nitrotoluene, 121-86-8; 2,3,5,6-tetrachloro-4-nitrotoluene, 22490-21-7; pentachloronitrobenzene, 82-68-8; dimethyl tetrachlorophthalate, 20098-41-3; *p*-chlorotoluene, 106-43-4; methyl *p*-toluate, 99-75-2; *p*-(trifluoromethyl)toluene, 6140-17-6; α,α-dichloro-*p*-nitrotoluene, 619-78-3; 1-(dichloromethyl)-4-(trifluoromethyl)benzene, 82510-98-3; p,α,α-trichlorotoluene, 13940-94-8; toluene, 108-88-3; *o*-chlorotoluene, 95-49-8.

Phospholipids Chiral at Phosphorus. 2. Preparation, Property, and Application of Chiral Thiophospholipids[1]

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Recently we have reported the synthesis of chiral [$^{18}O_1$]1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) and its application in studying the stereochemistry of transphosphatidylation catalyzed by phospholipase D.[2] By similar procedures, we have synthesized chiral [$^{17}O_1$]DPPC (as separate diastereomers and mixture), which are potentially useful in the chemical and physical study of the conformation and the motion of the phosphate head group of phospholipids in different phases and in protein–lipid interactions.[3-5]

However, [$^{18}O_1$]DPPC is useful only for phospholipases C and D and not for other phospholipases. Also, the ^{17}O NMR study of [$^{17}O_1$]DPPC is difficult in vesicles or lipid bilayers[7] due to large line widths and quadrupolar splittings. As an alternative and complementary approach, we now report synthesis and application of separate diastereomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylcholine (DPPsC).

The diastereomeric mixture of thiophospholipids (random configuration at both C-2 and P) has been synthesized recently[8] but has not been resolved into separate diastereomers either chemically or spectroscopically. Following the procedure of Nifant'ev et al.,[8a] we have synthesized DPPsC (**1**) from (*S*)-(−)-1,2-dipalmitin (synthesized from D-mannitol[9]). The structures of synthetic intermediates and the final DPPsC have been characterized by ^1H NMR, ^{13}C NMR, IR, and TLC, which are consistent with literature data.[8] ^{31}P NMR analysis of **1** in $CDCl_3$ (Figure 1A) showed two separate peaks due to two diastereomers (isomer A, lower field; isomer B, higher field). It was found that phospholipase A_2 (bee venom, Sigma, 1500 units/mg) preferentially hydrolyzes isomer B of DPPsC, which provides a convenient way to separate the two diastereomers, as outlined in Scheme I. Hydrolysis of DPPsC(A + B) in a mixture of 0.1 M Tris buffer

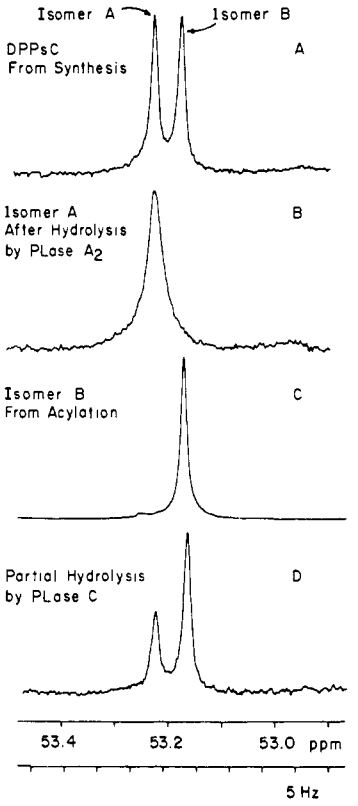
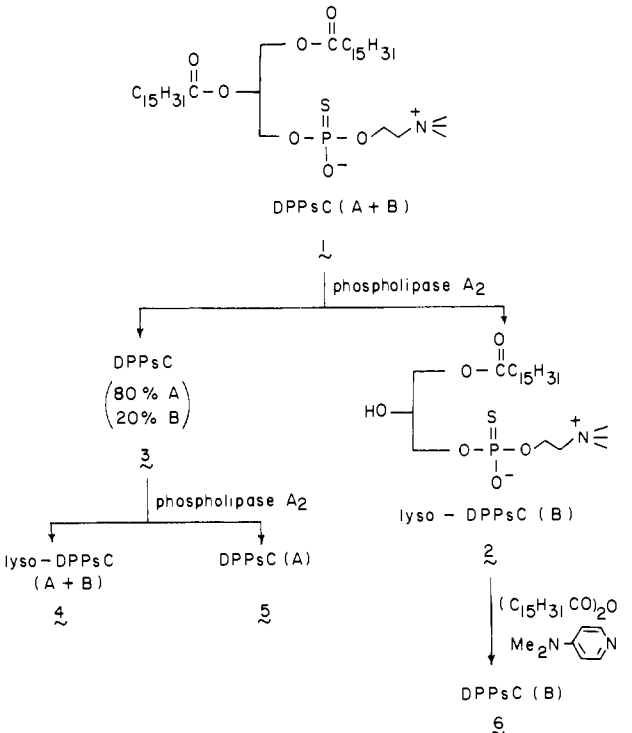


Figure 1. ^{31}P NMR spectra (81.0 MHz) of DPPsC (10 mM in $CDCl_3$): (A) mixture of diastereomers from chemical synthesis; (B) pure isomer A recovered from hydrolysis by phospholipase A_2; (C) pure isomer B (containing 3% isomer A) obtained from acylation of the product of phospholipase A_2 hydrolysis, lyso-DPPsC; (D) DPPsC after partial hydrolysis by phospholipase C. NMR parameters: spectral width 1000 Hz; acquisition time 4.1 s; ^1H decoupling; line broadening 0.1 Hz; pulse width 12 μs (90° pulse at 20 μs); number of scans 500 (A, D), 1000 (B), 2600 (C); temperature 30 °C. Chemical shifts are referenced to external 1 M H_3PO_4, with + indicating a downfield shift.

Scheme 1. Separation of Diastereomers of DPPsC



(1) Supported by a grant from NIH (GM30327) and in part by a grant from NSF (PCM 8140443). The NMR spectrometers used were funded by NIH GM 27431 and NSF CHE 7910019. The ^{14}N NMR was obtained with the help of Dr. C. Cottrell. Abbreviations used: NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylcholine; MOPS, 3-[*N*-morpholino]propanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Pi, inorganic phosphate; IR, infrared spectrum; TLC, thin-layer chromatography.

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(4) The ^{17}O NMR signal of [$^{17}O_1$]DPPC broadens from 440 Hz in CH_3OD (100 mg in 1.5 ml) to 3250 Hz in $CDCl_3$ (at 33 °C) (ref 5) as a result of the micelle formation in chloroform (ref 6). This can best be explained by a 7.4-fold increase in the rotational correlation time τ_r of phosphoryl oxygens, which suggests a restriction in the motion of the phosphate group upon aggregation, which is consistent with the recent finding in the restricted motion of the choline side chain due to aggregation (ref 6a).

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(7) We have so far not been able to observe any ^{17}O NMR signal of a sonicated or unsonicated dispersion of [$^{17}O_1$]DPPC in water. A possible solution would be to prepare oriented lipid bilayers.

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Table I. Summary of ^{31}P Chemical Shifts (81.0 MHz) for Thiophospholipids at 30 °C[a]

compd	$CDCl_3$ (10 mM)	CH_3OD (50 mM)	0.1 M tris/ Triton X (10 mM, pH 8.0)	0.1 M Pi/ deoxy-cholate (10 mM, pH 7.5)
DPPsC(A)	53.24	59.686	56.787	56.769
DPPsC(B)	53.19	59.665	56.859	56.843
DPPC	−2.70	0.96	−0.72	−0.52
lyso-DPPsC(A)			56.615	
lyso-DPPsC(B)			56.558	

[a] Chemical shifts are referenced to external 1 M H_3PO_4, with + indicating a downfield shift.

(pH 8.0, 20% D_2O) and Triton X-100 by phospholipase A_2[10] was followed by ^{31}P NMR. Under this condition both DPPsC and its product lyso-DPPsC give separate ^{31}P NMR signals for the two diastereomers (their chemical shifts are listed in Table I). When ca. 80% of isomer B of DPPsC (which resonates at *lower* field than isomer A in H_2O/triton X) had been hydrolyzed, only one isomer of lyso-DPPsC formed (this isomer, which comes from the isomer B of DPPsC, is designated as the isomer B of lyso-DPPsC). The reaction was stopped and the reaction mixture was chromatographed on silica gel (30 μm particles size) to give lyso-DPPsC (B) (**2**) and unreacted DPPsC (**3**, which contains ca. 80% isomer A and 20% isomer B). Further hydrolysis of **3** by the above procedure gave lyso-DPPsC (**4**) mixture of A and B; chemical shifts shown in Table I) and the pure diastereomer DPPsC(A) (**5**). Reacylation[11] of **2** gave the other pure diastereomer, DPPsC(B) (**6**). ^{31}P NMR analysis of **5** and **6** in $CDCl_3$ (Figure 1, B and C, respectively) showed an isomeric purity of >99% for **5** and 97% for **6**. Experiments are in progress in our laboratory to identify a phospholipase A_2 (from different sources) that is most stereospecific and to modify the reaction condition such that the chromatographic work can be minimized.

As the separate diastereomers of the sulfur analogues of adenine nucleotides had been used as a stereochemical probe to solve many important problems before their absolute configurations at phosphorus were determined,[12] the DPPsC isomers should be useful both in phospholipid enzymology and in probing the interaction between enzymes and phospholipids even before the absolute configuration of isomers A and B can be assigned. For example, we have found that phospholipase C (from *Bacillus cereus*, Sigma, 400–600 units/mg) is specific to the isomer A of DPPsC.[13] Figure 1D shows the ^{31}P NMR spectrum of a DPPsC sample after partial hydrolysis by phospholipase C.[13] This sample, which contains a ca. 2:1 isomer B/isomer A mixture, was used to assign ^{31}P chemical shifts (Table I) and ^{13}C chemical shifts (Table II). The relative ^{31}P chemical shifts of the two isomers show a large dependence on solvents. As expected, the ^{31}P chemical shifts of DPPsC are ca. 55–60 ppm downfield from that of DPPC, which makes it possible to monitor the property of a particular phospholipid in a mixture of phospholipids in different phases.[8b]

The requirement of a specific configuration at phosphorus in the phospholipase C catalysis is expectable since the reaction involves a P–O bond cleavage. However, the stereospecificity observed for phospholipase A_2 is suprising since it hydrolyzes the C-2 ester but not the phospho diester, and it can tolerate substitution of the choline side chain by other groups.[14]

Table II. ^{13}C Chemical Shifts of DPPsC and DPPC in CD_3OD[a]

	DPPsC(A)	DPPsC(B)	DPPC
choline chain			
Me_3N	54.93	54.93	54.80
$^1J_{CN}$	3.4	3.4	3.7
CH_2N	67.46	67.46	67.62
$^1J_{CN}$	2.7	2.7	3.3
$^3J_{CP}$	8.1	8.1	6.4
CH_2OP	60.81	60.81	60.47
$^2J_{CP}$	5.4	5.4	5.0
glycerol backbone			
1-CH_2O	63.80	63.80	63.77
2-CHO	71.88	71.66	71.93
$^3J_{CP}$	8.2	8.1	8.0
3-CH_2OP	65.13	65.05	64.91
$^2J_{CP}$	4.1	4.1	5.0
carbonyl			
C=O	174.9	174.9	175.2
	174.6	174.6	174.9

[a] Obtained at 50.3 MHz on WP-200 at 30 °C. Samples are 0.1 M in CD_3OD. Chemical shifts are referenced to Me_4Si. Coupling constants are in Hz.

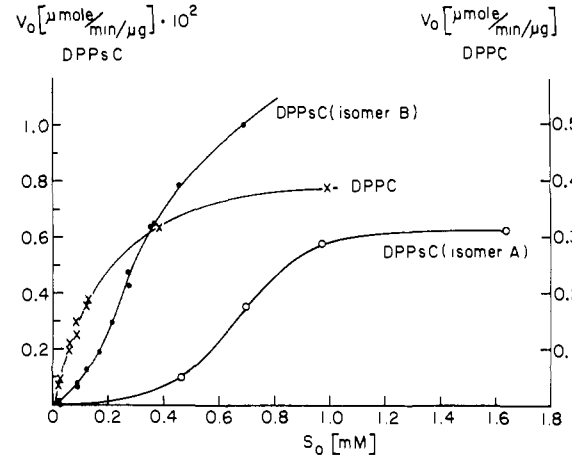

Figure 2. Plots of initial velocity vs. substrate concentration for DPPC (right vertical scale) and DPPsC (A) and DPPsC (B) (left vertical scale). The initial velocity was measured spectrophotometrically as described by Kupferberg et al. (ref 19). Substrates were sonicated in buffer at 50 °C and the rates measured at 37 °C. Buffer condition: 0.2 mM MOPS, 0.1 mM *p*-nitrophenol, 0.1 mM EDTA, 5 mM $CaCl_2$, 0.1% Triton X-100, pH 7.23 (measured at 23 °C).

The mechanism of phospholipase A_2 (from various sources) has been investigated extensively,[15–20] particularly in the role of Ca^{2+} in catalysis,[15] the existence of an activator site and a catalytic site,[16] the possible cooperative interactions between the two protomers,[17] and the interfacial interaction between the enzyme and the phospholipid.[18,19] A direct binding of Ca^{2+} with the phosphate group has been suggested,[15,21] but no direct evidence has been provided. The following results show that separate diastereomers of DPPsC are useful in probing the mechanism of phospholipase A_2. ^{31}P NMR study indicated that in addition to the bee venom

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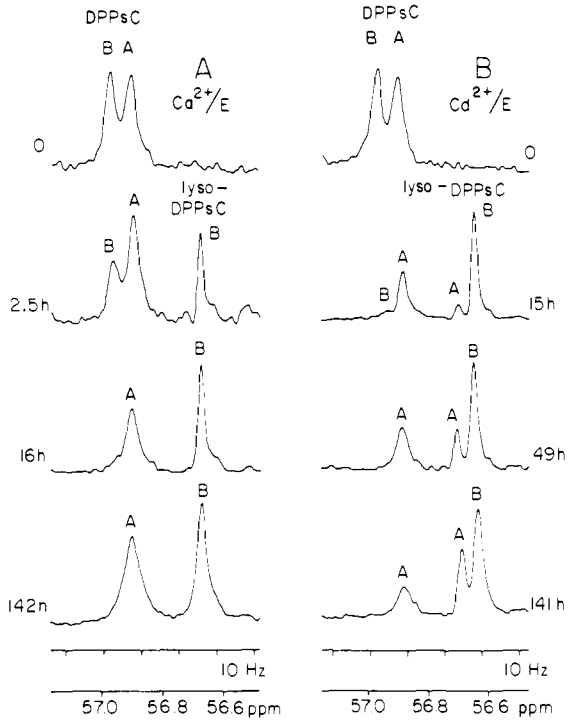

Figure 3. Hydrolysis of DPPsC(A + B) by bee venom phospholipase A_2 followed by ^{31}P NMR (81.0 MHz). Sample conditions: 20 μmol of DPPsC(A + B) in 2 mL of buffer containing 50 mM MOPS·Na, pH 7.2; 0.25 mM EDTA, 35% D_2O, 5% Triton X-100, and (A) 2.5 mM puratronic grade $Ca(NO_3)_2$ and 3.8 μg of enzyme, (B) 2.5 mM puratronic grade $Cd(NO_3)_2$ and 350 μg of enzyme. NMR parameters are same as in Figure 1 except for the number of scans. The probe temperature was set at 37 °C and samples were incubated at 37 °C. A small Gaussian multiplication (LB = −1, GB = 0.05) was applied (which did not effect relative intensities greatly) to resolve overlapped peaks.

enzyme, the phospholipases A_2 from *Naja naja* venom (Sigma, 200–600 units/mg) and from porcine pancreas (Sigma, 600 units/mg) also specifically hydrolyze the isomer B of DPPsC. Figure 2 shows the plot of initial velocity vs. substrate concentrations for DPPC, DPPsC(A), and DPPsC(B) in the reaction of bee venom phospholipase A_2 by use of the spectrophotometric assay of Kupferberg et al.[19] The results indicate that the reaction rates of DPPsC isomers are considerably slower (<10%) than that of DPPC, which is consistent with the properties of the sulfur analogues of adenine nucleotides.[12] In addition, the DPPsC isomers show "sigmoidal" curves which may support either a cooperative interaction between protomers[17] or the presence of an allosteric site and a catalytic site, if the effect of substrate concentration in mixed micelles (DPPsC/triton X-100 as described in the legend of Figure 2) is the same as that in normal homogeneous catalysis.

The observed stereospecificity of phospholipase A_2 in the experiments described above suggests that the phosphate group is involved in binding, most likely with Ca^{2+}. To further support this, we have investigated the metal–ion dependence in stereospecificity. Figure 3 shows ^{31}P NMR spectra of the reaction mixture at different time intervals for the hydrolysis of DPPsC(A + B) at a saturating concentration (10 mM) catalyzed by the bee venom phospholipase A_2 in the presence of Ca^{2+} (Figure 3A) and Cd^{2+} (Figure 3B).[22] In Figure 3A, approximately 50% of DPPsC(B) was hydrolyzed at 2.5 h, but no hydrolysis of DPPsC(A) can be detected up to 142 h. In Figure 3B (in which 92 times as much enzyme was used since Cd^{2+} substitution caused a substantial decrease in enzyme activity) an additional peak (0.057 ppm downfield from the isomer B of lyso-DPPsC) apeared even before DPPsC(B) is completely hydrolyzed (at 15 h). After 49 h it was quite obvious that this new peak was from the hydrolysis of DPPsC(A). On the basis of the work of Jaffe and Cohn,[23] the decreased stereospecificity of phospholipase A_2 toward isomer B in the presence of Cd^{2+} is a positive evidence for a direct coordination between the divalent metal ion and the phosphate group.

Proton-decoupled ^{31}P NMR (81.0 MHz) of an aqueous dispersion of DPPsC(A + B) at 45 °C shows a line shape characteristic of lipid bilayers, with a chemical shift anisotropy $\Delta\sigma$ = 32 ppm (compared to 47 ppm for DPPC under the same condition). ^{14}N NMR (21.7 MHz) of the same sample shows two quadrupolar splittings, $\Delta\nu_Q$ = 7.43 and 7.60 KHz, possibly due to the two diastereomers[24] (compared to 10.26 KHz for DPPC). When such NMR techniques (and ^2H NMR for ^2H-labeled DPPsC) and other physical methods are applied to study the interaction of *separate isomers* of DPPsC with other membrane components, valuable information concerning the biological role of the phosphate head group in membrane functions should be generated.

Registry No. 1, isomer 1, 82482-77-7; **1**, isomer 2, 82482-78-8; **2**, isomer 1, 82482-79-9; **2**, isomer 2, 82482-80-2.

(22) Although phospholipase A_2 is highly specific to Ca^{2+}, we have been able to remove Ca^{2+} by extensive dialysis against MOPS buffer (which resulted in complete deactivation) and reactivate the enzyme with Ca^{2+} or Cd^{2+}. The Cd^{2+} enzyme is only 1/120 as active as the Ca^{2+} enzyme based on the spectrophotometric assay (ref 19) at 1 mM DPPC. In Figure 3, the enzyme used was first dialyzed to remove Ca^{2+} and then added to the two reaction mixtures containing Ca^{2+} and Cd^{2+}. The activity of the enzyme (in units) was always assayed at 1 mM DPPC by the procedure of Figure 2.

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(24) An alternative but less likely interpretation is that there are two doublets with $\Delta\nu_Q$ = 7.52 KHz but separated by 85 Hz (3.9 ppm). The reduced $\Delta\nu_Q$ in DPPsC (relative to DPPC) suggests that the motion of the choline side chain is less ordered in DPPsC.

Dibenzyltetrakis(dimethylamido)dimolybdenum and -ditungsten (M≡M) Compounds and Their Reactions with Carbon Dioxide and 1,3-Diaryltriazenes. A Radical Difference

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Both molybdenum and tungsten have a rich dinuclear chemistry that in many ways, but by no means all ways, is very similar.[1] The search and ultimate discovery of a successful route to a tungsten $M_2(O_2CR)_4$ ($M\overset{4}{-}M$) compound is an interesting story[2] and reveals what is now generally accepted: the $(W\overset{4}{-}W)^{4+}$ unit is notably more reactive toward oxidative–addition reactions than the $(Mo\overset{4}{-}Mo)^{4+}$ unit. McCarley's[3] spectacular success in preparing pure $MoW(O_2C\text{-}t\text{-}Bu)_4$ ($M\overset{4}{-}M$) used this principle. We report here an intriguing difference between reactions involving $(M\equiv M)^{6+}$ units (M = Mo and W)[4] that reveals the comple-

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