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Structural Analysis of Glycosphinoglipids by High-Resolution ^{1}H Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The carbohydrate composition of eight glycosphingolipids–glucosylceramide, lactosylceramide, lacto-*N*-triaosylceramide, neolactotetraosylceramide, IV^{3}Galαneolactotetraosylceramide, globotriaosylceramide, globoside (P-antigen), and Forssman glycolipid (IV^{3}GalNAcα-GbOse$_4$Cer)—derived from different sources was analyzed by ^{1}H nuclear magnetic resonance at 360 MHz in dimethyl-d_6 sulfoxide as solvent. The resonances of all H-1 and H-2 protons as well as those of most of the H-3 and H-4, and of some H-5 protons, of the sugar rings were assigned with the aid of spin decoupling difference spectroscopy. They show regularities related to the type, anomeric configuration, site of glycosidic linkage, and sequence of the component sugars glucose, galactose, glucosamine, and galactosamine. These regularities are thus suited for elucidation of hitherto unknown structures of more complex glycosphingolipids.

Increasing knowledge of the biological functions of glycosphingolipids as compounds of cellular surfaces, particularly of their blood-group antigenicity (Hakomori & Kobata, 1974; Hanfland, 1975; Hanfland et al., 1978a; Koscielak et al., 1979; Watanabe et al., 1979), as well as of their role in cellular recognition, growth control, and malignant degeneration (Hakomori, 1975; Hanfland & Uhlenbruck, 1978) has promoted the development of microscale characterization methods; combined gas chromatography–mass spectrometry of partially methylated alditol acetates (Björndal et al., 1970; Stoffel & Hanfland, 1973; Stellner et al., 1973), direct-inlet mass spectrometry of permethylated glycolipids (Karlsson et al., 1974; Ledeen et al., 1974; Hanfland & Egge, 1976; Egge, 1978), and enzymatic degradation by specific glycosidases (Hakomori et al., 1971; Hanfland et al., 1978b) allow one to evaluate sugar linkage and sequence as well as anomeric configuration. However, regarding the tiny amount of material obtainable from some sources, its loss due to these destructive methods is a serious disadvantage. High-resolution ^{1}H NMR spectroscopy is a nondestructive method well suited for elucidation of complex structures. However, if the degree of complexity exceeds certain limits, the problem of overlapping multiplets must be solved. Although several recent investigations on oligosaccharides (Dorland et al., 1977) and glycosphingolipids (Martin-Lomas & Chapman, 1973; Falk et al., 1979) benefited by the enhanced spectral resolution of the high-field spectrometers now available, the source of information was still limited to signals which occurred outside of the bulk of overlapping resonances and could thus be found by inspection. In our work, we show that additional structural information can be obtained from signals extracted by means of spin-decoupling difference spectroscopy (SDDS;[1] Gibbons et al., 1975) from the originally uninterpretable signal agglomerates.

The glycosphingolipids **1**–**10** related to the Forssman glycolipid (IV^{3}GalNAcα-GbOse$_4$Cer) and the ceramide pentasaccharide IV^{3}GalαnLcOse$_4$Cer from rabbit erythrocytes with B-like blood group activity offer manifold combinations in which the sugars, their anomeric configuration, sequence, and the sites of glycosidic linkage are the variables. We demonstrate that some regularities concerning the chemical shifts of several of the sugar protons (Tables I–V) enable one to determine these variables.

Materials and Methods

Preparation of Glycosphingolipids. Glucosylceramide (**1**) obtained from IV^{3}Galα-nLcOse$_4$Cer (**10**) after repeated partial hydrolysis with 0.1 N HCl at 90 °C for 40 min was purified by preparative silica gel high-performance thin-layer chromatography with $CHCl_3$/MeOH/H_2O (75:16:2) as developing solvent (Hanfland, 1978).

Forssman glycolipid (**7**) was isolated from equine kidneys according to the procedures described for the glycosphingolipids B-I and B-II (Hanfland & Egli, 1975) and purified by preparative high-performance thin-layer chromatography (Hanfland, 1978). The identification as IV^3 GalNAcα-GbOse$_4$Cer (**7**) is based on sugar analysis (Hanfland et al., 1978b), analysis of the partially methylated alditolacetates (Stoffel & Hanfland, 1973), and two-dimensional immunodiffusion against *Dolichos biflorus* lectin.

Globotetraosylceramide from human erythrocyte membranes (**6**), globotriaosylceramide with both *n*-FA and α-hydroxy fatty acid (HFA) residues (**4**) and (**5**), and lactosylceramide, also with FA (**2**) and HFA (**3**) residues, from human plasma, as well as IV^{3}Galα-nLcOse$_4$Cer (**10**) from rabbit erythrocyte membranes were obtained by methods described earlier (Hanfland & Egli, 1975; Hanfland, 1978).

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[1] Abbreviations used: FT NMR, Fourier-transform nuclear magnetic resonance; SDDS, spin-decoupling difference spectroscopy; FA and HFA, fatty acid and α-hydroxy fatty acid of the ceramide part; INDOR, internuclear double resonance; SPI, selective population inversion; SECSY, spin-echo correlated spectroscopy; Me_2SO, dimethyl sulfoxide.

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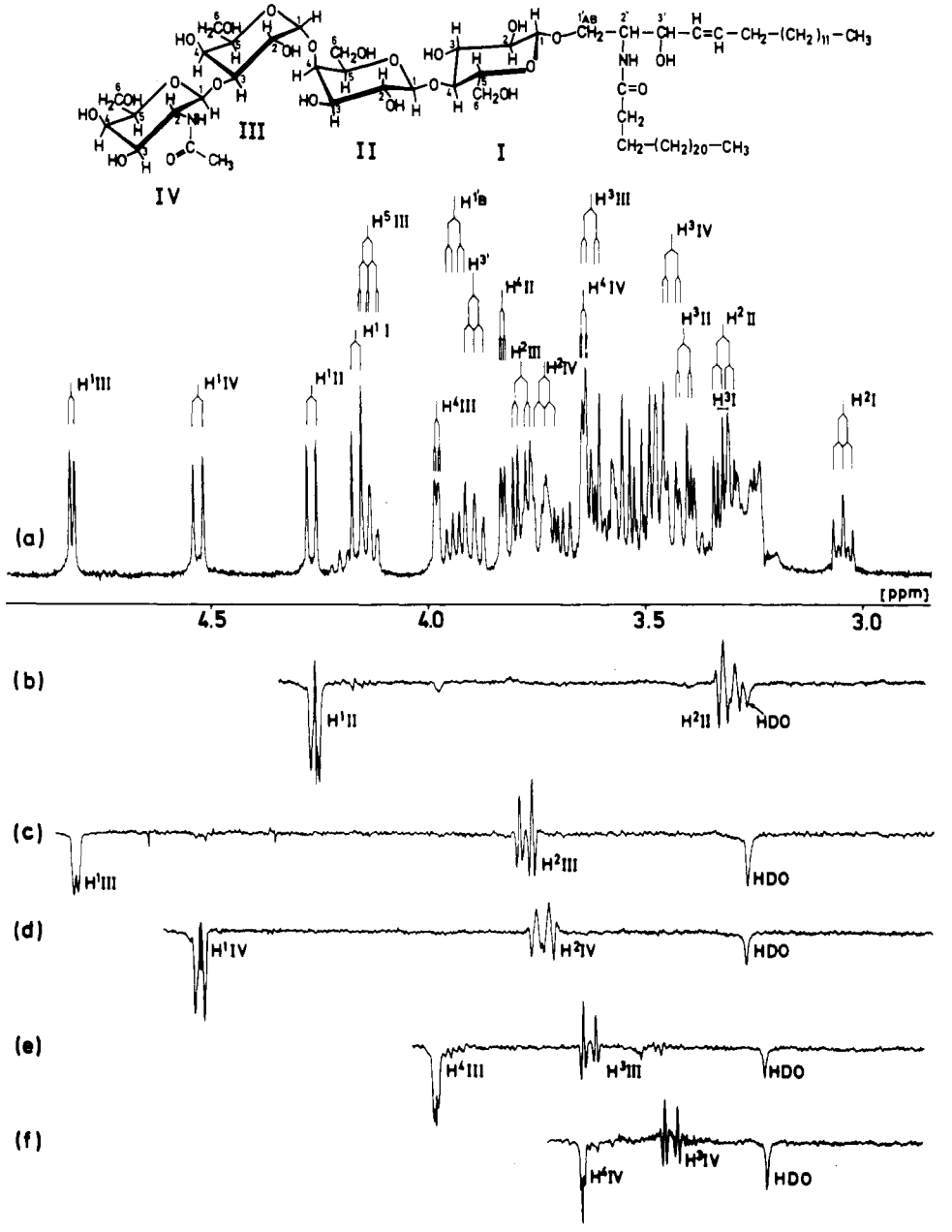

FIGURE 1: The sugar protons region of the 360-MHz ^{1}H NMR spectra of globotetraosylceramide (**6**). (a) The resolution-enhanced spectrum obtained with HDO peak suppression; (b–f) examples of SDDS spectra: low-field resonance irradiated, high-field one observed, e.g., H^2II {H^1II} in (b).

Neolactotetraosylceramide (**9**) and lactotriaosylceramide (**8**) were obtained from **10** after enzymatic degradation with α-galactosidase from coffee beans, followed by treatment with β-galactosidase from Jack beans in the case of **8** (Hanfland et al., 1978a). Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) and ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**12**) were taken from Kuhn's archives.

NMR Measurements. ^{1}H NMR spectra were obtained at 338 K on a Bruker HX-360 spectrometer in the Fourier-transform mode by using quadrature detection. The Bruker Aspect 2000 computer had 32K memory capacity. The operating frequency was 360 MHz, and the spectral widths amounted to 3.3 kHz. The deuterium-exchanged samples were dissolved in Me_2SO-d_6 containing 2% D_2O. The sample concentration amounted to about 0.2%. The free induction decays were multiplied by a resolution-enhancement function (Ernst, 1966) included into the Bruker software package. The overlapped signals were found with the aid of SDDS (Gibbons et al., 1975) (for examples, see Figure 1). Although the accuracy of the measurements was better than 0.001 ppm, the chemical shift values were rounded to 0.01 ppm for convenience of the discussion and because of their slight concentration dependence.

Results

The presentation of the material will be organized according to the following lines. At first, the resonances of the particular sugar protons will be treated separately in order to show what kind of structural information can be derived from the analysis of each. This will be followed by the discussion on the possibility of the elucidation of complex glycosphingolipid structures by combining the fragmentary information thus obtained.

^{1}H Resonances (Table I). Glucosylceramide (**1**) and lactosylceramide (**2**) have been investigated independently within both series presented, but since the chemical shifts were

Table I: Sugar H-1 Chemical Shifts (ppm from Me_4Si) and $^3J_{1,2}$ Coupling Constants (Hz) of Glycosphingolipids[a] in Me_2SO-d_6

	GalNAc(α1-3)		GalNAc(β1-3)		Gal(α1-4)		Gal(β1-4)		Glc(β1-1)Cer	
	δ	J	δ	J	δ	J	δ	J	δ	J
1									4.10	7.7
2 (FA)							4.22	7.3	4.17	7.7
3 (HFA)							4.23	7.7	4.17	7.7
4 (FA)					4.81	4.0	4.27	7.7	4.16	8.1
5 (HFA)					4.80	3.2	4.27	7.7	4.16	7.7
6			4.52	8.1	4.81	3.6	4.26	7.7	4.16	7.7
7	4.74	3.6	4.56	8.5	4.82	3.6	4.27	7.7	4.17	7.7
11[a]	4.55	3.5								

	Gal(α1-3)		Gal(β1-4)		GlcNAc(β1-3)		Gal(β1-4)		Glc(β1-1)Cer	
	δ	J	δ	J	δ	J	δ	J	δ	J
8					4.65	9.2	4.28	7.3	4.17	7.7
9			4.23	6.5	4.70	7.7	4.28	7.7	4.17	7.7
10	4.85	3.8	4.29	7.4	4.70	8.4	4.27	8.0	4.17	7.8
12[a]					4.29	7.9				

[a] Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) and ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**12**) were measured for comparison.

identical, they were listed only once.

When the present data are compared with those obtained for solutions of oligosaccharides or their derivatives in D_2O (De Bruyn et al., 1976) or $CDCl_3$ (Paulsen & Kolář, 1979), an upfield shift of ca. 0.3 ppm typical for Me_2SO solutions has to be taken into account.

The H-1 signals between 4.10 and 4.29 ppm ($J_{1,2} \approx 7$–8 Hz) have been assigned to β-glucose and -galactose units, the differentiation between them having been made by decoupling from H-2, the position of which is unambiguous (see Table II).

For *N*-acetyl-β-glucosamine and *N*-acetyl-β-galactosamine units, the anomeric proton signal is shifted downfield by ca. 0.5[2] and 0.3 ppm, i.e., to 4.65–4.70 and 4.52–4.56 ppm, respectively, obviously due to the deshielding effect of the vicinal 2-acetamido group. The vicinal coupling constants are slightly increased ($J_{1,2} \approx 8$–9 Hz).

The resonances of the equatorial H-1 of α-galactose units occupy a narrow region between 4.80 and 4.85 ppm ($J_{1,2} \approx$ 3–4 Hz). Interestingly, the same resonance of the 2-*N*-acetamido derivative is shifted to 4.74 ppm, i.e., upfield by 0.11 ppm (substance **7** vs. **10**). Since 2-*N*-acetamido-α-galactosyl in **7** and α-galactosyl in **10** are glycosidically linked to different sugars, it is not clear whether this is only induced by the acetamido group or rather by the different aglycon, or both. It seems, however, that the aglycon is immaterial here since replacing the 2-OH group by an acetamido group in an analogous stereochemical situation of α- and β-glucopyranosides resulted in a downfield shift of H-1 for the β anomer and an upfield shift for the α one (Perkins et al., 1977). It can be assumed that the interaction of the closely spaced α C-1–O-1 and 2-acetamido fragments results in their orientation which has a shielding component with respect to H-1. It should be added that the H-3 and H-5 chemical shifts of 2-*N*-acetamido-α-galactosyl of **7** also deviate from the expected values (vide infra).

Glycosylation of a terminal α-galactose unit leaves its H-1 resonance unchanged, as does the subsequent elongation of the oligosaccharide chain (substances **4**–**7**). In contrast, the glycosylation of any of the terminal β-anomeric sugar residues will shift its H-1 signal by 0.04–0.07 ppm to lower field; as with the α-galactose moiety, extension of the oligosaccharide chain has no further effect on this signal. This shift, though regular, is not specific with regard to the type of the substituting sugar or the site of its attachment (3 or 4 for the series investigated here).

The situation changes if a sugar linked directly to the anomeric center, i.e., an aglycon, is considered as a substituent. For example, the H-1 signal of an α-galactose occurs alternatively at 4.85[3] or 4.81 ppm when glycosidically linked to a 3- or 4-galactose unit, respectively. Hence, the differentiation between alternative sites of glycosylation can in principle be deduced from NMR spectra.

No analogous regularities have been found either for aqueous solutions of oligosaccharides or for solutions of permethylated glycosphingolipids in chloroform. The scrutiny of the data published by the Vliegenthart group (Dorland et. al., 1977) and by De Bruyn (1975) reveals that 3- or 4-glycosylation induces small and irregular shifts (±0.02 ppm) of these signals in the spectra of related β sugars. Also, for

Table II: Sugar H-2 Chemical Shifts (ppm from Me_4Si) of Glycosphingolipids[a] in Me_2SO-d_6

	GalNAc-(α1-3)	GalNAc-(β1-3)	Gal-(α1-4)	Gal-(β1-4)	Glc-(β1-1)Cer
1					2.98
2 (FA)				3.33	3.05
3 (HFA)				3.33	3.05
4 (FA)			3.65	3.34	3.05
5 (HFA)			3.65	3.32	3.04
6		3.73	3.78	3.31	3.05
7	4.09	4.02	3.77	3.31	3.04
11[a]	4.04				

	Gal-(α1-3)	Gal (β1-4)	GlcNAc-(β1-3)	Gal-(β1-4)	Glc-(β1-1)Cer
8			3.36	3.48	3.05
9		3.32	3.44	3.44	3.05
10	3.59	3.42	3.44	3.42	3.04
12[a]			3.36		

[a] Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) and ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**12**) were measured for comparison.

[2] This value would be reduced to ~0.3 ppm if the aglycon effect was taken into account for the reference glucose unit. Indeed, the relative shift resulting from the linkage to a glycosyl vs. a ceramide aglycon amounts to almost 0.2 ppm [Dabrowski et al. (1980) and the present data].

[3] This observation was corroborated by δ 4.84 (H-1) of two terminal α-galactosyls linked to 3-galactose residues in a branched ceramide decasaccharide (Hanfland et al., 1979).

the permethylated derivatives, irregular shifts of 0.01–0.02 ppm can be derived from the untabulated data presented by Falk et al. (1979).

H-2 Resonances (*Table II*). The H-2 signals of the glucose units occurred at the high-field limit of the spectral region of sugar protons and could be found by inspection, but those of all other sugar units had to be extracted from the agglomerates of overlapping resonances with the aid of SDDS.

Glycosylation of the terminal glucose unit at O-4 induces a downfield shift of its H-2 resonance from 2.98 (**1**) to 3.05 ppm (**2**, **3**), and the latter value is little changed upon elongation of the oligosaccharide chain (substances **4**–**10**). The H-2 signal of the terminal *N*-acetylglucosamine unit in **8** occurs further downfield (3.36 ppm) due to the strong deshielding effect of the acetamido group, but the shift induced by substitution at position 4 is of the same order of magnitude (3.44 ppm in **9** and **10**).

The H-2 resonances of β-galactose and β-*N*-acetylgalactosamine units occur downfield compared to those of β-glucose and β-*N*-acetylglucosamine units, respectively. The relative shifts of 0.34–0.37 ppm (**2** and **9** vs. **1**, and **6** vs. **8**) can be accounted for by the 1,3-syn-axial orientation (Lemieux & Stevens, 1966) of the C-2–H and C-4–O bonds in the galactose framework. These protons are even more deshielded in the α-galactose and α-*N*-acetylgalactosamine units due to the 1,2-antiperiplanar orientation (Lemieux & Stevens, 1966) of the C-2–H and C-1–O-1 bonds, the additional shifts amounting to 0.32–0.36 ppm (**4** vs. **2** or **9**, and **7** vs. **6**). The resulting location of some of the H-2 resonances outside of the main bulk of overlapping signals renders the former amenable to a simple spectral analysis. Moreover, since these signals are but slightly overlapped, they can serve as starting points for consecutive SDDS experiments aiming at detection of H-3 resonances.

As with *N*-acetylglucosamine, the 2-acetamido group has a strong deshielding effect on galactosamine H-2 resonances. By comparing the structurally related *N*-acetylgalactosamine and galactose fragments (**6** vs. **2** or **9** for β, and **7** vs. **10** for α), one obtains a shift of 0.40–0.50 ppm for this effect. It has to be mentioned, however, that an additional effect of a different aglycon may possibly be involved if **6** is compared with **2** or **9**.

The H-2 resonances of β-galactose units react in a different manner to the substitution than described above for the β-glucose unit. The attachment of a new sugar group at position 4 leaves these signals practically unaffected (**4**–**7** vs. **2** and **3**) whereas the substitution at O-3 induces a shift of 0.09–0.15 ppm (**8**–**10** vs. **2** for the inner galactose unit, and **10** vs. **9** for the penultimate galactose unit). Similarly, glycosylation of α-galactose at O-3 shifts the H-2 resonance by 0.12–0.13 ppm (**6** and **7** vs. **4**). This large shift caused by the close proximity of the substituent at O-3 to H-2 can have a diagnostic value for structure determination of complex oligosaccharide derivatives. Furthermore, the differentiation of these two types of substitution follows independently from the effect of the aglycon on the H-2 resonance of the terminal α-galactose residue (3.65 vs. 3.59 ppm in **4** and **10**, respectively). It should be recalled that the 4- vs. 3-substitution for **4** and **10**, respectively, has been deduced from the effect of the aglycon on the H-1 resonances of the terminal α-galactose units.

The largest of the shifts induced by glycosylation is that occurring on linking α-*N*-acetylgalactosamine to 0-3 of a β-*N*-acetylgalactosamine unit (0.29 ppm for **7** vs. **6**). This fact possibly points to an interaction between O-5 of the former and the 2-*N*-acetamido group of the latter unit and thus contains structural information concerning the conformation of this fragment of the oligosaccharide chain. Accordingly, the H-2 resonance of the β-galactose unit lacking the acetamido group experiences a much smaller shift on substitution by α-galactose at the same site (0.10 ppm for **10** vs. **9**).

Table III: Sugar H-3 Chemical Shifts (ppm from Me_4Si) of Glycosphingolipids[a] in Me_2SO-d_6

	GalNAc-(α1–3)	GalNAc-(β1–3)	Gal-(α1–4)	Gal-(β1–4)	Glc-(β1–1)Cer
1					3.16
2				3.32	3.31
4			3.56	3.40	3.33
6		3.44	3.62	3.41	3.32
7	3.56	3.52	3.62	3.41	3.32
11[a]	3.58				
	Gal-(α1–3)	**Gal-(β1–4)**	**GlcNAc-(β1–3)**	**Gal-(β1–4)**	**Glc-(β1–1)Cer**
8			*b*	3.46	3.32
9		3.32	*b*	3.46	3.33
10	3.64	3.48	*b*	3.48	3.32

[a] Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) was measured for comparison. [b] Not measured.

H-3 Resonances (*Table III*). The H-3 resonances which are always buried under the bulk of overlapping signals were found with the aid of SDDS by irradiation of the neighboring H-4 or H-2 resonances. In a few instances, a double check through both of these frequencies was performed.

A 2-*N*-acetamido group has a deshielding effect on H-3 if β-galactose and β-*N*-acetylgalactose units are compared (0.12 ppm for **6** vs. **2** or **9**), but for the corresponding α anomers either the shielding remains unchanged (**7** vs. **4**) or the shift is to higher field (0.08 ppm for **7** vs. **10**). As in the case of H-1, the cause of this different behavior can be looked for in the changed orientation of the 2-*N*-acetamido group due to its different interaction with the neighboring C-1–O-1 moiety in the alternative e,e and e,a situations.

The anomeric configuration of galactose units strongly affects their H-3 resonances, those of the α anomers being shifted downfield by 0.24–0.32 ppm due to the 1,3-syn-axial position of the C-1–O-1 bond (**4** vs. **2**, and **10** vs. **9** or **2**). The relative chemical shift is much smaller if the α- and β-*N*-acetylgalactosamine fragments are compared (0.12 ppm for **7** vs. **6**), this being another consequence of the interaction between the acetamido group and the C-1–O-1 fragment just discussed.

The H-3 resonances of the glucose units experience a shift of 0.15–0.17 ppm on glycosylation at C-4 (**2**, **4**, and **6**–**10** vs. **1**). For galactose units, in which the entering substituent occupies a remote position at the antiperiplanar O-4 position, the corresponding shift is smaller (0.08–0.09 ppm for **4**, **6**, and **7** vs. **2**).

Glycosylation of a β-galactose unit at C-3 by either β-*N*-acetylglucosamine or α-galactose induces a practically identical shift of the H-3 resonance in both cases (0.14–0.16 ppm for **8**–**10** vs. **2**, and **10** vs. **9**, respectively).

It seems opportune to compare the substitution of a β-galactose unit by an α-galactose just mentioned (**10** vs. **9**) with a situation when both these fragments have an *N*-acetamido group in the vicinal position at C-2 and the relative shift is twice as small (0.08 ppm for **7** vs. **6**). It should be recalled that the extreme *downfield* shift of H-2 of **7** was supposed to ensue from the interaction between O-5 of the α-*N*-acetylgalactose unit and the *N*-acetamido group of the β-unit. It seems feasible that the orientation of that group resulting from this interaction produces a *shielding* effect on H-3. Hence,

Table IV: Sugar H-4 Chemical Shifts (ppm from Me_4Si) of Galactoses and Galactosamines in Glycosphingolipids[a] (in Me_2SO-d_6)

	GalNAc-(α1-3)	GalNAc-(β1-3)	Gal-(α1-4)	Gal-(β1-4)	Glc-(β1-1)Cer
2				3.64	
4			3.76	3.81	
6		3.64	3.98	3.82	
7	3.73	3.76	3.98	3.82	
11[a]	3.73				
	Gal-(α1-3)	**Gal-(β1-4)**	**GlcNAc-(β1-3)**	**Gal-(β1-4)**	**Glc-(β1-1)Cer**
8				3.86	
9		3.64		3.86	
10	3.75	3.85		3.85	

[a] Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) was measured for comparison.

Table V: Sugar H-5 Chemical Shifts (ppm from Me_4Si) of α-Galactoses and α-Galactosamines in Glycosphingolipids[a] in Me_2SO-d_6

α-Gal		α-GalNAc
terminal	substituted	terminal
4, 4.05	6, 4.14	7, <3.80
10, 3.99	7, 4.14	11,[a] <3.55

[a] Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**) was measured for comparison.

the relatively small net downfield shift when going from **6** to **7** could be explained in this way.

Glycosylation of an α-galactose unit at C-3 by a β-*N*-acetylgalactosamine shifts the H-3 resonance of the former by 0.06 ppm (**6**, **7** vs. **4**), which is only about 40% of the value observed for the analogous substitution of a β-galactose by a β-*N*-acetylglucosamine (**8**–**10** vs. **2**; *vide supra*). Since the entering substituents only differ in those two cases in their configuration at the very remote C-4 center and their effect on H-3 must be virtually equal, these different shifts seem to ensue from a different interaction of the axial or equatorial C-1–O-1 bond of the galactose unit with those substituents, particularly with their *N*-acetylamino group. Although the named fragments are rather distant from each other, the interaction between them can be mediated by the galactose C-2 OH group—a kind of conformational transmission.

H-4 Resonances (*Table IV*). The equatorial position of H-4 of galactoses has two important consequences for its NMR signal. First, this signal is shifted downfield with respect to the conglomerate of the many axial nonanomeric proton resonances. Second, because of the two small equatorial–axial coupling constants ($J_{3,4} \approx 3$ Hz and $J_{4,5} \approx 1$ Hz; De Bruyn & Anteunis, 1976), this signal is rather high and can usually be found by inspection among the few other signals occurring in this part of the spectrum as those are split by larger couplings into components of lower intensity. The assignments thus made were cross-checked in several cases by SDDS. For example, a two-step procedure was applied for α-galactose and α-*N*-acetylgalactosamine units by taking advantage of the extreme downfield position of their H-2 resonances. First, the latter was irradiated and the H-3 difference signal observed. Next, the assumed H-4 resonance was irradiated, and, if the H-3 difference signal occurred at the same position, all three protons were related to the same sugar unit. Alternatively, H-4 could be identified in one step by correlating with H-5 in those cases when the position of the latter was unambiguous.

The H-4 resonances of β anomers of terminal galactose and *N*-acetylgalactosamine units occur at 3.64 ppm whereas those of the corresponding α anomers are located downfield, at 3.73–3.76 ppm, probably due to the deshielding effect of the free electron pairs of the α O-1. Hence, the difference of 0.09–0.12 ppm is characteristic of the above structural variation (**4** vs. **2**, **7** vs. **6**, and **10** vs. **9**).

Glycosylation of a β-galactose unit by an α-galactose unit at sites 3 and 4 gives rise to a downfield shift of H-4 of the former by 0.17–0.21 ppm (**4**, **6**, and **7** vs. **2**, and **10** vs. **9**).

The substitution at site 3 by a β-*N*-acetylgalactosamine or a β-*N*-acetylglucosamine unit produces a H-4 downfield shift of 0.21–0.22 ppm regardless of whether an α- or, β-galactose unit was subjected to this substitution (**6** and **7** vs. **4**, and **8**–**10** vs. **2**). In both these cases, the O-5 free electron pairs of the new substituent are very close to H-4 of the glycosylated galactose. The H-4 resonances thus shifted show a convincing constancy within the short series **6**, **7**, and **8**–**10** and seem to be useful for identification of the corresponding structural situations.

H-5 Resonances. The H-5 signals of the β-glucose group in **1** and the β-galactose group in **2** were but slightly masked by other multiplets and could be found by conventional spin-decoupling experiments at 3.10 and 3.35 ppm, respectively. The latter value is almost identical with that found in our previous work (Dabrowski et al., 1980) for β-galactosylceramide and β-methyl galactoside (both 3.34 ppm). The downfield shift of the β-galactose H-5 resonance with respect to that of β-glucose is readily explained by the 1,2-antiperiplanar orientation of the C-5–H and C-4–O bonds in the former.

The irradiation of H-4 protons of β-galactose and galactosamine units undertaken for detecting the overlapped H-3 signals of higher glycosphingolipids should theoretically have furnished the H-5 ones as well. However, the coupling constant, $J_{4,5} \approx 1$ Hz, is too small to serve as a basis for reliable difference spectra. With glucose and glucosamine units, the signals of their protons vicinal to H-5 are themselves overlapped; hence, they are also unsuitable for this purpose. In contrast, the H-5 signals of α-galactose units can easily be found, owing to their extreme downfield shift resulting from both the 1,3-syn-axial position of the C-1–O-1 bond and the 1,2-antiperiplanar orientation of the C-4–O bond. They are listed in Table V.

The H-5 resonance of the terminal α-galactose linked to a β-galactose unit at C-3 occurs at 3.99 ppm whereas a chemical shift of 4.05 ppm was found when the joint was at C-4 (**10** vs. **4**). This difference offers an additional possibility to distinguish between those two types of substitution. The H-5 signals of the inner α-galactose units are moved downfield by 0.09 ppm (**6**, **7** vs. **4**).

Judging by the extreme downfield position of the H-5 resonance of the α-galactose unit of **10**, one could anticipate a similar situation for the α-*N*-acetylgalactosamine unit of **7**. However, in the spectrum of **7**, no such signal occurs in the fully assigned region downfield from 3.80 ppm, and a control experiment showed that in the spectrum of α-methyl-*N*-acetylgalactosamine (**11**) this resonance must be located upfield from the 3.55-ppm mark. It can be concluded that the bulky 2-acetylamino group of the α-galactosamine unit affects the orientation of the C-1–O aglycon fragment in such a way as to greatly reduce its deshielding effect on the 1,3-synaxial H-5 proton.

Methyl resonances of the acetyl groups of substances **6**–**12** occur between 1.80 and 1.85 ppm. The assignment of all ceramide signals has been performed in our previous works (Dabrowski et al., 1980; Yamada et al., 1980). The oligo-

saccharide part of the glycosphingolipids has little effect on these signals.

Discussion

The structure elucidation of complex glycosphingolipids by NMR is a formidable task both in the purely technical aspect of obtaining spectral parameters and from the point of view of their interpretation in the sense of spectra–structure relationships.

To our experience, an unambiguous assignment of all ^{1}H NMR signals is a problem already on the level of a trisaccharide structure. The selective INDOR (De Bruyn et al., 1976) or SPI (Pachler & Wessels, 1974) techniques gradually loose their selectivity with increasing number of sugar residues in the oligosaccharide chain, and, even more important, they are impractical from the point of view of measuring time. The powerful two-dimensional J-resolved NMR spectroscopy is applicable to first-order spectra only; besides, it does not provide the information about the connectivities between spin-coupled nuclei. The recent SECSY modification of the two-dimensional spectroscopy (Nagayama et al., 1979) has removed, in principle, the latter limitation, but its applicability to spectral regions of a degree of complexity discussed here remains to be demonstrated. In contrast, the SDDS technique applied in this work has the important advantage of (1) virtually retaining the sensitivity of the conventional FT NMR spectroscopy (diminished by the noise accumulated twice), (2) being applicable to second-order spectra, and (3) directly showing the couplings between the nuclei of interest. The third point is especially important, as signals occurring at close or even identical frequencies can be correlated in this manner with given protons of given sugar units. A particularly striking example is the SDDS spectra of substance **10** in which the values δ 3.42 (H-2), 3.48 (H-3), and 3.85 (H-4) occur twice, but since these twin signals show connectivities with different proton signals of ascertainable origin, they can unambiguously be ascribed to defined sugar units.

Although the approach based on the SDDS is not universal, it seems to be more effective than any other method known to date.

The choice of the solvent is a dilemma. The biologically relevant aqueous solutions are unacceptable for high-resolution NMR work as all glycosphingolipids form micelles in water. Nonpolar solvents, like chloroform, are also unsuitable for native glycosphingolipids because of solubility problems. We consider dimethyl sulfoxide (Me_2SO) superior to other solvents for the following reasons: (1) All native glycosphingolipids are very well soluble, and their derivatives and degradation products can be investigated for comparison in the same solvent, if desired. (2) The chemical shift changes upon structure variations described above have a particularly regular character in the case of Me_2SO solutions of native glycosphingolipids. The diagnostic value of our data is clearly demonstrated on comparison with those obtained in other laboratories for derivatized species (Falk et al., 1979) in chloroform solutions or for related oligosaccharides lacking the ceramide moiety investigated in aqueous solutions (Dorland et al., 1977).

On the other hand, one has to be aware of the possible conformational changes induced by solvation, particularly if the shielding effect of the strongly interacting acetamido group is concerned.

In the Results section, the stress was laid on chemical shift differences rather than on the chemical shifts themselves, as this seemed to be the best way to correlate the manifold structural characteristics with the limited set of so strongly differentiated NMR data. Such an advantageous approach is only possible if series of structurally related substances are available. The regularities thus derived can then be applied to rationalize the concrete chemical shifts of an unknown substance and, consequently, to identify it.

The structure of a glycosphingolipid can be deduced from its NMR spectrum in the following manner.

(1) The number of the sugar residues can be determined by integrating the H-1 signals. The number of the amino sugar units is given by the integral of the methyl signals of the acetamido groups.

(2) The anomeric configuration of a given sugar unit follows from its $J_{1,2}$ coupling constant, a value of ca. 3–4 Hz indicating an α and that of ca. 7–9 Hz a β isomer. The possible confusions between β-glucose and β-galactose, β-*N*-acetylgalactosamine and β-*N*-acetylglucosamine, and α-*N*-acetylgalactosamine and α-galactose units, respectively, can unambiguously be excluded by establishing the connectivities with the corresponding H-2 resonances with the aid of SDDS. The occurrence of galactose and *N*-acetylgalactosamine residues can additionally be confirmed by their H-4 signals, and an additional proof of α-galactose units follows from their H-5 resonances. As a consequence of this combined approach to establishing the anomeric structure, the sugar components of the oligosaccharide chain are simultaneously identified.

(3) The site of glycosidic linkage to galactose units (3 or 4) can be deduced from five sources of information, (a) the chemical shift of H-2 of the glycosylated unit and (b–e) the chemical shift of H-1, H-2, H-3, and H-5, respectively, of the glycosylating α-galactose unit.

(4) The knowledge of the sequence of sugar residues in an oligosaccharide chain obviously cannot be derived from any single fact but has rather to be built, step by step, on diverse pieces of information. Evidently, none of the protons is uniquely suited for this purpose, and no universal scheme can be offered; on the contrary, the approach to be chosen will depend not only on the substance in question and its similarity to substances already investigated but also on the fragment of the structure to be elucidated. For example, if data on substances **1–9** were available, the SDDS results on the pentasaccharide ceramide **10** would hardly leave any doubts with respect to the composition, anomeric structure, sites of glycosidic joints, and sequence of its first three sugar residues linked to ceramide. Further, the presence of two more sugars, viz., the α- and β-galactose units, would follow from the analysis of the H-1 and H-2 signals, and it would be obvious that β-galactose is the glycosylated one [δ 4.29 (H-1)], the glycosidic linkage being at its site 3 [δ 3.42 (H-2)]. As another example, the important question in sequence determination, the terminal vs. inner location of a sugar unit, can also be answered for β-galactose residues by analyzing the H-3 and H-4 resonances, whereas the H-4 and H-5 resonances answer the same question for α-galactose units. These examples were given to illustrate the approach, but they have by no means exhausted its potential. It should be added, however, that it is often not necessary to seek information on the succession of every two members of the oligosaccharide chain as knowledge concerning some fragments may be gained automatically by logic after the sequence of other fragments has been established.

Finally, the problems of secondary structure will briefly be touched. In a recent article on blood-group determinants, Lemieux (1978) interpreted the chemical shifts of some protons from the point of view of their deshielding by oxygen atoms of the neighboring sugar residues. The shifts were correlated

with interatomic distances computed by hard-sphere calculations. This very promising attempt to elucidate the relative orientations between sugar residues in the oligosaccharide chain was based on the signals of H-1, H-5, and methyl protons. No doubt, the H-2, H-3, and H-4 signals which can be found by SDDS are potentially no less useful for this purpose. It should be pointed out that on several occasions this approach was used in a qualitative sense when presenting the results in this work.

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