Cyclopentanoid analogs of phosphatidylcholine: susceptibility to phospholipase A_2

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Abstract Six isomers of dipalmitoylcyclopentanetriol phosphocholine (*cyclopentano*-lecithin) were tested as potential substrates for phospholipase A_2. Since each of these analogs possesses a configuration that mimics a narrow range of conformations of a glycerophospholipid molecule, the analogs were used to assess the enzyme's conformational requirements. Studies showed that all of the analogs containing the phosphocholine at the C-1 (or C-3) position could be hydrolyzed, while only one of the three analogs that contains the polar head group at the C-2 position was susceptible. Kinetic studies, however, revealed that only the all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin gave initial rates of hydrolysis that were measurable by pH-stat. Acyl group specificity of the enzyme towards the all-*trans* isomer was determined with an analog whose acyl groups were distinguishable. The synthesis of this mixed-acid-*cyclopentano*-PC is described herein. When this analog was enzymatically assayed, results unequivocally showed the enzyme to be specific for C-2 acyl hydrolysis. This specificity, and data showing that the all-*trans* analog is stereospecifically hydrolyzed, indicate that it is acted on in an analogous manner to dipalmitoylphosphatidylcholine. These studies indicate that although the configuration of the analog is not necessarily a prerequisite for hydrolysis, there does appear to be an optimal spatial orientation for enzymatic activity. The analogy between the susceptibilities of all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin and *glycero*-lecithin suggests that the conformation of the *glycero*-lecithin during phospholipase A_2-mediated hydrolysis may be best simulated by the all-*trans* orientation of C–O bonds in the artificial substrate. — **Lister, M. D., and A. J. Hancock.** Cyclopentanoid analogs of phosphatidylcholine: susceptibility to phospholipase A_2. *J. Lipid Res.* 1988. **29:** 1297–1308.

Supplementary key words *cyclopentano*-lecithin • mixed-acid-*cyclopentano*-lecithin • conformation of *glycero*-PC • phospholipase A_2 specificity • kinetic studies • mixed micelles

Phospholipase A_2, in common with other phospholipases, requires the presence of a lipid–water interface to function optimally. There have been various explanations given for this requirement (reviewed in ref. 1). Wells (2, 3) has suggested that one explanation for the high enzymatic activity observed at an interface may be that a uniform conformation is attained by phospholipids in the aggregated state. This conformation is not attained with the monomeric phospholipid. In an attempt to evaluate the conformational aspect with respect to phospholipase A_2 activity, we used as potential substrates an isomeric series of *cyclopentano*-lecithins. The rationale and synthesis of these compounds have been previously reported (4–6). The backbone of these lecithin analogs consists of a cyclopentane ring which severely constricts the radial mobility of the attached acyl chains and polar head group, unlike the situation for the glyceryl backbone of the natural counterpart. Each isomer, therefore, is believed to represent a relatively fixed range of conformations of a *glycero*-lecithin. These analogs allow, for the first time, an opportunity to investigate the contribution of conformation at the glycerol backbone to the activity of an extracellular phospholipase A_2. This study and a subsequent one describing the properties of a short chain cyclopentanoid lecithin (7) give credence to the hypothesis that conformational attitudes of the phospholipid backbone influence phospholipase activity (Barlow, P. N., M. D. Lister, E. A. Dennis, and P. B. Sigler. 1988 *J. Biol. Chem.* **263.** In press).

The studies that are described in the following section investigate the susceptibility of six lecithin analogs to phospholipase A_2. Three analogs bear the polar head group at the C-1 (or C-3) position and three at the C-2 position. These studies of the model system suggest that conformational attitudes required for efficient catalysis may have to include an all-*trans* arrangement. This arrangement is best represented by the *trans-cyclopentano*-lecithin isomer. Based on this finding, we developed a synthetic route for the corresponding mixed-acid-*cyclopentano*-lecithin. This synthesis provided the means to perform an unequivocal determination of the enzyme's ability to dis-

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; mixed-acid-*cyclopentano*-lecithin, DL-(1,3/2)-2-O-palmitoyl-3-O-myristoylcyclopentane-1-O-phosphocholine; homo-acid-*cyclopentano*-lecithin, DL-(1,3/2)-2,3-O-dipalmitoyl-cyclopentane-1-O-phosphocholine; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; NMR nuclear magnetic resonance; THF, tetrahydrofuran; IR, infrared; TPS, triisopropylbenzenesulfonyl chloride; FAMEs, fatty acid methyl esters.

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criminate between the two acyl groups, both of which are esterified to secondary alcohol groups in the parent triol.

MATERIALS AND METHODS

Phospholipase A_2 from rattlesnake venom (*Crotalus adamanteus*; claimed activity: 600 units/mg protein at pH 8.9, 25°C) and from bee venom (mixed subspecies of *Apis mellifica*; claimed activity: 1510 units/mg protein at pH 8.5, 37°C) was acquired from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) refers to synthetic *DL*-α-phosphatidylcholine, dipalmitoyl (Sigma Chemical Co.), which was used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel (adsorbent thickness, 250 μm; EM Laboratories, Inc., Elmsford, NY). Sodium deoxycholate (Sigma Chemical Co.) or Triton X-100 (Research Products International Corp., Elk Grove Village, IL) were used as detergents for solubilization of phospholipids to form mixed micelles. Optical rotation values were determined using a Perkin-Elmer Model 241 polarimeter (Norwalk, CT). The microanalysis was performed by Galbraith Laboratories, Inc., Knoxville, TN. Melting points were measured on a Thomas Hoover Unimelt capillary melting point apparatus. Infrared spectra were measured for KBr dispersions or for thin films with Perkin-Elmer 621 or 1320 spectrometers (Perkin-Elmer Corp., Norwalk, CT). NMR spectra were measured on a Bruker 300 MHz instrument. All solvents used were distilled over glass, a 10% forerun being discarded. Pyridine was distilled from potassium hydroxide and stored over molecular sieves (4 Å) and calcium hydride. Purification of compounds was performed by one of two methods: *1*) preparative TLC using 1500 μm layers of silica gel G (Analtech, Inc., Newark, DE), or *2*) by high pressure liquid chromatography on silicic acid using a Prep LC System 500 liquid chromatography (Waters Associates, Inc., Milford, MA). Phosphates were detected after analytical TLC by heating with the modified reagent (8) of Dittmer and Lester (9). Amberlite resins (IRA-410 and IR-120-P), used for purification of the *cyclopentano*-lecithin analogs, were obtained from Sigma Chemical Co. Diphenylphosphoric acid was generated by hydrolysis of diphenyl chlorophosphate (Aldrich Chemical Co., Inc., Milwaukee, WI) as follows: diphenyl chlorophosphate (25 g) was added to water (500 ml) and the mixture was heated to 65–70°C in a water bath and stirred vigorously until the oil dissolved. The solution was then cooled to room temperature and 6 N HCl was added to acidify the solution. Crystalline diphenylphosphoric acid dihydrate was obtained by chilling the solution on ice. The crystalline dihydrate (mp 45–50°C) was filtered, allowed to air-dry, and was then freed from water of hydration by vacuum treatment (0.2 torr, 56°C; mp 65–66°C). Palmitoyl chloride and myristoyl chloride were products of Nu-Chek Prep (Elysian, MN); analysis by GLC as their methyl ester derivatives showed them to be >99% pure. Hydrogenolysis experiments were carried out on a Parr (Model 3911) shaker-type hydrogenation apparatus for large samples (>1 g) and an all-glass atmospheric pressure hydrogenation apparatus for small quantities. Palladium on activated carbon (10%) was obtained from Alfa Products (Danvers, MA), platinum oxide from Engelhard Industries Co. (Newark, NJ), and the condensing agent 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) from Aldrich Chemical Co., Inc. Choline tosylate was prepared from N,N-dimethylethanolamine and methyl *p*-toluenesulfonate as described by Rosenthal (10), recrystallized from dry acetone, and stored in vacuo.

pH-Stat

Kinetic data was obtained using a pH-stat apparatus which consisted of the following Radiometer components: titrator TTT1C, titragraph recorder SBR-2c, autoburette ABU-12 equipped with a 0.25-ml burette assembly B230, and a microtitration assembly TTA31 equipped with a thermostatted jacket V526. The water jacket was maintained at a constant temperature (± 0.2°C) with a Haake constant temperature circulator type F. The titration assembly was equipped with a Radiometer calomel electrode type K4112 and glass electrode type G2222C. Electrodes were briefly dipped in bleach followed by a thorough rinsing with deionized water before each experimental measurement. This was done to maintain electrode sensitivity and reproducibility as recommended by Dennis (11). After each bleach treatment and rinse, electrode response was checked with two standard buffers (pH 6 and 9, Fisher Scientific Co.). Sodium hydroxide solution (0.01 N, Fisher Scientific Co.), standardized with potassium acid phthalate (Thorn Smith, Troy, MI), was used as titrant for pH-stat experiments. Phospholipids for assays were prepared as mixed micelles with Triton X-100 (2:1 detergent–PC), obtained by warming (50°C) and vortexing until a uniform, optically clear suspension was achieved.

Phospholipase A_2-PC analog incubations

Experiments that required the identification or isolation of enzymatic products were performed by incubation of enzyme with mixed-micelles of *cyclopentano*-lecithin. A 4.5-mM mixed micelle solution (1.5 ml) of the respective lecithin analog containing 9 mM sodium deoxycholate, 5 mM calcium acetate, 25 mM sodium chloride, and 50 mM sodium borate buffer (pH 8.9) was placed in a shaker bath (48°C) for 10 min to allow for micelle formation. Phospholipase A_2 (80 μg protein, *Crotalus* or 40 μg protein, *Apis*) was added to this optically clear solution and the incubation was allowed to proceed for 6 hr. Controls were run under identical conditions, except for the

addition of enzyme. In all controls, neither lyso-product nor free fatty acid could be detected by TLC during the incubation period.

Extraction and separation of enzymatic products

The organic-soluble components, fatty acid, lyso-product, and lecithin, were partitioned from the aqueous phase into chloroform as described by Bligh and Dyer (12). The phases were separated, and the lower chloroform phase was evaporated to dryness after the addition of an equal volume of benzene. The residual mixture was resolved by preparative TLC (1500 μm or 250 μm thick silica layers) using chloroform–methanol–water 65:35:5 (v/v/v). Bands were identified by iodine vapor on 2-cm margins. Iodine-free bands containing *cyclopentano*-lyso-lecithin and *cyclopentano*-lecithin were scraped from plates and eluted with methanol–chloroform 90:10 (v/v). The band containing fatty acid was eluted with chloroform–methanol–diethyl ether 1:1:1 (v/v/v). Silicic acid was removed by centrifugation, and solvents were removed by rotary evaporation followed by treatment in vacuo (0.2 torr, 56°C) for 24 hr.

Fatty acid identification by GLC

Fatty acids were converted to their methyl esters, as described by Kates (13), for GLC analysis. This procedure entails the use of 2.5% methanolic HCl to *1*) make the methyl ester of free fatty acids and *2*) for methanolysis of the *cyclopentano*-lyso-lecithin and the *cyclopentano*-lecithin. Fatty acid methyl esters (FAMEs) were extracted from the methanolic–HCl phase with hexanes; solvent was removed under a gentle stream of nitrogen. The residue was taken up in chloroform (0.2 ml) and analyzed by GLC (OV-17 column, chromosorb W), using a Varian Model 3700 gas chromatograph and integrator CDS-111 (Palo Alto, CA). The GLC was programmed for a temperature gradient as follows. Upon injection of the FAMEs, the temperature was maintained at 150°C for 1 min followed by a 25°C/min increase up to 225°C where the temperature was sustained for 4 min (retention times for methyl myristate and for methyl palmitate were 4.30 min and 5.50 min, respectively).

EXPERIMENTAL

DL-(1,3/2)-1-O-(Diphenylphosphoryl)-3-O-benzylcyclopentane-1,2,3-triol (3)

Isolation of isomerically pure anti-*epoxide (1).* The synthetic procedure for the epoxy benzyl ether, which produces a mixture of both *syn*- and *anti*-epoxide, has previously been reported (4, 14). This mixture of diastereomeric epoxides (approximately 10 g) was separated by preparative HPLC using hexane–ethyl acetate 95:5 (v/v) as eluant at a flow rate of 100 ml min^{-1}. The retention time for the *anti*-epoxide was 18–25 min (eluted in 700 ml of solvent) and for the *syn*-epoxide 31–40 min (eluted in 900 ml of solvent). Eluates containing the resolved *syn* and *anti*-epoxides were concentrated under reduced pressure to oils that were chromatographically homogeneous as determined by GLC (retention times at 200°C on an OV-17 column, Chromosorb W: *anti*-epoxide 2.4 min and *syn*-epoxide, 3.1 min).

Ring opening of anti-*epoxide (1).* Anhydrous conditions were essential for introduction of phosphate in good yield with minimal formation of cyclopentanetriol benzyl ethers. Therefore, the solvent tetrahydrofuran (THF) was dried over molecular sieves (4 Å) and anhydrous diphenylphosphoric acid was prepared as described in Materials and Methods. *Anti*-epoxide (**1**) (3.6 g, 18.9 mmol) (**Fig. 1**) and diphenylphosphoric acid (5.7 g, 22.8 mmol) were dissolved in THF (20 ml) and the solution was stirred for 1 hr at room temperature. The mixture was then diluted with chloroform and the solution was washed with sodium bicarbonate (5% aqueous solution) and water. The chloroform phase was then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. TLC analysis of the resulting oil (chloroform–diethyl ether 3:1 (v/v) showed two major phosphate-positive spots (R_f 0.55, 0.35) with the major spot of the two having the higher R_f. The mixture was separated by preparative TLC with the same solvent system. Bands corresponding to each isomer were visualized by exposure of 2-cm margins to iodine vapor. The remaining iodine-free bands were removed by scraping and the silicic acid was thoroughly extracted with chloroform–methanol–diethyl ether

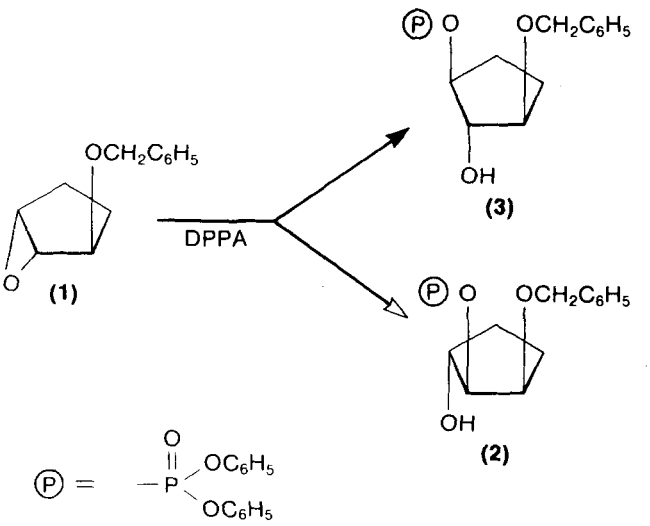

Fig. 1. Reaction sequence for (1,2/3)-2-O-(diphenylphosphoryl)-3-O-benzylcyclopentane-1,2,3-triol (**2**) and (1,3/2)-1-O-(diphenylphosphoryl)-3-O-benzylcyclopentane-1,2,3-triol (**3**) through a phosphoric acid, diphenyl ester-mediated oxirane ring opening of benzyl ether *anti*-anhydrocyclopentanetriol (**1**).

1:1:1 (v/v/v). The silica was removed by filtration and solvents were removed under reduced pressure to give oils. The all-*trans* isomer (**3**) (R_f 0.55) was obtained in a chromatographically pure state (52%; 4.33 g, 9.83 mmol). The other major phosphate-positive spot (R_f 0.35), which is believed to represent the *cis-trans* isomer (**2**), has not been characterized. IR and NMR spectral assignments for (**3**) are listed in **Table 1** and **Table 2**, respectively.

Anal. Calc. for (**3**), $C_{24}H_{25}O_6P$ (440.41): C, 65.45; H, 5.72; P, 7.03. Found: C, 65.46; H, 6.02; P, 6.35.

DL-(1,3/2)-1-O-(Diphenylphosphoryl)-2-O-palmitoyl-3-O-benzylcyclopentane-1,2,3-triol (4)

To a stirred, cool solution (0°C) of *DL*-(1,3/2)-1-O-(diphenylphosphoryl)-3-O-benzylcyclopentane-1,2,3-triol (**3**) (4.12 g, 9.35 mmol) (**Fig. 2**) in anhydrous pyridine (20 ml) was added palmitoyl chloride (3.86 g, 14.0 mmol). The ice bath was then allowed to reach room temperature as the mixture was stirred for 12–28 hr; about 15 g of ice was added to the reaction to hydrolyze excess palmitoyl chloride (30 min), and the mixture was extracted thoroughly with chloroform. The chloroform phase was washed with cold 1N H_2SO_4 (3 × 200 ml), saturated sodium bicarbonate solution (3 × 200 ml), water (3 × 200 ml), and dried over anhydrous sodium sulfate. Removal of solvent under reduced pressure gave an oily product from which the acylated material was isolated by preparative HPLC in the single silica column mode. The eluant was hexane–ethyl acetate 90:10 (v/v), solvent flow rate 100 ml min^{-1}. The acylated product (**4**) eluted at a retention of 18–40 min (2.2 l). Solvent was removed under reduced pressure to give an oil (4.00 g, 5.89 mmol, 63%) that was chromatographically homogeneous by TLC [R_f 0.40, hexane-ethyl acetate 60:40 (v/v)]. The IR assignments are listed in Table 1.

Anal. Calc. for (**4**) $C_{40}H_{55}O_7P$ (678.81): C, 70.77; H, 8.17; P, 4.56. Found: C, 70.83; H, 8.09, P, 4.25.

DL-(1,3/2)-1-O-(Diphenylphosphoryl)-2-O-palmitoylcyclopentane-1,2,3-triol (5)

Debenzylation of *DL*-(1,3/2)-1-O-(diphenylphosphoryl)-2-O-palmitoyl-3-O-benzylcyclopentane-1,2,3-triol (**4**) was carried out by hydrogenolysis as follows. Monopalmitoylbenzyl ether (1.0 g, 1.47 mmol) was dissolved in glacial acetic acid (100 ml). Palladium on charcoal (10%, 100 mg) was added and hydrogenolysis was effected at 50 p.s.i. hydrogen pressure (room temperature) for a period of 8–18 hr. The catalyst was then removed by filtration through Celite. The acetic acid was removed by rotary evaporation and the residual oil was stored in vacuo (0.2 torr) where it crystallized (0.82 g, 1.39 mmol, 95%). TLC developed in chloroform–diethyl ether 3:1 (v/v) showed one major spot (R_f 0.64) and one minor contaminant at the origin. An analytical sample was obtained by preparative TLC using the above solvent system and elution with chloroform–methanol–diethyl ether 1:1:1 (v/v/v) (mp (**5**), 65–65.5°C). IR and NMR assignments are listed in Tables 1 and 2, respectively.

Anal. Calc. for (**5**), $C_{33}H_{49}O_7P$ (588.79): C, 67.31; H, 8.40; P, 5.26. Found: C, 67.46; H, 8.67, P, 5.08.

TABLE 1. Assignment of infrared absorption bands for all-*trans*-(1,3/2-1P) *cyclopentano*-analog precursors in the synthetic route leading to the mixed-acid lecithin (see Figs. 1 and 2)

Assignment	2-Hydroxy-3-O-Bz (3)	2-O-Palmitoyl-3-O-Bz (4)	2-O-Palmitoyl 3-hydroxy (5)	2-O-Palmitoyl 3-O-myristoyl (6)
			cm^{-1}	
1) -OH	3410(s)	NA[a]	3450(s)	NA
2) Aromatics				
C-H bending	745(m)	745(m)	755(sh)	750(sh)
	680(m)	680(m)	690(m)	685(m)
C-H stretching	3030(w)	3030(w)		
C-C multiple bond stretch	1585(s)	1585(m)	1590(m)	1585(m)
	1480(s)	1480(s)	1490(s)	1485(m)
	1450(m)	1450(m)	1460(sh)	1450(sh)
3) P-O-ϕ				
C-O	1185(s)	1185(s)	1190(s)	1190(s)
P-O	950(s)	950(s)	965(s)	945(s)
4) P=O	1270(m)	1285(m)	1270	1295(s)
5) C=O	NA	1735(s)	1735	1735(s)
6) -CH_2 stretch	2920(w)	2920(vs)	2920(vs)	2920(vs)
	2880(sh)	2850(s)	2860(s)	2850(vs)
7) -CH_2 bending	NA	1460(sh)	1470(w)	1460(m)
	1355(vw)	1355(w)	1380(w)	1370(w)

[a]No absorption band observed.

TABLE 2. Assignment of ^{1}H NMR spectra for all-*trans*-(1,3/2-1P) *cyclopentano*-analog precursors in the synthetic route leading to the mixed-acid lecithin (see Figs. 1 and 2)

Compound	Aromatics	Methylenes			Methyl	Ring Protons			
						1	2	3	4,5
		CH_2 | C=O | O |	$-CH_2$	$-(CH_2)_{12}$ or $-(CH_2)_{10}$	CH_3	CH | O | P=O |	CH | $R^{a,b}$	CH | R^{a-c}	$-(CH_2)_2$
					δ				
2-Hydroxy-3-O-Bz (3)	7.32					4.69	4.20[a]	3.80[c]	1.82, 1.97
2-O-Palmitoyl-3-hydroxy (5)	7.26	2.25	1.58	1.25	0.88	4.96	4.80[b]	3.98[a]	1.88, 2.05
2-O-Palmitoyl-3-O-myristoyl (6)	7.26	2.27	1.58	1.25	0.88	4.91	5.31[b]	5.01[b]	1.84, 2.11

[a] -OH.
[b] -OCOR.
[c] -O-CH_2-ϕ.

DL-(1,3/2)-1-O-(Diphenylphosphoryl)-1-O-palmitoyl-3-O-myristoylcyclopentane-1,2,3-triol (*cyclopentano*-phosphatidic acid, diphenyl ester) (6)

The monoacylated triol diphenylphosphate ester (5) (638 mg, 1.08 mmol) was dissolved in pyridine (50 ml) and the solution was cooled to 0°C. Myristoyl chloride (600 mg, 2.4 mmol) was added with stirring. The crude product was obtained as described for (4), and the acylated material was isolated by preparative TLC. The plate was developed in hexane–ethyl acetate 70:40 (v/v) and the product was eluted with chloroform–methanol–diethyl ether 1:1:1 (v/v/v). Silica was removed by filtration and solvents were removed by rotary evaporation. The oily product obtained was then stored in vacuo (0.2 torr) where it crystallized (450 mg, 0.56 mmol, 52%; mp 47–48°C). TLC analysis of the *cyclopentano*-phosphatidic acid, diphenyl ester (hexane–ethyl acetate 60:40, v/v) revealed one spot (R_f 0.39). IR and NMR assignments are listed in Tables 1 and 2, respectively.

Anal. Calc. for (6) $C_{47}H_{75}O_8P$ (799.10): C, 70.65; H, 9.46; P, 3.88. Found: C, 70.58; H, 9.56; P, 3.85.

DL-(1,3/2)-1-O-Phosphorylcholine-2-O-palmitoyl-3-O-myristoylcyclopentane-1,2,3-triol (*cyclopentano*-lecithin) (7)

Synthesis of mixed-acid cyclopentano-*phosphatidic acid.* The phenyl groups were removed from the diphenylphosphoryl ester (6) (450 mg, 0.56 mmol) by hydrogenolysis

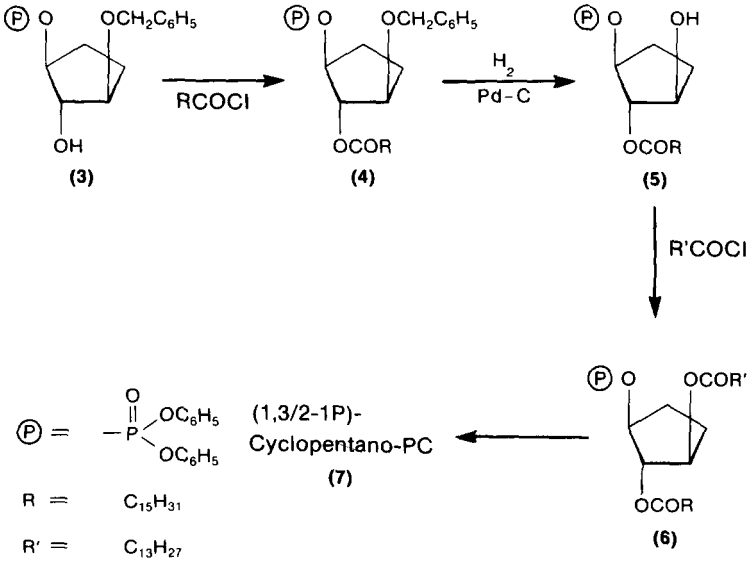

Fig. 2. Reaction sequence for the mixed-acid all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin.

over platinum (50 mg) in chloroform–glacial acetic acid 1:1 (v/v) (200 ml) at 50 p.s.i. hydrogen pressure (room temperature). The catalyst was removed by filtration through Celite and solvent was removed by rotary evaporation and subsequent drying over phosphorus pentoxide in vacuo (0.2 torr) (356 mg, 0.550 mmol, 98%). An analytical sample was prepared by precipitation as follows. The oil (50 mg) was dissolved in chloroform (0.5 ml). Methanol was added, the mixture was heated to 60°C, and the supernatant was decanted from the residual oil. After cooling (−15°C) the precipitate was recovered by centrifugation (mp 81–82°C). TLC (chloroform–methanol–water 65:25:4, v/v/v) showed a single phosphate-positive spot (R_f 0.54).

Anal Calc. for $C_{35}H_{67}O_8P$ (646.99): C, 64.96; H, 10.45; P, 4.79. Found: C, 65.03; H, 10.34; P, 4.78.

IR(KBr) cm^{-1}: 3680–3200, OH; 2920, 2850, CH_2 stretch; 1730, C=O; 1465, 1360, CH_2 bend; 1250, P=O; 1175, C-O-C stretch; 1110, PO_2^- stretch; 1085, P-O-C stretch; 720, CH_2 rock.

Synthesis of mixed-acid cyclopentano-*phosphatidylcholine.* *DL*-(1,3/2)-2-O-Palmitoyl-2-O-myristoyl-1-O-phosphoric acid (81 mg, 0.12 mmol) and choline tosylate (69 mg, 0.25 mmol) were dissolved in anhydrous pyridine (30 ml) with gentle warming (60°C) in a stoppered flask. TPS (114 mg, 0.375 mmol) was quickly added and the solution was stirred at room temperature for 12 hr. After addition of water (1 ml), the yellow solution was evaporated under reduced pressure to an oil that was further dried in vacuo (0.2 torr) in the presence of phosphorus pentoxide. Chloroform (100 ml) was added, and the resulting solution was successively stirred with Amberlite IRA-410 resin (3 × 10 mg) and IR-120 resin (3 × 10 mg). The resulting tan-colored low melting solid was further purified by preparative TLC (chloroform–methanol–water 65:35:4, v/v/v) and was eluted from the silica with methanol–chloroform 95:5 (v/v). Solvents were removed by rotary evaporation giving a white solid (44 mg, 0.060 mmol, 50%). TLC analysis (chloroform–methanol–water 65:25:4, v/v/v) showed a single phosphate-positive spot (R_f 0.60). NMR assignments are listed in **Table 3.**

Anal. Calc. for (7) $C_{40}H_{80}O_8PN$ (734.0): C, 65.45; H, 10.98; P, 4.22; N, 1.91. Found: C, 65.60; H, 10.94; P, 4.26; N, 1.77; N/P, 0.92.

IR(KBr) cm^{-1}: 2920, 2840, CH_2 stretch; 1740, C=O; 1465, 1375, CH_2 bending; 1260, P=O; 1050, P-O-C stretch; 1170, C-O-C stretch; 1090, PO_2^- stretch; 965, C-C-N^+.

RESULTS

Cyclopentano-lecithin susceptibility

Six cyclopentanoid analogs of DPPC, as mixed micelles, were assayed with phospholipase A_2 (*Crotalus adamanteus*). This study was designed as an initial survey to target those diastereoisomers that acted as substrate for further kinetic analysis. These incubations (described in Materials and Methods) were performed at 48°C, above the thermotropic phase transition of each of the *cyclopentano*-lecithin isomers (15) Enzymatic activity was determined qualitatively by TLC where the products of enzymatic hydrolysis could be identified (lyso-product, palmitic acid). Therefore, for these studies it was necessary to use a detergent that not only solubilized these lecithin analogs, but which possessed chromatographic mobility sufficiently different from that of the palmitic acid. We found sodium deoxycholate, an anionic detergent, well suited to this study. It gave an optically clear solution of

TABLE 3. Assignment of ^1H NMR spectra for all-*trans*-(1,3/2-1P) *cyclopentano*-homo-acid- and mixed-acid-PC

Compound	Polar Head Group			Methylene			Methyl	Ring			
								1	2	3	4,5
	$^+N(CH_3)_3$	N | CH_2 |	P | O | CH_2 |	CH_2 | C=O | O |	$-CH_2$	$-(CH_2)_{12}^-$ or $-(CH_2)_{10}^-$	CH_3	CH | O | P=O |	CH | R[a,b]	CH | O | C=O	$-(CH_2)_2^-$
						δ					
Glycero-PC[f]	3.32	3.32	4.30	2.30	1.58	1.27	0.88	4.08[c]	5.15[d]	4.35[c]	
Cyclopentano-homo-acid-PC	3.38	3.78	4.52	2.30	1.58	1.25	0.89	4.40	5.15[a]	4.96	2.10
Cyclopentano-mixed-acid-PC	3.37	3.80	4.58	2.28	1.57	1.25	0.88	4.35	5.13[a]	4.96	1.87, 2.04

[a]R = ester linkage.
[b]R = hydroxyl.
[c]Indicates protons on glycerol carbon ($-CH_2-O-P=O$).
[d]Indicates proton on glycerol carbon ($-CH-O-C=O$).
[e]Indicates protons on glycerol carbon ($-CH_2-O-C=O$).
[f]Model compound, *DL*-1,2-dipalmitoyl-3-phosphatidylcholine.

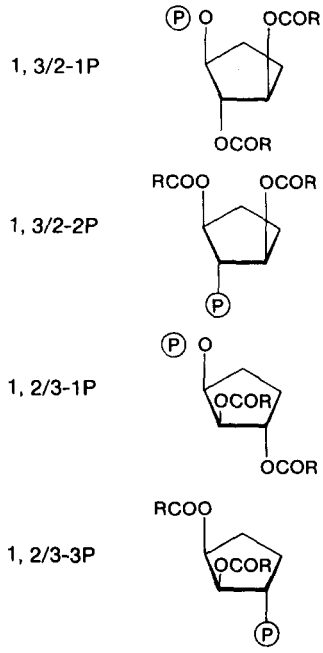

Fig. 3. *Cyclopentano*-PC isomers observed to be hydrolyzed by phospholipase A_2. The circled P represents the polar head group.

mixed micelles at 2:1 molar ratio (detergent/lipid) and possessed a chromatographic mobility (R_f 0.75) lower than that of palmitic acid (R_f 0.90).

The experiments showed that after an incubation of 6 hr, four of the six *cyclopentano*-lecithin isomers were observed to be effective substrates (**Fig. 3**). Each analog which contained the phosphocholine moiety in the C-1 (or C-3) position (1,2/3-3P; 1,3/2-1P; 1,2/3-1P) was a substrate, but when the phosphocholine occupied the C-2 position, only the all-*trans*-(1,3/2-2P)-*cyclopentano*-lecithin was found to be a substrate. The other two isomers (1,2,3/0-2P; 1,2/3-2P), even after extended incubation times (24 hr) and the addition of further enzyme, were not hydrolyzed. To ensure that nonspecific hydrolysis did not occur, the 1,2/3-3P analog was also tested with the enzyme in an assay mixture containing 5 mM EDTA but devoid of Ca^{2+}. No release of fatty acid was observed, indicating the relative stability of the analog under the experimental conditions.

pH-Stat assay

A 10 mM stock mixed micelle solution containing 20 mM Triton X-100 and 10 mM calcium chloride was prepared for DPPC and for each of those *cyclopentano*-lecithin substrates. After the foam had subsided, appropriate aliquots were taken, diluted (10 mM calcium chloride) to the desired concentration, and transferred to a pH-stat vessel where reactants were brought to assay temperature (41°C) and pH 8.9 under a nitrogen atmosphere. Assays were initiated by the addition of phospholipase A_2 (*Crotalus*) using a 10-μl syringe. Enzymatic release of fatty acid was automatically titrated with sodium hydroxide solution. Initial rates were obtained from the slopes of the recorder output (equivalents of base/time).

For DPPC, initial rates were obtained by adding 0.425 μg of enzyme protein to 2.5 ml of an appropriate mixed micelle concentration and titrating with 10 mM sodium hydroxide. To observe the initial rates for the all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin, it was necessary to add 17.0 μg protein to 5 ml of the appropriate mixed micelle concentration and to titrate with 5 mM sodium hydroxide. Double reciprocal plots for DPPC and the all-*trans*-lecithin analog are shown in **Fig. 4.** Kinetic constants determined from lines are seen in **Table 4.** For the other

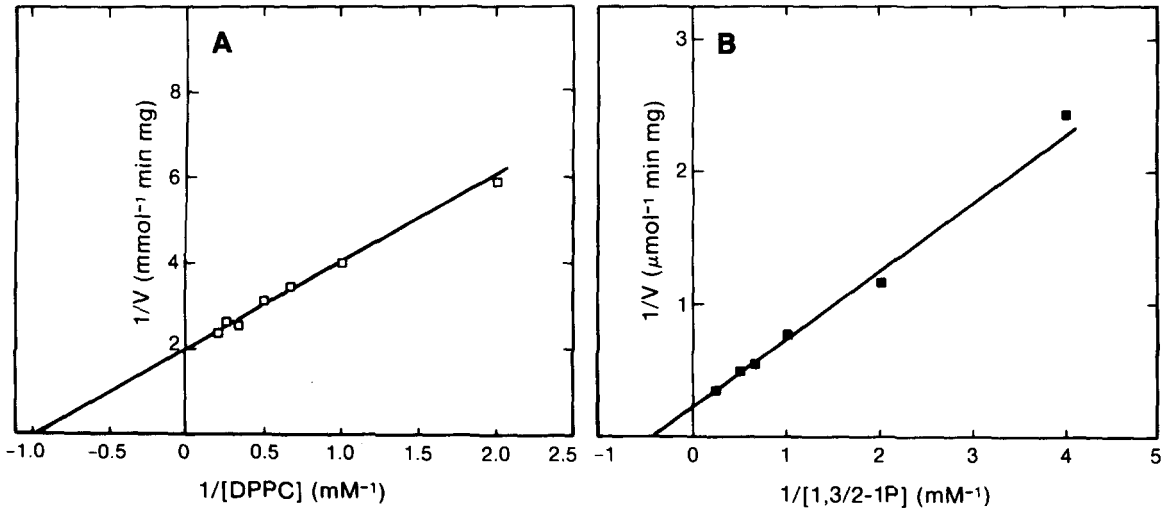

Fig. 4. Double reciprocal plot of phospholipase A_2 activity towards (A) DL-DPPC and the (B) all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin. Each substrate was in the form of Triton X-100 mixed micelles at a molar ratio of 2:1 (detergent:phospholipid).

TABLE 4. Kinetic constants for the phospholipase A_2-mediated hydrolysis of all-*trans cyclopentano*-lecithin and DL-α-DPPC

Substrate	App. K_M	App. V_{MAX}
	mM	μmol min^{-1} mg $protein^{-1}$
DPPC	1.0	500
(1,3/2-1P)-CPC	2.0	5.0

lecithin analogs, slopes obtained from the pH-stat were not sufficiently above background to yield reliable rates, even at the highest substrate concentrations employed (6 mM) and using twice the amount of enzyme used for the all-*trans*-(1,3/2-1P) isomer (34 μg protein). Addition of excessive amounts of enzyme resulted in a large initial fluctuation in pH followed by erratic electrode response. Furthermore, it was not possible to obtain a rate from a prolonged incubation because a sensitive electrode and stable baseline could not be maintained. Based on the rates obtained for the all-*trans* analog, the data suggest that at concentrations approaching the V_{max}, the rates of the other three hydrolyzable substrates must be at least 10 times lower.

Analysis of enzymatic products

A mixed micellar solution of the all-*trans* (1,3/2-1P)-*cyclopentano*-lecithin (30 ml, 4.5 mM) was incubated with phospholipase A_2 (*Crotalus*) after which products were separated by preparative TLC as described previously. The optical rotation values ($[\alpha]_D^{25°}$) of these compounds were +4.08 for the unhydrolyzed *cyclopentano*-lecithin and +9.82 for the lyso-product. The enzymatically derived lyso-product, a new compound, was further characterized by IR, NMR, and elemental analysis (see below). The IR spectrum was consistent with that expected for the acyl compound with distinctive hydroxyl absorption at 3400–3200 cm^{-1}. The NMR spectrum showed the expected upfield chemical shift of the CH ring protons relative to the corresponding protons of the diacyl compound (^{1}H NMR assignments for lecithin analog shown in Table 3) with the greatest shift observed for the ring proton on the carbon now containing the hydroxyl (>1.0 ppm shift). These chemical shift differences are analogous to those observed for the glycerol protons of the natural lyso-lecithin relative to those of PC (16, 17).

Anal Calc. for $C_{24}H_{48}O_7PN1/2H_2O$ (511.64): C, 56.35; H, 9.83; N, 2.79; P, 6.05; N/P, 1.00. Found: C, 57.17; H, 9.80; N, 2.68; P, 5.50; N/P, 1.08.

IR (cm^{-1}): 3400–3200, OH; 2920, 2850, CH_2 stretch; 1730, C=O; 1465, 1355, CH_2 bending; 1245, P=O; 1045, P–O–C stretch; 1175, C–O–C stretch; 1085, PO_2^- stretch; 970, C–C–N^+.

NMR: Polar head group: δ 3.35, N^+ $(CH_3)_3$; δ 3.83, NCH_2; δ 4.29, $POCH_2$; acyl group: δ 2.30, α CH_2; δ 1.58, β CH_2; δ 1.25, $(CH_2)_{12}$; δ 0.88 CH_3; ring: δ 4.29, CHOPO; δ 3.99, CHOH; δ 4.83, CHOCO; δ 1.80, 2.06 $(CH_2)_2$.

Synthesis of mixed acid-lecithin analog

The synthesis of a *cyclopentano*-lecithin with distinguishable acyl groups was undertaken so that enzyme specificity could be determined. The synthetic approach for the mixed-acid all-*trans*-DL-(1,3/2-1P)-*cyclopentano*-lecithin involved an oxirane ring opening. The method was based on two previous observations. Chan and Di Raddo (18) found that the reaction of a phosphodiester with either a *cis*- or *trans*-cyclohexanol-2,3-oxide was stereospecific for a *trans*-opening of the epoxide and regiospecific, with the phosphate attacking the oxirane carbon distal to the neighboring hydroxyl group. Wade and Hancock (K. C. Wade and A. J. Hancock, unpublished results) performed an analogous oxirane ring opening on *trans*-cyclopentanol 2,3-oxide (*anti*-epoxy alcohol) with diphenylphosphoric acid. The reaction was observed to be stereospecific with *trans*-opening, but not exclusively regiospecific. However, the phosphate did favor attack on the distal oxirane carbon (relative to the hydroxyl), yielding as the major product the all-*trans*-(1,3/2-1P) isomer.

For the synthesis of the mixed-acid *cyclopentano*-lecithin, a similar procedure was employed for the oxirane ring opening of the *anti*-epoxy alcohol benzyl ether (1) (Fig. 1). In this case, the benzyl ether provided an appropriate blocking group on the *trans*-anhydrotriol, permitting upon ring opening the availability of only one hydroxyl position for acylation. As similarly noted by Wade and Hancock (unpublished results), the formation of two products was observed, believed to be compounds (**2**) and (**3**). It was further noted that if reaction conditions were not maintained strictly anhydrous, an additional pair of products was formed. These were identified by TLC and GLC evaluation as triol benzyl ethers (1,2/3-1-0-Bz, and 1,3/2-1-0-Bz). Under anhydrous conditions, purification of the major phosphorylated product (R_f 0.55; see Experimental) was identified by ^{1}H NMR to be the all-*trans*-(1,3/2-1P) isomer (**3**). ^{1}H NMR homodecoupling study (**Fig. 5**) allowed identification of each of the CH ring protons and showed that the well-defined triplet centered at δ4.20 corresponded to the C-2 proton. This triplet for the C-2 proton, shown to be diagnostic for cyclopentanoid compounds with the all-*trans* configuration (19, 20), confirmed the isomer as compound (**3**). The nonequivalence of the benzylic CH_2 protons showed the expected AB quartet (21) centered at δ4.56, an observation analogous to that of Hancock, Stokes, and Sable (20) in the NMR spectrum for the all-*cis*- and all-*trans*-2,3-dipalmitoyl-1-benzyl ethers.

The synthetic route for the mixed-acid-*cyclopentano*-lecithin is shown in Fig. 2. The benzyl ether (**3**) was acylated with palmitoyl chloride to give the all-*trans* diphenyl ester of 1-O-benzyl-2-O-palmitoyl cyclopentanetriol-3-

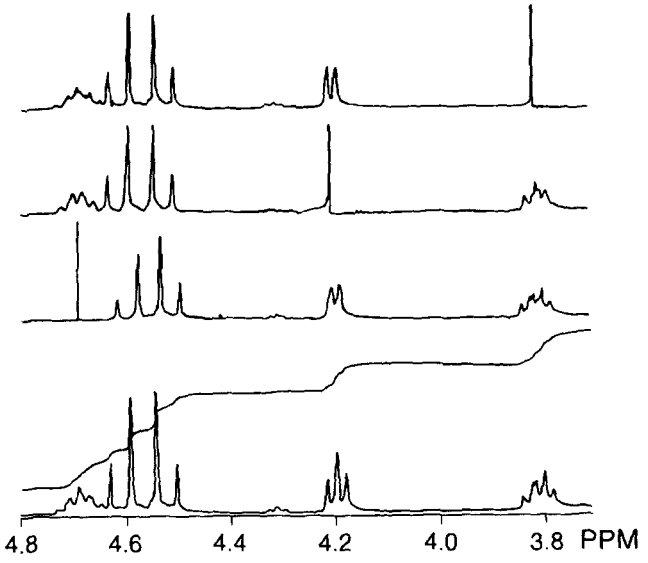

Fig. 5. Homodecoupled ^1H NMR spectra of CH ring protons of (1,3/2)-1-O-(diphenylphosphoryl)-3-O-benzylcyclopentane-1,2,3-triol (**3**). Spectra identify the triplet signal as the C-2 ring proton, characteristic of the all-*trans* ring configuration. The quartet is due to nonequivalence of the benzylic CH_2 protons.

phosphate (**4**). The formation of this monoacylated compound was apparent from the IR analysis (absence of -OH in the 3400 cm^{-1} region and the appearance of the C=O (1735 cm^{-1}) and typical CH_2 stretch (e.g., 2920 cm^{-1}, 2850 cm^{-1}) absorption bands (Table 1)). Debenzylation of this compound by hydrogenolysis over palladium gave the 2-O-acyltriol (**5**). Characterization of the lyso-PA, diphenyl ester by IR (Table 1) showed the presence of the -OH (3450 cm^{-1}) and a weakened intensity of some of the aromatic absorption bands. Furthermore, NMR deuterium exchange identified the presence of an -OH proton (δ3.26) by showing disappearance of its resonance after the addition of D_2O. The CH ring proton at C-2 was again easily identified by its well-defined triplet (δ4.80) as previously discussed. The peak assignments are given in Table 2. The lyso-compound was acylated with myristoyl chloride to give the mixed-acid-*cyclopentano*-phosphatidic acid, diphenyl ester (**6**). The NMR and IR spectra for the mixed-acid compound were found to be essentially identical to those measured in this laboratory for the homo-acyl compound (E. O. Lewis and A. J. Hancock, unpublished data). Diagnostic of this compound with respect to its precursor was the absence of the -OH band in the 3400 cm^{-1} region of the IR Spectrum (Table 1) and the chemical shifts of the CH ring protons in the NMR spectrum (Table 2). The expected triplet for the C-2 proton (δ5.31) was easily observed at a higher sensitivity. Of particular interest were the CH ring protons which gave identical signals (distinct from the other diastereoisomers) to that of the (1,3/2-1P) homo-acyl compound, consistent with the shifts expected for this structure. The diphenyl ester (**6**) was freed of phenyl groups by hydrogenolysis over platinum, and the *cyclopentano*-phosphatidic acid was condensed with choline tosylate to give the mixed-acid-*cyclopentano*-lecithin (7) in good yield as described previously for the homo-acid *cyclopentano*-lecithins (5). Elemental analytical data for (7) gave an empirical formula that was consistent with that calculated for a *cyclopentano*-lecithin devoid of hydration. Spectroscopic analytical data were almost indistinguishable from those obtained for the homo-acid all-*trans-cyclopentano*-lecithin. Comparison of the NMR assignments for the homo- and mixed-acid analogs as well as *glycero*-PC (17) is summarized in Table 3. The NMR spectrum for the mixed-acid lecithin analog clearly showed the expected triplet for the C-2 ring proton at δ5.13. The spectrum of the CH ring protons was, as with the phosphatidic acid, diphenyl ester analog, found to be essentially identical to that of the homo-acid.

Acyl group specificity

The mixed-acid-*cyclopentano*-lecithin (2-palmitoyl-3-myristoyl) (3 ml, 4.5 mM) was incubated with phospholipase A_2 from *Crotalus* and *Apis* for the purpose of determining acyl group specificity for each enzyme. Extraction from the aqueous phase and separation of the residual mixture was done as described above. The fatty acid composition of both the lyso-product and the parent mixed-acid-*cyclopentano*-lecithin was determined by transesterification in refluxing methanolic-HCl followed by GLC analysis of the resulting FAMEs. The identity of the free fatty acid was determined in an analogous manner, except that it was directly esterified by acid reflux. The data showed that the free fatty acid liberated in incubation with each enzyme was >97% palmitate while the acyl group retained on the lyso-product was >92% myristate (**Table 5**).

DISCUSSION

Six *cyclopentano*-lecithin isomers were tested qualitatively for their susceptibility to phospholipase A_2 hydrolysis by simple incubation studies. These experiments showed that four of the isomers were substrates. All analogs that contained the phosphocholine moiety in the C-1 (or C-3) position (1,2/3-3P, 1,3/2-1P, 1,3/2-1P) were substrates suggesting that given sufficient time, the configuration of the substrate is not a prerequisite for enzymatic hydrolysis. When the phosphocholine occupied the C-2 position, only the all-*trans*-(1,3/2-2P)-*cyclopentano*-lecithin was found to be a substrate. This would indicate that the enzyme acts only on a configuration where the acyl groups are nominally *trans* to the polar head group. Such an arrangement might be expected, since the configuration easily accommodates the polar head group in

TABLE 5. GLC analysis of fatty acids after phospholipase A_2-mediated hydrolysis of the mixed-acid *cyclopentano*-lecithin

Enzyme Source	Fatty Acid		Lyso-CPC[a]		Unhydrolyzed-CPC[a]	
	$\%C_{16}$	$\%C_{14}$	$\%C_{16}$	$\%C_{14}$	$\%C_{16}$	$\%C_{14}$
Apis mellifica (bee venom)	98.5	1.5	7.4	92.6	53.9	46.1
Crotalus adamanteus (rattlesnake venom)	97.7	2.3	7.0	93.0	52.8	47.2
Control (no PLA_2)					51.4	48.6

[a]CPC, *cyclopentano*-phosphatidylcholine.

a hydrophilic environment and the acyl groups in a hydrophobic environment, accommodations not so easily attained for the other two isomers (1,2,3/0-2P; 1,2/3-2P). A similar *trans* arrangement has been implicated for β-DPPC (polar group on C-2 of the glycerol backbone) in detergent micelles. Roberts, Bothner-By, and Dennis (22) have shown by ^1H NMR, that the α-methylene protons of both acyl groups of the β-DPPC are equally shielded and resonate to give a single peak (δ2.32), which indicates that both are in similar environments. While this does not confirm an all-*trans* conformation, it does appear to be a logical probability that would be consistent with the results from the *cyclopentano*-β-lecithin study.

In an attempt to measure the kinetic constants of phospholipase A_2 (*Crotalus adamanteus*) toward each of the *cyclopentano*-lecithin analogs identified as substrates, pH-stat experiments were performed under conditions optimal for the enzymatic hydrolysis of DPPC essentially as reported by Dennis (11, 23). Since each *cyclopentano*-lecithin used in these experiments was a racemic mixture (except the all-*trans*-(1,3/2-2P) isomer, a *meso* form), *DL*-α-DPPC was used as a control substrate. A substrate dependence curve was obtained for the all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin. For the other lecithin analogs, initial rates were too low to be measured by the pH-stat method. The results indicate that the all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin is hydrolyzed at a rate of at least an order of magnitude higher than the rates for the other analogs, suggesting that the enzyme does possess conformational requirements for maximal activity.

At this point, these findings could only construe information about the conformational requirements of phospholipase A_2 towards the *cyclopentano*-lecithins, and could only be directly extrapolated into requirements of the enzyme for the natural glyceryl counterpart, DPPC, if it could be shown that the analogs participated as an analogous substrate. Therefore, the products derived from enzymatic action on all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin were characterized. One of the enzymatic products was shown to be lyso-product by IR, NMR, and elemental analysis. The lyso-lipid was found to rotate the plane of polarized light, as did the residual unhydrolyzed diacyl compound. Since the *cyclopentano*-lecithin was synthesized from racemic intermediates and therefore was devoid of optical rotatory activity, these findings indicate that the enzyme discriminated against the two optical antipodes in the *DL-cyclopentano*-lecithin. Discrimination has also been observed for the short chain all-*trans-cyclopentano*-lecithin (7). It is assumed that hydrolysis occurs either exclusively at one chiral center, or at one center at a substantially faster rate than the other. At this time, it is not possible to identify the susceptible enantiomer, since optically pure *cyclopentano*-lecithins have not yet been synthesized.

The positional specificity of enzymatic hydrolysis could not be unambiguously determined from the lyso-product alone. In order to determine whether the enzyme was able to discriminate between the acyl groups of the *cyclopentano*-lecithin as it does in a natural glyceryl substrate, it was necessary to study the hydrolysis of an all-*trans-cyclopentano*-lecithin having distinguishable acyl groups. We have reported herein the synthesis of such a compound which contains palmitoyl and myristoyl at the C-2 and C-3 positions, respectively. Enzyme from two different sources (*Crotalus adamanteus; Apis mellifica*) was utilized in these experiments to observe whether the positional specificity of each enzyme was similar for this lecithin analog. The composition of the free fatty acid mixture liberated separately by each enzyme source was >97% palmitate and <3% myristate. Since intramolecular acyl migration is not expected and indeed has not been observed in the all-*trans-cyclopentano*-lecithin, we interpret this finding to indicate a positional specificity of phospholipase A_2 for attack at C-2 of the cyclopentanoid substrate. Analysis of the lyso-product after isolation from the reaction mixture lent support to this interpretation; the esterified fatty acid was found to be >92% myristate. In early experiments, van Deenen and de Haas (24) defined the minimal structural requirements for substrate hydrolysis by phospholipase A_2. For the class of α-glycerophospholipids, the authors demonstrated that the enzyme was specific only for the *L*-isomer (*sn*-3-phosphocholine) and that it hydrolyzed the acyl group at the C-2 position. Since we have shown that the all-*trans-cyclopentano*-lecithin is acted on stereospecifically and that it is preferentially hydrolyzed at the C-2 position, we believe that this analog mimics its glycerol-derived counterpart, DPPC, as a bona fide substrate.

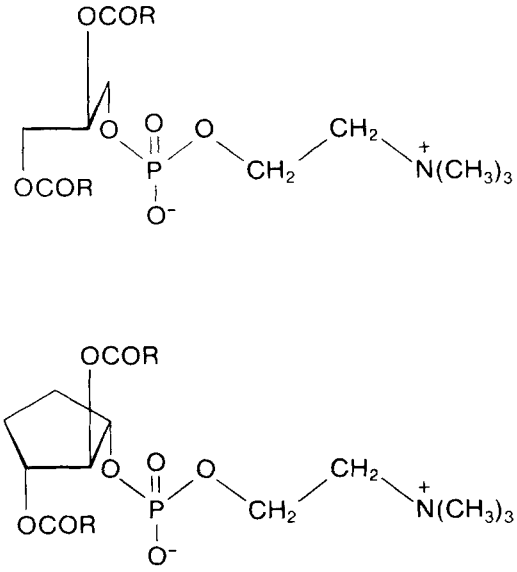

Fig. 6. Molecular representation of *glycero*-PC in a conformation resembling all-*trans*-(1,3/2-1P)-*cyclopentano*-lecithin. Neither structure is implied as fixed.

The all-*trans* lecithin analog, having a comparable K_m value, appears to be a poorer substrate than DPPC with respect to maximal velocity. The assays described here were performed under conditions optimal for DPPC (11, 23). Realizing that the cyclopentanoid analogs possess different physical properties (15, 17) due to restricted rotation of their substituents, assay temperatures and Triton X-100 concentrations were varied. No substantial increases in rates were obtained (M. D. Lister and E. A. Dennis, unpublished results). However, activity of phospholipase A_2 towards the optically active substrate alone remains to be determined since it is possible that the inactive stereoisomer may be a potent inhibitor. Other factors may contribute to the slower rates for the *cyclopentano*-lecithin, such as the presence of the ethylene bridge of the ring at the enzyme active site, a lack of a precise fit of analog configuration to binding site, or alternatively, an inability of the analog in attaining optimal transition state geometry. Despite the slower rates observed in these experiments, we believe that the all-*trans*-lecithin analog most accurately defines the conformational requirements of the glycerol backbone for phospholipase A_2 activity. **Fig. 6** depicts an illustration of how this lecithin analog and its glycerol counterpart might be compared. However, it is not intended to imply that either structure represents the known, fixed conformation of the compound.

X-ray diffraction (25, 26) studies on crystalline bilayers of phosphatidylcholine and phosphatidylethanolamine, and ^{2}H NMR studies (27) on phosphatidylethanolamine indicate that the beginning segment of the *sn*-2 chain (α-methylene group) is oriented parallel to the bilayer surface while the *sn*-1 chain is perpendicular to that surface. ^{1}H NMR studies by Roberts and co-workers (22) and De Bony and Dennis (28) have further indicated that the orientation of the acyl chains is similar in all aggregated states (crystal, bilayer and micelles), whereas the monomer form may not be as constrained. These findings suggest that one possibility for the dramatically increased rate of hydrolysis observed for micelles is the conformational requirements of phospholipase A_2, which are met in the aggregated state of the phospholipid, but are lacking in the monomer. This conclusion has also been drawn by Wells in earlier reports (2, 3). In this regard, phospholipase A_2 activity toward the recently synthesized short chain all-*trans* analog (7), in contrast to the short chain *glycero*-lecithin, has shown no dramatic increase in rates of hydrolysis when concentrations went from below to above the CMC of the analog (Barlow, P. N., J-C. Vidal, M. D. Lister, E. A. Dennis, and P. B. Sigler. 1988,*J. Biol. Chem.* **263** In press.). These results, coupled with those herein, suggest that phospholipase A_2 does require specific conformational states for optimal activity and that the range of states can be approximated by the all-*trans* arrangement represented by (1,3/2-1P)-*cyclopentano*-lecithin. We believe that continued studies of these rotationally restricted analogs will further reveal the role that conformation plays in the mechanism of action of this key enzyme.

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REFERENCES

1. Dennis, E. A. 1983. Phospholipases. *In* The Enzymes. Third Edition, Vol. 16. Lipid Enzymology, P. Boyer, editor. Academic Press, New York. 307–353.
2. Wells, M. A. 1974. The mechanism of interfacial activation of phospholipase A_2. *Biochemistry.* **13:** 2248–2257.
3. Wells, M. A. 1978. Interfacial activation of phospholipase A_2. *Adv. Prostaglandin Thromboxane Res.* **3:** 39–45.
4. Hancock, A. J. 1981. Synthesis of cyclopentanoid analogs of diacylglycerophosphate. *Methods Enzymol.* **72:** 640–672.
5. Hancock, A. J., M. D. Lister, and H. Z. Sable. 1982. Analogs of natural lipids. VII. Synthesis of cyclopentanoid analogs of phosphatidylcholine. *J. Lipid Res.* **23:** 183–189.
6. Lister, M. D., and A. J. Hancock. 1981. 17th Midwest Regional Meeting, American Chemical Society, Columbia, MO. Abstr. No. 207.
7. Barlow, P. N., J-C. Vidal, M. D. Lister, A. J. Hancock, and P. B. Sigler. 1988. Synthesis and some properties of constrained short-chain phosphatidylcholine analogs -(+)- and (−)-(1,3/2)-1-O-(phosphorylcholine)-2,3-O-dihexanoylcyclopentane-1,2,3-triol. *Chem. Phys. Lipids.* **46:** 157–164.
8. Ryu, E. K., and M. MacCoss. 1979. Modification of the Dittmer-Lester reagent for the detection of phospholipid derivatives on thin-layer chromatograms. *J. Lipid Res.* **20:** 561–563.
9. Dittmer, J. C., and R. J. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126–127.
10. Rosenthal, A. F. 1966. New, partially hydrolyzable synthetic analogues of lecithin, phosphatidylethanolamine, and phosphatidic acid. *J. Lipid Res.* **7:** 779–785.
11. Dennis, E. A. 1973. Kinetic dependence of phospholipase

A_2 activity on the detergent Triton X-100. *J. Lipid Res.* **14:** 152–159.
12. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
13. Kates, M. 1972. *In* Techniques of Lipidology. T. S. Work, editor. North-Holland Publishing Company, Amsterdam. 362–363.
14. Hancock, A. J., S. M. Greenwald, and H. Z. Sable. 1975. Analogs of natural lipids. I. Synthesis and properties of tris-homoacyl derivatives of cyclopentane-1,2,3-triols. *J. Lipid Res.* **16:** 300–307.
15. Singer, M. A., M. K. Jain, H. Z. Sable, H. J. Pownall, W. W. Mantulin, M. D. Lister, and A. J. Hancock. 1983. The properties of membranes formed from cyclopentanoid analogues of phosphatidylcholine. *Biochim. Biophys. Acta.* **731:** 373–377.
16. Lammers, J. G., T. J. Liefkens, J. Bus, and J. van der Meer. 1978. Synthesis and spectroscopic properties of α and β phosphatidylcholines and phosphatidylethanolamines. *Chem. Phys. Lipids.* **22:** 293–305.
17. Birdsall, N. J. M., J. Feeney, A. G. Lee, Y. K. Levine, and J. C. Metcalfe. 1972. Dipalmitoyl-lecithin: assignment of the ^1H and ^{13}C nuclear magnetic resonance spectra and conformational studies. *J. Chem. Soc. Perkin Trans. II.* 1441–1445.
18. Chan, T. H., and P. Di Raddo. 1979. Reaction of cyclohexene oxides with phosphodiesters—towards understanding the reaction of benzo[a]pyrene diol epoxide with DNA. *Tetrahedron Lett.* **22:** 1947–1950.
19. Steyn, R., and H. Z. Sable. 1971. Studies on cyclitols. XVI. Conformational analysis of substituted cyclopentanes, cyclopentenes and cyclopentane oxides. *Tetrahedron.* **27:** 4429–4447.
20. Hancock, A. J., M. H. Stokes, and H. Z. Sable. 1977. Analogs of natural lipids. IV. Synthesis and properties of cyclopentanoid analogs of phosphatidic acid. *J. Lipid Res.* **18:** 81–92.
21. Jackman, L. M., and S. Sternhell. 1969. Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry. Pergamon Press, Braunschweig, Germany. 129–130.
22. Roberts, M. F., A. A. Bothner-By, and E. A. Dennis. 1978. Magnetic nonequivalence within the fatty acyl chains of phospholipids in membrane models: ^1H nuclear magnetic resonance studies of the α-methylene gorups. *Biochemistry.* **17:** 935–942.
23. Dennis, E. A. 1973. Phospholipase A_2 activity toward phosphatidylcholine in mixed micelles: surface dilution kinetics and the effect of thermotropic phase transition. *Arch. Biochem. Biophys.* **158:** 485–493
24. van Deenen, L. L. M., and G. H. de Haas. 1963. The substrate specificity of phospholipase A_2. *Biochim. Biophys. Acta.* **70:** 538–553.
25. Pearson, R. H., and I. Pasher. 1979. The molecular structure of lecithin dihydrate. *Nature (London).* **281:** 499–501.
26. Hitchcock, P. B., R. Mason, K. M. Thomas, and G. G. Shipley. 1974. Structural chemistry of 1,2-dilauroyl-DL-phosphatidylethanolamine: molecular conformation and intermolecular packing of phospholipids. *Proc. Natl. Acad. Sci. USA.* **71:** 3036–3040.
27. Seelig, A., and J. Seelig. 1975. Bilayers of dipalmitoyl-3-*sn*-phosphatidylcholine conformational differences between the fatty acyl chains. *Biochim. Biophys. Acta.* **406:** 1–5.
28. DeBony, J., and E. A. Dennis. 1981. Magnetic nonequivalence of two fatty acid chains in the phospholipids of small unilamellar vesicles and mixed micelles. *Biochemistry.* **20:** 5256–5260.