J|A|C|S
ARTICLES
Published on Web 12/24/2005

# Chemical Synthesis and Molecular Recognition of Phosphatase-Resistant Analogues of Phosphatidylinositol-3-phosphate

Yong Xu,[†] Stephanie A. Lee,[‡] Tatiana G. Kutateladze,[‡] Diego Sbrissa,[§] Assia Shisheva,[§] and Glenn D. Prestwich*,[†]

*Contribution from the Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257, Department of Pharmacology, University of Colorado Health Sciences Center, Aurora, Colorado 80045, and Department of Physiology, Wayne State University School of Medicine, 540 East Canfield, Detroit, Michigan 48201*

Received August 22, 2005; E-mail: gprestwich@pharm.utah.edu

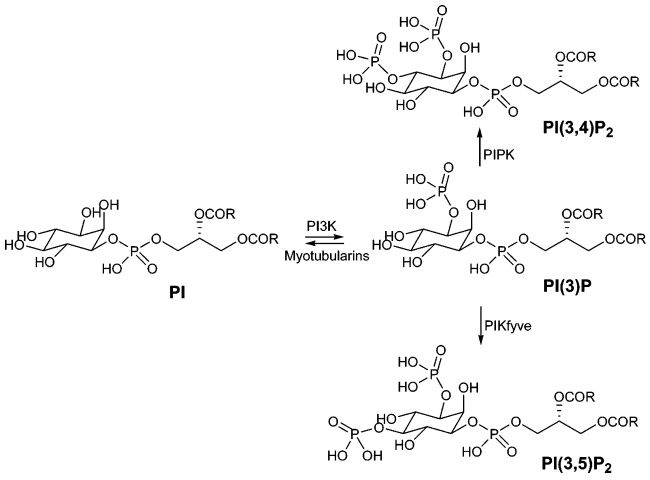
***Abstract:*** The remodeling of phosphatidylinositol polyphosphates in cellular membranes by phosphatases and kinases orchestrates the signaling by these lipids in space and time. To provide chemical tools to study the changes in cell physiology mediated by these lipids, three new metabolically stabilized (ms) analogues of phosphatidylinositol-3-phosphate (PtdIns(3)P) were synthesized. We describe herein the total asymmetric synthesis of 3-methylphosphonate, 3-(monofluoromethyl)phosphonate and 3-phosphorothioate analogues of PtdIns(3)P. From differentially protected D-*myo*-inositol key intermediates, a versatile phosphoramidite reagent was employed in the synthesis of PtdIns(3)P analogues with diacylglyceryl moieties containing dioleoyl, dipalmitoyl, and dibutyryl chains. In addition, we introduce a new phosphorylation reagent, (monofluoromethyl)phosphonyl chloride, which has general applications for the preparation of "p$K_a$-matched" monofluorophosphonates. These ms-PtdIns(3)P analogues exhibited reduced binding activities with $^{15}$N-labeled FYVE and PX domains, as significant $^{1}$H and $^{15}$N chemical shift changes in the FYVE domain were induced by titrating ms-PtdIns(3)P analogues into membrane-mimetic dodecylphosphocholine micelles. In addition, the PtdIns(3)P analogues with dioleoyl and dipalmitoyl chains were substrates for the 5-kinase enzyme PIKfyve; the corresponding phosphorylated ms-PI(3,5)$P_2$ products were detected by radio-TLC analysis.

## Introduction

Phosphoinositide (PtdIns$P_n$) signaling networks are dynamically modulated by proteins with lipid recognition motifs as well as kinase, phosphatase, and phospholipase enzymatic activities. Lipid−protein interactions form the cornerstone for many signaling pathways, and the new discipline of functional lipidomics[1] is defining many new targets for therapeutic interaction.[2] It is now accepted that 3-phosphorylated PtdIns$P_n$ lipids—PtdIns(3)P, PtdIns(3,4)$P_2$, PtdIns(3,5)$P_2$, and PtdIns(3,4,5)$P_3$—are intracellular signals in mammalian cells.[3] These signaling lipids have been implicated as activators of protein kinase C isoforms, and are putative messengers in cellular signal cascades pertinent to inflammation, cell proliferation, transformation, protein kinesis, and cytoskeletal assembly.[4,5]

PtdIns(3)P is produced by the action of phosphoinositide-3-kinase (PI 3-K)[3,6] on PtdIns (Scheme 1), and has cognate binding proteins, kinase and phosphatases that are important in cell physiology. The PI 3-K family of enzymes is an important

***Scheme 1.*** Metabolic Interconversions of PtdIns(3)P



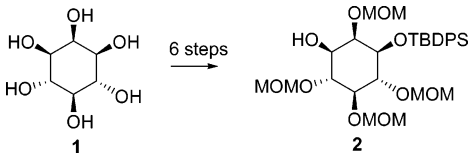

[†] The University of Utah.
[‡] University of Colorado Health Sciences Center.
[§] Wayne State University School of Medicine.

(1) Feng, L.; Ferguson, C. G.; Neilsen, P. O.; Chakravarty, L.; Rzepecki, P.; Prestwich, G. D. In *Functional Lipidomics*; Feng, L., Prestwich, G. D., Eds.; Dekker-CRC Press: New York, 2005; pp 189−210.
(2) Prestwich, G. D. *Chem. Biol.* **2004**, *11*, 619−637.
(3) Vanhaesebroeck, B.; Leevers, S. J.; Ahmadi, K.; Timms, J.; Katso, R.; Driscoll, P. C.; Woscholski, R.; Parker, P. J.; Waterfield, M. D. *Annu. Rev. Biochem.* **2001**, *70*, 535−602.
(4) Yin, H. L.; Janmey, P. A. *Annu. Rev. Physiol.* **2003**, *65*, 761−789.
(5) Wymann, M. P.; Bjorklof, K.; Calvez, R.; Finan, P.; Thomas, M.; Trifilieff, A.; Barbier, M.; Altruda, F.; Hirsch, E.; Laffargue, M. *Biochem. Soc. Trans.* **2003**, *31*, 275−280.
(6) Anderson, K. E.; Jackson, S. P. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 1028−1033.

therapeutic target for prevention of cancer, inflammation, allergy, thrombosis, and autoimmune disorders.[5,7−11] PtdIns-(3)P is also a substrate for PIKfyve, a 5-kinase required for endomembrane integrity and the formation of multivesicular bodies.[12] PtdIns(3)P binds FYVE domains,[13,14] and is thus an important player in phagocytosis[15] and the biochemistry of vesicular trafficking of proteins.[16] PX domains also bind PtdIns-(3)P with high affinity, and their spatiotemporal changes mediate important aspects of cell respiration and physiology.[17] The 3-phosphatase myotubularin (MTM) and MTM-related (MTMR) proteins[18] have been identified as important PtdIns(3)P phosphatases that contribute to lipid remodeling and are commonly mutated in genetic diseases.[19] Very recently, PtdIns(3)P phosphatase activity was observed for SapM, a *Mycobacterium tuberculosis* enzyme required for bacterial viability and inhibition of host cell phagolysosome biogenesis.[20] To gain deeper insights into these biological pathways, selective reagents that could block binding, inhibit enzyme activity, activate lipid−protein mediate pathways, or act as alternative substrates would be highly desirable. To date, 3-modified PtdIns(3)P analogues with these properties have not been reported.

To create metabolically stabilized analogues of lysophosphatidic acid (LPA), we prepared two kinds of phosphatase-resistant moieties—(fluoromethylene)phosphonates[21,22] and phosphorothioates[23]—which were found to be receptor subtype specific agonists. These two functional groups have second p$K_a$ values that are matched to the normal phosphomonoester p$K_a$ value of 6.5.[24] Phosphonate esters have been used extensively to stabilize naturally occurring phosphates, replacing the bridging oxygen or one of the phosphate oxygens with a methylene or methyl group. The resulting analogues were less susceptible to either acidic or enzymatic cleavage. Methylphosphonates have been studied as metabolically stable phosphate mimics, potential enzyme inhibitors, and probes for the elucidation of biochemical processes,[25] but these analogues have significantly higher second p$K_a$ values than the native phosphate group. Herein we describe the asymmetric total synthesis of methylphosphonate, (fluoromethyl)phosphonate, and phosphorothioate analogues of PtdIns-(3)P phosphatase-resistant analogues of PtdIns(3)P. The utility of these analogues in cellular, molecular, and structural biology will be described in due course.

***Scheme 2.*** Synthesis of the Enantiomerically Pure D-*myo*-Inositol Intermediate




**1** **2**

## Results and Discussion

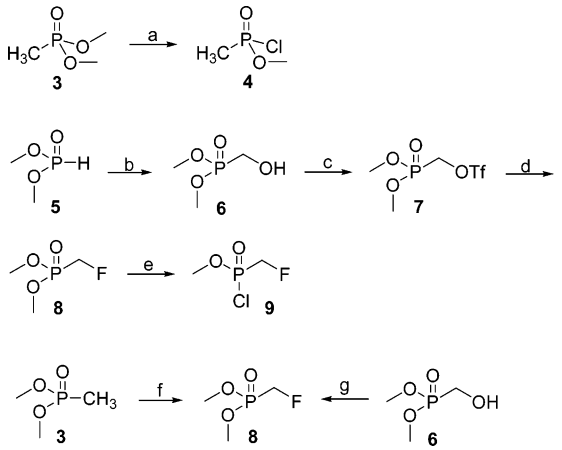
**Chemical Synthesis of Phosphatase-Resistant Analogues of Phosphatidylinositol-3-phosphate.** Several strategies have been employed for total asymmetric synthesis of phosphoinositides. One approach is the use of enantiopure natural precursors, e.g., D-glucose,[26−30] L-*chiro*-inositol derivatives,[31] and quinic acid.[32−34] Kinetic resolution or desymmetrization via enantioselective enzymatic acylations and nonenzymatic phosphorylation of protected *myo*-inositol derivatives is another common strategy.[35−38] The separation of diastereomeric derivatives of *myo*-inositol with chiral auxiliaries is also used in many synthetic routes.[39−41] In our early work, we developed the synthesis of enantiomerically pure PtdIns$P_n$ and a variety of derivatives from D-glucose via the Ferrier rearrangement.[28,41] In this paper, we have employed the more atom-efficient production of the enantiomeric inositol derivatives via the resolution of *myo*-inositol by crystallization of diastereomeric D-camphor ketals.[40]

Each of the phosphorylated phosphatidylinositols synthesized in this work employed the simple and elegant protection scheme developed by Bruzik.[40] The inositol 1-position was silylated with the TBDPS group, the phosphomonoester 3-position was protected as a benzoate group, and all the remaining hydroxyl groups were protected as methoxymethyl (MOM) ethers. In this way, 1-*O*-(*tert*-butyldiphenylsilyl)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol was synthesized from *myo*-inositol in six steps (Scheme 2).[40]

The phosphonochloridates derived from simple phosphonic acid alkyl esters are reliable and readily accessible reagents for the phosphonylation of alcohols to produce the corresponding phosphonates.[42,43] *O*,*O*-Dimethyl methylphosphonyl chloride can

(7) Drees, B. E.; Mills, G. B.; Rommel, C.; Prestwich, G. D. *Expert Opin. Ther. Pat.* **2004**, *14*, 703−732.
(8) Wetzker, R.; Rommel, C. *Curr. Pharm. Des.* **2004**, *10*, 1915−1922.
(9) Wymann, M. P.; Zvelebil, M.; Laffargue, M. *Trends Pharmacol. Sci.* **2003**, *24*, 366−376.
(10) Jackson, S. P.; Yap, C.; Anderson, K. *Biochem. Soc. Trans.* **2004**, *32*, 387−392.
(11) Workman, P. *Biochem. Soc. Trans.* **2004**, *32*, 393−396.
(12) Ikonomov O. C.; Sbrissa D.; Fligger, J.; Foti, M.; Carpentier, J.-L.; Shisheva, A. *Mol. Biol. Cell* **2003**, *14*, 4581−4591.
(13) Kutateladze, T.; Ogburn, K.; Watson, W.; de Beer, T.; Emr, S.; Burd, C.; Overduin, M. *Mol. Cell* **1999**, *3*, 805−811.
(14) Gaullier, J. M.; Simonsen, A.; D'Arrigo, A.; Bremnes, B.; Stenmark, H.; Aasland, R. *Nature* **1998**, *394*, 432−433.
(15) Gillooly, D. J.; Simonsen, A.; Stenmark, H. *J. Cell Biol.* **2001**, *155*, 15−17.
(16) Petiot, A.; Faure, J.; Stenmark, H.; Gruenberg, J. *J. Cell Biol.* **2003**, *162*, 971−979.
(17) Sato, T.; Overduin, M.; Emr, S. D. *Science* **2001**, *294*, 1881−1885.
(18) Wishart, M. J.; Dixon, J. E. *Trends Cell Biol.* **2002**, *12*, 579−585.
(19) Laporte, J.; Bedez, F.; Bolino, A.; Mandel, J. L. *Hum. Mol. Genet.* **2003**, *12*, R285−R292.
(20) Vergne, I.; Chua, J.; Lee, H.-H.; Lucas, M.; Belisle, J.; Deretic, V. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *202*, 4033−4038.
(21) Xu, Y.; Qian, L.; Prestwich, G. D. *J. Org. Chem.* **2003**, *68*, 5320−5330.
(22) Xu, Y.; Qian, L.; Prestwich, G. D. *Org. Lett.* **2003**, *5*, 2267−2270.
(23) Qian, L.; Xu, Y.; Hasegawa, Y.; Aoki, J.; Mills, G. B.; Prestwich, G. D. *J. Med. Chem.* **2003**, *46*, 5575−5578.
(24) Prestwich, G. D.; Xu, Y.; Qian, L.; Gajewiak, J.; Jiang, G. *Biochem. Soc. Trans.* **2005**, *33*, 1357−1361.
(25) Schmitt, L.; Spiess, B.; Schlewer, G. *Tetrahedron Lett.* **1993**, *34*, 7059−7060.
(26) Chen, J.; Profit, A. A.; Prestwich, G. D. *J. Org. Chem.* **1996**, *61*, 6305−6312.
(27) Chen, J.; Feng, L.; Prestwich, G. D. *J. Org. Chem.* **1998**, *63*, 6511−6522.
(28) Chen, J.; Prestwich, G. D. *J. Org. Chem.* **1998**, *63*, 430−431.
(29) Peng, J.; Prestwich, G. D. *Tetrahedron Lett.* **1998**, *39*, 3965−3968.
(30) Thum, O.; Chen, J.; Prestwich, G. D. *Tetrahedron Lett.* **1996**, *37*, 9017−9020.
(31) Qiao, L.; Hu, Y.; Nan, F.; Powis, G.; Kozikowski, A. P. *Org. Lett.* **2000**, *2*, 115−117.
(32) Reddy, K. K.; Saady, M.; Falck, J. R. *J. Org. Chem.* **1995**, *60*, 3385−3390.
(33) Reddy, K. K.; Ye, J.; Falck, J. R.; Capdevila, J. H. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2115−2116.
(34) Reddy, K. K.; Rizo, J.; Falck, J. R. *Tetrahedron Lett.* **1997**, *38*, 4729−4730.
(35) Sculimbrene, B. R.; Xu, Y.; Miller, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 13182−13183.
(36) Sculimbrene, B. R.; Miller, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 10125−10126.
(37) Gou, D.-M.; Chen, C.-S. *J. Chem. Soc., Chem. Commun.* **1994**, *18*, 2125−2126.
(38) Wang, D.-S.; Chen, C.-S. *J. Org. Chem.* **1996**, *61*, 5905−5910.
(39) Bruzik, K. S.; Tsai, M.-D. *J. Am. Chem. Soc.* **1992**, *114*, 6361−6374.
(40) Kubiak, R. J.; Bruzik, K. S. *J. Org. Chem.* **2002**, 960−968.
(41) Prestwich, G. D. *Acc. Chem. Res.* **1996**, *29*, 503−513.
(42) Denmark, S. E.; Chen, C.-T. *J. Org. Chem.* **1994**, *59*, 2922−2924.
(43) Malachowski, W. P.; Coward, J. K. *J. Org. Chem.* **1994**, *59*, 7625−7634.

***Scheme 3.*** Synthesis of (Fluoromethyl)phosphonate Chloride[a]



[a] Reagents and conditions: (a) $PCl_5$, benzene; (b) $(CH_2O)_n$, $K_2CO_3$, $CH_3OH$; (c) $Tf_2O$, 2,6-lutidine, $CH_2Cl_2$; (d) TBAF, THF; (e) *t*-$BuNH_2$; Dowex resin; then $(ClCO)_2$, DMF, $CH_2Cl_2$; (f) *n*-BuLi, THF; then Selectfluor; (g) DAST, $CH_2Cl_2$.

be readily prepared from the corresponding methylphosphonate by chlorination with $PCl_5$ at room temperature (rt).[44]

To prepare methyl (fluoromethyl)phosphonate, we first tried N−F "electrophilic" fluorinating agents to fluorinate dimethyl methylphosphonate **10**. The commercially available fluorinating agents *N*-fluoro-1,4-diazabicyclooctane bis(tetrafluoroborate) (F-TEDA-$BF_4$) and *N*-fluorobenzenesulfonimide (NFSI) were chosen for investigation.[45] A number of procedures for deprotonation of methylphosphonate, and its subsequent monofluorination, were evaluated. The only positive result was obtained using F-TEDA-$BF_4$ in anhydrous THF solvent to fluorinate the sodium enolate, but the product yields were too low (5%) to be practical. Thus, as illustrated in Scheme 3, we developed a new method to prepare methyl (fluoromethyl)phosphonic chloride (**9**).

Dimethyl hydroxymethylphosphonate (**6**) was prepared in quantitative yield by the reaction of dimethyl phosphite with paraformaldehdye and anhydrous potassium carbonate in methanol.[46] Direct fluorination of **6** with DAST at −78 °C gave a low yield (5%). In contrast, activation of the alcohol **6** as triflate **7** (with 2,6-lutidine as the base), followed by nucleophilic displacement with tetrabutylammonium fluoride (TBAF) in THF, proved to be a safe and simple route to the protected (monofluoromethyl)phosphonate **8**.[47] Direct chlorination of dialkyl phosphonate with oxalyl chloride[48] was unsuccessful. However, a two-step protocol was successful. Thus, aminolysis of **8** with *tert*-butylamine afforded the desired phosphonic acid monomethyl ester *tert*-butylamine salt in quantitative yield.[43] The *tert*-butylamine salt was then converted to the free monomethyl phosphonic acid by passage through a Dowex acidic resin. The monomethyl phosphonate was then treated with oxalyl chloride and catalytic DMF in $CH_2Cl_2$ initially at 0°C, followed by stirring for 2 h at rt.[49] Analysis of a reaction aliquot by $^{31}P$ NMR demonstrated complete conversion. Concentration in vacuo eliminated the volatile reagents and left the desired phosphonochloridate **9** as an oily yellow residue. The product was then used without further purification, as attempts at vacuum distillation resulted in decomposition.

Returning to the inositol ring intermediates, the secondary alcohol **10** was phosphorylated with methyl (fluoromethyl)-phosphonyl chloride in the presence of *t*-BuOK and gave the protected 3-(fluoromethyl)phosphonate **11a** in good yield (46−48%) (Scheme 4). Using amine bases commonly employed for this type of reaction, e.g., triethylamine and *N*-methylimidazole, resulted in poorer yields. The silyl group in the 1-position was removed with the neutral reagent TBAF−HOAc, and the resulting alcohols **11** were treated with one of the three diacylglyceryl phosphoramidite reagents **19a**−**19c** in the presence of tetrazole followed by mild oxidation with *n*-$Bu_4NIO_4$ to give the fully protected PtdIns(3)P derivatives **14**. Using the mild oxidation reagent *n*-$Bu_4NIO_4$ avoided the undesired epoxidation of the oleic acyl double bond.

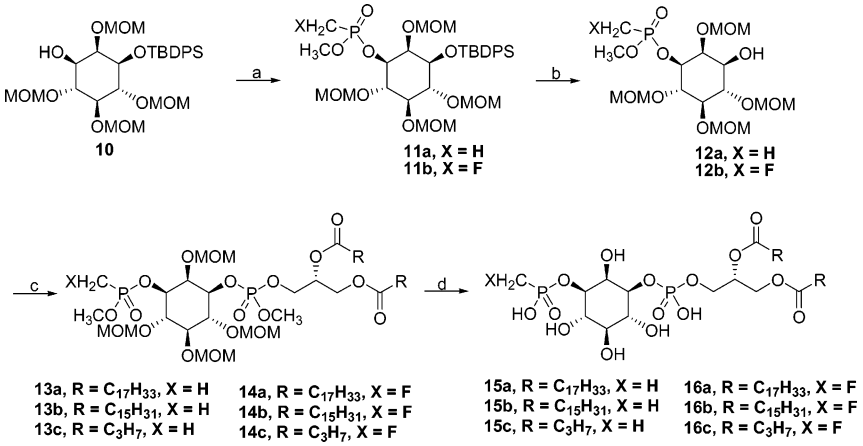
To prepare the PtdIns(3)P diesters, different phosphoramidites with preattached long, medium, and short diacyl chains were prepared (Scheme 5). For evaluation in different biological and biophysical systems, we prepared the dioleoyl, dipalmitoyl, and dibutyryl reagents. Following our previously reported procedures, the 3-*O*-PMB-*sn*-(2*R*)-glycerol was esterified with various fatty acids using DCC/DMAP to give compounds in high yields.[26] Oxidative removal of the PMB protective group with DDQ gave the corresponding 1,2-diacyl-*sn*-(2*S*)-glycerols in good yields without significant acyl migration. Although the 1,2-diacyl-*sn*-(2*S*)-glycerol was reasonably stable, slow purification on silica gel facilitated acyl chain migration. Therefore, rapid flash chromatography was essential to obtain homogeneous 1,2-diacyl-*sn*-(2*S*)-glycerols without acyl migration. Finally, coupling these alcohols with methyl *N*,*N*-diisopropylchlorophosphoramidite and cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite gave phosphorylation reagents **19** and **20** in high yields.

The removal of protective groups from the phosphate and hydroxyl of the fully protected inositol lipid intermediates was performed as follows. First, (TMS)Br was used to deprotect the phosphate methyl esters.[40] The fully protected derivatives were dissolved in (TMS)Br and $CH_2Cl_2$ (1:1, v/v) under strictly anhydrous conditions, and the resulting solution was stirred at rt for 1 h. After concentration in vacuo, the residue was dissolved in a methanol−water mixture (95:5, v/v) and the resulting solution then stirred for an additional 30 min to hydrolyze the silyl phosphate esters. After complete drying of the reaction mixture in vacuo, ethanethiol was added to remove all MOM groups and provide the final products in >98% purity.[40]

The reported methods of preparation of phosphorothioates vary considerably. Some methods that are suitable for specific compounds utilize conditions that would preclude application to the synthesis of derivatives bearing more labile functional groups. For example, methyl and ethyl esters have often been used as protecting groups; deprotection is then accomplished with (TMS)I or (TMS)Br. However, this approach fails to give practical yields with phosphonothioate or phosphorothioate derivatives.[50] Deprotection of the dibenzyl esters of phos-

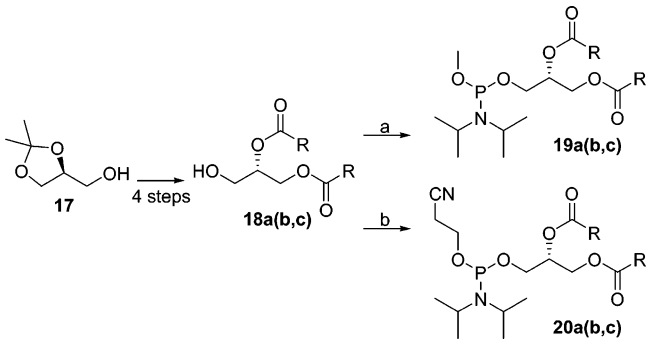
(44) Esalthazor, M.; Flores, R. A. *J. Org. Chem.* **1980**, *45*, 529−531.
(45) Lal, G. S.; Pez, G. P.; Syvret, R. G. *Chem. Rev.* **1996**, *96*, 1737−1755.
(46) Jeanmarie, T.; Hervaud, Y.; Boutevin, B. *Phosphorous, Sulfur Silicon* **2002**, *177*, 1137−1145.
(47) Hamilton, C. J.; Roberts, S. M. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1051−1056.
(48) Deussen, H. J.; Danielsen, S.; Breinholt, J.; Borchert, T. V. *Bioorg. Med. Chem.* **2000**, *8*, 507−513.
(49) Hirschmann, R.; Yager, K. M.; Taylor, C. M.; Witherington, J.; Sprengeler, P. A.; Phillips, B. W.; Moore, W.; Smith, A. B. I. *J. Am. Chem. Soc.* **1997**, *119*, 8177−8190.

***Scheme 4.*** Synthesis of 3-(Fluoromethyl)phosphonate Analogues of PtdIns(3)P[a]




[a] Reagents and conditions: (a) **4** or **9**, *t*-BuOK, $CH_2Cl_2$; (b) TBAF·$3H_2O$, HOAc, THF; (c) **19a**−**19c**, 1*H*-tetrazole, THF/$CH_3CN$; then *n*-$Bu_4NIO_4$, $CH_3CN$; (d) (TMS)Br, $CH_2Cl_2$; then $CH_3OH/H_2O$; then EtSH.

***Scheme 5.*** Synthesis of Glyceryl Phosphoramidites[a]




[a] Reagents and conditions: (a) methyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, $CH_2Cl_2$; (b) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, $CH_2Cl_2$.

phonothioic acids has been achieved with sodium in liquid ammonia,[51] but we sought milder reaction conditions. We turned to the cyanoethyl ester (CE), which is widely used for the synthesis of phosphonothioate or phosphorothioate acids. The CE group can be deprotected under mild basic conditions.[23,52] Primary, secondary, and tertiary amine, e.g., triethylamine and *tert*-butylamine, will readily remove the CE protection from phosphates and phosphorothioates at rt.

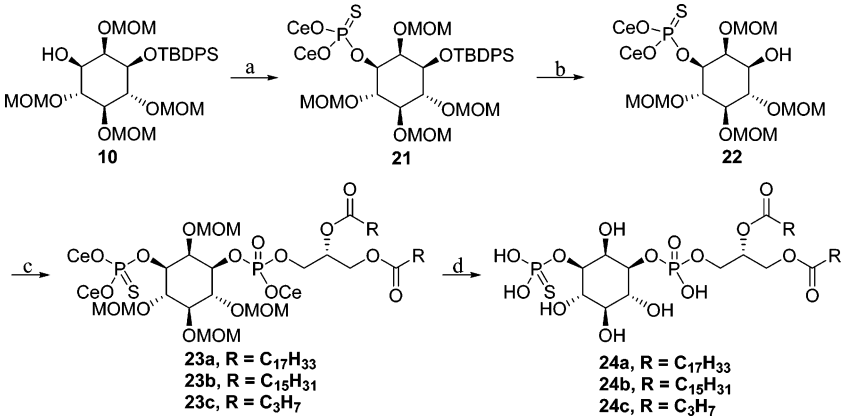
Alcohol **10** was then phosphorylated employing phosphoramidite methodology (Scheme 6). The resulting phosphoramidite triester was oxidized with elemental sulfur to yield the corresponding phosphorothioate triester. TBAF was frequently used to deprotect the TBDPS protective group. However, we found TBAF not only removed the TBDPS ether, but simultaneously removed the CE group, despite the fact that the TBAF reagent had been neutralized with 1 equiv of acetic acid. Apparently the basicity of TBAF was sufficient to cleave the CE phosphate linkage. Thus, to selectively deprotect the TBDPS group, we selected the HF− pyridine complex, which in Py−THF solution selectively removed the TBDPS ether. This deprotection reaction was very slow, and required three weeks to reach completion. Importantly, the reaction was conducted in a Teflon container. The reaction did not occur in common glassware, which was damaged in the process. The reaction rate could not be increased without also accelerating side reactions. Finally, TASF (tris(dimethylamino)sulfonium difluorotrimethylsilicate) was also explored for this deprotection,[50,53] but TASF was unable to cleave the secondary alcohol silyl ether **21**.

Three phosphoramidite reagents, **20a**−**20c**, were prepared by the reaction of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite with diacylglycerol preattached with different acyl chains using *N*,*N*-diisopropylethylamine (DIPEA) as the base.[54] The air-sensitive phosphoramidites were purified by rapid flash chromatography using a basic solvent system. The phosphoramidites were then condensed with secondary alcohol **22** in the presence of 1*H*-tetrazole to yield the phosphoramidite intermediates.

Oxidation of phosphoramidite triester proved to be problematic. The commonly used oxidation reagents, including MCPBA, *n*-$Bu_4NIO_4$, and $H_2O_2$, oxidized both the phosphoramidite triester and the 3-position phosphorothioate, even at low temperatures (−78 to −20 °C). Indeed, the phosphorothioate is readily oxidized to the corresponding phosphate when exposed to standard oxidation reagents. Therefore, a mild oxidation reagent was required to selectively oxidize the phosphoramidite triester without overoxidation of the phosphorothioate. Recently, *t*-BuOOSi$(CH_3)_3$ and *t*-BuOOH have been utilized to selectively oxidize phosphoramidite triesters.[55,56] Using *t*-BuOOH gave complete oxidation of the phosphoramidite triester to phosphate without overoxidation of the phosphorothioate. The CE groups on the phosphorothioate were removed by using triethylamine (TEA) plus bis((trifluoromethyl)silyl)acetamide (BTFSA) in anhydrous acetonitrile.[52] BTFSA was added to prevent the phosphorothioate anion from undergoing realkylation. The cleavage of the *O*-silyl derivatives was achieved by aqueous hydrolysis at neutral pH to give the MOM ether-protected intermediate. The MOM groups were removed using ethanethiol−$BF_3$ at rt to give the final products.[57]

(50) Borecka, B.; Chojnowski, J.; Cypryk, M.; Michalski, J.; Zielinska, J. *J. Organomet. Chem.* **1979**, *171*, 17−34.
(51) Piettre, S. R.; Raboisson, P. *Tetrahedron Lett.* **1996**, *37*, 2229−2232.
(52) Heeb, N. V.; Nambiar, K. P. *Tetrahedron Lett.* **1993**, *39*, 6193−6196.
(53) Kurosu, M.; Marcin, L. R.; Grinsteiner, T. J.; Kishi, Y. *J. Am. Chem. Soc.* **1998**, *120*, 6627−6628.
(54) Uhlman, E.; Engels, J. *Tetrahedron Lett.* **1986**, *27*, 1023−1026.
(55) Salamonczyk, G. M. *Tetrahedron Lett.* **2003**, *44*, 7449−7453.
(56) Sekine, M.; Tsuruoka, H.; Iimura, S.; Kusuoku, H.; Wada, T. *J. Org. Chem.* **1996**, *61*, 4087−4100.
(57) Kubiak, R. J.; Bruzik, K. S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1231−1234.

***Scheme 6.*** Synthesis of 3-Phosphorothioate Analogues of PtdIns(3)P[a]




[a] Reagents and conditions: (a) bis(2-cyanoethyl) diisopropylphosphorodiamidite, 1*H*-tetrazole, $CH_3CN$; then $S_8$, $CS_2$/Py; (b) HF·Py−Py, THF; (c) **20a**−**20c**, 1*H*-tetrazole, THF/$CH_3CN$; *t*-BuOOH, $CH_3CN$; (d) TEA, TFBSA, $CH_3CN$; then $NH_4OAc/H_2O$; then EtSH, $BF_3{\cdot}Et_2O$.

Consistent with the analytical TLC behavior of these molecules, each of the final amphiphilic ms-PtdIns(3)P derivatives could be purified by conventional chromatography using a $CHCl_3$−MeOH−$NH_4OH$ solvent system on silica gel.[58] The $^1H$ NMR of the PtdIns(3)P analogues illustrated the curious solubility of these compounds and the intrinsic difficulty in obtaining high-quality NMR spectroscopic data.[27] Only broad, poorly resolved $^1H$ NMR resonances could be detected in the single-solvent system $CDCl_3$ or $CD_3OD$. In contrast, in $CDCl_3$−$CD_3OD$ (3:2, v/v), the $^1H$ NMR resonances for the headgroup and acyl chains were well resolved.

**Binding to FYVE and PX Domains.** In cellular membranes, PtdIns(3)P is specifically recognized by a number of protein binding partners including FYVE and PX domains.[14,59] To test whether the PtdIns(3)P analogues are able to bind the physiological targets, we investigated interactions of human EEA1 FYVE and yeast Vam7 PX domains by NMR spectroscopy. Significant $^1H$ and $^{15}N$ chemical shift changes in the FYVE domain were induced by titrating $C_{16}$-PtdIns(3)P-$CH_2F$ (**16b**) embedded in membrane-mimetic dodecylphosphocholine (DPC) micelles (Figure 1A). These perturbations paralleled chemical shift changes seen in the FYVE domain as $C_4$-PtdIns(3)P-enriched DPC micelles were titrated in (Figure 1B). Thus, the PtdIns(3)P analogue and unmodified lipid are accommodated by the same binding pocket consisting of four Arg and two His residues of the FYVE domain.[59] However, the resonance perturbations observed during PtdIns(3)P-$CH_2F$ titration were smaller in magnitude, indicating weaker binding. These results reveal the importance of two negatively charged oxygen atoms of the 3-phosphate group of PtdIns(3)P and their hydrogen-bonding potential for the strong anchoring of the inositol ring by the basic residues of the FYVE domain. Similarly, when the PX domain was treated with PtdIns(3)P-$CH_2F$ **16b(c)**, which was prebound to the mixed diheptanoylphosphatidylcholine (DHPC)/CHAPS micelles, the observed chemical shift changes mirrored those seen upon binding of unmodified PtdIns(3)P. Addition of PtdIns(3)P(S) **24b(c)** and PtdIns(3)P-$CH_3$ **15b(c)** lipids or titrating the soluble $C_4$ forms of PtdIns(3)P analogues resulted in considerably smaller chemical shift changes in $^1H$−$^{15}N$ HSQC spectra of the proteins, although the pattern of resonance perturbations remained essentially unchanged (Figure 1C,D and data not shown).

**PIKfyve Uses ms-PtdIns(3)P Analogues as Substrates.** PtdIns(3,5)$P_2$ and PtdIns(5)P could be produced in vitro by two mammalian enzymes: type I PIP kinase[60] and PIKfyve.[61] Genetic and biochemical evidence has recently accumulated to implicate PIKfyve as the principal enzyme responsible for their biosynthesis in the cellular context.[62] We now provide evidence for specific and high-affinity interactions between the PIKfyve and metabolically stabilized analogues of PtdIns(3)P in vitro. PIKfyve activity was assayed as described previously by the [$\gamma$-$^{32}$P]ATP-dependent phosphorylation of PtdIns. It was found that each of the three PtdIns(3)P analogues having dioleoyl and dipalmitoyl chains were substrates for the kinase PIKfyve (Scheme 7), as phosphorylated products were detected by radio-TLC analysis (Figure 2). Since (monofluoromethyl)phosphonate and phosphorothioate are less polar than the phosphate group, the resulting [5-$^{32}$P]ms-PtdIns(3,5)$P_2$ analogues migrated faster than the unmodified [5-$^{32}$P]PtdIns(3,5)$P_2$ (Figure 2). Thus, the stabilized phosphorothioate and phosphonate groups at the 3-position of these PtdIns(3)P analogues are recognized by the nonclassical FYVE motif within the PIKfyve catalytic domain.[63]

**Conclusions**. In summary, we have developed a general approach to the synthesis of methylphosphonate, (monofluoromethyl)phosphonate, and phosphorothioate analogues of PtdIns(3)P. This strategy also is applicable to the synthesis of analogues of all other PtdIns$P_n$ and Ins$P_n$ compounds. In addition, our method enables synthesis of both saturated and unsaturated acyl analogues of PtdIns$P_n$. These PI(3)P analogues exhibited reduced binding activities with $^{15}N$-labeled FYVE and PX domains as significant $^1H$ and $^{15}N$ chemical shift changes in the FYVE domain were induced by titrating ms-PtdIns(3)P in membrane-mimetic DPC micelles. In addition, these PtdIns-(3)P analogues with dioleoyl and dipalmitoyl chains were recognized by PIKfyve, as phosphorylated ms-PtdIns(3,5)$P_2$

(58) Auger, K. R.; Carpenterg, C. L.; Cantley, L. C.; Varticovski, L. *J. Biol. Chem.* **1989**, *264*, 20181−20184.
(59) Kutateladze, T.; Overduin, M. *Science* **2001**, *291*, 1793−1796.
(60) Tollas, K. F.; Rameh, L. E.; Ishihara, H.; Shibashi, Y.; Chen, J.; Prestwich, G. D.; Cantley, L. C.; Carpenter, C. L. *J. Biol. Chem.* **1998**, *273*, 18040−18046.
(61) Sbrissa, D.; Ikonomov, O. C.; Shisheva, A. *J. Biol. Chem.* **1999**, *274*, 21589−21597.
(62) Shisheva, A. *Cell Biol. Int.* **2001**, *25*, 1201−1206.
(63) Sbrissa, D.; Ikonomov, O. C.; Shisheva, A. *J. Biol. Chem.* **2002**, *277*, 6073−6079.

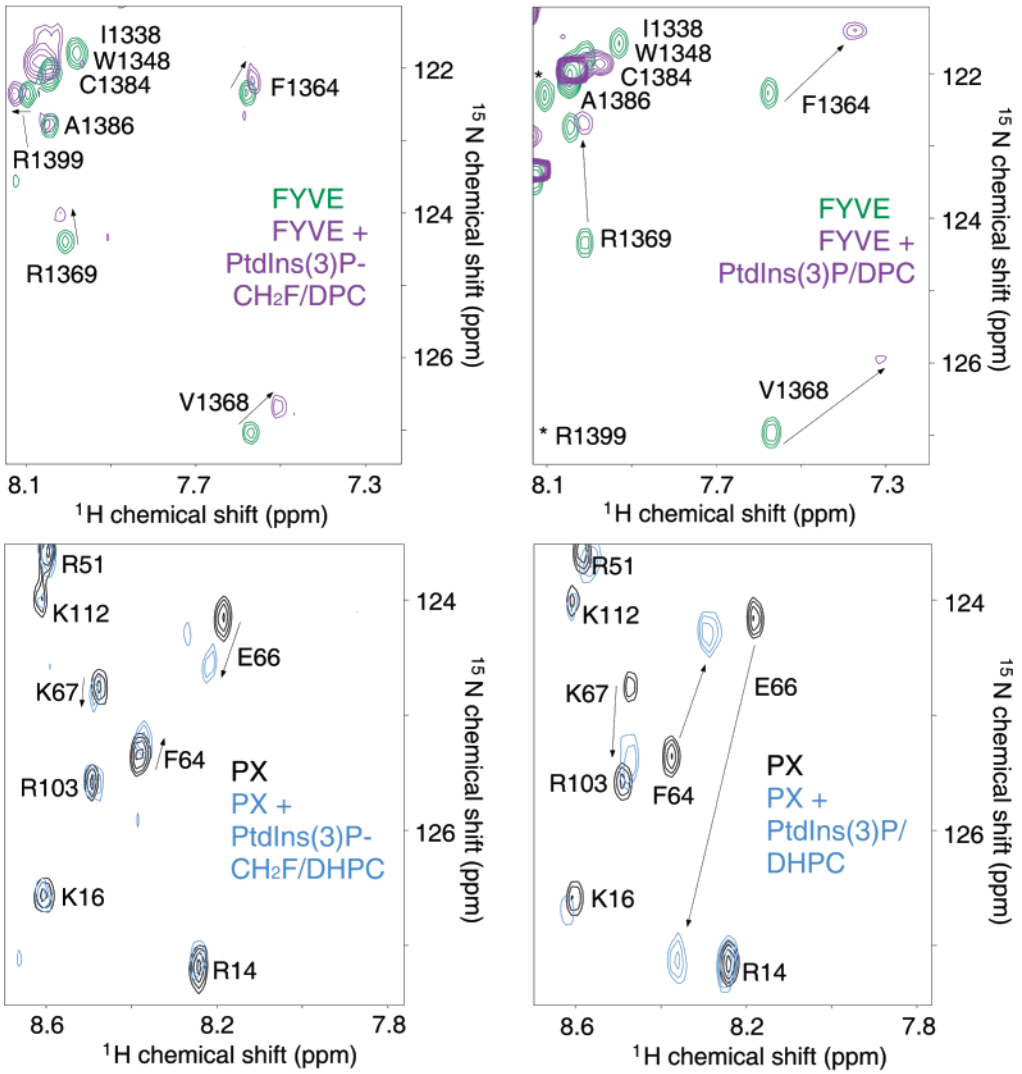
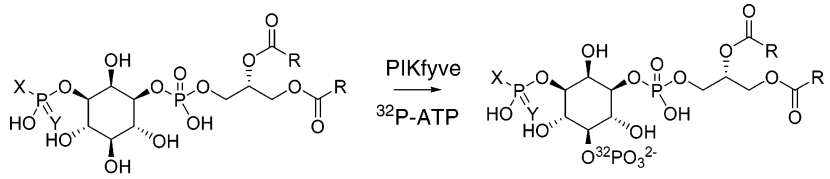



***Figure 1.*** PtdIns(3)P analogues are recognized by the FYVE and PX domains. Superimposed $^{1}H-^{15}N$ HSQC NMR spectra of the (A, B, upper left and right) 0.2 mM EEA1 FYVE domain and (C, D, lower left and right) 0.2 mM Vam7 PX domain collected before and after addition of (A) 4 mM $C_{16}$-PtdIns-(3)P-$CH_2F$ (**16b**) and 250 mM $d_{38}$-DPC, (B) 1 mM $C_4$-PtdIns(3)P and 250 mM $d_{38}$-DPC, (C) 2 mM $C_4$-PtdIns(3)P-$CH_2F$ (**16c**) in 100 mM DHPC and 33 mM CHAPS, and (D) 2 mM $C_4$-PtdIns(3)P in 100 mM DHPC and 33 mM CHAPS. Directions of the chemical shift changes are indicated by arrows.

***Scheme 7.*** Phosphorylation of PtdIns(3)P Analogues by PIKfyve




**15a, R = $C_{17}H_{33}$, X = H**
**15b, R = $C_{15}H_{31}$, X = H**

**16a, R = $C_{17}H_{33}$, X = F**
**16b, R = $C_{15}H_{31}$, X = F**

**24a, R = $C_{17}H_{33}$**
**24b, R = $C_{15}H_{31}$**

**25a, R = $C_{17}H_{33}$, X = H**
**25b, R = $C_{15}H_{31}$, X = H**

**26a, R = $C_{17}H_{33}$, X = F**
**26b, R = $C_{15}H_{31}$, X = F**

**27a, R = $C_{17}H_{33}$**
**27b, R = $C_{15}H_{31}$**

products were detected by radio-TLC analysis. The metabolically stabilized analogues of PtdIns(3)P reported herein provide new tools for the elucidation of the roles of these phosphoinositide monophosphates in cell signaling. Specific applications of these analogues in cell and molecular biology will be presented in due course.

## Experimental Section

**General Procedures.** Chemicals were purchased from Aldrich and Acros Chemical Corp. and used without prior purification. Solvents were reagent-grade and distilled before use: $CH_2Cl_2$ was distilled from $CaH_2$, and THF was distilled from sodium wire. TLC used precoated silica gel aluminum sheets (EM Science silica gel $60F_{254}$). Flash

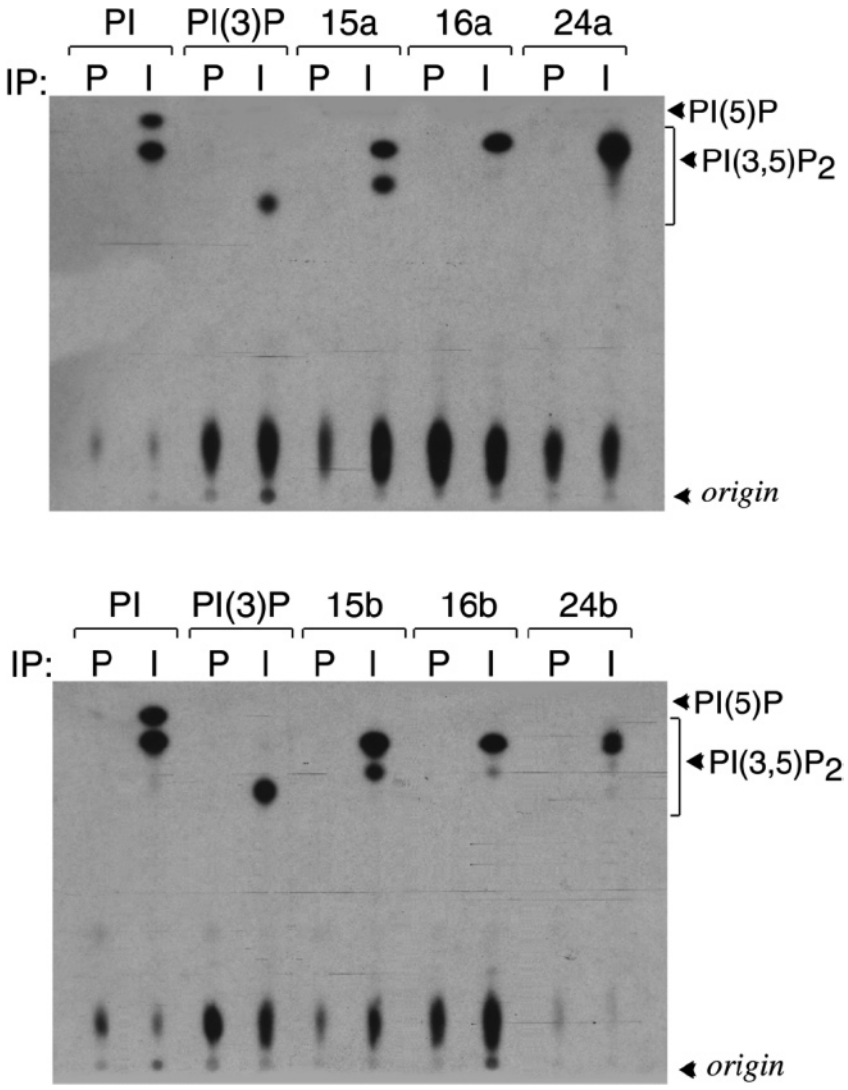



***Figure 2.*** Radio-TLC shows that PtdIns(3)P analogs are recognized and phosphorylated by PIKfyve. PIKfyve activity was determined in anti-PIKfyve immunoprecipitates with the indicated substrates and [$\gamma$-$^{32}$P]ATP. Refer to the Experimental Section for details. IP, immunoprecipitates; P, preimmune serum; I, immune anti-PIKfyve-serum.

chromatography (FC) employed Whatman 230−400 mesh ASTM silica gel. NMR spectra were recorded on a Varian INOVA 400 at 400 MHz ($^1$H), 101 MHz ($^{13}$C), 162 MHz ($^{31}$P), and 376 MHz ($^{19}$F) at 25 °C. Chemical shifts are reported in parts per million with TMS as the internal standard ($\delta$ = 0.00). The internal standard for $^{31}$P NMR is 85% $H_3PO_4$ ($\delta$ = 0.00), and that for $^{19}$F NMR is $CFCl_3$ ($\delta$ = 0.00). Low- and high-resolution mass spectra were obtained on HP5971A MSD and Finnigan MAT95 double-focusing mass spectrometer (MS) instruments, respectively. Dibutyryl- and dipalmitoyl-PtdIns(3)P were obtained from Echelon Biosciences (Salt Lake City, UT).

**Methyl Methylphosphonyl Chloride (4).** To 12.87 g (0.104 mol) of dimethyl methylphosphonate in 30 mL of anhydrous benzene was added 21.6 g (0.104 mol) of $PCl_5$ at 0 °C. The solution was stirred vigorously for 1 h at 0 °C. The solvent was removed under vacuum. Vacuum distillation (80 °C, 22 mmHg) gave 12.1 g (0.094 mol, 94%) of homogeneous product. $^1$H NMR ($CDCl_3$): $\delta$ 3.72 (d, $J$ = 6.0 Hz, 2H), 3.57 (d, $J$ = 10.5 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 56.40 (s), 54.78 (s), 53.14 (s), 53.07 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 28.18 (s). MS (CI): *m*/*z* 129.0 ($M^+ + 1$, 8.32). HRMS (CI) (*m*/*z*) ($M^+ + 1$): found, 128.9872; calcd for $C_2H_6ClO_2P$, 128.9871.

**(Dimethoxyphosphinyl)methyl Triflates (7).** To a stirred solution of dimethoxy (hydroxymethyl)phosphonate (30.6 mmol) and 2,6-lutidine (37.6 mmol) in anhydrous $CH_2Cl_2$ (50 mL) at −50 °C under $N_2$ was added trifluoromethanesulfonic anhydride (35.5 mmol) dropwise. The resulting mixture was allowed to warm to 0 °C over a period of 1.5 h, whereupon the dark brown solution was diluted with ether (300 mL). The precipitates formed were removed by filtration. The ethereal solution was successively washed with water, 1 N HCl, and brine and then dried over $Na_2SO_4$. After concentration, a yellow oil was obtained, which was used in the next step without further purification.

**Dimethyl (Fluoromethyl)phosphonate (8).** A solution of the triflate **7** (5.08 g, 0.015 mol) in THF (22 mL) was cooled to 0 °C before 20 mL (0.02 mol) of a 1 M solution of tetrabutylammonium fluoride in THF was added dropwise. The solution was stirred at 0 °C for 1 h. Solvents were then removed, and $CH_2Cl_2$ (35 mL) was added. The organic layer was washed ($H_2O$, 3 × 8 mL), dried ($MgSO_4$), and evaporated to a crude oil. This was purified by FC, using EtOAc−hexane (1:1) as the eluent, to give **8** as a pale yellow oil (1.67 g, 67%). $^1$H NMR ($CDCl_3$): $\delta$ 4.68 (dd, $J$ = 46.8, 4.8 Hz, 2H), 3.81 (d, $J$ = 10.8 Hz, 6 H). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.83 (d, $J$ = 63.0 Hz). $^{19}$F NMR ($CDCl_3$): $\delta$ −250.74 (dt, $J$ = 61.7, 47.0 Hz). MS (CI): *m*/*z* 143.0 ($M^+ + 1$, 100.00). HRMS (CI) (*m*/*z*) ($M^+ + 1$): found, 143.0262; calcd for $C_3H_9FO_3P$, 143.0267.

**Methyl (Fluoromethyl)phosphonate Chloride (9). 8** (0.221 g, 0.592 mmol) was dissolved in *tert*-butylamine (8 mL, 76 mmol) and the resulting solution was heated at reflux overnight. The reaction was concentrated and gave 0.256 g of product as a white salt (quantitative

yield). Methyl (fluoromethyl)phosphonate *tert*-butylamine salt (0.290 g, 0.670 mmol) was dissolved in $CHCl_3$ and the resulting solution treated with cation-exchange resin (Dowex 50W-X8 ($H^+$ form), 200−400 mesh). The Dowex resin was removed by filtration, and the filtrate was concentrated in vacuo to give 0.241 g (quantitative yield) of the oily phosphonate. Next, oxalyl chloride (0.132 g, 1.04 mmol) was added dropwise to a solution of the methyl (fluoromethyl)phosphonic acid (0.240 g, 0.668 mmol) and DMF (2.5 $\mu$L, 0.033 mmol) dissolved in $CH_2Cl_2$ at 0 °C. The solution was stirred at 0 °C for 20 min, then warmed to rt, and stirred for 1.5 h. The reaction was concentrated, dissolved in toluene (2 mL), and then reconcentrated in vacuo to remove the volatile reagents. This gave phosphonochloridate **9** as a yellow oil, which was then used immediately for reaction with the 3-hydroxy-containing protected inositol derivatives. $^1$H NMR ($CDCl_3$): $\delta$ 4.62 (dd, $J$ = 46.8, 4.4 Hz, 2H), 3.74 (d, $J$ = 11.2 Hz, 3 H). $^{13}$C NMR ($CDCl_3$): $\delta$ 75.72 (dd, $J$ = 180.2, 168.7 Hz), 53.14 (d, $J$ = 6.1 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.80 (d, $J$ = 63.0 Hz). $^{19}$F NMR($CDCl_3$): $\delta$ −250.98 (dt, $J$ = 62.8, 47.0 Hz). MS (CI): $m/z$ 147.0 ($M^+ + 1$, 100.00). HRMS (CI) ($m/z$) ($M^+ + 1$): found, 146.9759; calcd for $C_2H_6ClFO_2P$, 146.9778.

**1D 1-*O*-(*tert*-Butyldiphenylsilyl)-3-(methyl methylphosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (11a).** *t*-BuOK (18 mg, 0.163 mmol, 1.4 equiv) was added to a stirred solution (69 mg, 0.116 mmol) of **10** and methyl methylphosphonate chloride (18 mg, 0.139 mmol, 1.2 equiv) in $CH_2Cl_2$ (1 mL) at 0 °C, then the resulting solution was stirred for 2 h at rt, and the reaction was complete. A saturated aqueous solution of $NH_4Cl$ (1 mL) was added, the resulting solution was stirred for 10 min, the aqueous phase was extracted with $CH_2Cl_2$ (3 × 5 mL), the organic solution was dried with anhydrous $Na_2SO_4$, and the solvent was evaporated under reduced pressure. The crude product was purified by chromatography (*n*-hexane−acetone, 2:1, v/v) to afford a colorless liquid (37 mg, 0.054 mmol, 46%). $^1$H NMR ($CDCl_3$): $\delta$ 7.72−7.67 (m, 4H), 7.41−7.36 (m, 6H), 4.98 (d, $J$ = 4.4 Hz, 1H), 4.91 (d, $J$ = 4.4 Hz, 1H), 4.79 (d, $J$ = 4.4 Hz, 1H), 4.75 (m, 3H), 4.57 (d, $J$ = 4.8 Hz, 1H), 4.26 (d, $J$ = 4.8 Hz, 1H), 3.92−3.85 (m, 4H), 3.86 (d, $J$ = 6.0 Hz, 3H), 3.42−3.32 (m, 12H), 3.20 (d, $J$ = 23.0 Hz, 2H), 1.34 (d, $J$ = 17.6 Hz, 3H), 1.06 (s, 9H). $^{13}$C NMR ($CDCl_3$): $\delta$ 135.93 (s), 135.81 (s), 133.80 (s), 133.77 (s), 132.51 (s), 132.48 (s), 129.95 (s), 129.86 (s), 129.80 (s), 128.01 (s), 127.89 (s), 127.71 (s), 127.69 (s), 98.93 (s), 98.35 (s), 98.30 (s), 98.22 (s), 97.46 (d, $J$ = 14.58 Hz), 82.04 (d, $J$ = 8.45 Hz), 81.28 (d, $J$ = 5.43 Hz), 78.88 (d, $J$ = 34.49 Hz), 74.84 (s), 70.49 (s), 56.05 (dd, $J$ = 62.05, 24.54 Hz), 27.07 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 33.89 (s), 33.04 (s). MS (CI): $m/z$ 687.1 ($M^+ + 1$, 79.67), 655.1 ($M^+ - OCH_3$, 100.00). HRMS (CI) ($m/z$) ($M^+ + 1$): found, 687.2962; calcd for $C_{32}H_{52}O_{12}PSi$, 687.2966.

**1D 3-(Methyl methylphosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (12a).** A solution of **11a** (24 mg, 0.035 mmol) in THF (1 mL) was treated with tetrabutylammonium fluoride trihydrate (16 mg, 0.049 mmol) at rt. After the solution was stirred for 18 h, the reaction was complete (monitored by TLC). The solvent was evaporated under reduced pressure, and the crude product was purified by passage through a short silica gel column (*n*-hexane−acetone, 3:1, v/v) to afford 12 mg of a colorless liquid (0.027 mmol, 77%). $^1$H NMR ($CDCl_3$): $\delta$ 4.79−4.64 (m, 8H), 4.17 (t, $J$ = 10.4 Hz, 1H), 4.10 (d, $J$ = 20.4 Hz, 1H), 3.98 (dd, $J$ = 11.6, 4.4 Hz, 1H), 3.89 (dd, $J$ = 21.2, 9.6 Hz, 1H), 3.69 (dd, $J$ = 20.0, 9.2 Hz, 1H), 3.58 (td, $J$ = 9.2, 1.2 Hz, 1H), 3.42 (m, 1H), 3.41−3.34 (m, 15H), 1.46 (dd, $J$ = 18.0, 3.6 Hz, 3H). $^{13}$C NMR ($CDCl_3$): $\delta$ 98.52 (s), 98.35 (s), 98.31 (s), 98.26 (s), 98.17 (s), 98.11 (s), 97.97 (s), 82.93 (s), 79.11 (d, $J$ = 16.09 Hz), 78.41 (d, $J$ = 6.94 Hz), 76.50 (d, $J$ = 6.13 Hz), 74.82 (dd, $J$ = 29.06, 6.84 Hz), 70.40 (d, $J$ = 5.33 Hz), 52.03 (dd, $J$ = 40.63, 6.13 Hz), 10.97 (dd, $J$ = 146.52, 23.73 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 34.60 (s), 33.36 (s). MS (CI): $m/z$ 449.1 ($M^+ + 1$, 100.00). HRMS (CI) ($m/z$) ($M^+ + 1$): found, 449.1779; calcd for $C_{16}H_{34}O_{12}P$, 449.1788.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphospho)-3-(methyl methylphosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (13a).** *N*,*N*-Diisopropyl-*O*-methyl-*O*-(di-(2*S*)-oleoyl-*sn*-glycerol)phosphonamidite (0.187 g, 0.135 mmol) was added under an argon atmosphere to a solution of **12a** (110 mg, 0.089 mmol) and 1*H*-tetrazole (26 mg, 0.374 mmol) in 4 mL of dry $CH_2Cl_2$/THF (1:1, v/v). After the resulting solution was stirred for 20 h at rt, oxidation was performed with $(n\text{-}C_4H_9)_4NIO_4$ (78 mg, 0.180 mmol) at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min, and after aqueous workup, the crude product was purified by FC on silica gel (*n*-hexane/acetone, 3:1, v/v) to give 77 mg of a homogeneous colorless oil (0.068 mmol, 76%). $^1$H NMR ($CDCl_3$): $\delta$ 5.32 (m, 2H), 5.21 (m, 1H), 4.84−4.71 (m, 8H), 4.32−4.09 (m, 8H), 3.96−3.89 (m, 2H), 3.77−3.72 (m, 6H), 3.41−3.33 (m, 12H), 2.29 (m, 4H), 1.96 (m, 8H), 1.55 (m, 4H), 1.52 (dd, $J$ = 17.6, 6.8 Hz, 3H), 1.21 (m, 42H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.15 (s), 172.73 (s), 129.91 (s), 129.66 (s), 98.91 (s), 98.54 (s), 98.45 (s), 98.37 (s), 98.03 (s), 97.85 (s), 79.13 (d, $J$ = 13.78 Hz), 74.24 (dd, $J$ = 31.48, 6.13 Hz), 69.37 (s), 65.80 (s), 65.52 (s), 61.61 (s), 56.78 (s), 56.65 (s), 56.56 (s), 56.51 (s), 55.76 (s), 54.81 (s), 53.75 (s), 52.18 (dd, $J$ = 35.30, 6.13 Hz), 34.08 (s), 33.96 (s), 31.88 (s), 29.65 (s), 29.63 (s), 29.61 (s), 29.45 (s), 29.31 (s), 29.25 (s), 29.22 (s), 29.09 (s), 29.06 (s), 24.78 (s), 22.64 (s), 14.07 (s), 11.03 (dd, $J$ = 148.83, 12.27 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 34.60 (s), 33.49 (s), 0.74 (s), 0.52 (d, $J$ = 7.61 Hz). MS (CI): $m/z$ 1145.7 ($M^+ + 1$, 100.00). HRMS (CI) ($m/z$) ($M^+ + 1$): found, 1145.6897; calcd for $C_{56}H_{107}O_{19}P_2$, 1145.6882.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(methylphosphonate)-*myo*-inositol (15a).** The phosphate **13a** (22 mg, 0.019 mmol) in a 5 mL flask was dried in vacuo, and then anhydrous (TMS)Br (0.2 mL) and $CH_2Cl_2$ (0.2 mL) were added into the flask. The solution was stirred at rt for 30 min. (TMS)Br and volatile products were evaporated under high vacuum for 6 h. The residue was dissolved in MeOH−$H_2O$ (95:5, 1.0 mL) and the resulting solution stirred for 30 min at rt. The solution was thoroughly concentrated for an additional 3 h under high vacuum. Ethanethiol (1 mL) was added, and the solution was kept at rt for 1 h and then concentrated to yield the crude product. Chromatography on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) provided 16 mg of pure product **15a** (0.017 mmol, 89%). $^1$H NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 5.32 (m, 2H), 5.21 (m, 1H), 3.93 (dd, $J$ = 12.0, 3.6 Hz, 1H), 3.82 (m, 2H), 3.73−3.60 (m, 5H), 3.30 (m, 2H), 2.33 (m, 4H), 1.96 (m, 8H), 1.55 (m, 4H), 1.52 (dd, $J$ = 17.6, 6.8 Hz, 3H), 1.21 (m, 42H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 173.28 (s), 172.89 (s), 129.25 (s), 128.98 (s), 97.13 (s), 73.70 (s), 70.87 (s), 70.46 (s), 69.98 (s), 69.33 (s), 64.39 (s), 61.60 (s), 54.88 (s), 42.53 (s), 42.46 (s), 33.30 (s), 31.24 (s), 29.26 (s), 29.06 (s), 28.83 (s), 28.64 (s), 28.60 (s), 28.56 (s), 28.53 (s), 28.45 (s), 28.43 (s), 28.39 (s), 26.46 (s), 24.17 (s), 21.96 (s), 13.08 (s), 10.62 (d, $J$ = 140.80 Hz). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 32.56 (s), 32.40 (s), −0.51 (s). MS (ESI): $m/z$ 963.53 ($M^+$ + Na, $C_{46}H_{86}NaO_{15}P_2$, 100.0). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 963.5312; calcd for $C_{46}H_{86}NaO_{15}P_2$, 963.5340.

**1D *O*-(1,2-Di-*O*-palmitoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphospho)-3-(methyl methylphosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (13b)**. *N*,*N*-Diisopropyl-*O*-methyl-*O*-(di-(2*S*)-palmitoyl-*sn*-glycerol)phosphonamidite (91 mg, 0.125 mmol) was added under an argon atmosphere to a solution of **12a** (37 mg, 0.083 mmol) and 1*H*-tetrazole (25 mg, 0.349 mmol) in 4 mL of dry $CH_2Cl_2$−THF (1:1, v/v). After the resulting solution was stirred for 20 h at rt, oxidation was performed with $(n\text{-}C_4H_9)_4NIO_4$ (69 mg, 0.160 mmol) at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min, and after aqueous workup, the crude product was chromatographed on silica gel (*n*-hexane−acetone, 3:1, v/v) to give 67 mg of pure **13b** as a colorless oil (0.061 mmol, 73%). $^1$H NMR ($CDCl_3$): $\delta$ 5.21 (m, 1H), 4.84−4.71 (m, 8H), 4.34−4.07 (m, 8H), 3.96−3.89 (m, 2H), 3.77−3.72 (m, 6H), 3.41−3.33 (m, 12H), 2.29 (m, 4H), 1.55 (m, 4H), 1.52 (dd, $J$ = 17.6, 6.8 Hz, 3H), 1.21 (m, 48H), 0.84 (t, $J$ = 7.2 Hz, 6H).

$^{13}$C NMR ($CDCl_3$): $\delta$ 173.19 (s), 172.80 (s), 98.90 (s), 98.53 (s), 98.43 (s), 98.35 (s), 98.03 (s), 97.83 (s), 79.13 (d, $J$ = 13.78 Hz), 77.21 (s), 76.90 (s), 76.35 (s), 74.24 (dd, $J$ = 31.48, 6.13 Hz), 69.44 (s), 69.35 (s), 65.75 (s), 65.57 (s), 61.60 (s), 56.77 (s), 56.64 (s), 56.55 (s), 56.50 (s), 55.75 (s), 53.75 (s), 52.18 (dd, $J$ = 35.30, 6.13 Hz), 34.08 (s), 33.96 (s), 31.88 (s), 29.65 (s), 29.63 (s), 29.61 (s), 29.45 (s), 29.31 (s), 29.25 (s), 29.22 (s), 29.09 (s), 29.06 (s), 24.78 (s), 22.64 (s), 14.07 (s), 11.03 (dd, $J$ = 148.83, 12.27 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 34.60 (d, $J$ = 8.74 Hz), 33.51 (d, $J$ = 6.64 Hz), 0.74 (s), 0.53 (d, $J$ = 8.74 Hz). MS (CI): $m/z$ 1093.7 ($M^+ + 1$, 4.58). HRMS (CI) ($m/z$) ($M^+ + 1$): found, 1093.6583; calcd for $C_{52}H_{103}O_{19}P_2$, 1093.6569.

**1D *O*-(1,2-Di-*O*-palmitoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(methylphosphonate)-*myo*-inositol (15b).** The phosphate **13b** (29 mg, 0.027 mmol) in a 5 mL flask was thoroughly dried in vacuo, and then anhydrous (TMS)Br (0.2 mL) and $CH_2Cl_2$ (0.2 mL) were added. The solution was stirred at rt for 30 min. (TMS)Br and volatile products were evaporated under high vacuum for 6 h. The residue was dissolved in MeOH−$H_2O$ (95:5, 1.0 mL) and the resulting solution stirred for 30 min at rt. The solution was thoroughly concentrated for an additional 3 h under high vacuum. Ethanethiol (1 mL) was added, and the solution was kept at rt for 1 h and then concentrated to yield the crude product. Chromatography on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) provided 22 mg of pure product **15b** (0.025 mmol, 92%). $^1$H NMR ($CDCl_3$−$CD_3OD$, 3:2, v/v): $\delta$ 5.27 (m, 1H), 3.93 (dd, $J$ = 12.0, 3.6 Hz, 1H), 3.82 (m, 2H), 3.73−3.60 (m, 6H), 3.30 (m, 2H), 2.33 (m, 4H), 1.62 (m, 4H), 1.56 (dd, $J$ = 17.6, 6.8 Hz, 3H), 1.27 (m, 48H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 173.17 (s), 172.75 (s), 98.20 (s), 73.80 (s), 70.97 (s), 70.55 (s), 70.02 (s), 69.74 (s), 64.52 (s), 61.81 (s), 54.97 (s), 33.45 (s), 33.42 (s), 31.36 (s), 29.32 (s), 29.12 (s), 28.85 (s), 28.64 (s), 28.70 (s), 14.01 (s), 10.58 (d, $J$ = 138.20 Hz). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 32.45 (s), 32.30 (s), 0.74 (s), −0.55 (s). MS (ESI): $m/z$ 911.50 ($M^+$ + Na, $C_{42}H_{82}NaO_{15}P_2$, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 911.5045; calcd for $C_{42}H_{82}NaO_{15}P_2$, 911.5027.

**1D *O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphospho)-3-(methyl methylphosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (13c)**. To a solution of alcohol **12a** (17 mg, 0.038 mmol) in dry THF (0.5 mL) were added *N*,*N*-diisopropyl-*O*-(methyl)-*O*-di-*O*-butanoyl-*sn*-(2*S*)-glycerol)phosphonamidite (25 mg, 24 $\mu$L, 0.125 mmol) and 1*H*-tetrazole (26 mg, 0.374 mmol). The mixture was stirred at rt for 16 h. Then oxidation was performed with ($n$-$C_4H_9)_4NIO_4$ (76 mg, 0.174 mmol) at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min. The solution was diluted with methylene chloride (20 mL) and washed with 10% sodium bisulfite. The organic layer was concentrated, and the residue was chromatographed on silica gel (*n*-hexane−acetone, 1:1, v/v) to give 25 mg of pure **13c** as a colorless oil (0.033 mmol, 87%). $^1$H NMR ($CDCl_3$): $\delta$ 5.23 (m, 1H), 4.84−4.74 (m, 8H), 4.34−4.07 (m, 8H), 3.96−3.89 (m, 2H), 3.77−3.72 (m, 6H), 3.41−3.33 (m, 12H), 2.31 (m, 4H), 1.62 (m, 4H), 1.52 (dd, $J$ = 17.6, 6.8 Hz, 3H), 0.93 (t, $J$ = 7.2 Hz, 3H), 0.90 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.03 (s), 172.90 (s), 98.90 (s), 98.45 (s), 97.98 (s), 97.85 (s), 98.03 (s), 79.20 (m), 74.28 (d, $J$ = 31.48 Hz), 69.36 (s), 65.65 (d, $J$ = 23.03 Hz), 61.57 (s), 56.79 (s), 56.57 (s), 55.78 (s), 54.59 (s), 52.20 (dd, $J$ = 35.30, 6.13 Hz), 35.93 (s), 35.83 (s), 18.26 (s), 13.57 (s), 11.03 (dd, $J$ = 148.83, 12.27 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 34.65 (s), 33.54 (s), 0.75 (s), 0.52 (d, $J$ = 7.61 Hz). MS (ESI): $m/z$ 757.41 ($M^+ + 1$, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 779.2627; calcd for $C_{28}H_{54}NaO_{19}P_2$, 779.2632.

**1D *O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(methylphosphonate)-*myo*-inositol (15c).** The phosphate **13c** (12 mg, 0.016 mmol) was dried and reacted with (TMS)Br and the resulting compound then hydrolyzed in aqueous MeOH as described above for **15a**. Ethanethiol (1 mL) was added, and the solution was kept at rt for 1 h and then concentrated to yield the crude product. Chromatography on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) provided 8 mg of pure product **15c** (0.016 mmol, 94%). $^1$H NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 5.27 (m, 1H), 4.74 (s, 1H), 4.36 (dd, $J$ = 12.0, 3.2 Hz, 1H), 4.28 (m, 1H), 4.18−4.06 (m, 5H), 3.78 (m, 2H), 3.23 (t, $J$ = 9.2 Hz, 1H), 2.28 (m, 4H), 1.58 (m, 7H), 0.99 (m, 6H). $^{13}$C NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 174.32 (s), 173.94 (s), 98.25 (s), 78.74 (m), 78.36 (m), 76.19 (m), 74.75 (s), 71.99 (s), 71.57 (s), 71.08 (s), 70.32 (s), 65.56 (s), 62.60 (s), 56.07 (s), 36.42 (s), 36.29 (s), 18.72 (s), 13.73 (d, $J$ = 3.84 Hz). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 36.57 (s), 36.42 (s), 3.47 (s), 3.31 (s). MS (ESI): $m/z$ 575.13 ($M^+$ + Na, $C_{18}H_{34}NaO_{15}P_2$). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 575.1296; calcd for $C_{18}H_{34}NaO_{15}P_2$, 575.1271.

**1D 1-*O*-(*tert*-Butyldiphenylsilyl)-3-(methyl (fluoromethyl)phosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (11b)**. *t*-BuOK (230 mg, 2.31 mmol, 1.4 equiv) was added to a stirred solution of 980 mg (1.65 mmol) of 10 and methyl (fluoromethyl)phosphonate chloride (1.99 mmol, 1.2 equiv) in $CH_2Cl_2$ (10 mL) at 0 °C; stirring was continued for 2 h at rt to complete the reaction. A saturated aqueous solution of $NH_4Cl$ (1 mL) was added, the resulting solution was stirred for 10 min, and the aqueous phase was extracted with $CH_2Cl_2$ (3 × 5 mL). The combined organic phases were dried with anhydrous $Na_2SO_4$, and the solvent was removed in vacuo. The crude product was purified by chromatography (*n*-hexane−acetone, 3:1, v/v) to afford 552 mg of **11b** as a colorless liquid (0.784 mmol, 48%). $^1$H NMR ($CDCl_3$): $\delta$ 7.70−7.65 (m, 4H), 7.42−7.34 (m, 6H), 4.97 (d, $J$ = 2.0 Hz, 1H), 4.96 (d, $J$ = 2.4 Hz, 1H), 4.90 (d, $J$ = 6.0 Hz, 1H), 4.76 (m, 3H), 4.62 (m, 1H), 4.50 (m, 2H), 4.46 (m, 1H), 3.98−3.73 (m, 5H), 3.52 (m, 2H), 3.43−3.23 (m, 14H), 1.05 (s, 9H). $^{13}$C NMR ($CDCl_3$): $\delta$ 135.99 (s), 135.85 (s), 133.79 (s), 133.76 (s), 132.48 (s), 130.08 (s), 129.97 (s), 129.91 (s), 129.89 (s), 128.10 (s), 127.99 (s), 127.81 (s), 127.77 (s), 99.14 (s), 99.09 (s), 98.86 (s), 98.67 (s), 98.38 (s), 97.78 (s), 97.55 (s), 78.85 (m), 78.04 (s), 77.76 (s), 76.42 (m), 75.38 (s), 75.31 (s), 75.23 (s), 73.54 (d, $J$ = 7.68 Hz), 56.68 (d, $J$ = 3.84 Hz), 56.63 (s), 56.43 (s), 53.10 (d, $J$ = 6.16 Hz), 52.46 (d, $J$ = 6.97 Hz), 27.13 (s), 27.10 (s), 19.11 (s), 19.08 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.57 (d, $J$ = 61.88 Hz), 18.91 (d, $J$ = 64.15 Hz). $^{19}$F NMR ($CDCl_3$): $\delta$ −249.64 (m). MS (ESI): $m/z$ 705.3 ($M^+ + 1$, 100.00). HRMS (MALDI): ($m/z$) ($M^+$ + Na): found, 727.2685; calcd for $C_{32}H_{50}FNaO_{12}PSi$, 727.2691.

**1D 3-(Methyl (fluoromethyl)phosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (12b).** A solution of **11b** (40 mg, 0.057 mmol) in THF (1 mL) was treated with tetrabutylammonium fluoride trihydrate (36 mg, 0.114 mmol) and acetic acid (7 $\mu$L, 0.114 mmol) at rt. After the resulting solution was stirred for 12 h, TLC indicated that the reaction was complete; solvent was evaporated in vacuo, and the crude product was purified by passage through a short silica column (*n*-hexane−acetone, 1:1, v/v) to afford 15 mg of **12b** as a colorless liquid (0.032 mmol, 56%). $^1$H NMR ($CDCl_3$): $\delta$ 4.86−4.68 (m, 10H), 4.32 (m, 1H), 4.17 (m, 1H), 3.98 (m, 1H), 3.87 (dd, $J$ = 10.8, 3.2 Hz, 3H), 3.65 (t, $J$ = 9.6 Hz, 1H), 3.41−3.34 (m, 12H), 3.20 (m, 2 H). $^{13}$C NMR ($CDCl_3$): $\delta$ 98.73 (s), 98.66 (s), 98.48 (s), 98.38 (s), 98.18 (s), 83.40 (s), 83.00 (s), 78.72 (d, $J$ = 90.09 Hz), 78.33 (d, $J$ = 90.09 Hz), 76.10 (d, $J$ = 10.00 Hz), 70.43 (d, $J$ = 6.16 Hz), 58.68 (s), 56.65 (s), 56.52 (s), 56.37 (s), 56.12 (s), 55.91 (s), 55.85 (s), 53.52 (s), 53.11 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 34.60 (d, $J$ = 62.05 Hz), 33.36 (d, $J$ = 65.12 Hz). $^{19}$F NMR ($CDCl_3$): $\delta$ −249.36 (dt, $J$ = 60.19, 45.88 Hz), −250.08 (dt, $J$ = 62.78, 45.88 Hz). MS (CI): $m/z$ 467.2 ($M^+ + 1$, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 489.1540; calcd for $C_{16}H_{32}FNaO_{12}P$, 489.1508.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphospho)-3-(methyl (fluoromethyl)phosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (14a)**. *N*,*N*-Diisopropyl-*O*-methyl-*O*-(di-(2*S*)-oleoyl-*sn*-glycerol)phosphoramidite (77 mg, 0.093 mmol) was added under an argon atmosphere to a solution of **12b** (29 mg, 0.062 mmol) and 1*H*-tetrazole (0.73 mL, 3 wt %, 0.249 mmol) in 4 mL of dry $CH_3CN$−THF (1:1, v/v). After the resulting solution was stirred for 20 h at rt, oxidation was performed with ($n$-$C_4H_9)_4NIO_4$ (40 mg, 0.093 mmol)

at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min, and after aqueous workup the crude product was purified by FC (*n*-hexane−acetone, 2:1, v/v) to give 60 mg of pure product **14a** as colorless oil (0.052 mmol, 83%). $^1$H NMR ($CDCl_3$): $\delta$ 5.30 (m, 4H), 5.21 (m, 1H), 4.84−4.67 (m, 10H), 4.32−4.09 (m, 8H), 3.93 (m, 2H), 3.86 (d, $J$ = 11.2 Hz, 3H), 3.78 (dd, $J$ = 10.8, 3.2 Hz, 3H), 3.41 (m, 12H), 2.29 (m, 4H), 1.96 (m, 8H), 1.57 (m, 4H), 1.26 (m, 42H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.16 (s), 172.78 (s), 129.96 (s), 129.64 (s), 98.86 (s), 98.70 (s), 98.42 (s), 98.18 (s), 97.93 (s), 78.94 (d, $J$ = 6.16 Hz), 76.39 (m), 75.40 (d, $J$ = 22.32 Hz), 75.30 (d, $J$ = 22.42 Hz), 69.34 (m), 65.82 (s), 65.51 (s), 61.56 (s), 56.73 (s), 56.56 (s), 55.83 (s), 55.56 (s), 54.80 (d, $J$ = 6.16 Hz), 54.60 (d, $J$ = 8.08 Hz), 53.60 (d, $J$ = 6.06 Hz), 53.25 (d, $J$ = 6.16 Hz), 34.04 (s), 33.92 (s), 31.85 (s), 29.70 (s), 29.67 (s), 29.47 (s), 29.26 (s), 29.17 (s), 29.15 (s), 29.08 (s), 29.05 (s), 29.02 (s), 27.16 (s), 27.12 (s), 24.75 (s), 22.63 (s), 14.06 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.53 (d, $J$ = 63.02 Hz), 19.13 (d, $J$ = 67.39 Hz), 0.57 (s). $^{19}$F NMR ($CDCl_3$): $\delta$ −249.23 (m), −250.08 (m). MS (ESI): $m/z$ 1163.7 ($M^+$ + 1, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 1185.6608; calcd for $C_{56}H_{105}FNaO_{19}P_2$, 1185.6607.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-((fluoromethyl)phosphonate)-*myo*-inositol (16a).** The phosphate **14a** (32 mg, 0.028 mmol) was dried and reacted with (TMS)Br, the resulting compound then hydrolyzed in aqueous MeOH, and the crude product dried as described above for **15a**. Ethanethiol (1 mL) was added, and the solution was kept at rt for 1 h and concentrated to yield the crude product. Chromatography on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) provided 20 mg of pure product **16a** (0.021 mmol, 75%). $^1$H NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 5.29 (m, 4H), 5.21 (m, 1H), 4.78 (m, 1H), 4.73 (m, 1H), 4.66 (m, 1H), 4.14 (m, 4H), 3.77 (m, 2H), 3.38 (m, 2H), 2.28 (m, 4H), 1.96 (m, 8H), 1.56 (m, 4H), 1.24 (m, 42H), 0.83 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 174.26 (s), 173.87 (s), 130.27 (s), 129.97 (s), 98.10 (s), 71.70 (m), 71.24 (m), 70.12 (s), 70.04 (s), 65.37 (m), 62.43 (m), 56.01 (s), 34.36 (s), 30.03 (s), 29.80 (s), 29.60 (s), 29.58 (s), 29.42 (m), 27.47 (s), 27.44 (s), 25.12 (s), 22.9 (s), 14.22 (s). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 21.25 (d, $J$ = 63.02 Hz), 21.10 (d, $J$ = 67.39 Hz), 3.61 (s), 3.36 (s). $^{19}$F NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ −245.96 (m). MS (ESI): $m/z$ 1015.6 ($M^+$ + Na, $C_{46}H_{91}FN_2NaO_{15}P_2$). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 1015.5803; calcd for $C_{46}H_{91}FN_2NaO_{15}P_2$, 1015.5776.

**1D *O*-(1,2-Di-*O*-palmitoyl-*sn*-(2S)-glycerol-3-*O*-methylphospho)-3-(methyl (fluoromethyl)phosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (14b)**. *N*,*N*-Diisopropyl-*O*-methyl-*O*-(di-(2*S*)-palmitoyl-*sn*-glycerol)phosphoramidite (90 mg, 0.123 mmol) was added under an argon atmosphere to a solution of **12b** (38 mg, 0.082 mmol) and 1*H*-tetrazole (1.0 mL, 3 wt %, 0.320 mmol) in 4 mL of dry $CH_3$-CN−THF (1:1, v/v). After the resulting solution was stirrred for 20 h at rt, oxidation was performed with ($n$-$C_4H_9$)$_4NIO_4$ (60 mg, 0.123 mmol) at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min, and after aqueous workup the crude product was purified by FC (*n*-hexane−acetone, 2:1, v/v) to give 49 mg of pure **14b** as a colorless oil (49 mg, 0.044 mmol, 54%). $^1$H NMR ($CDCl_3$): $\delta$ 5.22 (m, 1H), 4.85−4.69 (m, 10H), 4.34−4.10 (m, 8H), 3.95 (t, $J$ = 9.6 Hz, 2H), 3.87 (d, $J$ = 10.8 Hz, 1H), 3.79 (d, $J$ = 11.2 Hz, 1H), 3.43−3.39 (m, 12H), 2.29 (m, 4H), 1.58 (m, 8H), 1.23 (m, 48H), 0.86 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.19 (s), 172.80 (s), 98.90 (s), 98.53 (s), 98.43 (s), 98.35 (s), 98.03 (s), 97.83 (s), 79.13 (d, $J$ = 13.78 Hz), 77.21 (s), 76.90 (s), 76.35 (s), 74.24 (dd, $J$ = 31.48, 6.13 Hz), 69.44 (s), 69.35 (s), 65.75 (s), 65.57 (s), 61.60 (s), 56.77 (s), 56.64 (s), 56.55 (s), 56.50 (s), 55.75 (s), 53.75 (s), 52.18 (dd, $J$ = 35.30, 6.13 Hz), 34.08 (s), 33.96 (s), 31.88 (s), 29.65 (s), 29.63 (s), 29.61 (s), 29.45 (s), 29.31 (s), 29.25 (s), 29.22 (s), 29.09 (s), 29.06 (s), 24.78 (s), 22.64 (s), 14.07 (s), 11.03 (dd, $J$ = 148.83, 12.27 Hz). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.53 (d, $J$ = 61.88 Hz), 0.74 (s). $^{19}$F NMR ($CDCl_3$): $\delta$ −249.09 (m). MS (ESI): $m/z$ 1133.6 ($M^+$ + 1, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 1133.6325; calcd for $C_{52}H_{101}FNaO_{19}P_2$, 1133.6294.

**1D *O*-(1,2-Di-*O*-palmitoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-((fluoromethyl)phosphonate)-*myo*-inositol (16b).** The phosphate **14b** (35 mg, 0.032 mmol) was dried and treated with (TMS)Br, the resulting ethers were hydrolyzed in aqueous MeOH, and the crude product was dried as described above for **15a**. Ethanethiol (1 mL) was added, and the solution was kept at rt for 1 h and concentrated to yield the crude product. FC on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) provided 21 mg of **16b** (0.022 mmol, 67%). $^1$H NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 5.21 (m, 1H), 4.78 (m, 1H), 4.73 (m, 1H), 4.66 (m, 1H), 4.14 (m, 4H), 3.77 (m, 2H), 3.38 (m, 2H), 2.28 (m, 4H), 1.56 (m, 4H), 1.24 (m, 48H), 0.83 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 174.26 (s), 173.87 (s), 98.10 (s), 71.70 (m), 71.24 (m), 70.12 (s), 70.04 (s), 65.37 (m), 62.43 (m), 56.01 (s), 34.36 (s), 30.03 (s), 29.80 (s), 29.60 (s), 29.58 (s), 29.42 (m), 27.47 (s), 27.44 (s), 25.12 (s), 22.9 (s), 14.22 (s). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 21.25 (d, $J$ = 63.02 Hz), 21.10 (d, $J$ = 67.39 Hz), 3.61 (s), 3.36 (s). $^{19}$F NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ −245.96 (m). MS (ESI): $m/z$ 959.63 ($M^+$ + $NH_4$, $C_{42}H_{91}FN_3O_{15}P_2$, 100.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 958.5884; calcd for $C_{42}H_{91}FN_3O_{15}P_2$, 958.5909.

**1D *O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphospho)-3-(methyl (fluoromethyl)phosphonate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (14c)**. To a solution of alcohol **12b** (12 mg, 0.026 mmol) in dry THF (0.5 mL) were added *N*,*N*-diisopropyl-*O*-(methyl-*O*-di-butanoyl-*sn*-(2*S*)-glycerol)phosphoramidite (145 mg, 0.039 mmol) and 1*H*-tetrazole (0.31 mL, 3 wt %, 0.104 mmol). The mixture was stirred at rt for 16 h. Then oxidation was performed with ($n$-$C_4H_9$)$_4$-$NIO_4$ (17 mg, 0.039 mmol) at −20 °C for 1 h. The reaction mixture was warmed to rt for an additional 30 min. The solution was diluted with methylene chloride (20 mL) and washed with 10% sodium bisulfite. The organic layer was concentrated, and the residue was purified by FC (*n*-hexane−acetone, 2:1, v/v) to give 14 mg of pure **14c** as a colorless oil (0.018 mmol, 70%). $^1$H NMR ($CDCl_3$): $\delta$ 5.23 (m, 1H), 4.85−4.69 (m, 10H), 4.34−4.07 (m, 8H), 3.94 (t, $J$ = 10.0 Hz, 2H), 3.87 (t, $J$ = 9.6 Hz, 3H), 3.97 (dd, $J$ = 5.6, 4.4 Hz, 3H), 3.42−3.37 (m, 12H), 2.29 (m, 4H), 1.62 (m, 4H), 0.93 (t, $J$ = 7.2 Hz, 3H), 0.90 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.06 (s), 172.90 (s), 98.89 (s), 98.73 (s), 98.46 (s), 97.96 (s), 79.01 (s), 76.44 (s), 75.54 (s), 69.34 (s), 65.85 (s), 61.56 (s), 56.81 (s), 56.59 (s), 55.87 (s), 35.94 (s), 35.83 (s), 18.28 (s), 13.60 (s), 13.54(s). $^{31}$P NMR ($CDCl_3$): $\delta$ 19.55 (dd, $J$ = 60.75, 3.24 Hz), 19.11 (dd, $J$ = 63.99, 5.34 Hz), 0.74 (s), 0.68 (s), 0.59 (s), 0.56 (s). $^{19}$F NMR ($CDCl_3$): $\delta$ −249.26 (m), −250.01 (m). MS (CI): $m/z$ 775.46 ($M^+$ + 1, 100.00). HRMS (CI) ($m/z$) ($M^+$ + 1): found, 775.2751; calcd for $C_{28}H_{54}FO_{19}P_2$, 775.2719.

**1D *O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-((fluoromethyl)phosphonate)-*myo*-inositol (16c).** The phosphate **14c** (17 mg, 0.022 mmol) was dried and treated with (TMS)Br, the silyl ethers were hydrolyzed in aqueous MeOH, and the crude product was dried as described above for **16a**. Ethanethiol (1 mL) was added, the solution was kept at rt for 1 h and concentrated, and the crude product was chromatographed on silica gel ($CHCl_3$−$CH_3OH$−$NH_4OH$ (2.0 M), 65:25:3, v/v/v) to give pure product as a white powder (9 mg, 0.015 mmol, 68%). $^1$H NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 5.18 (m, 1H), 4.77 (m, 1H), 4.66 (dd, $J$ = 12.0, 5.2 Hz, 1H), 4.43−4.15 (m, 2H), 4.12−4.07 (m, 4H), 3.95 (m, 1H), 3.74 (m, 1H), 3.15 (m, 2H), 2.23 (m, 4H), 1.56 (m, 4H), 0.86 (m, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 174.46 (s), 174.07 (s), 130.44 (s), 130.16 (s), 74.70 (d, $J$ = 9.16 Hz), 71.51 (s), 71.15 (s), 70.36 (d, $J$ = 7.64 Hz), 65.61 (s), 62.77 (s), 34.61 (s), 34.49 (s), 32.40 (s), 30.23 (s), 30.00 (s), 29.81 (s), 29.77 (s), 29.72 (s), 29.70 (s), 29.61 (s), 29.55 (s), 27.65 (s), 25.34 (s), 23.13 (s), 14.32 (s). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 21.49 (s), 21.09 (s), 3.35 (s), 3.32 (s). $^{19}$F NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ −246.28 (m). MS (ESI): $m/z$ 571.26 ($M^+$ + 1, 18.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 593.1199; calcd for $C_{18}H_{33}FNaO_{15}P_2$, 593.1176.

***N*,*N*-Diisopropyl-*O*-(cyanoethyl)-*O*-(dioleoyl-*sn*-(2*S*)-glycerol)-phosphonamidite (20a).** A solution of 53 mg of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.226 mmol) and 35 mg (47 $\mu$L, 0.271 mmol) of DIPEA in 3 mL of anhydrous $CH_2Cl_2$ was cooled to 0 °C under nitrogen. To this solution was added 140 mg (0.226 mmol) of 1,2-dioleoyl-*sn*-(2*S*)-glycerol with vigorous stirring. After being stirred at 0 °C for 1 h and at rt for 3 h under nitrogen, the solution was separated from the white precipitate. FC on silica gel (hexanes−EtOAc−TEA, 100:10:1, v/v/v) gave 96 mg of **20a** (0.118 mmol, 52%) as a colorless oil. $^1$H NMR ($CDCl_3$): $\delta$ 5.32 (m, 4H), 5.17 (m, 1H), 4.32 (m, 1H), 4.13 (m, 1H), 3.85−3.52 (m, 6H), 2.61 (t, $J$ = 6.8 Hz, 2H), 2.28 (m, 4H), 1.98 (m, 8H), 1.58 (m, 4H), 1.26 (m, 42H), 1.14 (m, 12H), 0.86 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.35 (s), 172.96 (s), 129.99 (s), 129.69 (s), 62.36 (s), 61.77 (s), 61.62 (s), 58.57 (s), 58.48 (s), 58.30 (s), 43.20 (s), 43.08 (s), 34.28 (s), 34.08 (s), 33.36 (s), 31.88 (s), 29.75 (s), 29.70 (s), 29.51 (s), 29.31 (s), 29.19 (s), 29.12 (s), 28.96 (s), 27.20 (s), 27.16 (s), 24.89 (s), 24.86 (s), 24.60 (s), 24.53 (s), 22.66 (s), 20.38 (s), 20.31 (s), 14.10 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 150.61 (s), 150.46 (s). MS (CI): *m*/*z* 821.6 ($M^+ + 1$, $C_{48}H_{90}N_2O_6P$, 41.22). HRMS (CI) (*m*/*z*) ($M^+ + 1$): found, 821.6562; calcd for $C_{48}H_{90}N_2O_6P$, 821.6537.

***N*,*N*-Diisopropyl-*O*-(cyanoethyl)-*O*-(dipalmitoyl-*sn*-(2*S*)-glycerol)-phosphonamidite (20b).** A solution of 38 mg (0.162 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite and 25 mg (34 $\mu$L, 0.194 mmol) of DIPEA in 3 mL of anhydrous $CH_2Cl_2$ was cooled to 0 °C under nitrogen. To this solution was added 92 mg (0.162 mmol) of 1,2-dipalmitoyl-*sn*-(2*S*)-glycerol with vigorous stirring. After being stirred at 0 °C for 1 h and at rt for 3 h under nitrogen, the solution was separated from the white precipitate. FC on silica gel (hexanes−EtOAc−TEA, 100:10:1, v/v/v) gave 67 mg of **20b** (0.087 mmol, 54%) as a white solid. $^1$H NMR ($CDCl_3$): $\delta$ 5.16 (m, 1H), 4.31 (m, 1H), 4.15 (m, 1H), 3.85−3.54 (m, 6H), 2.60 (t, $J$ = 6.8 Hz, 2H), 2.29 (m, 4H), 1.59 (m, 4H), 1.26 (m, 48H), 1.26 (m, 42H), 1.14 (m, 12H), 0.85 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.36 (s), 172.97 (s), 117.50 (s), 70.56 (d, $J$ = 3.84 Hz) 62.34 (s), 61.76 (s), 61.61 (s), 61.44 (s), 58.56 (s), 58.48 (s), 58.38 (s), 58.29 (s), 43.19 (s), 43.06 (s), 34.29 (s), 34.10 (s), 33.35 (s), 31.90 (s), 29.67 (s), 29.63 (s), 29.61 (s), 29.47 (s), 29.34 (s), 29.28 (s), 29.12 (s), 29.09 (s), 24.90 (s), 24.87 (s), 24.59 (s), 24.52 (s), 22.66 (s), 20.37 (s), 20.29 (s), 14.09 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 150.60 (s), 150.45 (s). MS (CI): *m*/*z* 769.6 ($M^+ + 1$, 22.44). HRMS (CI) (*m*/*z*) ($M^+$): found, 768.6141; calcd for $C_{44}H_{85}N_2O_6P$, 768.6145.

***N*,*N*-Diisopropyl-*O*-(cyanoethyl)-*O*-(dibutanoyl-*sn*-(2*S*)-glycerol)-phosphonamidite (20c)**. A solution of 220 mg (0.931 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite and 144 mg (200 $\mu$L, 1.117 mmol) of DIPEA in 5 mL of anhydrous $CH_2Cl_2$ was cooled to 0 °C under nitrogen. To this solution was added 1,2-dibutanoyl-*sn*-(2*S*)-glycerol (216 mg, 0.931 mmol) with vigorous stirring. After being stirred at 0 °C for 1 h and at rt for 3 h under nitrogen, the solution was separated from the white precipitate. FC on silica gel (hexane−EtOAc−TEA, 100:10:1, v/v/v) gave 285 mg of pure **20c** (0.661 mmol, 71%) as a colorless oil. $^1$H NMR ($CDCl_3$): $\delta$ 5.07 (m, 1H), 4.22 (m, 1H), 4.05 (m, 1H), 3.76−3.47 (m, 6H), 2.54 (t, $J$ = 6.4 Hz, 2H), 2.19 (m, 4H), 1.53 (m, 4H), 1.06 (m, 12H), 0.84 (t, $J$ = 7.2 Hz, 3H), 0.81 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$): $\delta$ 172.78 (s), 172.43 (s), 117.32 (s), 70.34 (dd, $J$ = 7.68, 3.84 Hz), 62.01 (d, $J$ = 1.52 Hz), 61.51 (s), 61.35 (s), 61.21 (s), 58.28 (d, $J$ = 7.68 Hz), 58.09 (d, $J$ = 8.48 Hz), 42.90 (s), 42.77 (s), 35.82 (s), 35.62 (s), 24.28 (s), 24.22 (s), 20.08 (s), 20.02 (s), 18.07 (s), 18.04 (s), 13.31 (s), 13.27 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 150.38 (s), 150.27 (s). MS (CI): *m*/*z* 433.3 ($M^+ + 1$, 100.00). HRMS (CI): (*m*/*z*) ($M^+$): found, 432.2408; calcd for $C_{20}H_{37}N_2O_6P$, 432.2389.

**1D 1-*O*-(*tert*-Butyldiphenylsilyl)-3-(bis(cyanoethyl) phosphothionate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (21)**. Bis(2-cyanoethyl) diisopropylphosphorodiamidite (28 mg, 0.104 mmol) was added under an argon atmosphere to a solution of **10** (56 mg, 0.094 mmol) and 1*H*-tetrazole (0.48 mL, 3 wt % in $CH_3CN$, 0.207 mmol) in 1 mL of dry $CH_3CN$. After the resulting solution was stirred at rt for 2 h, sulfur (100 mg) and $CS_2$/pyridine (1.0 mL, 1:1, v/v) were added. After being stirred at rt for 2 h, the reaction mixture was filtered, and the filtrate was washed with brine, dried over $Na_2SO_4$, and concentrated. FC (EtOAc−hexane, 1:3, v/v) gave 68 mg of **21** as a colorless oil (0.085 mmol, 91%). $^1$H NMR ($CDCl_3$): $\delta$ 7.71−7.65 (m, 4H), 7.44−7.34 (m, 6H), 4.97 (d, $J$ = 6.4 Hz, 1H), 4.92 (d, $J$ = 6.0 Hz, 1H), 4.85 (d, $J$ = 6.4 Hz, 1H), 4.78 (d, $J$ = 7.2 Hz, 1H), 4.73 (d, $J$ = 6.4 Hz, 1H), 4.53 (d, $J$ = 6.8 Hz, 1H), 4.43 (d, $J$ = 6.8 Hz, 1H), 4.22−3.86 (m, 8H), 3.44−3.34 (m, 12H), 3.23 (s, 3H), 2.67−2.58 (m, 4H), 1.06 (s, 9H). $^{13}$C NMR ($CDCl_3$): $\delta$ 136.00 (s), 135.89 (s),133.88 (s), 132.60 (s), 129.89 (s), 128.16 (s), 127.82 (s), 116.39 (s), 99.19 (s), 98.86 (s), 98.45 (s), 97.71 (s), 78.91 (s), 78.76 (s), 78.16 (d, $J$ = 5.45 Hz), 77.88 (s), 77.24 (d, $J$ = 6.16 Hz), 73.52 (s), 62.37 (d, $J$ = 3.84 Hz), 61.98 (d, $J$ = 3.13 Hz), 56.74 (s), 56.72 (s), 55.59 (s), 27.15 (s), 19.22 (s), 19.17 (s), 19.14 (s), 19.07 (s), 18.98 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 68.69 (s). MS (CI): *m*/*z* 797.3 ($M^+ + 1$, 43.15). HRMS (CI) (*m*/*z*) ($M^+$): found, 796.2845; calcd for $C_{36}H_{53}N_2O_{12}PSSi$, 796.2826.

**1D 3-(Bis(cyanoethyl) phosphothionate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (22).** A solution of 35 mg (0.035 mmol) of **21** in THF (1 mL) was added along with anhydrous pyridine (0.4 mL) and hydrogen fluoride−pyridine complex (70%, 0.2 mL) at rt to a Teflon container. After the resulting solution was stirred for 3 weeks, the reaction was complete as determined by TLC; the reaction was then diluted with ethyl acetate (30 mL) and washed with 10% sodium bicarbonate (8 mL × 2). The organic layer was washed with brine, dried over $Na_2SO_4$, and concentrated. FC (acetone−hexane, 1:3, v/v) gave 21 mg of **22** as a colorless liquid (0.038 mmol, 86%). $^1$H NMR ($CDCl_3$): $\delta$ 4.85−4.71 (m, 8H), 4.34 (m, 5H), 4.20 (s, 1H), 4.10 (s, 1H), 4.01 (t, $J$ = 10.0 Hz, 1H), 3.64 (t, $J$ = 10.0 Hz, 1H), 3.44 (m, 14H), 2.75 (m, 4H). $^{13}$C NMR ($CDCl_3$): $\delta$ 115.48 (s), 108.75 (s), 97.73 (s), 97.50 (s), 97.14 (s), 82.50 (s), 78.21 (d, $J$ = 1.62 Hz), 77.34 (d, $J$ = 6.87 Hz), 75.57 (d, $J$ = 6.97 Hz), 69.43 (s), 61.72 (d, $J$ = 3.84 Hz), 61.47 (d, $J$ = 3.94 Hz), 55.76 (s), 55.39 (s), 55.12 (s), 54.99 (s), 18.48 (s), 18.45 (s), 18.39 (s), 18.37 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 68.12 (s). MS (CI): *m*/*z* 559.2 ($M^+ + 1$, 17.87). HRMS (CI) (*m*/*z*) ($M^+$): found, 558.1686; calcd for $C_{20}H_{35}N_2O_{12}PS$, 558.1648.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-*O*-(cyanoethyl)phospho)-3-(bis(cyanoethyl) phosphothionate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (23a)**. To a solution of 12 mg of alcohol **22** (0.022 mmol) in dry THF (0.5 mL) were added *N*,*N*-diisopropyl-*O*-(cyanoethyl)-*O*-(dioleoyl-*sn*-(2*S*)-glycerol)phosphonamidite (25 mg, 0.030 mmol) and 1*H*-tetrazole (6 mg, 0.26 mL, 0.088 mmol). The mixture was stirred at rt for 16 h. Oxidation was then performed with *t*-BuOOH (9.9 mg, 11 $\mu$L, 0.110 mmol) at rt for 1 h. The solution was diluted with $CH_2Cl_2$ (20 mL) and washed with 10% sodium bisulfite. The organic layer was concentrated and the residue purified by FC (acetone−hexane, 1:3, v/v) to give 25 mg of **23a** as a colorless oil (0.019 mmol, 88%). $^1$H NMR ($CDCl_3$): $\delta$ 5.35 (m, 4H), 5.22 (m, 1H), 4.83−4.74 (m, 8H), 4.42−4.19 (m, 12H), 4.10 (m, 2H), 3.42 (m, 12H), 2.78 (m, 6H), 2.29 (m, 4H), 1.99 (m, 8H), 1.57 (m, 4H), 1.28 (m, 42H), 0.85 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.23 (s), 172.84 (s), 172.81 (s), 130.00 (s), 129.67 (s), 116.63 (s), 116.49 (s), 98.91 (s), 98.93 (s), 98.55 (s), 98.49 (s), 98.01 (s), 97.96 (s), 79.17 (s), 76.40 (m), 75.70 (d, $J$ = 19.29 Hz), 69.28 (d, $J$ = 5.35 Hz), 66.09 (d, $J$ = 5.35 Hz), 65.98 (d, $J$ = 5.45 Hz), 62.89 (d, $J$ = 3.84 Hz), 62.70 (m), 62.30 (d, $J$ = 5.35 Hz), 62.24 (d, $J$ = 4.55 Hz), 61.60 (s), 56.64 (s), 56.62 (s), 56.59 (s), 56.03 (s), 34.10 (s), 33.94 (s), 31.87 (s), 29.73 (s), 29.70 (s), 29.50 (s), 29.29 (s), 29.21 (s), 29.19 (s), 29.12 (s), 29.08 (s), 29.06 (s), 27.19 (s), 27.16 (s), 24.79 (s), 22.65 (s), 19.69 (s), 19.62 (s), 19.44 (s), 19.36 (s), 14.09 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 67.86 (s), 67.70 (s), −0.84 (s), −1.25 (s). MS (CI): *m*/*z* 1294.3 ($M^+$, 40.74), 1262.2 ($M^+ - OCH_4$, 100.00). HRMS (CI) (*m*/*z*) ($M^+$): found, 1293.6830; calcd for $C_{62}H_{109}N_3O_{19}P_2S$, 1293.6851.

**1D *O*-(1,2-Di-*O*-oleoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(phosphothionate)-*myo*-inositol (24a).** To a solution of 21 mg of compound **23a** (0.016 mmol) in $CH_3CN$ (1.0 mL) under $N_2$ was added triethyl-

amine (0.5 mL) followed by the addition of bis((trifluoromethyl)silyl)-acetamide (0.50 mL). After 24 h, the reaction mixture was concentrated, and the residue was dissolved in 30 mL of 8 mM ammonium acetate (pH 7.1). The water phase was lyophilized, and a white powder was obtained. The anhydrous white powder was dissolved in ethanethiol (1 mL) and the resulting solution treated with boron trifluoride diethyl etherate (13 $\mu$L). After 3 h, the reaction was stopped by adding dry triethylamine (20 $\mu$L). The thiol was removed by evaporation, and the semisolid residue was dissolved in ammonium acetate buffer. FC ($CHCl_3-CH_3OH-NH_4OH$ (2.0 M) 9:7:2, v/v/v) provided 15 mg of pure **24a** (0.015 mmol, 93%). $^1$H NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 5.32 (m, 4H), 5.24 (m, 1H), 4.20 (m, 3H), 4.04−3.95 (m, 5H), 3.77 (m, 2H), 3.20 (m, 2H), 2.29 (m, 4H), 2.00 (m, 8H), 1.57 (m, 4H), 1.27 (m, 42H), 0.86 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 175.15 (s), 174.88 (s), 130.71 (s), 130.43 (s), 76.82 (s), 75.73 (s), 75.13 (s), 72.63 (s), 72.27 (s), 71.61 (s), 71.42 (s), 64.53 (s), 63.86 (s), 34.95 (s), 34.83 (s), 32.60 (s), 30.50 (s), 30.47 (s), 30.44 (s), 30.19 (s), 30.06 (s), 30.00 (s), 29.97 (s), 29.90 (s), 29.87 (s), 29.82 (s), 27.91 (s), 27.88 (s), 25.67 (s), 25.57 (s), 23.35 (s), 14.58 (s). $^{31}$P NMR ($CDCl_3$/$CD_3OD$, 3:2, v/v): $\delta$ 48.96 (s), 0.74 (s). MS (ESI): $m/z$ 981.64 ($M^+ - NH_4^+$, 100.00). HRMS (MALDI) ($m/z$) ($M^+ + 2NH_4^+ + H^+$): found, 1046.6530; calcd for $C_{45}H_{102}NO_{15}P_2S$, 1046.6568.

**1D *O*-(1,2-Di-*O*-pamitoyl-*sn*-(2*S*)-glycerol-3-*O*-(cyanoethyl)phospho)-3-(bis(cyanoethyl) phosphothionate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (23b)**. To a solution of alcohol **22** (53 mg, 0.095 mmol) in dry THF (0.5 mL) were added *N*,*N*-diisopropyl-*O*-(cyanoethyl)-*O*-(dipalmitoyl-*sn*-(2*S*)-glycerol)phosphonamidite (102 mg, 0.133 mmol) and 1*H*-tetrazole (1.12 mL, 3 wt %, 0.380 mmol). The mixture was stirred at rt for 16 h. Then oxidation was performed with *t*-BuOOH (60 $\mu$L, 0.380 mmol) at rt for 1 h. The solution was diluted with methylene chloride (20 mL) and washed with 10% sodium bisulfite. The organic layer was concentrated, and the residue was purified by FC (acetone−hexane, 1:3, v/v) to give 95 mg of pure product (0.077 mmol, 81%) as a colorless oil. $^1$H NMR ($CDCl_3$): $\delta$ 5.24 (m, 1H), 4.83−4.72 (m, 7H), 4.39 (m, 1H), 4.31−4.13 (m, 12H), 3.93 (m, 2H), 3.44 (m, 2H), 3.40 (m, 12H), 2.77 (m, 6H), 2.28 (m, 4H), 1.56 (m, 4H), 1.21 (m, 48H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.23 (s), 172.84 (s), 116.61 (s), 116.48 (s), 98.87 (s), 98.51 (s), 98.45 (s), 97.97 (s), 97.92 (s), 79.14 (s), 76.34 (m), 75.77 (s), 75.56 (s), 69.29 (s), 66.11 (m), 62.71 (d, $J$ = 3.84 Hz), 62.20 (d, $J$ = 12.32 Hz,), 61.60 (s), 58.31 (s), 56.80 (s), 56.55 (s), 55.99 (s), 34.09 (s), 33.94 (s), 31.86 (s), 29.64 (s), 29.60 (s), 29.45 (s), 29.30 (s), 29.26 (s), 29.08 (s), 29.06 (s), 24.78 (s), 22.63 (s), 19.66 (s), 19.58 (s), 19.40 (s), 19.32 (s), 14.06 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 67.84 (s), 67.68 (s), −0.88 (s), −1.28 (s). MS (ESI): $m/z$ 1242.8 ($M^+$ + 1, 18.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na): found, 1264.6463; calcd for $C_{58}H_{105}N_3NaO_{19}P_2S$, 1264.6436.

**1D *O*-(1,2-Di-*O*-palmitoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(phosphothionate)-*myo*-inositol (24b).** To a solution of 35 mg of compound **23b** (0.028 mmol) in $CH_3CN$ (1.0 mL) under $N_2$ was added triethylamine (0.5 mL) followed by the addition of bis((trifluoromethyl)silyl)-acetamide (0.50 mL). After 24 h, the reaction mixture was concentrated, and the residue was dissolved in 30 mL of 8 mM ammonium acetate (pH 7.1). The water phase was lyophilized, and a white powder was obtained. The anhydrous white powder was dissolved in ethanethiol (2 mL) and the resulting solution treated with boron trifluoride diethyl etherate (14 $\mu$L). After 3 h, the reaction was stopped by adding dry triethylamine (30 $\mu$L). The thiol was removed by evaporation, and the semisolid residue was dissolved in ammonium acetate buffer. FC ($CHCl_3-CH_3OH-NH_4OH$ (2.0 M) 9:7:2, v/v/v) provided 22 mg of pure **24b** (0.022 mmol, 79%). $^1$H NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 5.27 (m, 1H), 3.93 (dd, $J$ = 12.0, 3.6 Hz, 1H), 3.82 (m, 2H), 3.73−3.60 (m, 5H), 3.30 (m, 2H), 2.33 (m, 4H), 1.62 (m, 4H), 1.56 (dd, $J$ = 17.6, 6.8 Hz, 3H), 1.27 (m, 48H), 0.84 (t, $J$ = 7.2 Hz, 6H). $^{31}$P NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 48.96 (s), 0.74 (s). MS (ESI): $m/z$ 981.64 ($M^+$ + Na, 17.00). HRMS (MALDI) ($m/z$) ($M^+ + Na^+$): found, 980.5410; calcd for $C_{41}H_{89}N_3NaO_{15}P_2S$, 980.5387.

**1D *O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-*O*-(cyanoethyl)phospho)-3-(bis(cyanoethyl) phosphothionate)-2,4,5,6-*O*-tetrakis(methoxymethylene)-*myo*-inositol (23c)**. To a solution of alcohol **22** (35 mg, 0.063 mmol) in dry THF (0.5 mL) were added *N*,*N*-diisopropyl-*O*-(cyanoethyl)-*O*-(dibutanoyl-*sn*-(2*S*)-glycerol)phosphonamidite (38 mg, 0.088 mmol) and 1*H*-tetrazole (0.74 mL, 3 wt %, 0.252 mmol). The mixture was stirred at rt for 16 h. Then oxidation was performed with *t*-BuOOH (33 $\mu$L, 0.300 mmol) at rt for 1 h. The solution was diluted with $CH_2Cl_2$ (20 mL) and washed with 10% sodium bisulfite. The organic layer was concentrated, and the residue was chromatographed (acetone−hexane, 1:3, v/v) on silica gel to give pure product as a colorless oil (49 mg, 0.054 mmol, 86%). $^1$H NMR ($CDCl_3$): $\delta$ 5.26 (m, 1H), 4.83−4.74 (m, 9H), 4.40 (d, $J$ = 2.4 Hz, 1H), 4.35−4.12 (m, 12H), 3.79 (m, 2H), 3.45−3.38 (m, 12H), 2.76 (m, 6H), 2.31 (m, 4H), 1.67 (m, 4H), 0.95 (t, $J$ = 7.2 Hz, 3H), 0.91 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$): $\delta$ 173.07 (s), 172.68 (s), 116.63 (s), 116.50 (s), 98.91 (s), 98.89 (s), 98.48 (s), 98.00 (s), 97.94 (s), 79.16 (m), 76.41 (m), 75.69 (d, $J$ = 19.91 Hz), 69.27 (d, $J$ = 6.13 Hz), 66.14 (dd, $J$ = 12.97, 7.64 Hz), 62.90 (d, $J$ = 3.82 Hz), 62.70 (s), 62.38 (d, $J$ = 4.63 Hz), 62.22 (d, $J$ = 4.20 Hz), 61.53 (s), 56.84 (s), 56.61 (s), 56.02 (s), 35.94 (s), 35.80 (s), 19.68 9s), 19.61 (s), 19.43 (s), 19.35 (s), 18.27 (s), 13.58 (s), 13.54 (s). $^{31}$P NMR ($CDCl_3$): $\delta$ 67.83 (s), 67.70 (s), −0.90 (s), −1.30 (s). MS (ESI): $m/z$ 906.41 ($M^+$ + 1, 30.00). HRMS (MALDI) ($m/z$) ($M^+$ + Na + $NH_4^+$): found, 946.2996; calcd for $C_{34}H_{61}N_4NaO_{19}P_2S$, 946.3024.

**1D-*O*-(1,2-Di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(phosphothionate)-*myo*-inositol (24c).** To a solution of 20 mg of compound **23a** (0.022 mmol) in $CH_3CN$ (1.0 mL) under $N_2$ was added triethylamine (0.5 mL) followed by the addition of bis((trifluoromethyl)silyl)-acetamide (0.50 mL). After 24 h, the reaction mixture was concentrated, and the residue was dissolved in 30 mL of 8 mM ammonium acetate (pH 7.1). The water phase was lyophilized, and white powder was obtained. The anhydrous white powder was dissolved in ethanethiol (1 mL) and the resulting solution treated with boron trifluoride diethyl etherate (11 $\mu$L). After 3 h, the reaction was stopped by adding dry triethylamine (20 $\mu$L). The thiol was removed by evaporation, and the semisolid residue was dissolved in ammonium acetate buffer. FC ($CHCl_3-CH_3OH-NH_4OH$ (2.0 M) 9:7:2, v/v/v) provided 11 mg of pure **24c** (0.018 mmol, 80%). $^1$H NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 5.26 (m, 1H), 3.93 (m, 2H), 3.82 (m, 2H), 3.73−3.60 (m, 5H), 3.30 (m, 2H), 2.31 (m, 4H), 1.67 (m, 4H), 0.95 (t, $J$ = 7.2 Hz, 3H), 0.91 (t, $J$ = 7.2 Hz, 3H). $^{31}$P NMR ($CDCl_3/CD_3OD$, 3:2, v/v): $\delta$ 48.96 (s), 0.74 (s). MS (ESI): $m/z$ 624.27 ($M^+$ + 3, 21.00), 667.28 ($M^+ + 2 + 2Na^+$, 100.00). HRMS (MALDI) ($m/z$) ($M^+ + Na^+$): found, 644.1652; calcd for $C_{17}H_{41}N_3O_{15}P_2S$, 644.1631.

**Protein Expression and Purification.** DNA fragments encoding residues 1325−1410 of human EEA1 FYVE and residues 2−122 of yeast Vam7 PX were cloned in pGEX-KG and pGEX-2T vectors (Amersham). The $^{15}$N-labeled proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS and BL21 Codon Plus RP strains in minimal media supplemented with $^{15}NH_4Cl$ (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with IPTG (0.5 mM) and lysed with a French press. The glutathione *S*-transferase (GST) fusion FYVE and PX were purified on a Glutathione Sepharose 4B column (Amersham). The GST tag was cleaved with thrombin (Sigma). The proteins were further purified by FPLC and concentrated in Millipore concentrators (Millipore). The buffers were exchanged into 20 mM $d_{11}$-Tris (FYVE) or 50 mM potassium phosphate (PX), pH 6.8, 100−200 mM KCl, 1−20 mM perdeuterated dithiothreitol, 50 $\mu$M 4-amidinophenylmethanesulfonyl fluoride, 1 mM $NaN_3$, and 7% $^2H_2O$.

**NMR Spectroscopy and Titration of PtdIns(3)P Analogues.** NMR spectra were recorded at 25 °C on a Varian INOVA 500 MHz spectrometer. The $^1$H−$^{15}$N heteronuclear single-quantum coherence

(HSQC) spectra of 0.2−0.3 mM uniformly $^{15}$N-labeled FYVE and PX domains were collected while dibutanoyl ($C_4$)- or dipalmitoyl ($C_{16}$)-PtdIns(3)P analogues (Echelon Biosciences) (up to 4 mM) embedded in micelles consisting of $d_{38}$-DPC (250 mM) (Cambridge Isotopes) or DHPC/CHAPS (100 mM/17 mM) (Avanti/Anatrace) were added stepwise.

**Phosphorylation of PtdIns(3)P Analogues by PIKfyve.** Phosphatase-resistant dipalmitoyl and dioleoyl analogues of PtdIns(3)P and di-$C_{16}$-PtdIns(3)P (Echelon Biosciences, Inc.) were separately prepared as 0.5 mM aqueous stocks and stored at −80 °C. Just before use as substrates in the phosphorylation reaction with PIKfyve immunoprecipitates, a 60 $\mu$L aliquot of PtdIns(3)P, the stabilized analogues, or soybean PtdIns (Avanti Polar Lipids, Inc.) was evaporated down to dryness with absolute ethanol (2 × 200 $\mu$L) under a stream of dry $N_2$. Lipids were then reconstituted in an equal volume of lipid buffer (20 mM, pH 7.5, HEPES, 1 mM EDTA) by sonication (2 × 30 s) in a bath sonicator at room temperature. A 10 $\mu$L aliquot of each 0.5 mM phosphoinositide reconstituted in lipid buffer was then phosphorylated with immunoprecipitates of PIKfyve immune (R7069) or preimmune sera derived from PC-12 cell lysates prepared in RIPA buffer (50 mM Tris−HCl, pH 8.0, 150 mM NaCl, 1% w/v Nonidet P-40, 0.5% w/v sodium deoxycholate) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 $\mu$g/mL leupeptin, 5 $\mu$g/mL aprotinin, 1 $\mu$g/mL pepstatin, and 1 mM benzamidine) and immunoadsorbed onto 10 $\mu$L of packed Protein A Sepharose beads, as previously described.[61] The reaction in 50 $\mu$L of assay buffer (50 mM, pH 7.5, Tris−HCl/2.5 mM $MnCl_2$/2.5 mM $MgCl_2$) containing 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (50 $\mu$M) was incubated for 15 min at 37 °C before being stopped with 200 $\mu$L of 1 N HCl and extraction with 160 $\mu$L of 1:1 (v/v) $CHCl_3$−$CH_3OH$. The lower chloroform layer containing the $^{32}$P-labeled lipid product was collected by centrifugation for 30 s at 5000 rpm in a microfuge and quickly rinsed twice with 100 $\mu$L of 1:1 (v/v) 1 N HCl−$CH_3OH$ before 50 $\mu$L was spotted onto a silica gel glass TLC plate (20 × 20 cm × 0.25 mm layer thickness, Merck) and developed up to the top (4−5 h) at room temperature with 65:35 (v/v) 1-propanol−2 M acetic acid. Following exposure with an X-omat autoradiography film for the appropriate length of time the radiolabeled spots were scraped into glass scintillation vials and deacylated by methylaminolysis at 54 °C for 50 min by the standard protocol.[61] Recovery of the aqueously soluble deacylation products was then analyzed by HPLC on a Whatman 5 $\mu$m Partisphere SAX ($H_2PO_4^-$) column as formerly described[61] and the $^{32}$P detected with a Radiomatic 525TR online flow scintillation analyzer and FLO-ONE radiochromatography software (Packard Instrument Co., Downers Grove, IL) by Cerenkov emission in the low-energy tritium channel.

**Acknowledgment.** We thank the NIH (Grant NS29632), the American Cancer Society (Grant RSG0513601) (T.G.K.), and the American Diabetes Association and NIH (Grant DK58058) (A.S.) for financial support of this work.

**Supporting Information Available:** Experimental procedures and characterization for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0554716