Phospholipids Chiral at Phosphorus. 18. Stereochemistry of Phosphatidylinositide-Specific Phospholipase C[1]

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Received January 18, 1989

Phosphatidylinositides-specific phospholipase C (PI-PLC), a key enzyme in the metabolism of phosphatidylinositides, catalyzes the formation of three second messengers: diacylglycerol, inositol 1,4,5-trisphosphate, and inositol 1,2-cyclic 4,5-trisphosphate.[2-4] Despite its biological significance and its mechanistic uniqueness in producing both cyclic and open inositol phosphates simultaneously, little mechanistic information about this enzyme has been available. We report the stereochemical mechanism of PI-PLC from *Bacillus cereus*.

Scheme I outlines the synthesis of R_p and S_p isomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol (DPPsI). The starting material **1** (DL) was synthesized from *myo*-inositol as described by Garegg et al.[5] Resolution of D and L enantiomers was achieved by derivatization with (−)-camphanic acid chloride

(1) Supported by research Grant GM 30327 from NIH. Paper 17: Sarvis, H. E.; Loffredo, W.; Dluhy, R. A.; Hernqvist, L.; Wisner, D.; Tsai, M.-D. *Biochemistry* **1988**, *27*, 4625–4631.
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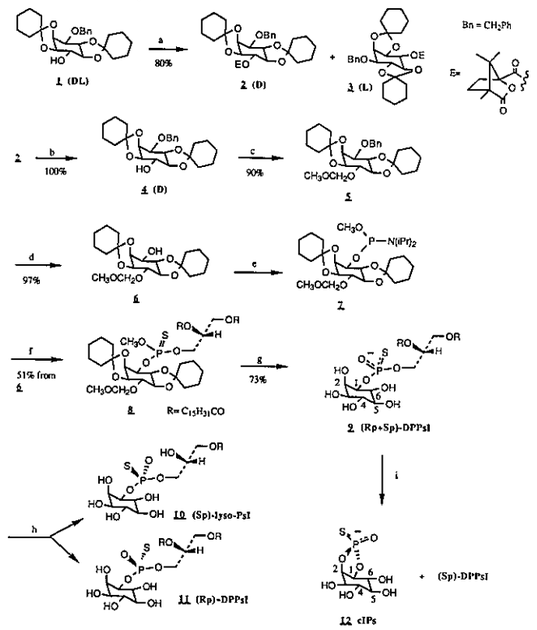
Scheme I. The Synthesis and Reactions of DPPsI[a]



[a] Reagents and conditions: (a) (−)-camphanic acid chloride, Et_3N, 4-(dimethylamino)pyridine, CH_2Cl_2, 25 °C, 7 h; (b) LiOH, THF–H_2O (2:1), 25 °C, 2 h; (c) CH_3OCH_2Cl, iPr_2NEt, CH_2Cl_2, 25 °C, 17 h; (d) Li, THF–NH_3, −78 °C, 0.5 h; (e) $ClP(OCH_3)N(iPr)_2$, Et_3N, CH_2Cl_2, 25 °C, 0.5 h; (f) (i) 1,2-dipalmitoyl-*sn*-glycerol, tetrazole, THF–CH_3CN, 25 °C, 24 h; (ii) S_8, toluene, 25 °C, 47 h; (g) (i) 80% HOAc, 90–100 °C, 2–3 h; (ii) NMe_3, toluene, 25 °C, 15 h; (h) PLA2; (i) PI-PLC.

followed by chromatographic separation of **2** and **3**.[6,7] Deprotection of **2** gave **4**, which was reprotected with chloromethyl methyl ether[8] to give **5**. Debenzylation of **5** gave **6**, which was phosphorylated with $ClP(OCH_3)N(iPr)_2$ to give **7**. The phosphite **7** was converted to **8** directly (without purification) by treating with 1,2-dipalmitoyl-*sn*-glycerol and tetrazole, followed with excess S_8 in toluene.[9] The presence of two diastereomers of **8** was demonstrated by two equal intensity resonances in ^{31}P NMR (101.256 MHz, $CDCl_3$) at 67.63 and 67.93 ppm. A separate sample of **8** derived from DL-**4** gave two additional signals at 67.69 and 67.86 ppm. All intermediates were characterized by ^1H and ^{13}C NMR. (R_p+S_p)-DPPsI (**9**) was obtained by deprotection of D-**8** with acetic acid followed with demethylation with trimethylamine and characterized by ^1H and ^{13}C NMR and fast atom bombardment mass spectroscopy. The ^{31}P NMR spectrum of **9** (δ 55.15 and 55.56 ppm) is shown in Figure 1A.

Assignment of the resonances in Figure 1A was based on the observation that the isomer at 55.56 ppm was hydrolyzed by bee venom phospholipase A_2 (PLA2), with concomitant formation of lyso-DPPsI (**10**) at 55.15 ppm (spectrum not shown). It has been established that PLA2 specifically hydrolyzes the R_p isomer of thiophosphatidylcholine and thiophosphatidylethanolamine.[10]

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(7) The percent diastereomeric excess (% de) of **2** was determined to be 91% from ^{13}C NMR (75.48 MHz) under nonsaturating conditions. However, the sample actually used for the large-scale synthesis of **9** was less pure (ca. 70% de).

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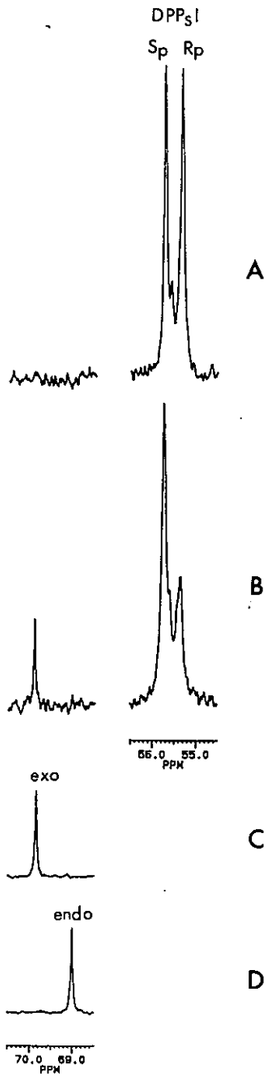


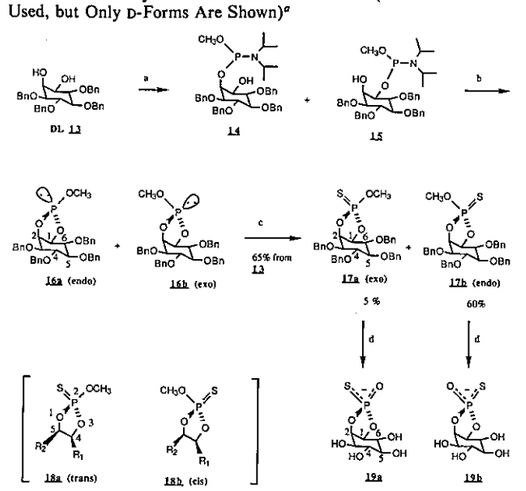
Figure 1. Use of ^{31}P NMR (101.2 MHz) to show the stereospecificity of PI-PLC. (A) 7.5 mg of (R_p+S_p)-DPPsI in D_2O containing 5% Triton X-100, 50 mM HEPES buffer, pD 7.2, 2.5 mM Ca^{2+}, and 0.25 mM EDTA. (B) After addition of PI-PLC. (C) **19a** (*exo*-DL-cIPs). (D) **19b** (*endo*-DL-cIPs). The minor peak in A and B (and another one with similar intensity, unresolved in the present spectra) can be attributed to a small amount of DPPsI derived from contaminating L-**3**.[7]

However, it should be noted that *due to a change in priority, the relative configurations of* (R_p)- *and* (S_p)-*DPPsI correspond to those of the* S_p *and* R_p *isomers, respectively, of thiophosphatidylcholine.* Thus the downfield resonance is assigned the S_p isomer.

Figure 1B shows that PI-PLC from *Bacillus cereus*[11-13] specifically converts the R_p isomer of DPPsI to inositol 1,2-cyclic thiophosphate (cIPs) (**12**) (^{31}P δ 69.89 ppm, characteristic of cyclic thiophosphates) as the predominant product. Thus despite differences in substrate specificity, structure, and function, PI-PLC exhibits the same stereospecificity as phosphatidylcholine-specific PLC (PC-PLC), which prefers the S_p isomer of thiophosphatidylcholine.[10b-d]

To elucidate the steric course of PI-PLC requires cIPs with known configuration. Thus, DL-cIPs was synthesized according to Scheme II. DL-1,4,5,6-Tetra-*O*-benzyl-*myo*-inositol (**13**; prepared by established procedures[14]) was phosphorylated by $ClP(OCH_3)N(iPr)_2$ to give **14** and **15**, which were then treated with tetrazole in THF–CH_3CN to produce **16(a+b)** via a novel intramolecular cyclization.[15] Without isolation, **16** was treated with an excess of S_8 in toluene to give **17a** (^{31}P δ 84.41 ppm, *exo*-DL, i.e. D-R_p + L-S_p)[16] and **17b** (^{31}P δ 82.65 ppm, *endo*-DL, i.e. D-S_p + L-R_p), which were separated by chromatography. Assignments of the configurations of **17a** and **17b** were based on four criteria, the first three of which had been established previously on model compounds **18a**, **18b**, and related systems: (i) The predominant form **17b** should be endo since the predominant form of the phosphite **16** should be the least sterically hindered form **16b**,[17] and oxidation by sulfur is known to proceed with retention of configuration at phosphorus.[18] (ii) The relative ^{31}P δ of **17a** and **17b** thus assigned are consistent with that of **18a** and **18b** (83.0 and 80.5 ppm, respectively, when $R_1 = R_2 = CH_3$) in that the trans (exo) form is more downfield.[17b,19] (iii) The three-bond coupling constants between P and 1-H are 18.4 and 9.7 Hz for **17a** and **17b**, respectively. These are consistent with the data for **18a**, **18b**, and related compounds ($^3J_{H-C(4)-O-P}$ is **a** > **b**), and with the empirical rule that the OCH_3 group is "axial seeking" in these systems.[19,20] (iv) Irradiation of 2-H resulted in detectable nuclear Overhauser effect on the methyl proton resonance in **17b** but not **17a**. Detailed NMR assignments and conformational analysis will be presented later.

Scheme II. The Synthesis of Endo and Exo cIPs (DL Mixtures Were Used, but Only D-Forms Are Shown)[a]



[a] Reagents and conditions: (a) 1.2 equiv $ClP(OCH_3)N(iPr)_2$, iPr_2NEt, CH_2Cl_2, 25 °C, 0.5 h; (b) 4 equiv tetrazole, THF–CH_3CN, 25 °C, 18 h; (c) excess S_8, toluene, 25 °C, 48 h; (d) 40 equiv Li, THF–NH_3, −78 °C, 5 min.

The synthesis was completed by treating **17a** and **17b** with Li in THF–NH_3(l) to give **19a** (exo[16], ^{31}P δ 69.85 ppm, Figure 1C) and **19b** (endo, ^{31}P δ 69.00 ppm, Figure 1D), respectively. The ^{31}P δ of **19a** coincides with that of **12**, which was further confirmed by addition of **19a** to the reaction mixture in Figure 1B (spectrum not shown). Thus the configuration of **12** should be D-R_p, and the steric course should be *inversion* at phosphorus. The result suggests that the conversion of PI to cIP catalyzed by PI-PLC from *B. cereus* involves direct attack of the 2-OH group to displace the diacylglycerol moiety of the substrate. The steric course of the formation of the noncyclic IP awaits future studies.

Application of phosphorothioates on PI-related systems has also been realized by other groups recently. Chemical synthesis of DL-cIPs[21] by a different procedure has been reported, but the configuration was not determined. The phosphorothioate analogues of DL-*myo*-inositol phosphates have been synthesized[22] and shown to be resistant to hydrolysis by phosphatases.[22c]

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(13) The PI-PLC used in this work was obtained from Sigma (which consists of a mixture of PC-PLC, PI-PLC, and sphingomyelinase) and further purified by fast protein liquid chromatography. Sundler, R.; Alberts, A. W.; Vagelos, P. R. *J. Biol. Chem.* **1978**, *253*, 4175–4179.

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(15) To the best of our knowledge, this is the first example of using $ClP(OCH_3)N(iPr)_2$ as a phosphorylating and intramolecular cyclization agent.

(16) The exo form of **17** and **19** is defined as the form in which sulfur and the inositol ring are on the opposite side of the five-membered ring. In the *R*/*S* designation, the axial position has higher priority than the equatorial position when all things are equal.

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