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Synthesis of Hybrid Lipid Probes: Derivatives of Phosphatidylethanolamine-Extended Phosphatidylinositol 4,5-Bisphosphate (Pea-PIP_2)

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The total asymmetric synthesis of a novel hybrid lipid possessing a 2,3-diacylthreitol backbone, rather than a 1,2-diacylglycerol backbone, is described. The title compound, Pea-PIP_2, possesses a phosphatidylethanolamine (PE) headgroup at the 1-position and a phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) headgroup at the 4-position. Reporters (biotin, fluorophores, spin label) were covalently attached to the free amino group of the PE, such that these reporters were targeted to the lipid−water interface. The diacyl moieties allow incorporation of Pea-PIP_2 into a lipid bilayer, while the PtdIns(4,5)P_2 moiety in the aqueous layer was specifically recognized by PtdIns(4,5)P_2-specific binding proteins.

Introduction

Phosphoinositides (PtdInsP_ns) are biosynthesized by the interplay of kinases[1] and phosphatases.[2] These charged lipids are minor components of cellular membranes but are vital as second messengers for diverse cellular functions.[3,4] PtdInsP_ns are essential elements in tyrosine kinase growth factor receptor and G-protein receptor signaling pathways.[5−7] Furthermore, these lipid signals have important roles in membrane trafficking,[8] including endocytosis, exocytosis, Golgi vesicle movement, and protein trafficking,[9,10] in cell adhesion and migration,[11] in remodeling of the actin cytoskeleton,[12] and in mitogenesis and oncogenesis.[6,7] Activation of cellular signaling pathways often results from production of one of eight specific PtdInsP_ns in response to a stimuli, and each PtdInsP_n has a specific role for a given signaling pathway in each cell-type.

Phosphatidylinositol 4,5-bisphosphate, or PtdIns(4,5)-P_2, has been found to play a central role in a variety of cellular functions, each utilizing different portions of the total molecule.[4,5] First, PtdIns(4,5)P_2 is a substrate for phospholipase C (PLC), a pleckstrin homology (PH)-domain-containing enzyme whose three-dimensional structure has been determined.[13] Hydrolysis of the phosphodiester linkage releases the calcium-mobilizing second messenger Ins(1,4,5)P_3.[14] Second, PtdIns(4,5)P_2 itself recruits PH-domain-containing proteins to membranes;[15] PH domains occur in over 120 mammalian proteins, and each exhibits distinctive PIP_n-binding affinities and selectivities.[16−18] Among these, the best example is the specific binding of PtdIns(4,5)P_2 to the PH domain of the δ_1 isoform of PLC.[19] Third, PtdIns(4,5)P_2 binds to non-PH-domain-containing proteins, e.g., gelsolin and profilin, and regulates actin polymerization.[5] PtdIns(4,5)P_2 binding also modulates the GTPase activity of adenosine ribosylation factor (ARF) and the phospholipase activity of phospholipase D,[20,21] recruits the human class II phosphoinositide (PI) 3-kinase to membranes via its C2-γ

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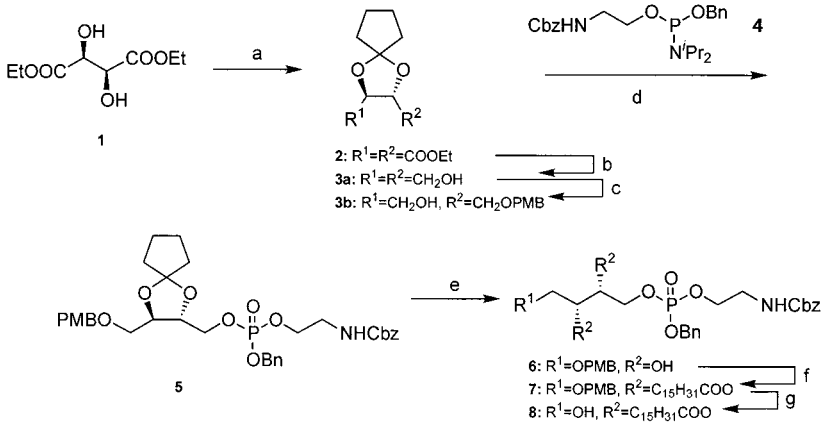
SCHEME 1. Synthesis of Differentially Functionalized 2,3-Diacylthreitol Backbone[a]



[a] Reagents and conditions: (a) cyclopentanone, *p*TSA, toluene, reflux; (b) $LiAlH_4$, THF; (c) NaH, PMBCl, DMF; (d) 1-*H*-tetrazole, phosphoramidite **4**, CH_2Cl_2; (e) 1 M HCl, THF; (f) $C_{15}H_{31}COOH$, DCC, DMAP, CH_2Cl_2; (g) DDQ, CH_2Cl_2/H_2O

PX domain,[22] and binds to the AP180 ENTH domain to mediate endocytosis via clathrin-coated pits.[23] The profilin[24] and gelsolin[25] interactions with PtdIns(4,5)P_2 have been characterized by photoaffinity labeling. Finally, PtdIns(4,5)P_2 can be converted by PI 3-kinase[26] to PtdIns(3,4,5)P_3, which interacts with many PH domains and activates the specific protein kinase PDK1.[3]

Chemically modified derivatives of the natural PtdInsP_ns[27,28] have proven extremely valuable in isolating new PtdInsP_n-binding proteins, identifying binding sites, and unraveling the roles of specific molecular species in cell signaling.[28] These derivatives have included photoaffinity analogues based on phosphotriesters[29] and acyl modifications.[27] In addition, fluorescently labeled analogues[30] have been employed for biophysical studies[31,32] as well as investigations of cellular uptake and subcellular localization.[33] Biotinylated derivatives have proven valuable as "bait" in fishing through expression libraries for novel high-affinity PtdInsP_n-specific binding proteins.[34] For each of these derivatives, the modification has involved either the P-1 phosphate, thus placing the reporter moiety in potential conflict with headgroup recognition, or the acyl chain, thus altering the partitioning of the analogue into a lipid bilayer environment.

In this paper, we describe the asymmetric total synthesis of a new class of functionalized PtdInsP_ns, the Pea-PIPs. Our strategy involves homologation of the 1,2-diacylglycerol backbone to a 2,3-diacylthreitol backbone. These hybrid lipids thus possess a phosphatidylethanolamine (PE, or Pea) headgroup at the 1-position and a PtdIns(4,5)P_2 headgroup at the 4-position. The reporter group, e.g., biotin, a fluorophore, or a spin label, could then be covalently attached to the free PE amino group. The reporter would thus be targeted to the lipid−water interface at a site distant from the key PtdIns(4,5)P_2 headgroup recognition features of the binding protein. The diacyl moiety should permit insertion and retention in a lipid bilayer to facilitate recruitment of PtdIns(4,5)P_2-specific binding proteins to a membrane surface environment.

Results and Discussion

Diethyl D-tartrate was chosen as the chiral precursor for the extended glycerol backbone of the target hybrid lipid. The absolute configuration of both stereogenic centers at C-2 and C-3 is identical to the configuration of glycerol *sn*-2 position in naturally occurring PtdInsP_ns and in natural PE. Moreover, the C_2 axis allowed the use of a monoprotection step in the early stages of the synthesis. As shown in Scheme 1, diethyl D-tartrate **1** was protected as a cyclopentylidene acetal, which was found to be most readily removed after backbone functionalization. Initially, an isopropylidene acetal was used, but scale-up of the deprotection led to unsatisfactory yields of the desired intermediate. Reduction of acetal **2** with lithium aluminum hydride afforded (2*R*,3*R*)-*O*-cyclopentylidene threitol **3a**, which was protected with 1 equiv of PMB-Cl to give the monobenzyl ether **3b**. The primary alcohol of **3b** was converted to a Cbz-protected PE headgroup by coupling to the phosphoramidite **4**,

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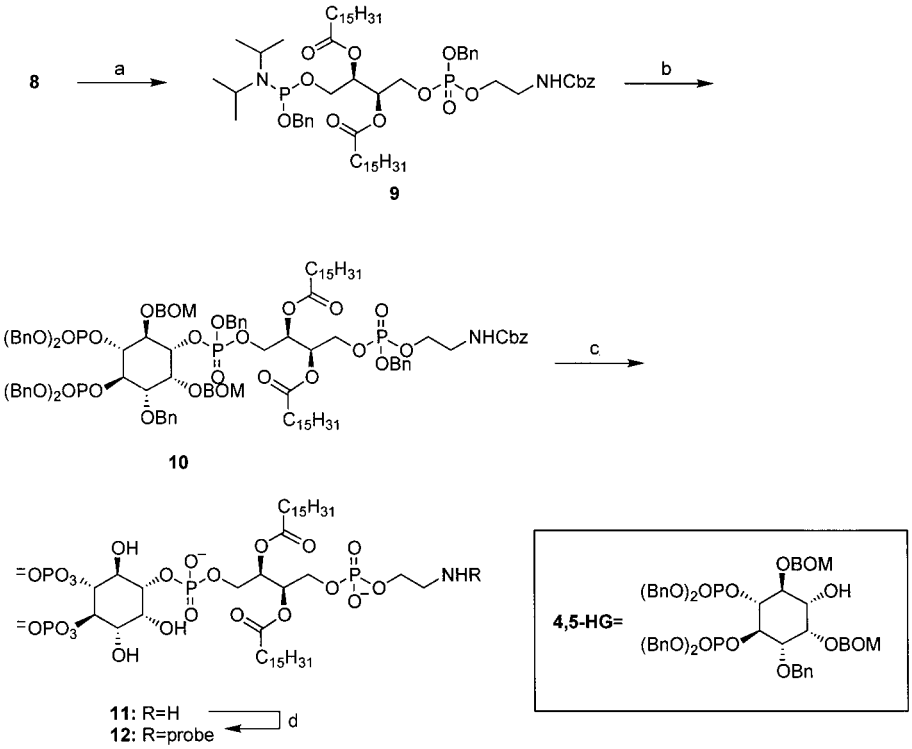
SCHEME 2. Backbone Phosphorylation and Synthesis of Pea-PIP$_2$ Derivatives 12[a]



[a] Reagents and Conditions: (a) BnOP(N*i*Pr$_2$)$_2$, 1-*H*-tetrazole, CH_2Cl_2; (b) 1-*H*-tetrazole, **4,5-HG**, CH_2Cl_2; (c) H_2 (60 psi), 10% Pd/C, THF/H_2O; (d) probe-NHS ester, 0.5 M TEAB, DMF

which after oxidation afforded the protected PE analogue **5**. Acidic hydrolysis yielded diol **6** in 65% yield after silica gel chromatography. Acylation with palmitic acid provided diester **7** and oxidative cleavage of the *p*-methoxybenzyl (PMB) ether with DDQ gave primary alcohol **8**.

Scheme 2 illustrates the installation of two different phosphorylated headgroups on the 2,3-diacylthreitol backbone. Thus, reaction of alcohol **8** with benzyloxybis(*N*,*N*-diisopropylamino)phosphine yielded a homologated PE-like phosphoramidite reagent **9**, which was coupled with the protected *myo*-inositol 4,5-bisphosphate headgroup obtained as previously described,[29] to give the fully protected Pea-PIP$_2$ precursor **10**. Global debenzylation of **10** was accomplished by hydrogenolysis to give the free phosphate monoesters and phosphodiesters in the hybrid lipid Pea-PIP$_2$ (**11**). Reaction of the free amino group of **11** with four *N*-hydroxysuccinimidyl (NHS) esters[35] afforded the corresponding biotinylated derivative **12a**, the fluorescent *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and 6-carboxyfluorescein derivatives **12b** and **12c**, and the spin-labeled 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXYL) derivative **12d**. Biological results are described below for biotinylated derivative **12a** and fluorescent analogue **12c**. Use of the spin-labeled derivative **12d** to probe interfacial protein−lipid interactions in liposomes will be described elsewhere, in analogy to the use of acyl spin-labeled probes to characterize the MARCKS peptide−PtdIns(4,5)P$_2$ interaction in liposomes.[36]

Two preliminary biological assays were conducted to establish the utility of this reagent in a biochemical context. First, we established biochemical relevance by demonstrating that the Pea-PIP$_2$ **12** derivatives could bind to the PtdIns(4,5)P$_2$-selective PH domain of the PLC δ_1 isoform. We employed a bioluminescence assay (AlphaScreen)[37] in which the biotinylated lipid **12a** was bound to a streptavidin-coated donor bead and the GST-tagged PLC δ_1-PH domain was attached to an anti-GST-coated acceptor bead. A luminescent signal quantitatively reported the interaction between the biotinylated lipid and the binding protein. Thus, in the absence of a lipid or a specific binding protein, no signal was seen. A biotinylated PtdIns(4,5)P$_2$ analogue[27] exhibited the benchmark binding interaction, and the Pea-PIP$_2$ analogue **12a** showed approximately 30% of the full PtdIns(4,5)P$_2$ response. As expected, the biotinylated InsP$_6$ derivative[38] did not bind to the GST-PLC δ_1-PH construct (Figure 1). Next, competitive binding assays were conducted (data not shown) in which the GST-PLC δ_1-PH protein was preincubated for 30 min with 10 nM to 10 fM of unlabeled di-C$_4$ PtdIns(4)P, PtdIns(3,4,5)P$_3$, and PtdIns(4,5)P$_2$ prior to addition of the other reagents, using biotinylated Pea-PIP$_2$ **12a** as the probe lipid. Over 100-fold selectivity was observed for displacement of the GST-PLC δ_1-PH from binding to **12a** by the di-C$_4$ PtdIns(4,5)P$_2$, relative to the other lipids.

Second, preliminary data were obtained with immobilized lipids.[18] Eight different quantities (from 200

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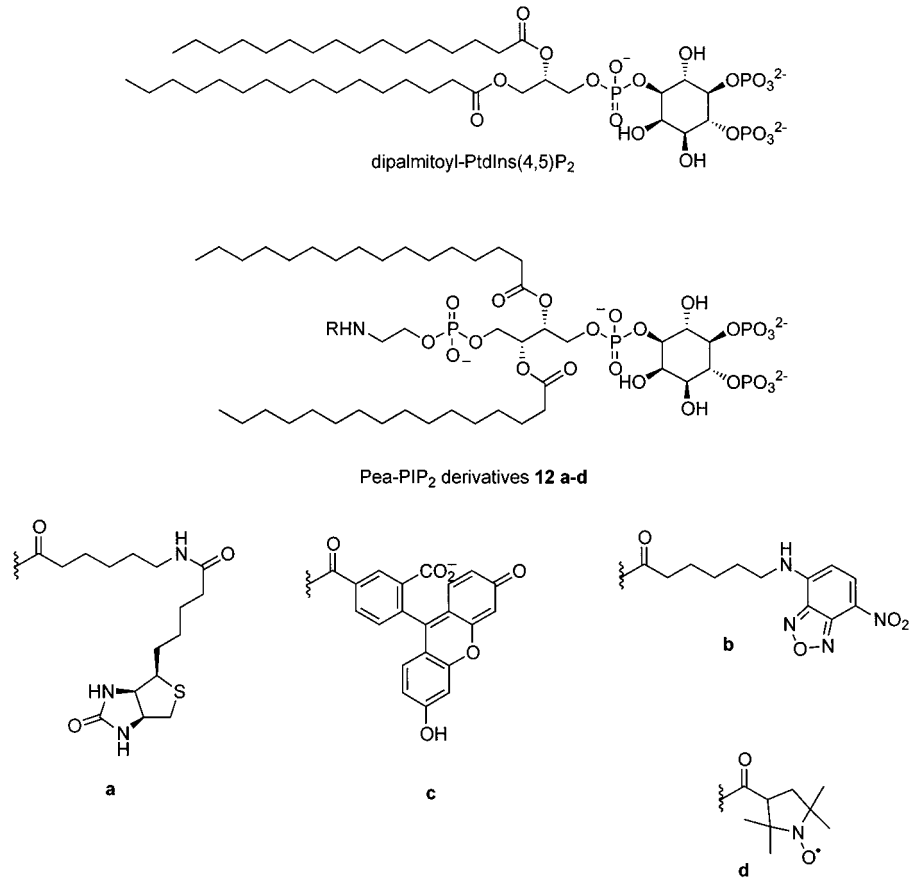
SCHEME 3. Structures of Dipalmitoyl-PtdIns(4,5)P_2 and Four Pea-PIP$_2$ Probes (12a−d) Prepared for Biochemical and Biological Studies



to 2 pmol) of PE, PtdIns(4,5)P_2, and biotinylated Pea-PIP$_2$ **12a** were spotted onto nitrocellulose, and the binding of an anti-PtdIns(4,5)P_2 IgM monoclonal antibody (Echelon) and the GST-PLC δ_1-PH construct were examined. Neither protein recognized the PE control lipid, but both proteins showed dose-dependent recognition of the immobilized lipids. The antibody recognition was more robust than that of the GST-PLC δ_1-PH protein. Full details for all biochemical assays will be published in due course.

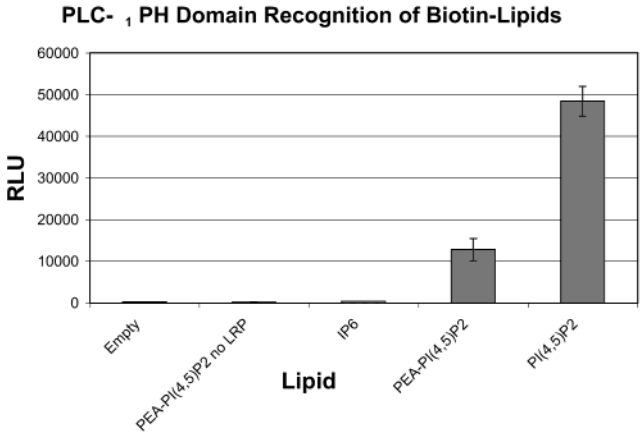


FIGURE 1. Binding of biotinylated Pea-PIP$_2$ **12a** and control biotinylated inositide lipids to GST-PLC δ_1-PH ("LRP").

Experimental Section

General. Reactions requiring anhydrous conditions were carried out in oven-dried glassware (2 h, 120 °C). Dry solvents were prepared using standard procedures. Concentration in vacuo refers to the use of a rotary evaporator for solvent removal, and purification on SiO_2 refers to flash chromatography on silica gel. Reactions were monitored by TLC (SiO_2) on Whatman glass plates using a mixture of $Ce(SO_4)_2$, MoO_3, and H_2SO_4 in water for visualization. NMR spectra were recorded on Varian INOVA instrument in chloroform-*d* or D_2O with TMS as internal reference. Coupling constants (*J*) are reported in Hz. IR spectra were recorded on JASCO FT/IR-420 apparatus (film on NaCl disk). Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ), and mass spectra were measured at The University of California at Riverside (Riverside, CA) using matrix-assisted laser desorption ionization (MALDI).

(2*R*,3*R*)-1,4-Dioxa-spiro[4.4]nonane-2,3-dicarboxylic Acid Diethyl Ester 2. Diethyl D-tartrate **1** (1.004 g, 4.87 mmol), cyclopentanone (2.2 mL, 24.35 mmol, 5 equiv), and *p*-toluenesulfonic acid (93 mg, 0.49 mmol, 0.1 equiv) were dissolved in toluene (75 mL) and stirred under reflux for 36 h with azeotropic removal of water using a Dean−Stark trap. Upon completion, the reaction mixture was cooled to room temperature and neutralized with solid $NaHCO_3$. Solid salts were removed by filtration, the filtrate was concentrated in vacuo, and the crude product was purified on SiO_2 (hexane/acetone 4:1 containing 10% v/v Et_3N) to give 1.166 g (4.28 mmol, 88%) of acetal **2** (enantiomer[39]) as a colorless oil, $[\alpha]^D = +30.5°$ (0.36 g/dL).^{1}H NMR (400 MHz, $CDCl_3$) δ 4.73 (s, 2H),

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4.28 (q, 4H, J = 7.2), 1.94−2.04 (m, 2H), 1.80−1.90 (m, 2H), 1.65−1.76 (m, 4H), 1.32 (t, 6H, J = 7.2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.67, 123.34, 77.08, 61.85, 36.66, 23.50, 14.15. IR 2980, 1756, 1337, 1119, 1115, 1023, 460, 453. Anal. Calcd for $C_{13}H_{20}O_6$: C, 57.34; H, 7.40. Found: C, 57.34; H, 7.21.

(2*R*,3*R*)-(3-Hydroxymethyl-1,4-dioxa-spiro[4.4]non-2-yl)-methanol 3a. A solution of diethyl ester **2** (1165 mg, 4.28 mmol, 1 equiv) in dry THF was transferred via cannula to a suspension of $LiAlH_4$ (244 mg, 6.42 mmol, 1.5 equiv) in dry THF that had been precooled in a brine/ice bath. The reaction mixture was stirred at room temperature for 24 h, and then saturated aqueous potassium sodium tartrate was added dropwise to decompose excess hydride reagent. The mixture was stirred for an additional 24 h and then extracted with three portions of CH_2Cl_2. The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo, and the crude product was purified on SiO_2 (hexane/acetone 3:2 containing 10% v/v Et_3N) to give 805 mg (4.27 mmol, 99%) of the diol **3a** as a colorless oil, $[\alpha]^D = +6.7°$ (4.08 g/dL).^{1}H NMR (400 MHz, $CDCl_3$) δ 3.85−3.95 (m, 2H), 3.60−3.80 (m, 4H), 1.73−1.85 (m, 4H), 1.60−1.73 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.36, 78.40, 62.46, 37.32, 23.44. IR 3390, 2956, 2875, 1434, 1335, 1204, 1112, 1041, 973. Anal. Calcd for $C_9H_{16}O_4$: C, 57.43; H, 8.57. Found: C, 57.48; H, 8.52.

(2*R*,3*R*)-[3-(4′-Methoxy-benzyloxymethyl)-1,4-dioxa-spiro[4.4]non-2-yl]-methanol 3b. To a suspension of NaH (175 mg, 4.38 mmol, 1 equiv) in dry DMF was added alcohol **3a** (824 mg, 4.38 mmol, 1 equiv) in dry DMF via cannula. The mixture was cooled to 0 °C, and then 4-methoxybenzyl chloride (0.65 mL, 4.82 mmol, 1.1 equiv) was added dropwise over 20 min. The ice bath was removed, and the mixture was stirred at room temperature for 18 h. Traces of NaH were decomposed by slow addition of water. The mixture was extracted 3× with CH_2Cl_2, dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified on SiO_2 (hexane/acetone 4:1 containing 10% v/v Et_3N) to give 879 mg (2.85 mmol, 65%) of compound **3b** as a colorless oil, $[\alpha]^D = -7.7°$ (3.82 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20−7.30 (m, 2H), 6.80−6.95 (m, 2H), 4.45−4.55 (m, 2H), 3.92−4.00 (m, 1H), 3.82−3.89 (m, 1H), 3.79 (s, 3H), 3.60−3.76 (m, 3H), 3.49 (dd, 1H, J_1 = 5.6, J_2 = 9.6), 2.45 (bs, 1H), 1.73−1.89 (m, 4H), 1.58−1.73 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.29, 129.70, 129.40, 128.52, 119.31, 113.82, 79.56, 76.58, 73.21, 70.14, 62.63, 55.19, 37.26, 37.20, 23.52, 23.40. IR 3466, 2955, 2872, 1612, 1514, 1248, 1101, 1035. Anal. Calcd for $C_{17}H_{24}O_5$: C, 66.21; H, 7.84. Found: C, 66.27, H, 7.76.

[2-(Benzyloxy-diisopropylamino-phosphanyloxy)-ethyl]-carbamic Acid Benzyl Ester 4.[40] To a solution of benzyloxybis(*N*,*N*-diisopropylamino)phosphine[41] (1.538 g, 4.54 mmol, 1.5 equiv) and 1-*H*-tetrazole (106 mg, 1.51 mmol, 0.5 equiv) in dry CH_2Cl_2 was added a solution of (2-hydroxyethyl)-carbamic acid benzyl ester (591 mg, 3.03 mmol) in CH_2Cl_2. The mixture was stirred at room temperature for 3 h and concentrated in vacuo, and the crude product was purified on SiO_2 (hexane/acetone/Et_3N 6:4:1) to give 886 mg (2.05 mmol, 68%) of compound **4** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20−7.40 (m, 10H), 5.21 (bs, 1H), 5.08 (s, 2H), 4.72 (dd, 1H, J_1 = 8.2, J_2 = 12.4), 4.63 (dd, 1H, J_1 = 8.2, J_2 = 12.4), 3.64−3.79 (m, 2H), 3.56−3.70 (m, 2H), 3.39 (m, 2H), 1.18 (d, 12H, J = 7.2). ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.1.

(2-{Benzyloxy-[(2*R*,3*R*)-3-(4′-methoxybenzyloxymethyl)-1,4-dioxa-spiro[4.4]non-2-ylmethoxy]-phosphoryloxy}-ethyl)-carbamic Acid Benzyl Ester 5. A solution of the monoprotected alcohol **3b** (486 mg, 1.58 mmol, 1 equiv) and tetrazole (331 mg, 473 mmol, 3 equiv) in dry CH_2Cl_2 (5 mL) was stirred under N_2 for 5 min at room temperature. Phosphoramidite **4** (886 mg, 2.05 mmol, 1.3 equiv) in dry CH_2Cl_2 (5 mL) was added via cannula, and the mixture was stirred for 2 h (until all starting material was consumed). After cooling to −40 °C, *m*CPBA (1.360 g, 6 mmol, 3 equiv, 60%) in CH_2Cl_2 (5 mL) was added, and the reaction mixture was stirred for 5 min. The cold bath was then removed and stirring was continued for an additional 1 h. The reaction was diluted with CH_2Cl_2, poured into saturated $NaHCO_3$, and after 20 min of vigorous stirring extracted 3× with CH_2Cl_2. Combined organic phases were dried ($MgSO_4$) and concentrated in vacuo, and the crude product was purified on SiO_2 (hexane/acetone 3:2) to give 1.033 g (1.57 mmol, 99%) of compound **5** as a colorless oil, $[\alpha]^D = +3.7°$ (4.83 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28−7.38 (m, 10H), 7.18−7.24 (m, 2H), 6.82−6.88 (m, 2H), 5.44−5.54 (m, 1H), 5.00−5.12 (m, 4H), 4.42−4.51 (m, 2H), 3.99−4.17 (m, 4H), 3.89−3.99 (m, 2H), 3.76 (s, 3H), 3.56 (dd, 1H, J_1 = 4.8, J_2 = 10.0), 3.43−3.50 (m, 1H), 3.33−3.41 (m, 2H), 1.70−1.84 (m, 4H), 1.54−1.70 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.30, 156.34, 136.47, 135.65, 135.58, 129.92, 129.79, 129.35, 128.68, 128.63, 128.48, 128.35, 128.07, 128.03, 119.95, 113.80, 77.24, 76.00, 73.18, 69.95, 69.53, 67.46, 66.94, 66.71, 55.21, 53.47, 41.30, 37.26, 37.20, 23.49, 23.42. ^{31}P NMR (162 MHz, $CDCl_3$) δ 2.23. IR 3316, 2956, 1722, 1514, 1250, 1023. Anal. Calcd for $C_{34}H_{42}NO_{10}P$: C, 62.28; H, 6.46; N, 2.14. Found: C, 62.38; H, 6.29; N, 2.18.

(2-{Benzyloxy-[(2*R*,3*R*)-2,3-dihydroxy-4-(4′-methoxy-benzyloxy)-butoxy]-phosphoryloxy}-ethyl)-carbamic Acid Benzyl Ester 6. To a solution of **5** (2.161 g, 3.3 mmol) in dry THF (100 mL) was added 100 mL of 1 M HCl. The mixture was stirred at room temperature for 3 h, and then saturated aqueous $NaHCO_3$ was added. The mixture was transferred to a separatory funnel and extracted with CH_2Cl_2. The crude product was purified on SiO_2 (toluene/acetone 1:4) to give 1.267 g (2.15 mmol, 65%) of the diol **6** as a colorless oil, $[\alpha]^D = 0.0°$ (2.14 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25−7−35 (m, 10H), 7.17−7.22 (m, 2H), 6.81−6.86 (m, 2H), 5.64−5.75 (m, 1H), 4.95−5.10 (m, 4H), 4.35−4.46 (m, 2H), 3.95−4.12 (m, 4H), 3.78−3.86 (m, 1H), 3.65−3.78 (m, 1H), 3.75 (s, 3H), 3.45−3.55 (m, 2H), 3.30−3.40 (m, 2H). ^{13}C NMR (100 MHz, CDCl3) δ 159.31, 156.51, 136.45, 135.50, 129.77, 129.42, 128.73, 128.64, 128.48, 128.06, 113.83, 73.14, 71.24, 70.50, 69.65, 69.25, 68.90, 66.95, 66.73, 55.22, 41.24. ^{31}P NMR (162 MHz, $CDCl_3$) δ 0.52. IR 3349, 2954, 1719, 1612, 1513, 1456, 1248, 1023, 821, 739, 698. Anal. Calcd for $C_{29}H_{36}NO_{10}P$: C, 59.08; H, 6.15; N, 2.38; O, 27.14; P, 5.25. Found: C, 58.96; H, 6.27; N, 2.44; P, 5.44.

Hexadecanoic Acid {(2*R*,3*R*)-3-[Benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxy]-2-hexadecanoyloxy-1-(4′-methoxy-benzyloxymethyl)}-propyl Ester 7. DCC (1.377 g, 6.67 mmol, 3 equiv) and DMAP (299 mg, 2.45 mmol, 1.1 equiv) were added in one portion to a solution of **6** (1.311 g, 2.22 mmol, 1 equiv) and hexadecanoic acid (1.901 g, 6.67 mmol, 3 equiv) in dry CH_2Cl_2. After stirring for 18 h, the reaction mixture was concentrated in vacuo and purified on SiO_2 (hexane/acetone 4:1) to give 1.68 g (1.58 mmol, 71%) of product **7** as a waxy solid, melting range 40−60 °C, $[\alpha]^D = -31.8°$ (0.93 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26−7.40 (m, 10H), 7.18−7.24 (m, 2H), 6.82−6.88 (m, 2H), 5.08 (s, 2H), 4.95−5.06 (m, 2H), 4.32−4.46 (m, 2H), 3.88−4.24 (m, 4H), 3.76 (s, 3H), 3.43−3.60 (m, 2H), 3.34−3.43 (m, 2H), 2.21−2.32 (m, 4H), 1.50−1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H, J = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.90, 172.77, 159.35, 129.41, 128.73, 128.65, 128.47, 128.08, 128.01, 113.80, 72.97, 70.04, 69.97, 69.74, 69.66, 69.62, 67.45, 67.02, 66.71, 65.65, 55.18, 41.30, 34.15, 31.94, 29.72, 29.67, 29.51, 29.38, 29.31, 29.15, 24.92, 24.86, 22.70, 14.12. ^{31}P NMR (162 MHz, $CDCl_3$) δ 0.26, 0.14. IR 2924, 2853, 1741, 1612, 1514, 1456, 1249, 1154, 1112, 1035, 736, 697. Anal. Calcd for $C_{61}H_{96}NO_{12}P$: C, 68.70; H, 9.07; N, 1.31. Found: C, 68.87; H, 8.81; N, 1.32.

Hexadecanoic Acid {(2*R*,3*R*)-3-[Benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxy]-2-hexadecanoyloxy-1-hydroxymethyl}-propyl Ester 8. To a solution of **7** (234 mg, 0.23 mmol, 1 equiv) in CH_2Cl_2 (23 mL) was added water (0.23 mL) followed by DDQ (103 mg, 0.46 mmol, 2 equiv).

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When TLC indicated that the reaction was complete, the mixture was transferred to a separatory funnel and washed with 5% Na_2SO_3 and saturated $NaHCO_3$ (2×). The aqueous phases were back-extracted once with CH_2Cl_2, the combined organic phases were dried ($MgSO_4$) and concentrated in vacuo, and product was purified on SiO_2 (hexane/acetone 4:1) to give 125 mg (0.13 mmol, 60%) of product **8** as a waxy solid, melting range 55−65 °C, $[\alpha]^D = -39.4°$ (1.09 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20−7.40 (m, 10H), 5.0−5.16 (m, 6H), 3.92−4.23 (m, 4H), 3.53−3.72 (m, 2H), 3.30−3.45 (m, 2H), 2.80−2.94 (m, 1H), 2.27−2.33 (m, 4H), 1.54−1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H, J = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.44, 173.16, 156.46, 136.45, 135.46, 128.81, 128.70, 128.51, 128.13, 128.06, 71.64, 70.05, 69.81, 67.08, 66.80, 65.79, 60.70, 41.30, 34.15, 31.94, 29.73, 29.68, 29.52, 29.38, 29.31, 29.16, 24.90, 22.70, 14.13. ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.68, 1.61. IR 2917, 2850, 1739, 1467, 1263, 1017. Anal. Calcd for $C_{53}H_{88}NO_{11}P$: C, 67.27; H, 9.37; N, 1.48. Found: C, 67.32; H, 9.49; N, 1.50.

Hexadecanoic Acid {(2*R*,3*R*)-1-[Benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxymethyl]-3-(benzyloxy-diisopropylamino-phosphanyloxy)-2-hexadecanoyloxy}-propyl Ester 9. To a solution of benzyloxybis(*N*,*N*-diisopropylamino)phosphine (1.164 g, 3.44 mmol, 1.5 equiv) and tetrazole (80 mg, 1.15 mmol, 0.5 equiv) in dry CH_2Cl_2 was added a solution of ester **8** (2.169 g, 2.30 mmol, 1 equiv) in CH_2Cl_2 via cannula. After 2 h, the reaction was concentrated in vacuo, and the residue was purified on SiO_2 (ethyl acetate/toluene 4:1 containing 5% Et_3N) to give 2.190 g (1.85 mmol, 81%) of phosphoramidite **9**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20−7.40 (m, 10H), 5.0−5.16 (m, 6H), 3.92−4.23 (m, 4H), 3.53−3.72 (m, 2H), 3.30−3.45 (m, 2H), 2.80−2.94 (m, 1H), 2.27−2.33 (m, 4H), 1.54−1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H, J = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.91, 172.75, 139.18, 135.58, 135.52, 128.74, 128.67, 128.49, 128.31, 128.26, 128.09, 128.02, 127.36, 127.29, 126.96, 126.93, 70.59, 69.72, 67.01, 66.72, 65.70, 65.37, 65.20, 61.69, 43.10, 34.18, 31.95, 29.73, 29.68, 29.52, 29.38, 29.33, 29.17, 24.88, 24.58, 22.70, 14.13. ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.18, 149.79, 0.32, 0.22.

Hexadecanoic Acid 1-[Benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxymethyl]-2-hexadecanoyloxy-3-[1-benzyloxy-phosphoryloxy-3-benzyloxy-2,6-bis-(benzyloxymethoxy)-4,5-bis-(bis-benzyloxy-phosphoryloxy)-D-*myo*-inositol]-propyl Ester 10. To a solution of 4,5-headgroup (**4**,**5-HG**, 250 mg, 0.24 mmol, 1 equiv) and tetrazole (51 mg, 0.73 mmol, 3 equiv) in dry CH_2Cl_2 was added a solution of phosphoramidite **9** (345 mg, 0.29 mmol, 1.2 equiv) in CH_2Cl_2. The mixture was stirred at room temperature for about 3 h and cooled to −40 °C, and *m*CPBA (210 mg, 0.73 mmol, 3 equiv, 60%) was added. After 15 min, the cold bath was removed, and stirring was continued for an additional hour. The reaction mixture was diluted with CH_2Cl_2 and poured into a solution of 5% Na_2SO_3 and saturated $NaHCO_3$. The mixture was extracted 3× with CH_2Cl_2, and the combined organic fractions were dried over $MgSO_4$, concentrated in vacuo, and purified on SiO_2 (hexane/acetone 3:2) to give 306 mg (0.14 mmol, 59%) of product **10** as a colorless oil, $[\alpha]^D = -14.43°$ (2.47 g/dL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00−7.35 (m, 50H), 5.55−5.95 (m, 1H), 5.15−5.35 (m, 2H), 4.40−5.15 (m, 27H), 4.20−4.40 (m, 2H), 3.90−4.20 (m, 6H), 3.45−3.60 (m, 1H), 3.25−3.45 (m, 2H), 2.10−2.30 (m, 4H), 1.40−1.60 (m, 4H), 1.05−1.35 (m, 48H), 0.88 (t, 6H, J = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.59, 138.09, 137.71, 137.25, 136.87, 136.56, 136.23, 136.17, 136.10, 136.06, 135.99, 135.90, 135.82, 135.51, 135.54, 128.65, 128.44, 128.34, 128.26, 128.02, 127.84, 127.75, 127.42, 96.64, 95.46, 78.99, 77.61, 77.34, 76.93, 76.60, 74.89, 72.79, 72.02, 70.45, 69.90, 69.70, 69.44, 69.12, 66.97, 66.62, 65.20, 41.29, 33.97, 31.92, 29.70, 29.67, 29.66, 29.51, 29.35, 29.29, 29.13, 24.78, 22.68, 14.12. ^{31}P NMR (162 MHz, $CDCl_3$) δ 0.35, −0.15, −0.56 (ratio 1:2:1). IR 2924, 2853, 1743, 1455, 1273, 1022. Anal. Calcd for $C_{117}H_{153}NO_{27}P_4$: C, 65.99; H, 7.24; N, 0.66; P, 5.82. Found: C, 65.75; H, 7.24; N, 0.73; P, 6.12.

1-[(2*R*,3*R*)-4-(2-Aminoethoxyphosphoryloxy)-2,3-di-*O*-palmitoylbutoxyphosphoryloxy]-4,5-*myo*-bisphosphate 11. To a solution of compound **10** (193.4 mg, 0.091 mmol) in a mixture of THF/water (4:1, v/v, 50 mL) was added 10% palladium on charcoal (387 mg). The mixture was shaken for 18 h at room temperature under 60 psi of H_2. The catalyst was removed by filtration, and solvent was removed in vacuo. The crude product was redissolved in water and stirred for 3 h with Dowex 50X-100 resin (Na^+ form). The resin was removed by filtration, and the filtrate was lyophilized to give 77.3 mg (0.062 mmol, 62%) as the sodium salt. The dried crude product was used for coupling with activated (NHS) esters as described below. ^{1}H NMR (400 MHz, D_2O) δ 5.15−5.30 (m, 2H), 4.10−4.30 (m, 2H), 3.85−4.10 (m, 7H), 3.75−3.85 (m, 1H), 3.6−3.7 (m, 1H), 3.4−3.5 (m, 1H), 3.18 (bs, 2H), 2.1−2.5 (m, 4H), 1.4−1.6 (bs, 4H), 1.18 (bs, 48H), 0.76 (bs, 6H). ^{31}P NMR (162 MHz, D_2O) δ 2.36, 1.80, 1.09, 0.56 (ratio 1:1:1:1). MS MALDI (free acid) 1146 (M + Na), 950 (M + 3Na − $C_{15}H_{31}CO$), 928 (M + 2Na − $C_{15}H_{31}CO$), 906 (M + Na − $C_{15}H_{31}CO$), 884 (M − $C_{15}H_{31}CO$).

General Procedure for Coupling with NHS Esters. To a solution of compound **11** (∼10 μmol, 1 equiv) in 0.5 M TEAB (0.5 mL, pH 7.5) was added a solution of appropriate NHS ester (∼12 μmol, 1.2 equiv) (three of which were obtained from Molecular Probes, Inc.) in 0.5 mL of DMF. PROXYL-SE was prepared as described.[36] The mixture was stirred at room temperature for 18 h, and solvents were then removed in vacuo. The residue was washed 4× with acetone and then purified on DEAE-cellulose column with a step gradient (0 to 2 M) of triethylammonium bicarbonate (TEAB). The desired fractions were pooled, lyophilized, converted by ion exchange into a sodium salt, and lyophilized again.

Biotin Derivative 12a. Reaction of **11** (9.4 mg, 7.5 μmol) with 6-[(biotinyoyl)amino]hexanoic acid, succinimidyl ester (Biotin-X, SE) (4.4 mg, 9.7 μmol) yielded 6.4 mg (4 μmol, 53%) of **12a**. ^{1}H NMR (400 MHz, D_2O) δ 5.20−5.40 (m, 4H), 4.50−4.60 (m, 1H), 4.30−4.45 (m, 1H), 3.70−4.45 (m, 9H), 3.60−3.70 (m, 1H), 3.30−3.45 (m, 1H), 3.05−3.20 (m, 4H), 2.65−2.95 (m, 2H), 2.00−2.50 (m, 8H), 1.40−1.80 (m, 12H), 0.90−1.40 (m, 52H), 0.70−0.90 (m, 6H). ^{31}P NMR (162 MHz, D_2O) δ 3.22, 2.32, 1.34, 0.41 (ratio 1:1:1:1). MS MALDI (free acid) 1528 (M + 3Na), 1506 (M + 2Na), 1484 (M + Na), 1462 (M − H), 1223 (M − H − $C_{15}H_{31}CO$), 1122 (M − H − biotin). HR MALDI $C_{60}H_{113}N_4O_{26}P_4S$ $[M - H]^-$ calcd 1461.60388, found 1461.60491.

NBD Derivative 12b. Reaction of **11** (9.9 mg, 7.9 μmol) with 6-NBD-aminohexanoic acid, succinimidyl ester (NBD-X, SE) (4.0 mg, 10.2 μmol) afforded 7.7 mg (5 μmol, 64%) of **12b**. ^{1}H NMR (400 MHz, D_2O) δ 8.10−8.30 (m, 1H), 6.00−6.20 (m, 1H), 5.10−5.30 (m, 2H), 3.65−4.20 (m, 12H), 3.20−3.65 (m, 5H), 2.95−3.25 (m, 4H), 2.20−2.40 (m, 2H), 2.15−2.25 (m, 4H), 1.65−1.80 (m, 2H), 1.50−1.65 (m, 2H), 1.30−1.50 (m, 4H), 0.80−1.30 (m, 50H), 0.50−0.80 (m, 6H). ^{31}P NMR (162 MHz, D_2O) δ 4.03, 2.92, 1.37, 0.47 (ratio 1:1:1:1). MS MALDI (free acid) 1161 (M − $C_{15}H_{31}CO$).

Fluorescein Derivative 12c. Reaction of **11** (9.5 mg, 7.6 μmol) with 6-carboxyfluorescein, succinimidyl ester (6-FAM-SE) (4.7 mg, 9.8 μmol) yielded 9.1 mg (5.6 μmol, 74%) of **12c**. ^{1}H NMR (400 MHz, D_2O) δ 7.30−7.50 (m, 8H), 5.20−5.40 (m, 2H), 3.10−4.30 (m, 14H), 2.20−2.40 (m, 4H), 1.40−1.60 (m, 4H), 0.90−1.40 (m, 48H), 0.60−0.90 (m, 6H). ^{31}P NMR (162 MHz, D_2O) δ 4.94, 4.33, 1.21, 0.65 (ratio 1:1:1:1). MS MALDI (free acid) 1243 (M − $C_{15}H_{31}CO$), 1123 (M − fluorescein), 884 (M − $C_{15}H_{31}CO$ − fluorescein), 841 (M − $C_{15}H_{31}CO$ − fluorescein − aminoethyl).

PROXYL Derivative 12d. Reaction of **11** (9.8 mg, 7.8 μmol) with PROXYL-SE (2.9 mg, 10.1 μmol) afforded 6.1 mg (4.3 μmol, 55%) of **12d**. MS MALDI (free acid) 1292 (M^-), 1123 (M − proxyl), 1053 (M − $C_{15}H_{31}CO$), 884 (M − $C_{15}H_{31}CO$ − proxyl). HR MALDI $C_{53}H_{103}N_2O_{25}P_4$ [M] calcd 1291.57950, found 1291.57679.

Binding Assays with 12a and Other Biotinylated Inositides. A competitive homogeneous assay (AlphaScreen,

Packard Biosciences) was employed. First, 0.2 pmol/well GST-PLC δ_1-PH domain protein was added to a white 384-well polystyrene plate, and then 0.4 pmol/well of biotinylated inositide, 5 μL of streptavidin donor beads, and 5 μL of anti-GST acceptor beads (25 μL final volume) were added. The biotinylated inositides tested were biotinylated Pea-PIP_2 (**12a**), biotinylated PtdIns(4,5)P_2, and biotinylated $InsP_6$. The contents of the wells were mixed, covered to shield from light, and incubated at room temperature for 2 h before being read on a Packard Fusion plate reader. For competition assays (data not shown), different concentrations (from 10 nM to 10 fM) of unlabeled di-C_4 PtdIns(4)P, PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2 were preincubated with the GST-PLC δ_1-PH for 30 min prior to addition of the other reagents. All biotinylated and short chain inositides were obtained from Echelon Biosciences, Inc.

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