Proton nuclear magnetic resonance of neutral and acidic glycosphingolipids

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Abstract A number of intact neutral glycosphingolipids (globo, asialoganglio, neolacto, and gala series), gangliosides, and sulfatide were analyzed by proton nuclear magnetic resonance (NMR) using dimethyl-d_6 sulfoxide as a solvent at different conditions of measurement. The chemical shifts of amide proton of ceramide, N-acetylhexosamine and sialic acid moieties were positioned with regularity, thus providing their molar composition. The chemical shifts of amide proton in ceramide moiety differed with respect to constituent fatty acids; δ7.45 to 7.52 ppm at 25°C for the nonhydroxy acids and 7.32 to 7.42 ppm for the hydroxy acids. The chemical shifts of methyl proton in N-acetyl group were distinguished between N-acetylhexosamine and N-acetylneuraminic acid, and those in N-acetylgalactosamine were discriminated between neutral glycolipids and gangliosides. In the presence or absence of D_2O in dimethyl sulfoxide at 110°C, the anomeric protons resonated with regularity characteristic of respective monosaccharide linkages, and the anomeric protons of N-acetylgalactosamine in neutral glycolipids and gangliosides were clearly distinguished. The present study thus demonstrates the general applicability of NMR procedure to glycosphingolipids, providing the determination of chemical composition of both the lipophilic and carbohydrate moieties and the structural elucidation.—**Gasa, S., T. Mitsuyama, and A. Makita.** Proton nuclear magnetic resonance of neutral and acidic glycosphingolipids. *J. Lipid Res.* **24:** 174–182.

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The biological role of glycosphingolipids is intimately correlated with their carbohydrate structure. Linkage position in the carbohydrate chain is almost exclusively determined by a methylation procedure. The sequence and anomeric configuration are determined by sequential hydrolysis with specific exoglycosidases. The anomeric structure can also be assayed by the procedure of chromium trioxide oxidation (1). In contrast to these destructive procedures, proton NMR spectroscopy is a nondestructive method. Alpha-galactosyl configuration in globotriaosylceramide was first demonstrated by NMR analysis on trihexosyl sphingenine derived from the glycolipid (2). The proton NMR technique has been applied to elucidation of the anomeric configuration of the sugar linkage in *a*) neutral glycosphingolipids with their pertrimethylsilylated derivatives (3), *b*) nonderivatized glycolipids (4, 5), and *c*) the oligosaccharides obtained from the glycosphingolipids (6–8). Although several recent studies on H-1 to H-6 protons in monosaccharide residues of methylated (9–11) and intact neutral glycosphingolipids (12) afforded abundant information on their carbohydrate structures, elucidation of other proton signals present in the complex structures and the category of glycosphingolipids examined so far are still restricted. There is little knowledge of the nature of amide proton in ceramide and carbohydrate moieties, and there have been only a few studies on acidic glycosphingolipids such as gangliosides and sulfatide.

In this communication, we demonstrate regularities of amide proton as well as anomeric and methyl protons in relation to the structures of 21 sphingolipids, using an aprotic and polar solvent, dimethyl sulfoxide (Me_2SO), which provides regular chemical shift of amide proton and has a wide range of solubility for various sphingolipids.

MATERIALS AND METHODS

Preparation of glycosphingolipids

Ceramide was prepared from equine kidney and separated into two classes containing nonhydroxy (FA) and hydroxy fatty acids (HFA) by means of silicic acid chromatography. The separation was confirmed by gas–liquid chromatographic analysis of their fatty acids. Glucosylceramide (GlcCer) and lactosylceramide (LacCer) were prepared from equine spleen. Hematoside (ganglioside G_{M3}) containing N-glycolylneuraminic acid (II^3NeuGc-LacCer) was prepared from equine kidney

Abbreviations: NMR, nuclear magnetic resonance; FA, nonhydroxy fatty acid; HFA, hydroxy fatty acid; TMS, tetramethylsilane; Me_2SO-d_6, 1,1,1,3,3,3-hexadeuterodimethylsulfoxide; Cer, ceramide.

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as described previously (13). Galactosylceramide (GalCer) and cerebroside sulfate were prepared from human brain. They were separated into FA- and HFA-containing fractions by silicic acid chromatography. Galabiosylceramide ($GaOse_2Cer$) and globotriaosylceramide ($GbOse_3Cer$) were isolated from Fabry kidney, the former being separated into FA- and HFA-containing species. Globoside (globotetraosylceramide, $GbOse_4Cer$), amino CTH (lactotriaosylceramide, $LcOse_3Cer$), and sialylparagloboside (IV^3NeuAc-$nLcOse_4Cer$) were isolated from human erythrocyte membranes (14); Forssman glycolipid (globopentaosylceramide, $IV^3GalNAc\alpha$-$GbOse_4Cer$) was isolated from goat erythrocytes (15), ganglioside G_{M2} (II^3NeuAc-$GgOse_3Cer$) and asialoganglioside G_{M2} (gangliotriaosylceramide, $GgOse_3Cer$) were from a Tay-Sachs brain (16), and ganglioside G_{M1} (II^3NeuAc-$GgOse_4Cer$) and ganglioside G_{D1a} (IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$) were from bovine brain. Asialoganglioside G_{M1} (gangliotetraosylceramide, $GgOse_4Cer$) was obtained by treatment of ganglioside G_{M1} with *Arthrobacter* neuraminidase (Nakarai Chem. Co., Kyoto).

NMR measurements

Proton NMR spectra of glycosphingolipids (1–8 mg) in 0.4 ml of Me_2SO-d_6 were obtained on a Varian JNM-FX 200 spectrometer in the Fourier-transform mode equipped with a JEC-980B computer which had 48k memory capacity. The frequency was 200 MHz, and the sweep width was 2 kHz. The operation was performed at 25°C and 110°C in Me_2SO-d_6, and then at 110°C in a Me_2SO-d_6 solution containing 50 μl of D_2O. Chemical shifts were indicated by δ(ppm) from distance of tetramethylsilane (TMS) as an internal standard. The materials were recovered by chromatography on a Sephadex LH-20 column (1 × 30 cm) eluting with chloroform–methanol–water 60:30:4.5 (by volume). The eluted glycolipids were monitored on a thin-layer plate by iodine vapor.

RESULTS

The whole NMR spectra of II^3NeuAc-$GgOse_3Cer$ at different conditions of measurement are presented in **Fig. 1.** Amide protons of N-acetylneuraminic acid, ceramide, and N-acetylgalactosamine are clearly demonstrated in the lowest field at 25°C with an equimolar intensity (Fig. 1-A). At a higher temperature, amide protons shift to upfield, and methyl protons at the N-acetyl group shift to downfield (Fig. 1-B). When D_2O is introduced, the amide protons completely disappear due to H-D exchange, being signals of anomeric protons clearer (Fig. 1-C).

Amide proton resonances

The chemical shifts of amide protons in ceramide and the ceramide moiety of several series of glycosphingolipids were systematically surveyed and the results are summarized in **Table 1** and **Table 2.** The resonances of the amide protons of all the glycosphingolipids with FA-containing ceramide are regularly observed in a range of 7.45–7.52 ppm, whereas those with HFA-containing ceramide shift to upfield (7.32–7.42 ppm). When the temperature is raised to 110°C, the ceramide protons of all the glycosphingolipids shift to 6.97–7.34 ppm.

On the other hand, the amide protons of N-acetylgalactosamine in neutral glycolipids resonate in a range of 7.62–7.79 ppm and shift to upfield by 0.28–0.42 ppm at 110°C (Table 1), while those in gangliosides have lower values ranging from 7.10 to 7.59 ppm and are essentially unaffected by raising the temperature, except for II^3NeuAc-$GgOse_4Cer$ (Table 2). The amide protons of N-acetylglucosamine (7.70–7.73 ppm) in neutral and acidic glycolipids are not distinguished from those of the N-acetylgalactosamine (7.62–7.79 ppm) in neutral glycolipids, but are clearly differentiated from amide protons in gangliosides (7.10–7.59 ppm). Since the amide protons of N-acetylgalactosamine resonate in a wide region, it is difficult to distinguish the protons of N-acetylgalactosamine from those of ceramide.

The amide protons of N-acetylneuraminic acid in gangliosides have the lowest chemical shift (8.02–8.20 ppm) at 25°C and migrate to upfield (7.71–7.76 ppm) at 110°C. The amide proton of N-glycolylneuraminyl residue in II^3NeuGc-LacCer at a low temperature is situated at 7.78 ppm in a similar range with N-acetylhexosamines, and also shifts to upfield (7.44 ppm) at a high temperature. The coupling constants of all the amide protons coupled to adjacent CH protons range from 6.3 to 10.3 Hz in doublet as summarized in **Table 3** and **Table 4.** No regularity of these coupling constants, however, is observed on any amide species described above.

Anomeric proton resonances

The chemical shifts of anomeric C-1-H doublet in the carbohydrate moiety were measured at a high temperature in the presence or absence of D_2O, which cancels the overlapping signals of the anomeric proton region. The results are summarized in **Table 5** (the data in the absence of D_2O are presented unless otherwise stated).

The anomeric protons of β-glucose bound directly to ceramide in all the glycosphingolipids occur consistently in a narrow range of 4.17 to 4.18 ppm except for FA-containing glucosylceramide (4.12 ppm) in which glucose is not substituted. The disparity of the chemical

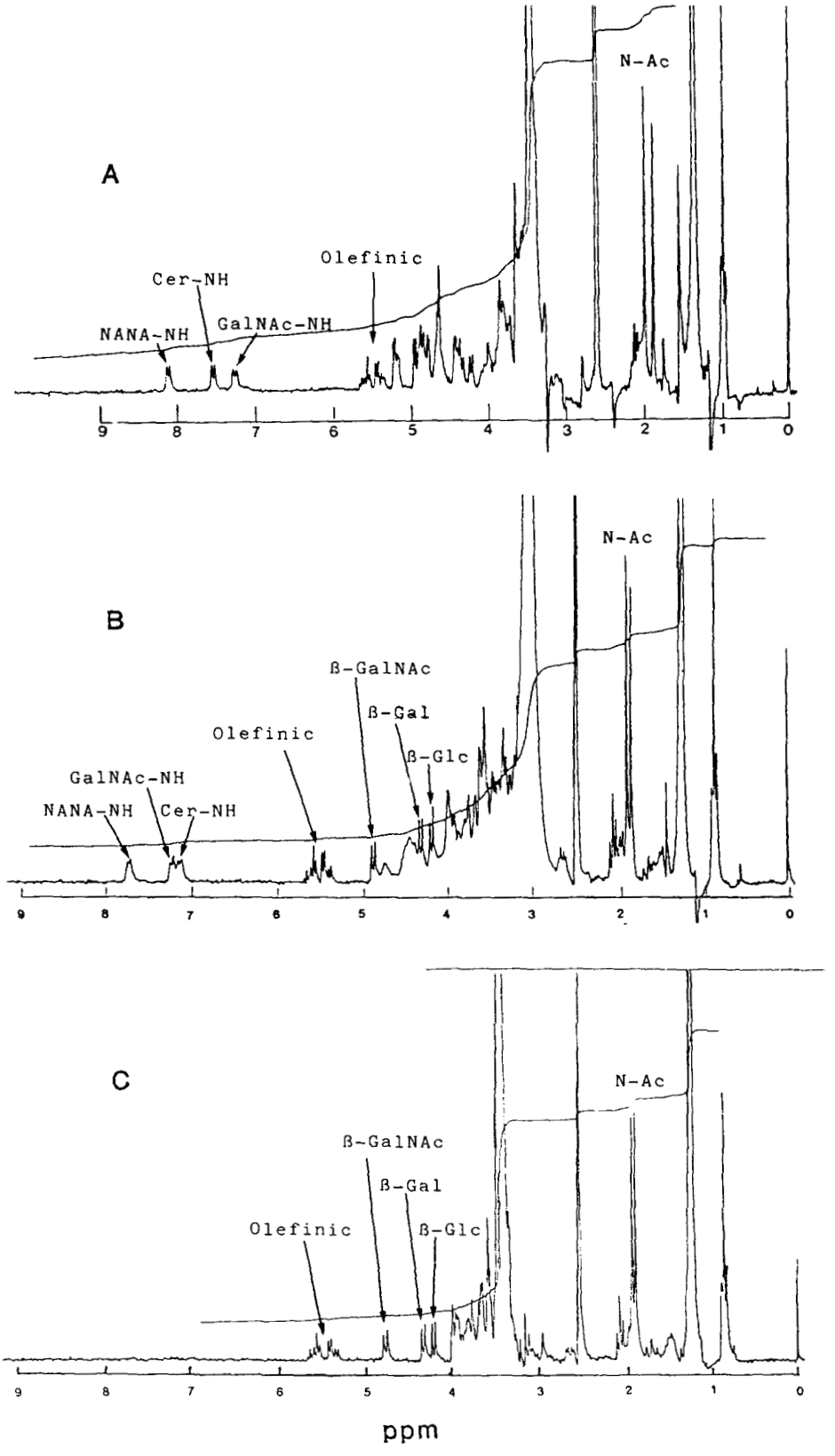

Fig. 1. Proton NMR spectra of II^{3}NeuAc-GgOse$_3$Cer in Me_2SO-d_6 at different conditions. A, Measured at 25°C; B, at 110°C; C, at 110°C in D_2O-Me_2SO-d_6 (1:9, v/v). The detail is described in Materials and Methods.

TABLE 1. Chemical shifts[a] of amide proton of ceramide and N-acetylhexosamine in neutral glycosphingolipids

Substances	Cer(FA)		Cer(HFA)		GalNAc		GlcNAc	
	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C
Cer	7.50	7.07	7.37	6.97				
GlcCer	7.45	7.08						
GalCer	7.51	7.12	7.38	7.19				
LacCer	7.47	7.14						
$GaOse_2Cer$	7.52	7.10	7.42	7.17				
$LcOse_3Cer$	7.51	7.11					7.71	7.44
$GgOse_3Cer$	7.49	7.07			7.79	7.51		
$GbOse_3Cer$	7.46	7.08						
$nLcOse_4Cer$	7.49	7.13					7.70	7.41
$GbOse_4Cer$	7.50	7.11			7.67	7.37		
$GgOse_4Cer$	7.52	7.15			7.66	7.25		
$IV^3GalNAc\alpha\text{-}GbOse_4Cer$	7.47	7.09			7.62	7.20		
Cerebroside sulfate	7.46	7.17	7.32	7.34				

[a] The chemical shifts (ppm) are expressed by deviations from internal standard (TMS) at 25°C and 110°C.

shifts observed between substituted and unsubstituted β-glucose residue is diminished by the addition of D_2O.

The anomeric signals (4.07 and 4.09 ppm) of β-galactose attached to ceramide (GalCer and $GaOse_2Cer$) shift to upfield as compared to those of the β-glucose in the lipids, while the anomeric proton (4.20 ppm) of cerebroside sulfate resonates at downfield, probably due to deshielding by anisotropy of sulfate ion or to electrostatic interaction with Me_2SO. The anomeric signals of β-galactose that is linked to C-4 of glucose or N-acetylglucosmine occupy a region between 4.24 to 4.30 ppm in the absence of D_2O. The anomeric signals of α-galactosyl 1 → 4 (globo series and $GaOse_2Cer$) and β-N-acetylgalactosaminyl 1 → 4 linkages (gangliosides, but not asialogangliosides) similarly appear in a narrow region of 4.83–4.89 ppm in the absence of D_2O, but deviations due to the addition of D_2O make characteristics clearer in each linkage, i.e., the protons of α-galactosyl linkage shift to downfield by 0.01–0.04 ppm while the protons of β-N-acetylgalactosamine in gangliosides shift to upfield by 0.07–0.08 ppm.

The anomeric protons of β-N-acetylhexosaminyl linkages in neutral glycolipids shift a little to downfield by 0.01–0.04 ppm or remain unchanged ($GgOse_4Cer$), in contrast to definite upfield shifting of those in the gangliosides by 0.07–0.08 ppm described above. The anomeric protons of β-N-acetylglucosamine (4.69–4.73 ppm) can be distinguished from those of β-N-acetylgalactosamine in neutral (4.52–4.64 ppm) or acidic glycolipid (4.84–4.89 ppm).

The coupling constants of anomeric protons at a high temperature are summarized in **Table 6.** The coupling constants of β-anomeric protons due to α-galactosides and α-N-acetylgalactosaminides in $GbOse_3Cer$, $GbOse_4Cer$, $IV^3GalNAc\alpha\text{-}GbOse_4Cer$, and $GaOse_2Cer$ are significantly smaller (3.2–3.9 Hz in the absence of D_2O) than those of α-anomeric protons (6.2–8.9 Hz), and these differences are diagnostic for anomeric configurations of glycosphingolipids and possibly other galactose-galactosamine-containing complex carbohydrates. The discrimination among β-Glc, β-GalNAc, and β-GlcAc by their coupling constants is not possible.

Methyl proton resonances in acetyl groups

The methyl protons of N-acetyl groups in N-acetylhexosamines and N-acetylneuraminic acid appear in higher resonance fields as shown in **Table 7.** The interpretation of the results was made on the values measured at 25°C unless otherwise stated.

The methyl protons (1.83–1.86 ppm) of the N-ace-

TABLE 2. Chemical shifts of amide protons of ceramide, N-acetylhexosamine, and sialic acid in gangliosides

Gangliosides	Cer(FA)		GalNAc		GlcNAc		NeuAc		NeuGc	
	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C
$II^3NeuGc\text{-}LacCer$	7.49	7.13							7.78	7.44
$II^3NeuAc\text{-}GgOse_3Cer$	7.47	7.11	7.21	7.21			8.02	7.71		
$II^3NeuAc\text{-}GgOse_4Cer$	7.50	7.12	7.59	7.28			8.16	7.76		
$IV^3NeuAc\text{-}nLcOse_4Cer$	7.50	7.09			7.73	7.36	8.10	7.73		
IV^3NeuAc, $II^3NeuAc\text{-}GgOse_4Cer$	7.50	7.12	7.10	7.06			8.20	7.72		

TABLE 3. Coupling constants of amide protons of ceramide and N-acetylhexosamines in neutral glycosphingolipids

Substances	Cer(FA)		Cer(HFA)		GalNAc		GlcNAc	
	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C
Cer	9.3	8.4	9.2	8.4				
GlcCer	7.3	7.5						
GalCer	7.6	7.2	7.5	7.3				
LacCer	7.8	9.3						
$GaOse_2Cer$	8.1	8.3	8.3	8.0				
$LcOse_3Cer$	8.6	8.2					8.5	8.1
$GgOse_3Cer$	8.3	7.8			6.3	8.3		
$GbOse_3Cer$	8.4	8.2						
$nLcOse_4Cer$	8.0	7.5					8.3	7.6
$GbOse_4Cer$	9.3	7.8			7.8	8.2		
$GgOse_4Cer$	8.0	7.8			8.1	9.0		
$IV^3GalNAc\alpha\text{-}GbOse_4Cer$	9.3	7.3			9.1	8.8		
Cerebroside sulfate	8.2	8.0	9.2	8.5				

The coupling constants are expressed by Hz.

tylgalactosamine residue resonate significantly in downfield in neutral glycosphingolipids (globo and asialoganglio series) as compared to those (1.75–1.77 ppm) of the ganglio series. By the addition of D_2O at 110°C, the methyl proton shifts to downfield by 0.04–0.07 ppm in neutral glycolipids and by 0.10–0.11 ppm in gangliosides. The methyl protons of N-acetylglucosamine residue resonate at 1.81 and 1.84 ppm ($LcOse_3Cer$) and shift to downfield by 0.06–0.09 ppm when measured at 110°C in the presence of D_2O.

Resonance of methyl protons of the N-acetylneuraminic acid residue exists at 1.88–1.90 ppm, shifting downfield by 0.02–0.04 ppm in the presence of D_2O at 110°C.

Olefinic proton resonance in ceramide moiety

The olefinic protons in ceramide moiety are located between 5.3–5.7 ppm, and were assigned previously (17).

Molar composition of ceramide and monosaccharides in glycosphingolipids by measurement of amide and anomeric protons

The molar composition of ceramide and carbohydrates on glycosphingolipids was measured by integrating the intensities of amide and anomeric protons, respectively, at 110°C in the absence of D_2O; the results are summarized in **Table 8.** All the molar ratios of respective monosaccharides to ceramide are reasonably consistent with their required molar composition.

In conclusion, the coupling constant of an anomeric proton elucidates the anomeric configuration of the sugar linkage; the chemical shifts of an amide proton as well as methyl protons differentiate ceramide, N-acetylhexosamines, and N-acetyl- and N-glycolylneuraminic acids; and a coupled measurement of intensities of amide and anomeric protons provides molar composition of ceramide and respective monosaccharides in glycosphingolipids.

DISCUSSION

The NMR analysis of amide protons of the complex carbohydrates and lipids provides valuable information on these molecular structures. The analysis of amide proton is suitable for the characterization and quantitation of ceramide and amino-containing carbohydrates (N-acetylhexosamines and sialic acids) in glycosphingolipids that have less amide as compared to other groups of glycoconjugates, glycoproteins and glycosaminoglycans. However, the use of deuterated protic sol-

TABLE 4. Coupling constants (Hz) of amide protons of ceramide, N-acetylhexosamines, and sialic acids in gangliosides

Gangliosides	Cer(FA)		GalNAc		GlcNAc		NeuAc		NeuGc	
	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C	25°C	110°C
$II^3NeuGc\text{-}LacCer$	7.8	8.3							6.8	6.8
$II^3NeuAc\text{-}GgOse_3Cer$	7.3	8.0	8.8	8.7			7.1	8.2		
$II^3NeuAc\text{-}GgOse_4Cer$	7.8	7.2	7.8	9.0			9.1	8.5		
$IV^3NeuAc\text{-}nLcOse_4Cer$	8.5	7.1			8.1	7.9	7.5	7.2		
IV^3NeuAc, $II^3NeuAc\text{-}GgOse_4Cer$	8.3	10.3	9.0	8.3			8.6	9.9		

TABLE 5. Chemical shifts of anomeric protons of glycosphingolipids[a]

Globo Series	GalNAc(α1 → 3)GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)				
GlcCer					4.12 (4.19)
LacCer				4.24 (4.27)	4.18 (4.21)
$GbOse_3Cer$			4.83 (4.85)	4.29 (4.31)	4.17 (4.18)
$GbOse_4Cer$		4.57 (4.58)	4.86 (4.88)	4.30 (4.34)	4.18 (4.23)
$IV^3GalNAc\alpha$-$GbOse_4Cer$	4.80 (4.85)	4.61 (4.64)	4.86 (4.84)	4.29 (4.33)	4.17 (4.22)

Ganglio Series	Gal(β1 → 3)GalNAc(β1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)			
	3 ↑ α2Sia[b]		3 ↑ α2Sia	
$GgOse_3Cer$		4.52 (4.56)	4.26 (4.30)	4.17 (4.24)
$GgOse_4Cer$	4.27 (4.27)	4.64 (4.64)	4.27 (4.27)	4.18 (4.22)
II^3NeuGc-LacCer			4.26 (4.34)	4.18 (4.25)
II^3NeuAc-$GgOse_3Cer$		4.84 (4.77)	4.29 (4.33)	4.17 (4.21)
II^3NeuAc-$GgOse_4Cer$	4.28 (4.33)	4.89 (4.82)	4.30 (4.35)	4.17 (4.22)
IV^3NeuAc, II^3NeuAc-$GgOse_4Cer$	4.28 (4.32)	4.85 (4.77)	4.29 (4.40)	4.18 (4.22)

Neolacto Series	Gal(β1 → 4)GlcNAc(β1 → 3)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)			
	3 ↑ α2Sia			
$LcOse_3Cer$		4.69 (4.66)	4.30 (4.31)	4.18 (4.22)
$nLcOse_4Cer$	4.24 (4.27)	4.73 (4.74)	4.28 (4.30)	4.18 (4.21)
IV^3NeuAc-$nLcOse_4Cer$	4.24 (4.33)	4.71 (4.67)	4.29 (4.33)	4.17 (4.21)

Gala Series	Gal(α1 → 4)Gal(β1 → 1)Cer(FA)		
GalCer			4.07 (4.14)
$GaOse_2Cer$	4.85 (4.89)	4.09 (4.19)	
Cerebroside sulfate			4.20 (4.28)

[a] Anomeric protons were measured at 110°C in the absence or presence (values indicated in parentheses) of D_2O.
[b] Sia, sialic acid.

vents such as D_2O and CD_3OD is not applicable for the analysis of amide proton because of the H-D exchange. Amide proton was not demonstrated in the oligosaccharides derived from glycosphingolipids in a D_2O solution (6–8), and disappeared in the permethylated glycosphingolipids that were derivatized under basic conditions (9–11). Although deuterated pyridine appears to be suitable, the peak of H_2O or HDO overlaps the anomeric proton region over a temperature range, and the chemical shift is not constant (4). Deuterated Me_2SO is an aprotic solvent and has a low exchange rate of H-H and H-D due to the rigid hydrogen bonding of polar Me_2SO both with amide and hydroxyl protons, as has been adopted in mono- (18) and polysaccharides (19). Dabrowski et al. (12, 20) demonstrated that Me_2SO was an effective solvent for examination of ^1H NMR of eight intact neutral glycosphingolipids (12) and ^1H and ^{13}C NMR of galactosylceramide (20). The conformation of glycosaminoglycans (21) and the whole structure of an O-acetylated ganglioside G_{M3} (22) were determined by measurement of the chemical shift of acetamido NH and by a spin decoupling procedure, respectively, in Me_2SO solution.

In the present study using Me_2SO, a number of glycosphingolipids including the globo, lacto, gala, and ganglio series, sulfatide, and ceramides were analyzed, without derivatization, for their amide protons as well as for anomeric and methyl protons. In most sphingolipids, the spectra of amide proton resonances present in a region more than δ7 ppm demonstrated not only the characteristic chemical shifts for ceramide, N-acetylhexosamines, and N-acylneuraminic acids (Tables 1, 2 and 8), but also allowed an estimation of the molar composition. Hexoses were quantitated by intensities of anomeric protons (Table 8). In the chemical composition of glycosphingolipids, the molar content of the lipophilic moiety has rarely been demonstrated owing to difficulty of its quantitation. The relative intensities of amide and anomeric protons in the glycosphingolipids provide a simultaneous estimate of the molar composition of lipid moiety (ceramide), N-acetylhexosamines, sialic acids, and hexoses. The respective monosaccha-

TABLE 6. Coupling constants of anomeric protons of glycosphingolipids[a]

Globo Series	GalNAc(α1 → 3)GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)				
GlcCer					7.3 (7.8)
LacCer				6.3 (7.0)	7.3 (7.3)
$GbOse_3Cer$			3.9 (3.6)	6.3 (6.9)	7.8 (7.1)
$GbOse_4Cer$		8.3 (7.8)	3.9 (3.4)	6.5 (7.3)	7.0 (7.7)
$IV^3GalNAc\alpha$-$GbOse_4Cer$	3.8 (3.5)	7.8 (7.9)	3.8 (3.5)	6.9 (7.5)	7.5 (7.8)
	Gal(β1 → 3)GalNAc(β1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)				
Ganglio Series	$\overset{3}{\underset{\alpha 2\text{Sia}^b}{\uparrow}}$		$\overset{3}{\underset{\alpha 2\text{Sia}}{\uparrow}}$		
$GgOse_3Cer$		8.0 (7.9)	7.8 (8.0)	7.4 (7.9)	
$GgOse_4Cer$	7.9 (7.3)	7.9 (7.7)	7.9 (7.2)	7.6 (7.8)	
II^3NeuGc-LacCer			7.3 (7.5)	7.2 (7.5)	
II^3NeuAc-$GgOse_3Cer$		8.3 (8.0)	8.3 (8.0)	7.3 (8.0)	
II^3NeuAc-$GgOse_4Cer$	6.9 (8.3)	7.8 (8.3)	6.8 (8.3)	7.3 (7.8)	
IV^3NeuAc, II^3NeuAc-$GgOse_4Cer$	7.0 (7.9)	7.6 (7.1)	7.0 (6.5)	7.3 (7.8)	
	Gal(β1 → 4)GlcNAc(β1 → 3)Gal(β1 → 4)Glc(β1 → 1)Cer(FA)				
Neolacto Series	$\overset{3}{\underset{\alpha 2\text{Sia}}{\uparrow}}$				
$LcOse_3Cer$		8.9 (9.0)	7.3 (7.4)	7.7 (7.9)	
$nLcOse_4Cer$	7.9 (7.5)	8.1 (7.9)	7.0 (7.0)	7.3 (7.8)	
IV^3NeuAc-$nLcOse_4Cer$	8.0 (7.2)	8.0 (8.0)	7.0 (6.9)	7.8 (7.6)	
Gala Series			Gal(α1 → 4)Gal(β1 → 1)Cer(FA)		
GalCer				7.6 (8.0)	
$GaOse_2Cer$			3.2 (3.0)	6.2 (7.0)	
Cerebroside sulfate				7.3 (7.5)	

[a] Anomeric protons were measured at 110°C in the absence or presence (values indicated in parentheses) of D_2O.
[b] Sia, sialic acid.

rides could be quantitatively differentiated. Since the materials after NMR analysis can be recovered, the proton NMR measurement provides a first choice for quantitation of ceramide and sugar components of glycosphingolipid. Although the chemical shifts of NH proton depend primarily on operating temperature and the concentration of a test material, these protons resonated with the same chemical shift with regularity in a range of 1–8 mg of sphingolipids (data not shown). Amide protons of ceramide in HFA-containing sphingolipids (free cermide and glycosphingolipids) resonated 0.01 to 0.14 ppm at a higher field than those in FA-containing sphingolipids, most probably due to a shielding effect of α-hydroxyl and Me_2SO. However, the regularity of

TABLE 7. Chemical shifts of methyl protons in acetyl group of glycosphingolipids in Me_2SO

	GalNAc			GlcNAc			NeuAc		
Glycosphingolipids	25°C	110°C	D_2O[a]	25°C	110°C	D_2O	25°C	110°C	D_2O
$GbOse_4Cer$	1.85	1.87	1.91						
$IV^3GalNAc\alpha$-$GbOse_4Cer$	1.83	1.85	1.90						
$GgOse_3Cer$	1.86	1.87	1.93						
$GgOse_4Cer$	1.83	1.85	1.87						
$LcOse_3Cer$				1.84	1.86	1.91			
$nLcOse_4Cer$				1.81	1.84	1.87			
IV^3NeuAc-$nLcOse_4Cer$				1.81	1.83	1.90	1.89	1.88	1.93
II^3NeuAc-$GgOse_3Cer$	1.77	1.83	1.88				1.88	1.88	1.90
II^3NeuAc-$GgOse_4Cer$	1.75	1.80	1.85				1.90	1.90	1.92
IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$	1.75	1.80	1.85				1.88	1.88	1.92

[a] Measured at 110°C in D_2O-containing solution.

TABLE 8. Molar composition of glycosphingolipids by measurement of intensities[a] of amide and anomeric protons

Glycolipids	Required						Analyzed							
	Cer	Glc	Gal	GalNAc	GlcNAc	Sia[b]	Cer	β-Glc	β-Gal	α-Gal	β-GalNAc	α-GalNAc	β-GlcNAc	Sia
GlcCer	1	1					1.0[c]	1.1[d]						
GalCer	1		1				1.0		0.8[d]					
LacCer	1	1	1				1.0	1.2	1.1					
$GaOse_2Cer$	1		2				1.0		1.1	1.1[d]				
$GbOse_3Cer$	1	1	2				1.0	1.2	1.1	0.9				
$GgOse_3Cer$	1	1	1	1			1.0	1.2	1.3		0.7[c] (1.3)[d]			
$LcOse_3Cer$	1	1	1		1		1.0	1.1	1.0				0.9[c] (1.0)[d]	
$GbOse_4Cer$	1	1	2	1			1.0	1.3	1.1	1.0	1.1[c] (1.1)[d]			
$GgOse_4Cer$	1	1	2	1			1.0	1.1	2.3		1.1[c] (0.9)[d]			
$nLcOse_4Cer$	1	1	2		1		1.0	1.4	2.2				1.1[c] (1.3)[d]	
$IV^3GalNAc\alpha$-$GbOse_4Cer$	1	1	2	2			1.0	1.2	1.0	0.9	0.9[c] (0.9)[d]	0.8[c] (0.9)[d]		
Cerebroside sulfate	1		1				1.0		1.1					
II^3NeuGc-LacCer	1	1	1			1	1.0	1.3	1.3					0.9[c]
II^3NeuAc-$GgOse_3Cer$	1	1	1	1		1	1.0	1.1	1.0		1.0[c] (0.9)[d]			1.0
IV^3NeuAc-$nLcOse_4Cer$	1	1	2		1	1	1.0	1.1	1.9				0.9[c] (1.0)[d]	1.0
II^3NeuAc-$GgOse_4Cer$	1	1	2	1		1	1.0	1.2	2.3		1.0[c] (1.0)[d]			1.1
IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$	1	1	2	1		2	1.0	1.0	1.9		1.3[c] (0.9)[d]			2.3

[a] The peak intensities were integrated in the spectra taken at 110°C.
[b] Sia = N-acetyl or N-glycolyl neuraminic acid.
[c] The value was from amide proton.
[d] The value was from anomeric proton.

an upfield shifting in HFA-containing sphingolipids at an increased temperature was not observed in FA-containing sphingolipids. On the other hand, amide proton of the N-acetylgalactosamine residue in gangliosides except for II^3NeuAc-$GgOse_4Cer$ shifted little at an elevated temperature, while the proton in neutral glycosphingolipids shifted to upfield by 0.33 to 0.42 ppm at a high temperature. Although the reason is not known, it is possible that the usual conformational change of ganglioside molecules caused by raising the temperature in Me_2SO solution is suppressed by the sialic acid moiety.

The chemical shifts and coupling constants of anomeric proton in globo- and lactoglycolipids were slightly different from those demonstrated previously (12) due to differences in the operating temperature, though they did not show the spectra of gangliosides. The coupling constants of α- and β-anomeric protons were clearly distinguished (Table 6). Moreover, the present study demonstrated that the chemical shifts of the anomeric protons in the nonreducing termini resonated in upfield as compared to those of the substituted sugars, while those of anomeric proton in sugars linked to sialic acid were not influenced by the terminal sialic acid (Table 5). The anomeric proton of β-linked N-acetylgalactosamine in gangliosides shifted markedly to lower field compared to that in asialogangliosides (Table 5). These phenomena suggest that sialic acid affects the anomeric proton of N-acetylgalactosamine considerably more than that of galactose to which sialic acid is linked.

In conclusion, the coupling constant of anomeric protons elucidates anomeric configuration of the sugar linkage; the chemical shifts of amide protons as well as methyl protons differentiate ceramide, N-acetylhexosamines, and N-acetyl- and N-glycolylneuraminic acids; and a coupled measurement of intensities of amide and

anomeric protons provides molar composition of ceramide and respective monosaccharides in glycosphingolipids.

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