Eur. J. Biochem. 228, 1009–1019 (1995)
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The major N-linked carbohydrate chains from human urokinase

The occurrence of 4-*O*-sulfated, (α2-6)-sialylated or (α1-3)-fucosylated *N*-acetylgalactosamine(β1-4)-*N*-acetylglucosamine elements

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(Received 6 October 1994) – EJB 94 1521/5

The primary structure of the major N-linked carbohydrate chains attached to Asn302 of urinary-type plasminogen activator (urokinase) have been determined. Urokinase was completely deglycosylated with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F from *Flavobacterium meningosepticum*. Released oligosaccharides were separated from the remaining protein using gel-permeation chromatography on Bio-Gel P-100, and then on Bio-Gel P-6. Fractionation of the oligosaccharides was achieved by a combination of FPLC anion-exchange chromatography on Mono Q HR 5/5 and amine-adsorption HPLC on LiChrospher 100-NH_2. Analysis by ^{1}H-NMR spectroscopy demonstrated that the collection of N-glycans comprises di-, tri-, and tri′-antennary structures. The glycans contain predominantly GalNAcβ1-4GlcNAcβ instead of Galβ1-4GlcNAcβ elements. The GalNAc residue is mainly sulfated at O4, or to a lesser extent it bears *N*-acetylneuraminic acid at O6; alternatively the GlcNAc residue can be fucosylated at O3. The major component, which accounts for more than 30 mol/100 mol of the total oligosaccharide pool, consists of an (α1-6)-fucosylated diantennary N-linked carbohydrate chain with (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-2 antennae.

Keywords. Urokinase; carbohydrate chains; sulfated oligosaccharides; glycoprotein; fucosylated *N,N′*-diacetyllactosediamine.

Urinary-type and tissue-type plasminogen activators are serine proteases that convert plasminogen into the fibrinolytic enzyme plasmin. Plasmin promotes the dissolution of blood clots by degradation of fibrin, and therefore plasminogen activators are potential therapeutic agents in the treatment of thrombotic occlusions, like myocardial infarction. Urinary-type plasminogen activator (u-PA) has gained biomedical interest, since it is also involved in physiological and pathological tissue destruction and cell migration processes, such as gland involution and tumor growth [1, 2]. Urinary-type plasminogen activator is predominantly produced by kidney cells [1]. It is secreted as a single-chain molecule, pro-u-PA or scu-PA, which can be converted by plasmin into a more proteolytically active two-chain form, urokinase [3, 4].

u-PA is O- and N-glycosylated. A Fuc residue is O-linked to Thr18 in the epidermal-growth-factor-like domain of urinary, recombinant, and cultured human-kidney-cell-derived u-PA [5, 6]. In addition, N-glycosylation of human u-PA occurs at Asn302 in the protease domain [7]. The N-glycans contain besides Man, Gal, Fuc, GlcNAc and Neu5Ac also GalNAc residues [7, 8]. The biological behavior of recombinant non-glycosylated scu-PA has been demonstrated to be similar to that of the glycosylated urinary and recombinant protein [9, 10]. However, it has also been shown that recombinant non-glycosylated scu-PA is more efficiently cleaved by plasmin, more proteolytically active, and more rapidly inactivated by plasminogen inhibitors than the glycosylated recombinant counterpart [11]. In addition, the sialic acid content of recombinant scu-PA was shown to affect the *in vivo* clearance significantly [12].

In view of the interest in urinary-type plasminogen activator in relation to its role in development of cancer and to its pharmaceutical application, the structure of the N-linked carbohydrate chains attached to u-PA was investigated.

EXPERIMENTAL PROCEDURES

Materials. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* was purchased from Boehringer Mannheim. Commercial urokinase (Ukidan), isolated from human urine, was obtained from Laboratoires Serono (Aubonne, Switzerland); each vial contained 500000 IU urokinase, 1.9 mg NaCl, 20 mg mannitol, 2 mg EDTA, and 1.5 mg Na_2HPO_4/NaH_2PO_4.

Liberation of the N-linked carbohydrate chains. Three urokinase batches, each of ten vials, were used for structural analysis. To remove additives, two procedures were followed. One batch was dissolved in 10 ml 50 mM Tris/HCl pH 8.1, con-

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Abbreviations. 1D, one dimensional; 2D, two dimensional; CHO, Chinese hamster ovary; HOHAHA, homonuclear Hartmann-Hahn; IU, international unit; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase F; K_2tu-PA, recombinant DNA plasminogen activator hybrid variant; MLEV, composite pulse devised by Malcom Levitt; Neu5Ac, *N*-acetylneuraminic acid; t-PA, tissue-type plasminogen activator; u-PA, urinary-type plasminogen activator.

Enzymes. PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (EC 3.5.1.52); urinary-type plasminogen activator (EC 3.4.21.73); tissue-type plasminogen activator (EC 3.4.21.68).

taining 0.5 M NaCl, then adsorbed to a column (1.5×10 cm) of benzamidine–Sepharose-6B (Pharmacia), which specifically binds urokinase, and eluted with 0.5 M arginine, at a flow rate of 60 ml/h. The urokinase-containing fractions (SDS/PAGE) were pooled, extensively dialysed against 100 mM NH_4HCO_3, and lyophilized. The other two batches were dissolved in 5 ml 50 mM NH_4HCO_3 pH 7.5 and then purified by gel-filtration chromatography on a column (1.5×47 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad), eluted with 50 mM NH_4HCO_3 pH 7.5 at a flow rate of 10 ml/h. The urokinase-containing void volume (SDS/PAGE) was collected and lyophilized. Each batch contained 35 mg protein as determined against bovine serum albumin, using the Pierce dye-reagent assay.

The N-linked carbohydrate chains were released enzymically from the glycoprotein with PNGase-F, essentially as described [13]. Briefly, each purified urokinase batch was dissolved in 4.2 ml 50 mM Tris/HCl pH 7.3 containing 50 mM EDTA, 1% (by vol.) 2-mercaptoethanol, and 1.4% (mass/vol.) SDS. The solution was kept for 1 h at 37°C, then 300 µl 10% (mass/vol.) aqueous Nonidet P-40 was added, and the mixture was incubated with 10 U PNGase-F for 4 h at ambient temperature. Subsequently, a fresh aliquot of 5 U PNGase-F was added, and the incubation continued for 16 h. The degree of de-N-glycosylation was checked by SDS/PAGE on a 12.5% slab gel (2.6% cross-linking) and amido-black staining.

Isolation and fractionation of the liberated carbohydrate chains. The digests were each applied to a column (2.6×58 cm) of Bio-Gel P-100 (200–400 mesh, Bio-Rad), which was eluted with 50 mM NH_4HCO_3, pH 7, at a flow rate of 19 ml/h. In each case the carbohydrate-positive fractions (resorcinol/sulfuric acid) were pooled, concentrated by lyophilization, and applied to a column (2.5×45 cm) of Bio-Gel P-6, eluted with 50 mM NH_4HCO_3 pH 7 at a flow rate of 16 ml/h. The effluent was monitored at 206 nm. The carbohydrate-positive fractions (resorcinol/sulfuric acid and 300-MHz ^{1}H-NMR) were pooled, concentrated to 5 ml by lyophilization and desalted on a column (1×28 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad), eluted with water, and the effluent was monitored at 206 nm. At this stage, the N-glycan fractions of each urokinase batch were pooled and concentrated to 1 ml by lyophilization. The oligosaccharides were fractionated by anion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia FPLC system), using a NaCl gradient as described [14], and detection at 214 nm. Collected fractions were lyophilized, desalted on a column (1×28 cm) of Bio-Gel P-2, eluted with water and lyophilized.

Subfractionation of the Mono Q fractions was carried out by HPLC on a 5-µm LiChrospher 100-NH_2 column (0.46×25 cm, Chrompack). Elutions were performed isocratically with 30 mM K_2HPO_4/KH_2PO_4, pH 7.0/acetonitrile (36:64, by vol.) at a flow rate of 90 ml/h at ambient temperature, and monitored at 205 nm. HPLC fractions were desalted on a Bio-Gel P-2 column (1×28 cm), eluted with water, and lyophilized.

Monosaccharide analysis. Monosaccharides were analyzed by GLC as described [15]. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis (1.0 M methanolic HCl, 24 h, 85°C), re-*N*-acetylation, and trimethylsilylation. The value of GlcNAc has been corrected for the relatively stable GlcNAc-Asn linkage [15].

^{1}H-NMR spectroscopy. The oligosaccharide samples were repeatedly exchanged in 99.8% ^2H_2O (MSD Isotopes) at p^{2}H 7 with intermittent lyophilization. Finally, for ^{1}H-NMR analysis, they were dissolved in 99.96% ^2H_2O [16]. The one-dimensional (1D) ^{1}H-NMR spectra were acquired using the pulse sequence and phase cycles described in [17]. The 300-MHz, 500-MHz and 600-MHz 1D/2D ^{1}H-NMR spectra were recorded on Bruker AC-300 (Department of Organic Chemistry, Utrecht University), AMX-500, AMX-600 (Bijvoet Center, Department of NMR spectroscopy, Utrecht University), and AM-600 (NSR center, SON NMR facility, Nijmegen University, The Netherlands) spectrometers, at a probe temperature of 300 K. Chemical shifts are expressed in ppm relative to internal acetone (δ 2.225).

The 600-MHz 2D HOHAHA spectrum was recorded using Bruker software with a MLEV-17 mixing sequence of 120 ms and a spin-lock field-strength corresponding to a 90° ^{1}H pulse-width of 23.3 µs. A data matrix of 424×2048 points was collected, representing a spectral width of 4800 Hz in each dimension. The ^{1}HO^{2}H signal was suppressed by presaturation for 1 s during the relaxation delay. Phase-sensitive handling of the data in the ω_1 dimension became possible by the time-proportional phase-incrementation method implemented in the Bruker software. The time domain data were zero-filled to a 1024×2048 data matrix prior to multiplication with a squared-bell function phase shifted by $\pi/3$.

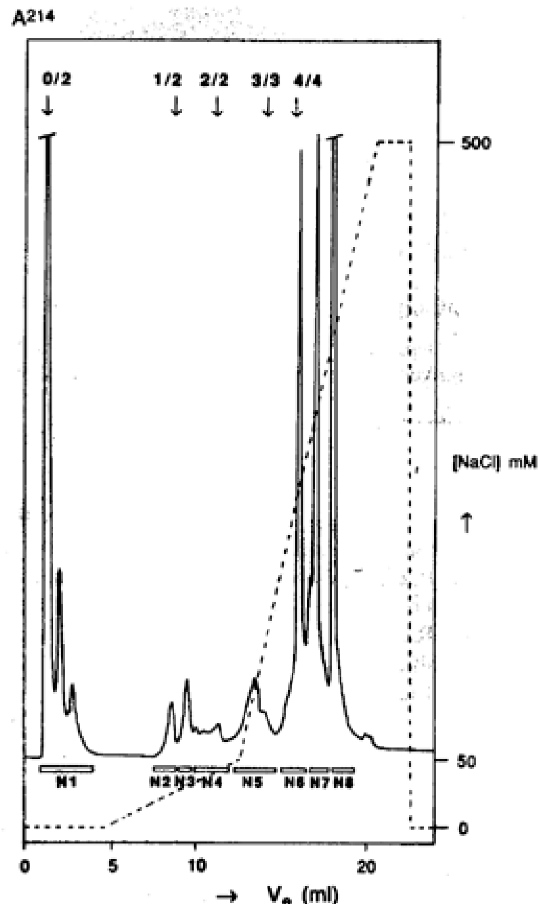


Fig. 1. Fractionation pattern at 214 nm of the carbohydrate chains derived from PNGase-F-treated human u-PA on a Mono Q HR 5/5 column. The column was first eluted with 5 ml H_2O, followed by a linear gradient (– – – –) of 0–50 mM NaCl in 8 ml H_2O, and finally by a steeper gradient of 50–500 mM NaCl in 8 ml H_2O at a flow rate of 1 ml/min. The fractions were collected as indicated. The arrows indicate the elution position of reference neutral (0/2), mono- (1/2) and di- (2/2) sialylated diantennary, trisialylated tri-/tri′-antennary (3/3) and tetrasialylated tetraantennary (4/4) carbohydrate chains [18].

RESULTS

The purity of the starting glycoprotein material, isolated from the commercial u-PA preparation, was indicated by amino

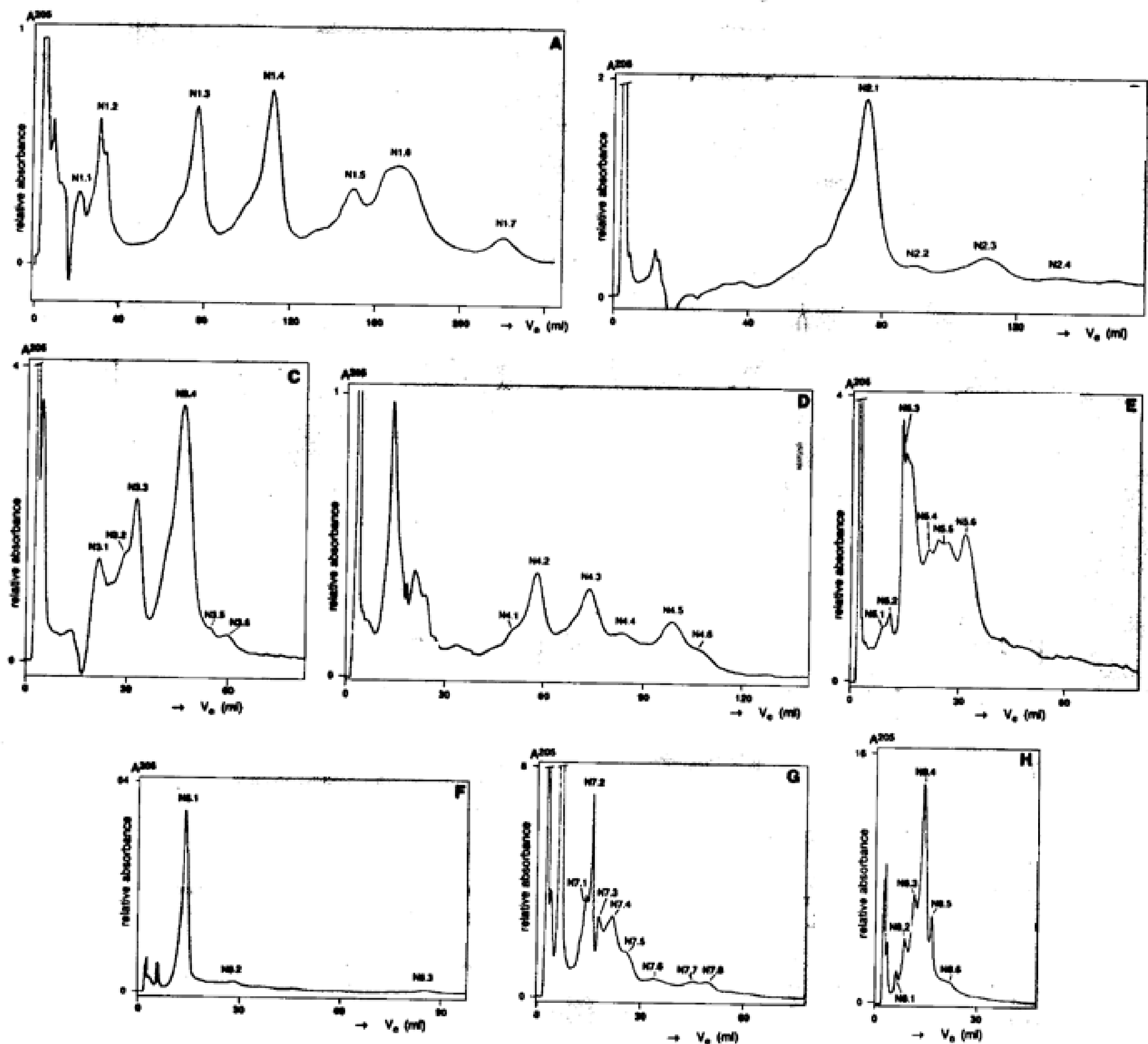


Fig. 2. Fractionation patterns at 205 nm of the Mono Q fractions N1–N8 from human u-PA on a 5-µm LiChrospher 100-NH_2 HPLC column. The column was eluted isocratically with a mixture of 30 mM K_2HPO_4/KH_2PO_4 pH 7.0/acetonitrile (36: 64, by vol.) at a flow rate of 90 ml/h at ambient temperature. (A) Mono Q fraction **N1**; (B) fraction **N2**; (C) fraction **N3**; (D) fraction **N4**; (E) fraction **N5**; (F) fraction **N6**; (G) fraction **N7**; (H) fraction **N8**.

acid sequence analysis of the first ten amino acids (Edman degradation), showing the exclusive presence of u-PA. Monosaccharide analysis of u-PA revealed the occurrence of Fuc, Gal, Man, GlcNAc, GalNAc and Neu5Ac in the molar ratio of 2.0:1.4:3.0:3.2:3.4:1.1. The carbohydrate content of u-PA was estimated to be 2% (by mass). SDS/PAGE on 12.5% slab gels of native u-PA showed two bands at apparently 33 kDa (B chain, containing the Asn302 glycosylation site) and 22 kDa (A chain), respectively, reflecting the presence of the two-chain form only. After PNGase-F treatment, the completely deglycosylated u-PA migrates as two bands at apparently 29 kDa and 22 kDa, respectively, in accordance with the removal of N-linked carbohydrate chains from the B chain only.

The pool of enzymically released N-linked carbohydrate chains was separated from the de-N-glycosylated protein by gel-permeation chromatography on Bio-Gel P-100, and further purified on Bio-Gel P-6 to remove residual detergents, which were added for the PNGase-F treatment. Subfractionation of the carbohydrate-containing fraction on Mono Q yielded eight carbohydrate-positive fractions, denoted **N1–N8** (Fig. 1). Each Mono Q fraction was further separated by HPLC on LiChrospher 100-NH_2. The collected subfractions were pooled as indicated in Fig. 2, and investigated by ^{1}H-NMR spectroscopy. Fractions not discussed below contained mixtures of compounds too complex for analysis, or did not contain enough material for structural analysis by ^{1}H-NMR spectroscopy, or consisted only of non-carbohydrate material. Relevant ^{1}H-NMR data are compiled in Tables 1 and 2. The structure determination of the various compounds will be presented in an order which is convenient for discussing the ^{1}H-NMR data. All discussed compounds have an (α1-6)-fucosylated *N,N'*-diacetylchitobiose core in common, as is evident from the corresponding ^{1}H-NMR structural-reporter-group signals [17].

Subjection of Mono Q fraction **N6** to HPLC (Fig. 2F) yielded one *prevalent subfraction* **N6.1**. *Although fraction* **N6** co-eluted with a reference tetrasialylated tetraantennary com-

```
SO4−-4GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
     GN'          5'        4'    \                    \
                                   Manβ1-4GlcNAcβ1-4GlcNAc
                                  /  3        2        1
SO4−-4GalNAcβ1-4GlcNAcβ1-2Manα1-3
     GN           5         4
```

Fig. 3. Structural-reporter-group-signal regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of fraction N6.1 derived from human u-PA, recorded in ^2H_2O at 300 K. The relative scale of the NAc protons region differs from that of the rest of the spectrum.

pound (Fig. 1) [18], the ^{1}H-NMR data of fraction **N6.1** (Fig. 3; Table 1) indicate the occurrence of an (α1-6)-fucosylated diantennary structure, containing a terminal (SO_4^-)-4GalNAc element in each antenna:

```
(SO4−)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
       GN'          5'        4'   \                    \
                                    Manβ1-4GlcNAcβ1-4GlcNAc
                                   /  3        2        1
(SO4−)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3
       GN           5         4                        [N6.1]
```

The ^{1}H-NMR data of the antennae are in good accordance with those of the corresponding oligosaccharide-alditol S2 [19]. Compound **N6.1** is the major N-linked oligosaccharide component occurring in u-PA, representing more than 30 mol/100 mol of the total carbohydrate pool. The remaining fractions, which are discussed contain not more than 7 mol/100 mol of the total carbohydrate pool each.

HPLC of the neutral Mono Q fraction **N1** gave rise to seven subfractions, denoted **N1.1–N1.7** (Fig. 2A). The ^{1}H-NMR spectrum of fraction **N1.3** indicates an (α1-6)-fucosylated diantennary compound, of which each antenna consists of a GalNAcβ1-4GlcNAcβ1-2 element (Table 1):

```
GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                           \                    \
                            Manβ1-4GlcNAcβ1-4GlcNAc
                           /
GalNAcβ1-4GlcNAcβ1-2Manα1-3                    [N1.3]
```

The diantennary character of compound **N1.3** was deduced from the structural-reporter-group signals for Man-**3/4/4'** H1 and H2 (Table 1), which are comparable to those observed for the disulfated [(SO_4^-)-4GalNAc] analogue **N6.1**. The GalNAcβ1-4GlcNAcβ1-2Manα1-3 branch is reflected by Man-**4** H1 at δ 5.107 and H2 at δ 4.174, GlcNAc-**5** H1 at δ 4.553 and NAc at δ 2.044, and GalNAc-**GN** H1 at δ 4.514 and NAc at δ 2.066, whereas the GalNAcβ1-4GlcNAcβ1-2Manα1-6 branch is characterized by Man-**4'** H1 at δ 4.912 and H2 at δ 4.090, GlcNAc-**5'** H1 at δ 4.559 and NAc at δ 2.039, and GalNAc-**GN'** H1 at δ 4.520 and NAc at δ 2.073. The specific assignment of the H1 signals of GlcNAc-**5** and GlcNAc-**5'** is based on 2D NOESY experiments of a corresponding glycopeptide (Mulder et al., unpublished results). For the assignment of the H1 and *N*-acetyl signals of both GalNAc-**GN** and GalNAc-**GN'**, the ^{1}H-NMR data of synthetic GalNAcβ1-4GlcNAcβ1-2Manα1-$(CH_2)_8COOCH_3$ and (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-$(CH_2)_8COOCH_3$ [20] have been used. Going from the non-sul-

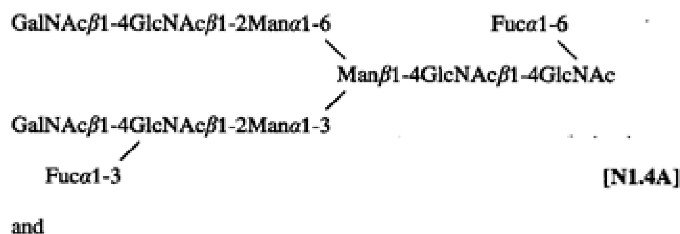
Table 1. ¹H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of neutral and sulfated, GalNAc-containing N-linked carbohydrate chains derived from human urokinase. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 300 K and at p²H 7 [16]. Compounds are represented by short-hand symbolic notation: (□) L-Fuc; (●) D-GlcNAc; (◆) D-Man; (◇) D-GalNAc; (S-◇) (SO_4^-)-4GalNAc. For numbering of the monosaccharide residues, see text. n.d., not determined. The subscripts α and β stand for the anomeric configuration of GlcNAc-**1**. The superscripts 3 and 6 stand for the linkage type of Fuc.

Reporter group	Residue	Chemical shift in						
		N1.3	**N1.4A**	**N1.4B**	**N3.3**	**N3.4A**	**N3.4B**	**N6.1**
		ppm						
H1	GlcNAc-**1**α	5.179	5.180	5.180	5.181	5.181	5.181	5.181
	GlcNAc-**1**β	4.694	4.694	4.694	4.692	n.d.	n.d.	4.693
	GlcNAc-**2**$_\alpha$	4.664	4.664	4.664	4.665			4.666
						4.666	4.666	
	GlcNAc-**2**$_\beta$	4.668	4.668	4.668	4.667			4.670
	Man-**4**	5.107	5.096	5.109	5.111	5.099	5.110	5.109
	Man-**4′**	4.912	4.914	4.896	4.916	4.910	4.898	4.911
	GlcNAc-**5**	4.553	4.546	4.553	4.555	4.556[a]	4.556[a]	4.557
	GlcNAc-**5′**	4.559	4.561	4.553	4.561	4.556[a]	4.556[a]	4.557
	GalNAc-**GN**	4.514	4.445	4.514	4.586	4.448	4.586	4.585
	GalNAc-**GN′**	4.520	4.521	4.452	4.523	4.592	4.456	4.591
	Fuc^3	–	5.127	5.127	–	5.128	5.132	–
	Fuc_α^6	4.892	4.892	4.892	4.893	4.894	4.894	4.898
	Fuc_β^6	4.899	4.900	4.900	4.899	n.d.	n.d.	4.906
H2	Man-**3**	4.241	4.245	4.245	4.245	4.246	4.246	4.244
	Man-**4**	4.174	4.164	4.175	4.174	4.166	4.175	4.177
	Man-**4′**	4.090	4.089	4.075	4.092	4.096	4.075	4.099
H4	GalNAc-**GN**	n.d.	n.d.	n.d.	4.692	n.d.	4.692	4.693
	GalNAc-**GN′**	n.d.	n.d.	n.d.	n.d.	4.692	n.d.	4.693
H5	Fuc^3	–	4.862	4.862	–	4.859	4.859	–
	Fuc_α^6	4.097	4.099	4.099	4.098	4.096	4.096	4.099
	Fuc_β^6	4.130	4.133	4.133	4.134	4.133	4.133	4.134
NAc	GlcNAc-**1**	2.039	2.038	2.038	2.039	2.038	2.038	2.040
	GlcNAc-**2**$_\alpha$	2.096	2.097	2.097	2.097			2.102
						2.102	2.095	
	GlcNAc-**2**$_\beta$	2.094	2.094	2.094	2.094			2.100
	GlcNAc-**5**	2.044	2.038	2.045	2.044	2.038	2.045	2.044
	GlcNAc-**5′**	2.039	2.038	2.038	2.039	2.038	2.038	2.040
	GalNAc-**GN**	2.066	2.038	2.066	2.069	2.038	2.069	2.068
	GalNAc-**GN′**	2.073	2.073	2.045	2.073	2.077	2.045	2.077
CH_3	Fuc^3	–	1.260	1.262	–	1.260	1.267	–
	Fuc_α^6	1.208	1.209	1.209	1.209	1.210	1.210	1.210
	Fuc_β^6	1.220	1.221	1.221	1.221	1.220	1.220	1.222

[a] Signals stemming from a broad signal.

fated to the sulfated reference compound, the chemical shift increments for H1 and NAc of GalNAc are $\Delta\delta$ +0.073 and $\Delta\delta$ +0.003, respectively. Applying these downfield shift effects in the comparison of the ¹H-NMR data for GalNAc-**GN** and GalNAc-**GN′** of **N1.3** and **N6.1**, the H1 and NAc resonances at δ 4.514 and 2.066, respectively, were assigned to non-sulfated GalNAc-**GN** and the H1 and NAc signals at δ 4.520 and 2.073, respectively, to non-sulfated GalNAc-**GN′**. It is interesting to note that recent 400-MHz (24°C) and 600-MHz (40°C) ¹H-NMR data of the 2-aminopyridyl derivative of **N1.3** showed identical chemical shift values for GlcNAc-**5** and GlcNAc-**5′** H1, as well as for GalNAc-**GN** and GalNAc-**GN′** H1 [21, 22].

¹H-NMR spectroscopic analysis of fraction **N1.4** revealed a mixture of two isomeric (α1-3)-fucosylated derivatives of compound **N1.3** (Table 1):



and

```
GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
            /                       \             \
  Fucα1-3                            Manβ1-4GlcNAcβ1-4GlcNAc
                                    /
GalNAcβ1-4GlcNAcβ1-2Manα1-3                          [N1.4B]
```

In the major component (80%), **N1.4A**, an αFuc residue is linked to GlcNAc-**5** at O3, whereas in the minor component (20%), **N1.4B**, GlcNAc-**5′** bears such a residue at O3. The ^{1}H-NMR data of these carbohydrate chains having a terminal GalNAcβ1-4[Fucα1-3]GlcNAcβ1-2 element in either the Manα1-3 (**N1.4A**) or the Manα1-6 (**N1.4B**) branch have been discussed recently [23].

HPLC of Mono Q fraction **N3** yielded six carbohydrate-positive subfractions, denoted **N3.1–N3.6** (Fig. 2C). ^{1}H-NMR spectroscopy of fraction **N3.3** indicated the presence of a monosulfated diantennary compound (Table 1):

```
        GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                                    \                   \
                                     Manβ1-4GlcNAcβ1-4GlcNAc
                                    /
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3                      [N3.3]
```

The structure of **N3.3** could be deduced by comparing the structural-reporter-group signals of **N3.3** with those of **N1.3** and **N6.1**. The ^{1}H-NMR data of the (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3 and GalNAcβ1-4GlcNAcβ1-2Manα1-6 branches fit those of the corresponding branches in compounds **N6.1** and **N1.3**, respectively. Comparison of the ^{1}H-NMR data of **N1.3** and **N6.1** reveals that replacement of two GalNAc residues by two (SO_4^-)-4GalNAc residues gives rise, in addition to the previously described chemical shift differences (see above), also to a downfield shift ($\Delta\delta$ +0.006) of the GlcNAc-**2** *N*-acetyl signal. As is evident from the ^{1}H-NMR data of **N3.3**, this phenomenon is not detected when a (SO_4^-)-4GalNAc residue is attached to the Manα1-3 branch in a diantennary carbohydrate chain. Hence, it is concluded that the appearance of the GlcNAc-**2** *N*-acetyl signal at δ 2.101 is indicative of the presence of a (SO_4^-)-4GalNAc residue in the Manα1-6 branch (see below).

^{1}H-NMR spectroscopic analysis of fraction **N3.4** revealed the occurrence of two isomeric monosulfated diantennary oligosaccharides, which can be considered as sulfated extensions of compound **N1.4A** (**N3.4A**) and compound **N1.4B** (**N3.4B**) (Table 1):

```
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                                    \                   \
                                     Manβ1-4GlcNAcβ1-4GlcNAc
                                    /
       GalNAcβ1-4GlcNAcβ1-2Manα1-3
                   /
           Fucα1-3                                       [N3.4A]
```

and

```
       GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                   /                \                  \
          Fucα1-3                    Manβ1-4GlcNAcβ1-4GlcNAc
                                    /
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3                      [N3.4B]
```

The presence of two species is evidenced by the occurrence of two different sets of structural-reporter-group signals for Man-**4**/**4′**, GalNAc-**GN**/**GN′**, and GlcNAc-**2**. The primary structures of **N3.4A** (40%) and **N3.4B** (60%) were elucidated by comparing each set of signals with those of compounds **N1.4A**, **N1.4B** and **N6.1**. The (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6 branch of compound **N3.4A** is indicated by the set of resonances of GalNAc-**GN′** H1 at δ 4.592, H4 at δ 4.692, and NAc at δ 2.077, together with GlcNAc-**5′** H1 at δ 4.556 and NAc at δ 2.038, Man-**4′** H1 at δ 4.910 and H2 at δ 4.096, and GlcNAc-**2** NAc at δ 2.102 (see discussion in relation to **N3.3**). In the case of compound **N3.4B**, the 4-*O*-sulfated GalNAc-containing Manα1-3 branch is shown by the set of resonances of GalNAc-**GN** H1 at δ 4.586, H4 at δ 4.692, and NAc at δ 2.069, together with GlcNAc-**5** H1 at δ 4.556 and NAc at δ 2.045, Man-**4** H1 at δ 5.110 and H2 at δ 4.175, and GlcNAc-**2** NAc at δ 2.095 (compare with **N3.3**). The presence of the (α1-3)-fucosylated Manα1-6 branch in compound **N3.4B** is reflected by Man-**4′** H1 at δ 4.898 and H2 at δ 4.075, GlcNAc-**5′** H1 at δ 4.556 and NAc at δ 2.038, GalNAc-**GN′** H1 at δ 4.456 and NAc at δ 2.045, and Fuc H1 at δ 5.132, H5 at δ 4.859 and CH_3 at δ 1.267. The (α1-3)-fucosylated Manα1-3 branch in compound **N3.4A** is shown by the reporters Man-**4** H1 at δ 5.099 and H2 at δ 4.166, GlcNAc-**5** H1 at δ 4.556 and NAc at δ 2.038, GalNAc-**GN** H1 at δ 4.448 and NAc at δ 2.038, and Fuc H1 at δ 5.128, H5 at δ 4.859 and CH_3 at δ 1.260. Remarkably, only Fuc (α1-3)-linked to GlcNAc-**5′** is affected by the introduction of a sulfate group in the other branch, since small chemical shift differences are observed for the structural-reporter-group signals of this residue going from **N1.4B** to **N3.4B**, namely H1 ($\Delta\delta$ +0.005) and CH_3 ($\Delta\delta$ +0.005).

HPLC of Mono Q fraction **N2**, co-eluting with reference monosialylated diantennary compounds (Fig. 1) [18], yielded one main carbohydrate-positive subfraction, denoted **N2.1** (Fig. 2B). The ^{1}H-NMR spectrum of **N2.1** shows the presence of an (α1-6)-fucosylated, (α2-6)-sialylated, GalNAc-containing diantennary carbohydrate chain (Table 2):

```
              GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                                          \                   \
                                           Manβ1-4GlcNAcβ1-4GlcNAc
                                           |
Neu5Acα2-6GalNAcβ1-4GlcNAcβ1-2Manα1-3                         [N2.1]
```

The ^{1}H-NMR data of the Neu5Acα2-6GalNAcβ1-4GlcNAcβ1-2Manα1-3 branch fit those reported for the identical branch in the diantennary reference oligosaccharide alditols N2/B and N1/B-6′ [24], whereas the ^{1}H-NMR data of the GalNAcβ1-4GlcNAcβ1-2Manα1-6 branch match those of compounds **N1.3**, **N1.4A** and **N3.3**.

Subjection of Mono Q fraction **N5** to HPLC yielded six subfractions, denoted **N5.1–N5.6** (Fig. 2E). The ^{1}H-NMR data of fraction **N5.3** (Table 2) fit those of the monosulfated, (α2-3)-monosialylated, (α1-6)-fucosylated diantennary oligosaccharide alditol S-[3]N [24]:

```
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
                                   \                   \
                                    Manβ1-4GlcNAcβ1-4GlcNAc
                                   /
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3                     [N5.3]
```

HPLC of Mono Q fraction **N8** gave rise to six subfractions, denoted **N8.1–N8.6** (Fig. 2H). The 1D ^{1}H-NMR spectrum of the major fraction **N8.4** indicated the occurrence of the following novel (SO_4^-)-4GalNAc-containing tri′-antennary carbohydrate chain:

```
(SO4-)-4GalNAcβ1-4GlcNAcβ1-6
        GN^           7'        \
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
        GN'           5'       4'   \                   \
                                     Manβ1-4GlcNAcβ1-4GlcNAc
                                    / 3        2         1
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3
        GN            5        4                         [N8.4]
```

The tri′-antennary character of **N8.4** is indicated by the presence of the structural-reporter-group signals of Man-**3** H1 at δ 4.751 (measured at 316 K) and H2 at δ 4.241, Man-**4** H1 at δ 5.118 and H2 at δ 4.184, and Man-**4′** H1 at δ 4.855 and H2 at δ

Table 2. ^{1}H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of sialylated and/or sulfated, GalNAc-containing N-linked carbohydrate chains derived from human urokinase. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 300 K and at p^{2}H 7 [16]. Compounds are represented by short-hand symbolic notation: (□) L-Fuc; (■) D-Gal; (●) D-GlcNAc; (◆) D-Man; (○) Neu5Acα2-6; (△) Neu5Acα2-3; (◇) D-GalNAc; (S-◇), (SO_4^-)-4GalNAc. For numbering of the monosaccharide residues, see text. n.d., not determined. The subscripts α and β stand for the anomeric configuration of GlcNAc-1.

Reporter group	Residue	Chemical shift in			
		N2.1	N5.3	N7.2	N8.4
		ppm			
H1	GlcNAc-1α	5.181	5.180	5.181	5.182
	GlcNAc-1β	4.694	n.d.	n.d.	n.d.
	GlcNAc-2$_α$	4.665			
			4.664	4.663	4.671[a]
	GlcNAc-2$_β$	4.670			
	Man-3	n.d.	n.d.	n.d.	4.751[a]
	Man-4	5.131	5.108	5.106	5.118
	Man-4′	4.917	4.922	4.895	4.855
	GlcNAc-5	4.583	4.557	4.543	4.563
	GlcNAc-5′	4.562	4.571	4.553	4.568
	GalNAc-GN	4.498	4.585	4.579	4.589
	GalNAc-GN′	4.523	–	4.592	4.589
	GalNAc-GN^	–	–	–	4.600
	Gal-6′	–	4.548	–	–
	GlcNAc-7	–	–	4.536[b]	–
	GlcNAc-7′	–	–	–	4.518
	Gal-8	–	–	4.543[b]	–
	Fuc$_α$	4.893	4.892	4.898	4.907
	Fuc$_β$	4.900	4.900	4.902	4.915
H2	Man-3	4.250	4.245	4.203	4.241
	Man-4	4.186	4.177	4.203	4.184
	Man-4′	4.090	4.12[c]	4.097	4.075
H3	Gal-6′	–	4.117	–	–
	Gal-8	–	–	4.112	–
H3a	Neu5Ac	1.718	1.802	1.800	–
H3e	Neu5Ac	2.660	2.759	2.757	–
H4	GalNAc-GN	n.d.	4.691	4.692	4.696
	GalNAc-GN′	n.d.	–	4.692	4.696
	GalNAc-GN^	–	–	–	4.696
	Man-4′	n.d.	n.d.	n.d.	3.389
H5	Fuc$_α$	4.097	4.097	4.098	4.100
	Fuc$_β$	4.134	4.132	4.133	4.136
H6	Man-4′	n.d.	n.d.	n.d.	4.208
NAc	GlcNAc-1	2.039	2.039	2.040	2.039
	GlcNAc-2$_α$	2.097	2.096		
				2.102	2.100
	GlcNAc-2$_β$	2.095	2.094		
	GlcNAc-5	2.069	2.044	2.040	2.048
	GlcNAc-5′	2.042	2.044	2.040	2.033
	GalNAc-GN	2.069	2.069	2.070	2.070
	GalNAc-GN′	2.073	–	2.077	2.077
	GalNAc-GN^	–	–	–	2.077
	GlcNAc-7	–	–	2.070	–
	GlcNAc-7′	–	–	–	2.033
	Neu5Ac	2.031	2.032	2.031	–
CH_3	Fuc$_α$	1.210	1.210	1.211	1.212
	Fuc$_β$	1.221	1.222	1.222	1.222

[a] Value measured at 316 K.
[b] Values may have to be interchanged.
[c] Value given with two decimals because of spectral overlap.

Table 3. Summary of ¹H-NMR chemical shifts of various H1, H2 and NAc protons in K₂tu-PA and u-PA. Protons considered are Man-3 H2, Man-4/4′ H1 and H2, GlcNAc-5/5′ H1 and NAc, and GlcNAc-7/7′ H1 and NAc signals stemming from the corresponding residues as part of (α2-3)-sialylated di- (N2.2), tri- (N3.2B) or tri′- (N3.2A) antennary carbohydrate chains from K_2tu-PA [25] and as part of disulfated di- (**N6.1**), disulfated, monosialylated tri- (**N7.2**) or trisulfated tri′- (**N8.4**) antennary carbohydrate chains from u-PA. Values given in parentheses are chemical shift differences (in ×1000 ppm) observed when going from disialylated (N2.2) [25] to disulfated (**N6.1**) diantennary compounds, going from trisialylated (N3.2B) [25] to monosialylated, disulfated (**N7.2**) triantennary compounds, and going from trisialylated (N3.2A) [25] to trisulfated (**N8.4**) tri′-antennary compounds. The oligosaccharide structures are represented by short-hand symbolic notation: (□) L-Fuc; (■) D-Gal; (●) D-GlcNAc; (◆) D-Man; (△) Neu5Acα2-3; (◇) D-GalNAc; (S-◇) (SO_4^-)-4GalNAc.

Residue	Proton	Chemical shift in					
		K_2tu-PA			u-PA		
		N2.2	N3.2B	N3.2A	**N6.1**	**N7.2**	**N8.4**
		ppm					
Man-3	H2	4.248	4.210	4.250	4.242(−6)	4.203(−7)	4.241(−9)
Man-4	H1	5.117	5.113	5.122	5.109(−8)	5.106(−7)	5.118(−4)
	H2	4.190	4.210	4.197	4.176(−14)	4.203(−7)	4.184(−13)
Man-4′	H1	4.922	4.903	4.871	4.910(−12)	4.895(−8)	4.855(−16)
	H2	4.117	4.105	4.090	4.098(−19)	4.097(−8)	4.075(−15)
GlcNAc-5	H1	4.573	4.560	4.577	4.556(−17)	4.543(−17)	4.563(−14)
	NAc	2.047	2.043	2.051	2.043(−4)	2.040(−3)	2.048(−3)
GlcNAc-5′	H1	4.573	4.577	4.587	4.556(−17)	4.553(−24)	4.568(−19)
	NAc	2.043	2.043	2.038	2.039(−4)	2.040(−3)	2.033(−5)
GlcNAc-7	H1		4.544			4.536(−8)	
	NAc		2.073			2.070(−3)	
GlcNAc-7′	H1			4.544			4.518(−26)
	NAc			2.038			2.033(−5)

4.075. In addition, the detection (2D HOHAHA) outside the bulk-region of Man-**4′** H4 at δ 3.389, and H6 at δ 4.208, characteristic for tri′- and tetraantennary structures [25], supports this assignment. It should be noted that the structural-reporter-group signals of Man-**3/4/4′** H1 and H2 in **N8.4** differ slightly from those observed for the (α2-3)-trisialylated tri′-antennary carbohydrate chain N3.2A [25]. As is evident from Table 3, the upfield shifts for these Man signals caused by the replacement of terminal Neu5Acα2-3Galβ1-4 elements by terminal (SO_4^-)-4GalNAcβ1-4 elements when going from the (α2-3)-trisialylated tri′-antennary chain N3.2A to the trisulfated tri′-antennary chain **N8.4** are similar to those going from the (α2-3)-disialylated diantennary chain N2.2 [25] to the disulfated diantennary chain **N6.1**.

The presence of three terminal (SO_4^-)-4GalNAcβ1-4 units in **N8.4** is deduced from the overlapping GalNAc-**GN**,**GN′**,**GN^** H4 signals at δ 4.696, the GalNAc-**GN** *N*-acetyl singlet at δ 2.070 and the GalNAc-**GN′**/**GN^** *N*-acetyl singlets at δ 2.077, which occur in the peak intensity ratio 3:3:6. The assignment of the GalNAc-**GN** and GalNAc-**GN′** *N*-acetyl signals is based on a comparison with the values of the corresponding signals in **N6.1**. In view of the doubled intensity of the *N*-acetyl signal at δ 2.077, not only the *N*-acetyl signal of GalNAc-**GN′**, but also that of GalNAc-**GN^** was assigned to this value. The GalNAc H1 signals at δ 4.589 and δ 4.600 (peak intensity ratio 2:1), traced by their HOHAHA connectivities with the coinciding GalNAc-**GN**/**GN′**/**GN^** H4 signals at δ 4.696, are tentatively assigned to GalNAc-**GN**/GalNAc-**GN′** and GalNAc-**GN^**, respectively. The criteria used for these assignments are the earlier observations that in *N*-acetyllactosamine-type tri′-antennary chains the Gal-**6** H1 and Gal-**6′** H1 signals coincide and resonate at a position upfield from Gal-**8′** H1 (cf. reference compound 11 [16] and (α2-3)-trisialylated reference compound N3.2A [25]). The remaining H1 signals at δ 4.563, δ 4.568 and δ 4.518 are assigned to GlcNAc-**5**, GlcNAc-**5′** and GlcNAc-**7**, respectively. In this case the assignments were made on guidance of the previous assignments for these residues in the reference compounds 11 [16] and N3.2A [25], taking into account their relative resonance positions. The same holds for the *N*-acetyl signals of these residues.

HPLC of Mono Q fraction **N7** yielded eight subfractions, denoted **N7.1**−**N7.8** (Fig. 2G). Investigation of the ¹H-NMR spectrum of fraction **N7.2** (Fig. 4) showed a novel triantennary oligosaccharide as major component:

```
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6              Fucα1-6
     GN'          5'        4'  \                    \
                                 Manβ1-4GlcNAcβ1-4GlcNAc
                                /  3          2         1
(SO4-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-3
     GN           5        / 4
Neu5Acα2-3Galβ1-4GlcNAcβ1-4
          8        7
                                                   [N7.2]
```

The presence of three antennae is reflected by one set of structural-reporter-group signals for a terminal Neu5Acα2-3Galβ1- unit and two sets of signals for two terminal (SO_4^-)-4GalNAcβ1- units, together with three sets of signals corresponding with GlcNAc-**5**, -**5′** and -**7**. As Man-**4′** H4 and H6 resonate inside the bulk region, a tri′-antennary structure for

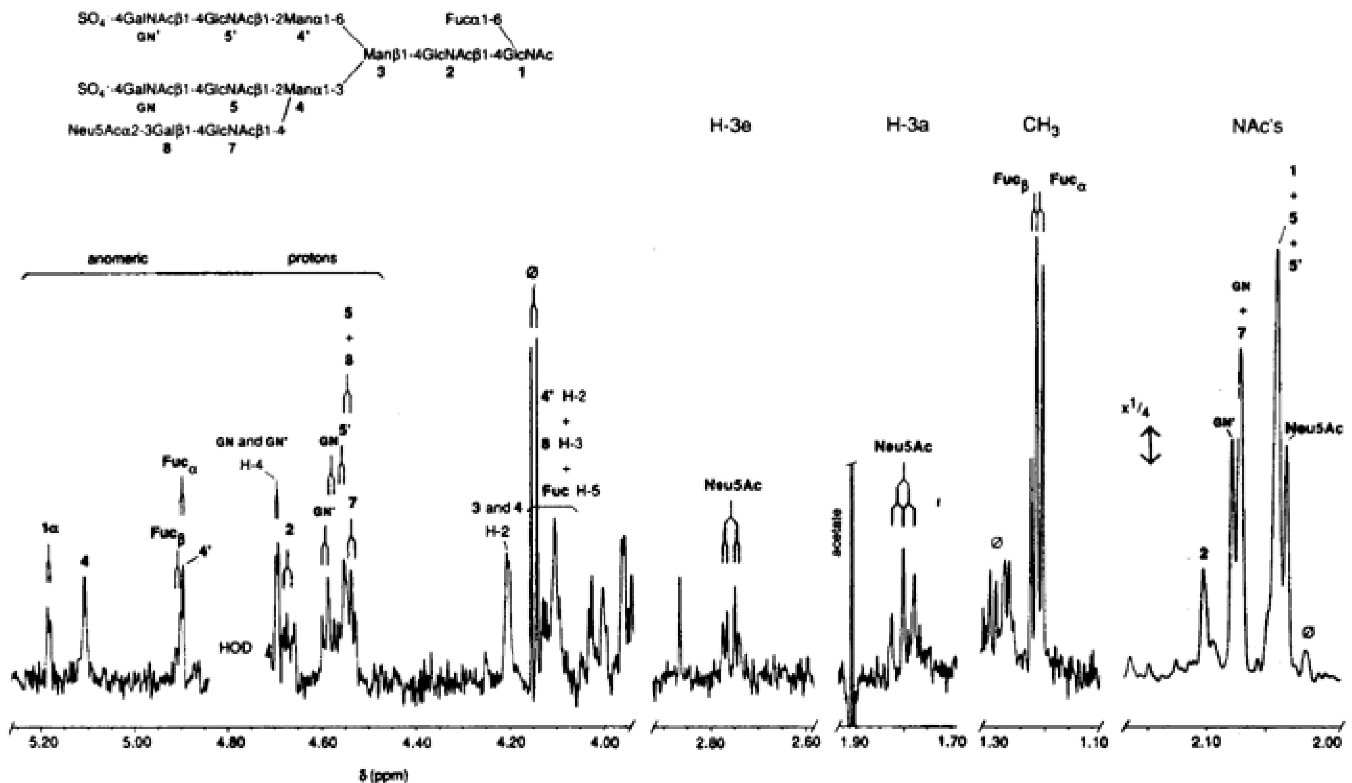


Fig. 4. Structural-reporter-group-signal regions of the resolution-enhanced 500-MHz ¹H-NMR spectrum of fraction N7.2 derived from human u-PA, recorded in ^2H_2O at 300 K. The relative scale of the NAc protons region differs from that of the rest of the spectrum.

N7.2 can be excluded [25]. The set of structural-reporter-group signals which indicate the triantennary character are found at δ 4.203 for Man-**3** H2, δ 5.106 for Man-**4** H1, δ 4.203 for Man-**4** H2, δ 4.895 for Man-**4'** H1, and δ 4.097 for Man-**4'** H2. Comparison of these values with those of the (α2-3)-trisialylated triantennary component N3.2B [25] shows comparable trends in chemical shift differences as observed when comparing **N6.1** with reference compound N2.2 and **N8.4** with reference compound N3.2A (Table 3).

The occurrence of a (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-2Manα1-6 branch is demonstrated by the set of GalNAc-**GN'** H1, H4 and NAc signals at δ 4.592, δ 4.692 and δ 2.077, respectively, in combination with the set of GlcNAc-**5'** H1 and NAc signals at δ 4.553 and δ 2.040, respectively, and the GlcNAc-**2** NAc singlet at δ 2.102 (compare with the corresponding data of **N6.1**; for a discussion of the δ value of the GlcNAc-**2** NAc signal, see above). The positioning of the two remaining antennae is based on the following. When going from reference compound N3.2A to **N8.4**, i.e. from Neu5Acα2-3Galβ1- to (SO_4^-)-4GalNAcβ1-, typical upfield shifts are observed for the H1 signals of the three antennary GlcNAc residues, namely GlcNAc-**5**, $\Delta\delta$ −0.014; GlcNAc-**5'**, $\Delta\delta$ −0.019; GlcNAc-**7'**, $\Delta\delta$ −0.026. Likewise, when going from reference compound N2.2 to **N6.1** the upfield shifts for GlcNAc-**5** and -**5'** H1 are both $\Delta\delta$ −0.017 (Table 3). Based on these observations, it is expected that, in the case of attachment of the second (SO_4^-)-4GalNAcβ1-4GlcNAcβ1- antenna at C4 of the Manα1-3 residue, GlcNAc-**7** H1 will resonate at approximately δ 4.52 (reference compound N3.2B: GlcNAc-**7** H1 at δ 4.544). The absence of a ¹H-signal around δ 4.52 suggests that the (SO_4^-)-4GalNAcβ1-4GlcNAcβ1-antenna is attached to C2 of the Manα1-3 residue. This assignment implies that the Neu5Acα2-3Galβ1-4GlcNAcβ1- antenna is attached to C4 of the Manα1-3 residue. Positioning the antennae in this way, which corresponds with the introduction of a Neu5Acα2-3Galβ1-4GlcNAcβ1-4 antenna in **N6.1**, an upfield shift of $\Delta\delta$ −0.013 is observed for GlcNAc-**5** H1, assigned at δ 4.543, in **N7.2** as compared to **N6.1**. An identical upfield shift for GlcNAc-**5** H1 is detected when going from reference compound N2.2 to reference compound N3.2B (Table 3). It should be noted that the set of GlcNAc-**7** NAc and Gal-**8** H1 are in good agreement with those in N3.2B, but an upfield shift is observed for GlcNAc-**7** H1 ($\Delta\delta$ −0.008). Likewise, the δ values of GalNAc-**GN** NAc in **N7.2** and **N6.1** are similar, but an upfield shift is detected for GalNAc-**GN** H1 ($\Delta\delta$ −0.006).

DISCUSSION

As has been shown in this study, the di-, tri- and tri'-antennary carbohydrate chains on u-PA are carriers of predominantly terminal non-reducing GalNAcβ1-4GlcNAcβ elements. The GalNAc residue is mainly sulfated at O4, or to a lesser extent it bears *N*-acetylneuraminic acid at O6; alternatively the GlcNAc residue can be fucosylated at O3. The large heterogeneity of N-glycans observed in u-PA, purified from a pool of human urine from numerous donors, does not necessarily mean that u-PA from a single individual will exhibit the same variation of N-glycans. The finding of GalNAc-containing oligosaccharides in human-urine-isolated u-PA implies the occurrence of a GalNAc:GlcNAcβ-R GalNAc-transferase activity in the kidney cells. In the context of an ongoing discussion concerning this type of transferase, it should be noted that, just like the human-urine-isolated GalNAc-containing N-glycoproteins Tamm-Horsfall glycoprotein [17] and kallidinogenase [22], u-PA does not contain a Pro-Xaa-Arg/Lys tripeptide, located 6−9 residues on the amino-terminal side of Asn302 [7]. This tripeptide motif has been proposed to be necessary for the action of GalNAc:GlcNAcβ-R GalNAc-transferase activity in certain glycoproteins

[26]. In other cases, a Pro-Xaa-Arg/Lys-independent GalNAc:GlcNAcβ-R GalNAc-transferase activity has been found, although not in (rat) kidney cells [27]. Further investigations are needed to characterize the GalNAc-transferase activity, which is involved in the incorporation of GalNAc into the N-glycans of u-PA and other urine-isolated glycoproteins. In earlier studies it was stated that the expression of 4-sulfo-transferase activity is co-ordinate with the expression of Pro-Xaa-Arg/Lys-dependent GalNAc-transferase activity, and not with that of Pro-Xaa-Arg/Lys-independent (transferrin-specific) GalNAc-transferase activity [27]. In view of the occurrence of (SO_4^-)-4GalNAcβ elements in u-PA and Tamm-Horsfall glycoprotein, this statement does not generally hold.

The appearance of the GalNAcβ1-4[Fucα1-3]GlcNAcβ element in u-PA and its relevance as potential immuno-determinant in humans has been discussed earlier [23, 28]. Interestingly, it has been suggested that this carbohydrate element is a ligand for E-selectin, and has thereby potential anti-inflammatory properties [29]. This element has also been detected in a glycoprotein pool from the parasite *Schistosoma mansoni* [30], in *Apis mellifera* honeybee venom phospholipase A_2 [31], in bovine pro-opiomelanocortin [32], and in recombinant protein C expressed in human kidney 293 cells [33]. Recently, a tyvelosylated variant, namely Tyv1-3GalNAc1-4[Fuc1-3]GlcNAc has been detected in tetraantennary N-glycans of the excretory/secretory antigens of the parasite *Trichinella spiralis* [34]. With respect to u-PA, besides the neutral GalNAcβ1-4[Fucα1-3]GlcNAcβ-containing diantennary N-glycans (**N1.4A/B**), also monosulfated analogues have been found, namely compounds **N3.4A** and **N3.4B**. Remarkably, (SO_4^-)-4GalNAc and Fuc residues are not occurring simultaneously in the same antenna. Moreover, the distribution of (α1-3)-linked Fuc over the branches of neutral (**N1.4A/B**) and sulfated (**N3.4A/B**) carbohydrate chains is different. The Fuc residue occurrs predominantly in the Manα1-3 branch of the carbohydrate chains in the neutral fraction **N1.4**, whereas this residue is mainly present in the Manα1-6 branch of the N-glycans in fraction **N3.4**. In monosulfated, diantennary carbohydrate chains, the (SO_4^-)-4GalNAc unit is detected in the Manα1-3 branch only (**N3.3** and **N5.3**), suggesting a Manα1-3 branch-specificity of the sulfo-transferase activity. The observed antennary distribution of (α1-3)-linked Fuc and (SO_4^-)-4GalNAc elements needs further studies addressing the enzyme kinetics and substrate specifities of the involved α-fucosyl- and sulfo-transferase activities.

The (SO_4^-)-4GalNAcβ1-4GlcNAcβ1- structural element has been found previously in pituitary glycohormones, like lutropin [24] or thyrotropin [35], but also in human urinary Tamm-Horsfall glycoprotein [17] and bovine pro-opiomelanocortin [32]. The element has been suggested as a modulator of the circulatory half-life of glycoproteins by recognition of a (SO_4^-)-4GalNAc-specific hepatic cell receptor [36]. It is therefore tempting to propose that human-urine-isolated urokinase, used as thrombolytic agent, is (partially) recruited to the liver by the (SO_4^-)-4GalNAc receptor, as described for the glycohormones [37]. Recently, it has also been demonstrated that terminal non-reducing βGalNAc, like that found in **N1.3** and **N2.1**, is a potent ligand for the hepatic asialoglycoprotein receptor [38]. In addition, it has been shown that the O-linked Fuc residue in tissue-type plasminogen activator (t-PA) may mediate its binding and degradation by hepatoma cells (HepG2), and it is suggested that this residue is involved in the uptake of the glycoprotein from the circulation [39]. These recognition processes may play a role in the relatively fast clearance of u-PA [40]. The fast clearance is (partly) responsible for the high clinical doses of u-PA which are generally needed to obtain and maintain vascular/arterial reperfusion. The high doses are associated with (life-threatening) systemic bleeding tendencies [41] and constitute a major drawback in the therapeutic application of u-PA.

In the framework of our program on the analysis of (recombinant) glycoprotein glycans, we recently finished a study on a human chimeric plasminogen activator, K_2tu-PA, expressed in CHO cells [25]. It consists of the kringle-2 domain of t-PA and the protease domain (B chain) of u-PA. The chimeric molecule was designed in order to combine the fibrin specificity of t-PA with the catalytic activity of u-PA. K_2tu-PA is partially glycosylated at Asn12 (originally Asn184 of t-PA) and completely glycosylated at Asn247 (originally Asn302 of u-PA), and bears exclusively N-linked (α2-3)-sialylated, (α1-6)-fucosylated carbohydrate chains of the *N*-acetyllactosamine type. Comparison of the collection of N-glycans of u-PA with that of the B chain of K_2tu-PA reveals dramatic differences. Unlike the carbohydrate chains on human-urine-isolated u-PA, the carbohydrate chains occurring on recombinant K_2tu-PA do not contain (sulfated) GalNAc residues. So far, GalNAc-containing N-linked carbohydrate chains have not been detected in recombinant glycoproteins produced by CHO cells [42], although recently a GalNAc-transferase activity has been detected in CHO cells [27]. The comparison illustrates that the B chain of K_2tu-PA is glycosylated in accordance with the known glycosylation capacity of the CHO cells used, which apparently show no action of a GalNAc-transferase. Consequently, the absence of terminal (SO_4^-)-4GalNAc, GalNAc and O-linked Fuc elements may increase the half-life of K_2tu-PA relative to that of u-PA. Moreover, K_2tu-PA lacks the potential immunogenic determinant GalNAcβ1-4[Fucα1-3]GlcNAcβ, which may otherwise be able to raise an immunogenic response. Such characteristics are probably profitable for the therapeutic efficiency and efficacy of K_2tu-PA.

We thank Mr René Knecht (Ciba-Geigy Ltd Basel, Switzerland) for performing the amino acid sequence analysis. This project was financially supported by Ciba-Geigy Ltd, the Netherlands Program for Innovation Oriented Carbohydrate Research (IOP-k), and the Netherlands Foundation for Chemical Research (NWO/SON).

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