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360-MHz ^{1}H Nuclear-Magnetic-Resonance Spectroscopy of Sialyl-Oligosaccharides from Patients with Sialidosis (Mucolipidosis I and II)

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360-MHz proton nuclear magnetic resonance spectra were recorded of 10 sialyl-oligosaccharides isolated from urine of sialidosis patients. Their structures are related to the complex asparagine-linked glycan chains of glycoproteins. By correlation of these spectra and comparison with spectra of reference glycopeptides and sialyl-lactose isomers it was possible to assign all signals belonging to anomeric, mannose H-2, sialic acid H-3 and *N*-acetyl protons. The number of the constituting monosaccharide residues of the oligomers can be obtained by integration of the above-mentioned signals. The chemical shifts of the anomeric and mannose H-2 protons give information about the type of glycan structure (mono-, bi-, triantennary) and the presence of terminal sialic acid at each of the antennas. The chemical shifts of sialic acid H-3 protons are typical for sialic acid residues in $2\rightarrow3$ or $2\rightarrow6$ linkage to galactose.

Recently it has been shown that the application of 360-MHz ^{1}H-NMR spectroscopy is a highly powerful technique in the structure elucidation of oligosaccharides and glycopeptides [1–5]. Most of the signals of anomeric protons in these high-resolution spectra are well separated. Their resonance positions are characteristic for the structure and in fact can be used as indicators for the type and position of the various glycosidic linkages and for the sequence of the constituting monosaccharide residues. The resonances of the non-anomeric protons can provide valuable additional information as has been demonstrated in particular for the H-2 protons of the mannose residues of the frequently occurring mannotriosido branching core of complex glycans [2–5].

In this paper the investigation of oligosaccharides and glycopeptides containing one or more sialic acid residues is described. The greater part of these compounds stem from the urine of patients suffering from inborn errors of metabolism associated with neuraminidase deficiency. These mucolipidosis (sialidosis) patients have increased levels of urinary sialyl-oligosaccharides [6] which originate from incomplete glycoprotein catabolism. The structures of these oligosaccharides have been determined by chemical methods [6, 7] and are related to the complex biantennary type of glycan chain [8].

Abbreviations. Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Man, mannose; AcNeu, *N*-acetylneuraminic acid (sialic acid); Asn, asparagine; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

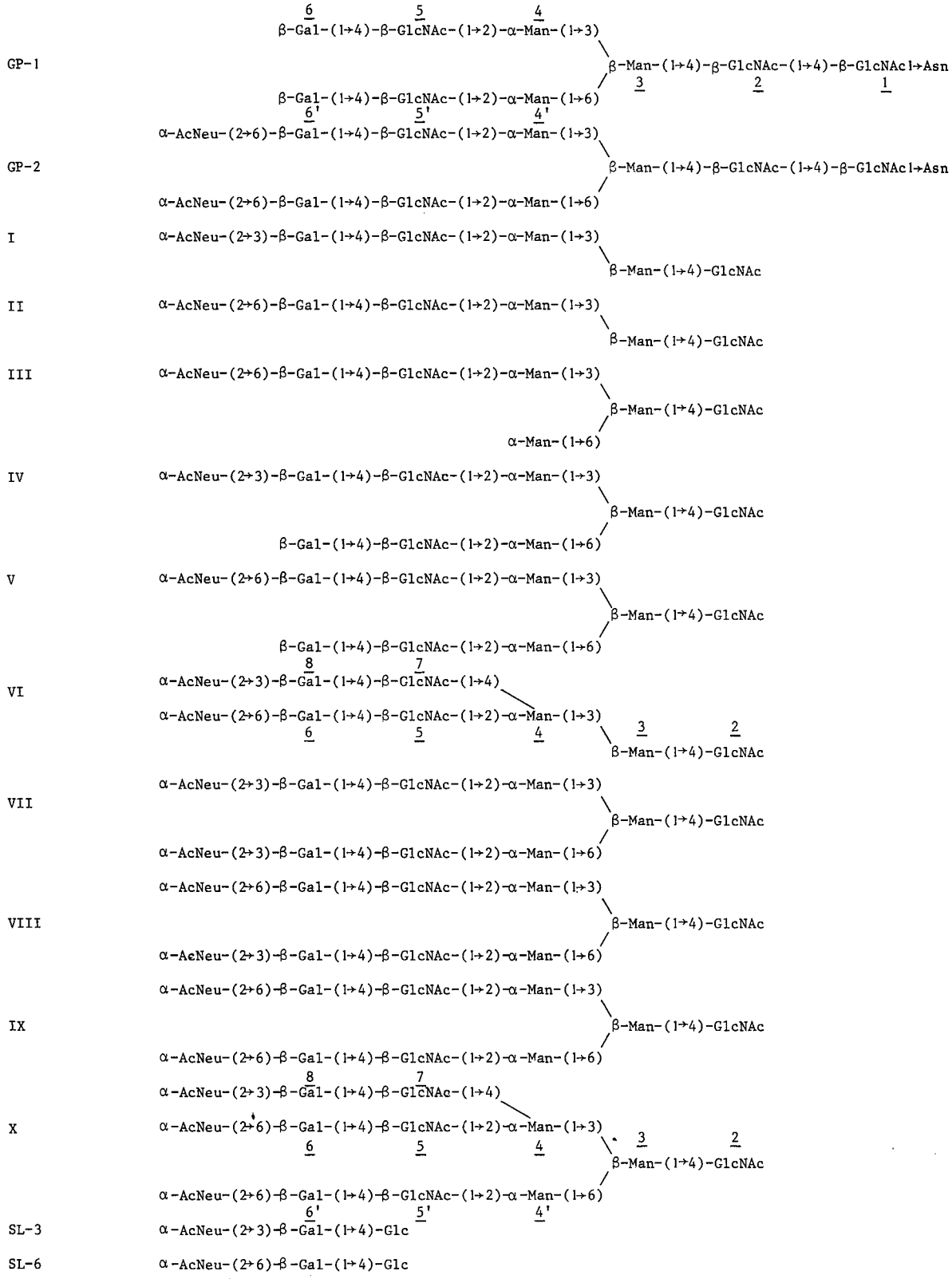
The isolation of oligosaccharides I–X from urine of patients with sialidosis [6, 7], of GP-1 and GP-2 from human serotransferrin [2, 9] and of sialyl-lactose isomers [10] has been described before. The structures of the compounds are presented in Table 1.

Solutions of the oligosaccharides and glycopeptides were neutralized if necessary and exchanged three times in ^2H_2O with intermediate lyophilization. Spectral analysis of 0.02–0.05 M solutions of the compounds in ^2H_2O (99.9%, Aldrich) was carried out on a Bruker HX-360 spectrometer, operating in the Fourier transform mode at probe temperatures of 25 °C or 60 °C. Chemical shifts at 25 °C are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O: $\delta = 2.225$ ppm).

RESULTS AND DISCUSSION

From all compounds listed in Table 1 360-MHz ^{1}H NMR spectra were recorded at 25 °C (δ HO^{2}H

Table 1. *Structures of the urinary sialyl-oligosaccharides and the reference compounds*
The coding of monosaccharide units in all compounds corresponds to those given for GP-1, VI and X



≈ 4.78 ppm) and at 60 °C (δ HO^2H shifted to ≈ 4.35 ppm). As a typical example of the compounds I–V and VII–IX which are all related to the biantennary structure GP-1, the spectrum of VIII is given in Fig. 1. The compounds VI and X have an additional sialyl-*N*-acetyllactosamine branch linked to mannose 4 (for coding of monosaccharide residues see Table 1) and are therefore derived from the triantennary type of complex glycan chains [8]. The spectrum of X is given in Fig. 2. The chemical shifts of the anomeric, *N*-acetyl, mannose H-2, and sialic acid H-3 axial and equatorial protons of all compounds are compiled in Table 2.

The assignment of the signals of the anomeric protons in the sialo compounds was made by using the resonance positions in the spectrum of GP-1 as reference data. Extension of both branches of GP-1 with sialic acid in 2 → 6 linkage to galactose (GP-2) introduces a few significant changes in the chemical shifts of the anomeric protons of the residues 6, 6′, 5, 5′, 4 and 4′. Deletion of the GlcNAcβ1 → Asn part which affords structure IX has no effect on the chemical shifts of the above-mentioned anomeric protons.

In compound V the sialic acid residue in the lower branch is missing. In comparison to the asialo structure GP-1 it is evident that sialic acid causes only shift increments for Gal-6, GlcNAc-5 and Man-4, leaving the anomeric protons of the lower branch unaffected. The same effects are observed in II and III wherein the lower branch is shortened up to residue 3 or 4′ respectively.

Compound VII differs from IX only in the type of linkage of the sialic acid residues to galactose, being 2 → 3 instead of 2 → 6. Attachment of sialic acid residues by 2 → 3 linkages to Gal-6 and 6′ introduces only shift increments for H-1 of Gal-6 and 6′ when compared to GP-1; the long-distance effects as described for a 2 → 6-linked sialic acid residue do not occur. From the spectral data of the partial structures I and IV it is clear that the 2 → 3-linked sialic acid residue affects only the H-1 of the directly attached galactose residue and not the H-1 of galactose in the other branch.

Both types of sialic acid linkages occur in VIII each giving rise to its typical effects as described above. The NMR data indicate immediately to which branch the 2 → 6-linked sialic acid residue is connected since the H-1 signal of Man-4 shows the characteristic shift increment (5.119 → 5.138 ppm) whereas the H-1 of Man-4′ is unaffected (4.926 ppm). For this kind of assignment the chemical shifts of the anomeric protons of Gal-6 and 6′ cannot be used because these protons are indistinguishable. The same holds for the anomeric protons of GlcNAc-5 and 5′. The influence on anomeric protons in the glycan chain of attachment of a sialic acid residue to the 3 or 6 position of galactose are summarized in Table 3. Sialic acid linked to galactose affects also the resonance positions of the *N*-acetyl protons of the *N*-acetylactosamine units but these effects are independent of the type of sialic acid linkage (see Table 3).

An interesting phenomenon in the spectra of the sialo compounds is the position of the axial and equatorial H-3 protons of sialic acid. These protons do not coincide with the bulk of the non-anomeric protons. Their chemical shifts are characteristic of the type of linkage of the sialic acid residue to any galactose moiety of *N*-acetyllactosamine as indicated in Table 4. From Table 2 it becomes clear that the resonance position of a sialic acid *N*-acetyl group depends on the type of glycosidic bond of the sialic acid residue as well as on the branch on which it is present.

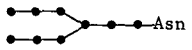
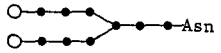
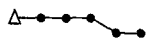
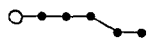
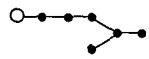
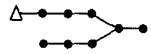
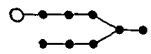
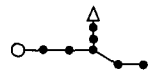
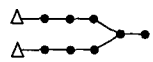
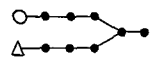
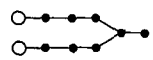
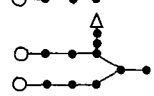
The biantennary structures (IV, V, VII, VIII, IX) can be recognized on the basis of the resonance pattern of the H-2 protons of the mannotriosido branching core (resonances at 4.12, 4.20 and 4.26 ppm, see Table 2). If the lower branch is completely absent, the resonance position of H-2 of Man-3 is changed (4.26 → 4.24 ppm, compounds I, II). In III the H-2 resonance of Man-4′ is buried in the bulk of the non-anomeric protons because Man-4′ is not glycosylated in position 2.

Extension of the biantennary structure with the additional sialyl *N*-acetyllactosamine chain as in X is expressed in the resonance position of H-2 of Man-3 and Man-4 (both at 4.22 ppm). It has been found that in other structures of this type the same set of parameters occurs (unpublished results). The determination of the positions of the 2 → 3 and 2 → 6-linked sialic acid residues in X can easily be carried out on the basis of the NMR data. Integration of the axial and equatorial H-3 protons of sialic acid shows that the molar ratio of 2 → 3-linked to 2 → 6-linked residues is 1:2. The chemical shifts of the H-1 protons of residues 4, 4′, 5, 5′, 6, 6′, 7 and 8 point to the presence of 2 → 6-linked residues in the upper (4, 5, 6) and the lower (4′, 5′, 6′) branch of the molecule, whereas the 2 → 3-linked sialic acid residue is attached to Gal-8. Structure VI can be conceived as derived from X by deletion of the lower (4′, 5′, 6′) branch. This is reflected by the absence of the signal at 4.94 ppm (H-1 of Man-4′) and by the integral values of the signals at 4.60, 4.44 and 4.12 ppm (see Table 2).

With regard to the sialyllactose isomers it has to be noted that the chemical shifts of the H-3 protons in SL-3 are identical to those for 2 → 3-linked sialic acid residues in the oligosaccharides I–X. However the H-3 protons in SL-6 occupy deviating resonance positions with regard to the corresponding protons in the oligosaccharides. The *N*-acetyl protons of the sialic acid residues in SL-3 as well as in SL-6 resonate at 2.030 ppm. This is in contrast to the positions of

Table 2. *^{1}H chemical shifts of anomeric, mannose H-2, sialic acid H-3 and* N*-acetyl protons for sialyl-oligosaccharides I–X and reference compounds*

Chemical shifts in ^2H_2O at 25 °C are given in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate. For coding of monosaccharide residues and complete structures see Table 1; ●— = neutral or amino sugar residue; △— = AcNeu(2→3); ○— = AcNeu-(2→6). The α and β anomeric protons of a reducing unit were in the molar ratio of 0.65:0.35. Values at ≈ 4.72 and ≈ 4.77 ppm cannot be determined more accurately (± 0.01 ppm) due to interference of the HO^{2}H line at 25 °C

Compound	Schematic Structure ·	H-1 of residue											
		1	2α	2β	3	4	4'	5	5'	6	6'	7	8
GP-1		5.072	-	4.616	∿4.77	5.119	4.926	4.581	4.581	4.470	4.470	-	-
GP-2		5.073	-	4.598	∿4.77	5.133	4.946	4.598	4.598	4.447	4.447	-	-
I		-	5.206	∿4.72	∿4.77	5.122	-	4.579	-	4.544	-	-	-
II		-	5.206	∿4.72	∿4.77	5.139	-	4.601	-	4.446	-	-	-
III		-	5.212	∿4.72	∿4.77	5.138	4.919	4.604	-	4.443	-	-	-
IV		-	5.212	∿4.72	∿4.77	5.123	4.930	4.578	4.578	4.552	4.471	-	-
V		-	5.213	∿4.72	∿4.77	5.131	4.929	4.603	4.583	4.447	4.468	-	-
VI		-	5.209	∿4.72	∿4.77	5.139	-	4.593	-	4.446	-	4.549	4.549
VII		-	5.211	∿4.72	∿4.77	5.117	4.920	4.571	4.571	4.546	4.546	-	-
VIII		-	5.212	∿4.72	∿4.77	5.138	4.926	4.609	4.578	4.447	4.546	-	-
IX		-	5.213	∿4.72	∿4.77	5.136	4.952	4.605	4.605	4.444	4.444	-	-
X		-	5.214	∿4.72	∿4.77	5.134	4.943	4.601	4.601	4.443	4.443	4.550	4.550
SL-3		-	-	-	-	-	-	5.221(α)[d] 4.663(β)[d]		4.531		-	-
SL-6		-	-	-	-	-	-	5.224(α)[d] 4.666(β)[d]		4.427		-	-

[a] Signal of two protons.
[b] In the case of an AcNeu residue linked to position 3 of galactose, the H-3 of that residue resonates in the range of 4.11–4.12 ppm which makes a more accurate calculation of δ for H-2 of Man-4' difficult.
[c] Values may be interchanged.
[d] Glucose residue.
[e] Signal of two methyl groups.

H-2 of Man			H-3 of AcNeu(2→3)		H-3 of AcNeu(2→6)		NAc of residue						
3	4	4'	eq.	ax.	eq.	ax.	1	2	5	5'	7	Acneu (2→3)	AcNeu (2→6)
4.244	4.188	4.107	-	-	-	-	2.013	2.076	2.047	2.047	-	-	-
4.254	4.196	4.115	-	-	2.671[a]	1.721[a]	2.013	2.079	2.030	2.030	-	-	2.068[e]
4.243	4.197	-	2.758	1.799	-	-	-	2.042	2.030	-	-	2.049	-
4.241	4.197	-	-	-	2.668	1.718	-	2.042	2.029	-	-	-	2.069
4.264	4.195	<4	-	-	2.670	1.719	-	2.058	2.030	-	-	-	2.070
4.258	4.194	4.11[b]	2.757	1.789	-	-	-	2.057	2.030	2.049	-	2.049	-
4.260	4.196	4.117	-	-	2.669	1.722	-	2.061	2.029	2.053	-	-	2.070
4.217	4.217	-	2.759	1.804	2.668	1.721	-	2.041	2.031	-	2.031	2.068[c]	2.074[c]
4.255	4.194	4.11[b]	2.757[a]	1.799[a]	-	-	-	2.058	2.029	2.029	-	2.045[e]	-
4.259	4.198	4.12[b]	2.758	1.799	2.672	1.716	-	2.061	2.031	2.031	-	2.046	2.070
4.263	4.198	4.120	-	-	2.667[a]	1.727[a]	-	2.067	2.029	2.029	-	-	2.067[e]
4.225	4.225	4.12[b]	2.757	1.804	2.672[a]	1.719[a]	-	2.067[c]	2.030	2.030	2.030	2.062[c]	2.067[c] 2.075[c]
-	-	-	2.757	1.799	-	-	-	-	-	-	-	2.030	-
-	-	-	-	-	2.712	1.739	-	-	-	-	-	-	2.029

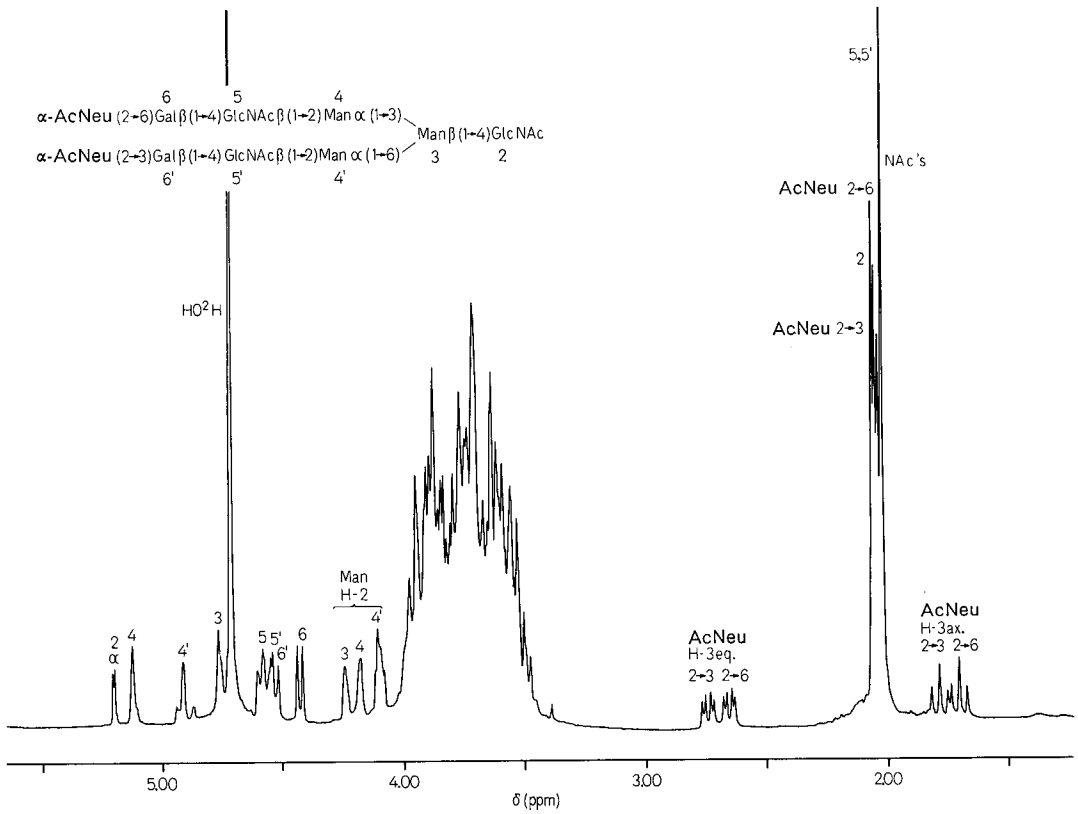


Fig. 1. *360-MHz ^{1}H-NMR spectrum of oligosaccharide VIII isolated from urine of a sialidosis patient*

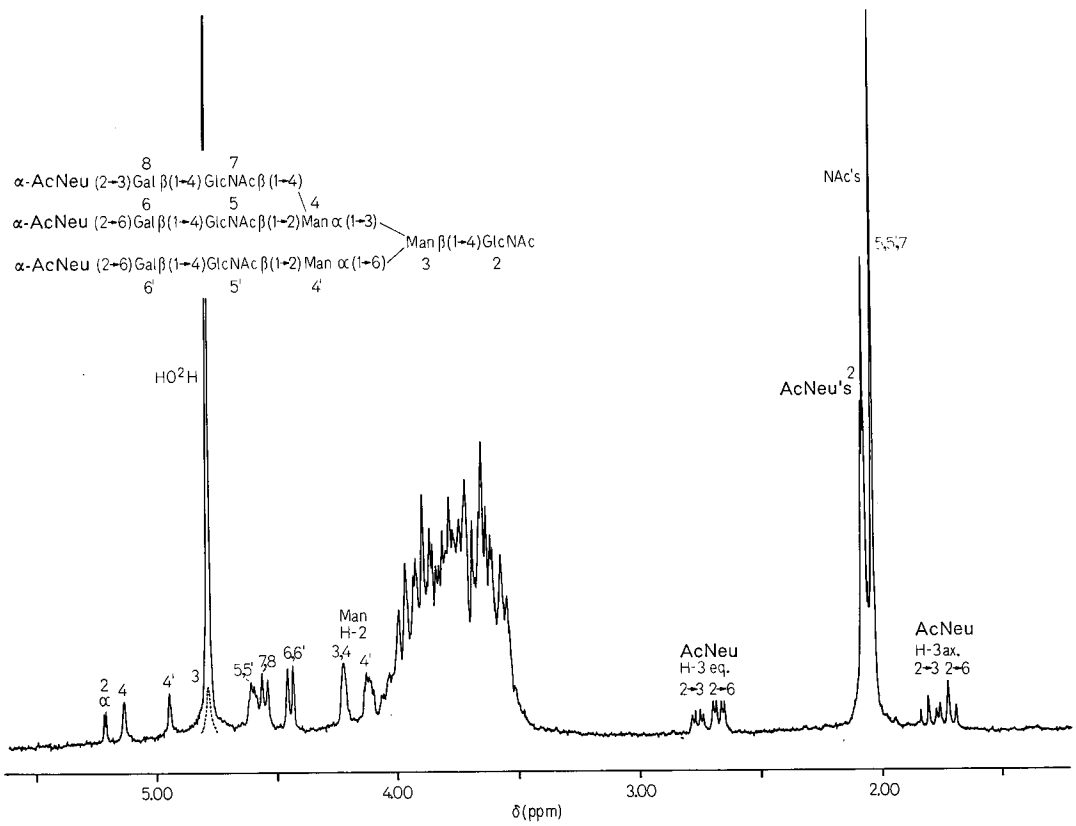


Fig. 2. *360-MHz ^{1}H-NMR spectrum of oligosaccharide X*

Table 3. *Influence of 2→3-linked and 2→6-linked terminal AcNeu residues on the chemical shift of anomeric and* N*-acetyl protons of other sugar residues in oligosaccharides I–X and GP-2*
All results are mean values ± S.D. calculated from data of Table 2 with the number of branches in which the effect is observed in parentheses. All reference values were from GP-1, except those for Gal-8 and NAc-7 which were from glycopeptides with carbohydrate structures identical to that of asialo-X (L. Dorland, J. Haverkamp & J. F. G. Vliegenthart, unpublished results)

AcNeu linkage	Residues influenced	Chemical shift of	
		asialo chain (reference)	sialo chain (observed)
		ppm	
(2 → 3)	Gal-6, 6′	4.470	4.548 ± 0.003 (7)
	Gal-8	4.469	
	NAc-5, 5′	2.047	2.030 ± 0.001 (7)
	NAc-7	2.078	
(2 → 6)	Gal-6, 6′	4.470	4.445 ± 0.002 (11)
	GlcNAc-5, 5′	4.581	4.602 ± 0.005 (11)
	Man-4	5.119	5.136 ± 0.003 (8)
	Man-4′	4.926	4.947 ± 0.005 (3)
	NAc-5, 5′	2.047	2.030 ± 0.001 (11)

Table 4. *Dependence of sialic acid H-3 chemical shifts on the type of glycosidic linkage of the sialic acid residue for oligosaccharides I–X and glycopeptide GP-2*
Results are mean values ± S.D. calculated from data of Table 2 with the number of branches in which these sialic acid residues are present in parentheses

Linkage	Chemical shift of H-3	
	equatorial	axial
	ppm	
α-AcNeu-(2 → 6)-Gal →	2.670 ± 0.002 (11)	1.721 ± 0.004 (11)
α-AcNeu-(2 → 3)-Gal →	2.758 ± 0.001 (7)	1.800 ± 0.003 (7)

these protons in the oligosaccharides I–X which depend on the type of the sialic acid linkage.

The results described in this study show that high-resolution ^{1}H-NMR spectroscopy is very suitable for characterizing sialo-oligosaccharides and glycopeptides which are related to the complex asparagine-bound carbohydrate chains of glycoproteins. In particular the number of constituting monosaccharides (integration), the type of branching of the glycan chain and the type of glycosidic linkage of sialic acid to the different branches can be determined in a convenient way. The NMR method is non-destructive and thus leaves open the possibility of subsequent chemical and enzymic investigation.

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