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Structural determination of novel tetra- and hexasaccharide sequences isolated from chondroitin sulfate H (oversulfated dermatan sulfate) of hagfish notochord

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Oversulfated chondroitin sulfate H (CS-H) isolated from hagfish notochord is a unique dermatan sulfate consisting mainly of IdoAα1-3GalNAc(4S,6S), where IdoA, GalNAc, 4S and 6S represent L-iduronic acid, *N*-acetyl-D-galactosamine, 4-*O*-sulfate and 6-*O*-sulfate, respectively. Several tetra- and hexasccharide fractions were isolated from CS-H after partial digestion with bacterial chondroitinase B to investigate the sequential arrangement of the IdoAα1-3GalNAc(4S,6S) unit in the CS-H polysaccharide. A structural analysis of the isolated oligosaccharides by enzymatic digestions, mass spectrometry and ^{1}H NMR spectroscopy demonstrated that the major tetrasaccharides shared the common disulfated core structure $\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4IdoAα1-3 GalNAc (4S) with 0 ~ 3 additional *O*-sulfate groups, where $\Delta^{4,5}$HexA represents 4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid. The major hexasaccharides shared the common trisulfated core structure $\Delta^{4,5}$HexAα1-3 GalNAc(4S)β1-4 IdoAα1-3 GalNAc(4S)β1-4IdoAα1-3 GalNAc(4S) with 1 ~ 4 additional *O*-sulfate groups. Some extra sulfate groups in both tetra- and hexasaccharides were located at the C-2 position of a $\Delta^{4,5}$HexA or an internal IdoA residue, or C-6 position of 4-*O*-sulfated GalNAc residues, forming the unique disulfated or trisulfated disaccharide units, IdoA (2S)-GalNAc(4S), IdoA-GalNAc(4S,6S) and IdoA (2S)-GalNAc(4S,6S), where 2S represents 2-*O*-sulfate. Of the demonstrated sequences, five tetra- and four hexasaccharide sequences containing these units were novel.

Keywords: **chondroitin sulfate H, dermatan sulfate, glycosaminoglycan, sulfated oligosaccharides**

Abbreviations: **CS, chondroitin sulfate; DS, dermatan sulfate; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; HexA, hexuronic acid; HexNAc, *N*-acetyl-D-hexosamine; ΔHexA or $\Delta^{4,5}$HexA, 4-deoxy-α-*threo*-hex-4-enepyranosyluronic acid; HCII, heparin cofactor II; COSY, correlation spectroscopy; 1 or 2D, one- or two-dimensional; ΔDi-0S, $\Delta^{4,5}$HexAα1-3GalNAc; ΔDi-4S, $\Delta^{4,5}$HexAα1-3 GalNAc(4-*O*-sulfate); ΔDi-6S, $\Delta^{4,5}$HexAα1-3 GalNAc(6-*O*-sulfate); ΔDi-diS$_B$, $\Delta^{4,5}$HexA (2-*O*-sulfate)α1-3GalNAc(4-*O*-sulfate); ΔDi-diS$_D$, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1-3GalNAc(6-*O*-sulfate); ΔDi-diS$_E$, $\Delta^{4,5}$HexAα1-3GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta^{4,5}$HexA(2-*O*-sulfate) α1-3GalNAc(4,6-*O*-disulfate); Δhexuronate-2-, CS-4- or CS-6-sulfatase stands for $\Delta^{4,5}$hexuronate-2-*O*-, chondro-4-*O*- or -6-*O*-sulfatase, respectively; ΔU, G, U, 2S, 4S and 6S denote $\Delta^{4,5}$HexA, GalNAc, IdoA, 2-*O*-, 4-*O*- and 6-*O*-sulfate, respectively.**

Introduction

Dermatan sulfate proteoglycans (DS-PGs) are widely distributed among various tissues including skin, tendon, sclera, cartilage, bone and blood vessel walls (see references in ref. [1]). They are components of cell surfaces and extracellular matrices, and are also found in intracellular granules of certain cells such as mast cells and macrophages [2]. There is increasing evidence that DS-PGs are involved in a number of biological activities including anticoagulant activity of vascular tissues [3,4], regulation of cell adhesiion [5], migration [6] and proliferation [7–9], and interaction with growth factors [7,10]. Although the molecular mechanisms of the expression of these activities of DS-PGs are not fully understood, at least some activities are expressed through DS side chains [3–5,9,10]. Difficulties in conduct-

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ing structural analyses have hampered the clarification of the structural basis of the various biological activities of DS chains.

DS was first isolated from pig skin [11], and is a stereoisoform of chondroitin sulfate (CS), differing at C-5 of the hexuronic acid moieties; CS contains D-glucuronic acid (GlcA), whereas DS contains L-iduronic acid (IdoA) as the major hexuronic acid. The disaccharide repeating units of DS are largely represented by the monosulfated unit, IdoAGalNAc(4-*O*-sulfate). However, like most other glycosaminoglycans, DS is intrinsically heterogeneous with some hexuronic acid residues being GlcA, as in the monosulfated unit GlcA-GalNAc(4S), and with some IdoA as in the disulfated units IdoA(2S)-GalNAc(4S) and IdoA-GalNAc(4S,6S), where 2S, 4S and 6S represent 2-*O*-, 4-*O*- and 6-*O*-sulfate, respectively (see ref. [12] for a review). Another disaccharide unit IdoA(2S)-GalNAc(6S) was recently reported for an ascidian DS [13]. However, the sequential arrangement of these DS disaccharide building units has not been extensively investigated. Reports on structurally defined oligosaccharide sequences are limited [4,14–17], and no detailed sequence information about biologically active domains is available except for the oversulfated hexasaccharide sequence interacting with heparin co-factor II (HCII): the DS sequence consisting of no less than three or four IdoA(2S)-GalNAc(4S) disaccharide units has been demonstrated to provoke an activation of HCII that inhibits thrombin [4]. Hepatocyte growth factor has recently been demonstrated to bind to DS with high affinity [10], where the smallest DS oligosaccharide with significant affinity for hepatocyte growth factor is an octasaccharide and a combination of non-sulfated IdoA and GalNAc(4S) is sufficient for high-affinity binding. These results imply that DS chains have the potential to interact with various growth factors and regulate their functions.

During the last few years we have conducted structural studies of oversulfated CS from cartilage of various animals including shark [18–21], squid [22] and king crab [23,24]. Oversulfated CS isoforms CS-D, CS-E and CS-K contain rare structural building units such as GlcA(2S)β1-3GalNAc(6S) [25], GlcAβ1-3GalNAc(4S,6S) [26] and GlcA(3S)β1-3GalNAc(4S) [27], respectively, which possibly form functional domain structures. The GlcA(3S)β1-3GalNAc(4S,6S) structure has recently been demonstrated in squid cartilage CS-E [22]. Shark cartilage CS-D and squid cartilage CS-E, which are characterized by the above-described disaccharide units, have recently been demonstrated to exhibit neurite outgrowth-promoting activity *in vitro* [28,29], and to inhibit the neural cell adhesion mediated by midkine [30], which is a heparin-binding growth/differentiation factor with neurite outgrowth-promoting activity [31,32]. Oversulfated DS is speculated to interact with biologically active proteins, in the light of its sulfated structure. In fact, it has been reported that DS interacts relatively weakly yet significantly with several HS/heparin-binding proteins, *e.g.,* basic fibroblast growth factor [33], platelet factor 4 [34], fibronectin [35], interleukin-7 [36], and protein C inhibitor [37]. The structural basis for the interaction of DS with such proteins is not yet well understood, partly because of the lack of DS structural information. As an extension of our structural studies, oversulfated DS was investigated in the present study.

Oversulfated DS (alternatively known as CS-H) was first isolated from the motochord of hagfish [38], which belongs to the most primitive phylogenetic branch of cyclostomes, during a series of comparative biochemical studies of sulfated glycosaminoglycans. CS-H is characterized by oversulfation (the molar ratio SO_4/hexosamine = 1.89) and a major disulfated disaccharide unit, IdoAα1-3GalNAc(4S,6S), with small proportions of IdoAα1-3GalNAc(4S) and IdoAα1-3GalNAc(6S). It was designated CS-H in relation to "hagfish". Hagfish skin was also reported to contain CS-H bearing three types of oversulfated disaccharide units, IdoA(2S)α1-3GalNAc(4S,6S), IdoA(2S)α1-3GalNAc(4S) and IdoAα1-3GalNAc(4S,6S) [39]. CS-H was demonstrated to exhibit considerable anticoagulant activity in an activated partial thromboplastin time system [40]. It specifically interacts with midkine *in vitro,* although the affinity is relatively lower than that between CS-E and midkine [30]. More recently it was also reported that DS with the structure IdoAα1-3GalNAc(4S,6S) potentiated HCII activity [41,42]. In the present study, we isolated and characterized several tetra- and hexasaccharides from CS-H of the hagfish notochord to obtain the sequence information of CS-H and to acquire authentic oligosaccharides for future structural studies of DS.

Materials and methods

CS-H was isolated from the notochord of hagfish (*Eptatretus burgeri*) as reported previously [38]. Briefly, a glycosaminoglycan fraction was obtained by the pronase digestion of acetone-dried notocord, followed by ethanol precipitation and then anion-exchange chromatography. The 3.0 M NaCl-eluted fraction was used for the structural analysis in this study. The following materials and enzymes were purchased from Seikagaku Corp., Tokyo, Japan: six unsaturated CS-disaccharide standards, chondroitinase B (EC 4.2.2) from *Flavobacterium heparinum,* chondroitinase AC-II (EC 4.2.2.5) from *Arthrobacter aurescens,* a conventional and a highly purified preparation (available as a protease-free preparation) of chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris,* chondro-4-*O*-sulfatase (EC 3.1.6.9) and chondro-6-*O*-sulfatase (EC 3.1.6.10) from *Proteus vulgaris,* which are abbreviated as CS-4-sulfatase and CS-6-sulfatase, respectively. $\Delta^{4,5}$Hexuronate-2-*O*-sulfatase (abbreviated as Δhexuronate-2-sulfatase) purified from *Flavobacterium heparinum* [43] was provided by Dr. K. Yoshida, Seikagaku Corp., Tokyo, Japan.

Enzymatic analysis of the isolated oligosaccharides

Enzymatic digestion with chondroitinase AC-II or chondroitinase ABC was carried out using 1.0 nmol of each oligosaccharide substrate and 10 mIU of the enzyme in a total volume of 30 µl of the appropriate buffer at 37 °C for 50 min as described [44]. Chondroitinase B treatment was performed using 1.0 nmol of each oligosaccharide and 2 mIU of the enzyme in a total volume of 30 µl of 0.05 M Tris-HCl buffer, pH 8.0 for 25 min at 37 °C [17]. CS-4- or 6-sulfatase treatment was carried out using 0.6 nmol of each oligosaccharide and 30 mIU of the enzyme in a total volume of 30 µl of 0.04 M Tris-HCl buffer, pH 7.5, containing 0.04 M sodium acetate and 100 µg/ml bovine serum albumin for 12 min at 37 °C [45]. ΔHexuronate-2-sulfatase (0.2 mIU) treatment was carried out using 0.6 nmol of each oligosaccharide and 0.2 mIU of the enzyme in a total volume of 20 µl of 20 mM sodium acetate buffer, pH 6.5, containing 0.15% bovine serum albumin at 37 °C for 10 min [43]. After each incubation, the reaction mixture was boiled at 100 °C for 1 min, cooled to room temperature, and diluted to 400 µl with 16 mM NaH_2PO_4; the products were then analyzed by HPLC.

HPLC

The fractionation and analysis of unsaturated oligosaccharides were carried out by HPLC on an amine-bound silica PA03 column (YMC Co., Kyoto, Japan) using a linear gradient of NaH_2PO_4 at a flow rate of 1 ml/min at room temperature as described for the separation of CS-di- and -tetrasaccharides [45–47]. Eluates were monitored by absorption at 232 nm. The separated fractions were concentrated and desalted through a column of Sephadex G-25. Capillary electrophoresis was carried out to examine the purity of each isolated fraction in a Waters capillary ion analyzer as reported previously [48]. The electrophoretic fractions were monitored by absorption at 185 nm with respect to carbonyl groups since its sensitivity was higher than that of detection at 232 nm.

Other analytical methods

Oligosaccharides produced by enzymatic digestions were quantified based on the absorbance (E_{232} = 5500 M^{-1} cm^{-1}) caused by the $\Delta^{4,5}$ sites of the uronic acid at the non-reducing ends [44].

Fast atom bombardment-mass spectrometry (FAB-MS)

FAB mass spectra of the tetrasaccharide samples were obtained using an Autospec OA-TOF mass spectrometer (Micromass, Manchester, UK) fitted with a cesium ion gun operated at 30 kV. Samples were dissolved in 5% acetic acid for loading onto the probe tip coated with monothioglycerol as matrix. Perdeuteroacetylation was performed by adding 1 µl of 1-methylimidazole and 5 µl of d_6-acetic anhydride directly to the sample. After being left standing at room temperature for 30 min, an aliquot of the reaction mixtures was analyzed directly by FAB-MS. Under the conditions employed, sulfate substituents were previously shown to remain intact whereas hydroxyl groups were efficiently perdeuteroacetylated [49].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The MALDI-TOF-MS spectrum of the hexasaccharide fraction 5 was acquired in the linear mode using a PerSeptive Biosystems (Framingham, Massachusetts) Voyager Elite reflectron time-flight instrument fitted with a 337-nm nitrogen laser. The ions were accelerated to 20 keV energy. Caffeic acid was used as a matrix at a concentration of 10 mg/ml in an acetonitrile/water mixture (1 : 1, v/v). A synthetic peptide $(Arg\text{-}Gly)_{15}$ was used as a complexing agent to shield the negatively charged groups of the sulfated oligosaccharide as previously reported [50] according to Juhasz and Biemann [51]. An aqueous solution of the hexasaccharide fraction 5 (10 pmol/µl) and the peptide (13 pmol/µl) were mixed in advance and then diluted with an equal volume of the matrix solution described above. Of this sample/matrix solution, a 1.2 µl aliquot was placed on the probe surface, dried under a stream of air and used for the measurement of the spectrum.

500-MHz ^{1}H NMR spectroscopy

Tetrasaccharide fractions were repeatedly exchanged in D_2O with intermediate lyophilization. Their ^{1}H NMR spectra were measured on a Varian VXR-500 (Varian Japan Ltd., Tokyo, Japan) at a probe temperature of 26 °C [20,21,52]. 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 D_2O [53].

Results

Preparation and isolation of oligosaccharides

When digested exhaustively with a conventional preparation of chondroitinase ABC, the CS-H yielded the following disaccharide units: $\Delta^{4,5}$HexA-GalNAc, $\Delta^{4,5}$HexA-GalNAc(4S), $\Delta^{4,5}$HexA-GalNAc(6S), $\Delta^{4,5}$HexA(2S)-GalNAc(4S), $\Delta^{4,5}$HexA-GalNAc(4S,6S) and $\Delta^{4,5}$HexA(2S)-GalNAc(4S,6S) in a molar ratio of 3.1 : 20.2 : 8.9 : 1.6 : 54.0 : 12.2, which is basically consistent with the previous results [38]. Although only the three major components $\Delta^{4,5}$HexA-GalNAc(4S), $\Delta^{4,5}$HexA-GalNAc(6S), $\Delta^{4,5}$HexA-GalNAc (4S,6S) were reported previously, it was presumably because a paper chromatographic technique was employed at

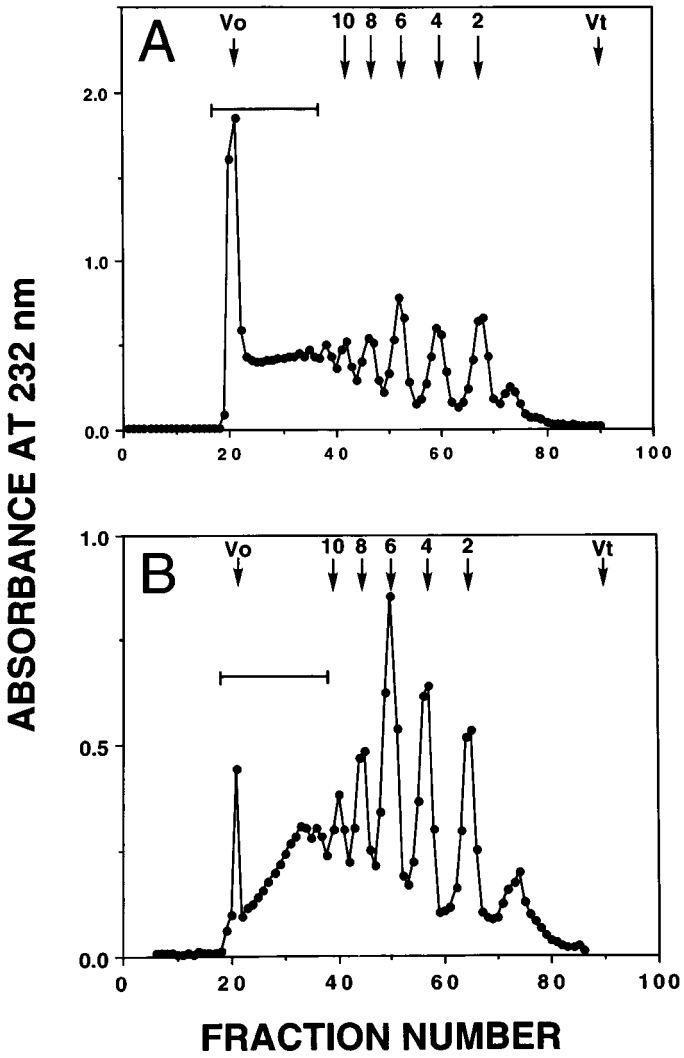

Figure 1. Gel filtration of the chondroitinase B digest of CS-H from hagfish notochord on Bio-gel P-10. (*A*) CS-H (103.8 mg) was digested with chondroitinase B. The digest was fractionated on a column (1.6 × 95 cm) of Bio-Gel P-10 using 1.0 M NaCl/10% ethanol as the eluent. Fractions (2 ml) were collected and monitored by absorbance at 232 nm. Di-, tetra-, hexa-, octa-, deca- and higher oligosaccharide fractions were pooled. (*B*) The higher oligosaccharide fraction indicated by the bar in Figure 1A was further digested with chondroitinase B, and the digest was size-fractionated using the same column. Arrows with numerals 2–10 indicate the elution positions of even-numbered unsaturated di- to decasaccharides, respectively, which were prepared by chondroitinase digestion of commercial shark cartilage CS-D [19].

the time whereas HPLC was used in the present study. For the preparation of oligosaccharides, CS-H from hagfish notochord was partially digested with *Flavobacterium* chondroitinase B until the digestion degree reached 10% of the complete digestion based on the absorbance at 232 nm, and the digest was size-fractionated by gel filtration into di-, tetra-, hexa-, octa-, deca- and higher oligosaccharides using a column of Bio-Gel P-10 (Fig. 1A). The separated fractions represented 15.0, 13.9, 14.3, 10.3, 8.6 and 37.9% of the resultant oligosaccharides, respectively, based on the absorbance at 232 nm. The higher oligosaccharide fraction indicated by the bar was further digested with chondroitinase B, and the digest was fractionated by gel filtration on the same Bio-Gel P-10 column (Fig. 1B). The di-, tetra-, hexa-, octa-, deca- and higher oligosaccharide fractions indicated by the bar represented 15.5, 15.5, 18.7, 11.8, 8.3 and 30.2% of the resultant oligosaccharides, respectively. The tetrasaccharide fractions obtained by the first gel chromatography were subfractionated into fractions I to VII by HPLC on an amine-bound silica column as indicated in Figure 2A. Among the isolated fractions, the major fractions I, II, III,

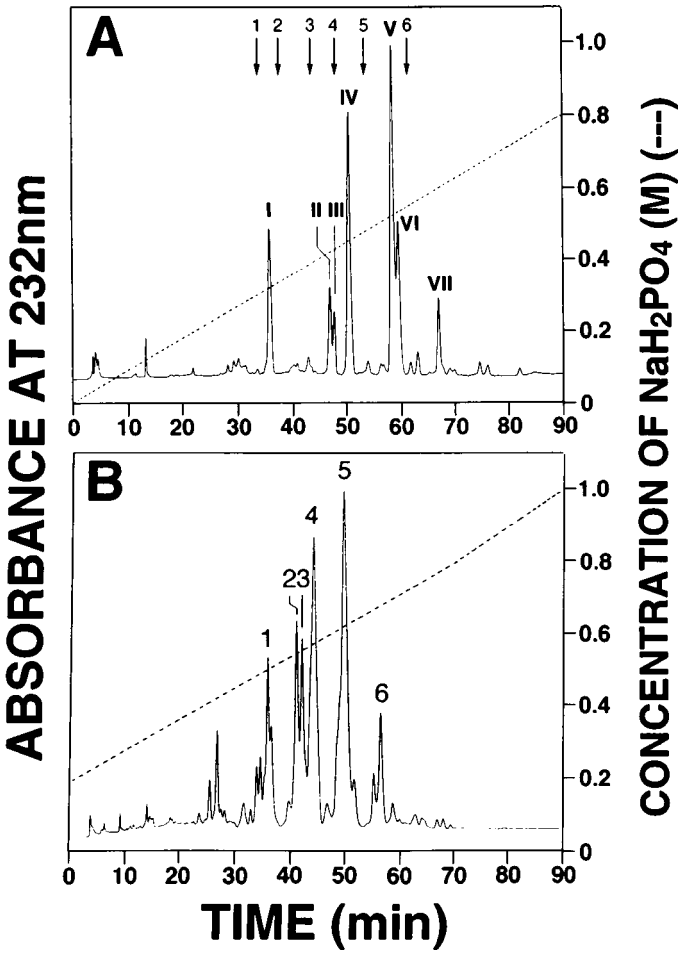

Figure 2. HPLC fractionation of the tetra- and hexasaccharide fractions. The tetra- and hexasaccharide fractions obtained by gel filtration (Fig. 1) were separated into fractions I–VII (*A*) or fractions 1–6 (*B*) on an amine-bound silica column using a linear NaH_2PO_4 gradient from 0.016 to 0.8 M over a 90-min period or 0.2 to 0.8 M over a 70-min period, respectively. For the experimental details, see Materials and methods. Arrows at the top of panel *A* indicate the elution positions of the following authentic compounds [45]: 1, $\Delta^{4,5}$HexAα1-3GalNAc(6S)β1-4GlcAβ1-3GalNAc(6S); 2, $\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4GlcAβ1-3GalNAc(4S); 3, $\Delta^{4,5}$HexA(2S)α1-3GalNAc(6S)β1-4GlcAβ1-3GalNAc(6S); 4, $\Delta^{4,5}$HexA(2S)α1-3GalNAc(6S)β1-4GlcAβ1-3GalNAc(4S); 5,$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4GlcAβ1-3GalNAc(4S); 6, $\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4GlcAβ1-3GalNAc(4S,6S).

IV, V, VI and VII were seemingly pure, giving a symmetrical single peak on HPLC and capillary electrophoresis (data not shown). The tetrasaccharide fraction from the second gel chromatography gave a very similar chromatographic pattern on HPLC (data not shown), suggesting that the first digestion step proceeded only partially, and that the corresponding fractions observed on gel filtration from both digestion steps contained structurally similar compounds. Each isolated fraction was subjected to a structural analysis as described below. Likewise, the hexasaccharide fraction obtained by gel filtration was subfractionated into fractions 1 to 6 by HPLC as indicated in Figure 2B. Each separated fraction gave a single symmetrical peak on HPLC, and the purity of each fraction checked by capillary electrophoresis was 70, 70, 82, 71, 100 and 64%, respectively (data not shown). They were also subjected to a structural analysis as described below.

FAB-MS analysis

FAB-MS analyses of the underivatized oligosaccharide fractions in the negative-ion mode defined their molecular weights, from which the composition and the maximum number of *O*-sulfate groups present in each fraction were inferred. In general, sodiated molecular ions of the type $[M - (x + 1)H + xNa]^-$ (where M represents the fully protonated acid forms of oligosaccharides) were preferentially observed in which the negative charges from both the uronic acids and sulfates were partly counter-balanced by the sodium cations. The high sodium content of the molecular ions afforded reflects the high salt content of the isolated fractions which also contributed to some sodium phosphate adducts detected at 120 u higher in some of the mass spectra acquired. Additional desalting steps or repeated drying and re-dissolving of the sample in 5% acetic acid resulted in the shift of the signal clusters to those carrying a lower sodium content but the $[M-H]^-$ signal was generally not observed for any of the samples. The assignments of the molecular ion signals afforded by each of the analyzed tetra- and hexasaccharide fractions are listed in Tables 1 and 2, respectively.

For the tetrasaccharides, fractions II, III and IV all gave similar molecular ion signal clusters at *m/z* 1085, 1107 and 1129 corresponding respectively to $[M-5H + 4Na]^-$, $[M-6H + 5Na]^-$ and $[M-7H + 6Na]^-$ of a tri-*O*-sulfated tetrasaccharide $\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_3$ (Fig. 3B). Likewise, fraction I was shown to be di-*O*-sulfated (Fig. 3A), whereas fractions V and VI were tetra-*O*-sulfated (Figs. 3C and D) and fraction VII was penta-*O*-sulfated (Fig. 3E). This assignment was further confirmed when, after perdeuteroacetylation, fraction IV afforded the expected molecular ion signal cluster at *m/z* 1311, 1333 and 1355 corresponding to $[M-3H + 2Na]^-$, $[M-4H + 3Na]^-$ and $[M-5H + 4Na]^-$, respectively, of a perdeuteroacetylated tri-*O*-sulfated tetrasaccharide $\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_3$ (data not shown). For the hexasaccharide fractions, the most highly sulfated species (fraction 6, hepta-*O*-sulfated) afforded molecular ions which carried up to nine sodium ions (Table 2). However, such highly sulfated and negatively charged molecules are generally less amenable to a FAB-MS analysis, resulting in weaker molecular ion signals and that fraction 5 failed to yield any signals despite repeated attempts. Instead, the major component in fraction 5 was detected by MALDI-TOF-MS when the sample was mixed with a basic peptide $(Arg\text{-}Gly)_{15}$ to form a noncovalent complex. The molecular weight of the detected hexasaccharide was calculated by subtracting the measured *m/z* value of the protonated peptide (*m/z* 3217.51) from the measured *m/z* value of the protonated peptide/hexasaccharide complex (*m/z* 4835.47). The measured average molecular weight of the major compound in fraction 5 was therefore 1618, which corresponded to a hexasulfated hexasaccharide $\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_6$.

Enzymatic characterization of the isolated tetrasaccharide fractions

The disaccharide composition of each of the isolated tetrasaccharide fractions was determined by digestion with chondroitinase ABC and B in conjunction with HPLC, and the results are summarized in Table 3. Tetrasaccharides in the isolated fractions except for fraction IV were completely digested into disaccharides by both enzymes, indicating that the internal uronic acid residue in the major component in each fraction was IdoA and not GlcA. In the case of fraction IV, 81 or 19% of the compounds therein was digested by chondroitinases B or AC-II, respectively, as judged by HPLC (data not shown), indicating that the compounds in the major and the minor subfraction designated as IVa and IVb had an IdoA and a GlcA residue as an internal uronic acid residue, respectively.

The results obtained from various enzymatic studies with fraction VI are described below as representative examples. The digestion of fraction VI with chondroitinase B gave two major products, ΔDi-4S and ΔDi-triS, in a molar ratio of 1 : 1 as determined by HPLC (Table 3 and Fig. 4A). The sequential arrangement of these disaccharide units was characterized using two types of sulfatases: Δhexuronate-2-sulfatase and CS-4-sulfatase. The enzyme Δhexuronate-2-sulfatase removes a sulfate group only from the C-2 position of a $\Delta^{4,5}$HexA residue located at the non-reducing terminus [43]. The enzyme CS-4-sulfatase acts predominantly on the GalNAc at the reducing end under limited incubation conditions, whereas it can remove sulfates not only from the GalNAc residue at the reducing end but also from internal GalNAc residues under harsh incubation conditions [54]. The major component in fraction VI was quantitatively digested by both Δhexuronate-2-sulfatase and CS-4-sulfatase under the limited incubation conditions, giving rise to a product which was eluted approximately 9 min earlier than

Table 1. FAB-MS analysis of the seven tetrasaccharide fractions isolated from CS-H of hagfish notochord

Fractions	*Yield*[a] *(nmol)*	*m/z for [M-4H + 3Na]⁻*	*m/z for [M-5H + 4Na]⁻*	*m/z for [M-6H + 5Na]⁻*	*m/z for [M-7H + 6Na]⁻*	*m/z for [M-8H + 7Na]⁻*	*m/z for [M-9H + 8Na]⁻*	*M.W.*[b]	*Assignment*
I	147	983	1005	1027				918	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_2$
II	183		1085	1107	1129			998	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_3$
III	87		1085	1107	1129			998	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_3$
IV	64		1085	1107	1129			998	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_3$
V	330			1187	1209	1231		1078	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_4$
VI	200		1165	1187				1078	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_4$
VII	84				1289	1311	1333	1158	$\Delta HexA_1HexA_1HexNAc_2(OSO_3H)_5$

[a]Nanomoles obtained from 103.8 mg of CS-H.
[b]Molecular weights are expressed as monoisotopic nominal masses for simplicity.

Table 2. FAB-MS analysis of the six hexasaccharide fractions isolated from CS-H of hagfish notochord

Fr.	*Yield*[a] *(nmoles)*	*m/z for [M-6H + 5Na]*	*m/z for [M-7H + 6Na]*	*m/z for [M-8H + 7Na]*	*m/z for [M-9H + 8Na]*	*m/z for [M-10H + 9Na]*	*m/z for [M-11H + 10Na]*	*M.W.*[c]	*Assignment*
1	59	1566	1646					1457	$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_4$
2	33		1646	1668	1690			1515	$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_5$
3	30		1646	1668	1690			1515	$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_5$
4	213		1646	1668	1690			1515	$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_5$
5[b]	119								$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_6$
6	37					1872	1894	1675	$\Delta HexA_1HexA_2HexNAc_3(OSO_3H)_7$

[a]Nanomoles obtained from 103.8 mg of CS-H.
[b]The analysis of fraction 5 by FAB-MS failed partly due to the high negative charge. Hence, fraction 5 was analyzed by MALDI-TOF-MS, which successfully determined the sugar and sulfate composition.
[c]Molecular weights are expressed as monoisotopic nominal masses for simplicity.

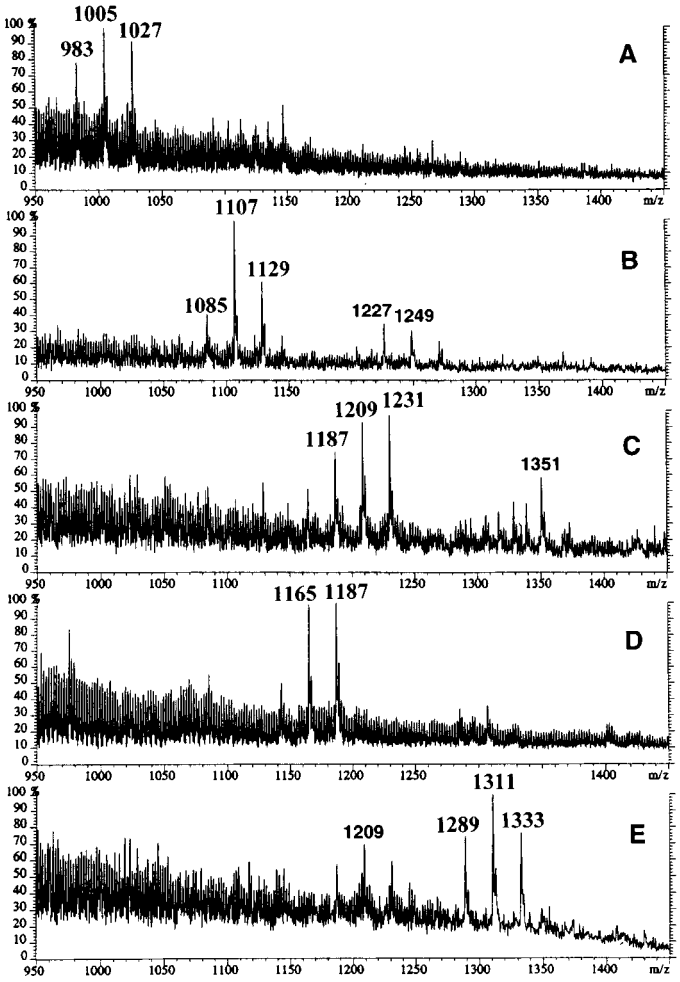

Figure 3. FAB-MS spectra of underivatized tetrasaccharide fractions. The FAB-MS spectra of underivatized tetrasaccharide fractions I (*A*), III (*B*), V (*C*), VI (*D*) and VII (*E*) were measured in the negative-ion mode. For the experimental details, see Materials and methods. Major molecular ion signals are assigned as summarized in Table 1.

the parent compound, respectively (Table 4 and Figs. 4B and C). The results indicated that the 2-sulfated $\Delta^{4,5}$HexA residue and the 4-sulfated GalNAc residue were located at the non-reducing and the reducing terminus, respectively. Hence, the major compound in fraction VI contained the following structure:

Fraction VI:
$\Delta^{4,5}$HexA(2S)α1-3GalNAc(4S,6S)β1-4IdoAα1-3 GalNAc(4S).

Likewise, fractions I, II, III, IV, V and VII were successfully analyzed by enzymatic digestions in conjunction with HPLC. The results obtained by chondroitinase ABC digestion showed the disaccharide composition of each fraction (Table 3), and those from sulfatase digestion experiments revealed the sequential arrangement of the respective disaccharide units in the major compound in each fraction (Table 4). The sensitivity to Δhexuronate-2-sulfatase indicated that fractions III and VII contained a $\Delta^{4,5}$HexA(2S) residue at the non-reducing end. The sensitivity to CS-4-sulfatase and CS-6-sulfatase suggested that fractions I, III and IV had a GalNAc(4S) residue whereas fractions II, V and VII had a GalNAc(4S,6S) at the reducing ends. All of the fractions except for fractions IV were completely degraded by chondroitinase B into disaccharides and were resistant to chondroitinase AC-II, indicating that the internal uronic acid was IdoA. In the case of fraction IV, although a majority, designated fraction IVa, was digested by chondroitinase B (Table 3), approximately 19% (designated IVb) was degraded by chondroitinase AC-II, yielding ΔDi-4S and ΔDi-diS_E in a molar ratio of 1.19 : 1.00 (data not shown). The results indicated the internal uronic acid of the compounds in fractions IVa and IVb were IdoA and GlcA, respectively. Based on these results, the following structures were deduced and were consistent with the results obtained by FAB-MS.

Fraction I:
$\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4IdoAα1-3GalNAc(4S).
Fraction II:
$\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4IdoAα1-3GalNAc (4S,6S).
Fraction III:
$\Delta^{4,5}$HexA(2S)α1-3GalNAc(4S)β1-4IdoAα1-3GalNAc (4S).
Fraction IVa:
$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc (4S).
Fraction IVb:
$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4GlcAβ1-3GalNAc (4S).
Fraction V:
$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc (4S,6S).
Fraction VII:
$\Delta^{4,5}$HexA(2S)α1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc (4S,6S).

500-MHz ^{1}H NMR spectroscopy of the isolated tetrasaccharide fractions

Tetrasaccharide fractions I, II, III, VI, V, VI and VII were obtained in amounts large enough to allow a solid structural determination by 500-MHz ^{1}H NMR spectroscopy. As representatives, results obtained with fraction VI are described below. The one-dimensional spectrum recorded at 26 °C is shown in Figure 5. Signals at 4.4–5.5 ppm were identified as H-1 resonances of the constituent saccharide residues by comparison with the NMR spectra of unsaturated CS-tetrasaccharides [45] and DS-tetrasaccharides [50]. Proton signals in the 1D spectra were assigned using two-dimen-

Table 3. Disaccharide composition of the isolated tetrasaccharide fractions[a]

Fr.	*Disaccharides formed*[b]	
	Chondroitinase ABC	*Chondroitinase B*
	%Recovery[c]	
I	ΔDi-4S (222%)	ΔDi-4S (203%)
II	ΔDi-4S (115%) + ΔDi-diS_E (117%)	ΔDi-4S (116%) + ΔDi-diS_E (113%)
III	ΔDi-4S (157%) + ΔDi-diS_B (144%)	ΔDi-4S (134%) + ΔDi-diS_B (145%)
IV[d]	ΔDi-4S (121%) + ΔDi-diS_E (109%)	ΔDi-4S (100%) + ΔDi-diS_E (100%)
V	ΔDi-diS_E (222%)	ΔDi-diS_E (216%)
VI	ΔDi-4S (111%) + ΔDi-triS (98%)	ΔDi-4S (108%) + ΔDi-triS (105%)
VII	ΔDi-diS_E (147%) + ΔDi-triS (125%)	ΔDi-diS_E (146%) + ΔDi-triS (119%)

[a]Each tetrasaccharide fraction was digested with chondoritinase ABC or B, and the products were identified and quantified by HPLC as described in Materials and methods.
[b]See footnote 1 for the abbreviations defining the disaccharides.
[c]The percent recovery was calculated based on the peak area on HPLC and is expressed in molar proportions, taking the amount of each parent tetrasaccharide fraction used for digestion to be 100%.
[d]Eighty-one % and 19% of fraction IV were digested by chondroitinases B and AC-II, respectively (see text).

sional correlation spectroscopy (2D COSY), which are shown in Figure 6, respectively. The assignment pathway from H-1 to H-4 signals for αGalNAc-*1* is shown in the COSY spectrum. Beginning with the H-1 signal at δ 5.213, a cross-peak showing the connectivity to the H-2 (δ 4.349) was found (Fig. 6). The connectivities of the H-2 to the H-3 (δ 4.15) and then to the H-4 (δ 4.74) were sequentially established (Fig. 6). The cross-peaks of the H-5 with the H-6 and H-6′ atoms were observed at δ 3.77 and 3.70, respectively, based on the comparison with the corresponding signals belonging to analogous tetrasaccharides such as GlcAβ1-3GalNAc(4S,6S)β1-4GlcAβ1-3GalNAc(4S) [22] and $\Delta^{4,5}$HexAβ1-3GalNAc(4S)α1-4IdoAβ1-3GalNAc(4S) [50]. Although the connectivity of the H-4 to the H-5 resonances was not observed, the H-5 signal (δ 4.28) was assigned based on the characteristic cross-peaks of the H-5 with the H-6 and H-6′ atoms. In a similar fashion, other proton signals were assigned starting with the H-1 resonance of each component sugar residue except for $\Delta^{4,5}$HexA-*4*. The $\Delta^{4,5}$HexA-*4* H-1 signal (δ 5.586) was identified by the sequential assignment of the cross-peaks starting with the characteristic H-4 signal (δ 6.042) in the COSY spectrum.

IdoA-*2* of each tetrasaccharide was readily identified by the anomerization effects caused by GalNAc-*1,* which resulted in doubling of the anomeric proton signal of this IdoA residue. For example, the anomeric resonances of IdoA-*2* of the tetrasaccharide in fraction VI were observed at δ 4.902 and 4.857. IdoA-*2* H-1 of the β-anomer was assigned to the latter rather than the former based on the closer resemblance to that of the IdoA residue attached to βGlcNAc through a β1-4 linkage as in IdoA-*2* of the β-anomer [50]. The remaining resonance at δ 4.902 was assigned to that of IdoA-*2* H-1 of the α-anomer.

The resonances observed around δ 2.1 are characteristic of the *N*-acetyl methyl protons of GalNAc. Two NAc signals belonging to the two GalNAc residues were found for each tetrasaccharide component. Generally, the NAc signal of the penultimate GalNAc residue substituted by $\Delta^{4,5}$HexA is located in a lower magnetic field than that of the other GalNAc residues in the same sequence, and that of the reducing GalNAc is observed in a higher magnetic field than those of the other GalNAc residues [18,19,45,55]. Thus, the NAc signal belonging to the GalNAc-*1* residue in each tetrasaccharide was readily assigned, and the remaining signal was assigned to the GalNAc-*3* residue.

Modification by *O*-sulfation causes downfield shifts of protons bound to the *O*-sulfated carbon atoms by approximately 0.4–0.7 ppm [56]. For example, downfield shifts of the H-6, 6′ (Δ δ 0.37 ppm) of GalNAc-*3,* the H-4 (Δ δ 0.56 ppm) of GalNAc-*1,* and GalNAc-*3* as well as the H-2 (Δ δ 0.65 ppm) of $\Delta^{4,5}$HexA-*4* of the tetrasaccharide in fraction VI were observed compared with those of the non-sulfated residues of authentic disaccharides [57], indicating the 6-*O*-sulfation of GalNAc-*3,* the 4-*O*-sulfation of GalNAc-*1* and GalNAc-*3* as well as the 2-*O*-sulfation of $\Delta^{4,5}$HexA-*4*. Thus, the structure of the compound in fraction VI was confirmed to be $\Delta^{4,5}$HexA(2S)α1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc(4S). Likewise, the sulfation profiles of the major compounds in the other tetrasaccharide fractions were firmly established by ^{1}H NMR spectroscopy (Table 5). The established structures were in good agreement with those proposed based on the enzymatic analysis described above, justifying the latter methodology for structural analysis.

Enzymatic characterization of the isolated hexasaccharide fractions

Hexasaccharide fractions 1 ~ 6 were analyzed by enzymatic digestions although they were not analyzed by ^{1}H

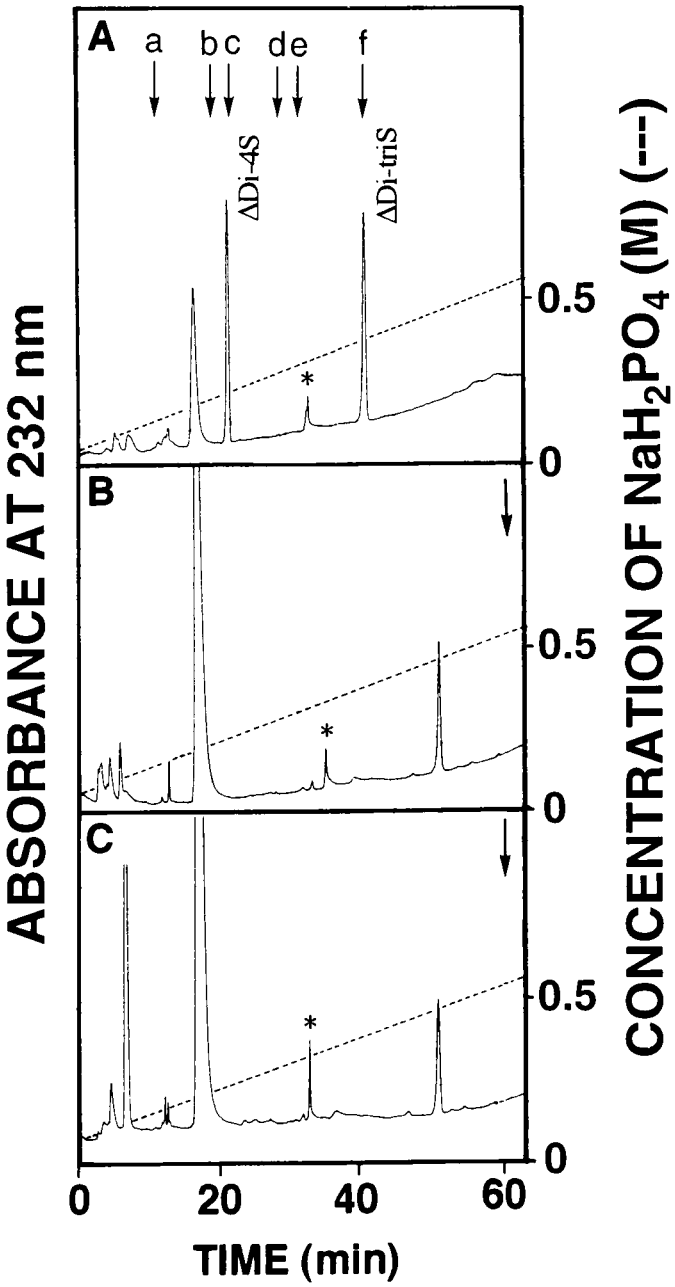

Figure 4. Enzymatic analysis of the isolated tetrasaccharide fraction VI. The isolated tetrasaccharide fraction VI was digested with chondroitinase B (*A*), Δhexuronate-2-sulfatase (*B*) or CS-4-sulfatase (*C*). Each digest was analyzed by HPLC on an amine-bound silica column using a linear NaH_2PO_4 gradient from 16 to 530 mM over a 60-min period. The peaks observed before 20 min are attributable to the buffer salts, and those marked by asterisks were often observed in high-sensitivity analyses and were due to an unknown substance eluted from the column resin. The arrow in Panels *B* and *C* indicates the elution position of undigested fraction VI. The elution positions of the authentic unsaturated disaccharides are indicated by arrows in Panel *A:* a, ΔDi-0S; b, ΔDi-6S; c, ΔDi-4S; d, ΔDi-diS_D; e, ΔDi-diS_E; f, ΔDi-triS.

Table 4. Action of sulfatases on the isolated tetra- and hexasaccharides[a]

Fr.	*ΔHexuronate-2-sulfatase*	*CS-4-sulfatase*	*CS-6-sulfatase*
Tetrasaccharides			
I	ND[c]	+[b]	−
II	ND	−	+
III	+	+	−
IV	ND	+	−
V	ND	−	+
VI	+	+	−
VII	+	−	+
Hexasaccharides			
1	ND	+	−
2	ND	−	+
3	ND	−	+
4	ND	+	−
5	ND	−	+
6	+	−	+

[a]Each tetra- or hexasaccharide was digested with Δhexuronate-2-, CS-4-, or CS-6-sulfatase, and the digest was analyzed by HPLC. The digestion with CS-4-sulfatase was carried out under limited incubation conditions, where the enzyme removes sulfate only from the reducing GalNAc(4S) [54].
[b]Symbols − and + represent the resistance and sensitivity, respectively, to each enzyme.
[c]ND, not determined.

NMR due to the limited amounts of the materials. These fractions were totally resistant to chondroitinase AC-II, suggesting that the internal uronic acid in the major compound in each fraction was IdoA rather than GlcA [50]. However, they were largely but not completely digested by chondroitinase B (data not shown), suggesting that the enzymatic action could be dependent on the substrate size as well as the sulfation pattern. These fractions were then analyzed using sulfatases and a conventional and a highly purified preparation of chondroitinase ABC in conjunction with HPLC, and the results are summarized in Tables 4 and 6, respectively. The conventional chondroitinase ABC completely degrades CS and DS into unsaturated disaccharides, whereas highly purified chondroitinase ABC acts on unsaturated hexasaccharides in an exolytic fashion, removing an unsaturated disaccharide unit from the non-reducing terminus of hexasaccharides irrespective of the sulfation profiles [19]. Since fraction 5 yielded only $\Delta^{4,5}$HexAα1-3GalNAc(4S,6S) upon digestion with a conventional preparation of chondroitinase ABC, the major compound in fraction 5 was the following hexasulfated hexasaccharide structure, in agreement with the composition determined by MALDI-TOF-MS (Table 2).

Fraction 5:
$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc(4S,6S)β-4IdoAα1-3GalNAc(4S,6S).

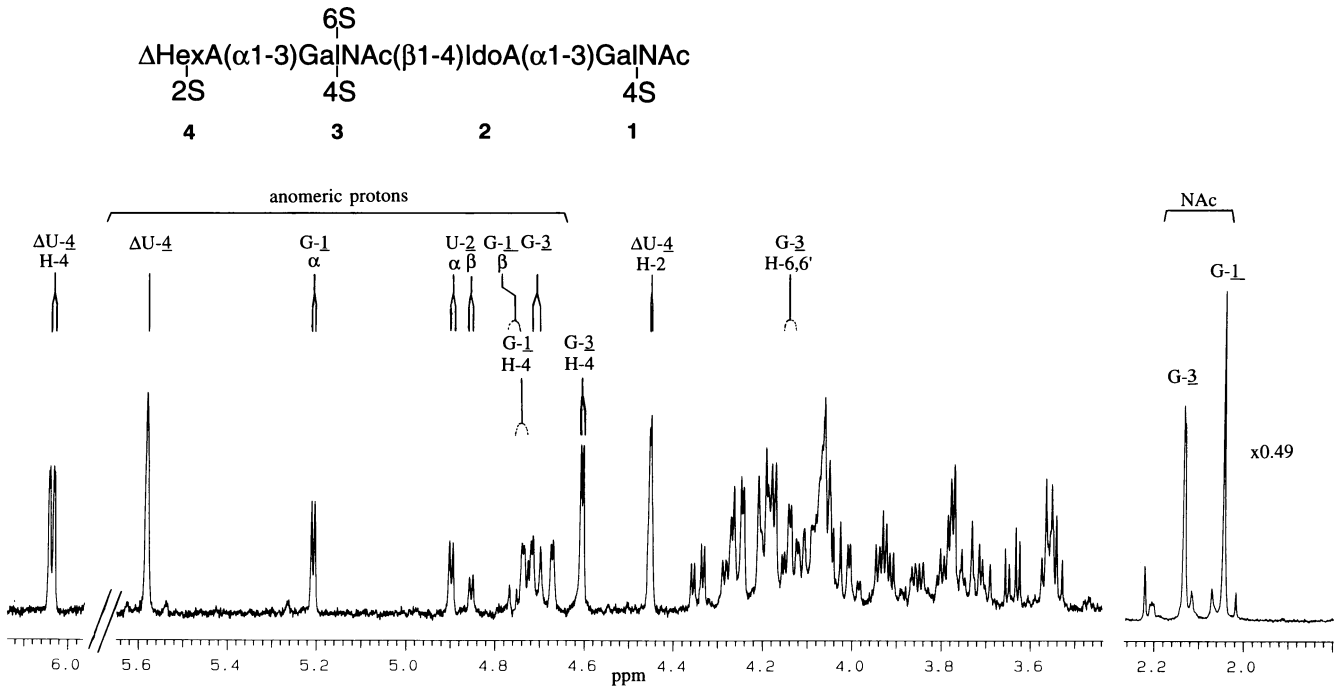

Figure 5. Structural-reporter-group regions of the 500-MHz ^1H NMR spectra of the tetrasaccharide fraction VI. The spectrum was recorded in ^2H_2O at 26 °C. The letters and numbers refer to the corresponding residues in the structures. U, IdoA; ΔU, $\Delta^{4,5}$HexA; G, GalNAc.

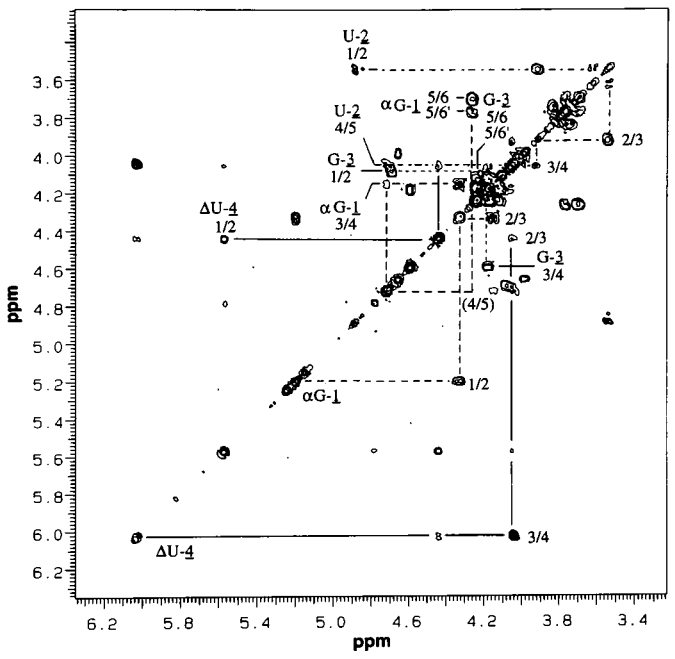

Figure 6. Two-dimensional COSY spectrum of tetrasaccharide fraction VI. The spectrum was recorded in ^2H_2O at 26 °C. The letters and numbers refer to the corresponding residues in the structures. U, IdoA; ΔU, $\Delta^{4,5}$HexA; G, GalNAc. The assignment pathways of proton signals for αG-*1*, U-*2*, G-*3*, ΔU-*4* are indicated. Although the cross peak between the H-4 to the H-5 resonances of αG-*1* was not observed, the connectivity of the H-4 and H-5 was inferred based on the characteristic cross-peaks of the H-5 with the H-6 and H-6′ atoms and those of the H-3 with the H-4 atoms (see text).

Fraction 1 yielded $\Delta^{4,5}$HexA-GalNAc(4S) and $\Delta^{4,5}$HexA-GalNAc(4S,6S) in a molar ratio of 2 : 1 upon digestion with conventional chondroitinase ABC (Table 6). When digested with a highly purified preparation of chondroitinase ABC, fraction 1 resulted in a disaccharide $\Delta^{4,5}$HexA-GalNAc(4S) and a tetrasaccharide $\Delta^{4,5}$HexA-GalNAc (4S,6S)-IdoA-GalNAc(4S) in a molar ratio of approximately 1 : 1 (Table 6). Fraction 1 was sensitive to CS-4-sulfatase but resistant to CS-6-sulfatase (Table 4), indicating that the compound in fraction 1 contained a disaccharide unit HexA-GalNAc(4S) at the reducing end. Together these results indicated that the parent compound in fraction 1 had the following tetrasulfated hexasaccharide structure, in agreement with the composition estimated by FAB-MS (Table 2).

Fraction 1:
$\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4IdoAα1-3GalNAc (4S,6S)β1-4IdoAα1-3GalNAc(4S).

Fraction 2 yielded $\Delta^{4,5}$HexA-GalNAc(6S), $\Delta^{4,5}$HexA-GalNAc(4S) and $\Delta^{4,5}$HexA-GalNAc(4S,6S) with recoveries of 80, 33 and 230% relative to the parent compound upon digestion with conventional chondroitinase ABC (Table 6). When digested with highly purified enzyme, the major products were a disaccharide $\Delta^{4,5}$HexA-GalNAc (4S,6S) and an unidentified trisulfated tetrasaccharide (Table 6). Fraction 2 was sensitive to CS-4-sulfatase but resistant to CS-6-sulfatase (Table 4), indicating that the major compound in fraction 2 contained a disaccharide unit HexA-GalNAc(4S) at

Table 5. ^{1}H-Chemical shifts of structural-reporter-groups of the constituent monosaccharides of the tetrasaccharides isolated from CS-H[a]

		Fr. II		*Fr. III*		*Fr. V*		*Fr. VI*		Fr. VI	
		ΔU–G–U–G(6S; 4S, 4S)		ΔU–G–U–G(2S, 4S, 4S)		ΔU–G–U–G(6S, 6S; 4S, 4S)		ΔU–G–U–G(6S; 2S, 4S, 4S)		ΔU–G–U–G(6S, 6S; 2S, 4S, 4S)	
Residue	*Reporter group*	*α*	*β*	*α*	*β*	*α*	*β*	*α*	*β*	*α*	*β*
GalNAc-*1*	H-1	5.217 (3.5)	ND[b]	5.210 (3.5)	ND	5.217 (4.0)	4.701 (8.5)	5.213 (3.5)	4.76 (ND)	5.221 (4.0)	4.70 (ND)
	H-2	4.361	ND	4.349	ND	4.357	4.02	4.349	4.02	4.360	4.01
	H-3	4.180	ND	4.167	ND	4.19	ND	4.15	ND	4.18	ND
	H-4	4.624	ND	4.72	ND	4.671	ND	4.74	ND	4.608	ND
	H-5	4.25	ND	4.26	ND	4.25	ND	4.28	ND	4.25	ND
	H-6	4.17	ND	3.76	ND	4.18	ND	3.77	ND	4.18	ND
	H-6′	4.11	ND	3.70	ND	4.18	ND	3.70	ND	4.18	ND
	NAc	2.049		2.049		2.045		2.048		2.046	
IdoA-*2*	H-1	4.921 (3.5)	4.878 (3.5)	4.910 (3.5)	4.865(4.0)	4.906 (4.0)	4.862 (4.5)	4.902 (4.0)	4.857 (4.0)	4.921 (4.0)	4.877 (4.5)
	H-2	3.539	3.562	3.52	ND	3.55	3.56	3.55	3.56	3.54	ND
	H-3	3.87		3.87		3.92	3.91	3.94	3.92	3.93	
	H-4	4.08		4.08		4.07	4.06	4.05		4.07	
	H-5	ND		4.74		4.73	4.72	4.70		ND	
GalNAc-*3*	H-1	4.688 (9.0)		4.695 (8.5)		4.701 (8.5)		4.711 (8.0)		4.711 (8.5)	
	H-2	4.06		4.06		4.08		4.08		4.08	
	H-3	4.14		4.107		4.18		4.19		4.27	
	H-4	4.518		4.567		4.515		4.610		4.517	
	H-5	3.86		3.85		4.16		4.23		4.25	
	H-6	3.76		3.75		4.26		4.14		4.18	
	H-6′	3.76		3.75		4.26		4.14		4.18	
	NAc	2.119		2.132		2.120		2.138		2.137	
ΔHexA-*4*	H-1	5.265 (3.0)		5.578 (<2.0)		5.266 (2.5)		5.586 (<2.0)		5.585 (<2.0)	
	H-2	3.83		4.456		3.828		4.457		4.458	
	H-3	3.939		4.05		3.94		4.04		4.055	
	H-4	5.964		6.043		5.957		6.042		6.043	

[a] ^{1}H-Chemical shifts of the consituent monosaccharides of the seven tetrasaccharides derived from CS-H are shown. Chemical shifts are given in ppm downfield from internal sodium 4, 4-dimethyl-4-silapentane-1-sulfonate [53] but were actually measured indirectly to acetone in D_2O (δ 2.225) at 26 °C. Coupling constants $J_{1,2}$ (in Hz) are given in parentheses.
[b] ND = not determined.

Table 6. Analysis of the chondroitinase ABC digests of the isolated hexasaccharide fractions[a]

	Products[b]	
Fr.	*Conventional chondroitinase ABC*	*Highly purified chondroitinase ABC*
	% Recovery[c]	
1	ΔDi-4S(247%) + ΔDi-diS$_E$ (133%)	ΔDi-4S (83%) + ΔDi-diS$_E$ (33%) + unidentified disulfated tetrasaccharide-1 (13%) + unidentified disulfated tetrasaccharide-2 (20%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S) (67%)
2	ΔDi-6S (80%) + ΔDi-4S (33%) + ΔDi-diS$_E$ (230%)	ΔDi-4S (27%) + ΔDi-6S (6%) + ΔDi-diS$_E$ (230%) + unidentified trisulfated tetrasaccharide (127%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S,6S) (7%)
3	ΔDi-4S (130%) + ΔDi-diS$_E$ (230%) + ΔDi-triS (22%)	ΔDi-4S (163%) + ΔDi-diS$_E$ (80%) + ΔDi-triS (25%) + unidentified trisulfated tetrasaccharide-1 (19%) + unidentified trisulfated tetrasaccharide-2 (7%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S,6S) (100%)
4	ΔDi-6S (7%) + ΔDi-4S (110%) + ΔDi-diS$_E$ (223%) + ΔDi-triS (6%)	ΔDi-diS$_E$ (80%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S) (67%)
5	ΔDi-4S (8%) + ΔDi-6S (7%) + ΔDi-diS$_E$ (173%)	ΔDi-diS$_E$ (90%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S,6S) (73%)
6	ΔDi-4S (30%) + ΔDi-6S (110%) + ΔDi-diS$_E$ (173%) + ΔDi-triS (167%)	ΔDi-4S (15%) + ΔDi-diS$_E$ (22%) + ΔDi-triS (100%) + ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S,6S) (53%) + unidentified tetrasulfated tetrasaccharide (57%)

[a]Each hexasaccharide fraction was digested with a conventional or a highly purified preparation of chondroitinase ABC, and the products were identified and quantified by HPLC as described in Materials and methods. These fractions were resistant to chondroitinase AC-II, indicating that the internal uronic acid in each component is IdoA rather than GlcA.
[b]See Footnote 1 for the abbreviations defining the disaccharides.
[7]he percent recovery was calculated based on the peak area on HPLC and is expressed in molar proportions taking the amount of each parent tetrasaccharide fraction used for digestion to be 100%.

the reducing end. Although these results indicated that fraction 2 contained at least two compounds, the major compound had the structure $\Delta^{4,5}$HexAα1-3GalNAc(4S,6S) β1-4IdoAα1-3GalNAc (6S) β1-4IdoAα1-3GalNAc(4S,6S) or $\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc (4S,6S)β1-4IdoAα1-3GalNAc(6S). However, due to the lack of authentic tetrasaccharides such as $\Delta^{4,5}$HexAα1-3 GalNAc(6S)β1-4IdoAα1-3 Gal NAc(4S,6S) and $\Delta^{4,5}$HexA α1-3GalNAc(4S,6S)β1-4IdoA α1-3GalNAc(6S), it was not possible to discriminate the two structures.

Fraction 3 yielded $\Delta^{4,5}$HexA-GalNAc(4S) and $\Delta^{4,5}$HexA-GalNAc(4S,6S) in a molar ratio of approximately 1 : 2 with a smaller proportion of a trisulfated unit upon digestion with conventional chondroitinase ABC (Table 6). When digested with highly purified enzyme, fraction 3 resulted in a major tetrasaccharide $\Delta^{4,5}$HexA-GalNAc(4S,6S)-IdoA-GalNAc (4S,6S) and two major disaccharides $\Delta^{4,5}$HexA-GalNAc (4S) and $\Delta^{4,5}$HexA-GalNAc (4S,6S). Since the results from FAB-MS indicated that the major compound in this fraction was a pentasulfated hexasaccharide, the parent hexasaccharide was concluded to contain the following structure with $\Delta^{4,5}$HexAα1-3GalNAc(4S) at the non-reducing end. The sensitivity of the major hexasaccharide to CS-6-sulfatase was consistent with the proposed structure.

Fraction 3:

$\Delta^{4,5}$HexAα1-3GalNAc(4S)β1-4IdoAα1-3GalNAc (4S,6S)β1-4IdoAα1-3GalNAc(4S,6S).

Fraction 4 yielded $\Delta^{4,5}$HexA-GalNAc(4S) and $\Delta^{4,5}$HexA-GalNAc(4S,6S) in a molar ratio of 1 : 2 upon digestion with conventional chondroitinase ABC (Table 6). When digested with highly purified chondroitinase ABC, fraction 4 resulted in a disaccharide $\Delta^{4,5}$HexA-GalNAc (4S,6S) and a tetrasaccharide $\Delta^{4,5}$HexA-GalNAc(4S,6S)-IdoA-GalNAc(4S) in a molar ratio of approximately 1 : 1 (Table 6). Fraction 4 was sensitive to CS-4-sulfatase but resistant to CS-6-sulfatase (Table 4), indicating that the compound in fraction 4 contained a disaccharide unit HexA-GalNAc(4S) at the reducing end. These results altogether indicated that the parent compound in fraction 4 had the following pentasulfated hexasaccharide structure, in agreement with the composition estimated by FAB-MS (Table 2).

Fraction 4:

$\Delta^{4,5}$HexAα1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc (4S,6S)β1-4IdoAα1-3GalNAc(4S).

Fraction 6 yielded the three major disaccharide units $\Delta^{4,5}$HexA-GalNAc(6S), $\Delta^{4,5}$HexA(2S)-GalNAc(4S,6S) and $\Delta^{4,5}$HexA-GalNAc(4S,6S) with a smaller proportion of $\Delta^{4,5}$HexA-GalNAc(4S) upon digestion with conventional chondroitinase ABC (Table 6). However, it was difficult to estimate the disaccharide composition of fraction 6, and it appeared to contain at least two different compounds. When digested with the highly purified enzyme, fraction 6 resulted in two putative trisulfated tetrasaccharides with recoveries of 53 and 57%, respectively, in addition to a major disaccharide $\Delta^{4,5}$HexA(2S)-GalNAc(4S,6S) (100% recovery) and two minor disaccharides (Table 6). One tetrasaccharide derived from a parent hexasaccharide designated 6-1 was identified as $\Delta^{4,5}$HexA-GalNAc(4S,6S)-IdoA-GalNAc(4S,6S) by co-chromatography with the authentic compound (Fraction V) (data not shown). The other tetrasaccharide derived from a parent hexasaccharide designated 6-2 eluted immediately after $\Delta^{4,5}$HexA-GalNAc(4S,6S)-IdoA-GalNAc(4S), indicating that it was a trisulfated tetrasaccharide. However, it remained unidentified because of the lack of an authentic compound. Both major hexasaccharides in fraction 6 were sensitive to Δ hexuronate-2-sulfatase and CS-6-sulfatase (Table 4), indicating that $\Delta^{4,5}$HexA(2S) and GalNAc(6S) were located at the non-reducing and reducing terminus of both hexasaccharides, respectively. Together these resulted indicated that the parent compound in fraction 6-1 had the following heptasulfated hexasaccharide structure, in agreement with the composition estimated by FAB-MS (Table 2).

Fraction 6-1:
$\Delta^{4,5}$HexA(2S)α1-3GalNAc(4S,6S)β1-4IdoA
α1-3GalNAc(4S,6S)β1-4IdoAα1-3GalNAc(4S,6S).

Discussion

The IdoAα1-3GalNAc(4S,6S) structure characteristic of CS-H is not limited to marine vertebrates such as hagfish; it has been identified in various mammalian tissues and cells. For example, it is present in significant amounts in various porcine tissues including skin, intestine, spleen, liver, lung and kidney [41,50,58,59]. Specific physiological functions of highly sulfated DS containing GalNAc(4S,6S) residue(s), which can be termed DS-E since it contains IdoA-GalNAc(4S,6S), have not been clarified, but have attracted increasing attention. A significant contribution to the HCII-mediated inhibitory activity against thrombin by DS with IdoA-GalNAc(4S,6S) sequences has been reported [41,42,60], although such sequences are less effective than those containing IdoA(2S)-GalNAc(4S) [59,60,61], and CS-H itself from hagfish notochord weakly exhibits the HCII-mediated inhibition of thrombin [60]. DS-E has been also found in guinea pig peritoneal macrophages [62] as well as in human monocyte-derived macrophages [63]. Edwards *et al.* [64] demonstrated that human macrophages exhibited the differentiation-associated expression of CS-E or DS-E, suggesting that the 4,6-*O*-disulfated GalNAc residue could be a marker for macrophage differentiation from monocytes. CS-E or DS-E specifically inhibits the function of activated properdin in the alternative complement pathway [65]. However, sequence information concerning DS chains containing IdoA-Gal NAc(4S,6S) is quite limited, and only two sequences found in the tetrasaccharide fraction I and the hexasaccharide fraction 1 have been reported [50].

In this study, seven tetra- and five hexasaccharide structures derived from CS-H of hagfish notochord were demonstrated. Of these structures, the tetarasccharides in fractions I and IVa have been isolated previously from DS of porcine intestine [50] and human umbilical cord [14], whereas the tetrasaccharide in fraction IVb has been isolated from CS-E of squid cartilage [45]. The other tetra- and hexasaccharides, which ranged from tri- to pentasulfated tetrasaccharides and from tetra- to heptasulfated hexasaccharides, were demonstrated for the first time in the present study. Since these structures were prepared by digestion with chondroitinase B, a $\Delta^{4,5}$HexA residue at the non-reducing end of each structure was derived from IdoA. The isolated sequences indicated that the enzyme catalyzed the eliminative cleavage of *N*-acetylgalactosaminidic linkages to IdoA residues in sequences containing GalNAc (4S or 4S,6S)β1-4IdoA(± 2S)α1-3GalNAc(4S or 4S,6S), yielding oligosaccharides with $\Delta^{4,5}$HexA or $\Delta^{4,5}$HexA(2S) at the non-reducing ends. Hence, the 4-*O*-sulfation of GalNAc residues flanking the cleavage site seems to be an essential requirement for the enzyme action. The isolated structures were composed of six kinds of disaccharide units: GlcAβ1-3GalNAc(4S), IdoAα1-3GalNAc(4S), IdoAα1-3GalNAc(6S), IdoA(2S)α1-3GalNAc(4S), IdoAα1-3GalNAc(4S,6S) and IdoA(2S)α1-3GalNAc(4S,6S), three of which were oversulfated containing 2 or 3 sulfate groups. The isolated sequences also indicated that an IdoA-GalNAc(4S,6S) unit characteristic of CS-H was located next to any one of the above six disaccharide units and at least three consecutive IdoA-GalNAc(4S,6S) units could be found, as in the hexasaccharide fraction 5.

Data concerning the biosynthetic mechanism of IdoA-GalNAc(4S,6S) are rather limited, although some information about CS biosynthesis has accumulated. Chondroitin 6-*O*-sulfotransferase, which transfers sulfate to the C-6 position of GalNAc residues of CS, has been purified, cloned and characterized [66,67]. It showed only slight activity toward DS, and had no ability to transfer sulfate to the C-6 position of a GalNAc(4S) residue of CS to form the GalNAc(4S,6S) structure. It should be noted that two other 6-*O*-sulfotransferases involved in the synthesis of CS-E have been reported. One that synthesizes internal GalNAc(4S,6S) units of CS has been demonstrated in squid cartilage [68]. The other that specifically transfers sulfate to the GalNAc(4S) residue at the non-reducing terminus of the newly synthe-

sized CS has been demonstrated in an organ culture of chick and rat embryo cartilage [69]. Chondroitin 4-*O*-sulfotransferase has been purified and characterized [70]; although it showed activity toward chondroitin but also chemically desulfated DS, it remains unclear whether it is involved in the DS biosynthesis. 4-*O*-Sulfotransferase acting on DS has been demonstrated in skin fibroblast microsomes [71], and 4-*O*-sulfation is a prerequisite for IdoA formation from GlcA by the action of C-5 epimerase, which has also been demonstrated in various porcine tissues [71]. However, neither enzyme has been well characterized. We recently found a novel sulfotransferase activity that transfers sulfate to the C-6 position of a GalNAc residue directly linked to an IdoA residue creating IdoAα1-3GalNAc(6S)β1-4IdoA [72], although it does not transfer sulfate to the C-6 position of a GalNAc(4S) residue linked to an IdoA residue to form IdoAα1-3GalNAc(4S,6S). No such sulfotransferase has been documented to date, to our knowledge. Therefore, a novel GalNAc-6-*O*-sulfotransferase is probably involved in the biosynthesis of CS-H or DS-E.

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