Synthesis of sulfated dendrimers and studies of their anticoagulant and antiinflammatory activity*

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Three hyperbranched dendrimers (polyglycidols) and the corresponding sulfated derivatives, differing in the average molecular weight, were synthesized. The compounds obtained were characterized in detail by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy, enabling estimation of the sizes of the corresponding molecules. Assignment of signals and identification of key structural blocks were performed using 2D homo- and heteronuclear spectroscopy (COSY, HSQC). The spectra of the sulfated derivatives showed the absence of the signals for the glycerol moiety with the free OH groups, that confirms exhaustive sulfation. The studies of antiinflammatory and anticoagulant activities of the polyanionic samples showed that all the compounds manifest weak antiinflammatory activity, however, their anticoagulant activity displayed in preliminary trials seems to be considerable. The results obtained indicate that it is reasonable to study in more detail biological activity of sulfated dendrimers of this type in terms of their anticoagulant properties.

Key words: dendrimer, O-sulfation, NMR spectroscopy, anticoagulant properties, antiinflammatory activity, selectin.

Sulfated polysaccharides possess a wide range of biological activity due to their strong electrostatic bonding with various proteins and protein receptors. For example, sulfated hexosaminoglycans can inhibit such physiological processes as blood coagulation, development of selectin-mediated inflammation, angiogenesis, cancer cell adhesion, binding a virus to a host cell, *etc.*[1—7] The highly sulfated polysaccharides, fucoidans, manifest activity similar to that of hexosaminoglycans (see Refs 8 and 9 and references cited therein). However, a therapeutical application of these macromolecules is limited by the low selectivity of their biological effect, largely resulted from the high heterogeneity and difficulties in standardization of such polysulfated polymers. In this connection, the search for the analogs of natural sulfated polysaccharides, whose action is selective and structural characteristics do not cause much difficulties, seems promising.

The so-called hyperbranched polyglycidols (HBP) are promising polyhydroxy matrices for the design of polyanionic agents. These macromolecules are built of the glycerol moieties bound to each other with the ether bonds. The thus formed branched chains are bound to trimethylolpropane, that results in the formation of a globular structure containing plenty of OH groups (Fig. 1). Hyperbranched polyglycidols belong to dendrimers, but form a separate group.[10] This is attributed to a number of distinctions: HBP have random branch-on-branch topology and contain extremely flexible aliphatic polyether chains and numerous hydroxy groups. This explains their complete solubility in water and excellent biocompatibility, that makes HBP very promising for biomedical application.[10]

Recently, we have developed a preparative approach to the exhaustive sulfation of polyhydroxy compounds upon the action of a $Et_3N \cdot SO_3$ complex in the presence of

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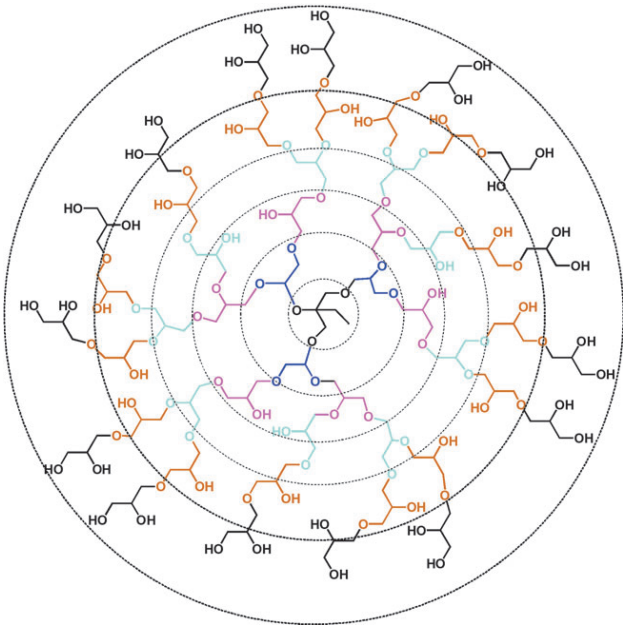


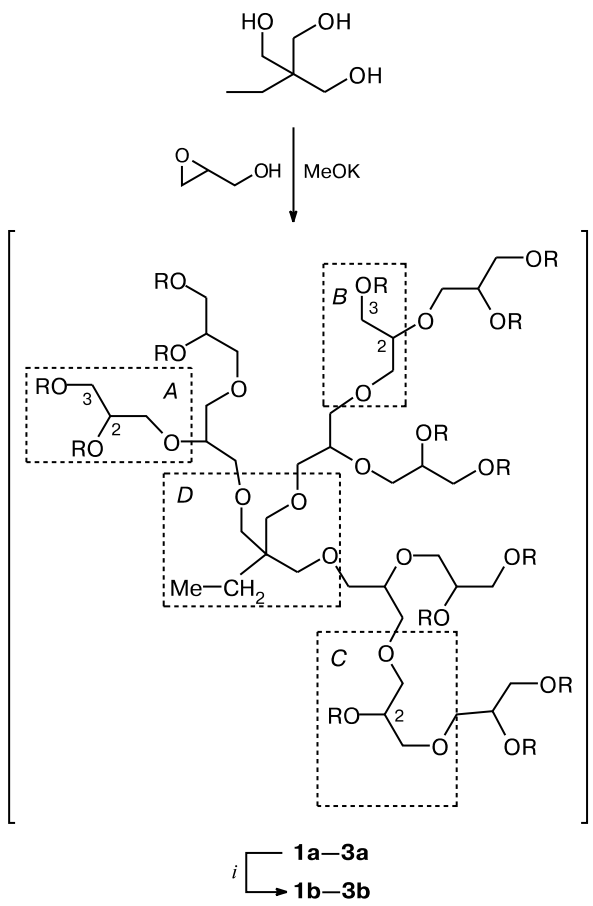
Fig. 1. The structure of hyperbranched polyglycidol dendrimers of different generations. *Note*. Figure 1 is available in full color in on-line version of the journal (http://www.springerlink.com/issn/1573-9171/current).

trifluoromethanesulfonic acid in DMF,[11,12] which has proved applicable for polyhydroxy dendrimers. In the present work, we consider synthesis, characteristics, and research results of anticoagulant and antiinflammatory activity of completely sulfated dendrimers differing in their molecular masses.

Results and Discussion

The starting HBP were synthesized by radical cyclopolymerization[13,14] (Scheme 1). Molecular weight of the final product was regulated by the change in the concentration ratios of 1,1,1-tris(hydroxymethyl)propane (trimethylolpropane) and 2,3-epoxypropanol (glycidol).

Scheme 1



R = H (**a**), SO_3Na (**b**)

i. 1) $Et_3N \cdot SO_3$, TfOH, DMF; 2) NaOH, H_2O.

Note. The following structural fragments, identified by NMR spectroscopy, are shown: 1-mono- (*A*), 1,2-di- (*B*), and 1,3-disubstituted (*C*) glycerol moieties and the trimethylolpropane core of the dendrimer (*D*). The number of glycerol moieties is ~6 (**1**), ~10 (**2**), >20 (**3**).

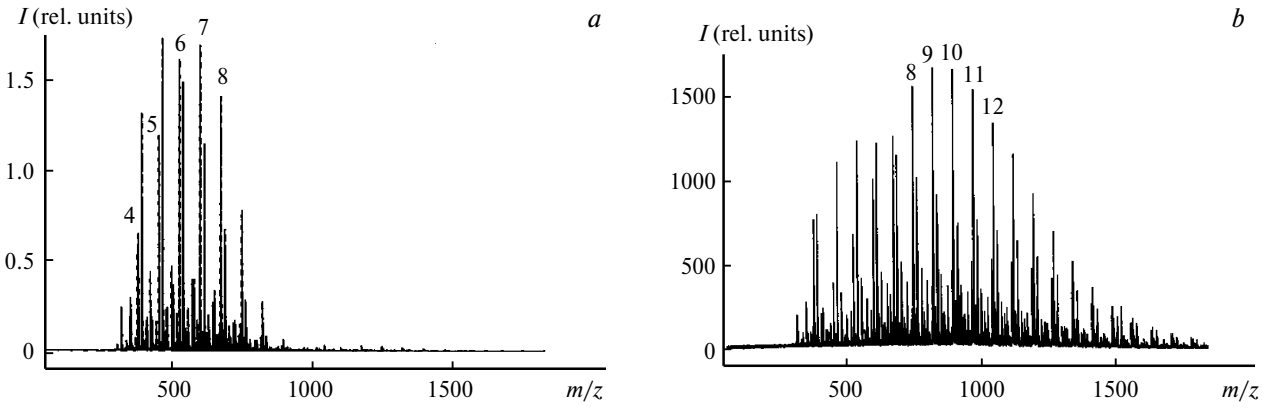


Fig. 2. The ESI mass spectra of dendrimers **1a** (*a*) and **2a** (*b*). The figures show the number of the glycerol units related to the corresponding peak.

An exhaustive sulfation of polyhydroxy compounds **1a—3a** with an $Et_3N \cdot SO_3$ complex in excess amount in DMF in the presence of TfOH (see Refs 11 and 12) with subsequent ionic exchange upon the action of aqueous NaOH efficiently led to the formation of sulfated products **1b—3b**, respectively. They were purified by gel permeation chromatography (refractometric detector). The first symmetric peak with the shortest retention time corresponded to the target sulfated derivatives.

The molecular mass distribution of the synthesized molecules was analyzed by mass spectrometry (Fig. 2). The spectra of compounds **1a** and **2a** contained peaks corresponding to the HBP with different number of the glycerol units, with the peaks corresponding to six and

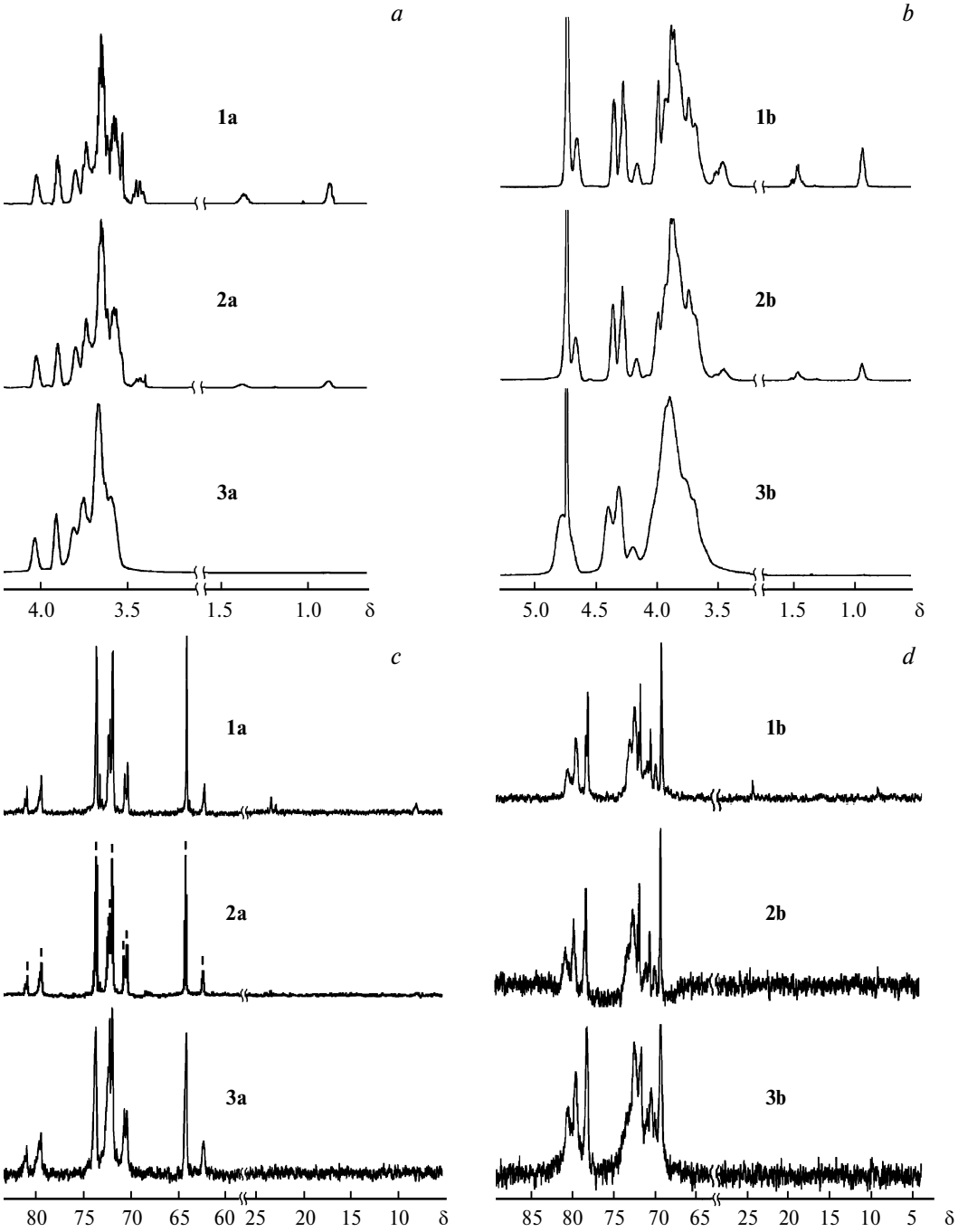


Fig. 3. The ^1H (*a*, *b*) and ^{13}C (*c*, *d*) NMR spectra (D_2O, 303 K) of the starting dendrimers **1a—3a** (*a*, *c*) and their sulfated derivatives **1b—3b** (*b*, *d*).

seven glycerol moieties being the most intensive in the sample **1a**. The sample **2a** was characterized by higher degree of polymerization: the maximum peaks corresponded to nine and ten glycerol units. In the mass spectra of polyhydroxy dendrimer **3a** and sulfated derivatives **1b**—**3b**, no signals corresponding to the molecular formulas of the target polymers were observed, which is attributed to the very high molecular masses of the samples under study.

^{1}H and ^{13}C NMR spectra were recorded for all the samples (Fig. 3). They were identical in the positions of signals, however, the integral intensities of the peaks were different. Analysis of the one-dimensional ^{1}H and ^{13}C NMR spectra (see Fig. 3) showed the same pattern in the change of the average molecular weight in the series of the samples under study, as was obtained from the mass spectrometric data. The ratio of integral intensities for the ^{1}H signals of the glycerol moieties (δ 4.10—3.50) and the dendrimer trimethylolpropane core (δ 0.88 ($CH_2C\underline{H}_3$), 1.38 ($C\underline{H}_2CH_3$), 3.43 ($C\underline{H}_2O$)) increased from samples **1** to samples **2**, that indicates an increase in the average molecular weight. It is important to note that the ratio did not change from sample **1a** to **1b** (neither from **2a** to **2b**), this indicates that no destruction of the polymer occurs during the acid-promoted sulfation. No signals for the trimethylolpropane core were found in the spectra of compounds **3**, that can indicate their considerably higher molecular weights.

The 2D ^{1}H/^{13}C HSQC spectra of unsulfated dendrimers showed the presence of two groups of signals related to the CH and CH_2 groups. Figure 4 demonstrates the data for the sample **2a**. According to the interpretation of the ^{13}C NMR spectra for a wide series of oligoglycerols suggested in the work,[15] the CH_2 fragments of the first type (δ_C 62—64) contain the free OH group as a substituent, while the CH_2 fragments of the second type (δ_C 70—74) are bound by an ether bond to other glycerol moieties. By analogy, the first group of the CH signals (δ_C 70—73) corresponds to the CH—OH fragments, whereas the second (δ_C 79—81) to the CH groups bound *via* an ether bond inside the polyglycerols. The strongest three signals in the ^{13}C NMR spectrum (δ_C 64.1, 71.9, and 73.6) belong to the 1-monosubstituted fragment of glycerol (fragment A in Scheme 1), that is confirmed by the presence of the correlation between protons A-2 and A-3 in the COSY spectrum, as well as by the complete agreement with the data for related structures.[15] In addition, the spectrum contained the signals related to 1,2-disubstituted (fragment B in Scheme 1) and 1,3-dis-

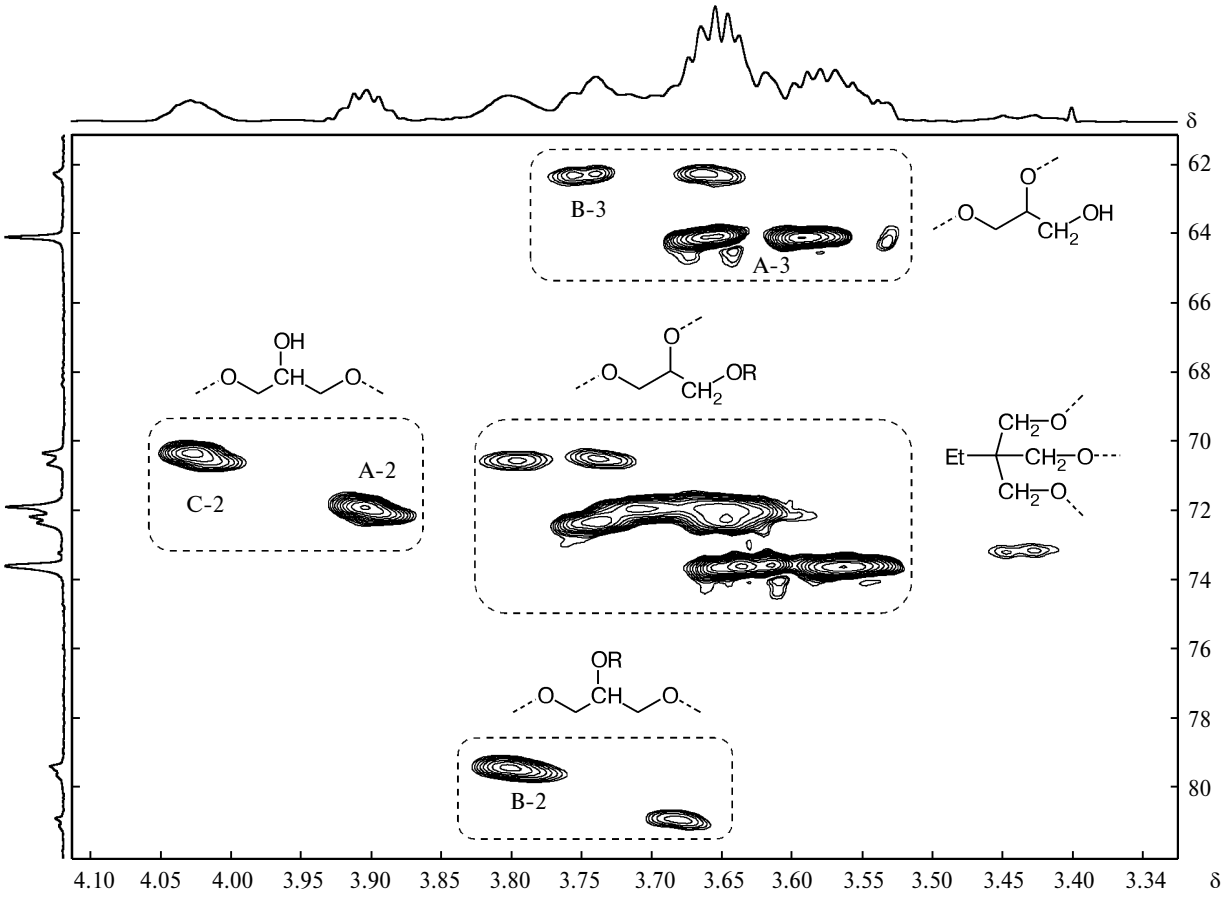


Fig. 4. The fragment of the HSQC spectrum of unsulfated dendrimer **2a**.

ubstituted (fragment C) glycerol moieties. The trisubstituted glycerol fragment was not unambiguously identified because of signal overlaps.

The 2D $^{1}H/^{13}C$ HSQC spectra of sulfated dendrimers **1b**—**3b**, like in the case of unsulfated precursors, exhibit two groups of the CH signals and two groups of the CH_2 signals (Fig. 5, the sample **2b** is considered as an example). The first group of the CH signals is shifted downfield as compared to the corresponding signals in the spectrum of the starting dendrimer, that confirms the introduction of the sulfate groups (δ_H 3.88—4.05 for **2a** → 4.62—4.78 for **2b**). As it was expected, the position of the second group of the CH signals, which are related to the bonds between units, remains virtually unchanged. A downfield shift is also observed for the signals of the CH_2 groups bound to the sulfate groups (δ_H 3.53—3.77 for **2a** and 4.12—4.41 for **2b**). Similarly to the unsulfated precursors, the key glycerol fragments A—C were identified in the sulfated derivatives, as well.

Anticoagulant and antiinflammatory activity was studied for the samples of sulfated dendrimers synthesized. The antiinflammatory activity was determined in the *in vivo* experiments on inhibation of the rat peritonitis. A peptone-induced acute rat peritonitis causes an increase in the number of neutrophils in the peritoneal cavities to $(74.0\pm12.5)\cdot10^6$ (a control group of nine animals). The intravenous administration of sulfated dendrimers led to an insignificant decrease in the number of neutrophils (Table. 1), which weakly depended on the dendrimer molecular weight. For all the compounds studied, the degree of inhibation was low and were in the 34—37% range.

The anticoagulant activity of the synthesized compounds was studied in the experiment on the increase in the activated partial thromboplastin time (APTT). The sulfated dendrimer **2b** with an intermediate molecular weight manifested the highest activity (see Table 1). For this compound, the concentration which causes the clotting time to double (the 2 APTT value) was 3.65 μg mL^{-1}. The low-molecular-weight **1b** and high-molecular-weight **3b** dendrimers possessed approximately the same activity (2 APTT was 7—8 μg mL^{-1}). A considerable anticoagulant activity of the sulfated dendrimers manifested in the preliminary biological tests made it reasonable to continue the search for the sulfated hyperbranched polyglycidol agents optimum in their structure and size.

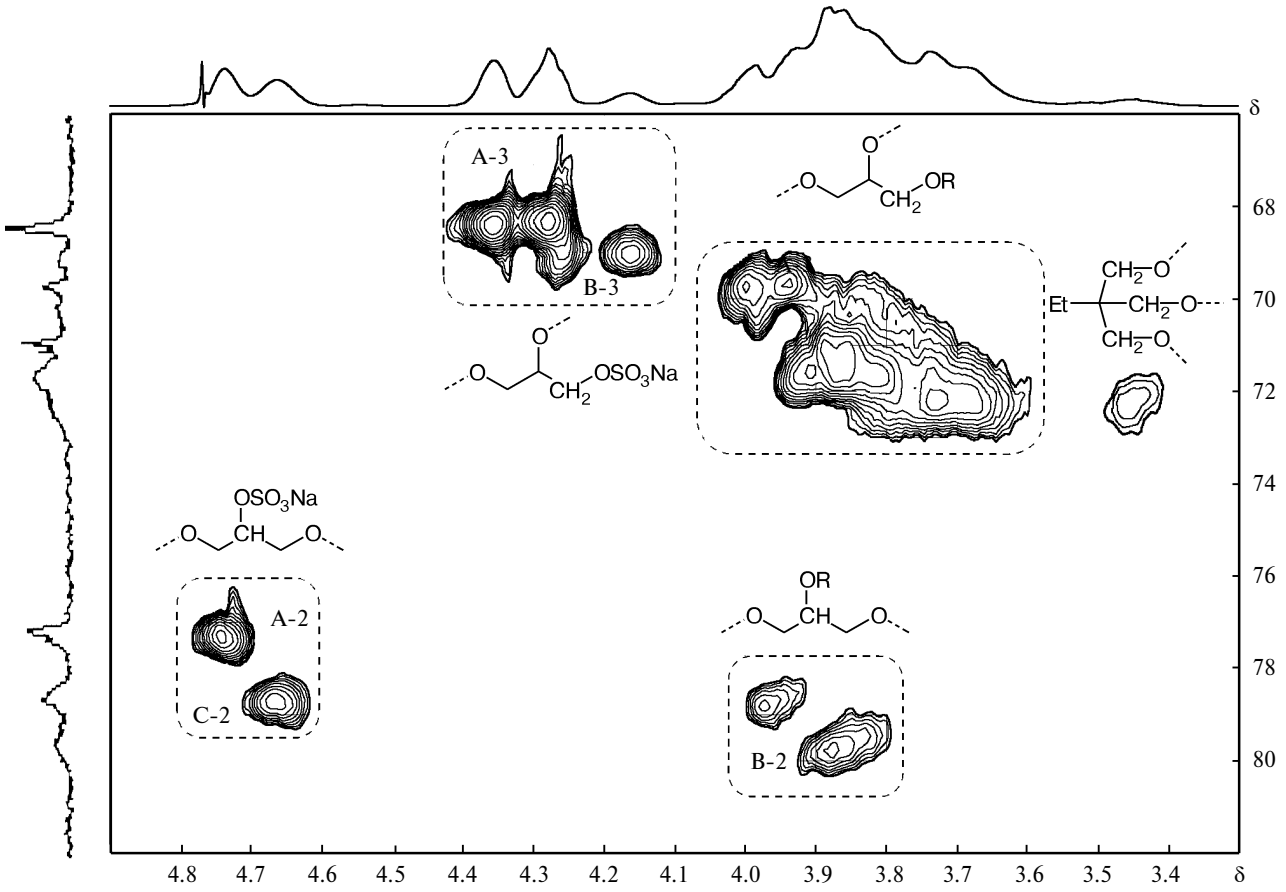


Fig. 5. The fragment of the HSQC spectrum of sulfated dendrimer **2b**.

Table 1. Antiinflammatory and anticoagulant activity of the dendrimer sulfated derivatives

Sample of dendrimer	Antiinflammatory activity			2 APTT[a]
	n^b	$N \cdot 10^{6\,c}$	I (%)[d]	
Control	9	74.0±12.5		
1b	3	46.5±9.3	37.2	7.02
2b	4	48.2±10.9	34.9	3.65
3b	3	48.5±8.4	34.4	8.09

[a] 2 APTT is the concentration of the sample causing doubling the value of the activated partial thromboplastin time (μg mL^{-1}).
[b] n is the number of laboratory animals (rats) in the group.
[c] N is the number of neutrophils per rat.
[d] I is the inhibition.

Experimental

Dimethylformamide was distilled over phthalic anhydride and then over CaH_2 under reduced pressure using a vacuum oil pump. NMR spectra were recorded on Bruker Avance 600 and Bruker DRX 500 spectrometers at 303—306 K after lyophilization of the samples once from D_2O and their subsequent dissolution in 99.96% D_2O (2—3% solutions). Assignment of the signals was performed using 2D homo- and heteronuclear COSY, TOCSY, and HSQC (edited HSQC) correlation spectra. Acetone (δ_H 2.225, δ_C 31.45) was used as the internal reference for recording the spectra in D_2O. High resolution mass spectra were recorded on a Bruker micrOTOF II instrument with the electrospray ionization (ESI).[16] The measurements were performed on cations, with the voltage on the capillary being 4500 V. The range of scanned masses m/z was 50—3000 Da with an external or internal calibration (Electrospray Calibrant Solution, Fluka). Solutions of compounds in acetonitrile were injected using syringes, 3 μL min^{-1} flow rate, nitrogen carrier gas (4 L min^{-1}), 180 °C interface temperature.

Synthesis of hyperbranched polyglycidols (1a—3a).[13,14,17] Glycidol (Aldrich, 50 mL, 0.851 mol) was slowly added (using a dispenser) into a reaction flask with trimethylolpropane (Aldrich, 16.2 g, 121 mmol for **1a**, 9.53 g, 71 mmol for **2a**, 5.5 g, 41 mmol for **3a**), which was partially deprotonated (10%) by addition of sodium methoxide (36.3 mmol for **1a**, 21.3 mmol for **2a**, 12.3 mmol for **3a**, Fluka, 3.7 mol L^{-1} in methanol). The following conditions were used for the synthesis: mechanical stirring, nitrogen atmosphere, 95 °C temperature, 12 h reaction time. After the synthesis reached completion, which was inferred from the absence of the epoxide in the reaction mixture, the reaction product was dissolved in methanol and neutralized by passing through a column with a Dowex 50(H^+) cationite. The polymer was precipitated thrice from methanol by addition of acetone, and then dried *in vacuo* at 80 °C for 20 h. ^{1}H NMR (300 MHz, DMSO-d_6), δ_H: 4.9 (s, OH); 3.7 (m, CH); 3.6—2.8 (m, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6), δ_C: 81 (CH); 80 (CH); 74 (CH_2); 73 (CH_2); 72.4 (CH_2); 71.2 (CH_2); 68.9 (CHOH); 63.8 (CH_2OH); 61.6 (CH_2OH).

Synthesis of sulfated dendrimers 1b—3b. Trifluoromethanesulfonic acid (39.9 μL, 0.45 mmol) was added to a cooled to 0 °C solution of the starting dendrimer (30.0 mg) in DMF (3.0 mL) containing an $Et_3N \cdot SO_3$ complex (407 mg, 2.25 mmol) under argon with stirring. The reaction mixture was kept at 0 °C for 90 min, followed by 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 water (2 mL), deposited on a column (3×40 cm) with a Sephadex® G-15 gel, and eluted with water. The target dendrimer, which was identified as a peak with the shortest retention time (refractometer as a detector), was collected and lyophilized to obtain a white amorphous product (43—46 mg). ^{1}H NMR (600 MHz, D_2O), δ_H: 4.74 (br.s, CH); 4.66 (br.s, CH); 4.37 (br.s, CH_2); 4.28 (br.s, CH_2); 4.27 (br.s, CH_2); 4.05—3.55 (m, CH, CH_2). ^{13}C NMR (125 MHz, D_2O), δ_C: 80 (CH); 79 (CH); 77 (CH); 72 (CH_2); 71 (CH_2); 70 (CH_2); 68.3 (CHOH).

Studies of anticoagulant activity of compounds on the increase in the activated partial thromboplastin time (APTT). An increase in the APTT, which reflects the change in the activity of factors of the so-called "intrinsic pathway" of blood coagulation, was performed using an APTT-test kit of reagents (NPO Renam, Russia). This experiment consisted in the addition of an aqueous solution of the compound under testing (20 μL, from 0.2 to 5 μg) to a control human blood plasma (80 μL) with the normal level of the haemostasis system, and the mixture was heated at 37 °C for 1 min. Then, a mixture of the soya phospholipids and an activator, ellagic acid, (100 μL) was added, followed by incubation at 37 °C for 2 min and addition of 0.025 M $CaCl_2$ (100 μL) heated to 37 °C, after which the clotting time was detected. The compounds under study were compared by the concentrations (μg mL^{-1}) at which the clotting time doubled (the 2 APTT values).

Studies of antiinflammatory activity in the rat acute peritonitis model. Peritonitis was induced according to the method described earlier.[18,19] Female rats of the Wistar line (about 250 g) under ether anaesthesia were intraperitoneally administered with a 9.0% solution of peptone (8 mL) (MKhK Laverna) in 0.9% aqueous NaCl. Solution of sulfated dendrimers (0.3 mL in the sterile 0.9% aqueous NaCl) were administered into the rat´s femoral vein under ether anaesthesia 15 min after the peptone injection. The control animals were intravenously administered with the sterile 0.9% NaCl (0.3 mL). After 3 h, the animals were decapitated under ether anaesthesia. The abdominal cavity was washed with a PBS buffer (30 mL) containing heparin (60 unit mL^{-1}), 0.02% EDTA, and 0.03% bovine serum with the intensive massage of the peritoneum. A Goryaev chamber was used for the calculation of the total number of cells in the washings. To calculate the number of neutrophils, a suspension of the cells was centrifuged at 400 g for 10 min. The concentrated suspension was diluted with the whole bovine serum (1 : 1), made smears and stained them using the Pappenheim method. Percentage of neutrophils in the smears was determined after calculation of 6—8 hundred of cells. The total number of neutrophils in the exudation was calculated proceeding from the percentage of neutrophils and the total number of cells.

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References

1. D. R. Coombe, W. C. Kett, *Cell. Mol. Life Sci.*, 2005, **62**, 410.
2. B. Lia, J. Suwanb, J. G. Martina, F. Zhangd, Zh. Zhanga, D. Hoppensteadte, M. Clarke, J. Fareede, R. J. Linhardt, *Bio. Chem. Pharm.*, 2009, **78**, 292.
3. *Chondroitin Sulfate: Structure, Role and Pharmacological Activity*, Ed. N. Volpi, Academic Press, Amsterdam—Boston—Heidelberg—London—New York—Oxford—Paris—San Diego—San Francisco—Singapore—Sydney—Tokyo, 2006.
4. J. M. Whitelock, R. V. Lozzo, *Chem. Rev.*, 2005, **105**, 2745.
5. V. B. Krylov, N. E. Ustyuzhanina, N. E. Nifantiev, *Bioorg. Khim.*, 2011, **37**, 745 [*Bioorgan. Chem.* (*Engl. Transl.*), 2011, **37**, 672].
6. V. B. Krylov, A. A. Grachev, N. E. Ustyuzhanina, N. A. Ushakova, M. E. Preobrazhenskaya, N. I. Kozlova, M. N. Portsel´, I. N. Konovalova, V. Yu. Novikov, Kh.-Kh. Ziebert, A. C. Shashkov, N. E. Nifantiev, *Izv. Akad. Nauk*, *Ser. Khim.*, 2011, 731 [*Russ. Chem. Bull., Int. Ed.*, 2011, **60**, 746].
7. R. Lever, C. P. Page, *Nature Rev. Drug Discovery*, 2002, **1**, 140.
8. A. Cumashi, N. A. Ushakova, M. E. Preobrazhenskaya, A. D´Incecco, A. Piccoli, L. Totani, N. Tinari, G. E. Morozevich, A. E. Berman, M. I. Bilan, A. I. Usov, N. E. Ustyuzhanina, A. A. Grachev, C. J. Sanderson, M. Kelly, G. A. Rabinovich, S. Iacobelli, N. E. Nifantiev, *Glycobiology*, 2007, **17**, 541.
9. D. O. Croci, A. Cumashi, N. A. Ushakova, M. E. Preobrazhenskaya, A. Piccoli, L. Totani, N. E. Ustyuzhanina, M. I. Bilan, A. I. Usov, A. A. Grachev, G. E. Morozevich, A. E. Berman, C. J. Sanderson, M. Kelly, P. Di Gregorio, C. Rossi, N. Tinari, S. Iacobelli, G. A. Rabinovich, N. E. Nifantiev, PloS ONE, 2011, **6** (2), e17283, doi:10.1371/journal.pone.0017283.13.
10. D. Wilms, S. E. Stiriba, H. Frey, *Accounts Chem. Res.*, 2009, **43**, 129.
11. V. B. Krylov, N. E. Ustyuzhanina, A. A. Grachev, N. E. Nifantiev, *Tetrahedron Lett.*, 2008, **49**, 5877.
12. V. B. Krylov, Z. M. Kaskova, D. Z. Vinnitskiy, N. E. Ustyuzhanina, A. A. Grachev, A. O. Chizhov, N. E. Nifantiev, *Carbohydr. Res.*, 2011, **346**, 540.
13. A. Sunder, R. Hanselmann, H. Frey, R. Mulhaupt, *Macromolecules*, 1999, **32**, 4240.
14. R. Haag, F. Jean, A. Sunder, H. Frey, A. Hebel, *Macromolecules*, 2000, **33**, 8158.
15. S. Cassel, C. Debaig, Th. Benvegnu, P. Chaimbault, M. Lafosse, D. Plusquellec, P. Rollin, *Eur. J. Org. Chem.*, 2001, 875.
16. P. A. Belyakov, V. I. Kadentsev, A. O. Chizhov, N. G. Kolotyrkina, A. S. Shashkov, V. P. Ananikov, *Mendeleev Commun.*, 2010, **20**, 125.
17. Haiqing Li, Lin Zhang, Jung Kyu Jo, Chang-Sik Ha, Y. A. Shchipunov, Il Kim, *J. Nanopart. Res.*, 2011, **13**, 2117.
18. M. E. Preobrazhenskaya, A. E. Berman, V. I. Mikhailov, N. A. Ushakova, A. V. Mazurov, A. V. Semenov, A. I. Usov, N. E. Nifantiev, N. V. Bovin, *Biochem. Mol. Bio. Intl.*, 1997, **43**, 443.
19. N. A. Ushakova, M. E. Preobrazhenskaya, N. E. Nifantiev, A. I. Usov, T. V. Pochechueva, O. E. Galanina, N. V. Bovin, *Vopr. Med. Khim.* [*Quest. Med. Chem.*], 1999, **45**, 375 (in Russian).

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