Glycoconjugate Journal (1996) **13:** 609–619

Preparation of a series of sulfated tetrasaccharides from shark cartilage chondroitin sulfate D using testicular hyaluronidase and structure determination by 500 MHz ^{1}H NMR spectroscopy

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Received 2 October 1995, revised 20 November 1995

Six tetrasaccharide fractions were isolated from shark cartilage chondroitin sulfate D by gel filtration chromatography followed by HPLC on an amine-bound silica column after exhaustive digestion with testicular hyaluronidase. Their structures were determined unambiguously by one- and two-dimensional 500 MHz ^{1}H NMR spectroscopy in conjunction with HPLC analysis of chondroitinase AC-II digests of the tetrasaccharides. One fraction was found to contain two tetrasaccharide components. All the seven tetrasaccharides shared the common core structure GlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc with various sulfation profiles. Four were disulfated comprising of two monosulfated disaccharide units GlcAβ1-3GalNAc(4-sulfate) and/or GlcAβ1-3GalNAc(6-sulfate), whereas the other three were hitherto unreported trisulfated tetrasaccharides containing a disulfated disaccharide unit GlcA(2-sulfate)β1-3GalNAc(6-sulfate) and a monosulfated disaccharide unit GlcAβ1-3GalNAc(4- or 6-sulfate). These sulfated tetrasaccharides were demonstrated to serve as appropriate acceptor substrates for serum α-*N*-acetylgalactosaminyltransferase, indicating their usefulness as authentic oligosaccharide substrates or probes for the glycobiology of sulfated glycosaminoglycans.

Keywords: chondroitin sulfate, sulfated tetrasaccharides, 500 MHz ^{1}H NMR

Abbreviations: NFU, National formulary unit; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn; 1D or 2D, one- or two-dimensional; IdoA, L-iduronic acid; ΔGlcA, D-*gluco*-4-enepyranosyluronic acid; ΔDi-0S, ΔGlcAβ1-3GalNAc; Di-4S, GlcAβ1-3GalNAc(4-sulfate); ΔDi-4S, ΔGlcAβ1-3GalNAc(4-sulfate); Di-6S, GlcAβ1-3GalNAc(6-sulfate); ΔDi-6S, ΔGlcAβ1-3GalNAc(6-sulfate); ΔDi-diS_D, ΔGlcAβ(2-sulfate)α1-3GalNAc(6-sulfate); ΔDi-diS_E, ΔGlcAβ1-3GalNAc(4, 6-disulfate); ΔU, G, U, 2S, 4S, and 6S represent ΔGlcA, GalNAc, GlcA, 2-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively.

Introduction

Accumulating evidence indicates that chondroitin sulfate proteoglycans are widely distributed among various tissues and exhibit a wide variety of biological functions. They are ubiquitous components of the extracellular matrix of connective tissues and are also found at the surface of many cell types and in intracellular secretory granules (for reviews see [1–3]).

Immunological studies using monoclonal antibodies directed against chondroitin sulfate have revealed the developmentally regulated expression of the epitopes in the rodent fetus [4, 5] (for a review see [6]). Some of these epitopes are distributed differentially in distinct tissues and in functionally distinct domains within these tissues such as adult human skin [7]. Furthermore, chondroitin sulfate proteoglycans are differentially expressed during rat central nervous system development [8]. Chondroitin/dermatan sulfate from mouse brain, which is recognized by a specific monoclonal antibody, promotes neurite outgrowth of neurons from the rat central nervous system [9].

Developmentally regulated expression and tissue-specific distribution of chondroitin sulfate isoforms suggest

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that chondroitin sulfate chains differing in degree and profile of sulfation perform distinct functions in development. In addition, structurally distinct epitopes have been demonstrated (using monoclonal antibodies) to be distributed non-randomly within the linear framework of native chondroitin sulfate chains of aggrecan isolated from embryonic chick chondrocyte cultures [10] and pig laryngeal cartilage [11]. However, the detailed structural and functional analysis of chondroitin sulfate chains and characterization of biosynthetic enzymes responsible for the controlled synthesis of these chains have hitherto been hampered by the lack of analytical tools.

Structurally defined oligosaccharide probes in addition to specific degradative enzymes and monoclonal antibodies are essential tools for microanalysis of biologically active domain structures and antibody epitopes of chondroitin sulfate chains. They would also be indispensable for screening and characterization of biosynthetic enzymes. Although various tri- and tetrasaccharides were prepared previously using bacterial chondroitinase [12, 13], their structures were converted to unsaturated forms by the action of eliminase and thus are not suitable acceptor substrates for biosynthetic enzymes.

In this study, we prepared seven sulfated tetrasaccharides after testicular hyaluronidase digestion of shark cartilage chondroitin sulfate D and characterized them by 500 MHz ^{1}H NMR spectroscopy. The tetrasaccharides were derived from the repeating disaccharide region and shared the common core structure, GlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc, with various sulfation profiles. These structurally defined sulfated tetrasaccharides were demonstrated to serve as appropriate acceptor substrates for serum α-*N*-acetylgalactosaminyltransferase.

Materials and methods

Materials

Shark cartilage chondroitin sulfate D, five unsaturated standard disaccharides derived from chondroitin sulfate, chondroitinase ABC (EC 4.2.2.4), and chondroitinase AC-II (EC 4.2.2.5) were purchased from Seikagaku Corp., Japan. Authentic saturated disaccharides, GlcAβ1-3GalNAc(4-sulfate) (Di-4S) and GlcAβ1-3GalNAc(6-sulfate) (Di-6S), were purchased from Funakoshi Co., Japan. Sheep testis hyaluronidase (EC 3.2.1.35) was obtained from Sigma, USA. Bio-Gel P-10 resin was obtained from Bio-Rad, USA.

Preparation of tetrasaccharide fraction

A commercial preparation (100 mg) of shark cartilage chondroitin sulfate D was digested with 10 mg (approx. 15000 NFU) of sheep testicular hyaluronidase in a total volume of 2.0 ml of 50 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl (1 NFU corresponds to the amount of the enzyme which hydrolyses 74 µg of hyaluronate per min) [14, 15]. Digestion was carried out at 37 °C for 20 h, then an additional 4 mg (6000 NFU) of the enzyme was added and the incubation proceeded for 5 h to complete the digestion. Following incubation, the digest was mixed with 0.42 ml of 30% trichloroacetic acid and centrifuged. The precipitate was washed with 0.5 ml of 5% trichloroacetic acid. The combined supernatant obtained from trichloroacetic acid precipitation was extracted with ether and the aqueous phase was neutralized with 1 M Na_2CO_3, then the sample was applied to a Bio-Gel P-10 column (1.6 × 95 cm) using 1.0 M NaCl/10% ethanol as the eluent. Eluates were monitored by absorption at 210 nm which is attributable mainly to the *N*-acetyl group of GalNAc. Tetra-, hexa-, octa, deca-, and dodecasaccharide fractions were pooled (Fig. 1), concentrated, desalted through a column (1.5 × 46 cm) of Sephadex G-25 (fine), and lyophilized. The smallest size fraction, which was presumed to contain tetrasaccharides, was subfractionated by HPLC on an amine-bound silica column as reported previously for unsaturated tetrasaccharides [12]. Each peak was purified by rechromatography under the same conditions as the first step and desalted by gel filtration. Quantification of each peak was performed by the carbazole method with glucuronic acid as a standard [16].

Digestion of the isolated tetrasaccharides with chondroitinase AC-II

Tetrasaccharides (5.4 nmol as GlcA) were digested using 10 mIU of chondroitinase AC-II as described previously [12]. Reactions were terminated by boiling for 1 min and the reaction mixture was analysed by HPLC as reported previously [12]. Eluates were monitored by absorption at 210 nm.

α-N-Acetylgalactosaminyltransferase assay

The enzyme reaction was carried out under standard conditions as described previously using heat-treated (50 °C, 60 min) fetal bovine serum as an enzyme source, UDP-[^{3}H]GalNAc as a sugar donor and various tetrasaccharide fractions as sugar acceptors [17]. Incorporation of [^{3}H]GalNAc into each acceptor substrate was determined by gel filtration followed by liquid scintillation counting.

500 MHz ^{1}H NMR spectroscopy

Five-hundred MHz ^{1}H NMR spectra of the tetrasaccharides were measured on a Varian VXR-500 at a probe temperature of 15, 26 or 60 °C as reported previously [12, 15]. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in ^2H_2O [18]. Tetrasaccharides for NMR analysis were repeatedly exchanged in ^2H_2O with intermediate lyophilization.

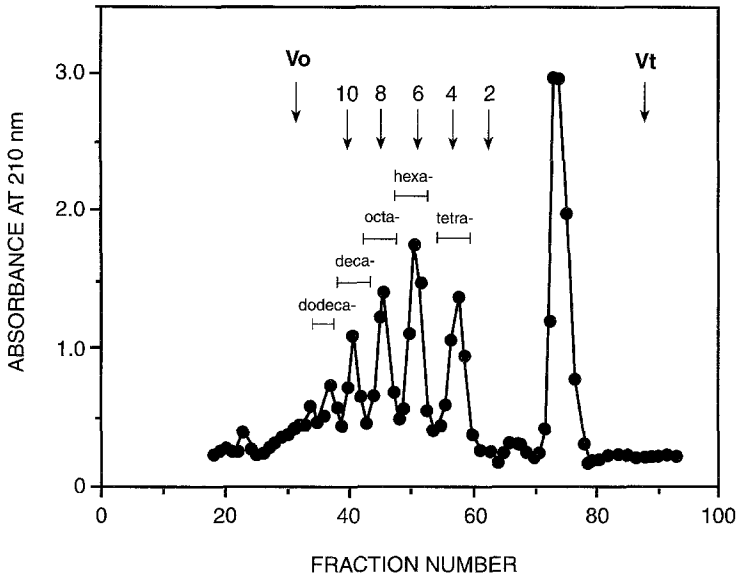

Figure 1. Gel filtration of the hyaluronidase digest of shark cartilage chondroitin sulfate D on Bio-Gel P-10. A commercial preparation (100 mg) of chondroitin sulfate D was exhaustively digested with sheep testicular hyaluronidase as described in Materials and methods. The digest was fractionated on a column (1.6 × 95 cm) of Bio-Gel P-10 using 1.0 M NaCl/10% ethanol as the eluent. Fractions (2 ml) were collected and monitored by absorbance at 210 nm. Tetra-, hexa-, octa-, deca- and dodecasaccharide fractions were pooled as indicated by bars. Arrows with Arabic numerals 2–10 indicate the elution positions of the even numbered unsaturated di- to decasaccharides, respectively, which were prepared by chondroitinase digestion of commercial shark cartilage chondroitin sulfate D.

Other analytical methods

The homogeneity of each fraction was judged by capillary electrophoresis using a fused silica capillary as reported previously in a Waters capillary ion analyzer [19]. Uronic acid was determined by the carbazole method [16]. Unsaturated disaccharides produced by the action of chondroitinase were quantified spectrophotometrically based upon an average millimolar absorption coefficient of 5.5 at 232 nm [20].

Results

Isolation of the tetrasaccharide fraction

A commercial preparation of shark cartilage chondroitin sulfate D was digested with sheep testicular hyaluronidase and the digest was subjected to fractionation by gel filtration on a column of Bio-Gel P-10. Several peaks were observed when monitored by absorbance at 210 nm caused primarily by *N*-acetyl groups and they were assigned as tetra-, hexa-, octa-, deca-, dodeca- and larger oligosaccharide fractions as indicated in Fig. 1 based upon the well-defined mechanism of action of testicular hyaluronidase [21, 22]. The elution position of each oligosaccharide fraction was similar to that of the corresponding even-numbered unsaturated oligosaccharide fraction obtained by limited chondroitinase ABC digestion of a commercial chondroitin sulfate D preparation (Sugahara K *et al.*, unpublished results). The large peak observed at around fraction number 75 was attributable to the buffer salts. The tetrasaccharide fraction was subfractionated into Fractions 1–6 by HPLC on an amine-bound silica column, Fractions 1–3 and 4–6 being eluted at the positions of authentic unsaturated tetrasaccharides with two or three sulfate groups, respectively, as indicated in Fig. 2. They were further purified by rechromatography to apparent homogeneity except for Fraction 1 as judged by HPLC and capillary electrophoresis (data not shown), and then subjected to structural analysis. Although Fraction 1 was resolved into two subfractions 1A (a faster migrating fraction) and 1B (a slower migrating fraction) by capillary electrophoresis in a molar ratio of 1.0:1.4, it was not possible to separate them on a preparative scale. Thus, Fraction 1 was analysed without further purification. Amounts of Fractions 1–6 isolated from 100 mg of the starting material are summarized in Table 1.

Enzymatic characterization of the tetrasaccharide fractions

All the isolated tetrasaccharide fractions were sensitive to chondroitinase AC-II, yielding two peaks with different absorbance intensities at 210 nm on HPLC. Only the major peak showed absorbance when monitored at 232 nm. Therefore, the major and the minor peak detected by absorbance at 210 nm were judged to be an unsaturated and a saturated disaccharide derived from the reducing and nonreducing side of the parent tetrasaccharide, respectively. Their carbohydrate backbone structures were assumed to be ΔGlcAβ1-3GalNAc and GlcAβ1-3GalNAc, respectively (ΔGlcAβ represents β-D-*gluco*-4-enepyranosyluronic acid: strictly α-L-*threo*-hex-4-enepyranosyluro-

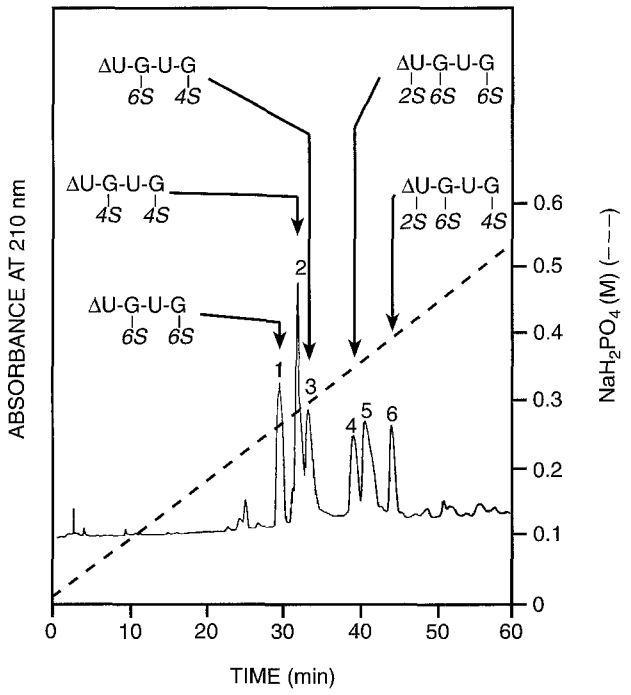

Figure 2. Subfractionation of the tetrasaccharide fraction by HPLC on an amine-bound silica column. The tetrasaccharide fraction obtained by gel filtration on Bio-Gel P-10 (Fig. 1) was chromatographed on an amine-bound silica column using a linear salt gradient, as indicated by the dashed line. Also indicated by arrows are the elution positions of the authentic unsaturated tetrasaccharides with the common structure ΔGlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc, which were prepared by chondroitinase digestion of various chondroitin sulfate isoforms [12].

nic acid (ΔHexAα) is more suitable since it is neither *gluco* nor *ido* due to the loss of two of its chiral centres. However, based on its origin and present practice ΔGlcAβ has been used here). The stronger intensity in UV-absorbance of the major peak was attributable to the double bond formed between C-4 and C-5 by the eliminase action of chrondroitinase AC-II [23]. Approximately one mole of an unsaturated disaccharide was produced from each tetrasaccharide fraction as judged from the absorbance at 232 nm although quantification of a saturated disaccharide was not achieved based on the absorbance at 210 nm because the peak area varied considerably from injection to injection for reasons which are as yet unclear.

Representative HPLC chromatograms obtained with Fractions 3 and 6 are presented in Fig. 3. When digested with chondroitinase AC-II, Fraction 3 yielded a major peak at the elution position of ΔGlcAβ1-3GalNAc(4-sulfate) (ΔDi-4S) and a minor peak at the position of the authentic saturated disaccharide GlcAβ1-3GalNAc(6-sulfate) slightly (0.48 min) ahead of ΔDi-6S when monitored by absorbance at 210 nm (panel A). Only the major peak showed absorbance at 232 nm (panel B). Another authentic saturated disaccharide, GlcAβ1-3GalNAc(4-sulfate), was eluted shortly (0.64 min) before ΔDi-4S being well separated from GlcAβ1-3GalNAc(6-sulfate), and standard monosulfated disaccharides were found to be eluted in the order of Di-6S, ΔDi-6S, Di-4S and ΔDi-4S. These results indicate that the compound in Fraction 3 was a disulfated tetrasaccharide composed of GlcAβ1-3GalNAc(4-sulfate) on the reducing side and most likely GlcAβ1-3GalNAc(6-sulfate) on the nonreducing side; i.e. GlcAβ1-3GalNAc(6-sulfate)β1-4GlcAβ1-3GalNAc(4-sulfate). Chondroitinase AC-II digestion of Fraction 6 yielded a major peak at the position of ΔDi-4S and two minor peaks at around the positions of ΔGlcA(2-sulfate)β1-3GalNAc(6-sulfate) (ΔDi-diS_D) and ΔGlcAβ1-3GalNAc(4,6-disulfate) (ΔDi-diS_E) (panel C). Only the major peak showed absorbance at 232 nm (panel D). The minor peak at the position of ΔDi-diS_E is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column resin. These results indicate that the compound in Fraction 6 was a trisulfated tetrasaccharide composed of a GlcAβ1-3GalNAc(4-sulfate) unit and an unidentified disulfated disaccharide unit on the reducing and nonreducing side, respectively; i.e.

Table 1. Disaccharide analysis of the isolated tetrasaccharides. Each tetrasaccharide was digested with chondroitinase AC-II and the digests were analysed by HPLC on an amine-bound silica column as described in Materials and methods.

		Reaction products		
Fraction	*nmol[a] isolated*	*Saturated disaccharide*	*Unsaturated disaccharide*	*Proposed tetrasaccharide structure*
1	435	Di-4S[b]	ΔDi-6S	U-G(4S)-U-G(6S)[c] and {monosulfated (U-G)}-U-G(6S)
2	607	Di-4S	ΔDi-4S	U-G(4S)-U-G(4S)
3	533	Di-6S	ΔDi-4S	U-G(6S)-U-G(4S)
4	346	disulfated disaccharide	ΔDi-6S	{disulfated (U-G)}-U-G(6S)
5	338	Di-4S	ΔDi-diS_D	U-G(4S)-U(2S)-G(6S)
6	103	disulfated disaccharide	ΔDi-4S	{disulfated (U-G)}-U-G(4S)

[a]nmol obtained from 100 mg of chondroitin sulfate D.
[b]Another monosulfated saturated disaccharide is also likely to be present, but is hidden under a large peak of ΔDi-6S (see text).
[c]G, U, 2S, 4S, and 6S represent GalNAc, GlcA, 2-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively.

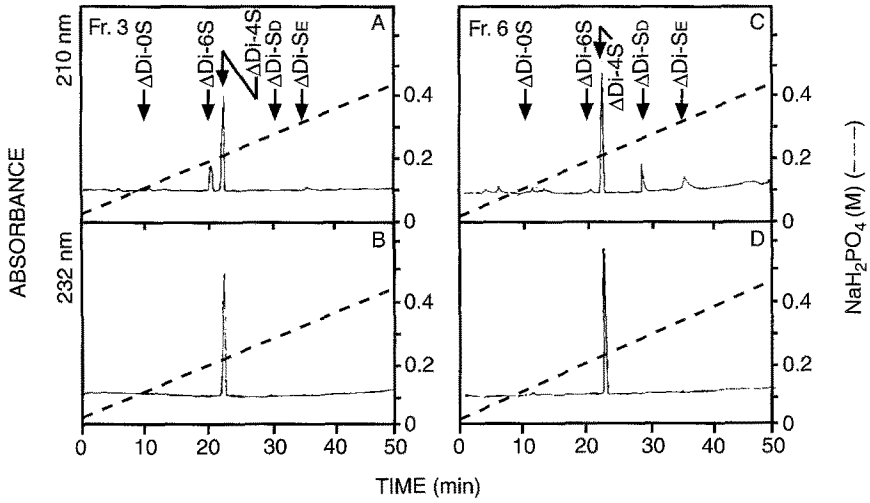

Figure 3. HPLC analysis of the chondroitinase AC-II digests of Fractions 3 and 6. The tetrasaccharide fractions 3 and 6 (2.7 nmol each) were digested with chondroitinase AC-II and analyzed by HPLC on an amine-bound silica column. Left panels, Fraction 3; right panels, Fraction 6. Chromatograms were monitored by absorbance at 210 nm (panels A and C) and 232 nm (panels B and D). The elution positions of chondroitin sulfate-derived authentic unsaturated disaccharides are indicated in the top panels. For disaccharide abbreviations see the title page.

{disulfated (GlcAβ1-3GalNAc)}β1-4GlcAβ1-3GalNAc(4-sulfate).

Chondroitinase AC-II digestion of Fraction 2 resulted in two discrete peaks, a minor and a major peak, at the positions of Di-4S and ΔDi-4S, respectively, in this order when monitored by absorbance at 210 nm (data not shown), and only the latter showed absorbance at 232 nm. Therefore, the compound in Fraction 2 was judged to be a tetrasaccharide of structure GlcAβ1-3GalNAc(4-sulfate)β1-4GlcAβ1-3GalNAc(4-sulfate). Chondroitinase AC-II digestion of Fraction 1 gave rise to a major and a minor peak at the positions of ΔDi-6S and Di-4S, respectively, when monitored by absorbance at 210 nm (data not shown). Only the former showed absorbance at 232 nm. Since Fraction 1 was dissolved into two subfractions by capillary electrophoresis, it was assumed that the fraction contained two components sharing the GlcAβ1-3GalNAc(6-sulfate) structure on the reducing side, but which differed in the structure of the disaccharide units on the nonreducing side. One tetrasaccharide component was assumed to contain GlcAβ1-3GalNAc(4-sulfate) on the nonreducing side based on the observation of Di-4S, and therefore GlcAβ1-3GalNAc(4-sulfate)β1-4GlcAβ1-3GalNAc(6-sulfate) is proposed for this tetrasaccharide structure. However, the other tetrasaccharide component in Fraction 1 could not be determined, and it was suspected that another monosulfated saturated disaccharide unit was hidden under the large peak of ΔDi-6S on HPLC. The structure determination of this tetrasaccharide had to await ^{1}H NMR analysis described below.

The above findings obtained by chondroitinase AC-II digestion in conjunction with HPLC are summarized in Table 1 with those obtained from the other tetrasaccharide fractions. Since all the tetrasaccharide fractions were sensitive to chondroitinase AC-II, the internal uronic acid of the tetrasaccharide in each fraction was confirmed as glucuronate but not iduronate. Sulfation patterns of the presumable disulfated saturated disaccharide portions of Fractions 4 and 6 could not be determined by HPLC because of the lack of corresponding authentic disaccharide standard(s).

500 MHz ^{1}H NMR analysis

Each isolated tetrasaccharide fraction was characterized by 500 MHz ^{1}H NMR spectroscopy. The 1D ^{1}H NMR spectra of Fractions 3 and 6 are shown as representatives in Fig. 4. Signals found in the anomeric proton region between 4.4 and 5.4 ppm were readily identified as H-1 resonances of the constituent saccharide residues by comparison with the NMR spectra of the unsaturated chondro-tetrasaccharides [12] and hyaluronic acid-derived tetrasaccharides [15]. Other proton signals in the 1D spectra were assigned using the 2D HOHAHA and COSY spectra (data not shown) as in the case of the oligosaccharides isolated previously from chondroitin sulfate [12, 24], dermatan sulfate [25], and hyaluronic acid [15]. Chemical shifts of most of the proton signals assigned for the tetrasaccharides in Fractions 3 and 6 are summarized in Tables 2 and 3, respectively, together with those of the reference compounds. Isomer types of the two uronic acid residues in each isolated tetrasaccharide were identified as GlcA based upon the chemical shifts (δ 4.482–4.734) of the anomeric proton signals. Anomeric proton signals of an αIdoA and a βGlcA in chondroitin sulfate/dermatan

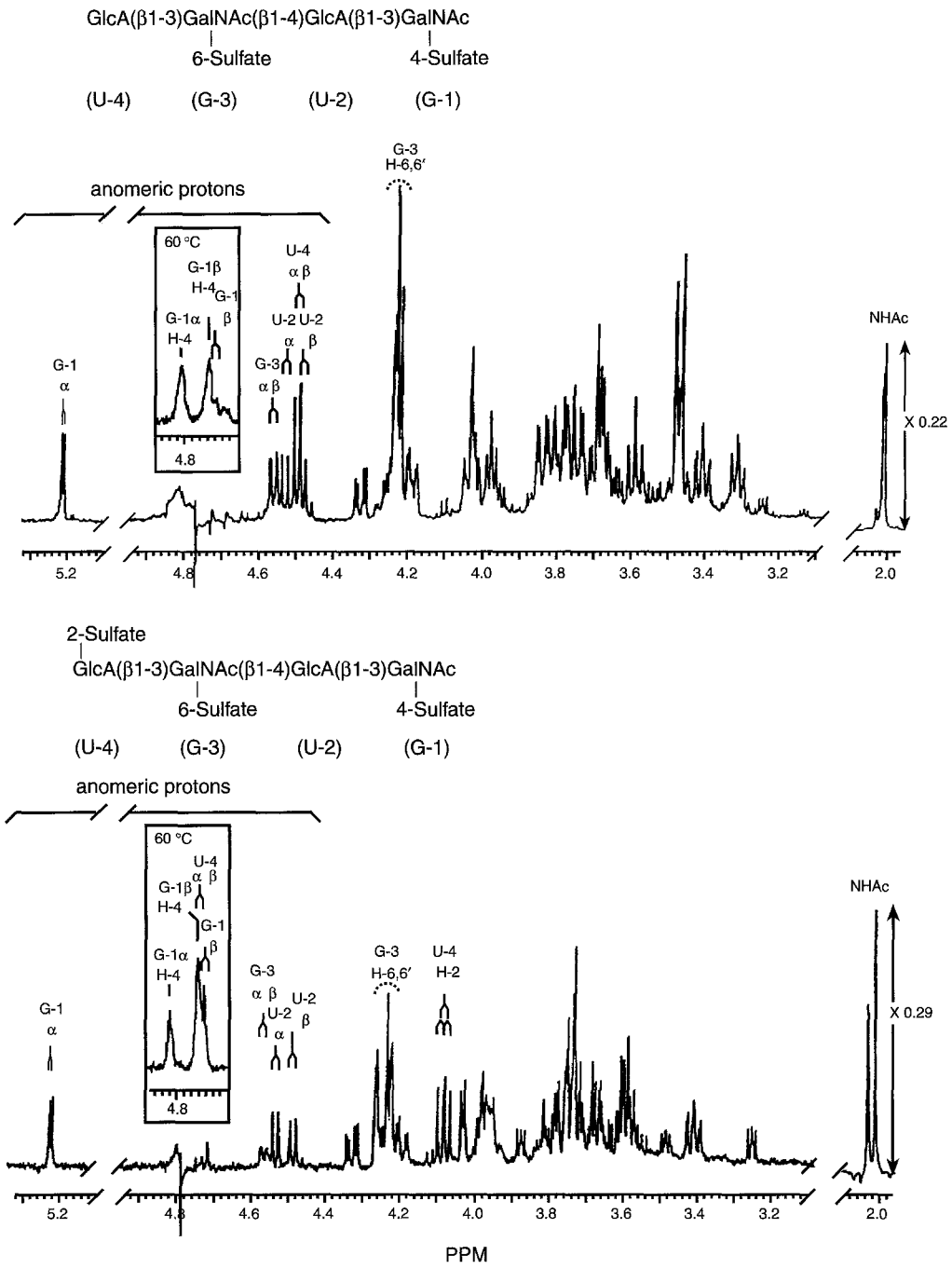

Figure 4. Structural-reporter group regions of the 500 MHz ^{1}H NMR spectra of Fractions 3 and 6 recorded in ^2H_2O at 26 °C. Upper panel, Fraction 3; lower panel, Fraction 6. The numbers and letters in the spectra refer to the corresponding sugar residues in the structure. The insets are the spectra recorded at 60 °C to suppress disturbance by the HOD line. U and G stand for GlcA and GalNAc, respectively.

sulfate oligosaccharides are observed at around δ 5.0–5.2 and 4.5–4.8, respectively [12, 13, 25, 26].

O-sulfation causes downfield shifts of protons bound to the *O*-sulfated carbon atoms by approximately 0.4–0.7 ppm [12, 24, 27]. Thus, sulfation positions of the saccharide residues were determined by comparison with the proton signals of nonsulfated saccharide residues [12, 24, 28]. In the spectrum of Fraction 3, downfield shifts of H-4 of GalNAc-1 and H-6 of GalNAc-3 by approx. Δ0.6 and 0.4 ppm were found, indicating 4- and 6-sulfation of GalNAc-1 and -3, respectively. Therefore, the structure of the compound in Fraction 3 was as follows:

Fraction 3: GlcAβ1-3GalNAc(6-sulfate)β1-4GlcAβ1-3GalNAc(4-sulfate).

Table 2. ^{1}H-Chemical shifts of the constituent monosaccharides of the disulfated tetrasaccharides derived from chondroitin sulfate D together with those of the reference compound R1. Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone (δ 2.225 ppm) in ^2H_2O at 26 °C. The estimated error for the values to two decimal places is only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places is ±0.002 ppm.

	Reporter group	*Fr. 1A* 4S 6S[b] U-G-U-G		*Fr. 1B* 6S 6S U-G-U-G		*Fr. 2* 4S 4S U-G-U-G		*Fr. 3* 6S 4S U-G-U-G		*R1*[a] 6S 4S ΔU-G-U-G	
Residue		α	β	α	β	α	β	α	β	α	β
GalNAc-1	H-1	5.215	4.683	5.215	4.683	5.210	4.722[d]	5.212	4.725[d]	5.212	4.712[e]
	H-2	4.284	4.006	4.284	4.006	4.329	4.054[d]	4.327	4.02	4.328	4.020
	H-3	4.022	3.840	4.022	3.840	4.184	4.022[d]	4.188	4.07	4.191	ND[f]
	H-4	4.234	4.17	4.234	4.17	4.815[d]	4.738[d]	4.812[d]	4.741[d]	4.810	4.743[e]
	H-5	4.347	3.93	4.347	3.93	4.254	ND	4.25	ND	4.254	3.82
	H-6	4.20	4.21	4.20	4.21	3.78	ND	3.79	ND	3.79	ND
	H-6′	4.14	4.16	4.14	4.16	3.70	ND	3.69	ND	3.71	ND
	NAc	2.017		2.017		2.023		2.020		2.018	
GlcA-2	H-1	4.565	4.509	4.565	4.509	4.521	4.476	4.529	4.482	4.530	4.483
	H-2	3.385		3.385		3.395	3.397	3.411		3.411	3.416
	H-3	3.593		3.593		3.584		3.593		3.591	
	H-4	3.71		3.71		3.785		3.760		3.76	
	H-5	3.63		3.63		3.67	3.65	3.68	3.65	3.689	3.680
GalNAc-3	H-1	4.560		4.543		4.572		4.560		4.593	
	H-2	4.041		4.022		4.038		4.025		4.03	
	H-3	4.068[d]		3.84		4.068[d]		3.843		3.941	
	H-4	4.802[d]		4.17		4.800[d]		4.23		4.180	
	H-5	ND		3.94		3.817[d]		3.98		ND	
	H-6	ND		4.22		ND		4.22		4.22	
	H-6′	ND		4.22		ND		4.22		4.22	
	NAc	2.041		2.023		2.043		2.023		2.056	
GlcA-4	H-1	4.463		4.494		4.465		4.497		5.184	
(or ΔGlcA-4[c])	H-2	3.333		3.306		3.340		3.317		3.77	
	H-3	3.45		3.47		3.466		3.46		4.105	
	H-4	3.52		3.68		3.525		3.686		5.877	
	H-5	ND		ND		3.664		ND		–[g]	

[a]R1, unsaturated tetrasaccharide in fraction 6 isolated from shark cartilage chondroitin sulfate D [12].
[b]ΔU, G, U, 2S, 4S, and 6S represent ΔGlcA, GalNAc, GlcA, 2-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively.
[c]ΔGlcA-4, R1 contains ΔGlcA-4 instead of GlcA-4.
[d]Values determined at 60 °C.
[e]Values determined at 15 °C.
[f]ND, not determined.
[g]–, not occurring.

In the spectrum of Fraction 6, downfield shifts of H-4 of GalNAc-1, H-6 of GalNAc-3, and H-2 of GlcA-4 by approx. Δ0.6, 0.4, and 0.7 ppm were found, indicating *O*-sulfation of the corresponding carbon atoms, respectively. Thus, the structure of the compound in Fraction 6 was:

Fraction 6: GlcA(2-sulfate)β1-3GalNAc(6-sulfate)β1-

4GlcAβ1-3GalNAc(4-sulfate).

Likewise, most of the proton chemical shifts of the other tetrasaccharides were also assigned, and their determined structures are shown below. It should be noted that the 1D ^{1}H NMR spectrum of Fraction 1 contained two series of signals differing in peak height, one being approximately 1.4-fold higher than the other (data not shown). Thus, most of the two series of structural-reporter group proton signals were distinctively assigned without difficulty. The NMR data clearly indicate that the following structures are present in the major (1B) and the minor fraction (1A), respectively:

Table 3. ^{1}H-Chemical shifts of the constituent monosaccharides of the trisulfated tetrasaccharides derived from chondroitin sulfate D together with those of the reference compounds R2 and R3. For details see the legend to Table 2.

	Reporter group	*Fr. 4* *2S 6S 6S* *U-G-U-G*		*R2*[a] *2S 6S 6S* *ΔU-G-U-G*		*Fr. 5* *4S 2S 6S* *U-G-U-G*		*Fr. 6* *2S 6S 4S* *U-G-U-G*		*R3*[a] *2S 6S 4S* *ΔU-G-U-G*	
Residue		α	β	α	β	α	β	α	β	α	β
GalNAc-1	H-1	5.214	4.682	5.216	4.684	5.323	4.73	5.209	4.72	5.211	4.711[e]
	H-2	4.280	4.00	4.283	4.01	4.179	4.13	4.325	4.00	4.328	4.03
	H-3	4.037	3.838	4.03	3.840	4.113	3.82	4.188	4.04	4.19	ND[f]
	H-4	4.207	4.153	4.184	4.17	4.291	ND	4.810[d]	4.75[d]	4.79	4.745[e]
	H-5	4.348	3.93	4.348	3.937	4.31	ND	4.252	ND	4.258	ND
	H-6	4.20	4.24	4.19	4.21	4.21	ND	3.78	ND	3.80	ND
	H-6′	4.13	4.18	4.14	4.13	4.15	ND	3.70	ND	3.74	ND
	NAc	2.016		2.015		2.039		2.020		2.018	
GlcA-2	H-1	4.566	4.509	4.568	4.510	4.837[b]		4.529	4.483	4.531	4.486
	H-2	3.377		3.386		4.132		3.404		3.413	3.418
	H-3	3.604		3.59		3.806		3.584		3.590	
	H-4	3.726		3.72		3.765		3.76		3.765	3.693
	H-5	3.68		ND		ND		3.67		3.684	
GalNAc-3	H-1	4.548		4.589		4.586		4.558		4.607	
	H-2	3.95		4.02		4.04		3.973		4.03	
	H-3	3.93		3.966		4.04		3.86		3.966	
	H-4	4.23		3.976		4.792		4.24		3.978	
	H-5	3.97		4.00		ND		3.93		4.00	
	H-6	4.23		4.20		ND		4.22		4.20	
	H-6′	4.23		4.20		ND		4.22		4.20	
	NAc	2.038		2.087		2.044		2.040		2.089	
GlcA-4	H-1	4.718		5.519		4.466		4.734		5.521	
(or ΔGlcA-4[c])	H-2	4.077		4.466		3.339		4.079		4.466	
	H-3	3.734		4.18		3.468		3.74		4.18	
	H-4	3.585		6.031		3.524		3.59		6.031	
	H-5	ND		–[g]		3.645		ND		–	

[a]R2 and R3, unsaturated tetrasaccharides in fractions 7b and 8a isolated from shark cartilage chondroitin sulfate D, respectively [12].
[b]Only one signal was observed due to disturbance by suppression of the HOD signal, and has not been assigned to that for α- and β-anomer.
[c]ΔGlcA-4, R2 and R3 contain ΔGlcA-4 instead of GlcA-4.
[d]Values determined at 60 °C.
[e]Values determined at 15 °C.
[f]ND not determined.
[g]–, not occurring.

Fraction 1A: GlcAβ1-3GalNAc(4-sulfate)β1-4GlcAβ1-3GalNAc(6-sulfate).

Fraction 1B: GlcAβ1-3GalNAc(6-sulfate)β1-4GlcAβ1-3GalNAc(6-sulfate).

Fraction 2: GlcAβ1-3GalNAc(4-sulfate)β1-4GlcAβ1-3GalNAc(4-sulfate).

Fraction 4: GlcA(2-sulfate)β1-3GalNAc(6-sulfate)β1-4GlcAβ1-3GalNAc(6-sulfate).

Fraction 5: GlcAβ1-3GalNAc(4-sulfate)β1-4GlcA(2-sulfate)β1-3GalNAc(6-sulfate).

The NMR data and the structures of the disulfated and trisulfated tetrasaccharides are summarized in Tables 2 and 3, respectively, with those of the standard unsaturated chondro-tetrasaccharides [12]. The sulfation sites determined by ^{1}H NMR of each tetrasaccharide in Fractions 1–6 were in good agreement with those assessed above by enzymatic analysis in conjunction with HPLC. Interestingly, the elution positions in the HPLC system

of the saturated tetrasaccharides characterized in this report were approximately the same as those of the corresponding unsaturated tetrasaccharides (see Fig. 2) as in the case of saturated and unsaturated disaccharides (see above and Fig. 3).

Anomerization effects resulted in doubling of the anomeric proton signal of the second constituent GlcA-2. For example, the anomeric resonances of GlcA-2 of the tetrasaccharide in Fraction 3 were observed at δ 4.529 and 4.482 (Fig. 4). GlcA-2 H-1 of the β-anomer was assigned to the latter rather than the former based on the closer resemblance to that of H-1 (δ 4.497) of GlcA-4 which is attached to βGalNAc through a β1-3 linkage as is GlcA-2 of the β-anomer. The remaining resonance at δ 4.529 was assigned to that of GlcA-2 H-1 of the α-anomer. Likewise, the two anomeric resonances of GlcA-2 of each tetrasaccharide in Fraction 1 or 2 were discriminated. The H-1 resonances of GlcA-2 of the tetrasaccharides in Fractions 4 and 6 were assigned based on the similarity in their chemical shifts to those of GlcA-2 of the tetrasaccharides in Fractions 1 and 3, respectively, since the disaccharide units on the reducing side of the tetrasaccharides in Fractions 1 and 4, or Fractions 3 and 6 share identical sulfated structures, respectively, as revealed by the enzymatic analysis above. The H-1 signal of GlcA-2 of the β-anomer is observed generally in the upper field than that of the α-anomer. The H-1 signal of GlcA-2 of the tetrasaccharide in Fraction 5 was readily identified based on the large downfield shift of its anomeric proton signal caused by 2-*O*-sulfation, but has not been assigned to that for α- or β-anomer (see footnotes to Table 3).

Two acetoamide group proton signals belonging to GalNAc-1 or GalNAc-3 were observed for each tetrasaccharide component. In a previous ^{1}H NMR study of the unsaturated chondro-tetrasaccharides, chemical shifts of the acetoamide group proton signals of GalNAc-3 resembled those of the corresponding unsaturated chondro-disaccharides [12]. The remaining acetoamide group proton signals of the tetrasaccharides were assigned to GalNAc-1 previously [12]. In turn, chemical shifts of those of GalNAc-1 of the saturated tetrasaccharides isolated in this study were judged to be similar to those of GalNAc-1 of the corresponding unsaturated tetrasaccharides. Thus, in the spectrum of Fraction 3 (Fig. 4), the acetoamide group proton signals observed at δ 2.020 and 2.023 were assigned to GalNAc-1 and GalNAc-3, respectively, based on comparison with the chemical shifts of the acetoamide group proton signals of the reference compound R1 (Table 2). Likewise, the acetoamide group proton signals of the tetrasaccharides in Fractions 4 and 6 were assigned by comparison with those of reference compounds R2 and R3, respectively (Table 3). Those of the tetrasaccharides in the other fractions could be assigned by comparison with the chemical shifts of the acetoamide group proton signals obtained for the compounds in Fractions 3, 4, and 6.

Acceptor activity of the tetrasaccharides towards serum α-N-acetylgalactosaminyltransferase

The tetrasaccharide fractions structurally characterized in this study were examined for their acceptor activities towards α-*N*-acetylgalactosaminyltransferase identified recently in fetal bovine serum [17]. Each tetrasaccharide fraction was incubated with heat-treated serum containing the above enzyme activity under the established incubation conditions (see Materials and methods) and the incorporation of [^{3}H]GalNAc from UDP-[^{3}H]GalNAc into the oligosaccharide was determined. It should be noted that the heat treatment completely abolishes the chondroitin β-*N*-acetylgalactosaminyltransferase activity in serum as reported previously [17]. Thus, under the conditions used the α-*N*-acetylgalactosaminyltransferase activity was specifically measured. The results summarized in Table 4 indicate that they served as suitable acceptors for this glycosyltransferase and the tetrasaccharides with different sulfation profiles exhibited different acceptor activities.

Discussion

In this study seven sulfated tetrasaccharides were isolated after testicular hyaluronidase digestion of shark cartilage chondroitin sulfate D. The tetrasaccharides in Fractions 1B, 2 and 3 have been reported previously [29–31], while those in Fractions 1A, 4, 5 and 6 are novel. It should be noted, however, that unsaturated counterparts of the tetrasaccharides in all fractions have been isolated previously [12, 32, 33]. Although it has been reported that shark cartilage chondroitin sulfate contains small proportions of the nonsulfated disaccharide unit GlcAβ1-

Table 4. Acceptor activity of the tetrasaccharides for α-GalNAc transferase. Various acceptor substrates (1 nmol each) were incubated with heat-treated fetal bovine serum containing α-*N*-acetylgalactosaminyltransferase and incorporation of [^{3}H]GalNAc into each acceptor substrate was determined as described in Materials and methods.

Acceptor	*Structure*	*[^{3}H]GalNAc incorporated pmol*
Fraction 2	GlcA-GalNAc-GlcA-GalNAc 4S 4S	1.97
Fraction 3	GlcA-GalNAc-GlcA-GalNAc 6S 4S	5.48
Fraction 4	GlcA-GalNAc-GlcA-GalNAc 2S 6S 6S	1.63
Fraction 5	GlcA-GalNAc-GlcA-GalNAc 4S 2S 6S	7.40
Fraction 6	GlcA-GalNAc-GlcA-GalNAc 2S 6S 4S	0.37

3GalNAc and the disulfated disaccharide unit GlcAβ1-3GalNAc(4,6-disulfate) [32], no such units were found at least in the major tetrasaccharide fractions analysed in this study. The structure of the tetrasaccharide in Fraction 5 is unique in that the disulfated disaccharide unit (D unit) characteristic of chondroitin sulfate D [31] is located on the reducing side, and thus contain glucuronate 2-*O*-sulfate at the internal position. Consequently, the structure may indicate that the D unit is located on the immediate reducing side of a GlcAβ1-3GalNAc(4-sulfate) unit in chondroitin sulfate D. This hypothesis remains to be clarified by isolating more oligosaccharide fragments containing an internal glucuronate 2-*O*-sulfate residue.

The isolated oligosaccharides were shown to serve as acceptor substances for serum α-*N*-acetylgalactosaminyltransferase which was discovered recently and discussed in view of its possible involvement in biosynthetic mechanisms of glycosaminoglycan chains [17]. Thus, the usefulness of tetrasaccharides prepared using testicular hyaluronidase has been demonstrated. A series of the above tetrasaccharides with different sulfation profiles will be valuable tools for specificity studies of such glycosyltransferases including the above-mentioned α-*N*-acetylgalactosaminyltransferase [17] and β-*N*-acetylgalactosaminyltransferase involved in chondroitin sulfate chain polymerization [34] and possibly chondroitin sulfate sulfotransferases as well.

Several monoclonal antibodies recognizing distinct epitopes on chondroitin sulfate chains have been developed. Some of these antibodies are directed against epitopes that contain unsaturated uronic acid residues created by chondroitinase digestion [35] whereas others were raised against those that are present in native chondroitin sulfate/dermatan sulfate chains [4, 7, 9, 32, 36, 37]. Although the use of these monoclonal antibodies has successfully demonstrated the developmentally regulated expression and tissue-specific distribution of chondroitin sulfate chains, the exact chemical structures have, as yet, to be defined for any of these epitopes. The tetrasaccharides described here may be useful for structural characterization of these epitopes. Since, however, they may be too small to exert biological activities, preparation of larger oligosaccharides is also in progress.

Recently, chondroitin 6-sulfate and heparin have been implicated in the mechanism of tumor cell recognition by natural killer cells [38]. Authentic sulfated oligosaccharide probes will also be useful for the developing new area of glycobiology of sulfated glycosaminoglycans.

Acknowledgements

We thank Dr Makiko Sugiura (Kobe Pharmaceutical University) for recording the NMR spectra and Shoko Shimoji for technical assistance in preparation of the oligosaccharides. This work was supported in part by the Science Research Promotion Fund from Japan Private School Promotion Foundation and Grants-in-Aid for Scientific Research on Priority Areas (05274107) from the Ministry of Education, Science and Culture of Japan.

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