Conformational Characterization of Ceramides by Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT Ceramide (Cer) has been identified as an active lipid second messenger in the regulation of cell growth, differentiation, and apoptosis. Its analog, dihydroceramide, without the 4 to 5 *trans* double bond in the sphingoid backbone lacks these biological effects. To establish the conformational features that distinguish ceramide from its analogs, nuclear magnetic resonance spectral data were acquired for diluted samples of ceramides (C2- and C18-Cer), dihydroceramide (C16-DHCer), and deoxydihydroceramide (C18-DODHCer). Our results suggest that in both C2- and C18-Cer, an H-bond network is formed in which the amide proton N*H* is donated to the OH groups on carbons C1 and C3 of the sphingosine backbone. Two tightly bound water molecules appear to stabilize this network by participating in flip-flop interactions with the hydroxyl groups. In DHCer, the lack of the *trans* double bond leads to a conformational distortion of this H-bonding motif. Without the critical double bond, the degree with which water molecules stabilize the H bonds between the two OH groups of the sphingolipid is reduced. This structural alteration might preclude the participation of DHCer in signaling-related interactions with cellular targets.

INTRODUCTION

Ceramide (Cer) has generated great interest since its participation as a signaling molecule in the "sphingomyelin cycle" was demonstrated (Ballou et al., 1996; Hannun, 1994, 1996; Merrill and Stevens, 1989; Merrill et al., 1997; Okazaki et al., 1989; Peña et al., 1997; Spiegel et al., 1996). Ceramide has been proposed to act as a lipid second messenger in a variety of important cellular functions, such as differentiation (Hannun, 1994; Kim et al., 1991; Okazaki et al., 1990), growth (Hauser et al., 1994; Olivera et al., 1992), and apoptosis (Cifone et al., 1994; Hannun, 1994; Heller and Kronke, 1994). In contrast to Cer, dihydroceramide (DHCer) without the *trans* double bond between carbons 4 and 5 of the sphingoid base, appears to be biologically inactive in most of these fundamental processes (Bielawska et al., 1993; Obeid et al., 1993). It has been postulated that the inactivity of DHCer is not due to its decreased uptake or increased metabolism (Bielawska et al., 1993) but rather to its inability to activate the Cer-activated protein phosphatase (CAPP) (Dobrowsky and Hannun, 1992), a potential cellular target for Cer action during signal transduction. In addition, C2-Cer with an abbreviated two-carbon long acyl chain has been shown to cause ADP-induced platelet aggregation and changes in platelet morphology, whereas C2-DHCer had no effect (Simon and Gear, 1998). Studies of the activity of a membrane-bound ceramidase purified from rat brain by Hannun and coworkers (El Bawab et al., 1999) using Cer and DHCer as substrates indicated that the enzyme hydrolyzes Cer preferentially. The intriguing differences between Cer and DHCer are puzzling from the chemical point of view because the only structural difference between them is the *trans* double bond between C4 and C5 of the sphingoid base. It is thus highly possible that this critical double bond serves to impart essential conformational features that make Cer biologically active (Hannun, 1994). Simon and Gear (1998) postulated the formation of an intramolecular H bond between the OH groups on carbons 1 and 3 of the sphingosine base and proposed the stabilization of this interaction by the presence of the double bond.

Biophysical techniques including x-ray diffraction (McIntosh et al., 1996; White et al., 1988), nuclear magnetic resonance (NMR) spectroscopy (Abraham and Downing, 1992; Kitson et al., 1994), differential scanning calorimetry (DSC) (White and Walker, 1990), atomic force microscopy (Grotenhuis et al., 1996), and Fourier transform infrared spectroscopy spectroscopy (Potts and Francoeur, 1990; Gay et al., 1994) have been applied to characterize the structure and phase behavior of lipids in the stratum corneum (SC). These lipids contain high amounts of Cer with acyl chains of 22 or more carbons. Some of these studies (Grotenhuis et al., 1996; Kitson et al., 1994) indicated the presence of ordered domains within model SC lipids under physiological conditions. DSC and x-ray diffraction techniques (Han et al., 1995; Shan et al., 1995a,b) on pure Cer revealed that hydrated Cer adopts a well-ordered lamellar phase. A detailed infrared spectral study (Moore et al., 1997) of the conformational order and molecular organization of hydrated Cer reported the main gel to a liquid-crystalline phase transition to occur at 80°C. This high transition temperature was attributed to the presence of strong intermolecular H bonds involving amide groups of neighboring molecules. At this time, however, no conformational studies have been carried out to establish the specific nature of the H bonds established between the amide and hydroxyl moieties of Cer. Furthermore, the conformational features that

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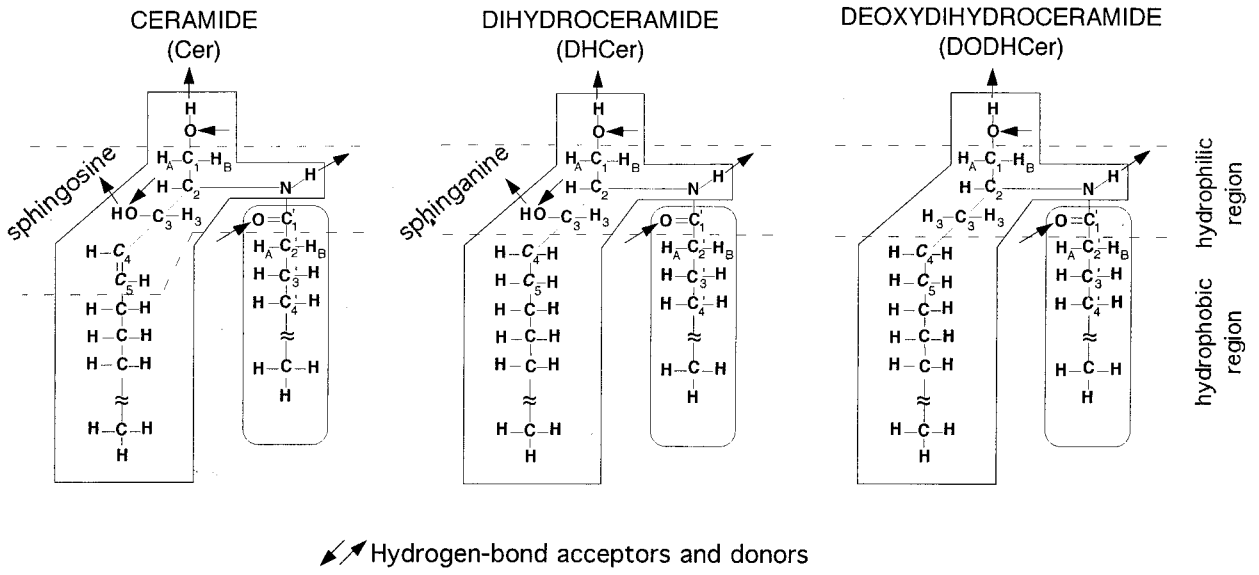


FIGURE 1 Components, regions, and atom numbering of ceramide (Cer), dihydroceramide (DHCer), and deoxy-dihydroceramide (DODHCer).

differentiate Cer from DHCer are not known. To address these issues we have used high-resolution NMR spectroscopy to investigate C2-Cer, the short alkyl chain Cer used in many model studies, C18-Cer, C16-DHCer, and C18-deoxydihydroceramide (DODHCer), a molecule without the OH group on carbon 3. Structures and labeling information on these molecules are presented in Fig. 1.

The NMR time scale is determined by the nuclear precession frequency. In a 500-MHz NMR spectrometer, the scale ranges from 10^{-4} to 10^{-1} s. These times are relatively long compared with those governing other spectroscopic methods, such as infrared, in which the vibrational transition occurs in a very short time, $\sim 10^{-13}$ s. As a result, infrared spectra provide instantaneous representations of each and every conformer in a molecular ensemble. The NMR spectrum, on the other hand, represents the time-averaged contributions of all conformers present. In the NMR studies described in this report, Cer and its analogs were dissolved in chloroform. Although it is clear that bilayer structures are not formed in chloroform, this solvent was chosen for the following reasons: 1) Cer and its analogs are nonswelling amphiphiles almost insoluble in aqueous media. As a result, severe spectral broadening occurs and prevents the observation of well-resolved NMR signals and precludes the measurement of coupling constants by high-resolution NMR spectroscopy. 2) Given the presence of glycosphingolipids and sphingomyelin in cholesterol-rich functional clusters or rafts (Brown, 1998; Brown and London, 1998; Rietveld and Simons, 1998), it is also possible that ceramides may be present in such hydrophobic aggregates. The conformational features exhibited by ceramides in chloroform thus may provide a better representation of the arrangements adopted in lipid rafts and/or caveolae that are enriched in cholesterol (Cameron et al., 1997; Harder and Simons, 1997; Hooper, 1999; Westermann et al., 1999). Furthermore, the low concentrations (below 10 μM) at which Cer interacts with CAPP suggest that the Cer is biologically active at concentrations below the CMC (Dobrowsky and Hannun, 1992). 3) Finally, and very importantly, the impact of bound water molecules and complete hydration on conformational preferences can be investigated readily by comparison of spectral features acquired for samples in chloroform with different hydration levels.

This report describes the unique average arrangements adopted by the hydrophilic region of these sphingolipids and the essential role of bound water molecules in the formation of extended and cooperative H-bonding networks.

MATERIALS AND METHODS

Chemicals

N-Acetyl-D-*erythro*-sphingosine (C2-Cer), *N*-palmitoyl-DL-*erythro* dihydrosphingosine (DHCer) were purchased from Sigma (St. Louis, MO). *N*-Stearoyl-D-*erythro* sphingosine (C18-Cer) was purchased from Matreya, Inc. (Pleasant Gap, PA) and used without further purification. 3-Deoxy-*N*-stearoyl-DL-sphinganine (DODHCer) was synthesized as described below. Deuterated chloroform, $CDCl_3$, deuterium oxide, D_2O, and deuterated dimethyl sulfoxide, DMSO-d6, were obtained from Sigma and used without further purification.

Sample preparation

Samples of C2- and C18-Cer, DHCer, and DODHCer in concentrations of 1 and 10 mM were prepared. For each sample, the proper weight was placed into a vial and either $CDCl_3$ or DMSO-d6 was added. The vial was then heated and vortexed for 2 min in a water bath at 50°C. The sample was

then cooled and heated again. This procedure was repeated until the sample was dissolved and the solution turned clear. Samples prepared in this way were partially hydrated. Based on the relative integration of the water resonance, the molar ratio of water to sphingolipid was estimated to be between 4:1 and 5:1. To investigate the effect of water on inter- or intramolecular hydrogen bond formation, spectral studies were also carried out using samples that were subjected to vacuum for 12 h for partial removal of bound water. Fully hydrated and partially deuterated samples were examined as well. After vacuum application, the samples contained two bound waters per lipid in the Cer (C2- and C18-Cer) and undetectable levels of bound water molecules per lipid in DHCer and DODHCer. Argon was used to void the vacuum, and $CDCl_3$ was immediately added, followed by heating and vortexing, as described for the partially hydrated samples. Fully hydrated samples were prepared by adding 50 μL D_2O to obtain a final molar ratio of D_2O to lipid of 500:1. Partially deuterated samples were obtained by adding minute amounts, between 2 and 4 μL, of D_2O directly into the NMR tubes containing the partially hydrated samples. The final D_2O to lipid molar ratio was between 20:1 and 40:1.

One-dimensional NMR studies

NMR experiments were performed on a Bruker AMX 500 spectrometer using 500.1 and 125.8 MHz for ^1H and ^{13}C frequencies, respectively. The temperature range investigated for each sample was chosen based on the sample solubility and solvent used. In $CDCl_3$, the temperature ranges were −10°C to 50°C for C2-Cer and 20°C to 50°C for C18-Cer, DHCer, and DODHCer as these lipids with longer acyl chains are less soluble than C2-Cer. In dimethylsulfoxide (DMSO), the temperature range investigated for C2-Cer, C18-Cer, and DHCer extended from 35°C to 70°C and it was changed to 50°C to 80°C for DODHCer because of the lower solubility of this lipid. All one-dimensional spectra were processed using Bruker WIN-NMR software on a personal computer.

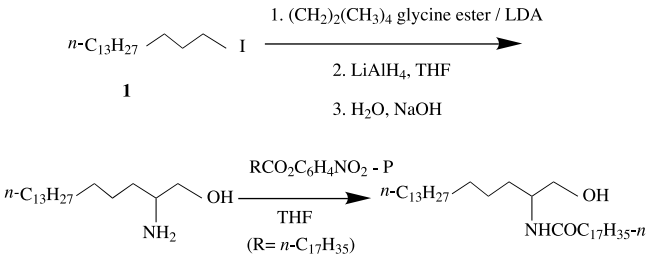
Two-dimensional NMR experiments

The ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe. The parameters used for the acquisition of COSY spectra were reported previously (Byrdwell et al., 1994). A total of 96 scans were accumulated per free-induction decay (FID). The HMQC spectral data were acquired with a phase-sensitive mode using time-proportional phase incremental and the BIRD preparation pulse (Bax and Subramanian, 1986). A total of 96 scans were collected per FID. The data for all two-dimensional spectra were recorded as 256 experiments with 512 complex points. Sweep widths of 6 ppm for ^1H and 70 ppm for ^{13}C were used. The acquired data were processed directly on a Bruker AMX 500 spectrometer.

Synthesis of 3-deoxy-*N*-stearoyl-DL-sphinganine

The two-step synthesis route is outlined in Scheme I and represents a modification of the approach published by Bittman's group (Kan et al., 1991) to prepare 3-deoxy-*N*-stearoyl-DL-SM. Because tris(trimethylsilyl) glycine ($(TMS)_3$ glycine), in which both NH_2 and OH groups were protected by TMS groups, appeared to be readily hydrolyzed in our hands, the synthesis was modified by reacting 1,2-bis(chlorodimethylsilyl) ethane with glycine ethyl ester, thereby forming a much more stable adduct (Djuric et al., 1981). Reaction products were monitored by thin-layer chromatography on silica gel 60 F254 plastic sheets (E. Merck). Spots were visualized under 254-nm ultraviolet light or by iodine. Column chromatography was carried out using Baker silica gel (60–200 mesh). Tetrahydrofuran (THF) was dried over anhydrous KOH overnight and then distilled from CaH_2.

Scheme I: Synthesis of 3-deoxy-N-stearoyl-DL-sphinganine



Step 1: preparation of 3-deoxy-DL-sphingosine

The synthesis of fresh lithium diisopropylamide was carried out according to the procedure used by Bittman's group (Kan et al., 1991). *N*-Butyllithium (3.1 ml, 1.4 M in hexane) was slowly added dropwise into diisopropylamine (6 μl, 4.77 mmol) in THF (5 ml) at −78°C. A white precipitate was formed immediately. After stirring the mixture for 30 min at −78°C, it was warmed to −30°C. Then a solution of the stable adduct made as above (600 mg, 2.45 mmol) in THF (1 ml) was added slowly to the mixture, resulting in a brown solution. The mixture was stirred for 2 h at −10°C. A solution of 1-iodo-hexadecane (809 mg, 2.30 mmol) in THF (1 ml) was added, and the mixture was stirred overnight at 0°C. Lithium aluminum hydride (450 mg, 11.8 mmol) was slowly added to the mixture. The suspension was refluxed for 24 h. Then, it was hydrolyzed with a small amount of water and 5.0 M NaOH. The mixture was filtered, and the solid residue was washed with $CHCl_3$. The combined organic solutions were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was purified by silica gel column chromatography (eluted with $CHCl_3$-MeOH, 2:1) to afford 250 mg (yield: 40%) of 3-deoxy-DL-sphinganine. The observed proton chemical shifts, in ppm, and the corresponding integration and assignments were: 0.84 to 0.87 (3H, CH_3), 1.23 (24H, $(CH_2)_{12}$), 1.32–1.37 (2H, CH_2-$CHNH_2$), 2.78–2.79 (1H, CH-NH_2), 3.21–3.24 (1H, CH_{1B}-OH), 3.54–3.57 (1H, CH_{1A}-OH).

Step 2: preparation of 3-deoxy-*N*-stearoyl-DL-sphinganine

The same procedure used by Bittman's group in the synthesis of 3-deoxy-*N*-stearoyl-DL-sphingosine was carried out. *p*-Nitrophenyl stearate (274 mg, 0.675 mmol) in THF (4 ml) was slowly added to 3-deoxy-DL-sphingosine (130 mg, 0.234 mmol) in THF (15 ml) under argon. The mixture was stirred overnight at room temperature. The solvent was evaporated under vacuum. The residue was dissolved in $CHCl_3$ and washed with 5 M NaOH and water. The organic layer was dried with anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was purified by silica gel column chromatography (eluted first with EtOAc/hexane, 1:6, then with MeOH/$CHCl_3$, 2:1) to give 150 mg (yield, 61%) of 3-deoxy-*N*-stearoyl-DL-sphingosine as a white solid product. The assignments for ^1H and ^{13}C resonances are reported in the next section.

RESULTS AND DISCUSSION

^1H NMR spectral assignments

The assignments of proton resonances observed for 10 mM samples of the sphingolipids in $CDCl_3$ at 25°C are listed in Table 1. The assignments reported previously for *N*-stearoyl-D-*erythro*-sphingosine (Julina et al., 1986) and 3-deoxy-*N*-stearoyl-DL-sphingosine (Kan et al., 1991) are also included in Table 1. The labeling of each resonance

TABLE 1 Spectral assignment of ^{1}H NMR resonances for 10 mM samples of Cer or its analogs in $CDCl_3$ at 25°C

Cer or analog	Chemical shift (ppm)*	Assignment	Observed shape multiplicity
C2-Cer, *C18-Cer*	0.86; *0.86* (0.85)	CH_3 (Terminal methyls)	Triplet
	1.24; *1.25* (1.23)	CH_2 (Methylenes)	Broad
	1.35; *1.35*	H7	Triplet
	2.02; *2.22* (2.21)	H′2	Singlet
	2.05; *2.04* (2.03)	H6	Quartet
	2.61; *2.67* (2.70)	OH	Broad
	3.70; *3.69* (3.68)	H1A	Quartet
	3.89; *3.90* (3.88)	H2	Decatet
	3.96; *3.95* (3.93)	H1B	Quartet
	4.32; *4.31* (4.31)	H3	Broad
	5.53; *5.52* (5.51)	H4	Quartet
	5.77; *5.76* (5.76)	H5	Pentet
	6.28; *6.21* (6.21)	NH	Doublet
C16-DHCer	0.86	CH_3 (Terminal methyls)	Triplet
	1.23	CH_2 (Methylenes)	Broad
	1.52	H4	Broad
	1.62	H′3	Broad quartet
	2.21	H′2	Triplet
	2.64, 2.52	OH	Broad
	3.75	H1A	Quartet
	3.76	H3	Broad
	3.81	H2	Decatet
	4.00	H1B	Quartet
	6.33	NH	Doublet
C16-DHDOCer†	0.86 (0.85 ~ 0.90)	CH_3 (Terminal methyls)	Triplet
	1.23 (1.25)	CH_2 (Methylenes)	Broad
	1.40, 1.51 (1.99 ~ 2.10)	H3	Multiplet
	1.61 (1.58 ~ 1.64)	H′3	Triplet
	2.18 (2.15 ~ 2.21)	H′2	Triplet
	3.55, 3.68 (3.58 ~ 3.66)	H1A, H1B	Quartet
	3.91 (3.09)	H2	Broad
	5.51	NH	Doublet

*Chemical shifts in parenthesis are those previously reported for C18-Cer (Kan et al., 1991).
†Chemical shifts in parenthesis are those previously reported for C16-DODHCer (Julina et al., 1986).

corresponds to the atom numbering shown in Fig. 1 and follows the same rationale described in previous works by our group (Ferguson-Yankey et al., 2000; Talbott et al., 2000). The assignment of proton resonances for DODHCer and DHCer was based on the correlations obtained by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC spectroscopy. The upfield shift of the H3 resonance for DHCer with respect to that in C2- and C18-Cer is related to the absence of the *trans* double bond between C4 and C5. The dramatic upfield shift of H3 resonance in DODHCer is due to the removal of the electron-withdrawing group OH from C3. The assignments for C2-Cer resonances were deduced by comparison with the previously reported values for C18-Cer (Julina et al., 1986).

^{13}C NMR spectral assignments

To investigate the effect of deuterium isotopic substitution on ^{13}C chemical shifts, the assignment of all carbon resonances for each lipid was pursued first. The assignments listed in Table 2 correspond to 10 mM samples of the lipids in $CDCl_3$. Assignments previously reported for sphinganine (Stoffel et al., 1972) and C18-Cer (Julina et al., 1986) are also included. Carbon-resonance assignments for DODHCer and DHCer were obtained by correlating the assigned proton resonances with the cross-peaks obtained in the two-dimensional ^{1}H-^{13}C HMQC NMR spectra. The assignments for C2-Cer were based on the values for C18-Cer reported previously (Julina et al., 1986).

Interface proton resonances

H1A, H1B, and H3

Proton resonances corresponding to the interface region of each lipid are shown in Fig. 2 for 10 mM samples in $CDCl_3$ and at 25°C. Similar spectral features were obtained for 1 mM samples (data not shown). As expected, large differences in chemical shift were observed in the H3 resonance. First, a 0.56 ± 0.02 ppm downfield shift was found in C2- and C18-Cer ($\delta = 4.32$) compared with DHCer ($\delta = 3.76$). Second, the two H3 resonances were nonequivalent in DODHCer and were shifted to 1.43 ppm and 1.52 ppm, significantly

TABLE 2 Spectral assignment of ^{13}C NMR resonances for 10 mM samples of Cer and its analogs in $CDCl_3$ at 25°C

Cer analog	Chemical shift* (ppm)	Assignment
C2-Cer; *C18-Cer*	14.10; *14.10* (13.7)	CH_3 (Terminal methyls) [C18 of sphingosine chain; and C′18 of acyl chain]
	22.68; *22.68* (22.4)	C17
	23.41; *36.84* (36.4)	C′2
	N/A; *25.74*	C′3
	29.66 ~ 29.10 (29.4 ~ 28.9)	CH_2 (intermediate methylenes of hydrophobic chains)
	31.92; *31.91* (31.7)	C16
	32.25; *32.27* (36.1)	C6
	54.40; *54.46* (54.7)	C2
	62.42; *62.52* (61.4)	C1
	74.78; *74.70* (73.1)	C3
	128.74; *128.75* (128.7)	C5
	134.32; *134.35* (133.7)	C4
	170.64; *173.95* (174.5)	O=C-NH
C16-DHCer†	14.10 (14.0)	CH_3 (Terminal methyls) [C16 of sphingosine chain and C′16 of acyl chain]
	22.68 (22.5)	C15, C′15
	25.76 (23 ~ 24)	C5
	25.94	C′3
	29.68 ~ 29.35 (28.8 ~ 29.7)	CH_2 (intermediate methylenes of hydrophobic chains)
	31.92 (31.9)	C14, C′15
	34.55	C4
	36.88	C′2
	53.71 (50 ~ 51)	C2
	62.53 (63)	C1
	74.29 (69.4)	C3
	173.57	O=C-NH
C18-DODHCer	14.12	CH_3 (Terminal methyls) in [C18 of sphinganine chain and C′18 of acyl chain]
	22.70	C17, C′17
	25.82	C′3
	26.12	C5
	29.26 ~ 29.70	CH_2 (intermediate methylenes of hydrophobic chains)
	31.18	C3
	31.93	C16, C′16
	36.92	C′2
	52.08	C2
	66.34	C1
	174.30	O=C-NH

*Chemical shifts listed in the parenthesis are those previously reported for C18-Cer (Kan et al., 1991).
†Chemical shifts listed in the parenthesis are those previously reported for sphinganine (Stoffel et al., 1972).

upfield with respect to the corresponding H3 resonances in the two analogs with the OH group attached to C3.

H1A and H1B are chemically nonequivalent protons connected to C1, to which an OH group is also attached. We have observed the nonequivalence of the H1A and H1B resonances in partially hydrated and dehydrated phosphosphingolipids (Ferguson-Yankey et al., 2000; Talbott et al., 2000). This was attributed to the restricted motion of C1 caused by internal H bonds formed between the NH and/or OH groups of the interface and the anionic and/or ester oxygens of the phosphorylcholine head group. To explore the effect of intramolecular H bonding on the spectral separation of the H1A and B resonances, preliminary results were obtained for a series of short chain diols in which the two OH groups are separated by 3, 4, 6, and 9 carbon atoms. In $CDCl_3$ at 25°C, the separation between the H1A and H1B bands was 0.06 ppm for 1,3-butanediol and decreased to 0.02 ppm for 1,4-butanediol. When the OH groups were separated by 6 carbon atoms, as in 1,6-hexanediol, or by 9 carbon atoms, as in 1,9-nonanediol, H1A and H1B became magnetically equivalent and a single resonance was detected. These results indicate that the extent of separation of the resonances of the geminal protons on C1 correlates well with the strength of intramolecular H bonding between the two OH groups of the diols.

The separation between the H1A and H1B bands was 0.26 ± 0.01 ppm for C2- or C18-Cer and 0.25 ± 0.01 ppm for DHCer. These large separations can be attributed to the average of conformations highly restricted by internal H bonds that may involve the two OH groups on C1 and C3 and/or the NH group of the amide moiety. For DODHCer, the chemical shifts of H1A and H1B were significantly upfield and exhibited a smaller separation of 0.13 ± 0.01 ppm. This points to the significant impact that the OH group on C3 has on the formation of internal H bonds.

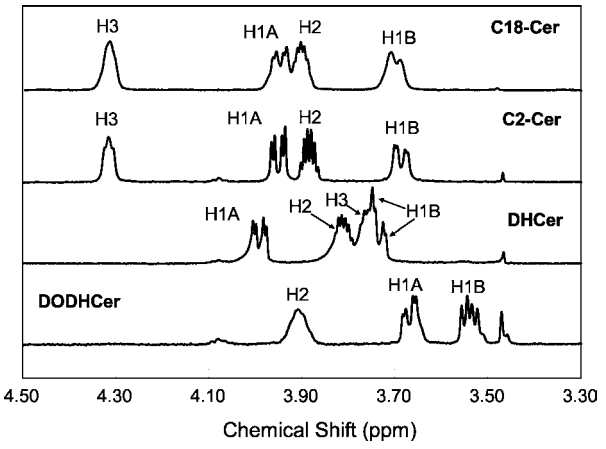


FIGURE 2 Interface proton resonances for 10 mM samples of ceramides in $CDCl_3$ at 25°C.

OH resonances

Rich structural information can be inferred from the coupling constants, chemical shifts, lineshapes, and isotopic shifts exhibited by OH resonances (Adams and Lerner, 1992, 1994; Pearce and Sanders, 1994; Poppe et al., 1990; Poppe and van Halbeek, 1991, 1994). Although these resonances may be difficult to detect due to fast intermolecular exchange, they can be observed when strong intramolecular H bonds are present and/or when highly pure solvents that prevent easy exchange are used. Fig. 3 (*a* (C2-Cer), *a′* (C18-Cer), and *b* (DHCer)) depicts the concentration and temperature dependence of OH resonances for each lipid.

In Cer, the secondary OH group on C3 is in an allylic position relative to the double bond between C4 and C5 and is, therefore, chemically different from the primary OH group on C1. Consequently, different chemical shifts would be expected for the corresponding OH resonances. However, for the 10 mM samples of partially hydrated C2-Cer or C18-Cer, a single OH resonance was observed (Fig. 3, *a* and *a′*), regardless of temperature. The resonance had an integrated area close to two protons (1.92 ± 0.01 ppm and 1.77 ± 0.06 ppm for the 1 mM and 10 mM samples at 50°C, respectively).

These chemical shifts are significantly higher from that corresponding to free OH, which was seen around 1.3 ppm. In addition, the observation of a single resonance indicates that, on the average, the two OH groups experience similar magnetic environments. Only when the temperature was lowered to −10°C in 1 mM C2-Cer (Fig. 3 *a*) or 25°C in 1 mM C18-Cer (Fig. 3 *a′*), a slight separation between the two OH resonances was seen. These observations could be explained by the formation of H bonds between these two OH groups with each other and/or with bound water molecules. This would lead to comparable levels of deshielding in the two OH protons and could result in a fast rate of exchange of the two protons at 25°C. At −10°C, the greater resolution

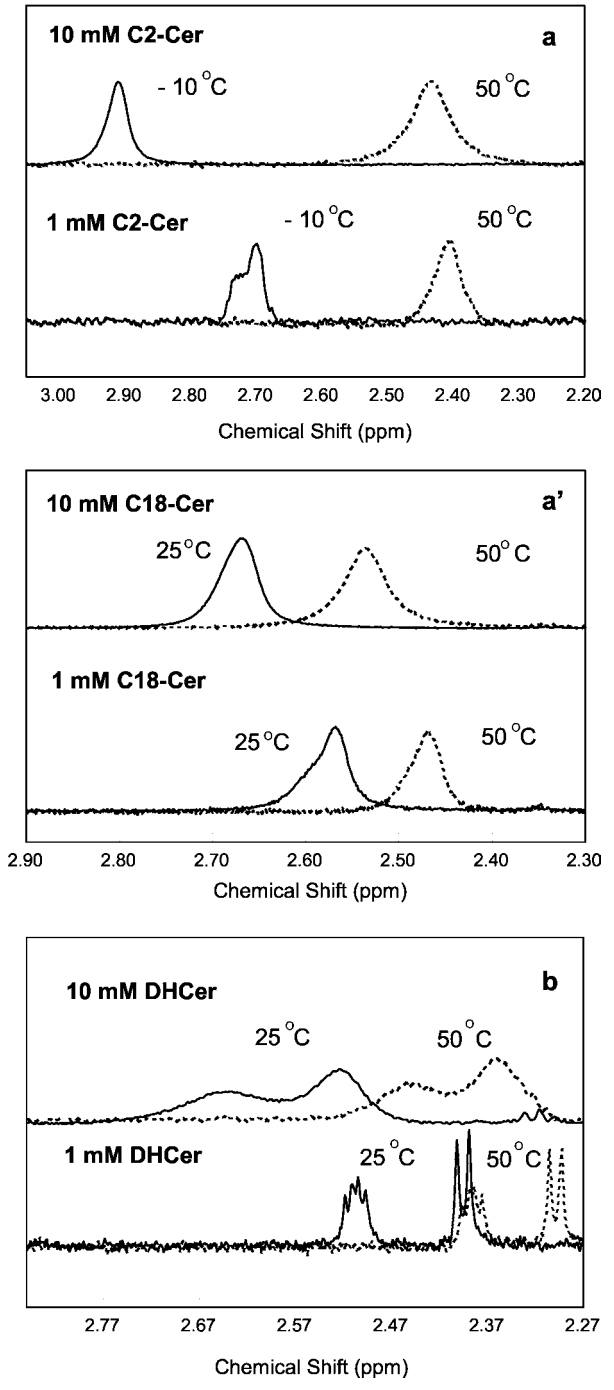


FIGURE 3 Changes in the OH resonance for 1 mM and 10 mM samples of (*a*) C2-Cer in $CDCl_3$ at −10°C (*solid lines*) and 50°C (*broken lines*), (*a′*) C18-Cer in $CDCl_3$ at 25°C (*solid lines*) and 50°C (*broken lines*), and (*b*) DHCer in $CDCl_3$ at 25°C (*solid lines*) and 50°C (*broken lines*).

of the two OH resonances of C2-Cer indicates that the rate of exchange slowed down. The lifetime of each arrangement was estimated to be 2.5 ms using the expression for the exchange rate proposed by Gutowsky and Holm (1956). For the 10 mM samples of C2-Cer and C18-Cer at high and low temperatures, downfield shifts were observed in the OH

TABLE 3 Temperature dependence for each lipid in $CDCl_3$ and in DMSO for OH and NH resonances

Lipid	$\Delta\delta/\Delta T$ (ppm/°C)		
	1 mM in $CDCl_3$	10 mM in $CDCl_3$	10 mM in DMSO
a) OH Resonance(s)			
C2-Cer	−0.0046 ± 0.0000	−0.0067 ± 0.0004	−0.0048 (C3-OH)
			−0.0053 (C1-OH)
C18-Cer	−0.0039 ± 0.0004	−0.0054 ± 0.0003	−0.0037 (C3-OH)
			−0.0043 (C1-OH)
C16-DHCer	0.0046 ± 0.0003 (C1-OH)	−0.0076 (C1-OH)	−0.0048 (C3-OH)
	0.0037 ± 0.0002 (C3-OH)	−0.0060 (C3-OH)	−0.0052 (C1-OH)
C18-DODHCer	−0.0088 ± 0.0006	−0.0099–0.0067	
b) NH Resonance:			
C2-Cer	−0.0041 ± 0.0001	−0.0050 ± 0.0000	−0.0062
C18-Cer	−0.0031 ± 0.0002	−0.0032 ± 0.0001	−0.0049
C16-DHCer	−0.0041 ± 0.0002	−0.0048 ± 0.0001	−0.0062
C18-DODHCer	−0.0026 ± 0.0001	−0.0028 ± 0.0001	−0.0076

resonance with respect to the 1 mM samples. A similar trend was also found for the water resonance that shifted from 1.59 ± 0.01 ppm (10 mM) to 1.54 ± 0.01 ppm (10 mM) at 25°C. This is a consequence of the greater level of aggregation at the higher concentration. The hydrophilic regions of neighboring lipids are in closer proximity, and water molecules are sequestered more efficiently and deshield more effectively the OH environment.

The OH resonances in 10 mM DHCer were broad and partially resolved from one another over the temperature range of 50°C to 25°C (Fig. 3 *b*). For the 1 mM sample, however, the OH resonances were well resolved and exhibited clear splitting patterns. The resonance with the higher chemical shift (more downfield) exhibited a quartet splitting pattern ($^3J_{OH/H1A(B)}$ = 6.8 Hz, $^3J_{OH/H1B(A)}$ = 4.0 Hz), whereas a doublet ($^3J_{OH/H3}$ = 6.0 Hz) could be seen in the other one. The quartet pattern indicates that the resonance located downfield corresponds to the C1-OH, and the splitting is caused by the vicinal nonequivalent protons H1A and H1B. The resonance upfield is assigned to C3-OH and the doublet results from the coupling with H3. Applying a similar rationale for the OH resonances in the 1 mM samples of C2-Cer at −10°C and C18-Cer at 25°C, the broader band component is assigned to C1-OH and the narrower one, slightly upfield, corresponds to C3-OH. Interestingly, the chemical shifts for the two OH protons in these sphingolipids oppose the expected upfield shift of the OH resonance in a primary alcohol with respect to that in a secondary alcohol. The behavior of the OH resonances supports the presence of an intramolecular H bonding involving OH groups on C1 and C3, similar to that in Cer but of lower strength.

If only the OH groups were involved in intramolecular H bonds in both Cer and DHCer, the removal of the C3-OH in DODHCer would free the C1-OH and lead to an upfield shift in the OH resonance. However, the OH resonance of DODHCer exhibited a slight downfield shift (δ = 2.68 ppm, 10 mM DODHCer at 25°C in $CDCl_3$) with respect to the other analogs, and the temperature coefficient ($\Delta\delta/\Delta T$) of the OH resonance was almost two times greater than those measured for C2-Cer, C18-Cer, and DHCer (Table 3). These observations suggest that the OH group in DODHCer is acting mainly as an H-bond donor to water molecules. In addition, it is possible that this OH group may be an H-bond acceptor with the NH moiety acting as donor.

NH resonance

The temperature dependence of the NH chemical shift ($\Delta\delta/\Delta T$) has been widely used as a tool for the study of intramolecular and intermolecular H bonding in peptides (Rose et al., 1985; Urry and Long, 1976). In a solvent with either weak or no ability to form H bonds, a small $\Delta\delta/\Delta T$ value for the NH resonance can indicate the participation of the NH proton in an internal H bond, or it may be also interpreted as an unbound NH that is completely exposed to the solvent. In a solvent capable of H-bond formation, small values of the temperature coefficient indicate an intramolecular H bond, and larger values suggest a solvent-exposed NH (Gellman et al., 1991).

When $CDCl_3$, a solvent with minimal H-bonding abilities, was used, the NH resonance in DODHCer was considerably upfield as compared with that of DHCer or the two Cers (see Fig. 4). All of the lipids exhibited small temperature coefficients in $CDCl_3$ ranging from −0.0026 to −0.0050 ppm/°C (Table 3b), implying either the presence of an intramolecular H bond involving the NH group as donor or, alternatively, a NH exposed to $CDCl_3$. The decrease in the temperature coefficient in C18-Cer with respect to that of C2-Cer is attributed to the formation of tighter H-bonding interactions that may result from a higher extent of micellization for the longer alkyl chain Cer. As a consequence, the hydrophilic sites are closer to each other and cooperative effects enhance the strength of H bonds.

The lowest temperature coefficient was seen for the NH resonance of DODHCer. This indicates that this proton is

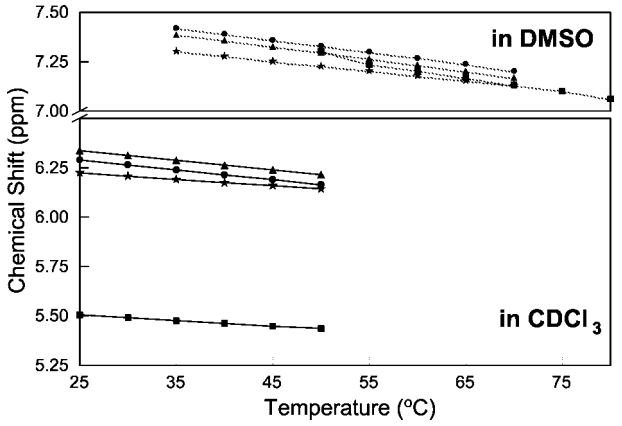


FIGURE 4 Temperature dependence of the NH resonance for 10 mM samples of C2-Cer (*solid circles*), C18-Cer (*stars*), DHCer (*triangles*), and DODHCer (*squares*) in $CDCl_3$ (*solid lines*) and in DMSO (*dotted lines*).

involved in an intramolecular H bond of greater strength as compared with the other analogs. The upfield shift of the NH resonance in DODHCer could result from the interaction of the amide proton with a single OH group, rather than two OH groups as is the case in Cers and DHCer. The relatively small variation in the NH chemical shifts with sample concentration (1 and 10 mM in Table 3b), as well as the extremely small rate of deuteration, favor the participation of the NH moiety in intramolecular H bonds for all the sphingolipids investigated. This possibility is further assessed below.

^{13}C NMR isotopic effects or secondary-isotope multiplets of partially labeled entities (SIMPLE) experiments

The OH and NH groups in all ceramides are potential participants in H bonds. Depending on the strength of these bonds, the partial deuteration of the samples could result in an isotope-induced shift. As a result, a splitting of the resonances of the carbon atoms bearing such H-bonding groups may be observed (Christofides and Davies, 1983a,b, 1984; Reuben, 1983, 1984, 1985, 1986). The magnitude of the two-bond isotope effect ($^2\Delta$) correlates with the strength of the H bond (Reuben, 1986). Fig. 5, *a*, *b*, and *c* illustrate the splitting patterns observed in the ^{13}C spectra of C2-Cer, DHCer, and DODHCer, respectively, in $CDCl_3$ at 25°C before (top trace) and after (bottom trace) partial deuteration. The levels of splitting of the C1 and C3 resonances were comparable for C2-Cer and C18-Cer. This indicates that regardless of the alkyl chain length, the two OH groups in Cers are involved in H bonds of similar strength.

The greater strength of H-bonding interactions postulated for Cers versus DHCer was confirmed by the larger splitting in the C1 and C3 resonances of C2-Cer and C18-Cer as compared with those for DHCer after partial deuteration of

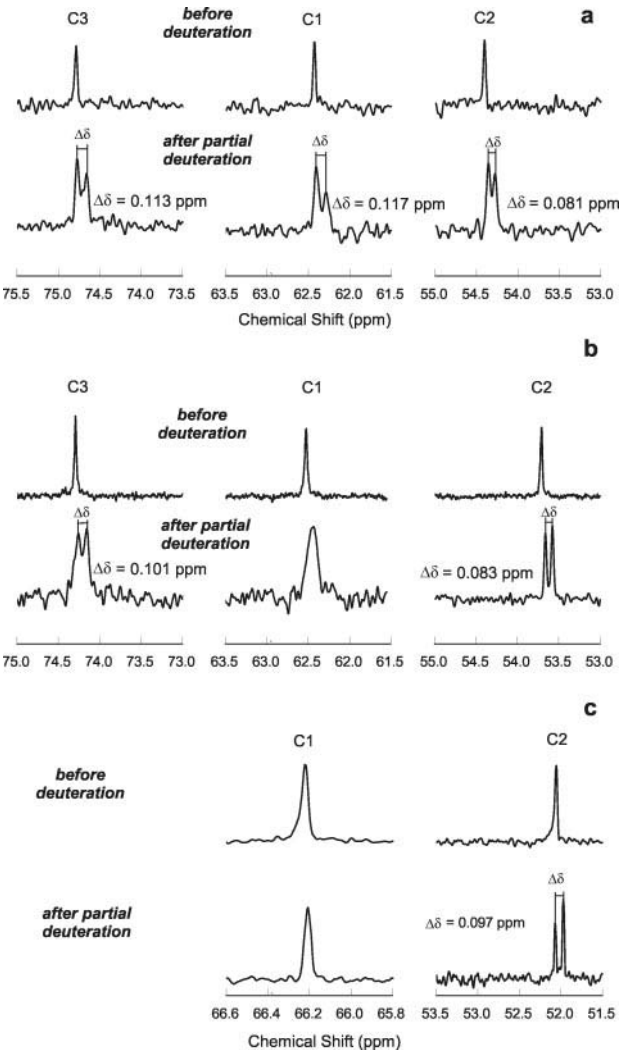


FIGURE 5 Isotopic effect on C1, C2, and C3 carbon-13 NMR resonances for a 10 mM sample of (*a*) C2-Cer in $CDCl_3$ at 25°C, (*b*) DHCer in $CDCl_3$ at 25°C, and (*c*) on C1 and C2 carbon-13 NMR resonance for a 10 mM sample of DODHCer in $CDCl_3$ at 25°C.

the samples. The average separations between the doublet for the C3 resonance was 0.123 ± 0.011 ppm for C2- and C-18 Cer and 0.104 ± 0.003 ppm for DHCer. In the case of the C1 resonance, an average splitting of 0.125 ± 0.014 ppm was observed for C2- and C18-Cer. Although the C1 resonance in DHCer was not split, a fourfold increase in the linewidth was observed upon partial deuteration. These observations lend further support to the postulated weakening on the intramolecular H bond that involves the OH group on C1 as a donor and the OH group on C3 as acceptor in DHCer. As a result, the OH group on C1 can, on the average, interact more efficiently with water molecules. This would allow for an easier exchange of the OH group on C1 with water or solvent. However, if the OH group on C1 interacted with water only, no such a broadening of the C1 resonance would be expected. This was the case for DODH-

Cer (Fig. 5 *c*), for which partial deuteration had little effect on the C1 resonance.

The participation of the NH group in an intramolecular H bond in every analog is supported by the splitting of the C2 resonance. The level of splitting for DODHCer was the largest (0.097 ± 0.004 ppm) compared with DHCer (0.083 ± 0.003), C18-Cer (0.084 ± 0.001), and C2- Cer (0.081 ± 0.002 ppm). The greater splitting of the C2 resonance in DODHCer points to the stronger interaction that the NH group can afford with the only OH group present in the molecule. For Cers and DHCer, the NH group is "shared" by the two OH groups and the timed-averaged strength of the net interaction is slightly weaker than for DODHCer.

Dehydration and hydration effects

NMR spectral studies were carried out for 10 mM samples of each lipid after 12 h of vacuum (10^{-2} Torr). Several changes were noted. First, the OH resonances in C2- and C18-Cer and DHCer could no longer be detected in the spectral region where they were observed in the partially hydrated samples. However, a single OH/H_2O band with an integrated area equivalent to six protons remained. This resonance exhibited a chemical shift of 1.86 ppm at room temperature and 1.74 ppm at 50°C. It was estimated, therefore, that two bound water molecules were still present even after 12 h of vacuum in 10 mM samples of Cers. For DHCer and DODHCer, however, no detectable amounts of water were present after vacuum. No changes were observed in the resonances for H1A, H1B, NH, H2, and H3 for any of the lipids. These trends suggest that in Cers, two bound water molecules are integral components of a tight network of H bonds. Their presence, however, is not essential for the formation of the H bonds connecting the two OH groups of Cers. The upfield shift seen in the OH/H_2O resonances of Cers upon partial removal of water suggests that the less bound water molecules enhance the deshielding of the OH protons.

To investigate the effect of complete hydration, 50-μl aliquots of D_2O were added to partially hydrated samples of the lipids in $CDCl_3$ and NMR spectra were acquired. Whereas the OH protons exchanged rapidly for both Cers and DHCer, the NH proton in all ceramides exchanged very slowly. Even after extended exposure (more than a day), more than one-half of the NH resonance still remained with no changes in chemical shift. This indicates the participation of the NH proton in a strong internal H bond. No changes were detected in any of the interface proton resonances in the presence of D_2O, suggesting that excess water did not disrupt the intramolecular H-bonding network established between the OH and NH moieties.

It is therefore proposed that the two nonremovable water molecules are strongly bound and serve as integral components of the H-bonding network in Cers. The removable water molecules contribute to the stabilization but are not required for the formation of the H-bonding network connecting the two OH groups of Cers. For DHCer and DODHCer, on the other hand, the absence of a water resonance after vacuum application indicates that water molecules are not as tightly bound as in Cers and are not required in the formation of the intramolecular H bonds.

TABLE 4 ^{1}H-^{1}H Coupling constants measured in Hz for 10 mM samples of Cer and its analogs in $CDCl_3$ at 50°C

Protons	C2-Cer	C18-Cer	C16-DHCer	C18-DODHCer
H1A-H1B	11.3	11.3	11.4	10.9
H1A-H2	3.9	3.7	3.5	3.3
H1B-H2	3.3	3.0	3.3	5.8
H2-H3	3.9	3.1	3.9	–
H3-H4	6.3	6.5	–	–
H2-NH*	7.2	7.3	7.6	7.3

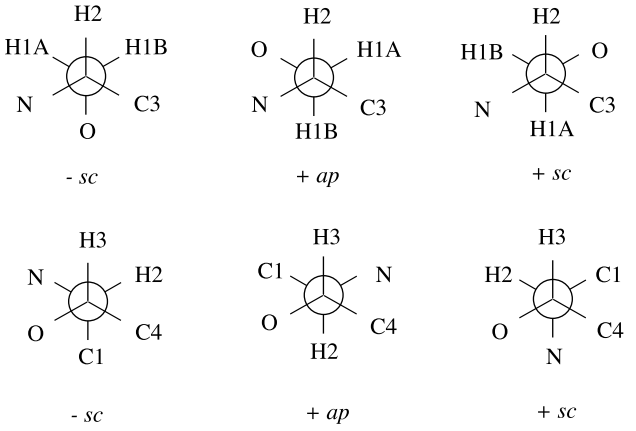
*These coupling constants were obtained at 25°C.

Vicinal proton-proton coupling constants and population analysis of conformers

Due to the high complexity of the splitting pattern for some resonances, only a limited number of vicinal ^{1}H-^{1}H coupling constants were obtained for 10 mM samples of each lipid in $CDCl_3$ at 50°C or 25°C, as shown in Table 4. Although the values for $^3J_{H1BH2}$ and $^3J_{H1AH2}$ were smaller for C18-Cer than for C2-Cer, the difference was ~0.6 Hz for both lipids. For DHCer this difference was reduced to 0.2 Hz but increased to 2.5 Hz for DODHCer. These trends reflect the conformational preferences that result from the different strength of intramolecular H bonding involving OH and NH groups, causing differences in the conformation about C1 and C2. Comparing the values of $^3J_{H2H3}$ for all ceramides with those reported for sphingomyelin (Bruzik, 1988), the drastic decrease in $^3J_{H2H3}$ indicates the dramatic change in the conformation about the C2-C3 bond as a result of the removal of the phosphate head group.

To analyze these conformational trends, we pursued the population analysis of the staggered conformers sketched in Scheme II. This analysis was based on the method published by Bruzik (1988), except that Pauling electronegativities were used in the calculation of the component coupling constants (Pauling, 1960). Molar fractions of the staggered conformers were calculated for θ_1 (O-C1-C2-C3) and θ_2 (C1-C2-C3-C4), the dihedral angles defined across C1-C2 and C2-C3, respectively. The estimated values are summarized in Table 5. Due to the uncertainty in the assignment of H1A (downfield resonance) and H1B (upfield resonance) as pro-R or pro-S, two sets of molar ratios about θ_1 can be obtained. For Cers and DHCer, the major rotamer for the conformation about C1-C2 (θ_1) is −sc, regardless of the assignment H1A and H1B as pro-R and pro-S or vice versa. This conformation may facilitate the formation internal H

Scheme II: Projections of the staggered conformers



bonds among the OH groups with each other and with the NH moiety. For DODHCer, the contributions from the −sc and +ap conformers are comparable and both support the formation of an intramolecular H bond between the NH moiety and the OH group on C1. In the analysis of the conformer population of θ_2 (C2-C3), rotamer +sc was discarded because it prevents the formation of an intramolecular H bond between the two OH groups. For the two Cers and for DHCer, the −sc conformer was predominant. This conformer favors the interaction of the NH group with the OH moieties on C3 and C1. The greater contribution of this rotamer for C18-Cer (compared with C2-Cer) supports the greater strength of the intramolecular bond involving the NH group, as postulated above.

POSTULATED CONFORMATIONAL PREFERENCES

The observations discussed above indicate that water molecules participate to different extents in the formation and/or extension of H-bonding networks. The OH groups of both, the sphingolipids and water, may act as H-bond donors and acceptors at different times. The transition from one state (flip) to another (flop) may occur at different rates and thus lead to changes in the spectral contour of the resonance bands. Based on the interpretation of neutron diffraction data obtained for β-cyclodextrin.12 H_2O, Saenger and coworkers proposed the participation of intramolecular H bonds in flip-flop interactions (Betzel et al., 1984; Saenger and Betzel, 1982; Steiner et al., 1991; Zabel et al., 1986). We believe that this type of interactions is operative in the hydrophilic regions of ceramides. The most likely conformational arrangements proposed for Cer, DHCer, and DODHCer are shown in Fig. 6 (*a*, *b*, and *c*), respectively, and are discussed below.

Ceramide

The presence of two nonremovable water molecules per Cer suggests the inclusion of these molecules in a chain-like cooperative H-bond network that involves the OH groups of the lipid (Fig. 6 *a*). The flip state shows the C1-OH participating in an intermolecular H bond with one of the water molecules, whereas C3-OH is involved in an intramolecular H bond with the OH group on C1. In addition, the OH group on C3 acts as an acceptor in the formation of another H bond with a water molecule tethered by a weak H bond to the double bond. The network is completed by the H bond between the two water molecules. The direction of the H bonds in this chain-like arrangement is reversed in the flop state (right side of figure). It is proposed also that this cooperative H-bonding network may be extended to include the amide NH group acting as an H-bond donor to the OH groups in C1 and C3, respectively, in the flip and flop states. This unique structural motif delineated by cooperative H bonds requires the proximity of the NH group and the OH on C3. Indeed, the small value of the 3-bond coupling constant $^3J_{H2\text{-}H3}$ supports the predominance of a gauche arrangement consistent with the conformation illustrated in Fig. 6 *a*.

According to Saenger's report, the flip and flop states of cyclodextrin were in thermodynamic equilibrium and the time scale of the interchange was evaluated to be $\sim 10^{-10}$ s from quasielastic incoherent neutron scattering data (Steiner et al., 1989). The fast dynamics of the exchange of the OH groups and water molecules between the two states made them indistinguishable on the NMR time scale. The tem-

TABLE 5 Populations of the staggered conformers based on the assignment of H1A to pro-*R* and H1B to pro-*S* configurations

Bond	Torsional angle		Relative population			
			C2-Cer	C18-Cer	DHCer	DODHCer
C1-C2	θ_1	−*sc*	0.69 (0.68)	0.73 (0.72)	0.72 (0.72)	0.50 (0.55)
		+*ap*	0.15 (0.24)	0.11 (0.23)	0.16 (0.19)	0.45 (0.08)
		+*sc*	0.16 (0.08)	0.15 (0.05)	0.12 (0.09)	0.05 (0.37)
C2-C3	θ_2	+*ap*	0.14	0.01	0.13	–
		−*sc*	0.86	0.99	0.87	–

θ_1 is the dihedral angle defined by O-C1-C2-C3.
θ_2 is the dihedral angle defined by C1-C2-C3-C4.
The populations for the reversed arrangements (H1A to pro-*S* and H1B to pro-*R*) are indicated in parenthesis.

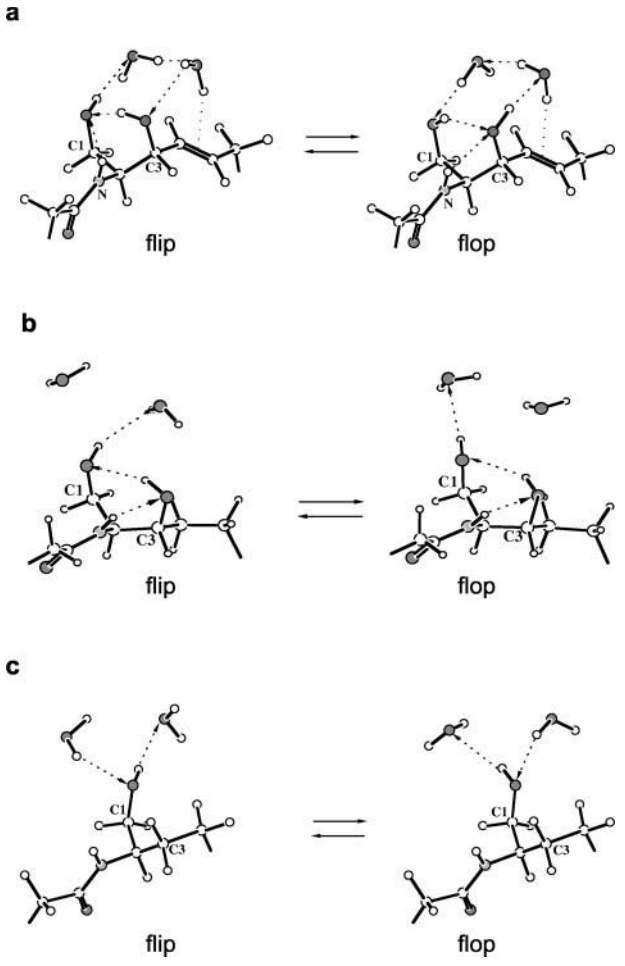


FIGURE 6 Proposed conformational arrangements and H-bonding interactions that affect the hydrophilic region of (*a*) Cers, (*b*) DHCer, and (*c*) DODHCer.

perature-dependent lineshape of the OH resonances shown in Fig. 3 *a* indicates that at 25°C the exchange between the two flip and flop states shown in Fig. 6 *a* for Cers is relatively fast. The lifetime of each state was estimated to be less than 1.3 ms using the maximal separation of the two resonances measured for Cer in DMSO-d6 in the expression for the coalescence lifetime (Slichter, 1990). The lineshape of the OH resonance in 10 mM C2-Cer and C18-Cer did not change appreciably over the temperature ranges investigated since at this concentration, reverse micelles are present with sequestered water molecules within the polar central core. As a result, water molecules form stronger H bonds with the OH groups, as supported by the downfield shift of the water resonance in the 10 mM sample of Cers as compared with the 1 mM sample. For 1 mM C2-Cer and at −10°C, the strength with which water and OH groups interact appeared to be somewhat weaker and from the separation of the OH resonances, a lifetime of ~2.5 ms for the flip and flop states was estimated.

From the results obtained for Cer and the comparison with those for DHCer discussed below, the presence of *trans* double bond between C4 and C5 could facilitate the stabilization of the arrangement illustrated in Fig. 6 *a* by anchoring a water molecule in the vicinity of the double bond. The water molecule could, in turn, serve as a unique link in the chain of H bonds that facilitates the interaction of the two OH groups with each other and with the bound water molecules. The cooperativity would be thus increased by the mutual polarization of the bonded OH groups (Koehler and Saenger, 1987; Gung et al., 1997; Lopez de la Paz et al., 1998). The formation of an H bond between water and the π-orbital of a double bond has been demonstrated experimentally for ethylene (Engdahl and Nelander, 1985). Both 1:1 and 2:1 water ethylene complexes were reported. The H bonds were significantly stronger in the ternary complex than in the binary complex. Computational studies in our group support this π-H bond (DuPré and Yappert, 2002).

DHCer

Compared with Cer, the greater resolution of the two OH resonances for the 10 mM DHCer sample and their complete separation at 1 mM suggest that the cooperative nature of flip-flop H bonds in DHcer is not as strong as in Cer. The exchange from the flip to the flop state in DHCer appears to occur more slowly than in Cer. The calculated lifetime of each state was estimated to be ~10 ms from the observed separations of the OH resonances (Michinori, 1985). In the absence of the C4-C5 double bond, the stabilization of the cooperative H-bonding network afforded by the localization of tightly bound water molecules in the vicinity of the OH groups and the double bond is eliminated. Consequently, we propose that the intramolecular C3-OH···OH-C1 H bond is slightly weaker than in Cer and the OH group on C1 may interact with water molecules that are no longer restricted by the formation of interlocking H bonds with the lipid interface. As a result, the OH group on C1 is, on the average, less bound to the oxygen of C3-OH and thus freer to interact with any water molecule. Overall, the extended array of flip-flop interactions proposed for Cer is no longer supported in DHCer. Thus, the stabilization induced by cooperative H bonds is reduced, and the energy barrier between flip-flop states is possibly higher than that in Cer.

DODHCer

The removal of OH group from C3 in DODHCer prevents the formation of the water-extended cooperative H bonds involving the OH groups of Cer or DHCer. However, given the low temperature coefficient of the NH resonance, its very low rate of deuterium exchange and the relatively large splitting of the C2 resonance upon partial deuteration, the

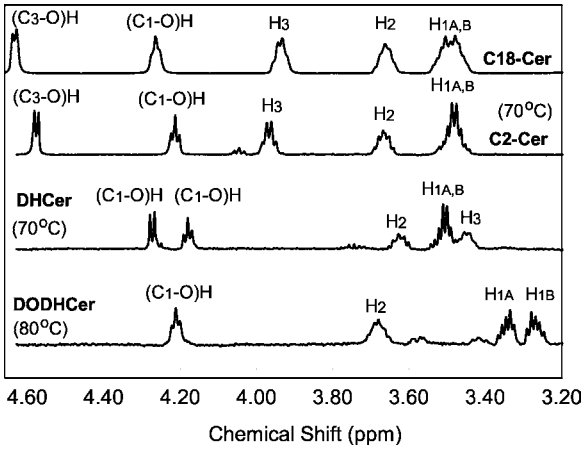


FIGURE 7 Interface proton resonances for 10 mM samples of Cer and analogs in DMSO.

intramolecular H bond between the NH (donor) and OH group on C1 appears to remain. In addition, the OH group is proposed to interact with neighboring water molecules both as an acceptor and as donor, as shown in Fig. 6 *c*.

Corroboration of the proposed arrangements

If the H-bonding networks proposed above are operative, their disruption should lead to significant changes in the resonances associated with hydrophilic sites of the ceramides. To check this possibility we investigated all analogs in DMSO, an aprotic solvent and a very strong H-bond acceptor capable of disrupting internal bonds and forming new ones (Bock, 1982; Pearce and Sanders, 1994). Indeed, in the presence of this solvent, the measured chemical shifts of the OH resonances in all three analogs were found to shift downfield from their positions in $CDCl_3$ (see Fig. 7). This is indicative of the formation of strong intermolecular H bonds between the OH groups and DMSO. The single resonance observed for the OH groups of Cers in $CDCl_3$ exhibited two very well separated peaks in DMSO.

The difference between the chemical shifts of the OH resonances was smaller in DHCer (0.09 ppm) than in Cers (0.37 ppm) because in the absence of internal H bonds, the primary and secondary OH groups in DHCer are less different chemically than the primary and allylic OH groups in Cer. The observed coalescence of the H1A and H1B resonances for both Cer and DHCer reflects the free rotation about the C1 atom, implying a breakage of the intramolecular H bond between the OH groups on C1 and C3. Interestingly, the H1A and H1B resonances in DODHCer exhibited a greater difference in chemical shift than in Cer and DHCer in DMSO. This suggests that an internal restriction still remained in DODHCer. The only possible internal H bond for DODHCer would be that formed between the NH group and OH moiety on C1. This possibility can be verified by the study of the temperature dependence of the NH resonance (Table 3b).

In DMSO, large downfield shifts were observed in the NH resonance of Cer and its analogs (see Fig. 4). This is the result of the rupture of internal H bonds and subsequent formation of strong intermolecular H bonds between the NH group and DMSO. The temperature coefficient for the NH resonance increased in DMSO as compared with that in $CDCl_3$ (see Table 3b). This is also indicative of a change from intra- to intermolecular H-bonding interactions.

CONCLUSIONS

Our interpretation of spectral trends points to a unique conformational arrangement adopted by the hydrophilic portion of Cers, regardless of acyl chain length. A network of cooperative H bonds that involves the NH and OH groups of Cer as well as two water molecules delineates a structural motif that is maintained even under conditions of complete hydration. This motif is distorted when the *trans* double bond between carbons 4 and 5 is absent and the strength with which the two OH groups interact is diminished.

It is recognized that these conformational preferences may differ from those of Cer in a bilayer arrangement. Yet, the structural differences proposed for Cer and DHCer in this report could be relevant to the understanding of the different activity that these two lipids display in biological processes such as cell signaling and trafficking. Indeed, the dose-dependent response of CAPP to stimulation by Cer indicates that this lipid activates the enzyme (Dobrowsky and Hannun, 1992) at concentrations below the CMC. The unique conformational arrangement and the interesting cooperative H-bonding network exhibited by Cer could be responsible for the specificity of the interactions between this sphingolipid and its targets in signaling pathways that control cell proliferation and differentiation.

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