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Defining the Dimensions of the Catalytic Site of Phospholipase A_2 Using Amide Substrate Analogues

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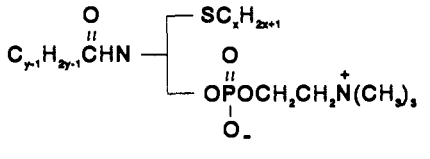
Abstract: Two series of phospholipid analogues, each containing a thioether function at the *sn*-1 position and an amide function at the *sn*-2 position, have been synthesized and evaluated as phospholipase A_2 inhibitors. The first series of analogues contained a hexyl group (C-6) at the *sn*-1 position and various acyl groups at the *sn*-2 position, ranging from formyl to dodecanoyl (1–12 carbons). The second series contained an *sn*-1 hexadecyl group (C-16) and various *sn*-2 acyl groups from formyl to eicosanoyl (1–20 carbons). Hydrophobic interactions of the enzyme with the amide analogues were studied using several different substrate forms including monomers, micelles, and mixed micelles with Triton X-100. The C-6 amide analogues were used for the monomeric study, while the C-16 analogues were used in the micellar studies. The inhibition studies with the monomeric amide analogues demonstrate that the *sn*-2 acyl chain is absolutely required for the binding of the analogue to the enzyme and that the catalytic site interacts with about the first 10 carbons of the *sn*-2 acyl chain. In addition, each methylene group of the *sn*-2 acyl chain from C5 to C10 provides about 665 cal/mol of binding energy. In contrast, the inhibition potency of the amide analogues in micellar states followed a quite different, more complex chain length dependency. The chain length of the *sn*-2 acyl group is much less important in the micellar systems than in the monomeric system, since the hydrophobic interactions between the *sn*-2 acyl chain and the enzyme are balanced by its interactions with the hydrophobic core of the micelle. The importance of double bonds in the *sn*-2 chain was also studied, but no correlation between the degree of unsaturation and the degree of inhibition was observed. These studies help delineate the mode of the interactions between enzyme and substrate.

The thioether amide phospholipid analogues **1** are potent, reversible inhibitors of phospholipase A_2 (PLA_2).[1,2] They offer



1

x = 6, y = 1 - 12
x = 16, y = 1 - 20

advantages over covalent inhibitors such as manoalogue[3] and SIBLINKS[4,5] for the study of enzyme/substrate interactions. X-ray crystallographic studies of porcine pancreatic PLA_2 complexed with a different alkylamide analogue have provided structural details about the enzyme/inhibitor interactions.[6] The amide analogue forms two hydrogen bonds with the enzyme: one from the amide hydrogen of the analogue to the Nδ1 atom of His 48, and the other from one of the nonbridging phosphate oxygens to the hydroxyl group of Tyr 69. The active-site calcium interacts electrostatically with the other nonbridging phosphate oxygen and with the *sn*-2 carbonyl oxygen of the analogue. In addition, the

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(1) Yu, L.; Deems, R. A.; Hajdu, J.; Dennis, E. A. *J. Biol. Chem.* **1990**, *265*, 2657–2664.
(2) Davidson, F. F.; Hajdu, J.; Dennis, E. A. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 587–592.
(3) Reynolds, L. J.; Morgan, B. P.; Hite, G. A.; Mihelich, E. D.; Dennis, E. A. *J. Am. Chem. Soc.* **1988**, *110*, 5172–5177.
(4) Washburn, W. N.; Dennis, E. A. *J. Am. Chem. Soc.* **1990**, *112*, 2040–2041.
(5) Washburn, W. N.; Dennis, E. A. *J. Am. Chem. Soc.* **1990**, *112*, 2042–2043.
(6) Thunnissen, M. M. G. M.; Ab, E.; Kalk, K. H.; Drenth, J.; Dijkstra, B. W.; Kuipers, O. P.; Dijkman, R.; de Haas, G. H.; Verheij, H. M. *Nature* **1990**, *347*, 689–691.

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fatty acyl chains make extensive contact with the hydrophobic wall of the catalytic site, which is formed by the side chains of highly conserved residues Leu 2, Phe 5, Ile 9, Phe 22, and Tyr 52 and the disulfide bridge between Cys 29 and Cys 45. Thus, the binding energy for the strong interaction between the enzyme and the amide analogue is derived from multiple weak noncovalent forces such as electrostatic attraction, hydrogen bonding, and hydrophobic interactions. Quantitative information about the energetics of the individual interactions between the enzyme and its substrate is very important for understanding the action of the enzyme as well as for designing potent inhibitors.

Although the interaction of PLA_2 with substrate and substrate analogues has been extensively studied by site-directed mutagenesis,[7,8] X-ray crystallography,[6] and substrate modification,[1,9,10] little has been done to quantitate the contributions of various individual interactions within the enzyme/ligand complex to the overall binding energy. Our recent studies of the pH dependence of cobra venom PLA_2 inhibition by the thioether amide analogue 1 suggest that the hydrogen bond formed between the amide hydrogen of the analogue and the Nδ1 atom of His 48 provides about 1.5 kcal/mol of binding energy.[11] In an attempt to quantitate the hydrophobic interactions within the enzyme/ligand complex and to define the effective size of the catalytic site of the enzyme, we have synthesized two series of structurally related amide substrate analogues which contain a thioether with a fixed alkyl chain of either 6 or 16 carbons at the *sn*-1 position and amides of varing chain lengths at the *sn*-2 position. The effect of the *sn*-2 chain length on the binding affinity of these amide analogues in both monomeric and micellar states is reported.

Experimental Procedures

Materials. Cobra venom PLA_2 (*Naja naja naja*) was purified as described previously.[12] 2-Bromoethyl phosphorodichloridate was prepared by the method of Hansen et al.[13] 1,2-Bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (DDPC) was synthesized using the method described by Yu et al.,[1] which is a modification of the method developed by Hendrickson and Dennis[14] and Hendrickson et al.[15] 1,2-Bis(hexanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (DHPC) was prepared as previously described.[16] Triton X-100 was obtained from Sigma. 4,4′-Dithiobipyridine (DTP), 6-(4-toluidino)-2-naphthalenesulfonic acid (TNS), D-cystine, iodohexane, and iodohexadecane were purchased from Aldrich, as were all acyl chlorides and carboxylic acids. Chloroform was distilled from phosphorus pentoxide prior to use.

General Methods. For the purification of crude reaction mixtures, flash chromatography was applied in almost all cases. Merck silica gel 60 (230–400 mesh) was used as a stationary phase. Thin-layer chromatography (TLC) was performed on Analtech silica gel G-250 glass plates. TLC plates were generally visualized by spraying with 2 M sulfuric acid, followed by charring on a hot plate. Trityl compounds were visualized by their bright yellow color after the sprayed plate was gently warmed. UV active compounds were viewed under ultraviolet light. Phospholipids were detected with a molybdate spray.[17] ^1H NMR spectra were obtained on an extensively modified Varian 360-MHz NMR spectrometer. Chemical shifts are recorded in parts per million (ppm) from tetramethylsilane (TMS). FAB mass spectra were obtained at the University of California Riverside mass spectrometry facility.

Phospholipase A_2 Assay. PLA_2 activity was determined by a thio assay according to the method reported previously.[1,16] The assay was carried out at 30 °C in a "standard Tris buffer" containing 100 mM KCl, 10 mM Ca^{2+}, and 25 mM Tris-HCl (pH 8.5). The enzyme activity was followed spectrophotometrically at 324 nm by monitoring the absorption change due to the formation of the chromophore 4-thiopyridine formed in the reaction of 4,4′-dithiobipyridine with the free thiol liberated upon substrate hydrolysis. An extinction coefficient of 13 400 M^{-1} cm^{-1} for 4-thiopyridine at pH 8.5 was used.[16] The activity of PLA_2 was measured using three different physical forms of substrate: monomeric, pure micellar, and Triton X-100 mixed micellar. DHPC, 0.5 mM and 4.0 mM, was used as a substrate in the monomeric and pure PC micellar systems, respectively. DDPC was employed in the Triton X-100 mixed micellar system, with 4.25 mM Triton X-100 and 0.5 mM DDPC. All substrate solutions were prepared fresh daily. The reaction was initiated by the addition of the enzyme to the assay buffer (50 ng for the micellar substrates and 500 ng for the monomeric substrate).

Inhibition Studies. The inhibition of PLA_2 by the amide phospholipid analogues was evaluated by measuring the concentration required for half maximum inhibition (IC_{50}). The inhibitor solutions were prepared by dissolving amide analogues in the standard Tris buffer for the monomeric substrate studies, in 4.0 mM DHPC solution for the micellar substrate studies, or in 4.25 mM Triton X-100 for the mixed micellar substrate studies. For each IC_{50} measurement, at least five inhibitor concentrations were employed and each point was determined in duplicate. IC_{50}'s were calculated by fitting the inhibition data to eq 1,[11,18] where v is the velocity

$$v = C/(IC_{50} + I) \quad (1)$$

at inhibitor concentration I. C is a function of the substrate concentration, the dissociation constant of the inhibitor, and the kinetic constants of the enzymatic reaction. At constant substrate concentrations, C is a constant. For monomeric inhibitors, IC_{50}'s are given in bulk concentration units (μM). For micellar inhibitors, IC_{50}'s are expressed in surface concentration units (mol fraction), assuming that all of the inhibitor is in the micelle.[11]

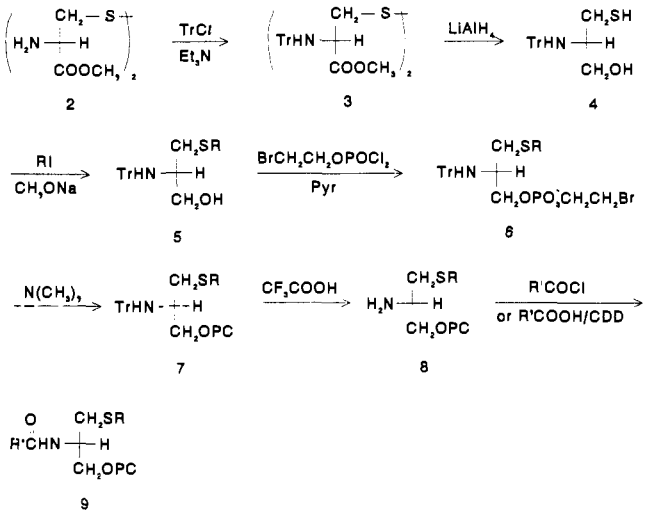
Cmc Determination. The critical micellar concentrations (cmc's) of the *sn*-1 hexylthio amide analogues were determined by fluorometric titration[19] at 30 °C in the standard Tris buffer. 6-(4-Toluidino)-2-naphthalenesulfonic acid (TNS) was used as a fluorescent probe. The quantum yield of TNS fluorescence increases when it is incorporated into the interior of a micelle. Due to the wide range of cmc's determined, three different concentrations of TNS, 10, 5, and 0.5 μM, were employed for the amide analogues with *sn*-2 acyl chains of 2–4, 4–9, and 10–12 carbons, respectively. Except for the acetyl- and propionylamide derivatives, the titrations were performed by stepwise additions of a concentrated solution of the amide analogue to the fluorescent probe solution (1.5 mL). To avoid dilution of the probe, the stock solutions of these amide analogues were prepared in the standard Tris buffer containing the corresponding concentration of TNS. For the acetyl and propionyl analogues, the starting solution contained 100 mM of the amide analogue and 10 μM TNS in 1.5 mL of standard Tris buffer. The cmc's were determined by stepwise dilution of the amide solution with 10 μM TNS in the standard Tris buffer. The cmc of DHPC was also determined using 5 μM TNS. Fifteen to twenty points were taken for each cmc determination.

Phosphate Assay. Phospholipid concentrations were determined by a modified ammonium molybdate phosphate assay.[20] To each dried sample was added 0.2 mL of concentrated sulfuric acid. The samples were digested at 200 °C for 2 h, by which time the solution became yellow to dark brown. After the samples had cooled to room temperature, 1 drop of 30% hydrogen peroxide was added. The samples were heated for another 30 min at 200 °C. In some cases, this step was repeated until the solutions cleared. After the solutions cooled to room temperature, 0.1 mL of saturated sodium sulfide was added to destroy any residual hydrogen peroxide. Color development was the same as in the original method.

Preparation of Amide Phospholipid Analogues. The synthesis of the thioether amide analogues was first reported by Bhatia and Hajdu.[21] The thioether amide analogues used in the present studies, which contain either hexylthio or hexadecylthio at the *sn*-1 position and various acyl chains at the *sn*-2 position, were synthesized as shown in Scheme I. This represents an alternate approach for the synthesis of thioether amide analogues. The synthesis of 1-(hexylthio)-2-acylamido-1,2-dideoxy-*sn*-glycerol-3-phosphocholine is used to illustrate the basic synthetic procedures.

(7) Kuipers, O. P.; Dijkman, R.; Rals, C. E. G. M.; Verheij, H. M.; de Haas, G. H. *Protein Eng.* **1989**, *2*, 467–471.
(8) Kuipers, O. P.; Kerver, J.; van Meersbergen, J.; Vis, R.; Dijkamn, R.; Verheij, H. M.; de Haas, G. H. *Protein Eng.* **1990**, *3*, 599–603.
(9) Lewis, K. A.; Bian, J. R.; Sweeney, A.; Roberts, M. F. *Biochemistry* **1990**, *29*, 9962–9970.
(10) de Haas, G. H.; Dijkman, R.; Ransac, S.; Verger, R. *Biochim. Biophys. Acta* **1990**, *1046*, 249–257.
(11) Yu, L.; Dennis, E. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9325–9329.
(12) Hazlett, T. L.; Dennis, E. A. *Toxicon* **1985**, *23*, 457–466.
(13) Hansen, W. J.; Murari, R.; Wedmid, Y.; Baumann, W. J. *Lipids* **1982**, *17*, 453–459.
(14) Hendrickson, H. S.; Dennis, E. A. *J. Biol. Chem.* **1984**, *259*, 5734–5739.
(15) Hendrickson, H. S.; Hendrickson, E. K.; Dybvig, R. H. *J. Lipid Res.* **1983**, *24*, 1532.
(16) Yu, L.; Dennis, E. A. *Methods Enzymol.* **1991**, *197*, 65–75.
(17) Ryn, E. K.; BacCoss, M. *J. Lipid Res.* **1979**, *20*, 561–562.
(18) Yu, L. Ph.D. Thesis, University of California at San Diego, San Diego, CA, 1991.
(19) Nakagaki, M.; Komatsu, H.; Handa, T. *Chem. Pharm. Bull.* **1986**, *34*, 4479–4485.
(20) Eaton, B. R.; Dennis, E. A. *Arch. Biochem. Biophys.* **1976**, *176*, 604–609.
(21) Bhatia, S. K.; Hajdu, J. *Tetrahedron Lett.* **1988**, *29*, 31–34.

Scheme I. Synthesis of 1-(alkylthio)-2-(acylamino)-1,2-dideoxy-*sn*-glycero-3-phosphocholine



D-**Cystine Dimethyl Ether Dihydrochloride (2).** Thionyl chloride (3.8 mL, 6.2 g, 52 mmol) was added dropwise to 80 mL of methanol which was stirring in an ice bath. D-Cystine (5 g, 20.8 mmol) was then added, and the reaction mixture was refluxed overnight. After the volatile chemicals and solvent were removed from the reaction mixture in vacuo, a white solid was obtained. The resulting residue was kept over sodium hydroxide overnight to remove hydrogen chloride absorbed in the solid and was used directly in the next step without further purification.

***N,N'*-Ditritylcystine Dimethyl Ester (3).** To a suspension of D-cystine dimethyl ester dihydrochloride (**2**), from the previous step, in 100 mL of chloroform and 25 mL of triethylamine (179 mmol) was added trityl chloride (12.5 g, 44.8 mmol) with stirring at room temperature. The heterogeneous mixture was stirred at room temperature for 24 h, by which time all the solid had dissolved and the solution had become brown. The reaction mixture was washed twice with 0.5 N HCl, followed by washing with saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulfate, and then the solvent was removed in vacuo. The resulting residue was purified by crystallization in methanol with a trace amount of water to give tritylated cystine **3** (13.5 g, 86% from cystine **1**) as a yellowish solid. R_f 0.46 in hexanes/acetone (7:3); ^{1}H NMR ($CDCl_3$) δ 2.831 (q, 4 H), 3.023 (q, 2 H), 3.205 (s, 6 H), 3.625 (m, 2), 7.184–7.304 (m, 18 H), 7.420–7.496 (m, 12 H). HRMS (FAB, MH^+) Calcd for $C_{46}H_{45}H_2O_4S_2$: 753.2821. Found: 753.2805.

1-Thio-2-(tritylamino)-1,2-dideoxy-*sn*-glycerol (4). To a solution of compound **3** (9.5 g, 12.6 mmol) in 50 mL of anhydrous THF was added 30 mL of 1 M $LiAlH_4$ (30 mmol) in THF slowly during a period of 1 h. The solution was stirred under nitrogen at room temperature for another hour. The reaction was then cooled in an ice bath and quenched by addition of 1 N HCl. The mixture was extracted with methylene chloride twice. The combined organic phases were washed with 5% $NaHCO_3$ and with water. The organic layer was dried over sodium sulfate; then the solvent was removed in vacuo. A yellowish oil was obtained. This viscous liquid was used directly in the next step without further purification, and the product had an R_f of 0.44 in hexanes/acetone (7:3).

1-(Hexylthio)-2-(tritylamino)-1,2-dideoxy-*sn*-glycerol (5). A methanol solution (60 mL) of iodohexane (5.4 g, 25.5 mmol), sodium methoxide (1.5 g, 26.4 mmol), and compound **4** from the previous step was stirred at room temperature overnight. After the solvent was removed under reduced pressure, methylene chloride was added and the solution was filtered. The filtrate was concentrated, dissolved in hexanes/ethyl acetate (9:1), applied to a flash silica gel column, and eluted with hexanes/ethyl acetate (9:1) to give a colorless oil (8.5 g, 78% from **3**). R_f 0.56 in hexanes/acetone (7:3); ^{1}H NMR ($CDCl_3$) δ 0.884 (t, 3 H), 1.277 (m, 6 H), 1.399 (m, 2 H), 2.200 (m, 4 H), 2.342 (q, 1 H), 2.786 (m, 1 H), 3.191 (q, 1 H), 3.522 (q, 1 H), 7.189–7.323 (m, 9 H), 7.554 (d, 6 H).

1-(Hexylthio)-2-(tritylamino)-1,2-dideoxy-3-((2'-bromoethyl)-phospho)-*sn*-glycerol (6). To a solution of 2-bromoethyl phosphorodichloridate (3.0 g, 12.4 mmol) in 30 mL of dry chloroform solution was added compound **5** (3.0 g, 6.9 mmol) in 40 mL of dry chloroform and 3 mL of pyridine dropwise at 0 °C. After stirring at room temperature for 4 h, the reaction was quenched by the addition of saturated $NaHCO_3$ and stirred an additional 10 min. The aqueous layer was extracted with methylene chloride. The combined organic portions were dried over Na_2SO_4, and the solvents were removed under reduced pressure. The residual oil was flash chromatographed on silica gel and eluted with hexanes/acetone (10:1) to give a colorless oil (3.2 g, 75%). R_f 0.35 in hexanes/acetone (7:3); ^{1}H NMR ($CDCl_3$) δ 0.884 (t, 3 H), 1.278 (m, 6 H), 1.401 (m, 2 H), 2.057 (m, 2 H), 2.336 (q, 1 H), 2.728 (t, 1 H), 3.457 (t, 2 H), 3.685 (m, 1 H), 3.890 (m, 1 H), 3.945–4.197 (m, 3 H), 7.225–7.398 (m, 9 H), 7.583 (d, 6 H).

1-(Hexylthio)-2-(tritylamino)-1,2-dideoxy-*sn*-glycerol-3-phosphocholine (7). A solution of compound **6** (3.2 g, 5.2 mmol) and 40% trimethylamine (40 mL) in 80 mL of chloroform/2-propanol/dimethylformamide (3:5:5) was incubated at 50 °C for 6 h. After the reaction was allowed to cool to room temperature, the volatiles were removed in vacuo and the remaining solvents were removed by lyophilization. The residue was taken up in chloroform and flash chromatographed on silica gel, eluting with chloroform/methanol (8:2) to give a white solid (2.2 g, 71%). ^{1}H NMR ($CDCl_3$) δ 0.875 (t, 3 H), 1.257 (m, 6 H), 1.392 (m, 2 H), 1.901 (m, 1 H), 1.998 (m, 1 H), 2.151 (d, 2 H), 2.497 (t, 1 H), 3.427 (s, 9 H), 3.896 (m, 3 H), 4.039 (m, 2 H), 4.170 (m, 1 H), 4.225 (m, 1 H), 7.251–7.413 (m, 9 H), 7.480 (d, 6 H).

1-(Hexylthio)-2-amino-1,2-dideoxy-*sn*-glycerol-3-phosphocholine (8). Compound **7** (2.1 g, 3.4 mmol) was dissolved in 40 mL of dry trifluoroacetic acid and allowed to stand at room temperature for 0.5 h. The volatile trifluoroacetic acid was removed under reduced pressure. Toluene was added to assist the removal of the remaining trace trifluoroacetic acid. The residue was dissolved in chloroform/methanol (7:3) and applied to a flash silica gel column. The column was first eluted with chloroform/methanol (7:3) to remove trityl compounds, followed by chloroform/methanol water (65:25:5) to give a white solid (1.24 g, 99%). ^{1}H NMR ($CDCl_3/CD_3OD$ 7:3) δ 0.905 (t, 3 H), 1.322 (m, 4 H), 1.410 (m, 2 H), 1.610 (m, 2 H), 2.590 (t, 2 H), 2.816 (m, 2 H), 3.232 (s, 9 H), 3.487 (m, 1 H), 3.664 (m, 2 H), 4.069 (m, 1 H), 4.732 (m, 1 H), 4.299 (m, 2 H). HRMS (FAB, MH^+) Calcd for $C_{14}H_{34}N_2O_4PS$: 357.1977. Found: 357.1963.

1-(Hexylthio)-2-(acylamino)-1,2-dideoxy-*sn*-glycerol-3-phosphocholine (9). Fatty acids of different chain length can be incorporated into the amide analogue at this stage. Acylation was accomplished by either one of the following methods based on the availability of acylation reagents: *Method A.* A solution of lysophospholipid amine analogue **8** (80 mg, 0.22 mmol), the appropriate fatty acyl chloride (0.34 mmol), and triethylamine (34.4 mg, 0.34 mmol) in 2 mL of anhydrous chloroform was stirred at room temperature until TLC indicated completion of the reaction (about 24 h). *Method B.* A solution of a fatty acid (0.44 mmol) and 1,1'-carbonyldiimidazole (71.35 mg, 0.44 mmol) was stirred at room temperature in a sealed vial. Since this reaction generates carbon dioxide, pressure builds up inside of the vial. The pressure was released three to four times, and the reaction was allowed to continue until no further pressure build up was observed (about 1 h). This mixture was then added to the mixture of compound **8** and triethylamine (44.5 mg, 0.44 mmol) in 1.5 mL of dry chloroform, and the resulting mixture was stirred at room temperature for 24 h. Without further treatment, the reaction mixture from either *method A* or *method B* was applied directly to a flash silica gel column and eluted first with chloroform/methanol (65:25) to remove the impurities, then with chloroform/methanol/water (65:25:4) to give the desired chain length amide analogue. All of the NMR and mass spectra data for the amide analogues are listed in the supplementary material.

Results

Dependence of the Cmc on the *sn*-2 Acyl Chain Length. The critical micellar concentrations of the amide analogues containing a hexylthio group (C-6) at the *sn*-1 position and various acyl chains (y = 2–12) at the *sn*-2 position were determined by the fluorescence method using TNS as a fluorescent probe.[19] The dependence of the cmc's of these amide analogues on the *sn*-2 acyl chain length, y, is shown in Figure 1. The cmc values varied from 14 μM for the dodecanoyl analogue to 60 mM for the acetyl analogue. The cmc increases as the *sn*-2 acyl chains decrease in length and plateaus at chain lengths less than 4 carbons. For chain lengths between 5 and 12 carbons, the log of the cmc decreases linearly with the number of methylene groups in the *sn*-2 acyl chain. Approximately a 10-fold decrease in cmc is observed for every 2 methylene groups added to the *sn*-2 acyl chain.

Dependence of Inhibition Potency of Monomeric Amide Analogues on the *sn*-2 Chain Length. The interaction of PLA_2 with monomeric substrate analogues was studied using the short-chain alkylthio amide analogues (x = 6). 1-(Hexylthio)-2-amino-1,2-dideoxy-*sn*-glycero-3-phosphocholine, a lysophospholipid analogue,

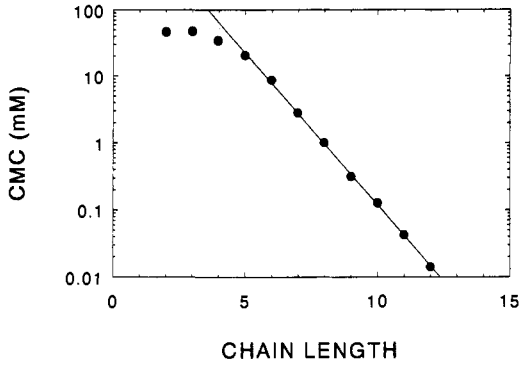


Figure 1. Dependence of the cmc on the *sn*-2 acyl chain length. The cmc's of the 1-(hexylthio)-2-(acylamino) analogues were determined by fluorometric titration at 30 °C in the standard Tris buffer.

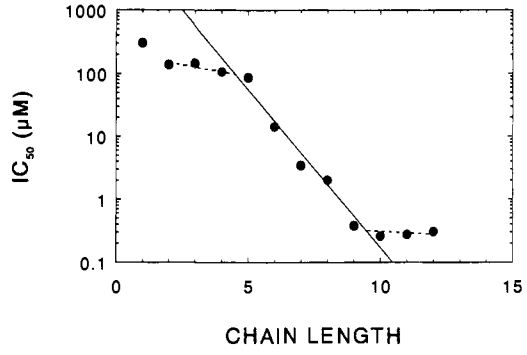


Figure 2. Inhibitory potencies of the short-chain amide analogues as a function of number of carbons in the *sn*-2 chain. The amide analogues contained a hexylthio at the *sn*-1 position and a variable *sn*-2 acyl chain. The inhibition potency was evaluated by its IC_{50}, which was determined at pH 8.5 with 0.5 mM DHPC as substrate. The *solid line* is a least squares fit to the points between 4 and 10; the *dashed lines* are least squares fits to the remaining points (2 to 4 and 10 to 12), which do not fall on the *solid line*. The error bars are smaller than the size of the symbols.

was also included in this study to determine the effect of the *sn*-2 carbonyl group. The effect of chain length on the enzyme/inhibitor interaction was investigated by comparing the inhibition potencies of these amide analogues on the hydrolysis of monomeric substrate DHPC. The inhibition potencies of the amide analogues were evaluated by measuring their IC_{50}'s.

The concentration of DHPC used in the IC_{50} determination was 0.5 mM, which is about 3 times lower than its cmc (1.3 mM). Even though the maximum concentration of the amide analogues employed was 15–1200 times lower than their cmc's, it is still possible that the amide analogue aggregates in the presence of the monomeric substrate to form a mixed micelle. To ascertain if this was occurring, the cmc of the most hydrophobic amide analogue, 1-(hexylthio)-2-(dodecanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphocholine, was determined in the standard Tris buffer solution containing 0.5 mM DHPC. The cmc of this DHPC/dodecanoyl analogue mixed micelle is 7.0 μM, which is half of that observed with the amide analogue alone but is still 7 times higher than the maximum analogue concentration used in the inhibition studies. If the enzyme itself does not dramatically influence the cmc's of both substrate and amide analogues, then all inhibitors and the substrate in the assay should exist in the monomeric state.

The inhibitory potencies of the monomeric amide analogues are strikingly dependent on the chain length of the *sn*-2 acyl group. A plot of the IC_{50}'s of the amide analogues versus the number of carbons in the *sn*-2 acyl chain (y) illustrates the relationship between the *sn*-2 acyl chain length and the affinity of the amide for the enzyme. Due to the wide range of IC_{50}'s displayed by the monomeric amide analogues, a semilog plot was employed. As shown in Figure 2, the binding affinity of the amide analogues increases as the *sn*-2 acyl chain length increases. Two plateaus

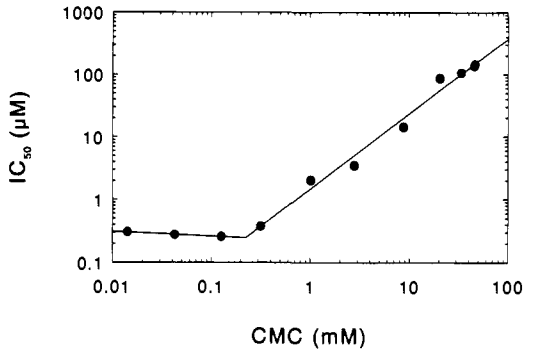


Figure 3. Inhibitory potencies of the short-chain amide analogues as a function of their cmc's. The amide analogues contained a hexylthio at the *sn*-1 position and a variable *sn*-2 acyl chain. The inhibition potency was evaluated by its IC_{50}, which was determined as in Figure 2. The cmc's of the amide analogues, a measure of their hydrophobicities, were determined as in Figure 1.

were observed: one at short chain lengths ($y < 5$), and the other at long chain lengths ($y > 9$). The formyl analogue ($y = 1$) had an IC_{50} of 0.3 mM. The lyso analogue produced only a 20% inhibition at 1 mM. This is consistent with the previous findings that lysophospholipids do not bind to the enzyme.[22] This is probably due to the fact that the carbonyl oxygen of the *sn*-2 acyl group binds to the calcium ion at the catalytic site of the enzyme.[6]

To quantitatively study the relationship between the binding affinity of an inhibitor and its hydrophobicity, the IC_{50} values of the amide substrate analogues were also plotted as a function of their cmc's, since the cmc is characteristic of the hydrophobicity of a compound. As shown in Figure 3, the binding affinities of these analogues correlate strongly with their hydrophobicities. The more hydrophobic the amide analogue, the tighter it binds to the enzyme. Below a cmc of 0.1 mM, the affinities leveled off.

Dependence of Inhibition Potency of Micellar Amide Analogues on the *sn*-2 Chain Length. The effect of the *sn*-2 acyl chain length on the interaction of PLA_2 with micellar inhibitors was studied by measuring the inhibition of the enzyme by amide analogues containing a hexadecylthio moiety ($x = 16$) at the *sn*-1 position and various acyl groups ($y = 2$–20) at the *sn*-2 position. Two different micellar systems were studied: phosphatidylcholine micelles (PC micelles) and Triton X-100/phosphatidylcholine mixed micelles (Triton X-100 mixed micelles). DHPC, at a concentration (4.0 mM) above its cmc, was used as a substrate for the PC micellar studies, whereas DDPC (0.5 mM) was used in Triton X-100 mixed micelles containing 4.0 mM Triton X-100. Since these micelles are composed predominantly of the substrate or the nonionic detergent, any effect of an inhibitor on the enzyme's activity is most likely due to a direct interaction between the enzyme and its inhibitor and not due to an effect of the inhibitor on the substrate's physical form. Due to the long *sn*-1 fatty acyl chain ($x = 16$), these amide analogues will presumably appear only in the micelle. Thus, the inhibition of PLA_2 should occur only at the lipid/water interface and not with free inhibitors in solution. The IC_{50}'s with these micellar systems are expressed in surface concentration terms (mol fraction) as we first employed.[2,14]

The dependence of the inhibition potencies on the chain length of the amide analogue in the PC micellar system is illustrated in Figure 4A. The most potent inhibition was observed with the decanoyl derivative. The binding affinity decreases gradually when the *sn*-2 chain length is either increased or decreased relative to the decanoyl analogue. The dependence of binding affinity on chain length was also evaluated in Triton X-100 mixed micelles and is shown in Figure 4B. In the plot of the IC_{50} vs the *sn*-2 chain length, two minimums were observed: one with the acetyl analogue, and the other with the decanoyl analogue. The effect of the formyl analogue on the enzyme activity was also studied in both PC and Triton X-100 mixed micelles. The formyl analogue

(22) Plückthun, A.; Dennis, E. A. *J. Biol. Chem.* **1985**, *260*, 11099–11106.

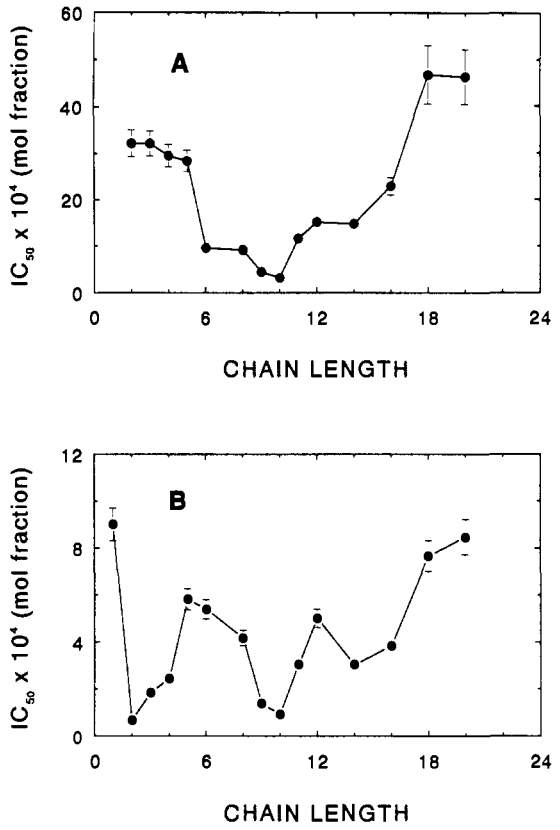


Figure 4. Dependence of the inhibition potency of the amide analogues on the *sn*-2 chain length in PC micelles (A) and in Triton X-100 mixed micelles (B). The amide analogues contained a hexadecylthio at the *sn*-1 position and a variable *sn*-2 acyl chain. The inhibition potency was evaluated by its IC_{50}, which was determined with 4 mM DHPC in PC micellar system and with 0.5 mM DDPC and 4.25 mM Triton X-100 in Triton X-100 mixed micellar system.

had an IC_{50} of 2×10^{-2} mol fraction in PC micelles and 9×10^{-4} mol fraction in Triton X-100 micelles.

Effect of Double Bonds on the Interaction of PLA_2 with Substrate in Triton X-100 Mixed Micelles. Six amide analogues were synthesized which contain a hexadecanylthio group ($x = 16$) at the *sn*-1 position and a 20-carbon acyl group at the *sn*-2 position with a varying number of double bonds, including eicosanoyl-, *cis*-11-eicosenoyl-, 11,14-eicosadienoyl-, *cis*-8,11,14-eicosatrienoyl-, *cis*-5,8,11,14-eicosatetraenoyl- (arachidonoyl-), and *cis*-5,8,11,14,17-eicosapentaenoylamide derivatives. The chain lengths of these analogues required that the inhibitory potency be measured in Triton X-100 mixed micelles using DDPC as substrate. Interestingly, the analogues all exhibit an IC_{50} between 6.7×10^{-4} and 12.9×10^{-4} mol fraction. No significant difference in IC_{50} was observed between analogues with different numbers of double bonds.

Discussion

Hydrophobicity of the *sn*-2 Chain. We have previously shown that the hydrophobicity of the *sn*-1 functional group of phospholipids plays a significant role in the interaction of PLA_2 with phospholipids.[1] Binding affinities are significantly enhanced by increasing the hydrophobicity of the *sn*-1 functional group. Changing the *sn*-1 functional group from an ester to a thioether produced a 100-fold increase in binding affinity. The present study examines the contribution of the *sn*-2 alkyl chain to binding affinity.

In order to study the relationship between binding affinity and hydrophobicity, the hydrophobicity of the analogues was characterized first. By comparing the cmc's of the series of analogues containing different *sn*-2 chain lengths, we were able to measure the contribution of each additional methylene group to the hydrophobic interactions within a micelle. The relationship between the cmc of an amide analogue and its *sn*-2 chain length is expressed in eq 2,[23] where R is the ideal gas constant, T is the absolute

$$\ln \text{cmc} = \Delta G_{tr}/RT = 0.41 - 1.05n_{CH} \quad (2)$$

temperature, n_{CH} is the number of aliphatic carbons ($y - 1$) in the *sn*-2 acyl chain, and ΔG_{tr} is the free energy of transfer of 1 mol of lipid into the micelle from aqueous solution. The free energy of transfer is calculated from the gradient of the chain length dependence in Figure 1, which is approximately $-1.05RT$ per mol per methylene group. This corresponds to a free energy of transfer of −633 cal/mol per methylene at 30 °C. This result is very similar to those reported in the literature for lysophosphatidylcholines[24] and for phosphatidylcholines.[25]

Interestingly, a deviation from eq 2 is observed with acyl chains shorter than five carbons ($n_{CH} = 4$), where the cmc is almost independent of the chain length. Various techniques have shown that the *sn*-2 acyl chain has a kink of about 90° at the C2 position. Thus, the carbonyl and C2 methylene carbons are parallel to the lipid/water interface[26-28] and do not contribute to the hydrophobic interactions. The C3 and C4 methylene groups are presumably located at the surface of the hydrophobic core of the micelle and are more or less perpendicular to the surface. Thus, these two methylene groups make less of a contribution to the hydrophobic interaction within the micelle than normal methylene groups. The observation that the few methylene groups close to the polar headgroup make less of a contribution to the cmc and hence presumably to hydrophobic interactions has been observed previously in several different micellar systems.[29-31]

Binding of Monomeric Substrates. The inhibitory potency of the monomeric amide analogues correlates with their hydrophobicity as shown in Figure 3. The inhibitory potency, and hence, the affinity of the analogue for the enzyme, increases as the *sn*-2 chain's hydrophobicity increases, reaching a plateau at the decanoyl derivative. The maximum hydrophobic interaction between the analogue and the enzyme is achieved with about a 10-carbon *sn*-2 acyl chain. Any methylene group beyond this length has little effect on the affinity of the amide analogue. This implies that the effective size of the enzyme catalytic site is about 10 carbons in length with respect to the *sn*-2 acyl group.

By analogy to the determination of the hydrophobicity of the interior of micelles,[23] the hydrophobicity of the catalytic site of the enzyme can also be determined by plotting the log of the IC_{50} values versus the number of methylene groups on the *sn*-2 acyl chain as shown in Figure 2. All of the monomeric amide analogues used in this study contain a thioether function at the *sn*-1 position, an amide function at the *sn*-2 position, and a phosphocholine headgroup but differ only in the chain length of the *sn*-2 fatty acyl group. Therefore, they presumably inhibit the enzyme through the same mechanism. In addition, their IC_{50}'s were determined under identical conditions. Therefore, the ratio of the IC_{50}'s of two amide analogues is equal to the ratio of their dissociation constants.[32]

The free energy, ΔG, for the binding of an inhibitor to the enzyme is related to its dissociation constant by eq 3, where K_i

$$\Delta G = -RT \ln K_i \quad (3)$$

is the dissociation constant. The ΔG for each inhibitor can be

(23) Tanford, C. *The Hydrophobic Effect* (2nd ed.); Wiley: New York, 1980; pp 66–70.
(24) Marsh, D.; King, M. D. *Chem. Phys. Lipids* **1986**, *42*, 271–277.
(25) King, M. D.; Marsh, D. *Biochemistry* **1987**, *26*, 1224–1231.
(26) Hitchcock, P. B.; Mason, R.; Thomas, K. M.; Shipley, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *72*, 3036–3040.
(27) Oldfield, E.; Meadows, M.; Rice, D.; Jacobs, R. *Biochemistry* **1978**, *17*, 2727–2740.
(28) Roberts, M. F.; Bothner-By, A. A.; Dennis, E. A. *Biochemistry* **1978**, *17*, 935–942.
(29) Tanford, C. In *Micellization, Solubilization and Microemulsions*; Mittal, K., Ed.; Plenum: New York, 1977; pp 119–131.
(30) Klevens, H. B. *J. Am. Oil Chem. Soc.* **1953**, *30*, 74.
(31) Evans, H. C. *J. Chem. Soc.* **1956**, 579.
(32) Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

broken into two parts as shown in eq 4. ΔG_i is the *sn*-2 chain

$$\Delta G = \Delta G_i + n\Delta G_{CH} = RT \ln K_i \quad (4)$$

length independent energy contributed by interactions of the enzyme with the polar headgroup, the glycerol backbone, the *sn*-1 alkyl thioether group, and the *sn*-2 amide functional group of the amide analogue, all of which are the same for this series of amide analogues. ΔG_{CH} is the energy contributed by each methylene group of the *sn*-2 acyl chain, and n is the number of methylene groups in the *sn*-2 acyl chain. ΔG_{CH} can be calculated from the slope of the semi log plot of the IC_{50} values versus chain length. For the linear region between butyryl ($n = 3$) and decanoyl ($n = 9$), the contribution of each methylene group to the binding energy was calculated to be −665 cal/mol at 30 °C. This value is comparable to the free energy calculated above for the transfer of a methylene group from aqueous solution to a phospholipid micelle. This result suggests that the interior of the catalytic site is as hydrophobic as the interior of a phospholipid micelle.

The maximum contribution of the alkyl chain on the *sn*-2 acyl group can also be determined. Among these monomeric amide analogues, the formylamide analogue is the weakest binding analogue whereas the decanoyl analogue is the tightest. These two analogues give the lower and upper limits for the hydrophobic interactions between the enzyme and the *sn*-2 alkyl chain. Compared with the formyl derivative, the decanoyl analogue has eight more methylene groups and one more methyl group. The total energy arising from the interaction of these groups can be calculated from the ratio of the IC_{50} of the decanoyl analogue to that of the formyl analogue. The maximum contribution of the hydrocarbons of the *sn*-2 acyl group to the binding energy is calculated to be 4.3 kcal/mol, which corresponds to a 3 orders of magnitude difference in binding affinity. Most of this binding energy comes from the hydrophobic interactions of the enzyme with the C5 to C10 methylenes. The C2 methylene provides about 460 cal/mol of binding energy, since the binding affinity is increased about 2-fold in going from the formyl to the acetyl analogues. However, the C3 and C4 methylene groups hardly provide any binding energy.

Binding of Micellar Substrates. To further explore the importance of hydrophobic interactions within the enzyme/inhibitor complex, the effect of the *sn*-2 chain length on the affinities of the amide analogues was studied using PC micellar and Triton X-100 mixed micellar substrates. As shown in Figure 4, the binding affinities of the micellar amide analogues to the enzyme were much less dependent on the *sn*-2 chain length than were those of the monomeric analogues. The maximum difference in the binding affinity of these amide analogues was less than 9-fold, compared with a 3 orders of magnitude difference observed with monomeric analogues. The chain length dependence was also much more complicated in micellar systems (Figure 4). The inhibitory potencies of these amides do not simply increase with the *sn*-2 acyl chain length.

Since the inhibition studies were conducted in micellar systems, it is important to consider the possible effect of these amide analogues on the lipid/water interface. This is because the activity of PLA_2 is very sensitive to the properties of the interface. Any molecule which perturbs the lipid/water interface could lead to the "inhibition" of PLA_2. Thus, the different inhibitory potencies of these micellar amides could be due to their effect on the interface. The strong argument against this "inhibition" mechanism comes from the composition of the micelles. The number of micelles in the solution can be calculated by assuming that the size of the micelle is similar with or without the presence of inhibitor. Each Triton X-100 mixed micelle contains about 140 Triton X-100 molecules[33] and 28 substrate molecules. Under the experimental conditions used, the concentration of the micelles is about 28.6 μM.

The least potent inhibitor studied in the Triton X-100 micellar system is the formyl analogue with an IC_{50} of 9×10^{-4} mol fraction, which corresponds to a bulk concentration of 4 μM. At this inhibitor concentration, only 14% of the micelles contain an inhibitor molecule. The number of micelles containing an inhibitor in the PC micellar system could also be calculated by assuming that the size of dihexanoyl thio PC micelles is the same as that of dihexanoyl PC micelles, each of which contains about 30 substrate molecules.[34] For the PC micellar system, less than 20% of the micelles contain an inhibitor. Due to their high affinities, these amide analogues are present in only a small portion of the micelles, even assuming that each micelle contains only one inhibitor molecule. Thus, it is unlikely that the inhibition results from the effect on the lipid/water interface.

One concern is that the long-chain inhibitors could phase separate rather than homogenously mix with the PC micelles or the Triton X-100 mixed micelles. However, mixtures of short-chain PC micelles with long-chain PC have been shown to phase separate only at very high mol fractions of long-chain PC.[35] Furthermore, in the experiments reported herein which used such low concentrations of inhibitor in the surface, the inhibitor molecules are so dilute that phase separation is even more unlikely. Nonetheless, the mixed micelles are probably not perfectly homogeneous, and it cannot be ruled out that some segregation or phase separation occurs at certain chain lengths, giving rise to the nonuniform dependencies of IC_{50}'s observed in DHPC micelles and Triton X-100 mixed micelles.

The reduced effect seen for the *sn*-2 acyl chain in micellar systems can be explained if the catalytic site of the enzyme and the core of micelles have similar hydrophobic characteristics. In the binding of a micellar inhibitor to a micelle-bound enzyme, the methylene groups are no longer being transferred from an aqueous environment to the hydrophobic one of the enzyme. In fact, the hydrophobicity of the interior of micelles is very similar to that of the enzyme, as indicated by the fact that the energy for transferring a methylene group from water to either a micelle or the enzyme is almost the same, about 700 cal/mol.[23,36] Thus, little change in free energy should occur on transferring an inhibitor from the micelle to the catalytic site, and little chain length dependence should be observed in the micellar systems. The chain length effect is indeed much smaller in micellar systems than in the monomeric system. In addition, the two chain length dependence curves obtained follow different patterns. There is no obvious correlation between the binding affinities of these micellar analogues and their *sn*-2 acyl chain length.

Unsaturated Fatty Acid Chains. Due to the biological importance of arachidonic acid in eicosanoid biosynthesis,[37,38] the effect of double bonds on the interaction of the enzyme with its substrate was investigated. Altering the number of double bonds in the *sn*-2 acyl chain had little effect on the affinity of the amide analogues for the enzyme. This result suggests that these double bonds simply function as hydrophobic identities and do not have any special structural interactions with the enzyme. The absence of a double-bond effect can be explained by the hydrophobic interactions of the analogue with the enzyme and with the Triton X-100 mixed micelle. As indicated earlier, the hydrophobicity of the enzyme active site is about the same as that of the Triton X-100 mixed micelle. The transfer of a double bond from one hydrophobic environment to another environment with similar hydrophobicity should not cost much energy.

Conclusion. In the present study, the hydrophobic interactions of PLA_2 with its substrate have been investigated by examining the dependence of the IC_{50}'s of the amide substrate analogues on their *sn*-2 acyl chain length. This study has delineated structural features of both the substrate analogue and the enzyme which are important for substrate binding. The *sn*-2 acyl group is required for the binding of the amide analogues to the enzyme. The catalytic site of the cobra venom PLA_2 can effectively interact

(33) Robson, R. J.; Dennis, E. A. *J. Phys. Chem.* **1977**, *81*, 1075–1078.

(34) Tausk, R. J. M.; Karmiggelt, J.; Oudshoorn, C.; Overbeek, J. Th. G. *Biophys. Chem.* **1974**, *1*, 175–183.

(35) Bian, J.; Roberts, M. F. *Biochemistry* **1990**, *29*, 7928–7935.

(36) Becher, P. In *Nonionic Surfactants*, Schick, M. J., Ed.; Marcel Dekker: New York, 1967; pp 478–515.

(37) Dennis, E. A. *Bio/Technology* **1987**, *5*, 1294–1300.

(38) Smith, W. *Biochem. J.* **1989**, *259*, 315–324.

with about the first 10 carbons on the *sn*-2 acyl chain of phospholipids; these ten or so carbons are exposed to an environment in the enzyme that is very hydrophobic. Each methylene on the *sn*-2 acyl chain provides a binding energy of about 655 cal/mol. The hydrophobic interaction between the enzyme and the *sn*-2 chain is much more important for the monomeric inhibitors than for the micellar inhibitors. Binding of a monomeric substrate dissolved in water is more favorable the more hydrophobic (i.e., longer) its *sn*-2 fatty acid chain is, as this represents a net transfer from an aqueous environment to a hydrophobic one. In contrast, for similar substrates which are micellar, the hydrophobic *sn*-2 chain is transferred from the hydrophobic environment of a micelle to a similar (energetically) hydrophobic environment in the active site of the enzyme. The small, but significant, effect of the fatty acyl chain length on the interaction of the enzyme with the micellar substrate is not well understood. We are currently trying to model these interactions.

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Supplementary Material Available: Listings of NMR and mass spectra data (8 pages). Ordering information is given on any current masthead page.

Unmasking the Chemistry of DNA Cleavage by the Esperamicins: Modulation of 4′-Hydrogen Abstraction and Bistranded Damage by the Fucose–Anthranilate Moiety

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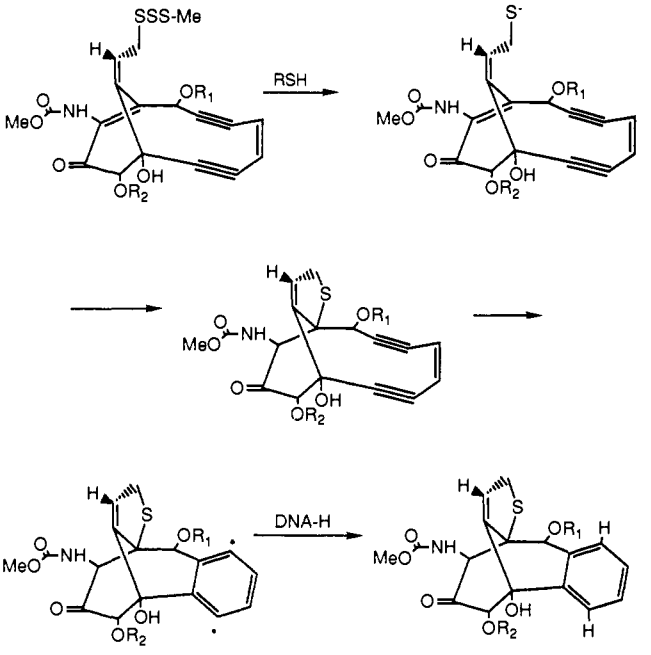
Abstract: The chemistry of DNA cleavage by the esperamicins A_1, C, D, and E (esp A, C–E) has been examined. High-resolution gel electrophoresis reveals that esp A, a known single-strand cleaver, affords fragmentation products consistent with exclusive 5′-hydrogen abstraction. In contrast, esp C–E, analogs that produce significant double-strand cleavage, generate fragmentation products consistent with both 5′- and 4′-hydrogen abstraction. On the basis of these observations and other findings reported for a related enediyne antibiotic, calicheamicin γ_1[1] (Townsend, C. A.; DeVoss, J. J.; Ding, W.-D.; Morton, G. O.; Ellestad, G. A.; Zein, N.; Tabor, A. B.; Schreiber, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 9669), we conclude that 4′-hydrogen abstraction and bistranded DNA cleavage are directly related to the reactivity of the C-7 radical as modulated by the fucosyl–anthranilate group.

The enediyne antibiotics, a potent class of antitumor compounds and DNA cleavers, represented by the esperamicins A_1, C, D, and E (esp A, C–E)[1] and by calicheamicin γ_1[12] (Figure 1), have been the subject of intense mechanistic and synthetic study.[3] A key mechanistic feature of the activation of these compounds toward DNA cleavage is a Bergman cyclization of the enediyne core,[4] triggered by an intramolecular conjugate addition of thiolate derived by reduction of the intrinsic trisulfide, to give the highly reactive bis radical 1,4-benzyne localized at C-7 and C-10 of the esperamicin core (Scheme I). The exquisite positioning of the benzyne moiety in the minor groove of DNA has been postulated to lead to double-strand scission by synchronous homolytic hydrogen abstractions from deoxyribose groups of nucleotide residues on opposing strands.[1-3]

The esperamicins and calicheamicins bear a number of structural similarities and differences (Figure 1). The bicyclo[7.3.1]tridecadiynediene ring system which constitutes the reactive core is identical for the two classes with the exception of the occurrence of a hydroxyl group at C-4 of esperamicin.[5] In the fully elaborated esp A, the C-4 hydroxyl group serves as the site of attachment of a fucosyl–anthranilate moiety. Esp C–E are derived from esp A by chemical hydrolysis and lack the fucosyl–anthranilate moiety at C-4. Esp D and E are derived by additional hydrolytic deletions from the oligosaccharide group appending C-12.[5] In the calicheamicins a single oligosaccharide

Scheme I. Mechanism of Activation of the Esperamicins by Reduction of the Trisulfide



array is attached to the core via the C-8 hydroxyl. Thus, esp C–E and the calicheamicins are most closely related, although structural

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