Reprinted from the Journal of the American Chemical Society, **1982,** *104,* 863.

Phospholipids Chiral at Phosphorus. 1. Stereochemistry of Transphosphatidylation Catalyzed by Phospholipase D[1]

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Received October 8, 1981

The recent research activity concerning the stereochemistry of biological processes at phosphorus features the following: introduction of phosphorothioates;[2] synthesis and application of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters,[3] chiral inorganic [^{16}O,^{17}O,^{18}O]thiophosphates,[4] and ATP chirally labeled at all positions;[5] preparation of "substitution-inert" metal–nucleotide complexes of known stereochemical structures;[6] use of metal ion dependence in stereospecificity to assess binding stereochemistry;[7] the development of ^{31}P NMR methods based on an ^{18}O isotope effect[8] and an ^{17}O quadrupolar effect[9] for configurational analysis.

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(1) Supported in part by grants from NIH (GM 30327) and NSF (PCM 8140443). The NMR spectrometer used (WP-200) was funded by a NIH grant (GM 27431). Abbreviations used: ATP, adenosine 5′-triphosphate; DPPE, dipalmitoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; Me_4Si, tetramethylsilane; TLC, thin layer chromatography; NMR, nuclear magnetic resonance.

(2) (a) Eckstein, F. *Acc. Chem. Res.* **1978,** *12,* 204. (b) Eckstein, F. *Methods Enzymol.* in press. (c) Sheu, K.-F. R.; Frey, P. A. *J. Biol. Chem.* **1978,** *253,* 3378. (d) Villafranca, J. J.; Raushel, F. M. *Annu. Rev. Biophys. Bioeng.* **1980,** *9,* 363.

(3) (a) Knowles, J. R. *Annu. Rev. Biochem.* **1980,** *49,* 877. (b) Coderre, J. A.; Medhi, S.; Gerlt, J. A. *J. Am. Chem. Soc.* **1981,** *103,* 1872. (c) Jarvest, R. L.; Lowe, G.; Potter, B. V. L. *J. Chem. Soc., Chem. Commun.* **1980,** 1142.

(4) (a) Webb, M. R. *Methods Enzymol.*, in press. (b) Tsai, M.-D. *Biochemistry* **1980,** *19,* 5310.

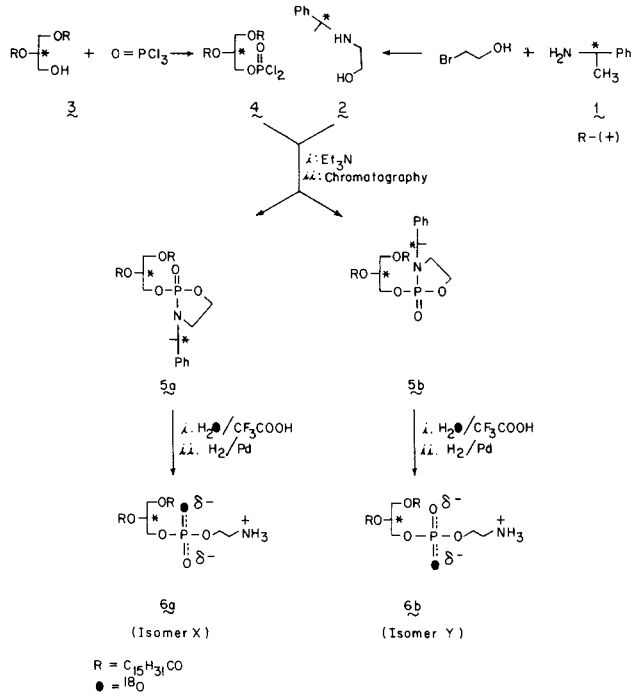
(5) (a) Buchwald, S. L.; Hansen, D. E.; Hassett, A.; Knowles, J. R., *Methods Enzymol.*, in press. (b) Frey, P. A. Ibid., in press. (c) Frey, P. A. *Tetrahedron,* in press. (d) Coderre, J. A.; Gerlt, J. A. *J. Am. Chem. Soc.* **1980,** *102,* 6594.

(6) Cleland, W. W. *Methods Enzymol.*, in press.

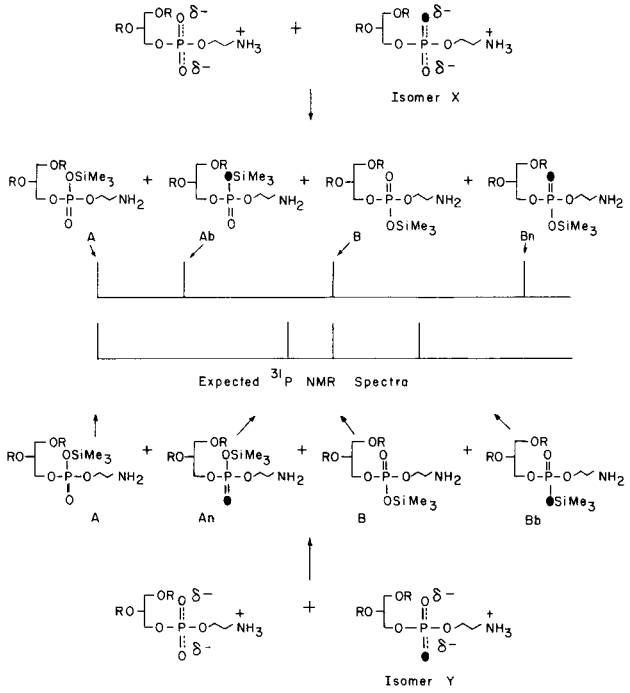
(7) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1979,** *254,* 10839.

(8) Cohn, M. *Annu. Rev. Biophys. Bioeng.*, in press.

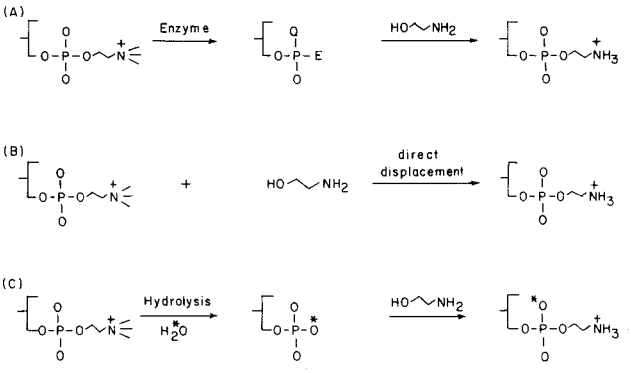
Scheme I. Synthesis of P-Chiral Phosphatidylethanolamines



Scheme II. Analysis of Relative Configurations



Scheme III. Possible Mechanisms of Transphosphatidylation[a]



[a] Charges at oxygen are omitted.

However, an important class of biophosphates, the phospholipids, have been ignored. We have therefore initiated the stereochemical study of phospholipids, aiming at probing the mechanism of phospholipase-catalyzed reactions and the role of the phosphate head group of phospholipids in various membrane functions. We now report the results of our initial study on the elucidation of the stereochemical course of the transphosphatidylation catalyzed by phospholipase D.[10]

Scheme I outlines the synthesis of the two diastereomers of [$^{18}O_1$]dipalmitoylphosphatidylethanolamine (DPPE) (**6a** and **6b**) of unknown configuration. Alkylation of (*R*)(+)-1-methylbenzylamine (**1**)([α]$^{20}_D$ +39.2°, neat) with 2-bromoethanol gave *N*-(1-methylbenzyl)-2-aminoethanol (**2**). Treatment of (*S*)-(−)-1,2-dipalmitin (**3**) (Sigma) with $POCl_3/Et_3N$ mixture (1.5 equiv) gave the phosphorodichloridate **4** which was subjected to condensation with **2** in the presence of Et_3N.[11] The resulting diastereomeric mixture of oxazaphospholidines **5a** and **5b** was then separated by column chromatography on the silica gel (60-μm particle size, using ether as an eluent) which yielded separate diastereomers **5a** and **5b**.[12] Hydrolysis of **5a** and **5b** separately with $H_2{}^{18}O/CF_3COOH$ (99 atom % ^{18}O) in dimethoxyethane[13] followed by hydrogenolysis with H_2/Pd (10% on charcoal, in ethanol, 40–50 °C at atmospheric pressure) gave **6a** and **6b**, respectively. The overall yield is 6.5% for each isomer.

Although the acidic hydrolysis of **5a** and **5b** is expected to proceed with inversion of configuration,[13] the "absolute" configuration of **6a** and **6b** remains to be established since the configuration of **5a** and **5b** has not been determined. However, we developed a procedure (Scheme II) which allows determination of the "relative" configuration and the ^{18}O enrichment based on the ^{18}O isotope shift observed by ^{31}P NMR spectroscopy.[8] It is known that in a P–^{18}O–P or a P–^{18}O–C bridge, ^{18}O causes a smaller isotope shift (0.01–0.02 ppm) than a nonbridging ^{18}O atom does (0.03–0.04 ppm).[8] As shown in Scheme II, silylation of an arbitrary isomer X of [$^{18}O_1$]DPPE (containing unlabeled DPPE) gives four different 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 which result from silylation at the pro-*R* and the pro-*S* oxygen of unlabeled DPPE. 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 Y gives the four species A, An, B, and Bb. The isomers X and Y are therefore expected to show the ^{31}P NMR patterns shown in Scheme II. It should be noted that all formulas in Scheme II describe only *relative* configurations at phosphorus.

Table I. Summary of Configurational Analysis by ^{31}P NMR Spectroscopy

DPPE samples	% species[a] A	Ab	An	B	Bb	Bn	% ^{18}O enrichment	% isomer X	Y
6a	22.0	21.5	5.7	23.4	6.2	21.0	55	72	28
6b	18.1	5.6	27.9	19.1	25.6	3.7	63	17	83
8a	22.4	20.4	8.2	23.1	4.8	21.1	55	69	31
8b	17.0	5.8	26.7	18.4	26.7	5.3	65	21	79

[a] Calculated on the basis of integrals. Estimated relative error ±5%.

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(10) (a) Heller, M. *Adv. Lipid Res.* **1978**, *16*, 267. (b) Saito, M.; Borque, E.; Kaufer, J. *Arch. Biochem. Biophys.* **1974**, *164*, 420. (c) Yang, S. F.; Freer, S.; Benson, A. A. *J. Biol. Chem.* **1967**, *242*, 477. (d) Tzur, R.; Shapiro, B. *Biochim. Biophys. Acta* **1972**, *280*, 290. (d) Heller, M.; Moses, N.; Maes, E. *Methods Enzymol.* **1975**, *35B*, 226.

(11) Kinas, R.; Pankiewicz, K.; Stec, W. J. *Bull. Acad. Pol. Sci., Ser. Sci. Chim.* **1975**, *23*, 981.

(12) The isomer **5a** was eluted off faster; ^{31}P chemical shift 17.22 ppm ($CDCl_3$, relative to external 1 M H_3PO_4), ≥98% isomeric purity, 20% yield from **3**. The isomer **5b** shows ^{31}P δ 16.90, ≥98% isomeric purity, 20% yield.

(13) Abbott, S. J.; Jones, S. R.; Weinman, S. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1978**, *100*, 2558.

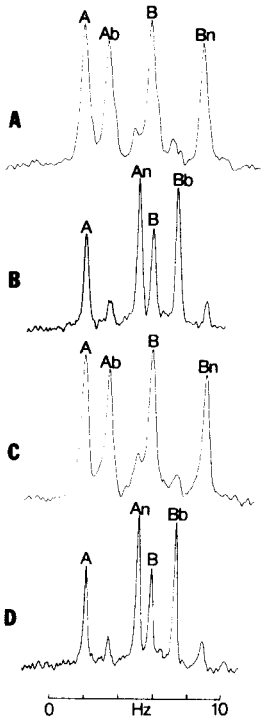
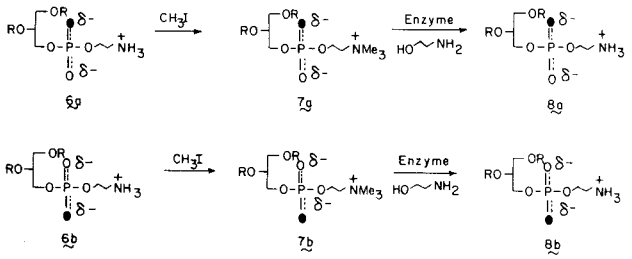


Figure 1. ^{31}P NMR spectra (at 81.0 MHz) of the silylated products of DPPE from **6a** (A, 36 μmol, 500 scans, line broadening 0.2 Hz), **6b** (B, 24 μmol, 1100 scans, line broadening 0.1 Hz), **8a** (C, 10 μmol, 2000 scans, line broadening 0.2 Hz), and **8b** (D, 20 μmol, 3500 scans, line broadening 0.1 Hz). Spectral parameters: Spectral width 500 Hz, 16K data points, ^1H decoupling, 60° pulse, repetition time 16 s.

The observed ^{31}P NMR spectra (at 81.0 MHz) of the silylated products[14] of **6a** and **6b** are shown in Figure 1,A and B, respectively, which shows a 1.45 Hz (0.018 ppm) and a 3.1 Hz (0.038 ppm) shift for bridge and nonbridge ^{18}O, respectively. The spectral analysis is summarized in Table I, which shows that **6a** contains 55% ^{18}O, 72% isomer X, and 28% isomer Y, whereas **6b** contains 63% ^{18}O, 17% isomer X, and 83% isomer Y. The optical purity remains to be improved by a detailed investigation.[15] Samples of higher isotopic purity (>95%) have been obtained, but the partially enriched samples were preferred for the present study.

Despite the imperfect optical purity and the indeterminate configuration of **6a** and **6b**, the stereochemical course of transphosphatidylation catalyzed by phospholipase D could be elucidated. Scheme III shows three most likely mechanisms for this conversion: (A) The reaction proceeds by a two-step process involving a phosphatidyl–enzyme intermediate; (B) the reaction proceeds by a single displacement; (C) the reaction is a reversal of hydrolysis. Although studies on the base exchange reaction have supported mechanism A,[10] there is no direct kinetic or stereochemical evidence to support A or to rule out B or C. A kinetic analysis is complicated by the fact that the reaction medium is heterogeneous.

Scheme IV. Stereochemistry of Transphosphatidylation Catalyzed by Phospholipase D



Our approach is outlined in Scheme IV. The [$^{18}O_1$]DPPE **6a** and **6b** were methylated with CH_3I to give [$^{18}O_1$]dipalmitoylphosphatidylcholine (DPPC) (**7a** and **7b**, respectively)[16] without affecting the configuration at phosphorus. The complete quaternization at nitrogen was characterized by ^1H NMR spectroscopy and TLC by comparing with authentic samples of DPPE and DPPC. Reaction of **7a** and **7b** separately with ethanolamine in H_2O/ether catalyzed by phospholipase D[18] gave DPPE **8a** and **8b**, respectively. The ^{31}P NMR spectra of the silylated products of **8a** and **8b** are shown in Figure 1,C and D, respectively. The spectral analysis in Table I indicates that the transphosphatidylation proceeds with complete retention of configuration and without detectable oxygen exchange.

On the basis of our results, mechanism C in Scheme III can be ruled out since it predicts an exchange of oxygen, assuming free rotation of the phosphoryl group. Mechanism B would predict an inversion of configuration based on the fact that all single-step phosphoryl-transfer reactions which have been studied proceed with inversion of configuration,[2-9] unless the mechanism in phospholipase is an exception which involves pseudorotation. On the basis of the overall retention in the stereochemistry, mechanism A seems to be the most probable mechanism for transphosphatidylation.

(14) Although the ^{31}P NMR signal of DPPE is very broad due to aggregation, the silylated product gives very sharp signals since the O-silylated head group is no more amphiphilic. In Figure 1A–D, the silylation was performed with hexamethyldisilazane (e.g., 25 μmol of DPPE in 2.5 mL of $CDCl_3$, added with 50 μL of reagent) which gives exclusively O silylation as shown by ^{29}Si NMR Spectroscopy at 39.73 MHz (24.66 ppm relative to Me_4Si). The samples can easily be recovered after the NMR experiments.

(15) The loss of diastereomeric purity occurs most likely at the acid hydrolysis of **5a** and **5b**. However, incomplete isomeric purity of starting materials and incomplete separation of **5a** and **5b** may also contribute to a small extent.

(16) The methylation of DPPE under the condition described in ref 17 was found to give a wrong product. Complete and quantitative quaternization of DPPE was achieved by the methylation in a heterogeneous system: DPPE + CH_3I in $CHCl_3$/2 M aqueous K_2CO_3 + $Et_3N^+CH_2C_6H_5Cl^-$.

(17) Eibl, H. *Chem. Phys. Lipids* **1980**, *26*, 239.

(18) A typical reaction mixture contains, in 30 mL of H_2O, 30 μmol of DPPC, 1.2 mmol of $CaCl_2$, 1.2 g of ethanolamine, pH adjusted to 5.6 with HCl, 15 mL of ether, and proper amount of phospholipase D (cabbage, 1–2 IU/mg, Sigma). Stirring at room temperature overnight gives ca. 50% DPPE plus a small amount of phosphatidic acid. No reaction occurs in the absence of the enzyme.