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A full assignment of proton resonances for an α(1-3)-linked fucose residue in keratan sulphate from bovine articular cartilage

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Full proton NMR assignments have been achieved for the α(1-3)-linked fucose residues contained in alkaline borohydride reduced keratan sulphate chains derived from bovine articular cartilage. This involved 500 MHz spectroscopy at 60°C and included COSY and RELAYED-COSY determinations.

Abbreviations: KS, keratan sulphate; TSP, sodium 3-trimethylsilylpropionate; Fuc, α-L-fucose; Gal, β-D-galactose; GalNAc-ol, *N*-acetylgalactosaminitol; GlcNAc, β-*N*-acetyl-D-glucosamine

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Many examples are now known of glycoconjugate-derived oligo- and polysaccharides which contain α-linked L-fucose residues attached to positions 2, 3 or 4 in a neighbouring sugar. The keratan sulphate system was first recognized as a carbohydrate polymer when derived [1] from bovine cornea in 1953; it has subsequently been found in many tissues including human cartilage [2] and, as early as 1955, in intervertebral disc nucleus pulposus [3], analysis [4] of which showed the presence of significant levels of fucose. Later, Seno *et al.* [5] demonstrated that several types of keratan sulphate contained both sialic acid and "methylpentose" (i.e. fucose) residues as minor components.

Keratan sulphates have structures whose details are even now poorly understood. They are, however, known [6] to be based upon a repeating (1-3)-β-D-galactose-(1-4)-β-D-*N*-acetylglucosamine (or *N*-acetyllactosamine) sequence where there is generally sulphation at C-6 of the hexosamine together with further ester sulphates at some galactose C-6 positions.

It is well known that sequences containing fucosylated -Gal-GlcNAc- units are of considerable significance in conferring antigenic properties. For example, Le^a activity has been associated [7] with the β(1-3) linkage joining the β-Gal(1-3)[Fucα(1-4)]β-GlcNAc sequence to the next residue, and the stage-specific embryonic antigen (SSEA-1) involves the substituted *N*-acetyllactosamine Galβ(1-4)[Fucα(1-3)]-GlcNAc moiety [8]. The monoclonal antibody anti-SSEA-1 can discriminate [9] between such fucosylated type 2 blood group chains possessing a Galβ(1-4)GlcNAc linkage and the alternate Le^a type 1 blood group chains with Galβ-(1-3)GlcNAc sequences.

Characterization of these fucosylated sugar sequences has been performed using various approaches. These may involve chemical and enzymatic degradations combined with methylation analysis as used in the study of a sialylated and fucosylated lactosamine sequence from rat brain glycoproteins [10] or a more sophisticated methylation and fast-atom-bombardment mass spectrometry approach used, for example, in the examination [11] of sialylated and fucosylated oligosaccharides from mucus glycoproteins of human seminal plasma, where anomeric configurations were then derived via exoglycosidase digestion and chromium trioxide oxidation.

Undoubtedly, the most powerful and direct approach to the identification and characterization of fucose residues in oligo- and polysaccharides derives from the use of high-field proton NMR spectroscopy. Extensive work concerning the presence of fucose linked glycosidically to C-2 and C-3 sites in an adjacent sugar has been presented by Vliegenthart and collaborators. Examples include neutral [12, 13] and sialylated [14, 15] oligosaccharides derived from the bronchial mucus glycoproteins of cystic fibrosis patients, and neutral oligosaccharides derived from the tracheobronchial mucus of a patient with bronchiectasis due to Kartageners'

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syndrome [16, 17]. The characterization of antigens containing the α(1-3)-fucosylated *N*-acetyllactosamine sequence has been reviewed by Hounsell [18] who also considered systems containing other fucosyl linkages including the α(1-4) attachment observed in some milk oligosaccharides. These latter data have been described in further detail [19]. A series of neutral and monosialylated oligosaccharides *O*-glycosidically linked to the human secretory immunoglobulin A hinge region has recently been described [20] in which α(1-2)-, α(1-3)- and α(1-4)-linked fucoses were all represented.

In each of these studies reliance has been placed on the use of "reporter groups"; that is, signals which from their form and position may clearly be recognized on a one-dimensional proton NMR spectrum when this is determined at a sufficiently high field strength. Thus, in fucose there are five ring protons, one of which is at the anomeric site, H-1, together with a methyl residue (H-6 protons). However, determinations of the nature of the linkage attachment have relied on the observation of positions for these latter two resonances, together with partial data from H-5, the multiplet from which may often be obscured, either by other ring proton resonances or, in some instances, when determinations are made at ambient temperature, through the presence of a residual HO^2H response from the solvent.

The observed shift value ranges [12–20] may be summarized as follows (all have been changed to a common reference scale, relative to internal TSP-d_4 at 0.0 ppm for convenience). For fucose α(1-2)-linked: H-1, 5.163–5.327 ppm; H-5, 4.235–4.357 ppm; CH_3, 1.243–1.292 ppm; α(1-3)-linked: H-1, 5.101–5.156 ppm; H-5, 4.817–4.890 ppm; CH_3, 1.163–1.254 ppm (mostly <1.20 ppm); α(1-4)-linked: H-1, 5.038–5.047 ppm; H-5, 4.876–4.890 ppm; CH_3, 1.194–1.274 ppm. Within these ranges, which are sufficiently discrete to permit assignment of linkage type, could be observed predictable shift perturbations related to the presence of specific neighbouring sugar residues. One example [19] which showed a dramatic variation from the normal range was the reduced trisaccharide

Gal(1-4)[Fucα(1-3)]GalNAc-ol

where the totally different character of the open chain structure perturbed the shifts to 5.081 (H-1), 4.317 (H-5) and 1.227 (CH_3) ppm, respectively. Variations in shift positions due to the presence of ester sulphate groups in neighbouring residues introduces a factor which is as yet unexplored.

As part of extensive studies on the structure and the immunological characteristics of keratan sulphate chains, recent work has involved the isolation, sub-fractionation and characterization of KS from a variety of tissue sources including mature bovine articular cartilage [21] where significant fucose levels were observed via ^{13}C-NMR spectroscopy. In the examination by ^{1}H-NMR of discrete Mono-Q ion-exchange chromatography-derived fractions, partial analysis [22] of a COSY-45 spectrum suggested, by comparison with the known fucose chemical shift ranges (as discussed above), that this residue was attached to the main KS chain via an α(1-3) linkage.

KS chains have been classified into two groups, namely KS-I (*N*-linked to peptide in the proteoglycan as in corneal KS) and KS-II (*O*-linked as in skeletal KS) [23]. High-field ^{1}H-NMR studies supported by chemical carbohydrate analyses have shown [24] that skeletal keratan sulphates, KS-II (of bovine origin), may themselves be further subclassified into two distinct groups. Those from articular and from intervertebral disc cartilage (KS-II-A) contain two structural features, namely α(1-3)-linked fucose and also α(2-6)-linked sialic acid residues, that are absent from KS chains derived from tracheal or nasal septum cartilage.

Detailed carbohydrate analyses for a series of mature bovine articular cartilage KS fractions of increasing molecular weight showed [25] that above a nominal minimum chain size (below which no fucose was present) the content of fucose increased linearly with molecular size. It was therefore concluded that the α(1-3)-linked fucose was located within the poly-*N*-acetyllactosamine repeat sequence and therefore must be linked to *N*-acetylglucosamine as galactose has no available 3-position. This site is thus identical to that of the SSEA-1 antigen [18] but almost certainly the C-6 methylene groups will be sulphated, as the region has been found to be keratanase resistant (unpublished results).

In this work the first full ^{1}H-NMR assignment is presented for fucose α(1-3)-linked to the poly-*N*-acetyllactosamine repeat sequence region of a keratan sulphate chain of type KS-II-A.

Experimental

Keratan sulphate preparation

Bovine femoral head cartilage from 6–8-year-old animals was comminuted and extracted in 4 M guanidinium chloride in the presence of proteolytic inhibitors. Proteoglycan monomers were isolated after dialysis into associative conditions followed by associative then dissociative caesium chloride density gradient centrifugation as has been described in detail [21]. The A1D1 fraction was then digested with chondroitin ABC lyase followed by diphenylcarbamyl chloride-treated trypsin to yield peptido-keratan sulphate fragments which were then isolated on Sepharose CL-6B and subsequently alkaline borohydride reduced [26]. The resulting keratan sulphate chains were subjected to chromatography on Sephadex G-50, then further purified on Mono Q as previously described [22], except that all KS chains eluting between 0.10 and 0.25 M $LiClO_4$ were pooled and desalted.

NMR spectroscopy

Spectra were determined at 500 MHz on a Bruker AM500 spectrometer using a 5 mm V.T. probe at 60°C. The sample (5 mg) was buffered to pH 7 with sodium phosphate, filtered

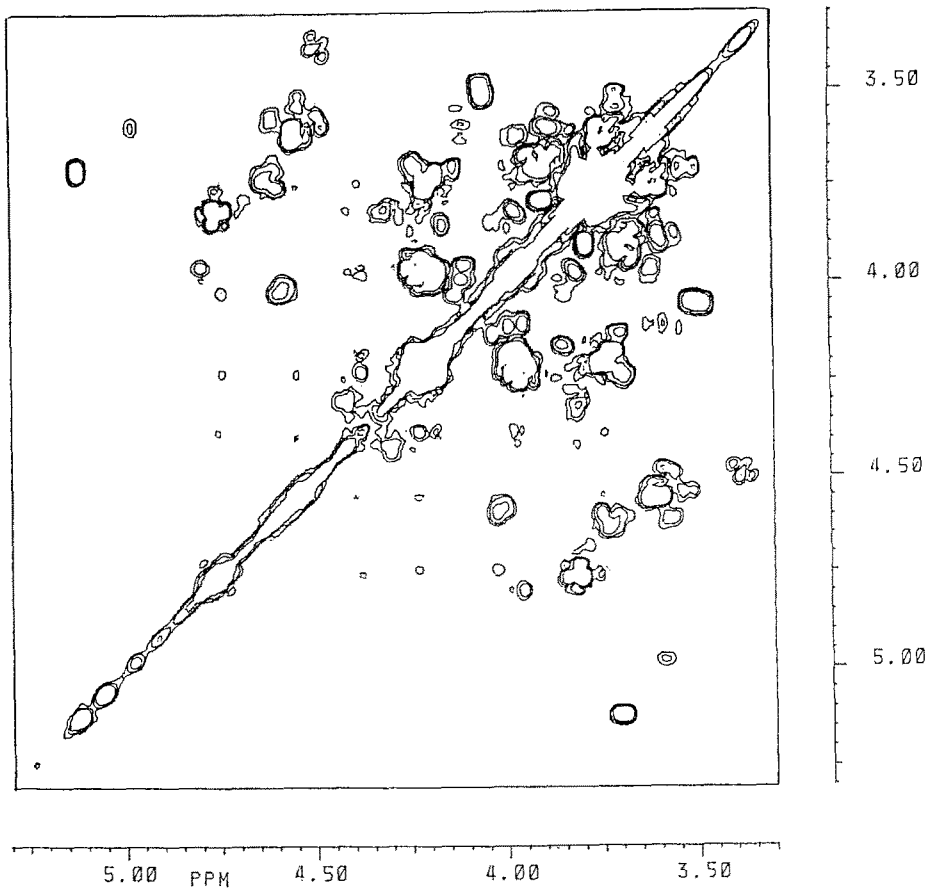

Figure 1. Partial COSY-45 spectrum from bovine femoral head KS, showing major ring proton resonances (3.3–5.3 ppm).

and referenced with internal TSP-d_4 for ^{1}H-NMR spectroscopy as previously described [27], and dissolved in 99.96% ^2H_2O (0.5 ml) after several exchanges with 99.8% and then finally with 99.96% ^2H_2O.

The COSY-45 spectrum was determined in the magnitude mode using a spectral width of 2994 Hz. Each of 512 t_1 increments was the result of 64 acquisitions and contained 512 complex points. In order to minimize the response of the residual HO^{2}H signal, weak gated presaturation was employed during a 1 s delay between acquisitions. The matrix was zero-filled to 1K × 1K and processed with standard Bruker software using a double sinebell window, then symmetrized. The magnitude mode RELAYED-COSY measurement was performed under similar conditions, with 393 t_1 increments collected and a transfer delay of 25 ms. The data were then processed on a VAX 11/785 computer at Lancaster using the computer program suite NMR2 (Lab One NMR2 Spectroscopic Data Analysis System, Release 3.95; New Methods Research Inc., Syracuse, NY, USA), zero-filled to 2K × 2K and a $\pi/10$ offset sinebell window applied before each of the Fourier-transform stages. Noise ridge subtraction was then performed down the t_1 axis using a summed horizontal set of slices taken from a signal-free region, and the resultant matrix symmetrized.

Results and discussion

The fucose ring is structurally very closely related to galactose. The major difference is that at C-6 there is a -CH_3 group instead of a -CH_2OH residue; the chair conformations are also different. In systems containing unsulphated galactose, the -CH_2OH and also the H-5 protons are normally not identifiable since they commonly fall under the complex envelope of signals from other sugar resonances. Attempts to locate their precise positions via COSY or RELAYED COSY experiments frequently fail because the cross-peaks which would point to them are either extremely weak or non-existent and there are no other indicators of their location.

The same situation concerning connections between H-3, H-4 and H-5 must be expected also for fucose residues; that is, $J_{3,4}$ will be small but observable, while $J_{4,5}$ may be vanishingly small. Examination of proton resonances from the fucose monosaccharide itself, where the quartet of H-5 is clearly visible, suggests that for this molecule there may be a very small $J_{4,5}$ coupling since the H-5 signal is broadened but structure cannot adequately be resolved.

Investigation of the COSY-45 spectrum for an intact keratan sulphate polymer derived from bovine femoral head cartilage proteoglycan (Fig. 1) shows clear H-1 to H-2, and

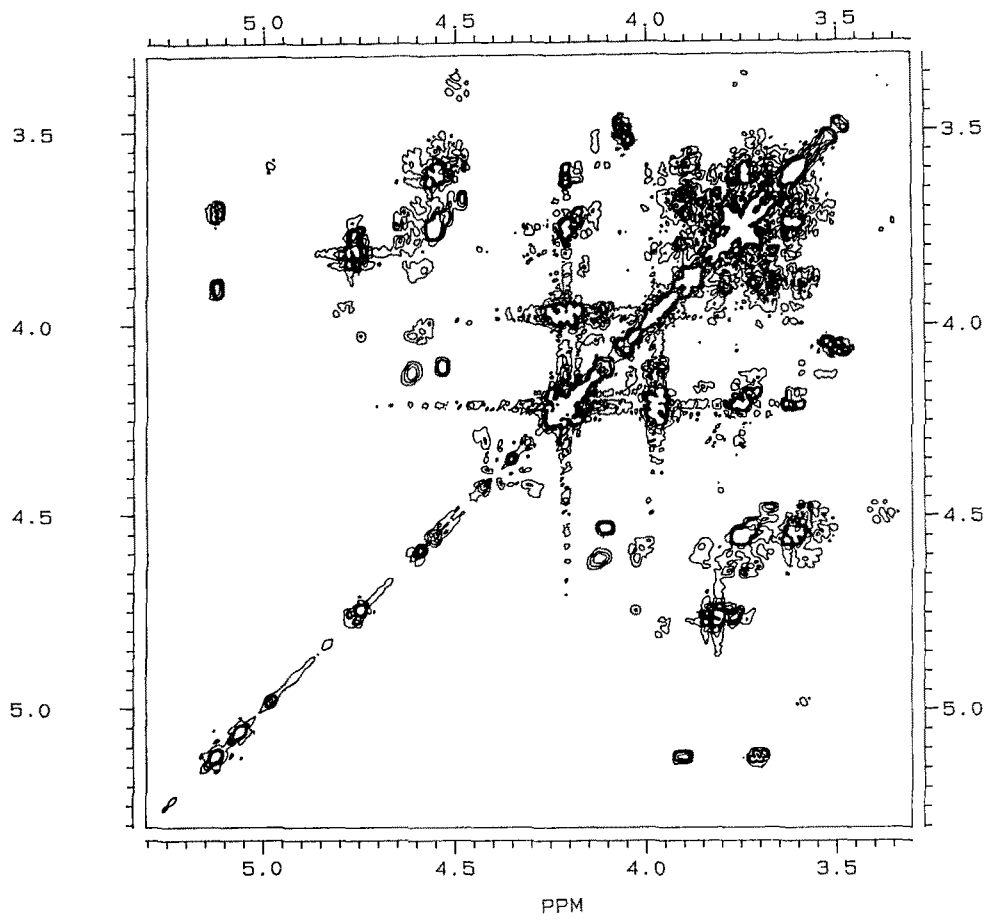

Figure 2. Partial RELAYED-COSY spectrum from bovine femoral head KS (3.3–5.3 ppm).

H-6 (CH_3) to H-5 connections for fucose, but the remaining cross-peaks, if present, are not identifiable amongst a complex array of correlations from other resonances.

RELAYED-COSY connections from fucose H-1 give the $1 \rightarrow 2 \rightarrow 3$ chain unambiguously, as is seen in Fig. 2, but leave the location of H-4 (via a $2 \rightarrow 3 \rightarrow 4$ chain) unclear since there is in that case a choice of cross-peaks. Neither COSY nor RELAYED-COSY spectra shows a connection from H-5 to H-4 since firstly the H-5 resonance is itself buried under a set of *N*-acetylglucosamine H-1 responses and secondly, the $J_{4,5}$ coupling will be very small, quite probably breaking the link.

Examination of the H-6 to H-5 line shows at first sight no extra connection. However, re-plotting with a careful choice of contour levels set very close to the baseline noise plane reveals a *very weak* connection between the methyl doublet at 1.17 ppm and a signal at ca. 3.79 ppm, as shown in Fig. 3. That this latter represents the location of H-4 is confirmed by the presence on the COSY spectrum (Fig. 1) of cross-peaks to H-3 from that shift location (with a narrow profile typical of those observed for the same cross-peak in a galactose residue), which could not otherwise have been unambiguously assigned. Data for the α(1-3)-linked fucose residue in bovine femoral head KS are summarized in Table 1.

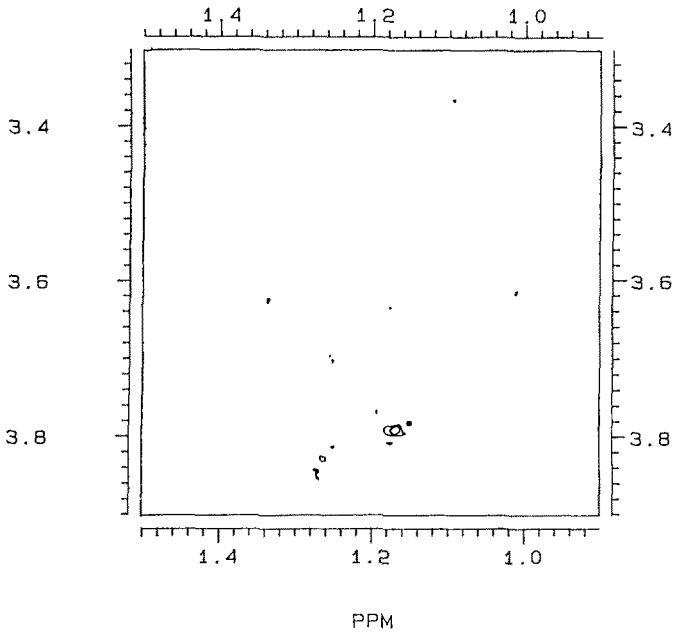

Figure 3. RELAYED-COSY H-6 to H-4 cross-peak for fucose in bovine femoral head KS plotted with contour levels set very close to the baseline noise plane.

Table 1. Chemical shifts for α(1-3)-linked fucose in ppm from interna internal TSP-d_4 at 60°.

Signal	*Chemical shift*
H-1	5.125
H-2	3.71
H-3	3.905
H-4	3.79
H-5	4.76
H-6 (CH_3)	1.17

This weak RELAYED-COSY connection was probably successfully achieved because of a fortuitous combination of factors. Firstly, the spectrum was obtained using a sufficient quantity of material to enable this exceedingly weak cross-peak to be discerned from the baseplane noise. Secondly, the cross-peaks are between a three-proton and a one-proton site which would be advantageous. There was also a clear line of sight along which to search for connections since the position of the -CH_3 resonance was known and was well clear of all other responses, unlike the situation for KS oligo- and polysaccharide unsulphated galactose responses where such a cross-peak would connect to what is probably a non-equivalent and hence complex pair of individual methylene protons with uncertain (and buried) proton chemical shift locations.

The values presented in Table 1, although generally similar in their positions to such other signals as have previously been observed for α(1-3)-linked fucose do show some deviations, which will derive from the proximity of ester sulphate residues probably situated both within the *N*-acetylglucosamine to which this moiety is attached and on the flanking galactose units. In particular, the H-5 position is shielded by more than 0.1 ppm compared with most α(1-3)-linked fucoses in unsulphated environments; the neighbouring CH_3 (H-6) signals are also slightly shielded. It is possible that these shift perturbations have arisen because this region of the fucose lies close to a sulphation site. The fucose resonances are simple in structure with little suggestion of environmental heterogeneity, quite unlike the typical structures observed, for example, for either galactose or *N*-acetylglucosamine H-1 protons, suggesting that fucoses within the extended poly-*N*-acetyllactosamine repeat sequence are closely similar in their structural environments.

Since the completion of this work a report [28] has appeared in which full ^{1}H assignments have been achieved for the α(1-3)-linked fucose residue in the carbohydrate part of the glycopeptide from the proteolytic enzyme bromelain. This fucose is also attached to *N*-acetylglucosamine, but in the slightly different microenvironment

-GlcNAcβ(1-4)[Fucα(1-3)]GlcNAc(1-*N*)Asn-.

The proton assignments were obtained by a different approach which combined 2D HOHAHA and ^{1}H-NOE measurements. It is significant that, for the bromelain system, NOE cross-peaks were observed not only between fucose H-1 and the *N*-acetylglucosamine H-3 but also between fucose H-5 and both the *N*-acetylglucosamine H-2 and an H-6 proton as well. The chemical shift for this fucose H-5, at 4.738 ppm (relative to TSP-d_4) is even more shielded than that in the KS system. The methyl resonance, however, at 1.301 ppm was remarkably strongly deshielded. (It should be noted that attempts to determine the precise site of attachment of fucose to the KS chain via 2D-NOE measurements failed, presumably because of unfavourable values for correlation times.)

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