One- and two-dimensional 90.5-MHz ^{13}C-NMR spectroscopy of the *N*-linked triantennary oligosaccharide units of calf fetuin

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Complete assignments of all anomeric resonances in the proton and carbon spectra of the *N*-linked oligosaccharide units of fetuin were made using one- and two-dimensional NMR spectroscopy. We are able to confirm the presence of microheterogeneity in the *N*-acetylneuraminic acid linkages to the galactose residues and the presence of a unique triantennary structure which carries a side chain: NeuAcα(1–3)Galβ(1–3)GlcNacβ(1–4)-. Anomeric carbon chemical shifts changes resulting from long-range conformational effects were observed.

Triantennary oligosaccharide *two-dimensional NMR spectroscopy* *^{13}C-NMR* *Fetuin* *Structural microheterogeneity* *(Calf)*

1. INTRODUCTION

Calf fetuin is a glycoprotein found in fetal calf serum [1], with three of its six carbohydrate side chains known to be *N*-linked to an asparagine residue. Two different isomeric side chain structures were proposed for these oligosaccharides [2,3]; but recently it was shown that only one of the proposed triantennary structures is correct [4–6]. Moreover, evidence has been accumulated for the presence of β(1–3) galactose residue directly linked to *N*-acetylglucosamine [5–7], as well as a ratio of ~1:1 between the NeuAcα(2–3) and NeuAcα(2–6) residues [5].

A study of the isolated fetuin triantennary glycopeptides using high magnetic field ^{13}C-NMR and 2D-NMR spectroscopy is reported. The results provide an additional proof for the presence of Galβ(1–3)GlcNAcβ(1–4) antenna in calf fetuin and correlates between the ^{13}C-NMR and ^{1}H-NMR chemical shifts of the anomeric resonances through heteronuclear shift-correlated NMR spectroscopy [8].

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Abbreviations: 2D, two-dimensional; Man, D-mannopyranose; Gal, D-galactopyranose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid

2. MATERIALS AND METHODS

An *N*-linked glycopeptide fraction of calf fetuin and its neuraminidase-treated glycopeptide fraction were isolated and purified from fetuin as described [2,3,5].

The 90.5-MHz ^{13}C-NMR (360 MHz for ^{1}H-NMR) spectra were recorded on a Bruker AM-360 spectrometer at ambient probe temperature. The fetuin glycopeptide sample was prepared as a 70 mM solution in D_2O at neutral pH (10 mm NMR tube). The asialo sample, from which the sialic acid residues were removed [5], was prepared in a similar manner (50 mM solution) in a 5 mm NMR tube. The heteronuclear shift-correlated 2D-

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NMR spectrum was obtained by setting the spectral windows to cover the regions of the anomeric protons (5.3–4.1 ppm) and anomeric carbons (109–95.5 ppm). The standard pulse sequence, as supplied by Bruker, was used with decoupling in the carbon dimension and setting the two delays just before and after the observed pulse to 0.003 s. The ^{13}C and ^{1}H chemical shift values were obtained from the cross sections of the 2D-NMR spectrum and are listed in table 1.

3. RESULTS AND DISCUSSION

A contour plot of the heteronuclear shift-correlated 2D-NMR spectrum for the anomeric carbons and protons is shown in fig.1, with the carbon chemical shifts along the horizontal axis and the corresponding proton chemical shifts along the vertical axis. The resonance corresponding to the anomeric carbon of residue 1 is outside the chemical shift range of the displayed spectrum. The signals of the non-protonated anomeric carbons of the sialic acid residues are also missing from the spectrum. This has reduced signal overlap in the spectrum and at the same time the induced substituent chemical shift effects on other anomeric carbons, due to the sialic acid substitution, could still be observed.

Using the anomeric carbon resonance assignments reported in [5], the assignments of the corresponding anomeric proton resonances were made through the 2D-NMR chemical shift connectivities. Excellent correspondence was found between these assignments, as obtained from the 2D-NMR, and those reported for similar triantennary structures [9–11]. The cross-peaks corresponding to the galactose residues (see 6, 6′, 8 and 8* in fig.1) are relatively strong due to both favourable

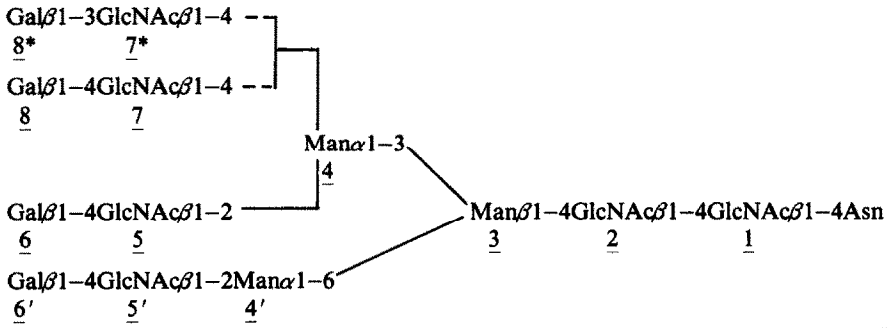
Table 1

^{13}C and ^{1}H chemical shifts (ppm from Me_4Si) of the *N*-linked glycopeptide fraction from calf fetuin, as obtained by 2D shift-correlated NMR spectroscopy[a]

Residue	2	3	4	4′	5	5′	7	6,6′	8
H1	4.62	4.76	5.13	4.90	4.59	4.60	4.56	4.43 4.44	4.53
C1	102.57	101.66	100.32	98.48 98.35	100.96	100.78	102.77	104.76 104.12 104.01	104.82[b]

[a] The sugar residue numbers are given below. The carbon chemical shifts for NeuAc are given elsewhere [5]

[b] Chemical shift of C1 of Gal-8*



Structure of the fetuin glycopeptide (the sialic acid residues linked to the galactose residues are not shown). The dashed lines represent alternate structures for the triantennary structure

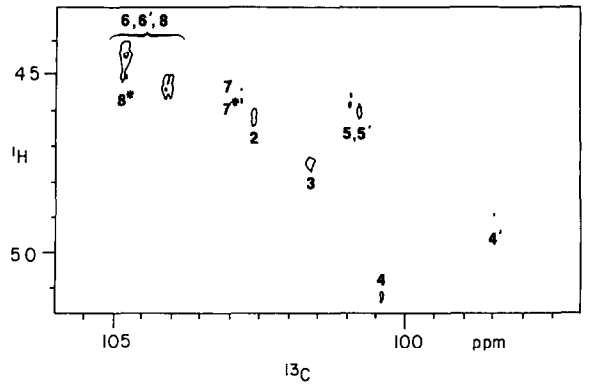


Fig.1. 2D chemical shift-correlated NMR contour plot (5.3–4.1 ppm for proton and 109–95.5 ppm for carbon) of the *N*-linked glycopeptides derived from calf fetuin. Residue numbering is given in the scheme shown at the bottom of table 1.

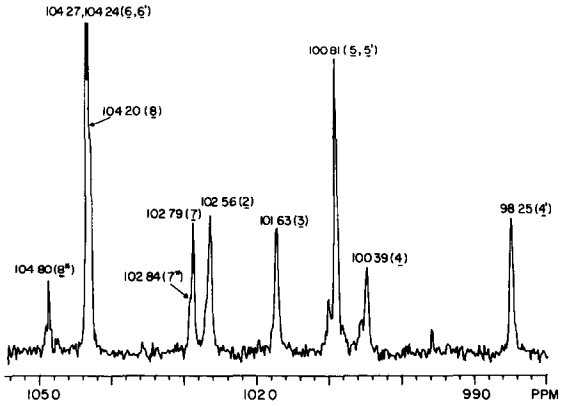


Fig.2. 90.5-MHz ^{13}C-NMR spectrum, showing the anomeric region of the asialylated *N*-linked glycopeptides derived from calf fetuin. Residue numbering corresponds to the scheme shown at the bottom of table 1.

relaxation rates and overlap of closely spaced resonances arising from the heterogeneity in the substitution by the sialic acid residues [5]. No carbon-proton connectivity, other than the connectivity for one of the α-mannose residues [9–11], was observed in the anomeric proton chemical shift region (>5.1 ppm) which correspond to α-galactose residue [12,13]. Thus it was concluded that all the galactose residues must have a β-anomeric configuration. In addition, two proton connectivities were observed for the anomeric carbon resonance of the β(1–4)-linked *N*-acetylglucosamine residue. Although it may arise from some long-range substituent chemical shift effects, it is more likely to be a result of different galactose substitutions [i.e. β(1–4) vs β(1–3)]. The problem was resolved when the ^{13}C-NMR spectrum of the asialofetuin glycopeptide was examined, where any long-range effects due to different sialic acid substitutions were removed from the spectrum (fig.2).

The 90.5-MHz ^{13}C-NMR spectrum of the anomeric carbons region of the fetuin triantennary units from which the α-linked sialic acid was removed enzymatically [5] is shown in fig.2. The assignments of the anomeric carbon resonances are based on an extensive chemical shift comparisons with model compounds and the analysis of the ^{13}C-NMR spectra of the corresponding sialyl and agalactosyl derivatives [5]. Unlike the lower magnetic field study [5], the improved spectral resolution enabled the observation of four anomeric carbon resonances which are associated with β-galactose residues. The smaller and most downfield resonance, at 104.82 ppm, was assigned previously to a Galβ(1–3) residue, (Gal-8*) [5]. The other, closely spaced, resonances at 104.30, 104.27 and a shoulder peak at 104.23 ppm, were assigned to residues 6, 6′ and 8 (see scheme in table 1). Integration of the carbon resonances appearing in the anomeric region, excluding the GlcNAc-1 residue, has indicated the presence of 10 sugar residues, as expected.

Two of the three resonances corresponding to Gal-6, -6′ and -8, are of equal intensity while the intensity of the third resonance is reduced by an amount approximately equal to the signal intensity of the peak assigned to Gal-8*. Therefore, one unique antenna in the triantennary structure must be associated with the β(1–3)-linked galactose. The carbon resonance of GlcNAc-7 is composed of two components, one at 102.84 (~1/3 carbon) and the other at 102.75 ppm (~2/3 carbon), so that it must be the GlcNAc-7 residue which is substituted by Galβ(1–3) as previously proposed [5]. Therefore, on average, one out of the three *N*-linked triantennary structures in calf fetuin carries this type of side chain.

4. CONCLUSIONS

The heteronuclear shift-correlated experiment confirmed the ^{13}C-NMR anomeric chemical shift assignments for the proposed triantennary structure of calf fetuin [5] via the correlation to the well-established proton chemical shift assignments [9–13]. This is the first report which directly correlates between these chemical shifts for the anomeric part of a triantennary oligosaccharide structure. It was used to establish the presence of a Galβ(1–3) residue in the *N*-linked side chain of calf fetuin.

Some long-range chemical shift effects were observed in the ^{13}C-NMR spectrum (fig.2), which are most likely conformational in origin. The two carbon peaks corresponding to GlcNAc-5 and -5′ in the sialylated glycopeptide (not shown) yielded a sharp resonance, 100.81 ppm, following the removal of the sialic residues (fig.2). In general, most of the anomeric resonances corresponding to the inner core *N*-acetylglucosamine and mannose residues undergo measurable chemical shift changes upon desialylation as observed for smaller model oligosaccharides [14,15].

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