Phospholipids Chiral at Phosphorus. Preparation and Spectral Properties of Chiral Thiophospholipids†

Karol Bruzik, Ru-Tai Jiang, and Ming-Daw Tsai*

ABSTRACT: The thiophospholipid 1,2-dipalmitoyl-sn-glycerol-3-thiophosphocholine (DPPsC) was shown to be a mixture of two diastereomers by $^{31}$P nuclear magnetic resonance. The isomer that resonates at the lower field in CDCl$_3$ (56.12 ppm) was designated as isomer A and the other (resonates at 56.07 ppm) as isomer B. Phospholipase A$_2$ from four different sources (bee venom, Naja naja venom, Crotalus adamanteus venom, and porcine pancreas) was shown to hydrolyze the isomer B of DPPsC specifically, whereas phospholipase C from two different sources (Bacillus cereus and Clostridium perfringens) hydrolyzes isomer A specifically. So that the two diastereomers could be separated, DPPsC(A+B) was first digested with phospholipase A$_2$ to give 1-palmitoyl-sn-glycero-3-thiophosphocholine (MPPsC) (which is designated as isomer B of MPPsC) and the unreacted DPPsC(A). Recyclation of MPPsC(B) gave pure DPPsC(B). The properties of DPPsC(A) and DPPsC(B) were investigated by $^{31}$P, $^{13}$C, $^1$H, and $^{14}$N nuclear magnetic resonance (NMR). $^1$H and $^{13}$C NMR showed that both isomers in methanol solution have conformational properties similar to those of the natural phospholipid, 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine. On the other hand, the two isomers A and B showed small but significant differences in the chemical shifts of the carbon in the chiral carbon center and the phosphorus in the chiral phosphorus center.

During the past 5 years, various chirally labeled nucleotides (in which P-O bonds are substituted by P-$^{17}$O, P-$^{18}$O, or P-S) have been synthesized and widely used in mechanistic studies of enzyme-catalyzed reactions (Buchwald et al., 1982; Cleland, 1982; Cohn, 1982; Eckstein, 1979; Eckstein et al., 1982; Frey, 1982; Frey et al., 1982; Knowles, 1980; Tsai, 1982; Tsai & Bruzik, 1983; Villafranca & Rauschel, 1980; Webb, 1982). However, an important class of biophosphates, the phospholipids, has been ignored. We have therefore initiated the stereochemical study of phospholipids, aiming at probing the mechanism of phospholipase-catalyzed reactions and the roles of the phosphate head group of phospholipids in protein-lipid interactions and in other membrane functions. In preliminary papers (Bruzik & Tsai, 1982; Bruzik et al., 1982; Tsai et al., 1982), we have reported synthesis of phospholipids chirally labeled with $^{18}$O and chiral thiophospholipids and use of these compounds to elucidate the stereochemical course of transphosphatidylolation catalyzed by phospholipase D and the stereospecificity of phospholipases A$_2$ and C toward the two diastereomers of DPPsC.† In this paper, we report detailed

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study in the synthesis and spectral properties of the two diastereomers of DPPsC and the stereospecificity of phospholipases A₂ and C toward the two diastereomers of DPPsC.

The synthesis of thiophospholipids was first reported by Nifant'ev et al. (1978). However, the compounds were not separated into diastereomers and have not been used in biochemical study. While our work was in progress, Vasilenko et al. (1982) reported an alternative synthesis of thiophospholipids and its application in ⁴³P NMR studies of lipid polymorphism. Since the compounds used were also diastereomeric mixtures, the validity of their results remains to be reestablished by use of pure diastereomers. Before the completion of this paper in our laboratory, Orr et al. (1982) also reported synthesis of DPPSe and stereospecific hydrolysis of one of the two diastereomers by phospholipases A₂ (bee venom) and C (Bacillus cereus). However, the separate diastereomers of DPPSe and the products of hydrolysis were neither isolated nor characterized. Our initial reports (Bruzik et al., 1982; Tsai et al., 1982) and the present paper represent the first complete study in the preparation and properties of chiral thiophospholipids.

Materials and Methods

Materials. The sources of enzymes and chromatographic products are described in later sections. Puratronic grade Ca(NO₃)₂ and NaOH were purchased from Ventron Co. Chelex-100 was obtained from Bio-Rad. DPPC, MPPC, Triton X-100, sodium deoxycholate, and other biochemicals were obtained from Sigma. General chemicals were of reagent grade or highest purity available.

Enzymes. The phospholipase A₂ used in the separation of diastereomers of DPPsC was purified from bee venom (Sigma, grade IV, lyophilized whole venom) according to the procedure of Cottrell and co-workers (Cottrell, 1981; Shipolini et al., 1971). The activity of the enzyme was assayed by a modified procedure of Kupferberg et al. (1981), which monitored the evolution of acid by a p-nitrophenol indicator, as described below. The stock buffer contained 0.2 mM Mops-Na, 0.1 mM p-nitrophenol, 5 mM CaCl₂, 0.1 mM EDTA, and 0.1% (w/v) Triton X-100, pH 7.23 (measured at 23 °C). The solution of DPPC was prepared fresh daily by sonicating 10 μmol of DPPC in 5 mL of the buffer for 10 min in a 50 °C water bath. The DPPC solution was kept at 37 °C right after sonication, while the stock buffer was preequilibrated at 37 °C before use, both under an atmosphere of argon. The enzyme solution, if necessary, was also diluted in the stock buffer (to a concentration of ca. 1-2 μg/mL) and preequilibrated at 37 °C under Ar. The cuvette (1 cm × 3 cm) was capped with a rubber septum and flushed with dry argon through needles. To start the reaction, 1 mL of each the stock buffer and the DPPC solution and 10 μL of the enzyme solution were transferred to the cuvette by syringes. After quick mixing by use of a micromagnetic stirring bar followed by transferring the cuvette to the cell holder (preequilibrated at 37 °C), the decrease in the absorbance at 400 nm was recorded. On the basis of calibration by back-titration of reaction mixtures with standardized KOH, a decrease of 0.01 in absorbance corre-

1 Abbreviations: DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPSe, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phospho-[choline; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPSe, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPsC, 1,2-

Scheme I: Synthesis of DPPsC(A+B) from Mannitol

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<th>Reaction</th>
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<td>1</td>
<td>CH₂OH + HOOC₁₈₂₀₂₃</td>
<td>HOOC₁₈₂₀₂₃</td>
</tr>
<tr>
<td>2</td>
<td>NaOH</td>
<td>NaOH</td>
</tr>
<tr>
<td>3</td>
<td>HOOC₁₈₂₀₂₃ + H₂O</td>
<td>HOOC₁₈₂₀₂₃</td>
</tr>
<tr>
<td>4</td>
<td>H₂O</td>
<td>H₂O</td>
</tr>
<tr>
<td>5</td>
<td>HOOC₁₈₂₀₂₃ + H₂O</td>
<td>HOOC₁₈₂₀₂₃</td>
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In the determination of specific activity, the protein concentration was determined by the procedure of Lowry et al. (1951), and 1 unit represented hydrolysis of 1 μmol of DPPC/min under above conditions. The purified PL A₂ from bee venom had a specific activity of 2000-2500 units/mg of protein.

The following phospholipases used in the study of stereo-specificity (Figures 3–6) were purchased from Sigma: phospholipase A₂ from bee venom (lyophilized powder, 1500 units/mg of protein), from Naja naja venom (lyophilized powder, 200–600 units/mg of protein), from porcine pancreas [suspension in 3.2 M (NH₄)₂SO₄ solution, 600 units/mg of protein], and from Crotalus adamanteus venom (lyophilized powder, 600–1200 units/mg of protein); phospholipase C from Clostridium perfringens [type X, lyophilized powder, 200–300 units/mg of protein] and from Bacillus cereus [type V, suspension in 3.2 M (NH₄)₂SO₄ solution, 400–600 units/mg of protein].

Chromatography. Thin-layer chromatography (TLC) of thiophospholipids was carried out in chloroform/methanol/water (70:30:4 by volume) on precoated plates (0.2 mm, aluminum support, E. Merck silica gel 60 F-254). The Rᵢ values are as follows: DPPsC, 0.4; MPPsC, 0.2; DPPe, 0.5; DPPsC, 0.2; MPPsC, 0.1. The phospholipids and thiophospholipids were visualized by spraying with phosphomolybdic acid (5 g in 100 mL of ethanol) followed by heating.

Column chromatography of thiophospholipids was also performed on silica gel columns (J. T. Baker TLC reagent, <40 μm) under moderate pressure (≥20 psi). A column with 1-in. diameter and 20-in. height is adequate for ca. a 1-g sample. Two solvent systems were used interchangeably. System A (same as the solvent used for TLC) was used in most crude separations. The fatty acid was eluted first, followed
by phospholipids and thiophospholipids in the order of $R_f$ values. System B (chloroform/methanol, 1:1 by volume) gave a slower but better separation and was used when a better purity was desired. Both UV absorption at 254 nm and TLC were used to monitor the position of compounds.

**Synthesis of DPPsC(A+B).** Scheme I summarizes the synthesis of DPPsC(A+B) from d-mannitol, which is a combination of literature procedures with some modification, as described below. d-Mannitol was acetatned (using ZnCl$_2$ as a catalyst) to form 1,2,5,6-diisopropylidene-d-mannitol (Baer, 1945; Jensen & Pitas, 1976). The yield of this step can be significantly improved (to 68%) if (i) both anhydrous ZnCl$_2$ and d-mannitol are dried at 110 °C under vacuum for 8 h immediately before use, (ii) passage of d-mannitol through a 200-mesh sieve is omitted to avoid moisture, and (iii) a molecular sieve (4 A) is used to replace K$_2$CO$_3$ in some drying steps to ensure a better drying of solutions. Treatment of 1,2,5,6-diisopropylidene-d-mannitol with Pb(OAc)$_4$ gave 1,2-isopropylidene-sn-glyceroldehyde (Baer & Fischer, 1945; Jensen & Pitas, 1976), which was then reduced to 1,2-isopropylidene-sn-glycerol by LiAlH$_4$/ether. After protection of the 3-OH group by treatment with Na followed by benzyl bromide, the isopropylidene group was hydrolyzed in 10% CH$_3$COOH/H$_2$O and the resulting benzyl-sn-glycerol was acetylated with palmitoyl chloride (Bird & Chadha, 1966). The resulting 1,2-dipalmitoyl-3-benzyl-sn-glycerol was stock in large quantity and was converted to d(-)-1,2-dipalmitin (1,2-dipalmitoyl-sn-glycerol) by hydrolysis in n-hexane catalyzed by Pd/C just before the next reaction. The overall yield of 1,2-dipalmitin was 23.4% of the theoretical yield from d-mannitol (1 g of mannitol gave 1.46 g of dipalmitin). All of the isolated intermediates and the product were characterized by mp, bp, $^1$H NMR, and $^{13}$C NMR and by comparing the above properties with authentic samples whenever possible.

The synthesis of DPPsC(A+B) from (d) 1,2-dipalmitin was performed first by the procedure of Nifant’ev et al. (1978) (route a in Scheme I) and then by the procedure of Vasilenko et al. (1982) (route b in Scheme I). Both previous reports used (±) 1,2-dipalmitin, which should give a mixture of four stereoisomers (due to the two chiral centers at C-2 and C-3), but a fact unnoticed by the authors. The DPPsC obtained by us gave $^1$H NMR and IR spectra consistent with previous reports (Nifant’ev et al., 1978; Vasilenko et al., 1982). Anal. Caled for C$_{90}$H$_{168}$O$_{36}$SN$_2$H$_2$: S, 4.12. Found: S, 4.21 (Galbraith Laboratories, Inc.). The specific rotations of DPPsC (c = 2.1 g/100 mL of chloroform) at 20 °C were [α]$_{20}^{D}$ +130.4°, [α]$_{20}^{D}$ +78.0°, [α]$_{20}^{D}$ +12.1°, [α]$_{20}^{D}$ +38.6°, and [α]$_{20}^{D}$ +58.5°, which was $^{31}$P and $^{13}$C NMR analysis indicated that DPPsC obtained was a mixture of two isomers (A + B), as described under Results.

**Separation of Diastereomers of DPPsC.** A typical reaction is described as follows. DPPsC(A+B) (1.0 g) was dissolved in 10 mL of chloroform (reagent grade) and then mixed with 50 mL of diethyl ether (reagent grade, redistilled). Approximately 1000 units of phospholipase A$_2$ (from venom) in 3.5 mL of buffer (10 mM Tris, pH 7.2, 2 mM CaCl$_2$, 0.2 mM EDTA) was added to the above solution of DPPsC. The resulting cloudy reaction mixture was stirred vigorously at room temperature by a magnetic stirrer. The reaction was followed by both TLC and $^{31}$P NMR (an aliquot of the reaction mixture was dried under vacuum and dissolved in 1.5 mL of D$_2$O containing 5% Triton X-100). $^{31}$P NMR analysis showed both the extent and the stereospecificity of the reaction. When ca. 80% of isomer B of DPPsC was hydrolyzed, the reaction mixture was concentrated and chromatographed on a column. The fatty acid was eluted first, followed by the unreacted DPPsC and then MPPsC, as monitored by TLC and by UV absorption at 254 nm. Possible byproducts in this reaction include a desulfurized phospholipid and an uncharacterized triphospholipid. The unreacted DPPsC was subjected to a second reaction by the same procedure, except that the reaction was allowed to proceed until formation of some MPPsC(A) can be observed (additional enzyme was added if necessary). After column chromatography, pure DPPsC(A) was obtained. The MPPsC(B) isolated from the first reaction should be free of MPPsC(A), but it is recommended that the purity be checked by $^{31}$P NMR in CH$_3$OD or in D$_2$O/Triton X-100. Recyclation of MPPsC(B) to DPPsC(B) was carried out according to the procedure of Gupta et al. (1977), by use of (C$_{15}$H$_{23}$CO$_2$)$_2$O and N,N-dimethyl-4-aminopyridine as a catalyst. The product DPPsC(B) was isolated from a column and checked again by $^{31}$P NMR. The whole procedure gave ca. 30–35% yield each of DPPsC(A) and DPPsC(B), relative to DPPsC(A+B).

The IR spectra showed a small difference between the two isomers of DPPsC (in a pellet with KBr): (isomer A) 2915, 2842, 1740 (C=O), 1470, 1183, 1095, 953, 965, 920, 872, 796, 765, 719, 617 (P-S), 430 cm$^{-1}$; (isomer B) 2918, 2845, 1738 (C=O), 1467, 1178, 1091, 1041, 969, 915, 870, 795, 769, 719, 610 (P-S), 440, 425 cm$^{-1}$. The detailed assignments and the significance of the difference remain to be established by further investigation.

**Instrumental and Spectroscopic Methods.** $^{31}$P, $^{13}$C, and $^1$H NMR were performed on a Bruker WP-200 NMR spectrometer, whereas a Bruker WM-300 was used for $^{14}$N NMR. Both spectrometers are equipped with a multinuclear probe. The probe sizes are 5 mm for $^1$H, 10 mm for $^{31}$P and $^{13}$C, and 15 mm for $^{14}$N. Deuterium lock was used in all experiments. The chemical shifts for $^{13}$C and $^1$H are referenced to internal Me$_2$Si, whereas $^{31}$P and $^{14}$N chemical shifts are referenced to external 85% H$_3$PO$_4$ at 25 °C and to 5.4 M NH$_4$Cl in 15% D$_2$O, respectively. The + sign indicates a downfield shift in all cases.

Spectrophotometric assays were performed on a Uvikon 820 UV–vis spectrophotometer (Kontron Analytic, Switzerland). A Perkin-Elmer 2383B IR spectrometer (4000–200 cm$^{-1}$) was used for IR experiments. Optical rotation was measured on a Perkin-Elmer 141 polarimeter in a 10-cm cell. Sonication was carried out in a Brownwill Biosonik.

**Results**

**Synthesis and Properties of DPPsC.** Following the procedures of both Nifant’ev et al. (1978) and Vasilenko et al. (1982) and using (S) 1,2-dipalmitin (synthesized from d-mannitol as described under Materials and Methods), we have synthesized 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPsC) (1). The presence of two diastereomers due to the chiral phosphorus center can be shown by both $^{31}$P NMR and $^{13}$C NMR. In $^{31}$P NMR (Figure 1a), two peaks separated by 0.05 ppm (in CDCl$_3$) can be resolved. In $^{13}$C NMR (Figure 2a), the two isomers of DPPsC show different chemical shifts (0.08 ppm) at C-2. Detailed assignments of $^{13}$C NMR peaks are further discussed in a later section.

We designate the isomer that resonates at lower field in $^{31}$P NMR (in CDCl$_3$) as isomer A and the one at higher field as isomer B. This convention will be used consistently until the absolute configuration can be assigned.

7 In the structure of DPPsC (1), the configuration at phosphorus is arbitrary, and the localization of the negative charge on the oxygen (rather than sulfur) is an assumption without experimental proof.
Stereospecific Hydrolysis of DPPsC by Phospholipase A₂. Phospholipase (PL) A₂ catalyzes hydrolysis of the 2-carboxylic ester of lecithin to form lysolecithin. A Ca²⁺ ion is usually required for the reaction (Volwerk & de Haas, 1982). Since the phosphorus atom is five bonds away from the carboxyl carbon and since the enzyme can tolerate substitution of the choline side chain by other groups (Van Deelen & de Haas, 1963a,b), the configuration at phosphorus may not be expected to be important in catalysis. However, by following the hydrolysis of DPPsC(A+B) by PL A₂ from bee venom using ³¹P NMR, we found that only one diastereomer was hydrolyzed (Figure 3). The reaction was carried out in a D₂O buffer containing 5% Triton X-100, in which phospholipids formed mixed micelles with Triton X-100 and gave relatively sharp ³¹P NMR signals (Roberts et al., 1979). As shown by Figure 3, the two isomers of DPPsC are separated by 0.07 ppm, and only the isomer that resonates at the lower field is hydrolyzed to form a single product. The hydrolysis of one isomer was complete at 16 h, and no appreciable hydrolysis of the other isomer occurred upon further incubation up to 142 h. The product and the unreacted DPPsC were then both isolated and characterized by ³¹P and ¹³C NMR (which are further discussed in later sections). The unreacted DPPsC was found to be isomer A of DPPsC. Thus, it is isomer B of DPPsC that is specifically hydrolyzed by PL A₂ from bee venom. The hydrolysis product of DPPsC(B) was designated as isomer B of MPPsC, which was further confirmed by recrystallization to DPPsC(B) by a procedure described in a later section.

To compare the catalytic property of PL A₂ from different sources, we have also investigated the specificity of PL A₂ from other sources. As shown in Figure 4, the PL A₂ from Naja naja venom, Crotaulus adamanteus, venom, and porcine pancreas are also specific to isomer B of DPPsC.

Stereospecific Hydrolysis of DPPsC by Phospholipase C. The PL C from B. cereus is a Zn²⁺ metalloenzyme that is also stabilized by some free metal ions such as Zn²⁺ or Ca²⁺ (Little, 1981). Since the enzyme hydrolyzes phospholipids at the C₂-O-P bond, it can be expected to require a specific configuration at phosphorus in the enzyme active site. Figure 5 shows that the enzyme specifically hydrolyzes one of the two isomers of DPPsC in a mixture of D₂O/deoxycholate. A new resonance at 43.2 ppm (not shown in Figure 5) appeared concomitant with the decrease of one of the signals of DPPsC. The unreacted isomer was isolated and shown to be isomer B of DPPsC on the basis of ³¹P chemical shifts in various solvents. In addition, the reaction products, 1,2-dipalmitin (which precipitated out from the reaction mixture) and thio-
Figure 4: Stereospecificity of PL A₂ from different sources shown by \(^{31}\)P NMR: (a) DPPsC(A+B) before addition of enzyme, same conditions as in Figure 3; (b) 20 h after addition of 25 mg of PL A₂ from Naja naja venom; (c) 6 h after addition of ca. 5 mg of PL A₂ from C. adamanteus venom; (d) 50 h after addition of ca. 200 \(\mu\)g of PL A₂ from porcine pancreas. All spectra and reactions were done at 37 °C. NMR parameters are the same as in Figure 3.

Figure 5: Stereospecific hydrolysis of DPPsC(A) by phospholipase C (B. cereus) followed by \(^{31}\)P NMR: (a) 37 mg of DPPsC(A+B) in 2 ml of D₂O containing 80 mg of sodium deoxycholate (the sample has been filtered through Chelex-100); (b) 0.7 h after addition of 5 \(\mu\)g of the enzyme; (c) 6 h; (d) 13 h. The time indicated is the time in the middle of accumulation. A new peak at 43.2 ppm due to phosphocholine appeared (not shown in the figure) concomitantly with the decrease of DPPsC(A). The spectra and reaction were done at 37 °C. NMR parameters: spectral width 2000 Hz; acquisition time 2 s; pulse width 16 ms; \(^1\)H decoupling; line broadening 0.5 Hz; Gaussian broadening 0.02 Hz.

Phosphocholine (which resonates at 43.2 ppm in the reaction mixture), have both been isolated and characterized by \(^1\)H NMR. These experiments establish that the PL C from B. cereus specifically hydrolyzes isomer A of DPPsC to form 1,2-dipalmitin and phosphocholine.

The PL C from C. perfringens is also a Zn\(^{2+}\) metalloenzyme but requires Ca\(^{2+}\) as an activator (Takahashi et al., 1981). As shown in Figure 6, the enzyme also specifically hydrolyzes isomer A of DPPsC, with a new resonance appearing at 43.2 ppm due to thiophosphocholine. However, the reaction was slow and incomplete in this case.

Preparation of Chiral DPPsC and Chiral MPPsC. Although the separate isomers DPPsC(A) and DPPsC(B) can be obtained by digesting the mixture DPPsC(A+B) with phospholipase A₂ (which hydrolyzes isomer B) and with phospholipase C (which hydrolyzes isomer A), respectively, half of the sample would be wasted by such a procedure. For a better yield, we have used phospholipase A₂ to separate DPPsC(A+B) into DPPsC(A) and MPPsC(B), followed by reacylating the MPPsC(B) to DPPsC(B).

Since all four types of phospholipase A₂ described in the previous section are specific to isomer B, they are all suitable for the separation of diastereomers. However, the specificity of phospholipase A₂ is not 100% (Bruzik et al., 1982). If the reaction is allowed to proceed until the hydrolysis of isomer B is complete, the isolated MPPsC(B) may contain ca. 0–5% of MPPsC(A) (which may not be detected by \(^{31}\)P NMR as in Figures 3 and 4), depending on the experimental condition and the purity and source of the enzyme used. The exact reasons for the variation in stereospecificity remains to be established. So that one can ensure a good diastereomeric purity, the reaction should be stopped when ca. 80–90% of isomer B is hydrolyzed. Chromatographic separation gave pure MPPsC(B) and unreacted DPPsC(A), which was contaminated with ca. 10–20% of DPPsC(B). The unreacted DPPsC was then subjected to exhaustive digestion by phospholipase A₂ to give pure DPPsC(A) and a mixture of MPPsC(A+B). The pure MPPsC(B) was reacylated with palmitoyl anhydride (Gupta et al., 1977) to give pure DPPsC(B). \(^{31}\)P NMR spectra of DPPsC(A) (Figure 1b) and DPPsC(B) (Figure 1c) indicate >98% diastereomeric purity.

The H₂O/Triton X-100 system (as described in the legend to Figure 3) allows a direct \(^{31}\)P NMR analysis of the reaction mixture. Since both DPPsC isomers and MPPsC isomers can be resolved by \(^{31}\)P NMR, not only the extent of reaction but also the stereospecificity can be followed. However, such a system is not suitable for large-scale preparations since it requires expensive chromatographic work to separate Triton X-100 from phospholipids. The best solvent system we have
used is a mixture of chloroform/ether/buffer. A complete procedure for the separation of DPPsC(A+B) is described under Materials and Methods. In the reaction mixture, chloroform is required to dissolve DPPsC, ether is required for enzyme activity, and the buffer is required to minimize desulfurization caused by the acid generated in the reaction. Use of less buffer may improve the reaction rate but may also increase byproducts. Use of more buffer may slow down the reaction due to inhomogeneity. The use of organic solvents does not seem to change the stereospecificity of the enzyme.

As an alternative to the above two-step procedure, the first hydrolysis by phospholipase A₂ may be allowed to ca. 50% completion (i.e., isomer B is completely hydrolyzed as determined by ³¹P NMR). After chromatographic separation, the MPPsC(C) obtained was acetylated to DPPsC(C). The separate isomers thus obtained may look pure in ³¹P NMR analysis unless under a very good signal/noise ratio and resolution. However, since it has been shown that the stereospecificity of bee venom PL A₂ is not absolute (Bruzik et al., 1982), the samples obtained by such a one-step separation should not be assumed to be isomERICally pure. So that a good purity can be ensured, DPPsC(A) and DPPsC(C) can be further digested with PL A₂ and PL C, respectively.

Isomer A of MPPsC has only been identified as a mixture with MPPsC(C). Figure 7a shows the ³¹P NMR spectrum of pure MPPsC(C) in CD₃OD, whereas Figure 7b shows that of a mixture of B/A of MPPsC(C) in a ratio of ca. 3/1. Figure 7c shows the same mixture of MPPsC(C) in D₂O/Triton X-100. When this mixture was reacylated to DPPsC(C), it gave a mixture of DPPsC(C) (B/A ≃ 3/1), as shown by ³¹P NMR in CD₃OD (Figure 7d) and in D₂O/Triton X-100.

Spectral Properties of Chiral Thiophospholipids in Solution. ¹³C NMR. Some ³¹P NMR spectra of chiral thiophospholipids have already been shown in previous sections (Figures 1 and 3–5). Table I summarizes the ³¹P chemical shifts in various solvent systems in which the two diastereomers can be resolved. The ³¹P chemical shifts of thiophospholipids are ca. 50–60 ppm downfield from those of phospholipids, which is characteristic of thiophosphate compounds (TsaI, 1983). In addition, the chemical shift of DPPsC shows a greater solvent dependence than that of DPPPC (footnote a of Table I must be noted). As shown in Table I, the relative chemical shifts between the two diastereomers of DPPsC or MPpSC also show a significant solvent dependence. The water/detergent systems give good separation of diastereomers, thus suitable for directly monitoring the stereospecific hydrolysis of DPPsC by PL A₂ or PL C, as described in previous sections. The concentration of Triton X-100 should not exceed 5%; otherwise, signals are broadened, possibly due to the increased viscosity. For routine analysis of diastereomeric purity, chloroform seems to be a good solvent for DPPsC and methanol a good one for MPPsC. The samples can easily be recovered from organic solvents but not from detergents.

(2) ¹³C NMR. Figure 2 shows the ¹³C NMR spectra (in CD₃OD, 50.3 MHz) of DPPsC(A+B) (Figure 2a) and MPPsC(B) (Figure 2b) in the choline-chain and the glycerol-backbone regions. The chemical shifts and spin–spin coupling constants are summarized in Table II. By use of pure isomers, it has been established that the C-2 of DPPsC(A) resonates at 71.88 ppm, whereas that of DPPsC(B) resonates at 71.80 ppm. The resonance of C-3 also differs by 0.08 ppm between the two isomers. The differences in the chemical shifts of other carbons are too small to be detected.

The ¹³C NMR spectra of various phospholipids have been reported previously (Murari et al., 1982; Birdsall et al., 1972; Murari & Baumann, 1981). As shown in Table II, the spectral data of DPPsC and DPPPC are very similar. In particular, the two-bond and three-bond coupling constants between ¹³C and ³¹P are all the same within experimental error. Since it has been established that Tₐ (2–10 Hz) depends on the dihedral angle between C and P (Lapper & Smith, 1973; Smith et al.,

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<th>compd</th>
<th>CDCl₃ (10 mM)</th>
<th>CH₃OD (50 mM)</th>
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<th>H₂O/D₂O/0.1 M P₄/1%DOC (10 mM, pH 7.5)</th>
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<tr>
<td>DPPsC(A)</td>
<td>56.12 (±0.05)</td>
<td>60.822 (±0.021)</td>
<td>57.13 (±0.072)</td>
<td>57.115 (±0.074)</td>
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<tr>
<td>DPPsC(B)</td>
<td>56.07</td>
<td>60.801</td>
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<td>57.180</td>
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<tr>
<td>DPPC</td>
<td>0.18</td>
<td>2.09</td>
<td>-0.374</td>
<td>-0.174</td>
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<tr>
<td>MPPsC(A)</td>
<td>59.275 (±0.061)</td>
<td>59.691 (±0.057)</td>
<td>59.604</td>
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<tr>
<td>MPPsC(C)</td>
<td>59.336</td>
<td>59.604</td>
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<tr>
<td>MPPC</td>
<td>1.05</td>
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</table>

Note: Chemical shifts were measured at 30 °C for CDCl₃ and 37 °C for others. b 50 mM in CD₃OD. c No appreciable change in the chemical shifts of the two isomers occurs at 50 mM Mops NaCl buffer, pH 7.2, containing 5% Triton X-100. d No appreciable change in the relative chemical shifts of the two isomers occurs if the 0.1 M P₄ is omitted and the concentration of sodium deoxycholate is increased to 5%. e Numbers in parentheses indicate the difference in the chemical shifts between the two diastereomers (isomer B = isomer A).
<table>
<thead>
<tr>
<th>Choline Chain</th>
<th>DPPS(A)</th>
<th>DPPS(C)</th>
<th>DPPC</th>
<th>MPPS(B)</th>
<th>MPPC</th>
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<tr>
<td>Me₃N</td>
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<td>54.93</td>
<td>54.80</td>
<td>54.84</td>
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<tr>
<td>t-CH₃CN</td>
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<td>3.4</td>
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<td>β-CH₂N</td>
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<td>67.46</td>
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<td>α-CH₂OP</td>
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<td>t-OP</td>
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<th>¹⁴N NMR Chemical Shifts of DPPS and DPPC in CD₂OD⁺</th>
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</tr>
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<td>t-CH₃CN</td>
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<td>β-CH₂N</td>
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<tbody>
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<td>C=O</td>
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<td>174.9</td>
<td>175.2</td>
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<td>Acetyl Groups</td>
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Discussion

Stereochemistry and Mechanism of Phospholipase A₂.

The mechanism of phospholipase A₂ (from various sources) has been investigated extensively, particularly in the role of Ca²⁺ in catalysis (Verheij et al., 1980), the existence of an activator site and a catalytic site (Plückthun & Dennis, 1982), the possible cooperative interactions between the two proton sites (Keith et al., 1981), and the interfacial interaction between the enzyme and the phospholipid (Burrage & Bardes, 1981; Kuperberg et al., 1981). A direct binding of Ca²⁺ with the phosphate group has been suggested (Verheij et al., 1980; Wells, 1974), but no direct evidence has been provided. The observed stereospecificity of phospholipase A₂ in the experiments described above suggests that the phosphate group is involved in binding, most likely with Ca²⁺. The direct Ca²⁺ binding with the phosphate group has been further supported by a metal-ion dependence in stereospecificity (Bruzik et al., 1982), which will be described in detail in a later paper.

Although phospholipase A₂ from different sources shows different biochemical properties, they are all specific to the same isomer of DPPS, which suggests that the active site geometry is similar for PL A₂ from different sources. However, the stereospecificity of PL A₂ from bee venom has been shown not to be 100% (Bruzik et al., 1982), and PL A₂ from other sources seems to show different degrees of stereospecificity, which are to be further investigated. In addition, plots of initial velocities of DPPS(A) and DPPS(B) vs. enzyme concentration showed "sigmoidal" curves (Bruzik et al., 1982). All of these results seem to suggest that chiral phospholipids are useful probes for the mechanism of phospholipases.

Stereochemistry and Mechanism of Phospholipase C.

Phospholipase C has not been studied as extensively as PL A₂. Since it is a Zn²⁺ metalloenzyme, but requires free Zn²⁺ for stabilization in the case of B. cereus PL C (Little, 1981) and requires free Ca²⁺ for activation in the case of C. perfringens PL C (Takahashi et al., 1981), the detailed mechanism of catalysis seems complicated and relatively unestablished. Our
finding that both enzymes are specific to the same isomer (isomer A) of DPPS C suggests that the active site geometry for the two enzymes may not be different. However, since the stereospecificity may be dependent on metal ions (Jaffe & Cohn, 1979; Bruzik et al., 1982), any conclusion should await detailed investigation under conditions that are better defined.

Are Thiocephospholipids a Good Model for Phospholipids?

In the past 2 decades a large number of phospholipid analogues have been synthesized for various biochemical studies. Thiocephospholipids provide a new class of model phospholipids that have several advantages. The sulfur substitution at phosphorus has caused a very small, if any, perturbation in the structural properties, as suggested by $^{13}$C and $^1$H NMR results. The downfield shift of $^{31}$P NMR signals of thiocephospholipids makes it possible to monitor the physical properties of a particular phospholipid in a complex system, as reported recently by Vasilenko et al. (1982).

A more interesting feature of thiocephospholipids is the existence of two diastereomers that differ only in the configuration at phosphorus. In natural phospholipids, the phosphorus atom is a prochiral center, and the nonbridging oxygen atoms are diastereotropic. When interacting with a chiral component such as an enzyme, the prochiral phosphorus center most likely will become chiral due to stereospecific interaction of the enzyme with one of the two diastereotopic oxygens. This explains the stereospecificity of PL A$_2$ and C and makes the diastereomers of thiocephospholipids good probes for the mechanism of phospholipases and other phospholipid transport enzymes.

In solution, the two isomers of DPPS C show a small yet detectable difference in the chemical shifts of P, C-2, and C-3, which are characteristic of diastereomers. Such a small difference in solution may not necessarily suggest that the two isomers should also have similar properties in lipid bilayers. Diastereomers are known to have very different crystal properties; thus, they may also have quite different properties in the liquid-crystalline phase. The physical properties of separate diastereomers of chiral thiocephospholipids are under investigation in our laboratory. A very interesting problem to be answered is whether membrane proteins interact stereospecifically with the two isomers in lipid bilayers.

Conclusions

The thiocephospholipid DPPS C was shown to be a mixture of two diastereomers (A and B). Phospholipase A$_2$ from four different sources specifically hydrolyzes isomer B of DPPS C, whereas phospholipase C from two different sources specifically hydrolyzes isomer A of DPPS C. A procedure has been developed to separate DPPS C into pure isomer A and pure isomer B. The spectral properties of chiral thiocephospholipids have been investigated. Our results suggest that chiral thiocephospholipids are useful in studying the mechanism of enzyme reactions involving phospholipids and are potentially useful in probing the role of the phosphate head group of phospholipids in various membrane functions.

Acknowledgments

We thank Dr. Sanjeev M. Gupta for his initial study on the synthesis of DPPS C and Judy Hart for isolating phospholipase A$_2$ from bee venom.

Registry No. (R,R)-DPPS C, 82482-77-7; (S,R)-DPPS C, 82482-78-8; (R,R)-MPPS C, 82482-79-9; (S,R)-MPPS C, 82482-80-2; phospholipase A$_2$, 9001-84-7; phospholipase C, 9001-86-9; $\beta$-mannitol, 69-65-8; 1,2,5,6-diisopropyliden-$\beta$-mannitol, 1707-77-3; 1,2-diisopropyliden-sn-glyceraldehyde, 15186-48-8; 1,2-diisopropyliden-sn-glycerol, 22323-82-6; 3-benzyl-sn-glycerol, 56552-80-8; 1,2-di-

References


