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Structural Studies on Sulfated Oligosaccharides Derived from the Carbohydrate-Protein Linkage Region of Chondroitin 6-Sulfate Proteoglycans of Shark Cartilage

II. SEVEN COMPOUNDS CONTAINING 2 OR 3 SULFATE RESIDUES*

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Shark cartilage proteoglycans bear predominantly chondroitin 6-sulfate. After exhaustive protease digestion, reductive β-elimination and subsequent chondroitinase ABC digestion, 13 hexasaccharide alditols were obtained from the carbohydrate-protein linkage region and six of them contain 0 or 1 sulfate and/or 1 phosphate residue (Sugahara, K., Ohi, Y., Harada, T., de Waard, P., and Vliegenthart, J. F. G. (1992) *J. Biol. Chem.* 267, 6027-6035). The other seven compounds, which represent approximately 60% of the isolated linkage hexasaccharides, were analyzed by chondroitinase ACII digestion in conjunction with high performance liquid chromatography and by 500-MHz one- and two dimensional ^{1}H NMR spectroscopy. All seven compounds have the following conventional structure in common.

Δ4,5-GlcAβ1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl-ol
6 5 4 3 2 1

Two disulfated compounds have an *O*-sulfate on C-6 of the Gal-*2* residue attached to xylitol in combination with an *O*-sulfate on C-4 or on C-6 of the GalNAc residue. The third disulfated compound has *O*-sulfate on C-6 of Gal-*2*, and also on C-6 of Gal-*3*. Two of the trisulfated compounds also have *O*-sulfate on C-6 of both Gal-*2* and Gal-*3* with in addition sulfate on C-6 or C-4 of GalNAc. The other two trisulfated compounds have *O*-sulfate on C-6 of Gal-*2* and on C-4 of Gal-*3* in conjunction with sulfate on C-6 or C-4 of GalNAc.

Proteoglycans are macromolecules containing a protein core to which are covalently attached side chains of characteristic sulfated glycosaminoglycans including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate (1). Accumulating evidence indicates that from the wide variety of biological functions of proteoglycans some are attributable to glycosaminoglycan chains (reviewed in Refs. 2-4). Functions of hybrid proteoglycans bearing both galactosaminoglycan and glucosaminoglycan chains seem to be regulated at least in part, by the control of biosynthetic sorting of glycosaminoglycans. For example, syndecan from mouse mammary epithelial cells exhibits molecular polymorphism showing that the length and the number of galactosaminoglycan and heparan sulfate chains are distinct on simple and stratified epithelia (5). This proteoglycan exhibits an alteration in the proportion of galactosaminoglycan to heparan sulfate chains attached to the core protein in response to transforming growth factor-β (6). Although the basic structures and the biosynthetic principles of proteoglycans have been well established (1), the sorting mechanisms in biosynthesis of different glycosaminoglycan chains remain largely unsolved.

We have addressed the question how different glycosaminoglycans are synthesized on the common carbohydrate-protein linkage structure, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser (7) and have been working on the hypothesis that there may be some differences in the structures of the linkage regions among different glycosaminoglycans (8). Such differences, if any, could contribute to the determination of the glycosaminoglycan species to be synthesized. The structural study of the linkage region of chondroitin 4-sulfate proteoglycans from Swarm rat chondrosarcoma revealed the new structure containing Gal(4-*O*-sulfate)β1-3Galβ1-4Xylβ1-*O*-Ser (8). This prompted us to reinvestigate the structures of the linkage regions of various proteoglycans. We confirmed this 4-*O*-sulfated Gal structure in whale cartilage chondroitin 4-sulfate (9) and a nonsulfated linkage region, Galβ1-3Galβ1-4Xylβ1-*O*-Ser, in porcine intestinal heparin (10).

In the first article of this series we determined the structures of six linkage oligosaccharide alditols isolated from shark cartilage chondroitin 6-sulfate proteoglycans (11). These linkages, which were prepared using chondroitinase ABC, had the conventional core hexasaccharide structure in common: $\Delta_{4,5}$-GlcA[1]β(1-3)GalNAcβ(1-4)GlcAβ(1-3)Galβ(1-3)Galβ(1-4)

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[1] The abbreviations used are: GlcA, glucuronic acid; ΔUA, 4,5-unsaturated uronic acid; ΔDi-0S, $\Delta_{4,5}$-GlcA(β1-3)GalNAc; ΔDi-4S

Xyl-ol. One of the compounds had a newly found ester sulfate on C-6 of the penultimate Gal residue attached to Xyl-ol. Thus, it has become evident that there are differences in modification of the linkages among different glycosaminoglycans. Although the biological significances of the sulfated structures are yet unknown, they may have regulatory roles in the sorting and modification of sulfated glycosaminoglycan chains.

In the present study we will present the di- and trisulfated compounds derived from the linkage region of shark cartilage chondroitin 6-sulfate proteoglycans. Two hitherto unrecognized structures, Gal(6-*O*-sulfate)β1–3Gal(6-*O*-sulfate)β1–4Xyl-ol and Gal(4-*O*-sulfate)β1–3Gal(6-*O*-sulfate)β1–4Xyl-ol are identified.

EXPERIMENTAL PROCEDURES

Most of the materials and methods used in this report including preparation of linkage oligosaccharides were described in detail in the first article of this series (11).

HPLC—Fractionation of the linkage oligosaccharides and analysis of the chondroitinase ACII digests of the isolated oligosaccharides were carried out by HPLC (11). Chromatography was performed on a 4.6 × 250-mm polyamine-bound silica PA03 column (YMC Co., Kyoto) in an LC6A HPLC system (Shimadzu Corp., Kyoto) using a linear gradient from 16 to 530 mM NaH_2PO_4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. For separation of fraction 14 into fraction 14–1 and 14–2, a linear gradient from 311 to 538 mM NaH_2PO_4 over a 60-min period was used. Eluates were monitored by UV absorbance at 232 nm. Samples dissolved in 16 mM NaH_2PO_4 were treated with a 0.45-μm membrane filter and injected.

500-MHz ^1H NMR Spectroscopy—500-MHz ^1H NMR spectra of the linkage oligosaccharides were recorded basically as described in the first article of this series (11). Resolution enhancement of the 1D spectra was achieved by Lorentzian-to-Gaussian transformation (12). For the 2D spectra 380 (fraction 10), 350 (fraction 11), 300 (fractions 14–1 and 15), or 400 (fraction 16) experiments of 2048 data points were recorded. The HOD signal was suppressed by presaturation during 1 s, the 90° ^1H pulse width was 30 μs. 2D HOHAHA spectra (13) were recorded with 8 (fraction 14–1), 16 (fractions 10 and 15), 24 (fraction 11), or 80 (fraction 16) scans/t_1 value, using a MLEV-17 mixing sequence of 120 ms for isotropic mixing. 2D ROESY spectra (14) were recorded with 24 (fraction 15), 64 (fractions 10 and 14–1), or 200 (fraction 16) scans/t_1 value; the spinlock mixing pulse with a duration of 200 ms was given as a series of 32° pulses (10 μs) with delays in between (30 μs) to attenuate the power of the amplifier (15). Triple quantum filtered COSY (TQF-COSY) spectrum (16) of fraction 14–1 was recorded with 48 scans/t_1 value. The time domain data were multiplied with a phase-shifted sine-bell; phase-sensitive Fourier transformation was performed after zero filling to a 2048 × 1024 data matrix size.

RESULTS

Isolation of the Linkage Oligosaccharides—In the previous paper (11) we described the preparation of the linkage oligosaccharide alditols from shark cartilage chondroitin sulfate proteoglycans. Briefly, a peptidoglycan fraction was first obtained by exhaustive Actinase E digestions of shark cartilage. A polysaccharide alditol fraction thereof was prepared by β-elimination, which released at least 91% of Ser-linked chains from the peptides as judged by the remaining Ser content in the glycans. The fraction was mixed with the ^3H-labeled polysaccharide alditol fraction, a tracer of the carbohydrate-protein linkage region, and exhaustively digested with chondroitinase ABC, which yielded a fraction containing a mixture of ^3H-labeled hexasaccharide alditols. The linkage fraction was subjected to HPLC on an polyamine-bound silica column using a salt gradient elution system.

In the present study the fractions 10, 11, 15, and 16 were further purified several times by repeated preparative chromatography, yielding 123, 18, 108, and 33 nmol (as $\Delta_{4,5}$-GlcA), respectively, per 100 mg of the peptidoglycans containing 1.84 μmol of Ser. In addition fraction 14 was separated using a shallow gradient (see "Experimental Procedures") into two subfractions, a major radioactively labeled fraction 14–1 and a slower eluting minor unlabeled fraction 14–2, corresponding to 445 and 45 nmol (as $\Delta_{4,5}$-GlcA), respectively, per 100 mg of peptidoglycan. Fractions 10 and 16 tended to show double peaks while they were purified by repeated chromatography (data not shown). Since, however, they could not be sufficiently separated into subfractions even in a shallow gradient system, they were subjected to structural analysis without further separation.

Characterization of the Oligosaccharides—As shown in Table I, each fraction contains per mol of oligosaccharide approximately 1 mol of $GalNH_2$ and 2 mol of uronic acid, one of which is $\Delta_{4,5}$-GlcA, implying the presence of the common hexasaccharide backbone structure. Fractions 10 and 11 contain 2 mol, whereas fractions 14–1, 15, and 16 contain 3 mol of sulfate/mol of oligosaccharide.

Aliquots of fractions 10, 11, 14–1, 15, and 16 were subjected to chondroitinase ACII digestion followed by HPLC analysis. Fractions 11, 14–1, and 15 were each quantitatively degraded to equimolar amounts of an unsaturated disaccharide and a presumed unsaturated tetrasaccharide (Fig. 1). The major compound (75%) in fraction 11 was degraded into ΔDi-4S and a tetrasaccharide which has the same retention time as that obtained from fraction 6 (11), indicating sulfate on C-4 of GalNAc and on C-6 of the Gal attached to xylitol. A minor component (25%) in fraction 11 which remained undigested is unidentified. Fractions 14–1 and 15 gave rise to ΔDi-6S and ΔDi-4S, respectively, as unsaturated disaccharide units, and to a common unsaturated tetrasaccharide which is clearly different from that of the fractions discussed so far. Since the compounds in fractions 14–1 and 15 bear three sulfates, the unsaturated tetrasaccharides are assumed to be disulfated, which is in accordance with their retention times in HPLC.

Fraction 10 (0.4 nmol) yielded two unsaturated disaccharides, ΔDi-0S (0.20 nmol) and ΔDi-6S (0.17 nmol), and two presumed tetrasaccharides (Fig. 1) each one of which accounts for 0.17 and 0.20 nmol; they have the same retention times as those of the mono- and disulfated tetrasaccharide obtained from fraction 11 and 14–1 (or 15), respectively. Quantitative

TABLE I

Chemical composition of linkage oligosaccharides from shark cartilage chondroitin sulfate

The values are expressed by molar ratio to the $GalNH_2$ values which were determined using an amino acid analyzer and have been corrected for degradation (22.7%) during acid hydrolysis. $\Delta_{4,5}$-Glcc and GlcA were determined by UV absorption and the carbazole reaction, respectively. Sulfate was quantitated by ion chromatography.

Fraction	$GalNH_2$	$\Delta_{4,5}$-GlcA	GlcA[a]	Sulfate
10	0.78	1.08	*2.00*	1.66
11	0.97	1.02	*2.00*	2.22
14-1	0.91	0.75	*2.00*	3.02
15	0.91	0.90	*2.00*	2.95
16	0.96	1.10	*2.00*	2.60

[a] Including $\Delta_{4,5}$-GlcA.

and ΔDi-6S, $\Delta_{4,5}$-GlcA(β1–3)GalNAc(4-*O*-sulfate) and (6-*O*-sulfate), respectively; ΔDi-diS$_D$, $\Delta_{4,5}$-GlcA(2-*O*-sulfate)(β1–3)GalNAc(6-*O*-sulfate); ΔDi-diS$_E$, $\Delta_{4,5}$-GlcA(β1–3)GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta_{4,5}$-GlcA(2-*O*-sulfate)(β1–3)GalNAc(4,6-*O*-disulfate); HOHAHA, homonuclear Hartmann-Hahn; MLEV, Malcom Levitt; ROESY, rotating frame Overhauser enhancement spectroscopy; TQF-COSY, triple quantum filtered correlation spectroscopy; HPLC, high performance liquid chromatography; 1D, one-dimensional; 2D, two-dimensional.

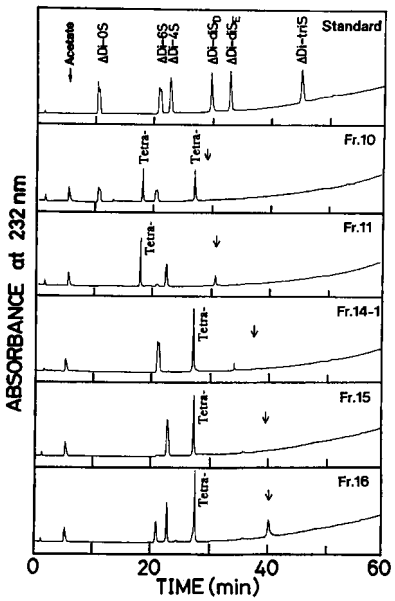
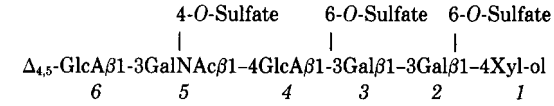


FIG. 1. **HPLC of the chondroitinase ACII digests of the isolated hexasaccharide alditols.** *Top column*, the authentic chondrodisaccharides; *lower columns*, the chondroitinase ACII digests of fractions 10, 11, 14–1, 15, and 16. The *arrows* indicate the elution positions of the corresponding intact hexasaccharide alditols.

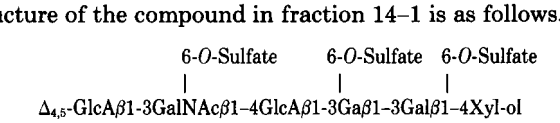
comparison of the yields of the four components indicates that ΔDi-6S and the monosulfated tetrasaccharide were derived from a disulfated hexasaccharide (fraction 10–1) accounting for 46% of fraction 10 while ΔDi-0S and the disulfated tetrasaccharides were produced from the other disulfated hexasaccharide (fraction 10–2) representing 54%. Thus, fraction 10–1 has a sulfate on C-6 of GalNAc and a sulfate on C-6 of the Gal attached to xylitol, similar to fraction 11. Fraction 10–2 has no sulfate on the repeating disaccharide unit, but two sulfates in the tetrasaccharide analogous to fraction 14–1 or 15. Fraction 16 (0.4 nmol) resulted in three components (Fig. 1): ΔDi-6S (0.15 nmol), ΔDi-4S (0.21 nmol), and a presumed disulfated tetrasaccharide (0.36 nmol). Therefore, it is reasonable to conclude that fraction 16 contains two trisulfated hexasaccharides which are composed of a disaccharide unit sulfated on C-6 or C-4 of GalNAc, respectively, and of a common disulfated tetrasaccharide with a retention time indistinguishable from that of the disulfated tetrasaccharide obtained from fraction 14–1 or 15. However, the tetrasaccharide core from the hexasaccharides in fraction 16 should be different from that of fraction 14–1 or 15 because fractions 16 and 14–1 (or 15) share the same monosulfated disaccharide unit, but have different retention times upon HPLC. The components sulfated on C-6 or C-4 of GalNAc were designated as 16–1 or 16–2, respectively. It is likely that 16–1 and 16–2 are eluted in this order when separated on HPLC as in the case of the 6-*O*- and 4-*O*-sulfated isomers in fractions 7 and 8–2 (Ref. 11) or 10–1 and 11, or 14–1 and 15. Compounds 16–1 and 16–2 represent 42 and 58% of fraction 16, respectively.

500-MHz ^1H NMR Spectroscopy—The ^1H NMR spectrum of fraction 15 is presented in Fig. 2*A*, and the ^1H NMR data are summarized in Table II. The GalNAc H-4 signal has shifted downfield to a position in the anomeric region in the same way as in the spectrum of fraction 8–2 (11). Furthermore, the chemical shifts of GalNAc H-1, H-2, H-3, and of $\Delta_{4,5}$-GlcA H-1, H-3, and H-4 are similar to those of fraction 8–2. By consequence, fraction 15 contains a 4-*O*-sulfated GalNAc residue (17). The partial subspectrum of this residue is indicated in the HOHAHA spectrum (Fig. 3). Comparison of the spectral data of fraction 15 with those of fraction 6 (11) indicates the presence of a 6-*O*-sulfated Gal-*2* residue. The assignment pathway for Gal-*2* from H-1 to the H-6/H-6′ signals, as shown in the 2D HOHAHA and 2D ROESY spectra of fraction 15 (Fig. 3), is similar to that of fraction 6 (11). The Gal-*2* H-4 signal can be assigned on the basis of the 2D ROESY spectrum, since the closeness in space of the H-4 and H-5 atoms affords a cross-peak in this spectrum. The connectivities of the H-4 with the Gal-*2* H-1 and of the H-5 with the H-6 and H-6′ signals are present in the 2D HOHAHA spectrum. The spectra of fraction 15 exhibit extra features not present in the spectra of the compounds discussed before (11). In the 1D spectrum (Fig. 2*A*) a strong coupling pattern is observed at δ 4.18. These signals, shifted out of the bulk region, stem probably from H-6 signals of another 6-*O*-sulfated residue. The H-1 of Gal-*3* has shifted downfield compared to the H-1 in fraction 7 (11). This downfield shift cannot be explained by the presence of 6-*O*-sulfate on Gal-*2*, as is evident from comparison with fraction 6, nor by the presence of 4-*O*-sulfate on GalNAc, as follows from comparison with the data of fraction 8–2 (11). In the 2D HOHAHA spectrum the extra H-6 signals at δ 4.18 are connected with an H-5 signal at δ 3.925 (Fig. 3). In the 2D ROESY spectrum a cross-peak between this H-5 and an H-4 is observable. The connectivity of this H-4 with Gal-*3* H-1 is present in the 2D HOHAHA spectrum. This leads for Gal-*3* to an assignment pathway from H-1 to H-6/H-6′, demonstrating that the compound in fraction 15 is also sulfated at C-6 of Gal-*3*.



It should be noted that the presence of the second sulfate group on the Gal-Gal-Xyl-ol core induces spectral alterations on neighboring residues. For example, the Gal-*2* H-4 and the GlcA-*4* H-1 have shifted downfield compared to those in fraction 6 (11). The resonance positions of the Gal-*2* H-6/6′ signals are also altered, showing no strong coupling.

The ^1H NMR spectrum of fraction 14–1 is given in Fig. 2*B*, and the corresponding ^1H NMR data are summarized in Table II. Comparison of the chemical shifts of the Gal residues in fractions 14–1 and 15 reveals that in both structures these residues are 6-*O*-sulfated. This was already indicated by similar retention times on HPLC of the unsaturated tetrasaccharides derived from fractions 14–1 and 15. Comparison of the chemical shifts of GalNAc and $\Delta_{4,5}$-GlcA of fraction 14–1 with those of fraction 7 (11) suggests that fraction 14–1 contains an *O*-sulfate on C-6 of GalNAc. In both the TQF-COSY spectrum (data not shown) and the 2D HOHAHA spectrum (Fig. 4) of fraction 14–1 cross-peaks are present between three sets of H-6/H-6′ and three H-5 signals, resonating in the range of δ 3.9–4.3. In the 2D ROESY spectrum (Fig. 4) cross-peaks between H-5 and H-4 atoms are observable. The connectivities of the different H-4 atoms with H-1 atoms are deduced from the 2D HOHAHA spectrum. The resulting three assignment pathways from H-1 to H-6/H-6′ indicate that Gal-*2*, Gal-*3*, and GalNAc are each sulfated at C-6. The structure of the compound in fraction 14–1 is as follows.



The interpretations of the spectra of the trisulfated compounds of 15 and 14–1 are substantiated by ROESY cross-

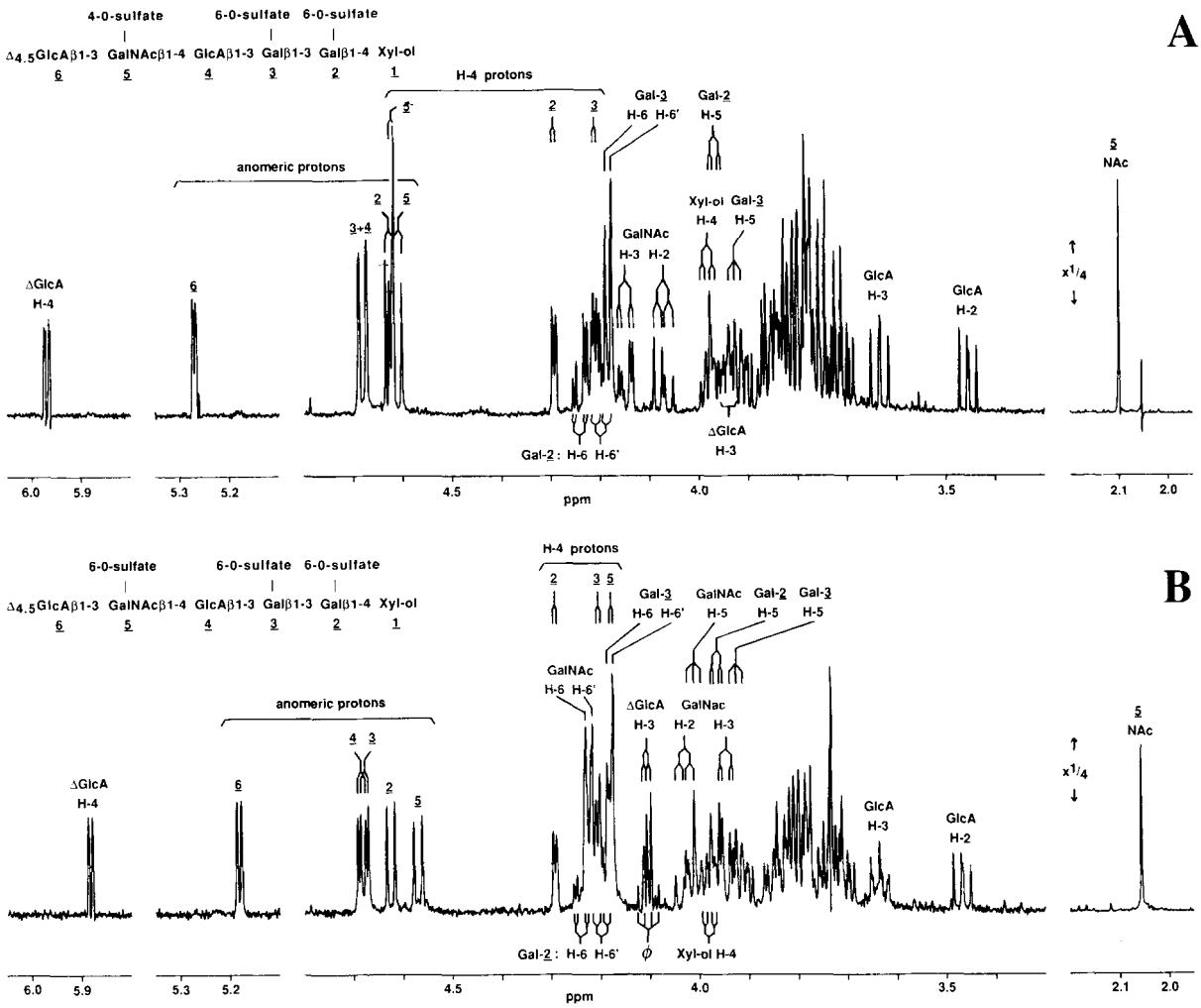
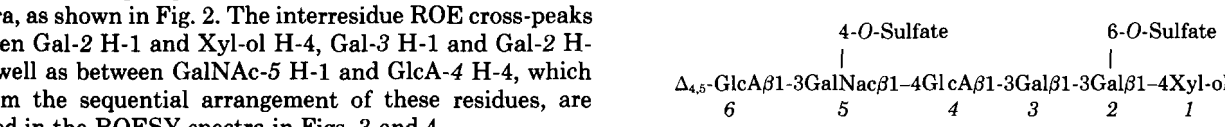


FIG. 2. **Structural reporter group regions of the resolution-enhanced 500 MHz ^{1}H NMR spectra of fraction 15 (*A*) and 14–1 (*B*) recorded in ^2H_2O at 15 °C.** The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures.

peaks between the corresponding H-1 and H-5 atoms of each of the 6-*O*-sulfated residues, as shown in Figs. 3 and 4. Although the presence of three different sulfate groups resulted in spectral overlap in the region between δ 3.9 and 4.3, all relevant multiplet patterns could be recognized in the 1D spectra, as shown in Fig. 2. The interresidue ROE cross-peaks between Gal-*2* H-1 and Xyl-ol H-4, Gal-*3* H-1 and Gal-*2* H-3, as well as between GalNAc-*5* H-1 and GlcA-*4* H-4, which confirm the sequential arrangement of these residues, are marked in the ROESY spectra in Figs. 3 and 4.

The ^{1}H NMR spectrum of fraction 11 is presented in Fig. 5*A* and the ^{1}H NMR data are summarized in Table II. The GalNAc H-4 of the major component of fraction 11 has shifted downfield to a position in the anomeric region in the same way as for fraction 15. Also the chemical shifts of GalNAc H-1, H-2, H-3, and $\Delta_{4,5}$-GlcA H-1, H-2, H-3, H-4 are similar to those of fraction 15. Therefore, fraction 11 contains a 4-*O*-sulfated GalNAc residue. The spectral data of the Gal-*2* residue of fraction 11, compared with those of fraction 6 (11), indicate the presence of a 6-*O*-sulfated Gal-*2* residue. The chemical shifts of the Gal-*3* residue are not altered, as was the case for those of fraction 15. Furthermore, the spectral alterations on neighboring residues due to a 6-*O*-sulfated Gal-*3* residue are not present. Therefore, the Gal-*3* residue bears no 6-*O*-sulfate group. The assignments of the major component in fraction 11 are substantiated by 2D HOHAHA spectroscopy (data not shown). The minor component in fraction 11 could not be assigned, but the presence of an anomeric signal at δ 4.42 suggests that this component does not belong to the linkage region. The structure of the main component in fraction 11 is as follows.



The ^{1}H NMR spectrum of fraction 10 is given in Fig. 5*B*, and the corresponding ^{1}H NMR data are summarized in Table II. From the structural reporter group regions it is evident that two components are present in fraction 10 in about equimolar quantities. Two sets of structural reporter groups for the GalNAc and $\Delta_{4,5}$-GlcA residues are present. One of the sets, representing a compound referred to as 10–1, is identical to that of fraction 7 (11) and fraction 14–1, indicating the presence of an *O*-sulfate on C-6 of GalNAc. The other set, belonging to compound 10–2, indicates the presence of a nonsulfated GalNAc residue, when compared to fractions 4 and 6 (11). These results are in good agreement with those obtained from chondroitinase ACII digestions. Two values are found for the chemical shift of Gal-*2* H-4, namely δ 4.291 which is characteristic for a structure bearing 6-*O*-sulfates on both Gal-*2* and Gal-*3* residues (compare with fractions 14–1

TABLE II

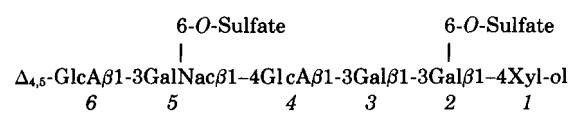
^1H chemical shifts of structural reporter groups of the constituent monosaccharides of linkage oligosaccharides

Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) at 15 °C. The compounds present in fractions 10 and 16 are referred to as 10-1 and 10-2, and as 16-1 and 16-2, respectively. The chemical shifts for the Gal-Gal-Xyl-ol core are identical for the two compounds present in fraction 16.

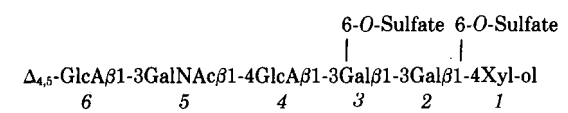
Residue	Reporter group	Chemical shift in						
		15	14-1	11	10-1	10-2	16-1	16-2
Xyl-ol	H-4	3.982	3.981	3.974	3.97	3.97	3.964	
Gal-*2*	H-1	4.627	4.625	4.630	4.629	4.626	4.627	
	H-2	3.713	3.712	3.722	3.721	3.714	3.722	
	H-3	3.859	3.856	3.863	3.863	3.860	3.868	
	H-4	4.293	4.293	4.252	4.251	4.291	4.237	
	H-5	3.966	3.965	3.963	3.965	3.965	3.965	
	H-6	4.241	4.241	4.203	4.203	4.240	4.206	
	H-6′	4.198	4.199	4.190	4.190	4.198	4.193	
Gal-*3*	H-1	4.681	4.678	4.670	4.668	4.679	4.701	
	H-2	3.758	3.754	3.75	3.748	3.759	3.777	
	H-3	3.832	3.829	3.805	3.810	3.832	4.015	
	H-4	4.210	4.205	4.164	4.161	4.207	4.752	
	H-5	3.927	3.925	ND[a]	ND	3.928	ND	
	H-6	4.189	4.187	ND	ND	4.186	ND	
	H-6′	4.177	4.175	ND	ND	4.174	ND	
GlcA-*4*	H-1	4.681	4.684	4.674	4.676	4.676	4.748	4.748
	H-2	3.455	3.468	3.455	3.469	3.454	3.450	3.439
	H-3	3.633	3.635	3.634	3.636	3.623	3.622	3.617
	H-4	3.742	3.74	ND	3.74	3.778	3.76	3.80
	H-5	3.782	3.74	ND	3.74	3.72	3.704	3.704
GalNAc-*5*	H-1	4.611	4.570	4.612	4.569	4.533	4.589	4.627
	H-2	4.072	4.030	4.072	4.032	4.003	4.031	4.073
	H-3	4.148	3.946	4.152	3.949	3.904	3.944	4.149
	H-4	4.623	4.178	4.624	4.180	4.096	4.185	4.630
	H-5	ND	4.010	ND	4.009	ND	4.014	ND
	H-6	ND	4.230	ND	4.232	ND	4.225	ND
	H-6′	ND	4.217	ND	4.220	ND	4.215	ND
	NAc	2.099	2.055	2.097	2.053	2.060	2.056	2.099
ΔGlcA-*6*	H-1	5.272	5.182	5.270	5.179	5.187	5.183	5.271
	H-2	3.835	3.781	3.836	3.79	3.79	3.782	3.835
	H-3	3.941	4.107	3.944	4.109	4.093	4.106	3.944
	H-4	5.970	5.883	5.970	5.883	5.899	5.880	5.969

[a] ND, not determined.

and 15), and δ 4.251 which belongs to a compound bearing a 6-*O*-sulfate on Gal-*2*, but not a 6-*O*-sulfate on Gal-*3* (compare with fraction 11 and with fraction 6 of Ref. 11). Based on the results discussed for Table I both compounds present in fraction 10 contain 2 sulfate groups, which is consistent with their identical retention times on HPLC. These results and those from the analysis by chondroitinase ACII strongly suggest that the monosulfated Gal-Gal-Xyl-ol core must belong to the compound containing sulfate on GalNAc, belonging to the following structure for compound 10–1.



The disulfated Gal-Gal-Xyl-ol core is assigned to the compound containing no sulfate on GalNAc. In accordance with the results from the analysis by chondroitinase ACII digestion (see above), compound 10–2 has the following structure.



The assignment pathways in the 2D HOHAHA and 2D ROESY spectra for the 6-*O*-sulfated residues of fraction 10 (data not shown) are identical to those discussed before (fractions 6 and 7 in Ref. 10 and fractions 14–1 and 15). The spectral alterations induced by the second sulfate group on the Gal-Gal-Xyl-ol core in compound 10–2 are comparable to those in fractions 14–1 and 15, resulting in a different data set for the chemical shifts of the Gal-*2*, Gal-*3*, and GlcA residues. The chemical shifts of the GlcA residue of compound 10–1 are identical to those of fraction 7 (11), reflecting the presence of a 6-*O*-sulfated GalNAc residue. The presence of 6-*O*-sulfate on Gal-*2* has no influence on the spectra of GlcA (see fraction 6 in Ref. 11). If the chemical shift effects of 6-*O*-sulfated GalNAc (compare fraction 7 with fraction 4 in Ref. 11) are subtracted from the values of GlcA in fraction 14–1, the chemical shift effects of 6-*O*-sulfation of Gal-*3* on GlcA are deduced. In this way, the proton chemical shifts for this residue in compound 10–2 can be explained. The assignments of the structural reporter groups of both compounds are indicated in the 1D spectrum (Fig. 5*B*).

The ^1H NMR spectrum of fraction 16 is given in Fig. 6, and the corresponding ^1H NMR data are summarized in Table II. From the structural reporter group regions it is evident that two major components are present in fraction 16 in about

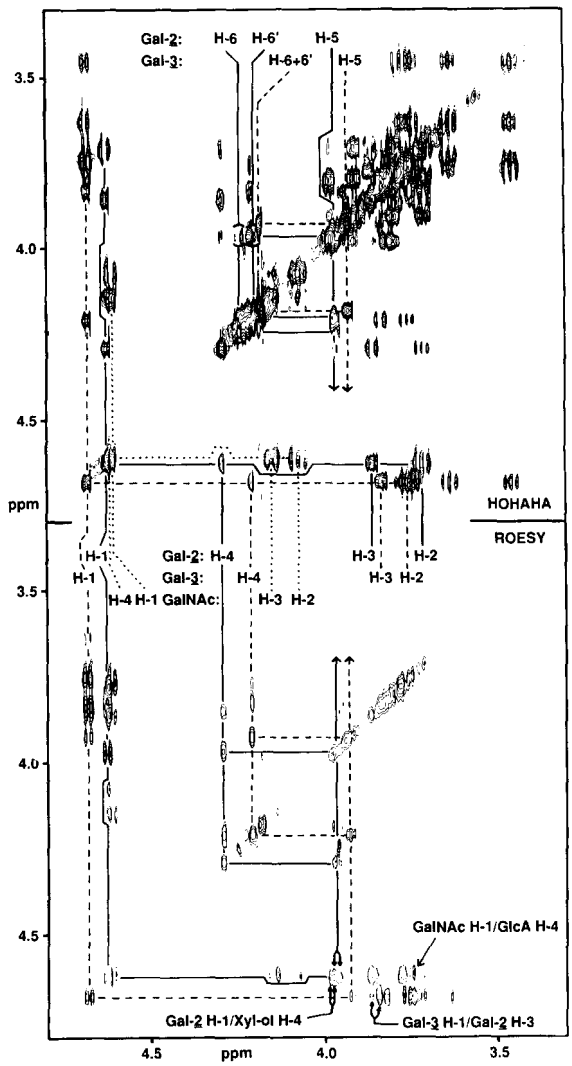


FIG. 3. **2D HOHAHA spectrum (*upper panel*) and 2D ROESY spectrum (*lower panel*) of fraction 15 recorded at 500 MHz in ^2H_2O at 15 °C.** The mixing time for the HOHAHA spectrum was 120 ms for the ROESY spectrum 200 ms. In the figure the assignment pathways are drawn for the Gal-*2* (——), the Gal-*3* (- - -), and the GalNAc (· · · ·) residues. Interresidue ROEs are indicated in the ROESY spectrum by *arrows*.

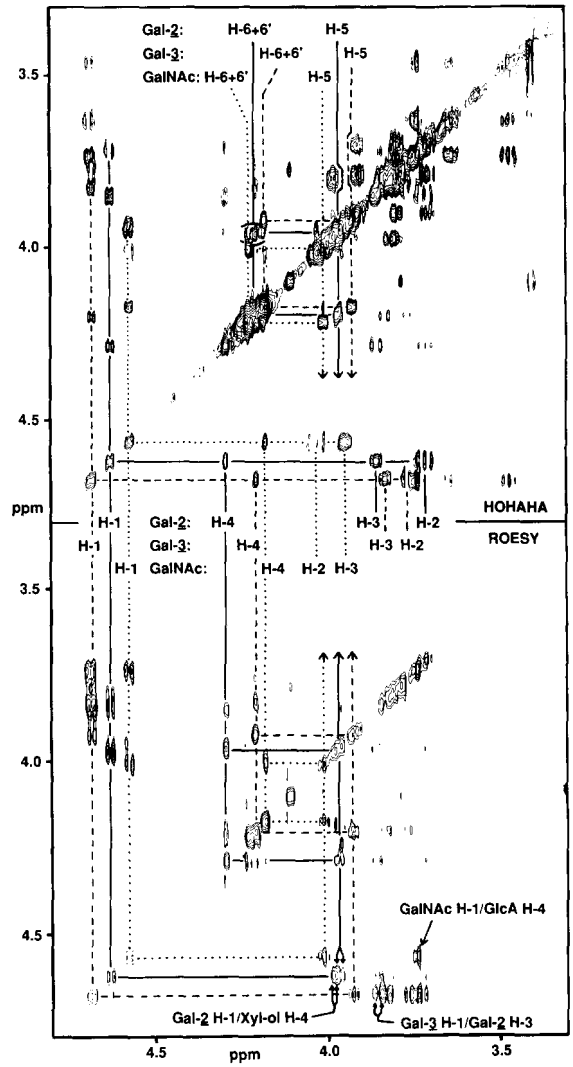
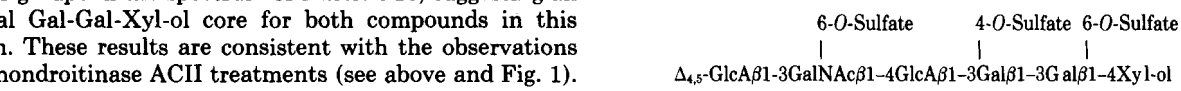
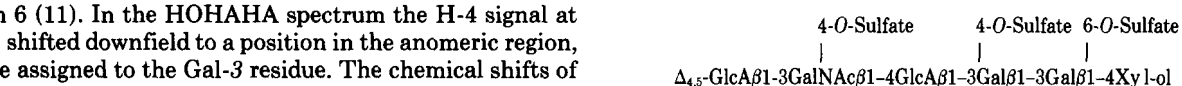


FIG. 4. **2D HOHAHA spectrum (*upper panel*) and 2D ROESY spectrum (*lower panel*) of fraction 14–1 recorded at 500 MHz in ^2H_2O at 15 °C.** The mixing time for the HOHAHA spectrum was 120 ms, for the ROESY spectrum 200 ms. In the figure the assignment pathways are drawn for the Gal-*2* (——), the Gal-*3* (- - -), and the GalNAc (· · · ·) residues. Interresidue ROEs are indicated in the ROESY spectrum by *arrows*.

equimolar quantities. As for fraction 10, two sets of structural reporter groups for the GalNAc and $\Delta_{4,5}$-GlcA residues are present. One of the sets, representing a compound referred to as 16–1, is identical to those of fraction 7 (Ref. 11), fraction 14–1, and compound 10–1, indicating the presence of an *O*-sulfate on C-6 of GalNAc. The other set, belonging to compound 16–2, indicates the presence of a 4-*O*-sulfated GalNAc residue, when compared to fractions 8–2 (11), 11, and 15. The Xyl-ol, Gal-*2*, and Gal-*3* residues have one set of structural reporter groups in the spectrum of fraction 16, suggesting an identical Gal-Gal-Xyl-ol core for both compounds in this fraction. These results are consistent with the observations upon chondroitinase ACII treatments (see above and Fig. 1). The presence of a 6-*O*-sulfated Gal-*2* residue is substantiated by assignment pathways in the 2D HOHAHA and 2D ROESY spectra (data not shown) for this residue similar to those in fraction 6 (11). In the HOHAHA spectrum the H-4 signal at δ 4.752, shifted downfield to a position in the anomeric region, could be assigned to the Gal-*3* residue. The chemical shifts of this 4-*O*-sulfated residue are similar to those of $\Delta_{4,5}$-GlcA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl-Ser (fraction D-4F in Ref. 8). If the chemical shift effect of 4-*O*-sulfation of Gal-*3* on the H-4 of Gal-*2*, being an upfield shift of δ Δ 0.01 (8), is added to the chemical shift of the H-4 in fraction 6 (11), the chemical shift of the H-4 in fraction 16 can be deduced. In the same way, the chemical shift effects of 4-*O*-sulfation of Gal-*3* on the GlcA and GalNAc residues are additive to the effects of 6-*O*-sulfation and 4-*O*-sulfation of the GalNAc residue in compounds 16–1 and 16–2, respectively. Therefore, the structure of compound 16–1 is



and the structure of compound 16–2 is



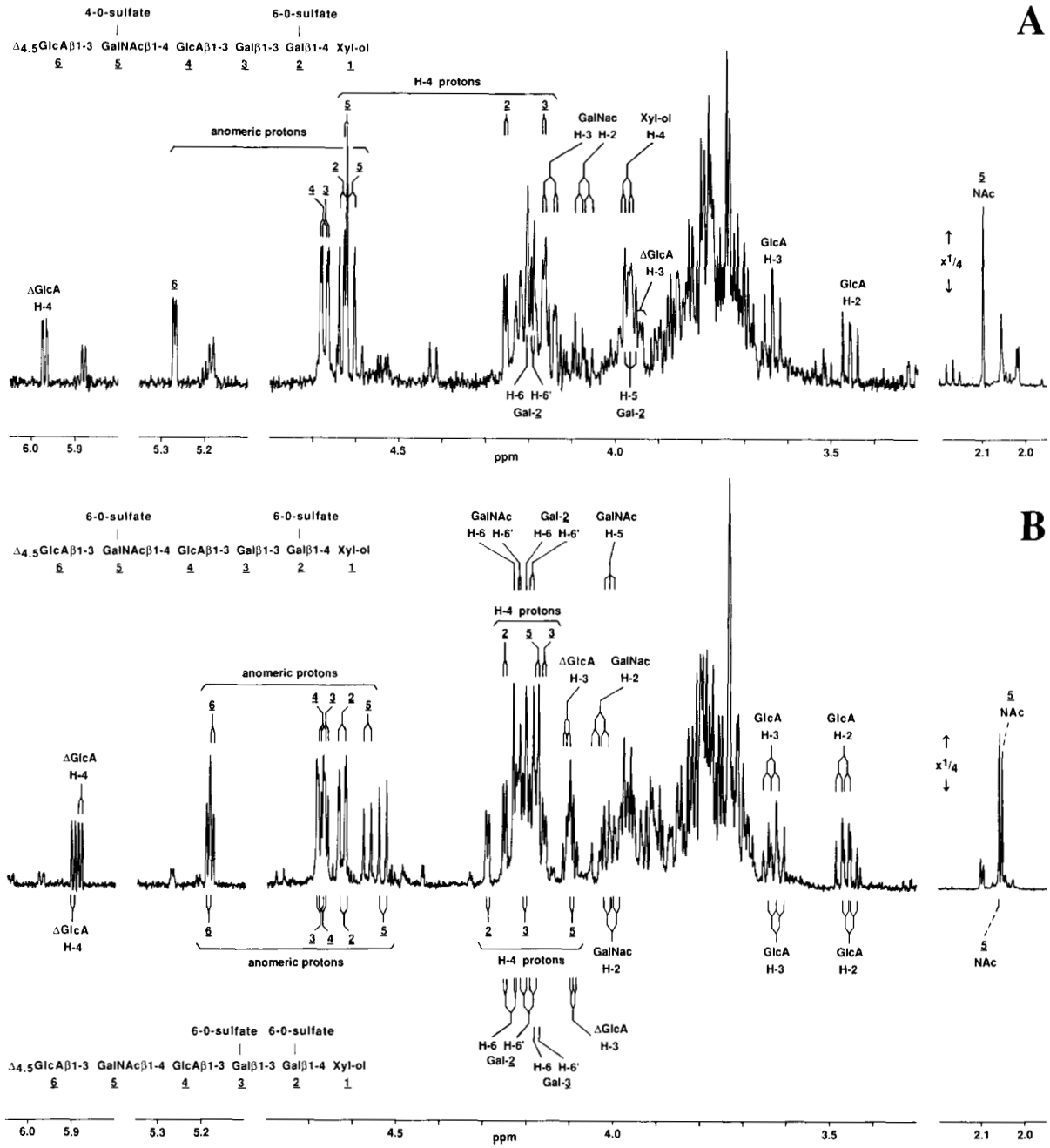


FIG. 5. **Structural reporter group regions of the resolution-enhanced 500 MHz ^{1}H NMR spectra of fraction 11 (*A*) and 10 (*B*) recorded in ^2H_2O at 15 °C.** The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures. The assignment of the compound referred to as 10–1 is depicted above the spectrum; the assignment of the compound referred to as 10–2 is depicted below the spectrum in *B*.

DISCUSSION

Investigation of the structure of the carbohydrate-protein linkage region of various proteoglycans revealed Gal(4-*O*-sulfate)β1–3Galβ1–4Xyl for chondroitin 4-sulfate (8, 9, 10) and Galβ1–3Gal(6-*O*-sulfate)β1–4Xyl for chondroitin 6-sulfate (11) and confirmed the conventional nonsulfated linkage structure for heparin (9). In the present study we determined additionally the structures of the di- and trisulfated hexasaccharide alditols isolated from the linkage region of chondroitin 6-sulfate proteoglycans of shark cartilage. Fraction 14 is the major fraction, comprising 26% of the ^{3}H label (Table I). ^{1}H NMR analysis of the purified fraction 14–1 revealed the presence of a novel, trisulfated compound, containing 6-*O*-sulfated Gal-*2* and Gal-*3* residues besides a 6-*O*-sulfated GalNAc residue. It is interesting that the disulfated Gal-Gal-Xyl-ol core is also found in combination with a 4-*O*-sulfated GalNAc residue in the compound of fraction 15. The two isomers seem to indicate that 6-*O*-sulfation of both Gal residues preferably, but not necessarily, dictate 6-*O*-sulfation of the first GalNAc residue. Another type of core is found in fraction 16 containing compounds with 6-*O*-sulfated Gal-*2* and 4-*O*-sulfated Gal-*3* in combination with a 4- or 6-*O*-sulfated GalNAc.

Comparison of the disulfated trisaccharide core structures with the previously demonstrated structures, Gal(4-*O*-sulfate)β1–3Galβ1–4Xyl and Galβ1–3Gal(6-*O*-sulfate)β1–4Xyl, may suggest several possible features of these sulfations although biological significances of the sulfated structures are unknown. 1) 6-*O*-Sulfation of Gal-*2* occurs prior to sulfation

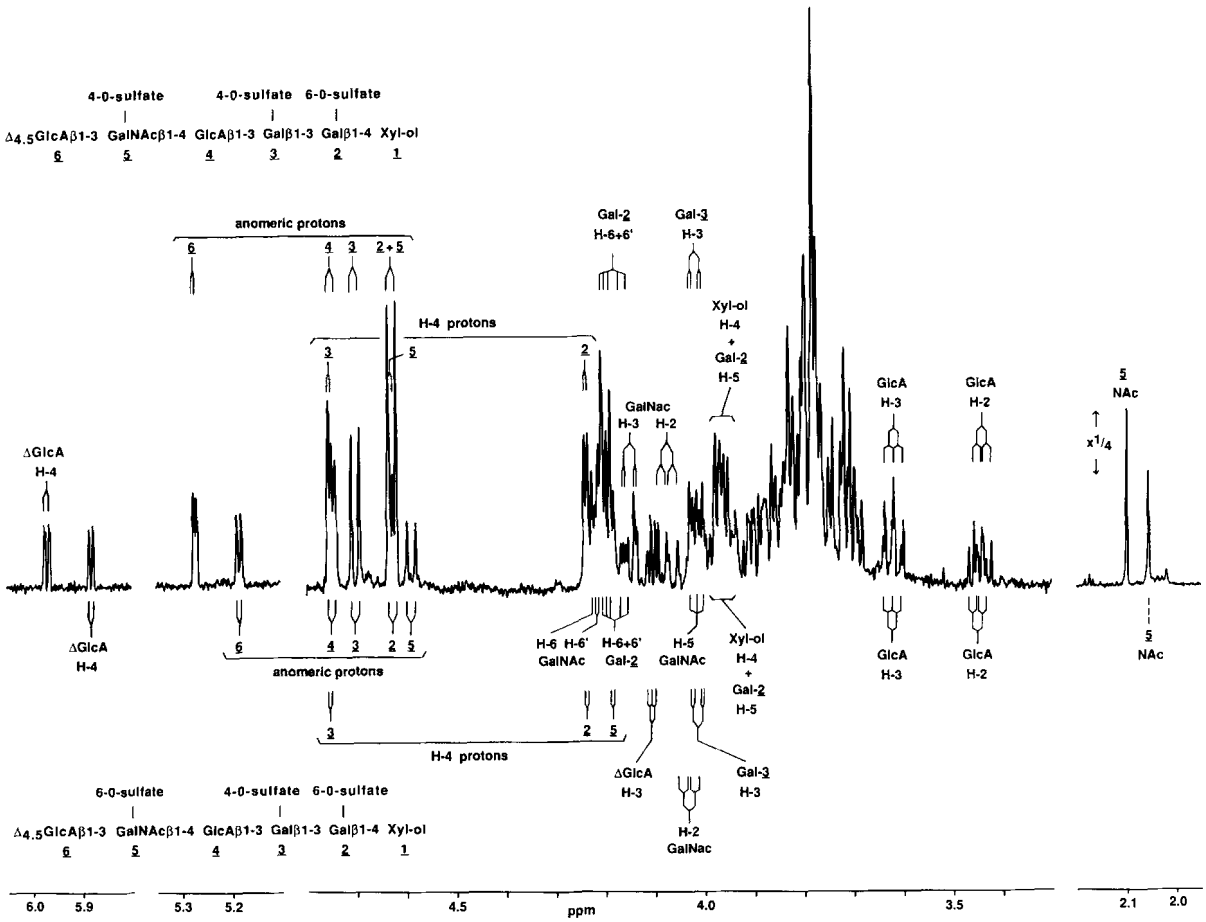


FIG. 6. **Structural reporter group regions of the resolution-enhanced 500 MHz ^{1}H NMR spectra of fraction 16 recorded in ^2H_2O at 15 °C.** The *numbers* and *letters* in the spectrum refer to the corresponding residues in the structures. The assignment of the compound referred to as 16-1 is depicted above the spectrum; the assignment of the compound referred to as 16-2 is depicted below the spectrum.

of Gal-*3*. 2) 4-*O*- or 6-*O*-sulfation of the Gal residues dictates the galactosaminoglycan synthesis and/or inhibit the glucosaminoglycan synthesis. Conversely, no modification of the Gal residues leads to the synthesis of a glucosaminoglycan chain. Thus, the possibilities exist that the sulfated Gal structures contribute to the segregation of galactosaminoglycans (chondroitin sulfate and dermatan sulfate) and glucosaminoglycans (heparin and heparan sulfate) as previously discussed in detail in relation to the sorting mechanisms in biosynthesis of different glycosaminoglycans (11). Evaluation of these possibilities requires characterization such as determination of the substrate specificities and subcellular localization of the responsible hexosaminyltransferases and sulfotransferases.

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