Structural and Hemostatic Activities of a Sulfated Galactofucan from the Brown Alga *Spatoglossum Schroederi*

**AN IDEAL ANTITHROMBOTIC AGENT?**

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The brown alga *Spatoglossum Schroederi* contains three fractions of sulfated polysaccharides. One of them was purified by acetone fractionation, ion exchange, and molecular sieving chromatography. It has a molecular size of 21.5 kDa and contains fucose, xylose, galactose, and sulfate in a molar ratio of 1.0:0.5:2.0:2.0 and contains trace amounts of glucuronic acid. Chemical analyses, methylation, and NMR spectroscopy showed that the polysaccharide has a unique structure, composed of a central core formed mainly by 4-linked β-galactose units, partially sulfated at the 3-O position. Approximately 23% of these units contain branches of oligosaccharides (mostly tetrascarbohydrates) composed of 3-sulfated, 4-linked α-fucose and one or two nonsulfated, 4-linked β-xylose units at the reducing and nonreducing end, respectively. This sulfated galactofucan showed no anticoagulant activity on several "in vitro" assays. Nevertheless, it had a potent antithrombotic activity on an animal model of experimental venous thrombosis. This effect is time-dependent, reaching the maximum 8 h after its administration compared with the more transient action of heparin. The effect was not observed with the desulfated molecule. Furthermore, the sulfated galactofucan was 2-fold more potent than heparin in stimulating the synthesis of an antithrombotic heparan sulfate by endothelial cells. Again, this action was also abolished by desulfation of the polysaccharide. Because this sulfated galactofucan has no anticoagulant activity but strongly stimulates the synthesis of heparan sulfate by endothelial cells, we suggested that this last effect may be related to the "in vivo" antithrombotic activity of this polysaccharide. In this case the highly sulfated heparan sulfate produced by the endothelial cells is in fact the antithrombotic agent. Our results suggested that this sulfated galactofucan may have a potential application as an antithrombotic drug.

The leading causes of death in the United States are diseases that involve heart and blood vessels and, consequently, thrombosis. The incidence of death because of thrombosis is almost two times higher than the next cause, cancer (1). Most thromboembolic processes require anticoagulant therapy. This explains the current efforts to develop specific and potent anticoagulant agents.

Unfractionated heparins and low molecular weight heparins are the only sulfated polysaccharides currently used as anticoagulant drugs. However, these compounds have several side effects such as bleeding and thrombocytopenia (2, 3). In addition, the commercial sources of heparins are mainly pig and bovine intestine. The possibility that prions and viruses could be carried by these molecules in addition to the increasing needs for antithrombotic therapies indicate the necessity to look for alternative sources of anticoagulant agents.

Marine brown algae are an abundant source of anticoagulant polysaccharides. They contain a variety of sulfated L-fucans with anticoagulant activity (4–11). The proposed mechanisms of action of these compounds are predominantly related to the "in vitro" inhibition of factors Xa and IIa mediated by antithrombin and heparin cofactor II. Besides the anticoagulant activity, some sulfated fucans possess other important pharmacological activities such as anticomplementary, anti-inflammatory, antiproliferative, antitumoral, antiviral, antiprptic, and antiadhesive activities (12–16).

Most of the structural requirements for the anticoagulant activity of sulfated fucans have not yet been determined; consequently, the structure-activity relationships remain to be elucidated. Most of the difficulties for these studies arise from the fact that these compounds are very heterogeneous polysaccharides, which give complex NMR spectra with broad signals hampering resolution (17). It is not always possible to define whether these algal polysaccharides have repetitive units. Furthermore, the structure of sulfated fucans varies according to the species of algae, as it is the case for heparan sulfates in vertebrates (5, 18). Thus, each new sulfated polysaccharide purified from a marine alga is a new compound with unique structures and, consequently, with potential novel biological activities.

Here we report the purification, structural characterization, and pharmacological activities of a new sulfated polysaccharide from the brown alga *Spatoglossum Schroederi*. This polysaccharide has a unique structure, composed of a central core of 4-linked, partially 3-sulfated β-galactose units. Approximately 25% of these units contain branches of oligosaccharides formed by nonsulfated β-xylose and 3-sulfated α-fucose units linked to the O-2 position of the central core. Of particular significance was the finding that this sulfated galactofucan has no anticoagulant activities but shows a potent antithrombotic activity with no hemorrhagic effect. We attributed the antithrombotic activity of this sulfated polysaccharide to its potent effect stimulating the synthesis of a
highly sulfated heparan sulfate by the endothelial cells of the vascular wall.

**MATERIALS AND METHODS**

**Reagents**—Chondroitin 4-sulfate was purchased from Miles Laboratories (Elkhart, IN). Heparan sulfate from bovine pancrease and heparin from bovine mucosa were gifts from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). Propylenediamine (1,3-diaminopropane) was purchased from Aldrich. Glucose, glucuronic acid, xylose, fucose, galactose, and chondroitinase AC and ABC were obtained from Sigma. Heparitinases I and II were prepared from induced fucose, xylose, and chondroitinase AC and ABC were obtained from Roche Applied Science. Human factor Xa (FXa) was purchased from Bio-Rad. Carrier-free [35S]inosinic sulfate was purchased from American Thiosemicarbazide Laboratories (São Paulo, SP Brazil). [3H]Methylthymidine (85 Ci/mmole) was purchased from American Signal Laboratories. Fucic acid was a gift from Dr. P. Bianchini (Opocrin). Dextran sulfate (MW 100,000) was obtained from Pharmacia, Uppsala, Sweden. Antithrombin was prepared as described (19).

**Extraction of Polysaccharides**—The marine alga *S. Schroederi* was collected on the seashore of Natal, RN, Brazil. Immediately after collection, the alga was dried at 50 °C under ventilation and was ground in a blender. The seaweed was then treated with acetone to eliminate lipids and pigments. One hundred grams of defatted, dried, and powdered alga was suspended in 500 ml of 0.25 M NaCl, and the pH was adjusted to 8.0 with NaOH. Twenty mg of maxatase, an alkaline protease from *Sporobacillus* (Biobras, MG, Brazil), was added to the mixture for proteolytic digestion. After 18 h of incubation at 60 °C under agitation, the mixture was filtered through cheesecloth. The filtrate was fractionated by precipitation with acetone as follows: 0.5 volumes of ice-cold acetone was added to the solution under gentle agitation and maintained at 4 °C for 24 h. The precipitate formed was collected by centrifugation (10,000 × g, 20 min), dried under vacuum, resuspended in distilled water, and analyzed. The operation was repeated by adding 0.6, 0.7, 0.9, 1.1, 1.3, and 2.0 volumes of acetone to the supernatant. The fraction precipitated with 0.9 volume of acetone (200 mg) contains the sulfated galactofucan used in the present work. This galactofucan was further purified by ion exchange chromatography (Lewatit from Bayer, São Paulo, Brazil) eluted stepwise with increasing concentrations of NaCl (0.25–3.0 M). The eluates were precipitated with 2 volumes of methanol (18 h, 4 °C). The precipitates were collected by centrifugation (10,000 × g, 15 min), dried, and resuspended in distilled water for subsequent analysis. The fraction eluted from the resin with 2 M NaCl was further purified by molecular sieving in Sephadex G-25 (120 × 1.8 cm). About 50 mg of sulfated galactofucan, dissolved in 2 ml of water, were applied to the column and eluted with a solution of 0.2 M acetic acid and 6 M urea, and fractions of 1 ml were collected and assayed by the phenol/ 

**Analysis of the Acidic Polysaccharides by Agarose Gel Electrophoresis**—Agarose gel electrophoresis of the acidic polysaccharides was performed in 0.6% agarose gels (7.5 × 10 cm, 0.2 cm thick) prepared in four different buffers as follows: 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0; discontinuous buffer 0.04 M barium acetate, pH 4.0; 0.05 M diaminopropane acetate, pH 9.0; 0.05 M KCI-HCl buffer, pH 2.0, or 0.06 M Tris acetate buffer, pH 8.0, as described previously (21, 22). Aliquots of the fractions (about 50 μg) were applied to the gel and subjected to electrophoresis. The gel was fixed with 0.1% cetyltrimethylammonium bromide solution for 4 h, dried, and stained for 15 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol. The gels were then destained with the same solution without the dye. The molecular weight was determined by HPLC in 0.2 M NaCl, 0.5% ethanol, using a GF-250 column (Asahipak GF series, Asahi Chemical Industry Co., Yokoh, Japan). The column was calibrated with standard glycosaminoglycans.

**Chemical Analyses**—The polysaccharides were hydrolyzed with 5 M trifluoroacetic acid. The resulting monosaccharides were converted to their alditol acetate derivatives and analyzed by gas chromatography. Fucose, xylose, and uronic acid content of the polymers were also estimated by the methods described by Dische (23–25). Total sugars were estimated by the phenol/H2SO4 reaction (26). After acid hydrolysis of the polysaccharides (6 M HCl, 100 °C, 4 h), the sulfate content was measured by the toluidine blue method, as described previously (27). The type of uronic acid was determined by electrophoresis in Whatman No. 3MM paper in 0.25 M ammonium formate buffer, pH 2.7 (28). The protein content was measured as described by Spector (29).

**Desulfation and Methylation of Fucan**—Desulfation of the polysaccharide was performed by solvolysis in dimethyl sulfoxide as used previously for desulfation of a sulfated fucan (30). The native and desulfated polysaccharides (10 mg) were subjected to three rounds of methylation, according to Patankar et al. (31). The methylated polysaccharides were hydrolyzed in 5 M trifluoroacetic acid for 5 h at 100 °C and reduced with borohydride, and the alditol acetates were acetylated with acetyl anhydride/pyridine (1:1, by volume) (32). The alditols acetates of methylated sugars were dissolved in ethanol and analyzed in a gas chromatography/mass spectrometer.

**NMR Experiments**—1H and 13C spectra of the fucan were recorded using a Bruker DRX 600 apparatus with triple resonance probe (30). About 15 mg of each sample was dissolved in 0.7 ml of 99.9% D2O (Cambridge Isotope Laboratory). All spectra were recorded at 60 °C with HOD suppression by presaturation. COSY, TOCSY, and 1H/13C HMOC spectra were recorded using states–times proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4096 × 400 points with a spin-lock field of 10 kHz and a mixing time of 80 ms. HMOC spectra were run with 1024 × 256 points and globally optimized alternating phase rectangular pulses for decoupling. NOESY spectra were run with a mixing time of 100 ms. Chemical shifts are relative to external trimethylsilylpropanionic acid at 0 ppm for 1H and to methyl for 13C.

**Anticoagulant Activity of the Galactofucan**—All the coagulation assays (prothrombin time, activated partial thromboplastin time; TT, thrombin time, and HEPTEST) were performed with a coagulometer, as described earlier (33), and were measured by using human plasma from Roche Applied Science. All assays were performed in duplicate and repeated at least three times on different days (n = 6).

**Antithrombotic Activity**—The inhibition of venous thrombosis produced after venae cavae ligature by sulfated polysaccharide was measured by the method of Rayers et al. (34). Briefly, the method consisted of exposing 1 cm of the inferior vein cavae of rats (below the left renal vein) and performing a ligature with cotton thread (number 8) 5 min after intravenous injection of the test substance. The abdominal cavity was then closed. After 2–24 h, the cavity was reopened, and the eventual thrombi formed were removed from the vein, washed, blotted with filter paper, dried under vacuum for 24 h, and weighed. At specific times, the sulfated polysaccharide was injected endovenously in a volume of 0.2 ml of saline. Ten determinations for each dose were performed. Heparin was used as control. The animal assays were approved by the Ethical Animal Research Committee of the Federal University of São Paulo.
Antithrombotic Agent without Anticoagulant Action

Hemorrhagic Effect—Hemorrhagic activity in a rat tail model of the polysaccharides was assayed as described previously (35). Following anesthesia with nembutal (40 mg/kg) and urethane (0.8 g/kg), scariﬁcation with a razor blade (1–2 mm deep and 5 mm long) was made 15 mm from the distal part of the rat tail (males, 3 months old). The tail was then immersed in isotonic NaCl, scraped with gauze, and immersed again in fresh saline to observe bleeding. The duration of bleeding of the control ranged from 30 to 60 s. Grazed tails were also immersed in saline solution containing sulfated galactofucan or heparin in different concentrations for 2 min and washed extensively with saline. The treated tails were then immersed in isotonic saline solution, and the amount of blood was measured by protein determination (29). The results were expressed as the sum of protein values of each tube minus the amount of blood present before exposure to the test substance.

Effect of Polysaccharides on the Synthesis of Heparan Sulfate by the Endothelial Cells—The effect of polysaccharide stimulation of the synthesis (36) of an antithrombotic heparan sulfate (37) by rabbit aorta endothelial cells (38) was performed essentially as described for heparin and other antithrombotic compounds (39). Briefly, at the end of the incubation, the culture medium was removed, and the cells were washed twice with serum-free F-12 medium. Protein-free heparan sulfate and chondroitin sulfate glycosaminoglycan chains were prepared from the culture medium by incubating the sample with 0.1 mg of SUPERase for 4 h at 60°C. At the end of the incubation, the mixture was heated for 7 min at 100°C, and the radiolabeled glycosaminoglycans were precipitated with 2 volumes of methanol in the presence of carrier heparan sulfate. The heparan sulfate and chondroitin sulfate synthesized by these cells and secreted to the medium were quantified and stained to a Kodak blue x-ray ﬁlm. The radioactive bands were characterized by their electrophoretic mobility in agarose gels and enzymatic degradation with glycosaminoglycan lyases (chondroitinases AC and ABC and heparitinases) as described previously (36, 39). The radiolabeled compounds were visualized by exposure of the gel after drying and staining to a Kodak blue x-ray ﬁlm. The radioactive bands were scraped from the gel and counted in a liquid scintillation counter using Ultima Gold (Packard Instrument Co.). Cell protein was estimated by a Coomassie Blue method. All the experiments were performed in triplicate for each data point. The bars of the ﬁgures indicate the ± S.E.

Effect of Polysaccharide on Cell Growth—Rabbit aorta endothelial cells were grown in F-12 medium (Invitrogen) supplemented with 10% FCS (Cultilab, São Paulo, Brazil), 100 µg/ml streptomycin, and 100 IU/ml penicillin (Sigma) at 37°C, 2.5% CO2. Endothelial cells, at 1 × 104 cell/plate, were seeded in 35-mm culture plates. The cells were maintained at G0 phase for 24 h by incubation in F-12 medium without serum. Cells were released from the G0 phase by addition of F-12 medium plus 10% FCS in the absence (control) or presence of 100 µg/ml sulfated galactofucan or heparin. Proliferation was measured by daily cell count (in triplicate) for 9 days. The cell viability was checked by trypan blue exclusion (40).

Thymidine Incorporation—The cell cycle was analyzed by [3H]thymidine incorporation. Quiescent cells were incubated with [3H]thymidine (0.25 µCi/ml) in the absence (control) or presence of sulfated galactofucan or heparin (100 µg/ml) for various times. The cells were then washed three times with phosphate-buffered saline and harvested with 3.5 M urea in 10 mM Tris-HCl buffer, pH 8.0. The incorporated radioactivity in the cells was determined by scintillation counting as described previously (40).

Assay for Factor Xa Activity—Confluent endothelial cells grown in 100-mm culture plates were incubated for 24 h (37°C, 2.5% CO2) in F-12 medium without phenol red in the presence or absence of sulfated galactofucan (100 µg/ml). At the end of the incubation, conditioned media were removed, and aliquots (10–100 µl) were assayed for FXa. F-12 medium not exposed to the cells was used as a negative control. The conditioned media were also tested after incubation with heparinases (50 µl to a final volume of 500 µl in the presence of 0.05 units of heparinases at 30°C, pH 7.0, overnight), and aliquots proportional to the original volume were assayed for FXa. These enzymes are free of proteolytic activity (20). Briefly, the assay for FXa consisted of preincubating 5 µl of FXa (20 nM) with the different media (10–100 µl) in 96-well plate for 5 min at 37°C. An aliquot of 5 µl of the synthetic substrate (S2222) (4.0 mM) was added to a ﬁnal volume of 200 µl and incubated at 37°C for 3000 s. The activity was continuously monitored by measurement of the absorbance at 405 nm (41) by using an enzyme-linked immunosorbent assay reader (Tecan, model Sunrise, Grödig, Austria) and the software Magellan version 5.01 (Tecan, Grödig, Austria). Three different sets of experiments were performed in duplicate for each condition investigated. The results were analyzed by nonlinear regression using GraphPad Prism version 3.0, and each point represents the mean ± S.E. The results are expressed as the ratio of the absorbance for the different experimental conditions relative to the negative control. Statistical analysis was performed using analysis of variance test of p < 0.05 and Student’s t test of p < 0.05.

RESULTS

Puriﬁcation of a Sulfated Galactofucan—In a previous work we puriﬁed a sulfated fucan from the marine alga S. Schroederi, designated as “fucan A” (6). However, we did not succeed in obtaining puriﬁed frac-
tions of another sulfated polysaccharide present in high amounts in the extracts from the alga, originally designated as “fucan B.” We now applied a simple methodology for the purification of these polysaccharides, based on precipitation with different concentrations of acetone. The electrophoretic mobility of the various fractions on agarose gel, using diaminopropane/acetate buffer, is shown in Fig. 1 A. Although the electrophoretic profiles show the presence of two or even three bands in the various fractions, clearly precipitation with 0.9% (v/v) acetone yields a single spot, whose electrophoretic mobility corresponds to fucan B, as we reported previously (5).

The chemical composition of the fractions obtained at various concentrations of acetone is shown in TABLE ONE. The relative proportions of sugars vary among the various fractions. Thus, uronic acid is the main sugar present in the polymers precipitated with 0.5 and 0.6% of acetone, possibly due to the presence of alginic acid. Neutral sugars and sulfate are found in greater amounts in the 0.7–2.0% acetone fractions. It is clear that the relative amounts of these sugars vary according to the fraction. Of course this variation in the sugar composition may be a consequence of different types of polysaccharides found in these fractions, except for the one obtained with 0.9% acetone. This fraction shows a single electrophoretic band on agarose gel, and the sugar composition reported in TABLE ONE possibly indicates the occurrence of a sulfated polysaccharide in S. Schroederi, containing galactose, fucose, sulfate, xylose, and traces of uronic acid, which we hereby will designate as sulfated galactofucan. These fractions were not contaminated with laminarans (a group of the reserve polysaccharide glucans found in brown algae) because glucose was not detected.

We further purified the sulfated galactofucan that is the fraction precipitated with 0.9% acetone, using ion exchange chromatography on a Lewatite resin. The polysaccharide was separated into three new fractions eluted with 1.5, 2.0, and 3.0 M NaCl. The fraction eluted with 2.0 M NaCl yielded 80% of the total polysaccharide. It was further subjected to gel chromatography on Sephadex G-75, revealing the presence of a single component. The electrophoretic migration of the sulfated galactofucan obtained after the ion exchange and gel filtration chromatography is shown in Fig. 1 B. Only a single band was obtained after these steps of purifications. Also a single component was observed in agarose gel electrophoresis in three different buffer systems, indicating that the sulfated galactofucan was essentially pure and free of other acidic polysaccharides (Fig. 1, C–E).

Chemical analysis of the sulfated galactofucan after the ion exchange and gel filtration chromatography confirms that this polysaccharide contains galactose, fucose, xylose, and sulfate in a molar ratio of ~2.0: 1.0: 0.5 and 2.0, besides minor amounts of uronic acid (TABLE TWO). A

![FIGURE 2.](image-url)
high content of sulfate esters confers a high negative charge density for this polysaccharide. In fact, the sulfated galactofucan was eluted from the Lewatite column at NaCl concentrations similar to the concentration used for the elution of heparin from this resin. The molecular mass of the fucan obtained by HPLC was 21.5 kDa.

NMR Spectroscopy—The $^1$H one-dimensional spectrum of the sulfated galactofucan is shown in Fig. 2. Two-dimensional assignment techniques of COSY, TOCSY, NOESY (Fig. 3), and HMQC (Fig. 4) were used to trace the spin systems. The chemical shifts in TABLE THREE are based on the interpretations of these spectra. At least seven distinguishable anomeric resonances were observed, two $\alpha$ systems (namely A and B) and five $\beta$ systems (C, D, E, F, and G).

The two main $\alpha$-anomeric protons were observed at 5.31 and 5.08 ppm, named as A and B units. The spin systems can be traced (Fig. 3A)
giving the values of TABLE THREE, which are compatible with 3-sulfated, 4-linked α-d-fucopyranoside residues. Thus, strong downshifts (approximately −0.40 ppm) of H-3 of these two residues relative to H-3 of nonsulfated fucose standards indicate that these two residues are sulfated at C-3. The strong downfield shift (approximately −13 ppm) of C-4 of C is compatible with 4-linked α-d-fucopyranoside residues. The difference between these two fucose units is clear on the NOESY spectrum (Fig. 3A), as NOEs between protons of different units can be seen. Thus, H-1 of A shows strong cross-peak to H-2 of residue D (β-galactose units glycosylated at C-2 and C-4, see below), providing evidence that this fucose unit is linked to the branching point of the central polysaccharide core. In contrast, H-2 of B shows cross-peaks to H-2, H-4, and H-5 of residue F (nonsulfated β-xylene units, see below).

In contrast with the simplicity of the two β-fucose residues, the β systems show a certain degree of multiplicity, probably due to diversity in the positions of interglycosidic linkages of sugar residues. The β-anomeric protons appeared as two unresolved multiplets centered at 4.5 and 4.7 ppm, together with signals of protons from sulfation sites. Residues C–E and G were assigned to β-galactose units. The downfield shift of H-3 in residues C and G indicates sulfation at this site. The preponderance of 4-linked units is indicated by the downfield shift of C-4. For residues of β-galactose, no unambiguous NOEs between different residues can be seen. However, the NOESY spectrum reveals the presence of cross-peaks between H-2 of F (terminal nonsulfated β-xylene) and H-2 of residue C (3-sulfated, 4-linked β-galactose).

Overall, NMR analysis of the sulfated galactofucan from S. schroederi is compatible with a polymer formed by a central core of 4-linked, partially 3-sulfated β-galactose units and branching oligosaccharides composed of 3-sulfated, 4-linked α-fucose residues, with nonsulfated β-xylene at the nonreducing terminals.

*Methylation Studies—* The native and desulfated galactofucan (loss of 75% sulfate groups) were submitted to three rounds of methylation (TABLE FOUR). The methylation data were not consistent with known polysaccharide structures. In fact, methylation of sulfated polysaccharides does not always yield reliable proportions of methylated alditols (7). This may be a consequence of steric hindrance because of 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. However, some conclusions are clearly derived from the methylation analysis.

After desulfation, the proportion of 2-O-methylfucose (26%) disappeared at the expense of 2,3-dimethylfucose (25%). This result indicates the presence of 4-linked fucopyranosyl units with sulfate groups at the C-3 position, as already indicated by NMR analysis. The proportions of methylated derivatives from xylose remain unchanged after desulfation, which indicates that these residues are not sulfated in the native
polysaccharide. Xylose (5.7%) and galactose (4.2%) as terminal units indicate that the sulfated galactofucan is a highly branched polysaccharide.

A large dispersion of galactose derivatives was observed. This may indicate that the galactose residues are highly heterogeneous, as already suggested by the NMR analysis. Nevertheless, some conclusions are

| Glycosyl residue | Position of O-methyl group | Deduced position of substitution | Fucan | Desulfated fucan |
|------------------|---------------------------|----------------------------------|-------|-----------------|
|                  |                           | mol %                            | mol % |
| Xylosyl          | 2,3,4                     | Terminal                         | 5.7   | 7.4             |
|                  | 2,3                       |                                  | 12.8  | 13.0            |
| Fucosyl          | 2,3,4                     | Terminal                         | 3.8   |                 |
|                  | 2,3                       |                                  | 2.5   | 25.2            |
|                  | 2,4                       |                                  | 2.9   | 2.8             |
|                  | 2                         |                                  | 26.0  |                 |
|                  | 0                         | 2,3,4                            | 1.7   |                 |
| Galactosyl       | 2,3,4,6                   | Terminal                         | 4.2   | 10.1            |
|                  | 2,3,6                     |                                  | 4     | 12.3            |
|                  | 2,6                       |                                  | 3.4   | 11.6            |
|                  | 2,3                       |                                  | 4.6   | 4.7             |
|                  | 3,6                       |                                  | 2.4   | 9.4             |
|                  | 2                         |                                  | 3.4,6 | 6.3             |
|                  | 3/4                       | 2,4,6/2,3,6                      | 10.1  | 9.4             |
|                  | 0                         | 2,3,4,6                          | 12.0  | 1.9             |

**TABLE FIVE**

**Anticoagulant activity of fucan**

| Compound, fucan | PT* | APTT | HEPTEST* | TT |
|-----------------|-----|------|----------|----|
| mg              | s   | s    | s        | s  |
| 0               | 12.4| 36.4 | 13.8     | 30.8|
| 0.2             | 12.3| 35.8 | 13.1     | 30.8|
| 0.4             | 11.9| 35.3 | 13.8     | 31.3|
| 0.8             | 11.8| 34.8 | 13.8     | 30.8|
| 1.5             | 12.9| 34.8 | 12.3     | 30.8|
| 3.1             | 11.8| 35.3 | 13.3     | 31.3|
| 6.2             | 12.4| 35.4 | 13.3     | 30.3|
| 12.5            | 12.4| 36.3 | 13.3     | 31.8|
| 25              | 12.3| 38.3 | 12.9     | 31.3|
| 50              | 12.2| 36.4 | 13.3     | 30.8|
| 100             | 13.3| 35.6 | 12.9     | 31.3|
| Heparin (0.1 mg)| >80 | >600 | >120     | >300|

* The abbreviations used are as follows: PT, prothrombin time; APTT, activated partial thromboplastin time; HEPTEST, heparin cofactor II time; TT, thrombin time.
Methylation analysis of desulfated fucan showed the disappearance of 2,6-di-O- and 2-O-methylgalactose at the expense of 2,3,6-tri-O- and 2,3-di-O-methylgalactose, indicating 1→4-linked galactose units with sulfate groups at C-3 and some substitutions at C-2, such as fucose or a branching point of galactoses, which is unlikely because of the NMR results. The relative amount of terminal galactose (4.2%) is consistent with a molecular mass of 20 kDa. The increase of terminal sugars after desulfation is probably related to some fragmentation during the desulfation process.

Fig. 5 summarizes a proposed structure for this sulfated galactofucan. NMR and methylation analysis suggest the polysaccharide has a linear chain of 4-linked, partially 3-sulfated β-galactose units, with branches of 3-sulfated, 4-linked α-fucose at the C-2 position of the central core. About half of the β-xylene residues but only minor amounts of fucose are at the nonreducing ends of the fucose side chains. NMR analysis suggests that xylose is also linked directly to galactose. Because glucuronic acid is only a minor constituent of the polysaccharide, which could result from minor contamination with other polymers such as fucan A (6), it was not included in the structural hypothesis.

Anticoagulant and Hemorrhagic Activities—No anticoagulant activity was found for the sulfated galactofucan in all the coagulation tests used (TABLE FIVE). Fig. 6 shows that the polysaccharide has no hemorrhagic activity when compared with heparin. Similar results were obtained with fucan A from the same brown seaweed.

In Vivo Antithrombotic Activity—A dose-response curve of the sulfated galactofucan in a venous model of thrombosis in rat is shown in Fig. 7A. No antithrombotic activity was found for all concentrations tested 1 h after administration of the polysaccharide. Most surprisingly, when the sulfated galactofucan was injected endovenously 24 h before the ligature of the vein cave, we observed a dose-dependent antithrombotic effect reaching saturation around 10 mg/kg of rat weight (Fig. 7B). Fig. 7C shows that this effect is also time-dependent, reaching the maximum antithrombotic effect around 8 h after administration of the sulfated galactofucan and remaining for more than 24 h. The antithrombotic activity was not detected with the desulfated fucan (data not shown).

Antithrombotic Agent without Anticoagulant Action

Sulfated Galactofucan Stimulates the Synthesis of Heparan Sulfate by Endothelial Cells—We have demonstrated previously that fucan A from S. schroederi was as effective as heparin in stimulating the synthesis of an antithrombotic heparan sulfate from endothelial cells (6, 36, 39). Thus, the effect of the present sulfated galactofucan was also investigated. The electrophoretic and enzymatic analysis of the sulfated glycosaminoglycans synthesized by endothelial cells showed that they are indeed heparan sulfate and chondroitin sulfate (data not shown). Nevertheless, the galactofucan specifically stimulated by 4-fold only the synthesis of heparan sulfate by endothelial cells in culture (Fig. 8). In addition, this experiment also shows that the sulfated galactofucan is 2-fold more potent than heparin itself in stimulating the synthesis of the antithrombotic heparan sulfate. Desulfation of the polysaccharide abolishes this effect (data not shown).
This effect of the sulfated galactofucan could be related to cell proliferation leading to an apparent increase of heparan sulfate synthesis. To rule out this possibility, the sulfated galactofucan was incubated with the endothelial cells for 9 days. Thereafter, the cells were harvested and counted. Fig. 9A shows that there are no differences between the number of cells in the presence or absence of the polysaccharide. Cell viability was 98%, indicating an absence of cytotoxic activity. In addition, it was observed that the sulfated galactofucan did not affect thymidine incorporation in various phases of the cell cycle (Fig. 9B).

We next focused on the effect of the conditioned media obtained from cells exposed or not exposed to the sulfated galactofucan upon thrombin (FIIa) and FXa activities. Fig. 10 shows that conditioned media regardless of the presence of the polysaccharide are capable of inhibiting FXa activity. It thus indicate that the endothelial cells synthesize compound(s) that are related to this action. Furthermore, the data also demonstrate that the media from cells exposed to the polysaccharide show higher inhibition of FXa activity when compared with the media from cells not exposed to the sulfated galactofucan. Thus, this result suggests that the polysaccharide enhances the synthesis of the compound(s) involved in this particular action. In addition, when galactofucan was added in vitro directly to the medium (negative control), no alteration in this inhibitory activity was observed (data not shown) (Fig. 10). On the other hand, this activity was totally abolished when the conditioned media were incubated with heparitinases (Fig. 10), thus indicating that the compound is heparan sulfate. Antithrombin had no influence on those effects (data not shown). Furthermore, no effect was observed when the conditioned media were incubated with thrombin both in the presence and absence of antithrombin (data not shown).

**DISCUSSION**

**Structural Features of the Sulfated Galactofucan**—The presence of sulfated fucans has been shown previously in different seaweeds such as *Sargassum vulgare*, *Dictyota mertensis*, *Padina gimnospora* (5), *Dictyota menstrualis* (8), and *Sargassum stenophyllum* (45). We were able to show that the brown alga *S. Schroederi* contains three main sulfated polysaccharides, named as fucan A, B, and C according to their relative migration in agarose gel electrophoresis in 1,3-diaminopropane acetate buffer (6). By a combination of ion exchange chromatography and electrophoresis, we have purified fucan A and proposed its structure after chemical analysis, methylation studies, NMR spectroscopy, and enzymatic degradation. Fucan A is a xylofucoglucuronan, with a molecular size of 21 kDa, containing a core oligosaccharide composed of 3-linked β-glucuronic acid and branches of 3-linked, mostly 4-sulfated α-fucose chains. The fucose units are also substituted at C-4 with chains of 3-linked β-glucuronic acid and branches of 3-linked, mostly 4-sulfated α-fucose chains. The fucan B, designated as sulfated galactofucan, is by using the same methodology but with an additional fractionation step. Interestingly, the present sulfated galactofucan contains...
galactose as its main constituent, which is absent in fucan A. The chemical analyses showed that the present fucan is indeed a sulfated xylogalactofucan. This leads to the conclusion that both polysaccharides, despite being present in the same seaweed, show striking structural differences regarding their core oligosaccharides as well as the type of sugar residues, position of glycosidic linkages, and sulfation sites. These differences certainly reflect their pharmacological activities.

Since the 1950s (46), several sulfated fucans containing xylose and galactose have been described, but only a few with galactose as the major component (45, 47, 48). The molecular weight of the present sulfated galactofucan (21.5 kDa) is similar to those encountered in other brown seaweed fucans (5, 48, 49), although in many cases products with values higher than 50 kDa were found (16, 48, 50, 51).

The structural features of several sulfated fucans have been investigated by using a complex sequence of procedures for the extraction and purification of the polysaccharide (45, 50, 52, 53). Usually the fractions selected for biological studies do not represent the major polymer biosynthesized by the seaweed, but the polysaccharides were selected because of their high content of fucose or sulfate and potent anticoagulant activity. In most cases these homofucans have an α(1 → 3) linkage or a repeating structure of alternate α(1 → 3) and α(1 → 4) glycosidic linkages. The position of the sulfate groups was found to be mainly in position O-4 and/or O-2.

Most of the difficulties for structural characterization of these polysaccharides arise from their heterogeneity, which give complex NMR spectra with broad signals hampering resolution. In fact, for these algal polysaccharides even high field NMR is of limited value, and complete descriptions of their structures are not available at present (50, 54).

The use of gel electrophoresis in different buffer systems, as a criteria of purity for these compounds, allowed us to isolate different sulfated polysaccharides in high yields. This approach differs from the methodologies used by different authors whose criteria are based mainly in the enrichment of a specific sugar or a biological activity.

Antithrombotic Agent without Anticoagulant Action

Dissociation of the Anticoagulant and Antithrombotic Activities—The sulfated galactofucan purified from the marine alga S. Schroederi showed no anticoagulant activity on several in vitro assays. This was a surprising result considering the high sulfate content of the polysaccharide. Possibly, the presence of nonsulfate xylose units at the nonreducing terminal ends of the branches found in the polysaccharide may prevent its interaction with coagulation cofactors and their target proteases. A similar observation was reported for a variety of sulfated galactans from marine invertebrates. In this case, insertion of nonsulfated galactose residues as branched units abolished the anticoagulant effect of the polysaccharide (55).

When the sulfated galactofucan was tested on an experimental model of thrombosis in rat immediately after its intravenous administration, it showed no antithrombotic activity. However, 8 h after its intravenous injection, and even after 24 h of administration, the sulfated galactofucan showed significant antithrombotic activity. This is unique because for the antithrombotic polysaccharides tested so far, like unfractionated heparin and its low molecular weight derivatives, the effect is more transient with a half-life of 3 and 12 h, respectively. This observation raises interesting questions concerning the mechanism of antithrombotic action of the algal polysaccharide. Is it the polysaccharide itself that yields the antithrombotic effect? Is it a metabolic derivative from the algal polysaccharide responsible for the biological activity? Or does the sulfated galactofucan induce metabolic modifications in the vessel walls, which ultimately prevent the formation of thrombus?

To clarify these aspects, we tested the effect of the sulfated galactofucan on endothelial cells grown in culture. The polysaccharide did not stimulate proliferation of the cells but induced the synthesis of a highly sulfated heparan sulfate, which may in fact be the antithrombotic agent. The sulfated galactofucan is 2-fold more potent than heparin. In addition, this effect was only observed for heparan sulfate synthesis similar to the effect of heparin (4, 36, 39, 56). Because the synthesis of the heparan sulfate by endothelial cells in culture exposed to the fucan requires ~4–6 h for the full effect (results not shown), it explains the delay to detect the antithrombotic effect of the algal polysaccharide on the experimental model of thrombosis. It requires the synthesis and accumulation of the highly sulfated heparan sulfate on the vessel wall.

In the 1980s, Colburn and Buonassisi (37) reported that rabbit aorta endothelial cells showed blood compatibility, i.e. the surface of these cells had antithrombotic activity. By using an antibody against heparan sulfate, they neutralized the anti-clotting activity of endothelial cells culture medium, showing that this effect was at least in part heparan sulfate-dependent. In the present work, by using another methodology, we show that the conditioned medium from endothelial cells show ant-FXa activity. This effect is increased when the cells are exposed to sulfated galactofucan. This anti-FXa activity disappeared after heparitinase treatment, showing that this activity may be related to the heparan sulfate produced by endothelial cells. It seems that the effect does not need the presence of antithrombin, because no differences were observed in the presence or absence of antithrombin. On the other hand, the media did not inhibit thrombin activity either in the presence or absence of antithrombin.

Possibly heparin prevents thrombosis because of its combined effect as an anticoagulant and an inducer of the synthesis of heparan sulfate by the endothelial cells. These two effects are difficult to distinguish. However, the algal sulfated galactofucan, which is devoid of anticoagulant action but has a potent action inducing the synthesis of heparan sulfate by the endothelial cells, allowed us to emphasize this last mechanism involved in the prevention of thrombosis.
Antithrombotic Agent without Anticoagulant Action

Finally, the findings that the sulfated galactofucan had no hemorrhagic activity or proliferative or cytotoxic actions make it an ideal candidate for further studies as an antithrombotic agent.

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