Study of sulfated derivatives of polyhydroxy compounds as inhibitors of blood coagulation

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Persulfated derivatives of natural polyhydroxy compounds, such as lignans secoisolariciresinol, and isolariciresinol, flavonoid dihydroquercetin, and *myo*-inositol, have been synthesized. The ability of these compounds to inhibit the intrinsic pathway of blood coagulation (APTT-test) and to reduce the activity of coagulation factor Xa in the presence of antithrombin(III) has been studied.

Key words: O-sulfation, anticoagulant, lignan, flavonoid, secoisolariciresinol, isolariciresinol, dihydroquercetin, *myo*-inositol.

A sulfated polysaccharide heparin and its lower molecular weight derivatives (low-molecular-weight heparin, LMWH) are currently the most actively used anticoagulation agents.[1] However, these substances possess substantial drawbacks. The clinical use of heparin may be accompanied by side effects, such as thrombocytopenia and hemorrhage development.[2,3] Such complications may be reduced by using LMWH,[4,5] however, the use of this product, as well as of the native heparin, carries the risk of the invasion of viruses and toxic proteins[7,6] that persist in porcine tissues, used as the raw material for the isolation of heparin. Nonregular structure of heparin, the variability of its composition, and the presence of foreign substances, which also show geographical diversity, make standardization extremely challenging. In addition, heparin can affect other physiological processes, not related directly to the blood coagulation, such as inflammation.[8] This determines the need for further search for the new types of anticoagulant agents free of the above shortages.

Other sulfated polysaccharides such as fucoidans and galactans, isolated from some species of algae and marine invertebrates, are currently being studied as the potential alternative to heparin. Some of these biopolymers demonstrate high anticoagulant activity,[9—11] however, their practical use in medicine is not feasible due to their irregular structure and related problems of structural characterization and standardization.

Detailed studies of the structure—properties relationship of heparin, including computer simulation, have revealed that a specific pentasaccharide unit in the structure of this polysaccharide plays the major role in the interaction of the factor Xa with antithrombin(III) (ATIII), the key stage of the blood coagulation cascade.[12—15] This allowed one to suggest that the low-molecular-weight synthetic analogs that efficiently bind to ATIII can act as the alternative to heparin. A recently synthesized analog of the pentasaccharide sequence of heparin (Fondaparinux sodium, Arixtra™) has several advantages as compared to the LMWH and was allowed for clinical use in many countries.[15—17]

The search for alternatives to heparin is carried out also among the noncarbohydrate structures. In particular, the anionic derivatives on the basis of low-molecular-weight polyhydroxy compounds containing hydrophobic fragments are promising compounds.[18,19] It is assumed that such drugs not only will be more bioavailable than heparin and its derivatives, but also possess higher stability against enzymatic degradation allowing their oral administration. Sulfated derivatives of plant polyphenols, particularly of flavonoids, have been formerly suggested as such anticoagulants,[20] however, the study of these compounds was not completed due to the inability to obtain persulfated derivatives of the polyols by the known methods of sulfation of alcohols and phenols.

We have recently suggested a preparative method for the persulfation of polyhydroxy compounds under the ac-

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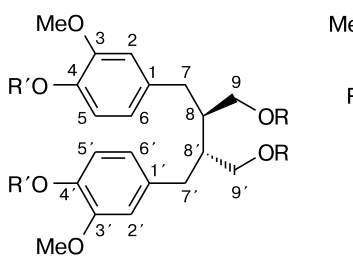
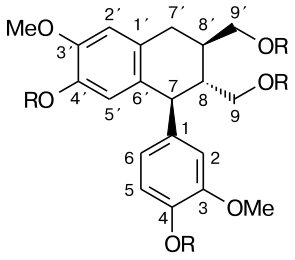
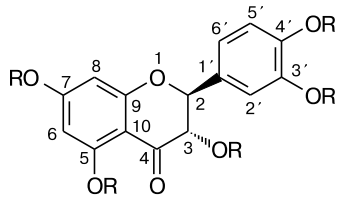
tion of the $Et_3N \cdot SO_3$ complex in the presence of trifluoromethane sulfonic acid in DMF, which turned out to be also suitable for the persulfation of plant polyphenols.[21] In the present work, we consider the synthesis and anticoagulant activity of persulfated derivatives of (−)-secoisolariciresinol (**1**) and (−)-isolariciresinol (**4**) belonging to the class of lignans,[22] as well as that of the flavonoid dihydroquercetin (**6**)[22] and *myo*-inositol (**8**).



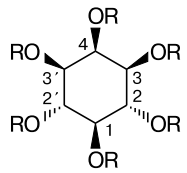
1—3



4, **5**



6, **7**



8, **9**

R′ = H (**1**, **2**), SO_3Na (**3**)
R = H (**1**, **4**, **6**, **8**), SO_3Na (**2**, **3**, **5**, **7**, **9**)

Results and Discussion

Persulfation of the polyhydroxy compounds **1**, **4**, **6,** and **8**, with an excess of the complex $Et_3N \cdot SO_3$ in DMF in the presence of TfOH efficiently led to persulfated products **3**, **5**, **7**, and **9**, respectively. In the absence of TfOH, the sulfation of flavonoid **6** and *myo*-inositol **8** does not go to completion and is accompanied by the formation of a mixture of products. As for lignan **1**, the substantial difference in the activities of the OH groups allows one to carry out regioselective 9,9′-disulfation in the absence of TfOH to form product **2**.

The structures of the synthesized sulfated products are established by ^1H and ^{13}C NMR spectroscopy and mass spectrometry. The position of the sulfate groups at the atoms C (9) and (9′) in disulfate **2** was confirmed by the downfield shift of the corresponding signals in the ^{13}C NMR spectrum with respect to the starting secoisolariciresinol (δ 60.3 → 70.6).[22] In contrast, the introduction of the sulfate group in the aromatic ring was accompanied by the upfield shift of the corresponding carbon atom. Thus for compound **3** an upfield shift of the signals for C (4) and C (4′) as compared to that of the original lignan **1** (δ 144.3 → 139.5) is observed. This pattern persisted for all the carbon atoms bearing the sulfate groups in the aromatic rings of the synthesized compounds **3**, **5**, and **7**. Compound **9** was previously obtained by the sulfation of *myo*-inositol with chlorosulfonic acid and oleum at elevated temperatures.[23,24]

The anticoagulant activity of the synthesized compounds was primarily studied by prolongation activated partial thromboplastin time (APTT test) (Fig. 1). Selectively disulfated lignan **2** showed no activity, while its persulfated analog **3** showed little anticoagulant effect. The concentration that doubled the time of the clot formation in the APTT test for compound **3** was more than 350 mg mL^{-1}. Its analog **5** and pentasulfated dihydroquercetin **7** showed somewhat higher activities (287 and 131 mg mL^{-1}, respectively), whereas the anticoagulant effect of the *myo*-inositol hexasulfate **9** was almost an order of magnitude higher (23 mg mL^{-1}). The prothrombin time remained virtually unchanged under the action of the studied compounds (data not shown).

The structure dependence of the inhibitory activity of the synthesized compounds with respect to the factor Xa was the same as that for the APTT (Fig. 2). The most effective inhibitor was *myo*-inositol hexasulfate **9**; dihydroquercetin pentasulfate **7** was substantially less active, and the sulfated lignans **2**, **3**, and **5** were inactive. The inhibitory effect of compounds **7** and **9** on the factor Xa was observed only in the presence of antithrombin(III), suggesting their indirect effect on the factor Xa *via* a complex with antithrombin(III). The synthesized compounds showed no anti-IIa activity (data not shown).

It may be noted that in the series of the investigated compounds the highest anticoagulant activity is shown by the compounds with the largest number of sulfate groups. This may be due to the fact that this factor is decisive in the stage of the interaction of the investigated compounds with ATIII. Therefore, in order to find the effective anti-

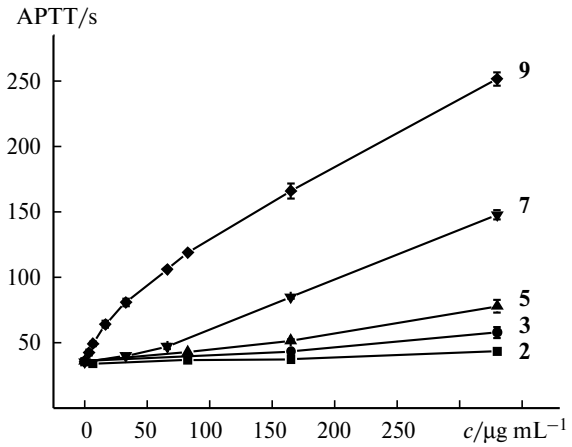


Fig. 1. The results of the determination of APTT for compounds **2**, **3**, **5**, **7**, and **9**.

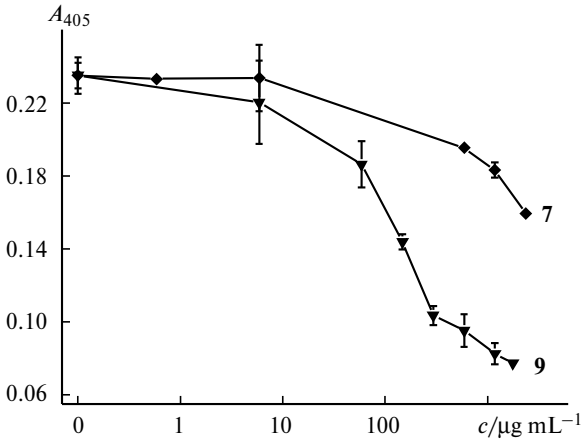


Fig. 2. Anti-Xa activity of compounds **7** and **9** in the presence of ATIII.

coagulants, the study of the compounds containing more than four sulfate groups seems to be the most promising.

Experimental

Dimethylformamide was distilled in an oil pump vacuum over phthalic anhydride and then over CaH_2. The ^1H and ^{13}C NMR spectra were recorded using Bruker DRX-500 and Bruker AM-300 spectrometers at 25 °C. The assignment of the signals was performed using the homo- and heteronuclear two-dimensional COSY, TOCSY, and HSQC correlation spectra. The residual signal of acetone was used as the internal standard for the registration of the spectra in D_2O. Mass spectra were recorded on Finnigan MAT LCQ spectrometer using electrospray ionization source (ESI) with methanol as the solvent, capillary voltage of +4.53 kV, and interface temperature of 220 °C. The optical rotation was measured using the PU-07 digital polarimeter (Russia) at 18—25 °C.

9,9´-Di-*O*-sulfosecoisolariciresinol (Na salt) (2). To a cooled (0 °C) solution of (−)-secoisolariciresinol (**1**)[22] (30.0 mg, 0.083 mmol) in DMF (3.0 mL), the complex $Et_3N \cdot SO_3$ (150.0 mg, 0.83 mmol) was added with stirring. The reaction mixture was kept for 40 min at 0 °C. Following addition of 0.5 *M* aqueous NaOH to pH 11, the aqueous phase was washed with CH_2Cl_2 (3×3 mL) and concentrated. The residue was dissolved in 2 mL of water and the solution was gel-chromatographed on a Sephadex® G-15 column (3×40 cm) with water as the eluent. The fractions containing product **2** (TLC control, silica gel, CH_2Cl_2—MeOH—Et_3N (5 : 2 : 0.1), UV-detection, R_f = 0.35), were combined and lyophilized. The residue was crystallized from EtOH yielding disulfate **2** (27 mg, 57%) as the white needle-shaped crystals, decomposition temperature >225 °C, $[\alpha]_D$ −30 (*c* 1.0, H_2O). ^1H NMR (D_2O, δ): 6.76 (d, 2 H, H(5, 5´), *J* = 8.6 Hz); 6.62 (br.s, 4 H, H(2, 2´); 4.21 (dd, 2 H, H(9a, 9´a), *J* = 5.1 Hz, *J* = 10.0 Hz); 4.06 (dd, 2 H, H(9b, 9´b), *J* = 6.8 Hz, *J* = 10.0 Hz); 3.70 (s, 6 H, CH_3); 2.72 (dd, 2 H, H(7a, 7´a), *J* = 5.1 Hz, *J* = 13.7 Hz); 2.57 (dd, 2 H, H(7b, 7´b), *J* = 9.4 Hz, *J* = 13.7 Hz); 2.07 (m, 2 H, H(8, 8´)). ^{13}C NMR, δ: 149.2 (C(3, 3´)); 145.9 (C(4, 4´)); 133.3 (C(1, 1´)); 123.7 (C(6, 6´)); 116.8 (C(5, 5´)); 114.5 (C(2, 2´)); 70.6 (C(9, 9´)); 57.1 (2 Me); 41.0 (C(8, 8´)); 35.7 (C(7, 7´)). MS-ESI: $[M + Na]^+$; found 589.04, calculated: 589.04, $C_{20}H_{24}Na_2O_{12}S_2$.

4,4´,9,9´-Tetra-*O*-sulfosecoisolariciresinol (Na salt) (3). A solution of tetrol **1** (30.0 mg, 0.083 mmol) in DMF (3.0 mL) containing the complex $Et_3N \cdot SO_3$ (300.0 mg, 1.65 mmol) was cooled to 0 °C and stirred under argon. To the solution TfOH (29 μL, 0.33 mmol) was added. The reaction mixture was kept 90 min at 0 °C followed by the addition of 0.5 *M* aquous NaOH to pH 11, the aqueous phase was washed with CH_2Cl_2 (3×3 mL) and concentrated. The residue was purified by gel-filtration in water, as described above, and then by crystallization from EtOH, yielding tetrasulfate **3** (48 mg, 75%), white needle-shaped crystals with the decomposition temperature > 230 °C, $[\alpha]_D$ −19 (*c* 1.0, H_2O). ^1H NMR (D_2O, δ): 7.29 (d, 2 H, H(5, 5´), *J* = 8.1 Hz); 6.83 (m, 4 H, H(2, 2´,6b´)); 4.25 (dd, 2 H, H(9a, 9´a), *J* = 5.1 Hz, *J* = 10.3 Hz); 4.13 (dd, 2 H, H(9b, 9´b), J = 5.9 Hz, *J* = 10.3 Hz); 3.77 (s, 6 H, 2 CH_3); 2.79 (m, 4 H, H(7, 7´)); 2.16 (m, 2 H, H(8, 8´)). ^{13}C NMR (125 MHz, D_2O, δ): 151.0 (C(3, 3´)); 139.5 (C(4, 4´)); 138.2 (C(1, 1´)); 122.3 (C(5, 5´)); 121.9 (C(6, 6´)); 113.8 (C(2, 2´)); 68.9 (C(9, 9´)); 55.9 (Me); 39.5 (C(8, 8´)); 34.4 (C(7, 7´)). MS-ESI: $[M + Na]^+$; found: 792.92, calculated: 792.92, $C_{20}H_{22}Na_4O_{18}S_4$.

4,4´,9,9´-Tetra-*O*-sulfoisolariciresinol (Na salt) (5). A solution of tetrol **4** (30.0 mg, 0.083 mmol) in DMF (3.0 mL) containing the complex $Et_3N \cdot SO_3$ (300.0 mg, 1.65 mmol) was cooled to 0 °C and stirred under argon. To the solution, TfOH (29 μL, 0.33 mmol) was added. The reaction mixture was kept for 90 min at 0 °C and Et_3N (50 μL) and MeOH (2 mL) were added. The mixture was stirred for 30 min at room temperature and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (Kieselgel 60, 40—63 μm, Merck) using the mixture CH_2Cl_2—MeOH—Et_3N (4 : 1 : 0.1) as the eluent. The fractions containing the target product were dissolved in water and stirred with the ion-exchange resin Amberlite IR-120(Na^+) (300 mg) for 2 h. The resin was filtered off, the filtrate was concentrated and the residue was purified by gel-filtration on Sephadex® G-15 in water to give the amorphous pale-yellow tetrasulfate **5** (33.9 mg, 53%), $[\alpha]_D$ −7 (*c* 1.0, H_2O). ^1H NMR: (D_2O, δ): 7.37 (d, 1 H, H(5), *J* = 8.3 Hz); 7.02 (s, 1 H, H(2´)); 6.99 (s, 1 H, H(2)); 6.89 (d, H, H(6); *J* = 8.3 Hz); 6.77 (s, 1 H, H(5´)); 4.24 (d, 2 H, H(9´), *J* = 4.6 Hz); 4.18 (d, 1 H, H(9a), *J* = 10.2 Hz); 4.13 (d, 1 H, H(7), *J* = 10.6 Hz); 3.90–3.84 (m, 7 H, 2 CH_3, H(9b)); 3.02 (m, 2 H, H(7´)); 2.37 (m, 1 H, H(8´)); 2.18 (t, 1 H, H(8), *J* = 10.2 Hz). ^{13}C NMR (125 MHz, D_2O, δ): 152.7 (C(3´)); 150.9 (C(3); 144.7 (C(6´)); 140.2 (C(4´)); 139.6 (C(4)); 136.7 (C(1)); 133.4 (C(1´)); 125.1 (C(5´)); 124.1 (C(5)); 123.7 (C(6)); 116.2 (C(2)); 114.5 (C(2´)); 71.71 (C(9´)); 68.3 (C(9)); 57.7 (Me); 57.6 (Me); 47.7 (C(7)); 43.8 (C(8)); 36.1 (C(8´)); 33.3 (C(7´)). MS-ESI $[M + Na]^+$, found 790.8, calculated 790.9, $C_{20}H_{20}Na_4O_{18}S_4$.

3,3´,4´,5,7-Penta-*O*-sulfodihydroquercetin (Na salt) (7). A solution of pentol **6** (20.0 mg, 0.066 mmol) in DMF (3.0 mL) containing the complex $Et_3N \cdot SO_3$ (300.0 mg, 1.65 mmol) was cooled to 0 °C and stirred under argon. To the solution TfOH (29 μL, 0.33 mmol) was added. The reaction mixture was kept for 24 h at 0 °C followed by addition of Et_3N (40 μL) and MeOH (3 mL), stirred for 30 min at room temperature and concentrated *in vacuo*. The residue was purified by column chromatography and gel-filtration as described above for the synthesis of tetrasulfate **5** yielding amorphous pale-yellow pentasulfate **7** (32.1 mg, 60%), $[\alpha]_D$ +17 (*c* 1.0, H_2O). ^1H NMR (D_2O, δ): 7.76

(d, 1 H, H(2´), J = 1.9 Hz); 7.62 (d, 1 H, H(5´), J = 8.5 Hz); 7.55 (dd, 1 H, H(6´), J = 1.9 Hz, J = 8.5 Hz); 7.20 (d, 1 H, H(6´), J = 2.2 Hz); 7.00 (d, 1 H, H(8), J = 2.2 Hz); 5.68 (d, 1 H, H(2), J = 11.0 Hz); 5.44 (d, 1 H, H(3), J = 11.0 Hz). ^{13}C NMR (125 MHz, D_2O, δ): 187.9 (C(4)), 162.3 (C(9)); 157.4 (C(7)); 151.7 (C(5)); 143.9 (C(3´)); 142.9 (C(4´)); 133.5 (C(1´)); 126.1 (C(6´)); 122.8 (C(2´)); 122.7(C(5´)); 110.5 (C(1´)); 108.7 (C(6)); 106.9 (C(8)); 80.8 (C(2)); 77.6 (C(3)). MS-ESI: $[M + Na]^+$, found 836.7, calculated 836.7, $C_{15}H_7Na_5O_{22}S_5$.

Hexa-*O*-sulfo-*myo*-inositol (Na salt) (9). Hexol **8** (10 mg, 0.056 mmol) was sulfated in DMF (3.0 mL) with $Et_3N \cdot SO_3$ (302 mg, 1.67 mmol) and TfOH (57 μL, 0.53 mmol) for 24 h and then purified as described above for the synthesis of compound **3** to yield white amorphous product **9** (26.8 mg, 61%). ^{1}H NMR (D_2O, δ): 5.13 (br.s, 1 H, H(4)); 5.07 (br.s, 2 H, H(2, 2´)); 4.87 (br.s, 2 H, H(3, 3´); 4.77 (br.s, 1 H, H(1)). ^{13}C NMR (125 MHz, D_2O): 76.1 (C(4)); 75.8 (C(2, 2´)); 75.1 (C(3, 3´)); 74.5 (C(1)). Found (%): C, 8.41; H, 1.93; S, 21.63; Na, 15.72. Calculated (%): C, 8.17; H, 1.83; S, 21.80; Na, 15.63, $C_6H_6Na_6O_{24}S_6 \cdot 5H_2O$.

The study of the anticoagulant activity of compounds 2, 3, 5, 7, and 9. ***A.*** *Determination of the activated partial thromboplastin time (APTT).* Prolonged APTT reflects the change in the activity factors of the so-called «intrinsic pathway» of blood coagulation, was determined using the APTT test reagents (Renam, Russia). In this experiment, 20 μL of an aqueous solution of the test compound (2 to 100 μg) was added to 80 μL of the control human blood plasma with the normal hemostasis system and the mixture was heated for 1 min at 37 °C. A mixture (100 μL) of soybean phospholipids and ellagic acid was added, the solution was incubated for 2 min at 37 °C and 100 μL of 0.025 M $CaCl_2$ preheated to 37 °C was added. The clot formation time was the parameter measured.

B. *Determination of the anti-Xa activity of polysulfates.* The effect of the studied compounds on the activity of the factor Xa in the presence and absence of the antithrombin(III) was determined using a set of reagents ReaChrom-Heparin (Russia). The buffer (0.15 mM Tris-HCl buffer, pH 8.4) or 50 μL of the antithrombin(III) solution (activity 0.5 units mL^{-1}) was added to 20 μL of the buffer solution containing different amounts of the tested compounds. The reaction was initiated by the addition of 50 μL of an aqueous solution of human factor Xa (with the activity of 2 units mL^{-1}) and the mixture was further incubated for 5 min at 37 °C. A solution of a synthetic chromogenic substrate (an oligopeptide p-nitroanilide) (50 μL) was added to the mixture, the mixture was incubated for 5 min, and the reaction was stopped by acidification with AcOH. The buffer (220 μL) was added and the optical density of free p-nitroaniline was measured on an Ultrospec II spectrophotometer (LKB, Sweden) at 405 nm. The data are presented as mean values of three measurements.

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