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Skeletal keratan sulphate chains isolated from bovine intervertebral disc may terminate in α(2→6)-linked N-acetylneuraminic acid

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Peptido-keratan sulphate fragments were isolated from the nucleus pulposus of bovine intervertebral discs (2-year-old animals) after digestion with chondroitin ABC lyase followed by digestion with diphenylcarbamoyl chloride-treated trypsin of A1D1 proteoglycans and gel-permeation chromatography on Sepharose CL-6B. The peptido-keratan sulphate fragments were subjected to alkaline borohydride reduction. The reduced chains were treated with keratanase in the presence of the sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, and the digest was subjected to alkaline borohydride reduction. This produced oligosaccharides with galactitol at their reducing ends. This reduced digest was chromatographed on a Nucleosil 5 SB anion-exchange column and individual oligosaccharides were isolated. One of these was shown by 600 MHz ^{1}H-n.m.r. spectroscopy to have the following structure:

NeuAcα2–6Galβ1–4GlcNAc(6-SO_4)β1–3Gal-ol

The structure of this oligosaccharide shows that keratan sulphate chains from bovine intervertebral disc have non-reducing termini with *N*-acetylneuraminic acid linked α(2→6) as well as α(2→3) to an unsulphated galactose.

INTRODUCTION

Keratan sulphate (KS) is a glycosaminoglycan that was initially isolated from the cornea (Meyer *et al.*, 1953), but later detected in human and bovine skeletal cartilage (Gardell & Rastgeldi, 1954; Meyer *et al.*, 1958; Furuhashi, 1961). More recently it has been found to be a component of rat brain (Krusius *et al.*, 1986), rabbit bone (Kinne & Fisher, 1987) and epithelial secretions from human endometrium (Hoadley *et al.*, 1990). Skeletal KSs occur natively in the form of proteoglycans, together with chondroitin sulphate chains and numerous *O*-linked and *N*-linked oligosaccharides (see Carney & Muir, 1988).

KS chains have been classified (Meyer, 1970) according to their linkage to protein, corneal KS (KS-I) being *N*-linked between *N*-acetylglucosamine and asparagine, and skeletal KS (KS-II) being *O*-linked between *N*-acetylglucosamine and serine or threonine. The KS isolated from brain tissue (Krusius *et al.*, 1986) possesses an *O*-glycosidic bond between mannose and threonine and may represent a third type (KS-III?).

Nieduszynski *et al.* (1990*a*) proposed that skeletal KS (KS-II) derived from bovine cartilage should be subclassified into two groups, KS-II-A for articular and intervertebral-disc-derived KS and KS-II-B for tracheal or nasal-septum-cartilage KS. The former contains α(1→3)-linked fucose and α(2→6)-linked *N*-acetylneuraminic acid residues, which are absent from KS-II-B.

The complete primary structure of skeletal KS is yet to be unravelled (see Nieduszynski *et al.*, 1990*b*). It is known that the *N*-acetylgalactosamine in the linkage region is substituted at position 6 with the main poly-*N*-acetyl-lactosamine chain (Lohmander *et al.*, 1980), and at position 3 with the disaccharide *N*-acetylneuraminylgalactose (Hopwood & Robinson, 1974). Fragments derived from the linkage region of bovine intervertebral-disk KS (Dickenson *et al.*, 1990) were shown to have the following structure:

```
GlcNAc(6-SO4)1–6GalNAc-ol
                3
                /
                1
(NeuAc)0-1α2–3Gal
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The α(2→3)-linked *N*-acetylneuraminic acids common to both KS-II-A and KS-II-B are thought to be located in the linkage region (as indicated above) and at the non-reducing terminal as reported by Dickenson *et al.* (1991). They isolated, from bovine tracheal-ring-cartilage KS, a capping (or chain-terminating) oligosaccharide, containing α(2→3)-linked *N*-acetylneuraminic acid with the structure shown below:

NeuAcα2–3Gal1–4GlcNAc(6-SO_4)1–3Gal-ol

The α(1→3)-linked fucose residue(s) found in KS-II-A occur(s) within the sulphated poly-*N*-acetyl-lactosamine repeat sequence (Tai *et al.*, 1991) with the structure shown below:

```
   αFuc
    1
    /
    3
–3Galβ1–4GlcNAcβ1–
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The present study has focused on identifying the location of an α(2→6)-linked *N*-acetylneuraminic acid found uniquely in KS-II-A.

Abbreviation used: KS, keratan sulphate.
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EXPERIMENTAL

Materials

Chemicals and enzymes used in this study are as described in Thornton *et al.* (1989), except that lithium perchlorate (ACS grade) was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.), and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid was from Boehringer (Mannheim, Germany). The Nucleosil 5 SB columns were manufactured by Macherey–Nagel (Düren, Germany) and purchased from H.P.L.C. Technology (Macclesfield, Cheshire, U.K.). Bio-Gel P-2 was purchased from Bio-Rad Laboratories (Watford, Herts., U.K.). The enzymes chondroitin ABC lyase (*Proteus vulgaris*, EC 4.2.2.4) and keratanase (*Pseudomonas* sp., EC 3.2.1.103) were purchased from ICN Biomedicals (High Wycombe, Bucks., U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.) respectively.

Preparation of peptido-KSs

Proteoglycan monomers were extracted from the comminuted nucleus pulposus of bovine intervertebral discs (2-year-old animals) in 4 M-guanidine hydrochloride in the presence of proteinase inhibitors, dialysed into associative conditions and subjected to associative followed by dissociative CsCl-density-gradient centrifugation (as described in detail in Thornton *et al.*, 1989). The A1D1 fraction was dialysed against 0.1 M-Tris/acetate buffer, pH 7.3, and then digested with chondroitin ABC lyase (0.5 unit/300 mg of proteoglycan) followed by diphenylcarbamoyl chloride-treated trypsin (2 mg/g of proteoglycan). The digest was partially freeze-dried and then chromatographed on a column (152 cm × 3.2 cm) of Sepharose CL-6B eluted with 0.5 M-sodium acetate/10 mM-EDTA buffer, pH 6.8. This produced the peptido-KS fragments (6B1 and 6B2) as described in Thornton *et al.* (1989).

Alkaline borohydride reduction

KS chains were prepared by alkaline borohydride reduction (Carlson, 1968) of peptido-KSs. These fragments (230 mg) were reduced with the use of 1 ml of reductant (1.0 M-$NaBH_4$ in 0.05 M-NaOH) per 5 mg of material. The reduction was terminated by the dropwise addition of acetic acid, the sample was then dialysed against water, and product (100 mg) was recovered by freeze-drying. The reduced KS chains were examined by 400 MHz ^{1}H-n.m.r. spectroscopy to assess their purity.

Keratanase digestion

Some batches of keratanase used in this laboratory have been shown also to have neuraminidase activity. Therefore, before proceeding to a large-scale digestion, an analytical keratanase digestion was performed, with 600 μg of the reduced 6B1-derived KS, which included the universal neuraminidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid in the digest medium. This proved successful in blocking sialidase activity. The analytical keratanase digest was chromatographed on a Nucleosil 5 SB column, eluted with a linear gradient of 0–0.5 M-lithium perchlorate, pH 5.0. The profile (Fig. 1) shows two peaks, labelled B and C, which are at the elution positions of the previously characterized sialylated tetrasaccharide capping fragment (Dickenson *et al.*, 1991) and sialylated linkage region oligosaccharide (Dickenson *et al.*, 1990). Experiments (results not shown) performed in the absence of the neuraminidase inhibitor produced Nucleosil 5 SB profiles devoid of peaks associated with these sialic acid-containing oligosaccharides. The oligosaccharide of interest in this work, peak A (see Fig. 1), contains an $\alpha(2\rightarrow6)$-linked sialic acid residue, and had been isolated previously but in quantities insufficient for structural analysis.

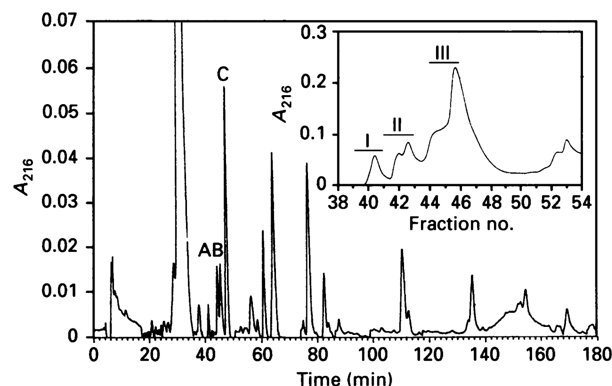


Fig. 1. High-performance anion-exchange chromatography profile of the keratanase digest of bovine intervertebral-disc KS chains

The Nucleosil 5 SB column (25 cm × 1 cm) was eluted at a flow rate of 2 ml/min. The column effluent was monitored on-line by using u.v. detection at 216 nm. The gradient programme was as follows: 10 min of buffer A (5 mM-lithium perchlorate, pH 5.0) and then 230 min of 0–100% (v/v) buffer B (0.5 M-lithium perchlorate, pH 5.0). Inset: the Nucleosil 5 SB column (25 cm × 4.6 mm) was eluted at a flow rate of 0.75 ml/min. The column eluate was monitored on-line by using u.v. detection at 216 nm. The gradient programme was as follows: 10 min of buffer A (5 mM-lithium perchlorate, pH 5.0) and then 230 min of 0–100% (v/v) buffer B (0.25 M-lithium perchlorate, pH 5.0). Fractions (0.75 ml) were pooled as indicated.

For larger-scale preparation of oligosaccharides, the remaining reduced KS chains (90 mg) were dissolved in 0.2 M-sodium acetate buffer, pH 7.4, containing 5 mM-2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid and digested with keratanase (1 unit/2.8 mg of KS) at 37 °C. The digest (2 ml) was then reduced by the addition of 2 ml of 2 M-$NaBH_4$ in 0.1 M-NaOH. The reduction was terminated by the careful addition of 4 M-acetic acid. The reduced digest was recovered by freeze-drying after desalting on a Bio-Gel P-2 column (90 cm × 1.5 cm), eluted with water.

H.p.l.c.

H.p.l.c. was performed on a Bio-Rad series 700 HRLC titanium gradient system with u.v. and refractive-index detectors.

The reduced keratanase digest was chromatographed in two separate runs (30 mg each) on a Nucleosil 5 SB column eluted with a linear gradient of 0–0.25 M-lithium perchlorate, pH 5.0. The region of the profile containing the oligosaccharide of interest is shown in Fig. 1 (inset). Fractions I, II and III (from both runs) were pooled as indicated, desalted on a Bio-Gel P-2 column (11.2 cm × 1 cm), eluted with water and freeze-dried. These fractions were then examined by 500 MHz ^{1}H-n.m.r. spectroscopy (results not shown). Fraction II was found to contain both $\alpha(2\rightarrow3)$- and $\alpha(2\rightarrow6)$-linked sialic acid. It was therefore rechromatographed on Nucleosil 5 SB and eluted with a shallower gradient, 0–0.125 M-lithium perchlorate, pH 5.0. This resolved the original fraction II into two components (Fig. 2). Fractions 57–58 were pooled, desalted and recovered by freeze-drying. The purified oligosaccharide (I) was then examined by 600 MHz ^{1}H-n.m.r. spectroscopy.

The KS chain preparation used in this study was not subjected to further purification, to remove any contaminating *O*-linked oligosaccharides, either by gel filtration on Sephadex G-50 or anion-exchange chromatography on a Pharmacia Mono Q column as described previously (Dickenson *et al.*, 1990). The

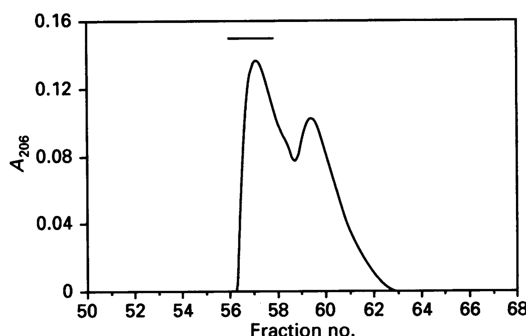


Fig. 2. High-performance anion-exchange chromatography profile of the rechromatographed fraction II

The Nucleosil 5 SB column (25 cm × 4.6 cm) was eluted at a flow rate of 0.75 ml/min. The column eluate was monitored on-line by using u.v. detection at 206 nm. The gradient programme was as follows: 10 min of buffer A (5 mM-lithium perchlorate, pH 5.0) and then 230 min of 0–100% (v/v) buffer B (0.125 M-lithium perchlorate, pH 5.0). Fractions (0.75 ml) were pooled.

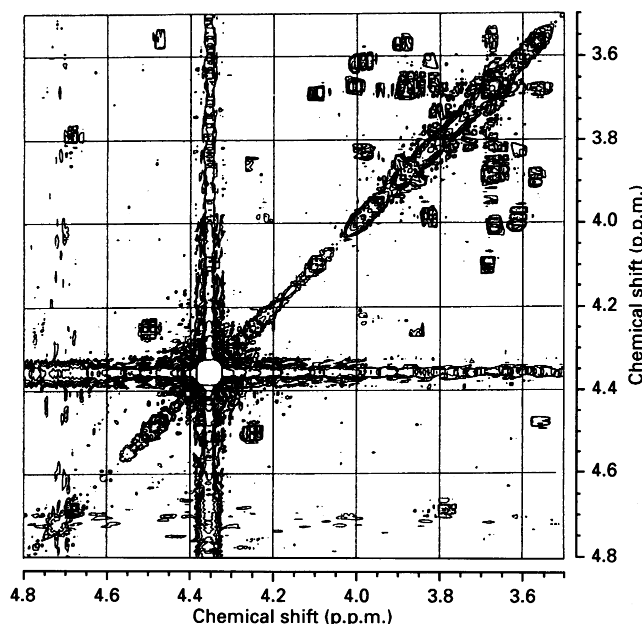


Fig. 3. Partial 600 MHz COSY-45 spectrum for tetrasaccharide (I) at 60 °C

The chemical shift scales are in p.p.m. from internal sodium trimethylsilyl[$^2H_4$]propionate.

oligosaccharide examined in this study had, however, previously been isolated from bovine intervertebral-disk KS chains that had been purified further to remove any *O*-linked oligosaccharides. Chromatographic and one-dimensional ^{1}H-n.m.r. profiles were identical with those for oligosaccharide (I); insufficient quantities were available for structural analysis by two-dimensional n.m.r.

N.m.r. spectroscopy

The oligosaccharide was dissolved in ^2H_2O (99.8%; 0.5 ml), buffered to pH 7, and referenced with sodium trimethylsilyl[$^2H_4$]propionate as internal standard for ^{1}H-n.m.r. spectroscopy as previously described (Sanderson *et al.*, 1987) after filtration by centrifugation in a microfilterfuge tube (0.45 μm pore size). The sample was exchanged three times with 99.8% ^2H_2O then once with 100% ^2H_2O before dissolution in 100% ^2H_2O (0.5 ml). Preliminary one-dimensional ^{1}H-n.m.r. spectra were determined at room temperature and 60 °C on a Bruker AM500 instrument operating at 500.14 MHz. Detailed measurements were performed at room temperature and at 60 °C with a Bruker AMX600 system operating at 600.14 MHz. A COSY-45 measurement was performed at 60 °C and 600 MHz with a spectral width of 3623 Hz with 128 pulses for each of 256 increments taken into 1024 complex points. The free induction decay responses were multiplied in each dimension with a $\pi/10$-offset sinebell window function before zero-filling and Fourier transformation to yield a matrix of 2k × 2k complex points, which was then symmetrized for presentation.

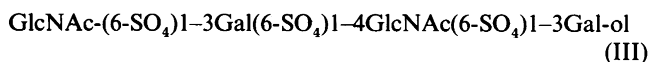
RESULTS AND DISCUSSION

Analytical and chromatographic data suggested that the oligosaccharide (I) was a tetramer containing sialic acid and one other negatively charged residue. The observation of characteristic H-3_a and H-3_e n.m.r. resonances at 1.718 and 2.680 p.p.m. (at 25 °C) indicated that the sialic acid was attached via an $\alpha(2\rightarrow6)$ linkage to the next sugar residue, probably galactose (Vliegenthart *et al.*, 1983; Breg *et al.*, 1989). Two methyl resonances, at 2.041 and 2.093 p.p.m. (60 °C), confirmed the presence of the *N*-acetylneuraminic acid residue, together with that of a second *N*-acetyl-containing unit.

Tetrasaccharide (I) shows the typical set of resonances found for a C-3-linked galactitol residue, with chemical-shift positions readily assignable via a 600 MHz COSY-45 measurement (Fig. 3), which are closely similar to those reported (Dickenson *et al.*, 1990) for the $\alpha(2\rightarrow3)$-linked sialic acid-containing chain-terminator tetrasaccharide (II) with internal β-linkages and the structure shown below:

NeuAcα2–3Gal1–4GlcNAc(6-SO_4)1–3Gal-ol (II)

Since (I) is derived from KS, which is a modified poly(*N*-acetyl-lactosamine), it would be expected that the moiety attached to the C-3 site of this galactitol residue should be *N*-acetyl-glucosamine. Examination of the one-dimensional and two-dimensional n.m.r. spectra from (I) permits identification of a set of resonances in accord with the presence of a C-6 ester-sulphated *N*-acetylglucosamine residue that is itself $\beta(1\rightarrow4)$-linked to a further unit (see Table 1). The chemical shifts are similar both to those observed for the corresponding residue in (II) and to those found for the internal sulphated glucosamine residue of the reduced KS tetrasaccharide repeat unit (III) (Huckerby *et al.*, 1990), which has the β-linked structure:


GlcNAc-(6-SO_4)1–3Gal(6-SO_4)1–4GlcNAc(6-SO_4)1–3Gal-ol (III)

This glucosamine residue has a set of closely coupled ring protons; it was not possible to identify the precise chemical-shift position for H-4. This could probably have been achieved via an additional RELAYED-COSY measurement, but this was not performed because of the limited quantity of (I) available.

By analogy with the internal repeat sequence in KS chains, the residue attached to the non-reducing terminus of this *N*-acetyl glucosamine residue is expected to be a galactose residue. A complete set of galactose ring proton signals can be identified from the COSY-45 spectrum, which are totally consistent with the presence of a 6-substituted galactose ring. As is commonly found, there is no readily observable cross-peak connecting H-4 to H-5 in this residue because of the small value for $J_{4,5}$ in this sugar. The derived chemical shifts bear a strong similarity to

Table 1. Spectral data for oligosaccharide I

Shifts are in p.p.m. from internal sodium trimethylsilyl[$^2H_4$]propionate and unless indicated are from COSY data. Couplings are first-order values in Hz at 60 °C.

	(I)			
Residue	60 °C	25 °C*	Couplings	(IV)§
Gal-ol				
H-1,1′	3.67			
H-2	4.007	4.019		
H-3	~3.90			
H-4	~3.85			
H-5	4.096*	4.135		
H-6,6′	3.685			
GlcNAc(SO_4)				GlcNAc (unsulphated)
H-1	4.682*	4.678	$J_{1,2}$ = 8.2	5.150
H-2	~3.782			3.987
H-3	~3.826			3.818
H-4	–			3.698
H-5	~3.855			3.701
H-6	~4.496*	4.514	$J_{5,6}$ = 2.0	3.945
H-6′	~4.248*	4.264	$J_{5,6'}$ = 6.6	3.844
CH_3	2.093		$J_{6,6'}$ = −11.2	2.058
Gal				
H-1	4.470*	4.468	$J_{1,2}$ = 7.9	4.463
H-2	3.555			3.553
H-3	3.677		$J_{3,4}$ = 3.3	3.684
H-4	3.942*†	3.936		3.933
H-5	3.828†		$J_{5,6}$ = 8.35	3.841
H-6	3.984	4.003	$J_{5,6'}$ = 3.85	3.996
H-6′	3.611		$J_{6,6'}$ = −10.1	3.544
NeuAc				
H-3_a	1.690*	1.718		1.733
H-3_e	2.692*	2.680		2.678
H-4	3.692			3.655
H-5	3.800			3.821
H-6	3.727‡	3.715		3.710
H-7	3.750‡			3.563
H-8	3.890			3.905
H-9	~3.87			3.891
H-9′	~3.67			3.643
CH_3	2.041			2.044

* From one-dimensional data.
† Connections between H-4 and H-5 are not seen because $J_{4,5}$ is small.
‡ Connections between H-6 and H-7 are not seen because $J_{6,7}$ is small.
§ At 8 °C; shifts are referenced to sodium trimethylsilyl[$^2H_4$]propionate at 0.0 p.p.m.

those observed (Breg *et al.*, 1989) for the 6-linked galactose residue within the tetrasaccharide portion of (IV), which has the structure:

The residue attached at C-6 of this galactose residue is the α(2→6)-linked sialic acid residue. A complete set of signal assignments can be achieved for this sugar from cross-peak information on the COSY-45 spectrum. Again, there is no link observable between H-6 and H-7 because the $J_{6,7}$ coupling is too small. The derived chemical shifts are again closely similar to the set of parameters reported by Breg *et al.* (1989) for (IV).

The spectral data for (I), which can therefore be seen to have the structure:

are summarized in Table 1, where they are compared with the corresponding values from the carbohydrate portion of (IV).

The identification of this oligosaccharide fragment as well as its α(2→3)-linked sialic acid partner shows that either different KS chains may have different non-reducing chain termini, or less probably, judged from the apparent sialic acid stoichiometries, individual KS chains may have branches in addition to that already characterized in the linkage region (Hopwood & Robinson, 1974; Dickenson *et al.*, 1990). The former hypothesis would indicate that discrete subpopulations of KS chains occur in articular cartilage.

The two structural features of KSs that are specific to articular cartilage (Nieduszynski *et al.*, 1990*a*), α(2→6)-NeuAc as well as the α(1→3)fucose, are both non-reducing termini, although the latter is clearly attached to the main poly(*N*-acetyl-lactosamine) repeat sequence of the chain (Tai *et al.*, 1991). The incorporation of these structures requires the presence of an α(2→6)-sialyltransferase and a fucosyltransferase in addition to the other enzymes required for the biosynthesis of non-articular KSs.

The distribution of the α(2→6)-*N*-acetylneuraminic acid residues within KS chains, proteoglycans or indeed within articular cartilage is unknown. Approximately one-third of the chains have this termination in the bovine system examined here, but the purification strategy employed biases the population recovered here towards higher M_r and higher charge density. There is an urgent need for monoclonal antibody and other probes that will recognize features such as the oligosaccharide characterized here.

Similarly, the function of the α(2→6)-*N*-acetylneuraminic acid residues is unknown, although it is possible that the differently linked sialic acid caps may have different labilities, thus affecting the rates of clearance of proteoglycan fragments via the asialoglycoprotein receptor system. This would be of significance to those studies of arthritic diseases that use KS concentration in body fluids as markers (e.g. Thonar *et al.*, 1985; Poole *et al.*, 1990). Alternatively, it seems likely that specific oligosaccharide sequences at the accessible chain caps (or terminators) might serve as recognition points for other macromolecules of the extracellular matrix.

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