Dihydroceramide Δ^4 Desaturase Initiates Substrate Oxidation at C-4

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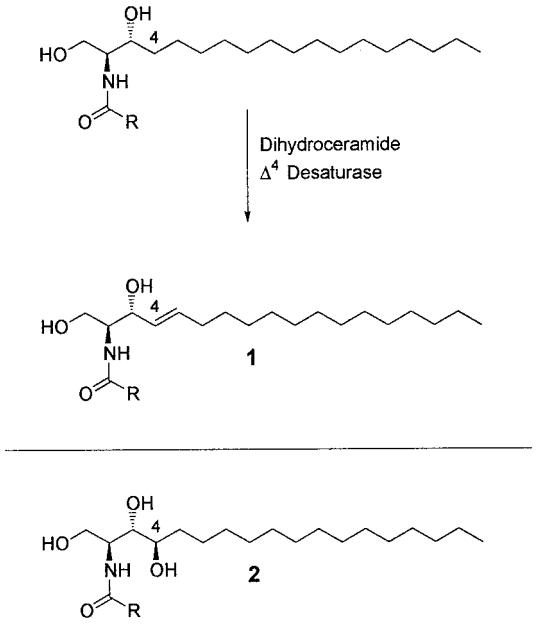
Abstract: The intermolecular primary deuterium isotope effects on the individual C−H bond cleavage steps involved in dihydroceramide Δ^4 desaturation have been determined for the first time by incubating rat liver microsomes with 1:1 mixtures of nonlabeled substrate and the appropriate regiospecifically dideuterated analogue. Analysis of the enzymatic products via gas chromatography coupled to mass spectrometry showed that the introduction of the (*E*) double bond between C-4 and C-5 occurs in two discrete steps: cleavage of the C_4−H bond was found to be very sensitive to isotopic substitution ($k_H/k_D = 8.0 \pm 0.8$), while a negligible isotope effect ($k_H/k_D = 1.02 \pm 0.07$) was observed for the C_5−H bond-breaking step. According to a mechanistic model that we have previously proposed, these results suggest that initial oxidation for this desaturation reaction occurs at C-4. This finding correlates nicely with the observation that 4-hydroxylated products are produced from a similar substrate by a closely related oxidative enzyme in yeast.

Introduction

The essential role of sphingolipids in the cell biology of both mammalian and yeast cells has been well documented.[1] In mammals, ceramide **1** (an important member of the sphingolipid family) acts as a second messenger in mediating antimitogenic effects, such as cell growth supression, cell cycle arrest, and programmed cell death or apoptosis. Ceramide is also an important regulator of cellular responses to stress. Sphingosine-1-phosphate, a deacylated ceramide derivative, is a potent mitogen that regulates mobilization of calcium, cell growth, differentiation, survival, and motility. In *Saccharomyces cerevisiae*, sphingolipids and their derivatives are known to participate in such functions as signal transduction during response to heat stress, regulation of calcium homeostasis, calcium-mediated signaling, and regulation of the cell cycle.[2]

Interestingly, some activities of mammalian sphingolipids depend critically[3] on the presence of an (*E*)-4,5 double bond in the long chain base structure of **1**. This functional group is formed by an unusual regio- and stereoselective dehydrogenation process catalyzed by a membrane-bound enzyme known as dihydroceramide desaturase.[4−6] Biochemical studies suggest that this enzyme is closely related to a 4-hydroxylase which is responsible for the biosynthesis of phytoanalogues of ceramide **1** such as 4-hydroxysphinganine **2**.[7] Indeed, desaturation can



be regarded as a mechanistic variation of hydroxylation and a generic scheme linking the two pathways has been proposed.[8] This features a common, initial C−H activation step to form a very short-lived radical intermediate or its organoiron equivalent (not shown)[9] which can collapse rapidly to give either alcohol or olefin (Scheme 1). The relative spatial relationship of the putative diiron oxidant[10a] to substrate is thought to be primarily

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(9) The precise nature of the intermediates in C−H activation reactions of this type remains obscure. Recently, Groves et al. have obtained some evidence for discrete carbon-centered radical intermediates in C−H activation reactions catalyzed by this class of enzyme: Austin, R. N.; Chang, H.; Zylstra, G.; Groves, J. T. *J. Am. Chem. Soc.* **2000**, *122*, 11747−11748.
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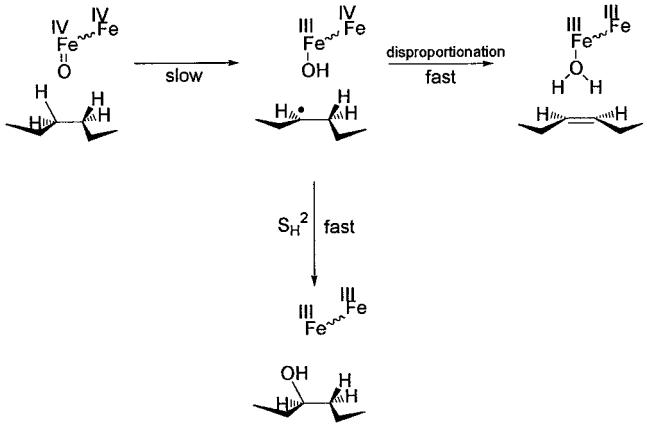
Scheme 1



responsible for the control of the reaction outcome.[10b,11] Minor changes in substrate structure can also effect a switch in the reaction pathway.[12]

Given the obvious importance of dihydroceramide Δ⁴ desaturase, the efforts devoted to its purification, and the ongoing search for inhibitors of this enzyme,[13] an examination of the mechanism of Δ⁴ desaturation is a worthy research objective. In particular, we were interested in determining which methylene group is attacked first in this enzymatic reaction since this is a key determinant for the regiochemical outcome. Here, we report the results of the first detailed mechanistic study of dihydroceramide Δ⁴ desaturase in which we show that the oxidation of substrate is initiated at C-4.

Materials and Methods

General Methods. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Brüker AMX 400 spectrometer with the use of dilute $CDCl_3$ or CD_3OD solutions. Chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane. *J*-values are reported in hertz (Hz). Deuterium isotope effects on ^{13}C NMR shifts were estimated by running spectra of mixtures (1:2) of labeled and unlabeled material. Mass spectra were obtained by GC/MS by using a Fisons MD-800 mass spectrometer coupled to a Fisons 8000 series GC equipped with a nonpolar Hewlett-Packard HP-1 capillary column (30 m × 0.20 mm), temperature programmed from 150 to 320 °C at 6 °C/min. Analyses were carried out under selected ion monitoring (SIM) mode. The deuterium content was estimated by using a dwell time of 0.02 s which resulted in 5−8 scans per GC peak; the integrated intensities of the individual ions in the pertinent ion cluster were recorded by using *Lab Base* software and have been corrected for natural isotopic abundances. Care was taken to include the entire GC peak in the integration procedure to prevent errors due to fractionation of isotopic species during chromatography. Isotopic ratios were determined by using the following ions: *m*/*z* 313, $((CH_3)_3Si{-}O{-}CH{-}(CH_2)_{14}CH_3)^+$ (*N*-octanoyl-D-*erythro*-sphinganine **3**), and *m*/*z* 311, $((CH_3)_3Si{-}O{-}CHCH{=}CH{-}(CH_2)_{12}CH_3)^+$ (*N*-octanoyl-D-*erythro*-sphingosine **4**). Each mass spectral measurement was repeated a minimum of three times to obtain an average value.

Flash chromatography with silica gel (230−400 mesh) was used to purify all intermediates. Chromatography with silica gel (70−230 mesh) was used to purify substrates. Visualization of UV-inactive materials was accomplished by using phosphomolybdic acid (PMA) followed by charring or a combination of I_2 vapor followed by a water spray.

All reagents and starting materials were purchased from Sigma-Aldrich Canada (Oakville, Canada) and used without purification. Tetrahydrofuran (THF), toluene, and diethyl ether (Et_2O) were freshly distilled from Na-benzophenone ketyl. All air- and moisture-sensitive reactions were performed under N_2. Organic extracts were typically shaken with saturated NaCl and dried over $MgSO_4$, and solvents were evaporated in vacuo on a Büchi RE 111 Rotavapor. [1,1-$^2H_2$]-1-Bromopentadecane was prepared by reduction[14] of pentadecanoic acid with $LiAlD_4$ followed by treatment with HBr/H_2SO_4.[15] [2,2-$^2H_2$]-1-Bromopentadecane was prepared by α-exchange of methyl pentadecanoate with Na in MeOD[16] followed by reduction with $LiAlH_4$[14] and treatment with HBr/H_2SO_4.[15]

All buffers and salts were purchased from Merck (Darmstadt, Germany); NADH, BSA, and other biochemicals were purchased from Sigma-Aldrich Química (Madrid, Spain). Protein concentrations were measured by the method of Bradford[17] using bovine serum albumin as standard protein. Male Sprague−Dawley rats were supplied by the animal breeding facility of the Centro de Investigación y Desarrollo (Barcelona, Spain).

Synthesis of Substrates. D-*erythro*-Sphinganine was prepared essentially as previously described[18,19] by Grignard addition[20] of pentadecylmagnesium bromide to the Garner aldehyde.[21] The deuterated analogues were synthesized in similar fashion by using the appropriately deuterated Grignard reagent. After deketalization of the Grignard adduct with *Amberlyst 15* catalyst,[19] the resultant *erythro* derivative was freed of the unwanted *threo* diastereomer by flash silica gel chromatography, using hexane/EtOAc (3:1 v/v) as eluent. Cleavage[19] of the carbamate moiety with 1 N HCl in dioxane afforded D-*erythro*-sphinganine. The analytical data are given below:

D-*erythro*-Sphinganine: mp 92−94 °C (lit.[22] mp 85−88 °C); ^{1}H NMR (*N*-acetyl derivative, 400 MHz) δ 0.89 (t, J = 6.7, 3H, $-CH_2C\mathbf{H_3}$), 1.28 (br s, 26 H, $-(C\mathbf{H_2})_{13}CH_3$), 1.37 (m, 1H, C(4)-**H**), 1.51 (m, 1H, C(4)−**H**), 1.97 (s, 3H, $-C(O)C\mathbf{H_3}$), 3.59 (ddd, J = 6.6, 2.7, 8.6, 1H, C(3)-**H**), 3.66 (dd, J = 6.3, 11.3, 1H, C(1)-**H**), 3.70 (dd, J 4.2, 11.3, 1H, C(1)-**H**), 3.81 (ddd, J = 4.2, 6.3, 6.4, 1H, C(2)-**H**); ^{13}C NMR (*N*-acetyl derivative, 100 MHz) δ 173.37 (*C*=O), 72.40 (C-3), 62.16 (C-1), 57.03 (C-2), 34.83 (C-4), 33.08 (C-16), 30.79, 30.76, 30.48 (C-6 to C-15), 26.85 (C-5), 23.74 (C-2′), 22.77 (C-17), 14.44 (C-18); MS (*N*-octanoyl derivative, EI, 70 eV) *m*/*z* 556 ($M^+ - CH_3$), 468 ($M^+ - (CH_3)_3Si{-}O{-}CH_2$), 313 $[((CH_3)_3Si{-}O{-}CH(CH_2)_{14}CH_3)^+]$, 258 $[((CH_3)_3Si{-}O{-}CH_2CH{-}NH{-}C(O)(CH_2)_6CH_3)^+]$.

D-*erythro*-[4,4-$^2H_2$]Sphinganine was synthesized from [1,1-$^2H_2$]-1-bromopentadecane. The spectral data of the title compound (*N*-acetyl derivative) were similar to those of the unlabeled parent except for ^{1}H NMR (400 MHz) 1.37, 1.51 (m, 2H, C(4)-$\mathbf{H_2}$, absent), 3.58 (d, J = 6.7, 1H, C(3)-**H**); ^{13}C NMR (100 MHz) 72.30 (C-3, upfield β-isotope shift (0.11 ppm)), 57.01 (C-2, upfield γ-isotope shift (0.02 ppm)), 34.83 (C-4, absent), 26.65 (C-5, upfield β-isotope shift (0.19 ppm)); MS (*N*-octanoyl derivative, EI, 70 eV) *m*/*z* 558 ($M^+ - CH_3$), 470 ($M^+ - (CH_3)_3Si{-}O{-}CH_2$), 315 $[((CH_3)_3Si{-}O{-}CHCD_2(CH_2)_{13}CH_3)^+]$, 258 $[((CH_3)_3Si{-}O{-}CH_2CH{-}NH{-}C(O)(CH_2)_6CH_3)^+]$.

D-*erythro*-[5,5-$^2H_2$]Sphinganine was synthesized from [2,2-$^2H_2$]-1-bromopentadecane. The spectral data of the title compound (*N*-acetyl derivative) were similar to those of the unlabeled parent except for ^{1}H NMR (400 MHz) 1.37 (dd, J = 13.8, 9.2, 1H, C(4)-**H**), 1.51 (dd, J = 3.3, 13.8, 1H, C(4)-**H**); ^{13}C NMR (100 MHz) δ 72.38 (C-3, upfield γ-isotope shift, (0.02 ppm)), 34.64 (C-4, upfield β-isotope shift (0.19 ppm)), 26.85 (C-5, absent); MS (*N*-octanoyl derivative, EI, 70 eV) *m*/*z*

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558 ($M^+ - CH_3$), 470 ($M^+ - (CH_3)_3Si-O-CH_2$), 315 [$((CH_3)_3Si-O-CHCH_2CD_2(CH_2)_{12}CH_3)^+$], 258 [$((CH_3)_3Si-O-CH_2CH-NHC(O)-(CH_2)_6CH_3)^+$].

Dihydroceramide Desaturase Assay. The procedure reported by Michel et al. was followed.[4] Rat liver microsomes were prepared as reported.[4] Synthetic D-*erythro*-sphinganine was acylated with octanoyl chloride[23] and purified by chromatography on silica ($CHCl_3$/MeOH 15:1 v/v) to give the corresponding *N*-octanoyl-D-*erythro*-sphinganine **3**. The semitruncated dihydroceramide probe was complexed with BSA as described for other lipids.[24] The solution contained 10 nmol of lipid substrate, 20 nmol of BSA, 10 μL of EtOH, and 90 μL of phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, pH 7.4). A rat liver microsomal suspension containing 300 μg of protein was added to the substrate/BSA complex and diluted to a volume of 320 μL with phosphate buffer. NADH (1 μmol) in 30 μL of phosphate buffer was added to the reaction mixture and incubated at 37 °C for 30 min.

To reduce mass spectral interferences from endogenous material, the mixture was cooled to 0 °C on ice and rapidly extracted with 500 μL of hexane. The reaction was terminated with the addition of 500 μL of $CHCl_3$. The phases were separated by centrifugation and the organic layer was collected. The extraction procedure was repeated twice with 250 μL of $CHCl_3$. The combined organics were evaporated under a steady stream of N_2 and derivatized with 100 μL of bis(trimethylsilyl)trifluoracetamide at 25 °C for 1 h. Excess reagent was evaporated under a stream of N_2 and the residue was diluted with 100 μL of $CHCl_3$ for analysis by GC/MS.

Results and Discussion

According to our mechanism (Scheme 1), the first C−H cleavage step in a desaturase-mediated reaction is energetically difficult and hence more sensitive to isotopic substitution than the collapse of the intermediate radical. Thus one way of determining the site of initial oxidative attack for dihydroceramide Δ^4 desaturase is via an unambiguous assessment of which C−H cleavage is rate-determining. This can be accomplished by a measurement of the intermolecular primary deuterium KIE associated with each C−H bond breaking step to assess its kinetic importance.[25] Our experimental approach involved incubating a roughly equimolar mixture of the appropriate regiospecifically dideuterated (−CD_2−) substrate analogue and the nondeuterated parent compound with a convenient source of the Δ^4 desaturase. Evaluation of the d_0/d_1 ratio in the olefinic products by mass spectral examination would then allow one to compute the competitive KIE isotope effect for each C−H bond rupture.

To examine the dihydroceramide Δ^4 desaturase system with this method, a reliable GC-MS assay of sufficient sensitivity had to be used. The procedure used by Michel[4] to measure desaturation of ^{14}C-labeled semitruncated dihydroceramide was followed for this purpose. In our case, the enzymatic reaction was monitored by GC-MS analyses of silylated extracts. Trimethylsilyl derivatives of ceramides give characteristic fragments in their mass spectra, with diagnostic ions arising from cleavage of the C2−C3 bond.[26a,b] For incubations with nonlabeled *N*-octanoyl-D-*erythro*-sphinganine **3**, the identity of both substrate and product was confirmed by mass spectral analysis of a silylated extract. Diagnostic fragment ions were *m*/*z* 258 and 313 (trimethylsilylated substrate) and *m*/*z* 258 and 311 (trimethylsilylated product) (Figure 1). In the competitive

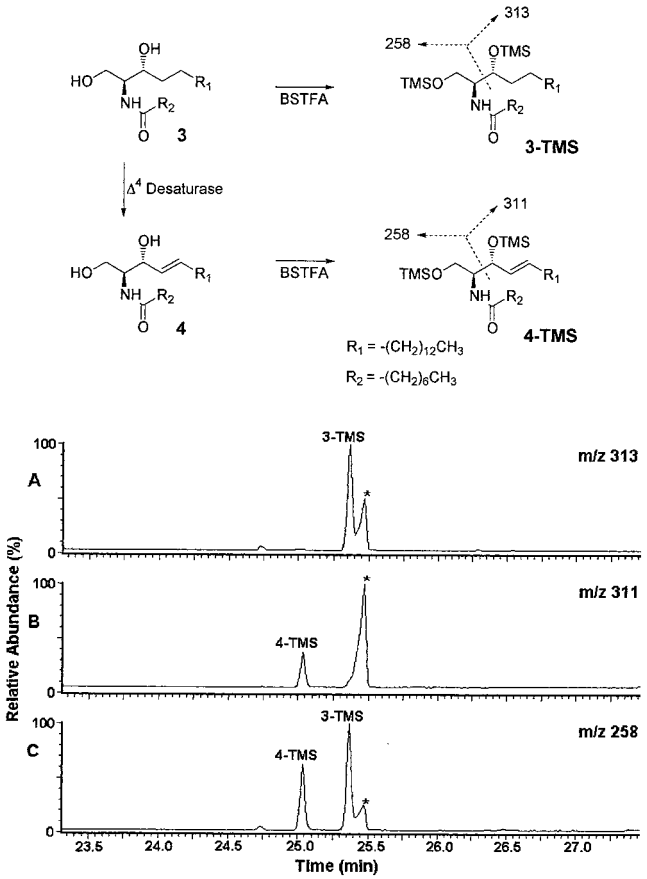

Figure 1. Formation of *N*-octanoyl-D-*erythro*-sphingosine **4** by rat liver microsomes as determined by SIM-GC/MS. The traces correspond to selection of ions at *m*/*z* 313(A), 311 (B), and 258 (C). The peaks are labeled as follows: **3**-TMS, trimethylsilyl derivative of **3** (reaction substrate) and **4**-TMS, trimethylsilyl derivative of **4** (reaction product). The peak marked with an asterisk is the trimethylsilyl derivative of cholesterol.

experiments (see below), selection of the appropriate diagnostic ions in the SIM-GC-MS analyses allowed us to make the measurements of isotopic content with high sensitivity and accuracy.

The two regiospecifically dideuterated substrates ([4,4-$^2H_2$]-**3**) and ([5,5-$^2H_2$]-**3**), which were required for this study, were synthesized de novo by well-precedented procedures involving Grignard addition to suitably protected L-serinal[18,19,20] followed by chromatographic separation of the *threo* and *erythro* adducts (1:2 ratio) as the t-Boc derivatives. The purified *erythro* isomers were deprotected and then acylated with octanoyl chloride to give ([4,4-$^2H_2$]-**3**) and ([5,5-$^2H_2$]-**3** in 4% and 5% overall yield, respectively (Scheme 2). The *erythro* and *threo* isomers could be distinguished on the basis of well-documented differences in the ^{1}H NMR of the *N*-acetyl derivatives.[27] GC/MS analysis of the final deuterated products revealed that each isotopomer consisted essentially entirely of dideuterated species.

In the competitive KIE experiments, a 1:1 mixture of each dideuterated substrate with its nondeuterated parent was incubated with the microsomes, containing the dihydroceramide desaturase, under conditions similar to those described by Michel et al.[4] Care was taken to maintain the level of conversion below 10% to prevent possible enhancement of the d_2/d_0 ratio in the substrates.[28] The deuterium content of the Δ^4 olefinic products was assessed by GC/MS as described in the Experimental

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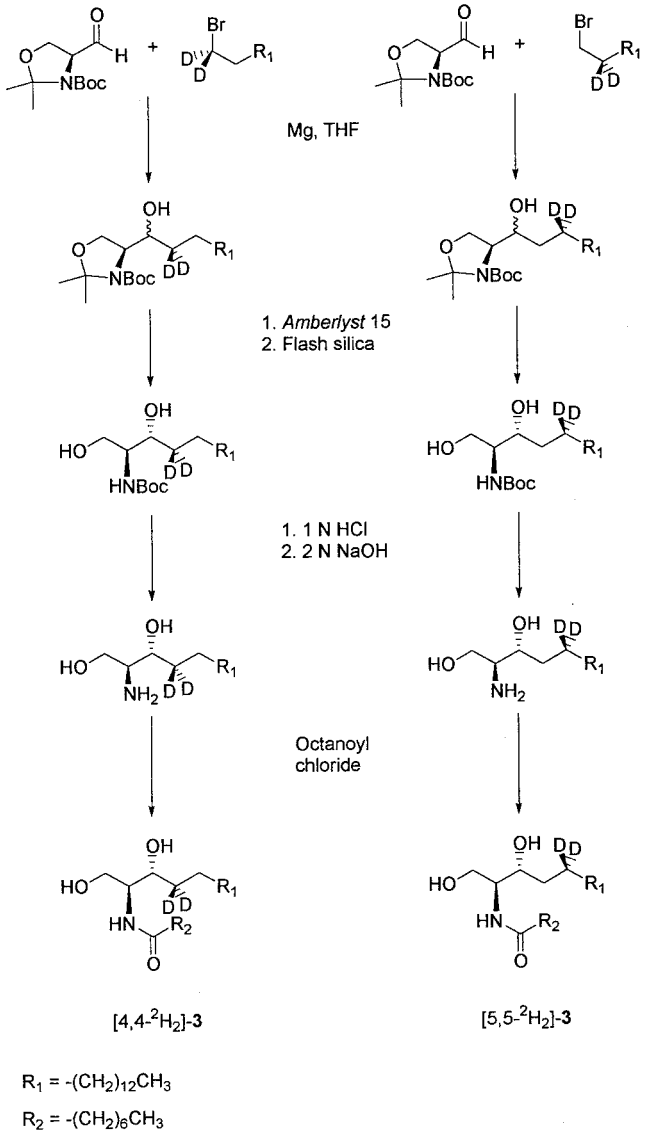
Scheme 2



Section. Product kinetic isotope effects (k_H/k_D) were calculated by using the following ratio: [% d_0(product)/% d_1(product)]/[% d_0(substrate)/% d_2(substrate)]. The d_0/d_2 ratio of the substrates was determined prior to incubation. Analysis of the products of Δ^4 desaturation **4** revealed a large primary deuterium isotope (8.0 ± 0.8) for the carbon−hydrogen bond cleavage at C-4 while the C5−H bond-breaking step was essentially insensitive to deuterium substitution (KIE = 1.02 ± 0.07). The analytical data are displayed in Table 1.

These results provide the first pieces of information on the mechanism of dihydroceramide Δ^4 desaturase. The KIE data clearly indicate that C−H cleavage at C-4 but not C-5 is kinetically important. According to our model (Scheme 1), this implies that initial oxidative attack occurs at C-4 and strengthens the notion that the production of phytosphingosines via 4-hydroxylation and formation of sphingosines via 4,5-desaturation are closely linked at the mechanistic level (see Introduction).

Table 1. Intermolecular Isotopic Discrimination in Δ^4 Desaturation of [4,4-$^2H_2$]-**3** and [5,5-$^2H_2$]-**3**

isotopic ratio				
substrates		products		
d_0:$4d_2$	d_0:$5d_2$	d_0:$4d_1$	d_0:$5d_1$	KIE[a]
1.04		8.28		8.0 ± 0.8
	1.32		1.34	1.02 ± 0.07

[a] The average KIE (three different incubations) ± standard deviation.

The stereochemical evidence also points to a common site of attack since it is the *pro*-(*R*) hydrogen at C-4 that is removed in both enzymatic reactions.[29,30] This picture is very strongly reminiscent of the connection that has been established between the structurally related castor oleate 12-hydroxylase and microsomal Δ^{12} oleate desaturase.[8,31,32] By using a KIE approach, it was shown that the latter enzyme initiated oxidation at the same carbon that is attacked by the corresponding 12-hydroxylase (KIE, Δ^{12} oleate desaturase: $k_H/k_D = 7.3$ (C-12); $k_H/k_D = 1.05$ (C-13)).[8] A similar trend in KIE values has also been observed in the cytochrome P450-catalyzed 4-desaturation of the anti-epileptic drug valproic acid (2-propylbutanoic acid)—a process that is accompanied by the formation of a 4-hydroxylated metabolite as the major product.[33]

In conclusion, the results presented in this paper have two important consequences for ceramide research. First, our data strongly support the use of existing sequence information for dihydroceramide 4-hydroxylase to probe for corresponding Δ^4 desaturase in mammalian systems. Second, knowing that the site of initial oxidative attack is at C-4 should aid in the design of and search for inhibitors of this enzyme. Progress at both of these fronts is crucial to a more complete understanding of ceramide biosynthesis and its role in apoptotic pathways.

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Supporting Information Available: Mass spectra of **3**-TMS and **4**-TMS showing the ions which were used for analytical purposes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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