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Fluorescent Phosphatidylinositol 4,5-Bisphosphate Derivatives with Modified 6-Hydroxy Group as Novel Substrates for Phospholipase C

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Abstract

The capacity to monitor spatiotemporal activity of phospholipase C (PLC) isozymes with a PLC-selective sensor would dramatically enhance understanding of the physiological function and disease relevance of these signaling proteins. Previous structural and biochemical studies defined critical roles for several of the functional groups of the endogenous substrate of PLC isozymes, phosphatidylinositol 4,5-bisphosphate (PIP_2), indicating that these sites cannot be readily modified without compromising interactions with the lipase active site. However, the role of the 6-hydroxy group of PIP_2 for interaction and hydrolysis by PLC has not been explored, possibly due to challenges in synthesizing 6-hydroxy derivatives. Here, we describe an efficient route for the synthesis of novel, fluorescent PIP_2 derivatives modified at the 6-hydroxy group. Two of these derivatives were used in assays of PLC activity in which the fluorescent PIP_2 substrates were separated from their diacylglycerol products and reaction rates quantified by fluorescence. Both PIP_2 analogues effectively function as substrates of PLC-δ1, and the K_M and V_{max} values obtained with one of these are similar to those observed with native PIP_2 substrate. These results indicate that the 6-hydroxy group can be modified to develop functional substrates for PLC isozymes, thereby serving as the foundation for further development of PLC-selective sensors.

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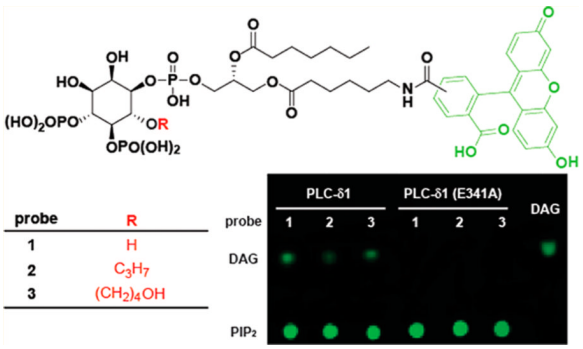
ASSOCIATED CONTENT

Supporting Information

Experimental procedures and NMR spectra of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Notes

The authors declare no competing financial interest.



The phospholipase C (PLC) family of enzymes catalyze the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3).[1] Diacylglycerol activates protein kinase C (PKC) while IP_3 activates calcium channels leading to the release of Ca^{2+} from endothelial reticulum to cytosol.[2] In addition, PLC action also regulates local PIP_2 pools leading to changes in subcellular localization and/or function of a broad range of PIP_2-interacting proteins, including numerous ion channels. Consequently, PLCs are essential signaling proteins that regulate diverse cellular processes, including proliferation[3] and differentiation,[4] vasculogenesis,[5] and fertilization.[6] Aberrant regulation of PLCs has been implicated in diseases including cancer,[7–13] heart diseases,[14] and neuropathic pain.[15]

The 13 mammalian PLC isozymes are broadly grouped into six families (−β, −γ, −δ, −ε, −ζ, −η) based on their sequence homologies and functions. Various extracellular stimuli activate different PLC isoforms through distinct mechanisms. For example, agonists of G-protein coupled receptors (GPCRs) activate PLC-β isozymes through Gαq- and Gβγ-subunits of heterotrimeric G proteins, while PLC-γ activity is enhanced through phosphorylation promoted by receptor and non-receptor tyrosine kinases. In addition, PLC-ε and certain members of the PLC-β and PLC-γ subclasses of isozymes are activated by Ras,[1] Rho,[17] and Rac subfamilies of small GTPases.[18] However, when, where, and how various PLC isoforms are activated under different extracellular stimuli are still not well understood. This is partly due to the lack of methods to monitor the spatiotemporal activities of cellular PLCs.

Standard, radioisotope-based assays are discontinuous and cannot be used to monitor the real-time dynamics of PLC activity in cells. Cell permeable dyes that increase in fluorescence upon binding calcium are also routinely used as complementary methods to monitor PLC activity. However, although such assays are simple and throughput is high, they are not a direct measure of PLC activity and often generate confounding data since other factors also contribute to intracellular calcium concentration.

With the long-term goal of spatiotemporal monitoring of inositol lipid signaling, we initiated studies to develop small molecule sensors of PLCs. Previous kinetic studies demonstrated that the 2-hydroxy (2-OH) in PIP_2 is essential[19–21] for its hydrolysis by PLCs and that 3-phosphoinositides are not PLC substrates.[22] Similarly, removal of the phosphate group from the 4- or 5-OH positions resulted in derivatives that are poor substrates for PLCs. Furthermore, the stereochemistry of the D-*myo*-insoitol headgroup, but not the diacylglycerol side chain, was shown to be critical for effective PLC-mediated catalysis.[23] Finally, alkyl substitutions at the *sn*-1 and *sn*-2 positions resulted in PIP_2 molecules that still function as PLC substrates.[24] In general, the longer the side chain, the more efficient the corresponding PIP_2 derivative was for PLC-promoted hydrolysis.[25] These results are supported by the crystal structure[26,27] of the catalytic domain of PLC-δ1 in complex with IP_3; the 1-, 4-, and

5-phosphates and the 2- and 3-hydroxyls of IP_3 interact with PLC-δ1, and mutation of these interacting residues greatly reduced the capacity of PLC-δ1 to hydrolyze PIP_2.[28] Consequently, modifications of PIP_2 to develop new PLC substrates have avoided targeting the inositol headgroup and have instead focused on the diacylglycerol side chain. For example, we[29] and others[30–33] have developed fluorogenic PLC reporters with modifications at the 1-phosphate to monitor PLC activity *in vitro*. However, these systems are unlikely to be optimal for studies in live cells since potential spatial information on PLC activity will be lost upon cleavage of the fluorophore from the PIP_2 derivative.

To our knowledge, selective modification of the 6-OH has not been explored despite extensive previous efforts synthesizing PIP_2 analogues for novel functions and assays. The structure of PLC-δ1 bound to IP_3 highlights a lack of direct interaction between the lipase active site and the 6-OH group, indicating that the 6-OH is solvent exposed and its modification in PIP_2 is likely tolerated within the active site of PLCs. Consequently, we designed and synthesized three PIP_2 derivatives (Figure 1) and investigated their capacity to be hydrolyzed by PLC-δ1. Probe **1** has a free 6-OH identical to endogenous PIP_2 while probes **2** and **3** have modified 6-OH groups, with a propyl modification in **2** and an extended hydroxy in **3**. The incorporation of a fluorescent group at the *sn*-1 position of the DAG side chain provides a sensitive detection of both the substrate and the product by fluorescence, thus avoiding the use of radioactive materials and providing a potential avenue for the use of realtime monitoring of phospholipase activity in cells with high spatiotemporal resolution and high throughput.

EXPERIMENTAL PROCEDURES

Kinetic Studies of Probes 1–3

Enzymatic Reactions—The fluorescent PIP_2 derivatives were dissolved in water to make working solutions at 432, 324, 216, 108, 54, 32, 24, or 12 μM. To these solutions (20 μL) were added a 6X buffer (5 μL) that contains HEPES (300 mM, pH 7.2), KCl (420 mM), $CaCl_2$ (17.8 mM), EGTA (18 mM), DTT (12 mM), and 3% cholate. The reaction was initiated by adding a solution (5 μL) that contains purified, full-length PLC-δ1 (0.05 or 0.1 ng/μL, final concentration) and BSA (1 mg/mL), and the reaction mixtures were incubated at 25 °C. At indicated time points, samples were taken out of the reaction mixtures with a multichannel pipet (1 μL) and spotted on TLC plates (Merck, Silica Gel-60). The TLC plates were then developed with $CHCl_3$:MeOH:H_2O (100:20:1) and scanned with a Typhoon 9400 Variable Mode Imager ($\lambda_{ex}/\lambda_{em}$ = 488 nm/520 nm). The fluorescence of DAG and PIP_2 derivatives on the TLC plate was quantified with ImageQuant software (V.5.0).

Fluorescence Calibration and Reaction Rate Calculation—To a set of water solutions (20 μL) containing various concentrations (432, 324, 216, 108, 54, 32, 24, and 12 μM) of PIP_2 derivative **1** was added the 6X buffer solution (5 μL) as defined previously and a PLC solution (5 μL) containing purified, full-length PLC-δ1 (10 ng/μL, final concentration) and BSA (1 mg/mL). The reaction mixtures were incubated at 25 °C for 2 h to completely convert probe **1** to DAG. As a control, the reactions were also carried out in the presence of BSA (PLC free). These two sets of reaction mixtures were spotted on TLC plates which were subsequently developed and the fluorescence was quantified. The fluorescence was plotted against concentration for probe **1** or DAG. The slope of the plot for probe **1** was 0.66-fold of that for DAG. Consequently, the amount of DAG that was generated from the reaction was calculated with the formula

$$\mathrm{DAG(pmol)} = \mathrm{initial\,PIP_2\,concentration}(\mu\mathrm{M}) \times \mathrm{volume}(\mu\mathrm{L}) \times (0.66 \times a)/(0.66 \times a + b)$$

where *a* = fluorescence of DAG and *b* = fluorescence of PIP_2.

Kinetic Studies of Endogenous PIP_2

A mixture of PIP_2 (Avanti Polar Lipids) and 30 000 cpm of [^{3}H]PIP_2 (Perkin-Elmer) was dried under a stream of nitrogen and resuspended in assay buffer that contains HEPES (50 mM, pH 7.2), KCl (70 mM), EGTA (3 mM), DTT (2 mM), $CaCl_2$ (2.96 mM), 0.5% cholate, and BSA (0.2 mg/mL). The resulting lipid stock was diluted to obtain final assay conditions with either 216, 144, 72, 36, 24, 16, 8, or 4 μM PIP_2 in assay buffer (50 μL, final volume). Assays were initiated by the addition of purified, full-length PLC-δ1 (0.075 ng) in assay buffer (10 μL). After incubation at 25 °C for 10 min, reactions were stopped by the addition of 10% (v/v) trichloroacetic acid (200 μL) and 10 mg/mL BSA (100 μL) to precipitate proteins and uncleaved lipids. Centrifugation of the reaction mixture isolated soluble [^{3}H]IP_3, which was quantified using liquid scintillation counting.

RESULTS AND DISCUSSION

1. Synthesis of the Fluorescent Probes

Fluorescent PIP_2 derivatives with free 6-OH such as 1 have been used to image cellular PIP_2 localization[34] and as probes for metabolic enzymes such as phosphoinositide 3-kinase (PI3K).[35] Consequently, the synthesis of **1** follows the literature procedure.[36–38] In contrast, PIP_2 analogues with 6-OH selectively modified have not been reported and their syntheses are challenging. To synthesize the probe **2** (Scheme 1) with 6-OH group protected as its propyl ether, we started with the known inositol derivative **4**. Alkylation of **4** with allyl bromide followed by deacetalization with trifluoroacetic acid (TFA) generated **5** in 74% yield. The diol in **5** was subsequently protected as the corresponding methoxymethyl (MOM) ethers. Next, the corresponding methoxymethyl (MOM) ethers. Next, the tetraisopropyldisiloxane (TIPDS) group was removed by treating with hydrofluoride (HF) in $CHCl_3/CH_3CN$. The resulting hydroxy groups were phosphorylated to form **7** in a two-step sequence: first reacted with dibenzyl diisopropylphosphoramidite in the presence of tetrazole and then oxidized by 3-chloroperoxybenzoic acid (*m*CPBA). The *tert*-butyldiphenylsilyl (TBDPS) protection was removed by tetrabutylammonium fluoride (TBAF) to form alcohol **8**, which was coupled to the diacylglycerol side chain **9** to form **10** in 90% yield through phosphorylation followed by oxidation with *tert*-butyl peroxide (*t*-BuOOH). To remove the MOM protective groups, compound **10** was treated with trimethylsilyl bromide (TMSBr) in CH_2Cl_2 followed by methanolysis. The benzyl protective groups were also partially removed during this process. Hydrogenolysis completed the deprotection of both benzyl and carbobenzyloxy (Cbz) groups and reduced the allyl to propyl group to form **11**. Finally, the terminal amine was acylated with activated fluorescein **12** to generate the fluorescent PIP_2 derivative **2** with 6-OH capped as a propyl ether. The overall yield of the synthesis was 37%.

To synthesize the probe **3** (Scheme 2), olefin metathesis of **6** with MOM-protected allylic alcohol was carried out. We did not attempt to quantify the *E/Z* ratio although the formed double bond had predominantly the *E* configuration as judged by NMR. Instead, the olefin was directly subjected to hydrogenation to form **13**. In a sequence that is analogous to the synthesis of probe **2**, the diol **13** was used to generate probe **3**. The overall yield of this sequence was 15%.

2. Thin-Layer Chromatography Coupled with Fluorescence Scanning as a New Assay for PLC Activity

The classical in-vitro assay of PLC activity requires the use of radioactive PIP_2, particularly tritium-labeled PIP_2, as the PLC substrate. To avoid the use of radioactive PIP_2, ^{31}P NMR

has been used to monitor PLC-catalyzed reactions.[39] However, the concentration of PIP_2 has to be high (>0.5 mM) to obtain accurate measurement, and these techniques are not amenable to large numbers of samples. Existing fluorogenic [31,32] and luminescence-based[33] reporters have the advantage of continuous monitoring of PLC activity with high sensitivity. However, these reporters typically replace the diacylglycerol unit connected to the 1-phosphate in PIP_2, thereby limiting their utility to faithfully recapitulate intracellular events. In contrast, 6-OH analogues provide unexplored potential to produce useful biosensors of PLC activity in cells.

The fluorescent PIP_2 derivative **1** has key structural features of endogenous PIP_2 including an identical inositol phosphate headgroup and a diacylglycerol side chain. Derivative **1** and its expected PLC-dependent hydrolysis products (Figure 2A) are predicted to be readily separable by either TLC or column chromatography, and subsequent detection by fluorescence should provide a new assay for PLC activity. To validate this format, purified PLC-δ1 was used to hydrolyze probe **1**. Based on sequence and structural similarities, the catalytic domain of PLC-δ1 is representative of the entire family of PLC isozymes. The enzymology of PLC-δ1 has also been extensively studied[2026,28,40–42], benefits from the unique structure of PLC-δ1 bound to IP_3 within its active site. The purified enzyme was incubated with probe **1** at room temperature for **10** min, and the mixture was separated on TLC and detected by fluorescence. Fluorescence at the origin of application represents probe **1** and a second fluorescent component appeared as a function of increasing amounts of time during the initial incubation (Figure 2B). This new component was confirmed by LC-MS analysis to be the diacylglycerol derivatives predicted to be generated upon hydrolysis of probe 1 by PLC-δ1. When probe **1** was incubated with catalytically inactive PLC-δ1 (E341A),[28] no new fluorescent components were formed (Figure 2B), indicating that the hydrolysis of **1** is dependent on the lipase activity of PLC-δ1.

Conditions were established to quantify the fluorescent DAG derivative formed during the reaction. Briefly, the linear plots of the fluorescence versus concentration for both **1** and the corresponding DAG product are shown in Figure 2C. The ratio of the slopes was used as the coefficient to normalize fluorescence readings for quantifications. Accordingly, 8% of probe **1** was converted to product in Figure 2B. Under identical conditions, 3% and 5% of probes **2** and **3** were converted to product, respectively. Like probe **1**, probes **2** and **3** were not cleaved by catalytically inactive PLC-δ1 (E341A) suggesting that both are selective substrates for PLC-δ1.

3. Kinetic Studies of Probes 1–3 with PLC-δ1

As highlighted in Figure 2B, probes **1–3** were cleaved by PLC-δ1 with different efficiencies. To further define these probes as PLC substrates for future development of PLC sensors, we carried out detailed kinetic studies to measure K_M and V_{max} of these probes in PLC-catalyzed reactions. For comparison, enzyme kinetics using PIP_2 as the substrate were quantified as previously described[43] by varying the bulk concentration of [^{3}H]PIP_2-containing vesicles.

To increase the efficiency and accuracy of the kinetic measurements, we carried out the experiments with probes **1–3** in a 96-well plate (Figure S1). At the indicated time, the samples were taken from reaction mixtures with a multichannel pipet and loaded directly onto the TLC plates. The fluorescent components in the reaction mixture were then separated and quantified by fluorescence. All the measurements were under initial rate conditions (Figures S2). K_M and V_{max} were then calculated by fitting the data to the Michaelis—Menten equation (Figure 3A,c,). The K_M for probe **1** was 39 ± 4.0 μM with a V_{max} of **94** ± 0.9 pmol/(ng min) while the K_M for probe **2** was 67 ± 9.6 μM with a V_{max} of

32 ± 1.8 pmol/(ng min). Capping of the 6-OH with a propyl group decreased V_{max} and increased K_M, suggesting that the modification at 6-OH might interfere with the key interactions of other substitutions in the inositol headgroup with PLC-δ1. In addition, the propyl group may increase the steric hindrance around the 1-phosphate, making the PLC-catalyzed nucleophilic addition of the 2-OH to the 1-phosphate a slower process. Interestingly, relative to probe **2**, probe **3** further increased K_M (128 ± 15.3 μM) while having a less detrimental effect on V_{max} (67 ± 9.6 pmol/(ng min)), suggesting that increasing the length of the substitution at the 6-OH disrupts interactions with PLC-δ1 while the terminal hydroxy of the substitution may participate in substrate-assisted catalysis to facilitate phospholipase activity. In both cases, the K_M and V_{max} of **2** and **3** are within a 3-fold range of equivalent values for probe **1**, which are similar to those for the endogenous PIP_2 (Figure 3B,C). Consequently, modifying the 6-OH generate functional PIP_2 derivatives that potentially can be applied to monitor PLC activity spatiotemporally.

CONCLUSION

In conclusion, we have developed an efficient synthesis to prepare PIP_2 derivatives modified at the 6-hydroxy group, making it possible to explore the role of the 6-OH in PIP_2 for PLC-catalyzed hydrolysis. Several of these derivatives have been characterized in a new TLC-based assay that features straightforward separation of products from the reaction mixture and sensitive detection for their capacity as substrates of PLC-δ1. These modifications at the 6-OH group result in derivatives that are excellent PLC substrates with marginally lower V_{max} and higher K_M values compared to the unmodified parent PIP_2 derivative and endogenous PIP_2. These results demonstrate that the 6-OH group can be modified for new functions, setting the stage for further development of sensors selective for PLC isozymes and useful for monitoring the spatiotemporal dynamics of these enzymes in living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PLC	phospholipase C
PIP_2	phosphatidylinositol 4,5-bi-sphosphate
IP_3	inositol 1,4,5-trisphosphate
DAG	diacylgly-cerol

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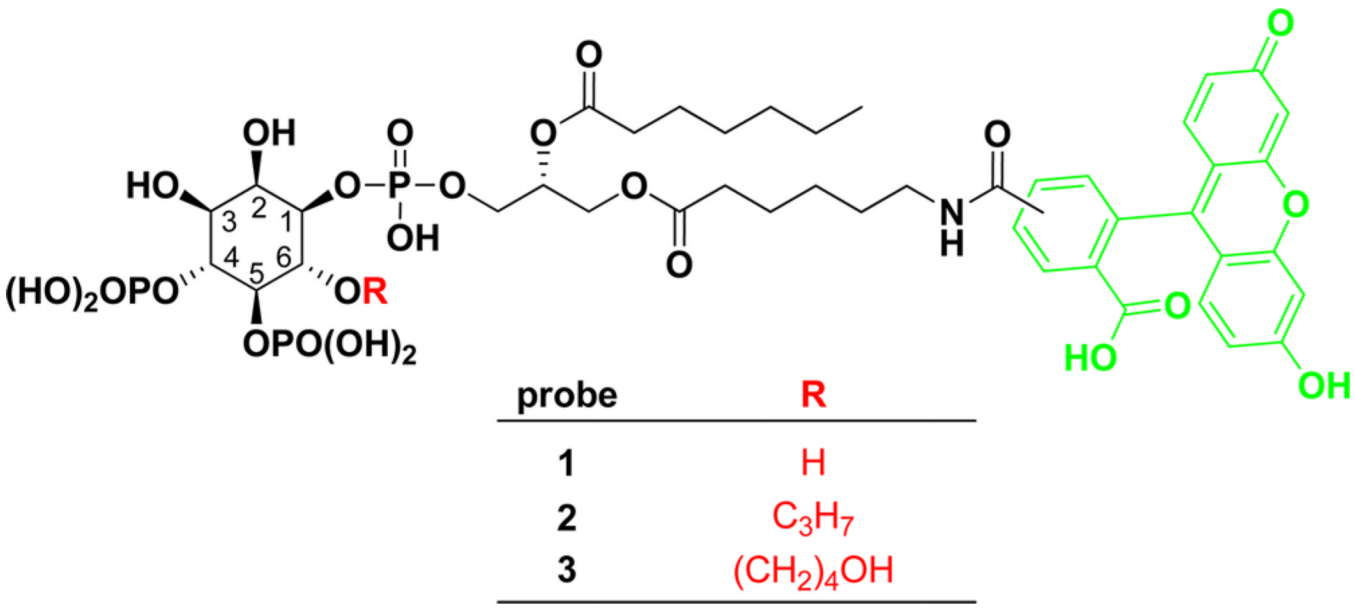


probe	R
1	H
2	C_3H_7
3	$(CH_2)_4OH$

Figure 1.
Chemical structures of the PIP_2 derivatives **1–3** as PLC substrates.

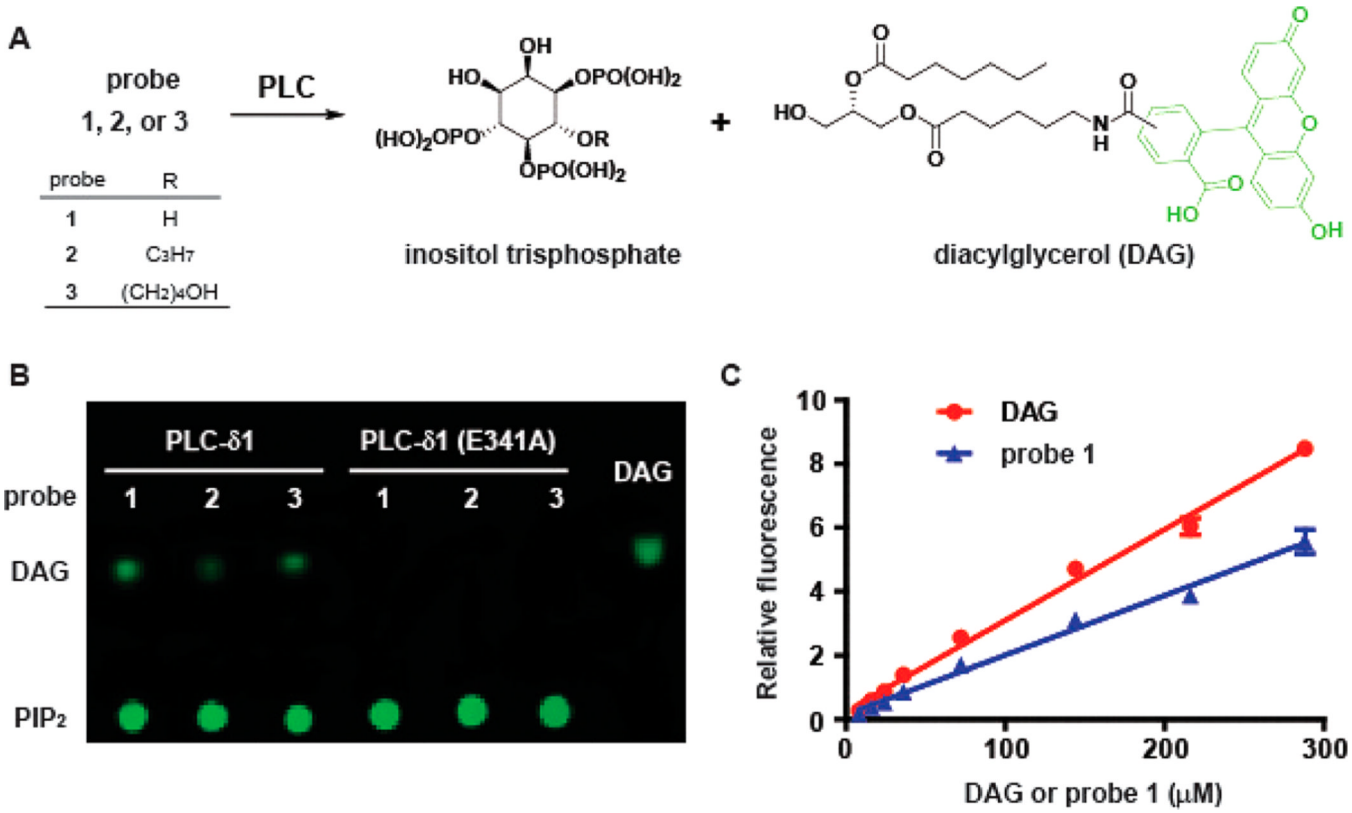
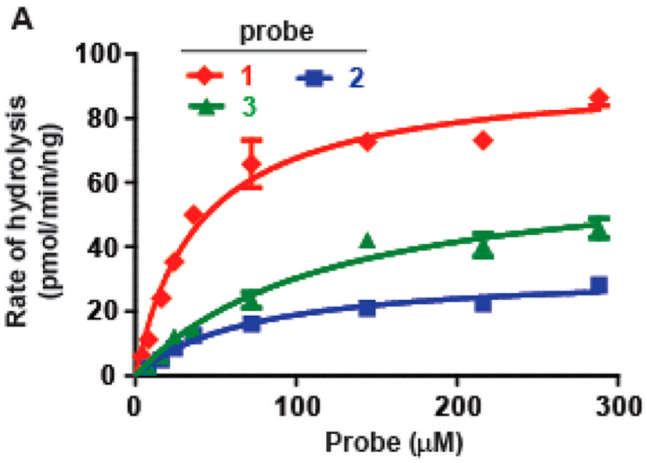
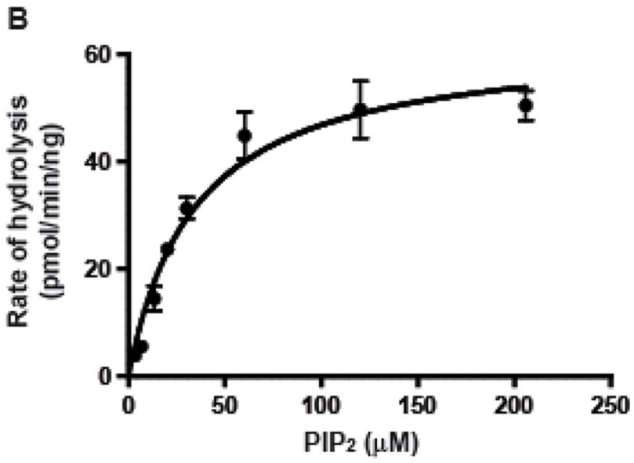


Figure 2.

Validation of the TLC-fluorescence assay. (A) Probes **1–3** are cleaved by PLC-δ1 to form inositol trisphosphate and diacylglycerol (DAG) derivatives. (B) The separation of PIP_2 derivatives **1–3** and their corresponding DAGs on thin-layer chromatography (TLC). The reaction mixture (1 μL) was spotted on the TLC plate and separated by $CHCl_3$:MeOH:H_2O (100:20:1). The fluorescent compounds were detected by a Typhoon 9400 variable mode imager ($\lambda_{ex}/\lambda_{em}$ = 488 nm/520 nm). (C) Plot of fluorescence versus concentration of DAG or probe **1**.



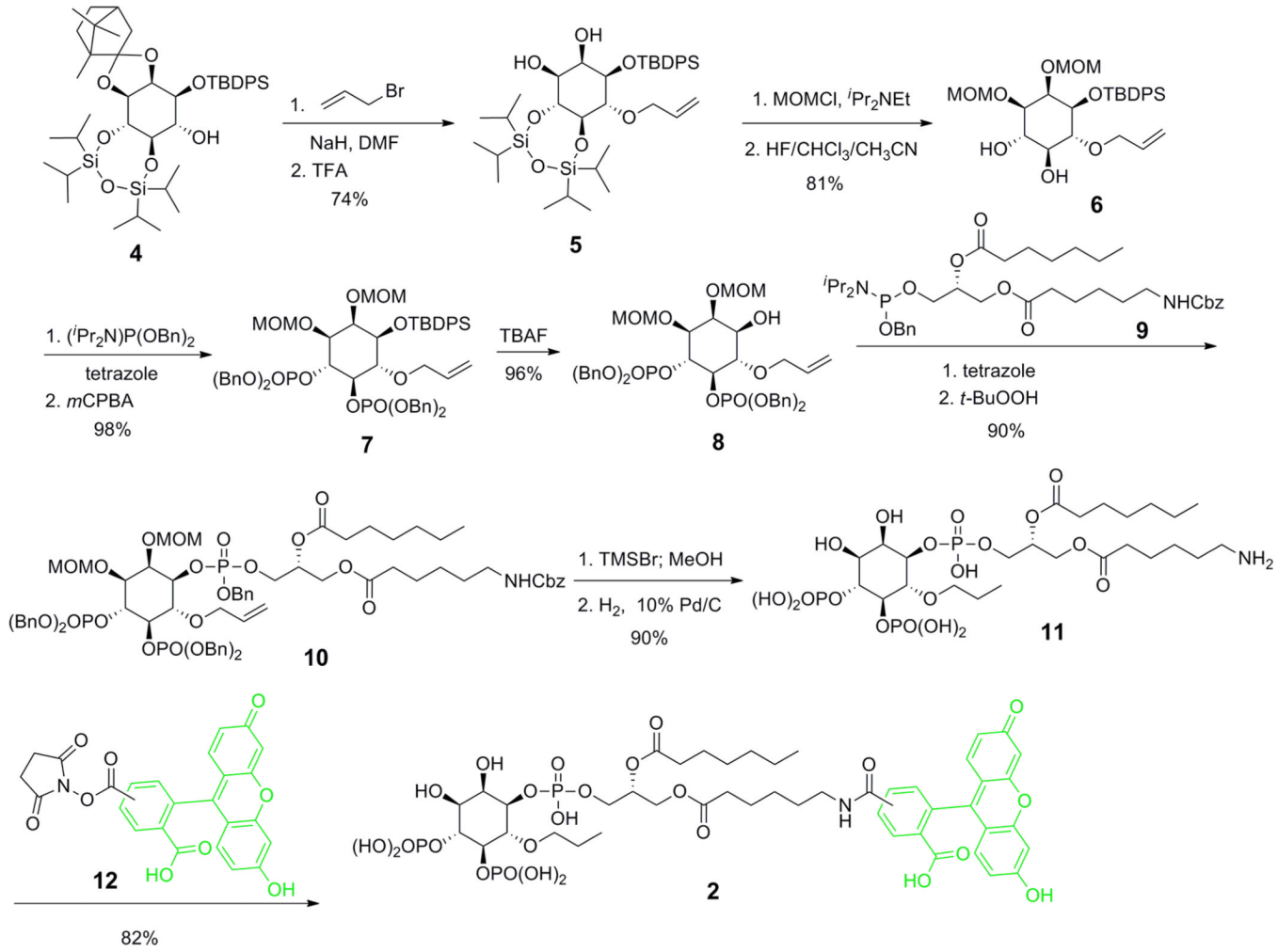


C

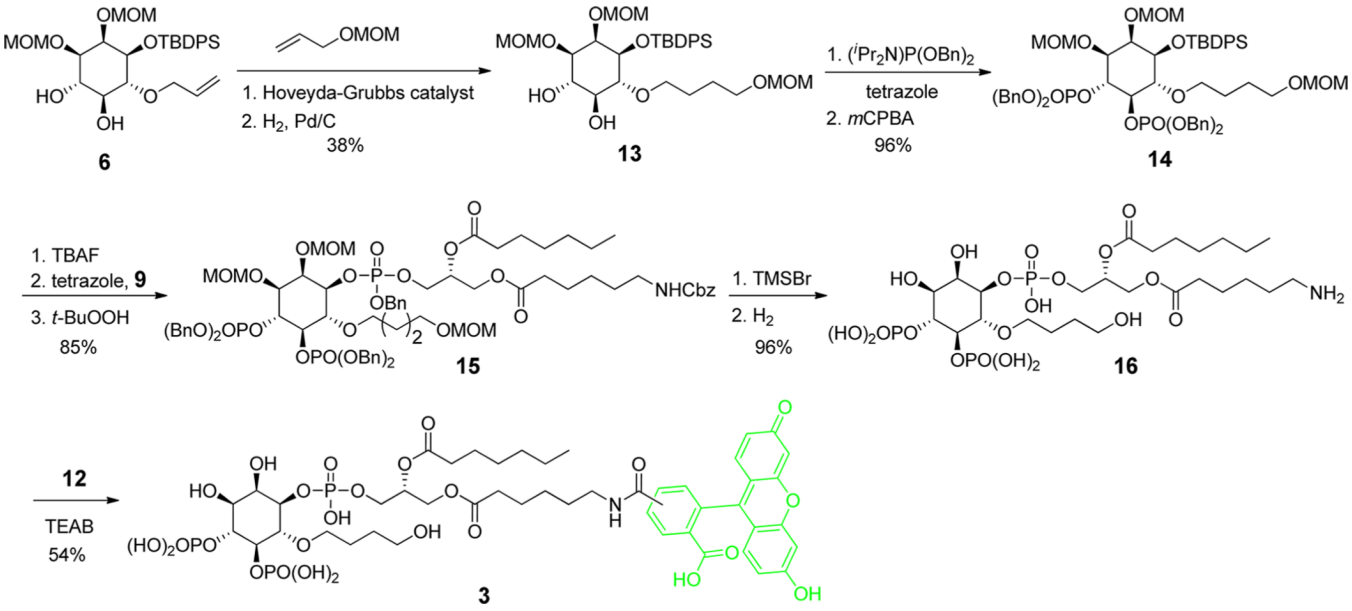
	Probe 1	Probe 2	Probe 3	PIP_2
K_M (μM)	39 ± 4.0	67 ± 9.6	128 ± 15.3	40 ± 4.7
V_{max} (pmol/ng/min)	94 ± 0.9	32 ± 1.8	67 ± 9.6	60 ± 3.8

Figure 3.

Kinetic studies of probes **1–3** or endogenous PIP_2 with PLC-δ1. (A) The initial velocities of hydrolysis of **1**, **2**, or **3** at various concentrations were fitted to the Michaelis—Menten equation. (B) Hydrolysis rates of PIP_2 at various concentrations were fitted to the Michaelis —Menten equation. (C) K_M and V_{max} of probes **1–3** and endogenous PIP_2 with PLC-δ1.



Scheme 1.
Synthesis of Probe 2



Scheme 2.
Synthesis of Probe 3