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Preparation and structure of heparin lyase-derived heparan sulfate oligosaccharides

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Porcine intestinal mucosal heparan sulfate was exhaustively depolymerized on a large scale using heparin lyase II (heparinase II) or heparin lyase III (heparitinase, EC 4.2.2.8). The oligosaccharide mixtures formed with each enzyme were fractionated by low pressure gel permeation chromatography. Size-uniform mixtures of disaccharides, tetrasaccharides, and hexasaccharides were obtained. Each size-fractionated mixture was then purified on the basis of charge by repetitive semipreparative strong-anion-exchange high-performance liquid chromatography. This approach has led to the isolation of 13 homogenous oligosaccharides. The purity of each oligosaccharide was demonstrated by the presence of a single peak on analytical strong-anion-exchange high-performance liquid chromatography and reversed polarity capillary electrophoresis. The structures of these oligosaccharides were established using 500 MHz one- and two-dimensional nuclear magnetic resonance spectroscopy. Three of the thirteen structures that were solved were novel while the remaining 10 have been previously described. All of the structures obtained using heparin lyase III contained a ΔUAp residue (where ΔUAp is 4-deoxy-α-L-*threo*-hex-4-eno-pyranosyluronic acid) at their nonreducing termini. Structures obtained using heparin lyase II contained both ΔUAp and ΔUAp2S (where S is sulfate) at their nonreducing termini. These results are consistent with the reported specificity of both enzymes.

Key words: heparan sulfate/heparinase/heparin lyase/1D and 2D NMR/sulfated oligosaccharides

Introduction

Glycosaminoglycan (GAG) heparan sulfate is a linear homocopolymer composed of glucosamine residues 1→4 linked to uronic acid residues (Gallagher *et al.*, 1992). It is found as a proteoglycan (PG) linked to a protein core in the extracellular matrix of most animal tissues (Gallagher *et al.*, 1992). The biological role of heparan sulfate is generally believed to be the control and regulation of cell–cell interaction (Gallagher *et al.*, 1990; Bernfield *et al.*, 1992). Heparan sulfate PG performs this role through the interaction of its GAG side chains with protein and peptide signaling molecules including growth factors (Walker *et al.*, 1994), chemokines (Witt and Lander, 1994), interferons (Lortat-Jacob and Grimand, 1991), and selectins (Norgard-Sumnicht *et al.*, 1993). Some of these protein–heparan sulfate interactions are known to involve specific sequences with the GAG side-chain (Lindahl and Kiellen, 1987; Marcum and Rosenberg, 1987; Ishihara and Conrad, 1989; Walker *et al.*, 1994). Thus, detailed knowledge of the structural variability of heparan sulfate is important and should lead to an improved understanding of these interactions and the biological role of heparan sulfate in cell–cell interactions.

Much of our knowledge of heparan sulfate structure and activity is based on the study of the closely related GAG, heparin. While heparan sulfate is composed of many of the same disaccharide building blocks as heparin, it is less highly sulfated and may not contain all of the same sequences that are present in heparan sulfate (Griffin *et al.*, 1995). For example, the antithrombin III binding site, a pentasaccharide containing an unusual 3-*O*-sulfated glucosamine residue responsible for antithrombotic activity, was first discovered in heparin (Lindahl *et al.*, 1983; Atha *et al.*, 1984) and later found only in the heparan sulfate GAG chains of proteoglycans that line the vascular endothelium (Lindahl and Kjellen, 1987; Marcum and Rosenberg, 1987).

Since heparin is used clinically as an anticoagulant and is manufactured as a bulk pharmaceutical (Linhardt, 1991), it is available in sufficiently large quantities to permit the spectroscopic determination of heparin-derived oligosaccharides (Pervin *et al.*, 1995). Recently, our laboratory reported the first large scale preparation of heparan sulfate (Griffin *et al.*, 1995). The multigram quantities obtained were sufficient for the enzymatic fragmentation of heparan sulfate into oligosaccharides for subsequent purification and structural characterization. This article describes the use of ^{1}H-nuclear magnetic resonance (NMR) spectroscopy for the characterization of thirteen heparan sulfate–derived oligosaccharides.

Results

Porcine intestinal heparan sulfate was exhaustively depolymerized in two separate reactions using heparin lyase II and heparin lyase III. The reactions were considered complete when the addition of more enzyme failed to increase the absorbance of the product mixtures at 232 nm. The two mixtures of oligosaccharides were fractionated by pressure filtration through a controlled pore membrane of 10,000 mol. wt cutoff (MWCO). This step results in the nearly complete removal of oligosaccharide and polysaccharide chains of mol. wt > 10,000 as well as bovine serum albumin (BSA). Next the heparan sulfate–derived oligosaccharide mixture (M_r < 10,000) was size fractionated by gel-permeation chromatography (GPC) on a Sephadex G-50 (superfine) column (4.8 cm × 1 m) eluted with 0.2 M sodium chloride (Figures 1a, 2a). This separation afforded fractions of size-uniform oligosaccharide mixtures ranging in size from disaccharides to hexasaccharides. After combining fractions corresponding to the peaks shown in Figures 1a and 2a, these oligosaccharide fractions were concen-

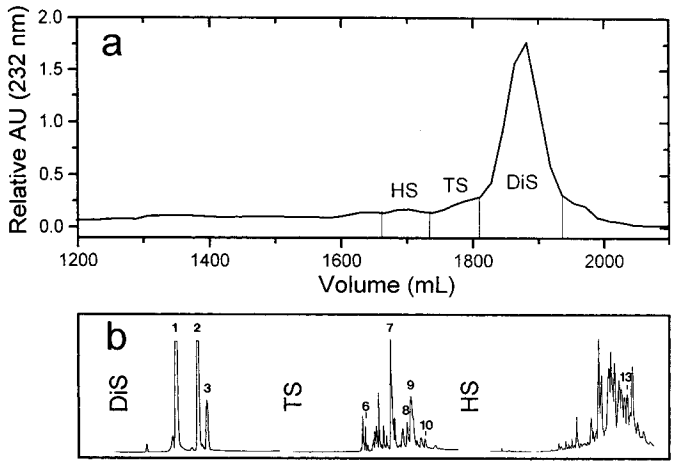

Fig. 1. Fractionation of heparin lyase III depolymerized heparan sulfate. (**a**) After pressure filtration to remove high molecular weight oligosaccharides and residual protein the reaction mixture was fractionated by GPC. Absorbance (232 nm) is plotted as a function of elution volume. Hexasaccharide (HS), tetrasaccharide (TS) and disaccharide (DiS) fractions were collected as indicated. (**b**) Semi-preparative SAX-HPLC fractionation of the size-fractionated mixtures shown in (**a**). Absorbance (at 232 nm at 0.02 absorbance units full scale (AUFS)) is plotted as a function of elution time (min) (see *Materials and methods* for details). The numbering of peaks correspond to the structures shown in Figure 6 and discussed in the text.

trated by rotary evaporation and desalted by GPC on Bio Gel P-2. Gradient PAGE analysis of these sized oligosaccharide fractions demonstrated that while they were primarily composed of the oligosaccharide of the expected size, each mixture was also contaminated with both larger and smaller oligosaccharides (data not shown).

The size fractions were next purified by semi-preparative SAX-HPLC (Figures 1b, 2b). Each fraction from the GPC column was then charge fractionated by multiple injections on a semipreparative column, like peaks were pooled, desalted by Bio Gel P-2 chromatography or dialysis, and lyophilized. Each peak was then examined by analytical SAX-HPLC to assess whether a second semipreparative SAX-HPLC separation step was necessary and to establish the optimum gradient for this separation. These separation steps resulted in the isolation of 13 heparan sulfate–derived oligosaccharides. The weight percentage of each oligosaccharide, found in both heparin lyase II and III depolymerized mixtures, are presented in Table I.

An assessment of oligosaccharide purity was also made using capillary electrophoresis (CE) with detection by absorbance at 232 nm. CE under reverse-polarity conditions showed a single major symmetrical peak for each of the 13 oligosaccharides (Figure 3). The purity of each oligosaccharide as determined by CE is presented in Table I.

The 500 MHz 1D-^{1}H-NMR spectra of all the oligosaccharides were acquired. Oligosaccharides **1–6, 8, 9, 11,** and **12** were for known structures whose spectral properties had been previously reported (Merchant *et al.*, 1985; Linhardt *et al.*, 1992; Yamada *et al.*, 1992; Sugahara *et al.*, 1994). The one-dimensional (1D) NMR spectra of **8** (Figure 4a; the NMR data are summarized with those of the other oligosaccharides in Table II and III) showed an unusual splitting in the ring protons of IdoAp2S residue. The H-1 and H-5 signals, assigned to the IdoAp2S ring protons (Table II), show a splitting pattern having the same ratios of the intensities as the anomeric α and β protons of the reducing end GlcNpAc residue. Probe temperature (298 - 333K) and ionic strength (by the addition of NaCl) had no effect on the splitting of the signals (data not shown).

Among the oligosaccharides isolated from heparan sulfate, three had 1D ^{1}H-NMR spectra that were dissimilar to any previously reported. The 1D-^{1}H-NMR spectra of oligosaccharides, **10** and **13** are presented in Figure 5. The structure of each oligosaccharide was assigned using a combination of 2D-^{1}H-NMR techniques. First, the individual saccharide residues were determined using correlation spectroscopy (COSY). The sequence of each oligosaccharide was then determined using nuclear Overhauser effect spectroscopy (NOESY). The ^{1}H-NMR assignments for these structures are presented in Tables II and III.

In the 1D spectra of **7** (Figure 4b) and **10** (Figure 5a), the integrations of signals observed in the anomeric region (4.5–5.5 ppm) show the presence of one unsaturated uronate, two glucosamine residues, and one iduronate residue, confirming that both structures were tetrasaccharides. Oligosaccharides containing *N*-sulfo glucosamine at their reducing termini typically show the proton signals corresponding to the α- but not the β-anomer, while oligosaccharides containing *N*-acetyl glucosamine at their reducing termini show signals corresponding to both anomers (Yamada *et al.*, 1994; Pervin *et al.*, 1995). Two multiplets, corresponding to the H-5 protons of α- and β-anomers are also observed at 3.95 and 3.55 ppm and demonstrate the presence of an *N*-acetylated, unsulfated glucosamine residue at the reducing termini. The proton signals for both α- and β-anomers are observed in the spectrum of **7** (Table II), but only the signals corresponding to the α-anomer are observed in the spectrum of **10** (Figure 5a). The small triplet, resonating at 3 ppm (Table II), which could be assigned as H-2 of β-*N*-sulfo glucosamine, was observed in the 2D-COSY spectrum obtained using low threshold plotting. The internal uronic acid was identified as IdoAp in both tetrasaccharides **7** and **10,** based on its small coupling constants (<1.5 Hz). The IdoAp residue in **10** contains a 2-sulfo group, based on the chemical shifts of H-1 and H-2 protons (5.4 ppm and 4.4 ppm, respectively). Thus, the structures of **7** and **10** presented in Figure 6 were confirmed.

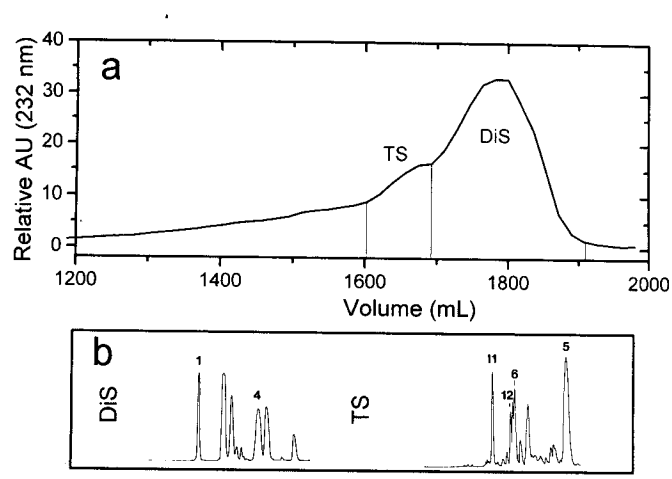

Fig. 2. Fractionation of heparin lyase II depolymerized heparan sulfate. (**a**) After pressure filtration fractionation by GPC afforded tetrasaccharide and disaccharide sized mixtures that were collected as indicated. (**b**) Semipreparative SAX-HPLC of size-fractionated tetrasaccharide (TS) and disaccharide (DiS) mixtures. The numbering of peaks correspond to the structures shown in Figure 6 and discussed in the text. For additional details see Figure 1 caption.

Table I. Purity and amount of each purified oligosaccharide isolated from heparin lyase treated heparin sulfate

Oligosaccharide #	Oligosaccharide structure	Heparin lyase used	% of Total reaction mixture	% Purity by CE
1	ΔUAp-GlcNpAc	III, II	14.3, 1.6	100, 98
2	ΔUAp-GlcNpS	III	2.3	98
3	ΔUAp-GlcNpAc6S	III	1.5	98
4	ΔUAp-GlcNpS6S	II	2.4	90
5	ΔUAp 2S-GlcNpS6S	II	1.2	83
6	ΔUAp-GlcNpS-GlcAp-GlcNpAc	III, II	0.1, 0.05	86, 82
7	ΔUAp-GlcNpS-IdoAp-GlcNpAc	III	0.6	89
8	ΔUAp-GlcNpS-IdoAp2S-GlcNpAc	III	0.1	88
9	ΔUAp-GlcNpS-IdoAp-GlcNpAc6S	III	0.5	84
10	ΔUAp-GlcNpS6S-IdoAp2S-GlcNpS	III	0.07	82
11	ΔUAp-GlcNpAc-GlcAp-GlcNpAc	II	5.9	88
12	ΔUAp-GlcNpAc-GlcAp-GlcNpS	II	5.0	95
13	ΔUAp-GlcNpAc6S-IdoAp-GlcNpS-GlcAp-GlcNpAc6S	III	0.1	92

The 1D 500 MHz ^{1}H NMR spectrum of oligosaccharide **13** is shown in Figure 5b and the assignments are presented in Tables II and III. Integration of anomeric protons show that **13** contains one unsaturated uronate (5.14 ppm), three glucosamine residues (5.18, 5.32, and 5.39 ppm), one iduronate residue (5.00 ppm), and one glucuronate residue (4.48 ppm). The two singlets at 2 ppm demonstrate the presence of two *N*-acetyl glucosamine residues, and the double doublets at 3.24 ppm demonstrate a single internal *N*-sulfo glucosamine. Oligosaccharide **13** contains 6-*O*-sulfo *N*-acetyl glucosamine at its reducing terminus based on the integration of the doublet at 5.18 ppm ($J_{1,2}$ = 3.6 Hz) and the absence of the multiplets at 3.55 and 3.95 ppm. The NOE signals between the H-1 of the glucosamine residues and H-4 of the uronate residues afforded a partial sequence for **13.** The overlap of H-4 signals from glucosamine residues (see Table II), made it difficult to completely assign the sequence of **13.** The correlation time for this oligosaccharide was too small to detect an NOE, even using ROESY with a spin lock pulse. 2D TOCSY and 2D NOESY (measured at mixing times of 150–500 ms) were performed at 298, 313 and 333 K. At higher temperatures (333 K), the intensity of NOE cross-peaks were reduced. However, the chemical shifts of the H-4 signals of IdoAp and GlcAp were resolved. This permitted the detection of NOE crosspeaks, affording the complete sequence of **13.** The assignments of methyl protons of *N*-acetyl glucosamine were made based on their chemical shifts.

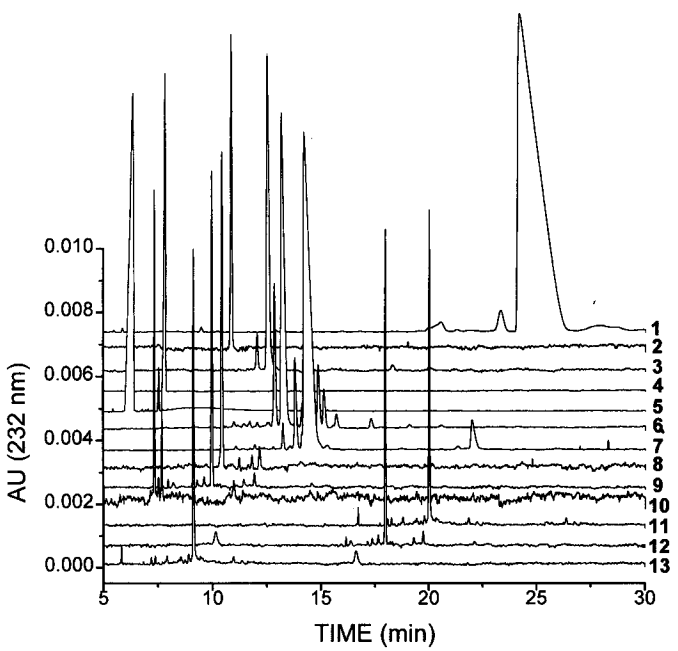

Fig. 3. Reverse polarity capillary electrophoresis analysis of the heparan sulfate-derived oligosaccharides. The corresponding oligosaccharides are numbered as shown in Figure 6.

Discussion

Porcine intestinal mucosal heparan sulfate is a structurally complex glycosaminoglycan that has important pharmaceutical applications (Griffin *et al.,* 1995; Linhardt and Toida, 1997). The →4)-α-D-GlcNpAc(1→4)-β-D-GlcAp (1→ linkage represents ~ 75% of the polymer's structure and the →4)-α-D-GlcNpS (1→4)-β-D-GlcAp (or α-L-IdoAp) (1→ and →4)-α-D-GlcNpAc6S (1→4)-β-D-GlcAp(or α-L-IdoAp) (1→ linkages represent 14% and 4% of porcine intestinal mucosal heparan sulfate, respectively (Griffin *et al.,* 1995). Heparin lyase II and III cleave heparan sulfate at →4)-α-D-GlcNp(S or Ac) 6 (S or OH) 3 (S or OH) (1→4) -β-D-GlcAp (or -α-L-IdoAp2(S or OH)) (1→ and →4)-α-D-GlcNp(S or Ac) 6 (S or OH) (1→4)-β-D-GlcAp (or -α-L-IdoAp) (1→ linkages, respectively (Linhardt 1994; Sugahara *et al.,* 1994). The oligosaccharides formed through the action of these enzymes have unsaturated uronic acid residues (ϵ_{232} = 5.2 × M^{-1} cm^{-1}) at their nonreducing termini that facilitate their detection (Rice and Linhardt, 1989). To improve further our understanding of heparan sulfate's structure, our laboratory has sought to prepare homogeneous oligosaccharides from heparan sulfate for structural characterization.

Porcine intestinal heparan sulfate was treated exhaustively with heparin lyase II and heparin lyase III in two separate reactions, and oligosaccharides were purified. Analyses of these oligosaccharides were next undertaken to establish their purity (Table I). All of the 13 oligosaccharides (**1–13**) gave a single major symmetrical peak on reverse polarity CE analysis (Figure 3). It should be noted that approximately 20 additional oligosaccharides, which were assessed as pure by analytical SAX-HPLC, gave multiple peaks on CE. These oligosaccharides were not carried forward for NMR structural characterization.

The structures of the purified oligosaccharides (Table I) were initially determined by high-field 1D NMR spectroscopy. The NMR spectroscopy previously performed by our research

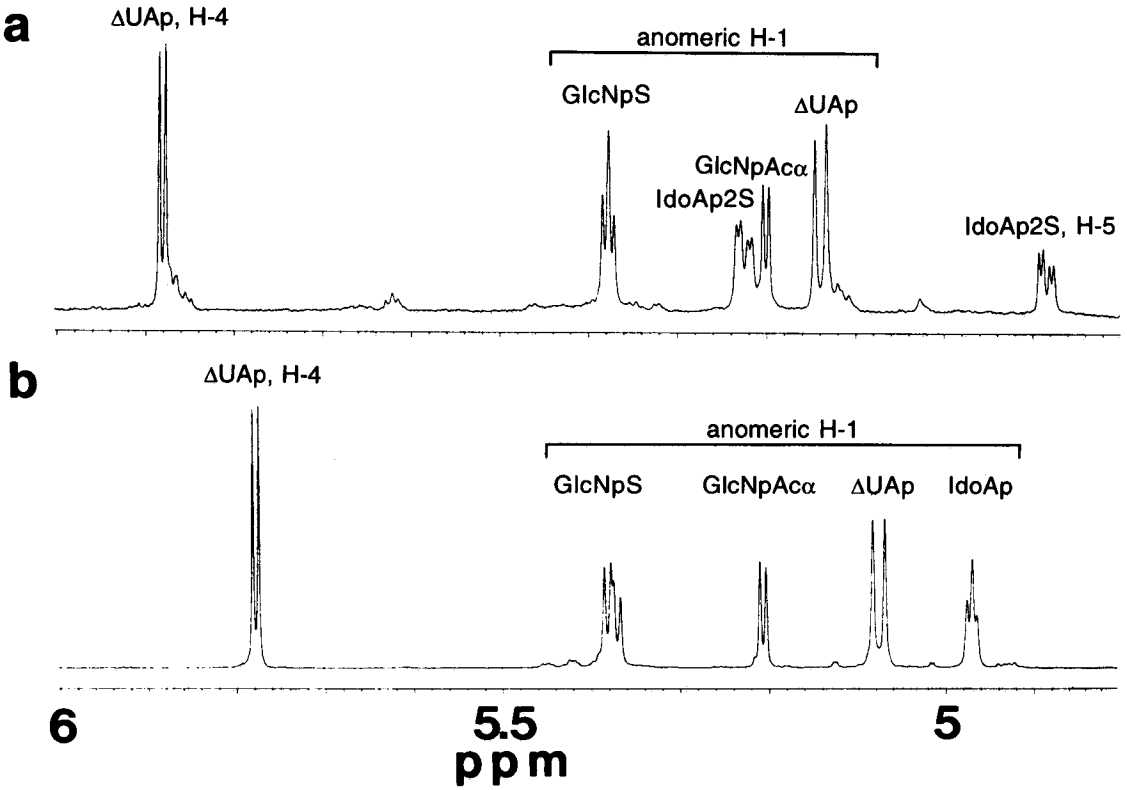

Fig. 4. The partial 1D 500 MHz ^{1}H-NMR spectra of (**a**) tetrasaccharide **8** and (**b**) tetrasaccharide **7.** The anomeric proton of the reducing end GlcNpAcβ residue, not shown in these partial spectra, resonates at 4.70 ppm (see Table II).

group (Merchant *et al.*, 1985; Loganathan *et al.*, 1990; Linhardt *et al.*, 1992; Desai *et al.*, 1993a; Pervin *et al.*, 1995) and others (Linker and Hovingh, 1984; Gettins, 1989; Yamada *et al.*, 1992; Sugahara *et al.*, 1994) on structurally related oligosaccharides was also helpful in determining the structure of these oligosaccharides. Tetrasaccharide **8,** corresponding to a known structure, ΔUAp (1→4)-β-D-GlcNpS(1→4)-α-L-IdoAp2S(1→4)GlcNpAc α,β (Sugarahara *et al.*, 1994), showed an interesting splitting pattern in the IdoAp2S residue in its 1D ^{1}H NMR spectrum (Figure 4a), suggesting that it exists in an unusual conformation. This splitting pattern is not observed in tetrasaccharide **7,** having the same structure but with an internal IdoAp residue that is unsulfated (Figure 4b). The unusual splitting pattern in **8** was unaffected by both probe temperature and ionic strength. Thus, neither restricted rotation nor internal hydrogen bonding could explain this observation. Further studies will be required to establish the reason for the unusual spectral properties of this tetrasaccharide.

1D ^{1}H-NMR spectroscopy indicated that several of the purified oligosaccharides were new structures and these were subjected to 2D spectral analysis. The ^{1}H-NMR spectra of these oligosaccharides were assigned by ^{1}H/^{1}H coupling interactions utilizing 2D homonuclear COSY. Since the cross-peaks observed in the COSY experiments are due to direct scalar (through-bond) coupling, the spin system present within each sugar residue of an oligosaccharide is easily assigned (Tables II, III). The positions of the saccharide residues present in each oligosaccharide were established using NOESY. Here cross-peaks result from dipolar (through-space) coupling of spatially adjacent nuclei which was used to assign sequence.

Previously, the isolation and structural characterization of disaccharides **1, 2, 3, 4,** and **5** and tetrasaccharides **6, 8, 9, 11,** and **12** (Figure 6) were reported (Merchant *et al.*, 1985; Yamada *et al.*, 1992; Linhardt *et al.*, 1994; Sugahara *et al.*, 1994). This study, using a combination of 1D and 2D NMR spectroscopy at 500 MHz, confirms these structures. Three new oligosaccharides were prepared from heparan sulfate and were completely characterized (Table I, Figure 6). The structures of these new oligosaccharides were unequivocally established using 2D ^{1}H-NMR spectroscopy.

It is of interest to note that the oligosaccharides prepared and characterized from porcine intestinal mucosal heparan sulfate using heparin lyase III contained ΔUAp (1→ at their nonreducing terminus while those prepared with heparin lyase II contained both ΔUAp and ΔUAp2S (1→. These results are consistent with the known specificity of heparin lyases II and III (Rice and Linhardt, 1989; Linhardt *et al.*, 1990; Desai *et al.*, 1993a,b; Linhardt, 1994; Sugahara, 1994).

Further work is required to isolate and characterize the large number of additional oligosaccharides afforded on heparin lyase depolymerization of heparan sulfate. Unusual structures including those associated with protein binding sites in heparan sulfate are expected based on the presence of many minor oligosaccharide components. Larger oligosaccharides might also be prepared by the partial depolymerization of heparan sulfate with heparin lyases (Pervin *et al.*, 1995).

Materials and methods

Porcine intestinal mucosal heparan sulfate, sodium salt (145 U/mg) was from Celsus Laboratories, Cincinnati, OH. Heparin lyase II and heparin lyase III used in structural studies were purified in our laboratory to homogeneity from

Table II. Proton NMR assignments of heparan sulfate–derived oligosaccharides

α-L-IdoAp(2S)/β-D-GlcAp/ΔUAp(2S)						α-D-GlcN(S)/α,β-D-GlcN(Ac)								
	1	2	3	4	5		1	2	3	4	5	6a	6b	*N*-Ac
Disaccharide (1)														
Residue						Residue								
A	5.156	3.805	4.244	5.826	—	B (α)	5.204	3.891	3.882	3.799	3.957	3.863	3.856	2.033
						(β)	4.703	3.712	3.714	3.791	3.583	3.924	3.822	2.033
Disaccharide (2)														
A	5.195	3.835	4.281	5.857	—	B (α)	5.495	3.296	3.786	3.824	3.988	3.931	3.881	—
						(β)	n.d.	3.082	n.d.	n.d.	n.d.	n.d.	n.d.	—
Disaccharide (3)														
A	5.244	3.881	4.236	5.878	—	B (α)	5.248	3.933	3.902	3.873	4.189	4.388	4.284	2.026
						(β)	4.733	3.755	3.712	3.855	3.836	4.351	4.233	2.026
Disaccharide (4)														
A	5.266	3.901	4.272	5.907	—	B (α)	5.519	3.344	3.776	3.878	4.204	4.421	4.286	—
						(β)	n.d.	3.125	n.d.	n.d.	n.d.	n.d.	n.d.	—
Disaccharide (5)														
A	5.482	4.541	4.315	5.933	—	B (α)	5.428	3.249	3.734	3.809	4.131	4.311	4.187	—
						(β)	n.d.	3.042	n.d.	n.d.	n.d.	n.d.	n.d.	—
Tetrasaccharide (6)														
A	5.096	3.875	4.245	5.828	—	B	5.649	3.336	3.701	3.854	3.955	3.875	3.864	—
C	4.551	3.412, 3.394	3.759	3.831	3.86	D (α)	5.211	3.884	3.955	3.802	3.926	3.85	3.81	2.041
						(β)	4.71	3.705	3.804	3.813	3.595	3.85	3.81	2.041
Tetrasaccharide (7)														
A	5.094	3.786	4.294	5.781	—	B	5.382, 5.379	3.278	3.733	3.848	4.022	3.91	3.91	—
C	4.982	3.732	4.128	4.081	4.75	D (α)	5.208	3.882	3.956	3.838	3.964	3.83	3.72	2.031
						(β)	4.702	3.704	3.803	3.813	3.592	3.92	3.81	2.031
Tetrasaccharide (8)														
A	5.139	3.581	4.266	5.882	—	B	5.381, 5.374	3.288	3.744	3.851	4.021	3.89	3.89	—
C	5.232	4.319	4.269	4.091	4.891	D (α)	5.201	3.879	3.904	3.82	3.953	3.87	3.79	2.033
	5.225	4.303	4.258	4.081	4.879	(β)	4.701	3.699	3.801	3.809	3.571	3.88	3.75	2.033
Tetrasaccharide (9)														
A	5.038	3.731	4.102	5.744	—	B	5.308, 5.304	3.226	3.703	3.811	3.882	3.84	3.81	—
C	4.978	3.733	4.102	4.028	4.75	D (α)	5.184	3.888	3.84	3.687	4.138	4.28	4.24	2.003
						(β)	4.698	3.692	3.812	3.803	3.76	4.28	4.24	2.003
Tetrasaccharide (10)														
A	5.038	3.721	4.251	5.739	—	B	5.401	3.238	3.662	3.813	4.104	4.324	4.219	—
C	5.404	4.445	4.307	3.83	4.75	D (α)	5.433	3.274	3.658	3.81	3.79	3.882	3.84	—
						(β)	4.75	3.021	n.d.	n.d.	n.d.	n.d.	n.d.	—
Tetrasaccharide (11)														
A	5.058	3.741	4.239	5.764	—	B	5.371, 5.364	3.894	3.81	3.85	3.844	3.826	3.755	2.019
C	4.504	3.328	3.661	3.806	3.842	D (α)	5.178	3.829	3.946	3.806	3.927	3.85	3.81	2.007
						(β)	4.681	3.656	3.789	3.792	3.561	3.85	3.81	2.007
Tetrasaccharide (12)														
A	5.049	3.764	4.239	5.759	—	B	5.369	3.895	3.812	3.812	3.899	3.84	3.82	2.042
C	4.507	3.327	3.644	3.734	3.81	D (α)	5.428	3.312	3.683	3.802	3.906	3.84	3.84	—
						(β)	4.75	2.944	n.d.	n.d.	3.532	n.d.	n.d.	—
Hexasaccharide (13)														
A	5.141	3.748	4.212	5.794	—	B	5.317	3.928	3.82	3.84	4.009	4.418	4.163	2.023
C	4.985	3.74	4.103	4.033	4.75	D	5.389	3.212	3.677	3.79	3.84	3.81	3.81	—
E	4.481	3.351	3.677	3.774	n.d.	F	5.178	3.902	3.83	3.84	4.124	4.294	4.144	2.029

n.d., Not determined because of overlapping signals.

F.heparinum (Lohse and Linhardt, 1992) and provided as a gift from Ibex, Montreal, Canada. Sterile Millex GS syringe filters (0.22 and 0.45 μm) were from Millipore, Millipore Product Division, Bedford, MA. BSA (biological grade) was from Sigma Chemical Co., St. Louis, MO. A 200 ml pressure filtration apparatus of 6.3 cm diameter, using diaflow ultrafiltration or a membrane of 10,000 MWCO, was from Amicon, W.R. Grace and Co., Beverly, MA. Size-exclusion chromatography was performed on Sephadex G-50 (superfine) from Pharmacia Biochemicals, Piscataway, NJ, on a glass column of dimensions 4.8 cm × 1 m from Kontes, Scientific Glassware, Morton, Grove, IL. Bio Gel P-2 (fine) from Bio-Rad, Richmond, CA, was used in a desalting column of dimensions 5 cm × 0.5 m. SAX-HPLC was performed on 5 μm Spherisorb columns from Phase separation, Norwalk, CT, of dimensions 0.64 × 25 cm (analytical) and 2.5 × 25 cm (semipreparative) using dual-face programmable LC-7A titanium-based pumps from Shimadzu, Kyoto, Japan. This system was equipped with a Rheodyne (Cotati, CA) titanium injector and a Pharmacia LKB variable-wavelength UV detector from Piscataway, NJ, and with a Shimadzu Chromatopac C-R2A integrating recorder.

CE was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, model I, equipped with high-voltage power supply capable of constant or gradient voltage control using a fused silica capillary, 75 μm i.d., 375 μm o.d., 65 cm long, from Dionex Corporation, Sunnyvale, CA. UV spectroscopy was performed on a Shimadzu model UV 160 spectrometer equipped with a thermostated cell. Heparin/heparan sulfate disaccharide standards were from Grampian Enzymes, Aberdeen, UK. All other reagents used were analytical grade. A Varian 500 MHz NMR spectrometer controlled by a SUN SPARC station 2 workstation was used for all 1D and 2D NMR experiments. The NMR experiments were performed in ^2H_2O (99.96 atom%) using 3-(trimethylsilyl) propionic *2,2,3,3-d*$_4$ acid, sodium salt (99+ atom%) as the internal reference, from Aldrich Chemical Col., Milwaukee, WI.

Enzymatic depolymerization of porcine intestinal mucosal heparan sulfate

Two solutions containing 2.5 g of porcine intestinal mucosal heparan sulfate and 2 mg/ml BSA in 100 ml of 50 mM sodium phosphate buffer at pH 7.6 for heparin lyase III and at pH 7.1 for heparin lyase II treatment. Heparin lyase II or III (1.25 U) in 20 μl of the same 50 mM sodium phosphate buffer was added to the heparan sulfate substrate solution, and the reactions were incubated in polyethylene vials at 30°C in a water bath that was gently shaken. At various time intervals, 10 μl aliquots were removed. A portion of each aliquot was

Table III. Coupling constants (Hz) of heparan sulfate–derived oligosaccharides

α-L-IdoAp(2S)/β-D-GlcAp/ΔUAp(2S)					α-D-GlcN(S)/α,β-D-GlcN(Ac)							
	J1,2	J2,3	J3,4	J4,5		J1,2	J2,3	J3,4	J4,5	J5,6a	J5,6b	J6a,6b
Disaccharide (1)												
Residue					Residue							
A	5.4	5.4	3.6	—	B (α)	2.2	9.4	5.6	10.2	3.6	3.5	−11.1
					(β)	8.2	9.2	4.8	10.2	2.2	5.1	−11.1
Disaccharide (2)												
A	6.1	5.4	3.8	—	B (α)	3.6	10.2	9.4	10.2	2.2	2.2	n.d.
					(β)	7.8	8.4	n.d.	n.d.	n.d.	n.d.	n.d.
Disaccharide (3)												
A	4.7	n.d.	3.2	—	B (α)	3.2	10.6	8.2	9.2	4.2	<1	−11.1
					(β)	8.2	10.2	7.4	9.4	3.8	1.8	−10.8
Disaccharide (4)												
A	5.4	4.5	4.2	—	B (α)	3.5	10.8	9.7	9.7	3.2	1.8	−10.8
					(β)	8.2	8.2	n.d.	n.d.	n.d.	n.d.	n.d.
Disaccharide (5)												
A	3.5	3.5	4.2	—	B (α)	2.4	9.6	8.4	9.6	3.8	2.2	−10.8
					(β)	7.8	8.2	n.d.	n.d.	n.d.	n.d.	n.d.
Tetrasaccharide (6)												
A	6.7	6.2	2.2	—	B	2.2	9.8	8.9	9.4	3.2	2.2	−11.1
C	7.9	8.2	9.4	n.d.	D (α)	2.8	9.7	10.2	9.4	3.4	<1	−10.6
					(β)	8.0	9.4	9.0	9.6	2.2	2.0	−12.0
Tetrasaccharide (7)												
A	6.5	6.2	2.0	—	B	2.2	9.4	10.4	9.4	2.2	<1	−10.6
C	1.8	<1	n.d.	n.d.	D (α)	2.2	9.0	10.4	9.4	3.2	1.8	−11.0
					(β)	7.8	8.0	8.6	9.7	2.0	2.0	−11.6
Tetrasaccharide (8)												
A	6.2	6.8	3.2	—	B	3.2	9.4	10.4	9.7	2.2	<1	−10.6
C	2.4	2.4	2.5	2.5	D (α)	3.3	9.0	10.4	9.4	3.2	1.8	−11.0
	2.4	2.4	2.5	2.5	(β)	7.8	8.2	8.6	9.7	2.2	1.8	−11.6
Tetrasaccharide (9)												
A	6.2	6.8	3.2	—	B	3.2	9.7	9.7	9.4	2.2	1.8	−11.1
C	<1	<1	<1	<1	D (α)	3.2	9.0	10.4	9.4	3.2	1.8	−10.8
					(β)	7.8	8.8	9.4	10.0	1.8	1.8	−11.6
Tetrasaccharide (10)												
A	6.3	6.8	3.3	—	B	2.4	9.7	9.7	9.4	2.3	2.2	−11.2
C	3.2	2.9	n.d.	3.83	D (α)	2.9	9.4	10.4	9.4	3.2	1.8	−10.8
					(β)	8.2	8.6	9.4	10.0	n.d.	n.d.	n.d.
Tetrasaccharide (11)												
A	5.9	6.8	3.0	—	B	3.2	9.7	10.4	9.7	4.5	2.2	−10.4
C	8.0	8.2	8.8	n.d.	D (α)	2.2	9.2	10.4	9.4	3.2	1.8	−10.8
					(β)	8.2	8.0	9.2	10.0	1.8	1.8	−11.6
Tetrasaccharide (12)												
A	6.0	6.4	3.2	—	B	2.4	9.4	n.d.	n.d.	3.2	2.6	n.d.
C	8.2	8.3	8.8	n.d.	D (α)	2.2	9.0	10.4	9.4	3.2	1.8	−10.8
					(β)	7.8	8.0	9.4	10.0	1.8	1.8	−11.6
Hexasaccharide (13)												
A	6.2	6.8	3.2	—	B	3.2	9.7	10.4	9.7	n.d.	n.d.	n.d.
C	<1	<1	<1	<1	D	3.2	9.7	10.4	n.d.	n.d.	n.d.	n.d.
E	8.2	8.2	8.8	n.d.	F	3.2	9.7	10.4	9.7	4.5	2.2	−10.4

n.d., Not determined because of overlapping signals.

used to monitor the reaction by diluting it in 1 ml of 0.03 M HCl and measuring the absorbance at 232 nm. After 6 h of incubation, an additional 1.25 U of each heparin lyase were added to their respective reactions, and during the remaining time course of the reaction aliquots continued to be removed. Reaction completion was defined by the failure of additional heparin lyase to result in increased absorbance at 232 nm.

Removal of high-molecular-weight oligosaccharides and protein residual by pressure filtration

The depolymerized heparan sulfate samples contain oligosaccharides of various sizes, buffer salts and BSA. A controlled pore 10,000 MWCO membrane using a 200 ml stirred pressure filtration cell was used with a gas pressure of 35 p.s.i. N_2 to remove oligosaccharides >10,000 as well as BSA. Pressure filtration concentrated the retentate to an 10 ml volume. This volume was readjusted to 150 ml with deionized, distilled water and pressure filtration was repeated three times. Both the retentate (10 ml) and the diffusate (510 ml) were collected and freeze-dried. The filtrate (MW <10,000) contained the oligosaccharides of interest as well as buffer salts.

Gel-permeation chromatography of oligosaccharides <10,000

The low-molecular-weight heparan sulfate oligosaccharides (M_r < 10,000) obtained from pressure filtration were fractionated on a Sephadex G-50 (superfine) column (4.8 cm × 1 m) eluted with 200 mM sodium chloride at a flow rate of 2 ml/min. Oligosaccharide mixture (~1.25 g corresponding to 50% of the total sample) was dissolved in 10 ml of deionized, distilled water and applied to this column, fractions were collected (10 ml/tube), and absorbance was measured at 232 nm. The fraction numbers were plotted versus absorbance, affording a chromatogram that showed a partial separation of disaccharide through octasaccharides. This separation was repeated giving a reproducible profile that permitted the combination of like fractions. Fractions consisting of disaccharides, tetrasaccharides, and hexasaccharides were obtained and evaporated to dryness.

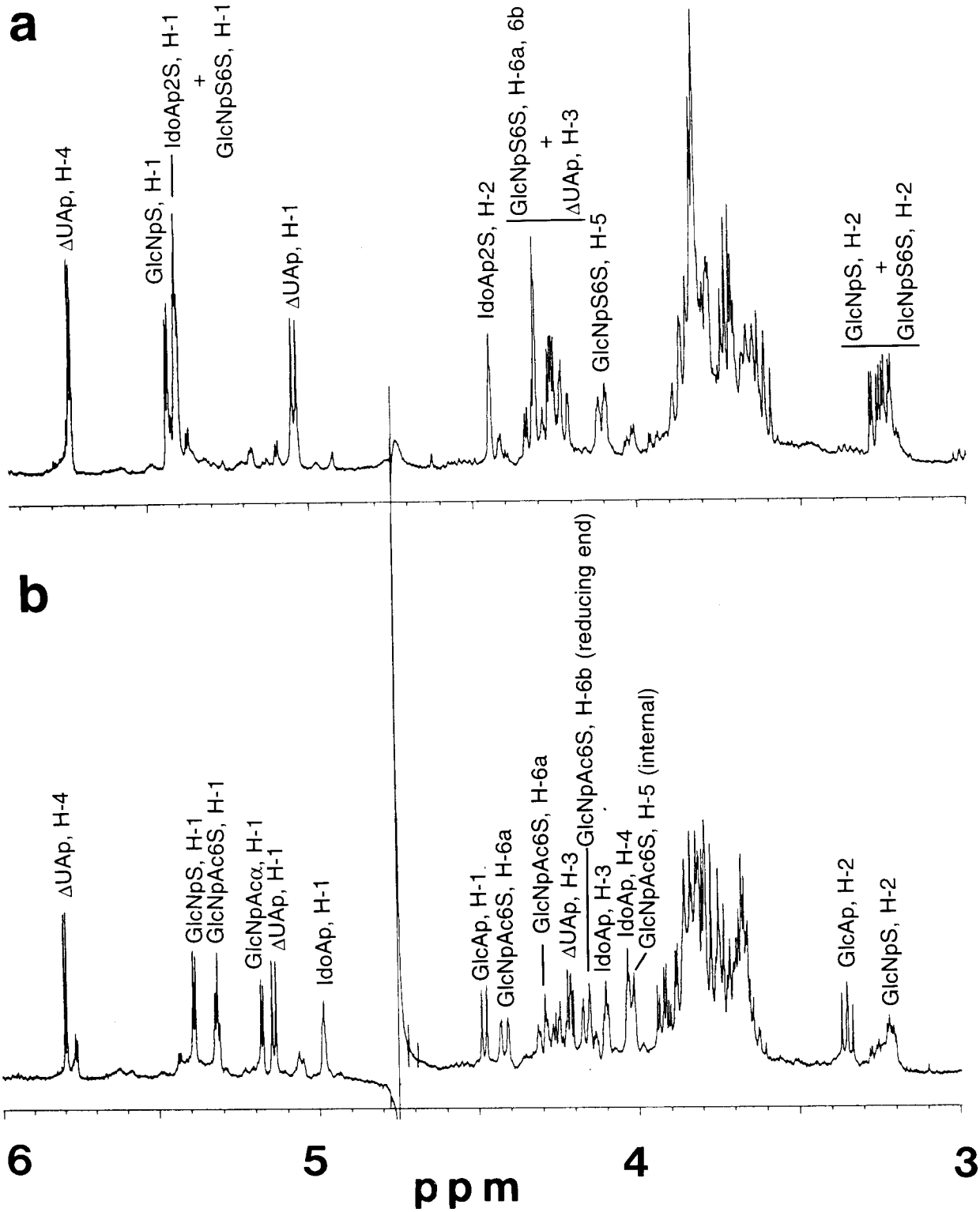

Fig. 5. The 1D 500 MHz ^{1}H-NMR spectra of heparan sulfate-derived oligosaccharides. (**a**) tetrasaccharide **10** and (**b**) hexasaccharide **13.**

Desalting the sized oligosaccharide fractions by GPC and dialysis

Sized oligosaccharide fractions were desalted by GPC on a Bio Gel P-2 column (5 cm × 0.5 m) eluted with water at 6 ml/min. The eluent was collected and the fractions containing oligosaccharides, having absorbance at 232 nm, were combined. The volume was reduced using a rotary evaporator and the samples were freeze-dried. The resulting sized oligosaccharide mixtures were light-yellow-colored powders and were stored at −60°C. Gradient PAGE (Pervin *et al.*, 1995) confirmed the oligosaccharide size in each mixture and also provided an estimation of its size purity.

Purification of sized oligosaccharides into homogenous oligosaccharides using semipreparative SAX-HPLC

Charge separation of sized oligosaccharide fractions was carried out by semi-preparative SAX-HPLC using a linear gradient of sodium chloride at pH 3.5. For example, 30 mg of the desalted tetrasaccharide mixture, obtained from the Sephadex G-50 column, was injected on a semi-preparative column equilibrated with water at pH 3.5. The sample was eluted from the column using a 120 min gradient from 0.0 to 0.8 M of sodium chloride (pH 3.5) at a flow rate of 4.0 ml/min. The elution profile was monitored by absorbance at 232 nm at

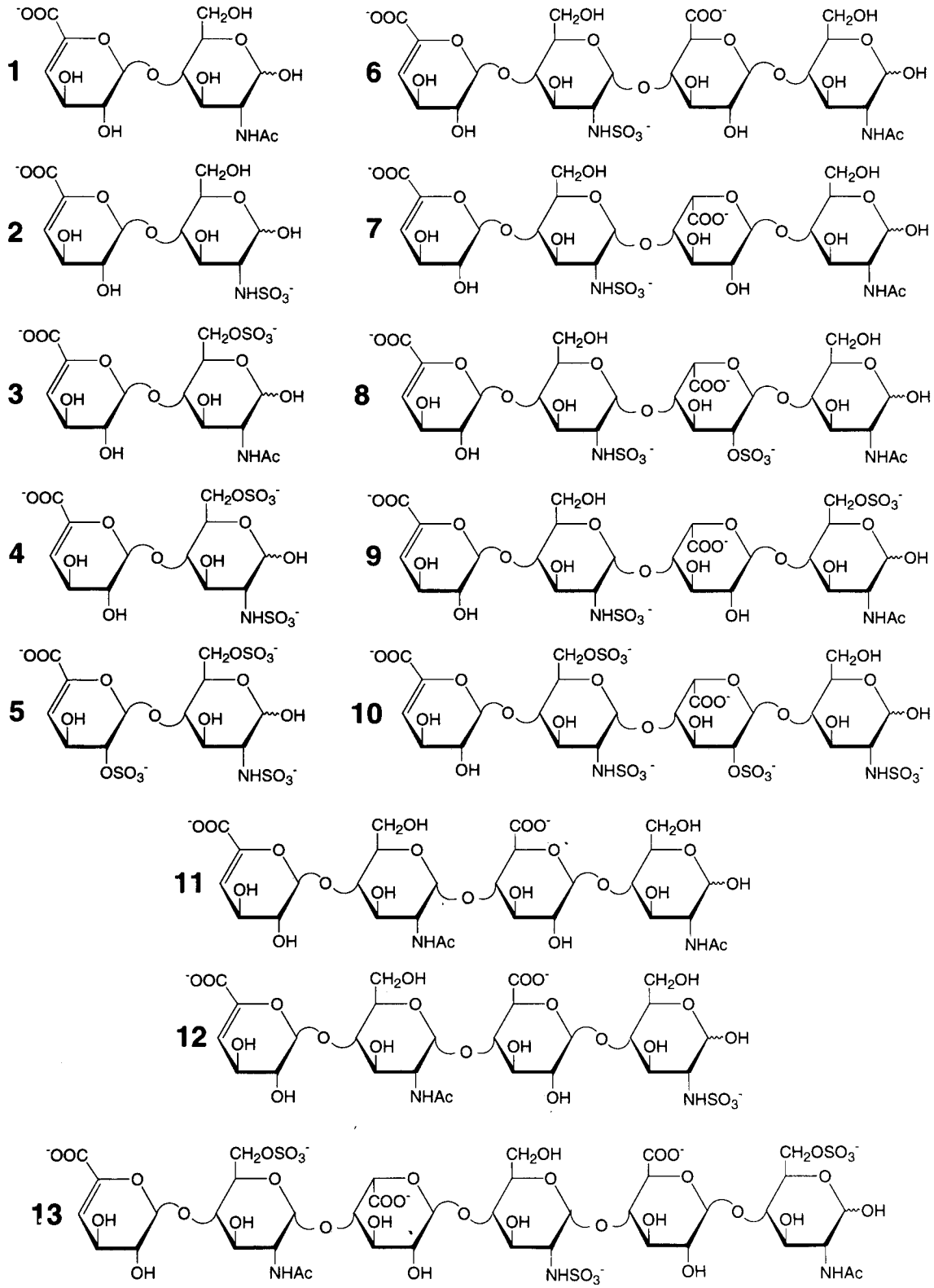

Fig. 6. Structure of heparan sulfate-derived oligosaccharides.

0.5–1.5 absorbance unit full scale (AUFS), and the sample eluted between 35 and 70 min (at 0.23–0.46 M salt). After each run, the column was washed with 2.0 M sodium chloride, followed by a water wash. The tetrasaccharide mixture was applied multiple times to the same column, resulting in nearly identical elution profiles. The major peaks were pooled, lyophilized, and desalted on a Bio-Gel P-2 column. Each tetrasaccharide peak corresponding primarily to a single tetrasaccharide (as demonstrated by capillary electrophoresis at this stage in purification) was applied to the analytical SAX-HPLC column pre-equilibrated to 0.2 M sodium chloride (pH 3.5). The sample was eluted from the column using a 120 min linear gradient from 0.2 to 0.6 M of sodium chloride (pH 3.5) at a flow rate of 1.0 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.5–1.0 AUFS, and the sample eluted at 30 min (0.3 M salt). The purified tetrasaccharide was desalted, lyophilized, and stored at −60°C. The disaccharide and hexasaccharide samples were initially

fractionated using the same gradient described above for the tetrasaccharide sample. The hexasaccharide and octasaccharide fractions were initially fractionated using a 120 min linear sodium chloride gradient from 0.2 to 1.0 M, and then each sized, desalted oligosaccharide fraction was treated as described for the tetrasaccharide fraction.

Analysis of oligosaccharides by analytical SAX-HPLC

Purified oligosaccharides were analyzed by analytical SAX-HPLC to confirm their purity. The SAX-HPLC column was equilibrated with 0.2 M sodium chloride at pH 3.5. Each oligosaccharide sample (10–100 μg) was analyzed using a 120 min linear gradient of 0.2–2 M sodium chloride at pH 3.5 at a flow rate of 1.0 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.02 AUFS. Sample purity was confirmed by the presence of a single symmetrical peak.

Analysis of oligosaccharides by capillary electrophoresis

The purity of each oligosaccharide was confirmed by the presence of a single major symmetrical peak on analysis using CE. The sample was separated and analyzed using a clean fused silica capillary (75 μm i.d., 375 μm o.d. 65 cm long) (Pervin *et al.*, 1994). The samples (1 mg/ml) were prepared in deionized, distilled water and loaded (8 nl) with gravity injection (hydrostatic pressure at a 45 mm head height for 15 s). Reverse-polarity analyses were carried out for 40 min at 18 kV using 20 mM phosphoric acid buffer adjusted to pH 3.48 with a saturated solution of sodium phosphate (Pervin *et al.*, 1994).

NMR spectral analysis of oligosaccharides

The pure oligosaccharide samples were dissolved in ^2H_2O (99.0%) filtered through a 0.45 μm syringe filter and freeze-dried to remove exchangeable protons. After exchanging the sample three times, the sample was dissolved in ^2H_2O (99.96% of atom) containing 0.01% 3-(trimethylsilyl) propionic acid-1 sodium salt (TSP) as internal reference and also measured indirectly relative to acetone (2.225 ppm). 1D NMR experiments were performed on a Varian VXR-500 spectrometer equipped with 5 mm triple resonance tunable probe with standard Varian software at 298K on 700 μl samples at 0.1–0.5 mM. The HO^2H signal was suppressed by presaturation during 3 or 1.5 s for 1 or 2D spectra, respectively. To obtain 2D spectra, 512 experiments resulting in 1024 data points for a spectral width of 2000 Hz were measured, and the time domain data were multiplied after zerofilling (data matrix size, 1K×1K) with shifted sine-bell window functions for 2D COSY, NOESY, or TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY and NOESY experiments by using 150, 250, and 500 ms as the mixing time were performed.

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Abbreviations

AUFS, absorbance unit full scale; BSA, bovine serum albumin; CE, capillary electrophoresis; COSY, correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional; dp, degree of polymerization; FIDs, free induction decays; GlcNp, 2-deoxy-2-aminoglucopyranose; GlcNpAc, 2-deoxy-2-acetamido-glucopyranose; GPC, gel-permeation chromatography; HPLC, high-performance liquid chromatography; MWCO, molecular weight cut-off; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; PAGE, polyacrylamide gel electrophoresis; S, sulfate; SAX, strong anion exchange; TSP, 3-(trimethylsilyl)propanoic acid-1 sodium salt; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-eno-pyranosyluronic acid.

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