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COMPARISON OF THE ^{13}C-N.M.R. SPECTRA OF GANGLIOSIDES G_{M1} WITH THOSE OF G_{M1}-OLIGOSACCHARIDE AND ASIALO-G_{M1}*

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ABSTRACT

The ^{13}C-n.m.r. spectra of asialo-G_{M1} and G_{M1}-oligosaccharide‡ are completely assigned and compared to those previously found for intact G_{M1} and for the series G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. Removal of the ceramide residue from G_{M1} liberated a free, reducing aldehyde group, which was reflected in a doubling of the ^{13}C-n.m.r. signals assignable to the D-glucose residue because of α,β equilibrium. The spectrum of asialo-G_{M1} lacks the resonances from the sialic acid residue, as expected; in addition, several resonances from the neutral gangliotetraglycosyl residue shifted to different field positions after removal of sialic acid from G_{M1}. These resonances include that of C-4 of the inner β-D-galactosyl residue, and C-1 of the 2-acetamido-2-deoxy-D-galactosyl residue that is near the site of attachment of the sialosyl residue. The differences between the chemical shifts of the carbon resonances of oligomeric and monomeric saccharides, termed linkage shifts, provide a quantitative assignment aid. They are $\sim 1/3$ of those for residues linked to sialic acid than those for residues linked to the neutral hexose chain. Correlations among linkage shifts for pairs of glycosidically-linked carbon atoms for asialo-G_{M1} and G_{M1}-oligosaccharide were compared with those for the series of gangliosides G_{M4} to G_{T1b}, and differences are noted for resonances for carbon atoms near the sialic acid residue. The spectrum of ganglioside G_{M1b}, a positional isomer of G_{M1} whose ^{13}C-n.m.r. spectrum has not yet been observed, is predicted.

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‡The following ganglioside nomenclature used in this report was recommended by the IUPAC–IUB commission on Biochemical Nomenclature: G_{M1}, II^{3}NeuAc-GgOse$_4$Cer (**1**); G_{M1}OS, II^{3}NeuAc-GgOse$_4$ (**2**); asialo-G_{M1} (ASG$_{M1}$), GgOse$_4$Cer (**3**); G_{M1b}, IV^{3}NeuAc-GgOse$_4$Cer (**7**); G_{M4}, I^3-NeuAc-GalCer (**8**); G_{M3}, II^{3}NeuAc-LacCer (**9**); G_{M2}, II^{3}NeuAc-GgOse$_3$Cer (**10**); G_{D1a}, IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer (**4**); G_{D1b}, II^3(NeuAc)$_2$-GgOse$_4$Cer (**5**); and G_{T1b}, IV^{3}NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer (**6**).

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INTRODUCTION

Interest in the sialic acid-containing, cell-surface glycolipids known as gangliosides stems from their diverse roles as receptors, antigenic attachment sites, and points of cell–cell interaction[1–3]. The best studied ganglioside to date is the monosialoganglioside G_{M1} (**1**) for which we[4], and others[5], have provided complete ^{13}C-n.m.r. peak-assignments. Recently, we have examined the ^{13}C-n.m.r. spectra of seven members of the ganglioside series, starting with G_{M4} (**8**) and progressing systematically to G_{T1b} (**6**), and were able to identify correlations and similarities among them[6]. Several unique features of the chemical-shift changes, due to the glycosidic linkage of the sialic acid residues to the neutral gangliotetraglycose, were interpreted in terms of primary and secondary structural effects. The basis for these effects could be further elucidated by examining the ^{13}C-n.m.r. spectra of additional, related compounds. We now report the confirmation of our previous proposals for the ^{13}C-n.m.r. spectra of the intact gangliosides. In addition, we present assignments for, and a complete analysis of, the ^{13}C-n.m.r. spectra of asialo-G_{M1} (**3**) and the oligosaccharide portion of G_{M1} (**2**). These results are compared with those obtained previously for the series of intact molecules. The data for $G_{M1}OS$ (**2**) will assist in the study of its interaction with cholera toxin[7].

Further interest in asialo-G_{M1} (ASG_{M1}, **3**) arises from the recent finding that it is a cell-surface marker of mouse, natural-killer cells[8,9], and it may be associated with natural, cell-mediated cytotoxicity[10]. It may also serve as a differentiation antigen of mouse[11] and rat[12,13] thymocytes. In addition, this glycolipid appears to be a cell-surface marker of leukemic cells from patients having acute, lymphoblastic leukemia[14]. The detection of asialo-G_{M1} (**3**) antibody in sera from patients having Graves' disease and Hashimoto's thyroiditis[15], and systemic lupus erythrematosus[16] suggests that this glycolipid may serve as an autoantigen in the pathogenesis of these diseases. Since ASG_{M1} (**3**) is more antigenic than most gangliosides, and is also capable of eliciting highly specific antibodies[17], a characterization of its properties by high-resolution, physical methods is necessary to enable studies of its interaction with immunoglobulins to proceed.

In addition, ASG_{M1} (**3**) is the neutral oligohexosylceramide backbone of both G_{M1} (**1**) and G_{M1b} (**7**); the latter compound is a minor, naturally occurring ganglioside, found in rat-ascites hepatoma cells[18] and human erythrocytes[19]. Assignments for ASG_{M1} (**3**) provide a basis for a prediction of the ^{13}C-n.m.r. spectrum of G_{M1b} (**7**) which has not been reported to date. This prediction should also provide a test of the generality of the assignment techniques used herein. A firm understanding of the relationship between structure and ^{13}C-n.m.r. spectrum is necessary for utilizing high-resolution spectroscopy alone to provide information about complex oligosaccharide structures.

EXPERIMENTAL

Materials. Deuterium oxide (100%), purified to remove paramagnetic im-

purities, was purchased from Stohler Chemical Inc. (Waltham, MA 02154), and 99.9% ^{13}C-depleted [^{2}H]methanol ($^{12}CD_3OD$) and [^{2}H]chloroform ($^{12}CDCl_3$) from Merck and Co. (Rahway, NJ 07065). A deuterated sodium phosphate buffer (pD 7.5, 200mM) was first prepared in distilled, filtered, and de-ionized water, and then lyophilized and reconstituted with deuterium oxide.

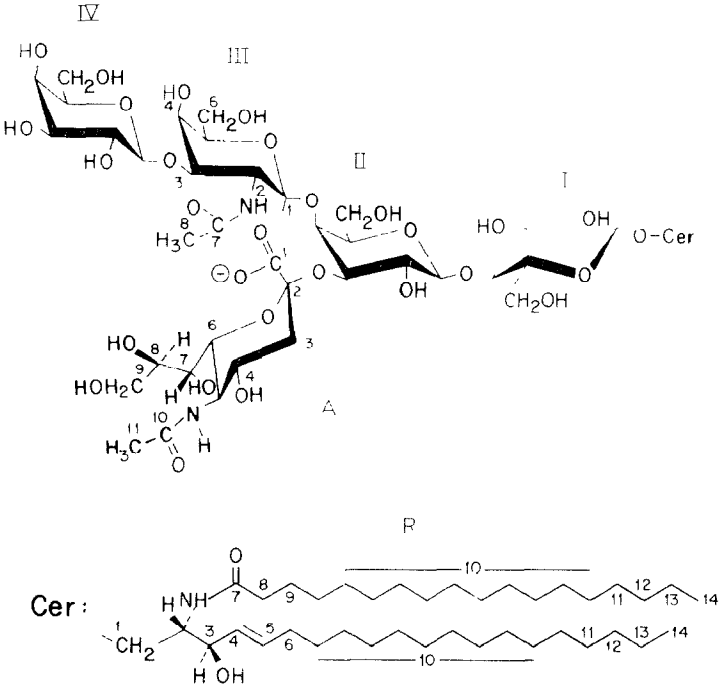
Purification of gangliosides. — Ganglioside G_{M1} (**1**, 105 mg) was prepared from bovine brain according to the method of Sillerud *et al.*[4], which involves neuraminidase treatment of a total ganglioside fraction. The oligosaccharide from G_{M1} was produced by ozonolysis and mild-base cleavage[7]. Both G_{M1} (**1**) and its oligosaccharide (**2**) were judged to be about 99% pure by t.l.c. on silica gel.

*Preparation of asialo-G_{M1} (ASG_{M1}, **3**).* — This compound was prepared from a bovine–brain ganglioside mixture by hydrolysis in 0.1M formic acid for 2 h at 100°. The liberated ASG_{M1} (**3**) was isolated and purified by Iatrobeads column chromatography. The ASG_{M1} (**3**) preparation was homogeneous as assessed by t.l.c. in several solvent systems. Details of the preparative method have been published[20].

^{13}C-N.m.r. spectrometry. — The spectrum of $G_{M1}OS$ (**2**) was recorded with a Bruker CXP200 spectrometer operating at 50.3 MHz in the Fourier-transform mode, according to the procedure of Sillerud *et al.*[7], as a 23mM solution in deuterated phosphate buffer, pD 7.5, in a 10-mm spinning tube. Spectra of intact G_{M1} (**1**) and ASG_{M1} (**3**) were recorded at 90.55 MHz with a Bruker, Fourier-transform WH 360 system, the former sample being in solution in 1:1 (v/v) $^{12}CD_3OD$–D_2O (deuterated phosphate buffer, pD 7.5), and the latter in solution in a similar mixture with the addition of 20% (v/v) $^{12}CDCl_3$. A 20-kHz sweep was collected in 16-k data points, following, at 45°, a 20-μs pulse. Complete proton-decoupling was accomplished by irradiation of the sample with 1 watt of noise-modulated, radio frequency centered 4 p.p.m. downfield from the signal of Me_4Si. The sample temperature was 20°. An excellent signal-to-noise ratio was obtained, after about 10 000 scans, from 75mM solutions. The chemical shift accuracy is limited to 0.013 p.p.m. by the digital resolution. The shifts are reported relative to the signal of Me_4Si at δ 0.00. They were referenced to the ω-1 carbon atom of the ceramide residue, set at δ 23.45, to coincide with our previous work[4]. We found that the chemical shifts thus determined for G_{M1} (**1**), at 90.55 MHz, were 0.05 $\pm$0.05 p.p.m. (N = 47) larger than those measured[6] at 67.89 MHz. The $G_{M1}OS$ spectrum was referenced to the $NHCOCH_3$ resonance of the 2-acetamido-2-deoxy-D-galactopyranosyl residue, also set to δ 23.45.

RESULTS

The nomenclature relating to the naming of the residues of ganglioside G_{M1} (**1**), and of its oligosaccharide (**2**) and asialo derivative (**3**) (See Scheme 1) was designed[6] for referring unambiguously to individual carbon atoms, since almost every carbon nucleus gives rise to a resolved resonance-signal. The free oligosaccharide consists of the five monosaccharide residues I–IV and A; with the free anomeric carbon C-1-I. We expected to find up to 12 resonances from residue I due to the α,β-D



Scheme I. Covalent structure of the compounds whose ^{13}C-n.m.r. spectra are compared in the present work. Ganglioside G_{M1} consists of residues I, II, III, IV, A, and R. Asialo-G_{M1} lacks residue A, and oligo-G_{M1} lacks residue R. In ganglioside G_{M1b}, the sialic acid residue is attached to C-3-IV instead of to C-3-II

equilibrium at C-1-I. ASG_{M1} (**3**) lacks residue A, which results in a poor solubility in methanol–water mixtures.

R″-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-R
|
R′

1 R = Cer, R′ = α-NeuAc-(2→3)-, R″ = H
2 R = H, R′ = α-NeuAc-(2→3)-, R″ = H
3 R = Cer, R′ = R″ = H
4 R = Cer, R′ = R″ = α-NeuAc-(2→3)-
5 R = Cer, R′ = α-NeuAc-(2→8)-α-NeuAc-(2→3)-, R″ = H
6 R = Cer, R′ = α-NeuAc-(2→8)-α-NeuAc-(2→3)-, R″ = α-NeuAc-(2→3)-
7 R = Cer, R′ = H, R″ = α-NeuAc-(2→3)-

α-NeuAc-(2→3)-β-D-Gal*p*-(1→1)-Cer
8

α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-Cer
9

α-NeuAc-(2→3)-[β-D-Gal*p*NAc-(1→4)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-Cer
10

TABLE I

^{13}C-CHEMICAL SHIFTS[a] (δ) AND OLIGOMER–MONOMER SHIELDING DIFFERENCES[b] (Δ) FOR GANGLIOSIDE G_{M1} (**1**), THE OLIGOSACCHARIDE PORTION OF G_{M1} (**2**), AND ASIALO-G_{M1} (**3**)

Carbon atom	G_{M1} (**1**)		$G_{M1}OS$ (**2**)		ASG_{M1} (**3**)		*Lactose*		*Monomers*
	δ	Δ	δ	Δ	δ	Δ	δ	Δ	(δ)
Residue I									D-*Glucose*
1α			92.63	−0.19			92.68	−0.14	92.82
β	103.47	6.76	96.61	−0.10	103.34	6.63	96.61	−0.10	96.71
2α			72.00	−0.25			72.03	−0.22	72.25
β	74.11	−0.86	74.59	−0.38	74.09	−0.88	74.70	−0.27	74.97
3α			72.21	−1.43			72.29	−1.35	73.64
β	75.24	−1.45	75.19	−1.50	75.57	−1.12	75.23	−1.46	76.69
4α			79.45	8.99			79.41	8.95	70.46
β	79.83	9.37	79.35	8.89	80.18	9.72	79.28	8.82	70.46
5α			70.81	−1.44			70.97	−1.28	72.25
β	75.76	−0.78	75.60	−0.94	75.95	−0.59	75.64	−0.90	76.54
6α			60.86	−0.69			60.90	−0.65	61.55
β	61.09	−0.59	60.96	−0.72	61.33	−0.35	61.02	−0.66	61.68
Residue II									β-D-*Galactose*
1	103.47	6.28	103.36	6.17	103.77	6.58	103.77	6.58	97.19
2	70.85	−1.82	70.81	−1.86	72.22	−0.45	71.84	−0.83	72.67
3	75.24	1.70	75.19	1.65	72.63	−0.91	73.41	−0.13	73.54
4	78.34	8.83	77.95	8.44	76.92	7.41	69.43	−0.08	69.51
5	75.00	−0.86	74.92	−0.94	75.73	−0.13	76.19	0.33	75.86
6	61.90	0.13	61.97	0.10	62.14	0.37	61.91	0.14	61.77
Residue III									*2-Acetamido-2-deoxy-β-D-galactose*
1	103.47	7.64	103.36	7.53	104.44	8.61			95.83
2	52.84	−1.29	52.47	−1.66	52.84	−1.29			54.13
3	81.58	10.05	81.17	9.64	81.23	9.70			71.53
4	68.75	0.46	68.73	0.44	69.18	0.89			68.29
5	75.76	0.24	75.19	−0.33	75.57	0.05			75.52
6	61.49	0.08	61.50	−0.09	61.41	0.00			61.41
7	175.65	0.81	175.62	0.78	175.70	0.86			174.84
8	23.45	0.73	23.45	0.73	23.45	0.73			22.72
Residue IV									β-D-*Galactose*
1	105.60	8.43	105.57	8.38	105.90	8.71			97.19
2	71.77	−0.90	71.54	−1.13	71.96	−0.71			72.67
3	73.65	0.11	73.35	−0.19	73.87	0.33			73.54
4	69.26	−0.25	69.44	−0.07	69.80	0.29			69.51
5	75.76	−0.10	75.72	−0.14	76.13	0.27			75.86
6	61.90	0.13	61.77	0.00	62.14	0.37			61.77

TABLE I (*continued*)

Carbon atom	G_{M1} (1)		$G_{M1}OS$ (2)		$4SG_{M1}$ (3)		*Lactose*		*Monomers*
	δ	Δ	δ	Δ	δ	Δ	δ	Δ	δ
Residue A									N-*Acetyl*-α-*neuraminic acid*
1	174.84	0.28	174.93	0.19					175.12
2	102.66	4.60	102.46	4.40					98.06
3	37.90	-3.72	37.84	-3.78					41.62
4	69.26	0.28	69.14	0.16					68.98
5	52.06	-0.76	52.01	0.67					52.68
6	73.65	0.32	73.88	0.55					73.33
7	69.26	0.28	68.86	-0.12					68.98
8	73.03	0.70	73.04	0.71					72.33
9	63.89	0.14	63.66	0.09					63.75
10	175.65	0.01	175.92	0.26					175.66
11	22.86	-0.25	22.94	0.17					23.11
Residue R									*Ceramide*
1	70.20	7.40			69.80	7.00			62.80
2	54.13	1.33			54.21	-1.25			55.46
3	72.22	-0.52			72.22	0.52			72.74
4	134.91	1.12			135.56	1.77			133.79
5	130.65	0.23			130.27	-0.61			130.88
6	33.40	0.29			33.26	0.15			33.11
7	175.22	-0.67			175.19	0.70			175.89
8	37.09	1.27			37.34	1.52			35.82
9	26.98	-0.87			26.87	0.98			27.85
10	30.92	0.44			30.54	0.06			30.84
11	30.35	0.27			30.16	0.08			30.08
12	32.83	0.14			32.73	0.04			32.69
13	23.45[c]				23.45[c]				23.45
14	14.58	-0.30			14.63	-0.25			14.88

[a]Chemical shifts relative to the signal of Me_4Si at δ 0.00. [b]Shielding difference in p.p.m. between resonances from the same carbon atoms in the oligomer and monomer. [c]Set to 23.45 p.p.m. to correspond with previous measurements[1].

Linkage shifts (Δ) were computed as $\Delta = \delta_o - \delta_m$, the difference between the chemical shifts of the oligomer and monomer. The values of Δ vary according to the distance between a carbon nucleus and the glycosidic linkage. Differences in Δ values may be used as an indication of altered conformations, or of interactions between residues, when comparisons among structurally related oligosaccharides are made. Empirically, it was found that signals from carbon atoms in positions β or β' to the linkage site (where O-1 is the α and C-1 the β atom, and the other carbon atom that participates in the glycosidic linkage is the β' atom) in neutral hexopyranosides show Δ_β and $\Delta_{\beta'}$ values of +6 and +9 p.p.m., respectively, whereas values of +2 to +5, and +2 to +6 p.p.m., respectively, were found where sialic acid residues are present

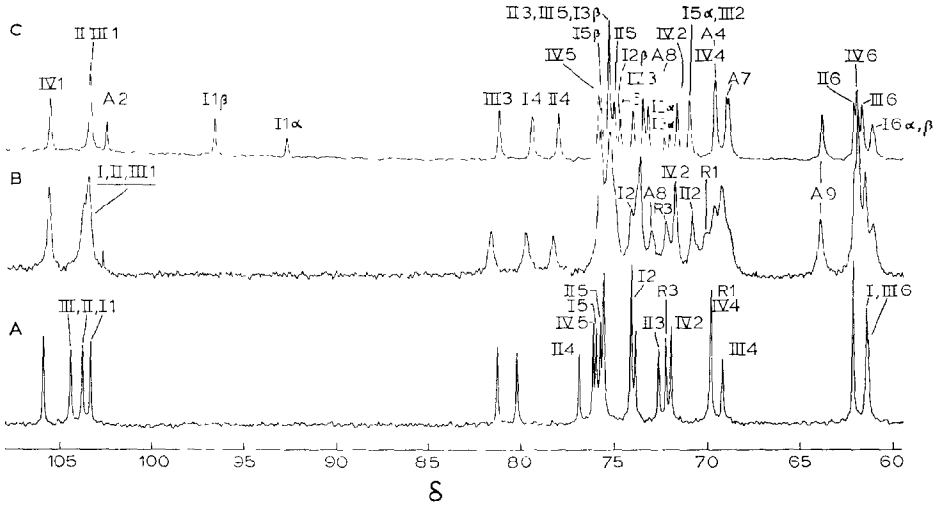


Fig. 1. Natural-abundance, ^{1}H-decoupled, ^{13}C-n.m.r. spectra of: (A) asialo-G_{M1} at 90.55 MHz, (B) G_{M1} at 90.55 MHz, and (C) G_{M1}OS at 50.3 MHz. The assignments are given above the peaks and refer to the numbering system shown in Scheme 1. The chemical shifts are reported in Table I. Most of the oligosaccharide carbons resonate between δ 60 and 86. The anomeric and ceramide carbon atoms resonate mainly outside this range.

Resonances from carbon atoms in position γ to the linkage site show small shifts ($\Delta_\gamma \sim -1$ p.p.m.). Carbon atoms in position δ or farther from the site of linkage show no significant shift. These guidelines are of general application; in case of failure, interesting new information about various linkage types may be obtained. For example, the small Δ_β values for C-2 of the sialic acid residue reflect the unusual electronic structure of these linkages arising from the adjacent carboxyl group.

A comparison of the central region (δ 60–85 p.p.m.) of the ^{13}C-n.m.r. spectra of the three compounds **1–3** is shown in Fig. 1; the complete assignments and chemical shifts are reported in Table I. The order of presentation of the data will follow the naming of the residues, *i.e.*, I, II, III, IV, A, and R, with the data for G_{M1}OS (**2**) preceding those for ASG_{M1} (**3**).

β-D-Glucopyranosyl residue (I). — Residue I, in the intact glycolipids, is attached by a β-D-glycosidic linkage to the ceramide moiety. Therefore, resonances from only the β-D anomer are expected for G_{M1} (**1**) and ASG_{M1} (**3**). On the other hand, G_{M1}OS (**2**) has an anomeric equilibrium for this reducing residue, with the result that resonances from both C-1-α and -β are expected. G_{M1} (**1**) and ASG_{M1} (**3**), as intact molecules, contribute six carbon resonances. G_{M1}OS (**2**) contributes twelve resonances, whose reduced intensities fall into a pattern characteristic of the equilibrium distribution of C-1-α and -β, namely, 60% of β and 40% of α at 20°. As a result, the resonances from residue I in G_{M1}OS (**2**) are smaller than those from the other residues, which are restricted to a single anomeric configuration. The resonances from residue I in G_{M1}OS (**2**) can be assigned on the basis of chemical shift, by use of α,β-D-glucose and lactose

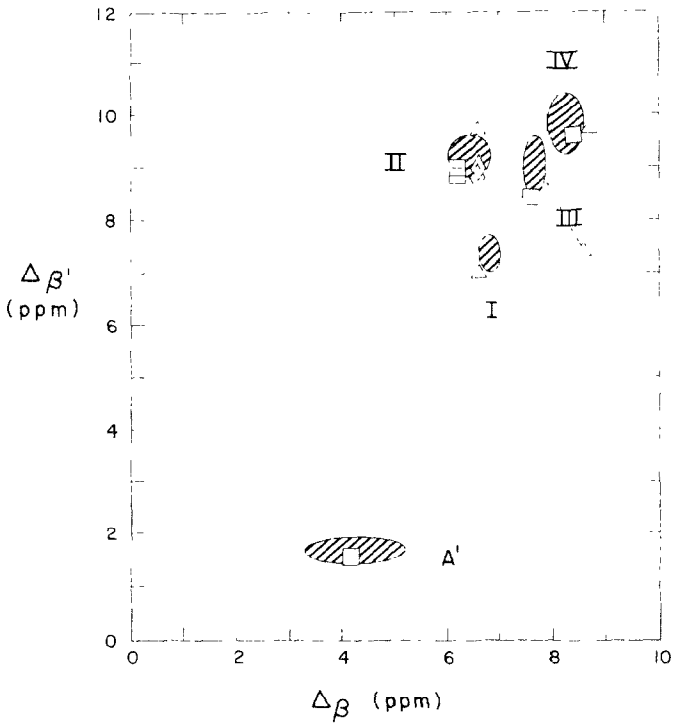


Fig. 2. Relationship between the oligomer–monomer shielding differences (Δ) for the pairs of ^{13}C resonances of the β and β' carbon atoms participating in glycosidic linkages, in the oligosaccharides of the gangliosides studied previously[6], and in the present G_{M1} derivatives. The shaded ellipses are centered at the means and have axes corresponding to two standard deviations for the data from gangliosides[6] G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. The symbols for residues from Scheme I are used to denote linkage-shift clusters. The clusters are identified by the residue contributing the β' carbon. Data from the present study are denoted by. . ASG_{M1}; | ', $G_{M1}OS$, and ◇, lactose.

(Table I) as models for the glycosidic-linkage shifts, and the α,β-D configurational and conformational effects. The free anomeric-equilibrium and the lack of a linkage shift (Δ_β) for C-1-I in $G_{M1}OS$ (**2**) are evident in the ratios of the intensities of the C-1-α and -β signals at δ 92.63 and 96.61, respectively (Fig. 2).

The intensity differences between resonances from carbon atoms in residue I, and those from carbon atoms in the other saccharide residues, are of continuing importance in the assignments for C-2-I through C-6-I in $G_{M1}OS$ (**2**). They also provide confirmation for the assignments for residue I in G_{M1} (**1**) and ASG_{M1} (**3**), where no such intensity differences exist to be utilized. Consequently, the assignments for $G_{M1}OS$ (**2**) will be presented before those for ASG_{M1} (**3**) and G_{M1} (**1**). The α,β intensity differences are clearly shown for the resonances from C-2β-I at δ 74.59 and C-2α-I at 72.00 (Table I) in $G_{M1}OS$ (**2**) (Fig. 2). The resonance due to C-3β-I at δ 75.10 overlaps several other resonances, but that for C-3α-I is distinct at δ 72.21 (Fig. 2). Lactose is an excellent model for the field position for C-4α,β-I. The high-

resolution ^{13}C-n.m.r. spectrum of lactose shows that the α,β-D-configurational and conformational effects are small, but present, at C-4-I. The separation is 0.13 in lactose, and 0.10 p.p.m. in $G_{M1}OS$ (**2**), the β resonance being at higher field in both cases (Table I). The resonance from C-5β-I is visible as a shoulder at δ 75.60. The CH_2OH resonances for C-6$\alpha(\beta)$-I at 60.96 (60.86) are resolved. They are shifted upfield from those of α,β-D-glucose by 0.7 p.p.m. This shift is also seen in the lactose spectrum.

The chemical-shift differences due to anomeric equilibrium at residue I in $G_{M1}OS$ (**2**) are virtually identical in magnitude and sign to those found for the spectra of D-glucose and lactose. These similarities indicate that residue I in $G_{M1}OS$ (**2**) is in the identical $^1C_4$(D) conformation to that found for the D-glucose residue in lactose and the monosaccharide D-glucose. The α and β resonances from $G_{M1}OS$ (**2**) C-3-I and -5-I are separated by 2.98 and 4.79 p.p.m., respectively, as compared with 2.94 and 4.67 p.p.m., respectively, for lactose, and 3.05 and 4.29 p.p.m., respectively, for D-glucose. Even C-6-I shows an α,β splitting of 0.12 $\pm$0.02 p.p.m. for $G_{M1}OS$ (**2**), D-glucose, and lactose. The assignments for residue I in $G_{M1}OS$ (**2**) then account in detail for all of the features, including the intensity pattern and the chemical-shift differences in the ^{13}C-n.m.r. spectrum. It is also clear that lactose serves as an excellent model for the linkage shifts for the resonances from each anomer of residue I in $G_{M1}OS$ (**2**). The linkage shift pattern for C-3-I, -4-I, and -5-I in lactose is very well correlated with that for $G_{M1}OS$ (**2**) (Table I).

Thus, the task of assigning the resonances of residue I in G_{M1} (**1**) and ASG_{M1} (**3**) was considerably simplified. The resonance from C-1-I in G_{M1} (**1**) at δ 103.47 (Table I) is not completely resolved from that for C-1-II and -III. In ASG_{M1} (**3**), the resonance for C-1-I was assigned to the peak at δ 103.34, which is the highest-field resonance of the spectral region characteristic for β-D-glycosyl-linked carbon atoms. This resonance appears in G_{M3} (**9**) at δ 103.64, indicating the most shielded, neutral-hexopyranoside, carbon resonance in this molecule[6]. The linkage shifts of C-1-I and -II of a size exemplified by Δ_β and Δ_γ should be present, and the remaining carbon resonances should be found at field positions characteristic for only the β-D anomer in the D-glucose residue in lactose and $G_{M1}OS$ (**2**). The linkage shifts for C-1 through 5-I, for G_{M1} (**1**) and ASG_{M1} (**3**) examined here, are anticipated to follow the Δ_β, Δ_γ, Δ_γ, $\Delta_{\beta'}$, and Δ_γ pattern resulting from the substitution at C-1-I and -4-I. For ASG_{M1} (**3**) and G_{M1} (**1**), the glycosidic-linkage shift (see below) of Δ_β 7.1 $\pm$0.2 p.p.m. found for C-1-I in ganglioside G_{M3} (**9**), for example[6], serves as a good model. The linkage shifts for C-2-I in G_{M1} (**1**) and ASG_{M1} (**3**) are expected to be small. The value of Δ_γ for C-2-I was found to be -0.86 p.p.m. for G_{M1} (**1**) and ASG_{M1} (**3**), suggesting a Δ_γ value for C-2-I for $G_{M1}OS$ (**2**) not much greater than a few tenths of a p.p.m. The pattern of Δ values for C-3-I, -4-I, and -5-I should be Δ_γ, Δ_β, and Δ_γ, respectively, in all three structures studied here. The results (Table I) bear out this expectation. It is gratifying to observe the differences in linkage Δ values for C-1-I and -2-I when the oligosaccharide data are compared with those for G_{M1} (**1**) and ASG_{M1} (**3**).

The present opportunity to examine the ^{13}C-n.m.r. spectra of ASG_{M1} (**3**) and $G_{M1}OS$ (**2**) has provided data that bear on the assignments originally proposed for ganglioside G_{M1} (**1**). When these new data are considered, together with the currently reported higher-field and higher-resolution spectrum for G_{M1} (**1**), several of the previous proposals for assignments of G_{M1} (**1**) ^{13}C-resonances may be put on a sounder experimental basis. On the whole, the original proposals for G_{M1} (**1**) have been confirmed, but some reassignments to slightly different chemical shifts are reported in Table I, owing to the increased resolution at 90.55 MHz. Other reassignments have been found to be necessary as a result of the discovery of unanticipated structural effects on the ^{13}C-n.m.r. spectra. A comprehensive examination of a complete series of gangliosides, ranging from G_{M4} (**8**) through G_{T1b} (**6**), also enabled us to reassign several G_{M1} (**1**) resonances[6]. In residue I, C-1-I, -5-I, and -6-I were reassigned to δ 103.47, 75.76, and 61.09, from δ 105.48, 75.17, and 61.46, respectively. The signal near δ 105.5 was not found[6] in the spectrum of G_{M2} (**10**) or other gangliosides that lack residue IV; therefore, it is assigned to C-1-IV. Reassignments for C-5-I and -6-I arose from the increased resolution at 90.55 MHz.

β-D-Galactopyranosyl residue (II). – The assignment techniques just utilized for residue I are applicable to the remaining residues as well. There is no free anomeric group in any residue other than in I. Consequently the ^{13}C-n.m.r. spectra of the other residues are simpler, and residue II in the β-D configuration should give 6 resonances. Linkage shifts for the resonances from C-1- through -5-II generally fall into the pattern Δ_β, $2\Delta_\gamma$, $\Delta_{\beta'}$, $\Delta_{\beta'}$, and Δ_γ for G_{M1} (**1**) and $G_{M1}OS$ (**2**), and into the pattern Δ_β, Δ_γ, Δ_γ, $\Delta_{\beta'}$, and Δ_γ for ASG_{M1} (**3**). Notable are the differences between the Δ values for C-2- through -5-II in ASG_{M1} (**3**), and in the other two glycolipids. These differences must reflect the effect of the sialic acid residue (A) on the chemical shifts of resonances from carbon atoms at or near the site of attachment of this residue. Of particular interest is the very small value of Δ_β for C-3-II in G_{M1} (**1**) and $G_{M1}OS$ (**2**). Instead of the ~9 p.p.m. expected, a value of Δ_β of only 1.67 p.p.m. was observed. Therefore, it must be emphasized that the effects of the linkages of the sialic acid residue do not follow the general rules for the linkage shifts for neutral hexose residues. Attachment of residue III to O-4-II shifted the resonance of C-4-II by 8.6 p.p.m. downfield for G_{M1} (**1**) and $G_{M1}OS$ (**2**), but only 7.4 p.p.m. for ASG_{M1} (**3**). This smaller $\Delta_{\beta'}$ for ASG_{M1} (**3**) may be a long-range effect[5] of the acetyl group C-7-III and -8-III of residue III, or an effect from the electric field of the nearby charged sialic acid carboxyl group. In any case, a pure Δ_γ of −1 p.p.m. would be expected at C-4-II due to the linkage at O-3-II; in fact, a difference of linkage shifts of +1.2 p.p.m. was observed between ASG_{M1} (**3**) and the other two structures **1** and **2**. It is unknown whether this anomalous effect on the values of Δ_γ is general for sialic acid residue linkages. A re-examination of the basis for the assignment for C-2-II may ultimately be necessary.

The resonance from C-5-II was found at δ 74.96 ±0.06 for the sialic acid-containing compounds **1** and **2**, and at δ 75.73 for ASG_{M1} (**3**). The difference of −0.77 p.p.m. is of the size expected for a Δ_γ as a result of substitution at O-4-II.

No effect of magnitude γ or larger is expected for C-6-II, and this is reflected by assignments. There are no significant differences between the chemical shifts for C-6-II for any of the gangliosides (δ 61.95 $\pm$0.17) or in model compounds, such as β-D-glucose, or lactose (δ 61.84 $\pm$0.10).

*2-Acetamido-2-deoxy-β-*D*-galactopyranosyl residue (III).* — The assignments for this sole 2-acetamido-2-deoxy sugar residue are not expected to differ, among any of the compounds studied, from those originally proposed[4] for G_{M1} (**1**). However, ASG_{M1} (**3**) and $G_{M1}OS$ (**2**) have structural features that give increased spectral resolution of the ^{13}C-n.m.r. spectra, which led, in a few instances, to improved chemical-shift measurements and more definitive assignments. The increased resolution of the spectrum of ASG_{M1} (**3**), at 90.55 MHz, and in a ternary solvent mixture (see Methods), is manifest in the anomeric-carbon region. Here are found, at δ ~103 three resonances, two of which have already been assigned to C-1-I and -1-II. The peak at δ 104.44 in the spectrum of ASG_{M1} (**3**) is now assigned to C-1-III. It is likely that the difference in chemical shift of 1.03 p.p.m. between the resonance for C-1-III of ASG_{M1} (**3**) and those of the other ganglioside oligosaccharides reflects a perturbation of electron density at this site by the adjacent sialic acid residue.

The resonance for C-2-III was originally[4] assigned to the peak at δ 51.86 in the spectrum of G_{M1} (**1**) on the basis of chemical and linkage shifts. Of the carbon atoms contributing signals in this spectral region, one is contributed by C-5-A and, hence, lacking in the spectrum of ASG_{M1} (**3**), and another one originates from C-2-R and is absent in the spectrum of $G_{M1}OS$ (**2**), whereas the last, from C-5-III, is present in all three oligosaccharide spectra. It is now clear that the resonance from C-2-III in the G_{M1} (**1**) spectrum occurs at δ 52.84, showing a γ-linkage shift of $-$1.29 p.p.m. due to substitution at both C-1-III and -3-III (Fig. 2). The resonances from C-3-III through -8-III are found at chemical shifts that vary little from those originally found[4] for G_{M1} (**1**). The higher-resolution data now available enable more accurate field-positions to be provided for the resonances from C-6-III and -8-III. The pattern of linkage shifts for C-1-III through C-4-III is Δ_β, $2\Delta_\gamma$,$\Delta_{\beta'}$, and Δ_γ = 7.8, $-$1.4, 9.8, and $-$0.6 p.p.m. in all three oligosaccharide spectra studied here.

*β-*D*-Galactopyranosyl residue (IV).* — Chemical shifts for this residue, and the changes due to the glycosidic linkage at C-1-IV, were initially expected to be quantitatively similar to those found for the β-D-galactopyranosyl residue in lactose. It was found[6], however, by comparing the spectrum of G_{M1} (**1**) with that of G_{M2} (**10**), in which residue IV is lacking, that the resonances from C-1-IV must be assigned to the peak at δ 105.60. The linkage shift Δ_β of 8.41 p.p.m. is larger than the 6.2 p.p.m. shift shown by C-1-II. This difference may reflect the difference between the β-D-(1→3) (between residues III and IV) and the β-D-(1→4) linkage (in lactose). The remaining resonances for C-2- through -6-IV follow the pattern of the resonances of the β-D-galactopyranosyl residue of lactose very well (Table 1). The signal of C-2-IV shows a γ-linkage shift of $-$0.89 $\pm$0.18 p.p.m. in lactose and in the oligosaccharide chains discussed here.

*5-Acetamido-3,5-dideoxy-*D*-*glycero*-α-*D*-*galacto*-nonulopyranosyl acid residue*

(*A*). — The assignments for this residue have proven to be the most difficult to establish, in spite of the anticipated, negligible linkage-shifts for C-4- through -11-A. Part of the problem stems from the lack of measurements for the chemical shifts of the α anomer of sialic acid, due to its low concentration in equilibrium solutions of α,β-sialic acid in deuterium oxide. Spectra having a high signal-to-noise ratio have now provided data[21] for all of the small γ resonances obtained from equilibrium mixtures, including the resonance from C-1α-A at δ 175.12 (Table 1). In addition, the assignments for both α- and β-sialic acid have been investigated[21] by means of differences in the deuterium-isotope shifts at the various ^{13}C-n.m.r. resonances[22]. These measurements provide a basis from which to propose better assignments for the resonances due to the γ-linked sialic acid residue in gangliosides. The average difference between the chemical shifts of resonances from C-4- through -11-A in the oligomers and monomer is 0.09 $\pm$0.44 p.p.m. Thus, for the aforementioned reasons, the signal at δ 51.96 in the spectrum of G_{M1} (**1**) has been reassigned from C-2-III to C-5-A, because residue A is absent in ASG_{M1} (**3**).

Resonances from C-1- through -3-A were expected to show a Δ_γ, Δ_β, and Δ_γ linkage-shift pattern as a result of the linkage of C-2-A to O-3-II. We found that the signal of the carboxyl group C-1-A only shifted upfield by Δ_γ 0.24 $\pm$0.06 p.p.m. Shifts Δ_β of 4.50 $\pm$0.14 and Δ_γ of $-$3.75 $\pm$0.04 p.p.m. were seen for C-2-A and -3-A, respectively. These last two values are quite different from the β- and γ-linkage shifts for neutral hexopyranosyl residues. It is probable that the electromagnetic field from the *N*-acetyl group in residue III perturbs[5,6] the local field at C-2-A and -3-A. Relatively large differences in chemical shift for the resonances from C-6-A and -8-A were observed between α-sialic acid and residue A of the oligosaccharide chains of the gangliosides.

Ceramide residue (*R*). — The hydrophobic portion of the gangliosides consist of a ceramide residue (R), whose chemical shifts have been previously reported[4,5,23]. This residue is not present in $G_{M1}OS$ (**2**). Inspection of the ^{13}C-n.m.r. spectrum of $G_{M1}OS$ (**2**) shows that the method of preparation of the oligosaccharide completely removed the ceramide residue, since no resonances from this residue were observed. Thus, the assignments of several ceramide resonances can be given with improved confidence. These include resonances from C-1- through -3-R, and -8-R. No significant deviation of the chemical shifts of the remaining ceramide-carbon resonances from those for either G_{M1} (**1**) or the ceramide residue alone were observed.

DISCUSSION

The high-field, high-resolution, ^{13}C-n.m.r. spectra of ganglioside G_{M1} (**1**), ASG_{M1} (**3**), and $G_{M1}OS$ (**2**) are presented herein. The large, chemical-shift range of the ^{13}C-n.m.r. spectra enabled us to resolve the resonances for almost every carbon nucleus of these molecules by comparing the spectrum of ganglioside G_{M1} (**1**) with the spectra of two previously unexamined derivatives, asialo-G_{M1} (**3**), which lacks only the sialic acid residue, and $G_{M1}OS$ (**2**), which lacks only the ceramide residue.

In the absence of ^{13}C-labeled compounds, the examination of such derivatives as **1**, **2**, and **3** is the only method to provide assignments that can show the effects of substitution at specific sites on the oligosaccharide. The proposed, complete assignments for ASG_{M1} (**3**) and $G_{M1}OS$ (**2**) illustrate the manner by which they may give information on the relationships between the spectra of complex oligosaccharides and their primary and secondary structures. These assignments will also be useful for interpreting ^{13}C-n.m.r. data from previously uncharacterized oligosaccharides. Thus, it will be possible to determine the conformation, anomeric configuration, linkage position, identity and number of sugar residues, and possibly the sequence of a new ganglioside.

The chemical shifts and α,β resonance-intensity pattern for the D-glucose residue of lactose were used to assign the resonances of residue I in $G_{M1}OS$ (**2**). The chemical shift difference between the α and β anomers of D-glucose is a parameter that is sensitive to the conformation of the sugar ring[24]. No significant difference in this parameter was observed among the spectra of the three compounds, D-glucose, lactose, and $G_{M1}OS$ (**2**) (see Table I). The proportion of β anomer was found to be $64 \pm 4\%$ for the D-glucose residue in $G_{M1}OS$ (**2**), a value similar to that of 63% for lactose and 61% for D-glucose, indicating that the conformation of residue I in $G_{M1}OS$ (**2**) is identical with that found for D-glucose, and for the D-glucose residue of lactose. Substitution of D-glucose at O-4 does not, therefore, alter the equilibrium between ring structures within the limits of the ^{13}C-n.m.r. data. Thus, the data obtained for C-1 in residue I of $G_{M1}OS$ (**2**) provide firm assignments for the same in ASG_{M1} (**3**), and confirm those for G_{M1} (**1**) as well. The data obtained for ASG_{M1} (**3**) allowed us to evaluate the effects of the sialic acid linkage, when compared with the data obtained[6] for G_{M3} (**9**) and G_{M2} (**10**), as well as with those from either G_{M1} (**1**) or $G_{M1}OS$ (**2**). Comparison of the chemical shifts and linkage shifts of ASG_{M1} with those of G_{M1} shows that removal of the sialic acid residue reduces the linkage shift $\Delta_{\beta'}$ of C-4-II by about 1 p.p.m. (Table I); in addition, the resonances from C-2-II and -3-II are displaced by -1.4 and $+2.6$ p.p.m., respectively. These shifts for the three carbon resonances that are expected to be most strongly influenced by the sialic acid residue linkage are quantitatively different from those found for the neutral hexose residue linkages. The shift for C-3-II is in the same direction as that caused by the neutral residues in G_{M1} (**1**) and its oligosaccharide portion, but it is 1/3 the size. On the other hand, the shift for C-4-II is of opposite sign from the Δ_γ of -1 p.p.m. shown by the shift for C-4 of residues I, III, and IV. It has previously been proposed[5,6] that the acetamido group of residue III perturbs the signals of C-2 and of the methylene carbon (C-3) of the sialic acid residue (A) of G_{M1} (**1**). Changes in the field positions of these resonances were observed[7] when cholera toxin binds $G_{M1}OS$ (**2**). The present data for the ^{13}C-n.m.r. spectrum of ASG_{M1} (**3**) indicate that the electromagnetic field originating from the sialic acid carboxyl group similarly perturbs the local nuclear field of the adjacent carbon atom C-4-II. Consequently, two sources of chemical-shift perturbation result from the branching in the oligosaccharide residue of G_{M1} (**1**). Examination of a Corey–Pauling–

Koltun model of $G_{M1}OS$ (**2**) shows that, when the carbonyl oxygen atom of residue III (O-7-III) is oriented toward the methylene carbon atom of the sialic acid residue, the carboxyl group is oriented in a position that deshields C-4-II and shields C-1-III. In this conformation, only a small effect on the resonance from C-4-II should be apparent. This effect on the resonance from C-4-II may be detected only by a specific ^{13}C-labeling experiment. As noted earlier, the resonance from C-4-III moves upfield, *i.e.*, is shielded, by 1.0 p.p.m. when the sialic acid residue is present as in G_{M1} (Table I). Thus, this residue perturbs both partners of the linkage pair C-1-III and C-4-II. Quantitative evaluation of this perturbation will be useful as a sensitive indicator of conformation for studies involving, for example, binding to proteins[5].

Accurate assignments for residue IV are of importance since binding by cholera toxin has been shown[25-27] at this site in G_{M1} (**1**). Examination of the ^{13}C-n.m.r. spectrum of ASG_{M1} (**3**) confirms previous proposals[4] for the assignments of the resonances from residue IV, because no significant shifts due to sialic acid are likely. The small difference of 0.2–0.4 p.p.m. between the chemical shifts of C-3- through -6-IV, and the corresponding shifts of D-galactose (Table I) may be explained by a solvent effect (See Methods).

It is possible now to ascertain the extent of the alteration of the magnetic environment of the sialic acid carbon nuclei by the gangliotetraglycosyl chain. The small downfield-shift Δ_β (4.5 p.p.m.) of C-2-A (Table I), and the upfield shift Δ_β (−3.8 p.p.m.) for C-3-A have been discussed earlier. They depend[6] on the presence of the 2-acetamido-2-deoxy-D-galactopyranosyl residue III. Correlated changes in these linkage shifts are conformational in origin, deriving from the relative orientation of residues III and A. The remainder of the ^{13}C resonances from carbons C-4- through -11-A are found at field positions that are close to those for the free monomer, those from C-6-A and -8-A being shifted downfield by 0.4 and 0.6 p.p.m., respectively. Salts[28], hydrogen bonding[29], and pH[21] affect the field positions of these carbon resonances.

It is of interest to compare the linkage shifts of G_{M1} (**1**), $G_{M1}OS$ (**2**), and ASG_{M1} (**3**). Correlations between the linkage shifts for the β and β' carbon resonances (Fig. 3) for the series[9] of gangliosides G_{M4}, G_{M2}, G_{M3}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} arise from covalent-electron-density changes accompanying formation of the oligosaccharides from the monosaccharides. The anomeric oxygen atom deshields both the β and β' carbon nuclei to a similar extent. The data in Fig. 3 for the series G_{M4} to G_{T1b} fall into distinct regions in the Δ_β and $\Delta_{\beta'}$ space, as indicated by shaded ellipses centered at the mean and extending by a value of two standard deviations on either side. Each type of linkage, for example, C-1-II→C-4-I (denoted by II in Fig. 3) is characterized by a unique region. There is no overlap among these regions. The linkage-shift pairs of the neutral hexose units are grouped around $\Delta_{\beta'} = 9$ p.p.m. The values for the sialic acid residues are strikingly smaller, however, clustering around Δ_β 4 and $\Delta_{\beta'}$ 2 p.p.m. The field on the sialic acid carboxyl group contributes a shielding effect that partially compensates for the deshielding observed in the neutral hexoses.

The data for the linkage-shift pairs of $G_{M1}OS$ (**2**) for residues A, II, III, and IV

are essentially identical to those found for G_{M1} (**1**) and the other gangliosides (Fig. 3). This is an indication that removal of the ceramide residue from G_{M1} (**1**) has no detectable long-range influence on the ^{13}C-n.m.r. properties of the oligosaccharide. We conclude that the chemical shifts and linkage shifts of $G_{M1}OS$ (**2**) are sensitive to conformation, like those of G_{M1} (**1**). The similarity of the chemical and linkage shifts of G_{M1} (**1**) and $G_{M1}OS$ (**2**), in spite of their marked difference in aggregation state, suggests also that the ^{13}C-n.m.r. chemical shifts of these molecules are insensitive to micelle formation.

No significant difference was found when the linkage shifts for ASG_{M1} (**3**) were compared to those for G_{M1} (**1**), except for the shift of the linkage C-1-III→O-4-II, which is adjacent to the site of attachment of the sialic acid residue in G_{M1} (**1**). Upon removal of residue A, the shifts for this linkage changed from 7.6 and 8.8, to 8.6 and 7.4 p.p.m., respectively. Thus, desialosylation resulted, for these linkage shifts, in changes, that are not in the expected directions of 0 for C-1-III and +1 p.p.m. for C-4-II, and the carboxyl group of residue A probably exerts long-range shielding effects.

Finally, the present data may be useful for predicting the ^{13}C-n.m.r. spectrum of ganglioside G_{M1b} (**7**), as the chemical shifts for residues I, II, and III would closely resemble those found for ASG_{M1} (**3**), and the shifts for residues IV and the sialic acid residue attached to O-3-IV would be very similar to those found[6] for residues IV and B in gangliosides G_{D1a} (**4**) and G_{T1b} (**6**). In particular, the linkage shifts for residue IV would fall into the pattern Δ_β, $2\,\Delta_\gamma$, $\Delta_{\beta'}$, Δ_γ, Δ_δ, and Δ_ε, where $\Delta_\beta = 8.15 \pm 0.32$, $2\Delta_\gamma = -1.83 \pm 0.73$, $\Delta_{\beta'} = 2.95 \pm 0.06$, Δ_γ (C-4-IV) $= -0.39 \pm 0.11$, $\Delta_\delta = -0.32$ p.p.m., and $\Delta_\varepsilon = 0.09 \pm 0.09$ p.p.m. The residue of sialic acid at position B would show a linkage shift pattern of $\Delta_\gamma, \Delta_\beta, \Delta_\gamma, \Delta_\delta, \ldots$, where $\Delta_\gamma = -0.23 \pm 0.21$, $\Delta_\beta = 2.63 \pm 0.04$, and Δ_γ (C-3-B) $= -0.92 \pm 0.02$ p.p.m., and the linkage shifts for C-4- through -11-B would be essentially 0 p.p.m.

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