Synthesis of a thiophosphate analog of dioctanoylphosphatidylcholine: a phospholipase C substrate

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Abstract
Dioctanoylthiophosphatidylcholine, a racemic thiophosphate analog of L-a-dioctanoylphosphatidylcholine, has been synthesized and isolated by flash chromatography. In contrast with the didecanoylthiophosphatidylcholine synthesized previously, the analog is easily dispersed on sonication in aqueous media and is rapidly hydrolyzed to produce a free thiol group in the presence of the extracellular phospholipase C from either Bacillus cereus or Clostridium perfringens. When 5,5'-dithiobis (2-nitrobenzoic acid) was included as a thiol reactive chromogenic agent, the resultant measurement of product release, as an increase in absorbance at 412 nm, showed a linear relationship with added enzyme.—Snyder, W.R. Synthesis of a thiophosphate analog of dioctanoylphosphatidylcholine: a phospholipase C substrate. J. Lipid Res. 1987. 28: 949–954.

Supplementary key words phospholipase C • spectrophotometric assay • phospholipid synthesis

Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) catalyzes the hydrolysis of the glycerophosphate bond in membrane phospholipids. The hydrolysis of phosphatidylcholine, for example, results in the production of a diglyceride and a water-soluble choline phosphomonoester. The resultant alteration of the amphipathic nature of the substrate phospholipid must result in a significant change in the nature of biological membranes.

In order to explore fully the reaction catalyzed by phospholipase C, it is advantageous to utilize an assay method that continuously measures product formation and also is highly specific for the hydrolysis of the glycerophosphate bond. To date, only the thiophosphate analog assay (1) satisfies both of these criteria.

Cox, Snyder, and Horrocks (1) synthesized phospholipid analogs where the glycerophosphate oxygen was replaced by a sulfur atom. After phospholipase C-catalyzed hydrolysis, a thiol-containing product (thiodiglyceride) was formed. Under mild aqueous conditions, this thiol could be reacted with a chromogenic agent to measure the continuous formation of product. The synthetic phosphatidylcholine analog substrates contained either one 16-carbon acyl chain (a mercaptoethanol derivative) or two 10-carbon acyl chains (a mercapto-glycerol derivative) (diC₁₀-S-PC). Although the latter analog has the advantage of being structurally more similar to the natural substrate for phospholipase C, it proved to be difficult to disperse and resulted in relatively slow hydrolytic rates.

A readily dispersible, rapidly hydrolyzed thiophosphate analog, dioctanoylthiophosphatidylcholine (diC₈-S-PC) has been synthesized and used to assay the extracellular C-specific phospholipases from Bacillus cereus and Clostridium perfringens. The organic synthesis, isolation by flash chromatography, and use as a phospholipase C substrate are reported here.

MATERIALS AND METHODS

Octanoylchloride, 3-mercapto-1,2-propanediol, dithiothreitol (DTT), 2-bromoethanol, anhydrous trimethylamine (TMA), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Aldrich Chemical Company. Phosphorous oxychloride (Baker) was distilled immediately before use (105–106°C). Triethyamine (TEA) (Eastman) was dried over Linde type 4A molecular sieve, refluxed with KOH pellets, and distilled (88–90°C). Pyridine (Aldrich Gold Label) was dried over Linde type 4A molecular sieve before use. Chloroform (Baker) was extracted with two volumes of water to remove the ethanol stabilizer, dried over CaCl₂, refluxed with CaSO₄, and distilled. MOPS, 3-(N-morpholino)propanesulfonic acid,

Abbreviations: diC₈-S-PC, dioctanoylthiophosphatidylcholine (ω-3-S-phosphocholine-1,2-O-dioctanoyl-3-mercaptopropanediol); diC₁₀-S-PC, didecanoylthiophosphatidylcholine; diC₈PC, L-a-dioctanoylphosphatidylcholine; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); MOPS, 3-(N-morpholino)propanesulfonic acid.

L-α-dioctanoylphosphatidylcholine (diC₈PC), phospholipase C (Bacillus cereus, 3,000 Units / mg of protein), and phospholipase C (Clostridium perfringens, 300 Units / mg of protein) were obtained from Sigma Chemical Company and used without further purification. The activity units quoted for the phospholipases are based on the supplier's specifications and were not obtained during this study.

Elemental analyses (C, H, N) were performed on a Perkin Elmer 240 Elemental Analyzer. Infrared spectra were recorded on a Mattson Cygnus 25 FT-IR, and NMR spectra (¹H and ³¹P) were obtained from an IBM WP-200SY 200 MHz FT-NMR using tetramethylsilane and 85% H₃PO₄ as the respective standards. Thin-layer chromatography was carried out on glass-backed silica gel 60 plates (E. Merck) and material was generally detected by exposure to I₂ vapor. Phosphate-containing compounds were stained with a molybdate spray (2), thiols were detected with a DTNB spray (1), and choline-containing materials were identified with Dragendorff reagent (2).

**Preparation of diC₈-S-PC**

The dioctanoyl phosphatidylcholine thiophosphate analog (VI) (Scheme 1) was prepared as a racemic mixture by a modification of the procedure of Cox et al. (1).

Compound I, rac-3,3'-dithiobis (1,2-propanediol) was prepared by a variation of the method of Danehy and Hunter (3). Eighty grams (0.74 mol) of 3-mercapto-1,2-propanediol was placed in a flask and cooled in an ice bath. Forty two ml of 30% hydrogen peroxide was added dropwise to the rapidly stirring thiol while the temperature was maintained between 30° and 40°C. When the exothermic reaction was complete, the mixture was stirred overnight at room temperature. Water was removed from the product in a rotary evaporator (40°C) with successive additions of absolute ethanol. The dried product was a chalky white solid which was used without further purification. Anal. calcd. for C₂₈H₄₄S₂O₄: C, 33.63; H, 6.58. Found: C, 33.60; H, 6.54.

The preparation of rac-3,3'-dithiobis (1,2-O-dioctanoyl-1,2-propanediol) (compound II) was carried out by a modification of the procedure used by Cox et al. (1) for the synthesis of diC₈-S-PC. Thirty mmol of the disulfide (I) was dissolved in 200 ml of ethanol-free chloroform and 180 mmol of dry pyridine. Octanoylchloride (150 mmol) was added with rapid stirring, and the mixture was stirred overnight at room temperature. The workup of the mixture and purification by basic alumina chromatography

![Scheme 1. Synthetic scheme.](image-url)
have been reported (1). FT-IR (thin film): 2950, 2920, 2850, 1460, 1375 cm⁻¹ (C-H); 1740, 1150 cm⁻¹ (ester); 1105 cm⁻¹ (secondary ester); 1055 cm⁻¹ (primary ester). ¹H-NMR (CDCl₃); δ (ppm): 0.9, [6H, apparent triplet, (CH₃)₄; 1.3, [16H, apparent singlet, -(CH₂)₄-CH₃]; 1.6, [4H, multiplet, -(CH₂)₄-CH₂-]; 2.3, [4H, multiplet, C(O)-CH₂-CH₃]; 2.9, [2H, doublet, -CH₂-S-CH₂-]; 4.0-4.5, [2H, complex, -CH₂-O-(O)-]; 5.3, [1H, multiplet, H-C-O-].

Enzyme assay

An aliquot of diC₈-S-PC in chloroform was placed in a glass vial, and the solvent was removed under a stream of N₂(g). The substrate was dispersed on a vortex mixer at room temperature in 0.05 M MOPS buffer (pH 7.3) at a final concentration of 2.5 mM diC₈-S-PC. The stock solution was sonicated (150 watts) for 30 sec with a Branson Sonifier cell disruptor Model 350A equipped with a 0.5-inch disruptor horn.

Into a 1.2-ml optical glass cuvette were added 200 µl of the diC₈-S-PC stock solution, 200 µl of 5.0 mM DTNB in 0.05 M MOPS buffer (pH 7.3), and sufficient volume of additional 0.05 M MOPS buffer (pH 7.3), to result in a final assay volume of 1.0 ml after enzyme addition. When the phospholipase C from Clostridium perfringens was utilized, the assay mixture included 25 mM CaCl₂. The cuvette was placed in the thermostated (37°C) sample compartment of an LKB Ultraspec 4050 UV-Vis Spectrophotometer. After temperature equilibration and observation of a stable baseline absorbance, an aliquot of phospholipase C was added. The resultant absorbance change at 412 nm was recorded, and the rate of hydrolysis was determined using a molar absorbtivity of 12,800 M⁻¹cm⁻¹ (5,7) for the 2-nitro-5-thiobenzoate dianion produced.

RESULTS AND DISCUSSION

The thio phosphate derivative of dioctanoylphosphatidylcholine, diC₈-S-PC, has been synthesized by a modification of the method reported by Cox et al. (1). The use of H₂O₂ for the oxidation of 3-mercapto-1,2-propanediol has proven to be an excellent method by which large quantities of the disulfide (compound I) can be prepared. The essentially odorless solid has been stored (desiccated at room temperature) for at least 6 years without apparent degradation. The procedure of Eibl and Nicksch (4) for the preparation of bromoethylphosphoric acid dichloride has led to improved yields of the low yield thiol phosphorylation step have been over-
come by the utilization of a silica gel flash column chromatography system. Fig. 1 shows thin-layer chromatograms obtained by spotting fractions eluted from the silica gel column. At the origin (labeled L), a sample of the trimethylation product (compound VI) was spotted after drying and dissolving in chloroform-methanol 1:4. This multiple component mixture was applied to the silica column. The remaining numbered lanes (5-185) were obtained from the eluted fractions (chloroform-methanol 1:4) that were subsequently collected. While the early fractions contained substantial contaminants, fractions 75-140 contained essentially pure diCE-S-PC. The same column technique has been used to purify several different thiophosphorylcholine analogs with equally successful results.

The IR spectrum for diCE-S-PC was virtually identical to that obtained with a similar thin film of diCEPC. The only significant difference was an extra signal at 824 cm⁻¹, which was present with diCEPC, but absent in the spectrum of the thiophosphate analog (diCE-S-PC). The observation is analogous to that observed by Cox et al. (1), and the band has been attributed to an -O-P-O- antisymmetric stretch (8), which should be absent in the thiophosphate analog.

A single difference was also observed when the ¹H NMR spectra of diCEPC and diCE-S-PC were compared. A two-proton multiplet at δ = 4.0 ppm was observed in the spectrum of diCEPC which can be assigned to the -CH₂-O-P protons (9). This signal was absent in the ¹H NMR spectrum of the thiophosphate analog (diCE-S-PC) and was replaced by a two-proton multiplet at δ = 3.0 ppm, which is consistent with the replacement of oxygen with sulfur (-CH₂-S-P). In the ³¹P NMR, diCEPC appeared slightly upfield from the reference (85% H₃PO₄) at δ = -0.21 ppm, while the signal obtained for the thiophosphate (diCE-S-PC) was shifted downfield to δ = 15.7 ppm. The magnitude of this chemical shift is consistent with that observed for other thiophosphate analogs (10, 11).

The products resulting from phospholipase C-catalyzed hydrolysis of diCE-S-PC have been identified by thin-layer chromatography on silica gel 60 plates. After incubation with the Bacillus cereus enzyme, a product extracted with chloroform–methanol 2:1 was shown to cochromatograph (chloroform–diethylether 9:1) with a sample of compound III, both staining positively with DTNB spray reagent (1). The water-soluble reaction product cochromatographed (butanol–acetic acid–water 5:3:1) with a sample of phosphocholine, and stained with molybdate spray (2) as well as Dragendorff reagent (2). The hydrolysis of diCE-S-PC catalyzed by phospholipase D was shown to produce a relatively unstable thiophosphate analog of phosphatidic acid which is currently under investigation (Nyquist, D.A., and Snyder, W.R., unpublished results).

The use of diCE-S-PC as a phospholipase C substrate has proven to be a significant improvement over the diC₁₀-S-PC analog. The diC₈-S-PC substrate is easily dispersed with a 30-sec sonication to provide a clear suspension, which is hydrolyzed in the presence of the C-specific phospholipases at rates that are at least five times greater than those observed with diC₁₀-S-PC. Fig. 2 shows representative time-course data for the assay of the phospholipases C from Bacillus cereus (Fig. 2A) and Clostridium perfringens (Fig. 2B) using diCE-S-PC in the presence of DTNB. After essentially no change in absorbance at 412 nm with substrate and DTNB alone, the resulting changes in the absorbance following the addition of the enzymes are indicated by the arrows. Under these assay conditions (pH 7.3, 37°C), both of the enzymes show an initial lag phase which is more pronounced with the enzyme from Clostridium perfringens (Fig. 2B). While conditions can be varied to eliminate the lag phase with
Fig. 2. Time courses for the hydrolysis of diC₈-S-PC by phospholipase C in the presence of DTNB in a total assay volume of 1 ml. A. Incubation mixture contained 200 μM diC₈-S-PC and 1.0 mM DTNB in 0.05 M MOPS buffer, pH 7.3. The arrow indicates the addition of 0.5 Units (Sigma Chemical) of the Bacillus cereus phospholipase C. B. Incubation mixture contained 200 μM diC₈-S-PC, 1.0 mM DTNB, and 25 mM Ca²⁺ in 0.05 M MOPS buffer, pH 7.3. The arrow indicates the addition of 0.5 Units (Sigma Chemical) of the Clostridium perfringens phospholipase C. Both reactions were carried out at 37°C.

The initial rate for each of the phospholipase C-catalyzed reactions is defined as the maximal velocity attained following the lag period and prior to the eventual rate decrease resulting from substrate depletion. Fig. 3 demonstrates these measured initial velocities as a function of phospholipase C concentration for the two extracellular bacterial enzymes. Both show a linear relationship between initial velocity and the amount of enzyme added, indicating that the thiophosphate analog spectrophotometric assay is a viable measure of phospholipase C activity for the enzymes from Bacillus cereus and Clostridium perfringens.

Relative hydrolytic rates were determined for diC₈PC and diC₈-S-PC using the traditional pH stat method (pH 8.0) originally described by Dennis (12) for the assay of phospholipase A₂. The results obtained with the Bacillus cereus enzyme showed that the diC₈PC substrate was hydrolyzed approximately 200 times faster than the thiophosphate analog under identical pH stat assay conditions. The rate obtained for the pH stat assay using diC₈PC was 100 times faster than the rate observed for diC₈-S-PC hydrolysis in the spectrophotometric assay. The twofold discrepancy appears to involve a stimulation of the enzyme-catalyzed reaction by DTNB and has been observed with both substrates and assay methods.

The loss in absolute hydrolytic rate observed when the thiophosphate analog is compared with diC₈PC hydrolysis appears to be offset by the gain in sensitivity resulting from the DTNB thiol reaction when diC₈-S-PC is used in the spectrophotometric assay. This coupled with the high degree of assay specificity, and the convenience afforded by a spectrophotometric method makes the technique a viable assay for phospholipase C. Studies are currently in progress to optimize the assay conditions, to investigate the involvement of DTNB in the reaction, and to explore the use of the method for the assay of other C-specific phospholipases.

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REFERENCES


