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A 500-MHz ^{1}H-N.M.R. STUDY OF OLIGOSACCHARIDES DERIVED FROM GANGLIOSIDES BY OZONOLYSIS–ALKALINE FRAGMENTATION*

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ABSTRACT

The structural-reporter-group resonances in the ^{1}H-n.m.r. spectra of a series of ganglioside-derived oligosaccharides are completely assigned. The three different types of sialic acid residues which may occur in these compounds could be adequately characterized. They are distinguishable on the basis of the set of chemical shifts of their H-3*a* and H-3*e* atoms. The mutual influence of GalNAc β-(1→4) and Neu5Ac α-(2→3), both linked to the same Gal residue, on the ^{1}H-n.m.r. parameters of each other, reflects a well-defined solution conformation, which is held to be responsible for the resistance of this "internal" sialic acid towards various sialidases.

INTRODUCTION

During the past decade, high-resolution ^{1}H-n.m.r. spectroscopy has become a powerful method in the structure determination of glycans derived from glycoproteins. Thanks to the availability of a consistent series of glycopeptides and oligosaccharides of gradually increasing structural complexity, we were able to develop empirical rules, correlating selective ^{1}H-n.m.r. features with structural parameters: the "*structural-reporter-group concept*"[1,2]. When aiming to extend such ^{1}H-n.m.r. studies to the field of glycolipid–ganglioside oligosaccharide chains, it should be

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kept in mind that the aforementioned compounds were investigated underivatized, using D_2O as a solvent, at ambient temperature, limiting the strict validity of the rules to these experimental conditions. To take advantage of the data from the glycoprotein studies for structural characterization of glycolipid oligosaccharides, it is necessary to have a method to split the lipid part from the oligosaccharide, while the latter remains intact. The ozonolysis–alkaline fragmentation[3] is an example of such a procedure yielding from the glycolipid the intact carbohydrate chain as a reducing oligosaccharide. Here, we report the exploration of the usefulness of the ^{1}H-n.m.r. structural-reporter-group concept in the field of oligosaccharides derived from gangliosides by the ozonolysis procedure.

EXPERIMENTAL

Materials. — Gangliosides of bovine brain were isolated by standard procedures[4]. Single ganglioside components were obtained from the mixture by high-pressure liquid chromatography (column, 1.5 cm inner diameter, 50 cm length; packing, Silica H, Merck, Darmstadt, F.R.G.; eluting system: linear gradient of chloroform–methanol–hexane–water (55:45:10:1–8, v/v).

Ganglioside-derived sialyl-oligosaccharides. — These were obtained according to Wiegandt and Bücking[3]. They were purified by preparative paper chromatography (paper: Schleicher & Schüll, Dassel, F.R.G., 2043 mgl, solvent: 5:5:1:3 (v/v) pyridine–ethyl acetate–acetic acid–water, followed by preparative paper electrophoresis (paper: Schleicher & Schüll, Dassel, F.R.G., 2043 mgl, pH 6.5, buffer: 100:10:890 (v/v) pyridine–acetic acid–water. The sialyl-oligosaccharides were identified by their behaviour against *Vibrio cholerae* sialidase and the chromatographic characterization of the products of partial, as well as total hydrolysis by mineral acid.

The neutral oligosaccharides. — Gangliotriaose ($GgOse_3$) and gangliotetraose ($GgOse_4$) were obtained from the corresponding glycosphingolipids (Gg_3Cer and Gg_4Cer, respectively), as described earlier[5].

500-MHz ^{1}H-N.m.r. spectroscopy. — Prior to ^{1}H-n.m.r. spectroscopic analysis, the oligosaccharides (100–500 μg) were repeatedly treated with D_2O at neutral pD, and at room temperature, with intermediate lyophilization. Finally, the samples were dissolved in 0.4 mL of D_2O (99.96 atom % D, Aldrich, Milwaukee, WI, U.S.A.).

500-MHz ^{1}H-N.m.r. spectroscopy was performed on a Bruker WM-500 spectrometer (S.O.N. hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands), operating in the Fourier-transform mode, and equipped with a Bruker Aspect-2000 computer. The spectra were obtained with quadrature phase-detection using a 90° pulse, and taken up in 16K data points with an acquisition time of 3.28 s at a spectral width of 2600 Hz. In general, a few hundred acquisitions were accumulated for each sample. The probe temperature was 27.0 (±0.1)°. Data processing involved resolution enhancement

by Lorentzian-to-Gaussian transformation, followed by a 32K Fourier transformation.

The chemical shifts are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 p.p.m.

RESULTS

Purification and characterization of ganglioside oligosaccharides. — Sialyloligosaccharides that were obtained from known, purified single ganglioside components could be completely freed from trace impurities by consecutive preparative paper-chromatography and -electrophoresis. The ganglioside sugars showed characteristic paper-electrophoretic properties, as described earlier[4]. A further identification was by their cleavage by (or resistance to) *Vibrio cholerae* sialidase and the chromatographic identification of the degradation products.

500-MHz ^{1}H-N.m.r. spectroscopic studies. — 500-MHz ^{1}H-N.m.r. spectra were recorded of the 7 ganglioside-derived oligosaccharides listed in Table I. The relevant ^{1}H-n.m.r. data for these compounds, together with those for Lac as a reference, are summarized in Table II. The spectral features of the compounds are arranged into three sections, namely, for the lactose (Lac) series, for the triaose ($GgOse_3$) series, and for the tetraose ($GgOse_4$) series, respectively.

The spectrum of II^3(Neu5Ac)$_2$Lac is given in Fig. 1, as an example of the Lac series. This disialyllactose may be conceived as an extension of II^{3}Neu5AcLac with a second Neu5Ac residue in α-(2→8) linkage to the α-(2→3)-linked one. The ^{1}H-n.m.r. characteristics of Lac and II^{3}Neu5AcLac have been described[6–9]. The α-(2→3) type of linkage between Neu5Ac and Gal is reflected by the typical chemical-shift values of Neu5Ac H-3*a* (δ 1.799) and H-3*e* (δ 2.757), and also by the shift effects on H-1 ($\Delta\delta$ 0.081) and H-3 ($\Delta\delta$ 0.47) of Gal, in comparison to Lac (see Table II). In the spectrum of II^3(Neu5Ac)$_2$Lac (Fig. 1), two sets of Neu5Ac structural-reporter group resonances are observed, namely, two nearly coinciding H-3*a* triplets at δ 1.74, two H-3*e* doublets of doublets at δ 2.68 and 2.78, and two *N*-acetyl singlets at δ 2.068 and 2.031, respectively. The assignment of the H-3 signals within each pair to one of the Neu5Ac residues is based on the differences in line width (H-3*a*) and/or in anomerization effect (H-3*e*). It is reasonable to assume that the chemical shifts of Neu5Ac in α-(2→3) linkage are more sensitive to the anomeric configuration of the reducing Glc residue than those of Neu5Ac in α-(2→8) linkage. The latter is likely to be further remote from this anomeric center. The assignment of the *N*-acetyl signals is based on the observation that, for terminal Neu5Ac residues, the *N*-acetyl singlet is always found at δ 2.030 ($\pm$0.003) irrespective of the type of linkage[2,6,7]. Thus, it may be concluded that attachment of Neu5Ac in α-(2→8) linkage to another Neu5Ac results in a set of reporter-group resonances typical for the α-(2→8) type of linkage of the residue itself, namely, H-3*a* at δ 1.741, H-3*e* at δ 2.779 and 5Ac at δ 2.031. These chemical-shift values for

TABLE I

STRUCTURES OF THE GANGLIOSIDE-DERIVED OLIGOSACCHARIDES USED FOR 500-MHz ^{1}H-N.M.R. STUDIES. THE NUMBERING SYSTEM FOR THE NEUTRAL MONOSACCHARIDE RESIDUES IS INDICATED IN THE LAST STRUCTURE

Abbreviation[a]	*Structure*
II^{3}Neu5AcLac	βGal(1→4)Glc / αNeu5Ac(2→3)
II^3(Neu5Ac)$_2$Lac	βGal(1→4)Glc / αNeu5Ac(2→8)αNeu5Ac(2→3)
GgOse$_3$	βGalNAc(1→4)βGal(1→4)Glc
II^{3}Neu5AcGgOse$_3$	βGalNAc(1→4)βGal(1→4)Glc / αNeu5Ac(2→3)
GgOse$_4$	βGal(1→3)βGalNAc(1→4)βGal(1→4)Glc
II^{3}Neu5AcGgOse$_4$	βGal(1→3)βGalNAc(1→4)βGal(1→4)Glc / αNeu5Ac(2→3)
IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$	IV III II I αNeu5Ac(2→3)βGal(1→3)βGalNAc(1→4)βGal(1→4)Glc / αNeu5Ac(2→3)

[a]Nomenclature recommended by the IUPAC–IUB commission on Biochemical Nomenclature.

H-3*a* and H-3*e* are in complete accord with those described for Neu5Gc α-(2→8)-linked to αNeu5Gc(2→6)GalNAc-ol[7,10]: δ H-3*a* 1.743 and δ H-3*e* 2.779. Moreover, shift effects are induced on the reporter groups of the substituted Neu5Ac residue (for H-3*a*), $\Delta\delta$ −0.06; for H-3*e*, $\Delta\delta$ −0.08; and for 5Ac, $\Delta\delta$ 0.038, respectively). The resonance positions of the Gal-II reporter groups are only moderately affected by the elongation (see Table II), whereas those for Glc-I are not influenced. It should be noted that, because of the α-(2→8) substitution, the H-8 and H-9 signal of II^{3}Neu5Ac undergo considerable downfield shift-effects, from δ ~3.9 to 4.14, and from δ ~3.9 to 4.18, respectively. The assignment of these signals, outside the bulk region of the II^3(Neu5Ac)$_2$Lac spectrum (see Fig. 1) was proved by selective-irradiation experiments.

In comparison with the spectrum of Lac, that of GgOse$_3$ shows additional reporter-group signals for GalNAc-III in β-(1→4) linkage to Gal, namely, H-1 (δ ~4.63), H-4 (δ ~3.912), and NAc (δ ~2.055). The H-1 and NAc signals appear to be sensitive to anomerization; they are doubled in the anomeric ratio α:β = 7:10 (compare ref. 6). The β linkage of GalNAc-III may be inferred from its $J_{1,2}$ value, being 8.4 Hz. Attachment of GalNAc-III in β-(1→4) linkage to Gal-II leads to a

TABLE II

PERTINENT ^{1}H-N.M.R. PARAMETERS OF STRUCTURAL-REPORTER-GROUP PROTONS OF CONSTITUENT MONOSACCHARIDES FOR A SERIES OF OLIGOSACCHARIDES DERIVED FROM GANGLIOSIDES[a]

Residue	*Reporter group*	*(Coupling constant)*	*Anomer of oligo-saccharide*	*Chemical shift (coupling constant) in*[b]							
				Lac	*II^{3}Neu5AcLac*	*II^3(Neu5Ac)$_2$Lac*[c]	*GgOse$_3$*	*II^{3}Neu5AcGgOse$_3$*	*GgOse$_4$*	*II^{3}Neu5AcGgOse$_4$*	*IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$*
Glc-I	H-1	($^3J_{1,2}$)	α	5.221 (3.8)	5.220 (3.8)	5.220 (3.7)	5.219 (3.8)	5.218 (3.7)	5.220 (3.7)	5.218 (3.8)	5.217 (3.8)
			β	4.663 (8.0)	4.661 (7.9)	4.659 (7.9)	4.665 (7.9)	4.665 (7.9)	4.665 (8.0)	4.664 (7.9)	4.667 (8.1)
	H-2	($^3J_{2,3}$)	β	3.284 (8.5)	3.281 (8.5)	3.287 (9.3)	3.272 (9.2)	3.275 (9.3)	3.273 (9.3)	3.275 (9.5)	3.272 (9.5)
Gal-II	H-1	($^3J_{1,2}$)	α	4.448 (7.8)[d]	4.530 (7.8)	4.527 (7.8)	4.441 (7.8)	4.530 (8.1)	4.443 (7.8)	4.532 (7.8)	4.529 (7.9)
			β		4.528 (7.8)	4.523 (7.9)	4.439 (7.9)	4.528 (7.9)	4.441 (7.9)	4.530 (7.9)	4.525 (7.9)
	H-2	($^3J_{2,3}$)	α	3.536 (9.9)	3.6–3.9 (9.9)[d]	3.5–3.9 (9.9)	3.419 (9.9)	3.370 (9.6)	3.422 (9.9)	3.370 (9.6)	3.385 (9.8)
			β	3.528 (9.9)			3.410 (10.1)	3.361 (9.8)	3.412 (9.9)	3.361 (9.6)	3.377 (9.8)
	H-3	($^3J_{3,4}$)	α	3.64 (3.4)[d]	4.114 (3.3)	4.095 (3.1)	3.5–3.9 (3.1)	4.153 (2.9)	3.5–3.9 (3.2)	4.146[e] (≈3)[e]	4.13[e] (≈3)[e]
			β		4.110 (3.3)	4.090 (3.1)		4.147 (3.1)		4.140[e]	
	H-4	($^3J_{4,5}$)	α,β	3.910 (1.0)	3.959 (≤1.0)	3.969 (≤0.9)	4.096 (≤0.9)	4.116 (≤0.9)	4.110 (≤0.9)	4.129[e] (≤1)[e]	4.118[e] (≤1)[e]
GalNAc-III	H-1	($^3J_{1,2}$)	α				4.638 (8.4)	4.744 (8.4)	4.706 (8.5)	4.787 (8.5)	4.778 (8.7)
			β				4.633 (8.4)	4.741 (8.4)	4.700 (8.5)	4.784 (8.6)	4.776 (8.4)
	H-2	($^3J_{2,3}$)	α				3.5–3.9 (n.d.)	3.5–3.9 (n.d.)	3.5–3.9 (n.d.)	4.049 (10.7)	4.041 (9.6)
			β							4.045 (10.3)	4.038 (9.9)
	H-4	($^3J_{3,4}$)	α,β				3.912 (3.1)	3.920 (3.0)	4.157 (3.0)	4.163 (3.5)	4.165 (3.8)
	NAc		α				2.057	2.018	2.044	2.006	2.011
			β				2.054	2.015	2.042	2.004	2.008
Gal-IV	H-1	($^3J_{1,2}$)	α,β						4.449 (7.8)	4.540 (7.8)	4.607 (7.9)
	H-2	($^3J_{2,3}$)	α,β						3.532 (10.0)	3.520 (9.9)	3.540 (9.7)
	H-3	($^3J_{3,4}$)	α,β						3.5–3.9 (3.0)	3.5–3.9 (3.1)	4.090 (3.3)
	H-4	($^3J_{4,5}$)	α,β						3.908 (0.9)	3.920 (0.9)	3.951 (≤0.9)
II^{3}Neu5Ac[c]	H-3*a*	($^2J_{3a,4}$)	α,β		1.799 (12.1)	1.739 (11.9)		1.923 (11.9)		1.929 (11.9)	1.913 (11.9)
	H-3*e*	($^3J_{3e,4}$)	α		2.757 (4.9)[d]	2.682 (4.8)[f]		2.666 (4.8)		2.664 (4.8)	2.683 (4.7)
			β			2.679 (4.5)[f]		2.664 (4.7)		2.662 (4.5)	2.681 (4.7)
	5Ac	($^2J_{3a,3e}$)	α,β		2.030 (−12.4)	2.068 (−12.3)[g]		2.032 (−12.5)		2.033 (−12.8)	2.034 (−12.4)
αNeu5Ac(2→8)	H-3*a*	($^2J_{3a,4}$)	α,β			1.741 (11.9)					
	H-3*e*	($^3J_{3e,4}$)	α,β			2.779 (4.7)					
	5Ac	($^2J_{3a,3e}$)	α,β			2.031 (−12.5)					
IV^{3}Neu5Ac	H-3*a*	($^2J_{3a,4}$)	α,β								1.798 (12.1)
	H-3*e*	($^3J_{3e,4}$)	α,β								2.750 (4.7)
	5Ac	($^2J_{3a,3e}$)	α,β								2.029 (−12.4)

[a]Data were acquired at 500 MHz, for neutral solutions in D_2O at 27°. Chemical shifts are in p.p.m. relative to internal DSS (using internal acetone at δ 2.225); coupling constants (in parentheses) are in Hz. [b]For complete structures of oligosaccharides, see Table I. [c]For this compound, H-8 of II^{3}Neu5Ac is found at δ 4.138, and its H-9 at δ 4.181 and 4.174 for the α and β anomers of the oligosaccharide, respectively. [d]An average value listed for the chemical shift of a proton signal with respect to the α and β anomer of the oligosaccharide means that it could not be localized by inspection, or that its $|\Delta\delta_{\alpha,\beta}|$ is smaller than 0.001 p.p.m. and therefore not observable at 500-MHz. [e]Values could not be determined more accurately, because of higher-order patterns of the strongly coupled H-3 and H-4 resonances. [f,g]Values are interchanged with those of αNeu5Ac(2→8) in ref. 22. Probably because of the lower magnetic field, anomerisation effects are absent in the spectra of ref. 22 leading to a different assignment.

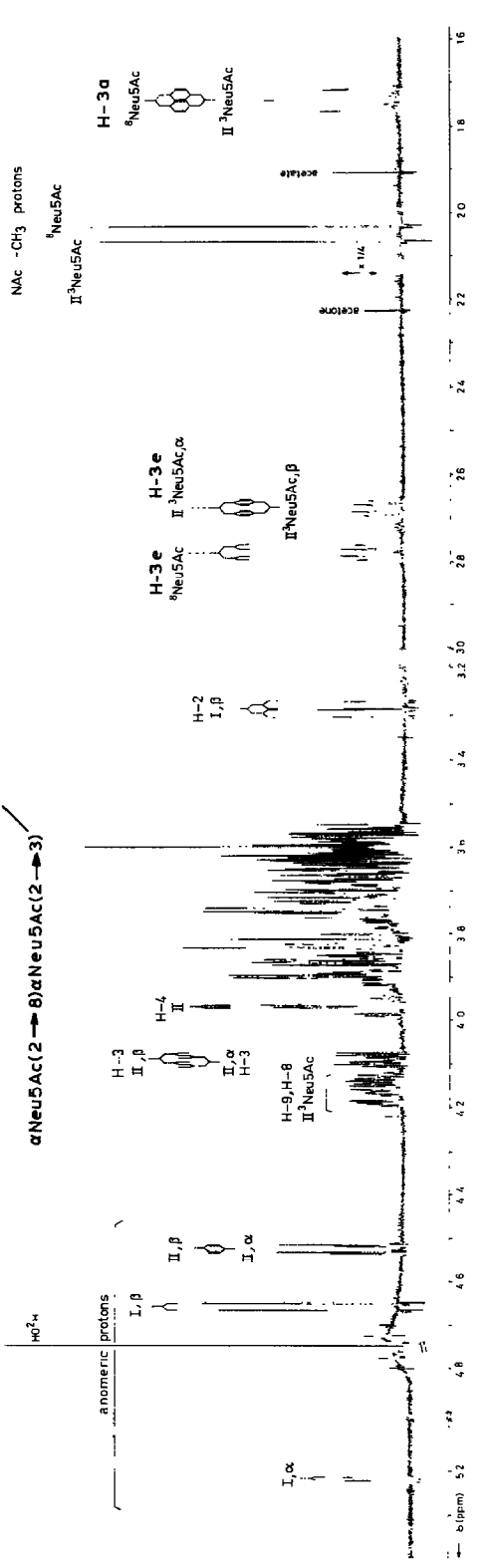

Fig. 1. 500-MHz ^{1}H-N.m.r. spectrum (D_2O; pD 7; 27°) of II^3(Neu5Ac)$_2$Lac. Roman numbers refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated.

significant downfield shift for H-4 of Gal (from δ 3.91 to 4.096). Furthermore, the H-2 signal of Gal-II is shifted upfield to $\delta \sim 3.41$, resonating clearly apart from the bulk of the skeleton protons. Also this signal is doubled because of anomerization (see Table II). The position of H-1 of Gal-II is not affected. These features are in line with the shifts observed for substitution of βGal(1→3)GalNAc-ol by GalNAc in β-(1→4) linkage, resulting in the blood-group Sd^a/Cad-specific determinant[11,12].

The spectrum of II^{3}Neu5AcGgOse$_3$, as a representative of the gangliotriaose series, is shown in Fig. 2. The presence of Neu5Ac is evident from the H-3*a* and H-3*e* signals, and of an additional *N*-acetyl signal, as compared to GgOse$_3$. The strong deviations in chemical shift of this so-called[13,14] internal Neu5Ac residue as compared to the external one in II^{3}Neu5AcLac, are ascribed to the presence of GalNAc-III in β-(1→4) linkage to the same Gal-II. The set of δ H-3*a* 1.92 and δ H-3*e* 2.665 is proposed to be typical for the structural element βGalNAc(1→4)-[αNeu5Ac(2→3)]βGal(1→·) (compare refs. 11 and 12). It should be mentioned that the chemical shift of H-3*e* for the internal, α-(2→3)-linked Neu5Ac ($\delta \sim 2.67$) is in the range of values usually observed for Neu5Ac residues in α-(2→6) linkage to Gal[2,7]. However, the value for H-3*a* (δ 1.92) is thus far unique. It should be mentioned that, for another internal, sialidase-resistant, Neu5Gc residue, namely that linked to GalNAc in *O*-glycosylic structures from trout-egg glycoproteins[15] that is, βGalNAc(1→4)βGal(1→4)[αNeu5Gc(2→3)]βGalNAc(1→3)βGal(1→3)GalNAc-ol, the set of δ H-3*a* 1.855 and δ H-3*e* 2.550 has been found. The introduction of Neu5Ac into GgOse$_3$ gives rise to shift effects on reporter groups of Gal-II as well as of the terminal GalNAc-III. Substitution of Gal by Neu5Ac leads to effects on H-1, H-2, H-3, and H-4 of Gal (see Table II). As far as H-1 and H-3 are concerned, the effects are the same as observed for extension of *N*-acetyllactosamine-type and mucin-type oligosaccharides derived from glycoproteins, with Neu5Ac in α-(2→3) linkage to Gal[2,7]. For H-2 and H-4, the effects could not be traced, until now. Among the GalNAc-III reporter groups, H-1 shows a significant downfield shift ($\Delta\delta \sim 0.11$), whereas the NAc signal shifts upfield ($\Delta\delta \sim -0.04$) (see Table II, and compare refs. 11 and 12).

The GgOse$_4$ oligosaccharide may be conceived as an extension of GgOse$_3$, with a Gal-IV residue in β-(1→3) linkage to GalNAc-III. This is reflected in the ^{1}H-n.m.r. spectrum by an additional H-1 doublet at δ 4.449 (showing no anomerization effect), and an H-4 signal at δ 3.908. Owing to the presence of Gal-IV, the H-1 and H-4 of GalNAc-III are shifted downfield. These effects are similar to those described for attachment of Gal in β-(1→3) linkage to another Gal in proteoglycan oligosaccharides[16]. The NAc signal of GalNAc-III is shifted upfield ($\Delta\delta$ −0.012). Furthermore, H-4 of Gal-II is shifted slightly downfield (see Table II).

The spectrum of II^{3}Neu5AcGgOse$_4$ is depicted in Fig. 3. The shifts and shift effects observed for the structural-reporter groups of constituent monosaccharides Neu5Ac, Gal-II, and GalNAc-III in the step from GgOse$_4$ to II^{3}Neu5AcGgOse$_4$ are completely analogous to those for the step from GgOse$_3$ to II^{3}Neu5AcGgOse$_3$. In addition, it should be mentioned that the presence of the internal sialic acid (δ

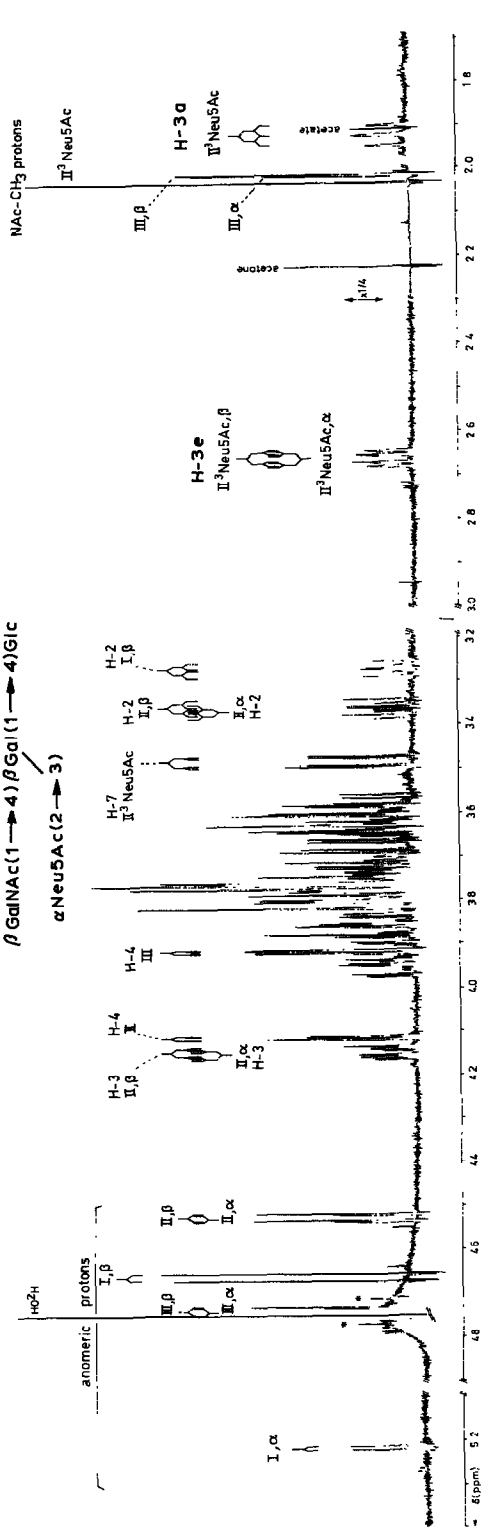

Fig. 2. 500-MHz ^{1}H-N.m.r. spectrum (D_2O; pD 7; 27°) of II^{3}Neu5AcGgOse$_3$. Roman numbers refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated.

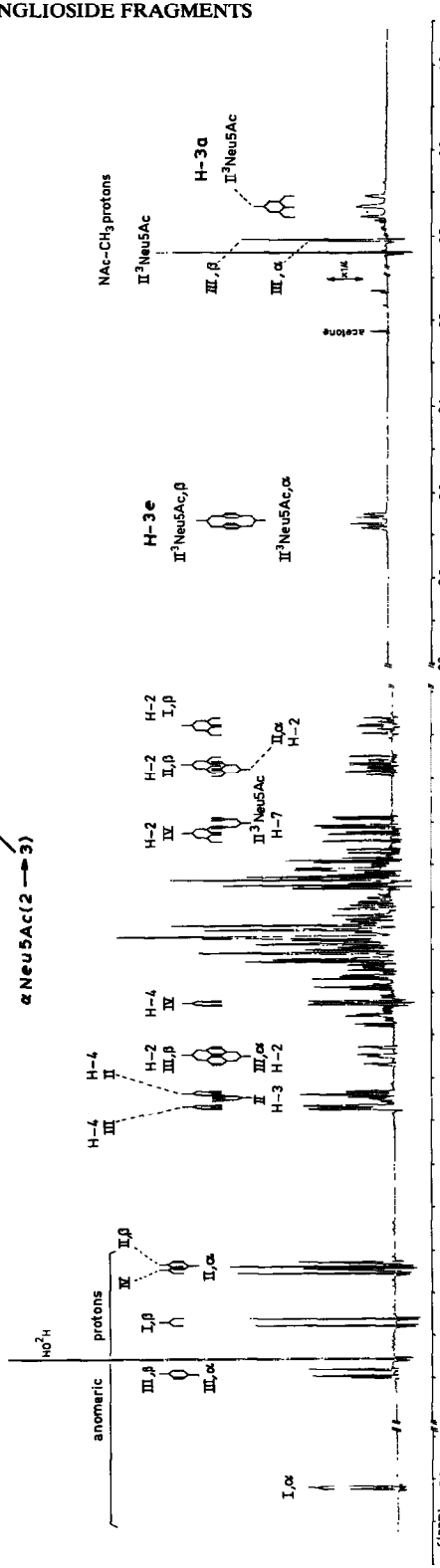

Fig. 3. 500-MHz ^{1}H-N.m.r. spectrum (D_2O; pD 7; 27°) of II^{3}Neu5AcGgOse$_4$. Roman numbers refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated.

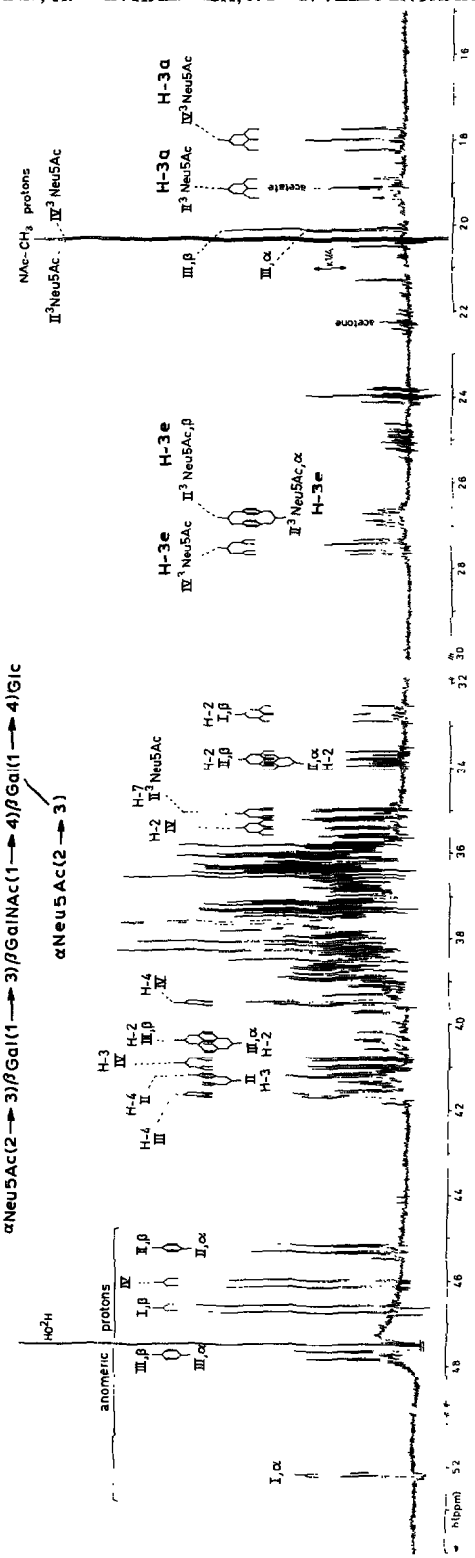

Fig. 4. 500-MHz ^{1}H-N.m.r. spectrum (D_2O; pD 7; 27°) of IV^{3}Neu5AcII^3 Neu5AcGgOse$_4$. Roman numbers refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated.

H-3*a* ~1.93) influences the positions of the Gal-IV reporter groups (e.g. $\Delta\delta$ for H-1, 0.09), but not vice versa (see Table II).

The spectrum of IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$ is shown in Fig. 4. Two sets of Neu5Ac structural-reporter groups are observed. The chemical shifts of H-3*a*, H-3*e*, and 5Ac of the IV^{3}Neu5Ac are identical to those observed for II^{3}Neu5AcLac (Table II), and, in general, for αNeu5Ac(2→3)βGal(1→·)[2,7], irrespective of the sugar adjacent to Gal (Glc[8,9], GlcNAc[2,7], GalNAc-ol[7,17], or GalNAc[18]). The second set of H-3*a*, H-3*e* and 5Ac chemical shifts is essentially the same as that mentioned already as being typical for internal II^{3}Neu5Ac. Regarding the effects of introduction of IV^{3}Neu5Ac, the H-1 and H-3 resonances of Gal-IV are shifted downfield, as could be expected from the well-documented effects of elongation of Gal by Neu5Ac in α-(2→3) linkage[2,7]. The attachment of IV^{3}Neu5Ac hardly influences the chemical shifts of the reporter groups of II^{3}Neu5Ac. It is remarkable that the linewidth of the H-3 signals of the internal II^{3}Neu5Ac are significantly larger than those of the corresponding IV^{3}Neu5Ac signals (see Fig. 4). To gain more insight into the origin of this phenomenon, the ^{1}H-n.m.r. spectrum of the reduced analogue of the disialylgangliotetraose was recorded. The spectrum of the alditol showed a pair of equally sharp H-3*e* signals, at δ 2.749 and 2.688, indicating that the line-broadening of H-3*e* of II^{3}Neu5Ac in IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$ is predominantly because of anomerization. However, the difference in linewidth between the H-3*a* signals, at δ 1.916 and 1.799, remained unaltered after reduction. The latter may be ascribed to steric hindrance of rotational freedom of II^{3}Neu5Ac, as suggested in ref. 10. Otherwise, in addition to the disappearance of all doublings of signals in the anomeric ratio, and the replacement of Glc signals by Glc-ol signals, the H-1 doublet of Gal-II was mainly influenced by the reduction, both as to chemical shift (for the alditol, δ 4.596) as well as to the $J_{1,2}$ value (7.3 Hz). This result is in line with the changes observed in the step from Lac to Lac-ol (H-1 of Gal-II for Lac-ol, δ 4.504; $J_{1,2}$ 7.2 Hz). The NAc signal of GalNAc-III shifted to δ 2.014. The difference in linewidth between the II^{3}Neu5Ac and IV^{3}Neu5Ac signals in the reducing oligosaccharide permits assignment of the observed 5Ac signals: the broader one at δ 2.034 is attributed to the internal II^{3}Neu5Ac.

DISCUSSION

Among the sialic acid-containing glycoconjugates, the sialylglycolipids, for instance the gangliosides of the brain of vertebrates, show a high degree of structural complexity (for a review, see ref. 4). For sialic acid, three major linkage-types may be distinguished: (*a*) sialic acid, α-(2→3)-linked to Gal without any other substituent being present at Gal, (*b*) "internal" sialic acid, that is Neu5Ac α-(2→3)-linked to Gal that is substituted at the adjacent O-4 position by GalNAc, and (*c*) sialic acid linked to another sialic acid by an α-(2→8) linkage (see Table I).

The constituent sialic acid residues in these compounds display properties that depend on their position along the carbohydrate backbone and on the

neighbouring saccharide. In particular, the "internal" sialic acid in such gangliosides as II^{3}Neu5Ac-Gg$_3$Cer, II^{3}Neu5Ac-Gg$_4$Cer, and IV^{3}Neu5AcII^3-Neu5Ac-Gg$_5$Cer, shows "anomalous" behaviour in several respects, for instance, it is resistant toward many bacterial and viral sialidases, and it has a vertical dipole moment opposite to that of a terminal sialic acid. Both properties could be due to steric hindrance caused by neighbouring groups (see ref. 10).

To analyse further the environment of the sialic acid groups in the hydrophilic part of the molecules, we have established the detailed ^{1}H-n.m.r. parameters of the structural-reporter groups of ganglioside-derived oligosaccharides of increasing structural complexity. As expected, great advantage in the interpretation of the ^{1}H spectra could be taken from the availability of a catalogue of ^{1}H spectra of glycoprotein glycans, also recorded in aqueous solution[2]. The three aforementioned types of sialic acid may readily be distinguished on the basis of the chemical shifts of their H-3*a* and H-3*e* signals. For terminal αNeu5Ac(2→3)βGal(1→·), we observed δ H-3*a* 1.80, δ H-3*e* 2.76; for "internal" αNeu5Ac(2→3)[βGalNAc(1→4)]β-Gal(1→·), δ H-3*a* 1.92 and δ H-3*e* 2.67 were found, whereas for the Neu5Ac residues in the sequence αNeu5Ac(2→8)αNeu5Ac(2→3)βGal(1→·), δ H-3*a* 1.74 for both and δ H-3*e* 2.78 and 2.68, respectively, are observed. In addition, the shift effects upon stepwise extension of Lac to, finally, IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$, could be traced.

In particular, the effects on the chemical shifts of the Neu5Ac reporters in the step from II^{3}Neu5AcLac to II^{3}Neu5AcGgOse$_3$ by attachment of GalNAc-III in β-(1→4) linkage to Gal-II, and the mutual influences of α-(2→3) attachment of Neu5Ac to Gal-II upon the positions of the H-1 and NAc signals of GalNAc-III (in the step from GgOse$_3$ to II^{3}Neu5AcGgOse$_3$) reflect a specific through-space interaction between these Neu5Ac and GalNAc residues (compare ref. 12). As far as II^{3}Neu5AcGgOse$_3$ and II^{3}Neu5AcGgOse$_4$ are concerned, our data show excellent agreement with those acquired by Sabesan *et al.*[20] at 400 MHz, 5°. In the latter report, in conjunction with the paper by Sillerud and Yu[21], a molecular model for the II^{3}Neu5AcGgOse$_4$ was proposed, based on ^{1}H- and ^{13}C-n.m.r. measurements and computer energy-calculations. Our results, in terms of mutual influences and shift-effects qualitatively expressed, fit well with the model proposed. It is of interest for future research to compare the conformational structure and the dynamic behaviour of the "internal" sialic acid in intact gangliosides with its behaviour in free oligosaccharides.

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