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Sulfated di-, tri- and tetraantennary N-glycans in human Tamm-Horsfall glycoprotein

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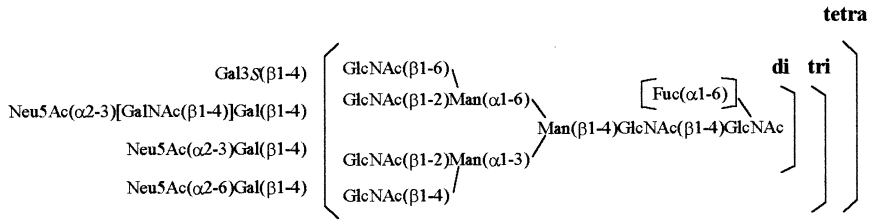
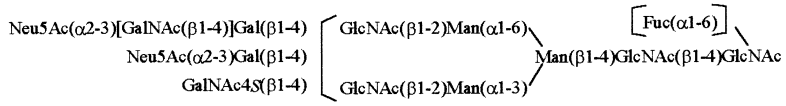
The primary structures of 32 sulfated di-, tri- and tetraantennary N-glycans of human Tamm-Horsfall glycoprotein (THP) have been determined. THP was isolated from the urine of one healthy male donor. The intact carbohydrate chains were released by PNGase-F and fractionated via FPLC on Resource Q, HPLC on LiChrosorb-NH_2, and high-pH anion-exchange chromatography on CarboPac PA-1. Characterizations were performed using 500-MHz and 600-MHz ^{1}H-NMR spectroscopy, in combination with sialidase treatments. The type of characterized N-glycans ranged from monosulfated to trisulfated N-glycans, whereby the sulfate groups were present as 3-*O*-sulfated Gal (Gal3*S*) and 4-*O*-sulfated GalNAc (GalNAc4*S*). A compilation of the established structures is shown below.





Keywords: Tamm-Horsfall glycoprotein; carbohydrate; NMR; sulfated N-glycan.

Human Tamm-Horsfall glycoprotein (THP) is the most abundant N-glycoprotein in normal human urine, being excreted in quantities of up to 100 mg/day (Tamm and Horsfall, 1950). THP is produced in the kidney where it is expressed via a phosphatidylinositol anchor on the endothelium of the thick ascending limb of the loop of Henle (Sikri et al., 1979; Rindler et al., 1990). The physiological function of THP is still subject of investigation. Several studies have indicated that THP acts as a natural inhibitor of microbial infections of the urinary tract and bladder (Duncan, 1988; Parkkinen et al., 1988). Uromodulin, a glycoprotein isolated from pregnant women that has the same amino acid sequence as THP, has the ability to inhibit antigen-specific T-cell proliferation *in vitro*, probably due to the glycan part of the protein (Muchmore et al., 1987). Furthermore, the carbohydrate chains of THP are implicated in the binding to neutrophils (Toma et al., 1994) and in immunosuppressive properties displayed by THP (Sathyamoorthy et al., 1991).

Previously, more than 150 carbohydrate-containing fractions were generated by peptide-N^4-(*N*-acetyl-*β*-glucosaminyl)asparagine amidase-F (PNGase-F) digestion of THP of one healthy male donor (Hård et al., 1992), and the primary structures of 30 N-glycans were determined, yielding di-, tri- and, most of all, tetraantennary structures, which can be fucosylated, sialylated, sulfated and/or contain the Sd^a determinant. It has been demonstrated that the Sd^a determinant is also expressed via an oligo(*N*-acetyllactosamine) unit on N-glycans of THP (Donald et al., 1983), and recently the characterization of five tetraantennary N-glycans, containing additional *N*-acetyllactosamine units with or without the Sd^a-determinant, has been reported (van Rooijen, J. J. M., Kamerling, J. P. and Vliegenthart, J. F. G., unpublished results). As shown by Hård et al. (1992), THP contains sulfated carbohydrate chains with 3-*O*-sulfated Gal (Gal3*S*) and/or 4-*O*-sulfated GalNAc (GalNAc4*S*) residues, and six diantennary and one triantennary compounds have been identified.

This report delineates in further detail the complexity of the glycan moiety of THP in terms of sulfation patterns, whereby the primary structures of 32 sulfated oligosaccharides, including 27 novel multisulfated di-, tri- and tetraantennary carbohydrate chains with or without the Sd^a determinant, were characterized.

EXPERIMENTAL PROCEDURES

Materials. THP was isolated from pooled morning urine of one single healthy male donor as described (Serafini Cessi et al., 1989), the donor being different from the one reported previously by Hård et al. (1992). Recombinant PNGase-F from *Fla-*

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Abbreviations. PNGase-F, peptide-N^4-(*N*-acetyl-*β*-glucosaminyl)-asparagine amidase-F; HPAEC, high-pH anion-exchange chromatography.

Enzymes. Peptide-N^4-(*N*-acetyl-*β*-glucosaminyl)asparagine amidase-F (EC 3.5.1.52); acylneuraminylhydrolase (sialidase) (EC 3.2.1.18).

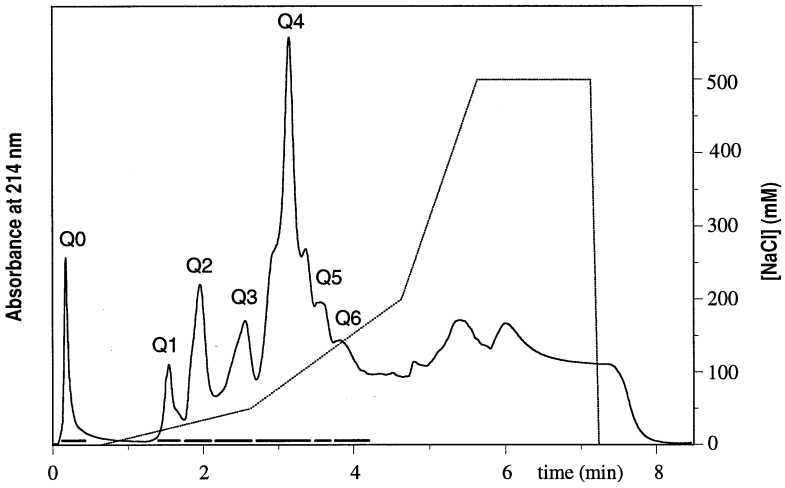


Fig. 1. Fractionation pattern at 214 nm on a FPLC Resource Q column of the carbohydrate-containing Superdex 75 fraction, derived from PNGase-F-treated human THP. Elutions were performed with linear concentration gradients of H_2O and NaCl as indicated in the figure, at a flow rate of 4 ml/min.

vobacterium meningosepticum and sialidase from *Vibrio cholerae* were purchased from Boehringer Mannheim.

Liberation of the carbohydrate chains. The N-linked carbohydrate chains were enzymatically released from THP according to a slightly modified version of a previously described protocol (Hård et al., 1992). Eight batches, each containing approximately 80−90 mg glycoprotein, were each dissolved in 8 ml H_2O. After addition of 0.8 ml 10% (by vol.) 2-mercaptoethanol and 1.6 ml 10% (mass/vol.) SDS, the solution was boiled for 3 min. After cooling to room temperature, 8 ml 7.1% (mass/vol.) octyl α-D-glucopyranoside in 50 mM Tris/HCl, pH 7.1, 50 mM EDTA, was added, and the solution was incubated with 24 U PNGase-F for 24 h at room temperature. Then, the mixture was boiled for 3 min, cooled to room temperature, and incubated again with 24 U PNGase-F for 24 h. After boiling for 3 min, the deglycosylation was assessed by SDS/PAGE on a 10% slab gel with Coomassie brilliant blue staining. All the batches were pooled, and aliquots of 6 ml of the total pool were fractionated on a Superdex 75 column (60 cm×2.6 cm, Pharmacia), eluted with 100 mM NH_4HCO_3, pH 7.0, at a flow rate of 4 ml/min. The effluent was monitored at 206 nm (Uvicord, LKB). Carbohydrate-positive fractions (orcinol/H_2SO_4) and fractions containing the deglycosylated protein were pooled, lyophilized, desalted by HiTrap (Pharmacia FPLC system; four 5-ml columns connected; eluent, 5 mM NH_4HCO_3; flow rate, 3 ml/min; detection, 214 nm), and lyophilized. Monosaccharide analyses of liberated carbohydrate chains and deglycosylated and glycosylated protein fractions were performed as described (Kamerling and Vliegenthart, 1989).

FPLC fractionation. The enzymatically released carbohydrate chains were fractionated by anion-exchange chromatography on a Resource Q column (1 ml, Pharmacia FPLC system) (Damm et al., 1987), at a flow rate of 4 ml/min. The elution was performed with 2.8 ml H_2O, followed by linear gradients from 0 to 50 mM NaCl in 8 ml H_2O, from 50 mM to 200 mM NaCl in 8 ml H_2O, and from 200 mM to 500 mM NaCl in 4 ml H_2O. The fractionation was monitored at 214 nm. Carbohydrate-positive fractions (orcinol/H_2SO_4) were lyophilized, desalted by HiTrap (Pharmacia), lyophilized again, and screened by ^{1}H-NMR spectroscopy for the presence of sulfated N-glycans.

HPLC fractionation. HPLC of FPLC fractions was carried out as described (Hård et al., 1992) using a Kratos SF 400 HPLC system (ABI Analytical, Kratos Division) equipped with a 5-µm LiChrosorb-NH_2 column (25 cm×0.4 cm, Merck) at a flow rate of 1.5 ml/min. Elutions were performed with linear acetonitrile gradients in 30 mM K_2HPO_4/KH_2PO_4, pH 6.8, as indicated in the figures. The effluent was monitored at 206 nm and relevant fractions were concentrated under a stream of nitrogen. These fractions were lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

High-pH anion-exchange chromatography (HPAEC). Subfractionation of the relevant HPLC fractions was performed by HPAEC with pulsed amperometric detection on a Dionex LC system consisting of a Dionex Bio LC quaternary gradient module, a PAD 2 detector, and a CarboPac PA-1 pellicular anion-exchange column (25 cm×0.9 cm, Dionex). Elutions were carried out with gradients of NaOAc in 0.1 M NaOH as indicated in the figures at flow rates of 4 ml/min. Detection was performed using the following pulse potentials and durations: E_1 = 0.05 V (300 ms); E_2 = 0.65 V (60 ms); E_3 = −0.95 V (180 ms). Collected fractions were immediately neutralized by 5 M HOAc, lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

Desialylation of N-glycans. Oligosaccharides were dissolved in 200 µl 100 mM NaOAc, pH 5.6, 1 mM $Ca(OAc)_2$ and 100 mM NaCl, and treated with 5 mU sialidase (*V. cholerae*). Each sample was incubated for 6 h at 37°C and separated by FPLC as described above. Carbohydrate-positive fractions were lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

^{1}H-NMR spectroscopy. Prior to ^{1}H-NMR analysis, samples were exchanged twice in 99.9% ^2H_2O with intermediate lyophilization, and dissolved in 450 µl 99.96% ^2H_2O (Isotec Inc). 500-MHz ^{1}H-NMR spectra were recorded on a Bruker AMX 500 instrument (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University, The Netherlands), and 600-MHz ^{1}H-NMR spectra on a Bruker AMX 600 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). The probe temperature was 300 K unless indicated otherwise. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225 in ^2H_2O at 300 K) (Vliegenthart et al., 1983). Typically, one-dimensional spectra were recorded with a spectral width of 6500 Hz, collecting 1000−4000 free induction decays of 16k complex data points. Suppression of the residual water signal was achieved by applying the WEFT pulse sequence as described (Hård et al., 1992). The resolution of the one-dimensional spectra was enhanced by

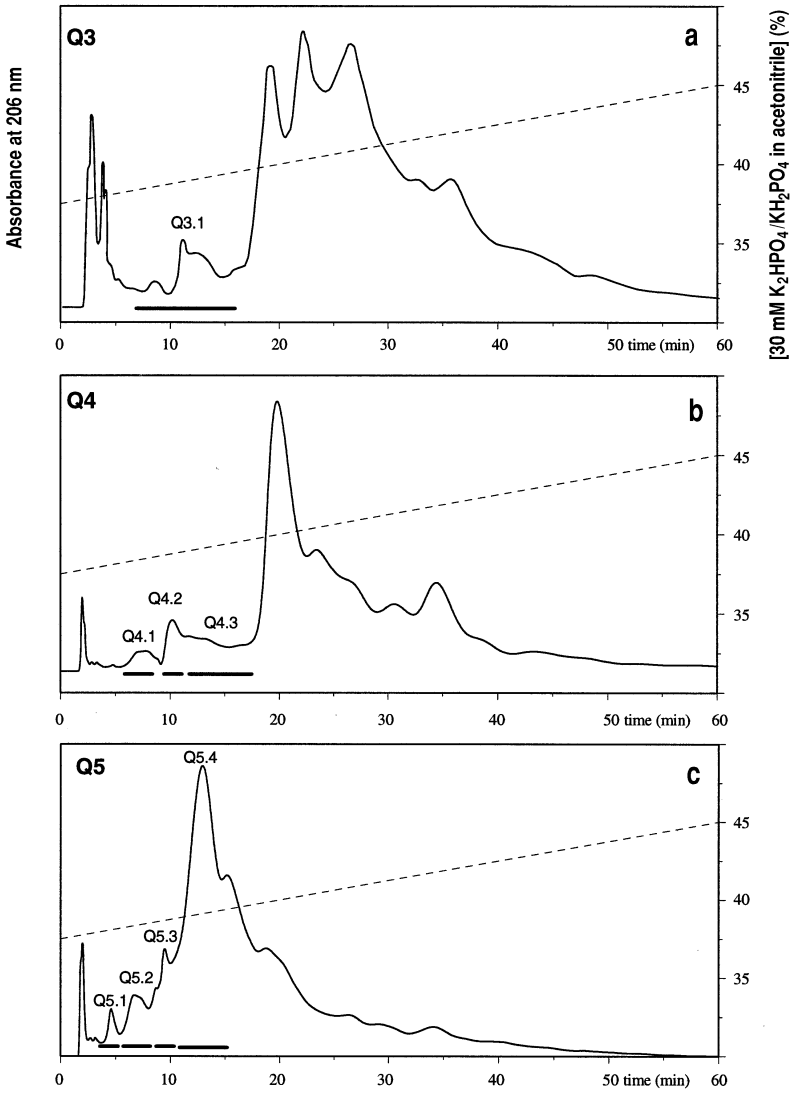


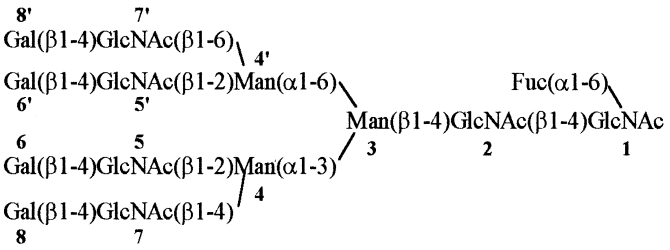
Fig. 2. Fractionation patterns at 206 nm on a HPLC LiChrosorb-NH_2 column of FPLC oligosaccharide fractions Q3–Q5, obtained from human THP. Elutions were performed with linear concentration gradients of 30 mM K_2HPO_4/KH_2PO_4, pH 6.8, and acetonitrile as indicated in the figures, at a flow rate of 1.5 ml/min. (a) FPLC fraction Q3; (b) fraction Q4; (c) fraction Q5.

Lorentzian-to-Gaussian transformation, and the final spectra were baseline corrected with a fourth-order-polynomial function.

RESULTS

General. Isolated THP (700 mg) appeared as a single band at 94 kDa on SDS/PAGE. Monosaccharide analysis of THP revealed the presence of Fuc, Man, Gal, GalNAc, GlcNAc and Neu5Ac in the molar ratio 0.9:3.0:3.7:1.1:5.5:2.9. The carbohydrate content was determined to be 28% (by mass). After PNGase-F treatment, THP migrated as a single band at 68 kDa on SDS/PAGE, indicating complete deglycosylation. The pool of enzymatically released N-linked carbohydrate chains was separated from the deglycosylated protein by gel-permeation chromatography on Superdex 75 (data not shown). Subfractionation of the carbohydrate-containing fraction on Resource Q yielded seven carbohydrate-positive fractions, denoted Q0−Q6 (Fig. 1). Each fraction was examined by ^{1}H-NMR spectroscopy for Gal3*S* and/or GalNAc4*S* residues (for specific structural-reporter-group signals, see Hård et al., 1992), affording Q3−Q6 as sulfate-containing fractions.

To separate sulfate-containing N-glycans from non-sulfate-containing N-glycans, fractions Q3, Q4 and Q5 were subfractionated by HPLC on LiChrosorb-NH_2, yielding the sulfate-containing fractions Q3.1, Q4.1−Q4.3, and Q5.1−Q5.4 (Fig. 2), respectively. Fraction Q6 contained only sulfated N-glycans and was, like fractions Q3.1, Q4.1−Q4.3 and Q5.1−Q5.4, subfractionated by HPAEC on CarboPac PA-1 (Fig. 3), affording a large series of subfractions. Only the fractions indicated in Fig. 3 were not too heterogeneous for structure determination or contained enough material for structural characterization by ^{1}H-NMR spectroscopy. Relevant ^{1}H-NMR data are compiled in Tables 1−3. The numbering of the monosaccharide residues is exemplified below.



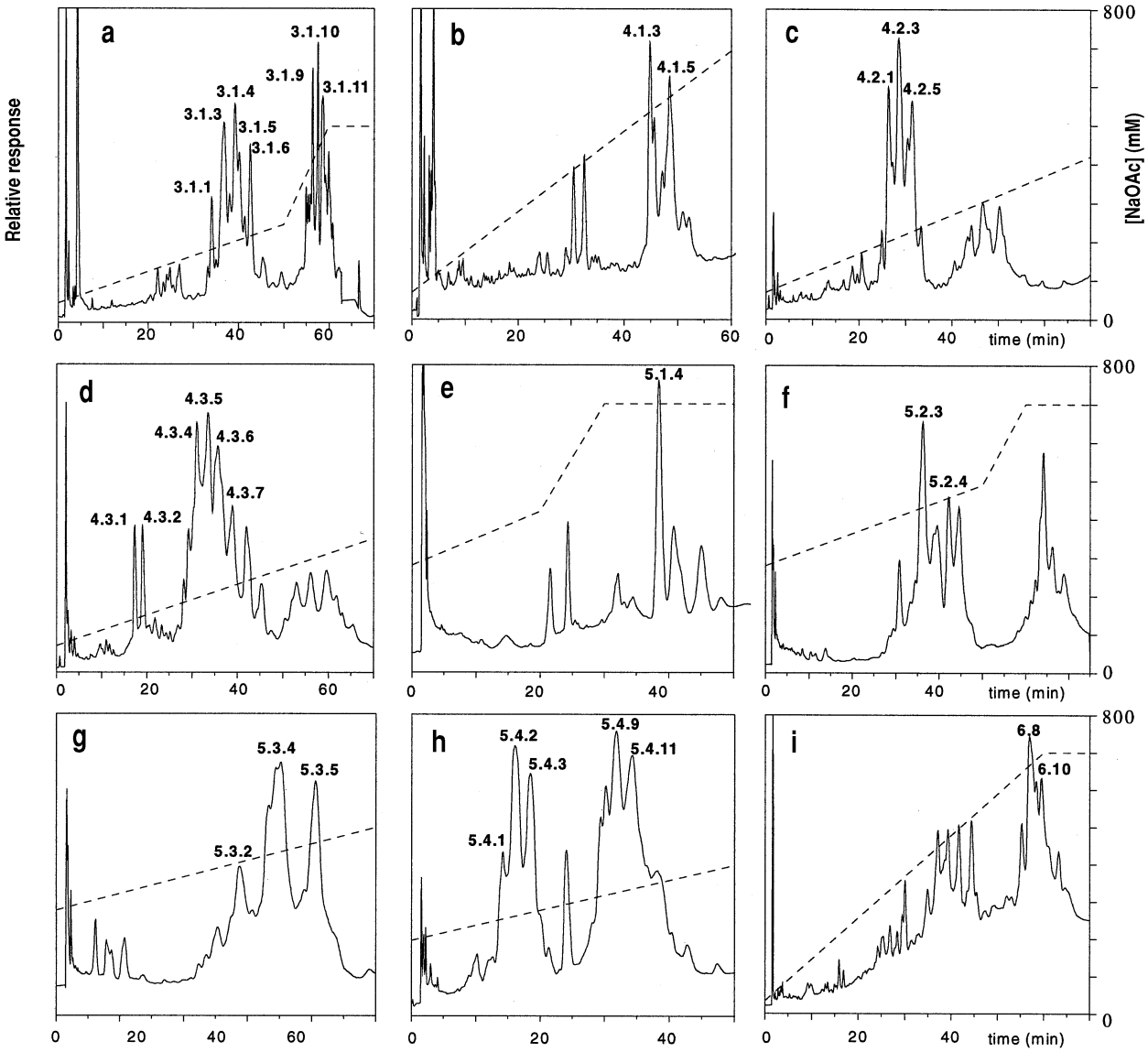


Fig. 3. Fractionation patterns of sulfate-containing HPLC fractions by HPAEC on CarboPac PA-1 with pulsed amperometric detection. Elutions were carried out with linear concentration gradients of NaOAc in 0.1 M NaOH as indicated in the figures, at a flow rate of 4 ml/min. (a) Q3.1; (b) Q4.1; (c) Q4.2; (d) Q4.3; (e) Q5.1; (f) Q5.2; (g) Q5.3; (h) Q5.4; (i) Q6.

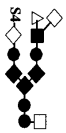
All identified N-linked oligosaccharides have one or more Gal3*S* or GalNAc4*S* residues at their non-reducing end. In general, 3-*O*-sulfation of Gal-**6**/**6′**/**8** residues is characterized by chemical shifts for Gal H3 at δ 4.335 (Gal-**6**/**8**) or δ 4.341 [Gal-**6′**; previously, a chemical shift value of δ 4.441 for N3.2.2B was tabulated in Hård et al. (1992)], and for Gal H4 at δ 4.291−4.296 (Gal-**6**/**6′**/**8**) [compare compounds N3.2.2A, N3.2.2B and N3.6.5 (Hård et al., 1992), and compound S_1-1 (de Waard et al., 1991)]. Gal H3 and Gal H4 resonate as a doublet of doublets [Gal H3 $J_{3,4}$ = 3.2 Hz, $J_{2,3}$ = 10 Hz; Gal H4 $J_{3,4}$ = 3.2 Hz, $J_{4,5}$ = 1 Hz (Kamerling et al., 1988)]. 3-*O*-sulfation of Gal leads to a downfield shift for Gal3*S* H1 of $\Delta\delta$ 0.04 compared with that of Gal H1 in a Neu5Ac(α2-3)Gal element. Furthermore, replacement of an (α2-3)-linked Neu5Ac residue by a sulfate group in a Gal(β1-4)GlcNAc(β1-*x*)Man sequence leads to a downfield shift of the *N*-acetyl signal of GlcNAc of $\Delta\delta$ 0.003−0.005 (Hård et al., 1992).

In diantennary N-glycans of THP, the presence of 4-*O*-sulfated GalNAc as part of the GalNAc(β1-4)GlcNAc(β1-2)Man(α1-3) element is indicated by reporter-group signals for GalNAc4*S* H1 at δ 4.586, H4 at δ 4.692, and NAc at δ 2.069 (Hård et al., 1992). When 4-*O*-sulfated GalNAc occurs at the Man(α1-6) branch, GalNAc4*S* H1 resonates at δ 4.591, H4 at δ 4.693, and NAc at δ 2.077 (Bergwerff et al., 1995).

The Sd[a] determinant {Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β-} is recognized from the typical signals for Neu5Ac H3a at δ 1.93 and H3e at δ 2.66. Furthermore, GalNAc H1 is observed at δ 4.73−4.76 (42°C), while its NAc signal resonates at δ 2.015−2.019. Due to the attachment of GalNAc to Gal via a (β1-4) linkage, Gal H4 resonates at δ 4.11−4.12 and H3 at δ 4.15 (Williams et al., 1984; Donald and Feeney, 1986; Hård et al., 1992).

The N-linked oligosaccharides identified have either an (α1-6)-fucosylated or a non-fucosylated *N*,*N′*-diacetylchitobiose element at their reducing ends. These N-glycans can be distinguished by the anomeric signals of αGlcNAc-**1** and GlcNAc-**2**, and the *N*-acetyl methyl signals of GlcNAc-**1** and GlcNAc-**2** in combination with the presence or absence of the H1 and CH_3

Table 1. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of sulfated diantennary N-linked oligosaccharides, derived from human THP. Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225). △, Neu5Ac(α2-3); ○, Neu5Ac(α2-6); ●, GlcNAc; ◆, Man; ■, Gal; ◇, GalNAc; □, Fuc. For numbering of the monosaccharide residues, see text. α and β represent the anomeric configuration of GlcNAc-**1**. n.d., not determined. −, not applicable. Some values are given with only two decimal figures because of spectral overlap. Neu5Ac′ is linked to Gal-**6′**.

Reporter signal	Residue	Chemical shift in					
		Q3.1.3 	**Q3.1.5** 	**Q3.1.4** 	**Q3.1.6** 	**Q3.1.1** 	**Q3.1.11A** 
		ppm					
H1	GlcNAc-**1*α***	5.180	5.190	5.180	5.189	5.180	5.180
	GlcNAc-**2**	4.666	4.613	4.665	4.614	4.667	4.663
	Man-**4**	5.110	5.109	5.118	5.118	5.109	5.113
	Man-**4′**	4.924	4.923	4.924	4.924	4.926	4.923
	GlcNAc-**5**	4.559	4.557	4.583	4.582	4.558	4.582
	GlcNAc-**5′**	4.572	4.571	4.572	4.571	4.570	4.577
	Gal-**6**	−	−	4.583	4.582	−	4.582
	Gal-**6′**	4.549	4.549	4.549	4.550	4.546	4.588
	GalNAc	−	−	−	−	4.73	−
	GalNAc4*S*	4.587	4.584	−	−	4.586	−
	Fuc_α	4.892	−	4.892	−	4.889	4.889
	Fuc_β	4.898	−	4.900	−	4.898	4.898
H2	Man-**3**	4.245	4.244	4.246	4.247	4.244	4.245
	Man-**4**	4.180	4.178	4.191	4.192	4.177	4.188
	Man-**4′**	n.d.	n.d.	4.11	4.11	n.d.	n.d.
H3	Gal-**6**	−	−	4.336	4.335	−	4.336
	Gal-**6′**	n.d.	n.d.	4.11	4.11	n.d.	4.340
H3a	Neu5Ac′	1.802	1.800	1.801	1.801	1.926	−
H3e	Neu5Ac′	2.758	2.758	2.757	2.758	2.662	−
H4	Gal-**6**	−	−	4.292	4.292	−	4.295
	Gal-**6′**	n.d.	n.d.	n.d.	n.d.	n.d.	4.295
	GalNAc4*S*	4.693	4.691	−	−	4.692	−
NAc	GlcNAc-**1**	2.04	2.038	2.04	2.04	2.038	2.038
	GlcNAc-**2**	2.094	2.080	2.094	2.081	2.094	2.094
	GlcNAc-**5**	2.04	2.043	2.050	2.050	2.043	2.050
	GlcNAc-**5′**	2.04	2.043	2.04	2.04	2.043	2.045
	GalNAc4*S*	2.069	2.068	−	−	2.067	−
	Neu5Ac	2.032	2.031	2.032	2.032	2.031	−
	GalNAc	−	−	−	−	2.015	−
CH_3	Fuc_α	1.210	−	1.209	−	1.209	1.208
	Fuc_β	1.222	−	1.222	−	1.220	1.220

signals of Fuc (Hård et al., 1992). The branching patterns of the elucidated N-glycans are deduced from the sets of chemical shifts of the H1 signals of Man-**4** and Man-**4′**, together with those of the H2 signals of Man-**3**, Man-**4** and Man-**4′** (Vliegenthart et al., 1983). Furthermore, Neu5Ac occurs in (α2-3) or (α2-6) linkages to Gal(β1-4)GlcNAc, as deduced from the H3e, H3a, and NAc signals of Neu5Ac and the H3 signal of Gal to which the sialic acid residue is linked (Vliegenthart et al., 1983).

Sulfated diantennary N-glycans. HPLC fraction Q3.1 contained mainly diantennary structures with Gal3*S* or GalNAc4*S* residues (Table 1). The glycans in the HPAEC fractions Q3.1.3, Q3.1.5, Q3.1.4, and Q3.1.6 have been established earlier for THP [compare compounds N3.2.1, N3.2.3, N3.2.2A, and N3.2.4, respectively (Hård et al., 1992)].

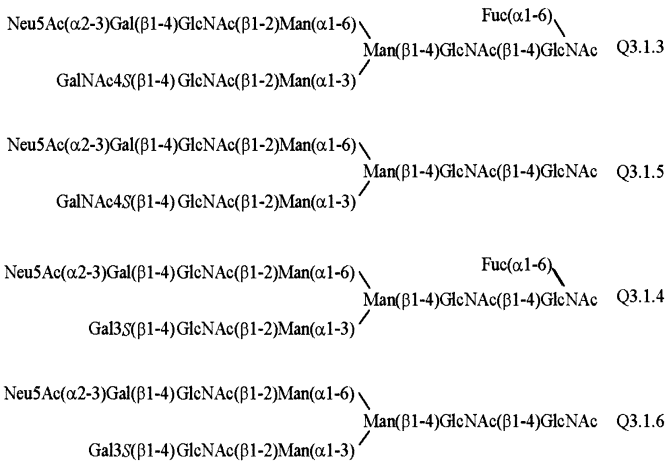
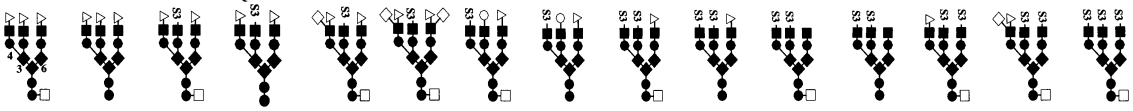
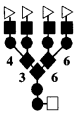
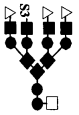
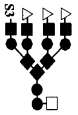
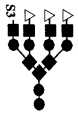
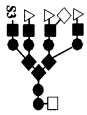
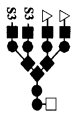
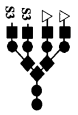
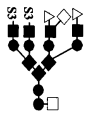
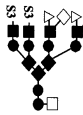
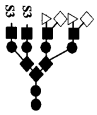


Table 2. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of sulfated triantennary N-linked oligosaccharides, derived from human THP. Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225). For explanation of symbols, see Table 1. For numbering of the monosaccharide residues, see text. α and β represent the anomeric configuration of GlcNAc-**1**. n.d., not determined. –, not applicable. Some values are given with only two decimal figures due to spectral overlap. For H3a,3e, Neu5Ac is linked to Gal-**6**, Neu5Ac′ to Gal-**6′** and Neu5Ac* to Gal-**8**.

Reporter signal	Residue	Chemical shift in														
		Q4.3.1	Q4.3.2	Q4.2.3	Q4.2.5	Q4.2.1	Q4.3.4	Q4.3.6	Q4.3.7	Q5.2.3	Q5.2.4	Q3.1.9	Q3.1.10	Q4.1.5	Q4.1.3	Q5.1.4
																
		ppm														
H1	GlcNAc-**1*α***	5.181	5.190	5.181	5.190	5.183	5.182	5.182	5.189	5.182	5.191	5.179	5.191	5.181	5.181	5.181
	GlcNAc-**1*β***	4.691	4.69	4.694	4.695	4.694	4.69	4.69	4.69	n.d.	4.69	n.d.	n.d.	n.d.	n.d.	4.69
	GlcNAc-**2**	4.665	4.611	4.664	4.611	4.665	4.67	4.665	4.617	4.663	4.612	4.666	4.612	4.665	4.665	4.666
	Man-**3**	n.d.	n.d.	4.76[a]	n.d.	4.76[a]	4.76[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Man-**4**	5.115	5.114	5.116	5.116	5.115	5.115	5.129	5.128	5.116	5.116	5.119	5.119	5.116	5.115	5.117
	Man-**4′**	4.906	4.908	4.904	4.908	4.906	4.909	4.908	4.911	4.907	4.911	4.923	4.923	4.911	4.911	4.912
	GlcNAc-**5**	4.559	4.560	4.571	4.571	4.572	4.573	4.597	4.596	4.570	4.571	4.570	4.570	4.570	4.570	4.570
	GlcNAc-**5′**	4.571	4.570	4.571	4.571	4.572	4.573	4.571	4.571	4.570	4.571	4.582	4.582	4.580	4.581	4.580
	Gal-**6**	4.542[b]	4.54	4.580	4.580	4.580	4.579	4.442	4.442	4.580	4.580	4.582	4.582	4.580	4.581	4.580
	Gal-**6′**	4.546[b]	4.54	4.545	4.546	4.542[b]	4.542[b]	4.547	4.546	4.548	4.548	4.474	4.474	4.589	4.589	4.587
	GlcNAc-**7**	4.546[b]	4.54	4.545	4.546	4.542[b]	4.542[b]	4.556	4.556	4.554	4.553	4.554	4.555	4.545	4.541	4.548
	Gal-**8**	4.542[b]	4.54	4.545	4.546	4.548[b]	4.547[b]	4.585	4.585	4.585	4.582	4.582	4.582	4.545	4.541	4.587
	GalNAc	–	–	–	–	4.735[a]	4.732[a,c]	–	–	–	–	–	–	–	n.d.	–
	Fuc_α	4.892	–	4.891	–	4.892	4.892	4.892	–	4.892	–	4.895	–	4.892	4.893	4.892
	Fuc_β	4.900	–	4.898	–	4.900	4.900	4.899	–	4.899	–	4.901	–	4.898	4.901	4.899
H2	Man-**3**	4.209	4.209	4.209	4.213	4.212	4.212	4.215	4.215	4.214	4.212	4.213	4.215	4.213	4.214	4.214
	Man-**4**	4.209	4.209	4.209	4.213	4.212	4.212	4.215	4.215	4.214	4.212	4.213	4.215	4.213	4.214	4.214
	Man-**4′**	4.106	4.106	4.106	4.108	4.105	4.105	4.105	4.105	4.106	4.107	4.105	4.105	4.106	4.105	4.105
H3	Gal-**6**	4.116	4.116	4.336	4.336	4.336	4.336	n.d.	n.d.	4.336	4.337	4.336	4.336	4.336	4.337	4.337
	Gal-**6′**	4.116	4.116	4.11	4.11	4.11	4.153	4.119	4.118	4.117	4.12	n.d.	n.d.	4.341	4.341	4.341
	Gal-**8**	4.116	4.116	4.11	4.11	4.153	4.153	4.336	4.336	4.336	4.337	4.336	4.336	4.11	4.15	4.337
H3a	Neu5Ac	1.802	1.801	–	–	–	–	1.718	1.719	–	–	–	–	–	–	–
	Neu5Ac′	1.802	1.801	1.801	1.801	1.800	1.927	1.802	1.801	1.801	1.801	–	–	–	–	–
	Neu5Ac*	1.802	1.801	1.801	1.801	1.927	1.927	–	–	–	–	–	–	1.801	1.926	–
H3e	Neu5Ac	2.756	2.756	–	–	–	–	2.664	2.665	–	–	–	–	–	–	–
	Neu5Ac′	2.756	2.756	2.757	2.756	2.757	2.663	2.756	2.756	2.758	2.758	–	–	–	–	–
	Neu5Ac*	2.756	2.756	2.757	2.756	2.660	2.663	–	–	–	–	–	–	2.754	2.660	–
H4	Gal-**6**	n.d.	n.d.	4.292	4.292	4.293	4.292	n.d.	n.d.	4.292	4.293	4.293	4.293	4.294	4.295	4.29
	Gal-**6′**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.294	4.295	4.29
	Gal-**8**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.292	4.292	4.292	4.293	4.293	4.293	n.d.	n.d.	4.29
NAc	GlcNAc-**1**	2.039	2.039	2.040	2.039	2.040	2.039	2.038	2.038	2.040	2.040	2.040	2.040	2.040	2.040	2.039
	GlcNAc-**2**	2.095	2.080	2.093	2.080	2.095	2.096	2.095	2.080	2.095	2.080	2.096	2.079	2.095	2.095	2.094
	GlcNAc-**5**	2.044	2.043	2.047	2.047	2.047	2.047	2.066	2.066	2.048	2.048	2.048	2.048	2.048	2.048	2.048
	GlcNAc-**5′**	2.044	2.043	2.044	2.043	2.044	2.044	2.043	2.042	2.044	2.044	2.044	2.043	2.048	2.048	2.048
	GlcNAc-**7**	2.074	2.072	2.073	2.073	2.074	2.074	2.077	2.077	2.078	2.078	2.079	2.079	2.074	2.074	2.078
	Neu5Ac	2.031[d]	2.031[d]	2.031[e]	2.031[e]	2.031[e]	2.032[e]	2.031[e]	2.030[e]	2.032	2.032	–	–	2.032	2.032	–
	GalNAc	–	–	–	–	2.015	2.015[c]	–	–	–	–	–	–	–	2.015	–
CH_3	Fuc_α	1.211	–	1.210	–	1.210	1.210	1.210	–	1.211	–	1.210	–	1.210	1.210	1.211
	Fuc_β	1.222	–	1.222	–	1.222	1.222	1.221	–	1.221	–	1.221	–	1.222	1.222	1.222

[a] Value obtained at 289 K.
[b] Assignments may have to be interchanged.
[c] Signal belonging to two GalNAc residues.
[d] Signal belonging to three Neu5Ac residues.
[e] Signal belonging to two Neu5Ac residues.

Table 3. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of sulfated tetraantennary N-linked oligosaccharides, derived from human THP. Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225). For explanation of symbols, see Table 1. For numbering of the monosaccharide residues, see text. α and β represent the anomeric configuration of GlcNAc-**1**. n.d., not determined. –, not applicable. Some values are given to two decimal figures due to spectral overlap. For H3a,3e, Neu5Ac is linked to Gal-**6**, Neu5Ac′ to Gal-**6′**, Neu5Ac* to Gal-**8** and Neu5Ac^ to Gal-**8′**. Data for N4.4.5 are from Hård et al. (1992).

Reporter signal	Residue	Chemical shift in										
		N4.4.5	**Q5.4.2A**	**Q5.4.2B**	**Q5.4.3A**	**Q5.4.3B**	**Q5.4.1**	**Q5.3.4**	**Q5.3.5**	**Q5.3.2**	**Q5.4.9**	**Q5.4.11**
												
		ppm										
H1	GlcNAc-**1*α***	5.181	5.183	5.183	5.192	5.192	5.181	5.182	5.190	5.182	5.180	5.189
	GlcNAc-**1*β***	4.689	4.689	4.689	4.692	4.692	n.d.	4.689	4.691	n.d.	4.695	4.694
	GlcNAc-**2**	4.66[a]	4.663	4.663	4.608	4.608	4.660	4.661	4.608	4.664	4.669	4.60
	Man-**3**	4.752	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Man-**4**	5.130	5.131	5.131	5.131	5.131	5.132	5.133	5.132	5.132	5.131	5.131
	Man-**4′**	4.857	4.857	4.857	4.860	4.860	4.860	4.858	4.859	4.860	4.860	4.860
	GlcNAc-**5**	4.564	4.576	4.56	4.577	4.56	4.565	4.579	4.578	4.579	4.578	4.578
	GlcNAc-**5′**	4.593	4.595	4.595	4.60	4.60	4.60	4.597	4.597	4.598	4.60	4.60
	Gal-**6**	4.542[b]	4.584	4.545	4.584	4.547	4.544	4.586	4.584	4.585	4.586	4.584
	Gal-**6′**	4.545[b]	4.545	4.545	4.547	4.547	4.544	4.546	4.547	4.547	4.54	4.54
	GlcNAc-**7**	4.542[b]	4.545	4.56	4.547	4.56	4.558	4.559	4.559	4.555	4.555	4.556
	GlcNAc-**7′**	4.545[b]	4.545	4.545	4.547	4.547	4.544	4.546	4.547	4.547	4.54	4.54
	Gal-**8**	4.542[b]	4.545	4.584	4.547	4.584	4.586	4.586	4.584	4.585	4.586	4.584
	Gal-**8′**	4.559	4.56	4.56	4.56	4.56	4.558	4.559	4.559	4.555	4.555	4.556
	GalNAc	–	–	–	–	–	n.d.	–	–	n.d.	n.d.	n.d.
	Fuc_α	4.900	4.900	4.900	–	–	4.901	4.900	–	4.900	4.900	–
	Fuc_β	4.908	4.910	4.910	–	–	4.910	4.908	–	4.907	4.909	–
H2	Man-**3**	4.204	4.202	4.202	4.203	4.203	4.207	4.204	4.205	4.207	4.204	4.205
	Man-**4**	4.220	4.219	4.219	4.221	4.221	4.219	4.220	4.220	4.219	4.220	4.217
	Man-**4′**	4.090	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10
H3	Gal-**6**	4.117	4.336	4.117	4.336	4.116	4.119	4.337	4.336	4.336	4.336	4.337
	Gal-**6′**	4.117	4.117	4.117	4.116	4.116	4.156	4.117	4.118	4.159	4.15	4.15
	Gal-**8**	4.117	4.117	4.336	4.116	4.336	4.336	4.337	4.336	4.336	4.336	4.337
	Gal-**8′**	4.117	4.117	4.117	4.116	4.116	4.119	4.117	4.118	n.d.	4.15	4.15
H3a	Neu5Ac	1.803	–	1.803	–	1.802	1.801	–	–	–	–	–
	Neu5Ac′	1.803	1.803	1.803	1.802	1.802	1.927	1.802	1.802	1.929	1.927	1.927
	Neu5Ac*	1.803	1.803	–	1.802	–	–	–	–	–	–	–
	Neu5Ac^	1.803	1.803	1.803	1.802	1.802	1.801	1.802	1.802	1.800	1.927	1.927
H3e	Neu5Ac	2.758	–	2.755	–	2.754	2.756	–	–	–	–	–
	Neu5Ac′	2.758	2.755	2.755	2.754	2.754	2.662	2.756	2.756	2.660	2.661	2.661
	Neu5Ac*	2.758	2.755	–	2.754	–	–	–	–	–	–	–
	Neu5Ac^	2.758	2.755	2.755	2.754	2.754	2.756	2.756	2.756	2.757	2.661	2.661
H4	Gal-**6**	n.d.	4.292	n.d.	4.293	n.d.	n.d.	4.293	4.293	4.293	4.291	4.292
	Gal-**6′**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Gal-**8**	n.d.	n.d.	4.292	n.d.	4.293	4.293	4.293	4.293	4.293	4.291	4.292
	Gal-**8′**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NAc	GlcNAc-**1**	2.038	2.040	2.040	2.039	2.039	2.038	2.039	2.039	2.038	2.038	2.038
	GlcNAc-**2**	2.092[a]	2.093	2.093	2.080	2.080	2.093	2.093	2.079	2.094	2.092	2.079
	GlcNAc-**5**	2.047	2.052	2.048	2.052	2.049	2.049	2.052	2.052	2.052	2.051	2.052
	GlcNAc-**5′**	2.038	2.040	2.040	2.039	2.039	2.038	2.039	2.039	2.038	2.038	2.038
	GlcNAc-**7**	2.074	2.075	2.079	2.074	2.078	2.079	2.079	2.079	2.079	2.079	2.079
	GlcNAc-**7′**	2.038	2.040	2.040	2.039	2.039	2.038	2.039	2.039	2.038	2.038	2.038
	Neu5Ac	2.031[c]	2.032[d]	2.032[d]	2.032[d]	2.032[d]	2.032[d]	2.031[e]	2.031[e]	2.031[e]	2.031[e]	2.031[e]
	GalNAc	–	–	–	–	–	2.015	–	–	2.015	2.019/2.015	2.019/2.015
CH_3	Fuc_α	1.211	1.210	1.210	–	–	1.210	1.209	–	1.209	1.209	–
	Fuc_β	1.221	1.222	1.222	–	–	1.222	1.222	–	1.222	1.222	–

[a] Average chemical shift.
[b] Assignments may have to be interchanged.
[c] Signal belonging to four Neu5Ac residues.
[d] Signal belonging to three Neu5Ac residues.
[e] Signal belonging to two Neu5Ac residues.

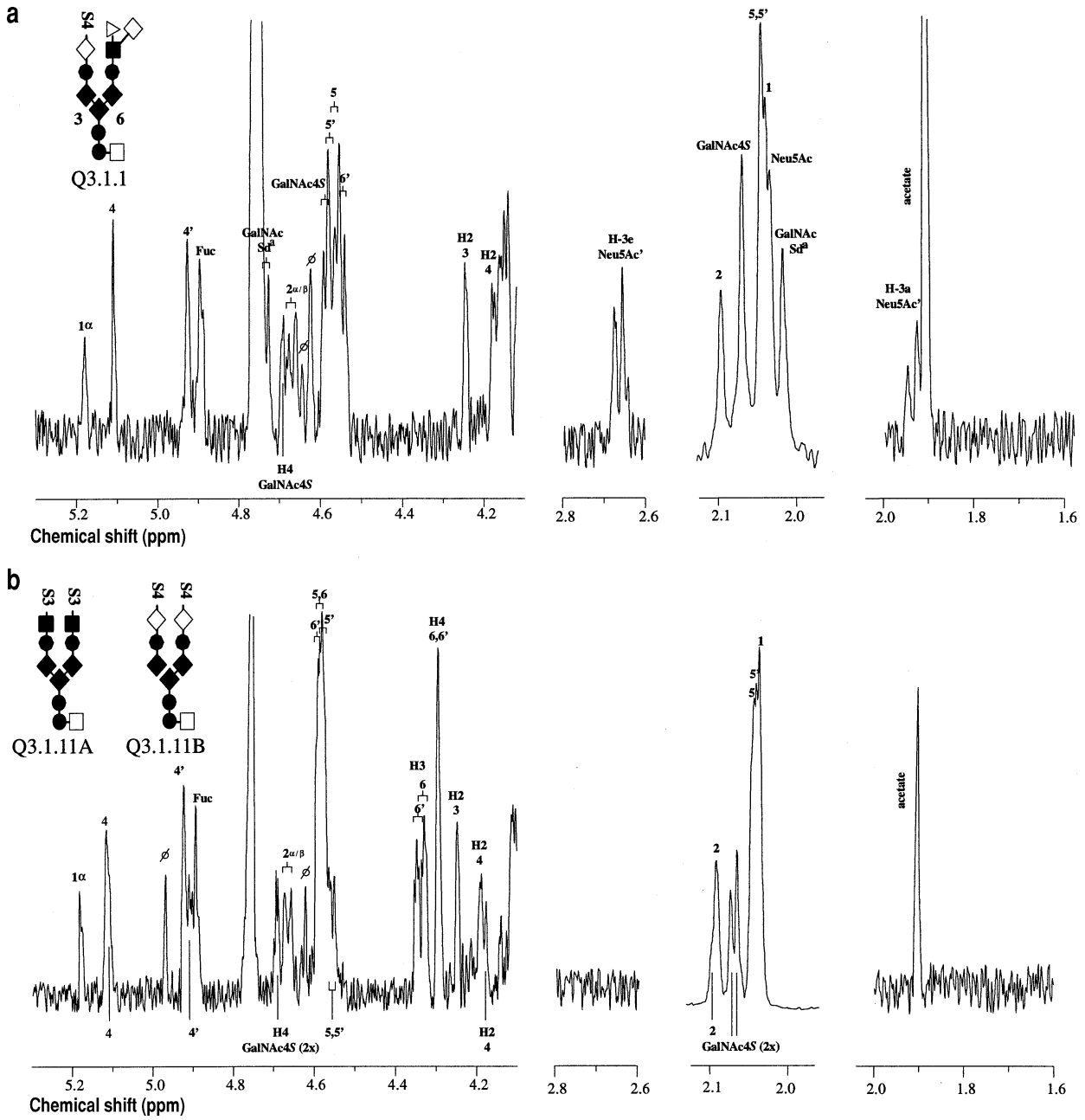


Fig. 4. Resolution-enhanced 500-MHz ^{1}H-NMR spectra at 300 K of sulfated diantennary oligosaccharides obtained from human THP. (a) Fraction Q3.1.1; (b) fraction Q3.1.11. Signals characteristic for Q3.1.11B are indicated below the spectrum. The numbering of the monosaccharide residues is indicated in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. △, Neu5Ac(*α*2-3); ○, Neu5Ac(*α*2-6); ●, GlcNAc; ◆, Man; ■, Gal; ◇, GalNAc; □, Fuc; ∅, contaminant.

Fraction Q3.1.1 contains a fucosylated diantennary structure having a GalNAc4*S* residue at the Man(*α*1-3) branch and an Sd^a determinant at the Man(*α*1-6) branch, as indicated by the structural-reporter-group signals for GalNAc4*S* (H1, *δ* 4.586; H4, *δ* 4.692; NAc, *δ* 2.067) and the Sd^a determinant (GalNAc H1, *δ* 4.73; GalNAc NAc, *δ* 2.015; Neu5Ac H3a, *δ* 1.926; Neu5Ac H3e, *δ* 2.662) (Fig. 4a).

Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\ Fuc(α1-6)\
Man(β1-4)GlcNAc(β1-4)GlcNAc Q3.1.1
GalNAc4*S*(β1-4)GlcNAc(β1-2)Man(α1-3)/

This is the only structure found combining the Sd^a determinant with a GalNAc4*S* residue.

Fraction Q3.1.11 consists of a mixture of two sulfated, non-sialylated diantennary structures (Fig. 4b).

Gal3*S*(β1-4)GlcNAc(β1-2)Man(α1-6)\ Fuc(α1-6)\
Man(β1-4)GlcNAc(β1-4)GlcNAc Q3.1.11A
Gal3*S*(β1-4)GlcNAc(β1-2)Man(α1-3)/

GalNAc4*S*(β1-4) GlcNAc(β1-2)Man(α1-6)\ Fuc(α1-6)\
Man(β1-4)GlcNAc(β1-4)GlcNAc Q3.1.11B
GalNAc4*S*(β1-4) GlcNAc(β1-2)Man(α1-3)/

The main compound, Q3.1.11A, is a fucosylated diantennary N-glycan terminated with a Gal3*S* residue at each branch, as indicated by the structural-reporter-group signals of Gal-**6** H3 at

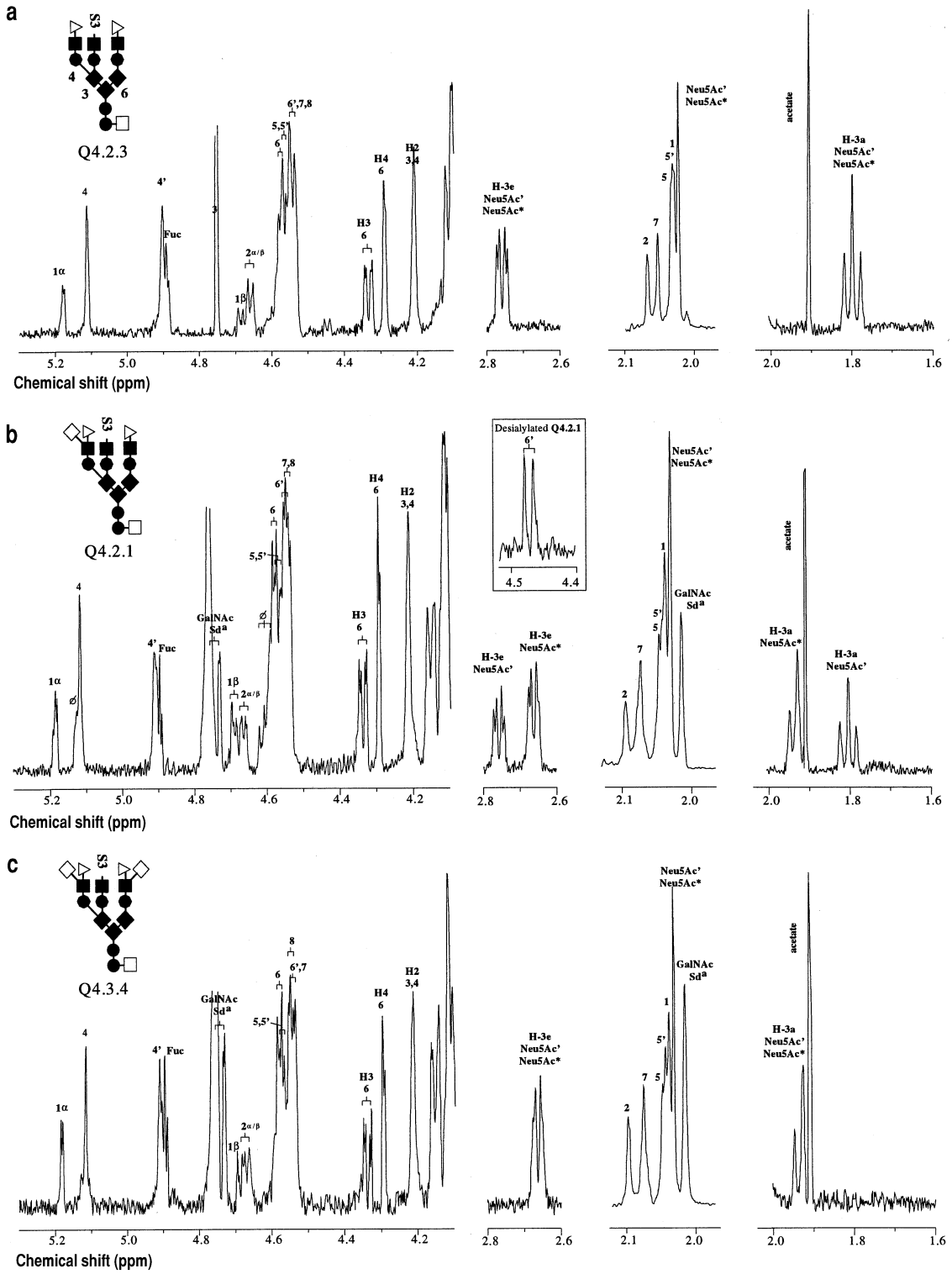


Fig. 5. Resolution-enhanced 600-MHz ^{1}H-NMR spectra at 300 K of sulfated triantennary oligosaccharides obtained from human THP. (a) Fraction Q4.2.3; (b) fraction Q4.2.1; (c) fraction Q4.3.4. The numbering of the monosaccharide residues is indicated in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. The symbols used are explained in the legend to Fig. 4.

δ 4.336, Gal-**6′** H3 at δ 4.340, and Gal-**6**/**6′** H4 at δ 4.295 [compare compound S_1-1 (de Waard et al., 1991)]. The minor compound, Q3.1.11B, is the sulfated GalNAc isomer with a GalNAc4*S* residue at each branch, indicated by the structural-reporter-group signals of GalNAc4*S* H4 at δ 4.692/4.692, and NAc at δ 2.068/2.075 [compare compound N6.1 (Bergwerff et al., 1995)].

Sulfated triantennary N-glycans. The ^{1}H-NMR data of the conventional (non-)fucosylated (α2-3)-trisialylated triantennary oligosaccharides present in fractions Q4.3.1 and Q4.3.2 [Table 2; compare compound N3.6.1 and N3.5 (Hård et al., 1992)] form the starting point for the analysis of the fractions containing sulfated triantennary N-glycans. The main component of HPLC fraction Q4.2 is HPAEC fraction Q4.2.3 (Fig. 5a, Table

2). The fucosylated triantennary compound contains one terminal Gal3*S* residue and two Neu5Ac(α2-3)Gal elements as shown by the relative intensities of Gal3*S* H3 and Neu5Ac H3e in the ^{1}H-NMR spectrum. The Gal3*S* H3 signal at δ 4.336 and the Gal3*S* H4 signal at δ 4.292 leaves two possibilities open for 3-*O*-sulfation, namely at Gal-**6** or at Gal-**8**. However, comparison of the *N*-acetyl regions of the ^{1}H-NMR spectra of Q4.3.1 and Q4.2.3 shows that the GlcNAc-**5** NAc signal shifts downfield, but not the GlcNAc-**7** NAc signal. This means that Gal-6 is 3-*O*-sulfated (H1, δ 4.580). Fraction Q4.2.5 is the non-fucosylated analogue of Q4.2.3 (Table 2).

```
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\                Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.2.3
                Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4)/

Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.2.5
                Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4)/

Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\                Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.2.1
                Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-4)/

Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\   Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.3.4
                Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-4)/
```

^{1}H-NMR analysis of HPAEC fraction Q4.2.1 shows that the major compound is an extension of Q4.2.3, wherein an (α2-3)-linked Neu5Ac residue is replaced by an Sd^a determinant (Fig. 5b, Table 2). Comparison of the chemical shifts of the GlcNAc-**5**, -**5′** and -**7** NAc signals in Q4.2.1 with those in Q4.3.1 and Q4.2.3 indicates the 3-*O*-sulfate group to be present at Gal-**6** in Q4.2.1. ^{1}H-NMR analysis of the sialidase-treated fraction Q4.2.1 revealed the structural-reporter-group signals Gal-**6′** H1 at δ 4.472 and Man-**4′** H1 at δ 4.923, indicating desialylated Gal-**6′** [compare compound N2.6B and N2.6C (Hokke et al., 1995), and compound 9 (Vliegenthart et al., 1983)]. This means that the structural element Neu5Ac(α2-3)Gal is located at the antenna containing Gal-**6′**, and the Sd^a determinant at the antenna containing Gal-**8**. The same compound was present in fraction Q4.3.5. The Sd^a determinant is resistant towards sialidase treatment (Donald et al., 1983).

Comparison of the ^{1}H-NMR spectrum of fraction Q4.3.4 with that of Q4.2.3 shows the replacement of both Neu5Ac(α2-3)Gal elements by Sd^a determinants (Fig. 5c, Table 2). The presence of one terminal Gal3*S* residue and two Sd^a determinants follows from the relative intensities of Gal3*S* H3 and Neu5Ac H3e. Based on the reasoning given for Q4.2.1, Gal-**6** is 3-*O*-sulfated. Compound Q4.3.4 gives new data about structural-reporter-group signals for the branch location of the Sd^a determinant. Hård et al. (1992) isolated a tetraantennary oligosaccharide containing four Sd^a determinants, wherein three GalNAc NAc signals resonated at δ 2.015, and one at δ 2.019 (compare compound N4.7.2). By comparing the resonance positions of the GalNAc NAc signals of compounds N4.9.2, N.4.7.2 and N3.9.2 in Hård et al. (1992) with those of compound Q4.3.4 in this report, it is evident that the Sd^a determinant located at the antenna containing the Gal-**8′** residue resonates at δ 2.019.

The main compound in fraction Q4.3.6 is a fucosylated triantennary oligosaccharide bearing one (α2-6)-linked Neu5Ac, one (α2-3)-linked Neu5Ac, and one terminal Gal3*S* residue, as shown by the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac H3e in the ^{1}H-NMR spectrum (Fig. 6a, Table 2).

```
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\                Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.3.6
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)/
        Gal3S(β1-4)GlcNAc(β1-4)/

Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q4.3.7
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)/
        Gal3S(β1-4)GlcNAc(β1-4)/
```

The observation of a Man-**4′** H1 signal at δ 4.908 instead of at δ 4.933 eliminates the possibility of an (α2-6)-linked Neu5Ac at Gal-**6′** [compare compounds N3.8 and N3.10.3 (Hård et al., 1992)]. Compared with compound Q4.3.1, GlcNAc-**7** NAc shifts from δ 2.074 to δ 2.077, which indicates the 3-*O*-sulfate group to be present at Gal-**8**. By consequence, Gal-**6** bears an (α2-6)-linked Neu5Ac and Gal-**6′** an (α2-3)-linked Neu5Ac residue. Therefore, the signal at δ 4.442 was assigned to Gal-**6** H1, and that at δ 2.066 to GlcNAc-**5** NAc. The assignment of δ 4.585 and δ 4.336 to Gal-**8** H1 and H3, respectively, fits the conclusions. Fraction Q4.3.7 is the non-fucosylated analogue of Q4.3.6 (Table 2).

HPAEC fraction Q5.2.3 is the most abundant subfraction of HPLC fraction Q5.2 and contains two terminal Gal3*S* residues and one (α2-3)-linked Neu5Ac residue, as shown by ^{1}H-NMR analysis of the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac H3e (Fig. 6b, Table 2). Gal-**6** and Gal-**8** are 3-*O*-sulfated, as indicated by the resonances of Gal-**6**/**8** H3 at δ 4.336 and H4 at δ 4.292, and those of GlcNAc-**5** NAc at δ 2.048 and GlcNAc-**7** NAc at δ 2.078. This means that the (α2-3)-linked Neu5Ac residue is attached to Gal-**6′**, which is in accordance with the structural-reporter-group signals for this antenna. Fraction Q5.2.4 contains the non-fucosylated analogue (Table 2).

```
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\                Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q5.2.3
        Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
        Gal3S(β1-4)GlcNAc(β1-4)/

Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q5.2.4
        Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
        Gal3S(β1-4)GlcNAc(β1-4)/

        Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\                     Fuc(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q3.1.9
        Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
        Gal3S(β1-4)GlcNAc(β1-4)/

Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q3.1.10
Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3)/
Gal3S(β1-4)GlcNAc(β1-4)/
```

The non-sialylated analogue of compound Q5.2.3 is present in fraction Q3.1.9 (Fig. 6c, Table 2). The proposed Gal(β1-4)GlcNAc(β1-2)Man(α1-6) sequence is supported by the set of structural-reporter-group signals of Man-**4′** H1 at δ 4.923, Gal-**6′** H1 at δ 4.474, and GlcNAc-**5′** H1 at δ 4.582 [compare compound N2.6B (Hokke et al., 1995)]. The non-fucosylated analogue of Q3.1.9 is present in fraction Q3.1.10 (Table 2).

^{1}H-NMR analysis of fraction Q4.1.5 reveals the presence of two terminal Gal3*S* residues and one (α2-3)-linked Neu5Ac resi-

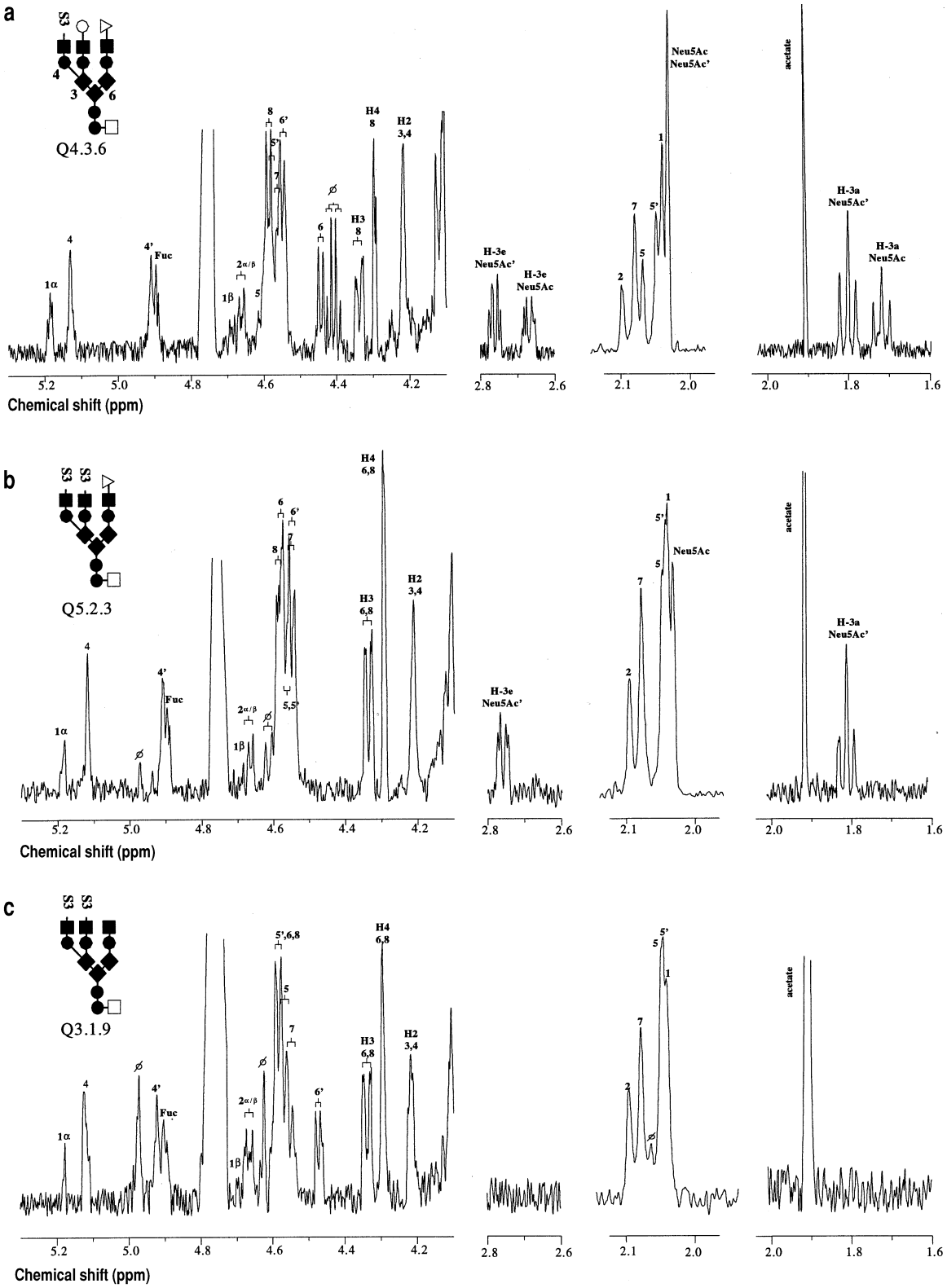


Fig. 6. Resolution-enhanced ¹H-NMR spectra at 300 K of sulfated triantennary oligosaccharides obtained from human THP. (a) Fraction Q4.3.6 (600-MHz); (b) fraction Q5.2.3 (600-MHz); (c) fraction Q3.1.9 (500-MHz). The numbering of the monosaccharide residues is exemplified in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. For short-hand symbolic notations, see Fig. 4.

due, as shown by the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac H3e (Fig. 7a, Table 2). In contrast to fraction Q5.2.3, the sulfate groups are linked to Gal-**6** and Gal-**6′**, as concluded from the NAc signals of GlcNAc-**5′** at δ 2.048, GlcNAc-**5** at δ 2.048 and GlcNAc-**7** at δ 2.074 (compare with Q4.3.1). This interpretation is supported by the signals of Gal-**6** H3 at δ 4.336 and Gal-**6′** H3 at δ 4.341, whereas both Gal H4 signals resonate at δ 4.294.

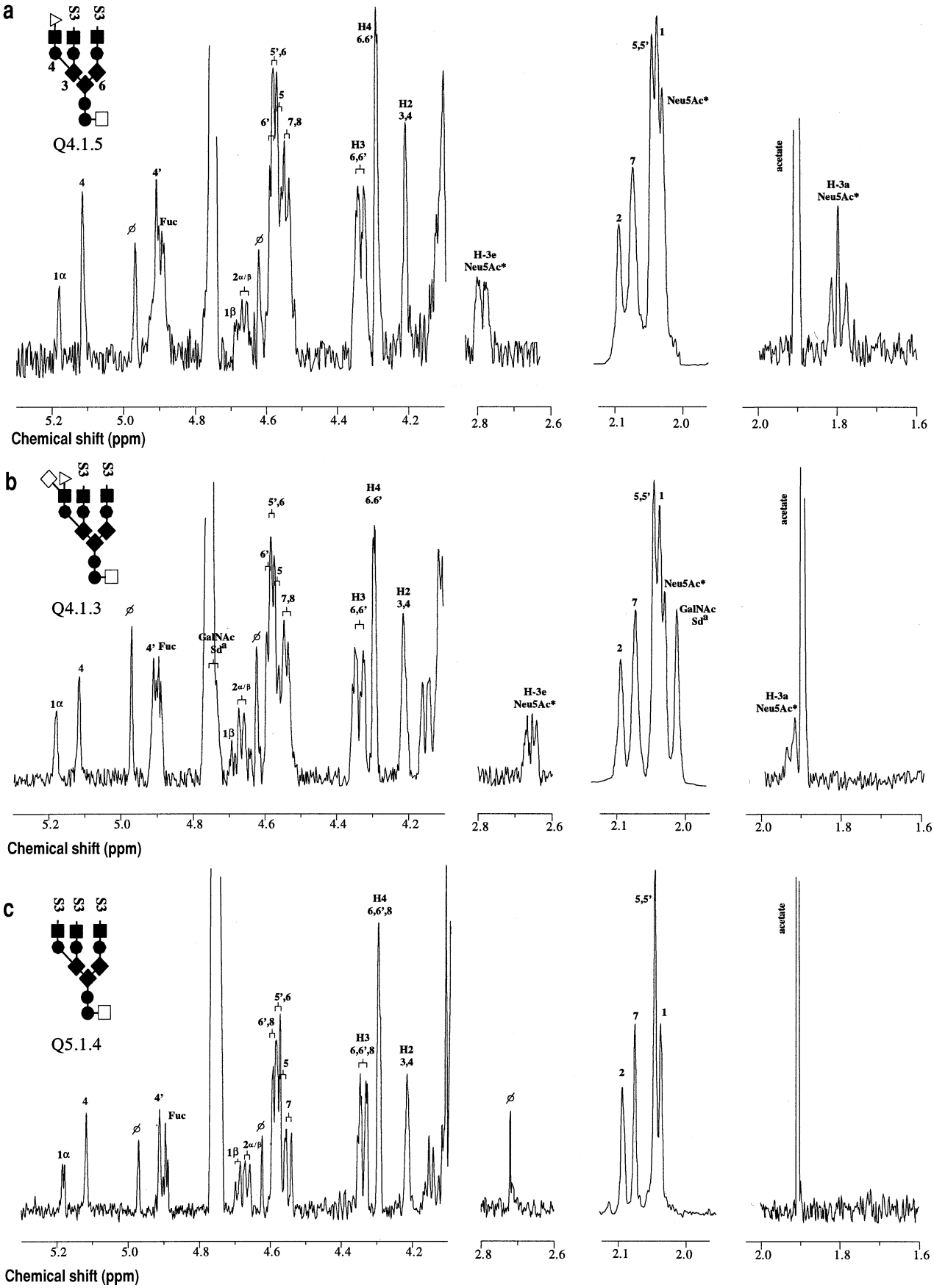


Fig. 7. Resolution-enhanced 600-MHz ^{1}H-NMR spectra at 300 K of sulfated triantennary oligosaccharides obtained from human THP. (a) Fraction Q4.1.5; (b) fraction Q4.1.3; (c) fraction Q5.1.4. The numbering of the monosaccharide residues is indicated in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. Symbols are explained in the legend to Fig. 4.

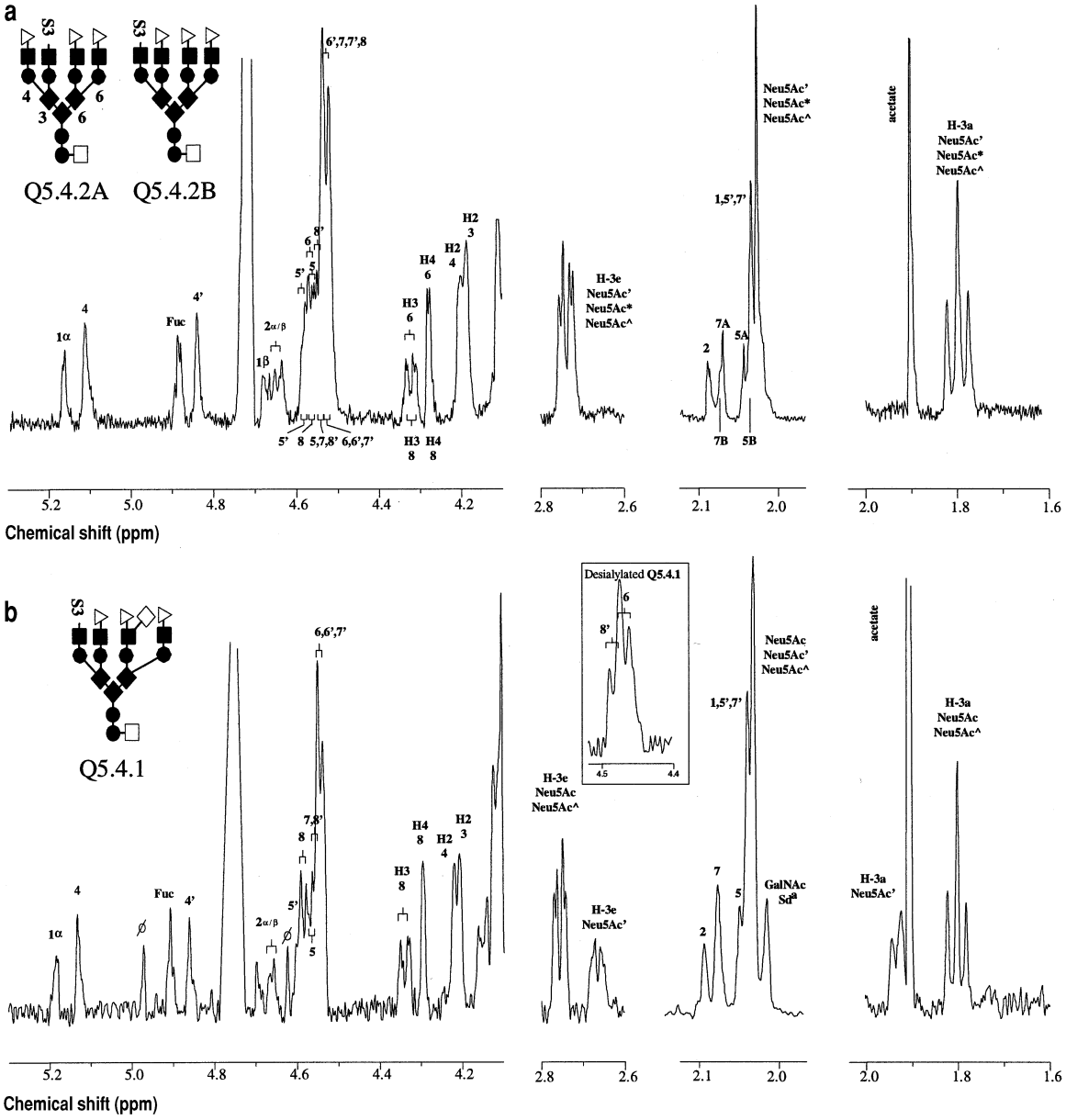
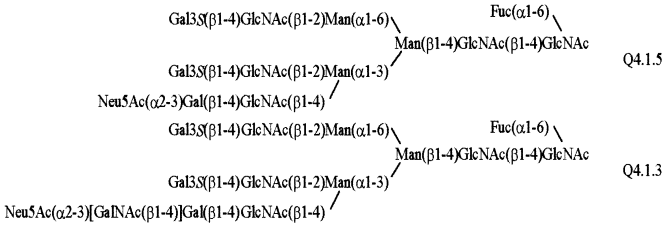
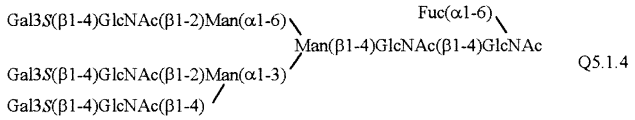


Fig. 8. Resolution-enhanced 500-MHz ¹H-NMR spectra at 300 K of sulfated tetraantennary oligosaccharides obtained from human THP. (a) Fraction Q5.4.2; (b) fraction Q5.4.1. Signals characteristic for Q5.4.2B are indicated below the spectrum. The numbering of the monosaccharide residues is explained in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. Symbols are explained in the legend to Fig. 4.

The analogue of Q4.1.5 containing the Sd[a] determinant is present in fraction Q4.1.3. This conclusion is based on the replacement of the structural-reporter-group signals of (α2-3)-linked Neu5Ac by those for the Sd[a] determinant (GalNAc NAc at δ 2.015, Neu5Ac H3a at δ 1.926, and Neu5Ac H3e at δ 2.660) (Fig. 7b, Table 2).



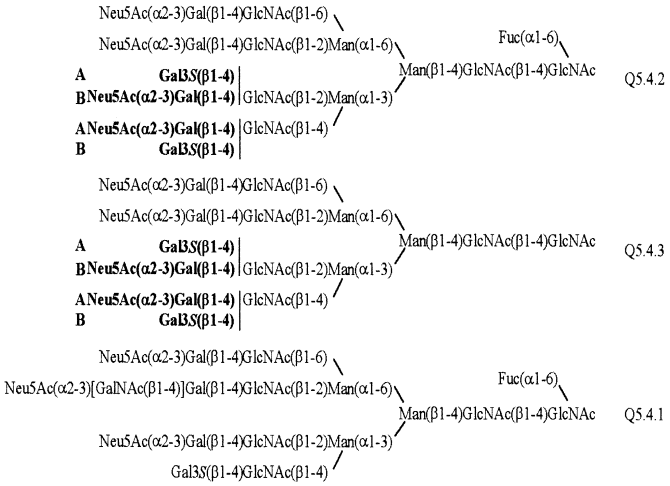
The ¹H-NMR spectrum of fraction Q5.1.4 shows a triantennary structure containing three Gal3*S* residues (Fig. 7c, Table 2) as indicated by the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Man-4 H1.



Compared with fraction Q4.3.1, downfield shifts of $\Delta\delta$ 0.004 for the NAc signals of GlcNAc-**5** (δ 2.048), GlcNAc-**5′** (δ 2.048) and GlcNAc-**7** (δ 2.078) are observed, indicating that Gal-**6**, Gal-**6′**, and Gal-**8** are 3-*O*-sulfated. This conclusion is supported by the presence of signals at δ 4.341 (Gal-**6′** H3), at δ 4.337 (Gal-**6** and Gal-**8** H3), and at δ 4.29 (Gal-**6**/**6′**/**8** H4).

Sulfated tetraantennary N-glycans. As a starting point for the analysis of the sulfated tetraantennary glycans, the ¹H-NMR data of reference compound N4.4.5 (Hård et al., 1992) were taken.

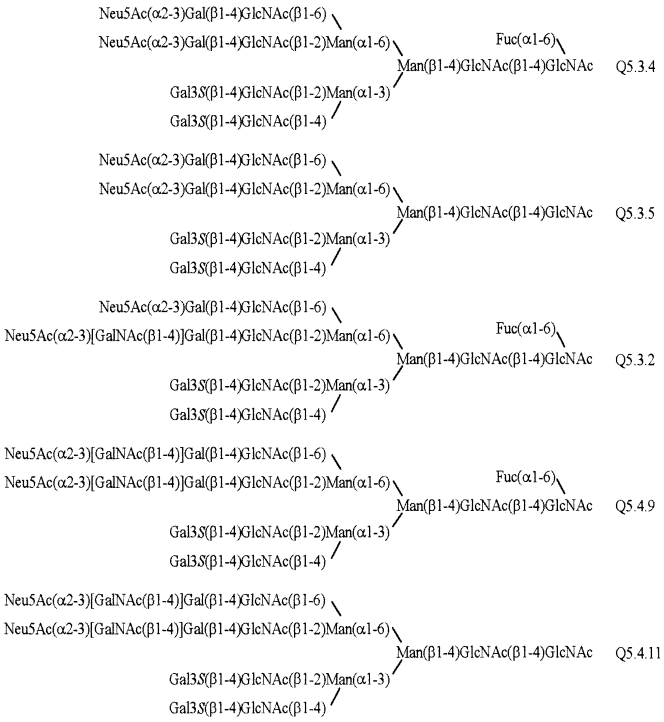
Based on the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac H3e, the ^{1}H-NMR spectrum of fraction Q5.4.2 suggests the occurrence of monosulfated, (α2-3)-trisialylated, fucosylated tetraantennary compounds (Fig. 8a, Table 3). As can be deduced from the reporter-group signals of GlcNAc-**7** NAc at δ 2.079/2.075, GlcNAc-**5** NAc at δ 2.052/2.048, and Gal3*S* H3 at δ 4.336/4.341 a mixture of isomers is present. Based on a comparison of the ^{1}H-NMR data of Q5.4.2 and N4.4.5, the main compound (Q5.4.2A) is readily recognized by the expected downfield shift of $\Delta\delta$ 0.004 for GlcNAc-**5** NAc towards δ 2.052, indicating the presence of a trisialylated tetraantennary oligosaccharide containing 3-*O*-sulfation at Gal-**6**. The signals of Gal-**6** H1 at δ 4.584, H3 at δ 4.336 and H4 at δ 4.292 are in agreement with this assignment. The shoulder present at the signal of GlcNAc-**7** NAc at δ 2.079 (δ 2.075) in that of Q5.4.2 stems from the isomer containing 3-*O*-sulfate at Gal-**8** (Q5.4.2B). Based on the relative intensities of GlcNAc-**7** at δ 2.079 and δ 2.075 compound Q5.4.2B is present for about 20%. It is evident from the resonance position of Gal3*S* H3 at δ 4.341, that also the isomer containing a sulfated Gal-**6′** (or Gal-**8′**; no reference data available) residue is present in trace amounts. Fraction Q5.4.3 contains the non-fucosylated analogues of the compounds found in fraction Q5.4.2 (Table 3). Evidence for the presence of such a mixture has been reported earlier (Hård et al., 1992; fraction N5.3.1).



Fraction Q5.4.1 contains one terminal Gal3*S* residue, one Sd^a determinant and two (α2-3)-linked Neu5Ac residues, based on the relative intensities of Gal3*S* H3, Neu5Ac(Sd^a) H3e, and Neu5Ac H3e (Fig. 8b, Table 3). Comparison of the chemical shift values of the GlcNAc-**5**, -**5′**, -**7**, and -**7′** NAc signals in Q5.4.1 with those in N4.4.5 and Q5.4.2B indicates that the 3-*O*-sulfate group is present at Gal-**8** in Q5.4.1. The ^{1}H-NMR spectrum of the sialidase-treated fraction Q5.4.1 showed no shift of Man-**4′** H1 from δ 4.860 to δ 4.867/4.870, indicating that Gal-**6′** was not desialylated [compare compounds N2.10.1, N2.10.2, N3.7.1A, N3.7.1B, N3.7.1C, and N3.7.2A (Hokke et al., 1995), N4.4.5 (Hård et al., 1992) and compound 13 (Vliegenthart et al., 1983)]. This observation and the resonance positions of Gal-**8′** H1 at δ 4.482 and Gal-**6** H1 at δ 4.470 (Fig. 8b) in the ^{1}H-NMR spectrum of the sialidase-treated compound confirmed that the Sd^a determinant is present at the antenna containing Gal-**6′**.

Based on the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac H3e, the fucosylated tetraantennary oligosaccharide in fraction Q5.3.4 contains two terminal Gal3*S* residues and two (α2-3)-linked Neu5Ac residues (Fig. 9a, Table 3). As shown by the reporter-group signals of GlcNAc-**5** NAc at δ 2.052, GlcNAc-**7** NAc at δ 2.079, Gal-**6**/**8** H3 at δ 4.337 and H4 at δ 4.293 the main component contains 3-*O*-sulfated Gal-**6** and Gal-**8**. Therefore, the Gal-**6′** and -**8′** residues are (α2-3)-sialylated. The ^{1}H-NMR spectrum of the sialidase-treated fraction Q5.3.4 revealed a shift for Man-**4′** H1 from δ 4.858 to δ 4.868, confirming that Gal-**6′** was sialylated. Taking into account this feature, the H1 signal pattern between δ 4.4–4.5 (Fig. 9a) supports the proposal that Gal-**6′** and Gal-**8′** are the main desialylated residues [Gal-**6′** at δ 4.470, Gal-**8′** at δ 4.482; compare compound N2.10.2 (Hokke et al., 1995)]. Fraction Q5.3.5 contains the non-fucosylated analogue of compound Q5.3.4 (Table 3).

The relative intensities of the structural-reporter-group signals Gal3*S* H3, Neu5Ac H3e, and Neu5Ac(Sd^a) H3e in the ^{1}H-NMR spectrum of fraction Q5.3.2 show that the major compound is an extension of Q5.3.4, wherein an (α2-3)-linked Neu5Ac residue is replaced by an Sd^a determinant (Fig. 9b, Table 3).



Both Gal-**6** and Gal-**8** are 3-*O*-sulfated, as indicated by the signals of GlcNAc-**5** NAc at δ 2.052 and GlcNAc-**7** NAc at δ 2.079, respectively. ^{1}H-NMR analysis of the sialidase-treated fraction revealed that Neu5Ac is (α2-3)-linked to Gal-**8′**. Going from Q5.3.2 to sialidase-treated Q5.3.2, Man-**4′** H1 (δ 4.860) does not shift downfield, eliminating Gal-**6′** as terminal residue, whereas the appearance of a H1 signal at δ 4.483 reflects a terminal Gal-**8′** residue [compare compound N3.7.1A (Hokke et al., 1995)]. These conclusions are confirmed by the resonance position of GalNAc NAc at δ 2.015, reflecting the position of the Sd^a determinant at the antenna containing Gal-**6′** and not Gal-**8′**.

The ^{1}H-NMR spectrum of fraction Q5.4.9 showed a fucosylated tetraantennary oligosaccharide containing two Gal3*S* residues and two Sd^a determinants, as deduced from the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac(Sd^a) H3e (Fig. 9c, Table 3). Comparison of the chemical shifts of the GlcNAc-**5**, -**5′**, -**7**, and -**7′** NAc signals in Q5.4.9 with those in N4.4.5, Q5.3.4, and Q5.3.2, indicate that the 3-*O*-sulfate groups are present at Gal-**6** and Gal-**8** in Q5.4.9 (GlcNAc-**5**, δ 2.051; GlcNAc-**7**, δ 2.079). Therefore, the Sd^a determinants are present at the antennae containing Gal-**8′** and Gal-**6′**, as confirmed by the signals of GalNAc NAc at δ 2.019

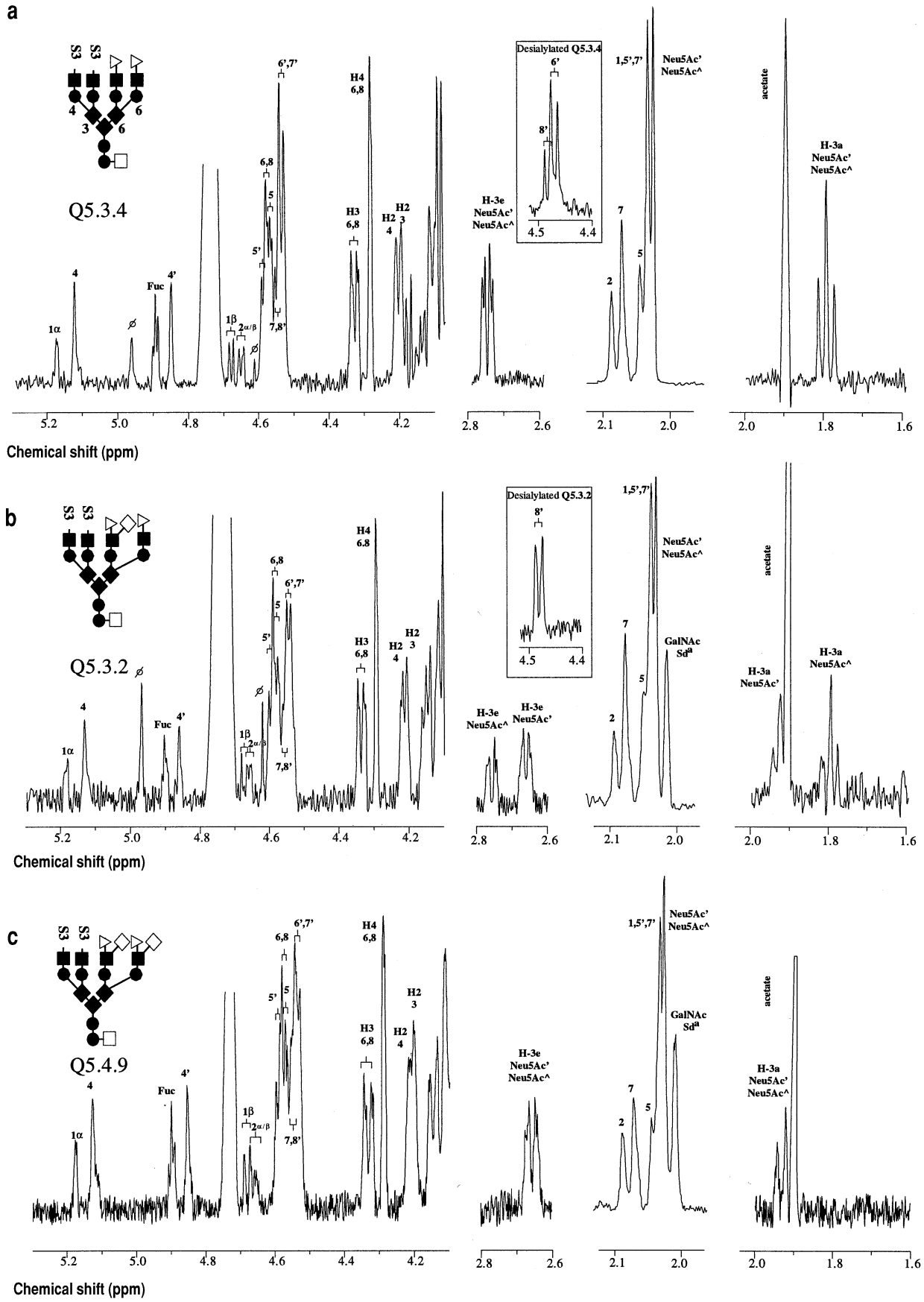


Fig. 9. Resolution-enhanced ¹H-NMR spectra at 300 K of sulfated tetraantennary oligosaccharides obtained from human THP. (a) Q5.3.4 (600-MHz); (b) fraction Q5.3.2 (600-MHz); (c) fraction Q5.4.9 (500-MHz). The numbering of the monosaccharide residues is explained in the text. Due to the Lorentzian-to-Gaussian transformation and baseline correction the GlcNAc-**1*β*** signal could be disturbed. Symbols are explained in the legend to Fig. 4.

and δ 2.015, respectively. The non-fucosylated analogue is present in fraction Q5.4.11 (Table 3).

Based on the relative intensities of the structural-reporter-group signals of Gal3*S* H3 and Neu5Ac(Sd^a) H3e, the ^{1}H-NMR spectrum of fraction Q6.8 suggests the presence of a mixture of isomeric fucosylated tetraantennary oligosaccharides terminated with three Gal3*S* residues and one Sd^a determinant.

```
Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-6) \
                                                  Fuc(α1-6) \
            Gal3S(β1-4)GlcNAc(β1-2)Man(α1-6) \
                                              Man(β1-4)GlcNAc(β1-4)GlcNAc    Q6.8
            Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3) /
            Gal3S(β1-4)GlcNAc(β1-4) /

Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-6) \
            Gal3S(β1-4)GlcNAc(β1-2)Man(α1-6) \
                                              Man(β1-4)GlcNAc(β1-4)GlcNAc    Q6.10
            Gal3S(β1-4)GlcNAc(β1-2)Man(α1-3) /
            Gal3S(β1-4)GlcNAc(β1-4) /
```

One of the compounds displays a signal of GalNAc NAc at δ 2.018, indicating that the antenna bearing Gal-**8′** contains the Sd^a determinant. Evidence for isomeric compounds is found by the resonance of GalNAc NAc at δ 2.015, indicating the presence of the Sd^a determinant at the antenna containing Gal-**8**, Gal-**6** or Gal-**6′**. The non-fucosylated analogues of fraction Q6.8 are present in fraction Q6.10.

Concluding remarks. The structural analysis of complex sulfated high-molecular-mass carbohydrate chains from THP has resulted in the elucidation of 4 GalNAc4*S*-containing N-glycans and 28 Gal3*S*-containing N-glycans. For the structures bearing GalNAc4*S*, only the diantennary N-glycans could be elucidated, but evidence was found for the presence of GalNAc4*S*-containing triantennary oligosaccharides (data not shown). Novel Gal3*S*-containing di-, tri- and tetraantennary carbohydrate structures containing additionally the Sd^a determinant, (α2-3)-linked and/or (α2-6)-linked Neu5Ac, were characterized. The sulfation pattern ranged from monosulfated to trisulfated structures, but evidence was obtained also for the occurrence of a tetrasulfated tetraantennary N-glycan (data not shown). No N-glycans were detected containing both Gal3*S* and GalNAc4*S* residues.

The biological role of THP remains to be clarified. Several studies have indicated that THP is possibly a urinary inhibitor of the binding of *Escherichia coli* to epithelial cells of the lower urinary tract and bladder by binding via its carbohydrate moiety to several types of carbohydrate receptors on the fimbriae of *E. coli* (Parkkinen et al., 1983, 1988; Moch et al., 1987; Sajjan and Forstner, 1990). An additional defence mechanism is possibly present in the upper urinary tract where THP is expressed on the endothelium of the thick ascending limb of the loop of Henle. It is evident that the large amount of free THP present in the urine is generated by a continuous release of bound THP from the endothelium. Therefore, when glycosylphosphatidylinositol-anchored THP binds to *E. coli* at the surface of the endothelium of the thick ascending limb of the loop of Henle, it is likely that the THP×*E. coli* complex is released from this cell surface. It is hypothesized that both free and bound THP are involved in a 'wash' mechanism to prevent interaction by *E. coli* in the urinary system. In this context, it can be questioned which oligosaccharide elements could play a role in the binding of *E. coli* to THP. Earlier, terminal Neu5Ac(α2-3)Gal and GalNAc(β1-4)Gal elements were shown to be substrates for these lectin-like regions (Parkkinen et al., 1983; Howard et al., 1988). It is known that the filamentous hemagglutinin of *Bordetella pertussis*, which is the major adhesin of this organism, exhibits a lectin-like activity for heparin and dextran sulfate (Menozzi et al., 1994). It is therefore interesting to consider the possibility that for Gal3*S* and/or GalNAc4*S* lectin-like receptors occur in pathogens. With the detailed structural knowledge of the sulfated di, tri- and tetraantennary N-glycans known (Hård et al., 1992; this paper), further studies with respect to biosynthetic pathways and biological significance are feasible.

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