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Phospholipids Chiral at Phosphorus. Stereochemical Mechanism of Reactions Catalyzed by Phosphatidylinositide-Specific Phospholipase C from *Bacillus cereus* and Guinea Pig Uterus†

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ABSTRACT: (R_p)- and (S_p)-1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol (DPPsI) were synthesized as a mixture and their configurations assigned on the basis of the stereospecific hydrolysis catalyzed by phospholipase A_2 (PLA2) from bee venom. PLA2 is known to be stereospecific to the R_p isomer of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) and 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE). Since the configurations of (R_p)- and (S_p)-DPPsI correspond to those of (S_p)- and (R_p)-DPPsC, respectively, due to a change in priority, the isomer specifically hydrolyzed by PLA2 was assigned to (S_p)-DPPsI. The DPPsI analogues were then used to probe the mechanism and to elucidate the steric course of the reaction catalyzed by phosphatidylinositide-specific phospholipase C (PI-PLC) from *Bacillus cereus* and for both isozyme I and isozyme II of PI-PLC from guinea pig uterus. It was found that the R_p isomer of DPPsI is the preferred substrate for all three PI-PLCs. Thus PI-PLC shows the same stereospecificity as phosphatidylcholine-specific PLC (PC-PLC), which prefers the S_p isomer of DPPsC. The ratio of the two products inositol 1,2-cyclic phosphorothioate (cIPs) and inositol phosphorothioate (IPs) was not significantly perturbed by the use of phosphorothioate analogue for all three PI-PLCs, which implies that IPs is not produced by enzyme-mediated ring opening of cIPs and supports a parallel pathway for the formation of both products. In order to elucidate the steric course of the cyclization reaction, exo and endo isomers of cIPs were synthesized and their absolute configurations at phosphorus were determined by nuclear magnetic resonance and other techniques. It was found that *exo*-cIPs is the product produced by all three PI-PLCs. Thus the steric course of the conversion DPPsI to cIPs catalyzed by all three PI-PLCs was inversion of configuration at phosphorus. These results taken together suggest that the reaction catalyzed by PI-PLC most likely proceeds via direct attack by the 2-OH group to generate the cyclic product, and parallelly by water to generate the noncyclic inositol phosphates, without involving a covalent enzyme–phosphoinositol intermediate.

Phosphatidylinositides are important phospholipids since their metabolism is highly responsive to various extracellular stimuli acting on the cell (Michell, 1975). A key enzyme for this metabolism is phosphatidylinositide-specific phospholipase C (PI-PLC)[1] [for recent reviews see Majerus et al. (1986) and Shukla (1982)]. PI-PLC has been found in many mammalian tissues and in some bacteria. It uses phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), or phosphatidylinositol 4,5-bisphosphate (PIP_2), and possibly other phosphatidylinositides as a substrate. The relative specificity of these substrates varies with the source of the enzyme, but PI is an acceptable substrate in most cases and is the substrate used in this study. The enzyme is biologically significant since it generates three second messengers from PIP_2: diacylglycerol, inositol 1,4,5-trisphosphate, and inositol 1,2-cyclic 4,5-trisphosphate (Berridge, 1984, 1987). It is mechanistically unique in that it produces both inositol phosphates and inositol cyclic phosphates simultaneously, at a ratio dependent on the source

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[1] Abbreviations: cIP, inositol 1,2-cyclic phosphate; cIPs, inositol 1,2-cyclic phosphorothioate; de, diastereomeric excess; COSY, correlated spectroscopy; DEPT, distorsionless enhancement by polarization transfer; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-1-*myo*-inositol; EDTA, ethylenediaminetetraacetate; FAB, fast atom bombardment; GC, gas chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IP, inositol 1-phosphate; IPs, inositol 1-phosphorothioate; MPPsI, 1-palmitoyl-*sn*-glycero-3-thiophosphoinositol; MOMCl, chloromethyl methyl ether; MPLC, medium-pressure liquid chromatography; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositide-specific phospholipase C; PC-PLC, phosphatidylcholine-specific PLC; PLA2, phospholipase A_2.

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of the enzyme and other conditions such as pH (Dawson et al., 1971; Michell, 1975; Majerus et al., 1986, 1988).

The enzymes used in this study are PI-PLC from *Bacillus cereus* and isozyme I and isozyme II of the PI-PLC from guinea pig uterus. The *Bacillus* PI-PLC [for review see Shukla (1982); Ikezawa & Taguchi, 1986] is a metal ion independent enzyme whereas the mammalian PI-PLC requires Ca^{2+} (Majerus et al., 1986). Little mechanistic information about PI-PLC has been available. Two basic mechanistic questions for the catalysis of this enzyme are whether the reactions involve a covalent phosphoinositol–enzyme intermediate, and whether the two products are formed from a parallel or sequential pathway. We set out to probe these and other questions by the following sequence: (i) synthesis of the phosphorothioate analogues of phosphatidylinositol, (R_p+S_p)-DPPsI; (ii) assignment of their configurations; (iii) determination of the stereospecificity of PI-PLC toward DPPsI; (iv) synthesis of the two possible isomers of the cyclic product of PI-PLC, *exo*- and *endo*-cIPs; (v) elucidation of the configurations of cIPs; and (vi) elucidation of the steric course of PI-PLC. A preliminary account of part of this work (*B. cereus* enzyme only) has been published recently (Lin & Tsai, 1989).

Materials and Methods

Materials. PI (sodium salt) from bovine liver was purchased from Avanti Polar Lipids. Bee venom PLA2 was purchased from Boehringer Mannheim. Other biochemicals were purchased from Sigma. All other chemicals were reagent grade. Silica gel used in liquid chromatography (Licorpre Silica 60, 200–400 mesh) and thin-layer chromatography (60 F_{254}) was obtained from EM reagent (Merck). Isozyme I [60% pure, 210 nmol/(mg·min)] and isozyme II [80% pure, 10 nmol/(mg·min)] of PI-PLC from guinea pig uterus were purified as described by Bennett and Crooke (1987). PI-PLC from *B. cereus* was purified from PLC purchased from Sigma, which contained three enzymes: PC-PLC, sphingomyelin-specific PLC, and PI-PLC (Sundler et al., 1978).

Chromatographic and Instrumental Methods. Most liquid chromatography was performed under medium pressure (ca. 20 psi) on a Licorpre silica 60 column. ^{1}H NMR spectra were recorded on a Bruker WM-300, AM-250, or AM-500 spectrometer. With Waltz-16 ^{1}H decoupling, ^{13}C NMR spectra were obtained at 75.44, 62.90, and 125.76 MHz on a Bruker WM-300, AM-250, or AM-500 spectrometer, and ^{31}P NMR spectra were obtained at 101.25 and 121.50 MHz on a Bruker AM-250 or WM-300 spectrometer. ^{31}P chemical shifts are referenced to external 85% H_3PO_4. The ^{1}H and ^{13}C chemical shifts are reference to external sodium 3-(trimethylsilyl)-1-propanesulfonate for D_2O samples and to Me_4Si for organic solvents. Assignments of proton chemical shifts and coupling constants were routinely assisted by homonuclear decoupling, and occasionally by other specified experiments. FAB mass spectra were recorded on a VG-70-250S mass spectrometer. Melting point was recorded on a Thomas-Hoover capillary melting point apparatus without calibration. Gas chromatography was performed on a Hewlett-Packard 5890 machine. The MM-2 energy minimization was calculated by using MODEL, CHEMX, MacroModel, or Chem 3D programs.

Synthesis of (R_p+S_p)-DPPsI. The starting compound **1** was prepared according to Garegg et al. (1984). D-1-*O*-Benzyl-4-*O*-(−)-camphanoyl-2,3:5,6-di-*O*-cyclohexylidene-*myo*-inositol (**2**) and L-3-*O*-benzyl-6-*O*-(−)-camphanoyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**3**) were obtained from **1** according to the procedure of Billington et al. (1987). The L form (**3**) was more polar (R_f = 0.55, petroleum ether–ethyl acetate, 3/1 v/v) and was obtained by recrystallization (petroleum ether–ethyl acetate, 2/1 v/v) four times from the mixture (628 mg). The D form (**2**) was less polar (R_f = 0.62, petroleum ether–ethyl acetate, 3/1 v/v) and was recovered from combined mother liquors by liquid chromatography on silica gel (petroleum ether–ethyl acetate, 3/1 v/v) (797 mg, 44%). D form (**2**): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.43–7.26 (m, 5 H, phenyl of 1-*O*-benzyl), 5.36 (dd, $J_{H\text{-}C(6)\text{-}C(1)\text{-}H}$ = 7.0 Hz, $J_{H\text{-}C(6)\text{-}C(5)\text{-}H}$ = 11.2 Hz, 1 H, 6-H), 4.94 and 4.86 (AB, J = 12.4 Hz, 2 H, CH_2 of 1-*O*-benzyl), 4.36 (t, $J_{H\text{-}C(2)\text{-}C(1)\text{-}H}$ = $J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 4.4 Hz, 1 H, 2-H), 4.15 (dd, $J_{H\text{-}C(4)\text{-}C(5)\text{-}H}$ = 9.6 Hz, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = 10.2 Hz, 1 H, 4-H), 4.11 (dd, $J_{H\text{-}C(1)\text{-}C(2)\text{-}H}$ = 4.1 Hz, $J_{H\text{-}C(1)\text{-}C(6)\text{-}H}$ = 7.2 Hz, 1 H, 1-H), 3.78 (dd, $J_{H\text{-}C(3)\text{-}C(2)\text{-}H}$ = 4.1 Hz, $J_{H\text{-}C(3)\text{-}C(4)\text{-}H}$ = 10.3 Hz, 1 H, 3-H), 3.38 (dd, $J_{H\text{-}C(5)\text{-}C(4)\text{-}H}$ = 9.4 Hz, $J_{H\text{-}C(5)\text{-}C(6)\text{-}H}$ = 11.1 Hz, 1 H, 5-H), 2.48–1.70 (m, 4 H, CH_2 of camphanoyl), 1.58–1.42 (m, 20 H, CH_2 of 2,3:5,6-di-*O*-cyclohexylidene), 1.11 (s, 3 H, camphanoyl 9-CH_3), 1.04 (s, 3 H, camphanoyl 8-CH_3), 0.99 (s, 3 H, camphanoyl 10-CH_3); ^{13}C NMR ($CDCl_3$, 75.44 MHz) δ 178.27 (camphanoyl C-11), 166.44 (camphanoyl C-2), 137.83 (phenyl C-1 of 1-*O*-benzyl), 128.33 and 128.15 (phenyl C-3 and C-5 of 1-*O*-benzyl), 127.95 (phenyl C-2 and C-6 of 1-*O*-benzyl), 127.46 (phenyl C-4 of 1-*O*-benzyl), 113.22 (tertiary C-1 of 5,6-*O*-cyclohexylidene), 111.10 (tertiary C-1 of 2,3-*O*-cyclohexylidene) (the assignment of this resonance was made by reference to the compound containing only a 2,3-cyclohexylidene group), 91.10 (camphanoyl C-4), 76.90, 76.32, 78.25, 75.98, 75.74, 74.38, and 71.71 (CH_2 of 1-*O*-benzyl and six inositol carbons), 54.78 and 54.68 (camphanoyl C-1 and C-7), 37.50, 36.33, 36.18, and 35.20 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene); 24.91, 24.86, 23.78, 23.60, and 23.58 (C-3, C-4, and C-5 of 2,3:5,6-di-*O*-cyclohexylidene), 30.28 (camphanoyl C-6), 28.90 (camphanoyl C-5), 16.54 (camphanoyl C-9), 16.33 (camphanoyl C-8), 9.67 (camphanoyl C-10). L form (**3**): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.36–7.20 (m, 5 H, phenyl of 1-*O*-benzyl), 5.32 (dd, $J_{H\text{-}C(6)\text{-}C(1)\text{-}H}$ = 7.0 Hz, $J_{H\text{-}C(6)\text{-}C(5)\text{-}H}$ = 11.2 Hz, 1 H, 6-H), 4.82 and 4.73 (AB, J = 12.4 Hz, 2 H, CH_2 of 1-*O*-benzyl), 4.27 (t, $J_{H\text{-}C(2)\text{-}C(1)\text{-}H}$ = $J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 4.1 Hz, 1 H, 2-H), 4.08 (t, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = $J_{H\text{-}C(4)\text{-}C(5)\text{-}H}$ = 9.8 Hz, 1 H, 4-H), 3.97 (dd, $J_{H\text{-}C(1)\text{-}C(2)\text{-}H}$ = 4.9 Hz, $J_{H\text{-}C(1)\text{-}C(6)\text{-}H}$ = 6.7 Hz, 1 H, 1-H), 3.71 (dd, $J_{H\text{-}C(3)\text{-}C(2)\text{-}H}$ = 4.0 Hz, $J_{H\text{-}C(3)\text{-}C(4)\text{-}H}$ = 10.2 Hz, 1 H, 3-H), 3.31 (t, $J_{H\text{-}C(5)\text{-}C(4)\text{-}H}$ = $J_{H\text{-}C(5)\text{-}C(6)\text{-}H}$ = 10.6 Hz, 1 H, 5-H), 2.39 and 2.12–1.70 (m, 4 H, CH_2 of camphanoyl), 1.53–1.40 (m, 20 H, CH_2 of 2,3:5,6-di-*O*-cyclohexylidene), 1.04 (s, 3 H, camphanoyl 9-CH_3), 0.98 (s, 3 H, camphanoyl 8-CH_3), 0.93 (s, 3 H, camphanoyl 10-CH_3); ^{13}C NMR ($CDCl_3$, 62.90 MHz) δ 178.05 (camphanoyl C-11), 166.21 (camphanoyl C-2), 137.80 (phenyl C-1 of 1-*O*-benzyl), 128.31 (phenyl C-3 and C-5 of 1-*O*-benzyl), 128.14 (phenyl C-2 and C-6 of 1-*O*-benzyl), 127.77 (phenyl C-4 of 1-*O*-benzyl), 113.30 (tertiary C-1 of 5,6-*O*-cyclohexylidene), 111.16 (tertiary C-1 of 2,3-*O*-cyclohexylidene), 91.03 (camphanoyl C-4), 78.38, 77.03, 76.31, 75.96, 75.64, 74.18, and 71.70 (CH_2 of 1-*O*-benzyl and six inositol carbons), 54.69 (camphanoyl C-1 and C-7), 37.21, 36.28, 36.20, and 35.28 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene), 24.89, 24.84, 23.78, and 23.58 (C-3, C-4, and C-5 of 2,3:5,6-di-*O*-cyclohexylidene), 30.16 (camphanoyl C-6), 28.98 (camphanoyl C-5), 16.47 (camphanoyl C-9), 16.22 (camphanoyl C-8), 9.63 (camphanoyl C-10). The % de of these two diastereomers was calculated by the relative intensities of the camphanoyl C-11 resonance from ^{13}C NMR spectra under the following nonsaturating conditions: 75.44 MHz, $CDCl_3$, 45° pulse, and 5-s delay. The % de was also calculated by the relative intensities from the GC traces. The

% de of these products after one fractional recrystallization and one MPLC purification is about 75–90. The % de of these products after two fractional recrystallizations and two MPLC purifications is about 90–100. MS (for both isomers): m/z 610 (M^+), 397, 199, and 91.

D-*1-O-Benzyl-2,3:5,6-di-O-cyclohexylidene-myo-inositol* (**4**). A mixture of 145 mg (0.241 mmol) of **2**, 25 mL of tetrahydrofuran, 100 mg (10 equiv) of $LiOH·H_2O$, and 12.5 mL of water was stirred at room temperature for 25 h. To the reaction mixture were added 100 mL of dichloromethane and 100 mL of water. The organic layer was washed with 100 mL of saturated NaCl, 100 mL of saturated $NaHCO_3$, and 100 mL of water. The crude product was further purified by flash chromatography on silica gel (chloroform–acetone, 10/1 v/v) to give a semisolid (100 mg, 98.8% yield): TLC R_f = 0.53 (chloroform–acetone, 10/1 v/v); ^1H NMR ($CDCl_3$, 250 MHz) δ 7.38–7.19 (m, 5 H, phenyl of 1-*O*-benzyl), 4.82 and 4.74 (AB, J = 12.5 Hz, 2 H, CH_2 of 1-*O*-benzyl), 4.29 (t, $J_{H\text{-}C(2)\text{-}C(3)\text{-}H} = J_{H\text{-}C(2)\text{-}C(1)\text{-}H}$ = 4.4 Hz, 1 H, 2-H), 3.96 (t, $J_{H\text{-}C(6)\text{-}C(1)\text{-}H} = J_{H\text{-}C(6)\text{-}C(5)\text{-}H}$ = 10.0 Hz, 1 H, 6-H), 3.85 (dd, $J_{H\text{-}C(1)\text{-}C(6)\text{-}H}$ = 9.5 Hz, $J_{H\text{-}C(1)\text{-}C(2)\text{-}H}$ = 4.9 Hz, 1 H, 1-H), 3.77 (ddd, $J_{H\text{-}C(4)\text{-}C(5)\text{-}H}$ = 9.6 Hz, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = 10.2 Hz, $J_{H\text{-}C(4)\text{-}O\text{-}H}$ = 2.5 Hz, 1 H, 4-H), 3.70 (dd, $J_{H\text{-}C(3)\text{-}C(2)\text{-}H}$ = 4.2 Hz, $J_{H\text{-}C(3)\text{-}C(4)\text{-}H}$ = 10.2 Hz, 1 H, 3-H), 3.18 (dd, $J_{H\text{-}C(5)\text{-}C(4)\text{-}H}$ = 9.6 Hz, $J_{H\text{-}C(5)\text{-}C(6)\text{-}H}$ = 10.1 Hz, 1 H, 5-H), 2.55 (d, $J_{H\text{-}C(4)\text{-}O\text{-}H}$ = 2.6 Hz, 1 H, OH), 1.58–1.35 (m, 20 H, CH_2 of 2,3:5,6-di-*O*-cyclohexylidene); ^{13}C NMR ($CDCl_3$, 62.90 MHz, DL form) δ 138.08 (phenyl C-1 of 1-*O*-benzyl), 128.35 (phenyl C-2 and C-6 of 1-*O*-benzyl), 128.15 (phenyl C-3 and C-5 of 1-*O*-benzyl), 127.74 (phenyl C-4 of 1-*O*-benzyl), 113.02 (tertiary C-1 of 5,6-*O*-cyclohexylidene), 110.76 (tertiary C-1 of 2,3-*O*-cyclohexylidene), 81.35 (CH_2 of 1-*O*-benzyl), 78.21, 76.78, 76.18, 74.99, 74.76, and 71.64 (six inositol carbons), 37.78, 36.55, 36.33, and 35.18 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene), 25.01, 24.97, 23.94, 23.77, and 23.59 (C-3, C-4 and C-5 of 2,3:5,6-di-*O*-cyclohexylidene).

D-*1-O-Benzyl-2,3:5,6-di-O-cyclohexylidene-4-(methoxymethyl)-myo-inositol* (**5**). To **4** (100 mg, 0.238 mmol) in dichloromethane (25 mL), 0.96 mmol of iPr_2NEt and 0.96 mmol of MOMCl were added and the mixture was stirred at room temperature for 4.5 h. Excess MOMCl, iPr_2NEt, and solvent were removed by rotary evaporation. The crude product was purified by flash chromatography on silica gel in hexane–ethyl acetate (5/1 v/v) to give a white solid (100 mg, 90%yield): mp 110–113 °C; TLC R_f = 0.5 (hexane–ethyl acetate, 5/1 v/v); ^1H NMR ($CDCl_3$, 250 MHz) δ 7.45–7.26 (m, 5 H, phenyl of 1-*O*-benzyl), 4.90 and 4.78 (AB, J = 6.4 Hz, 2 H, CH_2 of 4-*O*-MOM), 4.89 and 4.82 (AB, J = 12.5 Hz, 2 H, CH_2 of 1-*O*-benzyl), 4.33 (t, $J_{H\text{-}C(2)\text{-}C(1)\text{-}H} = J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 4.4 Hz, 1 H, 2-H), 4.10–3.88 (m, 3 H) and 3.73 (dd, J = 4.2 Hz, 10.1 Hz, 1 H) (1-H, 3-H, 4-H, and 6-H); 3.43 (s, 3 H, CH_3 of 4-*O*-MOM), 3.26 (dd, J = 10.3, 9.4 Hz, 1 H, 5-H), 1.82–0.77 (m, 20 H, CH_2 of 2,3:5,6-di-*O*-cyclohexylidene); ^{13}C NMR ($CDCl_3$, 62.90 MHz) δ 138.08 (phenyl C-1 of 1-*O*-benzyl), 128.32 (phenyl C-2 and C-6 of 1-*O*-benzyl), 128.16 (phenyl C-3 and C-5 of 1-*O*-benzyl), 127.71 (phenyl C-4 of 1-*O*-benzyl), 112.74 (C-1 of 5,6-*O*-cyclohexylidene), 110.50 (C-1 of 2,3-*O*-cyclohexylidene), 95.20 (CH_2 of 4-*O*-MOM), 80.26, 78.01, 76.94, 76.66, 76.18, 74.56, and 71.60 (CH_2 of 1-*O*-benzyl and six inositol carbons), 55.23 (CH_3 of 4-*O*-MOM), 37.57, 36.42, and 35.33 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene), 25.04, 24.98, 23.90, 23.78, and 23.58 (C-3, C-4, and C-5 of 2,3:5,6-di-*O*-cyclohexylidene).

D-*2,3:5,6-Di-O-cyclohexylidene-4-O-(methoxymethyl)-myo-inositol* (**6**). (*A*) *Hydrogenolysis Method.* To **5** (100 mg, 0.215 mmol) in 40 mL of ethyl acetate was added 159 mg of 10% Pd in activated carbon at room temperature. The solution was connected to hydrogen gas (1 atm) and stirred at room temperature for 24 h. The filtrate (from Celite) was evaporated to dryness, and the product was purified by flash chromatography on silica gel in chloroform–acetone (10/1 v/v) to give 80 mg (97% yield) of white solid.

(*B*) *Li or Na–NH_3 Method.* To a dry tetrahydrofuran (15 mL, over Na) solution of **5** (232 mg, 0.5 mmol), Li or Na (about 160 mg) was added at −78 °C (dry ice–tetrahydrofuran bath). Dry NH_3 (over Na) was distilled into the reaction mixture and stirred at −78 °C for 1 h. To this reaction mixture, 300 mg of NH_4Cl was added at −78 °C and stirred from −78 °C to room temperature. The reaction mixture was diluted with 200 mL of petroleum ether. The organic layer was washed with 3 × 50 mL of water, 100 mL of saturated NaCl, and 100 mL of water and dried over $CaCl_2$. The product was purified by flash chromatography on silica gel in chloroform–acetone (10/1 v/v) to give **6** (190 mg, 100% yield): TLC R_f = 0.14 (hexane–ethyl acetate, 5/1 v/v); ^1H NMR ($CDCl_3$, 250 MHz) δ 4.90 and 4.78 (AB, J = 6.4 Hz, 2 H, CH_2 of 4-*O*-MOM); 4.44 (t, $J_{H\text{-}C(2)\text{-}C(1)\text{-}H} = J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 4.7 Hz, 1 H, 2-H); 4.11 (dd, J = 5.1 Hz, J = 6.6 Hz, 1 H) and 3.94–3.79 (m, 3 H) (1-H, 3-H, 4-H, and 6-H); 3.43 (s, 3 H, CH_3 of 4-*O*-MOM); 3.31 (dd, J = 10.3, 9.4 Hz, 1 H, 5-H); 1.74–0.76 (m, 20 H, CH_2 of 2,3:5,6-di-*O*-cyclohexylidene); ^{13}C NMR ($CDCl_3$, 62.90 MHz) δ 112.99 (C-1 of 5,6-*O*-cyclohexylidene); 110.58 (C-1 of 2,3-*O*-cyclohexylidene); 95.18 (CH_2 of 4-*O*-MOM); 80.41, 77.92, 77.24, 77.09, 77.00, and 69.98 (six inositol carbons); 55.24 (CH_3 of 4-*O*-MOM); 37.59, 36.53, 36.29, and 35.18 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene); 24.97, 24.87, 23.84, 23.76, and 23.68 (C-3, C-4, and C-5 of 2,3:5,6-di-*O*-cyclohexylidene).

1,2-Dipalmitoyl-sn-glycero-3-(O-methylthiophospho)-2,3:5,6-di-O-cyclohexylidene-4-O-(methoxymethyl)-myo-inositol (**8**). Compound **6** (0.47 mmol) was dried three times by rotary evaporation with 5 mL of dry toluene. To this dry compound, 5 mL of dichloromethane (dried over Na_2SO_4) and an excess of triethylamine (dried over NaH) were added through vacuum transfer. To this reaction mixture, chloro-(*N*,*N*-diisopropylamino)methoxyphosphine (0.70 mmol) was added through a dry syringe under the argon atmosphere. After the reaction was complete (about 25 min) on the basis of the disappearance of **6** on TLC, the solvent and excess triethylamine were removed under vacuum, and the reaction mixture was further dried in vacuo (0.2 mmHg) for 4 h. In a separate flask, thoroughly dried tetrazole (1.88 mmol) and 1,2-dipalmitoyl-*sn*-glycerol (0.56 mmol) were dissolved in tetrahydrofuran–acetonitrile (1/1 v/v) through vacuum transfer. After being warmed to 40 °C to make sure all compounds were dissolved, the solution was added to the above reaction mixture by a syringe under argon. After stirring for 18 h at room temperature, the reaction was complete on the basis of TLC. The solvents were evaporated to dryness and replaced with 10 mL of dry toluene (distilled over NaH). To this heterogeneous mixture, an excess of S_8 (powder, sublimed) was added at room temperature. The suspension was stirred at room temperature for 2 days. The reaction mixture was then washed with 25 mL of saturated $NaHCO_3$, and the organic phase was evaporated to dryness. The crude product was purified by liquid chromatography on silica gel in dichloromethane–diethyl ether (95/5 v/v) to give **8** (an oil, 405 mg, 83% yield from **6**): TLC (dichloromethane–diethyl ether, 95/5 v/v) R_f = 0.7; ^1H NMR (CD_3OD, 250 MHz) δ 5.24 (m, 1 H, glycero-*sn*-2-CH); 4.88 and 4.76 (AB, J = 6.4 Hz, 2 H,

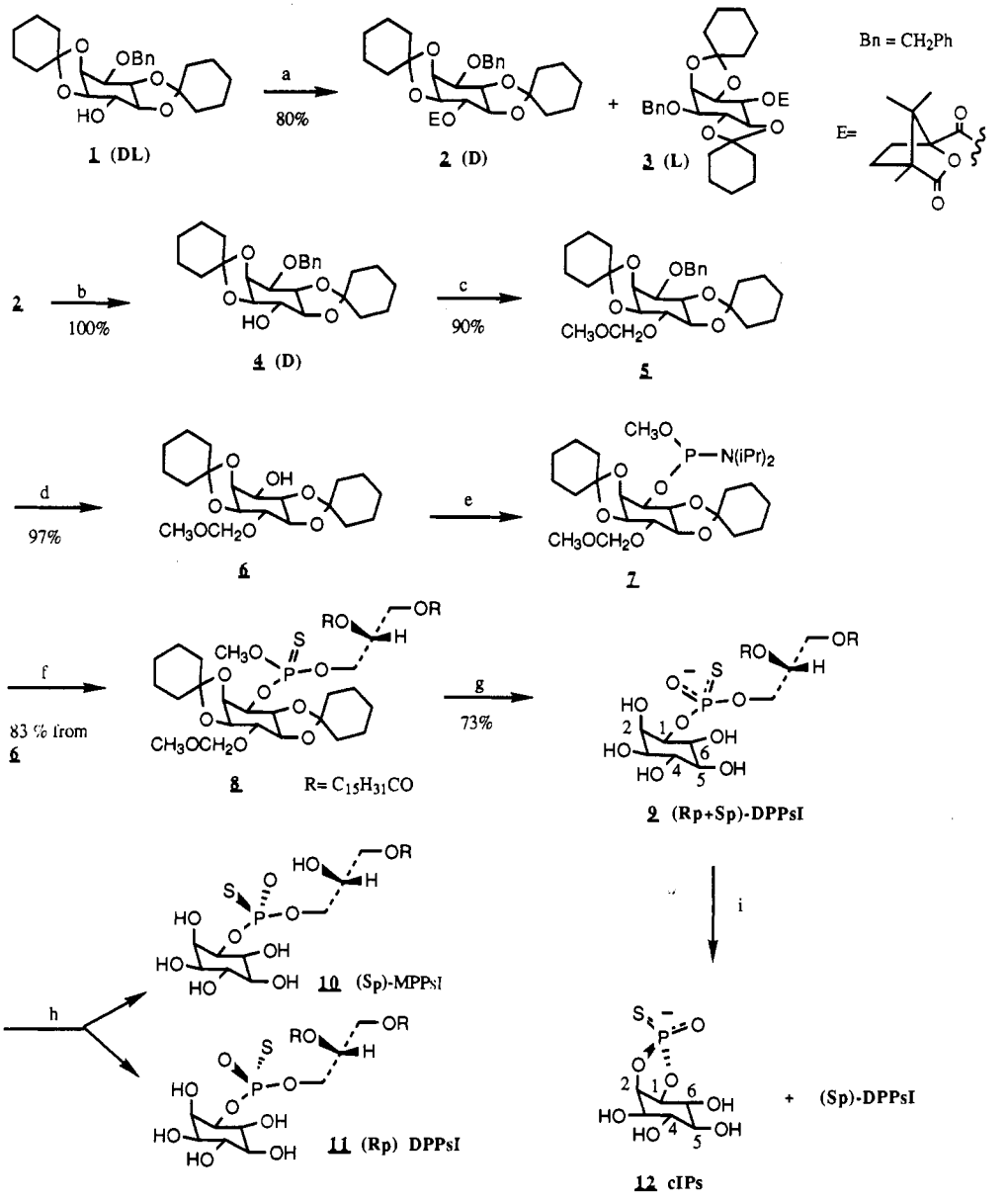
CH_2 of 4-*O*-MOM); 4.82 and 4.35 (m, 2 H, glycero-*sn*-1 CH_2); 4.52 (dd, J = 4.5, 9.0 Hz, 1 H, 1-H); 4.26–3.99 (m, 4 H, 4-H, 6-H, and glycero-*sn*-3 CH_2); 3.81 and 3.75 [two doublets, $^3J_{P-H}$ = 5.2 Hz, 3 H, (R_p)- and (S_p)-$POCH_3$]; 3.36 (t, J = 10.2 Hz, 1 H, 5-H); 2.31 (t, J = 7.5 Hz, 2 H, α-CH_2 of *sn*-2 acyl chain); 2.28 (t, J = 7.4 Hz, 2 H, α-CH_2 of *sn*-1 acyl chain); 1.60 (m, 4 H, acyl chain β-CH_2); 1.8–1.1 (m, 72 H, all the other CH_2 protons); 0.86 [t, J = 7.4 Hz, 6 H, acyl-chain 16(t)-CH_3]. The above assignments were made in reference to **6**, PI (Shibata et al., 1984), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Hauser et al., 1980), and by ^{31}P decoupling. ^{13}C NMR (CD_3OD, 62.90 MHz) δ 173.14 and 172.71 (acyl chain C=O); 113.20 and 110.77 (tertiary C-1 of 2,3:5,6-di-*O*-cyclohexylidene); 95.15 (CH_2 of 4-*O*-MOM); 80.31, 75.94, 75.65, 75.58, 75.09, and 74.99 (six inositol carbons); 69.15 (glycero-*sn*-2); 65.51 (glycero-*sn*-3); 61.81 (glycero-*sn*-1); 55.24 (CH_3 of 4-*O*-MOM); 54.76 and 54.44 (dd, $^2J_{POC}$ = 6.9 Hz, $POCH_3$); 37.55, 36.33, 36.12, and 35.15 (C-2 and C-6 of 2,3:5,6-di-*O*-cyclohexylidene); 34.15 (α-CH_2 of *sn*-2 acyl chain); 34.00 (α-CH_2 of *sn*-1 acyl chain); 31.68 (acyl chain 14-CH_2); 29.63, 29.43, 29.30, 29.24, and 29.08 (acyl chain 4-13 CH_2); 24.88, 23.87, 23.78, and 23.56 (C-3, C-4, and C-5 of 2,3:5,6-di-*O*-cyclohexylidene); 24.81 (acyl-chain β-CH_2); 22.63 (acyl-chain 15-CH_2); 14.04 (acyl-chain t-CH_3). ^{31}P NMR (CD_3OD, 101.26 MHz) showed two diastereomers: δ 67.93, 67.63. Two minor resonances arising from the L form of the inositol moiety was also detected by ^{31}P NMR (67.86 and 67.69 ppm).

(R_p+S_p)-DPPsI (**9**). To 110 mg of **8** were added 4 mL of tetrahydrofuran and 2 mL of 6 N HCl. The resulting solution was stirred at room temperature for 3 h. Solvents and HCl were removed by rotary evaporation. To this dry solid, toluene (over NaH, 10 mL) and anhydrous trimethylamine (over NaH, 5 mL) were added through vacuum transfer. The resulting solution was stirred at room temperature for 15 h. After the deprotection reaction was complete, the mixture was evaporated to dryness and dissolved in 100 mL of chloroform. The organic layer was washed with 3 × 50 mL of saturated NaCl and was concentrated to about 1 mL. This compound was purified by liquid chromatography on silica gel with chloroform–methanol (2/1 v/v) as the eluting solvent to give 62.4 mg of a white solid **9** (73% yield): TLC R_f = 0.2 (chloroform–methanol–H_2O, 66/33/4 v/v/v); ^1H NMR (500 MHz, CD_3OD–$CDCl_3$, 1/2 v/v) δ 5.25 (m, 1 H, *sn*-2 CH); 4.41 and 4.19 (m, 2 H, glycero-*sn*-1 CH_2); 4.23–4.09 (m, 4 H, 2-H, glycero-*sn*-3 CH_2 and 1-H); 3.78 (m, 1 H, inositol 6-H); 3.59 (m, 1 H, inositol 4-H); 3.42 (m, 1 H, inositol 3-H); 3.25 (m, 1 H, inositol 5-H); 2.31 (t, J = 7.4 Hz, 2 H, α-CH_2 of *sn*-2 acyl chain); 2.29 (t, J = 7.4 Hz, 2 H, α-CH_2 of *sn*-1 acyl chain); 1.58 (m, 4 H, acyl-chain β-CH_2); 1.26 (b, 48 H, all the other CH_2 protons); 0.86 (t, J = 7.4 Hz, 6 H, acyl-chain t-CH_3); ^{13}C NMR (CD_3OD, 125.76 MHz) δ 173.43 and 173.13 (acyl chain C=O); 76.53 (inositol C-1); 73.91 (inositol C-5); 71.92 (inositol C-4); 71.16 (inositol C-6); 70.91 and 70.78 (inositol C-2); 70.70 (inositol C-3); 69.94 and 69.85 (glycero-*sn*-2); 63.61 and 63.34 (glycero-*sn*-3); 62.13 (glycero-*sn*-1); 33.51 (acyl-chain *sn*-2 α-CH_2); 33.39 (acyl-chain *sn*-1 α-CH_2); 31.19 (acyl-chain 14-CH_2); 28.96, 28.79, 28.62, and 28.45 (acyl-chain 4–13 CH_2); 24.19 (acyl-chain β-CH_2); 21.91 (acyl-chain 15-CH_2); 12.96 (acyl-chain t-CH_3). The assignments of ^{13}C NMR spectrum were made by the $^1H/^{13}C$ COSY spectrum and by ^{13}C DEPT spectra (Lin, 1989). ^{31}P NMR (CD_3OD–$CDCl_3$, 1/2 v/v, 101.26 MHz) δ 55.934, 57.01. Two minor peaks were observed at δ 56.35 (possibly due to the contaminating L form) and 57.88 (possibly due to 1,2-migration). Detailed assignments in a different solvent have been described in the text. MS (FAB) m/z 850 ($[M + Na + H]^+$), 849 ($[M + Na]^+$), 551 (dipalmitoylglycerol – OH), 331 (inositol phosphorothioate + C_3H_5O), 315 (*O*-vinyl inositol phosphorothioate diester), 275 (inositol phosphorothioate), and 179 (inositol – H). The above assignments of MS fragments were made in reference to PI (Jugalwala, 1985; Jensen, 1987).

Hydrolysis by PI-PLC. The hydrolysis of DPPsI by *B. cereus* PI-PLC was followed by ^{31}P NMR as described in the legend of Figure 2. The hydrolysis of DPPsI by guinea pig uterus PI-PLC was followed similarly except for the presence of 3 mM deoxycholate, using 3 mg of substrate and 3.5 mg of enzyme in 1 mL of buffer in the case of isozyme I, and 1 mg of substrate and 2.4 mg of enzyme in 1 mL of buffer in the case of isozyme II. The reaction time was 3 days at room temperature. No quantitative kinetic data were obtained, but the reactivity of DPPsI is estimated to be <1% relative to the natural PI. In all cases ca. 50% of the R_p isomer was consumed in 3–4 days. As a control, we have shown that *exo*-cIPs (**18a**) incubated under identical conditions except the absence of PI-PLC gave no detectable ring-opened product IPs by ^{31}P NMR.

Synthesis of myo-Inositol 1,2-Cyclic Phosphorothioates. DL-*exo-3,4,5,6-Tetra-O-benzyl-myo-inositol 1,2-Cyclic O-Methylphosphorothioate* (**17a**) *and* DL-*endo-3,4,5,6-Tetra-O-benzyl-myo-inositol 1,2-Cyclic O-Methylphosphorothioate* (**17b**). **13** (0.156 mmol) [prepared by the procedures of Ozaki et al. (1986)] was dried three times by rotary evaporation with 2 mL of dry toluene. To this dry material, 5 mL of dichloromethane (over Na_2SO_4) and an excess of triethylamine (over NaH) were added through vacuum transfer. While the mixture was still cold, chloro(*N*,*N*-diisopropylamino)methoxyphosphine (1.25 equiv, 0.195 mmol) was added to the flask through a dry syringe. The reaction was completed about 30 min later on the basis of TLC, and the solvent and excess triethylamine were removed under vacuum. The reaction mixture was further dried in vacuo (0.01 mmHg) for $^1/_2$ h. Thoroughly dried tetrazole (4 equiv, 0.624 mmol, Aldrich gold label) was dissolved in tetrahydrofuran–acetonitrile (1/1 v/v, 20 mL, over NaH) through vacuum transfer. After being warmed to 40 °C to make sure all compounds were dissolved, this solution was added to the above reaction mixture through a syringe under the argon atmosphere. The reaction mixture was stirred at room temperature for 4 h and then evaporated to dryness. To this mixture, 50 mL of dry toluene (dried over NaH) and an excess of S_8 (powder, sublimed) were added at room temperature. The suspension was stirred at room temperature for 6 days. The reaction mixture was then washed with 25 mL of saturated $NaHCO_3$ and evaporated to dryness. The crude products were purified by MPLC on silica gel in hexane–ethyl acetate (5/1 v/v) to give an oil, **17a** [5 mg, 5% yield based on **13**; TLC R_f = 0.31 (hexane–ethyl acetate (5/1 v/v)] and an oil **17b** [42 mg, 60% yield based on **13**; TLC R_f = 0.26 (hexane–ethyl acetate, 5/1 v/v)]. Procedures and rationales for configurational assignments of the two isomers are described in the text. Detailed proton resonance assignments were made in reference to cIP (Cerdan et al., 1986), and by ^1H–^1H decoupling experiments, ^{31}P decoupling ^1H NMR, 2-D J-resolved ^1H NMR, and 2-D NOESY. **17a** (exo): ^{31}P NMR ($CDCl_3$, 101.256 MHz) δ 84.41; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.35–7.24 (m, 20 H, phenyl of 3,4,5,6-tetra-*O*-benzyl); 4.87 (ddd, $J_{H-C(2)-C(1)-H}$ = 6.5 Hz, $J_{H-C(2)-C(3)-H}$ = 4.0 Hz, $^3J_{P-O-C(2)-H}$ = 3.7 Hz, 1 H, 2-H); 4.85 and 4.75 (AB, J = 11.2 Hz, 2 H, CH_2 of 6-*O*-benzyl); 4.76 and 4.70 (AB, J = 11.0 Hz, 2 H, CH_2 of 5-*O*-benzyl); 4.77

Scheme I: Synthesis and Reactions of DPPsI[a]



[a] Reagents and conditions: (a) (−)-camphanic acid chloride, Et_3N, DMAP, 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) H_2/10% Pd–C, or Li, THF–NH_3, −78 °C, $^1/_2$ h; (e) $ClP(OCH_3)N(iPr)_2$, Et_3N, CH_2Cl_2, 25 °C, $^1/_2$ 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.

and 4.69 (AB, J = 11.0 Hz, 2 H, CH_2 of 3-*O*-benzyl); 4.68 and 4.66 (AB, J = 11.0 Hz, 2 H, CH_2 of 4-*O*-benzyl); 4.59 (ddd, $J_{H\text{-}C(1)\text{-}C(2)\text{-}H}$ = 6.3 Hz, $J_{H\text{-}C(1)\text{-}C(6)\text{-}H}$ = 8.1 Hz, $^3J_{P\text{-}O\text{-}C(1)\text{-}H}$ = 18.4 Hz, 1 H, 1-H); 4.20 (dd, $J_{H\text{-}C(6)\text{-}C(5)\text{-}H}$ = 9.7 Hz, $J_{H\text{-}C(6)\text{-}C(1)\text{-}H}$ = 7.9 Hz, 1 H, 6-H); 3.86 (dd, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = 7.3 Hz, $J_{H\text{-}C(4)\text{-}C(5)\text{-}H}$ = 7.1 Hz, 1 H, 4-H); 3.76 (d, $^3J_{P\text{-}O\text{-}C\text{-}H}$ = 15.0 Hz, 3 H, CH_3OP); 3.73 (dd, $J_{H\text{-}C(3)\text{-}C(4)\text{-}H}$ = 7.5 Hz, $J_{H\text{-}C(3)\text{-}C(2)\text{-}H}$ = 3.9 Hz, 1 H, 3-H); 3.45 (dd, $J_{H\text{-}C(5)\text{-}C(6)\text{-}H}$ = 9.7 Hz, $J_{H\text{-}C(5)\text{-}C(4)\text{-}H}$ = 7.0 Hz, 1 H, 5-H). **17b** (endo): ^{31}P NMR ($CDCl_3$, 101.256 MHz) δ 82.65; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.44–7.22 (m, 20 H, phenyl of 3,4,5,6-tetra-*O*-benzyl); 4.92 and 4.82 (AB, J = 11.1 Hz, 2 H, CH_2 of 6-*O*-benzyl); 4.83 and 4.73 (AB, J = 12.2 Hz, 2 H, CH_2 of 3-*O*-benzyl); 4.79 and 4.71 (AB, J = 11.1 Hz, 2 H, CH_2 of 5-*O*-benzyl); 4.73 (ddd, $J_{H\text{-}C(2)\text{-}C(1)\text{-}H}$ = 6.6 Hz, $J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 3.4 Hz, $^3J_{P\text{-}O\text{-}C(2)\text{-}H}$ = 5.5 Hz, 1 H, 2-H), 4.60 (ddd, $J_{H\text{-}C(1)\text{-}C(2)\text{-}H}$ = 6.6 Hz, $J_{H\text{-}C(1)\text{-}C(6)\text{-}H}$ = 8.2 Hz, $^3J_{P\text{-}O\text{-}C(1)\text{-}H}$ = 9.7 Hz, 1 H, 1-H); 4.56 (s, 2 H, CH_2 of 4-*O*-benzyl); 4.35 (dd, $J_{H\text{-}C(6)\text{-}C(5)\text{-}H}$ = 10.0 Hz, $J_{H\text{-}C(6)\text{-}C(1)\text{-}H}$ = 7.8 Hz, 1 H, 6-H); 3.87 (dd, $J_{H\text{-}C(4)\text{-}C(5)\text{-}H}$ = 6.3 Hz, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = 5.3 Hz, 1 H, 4-H); 3.85 (d, $^3J_{P\text{-}O\text{-}C\text{-}H}$ = 14.6 Hz, 3 H, CH_3OP); 3.76 (dd, $J_{H\text{-}C(2)\text{-}C(3)\text{-}H}$ = 3.2 Hz, $J_{H\text{-}C(4)\text{-}C(3)\text{-}H}$ = 5.7 Hz, 1 H, 3-H); 3.46 (dd, $J_{H\text{-}C(5)\text{-}C(6)\text{-}H}$ = 10.2 Hz, $J_{H\text{-}C(5)\text{-}C(4)\text{-}H}$ = 6.6 Hz, 1 H, 5-H).

DL-*exo-cIPs* (**18a**) *and* DL-*endo-cIPs* (**18b**). To a dry tetrahydrofuran (2 mL, over Na) solution of **17a** (14 mg, 0.022 mmol) was added Li (12 mg, 60 equiv) at −78 °C (dry ice–tetrahydrofuran bath). Dry NH_3 (over Na) was distilled into the above reaction mixture. After stirring at −78 °C for 5 min, 3 mg of NH_4Cl was added at −78 °C. The reaction mixture was stirred while warming up from −78 °C to room temperature and dissolved in 2 mL of D_2O. ^{31}P NMR (D_2O, 101.256 MHz) δ 69.85. **18b** was prepared by the same procedure in 60% yield from **17b**. ^{31}P NMR (D_2O, 101.256 MHz) δ 69.00.

Results

Synthesis of DPPsI. DPPsI was synthesized according to Scheme I. The overall procedure is novel and should be applicable to future synthesis of labeled or unlabeled phos-

phatidylinositides. However, most of the individual steps have been adopted from known procedures established for related systems. The starting compound **1**, DL form of a protected *myo*-inositol, was synthesized from *myo*-inositol as described by Garegg et al. (1984). Resolution of D and L enantiomers was achieved by derivatization with (−)-camphanic acid chloride followed by chromatographic separation of **2** and **3** (Billington et al., 1987; Vacca et al., 1987). The D form **2** was obtained in >99% diastereomeric excess (de) on the basis of gas chromatographic analysis. However, for practical reasons, the % de of the bulk sample of **2** used in the synthesis of DPPsI varied between 75 and 90%. The camphanyl group in **2** was then removed by basic hydrolysis, which yielded **4** in quantitative yield. Protecton of **4** with chloromethyl methyl ether (MOMCl) (Corey et al., 1982a,b; Stork & Takahashi, 1977) gave **5** in 90% yield. Debenzylation of **5** was achieved quantitatively by hydrogenolysis on 10% Pd–C, or by reductive debenzylation with Li or Na metal in tetrahydrofuran and liquid NH_3, a procedure modified from Potter and Lowe (1981). The product **6** has the correct OH group open for phosphorylation.

The phosphorylation steps were adapted from Bruzik et al. (1986). Thus, phosphitylation of **6** with chloro(*N*,*N*-diisopropylamino)methoxyphosphine gave phosphoramidite **7**. This phosphoramidite was condensed with 1,2-dipalmitoyl-*sn*-glycerol in the presence of tetrazole to give a phosphite triester intermediate, which was sulfurized with an excess of S_8 to give a phosphorothioate triester **8**, which is the precursor of DPPsI. The overall yield from 6 to 8 was 83%. The presence of two diastereomers of **8** was identified by two equal-intensity resonances in ^{31}P NMR at 67.63 and 67.93 ppm. Deprotection of two ketal groups and the methoxymethyl ether by acid hydrolysis, followed by demethylation with trimethylamine, gave (R_p+S_p)-DPPsI (**9**) in 73% yield. The presence of two diastereomers was characterized by two resonances in ^{31}P NMR at 55.71 and 55.32 ppm, as shown in Figure 1A. The pair of small peaks (55.56 and 55.40 ppm) labeled as a and b, respectively, can be attributed to DPPsI in which the configuration of the inositol moiety is L instead of D, since the intensity of these two peaks corresponds with the % de of **2**. A similar ^{31}P NMR pattern (two major peaks due to D form, two minor peaks due to L form) was also observed for the precursor **8** as described under Materials and Methods. Besides those peaks, two small peaks at 57.13 and 57.02 ppm (10–15%) in ^{31}P NMR were also observed (not shown), which were attributed to the products of 1,2-migration of the phosphatidyl moiety during the last acid deprotection step, since no such impurity was observed in the precursor **8**. To further confirm such an interpretation, we have found that treatment of commercial PI (12 mg in 0.6 mL of $CDCl_3$) (^{31}P δ 5.31 ppm) with concentrated HCl resulted in the formation of a new peak at 7.71 ppm. These two isomeric impurities (<15% in each case) were not separated from DPPsI. However, they presented no problem to the spectral assignments and the biochemical studies of DPPsI.

Configurational Assignment of DPPsI by Phospholipase A_2. It has been established previously that phospholipase A_2 (PLA2) from various sources specifically hydrolyzes the R_p isomer of phosphorothioate analogues of phosphatidylcholines and phosphatidylethanolamines (Orr et al., 1982; Bruzik et al., 1982, 1983; Jiang et al., 1984). Since PLA2 has a broad specificity toward different types of phospholipids, it can be used to assign the absolute configuration at phosphorus of DPPsI. It should be noted that, due to a change in priority, the relative configurations of (R_p)- and (S_p)-DPPsI correspond

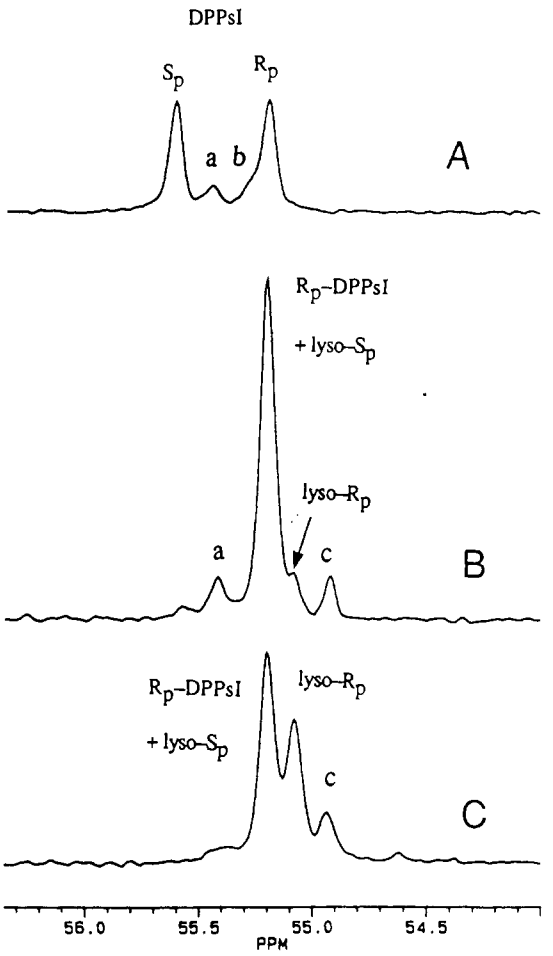


FIGURE 1: Use of ^{31}P NMR to monitor the hydrolysis of DPPsI by PLA2. (A) 10 mg of (R_p+S_p)-DPPsI in 50% D_2O containing 5% Triton X-100, 50 mM HEPES buffer, pH 7.2, 2.5 mM Ca^{2+}, and 0.25 mM EDTA. (B) Spectrum taken within the first 30 min after addition of 1.3 mg of PLA2 from bee venom. (C) After further incubation at 25 °C for 12 h.

to those of S_p and R_p isomers, respectively, of DPPsC. As shown in Figure 1B, upon addition of bee venom PLA2 to DPPsI, the downfield peak of ^{31}P resonances of DPPsI (55.71 ppm) disappeared and was thus assigned to (S_p)-DPPsI. The resonance of the product (S_p)-MPPsI (**10**) happened to coincide with that of (R_p)-DPPsI (**11**) (55.32 ppm). These assignments have been verified by independent experiments in which hydrolysis of (S_p)-DPPsI was only partially complete. The small peak c in Figure 1B can be attributed to the product from b, since PLA2, being insensitive to the type of the head group, is not expected to be sensitive to the configuration of the inositol ring. Such interpretation also yields assignment of a and b as R_p and S_p isomers, respectively. The less reactive isomer (R_p)-DPPsI can also be hydrolyzed by PLA2 upon further incubation, as shown in Figure 1C.

Hydrolysis of DPPsI Catalyzed by PI-PLC. It has been shown that PC-PLC specifically hydrolyzes the S_p isomers of DPPsC and DPPsE (Orr et al., 1982; Bruzik et al., 1982, 1983; Jiang et al., 1984). Bruzik (1988) also observed that PLC also specifically hydrolyzed one isomer of the phosphorothioate analogues of sphingomyelin. As shown in Figure 2A,B, the PI-PLC from *B. cereus* stereospecifically converts the R_p isomer of DPPsI to the product, cIPs at 69.89 ppm in ^{31}P NMR. Thus PI-PLC shows the same stereospecificity as PC-PLC despite the differences in structural and catalytic properties. Unlike PI-PLC from mammalian sources, the *Bacillus* enzyme gives only cyclic IP according to Michell

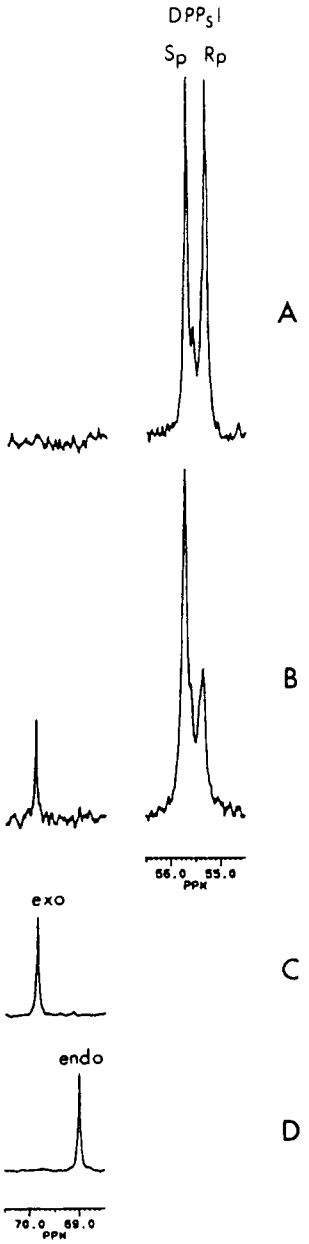


FIGURE 2: Use of ^{31}P NMR (101.2 MHz) to show the stereospecificity of PI-PLC. (A) 7.5 mg of (R_p+S_p)-DPPsI in 50% D_2O containing 5% Triton X-100, 50 mM HEPES buffer, pH 7.2, 2.5 mM Ca^{2+}, and 0.25 mM EDTA. (B) 72 h after addition of 2 mg of PI-PLC from *B. cereus*. (C) **18a** (*exo*-DL-cIPs). (D) **18b** (*endo*-DL-cIPs).

(1975). This was confirmed by our ^{31}P NMR analysis, which showed no detectable noncyclic product from either the natural PI or the synthetic DPPsI.

Essentially the same results were obtained for both isozyme I and isozyme II of the PI-PLC from guinea pig uterus, except that an additional noncyclic product IPs at 43.84 ppm was observed in both cases. On the basis of relative intensities, it can be concluded that the R_p isomer of DPPsI is the precursor for both cIPs and IPs. The ratio cIPs/IPs was ca. 2 for isozyme I and ca. 0.5 for isozyme II as determined by ^{31}P NMR. Separate ^{31}P NMR experiments using natural PI as substrate showed that the ratio cIP/IP was 1 for isozyme I and 0.5 for isozyme II. Thus the use of phosphorothioate analogue does not have a significant effect on the ratio of the two products. The mechanistic implication of this finding will be discussed later.

Synthesis of Inositol 1,2-Cyclic Phosphorothioate (cIPs). In order to elucidate the steric course of the reaction, it is necessary to determine the configuration of the product. Since

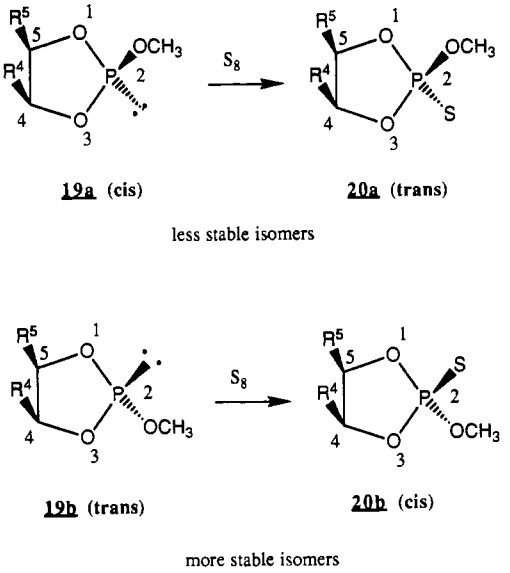


FIGURE 3: Structures of model phosphites **19a** and **19b** and products of sulfurization **20a** and **20b**.

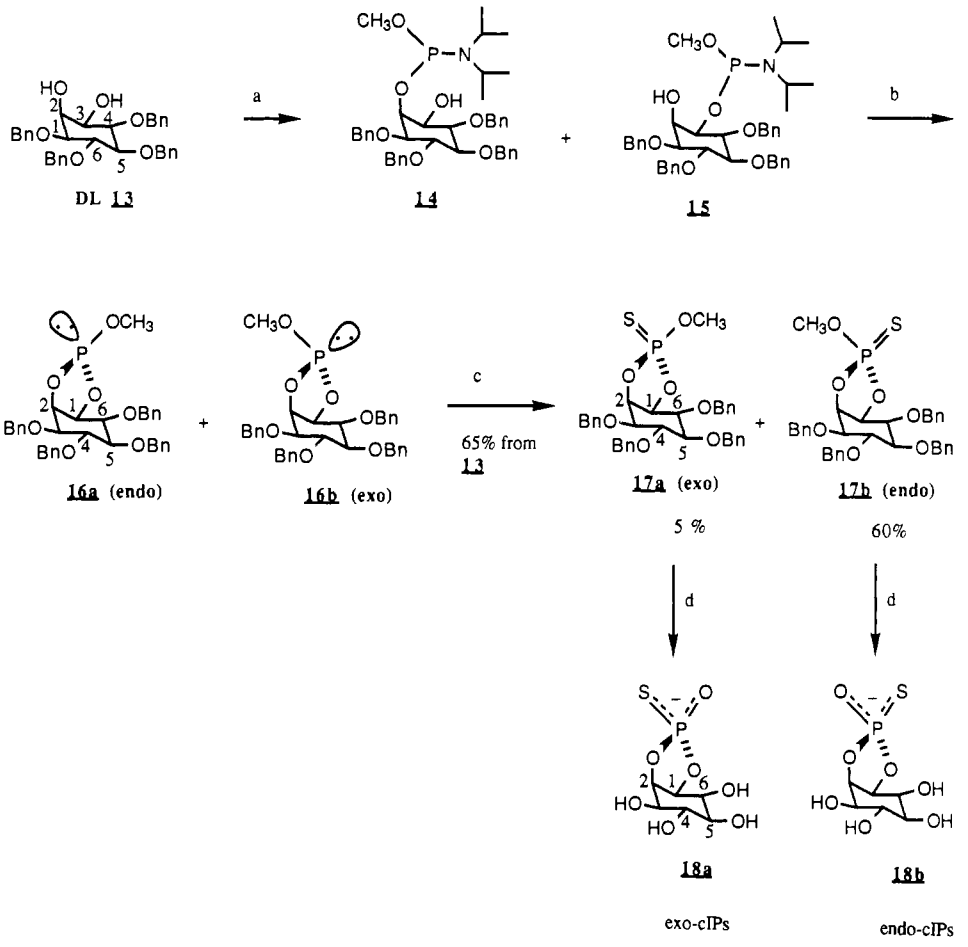
only one isomer of cIPs is generated and the quantity is very limited from enzymatic reactions, we set out to synthesize both isomers of cIPs according to Scheme II. The tetrabenzylated *myo*-inositol **13** was first prepared from established procedures (Angyal et al., 1961; Gigg & Warren, 1969; Watanabe et al., 1987). Monophosphitylation with 1.2 equiv of chloro(*N*,*N*-diisopropylamino)methoxyphosphine in the presence of 1.5 equiv of triethylamine gave phosphoramidites **14** and **15**. After removal of the excess triethylamine, 4 equiv of tetrazole in tetrahydrofuran–acetonitrile was added to the reaction mixture to form two cyclic phosphite triesters **16a** and **16b**. This procedure affords a novel and efficient synthesis of 1,3,2-dioxaphospholanes. These phosphite triesters were oxidized by elemental sulfur (S_8) to give **17a** and **17b**, which were separated by medium-pressure liquid chromatography and resulted in 5 and 60% yield, respectively, based on **13**. Deprotection of **17a** and **17b** with Li metal in tetrhydrofuran–liquid NH_3 at −78 °C for 5 min [modified from Potter and Lowe (1981)] gave the two isomers of cIPs **18a** and **18b**, respectively. It is believed that the benzyl groups are removed by a radical reductive cleavage and the methyl group is removed by an S_N2 nucleophilic attack of ammonia; neither would affect the configuration at phosphorus. However, it is important that the reaction time is kept within 5 min; otherwise ring opening and desulfurization products may dominate. It should be noted that throughout the above synthesis DL mixtures were used since it will have no effect on chemical shifts.

The two isomers of DL-cIPs have also been synthesized by Schultz et al. (1988) by a different procedure which gave lower yield (20%). In addition, Schultz et al. (1988) have not assigned the absolute configurations of cIPs. Our configurational analysis is described in the next section.

Configurational Assignment of cIPs. The phosphorus configurations of **18a** and **18b** were not assigned directly. Instead, they were derived from their precursors **17a** and **17b**, respectively. The absolute configuration at phosphorus of **17a** and **17b** was assigned on the basis of the following four criteria:

(1) It has been well established that the predominant conformation of the phosphite **19** in Figure 3 is the sterically less hindered trans form, **19b** (McEven, 1965; Denney et al., 1969; Bentrude & Tan, 1976). As also shown in Figure 3, oxidation (or sulfurization) of phosphite is known to proceed with retention of configuration at phosphorus; moreover, the cis/trans ratio of phosphite was the same as that of the resulting product

Scheme II: Synthesis of *exo*- and *endo*-cIPs[a]



[a] Only D-forms are shown. Reagents and conditions: (a) 1.2 equiv of ClP $(OCH_3)N(iPr)_2$, iPr_2NEt, CH_2Cl_2, 25 °C, $^1/_2$ h; (b) 4 equiv of tetrazole, THF–CH_3CN, 25 °C, 18 h; (c) excess S_8, toluene, 25 °C, 48 h; (d) 40 equiv of Li, THF–NH_3, −78 °C, 5 min.

(McEven, 1965; Denney et al., 1969; Bentrude & Tan, 1976; Mikolajczyk et al., 1976; Mikolajczyk & Witczak, 1977). Since the yield of **17b** (60%) is lareger than that of **17a** (5%), **17b** should correspond to **20b** (and **16b** should correspond to **19b**). Our MM-2 calculation also confirmed that **16b** (minimization energy 85.3 kcal/mol) is more stable than **16a** (88.5 kcal/mol). The configuration of **17b** can thus be established as endo (D-S_p + L-R_p), and that of **17a** as exo (D-R_p + L-S_p). It should be noted that in all the "b" series the OCH_3 group is located opposite to the other substituents on the five-membered 1,3,2-dioxaphospholanes ring, but the isomeric notation varies from compound to compound.

(2) The second evidence supporting these assignments was derived from the chemical shift difference in the ^{31}P NMR spectra. In ^{31}P NMR, the trans isomer **20a** resonates more downfield than the cis isomer **20b** by 0.5–2.5 ppm (Mikolajczyk et al., 1976; Mikolajczyk & Witczak, 1977). The ^{31}P resonances observed for **17a** and **17b** (84.41 and 82.65 ppm, respectively) are consistent with this qualitative trend.

(3) The assignments were further confirmed by the three-bond coupling constants between P and 1-H. The relationship between $^3J_{P\text{-}O\text{-}C\text{-}H}$ and the P–O–C–H dihedral angle (ϕ) can be written as (Bentrude & Setzer, 1987)

$$^3J_{POCH} = 15.3 \cos^2 \phi - 6.1 \cos \phi + 1.6 \qquad (1)$$

According to the structures after MM-2 energy minimization [the bond angle and length data were obtained from Mikolajczyk et al. (1976) and Cerdan et al. (1986)], the H–C(1)–O–P dihedral angles of **17a** and **17b** were 150° and 124°, respectively. The $J_{H\text{-}C(1)\text{-}O\text{-}P}$ values calculated from eq 1 are 18.3 and 9.8 Hz for **17a** and **17b**, respectively, which agree very well with the observed values (18.4 and 9.7 Hz, respectively).

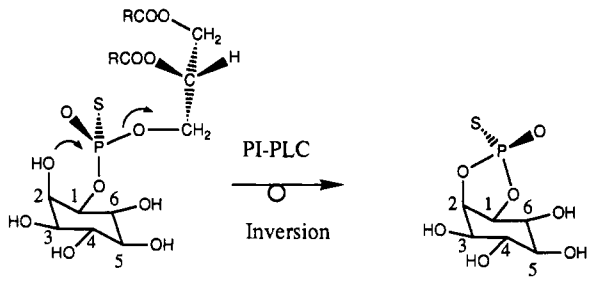
(4) The fourth evidence that supports these assignments is based on the analysis of the NOESY spectra of **17a** and **17b** (panels A and B, respectively, of Figure 4). In **17a** but not in **17b**, a detectable NOE is observed between 4-H and the *O*-methyl proton resonance, which suggests that the *O*-methyl group is at the same side with the 4-H of the inositol ring in **17a** and supports the assignment of exo to **17a**. On the other hand, in **17b** but not in **17a**, a detectable NOE is observed between 2-H and the *O*-methyl proton resonance. This suggests that the *O*-methyl group is at the same side with the 2-H of the inositol ring in **17b** and supports the assignment of endo to **17b**.

Steric Course of Phosphoinositide-Specific Phospholipase C. As shown in Figure 2, the cIPs produced from the reactions catalyzed by the three PI-PLCs have the same δ ^{31}P as the exo isomer of cIPs. This was further confirmed by addition of the endo isomer and the exo isomer independently to the reaction mixture followed by ^{31}P NMR analysis. Therefore, the stereochemical course of the conversion of PI to cIP catalyzed by PI-PLC was inversion in the configuration of phosphorus as shown in Scheme III.

Discussion

PI-PLC specifically catalyzes hydrolysis of the R_p isomer of DPPsI, which corresponds to the S_p isomer of the phosphorothioate analogues of phosphatidylcholines. It is inter-

Scheme III: Steric Course of Formation of cIPs Catalyzed by PI-PLC



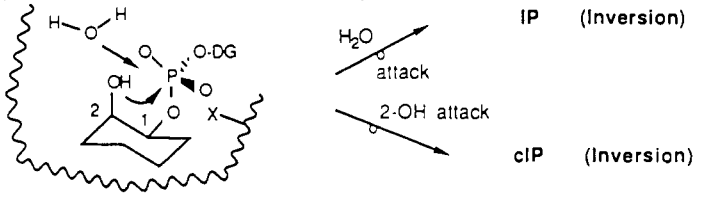
R_p-DPPsI exo-cIPs

esting that PC-PLC and the three different PI-PLCs, which differ greatly in catalytic and structural properties, all have the same stereochemical requirement at phosphorus.

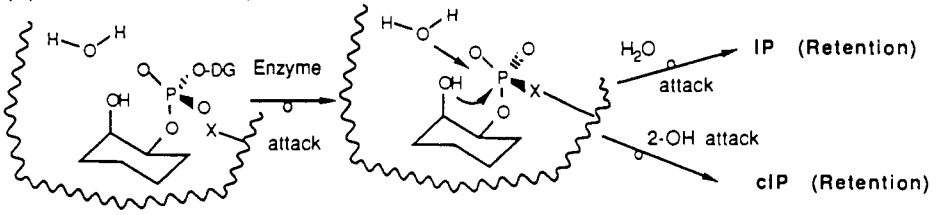
Scheme IV summarizes the six possible mechanisms for the formation of IP and cIP from DPPI, along with the predicted stereochemical outcomes (assuming every single substitution at phosphorus is inversion, as has been the case in most known enzymatic reactions). Mechanisms A and B are parallel reactions (at the same active site) and are favored by some in the field (Dawson et al., 1971; Michell, 1975; Quinn, 1978; Majerus et al., 1986). Mechanism B differs from A in that a covalent enzyme–phosphoinositol intermediate has been formed. In mechanisms C and D cIP is first formed and then converted to IP by an enzyme-mediated process. Nonenzymatic conversion of cIP to IP can be ruled out since when cIP is subjected to chemical hydrolysis, both IP and 2-IP should be obtained, as has been demonstrated by Pizer and Ballou (1959). Mechanism C mimics the mechanism of ribonuclease A, but ribonuclease A does not release the cyclic intermediate. Mechanism D differs from C in that a covalent enzyme–phosphoinositol intermediate has been formed. It has been shown that both IP and cIP are released from the substrate simultaneously and there is no subsequent interconversion of cIP into IP or vice versa (Dawson et al., 1971; Allan & Michell, 1974; Lapetina & Michell, 1973; Michell, 1975; Quinn, 1978; Majerus et al., 1986). Therefore, these two mechanisms (C and D) seem less likely. Mechanisms E and F differ from C and D, respectively, in the order of formation of IP and cIP. These two mechanisms (E and F) are unlikely and can be ruled out because no ^{18}O was incorporated into cIP

Scheme IV: Possible Mechanisms of PI-PLC

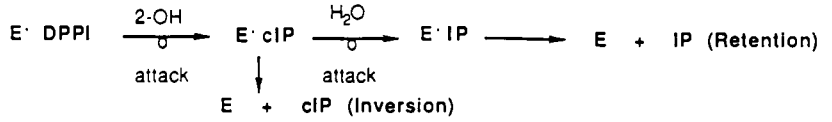
(A) Parallel reactions, Direct displacement



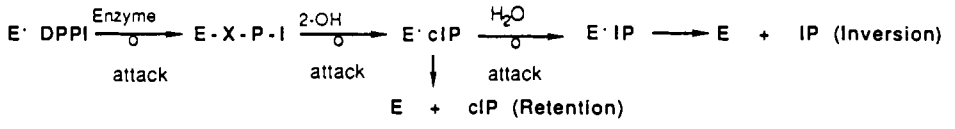
(B) Parallel reactions, Covalent E-S intermediate



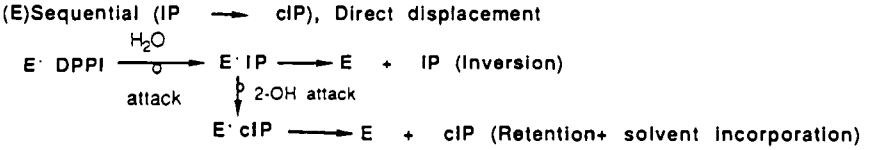
(C)Sequential(cIP → IP), Direct displacement



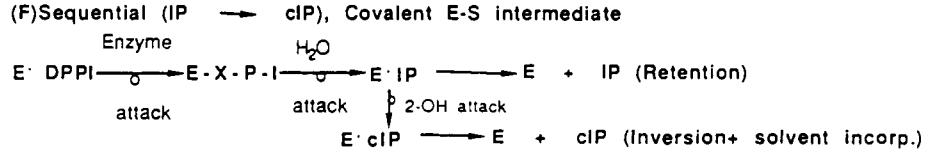
(D)Sequential(cIP → IP), Covalent E-S intermediate



(E)Sequential (IP → cIP), Direct displacement



(F)Sequential (IP → cIP), Covalent E-S intermediate



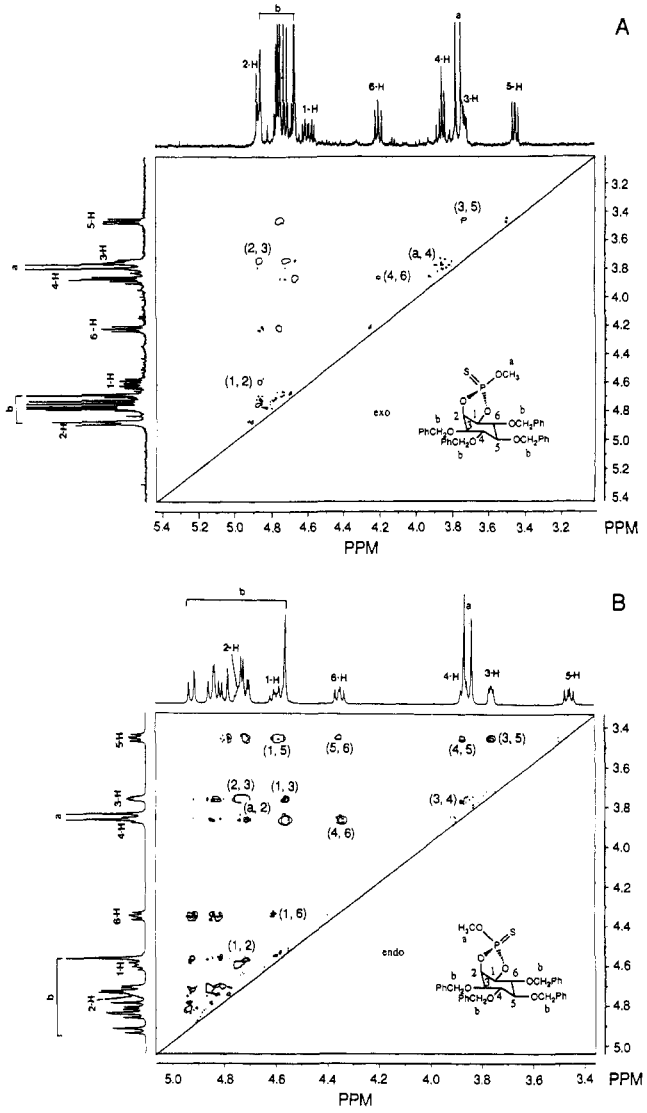


FIGURE 4: NOESY spectra of **17a** (A) and **17b** (B). The peaks labeled (a,4) in **17a** and (a,2) in **17b** represent the critical NOEs discussed in the text. The unlabeled cross peaks arise from the NOE between the benzyl CH_2 protons and various other protons.

when the reaction was carried out in [^{18}O]water (Wilson et al., 1984, 1985). The observed inversion in the formation of cIPs rules out mechanisms B and D. The remaining possibilities are mechanisms A and C. Mechanism C is less likely as mentioned above. Furthermore, mechanism C would predict that IPs is formed in much lower quantity than cIPs since there is one additional step in the formation of IPs which almost certainly will be slowed down by sulfur substitution. Our observation that the ratio cIPs/IPs remains similar to that of IP/cIP for the two mammalian PI-PLCs strongly argues against mechanism C. Thus mechanism A remains as the only feasible mechanism.

Another point of interest is why the *B. cereus* PI-PLC gives only cIP as the product. Since the *B. cereus* enzyme does not require a divalent metal ion for catalysis, we speculate that the divalent metal ion in the mammalian PI-PLC could mediate the water attack to give IP in mechanism A. Whether this is the case or not, it is intriguing how the enzyme mediates two competing reactions at the active site, with the water molecule and the 2-OH group both occupying optimal positions for "in-line" attack.

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Transient-State Kinetics of the Reactions of 1-Methoxy-4-(methylthio)benzene with Horseradish Peroxidase Compounds I and II†

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ABSTRACT: Transient-state reactions of horseradish peroxidase compounds I and II with 1-methoxy-4-(methylthio)benzene (a para-substituted thioanisole) were studied over the pH range from 3.4 to 10.5. The pH-jump technique was applied to the compound II reactions at pH values below 8.6. The reactions of both compound I and compound II with the para-substituted thioanisole consisted predominantly of an initial burst. The burst was followed by a steady-state phase that became more obvious at lower concentrations of the thioanisoles. The burst phase for both compounds I and II can be explained in terms of two independent transient-state reactions with 1-methoxy-4-(methylthio)benzene as follows: (i) a single reaction of compound I (or compound II) with the substrate and (ii) the formation of a complex between compound I or II and the substrate followed by reaction of the productive complex with another molecule of sulfide. The overall rate of reaction path ii is faster than that of path i. The preference for path i or ii is highly dependent upon the concentration of sulfide with step ii favored at higher sulfide concentrations. The experimental results obtained on the overall reaction under both pseudo-first-order and single-turnover conditions indicate that compound II reacts competitively with both the organic sulfide substrate and the sulfur cation radical produced from compound I oxidation of sulfide.

Despite the importance of the enzymatic oxidation of organic sulfides in mammalian detoxification processes, little information is available. Watanabe et al. (1980, 1981a,b) reported that oxidation of alkyl sulfides by mammalian liver microsomes and by purified cytochrome P-450 occurs via a sulfur cation radical intermediate. Both sulfur cation radical formation and sulfoxide formation were also noted with cytochrome P-450 by Guengerich and MacDonald (1984).

Kobayashi et al. (1986, 1987) reported evidence for oxygen atom transfer from H_2O_2 and from H_2O to organic sulfides (thioanisoles) catalyzed by HRP.[1] Their results were in accord with the formation of a cation radical intermediate in the rate-limiting step and with an overall mechanism always involving single-electron transfers.

More recently, Blée and Schuber (1989) demonstrated that the hemoprotein soybean sulfoxidase catalyzes oxygen transfer from 13(*S*)-hydroperoxylinoleic acid to para-substituted thioanisoles. On the basis of their results they suggested a

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[1] Abbreviations: HRP, horseradish peroxidase; HRP-I, horseradish peroxidase compound I; HRP-II, horseradish peroxidase compound II.

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