Structures of Asparagine-Linked Oligosaccharides of the Glycoprotein Fetuin Having Sialic Acid Linked to *N*-Acetylglucosamine†

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ABSTRACT: In the accompanying paper (Bendiak et al., 1989), the separation of a series of oligosaccharides released from asparagine residues of fetuin was described. A series of NMR experiments, which included one- and two-dimensional nuclear Overhauser enhancement, two-dimensional correlation spectroscopy, and two-dimensional relayed-coherence spectroscopy, as well as permethylation analyses, established a Galβ1→3(NeuAcα2→6)GlcNAcβ1→4Man unit common to a series of purified structures. These oligosaccharides contained either three, four, or five glycosidically linked sialic acid residues. The NeuAc residue in α2→6 linkage to GlcNAc gives rise to diagnostic chemical shift perturbations of particular proton signals in the oligosaccharides.

The asparagine-linked oligosaccharides of the glycoprotein fetuin have been under investigation for a considerable period of time (Spiro, 1962). Earlier studies have proposed triantennary structures that contained solely Galβ1→4GlcNAc branches in the periphery (Nilsson et al., 1979; Baenziger & Fiete, 1979). More recently, evidence was presented which indicated the presence of a population of molecules bearing a Galβ1→3GlcNAc moiety (Berman, 1986; Townsend et al., 1986; Takasaki & Kobata, 1986; Berman et al., 1988). However, either these studies did not address the fractionation of fully sialylated oligosaccharides, or they did not employ adequate means (NMR)[1] to assess isomeric purity of fractionated components. In the accompanying paper, we demonstrated that fetuin contains a mixture of many oligosaccharides and that evaluation of the purity of structural isomers required a combination of HPLC and high-field NMR. In this paper, we provide evidence for the structures of seven individual sialylated oligosaccharides isolated from fetuin. These oligosaccharides all contained the previously described Galβ1→3GlcNAc moiety, which we confirm by methylation analysis and NMR experiments. A NeuAcα2→6GlcNAc unit was also present on these structures; this specific sialic acid resulted in diagnostic changes in the chemical shift of several "structural reporter" protons on the oligosaccharides. Finally, as many as five NeuAc residues were found on one structure bearing two penultimate Galβ1→3GlcNAc units.

Materials and Methods

The isolation and desialylation of oligosaccharides from fetuin was described in the preceding paper (Bendiak et al., 1989). Acquisition of one-dimensional ^{1}H NMR spectra was also described therein. NOE difference spectra were collected as 1024 or 2048 difference transients with 32K real/imaginary points. Irradiated peaks were saturated at 2–20 μW for 2–5 s and transients collected. The receiver sign was negated, and decoupler power was applied off-resonance at the same power and duration; the transient was subtracted from the on-resonance transient. Difference free induction decays were processed with exponential apodization prior to Fourier transformation. Two-dimensional COSY and RECSY data were collected in both absolute magnitude and phase-sensitive mode. Averages of 32 or 64 transients with 2048 real/imaginary points were collected in the t_2 domain, and either 256 or 512 data points were collected in the t_1 domain. Data sets were transferred via an ethernet interface to a μVAX 2000 computer and processed with FTNMR(C) software developed by Dr. Dennis Hare. Individual rows were apodized with a skewed sine-bell window and optionally zero filled to 4096 complex points prior to Fourier transformation. In the t_1 dimension, data were again apodized with a sine-bell window and zero filled to obtain equal digital resolution in both dimensions. Phase-sensitive NOESY data were collected at selected mixing times of 200–500 ms. The data were processed as above except that a 30°–40° phase-shifted skewed sine-bell window was used. Final digital resolutions were 2.096 Hz/point and 1.048 Hz/point for zero-filled data.

Methylation was carried out as follows. Samples (100 μg) were dissolved in DMSO (0.5 mL) in a sonicator bath. Sodium dimethyl sulfoxide anion (0.5 mL, 2 M) was added (Hakomori, 1964). The samples were sonicated for 30 min and left at room temperature for 6 h. Methyl iodide (0.5 mL) was added, and samples were sonicated at 15 °C for 1 h (Hellerqvist et al., 1968). Solubility problems warranted partitioning the samples between chloroform–water rather than the use of Sep-PAK (Waters) units. Purified samples were redissolved in 1.8 mL of glacial acetic acid, and 0.20 mL of

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[1] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser enhancement; COSY, two-dimensional correlation spectroscopy; RECSY, two-dimensional relayed-coherence spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; MS, mass spectrometry; GLC, gas–liquid chromatography; GC, gas chromatograph; III^{6}NeuAc-LcOse$_4$, Galβ1→3(NeuAcα2→6)GlcNAcβ1→3Galβ1→4Glc.

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2 M sulfuric acid was added. After 9 h at 100 °C acetic acid was removed by codistillation with water. Water was added to a sulfuric acid concentration of 0.5 M, and the samples were hydrolyzed for an additional 2 h to quantitatively hydrolyze previously formed 1-*O*-acetyl groups (Hellerqvist & Lindberg, 1971; Nurminen et al., 1971). The acid was neutralized with barium carbonate, and the sample was reduced with borodeuteride (Hellerqvist & Lindberg, 1971), acetylated, and analyzed by GLC–MS with an OV-225 column and OV-101 column adapted to a Sigma 1 GC and a Perkin-Elmer Model 8500 GC with ion trap, respectively. GLC retention times were determined by use of 2,3,4,6-tetra-*O*-methylglucitol and 2,4-di-*O*-methylmannitol as early and late standards, respectively. MS data were recorded from derivatives separated on a gradient from 70 to 280 °C at 30 °C/min after an initial oven temperature of 70 °C.

RESULTS

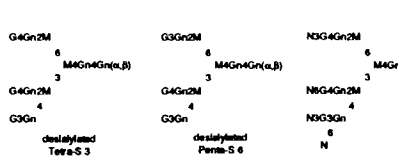
Structures of the Fetuin Oligosaccharides Having NeuAc Linked to GlcNAc. (A) Desialylated Compounds. In order to establish the linkage and branch location of all terminal β-Gal residues, it was necessary to desialylate a portion of each sialylated oligosaccharide. The structure of desialylated oligosaccharide Tri-S 2A (Bendiak et al., 1989) is shown in Figure 1. The region of its ^{1}H NMR spectrum containing the Gal H-1 and GlcNAc H-1 signals is shown in Figure 2a, and assignments for structural reporter protons on the molecule are shown in Table I. This structure is a standard triantennary oligosaccharide (Fournet et al., 1978; Carver & Grey, 1981) containing all galactose residues in β1→4 linkage to peripheral *N*-acetylglucosamine residues. It is presented here as a necessary comparison to structures bearing the presumptive Galβ1→3GlcNAc linkage, in order to firmly assign the branch location of this linkage. Due to previous disparate assignments of β-Gal H-1 signals to different branches in triantennary structures (Vliegenthart et al., 1983; Townsend et al., 1986; Joziasse et al., 1987), we performed additional NOE experiments on the pure desialylated compounds to unequivocally establish the GlcNAc H-1 assignments and, by comparison of the series, to provide evidence in support of the Gal H-1 assignments of the latter two papers. In Figure 2b, the H-2's of Man 4 and 3 were irradiated, and an observed NOE was seen across the glycosidic bond from the Man 4 H-2 to the GlcNAc 5 H-1 signal at δ = 4.569. Similarly, irradiation of the H-2 of Man 4′ (Figure 2c) resulted in a NOE across the glycosidic bond to the GlcNAc 5′ H-1 signal at δ = 4.583. The third GlcNAc H-1 signal (δ = 4.545) is therefore assigned to the GlcNAcβ1→4Man linkage. The Gal H-1 signals could only be assigned by a comparison of the above compound to the two related structures, below, having Galβ1→3GlcNAc linkages on either one or two of the branches.

The oligosaccharides Tetra-S 3 and Tetra-S 5 gave the same desialylated product, on the basis of their identical NMR spectra. The proposed structure of this product is shown in Figure 1 (desialylated Tetra-S 3). The region of its ^{1}H NMR spectrum containing Gal H-1 and GlcNAc H-1 signals is shown in Figure 2d, and assignments of structural reporter signals are presented in Table I. The spectrum reveals a new Gal H-1 signal at δ = 4.443, as compared to that of the desialylated Tri-S 2A (Figure 2a), and is missing the signal at δ = 4.470. In addition, the signal attributed to GlcNAc 7 H-1 in desialylated Tri-S 2A is missing in the spectrum of desialylated Tetra-S 3, and an additional signal is found at δ = 4.570. The upfield Gal signal has been attributed to a Galβ1→3GlcNAc linkage, on the basis of its similar chemical shift to the model compound Galβ1→3GlcNAcβ1→3Galβ1→4Glc, in which the nonreducing terminal Gal H-1 signal appears at δ = 4.439 (Bernard et al., 1984). However, it should be noted that, by classical chemical shift comparisons alone, this linkage could not be unambiguously assigned. It was also possible that a Galβ1→6GlcNAc linkage could, coincidentally, have a similar chemical shift if present in this compound. The Galβ1→6GlcNAc linkage could only be ruled out by permethylation analysis. Methylation studies of desialylated Tetra-S 3 (Table II) revealed the presence of the monodeuterated alditol acetate of 4,6-di-*O*-Me-GlcNAcMe, establishing a 3-substituted GlcNAc in the molecule. Additional NMR experiments reported later in this paper established an interresidue NOE between Gal 8 H-1 and GlcNAc 7 H-3, which added further evidence in support of a Galβ1→3GlcNAc linkage. The branch to which the Galβ1→3GlcNAc unit was attached was determined by NOE experiments (Figure 2e,f). Irradiating the Man 4 and 3 H-2's revealed an interresidue NOE from the Man 4 H-2 to the GlcNAc 5 H-1 signal at δ = 4.570 (Figure 2e). Similarly, irradiating the Man 4′ H-2 showed an interresidue NOE to the GlcNAc 5′ H-1 signal at δ = 4.583 (Figure 2f). The additional GlcNAc 7 H-1 signal (δ = 4.570), which overlaps the GlcNAc 5 H-1 doublet, is therefore assigned to the GlcNAcβ1→4Man linkage. As the GlcNAc 7 H-1 signal shifts considerably between desialylated Tri-S 2A and desialylated Tetra-S 3 (from δ = 4.545 to δ = 4.570) yet the other GlcNAc H-1 chemical shifts remain essentially identical (to within the experimental error of ±0.002 ppm), we conclude that the Galβ1→3GlcNAc is attached to the 4-position of Man 4 in desialylated Tetra-S 3. A similar comparison of the Gal H-1 signals enabled the Gal 8 H-1 doublet of desialylated Tri-S 2A to be assigned at δ = 4.470.

The proposed structure of desialylated Penta-S 6 is shown in Figure 1. The region of its ^{1}H NMR spectrum containing Gal H-1 and GlcNAc H-1 signals is presented in Figure 2g, and its chemical shift assignments are shown in Table I. This compound is similar to desialylated Tetra-S 3, but contains a Galβ1→3GlcNAc instead of a Galβ1→4GlcNAc on the Manα1→6 branch. Methylation analysis revealed the presence (Table II) of the monodeuterated alditol acetate derivatives of 4,6-di-*O*-Me-GlcNAcMe and 3,6-di-*O*-Me-GlcNAcMe, but not 3,4-di-*O*-Me-GlcNAcMe, indicating the presence of 3- and 4-substituted, but not 6-substituted, GlcNAc in desialylated Penta-S 6. In comparison of the NMR spectral data of desialylated Penta-S 6 with those of desialylated Tetra-S 3 (Table I), evidence for location of the additional Galβ1→3GlcNAc unit to the Manα1→6 branch was the upfield shift of the Man 4′ H-1 signal (from δ = 4.926 to δ = 4.915) and the changes in the chemical shifts of the GlcNAc 5′ H-1 doublet (from δ = 4.583 to δ = 4.603) and Gal 6′ H-1 doublet (from δ = 4.475 to δ = 4.456). Again, specific GlcNAc H-1 shifts were assigned by NOE experiments, i.e., irradiating Man H-2 protons and detecting interresidue NOE's to the GlcNAc H-1 protons (Figure 2h,i). The Gal 6′ H-1 signal could be assigned by the observed change in its chemical shift between desialylated Tetra-S 3 and Penta-S 6. Consequently, the Gal 6 H-1 doublet could be assigned in both compounds to the doublet at δ = 4.464 (within experimental error of ±0.002 ppm). By comparison of both these compounds to desialylated Tri-S 2A, all Gal H-1 signals were assigned.

With assignments for the Galβ1→3GlcNAc H-1 chemical shifts on two of the antennae, the branch location of the Galβ1→3GlcNAc could be determined in other desialylated compounds by comparison of spectra. In some of the compounds, this was not necessary, because of the unique chemical

Table I: Chemical Shifts of Structural Reporter Signals of Native and Desialylated Oligosaccharides from the Glycoprotein Fetuin

	oligosaccharide and schematic structure									
	 desialylated Tri-S 2A	 desialylated Tetra-S 3	desialylated Penta-S 6	Tetra-S 3	Tetra-S 5	Tri-S 3D	Tri-S 5C	Tri-S 6B	Tri-S 5D	Penta-S 6
proton										
H-1 of										
1	5.190 (α)	5.191 (α)	5.190 (α)	5.192 (α)	5.192 (α)	5.192 (α)	5.192 (α)	5.192 (α)	5.192 (α)	5.192 (α)
2	4.614 (α)	4.614 (α)	4.613 (α)	4.611 (α)	4.614 (α)	4.611 (α)	4.612 (α)	4.613 (α)	4.612 (α)	4.614 (α)
3	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]	~4.76[a]
4	5.118	5.118	5.118	5.126	5.126	5.112	5.114	5.126	5.123	5.126
4′	4.925	4.926	4.915	4.904	4.936	4.901	4.931	4.939	4.924	4.892
5	4.569	4.570	4.571	4.587	4.589	4.561	4.563	4.586	4.589	4.586
5′	4.583	4.583	4.603	4.573	4.603	4.571	4.602	4.603	4.580	4.586
6	4.464	4.463	4.464	4.449	4.448	4.471	4.471	4.449	4.451	4.447
6′	4.475	4.475	4.456	4.550	4.448	4.550	4.449	4.449	4.473	4.519
7	4.545	4.570	4.571	4.568	4.569	4.561	4.563	4.574	4.570	4.568
8	4.470	4.443	4.443	4.500	4.501	4.495	4.496	4.434	4.498	4.499
H-2 of										
3	4.216	4.216	4.217	4.221	4.223	4.216	4.216	4.224	4.221	4.221
4	4.216	4.216	4.217	4.221	4.223	4.216	4.216	4.224	4.221	4.221
4′	4.113	4.113	4.117	4.115	4.118	4.109	4.113	4.118	4.112	4.125
H-3 of										
4	4.047	4.051	4.050	4.086	4.087	4.083	4.083	4.099	4.091	4.087
6′	<4.0	<4.0	<4.0	4.119	<4.0	4.119	<4.0	<4.0	<4.0	4.087
8	<4.0	<4.0	<4.0	4.086	4.087	4.083	4.083	<4.0	4.085	4.087
H-3a of										
N				1.725	1.726			1.726	1.725	1.725
N′				1.803	1.719	1.802	1.718	1.719		1.787
N*				1.784	1.785	1.784	1.784		1.784	1.787
N^				1.764	1.766	1.760	1.761	1.768	1.767	1.764
N°										1.725
H-3e of										
N				2.668	2.670			2.670	2.670	2.671
N′				2.758	2.673	2.758	2.673	2.675		2.758
N*				2.758	2.760	2.758	2.760		2.759	2.758
N^				2.728	2.729	2.723	2.724	2.734	2.729	2.728
N°										2.742
NAc of										
1	2.040	2.039	2.040	2.039	2.039	2.039	2.039	2.037	2.038	2.039
2	2.082	2.081	2.081	2.082	2.084	2.081	2.084	2.085	2.081	2.087
5	2.050	2.050	2.051	2.068	2.069	2.049	2.051	2.070	2.068	2.068
5′	2.047	2.046	2.045	2.045	2.069	2.044	2.067	2.067[b]	2.047	2.042
7	2.077	2.067	2.068	2.068	2.069	2.066	2.067	2.070	2.068	2.068
N				2.030	2.030			2.031[c]	2.030	2.030
N′				2.030	2.030	2.031	2.030	2.031		2.030[c]
N*				2.030	2.030	2.031	2.030		2.030	2.030
N^				2.030	2.030	2.031	2.030	2.037[c]	2.030	2.030
N°										2.034[c]

[a] Signal assignment only approximate to 0.010 ppm due to $^1HO^2H$ overlap at 27 °C. [b] Signal assignment might be interchanged with GlcNAc 5 or 7. [c] Signal assignments might be interchanged.

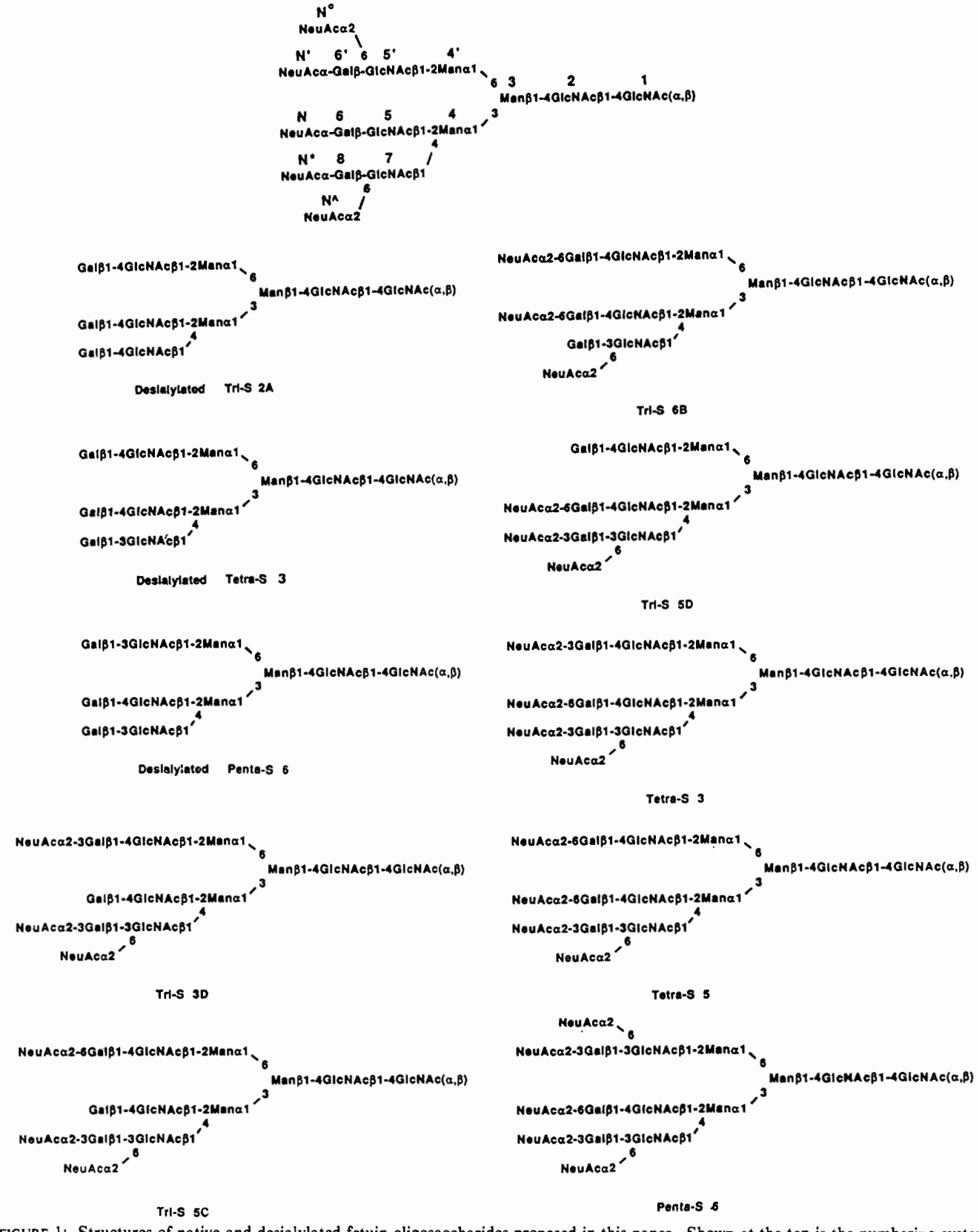


FIGURE 1: Structures of native and desialylated fetuin oligosaccharides proposed in this paper. Shown at the top is the numbering system of monosaccharide residues, after the general system proposed by Vliegenthart et al. (1983).

shift perturbation effects associated with a Galβ1→3-(NeuAcα2→6)GlcNAcβ1→4Man unit.

(*B*) *Sialylated Compounds.* The structure of sialylated compounds was determined by a combination of ^{1}H NMR and methylation analysis. Experiments are described as follows.

(*i*) *Tetra-S 3.* The proposed structure of Tetra-S 3 is shown in Figure 1, its ^{1}H NMR spectrum is shown in Figure 3a, and its chemical shift assignments are shown in Table I. The structure of the desialylated compound was determined as described above. Tetra-S 3 is therefore a triantennary oligosaccharide which contains only three β-galactoside residues but contains four sialic acid residues. All three of the galactose residues are substituted with one sialic acid, on the basis of methylation analysis of the sialylated compound; one GlcNAc

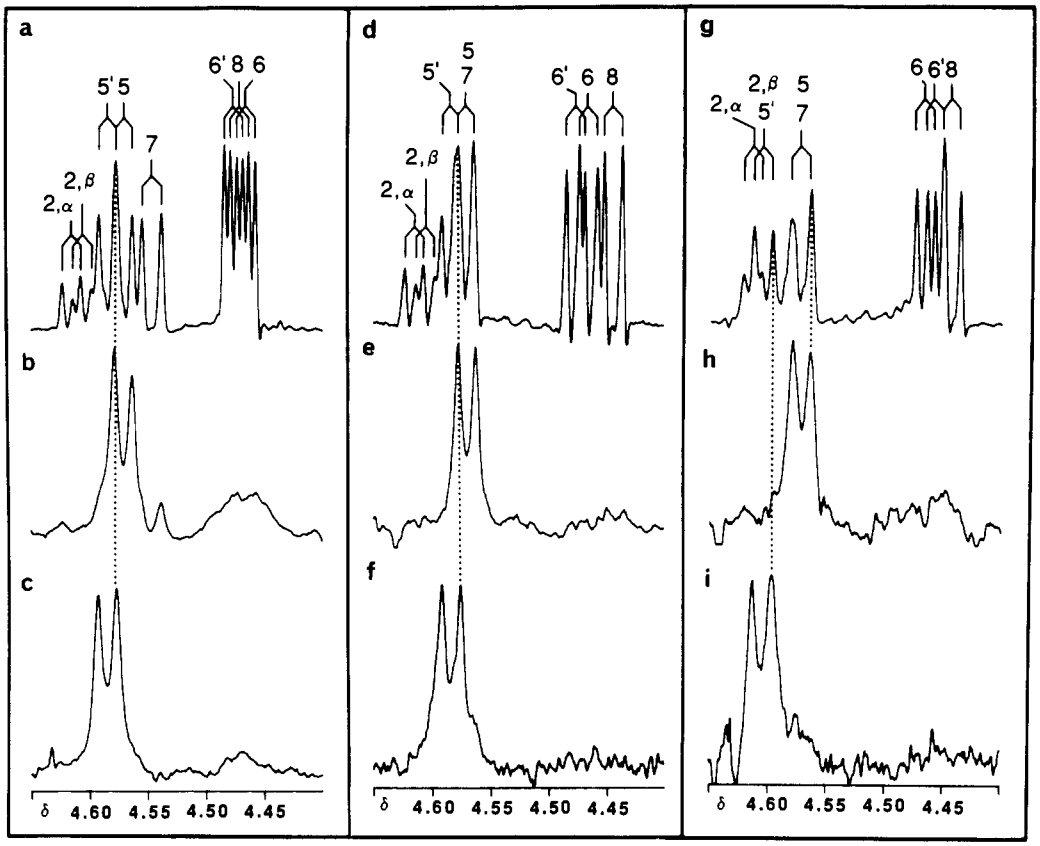


FIGURE 2: (a) Partial ^1H NMR spectrum of desialylated Tri-S 2A (Figure 1), showing the region containing the Gal and GlcNAc H-1 signals. (b) Partial NOE difference spectrum of desialylated Tri-S 2A, irradiating the Manα1→3 and β-Man H-2 protons overlapping at δ = 4.216. (c) Partial NOE difference spectrum of desialylated Tri-S 2A, irradiating the Manα1→6 H-2 proton at δ = 4.113. (d) Partial ^1H NMR spectrum of desialylated Tetra-S 3, (Figure 1), showing the region containing the Gal and GlcNAc H-1 signals. (e) Partial NOE difference spectrum of desialylated Tetra-S 3, irradiating the Manα1→3 and β-Man H-2 protons overlapping at δ = 4.216. (f) Partial NOE difference spectrum of desialylated Tetra-S 3, irradiating the Manα1→6 H-2 proton at δ = 4.113. (g) Partial ^1H NMR spectrum of desialylated Penta-S 6 (Figure 1), showing the region containing the Gal and GlcNAc H-1 signals. (h) Partial NOE difference spectrum of desialylated Penta-S 6, irradiating the Manα1→3 and β-Man H-2 protons having signals overlapping at δ = 4.217. (i) Partial NOE difference spectrum of desialylated Penta-S 6, irradiating the Manα1→6 H-2 proton having a signal at δ = 4.117. All spectra were recorded at a field strength of 500 MHz in ^2H_2O at 27 °C. Assignments are indicated.

Table II: Monodeuterated, Partially Methylated Alditol Acetates Derived from Permethylated Oligosaccharides[a]

methyl ethers	Tetra-S 3	Tetra-S 5	Penta-S 6	desialylated Tetra-S 3	desialylated Penta-S 6
2,3,4,6-Gal	0	0	0	3.1	5.8
3,4,6-Man	1.0	1.2	1.4	0.8	1.2
2,4,6-Gal	2.1	1.9	2.4	0	0
2,3,4-Gal	1.1	2.0	1.2	0	0
3,6-Man	1.0	1.0	1.0	1.0	1.0
2,4-Man	1.4	1.2	1.5	0.8	1.3
3,6-GlcNAcMe	3.8	4.3	2.6	4.9	5.2
4,6-GlcNAcMe	0	0	0	1.3	1.2
3-GlcNAcMe	0	0.3	0	0	0
4-GlcNAcMe	0.9	1.2	1.0	0	0

[a] Values represent integrations of the total ion current of GLC–MS peaks relative to that of the 3,6-di-*O*-Me-Man derivative. A correction factor (1.8) was applied to all hexosaminitol derivatives. Due to the nature of total ion current monitoring and the presence of contaminating compounds after permethylation which comigrate with partially methylated alditol acetates, values are semiquantitative. Conclusions drawn with regard to stoichiometry are based on the NMR spectral data.

residue was 3,6-disubstituted, indicating the presence of a NeuAc linked to the 6-position of GlcNAc 7 (Figure 4a, Table II). These results were corroborated by ^1H NMR; specific upfield and downfield shifts of the Gal H-1 signals were observed as compared to those of the desialylated compound (Vliegenthart et al., 1983). Assignment of NeuAc H3a/H3e pairs (Table I) was achieved by performing a two-dimensional correlation spectroscopy (COSY) experiment (Figure 5a).

It is evident from the chemical shifts of the β1→4-linked Gal H-1 signals (Table I) that one NeuAcα2→3Galβ1→4GlcNAcβ1→R branch and one NeuAcα2→6Galβ1→4GlcNAcβ1→R branch are present on the molecule. As there are just two Galβ1→4GlcNAcβ1→R branches in the desialylated molecule, it was necessary to assign the NeuAc linkages (α2→6 or α2→3) to a particular Galβ1→4GlcNAc branch location.

The NeuAcα2→3Galβ1→4GlcNAcβ1→R branch was linked to the 2-position of Man 4′, as indicated by the following evidence. First, the Man 4′ H-1 signal undergoes an upfield shift as compared to that of the desialylated compound (from δ = 4.925 to δ = 4.904). Therefore, a NeuAcα2→6Gal linkage on this branch is unlikely because this substitution leads to a well-documented downfield shift of the Man 4′ H-1 signal [Vliegenthart et al., 1983; also compare Tetra-S 3 to Tetra-S 5 and the set Tri-S 2A and Tri-S 4B (Bendiak et al., 1989)]. Second, the H-1 signals of GlcNAc 5′ and 5 (and 7, by elimination) were assigned in Tetra-S 3 by the performance of a NOESY experiment (Figure 6a). Two of the crosspeaks shown in the expanded section (Figure 6b) represent NOE's across the glycosidic bonds between GlcNAc 5 H-1 and Man 4 H-2 and between GlcNAc 5′ H-1 and Man 4′ H-2. NOE crosspeaks were also observed from these GlcNAc H-1 protons to the appropriate Man 4 and 4′ H-1 protons (Figure 6a). The third β-GlcNAc signal in the standard spectrum (Figure 3a) therefore represents GlcNAc 7 H-1. Third, the GlcNAc 5′ H-1 signal shifts upfield upon sialylation, in comparison to that of the desialylated compound (from δ = 4.583 to δ = 4.573).

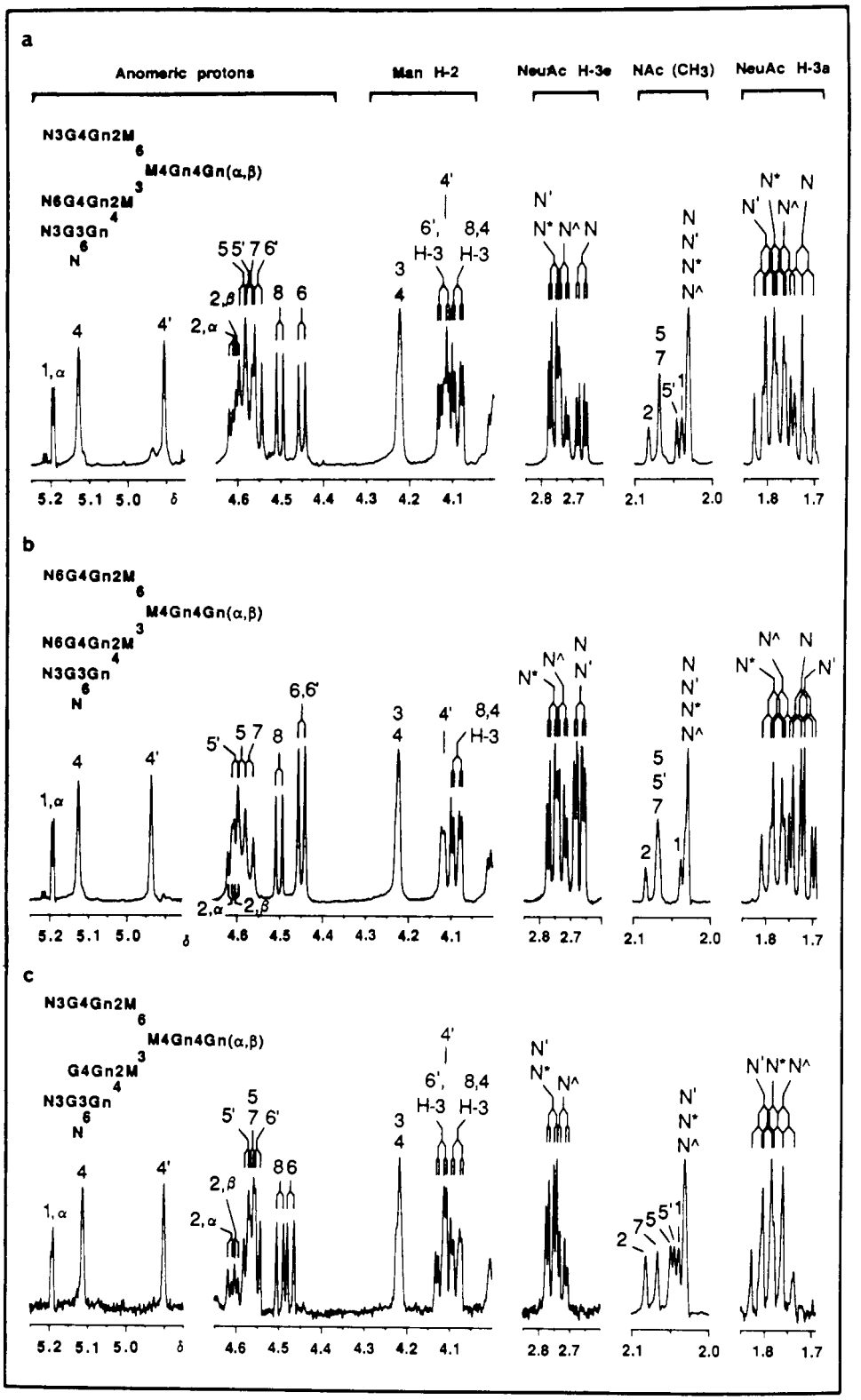


FIGURE 3: Partial 500-MHz ^1H NMR spectra of reducing oligosaccharides released from fetuin. Acquisitions were performed at 27 °C in ^2H_2O. Individual sections have been plotted with different intensities; therefore, signal integration as presented differs from section to section. Signals of diagnostic protons are labeled as depicted in the generalized structure in Figure 1. Schematic structures are shown with each spectrum; residues and linkages are shown in full in Figure 1 for each schematic structure. Shown are the spectra of oligosaccharides (a) Tetra-S 3, (b) Tetra-S 5, and (c) Tri-S 3D.

This is an established effect of $\alpha 2 \rightarrow 3$ substitution of a Gal$\beta 1 \rightarrow$4GlcNAc branch; sialylation at the 6-position of the Gal residue results in a major downfield shift of the GlcNAc H-1 (Vliegenthart et al., 1983). Fourth, the Gal 6′ H-1 signal is shifted significantly from δ = 4.475 in the desialylated compound to δ = 4.550 in Tetra-S 3. This standard downfield shift of the Gal H-1 doublet upon NeuAc$\alpha 2 \rightarrow 3$ substitution is accompanied by a major downfield shift of the Gal 6′ H-3 quadruplet to δ = 4.119. The Gal 6′ H-1/H-3 pair (δ = 4.550/4.119) was established by an observed H-1/H-3 cross-speak in a one-step RECSY experiment (Figure 7b) and in the NOESY experiment (Figure 6b). Again, these chemical shift effects are indicative of a NeuAc$\alpha 2 \rightarrow 3$ substitution of a terminal Gal$\beta 1 \rightarrow$4GlcNAc unit (Vliegenthart et al., 1983).

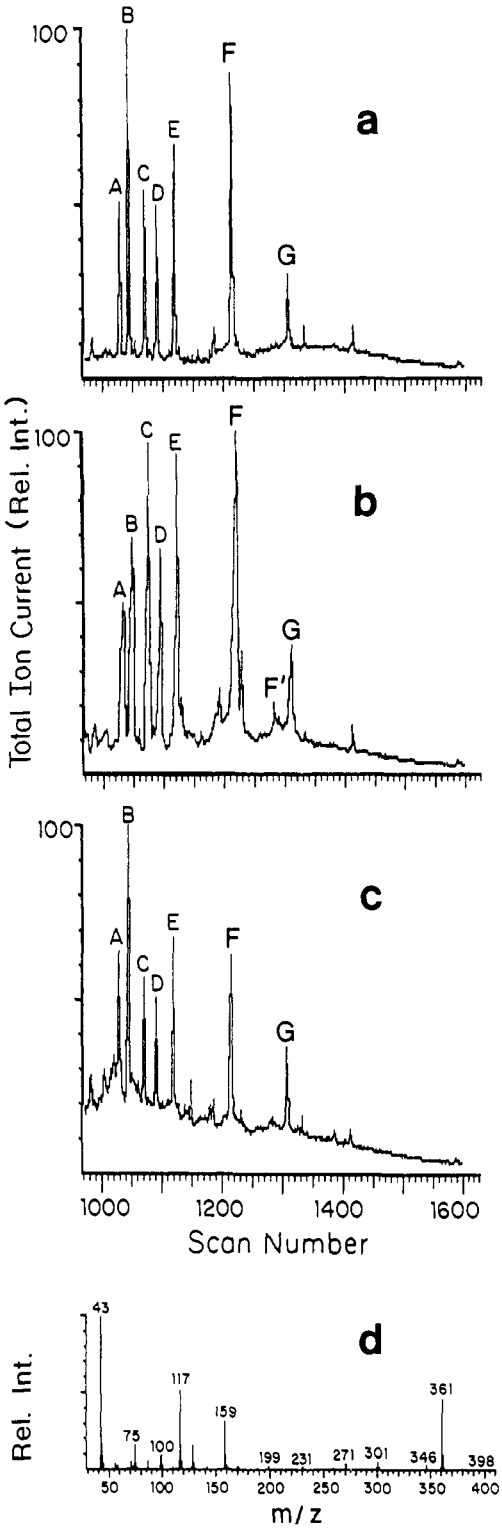


FIGURE 4: Methylation analysis of fetuin oligosaccharides. Panels a–c show the GLC profiles (total ion current) of monodeuterated partially methylated alditol acetate derivatives from (a) Tetra-S 3, (b) Tetra-S 5, and (c) Penta-S 6. Labeled on the profiles are the monodeuterated alditol acetate derivatives of (A) 3,4,6-tri-*O*-Me-Man, (B) 2,4,6-tri-*O*-Me-Gal, (C) 2,3,4-tri-*O*-Me-Gal, (D) 3,6-di-*O*-Me-Man, (E) 2,4-di-*O*-Me-Man, (F) 3,6-di-*O*-Me-GlcNAcMe, (F′) 3-*O*-Me-GlcNAcMe, and (G) 4-*O*-Me-GlcNAcMe. In panel d, the MS of the monodeuterated alditol acetate derivative G (4-*O*-Me-GlcNAcMe) is shown.

Fifth, the NeuAc N′ H-3a/H-3e signals were assigned as the pair 1.803/2.758, in agreement with the standard chemical shifts for NeuAc on a NeuAcα2→3Galβ1→4GlcNAcβ→R branch (Vliegenthart et al., 1983).

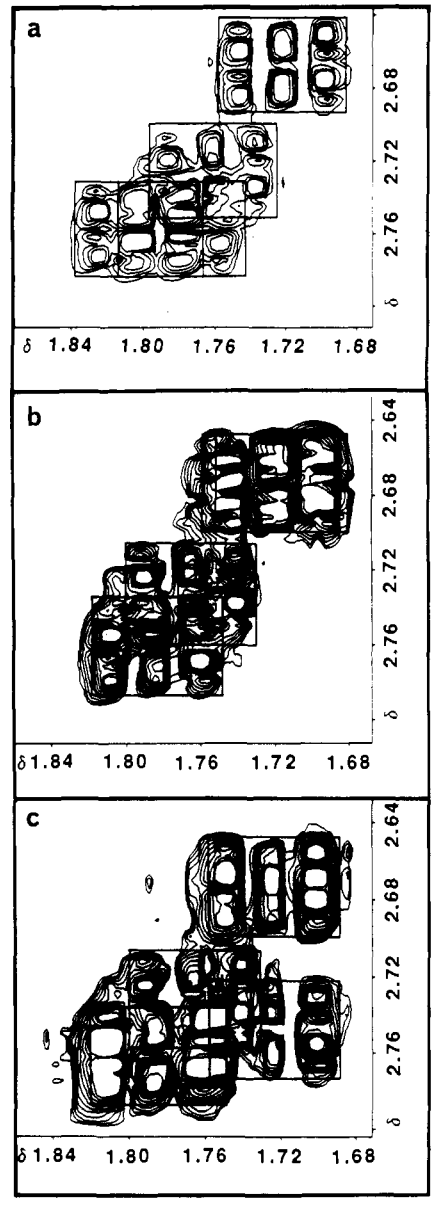


FIGURE 5: Expansions of two-dimensional correlation (COSY) ^{1}H NMR spectra of sialylated oligosaccharides showing the NeuAc H-3a/H-3e crosspeaks. Individual H-3a/H-3e signal pairs are indicated in the enclosed areas. Shown are spectra of (a) Tetra-S 3, (b) Tetra-S 5, and (c) Penta-S 6.

The NeuAcα2→6Galβ1→4GlcNAcβ1→R branch was linked to the 2-position of Man 4, on the basis of the established perturbations of structural reporter signals upon NeuAcα2→6 substitution of the terminal Gal on this branch (Joziasse et al., 1987). The GlcNAc 5 H-1 signal (δ = 4.587) displays a major downfield shift (+0.017 ppm) as compared to that of the desialylated compound (δ = 4.570). The Gal 6 H-1 signal is shifted upfield upon sialylation (from δ = 4.463 to δ = 4.449), another characteristic feature of a NeuAcα2→6 substitution. The NeuAc N H-3a/H-3e pair (δ = 1.725/2.668) is assigned to the NeuAcα2→6Gal linkage. The H-3a chemical shift is slightly perturbed from the standard shift (near δ = 1.720) in a NeuAcα2→6Galβ1→4GlcNAcβ1→R branch. The reason for this minor perturbation, however, is described further under Discussion; it is a result of an inter-branch effect caused by a NeuAcα2→6 linkage to GlcNAc 7.

The structure which extended from the 4-position of Man 4 was determined to be a NeuAcα2→3Galβ1→3-

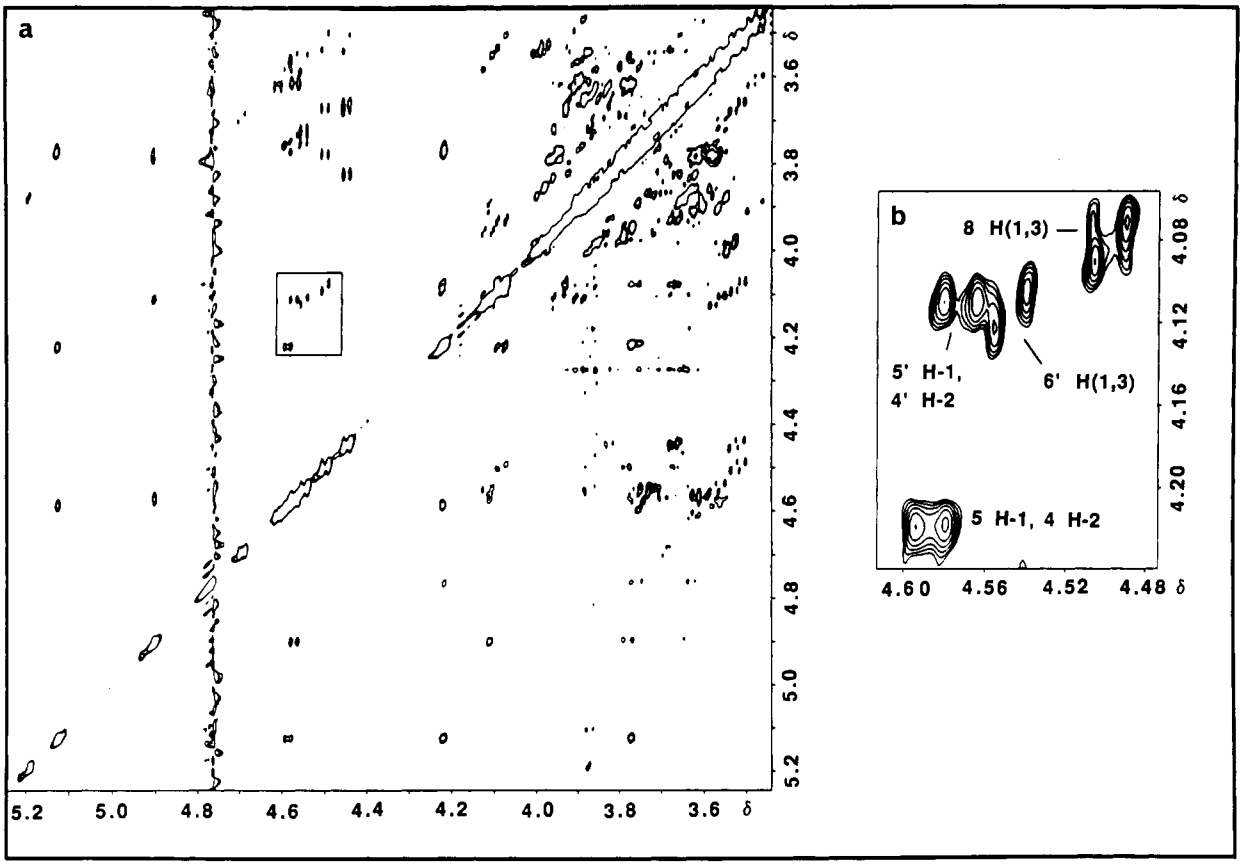


FIGURE 6: A two-dimensional nuclear Overhauser enhancement (NOESY) ^{1}H NMR experiment with oligosaccharide Tetra-S 3. (a) Region of the spectrum showing monosaccharide anomeric and skeletal proton NOE's. (b) An expansion of the boxed area in (a), showing the intraresidue NOE crosspeaks between Gal 8 H-1 and Gal 8 H-3 and between Gal 6′ H-1 and Gal 6′ H-3 as well as the interresidue NOE crosspeaks between GlcNAc 5 H-1 and Man 4 H-2 and between GlcNAc 5′ H-1 and Man 4′ H-2.

(NeuAcα2→6)GlcNAcβ1→R unit on the basis of the following data. Methylation analysis revealed the presence of the monodeuterated alditol acetate derivative of 4-*O*-Me-GlcNAcMe (Figure 4a, Table II). This left two possible alternatives for substitution on one peripheral GlcNAc residue, either Galβ1→3(NeuAcα2→6)GlcNAcβ1→R or NeuAcα2→3(Galβ1→6)GlcNAcβ1→R. The second alternative could be ruled out, because permethylation of the desialylated oligosaccharide revealed the presence of the monodeuterated alditol acetate derivative of 4,6-di-*O*-Me-GlcNAcMe and not the 3,4-di-*O*-Me isomer (Table II). Therefore, the structure of the desialylated compound and the above assignments having been established, only one possible branch location and substitution for this GlcNAc derivative remains, a Galβ1→3(NeuAcα2→6)GlcNAcβ1→4Man branch.

The assignment of the Galβ1→3GlcNAc linkage in the sialylated compound was further supported by the observation of a direct interresidue NOE between Gal 8 H-1 (δ = 4.500) and GlcNAc 7 H-3 (δ = 3.775) in Tetra-S 3 in the NOESY experiment (Figure 7a). The interresidue Gal 8 H-1/GlcNAc 7 H-3 NOE was confirmed in a one-dimensional NOE experiment in which Gal 8 H-1 was irradiated; an NOE to GlcNAc 7 H-3 was observed as a triplet centered at δ = 3.777 ($J_{2,3}$ and $J_{3,4}$ = 8.5 Hz; data not shown). The GlcNAc 7 H-3 was assigned by a COSY (Figure 7c) and a one-step RECSY (Figure 7b); these experiments permitted hexose and GlcNAc protons from H-1 to H-3 and some of the H-4 protons to be assigned (Table III).

On the basis of the permethylation data and the data above, a NeuAc residue was linked to the 3-position of Gal 8, since a significant amount of the monodeuterated alditol acetate derivative of 2,4,6-tri-*O*-Me-Gal appeared to be present in the molecule. This was corroborated by ^{1}H NMR studies. On the basis of the model compound NeuAcα2→3Galβ1→3-(NeuAcα2→6)GlcNAcβ1→3Galβ1→4Glc (disialyllacto-*N*-tetraose; Bernard et al., 1984), NeuAc residues were assigned to a similar structure present on the 4-position of Man residue 4: NeuAcα2→3Galβ1→3(NeuAcα2→6)GlcNAcβ1→R. This assignment was based on similarities in chemical shifts for the NeuAcα2→3 signals (H-3a/H-3e pair δ = 1.782/2.756 for the model compound and 1.784/2.758 for the Tetra-S 3 branch) and the Galβ1→3 H-1 signal (δ = 4.500 for the model compound and 4.500 for the Tetra-S 3 branch). The most convincing NMR evidence for the NeuAcα2→3 substitution on the Gal of the Galβ1→3GlcNAc unit came from the marked downfield shift of the Gal 8 H-3 quadruplet (δ = 4.086) as compared to that of the desialylated compound (δ < 4.0). This is a standard feature of NeuAcα2→3 substitution of Gal (Bernard et al., 1984). The additional NeuAc present on the Tetra-S 3 molecule (H-3a/H-3e δ = 1.764/2.728) was distinct from any previously reported sialic acid chemical shifts, including those of the model compound. This was not considered unusual, however, since this sialic acid was linked to a GlcNAc which in turn was linked to different aglycon structures in the two compounds. The linkage of this NeuAc could only be established by methylation analysis (Figure 4a, Table II). An interesting aspect of the NeuAcα2→6GlcNAc linkage was the specific perturbations in the observed chemical shifts of several protons that occurred when this residue was present (see below).

(*ii*) *Tetra-S 5.* The proposed structure of Tetra-S 5 is shown in Figure 1, its ^{1}H NMR spectrum is shown in Figure 3b, and its chemical shift assignments are shown in Tables I and III.

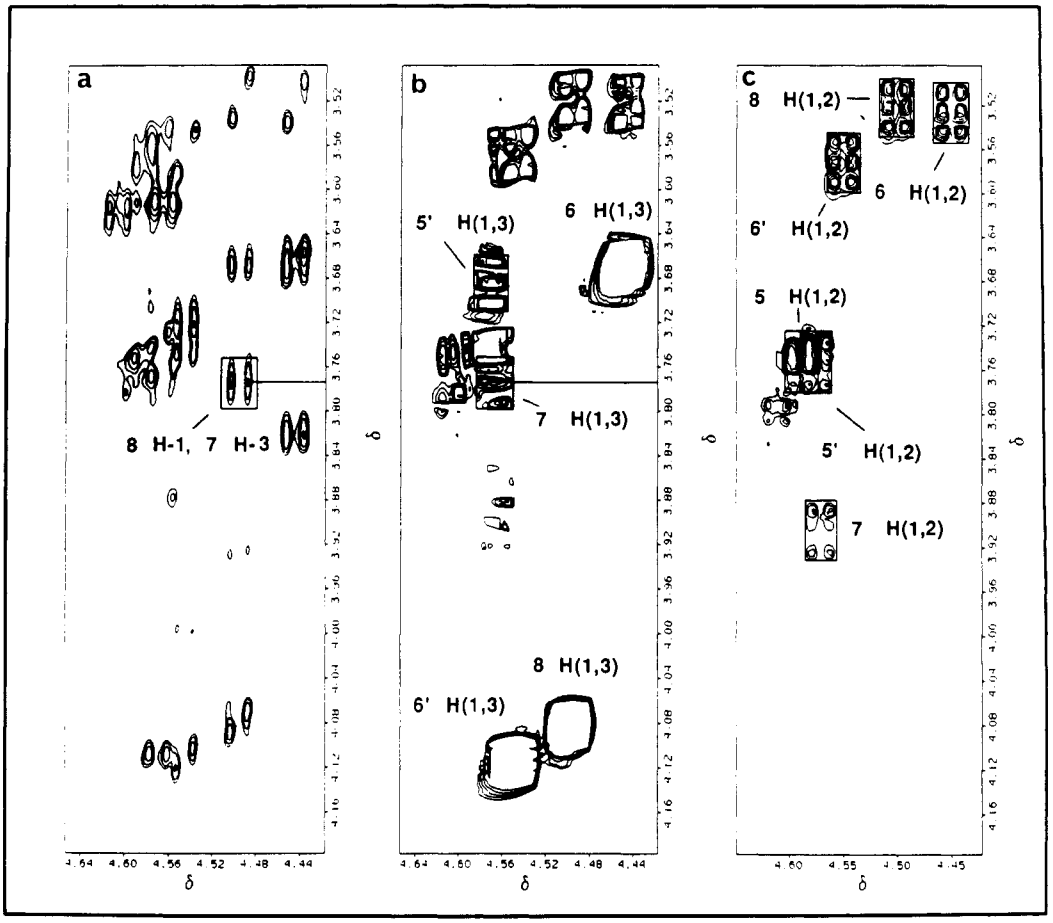


FIGURE 7: Expansions of two-dimensional ^{1}H NMR spectra of the compound Tetra-S 3. (a) Expansion of the NOESY spectrum (Figure 6). Labeled in the spectrum is the interresidue NOE (boxed) between Gal 8 H-1 and GlcNAc 7 H-3, having a chemical shift at δ = 3.775 (±0.005 ppm). (b) Expansion of the same region of the one-step RECSY spectrum, showing the Gal and GlcNAc H-1,H-3 crosspeaks. Boxed in the spectrum are the GlcNAc 7 H-1,H-3 crosspeaks, at δ = 3.775 (±0.005 ppm). (c) Expansion of the same region of the COSY spectrum, showing the Gal and GlcNAc H-1,H-2 crosspeaks. The crosspeaks of the GlcNAc 5 H-1,H-2 pair are distorted. This is probably due to a tight coupling of the GlcNAc 5 H-2 and H-3, which is supported by a presumptive GlcNAc 5 H-1,H-3 crosspeak of a near-identical shift observed in the one-step relay in (b).

Table III: Additional Assignments for Tetra-S 3, Tetra-S 5, and Penta-S 6 (from COSY and RECSY Experiments)[a]

	Tetra-S 3			Tetra-S 5			Penta-S 6		
residue	H-2	H-3	H-4	H-2	H-3	H-4	H-2	H-3	H-4
1α	3.87			3.87			3.87		
1β	3.69			3.69			3.69		
2	3.80	3.76		3.80			3.79		
3	4.22	3.77		4.22	3.77		4.22	3.77	
4	4.22	4.09	3.61	4.22	4.09	3.61	4.22	4.09	3.61
4′	4.12	3.90		4.12	3.90		4.12	3.91	
5	3.76	3.75[b]		3.76			3.76		
5′	3.76	3.69		3.76			3.85		
6	3.53	3.67		3.52[c]			3.53		
6′	3.57	4.12	3.96	3.54[c]			3.53	4.09	3.93
7	3.91	3.78		3.91			3.91		
8	3.52	4.09	3.93	3.52	4.09	3.93	3.52	4.09	3.93
N		*d*	3.66		*d*	3.66		*d*	3.66
N′		*d*	3.69		*d*	3.66		*d*	3.68
N*		*d*	3.68		*d*	3.68		*d*	3.68
N^		*d*	3.68		*d*	3.68		*d*	3.68
N°								*d*	3.68

[a] Due to the relatively large area of crosspeaks, experimental error in assignments is ±0.010 ppm. [b] GlcNAc **5** H-2 and H-3 appear to be tightly coupled in Tetra-S 3. [c] Assignments may need to be interchanged. [d] NeuAc H-3a/H-3e pairs are reported in Table I.

The desialylated compound had an identical ^{1}H NMR spectrum with that of desialylated Tetra-S 3 (Figure 2d, Table I). The sialylated oligosaccharide was closely related to Tetra-S 3; in fact, the only difference between the compounds was the linkage of sialic acid to the Manα1→6 branch. NeuAc H-3a/H-3e pairs were established by a COSY experiment (Figure 5b). All signals assigned to the Manα1→3 branch were identical within experimental error with those of Tetra-S 3, including all three unique sialic acid H-3a/H-3e pairs (δ = 1.726/2.670, 1.785/2.760, and 1.766/2.729) which defined the structure of this branch in Tetra-S 3. On the Manα1→6 branch, however, all chemical shifts were altered: the Man 4′ H-1 (from δ = 4.904 in Tetra-S 3 to δ = 4.936), the GlcNAc 5′ H-1 (from δ = 4.573 to δ = 4.603), the Gal 6′ H-1 (from

δ = 4.550 to δ = 4.448), and the NeuAc N′ H-3a/H-3e (from δ = 1.803/2.758 to δ = 1.719/2.673). These are standard chemical shift perturbations observed in a change from NeuAcα2→3Gal to a NeuAcα2→6Gal on a Manα1→6 branch (Vliegenthart et al., 1983). Methylation analysis of the sialylated compound (Figure 4b, Table II) established the presence of a monodeuterated alditol acetate derivative of 4-*O*-Me-GlcNAcMe and confirmed the other substituted sugar derivatives. A small amount of derivatized 3-*O*-Me-GlcNAcMe was also observed; its origin is unknown but may represent an oligosaccharide contaminant of Tetra-S 5 which was not observed in the NMR spectrum.

(*iii*) *Tri-S 3D.* The ^{1}H NMR spectrum of Tri-S 3D is shown in Figure 3c, its proposed structure is shown in Figure 1, and its chemical shift assignments are shown in Table I. The desialylated compound had an identical ^{1}H NMR spectrum with that of desialylated Tetra-S 3 and 5 (Figure 2d; see Figure 1 for structure), bearing a single Galβ1→3GlcNAcβ1→R unit linked to the 4-position of Man 4. The structure of Tri-S 3D is similar to that of Tetra-S 3, but lacks NeuAc residue N. Hence, the major chemical shift perturbations are associated with the branch attached to the 2-position of Man 4 and are characteristic changes associated with a loss of α2→6 sialylation of a Galβ1→4GlcNAcβ1→2Man branch (Vliegenthart et al., 1983). These changes include perturbations of the signals of Man 4 H-1 (−0.014 ppm), GlcNAc 5 H-1 (−0.026 ppm), and Gal 6 H-1 (+0.022 ppm) and the absence of the NeuAc residue N signals present in Tetra-S 3 (H-3a/H-3e δ = 1.725/2.668). The Manα1→6 branch is substituted with NeuAc in an α2→3 linkage to Gal, on the basis of the chemical shifts of the Man 4′, GlcNAc 5′, and Gal 6′ H-1 signals and the shifts of the NeuAc N′ H-3a/H3e signals. The chemical shifts of signals associated with residues of the Manα1→6 branch of Tri-S 3D were shifted negligibly as compared to those of Tetra-S 3. In further comparison of Tri-S 3D to Tetra-S 3, it is most interesting to note that the addition of NeuAc residue N to Gal 6 has minor perturbation effects on residues of the branch attached at the 4-position of Man 4. These are minor interbranch perturbations caused by the mutual presence of residues N and N^, which will be elaborated in greater detail under Discussion.

(*iv*) *Tri-S 5C.* The proposed structure of Tri-S 5C is shown in Figure 1, its ^{1}H NMR spectrum is shown in Figure 8a, and its chemical shift assignments are shown in Table I. The oligosaccharide contains a minor contaminant of unknown structure (signals marked with asterisks in Figure 8a). The compound is closely related to both Tri-S 3D, in having N′ in α2→6 linkage instead of α2→3 linkage, and Tetra-S 5, in lacking NeuAc residue N. A comparison to Tri-S 3D reveals major chemical shift changes in diagnostic signals of all residues associated with the Manα1→6 branch (Man 4′, GlcNAc 5′, Gal 6′, NeuAc N′), a result of the change in sialic acid substitution on this branch (Vliegenthart et al., 1983). Signals of other residues, associated with the Manα1→3 branch, were identical between the two compounds, within experimental error. Tri-S 5C is related to Tetra-S 5 in the same way as the pair Tri-S 3D and Tetra-S 3, and all observed perturbations due to the addition of NeuAc residue N, as discussed with the comparison of Tri-S 3D and Tetra-S 3, above, also apply to the comparison of Tri-S 5C and Tetra-S 5.

(*v*) *Tri-S 6B.* The ^{1}H NMR spectrum of compound Tri-S 6B is shown in Figure 8b, its proposed structure is shown in Figure 1, and its chemical shift assignments are shown in Table I. It is closely related in structure to Tetra-S 5, but lacks NeuAc N*. This oligosaccharide is unique among the proposed trisialylated oligosaccharides in having no NeuAcα2→3Gal linkage. On the basis of the chemical shifts of H-1 signals, Tri-S 6B and Tetra-S 5 can be seen to have an identical Manα1→6 branch. Most noticeable in the spectrum is the appearance of an upfield Gal H-1 appearing at δ = 4.434, a position that is assigned to an unsubstituted Gal in β1→3 linkage to GlcNAc on the basis of its similarity to a model compound, III^{6}NeuAc-LcOse$_4$ (Bernard et al., 1984). As compared to that in the spectrum of Tetra-S 5, the Gal 8 H-3 signal is also shifted upfield into the major proton envelope, adding further evidence that N* was linked to the 3-position of Gal 8. This is also supported by the lack of the N* H-3a/H-3e proton pair (normally near δ = 1.785/2.758). However, the NeuAc N^ is still present and continues to have a major effect on the chemical shift of the Man 4 H-3 signal and a minor effect on the chemical shift of the N H-3a signal, among other minor perturbations, as compared to the structure which lacks residue N^ (Tri-S 2B; Bendiak et al., 1989). On the basis of the spectral similarity of Tri-S 6B to Tetra-S 5, it is concluded that it represents a structure otherwise identical with Tetra-S 5 but lacking the NeuAcα2→3Gal linkage.

(*vi*) *Tri-S 5D.* The proposed structure of Tri-S 5D is shown in Figure 1, its ^{1}H NMR spectrum is shown in Figure 9a, and its chemical shift assignments are shown in Table I. This oligosaccharide is closely related to both Tetra-S 3 and Tetra-S 5 in that it lacks the NeuAc residue N′. Therefore, a chemical shift for an unsubstituted Gal 6′ H-1 signal appears at δ = 4.473, accompanied by a standard chemical shift (δ = 4.580) for a 4-substituted GlcNAc 5′ H-1 resonance and a standard shift (δ = 4.924) for the Man 4′ H-1 signal (Vliegenthart et al., 1983). Other signals assigned to residues attached to the Manα1→3-linked branch were characteristic of the same substructures present in Tetra-S 3 and Tetra-S 5; immediately apparent in the spectrum are the unique NeuAc H-3a/H-3e signals and the downfield position (δ = 4.091) of the Man 4 H-3 signal.

(*vii*) *Penta-S 6.* The ^{1}H NMR spectrum of Penta-S 6 is shown in Figure 9b, and its chemical shift assignments are shown in Tables I and III. The proposed structure is shown in Figure 1. The desialylated compound contained two Galβ1→3GlcNAc branches; on the basis of NOE experiments, these could be assigned to specific branch locations as described under Desialylated Compounds. In the sialylated compound, NeuAc H-3a/H-3e pairs were assigned by a COSY experiment (Figure 5c). Two of the residues had identical H-3a/H-3e signals (δ = 1.787/2.758), which were attributed to NeuAcα2→3Galβ1→3GlcNAc units on the molecule. Two other NeuAc residues (having H-3a/H-3e pairs at chemical shifts of δ = 1.764/2.728 and 1.725/2.742) were assigned to NeuAc residues N^ and N°, respectively, linked at the 6-positions of GlcNAc residues 7 and 5′ (Figure 1). These assignments are based on the absence of any observable monodeuterated alditol acetate derivatives of 4,6-di-*O*-Me-GlcNAcMe or 6-*O*-Me-GlcNAcMe but the presence of 4-*O*-Me-GlcNAcMe in the permethylation analysis (Figure 4c, Table II). This indicates that the Galβ1→3GlcNAc units must be substituted at the 6-positions of the GlcNAc residues by sialic acids. Although quantitation of the total ion current on the GC (Figure 4c) indicated one residue of the 4-*O*-Me-GlcNAcMe derivative (Table II), quantitation by total ion current is well-known to yield semiquantitative data; application of response factors can vary from compound to compound, and low yields of partially methylated hexosaminitol acetates are frequently encountered (Levery & Hakomori, 1987). Furthermore, non-sugar contaminating peaks can

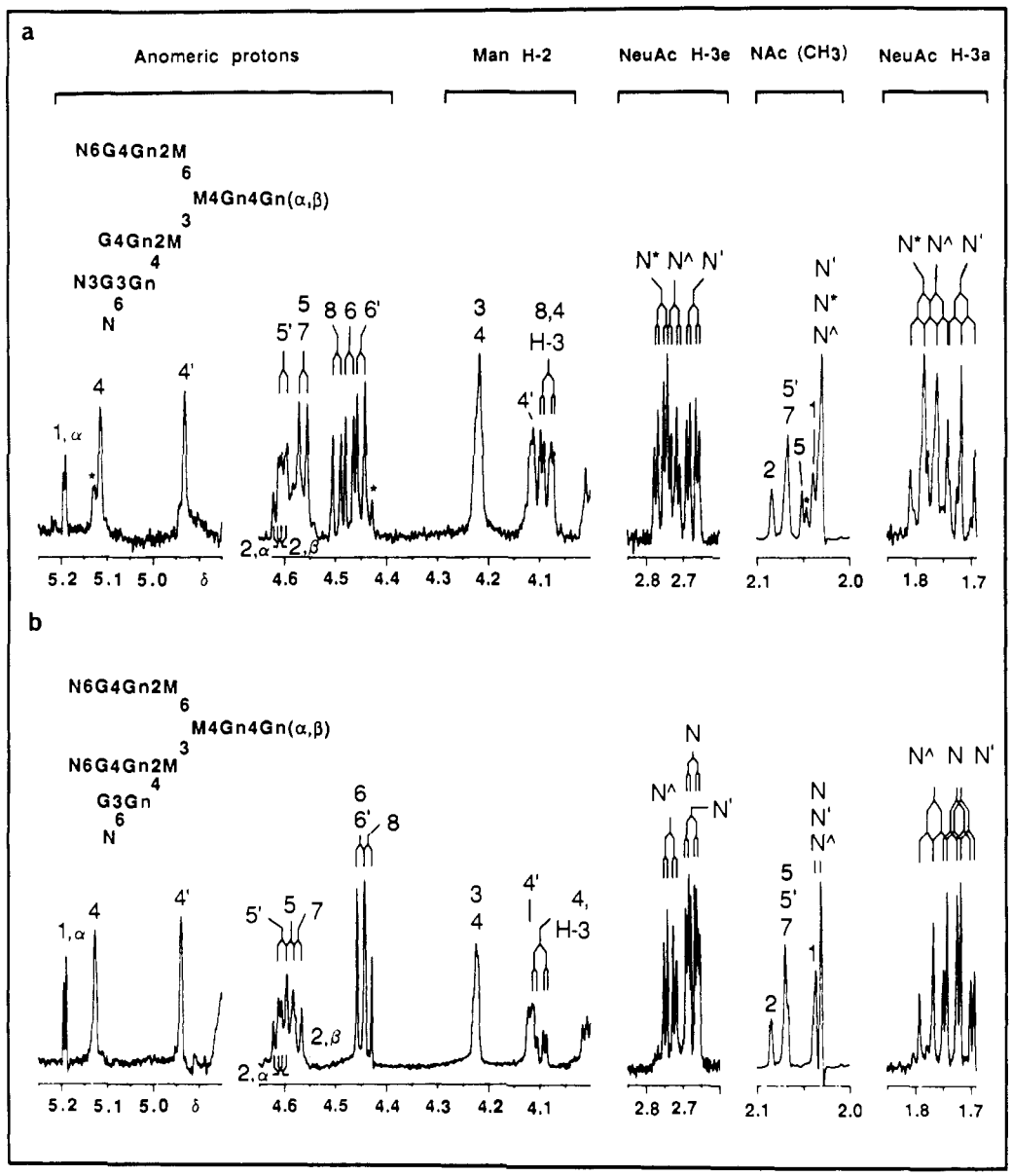


FIGURE 8: Partial ^1H NMR spectra (at 500 MHz) of reducing oligosaccharides released from fetuin. Acquisitions were performed at 27 °C in ^2H_2O. Shown are the sections of the spectra which include the structural reporter signals. As presented, sections vary in intensity, and therefore, integrations of signals as observed vary between sections. Assigned signals are indicated according to the generalized structure in Figure 1. Schematic structures presented with each spectrum are presented in full in Figure 1. (a) Spectrum of oligosaccharide Tri-S 5C. Signals of a minor component are shown with asterisks. (b) Spectrum of oligosaccharide Tri-S 6B.

present difficulties in quantitating by total ion current, which varies between individual samples.

An additional set of NeuAc H-3a/H-3e chemical shifts at δ = 1.725/2.671 was assigned to NeuAc residue N, present in α2→6 linkage to Gal 6. NeuAc shift assignments were made by comparison to those of Tetra-S 3 and Tetra-S 5, which displayed three of the characteristic sialic acid H-3a/H-3e pairs which, along with other characteristic proton signals, identified the structure present on the α1→3-linked mannose. The structure of the α1→6-linked branch was deduced from the knowledge that two NeuAcα2→3Galβ1→3-(NeuAcα2→6)GlcNAc units were present on the molecule.

Discussion

The asparagine-linked oligosaccharides of fetuin are a mixture of many structures; those reported herein include some of the major components. Besides the structures described in these papers, a number of other structures were also present, as indicated by NMR of other fractions. These were not sufficiently pure to unambiguously establish their structures as of yet. As shown in this paper, a number of these structures are related in having a Galβ1→3(NeuAcα2→6)-GlcNAcβ1→4Man moiety. Isolation of these structures demonstrates a complexity of sialylation patterns previously undetected (Takasaki & Kobata, 1986), a feature that was only made possible through the resolution afforded by two HPLC columns and evaluation of isomeric purity by high-field NMR.

It is interesting from a biosynthetic standpoint that in most oligosaccharides the Galβ1→3GlcNAc unit is located on the 4-position of Man 4 [also see Townsend et al. (1986)]. Therefore, it would appear that the galactosyltransferase that synthesizes the Galβ1→3GlcNAc linkage has a preference for the GlcNAcβ1→4Man branch in triantennary oligosaccharides. We did, however, isolate one oligosaccharide (Penta-S 6) having two Galβ1→3GlcNAc branches, which indicates that either the enzyme is not completely branch specific or that two such enzymes exist. In addition, preliminary NMR results of several other minor fractions indicate heterogeneity due to the presence of the Galβ1→3GlcNAc unit

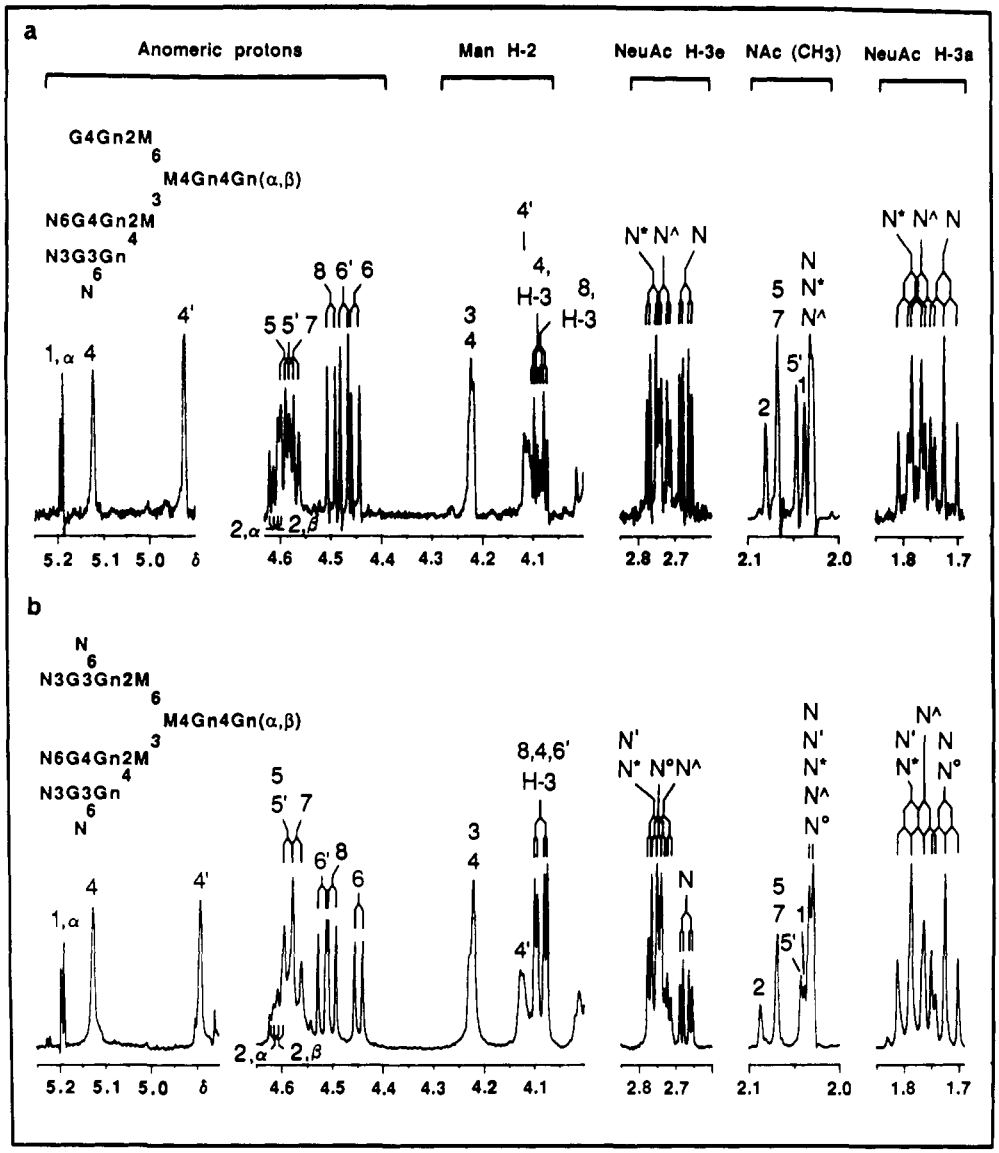


FIGURE 9: Partial 500-MHz ^1H NMR spectra of reducing oligosaccharides from the glycoprotein fetuin. Acquisitions were performed at 27 °C in ^2H_2O. The structural reporter regions of the spectra are shown in sections, which differ in intensity from section to section as plotted. Therefore, signal integrations vary between sections as shown in this figure. Labeled signals are indicated according to the generalized structure in Figure 1. Schematic structures shown with each spectrum are presented in full in Figure 1. (a) Spectrum of oligosaccharide Tri-S 5D. (b) Spectrum of oligosaccharide Penta-S 6.

on other branches of the basic triantennary structure.

Also of interest is structure Tri-S 6B, where an unsubstituted β-Gal is terminal in a Galβ1→3(NeuAcα2→6)GlcNAc unit. If the oligosaccharide does not represent an in vivo degradation product, this indicates that the sialyltransferase that catalyzes the NeuAcα2→6GlcNAc linkage may act on a free Galβ1→3GlcNAc linkage. Paulson et al. (1984) have studied this enzyme and reported that it was capable of acting on a terminal Galβ1→3GlcNAcβ1→R structure, although it had a preference for a NeuAcα2→3Galβ1→3GlcNAcβ1→R structure. However, as most purified glycoproteins, particularly plasma glycoproteins, reflect the structure of the molecule after exposure to in vivo synthetic as well as in vivo degradative processes, it is not possible to convincingly propose oligosaccharide biosynthetic pathways on the basis of observed structures present on glycoproteins. We believe that only studies of the glycosyltransferases themselves can demonstrate the specificity of biosynthetic pathways; attempts to do so with structural evidence alone is conjecture.

The ^1H NMR data indicate some interbranch perturbations caused by the presence of the NeuAc linked to the 6-position of GlcNAc residue 7. Most noticeable was the large downfield shift in the Man 4 H-3 signal (ca. +0.042 ppm), a change that was always correlated with the presence of NeuAc N^ and that was highly diagnostic for the Galβ1→3(NeuAcα2→6)-GlcNAcβ1→4Man unit. There are two interesting comparisons that can be made which indicate that NeuAc residues N and N^ give rise to mutual, interbranch perturbations. First, a comparison between the compound Tetra-S 3 and the identical compound lacking only residue N^ (Tri-S 2B; Bendiak et al., 1989) reveals the specific spectral shifts which occur upon addition of residue N^ to the molecule. These spectral shifts include the large shift of the Man 4 H-3 signal (from δ = 4.044 in Tri-S 2B to δ = 4.086 in Tetra-S 3). In addition, the NeuAcα2→6GlcNAc linkage had lesser effects on shifts of signals of the Man 4 H-1 (−0.006 ppm), GlcNAc 5 H-1 (−0.007 ppm), Gal 6 H-1 (+0.008 ppm), NeuAc N H-3a (+0.005 ppm), GlcNAc 7 H-1 (−0.005 ppm), Gal 8 H-1 (−0.010 ppm) and H-3 (−0.006 ppm), and N* H-3a (−0.003 ppm). The Man 4′ H-1 (−0.003 ppm), was also affected slightly. Clearly, the addition of the NeuAc residue N^ had significant effects on the induced magnetic environments of

protons in residues which seemed far removed (in primary structure) from the NeuAcα2→6GlcNAc linkage.

A second important comparison is between Tetra-S 3 and the identical structure lacking only NeuAc residue N, Tri-S 3D (an equally valid comparison with similar results can be made between the pair Tetra-S 5 and Tri-S 5C but will not be discussed further here). Quite evident in this comparison are the effects of the addition of NeuAc residue N upon the chemical shifts of other structural reporter signals. Included in these shifts are the signals assigned to Man 4 H-1 (+0.014 ppm), GlcNAc 5 H-1 (+0.026 ppm) and *N*-acetyl protons (+0.019 ppm), Gal 6 H-1 (−0.022 ppm), GlcNAc 7 H-1 (+0.007 ppm), Gal 8 H-1 (+0.005 ppm), and N^ H-3a (+0.004 ppm) and H-3e (+0.005 ppm). The Man 4′ H-1 signal (+0.003 ppm), H-2 signal (+0.006 ppm) and H-3 signal (+0.003 ppm) were also slightly affected.

On the basis of these comparisons, it is apparent that NeuAc residues N and N^ perturb each other's chemical shifts, as well as most other H-1 signals of the Manα1→3-linked branch. There are plausible hypotheses to explain this observation. It can be assumed, in the trisialylated molecules that lack either N^ or N [Tri-S 2B (Bendiak et al., 1989) and Tri-S 3-D] that the respective NeuAc residues which are present (N and N^) sample a space defined by rotations about the NeuAc glycosidic bond (ϕ and ψ torsion angles), rotation about the C-5 to C-6 bond of Gal 6 or GlcNAc 7 (ω), and the steric and energetic restraints dependent upon the rest of the molecule. Any space that could be independently occupied by either N^ or N in the trisialylated molecules would be a zone of potential steric contact in the tetrasialylated molecule (or at least a certain population of tetrasialylated molecules). Such steric contact might be avoided or minimized by the accommodation of minor changes in the population distribution about other glycosidic torsion angles, which could result in the observed minor changes in the chemical shifts of several protons which appear far removed from the immediate region of NeuAc substitution. Alternately, even without a region of potential steric contact of these NeuAc residues, averaged changes in torsion angles might occur for energetic reasons that we have not yet fully evaluated.

In addition, such proposals should be capable of explaining the large chemical shift observed in the Man 4 H-3 signal upon substitution of GlcNAc 7 with NeuAc N^. Preliminary potential energy minimization studies indicate a change in the calculated ensemble of ϕ,ψ glycosidic torsion angles for the Man 4 residue, but understanding this effect will clearly require additional studies of other conformational perturbations caused by the addition of NeuAc residue N^. What is evident from the data, however, is that highly characteristic perturbations (probably conformationally related) of the induced magnetic environment of several protons occur in the presence of this NeuAc residue and that residues N^ and N have mutual effects on the other's chemical shift.

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