Mechanism-Based in Vivo Inactivation of Lauric Acid Hydroxylases†

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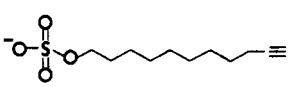
ABSTRACT: The hepatic cytochrome P-450 isozymes that catalyze ω- and (ω − 1)-hydroxylation of lauric acid are specifically inactivated in vitro but not in vivo by 10-undecynoic acid. The lack of in vivo activity may result from rapid degradation of the inhibitor by β-oxidation. Strategies for the construction of fatty acid analogues that retain the ability to inactivate fatty acid hydroxylases but are resistant to metabolic degradation have therefore been sought. Fatty acid analogues in which the carboxylic acid group is replaced by a sulfate moiety, or in which two methyl groups are placed vicinal to the carboxylic acid group, have been found to inactivate lauric acid hydroxylases in vitro and in vivo without causing time-dependent inhibition of ethoxycoumarin O-deethylation or *N*-methyl-*p*-chloroaniline N-demethylation.

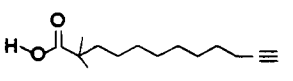
Fatty acids and their derivatives, including arachidonic acid and the physiologically important prostaglandins, thromboxanes, prostacyclins, and leukotrienes, are hydroxylated at the ω- and (ω − 1)-positions by cytochrome P-450 monooxygenases (Kupfer, 1980). The physiological roles of fatty acid ω-oxidations remain obscure, but the existence of isozymes that uniquely catalyze this reaction, the specific induction of one of these isozymes by clofibrate, and the enhanced excretion of dicarboxylic acids under conditions of high fatty acid flux suggest that ω-hydroxylation, at the very least, assists in the degradation of excess fatty acids and facilitates the degradation of fatty acids that are resistant to β-oxidation (Kupfer, 1980; Tamburini et al., 1984). The construction of mechanism-based agents that inactivate the ω-hydroxylases in vivo should help to clarify their physiological functions and to develop approaches for extending the half-life of therapeutic agents related to the prostaglandins and other arachidonate derivatives.

The inactivation in vitro of several fatty acid hydroxylases by fatty acid analogues bearing a terminal acetylenic group has been reported by this laboratory (Ortiz de Montellano & Reich, 1984; Shak et al., 1985). The lauric acid ω- and (ω − 1)-hydroxylases are irreversibly inhibited in vitro by 10-undecynoic acid (10-UDYA)[1] and 11-dodecynoic acid (11-DDYA), but neither of these agents is active in vivo below the concentration at which it becomes toxic (Ortiz de Montellano & Reich, 1984). The inactivity of these acetylenic agents in vivo presumably results from their rapid metabolic degradation via β-oxidation and their esterification and storage in the liver. These metabolic processes not only may eliminate the active agent but, in the case of β-oxidation, may generate propynoic acid or a related reactive metabolite. The formation of such an electrophilic species could explain the higher toxicity of the acetylenic than of the saturated fatty acids. We have therefore undertaken the development of agents that retain the ability to inactivate fatty acid hydroxylases but are not susceptible to β-oxidation.

Two different strategies have been investigated for the construction of agents able to inactivate fatty acid hydroxylases in vivo. The first is based on the fact that the degradation of alkyl sulfates in mammals involves oxidation of the terminal alkyl chain carbon to the corresponding carboxylic acid, presumably via initial ω-hydroxylation, followed by β-oxidation of the resulting "ω-sulfated" fatty acid. Hydrolysis of the sulfate ester, a reaction observed in the metabolism of alkyl sulfates by microorganisms (Denner et al., 1969; Burke et al., 1975; Maggs et al., 1984), appears not to be an important process in mammals. Alkyl sulfates with a double bond or a phenyl group at the alkyl chain terminus thus are not subject to β-oxidation (Burke et al., 1978). The possibility that ω-acetylenic sulfate esters may not be susceptible to rapid catabolism or storage, but may still be catalytically turned over by the ω-hydroxylases, has therefore been examined. The second approach is based on the observation that introduction of a *gem*-dimethyl group α or β to the carboxylic acid function inhibits fatty acid β-oxidation (Bergstrom et al., 1954; Tryding & Westoo, 1956; Bar-Tana et al., 1985; Fretland et al., 1985). We report here the synthesis of sodium 10-undecynyl sulfate (10-SUYS) and 2,2-dimethyl-11-dodecynoic acid (DMDYA)



10-SUYS



DMDYA

and demonstrate that these analogues specifically inhibit the isozymes of cytochrome P-450 that hydroxylate lauric acid in vivo as well as in vitro.

Experimental Procedures

Materials. NADP, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO); isobutyric acid, 7-ethoxycoumarin, and fluorescamine were from Aldrich (Milwaukee, WI); 10-undecynoic acid and 3-decyn-1-ol were from Farchan Laboratories (Willoughby, OH); radiolabeled [1-^{14}C]lauric acid

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[1] Abbreviations: 10-UDYA, 10-undecynoic acid; 11-DDYA, 11-dodecynoic acid; 10-SUYS, sodium 10-undecynyl sulfate; DMDYA, 2,2-dimethyl-11-dodecynoic acid; DETAPAC, diethylenetriaminepentaacetic acid; PCMA, *N*-methyl-*p*-chloroaniline.

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(26 mCi/mmol) was from Amersham (Arlington Heights, IL); lauric acid was from NuCheck Prep (Elysian, MN); sodium dodecyl sulfate was from Bio-Rad (Richmond, CA.).

Instrumentation. High-pressure liquid chromatographic analyses were carried out on a 15 cm × 4.6 mm Du Pont Zorbax ODS 5-μm C_{18} reverse-phase column with 63% methanol/water (1 mL/min) as the elution solvent. The eluent was monitored at 217 nm with a Hitachi Model 100-40 variable-wavelength detector. NMR spectra were obtained on a Varian FT-80 instrument. NMR spectra were recorded in deuterated chloroform with chloroform as internal standard unless indicated otherwise. Chemical shift values are reported in parts per million relative to tetramethylsilane. Fluorometric measurements were performed on a Perkin-Elmer Model 650-10S fluorescence spectrophotometer. Infrared spectra were obtained on a Nicolet 5DX FT-IR instrument. Liquid scintillation counting was performed with a Searle Mark III scintillation counter in Aquasol (New England Nuclear, Boston, MA). Cytochrome P-450 concentrations and binding assays were measured on an Aminco DW-2a UV–visible spectrophotometer. Electron-impact (70-eV) mass spectra were obtained on a Kratos AEI-MS 25 mass spectrometer coupled to a Varian 3700 gas chromatograph fitted with a 30-m DB-5 column programmed to rise from 100 to 200 °C at 5 °C/min.

Synthesis of 10-SUYS. 10-UDYA (6.37 g, 35 mmol) in 50 mL of ether was added dropwise over a period of 1 h at 0 °C to a stirred suspension of lithium aluminum hydride (2.66 g, 70 mmol) in 150 mL of ether. The reaction mixture was stirred for an additional hour, at which point 40 mL of 4 N NaOH followed by 70 mL of water was added. The product mixture was filtered, the aqueous layer was extracted with ether, and the ether layers were combined. The crude alcohol product obtained on solvent removal yielded, after vacuum distillation, 4.71 g (80.0%) of pure 10-undecyn-1-ol: ^{1}H NMR $CDCl_3$) δ 1.31 (m, 14 H, $-CH_2-$), 1.92 (t, J = 2.6 Hz, 1 H, HC≡C—), 2.16 (m, 2 H, —C≡C—CH_2—), and 3.62 ppm (t, J = 6.2 Hz, 2 H, $-CH_2OH$); IR (neat) 3300 cm^{-1} ($-CH_2OH$).

The sulfate ester was synthesized from the alcohol by a procedure based on that of Burke et al. (1978). A solution of freshly distilled chlorosulfonic acid (1.10 mL, 16.6 mmol) in 25 mL of dichloromethane was added dropwise, over a period of 1 h at 0 °C under a nitrogen atmosphere, to a solution of 10-undecyn-1-ol (2.80 g, 16.6 mmol) and pyridine (4 mL, 49.7 mmol) in 75 mL of dichloromethane. The reaction mixture was stirred for 30 min at 0 °C and then overnight at room temperature. The solvent was removed with a stream of nitrogen, and 75 mL of methanol was added. The resulting mixture was neutralized with 10% NaOH and was stirred overnight to ensure formation of the sodium salt. After solvent removal under vacuum, 200 mL of methanol was added to the crude product. The mixture was stirred for 2 h and filtered, and the solvent was removed. 10-SUYS was obtained in 89% yield: ^{1}H NMR (^2H_2O) δ 0.92 (m, 14 H, $-CH_2-$), 1.80 (m, 2 H, —C≡C—CH_2—), 1.91 (t, J = 2.6 Hz, 1 H, HC≡C—), and 3.64 ppm (t, J = 6.0 Hz, 2 H, —CH_2—OSO_3^-); IR (Nujol) 1250 cm^{-1} ($-R-O-SO_3^-$). Anal. Calcd for $C_{11}H_{19}O_4SNa$ (270.35): C, 48.87; H, 7.10; S, 11.86. Found: C, 48.86; H, 7.14; S, 11.80.

Synthesis of DMDYA. A solution of 3-decyn-1-ol (10 g, 65 mmol) in 150 mL of pyridine was cooled to 0 °C; tosyl chloride (24.8 g, 130 mmol) was added and the mixture stirred for 30 min (Fieser & Fieser, 1967). The reaction flask was kept at 4 °C overnight. The reaction mixture turned from bright yellow to a deep purple, and a white crystalline solid formed at the bottom. The entire mixture was then poured with stirring into 800 mL of ice–water and stirred for an additional 15 min. The aqueous layer was extracted 3 times with ether. The organic layers were combined and washed twice with cold 5% HCl to remove pyridine. Finally, the organic layer was washed with saturated NaCl solution and water and dried over K_2CO_3–Na_2SO_4, and the solvent was removed under reduced pressure. To purify the tosylate, the crude oil was dissolved in 30–60 °C petroleum ether, stirred with decolorizing carbon, and filtered through Celite. The resulting solution was chromatographed on silica gel. Elution of the column with ethyl acetate/hexane (5:95) yielded, after solvent removal, 19.12 g (95.3%) of 1-tosyl-3-decyne: ^{1}H NMR ($CDCl_3$) δ 0.85 (t, J = 6.0 Hz, 3 H, CH_3–R), 1.26 [m, 8 H, $CH_3-(CH_2)_4$–R], 2.01 (m, 2 H, R—CH_2—C≡C—R), 2.42 (s, 3 H, R–OSO_2–Ph–CH_3), 2.48 (m, 2 H, R—C≡C—CH_2—CH_2—OSO_2—R), 4.03 (t, J = 7.2 Hz, 2 H, R–CH_2–OSO_2–R), 7.30 (d, J = 8.5 Hz, 2 H, meta protons), and 7.78 ppm (d, J = 8.4 Hz, 2 H, ortho protons); IR (neat) 1187 and 1173 cm^{-1} (R–OSO_2–Ar).

Lithium bromide (10.74 g, 124 mmol) was added to a solution of the tosylate of 3-decyn-1-ol (19.12 g, 62 mmol) in 200 mL of acetone (Buchta & Merk, 1968). The mixture was heated to reflux under a nitrogen atmosphere for 4.5 h. The precipitate present after the mixture was cooled was removed by filtration, and the filtrate was added to 500 mL of water. The organic layer was separated from the aqueous layer, and the aqueous layer was washed with ether. The combined organic layers, after being dried with sodium sulfate and removal of the solvent, yielded 12.37 g (92.0%) of 1-bromo-3-decyne: ^{1}H NMR ($CDCl_3$) 0.89 (t, J = 5.4 Hz, 3 H, CH_3–R), 1.34 [m, 8 H, $CH_3-(CH_2)_4$–R], 2.14 [m, 2 H, R—CH_2—C≡C—$(CH_2)_2$—Br], 2.70 (m, 2 H, R—C≡C—CH_2—CH_2—Br), and 3.41 ppm (t, J = 7.2 Hz, 2 H, R–CH_2–Br).

The bromide was coupled to isobutyric acid according to the procedure of Creger (1970). Sodium hydride, 50% in mineral oil (2.32 g, 48.5 mmol), was suspended in 60 mL of dry THF in a dry three-necked round-bottom flask equipped with a thermometer, condenser, and dropping funnel. Diisopropylamine (6.15 mL, 43.9 mmol) was added via syringe to the stirred mixture, followed by the slow addition of isobutyric acid (4.08 g, 43.9 mmol). The reaction mixture was heated to reflux for 15 min and then cooled to 0 °C with an ice–salt bath. After the mixture was cooled, 17.58 mL of a 2.5 M solution of *n*-butyllithium in hexanes (43.9 mmol) was added dropwise at a temperature below 10 °C. The mixture was maintained at 0 °C for 15 min and then heated to 35 °C for 30 min. The resulting turbid solution was cooled and 1-bromo-3-decyne (9.54 g, 43.9 mmol) added dropwise over a period of 20 min at 0 °C. The mixture was kept at 0 °C for 30 min and then heated to 35 °C for 1 h. The mixture was then cooled below 15 °C, and 150 mL of water was added. The organic layer was separated and was washed with 75 mL of water. The aqueous layers were combined, back-extracted with 75 mL of ether, and acidified with 1 N HCl to pH 3–5 at 0 °C. After acidification, the aqueous mixture was extracted twice with 120 mL of ether, and the ether extracts were washed twice with 90 mL of saturated NaCl solution. The ether solution was dried and the solvent removed under vacuum. The crude product, chromatographed on silica gel with ethyl acetate/hexane (15:85), yielded 8.74 g (88.6%) of 2,2-dimethyldodecynoic acid (the formation of a mixture of isomers is indicated by gas–liquid chromatography): ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 5.9 Hz, 3 H, R–CH_3), 1.20 [s, 6 H,

R–C(CH_3)$_2$COOH], 1.28 [m, 10 H, CH_3–(CH_2)$_4$–R], and 1.85 and 2.09 ppm (m, 4 H, propargylic protons).

The internal acetylene was converted to the terminal acetylenic product according to the procedure of Macaulay (1980). Sodium hydride, 50% in mineral oil and washed with hexane (17.88 g, 0.37 mol), was combined with 140 mL of diaminopropane. The mixture was stirred 1 h at 70 °C and was then cooled to room temperature. A solution of 2,2-dimethyl-5-dodecynoic acid (5.28 g, 23.6 mmol) in 74.6 mL of diaminopropane was added dropwise. This mixture was stirred at 55 °C overnight and then cooled on ice. Concentrated HCl was added to neutralize the solution and the pH adjusted to 3–5 with 3 M HCl. The aqueous mixture was extracted 3 times with ether. The combined ether extracts were washed with water and saturated sodium chloride solution and then dried over sodium sulfate. Vacuum distillation after solvent removal yielded 3.78 g (71.6%) of 2,2-dimethyl-11-dodecynoic acid: ^{1}H NMR ($CDCl_3$) δ 1.19 [s, 6 H, R–C(CH_3)$_2$COOH], 1.29 [m, 14 H, R–(CH_2)$_7$–R], 1.93 (t, J = 2.5 Hz, 1 H, HC≡C—R), 2.16 ppm (m, 2 H, HC≡C—CH_2—R); IR (neat) 2116 cm^{-1} (HC≡C—R); mass spectrum, M_r 238, found 207 (M-31; loss of –OCH_3 from the methyl ester). Anal. Calcd for $C_{14}H_{24}O_2$ (224.38): C, 74.94; H, 10.80. found: C, 75.12; H, 10.78.

In Vitro Inactivation of Lauric Acid Hydroxylases. Microsomal incubations with the inhibitors were performed as described by Ortiz de Montellano and Reich (1984).

Binding Assay (Jefcoate, 1978). Aliquots (1 mL) of a 1.5 mg of protein/mL of microsomal suspension were placed in two cuvettes, and their base-line difference spectrum was recorded from 375 to 445 nm. Succesive aliquots (1–10 μL) of a solution of the inhibitor in Me_2SO were then added to the sample cuvette and equivalent amounts of Me_2SO to the reference cuvette. Difference spectra were thus obtained for inhibitor concentrations ranging from 5 to 500 μM.

In Vitro Cytochrome P-450 Destruction. Microsomes (1 mg/mL of microsomal protein) prepared from the livers of uninduced male Sprague-Dawley rats (200–220 g) were incubated with the inhibitor (150 μM), NADPH (1.0 mM), and DETAPAC (1.0 mM) in 0.1 M sodium phosphate/potassium phosphate buffer, pH 7.4. The incubation mixture was prepared by coating the inhibitor on the walls of the reaction vessel, adding the microsomal suspension, and preincubating the mixture for 5 min at 37 °C. NADPH was then added, and 3-mL aliquots, removed after 1, 15, and 30 min, were placed in an ice bath. Cytochrome P-450 levels were immediately measured from the dithionite vs. CO – dithionite difference spectra (Estabrook & Werringloer, 1978).

N-Methyl-p-chloroaniline (PCMA) N-Demethylase Assay. A sensitive fluorometric method was utilized to measure PCMA demethylase activities (Van der Hoeven, 1977). Microsomes (1 mg/mL) were preincubated with inactivator (150 μM) and NADPH (1.0 mM) for 0.5, 15, and 30 min at 37 °C. Aliquots (100 μL), removed at the indicated times, were transferred to 0.9 mL of a mixture containing PCMA (0.5 mM) and an NADPH generating system (0.5 mM NADP, 4.5 mM glucose 6-phosphate, 2.0 mM $MgCl_2$, and 2 units of glucose-6-phosphate dehydrogenase) in 0.1 M sodium phosphate/potassium phosphate buffer (pH 7.4) containing 0.1 mol of sodium acetate. The assay mixture was incubated for 30 min at 37 °C and the reaction stopped by addition of 0.2 mL of 30% trichloroacetic acid. After this was mixed, 1 mL of water was added and the denatured protein sedimented by centrifugation. The supernatant was separated and combined with 0.1 mL of 1.0 M sodium acetate (pH 4.0). A fluorescent derivative of the *p*-chloroaniline metabolite was formed by adding 0.025 mL of fluorescamine (9.0 mg in 5.0 mL of acetone) to the above solution with vigorous mixing. The aqueous solution was then extracted with 2 mL of ethyl acetate and the fluorescence measured with a Perkin-Elmer Model 650-10S fluorescence spectrophotometer. Excitation and emission wavelengths of 410 and 500 nm, respectively, and slit widths of 5 nm were used. Blanks were run as described above except that trichloroacetic acid was added to the assay mixture just prior to addition of the preincubation mixture.

Ethoxycoumarin O-Deethylase Assay. Ethoxycoumarin deethylase activity was measured by a fluorometric method (Guengerich, 1978). Mixtures consisting of microsomes (1 mg/mL), inactivator (150 μM), and NADPH (1 mM) in 0.1 M sodium phosphate/potassium phosphate buffer (pH 7.4) were incubated at 37 °C for 0.5, 15, and 30 min. Aliquots (100 μL) of this preincubation mixture were added to 0.9 mL of the assay mixture, which consisted of 0.5 mM NADPH, 5 mM $MgCl_2$, and 0.5 mM ethoxycoumarin in 65 mM sodium phosphate/potassium phosphate buffer, pH 7.4. This mixture was incubated for 10 min at 37 °C and was then quenched with 0.1 mL of 2 N HCl and 2 mL of chloroform. After this was mixed and centrifuged, 1 mL of the chloroform layer was combined with 2.5 mL of 30 mM sodium borate buffer, pH 9.2. The fluorescence of the aqueous layer was measured at an excitation wavelength of 368 nm and an emission wavelength of 458 nm with slit widths of 5 nm. Blanks were run in the same manner except that 2 N HCl was added to the assay mixture before preincubation.

In Vivo Inactivation of Lauric Acid Hydroxylases. Uninduced male Sprague-Dawley rats (200–220 g) were injected intraperitoneally with the inactivator dissolved in Me_2SO. After 4 h, the rats were decapitated and their livers perfused with isotonic KCl before they were removed. Microsomes were then prepared from each liver and examined for their lauric acid hydroxylase activities. The assay mixtures contained microsomal protein (1 mg), NADPH (1 mM), and DETAPAC (1.0 mM) in 0.1 M sodium phosphate/potassium phosphate buffer (pH 7.4). The mixtures were incubated for 5 min at 37 °C. NADPH or buffer was then added, and the mixture was incubated for an additional 10 min before 400 μL of 10% H_2SO_4 was added. Each assay mixture was worked up as described for the in vitro experiments.

Protein Determinations. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

In Vitro Inactivation of Lauric Acid Hydroxylases. Fatty acid analogues were screened for intrinsic activity as inhibitors of lauric acid hydroxylases in an in vitro microsomal system. DMDYA and 10-SUYS, as shown respectively in Figures 1 and 2, cause the NADPH- and time-dependent loss of both the ω and $\omega - 1$ lauric acid hydroxylase activities. In contrast, sodium dodecyl sulfate (SDS), a saturated homologue of 10-SUYS, decreases the hydroxylase activities by no more than about 5% in a time-independent manner (not shown). Both DMDYA and 10-SUYS have a more pronounced effect on ω- than on ($\omega - 1$)-hydroxylation. The inhibitory activity of 10-SUYS is nearly identical with that of 10-UDYA, with approximately 23% of the ω and 69% of the $\omega - 1$ activity remaining after 30 min. On the other hand, DMDYA discriminates less between the ω- and ($\omega - 1$)-hydroxylase activities, 31% of the former and 43% of the latter remaining after 30 min.

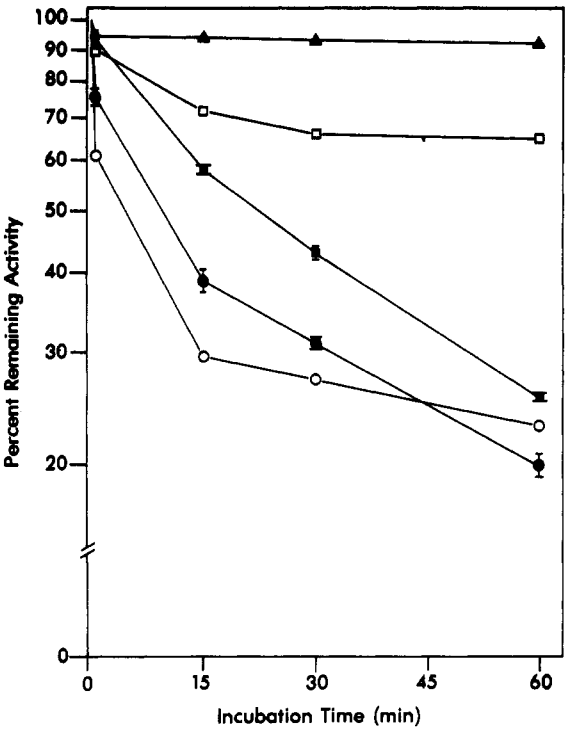

FIGURE 1: In vitro inactivation of lauric acid ω- (●) and (ω − 1)- (■) hydroxylases by DMDYA in the presence of NADPH. The in vitro inactivation of the ω- (○) and (ω − 1)- (□) hydroxylases by 10-UDYA is shown for comparison. In the absence of NADPH, neither compound had a measurable effect (▲). The activities of the ω- and (ω − 1)-hydroxylases that correspond to 100% activity were 0.78 and 0.84 nmol min^{-1} (mg of protein)$^{-1}$, respectively. The points are averages of three or more independent measurements. Standard deviations are indicated. Details of the incubation and assay procedures are provided under Experimental Procedures.

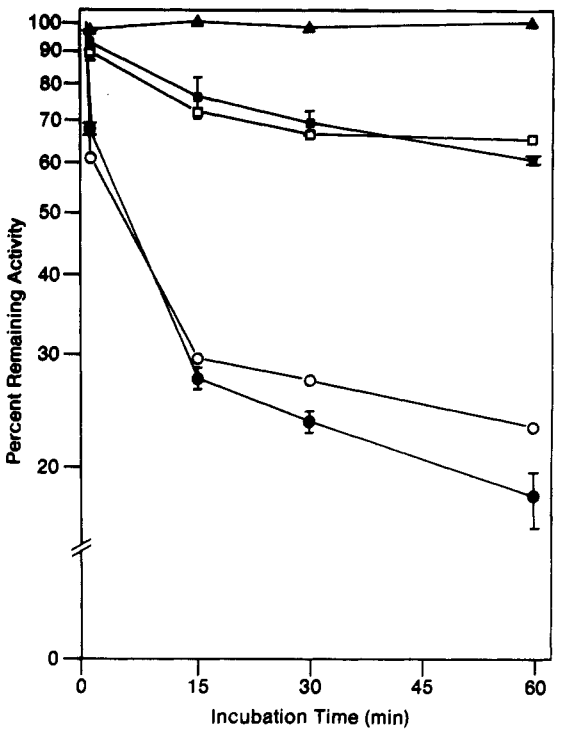

FIGURE 2: In vitro inactivation of lauric acid ω- (●) and (ω − 1)- (■) hydroxylases by 10-SUYS in the presence of NADPH. The in vitro inactivation of the ω- (○) and (ω − 1)- (□) hydroxylases by 10-UDYA is shown for comparison. In the absence of NADPH, neither compound had a measurable effect (▲). The activities of the ω- and (ω − 1)-hydroxylases that correspond to 100% activity were 0.69 and 0.79 nmol min^{-1} (mg of protein)$^{-1}$, respectively. The points are averages of three or more independent measurements. Standard deviations are indicated. The details of the incubation and assay procedures are provided under Experimental Procedures.

Table I: Binding of Acetylenic Fatty Acid Analogues to Cytochrome P-450[a]

agent	K_s (μM)[b]	A (30 μM)[c]	A (150 μM)
lauric acid	6.8	0.008	0.063
10-SUYS	4.9	0.012	0.017
DMDYA	10.7	0.006	0.012

[a] Procedure given under Experimental Procedures. [b] Binding constants determined from plots of the reciprocal of the absorbance difference vs. the reciprocal of the inhibitor concentration. [c] Absorbance difference at the indicated concentration of the agent.

Binding of Inhibitors to Cytochrome P-450. Type I difference spectra were observed when 10-SUYS and DMDYA were added to hepatic microsomes from uninduced rats. Table I lists the magnitude of the 385–420-nm peak-to-trough absorbance difference for 30 and 150 μM concentrations of the inhibitors and the spectroscopic binding constants (K_s) determined from plots of the reciprocal of the absorbance difference vs. the reciprocal of the inhibitor concentration. The spectroscopic binding constant for 10-SUYS is about half that of DMDYA. This suggests that 10-SUYS perturbs the hemoprotein spin equilibrium more effectively and possibly has a higher affinity for cytochrome P-450 than DMDYA. The spectroscopic binding constant for SDS in rat kidney cortex microsomes, reported as 5.0 μM (Ellin et al., 1973; Ellin & Orrenius, 1975), is quite similar to the value of 4.9 μM obtained here for 10-SUYS.

In Vitro Effects of Inhibitors on Other Cytochrome P-450 Isozymes. The specificity of the inactivators for the fatty acid hydroxylases was examined by measuring their effects on reactions catalyzed by other cytochrome P-450 isozymes. Sensitive methods for the determination of these activities are required because the microsomal content of each assay mixture is quite low after the 10-fold dilution of the preincubation mixture required to minimize competitive inhibitory effects. Fluorescence assays were therefore employed to measure the deethylation of ethoxycoumarin and the demethylation of *N*-methyl-*p*-chloroaniline. As shown in Tables II and III, 10-SUYS and DMDYA have a small NADPH-dependent

Table II: Inhibition of Ethoxycoumarin O-Deethylation Activity Remaining (%)

time (min)[a]	NADPH alone	inhibitor alone		inhibitor + NADPH[b]	
		10-SUYS	DMDYA	10-SUYS	DMDYA
0	100	96	98	102 ± 7	100 ± 2
15	97	88	89	96 ± 5	95 ± 2
30	87	86	85	96 ± 4	93 ± 2

[a] Incubation time prior to dilution and assay of the lauric acid hydroxylase activity. Details of the procedure are given under Experimental Procedures. [b] Corrected for loss due to NADPH and inhibitor alone.

Table III: Inhibition of *N*-Methyl-*p*-chloroaniline N-Demethylation Activity Remaining (%)

time (min)[a]	NADPH alone	inhibitor alone		inhibitor + NADPH[b]	
		10-SUYS	DMDYA	10-SUYS	DMDYA
0	100	100	99	92 ± 1	94 ± 1
15	97	85	82	95 ± 3	96 ± 3
30	98	82	79	90 ± 2	87 ± 6

[a] Incubation time prior to assay of lauric acid hydroxylase activity. Details of the procedure are given under Experimental Procedures. [b] Corrected for loss due to NADPH and inhibitor alone.

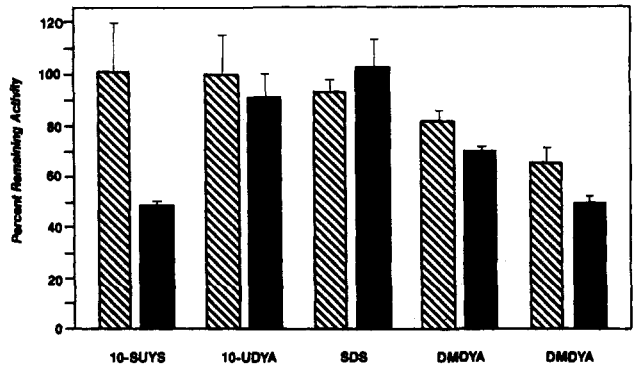

FIGURE 3: Effects of administering 10-SUYS, 10-UDYA, SDS, or DMDYA, all at 50 mg/kg, or a 500 mg/kg dose of DMDYA (bars at far right) on hepatic lauric acid hydroxylation. Rats were decapitated and microsomes prepared 4 h after administration of these agents. The hatched bars represent the ($\omega - 1$)-hydroxylase activity and the filled bars the ω-hydroxylase activity. The activities of the ω- and ($\omega - 1$)-hydroxylases that correspond to 100% activity were 1.34 and 1.79 nmol min^{-1} (mg of protein)$^{-1}$, respectively. The results are averages of four independent experiments. Standard deviations are given. The details of the incubation and assay procedures are provided under Experimental Procedures.

effect on the deethylation of ethoxycoumarin and the demethylation of PCMA. Some activity is lost even if NADPH or the inhibitory agent is withheld. SDS, the saturated homologue of 10-SUYS, has essentially no effect on either enzymatic activity. Correction of the enzyme losses observed in the complete system by subtraction of the losses observed in the absence of NADPH or substrate indicates that only about 5% of each of the two activities is lost in 30 min. These minor losses of activity are not time dependent and therefore probably do not result from mechanism-based inactivation of the isozymes in question. Approximately 50% of the fatty acid ω-hydroxylase activity is lost under identical conditions. DMDYA and 10-SUYS thus appear to be fairly specific inactivators of the fatty acid hydroxylases.

In Vitro Cytochrome P-450 Destruction. Incubation of 10-SUYS or DMDYA with a microsomal suspension in the presence of NADPH resulted in the loss of 15–20% of the total cytochrome P-450 as judged from the decrease in the 450-nm absorbance of the carbon monoxide complex (not shown). No chromophore loss was observed with either inhibitor in the absence of NADPH. SDS decreased the total cytochrome P-450 content by about 3%. The loss caused by 10-SUYS is virtually time independent, but that caused by DMDYA exhibits a small time dependence, the loss being 5% after 1 min but 20% after 15 or 30 min. The strong time dependence observed in the inactivation of the fatty acid hydroxylases suggests that the chromophore loss represents a process other than that responsible for inactivation of the fatty acid hydroxylases.

In Vivo Inactivation of Lauric Acid Hydroxylases. The ability of DMDYA and 10-SUYS to inactivate the lauric acid hydroxylases in vivo was examined once their intrinsic activity and specificity were established in vitro. The lauric acid hydroxylase activities of hepatic microsomes from rats pretreated with 50 mg/kg DMDYA or 10-SUYS are shown in Figure 3. The control (100%) activities are those obtained with microsomes from rats that only received dimethyl sulfoxide, the injection vehicle. Administration of 10-SUYS decreased the hepatic ω-hydroxylase activity by 50% but had little effect on the ($\omega - 1$)-hydroxylase activity 4 h after the agent was administered. Rats injected with a 10-fold higher dose (500 mg/kg) of 10-SUYS died within 1 h. SDS, the saturated homologue, did not reduce the hydroxylase activities when administered in a 50 mg/kg dose but was apparently as toxic as 10-SUYS when the dose was increased to 500 mg/kg.

DMDYA, the 2,2-dimethyl derivative, is also effective in vivo but, in contrast to 10-SUYS, has a measurable effect on both the ω- and ($\omega - 1$)-hydroxylase activities. This is consistent with the lower in vitro specificity of DMDYA than of 10-SUYS for the ω vs. $\omega - 1$ activity. A 50 mg/kg dose of DMDYA decreases the ω-hydroxylase activity by approximately 30% and the $\omega - 1$ activity by 20%. DMDYA, unlike 10-SUYS or 10-UDYA, is not observably toxic when administered at a dose of 500 mg/kg. Both hydroxylase activities are decreased a further 20% when a 500 mg/kg dose of DMDYA is employed (Figure 3). A dose of 1 g/kg was toxic, as was a similar dose of lauric acid itself.

Discussion

The introduction of a terminal triple bond into a fatty acid, as reported earlier (Reich & Ortiz de Montellano, 1984), transforms it into a mechanism-based inactivator of the cytochrome P-450 isozymes that hydroxylate lauric acid. These acetylenic fatty acids are active in vitro but not in vivo. Modification of the carboxylate terminus so that degradation of the acetylenic agents by β-oxidation is prevented, however, results in agents that irreversibly inhibit lauric acid hydroxylation in vivo. Replacement of the carboxylic acid group by a sulfate, or introduction of two methyl groups vicinal to the carboxylic acid, yields agents with in vitro activities similar to that of 10-undecynoic acid itself (Figures 1 and 2). This loss of activity, as expected for a mechanism-based process, is both NADPH and time dependent, although the time dependence is superimposed on a relatively minor, time-independent process. The time-independent component may include (1) competitive inhibition of the hydroxylation reaction despite the 10-fold dilution of the inhibitor concentration that precedes the hydroxylation assay, (2) slow inactivation of the hydroxylase during the assay incubation itself, and, possibly, (3) inactivation caused by the detergent action of these agents. Perturbation of the membrane by the detergent action of high concentrations of free fatty acids has been reported to inhibit mixed-function oxidases (Danis et al., 1985).

DMDYA appears to be less specific in vitro than 10-SUYS or 10-UDYA in that it discriminates less between the ω- and ($\omega - 1$)-hydroxylases. The bulky dimethyl substitution in DMDYA may alter its relative binding affinity for the different hydroxylase isozymes, particularly if the carboxylic acid moiety serves to anchor fatty acids in the active sites of these enzymes. Evidence was obtained in the previous study for the existence of at least three hepatic fatty acid hydroxylases: one ω-hydroxylase, presumably that specifically induced by clofibrate (Orton & Parker, 1982), and two ($\omega - 1$)-hydroxylases, at least one of which appears to be induced by phenobarbital (Bjorkhem & Danielsson, 1970; Okita & Masters, 1980). More classical inhibitors, including CO, SKF-525A, and metyrapone, have also provided evidence for distinct ω- and ($\omega - 1$)-hydroxylases (Okita & Masters, 1980). The differences in the binding of 10-SUYS and DMDYA to microsomal cytochrome P-450 suggested by the spectroscopic data do not correlate with the differences in substrate specificity (Table I). 10-SUYS has a lower spectroscopic binding constant than DMDYA but causes a larger perturbation of the chromophore in the difference spectrum. Difference spectra, however, are not a reliable measure of the binding of substrates to cytochrome P-450 because they only reflect changes in the equilibrium ligation state of the prosthetic heme group in the total microsomal cytochrome P-450 pool. Interaction of the

fatty acid analogues with isozymes other than those involved in lauric acid hydroxylation, suggested by the fact that lauric acid interferes with the type I spectral changes engendered by hexobarbital and ethylmorphine (Orrenius & Thor, 1969; Kupfer & Orrenius, 1970; Orrenius et al., 1970), in part explains the absence of a strict correlation between the spectroscopic binding constants and inactivation specificities.

DMDYA and 10-SUYS inactivate the lauric acid hydroxylases without similarly inactivating other cytochrome P-450 isozymes, as indicated by the absence of time-dependent inhibition of ethoxycoumarin O-deethylation and *N*-methyl-*p*-chloroaniline demethylation (Tables II and III). The small, time-independent inhibition of these two activities and the parallel, essentially time-independent decrease of the P-450 chromophore presumably stem from detergent-mediated changes in the microsomal membrane or other mechanism-independent effects. The possibility also exists that the Soret absorbance of the P-450 enzymes is decreased by the agents in question. Sodium dodecyl sulfate (SDS), for example, has been reported to alter the Soret band of cytochrome *c* (Burkhard & Stolzenberg, 1972; Takeda et al., 1985).

The absence of significant, time-dependent chromophore loss, in view of the strict time dependence of the decrease in lauric acid hydroxylation, indicates that catalytic inactivation of the fatty acid hydroxylases is not tied to destruction of a significant fraction of the total P-450 chromophore. One interpretation of this observation (Reich & Ortiz de Montellano, 1984) is that fatty acid hydroxylation is catalyzed by so small a fraction of the total cytochrome P-450 that its time-dependent loss is not detected experimentally. This interpretation is not consistent, however, with recent immunoquantitation studies, which suggest that the lauric acid hydroxylases amount to something on the order of 22% of the total microsomal cytochrome P-450 in uninduced rat liver microsomes (Bains et al., 1985). This dichotomy suggests that inactivation of the hydroxylases by acetylenic fatty acids, in contrast to the inactivation of other isozymes by a variety of acetylenes (Ortiz de Montellano, 1985), occurs without destruction of the prosthetic heme group. The observation that 1-ethynylpyrene decreases benzo[*a*]pyrene hydroxylase activity to a greater extent than it does the spectroscopically measured P-450 content of the microsomal membrane (Gan et al., 1984) is, as noted by the authors, also consistent with such a mechanism. Inactivation of the lauric acid hydroxylases, in analogy with the well-documented inactivation of cytochrome P-450 by chloramphenicol (Halpert & Neal, 1980; Halpert, 1981), may reflect alkylation of the protein by a catalytically activated species. Our earlier demonstration that terminal acetylenes are oxidized to ketenes by cytochrome P-450 (Ortiz de Montellano, 1985) provides an attractive candidate for the reactive species required by such a mechanism.

DMDYA and 10-SUYS, as already noted, are unique in that they inactivate the lauric acid hydroxylases when administered intraperitoneally to uninduced rats (Figure 3). The sulfate ester primarily inhibits the ω-hydroxylase, but the *gem*-dimethyl fatty acid indiscriminately inhibits both activities. These results correlated well with the in vitro finding that both agents inactivate ω-hydroxylase(s) more efficiently than ($\omega - 1$)-hydroxylase(s). Higher doses of DMDYA than of 10-SUYS are required to achieve comparable inhibition of the ω-hydroxylase, but DMDYA is less toxic than 10-SUYS and thus can be administered in the required amounts. The higher toxicity of the sulfate ester, presumably due to its detergent properties, is consistent with the fact that the LD_{50} in rats of intravenously administered SDS is approximately 118 mg/kg (Cascorbi et al., 1963).

The development of in vivo suicide substrates that are specific for the isozymes of cytochrome P-450 involved in fatty acid hydroxylation makes available new and powerful probes of their physiological role. It also confirms the feasibility of prolonging the biological half-life of therapeutic agents related to arachidonic acids and other fatty acids by introducing functionalities that specifically inactivate enzymes responsible for their rapid metabolic degradation.

Registry No. 10-SUYS, 103201-18-9; DMDYA, 103201-19-0; 10-UDYA, 2777-65-3; $HO(CH_2)_9C{\equiv}CH$, 2774-84-7; $HO(CH_2)_2C{\equiv}C(CH_2)_5Me$, 51721-39-2; $TsO(CH_2)_2C{\equiv}C(CH_2)_5Me$, 51721-40-5; $MeCH_2C{\equiv}C(CH_2)_2CHBrPr$, 66249-75-0; *i*-$PrCO_2H$, 79-31-2; $HO_2CCMe_2(CH_2)_2C{\equiv}C(CH_2)_5Me$, 103201-20-3; *p*-$MeNHC_6H_4Cl$, 932-96-7; laurate ω-hydroxylase, 78783-57-0; lauric acid, 143-07-7; cytochrome P-450, 9035-51-2; ethoxycoumarin, 31005-02-4; laurate ($\omega - 1$)-hydroxylase, 78783-56-9.

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Inactivation of *Escherichia coli* Glycerol Kinase by 5,5′-Dithiobis(2-nitrobenzoic acid) and *N*-Ethylmaleimide: Evidence for Nucleotide Regulatory Binding Sites†

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ABSTRACT: Glycerol kinase (EC 2.7.1.30, ATP:glycerol 3-phosphotransferase) from *Escherichia coli* is inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and by *N*-ethylmaleimide (NEM) in 0.1 M triethanolamine at pH 7 and 25 °C. The inactivation by DTNB is reversed by dithiothreitol. In the cases of both reagents, the kinetics of activity loss are pseudo first order. The dependencies of the rate constants on reagent concentration show that while the inactivation by NEM obeys second-order kinetics (k_2^{app} = 0.3 M^{-1} s^{-1}), DTNB binds to the enzyme prior to the inactivation reaction; i.e., the pseudo-first-order rate constant shows a hyperbolic dependence on DTNB concentration. Complete inactivation by each reagent apparently involves the modification of two sulfhydryl groups per enzyme subunit. However, analysis of the kinetics of DTNB modification, as measured by the release of 2-nitro-5-thiobenzoate, shows that the inactivation is due to the modification of one sulfhydryl group per subunit, while two other groups are modified 6 and 15 times more slowly. The enzyme is protected from inactivation by the ligands glycerol, propane-1,2-diol, ATP, ADP, AMP, and cAMP but not by Mg^{2+}, fructose 1,6-bisphosphate, or propane-1,3-diol. The protection afforded by ATP or AMP is not dependent on Mg^{2+}. The kinetics of DTNB modification are different in the presence of glycerol or ATP, despite the observation that the degree of protection afforded by both of these ligands is the same. Initial velocity studies show that the kinetics with respect to ATP are complex, in agreement with previous observations [Thorner, J. W., & Paulus, H. (1973) *J. Biol. Chem. 248*, 3922–3932]. AMP is an inhibitor and displays complex inhibition behavior with respect to ATP. These results suggest the presence of at least one regulatory binding site for adenine nucleotides on the enzyme.

Glycerol kinase (EC 2.7.1.30, ATP:glycerol 3-phosphotransferase) from *Escherichia coli* catalyzes the MgATP-dependent phosphorylation of glycerol to yield *sn*-glycerol 3-phosphate (Lin, 1976). It is an inducible catabolic enzyme whose metabolic role is to mobilize glycerol as a carbon source. The activity of glycerol kinase is rate-limiting in the metabolism of glycerol by *E. coli* (Zwaig et al., 1970). The reaction proceeds with net inversion of the configuration at the phosphorus, and it is postulated that this may require a general base to deprotonate the attacking glycerol hydroxyl group and a general acid to assist in the departure of ADP (Blättler & Knowles, 1979). The enzyme is subject to feedback inhibition by Fru-1,6-P_2[1] (Zwaig & Lin, 1966). Little is known, however, about roles of specific amino acids in the catalytic and regulatory properties of this enzyme. It has been reported that treatment of the enzyme with different sulfhydryl reagents results in different extents of inactivation at the end of an incubation period (Thorner & Paulus, 1973a). In the absence of ligands, it was reported that DTNB modifies three sulfhydryl groups per subunit, while only 0.5 group is modified in the presence of glycerol (Thorner & Paulus, 1973b). It was postulated that this change in the number of DTNB–titrable sulfhydryl groups upon glycerol binding reflects a substantial

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Fru-1,6-P_2, fructose 1,6-bisphosphate; Gdn-HCl, guanidine hydrochloride; MMTS, methylmethanethiosulfonate; NEM, *N*-ethylmaleimide; NTCB, 2-nitro-5-(thiocyano)benzoic acid; SDS, sodium dodecyl sulfate; TNB^-, 2-nitro-5-thiobenzoate.

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