mixture was slowly stirred at room temperature until the ester was consumed (~15 h). The product was isolated as above and purified by silica gel column chromatography (CH_2Cl_2:MeOH = 95:5, R_f 0.25): $[\alpha]_D$ = -11.92° (c 0.02, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.85 (d, 3 H, J = 6.8 Hz, Val γ-H), 0.92 (d, 3 H, J = 6.8 Hz, Val γ-H), 1.71 (m, 4 H, Adp β- and γ-H), 2.19 (m, 3 H, val β-H + Adp δ-H), 2.70 (dd, 1 H, J_1 = 5.6 Hz, J_2 = 14 Hz, Cys β-H), 2.89 (dd, 2 H, J_1 = 5.6 Hz, J_2 = 14 Hz, Cys β-H), 3.63 (s, 3 H, OMe), 3.78 (s, 2 H, SCH_2Ph), 4.38 (m, 1 H, Val α-H), 4.56 (m, 2 H, Cys α-H + Adp α-H), 5.11 (s, 2 H, Z-CH_2), 5.15 (m, 2 H, Val-OCH_2Ph), 5.62 (d, 1 H, J = 8.8 Hz), 6.33 (d, 1 H, J = 8.0 Hz, Adp NH), 6.82 (d, 1 H, J = 9.2 Hz, Cys NH), 7.33 (s, 15 H, PhH). Anal. Calcd for $C_{36}H_{43}N_3O_8S$: C, 63.81; H, 6.35; N, 6.20. Found: C, 63.90; H, 6.50; N, 6.18.

Bovine kidney γ-glutamyl transpeptidase (60 mg) also catalyzed the same reaction to give the product in 20% yield. When the nucleophile was replaced with Cys(SAcm)OMe-TFA, the yield of Z-Adp-Cys(SAcm)OMe was 45%: ^1H NMR ($CDCl_3$) δ 1.55-1.85 (m, 4 H, Adp β, γ-H), 2.05 (s, 3 H, Acm CH_3), 2.20 (m, 2 H, Adp γ-H), 3.15, 2.95 (ddd, 2 H, Cys β-H), 3.70 (s, 3 H, Adp OCH_3), 3.80 (s, 3 H, Cys OCH_3), 4.30 (m, 1 H, Adp α-H), 4.38 (d, 2 H, Acm CH_2), 4.5 (m, 1 H, Cys α-H), 5.05 (s, 2 H, Z-CH_2), 6.0 (d, 1 H, Adp NH), 6.8 (d, 1 H, Cys NH), 6.9 (m, 1 H, Acm NH), 7.3 (s, 5 H, Ph). Anal. Calcd for $C_{21}H_{29}O_8N_3S$: C, 52.17; H, 6.00; N, 8.70. Found: C, 52.30; H, 5.98; N, 8.80.

Enzymatic Synthesis of δ-[*N*-(Benzyloxycarbonyl)-L-α-(aminoadipyl)]-*S*-(acetamidomethyl)-L-cysteinyl-*o*-methyl-D-allothreonine Methyl Ester [Z-Adp-δ-Cys(SAcm)-D-alloThr(OMe)OMe]. This was prepared with the same procedure as above. The yield obtained from the papain-catalyzed reaction was 46% and that from the glutamyl transpeptidase reaction was 26%: ^1H NMR ($CDCl_3$) δ 1.80 (d, 3 H, Thr γ-H), 1.55-1.85 (m, 4 H, Adp, β, γ-H), 2.05 (s, 3 H, Acm CH_3), 2.10 (m, 2 H, Adp δ-H) 3.15, 2.95 (ddd, 2 H, Cys β-H), 3.10 (s, 3 H, OCH_3), 3.8 (s, 3 H, ester CH_3), 4.40 (d, 2 H, Acm CH_2), 4.45 (m, 1 H, Adp α-H), 4.6 (m, 1 H, Cys α-H), 5.15 (m, 4 H, Thr α, β-H, and Z-CH_2), 6.1 (d, 1 H, Thr NH), 6.35 (d, 1 H, Adp NH), 6.5 (d, 1 H, Cys NH), 7.25 (m, 1 H, Acm NH), 7.35 (s, 5 H, Ph). Anal. Calcd for $C_{26}H_{38}N_4O_{10}S$: C, 52.17; H, 6.35; N, 9.37. Found: C, 52.11; H, 6.33; N, 9.40.

Enzymatic Synthesis of *N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-glycyl-D-leucine Ethyl Ester (Z-Phe-Gly-D-Leu-OEt). Z-Phe-Gly-OEt (2 mmol) and D-Leu-OMe·HCl (4 mmol) were added to 10 mL of Tris (0.2 M) containing 0.02 M $CaCl_2$, 10 mM meraptoethanol, 40% dioxane, and 10% methyl isobutyl ketone by volume, pH 9. Papain (0.8 g) was added, and the mixture was stirred for 2.5 h. The product was extracted with $CHCl_3$, (3 × 50 mL) and washed with 0.1 N $NaHCO_3$ (2 × 20 mL), 0.1 N HCl (2 × 20 mL), and water, dried over Na_2SO_4, and evaporated to give a crystalline solid (0.87 g, 87% yield), which is pure by TLC and NMR: mp 115-116 °C; $[\alpha]_D$ = +9.6° (c 2.4, MeOH); ^1H NMR ($CDCl_3$) δ 3.40 (t, 1 H, Gly NH), 8.20 (d, 1 H, Leu NH), 7.25 (br, 8 H, Z and Phe), 4.91 (s, 2 H, Z-CH_2), 4.30 (br, α-CH of Phe + Leu), 3.90 (br, 2 H, Gly-CH_2), 3.61 (s, 3 H, OCH_3), 3.01 (dd, 2 H, Phe-CH_2), 1.55 (br, 2 H, Leu-CH_2), 0.85 (q, 6 H, Leu-CH_3). Anal. Calcd for $C_{27}H_{35}N_3O_6$: C, 64.80; H, 7.00; N, 8.40. Found: C, 65.18; H, 7.10; N, 7.78. Amino acid analysis: Phe (1) 1.01, Gly (1) 1.0, Leu (1) 1.0. In another synthesis, Z-Phe-OMe (1 mmol), Gly-OPr*i* (2 mmol), and D-Leu-OMe (2 mmol) were used and other conditions were the same. The same product was isolated in 70% yield.

Enzymatic Syntheses of *N*-(Benzyloxycarbonyl)-L-tyrosyl-L(or D)-arginine Methyl Ester (Z-Tyr-L(or D)-Arg-OMe). To 50 mL of sodium carbonate buffer (0.2 M) containing CH_3CN (50%, v/v) was added Z-Tyr-OMe (0.5 M), L- or D-Arg-OMe·HCl (1.5 M), and chymotrypsin (0.2 mM). The reaction was monitored by HPLC and stopped after 10 min when it reached the maximal yield. The mixture was then evaporated to an oily residue under vacuum and 1-butanol (400 mL) was added. The solution was washed with water, 5% $NaHCO_3$, and water and then concentrated. The solid was recrystallized from MeOH/ether to give the product in 75-80% yield. Z-Tyr-L-Arg-OMe: TLC (CH_3Cl_3:MeOH = 9:1 v/v) R_f 0.14; mp 118-121 °C. Z-Tyr-D-Arg-OMe: TLC (CH_3Cl_3:MeOH = 9:1 v/v), R_f 0.14; mp 120-124 °C. Both compounds were identical with those prepared previously.[15]

A New Class of Phospholipase A_2 Substrates: Kinetics of the Phospholipase A_2 Catalyzed Hydrolysis of 3-(Acyloxy)-4-nitrobenzoic Acids

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Abstract: 3-(Acyloxy)-4-nitrobenzoic acids were synthesized with acyl groups ranging from butyryl to dodecanoyl. All these compounds yielded monomeric solutions in water with 1.6% (v/v) acetonitrile in the neutral pH range, and they were hydrolyzed by catalytic amounts of phospholipases A_2 from a variety of sources as shown by the spectral change at 425 nm due to the appearance of nitrophenolate ion. Most of the kinetic studies were performed using *Agkistrodon piscivorus piscivorus* phospholipase A_2, but similar results were obtained with porcine pancreatic and *Crotalus atrox* phospholipase A_2. The catalytic reaction requires the presence of Ca^{2+}, but unlike the hydrolysis of lecithins, the hydrolysis of these substrates also occurs in the presence of Ba^{2+} and Sr^{2+}, while Mg^{2+} and Zn^{2+} are not catalytically competent. Increasing the acyl chain length increases the enzymatic rate mainly by enhancing the hydrophobic interaction in the E-Ca^{2+}-S complex. Among structural isomers of the octanoyl compound, 3-nitro-4-(octanoyloxy)benzoic acid shows the highest specificity toward the enzyme, suggesting that it is in this compound that the distance between the negatively charged carboxylate and the reactive ester approximates best that found in the lecithin-enzyme complex. All kinetic characteristics of the enzymatic hydrolysis indicate that the reaction occurs by the same mechanism as that of the hydrolysis of lecithins. The highest catalytic efficiency observed with this series of substrates occurs with 3-dodecanoyl-4-nitrobenzoic acid, and the second-order rate constant of this reaction (k_{cat}/K_m = 9.1 × 10^4 M^{-1} s^{-1}) is only 1 order of magnitude lower than that of the hydrolysis of egg phosphatidylcholine in unilamellar vesicles. The reactivity of all isomers, especially that of the *p*-carboxy ester, shows that Ca^{2+} does not act as a catalyst in the phospholipase A_2 catalyzed hydrolysis but rather serves to bind and orient the substrate at the active site of the enzyme. The octanoyl compounds, **1** and **2**, are ideally suited for a rapid and sensitive spectrophotometric assay of phospholipases A_2, and the conditions for the assay are described.

Phospholipases A_2 (PLA_2, EC 3.1.1.4) are a class of calcium-dependent enzymes that catalyze the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids (for a review, see ref 1). The essential structural elements of the substrate that appear to be required for recognition by PLA_2 are the glycerol backbone, the ester function in position 2, and the negative charge of the phosphate in position 3 (see Figure 1). While the enzyme has a pronounced preference for substrates aggregated into micelles,

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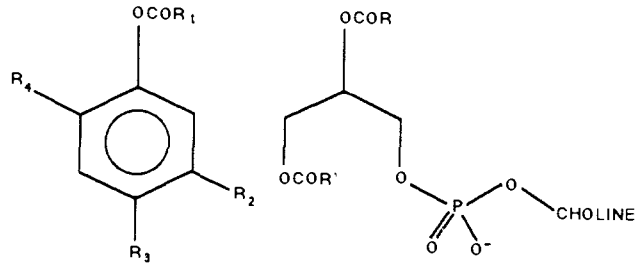

	R_1	R_2	R_3	R_4
1	$(CH_2)_6CH_3$	COOH	H	NO_2
2	$(CH_2)_6CH_3$	H	COOH	NO_2
3	$(CH_2)_6CH_3$	H	NO_2	COOH
4	$OCO(CH_2)_6CH_3$	COOH	H	NO_2

Figure 1. Structure of **1–4**. Essential structural features of a phosphatidylcholine are presented for comparison.

unilamellar vesicles, or monolayers, it will also react with water-soluble phospholipids, although at a slower rate. The chemical mechanism of the enzymatic catalysis is not fully understood yet. Wells has shown[2,3] that the order of addition in the case of the enzyme from the venom of *Crotalus adamanteus* is Ca^{2+} first, followed by the substrate, and that the hydrolysis occurs with acyl-oxygen cleavage. It has been proposed[4] that Ca^{2+} might act as a superacid in the hydrolysis of the substrate.

In order to investigate the mechanism of the PLA_2-catalyzed reaction, it would be advantageous to use substrates for which structural variations could be correlated with catalytic activity. We explored the possibility that perhaps the enzyme would complex in which the glycerol backbone and the phosphate diester are replaced by simpler structures readily of organic synthesis. Since a major role of the phosphate is to complex Ca^{2+} in the active site of the enzyme, it was reasonable to propose that a carboxylate group could replace the phosphate in this function. For this purpose, the distance between the reactive ester and the carboxylate group in the new substrate should be similar to that found in phospholipids in order to position properly the reactive ester moiety with respect to the catalytic apparatus. The glycerol would also be replaced by a nitrophenol, a much better leaving group. The release of the nitrophenol in the enzymatic reaction would provide a way to monitor the progress of the enzyme-catalyzed hydrolysis by spectrophotometry. The simplest molecule that fulfills these structural requirements is an ester of 3-hydroxy-4-nitrobenzoic acid, shown in Figure 1. In the same figure, the essential structural features of a phosphatidylcholine are presented for comparison. It should be noted that while the geometries of the molecules are different, the sums of the bond lengths between the reactive ester and the ligand of Ca^{2+} are approximately the same in both molecules. We have synthesized 4-nitro-3-(octanoyloxy)benzoic acid and have found it to be an excellent substrate for the enzyme. In order to characterize the chemical features of this type of substrate necessary for reactivity toward PLA_2, similar substrates were synthesized in which the distance between the reactive ester and the carboxylate group was varied and the reactivity of these compounds toward the enzyme was also determined. In addition, the dependence of the rate of the enzyme-catalyzed hydrolysis on the chain length of the reactive ester was investigated in order to assess the importance of the hydrophobic interaction between the enzyme and the substrate in this process. Finally, the role of the calcium ion in the catalysis was investigated in detail using these compounds.

Thus, we present in this paper a new class of substrates for PLA_2 that are excellent tools in the investigation of the mechanism of the enzyme.

Experimental Section

Materials. 3-Hydroxy-4-nitrobenzoic acid, 4-hydroxy-3-nitrobenzoic acid, and 2-hydroxy-5-nitrobenzoic acid were purchased from Aldrich Chemical Co. and recrystallized twice from water before use. They are pure by TLC and melting point criteria. Butyryl chloride (>99%), hexanoyl chloride (>99%), heptanoyl chloride (99%), octanoyl chloride (99%), nonanoyl chloride (98%), decanoyl chloride (98%), dodecanoyl chloride (98%), octanoic acid (>99%), undecanoic acid, isobutyl chloroformate, and methyl 4-nitrobenzenesulfonate (>99%) were obtained from Aldrich and used without purification. *p*-Nitrophenyl dodecanoate was purchased from Sigma Chemical Co. THF, acetonitrile, and hexane (HPLC grade) were purchased from Burdick and Jackson Co. THF was refluxed over $LiAlH_4$ and distilled. *N,N*-Diisopropylethylamine (Aldrich) was treated with solid KOH and distilled over ninhydrin. *N*-Methylmorpholine (Aldrich) was refluxed over BaO and distilled. 1,2-Dioctanoyl-*sn*-glycero-3-phosphorylcholine was purchased from Avanti Biochemicals, Inc. All other buffers and chemicals were of the highest purity commercially available. Water was deionized on a mixed-bed ion exchanger and then distilled in an all-glass apparatus. Elemental analyses were performed by Galbraith Laboratories, Inc.

4-Nitro-3-(octanoyloxy)benzoic Acid (1). Method 1. 3-Hydroxy-4-nitrobenzoic acid (183 mg, 1.0 mmol) and *N,N*-diisopropylethylamine (259 mg, 2.0 mmol) in 2 mL of dry THF was added dropwise to the cooled solution (0 °C) of octanoyl chloride (163 mg, 1.0 mmol) in 10 mL of dry THF. The reaction mixture was warmed slowly to room temperature and stirred overnight. The reaction mixture was filtered, and the precipitate was washed with ether (10 mL × 3). The filtrate was concentrated in vacuo, and the residue was taken up by 50 mL of ether–hexane mixture (2:1 (v/v)). The organic layer was washed with 0.01 N HCl (50 mL × 2) and water (50 mL × 2), dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was further purified by flash chromatography (Merck Kieselgel 60, hexane–ether–acetic acid, 2:1:0.1 (v/v/v) (solvent I)). The eluate was monitored by TLC. Evaporation of the pooled major fractions resulted in 280 mg of a slightly yellow solid (90%). Finally, the product was recrystallized from ethyl acetate–hexane (1:10 (v/v)): mp 142–143 °C; R_f 0.44 (solvent I); IR (neat) 3000–2750 (m), 1731 (s), 1642 (s), 1520 (s) cm^{-1}; ^1H NMR (determined on a Nicolet 500-MHz instrument in $CDCl_3$ with TMS as a standard) δ 0.91 (t, 3 H), 1.29–1.47 (m, 8 H), 1.79 (quintuplet, 2 H), 2.66 (t, 2 H), 7.94 (s, 1 H), 8.08 (d, 1 H), 8.12 (d, 1 H). Anal. Calcd for $C_{15}H_{19}NO_6$: C, 58.25; H, 6.19; N, 4.53. Found: C, 57.98; H, 6.30; N, 4.31. Finally, alkaline hydrolysis of a weighed amount of the compound released the expected amount of 3-hydroxy-4-nitrobenzoic acid, as determined spectrophotometrically at 425 nm.

Method 2. Octanoic acid (144 mg, 1.0 mmol) was dissolved in 10 mL of dry THF. The solution was stirred and cooled to 0 °C. Then, *N*-methylmorpholine (101 mg, 1.0 mmol) and isobutyl chloroformate (137 mg, 1.0 mmol) were added successively. The reaction mixture was stirred for 30 min at 0 °C during which time a white precipitate formed. Next, the reaction mixture was allowed to reach room temperature, and a solution of 3-hydroxy-4-nitrobenzoic acid (200 mg, 1.1 mmol) and *N*-methylmorpholine (222 mg, 2.2 mmol) in 5 mL of dry THF was added. The resulting mixture was stirred overnight at room temperature. The crude product was treated as described in method 1. Method 2 yielded two major products that were separated by flash chromatography. The first fractions proved to be the same as the ester prepared by method 1. The later fractions (R_f 0.29, solvent I) were collected and concentrated in vacuo, resulting in 140 mg of a light yellow solid that is pure by TLC and has a sharp melting point. Alkaline hydrolysis of the compound monitored spectrophotometrically at 425 nm showed a biphasic reaction, indicating the presence of two distinct nitrophenyl esters in the molecule in a strictly 1:1 ratio. The UV–vis spectrum and the absorbance at 425 nm of the completely hydrolyzed compound showed that this compound contained two 3-hydroxy-4-nitrobenzoic acids per octanoic acid. Thus, this compound was 4-nitro-3-[[4′-nitro-3′-(octanoyloxy)benzoyl]oxy]-benzoic acid (**4**): mp 141–142 °C; ^1H NMR ($CDCl_3$) δ 0.90 (t, 3 H), 1.32–1.45 (m, 8 H), 1.79 (quintuplet, 2 H), 2.66 (t, 2 H), 8.04–8.21 (m, 6 H); IR (neat) 3000–2650 (br), 1750 (s), 1700 (s), 1590 (s), 1485 (s) cm^{-1}. Anal. Calcd for $C_{22}H_{22}N_2O_{10}$: C, 55.70; H, 4.67; N, 5.91. Found: C, 56.17; H, 4.78; N, 5.62.

3-(Butyryloxy)-4-nitrobenzoic Acid. This compound was synthesized by method 1 from butyryl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 143–143.5 °C. Anal. Calcd for $C_{11}H_{11}NO_6$: C, 52.18; H, 4.38; N,

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5.53. Found: C, 52.26; H, 4.29; N, 5.46.

3-(Hexanoyloxy)-4-nitrobenzoic Acid. This compound was synthesized by method 1 from hexanoyl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 139–140 °C (lit.[5] mp 139–140 °C). Anal. Calcd for $C_{13}H_{15}NO_6$: C, 55.52; H, 5.38; N, 4.98. Found: C, 55.31; H, 5.21; N, 4.94.

3-(Heptanoyloxy)-4-nitrobenzoic Acid. This compound was prepared by method 1 from heptanoyl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 144.5–155.5 °C. Anal. Calcd for $C_{14}H_{17}NO_6$: C, 56.95; H, 5.80; N, 4.74. Found: C, 56.51; H, 5.54; N, 4.73.

4-Nitro-3-(nonanoyloxy)benzoic Acid. This compound was prepared by method 1 from nonanoyl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 143–144 °C. Anal. Calcd for $C_{16}H_{21}NO_6$: C, 59.43; H, 6.55; N, 4.33. Found: C, 59.18; H, 6.63; N, 4.11.

3-(Decanoyloxy)-4-nitrobenzoic Acid. This compound was synthesized by method 1 from decanoyl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 142.5–144 °C. Anal. Calcd for $C_{17}H_{23}NO_6$: C, 60.52; H, 6.87; N, 4.15. Found: C, 60.85; H, 6.87; N, 4.08.

4-Nitro-3-(undecanoyloxy)benzoic Acid. This compound was prepared by method 2 from undecanoic acid and 3-hydroxy-4-nitrobenzoic acid; mp 143–144 °C. Anal. Calcd for $C_{18}H_{25}NO_6$: C, 61.53; H, 7.17; N, 3.99. Found: C, 61.56; H, 7.01; N, 3.96.

3-(Dodecanoyloxy)-4-nitrobenzoic Acid. This compound was synthesized by method 1 from dodecanoyl chloride and 3-hydroxy-4-nitrobenzoic acid; mp 142–143 °C. Anal. Calcd for $C_{19}H_{27}NO_6$: C, 62.45; H, 7.45; N, 3.83. Found: C, 62.32; H, 7.13; N, 3.76.

3-Nitro-4-(octanoyloxy)benzoic Acid (2). This compound was synthesized by method 1 from octanoyl chloride and 4-hydroxy-3-nitrobenzoic acid; mp 67–68 °C. anal. Calcd for $C_{15}H_{19}NO_6$: C, 58.27; H, 6.19; N, 4.53. Found: C, 58.32; H, 6.17; N, 4.46.

4-Nitro-2-(octanoyloxy)benzoic Acid (3). This compound was synthesized by method 1 from octanoyl chloride and 2-hydroxy-5-nitrobenzoic acid; mp 35–36 °C. Anal. Calcd for $C_{15}H_{19}NO_6$: C, 58.27; H, 6.19; N, 4.53. Found: C, 58.05; H, 6.19; N, 4.25.

Agkistrodon piscivorus piscivorus PLA_2 (App-D-49) was purified as reported previously.[6] *Crotalus atrox* PLA_2 was purified according to the procedure described by Hachimori et al.[7] Porcine pancreatic PLA_2 was purchased from Sigma as a suspension in a 3.2 M $(NH_4)_2SO_4$ solution and used after dialysis and lyophilization. Enzyme concentrations were determined spectrophotometrically at 280 nm with values of molar absorptivity (M^{-1} cm^{-1}) of 3.5×10^4 for App-D-49 and 1.8×10^4 for pancreatic PLA_2. Concentration of *C. atrox* enzyme solution was determined by amino acid analyses. The enzymes were assayed with a monolayer of dioctanoyllecithin at the air–water interface.[8] In the following, PLA_2 refers to App-D-49 unless specified otherwise.

Methylation of PLA_2 was performed in the following manner: PLA_2 (10 mg, 0.7 μmol) was dissolved in 0.1 M cacodylate hydrochloride, pH 6.0 (2 mL). Methyl 4-nitrobenzenesulfonate[9] (7.6 mg, 35 μmol) in 50 μL of acetonitrile was added, and the reaction was allowed to proceed at room temperature until the enzymatic activity was reduced to less than 1% of the original (ca. 1.5 h), as measured by the monolayer assay. The reaction was then quenched by 1 mL of acetic acid, and the slight amount of precipitate formed was removed by centrifugation. The supernatant was chromatographed on a column (2 × 30 cm) of Sephadex G-25 (Pharmacia) with 5% (v/v) aqueous acetic acid as an eluent. The protein peak monitored spectrophotometrically at 280 nm, was collected, lyophilized, and purified further by HPLC using a Mono S HR 5/5 (Pharmacia) column equilibrated in 50 mM MES buffer, pH 6.5, containing 0.1 M NaCl. The column was developed with a linear gradient of NaCl concentration up to 0.5 M in the same buffer (total volume 30 mL), and the eluent was monitored at 280 nm. A major protein peak was collected and rechromatographed under the same condition. Desalting and lyophilization of the pooled protein fractions yielded 5.0 mg of modified PLA_2 (overall yield 50%). Amino acid analysis of the protein was performed on a Beckman Model 118 CL amino acid analyzer equipped with a Beckman W-3H column 0.6 × 23 cm). The amino acid standards were purchased from Pierce Chemical Co. A base-line separation of histidine, 1-methylhistidine, and 3-methylhistidine was achieved under the conditions employed. Analysis of the purified protein showed that there was no measurable change in the amino acid composition except for the complete conversion of one histidine per molecule of enzyme to 3-methylhistidine. 1-Methylhistidine was not detectable. The purified methyl-PLA_2 ($MPLA_2$) showed less than 0.5% of the original specific activity in the monolayer assay.

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Table I. Second-Order Rate Constants of PLA_2-Catalyzed Hydrolysis[a] of **1**

10^6E_0, M	10^5S_0, M	$10^3[Ca^{2+}]$, M	$10^{-3}(k_{cat}/K_m)$, M^{-1} s^{-1}
1.0	5.0	10.0	3.2 ± 0.02
4.9	5.0	10.0	3.2 ± 0.04
9.8	5.0	10.0	3.1 ± 0.04
4.9	7.5	10.0	3.0 ± 0.04
4.9	10.0	10.0	3.0 ± 0.04
3.7	7.2	0.0[b]	<0.05[c]
10.0[d]	7.2	0.0[b]	<0.05[c]
10.0[d]	7.2	10.0	<0.05[c]

[a] 37 °C, 0.1 M Tris-HCl buffer, pH 8.0, 0.1 M NaCl, 1.6% (v/v) CH_3CN. [b] [EDTA] = 0.1 mM. [c] Calculated from initial rate. [d] $MPLA_2$.

Kinetics. The kinetics of hydrolysis were monitored on a Perkin-Elmer Lambda-5 recording spectrophotometer equipped with a thermostated cell compartment and a magnetic stirrer for the cuvette. The progress of the reaction was monitored in the range 425–500 nm. The wavelength was chosen to limit the total spectral change during the reaction to less than 1 AU. A typical experiment is described for the hydrolysis of **1**: Three milliliters of 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ and 0.1 M NaCl was equilibrated at 37 °C in the cuvette in the sample compartment of the spectrophotometer. A 50-μL portion of the stock solution of the ester in acetonitrile was added with thorough mixing ($S_0 = 5 \times 10^{-5}$ M), and the slight buffer-catalyzed hydrolysis was monitored at 425 nm as a function of time for base-line correction. The enzymatic reaction was initiated by adding 50 μL of an enzyme stock solution, and the reaction was monitored for at least 4 half-lives. Correction for the nonenzymatic hydrolysis was negligible in the range pH 7.0–8.6. After completion of the reaction, the spectrum of the solution was measured and the amount of 3-hydroxy-4-nitrobenzoate was calculated. The products were extracted with ether after acidification with 1 N HCl and analyzed by TLC. Kinetic data were collected in the digitized form, and the kinetic parameters were calculated with a nonlinear least-squares analysis of the entire progress curve of the reaction. At least 40 datum points per experiment were used for the analysis.

Results

In preliminary experiments, we determined spectrophotometrically the ionization constant of the phenol group of 3-hydroxy-4-nitrobenzoic acid ($pK_a = 7.10 \pm 0.03$) and its molar absorptivity in the 425–500-nm range (4990 M^{-1} cm^{-1} at 425 nm at pH >9.0) under our experimental conditions. We also determined the solubility limit of each compound under the same conditions (see Table III). The existence of a clear solubility limit generally precludes aggregation in solution. To rule out the possibility that a compound already existed as an aggregate at its solubility limit, we ascertained that the conductivity and the UV absorbance of the compounds were linear with respect to the concentration up to the solubility limit (data not shown).

Enzymatic Hydrolysis of 4-Nitro-3-(octanoyloxy)benzoic Acid. Addition of catalytic amount of PLA_2 to solution of **1** resulted in complete hydrolysis as assessed spectrophotometrically. At $S_0 < 1.0 \times 10^{-4}$ M and $E_0 = 1.0 \times 10^{-6}$ to 1.0×10^{-5} M, the rate of phenolate formation obeyed first-order kinetics. The rate constants were calculated from the experimental absorbance vs time data by the equation $A = (A_f - A_i)(1 - e^{-kt}) + A_i$, where A_i and A_f are the initial and final absorbances of the reaction mixture at a given wavelength, respectively, and k is the apparent first-order rate constant. The value of k_{cat}/K_m was calculated by dividing k by the enzyme concentration. We could not determine values of k_{cat} and K_m, because we observed that at $S_0 > 0.5$ mM, the enzyme ($E_0 = 2 \times 10^{-6}$) underwent an irreversible change during the reaction.[10] This phenomenon was not observable under our experimental conditions as shown by the fact that the rate

(10) Using radioactivity labeled **1**, prepared from octanoic acid-*carboxy*-^{14}C, we found that the modification is due to the acylation of two Lys residues (Lys-7, Lys-10) in the N-terminal region of the enzyme by the octanoyl moiety of the substrate. The acylation enhances the specific activity of the enzyme toward **1** by a factor of ca. 3.

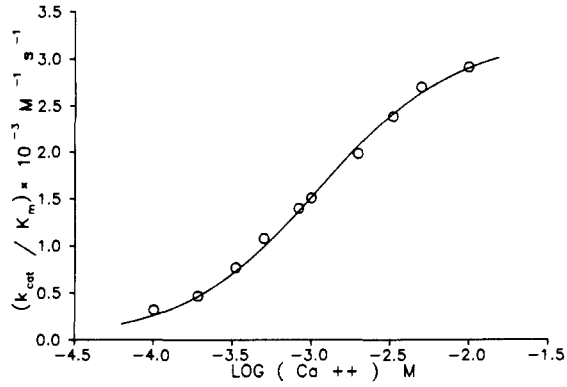


Figure 2. Calcium ion dependence of the second-order rate constant of the PLA_2-catalyzed hydrolysis of **1** at pH 8.0, 37 °C, $E_0 = 2.5 \times 10^{-6}$ M, $S_0 = 5.0 \times 10^{-5}$ M. Each point is the mean of three determinations. Theoretical curve is constructed with eq 1.

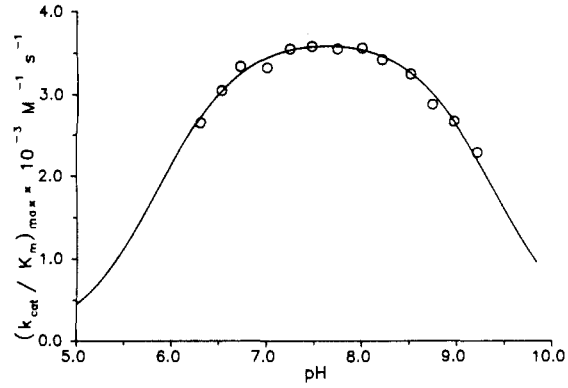


Figure 3. pH dependence of $(k_{cat}/K_m)_{max}$ for the PLA_2-catalyzed hydrolysis of **1** at 37 °C, $E_0 = 5.0 \times 10^{-6}$ M, $S_0 = 5.0 \times 10^{-5}$ M, $[Ca^{2+}]$ = 0.05–20 mM. Each point is the mean of three determinations. Theoretical curve is constructed from the parameters in Table II and eq 3.

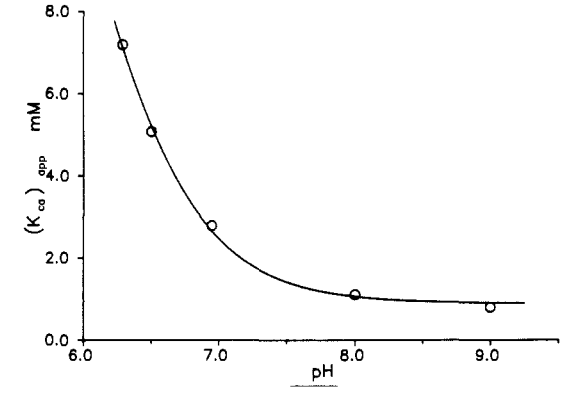


Figure 4. pH dependence of $(K_{Ca})_{app}$ for the PLA_2-catalyzed hydrolysis of **1** at 37 °C, $E_0 = 5.0 \times 10^{-6}$ M, $S_0 = 5.0 \times 10^{-5}$ M, $[Ca^{2+}] = 0.05$–20 mM. Each point is the mean of three determinations. Theoretical curve is constructed from the parameters in Table II and eq 5.

constant of a second reaction, initiated by adding 5 μL of the concentrated substrate stock solution to the reaction mixture ($S_0 = 5.0 \times 10^{-5}$ M) after completion of the hydrolysis, was always the same as that of the first reaction. The second-order rate constants determined under a variety of reaction conditions are summarized in Table I. They show that the rate of hydrolysis is indeed linearly proportional to the enzyme concentration in the range of 1–10 μM; i.e., the reaction is first-order with respect to the enzyme. The rate constant is independent of initial substrate concentration; thus, inhibition by the reaction products is not significant. The reaction requires the presence of Ca^{2+}, indicating that the reaction must occur at the active site of the enzyme. This conclusion is also supported by the fact that *p*-nitrophenyl dodecanoate, which lacks a carboxylic group, is not hydrolyzed by the enzyme. Also, methylated PLA_2 ($MPLA_2$) is inert toward **1** in the presence or absence of Ca^{2+}. Finally, we found that acetonitrile, which was added to assist the dissolution of substrates, competitively inhibited PLA_2 (data not shown). However, the inhibition is not significant when the concentration of acetonitrile is lower than 3% (v/v).

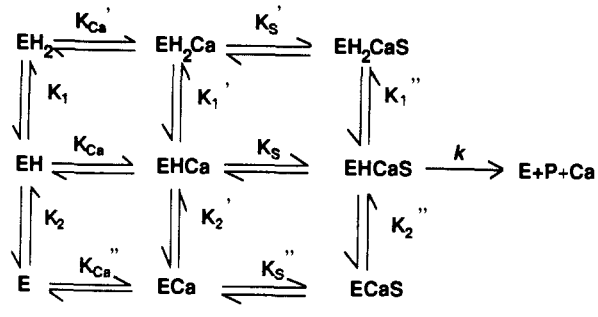
Calcium Dependence of k_{cat}/K_m at pH 8.0. Dibutyryllecithin is hydrolyzed by *C. adamanteus* PLA_2 through the ordered pathway where Ca^{2+} is added first and the substrate second.[2] We have observed[6] the same order of addition for *A. piscivorus piscivorus* PLA_2. If the addition of Ca^{2+} is a fast equilibrium, then the calcium dependence of the rate at a given pH is expressed by eq 1, where $(k_{cat}/K_m)_{max}$ is the specificity constant at saturating

$$-d[S]/dt = (k_{cat}/K_m)[E_0][S] = \frac{(k_{cat}/K_m)_{max}}{1 + K_{Ca}/[Ca^{2+}]}[E_0][S] \quad (1)$$

calcium concentration and K_{Ca} is the dissociation constant of the enzyme–Ca^{2+} complex. The calcium dependence of k_{cat}/K_m of **1** is shown in Figure 2. The data were analyzed by a nonlinear least-squares fit program using eq 1, and the agreement of the experimental points with the theoretical curve calculated from eq 1 justifies the assumption underlying eq 1. The validity of eq 1 implies that calcium binding is not rate limiting. The value of the dissociation constant, K_{Ca}, determined by this analysis (see Table V) is comparable to that determined from the hydrolysis of a dioctanoyllecithin monolayer at the air–water interface with the same enzyme,[6] $K_{Ca} = 1.9 \times 10^{-3}$ M.

pH Dependence of the Kinetic Parameters. The pH dependence of $(k_{cat}/K_m)_{max}$ and of K_{Ca} of the hydrolysis of **1** is shown in Figure 3 and 4. The pH dependence of the second-order rate constant is a typical bell-shaped curve (Figure 3), suggesting the involvement of one acidic ($pK_a \simeq 6.0$) and one basic ($pK_a \simeq 9.5$) group in the catalysis. Figure 4 shows that the calcium ion binding is hindered, albeit not precluded, by the protonation of a group whose pK_a value is lower than 6.5. The simplest mechanism that could account for these observations consists of the involvement of a cationic acidic group of $pK_a \simeq 6.0$ on the enzyme, which in its deprotonated form participates in the catalysis but, once protonated, repels electrostatically Ca^{2+}. In addition, a group of $pK_a \simeq 9.5$ is required for the catalysis but this group does not interfere with the binding of Ca^{2+} and hence should be located at the relatively large distance from Ca^{2+}. Such a pathway leads to the reaction scheme shown in Scheme I. When $S_0 \ll K_m$, Scheme I yields the rate equations 2–4.

Scheme I



$$-d[S]/dt = \frac{(k_{cat}/K_m)_{max}}{1 + (K_{Ca})_{app}/[Ca^{2+}]}[E_0][S] \quad (2)$$

$$(k_{cat}/K_m)_{max} = (k/K_s)/[1 + (K_2'/H) + (H/K_1')] \quad (3)$$

$$(K_{Ca})_{app} = \frac{K_{Ca}[1 + (H/K_1) + (K_2/H)]}{1 + (K_2'/H) + (H/K_1')} \quad (4)$$

Because calcium binding is not sensitive to the ionization of the group of $pK_a \simeq 9.5$ (i.e., $K_2 \simeq K_2'$) and since $K_2 \ll K_1$ and $K_2 \ll K_1'$, eq 4 is simplified to eq 5.

$$(K_{Ca})_{app} = \frac{K_{Ca}[1 + (H/K_1)]}{1 + (H/K_1')} \quad (5)$$

Table II. Dissociation Constants Calculated from the pH Dependence[a] of $(K_{Ca})_{app}$ (A) and $(k_{cat}/K_m)_{max}$ (B)

dissoc const	A	B
pK_1	7.30 ± 0.12	
pK_1'	5.89 ± 0.21	5.87 ± 0.05
pK_2'		9.38 ± 0.03
K_{Ca}, mM	0.89 ± 0.12	
K_{Ca}', mM	22.90 ± 7.10	

[a] 37 °C, 20 mM 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane hydrochloride buffer, pH 6.3–9.3, 1.6% (v/v) CH_3CN; ionic strength 0.15 M, $[Ca^{2+}]$ = 0.05–20 mM, [1] = 5.0 × 10^{-5} M, E_0 = 5.0 × 10^{-6} M.

Table III. Second-Order Rate Constants[a] of 3-(Acyloxy)-4-nitrobenzoic Acids[b]

acyl gp	10^5S_0, M	$10^{-3}(k_{cat}/K_m)$, M^{-1} s^{-1}
butyryl	10.0	0.01
hexanoyl	10.0	0.05
heptanoyl	9.0	0.19
octanoyl	5.0	2.64 ± 0.04
nonanoyl	3.8	6.53 ± 0.08
decanoyl	3.0	5.60 ± 0.09
undecanoyl	2.5	10.13 ± 0.38
dodecanoyl	2.2	91.12 ± 3.19

[a] 37 °C, 10 mM Tris-HCl buffer, pH 8.0, 0.1 M NaCl, 3.2% (v/v) CH_3CN; $[Ca^{2+}]$ = 10 mM, E_0 = (0.5–8.4) × 10^{-6} M. [b] Solubility limit for several compounds in the buffer solution used: hexanoyl, 4.0 mM; octanoyl, 2.2 mM; nonanoyl, 0.85 mM; decanoyl, 9.2 × 10^{-5} M; dodecanoyl, 3.0 × 10^{-5} M.

Equations 3 and 5 were used to analyze the experimental data with the help of a nonlinear least-squares fit program. Figures 3 and 4 show that the experimental data are indeed consistent with the theoretical curves calculated from eq 3 and 5. The values of the microscopic equilibrium constants determined from these data are summarized in Table II. The pH dependence of the specificity constant (eq 3) and the pH dependence of the dissociation constant (eq 5) yield the same value for K_1', thereby confirming the consistency of the data with the proposed reaction scheme. The value of pK_1 is higher than that of pK_1', indicating that the presence of calcium ion facilitates the deprotonation of the group of pK_a = 7.30. This acidic residue is most probably His-48 whose pK_a in several PLA_2 has been estimated to be in the range 5.5–7.0.[1] If indeed the pH dependence reflects the ionization of His-48, then the decreased calcium binding at low pH can be accounted for by unfavorable electrostatic interaction between Ca^{2+} and the protonated form of His-48, which is indeed located[11] within range for an appreciable electrostatic interaction[12] with Ca^{2+}. Assuming that the dielectric constant in the open active-site cavity is 80, one calculates that introduction of a divalent cation should lower the pK_a of a cationic acid from 7.3 to 5.9 when the distance between the ion and the acid is 3.5 Å. These results are similar to those observed for pancreatic PLA_2 where the binding of Ca^{2+} lowered the pK_a of His-48 from 7.0 to 5.7.[13] The identity of the residue of pK_a = 9.38 is uncertain at present. Its pK_a is higher than that of the structurally important α-ammonium group, which is estimated to be in the range 8.4–8.9 for PLA_2 from several species.[1] The high-pK_a group could perhaps be one of two conserved tyrosyl residues (Tyr-52, Tyr-73) proposed[11,14] to form a hydrogen-bond network in the active site.

Variation of the Acyl Chain Length. Because of the hydrophobic nature of the active-site cativty of PLA_2, the interaction between the active site and the alkyl chain of the substrate should play an important role in maintaining the proper geometry of the enzyme-Ca^{2+}-substrate complex (ECaS). In fact, monomeric dihexanoyllecithin reacts with PLA_2 ca. 8 times faster than monomeric dibutyryllecithin does.[15] Unfortunately, the high tendency of phospholipids with medium-length acyl groups to form micelles prevents one from exploring the feature of this interaction. With our substrates, we were able to evaluate the role of hydrophobic interactions for acyl chains containing as many as 12 carbon atoms. The second-order rate constants for these substrates are summarized in Table III. They show that an increase in chain length enhances monotonously the rate of the enzymatic reaction. The value of k_{cat}/K_m measures an enzyme's overall preference for a particular substrate because this quantity includes both the binding energy term and the activation energy term.[16] At a constant $[Ca^{2+}]$, $k_{cat}/K_m = k/K_s = A \exp[-(\Delta G_b^\circ + \Delta G^\ddagger)]$, where ΔG_b° is the free energy of binding of substrate to the enzyme–Ca^{2+} complex and $\Delta G^\ddagger$ is the free energy of activation. Thus, one can use eq 6 to estimate the incremental interaction energy contributed by an additional methylene unit in the acyl chain of the substrate.

$$\Delta\Delta G_{exp} = \Delta\Delta G_b^\circ + \Delta\Delta G^\ddagger = -RT \ln \frac{(k_{cat}/K_m)_{(S\text{-}CH_2\text{-}H)}}{(k_{cat}/K_m)_{(S\text{-}H)}} \quad (6)$$

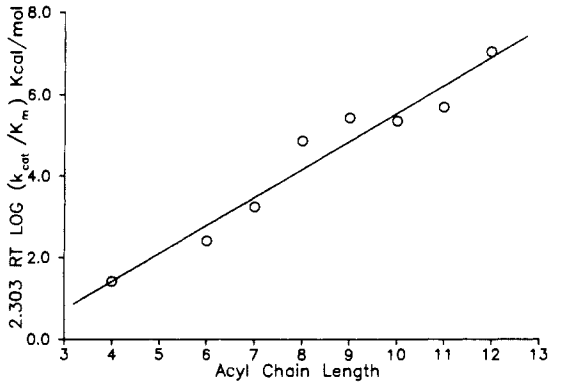


Figure 5. Dependence of log (k_{cat}/K_m) on the acyl chain length of 3-(acyloxy)-4-nitrobenzoic acids at pH 8.0, 37 °C, $[Ca^{2+}]$ = 10 mM, E_0 = (0.5–8.4) × 10^{-6} M, S_0 = (2.2–10.0) × 10^{-5} M. Each point is the mean of three determinations. The straight line represents the best fit of the data in Table III by the linear regression.

Table IV. Kinetic Parameters of the PLA_2-Catalyzed Hydrolysis[a] of **1**, **2**,[b] and **3**[b]

compd	10^3K_{Ca}, M	$10^{-3}(k_{cat}/K_m)_{max}$, M^{-1} s^{-1}
1	1.11 ± 0.05	3.23 ± 0.05
2	1.09 ± 0.06	21.00 ± 0.38
3	1.10 ± 0.10	8.50 ± 0.50

[a] 37 °C, 10 mM Tris-HCl, pH 8.0, 1.6% (v/v) CH_3CN; ionic strength 0.15 M, $[Ca^{2+}]$ = 0.05–20 mM, E_0 = 4.0 × 10^{-6} M, S_0 = 5.0 × 10^{-5} M. [b] Monitored at 410 nm.

Figure 5 shows the linearity of log (k_{cat}/K_m) as a function of the acyl chain length. The slope of the line, $\Delta\Delta G_{exp}$, is 0.69 ± 0.07 kcal/mol. It is noteworthy that the increment of free energy of transferring one methylene group from water to 1-octanol is 0.68 kcal/mol.[17] This suggests that the improved interaction for a longer chain substrate is mostly due to favorable hydrophobic interaction in the (ECaS) complex.

Structural Requirement for the Substrate. Although our substrates were designed to resemble a phospholipid molecule, they are still deficient in important structural features of the natural substrate. They do not contain a second acyl chain, and thus their binding mode in the active site of the enzyme should not be as productive as that of a phospholipid. In fact, k_{cat}/K_m for 3-(butyryloxy)-4-nitrobenzoic acid (see Table III) is 10 times smaller than that observed for the hydrolysis of monomeric dibutyryllecithin,[18] suggesting that the chemical activation of the reactive ester group is strongly counteracted by the loss of specificity. We

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Table V. Dissociation Constants (K_d) of PLA_2–M^{2+} Complex and Maximal Specificity Constants Determined from the PLA_2-Catalyzed Hydrolysis[a] of **1** in the Presence of Several Divalent Metal Ions

metal	10^3K_d, M	$10^{-3}(k_{cat}/K_m)_{max}$, M^{-1} s^{-1}
Ca[b]	1.11 ± 0.05	3.23 ± 0.05
Sr[b]	1.15 ± 0.05	4.69 ± 0.05
Ba[b]	1.02 ± 0.06	0.86 ± 0.01
Mg[c]		<0.05
Zn[d]		<0.05

[a] 37 °C, 10 mM Tris-HCl buffer, pH 8.0, 1.6% (v/v) CH_3CN; ionic strength maintained by addition of the appropriate amount of NaCl, [**1**] = 5.0×10^{-5} M, $E_0 = (4.0\text{–}7.0) \times 10^{-6}$ M. [b] $[M^{2+}]$ = 0.05–20 mM. [c] $[Mg^{2+}]$ = 20 mM. [d] $[Zn^{2+}]$ = 1 mM.

also found that **4** was hydrolyzed about 10 times faster than **1**, but TLC of the reaction product showed that the group hydrolyzed was exclusively the octanoyl ester. These results imply that the location of the ester function with respect to the carboxylate anion of **1** is not optimal for enzymatic catalysis. To estimate the optimal distance between the carboxylate anion and the reactive ester, structural isomers of 4-nitro-3-(octanoyloxy)benzoic acid were synthesized, and the kinetics of their enzymatic hydrolysis was determined. These results are summarized in Table IV. The values of K_{Ca} are identical for all three compounds, and **2** is the preferred substrate for the enzyme. The constancy of K_{Ca} implies that the order of addition of Ca^{2+} is the same for all three substrates. The fact that **2** is the best substrate suggests that the distance between the carboxylate anion and the carbonyl carbon of the reactive ester in this compound (ca. 4.5 Å) is the closest approximation of the actual distance between the phosphate anion and the reactive ester function in the enzyme–Ca^{2+}–phospholipid complex. This also eliminates the possibility that Ca^{2+} would be involved in the stabilization of the transition state through coordination to the negatively charged oxygen atom of the tetrahedral intermediate[4] because the rigidity of the benzene ring and unfavorable structural geometry of **2** do not allow Ca^{2+} to coordinate to the oxyanion of the tetrahedral intermediate of **2**. Finally, we observed that Ca^{2+} up to 4.0×10^{-2} M did not have any influence on the rate of nonenzymatic hydrolysis of these substrates, thereby showing that Ca^{2+} by itself does not catalyze at all the hydrolysis of these substrates.

Action of Divalent Ions Other than Ca^{2+}. No cation can substitute for Ca^{2+} in the PLA_2-catalyzed hydrolysis of lecithins although some cations such as Sr^{2+} and Ba^{2+} bind to the enzyme as effectively as Ca^{2+} does.[1] In contrast to lecithins, our substrates are hydrolyzed by the enzyme in the presence of the divalent ions other than Ca^{2+}. The kinetic parameters of these reactions for **1** are summarized in Table V. Sr^{2+} and Ba^{2+} show the same affinity toward the enzyme as Ca^{2+} does. It appears that Sr^{2+} (ionic radius 1.12 Å) is more conducive to catalysis than Ca^{2+} (ionic radius 0.99 Å) by a factor of 1.5, whereas Ba^{2+} (ionic radius 1.34 Å) is less efficient, although still catalytically competent. That the catalysis is still dependent on the exact chemical nature of the metal ion is shown by the fact that neither Mg^{2+} nor Zn^{2+} induces the hydrolysis. Therefore, the catalytic action of Sr^{2+} and Ba^{2+} must be due to the fact that they bind the substrate in such an orientation as to allow interaction of the catalytic groups with the reactive ester function.

Hydrolysis of 3-(Decanoyloxy)-4-nitrobenzoic Acid by Phospholipase A_2 from Various Sources. Table VI summarizes the kinetic parameters calculated from the hydrolysis of 3-(decanoyloxy)-4-nitrobenzoic acid by the enzyme from a variety of sources. *C. atrox* enzyme and porcine pancreatic enzyme represent two distinct families of PLA_2.[19] The calcium binding constants determined with 3-(decanoyloxy)-4-nitrobenzoic acid show a reasonable consistency with those reported for specific substrates,[1] and the specificity constant reflects the intrinsic activity of the different enzymes toward phospholipids. Thus, our synthetic substrates are recognized by all known classes of PLA_2.

Table VI. Kinetic Parameters of the Hydrolysis[a] of 3-(Decanoyloxy)-4-nitrobenzoic Acid by PLA_2 from a Variety of Sources

source	10^3K_{Ca}, M	$10^{-3}(k_{cat}/K_m)_{max}$, M^{-1} s^{-1}
A. piscivorus piscivorus venom	1.32 ± 0.17	7.55 ± 0.26
C. atrox venom	1.23 ± 0.17	17.94 ± 0.76
porcine pancreas	1.93 ± 0.15	1.53 ± 0.18

[a] 37 °C, 10 mM Tris-HCl buffer, pH 8.0, 0.1 M NaCl, 1.6% (v/v) CH_3CN; $[Ca^{2+}]$ = 0.05–20 mM, $S_0 = 2.5 \times 10^{-5}$ M, $E_0 = (1.0\text{–}3.0) \times 10^{-6}$ M.

Discussion

The preceding data establish that carboxylate-based compounds are true substrates for PLA_2. The synthetic substrates used in this study were hydrolyzed by a catalytic amount of enzyme at rates comparable to those for the hydrolysis of phospholipids. Enzymatic hydrolysis of these compounds showed all the kinetic characteristics of PLA_2-catalyzed hydrolysis of lecithins including Ca^{2+} dependence, pH dependence, order of addition of Ca^{2+} and substrate, acyl chain length dependence, and the suppression of the reaction by methylation of His-48. We showed that the reactive species of the substrate is the monomeric anionic form. It has been shown[20] that phospholipid analogue compounds where a sulfate anion replaces the phosphate are good substrates for PLA_2. Here, we show that the carboxylate anion is sufficient for recognition by PLA_2 and that the glycerol moiety is not an absolute requirement. It is understood that with such "nonspecific substrates", some flexibility in the molecule is essential for catalysis to occur since it is the flexibility that prevents the substrate from being locked into a nonproductive binding mode. We feel that with our substrates this flexibility is provided by the absence of the second acyl group, which in lecithins imposes a single well-defined location on the acyl group to be hydrolyzed. In other words, the single acyl chain of our substrates has several closely related binding modes, resulting in a diffuse location of the substrate bound in the active site. Loss of the catalytic efficiency is inevitable due to the fact that only a few of these binding modes are conducive to catalysis, but the mobility of the enzyme–substrate complex should then allow the substrate with suboptimal structure to react with enzyme. This point is illustrated by the fact that all three isomeric octanoyl ester are substrates and by the fact that with our substrates Sr^{2+} and Ba^{2+} are sufficient for the catalysis. Thus, we conclude that the minimum requirement for a PLA_2 substrate is a fatty acid ester located within 3–6 Å of an anionic group.

We were unable to observe pure Michaelis–Menten-type kinetics for the hydrolysis of our substrates because at high substrate concentration the enzyme was irreversibly acylated by the substrate, although only in the presence of Ca^{2+}. This modification produced an enzyme with enhanced reactivity toward all substrates. Detailed studies on this acylation are in progress.

Finally, our substrates are eminently suitable for a fast, spectrophotometric rate assay of PLA_2 in homogeneous solution for enzyme concentrations in the range 10^{-8}–10^{-7} M. Besides its experimental simplicity, this assay has the advantage over existing methods in rate constants can be readily obtained by analysis of the whole time course of the reaction.

In conclusion, we feel that our substrates will greatly facilitate the study of the specificity and mechanism of PLA_2. These compounds are much easier to synthesize and modify than phospholipids and they react with the enzyme through the same mechanism as lecithins do. Thus, it should be possible to synthesize a large number of substrates for which differences in structure can be correlated with enzymatic activity.

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