Structure and contribution to the heparin cofactor II-mediated inhibition of thrombin of naturally oversulphated sequences of dermatan sulphate

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Dermatan sulphate (DS) obtained from bovine and pig mucosa and pig skin, and charge-enriched fractions of a selected DS preparation, were characterized in terms of charge density, M_r and disaccharide composition of chondroitin ABC lyase digests, and by ^{13}C -n.m.r. spectroscopy. Besides the major IdoA-GalNAc4SO₃ sequences, all DS preparations contain about 10% disulphated disaccharide sequences (mostly IdoA2SO₃-GalNAc4SO₃, with minor amounts of IdoA-GalNAc4,6SO₃). DS fragments (prepared by radical-catalysed depolymerization of DS and retaining the internal structure of the parent polysaccharide) as well as Smith degraded fragments [SD-DS, obtained by controlled degradation of periodate-oxidized and borohydride-reduced DS (RO-DS)] with the general structure GalNAc4SO₃(IdoA2SO₃-GalNAc4SO₃)_n-R (where R is the

remnant of a glycol-split uronic acid, and $n = 2-3$ and $3-4$) were characterized by one- and two-dimensional ^1H -n.m.r., ^{13}C -n.m.r. and disaccharide composition analysis. In accordance with previous findings [Maimone and Tollefsen (1990) J. Biol. Chem. 265, 18263–18271], only fragments with $n \geq 3$ significantly enhance the heparin cofactor II-mediated inhibition of thrombin. In natural DS preparations and their fractions, this activity (as well as the antithrombotic activity in an animal model) appears to require IdoA2SO₃-containing sequences. The heparin cofactor II activity of DS, RO-DS and SD-DS fragments decreases with decreasing M_r . However, RO-DS fragments are more active than DS fragments of similar M_r , probably because of the extra flexibility endowed by glycol-split IdoA residues.

INTRODUCTION

Dermatan sulphate (DS) is a polysaccharide belonging to the family of glycosaminoglycans. As a proteoglycan, it is a component of the extracellular matrix of several tissues and is also present on the surface of endothelial cells, with physiological roles that are still not fully understood (Fransson, 1987; Heinegard and Sommarin, 1987; Kinsella and Wight, 1988). DS proteoglycans, as well as the DS polysaccharide chains detached from their original peptide matrix, bind to a number of proteins (Lindahl and Höök, 1978; Lane, 1989). Binding to heparin cofactor II (HC-II) is thought to contribute, at least in part, to the antithrombotic/thrombolytic properties of DS (Tollefsen et al., 1983; Pratt et al., 1989).

The structure of DS (see Scheme 1) is largely represented by the monosulphated disaccharide repeating unit 1 (IdoA-GalNAc4SO₃), where IdoA is α -1,4-linked to the amino sugar, and GalNAc is β -1,3-linked to the uronic acid. However, like most glycosaminoglycans, DS is intrinsically heterogeneous, with some of its uronic acid residues being β -D-GlcA as in monosulphated unit 2 ($R = \text{H}$) (GlcA-GalNAc4SO₃) and with some of its IdoA or GalNAc4SO₃ residues bearing an extra sulphate group, as in the disulphated units 3 (IdoA2SO₃-GalNAc4SO₃) and 1' (IdoA-GalNAc4,6SO₃) (Fransson, 1985).

The structure of the sequences most represented in DS chains is shown in Scheme 1(a). The relative content of various sequences varies widely according to the animal species and organ source of DS (Fransson, 1985, 1987; Poblacion and Michelacci, 1986; Yoshida et al., 1988). In DS preparations from bovine intestinal mucosa, GlcA-containing sequences (2) usually account for only a few per cent of the total (Bossennec et al., 1990) and the disulphated sequences (3) and (1') for not more than 15% altogether. DS from pig skin (another common source of this glycosaminoglycan) contains larger amounts of GlcA than does mucosal DS (Bossennec et al., 1990).

Although oversulphated sequences, especially those containing 4,6-disulphated GalNAc residues, have been reported to potentiate the HC-II activity of DS (Sié et al., 1986; Munakata et al., 1987; Scully et al., 1988; Linhardt et al., 1991a), the HC-II and antithrombotic activities of different DS preparations did not correlate with their contents of either GalNAc4,6SO₃ or IdoA2SO₃ residues, the activities being apparently associated with the highest non-sulphated IdoA contents (Linhardt et al., 1991b). The catalytic efficiency of heparin saccharides on the HC-II-mediated inhibition of thrombin has been reported to be governed mainly by the overall charge and by the molecular size of the chain (Sié et al., 1988). A hexasaccharide containing only IdoA2SO₃ as oversulphated residue, obtained by nitrous acid

Abbreviations used: DS, dermatan sulphate; HC-II, heparin cofactor II; h.p.s.e.c., high-performance size-exclusion chromatography; s.a.x.-h.p.l.c., strong anion exchange h.p.l.c.; g.p.c., gel permeation chromatography; IdoA, L-iduronic acid; GlcA, D-glucuronic acid; IdoA2SO₃, L-iduronic acid 2-sulphate; GalNAc4SO₃, N-acetyl-D-galactosamine 4-sulphate; GalNAc6SO₃, N-acetyl-D-galactosamine 6-sulphate; GalNAc4,6SO₃, N-acetyl-D-galactosamine 4,6-disulphate; $\Delta\text{Di-OS}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -threo-hex-L-4-enepyransyluronic acid)-D-galactose; $\Delta\text{Di-6S}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -threo-hex-L-4-enepyransyluronic acid)-6-sulpho-D-galactose; $\Delta\text{Di-4S}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-enepyransyluronic acid)-4-O-sulpho-D-galactose; $\Delta\text{Di-2,6diS}$, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho- α -L-threo-hex-4-enepyransyluronic acid)-6-O-sulpho-D-galactose; $\Delta\text{Di-2,4diS}$, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho- α -L-threo-hex-4-enepyransyluronic acid)-4-O-sulpho-D-galactose; $\Delta\text{Di-4,6diS}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -threo-hex-L-4-enepyransyluronic acid)-4,6-bis-O-sulpho-D-galactose; $\Delta\text{Di-triS}$, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho- α -L-threo-hex-4-enepyransyluronic acid)-4,6-bis-O-sulpho-D-galactose.

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Scheme 1 Major disaccharide sequences of DS (a), the corresponding glycol-split polysaccharide (RO-DS) (b), and products of Smith degradation (SD-DS) (c)

Arbitrary sequences are shown. $n \approx 40-60$; 1, R = H; 1', R = SO₃⁻.

deamination of partially *N*-deacetylated DS, has been reported to represent the minimal sequence necessary for high-affinity binding to HC-II (Maimone and Tollefsen, 1990).

The present work aimed to study further the relative importance of different DS sequences in determining the interaction with HC-II. The disaccharide composition and HC-II activity were determined for a number of unfractionated DS preparations and for some charge-enriched fractions of a selected preparation. Another aim was to characterize more specifically the structure and activities of IdoA2SO₃-containing sequences of different length, both at the polymer level and as isolated fragments. For this purpose, all the C-2-C-3 bonds of DS were split with periodate. On reduction with borohydride, the resulting polymers incorporating the intact IdoA2SO₃-containing sequences (RO-DS) were either isolated or hydrolysed with mild acid to give fragments typically obtained from Smith degraded DS (SD-DS). Comparison of structural features and activities of DS, RO-DS and SD-DS confirmed the importance of the IdoA2SO₃-containing sequences in the expression of both HC-II-mediated inhibition of thrombin and the antithrombotic properties of DS.

EXPERIMENTAL

Materials

Materials were obtained as follows: papain (EC 3.4.22.2) from

Merck; SG15 spiralled membranes and equipment for inverse osmosis from Osmonic (Minnetonka, MN, U.S.A.) and Stemar (Milan, Italy); Maxatase proteolytic enzyme from Gist Brokades (Delft, The Netherlands); Dicalite from Dicalite Sud (Milan, Italy); Lewatit MP 5080 resin from Bayer; Ultrogel AcA54 and AcA202 and Trisacryl GF 0.5 M from IBF; chromatographic columns AK 50 50/100 and XK 50/690 from Pharmacia; 10 μ m Spherisorb from Phase Separation GB; Stachrom DS kit from Diagnostica Stago (Asnières sur Seine, France); Chondroitin ABC lyase (EC 4.2.2.4) and unsaturated chondrodisaccharide kit from Seikagaku Kogyo.

Male Sprague-Dawley rats (from Nossan) weighing 280-300 g were maintained on Altromin (Rieper) rat chow and water *ad libitum* and were housed in Makrolon cages.

Preparation of DS

DS preparations from bovine intestinal mucosa (designated BM-1 and BM-2) and one from pig intestinal mucosa (PM-1) were obtained as byproducts of heparin production. The ground intestinal mucosa (65 kg), NaCl (430 g) and papain (150 g) were added to water (75 l), adjusted to pH 6.3 with sodium acetate, heated to 60 °C and stirred for 6 h. The mass, heated to 78 °C for 10 min, was filtered through a press filter on a filter aid (dicalite). The filtrate was percolated through a chromatography column (10 cm $\times$ 80 cm) containing Lewatit MP5080 in Cl⁻ form. The

percolate was discarded and the resin washed with 12 l of 0.6 M NaCl solution, then eluted with 18 l of 1.8 M NaCl (flow rate 2.5 bed vol./h). The solution was collected and concentrated to 5 l and to 1 M NaCl by ultrafiltration and inverse osmosis on SG15 spiralled membranes (cut-off M_r 2000). Then 0.6 vol. of methanol was added to this solution which was left for 10 h at 5 °C. The precipitate was removed by filtration, and the filtrate was added to 7 l of methanol, producing a precipitate of crude DS, which was purified through calcium salt by conventional methods (Rodén et al., 1972). To remove contaminating heparin, [checked by ^1H -n.m.r. (Perlin and Holme, 1989)] the DS preparation was treated with NaNO_2 in acetic acid medium (Abildgaard and Larsen, 1984). A further purification, mainly to remove bacterial endotoxins, was achieved by treatment with potassium permanganate in neutral medium (Bush et al., 1964).

DS from pig skin (designated PS-1, PS-2, PS-3, PS-4, PS-5 and PS-6) was obtained as follows. Ground pig skin (100 kg) was added to 0.5 M NaCl (100 l). The pH was adjusted to 7.8 with NaOH before the addition of 400 g of proteolytic enzyme (Maxatase). The reaction mixture was stirred for 8 h at 60 °C, heated to 80 °C and centrifuged in a liquid-liquid-separating centrifuge. The fat phase (approx. 35 kg) was discarded. The aqueous phase was treated as the above-mentioned intestinal mucosa DS. Yields were 0.6–1.0 g of DS per kg of intestinal mucosa or skin.

Preparation of charge-density-fractionated DS

The DS preparation BM-2 was fractionated through strong anion-exchange resin (10 μm Spherisorb) on a semi-preparative h.p.l.c. column (7.5 mm $\times$ 300 mm). BM-2 elution (750 mg in 50 ml of water applied to the column) was performed at 1.5 ml/min with increasing NaCl concentration (0.1–2 M) in a discontinuous way. The fractions were desalted by dialysis and lyophilized. Yields for fractions recovered at 1.25, 1.5 and 1.75 M NaCl were respectively 15.8, 8.4 and 1.2 %, the remainder being recovered mainly at 0.1 and 0.25 M.

Preparation of RO-DS and SD-DS

The BM-1 preparation of DS (100 g) was dissolved in water (1 l) and 0.5 M NaIO_4 (800 ml) was slowly added. The solution, stirred for 4 h, was cooled in a thermostatically controlled water-bath to 10 °C and adjusted to pH 8 with 1 M NaOH. NaBH_4 (80 g) was added with stirring during 4 h, the pH being kept within 7.5–8.5 with dilute acetic acid. After being left overnight, the pH was adjusted to 4 with 8 M HCl and stirred for 1 h at room temperature. The pH was then adjusted to 5.5 with NaOH, and the product precipitated twice with 3 vol. of ethanol. After filtration and drying, RO-DS BM-1 (80 g) was obtained: sulphur, 5.9 %; uronic acids, 32.4 %; $[\alpha]_D^{20}$, -8° . Other analytical data are given in Table 2. Using the same procedure, RO-DS PS-5 (sulphur, 5.7 %; uronic acids, 34.8 %; $[\alpha]_D^{20}$ -15.2°) was obtained from pig skin DS-PS-5.

RO-DS BM-1, obtained as above (5 g in 50 ml of 1 M NaCl), was fractionated by gel permeation on Ultrogel AcA54 on a 5 cm $\times$ 84 cm column (flow rate 2 ml/min). The effluents were monitored with a u.v. detector (205 nm). Fractions of volume 20 ml were collected and divided into seven pools of about 160–200 ml each (RO-DS BM-1/1 to RO-DS BM-1/7). The homogeneous fractions from five runs were concentrated in a rotary evaporator to 80–120 ml, and the precipitated NaCl was removed by filtering. The solutions were adjusted to pH 5.3 with

acetic acid and added to 2 vol. of ethanol. The oily precipitate was redissolved in water and reprecipitated twice more with ethanol and acetone; the products obtained were desalted on a GF 05 M Trisacryl column (5 cm $\times$ 38 cm) at a flow rate of 5 ml/min. The solutions were concentrated and the product was precipitated again with ethanol and dried. All fractions were free of NaCl (AgNO_3 test).

Optimal conditions for Smith degradation were first found following the reaction course with RO-DS BM-1; 300 mg was dissolved in 0.1 M HCl (15 ml) in a sealed test tube in a water-bath at 60 °C. Samples of 2.2 ml were taken at 0.5, 1, 1.5, 2, 4 and 6 h. The solutions were neutralized with NaOH at pH 7, then analysed by h.p.l.c. RO-DS BM-1 (13.12 g) obtained as described above was then dissolved in 0.1 M HCl (656 ml) and heated for 2 h in a water-bath at 60 °C, with stirring. The solution was neutralized with NaOH and concentrated in a rotary evaporator to 70 ml and to about 1 M NaCl. It was then fractionated by gel filtration on Ultrogel AcA202 in a 5 cm $\times$ 90 cm column (flow rate 2 ml/min) using 1 M NaCl solution as the mobile phase. Fractions of volume 20 ml were collected, the void volume being discarded. Eluates were monitored by a continuous-flow-cell u.v. detector (205 nm). Fractions 13–30 (360 ml; A), 31–44 (280 ml; B), 45–60 (320 ml; C) and 61–67 (140 ml; D) were pooled. Pool A was concentrated to 55 ml and passed through a Trisacryl GF 05 M column (5 cm $\times$ 38 cm), producing fractions SD-DS BM-1/1 and SD-DS BM-1/2, which were collected and lyophilized (yields 0.2 % and 3.4 % respectively of the parent RO-DS BM-1). Fractions SD-DS BM-1/3 and SD-DS BM-1/4 (yields 3 % and 3.3 % respectively) were similarly obtained from pool B. Fractions from pool C (yield > 50 %), largely consisting of monosaccharides + 'remnant' as estimated by n.m.r. analysis (Mascellani et al., 1989), are not described here. Pool D consisted of salts only.

RO-DS BM-1/5 (300 mg) was dissolved in 0.1 M HCl (15 ml) and heated for 3 h in a water-bath at 60 °C. The solution, neutralized with NaOH, was added to ethanol (5.3 vol.) and left overnight at 4 °C. The precipitate was isolated by centrifugation, dissolved in 5 ml of water and reprecipitated twice more: first under the same conditions as above, and then with 5 vol. of ethanol. The compound designated GC 80/4 was obtained (yield 6.4 %).

Size fractionation and further ion-exchange fractionation of SD-DS

The DS preparation from pig skin (DS PS-5) was oxidized with NaIO_4 and reduced with NaBH_4 as described above. The corresponding RO-DS derivative (20 g) was hydrolysed with 0.1 M HCl (1 l) for 2 h at 60 °C. The solution was treated as described above for the SD-DS BM-1 fragments, by chromatography on Ultrogel AcA202, and the fractions obtained were desalted on 0.5 M Trisacryl GF. The oligosaccharide-containing eluates (n.m.r. analysis) were monitored also by periodical sampling and by M_r control on analytical high-performance size-exclusion chromatography (h.p.s.e.c.). Corresponding core fractions (SD-DS PS-5/5/a, SD-DS PS-5/5/b, SD-DS PS-5/5/c and SD-DS PS-5/5/d; see Table 2) containing oligosaccharides with mean M_r values (h.p.l.c. analysis) of 2800, 2200, 1800 and 1100 respectively were isolated. N.m.r. analyses (^1H and ^{13}C) of the oligosaccharides revealed the presence of glucuronic acid residues, whereas analysis of the constitutive disaccharides produced by chondroitin ABC lyase degradation still exhibited more than 20 % of $\Delta\text{Di-4S}$. The SD-DS PS-5/5/b fraction (100 mg; 5 mg/ml in water) was further fractionated by strong anion exchange (s.a.x.)-h.p.l.c. on a 10 μm Spherisorb column (7.5 mm $\times$ 300 mm) at discontinuously increasing

concentrations of NaCl and a flow rate of 1.5 ml/min. The void volume (7 ml) was discarded. Fractions of 25, 55, 30 and 8 ml (corresponding to elution peaks) were collected, desalted and freeze-dried. The following fragments were obtained: SD-DS PS-5/6/0M, 0.5 M, 0.75 M and 2 M (yields: 1, 0.9, 1.1 and 0.45 % of their parent DS PS-5).

Preparation of low- M_r DS fractions

Low- M_r DS fractions BM-1/1, BM-1/3, BM-1/4, BM-1/6, BM-1/7, BM-1/8, BM-1/9 and BM-1/11 were obtained by radical depolymerization with H_2O_2 and cupric salts as described by Volpi et al. (1992). Size fractionation of low- M_r DS BM-1/3 on AcA202 produced low- M_r DS BM-1/2, BM-1/5 and BM-1/10 (yields $\approx$ 12, 33 and 18 % respectively) with the following dispersion: 1.2, 1.1 and 1.2 respectively.

Determination of oligosaccharide size (M_r), SO_3^-/COO^- molar ratio and sulphur and uronic acid contents

H.p.s.e.c. was used. A Waters h.p.l.c. chromatograph (pump model 510, injector U6K), with Protein Pak 60 and 125 columns assembled in series (Waters code 085250 and 084601) or Protein Pak 125 and 300 (Waters code 80013) according to the M_r range to be measured, refraction index (Waters model 401) and u.v. (Waters model 490) detectors and an integrator for gel permeation chromatography (g.p.c.) data module (Waters model 730) were used. M_r values were determined as described by Volpi et al. (1992) using a third-order polynomial calibration curve obtained using commercial unsaturated disaccharide standards (Seikagaku), heparin oligosaccharide and glycosaminoglycan working standards, the M_r values of which had been determined by analytical ultracentrifugation (Nieduszinski, 1989).

The SO_3^-/COO^- ratio, sulphur and uronic acid contents were determined by potentiometry (Mascellani et al., 1988) with a Metrohm 682 titroprocessor.

H.p.l.c. quantification of constituent disaccharides

Results of the analysis (Volpi et al., 1992) were expressed as percentages of the total peak area that could be assigned to known disaccharides (here defined as disaccharide quantification recovery). Reference standard disaccharides were assumed to have the same molar absorption coefficient (Yoshida et al., 1988).

N.m.r. spectroscopy

1H -n.m.r. spectra were obtained at 500 MHz with a Bruker AMX 500 spectrometer equipped with 5 mm $^1H/X$ inverse probe. ^{13}C -n.m.r. spectra were obtained at 300 MHz with a Bruker AC 300 equipped with 5 mm $^{13}C/^1H$ dual probe. Samples (about 15 mg for proton and about 100 mg for carbon spectra) were solubilized in 2H_2O (0.5 ml; 99.99 % 2H). Carbon and proton spectra were recorded respectively at 25 °C and 60 °C. All proton shifts are referenced to internal 4,4'-dimethyl 4-silapentane sodium sulphate, and all carbon shifts are related to external trimethylsilylpropionate (sodium salt).

HC-II-mediated inhibition of Factor IIa activity

The HC-II-mediated inhibition of thrombin (Factor IIa) activity was measured by colorimetric assay with Stachrom DS kit on CBS 34.47 substrate. Unlike the original method (Dupouy et al.,

1988), in the present work bovine HC-II instead of human HC-II was used. The colorimetric response of the assay with bovine HC-II was 13 % less than that using human HC-II. All the assays were carried out by comparison of parallel curves. The A_{405} -log(dose) correlation was linear. The activity of DS BM-2, considered as the working standard, was expressed in units/mg and calculated from a standard curve obtained using the IVth International WHO Heparin Standard (193.4 i.u./mg). Curves of DS and heparin were parallel. The HC-II-mediated inhibition of Factor IIa activity by DS BM-2 corresponded to 235 ± 10 i.u./mg. The potencies of the other samples analysed were determined from the working standard DS BM-2. The confidence limit of the assay is ± 10 %.

Antithrombotic activity

The *in vivo* antithrombotic activity was evaluated by using a stasis-induced thrombosis model (Reyers et al., 1980). The DS samples (three doses per sample) were injected by the intravenous route 10 min before induction of thrombosis in rats by ligation with cotton thread of the inferior vena cava below the branch to the left renal vein. Rats were subdivided into groups of 10 animals each after being anaesthetized with an intraperitoneal injection of 1 g/kg urethane.

The thrombi that developed below the ligation were withdrawn from the vena cava after 2 h, washed with water, dried for 24 h and then weighed. The average weights of the thrombi from each group of animals were expressed as percentages of the average weight of thrombi from control animals (treated with normal saline). From the resulting values, the ED_{50} was calculated by a sigmoid dose (mg/kg)-effect (thrombus weight %) curve corresponding to the following Hill's modified formula (Nimmo and Bauermeister, 1977):

$$y = \frac{ax^b}{1 + ax^b}$$

RESULTS

Physicochemical and structural characterization

Unfractionated and charge-density-fractionated DS

Table 1 reports the charge density (expressed as SO_3^-/COO^- molar ratios) and M_r of the unfractionated DS preparations BM-1 and BM-2, PM-1 and PS-1 to PS-6, and of three charge-density-fractionated DS preparations (BM-2/1.25, BM-2/1.50 and BM-2/1.75). Whereas the SO_3^-/COO^- ratios for the unfractionated preparations range from 0.92 to 1.10, those for the charge-enriched fractions are higher (up to 1.19). The M_r values range from about 20000 to about 34000 for the various preparations and fractions.

Table 1 also shows the disaccharide composition (determined by h.p.l.c. after exhaustive digestion with chondroitin ABC lyase). For unfractionated DS preparations, the average content of the major unsaturated monosulphated disaccharide, $\Delta Di-4S$, is 87.4 % of the total disaccharides in the enzyme digests, with a minimum of 82.9 % (for PM-1) and a maximum of 90.6 % (for PS-1). This content decreases (to 76 %) for the charge-density-enriched fraction BM-2/1.75. The monosulphated disaccharide 6S is present at an average concentration of 2.9 %, with a peak value for preparation PM-1 (6.8 %). Both non-sulphated (0S) and 2,6-disulphated disaccharides are present at very low levels. The most frequently represented among the minor disaccharides is $\Delta Di-2,4diS$ (average of 7.0 % for unfractionated preparations, and up to 11.0 % for BM-2/1.75). The other common disulphated

Table 1 M_r , disaccharide composition, HC-II-mediated inhibition of thrombin and antithrombotic activity of natural and charge-fractionated DS

The $\text{SO}_3^-/\text{COO}^-$ ratios were obtained by potentiometry and the M_r values by size-exclusion h.p.l.c. The values for the disaccharides are based on h.p.l.c. analysis of chondroitin ABC lyase digests and are expressed as percentages of total disaccharides in the enzyme digests. Recovery exceeded 85% for all the samples. HC-II-mediated inhibition of thrombin activity is expressed as potency, referred to DS BM-2 = 1. n.d., not determined.

Code	SO ₃ ⁻ / COO ⁻ ratios	10 ⁻³ × <i>M_r</i>	ΔDi- (%)							HC-II	ED ₅₀ (mg/kg)
			0S	6S	4S	2,6diS	4,6diS	2,4diS	triS		
Unfractionated											
DS BM-1	1.03	21.0	1.5	2.9	86.3	0	1.4	7.9	0	0.85	1.4
DS BM-2	1.10	26.3	1.8	2.5	86.0	0.4	1.4	7.8	0.1	1.0	1.3
DS PM-1	1.03	33.9	0.9	6.8	82.9	0.2	5.6	3.5	0	0.96	2.1
DS PS-1	0.93	26.4	0.5	2.0	90.6	0	0	6.8	0	0.71	2.2
DS PS-2	0.92	27.3	2.2	4.0	85.2	0.6	0.3	7.7	0	0.78	n.d.
DS PS-3	0.93	23.6	1.4	3.8	87.7	0.3	1.1	5.7	0	0.71	2.0
DS PS-4	0.94	26.8	2.0	2.0	87.7	0.2	0.1	7.9	0.1	0.86	1.4
DS PS-5	1.04	27.7	1.6	1.2	90.0	0	0	7.2	0	0.92	1.5
DS PS-6	1.05	19.9	0.3	1.2	90.2	0	0	8.3	0	0.90	1.6
Charge-density-fractionated											
DS BM-2/1.25	1.13	20.0	0.5	1.7	85.4	0.5	1.2	10.4	0.2	1.63	n.d.
DS BM-2/1.50	1.19	23.5	0.7	2.5	81.8	0.5	2.8	10.9	0.8	2.04	1.28
DS BM-2/1.75	1.17	33.3	1.0	3.0	76.0	0.8	8.0	11.0	0.2	2.00	0.94

disaccharide (4,6diS) is present in significant amounts only in the DS preparation PM-1 (5.6%), actually being the major disulphated disaccharide in this preparation. The content of 4,6diS is also high (up to 8%) in the charge-enriched fraction BM-2/1.75. The trisulphated disaccharide (triS) is present at very low concentrations (0.1%) with some increase (up to 0.8%) in charge-enriched fractions.

As illustrated in Figure 1(a) for BM-2, the ^{13}C -n.m.r. spectra of all the DS preparations essentially consist of the 12 major signals attributable to sequence 1 (Sanderson et al., 1989; Holme and Perlin, 1989); the low-field C=O signals of carboxylate and acetamido groups are not considered in the present context. In addition, a set of weak signals, labelled with asterisks in Figure 1(a), is evident in the spectra of all the DS preparations and fractions, with somewhat higher intensity in the spectra (not shown) of charge-enriched fractions.

Glycol-split DS (RO-DS) and corresponding fragments (SD-DS)

The bovine mucosal (BM-1) and pig skin (PS-5) DS preparations were periodate-oxidized and borohydride-reduced, as indicated in Scheme 1 (Fransson and Carlstedt, 1974). The corresponding oxidized-reduced products (RO-DS BM-1/1 and RO-DS PS-5) were isolated with a relatively high yield (80%). The M_r values of the present preparation are always lower and have a wider distribution than those of the original DS (15200 compared with 21000 for BM-1 and 18000 compared with 27700 for PS-5), indicating that the oxidation (or reduction) reactions also involved some depolymerization.

On size fractionation of RO-DS BM-1 (on Ultrogel Aca54), seven fractions (RO-DS BM-1/1 to BM-1/7) were obtained, with M_r values ranging from 25700 to 5800 and with yields of 7, 20, 42, 4, 18, 2 and 2.8% respectively of RO-DS BM-1.

The ^{13}C -n.m.r. spectra of RO-DS BM-1 and all its fractions clearly show that the oxidation and reduction reactions occurred at the level of practically all the non-sulphated uronic acid residues (mostly IdoA) of the parent mucosal DS. In fact, as illustrated by the spectrum in Figure 1(b), the prominent ^{13}C

Figure 1 Comparison of ^{13}C -n.m.r. spectra of DS BM-2 (a), RO-DS BM-1/5 (b) and SD-DS BM-1/2 (c)

Asterisks denote the carbon atoms of IdoA2SO₃ residues.

signals associated with DS sequences 1 disappear, and are replaced by new signals attributable to sequences 4 of RO-DS.

In contrast, the asterisked minor DS signals show up practically unaltered in RO-DS and its fractions.

Table 2 M_r values, disaccharide composition, HC-II-mediated inhibition of thrombin and antithrombotic activity of RO-DS, RO-DS gel-chromatographic fractions and some SD-DS fragments

See Table 1 for further details. The disaccharide recovery of RO-DS ranged from 7.5 to 8.2%, except for that of RO-DS PS-5, which was 6.4%. The disaccharide recovery of SD-DS ranged from 5 to 10% of the Smith-hydrolysed product.

Code	SO ₃ ⁻ / COO ⁻ ratio	M_r	Δ Di- (%)							HC-II	ED ₅₀ (mg/kg)
			OS	6S	4S	2,6diS	4,6diS	2,4diS	triS		
RO-DS BM-1	1.10	15200*	13.1	4.8	2.0	0.9	1.9	75.7	1.6	0.99	—
RO-DS BM-1/1	1.04	25700	13.1	6.5	2.2	1.3	3.7	72.1	1.0	0.99	2.7
RO-DS BM-1/2	1.08	15600	11.6	0	3.2	1.1	1.4	80.8	1.8	1.07	—
RO-DS BM-1/3	1.11	11000	10.4	3.0	3.0	1.0	1.4	79.0	2.2	1.16	—
RO-DS BM-1/4	1.08	7900	7.7	2.1	1.6	1.6	0	86.9	0	1.32	2.6
RO-DS BM-1/5	1.09	6700	11.5	0.7	1.7	1.7	0.4	83.5	0.5	1.09	—
RO-DS BM-1/6	1.03	6000	7.8	0.8	1.0	0.5	0.5	89.2	0.2	0.86	—
RO-DS BM-1/7	1.03	5800	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.78	—
RO-DS PS-5	0.99	18000*	9.5	3.0	12.8	0	0	71.4	3.3	n.d.	—
SD-DS BM-1/1	1.92	3000	1.4	1.3	17.3	0.9	3.4	74.2	1.5	0.70	—
SD-DS BM-1/2	1.78	2900	0.4	0.6	7.7	0.6	0.6	89.6	0.4	0.34	2.1
SD-DS BM-1/3	1.76	2300	0.2	1.1	9.6	0.7	0.7	87.2	0.5	0.13	—
SD-DS BM-1/4	1.65	1700	0.7	3.0	17.9	1.6	2.4	74.4	0	0.02	—
SD-DS PS-5/5/b	1.44	2200	3.1	3.0	27.2	0.9	0	65.8	0	0.11	—
SD-DS PS-5/6/2M	1.74	2500	5.5	1.2	2.9	0.2	0	88.2	2	n.d.	—

* Broad distribution of M_r .

Figure 2 Kinetics of the Smith degradation reaction (0.1 M HCl, 60 °C)

M_r profile (on Protein Pak 60–125) of RO-DS BM-1 (A) and SD-DS after 0.5 (B), 1 (C), 2 (D) and 4 (E) h of hydrolysis. The marks on the retention time axis correspond to M_r values of (from right to left) 460, 1100, 1700, 2300, 2900 and 15000.

Under the present experimental conditions, periodate oxidation was less effective in splitting non-sulphated uronic acid residues of the DS preparation from pig skin (PS-5), the GlcA content of which [about 15%, as estimated by ^1H -n.m.r., (Bossennec et al. (1990))] is no longer negligible. In fact, the ^{13}C -n.m.r. spectrum of RO-DS PS-5 (not shown) indicates that about 20% of the original DS sequences remained unsplit. Under the present experimental conditions, D-GlcA-containing segments resisted glycol splitting (Fransson, 1974; Aspinall, 1982).

The physicochemical and compositional characteristics of RO-DS preparations from BM-1 and PS-5, and of the seven gel-permeation fractions of RO-DS BM-1, are reported in Table 2.

The disulphated disaccharide Δ Di-2,4diS is by far the major component (71.4–89.2%) of the disaccharide pools obtained by digestion with chondroitin ABC lyase of the RO-DS preparations and their fractions. However, a significant amount (12.8%) of the monosulphated disaccharide, Δ Di-4S, is still contained in unfractionated RO-DS PS-5. To find the optimal experimental conditions for hydrolysis of all the glycol-split uronic acid residues while not affecting the glycosidic bonds of (non-split) IdoA2SO₃ residues, the progress of Smith degradation (Fransson and Carlstedt, 1974) of RO-DS BM-1 was monitored by size-exclusion chromatography on Protein Pak columns.

The elution profiles of reaction mixtures after 0.5, 1, 2 and 4 h of reaction are shown in Figure 2, which also shows the profile of the parent RO-DS BM-1. Well-defined chromatographic peaks of oligosaccharide fragments show up already after 0.5 h hydrolysis. After 2 h, most of the RO-DS is converted into monosaccharides plus remnants (structures 6 and 7 of Scheme 1), identified by ^{13}C -n.m.r. (Mascellani et al., 1989).

After 2 h, some oligosaccharides containing non-glycol-split uronic acid residues also begin to be hydrolysed. The reaction time required to obtain the desired fragments was accordingly set at 2 h.

Two sets of SD oligosaccharides were obtained, the first one from bovine mucosal DS BM-1, and the second one from pig skin DS PS-5. The reduced–oxidized derivatives of the two DS forms were hydrolysed with mild acid under conditions of optimized Smith degradation (2 h), then fractionated on Ultrogel AcA202 (twice for the BM product, and after desalting with Trisacryl GF 05 M for the PS product); four fractions were obtained (a, b, c and d).

All fractions were analysed by ^1H - and ^{13}C -n.m.r. spectroscopy. The carbohydrate-containing fractions a, b and c from the BM products were desalted, and further fractionated by chromatography on Trisacryl GF 05 M; subfractions SD-DS BM-1/1 and BM-1/2 were obtained from fraction a, BM-1/3 and BM-1/4

Figure 3 H.p.l.c. elution profile of the digest of SD-DS PS-5/6/2M with chondroitin ABC lyase in comparison with disaccharide standards

Retention times (min): Δ Di-OS, 3.17 (A); Δ Di-6S, 10.86 (B); Δ Di-4S, 12.53 (C); Δ Di-2,6diS, 24.3 (D); Δ Di-4,6diS, 25.5 (E); Δ Di-2,4diS, 28.72 (F); Δ Di-TriS, 39.5 (G).

from fraction b, and c1, c2 and c3 from fraction c. The oligosaccharide-containing fraction b from the PS products was further fractionated by s.a.x.-h.p.l.c. on a Spherisorb column. Selected subfractions (SD-DS BM-1/1, BM-1/2, BM-1/3, BM-1/4 and SD-DS PS-5/6/2M, as well as the parent fraction of the latter, SD-DS PS-5) were characterized by n.m.r. spectroscopy

(^1H and ^{13}C), analysed for their $\text{SO}_3^-/\text{COO}^-$ ratios, M_r and percentage constituent disaccharides from chondroitin ABC lyase-cleavable sequences, and tested for their activity in HC-II-mediated inhibition of thrombin. For subfraction SD-DS BM-1/2, the antithrombotic activity was also determined (Table 2). The SD-DS subfractions contained in some cases greater than 80% Δ Di2,4diS, as revealed by the analysis of constituent disaccharides from chondroitin ABC lyase digests (Figure 3).

As shown for RO-DS BM-1/5 in Figure 1(b), asterisked signals become major signals in the ^{13}C -n.m.r. spectra of these fragments. The ^1H -n.m.r. spectra of the same fragments are relatively well-defined (see examples in Figure 4), and show prominent low-field signals (at about 5.1 p.p.m.) attributable to H-1 of IdoA2SO₃ residues in DS (Bossennec et al., 1990). As shown in Figure 5 for fragment SD-DS BM-1/2, complete assignment of signals associated with these residues was made by homo- and hetero-nuclear correlation spectroscopy. ^1H -n.m.r. spectra indicated that the only uronic acid in most of the fragments is IdoA2SO₃. Fragments obtained from pig skin DS, originally also contained GlcA [^1H -n.m.r. analysis as described by Bossennec et al. (1990)] which was removed by chromatography on a s.a.x. column, producing the GlcA-free fragment SD-DS PS-5/6/2M.

The M_r values for the SD-DS fragments range from 1700 to 3000. Assuming that IdoA2SO₃ is the only uronic acid in these fragments (at least for those from mucosal DS), these values correspond to oligosaccharides **8** (Scheme 1) where p is 2–4. Although they produce single symmetrical peaks on h.p.s.e.c., none of the isolated fragments consist of individual oligosaccharides. In fact, minor signals in the ^{13}C -n.m.r. and ^1H -n.m.r. spectra, as well as preliminary m.s. data (L. Silvestro,

Figure 4 Comparison of the ^1H -n.m.r. spectra of GC 80/4 obtained by Smith degradation (3 h; 60 °C) in 0.1 M HCl of RO-DS BM-1/5 and by fractionation with ethanol (a) and SD-DS BM-1/2 (b)

Asterisks denote the carbon atoms of IdoA2SO₃ residues.

Figure 5 Two-dimensional ^1H correlation spectrum via double quantum coherence of SD-DS BM-1/2

unpublished work) suggest some structural heterogeneity. As illustrated in Figure 4, the I_1 and I_5 signals of the fragments (at about 5.1 and 4.75 p.p.m. respectively) clearly consist of resonances from IdoA2SO₃ residues with two different environments. The area ratio of these signals is reversed on going from the smaller to the larger oligosaccharide [i.e. from GC 80/4 (approx. 1:1) to SD-DS BM-1/2 (approx. 1:2)], suggesting for these two fragments a p value of at least 2 for the former, and at least 3 for the latter, without excluding the possibilities of a substantial contribution of oligosaccharides with $p = 4$.

M_r -dependence of biological activities

The HC-II-mediated inhibition of thrombin and the antithrombotic activity of DS preparations and fractions, and of

RO-DS preparations and corresponding fractions and fragments, are given in Tables 1 and 2 respectively. The *in vitro* HC-II activity of unfractionated DS varies by a maximum of 15% from average values (i.e. the activity of BM-1), and practically doubles for the charge-enriched DS fractions.

The *in vivo* antithrombotic activity of the various DS preparations (ED_{50} 1.3–2.2) varies more than the *in vitro* activity, with an apparent increase in charge-enriched fractions ($\text{ED}_{50} = 0.94$ and 1.28). There is no clear correlation between antithrombotic activity (with the present model of vena cava ligation in rat) and HC-II-mediated inhibition of thrombin in the present DS preparations and fragments. In contrast, a proportional correlation was found between the antithrombotic activity, assayed by using the Wessler thromboplastin model in rabbits, and the catalytic activity, assayed with HC-II *in vitro*, of a DS hexadecasaccharide fragment with high affinity for HC-II, obtained by partial Smith degradation of DS (Sié et al., 1993). The red thrombi induced by the stasis as well as by endothelial damage and resulting phlogosis were mostly composed of fibrin and erythrocytes. The antithrombotic action of DS preparations in the experimental protocol adopted could be explained by inhibition of thrombin by HC-II and to a lesser extent by their direct inhibitory action on thrombin generation (Van Ryn-McKenna et al., 1989).

The present experimental model, although adequately expressing heparin-like antithrombotic activities, may underestimate the contribution of thrombolytic properties typical of DS (Van Ryn-McKenna et al., 1989). This could explain the absence, in our case, of a clear correlation between *in vivo* and *in vitro* activity.

The activities of RO-DS derivatives are, on average, somewhat higher than those of unmodified DS. On the other hand, HC-II activity falls dramatically with decreasing size of the SD fragments.

To compare the M_r -dependence of the activities of DS with unmodified internal structure with those of RO-DS (where most of the uronic acid residues are glycol-split), a number of low- M_r DS forms, prepared by radical-catalysed depolymerization of DS BM-1 and DS PS-1 were characterized in the same way as the DS, RO-DS and SD-DS samples. The analytical data (Table 3) and the ^{13}C -n.m.r. spectra (Volpi et al., 1992) of low- M_r DS indicate that these DS fragments substantially retained the internal structure of the parent DS. As shown in Table 3 and

Table 3 M_r , disaccharide composition, HC-II-mediated inhibition of thrombin and antithrombotic activity of low- M_r DS fragments

For details, see Table 1.

Code	$\text{SO}_3^-/\text{COO}^-$ ratio	M_r	$\Delta\text{Di} (\%)$							HC-II	ED_{50} (mg/kg)
			OS	6S	4S	2,6diS	4,6diS	2,4diS	triS		
DS BM-1/1	1.04	2.0	—	—	—	—	—	—	—	0.13	—
DS BM-1/2	0.98	4.2	2.6	3.7	80.5*	0.6	2.8	8.9	0.7	0.38	—
DS BM-1/3	0.97	5.0	1.1	2.4	84.7	0.2	1.6	9.7	0	0.45	—
DS BM-1/4	0.99	5.0	3.0	5.6	80.5*	0.2	5.9	4.8	0	0.26	—
DS BM-1/5	0.92	5.5	3.0	2.1	85.1	0.4	2.1	7.2	0.1	n.d.	2.7
DS BM-1/6	1.05	5.5	1.2	2.2	83.9	0.3	1.6	10.8	0	0.45	—
DS PS-1/7	0.92	6.0	3.0	3.4	85.3	0.4	0.5	7.4	0	n.d.	1.7
DS BM-1/8	0.98	6.2	1.2	2.3	85.4	0	2.9	8.2	0	0.37	1.6
DS BM-1/9	n.d.	7.2	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.38	—
DS BM-1/10	1.04	7.8	1.8	3.4	85.4	0.4	2.3	6.7	0	0.49	—
DS BM-1/11	1.08	10.3	1.2	2.5	86.1	0.7	1.1	7.8	0.6	0.51	—

* The enzymic digestion was incomplete for about 20% of the total owing to the incomplete cleavage of the DS fragments with low M_r , tentatively identified as tetra- and hexa-saccharides.

Figure 6 Correlation between M_r and HC-II-mediated inhibition of thrombin by DS and its fragments (⊠) and by RO-DS fractions (□)

HC-II-mediated inhibition of thrombin activity is expressed as potency, with that of DS BM-2 equal to 1.0

Figure 6, the HC-II potency increases with increasing M_r , levelling off for M_r values higher than 25000.

Among the low- M_r DS fragments, except for the oligosaccharide fragment low- M_r DS BM-1/1, which is too small, and the low- M_r DS BM-1/4, which contains only small amounts of 2,4-diS disaccharide, the mean value of the HC-II-mediated inhibition of thrombin is about 0.43. This value is about half of that of the native DS except for the samples poor in 2,4diS disaccharide (Tables 1 and 3, and Figure 6). In contrast, the activity of the reduced-oxidized fragments (Table 2 and Figure 6) is consistently higher than that of the 'normal' DS fragments of comparable M_r .

DISCUSSION

Oversulphated disaccharide sequences have been reported to account for a minor but significant part of the structure of mammalian chondroitin sulphates and DS (Fransson, 1985; Poblacion and Michelacci, 1986; Linhardt et al., 1991a,b). Although oversulphation of segments of DS chains was not always apparent from values of the $\text{SO}_3^-/\text{COO}^-$ molar ratios, h.p.l.c. of disaccharides obtained by exhaustive digestion with chondroitin ABC lyase indicated that disulphated disaccharide sequences account for up to 10% of the present DS preparations (Table 1). IdoA2SO₃-GalNAc4SO₃ is the major oversulphated sequence, accounting for 5.7–8.3% of eight of the present DS preparations. The ninth preparation (DS BM-1) contained only 3.5% of this (2,4diS) disaccharide, together with an unusually high proportion of 4,6diS (IdoA-GalNAc4,6SO₃), the latter accounting for only 0–1.4% of the total disaccharides of the other eight preparations. No clear difference in sulphation patterns was evident between DS from mucosa (bovine or pig) and pig skin. As previously reported (Bossennec et al., 1990), the latter differ from mucosal preparations mainly in having a higher GlcA content (15–20% compared with 1–3%).

Both the $\text{SO}_3^-/\text{COO}^-$ ratios and contents of oversulphated disaccharides increased (up to about 1.2 and 20% respectively) in charge-enriched fractions of DS BM-2. Interestingly, such an increase is essentially due to the contribution of 4,6diS disaccharides, with the 2,4diS disaccharide content remaining essentially constant (Table 1).

Signals associated with 2,4-disulphated disaccharide sequences were identified in the ¹³C-n.m.r. spectra of the unfractionated DS preparations (Figure 1a). These signals became prominent in oligosaccharide fragments (SD-DS) obtained by mild acid hydrolysis of RO-DS. SD-DS fragments tightly retained on HC-II-affinity columns were shown by ¹H-n.m.r. to be enriched (up to 60%) in IdoA2SO₃, the smallest fragment with full HC-II activity being a hexadecasaccharide (Sié et al., 1993). The smallest SD-DS fragments in the present work (penta- to nona-saccharides plus remnants) contained 2-*O*-sulphated iduronic acid (IdoA2SO₃) as the only uronic acid. Recovery values of these SD-DS fragments accounted for practically all the IdoA2SO₃-containing disaccharides (7–8%) in the original unfractionated DS. As shown by Tollefsen (1992) for pig skin DS, about 80% of the DS chains contain at least one HC-II-binding site. The HC-II-binding sites should accordingly be composed of at least four disulphate disaccharides, unless some of the DS chains contain two, somewhat shorter, IdoA2SO₃-containing sequences.

Although Table 1 indicated a qualitative correlation between the HC-II-mediated inhibition of thrombin and 2,4-disulphate disaccharide content for unfractionated unmodified DS, a significant contribution to the activity by 4,6-disulphated sequences also cannot be ruled out. The latter were shown to greatly enhance the HC-II activity for natural oversulphated non-mammalian DS (Seno and Murakami, 1982; Scully et al., 1988) as well as for chemically oversulphated chondroitin sulphates and DS (Sié et al., 1986; Ogamo et al., 1990). Indeed, these sequences may account for a substantial part of the HC-II activity of DS PM-1. However, 2,4diS sequences appear to be more effective than 4,6diS sequences in eliciting the activity of charge-enriched BM fractions, the activity of fraction BM-2/1.75 being essentially the same as that of fraction BM-2/1.50, in spite of the much larger content of 4,6diS sequences in the former.

The important role of 2,4diS sequences as well as the non-critical role of non-sulphated unmodified IdoA residues is clearly indicated by the data in Table 2, showing retention of the HC-II activity on splitting of all the C-2–C-3 bonds of unsubstituted uronic acids of DS. In reduced-oxidized derivatives, as well as in SD fragments, the 2,4diS disaccharides become the major components of chondroitin ABC lyase digests (Figure 3), in accordance with an increase in intensity observed for ¹³C-n.m.r. signals associated with 2,4diS sequences before enzyme treatment (Figure 1c). As DS segments containing glycol-split uronic acid residues are not cleaved by lyases, 2,4diS disaccharides are the major components of chondroitin ABC lyase digests of RO-DS (Table 2).

A significant HC-II-mediated inhibition of thrombin requires chains longer than the actual minimal binding site of DS for HC-II, because of the need to form a DS–HC-II–thrombin complex (Tollefsen, 1992). The HC-II activity of the oligosaccharides is about half of that of the native DS. Even the length of the DS chain, as well as the oversulphated sequence, contributes to the thrombin inhibition.

However, whereas the activity of 10000- M_r DS is about half that of DS preparations or fractions of $M_r > 20000$, for RO-DS a significant activity decrease is observed only for fractions of $M_r < 6000$. The overall activity of RO-DS is indeed higher than that of DS preparations and fractions of comparable molecular size. Below M_r approx. 3000, the activity is very low for all types of fractions and fragments (Figure 6). Retention of activity by RO-DS indicates that unmodified IdoA residues, as well as a precise spacing between their carboxylate groups and the sulphate groups of the adjacent GlcNAc4SO₃ residues, are not strictly required for the HC-II-mediated inhibition of thrombin. On account of

Figure 7 Segments of DS chains incorporating an IdoA2SO₃-containing active site for HC-II before (a) and after (b) periodate oxidation/borohydride reduction

Arrows indicate points of cleavage of RO-DS with mild acid.

the conformational flexibility of IdoA residues and the expected energy-efficient adaptation of the shape of these residues for optimal binding to basic groups in receptors (Casu et al., 1988), a precise charge spacing could actually not be essential. Indeed, glycol-split uronic acid residues are expected to be even more flexible than unmodified IdoA residues (Casu, 1990), and such extra flexibility probably contributes to the overall activity of RO-DS. As shown in Figure 7, glycol splitting provides a method for providing extra flexibility for chains extending from the 'active' IdoA2SO₃-containing sequences, as well as, by mild acid hydrolysis at the level of the split residues, of 'concentrating' these active sequences in SD fragments.

Although they confirm some relationships between the HC-II activity *in vitro* and the antithrombotic activity *in vivo*, the present data are insufficient to establish a precise correlation between the two activities, probably because the antithrombotic activity of DS is also associated with the HC-II-mediated inactivation of thrombin generation (Ofosu et al., 1989). However, the present results indicate that the 2,4-disulphated disaccharide sequences are also important in the expression of antithrombotic activity.

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