A Fully Synthetic and Biochemically Validated Phosphatidyl Inositol-3-Phosphate Hapten via Asymmetric Synthesis and Native Chemical Ligation

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Ⓢ Supporting Information

ABSTRACT: We report the synthesis and biochemical validation of a phosphatidyl inositol-3 phosphate (PI3P) immunogen. The inositol stereochemistry was secured through peptide-catalyzed asymmetric phosphorylation catalysis, and the subsequent incorporation of a cysteine residue was achieved by native chemical ligation (NCL). Conjugation of the PI3P hapten to maleimide-activated keyhole limpet hemocyanin (KLH) provided a PI3P immunogen, which was successfully used to generate selective PI3P antibodies. The incorporation of a sulfhydryl nucleophile into a phosphoinositide hapten demonstrates a general strategy to reliably access phosphoinositide immunogens.
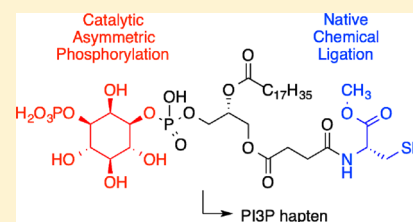
INTRODUCTION

Phosphoinositides are critical components of cellular signaling networks and are involved in regulating numerous membrane trafficking and cellular signaling events. In cells, phosphoinositides are reversibly generated by phosphorylation of phosphatidylinositol (PI) at positions 3, 4, and 5 of the inositol ring, giving rise to seven distinct phosphoinositide species with distinct functions. The various phosphoinositide species are minor constituents of phospholipid bilayers, occurring exclusively on the cytoplasmic side of the plasma membrane and on intracellular organelles where they serve as intermediates in a wide variety of intracellular signaling events. Several studies have indicated that PI3P is localized predominantly at early endosomes, PI4P at Golgi membranes, PI3,5P at late endosomes, and PI4,5P at the plasma membrane.[1]

While the field of phosphoinositide research has progressed significantly over the past decade, there is still a need for tools to probe spatial and temporal characteristics of these distinct and dynamic membrane components. GFP fusion proteins containing minimal phosphoinositide-binding domains such as PH (pleckstrin homology), PX (phox homology), and FYVE (Fab1, YotB, Vac1, EEA1) have been extremely useful probes to follow phosphoinositide dynamics.[2] However, this approach requires the derivatization of a cell line and therefore careful consideration of the effects of overexpression on competition with endogenous lipid binding proteins. Antibodies specific for individual species of phosphoinositides could facilitate monitoring endogenous phosphoinositides. The development of such antibody reagents has progressed slowly due to the apparent lack of immunogenicity of lipids in general. Limited success has been met in the past by inserting phospholipids into liposomes,[3,4] adsorption of phospholipids to bacteria,[5] or conjugation to a carrier protein.[6]

Here we describe the synthesis of a PI3P immunogen **1** (Scheme 1) that successfully elicited the generation of selective PI3P antibodies in immunized rabbits. Our construction of the PI3P immunogen (**1**) takes advantage of asymmetric catalysis[7] and native chemical ligation,[8] each of which provides unique and noteworthy benefits. First, asymmetric catalysis allows for rapid access to a single regio- and stereoisomeric inositol phosphate.[7] Second, the late-stage introduction of a sulfhydryl-bearing cysteine at the penultimate step through native chemical ligation provides a selective and efficient conjugation partner for a maleimide-activated carrier protein.[9] The combination of these two technologies provided rapid access to a successful immunogen for PI3P and demonstrates a general strategy for accessing phosphoinositide antibodies.
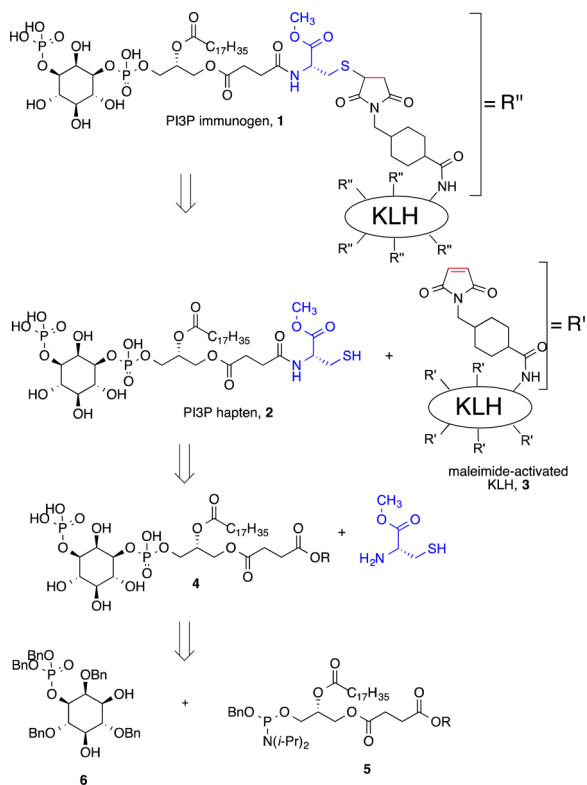
RESULTS AND DISCUSSION

Retrosynthetic Analysis of a PI3P immunogen. Our convergent synthesis of PI3P immunogen **1** (Scheme 1) was projected to involve the coupling of the PI3P hapten **2** and the maleimide-substituted KLH construct **3**. The synthesis of PI3P analogue **2** was envisioned to involve the straightforward coupling of a cysteine residue to an appropriately functionalized, enantio- and regioisomerically pure phosphoinositide such as **4**. Compounds like **4** were projected to be available in a streamlined fashion in analogy to prior studies.[10,11] Thus, we felt that phosphoramidite **5** (R = Ph or Bn) could be readily prepared and coupled with dibenzyl phosphate **6**. An *in situ* P(III) to P(V) oxidation and subsequent global deprotection could generate phosphoinositide **4** (R = H or Ph). The critical synthetic steps

Scheme 1

Scheme 2

outlined by Martin and co-workers.[15] The synthesis was initiated from *p*-methoxybenzyl (PMB)-protected diol **11**.[16] Dicyclohexyl carbodiimide (DCC)-activated **12** was treated with diol **11** which led to ester formation at the less hindered primary alcohol. The remaining secondary alcohol of **13** was then coupled to DCC-activated stearic acid in an analogous manner to generate diester **14**. Removal of the PMB group by a 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)-mediated cleavage in wet CH_2Cl_2 produced alcohol **15**. Alcohol **15** was then treated with freshly prepared $BnOP(N(i\text{-}Pr)_2)_2$ to give the desired phosphoramidite product **16** in four steps (Scheme 3).
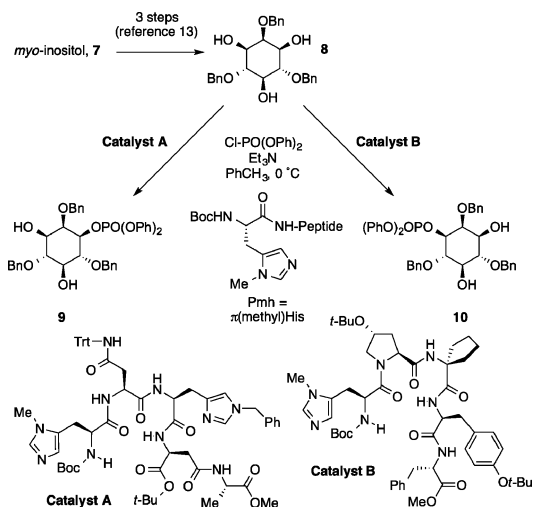
thus seemed to be the asymmetric phosphorylation chemistry we had previously developed,[7] and the subsequent incorporation of a cysteine residue to yield the PI3P hapten (**2**). Formation of an amide bond to a phosphoinositide like **4** (R = H) was not a straightforward process; substantial study and optimization were required. Ultimately, as will be detailed below, a native chemical ligation strategy accomplished formation of **2**.[8] In the end, access to the cysteine-functionalized PI3P analogue effectively set up the final coupling step to covalently bond the hapten (**2**) to KLH, providing the desired PI3P immunogen (**1**). The synthesis and biochemical validation of our synthetic hapten (**2**) through the elicitation and testing of antibodies from immunized rabbits are described below.

Synthesis of a PI3P Hapten. The first compound that we required to incorporate into our synthesis was the chiral dibenzyl phosphate **6** (Scheme 1). This intermediate is now readily accessible[10] using asymmetric group transfer catalysis developed in our laboratory as part of our long-standing interest in peptide-based catalysis.[12] Readily available, naturally occurring *myo*-inositol, **7**, can be readily transformed into 2,4,6-tribenzyl-*myo*-inositol derivative **8** through established procedures.[13] Then, asymmetric phosphorylation may deliver either enantiomer of the inositol-derived species, **9** or **10**, by choice of catalyst (Catalyst **A** or Catalyst **B**, Scheme 2).[7] The access to either stereoisomer of an inositol-1/3-monophosphate provides additional flexibility when planning a synthesis, and both have been utilized to prepare naturally occurring inositol phosphates and phosphoinositides as well as unnatural derivatives and their enantiomers.[7,10,14] We planned to take advantage of inositol-3-phosphate, **10**, as we require the naturally occurring enantiomer of a PI3P analogue for hapten-like properties.
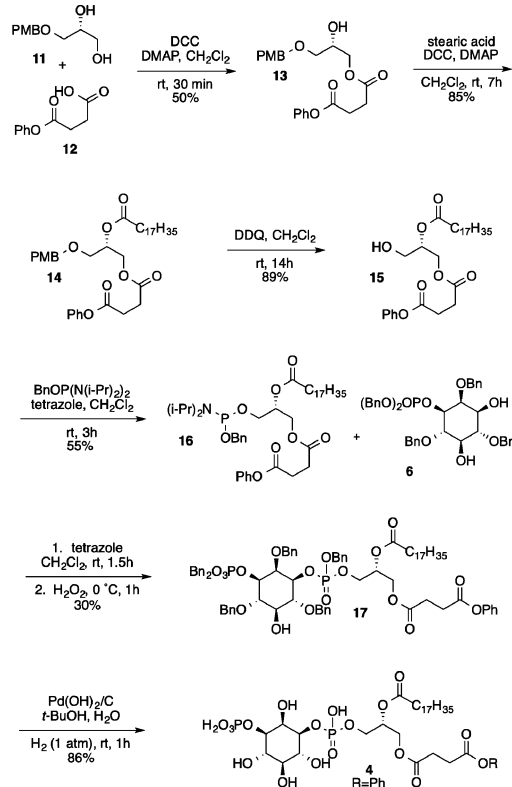
The second key intermediate we required was the phosphoramidite **5** as a coupling partner for the *myo*-inositol-derived headgroup **6**. Its preparation followed a strategy first

Scheme 3

With a scalable preparation of phosphoramidite **16** in hand, we were ready to pursue the preparation of the phosphoinositide

derivative **4**. The conversion of *myo*-inositol-derived diphenyl-phosphate **10** to its corresponding dibenzyl-protected derivative **6** is necessary to facilitate later protecting group manipulations, and was accomplished as in the past.[10] Treatment of phosphoramidite **16** with tetrazole and dibenzyl phosphate **6** followed by an *in situ* oxidation yielded the hexabenzyl phosphoinositide derivative **17** as an inconsequential mixture of diastereomers at phosphorus. Great care was taken to achieve very high purity of **17**, resulting in discarding mixed fractions that possessed any hint of minor isomers. Late-stage, further purification of phosphoinositides is often very difficult. The result was a rather modest isolated yield of **17**. Initial efforts to effect a hydrogenolysis of **17** in a sodium bicarbonate buffered solution led to significant hydrolysis of the phenyl ester. Fortunately, the hydrogenolysis proceeded in high yield in the absence of a buffer, and product **4** was isolated by filtration without the need for further purification (Scheme 3).

At this stage it is worth highlighting our initial, unsuccessful strategy for the preparation of hapten **2**. We successfully achieved conversion of a fully deprotected phosphoinositide free acid (**4**, R = H, eq 1) following a global benzyl protecting group
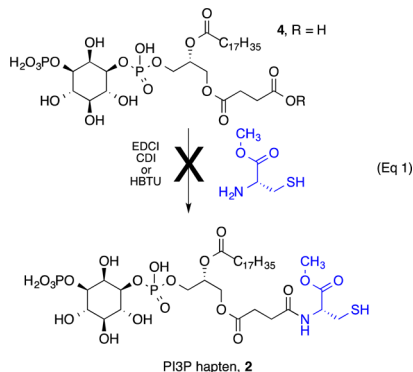

strategy analogous to our earlier studies of PIP synthesis.[10] Free acid **4** allowed us to study coupling with cysteine via activation with reagents such as EDCI, HBTU, and CDI. Unfortunately, in all instances no product formation was detected. Of note, LC/MS analysis revealed evidence of neither product formation nor decomposition of starting materials, with residual **4** (R = H) consistently present in the reaction mixture. At this point we considered the issues that might have led to a lack of reactivity in our system. We were unable to discount the surfactant-like nature of our starting material and propose that aggregation of the phosphoinositide intermediates in organic solvents may have led to the observed lack of reactivity. With these thoughts in mind, we turned our attention to native chemical ligation (NCL), the elegant strategy developed by Kent and co-workers.[8] What follows is a description of our successful implementation of this approach, which may be a unique application in the context of phosphoinositide synthesis.

In the traditional NCL reaction setup, a new peptide bond is formed between unprotected reacting peptides. The electrophile bears a thioester at the *C* terminus while the nucleophile bears an *N* terminal cysteine residue. The basis of the impressive reactivity profile for this coupling results from a kinetically favorable trans-thioesterification followed by an intramolecular rearrangement to generate a thermodynamically stable amide bond. The reaction employs aqueous conditions that incorporate chaotropic additives, which denature the reacting peptides. In addition, a reducing agent is incorporated to ensure that the thiol group is

not oxidized.[8,17] We hoped to take advantage of the combined benefits provided by the reaction conditions to successfully incorporate cysteine to produce PI3P hapten **2**.

A problem remained if we attempted to utilize the traditional NCL reaction. Specifically, the conventional usage of a *C*-terminal thioester would not survive the hydrogenolytic conditions required for our protecting group strategy, which employed benzyl ethers and esters. Fortunately, Liu and co-workers demonstrated, with imidazole activation, a *C*-terminal phenyl ester could successfully participate in an NCL in the place of a *C*-terminal thioester.[18]

We set out to test the Liu modification on our substrate. By treating the phenyl ester phosphoinositide derivative (**4**) with a solution of cysteine methyl ester, guanidine (Gn), triscarboxy-ethyl phosphine (TCEP), and imidazole in a solution of water and acetonitrile (4:1) buffered to pH = 7.1 we were able to prepare the desired hapten (**2**) in 46% yield (eq 2).
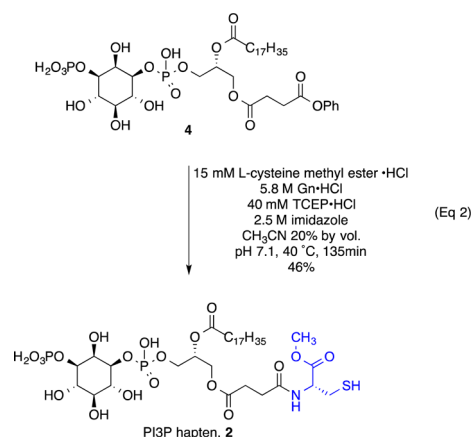
Successful Bioconjugation of the PI3P Hapten.

The final intermediate that we required to generate PI3P immunogen **1** was the commercially available maleimide-activated keyhole limpet hemocyanin (**3**, Scheme 1) as our carrier protein. The maleimide group provides an effective conjugation partner, especially when the hapten bears a sulfhydryl group distant from the epitope. The coupling is known to proceed in a selective and efficient manner,[9] and in addition, this strategy minimizes undesirable side reactions with the epitope portion of the hapten.[19]

The PI3P hapten **2** was successfully conjugated to maleimide-activated KLH **3** to generate the hapten−carrier protein complex (**1**) for immunization of rabbits.[20] The hapten (**2**) was also coupled to maleimide-activated ovalbumin (OVA) to generate a screening reagent, since the large molecular weight of KLH prevents its utility in subsequent analyses. To confirm that the conjugation reactions were effective, the PI3P-OVA reaction mix was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and two different evaluations were performed (Figure 1). The first method utilized Coomassie Blue staining of the polyacrylamide gel and demonstrated an increase in the molecular weight of the ovalbumin protein following the coupling reaction, implying the covalent attachment of hapten to carrier (Figure 1A). The second method was a Western blot procedure employing a lipid-binding probe to ascertain the covalent attachment of PI3P to the protein (Figure 1B). Proteins resolved by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. The PI3P-OVA conjugate was detected by the binding of GST-2X FYVE, a
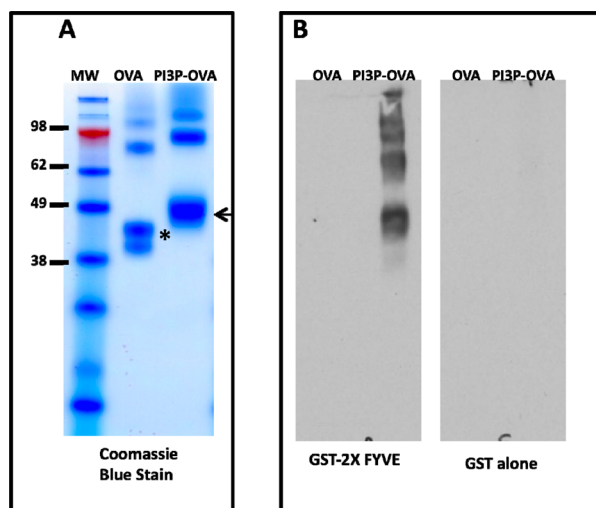
Figure 1. SDS-PAGE evaluation of the conjugation of PI3P to OVA. (A) Unconjugated ovalbumin (OVA) or PI3P-conjugated OVA was subjected to SDS-PAGE and Coomassie blue staining. Lane 1: molecular weight markers (MW, kilodaltons); lane 2: unconjugated OVA; lane 3: PI3P conjugated OVA. Asterisk indicates unconjugated OVA, arrow indicates PI3P-OVA. (B) OVA and PI3P-OVA were subjected to SDS-PAGE-Western blot analysis using GST-2X FYVE as a probe. GST protein was used as a control.

recombinant protein consisting of a glutathione-S-transferase (GST) fused to a phosphoinositide-binding domain selective for PI3P called FYVE (Fab1, YotB, Vac1, EEA1).[21] The GST-FYVE probe bound only to the PI3P-OVA and not to the unconjugated OVA, indicating that the coupling reaction occurred. In the control experiment, recombinant GST (without the lipid binding 2X FYVE domain) did not bind to the PI3P-OVA.

Biochemical Validation of the PI3P Immunogen. Five rabbits were immunized with PI3P immunogen **1**, sera were collected, and immunoglobulin G (IgG) fractions were purified by protein A affinity chromatography. IgG fractions were also purified from sera obtained from the rabbits prior to immunization (preimmune). The immune response in each rabbit was assessed by subjecting preimmune and immune IgG fractions to Western blot assays on OVA and PI3P-OVA. The immune response was further characterized with respect to phosphoinositide specificity through the utilization of PIP Arrays (Echelon Biosciences). PIP Arrays are membranes that have been spotted with a concentration gradient of eight phosphoinositides. The Western blot strips in Figure 2 indicate that each rabbit elicited a response to the PI3P hapten coupled to KLH. The IgG fractions from each immunized rabbit detected PI3P-OVA and not the carrier protein alone, while the preimmune IgG fractions were negative for detection of either forms of carrier protein. Data generated from probing PIP Arrays indicated that the immune IgG fractions from three of the rabbits (# 1, # 2 and # 4) exhibited high specificity for PI3P (see arrows). IgG fractions from rabbit # 3 is also quite PI3P specific, although a quite minor response to PI3,5P$_2$ is also evident. Immune IgG fractions from Rabbit # 5 are also PI3P responsive, with a somewhat more pronounced, but qualitatively still minor reaction to PI3,5P$_2$. There were no detectable signals when PIP Arrays were probed with preimmune IgG fractions (data not shown). It is quite striking that the response of the polyclonals is as PI3P-specific as it is. Since PI3,5P$_2$ also carries a phosphate at the 3-position of the inositide headgroup, it is also perhaps unsurprising that there is minor response to this phosphoinositde in a subset of the polyclonal-enriched rabbit sera.
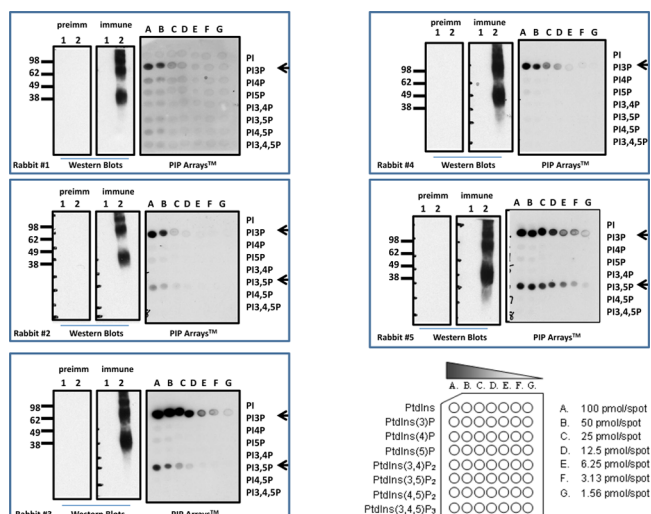

Figure 2. Evaluation of purified IgG fractions for PI3P specificity. Rabbit sera (preimmunization and postimmunization with PI3P-OVA) were analyzed by Western blots on OVA (lane 1) and PI3P-OVA (lane 2) resolved by SDS-PAGE or by PIP arrays. Configuration of the PIP arrays is indicated in lower right-hand corner of figure. Arrows indicate positive reactivity within the PIP arrays.

◼ CONCLUSIONS

The preparation of a PI3P hapten (**2**) was achieved in seven steps from known materials by incorporating cysteine in the final synthetic step by native chemical ligation. This hapten (**2**) was readily conjugated to a KLH carrier protein providing a PI3P immunogen (**1**). This immunogen was biochemically validated, and the approach that we describe provides a streamlined method to incorporate a sulfhydryl nucleophile to generate a phosphoinositide hapten. We anticipate the combined approach of "catalytic asymmetric phosphorylation + native chemical ligation" could be quite general for the synthesis of other PIP-based haptens based on other PIPs that have been prepared with asymmetric phosphorylation strategies.[10]

◼ EXPERIMENTAL SECTION

General Information. Proton NMR spectra were recorded on a Bruker 500 MHz spectrometer. Proton chemical shifts were reported in ppm (δ) with the residual protium in the NMR solvent as a reference (CHCl$_3$, δ 7.26 relative to tetramethylsilane, TMS). Spectral data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants (Hz), integration). Carbon NMR spectra were recorded on a Bruker 126 MHz spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to the solvent signal (CDCl$_3$, δ 77.16). NMR data were collected at 25 °C. Infrared spectra were obtained on a Nicolet 6700 ATR/FT-IR spectrometer and ν_{max} are partially reported (cm^{-1}). Analytical thin-layer chromatography (TLC) was performed using Silica Gel 60 Å F254 precoated plates (0.25 mm thickness). TLC R_f values are reported, and visualization was accomplished by irradiation with a UV lamp and/or staining with potassium permanganate (KMnO$_4$) solution. Flash column chromatography was performed using Silica Gel 60 Å (32−62 μm). Optical rotations were recorded on a Perkin-Elmer Polarimeter 341 at the sodium D line (1.0 dm path length). High-resolution liquid chromatography−mass spectrometry (HR-LC/MS) was performed on a Waters XEVO instrument equipped with ESI, a QToF mass spectrometer, and a photodiode array detector. The method of ionization is given in parentheses.

Acetonitrile and dichloromethane were purified by a Seca Solvent Purification System from GlassContour. Chloroform-*d* (CDCl$_3$) and methanol-*d*$_4$ (CD$_3$OD) were purchased from Cambridge Isotope

Laboratories without TMS internal standard and were used without further purification. All other chemicals were commercially available and used as received.

(S)-2-Hydroxy-3-(4-methoxybenzyloxy)propyl Phenyl Succinate (13). To a solution of **12**[22] (1.23 g, 6.33 mmol) in CH_2Cl_2 (70 mL) was added DCC (1.44 g, 6.97 mmol), which was stirred at ambient temperature for 5 min. **11**[16] (1.34 g, 6.33 mmol) and DMAP (77 mg, 0.63 mmol) were then added as a solution in CH_2Cl_2 (10 mL). The reaction was stirred at rt for 30 min. The reaction material was transferred to a separatory funnel and washed with 1N HCl and brine. The organic fraction was dried over Na_2SO_4, filtered, evaporated and purified by column chromatography (4:1, CH_2Cl_2/EtOAc). The product was isolated as a colorless viscous oil (1.24 g, 50% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 (t, J = 7.9 Hz, 2H), 7.29–7.23 (m, 3H), 7.12 (d, J = 7.7 Hz, 2H), 6.91 (d, J = 8.6 Hz, 2H), 4.50 (s, 2H), 4.26 (dd, J = 11.5, 4.4 Hz, 1H), 4.21 (dd, J = 11.5, 6.2 Hz, 1H), 4.08–4.02 (m, 1H), 3.83 (s, 3H), 3.55 (dd, J = 9.6, 4.4 Hz, 1H), 3.49 (dd, J = 9.6, 6.1 Hz, 1H), 2.91 (t, J = 6.8 Hz, 2H), 2.79 (t, J = 6.7 Hz, 2H), 2.56 (d, J = 4.9 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.3, 171.1, 159.5, 150.7, 129.9, 129.58, 129.56, 126.0, 121.6, 114.0, 73.3, 70.6, 68.9, 66.0, 55.4, 29.5, 29.2. ATR-IR ν_{max} (cm^{-1}) 3404, 2937, 1733, 1612, 1592, 1513, 1493, 1457, 1361, 1303, 1245, 1192, 1133, 1028, 815, 754, 691. TLC R_f = 0.36 (4:1 CH_2Cl_2/EtOAc, UV). UPLC/MS τ_r = 5.31 min; m/z for $[C_{21}H_{24}NaO_7]^+$ expected = 411.1414, found = 411.14 (ESI$^+$).

(S)-3-(4-Methoxybenzyloxy)-2-(stearoyloxy)propyl Phenyl Succinate (14). To stearic acid (0.82 g, 2.9 mmol) and DCC (0.59 g, 2.9 mmol) was added CH_2Cl_2 (30 mL), which was stirred at ambient temperature for 5 min. **13** (0.93 g, 2.39 mmol) and DMAP (29 mg, 0.24 mmol) were then added as a solution in CH_2Cl_2 (10 mL). The reaction was stirred at room temperature for 7 h. The solvent was evaporated and the crude material was purified by column chromatography (3:1 hexanes/EtOAc). The product was isolated as a white solid (1.33 g, 85% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (t, J = 7.9 Hz, 2H), 7.25–7.20 (m, 3H), 7.09 (d, J = 7.7 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 5.27–5.20 (m, 1H), 4.47 (d, J = 11.7 Hz, 1H), 4.43 (d, J = 11.7 Hz, 1H), 4.38 (dd, J = 11.9, 3.7 Hz, 1H), 4.24 (dd, J = 11.9, 6.4 Hz, 1H), 3.80 (s, 3H), 3.57 (dd, J = 8.8, 3.5 Hz, 1H), 3.54 (dd, J = 8.8, 3.6 Hz, 1H), 2.86 (td, J = 6.7, 2.5 Hz, 2H), 2.72 (t, J = 6.8 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H), 1.59 m, 2H), 1.32–1.21 (m, 28H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.3, 171.8, 170.9, 159.5, 150.8, 129.9, 129.54, 129.47, 126.0, 121.6, 114.0, 73.1, 70.0, 67.9, 63.5, 55.4, 34.4, 32.1, 29.9, 29.81, 29.78, 29.6, 29.5, 29.43, 29.36, 29.2, 29.1, 25.1, 22.8, 14.3 (several stearate peaks are unresolved). ATR-IR ν_{max} (cm^{-1}) 2911, 2847, 1735, 1723, 1612, 1518, 1470, 1518, 1470, 1304, 1192, 1166, 1139, 1114, 1051, 851, 818, 801. TLC R_f = 0.47 (3:1, hexanes/EtOAc, UV). UPLC/MS τ_r = 5.31 min; m/z for $[C_{39}H_{58}NaO_8]^+$ expected = 677.4024, found = 677.4 (ESI$^+$).

(S)-3-Hydroxy-2-(stearoyloxy)propyl Phenyl Succinate (15). To a solution **14** (750 mg, 1.14 mmol) in wet CH_2Cl_2 (30 mL) was added DDQ (546 mg, 2.41 mmol). The reaction was stirred for 16 h at rt. The solvent was evaporated and the crude material was purified by column chromatography (4:1, CH_2Cl_2/EtOAc). The product was isolated as a pink–orange solid (544 mg, 89% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (t, J = 7.9 Hz, 2H), 7.23 (t, J = 7.4 Hz, 1H), 7.09 (d, J = 7.7 Hz, 2H), 5.09 (p, J = 5.0 Hz, 1H), 4.37 (dd, J = 11.9, 4.5 Hz, 1H), 4.30 (dd, J = 11.9, 5.6 Hz, 1H), 3.76–3.69 (m, 2H), 2.93–2.86 (m, 2H), 2.79–2.73 (m, 2H), 2.33 (t, J = 7.6 Hz, 2H), 2.02 (t, J = 6.5 Hz, 1H), 1.65–1.57 (m, 2H), 1.33–1.20 (m, 28H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.6, 172.2, 171.0, 150.7, 129.6, 126.1, 121.6, 72.1, 62.8, 61.6, 34.4, 32.1, 29.84, 29.80, 29.76, 29.6, 29.5, 29.40, 29.36, 29.2, 29.09, 25.05, 22.8, 14.3 (several stearate peaks are unresolved). ATR-IR ν_{max} (cm^{-1}) 3529, 2958, 2916, 2850, 1732, 1712, 1494, 1472, 1196, 1147, 991, 892. TLC R_f = 0.46 (4:1, CH_2Cl_2/EtOAc, UV). UPLC/MS τ_r = 4.75 min; m/z for $[C_{31}H_{50}NaO_7]^+$ expected = 557.3449, found = 557.3 (ESI$^+$).

(2R)-3-(Benzyloxy(diisopropylamino)phosphinooxy)-2-(stearoyloxy)propyl Phenyl Succinate (16). To a solution BnOP-(N(i-Pr)$_2$)$_2$[23] (1.38 g, 4.07 mmol) in CH_2Cl_2 (15 mL) was added **15** (1.45 g, 2.71 mmol) as a solution in CH_2Cl_2 (30 mL) followed by 0.45 M tetrazole in CH_3CN (9.04 mL, 4.07 mmol). The reaction was stirred at rt

for 3 h. It was then evaporated and purified by column chromatography (85 hexanes/10 EtOAc/5 Et$_3$N). The product was isolated as a clear, colorless oil (1.14g, 55% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.30 (m, 6H), 7.28–7.19 (m, 2H), 7.09 (d, J = 7.6 Hz, 2H), 5.25–5.17 (m, 1H), 4.74 (ddd, J = 12.5, 8.4, 1.3 Hz, 1H), 4.69–4.63 (m, 1H), 4.41 (td, J = 12.3, 3.7 Hz, 1H), 4.24 (ddd, J = 11.8, 7.8, 6.4 Hz, 1H), 3.80 (ddd, J = 12.3, 7.1, 5.3 Hz, 1H), 3.77–3.69 (m, 1H), 3.68–3.59 (m, 2H), 2.89–2.84 (m, 2H), 2.74 (t, J = 6.9 Hz, 2H), 2.28 (t, J = 7.5 Hz, 2H), 1.63–1.55 (m, 2H), 1.33–1.22 (m, 28H), 1.20–1.17 (m, 12H), 0.89 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.2, 171.8, 170.9, 150.8, 129.5, 128.4, 127.4, 127.1, 126.0, 121.6, 70.9, 70.8, 70.7, 65.63, 65.59, 65.49, 65.45, 63.35, 63.31, 61.9, 61.8, 61.7, 61.6, 43.3, 43.2, 34.4, 32.0, 29.84, 29.80, 29.77, 29.6, 29.5, 29.42, 29.38, 29.3, 29.1, 25.0, 24.82, 24.77, 24.73, 24.70, 24.67, 24.65, 22.8, 14.3 (several stearate peaks are unresolved). ^{31}P NMR (121 MHz, $CDCl_3$) δ 146.91, 146.77. ATR-IR ν_{max} (cm^{-1}) 2964, 2923, 2853, 1741, 1494, 1456, 1364, 1197, 1184, 1162, 1135, 1060, 1023, 974, 753, 731, 692. TLC R_f = 0.37 (85:10:5, hexanes/EtOAc/Et$_3$N, UV). UPLC/MS compound hydrolyzed in MS no M + H detected.

(2R)-3-(Benzyloxy((1R,2R,3R,4R,5S,6R)-2,4,6-tris(benzyloxy)-3-((bis(benzyloxy)phosphoryl)methyl)-5-hydroxycyclohexyloxy)phosphoryloxy)-2-(stearoyloxy)propyl Phenyl Succinate (17). To **6**[10] (597 mg, 0.840 mmol) was added **16** (908 mg, 1.18 mmol) as a solution in CH_2Cl_2 (30 mL) followed by 0.45 M tetrazole in CH_3CN (10.1 mL, 4.54 mmol). The reaction was stirred at rt for 1.5 h and then cooled to 0 °C. H_2O_2 [30% in H_2O] (15 mL) was added, and the reaction was stirred an additional 1 h and then carefully treated with aq saturated Na_2SO_3 (exothermic bubbling occurs). The solution was extracted with EtOAc, washed with H_2O. The aqueous phase was back extracted with EtOAc (1×). The combined organic fractions were dried (Na_2SO_4), filtered, evaporated, and purified by flash chromatography (7:3, CH_2Cl_2/EtOAc) twice. The product was isolated as a clear, colorless oil (0.35 g, 30% yield) as a mixture of phosphorus diastereomers. ^{1}H NMR (500 MHz, $CDCl_3$, unresolved mixture of diastereomers) δ 7.38–7.18 (m, 33H), 7.07 (d, J = 7.9 Hz, 2H), 5.14–4.88 (m, 7H), 4.86–4.67 (m, 6H), 4.49–4.44 (m, 1H), 4.34–4.15 (m, 3H), 4.10–3.84 (m, 5H), 3.58–3.46 (m, 1H), 2.85–2.77 (m, 2H), 2.69–2.61 (m, 2H), 2.42–2.36 (m, 1H), 2.22–2.12 (m, 2H), 1.56–1.46 (m, 2H), 1.33–1.15 (m, 28H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$, unresolved mixture of diastereomers) δ 172.93, 172.87, 171.63, 171.60, 170.82, 150.76, 138.62, 138.62, 138.47, 138.43, 138.40, 129.54, 128.78, 128.74, 128.70, 128.69, 128.60, 128.56, 128.41, 128.37, 128.09, 128.06, 128.04, 128.01, 128.00, 127.97, 127.91, 127.86, 127.68, 127.63, 127.44, 127.38, 126.00, 121.60, 79.77, 79.71, 79.65, 79.59, 79.54, 79.49, 79.44, 78.73, 78.63, 78.06, 78.01, 77.94, 77.91, 77.89, 77.86, 77.81, 77.41, 77.16, 76.91, 75.91, 75.90, 75.39, 75.36, 75.34, 75.21, 74.54, 69.88, 69.68, 69.64, 69.48, 69.44, 69.24, 69.18, 65.69, 65.65, 65.50, 65.46, 62.24, 34.15, 34.13, 32.07, 29.85, 29.82, 29.80, 29.79, 29.64, 29.50, 29.42, 29.28, 29.21, 28.93, 24.86, 22.83, 14.26. (note several stearate peaks are not resolved). ^{31}P NMR (121 MHz, $CDCl_3$) δ −1.86, −1.91, −2.03. TLC R_f = 0.47 (7:3, CH_2Cl_2/EtOAc, KMnO$_4$). UPLC/MS τ_r = 5.70 min; m/z for $[C_{79}H_{99}O_{18}P_2]^+$ expected = 1397.6301, found = 1397.6007 (ESI$^+$).

(2R)-3-(Hydroxy((1S,2R,3S,4S,5R,6R)-2,3,4-tetrahydroxy-5-(phosphonooxy)cyclohexyloxy)phosphoryloxy)-2-(stearoyloxy)propyl Phenyl Succinate (4). To a solution **17** (177 mg, 0.127 mmol) in t-BuOH (30 mL) and H_2O (6 mL) was added Pd(OH)$_2$ on carbon (177 mg). H_2 was bubbled through the solution for 5 min, and the reaction was then left stirring under a balloon of H_2 at rt for 1 h. Ar was bubbled through the reaction mixture for 5 min, and it was then filtered through Celite, washing alternately with MeOH and H_2O. The solvent was evaporated to provide the title compound as a clear, colorless oil (94 mg, 86% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.03 (t, J = 7.9 Hz, 2H), 6.89 (t, J = 7.4 Hz, 1H), 6.74 (d, J = 7.7 Hz, 2H), 4.98–4.85 (m, 1H), 4.16–4.09 (m, 1H), 4.08–4.03 (m, 1H), 3.95–3.89 (m, 1H), 3.90–3.82 (m, 2H), 3.74–3.65 (m, 2H), 3.55–3.45 (m, 2H), 2.97–2.89 (m, 1H), 2.55 (t, J = 6.3 Hz, 2H), 2.42 (t, J = 6.5 Hz, 2H), 1.98 (t, J = 7.5 Hz, 2H), 1.30–1.20 (m, 2H), 1.01–0.84 (m, 28H), 0.54 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.2, 171.8, 171.0, 150.3, 128.9, 125.4, 121.0, 77.3, 76.8, 73.8, 71.0, 70.7, 69.7, 69.4, 64.4, 62.2,

33.6, 31.4, 29.2, 29.1, 29.0, 28.85, 28.8, 28.61, 28.58, 28.4, 24.3, 22.1, 13.3 (note several stearate peaks are not resolved). ^{31}P NMR (121 MHz, CDCl$_3$) δ 3.22, 2.10. UPLC/MS τ_r = 5.10 min; m/z for $[C_{37}H_{63}O_{18}P_2]^+$ expected = 857.3484, found = 857.3575 (ESI$^+$).

PI3P Hapten (2). Fifteen millimolar L-cysteine methyl ester solution preparation: To L-cysteine methyl ester·HCl (129 mg, 0.752 mmol) and imidazole (8.51 g, 125 mmol) was added 8 M guanidine·HCl (36 mL), 0.5 M tris(2-carboxyethyl)phosphine·HCl (4 mL) and CH$_3$CN (10 mL). The pH of the solution was adjusted to 7.1 with dropwise addition of concentrated trifluoroacetic acid. The 15 mM L-cysteine methyl ester solution (36.6 mL, 0.55 mmol) was added to **4** (94 mg, 0.11 mmol). Ar was carefully bubbled through the solution for 5 min (bubbles filled the flask headspace). This solution was heated to 40 °C under Ar for 135 min. The crude reaction mixture was chromatographed through a desalting Sephadex G-10 column, eluting with H$_2$O/CH$_3$CN (4:1). The fractions containing compound **2** were collected, evaporated, and purified with an Interchim PT-15C4/20 g column, eluting with CH$_3$CN/H$_2$0 with 0.1% formic acid (1:1 ramping to 9:1). The fractions identified as containing the desired product were combined and evaporated to yield a white solid (46 mg, 46%). ^{1}H NMR (500 MHz, CDCl$_3$/CD$_3$OD (1:1)) δ 4.88−4.82 (m, 1H), 4.33−4.29 (m, 1H), 4.06−4.00 (m, 2H), 3.84 (dd, J = 11.9, 5.9 Hz, 1H), 3.81−3.74 (m, 2H), 3.70−3.57 (m, 2H), 3.50−3.42 (m, 2H), 3.38 (s, 3H), 2.90 (t, J = 9.2 Hz, 1H), 2.56 (dd, J = 14.0, 4.8 Hz, 1H), 2.51 (dd, J = 13.9, 6.2 Hz, 1H), 2.33−2.18 (m, 4H), 1.96 (t, J = 7.5 Hz, 2H), 1.28−1.17 (m, 2H), 0.97−0.83 (m, 28H), 0.50 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, CDCl$_3$/CD$_3$OD (1:1)) δ 173.2, 172.3, 172.2, 170.42, 77.4 (d obscured by CDCl$_3$), 76.7 (d, J = 5.8 Hz), 73.9 (unresolved dd), 70.9 (d, J = 5.5 Hz), 70.8 (d, J = 5.7 Hz), 69.8, 69.6 (d, J = 8.7 Hz), 63.8 (d, J = 4.4 Hz), 62.0, 54.2, 51.7, 33.5, 31.4, 29.5, 29.10, 29.06, 28.9, 28.77, 28.75, 28.7, 28.5, 25.3, 24.3, 22.1, 13.1. (several stearate peaks are unresolved) ^{31}P NMR (121 MHz, CDCl$_3$/CD$_3$OD (1:1)) δ 4.08, 3.38. UPLC/MS τ_r = 3.72 min; m/z for $[C_{35}H_{66}NO_{19}P_2S]^+$ expected = 898.3419, found = 898.3394 (ESI$^+$).

Generation of Rabbit Polyclonal Antibodies to PI3P. PI3P hapten **2** was coupled to maleimide-activated KLH **3** (for immunogen), ovalbumin and bovine serum albumin (for screening reagents) using maleimide coupling chemistry (Thermo Scientific). Five New Zealand white female rabbits were immunized with PI3P immunogen **1**, sera was collected, and IgG fractions were purified by protein A affinity chromatography (Thermo Scientific). Rabbits were immunized four times at three-week intervals. **Western blot assay**: Ovalbumin (OVA) and PI3P-OVA were resolved by SDS-PAGE on 4−12% NuPAGE Bis-Tris 4−12% gels (Invitrogen) and transferred to 0.45 mm poly-vinylidene difluoride (PVDF) membrane (Millipore) by the semi-dry transfer method. Membranes were incubated overnight at 4 °C in a blocking buffer consisting of 3% (w/v) lipid-free bovine serum albumin (BSA) (Roche) in PBS-T (phosphate-buffered saline with 0.1% Tween-20) to block nonspecific binding sites. PI3P was detected using either purified GST-2X FYVE (a purified recombinant protein containing 2 FYVE domains from Hrs) or rabbit polyclonal antibodies generated to PI3P-KLH.

Detection with the lipid binding protein probe was performed by incubating blocked membranes with GST-2X FYVE (1 μg/mL in blocking buffer) overnight at 4 °C on a rocking platform. Membranes were washed three times for 5 min each in PBS-T, with agitation. GST-2X FYVE was detected by incubating membranes with rabbit anti-GST antibody coupled to horse radish peroxidase (HRP) at a dilution of 1:1000 (Novus Biologicals) for 1 h at room temperature, on a rocking platform. Membranes were washed three times for 5 min each in PBS-T. Detection was achieved using the chemiluminescent system ECL (GE Healthcare Life Sciences). Detection of PI3P with rabbit polyclonal antibodies was performed by incubating blocked membranes with purified IgG fractions of preimmune or immune sera at a concentration of 1 μg/mL in blocking buffer, overnight at 4 °C, on a rocking platform. Membranes were washed three times for 5 min each in PBS-T, with agitation and were then incubated in donkey antirabbit IgG coupled to HRP (GE Healthcare Life Sciences) at a dilution of 1:5000 in blocking buffer for 1 h at room temperature, on a rocking platform. Membranes were washed three times for 5 min each in PBS-T. Detection was achieved using the chemiluminescent system ECL (GE Healthcare Life Sciences).

Phosphoinositide Detection with PIP Array. PIP Array membranes were purchased from Echelon Biosciences, Inc. and were incubated in blocking buffer overnight at 4 °C on a rocking platform. Membranes were then incubated with purified IgG fractions of preimmune or immune sera at a concentration of 1 μg/mL in blocking buffer for 1 h at room temperature, on a rocking platform. Membranes were washed three times for 5 min each in PBS-T followed by incubation with donkey antirabbit IgG coupled to HRP (GE Healthcare Life Sciences) at a dilution of 1:5000 in blocking buffer for 1 h at room temperature, on a rocking platform. Membranes were washed three times for 5 min each in PBS-T. Detection was achieved using the chemiluminescent system ECL (GE Healthcare Life Sciences).

■ ASSOCIATED CONTENT

⑤ Supporting Information

Additional experimental procedures and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Mayinger, P. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2012**, *1821*, 1104−1113.

(2) Balla, T.; Szentpetery, Z.; Kim, Y. J. *Physiology* **2009**, *24*, 231−244.

(3) Wassef, N. M.; Roerdink, F.; Swartz, G. M., Jr; Lyon, J. A.; Berson, B. J.; Alving, C. R. *Mol. Immunol.* **1984**, *21*, 863−868.

(4) Atsuo, M.; Masato, U.; Tetsuro, H.; Keiji, Y.; Tohoru, Y.; Keizo, I. *Mol. Immunol.* **1988**, *25*, 1025−1031.

(5) Umeda, M.; Igarashi, K.; Nam, K. S.; Inoue, K. *J. Immunol.* **1989**, *143*, 2273−2279.

(6) Chen, R.; Kang, V. H.; Chen, J.; Shope, J. C.; Torabinejad, J.; DeWald, D. B.; Prestwich, G. D. *J. Histochem. Cytochem.* **2002**, *50*, 697−708.

(7) (a) Sculimbrene, B. R.; Miller, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 10125−10126. (b) Sculimbrene, B. R.; Morgan, A. J.; Miller, S. J. *J. Am. Chem. Soc.* **2002**, *124*, 11653−11656. (c) Sculimbrene, B. R.; Morgan, A. J.; Miller, S. J. *Chem. Commun.* **2003**, 1781−1785.

(8) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, 776−776. (b) Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640−6646. (c) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338−351.

(9) (a) Grayson, E. J.; Bernardes, G. J. L.; Chalker, J. M.; Boutureira, O.; Koeppe, J. R.; Davis, B. G. *Angew. Chem., Int. Ed.* **2011**, *50*, 4127−4132. (b) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem. Asian J.* **2009**, *4*, 630−640.

(10) (a) Sculimbrene, B. R.; Xu, Y.; Miller, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 13182−13183. (b) Xu, Y.; Sculimbrene, B. R.; Miller, S. J. *J. Org. Chem.* **2006**, *71*, 4919−4928. (c) Wang, Y. K.; Chen, W.; Blair, D.; Pu, M.; Xu, Y.; Miller, S. J.; Redfield, A. G.; Chiles, T. C.; Roberts, M. F. *J. Am. Chem. Soc.* **2008**, *130*, 7746−7755. (d) Kayser-Bricker, K. J.; Jordan, P. A.; Miller, S. J. *Tetrahedron* **2008**, *64*, 7015−7020.

(11) For representative syntheses of PI3P-compounds, see: (a) Bruzik, K. S.; Kubiak, R. J. *Tetrahedron Lett.* **1995**, *36*, 2415−2418. (b) Wang, D. S.; Chen, S. *J. Org. Chem.* **1996**, *61*, 5905−5910. (c) Chen, J.; Feng, L.;

Prestwich, G. D. *J. Org. Chem.* **1998**, *63*, 6511−6522. (d) Painter, G. F.; Grove, S. J. A.; Gilbert, I. H.; Holmes, A. B.; Raithby, P. R.; Hill, M. L.; Hawkins, P. T.; Stephens, L. R. *J. Chem. Soc., Perkin Trans.* **1999**, *1*, 923−936. (e) Falck, J. R.; Krishna, U. M.; Capdevila, J. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1711−1713. (f) Morisaki, N.; Morita, K.; Nishikawa, A.; Nakatsu, N.; Fukui, Y.; Hashimoto, Y.; Shirai, R. *Tetrahedron* **2000**, *56*, 2603−2614.

(12) (a) Davie, E. A. C.; Mennen, S. M.; Xu, Y.; Miller, S. J. *Chem. Rev.* **2007**, *107*, 5759−5812. (b) Miller, S. J. *Acc. Chem. Res.* **2004**, *37*, 601−610.

(13) Billington, D. C.; Baker, R.; Kulagowski, J. J.; Mawer, I. M.; Vacca, J. P.; deSolms, S. J.; Huff, J. R. *J. Chem. Soc., Perkin Trans.* **1989**, *1*, 1423−1429.

(14) (a) Morgan, A. J.; Komiya, S.; Xu, Y.; Miller, S. J. *J. Org. Chem.* **2006**, *71*, 6923−6931. (b) Longo, C. M.; Wei, Y.; Roberts, M. F.; Miller, S. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 4158−4161.

(15) Martin, S. F.; Josey, J. A.; Wong, Y.-L.; Dean, D. W. *J. Org. Chem.* **1994**, *59*, 4805−4820.

(16) Chen, J.; Profit, A. A.; Prestwich, G. D. *J. Org. Chem.* **1996**, *61*, 6305−6312.

(17) Macmillan, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 7668−7672.

(18) Fang, G. M.; Cui, H. K.; Zheng, J. S.; Liu, L. *ChemBioChem* **2010**, *11*, 1061−1065.

(19) (a) Betting, D. J.; Kafi, K.; Abdollahi-Fard, A.; Hurvitz, S. A.; Timmerman, J. M. *J. Immunol.* **2008**, *181*, 4131−4140. (b) Hashida, S.; Imagawa, M.; Inoue, S.; Ruan, K.; Ishikawa, E. *J. Appl. Biochem.* **1984**, *6*, 56. (c) Yoshitake, S.; Imagawa, M.; Ishikawa, E.; Niitsu, Y.; Urushizaki, I.; Nishiura, M.; Kanazawa, R.; Kurosaki, H.; Tachibana, S.; Nakazawa, N. *J. Biochem.* **1982**, *92*, 1413−1424. (d) Peeters, J.; Hazendonk, T.; Beuvery, E.; Tesser, G. *J. Immunol.* **1989**, *120*, 133−143.

(20) (a) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor: New York, 1988; Chapter 5; (b) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed.; Academic Press: New York, 2008; Chapter 19.

(21) Hammond, G. R. V.; Schiavo, G.; Irvine, R. F. *Biochem. J.* **2009**, *422*, 23−35.

(22) He, L.; Yan, F.; Cao, B.; Lu, H.; Wang, X.; Wang, E. *Jilin Daxue Xuebao, Lixueban* **2006**, *44*, 287.

(23) Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, 175−186.