A Unique Dermatan Sulfate-like Glycosaminoglycan from Ascidian

ITS STRUCTURE AND THE EFFECT OF ITS UNUSUAL SULFATION PATTERN ON ANTICOAGULANT ACTIVITY

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A dermatan sulfate, similar to the mammalian glycosaminoglycans but not identical with any of them, has been isolated from the body of the ascidian Ascidia nigra. Degradation with chondroitin ABC lyase, analysis of the disaccharide products by digestion with chondro-4- and -6-sulfatases, and 1H and 13C NMR data confirm that the predominant structure is [4-α-L-IdoA-(2SO4)3-1-β-D-GalNAc(4SO4)3-1]n. Mammalian dermatan sulfate is an anticoagulant due to its ability to potentiate inhibition of thrombin by heparin cofactor II. The structure in dermatan sulfate which binds to heparin cofactor II is [4-α-L-IdoA-(2SO4)3-1-3-β-D-GalNAc(4SO4)3-1]n, where n is a positive integer. We have compared the ascidian dermatan sulfate with mammalian dermatan sulfate and with chemically oversulfated mammalian dermatan sulfate for anticoagulant activity as measured by the activated partial thromboplastin time assay and for its ability to potentiate heparin cofactor II. In spite of its high content of 2-O-sulfated α-L-iduronic acid residues, the ascidian compound had no discernible anticoagulant activity and had low ability to potentiate heparin cofactor II. These results suggest that 4-O-sulfation of the N-acetyl-β-D-galactosamine residues is essential for the anticoagulant activity of dermatan sulfate.

The glycosaminoglycans are a group of structurally related polysaccharides found as the carbohydrate moieties of proteoglycans and sometimes as free polysaccharides (1). Their functions are diverse. Dermatan sulfate, chondroitin sulfate, and heparan sulfate may all be components of connective tissue (2) or cell surface carbohydrates involved in the cell’s interaction with and response to its surroundings (3). The anticoagulant glycosaminoglycan heparin is an important therapeutic agent used in the prophylaxis and treatment of thrombosis (4); dermatan sulfate is also an anticoagulant, although of lower potency than heparin (5-7).

Most interest, understandably, centers on the structures and functions of mammalian glycosaminoglycans, but these polysaccharides are widely distributed throughout the animal kingdom (8), and invertebrate species are a rich source of sulfated polysaccharides with novel structures (9-15). The ascidians (Chordata-Tunicata) are covered by an external supportive tissue called the tunic. This tissue contains large amounts of sulfated polysaccharides different from all previously described mammalian glycosaminoglycans and from the sulfated polysaccharides from marine algae (9, 10, 12, 14). The preponderant polysaccharide in the ascidian tunic is a high molecular weight sulfated α-L-galactan (10, 12, 16, 17). Recently, we reported the occurrence of glycosaminoglycans in the body of the ascidian Styela plicata (18). Small amounts of heparan sulfate and a large quantity of a dermatan sulfate-like glycosaminoglycan were found in the body of this ascidian. Preliminary analysis of this dermatan sulfate using degradation with chondroitin ABC lyase suggested structural differences when compared with mammalian dermatan sulfate (18).

In the present work, we report the isolation and structural characterization of a dermatan sulfate-like glycosaminoglycan from the body of the ascidian Ascidia nigra and a comparison of its structure and anticoagulant activity with those of mammalian dermatan sulfate. The ascidian polysaccharide has a distinctive structure, composed of repetitive disaccharide units of 6-O-sulfo-2-acetamido-2-deoxy-3-O-(2-O-sulfo-α-L-idopyranosyluronic acid)-β-D-galactose. It differs from mammalian dermatan sulfate in its sulfation at both the 2-position of the iduronic acid unit and the 6-position of the N-acetylgalactosamine unit, and the absence of sulfation at position 4 of the hexosamine residue. This unique glycosaminoglycan may help to determine the structural requirement for the anticoagulant activity of dermatan sulfate and specifically for the binding to heparin cofactor II.

EXPERIMENTAL PROCEDURES

Materials—Heparan sulfate from human aorta was extracted and purified as described previously (19). Dermatan sulfate was extracted...
and purified from bovine lung, and partially oversulfated mammalian
dermatan sulfate was prepared by reaction with sulfur trioxide-pyri-
dine, as described (20). Chondroitin 4-sulfate from shark cartilage,
chondroitin 6-sulfate from shark cartilage, heparan sulfate (HS
1.14), chondroitin 4-sulfate (disaccharide-4-sulfate sulfatase, EC
3.1.6.7), and heparan sulfate (HS 1.10) were applied to a 0.5% agarose
gel and run for 1 h at 110 V in 0.05 M 1.3-diaminopropane/acetate buffer (pH 9.0).
The sulfated polysaccharides in the gel were fixed with 0.1% N-cetyl-N,N-
trimethylammonium bromide solution. After 12 h, the gel was dried and
stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5,
V/V). In B, the ascidian dermatan sulfate, repurified on a new DEAE-
column (see Fig. 1B), before (-) and after (+) treatment with chon-
donitin AC or ABC lyase, or deaminative cleavage by nitrous acid, were
analyzed by agarose gel electrophoresis, as described under A.

**TABLE I**

| Sample                  | Sulfate/Galactosamine Ratio | [α]D20°C |
|-------------------------|-----------------------------|---------|
| Ascidian dermatan sulfate | 1.83                         | 54°     |
| Mammalian dermatan sulfate | 1.10                         | 61°     |
| Vertebrate chondroitin 4-sulfate | 1.14                         | 26°     |

**Amino sugars were identified in the acid hydrolysates by paper
chromatography in butanol:pyridine:water (3:2:1, v/v) for 48 h,
visualized by silver nitrate staining, and revealed exclusively galactosamine.
Sulfate was estimated by the BaCl2/gelatin method (25), and the molar
contents were normalized to total galactosamine, which was estimated
by a modified Elson-Morgan reaction (24).**

**Fig. 1.** Purification of the ascidian dermatan sulfate on DEAE-
cellulose. In A, the crude polysaccharides from the ascidian body
(100 mg) were purified on a DEAE-cellulose column (10 × 1.5 cm)
as described under “Experimental Procedures.” Fractions were assayed
by the carbazole reaction (C), for metachromasia (●), and NaCl concentra-
tion (–––). Three groups of fractions were pooled separately as indi-
cated by the horizontal bars in the figure, designated HS (heparan
sulfate), SG (sulfated glucan), and DS (dermatan sulfate), dialyzed
against distilled water, and lyophilized. In B, the DEAE-purified
dermatan sulfate (~40 mg) was rechromatographed on a new DEAE-
cellulose column, and fractions were assayed for metachromasia (●)
and NaCl concentration (–––). Those corresponding to the purified
ascidian dermatan sulfate, as indicated by the horizontal bar in the
panel, were pooled, dialyzed against distilled water, and lyophilized.

**Fig. 2.** Agarose gel electrophoresis of the purified sulfated
polysaccharides from ascidian body. In A, the purified sulfated polysaccharides (~20 μg) obtained from the DEAE-cellulose column
(see Fig. 1A) and a mixture of standard glycosaminoglycans containing
20 μg each of chondroitin 4-sulfate (CS), dermatan sulfate (DS), and
heparan sulfate (HS) were applied to a 0.5% agarose gel and run for 1
h at 110 V in 0.05 M 1.3-diaminopropane/acetate buffer (pH 9.0).
The sulfated polysaccharides in the gel were fixed with 0.1% N-cetyl-N,N-
trimethylammonium bromide solution. After 12 h, the gel was dried and
stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5,
V/V). In B, the ascidian dermatan sulfate, repurified on a new DEAE-
column (see Fig. 1B), before (-) and after (+) treatment with chon-
donitin AC or ABC lyase, or deaminative cleavage by nitrous acid, were
analyzed by agarose gel electrophoresis, as described under A.
The fractions were assayed by metachromasia. a Mono Q-FPLC, and the column was developed as described under Figure 1B. purified ascidian dermatan sulfate (3 mg from the experiment of Fig. 1B, before and after incubation with 0.2 unit of chondroitin ABC lyase 12 h, or a solution containing standard hyaluronic acid (250 µg), before and after incubation with 0.2 unit of chondroitin AC lyase, chondroitin ABC lyase, and chondroitinase ABC lyase (see above). The activity was expressed as units/mg using a parallel standard curve based on the International Hemarin Standard (193 units/mg).

Anticoagulant Action of the Ascidian Dermatan Sulfate Measured by Activated Partial Thromboplastin Time (APTT)—APTT clotting assays were carried out as described previously (31, 32). Normal human plasma (90 µl) was incubated with 10 µl of a solution of glycosaminoglycan (0–100 µg) and 100 µl of kaolin + bovine brain phospholipid reagent (National Institute for Biological Standard reference reagent). After 5 min of incubation at 37 °C, 100 µl of 0.25 mM CaCl₂ are added to the mixtures, and the clotting time is recorded. The specific activity was calculated as the difference in clotting time between the sample and the blank.

RESULTS AND DISCUSSION

Isolation of a Dermatan Sulfate-like Glycosaminoglycan from Ascidian—The sulfated polysaccharides from the body of A. nigra were separated by anion exchange chromatography on a DEAE-cellulose column into two major fractions, which eluted with different NaCl concentrations (H5 and DS in Fig. 1A). Additional metachromatic material eluted at ~0.5 M NaCl (SG in Fig. 1A).

Peak DS was identified as a heparan sulfate-like glycosaminoglycan since it migrates as a single homogeneous band on agarose gel electrophoresis and has the same mobility of standard mammalian heparan sulfate (Fig. 2A). In addition, this fraction is resistant to chondroitin AC and ABC lyases, but is cleaved by nitrous acid (not shown). The major fraction of the sulfated polysaccharides from the body of A. nigra (peak DS) was rechromatographed on another DEAE-cellulose column, now yielding a homogeneous peak (Fig. 1B). This polysaccharide migrates on agarose gel electrophoresis as a single homogeneous metachromatic band with the same mobility as standard mammalian dermatan sulfate (Fig. 2B). It is not degraded by chondroitin AC lyase² or by nitrous acid, but disappears after treatment with chondroitin ABC lyase. These results characterize fraction DS as a dermatan sulfate-like glycosaminoglycan.

The material under peak SG (Fig. 1A) contains the dermatan sulfate-like glycosaminoglycan, but is contaminated with two other polysaccharides (Fig. 2A). These polymers are resistant to

² Incubation with chondroitin AC lyase does not reduce the molecular weight of the ascidian dermatan sulfate, as revealed by polyacrylamide gel electrophoresis stained with toluidine blue.
chondroitin AC and ABC lyases and to deaminative cleavage by nitrous acid, treatments that specifically degrade chondroitin sulfate, dermatan sulfate, and heparan sulfate. Preliminary analysis indicates the major contaminant is a sulfated glucan.

Table I shows the chemical analysis and the specific optical rotation of the purified ascidian dermatan sulfate. The observation that the sulfate/galactosamine molar ratio is approximately 1.8 suggests that most of the disaccharide units in this glycosaminoglycan are disulfated. The specific rotation of the ascidian dermatan sulfate (\( [\alpha]_254° \)) is very close to that recorded for a solution of standard dermatan sulfate from the mammalian source (\( [\alpha]_261° \)).

Analysis of the ascidian dermatan sulfate by anion exchange chromatography on a Mono Q-FPLC column (Fig. 3) confirms the high negative charge density of this glycosaminoglycan. Thus, the ascidian dermatan sulfate (closed circle in Fig. 3B) was eluted from the column at a higher NaCl concentration than mammalian glycosaminoglycans (Fig. 3A), as expected from the data of Table I.3 The electrophoretic migration of glycosaminoglycans in 1,3-diaminopropane/acetate buffer (Fig. 2) depends on the structure of the polysaccharide, which forms a complex with the diame (33, 34). Thus, the ascidian dermatan sulfate (Fig. 3B) was eluted from the column at a higher NaCl concentration than mammalian glycosaminoglycans (Fig. 3A), as expected from the data of Table I.3 The electrophoretic migration of glycosaminoglycans in 1,3-diaminopropane/acetate buffer (Fig. 2) depends on the structure of the polysaccharide, which forms a complex with the diame (33, 34). 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The standard disulfated disaccharide D-GlcA-GalNAc, possibly as a product of incomplete action of the lyase. A small amount of tetrasaccharide was also detected, hexuronic acid. The predominant product was eluted from the column G-50. The fractions were assayed by the carbazole reaction (25) for different charge densities of these two glycosaminoglycans. The ascidian dermatan sulfate in this buffer is not totally unexpected, besides the similar electrophoretic mobility of the ascidian and mammalian dermatan sulfate. The results indicate the presence of a dermatan sulfate-like glycosaminoglycan in ascidian. The sulfate/galactosamine molar ratio (Table I) and the elution from a Mono Q-FPLC column (Fig. 3) suggest higher content of sulfate esters in this glycosaminoglycan than in the mammalian dermatan sulfate.

The Products Formed by Degradation with Chondroitin ABC Lyase. Indicate That the Ascidian Dermatan Sulfate Has an Unusual Sulfation Pattern—The products of the action of chondroitin lyases, chondro-4-sulfatase and chondro-6-sulfatase on the ascidian dermatan sulfate and on standard compounds were analyzed by paper chromatography (Fig. 4). The action of chondroitin ABC lyase on the ascidian dermatan sulfate produces two products of different mobility on paper chromatography.4 The one with low mobility has the same migration as the standard disulfated disaccharide \( \Delta \text{GlcA}(2\text{SO})_4\text{-GalNAc} \), and accounts for 90% of the reacted material. The high mobility product has the same migration as the standard \( \Delta \text{GlcA}-\text{GalNAc}(6\text{SO})_4 \) and represents −10% of reacted material. No product is formed by chondroitin AC lyase.

Further characterization of the disulfated disaccharide was obtained by incubation with specific sulfatases. It resists chondroitin-4-sulfatase but, when digested with chondroitin-6-sulfatase, it gives rise to \( \Delta \text{GlcA}(2\text{SO})_4\text{-GalNAc} \), with the same chromatographic mobility as that obtained from the ascidian dermatan sulfate. Chondroitin ABC lyase action on the desulfated ascidian dermatan sulfate produces exclusively the nonsulfated disaccharide \( \Delta \text{GlcA}-\text{GalNAc} \) (Fig. 4C), as expected.

The 1H spectra of the ascidian dermatan sulfate and of mammalian dermatan sulfate were analyzed by gel filtration on Sephadex G-50. The fractions were assayed by the carbazole reaction (25) for hexuronic acid. The predominant product was eluted from the column as disaccharide. A small amount of tetrasaccharide was also detected, possibly as a product of incomplete action of the lyase.

### Table II

| GAG            | Unit                     | % of total | Chemical shift |
|----------------|--------------------------|------------|---------------|
|                |                          |            | H1            | H2            | H3            | H4            | H5            |
| This work\(^a\) | \( \text{IdoA-(2-SO)}_4 \) | 80         | 5.14          | \textbf{4.14} | 4.32          | 4.03          | 4.83          |
|                | \( \text{IdoA} \)        | 20         | 4.90          | 3.52          | 4.06          | 4.06          | 4.80          |
| Oversulfated   | \( \text{IdoA-(2-SO)}_4 \) | 25         | 5.15          | \textbf{4.12} | ND            | ND            | ND            |
| mammalian DS   | \( \text{IdoA-(3-SO)}_4 \) | 55         | 4.94          | 3.68          | \textbf{4.72} | 4.30          | 4.75          |
| Mammalian DS   | \( \text{IdoA} \)        | >90        | 4.91          | 3.53          | 3.92          | 4.03          | 4.73          |
| Literature values |                          |            |               |               |               |               |               |
| Oversulfated   | \( \text{IdoA} \)        | NR         | 4.90          | 3.56          | 3.97          | 4.11          | 4.74          |
| mammalian DS\(^b\) | \( \text{IdoA-(2-SO)}_4 \) | NR         | 5.22          | \textbf{4.19} | 4.23          | ND            | ND            |
|                | \( \text{IdoA-(3-SO)}_4 \) | NR         | 4.99          | 3.75          | \textbf{4.77} | 4.35          | 4.78          |
|                | \( \text{IdoA-(2,3-diSO)}_4 \) | NR         | 5.23          | \textbf{4.37} | 4.96          | 4.29          | 4.89          |
| Mammalian DS\(^b\) | \( \text{IdoA} \)        | NR         | 4.88          | 3.53          | 3.90          | 4.10          | 4.72          |
|                | \( \text{GlcA} \)        | NR         | 4.76          | 3.38          | 3.58          | 3.78          | 3.66          |
| Mammalian DS\(^c\) | \( \text{IdoA} \)        | NR         | 4.90          | 3.54          | 3.92          | 4.11          | 4.72          |

\(^a\) 1H spectra were recorded at 500 MHz, 60 °C in D\(_2\)O. Chemical shifts are referenced to internal TSP at 0 ppm in the mammalian dermatan sulfate sample.

\(^b\) Bossenmee et al. (36). Chemical shifts relative to internal TSP; 37 °C.

\(^c\) Sanderson et al. (35).
oversulfated mammalian dermatan sulfate are based on the results of COSY and TOCSY spectra and on comparison with published results for a different sample of oversulfated dermatan sulfate (36) and for chondroitin 6-sulfate (37). These assignments and literature values are summarized in Table II.

In both COSY and TOCSY spectra, α-L-iduronate spin systems can be traced from their characteristic H1 resonances. Iduronic acid H1 signals have chemical shifts in the region 4.9–5.23 ppm and small JH1–H2 values, unresolved in the broad lines present in the spectra of these heterogeneous and polydisperse polysaccharides (Table II, Literature values). Bossennec et al. (36) identify four such systems in the 1H spectrum of a sample of oversulfated mammalian dermatan sulfate, corresponding to unsulfated IdoA, IdoA-(2SO4), IdoA-(3SO4), and IdoA-(2,3-diSO4).

We were able to recognize the first three of these systems in the TOCSY spectrum of our sample of partially oversulfated mammalian dermatan sulfate, but did not see a spin system for IdoA-(2,3-diSO4). Integration of H1 resonances in the one-dimensional spectrum (Fig. 5C, inset) indicated that the three types of residues are in the proportion IdoA/IdoA-(2SO4)/IdoA-(3SO4) of 55:25:20. Assignments for the IdoA-(2SO4) residue are incomplete both in this work and in Ref. 35, because of overlap with more intense galactosamine peaks in both one- and two-dimensional spectra, but the H2 signal at 4.12 ppm, 0.62 ppm downfield of H2 in unsulfated iduronic acid residue, is diagnostic of sulfation at the 2-position. The spin system for IdoA-(3SO4) is complete in both this work and in Ref. 36; for this residue, H2 is at 3.68 ppm and H3 at 4.72 ppm, 0.8 ppm downfield of H3 in unsulfated iduronic acid units.

In the TOCSY spectrum of the ascidian dermatan sulfate (Fig. 6A), it is possible to see two spin systems attributable to α-L-iduronic acid residues, in the proportion 80:20 (Fig. 5A, inset). The less abundant residue has chemical shifts which resemble those of unsulfated iduronic acid in mammalian and partially oversulfated mammalian dermatan sulfate, although the H3 signal at 4.06 is 0.14 ppm downfield of H3 in the mammalian compounds. The more abundant residue has chemical shifts which are close to those for IdoA-(2SO4) in mammalian dermatan sulfate and which agree with the chemical shifts for IdoA-(2SO4) in oversulfated mammalian dermatan sulfate so far as they can be determined. In particular, the H2 signal at 4.14 ppm indicates sulfation at this position; it can therefore be said that most of the α-L-iduronate residues in the ascidian

![Fig. 6. Part of the TOCSY spectrum of the ascidian dermatan sulfate. The dotted lines (A) show the points at which the two slices of the same spectrum are shown in B and C. These two slices are taken at the chemical shifts of IdoA-(2SO4) H5 and IdoA-(2SO4) H1, respectively. Cross-peaks from H5 to H4 and H3, and from H1 to H2 and H3 are displayed clearly in the slices. These peaks allow complete assignment of the IdoA-(2SO4) residue in this polysaccharide.](image)

### Table III

Proton chemical shift of N-acetyl-β-D-galactosamine residues in ascidian and mammalian dermatan sulfates

| GAG Unit | Chemical shift |
|----------|----------------|
|          | H1  | H2  | H3  | H4  | H5  | H6  | H6' | CH3  |
| This work<sup>a</sup> |      |     |     |     |     |     |     |      |
| Ascidian DS | GalNAc-(6-SO4) | 4.64 | 4.02 | 3.86 | 4.15 | 3.86 | 4.15 | 4.15 | 2.02 |
| Oversulfated mammalian DS | GalNAc-(4,6-diSO4) | 4.70 | 4.02 | 4.0–4.05 | 4.76 | 4.07 | 4.25 | 4.21 | 2.06 |
| Literature values |      |     |     |     |     |     |     |      |
| Chondroitin 6-sulfate<sup>b</sup> | GalNAc-(6-SO4) | 4.58 | 4.02 | 3.85 | 4.16 | 3.95 | 4.23 | 4.21 | 2.01 |
| Mammalian DS<sup>c</sup> | GalNAc-(4-SO4) | 4.70 | 4.05 | 3.95 | 4.65–4.7 | 3.80 | 3.75 | 3.80 | 2.08 |
| Mammalian DS<sup>d</sup> | GalNAc-(4-SO4) | 4.70 | 4.05 | 4.03 | 4.69 | 3.80 | 3.79 | 3.82 | 2.08 |

<sup>a</sup> 1H spectra were recorded at 500 MHz, 60 °C in D₂O. The chemical shifts are referenced to internal TSP at 0 ppm in the mammalian dermatan sulfate sample.
<sup>b</sup> Welti et al. (37).
<sup>c</sup> Sanderson et al. (35); chemical shifts relative to internal TSP; 60 °C.
<sup>d</sup> Bossennec et al. (36); chemical shifts relative to internal TSP; 37 °C.
dermatan sulfate is 2-0-sulfated.

The spin systems of N-acetyl-\(\beta\)-D-galactosamine residues in the \(^1\)H spectra are traced to the H1 signal at about 4.58–4.74 ppm through the COSY and TOCSY spectra. For the ascidian dermatan sulfate, the assignment given for GalNAc(6SO4) in Table III is consistent with the TOCSY spectrum, but for H4, H5, H6, and H6' it must be tentative as the cross-peaks (all between resonances at 3.86 and 4.15 ppm) overlap heavily. Although there is no sign of a signal for sulfated H4 at about 4.65–4.70 ppm, the possibility cannot be ruled out that H1 and H4 resonances are coincident.

A TOCSY spectrum of the partially oversulfated mammalian dermatan sulfate allowed complete assignment of the \(^1\)H spectrum of the doubly sulfated residue GalNAc(4,6-diSO4), partially assigned in Ref. 36. Both these data and the spectrum of GalNAc(6SO4) in chondroitin 6-sulfate (37) indicate that the downfield shift for H6 and H6' on sulfation at C6 is about 0.4 ppm. Although on this basis the H6 and H6' chemical shifts of ascidian dermatan sulfate are consistent with 6-O-sulfation of the galactosamine residues, the difficulties surrounding assignment are too great to make this deduction with certainty from the \(^1\)H NMR data.

\(^{13}\)C NMR Spectra Confirm 6-O-Sulfation of the N-Acetyl-\(\beta\)-D-galactosamine Residues—The \(^{13}\)C NMR spectra of ascidian dermatan sulfate, mammalian dermatan sulfate, and chemically oversulfated mammalian dermatan sulfate, are illustrated in Fig. 7, A, B, and C, respectively, with the corresponding DEPT spectra. Chemical shifts for mammalian dermatan sulfate were similar to literature values (35) as summarized in Table IV. With the exception of the C6 signals, the assignments of the \(^{13}\)C spectra of the ascidian and partially oversulfated mammalian dermatan sulfates summarized in Table IV are based on comparisons with literature values for mammalian dermatan sulfate (35), an oligosaccharide fragment of mammalian dermatan sulfate (SD-DS BM1/2) (38), and chondroitin 6-sulfate (39).

The DEPT spectra have been recorded in such a way that methine and methyl carbon signals are both 180° out of phase with methylene carbon signals, allowing the identification of C6 of galactosamine, which is the only methylene carbon in the dermatan sulfate structure. In the DEPT spectrum of mammalian dermatan sulfate (Fig. 7B), in which galactosamine is sulfated at C4 and linked at C3, the unsubstituted C6 resonating at 63.8 ppm is inverted. The DEPT spectrum of ascidian dermatan sulfate (Fig. 7A) has an inverted resonance at 69.7 ppm, shifted downfield by 5.9 ppm. This chemical shift is similar to that of C6 of chondroitin 6-sulfate (69.0 ppm) (39) and is confirmation that most of the galactosamine in the ascidian dermatan sulfate is sulfated at C6. A minor inverted signal in the \(^{13}\)C spectrum of ascidian dermatan sulfate at 64 ppm can be attributed to unsulfated galactosamine C6. The \(^{13}\)C spectrum of the oversulfated mammalian dermatan sulfate (Fig. 7C) was complex and overlapping and was not assigned, but the signal at 64.3 ppm from unsulfated C6 is minor in comparison to other peaks. The resonance from the majority of C6 of galactosamine must be in the group of overlapping peaks near 70.7 ppm and so indicates that C6 is largely substituted with sulfate.

Other signals in the \(^{13}\)C spectrum of ascidian dermatan sulfate correspond well with the IdoA-(2SO4) shifts from the mammalian dermatan oligosaccharide fragment or the GalNAc(6SO4) shifts from chondroitin sulfate, and all the signals in the spectrum can be accounted for on this basis. Thus, the spectrum confirms the linkage positions and configurations of the constituent sugars of the ascidian dermatan sulfate.

Overall, the proposed structure for the ascidian dermatan sulfate (Fig. 8A) and for the chemically oversulfated mammalian dermatan sulfate (Fig. 8C) is supported by degradation with chondroitin ABC lyase, analysis of the disaccharide products by digestion with chondro-4- and -6-sulfatases, and by \(^1\)H and \(^{13}\)C NMR data. Both compounds have the same backbone structure but have different patterns of sulfate substitution.

The Ascidian Dermatan Sulfate Has No Anticoagulant Activity and Reduced Capacity to Potentiate Heparin Cofactor II—The results of APTT assays in the absence, and in the presence, of an antithrombin III neutralizing antibody are listed for ascidian dermatan sulfate, mammalian dermatan sulfate, and chemically oversulfated mammalian dermatan sulfate, in Table V. The presence of the antithrombin III neutralizing antibody has the effect that the test measures only anticoagulant activity not mediated by antithrombin III; in practice, this indicates activity mediated by heparin cofactor II. Two extremes for the scale of APTT activity are the heparin standard used for the test (193 units/mg) and chondroitin 6-sulfate (<0.1 units/mg). Mammalian dermatan sulfate has a spe-
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Carbon chemical shifts for ascidian and mammalian dermatan sulfates

The abbreviations used are: ND, not determined; TSP, trimethylsilylpropionic acid; DS, dermatan sulfate. Values in boldface indicate positions bearing sulfate ester.

| GAG               | Unit          | Chemical shift (ppm) | Acetyl-CH₃ | Acetyl-CO   |
|-------------------|---------------|----------------------|------------|------------|
|                   |               | C1   | C2   | C3   | C4   | C5   | C6   |          |          |
| This work         |               |      |      |      |      |      |      |          |          |
| Ascidian DS       | IdoA-(2-SO₄) | 106.0| 75.4 | 71.0 | 80.3 | 69.7 | 175.4| 177.8    |          |
|                   | GalNAc-(6-SO₄)| 104.0| 54.2 | 82.5 | 70.4 | 76.5 | 69.7 | 25.6     |          |
| Mammalian DS      | IdoA         | 105.7| 72.1 | 73.7 | 82.7 | 72.1 | 176.6|          |          |
|                   | GalNAc-(4-SO₄)| 104.8| 54.8 | 78.2 | 78.9 | 77.4 | 63.8 | 25.4     | 177.8    |
| Literature values | Mammalian DS  | IdoA | 105.7| 72.0 | 73.7 | 82.7 | 72.0 | 176.6    |          |
|                   | GalNAc-(4-SO₄)| 104.7| 54.8 | 78.1 | 78.9 | 77.3 | 63.7 | 25.4     | 177.8    |
|                   | fragments     |       |      |      |      |      |      |          |          |
| Chondroitin-6-     |               | 102.7| 54.4 | 81.3 | 69.0 | 75.4 | 69.0 | 24.0     | 176.3    |
| sullfate          | GalNAc-(6-SO₄)²|     |      |      |      |      |      |          |          |

\[ ^{13}C \text{ spectra were recorded at } 125 \text{ MHz, } 60 \degree \text{C in } D_2O. \text{ Chemical shifts relative to internal TSP in the mammalian dermatan sulfate sample.} \]
\[ ^{1}H \text{ spectra were recorded at } 400 \text{ MHz, } 60 \degree \text{C in } D_2O. \text{ Chemical shifts relative to internal TSP; } 60 \degree \text{C.} \]
\[ ^{13}C \text{ spectra were recorded at } 125 \text{ MHz, } 60 \degree \text{C in } D_2O. \text{ Chemical shifts relative to internal TSP; } 60 \degree \text{C.} \]
\[ ^{1}H \text{ spectra were recorded at } 400 \text{ MHz, } 60 \degree \text{C in } D_2O. \text{ Chemical shifts relative to methanol at } 50.7 \text{ ppm; } 45 \degree \text{C.} \]

**CONCLUSIONS**

Ascidian dermatan sulfate has the same degree of sulfation as the sequence in porcine skin dermatan sulfate with high affinity for heparin cofactor II differing only in the position of sulfation of the galactosamine residues. In spite of this, it has lower activity with heparin cofactor II than mammalian dermatan sulfate (Fig. 9) and no measurable anticoagulant activity (Table V), thus establishing that 4-O-sulfation of N-acetyl-β-D-galactosamine residues is essential for interaction with heparin cofactor II and consequently for the anticoagulant activity of dermatan sulfate.

The degree of specificity of the interaction between heparin cofactor II and glycosaminoglycan has been the subject of argument. Sié et al. (42) concluded that for heparin there is no dependence of sulfation, but only on the degree of sulfation, for the interaction with heparin cofactor II. Our results indicate that the structural requirement of dermatan sulfate for the catalysis of thrombin inhibition by heparin cofactor II is not due to sulfate density alone, which is consistent with the observation that the binding sites in heparin cofactor II for heparin and dermatan sulfate are not identical (21). Ascidian dermatan sulfate is probably the most highly sulfated natural glycosaminoglycan reported to have undetectable APTT activity and very low effect on thrombin inhibition by heparin cofactor II (43). Studies of the anticoagulant activity of artificially oversulfated dermatan sulfates have all indicated increased activity on extra sulfation (40, 44, 45). The dependence of dermatan sulfate affinity for heparin cofactor II on sulfation of the iduronate residues has now been well established (41), but the importance of the sulfate group at the 4-position of the galactosamine units has not been explored as fully. Comparison of the activities of polysaccharides resembling dermatan sulfate extracted from hagfish demonstrate that sulfation at both the 4- and 6-positions of the galactosamine units does not confer heparin cofactor II affinity unless the iduronate residue is also sulfated (46).
Ascidian dermatan sulfate

![Chemical structure](image)

\(X = \text{OSO}_3^- (\sim 80\%) \) or \(\text{OH} (\sim 20\%)

Mammalian dermatan sulfate

![Chemical structure](image)

\(X = \text{OH} (\geq 95\%) \) or \(\text{OSO}_3^- (\leq 5\%)

Partially oversulfated mammalian dermatan sulfate

![Chemical structure](image)

\(Y = \text{OSO}_3^- (20\%) \) or \(\text{OH} (80\%)

\(X = \text{OSO}_3^- (25\%) \) or \(\text{OH} (75\%)

FIG. 8. Major repetitive disaccharide units of ascidian dermatan sulfate (A), mammalian dermatan sulfate (B), and chemically oversulfated mammalian dermatan sulfate (C). These glycosaminoglycans have the same backbone structure [4-\(\alpha-L-IdoA-1\rightarrow3\)-\(\beta-N\)-acetyl-\(\text{GalNAc-1}_m\)] but have different patterns of sulfate substitutions. The ascidian dermatan sulfate (A) is sulfated at both the 2-position of the \(\alpha-L\)-iduronic acid and the 6-position of the N-acetyl-\(\beta\)-D-galactosamine units. On the partially oversulfated dermatan sulfate (C), most of the galactosamine residues are sulfated at both 4- and 6-positions; the nonsulfated, 2-sulfated, and 3-sulfated \(\alpha-L\)-iduronic acid residues are in the proportion of 55:25:20. The repetitive disaccharide units of mammalian dermatan sulfate (B) are sulfated at carbon 4 of the hexosamine moiety; small amounts (<5%) of 2-O-sulfated \(\alpha-L\)-iduronic acid residues are also found in this mammalian glycosaminoglycan.

Our observation on the anticoagulant activity of the ascidian dermatan sulfate suggests that the particular disposition in space of the sulfate groups at the 2-position of \(\alpha-L\)-iduronic acid and 4-position of the N-acetyl-\(\beta\)-D-galactosamine residues is recognized by heparin cofactor II. As addition sulfate substitution does not appear to hinder the interaction, it may be possible for heavily sulfated polysaccharides such as pentosan polysulfate (with 4-sulfates per disaccharide) to mimic this spatial array, the high density of sulfation overwhelming any specificity of the binding site.

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TABLE V

| Glycan                  | APTT<sup>a</sup> | APTT without antithrombin III<sup>1i</sup> |
|------------------------|-----------------|-----------------------------------------|
| Mammalian dermatan sulfate | 4 units/mg | 71% original |
| Ascidian dermatan sulfate | <0.1 | 83% original |
| Oversulfated mammalian dermatan sulfate | 13 | 34% original |
| Chondroitin 6-sulfate | 0.1 | 83% original |
| Heparin | 193 | 34% original |

<sup>a</sup> The clotting times were recorded as described under “Experimental Procedures.” The activity is expressed as units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg).

<sup>1i</sup> APTT assay after the addition of antithrombin III neutralizing antibody.

FIG. 9. Activity of dermatan sulfate with heparin cofactor II. Heparin cofactor II (68 nM) was incubated with thrombin (15 nM) in the presence of various concentrations of mammalian dermatan sulfate (■), ascidian dermatan sulfate (●), chemically oversulfated dermatan sulfate (■), or mammalian dermatan sulfate plus an fixed concentration of ascidian dermatan sulfate (20 μg/ml) (□). After 60 s, the remaining thrombin activity was determined with a chromogenic substrate (A<sub>405</sub>/min).
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