Synthesis and Kinetic Evaluation of Inhibitors of the Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus*

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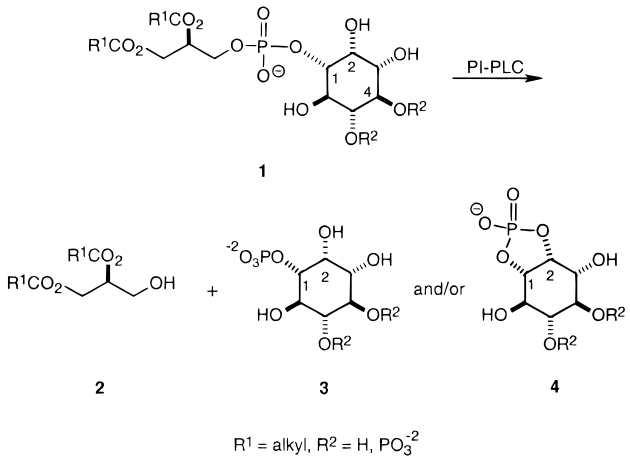
Received May 8, 1996[⊗]

Substrate analogues of phosphatidylinositol (**1**) were synthesized and evaluated as potential inhibitors of the bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus*. The chiral analogues of the water-soluble phospholipid substrate **5** were designed to probe the effects of varying the inositol C-2 hydroxyl group, which is generally believed to serve as the nucleophile in the first step of the hydrolysis of phosphatidylinositols by PI-PLC. In the analogues **6**−**9**, the C-2 hydroxyl group on the inositol ring of the phosphatidylinositol derivatives was rationally altered in several ways. Inversion of the stereochemistry at C-2 of the inositol ring led to the *scyllo* derivative **6**. The inositol C-2 hydroxy group was replaced with inversion by a fluorine to produce the *scyllo*-fluoro inositol **7** and with a hydrogen atom to furnish the 2-deoxy compound **8**. The C-2 hydroxyl group was *O*-methylated to prepare the methoxy derivative **9**. The natural inositol configuration at C-2 was retained in the nonhydrolyzable phosphorodithioate analogue **10**. The inhibition of PI-PLC by each of these analogues was then analyzed in a continuous assay using D-*myo*-inositol 1-(4-nitrophenyl phosphate) (**25**) as a chromogenic substrate. The kinetic parameters for each of these phosphatidylinositol derivatives were determined, and each was found to be a competitive inhibitor with K_i's as follows: **6**, 0.2 mM; **10**, 0.6 mM; **8**, 2.6 mM; **9**, 6.6 mM; and **7**, 8.8 mM. This study further establishes that the hydrolysis of phosphatidylinositol analogues by bacterial PI-PLC requires not only the presence of a C-2 hydroxyl group on the inositol ring, but the stereochemistry at this position must also correspond to the natural *myo*-configuration. For future inhibitor design, it is perhaps noteworthy that the best inhibitors **6** and **10** each possess a hydroxyl group at the C-2 position. Several of the inhibitors identified in this study are now being used to obtain crystallographic information for an enzyme-inhibitor complex to gain further insights regarding the mechanism of hydrolysis of phosphatidylinositides by this PI-PLC.

Introduction

Unraveling the intricate regulatory pathways of cellular signal transduction that involves the receptor-mediated turnover of inositol phospholipids by mammalian enzymes of the phosphatidylinositol-specific phospholipase C (PI-PLC) family has recently been the subject of unrivaled interest.[1,2] The intense attention accorded this class of enzymes was stimulated by the exciting discovery that when certain extracellular signaling molecules including hormones, neurotransmitters, and some growth factors bind to their G-protein linked surface receptors, a calcium-dependent PI-PLC is activated. The consequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (**1**, $R^2 = PO_3^{2-}$) by this enzyme produces 1,2-diacylglycerol (DAG) (**2**) and inositol 1,4,5-trisphosphate (IP_3) (**3**, $R^2 = PO_3^{2-}$) (Scheme 1). Both of these products function as intracellular second messengers that are integral to the regulation and stimulation of cellular metabolism, secretion, and proliferation. For example, IP_3 effects the release of calcium ions from the endoplasmic reticulum thereby activating numerous cytosolic enzymes, whereas DAG stimulates protein kinase C (PKC), a calcium- and phosphatidylserine-dependent enzyme that phosphorylates serine and threonine residues of the target enzymes.[3] Mammalian PI-PLC's also hydrolyze the unphosphorylated phosphatidylinositol **1** (R^2 = H) to give **2** together with mixtures of the inositol 1-phosphate (**3**) (R^2 = H) and cyclic inositol phosphate (IcP) (**4**) (R^2 = H). On the other hand, hydrolysis of **1** (R^2 = H) by bacterial PI-PLC produces only **2** and **4** (R^2 = H).

Scheme 1



[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

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(2) (a) Exton, J. H. *J. Biol. Chem.* **1990**, *265*, 1−4. (b) Billah, M. M.; Anthes, J. C. *Biochem. J.* **1990**, *269*, 281−291. (c) Liscovitch, M. *TIBS* **1992**, *17*, 393−399. (d) Cockcroft, S. *Biochem. Biophys. Acta* **1992**, *1113*, 135−160. (e) Lassegue, B.; Alexander, R. W.; Clark, M.; Akers, M.; Griendling, K. K. *Biochem. J.* **1993**, *292*, 509−517.

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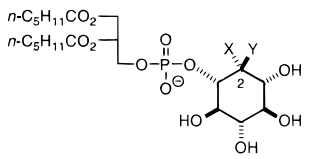
S0022-3263(96)00850-X CCC: $12.00

Despite the biological importance of PI-PLC's, mechanistic information and three-dimensional structural details that provide clues as to how these enzymes catalyze the hydrolysis of the phosphodiester bond of phospholipids are only recently becoming available. The fact that the cyclic phosphate **4** (R^2 = H) is produced upon hydrolysis of **1** (R^2 = H) by mammalian and bacterial PI-PLC suggests the C-2 hydroxyl group participates in the hydrolysis.[4] Supporting this hypothesis are the recent findings that modified phosphatidylinositol analogues lacking a free hydroxyl function at C-2 are inhibitors, not substrates, of PI-PLC.[5] The PI-PLC from *Bacillus cereus* has been cloned and over-expressed in *Escherichia coli*, and the crystal structure of this enzyme has been solved for the native enzyme and for the enzyme complexed with *myo*-inositol.[6] The recently reported X-ray structure of a catalytically-active deletion variant of the rat phosphatidylinositol-specific PLC-δ1 isozyme complexed with calcium ion and inositol trisphosphate provides further evidence for nucleophilic attack on phosphorus by the C-2 hydroxyl group and suggests mechanistic roles for several active site residues.[7]

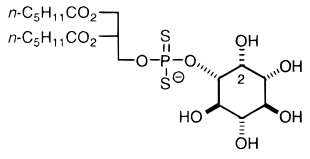
We have been engaged in a general program directed toward unveiling the mechanistic details in the hydrolysis of phosphatidylcholine by the PLC from *B. cereus* (PLC_{Bc}).[8] As a logical extension of this work, we became interested in the design and synthesis of novel inhibitors of PI-PLC to identify some structural features that are important for substrate specificity, binding, and catalysis. Ultimately, we hoped to gather details that would further illuminate the mechanistic features of PI-PLC-catalyzed hydrolysis of the phosphatidylinositols. As the first step in this endeavor, we focused upon the design, synthesis, and kinetic evaluation of a series of analogues of **1** (R^2 = H) that would be inhibitors of PI-PLC. Only one structural feature would be varied at a time so that the importance of each change could be reasonably quantified. X-ray crystallographic studies would then be undertaken with selected enzyme-inhibitor complexes in order to help identify the important active site amino acid residues and elucidate their roles in binding and catalysis. These studies would also provide insights into the function of the inositol C-2 hydroxyl group in enzyme/substrate recognition and enzymatic hydrolysis.

Results and Discussion

The weight of current experimental evidence suggests that the C-2 hydroxyl in phosphatidylinositol **1** (R^2 = H) is the nucleophile in the first step of the hydrolysis of **1** by bacterial PI-PLC (Scheme 1).[4-7] Thus, the design and synthesis of modified phosphatidylinositides in which the hydroxyl function at C-2 of the inositol ring is replaced, altered, or removed appeared to be the most direct route to developing competitive inhibitors of the enzyme. To test this hypothesis, we envisioned that the series of phosphatidylinositol derivatives **6**−**10**, which are patterned after the modified substrate **5**, would be interesting candidates as potential competitive inhibitors of bacterial PI-PLC. Each of these compounds contains *n*-hexanoyl side chains on the *sn*-glycerol moiety to confer water solubility so that the biological evaluation of these substrate analogues could be conducted below the critical micelle concentrations (CMC) of substrates and inhibitors, thereby simplifying analysis of the kinetic data. The reported CMC of **5** is 12.3 mM in pure water and 9.0 mM in Tris (tris(hydroxymethyl)aminomethane)acetate buffer at pH 7.5.[5c]



5: X = OH, Y = H
6: X = H, Y = OH
7: X = H, Y = F
8: X = H, Y = H
9: X = OMe, Y = H



10

We envisioned that the phosphatidylinositol targets **5**−**10** could be prepared by applying the general phosphite coupling techniques that had been developed in our laboratories for the synthesis of a diverse array of phosphodiesters. In each of these substrate analogues, the D-*myo*-inositol enantiomer was used since it had been determined that phospholipids incorporating L-*myo*-inositol are processed at only 0.2% the rate of those of D configuration.[9] Analogues **6**−**9** incorporated changes of the C-2 hydroxyl group of the *myo*-inositol moiety to help elucidate what alterations in the inhibitor would lead to optimal binding at the enzyme active site. The stereochemistry at C-2 in **6** was inverted to test the importance of stereochemistry at this position. In **7**−**9**, the hydroxyl group at C-2 was replaced so the nucleophile that has been proposed to initiate hydrolysis of the phosphodiester linkage is no longer available. Compound **10** maintains the natural integrity of inositol ring, but in place of the phosphate function, it possesses a phosphorodithioate group, which we anticipated would stabilize the phosphodiester linkage toward enzymatic hydrolysis.[10] While we were engaged in this effort, there were several reports of phosphatidylinositol analogues in which the C-2 hydroxyl group was removed or methylated; these compounds were found to be weak inhibitors, but no K_i's were determined.[5a-c]

Synthesis of Modified Phosphatidylinositols. The phosphatidylinositol derivatives **5**−**8** and **10** are known compounds that were prepared using a one-pot, chlorophosphite coupling protocol that had been developed in the our group.[11] In this procedure, which is summarized in Scheme 2, a chlorophosphite is sequentially treated

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(6) (a) Koke, J. A.; Yang, M.; Henner, D. J.; Volwerk, J. J.; Griffith, O. H. *Protein Expression Purif.* **1992**, *2*, 51−58. (b) Heinz, D. W.; Ryan, M.; Bullock, T. L.; Griffith, O. H. *EMBO J.* **1995**, 3855−3863.

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(8) (a) Hansen, S.; Hough, E.; Svensson, L. A.; Wong, Y.-L.; Martin, S. F. *J. Mol. Biol.* **1993**, *234*, 179−187. (b) Martin, S. F.; Wong, Y.-L.; Wagman, A. S. *J. Org. Chem.* **1994**, *59*, 4821−4831. (c) Martin, S. F.; Spaller, M.; Hergenrother, P. *Biochemistry*, in press.

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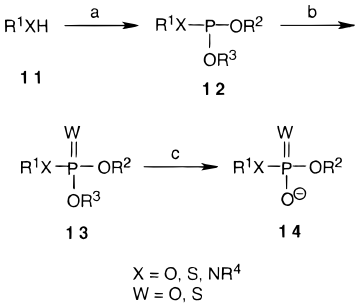
Scheme 2[a]



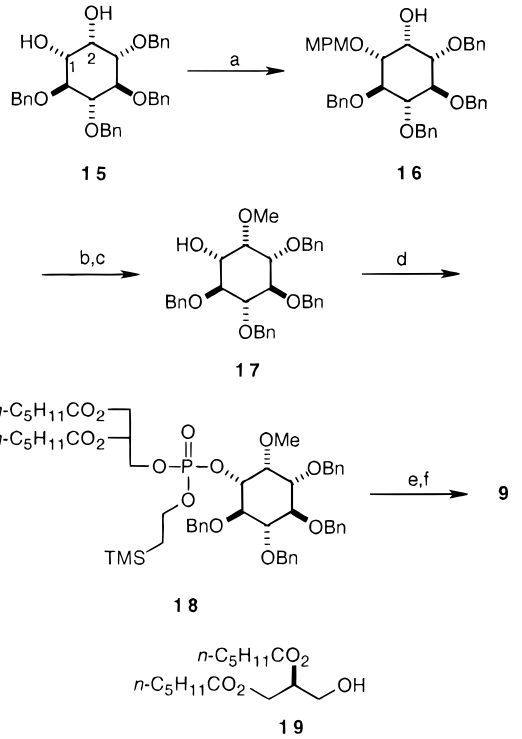
[a] Key: (a) R^3OPCl_2 (1 equiv); R^2OH (1 equiv); (b) [O] or S_8; (c) deprotect.

with two different nucleophiles to give the phosphotriester **12** that is oxidized to give the phosphate triester **13**. The desired phosphodiester **14** is then generated by deprotection of the phosphate. In many cases the chlorophosphite of choice is the commercially available methyl dichlorophosphite, but the 2-(trimethylsilyl)ethyl dichlorophosphite has been used as an alternative coupling reagent because the (trimethylsilyl)ethyl group may be removed under mildly acidic conditions.[12] A diverse array of phospholipid analogues with a variety of fatty acid side chains and different head groups are readily available by application of this method.

The synthesis of **9** by the same procedure used to prepare **5**−**8** required a modified protecting group strategy to manipulate the C-1 and C-2 hydroxyl groups of 3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (**15**) (Scheme 3). Reaction of **15** with dibutyltin oxide generated an intermediate stannylene ketal that was treated *in situ* with *p*-methoxybenzyl chloride in the presence of CsF to give **16** in 89% yield.[13,14] The selective alkylation of 1,2-diols mediated by a dialkyltin oxide is a common procedure in carbohydrate chemistry.[15] *O*-Methylation of **16** followed by removal of the *p*-methoxybenzyl protecting group with dichlorodicyanobenzoquinone (DDQ) gave **17** in 89% overall yield. The phosphate triester **18** was then prepared in 66% yield by coupling the protected inositol **17** with the diacylglycerol **19** using 2-(trimethylsilyl)ethyl dichlorophosphite followed by oxidation according to our standard protocol.[11c] Global deprotection of **18** by catalytic hydrogenolysis of the benzyl groups and removal of the trimethylsilylethyl group with aqueous HF gave **9** in 50% yield.

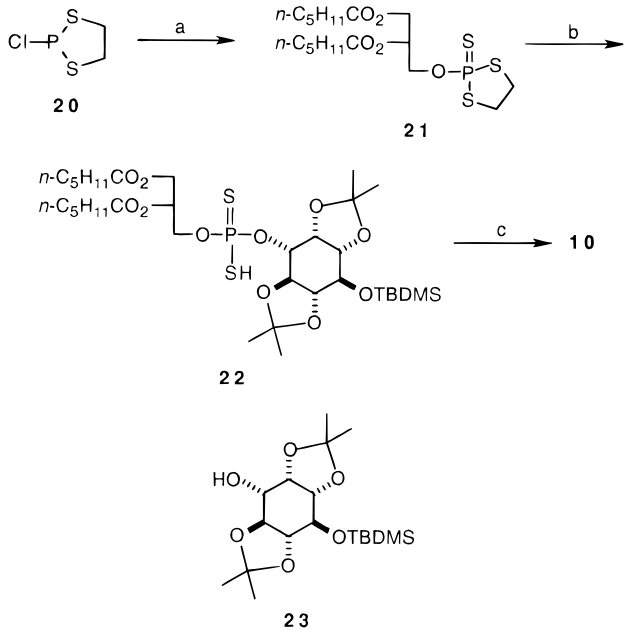
As part of a general program directed toward developing methods for the syntheses of isosteric phosphate replacements, we invented a facile method to prepare phosphorodithioates using the phosphatitylating reagent 2-chloro-1,3,2-dithiaphospholane (**20**).[11c,16] This protocol was applied to the synthesis of **10** (Scheme 4). Thus,

Scheme 3[a]



[a] Key: (a) *n*-Bu_2SnO, C_6H_6; Δ; *p*-$MeOC_6H_4CH_2Cl$; CsF, DMF; (b) NaH, THF; MeI; (c) DDQ, H_2O, CH_2Cl_2; (d) $TMSCH_2CH_2OPCl_2$, *i*-Pr_2NEt, THF, −78 °C; 19, −78 → 20 °C; *t*-BuOOH, CH_2Cl_2, 0 °C; (e) H_2 (500 psi), 20% $Pd(OH)_2$/C, EtOH, rt; (f) aqueous HF, MeCN/THF (2:1), rt.

Scheme 4[a]



[a] Key: (a) **19**, $EtN(i\text{-}Pr)_2$, CH_3CN, −38 °C → rt; S_8, CS_2, rt; (b) **23**, DBU, MeCN, rt; (c) aqueous HF, MeCN, rt.

reaction of **20** with 1,2-di-*n*-hexanoyl-*sn*-glycerol (**19**) followed by sulfurization with elemental sulfur gave the 2-alkoxy-2-thio-1,3,2-dithiaphospholane **21** in 84% yield. Reaction of **21** with the protected inositol **23** in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and elution of the intermediate DBU salt through an Amberlyst-15 ion exchange column (basic form) gave the protected phosphorodithioate **22** in 85% yield.[11c] Acid-

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(13) For a related example, see: Gigg, J.; Gigg, R.; Payne, S.; Conant, R. *J. Chem. Soc., Perkin Trans. 1* **1987**, 423426.

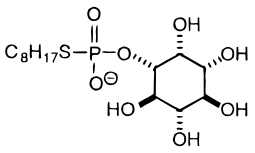
(14) Nagashima, N.; Ohno, M. *Chem. Lett.* **1987**, 141−144.

(15) (a) Nashed, M. A.; Anderson, L. *Tetrahedron Lett.* **1976**, *39*, 3503−3506. (b) Nashed, M. A.; Anderson, L. *Carbohydr. Res.* **1977**, *56*, 419−422. (c) Kong, X; Grindley, T. B. *Can. J. Chem.* **1994**, *72*, 2396−2404 and 2405−2415.

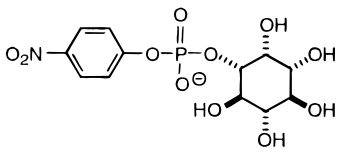
(16) Boudjebel, H.; Goncalves, H.; Mathis, F. *Bull. Soc. Chim. Fr.* **1974**, *5*, 1671−1674.

catalyzed deprotection of the inositol moiety of **22** furnished **10** in 93% yield.

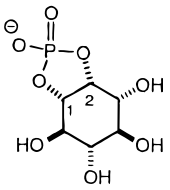
Biological Evaluation of 6−10 as Inhibitors of PI-PLC. The hydrolysis of phosphatidylinositol **1** (R^2 = H) by bacterial PI-PLC produces diacylglycerol **2** and the cyclic 1,2-phosphoinositol **4** (R^2 = H) (Scheme 1). Because no acid is produced by this enzymatic reaction, a pH titrimetric assay similar to that used for PLC_{Bc} cannot be employed,[11c,17] and alternate tactics have been developed to follow the hydrolysis of phosphatidylinositols by PI-PLC.[1f,18] After considering the available options, we decided to examine the inositol alkylthiophosphate **24**[19] and the nitrophenyl inositolphosphate **25**[9] as a potential water-soluble substrates to develop a continuous assay to measure the rates in the PI-PLC catalyzed hydrolysis of phosphoinositides in the presence of **6**−**10**. Cleavage of **24** by PI-PLC in the presence of 4,4′-dipyridyl disulfide produces pyridyl-4-thiol, the production of which would be monitored by UV spectrometry, and inositol-1,2-cyclic phosphate (**26**). Alternatively, hydrolysis of **25** by PI-PLC forms **26** and *p*-nitrophenol, whose formation may also be detected by UV.



24



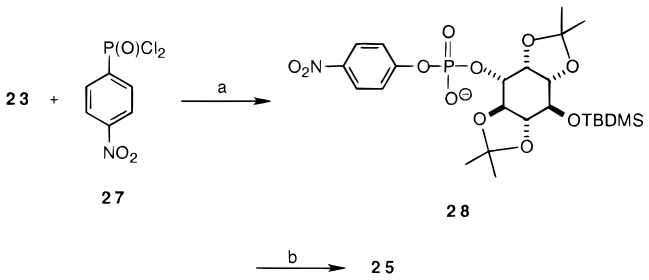
25



26

Preliminary attempts to develop a kinetic assay using **24** as a substrate for PI-PLC proved problematic owing to a significant background rate for the formation of pyridyl-4-thiol by the nonenzymatic, chemical hydrolysis of 4,4′-dipyridyl disulfide. Higher homologues of **24** are better substrates for the enzyme, but they have much lower CMC's and therefore were not examined. On the other hand, a continuous colorimetric assay using **25** as the substrate was simple to implement (*vide supra*). In the absence of PI-PLC, there was negligible hydrolysis of **25** under the assay conditions, so the change in UV absorbance over time was directly related to the amount of *p*-nitrophenol that was released by the enzymatic reaction. The only drawback to this assay is that the substrate **25** is significantly different from the natural phosphatidylinositol substrate in that *p*-nitrophenol is the leaving group rather than a hydrophobic diacylglycerol group. The natural phosphatidylinositol substrate has a K_m of 1−2 mM, whereas **25** has been reported to exhibit a relatively high K_m that varies from 5 to >15 mM depending upon buffer;[9] the V_{max} values of **25** and phosphatidylinositol were reported to be of the same order of magnitude. In exploratory experiments, we discovered that the rates of hydrolysis of **25** at low concentrations were easily measurable when using moderate (1−5 mM) concentrations of inhibitors having K_i's in the low millimolar range.

Scheme 5[a]



[a] Key: (a) pyridine, rt; H_2O; rt; (b) AcOH/H_2O (1:4), rt.

Compound **25** was prepared by the route outlined in Scheme 5, which is similar to the published procedure.[9] The protected *myo*-inositol **23** was coupled to commercially available 4-nitrophenyl phosphorodichloridate (**27**) to deliver the protected phosphate **28**, which was immediately deprotected to give **25** in 57% overall yield. Since the pure phosphate **25** hydrolyzes slowly in the presence of water or in aqueous base, it was dissolved in water and extracted with ether to remove traces of *p*-nitrophenol. The aqueous solution was then carefully freeze-dried to a glass, evaporated to dryness with toluene, dried *in vacuo*, and stored at −20 °C under argon. In this state, **25** could be kept for several months without decomposition. Upon dissolution of **25** in water at neutral or slightly acidic pH, no free *p*-nitrophenol was detected by UV spectrometry at 396 nm.

The rate of release of *p*-nitrophenol upon hydrolysis of **25** by PI-PLC at pH = 7.0 (100 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer) and 25 °C was followed by the change in the UV absorbance at 396 nm (see Experimental Section). The assays were performed at pH 7.0 as the best compromise for the stability of **25** to hydrolysis, the molar extinction coefficient of *p*-nitrophenol and the activity of the enzyme. The progression curves for the enzymatic hydrolysis of **25** were nearly linear for the first 60−70 s of the reaction for all substrate concentrations, [S], ranging from 1 to 25 mM so the initial rates, v_o, were determined by extrapolating a tangent to the curve back to the time at which the enzyme was added. The initial velocity data were then fit with a nonlinear least-squares algorithm, and the curved line was calculated from the Michaelis-Menten equation, $v_o = V_{max}[S](K_m + [S])^{-1}$ (Figure 1). The values for the kinetic parameters K_m and V_{max} for **25** that gave a reasonable fit to the experimental data were estimated to be 34 (±5) mM and 3800 (±500) μmol/min·mg, respectively.

At the outset of our work, the range of concentrations of **25** used in our assay was based upon the reported K_m of 5 mM (V_{max} = 650 μmol/min·mg) for **25**; this value had been determined in the same buffer system at concentrations of **25** ranging from 0.1 to 7.7 mM.[9b] Examination of Figure 1 reveals that even at 25 mM, the concentration of **25** is significantly below saturation for the enzyme. Because accurate kinetic parameters are most reliably obtained at substrate concentrations varying from 0.5 to

(17) For a facile chromogenic assay for PLC_{Bc}, see: Hergenrother, P. J.; Spaller, M. R.; Haas, M.; Martin, S. F. *Anal. Biochem.* **1995**, *229*, 313−316.

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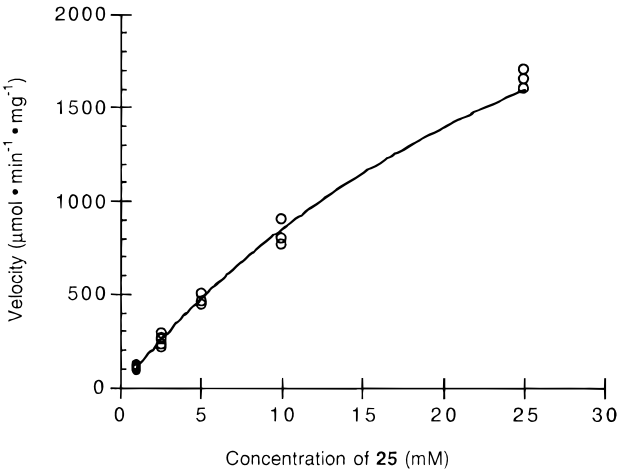

Figure 1. Velocity versus substrate concentration plot for the enzymatic hydrolysis of **25** by recombinant bacterial PI-PLC. The curved line is calculated from the Michaelis-Menten equation, $v_o = V_{max}[S](K_m + [S])^{-1}$.

5 K_m, all of the determinations of V_{max} and K_m are presumably subject to significant experimental error. Indeed, the measured K_m of **25** could be even higher if saturating substrate concentrations were used.

However, several factors conspired to limit the range of concentrations of **25** used in the subsequent parts of this study to 1−25 mM. First, the labor associated with synthesizing the quantities of **25** and **6**−**10** that would be required to evaluate each inhibitor at concentrations of **25** ranging from 15 to ≥150 mM seemed prohibitive. Furthermore, at higher concentrations of substrate and inhibitor, there loomed the danger of forming micelles or mixed micelles, and the analysis of the data obtained in the resulting heterogeneous system would not be amenable to simple treatment.

The water-soluble substrate analogues **6**−**10** were evaluated as inhibitors of bacterial PI-PLC in competition experiments in which enzyme was added to a stirred solution of substrate **25** and inhibitor under the standard assay conditions. The inhibitor concentration needed for the kinetic analysis was determined by adding different amounts of inhibitor to 2.5 mM solutions of **25**, and the inhibitor concentration [I] that gave a 30−50% rate reduction was used. The raw rate data were then fit using a nonlinear least-squares fit of the hyperbolic data of [S] vs v_o. The curved line for each inhibitor assay was calculated from the Michaelis-Menten equation ($v_o = V_{max}[S](K_m + [S])^{-1}$) giving directly the apparent K_m ($K_{m'}$). The values obtained for V_{max} for the enzymatic hydrolysis of **25** by PI-PLC in the presence of each inhibitor **6**−**10** were similar, but the measured apparent K_m's were always greater than for the substrate **25**. Although this behavior suggests that each inhibitor is competitive,[20] small components of noncompetitive inhibition would not be detected under the constraints of our assays. The initial velocities were used to generate double-reciprocal plots that also indicated that each of the compounds **6**−**10** was a competitive inhibitor. Using the expression $K_{m'} = K_m(1 + [I]/K_i)$, the K_i's were calculated as follows: **6**, 0.2 mM; **10**, 0.6 mM; **8**, 2.6 mM; **9**, 6.6 mM; and **7**, 8.8 mM. It should be noted that because these assays were not run at saturating substrate concentrations, there will be a tendency to underestimate the K_m and the apparent K_m's. Consequently, these values for the K_i's should be viewed as an indication of the relative binding affinities within this series of inhibitors rather than well-determined quantities.

(20) For a review of the concepts and the derivations for the equations for the different types of inhibition. see: (a) Segel, I. H. In *Enzyme Kinetics*; John Wiley & Sons: New York, 1975; pp 1−467. (b) Cornish-Bowden, A. In *Fundamentals of Enzyme Kinetics*; Butterworths, Inc.: Boston, 1979; pp 1−230.

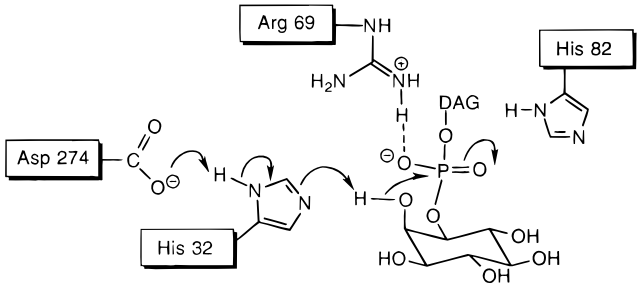

Figure 2. Proposed first step in catalytic mechanism for hydrolysis of phosphatidylinositol by PI-PLC.

These studies provide useful information that is relevant to the mechanism of the enzymatic hydrolysis of phosphatidylinositols by bacterial PI-PLC and to the future design of competitive inhibitors of this and related enzymes. Replacement of the axial hydroxyl group at C-2 of the inositol ring in compounds **7**−**9** leads to phosphatidylinositol derivatives that are inhibitors rather than substrates. This observation supports the mechanism for the hydrolysis of phospholipids by bacterial PI-PLC that was first proposed by Tsai,[4b,10a] who suggested that the inositol C-2 hydroxyl group attacks phosphorus in the first step of the reaction. Because **6** is an inhibitor rather than a substrate of PI-PLC, the enzymatic reaction is clearly sensitive to the precise orientation of the C-2 hydroxyl group as well as its presence. More specific details regarding this mechanism have recently emerged from the X-ray structural work of Heinz,[6b] who has suggested that this enzymatic reaction is catalyzed by His-32 and His-82, which serve as the general base and general acid, respectively (Figure 2). Site-directed mutagenesis has shown that both histidines are critical for catalytic activity. In this mechanistic model, Arg 69 activates the phosphate moiety for nucleophilic attack by neutralizing charge, and the developing charge on the imidazole of His-32 is neutralized by proton transfer to Asp-274. This type of relay mechanism is similar to those proposed for DNAse I[21] and mammalian PLC-δ1.[7]

The two most potent inhibitors **6** and **10** each possess a hydroxyl group at the C-2 position of the inositol ring that can participate as a hydrogen bond donor, whereas the weaker inhibitors **7**−**9** do not. In this context, it should be recognized that differences in the hydrogen-binding interactions at the active site are not the only factors affecting the K_i's, and solvation−desolvation effects may also play important roles. The fluoro *scyllo* analogue **7** was the worst inhibitor of all substrate analogues tested, but the efficacy this inositol replacement for inhibitor design should not yet be ruled out as 2-deoxy-2-fluoro-*scyllo*-inositol 1-*O*-dodecylphosphonate was the most potent of several phosphonate inhibitors of the bacterial PI-PLC from *B. cereus*.[5d] Substitution of the phosphodiester linkage in **5** with a phosphorodithioate group gave the nonhydrolyzable analogue **10**,

(21) Suck, D.; Oefner, C. *Nature* **1986**, *321*, 620−625.

so this replacement should be more broadly examined for the development of novel inhibitors. Because sulfur is less electronegative than oxygen, the ionic interaction of a phosphorodithioate moiety with a positive charge on the enzyme, which might be provided by either an arginine residue or a calcium ion, will be less than for a phosphate group. This reduced interaction not only decreases the susceptibility of phosphorous to nucleophilic attack, but it also suggests that inhibitors of PI-PLC that contain the phosphorodithioate moiety may bind less tightly than the corresponding phosphate analogues; this hypothesis remains to be tested. The importance of the hydroxyl groups at C-3, C-4, and C-5 in the design of inhibitors of bacterial PI-PLC must also be evaluated.

A number of mechanistic issues remain unresolved. The X-ray structure of PI-PLC with *myo*-inositol reveals only limited information regarding the specific roles of the various active site residues in binding and catalysis, and a structure of PI-PLC complexed with an inhibitor such as **6**−**10** is essential to developing more complete insights regarding the mechanism of hydrolysis of inositol phosphates by bacterial PI-PLC. Details regarding the kinetic mechanism are also lacking. Toward this end, it is necessary to determine the slow step and the order of product release in the enzymatic cycle. The nature of product inhibition by the enzyme must also be clarified. Resolution of these questions and the development of new kinetic assays for PI-PLC are in progress, and the results of these and related investigations will be reported in due course.

Experimental Section

General Procedures. Unless otherwise noted, all starting materials were obtained from commercial suppliers and were used without further purification. Reagent-grade solvents were dried according to established protocols by distillation under inert atmosphere from an appropriate drying agent immediately prior to use. Thus, tetrahydrofuran (THF) was distilled from the potassium/benzophenone ketal, and acetonitrile (MeCN) and benzene (C_6H_6) were distilled from CaH. Diisopropylamine and pyridine (Py) were distilled from calcium hydride and stored over 4 Å molecular sieves under argon. Ethanol (EtOH) was distilled from magnesium ethoxide and stored over 3 Å molecular sieves. Reactions involving air- and/or moisture-sensitive reagents were executed under an atmosphere of dry argon, and the glassware was flame dried under vacuum. Melting points and boiling points are uncorrected. Infrared (IR) spectra were recorded as solutions in $CHCl_3$. All spectra are reported in wavenumbers (cm^{-1}) and are referenced to the 1601 cm^{-1} absorption of a polystyrene film. Proton (^1H) (250 MHz), carbon (^{13}C) (63 MHz), and phosphorus (^{31}P) (120 or 146 MHz) nuclear magnetic resonance (NMR) spectra were obtained as solutions in deuteriochloroform ($CDCl_3$) unless otherwise indicated. ^1H and ^{13}C chemical shifts are reported in parts per million (ppm, δ) downfield relative to tetramethylsilane (TMS), which was referenced to the solvent, whereas ^{31}P chemical shifts are reported in parts per million (ppm, δ) downfield relative to external 80% phosphoric acid. Coupling constants are reported in hertz (Hz). Spectral splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; comp, complex multiplet; and br, broad. Flash chromatography was performed according to the established protocol with Merck silica gel 60 (230−400 ASTM).[22]

Recombinant bacterial PI-PLC was obtained from Professor O. H. Griffith (University of Oregon). The concentration of enzyme used in the kinetic assays was determined by the Bradford method.[23] The enzymatic hydrolysis reactions were carried out in disposable 0.5 mL methyl methacrylate cuvettes available from Fisher Scientific. The progression of the enzymatic reactions was monitored and recorded using a Hewlett-Packard 8452A UV diode array spectrophotometer. Compounds **5**−**8** and **10** were prepared according to literature procedures and were determined to be ≥95% pure by ^1H, ^{13}C, and ^{31}P NMR.[11]

D-1-*O*-(4-Methoxybenzyl)-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (16). A suspension of tetrabenzyl-*myo*-inositol (**15**)[24] (9.29 g, 17.0 mmol) and dibutyltin oxide (4.48 g, 17.0 mmol) in C_6H_6 (100 mL) was heated overnight at reflux using a Dean−Stark trap to remove water. After cooling, the solvent was removed under reduced pressure. The yellow residual oil was dissolved in DMF (60 mL) containing CsF (5.24 g, 34.5 mmol) and 4-methoxybenzyl chloride (2.3 mL, 2.66 g, 17.0 mmol). The reaction was stirred for 8 h, and the volatiles were removed *in vacuo* leaving a white crystalline solid, which was partially dissolved in EtOAc (50 mL). The solid was removed by filtration, and the filtrate was evaporated to give a residue that was purified by flash chromatography eluting with hexane/EtOAc (7:3) to deliver 9.95 g (89%) of **16** as a white solid: mp 109−111 °C; ^1H NMR δ 7.65−7.28 (comp, 22 H), 6.97 (d, J = 7.0 Hz, 2 H), 5.17−4.92 (comp, 6 H), 4.88−4.71 (comp, 4 H), 4.19 (app t, J = 2.4 Hz, 1 H), 4.18 (m, 2 H), 3.85 (s, 3 H), 3.63 (app t, J = 7.8 Hz, 1 H), 3.53 (app t, J = 2.4 Hz, 1 H), 3.50 (app t, J = 2.4 Hz, 1 H), 2.86 (br s, 1 H); ^{13}C NMR δ 159.3, 138.8, 138.7, 138.6, 137.9, 129.9, 129.4, 128.4, 128.3, 127.9, 127.8, 127.5, 113.8, 83.1, 81.1, 79.7, 79.4, 75.8, 72.6, 72.3, 67.4, 55.2; IR 3578 cm^{-1}; mass spectrum (CI(+)) m/z 659.3001 ($C_{42}H_{42}O_7$ + H requires 659.3009), 630 (base).

D-1-*O*-(4-Methoxybenzyl)-2-*O*-methyl-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol. A solution of **16** (0.69 g, 1.04 mmol) in THF (2 mL) was added to a 60% NaH oil dispersion (0.16 g, 4.0 mmol) in THF (10 mL). After 30 min, freshly distilled methyl iodide (0.5 mL, 1.1 g, 8.0 mmol) was added, and the mixture was stirred for 24 h at 40 °C. Water (10 mL) was cautiously added, and the reaction was then diluted with Et_2O (100 mL). The layers were separated, and the organic layer was washed with brine (1 × 30 mL), dried ($MgSO_4$), and evaporated. The residue was purified by flash chromatography eluting with hexane/EtOAc (7:3) to provide 0.79 g (ca 100%) of the product as a white solid: mp 151−153 °C; ^1H NMR δ 7.50−7.36 (comp, 22 H), 6.99 (d, J = 6.7 Hz, 2 H), 5.11−4.94 (comp, 6 H), 4.89−4.71 (comp, 4 H), 4.19−4.12 (m, 2 H), 3.90 (s, 3 H), 3.84 (app t, J = 2.4 Hz, 1 H), 3.81 (s, 3 H), 3.60 (app t, J = 9.2 Hz, 1 H), 3.49 (app t, J = 2.4 Hz, 1 H), 3.45 (app t, J = 2.4 Hz, 1 H); ^{13}C NMR δ 159.4, 139.1, 139.0, 138.4, 130.5, 129.5, 128.5, 128.4, 128.1, 127.9, 127.6, 113.9, 83.7, 81.8, 80.8, 80.5, 77.8, 77.3, 76.8, 76.0, 73.0, 72.7, 61.4, 55.3; mass spectrum (CI(+)) m/z 673.3165 ($C_{43}H_{44}O_7$ + H requires 673.3165), 553 (base).

D-2-*O*-Methyl-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (17). 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (0.39 g, 1.7 mmol) was added to a stirred solution of the product of the preceding experiment (0.79 g, 1.10 mmol) and water (0.5 mL) in CH_2Cl_2 (20 mL). The reaction was stirred for 30 min at rt and then diluted with CH_2Cl_2 (100 mL). The mixture was washed with saturated aqueous $NaHCO_3$ (1 × 30 mL) and brine (1 × 30 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash chromatography eluting with hexane/EtOAc (7:3) to provide 0.79 g (ca. 100%) of **17** as a white solid: mp 133−135 °C; ^1H NMR δ 7.38−7.21 (comp, 20 H), 4.96−4.67 (comp, 8 H), 4.97 (app t, J = 9.5 Hz, 1 H), 3.84−3.70 (comp, 2 H), 3.65 (s, 3 H), 3.51−3.38 (comp, 3 H), 2.23 (br s, 1 H); ^{13}C NMR δ 138.7, 138.6, 138.5, 138.0, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.4, 83.4, 82.0, 81.8, 81.0, 79.0, 75.8, 75.6, 75.5, 72.9, 72.2, 61.3; mass spectrum (CI(+)) m/z 555.2744 (base) ($C_{35}H_{38}O_6$ + H requires 555.2747), 463.

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(23) Bradford, M. M. *Anal. Biochem.* **1975**,*72*, 248−254.

(24) Bruzik, K. S.; Salamonczyk, G. M. *Carbohydr. Res.* **1989**, *195*, 67−73.

***O*-(1,2-Dihexanoyl-*sn*-3-glyceryl) *O*-(D-2-*O*-methyl-3,4,5,6-tetra-*O*-benzyl-*myo*-inositolyl) *O*-[2-(trimethylsilyl)ethyl] phosphate (18)**. A solution of the protected inositol **17** (150 mg, 0.28 mmol) in THF (1 mL), was added dropwise to a vigorously stirred solution of 2-(trimethylsilyl)ethyl dichlorophosphite (67 mg, 0.31 mmol) and *N*,*N*-diisopropylethylamine (60 μL, 44 mg, 0.78) in dry, deoxygenated THF (2 mL) at −78 °C. After stirring for 1 h, a solution of diacylglycerol **19** (97 mg, 0.34 mmol) in THF (1 mL) was slowly added, and the resulting suspension was stirred for 2 h at −78 °C. The cooling bath was removed, and stirring was continued for an additional 1 h at rt. The reaction was filtered through a plug of celite (1 g), which was subsequently washed with dry THF (2 mL). The filtrate and washings were combined and cooled to 0 °C, and a solution of 3 M *tert*-butyl hydroperoxide in isopentane (0.3 mL, 1.0 mmol) was added with stirring. After 0.5 h, the excess oxidizing agent was destroyed by adding trimethyl phosphite (2 mL). When the mixture no longer gave a positive indication on acidic starch/iodine paper, the solvents were removed under reduced pressure, and the residue was dried *in vacuo*. The crude phosphate triester was then purified by flash chromatography eluting with hexanes/EtOAc (2:1) to give 0.18 g (66%) of **18** as inseparable mixture of diastereoisomers and a colorless oil: ^{1}H NMR δ 7.42−7.18 (comp, 20 H), 5.11−5.09 (m, 1 H), 4.99−4.70 (comp, 8 H), 4.31−3.90 (comp, 10 H), 3.71 (s, 3 H), 3.51 (t, J = 7.0 Hz, 2 H), 2.42−2.23 (m, 4 H), 1.73−1.52 (m, 4 H), 1.41−1.22 (m, 8 H), 1.15 (t, J = 7.0 Hz, 1.1 H), 1.05 (t, J = 7.0 Hz, 0.9 H), 0.96−0.85 (m, 6 H), 0.06 (s, 5 H), 0.01 (s, 4 H); ^{13}C NMR δ 172.8, 172.4, 138.5, 138.3, 137.8, 128.2, 128.1, 127.7, 127.5, 127.4, 127.3, 127.3, 82.8, 81.1, 80.2, 79.8, 79.7, 78.3, 78.2, 77.9, 77.2, 75.6), 75.2, 72.7, 69.2, 69.1, 67.6, 66.8, 66.7, 66.6, 65.2, 65.1, 64.7, 33.8, 33.7, 31.0, 30.9, 24.3, 24.2, 22.0, 19.4, 19.3, 13.7, −1.7, −1.8; IR 1736 cm^{-1}; mass spectrum (CI(+)) m/z 1005.4939 (base) ($C_{55}H_{77}O_{13}PSi$ + H requires 1005.4949), 977, 289, 271.

1,2-Dihexanoyl-*sn*-3-glyceryl-3-phospho-D-2-*O*-methyl-*myo*-inositol (9). To a solution of **18** (0.18 g, 0.18 mmol) in absolute EtOH (10 mL) was added 20% $Pd(OH)_2$/C (0.15 g). The resulting suspension was stirred under an atmosphere of H_2 (500 psi) for 14 h. The catalyst was removed by filtration through a plug of celite, and the solvent was removed under reduced pressure and dried *in vacuo*. The residue was dissolved in MeCN/THF (2:1) (2.8 mL), and 2.8 N aqueous HF in THF (195 μL) was added. After stirring at rt for 1 h, the volatiles were removed under reduced pressure, and the residue was purified by flash chromatography eluting with $CHCl_3$/MeOH/H_2O (6.6:3.0:0.4) to obtained 49 mg (50%) of **9** as a colorless glass: ^{1}H NMR (CD_3OD) δ 5.31−5.19 (m, 1 H), 4.49−4.39 (m, 1 H), 4.25−3.88 (comp, 4 H), 3.90−3.81 (m, 2 H), 3.81−3.68 (m, 1 H), 3.68−3.57 (m, 3 H), 3.57−3.51 (m, 1 H), 3.51−3.38 (m, 1 H), 3.23−3.15 (m, 1 H), 2.43−2.26 (m, 4 H), 1.71−1.49 (m, 4 H), 1.43−1.20 (m, 8 H), 0.90 (t, J = 6.6 Hz, 6 H); ^{13}C NMR (CD_3OD) δ 175.0, 174.7, 83.3, 78.1, 76.2, 74.3, 73.5, 73.1, 71.9, 65.0, 63.7, 62.3, 35.0, 34.8, 32.3, 25.6, 23.3, 14.3); ^{31}P NMR (CD_3OD) δ 0.41; IR 3338, 1733, 1449, 1230 cm^{-1}; mass spectrum (FAB(−)) m/z 543.2208 (base) ($C_{22}H_{41}O_{13}P$ − H requires 543.2207).

2-Chloro-1,3,2-dithiophospholane (20).[16] 1,2-Ethanedithiol (85 mL, 1 mol) was added dropwise over 1.5 h with stirring to a solution of freshly distilled PCl_3 (90 mL, 1.0 mol) in degassed C_6H_6 (350 mL) at 50 °C (oil bath). After the addition was complete, the solution was heated for 2 h at which time the reaction flask was fitted for a simple distillation. The solvent was removed by distillation at atmospheric pressure (head temp ~ 61 °C), and the product was then distilled at 0.1 mm Hg (head temp, 48 °C). (**NOTE**: It is *important* to seal all joints with grease to exclude oxygen and to distill the product slowly (~5−6 h) to *avoid an explosion*! Signs of imminent danger include a darkening to a deep orange or red, and a thickening of the material in the distillation pot.) The crude distillation was stopped when the oil in the distillation pot becomes viscous. The crude product was then purified by fractional distillation using a 6 in. vigreux column (~0.1 mmHg, head temp 75 °C), and the vacuum is quenched with a stream of dry argon to yield 142.7 g (90%) of **31** as clear colorless liquid (which must be stored under argon and refrigerated): ^{1}H NMR δ 3.78−3.51 (comp, 4 H); ^{13}C NMR δ 42.8 (d, J_{CP} = 7.8 Hz); ^{31}P NMR δ 168.9; IR 3008, 1417, 1281, 1077, 988 cm^{-1}; mass spectrum (CI(+)) m/z 157.9181 (C_2H_4-PS_2 requires 157.9181), 125, 123 (base).

2-*O*-(1,2-Dihexanoyl-*sn*-3-glyceryl)-2-thio-1,3,2-dithiophospholane (21). 2-Chloro-1,3,2-dithiophospholane (**20**) (0.79 g, 5.00 mmol) in MeCN (1 mL) was added dropwise to a stirred solution of 1,2-di-*n*-hexanoyl-*sn*-3-glycerol (**19**) (1.44 g, 5.00 mmol) and Hünig's base (0.96 mL, 5.50 mmol) in dry, degassed MeCN (2 mL) at −38 °C. After stirring for 2 h at −38 °C, the reaction mixture was warmed to rt and stirred for an additional 1 h. A solution of S_8 (0.48 g, 15.00 mmol) dissolved in CS_2 (5 mL) was added, and the resultant light yellow heterogeneous mixture was stirred vigorously for 6 h. The reaction mixture was concentrated under reduced pressure, EtOAc (5 mL) was added, and the mixture was filtered through a plug of glasswool. The filtrate was concentrated under reduced pressure, and the residue was purified by flash chromatography eluting with hexanes/EtOAc (7:3) to deliver 1.87 g (84%) of **21** as a viscous oil: ^{1}H NMR δ 5.26−5.18 (m, 1 H), 4.31−4.09 (comp, 4 H), 3.70−3.52 (comp, 4 H), 2.29 (t, J = 7.3 Hz, 2 H), 2.27 (t, J = 7.3 Hz, 2 H), 1.64−1.51 (comp, 4 H), 1.31−1.20 (comp, 8 H), 0.84 (t, J = 6.7 Hz, 6 H); ^{13}C NMR δ 173.2, 172.7, 69.1 (d, J_{CP} = 10.1 Hz), 65.4 (d, J_{CP} = 8.6 Hz), 61.7, 41.5 (d, J_{CP} = 5.3 Hz), 34.1, 33.9, 31.2, 24.4, 22.2, 13.9; ^{31}P NMR δ 124.0; IR 1737, 1466, 1229, 1165, 1020 cm^{-1}; mass spectrum (CI(+)) m/z 443.1140 ($C_{17}H_{31}O_5PS_3$ + H requires 443.1150), 271 (base).

***O*-(1,2-Dihexanoyl-*sn*-3-glyceryl) *O*-(2,3:5,6-Bis-*O*-(1-methylethylidene)-4-*O*-(*tert*-butyldimethylsilyl)-D-*myo*-inositolyl) Phosphorodithioate (22)**. 1,8-Diazo[5.4.0]-bicycloundec-7-ene (DBU) (0.19 mL, 1.25 mmol) was added to a solution of the dithiophospholane **21** (0.63 g, 1.40 mmol) and the protected D-*myo*-inositol **23** (0.48 g, 1.25 mmol) in MeCN (50 mL), and the reaction was stirred for 1 h at rt. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography eluting first with MeOH/$CHCl_3$ (1:19) and then Me_2CO/$CHCl_3$/H_2O (6.7: 3.2: 0.1) to provide 0.80 g (85%) of the DBU salt of **22**. The free phosphorodithioate was isolated by ion exchange chromatography using an Amberlyst column (basic form), which was prepared by washing the hydrated resin (ca. 10g) with ethanolic NaOH and then absolute EtOH. The DBU salt of **22** was eluted through the basic resin using absolute EtOH. The eluant was acidified with dilute aq. HCl, and the solvents were removed *in vacuo* to yield 0.76 g (95%) **22** as a light yellow glass: ^{1}H NMR δ 5.26−5.18 (m, 1 H), 4.92−4.81 (m, 1 H), 4.72 (t, J = 4.5 Hz, 1 H), 4.40−4.03 (comp, 4 H), 3.93−3.84 (m, 2 H), 3.71 (dd, J = 9.8, 6.3 Hz, 1 H), 3.25 (t, J = 9.8 Hz, 1 H), 2.23 (t, J = 7.4 Hz, 2 H), 2.21 (t, J = 7.4 Hz, 2 H), 1.68−1.51 (comp, 4 H), 1.36−1.19 (comp, 8 H), 1.32 (s, 3 H), 1.23 (s, 6 H), 1.20 (s, 3 H), 0.84 (s, 9 H), 0.83 (t, J = 6.9 Hz, 6 H), 0.03 (s, 6 H); ^{13}C NMR δ 173.3, 172.9, 111.7, 109.6, 79.9, 82.5, 79.1, 77.3, 75.8, 72.9 (d, J_{CP} = 8.5 Hz), 70.2 (d, J_{CP} = 8.6 Hz), 66.2 (d, J_{CP} =7.7 Hz), 63.1, 34.2, 34.0, 31.1, 28.2, 26.8, 24.4, 22.2, 18.1, 13.8, −4.6, −4.8; ^{31}P NMR δ 115.6; IR 1731, 1456, 1170 cm^{-1}; mass spectrum (CI(+)) m/z 755.3092 ($C_{33}H_{59}O_{11}PSi_2$ + H requires 755.3084), 469, 271 (base), 173.

***O*-(1,2-Dihexanoyl-*sn*-3-glyceryl) *O*-(2,3:5,6-Bis-*O*-(1-methylethylidene)-4-*O*-(*tert*-butyldimethylsilyl)-D-*myo*-inositolyl) phosphorodithioate (10).** A mixture of the protected inositol dithioate **22** (0.40 g, 0.53 mmol) and aq. 2.5 M HF (0.6 mL, 0.74 mmol) in MeCN (10 mL) was stirred for 2 h at rt, whereupon the solvents were removed *in vacuo*. The residue was suspended in $CHCl_3$ (10 mL), and the insoluble solids were removed by vacuum filtration. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography eluting with $CHCl_3$/MeOH/H_2O (7: 3: 0.5) to provide 0.28 g (93%) of **10** as a yellow glass: ^{1}H NMR (DMSO-d_6) δ 5.17−5.04 (br s, 1 H), 4.67−4.63 (br s, 1 H), 4.52−4.49 (br s, 1 H), 4.46−4.42 (br s, 1 H), 4.39−4.35 (br s, 1 H), 4.31−3.90 (comp, 5 H), 3.56−3.46 (m, 1 H), 3.42−3.31 (m, 2 H), 3.13−3.04 (m, 1 H), 3.00−2.89 (m, 1 H), 2.34−2.21 (comp, 4 H), 1.60−1.44 (comp, 4 H), 1.37−1.15 (comp, 8 H), 0.83 (t, J = 6.9 Hz, 6 H); ^{13}C NMR (CD_3OD) δ 175.1, 174.1, 88.9 (d, J_{CP} = 6.0 Hz), 76.0, 73.9, 73.2, 73.1 (d, J_{CP} = 3.6 Hz),

72.7 (d, J_{CP} = 7.5 Hz), 71.7 (d, J_{CP} = 7.6 Hz), 64.9 (d, J_{CP} =5.6 Hz), 63.8, 35.1, 34.9, 32.3, 25.6, 23.4, 14.3; ^{31}P NMR δ 116.7; IR 3251, 1737, 1466, 1164 cm^{-1}; mass spectrum (CI(+)) *m/z* 561.1600 (base) ($C_{21}H_{37}O_{11}PS_2$ + H requires 561.1593), 257.

***myo*-Inositol 1-(4-Nitrophenylphosphate) (25).** A mixture of 4-nitrophenyl phosphorodichloridate (1.56 g, 6.00 mmol) and **23** (0.75 g, 2.00 mmol) in dry pyridine (10 mL) was stirred overnight at rt. Water (0.5 mL) was added, and the mixture was stirred for 1 h at rt. The solvents were removed *in vacuo*, and the residual oil was dissolved in CH_3CN/THF (2:1) (30 mL) containing 2.45 N aqueous HF (2 mL). After stirring for 2 h at rt, the volatiles were removed under reduced pressure, and the crude product was purified by flash chromatography eluting with $CHCl_3$/MeOH/H_2O (7:3:0.5) to provide 0.45 g (57%) of **25** as a yellow glass. Any traces of 4-nitrophenol were removed by extracting an aqueous solution of **25** with ether. The aqueous layer was then freeze-dried to yield **25** as a stable glass, the physical properties of which were identical to those reported.[9]

Standard Spectrophotometric Assay. The activity of recombinant bacterial PI-PLC (*B. cereus*) was assayed spectrophotometrically at 396 nm by measuring the rate of formation of 4-nitrophenol that was produced by the enzyme catalyzed hydrolysis of the chromogenic substrate **25.**[9] Stock solutions of substrate **25** were obtained by dissolving a weighed amount of dry **25** in the required amount of acidic water (pH 4.5−5) to give 1000 mM and 100 mM solutions. The concentrations of these stock solutions were then accurately determined by adding a small aliquot of the **25** solution to 0.01 N aq. NaOH and measuring the absorbance at 396 nm after complete hydrolysis (30 min).[9a] The stock solution of recombinant PI-PLC was prepared by diluting a small aliquot of enzyme in Tris buffer (20 mM, pH 8.6) in the required amount of 1% bovine serum albumin (BSA) at pH 7−8 to give a 5.25 μg/mL solution. Inhibitor stock solutions were made by dissolving a weighed amount of dry inhibitor with the required volume of water. During the kinetic evaluations the stock solutions were kept on ice. In a typical assay, the required amount of **25** (5−25 μL) and inhibitor stock solution (10 μL) were mixed in a cuvette containing the proper volume of 100 mM Hepes/1 mM ethylenediamine tetraacetic acid (EDTA) buffer at pH 7.0. The mixture was stirred in a constant temperature bath at 25 °C. After a background measurement was recorded by the spectrophotometer, the reaction was initiated by the addition of 0.01 μg of PI-PLC (10 μL) with stirring. The rate of release of 4-nitrophenol was followed continuously at 396 nm for 2 min. PI-PLC activity was assayed at [S]'s of 25.0, 10.0, 5.0, 2.5, and 1.0 mM in both the presence and in the absence of inhibitors **6**−**10**; only one [I] was assayed for each inhibitor. The assays conducted with 10 mM and 25 mM substrate concentrations were performed at least in duplicate, whereas those executed at lower substrate concentrations were done at least in triplicate. The specific activity of PI-PLC was determined by calculating the rate of product released over time by using the molar extinction coefficient of 4-nitrophenol, which is 7700 M^{-1} cm^{-1} at 396 nm at pH 7.0.[9a] The initial velocity was determined by fitting a tangent anchored at time zero to the initial linear region of the rate progression curve. This tangent line was calculated using the kinetics software provided by Hewlett-Packard that also provides the standard deviation of the data points in the fitted curve. The initial velocity data from each enzyme assay were examined both by non-linear least squares analysis and by extrapolation of the double reciprocal plot of velocity versus substrate concentration. The hyperbolic data in the plot of velocity versus concentration were fit with a non-linear least squares algorithm, and the curved line was calculated from the Michaelis-Menten equation $v_o = V_{max}[S](K_m + [S])^{-1}$ using the program KaleidaGraph. The values for K_m and V_{max} for the substrate **25** were estimated directly from the hyperbolic data. For the assays in which the hydrolysis of **25** was monitored in the presence of the inhibitors **6**−**10**, the apparent K_m's ($K_{m'}$) were determined by the same method assuming competitive inhibition for each compound.[20] The initial velocities were used to generate double-reciprocal plots from which the type of inhibition of each of the compounds **6**−**10** was also determined to be competitive. The K_i's were calculated using the expression $K_{m'} = K_m(1 + [I]/K_i)$.

Acknowledgment. We thank the National Institutes of Health and the Robert A. Welch Foundation for their generous support of this research. We also thank Mr. Daniel W. Dean for preparing compounds **6**−**8**, for the synthesis of a number of other intermediates, and for assistance in developing the assay. We are especially grateful to Professor O. Hayes Griffith (University of Oregon) for a generous gift of recombinant PI-PLC and to Dr. Dirk W. Heinz (Universität Freiburg) for helpful discussions.

Supporting Information Available: Copies of ^{1}H NMR spectra of all new compounds (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO960850Q