

**A FLUORESCENT SUBSTRATE FOR THE ASSAY OF
PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C:
4-(1-PYRENO)BUTYLPHOSPHORYL-1-*myo*-INOSITOL**

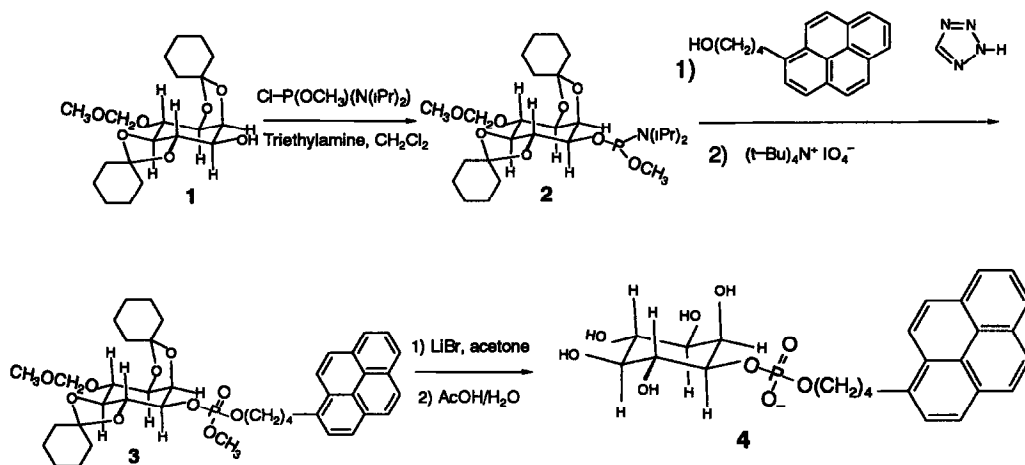
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Abstract: Racemic 4-(1-pyreno)butylphosphoryl-1-*myo*-inositol was synthesized from a pentaprotected inositol-1-dimethylphosphite by phosphite coupling with 4-(1-pyreno)butanol. It is a good substrate for a very sensitive assay of phosphatidylinositol-specific 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. We recently synthesized a thiophosphate analog of phosphatidylinositol, hexadecylthiophosphoryl-1-*myo*-inositol, which proved to be a good substrate for the continuous spectrophotometric assay of PI-PLC in which release of the thiol is determined by a coupled reaction with 4,4'-dithiopyridine.⁵ This assay, however, is not well suited for crude enzyme preparations since the presence of endogenous thiols creates a high background. We report here the synthesis of a pyrene-labeled fluorescent analog of phosphatidylinositol. Separation by HPLC of the fluorescent substrate and product (pyrenebutanol) with fluorescence detection provides a very sensitive assay for PI-PLC in crude preparations.



Scheme I. Synthesis of 4-(1-pyreno)butylphosphoryl-*myo*-inositol.

Racemic 4-(1-pyreno)butylphosphoryl-*myo*-inositol (**4**) 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*-inositol⁶ by specific formation of the *t*-butyldiphenylsilyl ether at the 3-position,⁷ formation of the methoxymethyl ether at the 6-position,⁸ and cleavage of the silyl ether at the 3-position.⁹ Compound **1** was coupled to 4-(1-pyreno)butanol by *N,N*-diisopropylmethylphosphonamidic chloride to give **3**.¹⁰ The methylphosphate of **3** was cleaved by refluxing with LiBr in dry acetone. The lithium salt which precipitated was dried and further deprotected (hydrolysis of the acetal and ketals) by heating at 95° C with 80% acetic acid in water for 45 min. The crude product (**4**) was dried and purified by chromatography on silica gel (8-35% CH₃OH in CHCl₃).¹¹

PI-PLC¹² was assayed with **4** by HPLC separation¹³ of the substrate and product with fluorescence detection using the general method described earlier for phospholipase A₂ assays with fluorescent substrates.¹⁴ Plots of nmol of pyrenebutanol released versus time were linear up to at least 5% hydrolysis. The presence of sodium deoxycholate resulted in a lower activity as

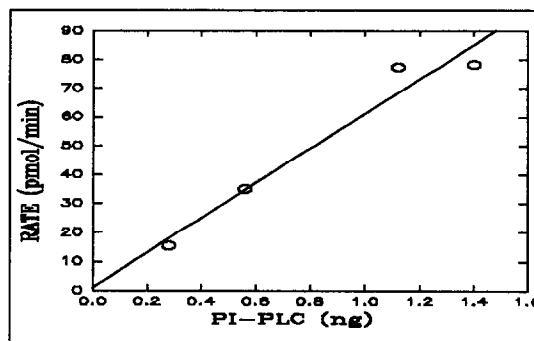


Figure 1. Initial rate versus amount of enzyme

compared to the reaction in the absence of detergent.¹⁵ Activity versus amount of enzyme gave a linear plot (Figure 1) with a slope equal to $60 \pm 9 \mu\text{mol min}^{-1} \text{mg}^{-1}$. This is about 4% of the activity with the natural substrate.¹ Rates as low as 10 pmol/min (about 0.2 ng of enzyme) could readily be detected. The detection limit for pyrenebutanol was estimated to be about 100 pmol. This assay should prove to be an attractive alternative to the radioisotope assay to measure PI-PLC activity in crude preparations during enzyme purification.

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10. Compound 1 (0.39 mmol in 4 ml of CH_2Cl_2 and 0.1 g of triethylamine) was reacted with *N,N*-diisopropylmethylphosphonamidic chloride under argon with stirring for 30 min. The reaction mixture was dried under vacuum. 1-H-Tetrazole (1.5 mmol) in 6 ml of tetrahydrofuran-acetonitrile (1:1) was added, followed by 4-(1-pyreno)butanol (0.45 mmol), and the reaction allowed to stir overnight. Pyridine (0.1 ml) and tetrabutylammonium periodate (0.78 mmol) in 2 ml of CH_2Cl_2 were added with stirring for 7 min. The reaction was diluted with CH_2Cl_2 and washed successively with 5% NaHSO_3 , saturated NaHCO_3 , and brine, dried over anhydrous Na_2SO_4 , and evaporated to dryness. Chromatography on a 5 g silica column (50% CHCl_3 in hexane to pure CHCl_3) gave 0.24 g (90% yield) of 3, which was contaminated with pyrenebutanol. The latter was removed after demethylation and precipitation of the lithium salt.

11. Compound **4**. MS(FAB): Calculated for $C_{26}H_{28}PO_9$, 515.1471 (M⁺); observed, 515.1489 (Mass Spectrometry Service Laboratory, Department of Chemistry, University of Minnesota).
12. Recombinant *B. cereus* PI-PLC was a generous gift from Dr. J. J. Wolwerk, Institute of Molecular Biology, University of Oregon, Eugene, OR. Dilute solutions of the enzyme (less than 1 mg/ml) were stabilized in the presence of 0.1% bovine serum albumin.
13. Conditions for reversed-phase ion-pair HPLC of **5** and pyrenebutanol were adapted from Abidi, S.L.; Mounts, T.L.; Rennick, K.A. *J.Liquid Chromatogr.* **1991**, *14*, 573-588.
14. Hendrickson, H.S. *Methods Enzymol.* **1991**, *197*, 90-94. The reaction mixture consisted of 50 μ l of a solution containing 50 mM 2-(N-morpholino)ethanesulfonate, pH 6.5, 1 mM **4**, and PI-PLC. The mixture was incubated at 25° C, and at various time intervals 5- μ l aliquots were removed and diluted to 100 μ l with HPLC solvent (5 mM tetrabutylammonium dihydrogenphosphate in acetonitrile/methanol/water, 70:10:20). Twenty μ l of diluted sample were chromatographed on a reverse phase silica column (5 μ m Spherisorb ODS, 25 cm x 4.6 mm) at 1 ml/min, and the eluate was analyzed by a fluorescence detector (excitation, 343 nm; emission >370 nm). Retention times for **4** and pyrenebutanol were 2.44 and 6.17 min respectively.
15. Substrate (2 mM) and 0.16% (w/v) sodium deoxycholate compared to 2 mM substrate with no deoxycholate.