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^{1}H-N.M.R. ANALYSIS OF GLYCOLIPIDS POSSESSING MONO- AND MULTI-MERIC X AND Y HAPTENS: CHARACTERIZATION OF TWO NOVEL EXTENDED Y STRUCTURES FROM HUMAN ADENOCARCINOMA*,§

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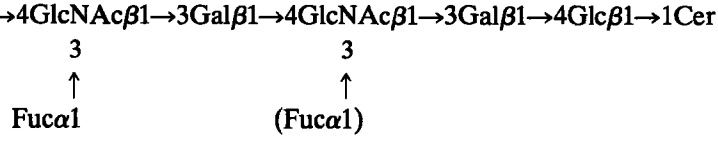
ABSTRACT

Analysis of glycolipids having repeating core-structures of type 2 chains ([Galβ1→4GlcNAcβ1→3]$_n$Galβ1→4Glcβ1→1Cer) by 1- and 2-dimensional high resolution ^{1}H-n.m.r. spectroscopy shows that fucosylation α1→3 to GlcNAc with or without fucosylation α1→2 to Gal produces predictable chemical shifts of anomeric protons and systematic, glycosylation-induced shift changes. These effects were analyzed for a series of glycolipids of known structure bearing mono- and multimeric X [Galβ1→4(Fucα1→3)GlcNAcβ1→3] and Y [Fucα1→2Galβ1→4-(Fucα1→3)GlcNAcβ1→3] determinants, and were subsequently used to elucidate the primary saccharide structure of two novel glycolipids isolated from human liver adenocarcinoma. They are proposed to be Y determinants carried on a norhexaosylceramide core, with the following structures:



INTRODUCTION

Studies of glycolipids of human cancer tissues have established that accumulation of highly fucosylated, extended polylactosamine (repeating *N*-acetyllactosamine) is highly characteristic for certain types of human cancers[1–7]. Although

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glycolipids having terminal X and Y structures ([Fuc*α1→2]Galβ1→4[Fucα1→3]-GlcNAcβ1→3) have been isolated from blood, serum, and some normal tissue, such as intestine[8–13], the degree of fucosylation (particularly at the GlcNAc III residue) and chain elongation[4,6,7,14] and significant accumulation[4,6,14,15] appear only in cancers and at certain stages of fetal organogenesis. Thus, malignant transformation may involve an increase in the amount or activity of the fucosyltransferase responsible[16], with a concomitant disruption of the normal cellular mechanisms for chain termination and branching[1,2]. These poly-X structure are associated also with a unique ceramide profile consisting of increased 2-hydroxy fatty acids and phytosphingosines[4,6,15].

Although monoclonal antibodies directed to X- and Y-terminated structures will react with a variety of normal and malignant tissues[8,17–22], antibodies directed to internal or multimeric epitopes are expected to provide much more-specific tools for immunohistochemistry and diagnosis[1,2]. Thus, we have given high priority to the isolation and characterization of glycolipids possessing such structures, with a view towards production of monoclonal antibodies directed to them[4–7]. In this effort, we have made increasing use of ^{1}H-n.m.r. spectroscopy. The inherent insensitivity of this technique (relative to mass spectrometry) is counterbalanced by its nondestructiveness and the greater amount of information obtained. This is fortunate, as the increasing size of the compounds of interest presents greater problems for isolating a homogeneous carbohydrate moiety in sufficient quantity for analysis and subsequent production and screening of monoclonal antibodies.

There is available a library of data sufficient for distinguishing X structures found on oligosaccharides and glycopeptides in D_2O[23], and more or less complete spectral assignments of the synthetic terminal tri- and tetra-saccharides of both the X and Y haptens[24] as well as for their positional isomers[25] Le^a and Le^b, sufficient for comparison of some secondary features in addition to primary structures. However, this does not constitute a convenient "data base" for recognition of such structures in Me_2SO, which is the solvent of choice for glycolipids[26]. This investigation was undertaken to provide a body of data useful for structure determination of glycolipids, and to demonstrate that glycolipids having single or repeating X haptens are particularly apt subjects for analysis by ^{1}H-n.m.r. spectroscopy because their regular structures reproducibly yield highly characteristic, identifiable proton resonances[4,27]. In addition, we have applied this method to the preliminary structural identification of two novel glycolipids, isolated from human adenocarcinoma, possessing Y haptens carried on extended type 2 chain-cores, with and without internal fucosylation.

RESULTS AND DISCUSSION

X-Pentaglycosyl ceramide (**2**). — The 1-D ^{1}H-n.m.r. spectrum of **2** (Fig. 1)

*This group may be present or absent.

illustrates some of the typical features associated with this series of compounds. The single α-anomeric signal (4.876 p.p.m.; $^3J_{1,2}$ 4.3 Hz) is obviously assignable to the Fucα1→3 residue, as is the characteristic broadened H-5 quartet at 4.590 p.p.m. coupled to the methyl doublet at 1.020 p.p.m. ($^3J_{5,6}$ 6.7; $^3J_{4,5}$ 1.5 Hz). Logically, one would assign the highest frequency β-anomeric resonance (4.778 p.p.m.; $^3J_{1,2}$ 7.9 Hz) to the GlcNAc residue, and the lowest (4.206 p.p.m.) to the Glc residue. This last assignment may be confirmed by decoupling from the Glc H-2 triplet at 3.048 p.p.m. ($^3J_{1,2}$ 7.9 Hz). This leaves the two remaining β-anomeric resonances (4.280 and 4.295 p.p.m.; $^3J_{1,2}$ 7.3 Hz) to be assigned to the Gal residues found in the X glycolipid. The assignment of the latter to the terminal Galβ1→4 unit is prompted by the following: (*a*) H-1 of Gal II typically resonates in the range of 2.6–2.8 p.p.m. for glycolipids of the lacto- and neolacto-series with three or more sugars[28]; (*b*) in the spectrum of Y hexaglycosylceramide (**9**), which is a Fucα1→2 to Gal IV analog of X (**2**), there is found a β-doublet at 4.276 p.p.m., while the resonance at 4.295 p.p.m. has been replaced by one at 4.404 p.p.m. indicating that this belongs to Gal IV (see subsequent section); (*c*) some additional evidence is provided by the distorted lineshape of the H-1 resonance at 4.280 p.p.m., which is suggestive of "virtual coupling" through near coincidence of the adjacent H-2 and H-3 chemical shifts. The data reported[29] for Le^a indeed show identical shifts for H-2 and H-3 of Gal II (although no reference is made to lineshapes); thus, 3,4-disubstitution of GlcNAc III, while having virtually no effect on H-1 of Gal II, does appear to have an effect on other protons of that residue (see next section). Be-

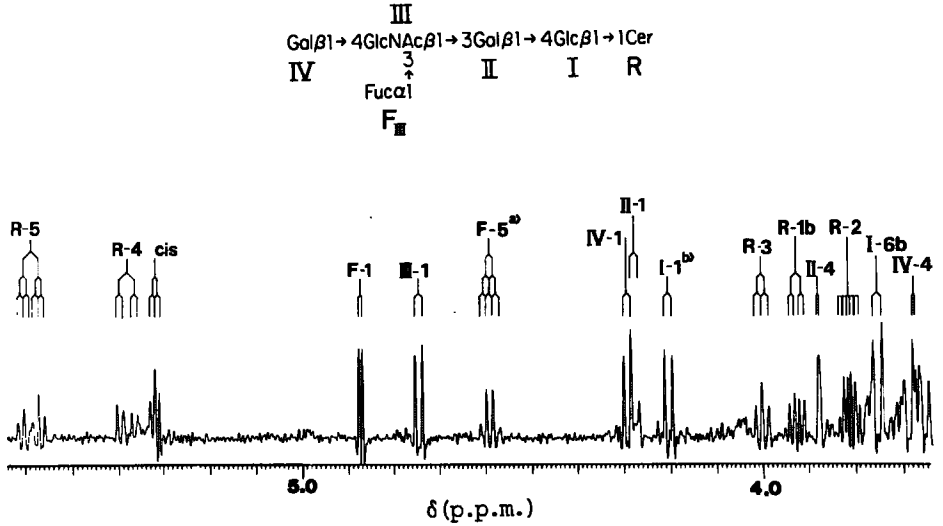

Fig. 1. Downfield region of resolution-enhanced 500 MHz ^{1}H-n.m.r.-spectrum of X-pentaglycosylceramide (**2**, III^{3}FucnLcCer) at 328 ±2K; conc. = mM in dimethyl sulfoxide-d_6–2% D_2O + 1% tetramethylsilane as chemical-shift reference; 100 f.i.d.s were accumulated. Arabic numerals refer to ring protons of residues designated by roman numerals in the corresponding structure; (*a*) confirmed by decoupling from CH_3 of Fuc at 1.020 p.p.m. (*b*) confirmed by decoupling from H-2 of Glc at 3.048 p.p.m. Resonances assigned as in text and confirmed by PS-COSY (see Fig. 2).

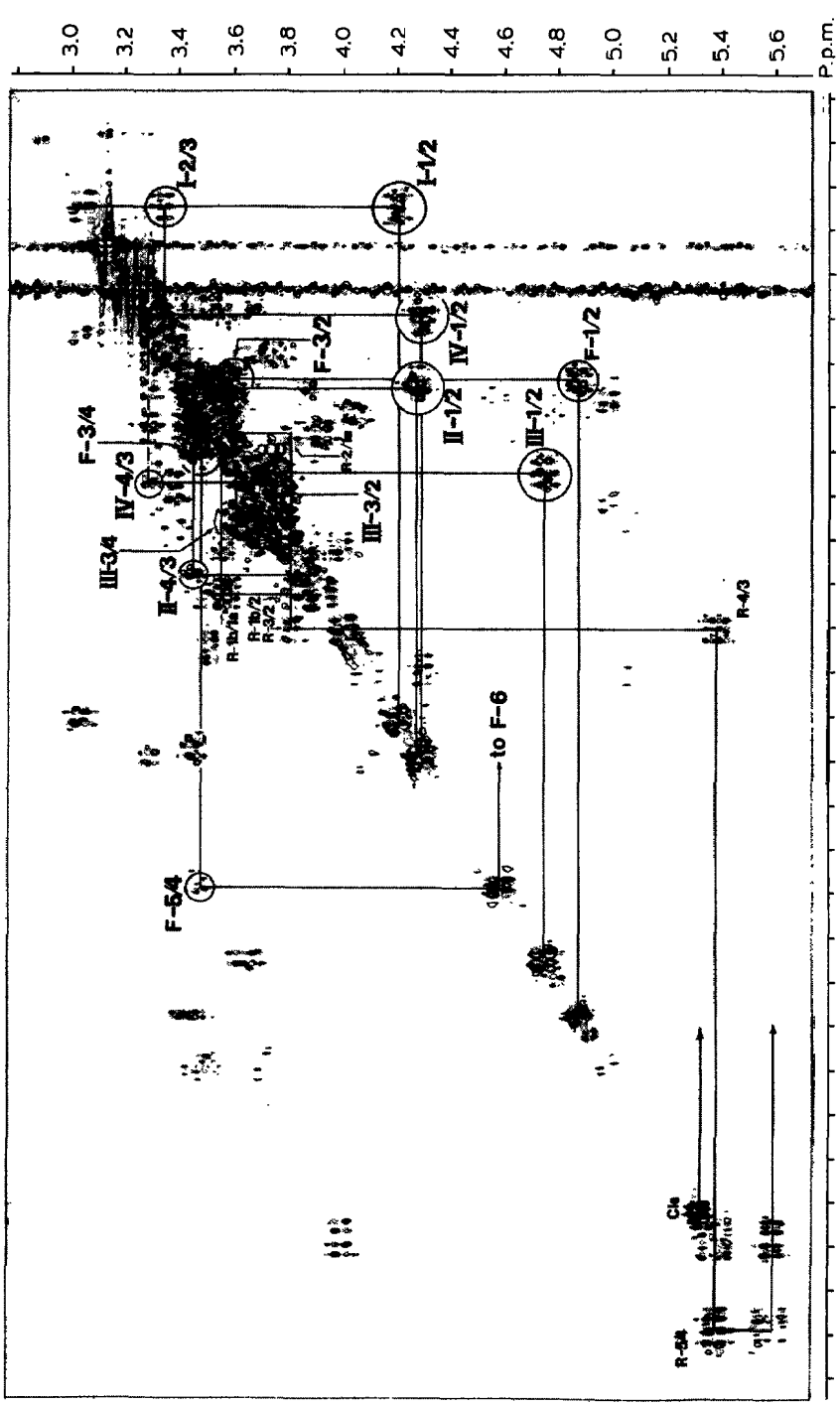

Fig. 2. Downfield region of phase-sensitive ^{1}H-COSY spectrum of X-pentaglycosylceramide taken at 300 MHz. Sample and conditions as given for Fig. 1. In the original spectrum, opposite-signed peaks were plotted in blue and red. The blue contours are reproduced somewhat lighter in this figure. The chemical-shift scale is not identical to that in Fig. 1.

cause of the expected conformational similarity of the terminal region of the Le^a and X haptens (the β-Gal and α-Fuc residues disposed on vicinal equatorial sites 3 and 4 of the β-GlcNAc residue), it is not surprising to find H-1 of Gal IV shifted downfield from its position in nLc$_4$Cer (**1**) to a position similar to that found[29] for Le^a. However, the NAc carbonyl group of the 2-deoxy-2-acetamido sugar to which they are linked has the effect of shifting H-1 of the α-Fuc to higher frequency in X relative to Le^a, while H-1 of β-Gal IV is found at slightly higher frequency in Le^a than in X. As is generally observed, the shift resulting from the conversion of type 1 chain Lc$_4$Cer to III^{4}FucLc$_4$Cer (Le^a) is greater than that found for the type 2 chain, nLc$_4$Cer (**1**) → III^{3}FucnLc$_4$Cer (**2**) conversion.

Other features that one might expect to stand out on inspection of glycolipid ^{1}H-n.m.r. spectra are galactosyl H-4 resonances, which, because of their equatorial orientation (which also gives[30] small vicinal couplings, $^3J_{3,4}$ 3 and $^3J_{4,5}$ 1 Hz), are characteristically seen at higher frequency compared to the bulk of axial non-anomeric proton signals, and as distinct, narrow doublets[26]. In glycolipids of the lacto- and neolacto-series, such a signal is commonly found at ~3.85–3.87 p.p.m. for H-4 of Gal II, due to the influence of the β1→3-linked GlcNAc. In the spectrum of **2**, there is a tall 1-proton signal at 3.881 p.p.m., which is close to the 3.87 p.p.m. reported for Le^a. Most often, with appropriate resolution enhancement, the $^3J_{3,4}$ splitting may be clearly discerned; for this resonance, such is not the case. However, if one again invokes the "virtual coupling" phenomenon, it may be taken as evidence that this H-4 is on the same ring as the H-1 at 4.280 p.p.m. Concerning the other Gal H-4 signal expected, Gal IV-4 of Le^a is reported[29] at 3.66 p.p.m.; no distinct resonance for X glycolipid is discernible at this temperature in the 1-D spectrum.

The phase-sensitive COSY spectrum of X-pentaglycosylceramide shown in Fig. 2 extends considerably the range and reliability of assignments that can be made. For example, galactosyl H-4/3 off-diagonal peaks appear to have a characteristic pattern, which makes it possible to locate H-3 even when the adjacent H-2 is too close in chemical shift for a corresponding H-2/3 cross peak to be seen. The Gal IV H-4/3 cross peak precisely locates H-4, as well, in a position not obvious from the 1-D spectrum (see Fig. 1). The chemical shift is close to that found for H-4 of Gal IV in Le^a. Separately tracing the connectivities for Gal II from H-4 and from H-1 confirms the chemical-shift equivalence of H-2 and H-3 in that residue, which was first suggested as the source of the distorted lineshapes for both H-4 and H-1. The connectivities for the fucosyl residue can similarly be traced from both ends of the extended spin-system.

The phase-selective detection mode used for the COSY spectrum provides a number of advantages over the usual COSY spectra, which produce a magnitude-spectrum representation. In PS-COSY, multiplet structure is preserved in the cross-peaks as a set of peaks of opposite sign separated by J-value multiples. As a result, specific types of cross-peaks may be readily recognized. This feature is particularly useful in moderately crowded areas of the spectrum. In very crowded areas contain-

ing multiple cross peaks close to the diagonal (such as those found between 3.4–3.6 and 3.6–3.8 on this spectrum), the mutual cancellation of oppositely-signed peaks occurs. In addition, the weak coupling between H-4 and H-5 of (NAc)galactosyl residues, and the weak H-5–H-6a coupling of (NAc)glucosyl residues, make it difficult to complete assignments of all spin systems, even though some H-5, H-6a, and H-6b cross peaks are clearly distinguishable in these region. These problems should be overcome by (*i*) going to higher fields; (*ii*) using pulse programs designed to suppress the diagonal (double quantum filters); (*iii*) relayed coherence transfer; or (*iv*) by tuning for smaller *J*-values.

Table I summarizes the chemical-shift data for X-pentaglycosylceramide. Direct comparison with the data of Dabrowski *et al.*[29] for Le^a is somewhat obscured by the temperature difference; allowing for this, there are enough shift similarities to argue for limited conformational homology, while most of the shift differences (for instance, for H-1 of α-Fuc) can probably be ascribed to proximity to the anisotropic NAc carbonyl group. Thus, the pronounced similarity of, for example, the α-Fuc H-5 chemical shifts (at the higher temperature reported by Dabrowski *et al.*[29], this resonance would be more upfield, almost coinciding with the same proton in Le^a), can be taken as an indication that the dispositions of the α-Fuc and β-Gal IV relative to each other are nearly the same in X as in Le^a under these conditions. This has been proposed previously by Lemieux and co-workers, based on hard sphere, exo-anomeric[31] calculations and ^{1}H- and ^{13}C-n.m.r. data[32] for the terminal trisaccharides in D_2O. The hard-sphere, exo-anomeric calculations show that the minimum-energy conformation of the X-trisaccharide has the α-Fuc and β-Gal residues directed at interglycosidic angles ϕ and ψ relative to the β-GlcNAc ring which are the same as those angles found for the minimum-energy conformation of the Le^a-trisaccharide[32]. Molecular modeling showed that H-5 of α-Fuc, which resonates in both cases at a rather high frequency, is deshielded in the former case by close proximity to O-4 of GlcNAc and O-5 of Gal (see Fig. 3) and in the latter case by proximity[24,25] to O-3 of GlcNAc and O-5 of Gal. In this arrangement, one would expect this proton to be particularly sensitive to conformational twisting of

TABLE I

GLYCOSYL CHEMICAL-SHIFTS (p.p.m. FROM TETRAMETHYLSILANE) AND $^3J_{1,2}$ COUPLING-CONSTANTS (Hz) FOR X PENTAOSYLCERAMIDE (**2**) IN DIMETHYL SULFOXIDE-d_6 AT 328 ±2K.

Parameter	*Galβ1* ⟶ *4GlcNacβ1* ⟶ *3Galβ1* ⟶ *4Glcβ1*⟶*1Cer*				
		Fucα1 ⟶ *3*			
H-1	4.295	4.4876	4.748	4.280	4.206
$J_{1,2}$	(7.3)	(4.3)	(7.9)	(7.3)	(7.9)
H-2	3.294	3.436	3.653	3.459	3.048
H-3	3.283	3.608	(3.760)[a]	3.458	3.350
H-4	3.672	3.482	(3.627)[a]	3.881	[b]

[a]Tentative assignment. [b]Not determined.

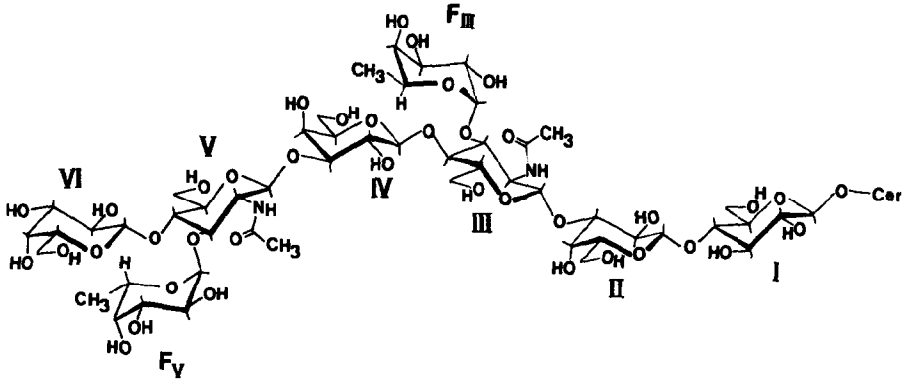

Fig. 3. Structure of III^{3}V^{3}Fuc$_2$nLc$_6$Cer (**6**), showing approximate disposition of core oligosaccharide and substituents. Relative conformation of the Fucα1→3 residues (F) adapted from Hindsgaul *et al.*[24]. The detailed conformation of the nLc$_6$ backbone is not depicted by this drawing.

the two terminal residues. The nearly identical shifts of H-5 of Fuc found for the synthetic X and Le^a trisaccharides in D_2O were taken as evidence supporting the calculated steric equivalence of residues disposed vicinally on the diaxial GlcNAc O-3–C-3–C-4–O-4 bond-framework[32]. The same result is found here for the ceramide pentasaccharides in Me_2SO. One could venture that an antibody able to distinguish between the two isomers would have to include, at a minimum, some other part of the GlcNAc residue in its epitope.

The large shift-difference found for H-1 of α-Fuc between X and Le^a is particularly helpful for qualitative analysis of these two positional isomers, as they co-migrate in many chromatographic systems and produce identical substituted sugar-derivatives in methylation analysis by g.l.c.–m.s. Although certain ions in the direct-probe mass spectra of their intact permethylated derivatives have been used to distinguish between the two[13,33–35], their frequent occurrence as mixtures can make such an analysis ambiguous. Thus, ^{1}H-n.m.r. is a reliable method for characterizing these glycolipids. The following section will demonstrate that application can be extended to large, multiply substituted glycolipids of the neolacto-series.

Complex analogs bearing one or more X-haptens. — Tables II and III summarize chemical-shift data for glycolipids of the neolacto-series. The first half (compounds **1–8**) includes five natural and modified analogs of X-pentaosylceramide (**2**). Examination of these data reveals the following systematic spectral correlations: (*a*) H-1 of Fucα1→3 linked to GlcNAc resonates at a characteristic frequency (4.877 ±0.002 p.p.m.) clearly distinguishable from other linkages (*i.e.*, Fucα1→4 in type 1 chain), and was insensitive to fucosyl substitutions elsewhere in the chain. It appeared also to be unaffected by NeuNAcα2→3 substitution at terminal Gal (see compound **8**). (*b*) H-1 of GlcNAc to which it is linked is shifted to higher frequency ($\Delta\delta$ = 0.060 ±0.002 p.p.m.); H-1 of the vicinally linked Galβ1→4 residue likewise is shifted ($\Delta\delta$ = 0.062 ±0.004 p.p.m.) regardless of whether it is terminal or internal. (*c*) Interestingly, as may be seen from the norhexaosyl-

TABLE II

CHEMICAL SHIFTS (p.p.m. FROM TETRAMETHYLSILANE) OF GLYCOSYL H-1 AND $^3J_{1,2}$ COUPLING-CONSTANTS (Hz) OF GLYCOLIPIDS IN DIMETHYL SULFOXIDE-d_6 AT 328 ±2K

Compound	*Fucα1→2*	*Galβ1→2*	*Fucα1→3*	*4GlcNAcβ1→3Galβ1→4*		*Fucα1→3*	*4GlcNAcβ1→3Galβ1→4*		*4Glcβ1→1R*
1		4.229(7.3)		4.687(7.9)				4.276(7.3)	4.173(7.9)
2		4.295(7.3)	4.876(4.3)	4.748(7.9)				4.280(7.3)	4.206(7.9)[a]
3		4.228(7.3)		4.682(8.5)	4.280(7.9)		4.682(8.5)	4.280(7.9)	4.172(7.9)
4		4.231(7.3)		4.710(8.5)	4.342(7.3)	4.875(7.9)	4.748(7.9)	4.279(7.3)	4.221(7.9)
5		4.291(7.3)	4.876(4.3)	4.744(7.9)	4.280(7.3)		4.681(8.5)	4.280(7.3)	4.172(7.9)
6 ($n = 1$)		4.300(7.3)	$4.878(3.7)_n$	$4.754(7.9)_n$	$4.345(6.7)_n$	4.878(3.7)	4.741(7.9)	4.276(7.3)	4.207(7.9)[a]
7 ($n = 2$)		4.299(7.3)	$4.878(3.7)_n$	$4.756(7.9)_n$	$4.346(6.7)_n$	4.878(3.7)	4.742(7.9)	4.276(7.3)	4.221(7.9)
8 (NeuNAcα2→3)		4.301(7.3)	4.875(3.7)	4.726(7.9)	4.345(6.7)	4.875(3.7)	4.739(7.9)	4.276(7.9)	4.222(7.9)[a]
9	5.045(2.5)	4.348(7.9)		4.662(8.5)				4.276(7.3)	4.174(7.9)
10	4.973(4.3)	4.404(6.7)	4.875(3.7)	4.712(7.9)				4.276(7.3)	4.223(7.9)[a]
11	5.044(2.5)	4.346(7.9)		4.659(8.5)	4.276(7.9)		4.679(8.5)	4.276(7.9)	4.176(7.3)
12	4.973(3.7)	4.401(7.3)	4.876(4.3)	4.714(7.3)	4.285(7.3)		4.684(7.9)	4.276(7.3)	4.172(7.9)
13	4.973(3.7)	4.407(6.7)	4.876(4.3)	4.728(7.3)	4.343(6.7)	4.876(4.3)	4.743(7.9)	4.279(7.9)	4.172(7.9)

[a]Predominant resonance only; more than one occur because of ceramide variants.

TABLE III

CHEMICAL SHIFTS[a] (p.p.m. FROM TETRAMETHYLSILANE) OF FUCOSE H-5 AND H-6 FOR GLYCOLIPIDS IN DIMETHYL SULFOXIDE-d_6 AT 328 ±2K

Compound	*Fucα1→2*		*Fucα1→3*		*Fucα1→3*	
	H-5	*H-6*	*H-5*	*H-6*	*H-5*	*H-6*
2			4.590	1.020		
4					4.588	1.013
5			4.589	1.020		
6 ($n = 1$)			4.604_n	1.020	4.592	1.015_n
7 ($n = 2$)			4.604_n	1.021	4.592	1.016_n
8			4.631	1.003	4.586	1.015
9	4.016	1.080				
10	4.027	1.103	4.644	1.060		
11	4.016	1.081				
12	4.027	1.103	4.640	1.072		
13	4.025	1.101	4.653	1.072	4.586	1.014

[a]All $^3J_{5,6}$ 6.7 Hz. All H-5, H-6 connectivities established by selective decoupling experiments.

ceramide series (**3–6**), H-1 of GlcNAc V showed a shift to higher frequency on fucosylation at GlcNAc III. This shift was somewhat larger for the conversion **3→4** ($\Delta\delta = 0.018$ p.p.m.) than for compounds already bearing fucose at GlcNAc V: *i.e.*, for **5→6** and **10→11** (see next section), $\Delta\delta = 0.012 \pm 0.002$ p.p.m. This means that the assignments of GlcNAc H-1 resonances given in ref. 4 are incorrect and should be reversed, as shown in Table II. Compound **7**, consistent with this scheme, had two protons resonating at the higher frequency *vs.* one at the lower, for β-GlcNAc VII + V, and III, respectively. (*d*) A small but measurable difference was found between CH_3 shifts of Fuc depending on substitution position at the "outer" (1.020 ±0.001 p.p.m.) or "inner"* (1.014 ±0.002 p.p.m.) GlcNAc for all neutral compounds **1–7**. These two different resonance positions are clearly discernible in the spectra of compounds **6** and **7** (Table III; these differences are not apparent in the n.m.r. figures of ref. 4). As compounds **2** and **5** have 1.020 p.p.m. (Fucα1→3GlcNAc III and V, respectively) and **4** has only 1.013 p.p.m. (Fucα1→3GlcNAc III), they are assigned as shown in Table III for compounds **6** and **7**, the latter having two "inner" methyl groups at 1.015 p.p.m. This small shielding-effect on the CH_3 of Fucα1→3GlcNAc III in the neutral compounds **4** and **6** may be rationalized by placement of the CH_3 within the shielding zone of the carbonyl group of GlcNAc V NAc . Construction of a physical model of compound **6**, using interglycosidic bond angles ϕ and ψ that approximate the values calculated by Bock, Lemieux, Thøgersen and associates for X-trisaccharide[32] and the

*The terms "outer" and "inner" are used in this paper to denote substitution on GlcNAc residues located toward the nonreducing and reducing end, respectively, of the oligosaccharide chain.

GlcNAcβ1→3Gal disaccharide[36], shows that the neolacto core-oligosaccharide (Galβ1→4GlcNAcβ1→3)$_n$ has a helical structure with each alternating GlcNAc β1→3 residue (and any side-chain substituents) offset 90–110° out of plane from the previous one as the chain is built towards the non-reducing end. These parameters make it quite possible for the methyl group protons of Fucα1→3GlcNAc III to approach to within 3–5 Å of the center of the NAc C=O bond attached to GlcNAc V (see Fig. 3). Ascribing the effect to the carbonyl group of GlcNAc NAc explains why the outer CH_3 of Fucα1→3GlcNAc V of **6** should have the same shift as that of Fucα1→3GlcNAc III of **2**, and why **7** should have two shielded CH_3 resonances. These small effects would scarcely be noticeable were it not for the occurrence of discrete chemical-shift positions for Fuc CH_3 in the spectra of single carbohydrate moieties (**6** and **7**), which rules out artifacts due to variations in temperature and concentration between samples. But the argument that has ensued over the significance of such small (<0.02 p.p.m.) shift-effects[29,37] suggests that it is advisable not to ascribe too much significance to these. (It is possible, an alternative and less conformationally informative explanation, that the α-Fuc CH_3 protons experience some shielding by interaction with the vicinally-linked β-Gal residue which undergoes some distortion upon substitution at O-3 of that unit.) Nevertheless, a more refined knowledge of the conformational properties of these structures, and of the anisotropic shielding expected at various distances and directions from the NAc carbonyl group, may yield further information about secondary structure in this case. To this end, we expect these extended structures to be worthwhile subjects for computer-aided, molecular modeling experiments. (*e*) In contrast to the pattern of α-Fuc CH_3 resonances just described, for neutral compounds containing a single X-hapten (**2, 4, 5**) H-5 of Fucα1→3 appears at 4.588 ±0.001 p.p.m. regardless of whether it was at GlcNAc III or V. However, when two or more Fucα1→3GlcNAc residues were present (compounds **6** and **7**), their H-5 resonances were found at two distinct shifts, 4.592 p.p.m. (for "inner" Fuc) and 4.604 p.p.m. (for "outer" Fuc). The assignments of H-5 resonances, incorrectly dsepicted in ref. 4, are corrected in this work (see Table III). Further, the decoupling experiment CH_3→H-5 on compound **7** showed two of the H-5 resonances at the deshielded (outer) position and one at the normally shielded (inner) position. In other words, for Fucα1→3GlcNAc VII, the H-5 and CH_3 are both deshielded relative to the same protons on Fucα1→3GlcNAc III, while for the middle Fucα1→3GlcNAc V, H-5 is deshielded while CH_3 is shielded. This discrepancy suggests that the chemical environment of Fuc H-5 may be modified by a set of structural features (*i.e.*, lone electron-pairs or anisotropic functional groups) that are independent of those modifying the chemical environment of the adjacent methyl group, such as already discussed in (*d*). In this case, the explanation may lie in small spatial adjustments in the Galβ1→4GlcNAc linkage-regions, including O-4 of GlcNAc, to which H-5 of Fuc may be sensitive, as described in the previous section concerning X-pentaglycosylceramide. However, the mechanism whereby α1→3 fucosylation of one GlcNAc might affect the conformation of residues attached to the next GlcNAc

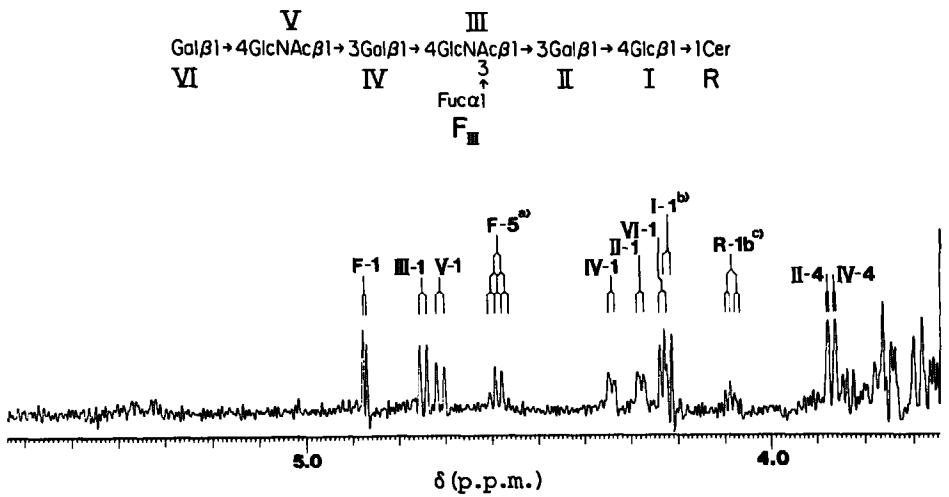

Fig. 4. Downfield region of resolution-enhanced 500-MHz ^{1}H-n.m.r. spectrum of desialylated "6C" ganglioside (**4**, III^{3}FucnLc$_6$Cer) at 328 ±2K; conc. = 0.1mM; 10,000 f.i.d.s were accumulated; (*a*) confirmed by decoupling from CH_3 of Fuc at 1.013 p.p.m.; (*b*) confirmed by decoupling from H-2 of Glc ast 3.029 p.p.m.; (*c*) unusual location for R-1b from ceramides containing phytosphingosine and 2-hydroxy fatty acid (see for example, Y.-T. Li *et al.*[40]).

down the chain is not clear. The approximate model described here in (*d*) does not indicate any direct interaction between fucose residues; a refined model based on molecular-mechanics calculations may provide insights into this effect. (*f*) The H-4 atom of β-Gal, substituted at O-3 by Galβ1→4GlcNAc only, was found at 3.866 ±0.001 p.p.m., whereas H-4 of Gal substituted at O-3 by Galβ1→4[Fucα1→3]-GlcNAcβ1 was consistently found at 3.881 ±0.001 p.p.m. (see previous section, Table I) throughout this series, integration of the two types providing verification for the number of internal Gal residues. (*g*) The NAc resonances of β-GlcNAc were found at 1.820 ±0.005 p.p.m. (not shown) and appeared to be little influenced by Fucα1→3 substitution. Integration provided verification for the number of β-GlcNAc residues.

The spectrum of compound **4** (Fig. 4) illustrates several of the features already discussed. For example, in addition to the H-4 resonance of Gal II at 3.881 p.p.m., another H-4 resonance is apparent at 3.886 p.p.m., assignable[29] to Gal IV of the nLc$_6$Cer core-structure. There are two NAc signals coincident at 1.820 p.p.m. (s, 6 H). The remainder of the glycosyl shifts determined are summarized in Tables II and III. It may be seen that the spectrum of **4** is quite distinct from that of **5** in a manner that is completely predictable. The distorted lineshape of the H-1 resonance of Gal II is expected as a result of "virtual coupling" because of equivalence of the H-2,3 shifts as discussed previously in connection with X-pentaglycosyl ceramide. The similar distortion of H-1 of Gal IV was not predicted, although its shift is (compared with compound **2** or **3**). The essential absence of signals between 5.3 and 5.6 p.p.m. shows that the ceramide sphingosine and fatty acid moieties are fully saturated. The R-1b signal at 4.082 p.p.m. shows that a high proportion of

these are constituted of phytosphingosine and 2-hydroxy fatty acids[38–40]. The ceramide composition has a measurable effect on the H-1 resonance of Glc I, which appears at the (less typical) high-frequency end of its range.

Compound **4** represents a novel, albeit artificially created structure, which would not normally be expected to occur in any significant quantity *in vivo*. Recent *in vitro* experiments have shown that processing of glycolipids of the neolacto series preferentially places Fucα1→3 residues starting at the outermost GlcNAc, unless the terminal Gal is "blocked" with a NeuNAcα2→6 residue[16]. The characterization of this type of structure (that is, as distinct from **5**) is therefore of interest.

Compound **8**, which is found together with the NeuNAcα2→6 glycosylated parent of **4** in large quantities in the monosialo fractions of adenocarcinoma glycolipids, appears to be a significant onco-developmental marker[41]. As the two gangliosides are difficult to separate, the isolation and characterization of pure **8** was fairly laborious[6,15]. Therefore, the ability to characterize such a compound with no loss of material is a significant achievement. Its n.m.r. spectrum is in fact highly confirmatory, and makes the identification of new preparations containing the same carbohydrate sequence a routine matter. The spectrum of **8** (Fig. 5), relative to **6**, shows typical[38] shielding of H-1 of GlcNAc V by NeuNAcα2→3Gal VI, as well as diagnostic resonances for H-3eq of NeuNAcα2→3 at 4.772 p.p.m. (dd, $^3J_{3eq,4}$ 5.2 Hz, $^2J_{3eq,3ax}$ −11.9 Hz), H-3ax at 1.358 (t, $^3J_{3ax,4}$ 11.5 Hz) H-3 of Gal VI at 3.980 p.p.m. (dd, $^3J_{2,3}$ 9.8 Hz, $^3J_{3,4}$ 3.4 Hz), and NAc of NeuNAc at 1.886 p.p.m. (s, 3 H)[38] [compare also[42] δ, J values for NeuNAc and Gal II of GM_3 (II^{3}NeuNAcLc$_2$Cer) at 303 K], and two distinct signals for NAc of GlcNAc at 1.820 p.p.m. (III) and 1.812 p.p.m.

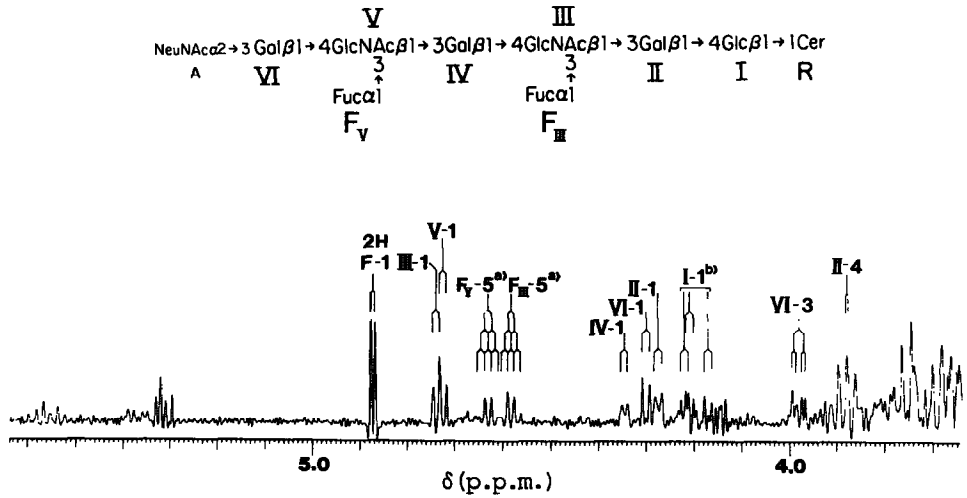

Fig. 5. Downfield region of resolution-enhanced 500 MHz ^{1}H-n.m.r. spectrum of "6B" ganglioside (**8**, VI^{3}NeuNAcIII^{3}V^{3}Fuc$_2$nLcCer) at 328 ±2K; conc. = 0.25mM; 1200 f.i.d.s were accumulated; (*a*) H-5 of III^3 and V^3 Fuc resonances confirmed by decoupling from CH_3 resonances at 1.015 and 1.003 p.p.m., respectively; (*b*) confirmed by decoupling from group of Glc H-2 resonances between 3.03–3.05 p.p.m. (decoupler centered at 3.040 p.p.m.).

(V). The data for ganglioside **8** also confirm the pattern of assignments of the Fuc H-5 and CH_3 shifts [earlier section (*e*)], as they show one set of coupled resonances relatively unshifted from those of the neutral compound **6** (assigned to Fucα1→3GlcNAc III), whereas a second set exhibits significantly shifted H-5 (to higher frequency) and CH_3 (to lower frequency) resonances (see Table III). These observations are consistent with the assumption that anisotropic groups on the NeuNAcα2→3 residue may affect protons on the "outer" Fucα1→3GlcNAc V but not those on Fucα1→3GlcNAc III, which is too far away. An expected small shielding[38] of H-1 of Gal VI (compare also[42] Gal II H-1 of GM_3 *vs.* Lc_2Cer at 303 K) was not detected (relative to compound **6**). The presence of multiple H-1 resonances for Glc demonstrates the extensive heterogeneity of ceramide moieties in this preparation.

Y-Hexaglycosylceramide (**10**). — The second halves of Tables II and III show chemical-shift data for glycolipids of the neolacto series (compounds **9**–**13**) bearing terminal Fucα1→2 to Gal structures (H or Y haptens), including Y-hexaglycosylceramide (**10**) and two putative analogs **12** and **13**. It was not practical to obtain a 2-D spectrum for **10** because of the limited quantity available. A one-dimensional spectrum at 303 K has been published[12], along with a brief comparison with that of Le^b hexaglycosylceramide. Although the spectra resulting for most of these compounds at 303 K can be interpreted, for **12** and **13** it was particularly advantageous to go to higher temperature to disperse the GlcNAc H-1 and Fuc H-5 resonances, taking advantage of the negative temperature–shift coefficients of the Fucα1→3 H-5 resonances. All compounds in this study were therefore analyzed at 328 K, to enable ready comparison for structure determination. This also gave a better comparison with the results of Dabrowski *et al.*[29], although their spectra were recorded at 338 K.

The effect of the Fucα1→2 residue on the terminal H-1 resonances of Gal and GlcNAc are roughly the same for **2**→**10** as for the conversion of **1**→**9**. Similarly, the effect of the Fucα1→3 residue on the same resonances is roughly the same for **9**→**10** as for **1**→**2**. That is, the effects are additive, in contrast to the analogous changes involving compounds having type 1 chains. Thus, use of the expression given by Dabrowski *et al.*[29] for differential chemical-shift, $\delta^{10} - (\delta^2 + \delta^9) + \delta^1$, gave for H-1 of Gal IV and GlcNAc III deviations from additivity of ± 0.01 p.p.m., whereas those values for the corresponding type 1 chain compounds were -0.08 and 0.07 p.p.m., respectively[29]. If this latter set of values is taken as evidence for a change in conformation of the oligosaccharide chain occurring on glycosylation of the monofucosylated, type 1 core-structure, then the smaller values for the type 2 chain analogs can be taken as an indication that those substitutions take place with considerably less reorientation of the core oligosaccharide.

In general, the glycosylation-induced shift changes for H-1, Fuc H-5, and CH_3 are similar to those found for type 1 chain analogs, but are smaller in magnitude. The H-1 resonances of Gal II and of Glc I are not affected measurably, but one interesting effect was noted on the lineshape of H-1 of Gal II: the "virtual coupling"

found for this resonance in the spectrum of **2** is almost eliminated by fucosylation of Gal IV. Logically, this must be the result of a change in shift of H-2 and/or H-3 of Gal II so that they no longer coincide. This could easily result from a small reorientation of either the core glycosyl chain or the anisotropic NAc carbonyl group. More-extensive assignment of proton shifts for **10** would obviously be needed for an accurate picture of these changes.

Complex analogs bearing Y-hapten at terminal. — Compounds **12** and **13** were detected in a glycolipid extract of human colonic adenocarcinoma as two h.p.t.l.c. bands in the 8–10 sugar region reactive with anti-Y antibodies on h.p.t.l.c. immunostaining. Details of their detection, isolation, and immunological characterization will be published separately[39]. Their H-1, Fuc H-5, and CH_3 resonance shifts obtained from 1-D ^{1}H-n.m.r. spectra are summarized in Tables II and III. In addition, two NAc resonances were found at 1.820 p.p.m. Comparison with data for compounds **10** and **11** makes it obvious that compound **12** is an analog of **10** containing an inserted *N*-acetyllactosamine unit. (It may also be thought of as compound **11** fucosylated α1→3 to GlcNAc V.) In particular, the detection of H-1, H-5, and CH_3 resonances for two fucose residues at the proper shifts for Y-terminal makes clear that there is only one possible arrangement of these relative to each other on the norhexaosylceramide core. The chemical shifts predicted for an isomer bearing the Fucα1→3 to GlcNAc III are not observed, eliminating that structure in this case. All other H-1 resonances are likewise at the shifts predicted for the proposed nLc_6 core structure. Furthermore, the expression for differential chemical-shift, $\delta^{12} - (\delta^5 + \delta^{11}) + \delta^3$ gave (for Gal VI and GlcNAc V) deviations from additivity $<\pm 0.01$ p.p.m., in agreement with the result for Y-hexaglycosylceramide.

Using the data for compound **12** as a basis, and the knowledge of glycosylation-induced shift effects, the structure of **13** was also elucidated from its ^{1}H-n.m.r. spectrum. Thus, the presence of the additional Fucα1→3 to GlcNAc III was detected not only by the additional H-1, H-5, and CH_3 resonances for this group, but by its predictable effect on H-1 of GlcNAc III ($\Delta\delta$ = 0.059 p.p.m.), Gal IV ($\Delta\delta$ = 0.058 p.p.m.), and GlcNAc V ($\Delta\delta$ = 0.014 p.p.m.), and H-5 of Fucα1→3GlcNAc V ($\Delta\delta$ = 0.013 p.p.m.). The shifts are equally in accord with the conversion of **6** by addition of a terminal Fucα1→2 group. In addition, the lineshapes for each of the Gal H-1 resonances are consistent with the "virtual coupling" phenomenon detected in the simpler analogs (not shown). The assigned structure is consistent with its antibody reactivity and h.p.t.l.c. behavior, and was confirmed subsequently by g.l.c.–m.s. of the hydrolysis products of the permethylated compound (data to be presented elsewhere, ref. 39).

In conclusion, we have shown that the application of 1-D ^{1}H-n.m.r. spectroscopy is sufficient, in the present cases of X- and Y-hapten-bearing glycolipids of the neolacto-series, for saccharide structure-determination using 100–200 μg of compound. Corroborative evidence is, of course, still highly desirable when obtainable. With useful g.l.c.–m.s. data available on ~20 μg of glycolipid and direct-probe m.s. data available in the 1–10 μg sample range, satisfactory chemical characteriza-

tion of such glycolipids can be accomplished on amounts small enough to leave sufficient sample for immunization, antibody screening, and specificity testing.

We have also shown that there are several chemical-shift effects that are detectable specifically on extended structures, which may give some indication of the secondary structure of these molecules. Currently we are pursuing computer-aided molecular modeling experiments to gain some insight into the possible significance of these effects, particularly with regard to the size and geometry of the epitope that is recognized by an antibody (FH4) specific[5] to molecules bearing a minimum of two Fucα1→3 residues, *i.e.*, **6** or **7**. The attempt to analyze such a system inevitably raises questions that are not necessarily answered by studies of the constituent trisaccharides. In the case of nLc$_8$Cer, for example, it may be worthwhile to consider the effect that three Fucα1→3GlcNAc substitutions (*i.e.*, to make **7**), might have on the global conformation of the carbohydrate chain. More importantly, it might be expected that the additional branching-residues have the effect of stabilizing the favored conformer.

Lemieux and Bock have stated that there is little justification for specifying interglycosidic bond-angles more precisely than ±5°, nor for considering the meaning[37] of substituent shift-effects ±0.02 p.p.m. However, for an extended epitope encompassing four to five residues, the cumulative effect of this uncertainty could lead to considerable variance in the calculated relative position of sugars at either end, with no connectivity available from n.O.e. data. Conceivably, by accounting for a set of long-range shift effects, it would be possible to decrease this variance in the expected conformational preference.

EXPERIMENTAL

General methods. — Thin-layer chromatography (t.l.c.) was performed with h.p.t.l.c. plates (silica gel 60, E. Merck, Darmstadt, W. Germany or Si-HPF, J. T. Baker, Phillipsburg, NJ); solvent *A*, 60:35:8 (v/v/v) $CHCl_3$–MeOH–H_2O; solvent *B*, 50:40:10 (v/v/v) $CHCl_3$–MeOH–H_2O + 0.5% $CaCl_2$. Ion-exchange chromatography was performed with DEAE-Sephadex (Pharmacia, Uppsala, Sweden) pre-equilibrated with solvent *A*. Glycolipids were solubilized for transfer with either 1:1 (v/v) $CHCl_3$–MeOH or solvent *C*, 2-propanol–hexane–H_2O 55:25:20 (v/v/v, upper phase discarded). Chloroform-*d*, 99.8 atom%, dimethyl sulfoxide-d_6, 99.9 and 99.96+ atom%, and deuterium oxide, 99.96+ atom% (low in paramagnetic ions) were purchased from Aldrich (Milwaukee, WI), and methanol-d_4, 99.5 atom%, from KOR Isotopes (Cambridge, MA). Samples were prepared for n.m.r. by deuterium-exchanging three times in chloroform-*d*–methanol-d_4 1:1 (v/v), with evaporation under dry nitrogen, and then dissolving them in 0.4 mL of dimethyl sulfoxide-d_6 containing 2% deuterium oxide[26] and 1% tetramethylsilane as chemical-shift reference.

Glycolipids. — nLc$_4$Cer (**1**) was prepared from O erythrocyte IV^{3}NeuNAc-nLc$_4$Cer by treatment with 20% acetic acid in solvent *C* for 48 h at 100° in a sealed

tube. After evaporation to dryness, free NeuNAc and unhydrolyzed starting material were removed by passage through DEAE-Sephadex in solvent *A*. The pass-through fraction was a single band by h.p.t.l.c. in solvents *A* and *B* and was used without further purification. III^{3}FucnLc$_6$Cer (**4**) was prepared from VI^{6}NeuNAc-III^{3}FucnLc$_6$Cer ("Fraction 6C, upper band") of human liver adenocarcinoma[14] by treatment with *Clostridium perfringens* neuraminidase X-A (insolubilized on beaded agarose; Sigma, St. Louis, MO) in a buffer consisting of PBS containing sodium acetate (50 mM) and $CaCl_2$ (mM), adjusted to pH 5.1. The glycolipid in 1 mL of buffer was sonicated for 2 days with the enzyme, which was then removed by centrifugation and washed twice with solvent *C*. The combined supernatants were evaporated to dryness, and the residue extracted with 1:1 $CHCl_3$–MeOH. The glycolipid extracted in this manner was further freed of salts by chromatography on LH-20 (0.9 × 30 cm) in 1:1 $CHCl_3$–MeOH, and then freed of unreacted 6C using DEAE-Sephadex.

nLc$_6$Cer (**3**) was prepared from O erythrocytes as described[43]. The isolation and characterization of V^{3}FucnLc$_6$Cer (**5**) from O erythrocytes[8], III^{3}FucnLc$_4$Cer (**2**), III^{3}V^{3}Fuc$_2$nLc (**6**), III^{3}V^{3}VI^{3}Fuc$_3$nLc$_8$Cer (**7**), III^{3}NeuNAcIII^{3}V^{3}Fuc$_2$nLc$_6$Cer (**8**), from human colonic and liver adenocarcinoma[4,6,14], and III^{3}IV^{2}Fuc$_2$nLc$_4$Cer (**10**) from[12] human colonic cancer cell-line MKN74 have been described previously. IV^{2}FucnLc$_4$Cer (**9**, H_1) and VI^{2}FucnLc$_6$Cer (**11**, H_2) were prepared as previously reported[44]. The isolation and further characterization of the title compounds **12** and **13** will be described elsewhere[39].

^{1}H-N.m.r. spectroscopy. — One-dimensional spectra were obtained with a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer, using quadrature detection and a 90° pulse-angle; the sweep width was 5 kHz collected over a 16K data-set (digital resolution 0.6 Hz/pt.), giving an acquisition time of 1.684 s, to which was added a 2-s delay. A total of 800–10,000 free-induction decays (f.i.d.s) were accumulated, depending on the sample concentration. Resolution enhancement employed a Gaussian-window function prior to transformation.

Two-dimensional correlated spectroscopy (2D-COSY) in the phase-sensitive mode[45] with presaturation of the residual HOD resonance, was performed on a Bruker AM-300 spectrometer equipped with an Aspect 3000 computer using a program included in the Bruker software package. A total of 512 measurements with t_1 values from 5 μs to 143.4 ms (in 0.28-ms increments) were made using a sweep width of 1786 Hz and a relaxation delay of 0.5 s, and 128 f.i.d.s were accumulated for each t_1. The data were transformed as a 2048 × 1024 matrix with phase-shifted sine-squared apodization applied to both the acquired f.i.d.s and the resulting t_1 interferogram prior to Fourier transformation.

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