# Structural Requirements of Ceramide and Sphingosine Based Inhibitors of Mitochondrial Ceramidase†

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ABSTRACT: The effects of structural analogues of ceramide on rat brain mitochondrial ceramidase (mt-CDase) were investigated. Design of target compounds was mainly based on modifications of the key elements in ceramide and sphingosine, including stereochemistry, the primary and secondary hydroxyl groups, the trans double bond in the sphingosine backbone, and the amide bond. Mt-CDase was inhibited by (1) all stereoisomers of D-*erythro*-ceramide (D-e-Cer) with an $IC_{50}$ of 0.11, 0.21, and 0.26 mol % for the L-*threo*, D-*threo*, and L-*erythro* isomers, respectively; (2) all stereoisomers of sphingosine with $IC_{50}$ ranging from 0.04 to 0.14 mol %, *N*-methyl-D-*erythro*-sphingosine (N-Me-Sph, $IC_{50}$ 0.13 mol %); and (3) D-*erythro*-urea-$C_{16}$-ceramide ($C_{16}$-urea-Cer $IC_{50}$ 0.33 mol %). The enzyme was not inhibited by *N*-methyl ceramide (N-Me-$C_{16}$-Cer), 1-O-methyl ceramide (1-O-Me-$C_{16}$-Cer), 3-O-methyl ceramide (3-O-Me-$C_{16}$-Cer), *cis*-D-*erythro* ceramide (*cis*-D-e-$C_{16}$-Cer) and 3-O-methyl-D-*erythro*-sphingosine (3-O-Me-Sph). It was less potently inhibited by D-*erythro*-sphinganine (D-e-dh-Sph, $IC_{50}$ 0.20 mol %), D-*erythro*-dehydro sphingosine (D-e-deh-Sph, $IC_{50}$ 0.25 mol %), (2S)-3-keto-sphinganine (3-keto-dh-Sph, $IC_{50}$ 0.34 mol %), (2S) 3-keto-ceramide (3-keto-$C_{16}$-Cer, $IC_{50}$ 0.60 mol %), and ceramine ($C_{18}$-ceramine, $IC_{50}$ 0.62 mol %), 1-O-methyl-D-*erythro*-sphingosine (1-O-Me-Sph), *cis*-D-*erythro*-sphingosine (*cis*-D-e-Sph), (2S)-3-keto-sphingosine (3-keto-Sph), (2S)-3-keto-dehyrosphingosine (3-keto-deh-Sph), and *N*,*N*-dimethyl-D-*erythro*-sphingosine (*N*,*N*-diMe-Sph) were weak inhibitors whereas ceramide-1-phosphate (Cer-1-P) and sphingosine-1-phosphate (Sph-1-P) stimulated the enzyme. Thus, for inhibition, the enzyme requires the primary and secondary hydroxyl groups, the C4−C5 double bond, the trans configuration of this double bond, and the NH-protons from either the amide of ceramide or the amine of sphingosine. Therefore, these results provide important information on the requirements for ceramide−enzyme interaction, and they suggest that ligand interaction with the enzyme occurs in a high affinity low specificity manner, in contrast to catalysis which is highly specific for D-erythro-ceramide (D-e-Cer) but occurs with a lower affinity. In addition, this study identifies two competitive inhibitors of mt-CDase; urea-ceramide ($C_{16}$-urea-Cer) and ceramine ($C_{18}$-ceramine) that may be further developed and used to understand the mechanism of mt-CDase in vitro and in biologic responses.

Ceramide is a potent signal transducer that affects cell growth, differentiation and death (*1*−*3*). It occupies a central position in sphingolipid metabolism. As an acceptor of carbohydrates, phosphorylcholine, and phosphate, it serves as precursor of the various complex sphingolipids. Alternatively, the enzymatic breakdown of these sphingolipids releases ceramide which may consequently be hydrolyzed into fatty acid and sphingosine; the latter exerting bioeffector functions on its own as well as acting as a precursor of sphingosine phosphate, another signal mediator and regulator of various cell functions. A controlled level of ceramide, therefore, reflects an intricate balance between the catabolic and anabolic pathways of ceramide.

Multiple enzymes are directly involved in regulating intracellular ceramide concentration. These include ceramide-generating enzymes such as ceramide synthase, cerebrosidase, sphingomyelinase, and ceramide-consuming enzymes such as cerebroside synthase, sphingomyelin synthase, ceramide kinase, and ceramidase (*4*).

Ceramidases are enzymes that hydrolyze ceramides at the amide bond linking the sphingosine moiety to the fatty acids. In that sense they provide a target site for regulating ceramide-sphingosine interconversion (*5*). At least three different types of ceramidases have been reported. A lysosomal acid ceramidase, the defect of which underlies the human disorder Farber's disease (*6*), was purified from human urine (*7*), and the cDNA encoding the enzyme was also cloned from mouse brain and human fibroblasts (*7*, *8*). Alkaline ceramidases, CDase-I and CDase-II, were purified from guinea pig skin (*9*) and from gram-negative bacterium *Pseudomonas aeruginosa* (*10*, *11*).

A nonlysosomal, ceramidase with a neutral to alkaline pH optimum was also purified to homogeneity from rat brain (*12*) and cloned from mouse (*13*) and human (*14*). The human form was found to localize to mitochondria (*14*). This mitochondria-associated ceramidase (mt-CDase)[1] specifically hydrolyzes the D-*erythro*-isomer of ceramide. These findings indicate that the mt-CDase harbors a very specific recognition

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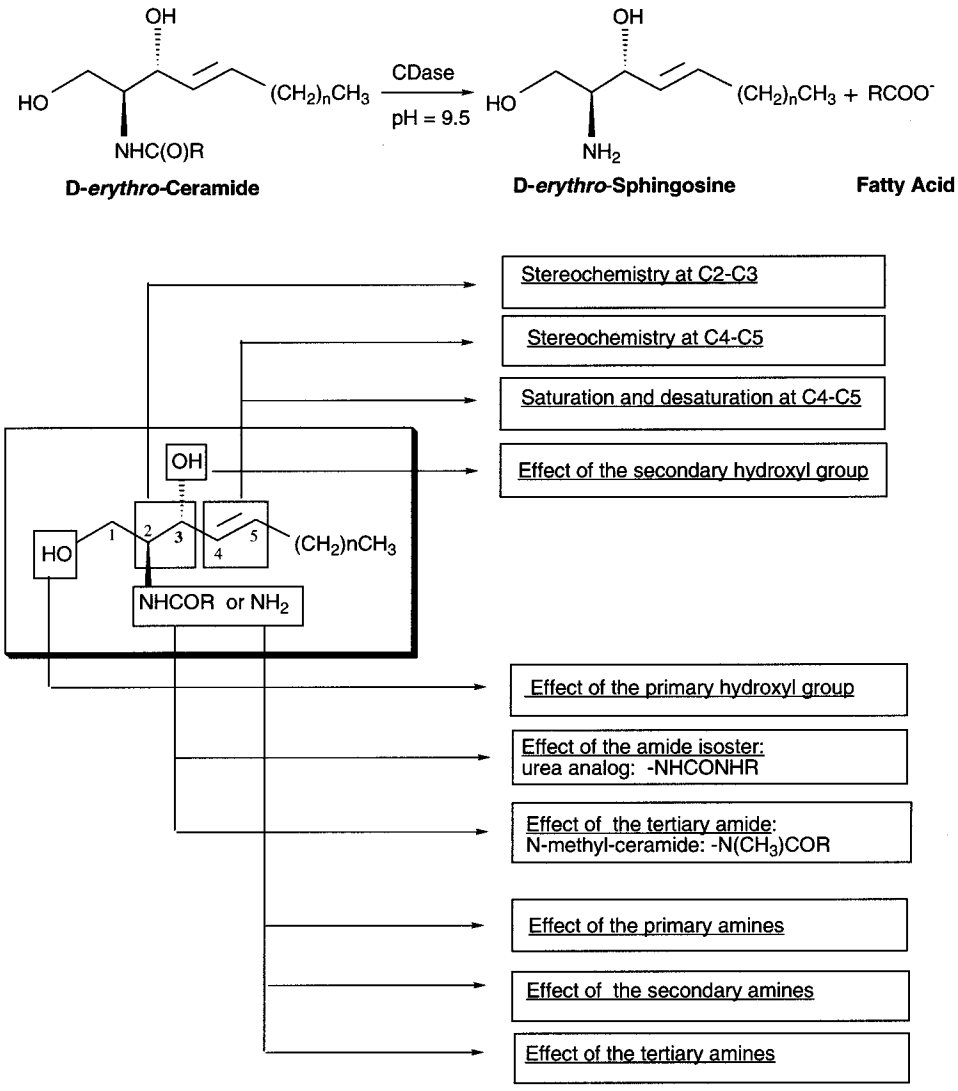


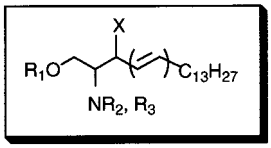

FIGURE 1: Scheme of structural modifications of ceramides and sphingosines.

site for its substrate, which renders it a ceramidase but not a generalized amidase. This high specificity also suggests that the enzyme is possibly geared to regulate the endogenous levels of ceramide and sphingosine. A better understanding of the role and mechanism of this mt-CDase would therefore require a detailed examination of ceramide structure−function relationships.

To test the importance of the various structural features required in the enzyme−substrate interaction, a series of related compounds based on ceramide or sphingosine structures were synthesized and tested for their effect on mt-CDase (Figure 1). The naturally occurring D-*erythro*-ceramide, [N-acyl-(2S,3R)-2-amino-1,3-dihydroxy-octadecene-4] exhibits several important features including the presence of the primary and secondary hydroxyl groups, two chiral centers, and at least two distinct elements of rigidity: the (4E) trans-alkenyl and the amide bond linkage to the (2S, 3R) chiral backbone of sphingosine (Figure 1). These structural features may be decisive elements in determining the proper conformation assumed by the substrate D-*erythro*-ceramide and its ability to interact effectively with the enzyme. Therefore, variation of these structural features result in analogues of ceramide and sphingosine with possible inhibitory effects on mt-CDase. In this study, we investigated the inhibition of mt-CDase activity by (i) stereoisomers of D-*erythro*-ceramide (the L-*erythro*-enantiomer, and the L-*threo*-, and the D-*threo*-diastereomers), (ii) *N*-methyl- and O-methyl-ceramides, (iii) 3-keto-ceramide, (iv) *cis*-ceramide, (v) all stereoisomers of sphingosine, (vi) *N*-methyl-and O-methyl-sphingosines, (vii) ceramine, (viii) *N*,*N*-dimethyl-sphingosine, (ix) sphinganine and dehydrosphingosine, (x) 3-keto-analogues of sphingosine, sphinganine and dehydrosphingosine, (xi) long chain hydrophobic primary amines,

[1] Abbreviations: mt-CDase, mitochondrial ceramidase; D-*erythro*-$C_{18}$-ceramide, D-e-$C_{18}$-Cer; L-*erythro*-$C_{18}$-ceramide, L-e-$C_{18}$-Cer; L-*threo*-$C_{18}$-ceramide, L-t-$C_{18}$-Cer; D-*threo*-$C_{18}$-ceramide, D-t-$C_{18}$-Cer; *cis*-D-*erythro*-$C_{16}$-ceramide, *cis*-D-e-$C_{16}$-Cer; 1-O-methyl-D-*erythro*-$C_{16}$-ceramide, 1-O-Me-$C_{16}$-Cer; 3-O-methyl-D-*erythro*-$C_{16}$-ceramide, 3-O-Me-$C_{16}$-Cer; 3-keto-$C_{16}$-ceramide, 3-keto-$C_{16}$-Cer; D-*erythro*-$C_{16}$-ceramide-1-phosphate, Cer-1-P; D-*erythro*-$C_{16}$-urea-ceramide, $C_{16}$-urea-Cer, *N*-methyl-D-*erythro*-$C_{16}$-ceramide, N-Me-$C_{16}$-Cer; D-*erythro*-sphingosine, D-e-Sph; L-*erythro*-sphingosine, L-e-Sph, L-*threo*-sphingosine, L-t-Sph; D-*threo*-sphingosine, D-t-Sph; *cis*-D-*erythro*-sphingosine, *cis*-D-e-Sph; D-*erythro*-dihysphingosine, D-e-dh-Sph; D-*erythro*-dehydrosphingosine, D-e-deh-Sph; 1-O-methyl-D-*erythro*-sphingosine, 1-O-Me-Sph; 3-O-methyl-D-*erythro*-sphingosine, 3-O-Me-Sph; (2S)-3-keto-sphingosine, 3-keto-Sph; (2S)-3-keto-dihydrosphingosine, 3-keto-dh-Sph; (2S)-3-keto-dehydrosphingosine, 3-keto-deh-Sph; D-*erythro*-sphingosine-1-phosphate, Sph-1-P; *N*-methyl-D-*erythro*-sphingosine, N-Me-Sph; *N*-stearyl-D-*erythro*-sphingosine, $C_{18}$-ceramine; *N*,*N*-dimethyl-D-*erythro*-sphingosine, *N*,*N*-diMe-Sph.

Table 1: Sructural Specificity for Inhibition of Mitochondrial Ceramidase by Ceramide Analogues




| compd | stereochemistry | substituents: X, $R_1$, $R_2$, $R_3$ | estimated $IC_{50}$ (mol %) |
|---|---|---|---|
| D-*erythro*-$C_{18}$-ceramide<br>**D-e-$C_{18}$-Cer** | (2S, 3R, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{16}CH_3$ | substrate |
| L-*erythro*-$C_{18}$-ceramide<br>**L-e-$C_{18}$-Cer** | (2R, 3S, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{16}CH_3$ | 0.26 |
| L-*threo*-$C_{18}$-ceramide<br>**L-t-$C_{18}$-Cer** | (2S, 3S, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{16}CH_3$ | 0.11 |
| D-*threo*-$C_{18}$-ceramide<br>**D-t-$C_{18}$-Cer** | (2R, 3R, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{16}CH_3$ | 0.21 |
| *cis*-D-*erythro*-$C_{16}$-ceramide<br>***cis*-D-e-$C_{16}$-Cer** | (2S, 3R, 4Z) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{14}CH_3$ | no effect |
| 1-O-methyl-D-*erythro*-$C_{16}$-ceramide<br>**1-O-Me-$C_{16}$-Cer** | (2S, 3R, 4E) | X = OH; $R_1 = CH_3$; $R_2$ = H<br>$R_3 = CO(CH_2)_{14}CH_3$ | no effect |
| 3-O-methyl-D-*erythro*-$C_{16}$-ceramide<br>**3-O-Me-$C_{16}$-Cer** | (2S, 3R, 4E) | X = $OCH_3$; $R_1$, $R_2$ =H<br>$R_3 = CO(CH_2)_{14}CH_3$ | no effect |
| 3-keto-$C_{16}$-ceramide<br>**3-keto-$C_{16}$-Cer** | (2S, 4E) | X = O; $R_1$, $R_2$ = H<br>$R_3 = CO(CH_2)_{14}CH_3$ | 0.6 |
| D-*erythro*-$C_{16}$-ceramide-1-phosphate<br>**Cer-1-P** | (2S, 3R, 4E) | X = OH; $R_1 = P(O)(OH)_2$<br>$R_2$ = H; $R_3 = CO(CH_2)_{14}CH_3$ | activator |
| D-*erythro*-$C_{16}$-urea-ceramide<br>**$C_{16}$-urea-Cer** | (2S, 3R, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3 = CONH(CH_2)_{15}CH_3$ | 0.33 |
| *N*-methyl-D-*erythro*-$C_{16}$-ceramide<br>***N*-Me-$C_{16}$-Cer** | (2S, 3R, 4E) | X = OH; $R_1$ = H; $R_2 = CH_3$<br>$R_3 = CO(CH_2)_{14}CH_3$ | no effect |

and (xii) a synthetic isoster of ceramide, $C_{16}$-urea-ceramide. In addition, we also report stimulation of ceramidase activity by sphingosine-1-phosphate, and ceramide-1-phosphate. In conclusion, we have identified structural features in natural substrates and synthetic compounds that inhibit mt-CDase. This allows better understanding of the key features in substrate required for interaction with enzyme, and will also help in designing and developing inhibitors of ceramidase.

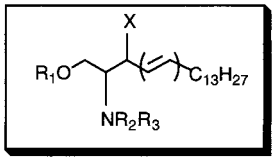
## EXPERIMENTAL PROCEDURES

*Materials and Methods.* Stearylamine, octylamine, dodecylamine, *N*,*N*-dimethylsphingosine and general chemicals were purchased from Sigma. [9,10-$^3$H]Palmitic acid was purchased from American Radiolabeled Chemicals. $^1$H NMR spectra were recorded using a Bruker AVANCE 500 MHz spectrometer equipped with Oxford Narrow Bore Magnet. Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane as internal standard and the listed *J* values are in hertz. Mass spectral data were obtained in positive ion electrospray ionization (ESI) mode on a Finnigan LCQ ion trap mass spectrometer. Samples were infused in methanol solution with an ESI voltage of 4.5 kV and capilary temperature of 200 °C. The purity of all synthesized lipids was >95% as estimated by TLC and $^1$H NMR analysis.

All stereoisomers of $C_{18}$-ceramide were prepared from their corresponding sphingosines as described previously (*15*, *16*). Sphingosines of specific stereochemical foundations [IUB nomenclature: D-*erythro*-(2S,3R); L-*threo*-; (2S,3S)] and the key intermediates: N- and 1,3-O-protected sphingosines, were utilized as basic substrates in the synthesis of the target compounds. Starting from known configurationally stable chiral auxiliary (Garner's aldehyde prepared from L-serine) we synthesized all regio-and diastereoisomeric 2S-sphingosines (*17*, *18*). Starting from D-serine, we had access to all remaining 2R-sphingosines (*17*, *19*). (2S)-3-Keto-sphingoid bases, (2S, 3R, 4E)-sphingosine-1-phosphate and (2S,-3R,4E)-$C_{16}$-ceramide-1-phosphate were synthesized and characterized as described (*20*). (2S,3R,4E)-1-O-methyl-sphingosine was synthesized following a previously described procedure (*21*). The other compounds listed in Tables 1 and 2 were prepared in our laboratory as shown below.

*Synthesis of (2S,3R,4Z)-sphingosine (cis-D-e-Sph).* This compound was prepared from *N*-Boc-4,5-dehydro-D-*erythro*-sphingosine in a two step synthetic sequence (68% overall yield) entailing catalytic hydrogenation of (2S,3R) *N*-Boc-4,5-dehydro-sphingosine over Ni−Raney catalyst in ethanol-pyridine solution (10:1, v/v) as described (*22*). After completion of the reduction, the reaction mixture was filtered and evaporated under reduced pressure, and the obtained crude mixture of cis/trans isomers of *N*-Boc-D-*erythro*-sphingosine was purified by crystallization from hexane. The final deprotection of sphingoid base was performed using trimethylchlorosilane in anhydrous methanol (*20*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH-concentrated $NH_4OH$, 5:1:0.05, v/v/v) and crystallized from hexane (mp 75−76 °C). TLC ($CHCl_3$-MeOH-concentrated $NH_4OH$, 5:1:0.05, v/v/v; $R_f$ 0.45); $^1$H NMR ($CDCl_3$) $\delta$ 5.66 (dtd, $J$ = 11.1, 8.9, 1.0, 5-H), 5.45 (ddt, $J$ = 11.2, 9.2, 1.5, 4-H), 4.43 (dd, $J$ = 8.9, 6.2, 3-H), 3.73 (dd, $J$ = 10.8, 4.5, 1-Ha), 3.68 (dd, $J$ = 10.8, 5.8, 1-Hb), 2.88 (q, $J$ = 5.6, 2-H), 2.15 [m, 2H, C(6)$H_2$], 1.39 [m, 2H, C(7)$H_2$], 1.29 (m, 20H, $CH_2$), 0.90 (t, $J$ = 7.1, $CH_3$); EI-MS ($CH_3OH$, relative intensity, %) *m*/*z* 599.0 ($2M + H^+$, 9), 300.1 ($MH^+$, 100), 282.3 ($MH^+ - H_2O$, 33), ($MH^+ - 2H_2O$, 6). Calcd for $C_{18}H_{37}NO_2$ *m*/*z* 299.3.

Table 2: Structural Specificity for Inhibition of Mitochondrial Ceramidase by Sphingosine Analogues




| compd | stereochemistry | substituents: X, $R_1$, $R_2$, $R_3$ | estimated $IC_{50}$ (mol %) |
|---|---|---|---|
| D-*erythro*-sphingosine<br>**D-e-Sph** | (2S, 3R, 4E) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.04 |
| L-*erythro*-sphingosine<br>**L-e-Sph** | (2R, 3S,4E) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.09 |
| L-*threo*-sphingosine<br>**L-t-Sph** | (2S, 3S, 4E) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.14 |
| D-*threo*-sphingosine<br>**D-t-Sph** | (2R, 3R, 4E) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.11 |
| *cis*-D-*erythro*-sphingosine<br>**cis D-e-Sph** | (2S, 3R, 4Z) | X = OH; $R_1$, $R_2$, $R_3$ = H | weak inhibitor |
| D-*erythro*-dihydrosphingosine<br>**D-e-dh-Sph** | (2S, 3R) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.34 |
| D-*erythro*-dehydrosphingosine<br>**D-e-deh-Sph** | (2S, 3R) | X = OH; $R_1$, $R_2$, $R_3$ = H | 0.25 |
| 1-O-methyl- D-*erythro*-sphingosine<br>**1-O-Me-Sph** | (2S, 3R, 4E) | X = OH; $R_1$ = $CH_3$; $R_2$, $R_3$ = H | weak inhibitor |
| 3-O-methyl-D-*erythro*-sphingosine<br>**3-O-Me-Sph** | (2S, 3R, 4E) | X = $OCH_3$; $R_1$,$R_2$, $R_3$ = H | no effect |
| 3-keto-sphingosine<br>**3-keto-Sph** | (2S, 4E) | X = O; $R_1$, $R_2$, $R_3$ = H | weak inhibitor |
| 3-keto-dihydrosphingosine<br>**3-keto-dh-Sph** | (2S) | X = O; $R_1$, $R_2$, $R_3$ = H | 0.2 |
| 3-keto-dehydrosphingosine<br>**3-keto-deh-Sph** | (2S) | X = OH; $R_1$, $R_2$, $R_3$ = H | weak inhibitor |
| D-*erythro*-sphingosine 1-phosphate<br>**Sph 1-P** | (2S, 3R, 4E) | X = OH; $R_2$, $R_3$ = H<br>$R_1$ = P(O)$(OH)_2$ | activator |
| *N*-methyl-D-*erythro*-sphingosine<br>**N-Me-Sph** | (2S, 3R, 4E) | X = OH<br>$R_1$, $R_2$ = H; $R_3$ = $CH_3$ | 0.13 |
| *N*-stearyl-D-*erythro*-sphingosine<br>**$C_{18}$-Ceramine** | (2S, 3R, 4E) | X = OH; $R_1$, $R_2$ = H<br>$R_3$ = $(CH_2)_{17}CH_3$ | 0.5 |
| *N*,*N*-dimethyl-D-*erythro*-sphingosine<br>***N*,*N*-diMe-Sph** | (2S, 3R, 4E) | X = OH; $R_1$ = H; $R_2$, $R_3$ = $(CH_3)_2$ | weak inhibitor |

*Synthesis of (2S,3R,4E)-N-methyl-sphingosine (N-Me-Sph).* This compound was prepared by reduction of (2S,3R)-*N*-Boc-4,5-dehydro-sphingosine with $LiAlH_4$ (*23*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH-concentrated $NH_4OH$, 4:1:0.05, v/v/v) and crystallized from cold hexane (mp 59−60 °C), TLC ($CHCl_3$-MeOH-concentrated $NH_4OH$, 5:1:0.05, v/v/v; $R_f$ 0.38); $^1$H NMR ($CDCl_3$) $\delta$ 5.89 (dtd, $J$ = 15.4, 6.8, 1.0, 5-H), 5.49 (ddt, $J$ = 15.3, 5.6, 1.1, 4-H), 4.75 (m, 1H, 3-H), 4.10 (dd, $J$ = 13.1, 4.6, 1-Ha), 3.97 (dd, $J$ = 13.0, 3.2, 1-Hb), 2.98 (m, 1H, 2-H), 2.85 (s, 3H, $NCH_3$), 2.07 [q, $J$ = 7.1, C(6)$H_2$], 1.37 [m, 2H, C(7)$H_2$], 1.28 (m, 20H, $CH_2$), 0.88 (t, $J$ = 7.1, $CH_3$). EI-MS ($CH_3OH$; relative intensity, %) *m*/*z* 649.1 ([2M + Na]$^+$, 5) 325.2 ([$C_{20}H_{39}NO_2$]$^+$, 8), 315.3 ([M + 2H ]$^+$, 20), 314.2 (MH$^+$, 100), 296.3 ([MH$^+$ − $H_2O$, 28). Calcd for $C_{19}H_{39}NO_2$ *m*/*z* 313.3.

*Synthesis of (2S,3R,4E)-3-O-methyl-sphingosine (3-O-Me-Sph).* This compound was prepared from 1-O-*tert*-butyl-diphenylsilyl-*N*-t-Boc-D-*erythro*-sphingosine (*21*) in a two step synthetic sequence following methods previously described for a similar class of compounds (*20*, *24*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH-concentrated $NH_4OH$, 6:1:0.05, v/v/v) and isolated as a waxy semisolid (54% yield). TLC ($CHCl_3$-MeOH-concentrated $NH_4OH$, 5:1:0.05, v/v/v; $R_f$ 0.66); $^1$H NMR ($CDCl_3$) $\delta$ 5.65 (dtd, $J$ = 15.3, 8.3, 1.2, 5-H), 5.22 (ddt, $J$ = 15.2, 8.2, 1.1, 4-H), 3.59 (dd, $J$ = 10.9, 4.7, 1-Ha), 3.52 (dd, $J$ = 10.7, 5.8, 1-Hb), 3.42 (dd, $J$ = 8.3, 6.2, 3-H), 3.18 (s, 3H, $OCH_3$), 2.79 (q, $J$ = 5.7, 2-H), 2.0 [m, 2H, C(6)$H_2$], 1.42 [m, 2H, C(7)$H_2$], 1.19 (m, 20H, $CH_2$), 0.79 (t, $J$ = 7.1, $CH_3$); EI-MS ($CH_3OH$, relative intensity, %) *m*/*z* 608.5 (2M$^+$ − $H_2O$, 17), 325.2 ([$C_{20}H_{39}NO_2$]$^+$, 17), 314.1 (MH$^+$, 100), 282.2 (MH$^+$ − $CH_3O$, 65), 264.3 ([M + 2H]$^+$ − 2$H_2O$ − $CH_3$, 5). Calcd for $C_{19}H_{39}NO_2$ *m*/*z* 313.3.

*Synthesis of (2S,3R,4Z)-N-palmitoyl-sphingosine (cis-$C_{16}$-Cer).* This compound was prepared from (2S,3R,4Z)-sphingosine and palmitoyl chloride following a general acylation procedure described previously (*16*, *25*, *26*). The crude was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 94:6, v/v/) and crystallization from ethyl acetate−hexane (6:1, v/v; mp 83−85 °C, 75% yield). TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.41); $^1$H NMR ($CDCl_3$), $\delta$ 6.23 (d, $J$ = 7.3, NH), 5.61 (dtd, $J$ = 11.1, 8.9, 1.0, 5-H), 5.49 (ddt, $J$ = 11.2, 9.2, 1.5, 4-H), 4.63 (dd, $J$ = 8.5, 4.2, 3-H), 4.0 (dd, $J$ = 11.3, 3.6, 1-Ha), 3.84 (m, 2-H), 3.73 (dd, $J$ = 11.4, 3.3, 1-Hb), 2.22 (t, $J$ = 7.6, COC$H_2$), 2.1 [m, 2H, C(6)-$H_2$], 1.64 [m, 2H, C(7)$H_2$], 1.28 (m, 46H, $CH_2$), 0.88 (t, 6H, $J$ = 7. 1, $CH_3$); EI-MS ($CH_3OH$; relative intensity, %) *m*/*z* 1097.6 ([2M − H + Na]$^+$, 100), 826.5 (12), 560.6 (MNa$^+$, 5), 538.2 (MH$^+$, 25), 520.4 (MH$^+$ − $H_2O$, 12). Calcd for $C_{34}H_{67}NO_3$ *m*/*z* 537.5.

*Synthesis of (2S,3R,4E)-1-O-methyl-$C_{16}$-ceramide (1-O-Me-$C_{16}$-Cer).* This compound was prepared from (2S,3R,-4E)-1-O-methyl-sphingosine and palmitoyl chloride follow-

ing the general acylation procedure described previously (*16*, *25*, *26*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and was isolated as a waxy semisolid (66% yield). TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.75); $^1$H NMR ($CDCl_3$) $\delta$ 6.12 (d, $J$ = 6.9, NH), 5.85 (dtd, $J$ = 11.8, 7.9, 1.6, 5-H), 5.45 (ddt, $J$ = 11.2, 9.6, 1.3, 4-H), 4.13 (m, 1H, 3-H), 4.02 (m, 1H, 2-H), 3.69 (m,1H, 1-Ha), 3.55−3.46 (m,1H, 1-Hb), 3.32 (s, 3H, $OCH_3$), 2.21 (m, 2H, $COCH_2$), 2.08 [m, 2H, C(6)$H_2$], 1.62 (m, 2H, $COCH_2CH_2$), 1.24 (m, 46H, $CH_2$), 0.86 (t, 6H, $J$ = 6.9, $CH_3$); EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 1125.6 ([2M − H + Na]$^+$, 100), 552.3 ($MH^+$, 50), 534.4 ($MH^+$ − $H_2O$, 28), 413.2 (15), 325.2 ([$C_{20}H_{39}NO_2$]$^+$, 11), 314.2 ([M + 2H]$^+$ − $COC_{15}H_{31}$, 22), 296.3 ([M + 2H]$^+$ − $H_2O$ − $COC_{15}H_{31}$, 12). Calcd for $C_{35}H_{69}NO_3$ $m/z$ 551.5.

*Synthesis of (2S,3R,4E)-3-O-methyl-$C_{16}$-ceramide (3-O-Me-$C_{16}$-Cer).* This compound was prepared from (2S,3R,-4E)-3-O-methyl-sphingosine and palmitoyl chloride following the same general acylation procedure (*16*, *25*, *26*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and isolated as a waxy semisolid (61% yield). TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.70); $^1$H NMR ($CDCl_3$) $\delta$ 6.20 (d, $J$ = 7.8, NH), 5.74 (dtd, $J$ = 11.1, 8.9, 1.0, 5-H), 5.35 (ddt, $J$ = 11.2, 9.2, 1.5, 4-H), 3.95 (m, 1-Ha), 3.90 (m, 2-H), 3.83 (dd, $J$ = 7.8, 3.7, 3-H), 3.55 (m, 1-Hb), 3.25 (s, 3H, $OCH_3$), 2.98 (dd, $J$ = 10.2, 2.4. 1-OH), 2.21 (t, $J$ = 7.6, $COCH_2$), 2.06 [q, $J$ = 7.1, C(6)-$H_2$], 1.62 (m, 2H, $COCH_2CH_2$), 1.38 (m, 2H, C(7)$H_2$), 1.21 (m, 46H, $CH_2$), 0.87 (t, 6H, $J$ = 7.0, $CH_3$). EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 1125.6 ([2M − H + Na]$^+$, 100), 552.3 ($MH^+$, 50), 534.4 ($MH^+$ − $H_2O$, 28), 314.2 ($M^+$ − [$COC_{15}H_{31}$], 22). Calcd for $C_{35}H_{69}NO_3$ $m/z$ 551.5.

*Synthesis of (2S,4E)-3-keto-$C_{16}$-ceramide (3-keto-$C_{16}$-Cer).* This compound was prepared from D-*erythro*-$C_{16}$-ceramide by the selective oxidation of its secondary hydroxyl group following the procedure described for the *N*-acetyl derivative (*16*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and crystallized from acetone (mp 74−76 °C, 75% yield). TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.73); $^1$H NMR ($CDCl_3$). $\delta$ 7.09 (dt, $J$ = 15.8, 7.2, 5-H), 6.71 (d, $J$ = 6.1, NH), 6.25 (d, $J$ = 15.8, 4-H), 4.88 (m, 1H, 2-H), 3.94 (m, 1H, 1-Ha), 3.79 (m, 1H, 1-Hb), 3.33 (m, 1H, OH), 2.25 [m, 4H, C(6)$CH_2$, $COCH_2$], 1.63 (m, 2H, $CH_2$), 1.46 (m, 2H, $CH_2$), 1.24 (m, 44H, $CH_2$), 0.87 (t, 6H, $J$ = 7.0, $CH_3$); EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 1094.5 ([2M + Na]$^+$, 60), 1093.4 ([2M − H + Na]$^+$, 100), 948.2 (22), 558.5 ($MNa^+$, 8), 535.9 ($M^+$, 5). Calcd for $C_{34}H_{65}NO_3$ $m/z$ 535.5.

*Synthesis of (2S,3R,4E)-N-methyl-$C_{18}$-ceramide (N-Me-$C_{16}$-Cer).* This compound was prepared from (2S,3R,4E)-*N*-methyl-sphingosine and stearoyl chloride following a general acylation procedure described previously (*16*, *25*, *26*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and crystallized from ethyl acetate (mp 79−81 °C, 55% yield). TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.42); $^1$H NMR ($CDCl_3$); mixture of two conformers, $\delta$ 5.63 (m, 1H, 5-H), 5.40 (m, 1H, 4-H), 4.43 (m, 0.45H, 2-H), 4.12 (t, 0.55H, $J$ = 10.2, 3-H), 4.75 (t, 0.45H, $J$ = 10.2, 3-H), 3.97 (dd, 0.45H, $J$ = 3.6, 10.8, 1-Ha′), 3.91 (dd, 0.55H, $J$ = 3.0, 8.2, 1-Ha″), 3.83 (m, 1H, 1-Hb″, 2-H), 3.75 (dd, 0.55H, $J$ = 8.9, 10.7, 1-Hb′), 2.93 (s, 1.65H, $NCH_3$), 2.77 (s, 1.35H, $NCH_3$), 2.44 (m, 0.9H, $COCH_2$), 2.33 (m, 1.1H, $COCH_2$ ), 2.03 [m, 2H, C(6)$H_2$], 1.61 (m, 2H, $COCH_2CH_2$), 1.27 (m, 50H, $CH_2$), 0.86 (t, 6H, $J$ = 7.0, $CH_3$); EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 1160.4 ([2M + H]$^+$, 15), 1159.3 ($2M^+$, 20), 893.2 ([2M + H]$^+$ − $COC_{17}H_{35}$, 22), 580.3 ($MH^+$, 100), 562.3 ($MH^+$ − $H_2O$, 21), 325.2 ([$C_{20}H_{39}NO_2$]$^+$, 14), 314.1([$MH^+$ − $COC_{17}H_{35}$, 52), 264.5 ([M + 2H]$^+$ − $2H_2O$ − $CH_3$ $COC_{17}H_{35}$, 5). Calcd for $C_{37}H_{73}NO_3$ $m/z$ 579.5.

*Synthesis of (2S,3R,4E)-N-stearyl-sphingosine ($C_{18}$-ceramine).* This compound was synthesized by the reduction of the amido group of (2S,3R,4E)-$C_{18}$-ceramide following the procedure described previously (*27*). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH-concentrated $NH_4OH$, 8:1:0.05, v/v/v) and isolated as a waxy semisolid (54% yield). TLC ($CHCl_3$-MeOH-concentrated $NH_4OH$, 5:1:0.05 v/v/v; $R_f$ 0.83); $^1$H NMR ($CDCl_3$), $\delta$ 5.75 (dtd, $J$ = 15.3, 6.3, 1.2, 5-H), 5.48 (ddt, $J$ = 15.2, 6.6, 1.1, 4-H), 4.23 (m, 1H, 3-H), 3.69 (m, 2H, 1-H), 2.67 (m, 2H, $NHCH_2$), 2.61 (m, 1H, 2-H), 2.05 [q, 2H, $J$ = 7.1, C(6)$H_2$], 1.50 (m, 2H, $NHCH_2CH_2$), 1.25 (m, 52H, $CH_2$), 0.86 (t, 6H, $J$ = 6.9, $CH_3$). EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 552.6 ($MH^+$, 100), 325.2 ([$C_{20}H_{39}$-$NO_2$]$^+$, 12). Calcd for $C_{36}H_{73}NO_2$ $m/z$ 551.5.

*Synthesis of (2S,3R)-N-[2-(1,3-dihydroxy-4E-octadecene)]-N′-hexadecane-urea ($C_{16}$-urea-Cer).* To a solution of (2S,-3R,4E)-sphingosine (43.5 mg, 0.145 mmol) in anhydrous acetonitrile (4 mL) and anhydrous diethyl ether (3 mL), hexadecyl isocyante (49.2 mg, 0.181 mmol) was added, and the mixture was stirred at room temperature under nitrogen for 4 h. After evaporation of the solvents under a reduced pressure, residue was crystallized from acetone-methanol (1:3; v/v) to give a pure urea isoster of ceramide as a white microcrystalline powder (mp 105−106 °C, 68.1 mg, 83% yield); TLC ($CHCl_3$-MeOH, 10:1, v/v; $R_f$ 0.39); $^1$H NMR (MeOD-$CDCl_3$), $\delta$ 5.46 (dtd, $J$ = 15.4, 6.7, 1.4, 5-H), 5.35 (ddt, $J$ = 15.4, 6.9, 1.3 4-H), 3.87 (m, 1H, 3-H), 3.41 (m, 2H, 1-Ha, 2-H), 3.37 (dd, $J$ = 10.2, 3.4, 1-Hb), 2.85 (m, 2H, $NHCH_2$), 1.78 (m, 2H, C(6)$H_2$), 1.20 (m, 2H, $NHCH_2$-$CH_2$), 1.0 (m, 48H, $CH_2$), 0.62 (t, 6H, $J$ = 7.2, $CH_3$). EI-MS ($CH_3OH$; relative intensity, %) $m/z$ 1134.4 ([2M + H]$^+$, 31), 1133.4 ($2M^+$, 43), 567.3 ($MH^+$, 100), 549.4 ($MH^+$ − $H_2O$, 67), 409.3 (18), 264.3 ([M + 2H]$^+$ − $2H_2O$ − $CONHC_{16}H_{33}$, 8). Calcd for $C_{35}H_{70}N_2O_3$ $m/z$ 566.5.

*Synthesis of Radiolabeled Compounds. (1) (2S,3R,4E) [N-9,10-$^3$H]-Palmitoyl-sphingosine.* [N-$^3$H]-$C_{16}$-ceramide was prepared as described (*28*).

*(2) [3-$^3$H] (2S,3R)-N-[2-(1,3-dihydroxy-4E-octadecene)], N′-hexadecanene-urea.* [3-$^3$H]-$C_{16}$-urea-Cer was prepared from [3-$^3$H]-sphingosine (*29*) and hexadecyl isocyanate following the procedure described for its nonradioactive analogue.

*Enzymatic Assays. (1) Ceramidase.* mt-CDase was isolated and purified from rat brain as described (*12*). Enzyme activity was determined by either one of the two following methods:

*(a) Radioactive Assay.* mt-CDase activity was determined by measuring the release of radioactive fatty acid from tritiated ceramide ([$^3$H]-$C_{16}$-Cer), labeled in the acyl chain. Briefly, organic solutions of ceramide and its analogues were initially mixed together, solvents were completely evaporated under nitrogen, then the dried lipids were dispersed in Triton-X 100 by sonication and vigorous vortexing. The reaction assay contained (in a final volume of 200 $\mu$L)

enzyme (5−10 ng) and 10 nmol of tritiated $C_{16}$-ceramide ($1 \times 10^5$ dpm) delivered in 100 $\mu$L of 1% Triton X-100 micelles in a glycine buffer pH 9.5 (200 mM). The reaction mixture was incubated at 37 °C for 1 h (the reaction was linear for at least 2 h) and terminated with the consecutive addition of 2 mL of isopropyl alcohol:heptane:1 N NaOH solution (4:1:0.1 v/v/v), water (1 mL), and heptane (1 mL). Following phase separation by centrifugation, the lower layer was washed twice with 1 mL of heptane, then sulfuric acid (1 mL, 1 N) and heptane (2 mL) were added, and the upper phase containing the released fatty acid was counted (hydrolyzed [$^3$H]$C_{16}$-ceramide was always less than 10%).

*(b) HPLC-Assay*. Enzyme activity was also determined by measuring the amount of sphingosine released. Basically, the assay was initially the same as described above but using nonradioactive ceramide. The reaction was stopped with 800 $\mu$L of chloroform:methanol (2:1 v/v) mixture followed by the addition of phyto-sphingosine (0.5 nmol) and dihydrosphingosine (1 nmol) as internal standard and carrier, respectively. The reaction mix was then extracted with NaOH (50 $\mu$L, 1 N), and the lower chloroform layer containing the sphingosine was further washed with water (100 $\mu$L) and dried. The released sphingosine was quantified as the orthophthaldehyde (OPA) derivatives as described (*30*). Briefly, HPLC analysis was conducted using a Waters 501 HPLC pump model with a 5 $\mu$m $C_{18}$ Ultrasphere ODS Beckmann column (4.6 cm × 25 cm) with a $C_{18}$-guard column. The solvent system was methyl alcohol:potassium phosphate buffer (5 mM), pH 7.0 (90:10 v/v) at a flow rate of 1 mL/min. A Shimadzu RF-551 spectrofluorometer detector was used with excitation and emission wavelengths of 340 and 455 nm, respectively. The retention times were 12, 18, and 26 min for phyto-sphingosine, sphingosine and dihydrosphingosine, respectively. Activity was determined relative to the phytosphingosine OPA peak.

*(2) Effect of Various Compounds on Ceramidase Activity*. The effects of stereochemical and structural analogues of ceramide and sphingosine on mt-CDase were investigated. Hydrolysis of D-*erythro*-$C_{16}$ or D-*erythro*-$C_{18}$-ceramide at a concentration of 0.625 mol % (50 $\mu$M) by mt-CDase was determined in the presence of different concentrations [0−1.25 mol % (0−100 $\mu$M)] of compounds listed in Tables-1 and 2. Results were expressed as percent of the control ceramidase activity.

## RESULTS

*Modifications of Ceramide. (1) Stereochemical Modification at the C2−C3-Chiral Centers; Effects of the Optical Isomers.* Initially, we investigated the effect of ceramide stereoisomers on the hydrolysis of D-e-Cer by mt-CDase. The enzyme was assayed at a constant substrate (D-e-$C_{18}$-Cer) concentration, 0.625 mol % (50 $\mu$M), and in the presence of varying concentrations, 0−1.25 mol % (0−100 $\mu$M), of ceramide stereoisomers. Enzyme activity was determined by measuring the amount of sphingosine released using the HPLC assay. All ceramide stereoisomers at 1.25 mol % (100 $\mu$M) inhibited mt-CDase activity to the same extent (77%) (Figure 2a). At lower concentrations, however, the *threo* isomers showed more inhibitory effects than the *erythro* isomer a 50% inhibition of mt-CDase activity was obtained at 0.11, 0.21, and 0.26 mol % (8.8, 16.6, and 20 $\mu$M) for L-*threo*, D-*threo*, and L-*erythro*, respectively (Table 1). We also tested the type of the inhibition demonstrated by the enantiomer of the natural substrate. Ceramidase was then assayed in the presence of different constant concentrations of L-*erythro*-$C_{18}$-ceramide, 0.125 and 0.314 mol % (10 and 25 $\mu$M), while varying D-e-$C_{18}$-Cer concentration from 0 to 2.5 mol % (0−200 $\mu$M). An increase in the $K_m$ value was observed with no change in $V_{max}$ (Figure 2b), indicating a competitive type of inhibition by the L-*erythro*-$C_{18}$ enantiomer. Therefore, whereas mt-CDase only hydrolyzes the natural D-*erythro* isomer, all other stereoisomers were inhibitory with the L-*threo*-isomer being the most potent. These findings confirm further the stereospecificity of the enzyme to its substrate.

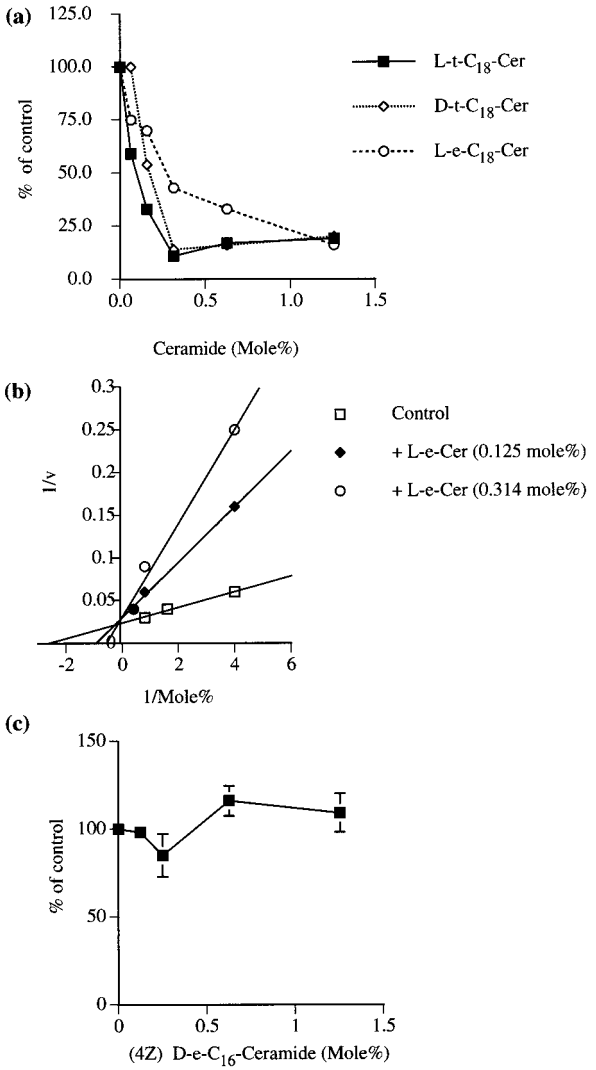



FIGURE 2: Effects of ceramide stereoisomers on mt-CDase activity. (a) Mt-CDase activity was determined using the HPLC assay for sphingoid products as described in the Experimental Procedures at constant D-*erythro*-$C_{18}$-ceramide (0.625 mol %) while varying the concentration of ceramide stereoisomers. Results are means of 2 separate experiments. (b) Double reciprocal representation of L-*erythro*-$C_{18}$-ceramide at 0.125 and 0.314 mol %. (c) Effect of *cis*-D-*erythro*-ceramide on mt-CDase activity. Results are average ± SD of 3 different determinations.

*(2) Effect of the Geometrical C4−C5 cis Isomer*. Next, we evaluated the effects of the *cis*-D-*erythro*-$C_{16}$-ceramide, the 4-Z isomer, on ceramidase activity. This compound was prepared as described in the Experimental Procedures. Using

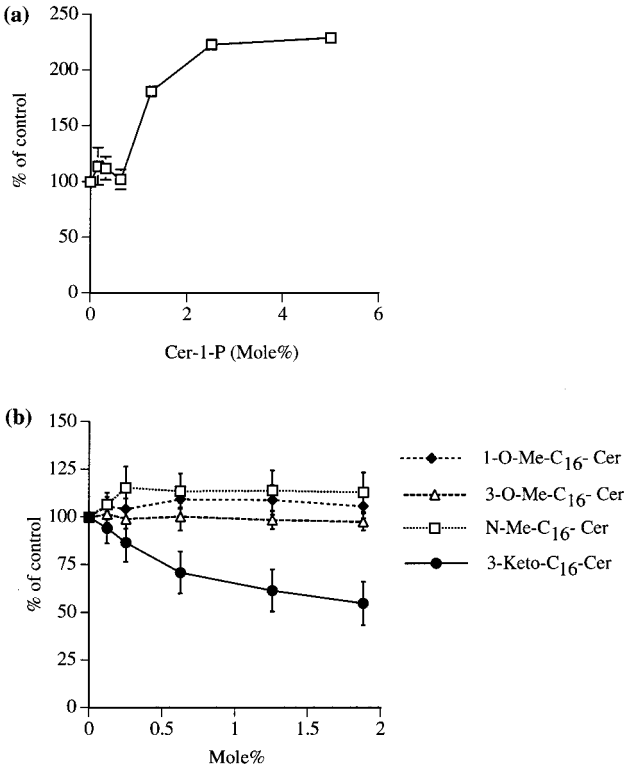



FIGURE 3: Effects of functional group modification of ceramide on mt-CDase. Mt-CDase activity was carried out as described in the Experimental Procedures at constant [$^3$H]-D-*erythro*-$C_{16}$-ceramide while varying the concentrations of (a) Cer-1-P; and (b) 1-O-methyl-ceramide (1-O-Me-$C_{16}$-Cer), 3-O-methyl-ceramide (3-O-Me-$C_{16}$-Cer), *N*-methyl-ceramide (N-Me-$C_{16}$-Cer), and 3-keto-ceramide (3-keto-$C_{16}$-Cer). Results are means ± SD of three separate experiments.

HPLC analysis, ceramidase was assayed with (4E) D-*erythro* $C_{16}$-ceramide (0.625 mol %) in the presence of varying amounts of the 4Z isomer: 0−1.25 mol % (0−100 $\mu$M). Results presented in Figure 2c show no significant effect of the cis isomer on substrate hydrolysis, suggesting an important role for the trans double bond in the interaction of ceramide with ceramidase.

*(3) Modification of the Functional Groups.* Ceramide is characterized by several potentially reactive centers, which include two hydroxyl groups, a double bond in the sphingosine backbone and an amide bond. Hence we investigated the role of these structural elements on ceramidase activity.

*(a) Phosphorylation*. Ceramide has a primary ($C_1$) and a secondary ($C_3$) hydroxyl groups. The phosphorylation of the primary hydroxyl group yields ceramide-1-phosphate (Cer-1-P), which appears to exist in vivo. The effects of varying concentrations (0−5 mol %; 0−400 uM) of Cer-1-P on the hydrolysis of [$^3$H]-$C_{16}$-ceramide (0.625 mol %) were determined. A significant increase (220%) in ceramide hydrolysis was observed (Figure 3a), indicating that phosphorylation of the primary hydroxyl group not only prevents hydrolysis of the molecule, but actually enhances ceramidase activity.

*(b) O-Methylation*. Next, we studied the effect of the O-methylated-ceramides on mt-CDase activity. Both, 1-O-methyl and 3-O-methyl ceramides were synthesized as described in the Experimental Procedures. Their effect on mt-CDase activity was tested at constant D-e-$C_{16}$-Cer (0.625 mol %) while varying the concentration of 1- and 3-O-methyl ceramides from 0 to 1.88 mol % (0−150 $\mu$M). Data (Figure 3b) show no effect of either compound on ceramidase activity, indicating that methylation of either of the hydroxyl groups inhibits the interaction of ceramide with the enzyme.

*(c) Oxidation*. We then investigated the effect of the oxidized secondary hydroxyl group (C3) of ceramide on ceramidase activity. 3-Keto analogue of D-e-$C_{16}$-Cer served as a substrate (data not shown), and when tested against ceramide as a substrate, it behaved as a competitive substrate with a maximal inhibition (53%) at 0.625 mol % (50 $\mu$M) of 3-ketoceramide (Figure-3b).

*(d) N-Methylation*. We also tested the effect of the introduction on of *N*-methyl group into the secondary amide function of ceramide on mt-CDase. Enzyme activity was assayed in the presence of constant [$^3$H]-D-*erythro*-$C_{16}$-ceramide (0.625 mol %) while varying the concentration of *N*-methyl-$C_{16}$-ceramide (0−1.8 mol %). Results shown in Figure 3b indicate that *N*-methyl-ceramide did not affect ceramidase activity. Thus, replacement of hydrogen in NHCO-function of ceramide with a methyl group prevented its interaction with mt-CDase. This may suggest an important role of the free amido hydrogen in ceramide hydrolysis, but also can raise the issue of steric effects imparted by the bulkier methyl group.

In conclusion our data suggest the involvement of the primary and the secondary hydroxyl groups and the free amido hydrogen of ceramide in the interaction of ceramide with ceramidase.

*Effects of Products of Ceramidase on mt-CDase Activity*. Ceramidase action on D-*erythro*-$C_{16}$-ceramide yields palmitic acid and sphingosine, hence their effects on mt-CDase activity were investigated.

*(1) Effect of Palmitic Acid*. The enzyme was assayed using [$^3$H]-D-*erythro*-$C_{16}$-ceramide 0.625 mol % (50 $\mu$M) as a substrate and in the presence of varying palmitic acid concentrations: 0−1.25 mol % (0−100 $\mu$M). Activity was then determined by measuring the amount of [$^3$H]-fatty acid released. Results (Figure 4a) showed no significant variation in the fatty acid released with increasing concentration of palmitate, indicating that fatty acids exert no negative feedback effects on ceramidase.

*(2) Effect of Sphingosine.* Next we studied the effects of D-*erythro*-sphingosine (0−0.625 mol %), the second product of ceramide hydrolysis, on ceramidase activity. Unlike palmitic acid, D-*erythro*-sphingosine inhibited ceramide hydrolysis in a concentration dependent manner with an $IC_{50}$ of 0.04 mol % (3.3 $\mu$M), and a maximal inhibition (98%) at D-*erythro*-sphingosine concentration of 0.625 mol % (50 $\mu$M) (Figure 4b). To determine whether the inhibitory effect of D-*erythro*-sphingosine displays stereospecificity, we tested the other sphingosine stereoisomers. Figure 4b shows that whereas all stereoisomers of D-*erythro*-sphingosine followed a similar inhibitory pattern at low concentrations, 0.015−0.125 mol % (1−10 $\mu$M), the extent of inhibition, however, by the L-*erythro* (98%) isomer was more than the *threo* diastereomers (60% for L-*threo* and 75% for D-*threo*) at higher concentrations.

In conclusion, whereas fatty acids have no effect, all sphingosine stereoisomers inhibited ceramidase suggesting a possible negative feed back regulatory role of sphingosine on the enzyme.

*Modifications of Sphingosine. (1) Effect of Saturation and Desaturation at the C4−C5 Position*. A distinctive feature

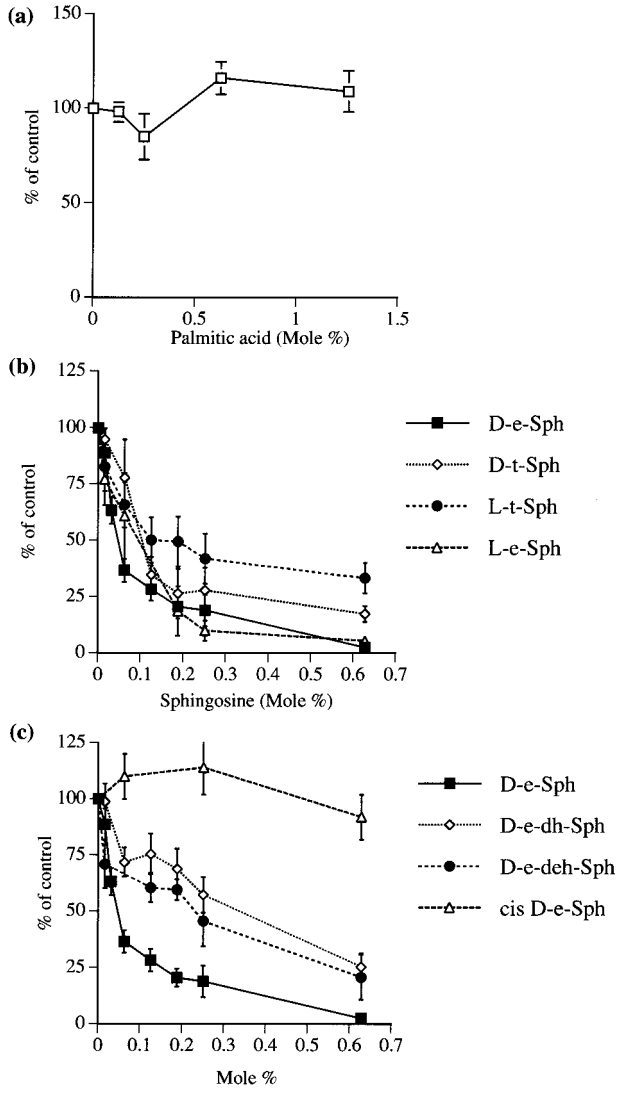



FIGURE 4: Effects of products of the ceramidase reaction on mt-CDase activity. The effects of increasing concentrations of palmitate (a) and sphingosine stereoisomers (b) on mt-CDase activity were determined using radiolabeled ceramide substrate. (c) The effects of desaturation, saturation, and configuration of the 4−5 double bond on ceramidase activity. Enzyme activity was determined at constant D-*erythro*-ceramide while varying the concentration of D-*erythro*-sphingosine, D-*erythro*-dihydrosphingosine, D-*erythro*-dehydrosphingosine, and *cis*-D-*erythro*-sphingosine. Results are means ± SD of three separate experiments.

of sphingosine and ceramide is the rigid 4E trans double bond that joins the carbon chain to the chiral backbone. Consequently, we investigated the role of this double bond. Toward this objective, three different variations of the C4−C5 position were synthesized and examined: the alkene-containing D-*erythro*-sphingosines (E, C4═C5, and Z, C4═C5); the reduced sphingosine (C4−C5); namely, D-*erythro*-dihydrosphingosine (D-e-dh-Sph); and the alkyne (C4≡C5) analogue of sphingosine, D-*erythro*-dehydrosphingosine (D-e-deh-Sph). All analogues of sphingosine inhibited the mt-CDase activity in a concentration dependent manner, 0−0.625 mol % (0−50 $\mu$M), with D-e-Sph being the most potent and D-e-dh-Sph the least potent (Figure 4c). The estimated $IC_{50}$ values were 0.04, 0.25, and 0.34 mol % for D-e-Sph, D-e-deh-Sph, and D-e-dh-Sph, respectively. These results indicate that the presence of the double bond, though not necessary, increases significantly the extent of inhibition.

*(2) Effect of the cis Isomer of* D-*erythro-Sphingosine.* Since the naturally occurring sphingosine is the trans isomer, we tested the effect of the cis isomer of sphingosine on mt-CDase activity. Contrary to the significant inhibition in ceramidase activity by the trans isomer, the cis isomer had no effect at concentrations lower than 0.5 mol % (Figure 4c). Results (Figure 4c) show that at equimolar concentration 0.625 mol % (50 $\mu$M), the trans isomer inhibited significantly (98%) ceramide hydrolysis whereas the cis isomer resulted only in 10% inhibition. A maximal inhibition of 32% was obtained by the cis isomer at higher concentrations, greater than 2 mol % (data not shown), indicating that the cis isomer is a less potent inhibitor than its natural geometric isomer and suggesting a role for the orientation of the trans double bond in interaction with the enzyme.

*(3) Effect of the Modified Hydroxyl Groups. (a) Phosphorylation*. Sphingosine-1-phosphate (S-1-P) is a natural product of sphingolipid metabolism, thus its effect on the hydrolysis of tritiated $C_{16}$-ceramide (0.625 mol %) was investigated. Similar to Cer-1-P, S-1-P tested over a wide range of concentrations 0−5 mol % (0−400 $\mu$M) increased (180%) the hydrolysis of ceramide (Figure 5a). Therefore, contrary to the effect of sphingosine on ceramidase activity, phosphorylation of the primary hydroxyl group of sphingosine not only abolishes the inhibitory effect by sphingosine but also enhances ceramide hydrolysis.

*(b) O-Methylation*. Modification of the primary and the secondary hydroxyl groups of sphingosine by methylation into the corresponding 1-O-methyl and 3-O-methyl sphingosines, respectively was performed as described in the Experimental Procedures. Both compounds were then tested for their effect on mt-CDase at 0−1.25 mol % (0−100 $\mu$M). No significant inhibition by either compound was observed (Figure 5b). At 0.6 mol %, a concentration where D-e-sphingosine totally inhibited the activity, 3-O-Me-Sph had no effect whereas 1-O-Me-Sph inhibited only by ∼25%. In conclusion, O-methylation of sphingosine at the hydroxyl group in position 3 abolishes the inhibitory effect of sphingosine, and the O-methylation at the C1 position severely affects the inhibitory effect of sphingosine.

*(c) Oxidation.* Results in Figure 3b showed that the 3-keto-ceramide inhibits ceramidase activity. To determine whether this is due to the keto-sphingoid backbone, the corresponding keto analogues of D-e-Sph, D-e-dh-Sph, and D-e-deh-Sph were synthesized (refer to the Experimental Procedures), and their effects on ceramidase were determined. Surprisingly, compared to their non-oxidized analogues, the keto-compounds followed an opposite profile. Whereas D-e-dh-Sph was the least potent (Figure 4c), its keto analogue had the most potent effect among the keto-compounds tested. A maximal inhibition of 75% was obtained at 0.625 mol %, (Figure 5c). On the other hand, contrary to the significant inhibitory effect of sphingosine and dehydrosphingosine (98 and 75%; Figure 4c) on mt-CDase, their corresponding 3-keto derivatives had relatively small effects (Figure 5c). A maximal inhibition of 15% and 35% for 3-keto-deh-Sph and 3-keto-Sph, respectively, was obtained at 0.625 mol % (50 $\mu$M). Our results indicate that the oxidation of the secondary alcohol to the keto group reduces the inhibitory effect of sphingosine but enhances that of dihydrosphingosine.

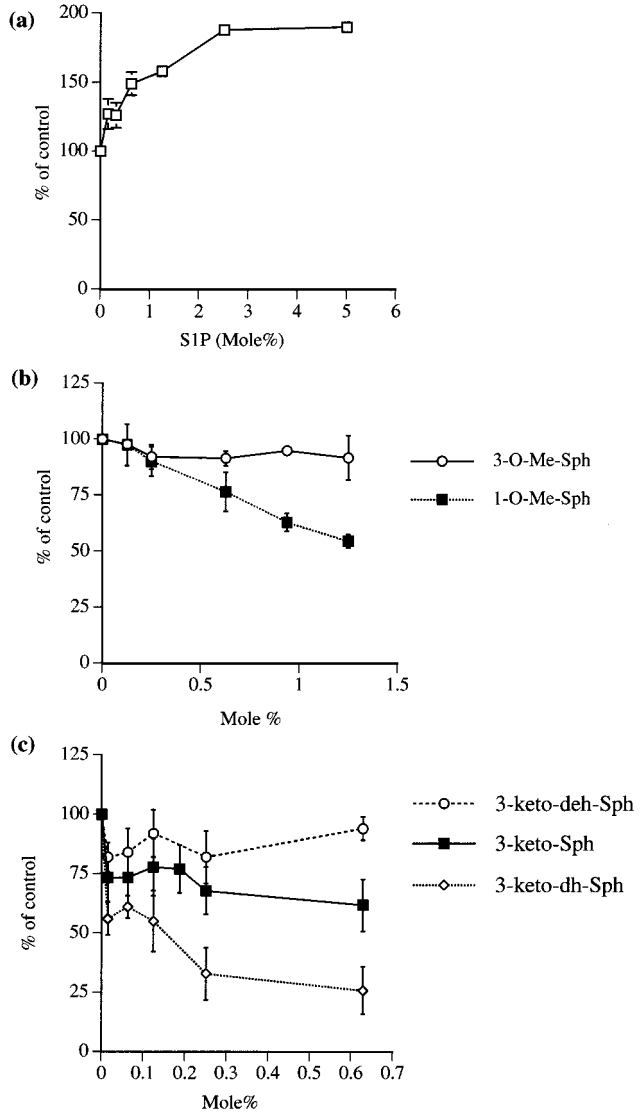



FIGURE 5: Effects of the modified hydroxyl groups of sphingosine on mt-CDase. Mt-CDase activity was carried out as described in the Experimental Procedures at constant D-*erythro*-ceramide while varying the concentrations of (a) Sph-1-P; (b) D-e-Sph, 1-O-Me-Sph, 3-O-Me-Sph; or (c) 3-keto-Sph, 3-keto-dh-Sph, and 3-keto-deh-Sph. Results are expressed as means ± SD of three separate experiments.

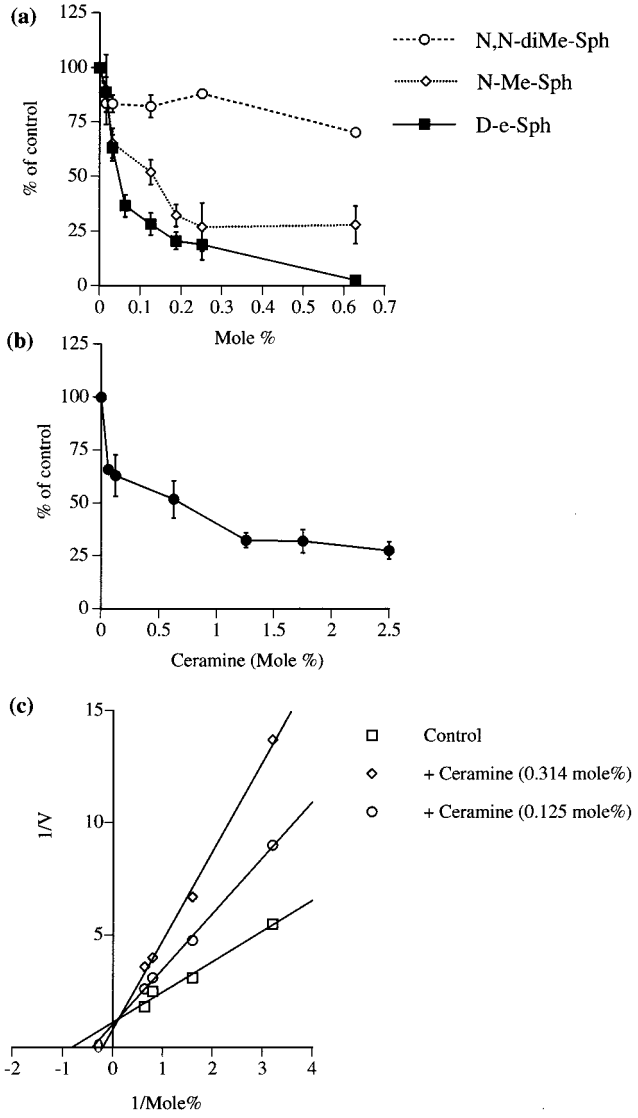



FIGURE 6: Effects of N-alkyl-sphingosines on mt-CDase. Mt-CDase activity was determined as described in the Experimental Procedures at constant D-*erythro*-ceramide while varying the concentration of (a) D-e-Sph (replotted from Figure 4b for comparison), N-Me-Sph, and *N*,*N*-diMe-Sph. (b) dose response effect of ceramine on mt-CDase activity. (c) Double reciprocal representation of ceramine effect at 0. 125 and 0. 314 mol %. Results are expressed as means ± SD of three separate experiments for panels a and b, and as means of two different experiments for panel c.

*(d) Role of the Free NH-Amino-Function on CDase Activity: Effect of N-Alkyl-sphingosines.* Our results on mt-CDase inhibition by sphingosine raise the question of whether the inhibition may be attributed to the primary amine group of sphingosine. Consequently, we investigated the effect of N-alkylated sphingosines on enzyme activity. Results (Figure 6a) show that whereas N-Me-Sph exhibited an inhibitory effect (75%), further methylation reduced the extent of inhibition significantly. Even at a concentration 10-fold that of *N*-methyl-sphingosine (2.5 mol %), *N*,*N*-diMe-Sph resulted in a maximal inhibition of only 30% (data not shown). Therefore, inhibition by sphingosine is significantly reduced by *N*,*N*-dimethylation. It may suggest an important role of the free amino hydrogen of sphingosine in the interaction with the enzyme, but also can raise the issue of steric effects of the larger methyl groups.

*N*-Stearyl-sphingosine ($C_{18}$-ceramine), a long-chain homologue of *N*-methyl-sphingosine also inhibited ceramidase activity significantly (75%) at 1.25 mol % (Figure 6b). The inhibition by ceramine (0.125 and 0.314 mol %) increased the Km but had no effect on the $V_{max}$, indicating that it is a competitve inhibitor of ceramidase (Figure 6c). These results confirm further the role of the free N−H group and identify ceramine as a competitive inhibitor of mt-CDase.

*Effect of Urea Isoster of Ceramide on mt-CDase Activity.* The amide bond in ceramide introduces another rigid element to the molecule linking the sphingosine backbone to the fatty acyl chain. Moreover, the possible existence of inter-/intramolecular hydrogen bonding between the hydrogen of the amido group NHCOR and the hydroxyl groups in ceramide may influence ceramide−enzyme interactions as shown for *N*-methylated ceramide. We further modified amide function of ceramide: NHCOR to its urea isoster NHCONHR, namely urea-ceramide ($C_{16}$-urea-Cer). Ceramidase activity was determined at constant D-e-$C_{16}$-cer 0.625 mol % (50 $\mu$M) while varying the concentration of $C_{16}$-urea-

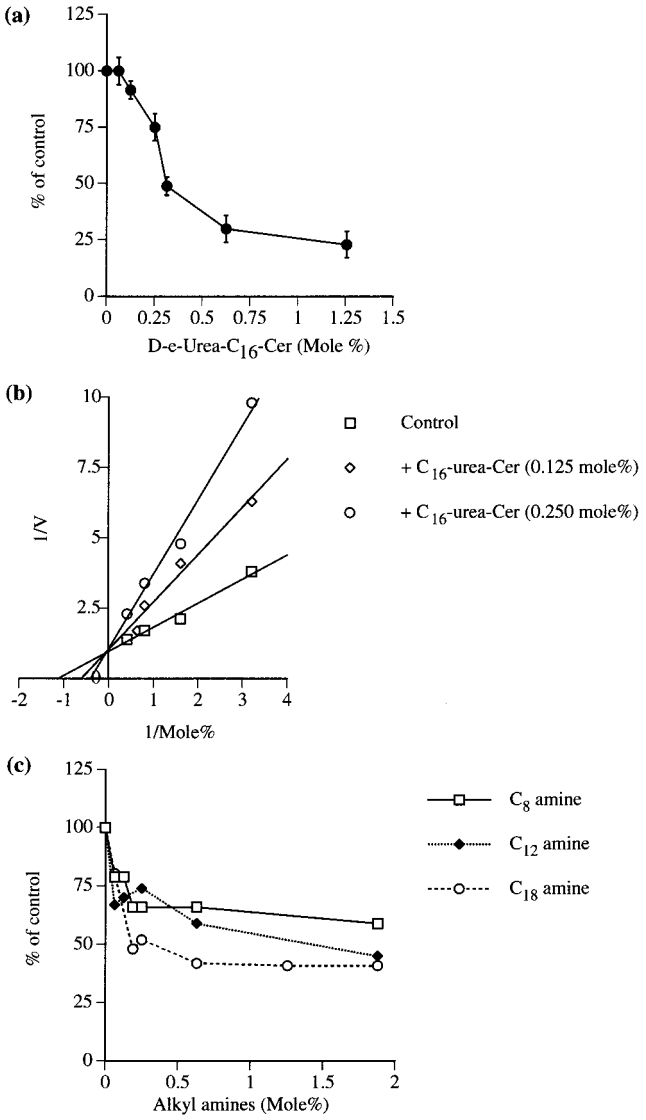



FIGURE 7: Effects of the urea isoster of ceramide on mt-CDase. Mt-CDase activity was determined using the radiolabeled substrate. (a) Inhibition of mt-CDase by $C_{16}$-urea-Cer. (b) double reciprocal representation of $C_{16}$-urea-Cer effect at 0.125 and 0.251 mol %. (c) effects of increasing concentration of alkylamines: $C_8$, $C_{12}$, and $C_{18}$ on mt-CDase. Results are means ± SD of three separate experiments for panel a, and means of two different experiments for panels b and c.

Cer 0−1.25 mol % (0−100 $\mu$M). A significant inhibition (75%) in ceramidase activity was obtained at 0.625 mol % (50 $\mu$M) with an $IC_{50}$ of 0.31 mol % (25 $\mu$M) (Figure 7a). To determine the type of inhibition, ceramidase was assayed in the presence of 0.125 and 0.25 mol % (10 and 20 $\mu$M) of $C_{16}$-urea-Cer while varying D-e-$C_{16}$-Cer concentration (0.1−2.5 mol %). Kinetic analysis by double reciprocal plots (Figure 7b) showed an increase in the Km value with no variation in the maximal velocity, indicating that $C_{16}$-urea-Cer is a competitive inhibitor of ceramidase with respect to the ceramide substrate.

However, the presence of the two potentially hydrolyzable amide bonds [Sph-$NH^a$-$CO^b$-NH-R] in the urea-isoster raises the question if the observed inhibition is due to D-*erythro*-$C_{16}$-urea-ceramide acting as a competitive substrate or possibly even due to the released sphingosine or N-palmitylamine. Consequently, [$^3$H]-$C_{16}$-urea-Cer labeled in the sphingosine backbone was synthesized as described in the Experimental Procedures. Should mt-CDase hydrolyze the amide bond at position "a", then radioactive sphingosine would be released. No [$^3$H]sphingosine was detected after TLC separation, (data not shown) indicating that ceramidase does not hydrolyze the "a" amide bond of urea ceramide.

On the other hand, if hydrolysis occurs at position "b" of urea ceramide it will yield N-alkylamine, which at the expected released levels was not detectable by HPLC. To further eliminate that this potential product may be an inhibitor, we examined the effect of N-alkylamines: $C_8$ (octyl), $C_{12}$ (dodecyl), and $C_{18}$ (stearyl) on mt-CDase activity. All tested amines at 0−1.88 mol % (0−150 $\mu$M) inhibited the release of [$^3$H]palmitic acid from D-*erythro*-$C_{16}$-ceramide (0.628 mol %, 50 $\mu$M). N-octylamine was the least potent showing a maximal inhibition of 40% over a wide range of concentrations [0.25−1.88 mol % (20−150 $\mu$M)]. On the other hand, *N*-stearylamine ($C_{18}$) exhibited the highest inhibitory effect (54%) on mt-CDase (Figure 7c) but was significantly less than that obtained by the urea isoster (85%), thus ruling out the possibility of bond hydrolysis at the "b" amide bond.

These results indicate that urea ceramide is not a substrate but is a competitive inhibitor of the enzyme.

## DISCUSSION

The regulation of ceramide and sphingosine levels in vivo is achieved by many enzymes, particularly ceramidases. A nonlysosomal ceramidase was isolated and characterized from rat brain (*12*). The purified enzyme showed exquisite stereospecificity for the hydrolysis of D-*erythro*-ceramides, suggesting stringent structural requirements for the substrate. In this study, we investigated the key features of ceramide structure necessary for recognition by and interaction with mt-CDase.

Toward this objective, analogues of ceramide were synthesized and tested for their effects on mt-CDase. In addition, analogues of sphingosine were also developed since sphingosine is a defining component of ceramide, a product of the reaction and a competitive inhibitor of ceramidase (*12*). The design of compounds was based mainly on investigation of (1) the stereochemistry at C2 and C3 positions; (2) the primary and secondary hydroxyl groups; (3) the 4−5 trans double bond; (4) the amide bond; and (5) the $NH_2$ function of sphingosine.

The results from this study disclose that even small modifications of ceramide can either prevent interaction with the enzyme or convert the substrate into an inhibitor. These results provide significant insight into the molecular interactions of substrate (and product) with the enzyme.

Indeed, the determination of requirements for hydrolysis of ceramide by the enzyme, shows that of all four optical isomers, only the D-*erythro*-ceramide is a substrate (unpublished data). Also, the enzyme requires the amide bond bearing a long fatty acid, and the free primary and secondary hydroxyls. The enzyme also shows significant preference for the 4−5 trans double bond of the sphingoid backbone. Indeed, the enzyme does not tolerate most modifications in the ceramide structure.

Modifications that allow hydrolysis include (i) variations in the chain length of the fatty acids ($C_{12}$−$C_{24}$); (ii) replacement of saturated fatty acids with mono-unsaturated

fatty acids; the latter were shown to exhibit a higher affinity to mt-CDase than their saturated counterparts; (iii) ceramides with a shorter sphingosine ($C_{10}$); and (iv) oxidation of the 3-hydroxyl group into the 3-keto-ceramide yielded a competitive substrate.

In contrast, the results from this study show that many of the analogues of ceramide or sphingosine inhibit the enzyme, demonstrating that the enzyme recognizes these structures as ligands but not as substrates. Examination of the ability of these ceramide and sphingosine analogues to inhibit the enzyme, therefore, provides important information on the requirements for interaction of the enzyme with ceramide.

A few structural modifications resulted in a significant loss of affinity of enzyme to D-erythro-ceramide as judged by loss of inhibition. Neither the N-Me-ceramide, the 1-O-Me-ceramide, the 3-O-Me-ceramide, the *cis*-D-e-ceramide, nor the ceramide-1-phosphate exhibited any inhibitory effects, demonstrating that these analogues do not interact with the enzyme. The lack of inhibition for these O- and N-substituted analogues may suggest an important role of the free hydrogens in the functional groups of ceramide (relative interactions of the functional groups or interaction of a specific group with the enzyme) but also can be related to steric effects that prevent or hinder the interaction of the analogues with the larger substituents with the ezyme.

The ability of Cer-1-P to stimulate the enzyme is probably similar to the effects of other anionic phospholipids (*12*).

In addition, methylation of the primary and secondary hydroxyl groups of sphingosine, reduced severely the potency of sphingosine, again suggesting a critical role for the primary and the secondary hydroxyl groups in interaction and recognition.

Further insight into the possible role of the 3 hydroxy group in interacting with the enzyme came with studies based on 3-keto sphingoid bases. (2S)-3-Keto-Sph displayed significant loss of inhibition when compared to D-e-Sph. On the other hand, 3-keto-dh-Sph was a potent inhibitor whereas 3-keto-dehsph was not. The keto-group of 3-keto-dh-Sph has a higher electron density compared to either 3-keto-sph or to 3-keto-deh-Sph as a result of the lack of conjugation with the electron deficient system (double or triple bond, respectively). This may facilitate formation of a stronger hydrogen acceptor bond between the carbonyl group in 3-keto-dihydrosphingosine and ceramidase. Taken together, the hydrogen-donor properties of the 3-OH are important for the interaction of the enzyme with sphingosines.

The results from this study also provide insight into the role of the trans 4−5 double bond in the sphingosine backbone. The *cis*-D-*erythro*-ceramide did not inhibit the enzyme, and thus did not demonstrate any interaction. Similarly, the 4,5-cis isomer of sphingosine was a weak inhibitor. Therefore the enzyme recognizes specifically the trans orientation. Since the cis bond introduces a kink in the hydrophobic chain, this may create a steric effect, possibly preventing ceramide (and sphingosine) from fitting into the catalytic site. Moreover, reduction of the C4−C5 double bond in ceramide produces dihydroceramide, which displayed significant loss of activity as a substrate. Similarly, reduction of this bond in sphingosine reduced the extent of inhibition and increased the $IC_{50}$ from 0.04 to 0.34 mol % for D-*erythro*-dihydrosphingosine when compared to D-*erythro*-sphingosine. Therefore, the enzyme shows preferential requirement for the trans-double bond which is a component of a rigid intramolecular allylic system that may facilitate interaction with the enzyme.

Introduction of a methyl group into the secondary amido-function of D-*erythro*-ceramide (N-Me-Cer) also resulted in loss of the interaction of ceramide with the enzyme, and N-alkylation of sphingosine (*N*-methyl- and *N*-stearyl-homologues) also attenuated inhibition by sphingosine. However, *N*,*N*-dimethylation of sphingosine resulted in profound loss of activity. Since *N*-Me-Cer and *N*,*N*-diMe-Sph do not have the proton donor activities of the amido and amino groups of ceramide and sphingosine, respectively, these results suggest that the NH protons contribute significantly to the interaction of ceramide and sphingosine with the enzyme.

On the other hand, there were several modifications that generated potent inhibitors of the enzyme, and these included (1) amide bond modification into the urea analogue ($IC_{50}$: 0.33 mol %) and (2) chiral modifications at the C2 and C3 positions. Since all these modifications were inhibitory, we may conclude that they did not disrupt critical features required for interaction with the enzyme.

The urea ceramide was a potent inhibitor of the enzyme, though not quite as potent as the stereoisomers of ceramide. The diminished effectiveness may be related to the lower polarity of the carbonyl group in the urea moiety as the result of the extended delocalization of the electron density of the carbonyl group over the two neighboring nitrogen atoms. This factor can diminish effectiveness of the hydrogen-acceptor properties of the carbonyl group in the formation of a lipid−enzyme complex.

One of the more intriguing results stems from the ability of all stereoisomers of ceramide (and sphingosine) to interact with the enzyme, with only the natural D-*erythro*-ceramide functioning as a substrate. These results suggest that D-*erythro*-ceramide retains a unique configuration required for catalysis but not for binding. Support for these conclusions also derived from studies utilizing analogues of sphingosine. As with ceramide isomers, stereochemistry had no impact on the ability of sphingosine to inhibit the enzyme, again supporting the conclusion that this interaction is not stereospecific. We are not familiar with many examples of such stereochemical interactions whereby one enantiomer is a substrate and the other is an inhibitor, even though enantiomeric selectivity is the rule in enzyme-mediated catalysis. Recently, a similar situation was reported with diastereoisomers of α-D-mannosyl transferase substrates with enantiomeric configuration at the polar headgroup (phosphoinositols), but without discussion of possible mechanisms (*31*).

This ability of the enzyme to recognize ceramide isomers and analogues as ligands (inhibitors) but not substrates suggests important conclusions on the mechanisms of interaction and on the respective structural requirements. Indeed, there are two main possibilities that could explain the different structural features required for inhibition and those required for catalysis. The first possibility suggests that the enzyme has two sites: (1) a catalytic site that recognizes D-*erythro*-ceramide in a highly stereospecific manner; and (2) a regulatory (allosteric) site that allows interaction with all stereoisomers of ceramide and sphingosine. According to this model, the unnatural isomers of ceramide (as well as sphingosine) interact at a distant site, inducing conformational

changes in the enzyme that prevent interaction of the enzyme with substrate (a $K_M$ type allosteric regulator). The other possibility suggests that catalysis of ceramide occurs by two steps. In the first step, ceramide interacts with the enzyme in a high affinity low specificity mechanism. This is supported by the ability of all stereoisomers to interact with relatively high affinity ($IC_{50}$ ranges between 0.11 and 0.26 mol %) compared to $K_m$ of hydrolysis (1.3 mol %). In the second step, catalysis occurs in a very stereochemically specific manner such that only the D-*erythro* configuration allows productive interaction and catalysis. The competitive nature of inhibition by the inhibitors evaluated in this study favors the second possibility but does not rule out the first.

What follows from either possibility is that the structural features required for initial interaction (whether at the catalytic site or at an allosteric site) are different (more general and less restrictive) than the structural features required for catalysis. If these interactions occur at the catalytic site (second possibility), then it follows that the requirements for catalysis should form a subset of the requirements for initial recognition. Thus, analogues that maintain the ability to interact (substrates and/or inhibitors) reveal modifications that are tolerated by the enzyme for initial interaction. On the other hand, analogues that function as neither substrates nor inhibitors do not interact with the enzyme, and they inform us of features that are critical for molecular recognition of these sphingolipids by mt-CDase. Thus, for inhibition, the enzyme requires the primary and secondary hydroxyl groups, the 4−5 double bond, the trans configuration of this double bond, and either the amide or free amine.

At a practical level, this study also disclosed some interesting inhibitors of ceramidase. Both urea-ceramide and ceramine, which are structurally highly analogous to ceramide and sphingosine, competitively inhibited the enzyme. Therefore, these modifications generated a new class of inhibitors. Also, the unnatural optical isomers of D-*erythro*-ceramide and sphingosine were highly potent inhibitors of mt-CDase. Thus, these compounds may serve as templates for further development of inhibitors of mt-CDase.

In conclusion, this study provides significant insight into the mechanisms and specificity of interaction of ceramidase with its substrates, products, and regulators. This information can also be used to develop the next generation of molecular tools that will provide further understanding of the mechanism of ceramidase action and evaluating its biological roles. Further studies will also require direct structural information on the enzyme itself.

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