Design, Synthesis, and Evaluation of Phospholipid Analogues as Inhibitors of the Bacterial Phospholipase C from *Bacillus cereus*

Stephen F. Martin,* Yue-Ling Wong, and Allan S. Wagman

Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas 78712

Received January 3, 1994 (Revised Manuscript Received May 26, 1994®)

Enzymes belonging to the phospholipase C (PLC) family hydrolyze the phosphodiester bond of phospholipids to give a diacylglycerol and a phosphorylated head group. The bacterial phospholipase C from *Bacillus cereus* (PLC_{Bc}) has been studied extensively, and there is a wealth of information regarding those structural features that are important for substrate activity. In contrast, there is virtually no data available regarding structure–activity relationships for inhibitors of this enzyme. To address this shortcoming, a series of optically pure analogues of 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (**2**) containing different replacements of the phosphate group were first synthesized including the phosphoramidates **4** and **8**, the phosphonate **5**, the (difluoromethylene)phosphonate **6**, the thiophosphate **7**, the diastereomeric phosphorothioates **9** and **10**, and the phosphorodithioate **11**. Each of these phosphatidylcholine derivatives was tested for inhibitor or substrate activity with PLC_{Bc} using the water-soluble phosphatidylcholine **2** as the monomeric substrate. The measurements were conducted below the critical micellar concentrations of both **2** and the inhibitor. Of the analogues, only **7** and **9** underwent observable enzymatic hydrolysis under the assay conditions used. The k_{cat} of the (S_P)-phosphorothioate **9** was approximately one-fifth that of **2**, and when compared to **2**, **7** was hydrolyzed only very slowly by the enzyme. Kinetic studies indicated that the phospholipid analogues tested were competitive inhibitors with increasing K_i's as follows: **7** ≈ **11** ≈ **10** < **4** ≈ **8** < **5** ≈ **6**.

Introduction

The phospholipase C (PLC) class of enzymes has attracted intense interest in the biological and chemical communities because of the central role these enzymes play in the regulatory functions related to agonist–receptor interaction and transmembrane signaling.[1-4] In the first stage of this process, agonists including hormones, neurotransmitters, and some growth factors bind to a G-protein linked receptor in the membrane to initiate a cascade that leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a phosphatidylinositol-specific PLC (PI-PLC) and consequent release of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The DAG then activates protein kinase C (PKC), which is important in regulating cell growth and transformation,[5] and IP_3, which promotes the release of intracellular calcium from the endoplasmic reticulum. Although the breakdown of PIP_2 by PI-PLC generates the DAG that initially activates PKC, the sustained activation of PKC that is required for a long term physiological response is thought to be provided by the DAG formed upon hydrolysis of the more abundant phosphatidylcholine (PC). Recent reports have indicated that hydrolysis of phosphatidylcholine by both a phosphatidylcholine-specific PLC (PC-PLC) and phospholipase D (PLD) may contribute to the production of the second phase of DAG in the stimulated cell. The hydrolysis of PC by PC-PLC provides DAG directly, whereas hydrolysis by PLD provides phosphatidic acid that is further processed by phosphatidic acid phosphohydrolase to yield DAG.

Recent experiments suggest that the PLC from *Bacillus cereus* (PLC_{Bc}) may be a useful model for mammalian PC-PLCs.[6-8] This bacterial PLC has been well characterized,[9] and its high-resolution (1.5 Å) X-ray structure has recently been disclosed.[10] Owing to these considerations coupled with the ready availability of PLC_{Bc}, we embarked upon an investigation to elucidate the mechanism of action of this enzyme to gain insights into the mechanistic details of the hydrolysis of the phosphodiester bond by enzymes of the PLC family. The first phase of this undertaking necessarily involved the design, preparation, and biological assay of a series of phosphati-

® Abstract published in *Advance ACS Abstracts*, July 1, 1994.

(1) For reviews of signal transduction involving degradation of phospholipids, see: (a) Berridge, M. J. *Scientific American* **1985**, *253*, 142. (b) Nishizuka, Y. *Science* **1986**, *233*, 305. (c) Nishizuka, Y. *Nature* **1988**, *334*, 661. (d) Cockroft, S. *Biochim. Biophys. Acta* **1992**, *1113*, 135. (e) Liscovitch, M. *Trends Biochem. Sci.* **1992**, *17*, 393. (f) Nishizuka, Y. *Science* **1992**, *258*, 607.

(2) For reviews of the roles of DAG and IP_3 as second messengers, see: (a) Berridge, M. J. *Biochem. J.* **1984**, *220*, 345. (b) Rana, R. S.; Hokin, L. E. *Physiol. Rev.* **1990**, *70*, 115.

(3) (a) Rhee, S. G.; Suh, P.-G.; Ryu, S.-H.; Lee, S. Y. *Science* **1989**, *244*, 546. (b) Rhee, S. G.; Choi, K. D. *J. Biol. Chem.* **1992**, *267*, 12393.

(4) (a) Pelech, S. L.; Vance, D. E. *Trends Biochem. Sci.* **1989**, *14*, 28. (b) Billah, M. M.; Anthes, J. C. *Biochem. J.* **1990**, *269*, 281. (c) Exton, J. H. *J. Biol. Chem.* **1990**, *265*, 1.

(5) (a) Larroders, P.; Cornet, M. E.; Diaz-Meco, M. T.; Lopez-Barahona, M.; Diaz-Laviada, I.; Guddal, P. H.; Johnsen, T.; Moscat, J. *Cell* **1990**, *61*, 1113. (b) Garcia de Herreros, A.; Dominguez, I.; Diaz-Meco, M. T.; Graziani, G.; Cornet, M. E.; Guddal, P. H.; Johnsen, T.; Moscat, J. *J. Biol. Chem.* **1991**, *266*, 6825.

(6) Levine, L.; Xiao, D. M.; Little, C. *Prostaglandins* **1988**, *34*, 633.

(7) Diaz-Meco, M. T.; Dominguez, I.; Sanz, L.; Municio, M. M.; Berra, E.; Cornet, M. E.; Garcia de Herreros, A.; Johansen, T.; Moscat, J. *Mol. Cell Biol.* **1992**, *12*, 302.

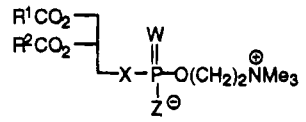
(8) Clark, M. A.; Shorr, R. G. L.; Bomalski, J. S. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 114.

(9) For some leading references, see: (a) Little, C. *Acta Chem. Scand.* **1977**, *B31*, 267. (b) Little, C. *Methods Enzymol.* **1981**, *71*, 725. (c) Orr, G. A.; Brewer, C. F.; Henney, G. *Biochemistry* **1982**, *22*, 3202. (d) Bruzik, K.; Jiang, R.-T.; Tsai, M.-D. *Biochemistry* **1983**, *22*, 2478. (e) Jiang, R.-T.; Shyy, Y.-J.; Tsai, M.-D. *Biochemistry* **1984**, *23*, 1661. (f) El-Sayed, M. Y.; DeBose, C. D.; Coury, L. A.; Roberts, M. F. *Biochim. Biophys. Acta* **1985**, *837*, 325. (g) Bicknell, R.; Hanson, G. R.; Holmquist, B.; Little, C. *Biochemistry* **1986**, *25*, 4219. (h) Gabriel, N. E.; Agman, N. V.; Roberts, M. F. *Biochemistry* **1987**, *26*, 7409. (i) Snyder, W. R. *J. Lipid Res.* **1987**, *28*, 949. (j) Johansen, T.; Holm, T.; Guddal, P. H.; Sletten, K.; Haugli, F. B.; Little, C. *Gene* **1988**, *65*, 293. (k) Lewis, K. A.; Bian, J.; Sweeney, A.; Roberts, M. F. *Biochemistry* **1990**, *29*, 9962. (l) Roberts, M. F. *Methods Enzymol.* **1991**, *197*, 95.

(10) Hough, E.; Hansen, L. K.; Birknes, B.; Jynge, K.; Hansen, S.; Hordvik, A.; Little, C.; Dodson, E.; Derewenda, Z. *Nature* **1989**, *338*, 357.

0022-3263/94/1959-4821$04.50/0

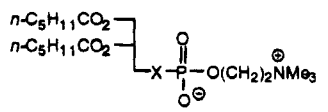
dylcholine analogues of the general structure **1** that would not undergo enzymatic hydrolysis and therefore serve as competitive inhibitors. Despite the abundance of data accumulated regarding the structural features that are important for substrate activity,[9] there are few reports of structure–activity relationships for inhibitors of bacterial PLC.[11] Once a series of inhibitory substrate analogues had been identified, X-ray crystallographic studies of selected enzyme–inhibitor complexes would be undertaken as a step toward identifying the active site amino acid residues and elucidating their roles in binding and catalysis.



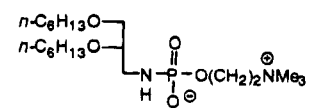
1: X = O, NR, S, CH_2, CF_2
W, Z = O, S combinations

Design of Inhibitors. Prior to designating what phospholipid analogues would be prepared as potential inhibitors for the enzyme kinetic studies, it was necessary to select the parent phospholipid that would serve as the substrate in the assays. This substrate should have a high critical micelle concentration (cmc), a comparatively low K_m, and a V_{max} to ensure that the rates of enzymatic processing of the substrate would be easily measurable in the presence of inhibitors. A high cmc is mandated because the conditions for Michaelis–Menten analysis require that the enzymatic reaction be conducted under homogeneous conditions. When the phospholipid concentration is above its critical micellar concentration (cmc), the enzyme kinetics are complicated by interfacial catalysis—a phenomenon that has been well characterized in elegant mechanistic studies of phospholipase A_2 (PLA_2).[12] Determining the precise nature of inhibition of enzymatic activity in a micellar system is problematic, since the inhibitor could form a mixed micelle with the substrate, thereby changing both the organization of the micelle and the interactions of the enzyme with the substrate. In such cases, enzyme inhibition would be nonspecific since it would not arise from the competitive binding of the inhibitor at the enzyme active site. Micellar substrates are hydrolyzed faster than monomolecularly dispersed ones,[9] but soluble phosphatidylcholine analogues are good substrates provided the fatty acid side chains contain at least six carbons each for symmetrical lecithins[9f] or a total of more than 12 for asymmetric ones.[9k] The desirability of a low K_m arises from the fact that the apparent K_m of a substrate increases in the presence of a competitive inhibitor, thereby giving the illusion that [S] is less than it actually is, and higher concentrations of the substrate are required. Thus, the quantity of substrate that must be synthesized to perform the enzyme assays decreases with a lower K_m. In consideration of these criteria, dihexanoylphosphatidylcholine (**2**), which has a cmc of 14 mM and a K_m of 0.36 mM,[9f] emerged as the substrate of choice.

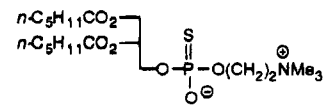
A series of analogues of **2**, each of which contained a different phosphate group replacement, was then designed to establish a pattern of structure–activity relationships and to identify those compounds that might be inhibitors of PLC_{Bc}. The general requirement for an inhibitor would be the presence of a phosphate group replacement in which the normal scissile P–O bond of the phosphodiester linkage is not hydrolytically labile. Toward this end, the analogues **3**–**7** in which the glyceryl oxygen is substituted with a group X (X = NH, NBn, S, CH_2, and CF_2) were identified. Since we were unable to prepare the phosphoramidate **3**, the dihexyl ether analogue **8** was selected as an alternative target. Other phosphate analogues include **9**–**11** in which the nonbridging oxygen atoms are replaced with sulfur atoms. To provide a brief overview of the area and to set the stage for future discussions, several introductory comments regarding these phospholipid analogues are warranted.



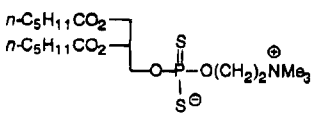
2: X = O
3: X = NH
4: X = NBn
5: X = CH_2
6: X = CF_2
7: X = S



8



9: S_P
10: R_P



11

Although phosphoramidate analogues of oligonucleotides may be substrates for phosphodiesterases,[13] the reactivity of bacterial PLC toward phospholipid-derived phosphoramidates such as **4** or **8** has not been examined. Phosphonates are well known to be hydrolytically stable, isosteric replacements for the phosphate group.[14] Indeed, racemic and achiral phosphonate derivatives related to **5** have been shown to inhibit the PLC from *Cl. perfringens*.[15] Difluorophosphonates have been recently suggested to be superior to phosphonates as replacements of the phosphate group because the CF_2 moiety is a better mimic of the polar character of the phosphate group.[16–18] The difluorophosphonate **6** was thus selected to explore the validity of this hypothesis in the context of inhibitors

(11) (a) Rosenthal, A. F.; Pousada, M. *Biochim. Biophys. Acta* **1968**, *164*, 226. (b) Rosenthal, A. F.; Chodsky, S. V. *Lipids* **1974**, *9*, 77. (c) Schwartz, P. W.; Tropp, B. E.; Engel, R. *Chem. Phys. Lipids* **1988**, *48*, 1.

(12) For some leading references, see: (a) Verheij, H. M.; Slotboom, A. J.; de Haas, G. H. *Rev. Physiol. Biochem.* **1981**, *91*, 91. (b) Jain, M. K.; Berg, O. G. *Biochim. Biophys. Acta* **1989**, *1002*, 127. (c) Jain, M. K.; Yuan, W.; Gelb, M. H. *Biochemistry* **1989**, *28*, 4135. (d) Scott, D. L.; White, S. P.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1541. (e) Jain, M. K.; Yu, B.-Z.; Rogers, J.; Gelb, M. H.; Tsai, M.-D.; Hendrickson, E. K.; Hendrickson, H. S. *Biochemistry* **1992**, *31*, 7841.

(13) Letsinger, R. L.; Mungall, W. S. *J. Org. Chem.* **1970**, *35*, 3800.

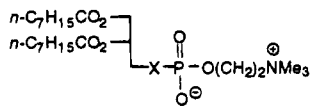
(14) For review, see: Engel, R. *Chem. Rev.* **1977**, *77*, 349.

(15) (a) Rosenthal, A. F.; Pousada, M. *Biochim. Biophys. Acta* **1968**, *164*, 226. (b) Rosenthal, A. F.; Chodsky, S. V. *Lipids* **1974**, *9*, 77.

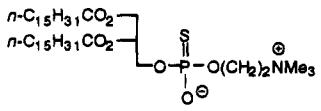
(16) (a) Blackburn, G. M. *Chem. Ind. (London)* **1981**, 134. (b) Blackburn, G. M.; Eckstein, F.; Kent, D. E.; Perree, T. D. *Nucleosides Nucleotides* **1985**, *4*, 165. (c) Blackburn, G. M.; Kent, D. E.; Kolkmann, F. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1119. (d) Blackburn, G. M.; Kent, D. E. *J. Chem. Soc., Perkin Trans. 1* **1986**, 913. (e) Thatcher, G. R. J.; Campbell, A. S. *J. Org. Chem.* **1993**, *58*, 2272.

(17) (a) Biller, S. A.; Forster, C.; Gordon, E. M.; Harrity, T.; Scott, W. A.; Ciosek, C. P., Jr. *J. Med. Chem.* **1988**, *31*, 1869. (b) Arabshahi, L.; Khan, N. N.; Butler, M.; Noonan, T.; Brown, N. C.; Wright, G. E. *Biochemistry* **1990**, *29*, 6820. (c) Kim C. U.; Luh, B. Y.; Misco, P. F.; Bronson, J. J.; Hitchcock, M. J. M.; Ghazzouli, I.; Martin, J. C. *J. Med. Chem.* **1990**, *33*, 1207.

of PLC_{Bc}. A number of phospholipid derivatives related to **7**, **9**, and **10** that contain sulfur atom replacements for the phosphate oxygens have been studied. For example, the dioctanoyl thiophosphate **12** (X = S) is a substrate for the extracellular PLC from both *B. cereus* and *Cl. perfringens*.[19] Although **12** (X = S) underwent hydrolysis 100–200 times slower than **12** (X = O) in separate assays, there was no reported comparison of the K_m and V_{max} of **12** (X = S) and **12** (X = O), and the possibility that **12** (X = S) might be a competitive inhibitor was not discussed. In an elegant series of experiments, Tsai demonstrated that the phosphodiester bond of the S_P isomer of **13** was selectively hydrolyzed by PLC_{Bc}.[20] However, the K_m and V_{max} of **13** (S_P) were not compared with the corresponding phosphate derivative, and whether the non-hydrolyzable diastereoisomer **13** (R_P) exhibited any inhibitory effects on PLC_{Bc} was not determined. Phosphorodithioate analogues of phospholipids such as **11** are virtually unknown, although oligonucleotide phosphorodithioates are resistant to nuclease degradation.



12: X = S, O

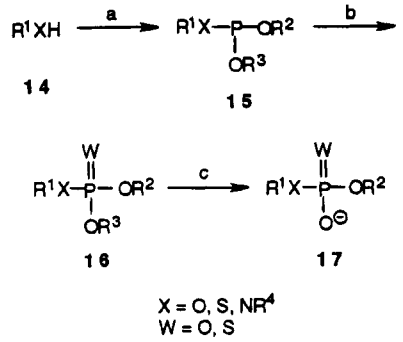


13: S_P and R_P

Results

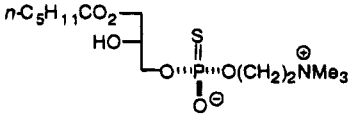
Synthesis of Phosphatidylcholine Analogues. When this study was initiated, a survey of the available methods for the synthesis of phospholipids and their analogues revealed that there were limitations inherent in many of the techniques, and the development of new procedures emerged as a prerequisite to the biological studies. To address this deficiency, we set to the task of examining the applicability of phosphite coupling procedures, which have been extensively exploited for the synthesis of oligonucleotides,[21] to the preparation of phospholipids.[22,23] We discovered that several alkyl dichlorophosphites could be effectively employed as reagents to couple diacylglycerol derivatives with a variety

Scheme 1[a]



[a] (a) R_3OPCl_2 (1 equiv); R_2OH (1 equiv); (b) [O] or S_8; (c) deprotect.

of different head groups to give members of four major classes of phospholipids and their analogues in excellent overall yields.[24] This general protocol, which is outlined in Scheme 1, was successfully applied to the syntheses of **2**, **7**, **8**, and a mixture of the two diastereoisomeric phosphorothioates **9** and **10**. A variant of this procedure was employed for the synthesis of **4**.[24b] Treatment of the mixture of **9** and **10** with bee venom phospholipase A_2 (PLA_2), which preferentially hydrolyzes the R_P isomer of diacyl phosphorothioate derivatives of phosphatidylcholine and phosphatidylethanolamine,[20] gave a separable mixture of the S_P diastereomer **9** and the *lyso* derivative **18**. Subsequent acylation of the *sn*-2 hydroxy group of **18** with *n*-hexanoyl chloride then regenerated the R_P isomer **10**.



18

We were concerned that the *N*-benzyl group in **4** might interfere with its binding at the enzyme active site owing to the proximity of the aromatic ring to the bond that would normally be cleaved by the enzyme; however, we were unable to prepare the *N*-H substituted analogue **3** using the protocol depicted in Scheme 1 or by catalytic *N*-debenzylation of **4**. To circumvent this problem, the dihexyl ether analogue **8** was selected as an alternate target. The primary amine **21** was first prepared from the known tosylate **19**[25] via the azide **20**. Subsequent phosphite coupling of **21** with 2-bromoethanol followed by oxidation gave the intermediate phosphate triester **22** that was converted directly into **8** upon treatment with trimethylamine (Scheme 2). The 1,2-dihexanoyl analogue of the diether **21** could not be made from **20** by modifying this route, because the *sn*-2 acyl group under-

(18) (a) Chambers, R. D.; Jaouhari, R.; O'Hagan, D. *Tetrahedron* **1989**, *45*, 5101. (b) Halazy, S.; Ehrhard, A.; Danzin, C. *J. Am. Chem. Soc.* **1991**, *113*, 315. (c) See also: Stremler, K. E.; Poulter, C. D. *J. Am. Chem. Soc.* **1987**, *109*, 5542 for a related example.

(19) (a) Riedy, G. P.; Snyder, W. R. *Arch. Biochem. Biophys.* **1987**, *258*, 504. (b) Snyder, W. R. *Biochim. Biophys. Acta* **1987**, *920*, 155. (c) Snyder, W. R. *Anal. Biochem.* **1987**, *164*, 199. For related work, see: (d) Young, P. R.; Snyder, W. R.; McMahon, R. F. *Biochem. J.* **1991**, *280*, 407.

(20) Bruzik, K.; Gupte, S. M.; Tsai, M.-D. *J. Am. Chem. Soc.* **1982**, *104*, 4682. See also refs 9d,e.

(21) For a review, see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223.

(22) For some leading reviews of phospholipid synthesis, see: (a) Eibl, H. In *Liposomes: From Physical Structure to Therapeutic Applications*; Knight, C. G., Ed.; Elsevier/North-Holland Biomedical: Amsterdam. 1981; p 19. (b) Ramirez, F.; Marecek, J. F. *Synthesis* **1985**, 449. (c) Stepanov, A. T. Shvets, V. I. *Chem. Phys. Lipids* **1986**, *46*, 1.

(23) For some other applications phosphite coupling techniques in phospholipid synthesis, see: (a) Bruzik, K. S.; Salamonczyk, G.; Stec, W. J. *J. Org. Chem.* **1986**, *51*, 2368. (b) Lamant, V.; Chap, H.; Klaébé, A.; Périé, J. J.; Willson, M. *J. Chem. Soc., Chem. Commun.* **1987**, 1608. (c) Elie, C. J. J.; Dreef, C. E.; Verduyn, R.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron* **1989**, *45*, 3477. (d) Salamonczyk, G. M.; Bruzik, K. S. *Tetrahedron Lett.* **1990**, *31*, 2015. (e) Garcia, M. L.; Pascual, J.; Andreu, J. A.; Fos, E.; Mauleón, D.; Carganico, G.; Arcamone, F. *Tetrahedron* **1991**, *47*, 10023. (f) Kazi, A. B.; Hajdu, J. *Tetrahedron Lett.* **1992**, *33*, 2291. (g) Seitz, S. P.; Kaltenbach, R. F., III; Vreekamp, R. H.; Calabrese, J. C.; Perrella, F. W. *BioMed. Chem. Lett.* **1992**, *2*, 171. (h) McGuigan, C.; Anson, M. S.; Swords, B. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2075.

(24) (a) Martin, S. F.; Josey, J. A. *Tetrahedron Lett.* **1988**, *29*, 3631. (b) Martin, S. F.; Josey, J. A.; Wong, Y.-L.; Dean, D. W. *J. Org. Chem.*, previous paper in this issue.

(25) Baer, E.; Fischer, H. O. L. *J. Chem. Soc.* **1948**, *70*, 609.

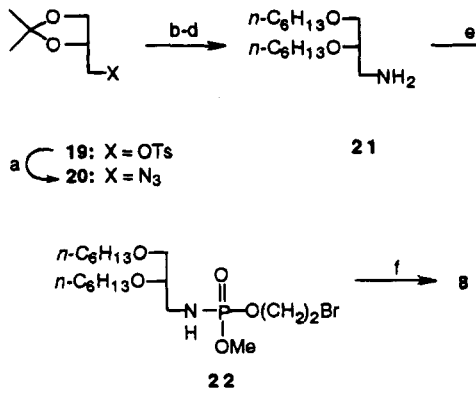
(26) (a) Tang, T.-C.; Tropp, B. E.; Engel, R.; Rosenthal, A. F. *Chem. Phys. Lipids* **1976**, *17*, 169. (b) Braksmayer, D.; Tropp, B. E.; Engel, R. *Chem. Phys. Lipids* **1977**, *19*, 93. (c) Schwartz, P. W.; Tropp, B. E.; Engel, R. *Chem. Phys. Lipids* **1988**, *48*, 1.

(27) Chambers, R. D.; Jaouhari, R.; O'Hagan, D. *J. Chem. Soc., Chem. Commun.* **1988**, 1169.

(28) Hayashi, H.; Nakanishi, K.; Brandon, C.; Marmur, J. *J. Am. Chem. Soc.* **1973**, *95*, 8749.

(29) (a) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-C. *Tetrahedron Lett.* **1977**, 155. (b) McKenna, C. E.; Schmidhauser, J. *J. Chem. Soc., Chem. Commun.* **1979**, 739.

Scheme 2[a]

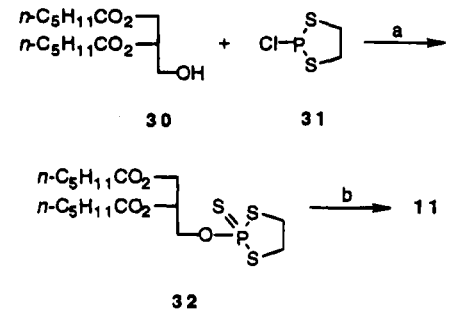


[a] (a) NaN_3, MeCN, Δ; (b) *p*-TsOH, MeOH, 25 °C; (c) NaH, DMSO; *n*-C_6H_{13}-Br, 25 °C; (d) H_2, 10% Pd–C, HOAc, EtOH; (e) $MeOPCl_2$, *i*-Pr_2NEt, THF, −78 °C; $BrCH_2CH_2OH$; 30% aqueous H_2O_2; (f) NMe_3, toluene, 50 °C.

went remarkably facile and unavoidable $O \rightarrow N$ acyl migration, even in the presence of acid, as soon as the azide was reduced.

Although the syntheses of racemic compounds related to **5** and **6** have been reported,[26,27] enantiomerically pure phosphonate and difluorophosphonate analogues of phospholipids are not well documented. In several preliminary experiments, we surveyed the reactions of the phosphonate anions $LiCH_2P(O)(OMe)_2$ and $LiCF_2P(O)(OEt)_2$ with a series of chiral electrophiles such as the tosylate **19** and the derived iodide, but the desired nucleophilic substitution reactions could not be efficiently induced. A completely different approach was then devised that is outlined in (Scheme 3). The phosphonate **24** was prepared by the Arbuzov reaction of the known bromide **23**[28] with triethyl phosphite. Acid-catalyzed cleavage of the acetonide protecting group followed by diacylation of the intermediate diol **26** then gave **28**. Selective hydrolysis of the phosphonate diester moiety[29] provided an intermediate phosphonic acid, which was coupled with choline chloride in the presence of trichloroacetonitrile/pyridine to furnish **5**. Application of a similar sequence of reactions starting with the difluorophosphonate **25**, which was prepared following a general procedure developed in our laboratories for the synthesis of difluorophosphonates,[30] lead to the difluoro analogue **6**.

Scheme 4[a]

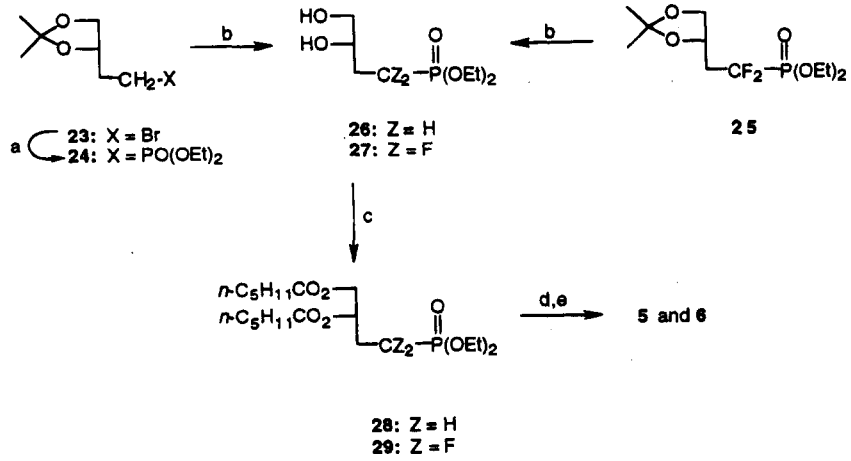


[a] (a) *i*-Pr_2NEt, MeCN, −35 °C; S_8, Pyr, CS_2; (b) DBU, $HO(CH_2)_2NMe_3^+TsO^-$, MeCN, rt.

Toward the objective of preparing phosphorodithioates of phospholipids, we examined several methods that had been used to synthesize phosphorodithioates of oligonucleotides, but each of these proved problematic. Eventually we developed a general and efficient route to phospholipid phosphorodithioates that was applied to the facile synthesis of **11** as shown in Scheme 4.[31] The readily available phosphatitylating reagent 2-chloro-1,3,2-dithiaphospholane (**31**) was allowed to react with the 1,2-dihexanoyl-*sn*-glycerol **30** and the intermediate phosphite was sulfurated to provide the 2-alkoxy-2-thio-1,3,2-dithiaphospholane **32**. Reaction of **32** with choline tosylate in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) then delivered **11**.

Enzyme Assays. With the phospholipid analogues **2** and **4**–**11** in hand, it remained to perform the enzyme assays and establish the relevant kinetic constants for each by Michaelis–Menten data treatment. The substrate **2** exhibited a sufficiently high activity that the initial velocity of the enzymatic reaction, V_{max} = 230–660 μmol/min·mg depending upon the batch of PLC_{Bc} used, could be easily measured. The rates of hydrolysis were then expressed in units of specific activity (V_{sp}), which normalizes the data according to the purity of the protein as evaluated by a standard egg yolk L-α-phosphatidylcholine assay. The double reciprocal (Lineweaver–Burk) plot for **2** (Figure 1) was generated using the

Scheme 3[a]



[a] (a) $P(OEt)_3$, Δ; (b) *p*-TsOH, MeOH, 25 °C; (c) *n*-$C_5H_{11}CO_2H$, DCC, DMAP, CH_2Cl_2; (d) TMS-Br, CH_2Cl_2; 10% aqueous THF; (e) $HOCH_2CH_2NMe_3^+Cl^-$, Cl_3CCN, Pyr, 60 °C.

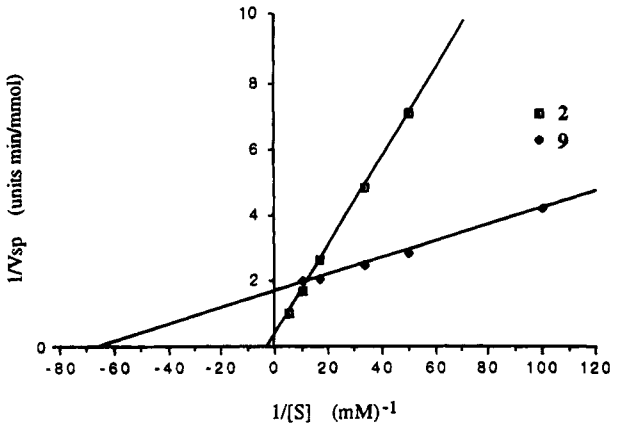


Figure 1. Lineweaver–Burk plots for 1,2-*O*-di-*n*-hexanoyl-*sn*-glycero-3-phosphocholine (**2**) (K_m = 0.39 ± 0.02 mM), which is derived from *average* specific activity data for all three batches of enzyme that were used, and 1,2-*O*-di-*n*-hexanoyl-*sn*-[3(S_P)-thioglycero]phosphocholine (**9**) (K_m = 15 ± 1 μM).

averages of the initial rates that were determined at each substrate concentration from three different batches of PLC_{Bc}. Data to generate this plot were collected over a range of substrate concentrations varying from approximately 0.05 to 1 K_m. Higher concentrations of **2** were avoided in all assays to ensure that the medium would remain homogeneous during the enzyme assays. The measured value of K_m for **2** was 0.39 mM (V_{max} = 2.88 μmol/min·U), which is in excellent agreement with the literature value of 0.36 mM.[9f] The phosphorothioate **9** was also a substrate for PLC_{Bc} exhibiting a K_m of 0.02 mM (V_{max} = 0.60 μmol/min·U). Consistent with the findings of Snyder,[19] the thiophosphate **7** underwent very slow hydrolysis (0.005 μmol/min using 0.1 μg of PLC_{Bc} at [**7**] = 1.6 mM), but it was not possible to determine a K_m or V_{max} because the rate of its hydrolysis under normal assay conditions could not be accurately detected by changes in the pH. Preliminary experiments revealed that even though **7** hydrolyzed slowly, it had a high affinity for the enzyme as measured by its potency as an inhibitor (*vide infra*).

The phosphatidylcholine analogues **4**–**8**, **10**, and **11** were each found to inhibit PLC_{Bc}, and the type of inhibition and the K_i were determined by examination of the corresponding Lineweaver–Burk plots, which are displayed in Figure 2. Only the double reciprocal plot of the substrate from the most active batch of enzyme is shown since the plots for each of the phospholipid analogues using other batches of enzymes were normalized to the activity of this batch. The Lineweaver–Burk plots of the sulfur-containing analogues **7**, **10**, and **11**, which were the most potent inhibitors of PLC_{Bc}, deviated somewhat from linearity in a fashion that suggests that the potency of the inhibitor increases at higher concentrations of substrate **2**.[32] Although the basis for this unusual observation is presently unknown, one possibility is that these inhibitors begin to aggregate with substrate at these concentrations. The resolution of this issue will be the focus of future investigations. The values for the relative constants K_m and K_i of the phospholipid analogues **2** and **4**–**11** are summarized in Table 1. Since these data were collected using three different batches of PLC_{Bc}, there was some incongruity in the measured maximal velocities. However, despite this variation, the different kinetic constants could be reliably calculated with less than 10% error.

Table 1. Summary of Results of Kinetic Studies on PC Analogs

compd	K_i (mM)	K_m (mM)	type of inhibition
2		0.39 ± 0.02[a–c]	
9		0.015 ± 0.001[c]	
7	0.007[a]		competitive
11	0.010[a]		competitive
10	0.013[a]		competitive
4	0.15[b]		competitive
8	0.46[b]		competitive
5	1.15[b]		competitive
6	2.68[c]		competitive

[a] Assay performed using PLC_{Bc} with activity of 1860 units/mg. [b] 930 units/mg. [c] 423 units/mg. The kinetic constants were determined using the raw data for each batch of enzyme.

Discussion

The phosphoramidates **4** and **8** did not undergo observable hydrolysis under the assay conditions,[33] and both were competitive inhibitors. The diether phosphoramidate **8** exhibited significant binding affinity for PLC_{Bc}, having a K_i comparable to the K_m of the substrate **2**. This finding is noteworthy since previous studies had revealed that relative to the corresponding diacyl compounds diether phospholipids are poor substrates for PLC_{Bc}. Since these diether phospholipids also did not inhibit PLC_{Bc},[9f,34] it appeared from these earlier investi-

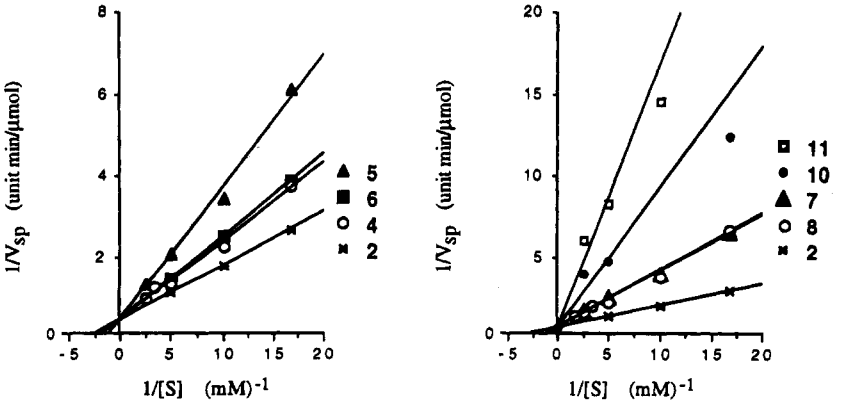


Figure 2. Lineweaver–Burk plots for substrate **2** (from the most active batch of enzyme) and inhibitors (normalized to **2**): **4** (0.05 mM), **5** (0.2 mM), and **6** (0.1 mM), **7** (0.05 μM), **8** (0.2 mM), **10** (0.2 mM), and **11** (0.05 mM). These plots have been truncated for better visualization of the *y*-axis, and two data points at lower [S] are not shown. See supplementary material for complete plots.

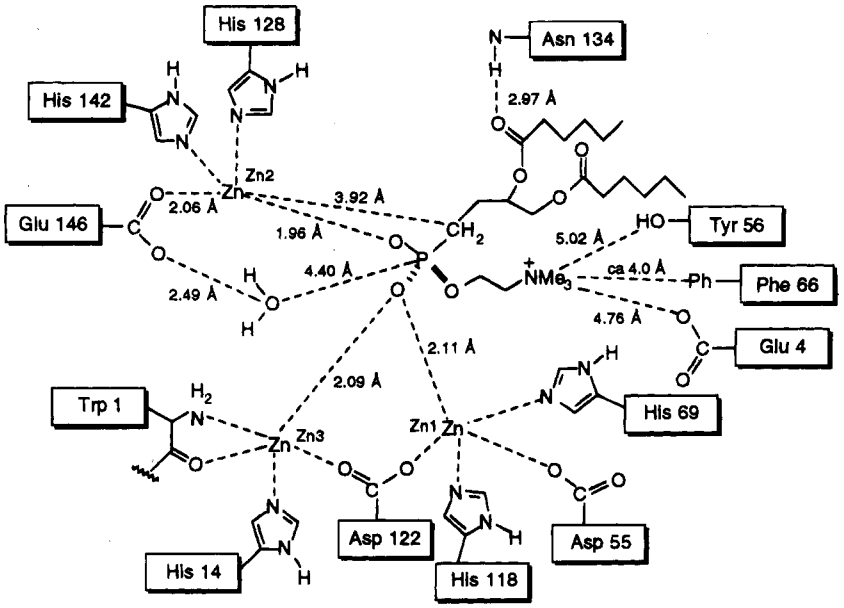


Figure 3. Summary of interactions and some interatomic distances at the active site of PLC_{Bc} complexed with **5**.

gations that one or more of the ester carbonyl groups of the phospholipid were critical for binding to the enzyme active site. We recently conducted an X-ray analysis (1.9-Å resolution) of the complex of PLC_{Bc} and the phosphonate inhibitor **5** and confirmed this hypothesis by observing a hydrogen bonding interaction between the backbone N—H of the Asn_{134} residue of PLC_{Bc} with the *sn*-2 ester carbonyl group of **5** (Figure 3).[35] It therefore appears that any loss of binding affinity that might result from deletion of the carbonyl groups in the glycerol side chains is balanced by replacing the scissile P—O bond of the substrate with a P—NH bond. An X-ray structure of PLC_{Bc} complexed with **8** would reveal any significant binding interactions between the NH of **8** and active site residues and lead to a better understanding of the unexpected potency of **8**. Since the phosphoramidate **4** bears a bulky *N*-benzyl group close to the bond that would be cleaved in the normal hydrolysis of a phospholipid, it is somewhat surprising that **4**, which has a K_i of 0.15 mM, is such an effective inhibitor. Examination of the crystal structure of the enzyme–inhibitor complex of PLC_{Bc} with **5** reveals that there is a hydrophobic pocket at the active site of the enzyme that might accommodate the aromatic ring of **4** without significant adverse effect. Molecular modeling studies are in progress to ascertain whether second-generation inhibitors that will exploit this ancillary binding pocket might be designed, leading to more potent inhibitors.

Both the phosphonate **5** and the difluorophosphonate **6** were competitive inhibitors of PLC_{Bc}, with **5** being marginally better than **6**. Thus, for the case of bacterial PLC_{Bc}, methylene and difluoromethylene replacements for the oxygen of the scissile P—O bond lead to approximately equipotent inhibitors. This observation does not support the hypothesis that a difluoromethylene group is superior to a methylene group as an isopolar replacement for an oxygen atom of a biological phosphate.[16] However, in the present instance it should be recognized that the active site of PLC_{Bc} has likely been engineered to facilitate cleavage of the phosphodiester P—O bond by coordination of the leaving oxygen with an acid, perhaps Zn_2 itself or a zinc-bound water. The atom at this position in an inhibitory ligand should presumably be a Lewis base for optimal interaction with the enzyme. The similarity of **5** and **6** as inhibitors of PLC_{Bc} may then simply result from the inability of both methylene and difluoromethylene groups to interact significantly with the general acid at the enzyme active site. Furthermore, the X-ray structure of **5** complexed with PLC_{Bc} reveals that a close contact could arise between the difluoromethylene group and Zn2 since the atomic radius of fluorine is larger than that of hydrogen. The resulting nonbonded repulsion could disfavor **6** relative to **5** without gaining the polar benefits normally ascribed to difluoro substitution.

The phospholipid analogues **7**, **10**, and **11**, which contain a sulfur atom as a replacement for one of the phosphate oxygens, were highly effective inhibitors of PLC_{Bc} with **7** being the most potent. For example, after preincubating **7** at concentrations ≥ 4 μM with PLC_{Bc}, virtually no enzymatic hydrolysis of **2** could be detected, even when the concentration of **2** was raised to 0.8 mM. When PLC_{Bc} was added to a solution of **7** (2 μM) and **2** (0.1 mM), the initial velocity of the enzymatic reaction was not constant. The velocity slowed after a fast start and became constant after a few minutes, so the time course plot was slightly concave. This observation indicated that steady-state conditions had not been estab-

(30) Martin, S. F.; Dean, D. W.; Wagman, A. S. *Tetrahedron Lett.* **1992**, *33*, 1839.

(31) Martin, S. F.; Wagman, A. S. *J. Org. Chem.* **1993**, *58*, 5897 and references therein.

(32) Because the double reciprocal plot line generated from the nonlinear raw data for **10** intercepted the *y*-axis below the value for $1/V_{max}$ for **2**, it was necessary to anchor this line by weighting the expected *y*-intercept ten times more than any other point. This constraint altered the slope from which the K_i was determined by only about 1%.

(33) No pH changes were observed for about 5 min after addition of the enzyme to the assay solution containing the phospholipid analogue. Initial velocities of enzymatic hydrolysis of substrate **2** (DHPC) were determined within the first 3 min after addition of the enzyme.

(34) Burns, R. A., Jr.; Friedman, J. M.; Roberts, M. F. *Biochemistry* **1981**, *20*, 5945.

(35) Hansen, S.; Hough, E.; Svensson, L. A.; Wong, Y.-L.; Martin, S. F. *J. Mol. Biol.* **1993** *234*, 179.

lished when the initial velocity was measured, and it suggested that **7** may be a slow-binding inhibitor since it was present in ≥10000-fold excess over the enzyme.[36] Analysis of the data for **7** by the Michaelis–Menten treatment required that the normal steady-state assumptions be in force, so the assays were run with **7** at a concentration of 0.05 μM, which is approximately 100-fold greater than that of PLC_{Bc}. Under these conditions, the enzymatic hydrolysis of **2** was >2000 times faster than that of **7**, so any hydrolysis of **7** should not interfere significantly with determining the initial rates for the hydrolysis of **2**. The ratio of enzyme concentration to inhibitor concentration during these assays was near the borderline for a non-steady-state situation, but it was nevertheless possible to classify **7** as a competitive inhibitor and calculate a K_i using standard methods.

Correlation of the behavior of the two phosphorothioates **9** and **10** with the crystal structure of the enzyme–inhibitor complex in Figure 3 reveals an interesting stereochemical insight regarding the different roles played by the nonbridging heteroatoms on phosphorus. Namely, **9**, which has the *S* configuration at phosphorus, is a substrate that must bind to the active site with the sulfur atom serving as a ligand for the two structural and cocatalytic zinc ions Zn1 and Zn3, which appear to bind the substrate and assist in charge neutralization of the phosphate group. On the other hand, **10**, which has the *R* configuration at phosphorus, must bind so that the sulfur atom is coordinated to the zinc ion (Zn2) that seems to be involved in catalysis. Thus, binding of a sulfur ligand to the catalytic zinc ion critically affects the ability of the enzyme to hydrolyze the phosphodiester linkage. The phosphorodithioate **11** in which sulfur atoms coordinate to all three zinc ions was found to be an inhibitor equipotent with **10**.

Conclusions

A series of short-chain phosphatidylcholine analogues were prepared and evaluated as potential inhibitors of the bacterial PLC from *B. cereus* (PLC_{Bc}). In the substrate analogues **4**, **5**, **6**, and **7**, the oxygen atom of the scissile P–O bond in the water-soluble phospholipid substrate **2** is replaced by NBn, CH_2, CF_2, and S, respectively. The related analogue **8**, which contains a NH replacement for the phosphodiester oxygen and ether side chains in place of the usual acyl side chains, was also prepared. Novel methods for the synthesis of phospholipid analogues bearing replacements of the phosphate moiety were developed during these studies. These procedures typically involved the use of reactive phosphite coupling agents and should be of considerable general utility. To assess the effect of replacing the nonbridging oxygen atoms of **2** with sulfur atoms, the phosphorothioates **9** and **10** and the phosphorodithioate **11** were prepared. The relevant kinetic constants K_m or K_i for **2** and **4**–**11** were then determined according to the Michaelis–Menten data treatment of the enzyme assays. Of the phospholipid analogues, only **7** and **9** underwent observable hydrolysis by the enzyme. The S_P-phosphorothioate **9** displayed a V_{max} and K_m substantially lower than that of **2**. Although the thiophosphate **7** was processed very slowly under the assay conditions, it was still a potent, competitive inhibitor. On the basis of Lineweaver–Burk analysis, each inhibitor was shown to be competitive with the order of binding affinity being: **7** ≈ **11** ≈ **10** < **4** ≈ **8** < **5** ≈ **6**. One of the objectives of this study was to identify inhibitors that would be candidates for X-ray crystallographic studies, because the complex of PLC_{Bc} with an inhibitor should reveal some useful insights regarding the mechanism of hydrolysis of the phosphodiester bond by PLC_{Bc}. Significantly, the X-ray study of PLC_{Bc} with the phosphonate **5** bound at the active site has been completed,[33] and it is now possible to identify the amino acid residues involved in binding and catalysis. Despite this important advance, mechanistic and stereochemical questions remain regarding the details of phosphodiester hydrolysis, and further studies are in progress. The results of these and related investigations will be reported in due course.

(36) Morrison, J. F.; Walsh, C. T. *Advances in Enzymology and Related Areas of Molecular Biology*; Meister, Alton, Ed.; John Wiley & Sons: Interscience. New York, 1988; Vol. 61, pp 201–301.

Experimental Section

General. Unless noted otherwise, all starting materials were obtained from commercial suppliers and were used without further purification. The dihexanoyl phosphatidylcholine **2** was synthesized, but it is also available from Avanti Polar Lipid, Inc. Tetrahydrofuran (THF) and diethyl ether (ether) were distilled from potassium/benzophenone ketyl under nitrogen immediately prior to use. Toluene was distilled from and stored over sodium under argon. Acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2), and chloroform ($CHCl_3$) were distilled from CaH_2 under nitrogen immediately prior to use. Diisopropylethylamine and pyridine were distilled from CaH_2 and stored over 4-Å molecular sieves under argon. Choline chloride was recrystallized in ethanol/ether, dried in vacuo for 48 h, and stored in a desiccator until use. Reactions involving air- and/or moisture-sensitive reagents were executed under an inert atmosphere of dry argon, and the glassware was flame-dried under vacuum. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM) and the indicated solvents. Infrared (IR) spectra were recorded as solutions in $CHCl_3$ or as indicated. All spectra were reported in wavenumbers (cm^{-1}) and referenced to the 1601.8 cm^{-1} absorption of a polystyrene film. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained as solutions in deuteriochloroform ($CDCl_3$) unless otherwise indicated. Chemical shifts were reported in parts per million (ppm, δ) downfield relative to tetramethylsilane (TMS) and were referenced to the $CHCl_3$ at δ 7.26 for ^1H NMR or to the center line of the $CDCl_3$ triplet at δ 77.0 for ^{13}C. Coupling constants were reported in hertz (Hz). Spectral splitting patterns were designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; comp, complex multiplet; and br, broad. Phosphorus (^{31}P) NMR spectra were obtained as solutions in $CDCl_3$ unless otherwise indicated. Chemical shifts were reported in parts per million (ppm, δ) downfield relative to the external 80% phosphoric acid.

Phospholipase C (*B. cereus*) was purchased from Sigma Chemical Co. as a suspension in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.0; both Type V at pH 7.5 with an activity of 930 or 423 units/mg protein or Type XI at pH 6 with an activity of 1860 units/mg protein were used. A unit is defined as the ability to liberate 1.0 μmol of water-soluble organic phosphorus from egg yolk L-α-phosphatidylcholine per minute at pH 7.3 at 37 °C. All aqueous solutions were prepared with distilled water, which was distilled from chromic acid. The pH of the assay system was determined with a Orion SA520 pH meter using a pH glass electrode (Aldrich 3.5 mm ultra thin or Cole-Parmer). The buffers (pH 4.00, 7.00, and 10.00) used for the pH calibration were from Fisher Scientific. The enzymatic hydrolysis of **2** by PLC_{Bc} was followed by constant pH titration at pH 8.00 using an aqueous 5.0 mM NaOH solution as titrant in a stirred, unbuffered aqueous assay solution that was carefully maintained under an argon atmosphere to exclude carbon dioxide. An Orion SA 520 or Corning 350 pH meter

fitted with a Cole-Parmer glass-body Ag/AgCl reference electrode was employed to monitor the pH. Blank runs indicated that under an argon atmosphere, a maximum of 0.001 μmol H^+/min were required to maintain a pH of 8; this background rate is well below the rates of the enzymatic reactions. The enzyme stock solution was prepared 1 h in advance in H_2O at an appropriate concentration to deliver 0.1 μg of protein in 10 μL to the assay solution. The inhibitor stock solutions were prepared by dissolving the appropriate phospholipid analogue in either H_2O or a small amount of MeOH by sonication in a sonicating bath; the presence of small amounts of MeOH in the media had a negligible effect on the rate. The inhibitor concentration used in each assay was determined as that amount that would reduce the rate of hydrolysis observed at a substrate concentration of 0.2 mM by 10 to 50%. The substrate **2** was dissolved in H_2O by sonication to produce a substrate stock solution of a convenient concentration to attain a final concentration of 0.03, 0.06, 0.1, 0.2, and 0.4 mM of substrate in a total assay volume of 5 mL. Just prior to mixing, the inhibitor and substrate solutions were always sonicated. A 0.2 M aqueous NaOH stock solution that was standardized with potassium hydrogen phthalate and stored under argon was used to prepare the titrant solution of 5.0 mM aqueous NaOH, which was also stored under argon. The rate of the enzymatic reaction was followed by titration of the acid liberated over a 3-min period. The rate data was plotted as min *vs* [OH^-] to determine the initial velocities. The Lineweaver–Burk (double reciprocal) plots used to calculate the K_m, K_i, and V_{max} were constructed from the initial velocities of at least two or three assays at each substrate concentration. Competitive inhibition from examination of all plots was assumed in calculating K_i.

Enzymatic Separation of 9 and 10. A solution of phospholipase A_2 from bee venom (Sigma) (*ca.* 0.3 mg) in Tris buffer (150 μL) was added to a solution of a diastereoisomeric mixture of **9** and **10**[24b] (45 mg, 0.096 mmol) in $CHCl_3/Et_2O$ (0.8 mL/3.5 mL) at room temperature. The reaction mixture was stirred vigorously at room temperature for 20 h, at which time the solvent was removed under reduced pressure, and the residue was then dried *in vacuo* for 30 min. The resulting mixture was then separated by flash column chromatography eluting with $CHCl_3/CH_3OH/H_2O$ (7:3:0.4) at a rate of 4 mL/min gave 19 mg (84%) of **9** as a white foam, which was assigned as having the S_P configuration based on Tsai's studies,[9d,e] and 16 mg (89%) of *lyso*-thiophospholipid **18**.

For 1,2-*O*-di-*n*-hexanoyl-*sn*-glycero-3(S_P)-phosphothiocholine (**9**): ^1H NMR (300 MHz) δ 5.28–5.21 (m, 1 H, 4.51 (m, 2 H), 4.36 (dd, 1 H, J = 12.0, 3.0 Hz), 4.15 (dd, 1 H, J = 12.0, 7.1 Hz), 4.10–4.05 (m, 1 H), 3.97–3.79 (m, 2 H), 3.39 (s, 9 H), 2.33–2.26 (comp, 4 H), 1.63–1.54 (comp, 4 H), 1.31–1.25 (comp, 8 H), 0.89 (t, 6 H, J = 6.7 Hz); ^{13}C NMR (75 MHz) δ 173.5, 173.1, 70.3 (d, J_{CP} = 9.0 Hz), 66.1 (d, J_{CP} = 6.9 Hz), 63.7 (d, J_{CP} = 4.3 Hz), 62.9, 59.6 (d, J_{CP} = 4.3 Hz), 54.7, 34.3, 34.0, 31.2, 24.6, 24.5, 22.2, 13.8; ^{31}P NMR (146 MHz) δ +56.0; mass spectrum (CI, methane) m/z 470.2343 ($C_{20}H_{40}NO_7PS$ + H requires 470.2341), 271 (base).

For **18**: ^1H NMR (300 MHz) δ 4.42–3.79 (comp, 10 H), 3.37 (s, 9 H), 2.31 (t, 2 H, J = 7.6 Hz), 1.63–1.53 (comp, 2 H), 1.34–1.21 (comp, 4 H), 0.88 (t, 3 H, J = 6.8 Hz); ^{13}C NMR (75 MHz) δ 173.9, 68.6 (d, J_{CP} = 7.1 Hz), 67.5, 66.1 (d, J_{CP} = 5.6 Hz), 65.0, 59.7, 54.6, 34.3, 34.1, 24.5, 22.5, 13.9; ^{31}P NMR (146 MHz) δ +57.8; mass spectrum (CI, methane) m/z 336, 267, 251 (base).

1,2-*O*-Di-*n*-hexanoyl-*sn*-glycero-3(R_P)-phosphothiocholine (10). To a solution of **18** (25 mg, 0.067 mmol) and DMAP (20 mg, 0.16 mmol) in $CHCl_3$ (1.2 mL) and pyridine (0.2 mL) at room temperature was added hexanoyl chloride (30 μL, 0.20 mmol). The reaction mixture was stirred overnight at room temperature. The solvent was removed first under reduced pressure and then *in vacuo*. The residue was purified by flash column chromatography eluting with $CHCl_3/CH_3OH/H_2O$ (2:1:0.15) at a rate of 10 mL/min to afford 15 mg (47%) of **10** as a white foam: ^1H NMR (300 MHz) δ 5.25–5.18 (m, 1 H), 4.50–4.33 (comp, 1 H), 4.39 (dd, 1 H, J = 12.0, 3.0 Hz), 4.12 (dd, 1 H, J = 12.0, 7.2 Hz), 4.03 (dd, 2 H, J = 8.5, 5.7 Hz), 3.96–3.79 (m, 2 H), 3.39 (s, 9 H), 2.32–2.25 (comp, 4 H), 1.61–1.55 (comp, 4 H), 1.34–1.21 (comp, 8 H), 0.88 (t, 6 H, J = 6.8 Hz); ^{13}C NMR (75 MHz) δ 173.5, 173.1, 70.2 (d, J_{CP} = 7.4 Hz), 66.1 (d, J_{CP} = 6.9 Hz), 63.7 (d, J_{CP} = 3.6 Hz), 62.9, 59.5, 54.7, 34.2, 34.1, 31.2, 24.6, 24.5, 22.3, 13.9; ^{31}P NMR (146 MHz) δ +56.2; mass spectrum (CI, methane) 470, 383, 382.

3-Azido-1,2(*S*)-propanediol. A mixture of tosylate **19**[25] (2.50 g, 8.73 mmol), NaN_3 (1.50 g, mmol), and 18-crown-6 (260 mg, 0.98 mmol) in CH_3CN (35 mL) was heated at reflux for 2 d. The solid was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. The residue was partially dissolved in CH_2Cl_2 (5 mL), and the remaining solid was removed by filtration through Celite again. Concentration of the filtrate gave crude azide **20** as a light yellow liquid: IR ν 2850 (br), 2000, 1400 cm^{-1}; ^1H NMR (300 MHz) δ 4.26 (m, 1 H), 4.05 (dd, 1 H, J = 8.3, 6.5 Hz), 3.77 (dd, 1 H, J = 8.3, 5.9 Hz), 3.39 (dd, 1H, J = 12.8, 4.7 Hz), 3.29 (dd, 1 H, J = 12.8, 5.6 Hz), 1.46 (s, 3 H), 1.36 (s, 3 H); ^{13}C NMR (75 MHz) δ 109.7, 74.4, 66.3, 52.6, 26.4, 25.0; mass spectrum (CI, methane) m/z 157.0861 ($C_6H_{11}N_3O_2$ requires 157.0851), 201, 142, 130, 115, 101 (base). The crude product obtained above was then dissolved in CH_3OH (30 mL) and *p*-TsOH·H_2O was added until the pH <2. The solution was stirred at room temperature overnight, whereupon Na_2CO_3 (s) was added to neutralize the solution. The excess solvents were removed under reduced pressure to give an oil, which was purified by flash chromatography, eluting with EtOAc to afford 0.95 g (89%) of 3-azido-1,2(*S*)-propanediol: IR ν 3400, 2120, 1280 cm^{-1}; ^1H NMR (300 MHz) δ 3.89–3.84 (comp, 1 H), 3.69 (dd, 1 H, J = 11.5, 3.6 Hz), 3.59 (dd, 1 H, J = 11.5, 6.3 Hz), 3.40–3.38 (comp, 2 H), 3.18 (s, 2 H); ^{13}C NMR (75 MHz) δ 70.9, 63.9, 53.4; mass spectrum (CI, methane) m/z 118.0616 ($C_3H_7N_3O_2$ + H requires 118.0616) (base), 117, 61, 60.

3-Azido-1,2(*S*)-bis(*n*-hexyloxy)propane. To a suspension of NaH (0.69 g, 28.75 mmol) in dry DMSO (3 mL) at room temperature was added a solution of the diol from the previous experiment (0.88 g, 7.51 mmol) in dry DMSO (2 mL). The mixture was stirred at room temperature for 1 h, at which time 1-bromohexane (3.20 g, 19.38 mmol) was added and the mixture stirred overnight at room temperature. Brine (30 mL) was added to the reaction mixture, and the aqueous solution was extracted with ether (40 mL). The ethereal solution was washed with brine (2 × 30 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography, eluting with hexanes/EtOAc (15:1) to afford 1.29 g (60%) of the diether as a colorless oil: IR ν 2920, 2860, 2105, 1470, 1280, 1130 cm^{-1}; ^1H NMR (300 MHz) δ 3.59–3.38 (comp, 7 H), 3.33–3.30 (comp, 1 H), 1.61–1.49 (comp, 4 H), 1.38–1.21 (comp, 12 H), 0.87 (t, 6 H, J = 6.7 Hz); ^{13}C NMR (75 MHz) δ 77.8, 71.5, 70.3, 69.9, 51.8, 31.5, 29.8, 29.4, 25.6, 25.5, 22.4, 13.8; mass spectrum (CI, methane) m/z 286.2488 ($C_{15}H_{31}N_3O_2$ + H requires 286.2494) (base), 258, 256, 229, 145.

3-Amino-1,2(*S*)-bis(*n*-hexyloxy)propane (21). A solution of the diether prepared in the preceding experiment (360 mg, 1.26 mmol) in a mixture of CH_3OH (4.5 mL) and glacial acetic acid (1.0 mL) containing 10% Pd/C (36 mg) was shaken under H_2(g) (50 psi) at room temperature overnight. The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. Purification of the residue by flash chromatography eluting with $CHCl_3/CH_3OH$ (5:1) afforded 240 mg (72%) of **21** as a colorless oil: IR ν 2920, 2850, 1570, 1470, 1120 cm^{-1}; ^1H NMR (300 MHz) δ 3.61–3.36 (comp, 9 H), 2.92–2.87 (m, 1 H), 2.80–2.74 (m, 1 H), 1.58–1.46 (comp, 4 H), 1.40–1.12 (comp, 12 H), 0.84 (t, 6 H, J = 6.7 Hz); ^{13}C NMR (75 MHz) δ 78.1, 71.2, 70.5, 69.8, 42.3, 31.2, 29.6, 29.1, 25.3, 22.1, 13.5; mass spectrum (CI, methane) m/z 260.2545 ($C_{15}H_{33}NO_2$ + H requires 260.2589) (base), 258, 244, 188, 158.

***O*-(2-Bromoethyl) *O*-Methyl *N*-[2′(*S*),3′-Bis(*n*-hexyloxy)propyl] phosphoramidate (22).** To a solution of methyl dichlorophosphite (70 μL, 0.74 mmol) and *i*-Pr_2NEt (220 μL, 1.72 mmol) in THF (2 mL) at −78 °C under argon was added a solution of 2-bromoethanol (94 mg, 0.75 mmol) in THF (2 mL). The reaction mixture was stirred at −78 °C for 45 min, at which time a solution of **21** (194 mg, 0.75 mmol) in THF

(1.5 mL) was added. The resulting solution was stirred at −78 °C for 45 min and then at room temperature for another 45 min, whereupon the white solid was removed by filtration through Celite. To the filtrate was added 30% aqueous H_2O_2 (0.5 mL, 4.41 mmol), and after 15 min at room temperature, the solution was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (5 mL), and the resulting solution was dried (Na_2SO_4 and Na_2CO_3), filtered, and concentrated under reduced pressure. The residue was purified by column chromatography, eluting with hexanes/EtOAc (1:2) to afford 135 mg (40%) of **22** as a colorless oil: IR ν 3180, 2880, 2820, 1250 cm^{-1}; ^1H NMR (300 MHz) δ 4.34−4.18 (comp, 2 H), 3.73 (d, 3 H, J = 10.8 Hz), 3.53 (t, 2 H, J = 6.1 Hz), 3.60−3.37 (comp, 7 H), 3.21−3.04 (comp, 2 H), 3.04−2.89 (m, 1 H), 1.58−1.49 (comp, 4 H), 1.35−1.24 (comp, 12 H), 0.87 (t, 6 H); ^{13}C NMR (75 MHz) δ 77.7 (d, J_{CP} = 6.9 Hz), 71.8, 70.6 (d, J_{CP} = 6.4 Hz), 70.3, 65.4 (d, J_{CP} = 3.4 Hz), 53.2, 42.7, 31.6, 30.0, 29.5, 25.7, 22.6, 29.5, 14.0; ^{31}P NMR (146 MHz) δ −0.9; mass spectrum (CI, methane) m/z 460.1774 ($C_{17}H_{39}BrNO_5P$ requires 460.1827), 462 (base), 380.

1,2-Bis(*n*-hexyloxy)-*sn*-(3-aminoglycero)phosphoramidocholine (8). Trimethylamine was passed through a solution of phosphoramidate from the preceding procedure (130 mg, 0.28 mmol) in toluene (3 mL) in a heavy-walled vessel at −40 °C until the volume of the reaction mixture was approximately doubled. The reaction mixture was stirred at 60 °C for 30 h, the excess trimethylamine was evaporated under a stream of N_2(g), and the solvent was removed *in vacuo*. The residue was purified by column chromatography, eluting with $CHCl_3/CH_3OH/H_2O$ (2.8:1:0.2) to afford 52 mg (43%) of **8** as a white foam: IR ν 3260 (br), 1580, 1410, 1250 cm^{-1}; ^1H NMR (300 MHz) δ 4.21−4.09 (comp, 2 H), 3.78−3.70 (comp, 2 H), 3.53−3.23 (comp, 16 H), 3.00−2.72 (comp, 2 H), 2.69−2.58 (comp, 1 H), 1.52−1.38 (comp, 4 H), 1.31−1.09 (comp, 12 H), 0.83−0.79 (comp, 6 H); ^{13}C NMR (75 MHz) δ 79.2 (d, J = 7.3 Hz), 71.5, 70.1, 66.3 (d, J_{CP} = 5.0 Hz, 58.2 (d, J_{CP} = 3.8 Hz), 54.2, 43.3, 31.6, 30.1, 29.6, 25.7, 22.5, 13.9; ^{31}P NMR (146 MHz) δ +6.7; mass spectrum (CI, methane) m/z 366.2404 ($C_{17}H_{36}N_2O_5P$ + H requires 366.2409), 286, 260 (base).

Diethyl [*O*-3,4-Isopropylidene-3(*S*),4-dihydroxybutyl]phosphonate (24). A mixture of 4-bromo-*O*-isopropylidenebutane-1,2(*S*)-diol (**23**)[28] (1.00 g, 4.78 mmol) and triethyl phosphite (4.1 mL, 23.91 mmol) was stirred at 165−175 °C for 6 h, at which time the excess triethyl phosphite was removed *in vacuo*. The residual oil was purified by flash chromatography, eluting with EtOAc to afford 1.09 g (86%) of **24** as a colorless oil: IR ν 2980, 1450, 1375, 1255, 1165, 1070, 1040 cm^{-1}; ^1H NMR (300 MHz) δ 3.85−3.67 (comp, 6 H), 3.21 (t, 1 H, J = 7.1 Hz), 1.63−1.35 (comp, 4 H), 1.05 (s, 3 H), 1.00 (s, 3 H), 0.98 (t, 6 H, J = 7.1 Hz); ^{13}C NMR (75 MHz) δ 108.3, 74.9 (d, J_{CP} = 17.0 Hz), 68.2, 60.8 (d, J_{CP} = 6.7 Hz), 26.2 (d, J_{CP} = 6.2 Hz), 24.9, 21.3 (d, J_{CP} = 142.7 Hz), 15.8 (d, J_{CP} = 4.7 Hz); ^{31}P NMR (146 MHz) δ +31.5; mass spectrum (CI, methane) m/z 267.1331 ($C_{11}H_{23}O_5P$ + H requires 267.1361) (base), 251, 237, 209.

Diethyl [3(*S*),4-Dihydroxybutyl]phosphonate (26). A solution of **24** (1.14 g, 4.28 mmol) and *p*-TsOH·H_2O (120 mg, 0.63 mmol) in CH_3OH (40 mL) was stirred overnight at room temperature. $NaHCO_3$(s) was added to neutralize the solution, and the stirring was continued for another 10 min. The mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (10 mL) and filtered through Celite. Concentration of the filtrate left a colorless oil, which was purified by flash chromatography, eluting with $CHCl_3/CH_3OH$ (5:1) to give 157 mg (14%) of recovered **24** and 794 mg (82%; 95% based on recovered **24**) of **26** as a colorless oil: IR ν 3400 (br), 2900, 1210, 1030 cm^{-1}; ^1H NMR (300 MHz) δ 4.21 (s, 2 H), 3.98−3.81 (m, 4 H), 3.56−3.46 (m, 1 H), 3.40 (dd, 1 H, J = 11.6, 3.4 Hz), 3.26 (dd, 1 H, J = 11.4, 6.74 Hz), 1.90−1.40 (comp, 4 H), 1.29 (t, 6 H, J = 7.1 Hz); ^{13}C NMR (75 MHz) δ 71.4 (d, J_{CP} = 14.9 Hz), 65.8, 61.4 (d, J_{CP} = 6.5 Hz), 25.6, 21.3 (d, J_{CP} = 142.1 Hz), 16.0 (d, J_{CP} = 4.9 Hz); ^{31}P NMR (146 MHz) δ +34.3; mass spectrum (CI, methane) m/z 227.1050 ($C_8H_{19}O_5P$ + H requires 227.1048), 209, 195, 183, 167.

Diethyl [3(*S*),4-Bis(*n*-hexanoyloxy)butyl]phosphonate (28). To a solution of **26** (337 mg, 1.49 mmol), *n*-hexanoic acid (0.39 mL, 3.11 mmol), and DMAP (38 mg, 0.31 mmol) in dry CH_2Cl_2 (6 mL) was added a solution of DCC (65 mg, 3.15 mmol) in dry CH_2Cl_2 (4 mL), and the resulting mixture was stirred for 5 h at room temperature. The white solid was removed by filtration through Celite, and the filtrate was evaporated to furnish an oil, which was purified by flash chromatography on silica gel using hexanes/EtOAc (1:1) to afford 490 mg (78%) of **28** as a colorless oil: IR ν 2910, 1730, 1440, 1370, 1240, 1160, 1100 cm^{-1}; ^1H NMR (300 MHz) δ 5.01 (m, 1 H), 4.16 (dd, 1 H, J = 11.9, 3.8 Hz), 4.07−3.92 (comp, 3 H), 2.22 (t, 1 H, J = 7.5 Hz), 2.21 (t, 1 H, J = 7.5 Hz), 1.87−1.62 (comp, 4 H), 1.60−1.48 (comp, 4 H), 1.24 (t, 6 H, J = 7.1 Hz), 1.32−1.10 (comp, 8 H), 0.81 (t, 6 H, J = 6.6 Hz); ^{13}C NMR (75 MHz) δ 173.2, 173.0, 70.7 (d, J_{CP} = 17.5 Hz), 64.1, 61.5 (d, J_{CP} = 7.1 Hz), 34.1, 33.9, 31.1, 24.5, 24.4, 22.5, 23.9 (d, J_{CP} = 4.2 Hz), 21.4 (d, J_{CP} = 117.2 Hz), 16.3 (d, J_{CP} = 6.9 Hz), 13.7; ^{31}P NMR (146 MHz) δ +30.8; mass spectrum (CI, methane) m/z 423.2508 ($C_{20}H_{39}O_7P$ + H requires 423.2512), 424, 421, 325, 307.

1-[3(*S*),4-Bis(*n*-hexanoyloxy)butyl]phosphonocholine (5). To a solution of **28** (490 mg, 1.16 mmol) in dry CH_2Cl_2 (1 mL) at room temperature was added slowly over 45 min bromotrimethylsilane (400 μL, 3.03 mmol). After the resulting mixture was stirred at room temperature for 6 h, the solvent was evaporated under reduced pressure. The residue was dissolved in 10% aqueous THF (1 mL), and the solution was heated at reflux for 1 h. The solvent was removed under reduced pressure, and the residue was dissolved in $CHCl_3$ (5 mL). The $CHCl_3$ solution was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure to afford 403 mg (95%) of the phosphonic acid as a colorless viscous oil that was utilized in the next step without further purification.

To a suspension of the phosphonic acid (341 mg, 0.93 mmol) obtained above and choline chloride (recrystallized from 3:1 absolute EtOH/ether) (600 mg, 4.30 mmol) in dry pyridine (25 mL) at 60 °C was added trichloroacetonitrile (4 mL, 40 mmol), and the reaction mixture was stirred at 60 °C for 50 h. The solvent was removed by vacuum distillation, and the dark orange residue was dissolved in CH_2Cl_2 (5 mL). The solution was treated with activated carbon, which was then removed by filtration through Celite, and the filtrate was concentrated under reduced pressure to afford a pale orange residue. Purification of the residue by chromatography on silica gel eluting with $CHCl_3/CH_3OH/H_2O$ (2:1:0.15) gave 155 mg (37%) of **5** as a white foam: IR ν 3360, 2940, 2860, 1725 cm^{-1}; ^1H NMR (300 MHz) δ 5.20−5.03 (m, 1 H), 4.32−4.20 (comp, 2 H), 4.18 (dd, 1 H, J = 12.1, 2.8 Hz), 3.94 (dd, 1 H, J = 11.8, 7.0 Hz), 3.78 (comp, 2 H), 3.39 (s, 9 H), 2.23 (t, 2 H, J = 7.6 Hz), 2.22 (t, 2 H, J = 7.6 Hz), 1.79 (td, 2 H, J = 16.4, 8.2 Hz), 1.58−1.45 (comp, 6 H), 1.42−1.18 (comp, 8 H), 0.84 (t, 6 H, J = 6.6 Hz); ^{13}C NMR (75 MHz) δ 173.1, 173.0, 71.8 (d, J_{CP} = 14.6 Hz), 66.5, 64.5, 57.6 (d, J_{CP} = 3.1 Hz), 54.1, 34.1, 33.8, 30.9, 24.4, 24.2, 21.9, 25.7 (d, J_{CP} = 3.1 Hz), 22.2 (d, J_{CP} = 50.7 Hz), 13.5; ^{31}P NMR (146 MHz) δ +23.6; mass spectrum (CI, methane) m/z 452.2753 ($C_{21}H_{42}NO_7P$ + H requires 452.2777), 438, 421, 395, 393, 297, 265, 117, 100, 88, 72 (base).

Diethyl [1,1-Difluoro-*O*-3,4-isopropylidene-3(*S*),4-dihydroxybutyl]phosphonate (25). To a solution of lithium diisopropylamide (7.14 mmol) in THF/hexane (15 mL, 2:1) cooled to −78 °C was added *via* cannula over 5 min a solution of diethyl (difluoromethyl)phosphonate[37] (1.27 g, 6.75 mmol) in THF (5.0 mL) that had been precooled to −78 °C. The lemon yellow solution was stirred at −78 °C for an additional 45 min, whereupon a solution of 2,3-*O*-isopropylidine-D-glyceraldehyde (0.95 g, 7.27 mmol) in THF (5.0 mL) that had been precooled to −78 °C was added *via* cannula over 10 min. The resulting lemon yellow solution was then stirred at −78 °C for 6 h, at which time phenyl chlorothiocarbonate (2.0 mL, 14.5 mmol) was added in one portion. Stirring was continued at −78 °C for an additional 45 min, and the mixture was allowed to warm to room temperature. The mixture was poured into ether (100

(37) Obayashi, M.; Ito, E.; Matsui, K.; Kondo, K. *Tetrahedron Lett.* **1982**, *23*, 2323.

mL), and the mixture was washed with 50% saturated aqueous NH_4Cl (3 × 30 mL). The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography, eluting with hexanes/EtOAc (7:1) to produce a colorless oil in 80% yield: ^1H NMR (250 MHz) δ 7.49 (t, J = 10.2 Hz, 2 H), 7.31 (t, J = 10.1 Hz, 1 H), 7.11 (t, J = 9.9 Hz, 2 H), 6.49–6.31 (m, 0.67 H), 6.15–5.95 (m, 0.33 H), 4.92–4.78 (m, 4 H), 4.19–4.15 (m, 1.44 H), 4.11–4.07 (m, 0.66 H), 1.52–1.35 (m, 12 H); ^{13}C NMR (63 MHz) δ 194.9, 194.8, 153.7, 153.5, 129.5, 126.7, 126.6, 121.6, 121.5, 118.4, 110.1, 109.3, 79.0, 78.8, 78.7, 78.4, 78.0, 76.5, 73.1, 73.0, 72.3, 65.9, 65.2, 65.1, 654.4, 64.3, 25.8, 25.4, 25.3, 16.3, 16.2; ^{31}P NMR (202 MHz) δ +3.7 (t, J_{PF} = 99.0 Hz), +3.6 (t, J_{PF} = 99.0 Hz); IR (neat) 2940, 1590, 1490, 1380 cm^{-1}; mass spectrum (CI(+)) m/z 455.1098 ($C_{18}H_{27}F_2O_7PS$ requires 455.1105), 425, 397, 377, 301, 259, 243.

A solution of the purified thiocarbonate from the previous experiment (2.43 g, 5.0 mmol) in dry toluene (25 mL) containing freshly distilled tri-*n*-butyltin hydride (0.55 mL, 5.0 mmol) and AIBN (0.025 g, 0.5 mmol) was heated at reflux for 2 h. At this time the mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by flash chromatography, eluting with hexanes/EtOAc (7:1) to furnish **25** as a colorless oil in 83% yield: ^1H NMR (250 MHz) δ 4.55–4.40 (m, 1 H), 4.35–4.20 (comp, 4 H), 4.15 (dd, J = 9.2, 6.1 Hz, 1 H), 3.62 (t, J = 9.3 Hz, 1 H), 2.70–2.15 (m, 2 H), 1.49–1.30 (comp, 12 H); ^{13}C NMR (63 MHz) δ 120.1 (ddd, J_{CF}, J_{CP} = 260.1, 215.4 Hz), 108.7, 69.5 (comp), 64.4 (comp), 37.9 (comp), 26.6, 25.5, 16.2 (d, J_{CP} = 4.8 Hz); ^{31}P NMR (202 MHz,) δ +6.38 (J_{PF} = 106.4 Hz); mass spectrum (CI(+)) m/z 303.1155 ($C_{11}H_{21}F_2O_5P$ + H requires 303.1173), 287, 245, 217, 183.

Diethyl [1,1-Difluoro-3(*S*),4-dihydroxybutyl]phosphonate (27). A solution of **25**[30] (0.3 g, 0.99 mmol) and *p*-TsOH·H_2O (20 mg, 0.10 mmol) in CH_3OH (10 mL) was stirred at room temperature overnight. $NaHCO_3$(s) was added to neutralize the solution. The mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (10 mL), and the solution was filtered through Celite and a small amount of silica gel. Concentration of the filtrate left 0.25 g (98%) of **27** as a colorless oil, which was utilized for the next step without further purification: IR ν 3360 (br), 2900, 1360, 1250, 1150 cm^{-1}; ^1H NMR (300 MHz) δ 4.32–4.21 (m, 4 H), 4.14–4.08 (m, 1 H), 3.62 (dd, 1 H, J = 11.2, 3.6 Hz), 3.49 (dd, 1 H, J = 11.3, 6.6 Hz), 3.46 (s, 2 H), 2.32–2.12 (comp, 2 H), 1.36 (t, 6 H, J = 7.0 Hz); ^{13}C NMR (300 MHz) δ 120.1 (td, J_{CF}, J_{CP} = 260.0, 214.7 Hz), 66.2, 65.9 (d, J_{CP} = 7.5 Hz), 65.2–64.9 (m), 38.8 (td, J_{CF}, J_{CP} = 19.4, 15.1 Hz), 16.3 (d, J_{CP} = 4.8 Hz); ^{31}P NMR (90 MHz) δ +6.60 (t, J_{PF} = 107.3 Hz); mass spectrum (CI, methane) m/z 263.0907 ($C_8H_{17}F_2O_5P$ + H requires 263.0860) (base), 245, 217.

Diethyl [1,1-Difluoro-3(*S*),4-Bis(*n*-hexanoyloxy)butyl]phosphonate (29). To a solution of **27** (310 mg, 1.18 mmol), hexanoic acid (270 mg, 2.32 mmol), and DMAP (30 mg, 0.24 mmol) in CH_2Cl_2 (4 mL) was added a solution of DCC (520 mg, 2.52 mmol) at room temperature. The reaction mixture was stirred at room temperature overnight, and the white solid was removed by filtration through Celite. Concentration of the filtrate left an oil, which was purified by column chromatography, eluting with hexanes/EtOAc (2:1) to afford 370 mg (69%) of **29** as a colorless oil: IR ν 2900, 2840, 1730, 1430, 1360, 1270, 1150, 1090, 1020 cm^{-1}; ^1H NMR (300 MHz) δ 5.47–5.43 (comp, 1 H), 4.31–4.16 (comp, 5 H), 4.03 (dd, 1 H, J = 11.9, 5.4 Hz), 2.48–2.22 (comp, 2 H), 2.27 (t, 2 H, J = 7.5 Hz), 2.24 (t, 2 H, J = 7.6 Hz), 1.61–1.51 (comp, 4 H), 1.33 (t, 6 H, J = 7.1 Hz), 1.29–1.18 (comp, 8 H), 0.86–0.82 (comp, 6 H); ^{13}C NMR (300 MHz) δ 173.1, 172.5, 119.2 (td, J_{CF}, J_{CP} = 261.3, 217.3 Hz), 65.0–64.8 (m), 64.6 (d, J_{CP} = 5.5 Hz), 64.5, 35.2–34.5 (m), 34.1, 33.9, 31.2, 31.0, 24.4, 24.3, 22.2, 16.3 (d, J_{CP} = 6.6 Hz), 13.8; ^{31}P NMR (146 MHz) δ +6.1 (t, J_{PF} = 105.7 Hz); mass spectrum (CI, methane) m/z 459.2319 ($C_{20}H_{37}F_2O_7P$ + H requires 459.2323) (base), 379, 344, 343.

1-[1,1-Difluoro-3(*S*),4-bis(*n*-hexanoyloxy)butyl]phosphonocholine (6). To a solution of **29** (310 mg, 0.68 mmol) in CH_2Cl_2 (3 mL) was added dropwise over 20 min bromotrimethylsilane (190 μL, 1.44 mmol) at room temperature. The resulting solution was stirred at room temperature for 3 d. After removal of the solvent under reduced pressure, the residue was dissolved in 10% aqueous THF (2 mL), and the solution was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (5 mL), and the solution was dried (Na_2SO_4), filtered, and concentrated under reduced pressure to afford 250 mg (92%) of the difluorophosphonic acid as a viscous colorless oil that was utilized in the next step without further purification.

To a suspension of the difluorophosphonic acid (250 mg, 0.62 mmol) obtained above and choline chloride (recrystallized from 3:1 absolute EtOH/ether) (340 mg, 2.43 mmol) in dry pyridine (25 mL) at 60 °C was added trichloroacetonitrile (2.0 mL, 19.94 mmol). The reaction mixture was stirred at 60 °C for 7 d. The solvent was removed by vacuum distillation. After having been dried *in vacuo* overnight, the dark orange residue was dissolved in CH_2Cl_2 (5 mL). The CH_2Cl_2 solution was treated with activated carbon, which was then removed by filtration through Celite. The filtrate was concentrated under reduced pressure to give a pale orange residue which was purified three times by short column chromatography on silica gel, eluting with $CHCl_3/CH_3OH/H_2O$ (2:1:0.15) to afford 56 mg (19%) of **6** as a white foam: IR ν 2900, 2840, 1730 cm^{-1}; ^1H NMR (300 MHz) δ 5.60–5.48 (m, 1 H), 4.54–4.40 (m, 2 H), 4.37–4.33 (m, 1 H), 4.02 (dd, 1 H, J = 12.0, 7.2 Hz), 3.89–3.80 (comp, 2 H), 3.34 (s, 9 H), 2.38–2.20 (comp, 2 H), 2.26 (t, 4 H, J = 7.2 Hz), 1.62–1.52 (comp, 4 H), 1.33–1.22 (comp, 8 H), 0.88 (t, 6 H, J = 6.7 Hz); ^{13}C NMR (75 MHz) δ 173.4, 172.9, 66.7 (d, J_{CP} = 3.6 Hz), 66.2, 65.4, 60.1, 54.5, 34.4, 34.1, 31.3, 24.5, 24.6, 22.3, 13.8; ^{31}P NMR (146 MHz) δ +2.7 (t, J_{PF} = 86.1 Hz); mass spectrum (CI, methane) m/z 487.2495 ($C_{21}H_{40}F_2NO_7P$ requires 487.2510), 429, 315, 313 (base), 301.

2-(1′,2′-*O*-Di-*n*-hexanoyl-*sn*-3′-glyceryl)-2-thio-1,3,2-dithiaphospholane (32). To a stirred solution of alcohol **30** (0.29 g, 1.0 mmol), and diisopropylethylamine (1.1 mmol) in dry, oxygen-free MeCN (10 mL) at −38 °C was added dropwise 2-chloro-1,3,2-dithiaphospholane (**31**)[38] (0.16 g, 1.0 mmol) in MeCN (1 mL). After stirring for 2 h, the reaction mixture was warmed to room temperature and stirring continued for an additional 1 h. A solution of S_8 (0.16 g, 5 mmol) in CS_2 (5 mL) was then added, and the resultant light yellow heterogeneous mixture was stirred vigorously for 6 h. The reaction mixture was concentrated under reduced pressure and then dissolved in EtOAc (5 mL). The yellow flocculant solid that formed was removed by filtration through a plug of glasswool. The filtrate was concentrated under reduced pressure, and the residue was purified by flash chromatography, eluting with $Me_2CO/CHCl_3/H_2O$ (6.7:3.2:0.1) to deliver **32**, as a viscous oil: ^1H NMR (250 MHz) 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, 2 H, J = 7.3 Hz), 2.27 (t, 2 H, J = 7.6 Hz), 1.64–1.51 (comp, 4 H), 1.31–1.20 (comp, 8 H), 0.84 (t, 6 H, J = 6.7 Hz); ^{13}C NMR (63 MHz) 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, 31.1, 24.4, 22.2, 13.9; ^{31}P NMR (120 MHz) δ +124.0; IR ν 2933, 1737 cm^{-1}; mass spectrum (CI(+), methane) m/z 443.1140 ($C_{20}H_{40}NO_7PS$ + H requires 443.1150), 271 (base).

1,2-*O*-Di-*n*-hexanoyl-*sn*-glycero-3-phosphodithiocholine (11). To a stirred solution of choline tosylate (0.28 g, 1.0 mmol) and **32** (0.44 g, 1.0 mmol) in MeCN (10 mL) at room temperature was added DBU (1.0 mmol) in one portion. The solution was stirred at room temperature until the reaction was complete, generally ca. 15 min. The mixture was concentrated under reduced pressure. The crude product was purified by flash chromatography, eluting with $Me_2CO/CHCl_3/H_2O$ (6.7:3.2:0.1). Excess choline tosylate was removed by extraction of the product from the insoluble amine salts with $CHCl_3$ to provide pure **11** as a yellow glass: ^1H NMR (250 MHz) δ 5.21–5.12 (m, 1 H), 4.58–4.45 (comp, 2 H), 4.43–4.34 (m, 1 H), 4.19–4.08 (comp, 3 H), 3.99–3.91 (comp, 2 H), 3.42 (s, 9 H), 2.30 (t, 2 H, J = 7.4 Hz), 2.27 (t, 2 H, J = 7.6 Hz), 1.63–1.53 (comp, 4 H), 1.38–1.20 (comp, 8 H), 0.87 (t, 6 H, J

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

= 6.7 Hz); ^{13}C NMR (63 MHz) δ 173.4, 173.0, 70.5 (d, J_{CP} = 6.9 Hz), 66.5 (d, J_{CP} = 6.4 Hz), 63.3, 62.8, 59.3 (d, J_{CP} = 3.7 Hz), 54.8, 34.1, 33.9, 31.0, 2.38, 24.3, 22.1, 13.7; ^{31}P NMR (120 MHz) δ +116.9; IR ν 2961, 1733 cm^{-1}; mass spectrum (CI, methane) m/z 486.2103 ($C_{20}H_{41}NO_6PS_2$ + H requires 486.2113), 427, 271 (base).

Enzyme Assays. Enzyme activity of bacterial PLC_{Bc} was assayed by pH stat titration of the monoester of phosphoric acid that was liberated upon hydrolysis of the soluble substrate **2** with 5.0 mM aqueous NaOH. A circulating constant-temperature bath connected to a water-jacketed sample holder was used to maintain the temperature at 25 ± 0.5 °C. A 9-mL glass vial was used as the reaction vessel for the assay. The vial was cleansed by soaking in a base-bath for at least 10 h; the vial was then rinsed with 1 M aqueous HCl, cleansed with detergent, rinsed thoroughly with tap water and distilled water, and then dried. The stock solution of the appropriate phospholipid analogue was prepared by dilution of a weighed sample of the analogue with distilled water. The enzyme solution (0.02 $\mu g/\mu L$) was prepared, on the same day of the assay, by diluting 10 μL of the enzyme suspension (2 mg/mL) with 1.0 mL of distilled water. The exact concentration of the enzyme stock solution was not determined, but enzymatic reaction velocities were compared to that of **2** at concentrations of 0.2–0.4 mM that were used in previous assays to test the enzyme activity. The enzymatic reaction was initiated employing one of two procedures. **Method A.** The assay solution containing the enzyme was adjusted to pH = 8.00 by adding a standard amount of 5.0 mM aqueous NaOH prior to introduction of the inhibitor and substrate. **Method B.** The enzyme was added to a solution of substrate and inhibitor, and a standard amount of 5.0 mM aqueous NaOH, which was determined by a blank measurement and corresponds to the amount required to neutralize the acid in the stock solution of enzyme, so the pH of the resulting solution was 8. Initial velocities were determined for ≤10% of the total reaction. At each concentration of substrate, the assay was done in duplicate or triplicate at least, and the average standard derivation (or range) of each assay point was about ±5%. The range of substrate concentrations used in the assays was $0.05K_m$ – $1.0K_m$.

Determination of Kinetic Parameters (K_m and V_{max}) for PLC_{Bc} Hydrolysis of Substrates 2 and 9. Biological testing of **2** was carried out using method A as the procedure to initiate the enzymatic reaction, whereas the testing of **9** employed method B. A total volume of 5.0 mL was used in all assays. The resulting mixture was stirred and titrated continuously with 5.0 mM aqueous NaOH under argon to maintain pH = 8.00. Initial velocities were obtained, and V_{max} and K_m were determined from Lineweaver–Burk plots. Initial velocities were obtained by extrapolating to zero time from the observed times of 0.08–3 min. There was no observable hydrolysis of substrates in the absence of enzyme.

Determination of K_i's of Inhibitors 4–8, 10, and 11. Biological testing of inhibitors **10** and **11** was carried out using method A as the procedure to initiate the enzymatic reaction, whereas the testing of **4–6** and **8** employed method B. A total volume to 5.0 mL was used in all assays. The concentration of **2** was varied from about $0.05K_m$ to $1.0K_m$, and the kinetic data for the PLC_{Bc} hydrolysis of **2** in the presence of inhibitor were determined as described above. There was no observable hydrolysis of **4–6**, **8**, **10**, or **11** under the assay conditions;[32] although **7** did undergo slow hydrolysis under the assay conditions, the rate was sufficiently slow that little hydrolysis occurred during the time required for the assay. Lineweaver–Burk plots of the results demonstrated that all were competitive inhibitors. The K_i values were determined by comparing the line equations from the Lineweaver–Burk plots for each inhibitor to that of the pure substrate **2** via eq 1.

$$\frac{v_o}{v_i} = \left(1 + \frac{[I]}{K_i}\right) \quad (1)$$

Acknowledgment. We wish to thank the Robert A. Welch Foundation, the National Institutes of Health (GM 42763), E. I. DuPont de Nemours (Medical Products Division), and the Texas Advanced Research Program for financial support of this research. We are also grateful to Professor Daniel M. Ziegler and Dr. Larry Poulsen (The University of Texas, Austin) for providing extensive technical advice.

Supplementary Material Available: Copies of ^1H and ^{31}P NMR spectra of all new compounds and a complete Figure 2 (41 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.