Carbohydrate Research, 255 (1994) 303–309
Elsevier Science B.V., Amsterdam

Note

600 MHz ^{1}H NMR study of a fucose-containing heptasaccharide derived from a keratanase digestion of bovine articular cartilage keratan sulphate †

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(Received March 8th, 1993; accepted August 12th, 1993)

Keratan sulphate (KS) is a glycosaminoglycan that has been classified[1] into two major types: KS-I which is *N*-linked and is found in cornea, and KS-II which is *O*-linked to protein and occurs in skeletal tissues such as cartilage. Furthermore, skeletal keratan sulphates have been sub-classified[2] into KS-II-A (articular) which occurs in load-bearing cartilages, and KS-II-B (basic) that occurs in nonload-bearing cartilages such as nasal septum. The distinction arises from the additional presence of the structural components, α-L-fucose and α-(2 → 6)-linked *N*-acetylneuraminic acids, in KS-II-A. These components have both been shown to occur in nonreducing terminal positions, with the fucose[3–5] being a pendant group on the main poly-*N*-acetyllactosamine repeat sequence and the α-(2 → 6)-linked sialic acid being a chain terminus or cap[6,7].

Studies of keratan sulphates have been of particular significance in recent years because the molecules, or antibodies that recognise structures within them, have been investigated as potential early markers for the disease of osteoarthritis[8,9].

To gain further insights into KS structure there have been a number of structural[10] and NMR spectroscopic studies[11], as well as further emphasis upon the development and understanding of KS fragmentation methods and their products that include the chemical treatment, hydrazinolysis–nitrous acid[5,12], and the enzymes, keratanase[13–16] and keratanase II[17].

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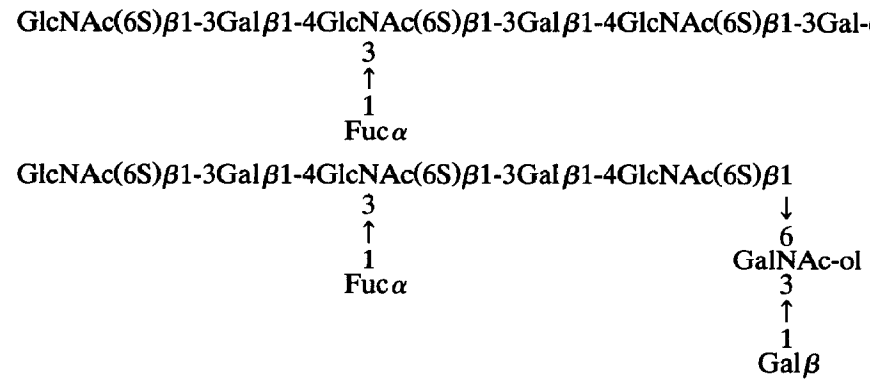
† Abbreviations: KS, keratan sulphate; 6S, 6-sulphate; COSY, two-dimensional homonuclear chemical-shift correlation spectroscopy; TSP, sodium 3-trimethylsilyl[$^2H_4$]propionate.

SSDI 0008-6215(93)E0374-A

In a recent study[16] of two large fucose-containing oligosaccharides derived by keratanase digestion of bovine femoral head cartilage keratan sulphates were shown to have the following structures:



In this investigation a third fucose-containing keratanase fragment is characterised.

EXPERIMENTAL

Materials.—The chemicals and enzymes used in this study were as previously described[15], except that the sialidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, was from Boehringer Mannheim UK (Lewes, East Sussex, UK) and chondroitin AC lyase (*Arthrobacter aurescens*) was from Sigma (Dorset, UK).

Methods.—High performance liquid chromatography (HPLC) was carried out on a Bio-Rad series 700 HRLC gradient and isocratic system using UV and refractive index detectors.

Keratan sulphate preparation.—Keratan sulphate chains were prepared from bovine femoral head cartilage (6–8 year-old animals) as previously described[14,16]. Briefly, this involved 4 M guanidine hydrochloride extraction of the proteoglycans in the presence of protease inhibitors, plus associative CsCl density gradient centrifugation. Keratan sulphate-rich region fragments were isolated after proteoglycan aggregate digestion with chondroitin ABC lyase followed by trypsin treatment and chromatography on a Sepharose CL-6B column. The KS-rich regions were separated and the KS chains released by alkaline-borohydride reduction. After a further treatment with chondroitin AC lyase and size-exclusion chromatography on Sephadex G-50 followed by ion-exchange chromatography on a Mono-Q MR 10/10 column a pure KS chain preparation was obtained.

Keratanase digestion and oligosaccharide isolation.—Keratan sulphate (90 mg) was dissolved in 0.2 M sodium acetate, pH 7.4, containing 5 mM sialidase inhibitor and incubated with 30 units (an excess) of keratanase (*Pseudomonas sp.*) at 37°C for 24 h. The digest was reduced with sodium borohydride and then recovered by desalting on a Bio-Gel P2 column and lyophilisation. The reduced keratanase digest was then chromatographed upon a Mono-Q MR 10/10 column, and the

oligosaccharides eluting between 0–0.1 M $LiClO_4$ were recovered and further fractionated on a Nucleosil 5SB column (250 × 10 mm), eluting with a 0–0.5 M $LiClO_4$ gradient over 240 min. Twelve fragments were recovered and the oligosaccharide I is that represented as number 12 (ref 16).

NMR spectroscopy.—Proton NMR spectra were measured at 55°C on a Bruker AMX600 spectrometer operating at 600.14 MHz. The sample (130 μg) was buffered to pH 7 with phosphate and referenced with TSP-d_4. Following microfiltration, several exchanges with 99.8% D_2O and one with 99.96% D_2O, the final dissolution was in 0.5 mL of 99.96% D_2O. For 1D measurements, the residual HOD resonance was suppressed using gated decoupling. A two-dimensional COSY-45 spectrum was measured with a spectral width of 2551 Hz, and 64 acquisitions of 496 increments were sampled into 1024 complex points. Data were transferred to a VAX 4000 computer and processed for presentation and detailed analysis using the software package NMR2 (New Methods Research Inc., Syracuse, NY, USA) The array was zero-filled to 2048 × 2048 complex points, and transformed in each dimension after application of a 5% offset sine bell-squared window function.

RESULTS AND DISCUSSION

A partial ^1H NMR spectrum for oligosaccharide I is shown in Fig. 1, and a partial 2D COSY-45 spectrum for the same spectral range is given in Fig. 2. Evidence for the presence of a single α-(1 → 3)-linked fucose residue is provided by resonances at 1.166 ppm (H-6) and 5.121 ppm (H-1) (not shown). On the COSY-45 spectrum, connections round the complete fucose ring are observable; values for these and other chemical shifts are summarised in Table I. This oligosaccharide is derived from the KS polymer by keratanase digestion followed by reduction and consequently will contain a galactitol reducing terminal residue if it has been cleaved from within the chain. Characteristic resonances for such a residue are clearly observed on the 2D spectrum[15]. Similarly, a full set of signals corresponding to a nonreducing terminal sulphated *N*-acetylglucosamine residue can be assigned[18]. Between 2.0 and 2.1 ppm, three well resolved *N*-acetyl methyl signals are seen. This provides strong evidence that the structure for I is a heptasaccharide, containing one fucose, three *N*-acetylglucosamines, two galactoses, and one galactitol residue. Indeed, six anomeric resonances are found in the range 4.5 to 5.2 ppm

By analogy with other keratanase-derived oligosaccharides[15,16] a connected set of signals starting from an anomeric proton at 4.651 ppm may be assigned to the *N*-acetylglucosamine residue adjacent to the reducing terminal galactitol. Also, a connected series of H-1 (at 4.540 ppm) to H-5 resonances correspond to a galactose sited next to the nonreducing terminal *N*-acetylglucosamine. Two residues remain to be considered. The first is the *N*-acetylglucosamine to which the fucose must be attached. The chemical shift positions observed for this are similar to

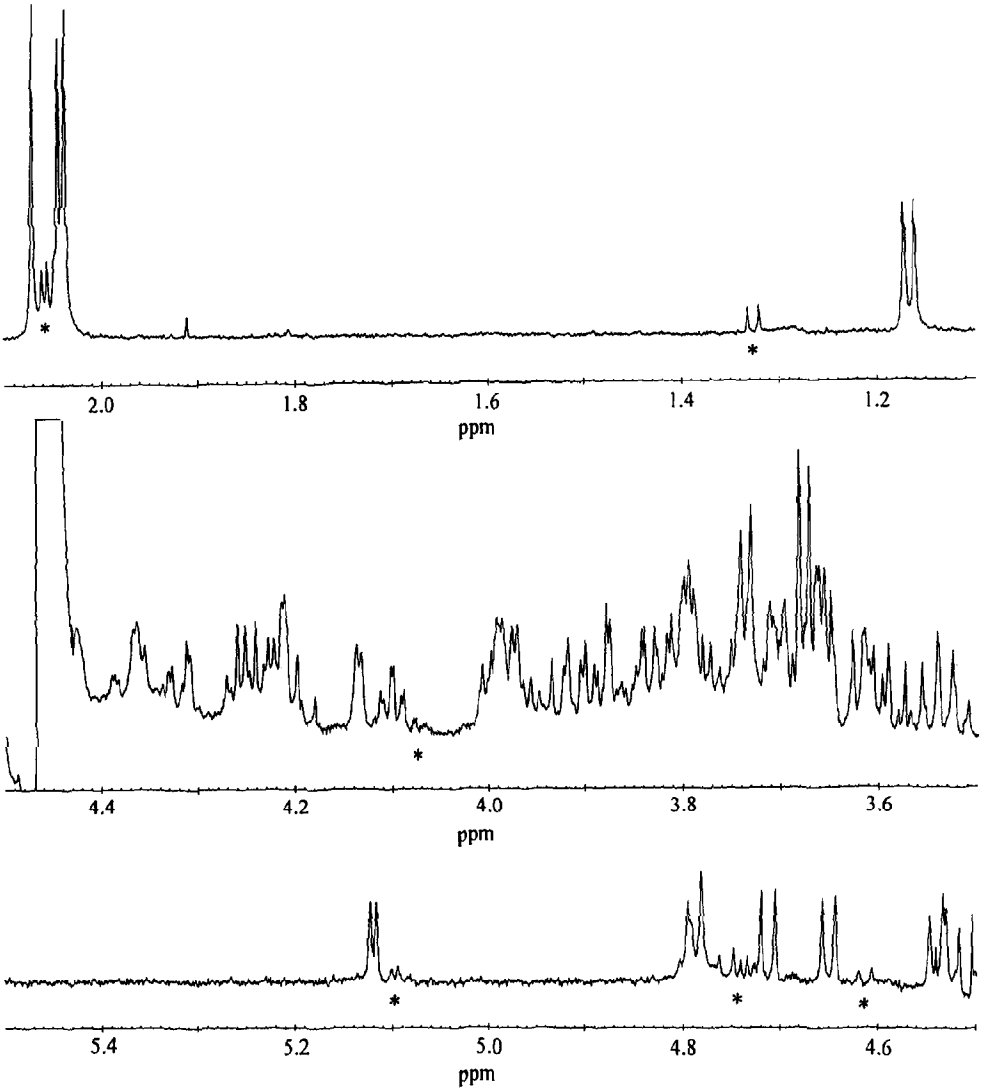


Fig. 1. Partial 600 MHz NMR spectrum for oligosaccharide I at 55°C. *, Signals derived from a contaminant.

those already found for the corresponding residue in a trisulphated fucose-containing heptasaccharide[16], and also for that in the two fucosylated pentasaccharides[5] below,

Galβ1-4GlcNAc(6S)β1-3Gal(6X)β1-4AnMan-ol(6S) (X = OH or SO_4)

3
↑
1
Fucα

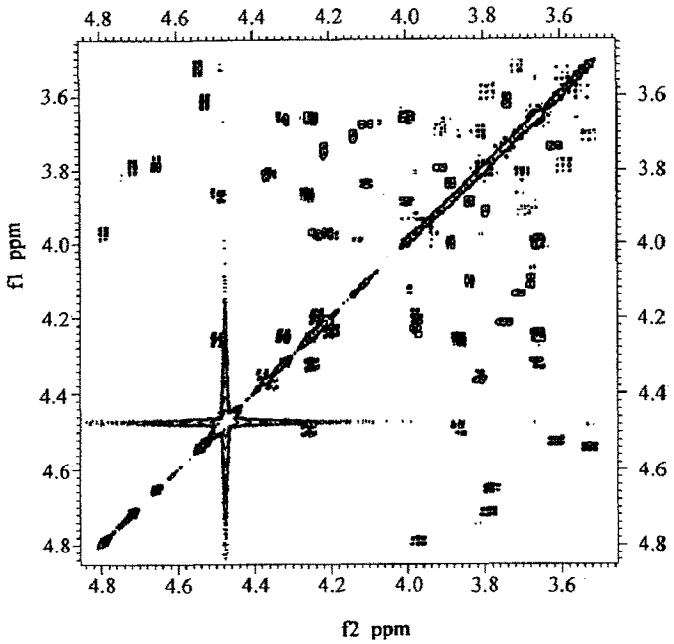


Fig. 2. Partial COSY-45 spectrum for oligosaccharide I at 55°C.

derived from KS by hydrazinolysis and nitrous acid cleavage. The other is a galactose, and a complete set of interproton connections demonstrate that this residue is 6-sulphated[5]. Overall, the observed shift values are similar to those found for the trisulphated fucose-containing heptasaccharide[16], except that one galactose is now seen to be sulphated. The fucosylated *N*-acetylglucosamine is attached at C-1 to this sulphated galactose, and this disaccharide unit is sited at the centre of oligosaccharide **I**, which has, therefore, the complete structure given below.

GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)β1-3Gal-ol **I**

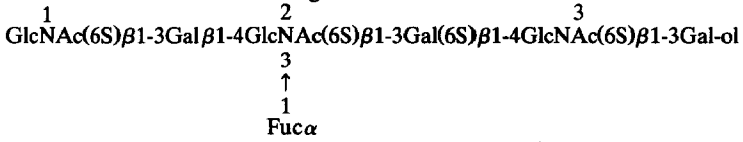
3
↑
1
Fucα

The significance of this structure and that of the other two characterised fucose-containing oligosaccharides[16] is that the enzyme keratanase produces two types of large keratanase-resistant fragments as a result of both galactose sulphation and the presence of fucose, which inhibits keratanase cleavage at the galactose residues on either side of the *N*-acetylglucosamine residue to which the fucose is attached.

Characterised KS fragments can be used as standards in the development of structural fingerprinting methods for keratan sulphates.

TABLE I

^{1}H NMR chemical shifts for oligosaccharide I



Proton	Chemical shifts [a,b] (ppm)		
	GlcNAc(6S) 1	GlcNAc(6S) 2	GlcNAc(6S) 3
H-1	[c] 4.714	4.790	4.651
H-2	3.788	3.972	3.781
H-3	3.591	3.918	3.813
H-4	3.540	3.994	3.697
H-5	3.661	3.810	3.865
H-6a	4.245	4.350	4.259
H-6b	4.320	4.375	4.494
	Gal	Gal(6S)	
H-1	[c] 4.540	4.524	
H-2	3.525	3.612	
H-3	3.709	3.740	
H-4	4.138	4.213	
H-5	~3.72	3.974	
H-6a		4.197	
H-6b			
	Fuc		
H-1	[c] 5.121		
H-2	3.694		
H-3	3.911		
H-4	3.793		
H-5	4.788		
H-6	[c] 1.166		
	Gal-ol		
H-1,1′	~3.657		
H-2	4.000		
H-3	3.884		
H-4	3.835		
H-5	4.102		
H-6,6	~3.677		

[a] Chemical shifts were measured at 55°C relative to internal TSP-d_4. [b] *N*-Acetyl methyl signals: 2.037, 2.044, and 2.071 ppm. [c] Shifts determined from 1D spectrum; all other values derive from 2D cross-peak positions.

ACKNOWLEDGMENTS

We thank the Medical Research Council and the Arthritis and Rheumatism Council for financial support. The Science and Engineering Research Council is thanked for time and for travel funds in respect of use of their Biological NMR Facility at Leicester University. Bev Abram is thanked for technical assistance.

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