Active-Site-Directed Specific Competitive Inhibitors of Phospholipase A_2: Novel Transition-State Analogues†

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ABSTRACT: More than 100 amphiphilic phosphoesters, possible tetrahedral transition-state analogues capable of coordinating to the calcium ion at the active site of phospholipase A_2, were designed, synthesized, and tested as inhibitors for the hydrolysis of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol vesicles in the scooting mode. This assay system permits the study of structurally diverse inhibitors with phospholipase A_2s from different sources, and it is not perturbed by factors that change the quality of the interface. As a prototype, 1-hexadecyl-3-trifluoroethylglycero-2-phosphomethanol (MJ33) was investigated in detail. Only the (*S*)-(+) analogue of MJ33 is inhibitory, and it is as effective as the *sn*-2 phosphonate or the *sn*-2 amide analogues of *sn*-3 phospholipids. The inhibitory potencies of the various phosphoesters depended strongly on the stereochemical and structural features, and the mole fractions of inhibitors required for 50% inhibition, $X_I(50)$, ranged from more than 1 to less than 0.001 mole fraction. The affinity of certain inhibitors for enzymes from different sources differed by more than 200-fold. The inhibitors protected the catalytic site residue His-48 from alkylation in the presence of calcium but not barium as expected if the formation of the EI complex is supported only by calcium. The equilibrium dissociation constant for the inhibitor bound to the enzyme at the interface was correlated with the $X_I(50)$ values, which were different if the inhibition was monitored in the pseudo-zero-order or the first-order region of the progress curve. These results show that the inhibitors described here interfered only with the catalytic turnover by phospholipase A_2's bound to the interface, their binding to the enzyme occurred through calcium, and the inhibitors did not have any effect on the dissociation of the enzyme bound to the interface.

Specific inhibitors of phospholipase A_2 (PLA2)[1] are of interest not only to resolve the mechanistic subtleties of interfacial catalysis (Verger & de Haas, 1977; Jain & Berg, 1989; Berg et al., 1991; Jain et al., 1986c, 1991a) and to establish the biological functions of PLA2 in an organism (Gallin et al., 1988) but also as potential therapeutic agents for inflammatory disorders (Mobilio & Marshall, 1989). Yet, the commonly used assay protocols for evaluation of inhibitors of PLA2 or other interfacial enzymes do not adequately distinguish between an active-site-directed inhibitor from a nonspecific inhibitor that perturbs the binding equilibrium of the enzyme to the interface by modulating the substrate interface. We have demonstrated that such difficulties can be eliminated by monitoring the effect of potential inhibitors on the kinetics of interfacial catalysis in the scooting mode (Jain et al., 1986c, 1989). As our understanding of the possible artifacts encountered in the assays of inhibitors of interfacial catalysis comes into focus (Jain & Jahagirdar, 1985; Jain & Berg, 1989), the search for specific competitive inhibitors of PLA2 has gained momentum. The analogues of the substrate in which the ester bond has been replaced by an ether (Jain et al., 1986c), phosphonate (Jain et al., 1989), or an amide bond (Bonsen et al., 1972; de Haas et al., 1990) have been shown as potent inhibitors of PLA2.

One of the major difficulties with the dialkyl- or diacylphospholipids as inhibitors of PLA2 is that they can not be readily incorporated into the preformed substrate interface such as bilayers or biomembranes because they have to be codispersed with the substrate. To some extent this difficulty is obviated with the short-chain analogues which form micellar solutions (Jain et al., 1989). Yet another difficulty is that the *sn*-glycero-3-phosphate head group present in these phospholipid analogues makes them potentially susceptible to the catalytic action of phospholipase C and D and other lipolytic enzymes found in living organisms. In this paper we describe a novel class of the tetrahedral transition-state analogues as competitive inhibitors of PLA2. These inhibitors do not suffer from the limitations outlined above, and they could be useful for developing the kinetic and mechanistic understanding of PLA2's. Inhibitors are also useful for ascertaining and modulating the function(s) of PLA2's in vivo, and the design principles developed in this paper can be potentially useful for developing inhibitors that may cross cell membranes.

Materials and Methods

General protocols for the isolation of PLA2's (Van den Bergh et al., 1989a; Jain et al., 1991b), for the synthesis of DMPM and the preparation of substrate vesicles (Jain et al., 1986a; Jain & Gelb, 1991), and the protocols for monitoring the kinetics of hydrolysis in the scooting mode have been described (Berg et al., 1991; Jain et al., 1986a,c, 1991a,c). Inhibitors H-18 (de Haas et al., 1990), MG-14 (Yuan et al., 1989; Jain et al., 1989), and RM-2 (Magolda et al., 1987) were

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[1] Abbreviations: See Figure 1 for the structures of H-18, MG-14, MJ33, and RM-2; k_i, first-order relaxation rate for the hydrolysis of all the substrate molecules on the outer monolayer of a vesicle; K_{MS}, interfacial Michaelis–Menten constant for the substrate [$= (k_2 + k_{-1})/k_1$]; K_p, equilibrium dissociation constant for products from E*P; K_I, equilibrium dissociation constant for the inhibitor from E*I; N_S, total number of lipid molecules in the outer layer of a vesicle; PLA2, phospholipase A_2 from pig pancreas unless indicated otherwise; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol.

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Specific experimental conditions for monitoring the kinetics of hydrolysis of the sonicated vesicles of DMPM in the presence of an inhibitor are given in the figure legends. Typically, the kinetics of hydrolysis by PLA2 was continuously monitored by pH-stat titration in a stream of nitrogen at pH 8.0 and 23 °C in a reaction mixture containing 0.5 or 2.5 mM $CaCl_2$, 1 mM NaCl, 0.28 mM DMPM as sonicated vesicles, and an appropriate amount of the inhibitor, if present. The reaction was initiated by adding 1–30 pmol of PLA2. The first-order progress curves (cf. Figure 3a) were obtained at 0.5 mM calcium. At 2.5 mM calcium the substrate vesicles fuse; therefore, it was necessary to start the reaction within 2 min after the addition of the substrate. Under these conditions, the initial rate of hydrolysis was linear for about 2 min, and as shown elsewhere (Jain et al., 1991c) it could be extended to more than 5 min if the intervesicle exchange of phospholipids was promoted by adding 10 μg of polymyxin B sulfate (Sigma) to the reaction mixture. As shown in Figure 3b, under these assay conditions, with appropriate controls, a water-soluble or micellar inhibitor could be added to the reaction mixture after the initial rate has been determined in the absence of the inhibitor. Virtually all the inhibitors described here have very high partition coefficients in the favor of bilayers; therefore, the total inhibitor concentration was used for the calculation of the mole fraction of the inhibitor at the interface. This was also confirmed by the fact that the dilution of the reaction mixture in the presence of an inhibitor did not noticeably influence the rate of hydrolysis. Thus the surface density of the components on the substrate interface did not change on dilution (Jain et al., 1989). The critical micelle concentration of the substrate was very low. The bilayer structure was not disrupted in the presence of more than 0.1 mole fraction of any of the inhibitors as indicated by the fact that the extent of hydrolysis in the first-order progress curve (cf. Figure 3) did not change in the presence of the inactive enantiomers. In this assay system one need not consider the problems associated with changes in the quality of the interface or those arising from the dispersity in the composition and size of the substrate aggregates at intermicellar concentrations (Jain & Berg, 1989; Berg et al., 1991). This is because under these conditions all the enzyme remains bound to the interface under virtually all the conditions used here.

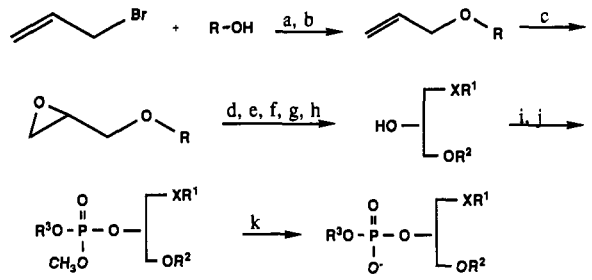
The procedure for obtaining the interfacial dissociation constant for an inhibitor bound to PLA2 at the interface, K_I, has been described (Jain et al., 1991a). Typically, the half-time for inactivation of His-48 of PLA2 (16 μM) bound to micelles of 2-hexadecyl-*sn*-glycero-3-phosphocholine (3 mM) by an alkylating agent such as 2-bromonitroacetophenone (0.05–2 mM) was measured in the presence and in the absence of the ligand. The K_I value for the ligand bound to the active site of PLA2 at the interface of the neutral diluent was calculated from the ratio of the half-times for the inactivation in the absence (t_f) and in the presence (t_L) of a ligand:

$$1/[1 - t_L/t_f] = [1 + K_I/X_I]/(1 - k_L/k_f]$$

This relationship is simplified considerably when the E*I complex is not susceptible to alkylation (i.e., $k_L/k_f \ll 1$), as is the case with all the ligands that bind to the catalytic site of PLA2.

Synthesis of Inhibitors. More than 100 amphiphilic phosphoesters (Tables IV–VI) used in this study were synthesized by procedures adopted from the well-established and published protocols. All samples were homogeneous. Unless stated otherwise, only the racemic mixtures of the amphiphiles were synthesized and assayed. Typical synthetic protocols are summarized in Schemes I–V and briefly described below for specific compounds. As indicated, these procedures were also used for the synthesis of related compounds with appropriate changes. In most cases the isolated yields were over 60%; however, no attempt was made to optimize the synthetic protocols. The structures of the intermediates and the final products were consistent with their ^1H NMR spectra ($CDCl_3$) and with other spectral data (mass, IR). All reagents and chromatographic silica gel (60A, 230–400 mesh) were from Aldrich. TLC plates were from Analtech (Newark). Sources of other reagents are given in the text. The basic protocol for the synthesis of most of the *sn*-glycero-2-phosphoesters (33–79) is outlined in Scheme I, and the specific protocols are described below.

Scheme I[a]



X= CH_2, O, S, NH.

[a] Conditions: a. NaH, DMF, room temperature; b. TBAH, 50% NaOH, 40 °C; c. MCPBA, CH_2Cl_2, room temperature; d. NaOH, THF, reflux; e. BF_3Et_2O, CH_2Cl_2, room temperature, N_2; f. H_2O, THF, reflux; g. NaH, DMF, room temperature; h. Mg, Et_2O, 0 °C; i. $POCl_3$, MeOH, Et_3N, THF, 0–40 °C; j. $R^3OP(OC)Cl_2$ or $CH_3P(O)Cl_2$, MeOH, Et_3N/THF, 0–40°C; k. LiBr, acetone, reflux.

General Procedures for the Preparation of Allyl Alkyl Ethers. Method a [adopted from Van Boecka et al. (1982)] was used for the precursors of MJ33, 40–47, 49, 51, 53–55, 58, 59, 61–63, 71–73, and 80–83. To a properly equipped three-neck flask containing 40 mmol of hexadecanol in 100 mL of DMF was added 60 mmol of sodium hydride. The mixture was stirred mechanically at room temperature. When the evolution of hydrogen ceased, a solution of 60 mmol of allyl bromide in 20 mL of DMF was added dropwise. The mixture was stirred for another 20 h, and the progress of the reaction was monitored by TLC in hexane/ether (70:30 v/v). After the completion of the reaction, 2 mL of methanol followed by 150 mL of water was added to destroy the excess sodium hydride. The mixture was extracted twice with ether, and the combined extract was dried over $MgSO_4$. The solvent was removed, and the crude product was chromatographed on silica gel where the allyl alkyl ether (**Ib**) was eluted with hexane. ^1H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.65 (m, 2H), 3.42 (t, 2H), 3.95 (dd, 2H), 5.24 (dd, 2H), 5.91 (m, 1H).

Method b [adopted from Boutevin et al. (1987)] was used for the precursors of MJ48, 66–70, 74, 75, and 81. To a two-neck flask equipped with a mechanical stirrer and a thermometer were added 35 mmol of trifluoroethanol, 50 mL of allyl chloride, 1.2 g of tetrabutylammonium hydrogen sulfate, and 37.5 mL of 50% sodium hydroxide. The mixture was stirred at 40 °C for 6 h. After the reaction mixture was cooled to room temperature, 20 mL of water and 20 mL of methylene chloride were added. The organic layer was separated, washed with water until the pH was 7, dried over $MgSO_4$, and concentrated. The trifluoroallyl ether was collected by fractional distillation at 60–65 °C as a colorless liquid. ^1H NMR: 3.81 (q, 2H), 4.25 (d, 2H); 5.30 (dd, 2H), 5.91 (m, 1H).

Procedure for Epoxidation (Scheme I). Method c [adopted from Paquette et al. (1969)] was used for the precursors of MJ33, 40–55, 58, 59, 61–63, 66–75, and 80–83. A solution of 10 mmol of allyl hexadecyl ether (**Ib**) and 10 mmol of *m*-chloroperoxybenzoic acid (Sigma) in 30 mL of methylene chloride was stirred magnetically at room temperature for 24 h. After the reaction was completed, as monitored by TLC (in hexane/ethyl acetate, 80:20 v/v), 10 mL of 10% Na_2SO_3 was added to remove the excess peracid, and then 10% NaOH was added to raise the pH to 10. The organic layer was separated, washed twice with water, and dried over $MgSO_4$. The residue after the removal of the solvent was crystallized from pentane at −20 °C to give hexadecyl glycidyl ether (**Ic**) as a white solid. ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.60 (m, 2H), 2.61 (dd, 2H), 2.80 (dd, 2H), 3.16 (m, 1H), 3.52 (m, 1H), 3.71 (dd, 1H).

Procedures for 1-3-Dialkyl-sn-glycerol (Scheme I). Method d was used for the precursors of MJ33, 43, 49, 53–55, 58, 59, 61, 62, and 67. A mixture of 5 mmol of hexadecyl glycidyl ether (**Ic**), 10 mL of trifluoroethanol, and 10 mmol of NaOH was refluxed overnight. After the completion of the reaction (monitored by TLC in hexane/ethylacetate, 80:20 v/v), the reaction mixture was poured into water and extracted with ether. The ether layer was washed twice with water and dried over $MgSO_4$. The residue after the removal of the solvent was crystallized from pentane at −20 °C. ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.59 (m, 2H), 2.65 (br, 1H), 3.47 (m, 4H), 3.68 (m, 2H), 3.90 (q, 2H), 3.98 (m, 1H). This procedure did not work with trichloroethanol. Method e was used for the preparation of trichloroethyl ethers.

Method e [adopted from Bittman et al. (1989)] was used for the precursors of MJ36, 48, 69, and 75. To a solution of 10 mmol of trichloroethyl glycidyl ether (from method c) and 11 mmol of hexadecanol in dry distilled methylene chloride in a round bottom flask were added five drops of BF_3 in diethyl ether (Fluka; distilled), and the mixture was then stirred at room temperature under nitrogen for 24–48 h. The solvent was removed, and the residue was chromatographed on silica gel, where 1-hexadecyl-3-trichloroethyl-*sn*-2-glycerol (**Id**) was eluted in hexane/ethyl acetate (80:20 v/v). ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.58 (m, 2H), 2.55 (br, 1H), 3.50 (m, 4H), 3.84 (m, 2H), 4.0 (m, 1H), 4.18 (s, 2H).

Method f [adopted from Klunder et al. (1989)] was used for the precursor of MJ67. A solution of 10 mmol of trifluoroethyl glycidyl ether, 15 mmol of 1-hexadecylamine, 2 mL of water, and 30 mL of THF were refluxed for 6 h. After the completion of the reaction (TLC in hexane/ethyl acetate, 80:20 v/v), the solvent was removed and the residue was chromatographed on silica gel. The desired product was eluted with $CHCl_3$/MeOH/NH_4OH (95:5:1 v/v). ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.45 (m, 2H), 2.67 (m, 4H), 3.64 (m, 2H), 3.82 (m, 1H), 3.90 (q, 2H), 3.98 (m, 1H).

Method g [adopted from Winstein and Ingrahm (1952)] was used for the precursors of MJ78 and 79. To a three-neck flask containing a solution of 50 mmol of octanol and 100 mL of dried DMF was added 50 mmol sodium hydride with mechanical stirring. When evolution of hydrogen ceased, a solution of 50 mmol of *O*-2-ethylhexyl glycidyl ether (**Ic**) in 20 mL of dry DMF was added dropwise and stirred overnight at room temperature. Water was added to remove excess sodium hydride, and the mixture was extracted with ether, washed well with water, and dried over $MgSO_4$. The residue after the removal of ether was chromatographed on silica gel. The column was eluted with hexane/ethyl acetate (90:10) to yield the product (**Id**) as a yellow liquid. ^{1}H NMR: 0.87 (m, 9H), 1.30 (m, 18H), 1.51 (m, 3H), 2.56 (d, 1H), 3.36 (d, 2H), 3.48 (m, 6H), 3.95 (m, 1H).

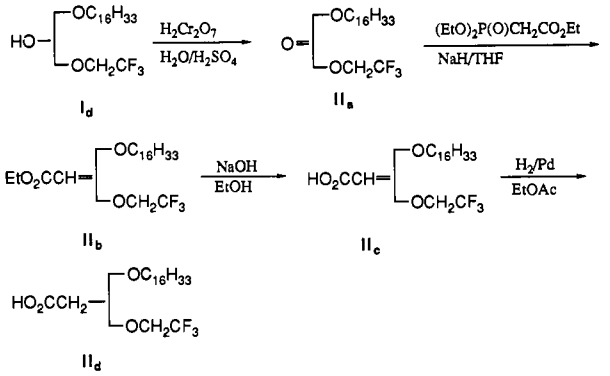
Method h [adopted from Dreger (1932)] was used for the precursors of MJ41 and 42. To a three-neck flask containing 3.6 g of magnesium, 20 mL of ether (sodium-dried) and about 10 mg of iodine was added 2.5 mL of a solution of 1.8 g of allyl bromide in 10 mL of dry ether. As soon as the reaction started, the rest of the solution of allyl bromide was added dropwise at a rate to maintain a gentle reflux. After the addition was completed, the mixture was refluxed on a hot water bath until all the magnesium had reacted (about 30 min). After the mixture was cooled in an ice bath, a solution of 10 mmol of hexadecyl glycidyl ether (Ic) in 10 mL of dry ether was introduced dropwise and then stirred for 2 h. After the reaction was completed (TLC in hexane/ethyl acetate 80:20), crushed ice and 30% sulfuric acid to dissolve the $Mg(OH)_2$ were added. The mixture was extracted with ether, and the extract was washed with water and dried over Na_2SO_4. The residue after the removal of the solvent was chromatographed on silica gel. The product (**Id**) was eluted with hexane/ethyl acetate (90:10 v/v). ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.58 (m, 4H), 2.26 (m, 2H), 3.25 (dd, 1H), 3.42 (dd, 1H), 3.42 (t, 2H), 3.70 (m, 1H), 5.01 (dd, 2H), 5.84 (m, 1H).

Procedures for Phosphorylation of 1,3-Dialkyl-sn-glycerol (Scheme I). Method i [adopted from Eibl & Woolley (1988)] was used for the precursors of MJ33-51, 62, 64–71, 75, 77–78, and 92–97. To a three-neck flask (placed in an ice bath and equipped with a dropping funnel and a drying tube) containing 6 mmol of $POCl_3$ (Aldrich, freshly distilled) was added a solution of 5 mmol of 1-hexadecyl-3-trifluoroethylglycerol (**Id**) and 12 mmol of triethylamine (dried over CaH_2 and distilled) in 15 mL of dry THF. The temperature was kept below 6 °C, and after the addition was completed the mixture was stirred for 30 min and then warmed to room temperature and stirred for 2 h. After the completion of the reaction, excess methanol and 8 mmol of triethylamine were added and the mixture was warmed at 60 °C for 30 min. The mixture was extracted with hexane after adding 10 mL of brine, and the top layer was dried over $MgSO_4$ and filtered through a short column of silica gel (3 × 3 cm) by suction. Hexane was removed to yield a viscous liquid (**Ie**) which was demethylated without purification.

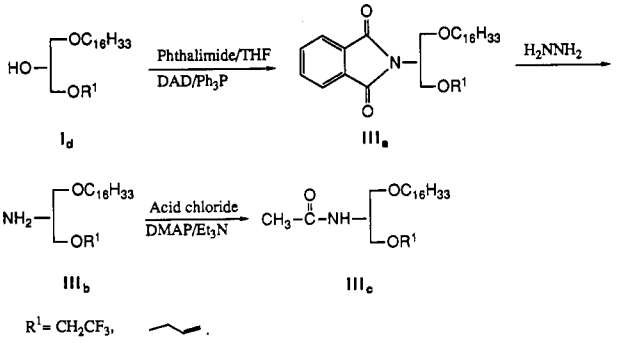
Method j was used for the precursors of MJ52–55, 72–74, 76, 79, and 99. To a three-neck flask (in an ice bath) containing 8 mmol of dichloroalkyl phosphate [e.g., dichlorooctyl phosphate prepared as described by Eibl et al. (1978)] was added dropwise a solution of 5 mmol of alcohol (**Id**) and 10 mmol of triethylamine in 15 mL of dry THF. After the addition, the mixture was stirred at 0 °C for 30 min and then warmed to room temperature and stirred for 12 h. After the completion of the reaction, excess methanol and 10 mmol of triethylamine were added, and the mixture was stirred for 2 h at 60 °C. The reaction mixture was cooled and extracted with hexane after the addition of brine. The extract was washed with brine and dried over $MgSO_4$. After filtration through a short column of silica gel (3 × 3 cm), the solvent was removed to yield a viscous liquid which was used for demethylation without purification.

Method j′ [adopted from Bittman et al. (1984)] was used for the precursor of MJ98. A mixture of 2 mmol of 2-hexadecylbenzyl alcohol, 5 g dimethylchlorophosphate, 20 mL of pyridine, and 10 mL of methylene chloride was stirred overnight at room temperature. Removal of the solvent after coevaporation (2×) with isopropanol gave a viscous liquid (**Ie**)

Scheme II



Scheme III



that was used for demethylation without purification.

General Procedure of Demethylation (Scheme I). Method k [adopted from Eibl & Woolley (1988)]. The solution of 1 mmol of phosphodimethyl ester (**Ie**) and 1.2 mmol of lithium bromide in 10 mL of acetone was refluxed overnight. A white solid precipitated on cooling to −20 °C was filtered and washed with cold acetone. If no solid precipitated, the mixture was concentrated and the residue was chromatographed on silica gel with $CHCL_3/CH_3OH$/concentrated NH_4OH (80:20:4 v/v) to give a waxy solid as the product (**If**). ^1H NMR data for MJ33: 0.87 (t, 3H), 1.25 (s, 26H), 1.55 (m, 2H), 3.45 (t, 2H), 3.55 (m, 2H), 3.62 (d, 3H, J = 10.2 Hz), 3.90 (q, 2H), 4.40 (m, 1H). ^1H NMR data for MJ96: 0.87 (t, 3H), 1.25 (s, 26H), 1.60 (m, 6H), 2.06 (br, 4H), 3.45 (m, 2H), 3.60 (d, 3H), 3.70 (dd, 1H), 3.95 (m, 1H).

Preparation of the Propenyl Derivative MJ44 [Adopted from Eibl et al. (1986)]. A solution of 4 mmol of 1-hexadecyl-3-allylglycerol and 8 mmol of potassium *tert*-butoxide in 15 mL of DMF was heated at 100–110 °C for 1 h. The solution was cooled to room temperature, and 40 mL of water and 40 mL of isopropyl ether were added. The organic layer was washed with water until the pH was about 6 and dried over $MgSO_4$, and then the solvent was removed. The residue was chromatographed on silica gel. The 3-propenyl derivative eluted with hexane/ethyl acetate (90:10 v/v). ^1H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.58 (m, 5H), 2.48 (br, 1H), 3.48 (m, 4H), 3.67 (dd, 2H), 4.42 (dt, 1H), 5.98 (m, 1H).

Preparation of the Trityl Precursor of MJ51 [Adopted from Bittman et al. (1986)]. A solution 7 mmol of 1-hexadecylglycerol, 8 mmol of trityl chloride, and 10 mmol triethylamine in 20 mL of toluene was heated overnight at 60 °C. The trityl derivative in the filtrate was chromatographed on silica gel and was eluted in hexane/ethyl acetate (80:20 v/v). ^1H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.52 (m, 2H), 2.45 (d, 1H), 3.18 (m, 2H), 3.47 (m, 4H), 3.95 (m, 1H), 7.20–7.48 (m, 15H).

1-(1-Hexadecyl-3-trifluoroethyl-2-deoxy-*sn*-glycero)acetic acid (MJ88) and its precursors (MJ86 and 87) were prepared as outlined in Scheme II and described below [adopted from Vogel (1979) and Wadsworth et al. (1965)]. To 33 mmol of sodium dichromate in 30 mL of water, 0.13 mol of sulfuric acid (98%) was added. The solution was cooled to room temperature and diluted to 50 mL with water to give a chromic acid solution; 10.4 mL of this solution was added to a round bottom flask containing 10 mmol of 1-hexadecyl-3-trifluoroethyl-*sn*-glycerol (**Id**) in 10 mL of ether at 0 °C. The reaction mixture was then warmed to room temperature and stirred overnight; during this time the color of the mixture changed from red to dark green. After the completion of the reaction, the organic layer was separated and the aqueous layer was extracted with ether (30 mL × 3). The combined organic layers were washed with 20 mL of saturated $NaHCO_3$ and brine and then dried over $MgSO_4$. The residue after the removal of the solvent was chromatographed on silica gel, and the ketone (**IIa**) was eluted with hexane/ethyl acetate (90:10 v/v), yield 44%. ^1H NMR: 0.87 (t, 3H), 1.30 (br, 24H), 1.60 (m, 4H), 3.48 (t, 2H), 3.95 (q, 2H), 4.16 (s, 2H), 4.50 (s, 2H).

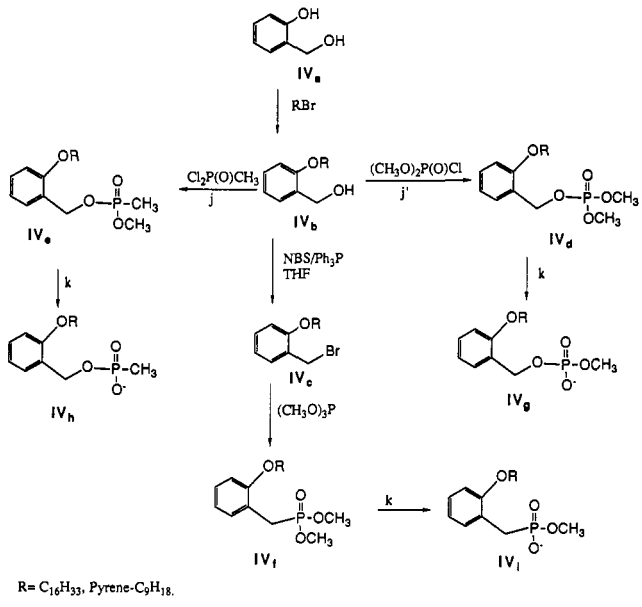
To a three-neck flask containing 3.0 mmol of NaH and 20 mL of dry THF was added dropwise a solution of 3.0 mmol of triethylphosphoroacetate in 10 mL of dry THF at room temperature under nitrogen. The mixture was stirred at room temperature until evolution of hydrogen stopped; then a solution of 2.9 mmol of the ketone (**IIa**) obtained in the preceding step in 10 mL of dry THF was added dropwise at a rate to maintain the temperature below 35 °C. The mixture was stirred overnight at room temperature. After the completion of the reaction, the mixture was poured into a large excess of water and extracted with ether. The extract was washed with water and dried over $MgSO_4$, and the residue after the removal of the solvent was chromatographed on silica gel. The product (**IIb** or MJ86) eluted with hexane/ethyl acetate (90:10), 46% yield. ^1H NMR: 0.87 (t, 3H), 1.30 (br, 24H), 1.60 (m, 7H), 3.40 (t, 2H), 3.95 (q, 2H), 4.20 (q, 2H), 4.40 (s, 2H), 4.80 (s, 2H), 6.80 (s, 1H).

The product (**IIb**) from the preceding step (1.2 mmol), 0.12 g of sodium hydroxide, and 15 mL of ethanol were refluxed overnight. After the reaction was completed, the solvent was evaporated and the waxy solid (**IIc** or MJ87) was hydrogenated without purification. ^1H NMR: 0.87 (t, 3H), 1.20–1.80 (br, 28H), 3.20 (t, 2H), 3.95 (q, 2H), 4.40 (s, 2H), 4.80 (s, 2H), 6.25 (s, 1H).

A flask with a solution and a gas inlet was set up for hydrogenation. To this flask was added 0.3 g of 5% of palladium on active carbon and 5 mL of THF. The flask was evacuated with a water pump, and a solution of 0.5 g of the acid (**IIc**, obtained above) in 20 mL of ethyl acetate was introduced through the solution inlet. Hydrogen was introduced, and the mixture was stirred vigorously. When the uptake of hydrogen stopped, palladium on active carbon was filtered off through Celite. The filtrate was concentrated, and the residue was chromatographed on silica gel, where the product (**IId** or MJ88) eluted with chloroform/methanol/ammonium hydroxide (80:20:4), yield 63%. ^1H NMR: 0.87 (t, 3H), 1.20–1.80 (br, 28H), 2.40 (s, 2H), 3.40 (m, 4H), 3.65 (m, 2H), 3.95 (q, 2H), 4.10 (m, 1H).

N-(1-Hexadecyl-3-alkyl-2-deoxy-*sn*-glycero)acetamide was prepared as outlined in Scheme III [adopted from Mitsunobu et al. (1970), Smith et al. (1955), and Yu et al. (1990)]. A solution of 5 mmol of 1-hexadecyl-3-trifluoroethyl-*sn*-glycerol, 5 mmol of diethylazodicarboxylate, 5 mmol of triphenyl phosphite, and 5 mmol of phthalimide in 30 mL of dry THF was stirred at room temperature under nitrogen for 24 h. After the completion of the reaction, the solvent was removed. The residue was dissolved in ether and hexane was added to pre-

Scheme IV



cipitate a white solid [$(Ph)_3PO$]. The filtrate was concentrated and the residue crystallized with 95% ethanol to give the phthalimide derivative **IIIa**. ^{1}H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.52 (m, 2H), 4.42 (t, 2H), 3.79 (m, 2H), 3.84 (q, 2H), 4.0 (dd, 1H), 4.16 (dd, 1H), 4.74 (m, 1H), 7.70 (m, 2H), 7.85 (m, 2H).

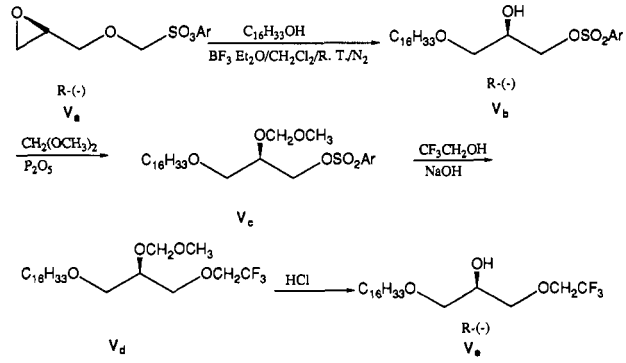
A suspension of 4.5 mmol of the *N*-alkyl phthalimide derivative (**IIIa**), 5 mmol of 95% hydrazine (Kodak), and 20 mL of 95% ethanol was refluxed for 1 h. A white solid precipitated as the reaction progressed. The mixture was cooled to room temperature, and 2 mL of 12 N HCl was added to bring the pH to 2. The precipitated solid was discarded, and the filtrate was evaporated to yield a pale yellow solid, **IIIb**, giving a single spot on TLC. ^{1}H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.58 (m, 2H), 3.48 (t, 2H), 3.58 (m, 1H), 3.68 (d, 2H), 3.90 (m, 2H), 3.98 (q, 2H).

In a three-neck flask containing 2 mmol of the amine derivative (**IIIb**), 0.36 g of 4-dimethylaminopyridine, 0.4 mL of triethylamine, and 10 mL of chloroform were added to a solution of 3.0 mmol of acetyl chloride in 5 mL of chloroform. The mixture was stirred at room temperature until **IIIb** could not be detected by TLC (about 1 h). The mixture was quenched with 20 mL of water, extracted with hexane, washed, and dried over $MgSO_4$. The residue (**IIIc**) was homogeneous on TLC. ^{1}H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.58 (m, 2H), 2.00 (s, 3H), 3.42 (t, 2H), 3.55 (d, 2H), 3.70 (m, 2H), 3.88 (q, 2H), 4.21 (m, 1H), 5.90 (br, 1H).

Preparation of 2-O-Hexadecylbenzyl Alcohol, IVb [Adopted from Vyas (1963)]. A mixture of 20 mmol of 2-hydroxybenzyl alcohol (**IVa**), 25 mmol of 1-bromohexadecane, 25 mmol of potassium carbonate, and 100 mL of ethanol was refluxed for 10 h. After the completion of the reaction, the mixture was poured in water, extracted with ether, washed, and dried over sodium sulfate. The residue after the removal of the solvent was flash chromatographed on silica gel and eluted with hexane/ethyl acetate (80:20 v/v) to yield (80%) the product (**IVb**). ^{1}H NMR: 0.87 (t, 3H), 1.28 (br, 26H), 1.72 (m, 2H), 4.02 (t, 2H), 4.70 (dd, 2H), 6.88 (d, 1H), 6.92 (d, 1H), 7.25 (m, 2H).

Preparation of 2-O-Hexadecylbenzyl Bromide, IVc [Adopted from Schweizer et al. (1969)]. To a three-neck flask with a reflux condenser was added 10 mmol of 2-*O*-hexadecylbenzyl alcohol (**IVb**), 11 mmol of triphenyl phosphite in

Scheme V



20 mL of THF, and then 10 mmol NBS was introduced slowly with stirring. The reaction is exothermic. The mixture was stirred at room temperature for 1 h. After the completion of the reaction, the solvent was evaporated and the residue was extracted with ether. The residue was chromatographed on silica gel and eluted with toluene to yield a purple solid (**IVc**), 71% yield. ^{1}H NMR: 0.87 (t, 3H), 1.28 (br, 26H), 1.82 (m, 2H), 4.02 (t, 2H), 4.58 (dd, 2H), 6.85 (dd, 2H), 7.329 (dd, 2H).

A solution of the bromide (**IVc**, 3 mmol in 10 mL of trimethyl phosphite) was refluxed overnight (Ford-Moore et al., 1963). After the completion of the reaction (TLC in toluene), excess trimethyl phosphite was removed under reduced pressure and the residue was chromatographed on silica gel. The desired product (**IVf**) was eluted with ethyl acetate/chloroform (50:50 v/v). ^{1}H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.70 (m, 2H), 3.30 (d, 2H J = 21.77 Hz), 3.68 (d, 6H j = 11.08 Hz), 4.00 (t, 2H), 6.89 (dd, 2H), 7.26 (m, 2H).

(*R*)-(−)- or (*S*)-(+)-1-hexadecyl-3-trifluoroethyl-*sn*-glycerol for the synthesis of enantiomeric MJ33 was obtained as outlined in Scheme V [adopted from Auerbach et al. (1974), Bittman et al. (1989), Fuji et al. (1975), and Sharpless et al. (1989)]. (2*R*)-(−)-Glycidyl-3-nitrobenzenesulfonate (Aldrich) was recrystallized three times from ethanol to yield pure crystals $[\alpha]_D = -21.1$ (c 16.11, $CHCl_3$). To a round bottom flask containing a solution of 5.8 mmol of recrystallized (2*R*)-(−)-Glycidyl-3-nitrobenzenesulfonate (**Va**), 6.9 mmol of 1-hexadecanol, and 10 mL of dry methylene chloride was added 10 drops of BF_3·Et_2O. This mixture was stirred at room temperature under nitrogen for 24 h. After the reaction was completed, the solvent was removed and the residue was chromatographed on silica gel. The product (**Vb**) eluted with hexane/ethyl acetate (80:20, v/v): $[\alpha]_D = -7.4$ (c 10, $CHCl_3$). ^{1}H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.50 (m, 2H), 3.45 (m, 4H), 4.15 (m, 3H), 7.80 (dd, 1H), 8.25 (d, 1H), 8.52 (d, 1H), 8.80 (s, 1H).

A suspension of 1 g of **Vb**, 25 mmol of dimethoxymethane, 20 mL of dry chloroform, and 30 mmol of P_2O_5 was stirred at room temperature under nitrogen for 24 h. After the completion of the reaction, the mixture was cooled to 0 °C, and 10 mL of 10% Na_2CO_3 was added dropwise to remove excess P_2O_5. The mixture was then extracted with chloroform, washed with water, dried over K_2CO_3, and evaporated to yield **Vc** (97%). ^{1}H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.55 (m, 2H), 3.32 (s, 3H), 3.50 (m, 4H), 3.85 (m, 2H), 4.30 (m, 1H), 4.72 (s, 2H), 7.80 (dd, 1H), 8.25 (d, 1H), 8.52 (d, 1H), 8.80 (s, 1H).

A solution of 1 g of **Vc** in 10 mL of trifluoroethanol and 1 g of NaOH was refluxed for 3 h. After the completion of the reaction, the mixture was cooled to room temperature, poured in water, and extracted with ether. The washed extract

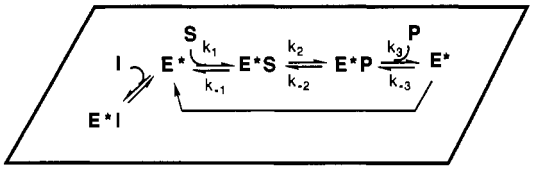


FIGURE 1: Kinetic scheme for interfacial catalysis. In the scooting mode virtually all the enzyme is bound to the interface where it undergoes catalytic turnover according to the steps shown in the box. For complete quantitative analysis of this scheme, see Berg et al. (1991).

was dried over $MgSO_4$. The residue after the removal of the solvent was chromatographed on silica gel, and the trifluoroethyl ether derivative was eluted with hexane/ethyl acetate (90:10) to yield (65%) a colorless liquid, **Vd**. ^1H NMR: 0.87 (t, 3H), 1.30 (s, 26H), 1.55 (m, 2H), 3.38 (s, 3H), 3.50 (m, 4H), 3.75 (m, 2H), 3.82 (q, 2H), 3.92 (m, 1H), 4.75 (s, 2H).

The solution of 0.57 g of **Vd** in 1.5 mL of 12 N HCl and 10 mL of THF was stirred at room temperature for 24 h. After the completion of the reaction, the mixture was extracted with ether and water. The organic layer was washed with water and dried over $MgSO_4$, and the residue was chromatographed on silica gel. The enantiomeric 1-hexadecyl-3-trifluoroethylglycerol (**Ve**) was eluted with hexane/ethyl acetate (80:20), yield 80%. ^1H NMR: 0.87 (t, 3H), 1.25 (s, 26H), 1.59 (m, 2H), 2.65 (br, 1H), 3.47 (m, 4H), 3.68 (m, 2H), 3.90 (q, 2H), 3.98 (m, 1H). $[\alpha]_D = -0.58°$ (c, 7.23; $CHCl_3$).

RESULTS

The analytical formalism to characterize the kinetics of competitive inhibition of the interfacial catalysis in the scooting mode has been described (Berg et al., 1991) and its feasibility demonstrated (Jain et al., 1986c, 1989, 1991a). The kinetic criteria for the competitive inhibition of interfacial catalysis are based on the minimal model shown in Figure 1 for the effect of competitive inhibitors on the kinetics of interfacial catalysis in the scooting mode. In this adaptation of Michaelis–Menten formalism, the inhibitor competes with the substrate monomer for the binding to the active site of the enzyme in the interface. In the highly processive scooting mode of interfacial catalysis, the enzyme remains bound to the anionic interface for several thousand catalytic turnover cycles. Therefore, in the presence of a competitive inhibitor, only the catalytic turnover in the interface would decrease with the increasing mole fraction of an inhibitor incorporated into the interface. In this paper we characterize the inhibition of PLA2 by a novel class of potential transition-state analogues. First we characterize the effects of MJ33 on the interfacial catalysis in the scooting mode and then discuss the structural features that establish this series of compounds as the transition-state analogues. The structure of MJ33 in Figure 2 is compared with other known inhibitors which are also used for certain comparative studies in this paper.

Effect of MJ33 on the Reaction Progress Curve. The progress curve for the hydrolysis of DMPM vesicles in the scooting mode has a pseudo-zero-order region and the logarithmic first-order region. As shown in Figure 3a, the progress curve for the hydrolysis of small sonicated vesicles of DMPM by PLA2 was essentially completely dominated by the first-order region. Such progress curves were obtained under the conditions where the intervesicle exchange of the enzyme, substrate, and products was negligible; and the vesicle population had the size dispersity <0.2 (Berg et al., 1991; Jain et al., 1986a; Jain & Berg, 1989). In the presence of at most one enzyme per vesicle, the progress curve was first-order, and

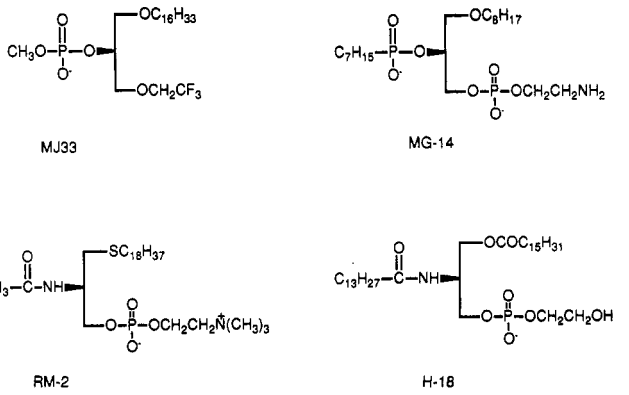


FIGURE 2: Structures of four potent competitive inhibitors of PLA2. RM-2 (Magolda et al., 1987), H-18 (de Haas et al., 1990a), and MG-14 (Yuan et al., 1988) have been described from other laboratories.

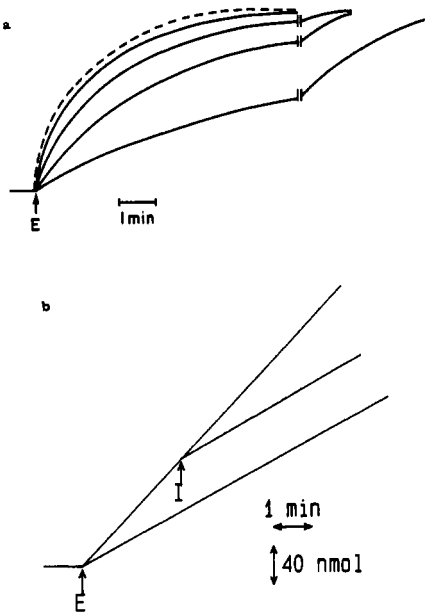


FIGURE 3: (a) First-order reaction progress curves for the hydrolysis of DMPM vesicles in the presence of the increasing (from top) mole fractions of MJ33. The sequence of addition to the reaction mixture was inhibitor, substrate vesicles, and then the enzyme to initiate the reaction. The reaction mixture contained 0.5 mM $CaCl_2$, 1 mM NaCl, 0.35 mM substrate, and 0.4 μg of PLA at pH 8 and 25 °C. The k_i values obtained from such curves are used for generating the plot shown in Figure 4. (b) Zero-order reaction progress curve for the hydrolysis of DMPM vesicles in the presence of 2.5 mM calcium and 10 μg of polymyxin B by 0.02 μg of PLA2. Other conditions as in panel a. MJ33 was added at I in the top curve or before the addition of the enzyme in the bottom curve. The initial slope of the lines is the initial rate, v_0, which is plotted in Figure 4 as a function of the mole ratio of the inhibitor in the interface. These progress curves do not change on dilution.

it was essentially completely described by a single relaxation constant, k_i, by which all the substrate molecules on the outer monolayer of the vesicle, N_S, were hydrolyzed. The rate parameter $k_iN_S = k_{cat}/K_{MS}(1 + 1/K_P)$ is the first-order relaxation rate for the substrate-limited rate of hydrolysis per enzyme (expressed as the turnover number) on a vesicle at the maximum mole fraction of the substrate (= 1). Under these conditions the effect of changing the mole fraction of the inhibitor, X_I, on the rate parameter N_Sk_i is described by the relationship (Berg et al., 1991)

$$(k_iN_S)^0/(k_iN_s)^I = 1 + [(1 + 1/K_I)/(1 + 1/K_P)][X_I/(1 - X_I)] \quad (1)$$

As shown in Figure 3a, the value of k_i decreased with in-

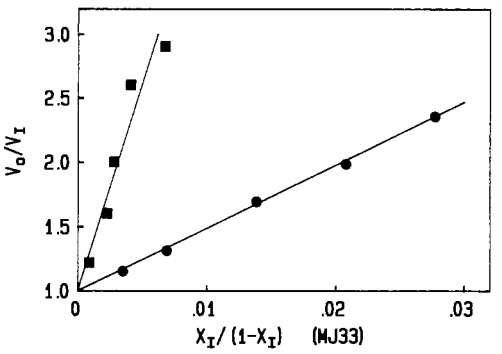


FIGURE 4: Plot of v_0/v_I (squares) or $(N_s k_i)_0/(N_S k_i)_I$ (circles) versus the mole ratio of MJ33 in the substrate interface.

creasing mole fraction of MJ33 without any change in the value of N_S. As shown elsewhere (Jain et al., 1986a; Berg et al., 1991a), under these conditions only the substrate on the outer monolayer was hydrolyzed by the enzyme molecule bound to the target vesicle. Since only a small proportion of the total substrate present in the reaction mixture was hydrolyzed at the end of the reaction, it can also be concluded that the kinetics of intervesicle exchange of the substrate, products, or the enzyme was not noticeably altered in the presence of the inhibitor. The mole fraction of (*S*)-(+)-MJ33 required for a 50% decrease in the value of k_i, $n_I(50) = 0.016$ mole fraction, was obtained from the linearized plot shown in Figure 4. Although it is conventional to express the inhibitory concentrations in molar units, for interfacial catalysis it is more meaningful to express such values as mole fractions because the enzyme, products, substrate, and the inhibitor remain in the interface during and between the catalytic turnover cycles. Also for the interfacial catalysis in the scooting mode the concentration of the bulk substrate or inhibitor has no mechanistic significance.

As shown in Figure 3b, MJ33 also inhibited the pseudo-zero-order region of the progress curve, i.e., the initial rate, v_0. The MJ33-induced inhibition was observed immediately after the addition of the inhibitor, and the rate of hydrolysis was the same whether the inhibitor was added before the addition of E or during the progress of the reaction. For these assays during the progress of the reaction, the substrate was replenished by the calcium-induced fusion of vesicles (Jain et al., 1986a) or by the intermixing of lipids promoted by polymyxin B (Jain et al., 1991). Under these conditions the ratio of the initial rates in the absence and the in the presence of an inhibitor is given by (Berg et al., 1991)

$$(v_0)^0/(v_0)^I = 1 + [(1 + 1/K_I)/(1 + 1/K_{MS})][X_I/(1 - X_I)] \quad (2)$$

Indeed, as shown in Figure 4, the $(v_0)^0/(v_0)^I$ ratio increased linearly with the mole ratio of the inhibitor. The slope of this curve was about 8-fold larger and the mole fraction for 50% inhibition, $X_I(50)$, was 0.003 for (*S*)-(+)-MJ33. The difference in the $X_I(50)$ and the $n_I(50)$ values for a competitive inhibitor of interfacial catalysis is predicted theoretically (Berg et al., 1991) as

$$[1/X_I(50) - 1]/[1/n_I(50) - 1] = [1 + 1/K_P]/[1 + 1/K_{MS}] \quad (3)$$

Indeed, the same ratio was predicted from the experimentally measured values of $K_P = 0.025$ and $K_{MS} = 0.35$ for the action of pig pancreatic PLA2 on DMPM vesicles (Jain et al., 1991a).

Equilibrium Dissociation Constant (K_I) for MJ33 from the Protection Studies. According to eqs 1 and 2, the $n_I(50)$ and $X_I(50)$ values are related to K_I and K_P or K_{MS} values, and the dilution of the substrate by the inhibitor in the interface was already taken into consideration in the derivation of these equations. As described elsewhere (Jain et al., 1991a), the K_I values can be determined independently from the protection experiments, i.e., from the kinetics of alkylation of His-48 in the presence or in the absence of an inhibitor at the active site. The K_I values for several inhibitors are summarized in Table I and III. In conformity with eqs 1 and 2, the K_I value of 0.0009 for (*S*)-(+)-MJ33 was consistent with the observed values of $n_I(50)$ and $X_I(50)$ and the values of K_P (= 0.025) and K_{MS} (0.35) obtained by other methods (Jain et al., 1991a). Thus a consistency of K_I, K_P, and K_{MS} values for an inhibitor–substrate system provided a kinetic proof for the competitive nature of inhibition by (*S*)-(+)-MJ33 (Berg et al., 1991).

MJ33 as a Transition-State Analogue. Calcium is an essential cofactor for PLA2, and barium does not support catalysis. These two cations also behave differently in the binding experiments. The measured dissociation constant for barium was more than 1 mM compared to 0.17 mM for calcium. Also as shown in Table I, only calcium (but not barium) synergistically promoted binding of the three inhibitors. Moreover, the role of His-48 in the binding of MJ33 to PLA2 is indicated by the fact that the K_I and $I(50)$ values are smaller by more than an order of magnitude at pH 6.0 than they are at pH 8.0 (to be published).

Table I: K_I and Related Parameters for Inhibitors of PLA2 (PP)

	K_I	DMPM		DMPC		K_I
inhibitor	(E*Ca·I)	$X_I(50)$	K_MS	$X_I(50)$	K_{MS}	(E*Ba·I)
(*S*)-(+)-MJ33	0.0008	0.0028	0.35	0.0012	1.2	>0.05
RM-2	0.0025	0.009	0.38	0.005	1.0	>0.05
MG-14	0.0011	0.004	0.38	0.002	1.5	>0.012
H-18	0.0016	0.0037	0.45			

Table II: Inhibition of PLA2 from Different Sources by MJ33

source	$n_I(50)$	$X_I(50)$
pig pancreas	0.028	0.003
proPLA		0.010
horse pancreas	0.076	0.0045
bovine pancreas	0.034	0.009
human pancreas	0.06	0.0065
sheep pancreas	0.06	0.005
bee venom	0.015	0.0015
Naja mosambica CM2	0.03	0.0045
Naja mosambica CM3	0.06	0.0055
Naja naja naja	0.08	0.006
Naja nigricola I	0.06	0.003
Naja melanoluca DE1	0.036	0.009
Naja melanoluca DE2	0.04	0.0043
Naja melanoluca DE3	0.044	0.0033
Agkistrodon halys Blomhoffi basic	0.044	0.015
Crotalus terrificus	0.04	0.036

Table III: Equilibrium Dissociation Constants, K_I, for the Amphiphile Bound to Pig Pancreatic PLA2 (E*Ca·L)

amphiphile	K_I
hexadecylphosphocholine	0.75
2-hexadecyl-*rac*-glycero-3-PC[a]	>1
1-hexadecyl-*sn*-glycero-3-PC	0.28
3-hexadecyl-*sn*-glycero-1-PC	0.9
1-oleyl-*rac*-glycero-3-PC	0.16
2-hexadecylpropanediol-1-PC	0.45
3-palmitoylpropanediol-1-PC	>1
3-hexadecylpropanediol-1-PC	>1
dodecyl sulfate	0.07
1-myristoyllysoPC	0.07
1-myristoyllysoPM	0.025
myristic acid	0.15
hexadecylphosphomethanol	0.04

[a] PC, phosphocholine; PM, phosphomethanol.

Table IV: $X_I(50)$ Values for Alkylphosphoesters as Inhibitors of Interfacial Catalysis by Porcine Phospholipase A_2

MJ no.	amphiphile	$X_I(50)$
1	CH_3–OPO_3CH_3	>2
	C_8H_{17}–OPO_3CH_3	0.24
	$C_{12}H_{23}$–OPO_3CH_3	0.12
	$C_{16}H_{33}$–OPO_3CH_3	0.08
	$C_{18}H_{37}$–OPO_3CH_3	0.04
	$C_{20}H_{41}$–OPO_3CH_3	0.037
2	$C_{18}H_{37}$–OPO_3H	>0.3
3	$C_{16}H_{33}$–SO_3H	0.05
	$C_{12}H_{25}$–SO_3H	0.24
4	$C_{16}H_{33}$–OPO_3–choline	>0.4
5	oleyl–OPO_3CH_3	0.03
6	phytyl–OPO_3CH_3	0.08
7	retinyl–OPO_3CH_3	>0.5
8	$C_8H_{17}SC_8H_{16}$–OPO_3CH_3	0.08
9	$C_{18}H_{37}$–$OPO_3CH_2CF_3$	0.3
10	$C_{16}H_{33}OPO_3$–CH_2CH_2OH	>0.5
11	$C_{16}H_{33}OPO_3$–1′-glycerol	>0.5
12	$C_{18}H_{37}OPO_3$–1′-glycerol	>0.5
13	$C_{18}H_{37}OPO_3$–1′-mannitol	0.20
14	$C_{16}H_{33}OPO_3$–1′-myoinositol	0.24
15	$C_{16}H_{33}OPO_3CH_2CH_2Br$	0.03
16	$C_{16}H_{33}$–NH–CH_2CH_2–OPO_3CH_3	0.15

Effect of MJ33 on PLA2s from Other Sources. As summarized in Table II, the $n_I(50)$ and $X_I(50)$ values for MJ33 for PLA2's from several sources are appreciably different. The $X_I(50)$ values for all the compounds of the MJ33 series with three different sources are summarized in Table IV–VI. These inhibitors did not change the shape of the first-order progress curve, which demonstrates that the bound enzyme is not desorbed from the interface in the presence of the inhibitor. The concentration dependence of the inhibition by MJ33 and its analogues for the enzyme was similar to that shown in Figures 3 and 4, and the results summarized in Table V showed that the inhibitory discrimination of more than 200-fold for different enzymes was achieved with some of the inhibitors. These results demonstrate the power of the inhibition assay based on the kinetics in the scooting mode: not only inhibitors but the enzymes from different sources can be compared on the same substrate system. This eliminates not only the need for matching the quality of the interface, but the $X_I(50)$ and $n_I(50)$ values also relate to the K_I values. It may be recalled that according to eq 2 a difference in the $X_I(50)$ values arises from an intrinsic difference in the K_I values of the inhibitor. On the other hand, according to eqs 1–3 the difference in the $X_I(50)$ and the $n_I(50)$ values are due to intrinsic differences between the K_{MS} and K_P values.

Inhibition of Hydrolysis of Zwitterionic Vesicles and Other Forms of Substrates. As expected for a competitive inhibitor of interfacial catalysis, MJ33 inhibited the hydrolysis of zwitterionic substrates by PLA2. The progress curve for the hydrolysis of DMPC vesicles has a latency period that was followed by a steady-state phase of hydrolysis (Apitz-Castro et al., 1982). In the presence of MJ33 the latency period increased and the steady-state rate of hydrolysis decreased with the increasing mole fraction of the inhibitor. In order to eliminate possible complications of the varying mole fractions of MJ33 on zwitterionic interface of DMPC vesicles, we examined the effect of MJ33 only on the catalytic parameters (Jain et al., 1982; Jain & Berg, 1989). This was accomplished by monitoring the initial rate of hydrolysis of the ternary codispersions of DMPC and both the products of hydrolysis (5:1:1 mole ratio) in the presence of varying mole fractions of MJ33. The mole fraction of MJ33 required for 50% inhibition of the steady-state rate of hydrolysis of DMPC vesicles was 0.0012. Since the K_I value of an inhibitor should not change with the nature of the substrate interface, according to eq 2, such a behavior would be consistent with a difference in the relative affinity of the inhibitor compared with that of the two substrates. The K_{MS} values for DMPC predicted from the $X_I(50)$ values are about mole fraction 1 (Table I), which is somewhat larger than the value of 0.35 mole fraction for DMPM. A corresponding difference in the relative K_{MS}/k_{cat} for these two substrates was also observed on the basis of the substrate specificity studies (Ghomashchi et al., 1991).

MJ33 also inhibits the hydrolysis of all other substrates that we have tested. In these cases we have not examined the detailed kinetics; however, the $X_I(50)$ values did change with the nature of the substrate and in some cases with the nature of the interface as modulated by the amphipathic additives.

Additional Controls. We also carried out additional experiments to verify certain assumptions that were necessary for the interpretation of the kinetics of interfacial catalysis in the scooting mode in the presence of inhibitors:

(1) The enzyme was not covalently modified by MJ33 as shown by the fact that the excess substrate vesicles were hydrolyzed at the end of the first-order reaction progress curves obtained in the presence of MJ33. These results were obtained if intervesicle exchange of the enzyme was promoted by salt, if the fusion of vesicles was induced by calcium, or if the intervesicle exchange of lipids was promoted by polymyxin B (Jain et al., 1991c). Also, PLA2 preincubated with MJ33 remained catalytically active when it was added with sufficient dilution to the substrate vesicles.

(2) As shown in Figure 3b, the rate of incorporation of MJ33 in preformed vesicles was rapid (<5 s). In addition, all the inhibitor added to the reaction mixture was incorporated in the vesicles because the dilution of the reaction mixture did not change the shape of the progress curve for the hydrolysis of the vesicles, nor did the $X_I(50)$ or the $n_I(50)$ values change by changing the total lipid present in the reaction mixture. The $n_I(50)$ and $X_I(50)$ values reported here are corrected for the inhibitor to phospholipid ratio in the outer monolayer of the vesicles. Since vesicles were added to a micellar solution of the inhibitor, it was assumed that the inhibitor was uniformly distributed in all the vesicles and that it was present only on the outer monolayer of each vesicle. The rationale for this assumption was that the rate of transbilayer movement of the amphiphiles like MJ33 is expected to be slow (Jain et al., 1985). This conclusion was also supported by the fact that the apparent $X_I(50)$ values increased approximately by a factor of 2 if the inhibitor was codispersed with the substrate during the formation of the vesicles.

(3) Factors that normally modulate the "quality of interface" (such as critical micelle concentration, phase properties, "fluidity") had little effect on the inhibition of interfacial catalysis in the scooting mode. For example, the $X_I(50)$ or $n_I(50)$ values for MJ33 did not show any anomalous effects at the gel–fluid transition temperature or in the presence of lipophilic additives that alter the phase properties of bilayers, i.e., MJ33 did not interfere with the E to E* equilibrium on DMPM vesicles. Indeed, direct binding studies according to the protocol described elsewhere (Jain et al., 1986a) showed that the binding of PLA2 to DTPM vesicles was not noticeably altered in the presence of MJ33. Similarly, binding of PLA2 to DTPC vesicles, which do not bind the enzyme, did not change noticeably in the presence of MJ33.

(4) We could not detect any inhibitory (or activating) effect of MJ33 at 0.05 mole fraction in their substrate interfaces on the catalysis by phospholipase C (*Bacillus cereus*, *Clostridium welchii*), phospholipase D (savoy cabbage), glyceride lipase

Table V: Glycerol Derivatives as Potential Inhibitors of PLA2 from Pig Pancreas (PP), *Agkistrodon haly* Blomhoffi (AHB), and *Cortalus atrox* (CA)

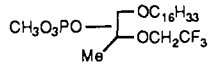
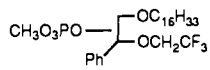
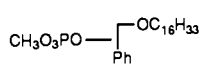
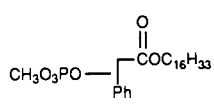
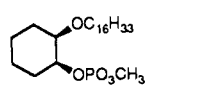
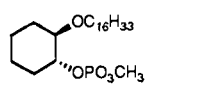
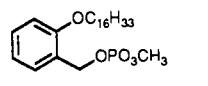
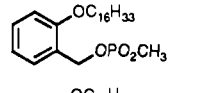
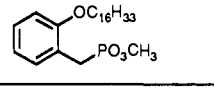
	amphiphile			$X_I(50)$		
MJ no.	$^1CH_2$	^2CH	$^3CH_2$	PP	AHB	CA
17	$OC_{12}H_{25}$	H	$-OPOCH_2CH_2Br$	>0.2		
18	$OC_{16}H_{33}$	H	$-OPO_3H$	>0.2		
19	$OCOC_{17}H_{35}$	H	$-OPO_3CH_2CH_2Br$	0.16		
20	$OC_{18}H_{37}$	H	$-OPO_3(CH_2)_6Br$	0.08		
21	$OC_{18}H_{37}$	H	$-OPO_3CH_3$	0.09		
22	$OC_{22}H_{45}$	H	$-OPO_3CH_2CH_2Br$	>0.2		
23	$OCOC_{15}H_{33}$	H	$-OPO_3H$	>0.2		
24	H	$OC_{16}H_{33}$	$-OPO_3CH_3$	0.08		
25	H	$OC_{16}H_{33}$	$-OPO_3CH_2CH_2Br$	0.09		
26	OCH_3	$OC_{16}H_{33}$	$-OPO_3CH_3$	0.06		
27	OH	$OC_{16}H_{33}$	$-OPO_3CH_3$	0.08		
28	$OCH_2C_6H_5$	$OC_{16}H_{33}$	$-OPO_3CH_2CH_2Br$	0.2		
29	$OC_{18}H_{37}$	OCH_3	$-OPO_3CH_3$	0.1		
30	$OC_{18}H_{37}$	OCH_3	$-OPO_3CH_2CH_2Br$	0.16		
31	$OCOC_{15}H_{31}$	OH	$-OPO_3CH_2CH_2Br$	0.04		
32	$OC_{14}H_{29}$	OH	$-OPO_3CH_2CH_2Br$	0.07		
33	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CF_3$ *rac*	0.0060	0.033	0.036
34	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CF_3$ (*S*)-(+)	0.0028	0.017	0.016
35	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CF_3$ (*R*)-(−)	>0.1	>0.1	>0.1
36	$OCOC_{15}H_{31}$	$-OPO_3CH_3$	-H	0.28	0.16	>0.3
37	$OCOC_{15}H_{31}$	$-OPO_3CH_3$	$-OCH_2CF_3$		a weak substrate	
38	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_3$	0.013	0.027	0.021
39	$OC_{16}H_{33}$	$-OPO_3CH_3$	-OH	0.042	0.038	0.02
40	$OC_{16}H_{33}$	$-OPO_3CH_3$	-Br	0.016	0.014	0.05
41	$OC_{16}H_{33}$	$-OPO_3CH_3$	-CN	>0.3	>0.3	>0.3
42	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OPO_3CH_3$	0.18	0.046	>0.2
43	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-CH_2COOH$	0.02	0.1	0.015
44	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-CH_2CH{=}CH_2$	0.04	0.05	0.02
45	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CH{=}CH_2$	0.0023	0.0028	0.026
46	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH{=}CHCH_3$	0.14	0.21	0.27
47	$OC_{16}H_{33}$	$-OPO_3CH_3$	-O-tosyl[a]	0.0048	0.014	0.004
48	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CH(-O-)CH_2$	0.011	0.08	0.35
49	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CHBrCH_2Br$	0.0019	0.0040	0.011
50	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CCl_3$	0.00042	0.0023	0.02
51	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CH{=}CH(Ph)$[a]	0.012	0.034	0.06
52	$OC_{16}H_{33}$	$-OPO_3CH_3$	$-OC(Ph)_3$	0.022	0.03	>0.5
53	$OC_{16}H_{33}$	$-OPO_3CH_2CH_2Br$	$-OCH_3$	0.09	0.08	0.05
54	$OC_{16}H_{33}$	$-OPO_3CH_2CH_2Br$	$-OCH_2CH{=}CH_2$	0.08	0.06	0.09
55	$OC_{16}H_{33}$	$-OPO_3C_6H_{13}$	$-OCH_2CH{=}CH_2$	0.06	>0.2	0.09
56	$OC_{16}H_{33}$	$-OPO_2CH_3$	$-OCH_2CH{=}CH_2$	0.0015	0.04	0.004
57	$OC_{16}H_{33}$	$-NHPO_2CH_3$	$-OCH_2CH{=}CH_2$	0.15	0.16	0.12
58	$OC_{16}H_{33}$	$-NHPO_3CH_3$	$-OCH_2CF_3$	0.1	0.1	0.02
59	$OC_{16}H_{33}$	$-OPO_2NH_2$	$-OCH_2CF_3$	0.006	0.03	0.08
60	$OC_{16}H_{33}$	$-OPO(NH_2)_2$	$-OCH_2CF_3$	0.08	0.1	0.08
61	$OC_{16}H_{33}$	$-OPO_3H$	$-OCH_3$	>0.3	>0.3	>0.3
62	$OC_{16}H_{33}$	$-OPO_3H$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
63	$OC_{16}H_{33}$	$-OPO_3(CH_3)_2$	$-OCH_2CF_3$	0.018	0.3	>0.5
64	$OC_{16}H_{33}$	$-OSO_3H$	$-OCH_2CF_3$	0.11	0.06	0.025
65	$-C_{15}H_{31}$	$-OSO_3H$	$-OCH_2CF_3$	0.07	0.028	0.03
66	$-C_{15}H_{31}$	$-OPO_3CH_3$	$-OCH_2CF_3$	0.01	0.06	0.05
67	$-NHC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CF_3$	0.27	0.26	0.036
68	$-SC_{16}H_{33}$	$-OPO_3CH_3$	$-OCH_2CF_3$	0.026	0.1	>0.3
69	-oleyl	$-OPO_3CH_3$	$-OCH_2CF_3$	0.0014	0.13	0.14
70	-dibromooleyl	$-OPO_3CH_3$	$-OCH_2CF_3$	0.006	0.1	0.6
71	$-OCH_2CH{=}CH(Ph)$	$-OPO_3CH_3$	$-OCH_2CF_3$	>0.5	>0.5	>0.5
72	$-OC_8H_{17}$	$-OPO_3CH_3$	$-OCH_2CF_3$	0.056	0.13	0.26
73	$-OC_8H_{17}$	$-OPO_3C_8H_{17}$	$-OCH_2CF_3$	0.08	0.05	0.09
74	$-OC_8H_{17}$	$-OPO_3C_6H_{13}$	$-OCH_2CF_3$	0.11	>0.2	>0.2
75	$-C_6H_{13}$	$-OPO_3C_8H_{17}$	$-OCH_2CCl_3$	0.021	0.5	0.13
76	-O-oleoyl	$-OPO_3CH_3$	$-OCH_2CCl_3$	0.0003	0.0045	0.003
77	-O-IOct[a]	$-OPO_3CH_3$	$-OCH_2CH{=}CH_2$	0.13	>0.4	>0.4
78	-O-IOct	$-OPO_3CH_3$	$-OC_8H_{17}$	0.0004	0.014	0.017
79	-O-IOct	$-OPO_3C_8H_{17}$	$-OC_8C_{17}$	0.17	0.23	0.09
80	$-OC_8H_{17}$	$-OCOC_7H_{15}$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
81	$-SC_{16}H_{33}$	$-OCOCH_3$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
82	$-OC_{16}H_{33}$	$-OCOCH_3$	$-OCH_2CF_3$	0.2	>0.3	>0.3
83	$-OC_{16}H_{33}$	-O-tosyl	$-OCH_2CF_3$	0.08	>0.3	>0.08
84	$-OC_{16}H_{33}$	-CN	$-OCH_2CF_3$	>0.3	>0.3	>0.3
85	$-OC_{16}H_{33}$	$=N-OH$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
86	$-OC_{16}H_{33}$	$=CHCOOCH_2CH_3$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
87	$-OC_{16}H_{33}$	$=CHCOOH$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
88	$-OC_{16}H_{33}$	$-CH_2COOH$	$-OCH_2CF_3$	>0.3	>0.3	>0.3
89	$-OC_{16}H_{33}$	$-NHCOCH_3$	$-OCH_2CF_3$	0.2	>0.3	0.2

Table V (Continued)

MJ no.	amphiphile $^1CH_2$	amphiphile ^2CH	amphiphile $^3CH_2$	$X_I(50)$ PP	$X_I(50)$ AHB	$X_I(50)$ CA
90	$-OC_{16}H_{33}$	$-NHCOCH_3$	$-OCH_2CH{=}CH_2$	>0.3	>0.3	>0.3
91	$-OC_{16}H_{33}$	$-NHCOCH_3$	$-OCH_2COOH$	0.07	>0.3	0.08
RM2	$-SC_{18}H_{37}$	$-NHCOCH_3$	-PC[a] *sn*-3	0.0094	0.014	0.017
H18	$-OCOC_{13}H_27$	$-NHCOC_{13}H_{27}$	$-CH_2CH_2OH$	0.0037		
MG14	$-OC_8H_{17}$	$-OPO_2C_7H_{15}$	-PE[a]	0.0034	0.02	0.0084

[a] Abbreviations: Ph, phenyl; IOct, 2-ethylhexyl; PC, phosphocholine; PE, phosphoethanolamine; tosyl, toluenesulfonyl.

Table VI: Inhibitors with a Modified Glycerol Backbone

MJ no.	amphiphile	$X_I(50)$ PP	$X_I(50)$ AHB	$X_I(50)$ CA
92		0.0031	0.17	0.17
93		0.037	0.14	>0.4
94		0.009	0.07	0.034
95		0.016	0.02	0.08
96		0.0005	0.008	0.0018
97		0.015	0.08	0.015
98		0.003	0.01	0.006
99		0.003	0.008	0.08
100		0.11	0.07	0.12

(*Rhizobium*), lipoxygenase (soya bean), cyclooxygenase (seminal vesicles), and acyl-CoA acyl transferase (rat liver). Similarly, MJ33 was not a substrate for phospholipase C and phospholipase D, even when incubated at a 50:1 mole ratio with the enzyme.

(5) MJ33 also inhibited proPLA2 with $X_I(50) = 0.01$ on DMPM. It means that the inhibitory effect is due to interaction at the catalytic site rather than on the interfacial binding. The precursor of pig pancreatic PLA2 hydrolyzes DMPM vesicles only in the hopping mode because its affinity for the interface is poor, i.e., dissociation for E* to E is favored (Jain & Vaz, 1987; Jain et al., 1988). Thus, the relative affinity of the substrate or the inhibitor for proPLA2 at the interface does not seem to be appreciably different than that for PLA2. This would be expected only if the binding of the substrate or inhibitor to the active site of the enzyme is not altered on binding of the enzyme to the interface.

(6) As summarized in Table III, the K_I values for several amphiphiles were also measured. Although most of the zwitterionic amphiphiles are weak inhibitors with $X_I(50)$ values >0.5 mole fraction, as described elsewhere, the weakest of these could be used as neutral diluents (Jain et al., 1991a).

Structure–Activity Correlation. In order to further establish the nature of inhibition of PLA2 by MJ33, we investigated the effect of changing the structure. As summarized in Tables IV–VI, over 100 phosphoesters and analogues without and with the glycerol backbone were synthesized as potential transition-state analogues that may coordinate to calcium at the active site of PLA2 and thus act as active-site-directed inhibitors. As summarized in Table IV, the $X_I(50)$ values for the simple alkylphosphoesters (MJ1 through 16) were >0.03 mole fraction. They exhibited only a modest (<10-fold range) dependence on the structure. For example, the inhibitory potency increased with the alkyl chain length, whereas the $X_I(50)$ values increased if a large polar substituent was also present on the phosphate. Although these amphiphiles were considerably poorer inhibitors compared to their anionic analogues, they served as controls to demonstrate any possible nonspecific inhibitory effects of such amphiphilic compounds on the substrate interface.

For several sn-glycero-3-phosphoesters (MJ17–32, Table V), the $X_I(50)$ values were also in the range of 0.03–0.2 mole fraction. The somewhat improved affinity with these derivatives suggested that the presence of the glycerol backbone provided a better fit at the active site. This could not be entirely due to the presence of the glycerol backbone, because many of the phosphocholine analogues of these compounds had considerably lower affinity [$X_I(50) > 0.5$] even though PLA2 could bind to the aqueous micellar dispersions of these zwitterionic amphiphiles (Jain et al., 1986b). Such a distinction between the ability of PLA2 to bind to dispersions of an amphiphile and the ability of the amphiphile to bind to the active site of the bound enzyme is the hallmark of the kinetic scheme in Figure 1 for interfacial catalysis (Jain & Berg, 1989; Berg, et al., 1991).

The most potent inhibitors of PLA2 that we have examined were *sn*-glycero-2-phosphodiesters with hydrophobic substituents in positions 1 and 3. Many of the compounds summarized in Tables V and VI (MJ33 to MJ100) had $X_I(50)$ values below 0.001, and they showed considerably different affinities for PLA2s from different sources. Additional features that provide insights into the structural requirement for the inhibition of PLA2 are summarized below:

(1) The $X_I(50)$ values for racemic MJ33 compared to that for the two enantiomers showed that only the (*S*)-(+) isomer was inhibitory. In the (*S*)-(+) analogue the hexadecyl chain is in the same spatial position as the *sn*-1 acyl chain in the *sn*-glycero-3-phospholipid substrates. This suggested that the glycerol backbone of MJ33 and the substrate compete for the same site on PLA2.

(2) A significant inhibition was observed only with the analogues containing a phosphate group in the *sn*-2 position but not in the *sn*-1 or *sn*-3 positions (MJ17 through MJ32). This suggested that the inhibitory effect of MJ33 was most probably due to a structural similarity of the phosphodiester bond to the tetrahedral transition state formed during the hydrolytic reaction of the *sn*-2 ester bond. The sulfonate (MJ64), amide (89, 90, and 91), carboxy (87 and 88), oxime (85), and dianionic phosphomonoester (MJ61 and 62) analogues do not appear to be as effective as the corresponding

phosphomethyl esters. It is intriguing that MJ42 with two phosphomethyl ester groups in positions 2 and 3 was a poor inhibitor.

(3) MJ63, the methyl ester of MJ33, was a considerably weaker inhibitor, which suggested that the negative charge played a role in its binding, presumably to the calcium ion bound near the active site. This is consistent with the observation that MJ33 binds to the active site only in the presence of calcium (Table I).

(4) As summarized in Table III, the *sn*-2 amido (H-18 or RM-2) and *sn*-2 phosphonate (MG14) analogues of phospholipids had K_I, $X_I(50)$, and $n_I(50)$ values comparable to those with MJ33. This suggested that the *sn*-3 phosphodiester group in these inhibitors did not necessarily provide additional stability for the inhibitor bound to the active site. It is however intriguing that MJ37 is a weak substrate with the maximum rate of hydrolysis 0.6 s^{-1} with pig pancreatic PLA2, compared to 7 s^{-1} for the hydrolysis of 1,3-dimyristoyl-*sn*-glycero-3-phosphomethanol (Jain & Rogers, 1989). The turnover numbers with *Agkistrodon halys* Blomhoffi and *Crotalus atrox* PLA2's were 0.46 and 0.26 s^{-1}, respectively. The $X_I(50)$ for MJ33 with MJ37 as a substrate for pig pancreatic PLA2 was 0.22 mole fraction. According to eq 2, the K_{MS} for MJ37 would be about 0.005, which implies that the lower rate of hydrolysis of MJ37 is largely due to a lower k_2 value.

(5) The *sn*-1 deoxy analogue of MJ33 with a methylene linkage instead of an alkoxy linkage in the *sn*-1 position was a weaker inhibitor. Similarly, the analogues with a H (MJ36), OH (MJ39), or OCH_3 (MJ38) group instead of the trifluoroethyl group of MJ33 were also weaker inhibitors.

(6) One of the more interesting inhibitors in this series is the cyclohexane analogue (MJ96). It may be viewed as a conformationally restricted analogue for the glycerol backbone during the formation of the transition state.

Numerous subtle features of the structure–activity correlation can be gleaned from the results summarized in Tables IV–VI, and these should be ultimately useful for the modeling of the transition state and for the drug design studies supported by the X-ray crystallographic studies.

Discussion

Evaluation of inhibitors of lipolytic enzymes is a challenging undertaking, stemming from the fact that these enzymes operate on substrates present at organized interfaces and the kinetics can be quite complex. The progress in our understanding of the kinetic basis of interfacial catalysis and inhibition (Jain & Berg, 1989; Berg et al., 1991) and the success in establishing protocols for monitoring interfacial catalysis in the scooting mode (Jain et al., 1986a, 1991a,c) have provided a rational basis for the design and characterization of specific inhibitors of PLA2. Only the broader significance of these results is discussed below.

The assay protocol for the characterization of competitive inhibitors of interfacial catalysis in the scooting mode as described in the preceding section and elsewhere (Jain & Jahagirdar, 1985; Jain et al., 1986b, 1989, 1991a) could be generally useful. It has a well-established kinetic basis (Berg et al., 1991), and the practical advantages include the following: it can be used for structurally diverse inhibitors; the bilayer vesicles used for these studies do not contain any additive; the same substrate system can be used with phospholipase A_2's from different sources without having to match the quality of the interface; the K_I values can be measured directly or calculated from the kinetic results; the competitive nature of inhibition can be unequivocally established on the basis of the kinetic criteria; and the specific inhibitors can be unambiguously distinguished from the nonspecific perturbers of the quality of the interface. This is because in this assay system the quality of the substrate interface is not noticeably perturbed by nonspecific amphiphilic additives as long as the bilayer organization is retained. Therefore virtually all the anomalous effects related to the phase properties of the bilayer can be neglected. Also the kinetics in the scooting mode permits a comparison of the inhibition under a much wider range of conditions including those that perturb the quality of the interface. As discussed elsewhere (Jain & Berg, 1989; Jain et al., 1991a), such advantages are not shared by any other protocol for the characterization of specific inhibitors of PLA2, and the scooting mode analysis does not give "false-positive" inhibitors (Jain et al., 1991a).

The quantitative analysis for the characterization of inhibitors is based on the Michaelis–Menten formalism adopted for the interface (Figure 1). Here all the rate and equilibrium parameters have well-established mechanistic significance (Berg et al., 1991). For example, it may be recalled that the K_I value of an inhibitor for an enzyme does not depend on the nature of the substrate or the interface as modulated by the composition, temperature, or the presence of other additives. The kinetic parameters to characterize the inhibition of interfacial catalysis correlate well with the K_I values obtained from the protection experiments. According to eqs 1 and 2, the K_I values can be computed from the $X_I(50)$ or $n_I(50)$ values if the K_{MS} and K_P values are established independently for a given substrate–enzyme system (Jain et al., 1991a). Otherwise the K_I values can be obtained directly by the protection studies. It may also be pointed out that the relationship between $n_I(50)$ and $X_I(50)$, as described by eq 3, provides the kinetic proof for the competitive nature of inhibition by MJ33 (Berg et al., 1991).

Inhibitors are useful for obtaining the mechanistic information from the kinetic (Jain et al., 1989) and structural studies with cocrystals (Scott et al., 1990; Thunnissen et al., 1990). The design principle for the inhibitors reported here was guided by the belief that the phosphomethyl group is analogous to, or at least mimics at some stage, the putative tetrahedral transition state for the hydrolysis of the *sn*-2 acyl group of the substrate. This is consistent with the observation that the binding of MJ33 to the enzyme is promoted only in the presence of calcium but not barium, that the *sn*-2 analogues are more potent, that they protect His-48 from alkylation, that the analogues with weaker liganding properties to calcium are also weaker inhibitors (e.g., MJ57, 58, 60, and 64); and that MJ33 is a considerably more effective inhibitor at pH 6 than it is at pH 8, as would be expected if the *sn*-2 phosphate interacted with protonated His-48. The fact that the K_I values for the *sn*-3 phosphate based analogues (Figure 2 and Tables I and V) are virtually the same as the values for MJ33 strongly implies that energetically the *sn*-3 phosphate contributes only marginally toward the binding of the inhibitor. Such considerations are being developed further for the mapping and the modeling of the transition state.

Mechanistically, the MJ33 type of inhibitors reported here probably bind to the enzyme via an E–Ca···O=P–inhibitor type of linkage, which is appreciably modulated by the presence of other substituent groups on the inhibitor molecule. In this complex, substitution of O with S (Jain et al., 1989) or NH_2, or the substitution of O=P with O=C–O, or introduction of a second charge on the phosphate drastically lowers the affinity (compare MJ33 with MJ61–65, 75, and 85–88). Other structural features that contribute to the overall binding include chirality at the *sn*-2 position to which the phosphate

group is attached, the linkage at the *sn*-1 position to which the alkyl chain is attached (MJ65–81), and the presence of a medium-sized hydrophobic group at the *sn*-3 position (MJ38–41 and 51–53). Such substitutions could potentially change the conformation of the glycerol backbone.

A requirement of only one monoanionic *sn*-2 phosphate group for the activity raises several interesting questions regarding the nature of its binding to the catalytic site. The X-ray studies on cocrystals of PLA2 show that the oxygen of the *sn*-2 amide (Thunnissen et al., 1990) or phosphonate (Scott et al., 1990) and the oxygen from the *sn*-3 phosphate provide the sixth and the seventh ligands by substitution of the two water molecules coordinated to the calcium ion bound to PLA2. The unexpectedly lower K_I value of the amide is due to the hydrogen bonding of the amide N–H to the ring nitrogen of the imidazole of His-48. In pig pancreatic PLA2, the *sn*-3 phosphate also appears to interact with Tyr-69. The interactions involving the *sn*-3 phosphate are not possible with MJ33; however, as an inhibitor, MJ33 and some of its analogues are as effective as the *sn*-2 amide and phosphonate inhibitors with an *sn*-3 phosphate group. This difference cannot be attributed to a specific interaction of the *sn*-3 substituent of the MJ series. One of the possible explanations is that the interaction of the *sn*-3 phosphate with the Tyr-69 "flap" does not contribute to the binding energy, although it may be favored geometrically during the approach to the transition state. We are exploring the significance of this difference for the stabilization of the transition state for the esterolysis.

The inhibitors are useful for many purposes (Jain, 1981). The MJ series of inhibitors reported here have widely different specificities for PLA2's from different sources, and they have an appreciably diverse range of biophysical properties. The design principles elaborated from these inhibitors could be potentially useful for designing ligands for affinity chromatography of PLA2, as probes for the active site, and for X-ray crystallographic studies on the EI complexes. Potentially, the phosphate triesters which are hydrolyzable in vivo could be designed to understand the biological role of PLA2's. For the in vivo studies, an inhibitor should partition into membranes from the aqueous phase, it must be able to cross the cell membrane, and it should not be susceptible to the action of lipid degrading enzymes present in an organism, such as esterases and phospholipase C and D. Some of these difficulties are overcome with the inhibitors reported here. They form micellar solutions with reasonably high critical micelle concentrations, and therefore they can be readily incorporated in preformed bilayers or intact cells. Since these inhibitors do not have many polar functional groups, they are less likely to be susceptible to degradation by most of the commonly found lipolytic enzymes. These amphiphiles are considerably less polar than the usual phospholipid analogues; therefore, the rate of transbilayer movement is probably less than 5 h (Jain et al., 1985). Thus the water-soluble specific inhibitors of PLA2, such as MJ33, are attractive for the study of their effect on cellular processes. Indeed, MJ33 inhibits the formation of lysophospholipids in perfused lungs (Fisher et al., 1990). Ultimately, such inhibitors should facilitate metabolic studies with whole cells, tissues, and animals to provide insights into the biological role of PLA2. It may be noted that although the amphiphiles like MJ33 do not readily cross the bilayer, special treatments may facilitate their internalization in cells, e.g., by permeabilization, by fusion and endocytosis of vesicles containing these inhibitors, or with the aid of agents that promote transbilayer movement of amphiphilic solutes (Jain et al., 1985), or during a prolonged incubation so that the inhibitor is internalized by endocytosis or "flip-flop". Potentially, it is also possible to design labile triesters of the active phosphate analogues which on hydrolysis in vivo generate the active inhibitor.

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