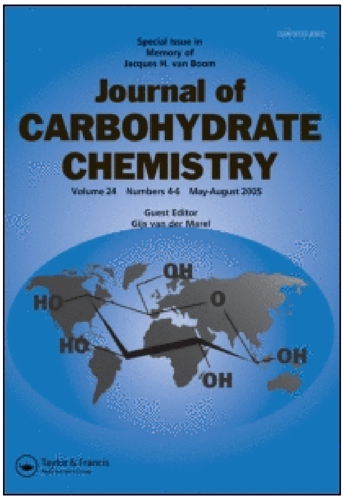


Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Active Sites of Dermatan Sulfate for Heparin Cofactor II. Isolation of a Nonasaccharide Fragment Containing Four Disaccharide Sequences [α-l-Iduronic Acid 2-*O*-Sulfate (1,3)-β-d-*N*-Acetylgalactosamine 4-Sulfate]

G. Mascellani[a]; L. Liverani[a]; A. Prete[a]; G. L. Bergonzini[a]; P. Bianchini[a]; L. Silvestro[b]; G. Torri[c]; A. Bisio[c]; M. Guerrini[c]; B. Casu[c]
[a] Opocrin S.p.A. R. & D. Laboratories, Modena, Italy [b] Res Pharma Pharmacological Research S.r.l., Turin, Italy [c] G. Ronzoni Institute for Chemical and Biochemical Research, Milan, Italy

To cite this Article Mascellani, G. , Liverani, L. , Prete, A. , Bergonzini, G. L. , Bianchini, P. , Silvestro, L. , Torri, G. , Bisio, A. , Guerrini, M. and Casu, B.(1995) 'Active Sites of Dermatan Sulfate for Heparin Cofactor II. Isolation of a Nonasaccharide Fragment Containing Four Disaccharide Sequences [α-l-Iduronic Acid 2-*O*-Sulfate (1,3)-β-d-*N*-Acetylgalactosamine 4-Sulfate]', Journal of Carbohydrate Chemistry, 14: 8, 1165 — 1177
To link to this Article: DOI: 10.1080/07328309508005402
URL: http://dx.doi.org/10.1080/07328309508005402

J. CARBOHYDRATE CHEMISTRY, 14(8), 1165-1177 (1995)

ACTIVE SITES OF DERMATAN SULFATE FOR HEPARIN COFACTOR II. ISOLATION OF A NONASACCHARIDE FRAGMENT CONTAINING FOUR DISACCHARIDE SEQUENCES [α-L-IDURONIC ACID 2-*O*-SULFATE (1,3)-β-D-*N*-ACETYLGALACTOSAMINE 4-SULFATE]

G. Mascellani,[a] L. Liverani,[a] A. Prete,[a] G. L. Bergonzini,[a] P. Bianchini,[a] L. Silvestro,[b]
G. Torri,[c] A. Bisio,[c] M. Guerrini[c] and B. Casu[c]

a Opocrin S.p.A. R. & D. Laboratories, Corlo di Formigine, Modena, Italy.
b Res Pharma Pharmacological Research S.r.l., Turin, Italy.
c G. Ronzoni Institute for Chemical and Biochemical Research, Milan, Italy.

Received November 25, 1994 - Final Form July 17, 1995

ABSTRACT

The active site of dermatan sulfate (DS) for heparin cofactor II (HCII) was isolated in a fragment obtained by periodate oxidation, borohydride reduction, mild acid hydrolysis, and SE- and SAX-chromatography of beef mucosal and pig skin DS preparations. Characterization by mass spectrometry, one- and two-dimensional NMR spectroscopy, and HPLC analysis of disaccharides, obtained by exhaustive digestion with chondroitinase-ABC, indicates that the fragment has the prevalent structure **1**, GalNAc-4SO_3-[IdoA-2SO_3-GalNAc-4SO_3]$_4$-R, where R is $CH(CH_2OH)CH(COO^-)$-OH. **1** is the largest DS fragment thus far isolated containing IdoA2SO_3 as the only uronic acid. Its lower activity (30%) with respect to the parent polymeric DS is explainable by Tollefsen model, requiring longer polyanionic chains for formation of ternary complex with thrombin.

INTRODUCTION

Dermatan sulfate (DS)[1] is a glycosaminoglycan with anticoagulant, pro-fibrinolytic and antithrombotic properties, associated, at least in part, with its capability to inhibit thrombin by potentiating heparin cofactor II (HCII).[2] The structure of DS is largely accounted for by the repeating disaccharide sequences [IdoA-GalNAc4SO_3], where IdoA is α-L-iduronic acid and GalNAc4SO_3 is *N*-acetyl-β-D-galactosamine 4-*O*-sulfate, linked 1,3 and 1,4, respectively. The activation of HCII, however, is mainly associated with minor, oversulfated sequences, which, in beef mucosal DS and in pig skin DS, are constituted of the disaccharide IdoA2SO_3-GalNAc4SO_3. The longest of these sequences so far isolated as a structurally homogeneous fragment contained at least three IdoASO_3 residues.[3,4]

The aim of the present work was to isolate and characterize the longest DS fragment containing IdoA2SO_3 as the only uronic acid residue. Controlled Smith degradation of IO_4^- oxidized and $NaBH_4$ reduced DS from beef mucosa (BM) and pig skin (PS) gave fragments that were separated by gel filtration. The fragments obtained from pig skin DS, structurally more heterogeneous because of the higher GlcA content in the parent DS, were further fractionated by ion-exchange HPLC. The longest fragments were characterized by HPLC analysis of digests with chondroitinase ABC, mass spectrometry and one- and two-dimensional NMR spectroscopy by complete assignment of ^1H and ^{13}C NMR signals through heteronuclear correlation methods.[5] Its activity on HCII was compared with that of the parent DS and with that of less sulfated fragments of different MW.

RESULTS AND DISCUSSION

BM- and PS-DS preparations were treated with periodate, reduced with borohydride and cleaved with mild acid as previously reported.[4] Fragments (typically consisting of GalNAc4SO_3[UA2SO_3-GalNAc4SO_3]$_n$R, where UA is either GlcA or - most frequently - IdoA, and R is the remnant of a glycol split UA) were fractionated as a function of size and charge density as described in the Experimental Part. Fractions eluted at the suitable size (BM-A2) and at the highest ionic strenght (PS-A2/2) were characterized in more detail. Their SO_3^-/COO^- molar ratios were 1.78 and 1.77, respectively (theoretical value for a nonasulfated nonasaccharide + R = 1.8).

Over 80% (wt) of fraction BM-A2 was cleaved by chondroitinase ABC. HPLC analysis of the constitutive disaccharides in the digests indicated a ΔDi-2,4-diS content

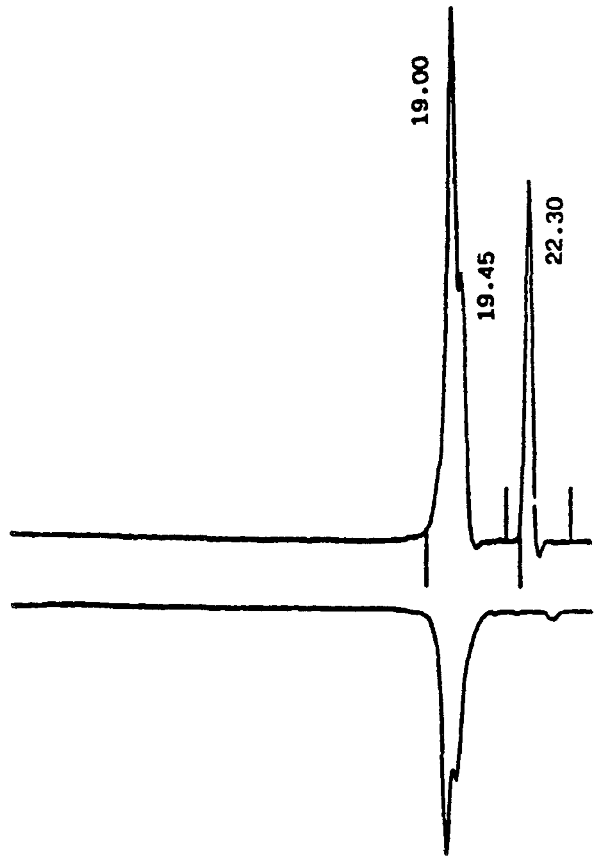


Figure 1. SE-HPLC profile of fraction BM-A2 (upper trace:RI detection; lower trace:UV detection) the peaks are nonasaccharide (RT 19.00), heptasaccharide (RT 19.45) and salt at 22.30 RT in the RI trace.

higher than 89%, with percent contents (wt) of the other disaccharides ΔDi-OS, ΔDi-6S, ΔDi-4S, ΔDi-2,6-diS, ΔDi-4,6-diS, ΔDi-triS of 0.4, 0.6, 7.7, 0.6, 0.6 and 0.4, respectively. The SE-HPLC profile of fraction BM-A2 (Fig. 1) consisted of two peaks corresponding to MW 2830 and 2300 Da. The smaller peak represented about 30% of the total.

The HPLC/MS traces (not shown) consist of three only partially resolved HPLC peaks (estimated molecular weights: 2036.8, 2494.4 and 2574.4 Da), corresponding to calculated MWs (free acid form) of a heptasulfated heptasaccharide, an octasulfated nonasaccharide and a nonasulfated nonasaccharide, respectively. Chromatograms obtained with tetra-propylammonium hydroxide in the mobile phase showed peaks at *m/z* values 1479.9, 1139.0 and 1288.8. These ions correspond to the $(M + 5TPA - 7H)^{-2}$ adduct of a compound with the MW of the heptasulfated heptasaccharide+R, to the $(M + 5TPA - 8H)^{-3}$ adduct of a molecule with the MW of the octasulfated nonasaccharide + R and to the $(M + 5TPA - 10H)^{-3}$ adduct of a molecule with the MW of the nonasulfated nonasaccharide + R, respectively. Interpretations of MS spectra were made comparing these results with those obtained from corresponding compounds as deuterated tetrapropylammonium salts.[6] Relative signal intensities of the main ions for each oligosaccharide (data not reported) indicated a nonasulfated nonasaccharide to be by far the major component, heptasulfated heptasaccharide, octasulfated nonasaccharide and nonasulfated nonasaccharide accounting for approximately 30%, 10% and 60% of fraction BM-A2.

Proton and carbon NMR chemical shifts of major signals are shown in Tables 1 and 2.

Major ^{1}H and ^{13}C NMR signals of the nonasaccharide fraction (Tables 1 and 2) were assigned by a combination of one- and two-dimensional techniques.

Signal assignment for the "internal" uronic acid and amino sugar residues (I and A) was straightforward, through homo- and heteronuclear correlation plots such as COSY and TOCSY (not shown) and HMQC[7] (Fig. 2A). Assignments reported in Fig. 2A include those for the remnant residue (R) and some for the amino sugar residue to which R is linked (A_R), obtained with the HMQC-COSY and HMBC[5] techniques (partial plots shown in Fig. 2B and 2C, respectively). The HMQC-COSY[8] technique permitted us to resolve closely spaced signals such as those from A_2 and $A_{2\text{-}R}$ (but not those of $A_{3\text{-}R}$ to $A_{6\text{-}R}$, almost superimposable on those of internal A residues) and the HMBC technique enabled us to correlate signals of protons and carbons (such as $A_{1\text{-}R}$ and R_4) across glycosidic bridges. The ^{1}H and ^{13}C chemical shifts of the amino sugar residue at the nonreducing end (Anr) are significantly different from those of the internal A residues. Also the ^{1}H chemical shifts of residue designated I_{nr} ($IdoA2SO_3$ linked to the $GlcNAc4SO_3$ residue at the nonreducing end, Anr) are different from those of the corresponding internal residues (signals from I_{nr} were distinguished from those of I essentially on the basis of their relative intensity).

^{13}C signals of I_{nr} were indistinguishable from those of I. No signals attributable to nonsulfated IdoA residues were detected in the spectra.

Table 1. ^{1}H chemical shifts of nonasaccharide fragment[a]

	A (internal)	Anr	AR	I (internal)	I(nr)	R
H1	4.71	4.65	4.62	5.17	5.15	-
H2	4.02	3.82	4.03	4.17	4.17	-
H3	4.08/4.06	3.87	4.08/4.06	4.21	4.19	4.77-4.68
H4	4.66/4.62	4.66/4.62	4.66/4.62	4.06	4.06	4.12
H5	3.86/3.80	3.86/3.80	3.86/3.80	4.82	4.80	4.01
H6	3.81	3.81	3.81			
H6'	3.74	3.74	3.74			

a. Given in ppm downfield from internal sodium-3-(trimethylsilyl)propionate.

Table 2. ^{13}C chemical shifts of nonasaccharide fragment[a]

	A (internal)	Anr	AR	I (internal)	I(nr)	R
C1	103.9	104.2	102.7	101.6	101.6	-
C2	53.0	54.0	53.0	73.8	73.8	-
C3	77.1	71.5	77.1	69.1	69.1	63.1
C4	77.0	77.0	77.0	79.3	79.3	83.2
C5	75.7	75.7	75.7	68.2	68.2	72.7
C6	62.1	62.1	62.1			

a. Given in ppm downfield from internal sodium-3-(trimethylsilyl)propionate.

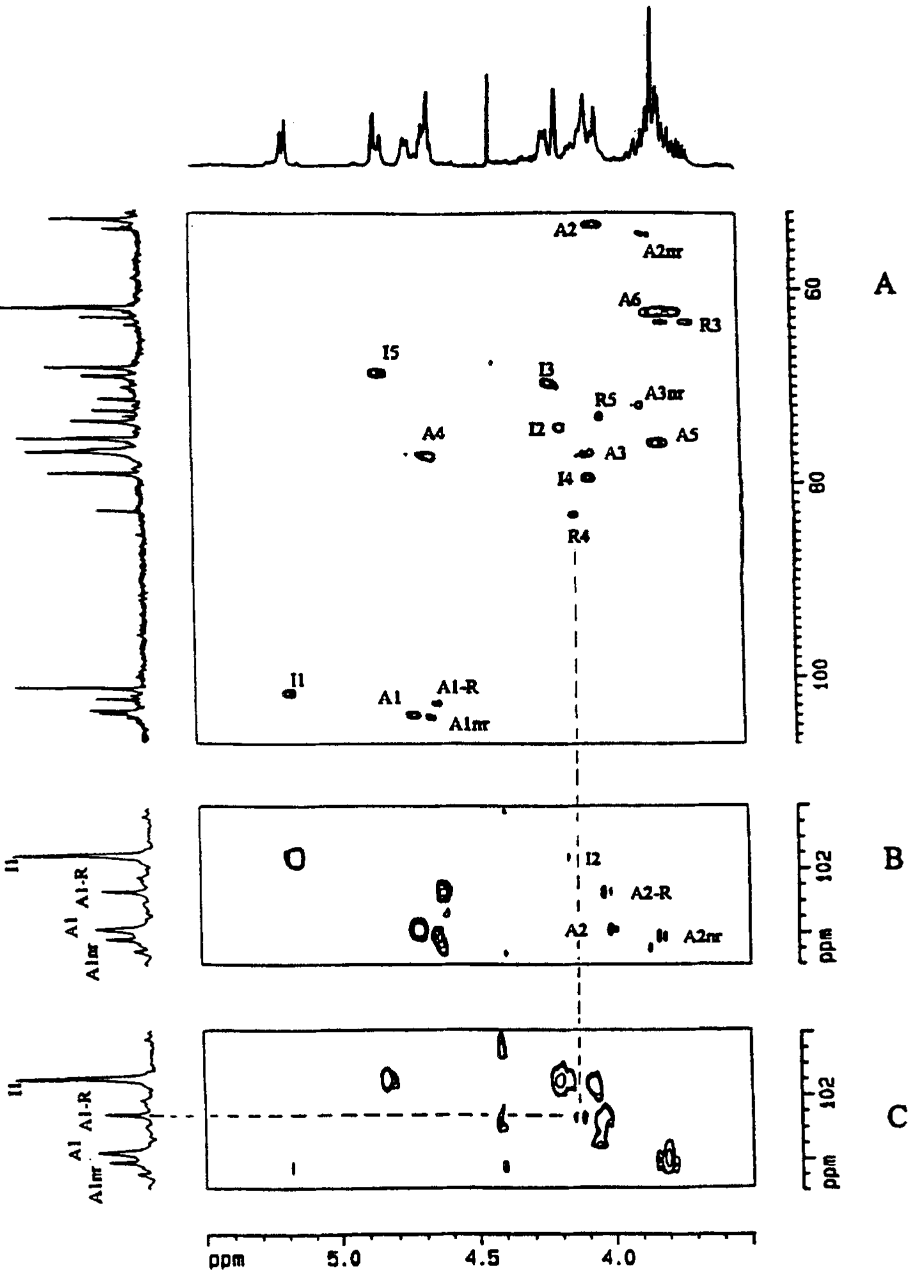
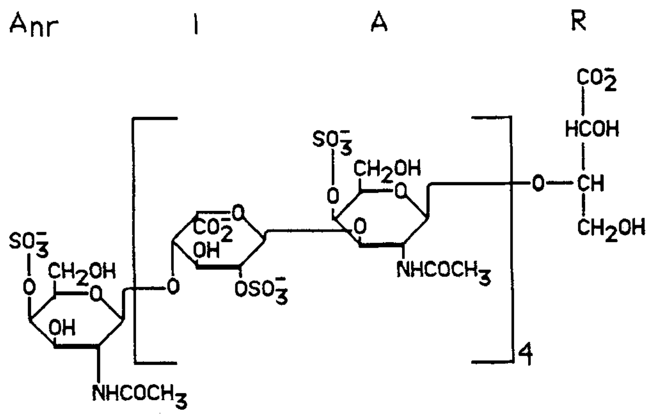


Figure 2. Two-dimensional NMR spectra of the nonasaccharide fraction (BM-A2) : (A) ^{1}H-^{13}C multiple quantum coherence shift correlation spectrum (HMQC). (B) 2D heteronuclear relayed coherence transfer partial spectrum (HMQC-COSY), with assignment for anomeric signals. (C) ^{1}H-^{13}C multiple bond shift correlation spectrum (HMBC), showing correlations between proton 4 of the remnant residue (R_4) and carbon 1 of galactosamine to which R is linked ($A_{1\text{-}R}$). The corresponding one-dimensional ^{1}H and ^{13}C spectra are shown at the top and at the left side of the two-dimensional spectra, respectively.

Integration of relevant signals in the low-field region of the ^1H NMR spectrum (Fig.3) indicates that the ratio between GalNAc4SO$_3$ and IdoA2SO$_3$ residues is approximately 5:4. Essentially the same value was obtained from the area ratio (15/14.34=1.05) of *N*-acetyl signals at 2.01-2.06 ppm and the sum of signals I$_5$, A$_1$, A$_1$-R, A$_{1nr}$, A$_4$, A$_4$-R, and A$_{4nr}$, at 4.6-4.9 ppm (theoretical ratio for the nonasaccharide + R = 15/14=1.07).

Combination of data obtained by HPLC/MS spectroscopy, NMR spectroscopy, HPLC analysis of constituent disaccharides and quantitation of ^1H NMR spectrum signals of SD-DS fraction indicates that the prevalent fragment in fraction BM-A2 is the nonasulfated nonasaccharide **1**, containing 2-*O*-sulfate iduronic acid as the only uronic acid. Similar results were obtained for fraction PS-A2/2 prepared from pig skin DS (see Experimental).



1

Sequences **1** are essential to the DS activity. In fact, their removal from DS causes a fall in DS activity on HCII.[9] Enrichment in these sequences by means of SAX-chromatography, involves an increase[10] in DS activity on HCII. Nonasaccharide **1** has an activity on HCII of 0.30, compared to 1.0 of the parent DS. The lower activity of the oligosaccharide as compared with the polysaccharides is explainable in terms of the known MW-dependence of the HCII activities. As suggested by Deerlin and Tollefsen,[11] DS chains longer than the actual binding site to HCII are essential for the interaction with both HCII and thrombin. Nonasaccharide **1** thus probably can still bind to the basic DS-binding domain of HCII, but it is not long enough to bind also to thrombin. The relationship between MW and the HCII-mediated inhibition of thrombin by the nonasaccharide and by

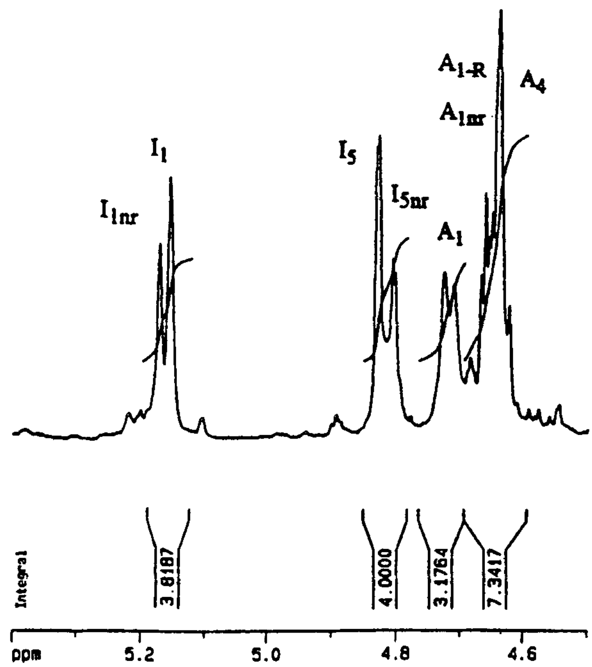


Figure 3. Anomeric region of the proton spectrum of the nonasaccharide fraction (BM-A2), with integration of relevant signals.

polysaccharides of different chain length, for a number of DS and DS fractions and fragments with the usual content of active sequences, is shown in Fig.4. Data from a DS and a LMW-DS, depleted of these sequences, are included as references. The aforesaid LMW-DS sample (C7/0.1) has a potency of 0.07 on HCII versus the DS BM-2, considered as a reference equal to 1. This fraction has a very low content in disulfated disaccharides (IdoA2SO_3-GalNAc4SO_3= 1.7%; IdoA-GalNAc4,6SO_3=1.3% the remaining part being IdoA-GalNAc6SO3=4.2%, IdoA-GalNAc=1.0% and IdoA-GalNAc4SO_3= 91.7%). These results confirm the correlation between MW and HCII activity for DS fractions and fragments containing IdoA2SO_3 residues.

EXPERIMENTAL

General Methods. The determinations of the molecular weight by SE-HPLC on TSK G2000 SWXL column (Toso Haas), of the SO_3^-/COO^- molar ratio by potentiometry, of

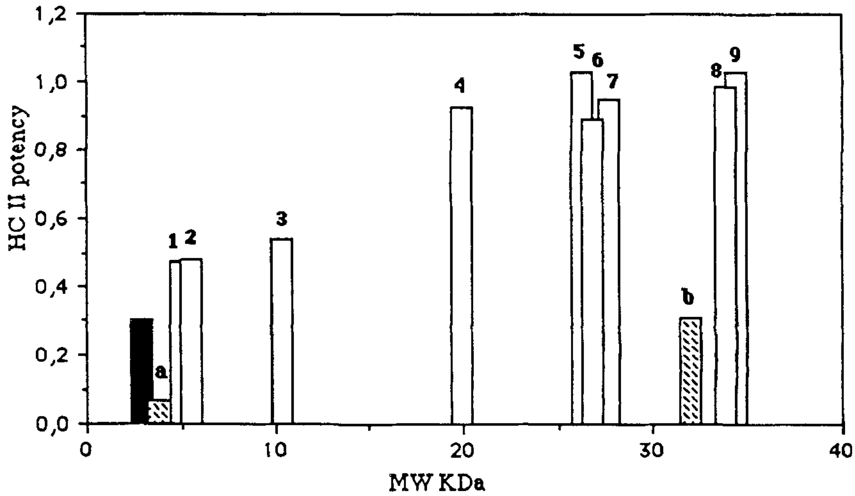


Figure 4. Correlation between MW(KDa) and HCII-mediated inhibition of thrombin by DSs and their fragments. ■ Nonasaccharide + R. ▧ Samples nearly depleted of oversulfated sequence; **a** : LMW-DS C7/0.1; **b**: DS PM-3 (Ref.9). □ LMW-DSs (obtained by radical depolymerization) and naturally occurring DSs;
1 : BM-1/15 (Ref.2); **2** : BM-1/6 (Ref.4); **3** : BM-1/11 (Ref.4);
4 : PS-6 (Ref.4); **5** : BM-2 (Ref.4); **6** : PS-4 (Ref.4);
7 : PS-5 (Ref.2); **8** : PM-1 (Ref.4); **9** : PM-2 (Ref.9).

the constitutive disaccharides by HPLC of the digests by ABC lyase, and of the HCII-mediated inhibition of Thrombin (Factor IIa) activity were performed as previously described.[4]

The HPLC separations for the mass spectroscopic (HPLC-MS) analyses were carried out with an Applied Biosystem 140A syringe pump equipped with a butyl reverse-phase microbore column (Hypersyl WP-Butyl 5 μm 250*1 mm). This system was interfaced to a mass spectrometer API III Serex by means of an ionspray source. The chromatographic separations were performed with a binary mobile phase gradient consisting of: A) 3.3 mM tetrapropylammonium hydroxide in water at pH 4.0 with formic acid; B) 3.3 mM tetrapropylammonium hydroxide in acetonitrile:water (90/10 v/v) at pH 4.0 with formic acid. The gradient program started from 100 % A then linearly decreased to 50 % A in 24 min and to 40 % A in other 16 min; the flow rate was 50 μL/min. Another series of HPLC/MS analyses was carried out under the same conditions but using a mobile phase buffer prepared with fully deuterated tetrapropylammonium hydroxide. This approach, with the two counterions, was used, as already described,[6] to identify the molecular ions from the sulfated oligosaccharides. The MS spectra were obtained by scanning negative ions in the range *m/z* 300-1800.

The ^{1}H NMR spectra were obtained at 500 MHz with a Bruker AMX 500 spectrometer equipped with 5 mm ^{1}H/X inverse probe. The sample (20 mg) was dissolved in D_2O (0.5 mL, 99.99 D%). Chemical shifts are given in ppm downfield from internal sodium-3-(trimethylsilyl)propionate at 25 °C. The spectra were obtained with presaturation of the HDO signal. COSY45 data were acquired using 32 scans per series in 1K x 512W data points with zero-filling in F1. A sine-bell function was applied before Fourier transformation. Two dimensional TOCSY spectra were measured in the phase sensitive mode using the TPPI (Time Proportional Phase Incrementation) with a mixing time of 75 ms. The spectra had 1K x 256W (F2 x F1) and before processing were zero filled to 2K x 512W; a squared sine-bell function was applied before Fourier transformation. The ^{1}H-^{13}C heteronuclear multiple-quantum coherence (HMQC)[7] and 2D heteronuclear relayed coherence transfer experiments (HMQC-COSY)[8] were made using 48 and 64 scans respectively. A matrix of 1K x 256W data points was applied using squared sine-bell functions prior to Fourier transformation.

Preparation of nonasaccharide+R from reduced-oxidized DS (RO-DS) and Smith-degraded DS (SD-DS).

RO-DS. DS (BM-1), obtained from beef mucosa and purified as previously described,[4] was dissolved (100 g/1000 mL) in water; a 0.5M $NaIO_4$ solution (800 mL) was slowly added. Four hours later the solution was cooled to 10 °C and its pH adjusted to 8. $NaBH_4$ (80 g) was added in small amounts over four hours at constant temperature (<=10 °C) and constant pH (8±0.5, by addition of 25% acetic acid). After a night's rest, the pH was adjusted to 4 with HCl and the solution stirred for 1 hour at room temperature. The pH was adjusted back to 5.5 and the product was precipitated twice with three volumes of ethanol. After filtration and drying, RO-DS BM-1 was obtained with an 80% yield.

Nonasaccharide + R. Nonasaccharide + R from beef mucosa (BM) and pig skin (PS) were prepared by Smith degradation (SD-DS) of the parent reduced-oxidized DSs.[4]

a. **From beef mucosa DS** (BM-A2) (Fig. 5a). A sample of RO-DS from DS BM-1 in 0.1 N HCl (13.12 g/656 mL) was hydrolyzed for 2 hours at 60 °C. The solution was neutralized with NaOH and concentrated to 1 M NaCl. The hydrolysis products were size-fractionated on Ultrogel AcA 202 (IBF, France; 5 x 90 cm column; eluent 1 M NaCl;

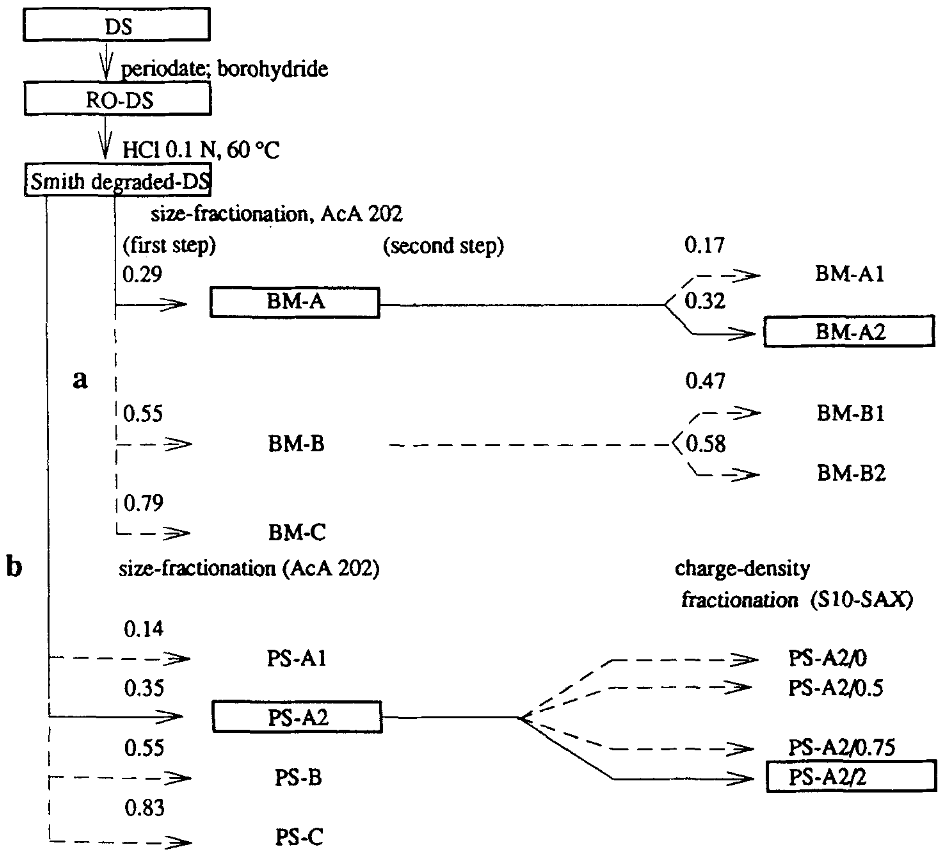


Figure 5. Scheme of degradation of DS, and fractionation of oligosaccharides from beef mucosa (**a**) and from pig skin (**b**). The figures above the arrows are the Kav values of each fraction.

flow rate 2 mL/min; 10 mL fractions). UV monitoring (254 nm) of eluate showed a single, broad and irregular peak with a maximum (Kav 0.90) due to products of Smith degradation.The void volume (400 mL) was discarded; fractions were sampled and molecular weight of oligosaccharides determined by SE-HPLC. Fractions containing oligosaccharides of comparable MW were pooled, Fig.5a. Three pools were collected: BM-A, BM-B, BM-C, constituted by fractions selected from fraction to fraction as follows and having Kav respectively: 17^{th}-52^{nd}, 0.29; 53^{rd}-79^{th}, 0.55; 80^{th}-110^{th}, 0.79. The pools were concentrated nearly to NaCl saturation. Pools BM-A and BM-B were desalted once on Trisacryl GF05M column (IBF-France 5 x 30 cm), concentrated and refractionated on the AcA 202 column (Second Step). Fractions from each pool were sampled and their MW determined : from pool BM-A, two new pools BM-A1 and BM-A2 constituted by fractions collected from fraction to fraction as follows and having Kav, respectively, 15^{th}-26^{th}, 0.17; 27^{th}-50^{th}, 0.32; were formed. The same for pool BM-B, obtaining BM-B1 and BM-B2, from fraction to fraction and Kav, respectively: 49^{th}-63^{rd}, 0.47; 64^{th}-75^{th}, 0.58. All new pools and pool BM-C were desalted on the Trisacryl column twice, concentrated and freeze-dried. The per cent yields of BM-A1, BM-A2, BM-B1, BM-B2 from parent RO-DS were respectively: 0.2; 3.4; 3.0; 3.3.

Pool BM-C was found to contain mostly a residue of hydrolysis, a mixture of monosaccharides + R. Fraction BM-A2 was found to contain mostly the nonasulfated nonasaccharide + R.

b. From pig skin DS (PS-A2/2) (Fig. 5b). A sample of RO-DS from PS-5 was Smith-degraded under the same conditions as above (2 hours, 60 °C). Solution containing the resulting mixture of oligosaccharides was size-fractionated on the same Ultrogel AcA 202 column and further processed as above.

Four fractions pools PS-A1, PS-A2, PS-B, PS-C; from fraction to fraction, respectively, as follows 5^{th} to 28^{th}; 29^{th} to 55^{th}; 56^{th} to 77^{th}; 78^{th} to 120^{th}, were collected.

Pool PS-A2, having a MW comparable with fraction BM-A2, was concentrated and desalted by three runs on the Trisacryl column. Fraction PS-A2 was then charge-density fractionated by strong anion exchange (SAX) HPLC. Two aliquots (100 mg/10 mL of water) of the product were loaded on a 7.5 x 300 mm Spherisorb 10 SAX column. The column was washed with water, then eluted with a 3-step gradient of NaCl (flow rate 1.5 mL/min; NaCl 0.5 M, 0.75 M, 2 M; 120 mL for each step). The elution was monitored by UV detector (214 nm) and SE-HPLC. The corresponding fractions from the two runs were pooled, concentrated, desalted and freeze-dried. PS-A2/0, -A2/0.5, -A2/0.75, -A2/2 fractions were obtained with percent yields, from parent RO-DS, respectively: 1.0; 0.9; 1.1; 0.5. The physico-chemical and spectroscopic characteristics of fraction PS-A2/2 (data not shown) correspond to those of a nonasulfated nonasaccharide + R, as for fraction BM-A2 obtained from beef mucosal DS.

Preparation of low-activity LMW-DS fraction

The low activity LMW-DS fraction C7/0.1 was prepared by repeated chromatographic runs on an anion exchange resin, of beef mucosa LMW-DS (obtained by radical depolymerization[12]). A sample of LMW-DS (5 g in 100 mL 0.1M NaCl) was loaded on Amberlite IRA 93 SP (Rohm & Haas, 2.5 x 10 cm column; eluent 0.1M NaCl flow rate 1.5 mL/min). The eluate was processed again twice, on a new column, affording fraction C7/0.1 (yield 63%, MW 3.7 KDa, potency 0.07).

ACKNOWLEDGMENTS

We thank Mrs. Sandra Carisio and Mrs. Stefania Dazzi for assisting in the preparation of the manuscript.

REFERENCES and NOTES

1. **Abbreviations**:

   IdoA2SO$_3$-GalNAc-4SO$_3$ = 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-threo-pyranosyl uronic acid)-4-*O*-sulfo-D-galactose.
   GlcA = glucuronic acid.

   ΔDi-2,4-diS = 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-threo-hex-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; the other ΔDi-abbreviations are related to the other 6 disaccharide-type obtainable by chondroitinase ABC digestion from DS.
   SE-HPLC = size exclusion high performance liquid chromatography.
   SAX-chromatography = strong anion exchange chromatography.
   RO-DS= reduced (with $NaBH_4$) oxidized (with IO_4^-)- DS.
   SD-DS= Smith-degraded DS.

2. P. Bianchini, G. L. Bergonzini, L. Liverani, G. Mascellani, B. Parma and A. Prete in: *Dermatan Sulphate Proteoglycans. Chemistry, Biology and Chemical Pathology*; J. E. Scott Ed.; Portland Press: London, 1993, p 249.
3. M. M. Maimone and D. M. Tollefsen, *J. Biol. Chem.*, **265**, 18263 (1990).
4. G. Mascellani, L. Liverani, P. Bianchini, B. Parma, G. Torri, A. Bisio, M. Guerrini and B. Casu, *Biochem. J.*, **296**, 639 (1993).
5. L. Lerner and E.A. Bax, *J. Magn. Reson.*, **69**, 375 (1986).
6. R. Da Col, L. Silvestro, A. Naggi, G. Torri, C. Baiocchi, D. Moltrasio, A. Cedro and I. Viano, *J. Chromatogr.*, **647**, 289 (1993).
7. A. Bax and S. Subramanion, *J. Magn. Reson .*, **67**, 565 (1986).
8. M. F. Summers, L. Marzilli and A. Bax, *J. Am. Chem. Soc.*, **108**, 565 (1986).
9. G. Mascellani, L. Liverani, A. Prete, P. A. Guppola, G. L. Bergonzini and P. Bianchini, *Thromb. Res.*, **74**, 605 (1994).
10. G. Mascellani, L. Liverani, A. Prete, G. L. Bergonzini, P. Bianchini, G. Torri, A. Bisio, M. Guerrini and B. Casu, *Anal. Biochem.*, **223**, 135 (1994).
11. V. M. D. Deerlin and D. M. Tollefsen, *J. Biol. Chem.*, **266**, 4285 (1986).
12. L. Liverani, N. Volpi, G. Mascellani and P. Bianchini, *Il Farmaco*, **47**, 841 (1992).