase was washed with 5% aqueous $NaHCO_3$, saturated NaCl solution, and then dried (Na_2SO_4). Evaporation of solvents left a white solid which was dissolved in a minimum volume of 15% ethyl acetate in light petroleum ether and purified by flash chromatography (elution with 15% EtOAc in light petroleum ether). There was isolated 1.24 g (84%) of (-)-**3** as a white solid, mp 46-48 °C; R_f 0.26 (30% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ -6.70° (*c* 7.20, C_6H_6). The literature value (Hirth and Barner 1982) of the enantiomer (+)-**3** is $[\alpha]^{25}_D$ +6.50° (*c* 10.0, C_6H_6). ^1H NMR ($CDCl_3$): δ 7.81-7.24 (m, $OC_6\underline{H}_4CH_3$, $C_6\underline{H}_5$, 9H), 4.49 (s, $C\underline{H}_2C_6H_5$, 2H), 4.10-4.01 (m, $C\underline{H}_2OSO_2Ar$, $CH_2C\underline{H}CH_2$, 3H), 3.52-3.49 (d, *J* = 4.7 Hz, $C\underline{H}_2OBn$, 2H), 2.44 (s,

$C_6H_4C\underline{H}_3$, 3H), 1.62 (s, OH, 1H). Anal. Calcd. for $C_{17}H_{20}O_5S$: C, 60.69; H, 5.99; S, 9.53. Found: C, 60.61; H, 6.06; S, 9.29.

1-*O*-Benzyl-*sn*-glycerol 3-*O*-*m*-Nitrobenzenesulfonate (3; Ar = $C_6H_4NO_2$-3). A solution of 200 mg (0.77 mmol) of (*R*)-(-)-glycidyl 3-nitrobenzenesulfonate, 125 mg (1.16 mmol) of benzyl alcohol, and a catalytic amount (4 drops, ~0.4mmol) of BF_3-etherate in 10 mL of dry CH_2Cl_2 was stirred at room temperature under nitrogen atmosphere for 2 h. The reaction mixture was extracted into ether (50 mL) and the ether layer was washed with 5% aqueous $NaHCO_3$, saturated NaCl solution, and dried (Na_2SO_4). Evaporation of solvents left an oil that was purified by flash chromatography with hexane:ether (1:1) to give 217 mg (77%) of **3** (Ar = $C_6H_4NO_2$-3) as a colorless oil; R_f 0.35 (Et_2O/hexane, 1:1); $[\alpha]^{25}_D$ -11.20° (*c* 6.0, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 8.77-7.78 (m, $C_6\underline{H}_4OSO_2Ar$, 4H), 7.26-7.23 (m, $C_6\underline{H}_5$, 5H), 4.45 (s, $C_6H_5C\underline{H}_2$, 2H), 4.20-4.30 (dd, *J* = 5.2 and 10.5 Hz, $C\underline{H}_2OSO_2Ar$, 2H), 4.10-4.05 (m, $CH_2C\underline{H}CH_2$, 1H), 3.50-3.45 (d, *J* = 4.7 Hz, $C\underline{H}_2OBn$, 2H). Anal. Calc. for $C_{16}H_{17}O_7NS$: C, 52.31; H, 4.66; N, 3.81; S, 8.72. Found: C, 52.42; H, 4.65; N, 3.65; S, 8.84. This compound was alkylated as described below using tosylate **3** (Ar = $C_6H_4CH_3$-4). Since higher yields of *O*-alkylation were obtained with the tosylate than with the 3-nitrobenzenesulfonate (**3**), the etherification procedure is described below. Nevertheless, it should be noted that glycidyl 3-nitrobenzenesulfonate (**2**, Ar = $C_6H_4NO_2$-3) is a valuable substrate for the preparation of 1(3)-*O*-alkyl-*sn*-glycerophospholipids; it is available in very high ee and its leaving group is readily displaced from the ring-opened intermediate (see Guivisdalsky and Bittman 1989c).

1-*O*-Benzyl-2-*O*-hexadecyl-*sn*-glycerol 3-*p*-Toluenesulfonate (4a, n = 15): A solution of 68 mg (0.28 mmol) of 1-hexadecanol and 115 mg (0.56 mmol) of 2,6-di-*tert*-butyl-4-methylpyridine in 10 mL of dry CH_2Cl_2 was cooled to -78 °C. After the solution was flushed with nitrogen several times, triflic anhydride (71 mg, 0.42 mmol) was added. The reaction temperature was brought to room temperature and stirred under nitrogen for nearly 1.5 h, during which time the solution turned clear. Tosylate **3** (50 mg, 0.14 mmol) was added, and the reaction mixture was refluxed at ~ 41 °C for 3 days. A white precipitate (triflic acid) appeared during the reaction, which was removed by filtration. Evaporation of the solvent left an oil that was purified by flash chromatography with 5% EtOAc in light petroleum ether to give 37 mg (43%) of **4a** as a white solid, mp 51-52 °C; R_f 0.58 (15% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ -2.16° (*c* 1.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.81-7.25 (m, $OC_6\underline{H}_4CH_3$, $C_6\underline{H}_5$, 9H), 4.48 (s, $C\underline{H}_2C_6H_5$, 2H), 4.25-4.00 (dd, J_{AC} = 4.3 Hz, J_{AB} = 10.3 Hz, $CH_CC\underline{H}_AH_BOSO_2Ar$, 1H, and dd, J_{BC} = 5.6 Hz, J_{AB} = 10.3 Hz, $CH_CH_A\underline{H}_BOSO_2Ar$, 1H), 3.66-3.61 (m, $CH_2C\underline{H}CH_2$, 1H), 3.48-3.41 (m, $C_{15}H_{31}C\underline{H}_2OCHC\underline{H}_2OBn$, 4H), 2.40 (s, $C_6H_4C\underline{H}_3$, 3H), 1.65-1.15 (br, $C\underline{H}_2$'s, 28H), 0.92-0.82 (t, *J* = 6.8 Hz, ω-CH_3, 3H).

1-*O*-Benzyl-2-*O*-decyl-*sn*-glycerol 3-*p*-Toluenesulfonate (4b, n = 9): This compound was prepared in 46% yield as described above for **4a**. There was obtained a colorless oil; $[\alpha]^{25}_D$ -1.57° (*c* 1.59, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.86-7.19 (m, $OC_6\underline{H}_4CH_3$, $C_6\underline{H}_5$, 9H), 4.48 (s, $C\underline{H}_2C_6H_5$, 2H), 4.24-4.05 (dd, J_{AC} = 4.3 Hz, J_{AB} = 10.3 Hz, $CH_CC\underline{H}_AH_BOSO_2Ar$, 1H, and dd, J_{BC} = 5.6 Hz, J_{AB} = 10.3 Hz, $CH_CH_A\underline{H}_BOSO_2Ar$, 1H), 3.67-3.57 (m, $CH_2C\underline{H}CH_2$, 1H), 3.52-3.40 (m, $C_9H_{19}C\underline{H}_2OCHC\underline{H}_2$, 4H), 2.43 (s, CH_3, 3H), 1.47-1.10 (br, $C\underline{H}_2$'s, 16H), 0.90-0.76 (t, *J* = 6.8 Hz, ω-CH_3, 3H).

1-*O*-Benzyl-2-*O*-hexyl-*sn*-glycerol 3-*p*-Toluenesulfonate (4c, n = 5): This compound was prepared in 73% yield as described above for **4a**. There was obtained a colorless oil; $[\alpha]^{25}_D$ -3.06° (*c* 2.10, $CHCl_3$). Anal. Calc. for $C_{23}H_{32}O_5S$: C, 65.68; H, 7.67; S, 7.62. Found: C, 66.00; H, 7.62; S, 7.34.

1-Hydroxy-2-*O*-hexadecyl-*sn*-glycerol 3-*p*-Toluenesulfonate (5a). A solution of **4a** (n = 15; 110 mg, 0.19 mmol) in 25 mL of MeOH:$CHCl_3$ (9:1) was stirred with 200 mg of Pearlman's catalyst under hydrogen atmosphere for 1 h. The reaction mixture was filtered through a Celite pad, which was washed with $CHCl_3$ (3 x 10 mL). The filtrate was evaporated under reduced pressure to give 93 mg (100%) of **5a** as an oil; R_f 0.11 (15% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ +12.69° (*c* 1.29, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.82-7.33 (dd, $C_6\underline{H}_4CH_3$, *J* = 8.3 and 8.1 Hz, 4H), 4.09-4.06 (d, *J* = 4.98 Hz, $C\underline{H}_2OH$, 2H), 3.57-3.49 (m, $C_{15}H_{31}C\underline{H}_2OC\underline{H}C\underline{H}_2OSO_2Ar$, 5H), 2.46 (s, $C_6H_4C\underline{H}_3$, 3H), 1.50-1.17 (br $C\underline{H}_2$'s, 28H), 0.91-0.84 (t, *J* = 6.2 Hz, ω-CH_3, 3H).

1-Hydroxy-2-*O*-decyl-*sn*-glycerol 3-*p*-Toluenesulfonate (5b). This compound was prepared in 94% yield as described above for **5a**. There was obtained an oil; R_f 0.13 (15% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ +13.39° (*c* 0.52, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.82-7.33 (dd, *J* = 8.3 and 7.9 Hz, $C_6\underline{H}_4CH_3$, 4H), 4.09-4.06 (d, *J* = 4.9 Hz, $C\underline{H}_2OH$, 2H), 3.67-3.44 (m, $C_{19}H_{19}C\underline{H}_2OC\underline{H}C\underline{H}_2OSO_2Ar$, 5H), 2.45 (s, $C_6H_4C\underline{H}_3$, 3H), 1.82-1.71 (br s, OH, 1H), 1.50-1.17 (br, $C\underline{H}_2$'s, 16H), 0.90-0.85 (t, *J* = 6.8 Hz, ω-CH_3, 3H).

1-Palmitoyl-2-*O*-hexadecyl-*sn*-glycerol 3-*p*-Toluenesulfonate (6a). To a stirred solution of 152 mg (0.58 mmol) of palmitic acid and 70 mg (0.32 mmol) dicyclohexylcarbodiimide in 20 mL of dry CH_2Cl_2 were added 93 mg

(0.19 mmol) of **5a** and 70 mg (0.58 mmol) of 4-(dimethylamino)pyridine. The reaction mixture was flushed with nitrogen and allowed to stir at room temperature for 2 h. A white precipitate of urea was formed, which was removed by filtration through Celite. The Celite pad was washed with $CHCl_3$, and the solvents were evaporated under reduced pressure. After flash chromatography with 2% EtOAc in light petroleum ether, 140 mg (100%) of **6a** was obtained as a white solid, mp 56-57 °C; R_f 0.61 (10% EtOAc in petroleum ether); $[\alpha]^{25}_D$ +2.06° (*c* 1.29, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 7.76-7.25 (dd, *J* = 8.0 and 8.3 Hz, $C_6\underline{H}_4CH_3$, 4H), 4.07-3.97 (m, $C\underline{H}_2CHC\underline{H}_2$, 4H), 3.62-3.57 (m, $CH_2C\underline{H}CH_2$, 1H), 3.42-3.35 (m, $C_{15}H_{31}C\underline{H}_2OCH$, 2H), 2.38 (s, $C_6H_4C\underline{H}_3$, 3H), 2.21-2.18 (t, *J* = 7.2 Hz, $C_{14}H_{29}C\underline{H}_2OCO$, 2H), 1.50-1.18 (br, $C\underline{H}_2$'s, 54H), 0.83-0.77 (t, *J* = 6.65 Hz, ω-CH_3, 6H).

1-Stearoyl-2-*O*-decyl-*sn*-glycerol 3-*p*-Toluenesulfonate (6b). This compound was prepared in 89% yield as described above for **6a**. There was obtained an oil which solidified on storage at -20 °C; R_f 0.61 (10% EtOAc in petroleum ether); $[\alpha]^{25}_D$ +2.40° (*c* 0.60, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 7.82-7.32 (dd, *J* = 8.2 and 7.7 Hz, $C_6\underline{H}_4CH_3$, 4H), 4.10-4.04 (m, $C\underline{H}_2CHC\underline{H}_2$, 4H), 3.70-3.67 (m, $CH_2C\underline{H}CH_2$, 1H), 3.46-3.42 (m, $C_9H_{19}C\underline{H}_2OCH$, 2H), 2.45 (s, $C_6H_4C\underline{H}_3$, 3H), 2.28-2.21 (t, *J* = 7.2 Hz, $C_{16}H_{31}C\underline{H}_2OCO$, 2H), 1.58-1.01 (br, $C\underline{H}_2$'s, 46 H), 0.88-0.84 (t, *J* = 6.9 Hz, ω-CH_3, 6H). Anal. Calcd. for $C_{38}H_{68}O_6S$: C, 69.89; H, 10.49. Found: C, 69.44; H, 10.53.

1-Palmitoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (1). Compound **6a** was converted to 3-palmitoyl-2-hexadecyloxy-1-iodopropane in 97% yield, then to diphenyl 1-palmitoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphate in 61% yield and finally to the corresponding phosphocholine **1** in 64% yield

using standard procedures (Ali and Bittman 1988). The pertinent spectral and physical constants are as follows: **3-palmitoyl-2-hexadecyloxy-1-iodopropane:** mp 36-37 °C; R_f 0.53 (5% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ -0.62°(*c* 1.33, $CHCl_3$). **Diphenyl 1-palmitoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphate:** mp 29-30 °C; R_f 0.52 (15% EtOAc in light petroleum ether); $[\alpha]^{25}_D$ +0.38° (*c* 3.25, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 7.38-7.20 (m, $C_6\underline{H}_5$, 10H), 4.33-4.11 (m, $C\underline{H}_2CHC\underline{H}_2$, 4H), 3.72-3.67 (m, $CH_2C\underline{H}CH_2$, 1H), 3.53-3.47 (m, $C_{15}H_{31}C\underline{H}_2OCH$, 2H), 2.33-2.25 (t, *J* = 7.3 Hz, $O_2CC\underline{H}_2C_{14}H_{29}$, 2H), 1.59-1.0 (br, $C\underline{H}_2$'s, 54H), 0.91-0.84 (t, *J* = 6.1 Hz, w-CH_3, 6H). **1:** R_f 0.49 ($CHCl_3$:MeOH:H_2O, 60:30:4); $[\alpha]^{25}_D$ +2.42° (*c* 0.59, $CHCl_3$:MeOH; 1:1); ^1H NMR ($CDCl_3$): δ 4.42-4.35 (m, $CHC\underline{H}_2O_2CC_{15}H_{31}$, 2H), 4.34-4.24 (m, $CH_2C\underline{H}CH_2$, 1H), 4.15-4.10 (t, *J* = 6.9 Hz, $C_{15}H_{31}C\underline{H}_2OCH$, 2H), 4.05-3.92 (m, $CHC\underline{H}_2OP$, 2H), 3.81-3.70 (m, $POC\underline{H}_2CH_2N$, 2H), 3.74-3.63 (m, $CH_2C\underline{H}_2N$, 2H), 3.34 (s, $C\underline{H}_3N$, 9H), 2.81 (br s, H_2O), 2.43 (t, *J* = 7.5 Hz, $OCOCH_2$, 2H), 1.71-1.22 (br, CH_2's, 54 H), 0.95-0.90 (t, *J* = 6.9 Hz, ω-CH_3's, 6H). Anal. Calcd. for $C_{40}H_{82}O_7NP \cdot 3.5\ H_2O$: C, 61.35, H, 11.45, N, 1.78. Found: C, 61.44, H, 10.20, N, 1.56.

(*R*)-a-Methoxy-a-(trifluoromethyl)phenylacetyl (MTPA) chloride. The MTPA chloride was prepared by adding 3 equiv of oxalyl chloride followed by one drop of dry DMF to 1 equiv of (*R*)-(+)-MTPA in 1 mL of hexane. After the mixture was stirred at room temperature for 30 min, and then was refluxed for 3 h, the solvents were removed under reduced pressure and (*R*)-(+)-MTPA chloride was distilled (bp 50°C, 0.6 mm).

3-[(*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetyloxy (MTPA)]-2-[(*R*)- or (*S*)-decyloxy]-1-iodopropane [(*R*)-7 or (*S*)-7]. Tosylate (*R*)-**4b** or

(*S*)-**4b** (prepared from (*S*)-**2**) was converted to the iodide using excess sodium iodide in refluxing acetone (Ali and Bittman 1988). Debenzylation of the iodide with Pearlman's catalyst ($Pd(OH)_2$-C) in methanol-chloroform (9:1) for 1 h gave the desired alcohol in 100% yield. The latter was filtered, dried (Na_2SO_4), and acylated with (*R*)-(+)-MTPA chloride as follows. To a solution of 10 mg (0.02 mmol) of (*R*)- or (*S*)-3-hydroxy-2-decyloxy-1-iodopropane, 5 mg (0.04 mmol) of 4-(dimethylamino)pyridine, and 30 μL of triethylamine in 1 mL of dry methylene chloride was added 30 μL of MTPA chloride. After the mixture had stirred at room temperature for about 30 min, TLC (20% ethyl acetate in hexanes) indicated that all of the starting material had been acylated. The reaction was quenched by adding 30 μL of 3-(dimethylamino)propylamine and the solvent was removed under reduced pressure. The residue was dissolved in 10 mL of spectral-grade hexane-ethyl acetate (4:1), and the solution was passed through a sintered-glass funnel packed with silica gel to remove polar impurities. The crude, light yellow MTPA ester [(*R*)-**7** or (*S*)-**7**] was obtained by evaporation of the solvents under reduced pressure. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of (*R*)-**7**: δ 7.55-7.39 (m, $C_6\underline{H}_5$, 5H), 4.50-4.46 (dd, J_{AC} = 4.67 Hz, J_{AB} = 11.72 Hz, MTPAOC$\underline{H}_A$H$_B$CH$_C$, 1H), 4.42-4.38 (dd, J_{BC} = 5.12 Hz, J_{AB} = 11.47 Hz MTPAOCH$_A\underline{H}_B$CH$_C$, 1H), 3.60-3.42 (m, $C_9H_{19}OC\underline{H}_2C\underline{H}$, $OC\underline{H}_3$, 6H), 3.20 (d, *J* = 6.0 Hz, $C\underline{H}_2I$, 2H), 1.60-1.20 (br m, $CH_3(C\underline{H}_2)_8$, 16H), 0.88 (t, *J* = 8.0 Hz, ω-CH_3, 3H). ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of (*S*)-**7**: identical to that of (*R*)-**7** except in the region of δ 4.3-4.5: δ 4.52-4.48 (dd, J_{AC} = 4.40 Hz, J_{AB} = 11.47 Hz, MTPAOC$\underline{H}_A$H$_B$CH$_C$, 1H), 4.38-4.34 (dd, J_{BC} = 5.61 Hz, J_{AB} = 11.59 Hz, MTPAOCH$_A\underline{H}_B$CH$_C$, 1H).

Evaluation of optical purity of 4. The 500-MHz ^{1}H NMR spectrum of the (*R*)-(+)-MTPA esters derived from (*R*)-**4** (Fig. 1A, δ 4.46-4.53) and from (*S*)-**4**

(Fig. 1B, δ 4.34-4.43) shows two sets of AB quartets. The lower field doublet of doublets (δ ~4.51) is assigned to H_a of the MTPA ester derived from stereoisomer (*S*)-4, whereas the higher field of doublet of doublets (δ ~4.36) is assigned to H_b of the MTPA ester derived from (*S*)-4. Note that each doublet of doublets of H_b is well resolved in Fig. 1B. The integrated areas of the two sets of doublets of doublets indicated that (*S*)-4 has 93% ee. The A proton segment (Fig. 1A) is not as readily separable as the B proton segment because the higher field doublet of (*S*)-H_a overlapped with the low field doublet of (*R*)-H_a. We estimated the integrated area of (*S*)-H_a from the area shown for the low-field doublet of (*S*)-H_a in Fig. 1A plus the equivalent area multiplied by the factor corresponding to the nonideal nature of the AB quartet; this factor was obtained from the ratio of the integrated areas of the high (δ ~ 4.47) and low (δ ~ 4.50) field doublets assigned to (*R*)-H_a. We thus calculated the ee of (*R*)-4 to be ~96%.

Discussion

Early approaches to the chemical synthesis of phospholipids involved the introduction of phosphate ester linkages into 1,2-diglycerides (e.g., Baer and Kates 1950; Baer 1951; Uhlenbroek and Verkade 1953; deHaas and van Deenen 1961) or into 2,3-diacyloxy-1-iodopropanes (Hessel *et al.* 1954; Stanacev and Kates 1960). Other approaches involve the simultaneous introduction of two identical fatty acids into glycerophosphocholine (e.g., Baer, Buchnea, and Newcombe 1956; Cubero Robles and van Berg 1969; Pugh and Kates 1975) and the acylation of lyso-PC (e.g., Gupta, Radhakrishnan, and Khorana 1977). The route from D-mannitol to (*R*)-2,3-*O*-isopropylidene-glyceraldehyde (Fischer and Baer 1937; Baer and Fischer 1939) and then to diacyl-PC (Baer and Kates 1950) may be regarded as the classical, but still

frequently used, approach for the synthesis of phospholipids. A disadvantage of this approach is that nine steps are required to convert D-mannitol into symmetric-acid phospholipids. Another disadvantage is that 1,2,5,6-diisopropylidene-D-mannitol is somewhat unstable on storage, apparently decomposing to D-mannitol and monoisopropylidene- D-mannitol (Rosenthal 1975). Furthermore, the enantiomer (*S*)-2,3-*O*-isopropylideneglyceraldehyde is not readily available (Jurczak, Pikul, and Bauer 1986), and has been made in reaction sequences starting from L-arabinose (Baer and Fischer 1939), L-inositol (Angyal and Hoskinson 1963), and ascorbic acid (Jung and Shaw 1980; Mikkilineni, Kumar, and Abushanab 1988). Since isopropylideneglycerol, which is prepared from diisopropylidene-D-mannitol (Baer 1952; LeCocq and Ballou 1964) and is the precursor of isopropylideneglyceraldehyde, undergoes self-catalyzed partial racemization during storage (Eibl 1984) and complete racemization in the presence of a trace of acidic impurity (Baldwin *et al.* 1978), starting materials with higher configurational stability are desired. 2,3-Isopropylidene-*sn*-glycerol tosylate provides such a stereochemically stable precursor (Jung and Shaw 1980; Jurczak, Pikul, and Bauer 1986; Bhatia and Hajdu 1989).

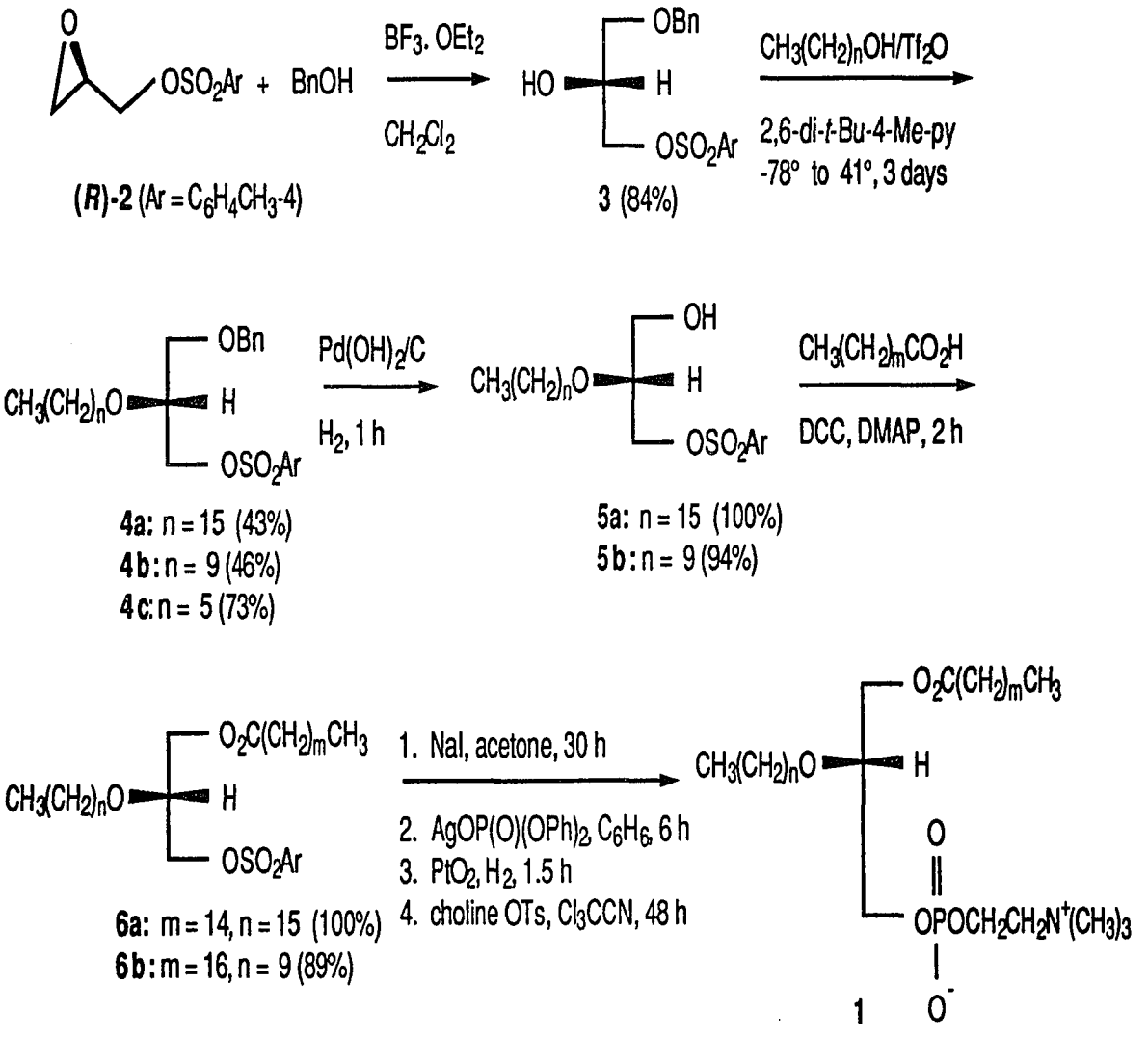
O-Alkylation of glycerol derivatives has generally been carried out with alkyl bromides (Kates, Chan, and Stanacev 1963; Palameta and Kates 1966) and alkyl methanesulfonates (Baumann and Mangold 1966; Baumann 1972) as the alkylating agent. Recently, the more reactive alkyl trifluoromethanesulfonate has been used to alkylate the C_2 hydroxyl group of a disubstituted glycerol in the presence of potassium hydride in tetrahydrofuran (Aoki and Poulter 1985). We report here a method using sufficiently mild basic conditions to alkylate the available hydroxyl group of **3** without formation of the epoxide via displacement of the tosylate group. Alkylation was carried out using alkyl

trifluoromethanesulfonates (triflates) in the presence of excess 2,6-di-*tert*-butyl-4-methylpyridine in methylene chloride. The yield of the alkylation of the C_2 hydroxyl group of **3** was 73% when 2 equiv of 1-hexanol and 3 equiv of fresh triflic anhydride were used. However, poorer yields were obtained when longer *n*-alkanols such as 1-decanol and 1-hexadecanol were used. The lower yields with the long-chain alcohols may arise from steric hindrance since prolonging the reaction time (e.g., 5 days in refluxing methylene chloride) did not improve the yields significantly.

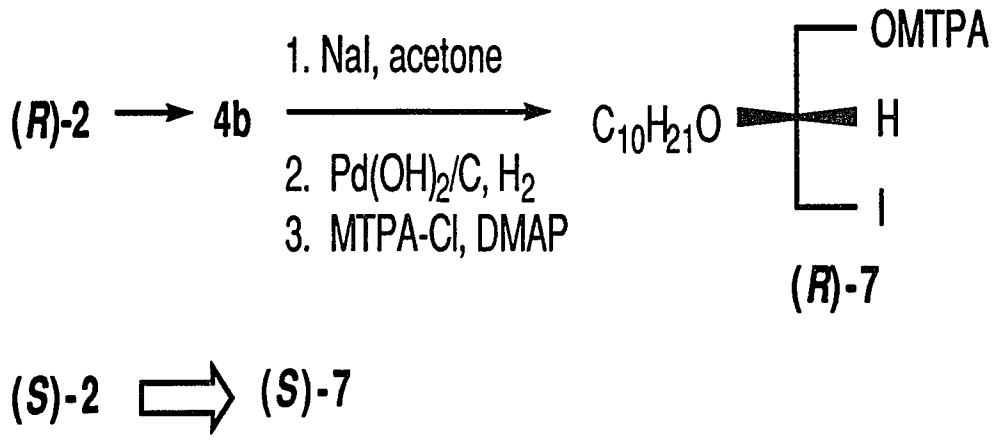
The acylation reaction is carried out with the disubstituted glycerol **5**; since the *O*-alkyl and arenesulfonate groups are not prone to intramolecular migration, acylation at C_1 gave the acylated product **6** uncontaminated by isomeric products. In order to complete the synthesis of **1**, it was necessary to remove the tolylsulfonyl group and replace it with the phosphocholine group. This was accomplished in satisfactory overall yield by displacement using sodium acetone in refluxing acetone, followed by standard procedures (Deroo *et al.* 1976; Ali and Bittman 1988).

We have recently synthesized both enantiomers of diester (Ali and Bittman 1988), diether (Guivisdalsky and Bittman 1989b), and ether/ester glycerophosphocholines (Guivisdalsky and Bittman 1988, 1989b,c) from stable, crystalline arenesulfonate derivatives of glycidol that are readily available in high enantiomeric purity. Although glycidol has been used as a precursor of 1-acyl-*sn*-glycerol (Burgos, Ayer, and Johnson 1987; Mank, Lok, and Ward 1989), it should be noted that the 3-arenesulfonate derivatives of glycidol offer the advantage of being synthetically more advanced in that they possess a "protecting group" at the *sn*-3 position. In this paper we have reported a new method for the synthesis of ester-ether PCs from (*R*)-glycidyl arenesulfonates (**2**) with different chains at the *sn*-1 and *sn*-2 positions. This method avoids the

possibility of acyl migration by introducing the ester into a difunctionalized glycerol derivative (**5**) and can also be used to prepare 3-acyl-2-*O*-alkyl-*sn*-1-glycerophosphocholines from (*S*)-**2**. The $BF_3 \cdot OEt_2$-mediated nucleophilic opening of (*R*)- and (*S*)-**2** gives the ring-opened intermediate **3** regio- and stereospecifically. After facile workup, **3** is alkylated with various alkyl triflates in the presence of excess 2,6-di-*tert*-butyl-4-methylpyridine. The optical purities of the PCs obtained by this method are very high (93-96% ee), as shown by 500-MHz ^{1}H-NMR spectroscopy of the Mosher ester derived from 2-*O*-alkyl-*sn*-glycerol 3-iodide (Fig. 1). Thus arenesulfonates derivatives of glycidol appear to be valuable C_3 synthons of general utility for the preparation of a variety of glycerolipids with both the natural and unnatural configuration.



Scheme I: Reaction sequence for the conversion of the chiral C_3 synthon (*R*)-**2** into 1-acyl-2-*O*-alkyl-*sn*-glycero-3-PC (**1**)



Scheme II: Conversion of (*R*)- (*S*)-**2** into the MTPA esters (*R*)- or (*S*)-**7**

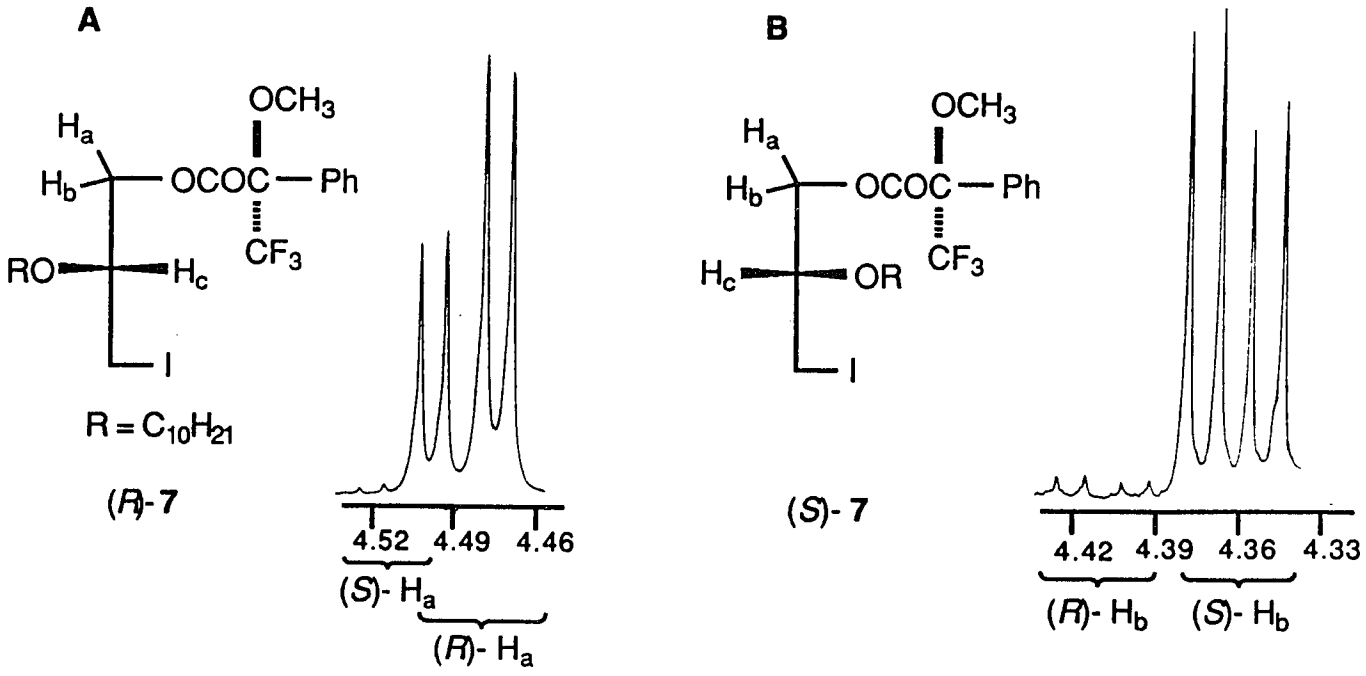


Fig. 1: ^{1}H NMR spectra (500 MHz) at δ 4.33-4.53 of the H_a and H_b protons at C-1 of the (*R*)-(+)-MTPA esters of (*R*)-**7** (panel **A**) and (*S*)-**7** (panel **B**)

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Chapter 4

Synthesis of diacylphosphatidylcholines with one and two long isopropenoid chains

Abstract: The syntheses of diacylphosphatidylcholines with one and two docosoyl chains containing four methyl branches are reported. The isopropenoid chain is derived from phytol, which is oxidized to phytenal using chromium trioxide-pyridine complex. The Wadsworth-Emmons modified Wittig reaction is used to extend the hydrocarbon chain of phytenal. The resulting homolog is converted into (5*R*/*S*,9*R*,13*R*)-5,9,13,17-tetramethyloctadecanoic anhydride, which is used in the diacylation of glycero-*sn*-3-phosphocholine-$CdCl_2$ complex, giving 1,2-bis[(5'*R*/*S*,9'*R*,13'*R*)-5',9',13',17'-tetramethyloctadecanoyl]-*sn*-glycero-3-phosphocholine in 58% yield. Hydrolysis of the *sn*-2 acyl chain by phospholipase A_2 catalysis gave the corresponding lysophosphatidylcholine, which is acylated using *n*-acyl anhydrides. The mixed-chain phosphatidylcholines with one C_{22}-isopropenoid acyl chain and one *n*-acyl chain may be useful in probing the hydrocarbon packing constraints imposed by multiple methyl branches in bilayers.

Introduction: The length, degree of saturation, and extent of branching of the hydrocarbon chains of phospholipids play important roles in determining the strength of lipid-lipid and lipid-protein interactions in membranes. Fully saturated branched-chain phospholipids and glycolipids, in which the hydrocarbon chains of isoprenoid alcohols are attached to glycerol through ether instead of through the usual ester linkages, occur in nature in archaebacteria, including extreme halophiles and various methanogens and thermoacidophiles [1-4 and references cited therein]. Diester and diether

phosphocholines bearing 20-carbon atoms including four methyl branches in each chain have been synthesized by chemical procedures. The synthesis of the diester, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, used phytanic acid as the hydrocarbon-chain precursor, which was derived from dihydrophytol (3, 7, 11, 15-tetramethylhexadecanol) [5]. The isopranyl glyceryl diether phospholipid, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphocholine, was synthesized in a semisynthetic approach by phosphorylation of natural 2,3-di-*O*-phytanyl-*sn*-glycerol, which was obtained from *Halobacterium halobium* [6]. The four methyl groups of each chain prevent close packing with neighboring fatty acyl chains, giving rise to an environment that resembles the fluidity of lipids having unsaturated chains [7]. Thus phosphatidylcholines with fatty acyl chains with multiple methyl branches form bilayers in the liquid-crystalline state at room temperature (unlike the corresponding *n*-acyl compounds); furthermore, they are not prone to oxidation, unlike the polyunsaturated analogs with the same number of carbon atoms. These properties have made fully saturated phospholipids with multiple methyl branches useful in many membrane studies, such as carrier-mediated ion transport [e.g., 5,8], water permeability [6], cholesterol movement [9], and reconstitution of membrane proteins [10]. Another advantage of the fully saturated phytanoyl and phytanyl groups is the absence of lipid unsaturation, which makes it possible to carry out NMR studies of vinyl groups in reconstituted membranes, as in the case of retinal in diphytanoyl-PC vesicles [11].

The effects of acyl chain length and position of methyl branching on the thermotropic properties and molecular areas of phosphatidylcholines have been studied in detail (see, for example, refs. 12, 13). No phase transitions were detected in diphytanoyl-PC bilayers over the temperature range -120 ° to 120

°C at atmospheric [7] and high pressure [14]. For the study of structural events associated with phase transitions, an increase in acyl chain length of the phytanoyl group and the presence of methyl branches in only one chain of the fully saturated phospholipid are needed. In this paper we report the synthesis of diester PC **7**, which has C_{22} chains bearing four methyl branches at both the *sn*-1 and *sn*-2 positions, and diester PC **9**, which has a *n*-acyl chain at the *sn*-2 position. The synthetic methods described here permit the chain lengths to be varied at both the *sn*-1 and *sn*-2 positions, making available a wide range of compounds for analysis of the influence of methyl branching on packing properties and phase transitions of saturated phosphatidylcholines.

Experimental Section

Materials and methods

Phytol (90-95% purity) was purchased from Pfaltz and Bauer (Waterbury, CT) and was used without further purification. Trimethyl phosphonoacetate was from Aldrich Chemical Co. (Milwaukee, WI). Glycero-*sn*-3-phosphocholine-$CdCl_2$ and dicyclohexylcarbodiimide (DCC) were from Sigma Chemical Co. (St. Louis, MO). Pyridine and benzene were distilled from CaH_2, and dichloromethane and chloroform were distilled from P_2O_5; these solvents were stored over type 4A molecular sieves. Acetone was dried over $CaSO_4$. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl immediately before use. 4-(Dimethylamino)pyridine (DMAP) was purchased from Aldrich and recrystallized from chloroform-ether (1:1). Phospholipase A_2 (*Naja naja*) was purchased from Sigma (catalog number P6139) and was used without further purification.

^1H NMR spectra were recorded on a 300-MHz GE Model QE or a 200-MHz IBM-Bruker spectrometer. Chemical shifts are reported in parts per

million downfield from tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer Model 598 spectrophotometer. Optical rotations were measured in a 1-dm cell on a JASCO Model DIP-140 digital polarimeter. Melting points were recorded on a Fisher-Johns hot stage and are uncorrected. Elemental analyses were performed by Desert Analytics (Tucson, AZ). Reactions were monitored using 0.25-mm thick silica gel G TLC plates (Analtech, Newark, DE). Silica gel 60 (230-400 mesh) from E. Merck was used for flash chromatography. Phosphorus- and nonphosphorus-containing compounds were detected by spraying the TLC plates with phosphomolybdic acid and sulfuric acid in ethanol as described previously [15].

Chemical syntheses

(7*R*, 11*R*)-3,7,11,15-Tetramethyl-2(*E*,*Z*)-hexadecen-1-al (2)

To a solution of 6.4 g (80 mmol) of dry pyridine in 20 mL of dry CH_2Cl_2 was added, in portions, 4.0 g (40 mmol) of chromium trioxide. [*Caution:* Addition of CrO_3 to pyridine is an exothermic reaction.] After the orange solution of CrO_3-pyridine complex (Collins' reagent) was stirred at room temperature for 15 min, a solution of 2.0 g (6.74 mmol) of phytol **1** in 10 mL of CH_2Cl_2 was added slowly through a pressure-equalizer funnel over a 15-min period. The reaction mixture was stirred for an additional 30 min, during which time the reduced CrO_3-pyridine complex turned black. Ether (20 mL) was added and the resulting solution was filtered through a sintered-glass funnel packed with silica gel to remove traces of pyridine and other colored material. The solvents were removed at reduced pressure to give 1.57 g (79%) of **2** as a light yellow oil; R_f 0.44 and 0.51(*cis/trans* mixture) in 5% EtOAc-95% light petroleum ether. Since attempted purification of the residue by chromatography on silica gel using 5% EtOAc in light petroleum ether resulted in polymerization and low yield of aldehyde, the crude oil **2** was used in the next step without purification. IR (neat)

2860, 2730, and 1690 (CHO); 1640 (vinyl) cm^{-1}. NMR ($CDCl_3$) δ 5.68 (CH=CH) and 9.90-10.04 (CHO) ppm.

Methyl (5*R/S*, 9*R*, 13*R*)-5,9,13,17-tetramethyl-2,4-octadidecen -oate (3) [Homologation of the isopropenoid chain by phosphonate Wittig reaction]

A two-neck oven-dried 50-mL round-bottom flask was charged with NaH (97%, 75 mg, 3.0 mmol) in 20 mL of dry THF. One side arm was fitted with a pressure-equalizer funnel and a nitrogen gas inlet, and a solution of 1.47 g (4.7 mmol) of **2** in 10 mL of THF was added to the funnel. The other arm was fitted with a glass-joint stopper. The flask was flushed with nitrogen several times. The phosphonate ester (0.55 g, 3.0 mmol, see Scheme 1) was added in portions, causing an immediate release of hydrogen gas. After addition of trimethyl phosphonoacetate was complete, the resulting white precipitate of the sodium salt of the phosphonoacetate was cooled to 0 °C and allowed to stir for 15 min. The solution of compound **2** in the pressure-equalizer funnel was added dropwise. As the reaction started to take place, the white precipitate disappeared and the solution became clear. After the reaction mixture was stirred overnight, 5 mL of water was added and the product was extracted with ether (3 x 100 mL). The ether layer was washed with 5% aqueous $NaHCO_3$ solution, saturated aqueous NaCl solution, and then dried (Na_2SO_4). Solvent evaporation left an oil that was purified by flash chromatography on silica gel with light petroleum ether giving 0.75 g (71%) of product **3** as an oil (mixture of *trans* and *cis* isomers); R_f 0.61 and 0.68 in 5% EtOAc-light petroleum ether. ^1H NMR ($CDCl_3$): δ 7.66-7.53 (m, 1 H, CHCO), 6.0-5.74 (m, 2 H, CH=CH), 3.74 (s, 3 H, CH_3), 2.14-0.85 (br, 36 H CH_2s and CH_3s).

Methyl (5*R/S*, 9*R*, 13*R*)-5,9,13,17-tetramethyloctadecanoate (4)

A suspension of 0.65 g of PtO_2 in 10 mL of glacial acetic acid was reduced in a two-neck flask with a hydrogen-filled balloon for nearly 0.5 h until PtO_2 turned to platinum black. A solution of 700 mg (1.9 mmol) of **3** dissolved in 10 mL of acetic acid was injected rapidly through the syringe, and the reaction mixture was allowed to stir at room temperature for 20 h. The mixture was filtered through a Celite bed, the Celite bed was washed with 20 mL of $CHCl_3$, and the combined filtrate was concentrated under reduced pressure. The residual acetic acid was removed by addition and reevaporation of acetone several times. The crude product was purified by flash column chromatography on silica gel with light petroleum ether to yield 490 mg (74%) of **4** as an oil. ^1H NMR ($CDCl_3$): δ 3.64 (s, 3 H, CH_3), 2.30-2.22 (t, J = 7.5 Hz, 2 H, CH_2CO), 1.58-0.80 (br s, 43 H, CH_2s and CH_3s).

(5*R/S*, 9*R*, 13*R*)-5,9,13,17-Tetramethyloctadecanoic acid (5)

To a solution of 460 mg (1.26 mmol) of methyl ester **4** in 35 mL of THF-MeOH (1:1) at 0 °C was added 2 mL of 10% aqueous NaOH. The reaction mixture was allowed to stir at room temperature for 24 h. After the mixture was cooled to 0 °C, 4*N* HCl (10 mL) was added dropwise to neutralize the excess of NaOH. The reaction mixture was extracted with ether (3 x 100 mL), washed with water, saturated aqueous NaCl, and dried (Na_2SO_4). Evaporation of the solvents left an oil that was purified by flash chromatography on silica gel with 20% EtOAc in light petroleum ether to give 410 mg (96%) of **5** as an oil. IR (neat): 3400-2500 (OH), 1710 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.37-2.29 (t, J = 7.47 Hz, 2 H, CH_2CO), 1.64-0.82 (br s, 43 H, CH_2s and CH_3s).

(5*R/S*, 9*R*, 13*R*)-5,9,13,17-Tetramethyloctadecanoic anhydride (6)

A mixture of 400 mg (1.1 mmol) of carboxylic acid **5** and 130 mg (0.60 mmol) of dicyclohexylcarbodiimide in 10 mL of dry CCl_4 was stirred at room temperature under nitrogen for 24 h. The white precipitate of dicyclohexyl urea was filtered through Celite, and the filtrate was concentrated. The anhydride was obtained as an oil (370 mg, 95%), which crystallized on standing overnight at -20 °C. The solid was dried over P_2O_5 in desiccator for several hours and was used directly in the next step without further purification.

1,2-Di-[(5'*R/S*,9'*R*,13'*R*)-5',9',13',17'-tetramethyloctadecanoyl]-*sn*-glycero-3-phosphocholine (7)

To a suspension of 75 mg (0.17 mmol) of GPC-$CdCl_2$ in 10 mL of dry $CHCl_3$ was added 370 mg (0.56 mmol) of fatty acid anhydride **6** and 50 mg (0.43 mmol) of dimethylaminopyridine. The flask was flushed several times with nitrogen, capped under a nitrogen-filled balloon, and wrapped with aluminum foil to exclude light. After the mixture was stirred for 2 days at room temperature, $CdCl_2$ was removed by filtration through a Celite bed, and the bed was washed with $CHCl_3$ (2 x 10 mL). The combined filtrate was concentrated. The residue was dissolved in 5 mL of $CHCl_3$ and purified by flash chromatography on a silica gel column (1.2 x 35 cm) using elution with $CHCl_3$, $CHCl_3$-MeOH (9:1), and finally $CHCl_3$-MeOH (6:4, 800 mL) to give the desired product **7** (R_f 0.41 in $CHCl_3$-MeOH-H_2O, 6:3:0.4, v/v/v). Phosphocholine **7** was dissolved in $CHCl_3$ and passed through a 0.45-μm Metricel® filter (Acrodisc-CR, Gelman Sciences) to remove suspended silica gel particles. Reevaporation of the solvent yielded 89 mg (58%) of **7** which was precipitated by addition and evaporation of acetone; mp 190-195° C (dec); $[\alpha]^{25}_D$ +5.07° (*c* 0.69, $CHCl_3$-MeOH, 1:1) ^1H NMR ($CDCl_3$-CD_3OD,1:1): δ 5.27-5.18 (m, 1 H,

$CH_2C\underline{H}CH_2$), 4.31-4.18 (m, 2 H, $OC\underline{H}_2CH$), 4.05-3.95 (m, 2 H, $CHC\underline{H}_2OP$), 3.65-3.57 (m, 2 H, $POC\underline{H}_2CH_2$), 3.38-3.32 (m, 2 H, CH_2N), 3.22 (s, 9 H, $N(CH_3)_3$), 2.40-2.28 (m, 4 H, $COCH_2$), 1.70-0.84 (br s, 82 H, CH_2s and CH_3s). Anal. Calcd for $C_{52}H_{104}O_8NP{\cdot}1.5H_2O$ (929.40): C, 67.20; H, 11.60; N, 1.50. Found: C, 67.20, H, 11.86; N, 1.38.

1,2-Di-[(5'R/S,9'R,13'R)-5',9'13',17'-tetramethyloctadecanoyl]-*sn*-glycero-3-phosphatidic acid monomethyl ester (7'):

A solution of di-C_{22}-PC (**7**) (230 mg, 0.2 mmol) in 10 ml of CH_3OH was stirred at room temperature for 2 days with phospholipase D (*Streptomyces chromofuscus*) (400 unit) and 5 ml of buffer containing 0.05 M NaOAc and 0.01 M $CaCl_2$. The methyl ester (**7'**) was extracted into 100 ml of $CHCl_3$:MeOH (2:1), and dried over Na_2SO_4. Solvent was evaporated and the product was purified on preparative TLC plate (1-mm, Analtech, Newark, DE) with a solvent composition, $CHCl_3$:MeOH:NH_4OH (6:2:0.3). The product on TLC plate was detected by spraying with water . The product was lyophilized from benzene to give 70 mg (34%) of **7'**; R_f 0.59 ($CHCl_3$:MeOH:NH_4OH, 6:2:0.3). ^1H NMR ($CDCl_3$:CD_3OD, 1:1) (200 MHz) δ 5.1 (m, $CH_2C\underline{H}CH_2$, 1 H), 4.5-3.5 (m, $C\underline{H}_2CHC\underline{H}_2OPOC\underline{H}_3$, 7 H), 2.2 (m, $COCH_2$, 4 H), 1.6-0.5 (br s, CH_2s and CH_3s, 86 H). Anal. cald. for $C_{48}H_{94}O_8P.NH_4^+$ (848.28); C, 67.96; H, 11.64; N, 1.65; P, 3.65; found C, 67.66; H, 11.49; N, 1.20; P, 3.49.

1-[(5'*R/S*,9'*R*,13'*R*)-5',9',13',17'-Tetramethyloctadecanoyl]-2-lyso-*sn*-glycero-3-phosphocholine (8)

The branched diacyl PC **7** (79 mg, 0.087 mmol) was dissolved in 20 mL of Et_2O-MeOH (49:1). Sodium borate buffer (0.1*M*, 2 mL) containing 4 mg/mL of calcium acetate, pH 7.4, was added and the reaction mixture was stirred until

the two phases were separated. Phospholipase A_2 (112.5 units, 0.12 mg) was added and the reaction mixture was stirred vigorously at 38 ± 2 °C for 5 days until phospholipase A_2 digestion was complete as monitored by TLC (**8**: R_f 0.24 in $CHCl_3$-MeOH-H_2O, 6:3:0.4, v/v/v). The reaction mixture was cooled to room temperature and the ether layer containing the fatty acid that was liberated from substrate **7** was separated. Water (10 mL) was added to the aqueous phase (which contains lyso-PC), and the aqueous layer was extracted with ether (3 x 10 mL) to ensure the complete removal of the hydrolyzed fatty acid. The aqueous phase was extracted with $CHCl_3$-MeOH (2:1, 3 x 50 mL). The organic layer was dried (Na_2SO_4) and the solvents were removed under reduced pressure. To remove traces of water, dry benzene - ethanol (1:1, v/v) was added to the residue and the solvents were removed under reduced pressure. This drying procedure was repeated several times. Lyso-PC **8** (53 mg, 100%) was obtained as a sticky white solid with a trace of fatty acid (~5%); $[\alpha]^{25}_D$ +20.74° (*c* 0.32, $CHCl_3$-MeOH, 1:1). To avoid possible acyl migration, the crude phospholipid **8** was used in the next step without purification by chromatography on silica gel.

1-[(5'*R/S*,9'*R*,13'*R*)-5',9',13',17'-Tetramethyloctadecanoyl]-2-decanoyl-*sn*-glycero-3-phosphocholine (9)

To a suspension of 43 mg (0.074 mmol) of lyso-PC **8** in 2.5 mL of dry $CHCl_3$-pyridine (4:1) was added 242 mg (0.74 mmol) of capric anhydride and dimethylaminopyridine (~5 mg). After the reaction mixture was flushed with nitrogen gas and stirred at room temperature for 20 h, the solvents were evaporated. The traces of pyridine were evaporated by addition and evaporation of $CHCl_3$-MeOH (1:1, 2 x 10 mL). The residue was purified by flash chromatography on a silica gel column (1.2 x 35 cm). Elution was first with

$CHCl_3$ (100 mL), then with $CHCl_3$-MeOH (9:1, 100 mL) to remove the nonpolar materials, and finally with $CHCl_3$-MeOH (6:4, 800 mL) to yield PC **9** (R_f 0.35 in $CHCl_3$-MeOH-H_2O, 6:3:0.4, v/v/v). Compound **9** was dissolved in $CHCl_3$ and passed through a 0.45-μm Metricel® filter to ensure the complete removal of suspended silica gel particles. Solvent evaporation gave 36 mg (67%) of product **9**; after lyophilization with benzene, **9** was obtained as a white sticky solid; $[\alpha]^{25}_D$ -11.60° (*c* 0.37, $CHCl_3$-MeOH, 1:1). ^1H NMR ($CDCl_3$): δ 5.25-5.15 (m, 1 H, CH), 4.33-4.27 (m, 2 H, $OC\underline{H}_2CH$), 4.13-4.06 (m, 2 H, $CHC\underline{H}_2OP$), 3.95-3.80 (br m, 2 H, $POC\underline{H}_2CH_2$), 3.78-3.74 (br m, 2 H, CH_2N), 3.37 (s, 9 H, $N(CH_3)_3$), 2.60 (br s, H_2O), 2.30-2.21 (m, 4 H, $COCH_2$), 1.59-0.74 (br s, 52 H, CH_2s and CH_3s). *Anal. Calcd for* $C_{40}H_{80}O_8NP{\cdot}0.5H_2O$ (743.06): C, 64.65; H, 10.99; N, 1.88. Found: C, 64.51; H, 11.07; N, 1.43.

Discussion

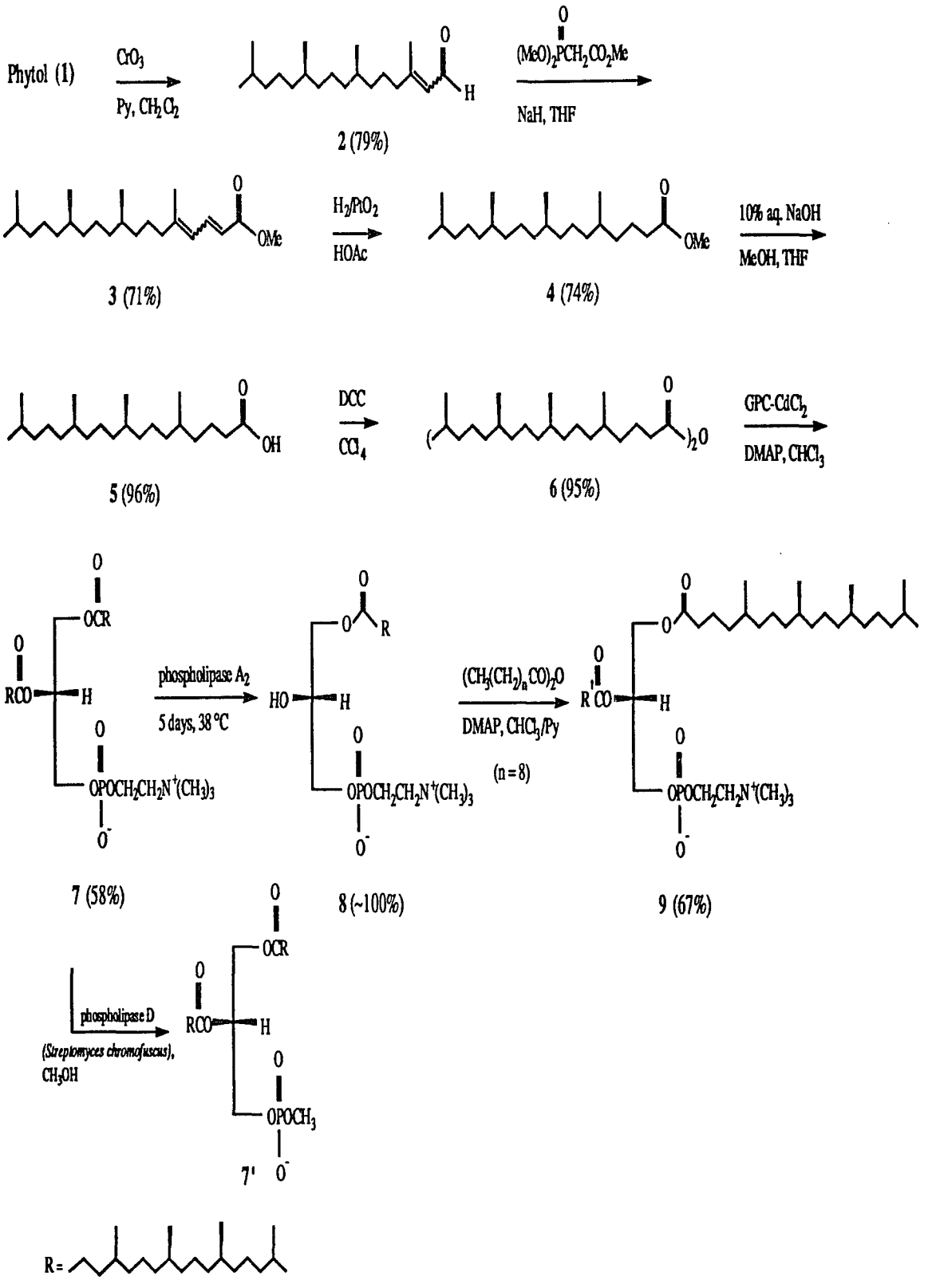
The procedure we describe introduces the multiply branched docosoyl chains into glycerophosphocholine in an efficient and convenient manner using dimethylaminopyridine as a catalyst. In contrast, a previous preparation of 1,2-diphytanoylglycero-*sn*-3-phosphocholine used the 3-(2',2',2'-trichloroethyl)-carbonate derivative of glycerol, which was acylated at 50 °C in 18 h to give 1,2-diphytanoyl-*sn*-glycero-3- (2',2',2'-trichloroethyl)carbonate in only 33% yield [5]. The trichloroethoxycarbonyl group was removed to give 1,2-diphytanoyl-*sn*-glycerol, which was converted into the desired PC. Our synthesis of the diisopropenoid-PC **7** is based on previously described methods involving glycerophosphocholine as the starting material [16,17]. This route is much shorter, and each step proceeds in good yield; furthermore, the deprotected 1,2-diacyl-*sn*-glycerol is not an intermediate in our synthetic scheme, so the possibility of acyl migration to give the 1,3-diacyl isomer is precluded. Another

synthesis of 1,2-diphytanoyl-PC was reported using glycero-*sn*-3-phosphocholine-$CdCl_2$ as the starting material; however, the diacylation step was accomplished using phytanoyl chloride during a 3-day period, and the yield of the desired PC was only 15% [18]. In our synthesis, the two highly branched acyl groups were introduced into glycerophosphocholine in 58% yield using fatty acid anhydride **6**.

1,2-Di-C_{22}-PC **7** was attacked sluggishly by venom phospholipase A_2, requiring about 5 days of incubation with an excess of the enzyme at 38 °C until digestion of the *sn*-2 chain reached completion. The resistance of **7** to oxidative degradation at elevated temperatures permits the prolonged incubation of **7** with the enzyme to take place without the formation of undesired byproducts. In contrast to the slow rate of hydrolysis of **7**, we found that distearoyl-PC was hydrolyzed completely to 1-stearoyl-2-lyso-PC in 3 h with lower concentrations of the same enzyme preparation at 38 °C. It is thus likely that steric hindrance accounts for the slow enzyme-catalyzed hydrolysis of the *sn*-2 chain of the bulky phosphocholine **7**. This conclusion is supported by the greatly diminished rate of pancreatic phospholipase A_2 catalyzed hydrolysis of short-chain phosphatidylcholines with methyl groups at the α and β positions of the *sn*-2 acyl group [19].

We have also found that PC **7** is susceptible to hydrolysis by phospholipase D from *Streptomyces chromofuscus* (Sigma, type VI), giving the corresponding phosphatidic acid in good yield; however, a poor yield of the methyl ester **7'** of phosphatidic acid was obtained in the single-step transphosphatidylation of **7** in the presence of excess methanol. Esterification of the phosphatidic acid by chemical means would afford isopropenoid-containing phospholipids with a variety of polar head groups, and specific probes can also be placed in the head group.

To summarize, we have presented a synthetic procedure that affords mixed-acid phosphatidylcholines with an isopropenoid chain at the *sn*-1 position in good yield. The C_{22} chain was prepared by two-carbon chain extension of phytenal (**2**) using the Wadsworth-Emmons modification of the Wittig reaction. Both C_{22} fatty acid ester linkages were introduced simultaneously into the $CdCl_2$ complex of glycerophosphocholine to provide the symmetric-chain PC **7**. Enzymatic hydrolysis of the 2-acyl bond of **7** gave lyso-PC **8**, which was acylated with fatty acid anhydride in chloroform-pyridine (4:1) in the presence of dimethylaminopyridine, affording the mixed-chain PC **9**. The semisynthetic approach of using phospholipase A_2 and phospholipase D permits changes to be made in the *sn*-2 acyl chain and polar head group of di-C_{22}-PC **7**, facilitating the study of the hydrophobic and polar regions of this class of lipids. Saturated mixed-chain phospholipids containing one branched acyl chain are expected to be useful for studies of the effects of methyl branches on the interchain packing and phase behavior of phospholipids in model membranes. Since diacylphosphatidylcholines with one acyl chain about twice as long as the other are known to form interdigitated bilayers in the gel phase [20], PC **9** will be useful in the analysis of the effects of methyl branching on the interdigitation process.



Scheme I: Reaction sequence for the synthesis of diacyl PCs (**7**, **9**) with one and two C22-isopropenoid chains and methyl ester (**7'**)

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Chapter 5

Mixed-chain PC analogs modified in the choline moiety: preparation of isomerically pure phospholipids with bulky head groups and one acyl chain twice as long as the other

Abstract: Diacylphosphatidylcholines are synthesized with widely different acyl chain lengths and bulky head groups. Lysophosphatidylcholine is acylated at room temperature within 6 hours with a 10-fold molar excess of fatty acid anhydride in dry, alcohol-free chloroform in the presence of 1.2 equivalents of 4-pyrrolidinopyridine as a catalyst, affording the mixed-acid phosphatidylcholines with widely different chain lengths in more than 90% yield and with less than 1% acyl migration. The syntheses of isomerically pure 1-stearoyl-2-decanoyl- and 1-stearoyl-2-undecenoyl (Δ^{10})-*sn*-glycero-3-phosphocholines (C(18:0)/C(10:0)-PC and C(18:0)/C(11:1Δ^{10})-PC, respectively), followed by conversion to various head-group analogs, are illustrated here. The transition peak widths at half-height of the endotherms obtained by differential scanning calorimetry are consistent with very high isomeric purity. Phospholipase D from *Streptomyces chromofuscus* was used as a catalyst in the hydrolysis of C(18:0)/C(10:0)-PC to give the corresponding phosphatidic acid in quantitative yield. The latter compound is condensed with 10 molar equivalents of various *N,N,N*-trialkylammonium alkanols (as their *p*-toluenesulfonate or tetraphenylborate salt) in the presence of trichloroacetonitrile in dry pyridine under nitrogen atmosphere to yield the C(18:0)/C(10:0) phospholipids bearing modified head groups, which are purified by flash chromatography.

Introduction: Many studies have used analogs of phosphatidylethanolamine and phosphatidylcholine in which the distance between the phosphoryl group and the amino group or trimethylammonium ion is varied. These compounds have been useful for determining the dependence of structural features in the phospholipid head group on physical properties. Some recent examples of phospholipid analogs whose head groups are modified by the interposition of additional methylene groups between the phosphorus and nitrogen functions or by substitution of *N*-alkyl groups include studies of the role of head group structure on the phase behavior and relative stabilities of lamellar and nonlamellar phases [1-4], on the interaction of phospholipids with phospholipid-requiring enzymes [5] or with cholesterol [6], on the rates of spontaneous transfer of phospholipids between membranes [7], and on the distribution of phospholipids between the leaflets of the bilayer of small unilamellar vesicles [8].

In order to examine whether increased steric bulk in the head group affects the ability of two phospholipid molecules from opposing leaflets of PC bilayers to interdigitate in the gel phase, we needed to prepare analogs of mixed-chain phosphatidylcholines (PCs) with modified choline head groups; moreover, we required the longer acyl chain in the fully extended conformation to be about twice as long as the shorter acyl chain, as in C(18:0)/C(10:0) phospholipids. In this paper we report an improved procedure for the acylation of 1-C(18:0)-lyso-PC to generate C(18:0)/C(10:0)-PC and C(18:0)/C(11:1Δ^{10})-PC in high yield and without significant contamination by mixed-chain isomers arising from acyl migration. Although efficient procedures for the synthesis of phospholipid head group analogs by enzymic transphosphatidylation have been reported [9, 10], the range of acyl groups in the phospholipid substrates reported in the literature is limited to symmetric

chains (in synthetic phospholipids) and to chains of similar lengths (as in naturally occurring PCs). We have found that many preparations of phospholipase D (such as chromatographically purified, lyophilized enzyme from peanut and cabbage) fail to hydrolyze mixed-chain PCs having widely different chain lengths at the *sn*-1 and *sn*-2 positions. In the present study we show that phospholipase D from *Streptomyces chromofuscus* does catalyze the hydrolysis of such mixed-chain PCs to the corresponding phosphatidic acids, but does not catalyze transesterification with bulky choline analogs. We report here an extremely efficient synthesis of mixed-chain PCs with modified head groups; the steps from the distearoyl-PC starting material to 1-stearoyl-2-decanoyl-*sn*-glycero-3-phosphatidic acid are essentially quantitative. The latter compound is coupled with the bulky, *N,N,N*-trialkylammonium alcohol (as the *p*-toluenesulfonate or tetraphenylborate salt). This route constitutes a general approach to a wide variety of head group analogs of phospholipids with various acyl chain lengths. Since calorimetric analysis of the phase behavior of mixed-chain phospholipids requires the absence of even small amounts of the mixed-chain isomer, we report the values of the transition peak widths at half-height ($\Delta T_{1/2}$) for the gel to liquid-crystalline phase transitions. These values indicate very high isomeric purity of the synthetic phosphatidylcholines prepared by the acylation of lyso-PC in about 6 h at room temperature.

Materials and methods

4-Pyrrolidinopyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was purified by recrystallization from pentane. 4-(*N,N*-Dimethylamino)pyridine (Aldrich) was recrystallized from chloroform-diethyl ether 1:1 (v/v). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylcholine, trichloroacetonitrile, and capric (C10:0) anhydride were

purchased from Sigma Chemical Co. (St. Louis, MO). For some reactions, capric anhydride was synthesized from capric acid and dicyclohexylcarbodiimide in carbon tetrachloride [11]. *N,N*-Dimethylamino-1 -propanol, *N,N*-diethylamino-1-propanol, *N,N*- dimethylamino-2-propanol, *N,N*-diethylamino-2-propanol, and ω-amino-1-pentanol were purchased from Aldrich. The *p*-toluenesulfonate salts were prepared in high yield by the reaction of the *N,N*-dialkylamino alcohols with methyl or ethyl *p*-toluenesulfonate (Aldrich) in dry THF as described previously [5]. Three equivalents of methyl *p*-toluenesulfonate were used in the reaction with 5-amino-1-pentanol to form the tosylate salt. The tetraphenylborate salts were obtained from the corresponding tosylate salts by addition of an equimolar amount of sodium tetraphenylborate (Aldrich) as described elsewhere [12]. The white crystalline *p*-toluenesulfonate and tetraphenylborate salts were washed with several portions of anhydrous ether to remove unreacted starting materials, and were then stored over P_2O_5 in a desiccator at room temperature. Phospholipase A_2 (*Naja naja*) (E.C. 3.1.1.4) was purchased from Sigma (catalog number P6139), as were preparations of phospholipase D (E.C. 3.1.4.4) (*Streptomyces chromofuscus*, type VI, catalog number P8023; cabbage, lyophilized powder; peanut, chromatographically purified, lyophilized powder). All of the commercial enzyme preparations were used without further purification.

Pyridine was refluxed over and distilled from calcium hydride, then stored over 4A molecular sieves. Hydrocarbon-stabilized chloroform was obtained from J.T. Baker (Phillipsburg, NJ) and was refluxed over and distilled from P_2O_5 before use. Anhydrous ether was purchased from J.T. Baker. Tetrahydrofuran (THF) (Aldrich) was refluxed over sodium benzophenone ketyl for several hours, and then used immediately.

TLC was carried out on silica gel G plates as described previously [13]. Detection of phosphorus-containing compounds with molybdate spray and charring of compounds on the TLC plates were described previously [13]. Flash chromatography was carried out on silica gel (230-400 mesh, 60 Å average pore diameter) purchased from Aldrich or Sigma.

Elemental analyses were performed by Desert Analytics (Tucson, AZ) and Schwarzkopf (Woodside, NY). Proton NMR spectra were recorded at 300.52 MHz on a GE Model QE spectrometer using tetramethylsilane as internal standard. Analysis of the fatty acid butyl esters was by gas chromatography - mass spectrometry on a Hewlett-Packard 5988A GC-quadrupole mass spectrometer equipped with a H-P 1000 data system.

Calorimetric measurements of the transition temperatures and $\Delta T_{1/2}$ for the main transitions of C(18:0)/C(10:0)-PC and C(18:0)/C(11:1Δ^{10})-PC were made on a high-resolution differential scanning microcalorimeter (Model MC-2, Microcal, Inc., Amherst, MA) [14].

Synthetic methods

Preparation of 1-stearoyllyso-PC **(2)**. To a suspension of 500 mg (0.63 mmol) of di-C(18:0)-PC in 50 mL of diethyl ether-methanol 49:1 (v/v) was added 3 mL of 100 mM sodium borate buffer, pH 7.4, containing 4 mg/mL of calcium acetate. The mixture was stirred at 38 ± 2°C until a clear solution was formed (about 15 min). Phospholipase A_2 (*Naja naja*) (250 units) was added, and the mixture was stirred vigorously for 3 h at the same temperature. The reaction mixture was washed with several portions of ether (1 x 50 mL, then 3 x 10 mL) to remove the fatty acid, and the aqueous phase was extracted with chloroform-methanol 2:1 (v/v) (3 x 100 mL) to isolate the lyso-PC. The chloroform-methanol extract was dried over Na_2SO_4 and the solvents were

evaporated. Residual water was removed by azeotropic distillation in a rotary evaporator with ethanol-benzene 4:1 (v/v), and 1-C(18:0)-lyso-PC (**2**) was obtained as a white solid (330 mg, 100%). TLC analysis showed that a trace of fatty acid was present; R_f of **2**, 0.16 in chloroform-methanol-water 6:3:0.4 (v/v/v), identical to authentic samples obtained from Avanti Polar Lipids (Birmingham, AL) and Sigma Chemical Co. No further attempt was made to remove the trace of fatty acid, since it is well known that column chromatography on silica gel causes significant acyl migration [see, for example, Refs. 15,16].

Acylation of 1-C(18:0)-lyso-PC. Capric anhydride (1.6 g, 4.7 mmol) and 4-pyrrolidinopyridine (85 mg, 0.56 mmol) were added to a suspension of 250 mg (0.47 mmol) of 1-C(18:0)-lyso-PC (**2**) in 35 mLof hydrocarbon-stabilized chloroform in an oven-dried 100-mL round-bottom flask capped with a nitrogen-filled balloon. The flask was flushed with nitrogen several times, and the reaction mixture was then stirred magnetically at room temperature for 5 - 6 h under a nitrogen atmosphere. The suspension of lyso-PC became a clear light yellow solution as the reaction proceeded. After the solvent was removed under reduced pressure, the residue was dissolved in a minimum volume of chloroform and applied to a column (1.2 x 40 cm) of silica gel 60 (230-400 mesh) for flash chromatography. Elution was first with chloroform (100 mL) to remove unreacted fatty acid anhydride and other nonpolar compounds, then with chloroform-methanol 9:1 (100 mL) and finally with chloroform-methanol 3:2 (about 800 mL). Fractions containing the pure product in the chloroform-methanol 3:2 (v/v) eluate were identified by TLC and combined. The solvents were evaporated, and the residue was dissolved in chloroform and filtered through a 0.45-μm Metricel filter (Acrodisc-CR, Gelman Sciences, Ann Arbor, MI) to remove the suspended silica gel particles. Evaporation of the

solvent left a light yellow residue, which was lyophilized with benzene (10 mL), affording 300 mg (93%) of C(18:0)/C(10:0)-PC (**3**) as a white solid. TLC: R_f 0.41 in chloroform-methanol-water 6:3:0.4 (v/v/v). Anal. Calc. for $C_{36}H_{72}O_8NP \cdot 3\ H_2O$ (731.95) C, 59.07; H, 10.74; N, 1.91; P, 4.23. Found: C, 59.42; H, 10.42; N, 1.76; P, 4.37. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, apparent triplet, ω-CH_3), 1.1-1.4 (40H, m, CCH_2C), 1.5-1.6 (4H, m, $C\underline{H}_2CH_2C(O)O$), 2.2-2.3 (4H, m, $C\underline{H}_2C(O)O$), 3.5 (9H, s, $N^+(CH_3)_3$), 3.9-4.1 (4H, m, $OC\underline{H}_2C\underline{H}_2N$), 4.25-4.44 (4H, m, OCH_2), 5.1-5.2 (1H, m, OCHC).

Synthesis of C(18:0)/C(11:1Δ^{10})-PC. To a suspension of 150 mg (0.29 mmol) of 1-C(18:0)-lyso-PC (Avanti Polar Lipids) in 30 ml of hydrocarbon-stabilized chloroform was added 1.0 g (2.9 mmol) of 10-undecenoyl (C11:1Δ^{10}) anhydride and 50 mg (0.34 mmol) of 4-pyrrolidinopyridine. The flask was flushed thoroughly with nitrogen, and the mixture was stirred at room temperature for 5 h. Removal of the solvent under reduced pressure left a residue that was purified by flash chromatography as described for the preparation of **3**; the yield of C(18:0)/C(11:1Δ^{10})-PC after flash chromatography was 190 mg (100%). To remove a trace of light yellow impurity, the product was further purified by preparative TLC. Anal. Calc. for $C_{37}H_{72}O_8NP \cdot 1.5H_2O$ (716.97) C, 61.98; H, 10.55; N, 1.95. Found: C, 61.83; H, 10.67; N, 2.07.

Synthesis of 1-stearoyl-2-decanoyl-sn-glycero-3-phosphatidic acid (**4**). To a solution of 100 mg (0.14 mmol) of C(18:0)/C(10:0)-PC (**3**) in 40 mL of ether was added 40 mL of 50 mM sodium acetate buffer, pH 5.6, containing 10 mM calcium chloride. After the mixture was stirred vigorously at 38 ± 2°C for 15 min, 500 units of phospholipase D (*Streptomyces chromofuscus*, type VI) was added and the reaction was stirred at the same temperature for an additional 6

h. The mixture was cooled to room temperature and ether was removed under reduced pressure. The aqueous layer was acidified with 5 mL of 0.6*N* HCl and the crude product was extracted with three 100-ml portions of ethyl acetate - acetone 3:2 (v/v). The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. Traces of water were removed by reevaporation in the presence of benzene. The residue was dissolved in chloroform and filtered through a capillary tube plugged with cotton to ensure removal of inorganic salts. Removal of the solvent gave ~ 90 mg (100%) of the crude phosphatidic acid **4**, which was used immediately in the next step without further purification. TLC: R_f ~ 0.5 in chloroform-methanol-formic acid 8.0:1.5:0.5 (v/v/v).

General procedure for the synthesis of mixed-acid phosphocholine analogues **(5 - 10)**. Phosphatidic acid **4** (~ 40-50 mg) was dissolved in 5-10 mL of dry pyridine in an oven-dried 50-mL round-bottom flask by heating and stirring at 50 ± 5°C for 30 min. Ten equivalents of the desired choline analog $HOCH(R)(CH_2)_nN^+R'_3$ (see Scheme 1) as the *p*-toluenesulfonate or tetraphenylborate salt (previously dried over P_2O_5) and 2-3 ml of trichloroacetonitrile were added. The reaction mixture was flushed with nitrogen (contained in a balloon fitted to the flask) and allowed to stir at 50 ± 5°C under nitrogen for 2 days. During the reaction, the mixture became brown. The solvent was removed under reduced pressure, leaving a brown residue; to assure removal of pyridine, 20 mL of chloroform-methanol 1:1 (v/v) was added to the residue, and the solvents were evaporated. The crude product was dissolved in 20 mL of THF-water 9:1 (v/v) and applied to a column of Amberlite MB-3 (20 g) that had been equilibrated with the same solvent system. After elution with about 400 mL of THF-water 9:1 (v/v), a brown residue was obtained on evaporation of the solvents. Water was removed by addition of 2-propanol

(3 x 100 mL) followed by reevaporation. The tan residue was dissolved in chloroform (2-5 mL) and purified by chromatography on silicic acid (Sigma, 325 mesh) using a 1.2 x 40 cm column that was eluted first with chloroform, then with chloroform-methanol 9:1 (v/v), and finally with chloroform-methanol 6:4 (v/v). As shown in Scheme 1, the yields of the bulky head-group analogues of C(18:0)/C(10:0)- PC varied between 30 and 59% (based on **4**), depending on the extent of steric bulk in the *N,N,N*-trialkylammonium alkanol. TLC: The R_f values of compounds **5-10** were in the range of 0.32-0.60 in chloroform-methanol-water 6:3:0.4 (v/v/v).

Elemental analysis and NMR data for compounds 5-10.

Compound **5:** Anal. Calc. for $C_{37}H_{74}O_8NP \cdot H_2O$ (709.97) C, 62.03; H, 10.78; N, 1.97. Found: C, 62.81; H, 10.22; N, 1.41. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, t, ω-CH_3), 1.0-1.4 (42H, m, CCH_2C and $CH_2C\underline{H}_2CH_2N^+Me_3$), 1.5-1.6 (4H, m, $C\underline{H}_2CH_2C(O)O$), 2.2-2.4 (4H, m, $CH_2C(O)O$), 3.4 (9H, broad singlet, N^+Me_3), 4.1-4.4 (8H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$, $OC\underline{H}_2CH_2$, and $CH_2C\underline{H}_2N^+Me_3$), and 5.2-5.3 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Compound **6:** Anal. Calc. for $C_{38}H_{76}O_8NP \cdot 1.5\ H_2O$ (733.02) C, 62.23; H, 10.86; N, 1.91. Found: C, 62.54; H, 10.28; N, 1.60. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, t, ω-CH_3), 1.1-1.5 (43H, m, CCH_2C, $N^+CH_2C\underline{H}_3$), 1.5-1.6 (4H, m, $C\underline{H}_2CH_2C(O)O$), 2.2-2.4 (4H, m, $CH_2C(O)O$), 3.25 (6H, broad singlet, $N^+(CH_3)_2$), 4.1-4.2 (2H, $N^+C\underline{H}_2CH_3$), 4.2-4.4 (8H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$, $OC\underline{H}_2CH_2$, and $CH_2C\underline{H}_2N^+Me_2Et$), and 5.17-5.25 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Compound **7:** Anal. Calc. for $C_{40}H_{80}O_8NP \cdot 1.5\ H_2O$ (761.07) C, 63.13; H, 10.99; N, 1.84. Found: C, 63.44; H, 10.96; N, 1.53. ^1H NMR ($CDCl_3$) δ (ppm):

0.9 (6H, t, ω-CH_3), 1.1-1.5 (51H, m, CCH_2C, $N^+(CH_2C\underline{H}_3)_3$, and $CH_2C\underline{H}_2CH_2N^+Et_3$), 1.5-1.7 (4H, m, $C\underline{H}_2CH_2C(O)O$), 2.2-2.4 (4H, m, $CH_2C(O)O$), 3.3-3.6 (8H, broad singlet, $N^+(C\underline{H}_2CH_3)_3$, $CH_2C\underline{H}_2N^+Et_3$), 3.6-3.8 (2H, m, $OC\underline{H}_2CH_2$), 4.1-4.4 (4H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$), 5.18-5.25 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Compound **8:** Anal. Calc. for $C_{37}H_{74}O_8NP \cdot 0.5\ H_2O$ (700.97) C, 63.39; H, 10.78; N, 1.99. Found: C, 63.10; H, 10.61; N, 1.42. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, t, ω-CH_3), 1.1-1.4 (43H, m, CCH_2C, $CHC\underline{H}_3$), 1.5-1.6 (4H, m, $C\underline{H}_2CH_2C(O)O$), 2.2-2.4 (4H, m, $CH_2C(O)O$), 3.4 (9H, broad singlet, $N^+(CH_3)_3$), 3.7 (2H, m, CH_2N^+), 4.0-4.2 (4H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$), 5.2-5.3 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Compound **9:** Anal. Calc. for $C_{40}H_{80}O_8NP \cdot 1.5\ H_2O$ (761.07) C, 63.13; H, 10.99; N, 1.84. Found: C, 63.79; H, 11.07; N, 1.70. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, t, ω-CH_3), 1.2-1.6 (56H, m, CCH_2C, $N^+CH_2C\underline{H}_3$), 2.2-2.3 (4H, m, $CH_2C(O)O$), 3.3-3.5 (2H, m, $CH_2C\underline{H}_2N^+Et_3$), 3.5-3.7 (6H, m, $N^+C\underline{H}_2CH_3$), 3.9-4.4 (4H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$), 4.8-4.9 (1H, $C\underline{H}CH_2N^+Et_3$) 5.1-5.3 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Compound **10:** Anal. Calc. for $C_{39}H_{78}O_8NP \cdot H_2O$ (738.04) C, 63.47; H, 10.92; N, 1.89. Found: C, 63.45; H, 10.40; N, 1.40. ^1H NMR ($CDCl_3$) δ (ppm): 0.8 (6H, t, ω-CH_3), 1.1-1.8 (50H, m, CCH_2C and $(C\underline{H}_2)_3CH_2N^+Me_3$), 2.2-2.4 (4H, m, $CH_2C(O)O$), 3.4 (9H, broad singlet, N^+Me_3), 3.8-4.4 (8H, m, $C\underline{H}_2CH(OCOR)C\underline{H}_2$, $OC\underline{H}_2CH_2$, and $CH_2C\underline{H}_2N^+Me_3$), and 5.1-5.3 (1H, m, $CH_2C\underline{H}(OCOR)CH_2$).

Evaluation of isomeric purity of mixed-acid chains

A solution of 10-20 mg of C(18:0)/C(10:0)-PC (**3**) in 5 mL of diethyl ether - methanol 4.9:0.1 (v/v) was digested with 1 mL of phospholipase A_2 (25 units/ml in 100 mM sodium borate buffer containing 4 mg/mL of calcium acetate, pH 7.4) at room temperature for 1.5 h. The ether layer was decanted and the solvent was removed under vacuum. The residue was applied to a silica gel G plate (10 x 20 cm) and developed in petroleum ether-ethyl acetate 7:3 (v/v) containing 1-2 drops of formic acid. The fatty acid zone was visualized by staining with iodine and scraped into a 25-mL flask. The *n*-butyl esters of the fatty acids were prepared without eluting the fatty acids from the silica gel [17] by heating the mixture scraped from the plate, together with the silica gel, with 1 mL of 1-butanol and 50 µL of 36*N* sulfuric acid at 80°C for 2 h. After the mixture had cooled to room temperature, ether (20 mL) was added and the mixture was filtered through a sintered-glass funnel. The filtrate was concentrated under reduced pressure, and the unreacted 1-butanol was removed by repeated addition of hexanes (Fisher Scientific) followed by reevaporation. The residue of fatty acid butyl esters was dissolved in 5 mL of ether and purified by TLC [developed in petroleum ether-ethyl acetate 95:5 (v/v)]. The fatty esters were scraped from the plate and eluted with chloroform. The chloroform was removed under reduced pressure, and the residue was dissolved in 0.1 mL of chloroform for GC/MS analysis. Gas chromatography was carried out on a 30 m x 0.25 mm i.d., 0.25 µm DB-5 bonded phase fused silica capillary column (J & W Scientific, Folsom, CA). The injection temperature was 250°C. The following temperature program was used: initial temperature, 35°C, 1 min; programmed at 20°C/min to 125°C; held for 1 min; programmed at 10°C/min to 225°C; held for 15 min. The ratio of fatty acid butyl esters was estimated from the integrated peak areas. The retention times of the *n*-butyl

esters prepared from the fatty acids released by phospholipase A_2 digestion of **3** were identical to those prepared by the reactions of authentic capric and stearic acids with 1-butanol (C(10:0) butyl ester, R_t = 13.1 min; C(18:0) butyl ester, R_t = 25.9 min). The retention time of C(11:1Δ^{10}) butyl ester was 14.6 min.

Since the acylation reaction does not affect the configuration at C-2, retention of the natural *sn*-3 configuration of di-C(18:0)-PC is expected. Confirmation of the optical purity of the glycerol backbone in product **3** was obtained by use of phospholipase A_2, which hydrolyzed **3** completely to 1-C(18:0)-lyso-PC (**2**).

Results and Discussion

$\Delta T_{1/2}$ values for gel to liquid-crystalline transitions of C(18:0)/C(10:0)- and C(18:0)/C(11:1Δ^{10})-PCs

Thermotropic properties of mixed-chain PCs may also reflect the isomeric purity of the synthetic lipids. Table I summarizes several calorimetric investigations which show large values of $\Delta T_{1/2}$ for the endothermic gel to liquid-crystalline transitions of mixed-chain PCs prepared by acylation at temperatures ≥35°C and/or prolonged reaction periods (≥24 h). These broad phase transitions may indicate the presence of substantial isomeric impurity in these PC preparations, even though use of phospholipase A_2 to cleave the fatty acid at the *sn*-2 position of the glycerol backbone indicated high isomeric purity [18, 19]. Table I shows that the $\Delta T_{1/2}$ values associated with the gel to liquid-crystalline transitions of the highly asymmetric mixed-acid phosphocholines, C(18:0)/C(10:0)- and C(18:0)/C(11:1Δ^{10})-PCs, prepared as described under Materials and Methods, are 0.23°C and 0.50°C, respectively. These values are consistent with the very high isomeric purities (>99%) we found using phospholipase A_2-catalyzed hydrolysis of the fatty acid at the *sn*-2

position. In contrast, much larger $\Delta T_{1/2}$ values (1.0-3.1°C) have been found for mixed-chain PCs prepared by acylation of lyso-PC at ≥35°C [18-20] or at room temperature for >24 h [21].

Acyl migration during acylation of lyso-PC

It is well known that some of the published methods leading to symmetric-chain (single acid) PCs from the reaction of *sn*-glycero-3-phosphocholine or its $CdCl_2$ adduct with fatty acid salt suffer from the need to use severe conditions to obtain a homogeneous acylation mixture, such as high temperatures (80°C) and long reaction times (2 days), resulting in poor yields [22] and contamination with 1,3-diacyl-2-PC [23]. Vigorous acylation of lyso-PC with 6 moles of fatty acid anhydride and 0.5 mole of sodium oxide per mole of lyso-PC at about 75°C for 6-8 h gave substantial amounts of acyl migration [24)]. It is thus necessary to prepare mixed-chain PCs by using efficient catalysts such as 4-(*N,N*-dimethylamino)pyridine and 4-pyrrolidinopyridine, which have been found to improve the synthesis of symmetric-chain PCs [15,25]. Unfortunately, these nitrogen-containing nucleophilic catalysts also promote acyl migration [26]. We obtained a relatively poor yield (approximately 50%) for acylation of 1-C(18:0)-lyso-PC at 25°C by the conditions used by Gupta et al. [15] even when 3-3.5 equivalents of capric anhydride and 2 equivalents of dimethylaminopyridine were used, together with prolonged reaction time (2-3 days). We were also not able to reproducibly obtain good yields of C(18:0)/C(10:0)-PC by acylation of 1-C(18:0)-lyso-PC using the Mason et al. procedure (5 equivalents of acid anhydride, 1 equivalent of 4-pyrrolidinopyridine, 35° C, 1.5 h) [27], and we detected 15-21% migration of acyl substituents. We obtained high yields (> 90%) when we modified the acylation procedure by performing the reaction with 3-3.5 equivalents of fatty

acid anhydride for 2 h in the presence of a catalytic amount of dimethylaminopyridine in chloroform/pyridine 4:1 (v/v) at 40°C. However, we found that appreciable amounts (about 8%) of acyl migration took place under these acylation conditions. The method shown in Scheme 1 for the conversion of **2** into **3** involves acylation of lyso-PC at *room temperature in 6 h* by using a 10-fold molar excess of fatty acid anhydride in the presence of a 20% molar excess of 4-pyrrolidinopyridine. This method represents a highly efficient synthesis with respect to the limiting reagent (lyso-PC), resulting in a 93% yield of **3** after purification by flash chromatography and lyophilization. The extent of acyl migration is apparently very low because the reaction takes place at room temperature in a relatively short time period; as noted above, we found that reaction at 35°C with 4-pyrrolidinopyridine as catalyst and reaction at 40°C with dimethylaminopyridine as catalyst resulted in appreciable acyl migration.

A disadvantage of the method reported here is the use of excess fatty acid anhydride, which is required to achieve rapid acylation at room temperature. An alternative procedure for the acylation of lyso-PC that avoids the use of a large excess of fatty acid anhydride involves the use of 2-pyridinethiol esters with excess Ag^+ as catalyst [28]. However, this method has the disadvantage of giving variable yields of PC, depending on the presence or absence of traces of moisture [28], and of requiring derivatization of the fatty acid to form an intermediate activated acylating agent (fatty acyl pyridine thioester).

Activity of phospholipase D on asymmetric mixed-acid PCs

Introduction of a short- chain acyl group at the *sn*-2 position of PC led to inhibition of both the transphosphatidylation and hydrolysis activities of phospholipases D from cabbage and peanut sources. Therefore, we modified the reaction conditions by adding various amounts of chloroform, *tert*-butyl

alcohol, or sodium dodecyl sulfate to the reaction mixture (16 mmol of substrate **3** dispersed in 2 mL of buffer, pH 5.6, 2 mL of ether, 50 units of cabbage phospholipase D) in attempts to activate the hydrolytic properties of the enzyme. These agents are known to serve as excellent activators for plant phospholipase D [e.g., 29]; however, cabbage phospholipase D still failed to catalyze significant hydrolysis of the highly asymmetric-chain substrate **3**. The enzyme preparation from *S. chromofuscus* could, however, convert C(18:0)/C(10:0)-PC (**3**) into the corresponding phosphatidic acid **4**.

The kinetic parameters the transphosphatidylation of PC to phosphatidylethanolamine catalyzed by various phospholipase D enzymes from *Streptomyces* sp. origin have recently been established [30]. Phospholipase D from *S. chromofuscus* catalyzed the conversion of plasmenylcholine to plasmenylethanolamine [31]. Unfortunately, efficient transphosphatidylation using alcohols bulkier than ethanolamine have not been achieved. No enzymic transphosphatidylation was observed with alkanols containing seven or more carbon atoms, and phosphatidic acid was either the major product or byproduct with alkanols with five or six carbon atoms [10]. Attempts to carry out transphosphatidylation of dimyristoyl-PC with the bulky alcohol 2-amino-2-methyl-1-propanol in the presence of phospholipase D were also not successful [1]. Synthesis of phosphatidylserine by the phospholipase D (cabbage) catalyzed reaction of PC with excess L-serine was also not efficient, resulting in substantial amounts of phosphatidic acid [9, 32]. Similar results were obtained in the attempted transesterification of dimyristoyl-PC with serine methyl ester in the presence of phospholipase D [33]. In agreement with these reports, we found that phospholipase D (*S. chromofuscus*) could not carry out the exchange reaction of substrate **3** with our bulky alkanols. Although phospholipase D from a microbial source (*Streptomyces* sp. from Toyo Jozo

Co., Tokyo) was reported recently to catalyze transphosphatidylation between di-C(16:0)-, di-C(18:0)-, and di-C(18:1)-PCs and the 5'-hydroxyl group of nucleosides in a chloroform-water two-phase system [34], we could not obtain transphosphatidylation of C(18:0)/C(10:0)-PC with bulky choline analogs in the same two-phase solvent system.

Coupling of bulky choline analogs to asymmetric mixed-acid phosphatidic acids

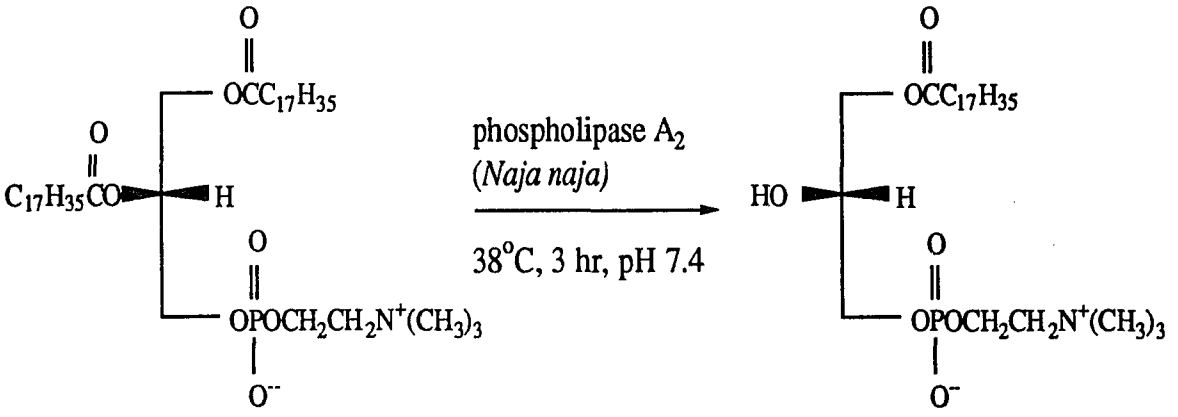
The yields of diacyl-PCs obtained by coupling of 2 molar equivalents of choline acetate to diacylphosphatidic acid are <60% [35], but can be improved to about 75% by the use of 2 molar equivalents of choline tetraphenylborate [12] or 8-10 molar equivalents of choline tosylate [13, 36]. Thus the relatively low yields (30-60%) found in the condensation reaction of phosphatidic acid **4** with bulky analogs of choline are not surprising, even though we used 10 molar equivalents of the alkanol salts to prepare compounds **5-10**. Similar yields for coupling of tosylate salts of bulky quaternary ammonium alcohols to dialkylphosphoric acid or to phosphatidic acid have been reported previously [5]. Yields in the range of 40-55% were reported for dipalmitoylphosphatidylethanolamine analogs having an ethyl, phenyl, or benzyl group at the carbon atom alpha to the amino group; the latter were formed by reactions of dipalmitoylphosphatidic acid dichloride with amino alcohols [8].

In conclusion, we have described a semisynthetic method for the preparation of mixed-chain diacyl-PCs having the natural *sn*-3 configuration and modified choline head groups. One of the advantages of this route is that only four steps are required to convert the di-C(18:0)-PC starting material into the desired C(18:0)/C(10:0) phospholipids bearing modified choline head groups.

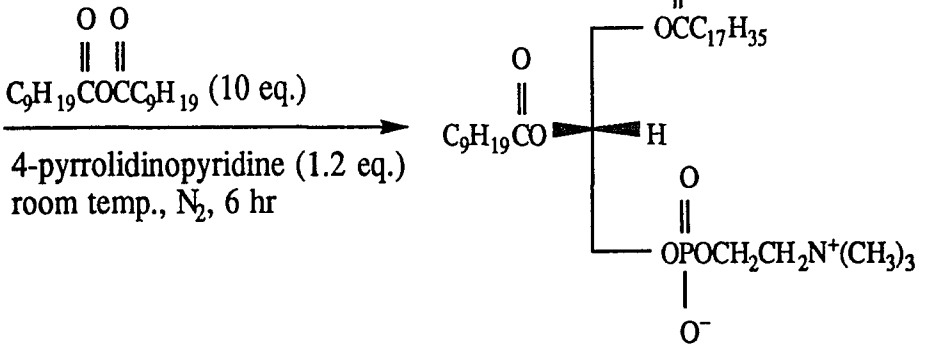
TABLE I: Differential scanning calorimetry data for gel to liquid-crystalline phase transitions of mixed-chain PCs prepared by acylation of lyso-PC at various temperatures and times.

PC	T_m (°C)	$\Delta T_{1/2}$ (°C)	Acylation conditions[a]	% of acyl migration reported	Ref.
C(18:0):C(18:1)	9.3	2.1	rt, 24-36 h	6	21
C(18:1):C(18:0)	8.9	1.3	rt, 24-36 h	6	21
C(20:0):C(20:1)	23.0	3.0	37°C, 24 h	6	20
C(20:0):C(20:3)	6.0	2.9	37°C, 24 h	34	20
C(18:0):C(10:0)	18.4	3.1	35°C	<5	19
C(18:0):C(10:0)	19.1	0.98	35°C	<2	18
C(18:0):C(10:0)	18.6	0.23	rt,<9 h[b]	-	14
C(18:0):C(11:1$_{\Delta^{10}}$)	13.3	0.50	rt, 5 h	<1	this paper

[a] The catalyst was 4-pyrrolidinopyridine for each entry in the table, except for C(18:0):C(18:1)- and C(18:1):C(18:0)-PCs; dimethylaminopyridine was used in the preparation of the latter compounds. Abbreviation: rt, room temperature.
[b]Prepared by H-n. Lin and C-h. Huang, University of Virginia.

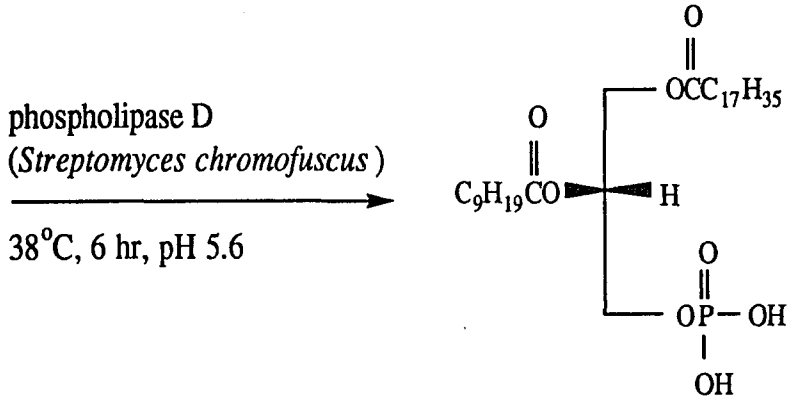


1 **2** (100%)

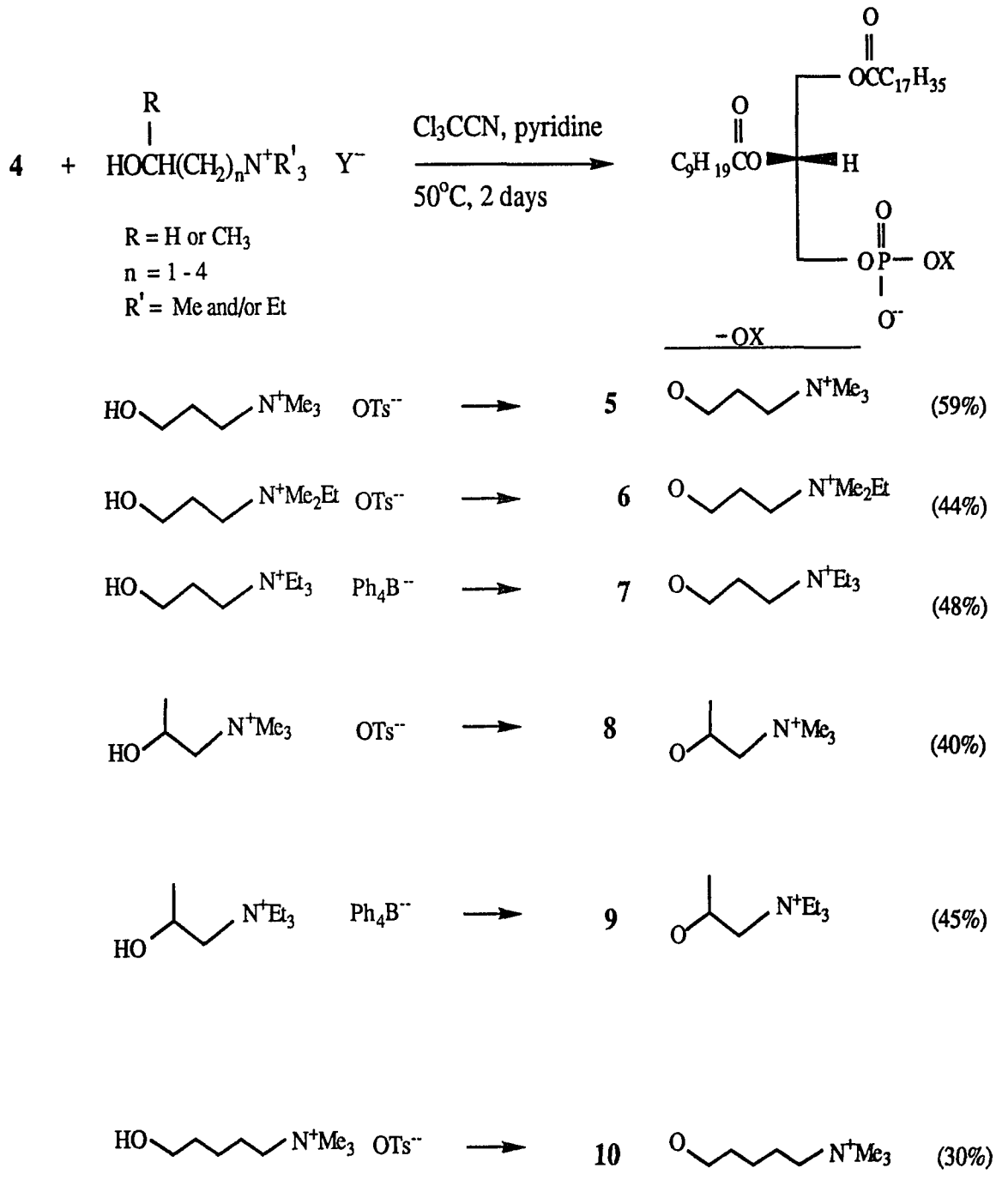


3

(93%) after flash chromatography and lyophilization



4 (100%)



Scheme I: Reaction sequence for the synthesis of analogs of asymmetric-chain PCs modified in the choline moiety

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Chapter 6

Binary mixtures of saturated and unsaturated mixed-chain phosphatidylcholines. A differential scanning calorimetry study

Abstract: High-resolution differential scanning calorimetry (DSC) has been used to study the aqueous dispersions of mixed-chain phosphatidylcholines prepared from colyophilized mixtures of C18:C(11:1Δ^{10})PC/C18:C10PC and C18:C(11:1Δ^{10})PC/C18:C11PC of various molar ratios. These mixed-chain phospholipids are characterized by a marked disparity in their acyl-chain lengths; however, the *sn*-1 acyl chain in the fully extended conformation is about twice as long as the *sn*-2 acyl chain. Their thermotropic behavior has been determined, and the phase diagrams of these two mixtures are constructed from the calorimetric data. Results indicate that C18:C(11:1Δ^{10})PC/C18:C10PC and C18:C(11:1Δ^{10})PC/C18:C11PC are miscible in all proportions with a near-ideal behavior of mixing in the gel and liquid crystalline phases. Equimolar mixtures of diC14PC/ C18:C(11:1Δ^{10})PC, diC14PC/C18:C10PC, and diC14PC/C18:C11PC have also been studied by DSC. These phosphatidylcholines in the 1:1 mixture differ in T_m by less than 11 °C; however, they exhibit gel-phase immiscibility in the plane of the bilayer. Taken together, these studies suggest that C18:C11PC and C18:C(11:1Δ^{10})PC are packed similarly to C18:C10PC in excess water as mixed interdigitated bilayers, at $T < T_m$, which transform into partially interdigitated bilayers when heated above T_m.

Introduction: In recent years, high-sensitivity differential scanning calorimetry (DSC) has been widely used to characterize the thermotropic behavior of lipid bilayers composed of binary mixtures in excess water. Most of the studies, however, have focused on mixtures of one type of saturated symmetric

diacylphosphatidylcholines such as diC14PC or diC16PC with other lipids. In general, the observed phase behavior of binary lipid mixture is rather complex, since diC14PC or diC16PC bilayers exhibit multiple phases such as L_c, $L_{\beta'}$, and $P_{\beta'}$ at temperatures below the gel to liquid-crystalline phase transition. Xu and Huang (1987) have recently reported on the phase behavior of a number of highly asymmetric phosphatidylcholines in which one acyl chain is approximately twice as long as the other. Fully hydrated bilayers of these purely asymmetric PCs exhibit only a single highly cooperative phase transition over a wide temperature range, indicating that only a single type of gel phase exists below the phase transition temperature. On the basis of data obtained with X-ray diffraction and other techniques, this gel phase is designated the mixed interdigitated phase, in which the long acyl chains from opposing monolayers interpenetrate fully across the entire hydrocarbon width of the bilayer, while the short acyl chain of one lipid molecule packs end-to-end with the short chain of another lipid molecule in the opposing bilayer leaflet [for reviews, see Huang and Mason (1986); Slater and Huang (1988)]. We have also studied the binary mixtures of C10:C22PC/C22:C12PC. These two lipid species are distinctively different in their acyl chain lengths esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone; however, one of the fully extended acyl chains in each of the lipid molecules is almost twice as long as the other. A simple phase diagram has been constructed for C10:C22PC/C22:C12PC mixtures on the basis of DSC data. This cigar-shaped phase diagram together with ^{31}P NMR results indicate that C10:C22PC and C22:C12PC can mix nearly ideally in the bilayer in the gel and liquid-crystalline phases (Xu et al., 1987). Such ideal mixing implies that the lipid-lipid lateral interactions for C10:C22PC/C22:C12PC mixtures in the plane of the membrane are virtually identical with those for the pure components. This phase behavior can thus be

used to support the conjecture that mixtures of C10:C22PC/C22:C12PC can form a mixed interdigitated phase at $T < T_m$ and that the total bilayer thickness for the C10:C22PC/C22:C12PC binary system at various molar ratios is most likely invariant.

In this paper, we have extended our previous DSC studies on binary mixtures of highly asymmetric phosphatidylcholines to include a species of monounsaturated PC. These PCs, C18:C11PC, C18:C10PC, and C18:C(11:1Δ^{10})PC, are characterized by a marked disparity in their acyl chain lengths. However, if a conformational bend at an α-methylene segment near the interface region is assumed to be present in the *sn*-2 acyl chain (Huang and Mason, 1986), the *sn*-1 acyl chain in the fully extended conformation is about twice as long as the zigzag *sn*-2 acyl chain for all of these asymmetric PCs.

Materials and Methods

1-Stearoyllysophosphatidylcholine and diC14PC, each with 99 mol% purity, were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Capric and undecanoic anhydrides with purity of greater than 99% were obtained from Nu Chek Prep, Inc. (Elysian, MN). C18:C10PC and C18:C11PC were prepared by acylation of 1-stearoyllysophosphatidylcholine with capric anhydride and undecanoic anhydride, respectively, at room temperature according to the modified semisynthetic method of Mason et al. (1981) as described elsewhere (Xu and Huang, 1987).

Synthesis of C18:C(11:1Δ^{10})PC: 1-StearoyllysoPC was acylated at room temperature with a 10-fold molar excess of 10-undecenylic anhydride in hydrocarbon-stabilized chloroform under a nitrogen atmosphere. The reaction mixture was stirred magnetically for 6 h in the presence of 1.2 equiv of 4-pyrrolidinopyridine as a catalyst. The fatty acid anhydride was prepared from

10-undecenoic acid (Sigma) and dicyclohexylcarbodiimide in carbon tetrachloride (Selinger and Lapidot, 1966). The product, C18:C(11:1Δ^{10})PC, was purified by flash chromatography on silica gel 60 by elution with chloroform, chloroform-methanol (9:1), and then chloroform-methanol (3:2). After suspended silica gel was removed by passing a chloroform solution of the product through a 0.45-μm Metricel filter, the product was further purified by preparative thin-layer chromatography followed by lyophilization with benzene, giving a white solid (62% yield). Elemental analysis (Desert Analytics, Tucson, AZ): Calcd for $C_{37}H_{72}O_8NP \cdot 1.5\ H_2O$ (716.97): C, 61.98; H, 10.54; N, 1.95. Found: C, 61.83; H, 10.67; N, 2.07. We have found that mixed-chain PCs synthesized by this acylation procedure are isomerically pure, with the extent of acyl migration less than 1% (Ali and Bittman, 1989).

Sample Preparation: Binary mixtures of C18:C(11:1Δ^{10})PC/ C18:C10PC and C18:C(11:1Δ^{10})PC/C18:C11PC of known molar ratios were prepared from the corresponding co-lyophilized lipid mixtures and suspended in 50 mM NaCl aqueous solution containing 5 mM phosphate buffer and 1mM EDTA at pH 7.4 to give a total lipid concentration in the range of 2.6-4.1 mM. Aqueous dispersions of 1:1 mixtures of diC14PC/C18:C(11:1Δ^{10})PC, diC14PC/C18:C(10)PC, and diC14PC/C18:C(11)PC were also prepared similarly from the corresponding co-lyophilized lipid mixtures. The exact lipid concentrations were determined by phosphorus analysis (Gomori, 1942). Detailed procedures for preparing binary mixtures have been given elsewhere (Xu et al., 1987). The binary lipid dispersion prepared from two different asymmetric PCs was first warmed to 35 °C, which is about 14 °C above the gel to liquid-crystalline phase transition temperature of the higher melting component of the lipid sample [C18:C10PC], followed by brief sonication under N_2 atmosphere using a bath-type sonicator (Branson, Model B-220, 100W).

The sample was then immediately cooled to 10 °C. The warming/sonication/cooling cycle was then repeated two more times. Finally, the dispersion was incubated at 0 °C for 2 days to allow thermal equilibration of the lipid components in the gel phase prior to calorimetry. The equimolar mixtures of diC14PC with C18:C(11:1Δ^{10})PC, diC14PC with C18:C11PC, and diC14PC with C18:C11PC were treated in the same way, except that the sample was warmed to 40 °C above the T_m of diC14PC.

DSC: The thermograms of the samples were recorded on a high resolution MC-2 differential scanning calorimeter equipped with the DA-2 digital interface and data acquisition utility for automatic data collection (Microcal Inc., Amherst, MA). The lipid dispersions were thermally equilibrated in the microcalorimeter at 0 °C for 1.5 h before heat was applied. Samples were heated at a constant rate of 15 °C/h in an ascending mode, starting from a common temperature of 0 °C and ending at 35 or 40 °C. All DSC experiments were repeated at least once to ensure reproducible results. After cooling, the sample was allowed to equilibrate thermally in the calorimeter for 90 min at 0 °C prior to the second heating scan. The onset and completion temperatures of the transition were determined by the extrapolations of the peak slopes with the base line. Phase diagrams were constructed on the basis of the onset and completion temperatures of the thermograms recorded for a series of the binary mixtures, as previously described (Xu et al., 1987). The transition temperatures and transition enthalpies were also determined as described previously (Xu and Huang, 1987). The experimental errors in ΔH were introduced primarily by the uncertainity in lipid concentrations, which were the average value of three determinations for each lipid sample.

Results

C18:C(11:1Δ^{10})PC/C18:C10PC Mixtures: The heating thermogram for C18:C(11:1Δ^{10})PC dispersions, as illustrated in the bottommost curve of Figure 1A, exhibits a single endothermic transition with a transition peak width at half-height ($\Delta T_{1/2}$) of 0.50 ± 0.01 °C; this transition is peaked at a T_m of 13.3 ± 0.1 °C with a well-defined calorimetric enthalpy (ΔH = 6.1 ± 0.5 kcal/m). The DSC scan of C18:C10PC dispersions, shown as the uppermost curve in Figure 1A, is characterized by a sharper endothermic transition with $\Delta T_{1/2}$ = 0.23 ± 0.01 °C, ΔH = 9.0 ± 0.6 kcal/m, and T_m = 18.6 ± 0.1 °C. This single transition has reported previously; it corresponds to the gel to liquid-crystalline phase transition (Huang and Mason, 1986; Xu and Huang, 1987).

Samples of C18:C(11:1Δ^{10})PC containing various molar ratios of C18:C10PC, which have been thermally equilibrated for at at least 48 h at 0 °C prior to DSC scans, exhibit single endothermic phase transitions (Figure 1A). Within experimental errors, the values of T_m appear to increase linearly with increasing C18:C10PC concentration (Figure 2). In fact, the values of T_m for the mixtures can be approximated to the sum of the partial molar fraction of the corresponding parameters for the pure components; namely, T_m(mixture) = $T_m^1X^1 + T_m^2X^2$, where X stands for the mole fraction and the superscripts 1 and 2 stand for the pure components of the binary mixture. Also shown in Figure 2 is the plot of transition enthalpy (ΔH) versus the mole fraction of C18:C10PC. Despite the large scatter of the data points, the values of ΔH for the binary mixtures are found to increase progressively with increasing mole fraction of C18:C10PC; moreover, the experimental values are all within 11% of the computed values obtained by assuming an ideal sum of the partial molar enthalpies. Thus, the calorimetric data of C18:C(11:1Δ^{10})PC/C18:C10PC mixtures shown in Figure 2 appear to be consistent with a complete miscibility

of the two component lipids over the entire composition range.

The temperature-composition phase diagram for C18:C(11:1Δ^{10})-PC/C18:C10PC mixtures is shown in Figure 3A. The solidus and liquidus lines of this phase diagram are constructed on the basis of onset and completion temperatures, respectively, of a series of transition curves (Figure 1) after correction for the finite width of the transition curves of the pure components (Mabrey and Sturtevant, 1976). It is evident that the solidus and liquidus lines do show smooth and continuous changes in the phase diagram; moreover, they do not display either a point of sharp inflection or a region of horizontal line. Also shown are the calculated solidus and liquidus lines (dotted curves) of the theoretical phase diagram expected for an ideal mixing of the lipid species in both the gel and liquid-crystalline phases using the procedure given by Mabrey and Sturtevant (1976). It can be seen that the experimental lines show slightly negative deviation from those theoretical lines. The cigar-shaped phase diagram for C18:C(11:1Δ^{10})PC/C18:C10PC shown in Figure 3A, therefore, reflects a complete miscibility of the component lipids with homogeneous, near-ideal mixing in both phases over the entire composition range.

C18:C(11:1Δ^{10})PC/C18:C11PC Mixtures. The uppermost DSC curve shown in Figure 1B illustrates a representive heating thermogram obtained with samples of pure C18:C11PC. The values of T_m, $\Delta T_{1/2}$, and ΔH for C18:C11PC are 21.4 °C, 0.4 °C, and 9.9 kcal/m, respectively; these values agree well with previously reported ones (Xu and Huang, 1987). Figure 1B also shows heating thermograms for series of C18:C(11:1Δ^{10})PC/C18:C11PC binary mixtures. The transition curves for the mixtures are slightly broadened in comparison with those of the pure component lipids. The largest values of $\Delta T_{1/2}$, 0.8 °C, are found for the sample of an equimolar mixture. The values of T_m for the binary mixtures are seen, within experimental error, to increase

linearly with increasing mole fraction of C18:C11PC between the pure C18:C(11:1Δ^{10})PC and C18:C11PC values (Figure 2). The values of ΔH for the mixtures presented in Figure 2 show less than 22% deviations from the sum of the partial molar enthalpies of the pure components.

In Figure 3B, the experimentally determined phase diagram (solid lines), derived from the transition curves shown in Figure 1B, and the theoretical calculated phase diagram (dotted lines), obtained on the basis of an ideal mixing of the component lipids in both the gel and liquid-crystalline phases, are presented for C18:C(11:1Δ^{10})PC/C18:C11PC mixtures. It is evident that the experimental lines of the equilibrium phase diagram exhibit slightly negative deviation from the theoretical phase boundaries. These results thus indicate that C18:C(11:1Δ^{10})PC and C18:C11PC are miscible in the bilayer over the entire composition range and that the behavior of mixing is nearly ideal in both the gel and liquid-crystalline phases.

Equimolar mixtures of DiC14PC with C18:C(11:1Δ^{10})PC, C18:C10PC, and C18:C11PC. Figure 4 represents the temperature dependence of excess heat capacity of equimolar mixtures of diC14PC/C18:C(11:1Δ^{10})PC, diC14PC/C18:C10PC, and diC14PC/C18:C11-PC. In contrast to 1:1 mixtures of C18:C(11:1Δ^{10})PC/C18:C10PC and C18:C(11:1Δ^{10})PC/C18:C11PC that exhibit single, sharp phase transitions shown in Figure 1, the calorimetric scans of equimolar mixtures of diC14PC with highly asymmetric PCs are characterized by multiple well-defined peaks as shown in Figure 4. In one case, the thermotropic behavior of the equimolar mixture depends on the thermal history of the sample.

The initial heating thermogram for an equimolar mixture of diC14PC/ C18:C(11:1Δ^{10})PC, which has been thermally equilibrated at 0 °C for 5 days prior to calorimetry, is presented in Figure 4a. Two highly cooperative

transitions and a broad overlapped endothermic transition are discernible in the temperature range 0-40 °C. The low-temperature endotherm is sharp and symmetric; it occurs over a narrow temperature range ($\Delta T_{1/2}$ = 0.75 °C) over which the maximal excess heat capacity is observed at a T_m of 10.8 °C. This transition corresponds closely to that recorded for dispersions of pure C18:C(11:1Δ^{10})PC, one of the components of the binary mixture; however, the value of T_m has dropped 2.5 °C in comparison with the pure component (Table I). The second broad endotherm, seen near the middle of the temperature profile shown in Figure 4a, appears to be a superposition of several minor transitions. This overlapping feature peaked at ~17 °C resembles the $L_c \rightarrow P_{\beta'}$ phase transition recorded for pure diC14PC dispersions at 16.2 °C (Lewis et al., 1987). Also shown in Figure 4a is a third endothermic transition occuring at a higher temperature of 23.7 °C. This highly cooperative transition ($\Delta T_{1/2}$ = 0.58 °C) corresponds closely to the main $P_{\beta'} \rightarrow L_\alpha$ phase transition exhibited by dispersions of pure diC14PC (Lewis et al., 1987; Xu and Huang, 1987); the value of T_m is only slightly downshifted (0.2 °C) in comparison with that of the pure component.

The two well-resolved major peaks observed in Figure 4a can be readily interpreted as indicating the coexistence of separate gel domains of nearly pure lipid components in 1:1 mixture of diC14PC/ C18:C(11:1Δ^{10})PC at temperatures below 10.8 °C. The calorimetrically observed sharp transitions of each of the PCs represent the chain melting of phospholipid in separate gel domains composed primarily of one kind of lipid component in the plane of the bilayer. The calorimetric results thus demonstrate that diC14PC and C18:C(11:1Δ^{10})PC at 1:1 molar ratio are immiscible in the gel state, and in this respect the nonmixing behavior of diC14PC with C18:C(11:1Δ^{10})PC is completely different from that of C18:C10PC or C18:C11PC with the same

second component lipid. It should be noted that separated gel domains in the same bilayer have been detected recently by DSC for a 1:1 mixture of diC14PC/diC20PC (Melchior, 1986).

After the initial heating scan, the sample was cooled from 40 to 0 °C in the calorimeter. Prior to the second heating scan, the sample was allowed to equilibrate at 0 °C for 90 min in the calorimeter. Figure 4b illustrates the second heating DSC curve of the 1:1 mixture of diC14PC/ C18:C(11:1Δ^{10})PC that has displayed the initial heating DSC curve shown in Figure 4a. This thermogram is characterized by two sharp well-defined endotherms with maxima occurring at 10.9 and 23.7 °C, respectively. These two prominent endotherms correspond to the low- and high- temperature transitions detected in the initial heating scan. The absence of a broad overlapping transition centered at ~ 17 °C in the second heating scan suggests that this broad endotherm between the two major endotherms observed in the initial scan is irreversible under the experimental conditions.

In Figure 4c, two prominent endothermic transitions with maxima occuring at 16.9 and 23.5 °C are observed in the initial heating scan of an equimolar mixture of diC14PC/C18:C10PC. This sample has been preincubated at 0 °C for 3 days. Lowering the temperature of the sample in the calorimeter to 0 °C, followed by incubating at 0 °C for 90 min and then rescanning, results in an identical thermogram. The thermal transition peaked at 16.9 °C is broad ($\Delta T_{1/2}$ = 1.1 °C), with a low- temperature shoulder at 13.5 °C. This shoulder persists in the second heating scan. Therefore, the broad thermal transition peaked at 16.9 °C is likely a composite, overlapping feature composed of a pretransition of lamellar diC14PCs and a gel to liquid-crystalline phase transition of lamellar C18:C10PCs. It is noted that the observed T_m of 16.9 °C is only 1.7 °C lower than the T_m value observed for the gel to liquid-

crystalline phase transition exhibited by the dispersions of pure C18:C10PC (Table I). The second prominent endotherm shown in Figure 4c is peaked at 23.5 °C with a $\Delta T_{1/2}$ of 0.8 °C. The T_m value is only 0.4 °C smaller than the typical value recorded for pure the $P_{\beta'} \rightarrow L_{\alpha}$ phase transition exhibited by dispersions of pure diC14PC (Lewis et al., 1987; Xu and Huang, 1987). The thermogram with distinct multiple transitions, shown in Figure 4c, can thus be taken as evidence to indicate that in the gel state of the 1:1 mixture of diC14PC/C18:C10PC the component lipids are mostly immiscible in the plane of the bilayer and that most of the component lipids exist as separate phases at equilibrium in the gel state.

A representative heating thermogram for aqueous dspersions of an equimolar mixture of diC14PC/C18:C11PC is presented in Figure 4d. This sample has been incubated at 0 °C for 3 days prior to DSC scan; however, repeated scans of the sample from 0 °C result in identical thermograms. Three endothermic transitions with maxima occurring at 13.4. 19.4, and 23.6 °C are observed in Figure 4d. The small endotherm at 13.4 °C appears to be the pretransition of diC14PC. The two major endotherms at 19.4 and 23.6 °C correspond closely to the gel to liquid-crystalline phase transition of each of the two components of the mixture, although there is clearly a small downshift in T_m and a broadening of the transition curve in comparison with the endotherm of each of the pure components. This is presumably due to the melting of the C18:C11PC [or diC14PC] enriched bilayers with relatively small amounts of diC14PC [or C18:C10PC] incorporated in separate domains. An additional noteworthy observation is the base line, which shifts upward continuously. It is not clear what causes this deviation from the horizontal base line.

In any case, the distinct transition peaks shown in Figure 4d again suggest that in the gel state there is immiscibility between the two component

phospholipids and that almost all phospholipids of each component are self-assembled into separate gel domains.

Discussion

In this sudy, the thermotropic phase behavior of C18C(11:1Δ^{10})PC dispersions is reported for the first time. A single endothermic phase transition is detected calorimetrically for C18C(11:1Δ^{10})PC dispersions between 0 and 35 °C. The thermodynamic parameters (T_m, $\Delta T_{1/2}$, ΔH, and ΔS) associated with the phase transition are listed in Table I together with those of the corresponding saturated asymmetric PCs. It is most interesting to note that the incorporation of a double bond into the C18C11PC molecule between carbon atoms 10 and 11 in the *sn*-2 acyl chains lower the values of T_m by only 8 °C. By contrast, the values of T_m for C18:C(18:1Δ^9)PC and C16:C(18:1Δ^9)PC lamellae are about 48 and 52 °C lower than the corresponding values for C18:C18PC and C16:C18PC lamellae, respectively (Keough, 1986; Xu and Huang, 1987). On the basis of calorimetric studies of a series of symmetric PCs with a *cis* double bond at different positions along each acyl chain, Barton and Gunstone (1975) have shown that the value of T_m for the unsaturated PC lamella is influenced by the location of the *cis* double bond. The value of T_m is minimal, that is, the shift relative to the corresponding saturated PC is maximal, when each *cis* double bond moves toward either end of the fatty acyl chain (Barton and Gunstone, 1975). The observed 8 °C shift in T_m for C18C(11:1Δ^{10})PC relative to C18:C10PC may, therefore, be attributed to the location of the double bond, which is placed at the chain end between carbon atoms 10 and 11 in the *sn*-2 acyl chain.

Since the molecular conformation and packing properties of C18:C10PC in the bilayer at temperatures above and below T_m are well characterized

(McIntosh et al., 1984; Hui et al., 1984; Mattai et al., 1987), our DSC results with C18:C(11:1Δ^{10})PC/C18:C10PC binary mixtures can be used to postulate the molecular packing of C18:C(11:1Δ^{10})PC in bilayers. At $T < T_m$, C18:C10PC molecules with acyl chains in a nearly all *trans* conformation are packed in a mixed interdigitated bilayer in the presence of excess water; this packing mode is characterized by having the area per phospholipid molecule at the lipid/water interface encompass three acyl chains. At T > Tm, C18:C10PC molecules in the bilayer are thought to pack into a partially interdigitated mode. In this state, the short chain of one lipid in one leaflet packs end-to-end with the long chain of another lipid in the oppossing leaflet of the bilayer. Furthermore, the acyl chains are no longer in nearly all *trans* conformation, but they contain several *gauche* rotamers. In the present work, C18:C(11:1Δ^{10})PC in the bilayer is shown to be homogeneously miscible with C18:C10PC in both the gel and liquid-crystalline phases. In order to achieve a complete miscibility of both lipid species (say, A and B) with near-ideal mixing in the gel and liquid-crystalline phases, the lipid-lipid lateral interactions between the two component lipids (E_{AB}) must be similar to those between the pure components (E_{AA} and E_{BB}) at temperatures both above and below the T_m. These similar lipid-lipid lateral interactions can, in turn, be interpreted to imply that these two kinds of lipid molecules are most likely to be very similar in their packing properties in bilayers. Our DSC results (Figure 2) and the equilibrium phase diagram (Figure 3A) can thus be taken to suggest that C18:C(11:1Δ^{10})PC molecules, in excess water, also adopt a mixed interdigitated packing mode at $T < T_m$ and a partially interdigitated packing mode at $T > T_m$ in the bilayer.

Our DSC studies also indicate that C18:C(11:1Δ^{10})PC and C18:C11PC are completely miscible in all proportions in both the gel and liquid-crystalline phases, and the mixing behavior is near ideal. This near ideal behavior of

complete miscibility is undoubtedly due to the similar lipid-lipid lateral ineractions between the two components in comparison with those between pure components. Again, we can attribute these similar lipid-lipid lateral interactions to packing similiarities of these two lipid species in the bilayer at temperatures above and below T_m. Although we know of no direct experimental data that describe the packing properties of C18:C11PC in the bilayer, two lines of evidence strongly suggest that C18:C11PC molecules adopt the mixed and partially interdigitated modes at temperatures below and above T_m, respectively. (1) Previous X-ray diffraction studies indicate that both C18:C10PC and C18:C12PC molecules are packed nearly identically in gel and liquid-crystalline states (Hui et al., 1984). In line with the data obtained with C18:C10PC and C18:C12PC, the C18:C11PC molecules are most likely to pack into the mixed interdigitated and partially interdigitated modes at temperatures below and above T_m, respectively. (2) Experimental data from a series of comparative DSC studies on interdigitated and noninterdigitated bilayer systems show that the thermodynamic parameters associated with the main transition are distinctively different for the two systems. Thermodynamic parameters obtained with C18:C11PC lamellae are consistent with the packing model whereby the C18:C11PC molecules are self-assembled into a mixed interdigitated bilayer at $T < T_m$ (Xu and Huang, 1987). On the basis of these two lines of indirect evidence, lamellar C18C11PC can be reasonably assumed to have packing properties similar to C18:C10PC in the bilayer; hence, a complete miscibility between C18:C(11:1Δ^{10})PC and C18:C11PC with near-ideal mixing behavior in both phases can be taken as evidence to argue in favor that C18:C(11:1Δ^{10})PC molecules adopt a mixed interdigitated packing mode in the bilayer at $T < T_m$ and a partially interdigitated packing mode at $T > T_m$.

The excess heat capacity profiles for equimolar mixtures of diC14PC with C18:C(11:1Δ^{10})PC, C18:C10PC, and C18:C11PC, shown in Figure 4, can be used to further substantiate the foregoing suggestion that C18:C(11:1Δ^{10})PC molecules are most likely packed into a mixed interdigitated bilayer at $T < T_m$. Before discussing the significance of the thermograms, it should be noted that Curatolo et al. (1985) concluded, on the basis of calorimetric data obtained with 21 pairs of binary PC mixtures, that binary mixtures of PCs exhibit gel-state miscibility if the difference in the transition temperature between the component PCs (ΔT_m) is less than 33 °C. DiC14PC and diC16PC, for instance, differ in T_m by 17.5 °C; they mix nearly ideally in the bilayer in both the gel to liquid-crystalline states (Mabrey and Sturtevant, 1976). When the value of ΔT_m is greater than 33 °C, binary mixtures of PCs exhibit gel-state immiscibility (Curatolo et al., 1985). The value of ΔT_m for diC14PC and diC20PC, for instance, is 41 °C, and the 1:1 mixture of diC14PC and diC20PC exhibits calorimetrically two major endotherms that correspond to the melting of separate gel domains of each lipid species (Melchior, 1986). The values of ΔT_m between two component PCs in diC14PC/C18:C(11:1Δ^{10})PC, diC14PC/C18:C10PC, and diC14PC/C18:C11PC mixtures are 10.6, 5.3, and 2.5 °C, respectively. These mixtures would be expected to exhibit gel-state miscibility on the basis of ΔT_m according to the general conclusion of Curatolo et al. (1985). The thermograms shown in Figure 4, however, demonstrated convincingly that diC14PC molecules are not completely miscible in the gel state with C18:C(11:1Δ^{10})PC, C18:c10PC, and C18:C11PC, respectively, in the 1:1 mixtures. An interesting question therefore arises as to why the three binary mixtures shown in Figure 4 exist as two separate phases at equilibrium in the gel state.

It is well known that diC14PC molecules are self-assembled into

noninterdigitated lamellae in excess water. In contrast, C18:C10PC or C18:C12PC molecules in the presence of excess water are packed into a mixed interdigitated bilayer at $T < T_m$. Because of their different packing modes, the bilayer thickness for noninterdigitated diC14PC lamellae at $T < T_m$ is substantially greater than that for mixed interdigitated C18:C10PC lamellae. At 10 °C, for instance, the bilayer thickness for diC14PC lamellae is 43 Å (Janiak et al., 1976), whereas a bilayer thickness of 33 Å is known for C18:C10PC lamellae (Hui et al., 1984). This 10 Å difference in the bilayer thickness contributes to a considerably larger area of van der Waals contact surface between diC14PC molecules in the plane of the bilayer. This expectation is indeed borne out by the thermogram shown in Figure 4c. The present study thus suggests that in order to have a gel-state miscibility for binary PC mixtures, a ΔT_m values of less than 33 °C is not sufficient. It is necessary that the lipid components must also have similar packing modes in the bilayer.

The observed gel-state immiscibility of diC14PC with C18:C11PC and C18:C(11:1Δ^{10})PC can be argued similarly as due to the relatively stronger attractive forces between diC14PC molecules in the gel-state bilayer. The strong lipid-lipid lateral interactions between diC14PCs in the plane of the gel-state bilayer have become possible because C18:C11PC and C18:C(11:1Δ^{10})PC, similarly to C18:C10PC, are most likely packed in a totally different mode in the mixtures. We believe that these highly asymmetric PCs are all packed in a mixed interdigitated bilayer, at $T < T_m$, in the presence of excess water. However , we should emphasize that X-ray diffraction or other spectroscopic studies are ultimately required to prove definitively the packing models of C18:C11PC and C18:C(11:1Δ^{10})PC bilayers as suggested by the present work.

In summary, results of DSC experiments on binary lipid mixtures can

provide information about the lipid-lipid lateral interactions. The present study reveals that C18:C(11:1Δ^{10})PC/C18:C10PC and C18:C(11:1Δ^{10})PC/C18:C11-PC mixtures are miscible in all proportions, with a near-ideal behavior of mixing in the gel and liquid-crystalline phases. This behavior is taken to reflect that the incorporation of a double bond at the end of the *sn*-2 acyl chain does not exert a pronounced disruptive effect on the overall lipid-lipid lateral interactions for highly asymmetric PCs in the bilayer. Consequently, the overall packing mode of the highly asymmetric PC molecule with a terminal double bond in the binary mixtures used in Figure 1 can be viewed to resemble closely that of the bilayer composed of single, saturated lipid component. Thus the C18:C(11:1Δ^{10})PC molecules, in either the pure one-component system or the two-component system with C18:C10PC or C18:C11PC, are most likely to assemble at $T < T_m$ into a mixed interdigitated bilayer that, when heated above the T_m, converts to a partially interdigitated phase. We have also demonstrated for the first time that equimolar mixtures of diC14PC/C18:C(11:1Δ^{10})PC, diC14PC/C18:C10PC, and diC14PC/ C18:C11- PC exhibit gel-state immiscibility in the plane of bilayer. This is extremely interesting, since the component PCs in each of the three binary mixtures differ in T_m by less than 11 °C. This immiscibility is attributed to the very strong attractive forces between diC14PC molecules in the 1:1 mixtures reflect that the packing of the highly asymmetric PCs differ markedly from that of diC14PCs in the gel-state bilayer. On the basis of the known packing mode of C18:C10PC in the bilayer at $T < T_m$ and the similar nonmixing behavior of C18:C10PC, C18:C(11:1Δ^{10})PC, and C18:C11PC with diC14PC at $T < T_m$, we suggest that C18:C(11:1Δ^{10})PC and C18:C11PC in the equimolar mixtures with diC14PC are assembled at $T < T_m$ into separate gel domains in which these highly asymmetric PCs are most likely to adopt a mixed interdigitated packing mode. At $T > T_m$, however, these binary mixtures are expected to be

miscible due to their similar packing properties. Further structural studies are required to varify the packing models proposed by this work.

Table I: Thermodynamic Parameters Associated with the phase Transition of Highly Asymmetric Phosphatidycholine dispersions

Phospholipid dispersions	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kcal/m)	ΔS (eu/mol)
C18:C(11:1Δ^{10})PC	13.3[a]	0.50[a]	6.1[a]	21.3[a]
C18:C11PC	21.4[a]	0.40[a]	9.9[a]	33.6[a]
	21.3[b]	0.34[b]	9.2[b]	31.3[b]
C18:C10PC	18.6[a]	0.23[a]	9.0[a]	31.0[a]
	19.2[b]	0.63[b]	8.9[b]	30.3[b]

a. Data from the present work. The experimental errors in T_m, $\Delta T_{1/2}$, and ΔH are within 1%, 5%, and 15%, respectively. The values of ΔS, the transition entropy, estimated from the Clausius equality as $\Delta S = \Delta H/T_m$, assuming a first-order equilibrium transition.

b. Taken from Xu and Huang (1987).

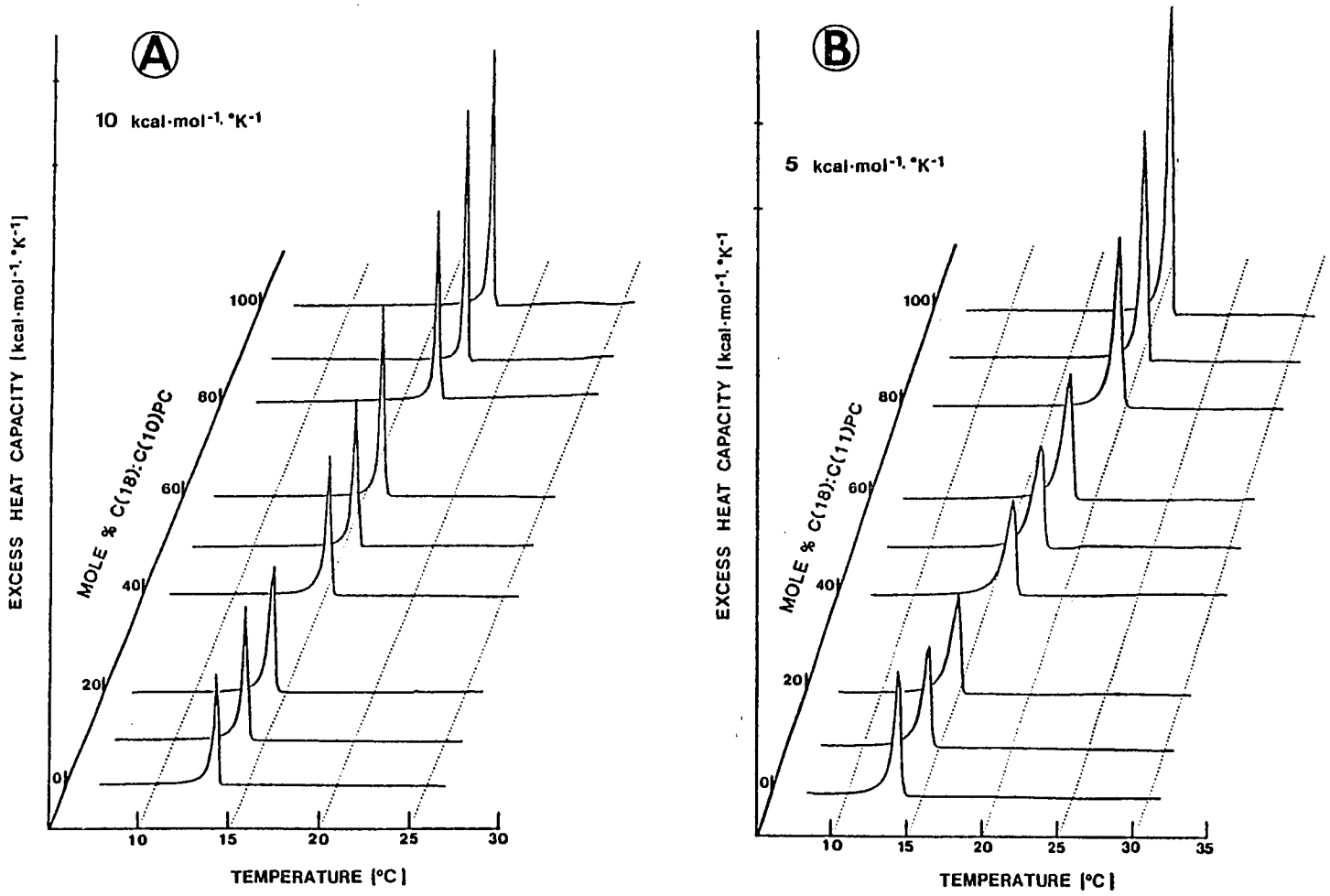

Fig. 1: Representative DSC heating thermograms for fully hydrated samples of C18:C(11:1Δ^{10})PC containing various concentrations of (**A**) C18:C10PC and (**B**) C18:C11PC. Samples of binary mixtures were preequilibrated at 0 °C for a minimum of 2 days prior to calorimetric scans. The curves are plotted relative to each other according to their mole percentages of C18:C10PC and C18:C11PC as indicated on the slanted axis in (**A**) and (**B**), respectively.

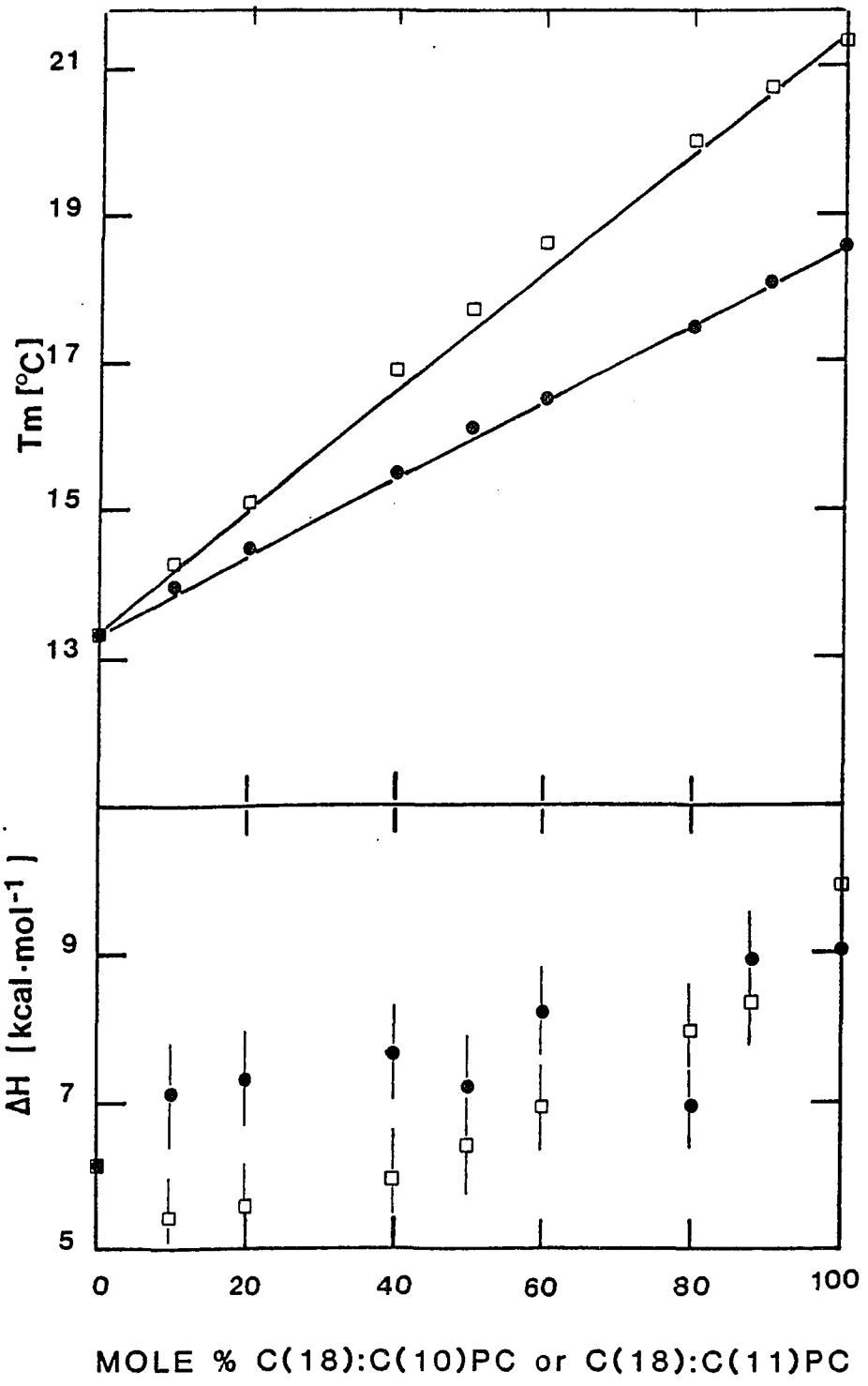

Fig. 2: Plots of transition temperature (T_m) and transition enthalpy (ΔH) of the phase transition versus the mole fraction of C18:C10PC (solid circles) or C18:C11PC (open squares) in C18:C(11:1Δ^{10})PC/C18:C11PC or C18:C(11:1Δ^{10})PC/C18:C10PC binary mixtures.

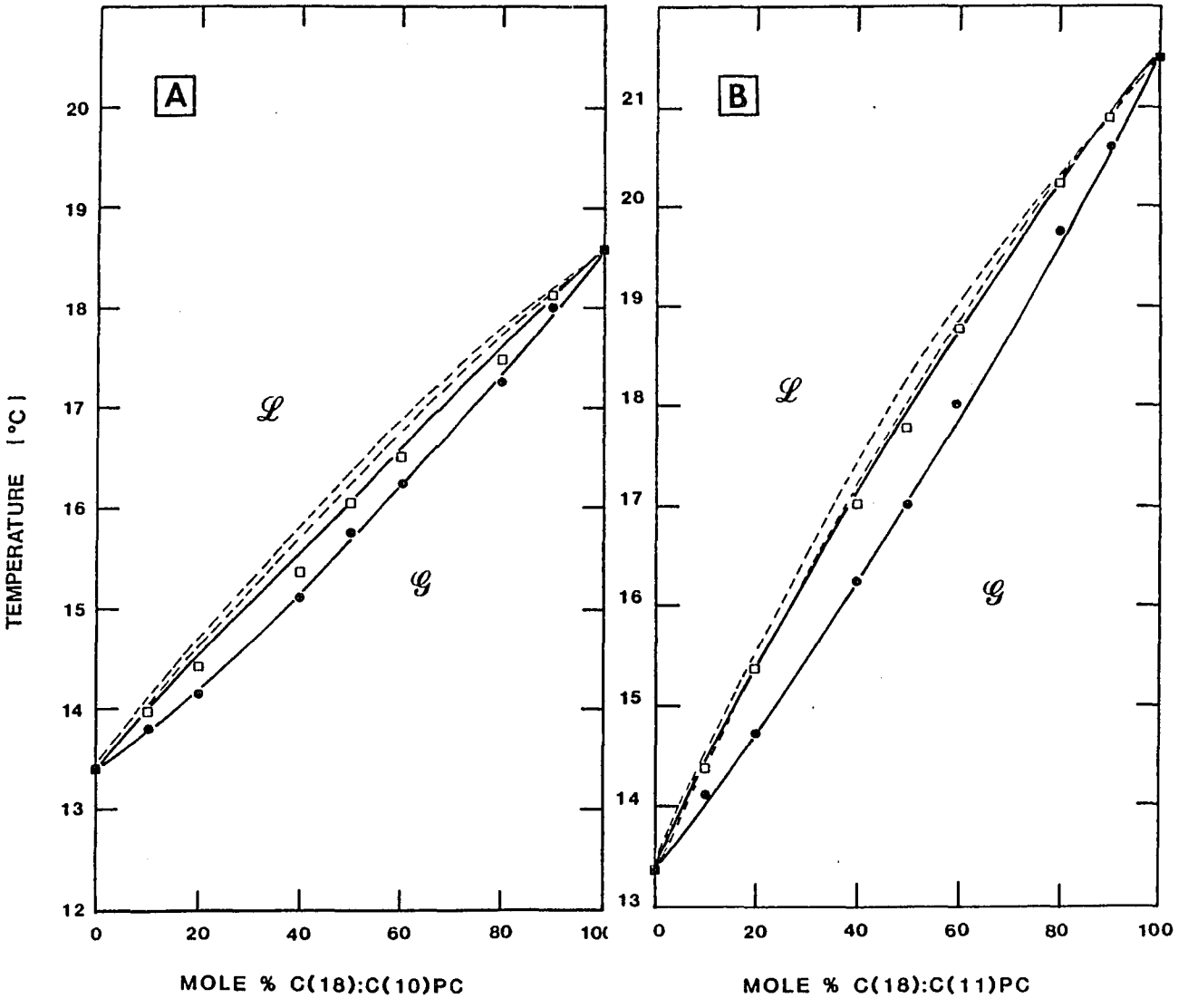

Fig. 3: Phase diagrams for (**A**) the C18:C(11:1Δ^{10})PC/C18:C10PC system and (**B**) the C18:C(11:1Δ^{10})PC/C18:C11PC system. The phase boundaries (solid lines) were constructed from the calorimetric data by connecting the onset temperatures (closed circles) and completion temperatures (open circles) of the phase transitions. The dotted lines are the phase boundaries of the calculated phase diagrams expected for ideal mixing of the component lipids. G and L denot the gel and liquid-crystalline phase, respectively.

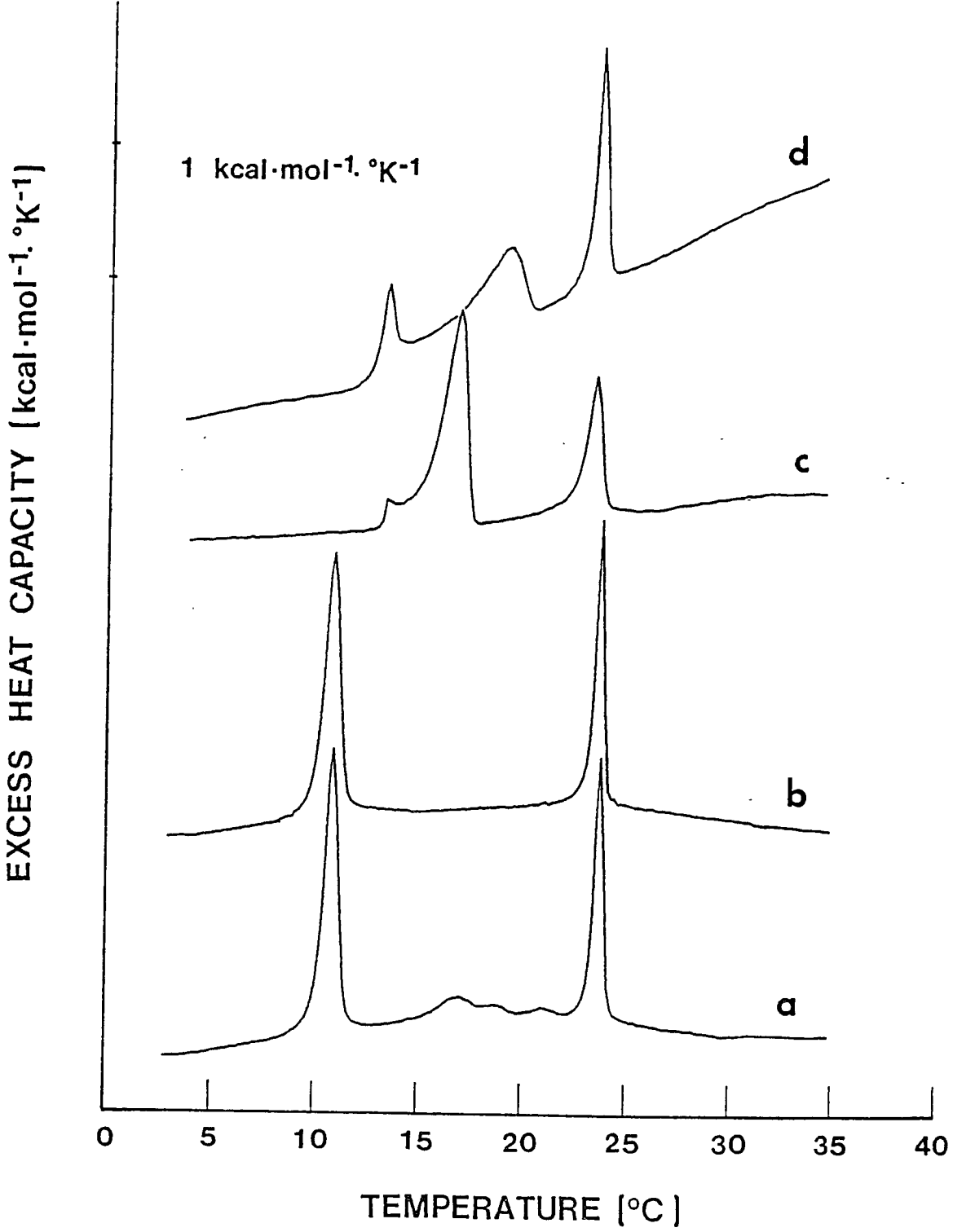

Fig. 4: Excess heat capacity versus temperature for equimolar mixtures of diC14PC with C18C(11:1Δ^{10})PC (curves a and b), C18:C10PC (curve c) and C18:C11PC (curve d). Curve b is the second DSC heating curve following the initial heating scan (curve a). Then preincubation time at 0 °C for samples prior to the initial heating run is (a) 5 days (c) 3 days and (d) 3 days.

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Chapter 7

Calorimetric investigations of mixing behavior of highly asymmetric-chain PCs: Evidence of gel phase immiscibility

Introduction: Differential scanning calorimetry (DSC) has provided a wealth of information about lipid-lipid and lipid-protein interactions (for reviews see: Huang and Mason (1986), Slater and Huang (1988); McElhaney (1986)). The mixing behavior of two or more lipid components in the plane of bilayers has attracted considerable attention in recent years (Slater and Huang, 1988). However, none of the studies reported so far has dealt with the mixing behavior of two highly asymmetric-chain PCs which are modified (i) in the acyl chain at the *sn*-2 position with a terminal ester moiety (ω-CH_3CO_2), and (ii) in the acyl chain at the *sn*-2 position with a terminal ketone moiety (ω-CH_3CO).

Naturally occurring phospholipids such as 1-palmitoyl-2-oleoyl-PC and other 1-saturated-2-unsaturated phospholipids can autooxidize to yield 1-palmitoyl-2-nonal-*sn*-3-glycero-PC, a highly asymmetric-chain lipid with an *sn*-2 acyl chain terminating in an aldehyde group. Phospholipids with highly disparate chain lengths are known to self-assemble in excess water to form a mixed interdigitated bilayer at low temperatures (Xu and Huang, 1987). All of the phospholipids examined to date bear acyl or alkyl chains (Huang and Mason, 1986; Mattai et al., 1987). In this work we wish to study whether a polar oxygen atom at the end of a short acyl chain perturbs the packing of deeply penetrated hydrocarbon chains in the interdigitated gel state. The carbonyl function introduces a dipole which may not be accommodated into the hydrophobic interior of the bilayer. Since the aldehyde is unstable and may undergo oxidation during the calorimetric studies, we prepared PCs **1** and **2**, in which the acyl chain at the *sn*-1 position is about twice long as the *sn*-2-acyl

chain that terminates in a ketone or an ester moiety.

In this paper we report the thermotropic phase behavior of two pairs of binary systems, C18:C10PC (**3**)/**1** and C18:C10PC (**3**)/**2**, by DSC. Since most of the properties of **3** have been investigated (Huang and Mason, 1986), the effects of adding another PC on **3** can be interpreted. From the DSC results it is suggested that these two pairs of highly asymmetric-chain PCs are immiscible at $T < T_m$ and can exist as separate domains with **3** in the gel phase within a wide composition range. However, **3**/**1** and **3**/**2** mixtures are miscible in the liquid-crystalline phase ($T > T_m$). The DSC results suggest that **1** and **2** both can perturb the gel-state packings at $T < T_m$, but have little effect on the packing in the liquid-crystalline state. The pertubing effect on the behavior of **3** is more pronounced in **1** as than in **2**.

Materials and Methods

1-Stearoyl-2-lyso-PC (C18:C0PC) with purity of >99% was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). 4-Pyrrolidinopyridine was purchased from Aldrich, and was purified by crystallization from chloroform:diethyl ether (1:1). The acylation of C18:C0PC was carried out by using the fatty acid anhydrides and 4-pyrrolidinopyridine as a catalyst by as reported previously (Ali and Bittman, 1989). The fatty acid anhydrides were synthesized from the fatty acids and dicylohexylcarbodiimide in CCl_4 according to the procedure of Selinger and Lapidot (1966). C18:C10PC (**3**) was synthesized according to the procedure described in Chapter 5. The purity of each of the phospholipids was checked by TLC (Analtech, Newark, DE) using $CHCl_3$:MeOH:H_2O (6:3:0.4) as the eluting solvent system. The phospholipids plates were detected on the TLC by spraying with molybdate solution (Witzke and Bittman, 1986). Chloroform was distilled from P_2O_5 and stored over 4 A

molecular sieves.

C18:C(10:ω-CH_3CO_2)-PC (1): To a suspension of C18:COPC (100 mg, 0.18 mmol) in 5 mL of dry, ethanol-free $CHCl_3$ at room temperature under nitrogen atmosphere were added sebacic acid anhydride (500 mg, 1.8 mmol) and 4-pyrrolidinopyridine (41 mg, 0.28 mmol). After the mixture had stirred for 6 h, the solvent was evaporated. The crude product was purified by flash chromatography using elution with $CHCl_3$, then with 100 mL of $CHCl_3$:MeOH (9:1), and finally with 700 mL of $CHCl_3$:MeOH (1:1). The product was further purified by preparative TLC using $CHCl_3$:MeOH:H_2O (6:3:0.4) as eluant. Finally, lyophilization with benzene gave 80 mg (50%) of **1** (Scheme I) as a white glue; R_f 0.34 ($CHCl_3$:MeOH:H_2O, 6:3:0.4). ^{1}H NMR ($CDCl_3$) (200 MHz): δ 5.17-5.15 (m, $CH_2C\underline{H}CH_2$, 1 H), 4.36-4.31 (m, $C\underline{H}_2CO_2C_{17}H_{35}$, 2 H), 4.12-3.83 (m, $C\underline{H}_2OP(O)(O^-)OC\underline{H}_2C\underline{H}_2N$, 6 H), 3.62 (s, CH_3, 3 H), 3.32 (s, $N(CH_3)_3$, 9 H), 2.30-2.21 (m, CH_2CO, 6 H), 1.57-1.16 (br m, $(CH_2)_{21}$, 42 H), 0.87-0.81 (t, J = 6.4 Hz, ω-CH_3, 3 H). Anal. Calcd. for $C_{37}H_{72}O_{10}NP\cdot H_2O$ (739.96): C, 60.06; H, 10.08; N, 1.89. Found: C, 60.28; H, 10.00; N, 2.22.

C18:C(10:ω-CH_3CO)-PC (2): **2** was prepared from C18:COPC (150 mg, 0.28 mmol) and 9-oxo-decanoic acid anhydride (600 mg, 1.45 mmol) using 4-pyrrolidinopyridine (51 mg, 0.34 mmol) as a catalyst as described above for the preparation of **1**. The PC was purified by flash chromatography using the same solvent gradients as used for the **1**; finally lyophilization with benzene gave 24 mg (40%) of **2** (Scheme I) as a white glue; R_f 0.32 ($CHCl_3$:MeOH:H_2O, 6:3:0.4). ^{1}H NMR ($CDCl_3$) (300 MHz): δ 5.22-5.19 (m, $CH_2C\underline{H}CH_2$, 1 H), 4.52-4.47 (m, $C\underline{H}_2O_2CC_{17}H_{35}$, 2 H), 4.35-3.90 ($C\underline{H}_2OPO(O^-)OC\underline{H}_2C\underline{H}_2N$, 6 H), 3.41 (s, $N(CH_3)_3$, 9 H), 2.42-2.37 (t, J = 6.8 Hz, $CH_3COC\underline{H}_2$, 2 H), 2.32-2.25 (m, $COCH_2$, 4 H), 2.10 (s, $C\underline{H}_3COCH_2$, 3 H), 1.58-1.14 (br m, $(CH_2)_{19}$, 38 H), 0.86-0.83 (t, J = 5.0 Hz, ω-CH_3, 3 H). Anal.

Calcd. for $C_{36}H_{70}O_9NP$. 0.5 H_2O (700.93): C, 61.68; H, 10.21; N, 1.99. Found: C, 61.61; H, 10.47; N, 2.24.

Preparation of Samples for Calorimetric Studies: Stock solutions of all of the phospholipids were made in spectral-grade chloroform and stored at -20 °C. The molecular weights used to determine the PC concentrations were based on the elemental analyses (Desert Analytics, Tucson, AZ) that indicated the content of bound water. Binary mixtures of two PCs were prepared by withdrawing the desired volumes from the stock solutions; the chloroform solution containing the PCs was mixed, then the chloroform was removed by using a stream of nitrogen. About 1 mL of spectral-grade benzene was added to the thin film formed on the walls of the centrifuge tube. The solution was frozen and lyophilized, giving a white powder that was suspended in 1 mL of 5 mM sodium phosphate buffer containing 50 mM NaCl solution and 1 mM EDTA, pH 7.4. The total phospholipid concentration was ~ 7 mg per mL of buffer. The aqueous suspension was vortexed, heated to ~ 35 °C (>10 °C above the T_m of pure C18:C10PC), and cooled to 0 °C. This annealing process was repeated 3-4 times to ensure the homogeneous mixing and complete incorporation of lipid components into the liposomes. The lipid dispersions were then incubated for nearly 2 days at 0 °C in an ice-water bath to permit the complete mixing of the PCs in the lipid bilayers.

DSC: DSC traces were recorded on a Hart Scientific Model 707 calorimeter (Provo, Utah) equipped with a Dell 386 computer for data acquisition and analysis. Liposome suspensions (0.6 mL) were loaded at room temperature in separate ampoules which had a capacity of 1 mL each. The fourth ampoule, composed of aluminum metal, was taken as a reference for the instrument calibration, and was used for all the samples throughout the DSC runs. Base-lines were obtained by using buffer solutions which were run in the same

way (rate, temperature range) and in the same ampoules as used for the lipid components. To obtain heat traces of the lipids, the values of the base-line of the buffer were subtracted from the values of the excess heat capacities of the lipid samples. After the samples were loaded at room temperature, the instrument was programmed to cool at a rate of 15 °C/h to -3 °C for **1** and to -8 °C for **2**, then held for 200 sec before the start of data accumulation in the ascending mode at a scan rate of 15 °C/h. For mixtures of **1/3**, the DSC traces were recorded from -3 ° to 50 °C, whereas for mixtures of **2/3** the temperature range was from -8 ° to 35 °C. The samples were again held for 200 sec for thermal equilibration at the high temperature limit (35 ° or 50 ° C) before the descending mode was begun, and data acquisition was carried out at the same scan rate, as in the ascending mode. Ascending and descending modes were repeated for each liposome preparation one or two times to check the reproducibility of the DSC runs. In certain preparations, air bubbles were present in the lipid suspensions; the ampoules were flushed with a stream of helium to displace the air bubbles. The weights of the ampoules with the lipid and the buffer were recorded before and after the DSC runs. If there was a significant difference between the two weights (before and after), the experiment was repeated.

The calorimetric data were analyzed by using software provided by Hart Scientific to yield excess heat capacity as a function of temperature and time. ΔH values were calculated by a program provided by Hart. The instrument was calibrated for ΔH values by employing PC different concentrations (1.2-2.5 mg/mL) of DPPC liposomes. For a single component phospholipid, a concentration of ~ 2.5 mg/mL was required. Occasionally pure component phospholipids were checked by TLC for any decomposition after the DSC run; no changes of R_f values of the PCs were found after the repeated

heating-cooling cycles. All of the DSC data were transferred to a Macintosh II computer, and graphs were made using Edit 2.1, Evolution, and Kaleidagraph programs of Macintosh. The values of T_o and T_c were calculated from the point of first deviation from the base-line by zooming the portion of the curve in the onset and completion modes and then extrapolating the pre- and post-transition slopes to the base-line (Fig. 1). The values of T_o and T_c calculated at different mol % of PCs in binary mixtures were corrected with respect to the finite widths of the pure component PC (Mabrey & Strutevant, 1976). It is to be noted that the finite width of the pure component PCs was taken as their real T_m values and the deviations from T_m with respect to T_o and T_c values were added and subtracted, respectively, for each of the thermograms at different mol % of **3** incorporated into **1** and **2**, and the corrected values were used to construct the phase diagrams shown in Figs. 3 and 5.

Results and Discussion

C18:C10PC/C18:C(10:ω-CH_3CO_2)PC (3/1) *Liposomes:* Excess heat capacity profiles of multibilayer dispersions of binary mixtures of C18:C10PC/ester-PC are shown in Fig. 2. Liposomes prepared from pure C18:C10PC show T_m = 20.4 °C and ΔH = 8.7 kcal/mol, which are similar to the values for C18:C10PC by Mason et al. (1981), McIntosh et al. (1984), Xu and Huang (1987), Mattai et al. (1987), and Mason (1988). The T_m and ΔH of **1** are 7.5 °C and 7.0 kcal/mol, respectively. The endotherms of both **1** and **3** are sharp and symmetric (Fig. 2). For binary mixtures in the composition range ~30-70% of **3** in **1**, the DSC profiles are broad and asymmetric, whereas the endotherms obtained with ~10-20% and ~80-90% of **3** incorporated into **1**, are relatively broad compared with the pure phospholipids (Fig. 1). T_o values define the solidus line; at and below this line the bilayer formed by the binary lipid components coexists as a single gel phase. T_c values define the fluidus

line; at and above this line the bilayer is composed of a liquid phase. The area under these two lines defines the coexistence region, where the bilayer consists of solid and fluid domains formed in the plane of the bilayer (Shimshick and McConnell, 1973).

The non-ideal mixing of **3/1** binary mixtures at different compositions is shown by the phase diagram (Fig. 3). Over a composition range of ~10-60 mol % of **3** in **1**, a region of isothermal melting is observed at ~7.5 °C, suggesting that a region of three phases exists: the solid phases of **3**, of **1**, and of a solid solution of each lipid component. The isothermal temperature (~7.5 °C) is almost the same as the main phase transition temperature (T_m = 7.5 °C) for **1**, indicating that at 7.5 ° C or above the binary mixtures of **3** in **1** exhibit the melting of the solid solution. This phenomenon is a characteristic of a system exhibiting partial immiscibility of the binary lipid components (Mason, 1988). For the composition range ~80-90%, the solid solutions are partially miscible at 10 °C or above. The liquidus line, on the other hand, at all compositions of **3** in **1** investigated, is smooth, suggesting that both components in the binary mixtures are miscible at temperatures above T_m.

C18:C10PC/C18:(C10:ω-CH$_3$CO)PC (3/2) Liposomes: Excess heat capacity profiles for the binary mixtures of C18:C10PC/methyl ketone-PC at different compositions are shown in Fig. 4. Pure **2** exhibits a single and sharp phase transition temperature, T_m = 0 °C and ΔH = 15.7 kcal/mol. The DSC thermograms recorded in composition range ~15-80% of **3** incorporated in **2** are broad and asymmetric. The lower boundary temperatures (T_o values) connected for all compositions, form an irregular solidus line, indicating a solid phase immiscibility at low temperatures. The upper boundary line constructed by connecting the completion temperatures (T_c values) at all compositions of **3**

in **2** shows a smooth curve, suggesting that both the lipids **2** and **3** are miscible in the liquid-crystalline phase at temperatures above T_m.

The phase diagram constructed for the **3/2** binary mixture from the T_o and T_c values is shown in Fig. 5. Again, like **3/1**, this binary system exhibits a nonideal mixing behavior. Over the composition range of ~10-50 mol% of **3** incorporated in **2**, a region of isothermal melting is observed nearly at 0 °C, the transition temperature of **2**. Again, this is indicative of a region of the three-phase coexistence. Over the composition range of ~60-80% of **3** in **2**, the DSC thermograms are asymmetric and relatively sharp compared with the previous ones at ~10-50%. This observation sugggests that the solid solutions are partially immiscible in the gel state, a behavior that is seen for **3/1** lipid mixtures. The liquidus line, on the other hand, is smooth at all compositions, suggesting that the both the lipids are miscible in the liquid-crystalline phase at temperatures above T_m.

Recently, it has been observed that when **3** is mixed with symmetric-chain PCs (e.g. DSPC or DMPC) solid phase immiscibility is found at low temperatures and it forms separate domains within the bilayers of DSPC and DMPC (Mason, 1988; Lin and Huang, 1988). This change in the chain length asymmetry has an implication on the mixing behavior of two lipids in the binary system. Curatolo et al. (1985) studied 21 pairs of PCs by DSC and suggested that if the difference in ΔT_m is less than 33 °C for a pair of lipids, both the PCs are miscible in the gel state, and if the difference in ΔT_m is greater than 33 °C, both the lipids are immiscible at low temperatures. However, this rule does not apply in very highly asymmetric-chain PCs (Ali et al., 1989). It appears also from our DSC results for **3/1**and **3/2** that although the ΔT_m values are 12.9 ° and 20.4 °C for the two binary systems, respectively, both PCs are partially immiscible at low temperatures, below T_m, and completely miscible at

all compositions in the liquid-crystalline state, above T_m.

Conclusions

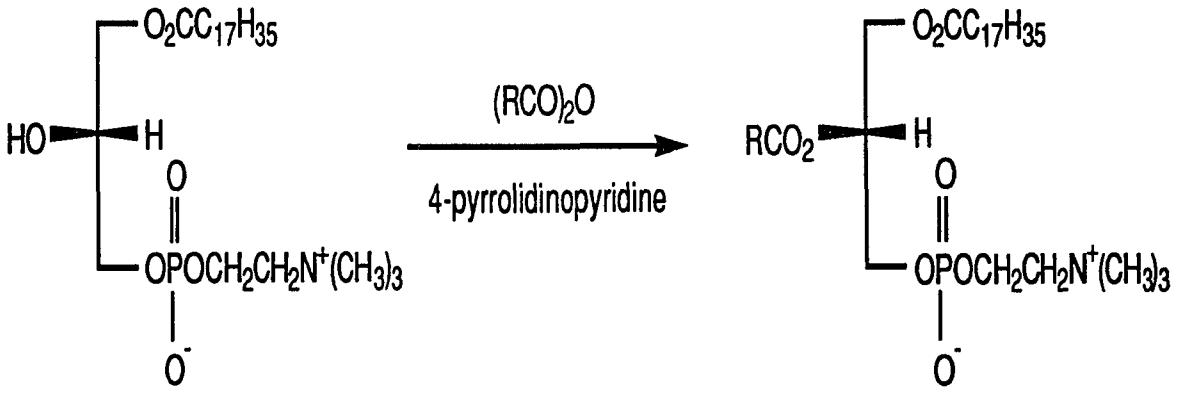
From the calorimetric studies of the binary mixtures of **3/1** and **3/2**, we the following conclusions are made:

1. The solid solutions of ~10-60% and ~10-50% of **3** in **1** and **2**, respectively, are apparently immiscible.

2. Since C18:C(10:1Δ^{10})PC, which has a ω-CH_2=CH moiety, is miscible in **3** at all compositions, indicated by an ideal mixing behavior, it is suggested that the mixed interdigitated bilayers are formed at low temperatures within the bilayers of **3**; the ω-CH_2=CH moiety has no perturbing effect on the packing of hydrocarbon chains (see Chapter 6, p.106; Ali et al., 1989). **1** and **2** form multilamellar bilayers, but appear not to exist as mixed interdigitated bilayers at low temperatures in excess water, unlike **3** and C18:C(10:1Δ^{10})PC, suggesting that the presence of a ω-CH_3CO_2 or a ω-CH_3CO moiety at the end of the *sn*-2 acyl chain indeed perturb significantly the packing of the hydrocarbon chains in the gel state.

3. In the liquid-crystalline state, **1** and **2** both are miscible at temperatures above T_m, which indicates that there is no constraint in the mixing of these lipid components in **3**.

4. **1** and **2** both form separate domains in the plane of the bilayers with **3** in a wide composition range at temperature below T_m. Domain formation in the gel state is most presumably due to a strong attractive force (caused by polarity of the oxygen atom(s) at the end of the *sn*-2 acyl chain) in the hydrocarbon chains of **1** and **2** compared to **3**.



1 $R=CH_3CO_2(CH_2)_8$
2 $R=CH_3CO(CH_2)_7$
3 $R=CH_3(CH_2)_9$

Scheme I: Reaction sequence for the synthesis of PCs (**1-3**).

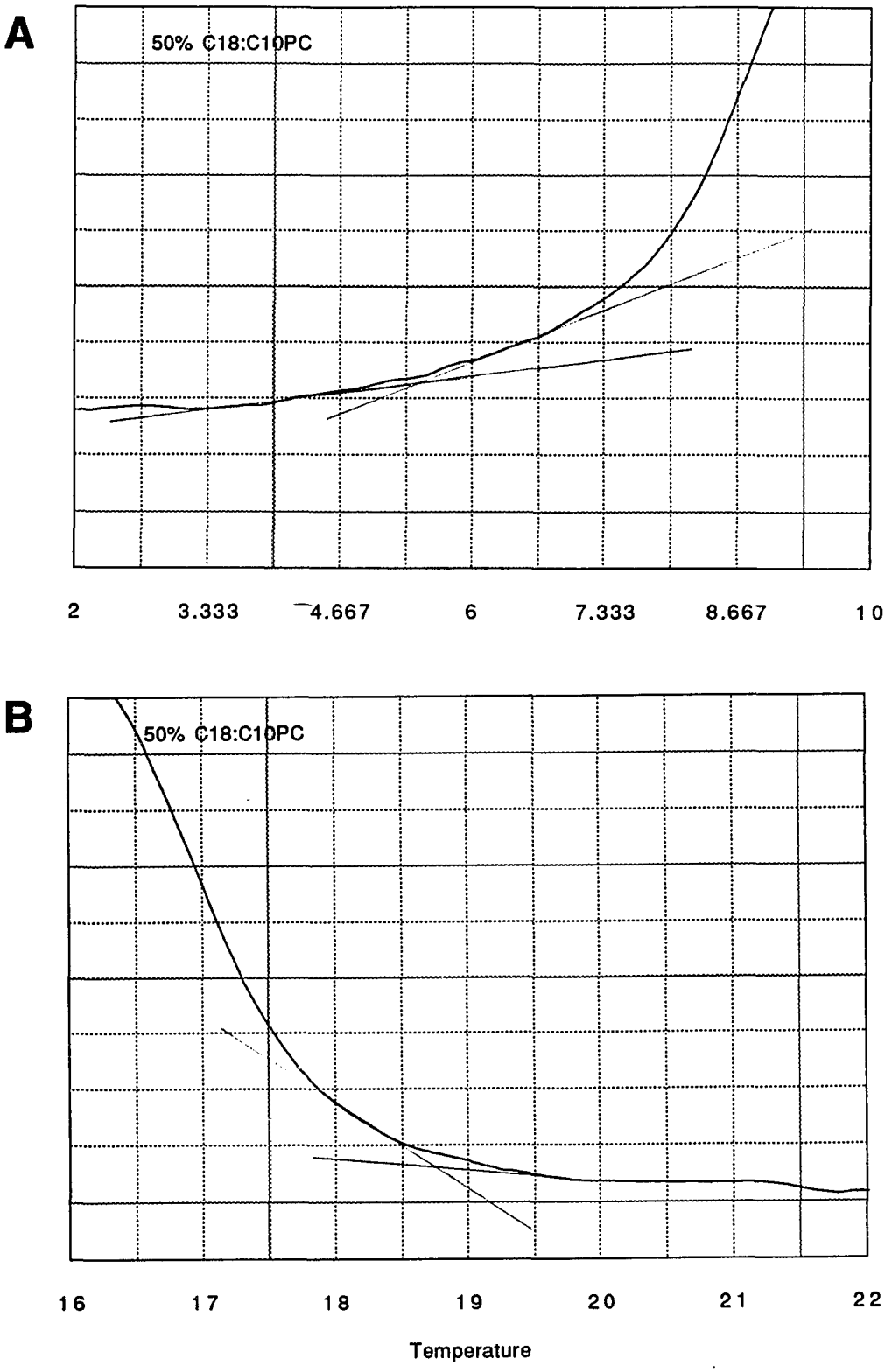


Fig. 1: The values of T_o (**A**) and T_c (**B**) calculated by the extrapolation procedure of Mabrey and Sturtevant (1976); T_o and T_c values are corrected further for the finite widths of pure component PCs.

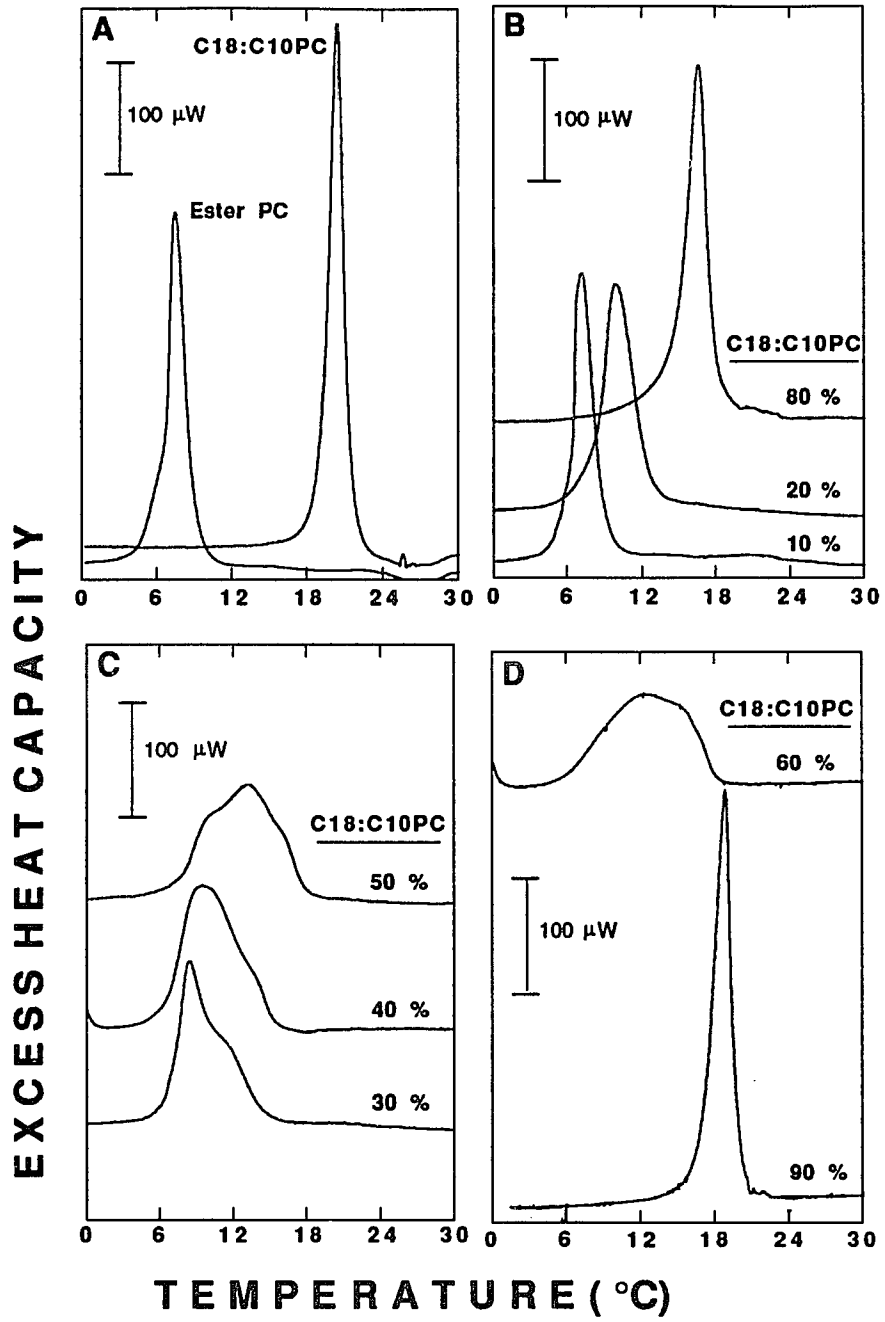


Fig. 2: Thermograms obtained with C18:C10PC/C18:C(10:ω-CH_3CO_2)PC multilamellar bilayers.

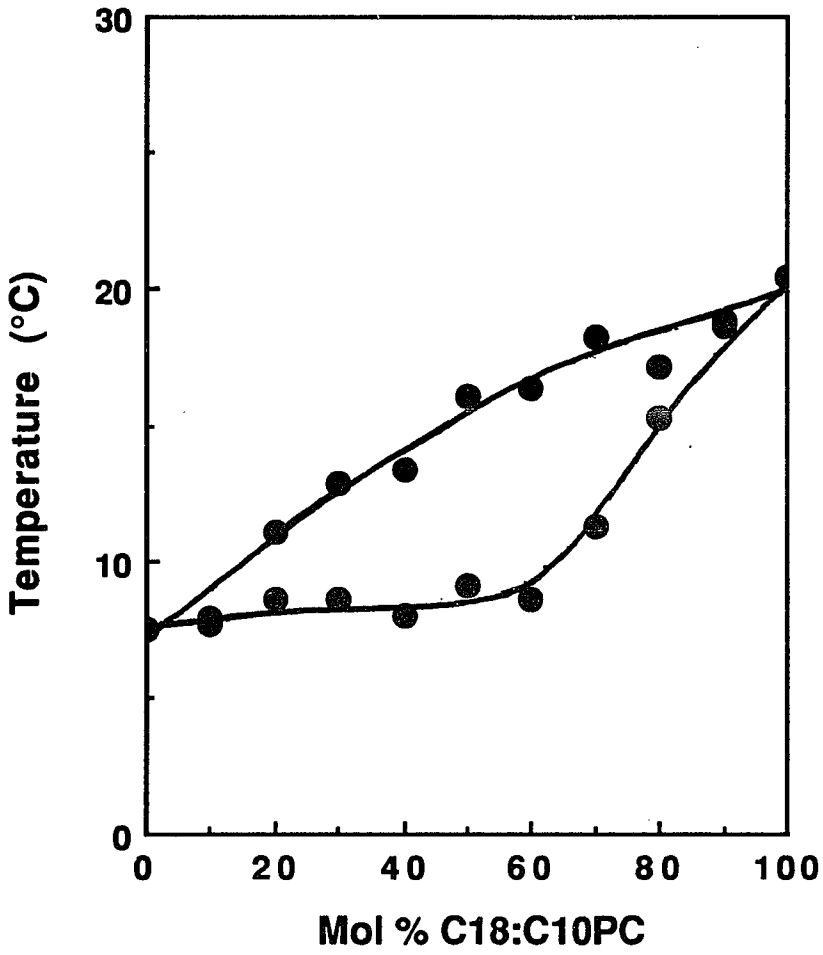


Fig. 3: Phase diagram for the C18:C10PC/C18:C(10:ω-CH_3CO_2)PC binary system; T_o and T_c values are corrected (Mabrey and Sturtevant, 1976).

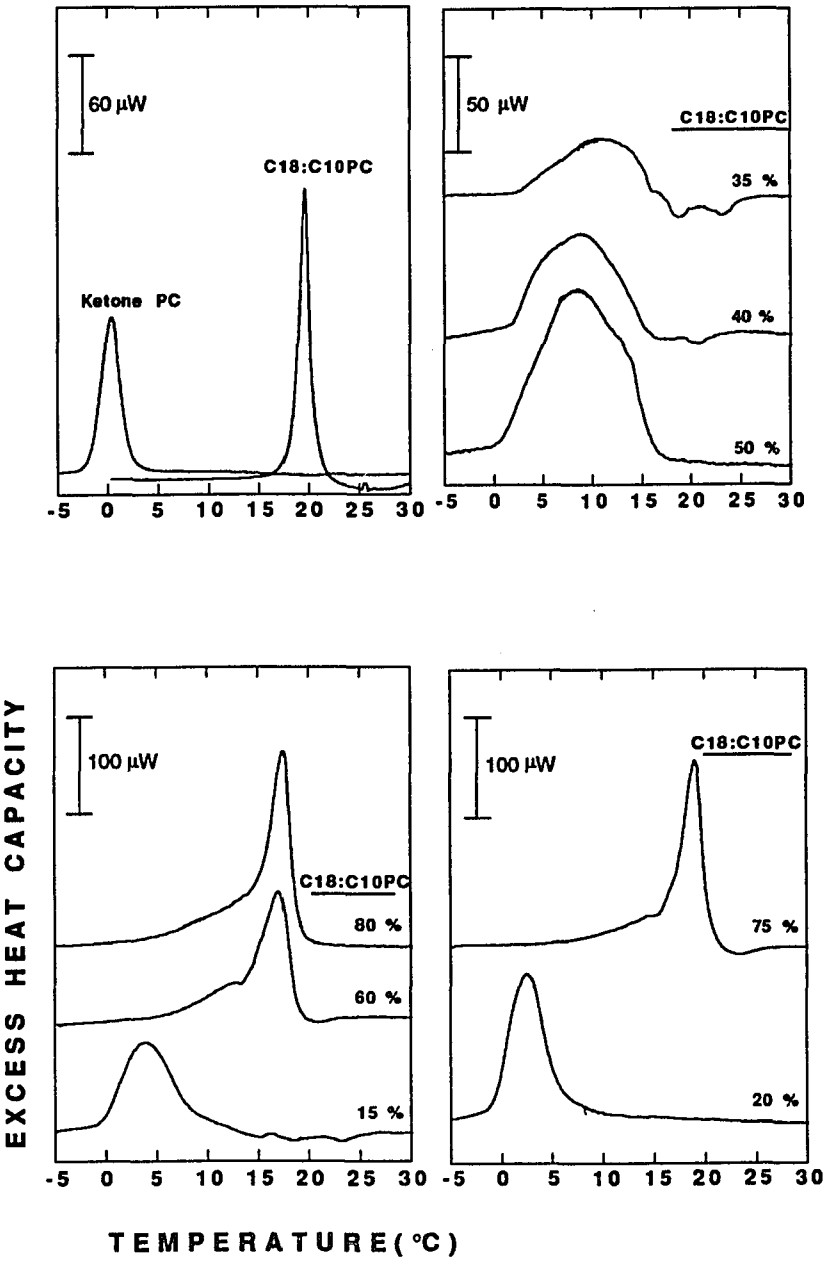


Fig. 4: Thermograms obtained with C18:C10PC/C18:C(10:ω-CH_3CO)PC multilamellar bilayers.

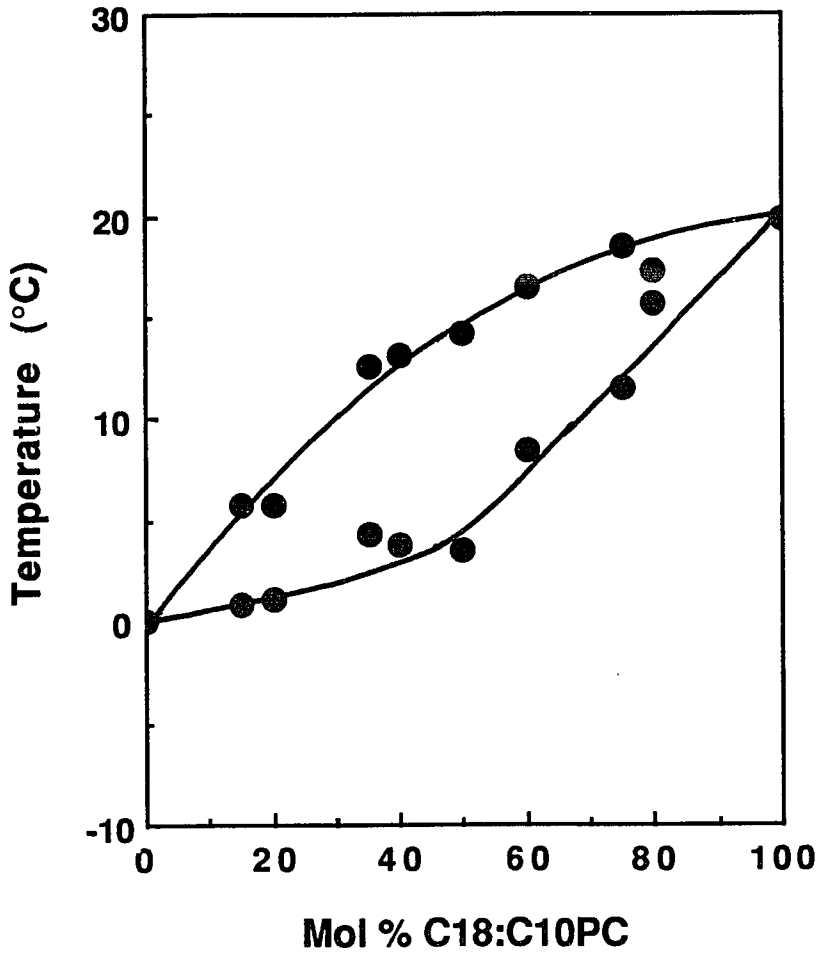


Fig. 5: Phase diagram for the C18:C10PC/C18:C(10:ω-CH_3CO)PC binary system; T_o and T_c values are corrected (Mabrey and Sturtevant, 1976).

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