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Synthesis of a New Fluorogenic Substrate for the Assay of Phosphoinositide-Specific Phospholipase C.

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Abstract: The synthesis of a fluorescein-derived fluorogenic substrate, D,L-*myo*-inositol 1-([6'-hexyloxy-3-oxo]spiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3'-yl hydrogen phosphate) sodium salt **9**, for assay of activity of phosphatidylinositide-specific phospholipase C (PI-PLC), is described. This substrate is shown to be a sensitive substrate for bacterial PI-PLC in a continuous, non-radioactive assay.

Phosphatidylinositide-specific phospholipase C (PI-PLC) excreted by *Bacillus cereus* and the human pathogens *Staphylococcus aureus* and *Listeria monocytogenes* catalyzes the cleavage of phosphatidylinositol to produce diacylglycerol and *myo*-inositol 1,2-cyclic-phosphate, which then is slowly hydrolyzed to *myo*-inositol 1-phosphate.[1] Bacterial PI-PLC is also widely used to release glycosylphosphatidylinositol (GPI) linked proteins from mammalian cells and the variable surface glycoprotein of the parasite *Trypanosoma brucei*.[1] Mammalian PI-PLC isozymes play a key role in signal transduction.[2]

The importance of PI-PLCs has led to a search for chromogenic and fluorogenic substrate analogues[1] which can be used to continuously monitor PI-PLC activity, as an alternative to the widely used radioactive assays, which are discontinuous and slow.[3] Continuous spectrophotometric assays for bacterial[4] and mammalian[5] PI-PLCs have been previously described. However, progress has been slower with the development of fluorogenic substrates. Of the two reported fluorometric assays, one was discontinuous, requiring the separation of substrate and product because of their similar fluorescent properties,[6] and the second, while being a continuous assay, suffered from low enzyme turnover.[7] In this communication, we describe the synthesis of a new fluorogenic PI-PLC substrate **9** (Scheme 1) which overcomes these two difficulties. The new assay is based on fluorescein, one of the most widely used compounds in fluorogenic assays because of its desirable fluorescent properties.[8]

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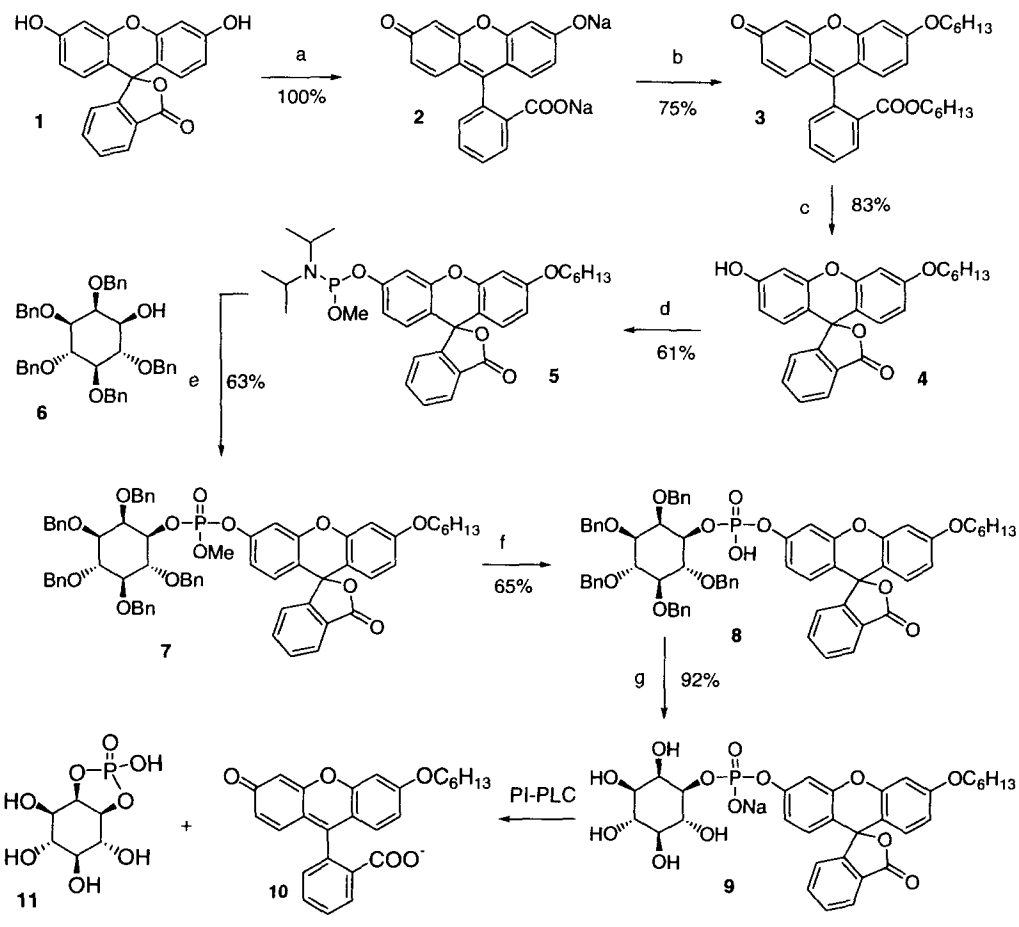
Disubstituted fluorescein compounds such as **9** are particularly attractive as fluorogenic substrates because they exist in the nonfluorescent lactoid form until cleaved by an enzyme. After cleavage, a strong green fluorescence is observed.

The target molecule, phosphate **9** was synthesized from commercially available fluorescein **1** (Scheme 1).

Scheme 1



Reagents and conditions: (a) 2 equiv of NaOH, MeOH, rt, 30 min; (b) 4 equiv of $C_6H_{13}Br$, DMF, 120 °C, 4 h; (c) 4% NaOH, MeOH/H_2O (3:1, v/v), rt, 2 h, then diluted HCl; (d) 1.57 equiv of $^iPr_2NEt$, 1.5 equiv of $MeOPCl N^iPr_2$, CH_2Cl_2, rt, 35 min; chromatography on silica gel with hexane/ether/Et_3N 50:50:1 (v/v); (e) 2 equiv of tetrazole, 1 equiv of **6**, CH_2Cl_2, rt, 2 h, then 3 equiv of ^tBuOOH (3 M in isooctane), rt, 1 h; (f) 1.5 equiv of Me_3SiBr, CH_2Cl_2, rt, 3 h, then acetone/water, 5:1 (v/v), rt, 30 min; (g) 20% $Pd(OH)_2$ on carbon, 50 psi H_2, MeOH/CH_2Cl_2 (25:1), rt, 4 h, then Amberlite CG-120 (Na^+ form).

Treatment of fluorescein disodium salt **2**, prepared from fluorescein as described by Kasai et al.[9] with hexyl bromide in DMF gave ester-ether **3** which was hydrolyzed to provide hexylsubstituted fluorescein **4**.[10] Reaction of **4** with $MeOPClN^{i}Pr_2$ yielded phosphoramidite **5** which was then coupled[11] with pentabenzyl protected inositol **6**.[12] The resulting phosphite triester was oxidized with *tert*-butyl hydroperoxide to give phosphate triester **7**. Pentabenzylprotected inositol **6** was prepared in 7 steps from *myo*-inositol according to the published procedure.[13] Phosphate **7**[14] was deprotected using Me_3SiBr followed by hydrolysis in aqueous acetone[15] to form phosphate diester **8**.[16] The OH group of phosphate **8** provides acidity that is essential for easy and complete removal of benzyl protective groups from inositol backbone. So, hydrogenolysis of compound **8** in methanol was completed in a short period of time (4 h) without any acids and inorganic buffers that reduces decomposition of phosphate **9** and gave, after treatment with Amberlite CG-120, deprotected compound **9**[17,18] as a sodium salt in the form of a yellowish non-hygroscopic powder.

The total yield of the target phosphate **9** was 14% on 7 steps starting from fluorescein, which is sufficient to produce the quantities necessary for biochemical experiments. The scheme is versatile: the hydrophobicity of the fluorgenic reagent may be varied by substituting other alkylhalides for the hexylbromide in Scheme 1.

The PI-PLC activity toward substrate **9** in aqueous buffer was tested with enzyme from *Bacillus cereus* (Figure 1). The substrate was readily cleaved by *B. cereus* PI-PLC, producing **10** (the open ring form of **4**) which has an easily monitored emission at 520 nm, and known compound *myo*-inositol 1,2-cyclic phosphate, **11**. The specific activity of *B. cereus* PI-PLC toward 50 μM racemic **9** was 780 μmol min^{-1} mg^{-1} at 22°C. This represents more than a ten thousand-fold improvement over the previously reported continuous fluorogenic assay.[7]

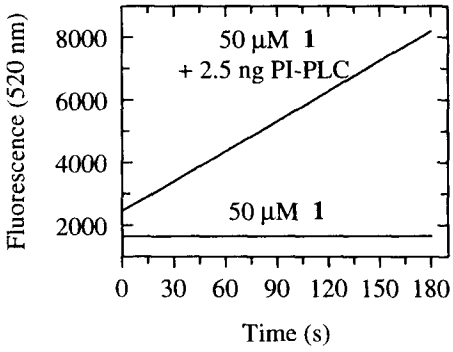


Figure 1. Fluorescence assay of PI-PLC activity using substrate **1** (upper curve). The assay was carried out in a total volume of 0.7 ml in a polymethacrylate cuvette using a Hitachi F-4500 fluorescence spectrometer with excitation at 476 nm and 10 nm slits. In the absence of enzyme, there is a low level of fluorescence which is constant with time (lower curve). The assay was carried out in 100 mM HEPES (pH 7.0), 1 mM EDTA, 0.008% PEG-8000. Substrate concentration was 50 μM. Fluorescence is in arbitrary units, and the lines are direct instrument output.

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10. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.91 (t, 3H, J = 6.6 Hz); 1.32 (m, 6H); 1.77 (m, 2H); 3.96 (t, 2H, J = 6.7 Hz); 6.68-6.50 (m, 4H); 6.76-6.72 (m, 2H); 7.16 (d, 1H, J = 7.2 Hz); 7.66 (m, 2H); 8.02 (d, 1H, J = 6.9 Hz).
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14. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.92 (t, 3 × 4H, J = 6.6 Hz); 1.23-1.60 (m, 6 × 4H); 1.68-1.85 (m, 2 × 4H); 3.48-3.56 (m, 2 × 4H); 3.70 (d, 3 × 2H, J = 11.4 Hz); 3.74 (d, 3H, J = 11.7 Hz); 3.77 (d, 3H, J = 11.4 Hz); 3.95-4.15 (m, 5 × 4H); 4.26 (brs, 1 × 2H); 4.30-4.34 (m, 1 × 2H); 4.35-4.45 (m, 1 × 4H); 4.65-5.00 (m, 10 × 4H); 6.45-7.10 (m, 7 × 4H); 7.10-7.43 (m, 25 × 4H); 7.50-7.70 (m, 2 × 4H); 8.02-8.05 (m, 1 × 4H) - 4 diastereomers.
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16. ^{1}H NMR ($CDCl_3$-CD_3OD 1:4, 200 MHz) δ 0.89 (t, 3 × 2H, J = 6.9 Hz); 1.20-1.45 (m, 6 × 4H); 1.65-1.80 (m, 2 × 2H); 3.41 (brt, 1 × 2H, J = 9.0 Hz); 3.49 (brd, 1 × 2H, J = 9.9 Hz); 3.90 (t, 2 × 2H, J = 6.6 Hz); 3.96 (t, 1 × 2H, J = 9.9 Hz); 4.02 (t, 1 × 2H, J = 9.6 Hz); 4.20-4.38 (m, 1 × 2H); 4.56 (brs, 1 × 2H); 4.63-5.00 (m, 10 × 2H); 6.40-7.00 (m, 7 × 2H); 7.00-7.40 (m, 25 × 2H); 7.40-7.65 (m, 2 × 2H); 7.96-8.00 (m, 1 × 2H) - 2 diastereomers.
17. ^{1}H NMR (CD_3OD-D_2O, 200 MHz) δ 0.92 (t, 3 × 2H, J = 6.3 Hz); 1.25-1.60 (m, 6 × 2H); 1.65-1.80 (m, 2 × 2H); 3.42 (brt, 1 × 2H, J = 8.4 Hz); 3.38 (brd, 1 × 2H, J = 9.3 Hz); 3.64 (brt, 1 × 2H, J = 9.6 Hz); 3.83 (brt, 1 × 2H, J = 9.6 Hz); 3.92 (t, 2 × 2H, J = 6.3 Hz); 4.02-4.13 (m, 1 × 2H); 4.17 (brs, 1 × 2H); 6.35 (s, 1 × 2H); 6.52 (dd, 1 × 2H, J = 2.4, 8.7 Hz); 6.61 (s, 1 × 2H); 6.80-7.00 (m, 3 × 2H); 7.09 (s, 1 × 2H); 7.18 (t, 1 × 2H, J = 6.9 Hz); 7.28 (t, 1 × 2H, J = 6.9 Hz); 7.82 (d, 1 × 2H, J = 6.9 Hz) - 2 diastereomers.
18. Satisfactory analytical data (C, H, ±0.4% of calcd values) was obtained for **9**.