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Design, synthesis, and characterization of bis-phosphatidylcholine, a mechanistic probe of phosphatidylcholine transfer protein catalytic activity

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The design, synthesis, and characterization of 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycero-3-phosphocholine) is described. Bis-phosphatidylcholine is a dimeric phospholipid comprised of two glycerophosphocholine groups linked together by a disulfide bond at the distal ends of the *sn*-1 fatty acyl chains. Electron microscopy and [^{14}C]glucose trapping studies indicate that hydrated dispersions of bis-phosphatidylcholine form closed, spherical structures which have diameters in the range of 125–500 nm. Sensitivity to phospholipase hydrolysis suggests that this bipolar lipid is organized in a membrane such that the two polar head groups of the molecular are oriented at the same surface of the membrane. Using conditions in which bovine liver phosphatidylcholine transfer protein transfers both unsaturated and saturated diacyl phosphatidylcholines between fluid phosphatidylcholine vesicles, no transfer of the bipolar phospholipid is observed. The lack of activity toward bis-phosphatidylcholine suggests that this molecule may be a useful tool for elucidating the role of membrane phosphatidylcholine in the catalytic mechanism of the phosphatidylcholine transfer protein.

Introduction

Phosphatidylcholine transfer protein from bovine liver specifically mediates the transport of PC between membrane populations, a process which may be either an exchange or a net transfer of lipid molecules (for recent reviews, see Refs. 1–3). PC-TP transfers, with varying levels of activity, a wide spectrum of saturated and unsaturated PC acyl chain species [4,5] as well as PC molecules containing chemically modified acyl chains [6–8]. Considerable emphasis has been placed on the mechanism of protein-mediated PC movement with regard to the effects on membrane lipid composition [9–11] and substrate organization within the membrane [11–14]. However, the relationship between protein-mediated PC transport and the involvement of the phosphorylcholine polar head group at the lipid/water interface has not been addressed. Detailed aspects of this relationship should help clarify the dual catalytic activities of PC-TP to mediate both an exchange and

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Abbreviations: PC, phosphatidylcholine; PC-TP, bovine liver phosphatidylcholine transfer protein; GPC-$CdCl_2$, L-α-glycero-3-phosphocholine-cadmium chloride; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectroscopy; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bis-PC, 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycero-3-phosphocholine).

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net transfer of PC. One critical problem in defining the role of membrane PC on PC-TP catalytic activity is to distinguish between PC molecules which are transferable substrates and those which are membrane components. Our approach has been to design a PC molecule which is not a substrate for PC-TP. Taken into account were the observations that PC-TP has a single binding site for PC [15] and that PC-TP exchanges its endogenous PC for a PC molecule in the outer monolayer of bilayer membranes [16,17]. Based on these criteria a dimeric phospholipid, 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycero-3-phosphocholine) (bis-PC), was synthesized. A principal feature of bis-PC is its chemical similarity to the saturated PC substrate for PC-TP, dipalmitoylPC, differing only at the terminal methyl region of the *sn*-1 acyl chain where a sulfhydryl group is placed in order that two PC molecules can be linked by a disulfide bond. Characterization of chemical and physical properties of bis-PC is described; evidence is presented that bis-PC is not transferred by PC-TP. In the accompanying paper bis-PC is used to examine the role of membrane PC in protein-mediated PC transfer [18].

Materials and Methods

Materials. L-α-Glycerophosphocholine-cadmium chloride adduct was prepared from egg PC [19], and 16-mercaptohexadecanoic acid was prepared from 16-bromohexadecanoic acid [20]. Acyl anhydrides and diacylglycerophosphocholines were synthesized according to published procedures [21,22]. DipalmitoylPC, dioleoylPC, phosphatidic acid, *Crotalus adamanteus* and *Naja naja* venoms, and methyl methanethiosulfonate were products of Sigma Chemical Co., St. Louis, MO. Tributylphosphine, dicyclohexylcarbodiimide, 1,12-dodecanedicarboxylic acid, 3-carboxypropyl disulfide, and 4-dimethylaminopyridine were purchased from Aldrich Chemical Co., Milwaukee, WI. Hexadecanoic acid and 10-heptadecenoic acid were obtained from Nu Chek Prep, Elysian, MN. D-[U-^{14}C]Glucose, 325 Ci · mol^{-1}, was purchased from American Radiolabeled Chemicals, St. Louis, MO. Sephadex G-75 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, and lactosylceramide was purchased from Calbiochem-Behring Diagnostics, La Jolla, CA. TLC was performed on silica gel G plates obtained from Analtech, Inc. Newark, DE. The silica gel G used for column chromatography was purchased from American Scientific Products, Kansas City, MO, and Rexyn I-300 was obtained from Fisher Scientific Co., St. Louis, MO. PC-TP was purified to homogeneity from fresh bovine liver and characterized as described in the accompanying paper [18].

Analytical methods. Fatty acid methyl esters were prepared using 1.5 M methanolic HCl [23] and were analyzed by GLC with a Hewlett-Packard model 5830A instrument equipped with flame ionization detector and electronic integrator. A glass column (1/8 × 3 ft) of 3% SP-2100 on 80/100 mesh Supelcoport (Supelco Inc., Bellefonte, PA) was operated between 150 and 310 °C at 10 C° · min^{-1}, and isothermally thereafter. Helium was the carrier gas. Fatty acids were quantitated using 1,12-dodecanedicarboxylic acid as an internal standard. Proton NMR spectra were recorded on a Bruker WM-200 MHz instrument at room temperature in deuterated chloroform. Mass spectral analyses were performed on a Nermag R10-10 quadrapole spectrometer operated in the electron impact ionization mode. Phosphorus analyses were performed by the method of Rouser et al. [24]. Fatty acyl and phospholipid sulfhydryl groups were quantitated using 5,5′-dithiobis(2-nitrobenzoic acid) [25]; in these analyses absorbances were measured at 410 nm and concentrations were determined using a molar absorption coefficient of 13600 for the 2-nitro-5-thiobenzoate anion [26].

Synthesis and purification of 16-(S-methyldithio)-hexadecanoic acid. 16-(*S*-methyldithio)hexadecanoic acid (II) was synthesized from 16-mercaptohexadecanoic acid (I) to protect the sulfhydryl group during the subsequent acylation reactions. A mixture of 16-mercaptohexadecanoic acid (9.7 mmol, 2.8 g), methyl methanethiosulfonate (14.6 mmol, 1.43 ml), and pyridine (14.6 mmol, 1.17 ml) in 26 ml of chloroform was stirred at room temperature for 20 min. Upon removal of the solvent by evaporation under reduced pressure, the residue was washed with cold ethanol followed by recrystallization in ice-cold hexane.

The product was dried in vacuo over P_2O_5 to yield 1 g of product with a purity of greater than 85% as assessed by GLC. GC-MS analysis of the methyl ester of the product gave the expected molecular ion peak at m/z 348; the fragmentation ion at m/z 269, indicating the loss of -S-S-CH_3, supported the predicted structure.

Synthesis and purification of 1,2-di(16-(S-methyldithio)hexadecanoyl)PC (III). The procedure used to acylate GPC-$CdCl_2$ with 16-(*S*-methyldithio)hexadecanoic acid has been reported [25]. To insure anhydrous conditions both GPC-$CdCl_2$ and 16-methyldithiohexadecanoic acid were dried for at least 12 h under vacuum over P_2O_5 prior to the acylation reaction; GPC-$CdCl_2$ was heated to 80°C during this process. To GPC-$CdCl_2$ (1.65 mmol, 0.73 g), 16-(*S*-methyldithio)hexadecanoic acid (6.6 mmol, 2.21 g), and 4-dimethylaminopyridine (3.3 mmol, 0.4 g) in 17 ml anhydrous chloroform was added dicyclohexylcarbodiimide (6.6 mmol, 1.36 g). The mixture was protected from light and stirred for 48 h at room temperature under nitrogen. After removal of the solvent, 20 ml of chloroform/methanol/water (4 : 5 : 1, v/v) was added and the resulting mixture was filtered to remove insoluble material. The filtrate was applied to a 50 ml column of Rexyn I-300 resin. Two bed volumes of the same solvent were used to elute the product. The crude product was then applied to a dry silica gel G column (3 × 40 cm) and eluted with chloroform/methanol/water (65 : 25 : 4, v/v). The yield, based on phosphorus analysis, was 36%. The product comigrated with egg PC on TLC analysis in above solvent system.

Synthesis and purification of lyso-(16-(S-methyldithio)hexadecanoyl)PC (IV). 1,2-Di(16-(*S*-methyldithio)hexadecanoyl)PC (307 μmol) was dissolved in 38 ml ether/methanol/chloroform (16 : 2 : 1, v/v). To this mixture was added 37 mg *Crotalus adamanteus* venom dissolved in 18 ml of 10 mM Tris-HCl, 20 mM $CaCl_2$ (pH 7.4). The mixture was stirred vigorously at 35°C for 2 h after which more venom (38 mg/2 ml) was added. The reaction was continued until completion (approx. 2.5 h later). The solvent was removed by rotary evaporation, using benzene and ethanol to dispose of the final traces of water. The product was dissolved in chloroform/methanol (2 : 1, v/v) and filtered (Whatman No. 3) to remove the protein. To separate the desired product from free fatty acid and a slight amount of unhydrolyzed PC, the residue was applied to a dry silica gel column and eluted with chloroform/methanol/water (65 : 25 : 4, v/v). The yield, based on phosphorus, was 86%.

Acylation of lyso-(16-(S-methyldithio)hexadecanoyl)PC with hexadecanoic anhydride. Lyso-(16-(*S*-methyldithio)hexadecanoyl)PC was acylated with hexadecanoic anhydride as described by Gupta et al. [22] with minor modifications. Both lysoPC and anhydride were dried over P_2O_5 for at least 12 h prior to starting the reaction, dicyclohexylcarbodiimide was added to the final reaction mixture, and the reaction was allowed to proceed for 48 h. The quantity of dicyclohexylcarbodiimide added was such that the final dicyclohexylcarbodiimide/anhydride mole ratio was 1 : 1. These modifications were employed to minimize the conversion of the fatty acid anhydride to the free fatty acid. 1-(16-(*S*-Methyldithio)hexadecanoyl)-2-hexadecanoylPC (V) was purified as described above for the synthesis of 1,2-di(16-(*S*-methyldithio)hexadecanoyl)PC, with yields which averaged 60%.

Removal of the blocking group and sulfhydryl oxidation. 1-(16-(*S*-Methyldithio)hexadecanoyl)-2-hexadecanoylPC (94 μmol) was dissolved in 1.5 ml ethanol to which 1.5 ml water and tributylphosphine (400 μmol, 0.1 ml) were added. The reaction mixture was stirred for 11 h at room temperature, protected from light and under N_2. The product was purified by silica gel G column chromatography. The ratio of free sulfhydryl to phosphorus in the product was found to be 0.7. Incomplete removal of the locking group interfered with neither the subsequent reaction nor the purification of the final product. Iodine was employed to oxidize 1-(16-mercaptohexadecanoyl)-2-hexadecanoylPC. The general procedure involved dissolving the phospholipid in chloroform to which was added an equivalent volume of water. While this mixture was stirring, chloroform saturated with iodine was added dropwise until the system remained light yellow in color, indicating excess iodine. The mixture was then stirred under N_2 for an additional 30 min at room temperature. Excess iodine was removed by silica gel

G column chromatography using chloroform/methanol/water (65 : 25 : 4, v/v) as the elution solvent. All intermediates shown in Scheme I as well as the final product were stored in chloroform/methanol (2 : 1, v/v) at −20 °C. The synthesis of radiolabelled bis-PC, 1-(17,18-dithiatetratriacontandioyl)-bis(2-[9,10-^{3}H]hexadecanoyl-PC), is described in the accompanying paper [18].

Lipid dispersions. Phospholipids in chloroform/methanol (2 : 1, v/v) were taken to dryness under N_2 and dispersed in 10 mM sodium borate, 140 mM NaCl, 2 mM NaN_3 (pH 8.4), containing 2-5 μCi [^{14}C]glucose. Dispersions of 0.5 mM bis-PC or 1 mM egg PC were prepared by bath sonication in a cup horn (Heat Systems - Ultrasonics Inc. model W 185 F) at 50–60 °C under nitrogen. Sonication was continued until the solution appeared clear and no further detectable change in turbidity was observed. Analysis of the fatty acid methyl eseers by GLC following sonication indicated that no modification of the disulfide had occurred. Lipid dispersions used in the transfer protein vesicle-vesicle assay were prepared by injection of ethanolic phospholipid solutions [27] into 10 mM Hepes, 50 mM NaCl, 1 mM Na_2EDTA, and 0.02% NaN_3 (pH 7.4). Donor vesicles contained PC/lactosylceramide/phosphatidic acid (92 : 6 : 2, mol%); acceptor vesicles contained PC/phosphatidic acid (98 : 2, mol%).

Trapped volume studies. An aliquot (100 μl) of the lipid dispersion prepared with [^{14}C]glucose was applied to a 0.9 ml column of Sephadex G-75. Vesicles were eluted by centrifugation (733 × g) for 4 min, followed by two successive additions of borate buffer (100 μl each) and centrifugation. Under these conditions, none of the free glucose eluted from the column. The eluate was assayed for phosphorus and radioactivity.

Electron microscopy. Lipid dispersions were placed on formbar-coated grids and stained with 2% aqueous uranyl acetate. Electron micrographs were recorded on a JEOL 100 S instrument.

Differential scanning calorimetry. Differential scanning calorimetry was performed with a Perkin-Elmer model DSC-1B calorimeter. Lipid samples in chloroform/methanol (2 : 1, v/v) were dried under N_2, lyophilized overnight, and then dispersed in 10 mM Tris-Cl, 100 mM NaCl, 0.2 mM Na_2EDTA (pH 7.0). The suspension was vortexed at room temperature for 3–4 min. Liposome preparations (0.6–1.0 mg phospholipid) were scanned in an ascending temperature mode, dipalmitoylPC at a rate of 5 C° · min^{-1} and bis-PC at a rate of 10 C° · min^{-1}. The faster scan rate employed for the bis-PC sample was necessary to maximize the signal-to-noise ratio for the broad transition of this lipid. The water melting endotherm was used for temperature calibration. Quantitation of the phospholipids used for analysis was determined by extracting the sample from the aluminum pans after the calorimetric scan using chloroform/methanol (2 : 1, v/v). The enthalpy of the main transition peaks was calculated by gravimetric analysis of the recordings, using the heat of fusion for water as a standard.

Phospholipase A_2 hydrolysis. Bath-sonciated lipid dispersions of bis-PC, radiolabelled with [9,10-^{3}H]palmitic acid in the *sn*-2 position, were prepared in 20 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.4). These dispersions, 1 mM in lipid phosphorus, were incubated with phospholipase A_2 (*Naja naja* venom) in a 37 °C shaking water bath. The enzyme was stored and used directly from a stock solution which was 10 I.U. · ml^{-1} in the above buffer; one I.U. will hydrolyze 1 μmol of PC to lysoPC and free fatty acid per min at 25 °C. The quantities of enzyme used are described in the text. Aliquots were withdrawn at various times during incubation, lyophylized, dissolved in chloroform/methanol (2 : 1, v/v), and filtered to remove protein. The products were then separated by TLC, using silica gel G plates and a solvent system of chloroform/methanol/water (40 : 28 : 8, v/v); under these conditions, the following R_f values were noted: fatty acid, 0.95, bis-PC, 0.47, mono-lyso-bis-PC, 0.41, and lyso-bis-PC, 0.35. After visualization with I_2 vapors, the plate was divided into five regions and analyzed for radioactivity.

Vesicle-vesicle assay of phospholipid transfer activity. PC transfer activity of bovine liver PC-TP was determined by measuring the rate of PC transfer from donor vesicles to acceptor vesicles, as described previously [12]. Transfers of chemically-defined PC molecules were determined by GLC [5]. Donor vesicles (400 nmol lipid) and acceptor vesicles (200 nmol lipid) were incubated in the presence or absence of 1 μg PC-TP. The

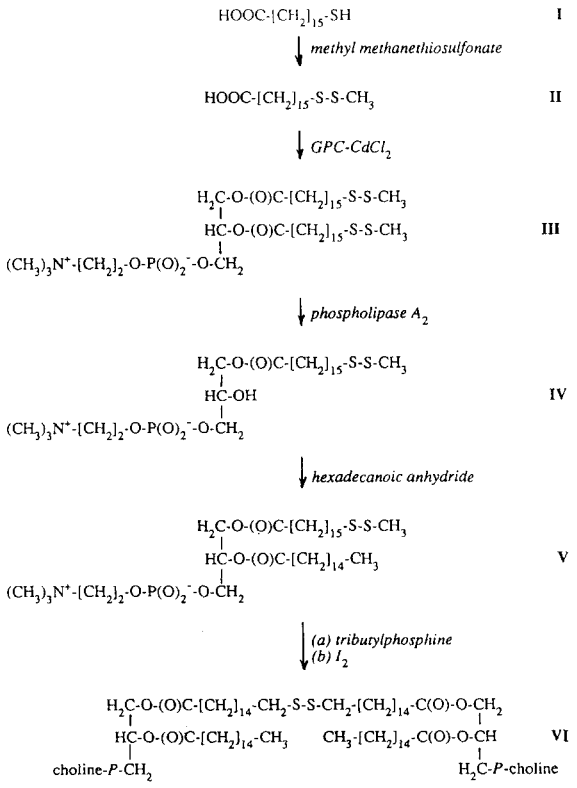
assay buffer was 10 mM Hepes, 50 mM NaCl, 1 mM Na_2EDTA, and 0.02% NaN_3 (pH 7.4).

Results

Synthesis of bis-PC

The synthetic pathway for the preparation of 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycerol-3-phosphocholine) is outlined in Scheme I. The dimeric phospholipid was judged to be pure based upon analysis by TLC and detection by charring. As shown in Table I, analyses of the fatty acyl chain composition and the phosphorus content of bis-PC are in strong agreement with the composition predicted. The identification of the disulfide dicarboxylic acid peak in the GLC system was based upon ^{1}H-NMR and GC-MS analysis of a standard obtained by oxidizing 16-mercaptohexadecanoic acid with iodine in a two-phase solvent system. Analysis of 17,18-dithiatetratriacontandioic acid by ^{1}H-NMR (Fig. 1a) gave a value of 0.91 for the ratio, CH_2-C(O)/CH_2-S (theoretical, 1.0). GC-MS of the corresponding methyl ester of disulfide dicarboxylic acid gave a peak at *m/z* 602, corresponding to the predicted molecular ion. Other fragmentation ions at *m/z* 301 and 269, corresponding to loss of -S-$[CH_2]_{15}$-C(O)-O-CH_3 and a further loss of S, respectively, were observed. Fragmentation of a commercially available disulfide dicarboxylic acid methyl ester, 3-carboxypropyl disulfide, gave a similar fragmentation pattern. This model compound gave a molecular ion peak at 266 and fragmentation ions at *m/z* 133 and 101, corresponding to a loss of -S-$[CH_2]_3$-C(O)-O-CH_3) and a further loss of S, respectively. These results convincingly support the predicted structure for 17,18-dithiatetratriacontandioic acid.

In analyzing the ^{1}H-NMR spectrum of bis-PC (Fig. 1b, inset), the observed value of 1.96 for the ratio CH_2-C(O)/CH_2-S (theoretical, 2.0) was in keeping with a disulfide linkage in the final product. The magnitude of this ratio also indicated that no detectable oxidation of the disulfide had occurred. Phospholipase A_2 (*Crotalus adamanteus* venom) hydrolysis of bis-PC and subsequent GLC analysis of the fatty acids released indicated only hexadecanoic acid, verifying that no acyl chain migration had taken place during the synthesis.



Scheme I. Steps in the synthesis of 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycero-3-phosphocholine).

Formation of vesicles containing bis-PC

Bis-PC was quantitatively formed into vesicles by ultrasonication. Electron micrographs of these bis-PC dispersions demonstrated closed vesicular structures (Fig. 2a). The average diameter of bis-PC vesicles was approximately 6 times larger than that of egg PC vesicles as determined from the micrographs (Fig. 2b and Table II). The trapped, internal volume of bis-PC and egg PC vesicles was determined using [^{14}C]glucose. Vesicles derived from bis-PC had a trapped volume which was approx. 25-times greater than egg PC vesicles made under the same conditions (Table II). Knowledge of the trapped volume makes it possible to estimate the diameter of a single membrane vesicle [28]. A molar volume of 0.75 $l \cdot mol^{-1}$ for egg PC and 1.47 $l \cdot mol^{-1}$ for bis-PC, a membrane thickness of 3.7 nm, and a hydration layer of 0.6 nm were assumed in calculating the diameters shown

TABLE I

PHOSPHORUS AND FATTY ACID ANALYSIS OF bis-PC

GLC and phosphorus analyses were performed on bis-PC as described in Materials and Methods; $34:0_{SS}$ and 16:0 refer to 17,18-dithiatetratriacontandioic acid and palmitic acid, respectively; experimental values presented are the mean ± S.D. of three determinations.

	$16:0/PO_4$	$34:0_{SS}/PO_4$	$34:0_{SS}/16:0$
Theoretical value	1.0	0.5	0.5
Experimental value	1.13 ± 0.15	0.45 ± 0.13	0.40 ± 0.12

in Table II. Diameters calculated from the observed trapped volumes were in approximate agreement with the particle diameters obtained by electron microscopy and suggested that the majority of the vesicles are unilamellar.

TABLE II

TRAPPED VOLUME AND DIAMETER OF VESICLES

Trapped volumes of bath-sonciated vesicles were determined using [^{14}C]glucose; particle diameters were calculated from the trapped volume as described in the text; values are the mean ± S.D. of three separate experiments. Phospholipid dispersions were examined by electron microscopy and the mean diameters ± S.D. were determined; the number of vesicles examined was approximately 30 for each lipid dispersion.

Vesicle PC composition	Trapped volume (μl water μmol lipid)	Calculated particle diameter (nm)	
		trapped volume	electron microscopy
Bis-PC	16.13 ± 11.3	179 ± 113	310 ± 163
Egg PC	0.61 ± 0.03	28 ± 0.6	51 ± 17

Phase transition behavior

Differential scanning calorimetry was used to monitor the thermotropic phase transition behav-

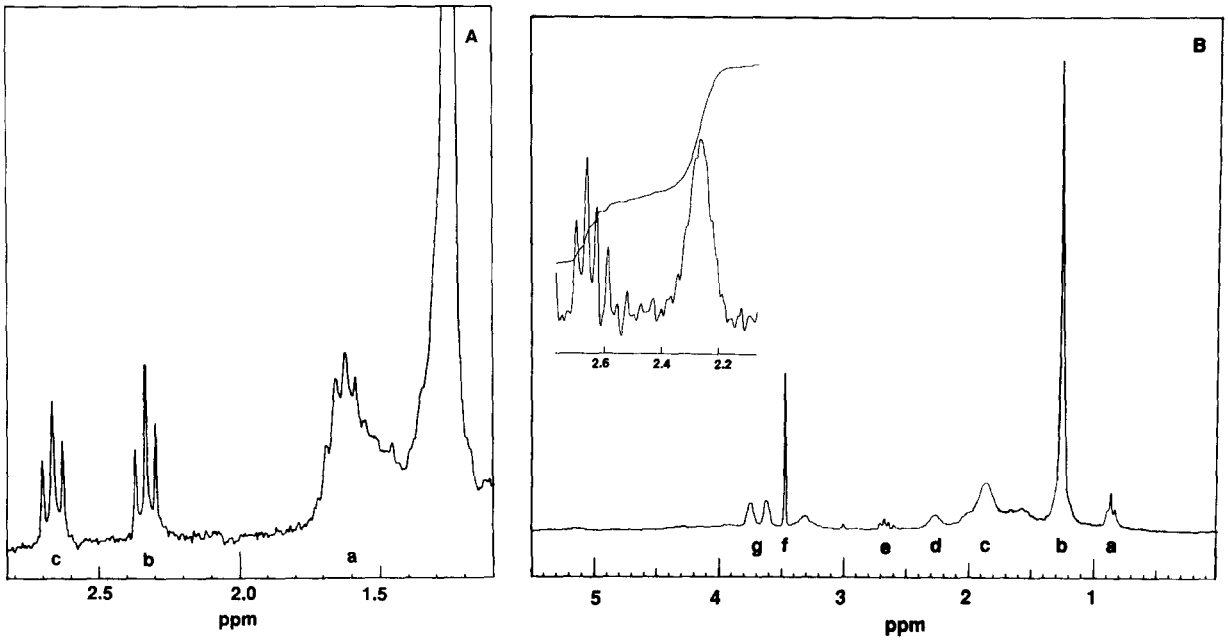


Fig. 1. (A) ^{1}H-NMR spectrum of 17,18-dithiatetratriacontandioic acid in C^2HCl_3: a, δ 1.24–1.7 (CH_2, 52H); b, δ 2.33 (CH_2-C(O), 4H); c, δ 2.66 (CH_2-S, 4H). (B) ^{1}H-NMR spectrum of 1-(17,18-dithiatetratriacontandioyl)-bis-(2-hexadecanoyl-*sn*-glycerol-3-phosphocholine), 4 mg/ml in C^2HCl_3: a, δ 0.86 (CH_3); b, δ 1.23 (CH_2); c, δ 1.88 (CH_2-CH_2-S and CH_2-CH_2-C(O)); d, δ 2.25-2.32 (CH_2-C(O)); e, δ 2.7 (CH_2-S); f, δ 3.46 (N-$(CH_3)_3$); and g, δ 3.6–3.75 (CH_2-N, CH_2-O-P, and CH_2-O). The ratio of protons included in peaks a, b, and c to protons found in peaks d, e, f, and g was found to be 2.7; the predicted theoretical value was 2.5. The inset shows a higher magnification of peaks d and e, with the proton integration; the observed ratio of 1.96 agrees with the predicted theoretical value of 2.0.

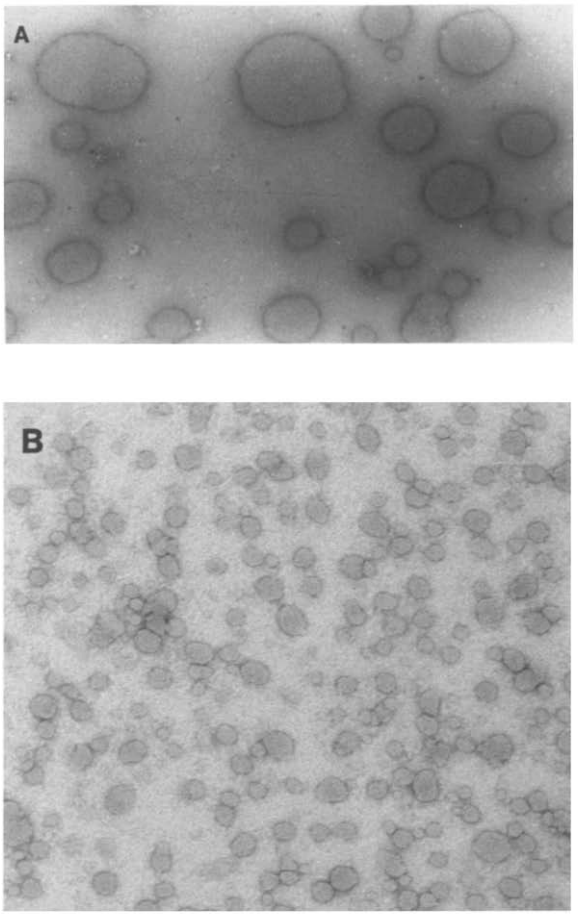


Fig. 2. Electron micrographs of sonicated phospholipid dispersions. Vesicles were prepared from bis-PC (A) and egg PC (B); magnification factors are 12600× and 60800×, respectively.

ior of bis-PC. The phase transition parameters of bis-PC and dipalmitoylPC dispersions are summarized in Table III. In comparison to dipalmitoylPC liposomes, the transition width and magnitude of bis-PC dispersions are considerably larger, and the midpoint of the main transition is increased. These differences perhaps may reflect the presence of the disulfide linkage located at the termini of the *sn*-1 chains. The thermodynamic parameters obtained in this study for dipalmitoylPC agree well with previous data (summarized in Ref. 29). Polymeric disulfide vesicles derived from 1,2-di(16-mercaptohexadecanoyl)PC, a lipid structurally related to bis-PC, have no

TABLE III

PHASE TRANSITION PARAMETERS OF PC DISPERSIONS DETERMINED BY DIFFERENTIAL SCANNING CALORIMETRY

Parameters obtained from calorimetric scans of samples prepared and analyzed as described in the text; T_{M1} and T_{M2}, the main transition and pretransition temperatures (°C), respectively, were measured at maximal heat capacity using water as the temperature reference; T_O and T_C are defined as the onset and completion temperatures (°C) of the main transition, respectively; and ΔH is the transition enthalpy. Di-16:0 PC represents dipalmitoylPC.

PC species	T_O	T_C	T_{M1}	T_{M2}	ΔH (kcal·mol^{-1})
Bis-PC	37.8	53.4	49.3±0.29	34.0±0.34	13.0
Di-16:0-PC	40.3	44.2	41.7±0.13	34.1±0.13	8.8

detectable phase transition as determined by absorbance change [25]; the absence of phase transition behavior of these polymeric vesicles might result from the disulfide linkages on both the *sn*-1 and *sn*-2 positions, a situation which apparently restricts the *trans* to *gauche* reorganization.

Orientation of bis-PC in membranes

The topological orientation of bis-PC in a lipid membrane was investigated enzymatically. Phospholipase A_2 attacks only those phospholipids located within the outer monolayer and has been utilized extensively as a tool to examine membrane phospholipid distribution [30]. In this context, this enzyme may also be used to examine the distribution of a bipolar phospholipid between the outer and inner surfaces. If the long, dicarboxylic acyl chain of bis-PC spans the lipid membrane, a *trans* orientation, then only one of the palmitoyl acyl chains should be accessible to phospholipase A_2 hydrolysis. Conversely, if bis-PC was oriented such that both polar headgroups of a single molecule were on the same side of the membrane, a *cis* orientation, then both palmitoyl acyl chains in a single bis-PC molecule, should be released upon phospholipase A_2 hydrolysis. Treatment of ^{3}H-labelled bis-PC vesicles with phospholipase A_2 released 55% of the radioactive palmitoyl residues from bis-PC (Fig. 3) during 2 h of incubation. All remaining radioactivity was found to co-migrate

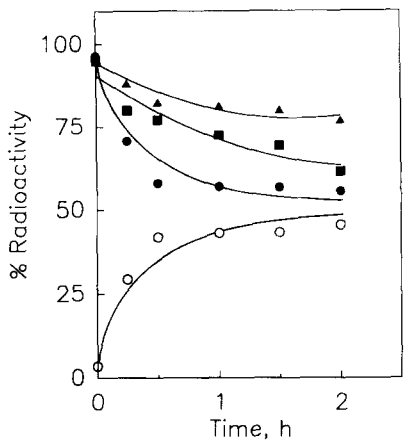


Fig. 3. Phospholipase A_2 hydrolysis of bis-PC. Phospholipid vesicles were incubated with phospholipase A_2 at an enzyme level of 0.1 I.U. per 100 nmol lipid phosphorus, using conditions described in Materials and Methods. Free fatty acid (open data points) and bis-PC (closed data points) were separated by TLC and analyzed by liquid scintillation spectrometry. Vesicles were prepared from bis-PC (●, ○), bis-PC/di-*O*-hexadecyl PC (50:50, mol lipid phosphorus) (■), or bis-PC/di-*O*-hexadecyl PC (5:95, mol lipid phosphorus) (▲).

with unreacted bis-PC, indicating that little, if any, of the possible mono-lyso-bis-PC product, i.e., bis-PC with the only one *sn*-2 palmitoyl acyl chain, had been formed, even at the early points of the kinetic analysis. The identity of the major phospholipid hydrolysis product, lyso-bis-PC, was confirmed by GLC analysis of the residual phospholipids after the 2 h incubation; the ratio of 17,18-dithiatetratriacontandioic acid to palmitic acid was 4.5. Had mono-lyso-bis-PC been a significant product, this ratio would have been closer to unity.

When bis-PC vesicles were incubated for 2 h with different amounts of phospholipase A_2 (0.05, 0.1, or 0.3 I.U. per 100 nmol lipid phosphorus), comparable extents of hydrolysis were observed: 42, 45, and 50%, respectively. Our data do not rule out the possibility that mono-lyso-bis-PC is an intermediate in the enzymatic conversion of bis-PC to lyso-bis-PC and palmitic acid. Since it is reasonable to expect that phospholipase A_2 will attack only one *sn*-2 ester bond at a time, monolyso-bis-PC should be on the reaction pathway; however, our inability to detect its presence would suggest that it is a much better substrate for the enzyme.

To determine whether the organization of bis-PC is influenced by other lipid components, vesicles were prepared from mixtures of bis-PC and di-*O*-hexadecyl PC, a phospholipid which contains ether rather than ester bonds at its *sn*-1 and *sn*-2 positions and consequently is not a substrate of phospholipase A_2. Although the rate of hydrolysis of bis-PC was retarded by the presence of di-*O*-hexadecylPC (Fig. 3), the distribution of ^{3}H-labelled palmitoyl residues was still restricted to either unreacted bis-PC, indicated by the data points, or the free fatty acid. It should be pointed out that these experiments were performed in the absence of bovine plasma albumin, a precaution taken to preserve vesicle integrity during the course of bis-PC hydrolysis. These results argue that the acyl chain moiety in the *sn*-1 positions of bis-PC does not traverse the vesicle membrane and that a bilayer lipid organization is likely to exist. Addition of Triton X-100 (0.1%, w/v) at the 2-h time point in Fig. 3, followed by further incubation for 1 h, resulted in the hydrolysis of bis-PC to the

TABLE IV

PROTEIN-MEDIATED TRANSFER OF PHOSPHATIDYLCHOLINE SPECIES

Donor vesicles of the indicated composition and diheptadecenoylPC acceptor vesicles were incubated in the presence of bovine phosphatidylcholine transfer protein at 37°C for 30 min. The phospholipid species in each mixture whose transfer was monitored is indicated by an asterisk (*); each determination was performed in triplicate and reported as a mean ± S.D. The following fatty acyl chain species were examined by GLC: 16:0, palmitoyl; 17:1, heptadecenoyl; and 18:1, oleoyl.

Donor vesicle PC composition (mol ratio) [a]	PC transferred to acceptor vesicles (nmol)
Di-18:1-PC */di-17:1-PC (1:6)	7.49 ± 0.41
Di-16:0-PC */di-17:1-PC (1:6)	6.51 ± 0.67
Bis-PC */di-17:1-PC (1:6) [b]	0.15 ± 0.18

[a] Based on lipid phosphorus and taking into account that 1 mol of bis-PC contains 2 mol lipid phosphorus.

[b] The formation of closed vesicles was confirmed by [^{14}C]glucose trapping as described in Materials and Methods; the trapped volume of these vesicles did not differ appreciably from vesicles derived from diheptadecenoylPC (data not shown).

extent of 68–73% for bis-PC and the bis-PC/di-*O*-hexadecylPC mixtures. The disruption of the bilayer organization by this non-ionic detergent exposed additional molecules of bis-PC to phospholipase A_2.

Transfer of bis-PC by PC-TP

The activity of PC-TP toward dioleoylPC, dipalmitoylPC, and bis-PC was compared using a vesicle-vesicle phospholipid transfer system. Donor vesicles contained diheptadecenoylPC and one of the other PC's, while acceptor vesicles contained only diheptadecenoylPC. Quantitation of phospholipids of donor membrane origin in the acceptor membranes was made by GLC analysis of fatty acid methyl esters. As shown in Table IV, significant transfer of both dioleoylPC and dipalmitoylPC was observed, but no transfer of bis-PC was detected.

Discussion

This report describes the synthesis of bis-PC, a phospholipid molecule comprised of two glycerophosphorylcholine groups linked together through a disulfide bond in the two *sn*-1 positions. The synthetic scheme employed minimized acyl chain migration and optimized the formation of a dimeric PC. The approximate overall yield of bis-PC was 8%. Characterization of the product included homogeneity by TLC, functional group analysis, GC-MS, and ^{1}H-NMR, all of which were consistent with a dimeric PC structure. Alternative synthetic approaches to bis-PC could have included (1) synthesis of dipalmitoylPC, followed by hydrolysis with a phospholipase A_1 and reacylation with 17,18-dithiatetratriacontandioic acid, or (2) synthesis of 1,2-di(17,18-dithiatetratriacontandioyl)-bis-PC, followed by hydrolysis with a phospholipase A_2 and then reacylation with hexadecanoic anhydride. Major problems associated with these alternative schemes are acyl chain migration, an unavoidable problem associated with phospholipase A_1 products [31], and the probability of obtaining significant amounts of the intermediate 1,2-di(17,18-dithiatetratriacontandioyl)-bis-PC.

Electron microscopy and glucose trapping studies suggested that bis-PC can form closed vesicles, which are approx. 6-times larger than those derived from egg PC. The unusually large size of these vesicles may reflect the inability of bis-PC to pack into highly curved, small unilamellar vesicles [32,33] or a slower kinetic approach to a limiting size using the ultrasonication technique. It would be informative to prepare membrane structures from bis-PC by other well-accepted methods, including more prolonged sonication, detergent dialysis, and high-pressure extrusion.

Phospholipase A_2 hydrolysis of bis-PC in vesicular structures indicated that approximately half of the total palmitic acid is released, a proportion which presumably is associated with those phospholipid molecules exposed on the outer surface of the membrane. Taken alone, this observation is consistent with two different orientations of bis-PC in membranes. In the *cis* conformation both polar headgroups of a single molecule are oriented on the same surface of a membrane; in the *trans* conformation the polar headgroups of a single molecule are located on the inner and outer surfaces. However, the additional experimental evidence that the remaining radiolabelled palmitoyl moieties migrated with unhydrolyzed bis-PC is consistent only with the *cis* conformation. Thus, the long acyl chain of bis-PC apparently can bend at the disulfide function to allow both polar headgroups to be oriented on the same side of the membrane. Further verifification of this orientation needs to be established by the use of other hydrolytic enzymes, such as phospholipases C and D, and the appropriate separation and analysis of bis-PC degradation products. Additional experimental support of a *cis* orientation is the observation that bis-PC is able to form closed vesicles. The isolated bipolar lipids of the archaebacterium *Cardariella acidophila*, which have two polar headgroups linked together by two 40-carbon isoprenoid chains, are unable to form closed vesicular structures [34]. As demonstrated by freeze-fracture electron microscopy [35] and glycosidase hydrolysis [36], the isoprenoid chains in these open structures stretch across the membrane.

The dimeric phospholipid described in this communication is not a transferable substrate of bovine liver PC-TP under conditions where significant transfer of dioleoylPC and dipalmitoylPC

was observed. This finding may be explained, in part, by the earlier observation [15] that PC-TP has a single PC binding site, one which presumably cannot accommodate the unusually large bis-PC molecule. Furthermore, the lack of transfer of bis-PC may result from a hydrophobic interaction of bis-PC with other membrane lipids which is substantially greater than the association of bis-PC with the protein's lipid binding site. The structural organization of bis-PC in model membranes, its retention by such membranes in the presence of PC-TP, and its chemical similarity to typical saturated PC-TP substrates have been exploited in an investigation of the role of membrane PC in the catalytic mechanism of protein-mediated PC transfer and exchange [18].

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