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Preparation and structural determination of dermatan sulfate–derived oligosaccharides

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Eight oligosaccharides were prepared from dermatan sulfate (DS) and their structures were elucidated. Porcine intestinal mucosal DS was subjected to controlled depolymerization using chondroitin ABC lyase (chondroitinase ABC). The oligosaccharide mixture formed was fractionated by low-pressure gel permeation chromatography (GPC). Size uniform mixtures of disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, and dodecasaccharides were obtained. Each size-fractionated mixture was then purified on the basis of charge by repetitive semi-preparative strong-anion-exchange (SAX) high-performance liquid chromatography (HPLC). This approach has led to the isolation of six homogeneous oligosaccharides. The size of the oligosaccharides were determined using GPC-HPLC. Treatment of tetrasaccharide and hexasaccharide fragments with Hg(OAc)2 afforded trisaccharide and pentasaccharide products, respectively. The purity of the oligosaccharides obtained was confirmed by analytical SAX-HPLC, and capillary electrophoresis (CE). The molecular mass and degree of sulfation of the eight purified oligosaccharides were elucidated using electrospray ionization (ESI) mass spectrometry and their structures were established with high field nuclear magnetic resonance (NMR) spectroscopy. These DS-oligosaccharides are currently being used to study for interaction of the DS with biologically important proteins.

Key words: dermatan sulfate/oligosaccharide structure/chondroitinase ABC/chondroitin ABC lyase/electrospray ionization mass spectrometry

Introduction

Dermatan sulfate (DS) is an important glycosaminoglycan found in a wide variety of tissues in virtually all animals. DS is a microheterogenous, polydisperse, linear copolymer of 2-acetamido-D-galactopyranose (D-GalpNAc), L-idopyranosyluronic acid (L-IdoAp) and D-glucopyranosyluronic acid (D-GlcAp) residues with *O*-sulfo groups most commonly found at the 4-position of D-GalpNAc residues and occasionally at the 6-position of D-GalpNAc and the 2-position of L-IdoAp residues. DS shows anticoagulant and antithrombotic activities and displays less hemorrhagic effects than unfractionated heparin (Maiamone and Tollefsen , 1990; Linhardt *et al.*, 1991; Ryan *et al.*, 1992; Linhardt and Hileman, 1995). The relatively high molecular weight of DS has limited its clinical applications (Barbanti *et al.*, 1993; Linhardt *et al.*, 1994). Therefore, low molecular weight-dermatan sulfate (LMW-DS) has been applied for clinical use as an anticoagulant and antithrombotic agent (Dettori *et al.*, 1995; Miglioli *et al.*, 1997).

The present study is aimed at gaining insights into the chemical structure of DS through the preparation and structural characterization of homogenous DS-oligosaccharides. Similar studies from our laboratory on heparin (Pervin *et al.*, 1995), heparan sulfate (Hileman *et al.*, 1997) and acharan sulfate (Kim *et al.*, 1998) has improved the understanding of these related families of glycosaminoglycans. DS-oligosaccharide standards might be useful in facilitating the development of new glycosaminoglycan sequencing technologies (Liu *et al.*, 1995; Merry *et al.*, 1999; Turnbull *et al.*, 1999; Venkataraman *et al.*, 1999). Homogenous, structurally characterized DS-oligosaccharides might also aid in studying the interaction of DS with biologically important proteins (Hardingham and Fosang, 1992; Templeton and Wang, 1992; Hileman *et al.*, 1998). For example, DS is known to interact with high specificity to hepatocyte growth factor (Lyon *et al.*, 1998) and annexin V (Ida *et al.*, 1999) as shown by surface plasmon resonance. Homogeneous, structurally characterized DS-oligosaccharides might be useful in better understanding the specificity, and in particular the size of the binding site within DS responsible for these interactions. Finally, DS-oligosaccharides should help in better understanding the specificity of enzymes acting on DS such as the chondroitin lyases and hydrolases (Yamagata *et al.*, 1968; Ototani and Yosizawa, 1979). Recently, a chondroitin sulfate-derived disaccharide prepared in our laboratory has been used to obtain the high-resolution x-ray co-crystal structure with chondroitin B lyase (Huang *et al.*, 1999). The current study describes the application of chondroitin ABC lyase to prepare DS-derived disaccharide, tetrasaccharide, hexasaccharide, octasaccharide, decasaccharide, and dodecasaccharide. Further chemical treatment with mercuric acetate was also used to afford DS oligosaccharides having an odd number of saccharide residues. The structures of these purified oligosaccharides were unequivocally established

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by using electrospray ionization (ESI) mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

Results and discussion

A number of DS samples from various tissues and species were initially examined by 500 MHz ^{1}H-NMR spectroscopy. The DS from porcine intestine was selected to prepare oligosaccharides as it had an extremely simple structure composed of >95 % →4)-α-L-IdoAp (1→3) β-D-GalpNAc4S (1→ (Figure 1). A small quantity of this porcine intestinal DS was exhaustively depolymerized in the reaction using chondroitin ABC lyase. Next, a controlled, partial depolymerization was performed to obtain the UV absorbance value at 232 nm corresponding to a 50% completion of the depolymerization reaction. The partial (50%) depolymerization reaction was next scaled-up to obtain a multi-gram mixture of DS-oligosaccharides. Analysis of this mixture on analytical SAX-HPLC and CE (under reverse polarity) demonstrated the presence of a complex mixture of oligosaccharide components grouped into clusters. It should be noted that, on reverse polarity CE the most highly charged components elute first but on SAX-HPLC the opposite is true (Figure 2A and B). Each group of clusters appeared to be dominated by a major component, presumably ΔUAp (1→[3) β-D-GalpNAc4S (1→4)-α-L-IdoAp (1→]$_n$3) β-D-GalpNAc4S (where n = 0, 1, 2, …). The oligosaccharide mixture was next size fractionated by GPC using a Bio-Gel P6 column. This separation afforded size-uniform oligosaccharide mixtures corresponding to oligosaccharides ranging in size from disaccharides to dodecasaccharides. After combining fractions under

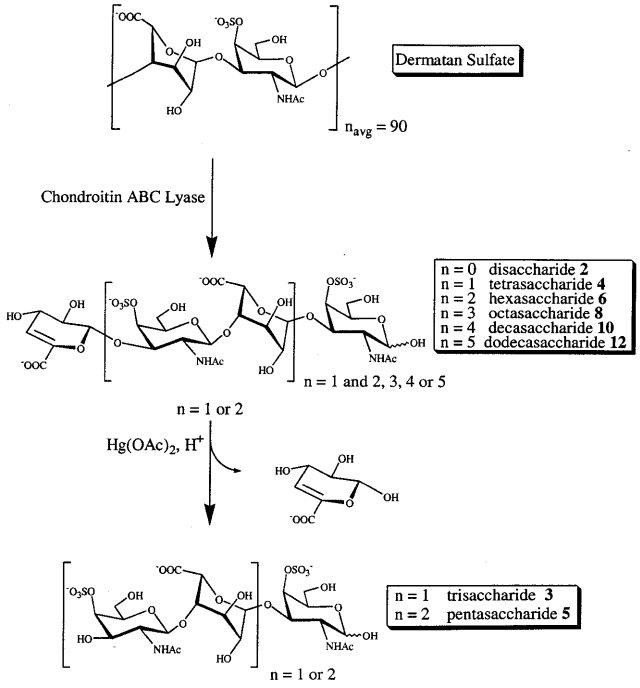

Fig. 1. Controlled enzymatic depolymerization of dermatan sulfate by chondroitin ABC lyase and mercuric acetate treatment to remove the unsaturated nonreducing terminal residue. The structures of oligosaccharides **2**–**12** are shown.

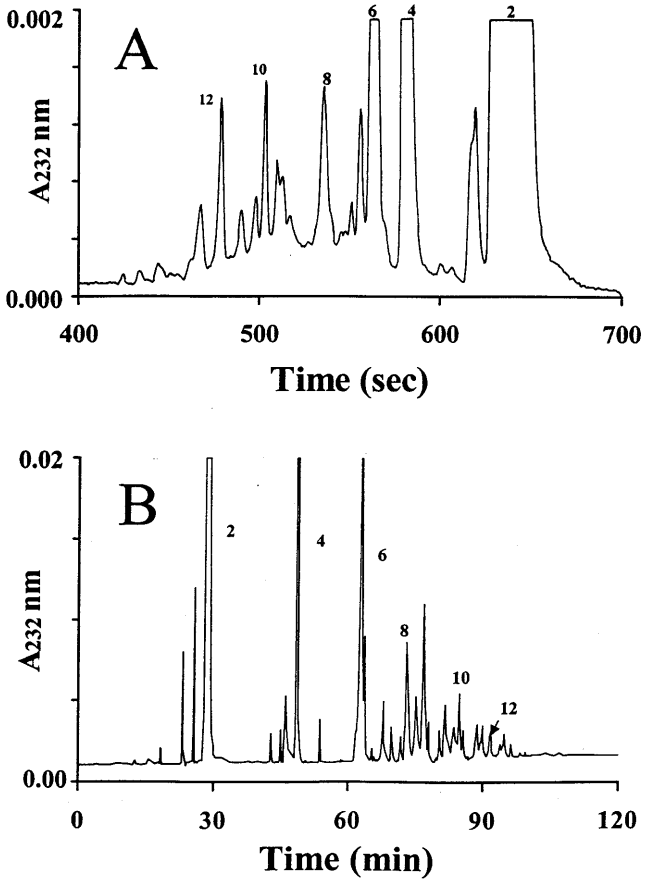

Fig. 2. SAX-HPLC analysis and CE analysis of dermatan sulfate derived oligosaccharide mixture. (**A**) CE analysis and (**B**) analytical SAX-HPLC of dermatan sulfate partially (50% reaction completion) depolymerized by chondroitinase ABC. The dp of each peak is given corresponding to the structures shown in Figure 4.

each peak, the size-uniform oligosaccharide mixtures were concentrated by rotary evaporation and desalted on a Bio-Gel P2 column. Gradient PAGE analysis of these size-fractionated oligosaccharides 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. Each fraction from the GPC column was then fractionated on the basis of charge by multiple injections on a semi-preparative column, like peaks were pooled, desalted by Bio-Gel P2 chromatography or dialysis, and lyophilized. Each peak was then examined by analytical SAX-HPLC to assess whether a second semi-preparative SAX-HPLC separation step was necessary and to establish the optimum gradient for this separation. These separation steps resulted in the isolation of six dermatan sulfate-derived oligosaccharides. The percentage of each oligosaccharide, found in the depolymerized mixture is presented in Table I. An assessment of oligosaccharide purity with detection by absorbance at 232 nm was made by analytical SAX-HPLC (Figure 3B) and CE under reverse-polarity conditions (Pervin *et al.*, 1994). Both analyses showed a single

Table I. Structure, purity, and amount of dermatan sulfate-derived oligosaccharides

		% Purity		Molecular weight	
Oligosaccharide	Mole %	SAX-HPLC	CE	GPC	Calculated
2	54.5	>99	>99	498	459
3	—	n.d.[a]	n.d.	n.d.	760
4	14.8	>99	>96	867	918
5	—	n.d.	n.d.	n.d.	1218
6	10.9	>99	90	1215	1377
8	1.8	>95	80	1360	1836
10	0.7	87	80	1906	2295
12	0.3	95	90	2687	2754

[a]n.d., Not determined.

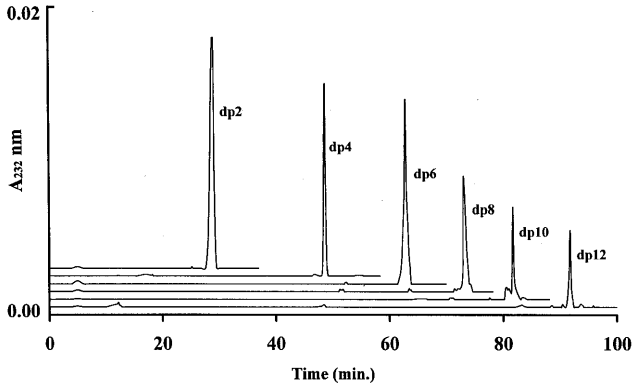

Fig. 3. Analytical SAX HPLC analysis of individual dermatan sulfate derived oligosaccharides used to calculate purity (see Table I).

major symmetrical peak for each of the six oligosaccharides and demonstrated oligosaccharide purities of from 80 to >99% (Table I). The molecular weights of purified seven oligosaccharides were next measured by using analytical GPC-HPLC. A calibration curve of log (M_r) vs. K_{avg} was prepared using heparin-derived oligosaccharide standards of dp 2, 4, 8, 10, 12, and 14 (Pervin *et al.*, 1995). The standard curve had the equation $y = –3.79x + 5.39$ with a correlation value of $r^2 = 0.988$. The molecular weights estimated by GPC-HPLC are presented in Table I. The results of this analysis suggested that we had isolated a disaccharide **2**, tetrasaccharide **4**, hexasaccharide **6**, octasaccharide **8** decasaccharide **10** and dodecasaccharide **12** (Table I).

The ESI-MS analysis was next performed on these six oligosaccharides. The parent ions for each of these oligosaccharides are given in Table II. The ESI mass spectrum of dodecasaccharide **12** is shown in Figure 4. Multiple molecular ions were observed for each of these oligosaccharides in negative ion ESI-MS corresponding to species carrying from 1 to 8 negative charges. Furthermore at each charge multiply sodiated species were observed. The presence of these multiple molecular ions afforded the molecular mass of each oligosaccharide with a high level of accuracy. The purity of each oligosaccharide was also assessed by carefully examining each spectrum for the presence of undersulfated and oversulfated oligosaccharides as well as ones having higher or lower degree of polymerization (dp). No such ions were observed for the six oligosaccharides studied. Dodecasaccharide **12**, shown in Figure 4, is among the largest glycosaminoglycan-derived oligosaccharides to be determined using ESI-MS. The molecular weights of the oligosaccharides obtained by ESI-MS were higher than those estimated by GPC-HPLC(based on a heparin-derived oligosaccharide standard curve) (Table I) suggesting that the DS oligosaccharides had lower molecular

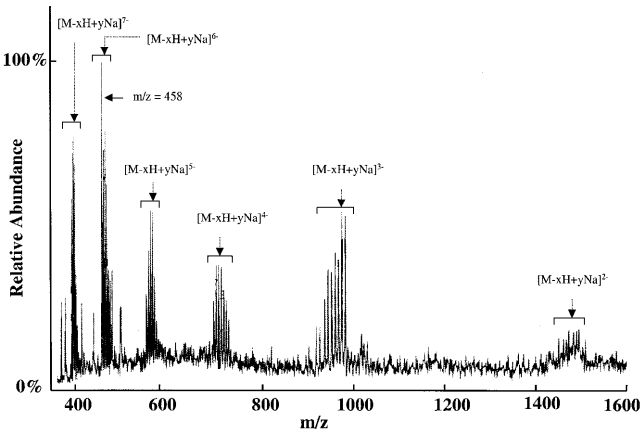

Fig. 4. ESI-MS analysis of dermatan sulfate dodecasaccharide **12**. Six ion clusters are marked with charges ranging from –7 to –2. The number of protons lost (x) ranged from 1 to 12 and the number of sodium atoms added (y) ranged from 0 to 9.

Table II. Selected electrospray ionization mass spectral data for dermatan sulfate derived oligosaccharides

	2	**3**	**4**	**5**	**6**	**8**	**10**	**12**
Parent ion[a]	$[M-H]^{1-}$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	$[M-3H]^{3-}$	$[M-4H]^{4-}$	$[M-4H]^{4-}$	$[M-6H]^{6-}$	$[M-6H]^{6-}$
m/z	458	379	305	405	343	458	382	458
Mass	459	760	918	1218	1377	1836	2295	2754

[a]Highest abundance ion reported. Clusters of peaks corresponding to multiply sodiated ions of different net charges are observed in each spectrum. Each peak could also be used to calculate a molecular mass consistent with that calculated for the parent ion.

volume per unit mass than did the heparin-derived oligosaccharides.

The DS tetrasaccharide **4** and hexasaccharide **6** were next treated with mercuric acetate to remove the unsaturated uronic acid introduced into their nonreducing end through the eliminative action of chondroitin ABC lyase (LeBrun and Linhardt, 2000). This treatment resulted in the nearly quantitative yield of a DS trisaccharide **3** and pentasaccharide **5**, thus affording convenient access to DS oligosaccharides having an odd number of saccharide residues. ESI-MS analysis confirmed the molecular mass of these newly formed oligosaccharides (Table II).

The 500 MHz 1D ^{1}H-NMR spectra of all the oligosaccharides were acquired for their structural elucidation. Oligosaccharides **2**, **4**, **6**, and the reduced analog of trisaccharide **3** had been previously prepared and their NMR structural data reported (Sanderson *et al.*, 1989; Yamada *et al.*, 1998). The spectra of oligosaccharides **2**, **4**, and **6** were identical to those already reported for these compounds. The complete removal of the unsaturated uronate (Figure 1) with mercuric acetate to form trisaccharide **3** could be demonstrated through the disappearance of the signals corresponding to this residue, in particular the H-4 signal of the ΔUAp residue at 5.9 p.p.m. The NMR spectrum of trisaccharide **3** was easily assigned based on the previously published spectrum of the reduced form of this trisaccharide (Sanderson *et al.*, 1989). The structure of octasaccharide **8** and decasaccharide **10** (Tables III and IV) were assigned based on the chemical shifts previously reported for the smaller oligosaccharide analogs (Sanderson *et al.*, 1989; Yamada *et al.*, 1998). Because of the complexity of the 1D ^{1}H-NMR spectra of pentasaccharide **5** and dodecasaccharide **12**, two dimensional (2D) spectroscopy was required for the complete assignment of these compounds. The 2D ^{1}H-NMR TOCSY spectra of oligosaccharides **5** and **12** are presented in Figures 5 and 6, respectively. These spectra were assigned based on the combination of COSY, NOESY and TOCSY experiments. The 2D TOCSY spectrum (Figure 5) shows prominent off-diagonal cross-peaks labeled 1–10 in Figure 5. Starting on the upper right of the diagonal, the peak at 5.199 can be assigned to the H1 proton of the reducing end GalpNAc4S residue. Off-diagonal cross-peaks 1–3 correspond to other protons in this residue that show scalar connectivity to the H1 proton. The off-diagonal cross-peaks labeled 4–7 are used to deduce the scalar connectivity in the adjacent IdoAp residue. Off-diagonal cross-peaks 8 and 9 correspond to connectivity within the single internal GalpNAc4S residue. Finally, off-diagonal cross-peak 10 corresponds to the H5/H6 connectivity in the GalpNAc4S residue at the reducing end. It was possible to completely assign the ^{1}H-NMR spectrum for pentasaccharide **5** based on these and other 2D experiments (Table III). The chemical shifts of the signals of GalpNAc at nonreducing end in pentasaccharide **5** (Figure 5) are very similar to those of penultimate GalpNAc residue, next to unsaturated uronate in hexasaccharide **6**. These results suggest that the magnetic anisotropic effects of an adjacent unsaturated uronate residue are not strong. Next the dodecasaccharide **12** was examined by 2D NMR spectroscopy. The 2D TOCSY spectrum is shown in Figure 6. Assignments were made in the same way as described above for pentasaccharide **5**. The chemical shifts of the internal GalpNAc4S and IdoAp residues of dodecasaccharide **12** are very similar suggesting that in solution dodecasaccharide **12** may exist in an extended linear conformation. The similar chemical shifts observed for the signals of the internal sequence suggest such a stable conformation of each carbohydrate residue within dodecasaccharide **12**. The complete assignment of dodecasaccharide **12** is presented in Table VI. By obtaining spectra at elevated temperature (45°C) and eliminating the overlap from the HOD signal, the H-5 proton signal of the IdoAp residue 2 and the H-1 and H-4

Table III. Chemical shifts of dermatan sulfate-derived octasaccharide 8

		1 α	1 β	2	3	4
GalpNAc4S	H-1	5.186	4.739*	4.66	4.68*	4.68*
	H-2	4.329	4.02	4.03	4.03	4.03
	H-3	4.143	4.02	4.03	4.14	4.14
	H-4	4.682*	4.71*	4.68*	4.645	4.597
	H-5	4.256	3.84	3.82	3.84	3.84
	H-6a	3.76	3.78	3.77	3.76	3.76
	H-6b	3.68	3.78	3.77	3.76	3.76
	N-Ac	2.028	2.028	2.049	2.049	2.092
IdoAp(ΔUAp)	H-1	4.889	4.840	4.860*	4.860*	5.243
	H-2	3.502	3.502	3.509	3.509	3.82
	H-3	3.82	3.82	3.89	3.89	3.94
	H-4	4.03	4.03	4.08	4.08	5.941
	H-5	4.71*	4.71*	4.71*	4.71*	—

*, Determined at 45°C.

Table IV. Chemical shifts of dermatan sulfate-derived decasaccharide 10

		1 α	1 β	2	3	4	5
GalpNAc4S	H-1	5.184	4.74*	4.66	4.68*	4.68*	4.68*
	H-2	4.322	4.02	4.02	4.02	4.02	4.02
	H-3	4.12	4.02	4.02	4.02	4.12	4.12
	H-4	4.69*	4.69*	4.69*	4.645	4.645	4.598
	H-5	4.26	3.84	3.82	3.84	3.84	3.84
	H-6a	3.76	3.77	3.77	3.77	3.77	3.77
	H-6b	3.76	3.77	3.77	3.77	3.77	3.77
	N-Ac	2.029	2.029	2.050	2.050	2.050	2.091
IdoAp(ΔUAp)	H-1	4.910*	4.863*	4.863*	4.863*	4.863*	5.242
	H-2	3.502	3.502	3.506	3.506	3.506	3.82
	H-3	3.82	3.82	3.84	3.84	3.84	3.911
	H-4	4.033	4.033	4.064	4.064	4.064	5.940
	H-5	4.69*	4.71*	4.71*	4.71*	4.71*	—

*, Determined at 45°C.

Table V. Chemical shifts of dermatan sulfate-derived pentasaccharide 5

	Penta	1 α	1 β	2	3
GalpNAc4S	H-1	5.199	4.739*	4.601	4.663
	H-2	4.348	4.06	4.12	4.06
	H-3	4.181	4.01	4.03	4.14
	H-4	4.736	4.66*	4.68*	4.655
	H-5	4.256	3.86	3.86	3.86
	H-6a	3.76	3.81	3.81	3.81
	H-6b	3.71	3.81	3.81	3.81
	N-Ac	2.043	2.043	2.069	2.070
IdoAp	H-1	4.889	4.840	4.860*	
	H-2	3.502	3.502	3.509	
	H-3	3.88	3.88	3.89	
	H-4	4.122	4.122	4.088	
	H-5	4.69	4.69	4.71	

*Determined at 45°C.

proton signals of GalpNAc4S residues were determined without ambiguity.

The structures of the oligosaccharides prepared through partial, controlled digestion with chondroitin ABC lyase (Figure 1) are consistent with the uniform →4)-α-L-IdoAp (1→3) β-D-GalpNAc4S (1→ sequence present in the dermatan sulfate prepared from porcine intestinal mucosa. Dermatan-derived oligosaccharides appear to occupy a smaller molecular volume than heparin-derived oligosaccharides. Furthermore, the conformational properties of the dermatan-derived dodecasaccharide suggests that it exists in an extended linear conformation in solution. The current study also demonstrates that it is possible to prepare large dermatan sulfate-derived oligosaccharides in sufficient purity and quantities required for full structural characterization. Work is currently underway to determine the biological activities of these large oligosaccharides and to examine their interaction with various glycosaminoglycan-binding proteins, including the polysaccharide lyases.

Materials and methods

Materials

Dermatan sulfate from porcine intestinal mucosa was purchased from Celsus Laboratories (Cincinnati, OH). Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) from *Proteus vulgaris* was purchased from Sigma Chemical Co. (St. Louis, MO). Gel permeation chromatography was performed on Bio-Gel P2 and P6 (superfine) from Bio-Rad (Richmond, CA). All other reagents used were analytical grade. SAX-HPLC was performed on 5 μm Spherisorb columns of dimensions 2.0 × 25 cm and 0.46 × 25 cm from Waters (Milford, MA). GPC-HPLC used a TSK-Gel 2000SW (10 μm) column of dimensions 0.75 × 30 cm from Phenomenex (Torrance, CA). HPLC was performed on LC-10A*i* pumps from Shimadzu (Kyoto, Japan) with a Shimadzu SPD-10A*i* variable-wavelength UV detector and data were processed using Shimadzu Class-Vp 4.03 chromatography data system running Windows based acquisition and control software.

CE was performed using a 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 from Dionex Corporation (Sunnyvale, CA). UV spectrometer was equipped with a thermostatted cell or a JASCO model V550 (Tokyo, Japan). A Varian 500 MHz NMR spectrometer controlled by a

Table VI. Chemical shifts of dermatan sulfate–derived dodecasaccharide 12

		1 α	1 β	2	3	4	5	6
GalpNAc4S	H-1	5.184	4.72*	4.66	4.68*	4.68*	4.68*	4.68*
	H-2	4.321	4.02	4.03	4.03	4.03	4.03	4.03
	H-3	4.141	4.02	4.03	4.14	4.14	4.14	4.14
	H-4	4.68*	4.68*	4.68*	4.642	4.642	4.642	4.596
	H-5	4.256	3.84	3.84	3.84	3.84	3.84	3.84
	H-6a	3.76	3.76	3.76	3.76	3.76	3.76	3.76
	H-6b	3.76	3.76	3.76	3.76	3.76	3.76	3.76
	N-Ac	2.027	2.027	2.047	2.047	2.047	2.047	2.091
IdoAp(ΔUAp)	H-1	4.889	4.841*	4.839*	4.839*	4.839*	4.839*	5.242
	H-2	3.502	3.502	3.509	3.509	3.509	3.509	3.82
	H-3	3.84	3.84	3.89	3.89	3.89	3.89	3.910
	H-4	4.03	4.03	4.08	4.08	4.08	4.08	5.940
	H-5	4.71*	4.71*	4.71*	4.71*	4.71*	4.71*	-

*Determined at 45°C.

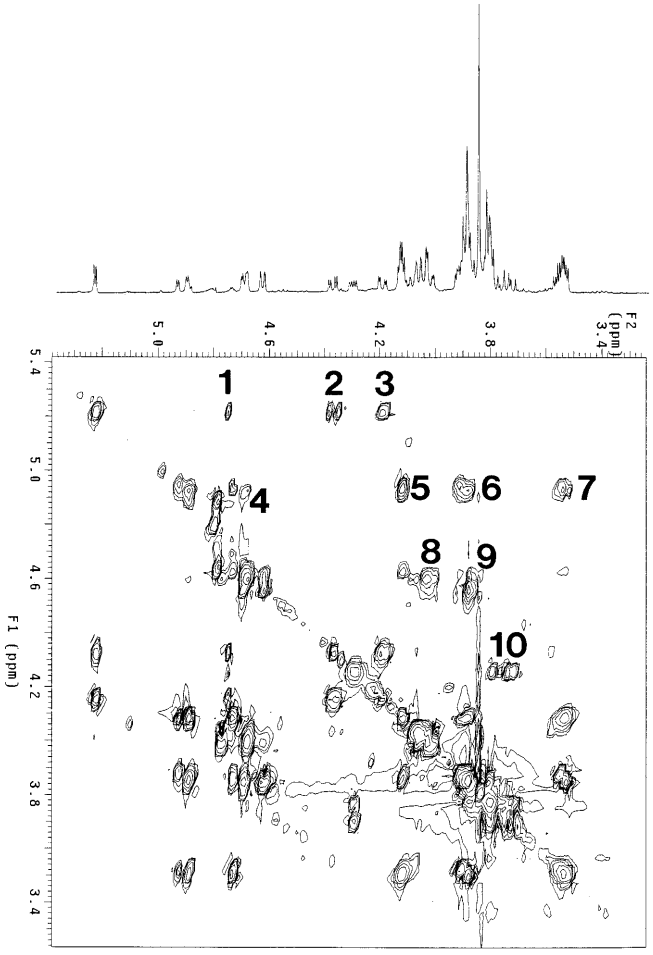

Fig. 5. 2D ^{1}H-NMR TOCSY spectrum of DS pentasaccharide **5** is presented. The cross-peaks are assigned as: 1, α-GalpNAc H1/H4; 2, α-GalpNAc H1/H2; 3, α-GalpNAc H1/H3; 4, IdoAp H1/H5; 5, IdoAp H1/H4; 6, IdoAp H1/H3; 7, IdoAp H1/H2; 8, β-GalpNAc H1/H2,H3; 9, β-GalpNAc H4/H5 (inner); and 10, α-GalpNAc H5/H6 (reducing end).

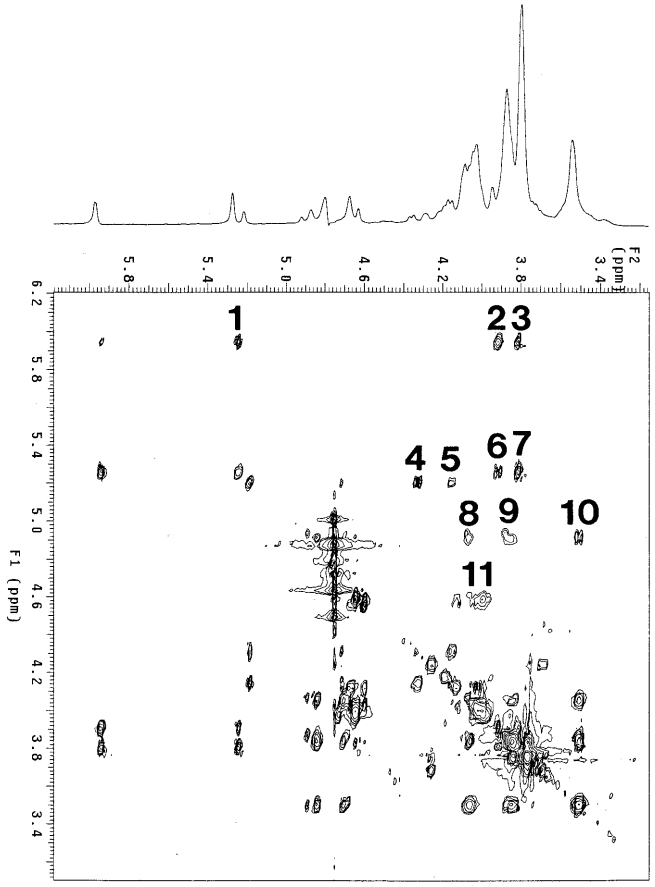

Fig. 6. 2D ^{1}H-NMR TOCSY spectrum of DS dodecasaccharide **12** is presented. The cross-peaks are assigned as: 1, ΔUAp H1/H4; 2, ΔUAp H3/H4; 3, ΔUAp H2/H4; 4, α-GalpNAc H1/H2; 5, α-GalpNAc H1/H3, H6; 6, ΔUAp H1/H3; 7, ΔUAp H1/H3; 8, IdoAp H1/H4; 9, IdoAp H1/H3; 10, IdoAp H1/H2; and 11, β-GalpNAc H1/H2,H3.

SUN SPARC station 2 workstation was used for all 1D and 2D NMR experiments.

Enzymatic depolymerization of porcine intestinal mucosal dermatan sulfate

Dermatan sulfate (500 ml, 20 mg/ml) in 50 mM Tris–HCl-sodium acetate buffer, pH 8.0 was treated with chondroitin ABC lyase (20 U) at 37°C. At various time intervals, 10 µl aliquots were removed and each aliquot was used to monitor the reaction by diluting it in 1 ml of 0.03 M HCl and measuring the absorbance at 232 nm. When the absorbance at 232 nm indicated the digestion was 50% completed, the digestion mixture was heated at 100°C for 3 min. The resulting oligosaccharide mixture was concentrated, by rotary evaporation with warming at 40°C, to 100 ml for fractionation by low pressure GPC.

Low pressure GPC fractionation of oligosaccharides

A portion (1.25 g in 12.5 ml) of the oligosaccharide mixture was fractionated on a Bio-Gel P6 (superfine) column (4.8 × 100 cm) eluted with 100 mM sodium chloride at a flow rate of 1.5 ml/min, 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 dodecasaccharides. This separation was repeated (eight times) giving reproducible profiles that permitted the pooling of like-fractions. Fractions consisting of disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides and dodecasaccharides were obtained and concentrated by rotary evaporation.

Desalting the size fractionated oligosaccharides

Sized oligosaccharide fractions were next desalted by GPC on a Bio-Gel P2 column (4.8 × 70 cm) eluted with water at a flow rate of 1 ml/min, fractions were collected (5 ml/tube). The eluent was collected and the fractions containing oligosaccharides, having absorbance at 232 nm, were combined. Each fraction was concentrated using a rotary evaporator and the samples were freeze-dried. The resulting size fractionated oligosaccharide mixtures were light yellow colored powders and were stored at –20°C.

Purification of size fractionated oligosaccharides using semipreparative SAX-HPLC

Charge separation of sized oligosaccharide fractions was carried out by semipreparative SAX-HPLC using a linear gradient of sodium chloride at pH 3.5. The desalted, sized oligosaccharide fractions were injected on a semipreparative column equilibrated with water at pH 3.5. The sample was eluted from the column using a 150 min gradient from 0 to 2 M 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 0.5–1.5 absorbance units full scale (AUFS), and the sample eluted between 30 and 80 min (at 0.5–1.3 M salt). After each run, the column was washed with 2.0 M sodium chloride, followed by a water wash. Each fraction 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 P2 column. Each peak was next analyzed by analytical SAX-HPLC and CE.

Analysis of oligosaccharides by analytical SAX-HPLC

Purified oligosaccharides were analyzed by analytical SAX-HPLC to confirm their purity. The SAX-HPLC column was washed as described above and equilibrated with 0.2 M sodium chloride at pH 3.5. Each oligosaccharide sample (5–20 μg) was analyzed using a 120 min linear gradient of 0–1.2 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 CE

The purity of each oligosaccharide was next confirmed by the presence of a single major symmetrical peak on analysis using CE. Experiments were performed on an ISCO capillary electrophoresis system equipped with a variable wavelength ultraviolet detector set at 232 nm. System operation and data handling were fully computer controlled. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with saturated dibasic sodium phosphate as described previously (Pervin *et al.*, 1994). Separation and analysis were carried out in a fused-silica capillary tube. This capillary was 50 μm inner diameter, 360 μm outer diameter, and 62 cm long, with a 42 cm effective length and was externally coated except where the tube passed through the detector. The capillary was washed extensively with 0.5 M sodium hydroxide followed by deionized distilled water, and then running buffer. Samples were injected by vacuum injection (vacuum level 2, 12.79 kPa.s). Each experiment was performed at 20 kV constant voltage.

Analysis of oligosaccharides by analytical GPC-HPLC

Samples (100 μl at 100 pM concentration) were injected into a 20 μl loop connected to a TSK-Gel 2000SW column. Analysis was performed by isocratic elution with 0.2 M NaCl at pH 3.5 and oligosaccharides were detected at 232 nm. The column void and total volumes were determined using blue dextran and sodium azide.

ESI-MS analysis

Negative-ion spectra were performed by using a Micromass, Inc. (England) Autospec equipped with an electrospray interface as described previously (Kim *et al.*, 1998). Nitrogen gas was used both as bath and nebulizer gas, at flow rates of 250 l/h and 12 l/h, respectively. The electrospray ion source was held at 80°C and the spray needle was held at 7.7 kV. Tetraethylammonium iodide in acetonitrile was used as the calibrant. The solutions of dermatan-derived oligosaccharides were prepared for negative ESI-MS by dissolving the solid sample in 1:1 water/acetonitrile with 0.05% NH_4OH which was also used for the mobile phase. Spectra were obtained by injecting 20 μl of each solution. Multiple injections were performed. The spectra were obtained by 30–40 scans with the use of manufacturer's OPUS software.

NMR spectral analysis of oligosaccharides

The pure oligosaccharide samples were dissolved in D_2O (99.0 atm %) 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 D_2O (99.96% of atom). 1D ^{1}H-NMR experiments were performed on a Varian VXR-500 spectrometer equipped with 5 mm triple resonance tunable probe with standard Varian software at 298°K and 313°K on 700 μl samples at 0.1–0.5 mM. The HOD signal was suppressed by presaturation during 3 and 1.5 s for 1D and 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 zero filing (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 mixing times.

Cleavage of terminal unsaturated uronic acid residue with mercuric acetate

DS tetrasaccharide and hexasaccharide were each dissolved at a concentration of 1 mg/ml in double distilled deionized water. Mercuric acetate reagent (35 mM) was prepared by dissolving 113.3 mg of $Hg(OAc)_2$ in 10 ml of distilled water adjusted to pH 5 with a few drops of acetic acid. In a typical experiment, 1 ml of oligosaccharide solution (1 mg/ml) was treated with 1 ml of mercuric acetate reagent, stirred for 10 min at room temperature. The reaction mixture was passed over a pre-washed Dowex 50W-X8 H^+ column (1 × 5 cm), and then washed with 5 column volumes of distilled water. The total effluent was adjusted to pH 7 using sodium bicarbonate solution, and freeze-dried. The resulting oligosaccharide was redissolved in minimal quantity of water and then was applied to a Sephadex G-25 column (2.5 × 50 cm). Fractions were analyzed by UV spectrometer for absorbance at 210 nm and 232 nm.

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Abbreviations

DS, dermatan sulfate; SAX, strong anion exchange; HPLC, high performance liquid chromatography, CE, capillary electrophoresis, ESI, electrospray ionization, LMW low molecular weight, ΔUAp, 4-deoxy-α-L-*threo*-hex-enopyranosyluronic acid, GalpNAc, *N*-acetylgalactopyranose; IdoAp, idopyranosyluronic acid, GlcAp, glucopyranosyluronic acid; S, sulfate COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser spectroscopy; dp, degree of polymerization; GPC, gel permeation chromatography.

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