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Regioselective Approach to Phosphatidylinositol 3,5-Bisphosphates: Syntheses of the Native Phospholipid and Biotinylated Short-Chain Derivative

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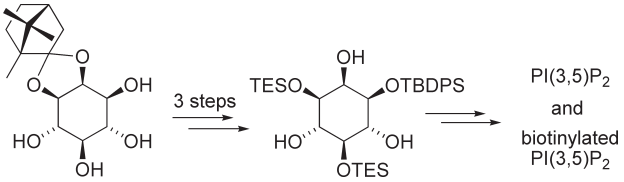

A selective bis-silylation of 1D-*O*-TBDPS-*myo*-inositol leads to a 1,3,5-trisubstituted inositol, which can be advanced to the headgroup of phosphatidylinositol-3,5-bisphosphate [PI(3,5)P_2]. A mild, regioselective method for construction of the diacylglycerol moiety containing differing fatty acid chains, including the naturally occurring lipids, was developed. Their union in the synthesis of the cell-signaling molecule PI(3,5)P_2 containing the *sn*-1-stearoyl and *sn*-2-arachidonoyl groups is described. The methodology was also used to generate dioctanoyl-PI(3,5)P_2 and a previously unreported biotin-PI(3,5)P_2 conjugate, which was coupled to neutravidin beads and used to pull down PI(3,5)P_2-binding proteins from the cytosolic extract of adrenal neurosecretory cells. We report the specific pull-down of the PI(3,5)P_2-binding protein svp1p, a known PI(3,5)P_2 effector involved in membrane trafficking.

Introduction

Phosphatidylinositol phosphates (PIPs) are intracellular amphiphilic molecules implicated in numerous cellular responses including growth,[1] division,[2] movement,[3] differentiation,[4] neuro-exocytosis,[5] and survival.[6] There are a number of forms that differ only in the phosphorylation state of the 3-, 4-, and 5-hydroxyls on the *myo*-inositol headgroup. The intracellular concentrations and dynamic relationships between the various PIP members are under tight control from a series of kinases and phosphatases. The 3-phosphorylated PIPs PI(3,4)P_2, PI(3,5)P_2, and PI(3,4,5)P_3 are intracellular second messengers that are synthesized by the action of PI-3-kinase, and in vivo, their half-life is relatively short as the active signal is under tight control (see above). The metabolic instability and relatively low concentrations of PIPs (especially the 3-phosphorylated forms) are factors contributing to the inability to isolate useful amounts of these compounds in pure form for biological studies. For this reason, the chemical synthesis of PIPs and derivatives remains an attractive endeavor. However, while the 3,4- and 3,4,5-headgroups have received considerable attention, approaches to PI(3,5)P_2 have been fewer due to its later discovery.[7] Furthermore, there

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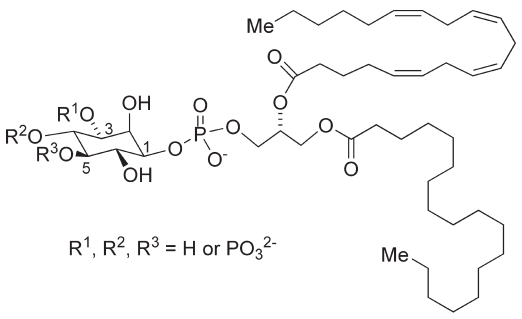
are few syntheses of PIPs containing unsaturated fatty acids, so new complementary methods are still needed.



The phosphatidylinositol phosphates

The signaling properties of phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2] were first characterized in yeast when its concentration was shown to dramatically rise following the application of hyperosmotic stress.[7] In yeast, PI(3,5)P_2 is synthesized from PI(3)P by the PI(3)P 5-kinase, Fab1p. Knockouts deficient in this enzyme exhibit vacuolar defects, indicating the importance of PI(3)P 5-kinase activity, and hence PI(3,5)P_2, in membrane trafficking events. Other work, utilizing siRNA, dominant negative constructs, and chemical inhibitors, implies that PIKfyve (the murine orthologue of Fab1p) is required for late endosome/lysosome function[8] and the full insulin stimulation of glucose transport in adipose and muscle tissue.[9] In contrast, PIKfyve has also been implicated in the negative regulation of exocytosis in neurosecretory cells.[10] The details of this mechanism are not currently known, but it is possible that PIKfyve activity contributes to the negative regulation of exocytosis by decreasing the levels of PI(3)P, as PI(3)P is known to promote large dense core vesicle (LDCV) priming for exocytosis.[5a,c]

To conduct further investigations into the role played by PI(3,5)P_2 in membrane trafficking, we sought a practical route to this phosphoinositide and its derivatives. Entry into the required D-enantiomeric series of PI(3,5)P_2 has most commonly involved the use of chiral auxiliaries, including camphor acetals,[11,12] camphanate esters,[13,14] *O*-acetylmandelate esters,[15] and 5-oxo-2-tetrahydrofurancarboxylate esters,[16] attached to the *myo*-inositol core. More recently, *meso*-triols have been desymmetrized by asymmetric phosphorylation, catalyzed by enantiodivergent pentapeptides.[17] Two chiral pool approaches have been reported, involving Ferrier rearrangement of a glucose derivative[18] or ring-closing metathesis of a glucose-derived 1,7-diene followed by dihydroxylation.[19] Although lengthy sequences are sometimes required to access the 1,3,5-substitution pattern found in PI(3,5)P_2, several of the above approaches have utilized tricyclic orthoesters of *myo*-inositol which provide rapid differentiation of the 1-, 3-, and 5-hydroxyl groups from the 2-, 4-, and 6-positions.[20]

In this work, we present novel syntheses of the PI(3,5)P_2 headgroup and mixed diacylglycerol moieties utilizing, respectively, the regioselective bis-silylation of pentol **2** with chlorotriethylsilane (TESCl) and the regioselective opening of acetonide **12** with triethylsilyl trifluoromethanesulfonate (TESOTf)/Hünig's base. The novel methodologies were used to prepare the natural *sn*-1-*O*-stearoyl-2-*O*-arachidonoyl PI(3,5)P_2, dioctanoyl PI(3,5)P_2 and a biotin-tagged analogue **30**. The biotin-tagged analogue was successfully employed in an affinity pull-down strategy to isolate PI(3,5)P_2 binding proteins from the cytosolic extract of adrenal neurosecretory cells.

Results and Discussion

Synthesis of Inositol Headgroup. The synthetic method developed by Bruzik's group represents a direct entry into useful homochiral starting materials for the synthesis of PIPs.[21] *myo*-Inositol is condensed with D-camphor dimethyl acetal to give camphanylidene acetal **1** (Scheme 1) as the major product, which is obtained in pure form after partial hydrolysis and recrystallization without recourse to chromatography. The strategy is applicable on a large scale and may be used to access all the naturally occurring members of the PIP family.[11] However, the approach is not equally efficient in accessing all the different phosphorylation patterns. In the synthesis of PI(3,5)P_2, dibenzoate **3** was obtained in only modest yield ($\sim$40%) upon treatment of **2** with benzoyl chloride (2 equiv) and proved difficult to separate from the 3,4-isomer. In our hands, altering the temperature and rate of addition did not improve the selectivity for the 3,5-isomer, so we decided to investigate alternative protecting group strategies to access this substitution pattern. The large silyl group at position 1 in compound **2** effectively blocks reaction at the two neighboring hydroxyl groups, and among the remaining three hydroxyl groups the 3-OH is usually the most nucleophilic, as has been amply demonstrated by various researchers.[22] Reaction of pentol **2** with a bulky group would initially form the 3-*O*-protected intermediate, which should preferentially undergo further reaction at the 5-OH to minimize steric interactions with the groups at positions 1 and 3. We initially examined the

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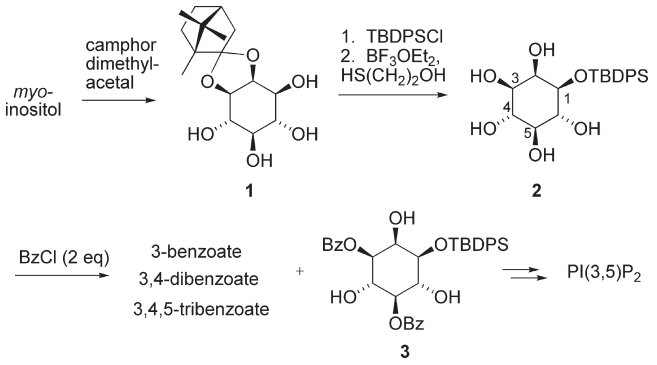
SCHEME 1. Bruzik's Synthesis of 3,5-Dibenzoate 3



reaction of **2** with a slight excess (1.2 equiv) of TESCl. As expected, the 3-*O*-silylated inositol **4** was the major product, isolated in 71% yield after column chromatography (Scheme 2). Lesser amounts of **5**−**7** were also isolated, along with a trace of starting material. Pleasingly, treatment of **2** with 2.5 equiv of TESCl reproducibly led to the formation of the 3,5-bis-silylated product **6** in 71% isolated yield, together with 14% of the 3,4-isomer **7** which was readily separated by chromatography on a multigram scale. The specific sites of silylation in products **4**−**7** were deduced from ^{1}H NMR coupling patterns and 2D-COSY analysis. Of particular importance to the regiochemical assignments were the well-known splitting patterns of *myo*-inositol ring-protons and cross-couplings from these signals to the hydroxyl protons in the 2D-COSY spectra (see the Supporting Information).

Among the TES, triphenylsilyl, and *tert*-butyldimethylsilyl protecting groups, TES was the most convenient in terms of 3,5-selectivity, product purification, and subsequent facile protection of the remaining hydroxyl groups. Although the addition of a third silyl group to **6** or **7** is strongly retarded due to the steric encumbrance around the remaining hydroxyl groups, exhaustive MOM protection of **6** could be readily achieved, followed by desilylation with acid to give the 3,5-diol **8** in excellent yield. Conversion of **8** to alcohol **9** followed Bruzik's reported procedure.[11] In a similar manner, **4** was transformed to alcohol **11**, which has previously been used as a precursor to PI(3)P.[11] Incidentally, silylation of diol **8** with TESCl was found to be completely selective for the 3-hydroxyl group to give compound **10**, in which the 1-, 3-, and 5-positions are all differentiated. This compound could provide a route into a series of PI(3,5)P_2 analogues with differing groups at positions 3 and 5.

Synthesis of Mixed Diacylglycerol. Native PIPs generally incorporate a mixed diacylglyceryl phosphate containing unsaturation at the *sn*-2 position. The 2-*O*-arachidonyl-1-*O*-stearoyl-*sn*-glycerol **16** is generally considered to be the most abundant form of the diacylglycerol moiety in mammalian-derived PIPs.[23] We chose to modify the reported[11,24] synthesis of **16** to allow for selective functionalization of each position on the glycerol and to minimize potential problems of acyl migration under acidic or basic conditions. Migration of acyl groups can occur at both the monoacyl- and diacylglycerol stages (vide infra). The isopropylidene group of 1-*O*-stearoyl-*sn*-glycerol **12** (Scheme 3) has previously been removed by refluxing in MeOH with Amberlyst-H$^+$ resin.[11] While no mention of any acyl migration was made, the ^{1}H NMR spectrum of the diol product indicated the presence of some *sn*-2-*O*-acylated isomer (5−10%). Others have reported similar results under these conditions.[25] Also of concern is the possible presence of 3-*O*-stearoyl-*sn*-glycerol (the enantiomeric product), which would be expected to form under conditions that promote acyl migration. Since the equilibrium ratio of 1-monoacylglycerol to 2-monoacylglycerol isomers is reported to be ~9:1,[26] the presence of 10% of the 2-acylated isomer would indicate a near-equilibrium mixture, suggesting that significant racemization may have taken place. Gaffney and Reese[24] have used a combination of TFA, triethyl borate and trifluoroethanol to effect this transformation, which relies on the formation of an intermediate 2,3-borate ester to prevent acyl migration. We, however, considered an alternative method for the opening of terminal acetonides, described by Rychnovsky and coworkers.[27] Although this method has received little attention since its initial report, we felt it would be well-suited to the synthesis of mixed diacylglycerols. Thus, heating acetonide **12** with TESOTf in the presence of a slight excess of Hünig's base led to a labile 2-*O*-isopropenyl-3-*O*-triethylsilyl diether **13** which, without purification, was converted to the secondary alcohol **14** under the mild conditions reported by Rychnovsky. Although a small amount (15%) of unreacted acetonide **12** was recovered from the crude reaction mixture, we observed no isomeric impurities in the crude mixture from this reaction and alcohol **14** showed no propensity for either silyl or acyl migration on chromatographic purification or in subsequent derivatization. Thus, *sn*-1 ↔ *sn*-2 ↔ *sn*-3 acyl migration which can occur during conventional acidic hydrolysis of the acetonide is completely avoided by this procedure, and regioselective introduction of the second fatty acid is ensured. Coupling with arachidonic acid was followed by desilylation using catalytic $FeCl_3$,[28] which provided the desired diacylglycerol **16** in excellent yield and purity. By comparison, removal of the bulkier TBDPS or TBDMS groups often leads to extensive acyl migration or requires reagents incompatible with unsaturated substrates.[29] The mild conditions used here for the removal of the TES group, combined with the regioselective introduction of fatty acids at each position of the glycerol unit, should engender further use of this protocol in di- and triacylglycerol syntheses. Conversion to the phosphoramidite **17** was achieved in the usual manner.

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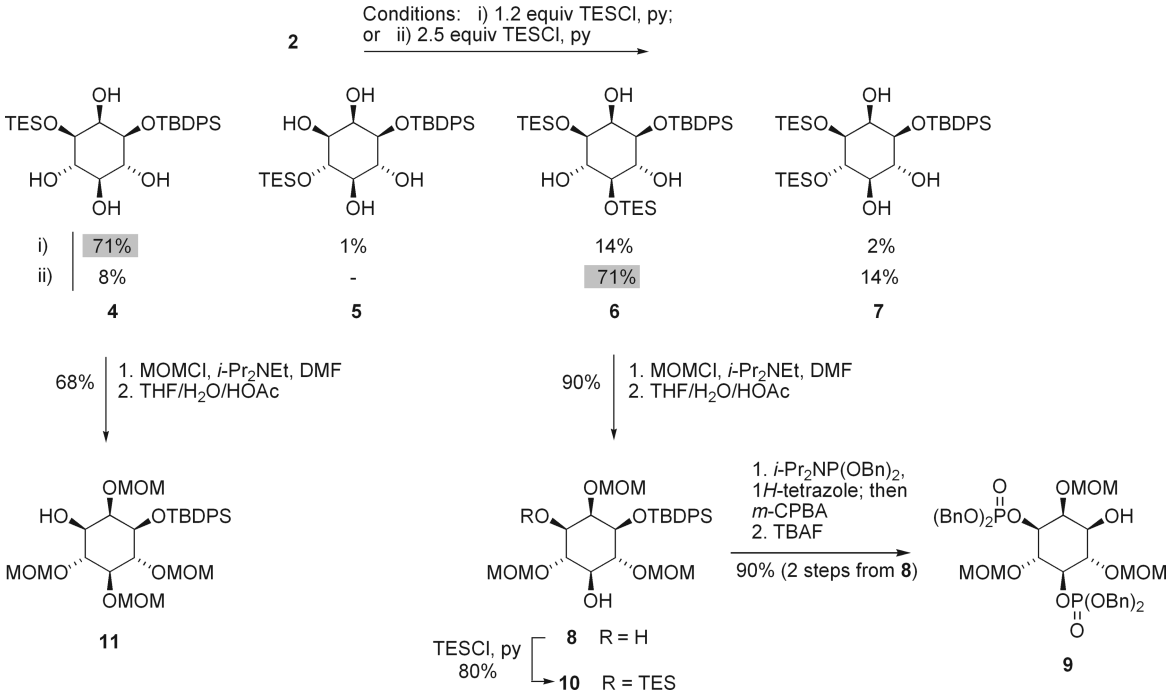
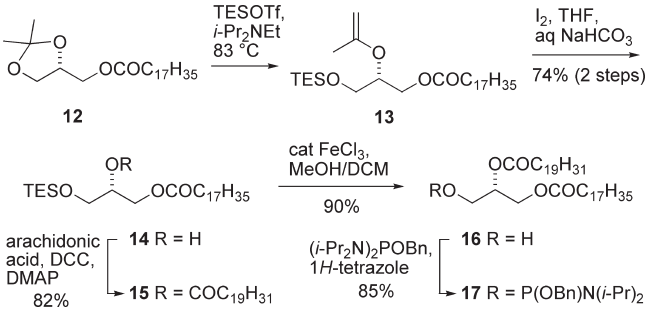
(29) For TBDPS deprotection, see: Greimel, P.; Ito, Y. *Tetrahedron Lett.* **2008**, *49*, 3562–3566. For TBDMS deprotection, see: Burgos, C. E.; Ayer., D. E.; Johnson, R. A. *J. Org. Chem.* **1987**, *52*, 4973–4977.

SCHEME 2. Synthesis of the Inositol Head Group 9



SCHEME 3. Synthesis of Phosphoramidite 17 Containing Mixed Diacylglycerol



Completion of the Synthesis of PI(3,5)P_2. Coupling of phosphoramidite **17** (2.2 equiv) with alcohol **9**, followed by oxidation of the intermediate phosphite with tetrabutylammonium periodate,[11] gave fully protected PI(3,5)P_2 **18** in 79% yield (Scheme 4). With slight modifications to Bruzik's deprotection protocol,[11] debenzylation was accomplished with bromotrimethylsilane (TMSBr) in the presence of 2-methylbut-2-ene. The latter was included in the reaction as a scavenger of adventitious HBr, the presence of which can cause undesired side reactions.[30] The 2-bromo-2-methylbutane formed is simply removed under high vacuum. Finally, treatment with ethanethiol[31] removed any remaining MOM groups to yield the target molecule PI(3,5)P_2 (**19**). Synthetic PI(3,5)P_2 containing the naturally occurring lipid chains has been described only once before in the literature;[17] the spectroscopic properties of our material compare well to the published data.

We also chose to synthesize dioctanoyl PI(3,5)P_2 (**22**) as its increased water solubility, as compared with longer chain derivatives, was expected to facilitate some biological studies. Coupling of phosphoramidite **20**[32] with **9** gave fully protected dioctanoyl PI(3,5)P_2 (**21**) which, upon deprotection,[33] afforded **22** (Scheme 5) whose spectroscopic properties compared well to previously reported data.[14,17] The difference in physical behavior between **19** and **22** was apparent from their NMR spectra in D_2O. Whereas **19** gave rather broad, poorly defined peaks (^1H and ^{31}P) indicative of solution aggregation behavior, the short-chain analogue **22** produced excellent ^1H, ^{31}P and ^{13}C spectra, with both the lipid and inositol headgroup regions of the ^1H spectrum well resolved. Good ^1H and ^{31}P spectra of **19** could be obtained from a dilute CD_3OD solution.

Synthesis of Biotin−PI(3,5)P_2 Affinity Probe. Our synthesis of affinity probe **30** began with Cbz protection of 8-aminooctanoic acid, followed by esterification with L-2,3-*O*-isopropylidene-*sn*-glycerol to afford acetonide **23** (Scheme 6). Opening of the acetonide and subsequent hydrolysis of the isopropenyl ether gave secondary alcohol **25**

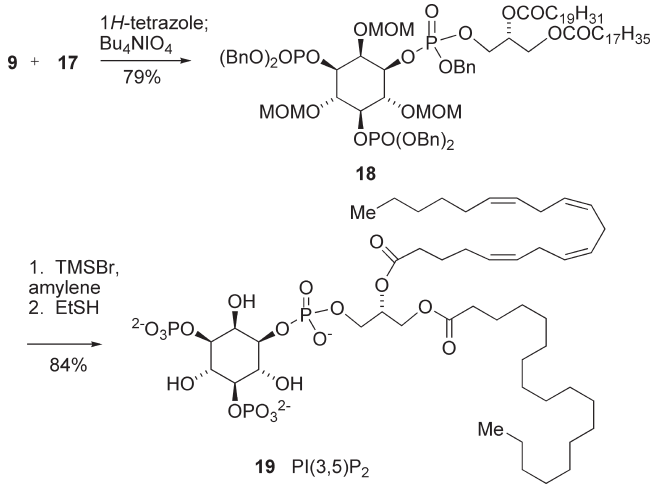
(30) The side product(s) appeared in the ^1H NMR as closely related minor signals on the shoulders of some of the main signals. We did not attempt to characterize them; however, Falck and co-workers have reported the migration of the *sn*-2-glycerylacyl group to an inositol hydroxyl group under acidic conditions, and it is conceivable that, in our case, a similar process may be promoted by HBr. See: Reddy, K. K.; Saady, M.; Falck, J. R. *J. Org. Chem.* **1995**, *60*, 3385–3390.

(31) A reviewer questioned whether, given the relatively weak Si−S bond strength, EtSH is capable of solvolyzing the intermediate silyl phosphate esters (which is required to provide the acidity necessary for the subsequent MOM deprotection). We can only say that our experience in the deprotection of compounds **18** and **29** and similar compounds (not reported here) has been that solvolysis of the interemediate silyl phosphates with water or methanol has not been necessary. EtSH has been, in our experience, adequate for this purpose.

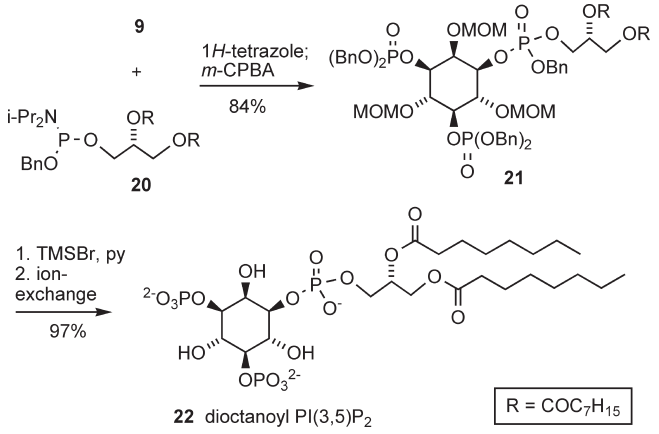
(32) Ali, S. M.; Ahmad, M. U.; Koslosky, P.; Kasireddy, K.; Krishna, U. M.; Ahmad, I. *Tetrahedron* **2006**, *62*, 6990–6997.

(33) In contrast to the deprotection of compound **18**, passage through strongly acidic ion-exchange resin was sufficient to remove any MOM groups remaining after TMSBr treatment of compound **21** (see the Experimental Section).

SCHEME 4. Synthesis of Natural PI(3,5)P_2 (19)



SCHEME 5. Synthesis of Dioctanoyl PI(3,5)P_2 (22)



which was esterified with octanoic acid and desilylated to furnish alcohol **27**. The sequence **23** → **27** could be conveniently performed without purification of intermediates, giving an improved yield of 51% over the four steps. The fully elaborated compound **29** was obtained upon coupling of phosphoramidite **28** with the inositol headgroup **9**. A stepwise deprotection of **29** in which the Cbz group is removed last was employed as, otherwise, the free amino group would buffer the acidity of the phosphates which is required for MOM-deprotection.[34] For the hydrogenolytic removal of the Cbz group, we found the addition of dilute aqueous ammonia to be beneficial with respect to reaction time and product recovery from the catalyst. No purification of the intermediates or the amine product was necessary, and the latter was allowed to react with the NHS ester of biotin to furnish the desired affinity probe **30**. The synthesis of **30** has, to our knowledge, not previously been reported in the literature, and we include here its full characterization by standard methods, including HPLC.

(34) While treatment of compound **29** with TMSBr/2-methyl-2-butene at rt led to partial removal of the Cbz group, this could be kept to a minimum by using a brief reaction time (35 min). Under more forcing conditions (40 °C, 19 h), products resulting from cleavage of the diacylglycerol moiety by Br^- attack at the *sn*-1 position were detected by MS and ^1H NMR (~10−15%). The Cbz group was ~90% deprotected in this experiment.

Identification of PI(3,5)P_2-Binding Proteins from Bovine Adrenal Chromaffin Cytosolic Fractions. PI(3,5)P_2 is an essential phospholipid in membrane-trafficking processes, but the specific details of its involvement on a molecular level remain unclear.[35] The use of biotin-tagged affinity probes can help to elucidate biochemical pathways by identifying effectors of a given ligand. Isolation of bound effectors is achieved using neutravidin-coated Sepharose beads, relying on the tight biotin−neutravidin binding interaction. Biotinylated PI(4,5)P_2 has previously been used in this manner to identify PI(4,5)P_2-binding proteins from subcellular fractions of bovine adrenal chromaffin cells.[5b] While many PI(4,5)P_2 binding proteins involved in a plethora of cellular functions have been identified, very little is known about potential PI(3,5)P_2 effectors. We report here our initial results using the biotinylated 3,5-phosphorylated isomer **30** and demonstrate its successful application in affinity purification, combined with subcellular fractionation, to pull down PI(3,5)P_2 binding proteins from bovine adrenal chromaffin cytosolic extract. We have chosen to use bovine adrenal chromaffin cells as a model system due to the involvement of PI(3,5)P_2 in constitutive membrane trafficking and also regulated secretion in these cell types.[10]

Biotinylated PI(3,5)P_2 was immobilized on beads and incubated with bovine adrenal chromaffin cytosol, and bound proteins were analyzed by SDS-PAGE and Western blotting, using antibodies against svp1p (also known as WIPI1, WIPI49, Atg18, and Aut10) and EEA1 (Figure 1A) as a means of identification. Svp1p has previously been shown to bind PI(3,5)P_2 in yeast[35] and mammalian cells[36] and has been implicated in the regulation of the endosomal pathway and autophagy[36,37] (the mechanism underpinning the degradation of intracellular components). EEA1, on the other hand, is a FYVE domain-containing protein that binds specifically to PI(3)P[38] and is used here to demonstrate the specificity of the pull-down for PI(3,5)P_2 binding proteins. As expected, svp1p immunoreactivity is enriched in the PI(3,5)P_2 lane compared to the control pull-down, while EEA1 is not associated with either control or PI(3,5)P_2 beads. These two results demonstrate the capacity of affinity probe **30** to specifically recruit PI(3,5)P_2 effectors. We further demonstrated that svp1p binding to PI(3,5)P_2 is sensitive to altered pH (Figure 1B). Significantly higher binding is observed at pH 7.3 than pH 7.6. A similar dependence on pH has previously been observed for EEA1 binding to PI(3)P and these results may indicate that binding of svp1p to membranes can be modulated by intracellular pH, as demonstrated for EEA1.[39]

In summary, we present concise syntheses of native, dioctanoyl, and biotin-tagged PI(3,5)P_2 targets **19**, **22**, and **30** utilizing novel chemical methodologies. Key steps in the

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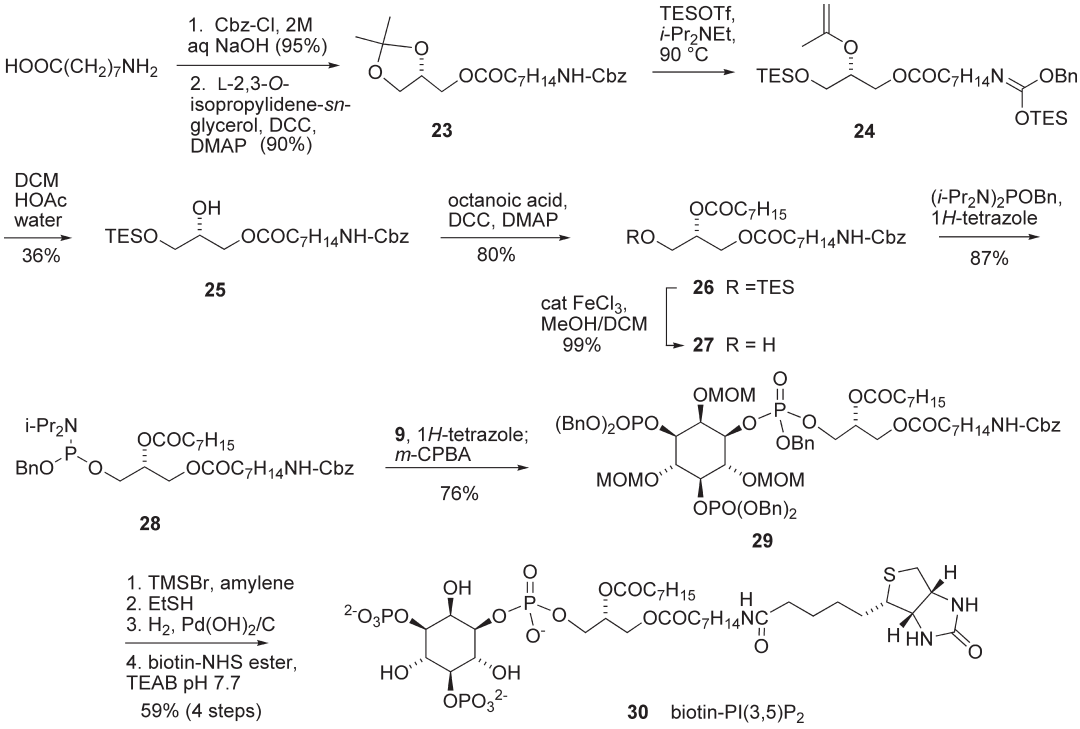
SCHEME 6. Synthesis of Biotin-PI(3,5)P_2 Affinity Probe 30



synthetic protocol included regioselective silylation of pentol **2** with TESCl to give the 3,5-protected intermediate **6** in good yield and a regioselective TESOTf-mediated acetonide opening to access mixed diacylglycerols **16** and **27**. The successful application of the biotin-tagged derivative **30** was demonstrated by its ability to specifically recruit a known PI(3,5)P_2-binding protein from a cytosolic fraction of adrenal neurosecretory cells. Further studies will utilize the affinity purification described here combined with mass spectrometry to identify novel PI(3,5)P_2 effectors.

Experimental Section

(i) Monosilylation of Pentol 2. Pentol **2**[21] (590 mg, 1.41 mmol) was dried by azeotropic distillation with pyridine under reduced pressure and thoroughly concentrated under high vacuum. The resultant foam was dissolved in pyridine (2.3 mL) and CH_2Cl_2 (1.0 mL) and stirred under argon at −78 °C while a solution of TESCl (0.29 mL, 1.7 mmol) in CH_2Cl_2 (1.0 mL) was added over 15 min. After 3 h at −78 °C, the reaction was allowed to warm to rt. The mixture was diluted with EtOAc, washed with water and brine, and dried over $MgSO_4$. Flash chromatography was carried out on silica gel, using 10%, 12%, 20%, and 65% EtOAc/petroleum ether as eluants, to give silyl ethers **6** (127 mg, 14%), **7** (20 mg, 2%), **5** (8 mg, 1%), and **4** (534 mg, 71%), respectively. Compounds **5**−**7** were isolated as colorless gums, while compound **4** was obtained as a white foam. **(ii) Bis-silylation of Pentol 2.** A solution of **2** (6.18 g, 14.8 mmol, dried using the above procedure) in CH_2Cl_2 (15 mL) and pyridine (10 mL) was stirred under argon at −78 °C while a solution of TESCl (4.52 g, 30.0 mmol) in CH_2Cl_2 (10 mL) was added over 15 min. The reaction was stirred for a further 1 h at −75 °C and then allowed to warm to 0 °C over 3 h. Workup and purification, as described above, gave compounds **6** (6.78 g, 71%), **7** (1.32 g, 14%), and **4** (0.62 g, 8%).

1-D-1-*O*-*tert*-Butyldiphenylsilyl-3-*O*-triethylsilyl-*myo*-inositol (4): mp 55−57 °C; $[\alpha]^{20}_D$ −10.4 (*c* 2 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.42−0.54 (m, 6 H), 0.86 (t, J = 7.9 Hz, 9 H), 1.11 (s, 9 H), 2.29 (d, J = 2.3 Hz, 1 H, 4-OH), 2.32 (s, 1 H, 2-OH), 2.36 (d, J = 2.6 Hz, 1 H, 6-OH), 2.66 (d, J = 2.0 Hz, 1 H, 5-OH), 3.17 (dd, J = 2.8, 9.2 Hz, 1 H, H-3), 3.22 (dt, J = 2.0, 9.3 Hz, 1 H, H-5), 3.54 (dd, J = 2.8, 9.3 Hz, 1 H, H-1), 3.57 (t, J = 2.8 Hz, 1 H, H-2), 3.74 (dt, J = 2.3, 9.3 Hz, 1 H, H-4), 3.97 (dt, J = 2.6, 9.3 Hz, 1 H, H-6), 7.37−7.41 (m, 4 H), 7.43−7.47 (m, 2 H), 7.72−7.76 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 6.6, 19.4, 27.0, 72.8, 72.9, 73.0, 73.2, 73.9, 74.2, 127.8, 127.9, 130.0, 133.1, 133.4, 135.8; HRMS(ESI) calcd for $C_{28}H_{44}NaO_6Si_2$ $[M + Na]^+$ 555.2574, found 555.2562.

1-D-1-*O*-*tert*-Butyldiphenylsilyl-4-*O*-triethylsilyl-*myo*-inositol (5): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.66 (q, J = 7.9 Hz, 6 H), 0.95 (t, J = 7.9 Hz, 9 H), 1.10 (s, 9 H), 2.18 (br, 1 H, 6-OH), 2.26 (d, J = 5.8 Hz, 1 H, 3-OH), 2.39 (br, 1 H, 5-OH), 2.44 (br, 1 H, 2-OH), 3.12 (t, J = 9.1 Hz, 1 H, H-5), 3.18 (ddd, J = 3.1, 5.8, 9.1 Hz, 1 H, H-3), 3.56 (dd, J = 2.9, 9.2 Hz, 1 H, H-1), 3.76 (t, J = 9.1 Hz, 1 H, H-4), 3.85−3.89 (m, 2 H, H-2 and H-6), 7.38−7.41 (m, 4 H), 7.43−7.47 (m, 2 H), 7.69−7.73 (m, 4 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 5.2, 6.8, 19.4, 27.0, 72.0, 72.4, 73.4, 74.4, 74.57, 74.64, 127.9, 130.1, 130.2, 132.9, 133.2, 135.7, 135.8; HRMS(ESI) calcd for $C_{28}H_{44}NaO_6Si_2$ $[M + Na]^+$ 555.2574, found 555.2571.

1-D-3,5-*O*-Bis(triethylsilyl)-1-*O*-*tert*-butyldiphenylsilyl-*myo*-inositol (6): $[\alpha]^{20}_D$ +1.35 (*c* 2 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.39−0.51 (m, 6 H), 0.66 (q, J = 7.9 Hz, 6 H), 0.85 (t, J = 7.9 Hz, 9 H), 0.96 (t, J = 7.9 Hz, 9 H), 1.11 (s, 9 H), 2.09 (d, J = 2.1 Hz, 1 H, 4-OH), 2.23 (d, J = 2.3 Hz, 1 H, 6-OH), 2.28 (s, 1 H, 2-OH), 3.11 (dd, J = 2.8, 9.2 Hz, 1 H, H-3), 3.18 (t, J = 9.1 Hz, 1 H, H-5), 3.49−3.54 (m, 2 H, H-2 and H-1), 3.64 (dt, J = 2.1, 9.2 Hz, 1 H, H-4), 3.89 (dt, J = 2.3, 9.1 Hz, 1 H, H-6), 7.36−7.46 (m, 6 H), 7.74−7.77 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.8, 5.3, 6.7, 6.9, 19.5, 27.0, 72.8, 73.1, 73.3, 73.4, 74.2, 76.0, 127.7, 129.8, 129.9, 133.3, 133.8, 135.8, 136.0; HRMS(ESI) calcd for $C_{34}H_{58}NaO_6Si_3$ $[M + Na]^+$ 669.3439, found 669.3428.

1-D-3,4-*O*-Bis(triethylsilyl)-1-*O*-*tert*-butyldiphenylsilyl-*myo*-inositol (7): $[\alpha]^{20}_D$ −13.2 (*c* 2 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR

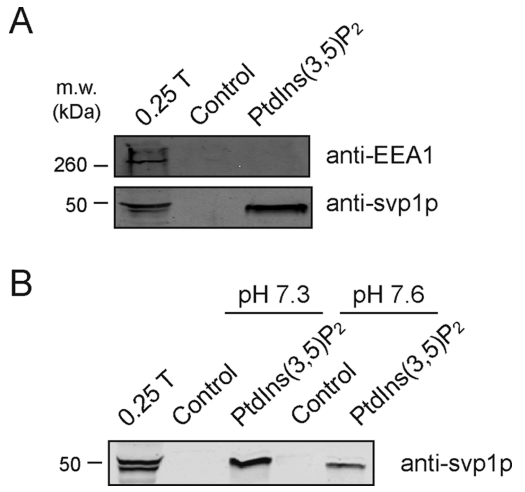


FIGURE 1. (A) Bovine adrenal chromaffin cytosol extract was incubated with biotin−PI(3,5)P_2 conjugated to Neutravidin Plus beads [PtdIns(3,5)P_2] or beads alone (control). Beads were washed extensively, and associated proteins were analyzed by SDS-PAGE and Western blotting together with an untreated portion of the cytosolic extract (0.25 T). Antibodies against svp1p and EEA1 were used to identify these proteins. (B) Biotin−PI(3,5)P_2 pull-downs were performed at pH 7.3 or 7.6 and associated proteins analyzed by SDS-PAGE and Western blotting.

(300 MHz, $CDCl_3$) δ 0.47 (q, J = 8.0 Hz, 6 H), 0.63 (q, J = 8.0 Hz, 6 H), 0.87 (t, J = 8.0 Hz, 9 H), 0.94 (t, J = 8.0 Hz, 9 H), 1.11 (s, 9 H), 2.33 (d, J = 1.5 Hz, 1 H, 2-OH), 2.42 (d, J = 3.0 Hz, 1 H, 6-OH), 2.45 (d, J = 3.1 Hz, 1 H, 5-OH), 3.16 (dt, J = 3.1, 8.7 Hz, 1 H, H-5), 3.25 (dd, J = 2.8, 8.5 Hz, 1 H, H-3), 3.57 (dd, J = 2.8, 8.8 Hz, 1 H, H-1), 3.62 (m, 1 H, H-2), 3.79 (t, J = 8.5 Hz, 1 H, H-4), 3.94 (dt, J = 3.0, 8.8 Hz, 1 H, H-6), 7.35−7.47 (m, 6 H), 7.70−7.77 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.9, 5.2, 6.8, 6.9, 19.4, 27.0, 72.4, 72.9, 73.7, 74.2, 74.4, 74.5, 127.7, 127.8, 129.89, 129.93, 133.2, 133.5, 135.8, 135.9; HRMS(ESI) calcd for $C_{34}H_{58}NaO_6Si_3$ $[M + Na]^+$ 669.3439, found 669.3422.

1-D-3,5-*O*-Bis(triethylsilyl)-1-*O*-*tert*-butyldiphenylsilyl-2,4,6-*O*-tris(methoxymethylene)-*myo*-inositol. To a solution of triol **6** (2.48 g, 3.83 mmol, previously dried by azeotropic distillation with toluene under reduced pressure) in DMF (9 mL) was added *N,N*-diisopropylethylamine (7.0 mL, 40 mmol) followed by MOMCl solution[40] (~6 M in MeOAc, 4.3 mL, 26 mmol). The reaction vessel was sealed and heated at 65 °C for 64 h. After being cooled to rt, the mixture was partitioned between petroleum ether and water, and the organic phase was washed with 0.1 M aq HCl, water, and brine and dried over $MgSO_4$ to give the crude MOM-protected product (3.3 g). A comparable sample was purified by flash chromatography on silica gel, using 8% EtOAc/petroleum ether as eluant, to afford the title compound as a colorless gum: $[\alpha]^{20}_D$ +18.6 (c 2 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.29−0.38 (m, 6 H), 0.72 (q, J = 7.9 Hz, 6 H), 0.82 (t, J = 7.9 Hz, 9 H), 1.00 (t, J = 7.9 Hz, 9 H), 1.09 (s, 9 H), 3.10 (dd, J = 2.2, 9.5 Hz, 1 H), 3.17 (t, J = 2.2 Hz, 1 H), 3.30 (t, J = 9.2 Hz, 1 H), 3.34 (s, 3 H), 3.39 (s, 3 H), 3.47 (s, 3 H), 3.62 (t, J = 9.4 Hz, 1 H), 3.74 (dd, J = 2.2, 9.8 Hz, 1 H), 3.90 (t, J = 9.5 Hz, 9 H), 4.60 (d, J = 6.0 Hz, 1 H), 4.64 (d, J = 6.0 Hz, 1 H), 4.72 (d, J = 5.8 Hz, 1 H), 4.74 (d, J = 5.8 Hz, 1 H), 4.87 (d, J = 5.7 Hz, 1 H), 4.94 (d, J = 5.7 Hz, 1 H), 7.35−7.40 (m, 4 H), 7.42−7.46 (m, 2 H), 7.72 (m, 2 H), 7.86 (m, 2 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.7, 5.2, 6.8, 7.1, 19.2, 27.3, 55.7, 56.5, 56.9, 73.0, 73.9, 75.0, 78.7, 79.0, 79.8, 97.7, 98.4, 99.0, 127.6, 127.8, 129.6, 129.9, 133.1, 134.4, 136.0, 136.4; HRMS-(ESI) calcd for $C_{40}H_{70}NaO_9Si_3$ $[M + Na]^+$ 801.4225, found 801.4221.

1-D-1-*O*-*tert*-Butyldiphenylsilyl-2,4,6-*O*-tris(methoxymethylene)-*myo*-inositol (8).[11] The crude MOM-protected product obtained above (3.3 g) was dissolved in 3:3:1 THF/HOAc/water (28 mL) and heated at 40 °C for 48 h. After concentration of the solvents under reduced pressure, the crude product was purified by flash chromatography on silica gel, using 40−45% EtOAc/petroleum ether as eluant, to afford diol **8** as a white crystalline solid (1.90 g, 90% over two steps): mp 116−117 °C; $[\alpha]^{20}_D$ +77.8 (c 2 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.07 (s, 9 H), 3.19 (ddd, J = 2.5, 7.0, 9.7 Hz, 1 H), 3.25 (m, 1 H), 3.33 (s, 3 H), 3.41 (s, 3 H), 3.43 (s, 3 H), 3.57−3.61 (m, 2 H), 3.70−3.76 (m, 3 H), 4.32 (d, J = 1.2 Hz, 1 H), 4.34 (d, J = 6.8 Hz, 1 H), 4.53 (d, J = 6.5 Hz, 1 H), 4.62 (d, J = 6.8 Hz, 1 H), 4.76 (d, J = 6.5 Hz, 1 H), 4.80 (d, J = 6.6 Hz, 1 H), 4.84 (d, J = 6.6 Hz, 1 H), 7.36−7.45 (m, 6 H), 7.68−7.71 (m, 4 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 19.3, 27.0, 55.7, 55.8, 56.0, 70.4, 72.6, 73.5, 81.91, 81.93, 85.0, 98.1, 98.60, 98.63, 127.6, 127.7, 129.7, 130.0, 133.6, 133.7, 135.8, 135.9; HRMS-(ESI) calcd for $C_{28}H_{42}NaO_9Si$ $[M + Na]^+$ 573.2496, found 573.2491.

1-D-2,4,6-*O*-Tris(methoxymethylene)-*myo*-inositol 3,5-Bis(dibenzyl phosphate) (9). Alcohol **9** was obtained from diol **8** in two steps (90%) according to the literature procedure.[11] ^{1}H and ^{13}C NMR data were in good agreement with those previously reported. Additional spectroscopic data: $[\alpha]^{20}_D$ −8.4 (c 2.4 g 100 mL^{-1}, $CDCl_3$); ^{31}P NMR (121 MHz, $CDCl_3$) δ −1.3; HRMS(ESI) calcd for $C_{40}H_{50}NaO_{15}P_2$ $[M + Na]^+$ 855.2523, found 855.2529.

1D-1-*O*-*tert*-Butyldiphenylsilyl-3-*O*-triethylsilyl-2,4,6-*O*-tris-(methoxymethylene)-*myo*-inositol (10). A solution of diol **8** (1.86 g, 3.38 mmol) in dry pyridine (3.4 mL) and CH_2Cl_2 (3.4 mL) was stirred at −90 °C while a solution of TESCl (0.67 mL, 4.0 mmol) in CH_2Cl_2 (1.5 mL) was added over 2 min. After being stirred at −78 °C for 3 h, the mixture was allowed to warm to rt overnight. The reaction was diluted with EtOAc, washed with water and brine, and dried over $MgSO_4$. Purification by flash chromatography on silica gel, using 15−20% EtOAc/petroleum ether as eluant, gave **10** as a colorless gum (1.79 g, 80%): $[\alpha]^{20}_D$ +10.7 (c 2.5 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.44 (q, J = 7.9 Hz, 6 H), 0.86 (t, J = 7.9 Hz, 9 H), 1.08 (s, 9 H), 3.20−3.25 (m, 2 H), 3.34 (s, 3 H), 3.39 (s, 3 H), 3.41 (m, 1 H), 3.43 (s, 3 H), 3.66 (t, J = 9.2 Hz, 1 H), 3.73 (dd, J = 2.0, 9.6 Hz, 1 H), 3.84 (t, J = 9.1 Hz, 1 H), 4.24 (d, J = 1.0 Hz, 1 H, exch), 4.51 (d, J = 6.5 Hz, 1 H), 4.66 (d, J = 6.1 Hz, 1 H), 4.69−4.73 (m, 2 H), 4.75−4.78 (m, 2 H), 7.33−7.46 (m, 6 H), 7.68−7.76 (m, 4 H); ^{1}H NMR (300 MHz, d6-acetone) δ 0.49 (q, J = 7.9 Hz, 6 H), 0.88 (t, J = 7.9 Hz, 9 H), 1.10 (s, 9 H), 3.26 (m, 1 H), 3.33 (s, 3 H), 3.37 (s, 3 H), 3.38 (s, 3 H), 3.47 (dd, J = 2.2, 9.4 Hz, 1 H), 3.51 (t, J = 2.2 Hz, 1 H), 3.64 (t, J = 9.4 Hz, 1 H), 3.81 (t, J = 9.4 Hz, 1 H), 3.97 (dd, J = 2.2, 9.4 Hz, 1 H), 4.15 (d, J = 2.2 Hz, 0.7 H, exch), 4.62 (d, J = 6.4 Hz, 1 H), 4.69 (d, J = 6.0 Hz, 1 H), 4.72−4.80 (m, 4 H), 7.40−7.51 (m, 6 H), 7.75−7.83 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 6.7, 19.3, 27.1, 55.7, 55.8, 72.4, 73.1, 73.3, 79.6, 81.5, 83.3, 97.5, 98.3, 98.5, 127.5, 127.7, 129.6, 129.8, 133.5, 133.9, 136.0, 136.1; HRMS(ESI) calcd for $C_{34}H_{56}NaO_9Si_2$ $[M + Na]^+$ 687.3361, found 687.3340.

1D-1-*O*-*tert*-Butyldiphenylsilyl-2,4,5,6-*O*-tetrakis(methoxymethylene)-3-*O*-triethylsilyl-*myo*-inositol. To a solution of tetrol **4** (687 mg, 1.29 mmol) in DMF (3 mL) in a pressure vessel was added *N,N*-diisopropylethylamine (2.2 mL, 13 mmol) followed by MOMCl solution[40] (~6 M in MeOAc, 1.8 mL, 11 mmol). The reaction vessel was sealed and heated at 60 °C for 4 days. After being cooled to rt, the mixture was partitioned between petroleum ether and water, and the organic phase was washed with 0.1 M aq HCl, water and brine and dried over $MgSO_4$ to give the crude MOM-protected product (996 mg). A comparable sample was purified by flash

(40) Amato, J. S.; Karady, S.; Sletzinger, M.; Weinstock, L. M. *Synthesis* **1979**, 970.

chromatography on silica gel, using 15% EtOAc/petroleum ether as eluant, to afford the title compound as a colorless gum: $[\alpha]^{20}_D$ +10.9 (*c* 1.8 g 100 mL^{-1}, $CDCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.28−0.39 (m, 6 H), 0.82 (t, J = 8.0 Hz, 9 H), 1.08 (s, 9 H), 3.16 (dd, J = 2.1, 9.5 Hz, 1 H), 3.19 (t, J = 2.1 Hz, 1 H), 3.29−3.32 (m, 4 H), 3.42 (s, 3 H), 3.48 (s, 3 H), 3.49 (s, 3 H), 3.74−3.79 (m, 2 H), 4.01 (t, J = 9.6 Hz, 1 H), 4.57 (d, J = 6.0 Hz, 1 H), 4.62 (d, J = 6.0 Hz, 1 H), 4.72 (d, J = 6.1 Hz, 1 H), 4.79 (d, J = 6.1 Hz, 1 H), 4.83 (d, J = 6.0 Hz, 1 H), 4.91 (d, J = 6.0 Hz, 1 H), 4.94 (d, J = 6.0 Hz, 1 H), 5.00 (d, J = 6.0 Hz, 1 H), 7.35−7.46 (m, 6 H), 7.71 (m, 2 H), 7.79 (m, 2 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.7, 6.8, 19.3, 27.3, 55.7, 56.5, 56.6, 56.7, 73.2, 74.1, 78.6, 79.0, 79.2, 79.7, 97.7, 98.66, 98.69, 99.3, 127.7, 127.8, 129.7, 129.9, 133.1, 134.1, 136.0, 136.2; HRMS(ESI) calcd for $C_{36}H_{60}NaO_{10}Si_2$ [M + Na]$^+$ 731.3623, found 731.3622.

1D-1-*O*-*tert*-Butyldiphenylsilyl-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (11).[11] The crude MOM-protected product obtained above (996 mg) was dissolved in 3:3:1 THF/HOAc/water (8.4 mL) and stirred at 40 °C for 24 h. The volatiles were concentrated under reduced pressure, and the residue was coevaporated with toluene twice to remove residual HOAc. Purification by flash chromatography on silica gel, using 20% acetone/petroleum ether as eluant, gave **11** as a colorless gum (521 mg, 68% over two steps): $[\alpha]^{20}_D$ +52 (*c* 2.0 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 1.09 (s, 9 H), 3.05 (ddd, J = 2.3, 7.1, 9.4 Hz, 1 H), 3.21 (t, J = 2.3 Hz, 1 H), 3.34−3.38 (m, 4 H), 3.39 (s, 3 H), 3.44 (s, 3 H), 3.45 (s, 3 H), 3.60 (t, J = 9.4 Hz, 1 H), 3.81 (dd, J = 2.3, 9.5 Hz, 1 H), 3.85 (d, J = 7.1 Hz, 1 H), 3.98 (t, J = 9.5 Hz, 1 H), 4.30 (d, J = 6.5 Hz, 1 H), 4.60 (d, J = 6.5 Hz, 1 H), 4.74−4.78 (m, 3 H), 4.82 (d, J = 6.3 Hz, 1 H), 4.92 (d, J = 6.3 Hz, 1 H), 4.99 (d, J = 6.2 Hz, 1 H), 7.36−7.40 (m, 4 H), 7.43−7.46 (m, 2 H), 7.70 (m, 2 H), 7.73 (m, 2 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 19.2, 27.1, 55.8, 55.9, 56.3, 56.5, 70.6, 73.9, 78.9, 79.1, 81.2, 81.4, 98.3, 98.36, 98.44, 99.0, 127.77, 127.83, 129.9, 130.1, 132.9, 134.0, 135.9, 136.0; HRMS(ESI) calcd for $C_{30}H_{46}NaO_{10}Si$ [M + Na]$^+$ 617.2758, found 617.2756.

1-*O*-Stearoyl-3-*O*-triethylsilyl-*sn*-glycerol (14). To a solution of acetonide **12**[24] (1.02 g, 2.56 mmol) in dry DCE (10 mL) was added *N*,*N*-diisopropylethylamine (0.90 mL, 5.2 mmol) followed by TESOTf (0.70 mL, 3.1 mmol). The mixture was stirred at reflux for 12 h, after which time further TESOTf (0.30 mL, 1.3 mmol) was added and heating was continued for an additional 22 h. After being cooled to rt, the reaction was diluted with petroleum ether, washed with 0.1 M aq HCl, water, and brine, and dried over $MgSO_4$ to afford the intermediate 2-*O*-isopropenyl-1-*O*-stearoyl-3-*O*-triethylsilyl-*sn*-glycerol (**13**), together with ∼15% unreacted starting material. This material was dissolved in THF (21 mL), and 10% aq $NaHCO_3$ (9 mL) was added, followed by iodine (956 mg, 3.77 mmol). The mixture was stirred vigorously at rt for 80 min, at which time TLC analysis indicated that the isopropenyl ether had been completely consumed. After being quenched with saturated aq $Na_2S_2O_3$, the product was extracted with Et_2O, washed with brine, and dried over $MgSO_4$. Flash chromatography on silica gel, using 3−5% acetone/petroleum ether as eluant, gave the pure secondary alcohol **14** as a colorless low-melting solid (890 mg, 74%, two steps): $[\alpha]^{20}_D$ +1.1 (*c* 4.0 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.62 (q, J = 8.0 Hz, 6 H), 0.88 (t, J = 6.9 Hz, 3 H), 0.96 (t, J = 8.0 Hz, 9 H), 1.25−1.33 (m, 28 H), 1.62 (pentet, J = 7.5 Hz, 2 H), 2.33 (t, J = 7.5 Hz, 2 H), 2.53 (d, J = 5.2 Hz, 1 H), 3.60 (dd, J = 5.8, 10.1 Hz, 1 H), 3.67 (dd, J = 4.6, 10.1 Hz, 1 H), 3.87 (m, 1 H), 4.12 (dd, J = 4.9, 11.5 Hz, 1 H), 4.15 (dd, J = 6.0, 11.5 Hz, 1 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.3, 6.7, 14.1, 22.7, 25.0, 29.2, 29.3, 29.4, 29.5, 29.6, 29.65, 29.67, 29.69, 31.9, 34.2, 63.4, 65.0, 70.1, 173.9; HRMS(ESI) calcd for $C_{27}H_{56}NaO_4Si$ [M + Na]$^+$ 495.3846, found 495.3846.

2-*O*-Arachidonoyl-1-*O*-stearoyl-3-*O*-triethylsilyl-*sn*-glycerol (15). To a solution of alcohol **14** (890 mg, 1.88 mmol) in dry CH_2Cl_2 (10 mL) were added DCC (564 mg, 2.73 mmol), arachidonic acid (630 mg, 2.07 mmol), and DMAP (50 mg, 0.41 mmol). After being stirred at rt for 2.5 h, the reaction was quenched by the addition of HOAc (50 μL) followed by water (20 μL). The mixture was diluted with petroleum ether, filtered, and concentrated to give an oil. Flash chromatography on silica gel, using 1.5−2% acetone/petroleum ether as eluant, followed by a second column using 5−7% Et_2O/petroleum ether as eluant, gave the protected diacylglycerol **15** (1.178 g, 82%): $[\alpha]^{20}_D$ +8.1 (*c* 4.0 g 100 mL^{-1}, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.60 (q, J = 8.0 Hz, 6 H), 0.87−0.91 (m, 6 H), 0.95 (t, J = 8.0 Hz, 9 H), 1.26−1.39 (m, 34 H), 1.61 (pentet, J = 7.3 Hz, 2 H), 1.70 (pentet, J = 7.5 Hz, 2 H), 2.06 (q, J = 7.1 Hz, 2 H), 2.12 (q, J = 6.9 Hz, 2 H), 2.28−2.34 (m, 4 H), 2.80−2.85 (m, 6 H), 3.69−3.75 (m, 2 H), 4.16 (dd, J = 6.2, 11.8 Hz, 1 H), 4.35 (dd, J = 3.7, 11.8 Hz, 1 H), 5.07 (m, 1 H), 5.31−5.43 (m, 8 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.3, 6.6, 14.0, 14.1, 22.6, 22.7, 24.8, 24.9, 25.6, 25.7, 26.5, 27.2, 29.1, 29.30, 29.32, 29.4, 29.5, 29.6, 29.65, 29.70, 31.5, 31.9, 33.7, 34.2, 61.2, 62.4, 71.9, 127.6, 127.9, 128.1, 128.3, 128.6, 128.9, 130.5, 172.8, 173.4; HRMS(ESI) calcd for $C_{47}H_{86}NaO_5Si$ [M + Na]$^+$ 781.6142, found 781.6154.

2-*O*-Arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (16).[24] The protected diacylglycerol **15** (1.09 g, 1.43 mmol) was stirred at rt with a solution of $FeCl_3$ in 3:1 reagent-grade MeOH/CH_2Cl_2 (5 mM, 14 mL). The starting material fully dissolved after 25 min, and after 8.5 h the solution was partitioned between Et_2O and water. The organic phase was washed with brine and dried over $MgSO_4$. Flash chromatography on silica gel, using 25−35% Et_2O/petroleum ether (containing 0.05% HOAc) as eluant, gave the desired product **16** as an oil (836 mg, 90%): $[\alpha]^{20}_D$ +2.6 (*c* 4.0 g 100 mL^{-1}, pyridine), −0.45 (*c* 3.42 g 100 mL^{-1}, toluene) [lit.[24] $[\alpha]^{20}_D$ −0.4 (*c* 3.42 g 100 mL^{-1}, toluene)]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87−0.90 (m, 6 H), 1.26−1.39 (m, 34 H), 1.61 (pentet, J = 7.4 Hz, 2 H), 1.72 (pentet, J = 7.5 Hz, 2 H), 2.00 (t, J = 8.0 Hz, 1 H), 2.06 (m, 2 H), 2.13 (m, 2 H), 2.32 (t, J = 7.4 Hz, 2 H), 2.37 (t, J = 7.5 Hz, 2 H), 2.80−2.85 (m, 6 H), 3.69−3.77 (m, 2 H), 4.24 (dd, J = 5.6, 11.9 Hz, 1 H), 4.32 (dd, J = 4.6, 11.9 Hz, 1 H), 5.08 (m, 1 H), 5.31−5.44 (m, 8 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.0, 14.1, 22.6, 22.7, 24.8, 24.9, 25.62, 25.64, 26.5, 27.2, 29.1, 29.26, 29.32, 29.35, 29.5, 29.6, 29.65, 29.69, 31.5, 31.9, 33.7, 34.1, 61.6, 62.0, 72.2, 127.5, 127.8, 128.1, 128.3, 128.6, 128.8, 129.0, 130.5, 173.1, 173.8; HRMS(ESI) calcd for $C_{41}H_{72}NaO_5$ [M + Na]$^+$ 667.5277, found 667.5271.

2-*O*-Arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-(benzyl diisopropylphosphoramidite) (17).[17] Diacylglycerol **16** (798 mg, 1.24 mmol) and 1*H*-tetrazole (88 mg, 1.26 mmol), dried by azeotropic distillation of toluene on a rotary evaporator, were suspended in dry CH_2Cl_2 (12 mL) and stirred in an ice bath under Ar while benzyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (540 mg, 1.60 mmol) was quickly added. After 10 min, the ice bath was removed, and stirring was continued at rt for 45 min. The mixture was concentrated under reduced pressure, suspended in petroleum ether, and immediately purified by rapid flash chromatography on Et_3N-neutralized silica gel, using 10:90:3 EtOAc/petroleum ether/Et_3N as eluant, to afford the phosphoramidite **17** (1:1 mixture of diastereomers) as a colorless oil (929 mg, 85%). The oil was stored under argon at −20 °C until ready for use: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87−0.90 (m, 6 H), 1.17−1.19 (m, 12 H), 1.25−1.39 (m, 34 H), 1.57−1.63 (m, 2 H), 1.66−1.72 (m, 2 H), 2.03−2.08 (m, 2 H), 2.08−2.12 (m, 2 H), 2.27−2.33 (m, 4 H), 2.79−2.85 (m, 6 H), 3.60−3.67 (m, 2 H), 3.69−3.81 (m, 2 H), 4.15−4.19 (m, 1 H), 4.32−4.37 (m, 1 H), 4.63−4.68 (m, 1 H), 4.71−4.76 (m, 1 H), 5.17−5.21 (m, 1 H), 5.31−5.43 (m, 8 H) 7.24−7.27 (m, 1 H), 7.31−7.35 (m, 4 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.05, 14.09, 22.6, 22.7, 24.5−24.59 (2 × d), 24.63, 24.68, 24.8, 24.9, 25.62, 25.63, 25.65, 26.6, 27.2, 29.2, 29.30, 29.32, 29.36, 29.5, 29.64, 29.66, 29.67, 29.70, 31.5, 31.9, 33.8, 34.1, 43.1−43.2 (2 × d), 61.5−61.8 (2 × d), 62.46, 62.51, 65.3−65.5 (2 × d), 70.9−71.0 (2 × d), 127.0, 127.3, 127.6, 127.9, 128.2, 128.3, 128.6, 128.87, 128.91, 130.5, 139.3−139.4 (2 × d),

172.7, 173.4; ^{31}P NMR (202 MHz, $CDCl_3$) δ 148.9, 149.1; HRMS(ESI) calcd for $C_{54}H_{92}NNaO_6P$ $[M + Na]^+$ 904.6560, found 904.6554.

1D-1-*O*-(2-*O*-Arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-*O*-benzylphospho)-2,4,6-*O*-tris(methoxymethylene)-*myo*-inositol 3,5-Bis(dibenzyl phosphate) (18). A mixture of inositol **9** (160 mg, 0.192 mmol) and 1*H*-tetrazole (41 mg, 0.59 mmol), dried by azeotropic distillation of toluene under reduced pressure, was stirred in dry CH_2Cl_2 (1 mL) at rt while neat phosphoramidite **17** (373 mg, 0.423 mmol) was added from a syringe over 1 h. The reaction was stirred at rt for a further 17 h and then cooled to −25 °C, and the phosphite intermediate was oxidized with a solution of Bu_4NIO_4 (220 mg, 0.508 mmol) in CH_2Cl_2 (1.5 mL). After being warmed to rt over 1.5 h, the reaction was quenched with 10% aq $Na_2S_2O_3$ and diluted with EtOAc. The organic phase was washed with water and brine and dried over $MgSO_4$. Purification by flash chromatography on silica gel, using 20%−25% acetone/petroleum ether as eluant, gave the fully protected PI(3,5)P_2 **18** (mixture of diastereomers) as a colorless gum (247 mg, 79%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87−0.90 (m, 6 H), 1.25−1.40 (m, 34 H), 1.53−1.60 (m, 2 H), 1.62−1.70 (m, 2 H), 2.03−2.10 (m, 4 H), 2.23−2.32 (m, 4 H), 2.77−2.84 (m, 6 H), 3.31 (s, 1.5 H), 3.317 (s, 1.5 H), 3.319 (s, 1.5 H), 3.34 (s, 1.5 H), 3.35 (s, 1.5 H), 3.37 (s, 1.5 H), 4.02−4.30 (m, 9 H), 4.50 (m, 1 H), 4.67−4.78 (m, 6 H), 5.00−5.09 (m, 9 H), 5.12 (m, 1 H), 5.17 (m, 1 H), 5.30−5.42 (8 H), 7.27−7.38 (m, 25 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.0, 14.1, 22.6, 22.7, 24.7, 24.8, 25.59, 25.61, 25.64, 26.5, 27.2, 29.1, 29.29, 29.30, 29.34, 29.5, 29.64, 29.67, 29.69, 31.5, 31.9, 33.49, 33.51, 34.0, 55.9, 56.58, 56.64, 56.65, 61.6, 65.53, 65.57, 65.74, 65.79, 69.38, 69.42, 69.50, 69.52, 69.55, 69.56, 69.62, 69.67, 69.85, 69.90, 75.45, 75.50, 75.64, 75.67, 75.82, 75.86, 75.95, 75.98, 76.02, 76.06, 79.7, 79,75, 79.79, 98.08, 98.10, 98.48, 98.53, 127.5, 127.8, 127.95, 127.96, 127.98, 128.01, 128.03, 128.1, 128.3, 128.5, 128.55, 128.60, 128.7, 128.8, 128.90, 128.91, 130.5, 135.5, 135.6, 135.65, 135.69, 135.71, 135.75, 135.77, 135.88, 135.93, 172.48, 172.51, 173.1; ^{31}P NMR (202 MHz, $CDCl_3$) δ −1.42, −1.38, −1.373, −1.366, −1.18, −1.17; HRMS(ESI) calcd for $C_{88}H_{127}NaO_{22}P_3$ $[M + Na]^+$ 1651.7930, found 1651.7916.

L-α-Phosphatidyl-D-*myo*-inositol 3,5-Bisphosphate (19).[17] Under an atmosphere of dry argon, TMSBr (∼0.5 mL) was distilled from calcium hydride directly into an ice-cooled solution of the fully protected PI(3,5)P_2 **18** (31 mg, 0.019 mmol) in 2-methylbut-2-ene (0.5 mL). The solution was protected from light and stirred at rt for 1.5 h and then, without exposure to air, concentrated under high vacuum overnight. The solid was dissolved in freshly distilled EtSH (0.5 mL) at rt. After 20 h, the solution was concentrated under reduced pressure. The resulting solid was dissolved in dilute aq NH_3 (pH 8.5), and the product was lyophilized. Conversion to the sodium salt by ion-exchange (Na form) gave PI(3,5)P_2 (**19**) as a white powder (17.7 mg, 84%): ^{1}H NMR (500 MHz, CD_3OD, ammonium salt) δ 0.89−0.92 (m, 6 H), 1.29−1.39 (m, 34 H), 1.59 (m, 2 H), 1.68 (m, 2 H), 2.07 (q, J = 6.9 Hz, 2 H), 2.13 (q, J = 6.0 Hz, 2 H), 2.30 (t, J = 7.4 Hz, 2 H), 2.36 (m, 2 H), 2.81−2.86 (m, 6 H), 3.91−4.15 (m, 7 H), 4.21 (dd, J = 6.8, 12.1 Hz, 1 H), 4.37 (t, J = 2.4 Hz, 1 H), 4.48 (dd, J = 2.9, 12.1 Hz, 1 H), 5.25 (m, 1 H), 5.33−5.39 (m, 8 H); ^{31}P NMR (202 MHz, CD_3OD, ammonium salt) δ −0.1, 1.3, 2.1; HRMS(ESI) calcd for $C_{47}H_{84}O_{19}P_3$ $[M - H]^-$ 1045.4820, found 1045.4828.

1D-1-*O*-(1,2-*O*-Dioctanoyl-*sn*-glycero-3-*O*-benzylphospho)-2,4,6-*O*-tris(methoxymethylene)-*myo*-inositol 3,5-bis(dibenzyl phosphate) (21). A mixture of inositol **9** (301 mg, 0.36 mmol) and 1*H*-tetrazole (75 mg, 1.1 mmol), dried by azeotropic distillation with toluene under reduced pressure, was stirred in dry CH_2Cl_2 (3.5 mL) at 0 °C while neat phosphoramidite **20**[32] (376 mg, 0.65 mmol) was added from a syringe. After the mixture was warmed to rt and stirred for 15 h, TLC analysis indicated some starting material remained, and the reaction was treated with a further portion of **20** (62 mg, 0.11 mmol). After 3 h, the mixture was cooled to −25 °C, and the phosphite intermediate was oxidized with *m*-CPBA (1.0 mmol, prepared by dissolving 350 mg of 50−55% *m*-CPBA in $CHCl_3$, drying over $MgSO_4$, filtering, and concentrating to ∼4 mL). After being stirred at rt overnight, the solution was diluted with EtOAc, washed with 10% aq $Na_2S_2O_3$, 10% aq $NaHCO_3$, and brine, and dried over $MgSO_4$. Flash chromatography on silica gel, using 20−30% acetone/petroleum ether as eluant, afforded the fully protected dioctanoyl PI(3,5)P_2 **21** (mixture of diastereomers) as a colorless gum (404 mg, 84%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85−0.89 (m, 6 H), 1.26, (m, 16 H), 1.57 (m, 4 H), 2.22−2.29 (m, 4 H), 3.31 (s, 1.5 H), 3.318 (s, 1.5 H), 3.319 (s, 1.5 H), 3.34 (s, 1.5 H), 3.35 (s, 1.5 H), 3.37 (s, 1.5 H), 4.03−4.30 (m, 9 H), 4.50 (m, 1 H), 4.67−4.78 (m, 6 H), 5.01−5.14 (m, 10 H), 5.17 (m, 1 H), 7.28−7.38 (m, 25 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.0, 22.6, 24.8, 28.9, 29.00, 29.04, 31.6, 33.9, 34.1, 55.9, 56.56, 56.62, 61.7, 65.5−65.8 (m, 4 peaks), 69.3−69.7 (m, 7 peaks), 69.8, 69.9, 75.3−76.1 (m, 12 peaks), 79.6−79.8 (m, 6 peaks), 98.06, 98.09, 98.46, 98.50, 127.95, 128.02, 128.47, 128.53, 128.6, 128.7, 135.5, 135.56, 135.59, 135.65, 135.74, 135.8, 135.9, 172.7, 173.1; ^{31}P NMR (121 MHz, $CDCl_3$) δ −1.43 (0.5 P), −1.40 (1.5 P), −1.2 (1 P); HRMS(ESI) calcd for $C_{66}H_{91}NaO_{22}P_3$ $[M + Na]^+$ 1351.5113, found 1351.5083.

Dioctanoyl L-α-Phosphatidyl-D-*myo*-inositol 3,5-Bisphosphate (22).[17] Under an atmosphere of dry argon, TMSBr (∼1 mL) was distilled directly into a solution of the fully protected dioctanoyl PI(3,5)P_2 **21** (50 mg, 0.038 mmol) in dry pyridine (0.2 mL) cooled to −78 °C. The reaction was stirred at rt for 50 min and then, without exposure to air, concentrated under high vacuum for 16 h. The residue was dissolved in 8 mM aq NH_4OAc (50 mL) and lyophilized to give a white solid (47 mg). The solid was dissolved in $CHCl_3$−MeOH and passed through Amberlite IR-120-H^+ resin to remove pyridinium salts. After concentration under reduced pressure, the product was dissolved in water, passed through Amberlite IR-120-Na^+, and finally lyophilized to give dioctanoyl PI(3,5)P_2 **22** (sodium salt) as a white powder (29 mg, 97%): ^{1}H NMR (300 MHz, D_2O) δ 0.94 (m, 6 H), 1.36, (m, 16 H), 1.68 (m, 4 H), 2.49 (m, 4 H), 3.94−4.05 (m, 3 H), 4.08−4.19 (m, 4 H), 4.36 (dd, J = 7.1, 12.3 Hz, 1 H), 4.50−4.56 (m, 2 H), 5.38 (m, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 14.1, 22.7, 25.0, 25.1, 28.8, 28.87, 28.91, 31.7, 34.5, 34.6, 63.6, 64.5 (d, J = 5.5 Hz), 70.7, 71.3−71.6 (m, ≥ 6 peaks), 75.5 (d, J = 5.6 Hz), 76.0 (d, J = 5.8 Hz), 79.5 (d, J = 6.1 Hz), 176.7, 177.1; ^{31}P NMR (121 MHz, D_2O) δ −0.6, 0.0, 0.9; HRMS(ESI) calcd for $C_{25}H_{48}O_{19}P_3$ $[M - H]^-$ 745.2003, found 745.2003.

8-(Benzyloxycarbonylamino)octanoic Acid.[41] To a solution of 8-aminooctanoic acid (1.00 g, 6.28 mmol) in 2 N aq NaOH (3.4 mL) were added benzyl chloroformate (0.97 mL, 6.8 mmol) and 2 N aq NaOH (3.4 mL) simultaneously over 1 min, with vigorous stirring. Additional water (10 mL) was added, and after 20 min, the solution was washed with Et_2O. The aqueous layer was acidified to pH 1−2 with 1 M aq HCl, and the resulting white precipitate was extracted with EtOAc. The organic extracts were dried with brine and $MgSO_4$ and concentrated under reduced pressure to give the title compound as a white solid (1.75 g, 95%): mp = 63−64 °C (lit.[42] mp 63−64 °C [EtOAc/petroleum ether]).

1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-2,3-*O*,*O*′-isopropylidene-*sn*-glycerol (23). To a solution of Cbz-protected 8-aminooctanoic acid (1.75 g, 5.96 mmol) in dry CH_2Cl_2 (12 mL) were added DCC (1.23 g, 5.96 mmol), L-2,3-*O*-isopropylidene-*sn*-glycerol (0.73 g, 5.52 mmol), and DMAP (0.22 g, 1.8 mmol). After being stirred for 2 h at rt, the reaction mixture was concentrated under reduced pressure and the residue was taken up in Et_2O. The dicyclohexyl urea was removed by filtration,

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and the filtrate was purified by flash chromatography on silica gel, using 25−30% EtOAc/petroleum ether as eluant, to give acetonide **23** as a white solid (2.035 g, 90% from isopropylidene glycerol): mp 38−39 °C; $[\alpha]^{20}_D$ +1.5 (c 2.0 g 100 mL^{-1}, chloroform); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (m, 6 H), 1.36 (s, 3 H), 1.43 (s, 3 H), 1.49 (m, 2 H), 1.62 (m, 2 H), 2.33 (t, J = 7.5 Hz, 2 H), 3.18 (m, 2 H), 3.73 (dd, J = 6.2, 8.5 Hz, 1 H), 4.07 (dd, J = 6.5, 8.5 Hz, 1 H), 4.09 (dd, J = 5.9, 11.5 Hz, 1 H), 4.15 (dd, J = 4.7, 11.5 Hz, 1 H), 4.31 (m, 1 H), 4.52 (br, 0.15 H), 4.74 (br, 0.85 H), 5.09 (s, 2 H), 7.29−7.36 (m, 5 H); ^{13}C NMR (126 MHz, $CDCl_3$) 24.7, 25.4, 26.5, 26.7, 28.8, 28.9, 29.9, 34.0, 41.0, 64.5, 66.3, 66.6, 73.7, 109.8, 128.0, 128.1, 128.5, 136.7, 156.4, 173.5; HRMS(ESI) calcd for $C_{22}H_{33}NNaO_6$ $[M + Na]^+$ 430.2206, found 430.2204.

1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-3-*O*-triethylsilyl-*sn*-glycerol (25). To a solution of acetonide **23** (700 mg, 1.72 mmol) in DCE (7 mL) was added *N,N*-diisopropylethylamine (1.2 mL, 6.9 mmol), followed by TESOTf (1.2 mL, 5.3 mmol), and the mixture was stirred at 90 °C for 6 h. After being cooled to rt, the mixture was diluted with petroleum ether, washed with 0.1 M aq HCl, water, and brine, and dried over $MgSO_4$. The resulting oil was dissolved in CH_2Cl_2 (20 mL) and stirred vigorously with 1:1 HOAc/water (20 mL) at rt, following the progress of the reaction by TLC (25% EtOAc/petroleum ether, product R_f 0.15). At approximately 75% conversion, the reaction mixture was extracted with petroleum ether (×3), and the extracts were dried with brine and $MgSO_4$. Flash chromatography on silica gel, using 25−30% EtOAc/petroleum ether as eluant, gave the secondary alcohol **25** as a gum (296 mg, 36%): $[\alpha]^{20}_D$ +1.9 (c 5.0 g 100 mL^{-1}, $CDCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.62 (q, J = 8.0 Hz, 6 H), 0.96 (t, J = 8.0 Hz, 9 H), 1.31 (m, 6 H), 1.49 (m, 2 H), 1.62 (m, 2 H), 2.33 (t, J = 7.5 Hz, 2 H), 2.60 (d, J = 5.2 Hz, 1 H), 3.17 (m, 2 H), 3.60 (dd, J = 5.7, 10.1 Hz, 1 H), 3.67 (dd, J = 4.7, 10.1 Hz, 1 H), 3.87 (m, 1 H), 4.11 (dd, J = 6.1, 11.5 Hz, 1 H), 4.15 (dd, J = 4.7, 11.5 Hz, 1 H), 4.58 (br, 0.15 H), 4.77 (br, 0.85 H), 5.09 (s, 2 H), 7.28−7.35 (m, 5 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.3, 6.6, 24.8, 26.4, 28.8, 28.9, 29.9, 34.1, 41.0, 63.4, 65.1, 66.5, 70.0, 128.01, 128.05, 128.5, 136.7, 156.4, 173.8; HRMS(ESI) calcd for $C_{25}H_{43}NNaO_6Si$ $[M + Na]^+$ 504.2757, found 504.2756.

1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-2-*O*-octanoyl-3-*O*-triethylsilyl-*sn*-glycerol (26). To a solution of alcohol **25** (296 mg, 0.61 mmol) in CH_2Cl_2 (6 mL) were added DCC (154 mg, 0.75 mmol), octanoic acid (99 mg, 0.69 mmol), and DMAP (20 mg, 0.16 mmol). After being stirred for 5.5 h at rt, the reaction mixture was concentrated under reduced pressure and purified by flash chromatography on silica gel, using 8−10% acetone/petroleum ether as eluant, to give the diacylated product **26** as a colorless oil (298 mg, 80%): $[\alpha]^{20}_D$ +10.5 (c 3.5 g 100 mL^{-1}, $CDCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.59 (q, J = 8.0 Hz, 6 H), 0.88 (t, J = 7.0 Hz, 3 H), 0.95 (t, J = 8.0 Hz, 9 H), 1.26−1.32 (m, 14 H), 1.49 (m, 2 H), 1.57−1.64 (m, 4 H), 2.28−2.32 (m, 4 H), 3.18 (m, 2 H), 3.71 (dd, J = 5.6, 10.8 Hz, 1 H), 3.73 (dd, J = 5.1, 10.8 Hz, 1 H), 4.16 (dd, J = 6.4, 11.8 Hz, 1 H), 4.35 (dd, J = 3.7, 11.8 Hz, 1 H), 4.51 (br, 0.15 H), 4.74 (br, 0.85 H), 5.05−5.09 (m, 3 H), 7.29−7.36 (m, 5 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 4.3, 6.6, 14.0, 22.6, 24.7, 24.9, 26.5, 28.87, 28.90, 28.96, 29.02, 29.9, 31.6, 34.0, 34.3, 41.0, 61.3, 62.5, 66.6, 71.7, 128.0, 128.1, 128.5, 136.7, 156.4, 173.1, 173.3; HRMS-(ESI) calcd for $C_{33}H_{57}NNaO_7Si$ $[M + Na]^+$ 630.3802, found 630.3805.

1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-2-*O*-octanoyl-*sn*-glycerol (27). To silyl ether **26** (293 mg, 0.48 mmol) was added a solution of $FeCl_3$ in 3:1 reagent-grade MeOH/CH_2Cl_2 (4.9 mL, 5 mM). After 90 min at 17 °C, the solution was partitioned between Et_2O and water, and the organic phase was washed with brine and dried over $MgSO_4$. Flash chromatography on silica gel (deactivated with 5% v/w water), using 30−40% EtOAc/petroleum ether as eluant, gave diacylglycerol **27** as a gum (237 mg, 99%). The four-step sequence from **23** → **27** could also be conducted without chromatographic purification of intermediates, and with the replacement of EDCI for DCC, to give the product in 51% overall yield: $[\alpha]^{20}_D$ −3.2 (c 4.0 g 100 mL^{-1}, $CDCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J = 7.0 Hz, 3 H), 1.26−1.32 (m, 14 H), 1.49 (m, 2 H), 1.58−1.65 (m, 4 H), 2.27 (t, J = 6.2 Hz, 1 H), 2.31 (t, J = 7.5 Hz, 2 H), 2.34 (t, J = 7.5 Hz, 2 H), 3.18 (m, 2 H), 3.71−3.73 (m, 2 H), 4.22 (dd, J = 5.7, 11.9 Hz, 1 H), 4.32 (dd, J = 4.4, 11.9 Hz, 1 H), 4.64 (br, 0.15 H), 4.79 (br, 0.85 H), 5.06−5.10 (m, 3 H), 7.29−7.35 (m, 5 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.0, 22.5, 24.7, 24.9, 26.4, 28.8, 28.9, 29.0, 29.8, 31.6, 34.0, 34.3, 41.0, 61.5, 62.1, 66.6, 72.1, 128.0, 128.5, 136.6, 156.4, 173.4, 173.6; HRMS(ESI) calcd for $C_{27}H_{43}NNaO_7$ $[M + Na]^+$ 516.2937, found 516.2933.

1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-2-*O*-octanoyl-*sn*-glycero-3-(benzyl diisopropylphosphoramidite) (28). To an ice-cooled solution of diacylglycerol **27** (237 mg, 0.48 mmol, dried by azeotropic distillation of toluene on a rotary evaporator) in CH_2Cl_2 (2.5 mL) were added benzyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (214 mg, 0.63 mmol) and 1*H*-tetrazole (33 mg, 0.47 mmol). After 10 min, the ice bath was removed, and stirring was continued at room temperature for 100 min. The mixture was concentrated under reduced pressure and immediately purified by rapid flash chromatography on Et_3N-neutralized silica gel, using 10:88:2 to 15:83:2 EtOAc/petroleum ether/Et_3N as eluant, to afford the phosphoramidite **28** (1:1 mixture of diastereomers) as a gum (307 mg, 87%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (t, J = 6.9 Hz, 3 H), 1.17−1.19 (m, 12 H), 1.26−1.30 (m, 14 H), 1.48 (m, 2 H), 1.56−1.63 (m, 4 H), 2.26−2.30 (m, 4 H), 3.17 (m, 2 H), 3.59−3.67 (m, 2 H), 3.68−3.81 (m, 2 H), 4.14−4.19 (m, 1 H), 4.31−4.37 (m, 1 H), 4.50 (br, 0.15 H), 4.63−4.75 (m, 2.85 H), 5.09 (s, 2 H), 5.18 (m, 1 H), 7.23−7.27 (m, 1 H), 7.28−7.35 (m, 9 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.0, 22.6, 24.5−24.57 (2 × d), 24.60, 24.66, 24.73, 24.9, 26.5, 28.86, 28.90, 28.95, 29.03, 29.9, 31.6, 34.0, 34.3, 41.0, 43.0−43.2 (2 × d), 61.5−61.8 (2 × d), 62.5, 62.6, 65.3−65.5 (2 × d), 66.5, 70.8−70.9 (2 × d), 126.9, 127.3, 128.0, 128.1, 128.2, 128.5, 136.7, 139.3−139.4 (2 × d), 156.4, 173.0, 173.2; ^{31}P NMR (202 MHz, $CDCl_3$) δ 148.9, 149.1; HRMS(ESI) calcd for $C_{40}H_{63}N_2NaO_8P$ $[M + Na]^+$ 753.4220, found 753.4224.

1-D-1-*O*-[1-*O*-[8-(Benzyloxycarbonylamino)octanoyl]-2-*O*-octanoyl-*sn*-glycero-3-*O*-benzylphospho]-2,4,6-*O*-tris(methoxymethylene)-*myo*-inositol 3,5-Bis(dibenzyl phosphate) (29). To an ice-cooled suspension of inositol **9** (99 mg, 0.12 mmol) and 1*H*-tetrazole (15 mg, 0.21 mmol) in CH_2Cl_2 (0.5 mL) was added neat phosphoramidite **28** (96 mg, 0.13 mmol). After being stirred at rt for 2 h, the reaction mixture was cooled to −75 °C and the phosphite intermediate was oxidized with *m*-CPBA (0.18 mmol, prepared by dissolving 62 mg of 50% *m*-CPBA in CH_2Cl_2, drying over $MgSO_4$, filtering and concentrating to ~2 mL). After being warmed to rt, the solution was diluted with EtOAc, washed with 10% aq $Na_2S_2O_3$ (×3), 10% aq $NaHCO_3$ (×3), and brine, and dried over $MgSO_4$. Flash chromatography on silica gel, using 70−80% EtOAc/petroleum ether as eluant, afforded fully protected PI(3,5)P_2−NH(Cbz) **29** (mixture of diastereomers) as a colorless gum (134 mg, 76%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (t, J = 7.0 Hz, 3 H), 1.23−1.30 (m, 14 H), 1.47 (m, 2 H), 1.52−1.60 (m, 4 H), 2.22−2.29 (m, 4 H), 3.16 (m, 2 H), 3.306 (s, 1.5 H), 3.313 (s, 1.5 H), 3.32 (s, 1.5 H), 3.34 (s, 1.5 H), 3.35 (s, 1.5 H), 3.37 (s, 1.5 H), 4.03−4.29 (m, 9 H), 4.50 (s, 1 H), 4.60 (br, 0.2 H), 4.67−4.77 (m, 6 H), 4.82 (br, 0.8 H), 5.00−5.06 (m, 8 H), 5.07−5.13 (m, 4 H), 5.17 (m, 1 H), 7.26−7.38 (m, 30 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.0, 22.5, 24.6, 24.7, 26.5, 28.8, 28.85, 28.88, 29.0, 29.8, 31.6, 33.8, 34.01, 34.03, 41.0, 55.9, 56.5, 56.6, 61.6, 65.50, 65.54, 65.71, 65.75, 66.5, 69.29, 69.32, 69.36, 69.40, 69.45, 69.47, 69.49, 69.51, 69.54, 69.57, 69.59, 69.61, 69.79, 69.84, 75.4−75.5 (m), 75.59, 75.62, 75.77, 75.81, 75.89, 75.94, 75.96, 76.02, 79.65, 79.70, 79.76,

98.0, 98.1, 98.4, 98.5, 127.89, 127.91, 127.92, 127.96, 127.98, 128.03, 128.4, 128.50, 128.54, 128.6, 135.5, 135.55, 135.61, 135.65, 135.66, 135.70, 135.72, 135.8, 135.9, 136.7, 156.3, 172.69, 172.71, 172.96, 172.98; ^{31}P NMR (202 MHz, $CDCl_3$) δ −1.45, −1.41, −1.40, −1.39, −1.19, −1.18; HRMS(ESI) calcd for $C_{74}H_{98}NNaO_{24}P_3$ $[M + Na]^+$ 1500.5589, found 1500.5591.

Biotinylated L-α-Phosphatidyl-D-*myo*-inositol 3,5-Bisphosphate (30). Under anhydrous conditions, TMSBr (~1 mL) was distilled from CaH_2 directly into an ice-cooled flask containing fully protected PI(3,5)P_2−NH(Cbz) **29** (34.7 mg, 0.023 mmol) and 2-methyl-2-butene (0.3 mL), and the resulting solution was stirred at rt for 35 min. Without exposure to air, the solution was concentrated under reduced pressure and dried under high vacuum for 3.5 h. After the vacuum was replaced with Ar, the gummy product was dissolved in freshly distilled ethanethiol (1.5 mL) and stirred at 25 °C for 6 h before the solution was concentrated to a white solid. ^{1}H NMR spectroscopy at this stage indicated the complete removal of all MOM groups (absence of CH_3O signal at 3.49 ppm, solvent: D_2O). After dissolution in aq NH_3 (~8 mM, 20 mL) and subsequent lyophilization, the solid was stirred in 7:5 THF/0.5 M aq NH_3 (3 mL) with 20% $Pd(OH)_2$/C (20 mg) under H_2 (balloon pressure) at rt for 1 h. The mixture was filtered through a glass fiber filter paper, washing the catalyst thoroughly with MeOH, and the filtrate concentrated under reduced pressure to afford the primary amine product. The NH_4^+ counterions were exchanged for Et_3NH^+ counterions by dissolution in 1 M triethylammonium bicarbonate (TEAB) buffer (pH 8.5) and subsequent lyophilization.

To a solution of the resulting solid in 1 M TEAB (pH ~7.7, 1.0 mL) was added a solution of biotin−NHS ester (11.3 mg, 0.033 mmol) in DMF (0.9 mL). After 1 h at rt, the solution (whose pH was now 9.3) was diluted with water and lyophilized. The product was purified by flash chromatography on silica gel, using 9:7:2 $CHCl_3$/MeOH/water as eluant, followed by reversed-phase flash chromatography on C18 silica gel, using 20−35% MeOH/water as eluant, to furnish the title compound **30**. Conversion to the sodium salt by ion exchange (Na form), followed by lyophilization, gave a fluffy white solid (14.6 mg, 59%): ^{1}H NMR (500 MHz, D_2O) δ 0.94 (t, J = 6.9 Hz, 3 H), 1.34−1.41 (m, 14 H), 1.48 (m, 2 H), 1.58 (m, 2 H), 1.65−1.84 (m, 8 H), 2.32 (t, J = 7.1 Hz, 2 H), 2.47 (m, 2 H), 2.51 (t, J = 7.3 Hz, 2 H), 2.87 (d, J = 13.1 Hz, 1 H), 3.07 (dd, J = 5.0, 13.1 Hz, 1 H), 3.25 (m, 2 H), 3.39 (m, 1 H), 3.94−4.00 (m, 3 H), 4.09−4.20 (m, 4 H), 4.37 (dd, J = 7.2, 12.3 Hz, 1 H), 4.48−4.51 (m, 2 H), 4.54 (dd, J = 2.6, 12.3 Hz, 1 H), 4.69 (dd, J = 5.0, 7.9 Hz, 1 H), 5.39 (m, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 14.1, 22.7, 24.9, 25.1, 25.9, 26.6, 28.4, 28.5, 28.7, 28.8, 28.85, 28.89, 31.6, 34.5, 34.7, 36.2, 39.9, 40.3, 56.1, 60.9, 62.7, 63.6, 64.4 (d, J = 5.5 Hz), 70.8, 71.5, 71.6, 71.8 (m), 75.2 (d, J = 5.7 Hz), 76.2 (d, J = 5.9 Hz), 79.0 (m), 176.8, 177.09, 177.13; ^{31}P NMR (202 MHz, D_2O) δ −0.6, 1.0, 2.6; HRMS(ESI) calcd for $C_{35}H_{63}N_3O_{21}P_3S$ $[M - H]^-$ 986.2888, found 986.2874. HPLC analysis was carried out on a Kinetex 2.6 μm C18 column at 40 °C with a flow rate of 0.5 mL/min, eluting with a linear gradient of 10:80:10 to 60:30:10 MeCN/water/100 mM aq NH_4OAc over 20 min. Detection was by electrospray MS in the negative ion mode. Compound **30** had a retention time of 8.0 min and a purity of 95%. See the Supporting Information for trace.

Pull-down Experiments. Chromaffin cytosol[5b] was diluted in pull-down buffer (20 mM Hepes−NaOH, pH 7.3, 150 mM NaCl, 0.5% Nonidet P40) containing Complete protease inhibitor mixture to a final concentration of 2 mg/mL and incubated for 30 min on ice before preclearing by centrifugation for 15 min at 13000 rpm at 4 °C. The cytosolic extract was incubated for 3 h at 4 °C with either 1 nmol of biotinylated PI(3,5)P_2 (**30**) prebound to UltraLink Plus NeutrAvidin beads or beads alone. Beads were washed extensively in pull-down buffer and then resuspended in Laemmli sample buffer containing β-mercaptoethanol. For pH binding studies, chromaffin cytosol was prepared with the pH indicated (Figure 1B), and beads were washed in the respective pH pull-down buffers. Proteins associated with the pull-downs were analyzed by Western blotting with antibodies raised against svp1p (gift from Peter Parker (Cancer Research UK)).

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Supporting Information Available: General experimental details, ^{1}H, ^{13}C, and ^{31}P NMR spectra for all new compounds reported in the manuscript, COSY spectra for compounds **4**−**7**, and HPLC trace for compound **30**. This material is available free of charge via the Internet at http://pubs.acs.org.