Oligosaccharides Derived from Bovine Articular Cartilage Keratan Sulfates after Keratanase II Digestion: Implications for Keratan Sulfate Structural Fingerprinting†

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ABSTRACT: Keratan sulfate chains were isolated from bovine articular cartilage (6–8-year-old animals) and digested with keratanase II, an *endo*-β-*N*-acetylglucosaminidase [Nakazawa, K., Ito, M., Yamagata, T., & Suzuki, S. (1989) in *Keratan Sulphate: Chemistry, Biology and Chemical Pathology* (Greiling, H., & Scott, J. E., Eds.) pp 99–110, The Biochemical Society, London]. Twenty-five borohydride-reduced oligosaccharides were purified chromatographically and characterized by one- and two-dimensional NMR spectroscopy. From the structures of these oligosaccharides the following conclusions can be drawn about the mode of action of keratanase II: (1) The enzyme cleaves the β(1 → 3)-glycosidic bond between 6-*O*-sulfated *N*-acetyl-glucosamine and galactose, the major products being mono- and disulfated disaccharides. (2) Larger oligosaccharides containing keratanase II susceptible bonds are produced which are resistant to further degradation, e.g., tetrasaccharides from the sulfated poly(*N*-acetyllactosamine) repeat sequence, fucose-containing penta- and hexasaccharides, and hexa- and heptasaccharides from the linkage region. (3) The enzyme cleaves the β(1 → 3)-glycosidic bond of a fucosylated 6-*O*-sulfated *N*-acetylglucosamine. (4) Sialic acid-containing capping fragments are always recovered as pentasaccharides, despite the presence of an apparently susceptible bond. Two new elements of skeletal keratan sulfate structure, namely, the highly sulfated cap NeuAcα2–3Gal(6S)β1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)-ol and the difucosylated sequence Galβ1–4(Fucα1–3)GlcNAc(6S)β1–3Galβ1–4(Fucα1–3)GlcNAc(6S)-ol, have been identified. A structural model for articular cartilage keratan sulfate is proposed. The potential of the enzyme keratanase II for the structural fingerprinting of subnanogram quantities both of keratan sulfates and of sulfated oligosaccharide selectin ligands is discussed.

Keratan sulfate (KS)[1] was first isolated from the cornea by Meyer *et al.* (1953) and was later detected in the nucleus pulposus of human intervertebral disk (Gardell & Rastageldi, 1954). KS chains have since been classified [after the notation of Meyer (1970)] according to their linkage to protein, with KS-I (corneal KS) having alkali-stable bonds between *N*-acetylglucosamine and asparagine, and KS-II (skeletal KS) having an alkali-labile bond between *N*-acetylgalactosamine and serine or threonine. This skeletal type has been reclassified into articular, KS-II-A, and nonarticular, KS-II-B (Nieduszynski *et al.*, 1990), on the basis that the former contain α(1 → 3)-fucose and α(2 → 6)-*N*-acetylneuraminic acid, which are absent in the latter. A possible third type of KS has been isolated from brain tissue (Krusius *et al.*, 1986) which has an *O*-glycosidic linkage between mannose and serine or threonine.

Skeletal keratan sulfates have recently attracted considerable attention because of their potential as early markers for the disease of osteoarthritis (Thonar *et al.*, 1985). Similarly, both corneal KS (Oeben *et al.*, 1987) and the KS proteoglycans (Funderburgh *et al.*, 1991; Blochberger *et al.*, 1992), which are implicated in certain forms of macular corneal dystrophy (Nakazawa *et al.*, 1984), are the subject of active research.

Several fragmentation methods have been developed for structural studies of keratan sulfates. These include the use of the enzymes endo-β-galactosidase and keratanase (Fukuda & Matsumura, 1976; Scudder *et al.*, 1983; Dickenson *et al.*, 1991; Tai *et al.*, 1993) and the chemical depolymerization procedure of hydrazinolysis followed by nitrous acid treatment (Hopwood & Elliott, 1983; Brown *et al.*, 1992). Recently, a new keratan sulfate degrading enzyme, keratanase II, has been introduced which is reported to cleave the β(1 → 3)-glycosidic bond of a 6-*O*-sulfated *N*-acetylglucosamine (Nakazawa *et al.*, 1989).

In the present study, 25 oligosaccharides derived from skeletal keratan sulfates by keratanase II fragmentation have been characterized by ^{1}H-NMR spectroscopy. The substrate specificity of the enzyme and its potential for keratan sulfate fingerprinting are discussed.

EXPERIMENTAL PROCEDURES

Materials. Guanidine hydrochloride (practical grade) was obtained from the Sigma Chemical Co. (Poole, U.K.). Cesium

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[1] Abbreviations: KS, keratan sulfate; Gal, β-D-galactose; GlcNAc, N-acetylglucosamine (2-acetamido-2-deoxy-β-D-glucose); GalNAc-ol, *N*-acetylgalactosaminitol (2-acetamido-2-deoxy-β-D-galactitol); GlcNAc-ol, *N*-acetylglucosaminitol (2-acetamido-2-deoxy-β-D-glucitol); Fuc, α-L-fucose; NeuAc, sialic acid (*N*-acetylneuraminic acid); (6S), *O*-ester sulfate group on C(6); Le^x, Galβ1–4(Fucα1–3)GlcNAc; NMR, nuclear magnetic resonance; ppm, parts per million; TSP-d_4, sodium 3-(trimethylsilyl)-[^{2}H$_4$]propionate; 1-D, one-dimensional; 2-D, two-dimensional; COSY, correlated spectroscopy.

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chloride and disodium EDTA were purchased from BDH Chemicals (Poole, U.K.). Lithium perchlorate (ACS grade) and piperazine were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All other chemicals were of analytical grade.

Diphenylcarbamyl chloride-treated (DPCC-treated) trypsin (bovine pancreas, EC 3.4.21.4) was purchased from the Sigma Chemical Company Ltd. (Dorset, U.K.). Chondroitin ABC lyase (*Proteus vulgaris*, EC 4.2.2.4) and keratanase II (*Bacillus* sp.) were obtained from ICN Biomedicals Ltd. (High Wycombe, Bucks., U.K.)

The Nucleosil 5SB column was purchased from Technicol Ltd. (Stockport, Cheshire, U.K.), and the Mono-Q HR 10/10 column was from Pharmacia (Uppsala, Sweden). The Bio-Gel TSK 30 XL column was obtained from Bio-Rad Laboratories Ltd. (Watford, Herts., U.K.)

Isolation and Purification of KS Chains. Keratan sulfate chains were prepared from bovine femoral head cartilage (6–8-year-old animals) essentially by the method described by Dickenson *et al.* (1990). This involved the extraction of the proteoglycans from comminuted cartilage using 4 M guanidine hydrochloride including protease inhibitors followed by associative CsCl density gradient centrifugation. The proteoglycan aggregate fraction was digested with chondroitin ABC lyase followed by DPCC-treated trypsin and then subjected to gel-permeation chromatography on a column of Sepharose CL-6B (152 × 3.2 cm) eluted with 0.5 M sodium acetate/10 mM EDTA. KS chains were isolated from both the KS-rich and the CS-rich peptide fragments (Heinegård & Axelsson, 1977; Thornton *et al.*, 1989) by alkaline borohydride reduction (Carlson, 1968) followed by chondroitin ABC lyase digestion and gel-permeation chromatography on a column of Sephadex G-50 (82 × 1.5 cm) eluted with 0.15 M NaCl. After dialysis against water, the KS chains were recovered by lyophilization and then purified further from any remaining O-linked oligosaccharides by ion-exchange chromatography on a Pharmacia Mono-Q HR 10/10 column eluted with a linear gradient of 0–0.5 M $LiClO_4$/10mM piperazine.

Analytical Keratanase II Digestion. In order to establish conditions for a limit digest, KS chains (5 mg) were dissolved in 10 mM sodium acetate buffer, pH 6.5 (490 μL), and 0.01 unit of keratanase II was added (in 10 μL of 10 mM sodium acetate buffer, pH 6.5). Digestion was performed at 37 °C, and 1 μL solution aliquots were removed at 30-min intervals. The aliquots were assayed for reducing sugar essentially by the method described by Chaplin (1986). Briefly, samples (1 μL) were diluted to 25 μL with water, thoroughly mixed with 50 μL reagent A and 50 μL reagent B (see below), and heated in a water bath at 100 °C for 12 min. The solutions were then diluted with 125 μL of water, mixed well, and centrifuged for 2 min (at 7000 rpm) to spin down any condensate. Aliquots (200 μL) of the resulting solutions were transferred to a microtiter plate and assayed for their absorbance at 450 nm using a Bio-Rad Model 2550 EIA microtiter plate reader. The reagents used were as follows.

Reagent A: Anhydrous sodium carbonate (40 g), glycine (16 g), and copper sulfate pentahydrate (0.45 g) were dissolved in 600 mL of water, and the solution was made up to 1 liter with water.

Reagent B: Neocuproine hydrochloride (150 mg) was dissolved in 100 mL of water.

After 33 h, when the reducing sugar concentration had been constant for 3 h, an extra 0.01 unit of enzyme was added and the solution was left for a further 17 h, whereupon the reducing sugar concentration was found to be unchanged. It was deduced that 0.01 unit of enzyme is sufficient to completely digest 5 mg of KS in 30 h at 37 °C.

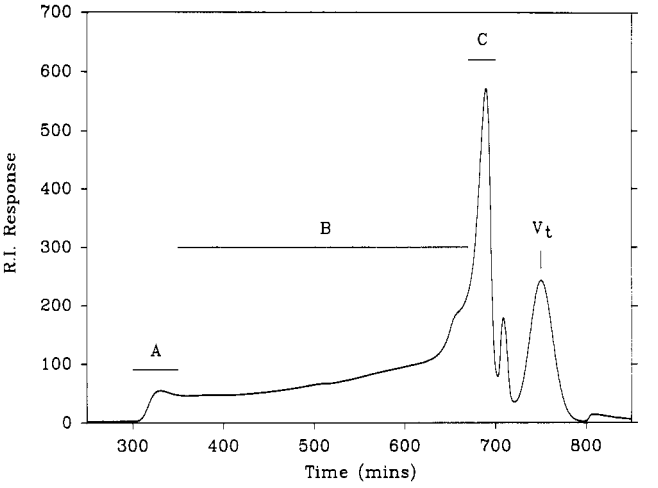


FIGURE 1: Bio-Gel P-6 gel-permeation chromatogram of reduced oligosaccharides produced by keratanase II digestion/borohydride reduction of bovine articular cartilage keratan sulfates. The column (100 × 1 cm) was eluted with water at a flow rate of 5.5 mL/h at 50 °C and monitored on-line using the refractive index. Fractions A, B, and C were pooled as shown. V_o and V_t were 310 and 750 min, respectively. Fraction A was composed of large oligosaccharides apparently deriving from the linkage region of N-linked keratan sulfates. Fraction C was a mixture of the mono- and disulfated disaccharides.

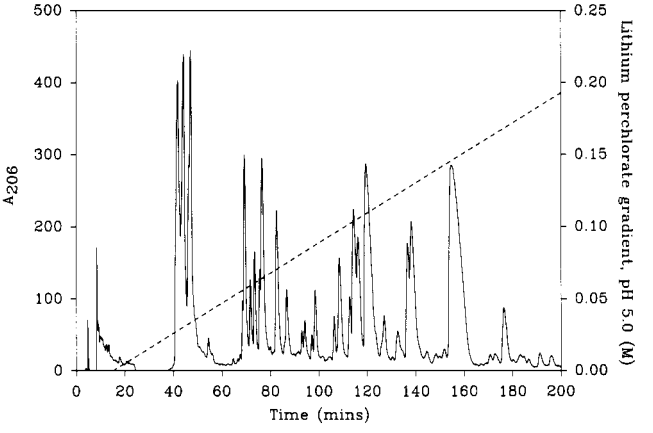


FIGURE 2: Nucleosil 5SB ion-exchange chromatogram of oligosaccharides produced by keratanase II digestion/borohydride reduction of bovine articular cartilage keratan sulfates. The column (25 × 1 cm) was eluted at a flow rate of 2 mL/min. The gradient program (shown by the dashed line) was as follows: 15 min of 2 mM $LiClO_4$, pH 5.0, and then 240 min of 0–100% 0.25 M $LiClO_4$, pH 5.0.

Preparative Keratanase II Digestion. KS chains (40 mg) from the KS-rich region were dissolved in 10 mM sodium acetate buffer, pH 6.5, (3.92 mL), and 0.08 unit of keratanase II was added (dissolved in 80 μL of 10 mM sodium acetate buffer, pH 6.5). Digestion was performed at 37 °C for 30 h.

Alkaline Borohydride Reduction. The pH of the digested mixture (4 mL) was raised to approximately pH 7.5 using 4 M NH_4OH, and 150 mg of sodium borohydride was added (equivalent to 1 M). The mixture was left at room temperature for 3 h. The reaction was terminated by the careful addition of 4 M CH_3COOH. The reduced oligosaccharides were desalted in several separate runs on a column of Bio-Gel P-2 (11.2 × 1.0 cm) and lyophilized.

Chromatographic Procedures. The reduced oligosaccharides were applied to a column of Bio-Gel P-6 (100 × 1 cm) and eluted with water (5.5 mL/h) at 50 °C. The column

Table 1: Oligosaccharide Elution Positions from the Nucleosil 5SB Strong Anion-Exchange Column

elution time (min)	code	oligosaccharide structure
44.3	F1	Galβ1–4(Fucα1–3)GlcNAc(6S)–ol
46.5	L1	Galβ1–4GlcNAc(6S)β1–6(Galβ1–3)GalNAc–ol
47.2	R1	Galβ1–4GlcNAc(6S)–ol
68.6	F2	Galβ1–4(Fucα1–3)GlcNAc(6S)β1–3Galβ1–4(Fucα1–3)GlcNAc(6S)–ol
69.5	L2	Galβ1–4GlcNAc(6S)β1–6(NeuAcα2–3Galβ1–3)GalNAc–ol
71.8	F3	Galβ1–4GlcNAc(6S)β1–3Galβ1–4(Fucα1–3)GlcNAc(6S)–ol
73.6	F4	Galβ1–4(Fucα1–3)GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)–ol
75.7	L3	Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)β1–6(Galβ1–3)GalNAc–ol
76.7	R2	Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)–ol
82.6	R3	Gal(6S)β1–4GlcNAc(6S)–ol
86.8	L4	Gal(6S)β1–4GlcNAc(6S)β1–6(Galβ1–3)GalNAc–ol
94.3	L5	Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)β1–6(NeuAcα2–3Galβ1–3)GalNAc–ol
97.2	C1	NeuAcα2–6Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)–ol
98.6	C2	NeuAcα2–3Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)–ol
106.4	L6	Gal(6S)β1–4GlcNAc(6S)β1–6(NeuAcα2–3Galβ1–3)GalNAc–ol
108.6	F5	Gal(6S)β1–4GlcNAc(6S)β1–3Galβ1–4(Fucα1–3)GlcNAc(6S)–ol
112.8	L7	Gal(6S)β1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)β1–6(Galβ1–3)GalNAc–ol
114.5	R4	Gal(6S)β1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)–ol
116.4	F6	Galβ1–4(Fucα1–3)GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol
119.7	R5	Galβ1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol
127.1	L8	Gal(6S)β1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S)β1–6(NeuAcα2–3Galβ1–3)GalNAc–ol
136.7	C3	NeuAcα2–6Galβ1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol
138.3	C4	NeuAcα2–3Galβ1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol
154.9	R6	Gal(6S)β1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol
176.4	C5	NeuAcα2–3Gal(6S)β1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S)–ol

eluate was monitored on-line using the refractive index. Fractions A–C were pooled as shown in Figure 1 and lyophilized.

Fraction B was dissolved in 100 μL of 2 mM $LiClO_4$, pH 5.0, and chromatographed on a Nucleosil 5SB strong anion-exchange column (25 × 1 cm), equilibrated in 2 mM $LiClO_4$, pH 5.0, and eluted with a linear gradient of 0–0.25 M $LiClO_4$, pH 5.0. The column eluate was monitored on-line at 206 nm (Figure 2). The 25 major peaks were pooled, and their structures were assigned using proton NMR spectroscopy. Where necessary, individual oligosaccharides were rechromatographed to remove any contaminants.

Further Keratanase II Treatment of Tetrasaccharide [R6]. The tetrasaccharide [R6] (for structure, see Table 1) was divided into two equal aliquots, and one aliquot was redigested with 0.004 unit of keratanase II in 20 μL of 10 mM sodium acetate buffer, pH 6.5, for 24 h. This sample and the undigested control were individually subjected to gel-permeation chromatography on a Bio-Gel TSK 30 XL column (30 cm × 7.8 mm) eluted with 0.2 M NaCl at 30 °C (Figure 3).

NMR Spectroscopy. Samples were dissolved in 0.5 mL of 99.8% ^2H_2O, buffered to pH 7 with phosphate (10 mM) and referenced with sodium 3-(trimethylsilyl)[$^2H_4$]propionate (TSP) as internal standard. After microfiltration through 0.45-μm nylon filters, samples were dried using either a freeze-drier or a rotary concentrator and exchanged several times with 0.5 mL of 99.8% ^2H_2O and then once with 99.96% ^2H_2O before final dissolution into 0.5 mL of 99.96% ^2H_2O.

Preliminary high-field ^{1}H-NMR spectra at 400 MHz were obtained on a Jeol GSX400 spectrometer fitted with a 5-mm probe. Very-high-field ^{1}H-NMR spectra at 600 MHz were acquired using a Bruker AMX600 spectrometer equipped with a 5-mm probe. Both 1-D and 2-D spectra were determined at 55–60 °C. All chemical shifts are quoted relative to internal TSP-d_4 at 0.0 ppm. Experimental details for 2-D spectra are given in the captions to figures.

Spectra were reprocessed for presentation using the software packages NMR1 and NMR2, Versions 1-4-1, supplied by New Methods Research Inc., Syracuse, NY.

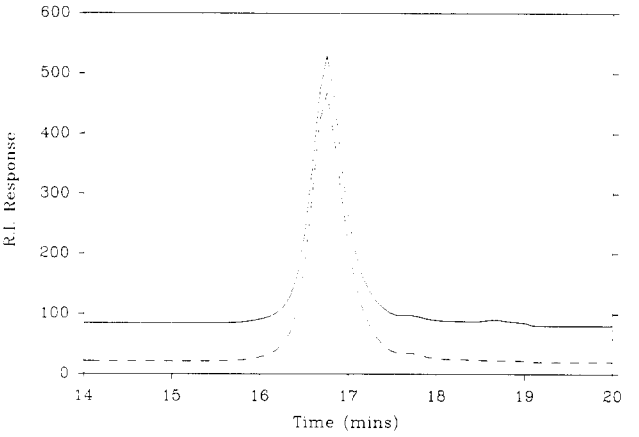


FIGURE 3: Comparison of the TSK 30 XL profiles for oligosaccharide [R6] (—) and oligosaccharide [R6] redigested with 0.004 unit of keratanase II (---). The column (30 × 7.8 cm) was eluted with 0.2 M NaCl at a flow rate of 0.5 mL/min at 30 °C and monitored on-line using the refractive index. V_o and V_t were 11.4 and 20.0 min, respectively.

RESULTS AND DISCUSSION

^{1}H-NMR Characterization of Selected Oligosaccharides. The 25 reduced keratanase II oligosaccharides that have been isolated from bovine articular cartilage keratan sulfate after keratanase II digestion and ion-exchange chromatography (Figure 2 and Table 1) and characterized by ^{1}H-NMR spectroscopy are shown in Figure 4. These have been categorized as deriving from the linkage [L] or the cap region [C] and from the non-fucosylated repeat [R] or the fucosylated repeat region [F]. In the present study no fucose-containing fragments are described that derive from the cap or linkage region, but in another study using the enzyme keratanase the latter type of fragment (Tai *et al.*, 1993) has been detected. The four oligosaccharides described below are a minimum subset which illustrate the mode of action of the enzyme.

The Monosulfated Disaccharide [R1]. From the findings of Nakazawa *et al.* (1989), it was anticipated that the major oligosaccharides produced by keratanase II digestion of articular cartilage keratan sulfates followed by borohydride

REPEAT SEQUENCE OLIGOSACCHARIDES	
Galβ1-4GlcNAc(6S)-ol	[R1]
Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol	[R2]
Gal(6S)β1-4GlcNAc(6S)-ol	[R3]
Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol	[R4]
Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[R5]
Gal(6S)β1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[R6]

FUCOSE-CONTAINING OLIGOSACCHARIDES	
Galβ1-4(Fucα1-3)GlcNAc(6S)-ol	[F1]
Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol	[F2]
Galβ1-4GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol	[F3]
Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol	[F4]
Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol	[F5]
Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[F6]

LINKAGE REGION OLIGOSACCHARIDES	
Galβ1-4GlcNAc(6S)β1-6(Galβ1-3)GalNAc-ol	[L1]
Galβ1-4GlcNAc(6S)β1-6(NeuAcα2-3Galβ1-3)GalNAc-ol	[L2]
Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-6(Galβ1-3)GalNAc-ol	[L3]
Gal(6S)β1-4GlcNAc(6S)β1-6(Galβ1-3)GalNAc-ol	[L4]
Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-6(NeuAcα2-3Galβ1-3)GalNAc-ol	[L5]
Gal(6S)β1-4GlcNAc(6S)β1-6(NeuAcα2-3Galβ1-3)GalNAc-ol	[L6]
Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-6(Galβ1-3)GalNAc-ol	[L7]
Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-6(NeuAcα2-3Galβ1-3)GalNAc-ol	[L8]

CAPPING OLIGOSACCHARIDES	
NeuAcα2-6Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol	[C1]
NeuAcα2-3Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol	[C2]
NeuAcα2-6Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[C3]
NeuAcα2-3Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[C4]
NeuAcα2-3Gal(6S)β1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol	[C5]

FIGURE 4: Structures of 25 reduced oligosaccharides isolated from bovine articular cartilage produced by keratanase II digestion/borohydride reduction.

reduction would be the disaccharides Galβ1–4GlcNAc(6S)-ol and Gal(6S)β1–4GlcNAc(6S)-ol.

The 600-MHz ^{1}H-NMR spectrum of oligosaccharide [R1] (Figure 5) shows the presence of a single anomeric resonance at 4.561 ppm and one *N*-acetyl methyl resonance at 2.064 ppm (not shown) suggesting that this oligosaccharide is a disaccharide (the *N*-acetylglucosaminitol residue has no anomeric resonance). COSY-45 data (Figure 6) show connections from this anomeric signal via H(2) at 3.575 ppm and H(3) at 3.673 ppm to a resonance at 3.942 ppm, which is readily identifiable as H(4) of a nonreducing terminal (i.e., C(3) unsubstituted) galactose residue (Dickenson *et al.*, 1991; Brown *et al.*, 1992). Although the $J(4,5)$ coupling is small (*ca.* 1 Hz), further connections can be seen to H(5) at 3.699 ppm and thence to H(6) and H(6′) at 3.779 and 3.814 ppm, respectively, the positions of which indicate that this residue is unsulfated on C(6) (Dickenson *et al.*, 1991). The nonequivalent methylene pair of protons occurring at 3.688 and 3.771 ppm can, therefore, be assigned to H(1) and H(1′), respectively, of *N*-acetylglucosaminitol. COSY-45 connections can be seen via H(2) at 4.293 ppm (perturbed downfield by substitution on C(2) by an *N*-acetyl group) through to the H(6) and H(6′) nonequivalent methylene protons at 4.265 and 4.330 ppm, respectively, these chemical shift positions

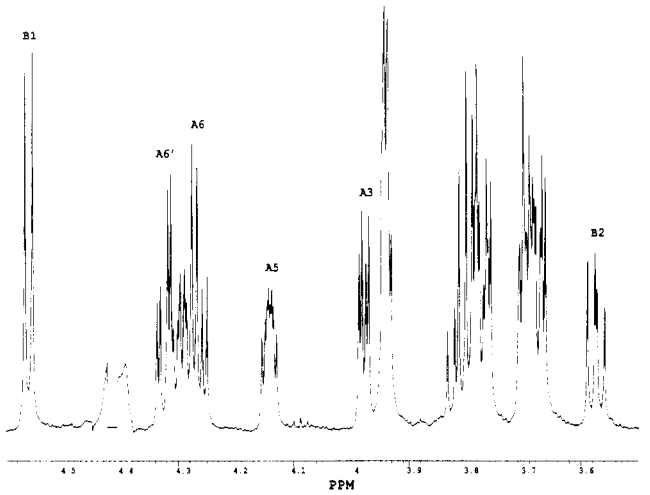


FIGURE 5: Partial 600-MHz ^{1}H-NMR spectra at 60 °C of oligosaccharide [R1], having the structure

Galβ-(1 → 4)-GlcNAc(6S)-ol
B A

The residual HO^{2}H signal has been artificially removed after processing for clarity.

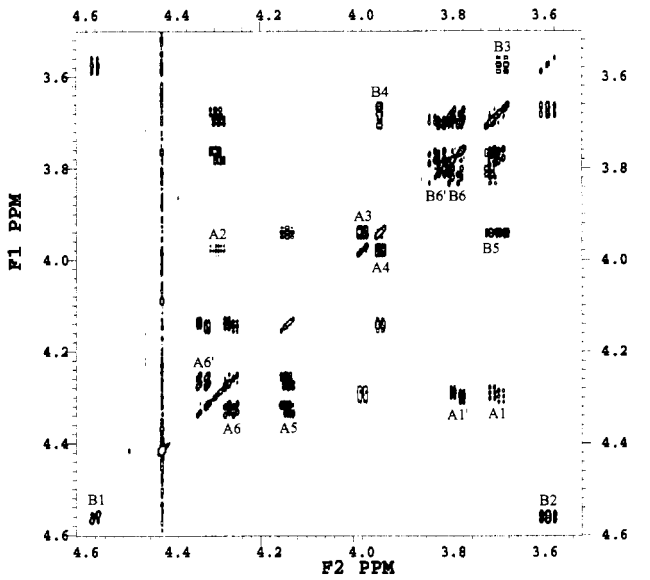


FIGURE 6: Partial 600-MHz COSY-45 spectrum of oligosaccharide [R1] at 60 °C.

Galβ-(1 → 4)-GlcNAc(6S)-ol
B A

The spectral width was 750.75 Hz, and 16 acquisitions for each of 256 increments were sampled into 512 complex points. Data were reprocessed for presentation and detailed analysis using the software package NMR2. The array was zero-filled to 1024 × 1024 complex points and transformed in each dimension after application of a (sinebell)^2 window function offset 2%.

being typical of a sulfated methylene group (Huckerby *et al.*, 1990).

Oligosaccharide [R1] is, therefore, the monosulfated disaccharide:

Galβ–(1 → 4)–GlcNAc(6S)–ol

The Difucosylated Hexasaccharide [F2]. The 600-MHz ^{1}H-NMR spectrum of [F2] (Figure 7) displays two *N*-acetyl methyl signals (not shown) and five anomeric resonances demonstrating that this is a hexasaccharide. The anomeric

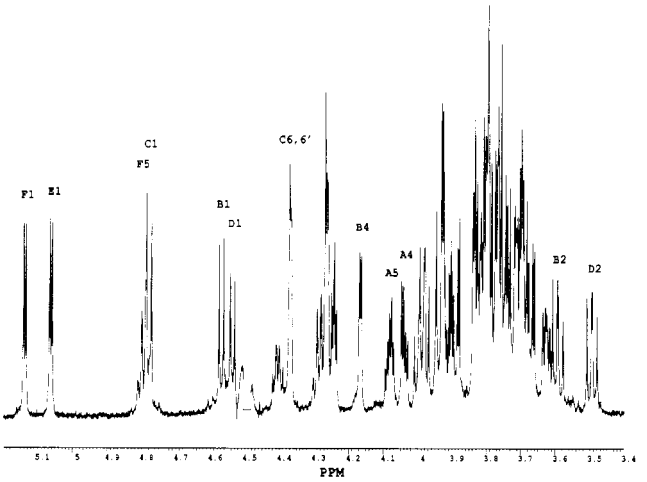


FIGURE 7: Partial 600-MHz ^{1}H-NMR spectrum at 55 °C of oligosaccharide [F2], having the structure

αFuc F αFuc E
1 1
↓ ↓
3 3
Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)-ol
D C B A

The residual HO^{2}H signal has been artificially removed after processing for clarity.

resonances at 5.066 and 5.141 ppm can be readily assigned to two α(1 → 3)-linked fucose residues (Vliegenthart *et al.*, 1983), and this is confirmed by COSY-45 data which indicate that the H(6) methyl protons resonate at 1.230 and 1.192 ppm, respectively. The chemical shifts for one of these residues (with H(1) at 5.141 ppm), assignable using COSY-45 data (Figure 8), are almost identical to those reported by Brown *et al.* (1992) for fucose in two pentasaccharides derived from hydrazinolysis/nitrous acid treatment of keratan sulfates, where the fucose residue is α(1 → 3)-linked to an internal (i.e., 4-*O*-substituted) *N*-acetylglucosamine residue. The presence of an internal *N*-acetylglucosamine residue is confirmed by 1-D and 2-D data, with the anomeric proton resonating at 4.785 ppm. The H(2) proton resonates at *ca.* 3.98 ppm, perturbed downfield by approximately 0.15–0.20 ppm from that in an *N*-acetylglucosamine which is substituted only (by galactose) on C(4) (Huckerby *et al.*, 1990, 1992). This indicates further substitution at C(3) by the fucose residue.

The H(1) and H(6) resonances of the second fucose (at 5.066 and 1.230 ppm, respectively) are noticeably different from those of fucose in intact keratan sulfate chains at 5.12 and 1.17 ppm (Thornton *et al.*, 1989; Huckerby *et al.*, 1991), indicating that the environment of this residue has changed. In addition, the H(5) resonance lies at 4.285 ppm, a perturbation upfield of almost 0.5 ppm from that usually found for fucose in KS (at *ca.* 4.76 ppm) (Huckerby *et al.*, 1991). Using 1-D and 2-D measurements, a complete set of chemical shift assignments can be identified for a 6-*O*-sulfated *N*-acetylglucosaminitol which is broadly comparable to that found for the monosulfated disaccharide [R1] (see above). However, H(2) (at 4.405 ppm), H(3) (at 4.244 ppm), and H(4) (at 4.040 ppm) all show a marked perturbation downfield, the largest of which is that for the H(3) proton, which has moved approximately 0.26 ppm. These data, together with the difference in the chemical shifts of the fucose protons, suggest that the *N*-acetylglucosaminitol residue is substituted at C(3) by the fucose.

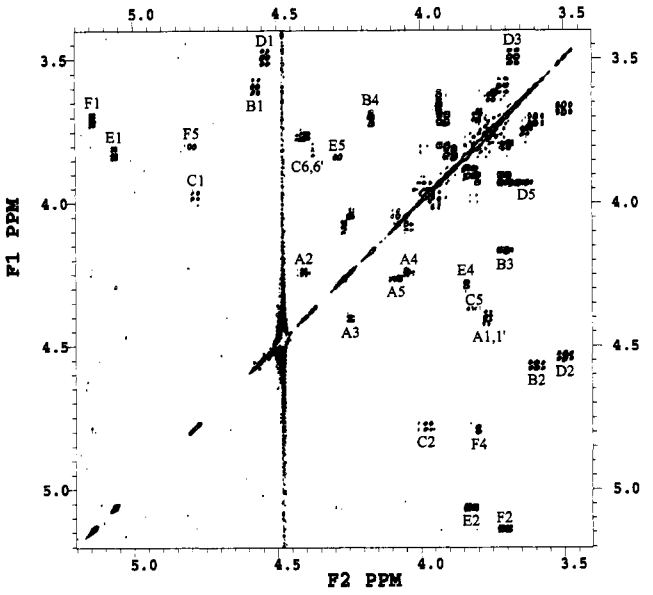


FIGURE 8: Partial 400-MHz COSY-45 spectrum of oligosaccharide [F2] at 55 °C.

αFuc F αFuc E
1 1
↓ ↓
3 3
Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)-ol
D C B A

The spectral width was 1679.5 Hz, and 32 acquisitions for each of 1024 increments were sampled into 1024 complex points. Data were reprocessed for presentation and detailed analysis using the software package NMR2. The array was zero-filled to 2048 × 2048 complex points and transformed in each dimension after application of a (sinebell)^2 window function offset 2%.

The resonance at 4.538 ppm can be readily identified as H(1) of a galactose residue, with COSY-45 data (Figure 8) providing almost complete chemical shift assignments for this residue. The H(4) resonance for this residue is located at 3.932 ppm, indicating that it occupies a nonreducing terminal (i.e., it is unsubstituted at C(3)) (Brown *et al.*, 1992; Huckerby *et al.*, 1992). A COSY-45 connection from H(5) (at *ca.* 3.63 ppm) locates H(6) at *ca.* 3.75 ppm, which by comparison with the data for [R1] confirms that this residue is unsulfated. The remaining anomeric resonance at 4.571 ppm can therefore be assigned to an internal galactose residue (between the *N*-acetylglucosamine and *N*-acetylglucosaminitol residues), and the location of H(4) at 4.168 ppm (shifted downfield by approximately 0.2 ppm due to substitution at C(3) by *N*-acetylglucosamine) confirms this assumption. This residue is also unsulfated, as evidenced by the H(5) and H(6) resonances at *ca.* 3.70 and *ca.* 3.80 ppm, respectively.

Oligosaccharide [F2] is therefore a difucosylated fragment with the structure

αFuc αFuc
1 1
↓ ↓
3 3
Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)-ol

The Hexasaccharide Linkage Region Fragment [*L7*]. Examination of the 600-MHz 1-D (Figure 9) and 2-D COSY-45 (Figure 10) spectra of oligosaccharide [L7] indicates the presence of three *N*-acetyl methyl resonances (not shown) and five anomeric resonances (one of which is obscured on the 1-D spectrum by the residual HO^{2}H signal). A complete set

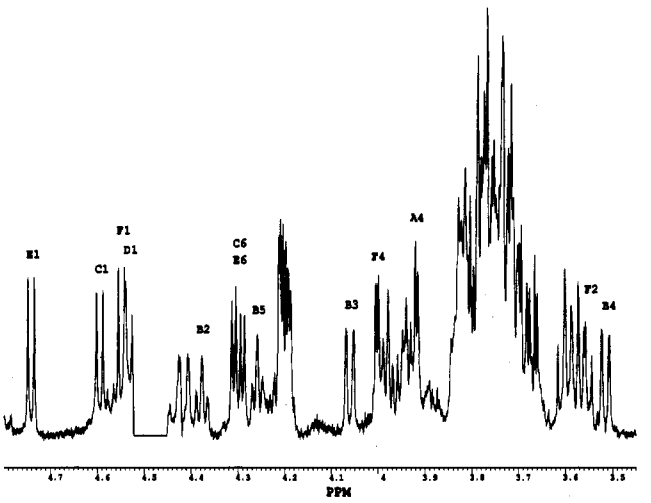


FIGURE 9: Partial 600-MHz ^{1}H-NMR spectrum at 55 °C of oligosaccharide [L7], having the structure

F E D C B
Gal(6S)β-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 6)-GalNAc-ol
3
↑
1
Galβ
A

The residual HO^{2}H signal has been artificially removed after processing for clarity.

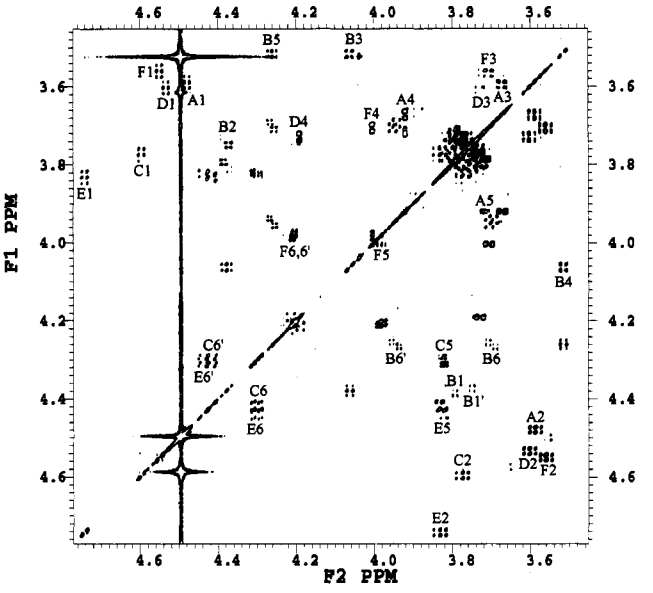


FIGURE 10: Partial 600-MHz COSY-45 spectrum of oligosaccharide [L7] at 55 °C.

F E D C B
Gal(6S)β-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 6)-GalNAc-ol
3
↑
1
Galβ
A

The spectral width was 1805.05 Hz, and 32 acquisitions for each of 808 increments were sampled into 1024 complex points. Data were reprocessed for presentation and detailed analysis using the software package NMR2. The array was zero-filled to 2048 × 2048 complex points and transformed in each dimension after application of a sinebell window function offset 2%.

of *N*-acetylgalactosaminitol signals can be identified with chemical shifts very similar to those reported in the literature [e.g., Tai *et al.* (1993), Dickenson *et al.* (1990), and Strecker

et al. (1987)] for an *N*-acetylgalactosaminitol residue linked at both C(6) and C(3). These findings suggest that [L7] is a hexasaccharide and is derived from the linkage region of the keratan sulfate chains.

The presence of an *N*-acetylglucosamine residue $\beta(1 \rightarrow 6)$-linked to *N*-acetylgalactosaminitol is indicated by the H(1) resonance at 4.595 ppm, and COSY data (Figure 10) provide a connection to H(2) at 3.775 ppm; however, further connections are lost within a complex envelope of resonances at *ca.* 3.7–3.8 ppm. This residue is clearly sulfated, as indicated by the relatively downfield location of the H(6) and H(6′) methylene proton resonances at 4.300 and 4.441 ppm, respectively (Huckerby *et al.*, 1992). The anomeric resonance at 4.481 ppm can be assigned to a galactose residue, and COSY-45 data provide connections as far as H(5) at *ca.* 3.715 ppm. The location of the H(4) resonance at 3.919 ppm confirms that this residue is unsubstituted at C(4). Comparison of the shift positions for H(1) to H(4) with those given by Dickenson *et al.* (1990) for a linkage region oligosaccharide indicate that this galactose is $\beta(1 \rightarrow 3)$-linked to the *N*-acetylgalactosamine residue.

The H(1) resonance at 4.742 ppm can be assigned to that of an internal *N*-acetylglucosamine residue. The H(6) and H(6′) methylene protons for this sugar resonate at 4.300 and 4.416 ppm, respectively, indicating that it is sulfated. Both 1-D and 2-D data permit identification of a galactose H(4) resonance at 4.003 ppm, this shift position being typical for a nonreducing terminal galactose (the anomeric resonance for this residue occurs at 4.549 ppm). A connection from H(4) to H(5) at 3.983 ppm is visible on the COSY-45 spectrum, with the H(6) and H(6′) methylene protons resonating at *ca.* 4.205 ppm. The relatively downfield location of these resonances indicates that this residue is sulfated on C(6) (Huckerby *et al.*, 1990). Finally, COSY-45 data show that the anomeric resonance at 4.533 ppm connects via H(2) and H(3) to an H(4) signal at 4.192 ppm, typical of an internal (i.e., 4-*O*-substituted) galactose (Huckerby *et al.*, 1992; Tai *et al.*, 1993). Unfortunately, a COSY-45 connection to H(5) is not observed; however, the complete assignment of all the resonances in the range 3.9–4.5 ppm indicates that no sulfated galactose methylene proton signals are present from this residue, suggesting that it is unsulfated.

Oligosaccharide [L7] is, therefore, the linkage region fragment:

Gal(6S)β-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 6)-GalNAc-ol
3
↑
1
Galβ

The Pentasaccharide Capping Fragment [*C5*]. Both the 1-D and 2-D spectra of oligosaccharide [C5] (Figures 11 and 12) display signals characteristic of a terminal *N*-acetylglucosaminitol residue with shift positions similar to those found for the monosulfated disaccharide [R1]. The H(6) and H(6′) protons resonate at 4.199 and 4.327 ppm, respectively, confirming that this residue is sulfated. In addition, the anomeric resonance at 4.763 ppm can be readily assigned to an internal *N*-acetylglucosamine, and COSY-45 data provide a connection to H(2) at *ca.* 3.825 ppm. As is often the case for such *N*-acetylglucosamine residues, further connections are lost within a complex envelope of resonances at 3.7–3.85 ppm; however, the COSY-45 spectrum clearly shows the presence of a nonequivalent methylene pair at 4.315 and 4.426 ppm which both connect to a resonance at *ca.* 3.825 ppm. These shift positions are typical of the H(6), H(6′), and H(5)

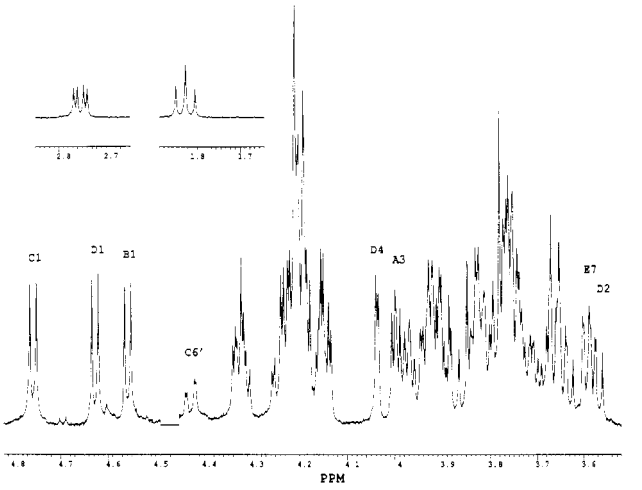


FIGURE 11: Partial 600-MHz ^{1}H-NMR spectrum at 60 °C of oligosaccharide [C5], having the structure

NeuAcα-(2 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)β-
E D C
(1 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)-ol
B A

Sialic acid H(3ax) and H(3eq) resonances are also shown (inset). The residual HO^{2}H signal has been artificially removed after processing for clarity.

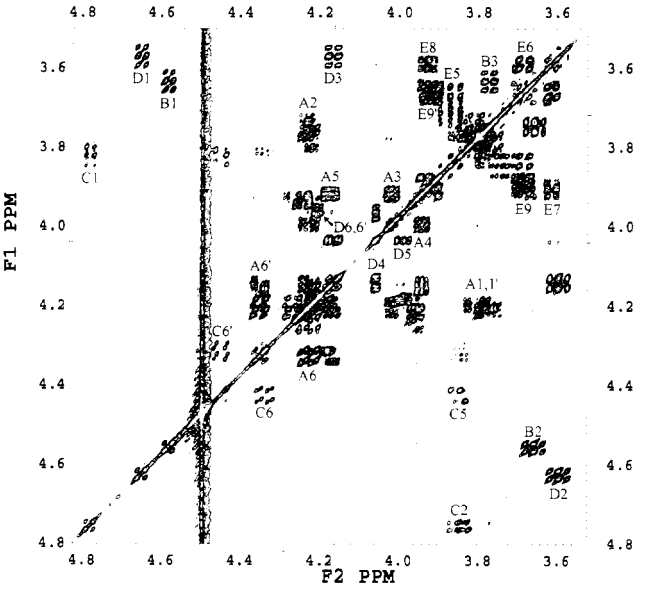


FIGURE 12: Partial 400-MHz COSY-45 spectrum of oligosaccharide [C5] at 60 °C.

NeuAcα-(2 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)β-
E D C
(1 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)-ol
B A

The spectral width was 1299.3 Hz, and 32 acquisitions for each of 880 increments were sampled into 1024 complex points. Data were reprocessed for presentation and detailed analysis using the software package NMR2. The array was zero-filled to 2048 × 2048 complex points and transformed in each dimension after application of a (sinebell)^2 window function offset 2%.

protons, respectively, of a 6-*O*-sulfated *N*-acetylglucosamine residue (Huckerby *et al.*, 1992).

The resonances at 1.814 and 2.754 ppm are typical of the H(3ax) and H(3eq) protons of sialic acid in an $\alpha(2 \rightarrow 3)$-linkage to galactose (Vliegenthart *et al.*, 1983; Dickenson *et al.*, 1991). This linkage type is confirmed by the identification

Table 2: Proton Chemical Shift Assignments for Oligosaccharides [R1] and [C5] at 60 °C and [F2] and [L7] at 55 °C

Galβ-(1 → 4)-GlcNAc-ol[R1]

B A

Gal B				GlcNAc–ol A			
H(1)	4.561	H(5)	3.699	H(1)	3.688	H(5)	4.142
H(2)	3.575	H(6)	3.779	H(1′)	3.771	H(6)	4.265
H(3)	3.673	H(6′)	3.814	H(2)	4.293	H(6′)	4.330
H(4)	3.942			H(3)	3.979	NAc	2.064
				H(4)	3.940		

αFuc F (1 → 3) and αFuc E (1 → 3)

Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)-ol [F2]

D C B A

Fuc F		Fuc E		Gal D		GlcNAc C		Gal B		GlcNAc–ol A	
H(1)	5.141	H(1)	5.066	H(1)	4.538	H(1)	4.785	H(1)	4.571	H(1)	~3.765
H(2)	3.703	H(2)	3.818	H(2)	3.497	H(2)	~3.98	H(2)	3.597	H(1′)	~3.765
H(3)	3.916	H(3)	3.893	H(3)	3.672	H(3)		H(3)	3.708	H(2)	4.405
H(4)	3.797	H(4)	3.836	H(4)	3.932	H(4)		H(4)	4.168	H(3)	4.244
H(5)	4.792	H(5)	4.285	H(5)	~3.63	H(5)	~3.81	H(5)	~3.70	H(4)	4.040
Me	1.192	Me	1.230	H(6)	~3.75	H(6)	~4.37	H(6)	~3.80	H(5)	4.083
				H(6′)		H(6′)	~4.37	H(6′)		H(6)	~4.265
						NAc	2.041			H(6′)	~4.265
										NAc	2.054

F E D C B

Galβ(6S)β-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Galβ-(1 → 4)-GlcNAc(6S)β-(1 → 6)-GalNAc-ol [L7]

1

3

Gal^{c}β

A

Gal F		GlcNAc E		Gal D		GlcNAc C		GalNAc–ol B		Gal A	
H(1)	4.549	H(1)	4.742	H(1)	4.533	H(1)	4.595	H(1)	3.740	H(1)	4.481
H(2)	3.559	H(2)	3.832	H(2)	3.603	H(2)	3.775	H(1′)	3.804	H(2)	3.588
H(3)	3.707	H(3)		H(3)	3.730	H(3)		H(2)	4.380	H(3)	3.674
H(4)	4.003	H(4)		H(4)	4.192	H(4)		H(3)	4.061	H(4)	3.919
H(5)	3.983	H(5)	3.832	H(5)		H(5)	3.825	H(4)	3.516	H(5)	~3.715
H(6)	~4.205	H(6)	4.300	H(6)		H(6)	4.300	H(5)	4.260	H(6)	
H(6′)	~4.205	H(6′)	4.416	H(6′)		H(6′)	4.441	H(6)	3.701	H(6′)	
		NAc	2.049			NAc	2.065	H(6′)	3.948		
							NAc	2.071			

NeuAcα-(2 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)β-(1 → 3)-Gal(6S)β-(1 → 4)-GlcNAc(6S)-ol [C5]

E D C B A

NeuAc E		Gal D		GlcNAc C		Gal B		GlcNAc–ol A	
H(3ax)	1.814	H(1)	4.626	H(1)	4.763	H(1)	4.560	H(1)	~3.75
H(3eq)	2.754	H(2)	3.581	H(2)	~3.825	H(2)	3.640	H(1′)	~3.78
H(4)	~3.715	H(3)	4.143	H(3)		H(3)	3.744	H(2)	~4.2
H(5)	~3.845	H(4)	4.039	H(4)		H(4)	~4.19	H(3)	3.999
H(6)	3.672	H(5)	~3.965	H(5)	~3.825	H(5)	3.935	H(4)	3.920
H(7)	3.590	H(6)	~4.18	H(6)	4.315	H(6)	~4.21	H(5)	4.155
H(8)	3.903	H(6′)	~4.18	H(6′)	4.426	H(6′)	~4.24	H(6)	4.199
H(9)	3.668			NAc	2.044			H(6′)	4.327
H(9′)	~3.89							NAc	2.071
NAc	2.039								

of a galactose residue (H(1) at 4.626 ppm) with an H(3) resonance at 4.143 ppm. This signal is perturbed characteristically downfield by approximately 0.4 ppm from that in either a terminal or an internal galactose residue (Huckerby *et al.*, 1991, 1992). Further connections around the ring are also visible on the COSY spectrum, and close examination indicates that the H(6) and H(6′) protons of this sialylated galactose both occur at *ca.* 4.18 ppm, thus demonstrating that this residue is sulfated. The remaining anomeric resonance at 4.560 ppm can therefore be assigned to an internal galactose residue, and the location of the H(4) resonance at *ca.* 4.19 ppm confirms that it is indeed substituted (by *N*-acetylglucosamine) on C(3). The H(6) and H(6′) signals for this residue occur at *ca.* 4.21 and *ca.* 4.24 ppm, respectively, indicating that this galactose is also sulfated.

Oligosaccharide [C5] is therefore a nonreducing terminal fragment with the structure

NeuAcα–(2 → 3)–Gal(6S)β–(1 → 4)–GlcNAc(6S)β–
(1 → 3)–Gal(6S)β–(1 → 4)–GlcNAc(6S)–ol

Proton chemical shift assignments for oligosaccharides [R1], [F2], [L7], and [C5] are given in Table 2.

Oligosaccharide Structures and Enzyme Substrate Specificity. Inspection of the structures shows that all oligosaccharides except those from the linkage region (which possess a terminal *N*-acetylgalactosaminitol) contain a reducing terminal sulfated *N*-acetylglucosaminitol deriving from the

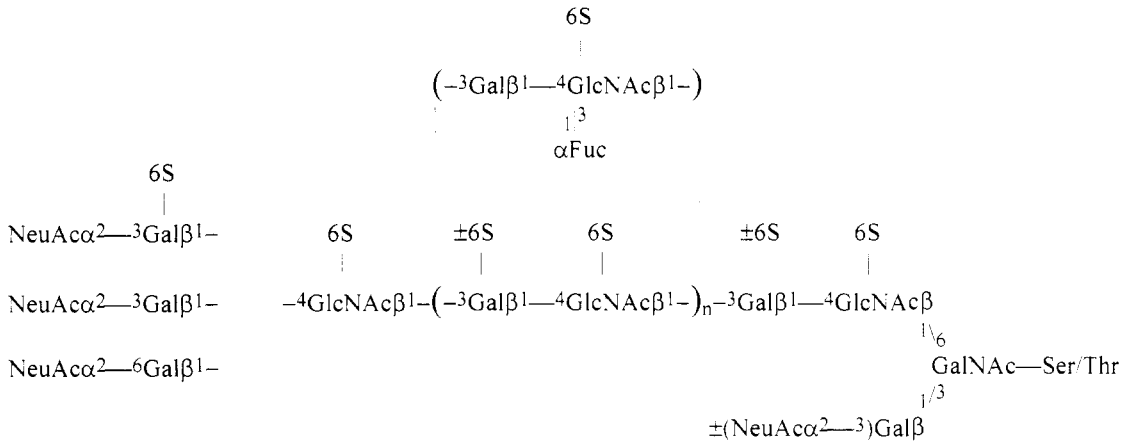


FIGURE 13: Structural model for skeletal keratan sulfate (KS-II-A) from articular cartilage.

N-acetylglucosamine at the site of cleavage. Similarly, all structures except those deriving from the caps contain a nonreducing terminal galactose or galactose 6-sulfate. It is, therefore, clear that the enzyme cleaves the $\beta(1 \rightarrow 3)$-glycosidic bond between *N*-acetylglucosamine 6-sulfate and galactose (sulfated or not). None of the oligosaccharides examined contain an unsulfated *N*-acetylglucosamine, so it must be assumed that this residue is predominantly sulfated in the parent keratan sulfate.

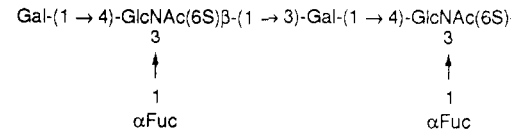
Further study of the structures shows that many of the oligosaccharides are larger than might have been expected. Thus, the repeat sequence (sulfated poly(*N*-acetyllactosamine)) oligosaccharides (see Figure 4) include four tetrasaccharides (in addition to the two disaccharides) which contain a potentially cleavable bond. Confirmation that these tetrasaccharides cannot be degraded further under these conditions comes from the experiment in which the tetrasaccharide [R6] was resubjected to keratanase II treatment (Figure 3). Clearly, therefore, the enzyme has a specific substrate size requirement, so that if a tetrasaccharide is produced, it is then too small to be degraded further. This is also observed for the fucosylated repeat region oligosaccharides where penta- and hexasaccharides (e.g., [F2]) occur in addition to the trisaccharide. It is noteworthy that the presence of an $\alpha(1 \rightarrow 3)$-linked fucose residue on the sulfated *N*-acetylglucosamine does not prevent keratanase II cleavage at this site, in contrast with the other keratan sulfate fragmentation procedures of hydrazinolysis/nitrous acid treatment (Brown *et al.*, 1992) and keratanase cleavage (Tai *et al.*, 1993) where the fucose protects the cleavage site, giving rise to larger fragments.

In the case of the sialic acid-containing capping oligosaccharides (e.g., [C5]) pentasaccharides are recovered which possess a potentially cleavable bond. Clearly, the *O*-glycosidic linkage of the internal sulfated *N*-acetylglucosamine is keratanase II resistant, presumably because of the proximity of the sialic acid residue.

Implications for Keratan Sulfate Structure. This study has revealed the presence of two new elements of KS structure, namely, the new chain capping sequence

NeuAcα–(2 → 3)–Gal(6S)–(1 → 4)–GlcNAc(6S)–
(1 → 3)–Gal(6S)–(1 → 4)–GlcNAc(6S)–

containing a sulfated galactose adjacent to the sialic acid, similar to a structure isolated from recombinant human tissue plasminogen activator expressed in mouse epithelial cells (Pfeiffer *et al.*, 1992), and the difucosylated sequence



High concentrations of this fragment would seem to be a good indicator of high fucosylation levels. It remains unclear whether or not the fucose is uniformly distributed among the whole of the KS chain population. An earlier study has shown that the fucose contents of chains capped with $\alpha(2 \rightarrow 6)$-linked sialic acid were identical to those of the remainder, which were predominantly capped with $\alpha(2 \rightarrow 3)$-linked sialic acid (Tai *et al.*, 1992).

None of the degradative methods, namely, keratanase II cleavage, keratanase cleavage, or hydrazinolysis/nitrous acid treatment, so far investigated in this laboratory have yielded oligosaccharides from skeletal KS which contain unsulfated *N*-acetylglucosamines.

The current model for articular cartilage keratan sulfate chains from bovine or human sources is shown in Figure 13.

Potential Keratan Sulfate Fingerprinting. Keratan sulfate structure is very similar to the core 2 (Carlsson *et al.*, 1986; Fukuda *et al.*, 1986) O-linked oligosaccharides found on leukocyte surfaces, but KS is highly sulfated on *N*-acetylglucosamine. Many of the structures identified in this study, such as the sulfated Le^x and di-Le^x as well as the sialylated oligosaccharides, closely resemble the ligands recognized by selectins (Varki, 1992). Indeed, the L-selectin recognizes sulfated, fucosylated, sialylated structures (Yasuyuki & Rosen, 1993). It therefore seems likely that the enzyme keratanase II could be used for structural examination in studies of sulfated carbohydrate ligands of selectins.

The three major methods available for fragmentation of keratan sulfates are hydrazinolysis/nitrous acid treatment, keratanase (or *endo*-β-galactosidase) cleavage, and keratanase II cleavage. Their comparative merits partially depend upon the structure of the KS to be studied, and the following comments refer to studies of skeletal KSs from articular cartilages.

First, the enzymatic fragmentation methods specifically degrade most keratan sulfates (although some chains will be resistant to either or both enzymes); however, the chemical hydrazinolysis/nitrous acid method will degrade any glycosaminoglycan, and hence a pure KS sample is a prerequisite. Second, the enzyme methods are convenient to use and do not require the extensive safety precautions necessary for the hydrazinolysis/nitrous acid procedure.

Hydrazinolysis/nitrous acid permits cleavage at each *N*-acetylglucosamine except where the initial de-*N*-acetylation step is prevented by the presence of fucose on this residue (Brown *et al.*, 1992). Consequently, this method produces a few very small fragments (i.e., 2–5 saccharides long). Analysis of these fragments permits good quantitation of gross chemical composition (e.g., fucose and sialic acid contents and galactose sulfation levels), but yields no information on KS block structure.

Keratanase cleaves at an unsulfated galactose if the galactose is not adjacent to a fucosylated *N*-acetylglucosamine (Tai *et al.*, 1993). Therefore, fragment size is critically dependent upon the levels of fucosylation and galactose sulfation. With articular cartilage KSs, fragment sizes are in a broad range (e.g., 2–24 saccharides long). In principle, keratanase fragmentation permits a study of the keratanase-resistant block structures. However, this procedure does not lend itself to analysis of gross chemical composition because there is more than one cleavage criterion (lack of galactose sulfation and lack of proximity to fucose) and a large number of fragments are formed.

Keratanase II cleaves at a sulfated *N*-acetylglucosamine (Nakazawa *et al.*, 1989). The keratanase II limit digest fragments from bovine articular cartilage KSs are in the size range of 2–7 saccharides long. No oligosaccharides containing unsulfated *N*-acetylglucosamines were isolated, and thus no information was obtained about such keratanase II-resistant block structures in this study. However, some fragments are recovered which clearly possess potentially susceptible bonds. Apparently, such oligosaccharides were too small to be adequate substrates for the enzyme, or in the cases of the pentasaccharide capping fragments the presence of a nearby sialic acid seems to have prevented cleavage. This aspect of the enzyme's action has the disadvantage that it is difficult to use digestion chromatograms for quantitation of gross chemical composition. However, larger elements of structure may be retained (e.g., the difucosylated hexasaccharide), and many of the fragments are similar in size to the epitopes recognized by antibodies. Thus, if a keratanase II fragment was shown to be a good marker for disease, then antibodies and antibody-based assays could be developed.

In summary, it is interesting to consider the relative merits of these methods for the structural "fingerprinting" of keratan sulfates. Hydrazinolysis/nitrous acid treatment requires pure KS samples and stringent safety precautions, but produces few oligosaccharides from which gross chemical composition can be obtained. Keratanase produces oligosaccharides with a wide range of sizes and charge densities posing formidable problems in their chromatographic resolution and identification. Keratanase II fragmentation yields a compromise between these two extremes, and the resultant oligosaccharides are sufficiently small to permit their chromatographic separation and identification.

Clearly, the enzyme keratanase II is of considerable value to further structural studies of keratan sulfates, because the availability of characterized fragments will permit the structural analysis of keratan sulfates (and, perhaps, cell-surface-derived sulfated poly(*N*-acetyllactosaminoglycans)) which are only available in very small quantities. Thus, the use of high-resolution chromatographic or electrophoretic methods coupled with the sensitivity provided by tritium incorporation upon oligosaccharide reduction should permit the structural fingerprinting of subnanogram quantities of KS.

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