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A CHROMOGENIC SUBSTRATE FOR THE CONTINUOUS ASSAY OF MAMMALIAN PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C

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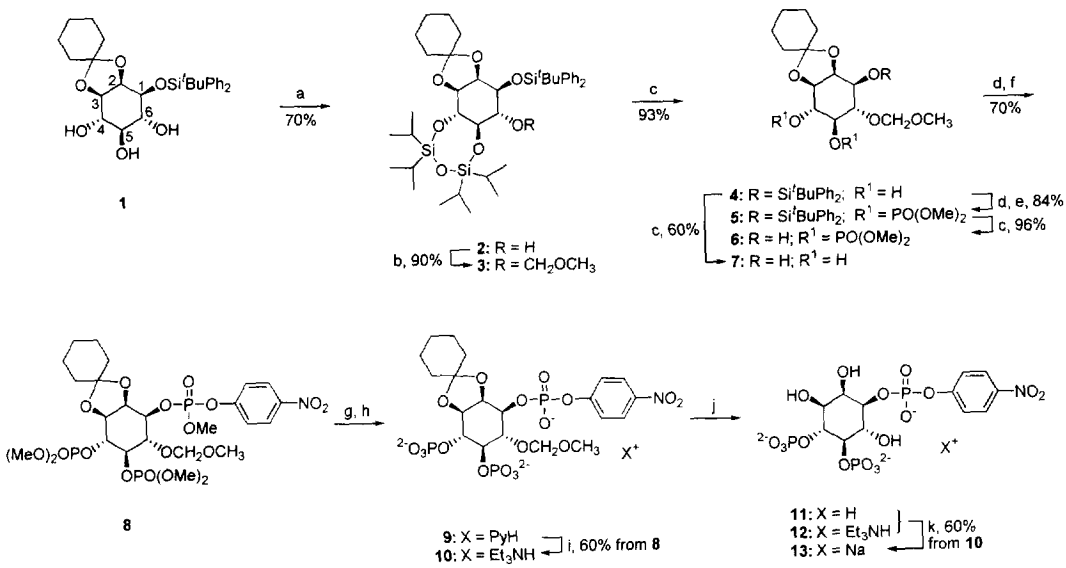
Abstract: The synthesis of a chromogenic substrate for mammalian PI-PLC, D,L-*myo*-inositol 4,5-bis(dihydrogen phosphate) 1-(4-nitrophenyl hydrogen phosphate) tetrasodium salt (**13**), is described. Phosphoinositol bisphosphate **13** (NPIP-P_2) is shown to be a substrate for human PI-PLCδ_1 in a non-radioactive, continuous assay. © 1997 Elsevier Science Ltd.

Growth factors, neurotransmitters, and mitogens bind to receptors on the external face of cells, initiating a chain of events that leads to profound changes such as cell proliferation and neuronal activity.[1] Determining the exact sequence of events in this signal transduction process is an active field of investigation with potential applications in cancer research and Alzheimer's disease.[2] Early in this signal transduction cascade the intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol are generated from the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2). The enzyme producing these second messengers is phosphoinositide-specific phospholipase C (PI-PLC), and the first X-ray crystal structure of a mammalian PI-PLC has recently been reported.[3] Progress in this field would be accelerated if chromophoric substrates for the PI-PLCs were available for structure-function studies on the isolated isozymes and for examining the time dependence of the sequence of events in receptor activation of PI-PLCs in reconstituted systems or by microinjection into cells. At present, the only general assay available is based on radiolabeled inositol phospholipids, which has the limitation of being discontinuous.[4] Herein we describe the synthesis of a chromogenic substrate for mammalian PI-PLC: D,L-*myo*-inositol 4,5-bis(dihydrogen phosphate) 1-(4-nitrophenyl hydrogen phosphate) tetrasodium salt (**13**), subsequently referred to as NPIP-P_2. We demonstrate that NPIP-P_2 is a substrate for human PI-PLCδ_1 and describe a rapid and sensitive continuous assay for this important class of second messenger generating enzymes.

Racemic triol **1** (Scheme 1), prepared[5] from *myo*-inositol, was treated with $(Cl\text{-}^iPr_2Si)_2O$ to give alcohol **2**[6,7] which was converted into MOM derivative **3**.[6,7] Selective desilylation of **3** with Bu_4NF gave diol **4**.[6] Regiochemistry was confirmed by desilylation with excess Bu_4NF to give the known triol **7**.[8] Reaction of **4** with $(MeO)_2PCl$[9] followed by oxidation with *m*-CPBA produced bisphosphate ester **5**[6,7] which was desilylated to give alcohol **6**.[6,7] Utilizing the two-step procedure of Nagai et al.,[9] **6** was treated with $(MeO)_2PCl$ followed by *p*-nitrophenol and I_2 to give trisphosphate **8**, obtained as a separable (silica gel) mixture of diastereomers involving the newly introduced P atom.[6] The mixture was demethylated with TMSBr and then hydrolyzed with aqueous pyridine to give crude pyridinium salt **9** which was separated from contaminating pyridinium bromide and concurrently ion exchanged to the Et_3NH^+ salt **10**[6] by chromatography over Cellex-D (elution with aqueous Et_3NH^+ HCO_3^-). The *O*-protecting groups were removed by mild hydrolysis (Amberlite IR-120 resin, H^+ form) and the resulting product was converted into Na^+ salt **13** by passage through a column of Amberlite CG-120 resin (Na^+ form). Chromatography over powdered cellulose (elution with 10–30% H_2O in EtOH) gave pure racemic *myo*-inositol trisphosphate tetra Na^+ salt NPIP-P_2 (**13**).[6,7,10]

Scheme 1



(a) 1.5 equiv of $(Cl^iPr_2Si)_2O$, Py, rt, 72 h; (b) 20 equiv of CH_3OCH_2Cl, $^iPr_2NEt/THF$, 55 °C, 68 h; (c) $^nBu_4NF$, THF, rt, 3 equiv, 30 min for **4** or 1.2 equiv, 15 h for **6** and **7**; (d) 50% excess of $(MeO)_2PCl$, $^iPr_2NEt/CH_2Cl_2$, 0 °C, 30 min; (e) 3 equiv of *m*-$ClC_6H_5CO_3H$, CH_2Cl_2, rt, 30 min; (f) 1 equiv of *p*-nitrophenol, 4 equiv of I_2, $^iPr_2NEt/CH_2Cl_2$, 0 °C, 1 h; (g) 15 equiv of Me_3SiBr, $CHCl_3$/Py, rt, 3 h; (h) H_2O/Py, rt, 30 min; (i) chromatography on Cellex-D with $Et_3NH_2CO_3$, 0.1 to 1 M; (j) excess of Amberlite IR-120 plus (H^+ form), H_2O, rt, 15 h; (k) column with Amberlite CG-120 (Na^+ form).

For the assay of PI-PLC,[11] NPIP-P_2 (6.3 mg) was dissolved in H_2O (0.12 mL; total vol, 0.123 mL) giving an optically clear solution, and the pH was adjusted to ~5 with solid $NaHCO_3$. Complete hydrolysis of a small aliquot in 0.01 *N* NaOH gave a total concentration of 70.2 mM *p*-nitrophenoxide ion (pNP), based on the molar absorbtivity at 399 nm (ε, 18,100 M^{-1} cm^{-1}) of pNP. A second aliquot was added to the assay buffer (see Figure 1 legend) and absorbance at 399 nm revealed that the stock solution contained 1.05 mM or 1.5 mol-% of pNP. Enzyme activities were measured using 1.0 mM NPIP-P_2 and varying concentrations of PI-PLC.[12] Under these conditions, the reaction rate is linear with time for at least 4 min at low (0.5 nM) enzyme concentrations (Figure 1A). Slight autolysis of substrate was detected (0.1 μM pNP/min) and subtracted from the enzyme-catalyzed hydrolysis curves before calculating the initial rates of enzymatic hydrolysis of NPIP-P_2. The rate of autolysis depends on the pH of the assay reactions and will increase at higher pH and decrease at lower pH. A linear correlation between enzyme concentration and initial hydrolysis rate was observed. The specific activity of the enzyme towards 1 mM racemic NPIP-P_2 is 28 μmol min^{-1} mg^{-1}, as calculated from the slope of the fitted curve (Figure 1B). This is comparable to the specific activity observed for the natural lipid substrate PIP_2 measured under similar conditions.[13] Kinetic constants for the hydrolysis of racemic NPIP-P_2 by PI-PLCδ_1 were determined from the initial rates of hydrolysis of substrate at concentrations between 0.1 and 20.0 mM giving K_M = 2.7 mM (assuming that only the D-isomer is a substrate,[12] K_M = 1.35 mM), V_{max} = 78.5 μmol min^{-1} mg^{-1} and k_{cat} = 112 s^{-1} (Figure 1C). The assay solutions are optically clear at all substrate concentrations, indicating the absence of possible aggregation of NPIP-P_2 with Ca^{2+}. The catalytic efficiency of the enzyme for the turnover of NPIP-P_2 gives k_{cat}/K_M = 8.3 × 10^4 $M^{-1}s^{-1}$. For the natural substrate PIP_2,

k_{cat}/K_M values range from 2.9 × 10^6 $M^{-1}s^{-1}$ to 1.7 × 10^7 $M^{-1}s^{-1}$ depending on assay conditions such as pH, temperature, concentration and type of detergent and source of the PI-PLCδ_1 enzyme. Thus, the efficiency of NPIP-P_2 catalysis is lower than that of PIP_2, but it is sufficiently high for enzyme kinetic studies of the mammalian PI-PLCs. The mammalian PI-PLCs are Ca^{2+}-dependent enzymes and enzymatic assays included 0.1 mM Ca^{2+}, sufficient to saturate the enzyme's requirement for this ion.[14] As expected, when Ca^{2+} was omitted and EDTA included in the reaction mixture, no enzymatic activity was detectable.

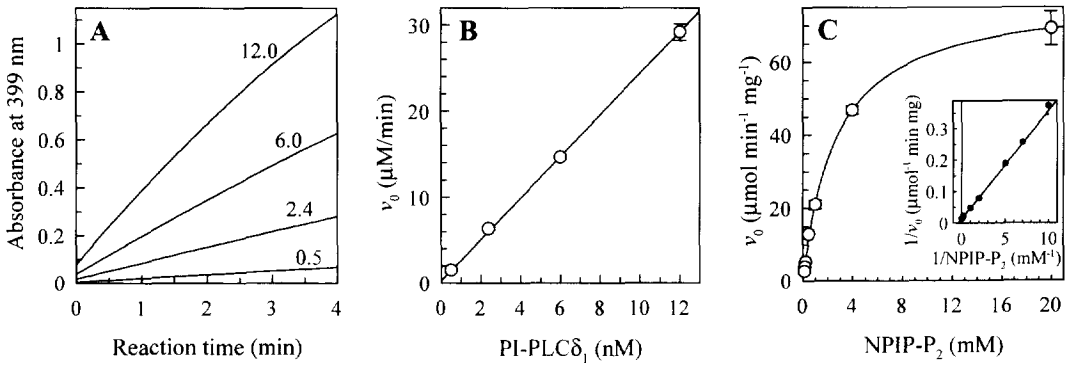


Figure 1. Assay of mammalian PI-PLC with the chromogenic substrate analog NPIP-P_2 (**13**). A. Catalysis monitored by spectrophotometric detection of the product pNP. The progress curves of a typical set of experiments are shown for 0.50–12.0 nM PI-PLC. B. After absorbance data were converted into [pNP] and corrected for pNP background, progress curves as shown in panel A were fitted by nonlinear regression to a first order rate equation to determine initial rates (v_0). The rates were plotted against enzyme concentration. Data points were fitted by linear regression. The mean of two experiments is presented. Assays were performed at 25 °C in assay buffer (25 mM HEPES (pH 7.2), 100 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM dithiothreitol, 0.01% gelatin (bovine skin, type B) with ε_{399} = 11,060 M^{-1} cm^{-1} for pNP) containing 1.0 mM racemic NPIP-P_2. The enzyme was added last and initiated the reaction. Gelatin was used in place of BSA to prevent non-specific adsorption of the enzyme to the vessel wall. C. K_M and V_{max} values were calculated from the initial rates measured at constant enzyme concentration (0.78 nM, 66.7 ng/ml) and substrate concentrations ranging from 0.1–20.0 mM racemic NPIP-P_2. Data points were determined in duplicate or triplicate and the mean was fitted by nonlinear regression to the Michaelis-Menten equation. The insert displays the fitted curve in the linear Lineweaver-Burk form. Assay conditions were as in panel A and B except the concentration of HEPES was raised to 100 mM and the pH of the stock solution of NPIP-P_2 was raised to about pH 7 in order not to perturb the pH of the reaction (pH 7.2) at high concentrations of substrate. Error bars are shown where larger than the symbol size.

In summary, the synthesis of the first chromogenic substrate analog NPIP-P_2 (**13**) specifically designed for mammalian PI-PLC is described. Phosphoinositol bisphosphate NPIP-P_2 is shown to be a substrate for human PI-PLCδ_1 with K_M = 1.35 mM V_{max} = 78.5 μmol min^{-1} mg^{-1}. This non-radioactive, continuous assay is both rapid and sensitive, and is conducted in the absence of interface-forming lipids or detergents.

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6. A satisfactory 300 MHz ^{1}H NMR spectrum has been obtained for **2** ($CDCl_3$) δ 0.90–1.10 (m, 24H); 1.11 (s, 9H); 1.20–1.80 (m, 10H); 2.55 (br.s, 1H); 3.24 (t, 1H, J = 9.1 Hz); 3.60–3.80 (m, 2H); 3.82 (m, 1H, $w_{1/2}$ = 11.0 Hz); 3.97 (t, 1H, J = 9.3 Hz); 7.39 (m, 6H, $w_{1/2}$ = 26.2 Hz); 7.86 (d, 4H, J = 8.0 Hz); **3** ($CDCl_3$) δ 0.90–1.08 (m, 24H); 1.11 (s, 9H); 1.20–1.80 (m, 10H); 3.35 (s, 3H); 3.46 (t, 1H, J = 8.3 Hz); 3.71 (m, 2H, $w_{1/2}$ = 28.2 Hz); 3.82 (t, 1H, J = 7.1 Hz); 3.93 (m, 1H, $w_{1/2}$ = 28.3 Hz); 4.04 (dd, 1H, J = 4.2, 8.1 Hz); 4.69 (m, 1H, $w_{1/2}$ = 16.3 Hz); 4.77 (m, 1H, $w_{1/2}$ = 16.0 Hz); 7.41 (m, 6H, $w_{1/2}$ = 24.1 Hz); 7.88 (d, 4H, J = 8.4 Hz); **4** ($CDCl_3$) δ 1.09 (s, 9H); 1.20–1.80 (m, 10H); 2.64 (br.s, 1H, $w_{1/2}$ = 17.6 Hz); 3.10 (t, 1H, J = 9.0 Hz); 3.40 (s, 3H); 3.60–3.75 (m, 3H); 3.89–3.95 (m, 2H); 4.14 (br.s, 1H, $w_{1/2}$ = 7.4 Hz); 4.66 (AB, 2H); 7.30–7.50 (m, 6H); 7.72 (d, 2H, J = 6.6 Hz); 7.77 (d, 2H, J = 6.9 Hz); **5** ($CDCl_3$) δ 1.12 (s, 9H); 1.15–1.85 (m, 10H); 3.17 (s, 3H); 3.6 (d, 3H, J = 11.4 Hz); 3.77 (d, 3H, J = 11.4 Hz); 3.79 (d, 3H, J = 11.1 Hz); 3.81 (d, 3H, J = 11.4 Hz); 4.14 (br.t, 1H, J = 7.6 Hz); 4.25 (m, 2H, $w_{1/2}$ = 8.0 Hz); 4.32 (dt, 1H, J = 2.7, 9.5 Hz); 4.44 (m, 2H, $w_{1/2}$ = 8.0 Hz); 5.12 (m, 1H, $w_{1/2}$ = 22.8 Hz); 7.30–7.45 (m, 6H); 7.75 (d, 2H, J = 7.5 Hz); 7.83 (d, 2H, J = 6.9 Hz); **6** ($CDCl_3$) δ 1.20–1.83 (m, 10H); 3.45 (s, 3H); 3.79 (d, 3H, J = 11.4 Hz); 3.81 (d, 3H, J = 11.1 Hz); 3.82 (d, 3H, J = 11.1 Hz); 3.83 (d, 3H, J = 11.4 Hz); 3.91 (m, 2H, $w_{1/2}$ = 8.2 Hz); 4.21 (br.t, 1H, J = 6.3 Hz); 4.39 (m, 1H, $w_{1/2}$ = 21.8 Hz); 4.50 (m, 1H, $w_{1/2}$ = 1.7 Hz); 4.67 (q, 1H, J = 7.5 Hz); 4.77 (m, 2H, $w_{1/2}$ = 5.4 Hz); **8** ($CDCl_3$), less polar isomer: δ 1.20–1.80 (m, 10H); 3.38 (s, 3H); 3.75 (d, 3H, J = 11.4 Hz); 3.79 (d, 3H, J = 9.9 Hz); 3.83 (d, 6H, J = 11.1 Hz); 3.94 (d, 3H, J = 11.7 Hz); 4.15 (dd, 1H, J = 4.5, 7.2 Hz); 4.34 (t, 1H, J = 6.9 Hz); 4.40–4.50 (m, 1H); 4.60–4.70 (m, 2H); 4.75 (AB, 2H); 4.80–4.87 (m, 1H); 7.42 (d, 2H, J = 8.7 Hz); 8.26 (d, 2H, J = 9.0 Hz); more polar isomer: 1.20–1.80 (m, 10H); 3.42 (s, 3H); 3.80 (d, 3H, J = 11.1 Hz); 3.81 (d, 3H, J = 11.1 Hz); 3.82 (d, 3H, J = 11.1 Hz); 3.83 (d, 3H, J = 11.1 Hz); 3.94 (d, 3H, J = 11.7 Hz); 4.25 (dd, 1H, J = 4.8, 7.2 Hz); 4.34 (t, 1H, J = 6.6 Hz); 4.49 (m, 1H, $w_{1/2}$ = 23.8 Hz); 4.62 (m, 1H, $w_{1/2}$ = 14.4 Hz); 4.79 (AB, 2H); 4.67–4.87 (m, 2H); 7.44 (d, 2H, J = 9.0 Hz); 8.24 (d, 2H, J = 9.0 Hz); **10** (D_2O) δ 1.23 (t, 27H, J = 7.2 Hz); 1.30–1.72 (m, 10H); 3.15 (q, 18H, J = 7.2 Hz); 3.47 (s, 3H); 3.96 (t, 1H, J = 8.7 Hz); 4.15 (q, 1H, J = 9.3 Hz); 4.25–4.39 (m, 2H); 4.56–4.65 (m, 2H); 4.86 (AB, 2H); 7.37 (d, 2H, J = 9.0 Hz); 8.24 (d, 2H, J = 9.0 Hz); **13** (D_2O) δ 3.69 (dd, 1H, J = 2.4, 9.6 Hz); 3.90 (t, 1H, J = 9.3 Hz); 4.00 (q, 1H, J = 9.0 Hz); 4.14 (ddd, 1H, J = 2.7, 7.5, 10.2 Hz); 4.22 (br.s, 1H, $w_{1/2}$ = 8.0 Hz); 4.27 (q, 1H, J = 9.3 Hz); 7.34 (d, 2H, J = 9.0 Hz); 8.21 (d, 2H, J = 9.0 Hz).
7. Satisfactory analytical data (±0.4% of calcd values) were obtained for **2** (C H), mp 114–115 °C (hexane); **3** (C H), mp 111–113 °C (hexane); **5** (C H), mp 138–139 °C (ether-chloroform); **6** (C H), mp 98 °C (ethyl acetate); **13** (C H N), white powder, dec > 120 °C.
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10. ^{31}P (δ, D_2O) -5.89 (d), 0.63 (br.s), 1.35 (br.s). λ_{max} 290 nm (ε, 8,900 M^{-1} cm^{-1}; ε399, background).
11. Human recombinant PI-PLCδ$_1$ was a generous gift from Dr. Mario J. Rebecchi, Health Sciences Center, State University of New York, Stony Brook, NY.
12. Earlier, we described the synthesis of *myo*-inositol 1-(4-nitrophenyl ammonium phosphate), a chromogenic substrate for *Bacillus cereus* PI-PLC and found that the bacterial enzyme showed activity only towards the D-isomer while the L-isomer was neither a substrate nor an inhibitor: Shashidhar, M. S.; Volwerk, J. J.; Griffith, O. H.; Keana, J. F. W. *Chem. Phys. Lipids* **1991**, *60*, 101 and Leigh, A. J.; Volwerk, J. J.; Griffith, O. H.; Keana, J. F. W. *Biochemistry* **1992**, *31*, 8978. We found that mammalian PI-PLCδ$_1$ does not hydrolyze D,L-*myo*-inositol 1-(4-nitrophenyl ammonium phosphate) under the conditions of our assay (0.5 mM substrate). Mammalian PI-PLCδ$_1$ has been shown to specifically bind D-IP_3 at its high affinity PIP_2 binding site on the PH domain (Cifuentes, M. E.; Delaney, T.; Rebecchi, M. J. *J. Biol. Chem.* **1994**, *269*, 1945) and structural evidence indicates the same is true for the catalytic site (Williams, R. L.; Katan, M. *Structure* **1996**, *4*, 1387). Thus the catalytic site shows the same enantioselectivity as that of the bacterial enzyme, in which case the concentration of the D-enantiomer of NPIP-P_2 represents the true substrate concentration.
13. Specific activities of PI-PLCδ$_1$ for the substrate PIP_2 have been reported under conditions similar to those of the assay used here, except that no lipids or detergents are added when using the non-interfacial substrate NPIP-P_2 whereas detergents or diluent lipids are present at the interface together with substrate in the assay with PIP_2. Taking into account this inherent difference between the assays, only approximate comparisons can be made. Specific activities towards PIP_2 were: 62.5 μmol min^{-1} mg^{-1} at 30 °C in 0.26 mM PIP_2, 1% octyl glucoside, 25 mM HEPES (pH 7.2), 100 mM KCl, 1.4 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.03% gelatin (Rebecchi, M.; Peterson, A.; McLaughlin, S. *Biochemistry* **1992**, *31*, 12742). 5.7 μmol min^{-1} mg^{-1} at 30 °C in 0.2 mM PIP_2, 3.8 mM dodecyl maltoside, 20 mM HEPES (pH 7,2), 100 mM NaCl, EGTA/$CaCl_2$ to 10 μM free Ca^{2+}, 5 mM dithiothreitol, 0.1% gelatin (Cifuentes, M. E.; Honkanen, L.; Rebecchi, M. J. *J. Biol. Chem.* **1993**, *268*, 11586). 53 μmol min^{-1} mg^{-1} at 30 °C in 0.5 mM PIP_2, 1% octyl glucoside, 50 mM bis-Tris (pH 7.0), 100 mM KCl, 1 mM free Ca^{2+}, 0.1 mM dithiothreitol (Rebecchi, M. J.; Rosen, O. M. *J. Biol. Chem.* **1987**, *262*, 12526).
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