Design and Synthesis of Some Substrate Analogue Inhibitors of Phospholipase A_2 and Investigations by NMR and Molecular Modeling into the Binding Interactions in the Enzyme–Inhibitor Complex

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A series of substrate analogue inhibitors of pancreatic phospholipase A_2 has been designed and synthesized. The compounds were tested in a novel dual-screening system based on parallel assays with monomeric and micellar substrates. Intermolecular nuclear Overhauser effects between vinylic protons on one inhibitor and identified active site residues on the bovine pancreatic enzyme have been observed in solution NMR studies of the enzyme–inhibitor complex. It can be deduced from both the biochemical results and the NMR data that the mode of interaction between this type of inhibitor and the active site of phospholipase A_2 is essentially the same, irrespective of the presence or absence of an aggregated phospholipid surface. A model of the binding between the enzyme and inhibitor which incorporates the two-dimensional NMR data has been developed. The model can account for the activity of modified inhibitor structures and can be extrapolated to an assessment of the mode of binding of the natural substrate itself.

The phospholipases A_2 (PLA_2's) are a ubiquitous group of enzymes which catalyze the hydrolysis of fatty acids, including arachidonic acid, from the sn-2 position of aggregated glycerophospholipids.[1] Subsequent metabolism of arachidonic acid by the cyclooxygenase and lipoxygenase enzymes results in the formation of a cascade of eicosanoid products, including the prostaglandins[2] and leukotrienes,[3] which are believed to play a major role in the inflammatory response. The other product of PLA_2 action is a lysophospholipid, which at low concentrations can give rise to changes in the properties of biological membranes.[4] *sn*-1-*O*-Alkyl-substituted lysophosphocholines can be further metabolized by acetylation at the sn-2 position to form platelet activating factor (PAF), another potent cellular mediator.[5]

As a consequence of its role in the formation of these products, the activity of PLA_2 has been widely associated with asthma,[6] psoriasis,[7] rheumatoid arthritis,[8] and other inflammatory conditions, and also with the damage caused by myocardial ischaemia and related cardiovascular diseases.[9] Indeed, elevated levels of PLA_2 activity have been detected in the synovial fluid and serum of patients with rheumatoid arthritis and in the plasma of patients suffering circulatory collapse as a result of endotoxin shock.[10] It is clear that a potent and selective inhibitor of PLA_2 would have great potential for the treatment of a wide range of common clinical disorders.

To date, the most widely studied PLA_2's are the low molecular weight (14 kDa), Ca^{2+} dependent, extracellular enzymes[1] which have been isolated from such sources as bee venom, snake venom, and mammalian pancreas and which are also secreted by other mammalian cell types such as platelets.[11] The aforementioned synovial fluid PLA_2 is considered to be a member of this class. At least 40 such proteins have been isolated and sequenced and a number have been structurally modified using the techniques of molecular biology.[12] Recently, the X-ray crystal structures of the human rheumatoid arthritic synovial fluid,[13] bee venom,[14] snake venom,[15] and pancreatic[16] PLA_2's, with or without inhibitors bound at the active site, have been reported. These studies have shown that the three-dimensional structures of the secreted PLA_2's are very similar to each other, particularly with respect to the active

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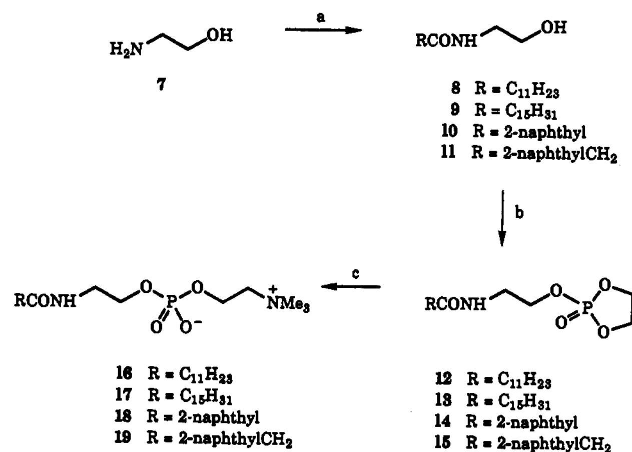
site residues, and have helped to indicate a possible catalytic mechanism for the hydrolysis of substrate.[17]

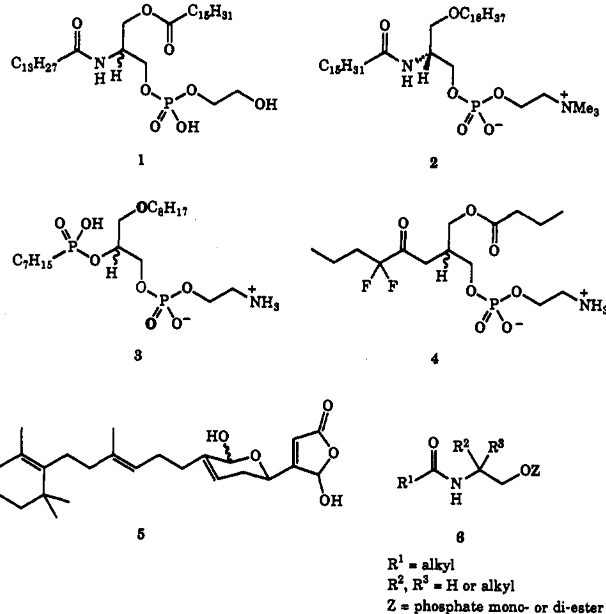
Evidence for the role of a secreted PLA_2 in the inflammation associated with conditions such as rheumatoid arthritis is provided by the observation[18] that a dramatic inflammatory response was produced when the pure, recombinant human synovial fluid enzyme was injected into the joint space of healthy rabbits.[19]

The biochemical properties of previously reported inhibitors of the secretory PLA_2's have been discussed in a number of recently published reviews.[21] Substrate analogue (or "transition state mimic") inhibitors including amides 1[22] and 2,[23] phosphonate 3,[24] and fluoro ketone 4[25]

Scheme I[a]



[a] Reagents: (a) RCOCl, Et_3N or RCOOH, DCC; (b) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N; (c) Me_3N, sealed tube, 65 °C.



appear to be reasonably potent and specific. The sesterterpenoid manoalide (5) and its analogues inhibit PLA_2 (especially that isolated from bee venom) irreversibly by covalent interaction with nucleophilic residues on the protein.[26] The inhibition by manoalide, however, is not

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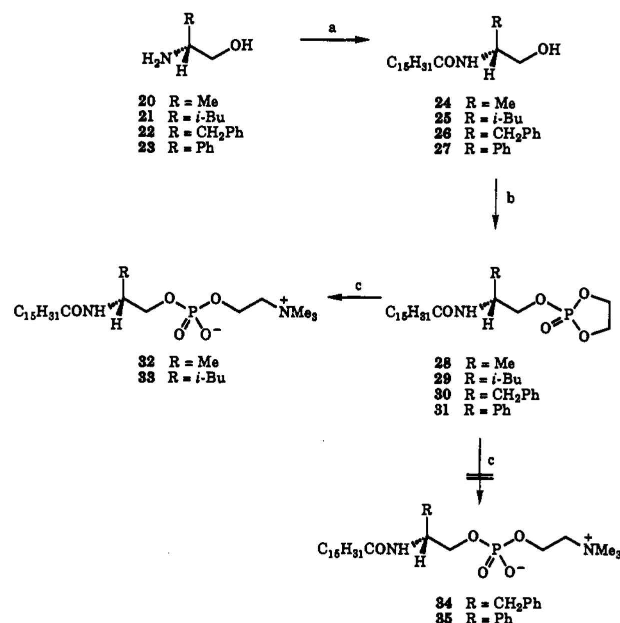
particularly specific, as other enzymes including phospholipase C[27] and 5-lipoxygenase[28] are also inhibited by this compound. Many other molecular species, for example cationic amphiphilic drugs, which have been claimed to inhibit PLA_2, owe their activity to their ability to alter the interfacial characteristics of the phospholipid bilayer or micelle substrate and not to a specific interaction with the enzyme itself.[29]

We now report[30] some of our preliminary studies on the design and synthesis of simplified substrate analogue inhibitors of pancreatic PLA_2. At the outset of the investigation, these particular enzymes were selected for study because of the availability of the corresponding X-ray crystal structures and the expectation that the pancreatic enzymes would provide a good model from a structural and mechanistic point of view for the secreted PLA_2 enzyme which is implicated in disease. This view has subsequently been confirmed by the publication of crystallographic data relating to the human synovial fluid enzyme.[13]

The inhibitor molecules of general structure **6** were designed after a consideration of the minimum substrate structure requirements of the porcine pancreatic enzyme as defined by de Haas and co-workers.[31] The compounds incorporate an amide bond as a replacement for the scissile ester bond of the glycerophospholipid and have a much reduced lipophilicity compared to that of the natural substrate and some inhibitors of related structure.[32]

We also describe a biochemical approach to the testing of inhibitors against the porcine enzyme using a dual-screening system based on parallel assays with monomeric and micellar substrates. This approach obviates the complexities associated with the nonspecific action of putative inhibitors on the aggregated natural substrate.[33] Finally,

Scheme II[a]



[a] Reagents: (a) RCOCl, Et_3N or RCOOH, DCC; (b) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N; (c) Me_3N, sealed tube, 65 °C.

we outline our approach to determining the mode of binding of our inhibitors to the active site of the bovine pancreatic enzyme[34] in solution, using two-dimensional (2D) NMR and molecular modeling techniques.

Chemistry

Compounds **16** and **17** have already been described as inhibitors of PLA_2 by Hajdu.[35] For their preparation, we have used a similar reaction sequence to that outlined previously (Scheme I). Thus, ethanolamine (**7**) was N-acylated either using an acid chloride with triethylamine as a base, or by using dicyclohexylcarbodiimide to couple the carboxylic acid to the amine. The resulting amido alcohols **8** and **9** were then phosphorylated using 2-chloro-2-oxo-1,3,2-dioxaphospholane[36] in dry benzene, with triethylamine as a base, to afford the cyclic phosphates **12** and **13**. These intermediates were found to be rather unstable and were normally treated immediately with a solution of trimethylamine in acetonitrile. Heating at 65 °C overnight in a sealed tube afforded the desired amido phosphocholines **16** and **17**, albeit only in low yield. The novel naphthyl analogues **18** and **19** were prepared by a similar sequence of reactions.

It was anticipated that compounds **32–35** (Scheme II)[37]

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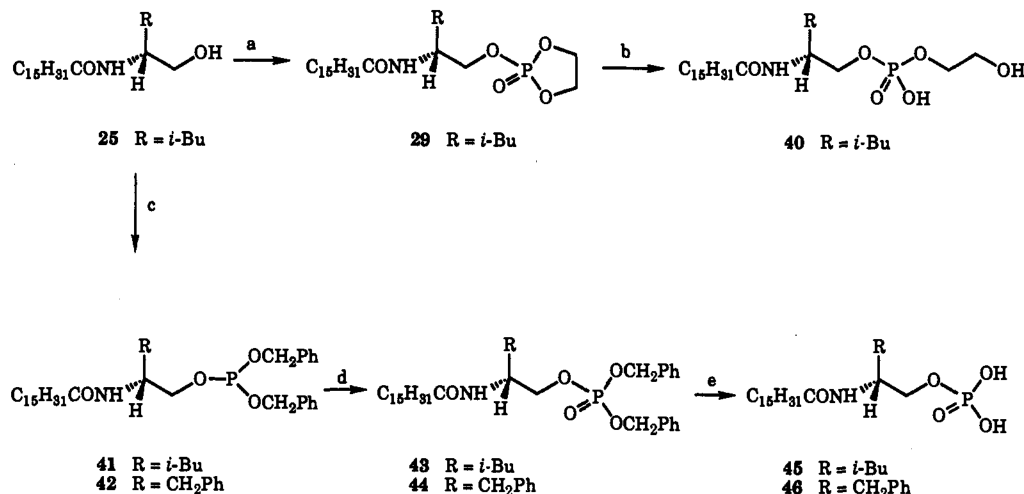
Table I. In Vitro Inhibition of Porcine Pancreatic Phospholipase A_2

	substituents				IC_{50},[a] μM	
	R^1	R^2	R^3	Z[b]	monomeric[c]	DOC-micelle[d]
1[e]	$C_{13}H_{27}$	$CH_2OCOC_{15}H_{31}$	H	PG	0.03	3.5
2	$C_{15}H_{31}$	$CH_2OC_{16}H_{31}$	H	PC	NT	15
16	$C_{11}H_{23}$	H	H	PC	NA	NA
17	$C_{15}H_{31}$	H	H	PC	4	300
18	2-naphthyl	H	H	PC	S	NA
19	2-naphthyl-CH_2	H	H	PC	>1000	NA
32	$C_{15}H_{31}$	Me	H	PC	1.4	189
33	$C_{15}H_{31}$	*i*-Bu	H	PC	0.23	30
36	$C_{15}H_{31}$	H	*i*-Bu	PC	4.5	NA
37	2-naphthyl-CH_2	*i*-Bu	H	PC	160	NA
38	*E*-3,4-C_9H_{17}	*i*-Bu	H	PC	6	NT
39	*E*-3,4-C_9H_{17}	H	*i*-Bu	PC	NA	NT
40	$C_{15}H_{31}$	*i*-Bu	H	PG	0.03	2.6
45	$C_{15}H_{31}$	*i*-Bu	H	PA	10	22% at 30
46	$C_{15}H_{31}$	CH_2Ph	H	PA	5	42% at 30

[a] NA, not active at the highest concentration tested; NT, not tested; S, stimulated. [b] Z, phospholipid headgroup; PG, phosphoglycol; PC, phosphocholine; PA, phosphate monoester. [c] The assay was performed with substrate **47** below its CMC as described in the Experimental Section. [d] The assay was performed using a phospholipid substrate dispersed in a mixed micelle with DOC as described in the Experimental Section. [e] Racemic.

Scheme III[a]



[a] Reagents: (a) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N; (b) dilute HCl, CH_3CN; (c) $(iPr)_2NP(OCH_2Ph)_2$, tetrazole; (d) MCPBA; (e) H_2, 10% Pd/C.

could be prepared from the commercially available D-amino alcohols **20–23** using a similar reaction sequence to that described above. Acylation of D-alaninol (**20**), D-leucinol (**21**), D-phenylalaninol (**22**), and D-phenylglycinol (**23**) with hexadecanoic acid afforded the required amido alcohols **24–27** in good yields. However, synthesis of the corresponding phosphocholines via the intermediate cyclic phosphates **28–31** was not straightforward. Although the methyl-substituted target **32** was prepared by this route, it was only isolated in poor yield and, despite various attempts, the benzyl- and phenyl-substituted targets **34** and **35** could not be obtained. In contrast, the isobutyl compound **33** was produced in a reasonable overall yield for this sequence.

Starting with L-leucinol, the enantiomer **36** (Table I) of the isobutyl compound **33** was synthesized in a similar manner to that already described. The (naphthylmethyl)amide **37** and the enantiomeric *trans*-dec-3-enylamides **38** and **39** (Table I) were also synthesized via the above route by coupling the appropriate acid to D- or L-leucinol followed by the standard phosphorylation and ring-opening procedure.

The phosphoglycol[22] analogue **40** (Scheme III) of phosphocholine **33** was prepared from the corresponding cyclic phosphate **29** by hydrolysis in acetonitrile with a few drops of dilute hydrochloric acid.

Synthesis of the corresponding phosphate monoester **45** utilized phosphoramidite methodology[38] (Scheme III). Thus, the alcohol **25** was reacted with dibenzyl *N,N*-diisopropylphosphoramidite using tetrazole as an activating agent. The resulting phosphite triester **41** was oxidized

(37) Since the completion of this work, a number of compounds of related structure were reported to be inhibitors of PLA_2 under micellar conditions; see: de Haas, G. H.; Dijkman, R.; Ransac, S.; Verger, R. Competitive Inhibition of Lipolytic Enzymes. IV. Structural Details of Acylamino Phospholipid Analogues Important for the Potent Inhibitory Effects on Pancreatic PLA_2. *Biochim. Biophys. Acta* **1990**, *1046*, 249–257.

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in situ with *m*-chloroperbenzoic acid and the dibenzyl phosphate **43** isolated in good yield. Catalytic hydrogenation provided the desired product **45**. In this series it was possible to prepare the analogous benzyl-substituted compound **46** (Table I).

Inhibitor Screening Strategy

PLA_2 activity has previously been measured in assays where the substrate was in the form of monomers, surface films, bilayer vesicles, micelles, or mixed micelles of phospholipid and detergent.[39] There are, however, specific problems associated with using each of these substrate forms in screening for inhibitors.

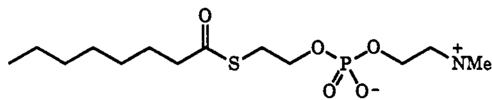
The ability of porcine pancreatic PLA_2 to bind to and hydrolyze phospholipids in a micelle or vesicle is strongly influenced by factors such as surface tension, phase transitions, and surface charge.[39] A number of compounds which have been reported to be inhibitors of PLA_2 have been found to change the properties of the interface in such a way that the enzyme binds less well, resulting in a decrease in catalytic activity. Inhibitors which have been shown to influence the association of the enzyme with the substrate interface in various ways are mepacrine, aristolochic acid, and lipocortin.[29] As only inhibitors which have their effect by binding directly to the enzyme are likely to be of therapeutic interest, it was considered necessary to design a screening procedure which would eliminate those compounds which influence PLA_2 activity indirectly.

Two approaches to solving this problem are employed by other workers. Firstly, dispersal of the phospholipid substrate in an excess of detergent is used to give a micelle interface which is less sensitive to disruption by added compounds than bilayers.[40] Secondly, bilayer vesicles can be prepared with dimyristoylphosphatidylmethanol which provides an interface for which PLA_2 has a very high affinity.[29] In this system PLA_2 is tightly bound and never leaves the surface of the vesicle (the so-called "scooting" mode of hydrolysis[29]), and the tendency of some compounds to cause dissociation of the enzyme from the surface is overwhelmed by this high affinity for the interface.

Although both these assay procedures have useful characteristics, inhibitor structure–activity relationships which are obtained may be distorted by extra complexities to which aggregated substrate assays are subject. For example, depending on its physicochemical properties, a compound will partition between the aggregated substrate and the aqueous phase. Therefore, when bound to the interface the enzyme may encounter locally high (or low) concentrations of inhibitor. As a result, inhibitor activity may depend both on intrinsic potency and on partition coefficient.

Clearly, all the complications arising from the aggregated nature of the substrate would be removed if a soluble, monomeric substrate were to be used in the assay. Unfortunately, however, the rate of PLA_2-catalyzed hydrolysis of monomeric substrates in solution is very slow compared to that when the substrate is present in an aggregated form.[39] Furthermore, there has been concern over whether the properties of the active site, such as hydration level and conformation, are the same when the enzyme is hydrolyzing monomers in solution as compared with a more physiological aggregated substrate.

In light of the foregoing considerations, the use of a single assay to screen for inhibitors of PLA_2 is likely to give misleading results. In this study, substrate analogue inhibitors of porcine pancreatic PLA_2 were evaluated in parallel in two complementary screens: (i) a monomeric assay employing the short chain thioester substrate **47**[41]

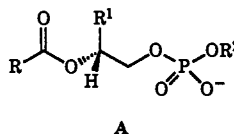


47

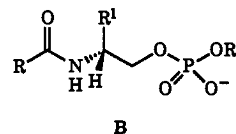
in which the enzyme obeyed Michaelis–Menten kinetics and (ii) a mixed micelle system in which substrate was dispersed in an excess of sodium deoxycholate (DOC) to provide a relatively robust interface, and the assay performed under conditions where the enzyme is believed to be able to move between micelles ("hopping" rather than "scooting" kinetics[29]). Although both assays are open to certain criticisms, these criticisms are different for each procedure. Therefore, if a series of compounds inhibited in both screens with a parallel structure–activity relationship, a greater degree of confidence could be placed in the conclusion that the inhibition was due to a direct interaction with the enzyme.

Inhibitor Design and Structure–Activity Relationships

The pioneering work of de Haas and his group[31] has helped to define the minimum structural features which a molecule requires in order to function as a substrate for PLA_2. It was shown that the enzyme would catalyze the hydrolysis of an acyl glycol moiety when appropriately substituted with an acidic function (general structure A, R^1 = H). Furthermore, it was found that replacement of the scissile ester bond of a phosphoglycerol substrate with an amide group, as in **1**,[22] produced a molecule which was a competitive inhibitor of the enzyme.



A



B

Our studies commenced with the assumption that the replacement of the scissile ester bond in the "minimum substrate structure" by an amide group would give rise to a "minimum inhibitor template" of general structure B (R^1 = H). Since a major problem associated with the design of drug molecules based on substrate analogues of PLA_2 is the high lipophilicity of the sn-1 and sn-2 alkyl chains of the natural phospholipid, it was anticipated that the "minimum inhibitor template" would represent a favorable starting point for the design of inhibitors with more suitable physicochemical properties.

Results of screening in both the monomeric and DOC-micelle assays are detailed in Table I. It can be seen from the activity of **17** that the simple aminoethanol-based phosphocholine derivative showed only weak inhibition when N-acylated with a C-16 fatty acid. Related compounds with shorter, less lipophilic substituents such as **16**, **18**, and **19** were inactive at the highest concentration tested.

Introduction of simple alkyl groups into a position in the inhibitor molecule which corresponds to the sn-1

(39) Verheij, H. M.; Slotboom, A. J.; de Haas, G. H. Structure and Function of PLA_2. *Rev. Physiol. Biochem.* **1981**, *91*, 91–103.

(40) Yuan, W.; Berman, R. J.; Gelb, M. H. Synthesis and Evaluation of Phospholipid Analogues as Inhibitors of Cobra Venom PLA_2. *J. Am. Chem. Soc.* **1987**, *109*, 8071–8081.

(41) Yuan, W.; Quinn, D. M.; Sigler, P. B.; Gelb, M. H. Kinetic and Inhibition Studies of PLA_2 with Short-Chain Substrates and Inhibitors. *Biochemistry* **1990**, *29*, 6082–6094.

Table II. Effect on the Chemical Shift of Selected Enzyme Resonances on Binding of Compounds 38 or 39 to Bovine Pancreatic Phospholipase A_2

residue and atom type	chemical shift[a] in native enzyme	change in shift on binding of compound **38**	change in shift on binding of compound **39**
Trp-3 C(4,5)H	7.60, 7.27	−0.03, −0.08	−0.01, −0.02
Trp-3 C(6,7)H	7.32, 7.51	−0.06, −0.03	−0.02, 0.00
Phe-5 H*o*,*m*,*p*	7.10, 6.96, 6.34	+0.06, 0.00, −0.14	0.00, −0.02, −0.10
Ile-9 CδH_3	0.02	−0.08	−0.07
Leu-41 CδH_3	0.06, 0.45	+0.04, +0.05	+0.03, −0.01
His-48 C(2,4)H	6.37, 6.63	−0.13, +0.07	+0.08, +0.01
Tyr-52 H*o*,*m*	6.71, 6.30	−0.05, +0.10	−0.03, −0.10
Tyr-69 H*o*,*m*	7.16, 6.81	+0.07, +0.28	+0.05, +0.07
Tyr-73 H*o*,*m*	6.78, 6.56	+0.07, 0.00	0.00, −0.01
Tyr-75 H*o*,*m*	6.95, 6.54	−0.01, −0.05	0.00, 0.00
Ala-102 Hα,β	4.17, 0.87	−0.11, −0.09	−0.02, +0.01
Ala-103 Hα,β	3.33, 1.07	−0.07, 0.00	+0.02, +0.01
Phe-106 H*o*,*m*,*p*	6.41, 6.87, 7.20	−0.11, −0.25, −0.34	−0.02, −0.09, −0.14
Tyr-111 H*o*,*m*	6.70, 6.13	+0.01, +0.08	+0.02, +0.07

[a] Measured in ppm relative to the CHα resonance of Cys-77 at 5.56 ppm. All spectra run in 50 mM $CaCl_2$, 200 mM NaCl, 10 mM d_{11}-Tris, pH 7.5 at 41 °C.

substituent of the natural substrate resulted in an improvement of activity. Potency increased as R^1 in general structure B was changed from hydrogen to methyl to isobutyl (compounds **17**, **32**, and **33**, respectively). However, it was particularly interesting to note that the activity of the simple isobutyl derivative **33** was very similar to that of the related inhibitor **2** bearing a long octadecyl ether chain. It can be seen from the results with compounds **37** and **38** that in the isobutyl series, activity decreased with less lipophilic or shorter and more bulky amide substituents.

A comparison of the activities of **33** with **45** (and **46**) showed that replacement of the zwitterionic phosphocholine headgroup by a dianionic phosphate severely reduced potency, while similar substitution by the monoanionic phosphoglycol (compare **33** with **40**) led to a 10-fold increase in activity. A similar result has been reported by de Haas[22] in a related series of phosphoglycerol inhibitors.

Evidence to support the belief that the inhibition of PLA_2 activity by this series of compounds was due to specific interaction with the enzyme was provided by the stereoselectivity of the inhibition. There was at least a 20-fold difference in the inhibitory activity of enantiomers **33** and **36**, with the more active isomer (**33**) possessing the *R*-stereochemistry associated with the natural substrate.

The data shown in Table I demonstrate that for this series of compounds there was a good correlation between the results in the monomeric and micellar assays. IC_{50}'s were approximately 100-fold lower in the simpler monomeric system, probably as a consequence of the lower enzyme binding affinity of the synthetic substrate.

NMR Studies of the Mode of Inhibitor Binding

In order to elucidate the mode of binding, complexes of substrate analogue inhibitors with bovine pancreatic PLA_2 in aqueous solution were analyzed by ^{1}H NMR spectroscopy. Enantiomeric phospholipids **38** and **39** were selected for study. Compound **38** contains a vinylic double bond at a position in the molecule which was, on the basis of inhibitor design, expected to bind within the active site of the enzyme. Such a species would give rise to signals in the ^{1}H NMR spectrum in a "window" which, from our previous work,[42] was found not to contain any signals from the protein itself. It was thus anticipated that identification of the vinylic proton resonances of the bound inhibitors would be straightforward, as indeed was the case. Two enantiomeric compounds were chosen in order that differences in their binding would provide information on the stereoselectivity of the enzyme.

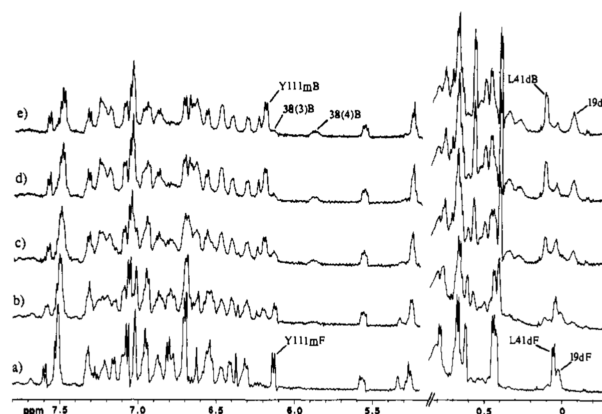


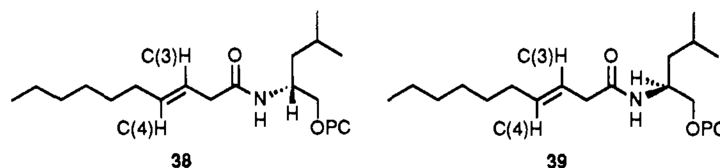
Figure 1. The aromatic, vinylic, and upfield methyl regions of the 1D ^{1}H NMR spectrum of an aqueous solution containing 2.0 mM bovine pancreatic PLA_2 and 50 mM Ca^{2+} in the presence of (a) 0, (b) 0.5, (c) 1.0, (d) 1.5 and (e) 2.0 mM **38**. Specific resonances are labeled as follows: Y111*m*, Tyr-111 H*m*; L41d, Leu-41 δ-methyl; I9d, Ile-9 δ-methyl; **38**(3), C(3)H of the inhibitor; **38**(4), C(4)H of the inhibitor. The suffixes F and B refer to signals from protons in the free enzyme or inhibitor and signals from the protons in the enzyme–inhibitor complex, respectively.

Addition of **38** to the enzyme caused considerable changes to the ^{1}H NMR spectrum of the protein (Figure 1). A large number of resonances in the aromatic region of the spectrum were affected, as might be expected given the preponderance of aromatic residues in the enzyme active site.[43] For example, as **38** was titrated into the solution of the enzyme, the NMR signal corresponding to Tyr-111 H*m* in the free protein was slowly replaced by a separate signal to lower field due to Tyr-111 H*m* in the enzyme–inhibitor complex (Figure 1). This behavior is characteristic of slow exchange on the NMR time scale, and indicates that the rate of dissociation of the complex is $<40\ s^{-1}$, corresponding to a binding constant of $>10^7\ M^{-1}$. In the aliphatic region of the spectrum, the two most upfield shifted resonances, which have been assigned[42] to the

(42) Fisher, J.; Primrose, W. U.; Roberts, G. C. K.; Dekker, N.; Boelens, R.; Kaptein, R.; Slotboom, A. J. ^{1}H NMR Studies of Bovine and Porcine PLA_2: Assignment of Aromatic Resonances and Evidence for a Conformational Equilibrium in Solution. *Biochemistry* **1989**, *28*, 5939–5946.

(43) Dijkstra, B. W.; Kalk, K. H.; Hol, W. G. J.; Drenth, J. Structure of Bovine Pancreatic PLA_2 at 1.7 Å Resolution. *J. Mol. Biol.* **1981**, *147*, 97–123.

Table III. Effect on the Chemical Shift of Selected Inhibitor Resonances on Binding of Compounds 38 or 39 to Bovine Pancreatic Phospholipase A_2



proton	chemical shift[a] in free inhibitor	change in shift on binding of compound 38	change in shift on binding of compound 39
$C(6)H_2$	1.33	−0.06	0.00
$C(5)H_2$	1.99	−0.03	+0.02
C(4)H	5.65	+0.29	0.00
C(3)H	5.52	+0.63	−0.01
$(CH_3)_3N^+$	3.20	+0.18	0.00

[a] Measured in ppm relative to the CHα resonance of Cys-77 at 5.56 ppm. All spectra run in 50 mM $CaCl_2$, 200 mM NaCl, 10 mM d_{11}-Tris, pH 7.5 at 41 °C.

δ-methyl protons of Leu-41 and Ile-9, were also perturbed, again showing slow exchange behavior. The changes in chemical shift for a number of functionally important protein residues are summarized in Table II. The resonances of the protein in the complex were assigned by semiquantitative use of the X-ray crystal structure[43] using 2D total correlation spectroscopy (TOCSY or HOHAHA) and nuclear Overhauser spectroscopy (NOESY) as reported previously.[42]

In order to assign unambiguously the resonances of the inhibitor (38) in its bound conformation with the enzyme, use was made of the Ca^{2+} dependence of the interaction. It has been postulated[44] that the role of Ca^{2+} in the binding and hydrolysis of the phospholipid substrate is 2-fold: firstly, the phosphate anion of the substrate coordinates to the bound Ca^{2+} at the enzyme active site and, secondly, the same Ca^{2+} ion acts as an electron sink for the developing negative charge on the scissile ester carbonyl in the transition state. It was anticipated that the binding of the substrate analogue inhibitors would also be Ca^{2+} dependent. In a sample containing a 1:1 mixture of 38 and bovine pancreatic PLA_2, but no Ca^{2+} ions, the exchange rate of the inhibitor between the enzyme bound and free conformations was sufficiently increased to allow the observation of exchange cross-peaks between free and bound inhibitor in a NOESY spectrum (data not shown). The assignment of the vinylic protons in the enzyme–inhibitor complex showed that there was a large downfield shift in the position of the C(3)H resonance (Table III). A limited number of other resonances of the bound inhibitor were also identified by this method. The majority remain unassigned, however, due to the small chemical shift dispersion of the protons on the alkyl chains of the inhibitor, and the complexity of the spectrum resulting from the loss of degeneracy on binding to the enzyme.

The NOESY spectrum of the complex between 38 and enzyme (Figure 2) in the presence of Ca^{2+} yielded information on the altered conformation that the enzyme adopts in order to facilitate binding, and also on the precise location of the bound inhibitor. A number of additional inter-residue nuclear Overhauser effects (NOE's), not seen in the native enzyme alone, appeared on binding 38. The aromatic rings of Tyr-52 and Tyr-69 moved closer together, as evidenced by the appearance of four strong NOE's between their protons (Figure 2). This showed that inhibitor

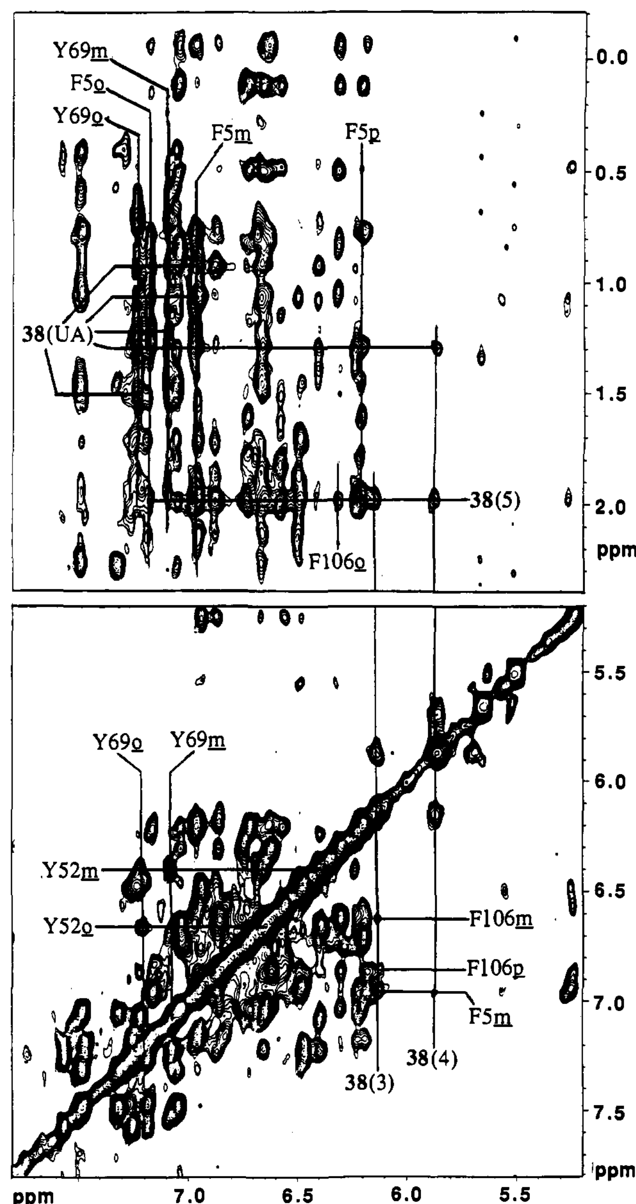


Figure 2. Portions of the 2D ^{1}H homonuclear NOESY spectrum of the 1:1 complex of bovine pancreatic PLA_2 and 38, showing correlations to aromatic, vinylic, and methyl protons. Intraprotein NOE's not present in the free enzyme are indicated, as well as NOE's between the protein and the inhibitor. Resonances are labeled as for Figure 1 as well as the following: F5, Phe-5; F106, Phe-106; 38(5), $C(5)H_2$ of the inhibitor; *o*, *m*, and *p* refer to $H_{2,6}$, $H_{3,5}$, and H_4 of aromatic residues, respectively; UA, unassigned.

(44) Verheij, H. M.; Volwerk, J. J.; Jansen, E. H. J. M.; Puyk, W. C.; Dijkstra, B. W.; Drenth, J.; de Haas, G. H. Methylation of His-48 in Pancreatic PLA_2. Role of Histidine and Calcium Ion in the Catalytic Mechanism. *Biochemistry* **1980**, *19*, 743–750.

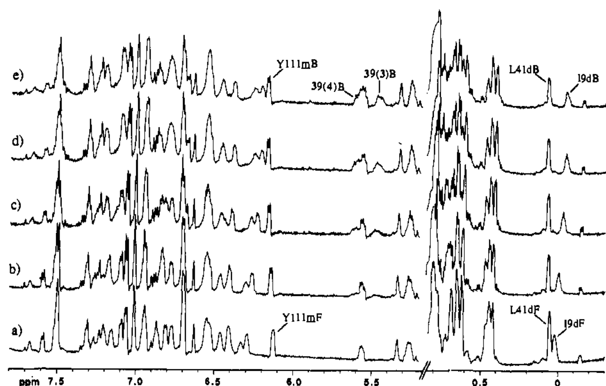


Figure 3. The aromatic, vinylic, and upfield regions of the 1D ^1H NMR spectrum of a solution of 2.0 mM bovine pancreatic PLA_2 and 50 mM Ca^{2+} in the presence of (a) 0, (b) 0.5, (c) 1.0, (d) 1.5, and (e) 2.0 mM **39**. Resonances are as labeled in Figure 1.

binding is accompanied by a movement of Tyr-69 across the mouth of the binding pocket. The shift is thought to be important in shielding the active site from solvent during catalysis and allowing the build up of charge separation during the formation of the tetrahedral oxyanion intermediate.[39] There was also a reduced number of NOE's between Phe-5 and Phe-106 compared to that observed in the enzyme alone.[42] These two residues make up the sides of a hydrophobic binding pocket within the enzyme, and appear to move apart in order to accommodate the binding of the ligand.

The ligand itself gave rise to a number of NOE's to protons of the protein (Figure 2). These showed that the C(3) vinylic proton on the inhibitor is bound close to one (or both) of Phe-5 H*m* and to Phe-106 H*m* and H*p*. The observations provide a rationalization for the large downfield shift of C(3)H on binding which resulted from the magnetic anisotropy of the aromatic rings. A very weak NOE was also seen between the inhibitor C(4)H and Phe-106 H*m*. The methylene protons of C(5) of the inhibitor gave rise to a strong NOE to Phe-5 H*p* and a weaker one to Phe-106 H*o* (top panel of Figure 2). Further NOE's from the inhibitor to Phe-5 and Tyr-69[45] were also observed, but unambiguous identification of the inhibitor protons responsible for these interactions has so far proved difficult. Nevertheless, the chemical shifts of these signals indicate that they arise from the isobutyl and/or decenyl substituents of the inhibitor. Subsequent modeling studies (see below) using distance restraints derived from the assigned NOE's showed that the isobutyl substituent binds close to Tyr-69, and that the unassigned NOE's are quite consistent with the derived structure.

The addition of **39** to a solution of the enzyme was also accompanied by changes in the ^1H NMR spectrum (Figure 3). However, in this case, as demonstrated by the behavior of the H*m* resonance of Tyr-111 (Figure 3), the spectra

(45) Previous work on the binding of various substrate analogue inhibitors to bovine PLA_2 suggests that these new NOE's are not intraprotein (Primrose, W. U.; Roberts, G. C. K., unpublished results). The binding of other compounds does not give rise to new NOE's to Phe-5 and Tyr-69 from other protein residues in this portion of the NOESY spectrum. The assumption that the structure of the protein in complex with 38 is not greatly different from that with any other bound compound is supported by the general similarity of the rest of the ^1H NMR spectrum to that of other PLA_2-inhibitor complex spectra. The large number of NOE's highlighted in the top panel of Figure 2 must therefore be due to close contacts between 38 and the enzyme. Exact identification of the nature of these interactions is currently underway using isotopically labeled inhibitors.

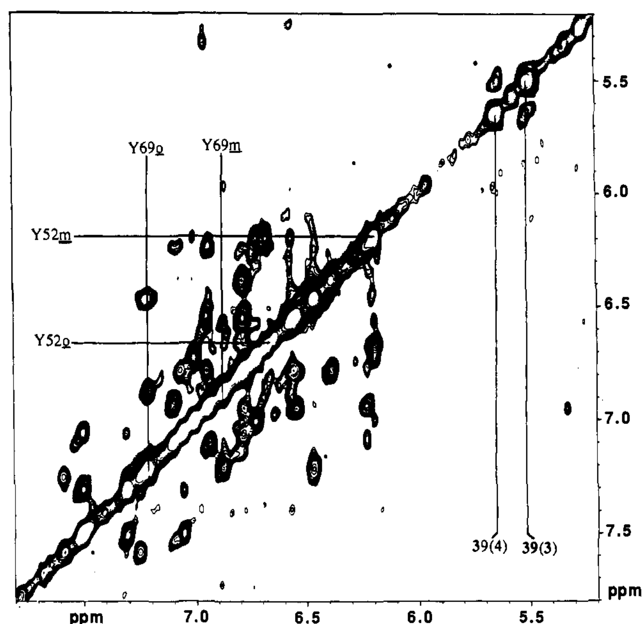


Figure 4. A portion of the 2D ^1H homonuclear NOESY spectrum of the 1:1 complex of bovine pancreatic PLA_2 and **39**, showing correlations between aromatic and vinylic protons. The lines indicate the expected positions of protein–inhibitor and new intraprotein NOE's as seen in Figure 2, but absent here. Resonances are as labeled in Figure 2.

represent a time average of those of the free enzyme and of the enzyme–inhibitor complex. This is indicative of fast exchange and weak binding. There was little or no change in the chemical shift of the inhibitor resonances on binding (Table III) and these appeared as fairly sharp lines in the spectrum of the 1:1 mixture of **39** and the enzyme. The changes in the protein spectrum (Table II) were much less widespread than those observed for the binding of **38**. The fact that any occurred at all was probably a consequence of nonspecific, hydrophobic interactions. The NOESY spectrum (Figure 4) of a 1:1 mixture of **39** and enzyme showed no evidence for NOE's between the inhibitor and the enzyme and no change in the NOE networks previously described for the free enzyme.[42] Crucially, there was no sign of the family of new NOE's between Tyr-52 and Tyr-69 that was observed on **38** binding. It can be concluded, therefore, that unlike the strong and specific binding between **38** and the enzyme, the interaction between **39** and the protein is both very weak and nonspecific.

Recently, a study has been reported[46] which describes the characterization by NMR of the binding of a related substrate analogue inhibitor to a pancreatic PLA_2 in the presence of a micellar lipid–water interface. Very similar intermolecular and intraprotein interactions to those described in our work were observed. An important point to note, however, is that our studies were performed in the absence of any form of lipid aggregate. Therefore, taken together, these findings indicate that the mode of inhibitor binding at the active site is essentially the same, irrespective of whether the enzyme is bound to a phospholipid surface or not. Furthermore, in the present work, the incorporation of the vinylic bond into **38** allowed the observation of NOE's between identified active site residues on the enzyme and specific protons on the inhibitor, thus

(46) Dekker, N.; Peters, A. R.; Slotboom, A. J.; Boelens, R.; Kaptein, R.; Dijkman, R.; de Haas, G. H. 2D ^1H-NMR Studies of PLA_2-Inhibitor Complexes Bound to a Micellar Lipid–Water Interface. *Eur. J. Biochem.* **1991**, *199*, 601–607.

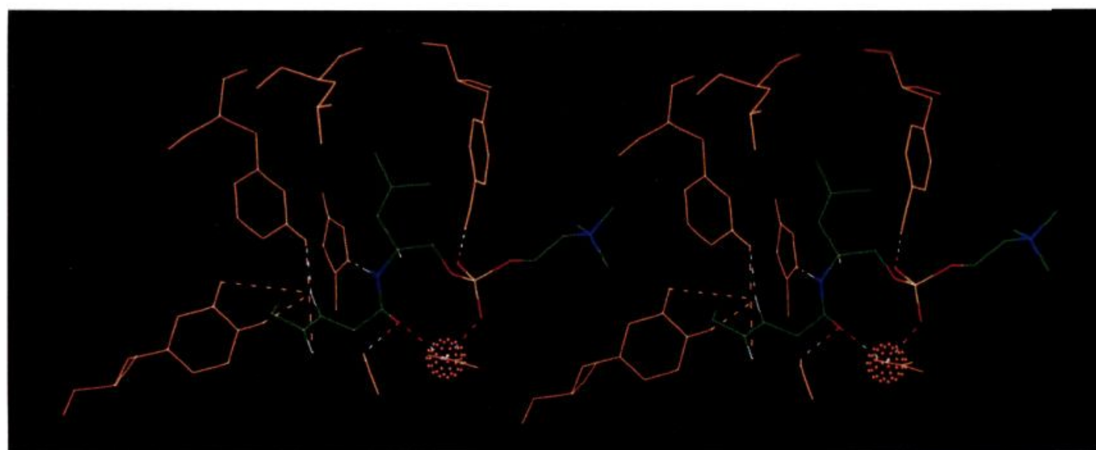


Figure 5. Stereoview of the energy-refined complex of inhibitor **38** (green) and key active site residues of bovine pancreatic PLA_2. The dotted lines represent interactions which are defined in the discussion and shown in Figure 6.

Table IV. Interproton Distances As Measured from the Computer Graphics Model of Compound **38** Complexed to Bovine Pancreatic Phospholipase A_2

proton of compound 38	protein residue and atom type	strength of observed NOE	distance, Å
C(3)H	Phe-5 H*m*	strong	2.2
C(3)H	Phe-106 H*m*	weak	3.5
C(3)H	Phe-106 H*p*	strong	2.5
C(4)H	Phe-5 H*m*	weak	4.6

defining the position of the hydrocarbon chain in the bound complex much more precisely.

A Model for Inhibitor Binding

A model was constructed for the complex of bovine pancreatic PLA_2 with inhibitor **38**. Molecular dynamics simulations commenced with the inhibitor outside the active site. Distance restraints were imposed in the modeling between C(3) and C(4) of the inhibitor and Phe-5 and Phe-106 on the enzyme to simulate the observed NOE interactions. The very weak distance restraints utilized during the molecular dynamics runs were sufficient to "dock" the inhibitor into the active site of the enzyme.

The structures were minimized using AMBER.[47] The measured distances between protons C(3)H and C(4)H and Phe-106 H*m* and H*p* and Phe-5 H*m* remained consistent with NMR NOE data (Table IV). The relative energies of minimized structures generated using distance geometry and molecular dynamics were used to evaluate and reject other possible modes of inhibitor binding.

Analysis of the results from these molecular modeling studies allows the identification of a number of key features which appear to be essential for good binding of the inhibitor (Figures 5 and 6). The phosphate anion provides an anchoring point for the inhibitor molecules. This group has one oxygen atom interacting with the essential Ca^{2+} ion at a distance of 2.25 Å and a second oxygen atom hydrogen bonding to the hydroxyl group of Tyr-69 at a distance of 1.85 Å. The carbonyl oxygen of the amide function interacts with the Ca^{2+} ion, with an interatomic distance of 2.4 Å, and forms a strong hydrogen bond with the amide hydrogen of Gly-30, the distance being 2.2 Å.

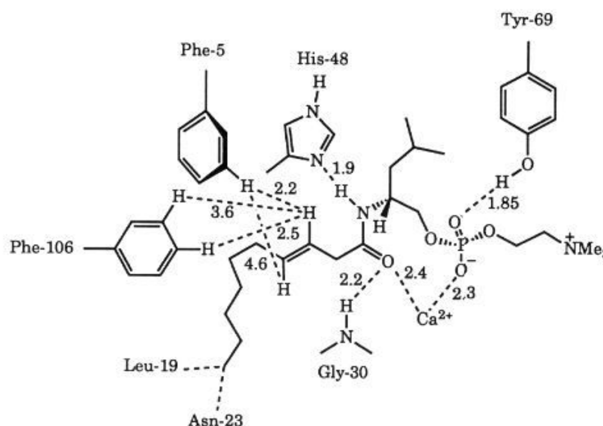


Figure 6. Schematic representation of the energy-refined complex of inhibitor **38** and bovine pancreatic PLA_2. Only the key residues of the enzyme active site have been included. Numbers indicate interatom distances in angstroms.

A specific aspect of the study design was the displacement of the "nucleophilic" water molecule found in the X-ray crystal structures of the native enzyme and the use of His-48 as a potential hydrogen-bonding site. In the model, His-48 was assumed to be unprotonated and under this condition the amide hydrogen of the inhibitor molecule formed a hydrogen bond to the N(δ)1-atom of this residue.

The first six carbon atoms of the hydrocarbon chain of **38** are buried deep within the active site of the enzyme. In this region, the chain turns back on itself to leave the active site adjacent to Leu-19 and Asn-23. This mode of binding positions the hydrogen atoms attached to C(3) and C(4) close to the hydrophobic pocket created by Phe-5 and Phe-106. The isobutyl moiety occupies the hydrophobic pocket created by Leu-2 and Tyr-69 (Figure 5), thus enhancing binding.

The results from this modeling study, which incorporates distance restraints indicated by the NOE's observed in the bovine enzyme–inhibitor complex, suggest that the native bovine pancreatic PLA_2–inhibitor interaction in aqueous solution is structurally very similar to that in the crystal state, as revealed in the recently published[48] X-ray structure of a related inhibitor complexed to a mutant form

(47) (a) Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P. A New Force Field for Molecular Mechanical Simulation of Nucleic Acids and Proteins. *J. Am. Chem. Soc.* **1984**, *106*, 765–784. (b) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. An All Atom Force Field for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* **1986**, *7*, 230–252.

(48) Thunnissen, M. M. G. M.; Eiso, A. B.; Kalk, K. H.; Drenth, J.; Dijkstra, B. W.; Kuipers, O. P.; Dijkman, R.; de Haas, G. H.; Verheij, H. M. X-Ray Structure of PLA_2 Complexed with a Substrate-Derived Inhibitor. *Nature* **1990**, *347*, 689–691.

of the enzyme. Furthermore, as can be deduced from Figures 5 and 6, the model indicates that the Ca^{2+} ion in the enzyme active site interacts with the *pro-S* oxygen atom of the phosphate group of the inhibitor. This observation is in accord with the stereoselectivity observed in kinetic studies of the bee venom PLA_2-catalyzed hydrolysis of phosphorothioate analogues of phosphocholines.[49]

If the natural substrate for PLA_2 adopts a similar conformation to that deduced for substrate analogue inhibitor 38 at the active site of the enzyme, then the hydrophobic chain of the scissile ester at the sn-2 position of the phospholipid must also come into close proximity with Phe-5 and Phe-106 and leave the active site near to Leu-19 and Asn-23. Furthermore, it is likely that the lipophilic chain at the sn-1 position of the substrate passes through the pocket created by Leu-2 and Tyr-69.

Conclusions

A series of substrate analogue inhibitors of pancreatic PLA_2 has been designed and synthesized. The molecules incorporate an amide bond as a replacement for the scissile ester bond of the substrate and have a much reduced lipophilicity compared to that of the natural phospholipid. Indeed, it has been demonstrated that within this series of inhibitors the small isobutyl group can substitute without loss of activity for the much larger and more lipophilic hexadecyloxymethyl group found in certain naturally occurring substrates. The resulting inhibitor is stereoselective with the more active enantiomer possessing the *R*-stereochemistry associated with the natural substrate for the enzyme.

A novel inhibitor screening strategy has been devised. The procedure, using porcine pancreatic PLA_2, is based on parallel monomeric and micellar substrate assays. This system obviates the complexities associated with the nonspecific interaction of compounds with the aggregated natural substrate and is able to identify and rank the activity of those compounds which bind directly to the enzyme. In light of the observed correlation of inhibitor potency in the monomeric and micellar substrate assays, it is surprising that other workers have reported[37] that related substrate analogue inhibitors completely lose their inhibitory power when using monomerically dispersed substrate assay systems. However, other monomeric assay systems differ extensively from ours in the nature of the substrate and the conditions used (particularly with respect to Ca^{2+} ion concentration), and these differences may be responsible for the apparent conflict of results.

Intermolecular NOE signals have been observed between vinylic protons of an inhibitor of PLA_2 and active site residues on the bovine pancreatic enzyme. This finding defines the position of the amide chain of the inhibitor in the active site pocket when the enzyme–inhibitor complex is in solution. In addition, the NMR data have shown that binding of the substrate analogue inhibitors to PLA_2 is accompanied by the movement of Tyr-69, such that its aromatic side chain partially occludes the entrance to the active site. We have earlier shown by NMR that the position of Tyr-69 is identical in the unliganded states of the bovine and porcine enzymes in solution.[42] Taken together, these observations clearly demonstrate that the difference in the position of Tyr-69 seen in the X-ray crystal structures of the two native enzymes[34] can be attributed to a crystal packing artifact.

Interestingly, both the correlation between the results in the monomeric and micellar substrate assays and the comparison of the NMR data presented here with that reported previously[46] indicate that, regardless of the presence or absence of an aggregated phospholipid surface, the mode of interaction between this type of inhibitor and the active site of PLA_2 is essentially the same.

A model of the binding between the bovine pancreatic enzyme and substrate analogue inhibitor which incorporates the 2D NMR evidence has been developed. It is of particular importance to note that only five restraints (four derived from NOE's and one between the phosphate of the ligand and the Ca^{2+} ion) were necessary to dock the inhibitor within the active site of the enzyme. The model can account for the activity of modified substrate analogues and can be extrapolated to an assessment of the mode of binding of the natural substrate itself. In particular, the model identifies a surprisingly small pocket in which the enzyme binds a portion of the substrate sn-1 acyl chain. The model is largely in accord with recently published X-ray data on a mutant pancreatic PLA_2 enzyme–inhibitor complex in the crystal state,[48] and with stereoselectivity findings derived from kinetic studies on the bee venom PLA_2-catalyzed hydrolysis of phosphorothioate analogues of phosphocholines.[49]

As a consequence of the close structural similarities between the pancreatic PLA_2's and the human rheumatoid arthritic synovial fluid enzyme, it is anticipated that this model of the enzyme–inhibitor complex will be of value for the design of other novel, potent inhibitors which are suitable for use in the treatment of inflammatory disorders in man.

Experimental Section

Chemistry. Melting points were determined in open capillary tubes with a Büchi melting point apparatus and are uncorrected. All compounds had elemental analyses for C, H, N within +/−0.4% of the theoretical value, except where indicated,[50] and structures are consistent with spectroscopic data (IR, ^{1}H NMR, and MS). Routine NMR spectra were recorded at 360 MHz on a Bruker AM360 spectrometer using TMS as standard. Mass spectra were recorded on a VG 70-250SEQ machine and optical rotations were measured with a Bendix Model 243 automatic polarimeter. Flash chromatography was performed on silica gel (Matrex Silica 60, 35–70 μm) according to the method of Still.[51] Acetonitrile, benzene, and triethylamine were distilled from CaH_2. Following aqueous extractions, organic solutions were dried over anhydrous magnesium sulfate.

General Procedure A. Preparation of Amido Alcohols 8–11 and 24–27. A solution of the amino alcohol (20 mmol) in CH_2Cl_2 (40 mL) was treated with either triethylamine (20 mmol) and the appropriate acid chloride (20 mmol) or dicyclohexylcarbodiimide (20 mmol) and the appropriate acid (20 mmol). The reaction mixture was stirred at room temperature for 1–18 h and filtered, and the resulting filtrate partitioned between 2 N HCl and $CHCl_3$. The organic layer was separated, washed with saturated aqueous $NaHCO_3$ solution and water, and dried. The solvent was removed by evaporation and the product purified by flash chromatography [C/solvent] or recrystallized [R/solvent].

***N*-(2-Hydroxyethyl)dodecanamide (8):** [R/acetone]; yield 57%; white solid; mp 90–92 °C (lit.[35] mp 83 °C); MS m/e (M^+) 243 ($C_{14}H_{29}NO_2$).

(50) The phosphocholine derivatives described in this work were isolated as extremely hygroscopic (and often deliquescent) solids or gums. We have often found during the nonencapsulation analysis technique that water of hydration is lost and this is reflected in a correspondingly low H analysis value.

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N-(2-Hydroxyethyl)hexadecanamide (9):[35] yield 80%; white solid; ^1H NMR ($CDCl_3$ + DMSO-d_6) δ 6.78 (bs, 1 H, NH), 4.20 (bs, 1 H, OH), 3.63 (m, 2 H, OCH_2), 3.35 (q, 2 H, NCH_2), 2.17 (t, 2 H, CH_2CO), 1.62 (m, 2 H, CH_2), 1.28 (m, 2 H, CH_2), 1.25 (s, 22 H, $(CH_2)_{11}$), 0.88 (t, 3 H, CH_3).

N-(2-Hydroxyethyl)naphthalene-2-carboxamide (10): [C/MeOH-NH_4OH, 20:1]; yield 29%; white solid; mp 138–40 °C (lit.[52] mp 132–4 °C); MS m/e (M^+) 215 ($C_{13}H_{13}NO_2$).

N-(2-Hydroxyethyl)naphthalene-2-acetamide (11): [R/EtOAc]; yield 32%; white solid; mp 136–7 °C (lit.[53] mp 125–7 °C); MS m/e (M^+) 229 ($C_{14}H_{15}NO_2$).

(R)-N-(2-Hydroxy-1-methylethyl)hexadecanamide (24):[54] [R/EtOAc]; yield 92%; white solid; ^1H NMR ($CDCl_3$) δ 5.63 (bd, 1 H, NH), 3.44 (m, 1 H, NCH), 3.59 (dq, 2 H, OCH_2), 3.29 (m, 1 H, OH), 2.19 (t, 2 H, CH_2CO), 1.63 (m, 2 H, CH_2), 1.26 (bs, 24 H, $(CH_2)_{12}$), 1.17 (d, 3 H, CH_3), 0.88 (t, 3 H, CH_3).

(R)-N-[1-(Hydroxymethyl)isopentyl]hexadecanamide (25): [C/EtOAc–hexane, 1:1]; yield 53%; white solid; mp 85–7 °C; ^1H NMR ($CDCl_3$) δ 5.50 (bd, 1 H, NH), 4.04 (m, 1 H, NCH), 3.62 (dq, 2 H, OCH_2), 2.78 (bs, 1 H, OH), 2.20 (t, 2 H, CH_2CO), 1.62 (m, 3 H, CH and CH_2), 1.38 (m, 2 H, CH_2), 1.25 (s, 22 H, $(CH_2)_{11}$), 0.94 (t, 6 H, $(CH_3)_2$), 0.88 (t, 3 H, CH_3).

(R)-N-[1-(Hydroxymethyl)-2-phenylethyl]hexadecanamide (26):[55] [R/EtOAc]; yield 90%; white solid; mp 100–101 °C; ^1H NMR ($CDCl_3$) δ 7.23 (m, 5 H, ArH), 5.67 (d, 1 H, NH), 4.17 (m, 1 H, NCH), 3.68 (bm, 2 H, OCH_2), 2.86 (m, 3 H, CH_2CO and OH), 2.12 (t, 2 H, CH_2), 1.55 (m, 2 H, CH_2), 1.25 (m, 24 H, $(CH_2)_{12}$), 0.88 (t, 3 H, CH_3).

(R)-N-(2-Hydroxy-1-phenylethyl)hexadecanamide (27): yield 85%; white solid; ^1H NMR ($CDCl_3$) δ 7.32 (m, 5 H, ArH), 6.08 (d, 1 H, NH), 5.07 (q, 1 H, ArCH), 3.89 (dq, 2 H, CH_2O), 2.25 (t, 2 H, CH_2CO), 1.65 (m, 2 H, CH_2), 1.25 (s, 24 H, $(CH_2)_{12}$), 0.88 (t, 3 H, CH_3).

General Procedure B. Preparation of Phosphocholines 16–19 and 32–33. A solution of the amido alcohol (1.97 mmol) in benzene (10 mL) was treated with 4-(dimethylamino)pyridine (0.7 mmol) and triethylamine (2.2 mmol) followed by 2-chloro-2-oxo-1,3,2-dioxaphospholane (2.3 mmol) under nitrogen. The mixture was stirred for 1–5 h and filtered and the solvent removed by evaporation. The residual gum was immediately treated with a solution of anhydrous 3 M triethylamine in acetonitrile (14 mL) and heated at 65 °C for 18 h. The solvent was removed by evaporation and the product purified by chromatography.

O-(2-Dodecanamidoethyl)phosphocholine (16):[35] [C/MeOH–NH_4OH, 20:1]; yield 12%; white solid; mp 88–91 °C; ^1H NMR (DMSO-d_6) δ 8.32 (t, 1 H, NH), 4.02 (bs, 2 H, OCH_2), 3.62 (m, 2 H, OCH_2), 3.50 (m, 2 H, NCH_2), 3.34 (s, 9 H, $(CH_3)_3$), 3.14 (m, 2 H, NCH_2), 2.02 (t, 2 H, CH_2CO), 1.46 (m, 2 H, CH_2), 1.23 (s, 16 H, $(CH_2)_8$), 0.85 (t, 3 H, CH_3). Anal. ($C_{19}H_{41}N_2O_5P{\cdot}C_2H_5OH{\cdot}1.5H_2O$) C, N; H: calcd, 10.46; found, 9.78.[50]

O-(2-Hexadecanamidoethyl)phosphocholine (17):[35] [C/$CHCl_3$–MeOH–H_2O, 65:25:4]; yield 17%; white foam; ^1H NMR (CD_3OD) δ 4.25 (m, 2 H, OCH_2), 3.90 (m, 2 H, OCH_2), 3.62 (m, 2 H, NCH_2), 3.4 (m, 2 H, NCH_2), 3.21 (s, 9 H, $(CH_3)_3$), 2.19 (t, 2 H, CH_2CO), 1.59 (bm, 2 H, CH_2), 1.28 (s, 24 H, $(CH_2)_{12}$), 0.89 (t, 3 H, CH_3). Anal. ($C_{23}H_{49}N_2O_5P{\cdot}5H_2O$) C, N; H: calcd, 10.72; found, 8.68.[50]

O-[2-(2-Naphthalenecarboxamido)ethyl]phosphocholine (18): [C/MeOH–NH_4OH, 20:1]; yield 29%; white foam; ^1H NMR (DMSO-d_6) δ 8.54 (s, 1 H, ArH), 8.00 (m, 3 H, ArH), 7.59 (m, 3 H, ArH), 4.10 (bs, 2 H, OCH_2), 3.93 (m, 2 H, OCH_2), 3.54 (m, 2 H, NCH_2), 3.46 (m, 2 H, NCH_2), 3.39 (s, 9 H, $(CH_3)_3$). Anal. ($C_{18}H_{25}N_2O_5P{\cdot}2H_2O$) C, H, N.

O-[2-(2-Naphthaleneacetamido)ethyl]phosphocholine (19): [C/MeOH–NH_4OH, 20:1], yield 56%; white foam; ^1H NMR (DMSO-d_6) δ 8.78 (t, 1 H, NH), 7.83 (m, 4 H, ArH), 7.48 (m, 3 H, ArH), 4.02 (bs, 2 H, OCH_2), 3.68 (m, 2 H, OCH_2), 3.58 (s, 2 H, CH_2CO), 3.46 (m, 2 H, NCH_2), 3.22 (m, 2 H, NCH_2), 3.11 (s, 9 H, $(CH_3)_3$). Anal. ($C_{19}H_{27}N_2O_5P{\cdot}C_2H_5OH{\cdot}0.6H_2O$) C, H, N.

(R)-O-(2-Hexadecanamidopropyl)phosphocholine (32): [C/$CHCl_3$–MeOH–H_2O, 65:25:4]; yield 15%; white solid; mp 73–5 °C; ^1H NMR ($CDCl_3$) δ 7.29 (bs, 1 H, NH), 4.32 (bs, 2 H, OCH_2), 4.06 (m, 1 H, NCH), 3.81 (bs, 4 H, OCH_2 and NCH_2), 3.37 (s, 9 H, $(CH_3)_3$), 2.13 (m, 2 H, CH_2CO), 1.58 (m, 2 H, CH_2), 1.25 (s, 24 H, $(CH_2)_{12}$), 1.15 (d, 3 H, CH_3), 0.88 (t, 3 H, CH_3). Anal. ($C_{24}H_{51}N_2O_5P{\cdot}1.4H_2O$) C, H, N.

(R)-O-(2-Hexadecanamidoisohexyl)phosphocholine (33): [C/$CHCl_3$–MeOH–H_2O, 65:25:4]; yield 27%; white foam; $[\alpha]^{26}{}_D$ +18.3° (c 0.37, MeOH); ^1H NMR (CD_3OD) δ 4.26 (bs, 2 H, OCH_2), 4.10 (m, 1 H, NCH), 3.78 (m, 2 H, OCH_2), 3.63 (bs, 2 H, NCH_2), 3.22 (s, 9 H, $(CH_3)_3$), 2.18 (m, 2 H, CH_2CO), 1.60 (m, 3 H, CH_2 and CH), 1.42 (m, 2 H, CH_2), 1.28 (s, 24 H, $(CH_2)_{12}$), 0.91 (m, 9 H, CH_3 and $(CH_3)_2$). Anal. ($C_{27}H_{57}N_2O_5P{\cdot}2H_2O$) C, H, N.

General Procedure C. Preparation of Phosphocholines 36–39. A solution of the amino alcohol (2.0 mmol) in CH_2Cl_2 (10 mL) was treated either with triethylamine (2.0 mmol) and the appropriate acid chloride (2.0 mmol) or with dicyclohexylcarbodiimide (2.0 mmol) and the appropriate acid (2.0 mmol). The reaction mixture was stirred at room temperature for 1–18 h and filtered, and the resulting filtrate partitioned between 2 N HCl and CH_2Cl_2. The organic layer was separated, washed with saturated aqueous $NaHCO_3$ solution and water, and dried. The solvent was removed by evaporation. A solution of the residue in benzene (10 mL) under nitrogen was treated with 4-(dimethylamino)pyridine (0.7 mmol) and triethylamine (2.2 mmol) followed by 2-chloro-2-oxo-1,3,2-dioxaphospholane (2.3 mmol). The mixture was stirred for 1–5 h and filtered and the solvent removed from the filtrate by evaporation. The residual gum was immediately treated with a saturated solution of anhydrous 3 M triethylamine in acetonitrile (14 mL) and heated at 65 °C for 18 h. The solvent was removed by evaporation and after purification by chromatography the required product was obtained as a hygroscopic solid.

(S)-O-(2-Hexadecanamidoisohexyl)phosphocholine (36): [C/$CHCl_3$–MeOH–H_2O, 65:25:4]; yield 19%; white foam; $[\alpha]^{26}{}_D$ −21.3° (c 0.16, MeOH); ^1H NMR identical to that of compound **33**. Anal. ($C_{27}H_{57}N_2O_5P{\cdot}2.2H_2O$) C, N; H: calcd, 11.04; found, 10.45.[50]

(R)-O-[2-(2-Naphthaleneacetamido)isohexyl]phosphocholine (37): [C/MeOH–NH_4OH, 100:1]; yield 23%; white foam; ^1H NMR ($CDCl_3$) δ 7.75 (m, 4 H, ArH), 7.55 (d, 1 H, NH), 7.43 (m, 3 H, ArH), 4.1 (m, 1 H, NCH), 4.07 (m, 2 H, OCH_2), 3.8 (m, 2 H, OCH_2), 3.67 (m, 2 H, CH_2CO), 3.35 (bs, 2 H, NCH_2), 2.98 (s, 9 H, $(CH_3)_3$), 1.57 (m, 1 H, CH), 1.41 (m, 1 H, CH_2), 1.26 (m, 1 H, CH_2), 0.83 (d, 6 H, $(CH_3)_2$). Anal. ($C_{23}H_{35}N_2O_5P{\cdot}1.2H_2O$) C, H, N.

(E)-(R)-O-(2-Dec-3-enamidoisohexyl)phosphocholine (38): [C/$CHCl_3$–MeOH–H_2O–HOAc, 65:25:4:1]; yield 36%; white foam; $[\alpha]^{26}{}_D$ +25.3° (c 0.29, MeOH); ^1H NMR ($CDCl_3$) δ 6.97 (bd, 1 H, NH), 5.54 (m, 2 H, CH=CH), 4.33 (bs, 2 H, OCH_2), 4.08 (bs, 1 H, NCH), 3.81 (bs, 4 H, OCH_2 and NCH_2), 2.93 (dq, 2 H, CH_2CO), 2.01 (m, 2 H, CH_2) 1.60 (m, 1 H, CH), 1.26 (m, 10 H, $(CH_2)_4$ and CH_2), 0.88 (m, 9 H, CH_3 and $(CH_3)_2$). Anal. ($C_{21}H_{43}N_2O_5P{\cdot}1.6H_2O$) C, H, N.

(E)-(S)-O-(2-Dec-3-enamidoisohexyl)phosphocholine (39): [C/$CHCl_3$–MeOH–H_2O–HOAc, 65:25:4:1]; yield 23%; white foam; $[\alpha]^{26}{}_D$ −22.3° (c 0.2, MeOH); ^1H NMR identical to that of compound **38**. Anal. ($C_{21}H_{43}N_2O_5P{\cdot}3H_2O$) C, N; H: calcd, 10.11; found, 9.07.[50]

Preparation of (R)-O-(2-Hexadecanamidoisohexyl)phosphoglycol (40). A solution of the amido alcohol **25** (1.1 g, 3 mmol) in benzene (100 mL) was treated with triethylamine (0.3 g, 3 mmol) followed by 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.4 g, 3 mmol) under nitrogen. After 2 h, a second equivalent each of triethylamine and the dioxaphospholane was added, and the mixture stirred for a further 1 h. The reaction mixture was filtered, the solvent removed by evaporation, and the residue dissolved in acetonitrile and treated with 2 M HCl (1 mL). After 16 h the solvent was removed by evaporation. A solution of the

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residue in ethyl acetate was washed with water and brine and dried and the solvent removed by evaporation to afford the crude product as an oil. Purification by flash chromatography (EtOAc–MeOH, 3:7) gave the required product as a hygroscopic white solid (0.4 g; 25%): mp 87–9 °C; ^1H NMR ($CDCl_3$) δ 5.7 (bs, 1 H, NH), 4.12 (bs, 1 H, NCH), 3.9 (bs, 2 H, OCH_2), 3.8 (bs, 2 H, OCH_2), 3.7 (bs, 2 H, OCH_2), 2.1 (m, 2 H, CH_2CO), 1.6 (bs, 5 H, 2 CH_2 and CH), 1.25 (s, 24 H, $(CH_2)_{12}$), 0.88 (m, 9 H, CH_3 and $(CH_3)_2$). Anal. ($C_{24}H_{49}NNaO_6P{\cdot}H_2O$) C, N; H: calcd, 9.89; found, 8.84 (the compound extracted sodium ions from the brine wash).

General Procedure D. Preparation of Dibenzyl Phosphates 43 and 44. A solution of the amido alcohol (2.6 mmol) and tetrazole (11 mmol) in $CHCl_3$ (50 mL) was treated with dibenzyl *N,N*-diisopropylphosphoramidite (3.8 mmol) under nitrogen. After 1 h the mixture was cooled to −40 °C and treated with a solution of *m*-chloroperbenzoic acid (3.8 mmol). The reaction was stirred at room temperature for 18 h, washed with saturated aqueous $Na_2S_2O_5$ solution, saturated $NaHCO_3$ solution, water, and brine, and dried. The solvent was removed by evaporation and the residue purified by chromatography to afford the product as an oil.

(*R*)-2-Hexadecanamidoisohexyl dibenzyl phosphate (43): [C/EtOAc–hexane, 4:6]; yield 82%; colorless oil; ^1H NMR ($CDCl_3$) δ 7.35 (s, 10 H, ArH), 5.61 (bd, 1 H, NH), 5.04 (m, 4 H, 2 $PhCH_2$), 4.18 (m, 1 H, NCH), 3.92 (dd, 2 H, OCH_2), 2.06 (t, 2 H, CH_2CO), 1.55 (m, 3 H, CH_2 and CH), 1.27 (s, 26 H, $(CH_2)_{12}$ and CH_2), 0.87 (m, 9 H, CH_3 and $(CH_3)_2$).

(*R*)-2-Hexadecanamido-3-phenylpropyl dibenzyl phosphate (44): [C/EtOAc–hexane, 1:2]; yield 82%; colorless oil; ^1H NMR ($CDCl_3$) δ 7.35 (m, 10 H, ArH), 7.1–7.3 (m, 5 H, ArH), 6.0 (d, 1 H, NH), 5.05 (m, 4 H, $PhCH_2$), 4.3 (m, 1 H, NCH), 3.9 (m, 2 H, OCH_2), 2.8 (m, 2 H, CH_2), 2.05 (m, 2 H, CH_2CO), 1.5 (m, 2 H, CH_2), 1.25 (m, 24 H, $(CH_2)_{12}$), 0.85 (t, 3 H, CH_3).

General Procedure E. Preparation of Phosphates 45 and 46. A solution of the dibenzyl ester in MeOH was treated with a catalytic amount of 10% Pd/C and the mixture hydrogenated at atmospheric pressure for 3 h. The catalyst was removed by filtration and the filtrate evaporated to dryness to give a viscous oil which was purified by crystallization from ether.

(*R*)-2-Hexadecanamidoisohexyl phosphate (45): isolated as the dicyclohexylamine (DCHA) salt; yield 65%; beige solid; mp 170–3 °C; ^1H NMR ($CDCl_3$) δ 6.9 (bd, 1 H, NH), 4.13 (m, 1 H, NCH), 3.77 (m, 1 H, OCH_2), 3.93 (m, 1 H, OCH_2), 2.9 (m, 2 H, DCHA), 2.2 (t, 2 H, CH_2CO), 2.1 (m, 4 H, DCHA), 1.8 (m, 4 H, DCHA), 1.5–1.6 (m, 10 H, CH_2 and DCHA), 1.24 (m, 33 H, $(CH_2)_{12}$ and CH_2 and CH and DCHA), 0.89 (m, 9 H, $(CH_3)_2$ and CH_3). Anal. ($C_{22}H_{46}NO_5P{\cdot}C_{12}H_{23}N{\cdot}1.1H_2O$) C, N; H: calcd, 11.27; found, 10.73.[50]

(*R*)-2-Hexadecanamido-3-phenylpropyl phosphate (46): yield 60%; white solid; mp 87–90 °C; ^1H NMR (CD_3OD) δ 7.25 (m, 5 H, ArH), 7.15 (bs, 1 H, NH), 4.25 (m, 1 H, NCH), 3.9 (m, 2 H, OCH_2), 2.85 (m, 2 H, $ArCH_2$), 2.1 (t, 2 H, CH_2CO), 1.45 (m, 2 H, CH_2), 1.3 (m, 24 H, $(CH_2)_{12}$), 0.90 (t, 3 H, CH_3). Anal. ($C_{25}H_{44}NO_5P{\cdot}H_2O$) C, H, N.

PLA_2 Screening Methods. Monomeric Substrate Assay. The monomeric assay contained 5.44 mM substrate 47 (CMC = 14 mM, determined by techniques which have previously been described[56]), 0.2 mM 5,5-dithiobis(2-nitrobenzoic acid), 0.25 mM $CaCl_2$, and 0.5 μg/mL porcine pancreatic PLA_2 (Boehringer) in 10 mM sodium phosphate buffer, pH 7.5. Assays were performed in 96-well, polypropylene microtiter plates in a final volume of 200 μL including addition of inhibitor or solvent (methanol) at 2.5% of assay volume. Reaction time courses were followed for 16 h at room temperature (ca. 22 °C) and rates calculated by a Thermomax plate reader. Percentage inhibitions relative to solvent controls were calculated after subtraction of low levels (10%) of nonenzymic hydrolysis.

Under the above assay conditions, the hydrolysis of 47 by pancreatic PLA_2 showed Michaelis–Menten kinetics indicating that the substrate was truly monomeric. Interfacial activation occurred at, and above, the substrate CMC. It has been reported[41] that microaggregate formation can occur between some PLA_2 enzymes and some substrates below the CMC. However, deviation of the plot of rate of reaction versus substrate concentration from Michaelis–Menten kinetics in those cases provided clear evidence for the aggregation. It was noted in the same publication[41] that premicellar aggregates do not usually occur when pancreatic PLA_2 acts on short acyl chain phosphocholine substrates.

Deviation from Michaelis–Menten kinetics in the case of the pancreatic PLA_2-catalyzed hydrolysis of a phosphocholine-containing substrate (the decanoyl analogue of 47) below the CMC has been reported.[57] Our studies indicate that this result was probably a consequence of the high Ca^{2+} concentrations used in that work, as similar behavior was observed in our screens when the Ca^{2+} concentration was increased to the same level.

Deoxycholate–Phospholipid Mixed Micelle Assay. The assay contained 0.074 μg/mL porcine pancreatic PLA_2, 2 mM $CaCl_2$, 2.4 mM sodium deoxycholate, and 100 μM 1-stearoyl-2-arachidonyl-L-phosphatidylcholine (Avanti Lipids) containing 22 000 dpm of 1-stearoyl-2-[1-^{14}C]arachidonyl-L-phosphatidylcholine (Amersham) in 50 mM Tris·HCl, pH 8.2. Inhibitors were added in 1% of the assay volume (500 μL) of methanol. [^{14}C]-Arachidonic acid released after 8 min at 37 °C was extracted as previously described[58] and measured by scintillation counting. Under these conditions it is expected that hopping kinetics will apply.

NMR Studies. Bovine PLA_2 was prepared from pancreas following the literature procedure.[60] The mixture of isoenzymes was further purified by FPLC using a Mono-S column and 10 mM sodium acetate buffer, pH 5.0, eluting with a 0–250 mM NaCl gradient.

Samples for NMR experiments used 2 mM enzyme in 99.96% D_2O (Aldrich) (0.4 mL), containing 50 mM $CaCl_2$, 200 mM NaCl, and 10 mM d_{11}-Tris (Aldrich). The pH was adjusted by adding microliter quantities of DCl or NaOD (Aldrich) to give a final pH* of 7.5, where pH* denotes a meter reading which was uncorrected for the deuterium isotope effect on the electrode. Substrate analogues were added to the enzyme solution as 60 mM solutions in D_2O (Aldrich).

One- and two-dimensional ^1H NMR spectra were recorded at 500 MHz using a Bruker AM500 spectrometer at a sample temperature of 41 °C. Chemical shifts were measured relative to the isolated CαH resonance of Cys-77, taken as 5.56 ppm, which has been found to be almost invariant to the sample conditions. A spectral width of 6 kHz was used with the transmitter placed on the water resonance, which was suppressed by low-power irradiation for 1.5 s prior to data acquisition. NOESY experiments with a 150 ms mixing time were carried out in the phase-sensitive mode using standard pulse sequences.[61] TOCSY spectra were carried out using an MLEV-17 pulse cycle[62] to give a spin lock time of 45 ms. The receiver phase was offset to allow data collection in sine-modulation mode.[63] A total of 512 and 2048 points were collected in f_1 and f_2, respectively. The data were zero filled once in f_1 and multiplied by a Gaussian weighting function in both

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dimensions prior to Fourier transformation.

Molecular Modeling. A series of complementary computational methods were utilized during the design cycle. Initially, possible sites of interaction between the enzyme active site and the inhibitor were identified using the program GRID.[64] The active site was evaluated for its ability to interact with a number of functional probes including hydroxyl, phosphate, and amino groups. The program GRID was also used to identify free volume in the minimized enzyme–inhibitor complex by probing the complex with a water probe and contouring at energy levels of 1 and 5 kcal/mol.

To evaluate how the substratelike inhibitor molecules interacted with the enzyme, distance geometry methodology as implemented in DISGEO[65] and DGEOM[66] was used to generate unbiased random structures. The distance geometry programs made use of a random number generator to generate internal distances that were transformed into a set of three-dimensional coordinates. The inhibitor–enzyme complex was then energy minimized to obtain an estimate of the energy of the system.

The enzyme used for the modeling studies, bovine pancreatic PLA_2, consists of approximately 960 heavy atoms. During the early energy refinement calculations the hydrogen atoms were incorporated into the heavy atoms using the AMBER[48] united atom force field parameters. Subsequent calculations on the Convex C220 allowed the AMBER all atom model to be utilized. All amino acid residues were held fixed except those making up the active site (i.e. residues Leu-2, Phe-5, Ile-9, Leu-19, Leu-20, Phe-22, Asn-23, Asn-24, Tyr-28, Cys-29, Gly-30, Leu-31, Gly-32, Cys-45, His-48, Asp-49, Tyr-52, Tyr-69, Tyr-73, Asp-99, Ala-102, Ala-103, and Phe-106).

The inhibitor was placed at multiple starting points, usually eight, and the enzyme plus inhibitor system minimized using AMBER. AMBER versions 3.0 and 3A were utilized to calculate the energies of the separate enzyme and inhibitor molecules and the bound complex of the enzyme with a series of inhibitor molecules.

Molecular dynamics simulations were performed to determine how the inhibitor molecule could interact with the enzyme. Simulation studies started with the inhibitor outside the active site. Weak distance restraints were placed on pairs of atoms to simulate the observed NMR NOE data. Restraints were applied between C(3) and C(4) and Phe-5 and Phe-106 to simulate the observed NOE's between H(3) and H(4) and those protein residues in order to be consistent when using the united atom force field approximation. Restraints in the form of an equilibrium distance and harmonic force constant were used. Depending on the strength of the NOE, different values were used. A strong NOE had an equilibrium distance of 3.5 Å and force constant (k) of 2 kcal/Å^2, for medium NOE's the distance was 5.0 Å and $k = 1.5$ kcal/Å^2, and for weak NOE's the distance was 6.0 Å and $k = 1.0$ kcal/Å^2. A weak restraint was applied between the phosphate group and the Ca^{2+} atom in the form of an equilibrium distance of 3.4 Å and a force constant of 2.0 kcal/Å^2.

In this way inhibitor molecules were docked into the active site while allowing the hydrocarbon chain to remain flexible and adopt a number of possible conformations. Results of the calculations were displayed using an Evans and Sutherland PS300 and interaction with the theoretical model was achieved using the modeling software package CHEMX.[67]

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