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SYNTHESIS OF A NEW FLUOROGENIC SUBSTRATE FOR THE CONTINUOUS ASSAY OF MAMMALIAN PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C

Aleksey V. Rukavishnikov,[a] Tatiana O. Zaikova,[a] G. Bruce Birrell,[a,b] John F. W. Keana,[a,*] and O. Hayes Griffith[a,b,*]

[a]*Department of Chemistry and* [b]*Institute of Molecular Biology University of Oregon, Eugene, OR 97403, U.S.A.*

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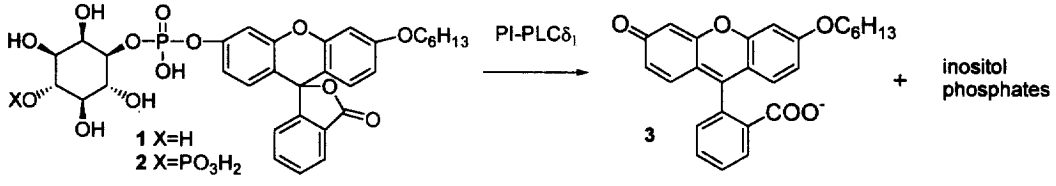
Abstract: The synthesis of a fluorogenic substrate for mammalian phosphoinositide-specific phospholipase C is described. The substrate, based on the widely used fluorescein molecule, is a water-soluble substrate analog of phosphatidylinositol-4-phosphate. The fluorogenic substrate **2** is shown to be a sensitive substrate for human PI-PLC-δ1 in a continuous assay.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide-specific phospholipase C (PI-PLC) is an early event in the regulation of cell functions by numerous extracellular signaling molecules.[1,2] The PI-PLC-catalyzed cleavage of PIP_2 results in the formation of the intracellular second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG and IP_3 mediate the activation of protein kinase C and the release of Ca^{2+} from internal stores, respectively. PIP_2 is the preferred substrate of PI-PLC. However, the less phosphorylated derivatives, phosphatidylinositol and phosphatidylinositol 4-phosphate, are also hydrolyzed by the enzyme, although to a lesser extent.[3]

PI-PLC activity is most commonly measured using radiolabeled phospholipid substrate molecules.[4] Although useful, activity assays based on radiolabeled substrates are tedious and discontinuous, requiring the separation of product and unreacted substrate prior to determination of the activity. The need for continuous assays of PI-PLC activity to provide information on enzyme kinetics has led to a search for substrates capable of producing colored or fluorescent products, whose formation can be monitored continuously. Although two colorimetric assays have been described for continuous assay of mammalian PI-PLC,[5,6] there have not yet been any reports of fluorogenic substrates which are suitable for this purpose. In a previous report,[7] we described the synthesis of a fluorogenic substrate **1** (Scheme 1) derived from fluorescein which was found to be effective for continuous measurement of *bacterial* PI-PLC activity.

Scheme 1



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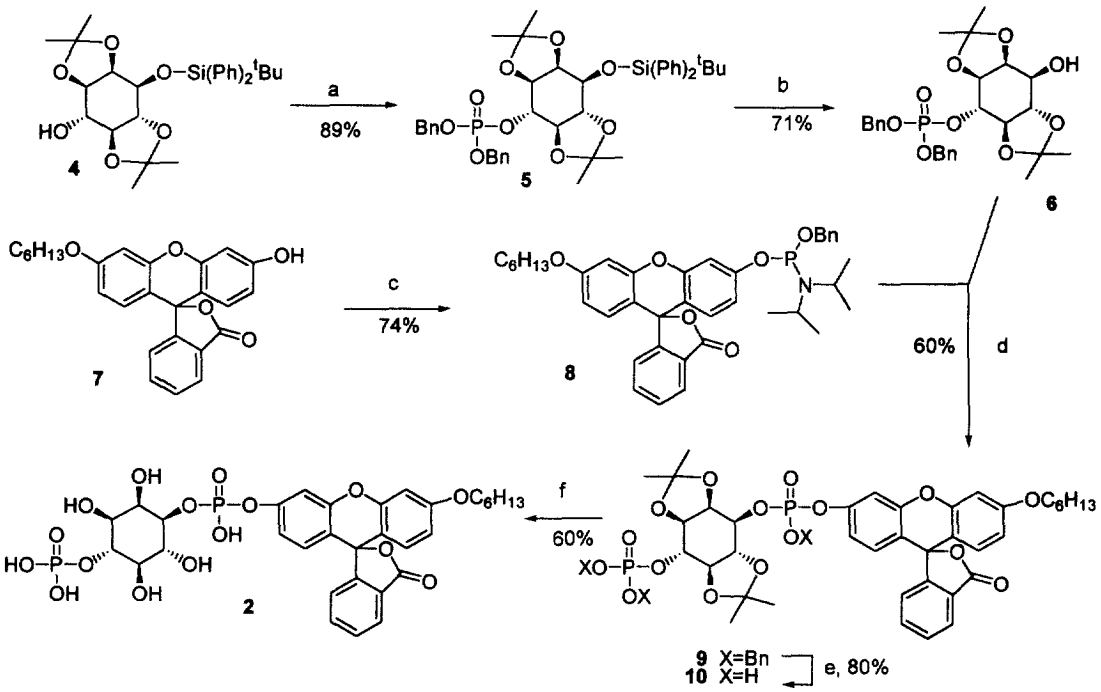
Since the initial report,[7] we have found that **1** is also cleaved, but rather inefficiently, by mammalian PI-PLC-δ1 (see below). Because the mammalian enzymes have a preference for inositol-containing substrates which are phosphorylated, we have undertaken the synthesis of a fluorogenic phosphorylated phosphoinositol substrate. In this communication we describe the synthesis of the new fluorogenic substrate **2**, and demonstrate its cleavage by mammalian PI-PLC-δ1.

Scheme 2



Reagents and conditions: (a) 1.5 equiv of $(BnO)_2PN^iPr_2$, 3 equiv of tetrazole, CH_2Cl_2/CH_3CN, rt, 3 h, then 3 equiv of ^{t}BuOOH (3 M in isooctane), rt, 2 h. (b) 1 equiv of ^{n}Bu$_4$NF (1 M solution in THF), rt, 5 h. (c) 2 equiv of $BnOP(Cl)N^iPr_2$,[8] 2 equiv of ^{i}Pr$_2$NEt, CH_2Cl_2, rt, 30 min. (d) 1 equiv of **6**, 1.5 equiv of **8**, 3 equiv of tetrazole, CH_2Cl_2, rt, 2 h, then 3 equiv of ^{t}BuOOH (3 M in isooctane), rt, 2 h. (e) 20% $Pd(OH)_2$ on carbon, H_2 (1 atm), MeOH, rt, 1 h. (f) MeOH, 24 h.

Racemic silylated inositol derivative **4**[9] was phosphorylated using commercially available dibenzyl diisopropylphosphoramidite followed by oxidation with *tert*-butyl hydroperoxide to provide phosphate **5** (Scheme 2).[10] Desilylation of **5** using 1 M THF solution of tetrabutylammonium fluoride gave alcohol **6**.[10] The coupling of **6** with phosphoramidite **8**, prepared from substituted fluorescein **7**[7] was achieved using tetrazole in dichloromethane solution and the coupled product was oxidized without isolation to produce diphosphate **9**.[10] The complete debenzylation of compound **9** was accomplished using catalyzed hydrogenolysis to give diphosphate **10**; the remaining protective groups underwent self-catalyzed hydrolysis in methanol solution to

give after chromatography (cellulose, elution with 2-propanol/ammonia/water, 70/10/20) the target substrate **2**, pure by TLC and NMR.[10,11] The overall yield of **2** was 18% based on **4**.

Under the assay conditions [25 °C; 25 mM HEPES (pH 7.2), 100 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM dithiothreitol, 0.01% gelatin] substrates **2** and **1**, in the absence of PI-PLC, exhibited a low level of fluorescence, which was stable with time (shown for **1** in Figure 1A: lower curve). Addition of recombinant human PI-PLC-δ1 to an aqueous solution of either substrate resulted in a steady rise in fluorescence due to the formation of fluorescent product **3**. There was a significant difference in the rate of cleavage of the two substrates by PI-PLC-δ1. While **1** was cleaved to some extent by PI-PLC-δ1 (Figure 1A), the hydrolysis of **2** occurred much more rapidly, and required much less enzyme to produce a measurable change in fluorescence (Figure 1B). Under the experimental conditions used for Figure 1B (5 μM substrate and 34 pM enzyme), the rate of hydrolysis of **2** was approximately 15-fold greater than that of **1**. Similar differences were observed for substrate concentrations in the range, 1–20 μM. We conclude that **2** is a sensitive fluorogenic substrate for continuous measurement of mammalian PI-PLC activity.

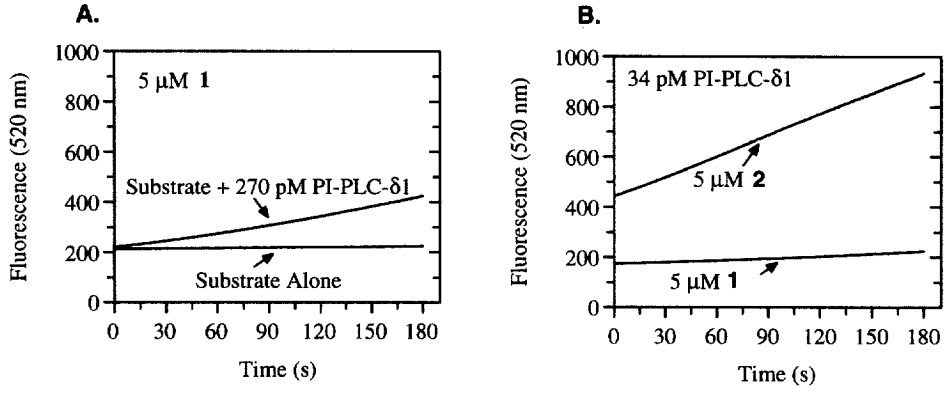


Figure 1. Fluorescence assays of mammalian PI-PLC-δ1 activity using fluorogenic substrates **2** and **1**. (A) Assay of PI-PLC-δ1 activity using 5 μM **1**. The lower and upper curves show the fluorescence behavior in the absence and presence of 270 pM enzyme, respectively. (B) Comparison of assays of PI-PLC-δ1 activity using substrates **2** and **1**. In each case the substrate concentration was 5 μM, and the enzyme concentration was 34 pM. The assays were carried out in a total volume of 0.7 mL in a polymethacrylate cuvette using a Hitachi F-4500 fluorescence spectrometer with excitation at 480 nm and 10 nm slits. The assay buffer consisted of 25 mM HEPES (pH 7.2), 100 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM dithiothreitol, 0.01% gelatin (Sigma, type B, bovine skin, 225 Bloom).

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References and Notes

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8. BnOP(Cl)N^{i}Pr$_2$ was prepared as follows: to a solution of PCl_3 in dry Et_2O a solution of benzyl alcohol in dry Et_2O (1 equiv) was added at –78 °C; stirring was continued for 1 h at –78 °C, after which the vessel was placed under reduced pressure (aspirator) and allowed to warm to rt until the Et_2O had evaporated; then it was held at 60 °C for 30 min. The residue ($BnOPCl_2$) was dissolved in CH_2Cl_2 and slowly treated with 2 equiv of ^{i}Pr$_2$NH at –20 °C. The mixture was stirred for 30 min and allowed to warm to rt over ~90 min, followed by filtration through a celite pad and concentration. The filtrate was suspended in hexane and stored at –6 °C for 8 h. The suspension was filtered through celite and the solvent removed by evaporation.
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10. A satisfactory 300 MHz ^{1}H NMR spectrum has been obtained: **5** ($CDCl_3$) δ 1.05 (s, 9H), 1.24 (s, 3H), 1.35 (s, 3H), 1.42 (s, 3H), 1.52 (s, 3H), 3.26 (dd, J = 8.8, 10.8, 1H), 3.84–3.92 (m, 2H), 3.99 (dd, J = 3.6, 6.4, 1H), 4.08 (t, J = 9.1, 1H), 4.59 (ddd, J = 5.9, 9.3, 11.1, 1H), 5.06 (d, J = 5.4, 4H), 7.20–7.40 (m, 16H), 7.66–7.83 (m, 4H); **6** ($CDCl_3$) δ 1.33 (s, 3H), 1.37 (s, 3H), 1.38 (s, 3H), 1.39 (s, 3H), 2.74 (brs, 1H), 3.92 (t, J = 10.0, 1H), 4.06 (dd, J = 4.1, 9.7, 1H), 4.17 (dd J = 5.6, 7.3, 1H), 4.44 (t, J = 5.0, 1H), 4.64 (ddd, 6.4, 8.8, 10.5, 1H), 5.04–5.15 (m, 4H), 7.22–7.40 (m, 10H); **9** ($CDCl_3$) δ 0.87–0.95 (m, 3H), 1.18–1.57 (m, 18H), 1.73–1.85 (m, 2H), 3.41–3.53 (m, 1H), 3.84–4.03 (m, 2H), 4.05–4.26 (m, 2H), 4.46–4.73 (m, 2H), 4.78–4.90 (m, 1H), 5.07–5.15 (m, 4H), 5.19–5.27 (m, 2H), 6.59–6.75 (m, 5H), 6.81–6.94 (m, 1H), 7.09–7.16 (m, 1H), 7.28–7.42 (m, 15H), 7.59–7.71 (m, 2H), 8.03 (brd, J = 7.3, 1H); **2** (CD_3OD) δ 0.89 (t, J = 7.1, 3H), 1.25–1.53 (m, 6H), 1.68–1.81 (m, 2H), 3.41 (brt, J = 9.7, 1H), 3.54 (dd, J = 2.9, 9.4, 1H), 3.88 (t, J = 9.7, 1H), 3.94 (t, J = 6.7, 2H), 4.06 (brt, J = 9.1, 1H), 4.18 (t, J = 2.3, 1H), 4.24 (q, J = 9.1, 1H), 6.52 (dd, J = 2.3, 8.5, 1H), 6.60 (d, J = 2.9, 1H), 6.77–6.84 (m, 1H), 6.84–6.91 (m, 1H), 7.02–7.11 (m, 3H), 7.12–7.24 (m, 2H), 7.41–7.48 (m, 1H).
11. HR FABMS: calculated for $C_{32}H_{37}O_{16}P_2$, 739.15569; found 739.15535 (M + H); Anal. calcd for $C_{32}H_{36}O_{16}P_2 \bullet 2H_2O \bullet 2NH_3$ (dihydrate of bisammonium salt of **2**): C, 47.53; H, 5.73; N, 3.46. Found: C, 47.69; H, 6.01; N, 4.01.