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Determination of the branch location of extra *N*-acetyllactosamine 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*-acetyllactosamine units in sialo N-linked carbohydrate chains from glycoproteins. Tetraantennary oligosaccharides containing extra *N*-acetyllactosamine units were digested with endo-β-galactosidase, followed by treatment with *N*-acetyl-β-glucosaminidase, yielding products which could be analysed by ^{1}H-NMR spectroscopy, thereby giving conclusive data about the location of the extra units in the intact structures.

Recombinant erythropoietin; Extra *N*-acetyllactosamine unit; ^{1}H-NMR spectroscopy

1. INTRODUCTION

High-resolution ^{1}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*-acetyllactosamine structural elements, hampering the determination of the branch location of *N*-acetyllactosamine repeating units.

In the framework of our program on the structural analysis of oligosaccharides by ^{1}H-NMR spectroscopy, we developed a method for the determination of the branch location of *N*-acetyllactosamine repeating units in tetraantennary sialo-oligosaccharides. The compounds are degraded with endo-β-galactosidase, followed by digestion with *N*-acetyl-β-glucosaminidase, to obtain oligosaccharides that can unambiguously be identified by high-resolution ^{1}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^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* and endo-β-galactosidase from *Bacteroides fragilis* were obtained from Boehringer Mannheim. *N*-Acetyl-β-glucosamidinase from jack beans was purchased from Sigma.

2.2. *500 MHz ^{1}H-NMR spectroscopy*

Prior to ^{1}H-NMR spectroscopy, samples were repeatedly treated with ^2H_2O, finally using 99.96 atom % ^2H_2O (MSD isotopes) at p^{2}H 7 and room temperature. 500 MHz ^{1}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 (δ 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_2 (Chrompack) [4] and further separated by HPAE-PAD chromatography on CarboPac PA-1 (Dionex) [5].

2.4. *Enzyme incubations*

Oligosaccharides (1 nmol/μl) were incubated with endo-β-galactosidase (1 mU/μl) in 50 mM NaAc, pH 5.8, containing bovine serum albumin (0.2 mg/ml), for 48 h at 37°C. The endo-β-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_2O, followed by a gradient from 50–500 mM NaCl in 8 ml H_2O, essentially as described [6]. Desalting of FPLC fractions was performed on Bio-Gel P-2 (Bio-Rad), using water as eluent.

Endo-β-galactosidase digested oligosaccharides (0.67 nmol/μl) were incubated with *N*-acetyl-β-glucosaminidase (2 mU/μl) in 0.2 M Na_2HPO_4/0.05 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_2, and CarboPac PA-1. In the context of this report, focused on a straightforward ^{1}H-NMR analysis

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Fuc, fucose; EPO, erythropoietin; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; HPAE-PAD, high-pH anion-exchange pulsed amperometric detection

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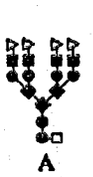
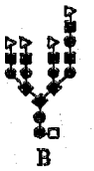
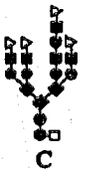
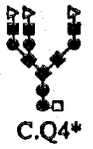
Table 1. *^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of compounds A, B, C and D and enzymatic breakdown products.* Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225) [2]. For numbering of the monosaccharide 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						
		 A	 B	 C	 D	 B.Q4*	 C.Q4*	 D.Q3*
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[a]	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[b]	4.564	4.563	4.561	4.560	4.557	4.559
	GlcNAc-5'	4.593	4.589	4.602[c]	4.60[c]	4.574	-	-
	Gal-6	4.542[d]	4.542[d]	4.542[d]	4.541[d]	4.547	4.542[d]	4.543
	Gal-6'	4.545[d]	4.547[d]	4.455	4.454	4.547	-	-
	GlcNAc-7	4.542[d]	4.542[d]	4.542[d]	4.541[d]	4.547	4.542[d]	4.543
	GlcNAc-7'	4.545[d]	4.547[d]	4.545[d]	4.546[d]	-	4.546[d]	-
	Gal-8	4.542[d]	4.542[d]	4.542[d]	4.541[d]	4.547	4.542[d]	4.543
	Gal-8'	4.559[b]	4.467	4.557	4.468	-	4.566	-
	$GlcNAc_{ext}$	-	4.701	4.699	4.700[e]	-	-	-
	Gal_{ext}	-	4.556	4.557	4.556[e]	-	-	-
H-2	Man-3	4.203	4.206	4.205	4.21[f]	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[g]	3.96[g]
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.[h]	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_{ext}	-	4.117	4.116	4.116[e]	-	-	-
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[i]	1.802[i]	1.803[i]	1.801[i]	1.802[j]	1.802[j]	1.800[e]
H-3e	Neu5Ac	2.756[i]	2.757[i]	2.757[i]	2.757[i]	2.756[j]	2.756[j]	2.756[e]
NAc	GlcNAc-1	2.038	2.037	2.038	2.037	2.040	2.037	2.038
	GlcNAc-2 α[a]	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	-
	$GlcNAc_{ext}$	-	2.037	2.038	2.037[k]	-	-	-
	Neu5Ac	2.031[l]	2.031[l]	2.031[l]	2.031[l]	2.031[m]	2.031[m]	2.031[k]
H-1	Fuc α[a]	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[a]	4.10[g]	4.10[g]	4.10[g]	4.10[g]	n.d.	4.10[g]	4.10[g]
	β	4.13[g]	4.13[g]	4.13[g]	4.13[g]	n.d.	4.13[g]	4.13[g]
CH_3	Fuc α[a]	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

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

of carbohydrate chains with additional *N*-acetyllactosamine 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 α2-3 linkage and having Fuc in α1-6 linkage to GlcNAc-1 (see coding system in structure for A).

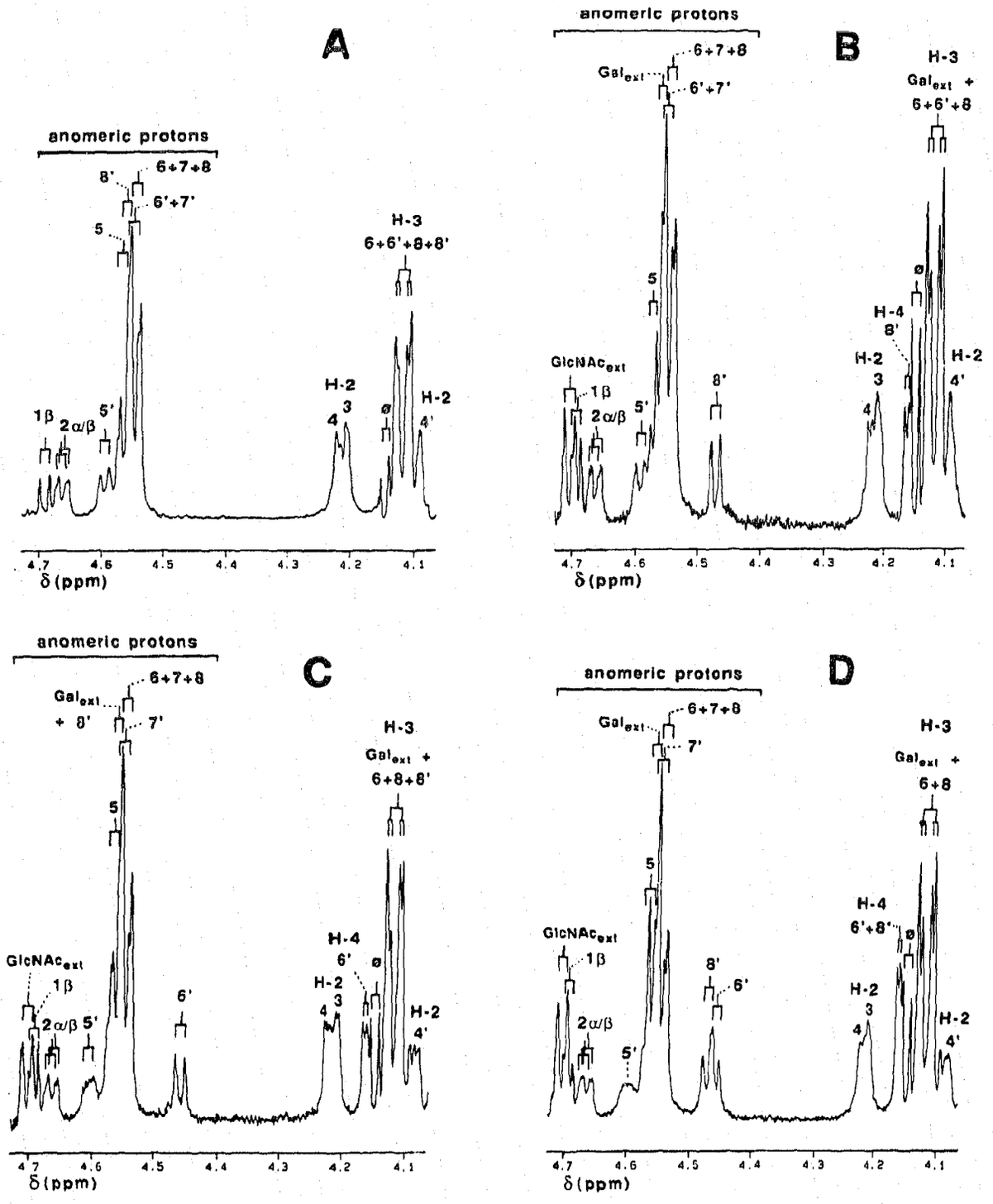
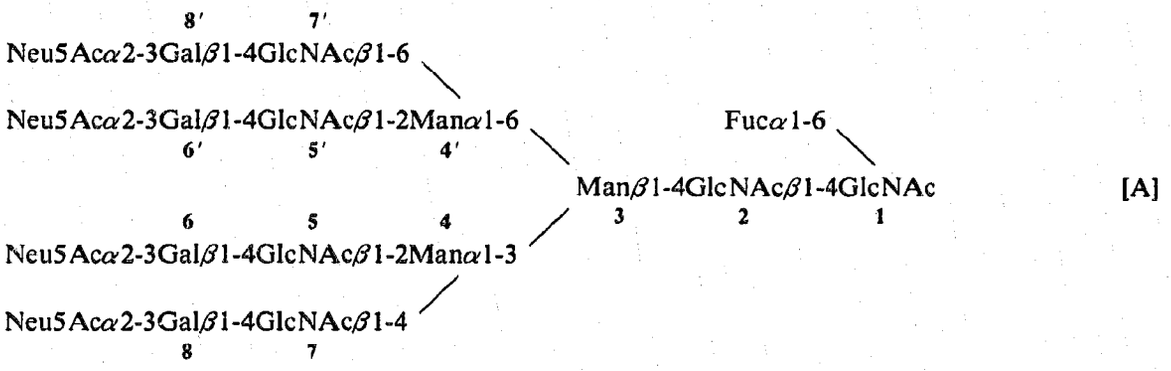


Fig. 1. Relevant sections of the 500 MHz ^{1}H-NMR spectra of compounds A, B, C and D, recorded in ^2H_2O at 300 K. For structures and numbering of the monosaccharide residues, see text. Ø denotes impurity.



3.1. *Oligosaccharides containing one extra N-acetyllactosamine unit*

The ^{1}H-NMR spectrum of B (Fig. 1) shows characteristic structural-reporter-group signals for an α2-3 tetrasialylated (Neu5Ac), α1-6 fucosylated tetraantennary oligosaccharide, and signals from one extra *N*-acetyllactosamine unit (Table I). The last feature is reflected by additional resonances at δ 4.701 and δ 4.467, corresponding to the H-1 signals of the extra GlcNAc and the internal Gal residues, respectively, and by their relative intensities (cf. compound 60 in [2] and 6a$_2$ in [8]). The H-4 resonance of the internal Gal residue is observed at δ 4.159, which is in agreement with the presence of a GlcNAcβ1-3Gal structural element (cf. compound 60 in [2] and 6a$_2$ in [8]). At this stage, no decision with respect to the antenna which contains the additional unit can be made.

Treatment of B (40 nmol) with endo-β-galactosidase and subsequent fractionation of the incubation mixture on Mono Q (Fig. 2) yielded peaks at the elution positions of mono-, di- and trisialo compounds. The compound eluting at the disialo position (B.Q3) was acetate. No tetrasialo material was present, indicating that the digestion was complete. The fraction containing the trisialo compound (B.Q4) was treated with *N*-acetyl-β-glucosaminidase, affording B.Q4*. The ^{1}H-NMR data of B.Q4* (Table I) fit those of the α1-6 fucosylated, α2-3 trisialylated (Neu5Ac) triantennary oligosaccharide from recombinant human follitropin [7] and human chorionic gonadotropin [9], which proves that the extra *N*-acetyllactosamine unit in B was attached to C-3 of Gal-8'.

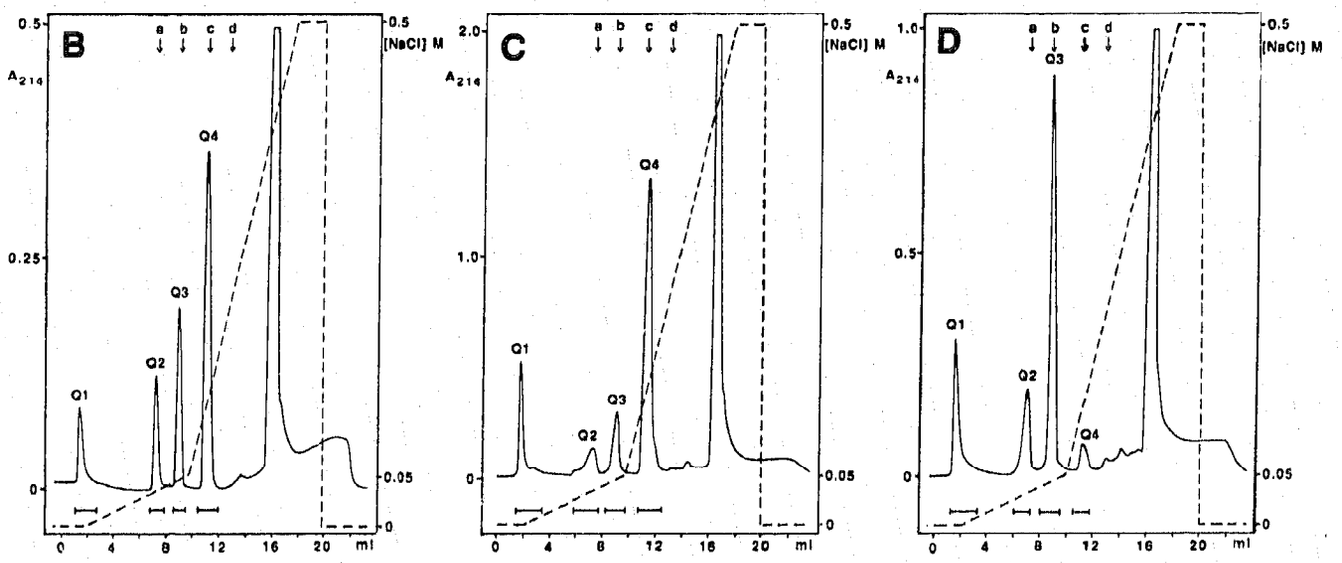
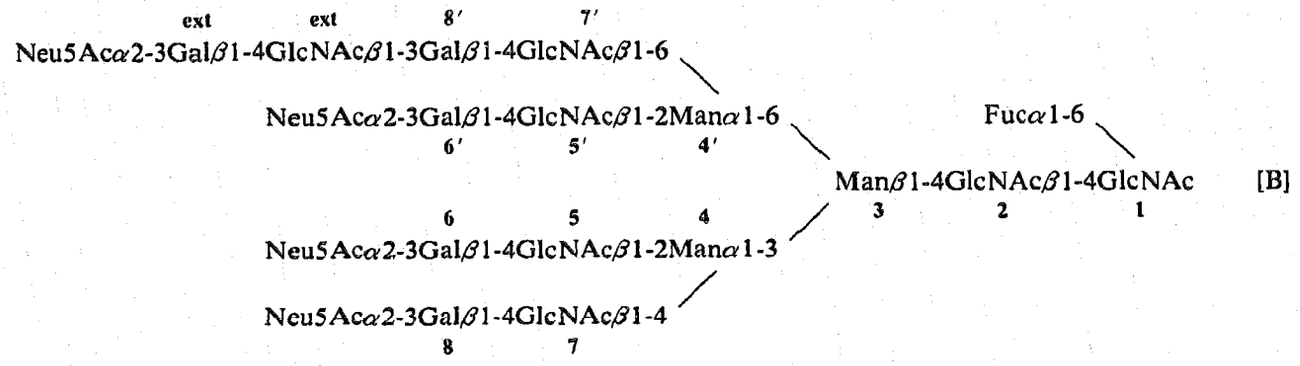


Fig. 2. Fractionation patterns at 214 nm of the endo-β-galactosidase digestion mixtures of compounds B, C and D on a FPLC HR 5/5 Mono Q column. The column was eluted with a gradient of NaCl in H_2O as indicated in the figure. Elution positions of mono (a), di (b), tri (c), and tetra (d) sialo complex oligosaccharides are marked by arrows. Fractions were collected as indicated. Fractions Q1 contain non-carbodydrate material.

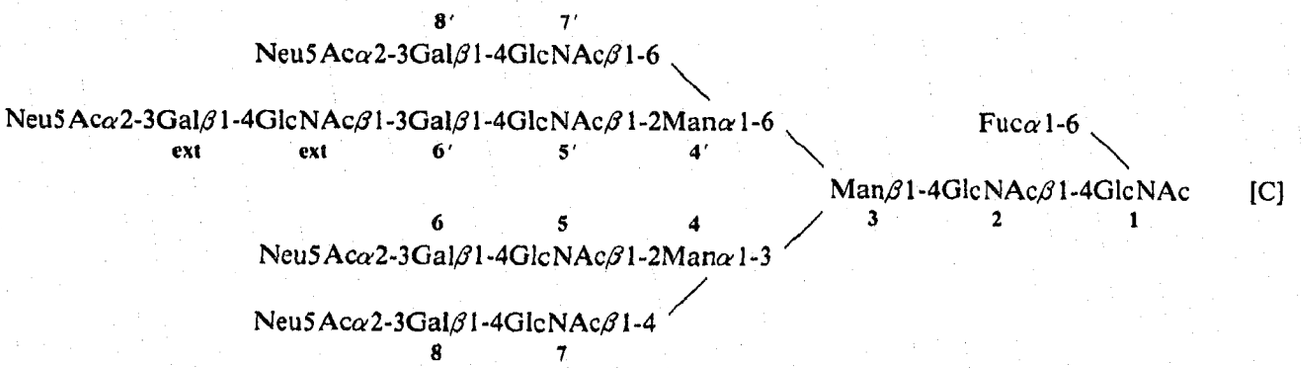


[B]

The Mono Q fraction containing the monosialo compound (B.Q2) was analysed by ^{1}H-NMR spectroscopy, showing the presence of the linear sialylated tetrasaccharide Neu5Acα2-3Gal$_{ext}$β1-4GlcNAcβ1-3Gal$_{red}$ (cf. compounds 60 in [2] and 6a$_2$ in [8]). The H-1 signals of Gal$_{ext}$ and βGal$_{red}$ are observed at δ 4.560 and δ 4.558, respectively, and the H-1 signal of αGal$_{red}$ 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$_{red}$) and δ 4.742 (βGal$_{red}$). The GlcNAcβ1-3Gal linkage is reflected by the presence of the H-4 signal of αGal$_{red}$ at δ 4.204 and of βGal$_{red}$ 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$_{ext}$ H-3 resonates at δ 4.115.

The ^{1}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*-acetyllactosamine 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*-acetyllactosamine 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 ^{1}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*-acetyllactosamine unit in C was located at C-3 of Gal-6′.



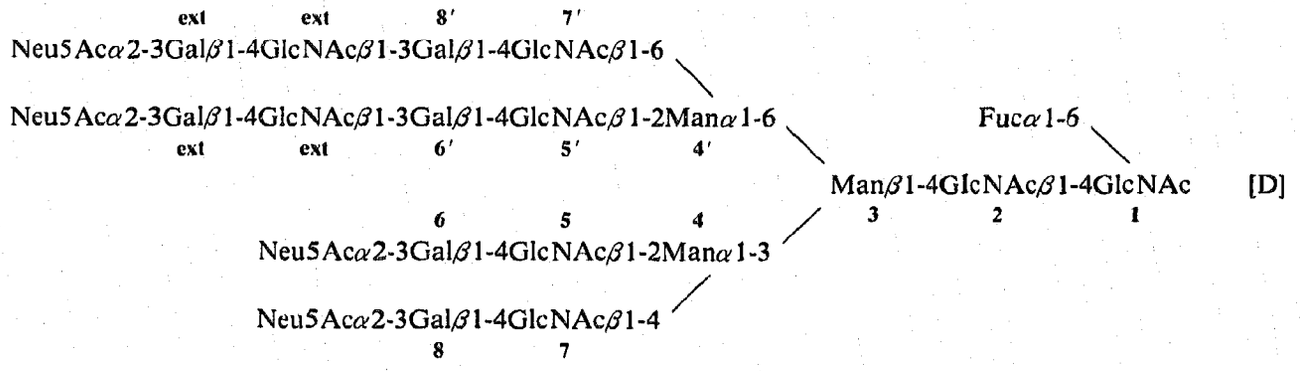
[C]

^{1}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-acetyllactosamine units*

The ^{1}H-NMR spectrum of D (Fig. 1) demonstrates the presence of an α2-3 tetrasialylated (Neu5Ac), α1-6 fucosylated tetraantennary compound containing extra *N*-acetyllactosamine units. The signals for GlcNAc H-1 at δ 4.700 and internal Gal H-4 at δ 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 δ 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*-acetyllactosamine 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-β-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 component. Tetrasialo compounds were absent, indicating that two sialic acid containing antennae had been split off. The minor peak at the trisialo position might originate from a small amount of isomers having both repeating units linked to one antenna. The Mono Q fraction containing the disialo compound and some acetate (D.Q3), was treated with *N*-acetyl-β-glucosaminidase, yielding D.Q3*. Comparison of the NMR data of D.Q3* and D, shows an upfield shift for the Man-4′ H-2 signal (δ 3.96; $\Delta\delta$ −0.12) and a downfield shift for the Man-4′ H-1 signal (δ 4.915; $\Delta\delta$ +0.060). These data reflect the presence of a terminal Man-4′ residue in D.Q3* (cf. compound 19 in [2], proving that the two extra *N*-acetyllactosamine units in D were attached at C-3 of Gal-8′ and Gal-6′, respectively. The Man-4′ H-1 signal in D. Q4* is observed at δ 5.114, a similar position as found for Man-4 H-1 in B.Q4* and C.Q4*.



From the ^{1}H-NMR spectrum of the monosialo Mono Q fraction of the endo-β-galactosidase digest of D (D.Q2) it is clear that it contains the tetrasaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal (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*-acetyllactosamine units. Oligosaccharides were treated with endo-β-galactosidase from *Bacteroides fragilis*, purified on Mono Q, and digested with *N*-acetyl-β-glucosaminidase. In this way the specific breakdown products can readily be identified by high-resolution ^{1}H-NMR spectroscopy.

Several reports on the use of bacterial endo-β-galactosidases as a tool to obtain structural information of poly-(*N*-acetyllactosamine) elements of N,O-glycoprotein glycans and glycolipids have been published [11-23]. In the case of N-linked carbohydrate chains, the endo-β-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*-acetyllactosamine) chains. So far, information concerning the activity of endo-β-galactosidases on the glycosidic linkage of 3,6-disubstituted β-Gal residues in *N*-acetyllactosamine sequences in N-linked oligosaccharides is somewhat confusing. It has been mentioned that the endo-β-galactosidase of *E. freundii* can hydrolyse the β-galactosidic linkage at 3,6-branching points, but a straightforward specificity is not easy to deduce [22,23]. The ^{1}H-NMR analysis procedure presented in this study can contribute to a further exploration of the substrate specificity of endo-β-galactosidases from different biological sources.

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