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Exploring the Hydroxylation−Dehydrogenation Connection: Novel Catalytic Activity of Castor Stearoyl-ACP Δ^9 Desaturase

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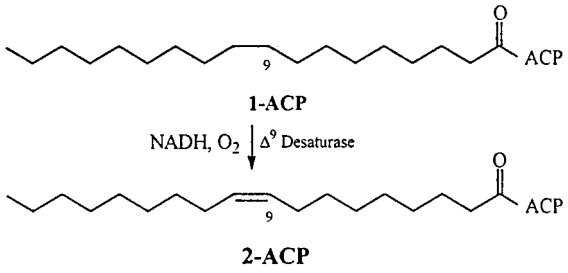
Abstract: The novel product profile obtained by incubating chiral fluorinated substrate analogues with castor stearoyl-ACP Δ^9 desaturase has been rationalized through a series of labeling studies. It was found that the introduction of the *Z*-double bond between C-9 and C-10 of the parent substrate occurs with pro-*R* enantioselectivity—a result that accounts for the observed stereochemistry of oxidation products derived from (9*R*)- and (9*S*)-9-fluorostearoyl-ACP. Oxidation of (9*R*)-9-fluorostearoyl-ACP occurs via at least two rapidly interchanging substrate conformations in the active site as detected by reaction pathway branching induced by deuteration at C-10 and C-11. Hydroxylation and desaturation of this substrate share the same site of initial oxidative attack.

Introduction

Non-heme diiron-containing enzymes have emerged as important catalysts for a wide variety of enzymatic processes.[1a−d] A prominent member of this family of proteins is the soluble, plant Δ^9 desaturase,[2] which carries out the chemo-, regio-, and stereoselective dehydrogenation (desaturation) of stearoyl-acyl carrier protein (ACP) **1**-ACP (Scheme 1). The product of this reaction, **2**-ACP is a key intermediate in the biosynthesis of cellular lipids.[3] Stearoyl-ACP Δ^9 desaturase is also significant because it is the only desaturase that has been purified in sufficient quantities to permit detailed structural characterization. Recent X-ray crystallographic studies[4] of the castor enzyme have determined that the catalytic diiron center is located midway along a narrow, hydrophobic binding pocket. The shape of the active site favors a gauche conformation at C9−C10 of the stearoyl substrate, thus facilitating formation of an olefinic product with *Z*-stereochemistry.

Considerable progress has been achieved in understanding the details of the overall catalytic cycle of stearoyl-ACP Δ^9 desaturase.[5a,b] A unique feature of this enzyme is the substrate-enhanced reactivity of its diiron core with O_2, which leads to the formation of a $(FeIII)_2$−peroxo intermediate. It is proposed that the high-valent iron−oxidant required for the desaturation reaction is similar to compound Q—an Fe(IV) Fe(IV) species involved in methane monooxygenase-mediated attack of unactivated C−H bonds.[5c]

Scheme 1



Recently, mechanistic probes originally developed for membrane-bound fatty acyl desaturases[6a−c] have been applied to the study of stearoyl-ACP Δ^9 desaturase. We have shown[7] that the C−H cleavage steps at C-9 and C-10 are not subject to an intermolecular primary deuterium kinetic isotope effect (KIE $\sim$ 1)—an observation that is consistent with a kinetic scheme

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dominated by other enzymatic events such as substrate binding or product release.[5b] We also discovered that the putative diiron oxidant acts as an oxygenase with thia analogues. Incubation of enzyme with 10-thiastearoyl-ACP leads to exclusive sulfoxide formation, while a mixture of 9-sulfoxide and an as yet unidentified product was obtained from the 9-thia analogue.[7] In a more recent development, it was demonstrated for the first time that stearoyl-ACP Δ^9 desaturase can act as an hydroxylase when presented with 9-fluorinated stearates.[8] This finding is particularly important since it points to a fundamental mechanistic link[6a] between dehydrogenation and the hydroxylation chemistry catalyzed by the structurally related enzyme, methane monooxygenase (MMO) alluded to above.[9a] Interestingly, the latter enzyme was recently shown to give a mixture of hydroxylated and desaturated products from benzylic substrates.[9b]

Revealing the latent hydroxylase capability of a structurally characterized desaturase through the use of a substrate analogue is intriguing and clearly deserves closer scrutiny.[10] In this paper, we document the results of deuterium-labeling experiments which demonstrate that the stereochemistry of the parent dehydrogenation reaction correlates with that of stearoyl-ACP desaturase-mediated oxidation of 9-fluorinated substrates. In addition, we show that hydroxylation and desaturation are initiated at the same carbon through the observation of isotopically induced reaction pathway branching.

Materials and Methods

General Methods. Except where noted otherwise, ^{1}H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, on a Brüker AMX 400 spectrometer with the use of dilute $CDCl_3$ solutions. Chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane. *J* values are reported in hertz (Hz). ^{1}H -Decoupled ^{19}F NMR (376.5 MHz) spectra were recorded on a Brüker AM 400 spectrometer with a dedicated 5-mm ^{19}F/^{1}H probe and ^{19}F-specific amplifier. Under these conditions, nanomolar quantities of enzymatic products dissolved in $CDCl_3$ could be analyzed. All ^{19}F NMR spectra were referenced to external trichlorofluoromethane at 0.00 ppm. Deuterium isotope effects on ^{13}C and ^{19}F NMR shifts were estimated by running spectra of mixtures (1:2) of labeled and unlabeled material.

Mass spectra were obtained by GC/MS using a HP5973 mass spectrometer in EI^+ mode (70 eV), coupled to a HP6890 GC equipped with a SP2340 capillary column (60 m × 0.25 mm), temperature programmed from 100 to 160 °C at 25 °C/min and 160 to 240 °C at 10 °C/min. Isotopic content of analytes was estimated by integrating the intensities of selected individual ions through the use of HP-ChemStation software and correcting for natural isotopic abundances (scan rate, 2 s^{-1}). Isotopic ratios for the stereochemical studies were determined using the corrected intensities for the ions: *m*/*z* 298 (299 for d_1 analogue), M^+ (**1**); *m*/*z* 325 (326 for d_1 analogue), $(M - 15)^+$ (**2**-1-OTMS).

Flash chromatography with silica gel (230−400 mesh) was used to purify all intermediates and substrates. Visualization of UV-inactive materials on TLC was accomplished by a combination of water spray and I_2 vapor.

All reagents and starting materials for organic synthesis were purchased from Sigma-Aldrich and used without purification. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled from sodium benzophenone ketyl. All air- and moisture-sensitive reactions were performed under N_2. Organic extracts were typically shaken with saturated NaCl and dried over Na_2SO_4, and solvents were evaporated in vacuo on a Büchi RE 111 Rotavapor. [1,1-$^2H_2$]-1-Bromononane was prepared by reduction[11] of nonanoic acid with $LiAlD_4$ followed by treatment with HBr/H_2SO_4.[12] [2,2-$^2H_2$]-1-Bromononane was prepared by α-exchange of methyl nonanoate with Na in MeOD[13] followed by reduction with $LiAlH_4$[11] and treatment with HBr/H_2SO_4.[12] *Dimorphetheca sinuata* seeds were obtained from Butchart Gardens, Victoria, BC, Canada. *Pseudomonas* cultures were obtained from the Center for Catalysis and Bioprocessing, University of Iowa, Iowa City, and cultured as previously described.[14]

All buffers and salts, NADPH, bovine serum albumin (BSA), and other biochemicals were purchased from Sigma-Aldrich. Protein concentrations were measured by the method of Bradford[15] with the use of BSA as standard protein. The purification of castor stearoyl-ACP Δ^9 desaturase, required cofactors and the synthesis of substrate ACP derivatives has been previously described.[16]

Synthesis of Substrates. Racemic 9-Fluorostearates. All racemic 9-fluorostearates were prepared by a Grignard addition reaction between the appropriate alkenal and a bromoalkane of the appropriate chain length followed by a sequence of reactions including fluorination, oxidative cleavage, and methylation as previously described.[17] The relevant analytical data are given below:

Methyl (*R,S*)-9-Fluorostearate. The analytical data for this compound have been reported previously[17] except for ^{13}C NMR δ 173.28 (*C*═O), 95.54 (C-9, J_{CF} 165.6), 51.44 (O*C*H$_3$), 35.21 (C-10, J_{CCF} 20.7), 35.16 (C-8, J_{CCF} 20.8), 34.09 (C-2), 31.92 (C-16), 29.57 (C-13), 29.55 (C-12), 29.33, 29.17, 29.07 (C-4 to C-6, C-14 to C-15), 25.17 (C-11, J_{CCCF} 4.4), 25.09 (C-7, J_{CCCF} 4.4), 24.94 (C-3), 22.70 (C-17), 14.12 (C-18).

Methyl [10,10-$^2H_2$]-(*R,S*)-9-Fluorostearate: from decen-9-en-1-al and [1,1-$^2H_2$]-1-bromononane. The spectral data of this compound were similar to those described for the parent unlabeled compound[17] except for the following: ^{1}H NMR δ 1.63 (m, 2H, C(10)-*H*$_2$, absent), 4.44 (ddd, 1 H, J_{HF} 49.2, J_{HH} 3.6, 7.9, C*H*F); ^{13}C NMR δ 94.47 (C-9, upfield β-isotope shift (0.07 ppm)), 35.21 (C-10, absent), 35.12 (C-8, upfield γ-isotope shift (0.04 ppm)), 29.51 (C-12, upfield γ-isotope shift (0.04 ppm)), 25.17 (C-11, upfield β-isotope shift (0.20 ppm)); ^{19}F NMR δ −180.60 (broadened, upfield β-isotope shift (0.41 ppm), unlabeled material: δ −180.19); MS (EI, 70 eV) *m*/*z* 298 (M^+ − HF), 266 (M^+ − HF, CH_3OH).

Methyl [11,11-$^2H_2$]-(*R,S*)-9-Fluorostearate: from decen-9-en-1-al and [2,2-$^2H_2$]-2-bromononane. The spectral data of this compound were similar to that described for the parent unlabeled compound[17] except for the following: ^{13}C NMR δ 35.02 (C-10, upfield β-isotope shift (0.19 ppm)), 29.53 (C-13, upfield β-isotope shift (0.04 ppm)), 29.36 (C-12, upfield β-isotope shift (0.19 ppm)), 25.17 (C-11, absent); ^{19}F NMR δ −180.20 (upfield γ-isotope shift (0.01 ppm), unlabeled material: δ −180.19); MS (EI, 70 eV) *m*/*z* 298 (M^+ − HF), 266 (M^+ − HF, CH_3OH).

Chiral Deuterated Substrates. All chiral deuterated substrates were prepared from samples of methyl (*R*)- or (*S*)-9-hydroxystearate and methyl (*R*)- or (*S*)-10-hydroxystearate.

Methyl (*R*)-9-Hydroxystearate. *D. sinuata* seeds (6 g) were ground in a mortar filled with liquid N_2. The resultant coarse powder was stirred

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under N_2 in 50 mL of $CHCl_3$/MeOH (2:1 v/v) for 24 h after which the slurry was filtered by suction and rinsed with $CHCl_3$/MeOH (2:1 v/v, 50 mL). The filtrate was acidified with 0.1 M HCl/0.1M NaCl and extracted with $CHCl_3$ (2 × 30 mL). The combined organics were dried and evaporated to give a yellow oil. This residue was treated with 20 mL of 1 M $NaOCH_3$ at 0 °C for 2 h. Addition of 1 N HCl (15 mL), followed by extraction with hexane (3 × 10 mL) gave crude methyl dimorphecolate (10(*E*),12(*E*)-(*R*)-9-hydroxyoctadecadienoate) as a clear viscous oil. Hydrogenation of this material over Pt (Adam's catalyst) in 95% ethanol and purification by flash chromatography (15% EtOAc/hexanes) gave the title compound as a white solid (225 mg): mp 50−52 °C (lit.[18]mp 49−51 °C); ^{1}H NMR δ 0.89 (t, *J* 6.7, 3H, CH_2CH_3), 1.27 (br s, 24H, methylenes), 1.58 (m, 4H, CH_2−CHOHCH_2), 2.30 (t, *J* 7.4, 2H, CH_2COOMe), 3.55 (p, 1H, C*H*OH), 3.67 (s, 3H, OCH_3); ^{13}C NMR δ 174.56 (C═O), 72.07 (C-9), 51.69 (OCH_3), 37.74 (C-8 or C-10), 37.65 (C-8 or C-10), 34.29 (C-2), 32.15 (C-16), 29.99, 29.91, 29.84, 29.73, 29.59, 29.31 (C-4 to C-6, C-12 to C-15), 25.93 (C-7 or C-11), 25.82 (C-7 or C-11), 25.14 (C-3), 22.93 (C-17), 14.36 (C-18); MS (TMS derivative, EI, 70 eV) *m*/*z* 259 ($TMSOC_8H_{15}COOMe$), 229 ($C_{10}H_{20}OTMS$); >98% ee (^{1}H NMR of (*S*)-1-(1-naphthyl)ethylcarbamate[19] or (*S*)-(+)-*O*-acetylmandelate derivative[14]).

Methyl (*R*)-10-Hydroxystearate. Oleic acid (1.0 g, 3.5 mmol) was incubated for 48 h with growing cultures (500 mL) of *Pseudomonas* NRLL 3266 in the presence of tergitol (1% w/v) according to the method of Rosazza.[14] The culture medium was adjusted to pH 2 with 50% HCl and extracted with ethyl acetate/propanol (9:1 v/v, 1 L). The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and concentrated in vacuo at 40 °C. The brown residue was methylated (BF_3/MeOH) and chromatographed on flash silica gel (15% EtOAc/hexanes) to give 105 mg of the title compound as a colorless solid: mp 53−55 °C (lit.[20a] mp 56.5−57.0). The spectral data were similar to those obtained for methyl (*R*)-9-hydroxystearate except for the following: ^{13}C NMR δ 174.33 (C═O), 71.88 (C-10), 51.46 (OCH_3), 37.56 (C-9 or C-11), 37.50 (C-9 or C-11), 34.10 (C-2), 31.94 (C-16), 29.80, 29.68, 29.46, 29.35, 29.25, 29.16 (C-4 to C-7, C-13 to C-15), 25.73 (C-8 or C-12), 25.68 (C-8 or C-12), 24.97 (C-3), 22.72 (C-17), 14.36 (C-18); MS (TMS derivative, EI, 70 eV) *m*/*z* 273 ($TMSOC_9H_{17}$-COOMe), 215 (C_9H_{18}-OTMS); % ee > 98% (^{1}H NMR of the corresponding (*S*)-(+)-*O*-acetylmandelate derivative).[14]

Methyl (*S*)-9-Hydroxystearate. Diethyl diazodicarboxylate (DEAD) (345 mg, 1.98 mmol) in 1 mL of dry THF was added to a solution of methyl (*R*)-9-hydroxystearate (205 mg, 0.65 mmol), benzoic acid (240 mg, 1.97 mmol), and triphenylphosphine (670 mg, 2.56 mmol) in 5 mL of anhydrous THF over 5 min at room temperature. The reaction was monitored by TLC (silica gel, 20% EtOAC/hexane), and additional DEAD was added as required (~200 mg) until starting material was consumed (2 h). The reaction mixture was concentrated in vacuo and flash chromatographed (7.5% EtOAc/hexane) to give the corresponding benzoate ester as an oil (125 mg, 46% estimated yield): ^{1}H NMR δ 0.87 (t, *J* 6.6, 3H, CH_2CH_3), 1.29 (br s, 24 H, methylenes), 1.59 (m, 4H, CH_2CH(OC(O)Ph)CH_2), 2.28 (t, *J* 7.4, 2H, CH_2COOMe), 5.11 (p, 1H, C*H*O(CO)Ph) 3.66 (s, 3H, OCH_3), 7.50 (m, 3H, phenyl), 8.04 (m, 2H, phenyl). Hydrolysis of this intermediate followed by methylation (BF_3/MeOH) and flash chromatography (15% EtOAc/Hexanes) yielded the title compound as a white solid (80 mg, 39% overall). The spectral data of this material were identical to those reported for the *R*-enantiomer. >98% ee (^{1}H NMR of (*S*)-(+)-*O*-acetylmandelate derivative[14]).

Methyl (*S*)-10-hydroxystearate was prepared from methyl (*R*)-10-hydroxystearate as described above. The title compound was obtained as a white solid (85 mg, 76%). The spectral data of this material were identical to those reported for the *R*-enantiomer. 50% ee (^{1}H NMR of the corresponding (*S*)-(+)-*O*-acetylmandelate derivative). The low % ee of this material was traced to the use of one batch of microbially produced (*R*)-10-hydroxystearate of exceptionally low stereochemical purity.

Methyl (9*S*)-[9-$^2H_1$]-Stearate ((9*S*)-[9-$^2H_1$]-1). A solution of methyl (*R*)-9-hydroxystearate (106 mg, 0.34 mmol) in dry pyridine was treated with TsCl (133 mg, 0.70 mmol) at 0 °C. The solution was stirred for 1 h at 0 °C and left at 4 °C until complete as determined by TLC (silica gel, 20% EtOAc/hexanes) (3−4 days). The reaction was quenched with H_2O (10 mL) and extracted with Et_2O (3 × 15 mL). The combined organics were washed with 1 N HCl (3 × 20 mL) and saturated NaCl (1 × 25 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification by flash chromatography (10% EtOAc/hexanes) gave the tosylate as a viscous oil: 92 mg, 58% yield; ^{1}H NMR δ 0.88 (t, *J* 6.6, 3H, CH_2CH_3), 1.20 (br s, 24 H, methylenes), 1.55 (m, 4H, CH_2CH(OTs)CH_2), 2.29 (t, *J* 7.4, 2H, CH_2COOMe), 3.67 (s, 3H, OCH_3), 4.53 (p, 1H, C*H*OTs), 7.31 (d, 2H, phenyl), 7.79 (d, 2H, phenyl). This intermediate (92 mg, 0.20 mmol) was dissolved in dry THF (2 mL), and a solution of 1.0 M lithium triethyl borodeuteride (1.5 mL) in THF was added at 0 °C over 10 min and stirred for 1 h at room temperature. The reaction was quenched with 1 N HCl (3 mL) and H_2O (3 mL) and extracted with Et_2O (3 × 10 mL). The combined ethereal layers were dried and concentrated in vacuo to give the crude deuterated alcohol (49 mg) as a low melting solid: ^{1}H NMR δ 0.88 (t, *J* 6.8, 3H, CH_2CH_3), 1.26 (br s, 29 H, methylenes), 1.51 (br t, 2H, CH_2CD_2OH). This intermediate was oxidized with 1.5 mL of Jones reagent (4 g of CrO_3, 15 mL of 3 M H_2SO_4, 2.5 mL of H_2O) in acetone (5 mL) for 4 h at room temperature. Reduction of excess oxidant with SO_2 gas and extraction with Et_2O gave the crude carboxylic acid. This material was subsequently methylated by BF_3/MeOH and purified by flash chromatography (1% EtOAc/hexanes) to give the title compound as a white solid (31 mg, 30% overall yield). The spectral data of the title compound were identical to that of methyl stearate except for the following: MS (EI, 70 eV) *m*/*z* 299 (M^+), 268 ($M^+ - CH_3O$).

Methyl (9*R*)-[9-$^2H_1$]-stearate ((9*R*)-[9-$^2H_1$]-1): from methyl (*S*)-9-hydroxystearate. This was obtained as a white solid (24 mg, 32% overall yield). The spectral data of the title compound were identical to that of corresponding *S*-enantiomer (see above).

Methyl (10*S*)-[10-$^2H_1$]-stearate ((10*S*)-[10-$^2H_1$]-1): from methyl (*R*)-10-hydroxystearate. This was obtained as a white solid (14 mg, 23% overall yield). The spectral data of the title compound were identical to those reported for methyl stearate except for the following: (EI, 70 eV) *m*/*z* 299 (M^+), 268 ($M^+ - CH_3O$).

Methyl (10*R*)-[10-$^2H_1$]-stearate ((10*R*)-[10-$^2H_1$]-1): from methyl (*S*)-10-hydroxystearate. This was obtained as a white solid (32 mg, 40% overall yield). The spectral data of the title compound were identical to those of corresponding *S*-enantiomer (see above).

Desaturase Assay. Reactions of acyl-ACP derivatives with Δ^9 desaturase were carried out at room temperature. Each reaction mixture consisted of Δ^9 desaturase (0.6 nmol), ferredoxin (1.0 nmol), NADPH: ferredoxin oxidoreductase (1.8 nmol), and acyl-ACP (8.1 nmol) in a total volume of 200 μL of buffer. The reaction was initiated by the addition of NADPH (1.0 mg, 1.2 μmol) in buffer (50 μL) and allowed to continue for 30 min. The reaction was terminated with the addition of THF (100 μL), and the thioester linkage of ACP derivatives was reduced to the corresponding primary alcohol with $NaBH_4$ at 37 °C for 15 min. The residue was diluted with H_2O (1 mL) and extracted with hexane (2 × 2 mL). (Similar product profiles were obtained when CH_2Cl_2 was used as the extracting solvent.) The phases were separated by centrifugation, and the organic layers were collected. The combined organics were evaporated under a steady stream of N_2 and derivatized with BSTFA/TMCS (50 μL) at 37 °C for 1 h. Excess reagent was

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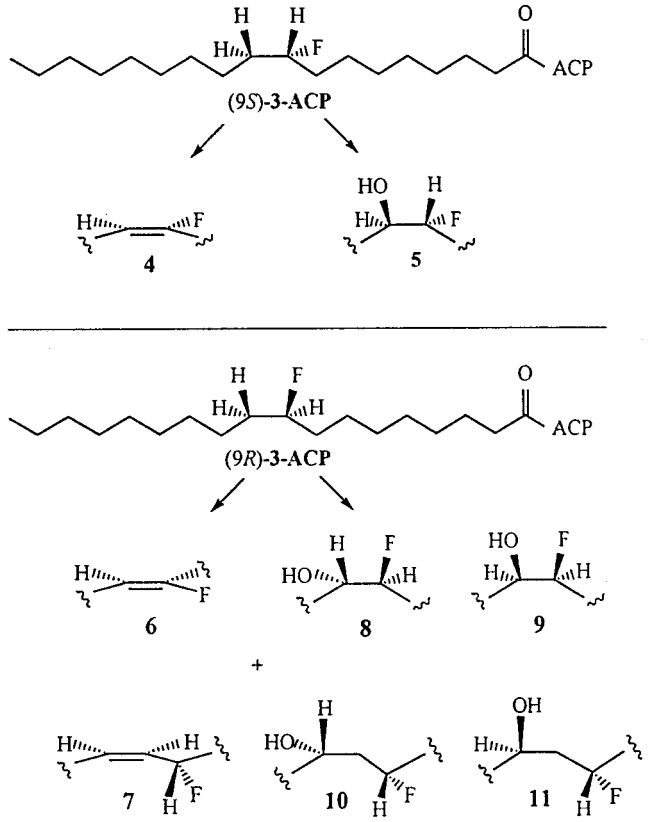
Scheme 2

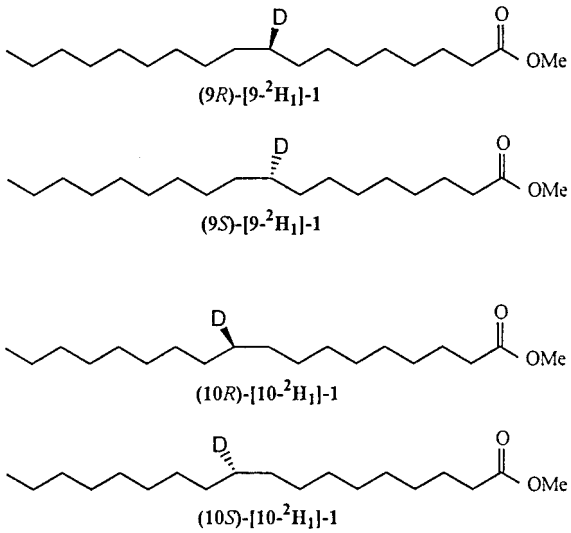


evaporated under a stream of N_2, and the residue was diluted with 50 μL of hexane for analysis by GC/MS.

Results and Discussion

Stereochemical Studies. We have previously shown[8] that separate incubation of (9*S*)-**3**-ACP and (9*R*)-**3**-ACP with stearoyl-ACP Δ^9 desaturase generates two different sets of oxidation products (Scheme 2).[21] A *threo*-9,10-fluorohydrin **5** along with a fluoroolefin of "normal" stereochemistry **4** was formed from (9*S*)-**3**-ACP. In contrast, a 1:2 mixture of *threo*- and *erythro*-9,10 fluorohydrins **8** and **9**[22] as well as traces of diastereomeric 9,11 fluoro alcohols **10** and **11**[23] accompanied two novel olefinic products **6** and **7** upon incubation of (9*R*)-**3**-ACP. Products attributable to hydroxylation at C-9 were not detected (detection limit, 0.1% of total products). Oxygenated products accounted for ~10% of the total product mixture in both incubations under conditions of partial and complete substrate conversion. The recovery of total products and remaining products was estimated to be ~70−90% of theoretical. The structural assignments of enzymatic products are based on a combination of ^{1}H-decoupled ^{19}F NMR and GC/MS analysis with the help of reference standards and deuterium-labeled substrates.[8]

To rationalize these results, we felt that it would be instructive to elucidate the enantioselectivity of the parent Δ^9 desaturation reaction.[20a−c] Our stereochemical study was accomplished by incubating ACP derivatives of four stearates that were stereospecifically deuterated at the C-9 and C-10 positions: (9*R*)- and (9*S*)-[9-^{2}H$_1$]-**1** and (10*R*)- and (10*S*)-[10-^{2}H$_1$]-**1**.



These compounds were prepared in straightforward fashion from the corresponding chiral hydroxystearates by a tosylation/superdeuteride displacement sequence as described in Materials and Methods. The source of (*R*)- and (*S*)-9-hydroxystearic acid (>98% ee) was identical to that employed for the chiral 9-fluorostearate syntheses.[8] (*R*)-10-Hydroxystearic acid was obtained by the known stereo- and regioselective hydration of oleic acid using growing cultures of *Pseudomonas* NRRL 3266.[14] The required (*S*)-10-hydroxystearic acid was prepared from the corresponding *R*-enantiomer by the same inversion sequence as was utilized for the 9-hydroxy series.[8] The outcome of the four incubations is shown in Table 1 and demonstrates clearly that Δ^9 desaturation catalyzed by stearoyl ACP desaturase involves syn removal of the pro-*R* hydrogens at C-9 and C-10 by the putative compound Q-type oxidant: Essentially complete loss of deuterium was observed upon desaturation of *R*-deuterated substrates while olefin produced from *S*-labeled stearates retained deuterium label (Scheme 3).

These findings account for the product profile obtained from the two 9-fluorostearoyl ACP derivatives (Scheme 2). We note that the desaturation of (9*S*)-**3**-ACP generates the fluoroolefinic product **4** with the "normal" configuration since the enzymic oxidant can carry out syn removal of the pro-*R* hydrogen at C-10 and the corresponding hydrogen at C-9 of substrate residing in a gauche conformation. Importantly, this picture is also consistent with the production of *threo*-fluorohydrin **5** from (9*S*)-**3**-ACP via an apparent MMO-type hydroxylation process which would be expected to proceed by attack at the pro-*R*

(21) A series of control experiments[8] have confirmed that the fluorohydrin products were not derived by hydration of fluoroolefin during the workup procedure. We have also demonstrated high (75−85%) incorporation of ^{18}O into the fluorohydrin products when incubations were carried out under an atmosphere of $^{18}O_2$ (unpublished experiments).

(22) As noted previously,[8] the *erythro*-9, 10-fluorohydrin **9** appeared to be accompanied by varying amounts of the regioisomeric *erythro*-10,9-fluorohydrin as evidenced by the appearance of two partially resolved components in the *erythro*-fluorohydrin region of the ^{19}F NMR spectrum and GC/MS chromatogram (SIMS mode). The presence of *erythro*-10,9-fluorohydrin was confirmed by the observation of a substantial upfield β-isotope shift (0.5 ppm) on one of the ^{19}F NMR signals due to the *erythro*-11-d$_2$-labeled fluorohydrins derived from 11-d$_2$-(*R,S*)-9-fluorostearoyl ACP. (This large isotope shift is not seen for the *threo* -fluorohydrin 11-d$_2$-**8**, as expected.) The incorporation of ^{18}O (see above, ref 21) into the fluorine-shifted compound was similar to that of the other fluorohydrins: ^{18}O incorporation, 85% ± 2 as measured at *m/z* 303/305. The origin of this product of fluorine migration is currently under investigation.

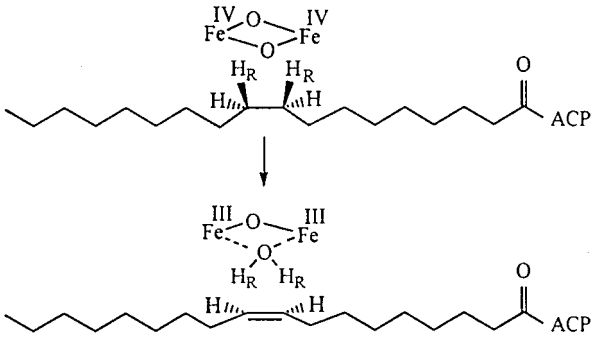
(23) These previously unreported compounds appeared as two very minor peaks (<1%) in the fluorohydrin region of the GC/MS chromatogram and were identified by their MS (diTMS derivatives: *m/z* 201 $(C_8H_{16}OTMS)^+$, 329 $[M - (C_7H_{15}) - HF]^+$). The correctness of the assignment of hydroxyl position was confirmed by subsequent deuterium labeling experiments (vide infra).

Table 1. Isotopic Content[a] of Stereospecifically Monodeuterated Stearates and Δ^9 Desaturated Products

	substrates		products		
	% d_0	% d_1	% d_0	% d_1	% retention of label[b]
(9*R*)-[9 -$^2H_1$]-**1**	4.5 ± 1.3	95.5 ± 1.3	96.4 ± 0.2	3.6 ± 0.2	3.8 ± 0.2
(9*S*)-[9 -$^2H_1$]-**1**	4.2 ± 0.7	95.8 ± 0.7	4.4 ± 0.6	95.6 ± 0.6	99.8 ± 0.9
(10*R*)-[10 -$^2H_1$]-**1**[c]	3.9 ± 0.6	96.1 ± 0.6	67.5 ± 1.4	32.5 ± 1.4[c]	2.2 ± 2.2[c]
(10*S*)-[10 -$^2H_1$]-**1**	6.2 ± 0.2	93.8 ± 0.2	8.2 ± 0.4	91.8 ± 0.4	97.9 ± 0.5

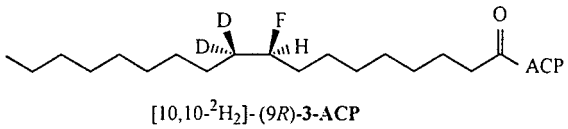
[a] Each incubation was repeated two times, and the deuterium content is given as an average value ± standard deviation. [b]Percent retention of label = [% d_1 (product)/(% d_1 (substrate) × 100]. [c]Calculation corrected for the fact that the deuterated substrate contained ~33% of the corresponding (10*S*)-enantiomer as assessed by the % ee of the synthetic precursor-methyl (10*S*)-10-hydroxystearate (see Materials and Methods).

Scheme 3

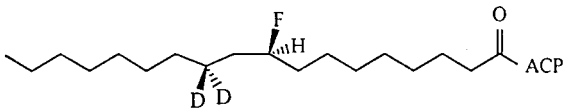


hydrogen at C-10 with predominately retention of configuration.[24a] An entirely different set of products is obtained upon oxidation of (9*R*)-**3**-ACP, because the C-9 hydrogen at the pro-*R* position of this substrate is replaced by fluorine: These include fluoroolefins with "abnormal" configuration **6** and regiochemistry **7** as well as a mixture of C-10- and C-11-hydroxylated products **8**−**11** (Scheme 2). Possible substrate conformations leading to these novel products are described in the next section.

The Hydroxylation/Desaturation Connection. The observation that both olefins *and* fluorohydrins derived from (9*R*)-**3**-ACP were obtained as regioisomeric pairs (Δ^9,Δ^{10}; 10-ol,11-ol) prompted us to examine how these products might be related in a mechanistic sense. According to a previously proposed model,[6a] hydroxylation and desaturation pathways share a common initial, irreversible, C−H activation step such as H-abstraction. The location of this event in the case of (9*R*)-**3**-ACP oxidation can potentially be probed through isotopically induced branching of the reaction pathway. That is, if this substrate presents two different methylene groups to enzymatic attack via rapidly interchanging conformations in the active site, and if the initial reaction leads to two different sets of products, then the presence of deuterium at either methylene position could, in principle, influence the proportion of product pairs obtained from each conformation. Precedent for this type of phenomenon has been established for other enzyme systems.[25] To test our hypothesis, two samples of labeled (9*R*)-**3**-ACP—one bearing deuterium at C-10 and the other at C-11—were required. The synthesis of these materials was accomplished by first synthesizing methyl [10,10-$^2H_2$]- and [11,11-$^2H_2$]-(*R*,*S*)-9-fluorostearate via a modification of a previously published synthesis[6c] (see Materials and Methods). We were then able to prepare the corresponding ACP derivatives, which were greatly enriched in the (*R*)-9-fluorostearoyl stereoisomer due to a substantial diastereoselective discrimination which occurs during the ACP synthase-mediated acylation of (*R*,*S*)-9-fluorostearic acid as previously noted.[8]



[10,10-$^2H_2$]-(9*R*)-**3**-ACP



[11,11-$^2H_2$]-(9*R*)-**3**-ACP

Each deuterated substrate, as well as a nonlabeled control, was incubated with stearoyl-ACP Δ^9 desaturase under conditions of high (>~85%) conversion. The product profiles obtained in these experiments are presented in Table 2 and displayed graphically in Figure 1. Examination of these data reveals that the amount of fluoroolefin 10-d_1-**6** and 9,10-fluorohydrins 10-d_1-**8** and 10-d_1-**9** derived from the [10,10-$^2H_2$]-(9*R*)-**3** was greatly reduced relative to the levels obtained from the corresponding d_0-substrate. In contrast, the intensity of peaks due to allylic fluoride 10-d_1-**7** and the 9,11-fluorohydrins, 10-d_2-**10** and 10-d_2-**11** (ratio of **10**/**11** ~1:3)[26] was substantially enhanced. Precisely the opposite trends were observed upon oxidation of the [11-$^2H_2$]-fluorostearate: The amount of 11-d_1-**7**, 11-d_1-**10** and 11-d_1-**11** produced was attenuated while the levels of 11-d_2-**6**, 11-d_2-**8** and 11-d_2-**9** increased.

Our interpretation of these results is presented in Scheme 4: Oxidation of (9*R*)-**3**-ACP proceeding via an anti conformation similar to **A** is initiated by the energetically difficult C−H cleavage at C-10 to give a short-lived intermediate, which collapses to olefinic product **6** by a relatively fast,[6b] second C−H bond breaking step at C-9. A closely related reaction pathway yields mainly *erythro*-fluoro alcohol **9**, assuming, as is reasonable, that hydroxylation occurs predominately with retention of configuration.[24a,b] In contrast, formation of allylic fluoride **7** and **11**, the putative,[26] major hydroxylated product, involves initial reaction at C-11 on a coiled (9*R*)-**3** (conformation similar to **B**).[27] In both cases, the point of attack is dictated by a strong regiochemical imperative. Because equilibration between alternate substrate conformations is faster than the initial C−H

(24) (a) Valentine, A. M.; Wilkinson, B.; Liu, K. E.; Komar-Panicucci, S.; Priestley, N. D.; Williams, P. G.; Morimoto, H.; Floss, H. G.; Lippard, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 1818−1827. (b) The production of the minor stereoisomers **8** (and **10**) is due to the fact that either hydroxylation proceeds with less than 100% stereospecificity for this substrate or that substrate conformations in addition to those depicted in Scheme 4 are involved in this reaction.

(25) Atkinson, J. K.; Hollenberg, P. F.; Ingold, K. U.; Johnson, C.; Le Tadic, M.; Newcomb, M.; Putt, D. A. *Biochemistry* **1994**, *33*, 10630−10637 and references therein.

(26) The assignment of 11(*R*)-configuration to the predominant stereoisomer **11** must be regarded as tentative at this time as it is based solely on the mechanistic considerations depicted in Scheme 4.

Table 2. Profile of Products Obtained by Oxidation of Predominately (9*R*)-3-ACP, [10,10 -$^2H_2$]-3-ACP, and [11,11-$^2H_2$]-3-ACP by Stearoyl-ACP Δ^9-Desaturase

	product profile[a]					
substrates	(Z)–9F-9-ene **6**	9F-10-ene **7**	9F-10-OH[b] **8, 9**	9F-11-OH[b] **10, 11**	(*E*)-9F-9-ene **4**	other[c]
3	28.7 ± 1.7	46.5 ± 1.4	11.6 ± 1.1	0.9 ± 0.4	6.0 ± 0.7	6.4 ± 1.0
[10 -$^2H_2$]-**3**	4.5 ± 0.2	71.5 ± 1.3	1.7 ± 0.3	11.0 ± 0.6	4.4 ± 1.0	6.9 ± 0.2
[11 -$^2H_2$]-**3**	51.4 ± 1.0	4.6 ± 0.8	24.5 ± 0.6	0.3 ± 0.1	4.6 ± 0.8	5.3 ± 0.3

[a] Each incubation was carried out to greater than 85% conversion and repeated at least three times; percent composition is given as an average value ± standard deviation and was determined by GC/MS analysis and corroborated by ^{19}F NMR. The mass spectra of analytes derived from deuterated substrates were in accord with the anticipated isotopic content. [b]Contributions of both diastereoisomers were combined since the ratios of **8**:**9** and **10**:**11** appeared to be invariant in all three experiments. Contribution of **5** from (*S*)-9F to amount of 9F-10-OH was neglected. [c] Total of unidentified peaks—assumed to be artifacts of workup procedure.

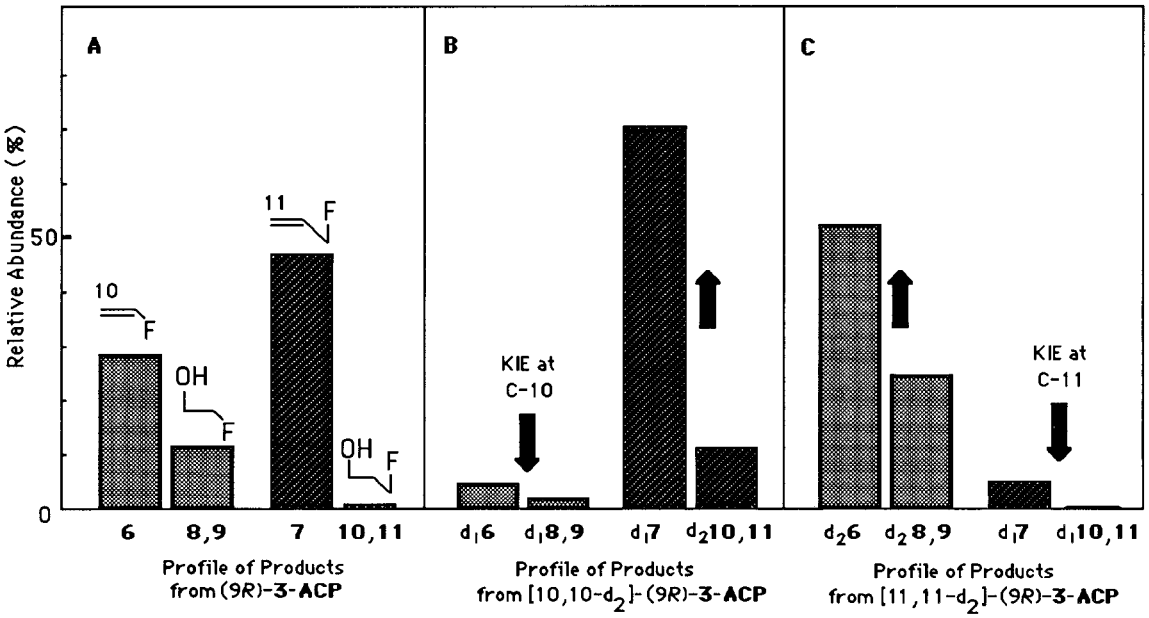
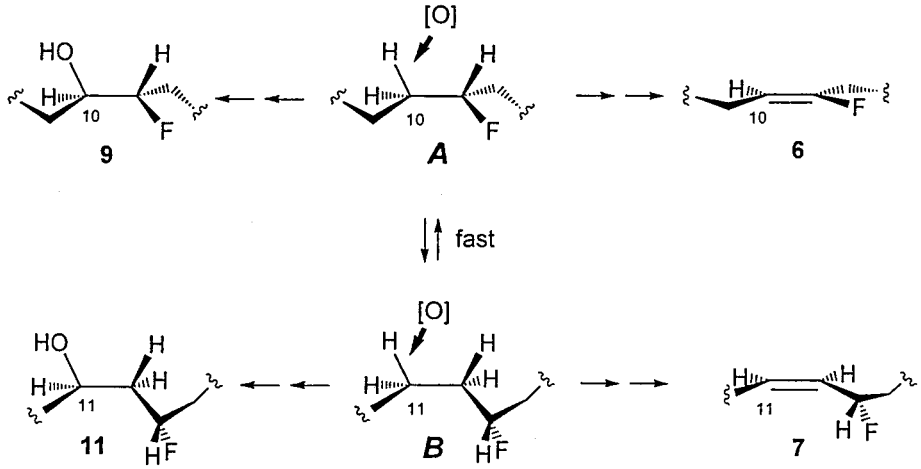


Figure 1. Isotopic discrimination in oxidation of [10,10 -$^2H_2$]-(9*R*)-**3**-ACP and [11,11-$^2H_2$]-(9*R*)-**3**-ACP by stearoyl-ACP Δ^9 desaturase. The structures of the analytes are given in Scheme 2.

Scheme 4



activation step, the oxidant can select the energetically lowest pathway when presented with a substrate that is dideuterated at C-10 or C-11—a phenomenon that leads to the manifestion of a kinetic isotope effect on the initial C−H cleavage at C-10 and C-11 leading to olefin formation. The intramolecular nature of this substantial isotope effect renders it less subject to masking by other enzymatic steps.[28,29] Large "intrinsic" primary[30] deuterium KIEs are typical of C−H cleavages involving

(27) There is some precedent for the production of a mixture of (*Z*)- Δ^9- and Δ^{10} -desaturated products in this system. Reaction of 7(*E*)-octadecenoyl-ACP by stearoyl-ACP Δ^9 desaturase gave a (4:1) mixture of (7*E*,9*Z*)-octadecadienoyl-ACP and (7*E*,10*Z*)-octadecadienoyl-ACP: Broadwater, J. A.; Laundre, B. J.; Fox, B. G. *J. Inorg. Biochem.* **2000**, *78*, 7−14.

(28) A detailed treatment of enzymatic kinetic isotope effects and the sensitivity to masking effects is given in: Miwa, G. T.; Garland, W. A.; Hodshon, B. J.; Lu, A. Y. H.; Northrop, D. B. *J. Biol. Chem.* **1980**, *255*, 6049−6054.

(29) Rataj, M. J.; Kauth, J. E.; Donnelly, M. I. *J. Biol. Chem.* **1991**, 18684−18690.

oxidation of unactivated C−H bonds and are reminiscent of values obtained for the initial oxidation step involved in a number of membrane-bound desaturase systems.[6b,31a−e]

Conclusions

The stereochemistry of C−H cleavage mediated by stearoyl-ACP Δ^9 desaturase has been unambiguously determined for the first time since the discovery of this class of enzymes some thirty-five years ago.[32] The observed pro-*R* enantioselectivity correlates well with the markedly different product profiles obtained by oxidation of stearoyl substrates bearing a (9*R*)- and (9*S*)-monofluorine substituent. These data are critical to the interpretation of ongoing biophysical studies involving substrate analogue−enzyme complexes.

9-Fluorinated substrates are processed by stearoyl-ACP Δ^9 desaturase to give novel oxygenated and olefinic products. In the case of (9*R*)-fluorostearoyl ACP oxidation, we were able to show that desaturation and hydroxylation are initiated at the same site and possibly via an identical transition state. How these two pathways diverge, and the effect of fluorine substitution on the ratio of the energy barriers to each route, remains to be identified; the use of emerging techniques for modeling the reactivity of diiron-containing enzymes may shed further light on this fascinating mechanistic problem.[9]

Acknowledgment. This work was supported by an NSERC PDF (to B.B.), NSERC Grant OGP-2528 (to P.H.B.), by the Office of Basic Energy Sciences of the United States Department of Energy, and in part by Dow and Dow Agrosciences (to J.S.). We are indebted to Dr. John Broadwater (BNL) for useful discussions and Jamie Coté and Christine Caputo at Carleton University for preparing some of the substrates. The technical assistance of Dr. Ted Edwards is also gratefully acknowledged. Cultures of *Pseudomonas* were a generous gift of Dr. John Rossaza, University of Iowa, Iowa City, IA.

JA012252L

(30) These values may include secondary kinetic isotope effects (~10% of primary KIE): Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171−2173.

(31) (a) Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1998**, *120*, 871−876. (b) Pinilla, A.; Camps, F.; Fabrias, G. *Biochemistry* **1999**, *38*, 15272−15277. (c) Meesapyodsuk, D.; Reed, D. W.; Savile, C. K.; Buist, P. H.; Covello, P. S. *Biochemistry* **2000**, *39*, 11948−11954. (d) Fauconnot, L.; Buist, P. H. *J. Org. Chem.* **2001**, *66*, 1210−1215. (e) Behrouzian, B.; Fauconnot, L.; Daligault, F.; Nugier-Chavin, C.; Patin, H.; Buist, P. H. *Eur. J. Biochem.* **2001**, *268*, 3545−3549.

(32) Nagai, J.; Bloch, K. *J. Biol. Chem.* **1966**, *241*, 1925−1927.