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Anticoagulant and antithrombotic activities of modified xylofucan sulfate from the brown alga *Punctaria plantaginea*

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ABSTRACT

Selectively and totally sulfated (1 → 3)-linked linear homofucans bearing ~20 monosaccharide residues on average have been prepared from the branched xylofucan sulfate isolated from the brown alga *Punctaria plantaginea*. Anticoagulant and antithrombotic properties of the parent biopolymer and its derivatives were assessed *in vitro*. Highly sulfated linear fucan derivatives were shown to inhibit clot formation in APTT assay and ristocetin induced platelets aggregation, while the partially sulfated analogs were inactive. In the experiments with purified proteins, fucan derivatives with degree of sulfation of ~2.0 were found to enhance thrombin and factor Xa inhibition by antithrombin III. The effect of sulfated fucans on thrombin inhibition, which was similar to those of heparinoid Clexane® (enoxaparin) and of a fucoidan from the brown alga *Saccharina latissima* studied previously, can be explained by the multicenter interaction and formation of a ternary complex thrombin–antithrombin III–polysaccharide. The possibility of such complexation was confirmed by computer docking study.

1. Introduction

Sulfated fucose-rich polysaccharides of marine origin are considered as very promising biologically active substances which can be used as bases for new drug development (Mourão, 2015; Pomin, 2012a). There are three classes of such polysaccharides: fucosylated chondroitin sulfates from sea cucumbers (Pomin, 2014), fucan sulfates from sea urchins (Pomin, 2009) and fucoidans from brown seaweeds (Usov & Bilan, 2009; Cumashi et al., 2007; Li, Lu, Wei, & Zhao, 2008; Ustyuzhanina et al., 2014a). These biopolymers were shown to inhibit blood coagulation, thrombus formation, inflammation, angiogenesis, adhesion of viruses and bacteria, but are of special interest as possible heparin substitutes in the therapy of thromboembolic diseases (Mourão, 2015). Many papers to date have been devoted to the elucidation of relationships between chemical structure and biological action of sulfated polysaccharides, and some important preliminary structure-activity conclusions have been made for polysaccharides from echinoderms, due to their more or less regularly constructed molecules (Pomin, 2009, 2012b, 2014).

It should be noted that fucans of invertebrates have very limited availability. Fucoidans from brown algae are available in unlimited amounts but possess significantly more complicated structure than fucans from echinoderms, due to non-regular sulfation pattern and branching, presence of non-fucose monosaccharide residues and acetyl groups. Therefore, the development of efficient approaches for the transformation of structurally irregular algal fucoidans into their derivatives with well-defined structure and required biological activity remains very challenging.

The anticoagulant and antithrombotic properties of sulfated polysaccharides are determined by their ability to potentiate interaction of thrombin with antithrombin (ATIII) and/or heparin cofactor II (HCII), but other mechanisms, such as direct thrombin inhibition, are also considered. The main structural features of sulfated polysaccharides, which should be taken into account regarding their anticoagulant and antithrombotic properties, include degree and pattern of sulfation as well as molecular weight (M_W). Fucans with higher M_W usually demonstrated greater anticoagulant effect than structurally similar polysaccharides having lower M_W. The sulfation level above 1.0 was shown to be important for high anticoagulant activity of sulfated fucans and galactans. It was found that 2-*O*-sulfated (1 → 3)-linked α-L-galactan, but not an α-L-fucan with similar sulfation pattern and molecular size, was a potent thrombin inhibitor mediated by ATIII

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or HCII (Pomin, 2012a,b). It was found that, besides ATIII- and HCII-mediated thrombin inhibition activities which are typical for sulfated fucans, algal fucoidans could be also direct inhibitors of thrombin (Pereira, Mulloy, & Mourão, 1999; Ushakova et al., 2009). In the case of HCII-mediated inhibition the major structural requirement for the activity was the presence of selectively 4-*O*-sulfated fucose units (Pereira, Melo, & Mourão, 2002). Additionally, it was shown that linear (1 → 3)-linked α-L-fucans enriched in 2,4-di-*O*-sulfated units had an amplifying effect on the ATIII-mediated anticoagulant activity (Fonseca, Santos, & Mourão, 2009).

In our previous studies we attempted to reveal the correlation between the chemical structure and biological properties of fucoidans by comparison of several native and chemically modified polysaccharides and synthetic sulfated oligosaccharides in a series of anticoagulation tests (Cumashi et al., 2007; Ustyuzhanina et al., 2013). In the present paper a unique sulfated xylofucan (**1**) isolated from the Pacific brown alga *Punctaria plantaginea* (Bilan, Shashkov, & Usov, 2014) and several more regular products of its chemical modifications have been studied as anticoagulants and antithrombotics in comparison with the most active fraction of a fucoidan (**SL**) from *Saccharina latissima* (Bilan et al., 2010) and with the totally and partially sulfated derivatives of synthetic *n*-propyl octa-(1 → 3)-α-L-fucoside (Ustyuzhanina, Krylov, Grachev, Gerbst, & Nifantiev, 2006; Krylov et al., 2011).

2. Experimental procedures

2.1. Materials and methods

Isolation of sulfated xylofucan **1** (designated as PPF) and analytical procedures were described (Bilan et al., 2014) along with the preparation of desulfated xylofucan **4** (designated as PPFdeS) and both Smith-degraded native **2** (designated as PPF-Sm2) and desulfated **5** (designated as PPFdeS-Sm) polysaccharides (for polysaccharide formulae see Scheme 1). Briefly, a sulfated polysaccharide was extracted from the algal biomass with hot diluted aqueous $CaCl_2$ solution, precipitated with cetyltrimethylammonium bromide and transformed into water-soluble sodium salt. Its desulfation was performed by solvolysis in DMSO–MeOH, and Smith degradation was carried out by conventional periodate oxidation, borohydride reduction and partial acid hydrolysis. Monosaccharide composition was determined by GLC of alditol acetates, and sulfate was estimated by turbidimetry.

2.2. Polyacrylamide gel electrophoresis (PAGE)

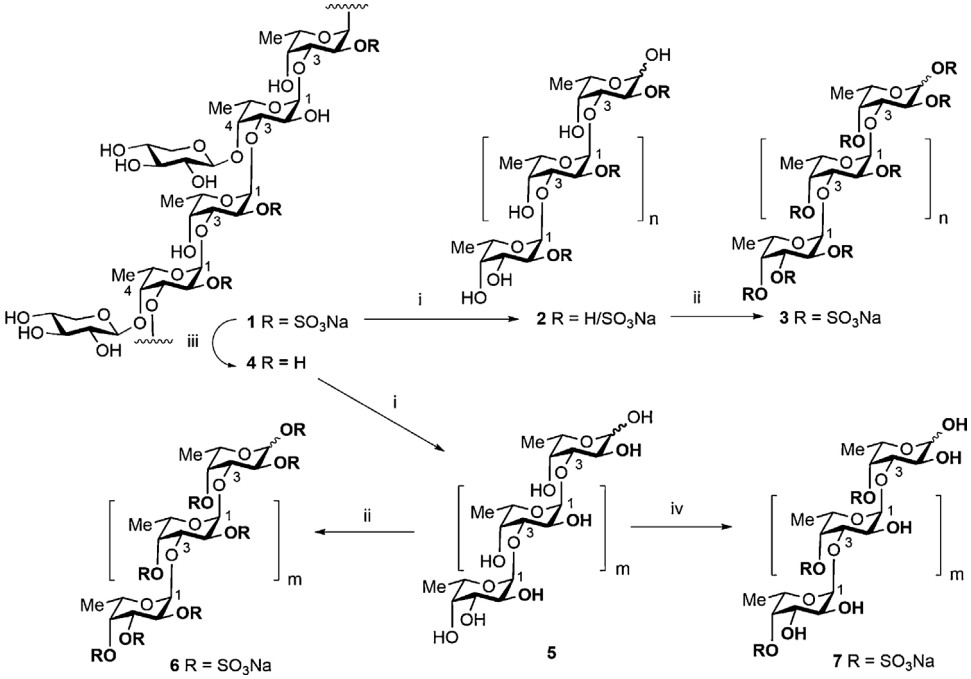
Each tested sample (25 μg) was applied to a 0.75-mm-thick 20% polyacrylamide (ICN Biochemicals) gel in a buffer (10 mM Tris-borate, pH 8.3) with 10% (w/v) of glycerol. Electrophoresis was run at 400 V in a buffer (100 mM Tris-borate, pH 8.3) during 1 h. The gel was stained with 0.1% toluidine blue (Merck, DE) in 1% acetic acid (Sigma, EUA). After staining the gel was washed overnight in 1% acetic acid. The results are presented in Fig. 1.

2.3. NMR spectroscopy

^{1}H and ^{13}C spectra of the saccharides were recorded using a Bruker AV-600 spectrometer. All spectra were recorded at 303 K with HOD suppression by pre-saturation. COSY and HSQC spectra were recorded using standard Bruker pulse sequences. Chemical shifts are relative to trimethylsilylpropionic acid at 0 ppm for ^{1}H and to acetone for ^{13}C at 29.9 ppm.

2.4. Preparation of fucoidan derivatives *3*, *6* and *7*

Transformation of fucoidan derivatives **2** and **5** into respective highly sulfated polymers **3** and **6** was performed under per-*O*-sulfation conditions as described previously (Krylov et al., 2011). Briefly, to a stirring solution of a polysaccharide (50 mg) in 3 mL of DMF (Sigma-Aldrich, DE) Py·SO_3 complex (450 mg, 2.8 mmol) and HSO_3Cl (6 μL, 0.09 mmol) were added at 0 °C under Ar atmosphere. The mixture was kept for 24 h at 0 °C, then $NaHCO_3$ (500 mg), Amberlite IR-120 (Na^+) and water (50 μL) were subsequently added at 0 °C, and the mixture was stirred for 1 h. Then the resin was filtered off, the filtrate was concentrated to 1 mL under reduced



Scheme 1. Reagents and conditions: (i) (1) $NaIO_4$, then $NaBH_4$, and H^+; (ii) Py·SO_3, HSO_3Cl, DMF, 0 °C; (2) $NaHCO_3$, Amberlite IR-120 (Na^+); (iii) MeOH, DMSO; (iv) (1) Py·SO_3, HSO_3Cl (excess), DMF, rt; (2) $NaHCO_3$, Amberlite IR-120 (Na^+).