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A THIOPHOSPHATE SUBSTRATE FOR A CONTINUOUS SPECTROPHOTOMETRIC ASSAY OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C: HEXADECYLTHIOPHOSPHORYL-1-*myo*-INOSITOL

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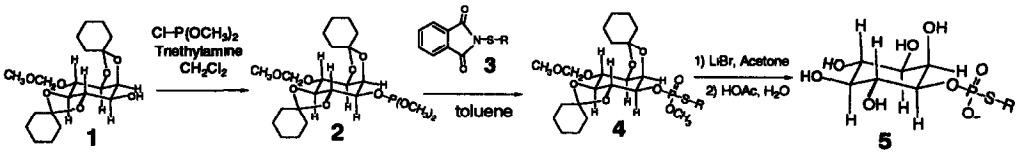
Abstract: Racemic hexadecylthiophosphoryl-1-*myo*-inositol was synthesized from a pentaprotected inositol-1-dimethylphosphite and hexadecylthiophthalimide in a Michaelis-Arbuzov reaction. It was shown to be a good substrate for the assay of phosphatidylinositol-specific phospholipase C.

Phosphatidylinositol-specific phospholipase C (PI-PLC; EC 3.1.4.10) from *Bacillus cereus* catalyzes the cleavage of phosphatidylinositol to a diglyceride and D-*myo*-inositol-1,2-cyclic phosphate. The latter is subsequently hydrolyzed slowly by the same enzyme to D-*myo*-inositol-1-phosphate.[1,2] This enzyme also catalyzes the release of a number of enzymes linked to glycosylphosphatidylinositol (GPI) membrane anchors.[3] Two different assay methods are commonly used to measure the activity of PI-PLC: 1) the determination of water-soluble inositol phosphate from radiolabeled phosphatidylinositol,[1] and 2) quantitation of the release of GPI-anchored enzymes from biological membranes.[4] The radioisotope-based assay is discontinuous, time-consuming, and expensive. Recently Shashidhar et al.[5] developed a continuous fluorometric assay using synthetic 2-naphthyl *myo*-inositol-1-phosphate. However, the specific activity of PI-PLC for this substrate was only about 0.003% that for the natural substrate. We have synthesized thiolester analogs of phospholipids as substrates for continuous spectrophotometric assays of phospholipase A_2, where the release of thiol is determined by coupled reaction with a colorometric thiol reagent.[6] Cox et al.[7] have synthesized thiophosphate analogs of phosphatidylcholine as substrates for the continuous assay of phosphatidylcholine-specific phospholipase C. We report here the first synthesis of a thiophosphate analog (C-S-P bond) of phosphatidylinositol as a substrate for the kinetic analysis of PI-PLC.



Scheme I. Synthesis of hexadecylthiophosphoryl-1-*myo*-inositol. R- = $C_{16}H_{33}$-

Racemic hexadecylthiophosphoryl-1-*myo*-inositol (**5**) was synthesized as shown in Scheme I. Racemic 1,2,4,5-biscyclohexylidino-6-methoxymethyl-*myo*-inositol (**1**) was synthesized from 1,2,4,5-biscyclohexylidino-*myo*-inositol[8] by specific formation of the t-butyldiphenylsilyl ether at the 3-position,[9] formation of the methoxymethyl ether at the 6-position,[10] and cleavage of the silyl ether at the 3-position.[11] Phosphitylation of **1** (1 mmol) with chlorodimethyl phosphite[12] (1.5 mmol) in the presence of 0.2 g of triethylamine in 3 ml of dry methylene chloride gave **2** (single spot on silica gel TLC with chloroform-methanol 1:1; washed with $NaHCO_3$, H_2O, dried over Na_2SO_4, evaporated to dryness with addition of toluene), which was used in the next reaction without purification. Hexadecylthiophthalimide[13] (**3**, 0.82 mmol in 1 ml toluene) was added dropwise to **2** in 2 ml of toluene at room temperature over 5 min.[14] There was a slight exothermic reaction which was complete (by TLC) after 15 min. The product (**4**) was purified on a silica column (20-50% $CHCl_3$ in hexane; 0.34 g, 60% yield). The methylphosphate of **4** was cleaved by refluxing with LiBr in dry acetone for 6 hr. The lithium salt which precipitated was filtered and washed with cold acetone. The salt was dissolved in acetic acid/water (4:1) and heated at 95° C for 40 min. The completely deprotected product **5** (silica gel TLC, R_f = 0.17, $CHCl_3/CH_3OH/H_2O$ 65:35:3) was purified on a silica column (8-35% CH_3OH in $CHCl_3$).[15]

PI-PLC[16] was assayed with **5**, using the spectrophotometric method[17] described by Hendrickson and Dennis,[6b] in the presence of detergent (hexadecylphosphorylcholine, HDPC), 50 mM 2-(N-morpholino)ethanesulfonate, pH 6.5, and 0.8 mM 4,4'-dithiopyridine at 20° C. In the presence of no detergent or with sodium deoxycholate,[18] the enzyme was considerably less active. Enzyme activity as a function of the amount of enzyme added gave a linear plot (Figure 1) with a slope equal to

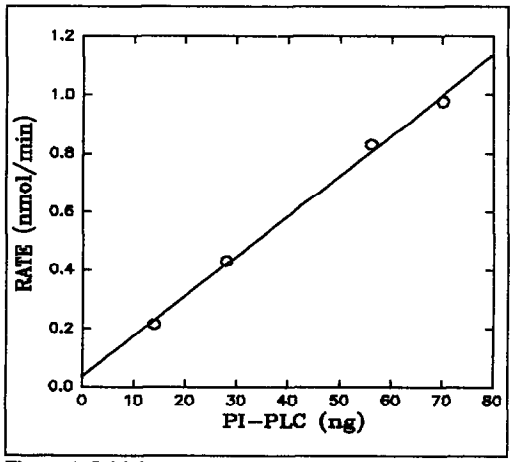


Figure 1. Initial rate versus enzyme concentration

13.8 ± 0.5 μmol min^{-1} mg^{-1} (0.2 mM substrate, 0.4 mM HDPC). Rates as low as 0.2 nmol/min (about 15 ng of enzyme) could easily be measured. Enzyme activity as a function of bulk substrate concentration at a constant surface concentration (Figure 2; constant ratio, 1:4, of substrate to detergent, HDPC), or surface concentration of substrate at a constant bulk concentration of 1 mM (Figure 3; varied ratio of detergent to substrate), as described by Hendrickson and Dennis,[6b] gave good hyperbolic plots from which the following kinetic constants could be calculated: K_M* (interfacial Michaelis constant) = 0.17 ± 0.01 mol fraction, K_S (dissociation constant for the initial enzyme-substrate complex) = 26 ± 4 μM, V_{max} = 16.9 ± 0.6 μmol min^{-1} mg^{-1}. This maximal activity is about 1% that for the natural substrate. Compound **5** is racemic and the unnatural enantiomer may act as a competitive inhibitor. We are in the process of synthesizing the chiral substrate and evaluating other interfacial assay systems in which the enzyme may be more active. We plan future studies with analogs of **5** in which the structure of the R-group is varied from a simple alkyl group to a more natural diglyceride. This new thiophosphate substrate analog should prove to be a good substrate with which to study the enzymatic properties of PI-PLC.

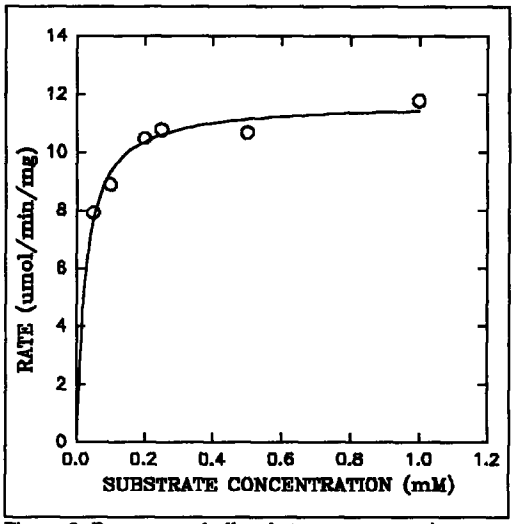


Figure 2. Rate versus bulk substrate concentration

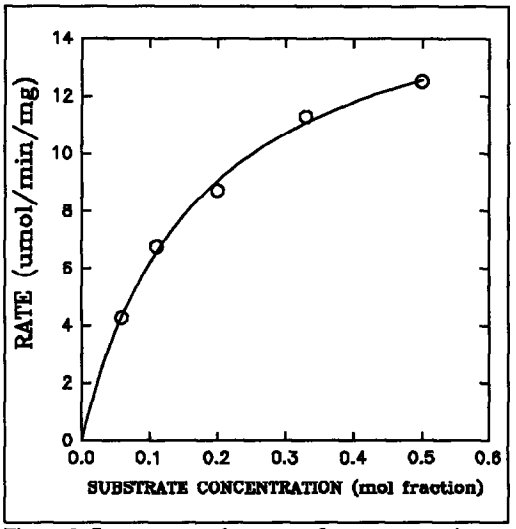


Figure 3. Rate versus substrate surface concentration

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15. Compound **5**. MS(FAB): Calculated for $C_{22}H_{44}PSO_8$, 499.2492 (M^-); observed, 499.2485 (Mass Spectrometry Service Laboratory, Department of Chemistry, University of Minnesota).
16. Recombinant *B. cereus* PI-PLC was a generous gift from Dr. J.J. Volwerk, Institute of Molecular Biology, University of Oregon, Eugene, OR. Dilute enzyme (less than 1 mg/ml) was stable in the presence of 5 mM hexadecylphosphorylcholine.
17. 4-Thiopyridine was determined using an extinction coefficient of 19,800 M^{-1} cm^{-1}.
18. Substrate (1 mM) and 0.16% (w/v) sodium deoxycholate.