

# Determination of the branch location of extra *N*-acetylglucosamine units in sialo *N*-linked tetraantennary oligosaccharides

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An approach is presented for the determination of the branch location of 1 or 2 extra *N*-acetylglucosamine units in sialo *N*-linked carbohydrate chains from glycoproteins. Tetraantennary oligosaccharides containing extra *N*-acetylglucosamine units were digested with endo- $\beta$ -galactosidase, followed by treatment with *N*-acetyl- $\beta$ -glucosaminidase, yielding products which could be analysed by <sup>1</sup>H-NMR spectroscopy, thereby giving conclusive data about the location of the extra units in the intact structures.

Recombinant erythropoietin; Extra *N*-acetylglucosamine unit; <sup>1</sup>H-NMR spectroscopy

## 1. INTRODUCTION

High-resolution <sup>1</sup>H-NMR spectroscopy is a powerful technique for the analysis of *N*- and *O*-linked carbohydrate chains from glycoproteins [1–3]. However, only limited NMR data are available on *N*-linked carbohydrate chains with extra *N*-acetylglucosamine structural elements, hampering the determination of the branch location of *N*-acetylglucosamine repeating units.

In the framework of our program on the structural analysis of oligosaccharides by <sup>1</sup>H-NMR spectroscopy, we developed a method for the determination of the branch location of *N*-acetylglucosamine repeating units in tetraantennary sialo-oligosaccharides. The compounds are degraded with endo- $\beta$ -galactosidase, followed by digestion with *N*-acetyl- $\beta$ -glucosaminidase, to obtain oligosaccharides that can unambiguously be identified by high-resolution <sup>1</sup>H-NMR spectroscopy, in this way providing conclusive evidence for the structure of the intact compound.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Recombinant human erythropoietin (EPO), expressed in Chinese hamster ovary cells, was a gift of Organon Teknika BV (Boxtel, The Netherlands). Peptide-*N*<sup>4</sup>-(*N*-acetyl- $\beta$ -glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* and endo-

*Abbreviations:* Neu5Ac, *N*-acetylneuraminic acid; Fuc, fucose; EPO, erythropoietin; PNGase-F, peptide-*N*<sup>4</sup>-(*N*-acetyl- $\beta$ -glucosaminyl)asparagine amidase F; HPAE-PAD, high-pH anion-exchange pulsed amperometric detection

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$\beta$ -galactosidase from *Bacteroides fragilis* were obtained from Boehringer Mannheim. *N*-Acetyl- $\beta$ -glucosaminidase from jack beans was purchased from Sigma.

### 2.2. 500 MHz <sup>1</sup>H-NMR spectroscopy

Prior to <sup>1</sup>H-NMR spectroscopy, samples were repeatedly treated with <sup>2</sup>H<sub>2</sub>O, finally using 99.96 atom % <sup>2</sup>H<sub>2</sub>O (MSD isotopes) at p<sup>2</sup>H 7 and room temperature. 500 MHz <sup>1</sup>H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR spectroscopy, Utrecht University) at a probe temperature of 27°C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone ( $\delta$  2.225) [2].

### 2.3. Liberation and purification of *N*-linked carbohydrate chains

*N*-Linked oligosaccharides were released from EPO with PNGase-F and fractionated by FPLC on Mono Q (Pharmacia) as described [4]. The Mono Q fraction containing the tetrasialylated carbohydrate chains was subfractionated by HPLC on Lichrosorb-NH<sub>2</sub> (Chrompack) [4] and further separated by HPAE-PAD chromatography on CarboPac PA-1 (Dionex) [5].

### 2.4. Enzyme incubations






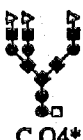

Oligosaccharides (1 nmol/ $\mu$ l) were incubated with endo- $\beta$ -galactosidase (1 mU/ $\mu$ l) in 50 mM NaAc, pH 5.8, containing bovine serum albumin (0.2 mg/ml), for 48 h at 37°C. The endo- $\beta$ -galactosidase digests were separated according to charge by FPLC on Mono Q (Pharmacia), using a linear gradient from 0–50 mM NaCl in 8 ml H<sub>2</sub>O, followed by a gradient from 50–500 mM NaCl in 8 ml H<sub>2</sub>O, essentially as described [6]. Desalting of FPLC fractions was performed on Bio-Gel P-2 (Bio-Rad), using water as eluent.

Endo- $\beta$ -galactosidase digested oligosaccharides (0.67 nmol/ $\mu$ l) were incubated with *N*-acetyl- $\beta$ -glucosaminidase (2 mU/ $\mu$ l) in 0.2 M Na<sub>2</sub>HPO<sub>4</sub>/0.05 M citric acid, pH 5.0, for 48 h at 37°C. The digests were desalted on Bio-Gel P-2 (Bio-Rad) with water as eluent.

## 3. RESULTS

The PNGase-F released *N*-linked carbohydrate chains from EPO were purified via a sequence of chromatographic procedures using Mono Q, Lichrosorb-NH<sub>2</sub>, and CarboPac PA-1. In the context of this report, focused on a straightforward <sup>1</sup>H-NMR analysis

Table 1. <sup>1</sup>H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of compounds A, B, C and D and enzymatic breakdown products. Chemical shifts are given at 300 K and were measured in <sup>2</sup>H<sub>2</sub>O relative to internal acetone (δ 2.225) [2]. For numbering of the monosaccharide residues, see text. Compounds are represented by short hand symbolic notation [2]; ●, GlcNAc; ◆, Man; □, Fuc; ■, Gal; ▲, α2-3Neu5Ac.

Reporter group	Residue	Chemical shift (ppm) in						
								
H-1	GlcNAc-1 α	5.182	5.182	5.182	5.182	5.182	5.181	5.181
	β	4.688	4.690	4.689	4.689	4.692	4.690	4.691
	GlcNAc-2 α <sup>a</sup>	4.659	4.659	4.659	4.659	4.664	4.663	4.663
	β	4.664	4.663	4.664	4.664	4.667	4.668	4.668
	Man-4	5.131	5.130	5.130	5.129	5.114	5.115	5.114
	Man-4'	4.857	4.854	4.858	4.855	4.905	4.884	4.915
	GlcNAc-5	4.563 <sup>b</sup>	4.564	4.563	4.561	4.560	4.557	4.559
	GlcNAc-5'	4.593	4.589	4.602 <sup>c</sup>	4.60 <sup>c</sup>	4.574	-	-
	Gal-6	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.541 <sup>d</sup>	4.547	4.542 <sup>d</sup>	4.543
	Gal-6'	4.545 <sup>d</sup>	4.547 <sup>d</sup>	4.455	4.454	4.547	-	-
	GlcNAc-7	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.541 <sup>d</sup>	4.547	4.542 <sup>d</sup>	4.543
	GlcNAc-7'	4.545 <sup>d</sup>	4.547 <sup>d</sup>	4.545 <sup>d</sup>	4.546 <sup>d</sup>	-	4.546 <sup>d</sup>	-
	Gal-8	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.542 <sup>d</sup>	4.541 <sup>d</sup>	4.547	4.542 <sup>d</sup>	4.543
	Gal-8'	4.559 <sup>b</sup>	4.467	4.557	4.468	-	4.566	-
GlcNAc <sub>ext</sub>	-	4.701	4.699	4.700 <sup>e</sup>	-	-	-	
Gal <sub>ext</sub>	-	4.556	4.557	4.556 <sup>e</sup>	-	-	-	
H-2	Man-3	4.203	4.206	4.205	4.21 <sup>f</sup>	4.212	4.21	4.213
	Man-4	4.220	4.219	4.219	4.22	4.212	4.21	4.213
	Man-4'	4.090	4.091	4.081	4.080	4.11	3.96 <sup>g</sup>	3.96 <sup>g</sup>
H-3	Gal-6	4.117	4.117	4.116	4.116	4.117	4.117	4.115
	Gal-6'	4.117	4.117	n.d. <sup>h</sup>	n.d.	4.117	-	-
	Gal-8	4.117	4.117	4.116	4.116	4.117	4.117	4.115
	Gal-8'	4.117	n.d.	4.116	n.d.	-	4.117	-
	Gal <sub>ext</sub>	-	4.117	4.116	4.116 <sup>c</sup>	-	-	-
H-4	Gal-6'	n.d.	n.d.	4.161	4.161	n.d.	-	-
	Gal-8'	n.d.	4.159	n.d.	4.161	-	n.d.	-
H-3a	Neu5Ac	1.804 <sup>i</sup>	1.802 <sup>i</sup>	1.803 <sup>i</sup>	1.801 <sup>i</sup>	1.802 <sup>j</sup>	1.802 <sup>j</sup>	1.800 <sup>c</sup>
H-3e	Neu5Ac	2.756 <sup>i</sup>	2.757 <sup>i</sup>	2.757 <sup>i</sup>	2.757 <sup>i</sup>	2.756 <sup>j</sup>	2.756 <sup>j</sup>	2.756 <sup>c</sup>
NAc	GlcNAc-1	2.038	2.037	2.038	2.037	2.040	2.037	2.038
	GlcNAc-2 α <sup>a</sup>	2.094	2.094	2.092	2.091	2.095	2.091	2.092
	β	2.090	2.090	2.088	2.088	2.095	2.087	2.092
	GlcNAc-5	2.047	2.047	2.047	2.047	2.044	2.044	2.043
	GlcNAc-5'	2.038	2.037	2.038	2.037	2.044	-	-
	GlcNAc-7	2.075	2.075	2.074	2.075	2.074	2.073	2.073
	GlcNAc-7'	2.038	2.037	2.038	2.037	-	2.052	-
	GlcNAc <sub>ext</sub>	-	2.037	2.038	2.037 <sup>k</sup>	-	-	-
	Neu5Ac	2.031 <sup>l</sup>	2.031 <sup>l</sup>	2.031 <sup>l</sup>	2.031 <sup>l</sup>	2.031 <sup>m</sup>	2.031 <sup>m</sup>	2.031 <sup>k</sup>
H-1	Fuc α <sup>a</sup>	4.901	4.900	4.898	4.897	4.893	4.888	4.886
	β	4.909	4.908	4.905	4.905	4.901	4.897	4.893
H-5	Fuc α <sup>a</sup>	4.10 <sup>g</sup>	4.10 <sup>g</sup>	4.10 <sup>g</sup>	4.10 <sup>g</sup>	n.d.	4.10 <sup>g</sup>	4.10 <sup>g</sup>
	β	4.13 <sup>g</sup>	4.13 <sup>g</sup>	4.13 <sup>g</sup>	4.13 <sup>g</sup>	n.d.	4.13 <sup>g</sup>	4.13 <sup>g</sup>
CH <sub>3</sub>	Fuc α <sup>a</sup>	1.211	1.211	1.210	1.210	1.211	1.210	1.209
	β	1.222	1.223	1.222	1.222	1.223	1.222	1.221

<sup>a</sup> α and β stand for the α and β anomers of GlcNAc-1; <sup>b</sup> Value obtained using 2D nuclear Overhauser enhancement spectroscopy; <sup>c</sup> Occurrence of virtual coupling; <sup>d</sup> Values may have to be interchanged; <sup>e</sup> Signal stemming from two protons; <sup>f</sup> Values given with only two decimals because of spectral overlap; <sup>g</sup> Value obtained from a 2D homonuclear Hartmann Hahn experiment; <sup>h</sup> n.d., not determined; <sup>i</sup> Signal stemming from four protons; <sup>j</sup> Signal stemming from three protons; <sup>k</sup> Signal stemming from two NAc groups; <sup>l</sup> Signal stemming from four NAc groups; <sup>m</sup> Signal stemming from three NAc groups

of carbohydrate chains with additional *N*-acetylactosamine units, four tetrasialo tetraantennary oligosaccharide chains, denoted A, B, C and D, will be discussed. The NMR data of these compounds are summarized in Table I. Relevant parts of the NMR spectra are shown in Fig. 1.

The structural-reporter-group signals of A are in agreement with those reported for the tetrasialo tetraantennary oligosaccharide from recombinant human follitropin [7], containing Neu5Ac in  $\alpha$ 2-3 linkage and having Fuc in  $\alpha$ 1-6 linkage to GlcNAc-1 (see coding system in structure for A).

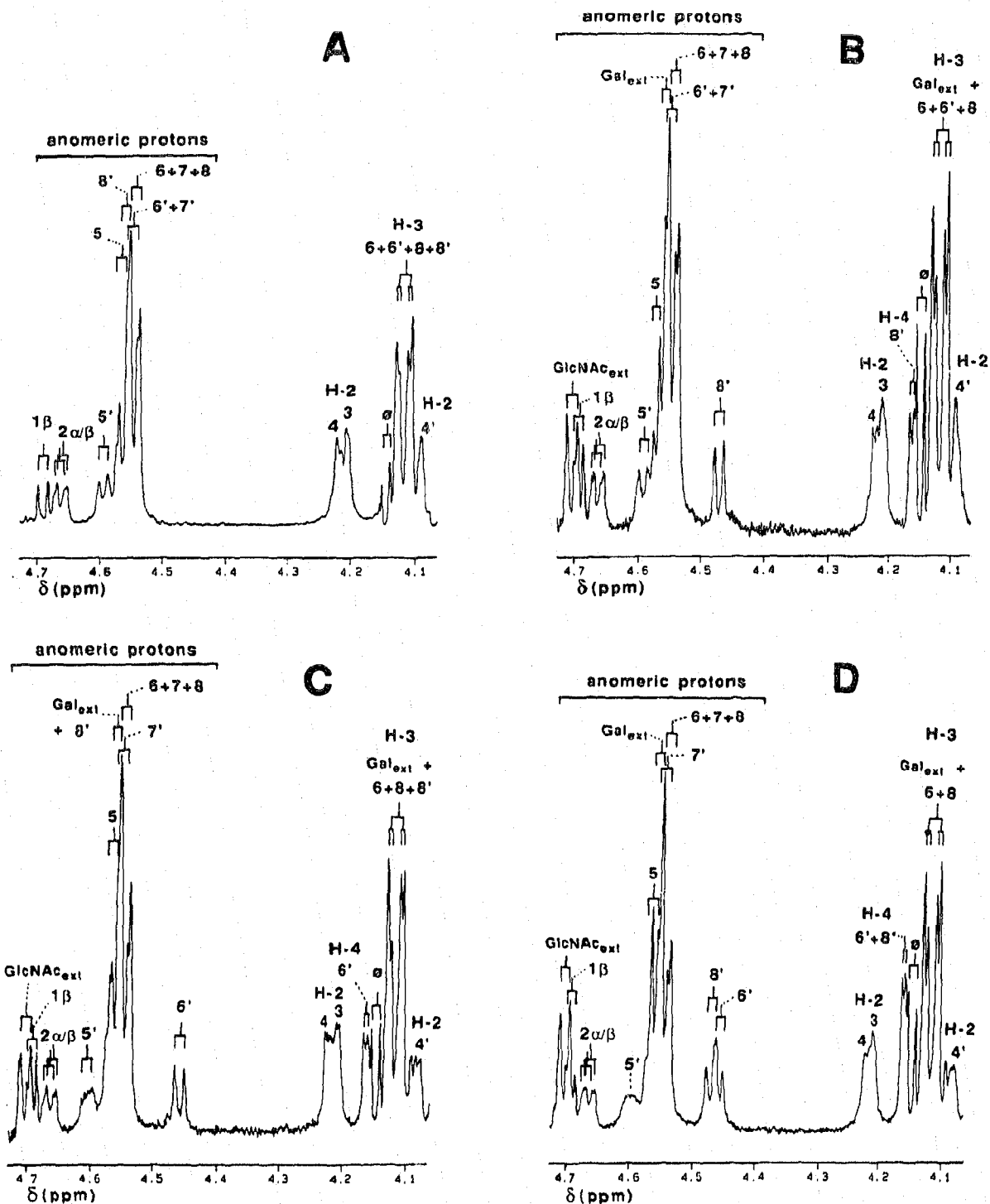
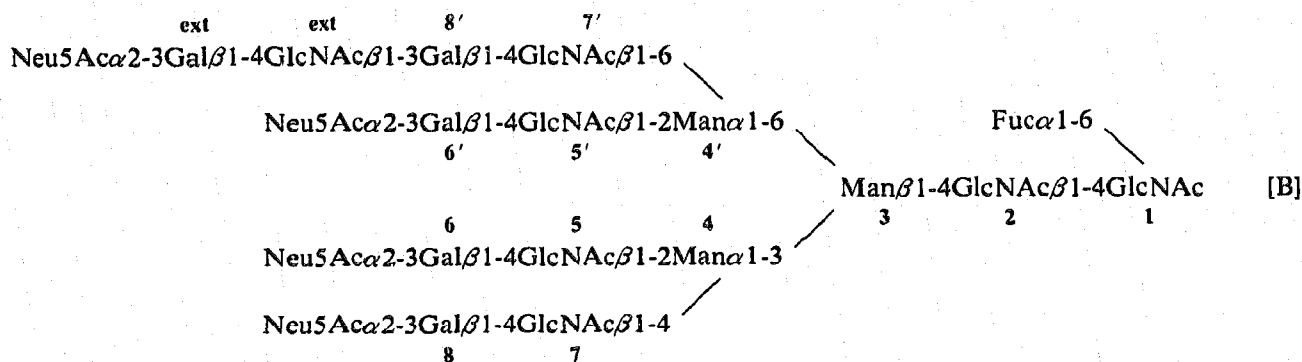


Fig. 1. Relevant sections of the 500 MHz <sup>1</sup>H-NMR spectra of compounds A, B, C and D, recorded in <sup>2</sup>H<sub>2</sub>O at 300 K. For structures and numbering of the monosaccharide residues, see text. Ø denotes impurity.

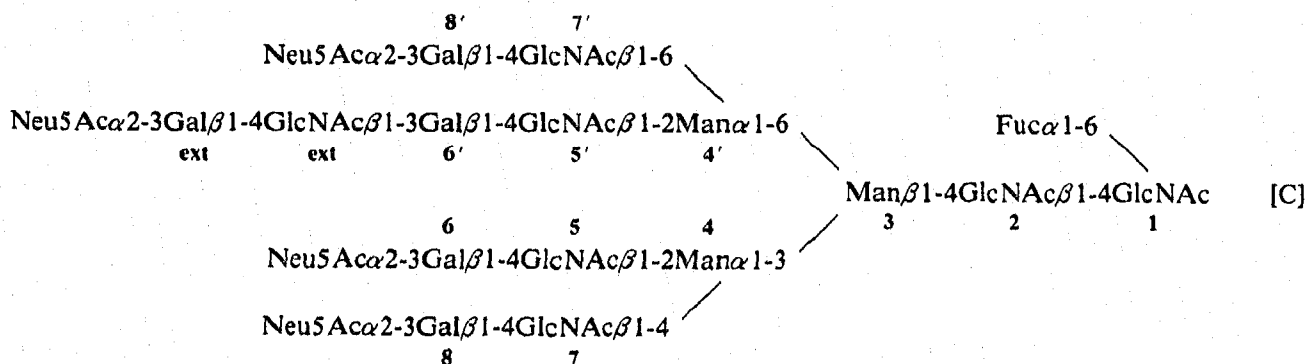




The Mono Q fraction containing the monosialo compound (B.Q2) was analysed by <sup>1</sup>H-NMR spectroscopy, showing the presence of the linear sialylated tetrasaccharide Neu5Acα2-3Gal<sub>ext</sub>β1-4GlcNAcβ1-3Gal<sub>red</sub> (cf. compounds 60 in [2] and 6a<sub>2</sub> in [8]). The H-1 signals of Gal<sub>ext</sub> and βGal<sub>red</sub> are observed at δ 4.560 and δ 4.558, respectively, and the H-1 signal of αGal<sub>red</sub> is present at δ 5.224 (cf. compound A in [10]). The chemical shift values of GlcNAc H-1 (obtained at 320K) are δ 4.758 (αGal<sub>red</sub>) and δ 4.742 (βGal<sub>red</sub>). The GlcNAcβ1-3Gal linkage is reflected by the presence of the H-4 signal of αGal<sub>red</sub> at δ 4.204 and of βGal<sub>red</sub> at δ 4.146 (cf. compound A in [10]). The α2-3 linked Neu5Ac gives rise to the characteristic set of structural-reporter-group signals at δ 2.033 (NAc), δ 1.797 (H-3a), and δ 2.758 (H-3e), whereas the Gal<sub>ext</sub> H-3 resonates at δ 4.115.

The <sup>1</sup>H-NMR spectrum of C (Fig. 1) reveals the presence of an α2-3 tetrasialylated (Neu5Ac), α1-6 fucosylated tetraantennary compound, containing one additional N-acetylglucosamine unit (Table I). The last feature is supported by the H-1 signal of the extra GlcNAc residue (δ 4.699), and the resonances of internal Gal H1 and H-4 (δ 4.455 and δ 4.161, respectively). The different chemical shift values for the internal Gal H-1 in B and C suggest that these compounds are isomers with the extra N-acetylglucosamine unit located on different antennae.

Treatment of C (40 nmol) with endo-β-galactosidase and subsequent fractionation of the resulting mixture on Mono Q (Fig. 2) gave rise to peaks at the elution positions of mono-, di- and trisialo, but not of tetrasialo compounds, proving that the digestion was complete. The disialo fraction (C.Q3) contained only acetate. The fraction containing the trisialo compound (C.Q4) was digested with N-acetyl-β-glucosaminidase, yielding C.Q4\*. The <sup>1</sup>H-NMR spectrum of C.Q4\* shows an unusual set of Man structural-reporter-group signals for an N-linked carbohydrate chain with three antennae (Table I). As compared with the Man structural reporters of B.Q4\*, the Man-4' H-1 and H-2 signals at δ 4.884 and δ 3.96, respectively, have shifted upfield (Δδ -0.021 and Δδ -0.15, respectively). This combination of δ-values deviates from that found for a terminal Man-4' residue (H-1, δ 4.915; H-2, δ 3.96; see below, and compound 19 in [2]). In both cases, the upfield shift of Man-4' H-2 indicates the absence of a GlcNAc residue in β1-2 linkage to Man-4', so that the third antenna in C.Q4\* is linked to C-6 of Man-4'. Together with the disappearance of the GlcNAc-5' H-1 signal at δ 4.602, found for C, this proves that the additional N-acetylglucosamine unit in C was located at C-3 of Gal-6'.



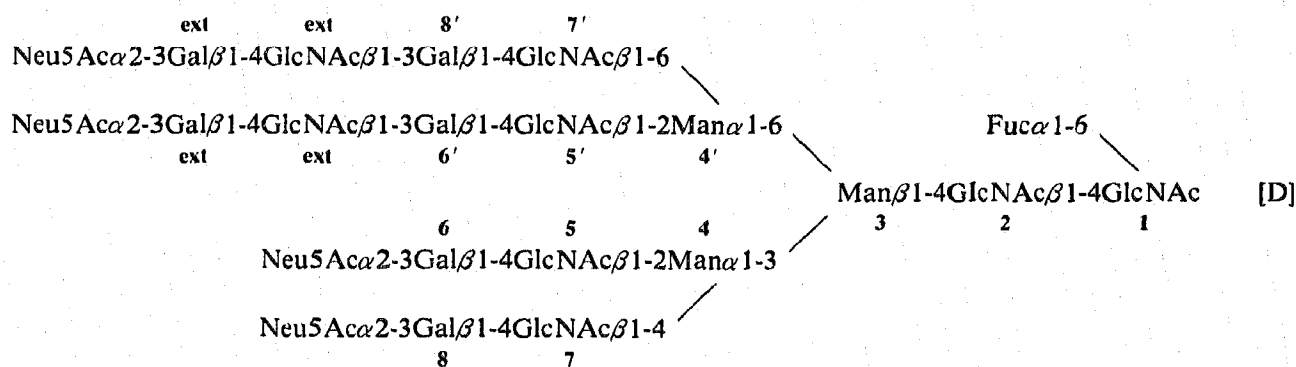
<sup>1</sup>H-NMR analysis of the monosialo Mono Q fraction of the endo-β-galactosidase digest of C (C.Q2) showed

the presence of the tetrasaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal (see B).

### 3.2. Oligosaccharide containing two extra *N*-acetylglucosamine units

The  $^1\text{H-NMR}$  spectrum of D (Fig. 1) demonstrates the presence of an  $\alpha$ 2-3 tetrasialylated (Neu5Ac),  $\alpha$ 1-6 fucosylated tetraantennary compound containing extra *N*-acetylglucosamine units. The signals for GlcNAc H-1 at  $\delta$  4.700 and internal Gal H-4 at  $\delta$  4.161 have doubled intensity compared with these signals in the spectra of B and C. Two extra Gal H-1 signals are found at  $\delta$  4.454 and 4.468, respectively, having intensities comparable to those of the Man-4 and Man-4' anomeric resonances of the tetraantennary structure, proving the presence of two additional *N*-acetylglucosamine units. To assign the position of the two units, the same strategy was followed as described for B and C.

Compound D (40 nmol) was treated with endo- $\beta$ -galactosidase. Then, the mixture was separated on Mono Q (Fig. 2), giving major fractions of mono- and disialo components and a minor fraction of a trisialo



From the  $^1\text{H-NMR}$  spectrum of the monosialo Mono Q fraction of the endo- $\beta$ -galactosidase digest of D (D.Q2) it is clear that it contains the tetrasaccharide Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal (see B).

## 4. DISCUSSION

In this paper we present a novel method to elucidate the primary structure of sialo *N*-linked tetraantennary oligosaccharides with 1 or 2 extra *N*-acetylglucosamine units. Oligosaccharides were treated with endo- $\beta$ -galactosidase from *Bacteroides fragilis*, purified on Mono Q, and digested with *N*-acetyl- $\beta$ -glucosaminidase. In this way the specific breakdown products can readily be identified by high-resolution  $^1\text{H-NMR}$  spectroscopy.

Several reports on the use of bacterial endo- $\beta$ -galactosidases as a tool to obtain structural information of poly-*(N*-acetylglucosamine) elements of *N,O*-glycoprotein glycans and glycolipids have been published [11-23]. In the case of *N*-linked carbohydrate chains, the endo- $\beta$ -galactosidases of *B. fragilis* (this study and [16]), *Escherichia freundii* [17-19], *Flavobacterium keratolyticus* [20], and *Diplococcus pneumoniae* [21]

have shown to be capable of cleaving linear oligo- and poly-*(N*-acetylglucosamine) chains. So far, information concerning the activity of endo- $\beta$ -galactosidases on the glycosidic linkage of 3,6-disubstituted  $\beta$ -Gal residues in *N*-acetylglucosamine sequences in *N*-linked oligosaccharides is somewhat confusing. It has been mentioned that the endo- $\beta$ -galactosidase of *E. freundii* can hydrolyse the  $\beta$ -galactosidic linkage at 3,6-branching points, but a straightforward specificity is not easy to deduce [22,23]. The  $^1\text{H-NMR}$  analysis procedure presented in this study can contribute to a further exploration of the substrate specificity of endo- $\beta$ -galactosidases from different biological sources.

In the investigated oligosaccharides, repeating *N*-acetylglucosamine units are located exclusively in the two antennae attached to Man-4'. In a  $^1\text{H-NMR}$  spectrum of the intact compound the extra *N*-acetylglucosamine unit attached to Gal-8' can be recognized by the characteristic Gal-8' H-1 signal at  $\delta$  4.467. Elongation at Gal-6' is characterized by its H-1 at  $\delta$  4.455. This approach allows the analysis of sialylated tetraantennary oligosaccharides with additional *N*-acetylglucosamine units by  $^1\text{H-NMR}$  spectroscopy, when the repeating units are attached to

Gal-8' and/or Gal-6'. It can be expected that in case of location at the other antennae the same approach can be followed.

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