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Phospholipids Chiral at Phosphorus. Synthesis of Chiral Phosphatidylcholine and Stereochemistry of Phospholipase D[†]

Karol Bruzik[‡] and Ming-Daw Tsai*

ABSTRACT: Chirally labeled 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholines (DPPC) with known configuration were synthesized by N-methylation of chirally labeled 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE). Transphosphatidylation of (R_P)- and (S_P)-[^{18}O]DPPC catalyzed by phospholipase D from cabbage gave (R_P)- and (S_P)-[^{18}O]DPPE, respectively, as indicated by ^{31}P nuclear magnetic resonance (NMR) analysis of [^{18}O]DPPE. Therefore, phospholipase D catalyzes transphosphatidylation with overall retention of configuration at phosphorus. The steric course of hydrolysis of DPPC catalyzed by the same enzyme was elucidated by the following procedures. Hydrolysis of (R_P)-[^{17}O,^{18}O]DPPC by phospholipase D gave 1,2-dipalmitoyl-*sn*-glycero-3-[^{16}O,^{17}O,^{18}O]phosphate ([^{16}O,^{17}O,^{18}O]DPPA) with unknown configuration. The latter compound was then converted to 1-[^{16}O,^{17}O,^{18}O]phospho-(*R*)-propane-1,2-diol by a procedure involving no P–O bond cleavage [Bruzik, K., & Tsai, M.-D. (1984) *J. Am. Chem. Soc. 106*, 747–754]. The configuration of the phosphopropane-1,2-diol was determined as R_P by ^{31}P NMR analysis following ring closure and methylation [Buchwald, S. L., & Knowles, J. R. (1980) *J. Am. Chem. Soc. 102*, 6601–6603]. The results indicated that hydrolysis of DPPC catalyzed by phospholipase D also proceeds with retention of configuration at phosphorus. Our results therefore support a two-step mechanism involving a phosphatidyl-enzyme intermediate in the reactions catalyzed by phospholipase D from cabbage.

The enzyme-catalyzed reactions involving a P–O bond cleavage can be categorized into the following types on the basis of the stereochemistry involved:

$$\mathrm{ROPO_2OR'}\ \text{(prochiral)} \rightleftarrows \mathrm{ROPO_2OR''}\ \text{(prochiral)} \qquad (1)$$

$$\mathrm{ROPO_2OR'}\ \text{(prochiral)} \rightleftarrows \mathrm{ROPO_3}\ \text{(pro-prochiral)} \qquad (2)$$

$$\mathrm{ROPO_3}\ \text{(pro-prochiral)} \rightleftarrows \mathrm{R'OPO_3}\ \text{(pro-prochiral)} \qquad (3)$$

$$\mathrm{ROPO_3}\ \text{(pro-prochiral)} \rightleftarrows \mathrm{PO_4}\ \text{(pro-pro-prochiral)} \qquad (4)$$

In the past 5 years, the steric course of a large number of enzymes has been elucidated by use of chirally labeled phosphates or phosphorothioates (Buchwald et al., 1982; Eckstein, 1983; Eckstein et al., 1982; Floss et al., 1984; Frey, 1982; Frey et al., 1982; Gerlt et al., 1983; Knowles, 1980; Lowe et al., 1981; Tsai, 1982; Webb, 1982). However, most of the reactions investigated previously involved nucleotides or sugar phosphates.

Recently, we have reported synthesis and configurational analysis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE)[1] chirally labeled with ^{17}O and/or ^{18}O at phosphorus (Bruzik & Tsai, 1984). These compounds and the methodology developed are useful in studying the stereochemistry of phospholipases. In this paper, we report the syntheses of chirally labeled 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholines (DPPC), as well as their application to elucidate the steric courses of transphosphatidylation (reaction in eq 1) and hydrolysis (reaction in eq 2) catalyzed by phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) from cabbage. Part of the work has been reported in a preliminary paper (Bruzik & Tsai, 1982) before the absolute configuration is known.

[†] From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received September 13, 1983.* This work was supported by National Institutes of Health Research Grant GM 30327. The NMR facilities used were funded in part by Grants NIH GM 27431 and NSF CHE 7910019. This is paper 6 in the series "Phospholipids Chiral at Phosphorus". For paper 5, see Bruzik & Tsai (1984). M.-D.T. is an Alfred P. Sloan Fellow, 1983–1985.

[‡] Present address: Center of Molecular and Macromolecular Studies, Polish Academy of Sciences, Boczna 5, 90-362 Lodz, Poland.

[1] Abbreviations: DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; EDTA, ethylenediaminetetraacetate; HMDSA, 1,1,1,3,3,3-hexamethyldisilazane; HMPA, hexamethylphosphoramide; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

0006-2960/84/0423-1656$01.50/0

Materials and Methods

Materials. Both $H_2{}^{18}O$ (97.5 atom % ^{18}O) and $H_2{}^{17}O$ (52.4 atom % ^{17}O, 35.1 atom % ^{18}O) used were obtained from Monsanto. Phospholipase D from cabbage was obtained from Sigma (1–2 units/mg) and Boehringer Mannheim (0.3 unit/mg). Benzyltriethylammonium chloride, methyl iodide, and HMDSA were purchased from Aldrich. Triton X-100 and other biochemicals were obtained from Sigma. Precoated TLC plates were purchased from Merck (0.2 mm, aluminum support, silica gel 60 F-254). Silica gel for column chromatography was obtained from Universal Scientific, Inc. (Woelm, 40–63-μm particle size), and Baker (silica gel 7, particle size <40 μm).

Synthesis of Chiral DPPE. The R_P and S_P isomers of [^{17}O,^{18}O]DPPE were synthesized as described elsewhere (Bruzik & Tsai, 1984), except that the acid-catalyzed ring opening of cyclic oxazaphospholidines was carried out with 13.5 equiv of 97.5% $H_2{}^{18}O$. The R_P and S_P isomers of [^{18}O]DPPE were synthesized according to the same procedure, except that unlabeled $POCl_3$ was used and that the ring opening of cyclic oxazaphospholidines was carried out in a relatively small excess of $H_2{}^{18}O$ (0.225 mmol was hydrolyzed with 20 μL of 99 atom % $H_2{}^{18}O$ and 5 μL of trifluoroacetic anhydride in dry dimethoxyethane).

Synthesis of Chiral DPPC from Chiral DPPE. A typical experiment is described as follows. [^{17}O,^{18}O]DPPE (**3**) (1.30 g, 1.87 mmol) and benzyltriethylammonium chloride (211 mg, 0.93 mmol) were dissolved in 40 mL of $CHCl_3$. To this solution was added 27 mL of 0.6 M Na_2CO_3, followed by 15 mL of methyl iodide. The mixture was stirred vigorously at room temperature, and the formation of DPPC was monitored by TLC (chloroform/methanol/water, 66/33/4; R_f 0.5 for DPPE, 0.25 for DPPC). After the reaction was complete (ca. 2 h), the chloroform layer was separated, and the aqueous layer was further washed with chloroform (50 mL). The chloroform solutions were combined and evaporated to dryness. Column chromatography on silica gel (Woelm, 2.5 × 50 cm column) yielded 1.13 g of [^{17}O,^{18}O]DPPC (**7**). The DPPC obtained gave identical TLC and ^1H and ^{31}P NMR results as authentic DPPC.

Transphosphatidylation of [^{18}O]DPPC. (R_P)-[^{18}O]DPPC (**5**) (65 μmol) was suspended in 65 mL of water containing 1.2 mmol of $CaCl_2$, 1.2 g of ethanolamine, and a sufficient amount of HCl to maintain the pH at 5.6. To start the reaction, 25 mg of phospholipase D (Sigma) was added, followed by 33 mL of alcohol-free ether (anhydrous grade, redistilled before use). The heterogeneous reaction mixture was stirred at room temperature for 3 h. An additional 10 mg of the enzyme was added, and stirring was continued for an additional 5 h. The formation of DPPE was monitored by TLC (chloroform/methanol/water, 70/30/4; R_f 0.2 for DPPC, 0.5 for DPPE). When the reaction was complete, both phases contained a thick colloidal precipitate. The ether layer was separated and the water layer extracted with chloroform. The organic fractions were pooled, evaporated to dryness, and chromatographed on silica gel (Baker, chloroform/methanol/water, 70/30/3, as the eluent). The product [^{18}O]DPPE (**9**) was isolated in 59% yield (26.5 mg). Ten milligrams of unreacted DPPC was also recovered. Transphosphatidylation of the other isomer, (S_P)-[^{18}O]DPPC (**6**), gave [^{18}O]DPPE (**10**).

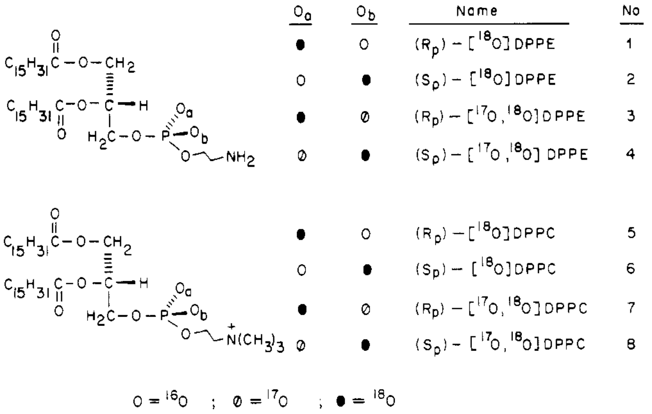
*Hydrolysis of (R_P)-[^{17}O,^{18}O]DPPC (**7**) by Phospholipase D.* The R_P isomer of [^{17}O,^{18}O]DPPC (**7**) (626 mg, 0.85 mmol) was suspended in an acetate buffer (500 mL, 0.2 M, pH 5.6) containing 0.1 M $CaCl_2$ (reagent grade). The resulting solution was mixed with 250 mL of ethanol-free ether and stirred for a few minutes until both phases became clear. Phospholipase D (100 mg, Boehringer) was then added, and the reaction mixture was stirred overnight at room temperature. Complete hydrolysis of DPPC was indicated by TLC (chloroform/methanol/water, 66/33/4; R_f 0.25 for DPPC, 0.46 for DPPA). The reaction mixture was then evaporated under reduced pressure to remove ether. The aqueous phase was acidified with 2 N HCl to pH 1.0 and shaken for 15 min. The resulting acid form of DPPA was extracted with a 2/1 mixture of chloroform/methanol. The organic extract was evaporated to dryness and chromatographed on silica gel (Woelm; chloroform/methanol/water, 66/33/4, as the eluent). The product [^{16}O,^{17}O,^{18}O]DPPA (**14**) was isolated in 74% yield (408 mg). The structure of **14** was identified by comparing its TLC and ^{31}P NMR results (δ 1.45 in D_2O containing 50 mM EDTA and 5% Triton X-100) with those of authentic DPPA (Sigma). The randomly labeled [^{18}O]DPPC (**11**) was hydrolyzed to [^{18}O]DPPA (**12**) by phospholipase D according to the same procedure.

Methylation of DPPA. The [^{16}O,^{17}O,^{18}O]DPPA obtained above was dissolved in chloroform and treated with dry, ethanol-free ethereal diazomethane until gas evolution ceased and a yellow color persisted. After being left at room temperature for 15 min, the mixture was evaporated to dryness and then loaded onto a silica gel column (Woelm, 50 × 2.5 cm column). Elution with ether gave 180 mg (42%) of the dimethyl ester **15**. The column was further washed with chloroform/methanol/water, 66/33/4, to elute off unreacted DPPA. After evaporation to dryness, the unreacted DPPA was dissolved in chloroform (50 mL) and shaken with 1 N HCl (5 mL) for 10 min. The organic layer was separated by centrifugation, evaporated to dryness, and then methylated with CH_2N_2 as described above. An additional 50 mg of **15** was isolated after the same chromatographic treatment as above. The product **15** gave identical ^1H NMR to that of the same compound obtained previously (Bruzik & Tsai, 1984). The randomly labeled [^{18}O]DPPA (**12**) was methylated to **13** by the same procedure.

Spectral Methods. ^{31}P and ^1H NMR were performed on Bruker WP-200 and Bruker WM-300 NMR spectrometers. Both spectrometers are equipped with a multinuclear probe. The probe sizes are 5 mm for ^1H and 10 mm for ^{31}P. Deuterium lock was used in all experiments. The chemical shifts for ^1H are referenced to internal Me_4Si, whereas ^{31}P chemical shifts are referenced to external 85% H_3PO_4 at 25 °C. The + signal indicates a downfield shift in all cases. The ^{31}P NMR analysis of DPPE was performed by addition of 1,1,1,3,3,3-hexamethyldisilazane (HMDSA) to silylate the DPPE, as described previously (Bruzik & Tsai, 1982, 1984).

Results

Synthesis of Chiral Phosphatidylcholines. According to the procedure described elsewhere (Bruzik & Tsai, 1984) for the synthesis of chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{17}O,^{18}O]phosphoethanolamine ([^{17}O,^{18}O]DPPE), we first synthesized (R_P)- and (S_P)-[^{18}O]DPPE (**1** and **2** of Figure 1, respectively) and (R_P)- and (S_P)-[^{17}O,^{18}O]DPPE (**3** and **4**, respectively). The chirally labeled 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholines (DPPC), (R_P)-[^{18}O]DPPC (**5**), (S_P)-[^{18}O]DPPC (**6**), (R_P)-[^{17}O,^{18}O]DPPC (**7**), and (S_P)-[^{17}O,^{18}O]DPPC (**8**), were obtained from N-methylation of the corresponding chiral DPPE, **1**–**4**, respectively. The N-methylation was achieved by treating DPPE with CH_3I in a heterogeneous system (chloroform/2 M K_2CO_3), with benzyltriethylammonium chloride as a phase-transfer catalyst.



Oa	Ob	Name	No
●	○	(R_P) – [^{18}O] DPPE	1
○	●	(S_P) – [^{18}O] DPPE	2
●	⊘	(R_P) – [^{17}O, ^{18}O] DPPE	3
⊘	●	(S_P) – [^{17}O, ^{18}O] DPPE	4
●	○	(R_P) – [^{18}O] DPPC	5
○	●	(S_P) – [^{18}O] DPPC	6
●	⊘	(R_P) – [^{17}O, ^{18}O] DPPC	7
⊘	●	(S_P) – [^{17}O, ^{18}O] DPPC	8

○ = ^{16}O ; ⊘ = ^{17}O ; ● = ^{18}O

FIGURE 1: Structures of chirally labeled DPPE (**1–4**) and chirally labeled DPPC (**5–8**).

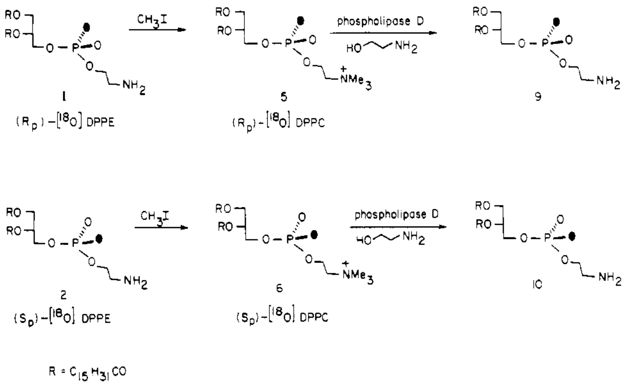


FIGURE 2: Procedures for the stereochemical study of transphosphatidylation catalyzed by phospholipase D.

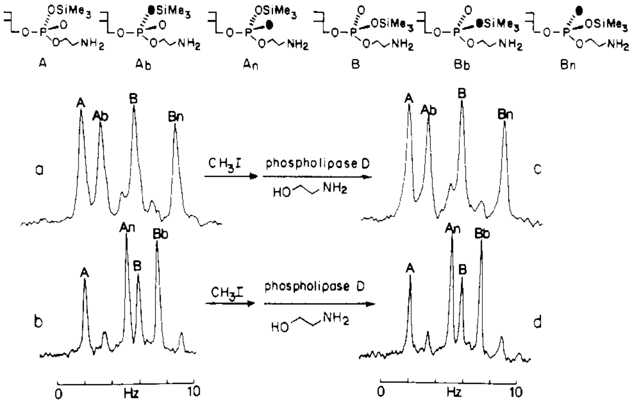


FIGURE 3: ^{31}P NMR spectra (at 81.0 MHz) of the silylated products of DPPE from **1** [(a) 36 μmol, 500 scans, line broadening 0.2 Hz], **2** [(b) 24 μmol, 1100 scans, line broadening 0.1 Hz], **9** [(c) 10 μmol, 2000 scans, line broadening 0.2 Hz], and **10** [(d) 20 μmol, 3500 scans, line broadening 0.1 Hz]. Spectral parameters were as follows: spectral width 500 Hz, 16K data points, ^{1}H decoupling, 90° pulse, and repetition time 16 s.

Table I: Stereochemical Analysis of Transphosphatidylation

DPPE samples	% species^a						% ^{18}O enrichment	% isomer	
	A	Ab	An	B	Bb	Bn		R_P	S_P
1	22.0	21.5	5.7	23.4	6.2	21.0	55	78	22
2	18.1	5.6	27.9	19.1	25.6	3.7	63	15	85
9	22.4	20.4	8.2	23.1	4.8	21.1	55	76	24
10	17.0	5.8	26.7	18.4	26.7	5.3	65	17	83

^{a} Calculated on the basis of integrals. Estimated relative error ±5%.

Under this condition, complete and quantitative quaternization of DPPE can be achieved without O-methylation. The procedure described previously by Eibl (1980) was found to be unsatisfactory in our laboratory.

Since all of the P–O bonds were not affected in the methylation, the chiral DPPC, **5–8**, should have the same isotopic enrichments and diastereomeric purity as the corresponding chiral DPPE, **1–4**, respectively, which can be analyzed by ^{31}P NMR following silylation (Bruzik & Tsai, 1982, 1984). However, it would be desirable to have a method to analyze the isotopic enrichments and diastereomeric purity of an unknown chiral DPPC obtained from a different source. This can be achieved by conversion of DPPC back to DPPE with defined stereochemistry, as described in the following section.

Stereochemistry of Transphosphatidylation. The two diastereomers of [^{18}O]DPPC, **5** and **6**, were converted back to DPPE, **9** and **10**, respectively, by transphosphatidylation catalyzed by phospholipase D (from cabbage) in the presence of ethanolamine as shown in Figure 2. To determine the stereochemical course of transphosphatidylation, it is only necessary to compare the configuration of **9** and **10** with that of the starting [^{18}O]DPPE, **1** and **2**, respectively, which can be achieved by ^{31}P NMR analysis following O-silylation of DPPE. Figure 3 shows the ^{31}P NMR analysis of the two isomers of starting [^{18}O]DPPE, **1** and **2** (spectra a and b of Figure 3, respectively), and the porducts of transphosphatidylation, **9** and **10** (spectra c and d of Figure 3, respectively). As shown in Figure 3a, silylation of (R_P)-[^{18}O]DPPE (**1**) gave four predominant species: A, Ab, B, and Bn, where A and B (separated by 0.048 ppm in the ^{31}P NMR spectrum) are the two diastereomers that result from silylation at the *pro-R* and the *pro-S* oxygens, respectively. Ab contains ^{18}O in a P–^{18}O–Si *b*ridge, while Bn contains a *n*onbridging ^{18}O, P=^{18}O. On the other hand, the opposite isomer, (S_P)-[^{18}O]DPPE (**2**), gave four predominant species: A, An, B, and Bb. Since a nonbridging ^{18}O causes a larger isotope shift in ^{31}P NMR (0.038 ppm) than a bridging ^{18}O does (0.018 ppm) (Cohn, 1982; Tsai & Bruzik, 1983), the two isomers of [^{18}O]DPPE can be easily distinguished on the basis of their ^{31}P NMR patterns. In addition, the isotopic enrichment can also be calculated from the ^{31}P NMR spectrum on the basis of relative intensities (listed in Table I). As shown in Table I, **1** contains 55 atom % ^{18}O, 78% R_P isomer, and 22% S_P isomer, while **2** contains 63 atom % ^{18}O, 15% R_P isomer, and 85% S_P isomer.

As shown in Figure 3 (c and d), the two isomers of DPPE obtained from transphosphatidylation (**9** and **10**) gave essentially the same ^{31}P NMR spectra as the two starting [^{18}O]-DPPE (**1** and **2**, respectively). The spectral analysis in Table I indicated that both isotopic enrichment and diastereomeric purity were unchanged within experimental error in the course of transphosphatidylation. The transphosphatidylation (from DPPC to DPPE) catalyzed by phospholipase D therefore proceeds with complete retention of configuration at phosphorus (98 ± 5% stereospecificity) and without detectable oxygen exchange. Such a conclusion is indeed independent of the configuration of **1** and **2**. Since the absolute configurations of **1** and **2** are known on the basis of previous studies (Bruzik & Tsai, 1984), the absolute configuration and isotopic enrichments of an unknown DPPC can be determined by conversion to DPPE followed by ^{31}P NMR analysis.

Stereochemistry of Hydrolysis of Phosphatidylcholine. The procedures for elucidating the steric course of hydrolysis of phosphatidylcholine are outlined in Figure 4. To make sure that the isotope label is not diluted by solvent during hydrolysis, we first investigated the hydrolysis of randomly labeled [^{18}O]DPPC (**11**) (85 atom % ^{18}O) [synthesized as described

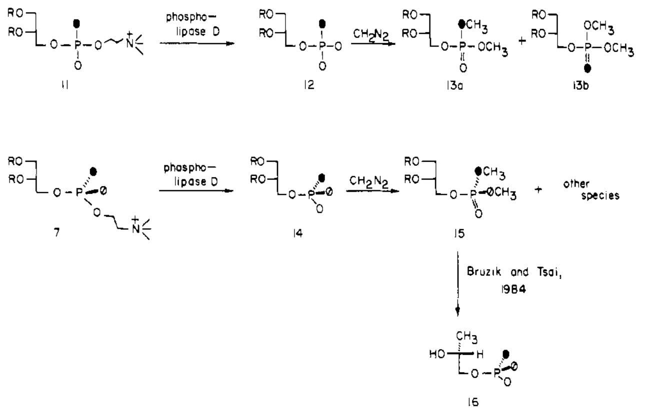


FIGURE 4: Procedures for the stereochemical study of the hydrolysis of DPPC catalyzed by phospholipase D.

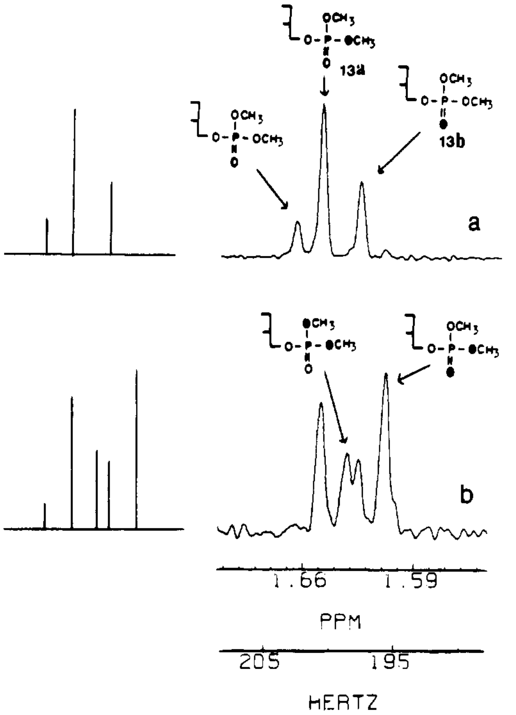


FIGURE 5: Observed and calculated ^{31}P NMR spectra ($CDCl_3$, 121.5 MHz) of the dimethyl esters of DPPA, **13** (a) and **15** (b). Spectral parameters were as follows: spectral width 500 Hz, 16K data points, ^{1}H decoupling, 90° pulse, line broadening −1.0 Hz, Gaussian broadening 0.07 Hz, and temperature 25 °C. Chemical shifts (ppm) were 1.664 (unlabeled), 1.648 (P–^{18}OCH$_3$), 1.631 (CH$_3$^{18}O–P–^{18}OCH$_3$), 1.624 (P=^{18}O), and 1.608 (^{18}O=P–^{18}OCH$_3$).

in Jiang et al. (1984)] catalyzed by phospholipase D in a mixture of ether/water. The resulting 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA) (**12**) gave a broad ^{31}P NMR signal in most solvent systems due to aggregation. So that the ^{18}O-induced isotope shift in ^{31}P NMR can be resolved, **12** was converted to **13** by treatment with diazomethane. Since **13** is randomly labeled, it should consist of a 2:1 mixture of **13a** (with a bridging ^{18}O, P–^{18}O–CH$_3$) and **13b** (with a nonbridging ^{18}O, P=^{18}O), respectively. Figure 5a shows the observed ^{31}P NMR spectrum of **13**, as well as the expected one calculated on the basis of 85 atom % ^{18}O. The good agreement between the two within experimental accuracy rules out a possible isotope dilution in the hydrolysis catalyzed by phospholipase D.

To elucidate the steric course of hydrolysis, (R_{P})-[^{17}O,^{18}O]DPPC (**7**), obtained from methylation of (R_{P})-[^{17}O,^{18}O]DPPE (**3**), was hydrolyzed by phospholipase D from cabbage. The resulting [^{16}O,^{17}O,^{18}O]DPPA (**14**) was isolated in 74% yield. The remaining task is to compare the isotopic enrichments, the configuration, and the diastereomeric purity between **7** and **14**.

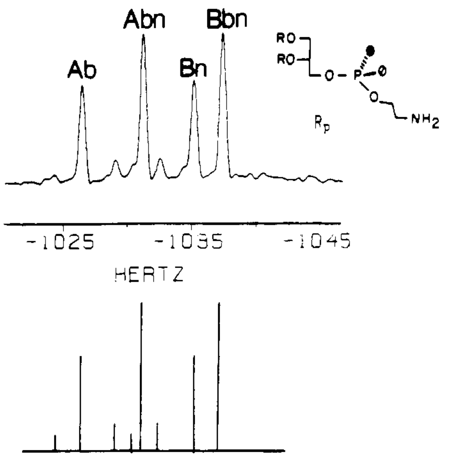


FIGURE 6: Observed and calculated ^{31}P NMR spectra ($CDCl_3$, 121.47 MHz) of the O-silylated products of (R_{P})-[^{17}O,^{18}O]DPPE (**3**). Spectral parameters are the same as in Figure 5.

Table II: Stereochemical Analysis of Hydrolysis of [^{17}O,^{18}O]DPPC

	[^{17}O,^{18}O]DPPE (**3**)	[^{16}O,^{17}O,^{18}O]phospho-(*R*)-propane-1,2-diol (**16**)
^{17}O position		
^{16}O (atom %)	21	21
^{17}O (atom %)	47	47
^{18}O (atom %)	32	32
^{18}O position		
^{16}O (atom %)	15	15
^{18}O (atom %)	85	85
diastereomeric composition		
R_{P} isomer (%)	95	88
S_{P} isomer (%)	5	12
purity (%)	40	40
chirality (%)	90	76
configuration	R_{P}	R_{P}

Figure 6 shows the ^{31}P NMR analysis of the *O*-(trimethylsilyl) derivative of the [^{17}O,^{18}O]DPPE (**3**). Since ^{17}O is known to quench ^{31}P NMR signals (Tsai, 1979; Tsai et al., 1980; Tsai & Bruzik, 1983) and since the starting ^{17}O-enriched water also contains ^{16}O and ^{18}O (^{16}O/^{17}O/^{18}O, 12.5/52.4/35.1), the observed ^{31}P NMR spectrum of **3** is due predominantly to [^{16}O,^{18}O]DPPE and [^{18}O,^{18}O]DPPE. The latter is achiral at phosphorus and gives rise to peaks Abn and Bbn (^{18}O at both bridging and nonbridging positions, shifted upfield by 0.057 ppm). [^{16}O,^{18}O]DPPE gives predominantly Ab and Bn (i.e., Ab > An; Bn > Bb), which is characteristic of R_{P} configuration. As described previously (Bruzik & Tsai, 1984), the isotopic enrichments and diastereomeric purity of **3** can be determined by simulating the observed ^{31}P NMR spectrum. The best fit calculated spectrum is also shown in Figure 6. The results of analysis are summarized in Table II.

For determination of the configuration of the chiral phosphoryl group, **14** was first methylated to **15**. The observed ^{31}P NMR spectrum of **15**, as well as the calculated one on the basis of the isotopic enrichments of **3**, is shown in Figure 5b. Again, the good agreement between the observed and the predicted spectra indicates complete retention of all isotopes up to this stage. The phosphotriester **15** was then converted by a six-step procedure (Bruzik & Tsai, 1984), without breaking any P–O bond, to 1-[^{16}O,^{17}O,^{18}O]phospho-(*R*)-propane-1,2-diol (**16**). The configuration (at phosphorus) of **16** was analyzed by ^{31}P NMR following chemical cyclization

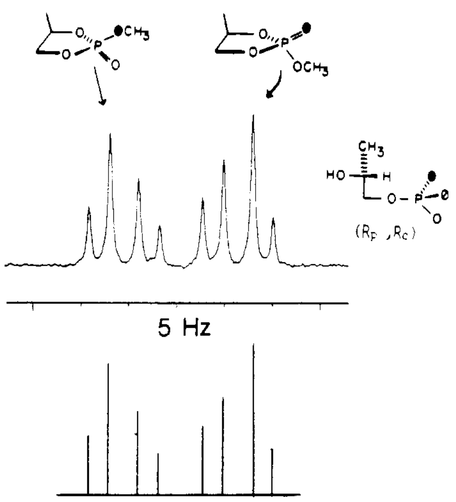


FIGURE 7: Observed and calculated ^{31}P NMR spectra (CD_3CN, 121.47 MHz) of the cyclic triesters derived from [^{16}O,^{17}O,^{18}O]-phospho-(*R*)-propane-1,2-diol (**16**). Spectral parameters were as follows: spectral width 500 Hz, 16K data points, ^1H decoupling, 90° pulse, line broadening 0.2 Hz, chemical shift +19.7 ppm, and temperature 25 °C.

and methylation, as previously described for chiral 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (Buchwald & Knowles, 1980; Buchwald et al., 1982). Figure 7 shows the ^{31}P NMR analysis of **16**. Since the configuration at C_2 is opposite between **16** and the phosphopropanediol of Knowles and co-workers, the ^{31}P NMR patterns for R_P and S_P isomers of **16** should correspond to S_P and R_P isomers, respectively, of Knowles' compound. The pattern of peaks in Figure 7 therefore indicates that **16** has R_P configuration. Therefore, the [^{16}O,^{17}O,^{18}O]DPPA (**14**) should also be R_P. The isotopic enrichments and the diastereomeric purity of **16** were again determined by simulating the spectrum. The results are listed in Table II (right column), and the calculated spectrum is also shown in Figure 7.

As shown in Table II, the starting [^{17}O,^{18}O]DPPE (**3**) and the final product **16** have, within experimental accuracy, the same "purity" (40%) (defined as the percentage of chirally labeled species, i.e., M + 3 species), as well as the same configuration (R_P). The "chirality" (defined as the optical purity of the chirally labeled species) is 90% for **3** and 76% for **16**. The results indicate that the hydrolysis of DPPC catalyzed by phospholipase D from cabbage proceeds with at least 84% retention of configuration at phosphorus. Since the ^{31}P NMR analysis of **16** involves a ring closure (Buchwald et al., 1982), which could possibly result in a small degree of racemization, it is not possible to quantitatively attribute the 16% racemization to the phospholipase D reaction, the ring closure, or experimental errors.

Discussion

Phospholipase D is widely distributed in plants and mammals. The enzyme catalyzes the hydrolysis of lecithin to phosphatidic acid and choline (the hydrolytic activity), as well as the exchange of the choline side chain by ethanolamine or other alcohols (the base-exchange activity or transphosphatidylation) (Heller, 1978; Hanahan & Chaikoff, 1947a,b, 1948). Although it has been suggested that in some mammalian sources the base-exchange activity and the hydrolytic activity may be due to different enzymes (Kanfer, 1980), the plant phospholipase D (from cabbage leaves or peanut) is believed to catalyze both hydrolysis and transphosphatidylation of lecithin (Yang et al., 1967; Yang, 1969; Tzur & Shapiro, 1972; Heller et al., 1975; Heller, 1978).

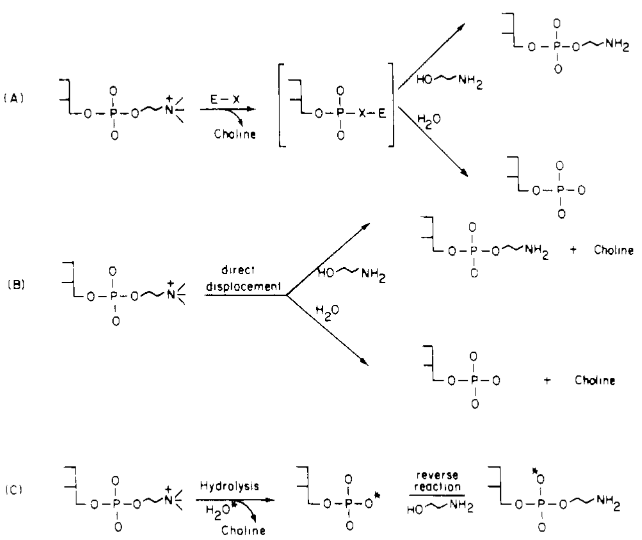


FIGURE 8: Possible mechanisms of the reactions catalyzed by phospholipase D.

The catalytic mechanism of phospholipase D had not been well characterized, possibly due to the fact that the enzyme is very unstable and has not been purified to homogeneity. In addition, kinetic studies of phospholipases are often complicated by interfacial interactions. Figure 8 shows three possible mechanisms for phospholipase D. In mechanism A, a phosphatidyl-enzyme intermediate is formed initially, which can then be transphosphatidylated to form phosphatidylethanolamine or hydrolyzed to phosphatidic acid. Mechanism B does not involve a phosphatidyl-enzyme intermediate. Direct displacement of the choline chain by H_2O or ethanolamine gives phosphatidic acid or phosphatidylethanolamine, respectively. In mechanism C, lecithin is first hydrolyzed to phosphatidic acid by either mechanism A or mechanism B. Reverse reaction between phosphatidic acid and ethanolamine gives the product of transphosphatidylation. The observed exchange between the choline group of phosphatidylcholine and free [^{14}C]choline has been used to support the possible existence of a phosphatidyl-enzyme complex (Yang et al., 1967). However, since the extent of exchange observed was low, mechanisms B and C cannot be ruled out on the basis of their results.

On the basis of recent stereochemical studies of enzyme-catalyzed reactions involving phosphorus (Buchwald et al., 1982; Eckstein et al., 1982; Frey, 1982; Frey et al., 1982; Gerlt et al., 1983; Lowe et al., 1981; Tsai, 1982; Webb, 1982), an overall retention of configuration at phosphorus can be expected for mechanism A, whereas mechanism B should result in inversion. Mechanism C predicts racemization as well as additional exchange of oxygen in transphosphatidylation. Since our results indicate that both transphosphatidylation and hydrolysis reactions catalyzed by cabbage phospholipase D proceed with an overall retention of configuration and without appreciable exchange of oxygen isotopes, mechanism A seems to be the most probable mechanism of phospholipase D.

Acknowledgments

We are indebted to R.-T. Jiang of this laboratory for synthesis of 1,2-dipalmitoyl-*sn*-glycerol and randomly labeled [^{18}O]DPPE.

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Phospholipids Chiral at Phosphorus. Absolute Configuration of Chiral Thiophospholipids and Stereospecificity of Phospholipase D[†]

Ru-Tai Jiang, Yeun-Jund Shyy, and Ming-Daw Tsai*

ABSTRACT: Separate diastereomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE) were prepared in 97% diastereomeric purity and characterized by ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance (NMR). The isomers hydrolyzed by phospholipases A_2 and C specifically were designated as isomer B (^{31}P NMR δ 59.13 in $CDCl_3$ + Et_3N) and isomer A (59.29 ppm), respectively, analogous to the isomers B and A of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) [Bruzik, K., Jiang, R.-T., & Tsai, M.-D. (1983) *Biochemistry 22*, 2478–2486]. Phospholipase D from cabbage was shown to be specific to isomer A of DPPsC in transphosphatidylation. The product DPPsE was shown to be isomer A. The absolute configuration of chiral DPPsE at phosphorus was elucidated by bromine-mediated desulfurization in $H_2{}^{18}O$ to give chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{18}O]phosphoethanolamine ([^{18}O]DPPE) followed by ^{31}P NMR analysis [Bruzik, K., & Tsai, M.-D. (1984) *J. Am. Chem. Soc. 106*, 747–754]. The absolute configuration of chiral DPPsC was elucidated by desulfurization in $H_2{}^{18}O$ mediated by bromine or cyanogen bromide to give chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{18}O]phosphocholine ([^{18}O]DPPC), which was then converted to [^{18}O]DPPE by phospholipase D with retention of configuration [Bruzik, K., & Tsai, M.-D. (1984) *Biochemistry* (preceding paper in this issue)]. The results indicate that isomer A of both DPPsE and DPPsC is S_P whereas isomer B is R_P.

Following the development of various nucleoside phosphorothioates (Eckstein, 1983), some thiophospholipids have been synthesized (Nifant'ev et al., 1978; Chupin et al., 1979) and used in biochemical and biophysical studies (Bruzik et al., 1982, 1983; Tsai et al., 1983; Vasilenko et al., 1982; Orr et al., 1982; Hui et al., 1983). However, most of the earlier work dealt with a diastereomeric mixture of thiophospholipids. Recently, we have reported (Bruzik et al., 1982, 1983) that 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC)[1] (**1**) exists in two diastereomers designated as isomer A (^{31}P NMR δ 56.12 in $CDCl_3$) and isomer B (^{31}P NMR δ 56.07 in $CDCl_3$). Phospholipases A_2 and C were shown to hydrolyze isomers B

[†] From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received September 13, 1983.* This work was supported by National Institutes of Health Research Grant GM 30327. The NMR facilities used were funded in part by Grants NIH GM 27431 and NSF CHE 7910019. This is paper 7 in the series "Phospholipids Chiral at Phosphorus". For paper 6, see Bruzik & Tsai (1984b). M.-D.T. is an Alfred P. Sloan Fellow, 1983–1985.

[1] Abbreviations: DOC, deoxycholate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; DPPsE, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine; EDTA, ethylenediaminetetraacetate; HMDSA, 1,1,1,3,3,3-hexamethyldisilazane; Mops, 3-(*N*-morpholino)propanesulfonic acid; MPPsE, 1-palmitoyl-*sn*-glycero-3-thiophosphoethanolamine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/84/0423-1661$01.50/0