Articles

Synthesis, Structure−Activity Relationships, and the Effect of Polyethylene Glycol on Inhibitors of Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus*

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Received June 12, 1996[⊗]

Substrate analog inhibitors of *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC) were synthesized and screened for their suitability to map the active site region of the enzyme by protein crystallography. Analogs of the natural substrate phosphatidylinositol (PI) were designed to examine the importance of the lipid portion and the inositol phosphate head group for binding to the enzyme. The synthetic compounds contained pentyl, hexyl, or hexanoyl and octyl lipid chains at the *sn*-1 and *sn*-2 positions of the glycerol backbone and phosphonoinositol, phosphonic acid, methyl phosphonate, phosphatidic acid, or methyl phosphate at the *sn*-3 position. The most hydrophobic compound, dioctyl methyl phosphate **14**, was also the best inhibitor with an IC_{50} of 12 μM. In a series of dihexyl lipids, compounds with phosphonoinositol head groups inhibited more strongly than those that do not contain inositol but are otherwise identical. Compound **29**, a short-chain lipid with a phosphonoinositol head group, was found to be a competitive inhibitor and the most potent in this series with an IC_{50} of 18 μM (K_i =14 μM). Analogs with dihexyl chains were better inhibitors than those with dihexanoyl chains, presumably because the ether-linked lipids are more hydrophobic than the ester-linked lipids. No appreciable difference in inhibition was found between a phosphonoinositol lipid and the corresponding difluorophosphonoinositol lipid. Inositols and inositol derivatives that do not contain lipid moieties show IC_{50}s about 3 orders of magnitude above those of the short-chain lipids. In this group, glucosaminyl(α1→6)-D-*myo*-inositol inhibited more strongly than *myo*-inositol, which in turn is a better inhibitor than inositol phosphate. The addition of polyethylene glycol (PEG-600) resulted in a marked decrease in inhibition by the short-chain lipids, but had little effect on the water-soluble head group analogs. This is accounted for in terms of solubilization of the amphipathic inhibitors by PEG. Since PEG is required in the crystallization, these data indicate that the best strategy for obtaining enzyme inhibitor complexes is to start by cocrystallizing PI-PLC with the head group analogs. The next step is to synthetically add the shortest possible hydrophobic moieties to the analogs and cocrystallize these with the enzyme. This strategy may be applicable to other lipolytic enzymes.

Introduction

Signaling molecules such as growth factors, neurotransmitters, and mitogens are received on the cell surface and, via heterotrimeric G proteins or tyrosine kinases, lead to the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) on the internal face of the cell membrane. PI-PLC produces the two second messengers *myo*-inositol 1,4,5-trisphosphate and diacylglycerol (DAG). These two molecules initiate signaling cascades that result in profound cellular changes such as cell proliferation and neuronal activity.[1,2] Links to cancer and Alzheimer's disease are being investigated.[3−5]

Mammalian PI-PLCs are large, Ca^{2+} dependent enzymes that contain one catalytic and several regulatory domains. The much smaller bacterial PI-PLCs consist only of the catalytic domain, and the crystal structure of *Bacillus cereus* PI-PLC[6] shows the same architecture as the catalytic domain of the recently solved structure of a mammalian PI-PLC.[7] Bacterial PI-PLCs do not require Ca^{2+} for activity and unlike mammalian PI-PLCs cleave the glycosylphosphatidylinositol (GPI) anchor of GPI-anchored proteins[8] in addition to phosphatidylinositol (PI). The GPI-anchor cleaving activity is of considerable medical interest as diagnostic tool for the analysis of proteins presented on the outer face of cell membranes. These GPI-anchored proteins include activation antigens of the immune system, scrapie prion protein, and the carcinoembryonic antigen, a human tumor marker.[9−11]

B. cereus PI-PLC is stereospecific for the D-*myo*-inositol enantiomer of its PI or GPI substrates. The corresponding L-enantiomers have been shown to be neither substrates nor inhibitors, which is fortunate since it is, therefore, possible to use racemic synthetic substrates.[12−14] The enzyme is insensitive to the stereochemistry around the *sn*-2 carbon of the lipid portion of the substrate molecule.[8,15] Variations in the lipid moiety are tolerated, e.g., ether- or ester-linked lipids, mono- or diacyl lipids, or ceramides, and are usually

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

S0022-2623(96)00434-7 CCC: $12.00

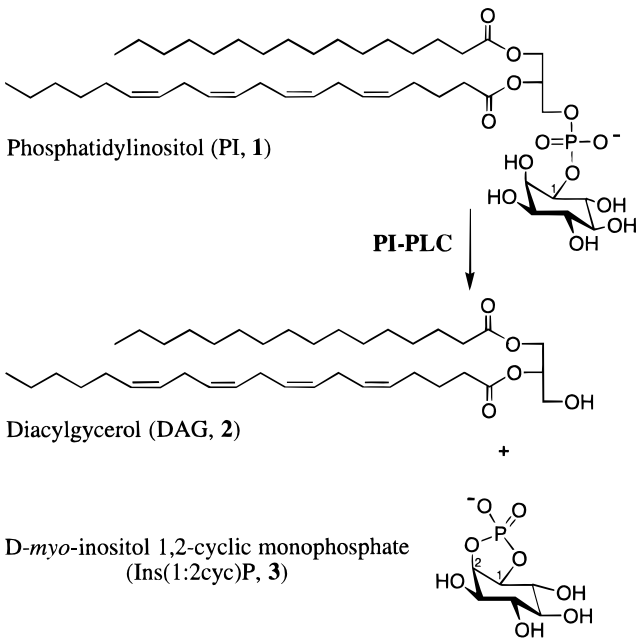
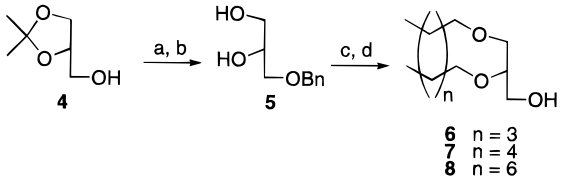


Figure 1. Reaction catalyzed by *B. cereus* PI-PLC. The substrate PI is cleaved by the enzyme's phosphotransferase activity into two components, diacylglycerol (DAG) and D-*myo*-inositol 1,2-cyclic phosphate (Ins(1:2-cyc)P). This cyclic phosphate is gradually hydrolyzed to *myo*-inositol 1-phosphate in a second reaction by PI-PLC. Mammalian PI-PLCs also cleave PI, but preferentially in a form phosphorylated at the 4 and 5 positions of *myo*-inositol.

equally good substrates.[16] *B. cereus* PI-PLC possesses phosphotransferase as well as phosphodiesterase activities, as shown by ^{31}P NMR.[12] Acting on the natural substrate PI, the phosphotransferase produces lipid-soluble DAG and water-soluble D-*myo*-inositol 1,2-cyclic phosphate (Ins(1:2-cyc)P) (Figure 1). In a second but much slower reaction, the phosphodiesterase activity of *B. cereus* PI-PLC opens the cyclic phosphate ring to form D-*myo*-inositol 1-phosphate (Ins(1)P). The reaction from PI to inositol 1-phosphate proceeds with overall retention of configuration at phosphorus.[15]

With the PI-PLC crystal structures now available,[6,7] it is important to design inhibitors for cocrystallization. Our goal here is to provide insight into the choice of inhibitors to map the active-site region of *B. cereus* PI-PLC, an important step toward arriving at a molecular mechanism. There are a number of reports of PI-PLC inhibition in the literature,[5,8,17−25] but because of varying assay conditions, the results are often difficult to compare. Most of these studies use assays based on the naturally occurring phospholipid substrate, PI, in a lipid-like environment, e.g., in detergent micelles or phospholipid vesicles. This strategy is appropriate for simulating physiological conditions, where the reference state is the lipid interface environment. However, to screen inhibitors for cocrystallization we have chosen a single-phase aqueous assay system to eliminate any secondary effects on the enzyme and added inhibitors which may be introduced by the presence of an interface. In this assay system the synthetic substrate *myo*-inositol 1-(4-nitrophenyl phosphate) (NPIP)[26] is used. With this approach, a panel of inhibitors can be compared with an aqueous environment as the reference state so that the results are applicable to the crystallization conditions. Here we report the synthesis of a series of short-chain phospholipids which have high monomeric solubility in water and compare the inhibition by these amphiphiles to that of a series of hydrophilic compounds resembling the head group of the natural substrate, PI. Since the crystallization of *B. cereus* PI-PLC is carried out in the presence of polyethylene glycol (PEG), the question of effects caused by this polymer is also addressed.

Scheme 1[a]



[a] Reagents: (a) NaH/THF, BnBr; (b) HOAc/MeOH/H_2O; (c) RBr, KOH/toluene; (d) H_2, 10% Pd/C, MeOH.

Results and Discussion

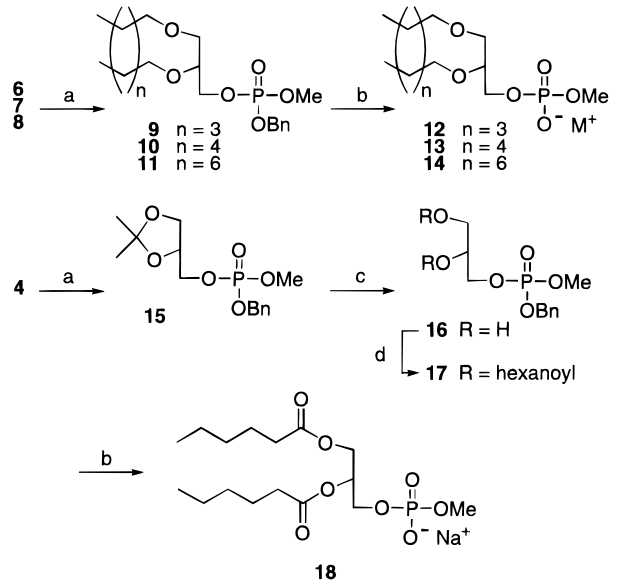
Synthesis. The commercially available racemic ketal **4** was selected as a starting point for the synthesis of the dialkylglycerols (Scheme 1). The known diol **5** is readily available from solketal **4** in high yield.[27,28] The diol **5** was readily alkylated with a variety of alkyl bromides using a modification of previously reported conditions[29] to afford the diethers in >90% yield. Hydrogenolysis[27] produced the desired di-*O*-alkylglycerols **6**, **7**, and **8** in 60−80% overall yield from **4**. While the synthesis of **7**[29] and **8**[30,31] by related procedures has been reported previously, the full characterization of these compounds is reported here for the first time.

We next undertook the phosphorylation of the short-chain dialkylglycerols. The importance of phospholipids in physiological processes, in particular as mediators in signal transduction, has stimulated the development of methods for the efficient chemical synthesis of structurally well defined phospholipids and their analogs as enzyme inhibitors and drug candidates.[32] These routes include the phosphorylation of alcohols using the phosphoramidites originally developed for oligonucleotide chemistry[33,34] and the use of activating reagents such as 2,4,6-triisopropylbenzenesulfonyl chloride (TPS).[14,35] These routes are somewhat limited by the availability of the required intermediates. To circumvent these problems the chlorophosphate route described by Eibl and Woolley[36] for the synthesis of long-chain diradylglycerol phospholipids was explored. However, in our hands this method did not produce satisfactory yields. The decreased steric bulk of the short-chain dialkyl glycerols employed in this study apparently greatly diminishes the reactivity differences between the successive electrophiles $OPCl_3$ and $OPCl_2(OR)$. Similarly the use of methyl dichlorophosphate[37,38] failed to afford the desired mixed phosphorus triesters in acceptable yields.

A modification of a procedure recently reported for the synthesis of diacylglyceryl phosphates[39] allowed the isolation of the desired mixed triester **10** as a mixture of diastereomers in 64% yield along with the corresponding symmetric diglyceryl phosphate in 31% yield. Extending these phosphorylation conditions to **6** and **8** gave the corresponding triesters (**9** and **11**) in 48 and 68% isolated yields, respectively (see Scheme 2).

Hydrogenolysis of the benzyl protecting group in **9**−**11** furnished the short-chain methyl phosphates **12**−**14** as

Scheme 2[a]



[a] Reagents: (a) $MeOPCl_2$, *i*-Pr_2NEt/CH_2Cl_2, then BnOH, *i*-Pr_2NEt/CH_2Cl_2, then *t*-BuOOH/isooctane; (b) H_2, 10% Pd/C, MeOH; (c) Dowex 50W-X8 (H^+ form), MeOH; (d) hexanoyl chloride, *i*-Pr_2NEt/CH_2Cl_2.

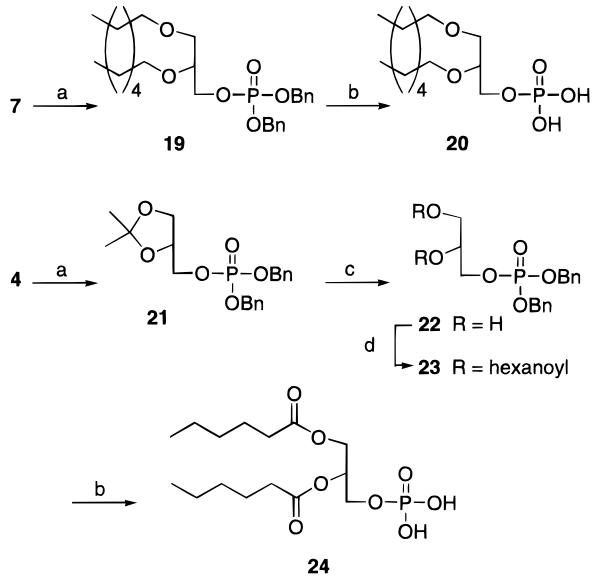
the free acids. Compound **12** could be readily purified by acid/base extraction and was isolated as the sodium salt. Methyl phosphates **13** and **14** could not be purified by this protocol and required column chromatography to afford **13** and **14**, isolated as the ammonium salts.

Dihexanoyl methyl phosphate **18** was prepared via an indirect route employing solketal **4** as the starting alcohol. This route avoids the potential complications arising from the 2 → 3 acyl migration in diacyl glycerols.[36] The phosphorylation of **4**, under the conditions reported above, afforded triester **15** in 48% yield. The isopropylidene protecting group was removed by the method of Heeb and Nambiar[40] to produce diol **16** in 53% yield after chromatographic isolation. Acylation of the hydroxyls in **16** afforded the fully protected **17** in good overall yield. On the basis of ^{13}C NMR analysis no 3 → 2 phosphoryl migration[41] under the conditions employed for the acylation reaction could be detected. The benzyl protecting group in **17** was then removed by hydrogenolysis, followed by ion exchange to afford the sodium salt of **18** in quantitative yield.

The dihexyl and dihexanoyl phosphatidic acid esters **20** and **24** were prepared as shown in Scheme 3. The intermediate dibenzyl triesters **19** and **21** were readily available in high yields (87 and 89%, respectively) using an excess (1.5 equiv) of phosphorus oxychloride followed by an excess of benzyl alcohol. By employing the conditions discussed above, the isopropylidene triester **21** was readily converted to the corresponding dihexanoyl analog **23**. Cleavage of the phosphate protecting groups in **19** and **23** afforded the acids **20** and **24** in nearly quantitative yields.

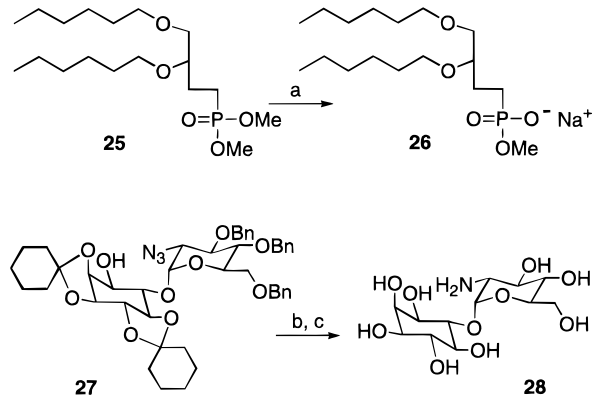
The phosphonate analog **26** of **15** was prepared (Scheme 4) from the dimethyl phosphonate **25**[25] by selective mono-demethylation employing NaI in methyl ethyl ketone (MEK). The remaining phosphonates employed in the present study (**29**−**32**) were prepared as previously described.[25] The glycosylinositol **28** was prepared from **27** by a modification of the procedure previously described.[42,43]

Scheme 3[a]



[a] Reagents: (a) $POCl_3$, Et_3N/Et_2O, then BnOH, Et_3N/Et_2O; (b) H_2, 10% Pd/C, MeOH; (c) Dowex 50W-X8 (H^+ form), MeOH; (d) hexanoyl chloride, *i*-Pr_2NEt/CH_2Cl_2.

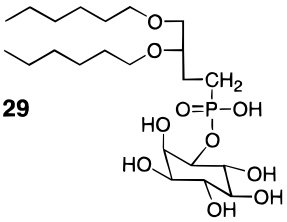
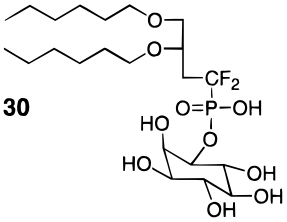
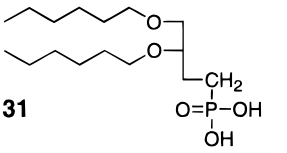
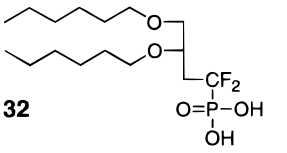
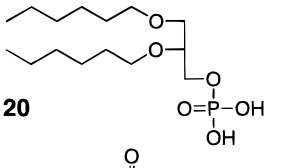
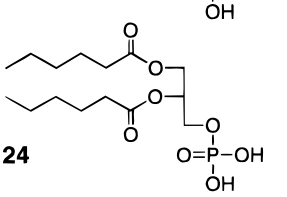
Scheme 4[a]



[a] Reagents: (a) NaI, MEK; (b) H_2, 20% $Pd(OH)_2/C$, MeOH; (c) AcOH/water, 1:1.

Critical Micelle Concentration (cmc). The short-chain lipid inhibitors are surfactants which will spontaneously aggregate into micelles above their cmc's. In order to design the inhibition experiments so that these molecules remained in their monomeric state, the cmc's were determined for representative inhibitor compounds under the condition of the enzyme assay. The values are listed in Tables 1 and 2. The lipid chain length has the largest effect on the cmc. The dependence of the cmc on the nature of the lipid head group effect is much smaller, and the values reported here are similar to published data on short-chain phosphatidylcholines,[44] phosphatidic acids,[45] and phosphatidylinositols.[14,46] Some variations are expected since the methods of measurements differ, and the cmc's of ionic amphiphiles are known to depend on the buffer concentration and pH.[45] Most of the representative compounds examined in Tables 1 and 2 are ether-linked lipids. In compounds containing the same number of carbon atoms in their lipid chains, ester-linked lipids are known to have cmc's higher than those of ether-linked lipids.[29] Thus, selecting concentrations below the cmc's of the ether-linked lipids will also be appropriate for the ester-linked lipids. In our enzyme assays, the concentrations of short-chain

Table 1. Inhibition of *B. cereus* PI-PLC by Amphiphilic Substrate Analogs with *myo*-Inositol Phosphate, Phosphonate or Phosphate Head Groups

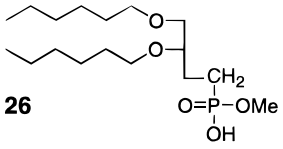
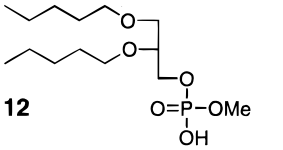
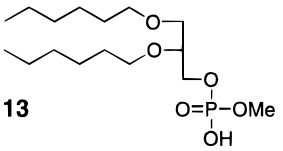
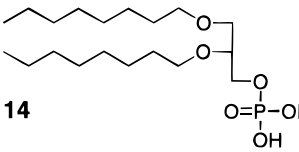
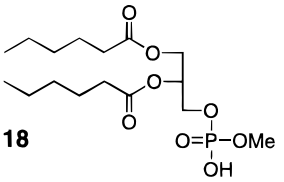
Inhibitor[a]	IC_{50} (μM)[b]	CMC (mM)[c]
29 	18 (± 2) K_i = 14 (±7)	14
30 	34 (± 23)	—
31 	60 (± 6)	11-15[d]
32 	45 (± 8)	—
20 	55 (± 4)	16-22[d]
24 	187 (± 33)	26-32[d]

[a] All compounds shown are racemic and were used as the free acids. [b] IC_{50} is the concentration of inhibitor which causes a 50% decrease in *B. cereus* PI-PLC activity and IC_{50} values are the mean (± average deviation) of two separate experiments using 5 nM PI-PLC and 0.25 mM NPIP substrate in assay buffer (100 mM HEPES, 1 mM EDTA; pH 7) at 25 °C. The K_i is the mean (± average deviation) of two determinations in assay buffer but varying the NPIP concentration from 0.1 mM to 0.5 mM. [c] The cmc values given for representative compounds were measured in assay buffer at 25 °C. [d] The cmc values were extrapolated as described in the Experimental Section, part II.

lipid inhibitors were maintained at least 1 order of magnitude below the cmc's, ensuring the inhibitors were present predominantly as monomers and not as micellar aggregates.

Structure−Activity Relationships. Our strategy was to synthesize a series of short-chain lipid inhibitors in which there are subsets with only one structural difference. For example, the three inhibitors **29**, **31**, and **26** are all phosphonates, i.e., the oxygen of the scissile oxygen−phosphorus bond has been replaced by CH_2. All three have in common the six carbon, ether-linked side chains. The only difference in these three molecules is the head group: *myo*-inositol-1-phosphonate, free phosphonic acid, and methyl phosphonate, respectively. The inhibition data of Table 1 show that **29** with the *myo*-inositol 1-phosphonate head group has the lowest IC_{50}, which is to be expected since this molecule most closely resembles the natural substrate, PI. We studied this model compound over a broad range of concentrations and determined that it is a competitive inhibitor with $K_i = 14\ \mu M$. Compounds with inositol head groups were synthesized as mixtures of diastereomers, and previous studies on the hydrolysis of the synthetic substrate NPIP showed that only the D-enantiomer is a substrate for PI-PLC, the L-enantiomer being neither a substrate nor an inhibitor.[13] Therefore, presumably only the D-enantiomer of **29** binds to the enzyme and the corrected IC_{50} and K_i values would be 9 and 7 μM, respectively. Surprisingly, **26** with the methyl group replacing the *myo*-inositol moiety is also an effective inhibitor. Removal of the methyl group as in the free phosphonic acid **31** increases the IC_{50} to 60 μM. The same trend, though to a smaller extent, is seen with the methyl phosphate **18** and the free phosphatidic acid **24**, two short-chain phospholipids with ester linkages. The corresponding lipid with a *myo*-inositol head group was omitted from this series, because it is expected to

Table 2. Inhibition of *B. cereus* PI-PLC by Amphiphilic Substrate Analogs with Methyl Phosphate or Methyl Phosphonate Head Groups

Inhibitor[a]	IC_{50} (μM)[b]	CMC (mM)[c]
26 	23 (± 2)	—
12 	235 (± 35)	55[d]
13 	53 (± 1)	15
14 	12 (± 2)	0.9
18 	118 (± 3)	—

[a] All compounds shown are racemic and were used as the sodium salts (**13** and **14** were converted from the ammonium to the sodium salt as described for **12** in Synthetic Procedures). [b] IC_{50} values are the mean (± average deviation) of two separate experiments using 5 nM PI-PLC and 0.25 mM NPIP substrate in assay buffer (100 mM HEPES, 1 mM EDTA; pH 7) at 25 °C. [c] The cmc values were measured in assay buffer at 25 °C. [d] This preparation was slightly less pure than the preparation used to determine the IC_{50}.

be a substrate rather than an inhibitor. In a third subset, consisting of the ether-linked phospholipids **20** and **13**, the difference between phosphate and methyl phosphonate head groups is not significant. Taken together, these data suggest that a component of the binding is due to an electrostatic interaction in the region of the phosphorous atom and that the optimal charge on this series of inhibitors is −1. A fraction of the free acids, under the conditions examined here, would be anions with a net charge of −2, which could account for the reduced inhibition compared to the corresponding methyl derivatives which are expected to have a net head group charge of −1 at neutral pH.[47]

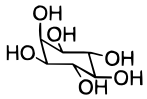
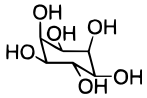
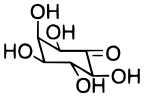
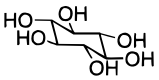
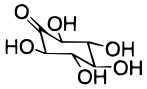
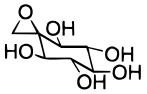
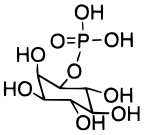
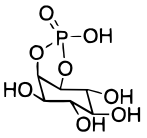
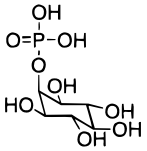
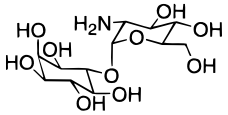
Another interesting trend is the effect of the length of the hydrocarbon chains on inhibition. The three ether-linked methyl phosphates, **12**−**14**, differ only in alkyl chain length, having both chains five, six, or eight carbons long, respectively. Inhibition by these compounds increases with the length of the lipid chains. The most potent inhibitor in Table 2 is the dioctyl lipid **14**, its IC_{50} of 12 μM is 20-fold lower than the IC_{50} for the dipentyl lipid **12**. These data underscore the importance of hydrophobic interactions in the active site region, identified in the crystal structure of *B. cereus* PI-PLC.[6] This trend is consistent with the results obtained for short-chain PI substrates of mammalian PI-PLCδ_1[46] and the substrates of other phospholipases,[32,48] where the activity is chain length dependent. Lipids with ether linkages are better inhibitors than the corresponding short-chain lipids with ester linkages (e.g., compare **20** with **24**; **26** with **18**), probably because the ether linkage conveys greater hydrophobicity.

Two modifications were designed to test for the importance of hydrogen bonding to the oxygen of the O−P bond cleaved by PI-PLC. One is the substitution of fluorine atoms for hydrogen atoms in the phosphonates (i.e., the pairs **29**, **30** and **31**, **32**). The second is the substitution of CH_2 for the oxygen, i.e., the pairs **20**, **31** and **13**, **26**. As can be seen from Tables 1 and 2, the differences in inhibition within these pairs are not significant within experimental error. We conclude that any loss of hydrogen-bonding potential when the oxygen atom of the scissile oxygen−phosphorus bond is replaced by CH_2 does not result in a large loss in binding. This is advantageous, as it indicates that phosphonate derivatives of PI are good routes to inhibitors of PI-PLCs. There appears to be little benefit in introducing CF_2 in place of CH_2.

A series of water-soluble molecules resembling the inositol head group were tested with the same assay as for the short-chain lipids so that the IC_{50}s would be directly comparable. The results are shown in Table 3. With IC_{50}s in the mM range, i.e., up to 3 orders of magnitude higher than many of the short-chain lipids of Tables 1 and 2, it would appear that these water-soluble molecules are of little interest because they are poor inhibitors. However, for reasons discussed below (i.e., the effect of PEG), these water-soluble molecules are important in developing a strategy for cocrystallization with the enzyme.

myo-Inositol is a convenient reference compound, as it is part of the natural substrate, PI. With an IC_{50} of 10 mM, it is a weak inhibitor, but nevertheless is able to displace the solvent molecules as cocrystals of PI-PLC in complex with *myo*-inositol have been obtained.

Table 3. Inhibition of *B. cereus* PI-PLC by Inositols and Related Compounds and Inositol Phosphates

Inhibitor[a]		IC_{50} (mM)[b]
	*myo*-Ins	10 (± 1)
	*epi*-Ins	2 (± 0.1)
	D,L-*epi*-inosose	3 (± 0.2)
	*scyllo*-Ins	410[c] (± 40)
	*scyllo*-inosose	133 (± 6)
	2-C-methylene-*myo*-inositol oxide	>50[d]
	D-*myo*-Ins(1)P	56 (± 1)
	D,L-*myo*-Ins(1:2-cyc)P	135[e] (± 3)
	*myo*-Ins(2)P	43 (± 7)
	GlcN(α1→6)Ins (**28**)	2 (± 0.2)

[a] The inositol phosphates were used as the cyclohexylammonium salts. [b] IC_{50} values are the mean (± average deviation) of two separate experiments using 5 nM PI-PLC and 0.25 mM NPIP substrate in assay buffer (100 mM HEPES, 1 mM EDTA; pH 7) at 25 °C. [c] The IC_{50} value was extrapolated from nonlinear regression analysis of the data with the highest concentration of *scyllo*-inositol, 85 mM, resulting in 78% of the uninhibited activity. [d] 50 mM inhibitor resulted in 73% of the uninhibited activity. [e] The IC_{50} was extrapolated from nonlinear regression analysis of the data with the highest inhibitor concentration used, 108 mM, resulting in 54% of the uninhibited activity.

epi-Inositol and *epi*-inosose have an axial C-6 hydroxyl or carbonyl group and are slightly better inhibitors. We have no explanation for the improved inhibition. We modified the C-6 position by adding glucosamine (**28**) to represent the proximal portion of a GPI anchor. The inhibition by glucosaminyl(α1→6)-D-*myo*-inositol (GlcN-(α1→6)Ins, **28**) is 5 times greater than by *myo*-inositol, arguing the presence of this portion of the GPI anchor improves binding to the enzyme. These results are in agreement with the observation that PI-PLC has a higher affinity for GPI substrates than for PI, demon-

strated by apparent K_M values for GPI substrates that are at least 2 orders of magnitude lower than the K_M for PI.[49]

scyllo-Inositol, *scyllo*-inosose, and 2-*C*-methylene-*myo*-inositol oxide are all poor inhibitors (Table 3). These molecules have alterations at the axial 2-hydroxyl group of *myo*-inositol, which is required for the formation of the cyclic phosphate ester, Ins(1:2-cyc)P (Figure 1). The crystal structure of PI-PLC in complex with *myo*-inositol shows the 2-hydroxyl at hydrogen-bonding distance to His32, one of the two histidines participating in catalysis. The loss of this hydrogen bond possibly in combination with steric hindrance by the altered arrangement around the 2-position would reduce binding to the enzyme. The three *myo*-inositol phosphates in Table 3 are also poor inhibitors; with IC_{50} values of about 50 mM they are 5 times weaker than *myo*-inositol. Two of these compounds, Ins(1:2-cyc)P and Ins(1)P, are products of reactions of *B. cereus* PI-PLC, and the third compound, *myo*-inositol 2-phosphate (Ins(2)P), is an isomer of a product. Thus, little or no product inhibition by the inositol phosphates is found, consistent with Ins(1:2-cyc)P acting as a very weak substrate for the phosphodiesterase activity of PI-PLC.[12]

The observation that short-chain methyl phosphates are inhibitors is consistent with results of a recent study on interfacial binding of *B. cereus* PI-PLC to phospholipid vesicles of ditetradecylglycerophosphomethanol (DTPM).[50] When *B. cereus* PI-PLC was exposed to DTPM vesicles, DTPM was found to act as a competitive inhibitor.[50] In fact, it was this report that encouraged us to synthesize and examine the short-chain methyl phosphates reported here.

Turning to the water-soluble inhibitors, we found in an earlier study of inhibition of hydrolysis of PI in the detergent deoxycholate that *myo*-inositol was an inhibitor but that *epi*-inositol was not.[24] Recently, Morris et al. examined the glycan requirements of GPI-PLC from *Trypanosoma brucei* and included a comparison with *B. cereus* PI-PLC.[21] Their assay was based on cleavage of GPI-anchored variant surface glycoprotein solubilized in deoxycholate micelles. With this assay, the glucosamine derivative GlcN(α1→6)Ins exhibited no inhibition of *B. cereus* PI-PLC.[21] We do not view these results as inconsistent with the data reported here, because *myo*-inositol and GlcN(α1→6)Ins are at best weak inhibitors. The differences serve primarily to point out the importance of considering the microenvironment in the assay when comparing inhibition data, e.g., phospholipid vesicles, detergent micelles, or monomers in an aqueous environment. For the purpose of identifying compounds for cocrystallizing with the enzyme, an aqueous reference state, as used here, is the appropriate choice since our crystallization conditions for PI-PLC do not include any lipid aggregates.

Effect of PEG on Inhibition. Polyethylene glycol, $HO(CH_2CH_2O)_nH$, is widely used as a precipitating agent in protein crystallography.[51,52] PEG lowers the solubility of proteins primarily by a volume-exclusion effect, more recently described as macromolecular crowding.[53,54] This same principle is involved in the enhancement of protein−protein and protein−nucleic acid association in the concentrated milieu of living cells, and PEG is used as an inert "crowding agent" to enhance these interactions *in vitro*.[53−55] Macromolecular crowding is an entropic effect, and it does not require any specific interactions between macromolecules. However, the effect of PEG on proteins is more complex. Other factors present include the water sorption by PEG[56] and weak attractive interactions of PEG with hydrophobic amino acid residues.[57]

Table 4. Effect of Polyethylene Glycol on Inhibition of PI-PLC

inhibitor	concn (mM)	activity remaining (%)[a] buffer[b,c]	PEG-600[c,d]
none		100 (±4)	100 (±5)
myo-Ins	40.0	18 (±0)	22 (±1)
myo-Ins(2)P	16.0	69 (±5)	56 (±3)
GlcN(α1→6)Ins (**28**)	0.8	71 (±0)	69 (±4)
14	0.17	14 (±1)	102 (±6)
29	0.25	25 (±5)	100[e]
29	0.5	ND	102[e]

[a] The assays contained 5 nM PI-PLC and 0.25 mM NPIP substrate and were performed as described in the Experimental Section. [b] Assays in the standard assay buffer (100 mM HEPES, 1 mM EDTA; pH 7) with no PEG present. [c] Values given are the mean (± average deviation) of at least two measurements from separate experiments. The data are normalized, so that the activity in the absence of inhibitor is 100% which corresponds to a turnover rate of about 40 μmol min^{-1} mg^{-1} in assay buffer and to about 90 μmol min^{-1} mg^{-1} in the 33% PEG solution. [d] Assays were in 90% precipitant solution, which contains 33% PEG-600, 0.16 M trisodium citrate−HCl, pH 7.35, and 46 mM HEPES, pH 7. This corresponds to 90% rather than 100% precipitant solution so as to avoid phase separation when inhibitor compound and substrate were added. [e] Single measurement at the given concentration. ND, not determined.

In contrast to the extensive literature on the effects of PEG on protein aggregation and crystallization, we were unable to locate any studies on the effect of PEG on enzyme inhibitors. For the present work, we selected PEG with an average molecular weight of 600, a relatively small polymer with an average *n* between 12.5 and 13.9,[58] because PEG-600 was used to grow crystals for the structure determination of *B. cereus* PI-PLC.[6,59]

For the purpose of measuring inhibition under crystallization conditions, a single concentration of inhibitor was added to reactions that simulate the conditions at which PI-PLC crystals were grown (i.e., close to 100% precipitant solution). The concentration of PEG present during the assays was 33% by volume. The results are summarized in Table 4. The control experiment (top row) shows that PEG has no inhibitory effect on the enzyme itself. Likewise, PEG has no effect on the inhibition of the enzymatic reaction by water-soluble molecules such as *myo*-inositol, *myo*-inositol 2 phosphate, or GlcN(α1→6)Ins. However, PEG causes a large *decrease* in the observed inhibition by the representative amphipathic inhibitors **14** and **29**, even though these molecules are present at concentrations that inhibit PI-PLC strongly in the absence of PEG (Tables 1, 2, and 4). This effect cannot be explained by the simple macromolecular crowding model. It can, however, be explained by the solubilizing action of PEG. For example, 20% PEG-400 is known to solubilize drugs and cosmetics that have low water solubility[60−62] and to interact with surfactants.[63,64] Thus, the PEG polymer molecules are excluding the protein and at the same time PEG is solubilizing, or increasing the aqueous solubility of, the short-chain amphipathic inhibitors. The net effect is that the inhibitor-binding equilibrium is shifted and less inhibitor is bound at the active site of the enzyme. This has important consequences in selecting a strategy for mapping the active site by means

of inhibitor−enzyme complexes. The molecules exhibiting the highest degree of inhibition in a conventional assay, i.e., the short-chain lipids, turn out to be the molecules most likely to be solubilized by PEG and fail in cocrystallization attempts. The water-soluble molecules representing the polar head group region of the substrate, although exhibiting only weak inhibition, are more likely to cocrystallize with the enzyme in an active site directed manner. This translates into a synthetic strategy for designing substrate analog inhibitors: instead of beginning with the lipid moiety of the substrate as the building block for inhibitors, a better strategy is to begin with the polar head group (i.e., *myo*-inositol) and then to synthetically add such residues as needed to mimic the essential features of the substrate or its transition state. This is the strategy we are pursuing because PEG is required for protein crystallization.[59] The first structure was of *B. cereus* PI-PLC complexed with *myo*-inositol.[6] The next logical step is to add glucosamine, since this increases the binding (Tables 3 and 4). Recently we have succeeded in cocrystallizing GlcN(α1→6)Ins with PI-PLC, which confirms this prediction.[65] Finally, the phosphate, and hydrophobic residues of minimum length will be added in order to complete the mapping of the active site. This strategy should be useful in studying other lipophilic enzymes where the presence of PEG is required for crystallization.

Conclusions

The synthesis of a series of amphiphilic PI-PLC substrate analogs with methyl phosphate, phosphate, methyl phosphonate, and phosphonate head groups was accomplished. The decreased steric bulk of the diradylglycerols precluded the use of standard methodologies for the synthesis of the intermediate phosphate triesters. A protocol that allowed for the efficient synthesis of short-chain dialkylglyceryl phosphates was developed. The inhibition of *B. cereus* PI-PLC by these short-chain lipids was measured below their cmc's in a single-phase aqueous assay using a water-soluble synthetic substrate. Inhibition was most strongly dependent on the hydrocarbon chain length of the inhibitors: among compounds with identical methyl phosphate head groups the potency of the inhibitor followed the order dioctyl > dihexyl > dipentyl. This trend is consistent with the structure of *B. cereus* PI-PLC and parallels observations for short-chain lipid substrates for mammalian PI-PLC.[46] Also supporting this trend are results showing ether-linked inhibitors are more potent than the corresponding ester-linked inhibitors. Together, these data stress the importance of hydrophobic interactions for inhibitor or substrate binding. The hydrophilic head group contributes to the inhibition, and the order of potency is *myo*-inositol phosphonate > methyl phosph(on)ate > phosph(on)ate. Consequently, the strongest inhibitor among the dihexyl compounds is the phosphonate most closely resembling the substrate, PI. In contrast, as a group, the water-soluble inositols and other molecules resembling the head group of PI exhibited a reduction in inhibition by two to three orders of magnitude. Surprisingly, the presence of PEG essentially abolished the inhibition by the short-chain lipids, but had little effect on the water-soluble inositol derivatives. Thus, when the presence of PEG is required, as in the crystallization of the *B. cereus* PI-PLC, the weakly inhibiting water-soluble derivatives are important. In the series examined, *myo*-inositol with an IC_{50} of 10 mM serves as a useful reference as it has been successfully cocrystallized with *B. cereus* PI-PLC. Adding a phosphate at the 1 or 2 position or the cyclic 1,2-phosphate decreases inhibition by a factor of about 5, whereas adding a glucosamine at the 6 position as in GlcN(α1→6)Ins increases inhibition by the same amount. This is consistent with the fact that the phosphotransferase activity is rapid, whereas the cyclic phosphodiesterase activity, which requires the binding of the water-soluble product Ins(1:2-cyc)P, proceeds very slowly. The increased inhibition observed for GlcN(α1→6)Ins is consistent with the GPI-cleaving activity of *B. cereus* PI-PLC, where the K_M of the GPI substrate < PI substrate.

Experimental Section

I. Synthetic Procedures. Melting points were obtained on a Thomas-Hoover apparatus and are uncorrected. Infrared spectra were recorded in KBr pellets (ca. 0.2%) or as thin films on NaCl plates on a Nicolet Magna-IR 550. ^{1}H and ^{13}C NMR spectra were taken on a General Electric QE-300 FT spectrometer, and ^{31}P NMR spectra were recorded on a Nicolet NT-360 spectrometer. All chemical shifts are reported in ppm relative to residual solvent peaks for ^{1}H and ^{13}C and 1% H_3PO_4 (external reference) for ^{31}P. The identity of all compounds were confirmed by their NMR and IR spectra as well as TLC R_f values. Analytical TLC utilized silica gel 60 F_{254} plates. Column chromatography was performed using Mallinckrodt silica gel, Davisil, grade 62 (60−200 mesh). Tetrahydrofuran (THF) was distilled under dry nitrogen from sodium benzophenone. Methylene chloride was distilled from calcium hydride. All reactions were carried out under a dry nitrogen atmosphere with anhydrous, freshly distilled solvents, unless otherwise stated.

(±)-3-*O*-Benzyl-1,2-di-*O*-pentyl-*sn*-glycerol. A suspension of (±)-1-(benzyloxy)propane-2,3-diol (**5**)[29] (10.3 g, 56.5 mmol), 1-bromopentane (34.1 g, 226 mmol), and powdered KOH (12.7 g, 226 mmol) in toluene (150 mL) was heated at reflux with a Dean−Stark drying tube attached for 24 h. The reaction mixture was cooled to 25 °C and washed with water (2 × 100 mL). The solvents were removed *in vacuo*, and the resulting residue was purified by column chromatography (9:1 hexanes/EtOAc) to afford (±)-3-*O*-benzyl-1,2-di-*O*-pentyl-*sn*-glycerol (17.4 g, 95%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 5 H), 4.56 (s, 2 H), 3.60−3.54 (m, 5 H), 3.43 (t, 4 H), 1.58 (m, 4 H), 1.31 (m, 8 H), 0.89 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 128.2, 127.5, 78.0, 73.3, 71.6, 70.8, 70.6, 70.4, 29.8, 29.4, 28.3, 22.5, 14.0.

(±)-3-*O*-Benzyl-1,2-di-*O*-hexyl-*sn*-glycerol. Prepared as described above, using 1-bromohexane, to afford (±)-3-*O*-benzyl-1,2-di-*O*-hexyl-*sn*-glycerol[29] as a colorless oil (91%): ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5 H), 4.57 (s, 2 H), 3.65−3.51 (m, 7 H), 3.44 (t, 2 H), 1.56 (m, 4 H), 1.30 (m, 12 H), 0.90 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 128.2, 127.5, 78.0, 73.3, 71.6, 70.8, 70.6, 70.4, 31.7, 30.1, 29.6, 25.8, 22.6, 14.0.

(±)-3-*O*-Benzyl-1,2-di-*O*-octyl-*sn*-glycerol. Prepared as described above, using 1-bromooctane, to afford (±)-3-*O*-benzyl-1,2-di-*O*-octyl-*sn*-glycerol[31] as a colorless oil (96%): ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5 H), 4.56 (s, 2 H), 3.68−3.35 (m, 9 H), 1.55 (m, 4 H), 1.27 (m, 20 H), 0.88 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 128.3, 127.5, 78.1, 73.3, 71.6, 70.8, 70.6, 70.3, 31.7, 30.2, 29.7, 29.4, 29.1, 26.0, 22.3, 13.7.

(±)-1,2-Di-*O*-pentyl-*sn*-glycerol (6). A suspension of (±)-3-*O*-benzyl-1,2-di-*O*-pentyl-*sn*-glycerol (13.8 g, 42.8 mmol) and 10% Pd/C (0.2 g) in MeOH (50 mL) was shaken under an H_2 atmosphere (50 psi) on a Parr hydrogenation apparatus for 2 h. The resulting suspension was filtered through a plug of Celite, and the solvents were removed *in vacuo* to afford **6** as a colorless oil (9.88 g, 99%): ^{1}H NMR ($CDCl_3$) δ 3.74−3.39 (m, 9 H), 2.36 (br s, 1 H), 1.58 (m, 4 H), 1.30 (m, 8 H), 0.89 (t, 6

H); ^{13}C NMR ($CDCl_3$) δ 78.6, 71.6, 70.7, 70.2, 62.6, 29.6, 29.2, 28.1, 22.3, 13.8. Anal. ($C_{13}H_{28}O_3$) C, H.

(±)-1,2-Di-*O*-hexyl-*sn*-glycerol (7). Prepared as described above for **6**, to afford **7**,[29] as a colorless oil (94%): ^{1}H NMR ($CDCl_3$) δ 3.69−3.36 (m, 9 H), 2.54 (br s, 1 H), 1.49 (m, 4 H), 1.29 (m, 12 H), 0.82 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 78.5, 71.7, 70.8, 70.3, 62.8, 31.7, 30.0, 29.5, 25.7, 22.5, 13.8.

(±)-1,2-Di-*O*-octyl-*sn*-glycerol (8). Prepared as described above for **6**, to afford **8**,[30] as a colorless oil (94%): ^{1}H NMR ($CDCl_3$) δ 3.61−3.31 (m, 9 H), 2.66 (br s, 1 H), 1.47 (m, 4 H), 1.20 (m, 20 H), 0.80 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 78.5, 71.6, 70.8, 70.3, 62.7, 31.7, 30.0, 29.6, 29.3, 29.1, 26.0, 22.5, 13.9.

(±)-Benzyl Methyl 1,2-Di-*O*-pentylglycer-3-yl Phosphate (9). Methyl dichlorophosphite (731 mg, 5.5 mmol) was added via syringe (ca. 3 min) to a rapidly stirred solution of *N*,*N*-diisopropylethylamine (1.94 g, 15 mmol) in CH_2Cl_2 (2.5 mL) at −78 °C. A solution of **6** (1.16 g, 5.0 mmol) in CH_2Cl_2 (25 mL) was then added dropwise over 45 min. After an additional 1 h at this temperature, a solution of benzyl alcohol (649 mg, 6.0 mmol) was added dropwise over 20 min. The resulting mixture was stirred at −78 °C for 1 h and then allowed to warm to 25 °C for 1 h. The solvents were removed *in vacuo*, and the residue obtained was suspended in EtOAc (10 mL). The resulting suspension was filtered through a plug of Celite, and the solids obtained were washed with EtOAc (2 × 5 mL). The combined filtrates were concentrated, and the residue was suspended in CH_2Cl_2 (10 mL) and cooled under N_2 to 0 °C. A 3 M solution of *t*-BuOOH in isooctane (1.5 mL) was then added dropwise over 5 min. The reaction mixture was then removed from the cooling bath and allowed to warm to room temperature over 2 h. The reaction was filtered through a pad of silica gel, and the filter was washed with hexanes/EtOAc (100 mL, 4:1). The combined filtrates were concentrated *in vacuo* to a yellow oil. This residue was purified by column chromatography (3:2 hexanes/EtOAc) to give **9** as a colorless oil (972 mg, 48%): ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 5 H), 5.05 (d, 2 H, J_{HP} 7.8 Hz), 4.20−3.99 (m, 2 H), 3.70 (d, 3 H, J_{HP} 11 Hz), 3.68−3.37 (m, 7 H), 1.52 (m, 4 H), 1.28 (m, 8 H), 0.85 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 135.8, 128.2, 126.7, 77.2 (m, obscured by solvent peak), 71.6, 70.6, 70.3, 69.2, 69.1, 67.1 (d, J_{CP} 11 Hz), 64.8, 54.1 (d, J_{CP} 12 Hz), 29.6, 29.2, 29.1, 28.2, 28.1, 22.4, 13.9; ^{31}P NMR ($CDCl_3$) δ 0.18 (m).

(±)-Benzyl Methyl 1,2-Di-*O*-hexylglycer-3-yl Phosphate (10). Prepared as described above for **9**. The crude residue obtained was purified by RPTLC (3:2 hexanes/EtOAc) to give **10** (541 mg, 64%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 5 H), 5.05 (d, 2 H, J_{HP} 7.8 Hz), 4.20−3.99 (m, 2 H), 3.70 (d, 3 H, J_{HP} 11 Hz), 3.68−3.37 (m, 7 H), 1.52 (m, 4 H), 1.28 (m, 12 H), 0.85 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 135.9, 128.5, 127.8, 77.2 (d, J_{CP} 7.0 Hz), 71.7, 70.6, 69.7, 69.2, 69.1, 67.1 (d, J_{CP} 5.0 Hz), 54.1 (m), 31.6, 29.9, 29.3, 25.7, 25.6, 22.5, 13.9; ^{31}P NMR ($CDCl_3$) δ 0.58 (br m), along with methyl bis(1,2-di-*O*-hexylglyceryl-3-yl) phosphate (177 mg, 31.2%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 4.19−4.01 (m, 4 H), 3.76 (d, 3 H, J_{HP} 11 Hz), 3.83−3.38 (m, 14 H), 1.57 (m, 8 H), 1.29 (m, 24 H), 0.83 (m, 12 H).

(±)-Benzyl Methyl 1,2-Di-*O*-octylglycer-3-yl Phosphate (11). Prepared as described above for **9**. The residue was purified by column chromatography (3:2 hexanes/EtOAc) to give **11** as a colorless oil (555 mg, 68%): ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 5 H), 5.06 (d, 2 H, J_{HP} 8.0 Hz), 4.20−3.95 (m, 2 H), 3.71 (d, 3 H, J_{HP} 11 Hz), 3.60−3.30 (m, 7 H), 1.52 (m, 4 H), 1.24 (m, 20 H), 0.86 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 135.9, 128.5, 127.8, 77.2 (m), 71.7, 70.6, 69.6, 69.2, 67.0 (m), 54.1 (m), 31.8, 30.0, 29.6, 29.4, 29.2, 26.0, 22.6, 14.0; ^{31}P NMR ($CDCl_3$) δ 0.23 (br m).

(±)-Methyl 1,2-Di-*O*-pentylglycer-3-yl Phosphate, Sodium Salt of 12. A suspension of **9** (560 mg, 1.38 mmol) and 20% $Pd(OH)_2$/C (150 mg) in MeOH (25 mL) was shaken on a Parr hydrogenation apparatus for 1.5 h under H_2 (50 psi). The resulting suspension was filtered through a plug of Celite, and the solvents were removed *in vacuo* to afford the crude free acid of **12** as a yellow oil. This crude residue was suspended in 2.5% aqueous $NaHCO_3$ (50 mL) and washed with CH_2Cl_2 (3 × 25 mL). The organic layers were discarded, and the aqueous solution was acidified to ca. pH = 1 with HCl and washed with CH_2Cl_2 (3 × 25 mL). The pooled organic extracts were concentrated *in vacuo* to afford the free acid of **12**. This residue was suspended in MeOH (5 mL) and stirred with ion exchange resin (BIO-REX 70, sodium form, 1.5 g). The suspension was filtered and stirred with a fresh sample of ion exchange resin. The suspension was filtered, and the solvents were removed *in vacuo* to afford the sodium salt of **12** (430 mg, 89%) as a clear colorless oil: ^{1}H NMR ($CDCl_3$) δ 3.85 (m, 2 H), 3.74−3.21 (m, 10 H), 1.54 (m, 4 H), 1.28 (m, 8 H), 0.87 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 77.9 (d, J_{CP} 4.7 Hz), 71.6, 70.5, 70.1, 64.9, 52.6 (d, J_{CP} 5.7 Hz), 29.6, 29.4, 28.3, 28.2, 22.5, 14.0; ^{31}P NMR ($CDCl_3$) δ −1.76 (m). Anal. ($C_{14}H_{30}O_6NaP$) C, H.

(±)-Methyl 1,2-Di-*O*-hexylglycer-3-yl Phosphate, Ammonium Salt of 13. The free acid of **13** was prepared as described above for the free acid of **12**. This colorless oil was purified by column chromatography ($CHCl_3$/MeOH/30% NH_4-OH, gradient) to afford the ammonium salt of **13** (301 mg, 52%), as a slightly yellow oil:[66] ^{1}H NMR ($CDCl_3$) δ 7.47 (br s, NH_4), 3.81 (m, 2 H), 3.72−3.34 (m, 10 H), 1.51 (m, 4 H), 1.24 (m, 12 H), 0.85 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 77.6 (d, J_{CP} 5.7 Hz), 71.3, 70.4, 70.1, 53.6 (m), 31.7, 29.9, 29.6, 25.7, 25.6, 22.6, 14.0; ^{31}P NMR ($CDCl_3$) δ −0.21 (m). Anal. ($C_{16}H_{38}O_6NP\cdot H_2O$) C, H.

(±)-Methyl 1,2-Di-*O*-octylglycer-3-yl Phosphate, Ammonium Salt of 14. The free acid of **14** was prepared as described above for the free acid of **12**. This colorless oil was purified by column chromatography (89:10:1 $CHCl_3$/MeOH/30% NH_4OH) to afford the ammonium salt of **14** (526 mg, 28%) as a colorless oil: ^{1}H NMR (D_2O) δ 3.80 (m, 2 H), 3.72−3.39 (m, 10 H), 1.55 (m, 4 H), 1.27 (m, 20 H), 0.85 (m, 6 H); ^{13}C NMR (D_2O) δ 78.3 (m), 71.7, 71.1, 70.6, 65.0, 53.0 (m), 32.2, 30.1, 29.9, 29.6, 26.4, 26.3, 22.8, 14.0; ^{31}P NMR (D_2O) δ 0.23 (m). Anal. ($C_{20}H_{46}O_6NP\cdot 0.5H_2O$) C, H.

(±)-Benzyl Methyl (2,2-Dimethyl-1,3-dioxolan-4-yl)-methyl Phosphate (15). Prepared as described above for **9**. The residue was purified by column chromatography (1:1 hexanes/EtOAc) to afford **15** (3.05 g, 48%) as a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5 H), 5.05 (d, 2 H, J_{HP} 8.4 Hz), 4.22 (m, 1 H), 3.96 (m, 3 H), 3.68 (d, 3 H, J_{HP} 11 Hz), 3.74 (m, 1 H), 1.37 (s, 3 H), 1.30 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 135.7, 128.5, 127.8, 109.7, 73.9 (d, J_{CP} 7.6 Hz), 69.4, 69.3, 67.4 (d, J_{CP} 5.8 Hz), 65.9, 54.2 (d, J_{CP} 5.6 Hz), 26.6, 25.9, 25.1; ^{31}P NMR ($CDCl_3$) δ 0.48 (m).

(±)-Benzyl Methyl Glycer-3-yl Phosphate (16). A suspension of **15** (1.49 g, 4.71 mmol) and ion exchange resin (Dowex 50W-X8, acid form, 1.25 g) was stirred at room temperature for 3.5 h under N_2. The suspension was filtered, and the solids obtained were washed with CH_2Cl_2 (2 × 5 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by column chromatography (9:1 EtOAc/MeOH) to afford **16** (693 mg, 53%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 5 H), 5.09 (d, 2 H, J_{HP} 13 Hz), 4.08 (m, 2 H), 3.87 (m, 1 H), 3.73 (d, 3 H, J_{HP} 11 Hz), 3.64 (m, 2 H), 3.00 (br s, 2 H).

(±)-Benzyl Methyl 1,2-Di-*O*-hexanoylglycer-3-yl Phosphate (17). Hexanoyl chloride (1.17 g, 6.86 mmol) was added dropwise to a rapidly stirred solution of **16** (600 mg, 2.17 mmol) and *N*,*N*-diisopropylethylamine (1.12 g, 8.68 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was allowed to warm to 25 °C over 14 h. The solution was flushed through a short column of silica gel (1:1 hexanes/EtOAc), and the solvents were removed *in vacuo*. The residue obtained was purified by column chromatography (3:1 hexanes/EtOAc) to afford **17** (833 mg, 81%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.37 (m, 5 H), 5.19 (t, 1 H), 5.07 (d, 2 H, J_{HP} 8.4 Hz), 4.27 (dd, 1 H), 4.13 (m, 3 H), 3.72 (d, 3 H, J_{HP} 11 Hz), 2.29 (t, 4 H), 1.60 (m, 4 H), 1.29 (m, 8 H), 0.88 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 173.0, 172.6, 135.7, 128.5, 127.8, 69.4 (m), 65.4 (d, J_{CP} 4.7 Hz), 61.5, 54.3 (m), 34.0, 33.9, 31.1, 31.0, 24.2, 22.1, 13.7; ^{31}P NMR ($CDCl_3$) δ 0.32 (m).

(±)-Methyl 1,2-Di-*O*-hexanoylglycer-3-yl Phosphate, Sodium Salt of 18. The free acid of **18** was prepared as described above for the free acid of **12**. This residue was stirred with ion exchange resin (Dowex 50W-X8, sodium form, 1.0 g) for 18 h and filtered. The solvents were removed *in vacuo* to afford a yellow oil. This oil was suspended in hexanes (50 mL) and filtered through a pad of Celite. The solvents

were removed *in vacuo*, and the residue was suspended in water (10 mL) and filtered through a pad of Celite. The resulting soapy solution was lyophilized to afford the sodium salt of **18** (468 mg, 100%) as a colorless leather:[66] ^{1}H NMR ($CDCl_3$) δ 5.32 (m, 1 H) 4.45 (dd, 1 H), 4.30 (m, 2 H), 4.02 (m, 2 H), 3.60 (d, 3 H, J_{HP} 10 Hz), 2.36 (m, 4 H), 1.63 (m, 4 H), 1.33 (m, 8 H), 0.91 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 173.5, 173.4, 70.8 (d, J_{CP} 7.7 Hz), 63.4, 62.7, 52.6 (d, J_{CP} 5.8 Hz), 34.2, 34.0, 31.2, 24.4, 22.2, 13.8; ^{31}P NMR ($CDCl_3$) δ 1.38 (m). Anal. ($C_{16}H_{30}O_8NaP$) C, H.

(±)-Dibenzyl 1,2-Di-*O*-hexylglycer-3-yl Phosphate (19). To a rapidly stirred suspension of $POCl_3$ (3.45 g, 22.5 mmol) and triethylamine (2.28 g, 22.5 mmol) in anhydrous ether (15 mL) at −15 °C was added a solution of **7** (3.26 g, 12.5 mmol) in anhydrous ether (5 mL) over 15 min. The reaction was stirred at this temperature for 1 h and filtered under N_2. The solids obtained were washed with toluene (25 mL), and the pooled filtrates were concentrated *in vacuo* at <40 °C. The resulting oil was suspended in anhydrous ether (40 mL), and triethylamine (8.34 g, 82.4 mmol) was added. The resulting mixture was cooled to 0 °C, and benzyl alcohol (8.93 g, 82.4 mmol) was added. The reaction was stirred at this temperature for 1.5 h, allowed to warm to 25 °C for 2 h and filtered, and the filtrates were concentrated to a red oil. This crude residue was purified by column chromatography (7:3 hexanes/EtOAc) to give **19** (5.63 g, 87%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 1 H), 5.05 (m, 4 H), 4.16−3.98 (m, 2 H), 3.58−3.48 (m, 3 H), 3.41−3.37 (m, 4 H), 1.52 (m, 4 H), 1.27 (m, 12 H), 0.89 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 135.9, 128.5, 128.4, 127.8, 71.7, 70.7, 69.7, 69.2, 69.1, 67.1 (d, J_{CP} 6.0 Hz), 31.6, 29.9, 29.6, 25.7, 22.6, 14.0; ^{31}P NMR ($CDCl_3$) δ −3.26 (m).

(±)-1,2-Di-*O*-hexylglycer-3-yl Phosphate, Free Acid of 20. The free acid of **20** was prepared as described above for **12**. The residue obtained (329 mg, 98%) was analytically pure without further purification: ^{1}H NMR ($CDCl_3$) δ 9.35 (br s, 2 H) 4.10 (m, 2 H), 3.70−3.55 (m, 3 H), 3.49−3.40 (m, 4 H), 1.56 (m, 4 H), 1.28 (m, 12 H), 0.88 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 72.1, 71.4, 69.8, 66.2, 31.6, 29.6, 29.4, 25.6, 25.5, 22.5, 14.0; ^{31}P NMR ($CDCl_3$) δ −0.97 (m). Anal. ($C_{15}H_{28}O_6P\cdot H_2O$) C, H.

(±)-Dibenzyl (2,2-Dimethyl-1,3-dioxolan-4-yl)methyl Phosphate (21). Prepared as described above for **19**. The residue was purified by column chromatography (3:2 hexanes/EtOAc) to afford **21** (8.72 g, 89%): ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 10 H), 5.05 (d, 4 H, J_{HP} 8.4 Hz), 4.21 (m, 1 H), 3.99−3.89 (m, 2 H), 3.80−3.70 (m, 2 H), 1.38 (s, 3 H), 1.33 (s, 3 H).

(±)-Dibenzyl Glycer-3-yl Phosphate (22). Prepared as described above for **16**. The crude residue obtained was purified by column chromatography (EtOAc) to afford **22** (1.83 g, 70.2%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 10 H), 5.06 (d, 4 H, J_{HP} 8.7 Hz), 4.03 (dd, 2 H), 3.82 (m, 1 H), 3.64−3.53 (m, 2 H), 2.75−2.60 (br s, 2 H).

(±)-Dibenzyl 1,2-Di-*O*-hexanoylglycer-3-yl Phosphate (23). Prepared as described for **17**. The crude residue obtained was purified by column chromatography (9:1 hexanes/EtOAc) to afford **23** (1.83 g, 83%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 10 H), 5.16 (m, 1 H), 5.07 (d, 4 H, J_{HP} 8.1 Hz), 4.27 (m, 1 H), 4.14 (m, 3 H), 2.30 (t, 4 H), 1.61 (m, 4 H), 1.31 (m, 8 H), 0.95 (t, 6 H); ^{13}C NMR ($CDCl_3$) δ 173.1, 172.8, 138.7, 128.7, 128.6, 127.9, 69.5 (m), 65.1, 61.6, 34.0, 33.9, 31.2, 31.0, 24.5, 22.2, 13.8; ^{31}P NMR ($CDCl_3$) δ −2.22 (m).

(±)-1,2-Di-*O*-hexanoylglycer-3-yl Phosphate, Free Acid of 24. The free acid of **24** was prepared as described above for **12**. The residue obtained (428 mg, 100%) was analytically pure without further purification: ^{1}H NMR ($CDCl_3$) δ 5.24 (m, 1 H), 4.37−4.10 (m, 5 H), 2.17 (m, 4 H), 1.59 (m, 4 H), 1.30 (m, 8 H), 0.86 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 174.0, 173.7, 69.7 (d, J_{CP} 13 Hz), 65.0, 64.9, 62.1, 34.1, 33.9, 31.2, 31.1, 22.4, 22.2, 13.8; ^{31}P NMR ($CDCl_3$) δ −2.37 (m).

Methyl [3,4-Bis(hexyloxy)butyl]phosphonate, Sodium Salt of 26. A suspension of **25**[25] (700 mg, 1.9 mmol) and NaI (2.0 g) in 5 mL of MEK was heated at reflux under N_2 for 9 h. The resulting mixture was poured into water (50 mL), and the resulting milky suspension was washed with CH_2Cl_2 (3 × 25 mL). The organic layers were discarded, and the aqueous suspension was acidified (HCl) to ca. pH = 1 and washed with CH_2Cl_2 (3 × 25 mL). The pooled organic phases were concentrated *in vacuo*, and the resulting residue was suspended in 1% $NaHCO_3$ (100 mL). The resulting milky suspension was washed with CH_2Cl_2 (3 × 25 mL). The organic layers were discarded, and the aqueous suspension was acidified (HCl) to ca. pH = 1 and washed with CH_2Cl_2 (3 × 25 mL). The pooled organic phases were concentrated *in vacuo*, and the resulting residue was suspended in water (10 mL), stirred with ion exchange resin (Dowex 50W-X8, sodium form, 2.0 g) for 2 h, and filtered. The filtrates were lyophilized to afford **26** (134 mg, 19%) as a white waxy residue: ^{1}H NMR (D_2O) δ 3.74−3.37 (m, 10 H), 1.87−1.64 (m, 8 H), 1.36 (m, 12 H), 0.86 (m, 6 H); ^{13}C NMR (D_2O) δ 179.5, 79.3, 73.3, 70.8 (d, J_{CP} 79 Hz), 51.3 (m), 31.6, 29.6 (d, J_{CP} 17 Hz), 26.7, 25.6, 25.4, 22.6, 13.8; ^{31}P NMR (D_2O) δ 28.9 (m). Anal. ($C_{17}H_{35}O_5NaP$) C, H.

II. Physical Properties. Determination of the cmc. The cmc of synthetic amphiphilic compounds was determined by recording the solute concentration dependent change in surface tension. The surface tension was measured by capillary rise[67] adapted to small volumes as described by Rebecchi and co-workers.[46] A glass cup (i.d. 21 mm) filled with at least 0.5 mL of the solution to be measured was placed onto a vertically adjustable table. The cup was raised to immerse the tip of a 10 μL glass capillary (Gold Seal glass capillaries, Clay Adams). The capillary had been clamped to a clear block of acrylic held by an adjustable arm and positioned perpendicular to the surface of the liquid. Latex tubing fitted to the distal end of the capillary served to apply suction as well as pressure to the liquid column risen in the capillary, allowing the inner wall of the capillary to be wetted before measuring falling and rising menisci. A cathetometer (Eberbach Corp.) was used to measure the distance between the surface of the solution and the meniscus of the liquid column in the capillary.

In a series of surface tension measurements, the highest concentration of solute was measured first, and subsequent dilutions were made directly in the glass cup. The height of the capillary rise resulting from at least one rising and one falling meniscus were recorded for each dilution after allowing the meniscus to stabilize for 10−20 min. A fresh capillary was used to measure each dilution.

Assuming a 0° contact angle between solution and capillary wall, the surface tension γ (mN m^{-1}) was calculated using the equation $\gamma = (1/2)hrpg$, where h (cm) is the distance between the surface of the solution in the cup and the meniscus of the solution in the capillary with the internal radius r (cm), p (g cm^{-3}) is the density of the solution, and g (cm s^{-2}) is the acceleration due to gravity. A graph of γ versus ln(solute concentration) gave the estimated cmc value as that concentration of solute above which the slope of the line fitted to the measurements is changed significantly.

Using this procedure, the cmc values determined for Triton X-100 (Boehringer, Mannheim) and SDS (BDH Chemicals Ltd.), 1.1 and 9.0 mM, were close to those reported in the literature,[68] 0.2−0.9 and 7−10 mM, respectively, and were higher than those we found using the du Nouy ring detachment method (Surface Tensiomat Model 21, Fisher), 0.3 and 5.0 mM, respectively.

For compounds **20**, **24**, and **31**, there was no clear transition in the slope of the curve of γ as a function of the concentration due to insufficient data at high concentrations. The estimated cmc's listed in Table 1 were obtained by placing γ = 25.5−28.5 mN m^{-1} as the surface tension for solute concentrations at the cmc that we have observed for other compounds listed in Tables 1 and 2 (see also results by Bian and Roberts[44] and Garigapati et al.,[45] obtained for similar compounds).

III. Biochemical Assays. Enzyme. The PI-PLC from *B. cereus* was isolated from recombinant *E. coli* over-expressing the cloned enzyme as described[69] but modifying the procedure as follows: the recombinant enzyme was released from the periplasmic space of the bacterial cells by treatment with lysozyme in combination with mild osmotic shock using the protocol given by Witholt et al.[70] The purified enzyme was stored at −20 °C in 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.01% NaN_3, and 50% glycerol. (Essentially the same enzyme preparation is now available from Molecular Probes Inc., Eugene, OR.) Enzyme dilutions were prepared in 0.1% PEG-8000 (Sigma), 6 mM HEPES, 0.06 mM EDTA, pH 7. HEPES,

free acid, was from Calbiochem and Tris base from Life Technologies.

Substrate. The chromogenic substrate, racemic *myo*-inositol 1-(4-nitrophenyl phosphate) (NPIP) was prepared as reported and used in all kinetic analyses.[26] Substrate concentrations given refer to the D-enantiomer only. The L-enantiomer was shown to be neither a substrate nor an inhibitor for PI-PLC.[13] The kinetic parameters for the hydrolysis of D-NPIP by *B. cereus* PI-PLC are $K_M = 5$ mM and $V_{max} = 650$ μmol min^{-1} mg^{-1}.[13]

Inhibitors. The short-chain phospholipids of Tables 1 and 2 and GlcN(α1→6)Ins (**28**) (Table 3) were synthesized as described above. All inositols, inososes, and inositol phosphates were commercial products obtained from Sigma Chemical Co. All compounds evaluated for their inhibitory properties were prepared in 1.25× assay buffer (125 mM HEPES, 1.25 mM EDTA; pH 7). These stock solutions were sonicated and stored at −20 °C. Dilutions of the stock solutions were prepared directly before use in the same buffer with 0.1% PEG-8000 added.

Enzyme Assays. The rate of substrate hydrolysis by PI-PLC was measured in 0.5 mL reactions thermostated at 25 °C. All reactions contained final concentrations of 5 nM PI-PLC and 0.25 mM NPIP substrate. Duplicate control reactions were included in each set of inhibition assays. In a disposable methacrylate semi-microcuvette 360 μL of 1.25× assay buffer (125 mM HEPES, 1.25 mM EDTA; pH 7), 40 μL of 0.1% PEG-8000 in 1.25× assay buffer and H_2O to 492.5 μL were combined. A few minutes before the reaction was started, 2.5 μL of 50 mM NPIP was added and mixed. The reaction was initiated by adding 5 μL of diluted enzyme and mixing immediately by rapid inversion. Reactions containing inhibitor were combined in the same fashion except inhibitor was added a few minutes prior to the addition of NPIP and enzyme. Of inhibitor stock solutions in 1.25× assay buffer up to 360 μL could be added or up to 40 μL of diluted inhibitor in 0.1% PEG-8000, 1.25× assay buffer. In each case, the 0.5 mL reactions contained final concentrations of 1× assay buffer and 0.008% PEG-8000.

When PI-PLC activity was measured under conditions routinely used to grow PI-PLC crystals, the reactions contained 450 μL of 100% precipitant solution (35.6% PEG-600 (Sigma), 0.18 M trisodium citrate−HCl, pH 7.35, and 40 mM HEPES, pH 7), 40 μL of 1.25× assay buffer with or without inhibitor, H_2O to 492.5 μL, 2.5 μL of 50 mM NPIP, and 5 μL of diluted enzyme. The reactions were combined as described above with the enzyme added last.

The progress of NPIP hydrolysis was followed by monitoring the accumulation of the *p*-nitrophenolate anion hydrolysis product in a spectrophotometer at 399 nm for 4 min, commencing within 15 s after addition of enzyme. The molar extinction coefficient of the *p*-nitrophenolate anion was determined for the different reaction conditions used. To obtain the initial velocity of the reaction, v_0, the progress curve was fitted by nonlinear regression to a first-order rate equation using GraFit Vs. 3.0 software.[71] To determine the IC_{50} of an inhibitor, v_0 values were obtained for uninhibited reactions and in the presence of at least five different concentrations of inhibitor. The plot of v_0 against inhibitor concentration was fitted by nonlinear regression to yield the concentration of inhibitor where $v_0 = 50\%$ of the uninhibited rate. When it was not possible to achieve inhibitor concentrations high enough to result in a 50% reduction of the initial rate, IC_{50} values were extrapolated from available data points if the trend of the curve was clearly defined.

The K_i was determined by measuring initial rates in the presence of varying concentrations of substrate without inhibitor added and in the presence of two different concentrations of inhibitor. Rate is monitored versus the concentration of substrate and inhibitor and the data fitted to a multidimensional equation for competitive inhibition using GraFit.[71] The K_i obtained in this fashion compared well with results obtained from linearized data in Lineweaver−Burke plots.

Acknowledgment. This work was supported by NIH Grants GM 25698 and GM 27137.

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JM960434Y