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Structure determination of the disialylated poly-(*N*-acetyllactosamine)-containing *O*-linked carbohydrate chains of equine chorionic gonadotropin

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The disialylated poly-(*N*-acetyllactosamine)-containing *O*-linked oligosaccharide alditols, released by alkaline borohydride treatment of the enzymically *N*-deglycosylated *β*-subunit of equine chorionic chonadotropin, were purified by fast protein liquid chromatography (FPLC) on Mono Q and analysed by fast ion bombardment mass spectrometry (FAB-MS) and ^{1}H-NMR spectroscopy. The identified oligosaccharide alditols have the following structure:

Neu5Acα2-3[Galβ1-4GlcNAcβ1-3]$_{0-4}$Galβ1-4GlcNAcβ1-6
GalNAc-ol
Neu5Acα2-3Galβ1-3

Keywords: equine chorionic gonadotropin, poly-(*N*-acetyllactosamine), *O*-linked carbohydrate chains

Abbreviations: CG, chorionic gonadotropin; eCG, equine chorionic gonadotropin; FAB-MS, fast atom bombardment mass spectrometry; FID, free induction decay; FSH, follicle stimulating hormone; FPLC, fast protein liquid chromatography; GLC, gas liquid chromatography; Hex, hexose; HexNAc, *N*-acetylhexosamine; LH, luteinizing hormone; NAc, *N*-acetyl; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid; PNGase-F, peptide-N^4-(*N*-acetyl-*β*-glycosaminyl)asparagine amidase F; WEFT, water eliminated Fourier transform.

Introduction

Equine chorionic gonadotropin (eCG) is a heterodimeric glycoprotein hormone, consisting of two non-covalently complexed subunits of molecular mass 17 kDa and 44 kDa, denoted α and β, respectively. The hormone is synthesized and secreted by placental cells in early pregnancy, and is involved in the stimulation of the corpus luteum to produce steroid sex hormones. eCG shows luteinizing hormone (LH) activity in the mare, but also elicits follicle stimulating hormone (FSH) and LH responses in species other than the horse (for a review, see [1]). Among all known glycoprotein hormones, eCG has the highest carbohydrate content, being more than 40% (by weight) [2]. The α-subunit (eCGα) contains two *N*-glycosylation sites (Asn-56 and Asn-82), and the β-subunit (eCGβ) has one *N*-glycosylation site (Asn-13) and at least four, but probably six *O*-glycosylation sites located in the C-terminal region of the protein [3, 4]. The major *O*-linked carbohydrate chains of eCGβ are identical to those of the β-subunit of human CG [5, 6]. However, it has been suggested that in eCGβ also larger *O*-linked carbohydrate chains with (branched) poly-(*N*-acetyllactosamine) peripheral extensions occur [5, 7]. We therefore reinvestigated the *O*-glycans of eCGβ, focusing on the isolation and structural analysis of poly-(*N*-acetyllactosamine)-containing oligosaccharides, and the results are presented in this paper.

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Materials and methods

Materials

Partially purified eCG (2480 IU mg^{-1}, ovarian augmentation test) was supplied by Diosynth BV (Oss, The Netherlands). Peptide-N^4-(*N*-acetyl-*β*-glucosaminyl)asparagine

amidase F (PNGase-F) from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim.

Purification of eCG and preparation of the subunits

Further purification of the partially purified eCG and preparation of the subunits was carried out essentially as previously described [5]. Briefly, the eCG sample (190 mg) was purified by gel-permeation chromatography on Sephacryl S-200 SF, whereas separation of the subunits was achieved by incubation for 1 h at 37 °C in 8 M urea pH 6.0, followed by gel-permeation chromatography on Sephacryl S-200 SF in the same urea buffer, adjusted to pH 7.4. The fractions containing the eCGβ subunit as determined by SDS-PAGE were pooled, then dialysed against water, and finally lyophilized. The presence of eCGβ, in this fraction only, was confirmed by monosaccharide analysis, using *N*-acetylgalactosamine as a guide. In total, 28 mg of purified eCGβ was obtained.

Liberation of the N- *and* O-*linked carbohydrate chains*

The *N*-linked carbohydrate chains were released from eCGβ essentially as previously described [5], but using 1 U of PNGase-F per mg protein, and instead of Nonidet P-40 the non-ionic detergent octylglucoside was added (7%, w/v). The *O*-glycoprotein was separated from the released oligosaccharides and detergents on a Bio-Gel P-100 column (51 × 2.6 cm, 200–400 mesh, Bio-Rad), eluted with 50 mM NH_4HCO_3, pH 7.0, at a flow rate of 10 ml h^{-1} and monitored at 206 nm. The void volume fraction, containing the *N*-deglycosylated eCGβ, was treated with alkaline borohydride as previously described [8] to yield the free *O*-linked carbohydrate chains. After work-up, the β-elimination reaction products were desalted on a Bio-Gel P-2 column (20 × 1 cm, 200–400 mesh, Bio-Rad), and lyophilized.

FPLC fractionation of the O-*linked oligosaccharide alditols*

Fractionation of the chemically released *O*-linked oligosaccharide alditols was carried out on a Mono Q HR 5/5 anion-exchange column as previously described [8], using gradients of NaCl in water as indicated in the Figures. Collected fractions were desalted on a Bio-Gel P-2 column (20 × 1 cm, 200–400 mesh, Bio-Rad), and lyophilized.

Monosaccharide analysis

Monosaccharide analysis of eCGβ (50 µg) was carried out using gas chromatography on a capillary CP-Sil 5 WCOT fused silica column (25 m × 0.32 mm, Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis, *N*-(re)acetylation, and trimethylsilylation as previously reported [9].

Linkage analysis

For linkage analysis, permethylation was carried out according to the method described in [10]. An aliquot of the permethylated oligosaccharide sample was hydrolysed, reduced, and acetylated as previously described [11]. GLC-MS analysis was carried out on a JEOL JMS-AX505W mass spectrometer with a Hewlett Packard 5980 gas chromatograph, fitted with a CP Sil 5CB column (25 m × 0.32 mm, Chrompack). The sample was injected on-column at 90 °C, after 2 min the temperature was increased to 140 °C at 30 °C min^{-1}, and then to 230 °C at 4 °C min^{-1}. The electron ionization mass spectra were recorded at an accelerating voltage of 3 kV.

Fast atom bombardment mass spectrometry

Positive-ion FAB mass spectra of the permethylated oligosaccharide alditols were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at an accelerating voltage of 10 kV (for the lower mass samples) or 6 kV (for the higher mass samples) in a matrix of thioglycerol. The FAB gun was operated at 6 kV with an emission current of 10 mA using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and were recorded and processed on a Hewlett Packard HP9000 series data system using the JEOL Complement software.

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, samples were exchanged twice in 99.9% ^2H_2O. Finally, samples were dissolved in 99.96% ^2H_2O (MSD Isotopes). ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer at a probe temperature of 22 °C, and with a spectral width of 5000 Hz, collecting 512–2500 free induction decays (FIDs) of 8 K complex data points. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) [12]. Suppression of the residual water signal was achieved by applying the WEFT pulse sequence as described [13]. The resolution of the spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomal function.

Results

Monosaccharide analysis of purified eCGβ revealed the presence of galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, mannose and *N*-acetylneuraminic acid in the molar ratio 29:6:21:3:27. The *N*-linked carbohydrate chains do not contain *N*-acetylgalactosamine and in the *O*-linked carbohydrate chains only the peptide-bound *N*-acetylgalactosamine is present ([5]; see also below). Therefore, mannose and *N*-acetylgalactosamine are the indicating factors for *N*- and *O*-glycosylation, respectively, showing that *N*- and *O*-linked carbohydrate chains are present in the molar ratio 1:6.

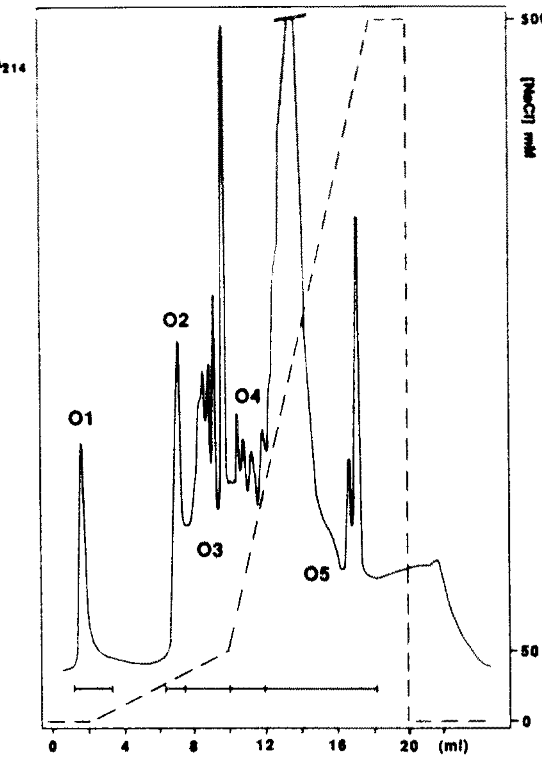

Figure 1. Fractionation pattern at 214 nm of the *β*-elimination products derived from *N*-deglycosylated eCG*β* on a FPLC HR 5/5 Mono Q column. The column was eluted with a linear concentration gradient of 0–50 mM NaCl in 8 ml H_2O, followed by a steeper gradient of 50–500 mM NaCl in 8 ml H_2O, at a flow rate of 1 ml min^{-1}. Fractions were collected as indicated.

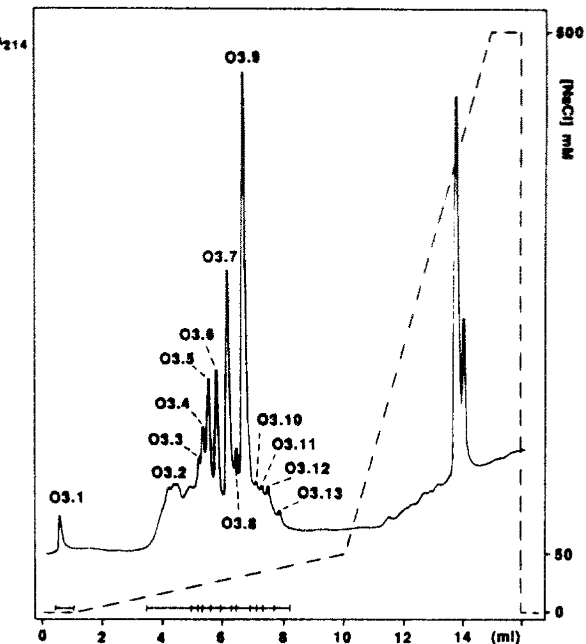

Figure 2. Fractionation pattern at 214 nm of FPLC fraction **O3** on Mono Q. The column was eluted with a linear concentration gradient of 0–50 mM NaCl in 18 ml H_2O, followed by a steeper gradient of 50–500 mM NaCl in 8 ml H_2O at a flow rate of 1 ml min^{-1}. Fractions were collected as indicated.

The *N*,*O*-glycoprotein was subsequently *N*-deglycosylated with PNGase-F and *O*-deglycosylated with alkaline borohydride. The mixture of *O*-linked oligosaccharide alditols was subfractionated on Mono Q (Fig. 1), and five fractions, denoted **O1–O5**, were collected as indicated. The Mono Q pattern closely resembles the previously reported chromatogram of the *O*-linked carbohydrate chains obtained from eCG*β* [5]. In the context of this study, fraction **O3**, which was suggested to contain disialylated poly-(*N*-acetyllactosamine)-containing oligosaccharides, was further investigated. Therefore, fraction **O3** was reapplied to Mono Q, now using a less steep gradient (Fig. 2), and thirteen subfractions, denoted **O3.1–O3.13**, were collected as indicated in the Figure.

The fractions **O3.4–O3.7** and **O3.9** were analysed by ^{1}H-NMR spectroscopy and positive-ion mode FAB-MS. Prior to FAB-MS, the samples were permethylated to increase the sensitivity [14], because of the limited amounts of material available. The ^{1}H-NMR data are compiled in Table 1.

Fraction **O3.9** contains the following oligosaccharide alditol (see [5]).

```
                3,4          4              6
O3.9     Neu5Acα2-3Galβ1-4GlcNAcβ1-6
                                            \
                                             GalNAc-ol
                      3,3       3           /
                  Neu5Acα2-3Galβ1-3
```

Positive-ion mode FAB-MS analysis of permethylated fraction **O3.9** yielded pseudomolecular ions corresponding to a major molecular species with the composition $Neu5Ac_2Hex_2HexNAc_2$-ol: *m*/*z* 1683 (M + H^+); *m*/*z* 1705 (M + Na^+); *m*/*z* 1721 (M + K^+). In addition, a single A^+-type fragment ion [14] was observed at *m*/*z* 825 corresponding to Neu5Ac-Hex-HexNAc$^+$.

The ^{1}H-NMR data of **O3.9** match those of compound 109 in [15]. In view of the extensions of **O3.9**, discussed below, it should be noted that the Galβ1-3(GlcNAcβ1-6)GalNAc-ol core is recognized from the combination of the GalNAc-ol H-2 and H-5 signals. The Neu5Acα2-3Galβ1-3 element is reflected by the Neu5Ac^{3,3} H-3a, H-3e and NAc signals (δ 1.801, δ 2.775 and δ 2.030, respectively) together with the Gal^3 H-1 (δ 4.532), H-3 (δ 4.116) and H-4 (δ 3.925) resonances. The Neu5Acα2-3Galβ1-4 element gives rise to the Neu5Ac^{3,4} signals at δ 1.801 (H-3a), δ 2.756 (H-3e) and δ 2.030 (NAc), and the Gal^4 signals at δ 4.549 (H-1), δ 4.116 (H-3) and δ 3.956 (H-4). It is clear that Neu5Ac^{3,3} and Neu5Ac^{3,4} can be distinguished from each other on the basis of their typical H-3e chemical shift values.

Fraction **O3.7** contains an oligosaccharide alditol which is an extension of compund **O3.9** with one *N*-acetyllactos-

Table 1. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of poly-(*N*-acetyllactosamine)-containing *O*-linked oligosaccharide alditols derived from the *β*-subunit of equine chorionic gonadotropin. Chemical shifts are given at 22 °C and were measured in ^2H_2O relative to internal acetone (*δ* 2.225 [12]). Compounds are represented by short-hand symbolic notation: ◇^{ol}, GalNAc-ol; ■, Gal; ●, GlcNAc; △, Neu5Acα2-3 [15]. For indexing of the monosaccharide residues, see text.

Residue	*Reporter Group*	*Chemical shift (ppm) in*				
		O3.9	**O3.7**	**O3.6**	**O3.5**	**O3.4**
GalNAc-ol	H-2	4.392	4.390	4.390	4.390	4.391
	H-3	4.069	4.068	4.069	4.069	4.069
	H-4	3.436	3.434	3.433	3.432	3.432
	H-5	4.269	4.271	4.271	4.271	4.271
	NAc	2.065	2.065	2.063	2.063	2.064
Gal^3	H-1	4.532	4.532	4.532	4.532	4.532
	H-3	4.116	4.116	4.116	4.116	4.116
	H-4	3.925	3.926	3.927	3.927	ND[a]
GlcNAc^6	H-1	4.549	4.552	4.551	4.553	4.552
	H-6	4.006	3.985	3.985	3.988	ND
	NAc	2.063	2.063	2.063	2.063	2.062
Gal^4	H-1	4.549	4.454	4.453	4.453	4.454
	H-3	4.116	ND[a]	ND	ND	ND
	H-4	3.956	4.155	4.155	4.153	4.154
GlcNAc^i	H-1	—	—	4.693	4.693[b]	4.694[c]
	NAc	—	—	2.030	2.030[d]	2.030[e]
Gal^i	H-1	—	—	4.464	4.464[b]	4.464[c]
	H-4	—	—	4.155	4.153[b]	4.154[c]
GlcNAc^t	H-1	—	4.692	4.693	4.693	4.694
	NAc	—	2.031	2.030	2.030	2.030
Gal^t	H-1	—	4.557	4.557	4.558	4.558
	H-3	—	4.116	4.116	4.116	4.116
	H-4	—	3.955	3.955	3.955	ND
Neu5Ac^{3,3}	H-3a	1.801	1.801	1.800	1.801	1.799
	H-3e	2.775	2.773	2.773	2.773	2.773
	NAc	2.030	2.031	2.030	2.030	2.030
Neu5Ac^{3,4}	H-3a	1.801	1.801	1.800	1.801	1.799
	H-3e	2.756	2.757	2.757	2.757	2.757
	NAc	2.030	2.031	2.030	2.030	2.030

[a] ND, not determined, [b] signal stemming from two protons; [c] signal stemming from three protons; [d] signal stemming from two NAc groups; [e] signal stemming from three NAc groups.

amine unit.

O3.7 Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Neu5Acα2-3Galβ1-3)GalNAc-ol (residue indices: Neu5Ac^{3,4}, Gal^t, GlcNAc^t, Gal^4, GlcNAc^6; Neu5Ac^{3,3}, Gal^3)

The positive-ion mode FAB mass spectrum of permethylated fraction **O3.7** contains an intense M + H$^+$ pseudomolecular ion at *m/z* 2132 corresponding to $Neu5Ac_2Hex_3$-$HexNAc_3$-ol. Fragment ions are observed at *m/z* 825 (for the A$^+$-type ion Neu5Ac-Hex-HexNAc$^+$) and *m/z* 1274 (for the A$^+$-type ion Neu5Ac-Hex-HexNAc-Hex-HexNAc$^+$), indicating that the two *N*-acetyllactosamine units are linearly arranged. An additional informative fragment ion is observed at *m/z* 793, generated by *β*-elimination of

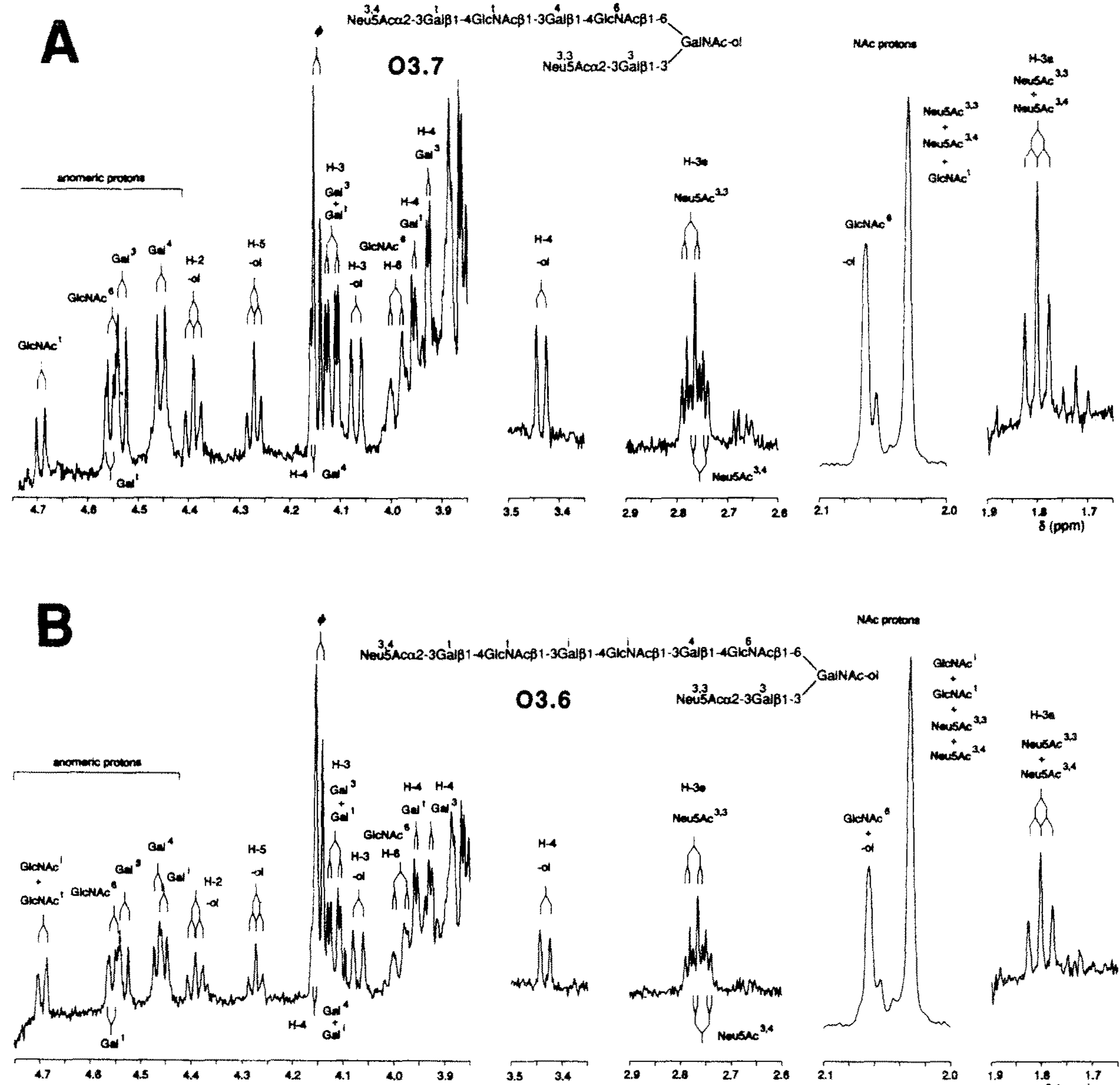

Figure 3. Structural-reporter-group regions of the resolution-enhanced 500 MHz ^{1}H-NMR spectra at 295 K of the fractions **O3.7** (A) and **O3.6** (B). ϕ indicates non-carbohydrate impurity.

methanol from the A^+-type ion at *m*/z 825, which indicates that C-3 of the HexNAc residue is unsubstituted [14].

In the ^{1}H-NMR spectrum of fraction **O3.7** (Fig. 3A), the presence of the additional Galβ1-4GlcNAcβ1-3 unit, compared with **O3.9**, is demonstrated by the set of Gal^4 H-1 and H-4 signals at δ 4.454 and δ 4.155, respectively, which is generally observed for a galactose residue to which an *N*-acetyllactosamine unit is linked [16, 17], and by the GlcNAc^t H-1 (δ 4.692) and Gal^t H-1 (δ 4.557) resonances. The last signal is typical for an α2-3-sialylated galactose residue in a poly-(*N*-acetyllactosamine) sequence [17]. The location of the extra *N*-acetyllactosamine unit in the upper branch is deduced from the Gal^4 H-4 signal (δ 4.155) which has a chemical shift value different from that of the Gal^3 H-4 resonance in case the *N*-acetyllactosamine repeat is attached to Gal^3 (δ 4.125; cf. compound N7 in [18]). Furthermore, the typical H-3e signal for a Neu5Acα2-3Galβ1-3 element is present.

Fraction **O3.6** contains the following oligosaccharide alditol in which the upper branch is elongated by the addition of one Galβ1-4GlcNAc repeat compared with **O3.7**.

```
3,4            t           t          i
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-
                 i          4          6
           4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6
                                       \
                                        GalNAc-ol
                       3,3          3  /
O3.6                   Neu5Acα2-3Galβ1-3
```

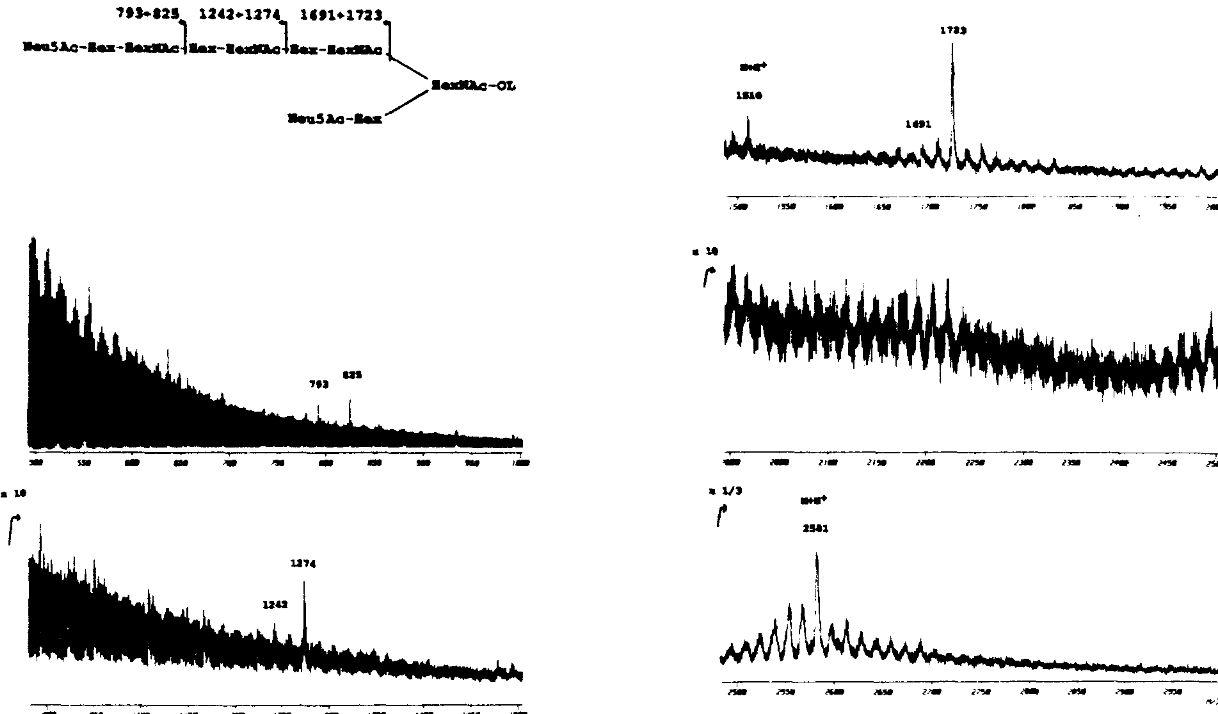

Figure 4. Positive-ion fast atom bombardment mass spectrum and fragmentation scheme of permethylated fraction **O3.6**. *m/z* values are quoted as nominal masses. The M + H^+ pseudomolecular ion observed at *m/z* 1510 arises from a minor species with the composition $Neu5Ac_1Hex_3HexNAc_2$.

The positive-ion FAB mass spectrum obtained from permethylated fraction **O3.6** is shown in Fig. 4. An intense M + H^+ pseudomolecular ion is observed at *m/z* 2581, corresponding to a $Neu5Ac_2Hex_4HexNAc_4$-ol species. A series of A^+-type fragment ions is observed at *m/z* 825 (Neu5Ac-Hex-$HexNAc^+$), *m/z* 1274 (Neu5Ac-Hex-HexNAc-Hex-$HexNAc^+$), and *m/z* 1723 (Neu5Ac-Hex-HexNAc-Hex-HexNAc-Hex-$HexNAc^+$), which indicates that the repeats form a linear sequence. The ions observed at *m/z* 793, 1242 and 1691 are formed from these A^+-type ions by β-elimination of the -O-CH_3 substituent present on C-3 of the charge-bearing HexNAc residue, demonstrating that the Hexl-3HexNAc element is absent from the elongated branch [14]. Linkage analysis, carried out on the permethylated fraction **O3.6**, shows the presence of 3-substituted hexose and 4-substituted HexNAc, which is consistent with the conclusions drawn.

The ^{1}H-NMR spectrum of fraction **O3.6** (Fig. 3B) shows, compared with that of fraction **O3.7**, additional signals at δ 4.464 (Gal^i H-1), δ 4.155 (Gal^i H-4) and δ 4.693 ($GlcNAc^i$ H-1). It should be noted that Gal^i H-1 has a chemical shift value different from that of Gal^4 H-1. The other structural-reporter-group signals remain unchanged.

Fraction **O3.5** indicates that it contains the following further extended oligosaccharide alditol.

O3.5

$Neu5Ac^{3,4}$α2-$3Gal^{t}$β1-$4GlcNAc^{t}$β1-3[Gal^{i}β1-
$4GlcNAc^{i}$β1-3]$_2$$Gal^{4}$β1-$4GlcNAc^{6}$β1-6
GalNAc-ol
$Neu5Ac^{3,3}$α2-$3Gal^{3}$β1-3

Positive ion FAB-MS analysis of permethylated fraction **O3.5** produced a spectrum containing an M + H^+ pseudomolecular ion at *m/z* 3030, corresponding to $Neu5Ac_2Hex_5$-$HexNAc_5$-ol. Because of the limited amount of sample available, no fragment ions were observed.

The ^{1}H-NMR data for fraction **O3.5** are similar to those of **O3.6**, but the additional *N*-acetyllactosamine unit gives rise to an increased intensity of the Gal^i H-1 and H-4, and the $GlcNAc^i$ H-1 and NAc signals.

Likewise, the ^{1}H-NMR data for fraction **O3.4** are comparable to those for fraction **O3.5**, but again the Gal^i and $GlcNAc^i$ signals have increased intensity. Together with the chromatographic behaviour of fraction **O3.4** (see discussion), this indicates the presence of the following oligosaccharide alditol.

```
3,4        t            t          i
Neu5Acα2-3Galβ1-4GlcNAcβ1-3[Galβ1-
         i          4          6
     4GlcNAcβ1-3]3Galβ1-4GlcNAcβ1-6
                                    \
                                     GalNAc-ol
                    3,3       3     /
O3.4          Neu5Acα2-3Galβ1-3
```

Discussion

In earlier investigations, it has been suggested that a portion of the *O*-linked carbohydrate chains of eCGβ should contain peripheral poly-(*N*-acetyllactosamine) extensions [5, 7]. In the present study, five disialylated *O*-glycans were identified, forming a series having zero to four repeating *N*-acetyllactosamine units linearly arranged in the branch attached to the *N*-acetylglucosamine residue of a Galβ1-3(GlcNAcβ1-6)GalNAc (type 2) core structure. The smallest oligosaccharide alditol (compound **O3.9**) was also identified in a previous study [5]. The other carbohydrate chains found in the present study represent minor *O*-glycans of eCGβ. The finding that the repeating *N*-acetyllactosamine units are exclusively located in the branch attached to C-6 of *N*-acetylgalactosamine is in agreement with the general observation that in type 2 core structures this specific branch is favoured for elongation with Galβ1-4GlcNAc elements [19, 20].

When applying alkaline borohydride treatment to release *O*-linked carbohydrate chains, *N*-acetyl-*O*-acyl-neuraminic acids are recovered as *N*-acetylneuraminic acid. It has been shown that a part of the sialic acid residues in the *N*-linked carbohydrate chains of eCGβ is Neu4,5Ac_2 [5]. Therefore, a portion of the sialic acids in the *O*-linked oligosaccharide alditols described in this study was probably present as Neu4,5Ac_2 in the native glycoprotein.

Fraction **O4** (see Fig. 1) elutes on Mono Q in the region for trisialylated carbohydrate chains. The ^{1}H-NMR spectrum of fraction **O4** (not shown) shows the presence of oligosaccharide alditols containing the same type 2 core structure as the disialylated carbohydrate chains, poly-(*N*-acetyllactosamine) extensions, and *N*-acetylneuraminic acid in α(2-3)-linkage to galactose only. Therefore, the presence of three sialic acids per molecule suggests that fraction **O4** contains sialylated branched poly-(*N*-acetyllactosamine) chains. Branching points in poly-(*N*-acetyllactosamine) chains, formed when a galactose residue is substituted at both C-3 and C-6 with *N*-acetylglucosamine, have previously been suggested to occur in the carbohydrate chains of eCG [7, 21]. Because of the low amount of material in combination with the complexity of the mixture, no further analysis of fraction **O4** could be carried out.

Finally, poly-(*N*-acetyllactosamine)-containing oligosaccharide alditols were separated by FPLC on Mono Q, according to their number of repeats. The introduction of a *N*-acetyllactosamine unit results systematically in a decreased retention time. This effect indicates that not only the molecular charge, but also the molecular mass is involved in the Mono Q separation of carbohydrate chains.

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