papers and notes on methodology

Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase A_2

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Abstract The synthesis of a dithiolester analog of phosphatidylcholine, 1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol-3-phosphocholine (thio PC), is described. Starting with 1-trityl-*sn*-glycerol (prepared from D-mannitol), tosylation followed by displacement with potassium methyl xanthate gave a trithiocarbonate. Reductive cleavage of the latter gave a 1,2-dithiol which was then acylated, detritylated, and esterified with choline phosphate. Hydrolysis of thio PC by phospholipase A_2 (*Naja naja*) indicated 95% chiral purity. The rate of hydrolysis as a function of substrate concentration showed a sharp increase at about 0.17 mM, the critical micellar concentration of the lipid. A spectrophotometric assay of phospholipase A_2 (by measurement of released thiol groups in the presence of dithionitrobenzoic acid) was quite sensitive. As little as 1 ng of enzyme was detected, representing a rate of about 0.2 nmol of substrate hydrolyzed per min.—**Hendrickson, H. S., E. K. Hendrickson, and R. H. Dybvig.** Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase A_2. *J. Lipid Res.* 1983. **24:** 1532–1537.

Supplementary key words {spectrophotometric assay • *Naja naja* • thiol

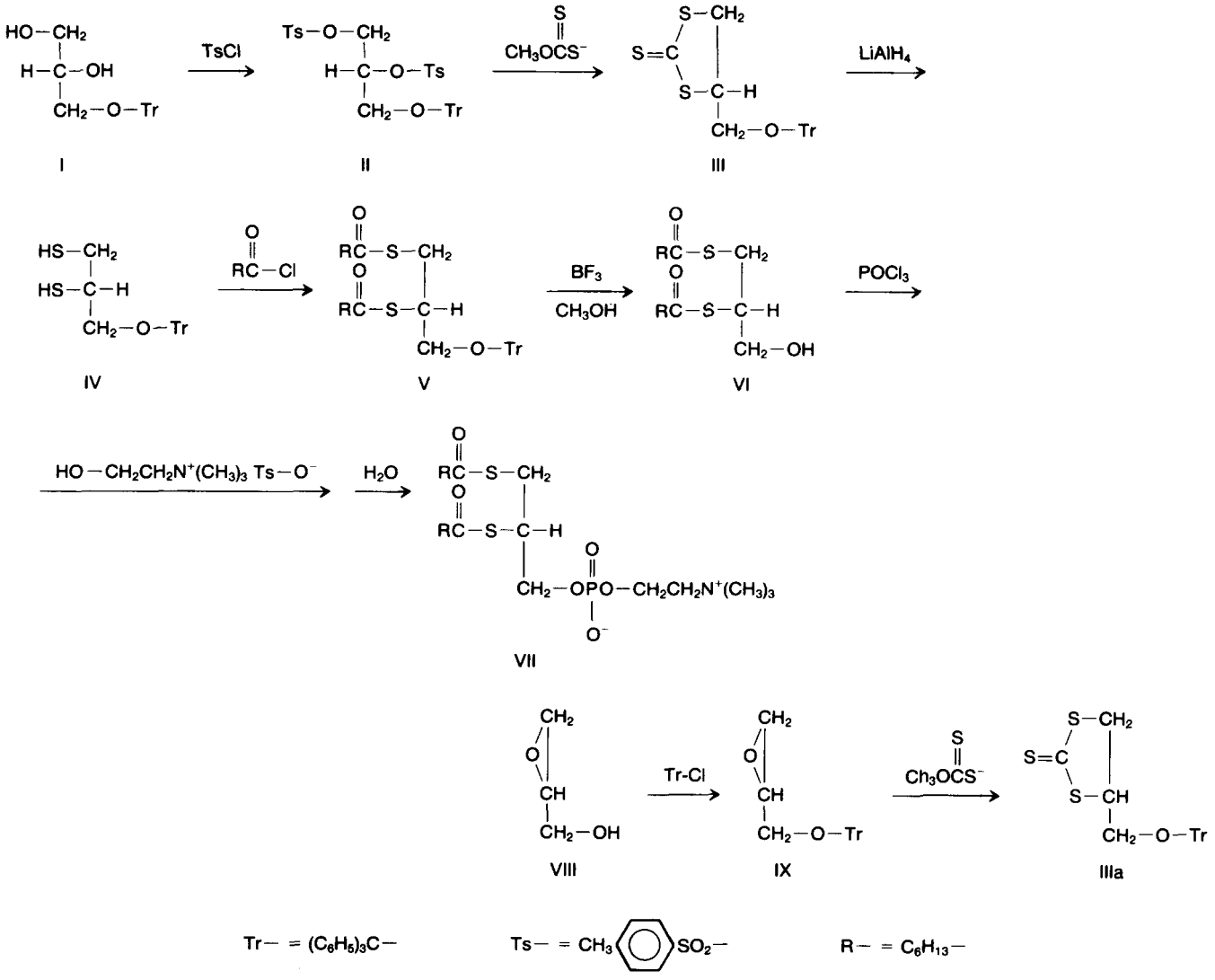
Phospholipase A_2 catalyzes the hydrolysis of the 2-acyl ester group of *sn*-3-phosphoglycerides. Sensitive assays are required for the detection of intracellular and membrane-bound enzymes which are found, prior to purification, at specific activities of only 1–10 nmol/min per mg. Continuous spectrophotometric assays have been developed involving fluorometric detection of a pyrene-labeled substrate (1) and colorimetric detection of thiolester substrates (2). The latter assay involves hydrolysis of a thiolester to produce a thiol that reacts with a colorimetric thiol reagent such as DTNB. The thioglycolecithin synthesized by Aarsman, van Deenen, and van den Bosch (2) is not a very good substrate for phospholipase A_2 since it is a monoacyl derivative and the enzyme prefers a diacyl substrate. Volwerk et al. (3) synthesized a short-chain dithiolester analog of phosphatidylcholine. This was a racemic substrate, synthesized from 1,2-dimercapto-3-propanol. Acylation, of this mercaptan gives a mixture of diacyl derivatives. We have observed that the desired dithiolester is obtained in very poor yields. In order to provide a better synthesis of the dithiolester analog of phosphatidylcholine and a chiral product, we developed a chiral synthesis starting with 1-trityl-*sn*-glycerol (prepared from D-mannitol) as shown in **Scheme 1.** This synthesis and the action of phospholipase A_2 on this substrate are reported here.

MATERIALS AND METHODS

Materials

Melting points were determined on a Kofler micro hot stage (A. H. Thomas) and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer model 467 spectrophotometer. Proton NMR spectra were taken on a Varian EM360L, 60 MHz instrument, using tetramethylsilane as an internal standard. Heptanoyl chloride, phosphorus oxychloride, and p-toluenesulfonyl (tosyl) chloride were obtained from Aldrich Chemical

Abbreviations: CMC, critical micellar concentration; diheptanoyl thio PC, 1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol-3-phosphocholine; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; tosyl, p-toluenesulfonyl; trityl, triphenylmethyl.



SCHEME 1.

Co., Milwaukee, WI. Boron trifluoride (14% in methanol), DTNB, and phospholipase A_2 (*Naja naja*, 700 units/mg protein) were obtained from Sigma Chemical Co., St. Louis, MO. 1-Trityl-*sn*-glycerol was prepared from D-mannitol according to the procedure of Virtanen et al. (4). Trityl glycidol was prepared from glycidol and trityl chloride according to the procedure of Lok (5). Choline p-toluenesulfonate was prepared according to Brockerhoff and Ayengar (6). Benzene was dried by azeotropic distillation, pyridine was distilled over barium oxide, and chloroform was dried by distillation over phosphorus pentoxide prior to use.

Chromatography

TLC was carried out on 2.5 × 10 cm glass plates coated with silica gel, type G, obtained from Sigma Chemical Co., St. Louis, MO. Sulfuric acid-dichromate spray (55% by weight H_2SO_4 containing 0.6% K_2CrO_3) was used for general detection. Trityl compounds gave a yellow color immediately, while other compounds were visualized by charring on a hot plate. Phospholipids were visualized by the molybdate spray (7). Preparative HPLC was accomplished on a 10 mm × 1 m stainless steel column slurry-packed with 10-micron silica gel (#10050, Analtech, Newark, DE). The flow rate was 8.7 ml/min. Fractions (17 ml) were collected and analyzed by TLC.

Analyses

Elemental analyses (C, H, N, S) were performed by Galbraith Laboratories, Inc., Knoxville, TN. Phosphorus was determined by the method of Bartlett (8).

Chemical syntheses

*Preparation of 2,3-bis(tosyl)-1-trityl-*sn*-glycerol (II).* To a solution of 5.4 g (16 mmol) of 1-trityl-*sn*-glycerol in 14 ml of dry pyridine at 10°C, 9.15 g (48 mmol) of tosyl chloride was added in small portions with stirring over 10 min. The reaction was stirred for 2 hr at room temperature. A solution of benzene–hexane 3:1 (75 ml) was added and the mixture was washed with ice water, 0.5 M HCl until acidic, water, 5% $NaHCO_3$, and water. The organic phase was dried over anhydrous $MgSO_4$ and evaporated in vacuo with additions of dry benzene to remove any trace of water. About 10 g of syrup was obtained. This product showed mostly a single spot on TLC with hexane–acetone 7:3 (R_f = 0.7). This was used without purification in the next reaction. Crystallization from ether–hexane was difficult, but pure crystals were obtained: mp = 103–105°C. IR (thin film): 3080, 3050 and 3030 cm^{-1} (aromatic C—H); 1360 and 1180 cm^{-1} (sulfonate ester); NMR ($CDCl_3$): 2.38 (6, s, CH_3—), 3.18 (2, d, H_2C—O—Tr), 3.71 (2, d, H_2C—O—Ts), 4.5 (1, m, HC—OTs), 7.2 (15, c, trityl), and 7.6 ppm (8, q, tosyl aromatic). Anal. calcd. for $C_{36}H_{34}S_2O_7$: C, 67.27; H, 5.33; S, 9.98. Found: C, 67.27; H, 5.42, S, 9.98.

*Preparation of 3-trityl-1,2-dideoxy-1,2-(thiocarbonyldithio)-*sn*-glycerol (III).* To a solution of 3.11 g (4.8 mmol) of 2,3-bis(tosyl)-1-trityl-*sn*-glycerol (II) in 30 ml of acetone, a solution of 2.3 g of KOH and 4.7 ml of CS_2 in 20 ml of methanol was added. The mixture was stirred at 40°C for 24 hr. A solution of benzene–hexane 2:1 (150 ml) was added and the mixture was washed four times with water. The organic phase was dried over anhydrous $MgSO_4$ and evaporated in vacuo. The solid (1.96 g) was dissolved in a small amount of benzene and hexane was added until cloudiness first appeared. Crystallization occurred slowly at room temperature (2–4 hr) after which the solution was cooled to 5°C overnight. Yellow crystals were obtained (1.07 g, 55% yield): mp = 155–157°C. More product was obtained by concentrating the mother liquor and chromatographing it on a column of silicic acid with hexane–acetone 10:1. TLC showed a single spot (R_f = 0.45) with hexane–acetone 7:1. UV-Vis (ethanol):λ max(ε) 453 nm (76 M^{-1} cm^{-1}), 317 nm (14,200 M^{-1} cm^{-1}), 298 nm (11,100 M^{-1} cm^{-1}). For ethylene trithiocarbonate (9): 460 nm (69 M^{-1} cm^{-1}), 311 nm (12,500 M^{-1} cm^{-1}), 292 nm (10,600 M^{-1} cm^{-1}). IR ($CHCl_3$): 3080, 3060, 3020 cm^{-1} (aromatic C—H); 1070 cm^{-1} (C=S). For ethylene trithiocarbonate, C=S is at 1080 cm^{-1} (10). NMR ($CDCl_3$): 3.4 (2, d, H_2C—O—Tr), 3.8 (2, d, H_2C—S), 4.2 (1, m, HC—S), and 7.3 ppm (15, c, trityl). Anal. calcd. for $C_{23}H_{20}S_3O$: C, 67.61; H, 4.93; S, 23.54. Found: C, 67.66; H, 5.04; S, 23.24.

*Preparation of 3-trityl-1,2-dideoxy-1,2-(thiocarbonyldithio)-*rac*-glycerol (IIIa).* Trityl glycidol was converted to the *rac*-trithiocarbonate by the same procedure as described for the chiral product above. This reaction required heating at 50°C for only 6 hr. The yield of product was 12.7 g (51% yield) from 19.2 g of trityl glycidol.

*Preparation of 3-trityl-1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol (IV).* A solution of 2.33 g (5.7 mmol) of 3-trityl-1,2-dideoxy-1,2-(thiocarbonyldithio)-*sn*-glycerol (III) in 10 ml of tetrahydrofuran was added under nitrogen with stirring to a solution of 0.22 g of $LiAlH_4$ in 10 ml of dry ether, at a rate such that no yellow color remained. The reaction was cooled in an ice bath and water was added slowly to decompose the excess $LiAlH_4$. The mixture was acidifed with cold 6 N HCl and extracted with ether. The ether extract was washed with 5% $NaHCO_3$, water, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The viscous liquid was not purified, but acylated as soon as possible. The crude mercaptan showed a major spot on TLC in hexane–acetone 7:1 (R_f = 0.65).

The crude mercaptan (IV) was dissolved in 20 ml of hexane and 3 ml of dry pyridine. A solution of 2.54 g (17.1 mmol) of heptanoyl chloride in 3 ml of hexane was slowly added to the mercaptan with stirring at room temperature. After stirring for 1 hr the reaction was judged complete by TLC in hexane–acetone 7:1 (R_f = 0.60). Benzene (50 ml) was added and the mixture was washed with water, 0.5 M NH_4OH in methanol–water 3:1 several times, and finally methanol–water 1:1. The organic phase was dried over anhydrous $MgSO_4$ and evaporated in vacuo to a yellow oil (3.0 g). The crude product was dissolved in 4 ml of hexane–acetone 25:1 and purified by HPLC in the same solvent in 2-ml portions. A clear oil was obtained (2.24 g, 66% yield) which gave a single spot on TLC in hexane–acetone 7:1 (R_f = 0.60). The infrared spectrum showed aromatic C—H at 3090, 3060, and 3030 cm^{-1}; C=O (thiolester) at 1685 cm^{-1} and a small amount of C=O (oxyester) at 1740 cm^{-1}. The latter is probably due to some triheptanoyl ester formed as a result of detritylation. This has nearly the same R_f value as the product and could not be separated by HPLC. This product was used without further purification in the next reaction.

*Preparation of 1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol (VI).* A solution of 2.24 g (3.8 mmol) of 3-trityl-1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol (V) in 125 ml of dry CH_2Cl_2 containing 1.84 ml (3.8 mmol) of a 14% BF_3—MeOH solution was stirred at 0°C (11). Detritylation was monitored by TLC in hexane–ethyl acetate 8:1 (detritylated product, R_f = 0.2). After 30–45 min, detritylation was complete. The mixture was extracted three times with ice water and the organic phase was dried over anhydrous Na_2SO_4. Evaporation in

vacuo gave about 2.2 g of oil. The oil was dissolved in 6 ml of hexane–ethyl acetate 9:1 and purified by HPLC with the same solvent in 2-ml portions. Pure (by TLC) product was obtained (0.37 g, 28% yield) as a clear liquid. IR (thin film): 3500 cm^{-1} (O—H), 1680 cm^{-1} (C=O, thiolester), no oxyester detected at 1740 cm^{-1}. This product was stored in the freezer and used within a few days in the next reaction.

Preparation of 1,2-bis(heptanoylthio)-1,2-dideoxy-sn-*glycerol-3-phosphocholine (VII).* A solution of 1.05 g (3 mmol) of 1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycerol (VI) and 0.32 ml (4 mmol) of dry pyridine in 40 ml of dry $CHCl_3$ was added to 0.55 g (3.6 mmol) of freshly distilled $POCl_3$ in 10 ml of dry $CHCl_3$ with stirring under nitrogen. The mixture was heated to 50°C for 25 min and then cooled to room temperature. The starting material had disappeared as judged by TLC in hexane–acetone 7:1 (R_f = 0.45). Choline p-toluenesulfonate (1.57 g, 4.2 mmol) and 0.9 ml (11 mmol) of dry pyridine were added and the mixture was stirred for 1 hr at room temperature. The product was visualized by TLC in chloroform–methanol–water–conc. ammonia 95:35:5.5:2 (R_f = 0.3). The solution was washed with two portions of water; methanol was added as necessary to break emulsions. The chloroform solution was dried over anhydrous Na_2SO_4 and evaporated in vacuo with the addition of benzene and ethanol to remove any traces of water. The residue (1.41 g) was dissolved in chloroform–methanol 96:4 and purified by HPLC with chloroform–methanol–water 65:25:4. Pure lipid (0.67 g, 47% yield) was obtained, after lyophilization from benzene, as a white solid. IR (thin film): 3400 cm^{-1} (bound water); 2960, 2930, 2860, 1470, 1380 cm^{-1} (C—H); 1690 cm^{-1} (C=O, thiolester); 1250, 1090 cm^{-1} (PO_2—). Anal. calcd. for $C_{22}H_{44}PNS_2O_6 \cdot H_2O$: C, 49.70; H, 8.72; S, 12.06; N, 2.63; P, 5.83. Found: C, 49.53; H, 8.72; S, 11.46; N, 2.63; P, 5.30.

Preparation of lipid dispersions

An aliquot of lipid in chloroform solution was dried under nitrogen and then high vacuum. The lipid was dispersed in 0.2 M Tris-maleate buffer (pH 7.5), containing 10 mM $CaCl_2$, by vortexing at room temperature and sonication in a sonic cleaner bath as necessary until a clear solution was obtained. The solution was then centrifuged for several minutes at 4,000 rpm.

Hydrolysis of lipid by phospholipase A_2

One ml of lipid dispersion was placed in a sample cuvette and 1 ml of buffer in a reference cuvette. The absorbance at 412 nm was adjusted to zero in a Cary model 210 double beam spectrophotometer. After addition of 100 μl of 10 mM DTNB in buffer to both cuvettes, the absorbance (0.1 full scale) was recorded until a stable baseline was obtained. The enzyme reaction was initiated by the addition of phospholipase A_2 to the sample cuvette and an equal volume of water to the reference cuvette. An extinction coefficient of 12,800 M^{-1} cm^{-1} (2) for the thionitrobenzoate anion was used to calculate the rate of hydrolysis. Assays were run at 29°C.

Determination of chiral purity

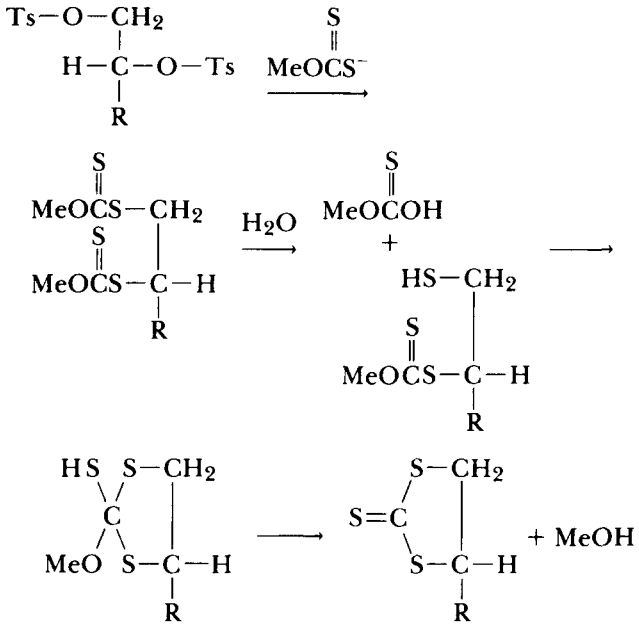
Lipid dispersions (0.1 mM) were hydrolyzed by the addition of 10 μg of phospholipase A_2 under the conditions of the enzyme assay as described above. The maximum absorbance was determined after the reaction reached completion (10–20 min). The percent chiral purity (P) was calculated by the equation: P = 100(At-0.5Ae)/0.5At, where At and Ae are the theoretical and experimental absorbance changes, respectively. The exact lipid concentration was determined by phosphorus analysis of the reaction mixture. The extinction coefficient for the thionitrobenzoic acid anion (12,800 M^{-1} cm^{-1}) was confirmed by reaction of DTNB with standard cysteine solution under the same conditions.

Determination of critical micelle concentration (CMC)

The CMC of the lipid was determined in buffer by measuring the surface tension (Wilhelmy plate method) as a function of concentration. A sharp break in the curve occurs at the CMC.

RESULTS AND DISCUSSION

The chirality of carbon 2 of glycerol was established from 1-trityl-*sn*-glycerol (prepared from D-mannitol). Inversion during the introduction of sulfur results in the *sn*-glycerol-3-phosphate configuration of natural glycerophospholipids. Tosylation of 1-trityl-*sn*-glycerol (I) afforded a good leaving group for the introduction of sulfur by displacement with potassium methyl xanthate. The expected product of this displacement, the 1,2-bis(methylxanthate), was not obtained. Instead, a yellow product was obtained, which was identified as the trithiocarbonate (III) by comparison of its infrared and UV-visable spectra with those of ethylene trithiocarbonate (see Chemical syntheses). Trithiocarbonates have been obtained from the reaction of epoxides with methyl xanthate (12). A possible mechanism for the reaction of the bistosylate (II) based on that proposed for the epoxide (10) is shown as follows:



Preliminary studies of this reaction with dry potassium ethyl xanthate in dry acetone showed a spot on TLC where one would expect the 1,2-bis(methylxanthate). This disappeared upon the addition of a trace of water as the trithiocarbonate (III) spot appeared. Reduction of the trithiocarbonate (III) with $LiAlH_4$ as described by Iqbal and Owen (10) gave the 1,2-dithiol (IV). Acylation of IV with an acyl chloride followed by detritylation with BF_3-methanol (11) gave the alcohol, VI. This was converted to the phosphocholine (VII) using the procedure of Brockerhoff and Ayengar (6).

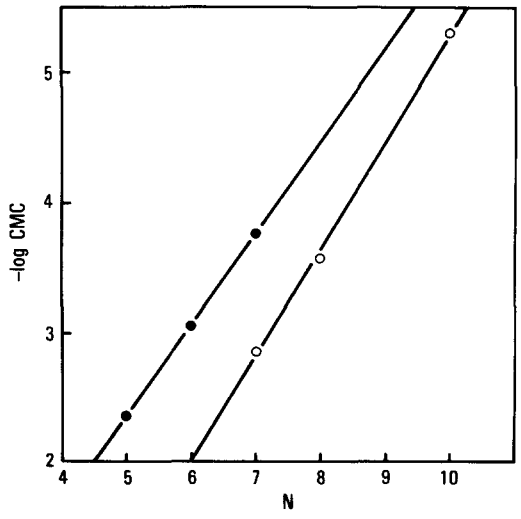


Fig. 1. Plots of −log CMC versus acyl chain length (N). Thio PC (●) (slope 0.71 ± 0.071, intercept −1.2 ± 0.3); dipentanoyl thio PC and dihexanoyl thio PC (3), diheptanoyl thio PC (this work). Normal oxy PC (○) slope 0.82 ± 0.45, intercept −2.96 ± 1.77); diheptanoyl PC and dioctanoyl PC (14), didecanoyl PC (15).

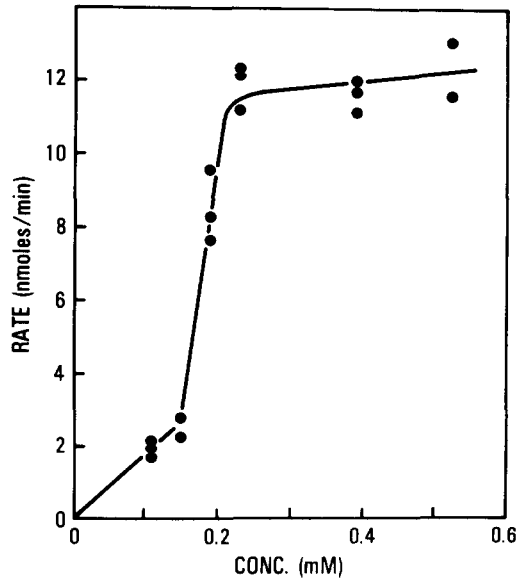


Fig. 2. Plot of hydrolysis rate with 50 ng of phospholipase A_2 (*naja naja*) versus chiral diheptanoyl thio PC (VII) concentration. Assay conditions described in text.

A racemic synthesis was carried out by the conversion of trityl glycidol (IX) to the racemic trithiocarbonate (IIIa) with potassium methyl xanthate under the same conditions as for the conversion of the bistosylate (II). Trityl glycidol (IX) is easily prepared from glycidol and trityl chloride (5).

The chiral purity of diheptanoyl thio PC (VII) was determined by complete hydrolysis with phospholipase A_2. Since this enzyme is specific for the *sn*-glycerol-3-phosphate configuration, 100% hydrolysis would indicate 100% chiral purity and 50% hydrolysis would indicate 0% chiral purity. Seven experiments showed a chiral purity of 95% ± 10% (SD).

The CMC for diheptanoyl thio PC was 0.17 mM as determined by surface tension measurements. This correlated well with the CMCs for the dihexanoyl thio PC and dipentanoyl thio PC analogs reported in the literature (3). A plot of log CMC versus acyl chain length (**Fig. 1**) shows a linear relationship for homologous thio PC analogs. When compared with the normal oxy lipids, similar slopes are obtained, indicating similar free energy changes for micelle formation per methylene group. The different intercepts indicate different head group contributions to micelle formation as expected, since sulfur is more hydrophobic than oxygen. Thus the CMC of a thio PC analog is equivalent to that of a normal oxy PC of about one more carbon in length.

The rate of hydrolysis of diheptanoyl thio PC (VII) by phospholipase A_2 (*Naja naja*) as a function of lipid concentration is shown in **Fig. 2.** The sharp increase between 0.15 mM and 0.19 mM correlates with the CMC

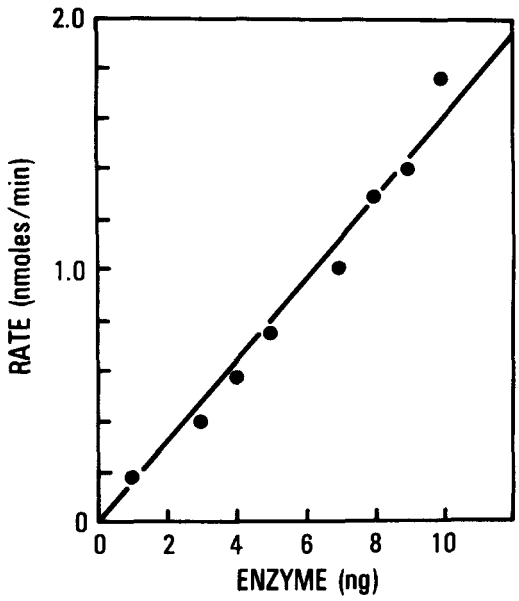


Fig. 3. Plot of hydrolysis rate of chiral diheptanoyl thio PC (VII) versus amount of phospholipase A_2 (*Naja naja*). Lipid concentration, 0.3 mM; assay conditions described in text.

as determined by surface tension measurements. This is expected since the enzyme prefers an interfacial substrate (13). For standard assays of phospholipase, a 0.3-mM concentration of substrate was used, since this is sufficiently above the CMC to give optimal rates and yet gives a clear dispersion in aqueous buffer. Above about 0.7 mM, the dispersions become turbid, particularly in the presence of DTNB before addition of enzyme, and are thus not suitable for spectrophotometric studies. The optimal homolog of thio PC as a substrate for the spectrophotometric assay of phospholipase A_2 appears to be diheptanoyl thio PC (VII). Chain lengths above this give turbid dispersions in the 0.1–1.0 mM concentration range suitable for enzyme assays, while chain lengths less than this have too high CMCs for micellar assays.

The sensitivity of this assay for phospholipase A_2 is shown in **Fig. 3.** There is a linear relationship between activity and amount of enzyme. The assay could detect as little as 1 ng of enzyme. This represents a rate of about 0.2 nmol of substrate hydrolyzed per min. This is at least two orders of magnitude better than the conventional titrametric assay (13) and slightly better than the fluorometric assay (1).

The chiral synthesis of a diacyl thio PC analog described here affords a substrate for a sensitive and continuous assay of phospholipase A_2. Use of a chiral substrate eliminates competitive inhibition by the other enantiomer which would be present in the racemic substrate, and thus simplifies kinetic studies with the enzyme. For routine assays where a kinetic analysis is not important, the racemic substrate may be preferred since its synthesis is shorter starting with *rac*-tritylglycidol, and it can be more readily obtained.

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