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Heparin-like compounds prepared by chemical modification of capsular polysaccharide from *E. coli* K5 ☆

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Abstract

O-Sulfation of sulfaminoheparosan SAH, a glycosaminoglucuronan with the structure →4)-β-D-GlcA(1→4)-β-D-GlcNSO$_3^-$-(1→, obtained by *N*-deacetylation and *N*-sulfation of the capsular polysaccharide from *E. coli* K5, was investigated in order to characterize the sulfation pattern eliciting heparin-like activities. SAH was reacted (as the tributylammonium salt in *N*,*N*-dimethylformamide) with pyridine–sulfur trioxide under systematically different experimental conditions. The structure of *O*-sulfated products (SAHS), as determined by mono- and two-dimensional ^{1}H and ^{13}C NMR, varied with variation of reaction parameters. Sulfation of SAH preferentially occurred at O-6 of the GlcNSO$_3^-$ residues. Further sulfation occurred either at O-3 or at O-2 of the GlcA residues, depending on the experimental conditions. Products with significantly high affinity for antithrombin and anti-factor Xa activity were obtained under well-defined conditions. These products contained the trisulfated aminosugar GlcNSO$_3^-$3,6SO$_3^-$, which is a marker component of the pentasaccharide sequence through which heparin binds to antithrombin.

Keywords: *E. coli* K5 polysaccharide; Chemical *N*- and *O*-sulfation; Sulfaminoheparosansulfates; Antithrombin

☆ *Symbols and abbreviations*: GlcA, D-glucuronic acid; GlcA2SO$_3^-$, D-glucuronic acid 2-*O*-sulfate; GlcA3SO$_3^-$, D-glucuronic acid 3-*O*-sulfate; GlcN, D-glucosamine; GlcNSO$_3^-$, D-glucosamine *N*-sulfate; GlcNSO$_3^-$6SO$_3^-$, D-glucosamine *N*,3,6-*O*-trisulfate; TBA, tributylammonium; TMA·SO$_3$, trimethylamine–sulfur trioxide; Py·SO$_3$, pyridine–sulfur trioxide; APTT, activated partial thromboplastin time; HC II, heparin cofactor; AT, antithrombin.

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1. Introduction

The capsular polysaccharide from *E. coli* K5, which has the same structure as the heparin precursor *N*-acetyl heparosan →4)-β-D-GlcA-(1→4)-α-D-GlcNAc-(1→, provides a substrate for the enzymes involved in heparin biosynthesis, including those required to generate products with high affinity for antithrombin (AT) [1]. In a combined chemical and enzymic approach involving partial or total conversion of *N*-acetylheparosan to sulfaminoheparosan (SAH) by *N*-deacetylation with hydrazine and *N*-sulfation with TMA·SO_3, partial conversion of GlcA of SAH to IdoA residues with a C-5 epimerase, and chemical or enzymic *O*-sulfation of epimerised SAH, products with structures and biological properties intermediate between those of heparin and heparan sulfate were obtained [2].

Parallel experiments indicated the possibility of obtaining biologically active species (especially in the inactivation of coagulation factor Xa by AT) by chemical sulfation alone, omitting the enzymic epimerization step [2]. In the present work direct *O*-sulfation of SAH was systematically studied with the aim of elucidating the sulfation pattern eliciting the activity.

2. Experimental

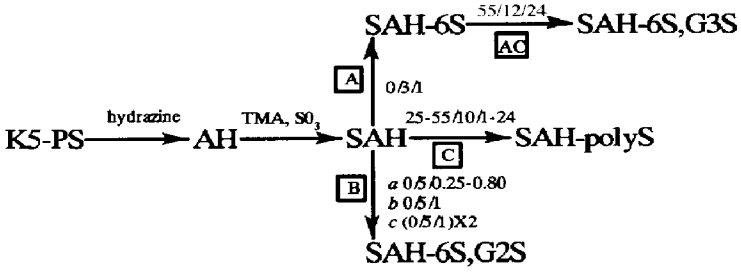
Materials and methods.—The K5-polysaccharide (K5-PS) was prepared (by V. Cavazzoni and M. Manzoni, Department of Industrial Microbiology, University of Milan) and purified as described by Vann et al. [3] K5-PS was fully *N*-deacetylated with hydrazine [4] and *N*-sulfated with TMA·SO_3 as described in detail elsewhere [2,5]. For this work, the hydrazinolysis reaction was performed with hydrazine and hydrazine sulfate for 5 h at 96°C to achieve extensive *N*-deacetylation (>95%, by ^{13}C NMR analysis), and sulfation of all free amino groups was performed for 4 h at pH 9 and 55°C. *O*-Sulfated sulfaminoheparosans (SAHS) with different degrees of sulfation and different sulfation patterns were obtained by reacting SAH (TBA salt) with Py·SO_3, essentially as described by Ogamo et al. for *O*-sulfation of glycosaminoglycans [6]. Typically, SAH (100 mg) was dissolved in H_2O, and the solution passed through a column of Amberlite IR-120 (H^+) resin. After adjusting the pH to 5.5 with 10% TBA in EtOH, the solution was lyophilised. The TBA salt of SAH thus obtained was dissolved in anhyd DMF (6 mg/mL), and excess amounts of Py·SO_3 complex in anhyd DMF (50 mg/mL) were added and allowed to react for different times (0.25 to 24 h). Reaction temperatures, amounts of reagent (equiv SO_3 per equiv free OH groups), and reaction times are as reported in Scheme 1. At the end of the reaction, the solutions were diluted with equal volumes of H_2O, and the pH adjusted to 9 with 2 M NaOH. Precipitations, filtrations, and purifications were as reported for SAH [2]. Occasional *N*-desulfation was observed (by ^{13}C NMR analysis) for some of the products; these partially *N*-desulfated SAHS were re-*N*-sulfated with TMA·SO_3 (pH 9, 6 h at 56°C). Yields were 80–90%.

The charge density of the products (expressed as sulfate-to-carboxylate molar ratios) was determined by conductimetry [7]. Mean molecular weights (expressed as weight-average molecular weights M_w, and molecular weight dispersion $Q = M_w/M_n$, where M_n is the number average molecular weight) were determined by HPLC essentially as described

in Ref [8] using Progel TSK PWXL 300 × 7.8 mm columns (a G3000 column and a G2500 column connected in series with a PWXL Guard pre-column; Supelco, USA). Calibration curves (3rd order fitting; correlation coefficient 0.996) were obtained using heparin and dermatan sulfate working standards. The M_w and Q values were calculated using the Bruker Chromstar 3.13 program, subroutine for gel-permeation chromatography.

NMR spectra.—The ^1H NMR spectra were obtained at 500 MHz with a Bruker AMX 500 spectrometer equipped with a 5-mm $^1H/X$ inverse probe, and the ^{13}C NMR spectra at 300 MHz with a Bruker AC 300 instrument equipped with a 5-mm $^{13}C/^1H$ dual probe. Each sample (~ 15 mg for ^1H and ~ 100 mg for ^{13}C measurements) was dissolved in D_2O (0.5 mL, 99.99% D). Chemical shifts are given in ppm downfield from internal sodium-3-(trimethylsilyl)-propionate but were actually measured indirectly with reference to acetone in D_2O (δ 2.235 for ^1H and δ 33.08 for ^{13}C) at 30°C. Mono-dimensional ^1H spectra were obtained with presaturation of the HDO signal with digitalisation of ~ 0.1 Hz/pt and ^{13}C spectra using ^1H composite pulse decoupling (CPD) without nuclear Overhauser effect. Typically, COSY 45 data were acquired using 48 scans per series in 1K × 512W data points with zero-filling in F1. Sine-bell function was applied before Fourier transformation. Two-dimensional TOCSY spectra were measured in the phase sensitive mode using the method of time-proportional phase increment (TPPI) with a mixing time of 75 ms. The spectra had 1K × 256W (F2 × F1) and before processing were zero filled to 2K × 512W; a squared sine-bell function was applied before Fourier transformation. ^1H–^{13}C chemical shift correlation data were acquired with ^1H detection via heteronuclear multiple-quantum coherence (HMQC). A matrix of 1K × 256W data points was applied using squared sine-bell functions prior to Fourier transformation. Quantitation of sulfation at different positions was made as described for heparin [9] from the area of relevant ^{13}C NMR signals (see Table 2 for assignments). Percent *N*-sulfation (NSO_3%, expressed as the molar ratio of NSO_3 to $NAc + NSO_3$ groups) was determined from the area of C-2 signals at 58 and 54 ppm, and percent 6-*O*-sulfation from the C-6 signals at ca. 68 and 62 ppm, respectively. Percent 2-*O*-sulfation was determined from the area of the C-1 signal of $GlcA2SO_3^-$ at 102.8 ppm, referred to half of the total area of C-1 signals (integration between 97 and 106 ppm).

Chromatography on immobilized antithrombin.—Affinity chromatography on antithrombin-Sepharose was performed essentially as described [10]. A preparation of SAHS (Bb-2), altogether 4 g, was subdivided into portions of 500 mg which were fractionated on a column (5 × 15 cm; ~ 300-mL bed volume) of the affinity matrix. Each portion, dissolved in 500 mL of 0.05 M Tris-HCl, was separately applied to the column (equilibrated



Scheme 1. Synthesis of SAH and of its *O*-sulfated derivatives (prevalent sequences). Numbers near the arrows indicate reaction temperatures (°C), reagent-to-reactant ratios (equiv SO_3/equiv available OH groups), and reaction times (h).

Table 1
Physico-chemical and biological data for SAHS

Sample	M_w [a]	Q [b]	SO_3^-/COO^- [c]	A6S(%) [d]	G2S(%) [e]	Anti-Xa [f]	APTT [g]	HC II [h]
SAH	13.3	2.07	n.d.	0	0	n.d.	n.d.	n.d.
A-1	n.d.	n.d.	1.9	95	<10	2	28	93
A-2	11.7	1.53	1.8	85	<5	1	28	58
Ba-1	12.2	1.57	1.6	71	<5	<1	7	2
Ba-2	17.6	2.49	1.7	41	<5	<1	3	14
Ba-3	13.4	1.5	1.7	74	8	1	13	50
Bb-1	17.7	1.49	2.3	100	40	16	39	63
Bb-2	18.2	1.69	n.d.	100	23	7	32	56
Bb-3	n.d.	n.d.	2.2	100	29	9	37	47
Bb-4	25.7	1.8	2.2	100	12	6	37	77
Bb-5	n.d.	n.d.	>2	95	25	6	32	68
Bb-2 HA	24.6	1.52	n.d.	98	25	29	52	71
Bb-6	14.8	1.8	2.5	92	25	11	33	57
Bb-7	14.9	1.8	2.2	81	27	15	37	62
Bb-8	16.9	1.7	2.3	98	20	9	45	75
Bb-9	16.1	1.7	2.3	79	15	7	27	62
Bb-10	17.8	1.7	2.1	81	18	9	35	66
Bb-11	15	1.9	2.1	100	27	13	40	63
Bc-1	17.1	1.6	2.5	98	58	22	48	51
Bc-2	11.3	2.96	2.5	98	59	28	29	44
Bc-3	21.5	n.d.	2.5	97	56	n.d.	n.d.	n.d.
AC-1	15.4	1.82	n.d.	96	<10	7	15	39
AC-2	14.3	1.91	2.8	90	<10	1	14	33
C-1	13.6	1.72	3.1	90	85	5	34	19
C-2	n.d.	n.d.	3.1	98	n.d.	14	36	30
C-3	11.9	1.48	2.9	95	n.d.	n.d.	n.d.	n.d.
C-4	10.8	2.09	2.8	95	n.d.	n.d.	n.d.	n.d.

[a] Mean molecular weight by HPLC.
[b] Molecular weight dispersion by HPLC.
[c] Sulfate-to-carboxylate molar ratio, by conductimetry.
[d] Percent 6-*O*-sulfation of GlcN residues (A) by ^{13}C NMR.
[e] percent 2-*O*-sulfation of GlcA residues (G) by ^{13}C NMR.
[f] Inhibition of Factor Xa.
[g] Activated partial thromboplastin time.
[h] Inhibition of thrombin by Heparin Cofactor II. n.d., Not determined. Activities are expressed as percent relative to heparin. Sample Bb-2 HA is the fraction of Bb-2 retarded on the AT affinity column that corresponds to fraction numbers 50–85 of Fig. 4.

with the same buffer) which was subsequently washed with the same buffer (~ 1 L), and then eluted using a salt gradient composed of 1.5 L of buffered 0.05 M NaCl (mixing vessel) and 1.5 L of 3 M NaCl (reservoir). Effluent fractions of ~30 mL were collected at a rate of ~90 mL/h, analysed for hexuronic acid by the carbazole reaction [11] and pooled as indicated in Fig. 4. Corresponding subfractions were combined and desalted on a column (7×100 cm) of Sephadex G-15, equilibrated with 10% aq EtOH.

Compositional analysis of saccharides.—The composition of the SAHS fractions, obtained by affinity chromatography on antithrombin-Sepharose, was investigated by HPLC

Table 2
^{1}H NMR and ^{13}C NMR chemical shifts of SAHS and their precursors [a]

Prevalent sequence	K5-PS (1)	AH (2)	SAH (3)	SAH-6S (4)	SAH-6S,G3S (5)	SAH-6S,G2S [b] (6)	SAH-polyS (8)
Glucosamine							
H-1/C-1	5.36/99.6	5.66/98.9	5.58/99.9	5.56/100.8	5.41/100.7	5.44/100.9	5.44/100.2
H-2/C-2	3.89/56.1	3.41/57.1	3.27/60.5	3.27/61.1	3.26/60.1	n.d./n.d.	3.48/58.4
H-3/C-3	3.86/73.5	3.99/70.9	3.68/72.4	3.67/73.1	3.75/71.9	n.d./n.d.	4.62/77.9
H-4/C-4	3.64/81.1	3.75/80.7	3.68/80.7	3.70/80.6	3.73/80.0	n.d./n.d.	3.95/77.0
H-5/C-5	3.82/72.0	3.76/74.7	3.79/73.3	3.96/72.3	3.99/71.8	n.d./n.d.	4.08/71.4
H-6; H-6′/C-6	3.84; 3.84/62.2	3.84; 3.90/62.2	3.81–3.83/62.4	4.37; 4.17/69.5	4.42; 4.24/68.1	n.d./n.d.	4.52; 4.31/67.6
Glucuronic acid							
H-1/C-1	4.48/105.2	4.61/105.5	4.55/105.1	4.58/105.5	4.69/104.6	4.74/102.8	4.91/102.4
H-2/C-2	3.37/76.3	3.46/76.1	3.41/75.6	3.36/76.4	3.64/76.9	4.14/82.3	4.39/79.3
H-3/C-3	3.69/78.9	3.83/78.6	3.83/78.7	3.82/79.5	4.52/82.7	3.98/77.3	4.65/80.8
H-4/C-4	3.78/79.1	3.44/79.7	3.90/78.8	3.84/79.8	4.12/77.6	3.85/79.1	4.17/79.7
H-5/C-5	3.78/79.1	4.14/76.7	3.83/79.3	3.78/80.3	3.91/79.5	3.88/79.5	3.88/79.5

[a] Chemical shifts are given in ppm downfield from internal sodium-3-(trimethylsilyl)-propionate, measured indirectly with reference to acetone in D_2O (2.235 for ^{1}H and 33.08 for ^{13}C) at 30°C.

n.d., Not determined.

[b] Prevalent disaccharide sequences of products obtained according to Procedures Bb and Bc. Products also contain sequences **5** (SAH-6S,G3S = GlcA3SO_3^- GlcNSO_3^- 6SO_3^-) and minor amounts of sequences **7** (SAH-3,6S = GlcA-GlcNSO_3^- 6SO_3^-) (see text).

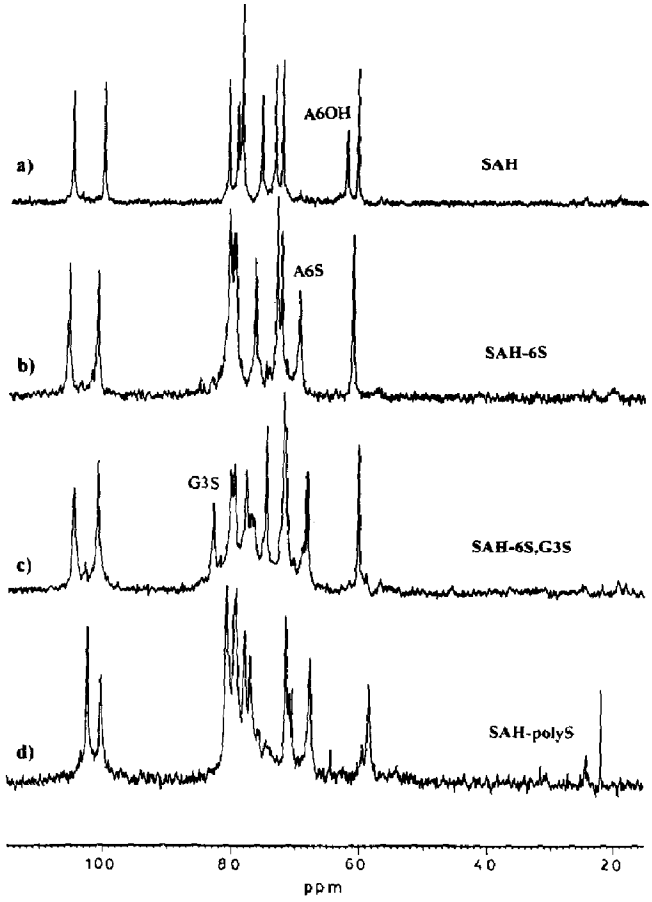


Fig. 1. ^{13}C NMR spectra of SAH (a) and some of its *O*-sulfated derivatives [b (preparation A-1) SAH-6S; c (preparation AC-2) SAH-6S,G3S; d (preparation C-2) SAH-polyS)]. Assignments are indicated for nonsulfated C-6 (A6OH) and sulfated C-6 (A6OS) of $GlcNSO_3$ residues, and for sulfated C-3 of GlcA residues (G3S). Full assignments are given in Table 2. The peak at 22 ppm is due to residual Na acetate.

analysis of ^3H-labeled disaccharides obtained by deaminative cleavage of the polysaccharides followed by reduction of the products with NaB^3H_4. The procedure employed was as described by Kusche et al. [9], with the exceptions that 20 μg (rather than 200 μg) of polysaccharide was subjected to deamination and 2.5 mCi (rather than 5 mCi) of NaB^3H_4 was used to reduce the products. Separation of the resulting labeled fragments on Sephadex G-15 afforded >95% labeled disaccharides, which were analyzed further by anion-exchange HPLC on a Partisil-10 SAX column (Whatman) as described [9].

Biological assays.—The in vitro anti-factor Xa (aXa) activity, expressing inhibition of factor Xa by antithrombin, was measured with a COATEST kit (Kabi-Vitrum). The in vitro inhibition of thrombin by heparin cofactor II (HCII activity) was determined with a STACHROM DS kit (Stago), and the in vitro APTT activity (a "global" coagulation test) with a "PTT"-Reagent (Boehringer Mannheim). The values were expressed as percent of heparin activity using a working standard sample (200 UI/mg) calibrated against the IV International Standard. Briefly, the amounts required to inhibit 50% of Xa and Thrombin

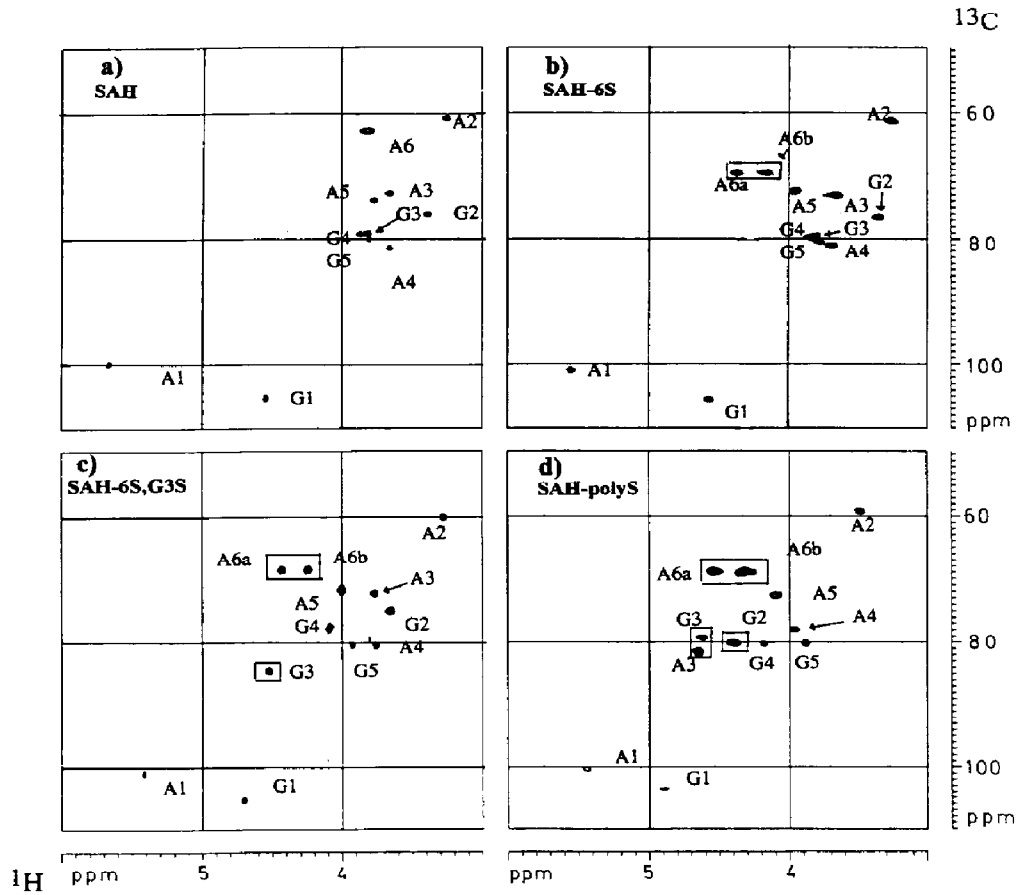


Fig. 2. Two-dimensional $^{13}C/^{1}H$ NMR plots of SAH and some of its *O*-sulfated derivatives (same preparations as for Fig. 1). A and G are signals from the amino sugar and glucuronic acid residues, respectively.

and to double the coagulation time (in the APTT test) were determined for the working standard and for each sample, and the specific activities of the samples were expressed (in %) relative to those of the standard. As a certain variability was obtained for different determinations of the standard at different days, the activity of the samples was calculated on the value of the standard measured in the same experiment. In the case of repeated measurements on the same sample, the activity was calculated from the average of the percent values.

3. Results and discussion

SAH was obtained as reported elsewhere [2] from a preparation of K5 polysaccharide (M_w 35 500, determined by HPLC), which was *N*-deacetylated with hydrazine, and *N*-sulfated with $Py \cdot SO_3$. In the present work, both reactions were brought to completion (> 95% *N*-deacetylation and corresponding *N*-sulfation, as evaluated by ^{13}C NMR). SAH was sulfated with $Py \cdot SO_3$ under different experimental conditions (summarised in Scheme 1) and isolated as described in the Experimental section.

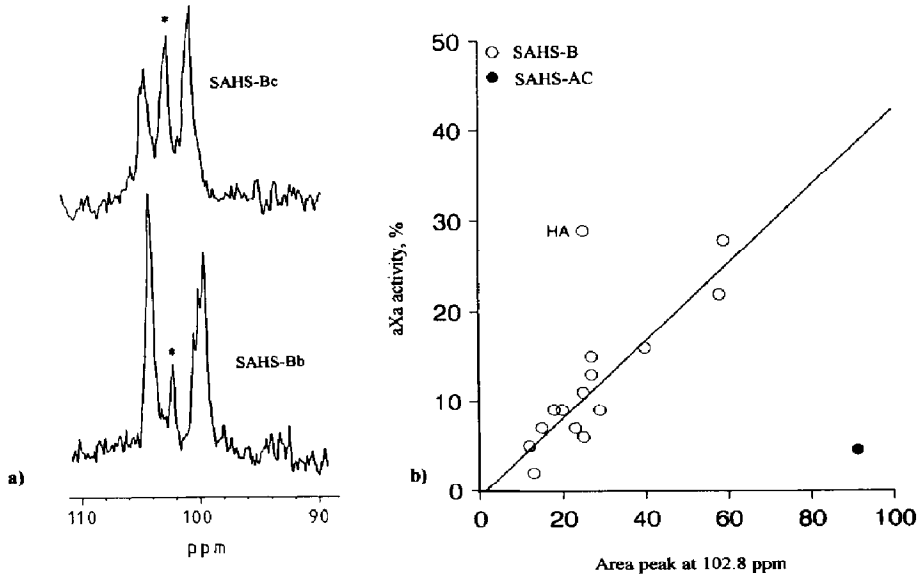


Fig. 3. (a) Partial ^{13}C NMR spectra (anomeric region) of typical preparations of SAH-B, and (b) relationship between the area of the peak at 102.8 ppm and aXa activity for SAHS preparations prepared according to procedures Bb (full circles), Bc (full squares), and C (open circle). The HA sample represents the fraction of preparation Bb-2 retarded on AT-Sepharose (corresponding to fraction numbers 50–85 of Fig. 4). Data for the HA and C preparations were not included in the calculation of the regression line.

Products (sulfaminoheparosan sulfates, SAHS) were analysed by HPLC for their molecular mass and by conductimetry for sulfate-to-carboxylate molar ratios, and their sulfation

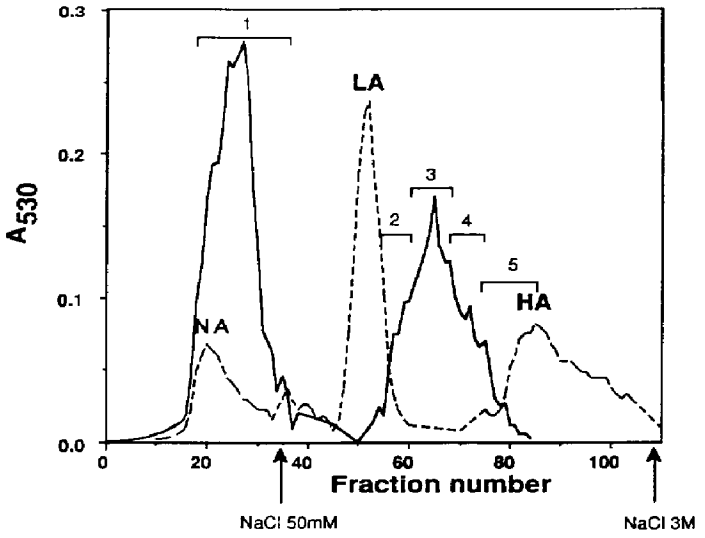
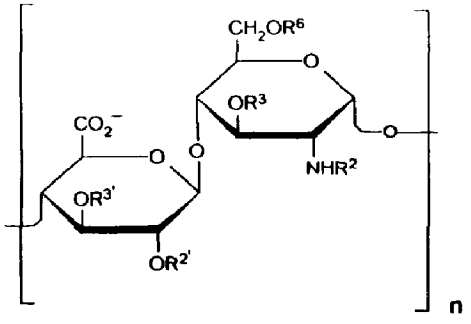


Fig. 4. Affinity chromatography of SAHS (preparation Bb-2) on immobilized antithrombin (AT). The modified polysaccharide was applied to a column of AT-Sepharose, and eluted using a NaCl gradient (extending between the two arrows), as described in detail in the Experimental section. Effluent fractions were analysed for hexuronic acid (solid line). Superimposed is a chromatogram of commercial pig mucosal heparin, which shows typical separation into fractions of no (NA), low (LA), and high (HA) apparent affinity for AT (broken line).

patterns were characterized by mono- and bi-dimensional ^1H and ^{13}C NMR spectroscopy. The M_w and SO_3^-/COO^- values of the products are reported in Table 1 together with the corresponding relative contents of 6-*O*-sulfate groups on glucosamine residues and 2-*O*-sulfate groups on glucuronic acid residues as determined by ^{13}C NMR spectroscopy (see later). The M_w values of the samples are consistently lower than those of the starting material, presumably due to limited depolymerisation during hydrazinolysis. Table 1 also reports the in vitro biological activities (anti-Xa, APTT, and HC-II) of the products. Table 2 reports the chemical shifts and signal assignments for ^1H and ^{13}C NMR signals of major sequences of products prepared according to procedures A, Ba–c, C, and AC of Scheme 1.

As illustrated in Fig. 1, the ^{13}C NMR spectra of products obtained following procedures A, AC, and C (spectra b, c, and d, respectively) are constituted of major repeating sequences, accounting for ca. 90% of the structure of the product. Analysis of the mono- and two-dimensional spectra (see Fig. 2 for heteronuclear, two-dimensional correlation plots) clearly indicates that the product obtained by Procedure A (0°C, 3 equiv SO_3, 1 h) is essentially a 6-*O*-sulfated SAH (SAH-6S), that obtained by Procedure AC (55°C, 12 equiv SO_3, 24 h) is essentially a 6-*O*-sulfated SAH further sulfated at O-3 of the GlcA residues (SAH-6,G3S), and that obtained by Procedure C (25–55°C, 10 equiv SO_3, 24 h) is extensively sulfated also at position 2 of GlcA, as well as at position 3 of GlcN residues. Starting from sequence **1** of *N*-acetyl heparosan (K5-PS) and through *N*-deacetylation (conversion to amino heparosan AH, sequence **2**) and *N*-sulfation (to sulfaminoheparosan SAH, sequence **3**), SAH derivatives regioselectively 6-*O*-sulfated (SAH-6S, sequence **4**) or further 3-*O*-sulfated at the GlcA residue (SAH-6S,G3S, sequences **5**) were thus obtained.



		sequences	abbreviations
1	R^2=Ac; R^3=R^6=$R^{2'}$=$R^{3'}$=H	GlcA-GlcNAc	K5-PS
2	R^2=R^3=R^6=$R^{2'}$=$R^{3'}$=H	GlcA-GlcN	AH
3	R^2=SO_3^-; R^3=R^6=$R^{2'}$=$R^{3'}$=H	GlcA-GlcNSO_3^-	SAH
4	R^2=R^6=SO_3^-; R^3=$R^{2'}$=$R^{3'}$=H	GlcA-GlcNSO_3^-6SO_3^-	SAH-6S
5	R^2=R^6=$R^{3'}$=SO_3^-; R^3=$R^{2'}$=H	GlcA3SO_3^--GlcNSO_3^-6SO_3^-	SAH-6S,G3S
6	R^2=R^6=$R^{2'}$=SO_3^-; R^3=$R^{3'}$=H	GlucA2SO_3^--GlcNSO_3^-6SO_3^-	SAH-6S,G2S
7	R^2=R^3=R^6=SO_3^-; $R^{2'}$=$R^{3'}$=H	GlucA-GlucNSO_3^-3,6SO_3^-	SAH-3,6S
8	R^2=R^3=R^6=$R^{2'}$=$R^{3'}$=SO_3^-	GlcA2,3SO_3^--GlcNSO_3^-3,6SO_3^-	SAH-polyS

Procedure B (reaction at 0°C with an excess of 5 equiv SO_3 per available OH groups) leads, for short reaction times (< 1 hr, version Ba), to products mainly consisting of partially

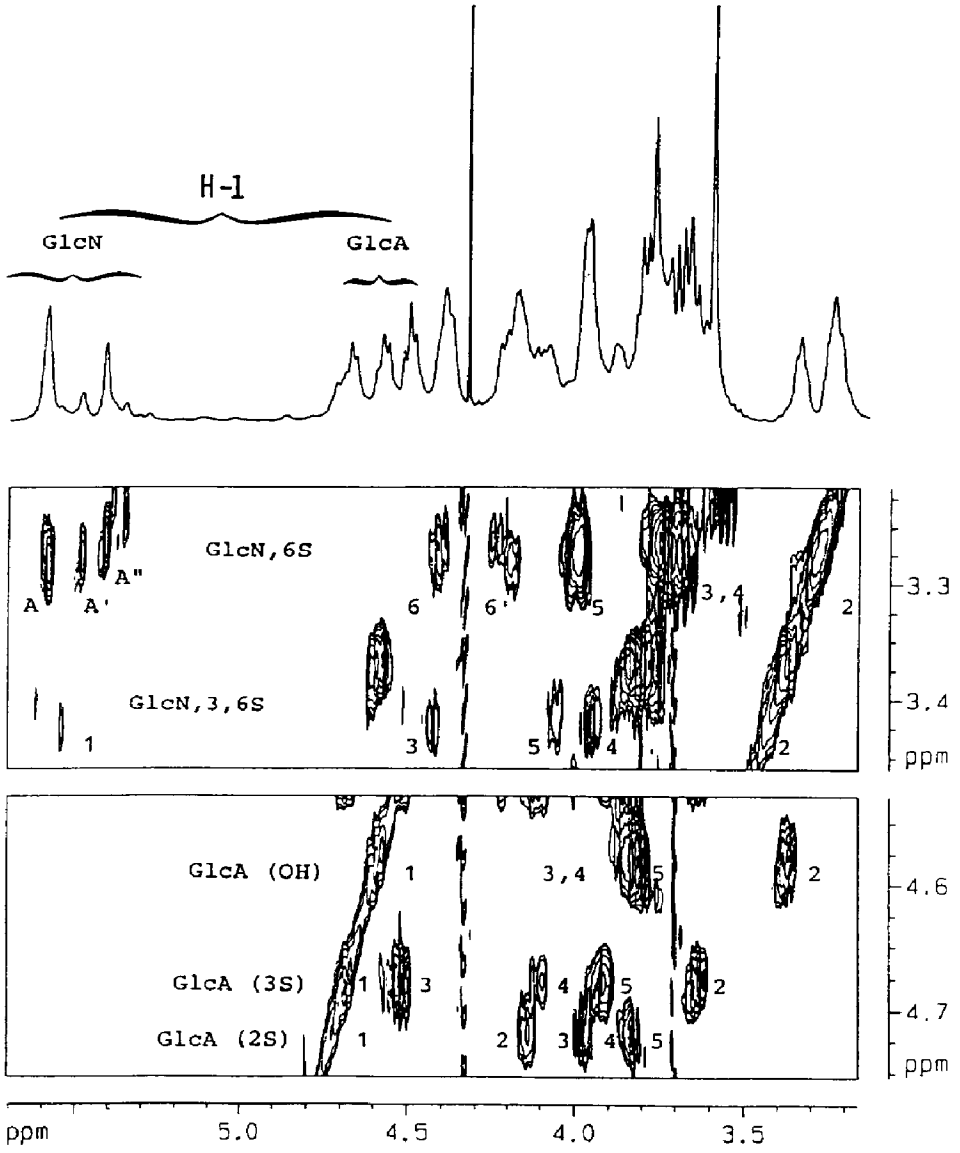


Fig. 5. Two-dimensional ^{1}H NMR TOCSY spectra of subfraction 3 of the fraction of SAHS Bb-2 retarded on the AT affinity column. A, A', and A" reflect $GlcNSO_3$ residues incorporated in different sequences.

6-*O*-sulfated SAHS. For longer reaction times (1 and 2×1 h, versions Bb and Bc, respectively), the NMR spectra of products become complex, and indicate sulfation also at position 2 and (to a somewhat lesser extent) at position 3 of the GlcA residues. Signals attributable to at least four types of sequences are present in products prepared according to procedure Bc, decreasing in relative intensity in the order: **6** (SAH-6S,G2S) – **5** (SAH-6S,G3S) – **4** (SAH-6S) – **7** (SAH-3,6S). Products prepared according to Procedure C largely consist of sequences **8** (SAH-polyS).

It is noteworthy that reactions with procedures A, AC, and C yield products which, though showing significantly high APTT and HC II activities, have generally low anti-Xa activity

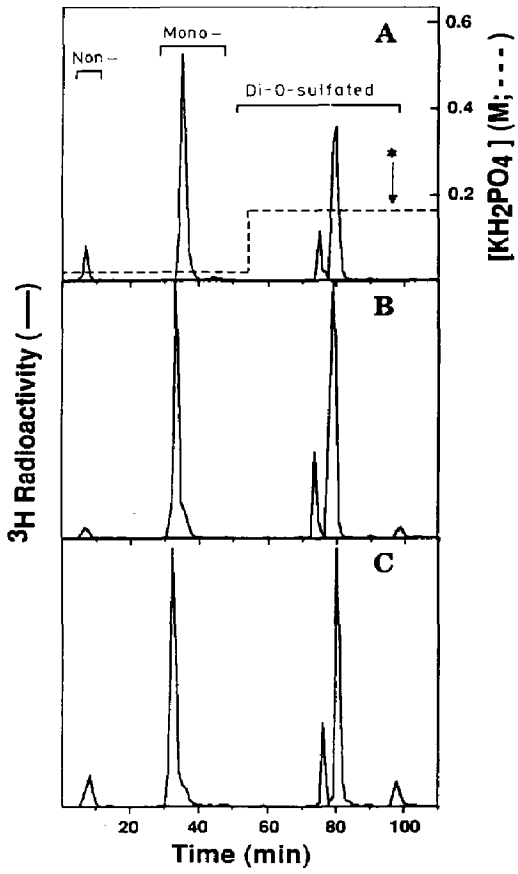


Fig. 6. Anion-exchange HPLC of ^{3}H-labeled disaccharides. Samples of fractions 1 (panel A), 3 (panel B), and 5 (panel C) of SAHS, following affinity chromatography on AT-Sepharose, were subjected to HNO_2 (pH 1.5)–$NaB^{3}H_4$ treatment, and the resulting labeled disaccharides were analyzed on a Partisil-10 SAX column (see Experimental section). The arrow show the elution position of standard glucuronosyl-anhydromannitol(3,6-di-*O*-sulfate) [9].

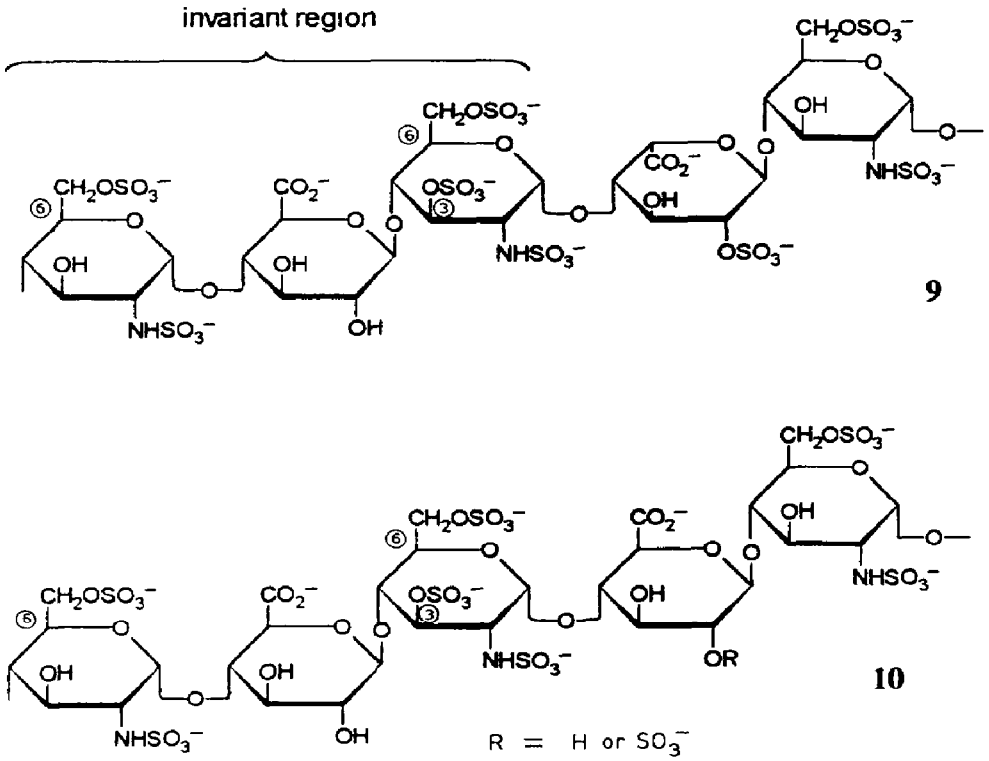
(Table 1). By contrast, products obtained with Procedure B in the versions Bb and Bc (i.e., obtained with longer reaction times), display, in addition, appreciable anti-Xa activity. As illustrated in Fig. 3b, the aXa activity appears to be correlated with the area of the ^{13}C NMR signal at 102.8 ppm (labeled with an asterisk in Fig. 3a). Analysis of two-dimensional spectra indicates that this signal is due to C-1 of 2-*O*-sulfated GlcA residues (Table 2).

To investigate the actual sulfation pattern(s) associated with activity, a preparation of the Bb series (Bb-2) was affinity-fractionated on a column of Sepharose-immobilized antithrombin-III (AT) (Fig. 4). Two major peaks were consistently observed, one which appeared unretarded under the c onditions employed (Fraction 1, ~40% of the total material, coinciding with the NA fraction of commercial heparin) and another (~60%) that emerged in a position intermediate to those of LA and HA heparin. The latter SAHS fraction was subdivided into 4 subfractions (numbered 2–5 in Fig. 4) that were separately subjected to structural analysis.

Subfraction 3 (corresponding to the central portion of the SAHS retarded on the AT-Sepharose column) was analysed by two-dimensional TOCSY ^{1}H NMR. As illustrated in

Fig. 5, the analysis indicates that this subfraction has a complex composition, with GlcA residues involved in at least three types of sequences (in one of which the uronic acid is unsubstituted, and the other two where it is 2-*O*-sulfated and 3-*O*-sulfated, respectively), and 6-*O*-sulfated GlcN residues in at least four sequences (one of which also bears a sulfated group at position 3 of GlcN).

For a more detailed compositional analysis in terms of disaccharide sequences, the SAHS subfractions obtained by affinity chromatography on AT-Sepharose were deaminated, and the products were reduced with NaB^3H_4. The resulting glucopyranosyl-anhydro[1-^{3}H]mannitol disaccharides were separated further by anion-exchange HPLC (Fig. 6). The chromatograms were largely similar, with a small peak of nonsulfated disaccharide, a major peak of mono-*O*-sulfated disaccharide, and a group of di-*O*-sulfated disaccharides. The most retarded of the latter components (marked with an asterisk) emerged at the same elution position as standard glucuronosyl-anhydromannitol (3,6-di-*O*-sulfate). This peak, contrary to the less retarded, major components, differed markedly in amount between the various fractions of affinity-separated SAHS. While the major fraction 1, unretarded by the immobilized AT, was virtually devoid of glucuronosyl-anhydromannitol (3,6-di-*O*-sulfate) (Fig. 6A), the two retarded fractions 3 and 5 showed significant amounts of this component (Figs. 6B and C).



Furthermore, the relative amounts of this disaccharide were larger in fraction 5 than in fraction 3, suggesting a correlation with the affinity of the parent polysaccharide for AT. This finding is in agreement with the notion, established through previous work [12], that theGlcA-GlcNSO$_3^-$3,6SO$_3^-$ [-glucuronosyl-glucosaminyl (*N*-sulfate-3,6-di-*O*-sulfate)-]

sequence is a marker group of the AT-binding region in the heparin molecule, represented by the pentasaccharide sequences **9** (where the sulfamino group of the residue at the nonreducing end could also be a NAc group). Accordingly, the aXa activity of the "high-affinity" subfraction (fraction 3 in Fig. 4) was 29% of that of the heparin standard, i.e., appreciably higher than that of the starting material.

Taken together, these results suggest that the aXa activity of the B-type SAHS is mainly associated with chains containing sequence **10**, which incorporates the trisaccharide structure $GlcNSO_3^-6SO_3^-$-GlcA-$GlcNSO_3^-3,6SO_3^-$, an essential, conformationally rigid [13,14] portion of the binding site of heparin for AT. (The second GlcA residue of **10** may be sulfated at either O-2 or O-3). Although the aXa activity of affinity-unfractionated SAHS prepared according to Procedure B increased linearly with increasing of the area of the ^{13}C NMR peak at 102.8 ppm (due to C-1 of 2-*O*-sulfated GlcA residues), for the HA fraction this activity is consistently higher than accounted for by its content of $GlcA2SO_3^-$ (Fig. 3b). Since 3-*O*-sulfated $GlcNSO_3^-6SO_3^-$ residues were not detected in the LA fraction of affinity-fractionated SAHS Bb-2, we conclude that increasing sulfation according to Procedure B involves formation of -$GlcA2SO_3^-$-$GlcNSO_3^-6SO_3^-$- as major sequences as well as of -GlcA-$GlcNSO_3^-3,6SO_3^-$- as minor sequences, of which the latter are the most important in eliciting the aXa activity (and, in part, the APTT activity).

A comment is warranted concerning the possibility that the observed aXa activities are associated with minor contents of IdoA residues (which arise from C-5 epimerization of GlcA in the hydrazinolysis reaction) [4] generating, in the subsequent *O*-sulfation reaction, -$GlcNSO_3^-3,6SO_3^-$-$IdoA2SO_3^-$- sequences as in pentasaccharide **9**. Such a possibility is suggested by the observation that substitution of the $IdoA2SO_3^-$ residue in the synthetic pentasaccharide **9** brings about a substantial loss of affinity for AT [17]. Indeed, weak signals in the 4.8–5.2 ppm region typical of H-5 and H-1 signals of IdoA and $IdoA2SO_3^-$ [18] are apparent in the monodimensional spectrum of subfraction 3 of the high-affinity fraction Bb2-HA (Fig. 5). However, area measurements from vertically expanded spectra (not shown) indicated that total (sulfated + nonsulfated) IdoA in this fraction with high affinity for AT corresponds to no more than 3% of total hexosamines (strong signals from 5.35 to 5.7 ppm). Further evidence that the overall contents of IdoA, if any, are low derives from the HPLC analysis of disaccharides generated by deaminative cleavage of the AT-binding polysaccharide fractions. Thus, the single mono-*O*-sulfated disaccharide detected (Fig. 6) emerged at the elution position of glucuronosyl-anhydro[1-^{3}H]mannitol 6-*O*-sulfate, clearly separated from any known IdoA-containing standard (data not shown). Further analysis of products generated on partial *O*-desulfation of the major di-*O*-sulfated fraction likewise failed to detect any significant amounts of IdoA-containing components (described elsewhere). It therefore seems highly improbable that any appreciable proportion of $IdoA2SO_3^-$ residues would occur adjacent to the few available residues of 3-*O*-sulfated $GlcNSO_3^-6SO_3^-$.

Further sulfation, such as achieved with Procedure C (leading to SAHS with SO_3^-/COO^- ratios >2.8) does not further increase the aXa and APTT activities. Also, SAHS prepared according to Procedure AC (consisting mainly of sequences -$GlcA3SO_3^-$-$GlcNSO_3^-6SO_3^-$-) have low aXa and APTT activities, implying that 3-*O*-sulfation of GlcA residues interferes with AT-mediated activities. Products with the highest degrees of sulfation have also somewhat low HC II activities, an unexpected finding on account of the

assumed nonspecificity of HC II-mediated thrombin inhibition [15]. In fact, the highest HC II activities were observed with simple 6-*O*-sulfation of SAH, as obtained with Procedure A. As for other classes of sulfated polysaccharides [16], heparin-like activities of SAHS are thus not a simple function of the degree of sulfation.

4. Conclusions

The present work indicates the possibility of obtaining a variety of sulfated sulfaminoheparosan sulfates with systematically varied sulfation patterns and biological properties by appropriate control of conditions of the chemical sulfation of *N*-deacetylated K5 polysaccharide. Among these properties, a potential antithrombotic activity (as indicated by AT-mediated inhibition of Factor Xa) is induced when some of the 6-*O*-sulfated $GlcNSO_3^-$ residues of sulfaminoheparosans are also sulfated at position 3, adjacent to unsubstituted GlcA residues. The resulting -$GlcNSO_3^-$ $6SO_3^-$ -GlcA-$GlcNSO_3^-$ $3,6SO_3^-$ - trisaccharide sequence constitutes an essential part of the pentasaccharidic active site of heparin for AT.

An alternative antithrombotic agent could be provided by simple 6-*O*-sulfation of $GlcNSO_3^-$ residues, inducing a HC II mediated inhibition of thrombin of the same order of magnitude as heparin. 3-*O*-Sulfation of GlcA residues appears to inhibit both activities.

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