Competitive Inhibition of Interfacial Catalysis by Phospholipase A_2: Differential Interaction of Inhibitors with the Vesicle Interface as a Controlling Factor of Inhibitor Potency

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Abstract: Phospholipid analogues containing a phosphonate in place of the ester at the *sn*-2 position have been previously shown to be tight-binding competitive inhibitors of secreted phospholipases A_2. Variants of these compounds in which the structure of the phospholipid polar head group has been changed were prepared and analyzed as inhibitors of the phospholipases A_2 from bee and cobra venom, porcine and bovine pancreas, and human synovial fluid. Kinetic measurements of inhibitor potencies were carried out using negatively charged substrate vesicles under conditions in which the enzyme undergoes catalysis without desorption from the vesicle (scooting mode). It is shown that this assay is useful in dissecting the contributions of enzyme-inhibitor affinities versus inhibitor aqueous phase-to-vesicle phase partitioning to the overall inhibitor potencies. Inhibition data were interpreted with the aid of the previously reported X-ray crystal structures of phospholipases A_2 containing bound phospholipid analogue inhibitors. Compared to inhibitors that have polar head groups containing unsubstituted alkyl chains or a hydroxylethyl chain attached to the *sn*-3 phosphate, an inhibitor with an ethylammonium chain in the same position was found to bind 5–14-fold more weakly to the pancreatic, bee venom, and synovial fluid enzymes. The X-ray structures of these enzymes reveal no interactions between the protein and the head group chain attached to the *sn*-3 phosphate. These results suggest that intermolecular interactions of the ethylammonium portion of the inhibitor polar head group with neighboring phospholipids in the interface ($-PO_2^-\cdots H-{}^+NH_2$) can reduce the inhibition potency relative to those inhibitors that cannot form this interaction. Thus, methodology developed in this study is useful in measuring the relative interaction free energies of phospholipids containing different polar head groups with neighboring phospholipids in the bilayer surface. In contrast to the other phospholipases A_2, the X-ray structure of the cobra venom enzyme reveals a geometrically ideal hydrogen bond between an asparagine residue and the polar head group ammonium of a bound phospholipid analogue inhibitor. Inhibitors with head group unsubstituted alkyl chains or an ethylammonium chain bind to the cobra venom enzyme with similar affinities; this is consistent with there being no net change in the number of hydrogen bonds when the inhibitors in the vesicle bind to the enzyme. An *sn*-2 phosphinate-containing phospholipid analogue was prepared and evaluated as a phospholipase A_2 inhibitor. The phosphinate was about 2 orders of magnitude less potent than the analogous phosphonate when tested in both a vesicle assay and in an aqueous solution assay with a water-soluble substrate. This result suggests that the bridging oxygen of the *sn*-2 ester of a phospholipid is well-solvated at the membrane surface. Since some of the inhibitors reported in this study are completely resistant to enzymatic degradation by all known types of phospholipases and are highly potent (producing 50% inhibition when present in the vesicles at a level of one inhibitor per several thousand substrates), they should be useful tools in the study of the roles of phospholipases A_2 in biological processes.

Introduction

Phospholipase A_2 (PLA2) catalyzes the stereospecific hydrolysis of the *sn*-2 ester of membrane phospholipids to release fatty acids and lysophospholipids.[1] There is mounting evidence that the release of arachidonic acid, the precursor of the eicosanoids, is mediated by PLA2,[2] and it is believed that inhibitors of PLA2 might exhibit a wide range of desirable pharmacological effects.[3] Since naturally-occurring phospholipids are virtually insoluble in water, PLA2s have evolved to be able to bind to the surface of a phospholipid aggregate as part of the catalytic reaction cycle.[4] Therefore, interactions that phospholipids experience both in the active site of the enzyme and in the vesicle will determine the equilibrium constants that describe the binding of these ligands to the enzyme bound to the interface.

Phospholipid analogues that contain an *sn*-2 phosphonate,[5a] phosphate,[5b] or amide[6] in place of the enzyme-susceptible ester linkage have been prepared and shown to be tight-binding competitive inhibitors of a number of different secreted PLA2s. The high-resolution X-ray crystal structures have been reported for complexes of short-chain phospholipid analogues with the enzymes from bee venom (*Apis mellifera*),[7] cobra venom (*Naja naja atra*),[8] human synovial fluid,[9] and porcine pancreas.[10] In

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(1) (a) van Deenen, L. L. M.; De Haas, G. H. *Biochim. Biophys. Acta* **1963**, *70*, 538–553. (b) Waite, M. *The Phospholipases*; Plenum: New York, 1987. (c) Gross, R. W. *Trends Cardiovasc. Med.* **1992**, *2*, 115–121.

(2) See, for example: (a) Lin, L.-L.; Lin, A. Y.; Knopf, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6147–6151 and references therein. (b) Clark, J. D.; Lin, L.-L.; Kriz, R. W.; Ramesha, C. S.; Sultzman, L. A.; Lin, A. Y.; Milona, N.; Knopf, J. L. *Cell* **1991**, *65*, 1043–1051. (c) Leslie, C. C. *J. Biol. Chem.* **1991**, *266*, 11366–11371.

(3) See, for example: (a) *Phospholipase A_2: Role and Function in Inflammation*; Dennis, E. A., Wong, P. Y. K., Eds.; Plenum: New York, 1990. (b) Dennis, E. A. *Drug. Dev. Res.* **1987**, *10*, 205–220.

(4) Ramirez, F.; Jain, M. K. *Proteins* **1991**, *9*, 229–239.

(5) (a) Jain, M. K.; Yuan, W.; Gelb, M. H. *Biochemistry* **1989**, *28*, 4135–4139. (b) Jain, M. K.; Tao, W.; Rogers, J.; Arenson, C.; Eibl, H.; Yu, B.-Z. *Biochemistry* **1991**, *30*, 10256–10268.

(6) (a) De Haas, G. H.; Dijkman, R.; Ransac, S.; Verger, R. *Biochim. Biophys. Acta* **1990**, *1046*, 249–257. (b) Yu, L.; Deems, R. A.; Hajdu, J.; Dennis, E. A. *J. Biol. Chem.* **1990**, *265*, 2657–2664. (c) Bennion, C.; Connolly, S.; Gensmantel, N. P.; Hallam, C.; Jackson, C. G.; Primrose, W. U.; Roberts, G. C. K.; Robinson, D. H.; Salich, P. K. *J. Med. Chem.* **1992**, *35*, 2939–2951.

(7) (a) Scott, D. L.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1541–1546. (b) Scott, D. L.; Otwinowski, Z.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1563–1566.

(8) White, S. P.; Scott, D. L.; Otwinowski, Z.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1560–1563.

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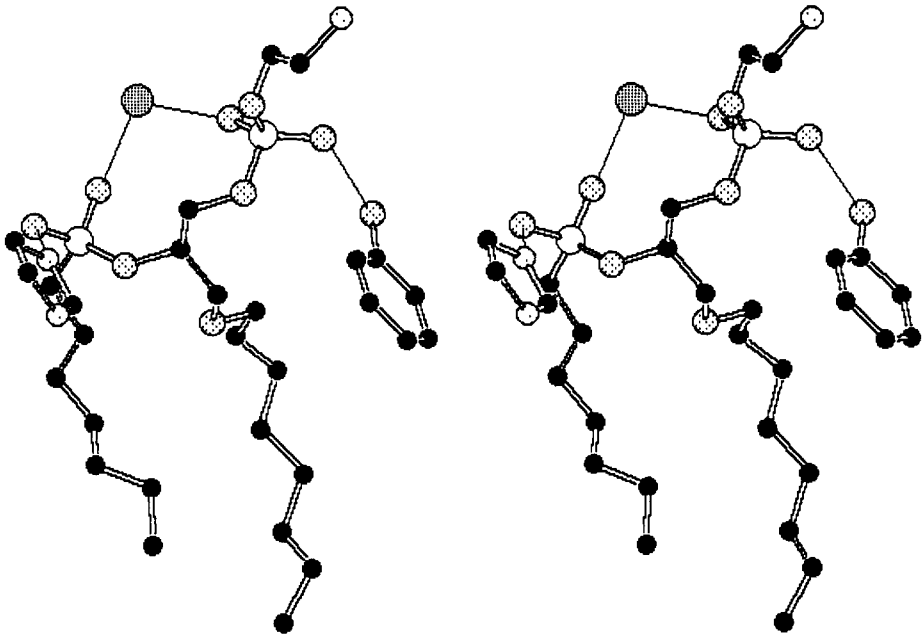


Figure 1. A portion of the X-ray crystal structure of the complex formed between **1** and the cobra venom PLA2.[8] Interactions of the inhibitor atoms with the active-site calcium ion, tyrosine, and histidine are shown by thin lines. The atom shading is as follows: carbon (black), calcium (dark gray), oxygen (middle gray), nitrogen (light gray), phosphorus (white).

all cases studied, the alkyl chains of the phospholipid analogues form hydrophobic interactions with nonpolar amino acid side chains that line the walls of the active-site cleft.

Figure 1 shows the noncovalent interactions formed between the polar groups of phosphatidylethanolamine analogue **1** and active-site residues of the cobra venom PLA2.[8] This inhibitor has an octyl chain at the *sn*-1 position that is oriented roughly parallel to the heptylphosphonyl chain at the *sn*-2 position. The *sn*-2 phosphonate is thought to mimic the high-energy tetrahedral intermediate that forms during the lipolysis reaction. This group is a ligand of the active site calcium ion and also accepts a hydrogen bond from a protonated histidine residue.[11] The *sn*-3 phosphate of the polar head group is also liganded to the calcium ion and accepts a hydrogen bond from a tyrosine hydroxyl group. The ethanolamine of the head group exists in an extended conformation that points away from the alkyl chains and toward the end of the active-site slot that is likely to be furthest from the membrane bilayer.[7a]

In this paper, the preparation of a number of variants of inhibitor **1** are described. These compounds were evaluated as competitive inhibitors of secreted PLA2s from bee and cobra venom, porcine and bovine pancreas, and human synovial fluid. The inhibition analyses were conducted under conditions such that the enzyme remains tightly bound to the surface of a substrate vesicle and hydrolyzes all of the substrate molecules in the outer leaflet of the vesicle without desorption into the aqueous phase. It has been extensively documented that this type of processive enzyme catalysis (termed scooting)[12] is a reliable method for detecting and analyzing true competitive inhibitors of interfacial catalysis.[5,13] That is, compounds that bind tightly and specifically to the enzyme in the interface can be detected. Importantly, there is no interference from numerous agents that inhibit PLA2 in a nonspecific fashion by changing the physical nature of the interface in a way that causes desorption of the enzyme into the aqueous phase under conditions in which the binding of the enzyme to the vesicle is of low affinity.[5,13] Results are presented showing that the scooting mode assay is useful in assessing whether differences in observed inhibitor potencies are due to differences in interfacial equilibrium dissociation constants for the enzyme-inhibitor interactions or due to differences in the aqueous phase-to-vesicle phase partition coefficients for the inhibitors under study. Additional data support the idea that differences in the interaction energetics of the inhibitors with adjacent phospholipid molecules in the vesicle interface can, in certain cases, modulate the relative inhibitor potencies. Finally, results with an *sn*-2 phosphinate-containing inhibitor suggest that the *sn*-2 ester of a phospholipid molecule either in the interface of a vesicle or dispersed as a monomer in aqueous solution is solvated by water to similar extents.

Results

Synthesis of Inhibitors.[14] The synthesis of the *sn*-2 phosphonate-containing phospholipid analogue with an ethanolamine-containing polar head groups is illustrated in Scheme I. Analogues with other head groups were prepared as described in the Experimental Section. Commercially available (*S*)-glycidol was converted to (*R*)-tritylglycidol, and the product was recrystallized four times to obtain material of high enantiomeric excess. The reaction of (*R*)-tritylglycidol with octanethiol in the presence of a catalytic amount of *n*-butyllithium provided thioglycerol analogue **3**.[15] This reaction sequence provides a rapid approach for preparing chiral phospholipid analogues. The enantiomeric excess of **3** was determined to be >98% when the ester formed between this compound and Mosher's acid was analyzed by

(9) (a) Scott, D. L.; White, S. P.; Browning, J. L.; Rosa, J. J.; Gelb, M. H.; Sigler, P. B. *Science* **1991**, *254*, 1007–1010. (b) Wery, J.-P.; Schevitz, R. W.; Clawson, D. K.; Bobbit, J. L.; Dow, E. R.; Gamboa, G. T.; Goodson, J.; Hermann, R. B.; Kramer, R. M.; McClure, D. B.; Mihelich, E. D.; Putnam, J. E.; Sharp, J. D.; Stark, D. H.; Teater, C.; Warrick, M. W.; Jones, N. D. *Nature (London)* **1991**, *352*, 79–82.

(10) Thunnissen, M. M. G. M.; Eiso, A. B.; Kalk, K. H.; Drenth, J.; Dijkstra, B. W.; Kuipers, O. P.; Dijkman, R.; De Hass, G. H.; Verheij, H. M. *Nature* **1990**, *347*, 689–691.

(11) Yu, L.; Dennis, E. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9325–9329.

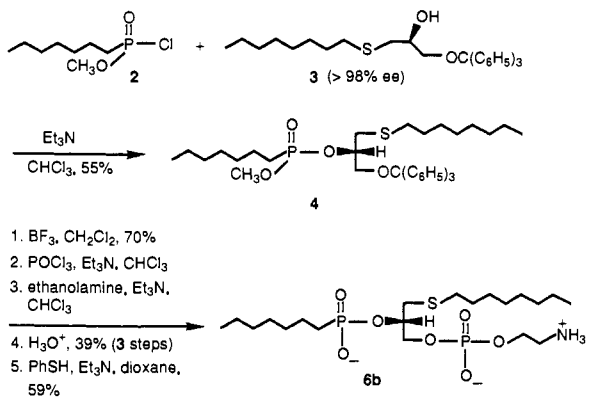
(12) Jain, M. K.; Rogers, J.; Jahagirdar, D. V.; Marecek, J. F.; Ramirez, F. *Biochim. Biophys. Acta* **1986**, *860*, 435–447.

(13) (a) Jain, M. K.; Jahagirdar, D. V. *Biochim. Biophys. Acta* **1985**, *814*, 319–326. (b) Jain, M. K.; Yu, B.-Z.; Rogers, J.; Ranadive, G. N.; Berg, O. *Biochemistry* **1991**, *30*, 7306–7317. (c) Jain, M. K.; Yu, B.-Z.; Gelb, M. H.; Berg, O. G. *Mediators Inflammation* **1992**, *1*, 85–100.

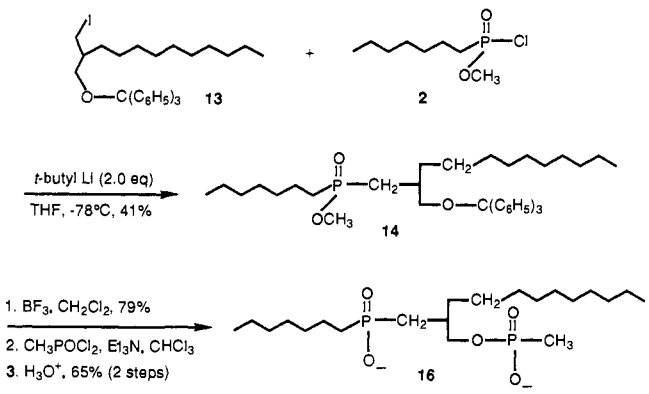
(14) All compounds were homogeneous as judged by TLC and gave high-resolution NMR spectra that were fully consistent with the indicated structures. The mass spectra of all inhibitors showed the predicted molecular ion; no other high mass ions were detected.

(15) Hendrickson, H. S.; Hendrickson, E. K. *Chem. Phys. Lipids* **1990**, *53*, 115–120.

Scheme I



Scheme II



NMR.[16] Compound **3** was reacted with heptylphosphonochloridate methyl ester **2** to give a mixture of diastereomeric phosphonyl esters **4**. Removal of the trityl group gave the mixture of diastereomeric alcohols. After introduction of the polar head group, the phosphonyl diesters were demethylated with thiophenol to afford a single stereoisomeric product **6b**. Phospholipid analogues containing phosphonates at both the *sn*-2 and *sn*-3 positions were prepared by treating the alcohol obtained after detritylation of **4** with the desired alkylphosphonic dichloride.

The synthesis of phosphinate **16**, which contains methylene groups in place of the bridging oxygen of the *sn*-2 ester and in place of the *sn*-1 sulfur, was prepared as outlined in Scheme II. Iodide **13**, made from diethyl malonate, was treated with *tert*-butyllithium to give the organolithium which reacts with phosphonochloridate **2** to furnish four stereoisomeric phosphinate esters **14**.[17] The alcohol obtained by removal of the trityl group was phosphonylated to generate phosphinate **16** as a racemate. The analogue of **16** that contains a phosphonate in place of the phosphinate, compound **17**, was prepared in racemic form using a method similar to that for the preparation of **6b** except that racemic tritylglycidol was ring opened with nonylmagnesium bromide.

PLA2 Inhibition Studies in the Scooting Mode. It has already been shown that secreted PLA2s bind irreversibly to vesicles of the negatively charged phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol (DMPM) and hydrolyze the vesicles in the scooting mode.[4,12] Scooting also occurs on covesicles containing zwitterionic phosphatidylcholine and a small amount of negatively charged phospholipid such as DMPM or phosphatidic acid.[18] With small vesicles (typically 10 000 DMPM molecules), the reaction progress curve has a first-order appearance since the depletion of substrate and the appearance of reaction products (fatty acid and lysophospholipid which remain in the DMPM vesicle) occurs relatively early in time.[19] With large vesicles (typically 100 000 DMPM molecules), a prolonged linear initial velocity (zero-order kinetics) is observed.[19]

The presence of a competitive inhibitor in the vesicle leads to a decrease in the initial velocity, and the amount of reduction is proportional to the concentration of inhibitor in the vesicle surface. The surface concentration of inhibitor is usually expressed as the mole fraction of inhibitor in the vesicle (X_I). The ratio of initial reaction velocities in the absence (v_o) and presence of a competitive inhibitor (v_I) is given by eq 1.[5,13b,c] Here, K_I^* is the interfacial

$$\frac{v_o}{v_I} = 1 + [(1 + 1/K_I^*)/(1 + 1/K_M^*)][X_I/(1 - X_I)] \quad (1)$$

equilibrium dissociation constant for the PLA2–inhibitor complex bound to the interface and K_M^* is the interfacial Michaelis constant for the DMPM substrate. Both constants are in units of mole fraction.

Phospholipid analogues **6b–10b** were tested as inhibitors of a number of different secreted PLA2 acting on large DMPM vesicles in the scooting mode. The parameter $X_I(50)$ is defined as the mole fraction of inhibitor required in the outer monolayer of the vesicles to reduce the initial reaction velocity by 50%. The results are summarized in Table I. The data in Table I are reported as the ratio of $X_I(50)$ values of the phosphatidylethanolamine analogue **6b** to that of the other inhibitors. Thus, the best inhibitors have the largest value of this ratio. In the comparison of $X_I(50)$ values for different inhibitors acting on the same PLA2, it is clear from eq 1 that the ratio of $X_I(50)$ values will be equal to the ratio of K_I^* values as long as $K_I^* \ll 1$, which is the case for all compounds listed in Table I. Because these short-chain phospholipid analogues are soluble in water at concentrations below 0.1 mM, they could be added to the aqueous reaction mixture containing preformed sonicated DMPM vesicles. With the low mole fractions of inhibitors utilized, the physical structure of the vesicles is not altered, and inhibitor–substrate rather than inhibitor–inhibitor intermolecular interactions are likely to dominate.

It can be seen from the results in Table I that the potencies of the inhibitors vary with the structure of the polar head group of the phospholipid analogues and with the source of the enzyme. Differences in the observed $X_I(50)$ values can arise, in part, from differences in the partition coefficients for the distribution of the inhibitors between the aqueous and vesicle phases since this would alter the mole fraction of inhibitors in the vesicle. Differences in $X_I(50)$ values can also arise from differences in the values of K_I^*. The scooting mode analysis provides a simple method for determining the contributions of these effects to the observed $X_I(50)$ values. Since the enzyme binds essentially irreversibly to DMPM vesicles,[4] the initial enzymatic velocity in the absence of the inhibitor is independent of the reaction volume.[5a,12] If the inhibitor is only partially partitioned into the vesicle, the initial reaction velocity in the presence of the inhibitor will increase as the reaction volume is increased, leading to a decrease in the mole fraction of inhibitor in the interface as it dissociates into the aqueous phase. The results of the dilution experiments are summarized in Table II. In these studies, all components of the assay were kept the same except for the volume of the reaction buffer which was varied from 4 to 16 mL. The degree of inhibition caused by addition of constant amounts of the inhibitors **6b**, **7b**, or **8b** to a constant amount of vesicles was found to be invariant to the reaction volume. This establishes that essentially all of the inhibitors are fully partitioned into the vesicles. For example,

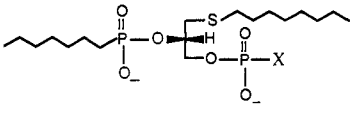
(16) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–2549.

(17) Karanewsky, D. S.; Badia, M. C.; Ciosek, C. P., Jr.; Robl, J. A.; Sofia, M. J.; Simpkins, L. M.; DeLange, B.; Harrity, T. W.; Biller, S. A.; Gordon, E. M. *J. Med. Chem.* **1990**, *33*, 2952–2956.

(18) Ghomashchi, F.; Yu, B.-Z.; Berg, O.; Jain, M. K.; Gelb, M. H. *Biochemistry* **1991**, *30*, 7318–7329.

(19) Berg, O. G.; Yu, B.-Z.; Rogers, J.; Jain, M. K. *Biochemistry* **1991**, *30*, 7283–7297.

Table I. $X_I(50)$ Values for the Inhibitiion of Secreted PLA2s by Phophonate-Containing Inhibitors with Phosphodiester Polar Head Groups 6b–10b[a]



6b X = $OCH_2CH_2NH_3^+$
7b X = OCH_2CH_2OH
8b X = $OCH_2CH_2CH_3$
9b X = OCH_2CH_3
10b X = OCH_3

X	porcine pancreatic DMPM	porcine pancreatic OPPC/DOPA	bovine pancreatic DMPM	bee venom DMPM	synovial fluid DMPM	cobra venom DMPM	cobra venom OPPC/DOPA
$OCH_2CH_2NH_3^+$	(0.0047) 1.0	(0.0043) 1.0	(0.0090) 1.0	(0.0076) 1.0	(0.028) 1.0	(0.0012) 1.0	(0.00033) 1.0
OCH_2CH_2OH	7.8	6.1	4.8	8.0	4.7	1.2	0.57
$OCH_2CH_2CH_3$	14	6.0	5.3	6.0	4.7	0.21	0.10
OCH_2CH_3	14	6.0	nd	nd	4.7	1.6	0.52
OCH_3	14	6.9	nd	nd	8.2	2.0	0.60

[a] The numbers in parentheses are the experimental $X_I(50)$ values in mole fraction for inhibitor **6b**, and all other values are listed as the $X_I(50)$ for this inhibitor divided by the $X_I(50)$ for the indicated inhibtor. The relative errors in all cases are less than 15% based on conducting each measurement in duplicate.

Table II. Percent Inhibition of the Porcine Pancreatic PLA2 by Inhibitors 6b, 7b, and 8b as a Function of Reaction Volume

reaction volume (mL)	6b	7b	8b
4	64	75	62
8	62	71	62
12	70	72	65
16	61	79	57

with a reaction volume of 4 mL, if the moles of inhibitor in the vesicle and aqueous phases are equal, the mole fraction of inhibitor in the vesicle would drop 2.5-fold in going to a reaction volume of 16 mL and the degree of inhibition by **8b**, for example, would drop from 62% to 30%. Thus, the data in Table I establish that the differences in the potency of the inhibitors with different head groups and with the source of the enzyme (Table I) is predominantly due to differences in the K_I^* values of the inhibitors.

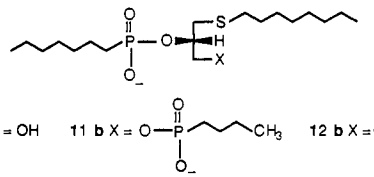
Certain trends are apparent from the inhibition data in Table I. For all enzymes except the cobra venom PLA2, replacement of the ammonium group of inhibitor **6b** with either hydroxyl (**7b**) or methyl (**8b**) results in an increase (4.8–14-fold) in the potency of the inhibitor. In the case of the porcine pancreatic enzyme, the potency of the inhibitors **8b**, **9b**, and **10b** with, respectively, X = $OCH_2CH_2CH_3$, OCH_2CH_3, and OCH_3 are the same within experimental error. The results with the cobra venom enzyme are markedly different. Inhibitors **6b**, **7b**, **9b**, and **10b** have similar potency, but **8b** is 5-fold less potent.

A long-chain analogue of **1** in which the *sn*-1 $S(CH_2)_7CH_3$ is replaced by $O(CH_2)_{15}CH_3$ and the *sn*-2 $CH_3(CH_2)_6PO_3^-$ is replaced by $CH_3(CH_2)_{15}PO_3^-$ was found to be 10-fold less potent than **1** when tested against the action of the porcine pancreatic PLA2 on DMPM vesicles. The effect of substituting the ether at the *sn*-1 position of **1** with sulfur (**6b**) is to decrease the potency by 2-fold against the porcine pancreatic PLA2 and to increase the potency by 5-fold for the cobra venom enzyme.

Inhibitors **6b–10b** were also tested against the porcine pancreatic and cobra venom enzymes using substrate vesicles composed of 7 mol % 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) in 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC). It has been previously shown that secreted PLA2s bind essentially irreversibly to these vesicles and operate in the scooting mode.[18] The results in Table I show that the trends in the inhibitor potencies in DOPA/OPPC vesicles are approximately the same as that measured in DMPM vesicles although the absolute values of $X_I(50)$ change slightly.

Phosphonate-containing phospholipid analogues were also prepared that contain a phosphonate in place of the *sn*-2 ester and in place of *sn*-3 phosphate diester (**11b**, **12b**). Inhibition results are summarized in Table III. Inhibitors **11b** and **12b** are considerably more potent than analogue **5b** which contains only an *sn*-3 hydroxyl group. Interestingly, inhibitors **11b** and **12b**

Table III. $X_I(50)$ Values for Inhibitors 5b, 11b, and 12b with and without an sn-3 Phosphonate Polar Head Group



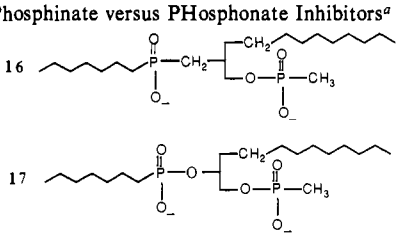
5 b X = OH 11 b X = $O-P(=O)(CH_3)-CH_3$ 12 b X = $O-P(=O)(CH_3)-CH_3$

$X_I(50)$ (mole fraction)

inhibitor	porcine pancreatic (DMPM)	cobra venom (DMPM)
5b	0.058	0.092
11b	0.00042	0.0036
12b	0.00020	0.00093

[a] The relative error in the values are less than 15% based on conducting the measurements in duplicate.

Table IV. Phosphinate versus PHosphonate Inhibitors[a]



16

17

inhibitor	porcine pancreatic PLA2 $X_I(50)$ (DMPM)	porcine pancreatic PLA2 IC(50) (diC_6thio-PM) (μM)	cobra venom PLA2 $X_I(50)$ (DMPM)	cobra venom PLA2 IC(50) (diC_6thio-PM) (μM)
16	0.023	>600[b]	0.12	≈360
17	0.00047	2.8	0.00093	4.2

[a] The relative errors in all numbers is less than 15% based on conducting the measurements in duplicate. [b] Less than 10% inhibition observed with 600 μM.

display similar potencies as the analogues that have an *sn*-3 phosphate diester head group (**8b**, **9b**, **10b**).

Phosphinate **16** has a methylene group replacing the bridging oxygen atom at the *sn*-2 position and a methylene group replacing the sulfur at the *sn*-1 position. This compound is 50–130-fold less potent as an inhibitor compared to phosphonate **17** when tested with the PLA2s from porcine pancreas and cobra venom on DMPM vesicles in the scooting mode (Table IV). Inhibition studies were also carried in the aqueous phase by using the water soluble substrate *rac*-1,2-bis(hexanoylthio)propane-3-phosphomethanol (diC_6thio-PM).[20] In this latter assay, the substrate concentration that is utilized (0.3 mM) is significantly below its

(20) Yuan, W.; Quinn, D. M.; Sigler, P. B.; Gelb, M. H. *Biochemistry* **1990**, *29*, 6082–6094.

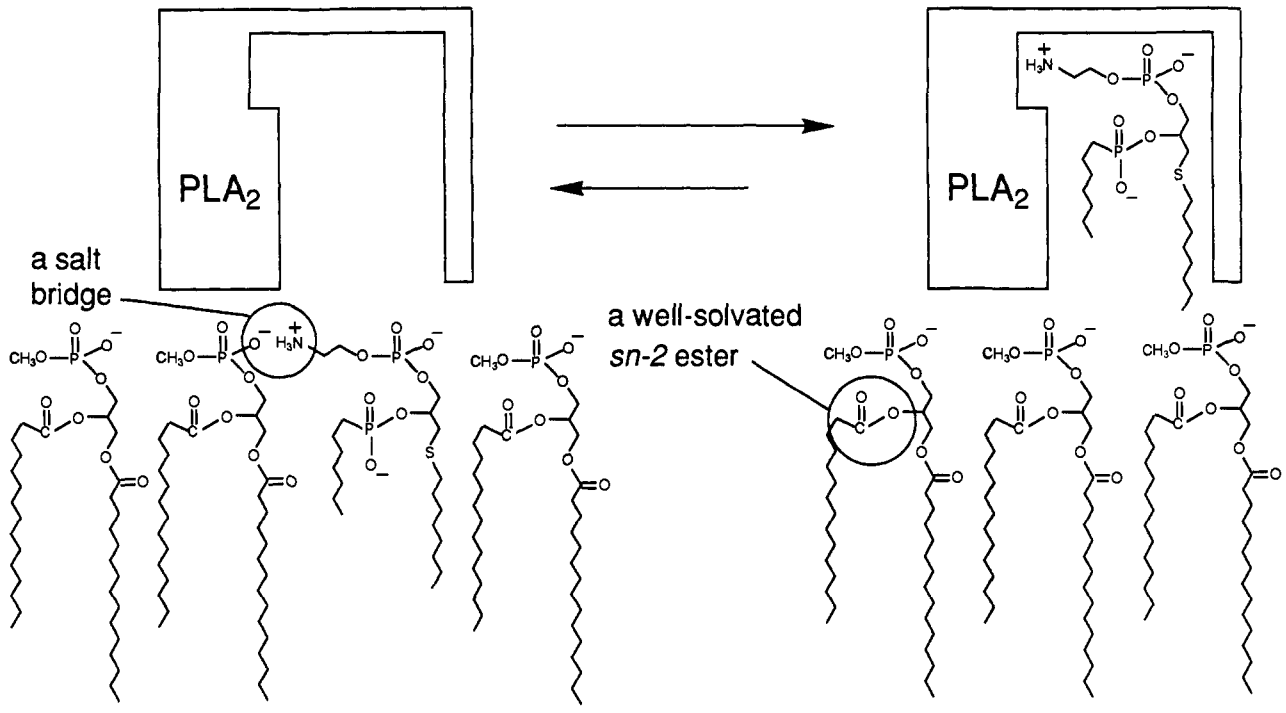


Figure 2. Schematic drawing of the PLA2 bound to the surface of a phospholipid aggregate. The inhibitor must be dislodged from the plane of the bilayer and diffuse into the enzyme's active site slot.

critical micelle concentration (2.5 mM).[20] Again, the results show that the phosphinate is a relatively poor inhibitor. For both enzymes, the relative potency of **16** compared to **17** is approximately the same in both the vesicle and the aqueous phase assays.

Discussion

PLA2 Inhibitors with an *sn*-2 Phosphonate. In this study, attempts have been made to understand some of the features that control the binding affinities of phospholipid analogues to 14-kDa secreted PLA2s. This is an ideal system to study since high-resolution X-ray crystal structures are available for a number of different PLA2s both with and without bound phospholipid analogues.[7-10] In all of these structures, the constellation of catalytic amino acid residues and the calcium ion are nearly superimposable, but there are structural differences in other regions of the active site, for example in the portion that surrounds the phospholipid polar head group. Recent NMR studies have shown that the porcine pancreatic PLA2 undergoes localized structural changes that occur when the enzyme in the water phase binds to micelles, but none of these changes occur in the region of the enzyme that is near the polar head group of an active-site-bound phospholipid.[21]

Prediction of absolute binding constants for the binding of a single inhibitor to different enzymes is a formidable problem that has never been solved. In the discussion that follows, the relative potencies of a series of inhibitors are compared for the different enzymes. It is likely that the different PLA2s have different interfacial Michaelis constants (K_M*) for the DMPM substrate. Only for the porcine pancreatic and synovial fluid PLA2s have the values of K_M* = 0.3 mole fraction and >1 mole fraction, respectively, been determined.[13b,22] The value of K_M* is needed to derive values of K_I* from the experimental values of $X_I(50)$ according to eq 1. However, as discussed in the previous section, the relative $X_I(50)$ values measured for a series of inhibitors against a single PLA2 are equal to the relative K_I* values. Thus, the relative energetics of inhibitor binding can be assessed from the relative $X_I(50)$ values (Table I) as long as the inhibitors under study are mostly partitioned into the vesicles, as is the case (Table II).

In the case of the bee venom PLA2, the 6–8-fold increase in inhibitor potency when the ammonium group of phosphonate **6b** is replaced by hydroxyl (**7b**) or methyl (**8b**) is interesting in light of the fact that the X-ray structure of the bee venom PLA2–**1** complex reveals no protein atoms within 4 Å of the ammonium group. The only candidate for possible interaction with the ammonium group is the carbohydrate chain attached to asparagine-13. The X-ray diffraction results indicate that the structure of the carbohydrate is ill defined, in part due to the chemical heterogeneity of the sugar chain.[7] However, the $X_I(50)$ values for inhibitor **6b**, **7b**, and **8b** with recombinant and nonglycosylated bee venom PLA2, produced in *Escherichia coli*,[23] are the same, within experimental error, as those measured with the glycosylated enzyme. This suggests that inhibitor–carbohydrate interactions do not occur. Thus, the change in $X_I(50)$ values for the bee venom enzyme observed with changes in the structure of the polar head groups must arise from differential interactions of the inhibitor polar head groups with the phospholipid bilayer rather than with enzymic residues. This is illustrated schematically in Figure 2.

It is expected that inhibitor **6b**, containing an ethanolamine head group, will interact more strongly with neighboring phospholipids in the DMPM vesicles because of the possibility of formation of an intermolecular ionic hydrogen bond ($-PO_2^-\cdots H-{}^+NH_2$). This is likely to be the reason that **6b** is the poorest inhibitor of the bee venom PLA2 since this additional hydrogen bonding will stabilize the unbound form of the inhibitor. The existence of such a bond has been extensively documented. The crystal structure of dilauroylphosphatidylethanolamine reveals this ionic hydrogen bond interaction.[24] Spectroscopic studies of noncrystalline aggregates of phosphatidylethanolamine have been reported. A hydrogen-bonded proton has been observed

(21) Peters, A. R.; Dekker, N.; van den Berg, L.; Boelens, R.; Kaptein, R.; Slootboom, A. J.; De Haas, G. H. *Biochemistry* **1992**, *31*, 10024–10030.
(22) Bayburt, T.; Yu, B.-Z.; Lin, H.-K.; Browning, J.; Jain, M. K.; Gelb, M. H. *Biochemistry* **1993**, *32*, 573–582.
(23) Dudler, T.; Chen, W.-Q.; Wang, S.; Schneider, T.; Annand, R. R.; Dempcy, R. O.; Crameri, R.; Gmachl, M.; Suter, M.; Gelb, M. H. *Biochim. Biophys. Acta* **1992**, *1165*, 201–210.
(24) Elder, M.; Hitchcock, P.; Mason, R.; Shipley, G. G. *Proc. R. Soc. London, Ser. A* **1977**, *354*, 157–170.

by proton NMR,[25] and infrared spectroscopic methods are consistent with head group hydrogen bond interactions.[26] In addition, Browning has provided multiple lines of evidence for the existence of this ionic hydrogen bond.[27a] The deuterium NMR spin–lattice relaxation time of head group deuterated phosphatidylethanolamine is shorter than that for deuterated phosphatidylcholine and phosphatidylpropanol aggregates.[27a] Similar trends are seen in the ^{31}P NMR spin–lattice relaxation rates. Addition of cholesterol to the phosphatidylethanolamine aggregate leads to an increase in the deuterium relaxation rate which suggests that the head group ionic hydrogen bond is intermolecular. Higher phase transition temperatures and smaller molecular surface areas for phosphatidylethanolamine aggregates compared to phosphatidylcholine aggregates are also consistent with the idea of an ionic hydrogen bond. This intermolecular head group interaction is also observed in covesicles of phosphatidylethanolamine and phosphatidylcholine.[27a] Indeed, in the present study, a similar trend in inhibitor potency was observed using either DMPM or OPPC/DOPA vesicles (Table I).

NMR relaxation studies have shown that phospholipid head groups containing a simple alkyl chain are less rigid than those containing ethanolamine.[27b] In the context of this model, it is predicted that the phospholipid analogue **8b**, in which the head group ammonium is replaced with methyl, will be a more potent inhibitor than **6b** of the bee venom enzyme, as is the case (Table I). With glycol-containing polar head groups, an intermolecular hydrogen bonding between the hydroxyl group and the phosphate diester of a neighboring phospholipid molecule may be possible, but such a hydrogen bond has never been detected. For example, the addition of a hydroxyl group to an alkyl chain in the polar head group does not significantly change the value of the phase transition temperature.[27c] The results in Table I show that the glycol-containing inhibitor **7b** displays a potency against the bee venom PLA2 similar to the simple alkyl chain-containing compound **8b**. This suggest that the hydroxyl of the polar head group is not engaged in hydrogen bonding with adjacent phospholipids in the vesicle interface.

Trends in inhibitor potencies measured with the synovial fluid PLA2 are similar to those measured with the bee venom enzyme (Table I). This is expected for the synovial fluid enzyme since the X-ray crystal structure of the PLA2–**1** complex clearly shows that the ethanolamine head group does not form contacts with the enzyme.[9a] As shown in Figure 3a, there is a glutamate side chain in the vicinity of the head group, but the observed conformation of the ethanolamine moiety places the ammonium group too far from the glutamate carboxyl group to form a hydrogen bond. The reason for this result is not entirely clear. It should be noted that if the ethanolamine is rotated about its C–C bond, this can bring the nitrogen atom within 2.8 Å of the carboxylate oxygen of the glutamate side chain. However, this will place the N–H vector of the ammonium group nearly perpendicular to the plane of the carboxylate. This geometry is far from ideal for a hydrogen bond since the overlap of the hydrogen with the lone pair of the accepting oxygen is minimal.[28a,b] It should also be noted that the structure shown in Figure 3a is identical in each of the two PLA2 molecules that make up the asymmetric unit in the crystalline lattice.[9a]

The trend in the inhibition data in Table I for the pancreatic PLA2s is similar to that measured for the bee venom and synovial fluid enzymes. Replacement of the ammonium group of **6b** with a hydroxyl (**7b**) or methyl (**8b**) results in an increase in inhibitor affinity. Similar trends have been reported in extensive studies by De Haas and co-workers[6a] and more recently by Bennion et al.[6c] These researchers have shown that choline-containing inhibitors are typically 10-fold less potent than glycol-containing compounds. According to the model developed above, such trends would be expected if the ammonium group in **6b** forms a strong interaction with phospholipids in the bilayer but only weakly interacts with residues of the pancreatic PLA2s. The latter statement is surported by the X-ray structure of the porcine pancreatic PLA2 with a bound short-chain amide-containing phospholipid analogue with a glycol head group. The structure reveals a possible hydrogen bond between the head group hydroxyl and a protein backbone carbonyl oxygen.[10] However, as shown in Figure 3c, it is likely that this hydrogen bond is weak since the O–H vector is nearly perpendicular to the plane containing the hydrogen bond-acceptoring carbonyl group.

Based on site-directed mutagenesis studies of the bovine pancreatic PLA2, it has been suggested that lysine-56 interacts

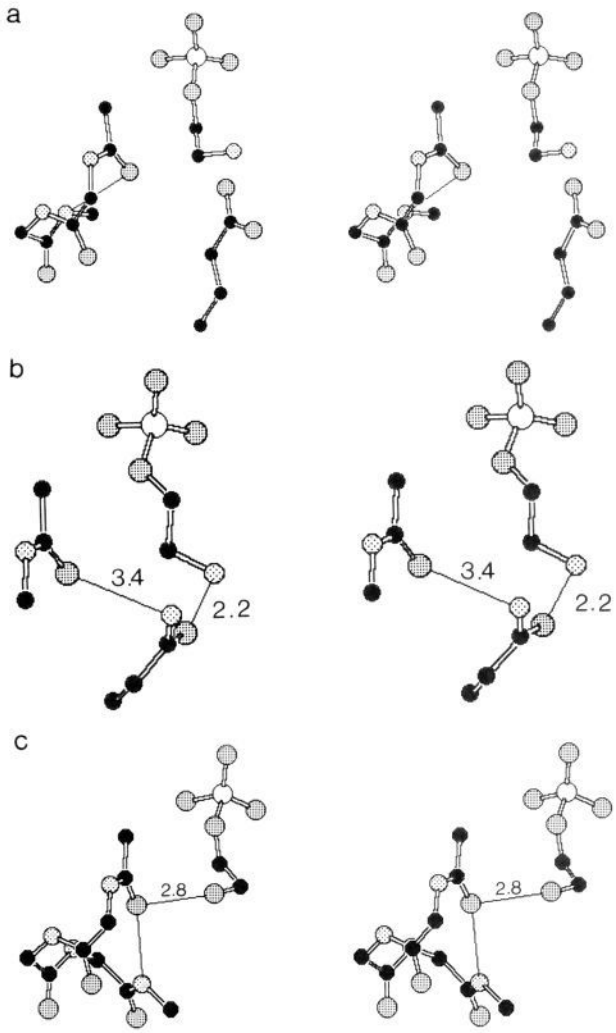


Figure 3. (a) Structural details of the phosphoethanolamine portion of **1** and nearby residues of the synovial fluid PLA2 (shown is the glutamate side chain and a portion of α-helical protein backbone).[9a] (b) Same except the enzyme is the cobra venom PLA2 (shown is the asparagine side chain and a portion of the peptide backbone).[8] (c) Structural details of the phosphoglycol portion of a short-chain *sn*-2 amide phospholipid analogue bond to the porcine pancreatic PLA2 (shown is a portion of α-helical protein backbone).[10] The atom shading is the same as in Figure 1.

(25) Seimiya, T.; Ashida, M.; Hayashi, M.; Muramatsu, T.; Hara, I. *Chem. Phys. Lipids* **1978**, *21*, 69–76.
(26) Akutsu, H.; Ikematsu, M.; Kyogoku, K. *Chem. Phys. Lipids* **1981**, *28*, 149–158.
(27) (a) Browning, J. L. *Biochemistry* **1981**, *20*, 7144–7151. (b) Browning, J. L. *Biochemistry* **1981**, *20*, 7123–7133. (c) Browning, J. L. *Biochemistry* **1981**, *20*, 7133–7143.
(28) (a) Buckingham, A. D.; Fowler, P. W. *Can. J. Chem.* **1985**, *63*, 2018–2025. (b) Baiocchi, F. A.; Klemperer, W. *J. Chem. Phys.* **1983**, *78*, 3509–3520.

with the trimethylammonium of phosphatidylcholine substrates.[29] Replacement of this residue with a negatively charged residue (glutamate) leads to a 4.4-fold increase in the apparent k_{cat} for the hydrolysis of micelles of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine.[29] It has been suggested that this effect may be due to electrostatic repulsion between lysine-56 and the trimethylammonium group of the substrate; this effect becomes an attractive interaction when the lysine is replaced with glutamate.[29] However, the kinetic parameters (k_{cat} and K_m) for the hydrolysis of the monomeric substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine are the same for the wild type and lysine-56 to glutamate mutant.[29] This type of electrostatic effect cannot be very important for the inhibitors reported in this study. If the conformation of the ethylammonium chain of **6b** is anti when bound to the bovine enzyme, as it is when bound to the bee and cobra venom, and synovial fluid PLA2s, the distance between the ammonium groups of lysine-56 and **6b** is 3.5 Å; this distance is larger for other head group conformations. The porcine pancreatic, bee venom, and human synovial fluid enzymes contain no positively charged groups that are less than 7 Å from the ammonium group of **6b**. Despite these difference in proximity of positively-charged groups, similar trends in inhibitor potencies are observed with all of these enzymes (Table I), suggesting that differential interaction of these inhibitors with vesicle phospholipids rather than differential electrostatic repulsion of the enzyme-bound inhibitors is the major reason for the inhibition results.

The cobra venom PLA2 is unique in that the X-ray structure of the enzyme–**1** complex reveals a potentially strong hydrogen bond between the head group ammonium group of the bound inhibitor and the carbonyl oxygen of an asparagine side chain (Figure 3c). The asparagine side chain is held in place by a second hydrogen bond between its side chain amino group and a peptide backbone carbonyl oxygen (Figure 3c). The geometry of the head group–enzyme hydrogen bond is ideal for maximum overlap between the hydrogen and the lone-pair electrons of the carbonyl oxygen. The N–H vector of the ammonium group is in the same plane as the asparagine side chain amide and the angle between the N–H and carbonyl C=O vectors is 140°. Interestingly, only for the cobra venom PLA2 did the replacement of the inhibitor head group ammonium with hydrogen (compound **6b** vs **9b**) result in no change in the observed inhibitor potencies. The alkyl chain of the head group of compound **9b** clearly cannot form a hydrogen bond with the enzyme and with other phospholipid molecules at the membrane surface, and so for both compounds **6b** and **9b**, there is no *net* change in the number of hydrogen bonds when the inhibitor in the interface binds to the enzyme. The same arguments apply for the comparison of **6b** and **10b**.

Compound **8b**, which has a methyl group in place of the head group ammonium of **6b**, is 5-fold poorer as an inhibitor of the cobra venom PLA2. This is most likely due to an unfavorable steric interaction of the propyl chain of **8b** with residues of the cobra venom PLA2 (possibly with the carbonyl oxygen that accepts hydrogen bond from **6b**). This suggestion seems most reasonable in light of the fact that compounds **10b**, **9b**, and **8b**, which have, respectively 1, 2, or 3 carbon atoms in the head group alkyl chain, display nearly equal affinity for the synovial fluid PLA2 (Table I). Since there are no head group–synovial PLA2 interactions, this result indicates that changing the length of the head group alkyl chain from 1 to 3 carbons does not differentially alter the energetics of phospholipid head group interactions in the interface.

Previous studies in which enzyme–ligand interactions are measured both before and after a single hydrogen bond donor or acceptor has been deleted either from the enzyme (by site-directed mutagenesis)[30a] or from the ligand (by chemical substitution)[30b,c] demonstrate that such a deletion results in a reduction of the enzyme–ligand affinity by about 10-fold (corresponding to a difference in free energy of only 1.4 kcal at ambient temperature). This has been rationalized with a simple model involving hydrogen bond counting. For example, reactions 2a,b can be used to describe the situation where a hydroxyl group on a ligand that is engaged in a hydrogen bond with an enzyme acceptor (A) is replaced by hydrogen. In this simple model, it can be seen that both reactions

$$\text{H–O–H···H–O–H + Enz–A···HO–ligand} \rightleftharpoons \text{Enz–A···H–O–H + H–O–H···HO–ligand} \quad (2a)$$

$$\text{H–O–H···H–O–H + Enz–A H–ligand} \rightleftharpoons \text{Enz–A···H–O–H + H–O–H + H–ligand} \quad (2b)$$

2a and 2b involve no net change in the number of hydrogen bonds, and this may be the reason why the loss of a hydrogen bond in the enzyme–ligand complex produces only a small effect. The same model can be applied to the present results. The only difference is that when the ammonium group of **6b** (hydrogen bond donor) is replaced with hydrogen (**8b**) it is the hydrogen bond of the inhibitor with the interface, rather than with the enzyme, that is disrupted. The approximately 10-fold difference in inhibitor potencies measured for **6b** compared to **8b** when acting on all of the PLA2s studied except the cobra venom enzyme (Table I) is congruent with this model and the previous studies.

The 10-fold higher inhibitor potency of **1** versus its long-chain analogue is interesting. The X-ray structural studies show that the short-chain inhibitor spans the length of the active site slot with the methyl groups at the ends of the alkyl chains near the surface of the enzyme.[7-9] This suggests that the long-chain analogue will not be able to form additional hydrophobic interactions with nonpolar amino acid side chains that line the active site. Thus, the inhibition results suggest that the relative potency of these compounds is controlled by differential interactions of the phospholipid analogues with neighboring phospholipids in the vesicle. For example, for the long-chain analogue complexed with PLA2 bound to the interface, one or two methylenes approximately midway down the length of the inhibitor *sn*-1 and *sn*-2 alkyl chains will be forced into unfavorable interactions with polar head groups on adjacent phospholipids. A second possibility is that the inhibitor **1** is less stable in the bilayer because its structure is not commensurate with the packing of the long-chain phospholipid molecules that form the ordered bilayer array. Interestingly, short-chain phospholipids present in vesicles composed mainly of long-chain phospholipids are preferred substrates for PLA2s.[31]

Inhibitors with *sn*-3-Phosphonate Head Groups. Phospholipid analogues **11b** and **12b** containing phosphonates groups at the *sn*-2 and *sn*-3 positions and an alkyl chain at the *sn*-1 position should be useful tools for probing the role of secreted PLA2s in biological processes since these analogues are resistant to hydrolytic breakdown by all known types of phospholipases. For example, prolonged treatment of these compounds with *Bacillus cereus* phospholipase C as described in the Experimental Section did not result in degradation of the inhibitors.

Phosphinate 16. Elegant studies by Bartlett and co-workers on phosphorus-containing inhibitors of zinc-dependent proteolytic enzymes have shown that peptidyl inhibitors with a phosphinate transition state mimic have a binding advantage over those with

(29) Noel, J. P.; Bingman, C. A.; Deng, T.; Dupureur, C. M.; Hamilton, K. J.; Jiang, R.-T.; Kwak, J.-G.; Sekharudu, C.; Sundaralingam, M.; Tsai, M.-D. *Biochemistry* **1991**, *30*, 11801–11811.

(30) (a) Fersht, A. R.; Shi, J.-P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Carter, P.; Waye, M. M. Y.; Winter, G. *Nature (London)* **1985**, *314*, 235–238. (b) Street, I. P.; Armstrong, C. R.; Withers, S. G. *Biochemistry* **1986**, *25*, 6021–6027. (c) James, M. N. G.; Sielecki, A. R.; Hayakawa, K.; Gelb, M. H. *Biochemistry* **1992**, *31*, 3872–3886.

(31) Gabriel, N. E.; Agman, N. V.; Roberts, M. F. *Biochemistry* **1987**, *26*, 7409–7418.

a phosphonate group.[32] This very likely stems from the fact that replacement of the bridging O with CH_2 destabilizes the unbound form of the inhibitor due to the relatively poor solvation of the methylene group.[32] The application of this idea to the case of PLA2 inhibition might be expected to give a more potent inhibitor when the phosphonate is replaced with a phosphinate. This is because the X-ray structure shows that the bridging oxygen of **1** does not accept a hydrogen bond from an enzymic residue, and thus, the replacement of this oxygen with methylene should not greatly effect the binding energetics in the enzyme–inhibitor complex. However, the results in Table IV clearly show that in the DMPM vesicle assay, phosphinate **16** is a much poorer inhibitor than phosphonate **17**. A possible reason for this result is that both the phosphonate and the phosphinate groups are desolvated in the membrane interface, and so the more hydrophobic phosphinate may interact more favorably with the bilayer than does the phosphonate. However, this cannot be the case since phosphinate **16** is also a relatively poor inhibitor of the PLA2-catalyzed hydrolysis of the water-soluble substrate diC_6thio-PM (Table IV). One must be a bit careful in this case as there is considerable evidence that PLA2s can interact with water-soluble short-chain phospholipid substrates to form an enzyme–phospholipid microaggregate in which several phospholipid molecules interact with the interfacial recognition surface of the enzyme.[20,33] However, this is not a concern in the present studies since the inhibitor-to-enzyme ratio is large, and even if some of the inhibitor molecules are present in the microaggregate, most of them will be present as solitary monomers in the aqueous phase. Thus, the relative aqueous solvation of these monomeric forms of **16** and **17** will influence the relative PLA2 binding affinities. The fact that the relative potency of **16** and **17** is similar in the vesicle and aqueous phase assays (Table IV) suggests that the bridging oxygen atom at the *sn*-2 position of a phospholipid in a bilayer aggregate is at least partially hydrated. Such a conclusion is consistent with ^{13}C-NMR measurements on phospholipid vesicles[34] and with ^{19}F-NMR measurements on *sn*-2 fluoro ketone phospholipid analogues.[35]

The likely reason for the relatively poor affinity of phosphinate **16** comes from modeling studies. Overlaying phosphinate **16** onto phosphonate **1** in the crystal structure of the bee and cobra venom PLA2s[7b,8] suggests that the *sn*-2 CH_2 group of **16** sterically clashes with the protonated $N_{\delta 1}$ of the active site histidine ring. The distance between these two hydrogens of 1.6 Å is considerably shorter than the sum of the van der Waals radii (2.4 Å).

Conclusions

Differential intermolecular interactions between PLA2 inhibitors and neighboring phospholipids in bilayered vesicles can modulate the dissociation equilibrium constants for PLA2–inhibitor complexes. The PLA2–inhibitor system described in this study should be useful for measuring the differential energetics of interaction of phospholipid polar head groups at membrane surfaces. The present studies show that the single interaction of the type $-PO_2^-\cdots H-{}^+NH_2-$ weakens the enzyme–inhibitor interaction by about 10-fold compared to an inhibitor in which this interaction cannot occur. The existence and magnitude of this effect is the same as that reported for enzyme–ligand interactions that occur with water-soluble enzymes and ligands.

Experimental Section

Materials and General Methods. Preparation of solvents, silica gel TLC and flash chromatography, and acquisition of spectra were carried out as described.[36] ^{1}H NMR data are presented as follows: chemical shift (multiplicity, number of protons, coupling constant in hertz). ^{31}P chemical shifts are reported downfield from trimethyl phosphate (3.086 ppm) or phosphoric acid (0.0 ppm). Except where noted, compounds on TLC plates were visualized by dipping into an aqueous solution containing 1% $KMnO_4$ and 0.08% NaOH. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Bovine pancreatic PLA2 and PLC type XI from *B. cereus* were obtained from Sigma Chemical Co. Bee venom PLA2 was obtained from Boehringer. Porcine pancreatic and *Naja naja atra* PLA2 were generous gifts from Prof. Mahendra K. Jain (University of Delaware) and Prof. Paul B. Sigler (Yale University). Compounds **1** and its long-chain analogue were prepared as previously described.[20,36]

Heptylphosphonochloridate Methyl Ester (2). Dimethyl heptylphosphonate was synthesized by the Michaelis-Becker reaction using reagent-grade DMF as solvent.[37] A fresh bottle of 80% NaH in a mineral oil dispersion (26.25 g, 0.876 mol) was dissolved in DMF (350 mL) under argon, and a thermometer was placed inside the reaction flask. Dimethyl phosphite (72.9 mL, 0.795 mol) was added gradually through an addition funnel with caution while maintaining the internal temperature of the solution below 30 °C with the aid of a large cold-water bath (temperature control is critical for preventing the formation of isomeric side products). The reaction was stirred at room temperature for 30 min or until the formation of H_2 gas had ceased. 1-Bromoheptane (135 mL, 0.86 mol) was added gradually through an additional funnel with caution, while the internal temperature of the solution was maintained below 30 °C. After the mixture was stirred at room temperature for 20 h, the trace amount of NaBr precipitate was filtered off, and most of the DMF was removed in vacuo. The resulting yellow residue was dissolved in 200 mL of water, followed by extraction with one 300-mL and four 100-mL portions of ether. The combined solvent was removed in vacuo after drying over anhydrous Na_2SO_4. The material was distilled under vacuum to afford 117 g (71% yield) of dimethyl heptylphosphonate as a colorless oil (bp 91 °C, 0.4 mmHg): R_f = 0.2 (50% EtOAc in petroleum ether); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.22–1.46 (m, 8), 1.47–1.71 (m, 3), 1.71–1.84 (m, 1), 3.72 (d, 6, J = 11).

Dimethyl heptylphosphonate was hydrolyzed to give heptylphosphonic acid monomethyl ester as described:[38] R_f = 0.2 (25% CH_3OH in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.18–1.47 (m, 8), 1.47–1.73 (m, 3), 1.73–1.85 (m, 1), 3.72 (d, 3, J = 11); ^{31}P NMR ($CDCl_3$) δ 37.1.

The phosphonic acid monomethyl ester (10 g, 51.5 mmol) was dissolved in dry benzene (30 mL), and 4 boiling chips were added to promote the removal of HCl gas generated during the reaction. Oxalyl chloride (11 mL, 128.8 mmol) was added slowly to the solution with caution, and the mixture was stirred at room temperature for 2 h with moisture protection provided by a drying tube filled with Ca_2SO_4. Benzene and oxalyl chloride were removed in vacuo at a temperature below 30 °C. Additional dry benzene (20 mL) was added and then removed in vacuo to ensure the complete removal of oxalyl chloride: ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.16–1.53 (m, 8), 1.58–1.87 (m, 2), 2.05–2.25 (m, 2), 3.87 (d, 3, J = 12); ^{31}P NMR ($CDCl_3$) δ 47.7.

Tritylated Alcohol (3). The (*R*)-tritylglycidol was prepared as follows: (*S*)-glycidol (25 g, 0.34 mol, ≈ 89% e.e., Aldrich) was added to a solution of triphenylmethyl chloride (103.8 g, 0.37 mol) and Et_3N (94 mL, 1.3 mol) in dry CH_2Cl_2 (700 mL). The reaction mixture was stirred at room temperature for 2 days and then washed with 500 mL of saturated $NaHCO_3$. The aqueous phase was extracted two more times with 70 mL of CH_2Cl_2, and the combined organic solvent was removed in vacuo after drying over anhydrous Na_2SO_4. After four rounds of recrystallization from absolute ethanol at room temperature, 38.3 g (36% yield) of needle-like (*R*)-tritylglycidol was obtained with an enantiomeric purity greater than 98% (see Mosher ester derivative of **3**). The observed spectral data is similar to the published values:[15] R_f = 0.78 (20% ether in CH_2Cl_2); mp 99–100 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.61 (dd, 1, J = 14, 8), 2.72 (dd, 1, J = 14, 5), 3.06–3.18 (m, 2), 3.24–3.38 (m, 1), 7.17–7.51 (m, 15).

The ring opening of (*R*)-tritylglycidol by 1-octanethiol was carried out essentially as described with minor changes on workup and reaction time.[15] An additional 21 h of reaction time was used to ensure complete reaction since the starting material and the product comigrated on the TLC plate. The reaction mixture was neutralized with 20% acetic acid

(32) Morgan, B. P.; Scholtz, J. M.; Ballinger, M. D.; Zipkin, I. D.; Bartlett, P. A. *J. Am. Chem. Soc.* **1991**, *113*, 297–307.
(33) (a) Rogers, J.; Yu, B.-Z.; Jain, M. K. *Biochemistry* **1992**, *31*, 6056–6062. (b) Hille, J. D. R.; Egmond, M. R.; Dijkman, R.; van Oort, M. G.; Jirgensons, B.; De Haas, G. H. *Biochemistry* **1983**, *22*, 5347–5353.
(34) Schmidt, C. F.; Barenholz, Y.; Huang, C.; Thompson, T. E. *Biochemistry* **1977**, *16*, 3948–3954.
(35) Yuan, W.; Berman, R. J.; Gelb, M. H. *J. Am. Chem. Soc.* **1987**, *109*, 8071–8081.
(36) Yuan, W.; Fearon, K.; Gelb, M. H. *J. Org. Chem.* **1989**, *54*, 906–910.
(37) Engel, R. *Synthesis of Carbon-Phosphorus Bonds*; CRC Press Inc: Boca Raton, FL, 1982, p 13.
(38) Yuan, W.; Gelb, M. H. *J. Am. Chem. Soc.* **1988**, *110*, 2665–2666.

in CH_3OH, the white precipitate was filtered off, and the organic layer was washed with saturated $NaHCO_3$. The solvent was removed in vacuo after drying over anhydrous Na_2SO_4, and the material was purified on a silica column with 2% ether in petroleum ether to afford a colorless oil in 90% yield: R_f = 0.40 (1:7 acetone/petroleum ether); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.12–1.41 (m, 10), 1.44–1.61 (m, 2), 2.47 (t, 2, J = 7), 2.59 (dd, 1, J = 14, 6), 2.65 (d, 1, J = 4.8), 2.74 (dd, 1, J = 13.4, 5.7), 3.14–3.29 (m, 2), 3.77–3.89 (m, 1), 7.20–7.38 (m, 9), 7.40–7.49 (m, 6).

(*S*)-Mosher Ester Derivative of Compound 3. The Mosher analysis was used to determine the enantiomeric purity of **3**.[16] The reaction mixture was washed with saturated NH_4Cl and saturated $NaHCO_3$, and the solvent was removed in vacuo after drying over anhydrous Na_2SO_4. Purification over silica was not used in order to avoid the possible removal of either one of the two diastereomers. On the basis of the integration of ^{1}H NMR spectrum, the OCH_3 resonance (δ 3.585 ppm) of the (*S**,*S**) ester was at least 99-fold higher than the OCH_3 resonance (δ 3.536 ppm) of the diastereomeric (*S**,*R**) ester. Thus, the enantiomeric excess of tritylglycidol is greater than 98%. The OCH_3 chemical shift of the (*S**,*R**) ester was confirmed by examining its enantiomeric (*R**,*S**) ester, synthesized by coupling (*R*)-Mosher acid chloride with **3**: R_f = 0.54 (1:7 acetone/petroleum ether).

Tritylated Diacylglycerol Analogue (4). Heptylphosphonochloridate methyl ester **2** (9.75 g, 44.4 mmol) was added to a solution of alcohol **3** (8.2 g, 17.8 mmol), Et_3N (12.3 mL, 88.8 mmol), and 4-(dimethylamino)pyridine (0.23 g, 1.78 mmol) in dry $CHCl_3$ (74 mL), and the mixture was stirred at room temperature in a capped flask overnight. The reaction mixture was washed with 70 mL of saturated $NaHCO_3$, and the aqueous phase was extracted three more times with 50-mL portions of EtOAc. The combined extracts were concentrated in vacuo after drying over Na_2SO_4, and the material was purified on silica with a gradient of 10–40% EtOAc in petroleum ether to afford 6.2 g (55% yield) of a mixture of diastereomeric products as a pale yellow oil: R_f (2 diastereomers) = 0.41 and 0.41 (30% EtOAc in petroleum ether); ^{1}H NMR (2 diastereomers, 200 MHz, $CDCl_3$) δ 0.80–0.95 (m, 6), 1.10–1.40 (m, 18), 1.40–1.88 (m, 6), 2.48 (t, 2, J = 7), 2.70–2.95 (m, 2), 3.21–3.44 (m, 2), 3.58 (d, 1.5, J = 10), 3.74 (d, 1.5, J = 10), 4.55–4.77 (m, 1), 7.17–7.38 (m, 9), 7.38–7.55 (m, 6).

Diacylglycerol Analogue (5a). The detritylation of **4** to alcohol **5a** was carried out essentially as described.[39] The compound was purified on silica with a gradient of 10–40% EtOAc in petroleum ether to afford 1.86 g (70% yield) of a mixture of products as a yellow oil: R_f (2 diastereomers) = 0.31 and 0.25 (25% EtOAc in petroleum ether); ^{1}H NMR (2 diastereomers, 500 MHz, $CDCl_3$) δ 0.85–0.95 (m, 6), 1.20–1.32 (m, 15), 1.32–1.43 (m, 3), 1.55–1.68 (m, 4), 1.75–1.86 (m, 2), 2.57 (t, 2, J = 7), 2.66–2.79 (m, 2), 3.70–3.87 (m, 2), 3.75 (d, 1.5, J = 11), 3.80 (d, 1.5, J = 11), 4.45–4.57 (m, 1).

Phosphatidylethanolamine Analogue (6a). The phosphoethanolamine group was introduced into **5a** as described,[34] except that Et_3N·HCl was precipitated by adding 20 mL of 20% ether in EtOAc and filtered before the addition of 2-propanol, $CHCl_3$, and 1 N HCl. The material was purified on silica with a gradient of 10–40% CH_3OH in $CHCl_3$ to afford 128 mg (39% yield) of the diastereomeric products as a yellow syrup-like material: R_f (2 diastereomers) = 0.21 and 0.13 (25% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 500 MHz, 60% CD_3OD in $CDCl_3$) δ 0.85–0.95 (m, 6), 1.22–1.35 (m, 15), 1.35–1.45 (m, 3), 1.51–1.67 (m, 4), 1.84–2.01 (m, 2), 2.59 (t, 2, J = 7), 2.67–2.79 (m, 1), 2.82–2.90 (m, 1), 3.19–3.24 (m, 2), 3.82 (d, 1.5, J = 11), 3.87 (d, 1.5, J = 11), 4.01–4.08 (m, 1), 4.10–4.22 (m, 3), 4.70–4.80 (m, 1).

Phosphatidylglycol Analogue (7a). The phosphorylation of alcohol **5a** was carried out essentially as described,[40] using 2-chloro-2-oxo-1,3,2-dioxaphospholane (Fluka). The material was purified by chromatography on silica with a gradient of 2–10% CH_3OH in $CHCl_3$ to afford 230 mg (58% yield) of the diastereomeric products as a colorless syrup-like material: R_f (2 diastereomers) = 0.39 and 0.26 (35% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 500 MHz, 66% CD_3OD in $CDCl_3$) δ 0.83–0.95 (m, 6), 1.25–1.35 (m, 15), 1.35–1.45 (m, 3), 1.51–1.67 (m, 4), 1.84–1.97 (m, 2), 2.54–2.66 (m, 2), 2.66–2.81 (m, 1), 2.81–2.93 (m, 1), 3.65–3.78 (m, 2), 3.81 (d, 1.5, J = 11), 3.85 (d, 1.5, J = 11), 3.91–4.02 (m, 2), 3.99–4.18 (m, 2), 4.71–4.82 (m, 1).

Phosphatidylpropanol Analogue (8a). **8a** was prepared in a similar manner as **6a**, except 1-propanol (1.1 equiv of alcohol/equiv of **8a**) was used instead of 2-aminoethanol. The material was purified on silica with a gradient of 3–20% CH_3OH in $CHCl_3$ to afford 123 mg (47% yield) of a mixture of diastereomeric products as a yellow syrup-like material: R_f (2 diastereomers) = 0.50 and 0.41 (35% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 500 MHz, 10% CD_3OD in $CDCl_3$) δ 0.82–0.97 (m, 9), 1.18–1.32 (m, 15), 1.32–1.42 (m, 3), 1.50–1.68 (m, 6), 1.76–1.87 (m, 2), 2.50–2.60 (m, 2), 2.60–2.73 (m, 1), 2.73–2.84 (m, 1), 3.72–3.84 (m, 5), 3.84–3.96 (m, 1), 3.96–4.09 (m, 1), 4.68–4.86 (m, 1).

Phosphatidylethanol Analogue (9a). **9a** was prepared in a similar manner as **8a**, except ethanol (1.1 equiv of alcohol/equiv of **8a**) was used instead of 1-propanol: yield, 56%; R_f (2 diastereomers) = 0.49 and 0.40 (35% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 200 MHz, $CDCl_3$) δ 0.81–0.96 (m, 6), 1.18–1.49 (m, 21), 1.49–1.76 (m, 4), 1.76–2.03 (m, 2), 2.53–2.66 (m, 2), 2.66–2.96 (m, 2), 3.76–3.99 (m, 5), 3.99–4.13 (m, 2), 4.70–4.88 (m, 1).

Phosphatidylmethanol Analogue (10a). **10a** was prepared in a similar manner as **8a** except excess methanol (4.0 equiv) was used instead to afford the compound with a dimethyl phosphate as the head group: yield, 38%; R_f (2 diastereomers) = 0.21 and 0.13 (25% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 200 MHz, $CDCl_3$) δ 0.81–0.93 (m, 6), 1.18–1.46 (m, 18), 1.46–1.68 (m, 4), 1.69–1.90 (m, 2), 2.52–2.63 (m, 2), 2.75–2.85 (m, 2), 3.68–3.85 (m, 9), 4.13–4.37 (m, 2), 4.58–4.76 (m, 1).

Phospholipid Analogue with a Butyl Phosphonate Head Group (11a). This compound was prepared in a similar manner as **8a**, except butylphosphonic dichloride (2.0 equiv) was used instead of phosphorus oxychloride and alcohol: yield, 48%; R_f (2 diastereomers) = 0.50 and 0.40 (35% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 500 MHz, 20% CD_3OD in $CDCl_3$) δ 0.81–0.96 (m, 9), 1.17–1.34 (m, 14), 1.34–1.46 (m, 6), 1.46–1.68 (m, 8), 1.78–1.98 (m, 2), 2.53–2.61 (m, 2), 2.61–2.74 (m, 1), 2.78–2.88 (m, 1), 3.72–3.88 (m, 3), 3.88–4.08 (m, 2), 4.61–4.69 (m, 0.5), 4.69–4.77 (m, 0.5).

Phospholipid Analogue with a Methyl Phosphonate Head Group (12a). This compound was prepared in a similar manner as **11a**, except methylphosphonic dichloride was used: yield, 58%; R_f (2 diastereomers) = 0.28 and 0.19 (35% CH_3OH in $CHCl_3$); ^{1}H NMR (2 diastereomers, 500 MHz, 10% CD_3OD in $CDCl_3$) δ 0.82–0.92 (m, 6), 1.16–1.32 (m, 17), 1.32–1.44 (m, 4), 1.52–1.67 (m, 4), 1.78–1.97 (m, 2), 2.49–2.60 (m, 2), 2.60–2.72 (m, 1), 2.76–2.87 (m, 1), 3.75–3.84 (m, 3), 3.84–4.10 (m, 2), 4.68–4.74 (m, 0.5), 4.74–4.83 (m, 0.5).

General Procedure for the Preparation of Inhibitors 5b–12b. The phosphonate methyl esters **5a–12a** were demethylated essentially as described.[36] A mixture of the phosphonate methyl ester (1.0 equiv, 0.17 M), thiophenol (10 equiv), and Et_3N (16 equiv) in dioxane was heated at 65 °C with stirring in a tightly sealed flask for 5 h. The solvent was removed under a stream of argon, and the material was purified on silica (a column of diameter 3.5 cm and length 2.5 cm was used with 75 mg of compound) with a gradient of 5–60% CH_3OH in $CHCl_3$. Trace amounts of silica were removed by dissolving the product in $CHCl_3$ containing a few drops of CH_3OH and filtering the mixture twice through a 0.45-μm disk (Gelman Acrodisc). Inhibitors **5b–12b** were lyophilized in benzene containing a few drops of CH_3OH and to give white powders, except for **5b** which was obtained as a yellow syrup-like material.

Diacylglycerol analogue 5b: yield 93%; R_f = 0.10 (25% CH_3OH in $CHCl_3$); IR ($CHCl_3$) 3252 br, 2928, 2852, 1459, 1173, 1054; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.78–1.00 (m, 6), 1.10–1.48 (m, 20), 1.45 (m, 4), 2.56 (t, 2, J = 7), 2.55–2.80 (m, 2), 3.55–3.75 (m, 1), 3.75–4.03 (m, 2), 4.03–4.21 (m, 1), 4.38–4.64 (m, 1); ^{31}P NMR ($CDCl_3$) 29.5; MS (FAB) calcd for $C_{18}H_{39}O_4PS$ 382.2, found 383 $(MH)^+$.

Phosphatidylethanolamine analogue 6b: yield 59%; R_f = 0.3 (streaking, 50% CH_3OH in $CHCl_3$); ninhydrin positive; IR (KBr) 3425 br, 3151 br, 2924, 2858, 1730, 1630, 1460, 1399, 1380, 1210, 1082, 1021, 959; ^{1}H NMR (500 MHz, 60% CD_3OD in $CDCl_3$) δ 0.82–0.94 (m, 6), 1.12–1.48 (m, 18), 1.48–1.78 (m, 6), 2.58 (t, 2, J = 7), 2.64–2.83 (m, 2), 3.06–3.26 (m, 2), 3.96–4.09 (m, 1), 4.09–4.32 (m, 3), 4.34–4.53 (m, 1); MS (FAB) calcd for $C_{20}H_{45}NO_7P_2S$ 505.2, found 506 $(MH)^+$.

Phosphatidylglycol analogue 7b: yield 67%; R_f = 0.65 (3:5:0.8:0.4 $CH_3OH/CHCl_3$/acetic acid/water); IR (KBr) 3418 br, 3208 br, 2936, 2848, 1736, 1639, 1459, 1402, 1380, 1214, 1091, 960; ^{1}H NMR (500 MHz, 70% CD_3OD in $CDCl_3$) δ 0.82–0.94 (m, 6), 1.12–1.46 (m, 18), 1.51–1.72 (m, 6), 2.58 (t, 2, J = 7), 2.66–2.82 (m, 2), 3.69–3.80 (m, 2), 3.95–4.06 (m, 2), 4.06–4.12 (m, 1), 4.12–4.23 (m, 1), 4.46–4.56 (m, 1); MS (FAB) calcd for $C_{20}H_{44}O_8P_2S$ 506.2, found 507 $(MH)^+$.

Phosphatidylpropanol analogue 8b: yield 94%; R_f = 0.5 (streaking, 50% CH_3OH in $CHCl_3$); IR (KBr) 3426 br, 3151 br, 2925, 2856, 1632, 1460, 1396, 1205, 1087, 856, 797; ^{1}H NMR (500 MHz, 10% CD_3OD in $CDCl_3$) δ 0.82–0.97 (m, 9), 1.18–1.32 (m, 15), 1.32–1.42 (m, 3),

(39) Hermetter, A.; Paltauf, F. *Chem. Phys. Lipids* **1981**, *29*, 191–195.
(40) Dijkman, R.; Dekker, N.; De Haas, G. H. *Biochim. Biophys. Acta* **1990**, *1043*, 67–74.

1.50–1.68 (m, 6), 1.76–1.87 (m, 2), 2.50–2.60 (m, 2), 2.60–2.73 (m, 1), 2.73–2.84 (m, 1), 3.72–3.84 (m, 5), 3.84–3.96 (m, 1), 3.96–4.09 (m, 1), 4.68–4.86 (m, 1); MS (FAB) calcd for $KC_{21}H_{45}O_7P_2S$ 542.3, found 543 $(MH)^+$.

Phosphatidylethanol analogue 9b: yield 83%; R_f = 0.21 (3:6.5:0.5 $CH_3OH/CHCl_3/NH_4OH$) IR (KBr) 3438 br, 3150 br, 2922, 2852, 1631, 1458, 1398, 1378, 1194, 1090, 956, 827, 797; ^1H NMR (500 MHz, 60% CD_3OD in $CDCl_3$) δ 0.82–0.98 (m, 6), 1.19–1.50 (m, 21), 1.50–1.74 (m, 6), 2.59 (t, 2, J = 7), 2.66–2.77 (m, 1), 2.77–2.90 (m, 1), 3.86–4.03 (m, 2), 4.03–4.21 (m, 2), 4.42–4.60 (m, 1); MS (FAB) calcd for $KC_{20}H_{43}O_7P_2S$ 528.3, found 529 $(MH)^+$.

Phosphatidylmethanol analogue 10b: yield 88%; R_f = 0.28 (4:5.5:0.5 $CH_3OH/CHCl_3/NH_4OH$); IR (KBr) 3438 br, 3150 br, 2922, 2852, 1631, 1458, 1398, 1378, 1194, 1090, 956, 827, 797; ^1H NMR (200 MHz, 60% CD_3OD in $CDCl_3$) δ 0.68–1.02 (m, 6), 1.02–1.50 (m, 18), 1.50–1.82 (m, 6), 2.60 (t, 2, J = 7), 2.67–2.90 (m, 2), 3.63 (d, 3, J = 11), 3.90–3.40 (m, 2), 4.40–4.71 (m, 1); MS (FAB) calcd for $KC_{19}H_{41}O_7P_2S$ 514.3, found 515 $(MH)^+$.

Phospholipid analogue with a butyl phosphonate head group (11b): yield, 81%; R_f = 0.25 (50% CH_3OH in $CHCl_3$); IR (KBr) 3438 br, 3136 br, 2951, 2922, 2854, 1639, 1459, 1396, 1382, 1177, 1075, 963, 817; ^1H NMR (500 MHz, 50% CD_3OD in $CDCl_3$) δ 0.81–0.90 (m, 6), 0.92 (t, 3, J = 7), 1.17–1.47 (m, 20), 1.47–1.73 (m, 10), 2.58 (t, 2, J = 7), 2.67–2.87 (m, 2), 3.94–4.17 (m, 2), 4.36–4.56 (m, 1); MS (FAB) calcd for $KC_{22}H_{47}O_6P_2S$ 540.4, found 541 $(MH)^+$.

Phospholipid analogue with a methyl phosphonate head group (12b): yield 77%; R_f = 0.15 (50% CH_3OH in $CHCl_3$); IR (KBr) 3429 br, 3140 br, 2923, 2850, 1629, 1459, 1392, 1304, 1185, 1076, 957, 895, 797; ^1H NMR (500 MHz, 50% CD_3OD in $CDCl_3$) δ 0.86–0.97 (m, 6), 1.24–1.52 (m, 21), 1.52–1.77 (m, 6), 2.58 (t, 2, J = 7), 2.67–2.84 (m, 2), 4.01–4.20 (m, 2), 4.43–4.59 (m, 1); ^{31}P NMR (50% CD_3OD in $CDCl_3$) δ 25.4, 26.6; MS (FAB) calcd for $KC_{19}H_{41}O_6P_2S$ 498.3, found 499 $(MH)^+$.

Iodide 13. Diethyl decylmalonate was prepared from 1-iododecane and diethyl malonate as described.[41] The compound was distilled to give a colorless oil in 66% yield (bp 135 °C, 0.6 mmHg): R_f = 0.52 (10% EtOAc in petroleum ether); IR (neat), 3476, 2943, 1740, 1471, 1417–987 br, 965, 869, 811, 792, 731, 605; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.20–1.45 (m, 22), 1.81–1.98 (m, 2), 3.31 (t, 1, J = 8), 4.20 (q, 4, J = 7).

The diethyl decylmalonate was reduced to 2-decyl-1,3-propanediol with $LiAlH_4$ in the usual manner as described.[42] The compound was recrystallized from petroleum ether to give colorless crystals in 68% yield (mp 59.5–60 °C): R_f = 0.18 (50% EtOAc in petroleum ether); IR (KBr) 3294 br, 2901 br, 1632, 1464, 1440, 1401, 1379, 1270–1200 multiples, 1132–890 multiples, 695; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.10–1.48 (m, 18), 1.67–1.88 (m, 1), 1.93–2.28 (br, 2), 3.66 (dd, J = 11, 12), 3.83 (dd, J = 11, 11).

The 2-decyl-1,3-propanediol was monotritylated to give the product in a similar manner as **2** except 1.0 equiv of triphenylmethyl chloride was used. The compound was purified on silica with a gradient of 15–40% EtOAc in petroleum ether to afford a pale yellow and viscous oil in 82% yield: R_f = 0.76 (50% EtOAc in petroleum ether); IR (neat) 3397 br, 3068, 3040, 2937, 2865, 1952, 1893, 1814, 1775, 1596, 1495, 1450, 1383, 1317, 1224, 1188, 1158, 1054 br; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.12–1.42 (m, 18), 1.70–1.90 (m, 1), 2.33–2.46 (m, 1), 3.07 (dd, 1, J = 9.2, 7.5), 3.31 (dd, 1, J = 9.2, 3.9), 3.63 (m, 2), 7.20–7.55 (m, 15).

The mono-tritylated alcohol was iodinated as described.[43] The reaction mixture containing alcohol (1.0 equiv), carbon tetraiodide (1.2 equiv), and triphenylphosphine (2.4 equiv) in pyridine (1.0 mL of pyridine/0.12 mmol of alcohol) was heated at 65 °C for 2 h. The compound was purified on silica with 1% EtOAc in petroleum ether to afford a dark red oil in 74% yield: R_f = 0.78 (15% EtOAc in petroleum ether); IR (neat), 3058, 3024, 2926, 2847, 1956, 1813, 1596, 1488, 1448, 1220, 1183, 1153, 1071, 984, 901, 763, 704; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3, J = 7), 1.02–1.41 (m, 18), 1.42–1.61 (m, 1), 2.95 (dd, 1, J = 10, 7), 3.13 (dd, 1, J = 10, 4), 3.39 (dd, 1, J = 10, 5), 3.49 (dd, 1, J = 10, 5), 7.18–7.57 (m, 15).

Phosphinate 14. The procedure for coupling phosphonochloridate methyl ester **2** with alkyl halide **13** was based on the described method.[17] *tert*-Butyllithium (1.7 M in pentane, 5.48 mmol) was slowly added to a solution of **2** (0.72 g, 3.43 mmol) and **13** (1.56 g, 2.74 mmol) in dry THF (15 mL) under argon at −78 °C (sufficient *tert*-butyllithium to cause the reaction mixture to change color from yellow to pink). The solution was stirred at −78 °C for 2 h and then stirred at room temperature for 1 h. The solution was washed with 20 mL of saturated $NaHCO_3$, and the aqueous layer was extracted with 20 mL of ether. The organic extracts were combined, and the solvent was removed in vacuo after drying over anhydrous $MgSO_4$. The compound was purified on silica with a gradient of 40–70% EtOAc in petroleum ether to afford a yellow oil in 41% yield: R_f = 0.16 (40% EtOAc in petroleum ether); IR (neat) 3045, 2928, 2850, 1489, 1454, 1406, 1376, 1215, 1059, 1035, 894, 825, 762, 703; ^1H NMR (200 MHz, $CDCl_3$) δ 0.78–0.97 (m, 6), 1.03–1.42 (m, 26), 1.42–1.75 (m, 7), 1.75–2.12 (m, 2), 3.00–3.18 (m, 2), 3.58 (d, 1.5, J = 10), 3.60 (d, 1.5, J = 10), 7.16–7.38 (m, 9), 7.38–7.55 (m, 6); ^{31}P NMR ($CDCl_3$) δ 59.7, 59.8; MS (FAB) calcd for $C_{40}H_{59}O_3P$ 618.4, found 619 $(MH)^+$.

Detritylated Phosphinate 15. The detritylation of compound **15** was similar to the one described for **4**. The compound was purified on silica with 1% CH_3OH in $CHCl_3$ to afford a yellow oil in 79% yield: R_f (2 spots) = 0.1 and 0.03 (50% EtOAc in petroleum ether); IR (neat) 3355 br, 2917, 2850, 1457, 1204, 1180, 1133, 1042, 818; ^1H NMR (200 MHz, $CDCl_3$) δ 0.78–0.97 (m, 6), 1.15–1.47 (m, 26), 1.47–2.10 (m, 7), 3.35–3.59 (m, 1), 3.63–3.70 (m, 1), 3.70 (d, 1.5, J = 11), 3.73 (d, 1.5, J = 11), 4.27–4.38 (m, 0.5), 4.42–4.53 (m, 0.5); ^{31}P NMR ($CDCl_3$) δ 61.7, 63.2; MS (FAB) calcd for $C_{21}H_{45}O_3P$ 376.3, found 377 $(MH)^+$.

Phospholipid Analogue 16. The coupling procedure of **15** with methylphosphonic dichloride was similar to the one described for **12a**. The phosphinyl methyl ester was then hydrolyzed by acidifying the reaction mixture to pH 2 with 1 N HCl. After being stirred at room temperature for 2 h, the reaction mixture was extracted twice with ether. The organic solvent was removed in vacuo after drying over anhydrous $MgSO_4$. The compound was purified in 65% yield in a similar manner as described for **6b–12b**, except a gradient of 3–50% CH_3OH in $CHCl_3$ was used: R_f = 0.20 (3:6.5:0.5 $CH_3OH/CHCl_3/NH_4OH$); IR (KBr) 3423 br, 3144 br, 2929, 2854, 1637, 1460, 1401, 1385, 1304, 1159, 1073, 896, 821; ^1H NMR (500 MHz, 40% CD_3OD in $CDCl_3$) δ 0.78–1.00 (m, 6), 1.11–1.44 (m, 29), 1.44–1.72 (m, 6), 1.89–2.05 (m, 1), 3.89–4.11 (m, 2); ^{31}P NMR (40% CD_3OD in $CDCl_3$) δ 24.9, 43.9; MS (FAB) calcd for $C_{21}H_{46}O_5P_2$ 440.3, found 441 $(MH)^+$.

Phospholipid Analogue 17. Compound **17** was prepared in a similar manner as that described for **12b** except the racemic tritylglycidol was ring-opened with nonylmagnesium bromide.[44] The racemic tritylglycidol was prepared in a similar manner as **3**. The product was purified on silica with CH_2Cl_2 to afford a pale yellow oil in 82% yield. A freshly prepared solution of nonylmagnesium bromide (40 mL, 1.7 M in dry THF) was added slowly to a solution of tritylglycidol (7.2 g, 22.7 mmol) and Cu(I)Br (228 mg, 1.59 mmol) in dry THF (100 mL) under N_2 at 0 °C. After being stirred at room temperature for 1 h, the solution was washed with 100 mL of saturated aqueous NaCl and extracted twice with 50-mL portions of ether. The solvent was removed in vacuo after drying over anhydrous Na_2SO_4. The material was purified on silica with 10–30% ether in petroleum ether to afford a wax-like material in 76% yield: R_f = 0.41 (CH_2Cl_2); IR ($CHCl_3$) 3592, 3063, 3022, 2928, 2856, 1598, 1491, 1449, 1219, 1073; ^1H NMR (200 MHz, $CDCl_3$) δ 0.87 (t, 3, J = 7.2), 1.08–1.47 (m, 18), 2.31 (d, 1, J = 3.6), 3.01 (dd, 1, J = 9.6, 7.8), 3.17 (dd, 1, J = 9.6, 3.4), 3.67–3.90 (m, 1), 7.1–7.7 (m, 15).

The alcohol was coupled to phosphonochloridate **2**: yield 48% (yellowish oil); R_f = 0.27 (50% EtOAc in petroleum ether); IR (neat) 3059, 3023, 2920, 2854, 1491, 1449, 1378, 1255, 1183, 1155, 1051, 993; ^1H NMR (200 MHz, $CDCl_3$) δ 0.78–0.98 (m, 6), 1.02–1.41 (m, 26), 1.43–1.84 (m, 4), 3.03–3.30 (m, 2), 3.58 (d, 1.5, J = 11), 3.69 (d, 1.5, J = 11), 4.49–4.70 (m, 1), 7.18–7.54 (m, 15).

The racemic phosphonate was detritylated to give the alcohol: yield 85% (yellowish oil); R_f (2 spots) = 0.1 and 0.05 (50% EtOAc in petroleum ether); IR (neat) 3369 br, 2919, 2848, 1458, 1217, 1054, 993, 824; ^1H NMR (200 MHz, $CDCl_3$) δ 0.78–0.98 (m, 6), 1.18–1.47 (m, 26), 1.47–1.88 (m, 4), 3.60–3.86 (m, 2), 3.76 (d, 1.5, J = 11), 3.77 (d, 1.5, J = 11), 4.29–4.48 (m, 1).

The alcohol was phosphonylated with methylphosphonic dichloride followed by demethylation of the phosphonyl methyl ester to obtain inhibitor **17** with an overall yield of 46% (viscous brown oil, free acid form): R_f = 0.1 (50% CH_3OH in $CHCl_3$); IR (neat) 3373 br, 2922, 2850, 2635 br, 2307 br, 1683 br, 1462, 1309, 1206, 991; ^1H NMR (200 MHz, 10% CD_3OD in $CDCl_3$) δ 0.78–0.96 (m, 6), 1.10–1.40 (m, 26),

(41) Stotter, P. L.; Hill, K. A. *Tetrahedron Lett.* **1972**, 4067–4070.
(42) Bailey, W. J.; Hermes, M. E.; Klein, W. A. *J. Org. Chem.* **1963**, *28*, 1724–1725.
(43) Anisuzzaman, A. K. M.; Whistler, R. L. *Carbohydr. Res.* **1978**, *61*, 511–518.
(44) Nájera, C.; Sansano, J. M. *Tetrahedron* **1990**, *46*, 3993–4002.
(45) Jain, M. K.; Gelb, M. H. *Methods Enzymol.* **1991**, *197*, 112–125.

1.40–1.82 (m, 7), 3.83–4.15 (m, 2), 4.39–4.59 (m, 1); ^{31}P NMR (10% CD_3OD in $CDCl_3$) δ 32.4 and 33.1; MS (FAB) calcd for $C_{20}H_{44}O_6P_2$ 442.3, found 443 $(MH)^+$.

Determination of the $X_I(50)$ Values. The preparation of DMPM, the preparation of sonicated vesicles, and the scooting mode assay were carried out as described.[44] For each assay, 60 μL of a 10 mg/mL solution of sonicated DMPM was added to a reaction cup containing 4 mL of solution containing 0.6 mM $CaCl_2$ and 1.0 mM NaCl. The solution containing $CaCl_2$ and NaCl was preequilibrated to pH 8.0 in the pH stat (Radiometer ETS822 system) before the addition of vesicles. The reaction was initiated by adding an appropriate amount of PLA2 followed by the immediate addition of 78 μL of 100 mM $CaCl_2$ (typically 0.04 μg of porcine pancreatic PLA2, 0.3 μg of bee venom PLA2, 0.75 μg of bovine pancreatic PLA2, and 0.8 μg of cobra venom PLA2). The reaction was maintained at pH 8.0 by continuous pH-stat titration with 3 mM NaOH. All reactions were carried out in a thermostated vessel at 21 °C. Inhibition studies were done by adding a few microliters of inhibitor solution (0.05–1.0 mM in methanol) prior to the addition of enzyme. The $X_I(50)$ values were calculated by dividing the moles of inhibitor needed to achieve 50% inhibition of the initial velocity by the number of moles of phospholipids in the outer layer of the vesicles (60% of the total phospholipid in the reaction mixture; previous studies have shown that short-chain phosphonate phospholipid analogues do not flip to the inner side of the bilayer during the time course of the experiments[5]). $X_I(50)$ values were obtained by fitting the inhibition data to eq 1. Data sets contained at least 5 values of X_I that produce 10–80% inhibition. Vesicles of 7 mol % DOPA and 93 mol % OPPC were prepared by dissolving both phospholipids (Avanti Polar Lipids) in $CHCl_3$/MeOH (1:1). After the solvent was removed in vacuo, water was added to the lipid film and the suspension was sonicated as above or until the solution became clear (typically several minutes).

PLA_2 Assays with diC_6thio-PM. Assays were carried out essentially as described.[20] Enzyme was added to 300 μL of a buffer solution (25 mM Tris·HCl, 0.1 M KCl and 10 mM $CaCl_2$ at pH 8.0) containing 0.8 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.3 mM of diC_6thio-PM in a microcuvette. The reaction progress was monitored at 412 nm at room temperature. Inhibition studies were done by adding an appropriate amount of inhibitor solution (0.05 to 2.0 mM) in methanol prior to the addition of enzyme. The enzyme activity was not significantly affected when less than 50 μL of methanol was present in the assay buffer.

Stability of Inhibitor 10b and 12b in the Presence of Phospholipase C. Phospholipase C type XI from *Bacillus cereus* (0.062 mg, Sigma) was added to a sonicated solution containing 2.3 mg of inhibitor in 0.15 M NaCl, 2.5 mM $ZnCl_2$, 25 mM Hepes, and 0.1 mM $CaCl_2$, pH of 7.5. The solution was stirred at 34 °C for 68 h and then extracted with 2 mL of 10% methanol in chloroform. TLC analysis with the solvent CH_3OH/$CHCl_3$/NH_4OH (3:6.5:0.5) revealed that no hydrolysis product (**5b**) was formed. Similar treatment of 1,2-dipalmitoylphosphatidylcholine (2.9 mg) gave the expected diglyceride product.

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