Structure and Anticoagulant Activity of Sulfated Galactans

ISOLATION OF A UNIQUE SULFATED GALACTAN FROM THE RED ALGAE BOTRYOCLADIA OCCIDENTALIS AND COMPARISON OF ITS ANTICOAGULANT ACTION WITH THAT OF SULFATED GALACTANS FROM INVERTEBRATES*

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We have characterized the structure of a sulfated p-galactan from the red algae Botryocladia occidentalis. The following repeating structure (4-α-D-Galp-1→3-β-D-Galp-1→) was found for this polysaccharide, but with a variable sulfation pattern. Clearly one-third of the total α-units are 2,3-di-O-sulfated and another one-third are 2-O-sulfated. The algal sulfated p-galactan has a potent anticoagulant activity (similar potency as unfractionated heparin) due to enhanced inhibition of thrombin and factor Xa by antithrombin and/or heparin cofactor II. We also extended the experiments to several sulfated polysaccharides from marine invertebrates with simple structures, composed of a single repeating structure. A 2-O- or 3-O-sulfated l-galactan (as well as a 2-O-sulfated l-fucan) has a weak anticoagulant action when compared with the potent action of the algal sulfated p-galactan. Possibly, the addition of two sulfate esters to a single α-galactose residue has an “amplifying effect” on the anticoagulant action, which cannot be totally ascribed to the increased charge density of the polymer. These results indicate that the wide diversity of polysaccharides from marine alga and invertebrates is a useful tool to elucidate structure/anticoagulant activity relationships.

Sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. These anionic polymers are widespread in nature, occurring in a great variety of organisms. In marine algae, the carrageenans and fucoids are composed mainly of sulfated galactose and fucose, respectively (1). Vertebrate tissues express abundant sulfated glycosaminoglycans (2). Invertebrate species are also a rich source of sulfated polysaccharides with novel structures (3–16).

We have isolated and characterized the structure of several sulfated galactans from marine invertebrates (3, 4, 6, 7, 9, 13). In contrast with the algal polysaccharides, invertebrate galactans have simple structures, composed of a single repeating unit. The specific pattern of sulfation and the position of the glycosidic linkage varies among different species. The sea urchin (Echinoderma Echinoidea) Echinometra lucunter contains a linear polysaccharide composed of 2-O-sulfated, 3-linked α-L-galactose (Fig. 1A) (13), while in the tunicate (Chordata Asciidae) Herdmania monus a similar polysaccharide is composed of 3-O-sulfated, 4-linked α-L-galactose (Fig. 1B) (9). The sea urchin Strongylocentrotus franciscanus contains a related polysaccharide composed of 2-O-sulfated, 3-linked α-L-fucose units (Fig. 1C) (15). In other species of tunicate, the sulfated l-galactans have more complex and branched structures (4, 6, 7, 9). In Styela plicata, non-sulfated l-galactose occurs as branched units linked to position O-2 of the central core (Fig. 1D) (6, 7).

One way to determine the relationship between structure and biological activity of sulfated polysaccharides is to compare their activity in various assays where the contributions of the polysaccharide backbone, and the extent and position of sulfation have been fully characterized. In this line of work, new sulfated galactans and the sulfated fucan from invertebrates constitute a valuable tool.

Anticoagulant and antithrombotic activities are among the most widely studied properties of sulfated polysaccharides. The anticoagulant glycosaminoglycan heparin is an important therapeutic agent for prophylaxis and treatment of thrombosis (17); dermatan sulfate is also anticoagulant, although of lower potency than heparin (18, 19). Other sulfated polysaccharides, either extracted from marine brown (16, 20–23) and red algae (24, 25), or obtained by chemical sulfation of natural polysaccharides (26), have been described as anticoagulant. In contrast with heparin and dermatan sulfate, the structural components of these algal sulfated polysaccharides have not been characterized for anticoagulant activity. Most of the difficulties arise from their heterogeneous chemical structures.

In the present study we isolated and characterized the structure of a sulfated p-galactan from the red algae Botryocladia occidentalis. This polysaccharide has the following repeating structure (4-α-D-Galp-1→3-β-D-Galp-1→). Besides its variable sulfation pattern, it clearly contains 2,3-di-O-sulfated p-galactose residues (approximately one-third of the total α-galactose units).

This algal sulfated galactan has a potent anticoagulant activity. Its action was compared with the sulfated galactans and

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the sulfated fucan shown in Fig. 1. Our results suggest that the 2,3-di-O-sulfated galactose residues have an "amplifying effect" on the anticoagulant activity of sulfated galactans.

**EXPERIMENTAL PROCEDURES**

**Sulfated Galactans and a Sulfated Fucan from Invertebrates**

Sulfated polysaccharides from marine invertebrates (A. E. lucunter; B. H. monus; and C. S. franciscanus) have a regular repeating structure, with a single sulfated monosaccharide unit, composed either of sulfated α-galactose or sulfated α-L-fucose. In the case of the galactan from the ascidian S. plicata (D) non-sulfated α-L-galactose residues occur as branched units.

![Fig. 1. Structures of the sulfated α-L-galactans and sulfated α-L-fucan from marine invertebrates. Sulfated polysaccharides from marine invertebrates (A. E. lucunter; B. H. monus; and C. S. franciscanus) have a regular repeating structure, with a single sulfated monosaccharide unit, composed either of sulfated α-galactose or sulfated α-L-fucose. In the case of the galactan from the ascidian S. plicata (D) non-sulfated α-L-galactose residues occur as branched units.](image)

**Anticoagulant Activity of Sulfated Galactans**

We extracted sulfated polysaccharides from 54 species of marine alga and tested their anticoagulant activity using clotting assays. The sulfated polysaccharide from *B. occidentalis* showed the highest anticoagulant activity among the species tested and therefore it was chosen for a detailed structural and anticoagulant analysis. The results of this preliminary screening analysis will be published elsewhere.  

**Desulfation and Methylation of the Galactan**

Desulfation of the sulfated fraction was obtained. The native and desulfated galactans were subjected to three rounds of methylation, as described (32) with the modifications suggested by Patankar et al. (33). The methylated polysaccharides were hydrolyzed with 6.0 M trifluoroacetic acid for 5 h at 100 °C, reduced with borohydride, and the alditoles were acetylated with 1:1 acetic anhydride/pyridine (30). The alditol acetates from the methylated sugars were analyzed in a Hewlett-Packard gas chromatography/mass spectrometry unit, model 5987-A. Injection was made in the splitless mode in a DB-1 capillary column (25 m × 0.3 mm). The column was programmed to run at 120 °C for 2 min, then raised to 230 °C at 2 °C/min, and held for 5 min.

**NMR Spectroscopy**

Proton and 13C spectra were recorded using a Bruker DRX 600 with a triple resonance probe. About 3 mg of each sample was dissolved in 0.5 ml of 99.9% D2O (CIL). All spectra were recorded at 60 °C with HOD suppression by presaturation. COSY, TOCSY, and 1H-13C heteronuclear correlation (HMOC) spectra were recorded using states-time proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4,096 × 400 points with a spin-lock field of about 10 KHz and a mixing time of 80 ms. HMOC were run with 1,024 × 256 points and globally optimized alternating phase rectangular pulses for decoupling. NOESY spectra were run with a mixing time of 100 ms. All chemical shifts were relative to external trimethylsilylpropanic acid and 13C-methanol.

**Anticoagulant Action Measured by APTT**

Activated partial thromboplastin time assays were carried out by the method of Anderson et al. (34). Normal human plasma (100 μl) was incubated with 10 μl of a solution of polysaccharide (0.05-5 μg) at 37 °C for 1 min. Then 100 μl of activated partial thromboplastin time reagent (Celite Biobal) was added to the mixture. The reaction was allowed to proceed for 2 min, and the end point was recorded as the time required for the APTT to double from the control. 

Effect spectroscopy; HMQC, 1H-13C heteronuclear multiple quantum coherence spectra.
were added and incubated at 37 °C. After 2 min of incubation 100 μl of 0.25 mM CaCl$_2$ were added to the mixtures and the clotting time recorded in a coagulometer (Amelung KC4A). The activity was expressed as international units/mg using a parallel standard curve based on the 4th International Heparin Standard (193 international units/mg).

Effect of Sulfated Polysaccharides on the Inactivation of Thrombin by Antithrombin—Sulfated polysaccharide solution (10 μl) and 5 μl of 1 unit/ml purified human antithrombin were mixed with 7 μl of 10 units/ml human purified thrombin in 66 μl of 0.015 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 1 mg/ml polyethylene glycol (Sigma). After a 1-min incubation (inhibition period), 500 μl of 0.24 mM chromogenic substrate S-2238 from Chromogenix AB (Molndal, Sweden) was added, and the remaining thrombin activity recorded for 2 min at 405 nm.

Effect of Sulfated Polysaccharides on the Inactivation of Thrombin by Heparin Cofactor II—This assay was just as described above except that heparin cofactor II (100 μg/ml, from Diagnostica Stago, Asnières, France) instead of antithrombin was added to the incubation mixtures.

Effect of Sulfated Polysaccharides on the Inactivation of Factor Xa by Antithrombin—Sulfated polysaccharide solution (10 μl) and 5 μl of 1 unit/ml purified human antithrombin were mixed with 7 μl of 4 units/ml purified bovine factor Xa (Chromogenix Molndal, Sweden) in 80 μl of 0.015 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 1 mg/ml polyethylene glycol. After a 1-min incubation (inhibition period), 500 μl of 0.24 mM chromogenic substrate S-2222 from Chromogenix AB (Molndal, Sweden) was added and the remaining factor Xa activity recorded for 2 min at 405 nm. In the incubation periods used, no inhibition occurred when thrombin or factor Xa was incubated at 37 °C with the sulfated polysaccharides or with the cofactors (antithrombin or heparin cofactor II) alone.

RESULTS AND DISCUSSION

Purification and Structural Analysis of the Sulfated Galactans from the Red Algae B. occidentalis

Purification of the Algal Sulfated Galactans—Anion exchange chromatography on Mono Q-FPLC separated the sulfated polysaccharides from red algae into three major peaks F1, F2, and F3, eluted from the column with ~1.0, ~2.2, and ~3.0 mM NaCl, respectively (Fig. 2A). All three fractions had strong metachromasia produced with 1,9-dimethylmethylene blue (open circles), and high hexose content, as revealed by the method of Dubois et al. (28) (closed circles). Agarose gel electrophoresis analysis revealed an increased mobility from F1 to F3 (Fig. 2B). Chemical analysis of the purified fractions showed galactose as the only sugar constituent and an increasing sulfate content from F1 to F3 (Table I). The trimethylsilylated (−)-2-butylyl galactosides obtained from the algal polysaccharides have the same retention times and peak area proportions on GC column as standard β-galactose (not shown). Therefore, galactose occurs on the B. occidentalis galactan as a β-antennamer. Overall, these results indicate that the red algae B. occidentalis contains three fractions of sulfated β-galactans that differ in their sulfate ester contents.

Fractions F2 and F3 Contain Approximately Equimolar Proportions of 3- and 4-Linked Units—When the three fractions of B. occidentalis-sulfated galactans were submitted to three rounds of methylation, a variety of methylated derivatives were obtained, mainly mono- and dimethylated derivatives (not shown). The methylation data were not consistent with known polysaccharide structures. Despite the observation that the proportions of methylated derivatives remain unchanged after an additional methylation cycle, we probably still have incomplete reaction. In fact, methylation of sulfated polysaccharides does not always yield reliable proportions of methylated aldito-ls (4, 33, 37, 38). This may be a consequence of steric hindrance due to the sulfate esters, which does not allow complete methylation of these polymers. The more drastic conditions necessary to remove sulfate esters may also destroy some of the methylated derivatives.

An alternative method to obtain information about the structure of these polymers is methylation of the desulfated polysaccharide. In fact methylation of the desulfated F2 and F3 fractions yields almost similar proportions of 2,3,6-tri-O- and 2,4,6-tri-O-methylgalactose, indicating 4-linked and 3-linked galactose residues, respectively (Table II). Small amounts of dimethyl derivatives were also obtained, especially from desulfated F2. They are not merely produced from an incomplete reaction since their proportions remain unchanged after an additional methylation cycle (not shown). Possibly these derivatives come from minor structural components and/or from units which were not desulfated. Desulfated F2 and F3 still contain ~0.18 and ~0.08 sulfate/sugar unit, respectively, which are close to the limit for detection of sulfate by the method we used (~0.1 sulfate/sugar, as molar ratio).

![Fig. 2. Purification of the sulfated galactans from B. occidentalis by Mono Q-FPLC (A) and analysis of the purified polysaccharides by agarose gel electrophoresis (B). A, fractions were collected and assayed for metachromasia (○), Dubois reaction (●), and NaCl concentration (—). Fractions indicated by horizontal bars were pooled, dialyzed against distilled water, and lyophilized. B, the three fractions of sulfated galactans obtained from the Mono Q-FPLC column (20 μg of each) were applied to a 0.5% agarose gel in 50 mM 1,3-diaminopropane/acetate (pH 9.0) and run at 110 V for 1 h. Gels were fixed with 0.1% N-ethyl-N,N,N-trimethylammonium bromide solution overnight, dried, and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

| Fraction | Composition (molar ratio)$^b$ | $\alpha_{20°C}$ | Elution from Mono Q-FPLC column (NaCl, $m^a$) |
|----------|-------------------------------|----------------|---------------------------------------------|
|          | Galactose | Sulfate | | |
| F1       | 1.00     | 0.69    | −8 $^c$ | 1.0  |
| F2       | 1.00     | 1.47    | −16 $^c$ | 2.2  |
| F3       | 1.00     | 2.08    | −10 $^c$ | 3.0  |

$^a$ See Fig. 2.

$^b$ Hexose was identified by its retention time on a gas chromatography/mass spectrometry unit of derived alditol acetate. The concentrations of hexose and sulfate on the acid hydrolysates obtained from the polysaccharides were determined by the phenol/H$_2$SO$_4$ and BaCl$_2$/gelatin reactions, respectively.

$^c$ See experiment shown in Fig. 2A.
Methylation of desulfated F1 fraction revealed a more complex mixture of methyl derivatives, which are not consistent with known polysaccharide structures. We made several attempts to methylate this polysaccharide using different rounds of the reaction and a variety of hydrolysis procedures to prepare the methylated derivatives. None of these attempts yielded reliable proportions of methyl derivatives. We cannot explain the difficulty in obtaining reliable proportions of methylated alditos from this polysaccharide. However, preliminary qualitative information can be obtained about the structure of this compound. In contrast with the other two fractions reported in Table II, desulfated F1 contains 2,3,6,8-tetra-O-methylgalactose (10% of the total methyl derivatives), indicative of a branched polysaccharide. However, similar to the other two fractions, it also yields 30 and 23% of 2,3,6-tri-O- and 2,4,6-tri-O-methylgalactose, respectively, originated from 4- and 3-linked galactose units.

Thus, methylation analysis indicates that F2 and F3 contain approximately equimolar proportions of 3-linked and 4-linked galactopyranose units. On the other hand, F1 has a more complex and branched structure, containing galactose at the non-reducing terminal.

**Table II**

Methylated (Me) galactose (Gal) derivatives obtained from native and desulfated galactans from the red alga *B. occidentalis*.

| Alditols<sup>a</sup> | $t_R$<sup>b</sup> | Desulfated F2 | Desulfated F3 |
|----------------------|-----------------|---------------|---------------|
| 2,3,6-Me<sub>3</sub>-Gal | 1.13            | 46%           | 49%           |
| 2,4,6-Me<sub>3</sub>-Gal | 1.16            | 36%           | 43%           |
| 2,6-Me<sub>2</sub>-Gal | 1.23            | 10            | 8             |
| 3,6-Me<sub>2</sub>-Gal | 1.28            | 4             | <1            |
| 2,4-Me<sub>2</sub>-Gal | 1.39            | 4             | <1            |

<sup>a</sup> The identity of each peak was established by mass spectrometry.

<sup>b</sup> Retention time ($t_R$) on DB-1 capillary column relative to 2,3,4,6-tetra-O-methylgalactitol.

**NMR Analysis Reveals Alternating α(1→3) and β(1→4) Units with a Variable Sulfation Pattern**

We employed NMR analysis to confirm the saccharide backbone structure of fractions F2 and F3 and also to determine the sulfation pattern of these polysaccharides. This last aspect was not possible to approach by methylation analysis, as discussed above.

The $^1$H one-dimensional spectra of the native and desulfated galactan from *B. occidentalis* (fraction F2) are shown in Fig. 3. The chemical shifts in Tables III and IV are based on the interpretations of TOCSY, COSY, and HMQC spectra.

The desulfated galactan shows two main anomeric resonances, one at 5.2 ppm (α unit) and another at 4.4 ppm (β unit) (Fig. 3B). Peak integration demonstrates a 1:1 ratio. The α and β spin systems can be traced based on TOCSY (Fig. 4B), COSY (not shown), and $^1$H/$^1$C HMQC (Fig. 5B) spectra, giving the values presented in Table III and IV (c and d). The C4 of α-galactose and C3 of β-galactose residues show strong downfield shifts (~10 ppm) indicating that the two residues are 4- and 3-linked, respectively (Table IV). Linkage information was also obtained in the NOESY spectrum (Fig. 6). A strong inter-residue NOE is seen between β-H1 and α-H4 and from α-H1 and β-H3, as expected for α-1→3 and β-1→4 linkages. NOEs also appear from the anomeric protons that are close in space to the linkage, but their intensity is smaller. These results are compatible with a polysaccharide with the following repeating structure: 4-α-d-Galp-1→3-β-d-Galp-1→ (Fig. 7).

The native polysaccharide has a very complex $^1$H NMR spectrum (Fig. 3A) due to its heterogeneous sulfation pattern. At least six distinguishable anomeric resonances were observed.

The 1H one-dimensional spectra of the native and desulfated galactans from the red alga *B. occidentalis* are shown in Fig. 3. Positions 2 and 3 of residue A are downshifted from the desulfated value both in the $^1$H (~0.88 and ~0.63 ppm, respectively) and $^1$C dimension, confirming sulfation in these two positions. Residue B is also sulfated at position 2, since it shows a $^1$H downshift of ~0.77 ppm. The chemical shifts are presented in Tables III and IV. For residues C, D, E, and F, no unambiguous cross-peak could be found. In this region several overlaps exist hampering the assignment strategy.

Three of them (A, B, and C) are between 5.6 and 5.2 ppm in agreement with α-anomeric protons while the three other (D, E, and F) are between 4.66 and 4.55 as expected for β-anomers. Integration of the two groups of anomers gave a 1:1 ratio.

Two-dimensional assignment techniques (TOCSY and COSY) were used to trace the spin systems but only two of them could be partially identified. Residue A is both 2-O and 3-O sulfated as seen by the unambiguous cross-peaks in the TOCSY spectrum (Fig. 4A). Positions 2 and 3 of residue A are downshifted from the desulfated value both in the $^1$H (~0.88 and ~0.63 ppm, respectively) and $^1$C dimension, confirming sulfation in these two positions. Residue B is also sulfated at position 2, since it shows a $^1$H downshift of ~0.77 ppm. The chemical shifts are presented in Tables III and IV. For residues C, D, E, and F, no unambiguous cross-peak could be found. In this region several overlaps exist hampering the assignment strategy.

The integrals of the three α-H1 resonances in the $^1$H spectrum suggest a 1:1:1 ratio of residues A:B:C. These integrals are derived from poorly resolved signals, and thus require careful interpretation. Nevertheless, they suggest that 2,3-di-O- and 2-O-sulfated residues account for one-third each of the total α-units in the sulfated β-galactan from *B. occidentalis*. The possible occurrence of 3,6-anhydrogalactose, a common component of algal polysaccharides, was excluded in the *B. occidentalis* galactan due to the absence of strong downshift of H6 in this type of residue (compare value for H6 in c and d with literature values for 3,6-anhydrogalactose in n and α, Table III).

Fraction F3 shows a $^1$H NMR spectrum similar to that obtained for F2 but with slight differences in the proportions of the various signals. In contrast, F1 has a much more complex spectrum which could not be resolved with the two-dimensional techniques.

In conclusion, methylation and NMR analyses indicate that fractions F2 and F3 obtained from *B. occidentalis* are linear polysaccharides, containing alternating residues of α(1→3)- and β(1→4)-d-galactopyranose. A variable sulfation pattern...
confers high heterogeneity to these polysaccharides. Nevertheless, it is clear that 2,3-di-O-sulfated α-D-galactopyranose residues occur as ~30% of the total α units of these galactans (Fig. 7). It was not possible to determine the structure of fraction F1. For this polysaccharide even high field NMR is at the limit of its powers and complete methylation was not achieved.

Anticoagulant Action of Sulfated Galactans and Sulfated Fucan from the Red Algae B. occidentalis and from Marine Invertebrates

Sulfated α-galactans from B. occidentalis are Potent Anticoagulant Polysaccharides—

### TABLE III

| Carbohydrate                      | Unit         | H1     | H2     | H3     | H4     | H5     | H6     |
|-----------------------------------|--------------|--------|--------|--------|--------|--------|--------|
| Native galactan from B. occidentalis | α, Unit A, see Fig. 3A | 5.62   | 4.73   | 4.58   |        |        |        |
| Desulfated galactan from B. occidentalis | c, 4-α-D-Gal-1 | 5.29   | 3.85   | 3.95   | 4.22   | 4.16   | 3.78   |
| Methyl α-β-galactopyranosides*   | e, α-β-Gal-1 | 4.84   | 3.82   | 3.81   | 3.97   | 3.90   | 3.74/3.75 |
| Native galactan from Clavelina sp.* | i, 4-α-L-Gal-1 | 5.03   | 3.89   | 3.95   | 4.13   | 4.34   | 3.81   |
| Desulfated galactan from Clavelina sp.* | j, 3-α-L-Gal-2(SO4)-1 | 5.47   | 4.65   | 4.23   | 4.35   | 3.85   | 3.82   |
| Native galactan from E. lucunter*   | k, 3-α-Gal-1  | 5.26   | 4.08   | 4.14   | 4.32   | 4.24   | 3.82   |
| Desulfated galactan from E. lucunter*   | l, 3-β-Gal-4(SO4)-1 | 4.85   | 3.62   | 4.00   | 4.89   | 3.78   | 3.80   |
| Native galactan from C. nipae*     | m, 3-β-Gal-1  | 4.61   | 3.62   | 3.82   | 4.10   | 3.65   | 3.86   |
| Desulfated galactan from C. nipae*   | n, 4-α-3,6-AnGal-2(SO4)-1 | 5.28   | 4.67   | 4.83   | 4.67   | 4.66   | 4.27   |
| Desulfated galactan from C. nipae*   | o, 4-α-3,6-AnGal-1 | 5.09   | 4.12   | 4.50   | 4.90   | 4.63   | 4.20   |

*Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicate positions bearing sulfate ester and those in italic indicate glycosylated positions.

Anticoagulant Activity of Sulfated Galactans and Sulfated Fucan from the Red Algae B. occidentalis. The assignment was based on TOCSY and COSY spectra. The values of chemical shifts in Tables III and IV are relative to external trimethylsilylpropionic acid at 0 ppm for 1H and methanol for 13C. The anomericities were identified by the characteristic carbon chemical shifts.
TABLE IV
Carbon chemical shifts for residues of α- and β-galactopyranosides in native and desulfated galactans

| Carbohydrate                        | Unit     | C1 | C2 | C3 | C4 | C5 | C6 |
|-------------------------------------|----------|----|----|----|----|----|----|
| Native galactan from *B. occidentalis* | a, Residue A, Fig. 3A | 100.5 | 74.5 | 78.2 | 78.2 | 78.2 | 78.2 |
| Desulfated galactan from *B. occidentalis* | c, 4-O-a-Gal-1 | 102.9 | 71.5 | 70.9 | 80.9 | 73.9 | 63.0 |
| Methyl α-L-galactopyranosides | d, 3-β-L-Gal-1 | 105.3 | 72.3 | 82.7 | 70.4 | 77.5 | 63.0 |
| Native galactan from *Clavelina sp.* | e, α-D-Gal-OMe | 100.7 | 69.5 | 70.8 | 70.5 | 72.0 | 62.5 |
| Desulfated galactan from *Clavelina sp.* | f, α-D-Gal-OMe-2(SO4) | 98.6 | 76.6 | 68.6 | 76.6 | 71.8 | 62.3 |
| Native galactan from *E. lucunter* | g, α-D-Gal-OMe-3(SO4) | 100.4 | 67.4 | 79.0 | 68.7 | 71.6 | 62.3 |
| Desulfated galactan from *E. lucunter* | h, 3-α-L-Gal-1 | 101.2 | 68.1 | 76.9 | 77.4 | 72.1 | 60.2 |
| Native galactan from *C. nipae* | i, 4-α-L-Gal-1 | 101.4 | 69.9 | 69.9 | 79.7 | 72.4 | 61.1 |
| Desulfated galactan from *C. nipae* | j, 3-α-L-Gal-2(SO4)-1 | 97.2 | 76.1 | 75.9 | 73.9 | 73.9 | 63.8 |
| Native galactan from *S. franciscanus* | k, 3-β-L-Gal-1 | 98.1 | 73.5 | 77.2 | 69.5 | 68.5 | 63.9 |
| Desulfated galactan from *S. franciscanus* | l, 3-β-Gal-4(SO4)-1 | 102.7 | 69.6 | 77.0 | 72.3 | 74.9 | 61.6 |

* Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicates positions bearing sulfated ester and those in italic refer to glycosylated positions.

§ Contreras et al. (39).
† Pavão et al. (40).
‡ Alves et al. (13).
* Falshaw et al. (41).

Fig. 7. Deduced preponderant structure of the sulfated D-galactan (fractions F2 and F3) from the red algae *B. occidentalis*. This polysaccharide has the following repeating structure (-4-α-D-Galp-1→3-β-D-Galp-1→), with a variable sulfation pattern. NMR analysis indicates the occurrence of 2,3-di-O-sulfated and 2-O-sulfated α-D-galactopyranose residues as one-third each of the total α units in the galactan. The structure of fraction F1 was not possible to determine.

The APTT assay (summarized in Table V) indicates that crude *B. occidentalis* polysaccharides have anticoagulant action (Table V, a). Purification of fractions F2 and F3 results in increased anticoagulant potency (Table V, c and d) comparable with that of unfractionated heparin (j). Fraction F1 has only a mild effect (Table V, b).

Comparison with sulfated polysaccharides from invertebrates shows that the sulfated L-galactan from *E. lucunter* has a significant anticoagulant action (Table V, h). Replacement of α-L-galactose by α-L-fucose (as in the case of the polysaccharide from *S. franciscanus*), sulfation in a different position, or the position of the glycosidic linkage (as in *H. monus*), decreases anticoagulant potency by severalfold (Table V, f and i). Insertion of non-sulfated L-galactose residues as branched units (as in *S. plicata*) abolishes the anticoagulant effect of the polysaccharide (Table V, g). Nevertheless, the sulfated galactans from *B. occidentalis* (fractions F2 and F3) are much more potent as anticoagulant than sulfated galactans/fucans from invertebrates. Possibly, this potent effect is related to the occurrence of 2,3-di-O-sulfated galactose units, since this type of unit is absent in the other galactans/fucan tested.

The sulfate content is important for anticoagulant action, since the desulfated galactan (Table V, e) lost the activity. However, it is not the only requirement since highly sulfated polysaccharides such as dextran sulfates have lower anticoagulant action than F2 and F3 (Table V, l and m). In addition, fractions F2 and F3 differ in sulfate content (Table I) but have

The sulfated galactans from red algae and from the marine invertebrates have similar molecular masses, as determined by polyacrylamide gel electrophoresis.
Anticoagulant properties of the sulfated polysaccharides derived from red algae and invertebrates, compared to standard compounds

| Source        | Species          | Polysaccharide                        | APTT \(^{a}\) |
|---------------|-----------------|---------------------------------------|---------------|
| Red algae     | B. occidentalis | a, Crude polysaccharides              | 30            |
|               |                 | b, Purified sulfated \(\beta\)-galactan, fraction F1 | 22            |
|               |                 | c, Purified sulfated \(\beta\)-galactan, fraction F2 | 150           |
|               |                 | d, Purified sulfated \(\beta\)-galactan, fraction F3 | 130           |
|               |                 | e, Desulfated \(\beta\)-galactan, fraction F2 | <1            |
| Tunicate      | H. monus        | f, Sulfated \(\beta\)-galactan, see Fig. 1B | 3             |
|               |                 | g, Sulfated \(\beta\)-galactan, see Fig. 1D | <1            |
| Echinoderm    | S. plicata      | h, Sulfated \(\beta\)-galactan, see Fig. 1A | 10            |
|               |                 | i, Sulfated \(\alpha\)-fucan, see Fig. 1C | 4             |
| Standard polysaccharides | | j, Unfractionated heparin\(^b\) | 193           |
|               |                 | k, Low molecular weight heparin\(^b\) | 30            |
|               |                 | l, Dextran sulfate, average mol. mass 50 kDa | 75            |
|               |                 | m, Dextran sulfate, average mol. mass 500 kDa | 82            |
|               |                 | n, \(\alpha\) Carrageenan            | 77            |

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

\(^b\) International standards.

**Fig. 8.** Dependence on the sulfated polysaccharide concentration for inactivation of thrombin (A and B) or factor Xa (C) by antithrombin (A and C) or heparin cofactor II (B). Antithrombin (50 nM) or heparin cofactor II (68 nM) were incubated with thrombin (15 nM) or factor Xa (15 nM) in the presence of various concentrations of the sulfated \(\beta\)-galactan from B. occidentalis (fraction F3) (●), unfractionated heparin (○), or mammalian dermatan sulfate (□). After 60 s, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate (\(A_{405 \text{ nm/min}}\)).

approximately the same potency as anticoagulant.

Thus, the data in Table V indicate marked differences in the anticoagulant effect of closely related sulfated galactans. This is not solely due to the sulfate content, but also to distinct structure and/or sulfation patterns of the polysaccharides.

**Sulfated \(\beta\)-Galactans from B. occidentalis Enhance Thrombin and Factor Xa Inhibition by Antithrombin and/or Heparin Cofactor II**—The sulfated \(\beta\)-galactan from B. occidentalis (fraction F3) enhances thrombin inhibition by antithrombin with an \(IC_{50}\) similar to that of unfractionated heparin (Fig. 8A). Replacement of antithrombin by heparin cofactor II results in a shift to the right of the F3 effect for thrombin inhibition, but this polysaccharide still has a \(IC_{50}\) 15-fold lower than that of mammalian dermatan sulfate (Fig. 8B). Finally, replacement of thrombin by factor Xa also decreases the inhibitory effect of fraction F3, compared with unfractionated heparin (Fig. 8C).

**Major Conclusions**

Our results indicate that the red algae B. occidentalis contains two fractions (F2 and F3) of regular sulfated \(\beta\)-galactans vesiculosus was recently reported (15), but in this case the amyloolytic assays were carried out at 25 °C. Under the same conditions the sulfated galactans from B. occidentalis also directly inhibit thrombin activity (not shown).

For comparison, we extended this set of experiments to the sulfated \(\beta\)-galactan/fucan from invertebrates (Table VI, d–f). The sulfated \(\beta\)-galactan from E. lucunter inhibits thrombin activity in the presence of either antithrombin or heparin cofactor II, but requires much higher concentrations than fractions F2 and F3 from B. occidentalis. Replacement of \(\alpha\)-l-galactose by \(\alpha\)-l-fucose (as in the polysaccharide from S. franciscanus; Table VI, f), sulfation in a different position, or the position of the glycosidic linkage (as in H. monus; Table VI, d), abolishes the inhibitory effect on thrombin.

The structural feature distinguishing B. occidentalis sulfated \(\beta\)-galactans is the occurrence of 2,3-dl-O-sulfated \(\beta\)-galactose units, which are absent in the other galactans or fucans tested in the experiment shown in Table VI. Possibly, this type of residue accounts for the potent thrombin inhibitory effect observed with these galactans.
composed of alternating α(1→3) and β(1→4) residues, but with a heterogeneous sulfation pattern. Clearly, 2,3-di-O-sulfated and 2-O-sulfated units account for approximately one-third each of the total α-residues. The structure of fraction F1 could not be determined.

Fractions F2 and F3 are potent anticoagulant polysaccharides due to enhanced thrombin/factor Xa inhibition by anti-thrombin and heparin cofactor II. Comparison with several well defined sulfated polysaccharides from marine invertebrates allows determination of some structure/biological activity relationships. Thus, galactans sulfated at either 2-O or 3-O positions of α-galactose residues (but not at both positions) have weak anticoagulant activity compared with B. *occidentalis* galactan. Presumably, the addition of two sulfate esters to a single α-galactose has an amplifying effect on anticoagulant activity. This is not merely a consequence of increased charge density. The anticoagulant activity increased 15-fold when comparing the 2-O-sulfated galactan of *E. lucunter* with *B. occidentalis* polysaccharide, fraction F2 (measured either as clotting time, Table V; or as IC₅₀ for thrombin inhibition, Table VI). On the other hand, the equivalent increase in sulfate content is ~50% (Table I). In addition, highly sulfated dextrans (~3 sulfate esters per glucose unit) have lower anticoagulant potency than the *B. occidentalis* galactan (Table V). Finally, the three linear and repetitive polysaccharides from invertebrates (Fig. 1, A-C) show dramatic differences in anticoagulant activity, despite their similar charge density. Therefore, the structural requirements for interaction of sulfated polysaccharides with coagulation factors are stereospecific. In agreement with this structural stringency, oversulfated dermatan sulfates show only discrete, selected sites competent for interaction with heparin cofactor II (14). A similar observation was extended for sulfated fucans (16).

The conformational analysis of these sulfated polysaccharides is an important route to follow. The differences in chemical structure may in fact determine spacing between sulfate groups required to match the interval between basic amino acid residues in the protein chain. Conformational analysis may explain the drastic differences in biological activity between a sulfated galactan and a sulfated fucan despite the same positions of sulfation and glycosidic linkage. Similarly, changes in biological activity may reflect dramatic modifications in the conformation of the polysaccharide as a consequence of the 2,3-di-O-sulfated α-galactose units.

Our results indicate that combining structural analysis of sulfated polysaccharides from marine algae and invertebrates with specific biological assays is a useful tool to investigate anticoagulant activity in mammals. These studies may help to delineate a closer relationship between structure and anticoagulant activity of sulfated polysaccharides, as already reported for heparin. New compounds with obvious practical applications may be found. Finally, the sulfated galactans from *B. occidentalis* are natural candidate molecules for testing in experimental thrombosis.

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Anticoagulant Activity of Sulfated Galactans

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