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^{1}H-N.M.R. ANALYSIS OF TYPE-2 CHAIN LACTO-GANGLIOSIDES. CONFIRMATION OF STRUCTURE OF A NOVEL CANCER-ASSOCIATED FUCOGANGLIOSIDE, α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-Cer (VI^{6}NeuAcIII^{3}FucnLc$_6$Cer)*,†

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ABSTRACT

Neolacto-glycosphingolipids, substituted with α-NeuAc-(2→3)- and -(2→6)-linked D-Gal*p* residues were analyzed by one- and two-dimensional ^{1}H-n.m.r. spectroscopy at 500 MHz in 49:1 (v/v) di($^2H_3$)methyl sulfoxide–deuterium oxide solution. For the simplest structures analyzed, nLc$_4$Cer, IV^{3}NeuAcnLc$_4$Cer, and IV^{6}NeuAcnLc$_4$Cer, sialosylation-induced changes in shifts of terminal and subterminal core residues were interpretable in terms of existing conformational models. Chemical shifts for H-3*e* and H-3*a* of NeuAc characteristic for the type of linkage, were also determined. In addition, regularly reproducible shifts were seen for H-1 and other resonances of terminal and subterminal core residues of all structures tested. Chemical-shift correlations proved to be useful in elucidating the structure of a unique ganglioside bearing an internal β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3) residue ("X-trisaccharide") with an α-NeuAc-(2→6)-substituted terminal group.

INTRODUCTION

Neolacto-gangliosides having an α-NeuAc-(2→6)-β-D-Gal*p* terminal group, a relatively recent addition to the list of known glycosphingolipid structures, appear to have a rather limited distribution in normal human tissues. α-NeuAc-(2→6)-lacto-*N-neo*-tetraosyl- and *-nor*-hexaosylceramides were characterized as minor components of human erythrocytes[1], and subsequently the former was found to be present in moderate quantities in human meconium[2]. In connection with the

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present study, significant quantities of these compounds, along with α-NeuAc-(2→3)-substituted analogs, were found in human granulocytes[3], in agreement with the results of Fukuda *et al.*[4]. However, the largest accumulation so far of α-NeuAc-(2→6)-substituted *neolacto*-gangliosides have been found in human colonic and liver adenocarcinoma[5,6]. In addition to large quantities of α-NeuAc-(2→6)-lacto-*neo*-tetraosylceramide (IV^{6}NeuAcnLc$_4$Cer) (**3**)*, two novel gangliosides, VI^{6}NeuAcIII^{3}FucnLc$_6$Cer (**10**) and VI^{3}NeuAcV^{3}III^{3}Fuc$_2$nLc$_6$Cer (**11**), were found and characterized[5,6,8]. Since these compounds were virtually absent in ganglioside fractions of normal colonic mucosa and liver tissue, they may be tumor-associated antigens. To facilitate the analysis of such compounds of interest, we have adopted the routine use of ^{1}H-n.m.r. spectroscopy to obtain structural information complementary to that obtained by other methods, *i.e.*, methylation analysis by m.s. and g.l.c.–m.s., t.l.c., enzymic and chemical degradation, and the more recent application of monoclonal antibody techniques. In the past few years, ^{1}H-n.m.r. spectroscopy has been of increasing importance in the elucidation of glycosphingolipid structures[9–12]. In particular, the use of two-dimensional methods[13,14] such as *J*-resolved[15,16], spin-echo correlated[12,17], and n.O.e.[12] spectroscopy have led to increased information. However, as has been pointed out[15], the chief limitations will be the time and amount of material available. It is apparent that many glycolipids, particularly those found in tumors, will be available in very limited quantities. Thus, it is impractical to expect to apply, in every case, two-dimensional n.m.r. techniques or to suggest establishing all connectivities between residues by sequential application of spin-decoupling difference spectroscopy[18,19] (s.d.d.s.) and n.O.e. experiments[9]. For this reason, it is anticipated that for some time to come, structural assignment will still depend heavily on correlative methods, *e.g.*, identifying resonances characteristic of particular structural features (linkage position, and anomeric configuration), and recognition of glycosidation-induced chemical shift changes of readily assignable resonances, provided that the regularity of such effects has been established with some degree of certainty. This appears to be particularly applicable to glycolipids of the Type 2-*lacto* series, which have a regular repeating backbone structure that is perturbed only within a limited domain by the addition of side-chains and terminal groups.

The complete assignment of resonances of asialyl- and monosialyl-ganglioseries glycolipids has been published[12]. However, with the exception[11] of IV^{3}NeuAcnLc$_4$Cer (**3**), no ^{1}H-n.m.r. data are currently available for *neolacto*-gangliosides. The purpose of this communication is to: (a) present data on resonances characteristic for this series to aid in identification of the particular substances studied; (b) identify some of the general trends associated with NeuAc

*Shorthand designations for glycolipids are used according to the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature[7]; however, the suffix "ose" is omitted in the text, while "Cer" is omitted as well in tables. The prefix α- is assumed for NeuAc and L Fuc residues and has also been omitted. The nonstandard designation "*iso*-nLc$_8$" is used in this paper for IV^6[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→]nLc$_6$, to distinguish it from the unbranched structure nLc$_8$.

Glycolipid nomenclature	*Abbreviated carbohydrate nomenclature**
1 nLc_4Cer	βGal4βGlcNAc3βGal4βGlc1Cer
2 $IV^3NeuAcnLc_4Cer$	αNeuAc3βGal4βGlcNAc3βGal4βGlc1Cer
3 $IV^6NeuAcnLc_4Cer$	αNeuAc6βGal4βGlcNAc3βGal4βGlc1Cer
4 nLc_6Cer	βGal4βGlcNAc3βGal4βGlcNAc3βGal4βGlc1Cer
5 $VI^3NeuAcnLc_6Cer$	αNeuAc3βGal4βGlcNAc3βGal4βGlcNAc3βGal4βGlcCer
6 $VI^6NeuAcnLc_6Cer$	αNeuAc5βGal4βGlcNAc3βGal4βGlcNAc3βGal4βGlc1Cer
7 *iso*-nLc_8Cer	βGal4βGlcNAc3(βGal4βGlcNAc6)βGal4βGlcNAc3βGal4βGlc1Cer
8 VI^3NeuAc-*iso*-nLc_8Cer	αNeuAc3βGal4βGlcNAc3(βGal4βGlcNAc6)βGal4βGlcNAc3βGal4βGlc1Cer
9 $VI^3NeuAcVIII^3NeuAc$-*iso*-nLc_8Cer	αNeuAc3βGal4βGlcNAc3(αNeuAc3βGal4βGlcNAc6)βGal4βGlcNAc3βGal4βGlc1Cer
10 $VI^6NeuAcIII^3FucnLc_6Cer$	αNeuAc6βGal4βGlcNAc3βGal4(αFuc3)βGlcNAc3βGal4βGlc1Cer
11 $VI^3NeuAcIII^3V^3Fuc_2nLc_6Cer$	αNeuAc3βGal4(αFuc3)βGlcNAc3βGal4(αFuc3)βGlcNAc3βGal4βGlc1Cer
12 $VI^3NeuAcIII^3FucnLc_6Cer$	αNeuAc3βGal4βGlcNAc3βGal4(αFuc3)βGlcNAc3βGal4βGlc1Cer

Scheme 1. Structures of glycolipids. In the abbreviated nomenclature (*), the pyranose form, the D configuration for the hexoses and 2-acetamido-2-deoxyglucose, the L configuration for fucose, and the linkage at C-1 (C-2 for NeuAc) are assumed; the configuration of the anomeric centre (α or β) is placed *before* the name of the sugar, as in the systematic nomenclature of carbohydrates.

substitution on the *neolacto* series; and (c) use this information to confirm the structure of a fucoganglioside, isolated from adenocarcinoma, with a minimum amount of time and material.

EXPERIMENTAL

^{1}H-N.m.r. spectroscopy. — Gangliosides were first converted to the Na^+ form by passing through ion exchange resin AG-50W-X8 (Bio-Rad) Na^+ form, in methanol–H_2O. Glycolipid samples (100–300 μg) were then deuterium exchanged by repeated addition and evaporation of 1:1 $CDCl_3$–CD_3OD and dissolved in $(CD_3)_2SO$ containing 2% D_2O (ref. 18) and 1% tetramethylsilane as chemical shift references. For very small samples (~100 μg), particularly those containing an α-NeuAc-(2→6)-linked group, it was desirable to perform a final lyophilization from a solution in $(CD_3)_2SO$–D_2O, and to dissolve the sample in the highest quality $(CD_3)SO$ (99.96 atom %) because, in these compounds, the H-3*e* resonance can be obscured by satellite peaks from a large residual Me_2SO peak.

500-MHz 1D ^{1}H-N.m.r. spectra were obtained at 308 and 328 ±2K with a Bruker (F.R.G.) WM-500 spectrometer–Aspect 2000 data system using quadrature detection, a spectral width of 5000 Hz over 16K data points, and a 2-s relaxation delay. The resolution of spectra was enhanced by application of a Lorentzian-to-Gaussian function[20], included in the Bruker software package, to the accumulated free-induction decays before Fourier transformation. The accuracy of the chemical shift measurements was ±0.001 p.p.m. Coupled resonances were determined conventionally by continuous irradiation of the appropriate resonance during acquisition.

Two-dimensional, phase sensitive COSY spectra[21] were obtained at 308 ±2K with a Bruker AM-300 spectrometer–Aspect 3000 data system under conditions previously described[22]. Additional COSY spectra in the magnitude mode were collected with a Bruker WM-500–Aspect 2000 instrument without quadrature in t_1 using established acquisition parameters.

Thin-layer chromatography. — Preparative and analytical t.l.c. was performed on Merck (Darmstadt, F.R.G.). Silica gel 60 HPTLC plates developed in the following solvents: (*A*) 50:40:11 (v/v) chloroform–methanol–water containing 0.05% $CaCl_2$; (*B*) 7:3:1 (v/v) propanol–water–conc. NH_3. Bands scraped from plates for preparative purposes were extracted with 11:5:4 (v/v) 2-propanol–hexane–water (upper phase discarded). For h.p.t.l.c. immunostaining, it was necessary to use a plate having a silica layer high in binder, such as that provided by J. T. Baker, which will not wash off in aqueous solutions.

Other analytical procedures. — Sphingosine bases were analyzed as long chain aldehydes according to the method of Sweeley and Moscatelli[23] after methanolysis with the modified reagent as described by Gaver and Sweeley[24]. G.l.c. was performed with a Hewlett–Packard 5890A using a 30M SPB-5 bonded-phase fused silica capillary column (temperature program, 140–180° at 2°/min). Identities of aldehyde peaks were verified (*vs.* synthetic standards) with a Finnigan 3300/6110 GC-MS/DS (DB-5 column, spitless injection) instrument.

Fatty acids were determined as methyl esters liberated by methanolysis (0.5M HCl, 80°, 24 h) of glycolipids. G.l.c. was performed with a DB-5 (similar to SPB-5) column (splitless injection, temperature program, 150–290° at 4°/min) with chemical-ionization detection (methane, 300 μm)[25].

Reagents. — Di($^2H_3$)methyl sulfoxide (99.9 atom %), (^{2}H)chloroform (99.9 atom %), and ($^2H_4$)methanol (99.5 atom %) were purchased from Stohler Isotope (Waltham, MA) or Aldrich (Milwauke, WI). Di($^2H_3$)methyl sulfoxide (99.96 atom %) and D_2O (99.96 atom %) were purchased from Aldrich.

Glycosphingolipids. — IV^{3}NeuAcnLc$_4$Cer (**2**), IV^{6}NeuAcnLc$_4$Cer (**3**), and VI^{3}NeuAcnLc$_6$Cer (**5**) were obtained from human neutrophil membranes according to methods previously described[26]. Further details concerning their characterization have been published elsewhere[3]. Compounds **2** and **3** were also obtained from human erythrocyte membranes[27–30]. nLc$_4$Cer (**1**) was obtained by treatment of **2** from human erythrocytes[27] with acetic acid in 20:20:1 (v/v) chloroform–methanol–water at 100° in a sealed test tube. nLc$_6$Cer (**4**) was obtained from human adult type A and O erythrocyte membranes[31]. VI^{6}NeuAcnLc$_6$Cer (**6**) was obtained from human liver adenocarcinomata by methods previously described[5,6]. This compound has the some carbohydrate structure as VI^{6}NeuAcnLc$_6$Cer (**6**) obtained from human erythrocytes[1]. VI^{3}NeuAc-*iso*-nLc$_8$Cer (**8**) was obtained from human type O and A erythrocytes[1]; VI^{3}NeuAcVIII^{3}NeuAc-*iso*-nLc$_8$Cer (**9**) was obtained from human type O erythrocytes as described by Kundu *et al.*[32]. *iso*-nLc$_8$Cer (**7**) was obtained by acetic acid treatment of **9**; its H-n.m.r. spectrum was essentially as described by Dabrowski *et al.*[33], but with shift differences due to differences in probe temperature. Structures of all glycolipids were confirmed by methylation analysis, including linkage analysis by g.l.c.–m.s. of reduced and acetylated hydrolyzates as described[34–38].

A preliminary account of the isolation and characterization of a fraction of VI^{6}NeuAcIII^{3}FucnLc$_6$Cer (**10**) has been published[5]. Subsequent to preliminary fractionation by l.c., the isolation of this glycolipid essentially involved preparative h.p.t.l.c. using solvent *A* (see Fig. 1A), followed by separation of the major band, migrating just below IV^{6}NeuAcnLc$_6$Cer (**6**), into two components by preparative h.p.t.l.c. in solvent *B* (Fig. 1B). The faster migrating band in solvent *B*, reactive with MAb FH6, was shown to have the structure, VI^{3}NeuAcV^{3}FucIII^{3}FucnLc$_6$Cer (**11**, "6B", ref. 8). To the slower component (Fraction "6C, upper"), reactive with MAb 1B9, was assigned[5] structure **10**. The band migrating just below Fraction 6B–6C upper in solvent *A* (Fig. 1A) was similarly separated into two components, by use of solvent *B*, the faster one reactive with FH6 (Fraction "7B"), the slower one with 1B9 (Fraction "6C, lower", see Fig. 1B). Analysis by ^{1}H-n.m.r. showed that Fraction 7B had carbohydrate resonances essentially identical with those found previously[22] for Fraction 6B. The complete analysis of the Fraction 6C components, as described below, is discussed in detail in the following sections.

Antibodies. — The production and determination of specificity of monoclonal antibodies (Mab) IB9 recognizing the terminal structure α-NeuAc-(2→6)-D-Gal,

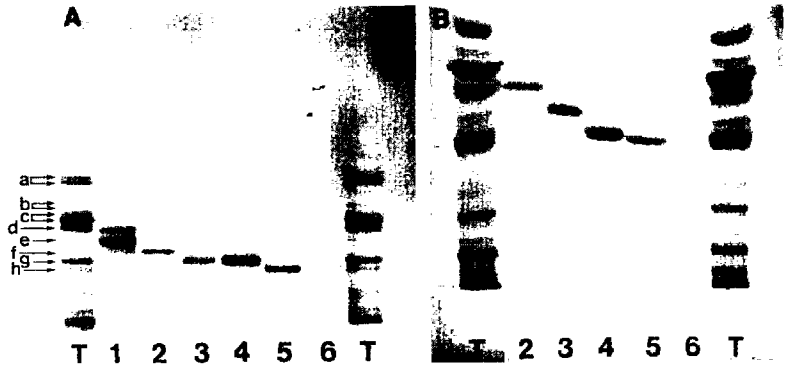


Fig. 1. H.p.t.l.c. of *lacto*-series gangliosides from adenocarcinoma FT75-620: (A) solvent *A*; (B) solvent *B*; (T) total monosialoganglioside fraction. Bands marked: (a) GM_3; (b) $IV^3NeuAcnLc_4Cer$ (**2**);(c) $IV^6NeuAcnLc_4Cer$ (**3**); (d–e) $VI^3NeuAcnLc_6Cer$ (**5**); (f) upper band of $VI^6NeuAcnLc_6Cer$ (**6**); (g) 6B + 6C upper; and (h) 7B + 6C lower. Lanes marked: (1) $VI^3NeuAcnLc_6Cer$ (**5**); (2) $VI^6NeuAcnLc_6Cer$ (**6**); (3) 6B; (4) 6C upper; (5) 6C lower; and (6) 7B.

and FH6 recognizing the structure, α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc, have been described previously[5,6,8]. The reactivity of glycolipids was determined by h.p.t.l.c.–immunostaining using the method of Magnani *et al.*[39], as modified by Kannagi *et al.*[40]

RESULTS AND DISCUSSION

The anomeric proton chemical shifts and $^3J_{1,2}$ coupling constants for compounds **1–9** are summarized in Table I. Additional ring proton assignments made from two-dimensional COSY spectra are given for compounds **1–3**. The phase-sensitive COSY spectrum of $IV^6NeuAcnLc_4Cer$ (**3**) is reproduced in Fig. 2 to illustrate the connectivities from which these assignments were extracted.

Results similar to those given for unsubstituted core structures **1**, **4**, and **7**, differing by temperature-dependent shift effects, have been previously obtained[9,33]. Results at a different temperature for anomeric protons of **2** have also been published[11]. *N*-Acetylneuraminic acid H-3*e* and H-3*a* resonances, recognizable by their characteristic multiplicities and easily verified mutual coupling, are listed in Table II along with NeuAc and GlcNAc N-acetyl methyl resonances, and NeuAc H-4 resonances found in COSY spectra of **2** and **3**. Values found for α-(2→3)-linked NeuAc are similar to those reported for α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-Cer (GM_3) obtained under similar conditions[12].

Unbranched analogs **1–6**. — The values found for anomeric protons (Table I) show good numerical agreement for substitution on nLc_4 and nLc_6 cores. Likewise, the results for H-3*e*, H-3*a*, and *N*-acetyl resonances, shown in Table II, are in excellent agreement. Thus, these resonances would appear to be suitable in this solvent system as "structural reporter groups"[41]. Interestingly, the shift changes

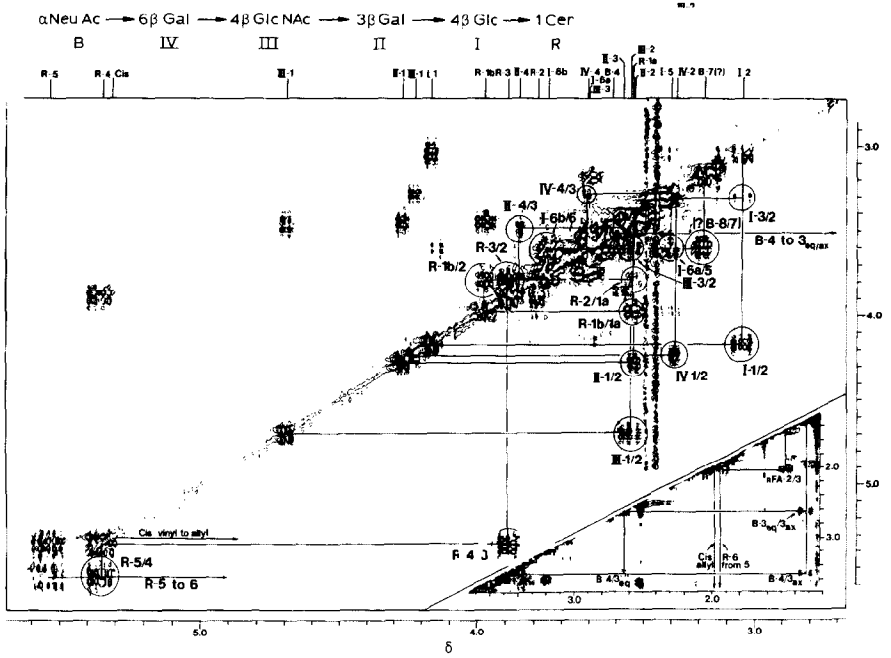


Fig. 2. Downfield region of two-dimensional, phase-sensitive ^{1}H-COSY at 300 MHz of IV^{6}NeuAcnLc$_4$Cer (**3**) from O erythrocytes. Conc. ~2mM in di($^2H_3$)methyl sulfoxide–2% D_2O. Inset: section of lower frequency region. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure. R refers to protons of sphingosine backbone only. *Cis*-vinyl and -allyl refer to protons of unsaturated fatty acids. nFA refers to protons of nonhydroxylated fatty acids.

seen for these compounds in dimethyl sulfoxide (Table III) are somewhat different from those reported for ^{1}H-n.m.r. of glycopeptides and oligosaccharides containing similar structures taken[41,42] in D_2O. The effects on the terminal Gal H-1 signal are smaller and opposite in sign, whereas the effects on the GlcNAc H-1 signal are of somewhat larger magnitude. Also, although the NAc signals of NeuAc occur at about the same location for α-(2→3) or α-(2→6) linkages for solutions in D_2O, they are sufficiently separated (by 0.010 ±0.001 p.p.m.) for solutions in di($^2H_3$)methyl sulfoxide to provide another basis for differentiation*.

Although less reliable as a structural marker, a characteristic feature of the α-NeuAc-(2→6) linkage is the appearance of the subterminal Gal H-1 resonance as

*Since the results reported by Sabesan and Paulson[42] for the structures, α-NeuAc-(2→3 or 6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, in D_2O are in essential agreement with those compiled by Vliegenthart *et al.*[41] for both branched and unbranched structures, α-NeuAc-(2→3 or 6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2 or 4)-α-D-Man*p*-(1→3 or 6)-β-D-Man*p*-(1→, it would appear that the differences with our results reflect differences in solvent characteristics (*e.g.*, solvation), or solvent-induced conformational changes, or both. It should be noted, however, that in the case of substitution with an α-(2→3)-linked NeuAc group, small but significant differences in shift changes were reported for β-D-GlcNAc H-1, dependent on which branch of the core structure is involved[41].

TABLE I

^{1}H CHEMICAL SHIFTS (δ)[a] AND $^3J_{1,2}$ COUPLING CONSTANTS (Hz)[b] FOR CORE SUGARS OF GLYCOSPHINGOLIPIDS[c]

		(A) Unbranched glycosphingolipids (groups or residues)					
Compound	*Proton*	*α-NeuAc*	*β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(2→1)-Cer*					
nLc_4 (**1**)	H-1	None	4.214 (6.7)	4.664 (8.5)			4.267 (7.3)	4.172 (7.9)[d]
	H-2		3.301	3.444			3.435	3.044
	H-3		3.291	3.556			3.447	3.314
	H-4		3.635				3.845	
$IV^3NeuAcnLc_4$ (**2**)	H-1	-(2→3)	4.201 (7.8)	4.642 (7.8)			4.262 (7.3)	4.166 (7.8)[e]
	H-2		3.291	3.449			3.430	3.047
	H-3		3.964	3.543			3.452	3.319
	H-4		3.700				3.849	
$IV^6NeuAcnLc_4$ (**3**)	H-1	-(2→6)	4.228 (7.9)	4.687 (8.5)			4.265 (7.9)	4.166 (7.9)
	H-2		3.284	3.449			3.433	3.046
	H-3		3.284	3.606			3.479	3.316
	H-4		3.614				3.850	
nLc_6[f] (**4**)		None	4.208 (6.1)	4.652 (8.5)	4.260 (7.3)	4.652 (8.5)	4.260 (7.3)	4.169 (7.9)[g]
$VI^3NeuAcnLc_6$[f] (**5**)		-(2→3)	4.200 (7.9)	4.636 (7.9)	4.262 (7.3)	4.652 (7.9)	4.263 (7.3)	4.168 (7.9)
$VI^6NeuAcnLc_6$[f] (**6**)		-(2→6)	4.223 (7.3)	4.691 (8.5)	4.264 (7.3)	4.656 (8.5)	4.262 (7.3)	4.164 (7.9)

Compound	(B) Branched glycosphingolipids (groups or residues)[h]								
	α-*NeuAc*[6]	β-D-*Galp*-(1		4)-β-D-*GlcpNAc*-(1		6)			
	α-*NeuAc*[3]		β-D-*Galp*-(1		4)-β-D-*GlcpNAc*-(1	3)	β-D-*Galp*-(1→4)-β-D-*GlcpNAc*-(1→3)-β-D-*Galp*-(1→4)-β-D-*Glcp*-(1→1)-*Cer*			
iso-nLc$_8$[f] (**7**)	None[6] None[3]	4.215 (7.3)	4.215 (7.3)	4.409 (7.9)	4.650 (8.5)		4.297 (7.9)	4.647 (8.5)	4.262 (7.3)	4.171 (7.9)[g]
VI^{3}NeuAc-*iso*-nLc$_8$[f] (**8**)	None[6] (2→3)[3]	4.214 (6.7)	4.198 (7.9)	4.416 (8.5)	4.662 (7.3)		4.301 (7.9)	4.646 (7.3)	4.264 (7.3)	4.169 (7.3)
VI^3, VIII^3-NeuAc$_2$-*iso*-nLc$_8$[f] (**9**)	(2→3)[6] (2→3)[3]	4.200 (7.9)	4.200 (7.9)	4.388 (7.9)	4.676 (7.9)		4.302 (7.3)	4.647 (8.5)	4.266 (6.7)	4.168 (7.9)

[a]From the signal of Me_4Si. [b]In parentheses. [c]For a solution in di($^2H_3$)methyl sulfoxide at 308 ±2 K. [d]Agrees with data of Dabrowski *et al.*[51] except for temperature-dependent shift. [e]Agrees with data of Gasa *et al.*[11] except for temperature-dependent shift. [f]H-1 Resonances only. [g]Agrees with data of Dabrowski *et al.*[33] except for temperature-dependent shift.

TABLE II

CHEMICAL SHIFTS (δ)[a] OF NAc GROUPS AND NeuAc H-3 OF GLYCOSPHINGOLIPIDS[b]

Compound	*α-NeuAc-(2→3)*		*α-NeuAc-(2→6)*		*α-NeuAc-(2→3) NAc_3*	*α-NeuAc-(2→6) NAc_3*	*GlcNAc- NAc_3*	*GlcNAc- NAc_3*	*GlcNAc- NAc_3*
	*H-3*e	*H-3*a	*H-3*e	*H-3*a					
nLc_4Cer (**1**)							1.817		
$IV^3NeuAcnLc_4$ (**2**)	2.752	1.367[c]			1.889			1.817	
$IV^6NeuAcnLc_4$ (**3**)			2.627	1.314[d]		1.878			1.833
nLc_6 (**4**)							1.822 (2)		
$VI^3NeuAcnLc_6$ (**5**)	2.750	1.368			1.888		1.820	1.820	
$VI^6NeuAcnLc_6$ (**6**)			2.624	1.314		1.878	1.817		1.835
iso-nLc_8 (**7**)							1.823	1.835	1.843
VI^3NeuAc-iso-nLc_8 (**8**)	2.750	1.366			1.887		1.816	1.834	1.841
VI^3, $VIII^3NeuAc_2$-*iso*-nLc_8 (**9**)	2.745	1.372 (2 ea)			1.887 (2)		1.815	1.836 (2)	

[a]From the signal of Me_4Si. [b]For solutions in di($^2H_3$)methyl sulfoxide at 308 ±2 K. [c]α-NeuAc-(2→3) H-4, δ 3.562. [d]α-NeuAc-(2→6) H-4, δ 3.513.

TABLE III

GLYCOSYLATION-INDUCED CHEMICAL SHIFT EFFECTS FOR α-(2→3) AND α-(2→6) SIALYLATION OF nLc_4 (**1**), nLc_6 (**4**), AND *iso*-nLc_8 (**7**) CORE GLYCOSPHINGOLIPIDS[a]

Group or residue	*α-(2→3)*	*α-(2→6)*
β-D-Gal*p* terminal		
H-1	−0.013[b]	+0.015[c]
H-2[d]	−0.010	−0.017
H-3[d]	+0.573	−0.007
H-4[d]	+0.065	−0.021
β-D-Glc*p*NAc subterminal		
H-1[e]	−0.019	+0.030
H-2[d]	[f]	[f]
H-3[d]	−0.013	+0.050
NAc[f]	[f]	+0.015
β-D-Gal*p* internal		
H-1[e]	[f]	[f]
H-2[d]	[f]	[f]
H-3[d]	[f]	+0.032
H-5[d]	[f]	[f]

[a]For a solution in di($^2H_3$)methyl sulfoxide at 308 K. [b]Average value for (**1**→**2**), (**4**→**5**), (**7**→**8**), and (**8**→**9**). [c]Average value for (**1**→**3**) and (**4**→**6**). [d]Single values for (**1**→**2**) or (**1**→**3**). [e]Average values for (**1**→**2**), (**4**→**5**), or (**1**→**3**), (**4**→**6**). [f]Not significant [≤0.005].

a pseudotriplet (see Fig. 3) due to virtual coupling between this proton and H-3 of the same sugar ring[43–46], a result of the coincidence of the chemical shifts of H-2 and H-3 (see Table I, compound **3**) and the strong coupling between them (compare also the computer-simulated line shapes in Dahmen *et al.*[47]).

Comparison of the data for IV^{3}NeuAcnLc$_4$ (**2**) with those of Koerner *et al.*[12] for the ganglioside GM_3, which shares the α-NeuAc-(2→3)-β-D-Gal*p*-(1→4) terminal group, suggests two other reporter groups, the subterminal Gal H-3 and H-4 resonances. As illustrated in Fig. 4 for VI^{3}NeuAcnLc$_6$Cer (**5**), they are easily found by inspection, which is also true for spectra of similar substances in aqueous media[41,42]. For these resonances, it does not appear to matter whether the adjacent sugar residue of the aglycon is D-glucose or 2-acetamido-2-deoxy-D-glucose.

There is essential agreement in values for chemical shift changes induced by an α-NeuAc-(2→3) group on the ring protons of a terminal β-D-galactopyranosyl group given in Table III and those reported for GM_3 by Koerner *et al.*[12], particularly the characteristically large shift for H-3, closest to the glycosylation site, and the somewhat smaller shift change for H-4. The negative shift change for H-2 is not as large as that reported for GM_3. No shift change of such magnitude was found for the same protons upon addition of an α-NeuAc-(2→6) group, as might be expected. Unfortunately, the effect of this residue on the β-GalIV H-5 and hydroxymethyl protons could not be tested because the appropriate connectivities could not be reliably traced on the COSY spectra available (Fig. 2).

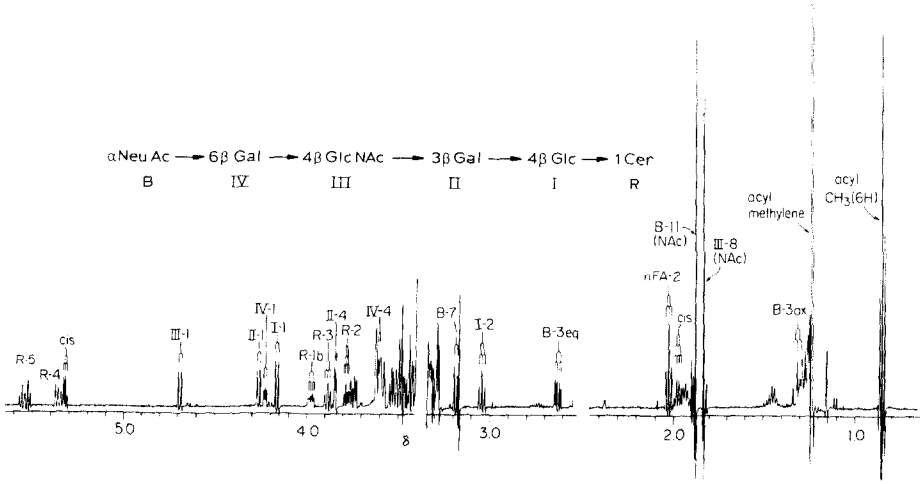


Fig. 3. Downfield region of resolution-enhanced 500-MHz ^{1}H-n.m.r. spectrum of granulocyte IV^{6}NeuAcnLc$_4$Cer (**3**) at 308 ±2K; conc. ~0.5mM in di($^2H_3$)methyl sulfoxide–2% D_2O; 936 f.i.d.s. were accumulated.

It is apparent (Table III) that α-(2→6) sialosylation affects the signal of the nearby GlcNAc NAc (Δδ 0.015 p.p.m.), whereas there is virtually no effect on this resonance from an α-NeuAc-(2→3)-linked group. The most substantial effect on the signals of the subterminal 2-acetamido-2-deoxy-β-D-glucopyranosyl residue is the shift of H-3 (Δδ 0.50 p.p.m.) upon α-(2→6) sialosylation. In addition, α-(2→6) sialosylation has an effect on the *internal* β-D-Gal H-3 that was as large as that on

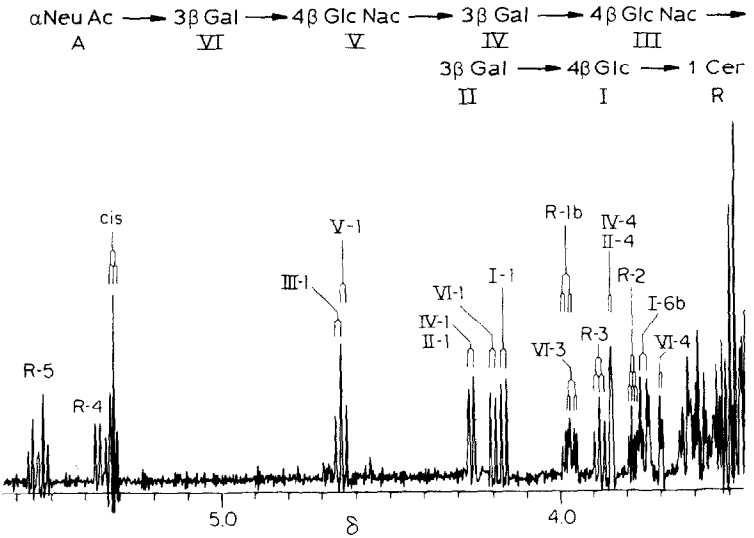
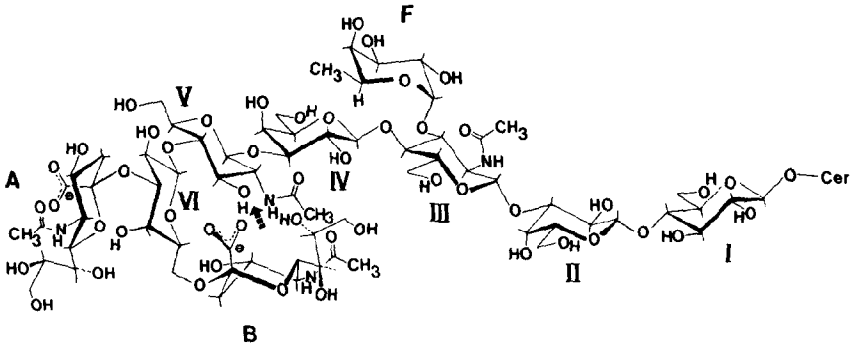


Fig. 4. Downfield region of resolution-enhanced, 500-MHz ^{1}H-n.m.r. spectrum of granulocyte IV^{3}NeuAcnLc$_6$Cer (**5**) at 308 ±2K; conc. ~0.5mM in di($^2H_3$)methyl sulfoxide–2% D_2O; 660 f.i.d.s. were accumulated.

the β-D-GlcNAc H-1 (Δδ 0.032 p.p.m.). The effects of α-(2→3) sialosylation on these protons are all considerably smaller, and, for H-1 and H-3 of β-D-GlcNAc, opposite in sign.

While no attempt was made to elaborate a detailed conformation of the *N*-acetylneuraminyl group from his ^{13}C-n.m.r. data on sialyl-*lacto*-oligosaccharides, Berman[48] proposed, on the basis of glycosylation-induced chemical shift effects, that while the α-(2→3) isomer extends from a lactosyl or lactosaminyl oligosaccharide to form an approximately linear extension of the chain, the α-(2→6) isomer is folded back over the oligosaccharide in close proximity to the subterminal glucosyl or 2-acetamido-2-deoxyglycosyl residues. Jennings *et al.*[49] proposed, based on their ^{13}C-n.m.r. data for the Type III group B streptococcal polysaccharide, which contains an internal (1→6)-[αNeuAc-(2→6)-β-D-Gal*p*-(1→4)]-β-D-Glc*p*-NAc-(1→3)-β-D-Gal*p* structure in its core, an interresidue hydrogen bond between the *N*-acetylneuraminic carboxyl group and the 2-acetamido-2-deoxyglucosyl 3-



Scheme 2. Composite structure of gangliosides derived from *nor*-hexaosylceramide showing approximate disposition of substituents on the nLc$_6$ core (residues I–VI). The relative conformation of the α-L-Fuc*p*-(1→3) group (F) was adapted from Hindsgaul *et al.*[50] for X trisaccharide, and that of the α-NeuAc-(2→6) group (B) is shown according to the proposal of Jennings *et al.*[49] for Type III Group B streptococcal polysaccharide.

hydroxy group, stabilizing the conformation of the oligosaccharide determinant. These proposals are illustrated in Scheme 2 of an α-NeuAc-(2→6) group attached to a poly-*N*-acetyllactosaminyl chain, *e.g.*, lacto-*N*-*nor*-hexaosylceramide. For this discussion, the terminal tetrasaccharide (residues IV, V, VI, and B) is pertinent. For contrast, the approximate position of an α-NeuAc-(2→3) terminal group (A) is also shown.

The data obtained for compound **3** can be interpreted in terms of this model, although qualifications should be noted regarding (a) the possible differences which may exist in conformation, or in solvation between dimethyl sulfoxide and water solutions of carbohydrates, or both; and (b) the degree to which shift changes <0.2 p.p.m. can be interpreted[51,52]. Nevertheless, the ^{1}H-n.m.r. data are consistent with this model in that the protons apparently experiencing the largest shifts to higher

frequency are all on the same side of the oligosaccharide chain, and that the large shift noted for GlcNAc H-3 can be attributed to the close approach of some anisotropic group, which *may* be the *N*-acetylneuraminyl carboxyl group, although there is no definite indication for or against the proposed hydrogen bond with HO-3. The shift of internal Gal IV H-3 might be caused by interaction with the *N*-acetylneuraminyl *N*-acetyl group. Alternatively, the shifts of Gal IV H-3 and GlcNAc H-3 might be caused indirectly by a change in relative conformation of the GlcNAc *N*-acetyl group as a result of its interaction with the α-NeuAc-(2→6) group. However, the lack of a shift change for GlcNAc H-2 is not consistent with this proposal. Interestingly, Jennings *et al.*[49] found that reduction of the sialic acid CO_2 to a CH_2OH group caused a shift of the Gal C-1 and GlcNAc C-4 signals of the *N*-acetyllactosamine residue to which it is linked. This was interpreted as resulting from a conformational change upon elimination of the hydrogen bonding interaction between the carboxyl group and HO-3 of the 2-acetamido-2-deoxyglucosyl residue. However, it is conceivable that the shift changes noted could be attributable solely to the change in chemical environment upon elimination of the anisotropic carboxyl group without invoking a conformational adjustment at those carbon atoms. Berman's conclusions express the same reservations[48]. Although not referring directly to Jennings *et al.*[49] results, Sabesan and Paulson[42] also have suggested that the unusual shift changes seen for α-NeuAc-(2→6) extension of Type 2 oligosaccharides are due to the anisotropic influence of the carboxylate group alone. The calculations performed by Sabesan *et al.*[53] on α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→ show no indication of a hydrogen bond able to control the trisaccharide conformation.

Branched analogs **7**–**9**. — The results in Table I showed that the effect of α-(2→3) sialosylation on the subterminal Gal H-1 resonances is conserved for the branched mono- and di-*N*-acetylneuraminyl compounds. In addition, the subterminal Gal H-3,4, NeuAc H-3*e*,*a*, and NAc resonances could be readily located (Fig. 5 and Table II). The shifts of the GlcNAc H-1 resonances appeared anomalous. Thus, for compound **8**, which had previously[1] been assigned the structure shown, the most noticeable effects was a shift to *higher* frequency (Δδ 0.01 p.p.m.) of the β-D-Glc*p*NAc-(1→3) H-1, and a somewhat smaller deshielding of the β-D-Glc*p*NAc-(1→6) H-1 resonance. Thus, although the existence of a single compound in this fraction could be established from the ^{1}H-n.m.r. data alone, it would be impossible to assign the NeuAc group to a particular branch, on the basis of a comparison with the spectra of simpler compounds. However, the problem could be apparently solved by comparison with the spectrum of **9**, which showed a "normal" shift to lower frequency for the β-D-Glc*p*NAc-(1→6) H-1 resonance, thereby suggesting that the precursor **8** had an α-NeuAc-(2→3)-linked to the (1→3)-branch, and confirming the original structural assignment, which had been made on the basis of analogy to other branched, monosialosylated gangliosides[1]. In addition, the β-D-Glc*p*NAc-(1→3) H-1 signal showed a *further* shift to higher frequency (Δδ 0.014 p.p.m.), suggesting that the expected shielding of that proton

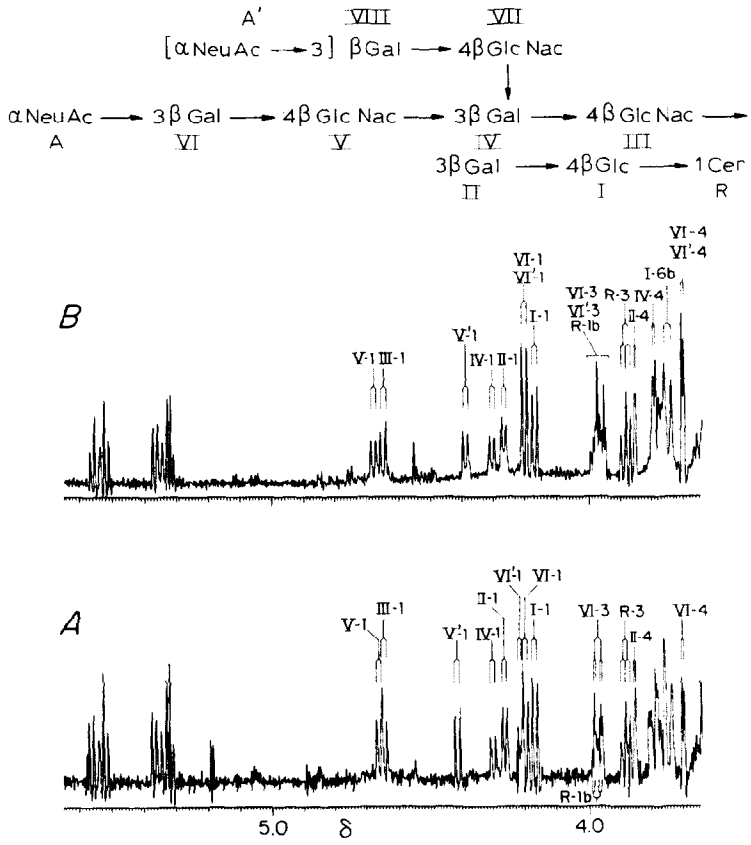


Fig. 5. Downfield region of resolution-enhanced, 500 MHz ^{1}H-n.m.r. spectra of O erythrocyte: (A) VI^{3}NeuAc-*iso*-nLc$_8$Cer (**8**); and (B), VI^{3}NeuAcVIII^{3}NeuAc-*iso*-nLc$_8$Cer (**9**) at 308 ±2K; conc. ~0.2 and 0.4mM, respectively, in di($^2H_3$)methyl sulfoxide–2% D_2O; 2000 and 800 f.i.d.s. were accumulated, respectively. A spectrum identical to that in (A) was obtained with **8** isolated from A erythrocyte membranes.

by the α-NeuAc group (2→3)-linked to the subterminal Gal residue is being counteracted by some other (deshielding) effect. This may be attributed to interchain interactions resulting from hydrogen bonding of the sialic acid groups, bringing about conformational changes relative to the unsubstituted *iso*-nLc$_8$ core structure, and locating a deshielding group (*i.e.*, the β-D-Glc*p*NAc-(1→6) NAc carbonyl group) near β-D-Glc*p*NAc-(1→3) H-1. Evidence for such interchain hydrogen-bonding has been published for the triantennary, N-linked oligosaccharide structure of fetuin[54] and assumed to control conformational changes in the branching arms of such structures. Data obtained by Vliegenthart *et al.*[41] also showed that, at least for glycopeptides and related oligosaccharides in D_2O, the effect of α-NeuAc-(2→3) substitution on a terminal Gal group in several cases causes deshielding rather than shielding effects on H-1 of the adjacent GlcNAc residue, depending on which branch is substituted. The problem of conformational changes in the system considered here deserves further study.

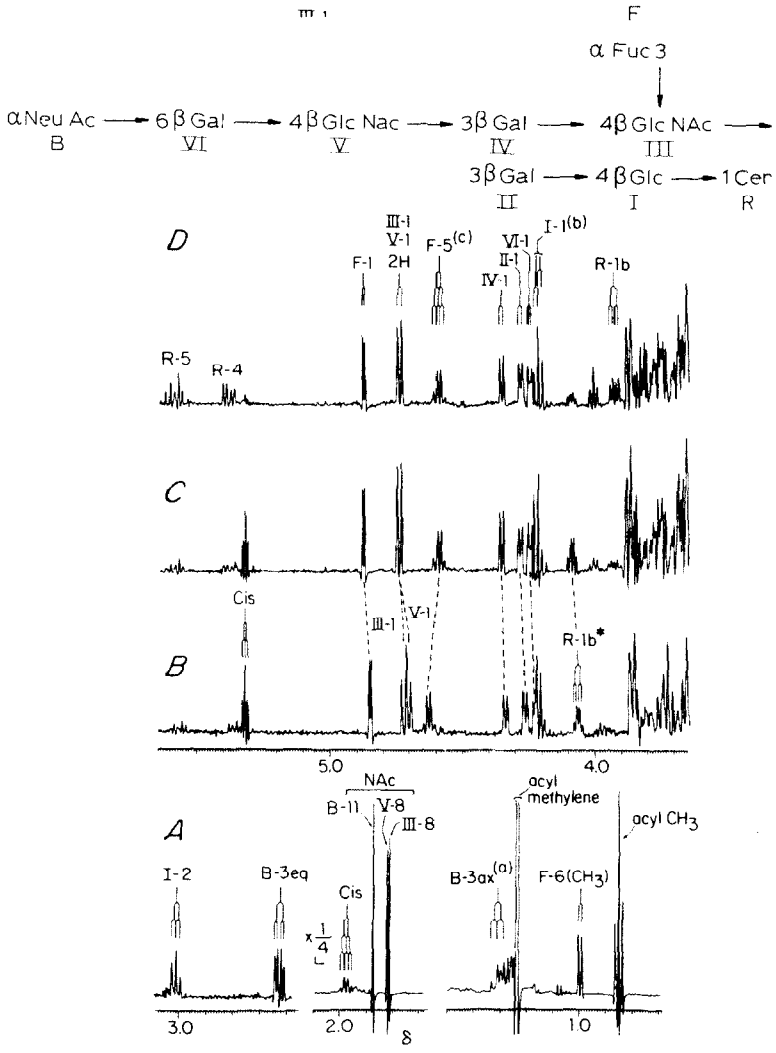


Fig. 6. (A) Upfield region of resolution-enhanced 500-MHz ^{1}H-n.m.r. spectrum of adenocarcinoma FT75-620 monosialo fraction 6C-upper band (VI^{6}NeuAcIII^{3}FucnLc$_6$Cer, **10**) at 308 ±2K; conc. ~mM in di($^2H_3$)methyl sulfoxide–2% D_2O; 400 f.i.d.s. were accumulated. (B) Downfield region of same spectrum. (C) Downfield region at 328 ±2K. (D) Downfield region of spectrum of fraction 6C-lower band isolated from same adenocarcinoma, at 328 ±2K; conc. ~0.8mM, 900 f.i.d.s. accumulated. (a) NeuAc H-3*a* confirmed by decoupling from H-3*e*; (b) Glc H-1 resonances confirmed by decoupling from Glc H-2; (c) Fuc H-5 confirmed by decoupling from Fuc H_3-6 doublet. The unmarked triplet at δ 4.010 p.p.m. in (C) and (D) was assigned to R-3 of sphingosine in combination with 2-hydroxyfatty acid[12,16,55,57].

"Fractions 6C". VI^{6}NeuAcIII^{3}FucnLc$_6$Cer (**10**). — Two monosialosyl-ganglioside fractions, migrating below VI^{6}NeuAcnLc$_6$Cer (**6**) on h.p.t.l.c. and reactive with monoclonal antibody IB9 (refs. 5, 6) were isolated as described in the Experimental section. On the basis of the results of methylation analysis, enzymic and chemical degradation, and h.p.t.l.c. immunostaining, the structure VI^{6}NeuAcIII^{3}FucnLc$_6$Cer (**10**) was tentatively assigned[5] to the upper band. ^{1}H-N.m.r. spectra of the two fractions were obtained, portions of which are displayed in Fig. 6 and summarized in Table IV. It can readily be seen that the spectra of both fractions represent identical carbohydrate components attached to different ceramide aglycons. The following discussion will illustrate how the ^{1}H-n.m.r. data can be used, with supporting methylation data, to determine the structure of a complex glycolipid without resorting to further degradative procedures. Methylation analysis had previously shown[5] that Fraction 6C (upper band) contained one 6-*O*- and two 3-*O*-linked Gal residues, one terminal Fuc group, 4-*O*- and 3,4-*O*-linked GlcNAc residues, and one 4-*O*-linked Glc residue. Inspection of the ^{1}H-n.m.r. spectrum revealed one α-anomeric resonance, which could be assigned with confidence to the α-L-Fuc*p* group; and six β resonances, which, taken with the methylation data, suggested a *nor*-hexaosylceramide core structure. The β-D-Glc H-1 resonance was not found in its more typical location around δ 4.16–4.18, but was easily found (by decoupling from the Glc H-2 triplet at δ 3.009) as the doublet at δ 4.213 ($^3J_{1,2}$ 7.3 Hz). At the other end of the saccharide chain, the characteristic NeuAc signals at δ 2.626 (H-3*e*), 1.308 (H-3*a*), and 1.876 (NAc) suggested an α-(2→6)-linked terminal NeuAc group, thereby confirming the origin of 2,3,4-tri-*O*-methyl galactitol (6-*O*-linked Gal residue) in the g.l.c.–m.s. analysis. This sugar residue was represented in the ^{1}H-n.m.r. spectrum by the β-D-Gal*p* H-1 resonance (δ 4.227) partially overlapped by the β-D-Glc H-1 resonance at 308 K [compare values for compounds **3** and **6** and lineshape in the ^{1}H-n.m.r. spectrum of **3** (Fig. 3)]. The H-1β resonance at δ 4.263 could be assigned to Gal II since this resonance is nearly invariant in the *lacto*-series ^{1}H-n.m.r. spectra for a given set of conditions[9,11,22]. The last H-1 resonance in the β-D-hexose region[18] could be assigned to the β-D-Gal*p* residue in the middle of the chain, with its exact location (δ 4.336) expected to reflect further structural features; the remaining downfield H-1 signals at δ 4.721 and 4.704 ($^3J_{1,2}$ 8.5 Hz) were assigned as β-D-Glc*p*NAc signals. Again, further structural information can be expected from detailed consideration of their chemical shifts. The presence of two 2-acetamido-2-deoxyglucose residues was further confirmed by a pair of NAc singlets at δ 1.813 and 1.822.

Further specific structural information could be obtained from the ^{1}H-n.m.r. spectra even in the absence of other data. The chemical shifts of the α-L-Fuc*p* H-1,5, and H_3-6 (δ 4.852, 4.628, and 0.996, respectively, at 308 K; δ 4.878, 4.589 and 1.014, respectively, at 328 K) are fully diagnostic for α-L-Fuc*p*-(1→3)-D-GlcNAc in Type 2 (X-haptenic) structures[22,25]. This establishes the origin of the 2-acetamido-2-deoxy-6-*O*-methylglucitol (3,4-*O*-linked GlcNAc residue) in the methylation analysis without the necessity of performing the same analysis after

TABLE IV

CHEMICAL SHIFTS (δ)[a] AND COUPLING CONSTANTS (Hz)[b] FOR SELECTED PROTONS OF VI^{3}NeuAcIII^{3}FucnLc$_6$ (**10**)[c]

Proton	*α-NeuAc-(2→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→1)-Cer*				α-L-*Fucp*-(1→3)			
H-1 ($^3J_{1,2}$)		4.227 (6.7)[d]	4.704 (8.5)	4.336 (7.3)	4.852 (3.9)	4.721 (8.5)	4.263 (7.3)	4.213 (7.3)[e]
H-2								3.009[e]
H-3*e*	2.626							
H-3*a*	1.308							
H-5 ($^3J_{5,6}$)					4.628 (6.7)			
H-6					0.996 (3H)			
NAc	1.876 (3H)		1.813 (3H)[f]			1.822 (3H)[f]		

[a]From the signal of Me_4Si. [b]In parentheses. [c]For a solution in di($^2H_3$)methylsulfoxide at 308 K. [d]At 338 K, $^3J_{1,2}$ 7.3 Hz. [e]Upper t.l.c. band major resonances (see Fig. 6B). Other β-D-Glc*p* H-1 resonances found at δ 4.200 and 4.169; H-2 resonances at δ 3.026 and 3.045. The proportions found for the lower t.l.c. band are different. [f]Assignment may be reversed.

defucosylation, as in the previous work[5], to ascertain whether an α-L-Fuc*p* or β-D-Gal*p* group is linked to O-3 of the β-D-Glc*p*NAc residue. Furthermore, the precise location of the α-L-Fuc*p*-(1→3) H_3-6 resonance suggested its attachment to the internal GlcNAc III residue, rather than penultimate GlcNAc V residue, since in the latter case a chemical shift of δ 1.020 ±0.001 at 328 K would be observed. Despite the small difference (0.006 p.p.m.), this resonance has been found, in all cases tested, to correlate with the relative position of α-L-Fuc*p*-(1→3) groups on a poly-*N*-acetyllactosamine chain[22]. We realize that the use of such a small effect (3 Hz) understandably invites skepticism, and we would not suggest as a general practice structural assignments based on such a criterion without supporting data. Nevertheless, we believe that the data here and in our previous publication[22] show that the difference is not artifactual, and suggests a secondary structural basis[22]. This assessment of which the D-Glc*p*NAc residue in the nLc_6 core has the α-L-Fuc-(1→3) group linked to it was confirmed by considering the alternative where this group is linked to GlcNAc V. Since α-(1→3) fucosylation of a Type 2 chain β-D-Glc*p*NAc residue does not affect the H-1 resonance of the β-D-Gal*p* residue to which the D-Glc*p*NAc residue is glycosidically linked[22], one would in this case expect the two internal β-D-Gal*p* II and IV H-1 resonances to coincide at their original positions relative to nLc_6 (**4**) at δ 4.26 p.p.m., as they do in Y_2 glycolipid[22] ($V^3FucnLc_6Cer$). On the other hand, addition of an α-L-Fuc*p*-(1→3) group to a β-D-Glc*p*NAc residue is known to induce the following chemical shift changes (for a solution in dimethyl sulfoxide–2% deuterium oxide at 328 K) for nearby residues in Type 2-chain structures[22]: for H-1 of the β-D-Glc*p*NAc residue to which it is linked, Δδ 0.060 ±0.002 p.p.m.; for H-1 of the vicinally linked β-D-Gal*p*-(1→4) residue, Δδ 0.062 ±0.005 p.p.m.; and for H-1 of the next β-D-GlcNAc residue linked (1→3) to that β-D-Gal*p* residue, Δδ 0.010–0.018 p.p.m. For an α-L-fucosyl group (1→3)-linked to GlcNAc III, addition of these shifts to those given in Table I for compound **6** would give, for H-1 of GlcNAc III, δ 4.716; for that of GlcNAc V, δ 4.701–4.719; and for that of Gal IV, δ 4.326. The values calculated for the two β-D-Glc*p*NAc H-1 resonances are sufficiently close to the experimental values to permit assignment as shown in Fig. 6 and Table IV. The value calculated for Gal IV H-1 is also reasonably close to the experimental value.

The structure is consistent with all data previously obtained[5]. In addition, the direct-probe, electron-impact mass spectrum of the permethylated compound (not shown) was characterized by an abundant ion pair *m/z* 825 and 793 representing the terminal sequence NeuAc→Hex→HexNAc→. Finally, the ^{1}H-n.m.r. spectrum of sialidase-treated **10** was found to be consistent with the expected asialo structure $III^3FucnLc_6$ and distinct from that of $V^3FucnLc_6$[22].

Ceramide of Fraction 6C glycolipid (**10**). — The ^{1}H-n.m.r. spectra of the two subfractions of 6C revealed some important structural information about the ceramide components of each. In the spectra of the upper band (Figs. 6A, B, and C), the near disappearance of the sphingosine *trans*-vinyl proton signals at δ 5.36 and 5.57, and the concomitant appearance of the major R-1b signal at δ 4.065 (δ

4.083 at 328 K) showed that the long-chain base is predominantly phytosphingosine[55]. Some degree of unsaturation of the fatty acids was indicated by the *cis*-vinyl proton signal at δ 5.321 and the *cis*-allyl proton signal at δ 1.977. The absence of any signal between δ 2.0 and 2.2 indicated that the fatty acids have a high proportion of α-hydroxylation, since the α-proton normally resonates in this region[12]. A similar ceramide ^{1}H-n.m.r. spectrum was recorded for GM_2 of mullet roe[55], although the degree of unsaturation of fatty acids was higher for that glycolipid. Previously, X-reactive (pentaglycosyl)ceramide isolated from adenocarcinoma was found to contain phytosphingosine almost exclusively, along with a high proportion of α-hydroxy fatty acids[56]. The spectra of di- and tri-meric X glycolipids from the same source also showed phytosphingosine signals, and the fatty acid analysis similarly revealed the presence of large proportions of α-hydroxy fatty acids[25]. This appears to be a rather general characteristic of the majority of adenocarcinoma fucolipids identified so far. Finally, the lower band could be seen (Fig. 6D) to contain a larger proportion of normal sphingosine species (R-1b at δ 3.925 at 328 K, and R-4 and 5 signals of greater intensity), along with a virtual absence of unsaturated fatty acid. The signal at δ 4.010 was assigned to R-3 of unsaturated sphingosine in combination with α-hydroxy fatty acid[12,16,55,57]. These conclusions were supported by the results of ceramide analysis by g.l.c.–m.s. Thus, estimation of sphingosines as long-chain aldehydes gave, for the upper band, 75% of C_{18} phytosphingosine (15:0 aldehyde), 25% of C_{18} sphingosine (16:1 aldehyde), and a trace of C_{20} phytosphingosine (17:0 aldehyde). For the lower band, the analysis gave 40% of C_{18} phytosphingosine and 60% of C_{18} sphingosine. Fatty acid analysis showed the upper band to contain 80% of α-hydroxy fatty acids, almost exclusively as long-chain compounds (22:1, 22:0, 24:1, and 24:0). The lower band yielded 42% of α-hydroxy fatty acids, almost exclusively short-chain compounds (16:0). The variation of the shift position for β-D-Glc H-1 and H-2 (Table IV)) showed that these nuclei are sensitive indicators of the differences in ceramide composition[16,57].

Structural basis for accumulation of Fraction 6C glycolipid (**10**). — The Fraction 6C glycolipid possesses a unique and exemplary structure. Whereas some colonic and liver adenocarcinomata are apparently characterized by induction of abnormal elevations of both α-NeuAc-(2→6)- and α-L-Fuc-(1→3)-transferases, the products that one might expect from the fucosylation of GlcNAc V, in addition to or instead of GlcNAc III, have not been found. The terminal tetrasaccharide structure, α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-, (sialosyl-X) has been found[5,8] in glycolipids of the same adenocarcinoma as that of Fraction 6C, in glycolipids of kidney[58], and in a variety of glycopeptide sources[59–61]. Thus, although cases are known where previous attachment of the α-L-Fuc*p*-(1→3) group will prevent α-(2→3) sialylation[62,63], the sialosyl-X structure can apparently be created by attachment of these sugars in reverse order. By contrast, the isomeric structure, α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3), has never been found. Paulson *et al.*[64] have demonstrated the mutually

exclusive glycosylation by α-NeuAc-(2→6)- and α-L-fucosyl-(1→3)-transferases of asparagine-linked oligosaccharides. In our laboratory, the same phenomenon has been observed with α-L-fuc-(1→3)-transferase (of human lung carcinoma) on α-NeuAc-(2→6)- and α-NeuAc-(2→3)-nLc$_6$Cer substrates[63]. Whereas the latter was observed to incorporate two fucosyl groups, the former incorporated only one to produce glycolipid 6C. Glycolipid 6C itself was not further fucosylated under the experimental conditions*.

The stereochemical model supported by n.m.r. data may shed some light on the structural basis for these phenomena. In Scheme 2, the carbohydrate structure of the ganglioside 6C is depicted by residues I–VI and groups B and F. The point of attachment for an incoming α-L-Fuc*p*-(1→3) group to GlcNAc V is illustrated by an arrow. It can be seen that, even in the absence of the proposed hydrogen-bond, the α-NeuAc-(2→6) group (B) could represent a barrier to the fucosyl group. The proposed preferred conformation would require the α-NeuAc-(2→6) and α-L-Fuc*p*-(1→3) groups to overlap in space in a hypothetical molecule containing both groups. It is apparent, on the other hand, that an α-NeuAc-(2→3) group (A) would offer no steric hindrance to the fucosylation of GlcNAc V, although it has been observed that previous attachment of the α-L-Fuc*p*-(1→3) group would prevent α-(2→3) as well as α-(2→6) sialosylation[62–64].

In this study, we have attempted to establish a body of ^{1}H-n.m.r. data useful for the systematic charaterization of glycolipids containing α-NeuAc groups (2→6) as well as (2→3)-linked to the terminal β-D-Gal*p* residue of Type 2-chain *lacto*-series core structures. Although an extensive compilation of data exists for such structures found in glycopeptides and free oligosaccharides (see for example refs. 41 and 42), the use of a different solvent[18] (dimethyl sulfoxide *vs.* deuterium oxide) would appear to call for additional data. We have demonstrated, in this series, the occurrence of chemical-shift correlations and systematic trends in glycosidation-induced shift changes which meet the criteria for useful primary structural-reporter groups[41]. Because of their regular repeating β-D-(1→3*e*)–β-D-(1→4*e*) structure, Type 2-chain oligosaccharides form an extended helix[66,67] on which terminal and side-chain substituents have effects confined locally (within three residues) and for which, therefore, the application of systematic chemical shift data is particularly useful. This applies also to branched structures, which have been shown to exhibit some specific correlations of their own[9,15,33]. In addition, in applying 2D n.m.r. methods to the simpler and more abundant analogs available, we have collected additional data consistent with previously proposed stereochemical models[48,49,53]. These provide not only a working three-dimensional structural hypothesis for the n.m.r. data, but also a basis for rationalizing the accumulation of certain oligosaccharide structures that reflects experimentally established specificities of

*The recent report[65] of a glycolipid having structure **12** (VI^{3}NeuAcIII^{3}FucnLc$_6$Cer) isomeric to glycolipid 6C) from chronic myelogenous leukemia cells may imply at least one case of inhibition of α-L-(1→3)-fucosylation of GlcNAc by previous α-(2→3)-sialosylation of the β-D-Gal*p* residue (1→4)-linked to it.

glycosyltransferases as obtained in several systems[63,64]. It is worth emphasizing that, in general, we agree with the proposition that the shift effects observed in this system are too small to support by themselves a detailed conformational model[52] (*i.e.*, glycoside torsion-angles specifying the preferred position of the α-(2→6)-linked NeuAc group). Only precise quantitative n.O.e. data or interresidue hetero-nuclear-coupling constants (or both) could provide the necessary desiderata. None of the n.m.r. data presented so far, here or elsewhere, categorically prove or exclude the existence of a hydrogen bond between the α-NeuAc-(2→6) carboxyl group and the nearby β-D-Glc*p*NAc HO-3 which could control the conformation of these sugars. Nevertheless, the grosser aspects of the stereochemistry are supported by ^{13}C- and ^{1}H-n.m.r. data[42,48,49,53], HSEA calculations[53], and enzyme studies[62–64]. In addition, although the argument may continue over the limit at which a change in chemical shift is a useful indicator specifying conformation, the usefulness of a chemical shift difference in specifying *primary* structure is determined more by its reproducibility than its magnitude. By this criterion, even the difference of 0.006 p.p.m. noted for some α-L-Fuc*p*-(1→3) H_3-6 resonances can be useful, providing the conclusion is consistent with whatever else is known about the structure. Furthermore, the small magnitude need not necessarily preclude some logical speculation on the source of the effect. This may at least suggest a hypothesis that can be tested by some other method.

Using the data collected for the present study, we have (a) confirmed the structure of two *lacto*-series gangliosides found in polymorphonuclear neutrophils[3,4] without the need for degradative studies (see Figs. 3 and 4); (b) confirmed details of the structure of the ganglioside G8 previously proposed only on the basis of analogy, but never established by chemical proof[1]; and (c) confirmed the structure of an adenocarcinoma fucoganglioside **10** (6C), particularly demonstrating the usefulness of 1D n.m.r. data in drawing conclusions about primary structure without resorting to numerous degradative experiments. In addition, we were able to identify solely on the basis of its ^{1}H-n.m.r. spectrum a second fraction of 6C not previously characterized and establish approximately the differences in ceramide structure between the two.

Frequently, significant glycolipids from cultured cells, tumors, or normal tissue samples are available only in small quantities (~100 μg). Thus, it has been necessary to develop methods of structural identification that are rapid and efficient in their use of material, in order to conserve material for other purposes, *i.e.*, production and screening of monoclonal antibodies. Methylation–hydrolysis–g.l.c.–m.s. can now routinely be done on 20–30 μg of material; if ^{1}H-n.m.r. observations are done first, this procedure could make further degradative procedures, *i.e.*, enzyme treatments, Smith degradation, desialosylation, defucosylation, etc., unnecessary. If an extensive data library is available, ^{1}H-n.m.r. data alone may be sufficient in certain cases.

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NOTE ADDED IN PROOF

Since the submission of this paper, a structural characterization of several *neolacto*-series gangliosides by methods including 1D ^{1}H-n.m.r. has been published (T. TAKI, K. MATSUMOTO, K. YAMAMOTO, T. MATSUBARA, A. HAYASHI, T. ABE, AND M. MATSUMOTO, *Lipids,* 23 (1988) 192–198). In addition, at least two extensive discussions concerning the application of 2D n.m.r. methods to glycoconjugate structure elucidation have become available, which interested readers are advised to consult (T. A. W. KOERNER, J. H. PRESTEGARD, AND R. K. YU, *Methods Enzymol.,* 138 (1987) 38–59; J. DABROWSKI, *Methods Stereochem. Analysis,* 9 [Two Dimensional N.M.R. Spectroscopy] (1987) 349–386).

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