High-Resolution Proton NMR Studies of Gangliosides. 1. Use of Homonuclear Two-Dimensional Spin-Echo *J*-Correlated Spectroscopy for Determination of Residue Composition and Anomeric Configurations[†]

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ABSTRACT: The proton nuclear magnetic resonance spectra of gangliosides G_{M4} (**6**), G_{M3} (**7**), G_{M2} (**8**), and G_{M1} (**9**) and their asialo derivatives (**3–5**) have been obtained at 500 MHz and 30 °C in dimethyl-d_6 sulfoxide–deuterium oxide (98:2 v/v). Through chemical shift analysis of the upfield (0.6–3.0 ppm), anomeric (4.0–5.0 ppm), and olefinic (5.2–5.6 ppm) regions, well resolved in the one-dimensional spectra, a quantitative determination of NeuAc/GalNAc/Gal/Glc/ceramide ratios is obtained within the series **3–9**. Through the use of homonuclear two-dimensional spin-echo *J*-correlated spectroscopy (2-D-SECSY), the *J* connectivities of **4–9** are revealed, allowing allocation of all resonances to subspectra. Each subspectrum is assigned to a specific residue through consideration of the number, *J*-connected pattern, and chemical shifts of its component protons and the magnitude and sequence of their vicinal coupling constants. Thus the monosaccharide composition, anomeric configurations, and aglycon structures of gangliosides and their derivatives may be obtained rapidly and nondestructively through high-field, proton 2-D-SECSY NMR, independent of congener series or other data. Formation of NeuAcα2→3Gal glycosidic linkages causes predictable 0.2–0.6 ppm deshielding of the proton directly attached to the glycosidation site. However, formation of GalNAcβ1→4Gal and Galβ1→3GalNAc glycosidic linkages causes irregular effects on the protons directly involved. The lack of chemical shift additivity in the latter cases indicates that glycosidation shift data can be unreliable in assignment of glycosidic linkage sites. Large, reciprocal long-range glycosidation shifts experienced by resonances of the GalNAc and NeuAc residues of **8** and **9** indicate through-space interactions exist between these residues, as they are disposed in these branched-chain gangliosides.

Gangliosides are sialic acid containing glycosphingolipids found in the plasma membrane of animal cells. First isolated from brain in which they are abundant, gangliosides have now been found in smaller quantities in extraneural tissues and body fluids (Ledeen & Yu, 1982). Due to the mounting evidence that gangliosides may function as membrane receptors, antigenic determinants, and cell–cell recognition sites (Hakomori, 1981; Fishman & Brady, 1976), there is a growing need to develop rapid micromethods for ganglioside structural analysis, especially in studies of tissues and fluids in which the amounts of gangliosides are limited, for example, human blood platelets and cerebrospinal fluid.

Proton nuclear magnetic resonance (NMR)[1] spectroscopy has always been a potentially powerful method for the structural analysis of gangliosides, since this method is rapid, quantitative, nondestructive, and sensitive. However, previous studies have only yielded fragmentary data concerning the primary structure of gangliosides (Harris & Thornton, 1978; Vliegenthart, 1980; Gasa et al., 1981; Koerner et al., 1982). This is largely due to the severe resolution problems that arise when all methine resonances of oligosaccharide rings, except anomeric resonances, fall within a 1 ppm chemical shift range. Previous solutions to this resolution or hidden-resonance problem (Dabrowski et al., 1982), which is a problem common to all oligosaccharide spectra (Hall, 1974), have been time consuming and only partially successful since coupling-constant data are lost; however, they have allowed the chemical shifts of most resonances of globo- and lacto-series glycosphingolipids to be obtained at 360 MHz (Dabrowski et al., 1980b, 1981; Hanfland et al., 1981).

Two advances now offer general and complete solutions to the hidden-resonance problem. These are the introduction of very high field NMR spectrometers (500 MHz) and the method of two-dimensional, homonuclear spin-echo *J*-correlated spectroscopy (2-D-SECSY), developed by Ernst and co-workers (Aue et al., 1976; Nagayama et al., 1980) and applied first to the analysis of polypeptide spectra by Wüthrich and co-workers (Nagayama et al., 1979; Nagayama & Wüthrich, 1981; Wagner et al., 1981). Recently 2-D-SECSY has been used to completely assign the proton spectrum of a derivatized monosaccharide (Bernstein et al., 1982) and a ceramide trisaccharide (**4**, Figure 1; Prestegard et al., 1982) and to partially assign the spectrum of a ceramide pentadecasaccharide (Dabrowski & Hanfland, 1982).

We now report the complete assignment of the proton spectra of the major monosialo gangliosides (**6–9**, Figure 1) and their asialo derivatives (**3–5**), using homonuclear 2-D-SECSY NMR at 500 MHz. We have carried out these studies with the idea of generating methods and a data base for future studies of gangliosides of unknown structure, es-

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[1] Abbreviations: NMR, nuclear magnetic resonance; 1-D, one-dimensional; 2-D-SECSY, two-dimensional spin-echo *J*-correlated spectroscopy; Me_2SO-d_6, dimethyl-d_6 sulfoxide; D_2O, deuterium oxide; Me_4Si, tetramethylsilane; NeuAc, *N*-acetylneuraminic acid; NeuGy, *N*-glycolylneuraminic acid; Glc, glucopyranose; GlcNAc, 2-acetamido-2-deoxyglucopyranose; Gal, galactopyranose; GalNAc, 2-acetamido-2-deoxygalactopyranose; L-Fuc, fucopyranose; G_{M1}, G_{M2}, G_{M3}, and G_{M4}, Svennerholm (1980) symbols for ganglioside structures (as shown in Figure 1); G_{A1}, asialo-G_{M1}; G_{A2}, asialo-G_{M2}; I, II, III, IV, and A, ganglioside residue structures (as shown in Figure 1); i, ii, iii, iv, and v, designators for ganglioside subspectra, prior to their assignment. All monosaccharides are assumed to be of the D configuration, unless indicated otherwise.

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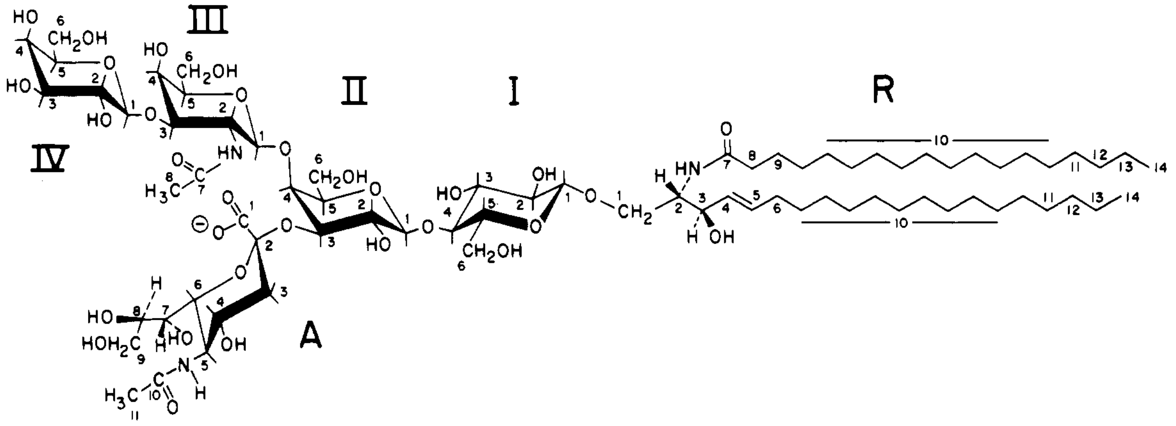

FIGURE 1: Structures, symbolism, and numbering for gangliosides (**6**–**9**), their asialo derivatives (**2**–**5**), glucosylceramide (**1**), and *N*-acetyl-α-neuraminic acid (**10**): **1** (GlcCer), R, I; **2** (GalCer), R, II; **3** (LacCer), R, I, II; **4** (G_{A2}), R, I, II, III; **5** (G_{A1}), R, I, II, III, IV; **6** (G_{M4}), R, II, A; **7** (G_{M3}), R, I, II, A; **8** (G_{M2}), R, I, II, III, A; **9** (G_{M1}), R, I, II, III, IV, A; **10** (NeuAc), A.

pecially those isolated only in small quantities. Thus we demonstrate that through analysis of the 2-D-SECSY-generated subspectra, the monosaccharide composition, anomeric configurations, and aglycon structure of a ganglioside may be assigned de novo.

Materials and Methods

Glycosphingolipids. Lactosyldihydroceramide (**3**) was purchased from Miles Laboratories (Elkhart, IN). Gangliotriaosylceramide or G_{A2} (**4**) and gangliotetraosylceramide or G_{A1} (**5**) were prepared by desialylation of human brain G_{M2} and G_{M1}, respectively, by employment of the procedure of Kasai et al. (1982). Gangliosides G_{M4} (**6**), G_{M2} (**8**), and G_{M1} (**9**) were isolated from human brain by column chromatography (Ando & Yu, 1977; Itoh et al., 1981). Ganglioside G_{M3} (**7**) was obtained from bovine adrenal medulla according to the procedure of Ariga et al. (1982) and kindly provided by Drs. T. Ariga and T. Miyatake (Tokyo Metropolitan Institute of Medical Science). All gangliosides were converted to their sodium salts during isolation.

Proton NMR Spectroscopy. For removal of exchangeable protons, each sample (2 mg) was placed in a 20 cm by 5 mm diameter NMR tube (PP-528; Wilmad Glass Co., Buena, NJ), suspended in D_2O (0.5 mL), completely solubilized through sonication and addition of Me_2SO-d_6 (between 0 and 0.6 mL), frozen, and lyophilized. The resulting residue was dissolved with sonication in 0.5 mL of freshly prepared Me_2SO-d_6–D_2O (98:2 v/v) containing Me_4Si reference (4 mM) to yield 2–3 mM sample solutions. Solvents were of highest purity and obtained from Aldrich (Milwaukee, WI) or Merck Isotopes (St. Louis, MO).

It should be pointed out that Me_2SO-d_6–D_2O (98:2 v/v) is a solvent as well suited for proton NMR studies of gangliosides as it is for neutral glycosphingolipids (Dabrowski et al., 1980b), because of not only its ability to produce well-dispersed spectra but also its ability to dissolve gangliosides and their asialo derivatives. It is also helpful that the solvent peaks are far upfield from the important anomeric resonances.

Proton spectra were obtained on a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer, operating in the Fourier-transform mode with quadrature detection. Probe temperature was 30 ± 2 °C, unless otherwise noted. Integrated, one-dimensional spectra were typically obtained with a 5000-Hz spectral width (0–10 ppm), 10-s cycle time, and 100–200 scans.

Two-dimensional spin-echo *J*-correlated spectroscopy (SECSY), which establishes scalar coupling (*J*) connectivities between peaks, was executed with two 90° pulses separated by a time $(1/2)t_1$. The first time domain was formed by incrementing t_1. FID's acquired at the end of time t_1 provide the second time domain. The data are displayed as a contour plot. Except for small displacements due to *J* coupling, the central horizontal region corresponds to a normal 1-D spectrum, and off-axis peaks occur at a vertical position corresponding to 0.5 the chemical shift distance to a spin-coupled resonance. Sequential construction of vertical, 135°, and vertical lines identifies coupled resonances. The software used to obtain the 2-D-SECSY spectra was obtained from Bruker Instruments, Billerica, MA (Manual for the Aspect 2000, FTNMR2D program, version 810515, developed by A. D. Bain, G. Balimann, and H. C. Jost). Each spectrum required a total of 32 pulses in a 256 × 2048 data set, which took approximately 2 h to acquire. Processing and plotting time for each spectrum was approximately 2 h.

We chose the SECSY experiments rather than the "2-D correlation spectroscopy" (COSY; Aue et al., 1976) because the former is advantageous in situations where differences in resonance frequencies of coupled pairs of nuclei are a small fraction of the total spectral width. This is particularly the case in gangliosides where differences in chemical shifts of ring protons are always less than 2 ppm. In these cases the SECSY experiment allows contraction of the ω_1 frequency domain to 0.5 the chemical shift difference with very substantial savings in data-set size, processing time, and acquisition time.

Results

One-Dimensional NMR Studies. Figure 2 shows the spectrum of ganglioside G_{M2} (**8**), an example of the 1-D spectra obtained at 500 MHz for gangliosides (**6**–**9**) and their asialo derivatives (**3**–**5**). Except for the region between 3 and 4 ppm, the combination of high-field strength and solution in Me_2SO-d_6–D_2O results in near complete spectral dispersion. Clearly resolved are several classes of protons, which are tentatively assignable on the basis of their chemical shifts and intensities. Found in the upfield part of the spectrum are the alkyl and acetamido methyl protons at 0.85 and 1.7–1.9 ppm, respectively, and the alkyl, allylic, α-carbonyl, and sialic acid H-3 methylene protons at 1.23, 1.93, 2.02, and 1.4–2.8 ppm, respectively. In the downfield part of the spectrum are found the oligosaccharide H-1 (anomeric) and the olefinic methine protons at 4.0–5.0 and 5.2–5.6 ppm, respectively. These well-resolved resonances will be analyzed first; then, those in the poorly dispersed 3–4 ppm region will be considered. In the following analysis and subsequent discussion the symbols and numbering used for individual protons of **1**–**10** are shown in Figure 1.

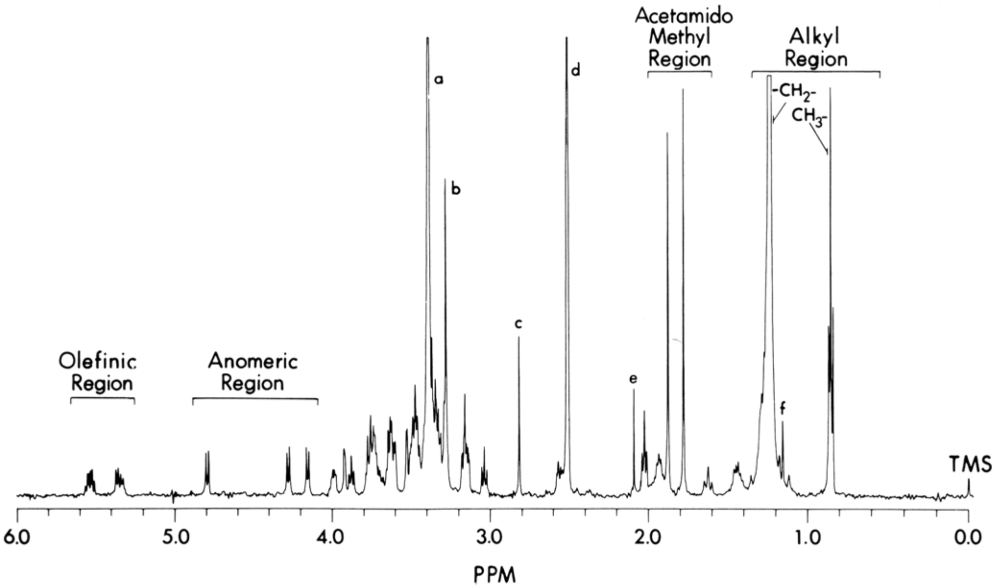

FIGURE 2: Proton NMR spectrum of ganglioside G_{M2} (**8**) obtained at 500 MHz and 30 °C. To be noted are the well-resolved olefinic, anomeric, acetamido methyl, and alkyl methyl and methylene regions and the following regularly observed nonganglioside resonances: (a) HOD; (b and c) EDTA methylenes; (d) Me$_2$SO-d_6 methyls; (e) acetone methyls; (f) stopcock grease methylenes; Me$_4$Si (TMS) reference.

Table I: Chemical Shifts and Integration Ratios of Methyl Proton Resonances of Gangliosides (**6–9**), Their Asialo Derivatives (**3–5**), and *N*-Acetyl-α-neuraminic Acid (**10**)

	proton chemical shifts (ppm)[a]			integration ratios[b]	
compd	A-11	III-8	R-14	A-11/R-14	III-8/R-14
3			0.852		
4		1.884	0.852		0.50 (0.50)
5		1.829	0.853		0.52 (0.50)
6	1.888		0.853	0.49 (0.50)	
7	1.888		0.853	0.49 (0.50)	
8	1.875	1.777	0.854	0.51 (0.50)	0.55 (0.50)
9	1.878	1.753	0.853	0.53 (0.50)	0.48 (0.50)
10[c]	1.881				

[a] Obtained in Me$_2$SO-d_6–D_2O (98:2 v/v) at 500 MHz and 30 °C, referenced to internal Me$_4$Si; error ±0.001 ppm. [b] Calculated value in parentheses; R-14 integration corrected for shorter relaxation time than for A-11 and III-8 (division by 1.3); error ±0.05. [c] Data obtained from a 2-D-SECSY study[2] of the α- and β-anomeric mixture of *N*-acetylneuraminic acid in Me$_2$SO-d_6–D_2O (98:2 v/v).

Upfield Spectrum. Shown in Figure 3 are the upfield halves of the spectra of **6–9**. The chemical shifts for the methyl proton resonances of **3–9** are reported in Table I. The alkyl methyl resonance (R-14) of **3–9** is assigned by its extreme upfield and regular shift (0.85 ppm). This is identical with the alkyl methyl resonance of other glycosphingolipids (Dabrowski et al., 1982). The methyl resonance of the GalNAc residue (III-8) of **4** and **5** and the NeuAc residue (A-11) of **6** and **7** is also readily assigned since each of structures **4–7** has only one acetamido methyl group and therefore has a unique resonance in the 1.7–1.9 ppm region of its spectrum. The chemical shifts of the two III-8 methyls of **4** and **5** are nearly identical with those found for the GalNAc and GlcNAc residues of globo- and lacto-series glycosphingolipids (Dabrowski et al., 1982). The 1.89 ppm chemical shift of A-11 of **6** and **7** is very close to that of the free residue (**10**) obtained in the same solvent.[2] In **8** and **9**, both A-11 and III-8 methyl groups are present and fairly close together in the acetamido methyl region. The downfield, structurally insensitive member of the pair (1.88 ppm) has been assigned to A-11, on the basis of the spectrum[2] of ganglioside G_{D1a}, which contains two methyl resonances near 1.88 ppm that arise from two NeuAc residues that resemble closely the NeuAc residues of **8** and **9**. Furthermore, carbon-13 NMR studies have indicated the chemical shift of the III-8 methyl group of gangliosides and glycopeptides is very structurally sensitive (Sillerud et al., 1982; Prohaska et al., 1981; Berman et al., 1980).

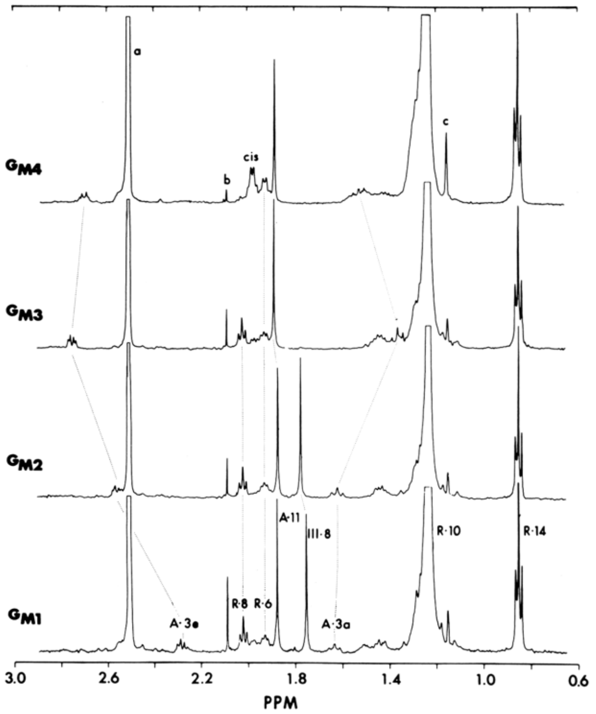

FIGURE 3: Upfield spectrum (0.6–3.0 ppm) of gangliosides G_{M4}–G_{M1} (**6–9**) obtained at 500 MHz and 30 °C. Resonance assignments running through all four spectra are labeled below. Also noted above are (cis) the resonance for methylene protons adjacent to the cis double bonds of unsaturated fatty acid groups and resonances due to the following impurities: (a) Me$_2$SO-d_6; (b) acetone; (c) stopcock grease. To be noted is the structural sensitivity of the A-3(a) and A-3(e) resonances (see Discussion).

The chemical shifts and coupling constants for the upfield methylene protons of **3–9** are reported in Tables II–V. The

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[2] T. A. W. Koerner, J. H. Prestegard, P. C. Demou, and R. K. Yu, unpublished data.

Table II: Chemical Shifts[a] for Ceramide (R) Methylene and Methine Protons of Gangliosides (**6–9**), Their Asialo Derivatives (**2–5**), and Glucosylceramide (**1**)

	proton chemical shifts (ppm)								
compd	H-1a	H-1b	H-2	H-3	H-4	H-5	H-6	H-8	H-10[b]
1[c]	3.469	3.922	3.782	3.898	5.366	5.548	1.937	2.030	1.20[d]
2[e]	3.451	3.937	3.784	3.918	5.369	5.562	1.936	2.038	1.25[d]
	3.539	3.909	3.820	4.001	5.383	5.582	1.936	3.820	1.25[d]
3[f]	3.478	3.957	3.751	3.30[g]	1.232	1.232	1.232	2.049	1.232
4	3.442	3.987	3.768	3.870	5.346	5.535	1.932	2.021	1.233
5	3.434	3.986	3.764	3.869	5.347	5.534	1.933	2.022	1.233
6[h]	3.509	3.962	3.795	3.926	5.346	5.546	1.919	2.020, 3.795[i]	1.233
7	3.432	3.993	3.769	3.876	5.343	5.533	1.931	2.024	1.234
8	3.421	3.987	3.774	3.875	5.346	5.534	1.932	2.023	1.235
9	3.432	3.988	3.765	3.873	5.345	5.533	1.928	2.022	1.235

[a] Obtained at 500 MHz at 30 °C in Me_2SO-d_6–D_2O (98:2 v/v), referenced to internal Me_4Si; error ±0.001 ppm. [b] Includes R-9, R-11, R-12, and R-13. [c] Values reported by Yamada et al. (1980). [d] Values not reported but estimated from published spectra. [e] Values reported by Dabrowski et al. (1980a). Lower set of values is for α-hydroxylated fatty acid component. [f] The aglycon is dihydroceramide. [g] Obscured by HOD resonance. [h] Also present were resonances at 5.320 and 1.977 ppm, assigned respectively to the *cis*-olefin methine and adjacent methylene of unsaturated fatty acid groups. [i] Value for R-8 of α-hydroxylated component.

Table III: Apparent Coupling Constants[a] for Ceramide (R) Methylene and Methine Protons of Gangliosides (**6–9**), Their Asialo Derivatives (**2–5**), and Glucosylceramide (**1**)

	proton–proton coupling constants (Hz)								
compd	$J_{1a,1b}$	$J_{1a,2}$	$J_{1b,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{8,9a}$	$J_{8,9b}$
1[b]	−10.5	4.0	5.0	6.4	7.0	15.3	6.9	7.2	7.2
2[c]	−10.2	3.9	5.1	6.9	7.4	15.4	6.5	7.5	7.5
	−10.4	4.2	5.5	6.9	7.0	15.4	6.7	(7.5)	(7.5)
3[d]	−10.3	3.0	5.1	7.5	*e*	*e*	*e*	7.1	7.1
4	−9.3	3.0	3.9	7.8	7.4	15.2	7.3	7.3	7.3
5	−9.5	3.3	4.0	7.9	7.2	15.7	7.4	7.4	7.4
6	−10.1	3.7	4.8	7.4	7.2	15.5	6.4	7.3	7.3
								7.5[f]	7.5[f]
7	−9.6	3.5	4.4	7.6	7.5	15.3	7.3	7.3	7.3
8	−9.6	3.3	4.1	7.7	7.4	15.7	6.0	7.4	7.4
9	−9.8	3.1	5.0	7.8	7.8	15.1	7.4	7.3	7.3

[a] Error estimated to be ±0.6 Hz. [b] Values as reported by Yamada et al. (1980). [c] Values as reported by Dabrowski et al. (1980a). Lower set of values is for α-hydroxylated fatty acid component; $J_{8,9}$ not reported but estimated from published spectra. [d] The aglycon is dihydroceramide. [e] Obscured by other resonances. [f] Value for α-hydroxylated fatty acid component.

Table IV: Chemical Shifts[a] for Methylene and Methine Protons of *N*-Acetylneuraminic Acid Residues (A) of Gangliosides (**6–9**) and Free *N*-Acetyl-α-neuraminic Acid (**10**)

	proton chemical shifts (ppm)								
compd	H-3(e)	H-3(a)	H-4	H-5	H-6	H-7	H-8	H-9a	H-9b
10[b]	2.494	1.505	3.608	3.468	3.600	3.221	3.421	3.368	3.600
6	2.658	1.536	3.571	3.450	3.564	3.180	3.39[c]	3.311	3.564
7	2.749	1.364	3.551	3.463	3.569	3.199	3.40[c]	3.377	3.569
8	2.558	1.621	3.700	3.39[c]	3.148	3.161	3.485	3.340	3.613
9	2.281	1.636	3.744	3.37[c]	3.116	3.184	3.495	3.335	3.619

[a] Obtained at 500 MHz at 30 °C in Me_2SO-d_6–D_2O (98:2 v/v), referenced to internal Me_4Si; error ±0.001 ppm. [b] Data obtained from a 2-D-SECSY study[2] of the α- and β-anomeric mixture of *N*-acetylneuraminic acid in Me_2SO-d_6–D_2O (98:2 v/v). [c] Obscured by HOD resonance.

Table V: Apparent Coupling Constants[a] for *N*-Acetylneuraminic Acid Residues (A) of Gangliosides (**6–9**) and Free *N*-Acetyl-α-neuraminic Acid (**10**)

	proton–proton coupling constants (Hz)									
compd	$J_{3(e),3(a)}$	$J_{3(e),4}$	$J_{3(a),4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9a}$	$J_{8,9b}$	$J_{9a,9b}$
10[b]	−11.7	5.0	11.7	10.7	9.4	2.2	9.6	6.5	2.6	−11.9
6	−11.8	4.7	11.6	9.9	8.9	<1.5	8.9	6.9	<1.5	−11.4
7	−11.8	5.0	11.6	9.6	9.0	<1.5	9.4	6.0	<1.5	−10.8
8	−12.4	5.1	10.6	10.6	9.1	1.9	9.8	6.0	<1.5	−10.8
9	−12.5	4.8	10.8	10.8	9.6	<1.5	9.0	6.0	<1.5	−10.8

[a] Error estimated to be ±0.6 Hz. [b] Data obtained from a 2-D-SECSY study[2] of the α- and β-anomeric mixture of *N*-acetylneuraminic acid in Me_2SO-d_6–D_2O (98:2 v/v).

alkyl (1.23 ppm), allylic (1.93 ppm), and α-carbonyl (2.02 ppm) methylene resonances of **3–9** are assigned to R-10, R-6, and R-8, respectively, by their similarity with the chemical shifts, multiplicities, and intensities of the analogous ceramide resonances of **1** and **2** (Tables II and III). In the upfield spectrum of **6** (Figure 3), an additional allylic resonance is

Table VI: Chemical Shifts[a] for Methine and Methylene Protons of Core Oligosaccharide Residues (I-IV) of Gangliosides (6-9), Their Asialo Derivatives (2-5), and Glucosylceramide (1)

		proton chemical shifts (ppm)						
compd	residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
1[b]	I	4.094	2.981	3.155	3.1	3.1	3.440	3.663
2[c]	II	4.053	3.321	3.285	3.653	3.336	3.491	3.546
		4.093	3.309	3.288	3.651	3.344	3.500	3.546
3	I	4.173	3.030	3.30[d]	3.30[d]	3.30[d]	3.584	3.741
	II	4.199	3.40	3.30[d]	3.613	3.40[d]	3.528	3.584
4	I	4.161	3.036	3.335	3.288	3.293	3.598	3.745
	II	4.222	3.245	3.519	3.789	3.500	3.478	3.604
	III	4.462	3.614	3.519	3.614	3.335	3.40[e]	3.478
5	I	4.159	3.033	3.325	3.289	3.296	3.612	3.741
	II	4.209	3.228	3.495	3.792	3.495	3.480	3.612
	III	4.566	3.768	3.647	3.792	3.341	3.435	3.498
	IV	4.216	3.325	3.245	3.604	3.341	3.388	3.512
6	II	4.063	3.269	3.919	3.684	3.213	3.468	3.530
7	I	4.156	3.037	3.328	3.292	3.297	3.606	3.741
	II	4.188	3.312	3.960	3.696	3.336	3.484	3.599
8	I	4.150	3.035	3.325	3.277	3.289	3.613	3.744
	II	4.274	3.158	3.744	3.920	3.470	3.463	3.613
	III	4.794	3.753	3.39[e]	3.524	3.642	3.39[e]	3.463
9	I	4.150	3.034	3.335	3.276	3.288	3.627	3.735
	II	4.275	3.131	3.732	3.935	3.486	3.471	3.627
	III	4.859	3.912	3.471	3.716	3.630	3.37[e]	3.471
	IV	4.212	3.37[e]	3.310	3.608	3.37[e]	3.411	3.538

[a] Obtained at 500 MHz and 30 °C in Me_2SO-d_6-D_2O (98:2 v/v), referenced to internal Me_4Si; error ±0.001 ppm. [b] Values reported by Yamada et al. (1980). [c] Values reported by Dabrowski et al. (1980a). Lower set of values is for α-hydroxylated fatty acid component. [d] Obscured by other resonances. [e] Obscured by HOD resonance.

Table VII: Apparent Vicinal Coupling Constants[a] for Methine and Methylene Protons of Core Oligosaccharide Residues (I-IV) of Gangliosides (6-9), Their Asialo Derivatives (2-5), and Glucosylceramide (1)

		proton-proton coupling constant (Hz)						
compd	residue	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
1[b]	I	7.6	9.4	9.0	c	5.6	2.4	−11.4
2[d]	II	6.9	9.8	2.9	1.2	6.4	6.2	−10.7
		7.0	9.4	3.1	1.2	6.2	5.8	−11.0
3	I	7.8	8.1	e	e	5.0	<1.5	−10.1
	II	7.8	e	2.0	<1.5	5.1	5.0	−10.7
4	I	7.8	8.2	9.4	10.3	6.3	<1.5	−10.8
	II	7.8	8.9	2.0	<1.5	5.0	5.0	−11.5
	III	8.4	10.0	2.5	<1.5	6.3	6.3	−10.5
5	I	7.8	8.2	9.3	10.5	6.1	1.5	−11.0
	II	7.7	9.5	2.0	<1.5	5.0	6.1	−11.5
	III	8.0	10.0	2.8	<1.5	6.5	5.5	−11.5
	IV	7.5	9.5	3.2	<1.5	6.0	6.0	−11.0
6	II	7.7	9.4	2.8	<1.5	6.2	6.2	−10.6
7	I	7.8	8.1	9.6	9.6	5.0	<1.5	−11.5
	II	7.9	9.9	3.2	1.5	5.7	6.0	−11.4
8	I	7.9	8.2	9.6	9.6	6.0	<1.5	−11.5
	II	9.6	8.7	<1.5	<1.5	6.0	5.6	−12.1
	III	8.8	9.6	1.5	<1.5	5.9	6.0	−12.0
9	I	7.9	8.2	9.6	9.6	6.0	1.5	−12.0
	II	7.7	9.6	2.4	<1.5	6.0	6.0	−11.5
	III	8.7	9.8	<1.5	<1.5	5.4	5.4	−11.0
	IV	7.3	10.3	2.4	<1.5	6.0	6.0	−11.2

[a] Error estimated to be ±0.6 Hz. [b] Values reported by Yamada et al. (1980). [c] Value not reported. [d] Values reported by Dabrowski et al. (1980a). Lower set of values is for α-hydroxylated fatty acid component. [e] Obscured by other resonances.

seen at 1.98 ppm. This resonance is assigned to methylene protons adjacent to cis double bonds, which are known to be abundant in **6** due to its large content of unsaturated fatty acyl groups (vide infra). Hydroxylation of most of the fatty acyl groups of **6** leads to the downfield shift of R-8 and its elimination from observation in Figure 3. The axial and equatorial H-3 methylene resonances [A-3(a) and A-3(e)] of the sialic acid residues of **6–9** are observed between 1.4 and 1.6 and 2.3 and 2.8 ppm, respectively, and are assigned (Tables IV and V) by comparison of their chemical shifts and coupling constants with those of the free monosaccharide residue αNeuAc (**10**)[2] and by the absence of these resonances in the spectra of **3–5**. The possible reasons for the structural sensitivity of the chemical shifts of the A-3(a) and A-3(e) resonances of **6–9** as noted in Figure 3 will be discussed below.

Anomeric Region. Inspection of the structures of **3–9** (Figure 1) reveals each oligosaccharide moiety contains between one and four neutral *aldo*-hexopyranosyl residues in its core oligosaccharide and either one (**6–9**) or no (**3–5**) attached sialic acid residue. As shown in Figure 4, one well-resolved H-1 (anomeric) doublet is present between 4 and 5 ppm for each core oligosaccharide residue present in **6–9**. The chemical shifts and coupling constants for the anomeric protons of **3–9** are reported in Tables VI and VII, respectively. It should be

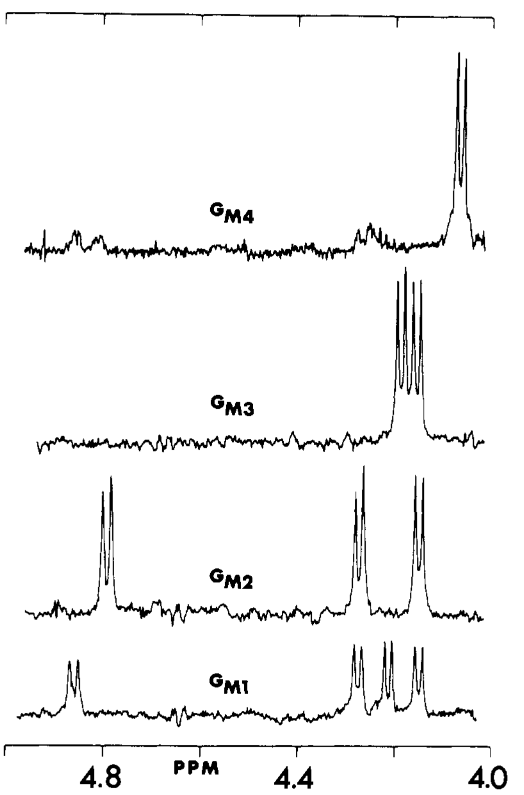

FIGURE 4: Anomeric resonance region (4.0–5.0 ppm) of gangliosides G_{M4}–G_{M1} (**6**–**9**) obtained at 500 MHz and 30 °C. From low to high field, assignments for oligosaccharide H-1 doublets are as follows: G_{M4}, II-1; G_{M3}, II-1, I-1; G_{M2}, III-1, II-1, I-1; G_{M1}, III-1, II-1, IV-1, I-1.

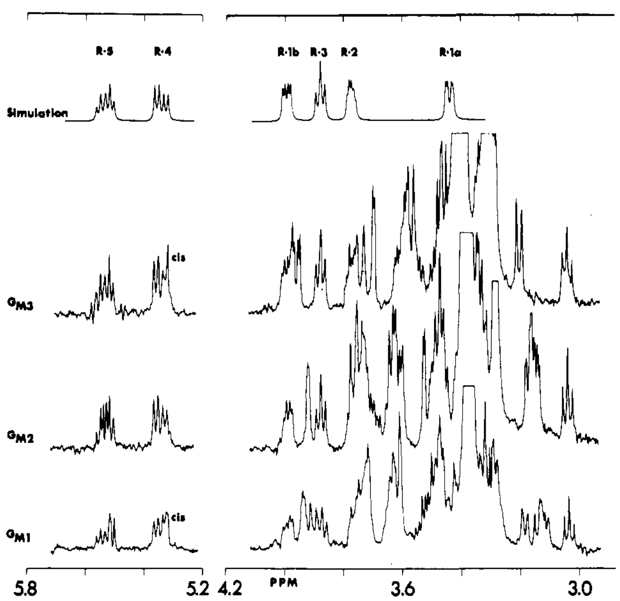

FIGURE 5: Olefinic (5.2–5.6 ppm) and overlapped 3–4 ppm region of gangliosides G_{M3}, G_{M2}, and G_{M1} (**7**–**9**) and computer simulation of ceramide eight-spin (AB)HMRSX$_2$ subspectrum for comparison. Resonance assignments are shown above; noted also are (cis) the resonances for methylene protons adjacent to the cis double bond of unsaturated fatty acid groups.

noted that sialic acid residues are ketosyl, rather than aldosyl residues, and manifest no anomeric proton. All anomeric resonances in the spectra of **3**–**9** have large coupling constants (7–9 Hz), consistent with the β-D configurations and ${}^4C_1$ conformations expected for the oligosaccharide residues of ganglio-series glycosphingolipids.

Except for II-1 of **6**, which uniquely resonates in the anomeric region, specific H-1 assignments may be made only if certain assumptions are made concerning the expected chemical shifts of anomeric protons (Tables VI and VII). If it is assumed that H-1 of *N*-acetyl-β-hexosamine residues resonates downfield from 4.35 ppm, then the doublet at 4.5 ppm in the spectra of **4** and **5** and at 4.8 ppm in the spectra of **8** and **9** may be assigned to III-1. This assumption is supported by studies of globo- and lacto-series glycosphingolipids (Dabrowski et al., 1982). If it is assumed that H-1 of Glc residues resonates regularly upfield and at a chemical shift similar to that of GlcCer (**1**; Yamada et al., 1980), then the doublet at 4.16 ppm in the spectra of **3**–**5** and **7**–**9** may be assigned to I-1. Due to their resulting uniqueness, the resonances between 4.19 and 4.28 ppm in the spectra of **3**, **4**, **7**, and **8** may then be assigned to II-1. However, in the spectra of **5** and **9**, two doublets appear in this same region (4.19–4.28 ppm). Unambiguous assignment of these signals to either II-1 or IV-1 requires establishing their *J* connectivities (see 2-D-SECSY Studies, below).

Olefinic Region. As shown in Figure 5 for **7**–**9**, each spectrum of **4**–**9** manifests a pair of mutually coupled (15.7-Hz) resonances at 5.3 and 5.5 ppm. These are doublets of doublets and doublets of triplets, respectively, as expected for the two methine protons of a trans double bond with neighboring methine and methylene groups, respectively. Since this functional group occurs only in the ceramide residue of **4**–**9**, these two resonances are assigned to R-4 and R-5. On the basis of their chemical shifts and multiplicities, the resonance at 5.3 ppm is assigned to R-4 and the resonance at 5.5 ppm to R-5 (Tables II and III). These assignments are supported by those made for **1** and **2** (Dabrowski et al., 1980a; Yamada et al., 1980). As expected, olefinic resonances do not appear in the spectrum of **3** where the aglycon is dihydroceramide. Observed slightly upfield from R-4 is the resonance of isochronous olefinic methine protons, assigned to the cis double bond(s) of unsaturated fatty acyl groups of ceramide. Consistent with previous carbon-13 NMR analysis (Sillerud et al., 1982), this cis resonance is intense for **6**, comparable to the trans resonances of **7** and **9**, and trace for **8**.

Potentially as useful as the information extracted from the upfield, anomeric, and olefinic regions is the constitutional and configurational data that are hidden in several closely overlapped envelops in the 3–4 ppm region. In these envelops are all core oligosaccharide methylene and methine protons, other than those at H-1, all but two of the NeuAc ring protons, and the four proximal ceramide protons. In an attempt to resolve the overlapped resonances of these protons, one-dimensional spin-decoupling experiments were carried out on G_{M1} (**9**), including spin-decoupling difference spectroscopy (SDDS) as suggested by Dabrowski et al. (1980b). We found that in cases where more than five SDDS experiments would have to be undertaken, a single 2-D-SECSY experiment was more practical, in that less operator and spectrometer time was required, and was more informative, in that accurate spin–spin coupling constants were obtainable in addition to chemical shift data. Since as many as 47 individual *J* connectivities must be established for gangliosides **6**–**9**, only 2-D-SECSY experiments were subsequently employed.

2-D-SECSY Studies. The overlapping envelops of resonances in the 3–4 ppm region of **3**–**9** were deciphered by consideration of integration data and establishment of the scalar coupling (*J*) connectivities of the component resonances of each envelop. The latter information was readily obtained through 2-D-SECSY studies. As an example, the 2-D-SECSY spectrum of the 3–4 ppm and anomeric regions of **8** is shown in Figure 6b, together with the corresponding segment of its

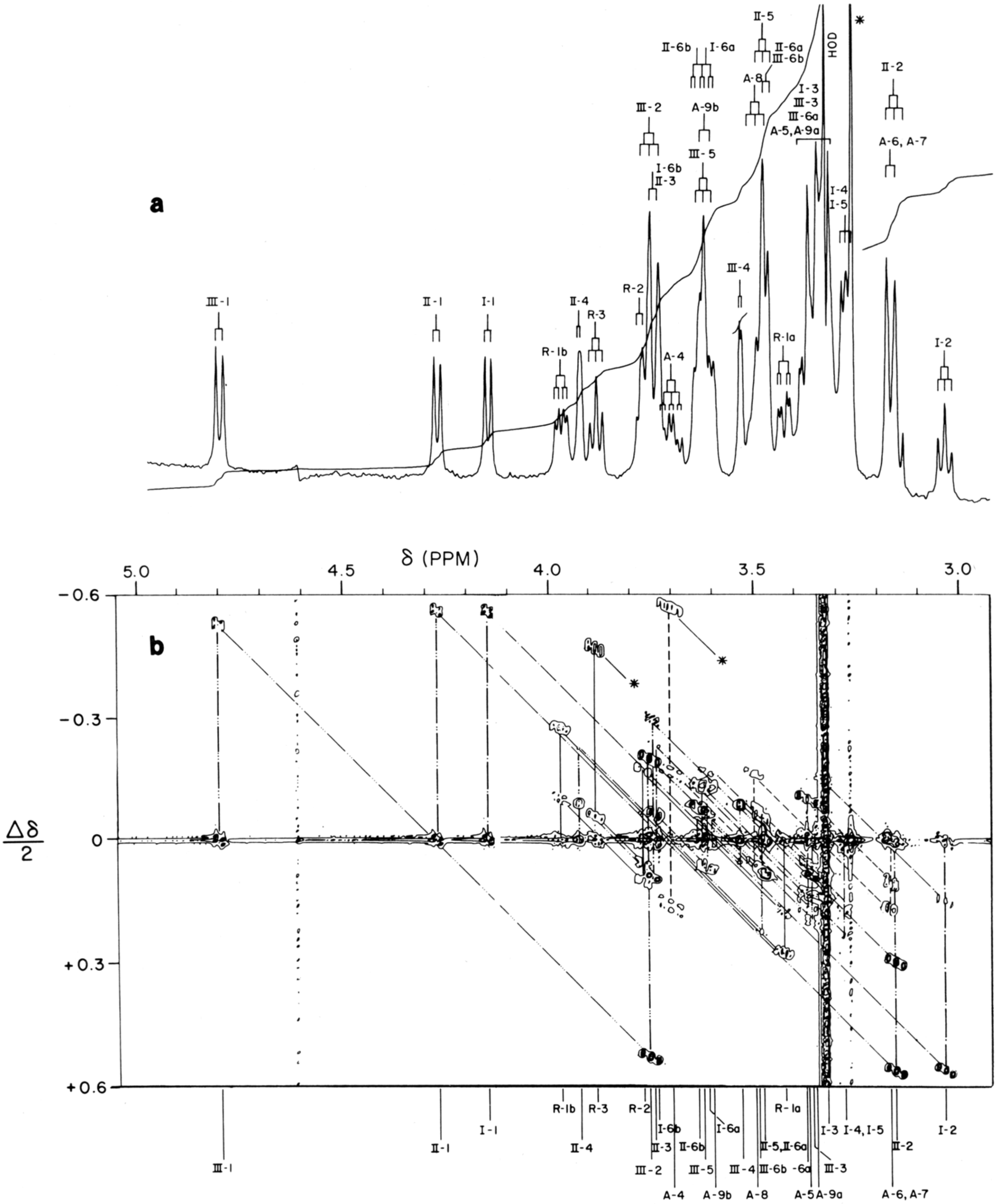

FIGURE 6: (a) Integrated, one-dimensional proton NMR spectrum of ganglioside G_{M2} (**8**) between 3 and 5 ppm. An asterisk (*) indicates EDTA methylene peak. (b) Two-dimensional proton spin-echo *J*-correlated (2-D-SECSY) spectrum of G_{M2} (**8**), revealing the *J* connectivities of its component residues in the oligosaccharide ring proton region (3–5 ppm). *J* connectivities are labeled for residues as follows: I, β-glucopyranosyl (–•–); II, β-galactopyranosyl (–••–); III, 2-acetamido-2-deoxy-β-galactopyranosyl (–•••–); R, ceramide (—); A, *N*-acetylneuraminyl (– –). An asterisk (*) indicates *J* connectivities to protons outside observed window.

one-dimensional spectrum (Figure 6a) for comparison. Analysis of this 2-D-SECSY spectrum of **8** will serve to illustrate the method. In this analysis each subspectrum will be temporarily labeled, in the order of consideration, with lower case Roman numerals (i, ii, iii, etc.) prior to its assignment.

Inspection of the anomeric region of **8** (Figure 6b) reveals three areas of contour density above the central axis. These represent cross peaks between the anomeric resonances and resonances for protons on adjacent carbons. Each anomeric resonance is arbitrarily assigned to subspectra i–iii according to increasing chemical shift. For anomeric protons (i-1, ii-1, iii-1), each is coupled to a single second proton (i-2, ii-2, iii-2, respectively) as indicated by the single area of off-axis contour density for each anomeric resonance. Resonances for coupled protons can be found at a position $2x$ upfield from the corresponding anomeric resonance, where x is the distance in hertz from the central axis to the area of cross-peak contour density. The assignment of i-2, ii-2, and iii-2 can be confirmed by reversal of the procedure and beginning with the cross-peak density located at the chemical shift of the coupled resonance but below the horizontal axis by distance x. It should be noted that the multiplicity of each resonance is usually recognizable in each area of contour density when projected onto the horizontal axis. Thus, the areas in the anomeric region, when projected, yield doublets, and the areas below the coupled protons, when projected, yield doublets of doublets (approximate triplets). While this is useful in assignment, one must be cautioned that not all components of a multiplet are accurately represented in the cross peaks; the central peak of an ideal triplet, for example, will be weak or missing.

If one continues the J-connectivity path as outlined above, it is seen that in addition to the H-1 coupling, each subspectrum H-2 resonance is coupled to a unique third resonance (i-3, ii-3, iii-3, respectively). Likewise, each H-3 leads to H-4, H-4 to H-5, and finally H-5 to H-6a and H-6b. With adequate resolution, this procedure is unambiguous, and a complete J-connectivity series H-1 → H-6b is revealed for each of the three subspectra that originates in the anomeric region of **8**. Thus each of these J-connectivities is seen to culminate in a seven-spin AHMRV(XZ) subspectral system, which are labeled i, ii, and iii in accordance with the labeling of the anomeric proton of each.

All of the other coupled protons of **8**, including those not shown in Figure 6, fall into one of two other subspectra, which differ primarily from i–iii in that their J-connectivity series must be entered via a region other than the anomeric region. One of these subspectra is picked up as the pair of trans double bond resonances in the olefinic region assigned on the basis of 1-D data to R-4 and R-5. Each of these olefinic resonances is seen to be coupled upfield, one to the isochronous methylene pair at 1.93 ppm (R-6) and the other to a chain of three resonances between 3.8 and 4.0 ppm, the last two of which are circuitally coupled to a fourth resonance at 3.4 ppm. The R-6 methylene is seen to be coupled upfield to the alkyl methylene resonance at 1.23 ppm. Taken together, this J-connectivity series is an eight-spin (AB)HMRSX_2 system (subspectrum iv), where RS represents R-4 and R-5 and X_2 represents R-6.

The last J connectivity of **8** is picked up as the methylene pair in the upfield region that has been assigned on the basis of 1-D data to A-3(a) and A-3(e). Each of these resonances is coupled downfield to a third resonance at 3.7 ppm, which begins a linearly coupled series of five protons with chemical shifts around the HOD solvent peak (3–4 ppm). The last of this linearly coupled series is circuitally coupled to two other resonances also in the 3.3–3.5 ppm region. Taken together, this last J-connectivity series is seen to be a nine-spin (AB)-HMRSV(XZ) system (subspectrum v).

Subspectral Assignments. After each resonance of **3**–**9** has been allocated to a specific spectrum via J connectivity, the identity of each subspectrum needs to be established. It should be noted that the lack of scalar (J) connectivity across the glycosidic linkage means that each subspectrum originates from, and is equivalent to, only one residue. When complete subspectra are available, assignment may be accomplished through consideration of three characteristics of each subspectrum. These characteristics, in order of their diagnostic utility, are (1) the number of protons and their pattern or network, (2) the sequence of vicinal coupling constants between component protons, and (3) the chemical shifts of component protons. With these characteristics, the subspectra of **3**–**9** may be unambiguously assigned, as will be illustrated with the subspectra of **8** as an example.

Subspectra i, ii, and iii. Inspection of the structure of **8** in Figure 1 reveals the only residues expected to manifest seven-spin subspectra with one noncircuital terminus are the core oligosaccharide residues (i–iii). Thus, the three AHMRV(XZ) subspectra (i–iii) are assigned collectively to residues I–III, and the assignment of the three doublets between 4 and 5 ppm of the 1-D spectrum to the anomeric protons of I–III is confirmed. Furthermore, the pattern of these AHMRV(XZ) subspectra independently establishes that I–III are *aldo*-hexosyl residues, since other subspectral patterns would have indicated other structures. For example, an AHMRVX_3 pattern would have indicated a 6-deoxy-*aldo*-hexosyl residue was present in **8** (e.g., L-fucose residue). Other possible core oligosaccharide residues and their expected patterns are as follows: 2-deoxy-*aldo*-hexosyl, A(HJ)MRV-(XZ); *aldo*-pentosyl, AHMR(XZ); and *aldo*-hexuronyl, AHMRV.

The constitution of each residue is further established by consideration of its H-1 and H-2 resonances (AH part of subspectrum). Thus the finding that all H-1 resonances are of equal intensity and no minor H-1 signal is present indicates all of the oligosaccharide residues are glycosidically-linked and none is free and mutarotating. The findings that the chemical shift of H-2 of iii is greater than 3.5 ppm but of i and ii is less than 3.3 ppm indicate that iii is an N-acylated hexosamine and i and ii are hexoses. The finding that H-1 resonances of i, ii, and iii all have large coupling constants and have a chemical shift less than 4.3 ppm for i and ii and less than 4.6 ppm for iii indicates they are all β-pyranosidically linked. In contrast, β-furanosidic linkages would be 0.4 ppm further downfield for hexose or *N*-acylhexosamines. The three-proton singlet at 1.777 ppm indicates iii is N-acetylated.

The configuration of each residue i–iii is established by consideration of the magnitude and sequence of coupling constants manifested by its ring protons (the AHMRV part of the subspectrum of each residue, H-1 through H-5). This configurational analysis is possible because aldopyranoside rings typically present in gangliosides are known to exist in rigid chair conformations and the Karplus relationships between the ring protons of such rings have been well studied (Hall, 1974; Altona & Haasnoot, 1980). Thus the series of coupling constants $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, $J_{4,5}$ for the β-*galacto* configuration would be L, L, S, S, where L and S are large (>6 Hz) and small (<4 Hz) coupling constants, respectively. For the α-*galacto*, β-*gluco*, and α-*gluco* configurations, the series would be as follows: S, L, S, S; L, L, L, L; and S, L, L, L, respectively. Inspection of Table VII reveals the coupling constant pattern for i is L, L, L, L and for ii and iii is L, L, S, S. Thus residue i has a β-*gluco* and residues ii and iii have a β-*galacto* configuration. Combining the constitutional and configurational data, it is concluded that i, ii, and iii of **8** are β-glucopyranosyl (I), β-galactopyranosyl (II), and 2-acetamido-2-deoxy-β-galactopyranosyl (III) residues, respectively.

Subspectrum iv. The characteristics of eight spins, two circuital termini, coupling to the alkyl methylene resonance, sequence of coupling constants (Table III), and inclusion of the trans olefinic resonances (R-4 and R-5) all clearly identify the (AB)HMRSX_2 subspectrum as arising from the proximal portion of the ceramide (R) residue of **8**. The number of protons and pattern of this subspectrum serves to independently establish its structure, since other possible aglycons would have manifest different subspectra. For example, dihydroceramide (R of **3**) or a steroid[2] would have displayed, respectively, six-spin (AB)HMX_2 or extended, complex (AB)(HI)M(RS)V... patterns. The appearance of an intense triplet at 2.02 ppm (R-8) but not at 3.80 ppm indicates no α-hydroxylated fatty acyl groups are present in **8**. The appearance of very weak resonances at 5.32 and 1.98 ppm indicates only trace fatty acid unsaturation (cis double bonds) are present.

Subspectrum v. The characteristics of nine spins, two circuital termini, sequence of coupling constants (Table V), and inclusion of the sialic acid H-3 methylene protons all identify the (AB)HMRSV(XZ) subspectrum as arising from the sialic acid (A) residue of **8**. The chemical shifts of the H, S, V, and XZ portions of this subspectrum indicate it is not *O*-acylated at these sites (A-4, -7, -8, and -9), which would have resulted in ca. 1.6 ppm downfield shifts for these protons.[2] The 3.4 ppm chemical shift of the M portion and the three-proton singlet at 1.875 ppm indicate that subspectrum v is N-acetylated, rather than N-glycolylated, which would have resulted in a two-proton AB pattern centered near 3.88 ppm.[2] Thus the sialic acid residue of **8** is independently verified as being *N*-acetylneuraminic acid (A).

A special problem in the assignment of the subspectra of **5** and **9** is the differentiation of the two galactopyranosyl residues (II and IV). This is accomplished by comparison of the chemical shifts of the H-3 and H-4 protons of these two residues, since II-3 and -4, but not IV-3 and -4, are involved in glycosidic linkages and are shifted downfield relative to the nonglycosylated residue. Also noteworthy is the observed regularity of the (AB)HMRSX_2 ceramide subspectrum throughout the series **4**–**9**, not only in the olefinic region (R-4 and -5) but also in the 3–4 ppm region (R-1b, -2, and -3). This is illustrated in Figure 5 by comparison of the appropriate regions of **7**–**9** with the computer-simulated subspectrum. Throughout the series **3**–**5** and **7**–**9**, a remarkable regularity is also observed for the AHMRV(XZ) subspectrum assigned to the Glc (I) residue (Tables VI and VII).

After all subspectra of **3**–**9** have been assigned in the manner illustrated above for **8**, complete resonance assignments can be made for all *J*-connected protons of each compound of the series. For the ceramide (R), sialic acid residues (A), and oligosaccharide (I–IV) residues of **3**–**9**, the chemical shifts and apparent vicinal coupling constants are reported in Tables II–VII. These assignments together with those made for the methyl protons (Table I) constitute complete assignments for all resonances of the series **3**–**9**, which includes a pentaglycosyl (**9**) and tetraglycosyl (**5** and **8**) ceramides. To our knowledge, these are the first assignments for ganglio-series glycosphingolipids and the first complete proton resonance assignments for glycosphinolipids more complex than monoglycosylceramides such as **1** and **2** (Dabrowski et al., 1980a; Yamada et al., 1980) or for oligosaccharides more complex than disaccharides (Hall et al., 1980).

Discussion

Anomeric Configurations. The anomeric form of each neutral oligosaccharide residue of a ganglioside is readily established by measurement of the vicinal coupling constant of the anomeric resonance of each residue. A large (>6 Hz) coupling indicates β-linkage, and a small (<4 Hz) coupling indicates α-linkage. This generality is supported by the data presented herein (Table VII), previous studies of gangliosides (Gasa et al., 1981; Koerner et al., 1982) and other glycosphingolipids (Dabrowski et al., 1982), and numerous studies of model compounds (Hall, 1981). However, it should be noted that the assumption is made that each oligosaccharide residue is a D-aldopyranoside set in a $^4C_1$ conformation. This assumption is certainly true for all known monosaccharide constituents of gangliosides, except L-fucose. This potential exception to the rule, however, should pose no problem when encountered, since the presence of fucose is clearly heralded by a distinct 3-proton doublet at 1.04 ppm due to its H-6 protons, as observed in the spectra of neutral glycosphingolipids (Dabrowski et al., 1981). Moreover, the combination of changes in conformation ($^4C_1 \rightarrow {}^1C_4$) and absolute stereochemistry (D → L) cancel each other, with the net result that the rule holds, namely, that small coupling constants (<4 Hz) indicate α-linkage.

The assignment of the anomeric form of the sialic acid residue(s) of a ganglioside can be made by determining the chemical shifts of its A-3 equatorial and axial protons, which are well resolved in the upfield spectrum. As shown[2] for the mutarotated mixture of *N*-acetylneuraminic acid (**10**) in Me_2SO-d_6–D_2O, A-3(a) and A-3(e) of the α-anomer are widely separated at 1.51 and 2.49 ppm, respectively; whereas for the β-anomer, they appear close together at 1.69 and 1.99 ppm, respectively. A similar difference in chemical shift for A-3(a) and A-3(e) resonances of α- vs. β-linked NeuAc residues has been observed by Vliegenthart (1980) in studies of sialooligosaccharides and sialoglycopeptides in D_2O.

Residue Composition. The composition of a ganglioside is established when the number, constitution and configuration of all component monosaccharide residues, and the structure of the aglycon are known. The determination of the composition of a ganglioside by proton NMR may be approached at two levels of complexity and confidence. One approach, similar to that historically employed, requires only 1-D data but is empiric and limited, whereas the other approach requires 2-D *J*-correlated data and should be generally applicable.

At the 1-D level of analysis, reliance is placed on the assumption that chemical shifts of certain resonances of each ganglioside residue are insensitive to, or vary regularly with, glycosidation in a variety of structures. Furthermore, the assumption is made that the ganglioside or asialoganglioside is a member of the ganglio series rather than one of the other four glycosphingolipid series (Hakomori, 1981; Ledeen & Yu, 1982) and contains oligosaccharide residues and an aglycon of previously encountered constitution and configuration (i.e., Glc, Gal, GalNAc, NeuAc, NeuGy, L-Fuc, and the various ceramide structures). Analysis of the composition of gangliosides then becomes a matter of (1) determination of the number of hexose and *N*-acylhexosamine residue(s), (2) determination of the number of sialic acid residue(s) if present, and (3) confirmation of the presence of ceramide or one of its derivatives. All necessary information for compositional analysis can then be obtained from the well-resolved olefinic, anomeric, and upfield regions of the high-field, one-dimensional proton NMR spectrum of an unknown ganglioside. When these data are compared with the data base of chemical shifts and coupling constants generated from the systematic series of ganglio-series glycosphingolipids (Tables I–VII),[2] the composition is readily obtained. For example, inspection of Tables VI and VII reveals **8** is composed of three β-linked oligosaccharide residues, one of them GalNAc by chemical shift. Tables II and III reveal the presence of ceramide in **8**. No resonances of L-Fuc or NeuGy are observed at 1.04 or 3.88 ppm, respectively. Finally, the integration data in Table I reveals III-8/R-14 and A-11/R-14 ratios both of 0.5. Thus the composition of **8** is found to be NeuAc/NeuGy/Gal-

Table VIII: Short-Range[a] Proton Glycosidation (Substituent) Shifts (Δ) for Gangliosides (6–9) and Their Asialo Derivatives (2–5)

substituent and linkage	acceptor residue	example	Δ for protons of acceptor residue					
			H-1	H-2	H-3	H-4	H-5	H-8
Aα2→3	II	6–2[b]	−0.03	−0.04	+0.63	+0.03	−0.13	
		7–3	−0.01	−0.09	+0.66	+0.08	−0.06	
		8–4	+0.05	−0.09	+0.23	+0.13	−0.03	
		9–5	+0.07	−0.10	+0.24	+0.14	−0.01	
IIIβ1→4	II	4–3	+0.02	−0.16	+0.22	+0.18	+0.10	
		8–7	+0.09	−0.15	−0.22	+0.22	+0.13	
IVβ1→3	III	5–4	+0.10	+0.15	+0.13	+0.18	+0.01	−0.06
		9–8	+0.07	+0.16	+0.08	+0.19	−0.01	−0.02

[a] Change in chemical shift (Δ) for protons of residue to which the added residue is *directly* linked; computed for each proton: chemical shift in linkage product *minus* chemical shift in precursor. [b] The values for the α-hydroxy fatty acid containing GalCer were used.

Table IX: Long-Range[a] Proton Glycosidation (Substituent) Shifts (Δ_ω) for Gangliosides (7–9) and Their Asialo Derivatives (4 and 5)

substituent, linkage, and acceptor residue	affected residue[b]	example	Δ_ω for protons of affected residue									
			H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-11
Aα2→3II	III	8–4	+0.33	+0.14	−0.1[e]	−0.09	+0.31	−0.0[e] −0.02				
		9–5	+0.29	+0.14	−0.18	−0.08	+0.29	−0.1 −0.03				
	IV	9–5	−0.00	+0.0[e]	+0.07	+0.00	+0.0[e]	+0.02 +0.03				
IIIβ1→4II	A	8–7			−0.19 −0.26	+0.15	−0.1[e]	−0.42	−0.04	+0.1	−0.04 +0.04	−0.01
IVβ1→3III	II	5–4	−0.01	−0.02	−0.02	+0.00	−0.01	+0.00 +0.01				
		9–8	+0.00	−0.03	−0.01	+0.02	+0.02	+0.01 +0.01				
	A	9–8			−0.28 +0.02	+0.04	−0.0[e]	−0.03	+0.02	+0.01	−0.01 +0.01	+0.00

[a] Change in chemical shift (Δ_ω) for protons of residue *other than* the one to which the added residue is directly linked; computed for each proton: chemical shift in linkage product *minus* chemical shift in precursor. [b] All long-range glycosidation shifts for Glc (I) residues of 4, 5, and 7–9 are insignificant ($|\Delta_\omega| < 0.1$ ppm). [c] For NeuAc residue (A) upper value is for H-3 equatorial resonance and lower value for H-3 axial resonance. [d] For core oligosaccharide residues (II–IV), upper value is for H-6a, and lower value is for H-6b. [e] One or both values used to compute chemical shift difference are obscured by HOD or other resonances.

NAc/Gal/Glc/L-Fuc/ceramide in a ratio of 1/0/1/1/1/0/1, as calculated from Figure 1 and previously established by chemical analyses (Makita & Yamakawa, 1963; Ledeen & Salsman, 1965).

At the 2-D level of compositional analysis, the 2-D-SECSY spectrum is obtained, *J*-connectivities for each residue are followed, and the complete proton NMR spectrum is assigned. From the resulting chemical shift and coupling constant data, the constitution and configuration of each residue may then be deduced and the structure of each residue assigned de novo, as demonstrated under Results. Integration of well-resolved resonances of each residue subspectra yields a quantitative composition, like that shown above for **8**.

The advantages of the 2-D-SECSY approach are that *no* assumptions are made concerning (1) the range of possible residue structures (i.e., confinement to ganglio series), (2) the chemical shifts of anomeric resonances (see Results), (3) the presence of a trans double bond in the aglycon, and (4) the number of methyl groups per hexosamine or sialic acid residue. More importantly, residue assignments never hinge on the uniqueness of a chemical shift or coupling constant of a single or small number of well-resolved resonances or on the availability of a systematic series of related structures (congeners). Thus, in the case of a newly isolated ganglioside or derivative that is suspected for any reason of not belonging to the ganglio series or of containing oligosaccharide or aglycon residues that are atypical, a 2-D-SECSY study should be undertaken to establish residue composition. Examples of findings that would exclude a ganglioside from 1-D level analysis would be the lack of an I-2 resonance at 3.02 ppm or the presence of α-anomeric resonances ($J < 4$ Hz).

Glycosidation Shifts. After the number and structure of all residues with the 2-D-SECSY approach have been established, the only information that remains to be obtained in order to establish the complete primary structure of a ganglioside is its sequence and glycosidic linkage sites of the core oligosaccharide and sialic acid residue(s). One might hope to obtain the latter information from a study of glycosidation shifts. We present such shifts in Table VIII to examine this possibility but also to illustrate the variation in chemical shifts that can occur for a given residue within the ganglioside series.

The chemical shift difference or substituent effect that occurs at a particular proton of an oligosaccharide after the addition of a new residue is termed a glycosidation shift (Δ). When a glycosidation shift occurs within the residue to which the new residue is directly linked, we term this a *short-range* glycosidation shift. If a glycosidation shift occurs in a residue more distant than the residue of direct linkage, we term this a *long-range* glycosidation shift. For the types of oligosaccharide residues discussed herein, the number of bonds involved in glycosidation shifts is 3–6 for short range and 6–10 for long range. Thus short-range Δ are due to expected local changes in proton shielding when a nearby new linkage is established, but long-range Δ will occur only if the new residue is juxtaposed to the effected area due to a through-space interaction. Such through-space interactions may originate in overcrowded primary structure or the existence of stabilized secondary structure.

For short-range Δ, we have employed a symbolism consistent with that previously used for carbon-13 NMR glycosidation shifts (Yu & Sillerud, 1982; Prohaska et al., 1982; Sillerud et al., 1982), in which the number of bonds is indicated in a subscript. For example, Δ_β, Δ_γ, and Δ_δ signify glycosidation shifts through 3, 4, or 5 bonds, respectively. Shown in Table VIII are three types of short-range Δ calculated from the oligosaccharide chemical shift data in Table VII. These Δ are for sialylation, N-acetylgalactosaminylation, and galactosylation, that is, the addition of NeuAc, GalNAc, and Gal residues, respectively. Except for sialylation, there is little regularity, either quantitatively or qualitatively, in these short-range glycosidation shifts. For example, the addition of a GalNAc residue to **3** causes a 0.22 ppm *downfield* shift at II-3 but, when added to **7**, causes a 0.22 ppm *upfield* shift at II-3. Glycosidation may have a greater effect on the protons adjacent to a linkage site (Δ_δ) than on the protons directly involved (Δ_γ). Similar failures of chemical shift additivity have also been noted in a series of neutral glycosphingolipids (Dabrowski et al., 1981) and are probably the result of long-range interactions occurring between oligosaccharide residues. This apparent lack of regularity in glycosidation shifts indicates that some other NMR method must be employed to establish the sequence and linkage sites of the core oligosaccharide residues (I–IV) of gangliosides. This finding also adds a note of caution to assignment schemes that rely too heavily on chemical shift correlations.

On the other hand, addition of a NeuAc residue consistently moves the proton at the linkage site (II-3) downfield between 0.2 and 0.6 ppm for the series **6**–**9**. Moreover, this value is much greater than for all other protons of the acceptor residue. Thus the Δ_γ sialylation shift is potentially useful in establishing the substitution site of sialic acid residues in a ganglioside.

Ganglioside Secondary Structure. Shown in Table IX are the long-range (Δ_ω) glycosidation shifts for **4**, **5**, and **7**–**9**. All are insignificant ($|\Delta_\omega| < 0.1$ ppm) except for GalNAc-1, -2, -3, and -5 protons after addition of the NeuAc (A) residue and for NeuAc-3(a), -3(e), -4, and -6 protons after addition of the GalNAc (III) residue. The lack of perturbation at other protons and the reciprocity of long-range shifts between the GalNAc and NeuAc residues of **8** and **9** suggest a specific through-space interaction occurs between these two residues. Since a space-filling model of a galactopyranose ring with attached NeuAcα2→3 and GalNAcβ1→4 residues does not seem to be a particularly crowded structure, it appears some secondary structure stabilizes the interactions of the GalNAc and NeuAc residues. Similar conclusions have been made on the basis of the results of carbon-13 NMR studies of gangliosides (Sillerud et al., 1978, 1982; Harris & Thornton, 1978). Likely possibilities for such secondary structure are (1) a dipole–charge interaction between the III-7 amide and the A-1 carboxylate anion, (2) hydrophobic interactions including the III-8 methyl and A-3 methylene groups, (3) one or more of several possible hydrogen bonds, including the A-4 hydroxyl and III-7 amide, (4) a counterion effect, and (5) combinations of the above four possibilities. Studies are currently in progress to characterize this apparent secondary structure between the GalNAc and NeuAc residues of **8** and **9**.

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High-Resolution Proton NMR Studies of Gangliosides. 2. Use of Two-Dimensional Nuclear Overhauser Effect Spectroscopy and Sialylation Shifts for Determination of Oligosaccharide Sequence and Linkage Sites[†]

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ABSTRACT: Homonuclear two-dimensional proton nuclear Overhauser effect (2-D-NOE) spectra have been obtained for asialo-G_{M2} (**4**) and gangliosides G_{M2} (**8**) and G_{M1} (**9**) at 500 MHz and 40 °C in Me$_2$SO-d_6–D$_2$O (98:2 v/v). The anomeric protons of each oligosaccharide residue of **4**, **8**, and **9** are observed to NOE couple via intraresidue 1,3- and 1,5-diaxial interactions and interresidue interactions across the glycosidic linkages. The former couplings are used to confirm the H-3 and H-5 assignments for each residue. From the latter couplings the *sequence* and glycosidic linkage sites of all oligosaccharide residues, except the sialic acid residues, are revealed. Sialic acid attachment sites are determined for the monosialogangliosides (**6**–**9**) through consideration of sialylation-induced glycosidation shifts. Combination of the above sequence and linkage-site data with the monosaccharide composition, anomeric configurations, and characterization of the aglycon (information obtained via two-dimensional spin-echo *J*-correlated spectroscopy or 2-D-SECSY) allows complete assignment of monosialoganglioside primary structure, independent of other methods of structural analysis. Compared to conventional chemical and enzymatic methods, high-resolution two-dimensional proton nuclear magnetic resonance spectroscopy has the advantages of speed, sensitivity, and sample preservation.

Previously, we have shown that the monosaccharide composition, anomeric configurations, and aglycon structure of gangliosides can be determined through homonuclear proton two-dimensional spin-echo *J*-correlated NMR[1] spectroscopy or 2-D-SECSY (Koerner et al., 1983). Through this 2-D NMR method, all information is obtained that is necessary to determine the complete primary structure of a ganglioside, except the sequence and linkage sites of the oligosaccharide residues. In theory, the potential proximity of the glycosidic-linkage protons of the core oligosaccharide residues should allow its sequence and glycosidation sites to be determined, if through-space couplings could be systematically measured. In fact, Ernst, Wüthrich, and co-workers (Jeener et al., 1979; Kumar et al., 1980a,b; Macura et al., 1981) have shown in studies of polypeptides that through-space couplings of protons can be systematically measured by another homonuclear proton NMR method, namely, two-dimensional nuclear Overhauser effect spectroscopy (2-D-NOE). Recently, Dabrowski et al. (1981) and Berstein & Hall (1982) have used 1-D NOE data to determine the sequence and linkage sites of neutral glycosphingolipids and an acetylated disaccharide, respectively. We have reported the use of 2-D-NOE data to determine the sequence and linkage sites of a ceramide trisaccharide (**4**; Prestegard et al., 1982).

In this paper we show how the *sequence* and linkage sites of the core oligosaccharide of gangliosides may be determined by 2-D-NOE and how their sialic acid residue linkage sites may be deduced from consideration of sialylation shifts. When this information is combined with composition data, generated through 2-D-SECSY, the complete primary structure of monosialogangliosides may be elucidated, nondestructively and independent of other methods of analysis. This study is an extension of our use of two-dimensional proton NMR methods for the complete analysis of oligosaccharide primary structure (Prestegard et al., 1982) and high-resolution proton NMR studies of gangliosides (Koerner et al., 1982). All numbering, symbols, and structures used in this paper are the same as those previously used [Figure 1 in Koerner et al. (1983)].

Materials and Methods

Asialo-G_{M2} (**4**) and gangliosides G_{M2} (**8**) and G_{M1} (**9**) were isolated from human brain and prepared for proton NMR

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[1] Abbreviations: 2-D-NOE, two-dimensional nuclear Overhauser effect; 2-D-SECSY, two-dimensional spin-echo *J*-correlated spectroscopy; Me$_2$SO-d_6, dimethyl-d_6 sulfoxide; D$_2$O, deuterium oxide; G_{M1}, G_{M2}, G_{M3}, G_{M4}, G_{A1}, and G_{A2}, symbols for ganglioside structures [as shown in Figure 1 of Koerner et al. (1983)]; I, II, III, IV, R, and A, ganglioside residue structures; NMR, nuclear magnetic resonance. All monosaccharides are assumed to be of the D configuration.

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