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Generation of anti-factor Xa active, 3-*O*-sulfated glucosamine-rich sequences by controlled desulfation of oversulfated heparins

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Abstract

In the framework of a project aimed at generating heparin-like sulfation patterns and biological activities in biotechnological glycosaminoglycans, different approaches have been considered for simulating the α(1→4)-linked 2-*O*-sulfated L-iduronic acid ($IdoA2SO_3$)→*N*,6-*O*-sulfated D-glucosamine ($GlcNSO_36SO_3$) disaccharide sequences prevalent in mammalian heparins. Since the direct approach of sulfating totally *O*-desulfated heparins, taken as model compounds for C-5-epimerized sulfaminoheparosan (*N*-deacetylated, *N*-sulfated *Escherichia coli* K5 polysaccharide), preferentially afforded heparins *O*-sulfated at C-3 instead than at C-2 of the iduronate residues, leading to products with low anticoagulant activities, the problem of re-generating a substantial proportion of the original $IdoA2SO_3$ residues was circumvented by performing controlled solvolytic desulfation (with 9:1 v/v DMSO–MeOH) of extensively sulfated heparins. The order of desulfation of major residues of heparin GlcN and IdoA and of the minor one D-glucuronic acid was: $GlcNSO_3 > GlcN6SO_3 > IdoA3SO_3 \cong GlcA2SO_3 \cong GlcN3SO_3 > IdoA2SO_3 \cong GlcA3SO_3$. Starting from a 'supersulfated' low-molecular weight heparin, we obtained products with up to 40% of iduronate residues *O*-sulfated exclusively at C-2 and up to 40% of their glucosamine residues *O*-sulfated at both C-6 and C-3. Upon re-*N*-sulfation, these products displayed an in vitro antithrombotic activity (expressed as anti-factor Xa units) comparable with those of current low-molecular weight heparins.

Keywords: Heparin oversulfation/desulfation; 3-*O*-Sulfation; NMR analysis; Anti-Xa activity

1. Introduction

Heparin, a glycosaminoglycan currently used as an anticoagulant and antithrombotic drug, is prevalently constituted by disaccharide sequences **1**, made up of α(1→4)-linked L-iduronic acid 2-*O*-sulfate ($IdoA2SO_3$) and *N*,6-*O*-sulfated D-glucosamine ($GlcNSO_36SO_3$) residues. Minor monosaccharidic components of heparin are D-glucuronic (GlcA) and *N*-acetylated D-glucosamine (GlcNAc), both reminiscent of the biosynthetic precursor *N*-acetylheparosan (**2**). Depending on tissue and animal species, and to some extent also on the isolation process, different heparins have somewhat different sulfation patterns and contain different proportions of minor residues such as 2-*O*-sulfated glucuronic acid ($GlcA2SO_3$) and 3-*O*-sulfated sulfamino-glu-

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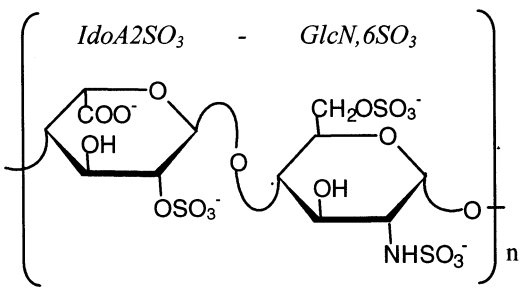
cosamine ($GlcNSO_3$, $3SO_3$), this latter often being also 6-*O*-sulfated ($GlcNSO_3 3$, $6SO_3$).[1] Sequences **1** usually account for more than 70% of the structure of mammalian heparins. However, the most important sequence for the expression of the antithrombotic and anticoagulant activities is the pentasaccharide **3**, representing the active site for antithrombin III (AT) (Scheme 1). This sequence, present in only about one third of the chains of mammalian heparins, contains a 3-*O*-sulfated glucosamine residue, considered a marker of this active site and correlated with the antithrombotic activity, usually expressed by the inhibition of coagulation factors Xa and IIa (thrombin).[1]

As part of a broader research project aimed at generating 'biotechnological' heparins and heparan sulfates (HS), we first investigated possible routes for regenerating heparin-like sulfation patterns starting from totally *O*-desulfated heparin as a model for *N*-deacetylated, *N*-sulfated and partially C-5-epimerized *N*-acetylheparosan produced by *Escherichia coli* K5 polysaccharide.[2] The original sulfation pattern of heparin was reported by Ogamo et al. to be regenerated by direct sulfation of completely *N*,*O*-desulfated, re-*N*-sulfated heparin using pyridine·SO_3 in aprotic solvents.[3] However, sulfation of 2-*O*-desulfated heparin under similar experimental conditions was shown by Perlin and coworkers to lead prevalently to O-sulfation at C-3 of the IdoA residue, together with some O-sulfation at C-3 of the $GlcNSO_3$ residues.[4] In line with synthetic heparin oligosaccharides structure–activity studies indicating that replacement of the $IdoA2SO_3$ residue in sequence **2** with an $IdoA3SO_3$ residue drastically decreases affinity for AT,[5] the aforementioned heparin prevalently sulfated at C-3 of IdoA residue have a much lower anti-Xa activity than the original heparin.[4] Indeed we have confirmed that direct sulfation also of totally *N*,*O*-desulfated, re-*N*-sulfated heparin leads to poorly active heparins, with prevalently 3-*O*-sulfated (instead of 2-*O*-sulfated) IdoA residues.[2]



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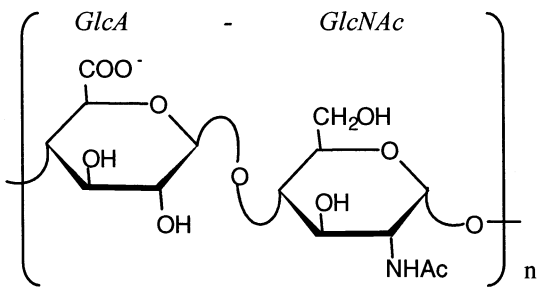

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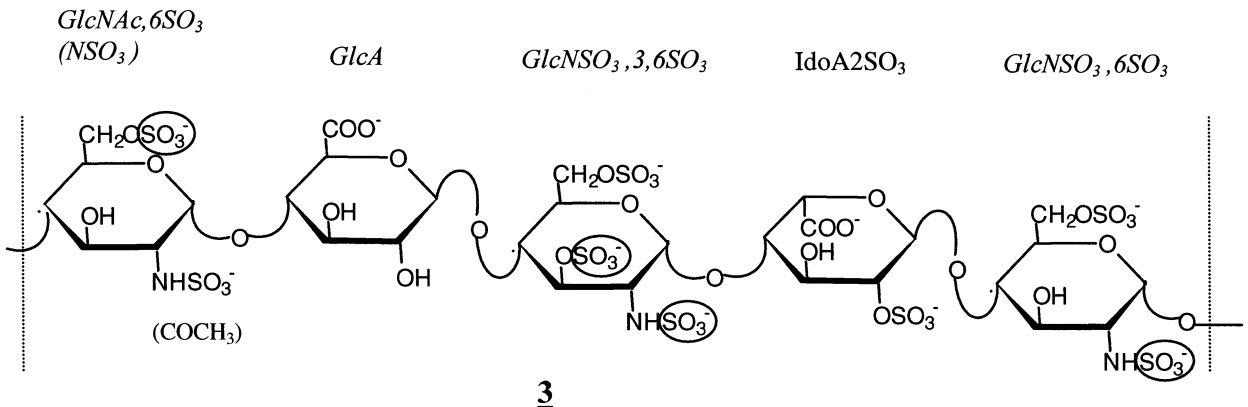

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Scheme 1.

The alternative approach described in this paper, based on controlled solvolytic desulfation of oversulfated heparin, permitted us to obtain heparins with up to 40% of their IdoA residues *O*-sulfated exclusively at C-2, with GlcA residues remaining prevalently 3-*O*-sulfated. Some of the products, also with up to 40% of 3-*O*-sulfated $GlcNSO_3$ residues, displayed in vitro anti-factor Xa activities even higher than those of conventional LMW-heparins.

2. Results and discussion

Heparin was exhaustively sulfated to totally sulfated heparin (ssH), both under conditions of controlled depolymerization with sulfuric acid–chlorosulphonic acid to generate a 'supersulfated' low-molecular weight heparin (ssLMW-H)[6] and with $Py{\cdot}SO_3$ in Me_2SO,[7] with substantial retention of the original molecular weight, to obtain totally sulfated heparin (ssH). In fact, conductimetric determination of the sulfate-to-carboxylate groups ratio[8] indicated, especially for ssH, that a minor proportion of the original free hydroxyl groups of heparin remained nonsulfated. Furthermore, as the final target of the study was a low-molecular weight heparin, most of the experiments were performed on ssLMW-H.

ssLMW-H was characterized by one- and two-dimensional NMR spectroscopy. The ^{13}C NMR spectrum (essentially as reported in Ref. 6) confirms complete N-sulfation and O-sulfation at C-6 of the glucosamine residues (characteristic signals at 59.5 and 68.7 ppm), and indicates almost complete sulfation of the other hydroxyl groups of the glucosamine (at C-3) and iduronic acid (at C-2 and C-3). The absence of signals in the ^1H NMR region 3.85–3.88 ppm confirms complete 6-*O*-sulfation of the glucosamine residues.[9] The ^1H COSY spectrum (not shown) indicates correlations between signals at 5.22 ppm (H-1), 5.33 ppm (H-2), and 4.38 (H-3), typically associated with 3-*O*-sulfated glucosamine.[10] Integration of the corresponding ^1H signal (see later) indicated that the GlcN residues of ssLMW-H were 3-*O*-sulfated only to the extent of about 60%. The correlations of the weak glucuronic acid signals in the COSY spectrum (not shown) are typical for glucuronic acid residues sulfated at both positions 2 and 3.[11] The anomeric signals of iduronic acid residues from the HMQC plots (not shown) correspond to 3-*O*-sulfated and 2,3-*O*-sulfated iduronic acid.[7]

The solvolysis reactions of ssLMW-H were carried out at 55, 65, 80, and 90 °C following a general procedure reported for glycosaminoglycans, leading to desulfated products with minimal depolymerization.[12] Products, recovered at several reaction times, were characterized directly by ^{13}C NMR spectroscopy as *N*-desulfated products. It is clearly seen from the ^{13}C spectrum of ssLMW-H made after only a few minutes desulfation (Fig. 1(A)), that the N-sulfate groups are removed almost immediately. Illustrated in Fig. 1(B) is a product of further desulfation that shows well

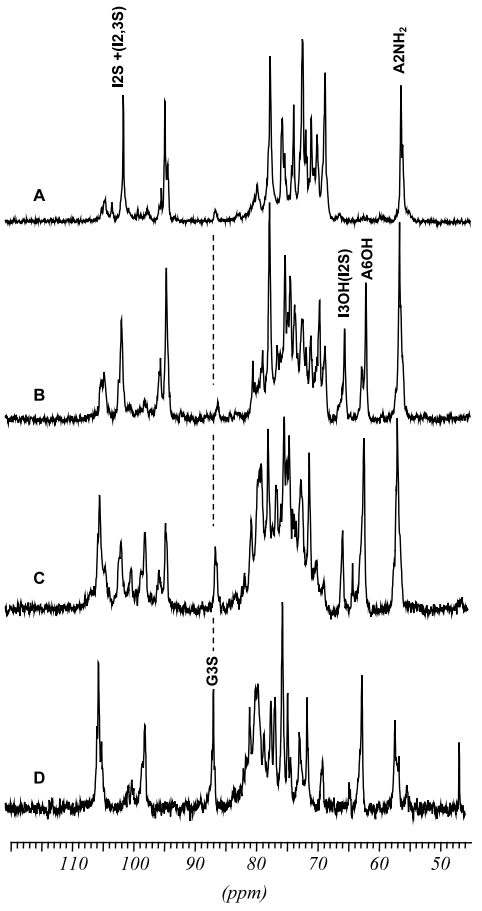

Fig. 1. ^{13}C NMR spectra of intermediate products of desulfation of ssLMW-H after 15 and 240 min desulfation at 65 °C (A and B, respectively); (C) ss-HS (65 °C, 250 min); (D) ss-K5-PS (80 °C, 45 min).

Table 1
Desulfation kinetics of supersulfated low-molecular weight heparin (ssLMW-H) in DMSO–MeOH 10% v/v at 65, 80, and 90 °C

Min	% A2NH$_2$			%A6OH			%U2OH			%I3OH-I2S			%A3S		
	65 °C	80 °C	90 °C	65 °C	80 °C	90 °C	65 °C	80 °C	90 °C	65 °C	80 °C	90 °C	65 °C	80 °C	90 °C
0	0	0	0	0	0	0	0	0	0	n.d.	n.d	n.d.	60	60	60
15		100			40			45			34			56	
30	100	100	100	15	60	37	53	52	45	0	35	36	54	n.d.	60
45	100	100	100	19	60	48	48	59	48	24	42	37	45	n.d.	56
90	100		100	32		82	52		54	27		41	28		39
120		100			90			61			35			n.d.	
150	100		100	34		95	51		62	27		34	25		32
240	100	100		75	100		61	69		46	34		22	32	
360		100			100			68.5			32			n.d.	
480		100			100			78			30			21	
1440		100			100			78			18			17	

A, GlcN; I, IdoA; U, GlcA + IdoA; n.d., not measured. % desulfation determined by ^{13}C and ^{1}H NMR.

recognizable signals of carbons bearing free hydroxyl groups. The ^{13}C signals at 59.5 and 68.7 ppm were used to estimate desulfation at A2 and A6, respectively. In order to focus on iduronic 2-*O*-sulfate residues (IdoA2SO$_3$), for the products obtained after 45 min solvolysis at 80 °C, two-dimensional NMR techniques were employed. HMQC spectra (not shown) show two sets of iduronic acid signals, the major one (5.15/101.3 or 101.9 ppm) attributable to IdoA2SO$_3$,[9] and the other (5.0/103.6 ppm) to IdoA3SO$_3$ (as well as to completely nonsulfated IdoA).[7] The COSY spectrum also gave signals at 4.56/77.5 ppm typical of H-3 of 3-*O*-sulfated glucosamines.

The signal at 65 ppm (specifically attributable to C-3 of 3-nonsulfated, IdoA2SO$_3$)[9] permitted us to quantify the relative content of iduronic acid residues sulfated only at C-2, as in the original heparins. The desulfation at A2 and A6 was estimated by the ^{13}C signals at 59.5 and 68.7 ppm comparing the areas of these signals with the total area of the A2 (NSO$_3$ + NAc) signals. The ratio between the total uronic acid residues sulfated and nonsulfated in position 2 has been obtained by the integration of signals in the 99–102 ppm and 102–105 ppm regions.

Due to signal overlapping with the ^{13}C and ^{1}H signals associated with 3-*O*-sulfated GlcN residues, O-sulfation at C-3 of these residues was determined indirectly through their quantitative conversion to *N*-sulfo-aziridine derivatives and quantification of ^{1}H signals of these residues.[13] Alternatively, the 3-*O*-sulfation percentage of GlcN residues was established by measuring the volume of pertinent signals in the HMQC spectra of nonderivatized products. Comparison of the data obtained with the two methods revealed the correspondence to be satisfactory (within 3%).

Table 1 shows, for ssLMW-H, the sulfation percentages at different positions of the IdoA and GlcN residues obtained by solvolytic desulfation at 65, 80, and 90 °C. For the reaction at 90 °C there is the steady release of the sulfate group at C-6 of GlcN, and this is paralleled by the release of the sulfate group at C-3 from IdoA2,3SO$_3$ (Fig. 2). In the first stages of solvolysis, the rate of 6-*O*-desulfation of glucosamine residues appears almost the same of the 3-*O*-desulfation of IdoA2,3SO$_3$. On the other hand, the sulfate

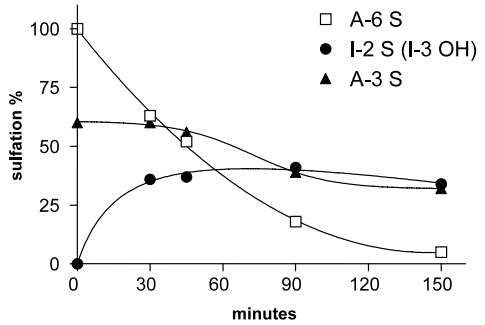

Fig. 2. Kinetics of solvolytic desulfation at 90 °C for ss-LMW-H, % of O-sulfation at different positions of IdoA and GlcN residues.

Table 2
Desulfation kinetics of supersulfated heparin (ssH) in DMSO–MeOH 10% v/v at 65 °C

Min	DS[8]	M_W[16]	%A2NH$_2$	%A6OH	%U2OH	%I3OH-I2S
0	3	12,000	100	0	0	n.d.
240	1.8	12,000	100	60	30	54
480	1.7	10,300	100	71	42	54
960	1.3	n.d.	100	95	50	54
1440	1	11,000	100	95	52	42

% desulfation determined by ^{13}C and ^{1}H NMR; DS by conductimetric titration; M_W by GPC–HPLC analysis.

Table 3
Desulfation kinetics of supersulfated heparansulfate (ssHS) and K5 polysaccharides (ssK5-PS) in DMSO–MeOH 10% v/v at 65 °C

Samples	°C	Min	%A2NH$_2$	%A6OH	%U2OH	%I3OH-I2S	%G3S
ssE$_{30}$HS	65	240	100	44	76	16	55
ssE$_{60}$HS	80	180	100	40	82	n.m.	n.m.
ssK5-PS	80	195	100	40	48		52
ssK5-PS	80	135	100	20	50		50

% desulfation determined by ^{13}C and ^{1}H NMR. Abbreviations as for Table 1. n.m., not measured.

group at C-3 of GlcN is resistant to solvolysis for up to about 40 min reaction time at all the tested temperatures and is released consistently more slowly than the 6-*O*-sulfate. The reaction temperature of 65 °C appeared the most convenient one, both to control the reaction kinetics and to achieve more than 50% 3-*O*-desulfation of IdoA2,3SO$_3$ residues while retaining almost 70% of the original 6-*O*-sulfate groups on GlcN residues. Notably, at temperatures as high as 90 °C and for reaction times up to 150 min, the levels of 3-*O*-sulfation were substantially higher than in natural mammalian heparins (more than 20% as compared with about 5% of total aminosugars). Desulfation experiments performed at 65 °C on 'supersulfated' heparin (ssH) generated sulfation patterns (Table 2) similar to those obtained with ssLMW-H. An experiment made on a supersulfated HS (ssHS) with an IdoA/GlcA ratio of about 3:7 yielded a product containing about 16% of IdoA2SO$_3$ calculated on total uronic acids.

Analysis of NMR signals associated with the minor GlcA residues of heparin indicated that the 3-*O*-sulfate groups are released from GlcA2,3SO$_3$ much more slowly than the 2-*O*-sulfate groups (Fig. 1(A and B)). Such a behavior, strikingly different from that of sulfated IdoA, was confirmed by experiments performed on HS and K5-PS, containing GlcA as, respectively, the prevalent and exclusive uronic acid (examples illustrated in Fig. 1(C) and (D)). In fact, this residue remained almost exclusively 3-*O*-sulfated even after 45 min at 80 °C (Table 3).

The in vitro anti-factor Xa activities, taken as predictive of the antithrombotic activity,[14] were measured for selected samples after their re-*N*-sulfation. As illustrated in Fig. 3 for products of solvolytic desulfation at 90 °C of ssLMW-H, the measured anti-Xa activity increases dramatically at the first stages of desulfation, before steadily decreasing for further desulfated products. The highest activity (after 30 min desulfation at 90 °C, and after

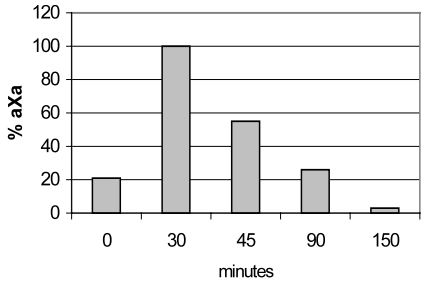

Fig. 3. Percent anti-Xa activity (referred to a conventional LMW-H) of re-*N*-sulfated ssLMW-H desulfated products at different times of solvolytic desulfation at 90 °C.

15 min at 80 °C and after 4 h at 65 °C) is also significantly higher than that of the typical LMW-heparin used for comparison purposes.

A major determinant for the anti-factor Xa activity of heparin and HS is the active site for AT (the pentasaccharide **3**).[1] Removal of critical sulfate groups (circled in formula **3**) is expected to impair the affinity for AT and the associated activities.[5,14] On the other hand, extra sulfate groups could have a quite different impact on the aXa activity. Sulfation at C-6 of the $GlcNSO_3$ (or the GlcNAc) at the nonreducing end of **3**, and at C-3 of the central $GlcNSO_3$ residue of this pentasaccharide sequence increase the activity,[5,15] which is further increased by 3-*O*-sulfation of the $GlcNSO_3$ residue at the reducing end.[5] On the other hand, 3-*O*-sulfation of the IdoA residue impairs activity,[4] as also suggested by the low aXa potency observed for 'supersulfated' heparins[16] and low-molecular weight heparins.[17] It is noteworthy that the activity of the partially *O*-desulfated, re-*N*-sulfated ssLMW-H is at least as high as that of the optimized LMW-H used for comparison purposes. Considering that even in the most active products of this study a significant proportion of IdoA residues (up to 20%) are still 3-*O*-sulfated (both as $IdoA2,3SO_3$ and $IdoA3SO_3$ residues), the simplest explanation of present results is that the positive effect of the high levels of 3-*O*-sulfation of the $GlcNSO_3$ residues overcomes the negative effect of the residual 3-*O*-sulfation of the IdoA residues. Such a tentative explanation is supported by previous finding that high affinity for AT (and high aXa activity) can be elicited by 3-*O*-sulfation of $GlcNSO_3$ residues in K5-derived products lacking $IdoA2SO_3$ residues.[11,18]

In conclusion, graded solvolytic desulfation of extensively sulfated heparins regenerate sulfation patterns very similar to those of the original glycosaminoglycan, but significantly richer in 3-*O*-sulfated glucosamine residues. Such residues appear to outweigh the anti-Xa activity-impairing effect of residual 3-*O*-sulfation of the iduronic acid residues. The present approach, followed by easy restoring of the original re-*N*-sulfation and 6-*O*-sulfation of the glucosamine residues, is therefore promising for upgrading current heparins and low-molecular weight heparins. The process is also amenable to application to C-5-epimerized sulfaminoheparosans derived from *E. coli* K5 polysaccharide,[19] to obtain biotechnological heparins and heparan sulfates.

3. Experimental

Materials and techniques.—Pig mucosal heparin (H) was a commercial preparation of unfractionated sodium heparin (Laboratori Derivati Organici S.p.A., Milan, Italy), Heparan sulfate ($E_{30}HS$; $E_{60}HS$), with IdoA + $IdoA2SO_3$ contents of 30 and 60%, respectively, from porcine pancreas were from Opocrin, Italy and K5 polysaccharide was an experimental preparation from RSM laboratory (Montale, Italy). LMWH (SO_3^-/COO^- 2; M_W 5000 Da), commercial sample obtained by nitrous acid deamination, was a gift from Professor J. Fareed, the ssLMW Heparin (apparent M_W 4000 Da; SO_3^-/COO^- 3.2) was prepared as described in Ref. 6, by reacting heparin with a 2:1 (v/v) mixture of H_2SO_4 and chlorosulfonic acid.

Ss-H and ss-HS were prepared as described in Ref. 12, starting from H, E_{30} and $E_{60}HS$. Briefly: 200 mg of de-N/*O*-sulfated, re-*N*-sulfated heparin or heparansulfate[21] was converted into the terabutylammonium salt, dried and dissolved in $HCONMe_2$. Twelve equiv of pyridine sulfur trioxide complex–disaccharide unit were added and the solution stirred at 55 °C for 18 h. The reaction products were purified by dialysis and dried under reduced pressure. The obtained *N*-desulfated, extensively *O*-sulfated product was re-*N*-sulfated essentially as described in Ref. 21. Supersulfated K5-PS were obtained from *E. coli* K5 capsular polysaccharides by O-sulfation with $Py{\cdot}SO_3$ as described in detail in Ref. 11.

The charge density of the products (expressed as sulfate to carboxylate molar ratios), determined by conductimetry,[8] was 3.2 and 2.9 for ss-LMW-H and ss-H, respectively.

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. 20, using Progel TSK PWXL 300 × 7.8 mm columns (a G3000 and a G2500 column connected in series with a PWXL Guard pre-column; Supelco, USA). Calibration curves (third order fitting; correlation coefficient 0.996) were obtained using heparin and dermatan sulfate house-working standards. The M_w and Q values were calculated using the Bruker CHROMSTAR 3.13 program, subroutine for gel-permeation chromatography. The average M_W were 4000 and 12,000 Da for ssLMW-H and ss-H samples, respectively.

Graded desulfations.—Time course reactions were performed at different temperature (55, 65, 80, 90 °C): 360 mg of ssH or ssLMWH were dissolved in water, and the solution passed through a column of Amberlite IR-120 (H^+) resin. After adjusting the pH to 5.5 with 10% tributylamine (TBA) in EtOH, the solution was lyophilized. The TBA salt thus obtained was dissolved in Me_2SO–MeOH 10% v/v solution (25 mL). The solution was heated for times between 15 min and 24 h. At the end of the reaction, the solutions were diluted with equal volumes of water, adjusted to pH 9 with 0.2 M NaOH, and precipitated with 4 vols of EtOH saturated with sodium acetate. The samples recovered by filtration were dissolved in water and dialyzed with 2000 Da cut-off membranes (Spectrapore) for 48 h against deionized water and finally desalted by gel permeation on Sephadex G25. The *N*,*O*-desulfated products were re-*N*-sulfated in water with $TMA{\cdot}SO_3$ (pH 9; 6 h at 55 °C) as described in Ref. 21. Total yields were 80–90%.

NMR spectroscopy.—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 100 MHz with a Bruker AMX400 instrument equipped with a 10 mm broad band probe. Each sample (15 mg for ^1H and 100 mg for ^{13}C measurements) was dissolved in D_2O (0.5 mL, 99.99%). Chemical shift given in ppm downfield from internal sodium-3-(trimethylsilyl)-propionate, was 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 digitalization of 0.1 Hz/pt and ^{13}C spectra using ^1H composite pulse decoupling (CPD) without nuclear Overhauser effect. COSY 45 data were acquired using 48 scans per series in 1Kx512W data points with zero-filling in F1. Sine-bell function was applied before Fourier transformation.

Quantification of sulfation patterns.—The area of the signals at 59.5 (A2NS) and 68.7 (A6S) ppm in the ^{13}C NMR spectra was compared to the total area of the A2 (59.5 ppm $NSO_3 + 55$ ppm NAc) and the A6OH (62 ppm) signals were used to estimate the extent of A2 and A6 desulfation, respectively.[22] The percentage of non 3-*O*-sulfated $IdoA2SO_3$ residues was determined from the ^{13}C NMR spectra of *N*-desulfated products by integration of the signal at 65 ppm[9] compared to the total area of A2 signals. The content of 2-*O*-sulfated uronic acid ($U2OS = IdoA2SO_3 + GlucA2SO_3$) was obtained from the ratio between the areas of anomeric signals in 99–102 ppm and 102–105 ppm regions. Value obtained includes contributions of U2,3S. The content of 2,3-sulfated uronic acid was obtained indirectly, from the difference between the areas of the U2S + U2,3S signals and that of the signal at 65 ppm.

A3 sulfation was evaluated by indirect measurements. Samples were treated with a base to convert, quantitatively, the $GlcN,3SO_3$ residues into the corresponding *N*-sulfated aziridine-containing residues.[10] Typically, a sample of chemically modified heparin (20 mg) was dissolved in 0.1 M NaOH (2 mL), frozen and lyophilized. The freeze-dried sample was dissolved in 1 mL of distilled water and neutralized with diluted HCl, purified by gel permeation on Sephadex G25 and analyzed by ^1H NMR spectroscopy. The evaluation of A3S residues was made from the ratio between the area of H-3 signal of the *N*-sulfated aziridine-containing residues (3.1 ppm) and that of the H-2 signals of both *N*-sulfated glucosamine and *N*-sulfated aziridine containing residues (3.3 ppm), adding of 1/3 of the area of signal at 2.1 ppm (CH_3 of GlcNAc residues).

Biological assay.—The in vitro anti-factor Xa (aXa) activity, expressing inhibition of factor Xa by antithrombin, was measured in purified system with a COATEST Heparin kit (Chromogenix).[14] The values were referred to the activity of a working standard heparin (192 IU/mg). It should be noted that as the slopes of the activity concentration curves are different for different sulfation patterns (Bisio et al., unpublished), the aXa activity in the present work should be regarded as only apparent.

Acknowledgements

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References

1. (a) Casu, B.; Lindahl, U. *Adv. Carbohydr. Chem. Biochem.* **2001**, *57*, 159–208;
(b) Conrad, E. *Heparin-Binding Proteins*; Academic Press: San Diego, 1998;
(c) Bourin, M. C.; Lindahl, U. *Biochem. J.* **1993**, *289*, 313–330.
2. Naggi, A.; Torri, G.; Casu, B.; Oreste, P.; Zoppetti, G.; Li, J.-P.; Lindahl, U. *Semin. Thromb. Hemost.* **2001**, *27*, 437–443.
3. Ogamo, A.; Metori, A.; Ukiyama, N. *Carbohydr. Res.* **1989**, *193*, 165–172.
4. Rej, R.; Ludwig-Baxter, K. G.; Perlin, A. S. *Carbohydr. Res.* **1991**, *210*, 299–310.
5. van Boeckel, C. A. A.; Petitou, M. *Angew. Chem. (Engl. Ed.)* **1993**, *32*, 1771–1818.
6. Naggi, A.; Torri, G.; Casu, B.; Pangrazzi, J.; Abbadini, M.; Zametta, M.; Donati, M. B.; Lansen, J.; Maffrand, J. *Biochem. Pharmacol.* **1986**, *36*, 1890–1895.
7. Yates, E. A.; Santini, F.; De Cristofano, B.; Payre, N.; Cosentino, C.; Guerrini, M.; Naggi, A.; Torri, G.; Hricovini, M. *Carbohydr. Res.* **2000**, *329*, 239–247.
8. Casu, B.; Gennaro, U. *Carbohydr. Res.* **1974**, *39*, 168–176.
9. Yates, E. A.; Santini, F.; Guerrini, M.; Naggi, A.; Torri, G.; Casu, B. *Carbohydr. Res.* **1996**, *294*, 15–27.
10. Santini, F.; Bisio, A.; Guerrini, M.; Casu, B.; Yates, E. A. *Carbohydr. Res.* **1997**, *302*, 103–108.
11. Casu, B.; Grazioli, G.; Razi, N.; Guerrini, M.; Naggi, A.; Torri, G.; Oreste, P.; Tursi, F.; Zoppetti, G.; Lindahl, U. *Carbohydr. Res.* **1994**, *263*, 271–284.
12. Nagasawa, K.; Inoue, Y.; Kamata, T. *Carbohydr. Res.* **1977**, *58*, 47–55.
13. Yates, E. A.; Santini, F.; Bisio, A.; Cosentino, C. *Carbohydr. Res.* **1997**, *298*, 335–340.
14. Teien, A. N.; Lie, M. *Thromb. Res.* **1977**, *10*, 399–410.
15. Thunberg, L.; Bäckström, G.; Lindahl, U. *Carbohydr. Res.* **1982**, *100*, 393–410.
16. Toida, T.; Maruyama, M.; Ogita, Y.; Suzuki, A.; Toyoda, M.; Imanari, I.; Linhardt, R. *Int. J. Biol. Macromol.* **1999**, *26*, 233–241.
17. Sissi, C.; Naggi, A.; Torri, G.; Palumbo, M. *Semin. Thromb. Hemost.* **2001**, *27*, 483–487.
18. Razi, N.; Feyzi, E.; Björk, I.; Naggi, A.; Casu, B.; Lindahl, U. *Biochem. J.* **1995**, *309*, 465–472.
19. Casu, B.; Grazioli, G.; Hannesson, H. H.; Jann, K.; Lindahl, U.; Naggi, A.; Oreste, P.; Razi, N.; Torri, G.; Tursi, F.; Zoppetti, G. *Carbohydr. Lett.* **1994**, *1*, 107–114.
20. Harenberg, J.; De Vries, J. X. *J. Chromatogr.* **1983**, *261*, 287–292.
21. Lloyd, A.; Emberg, G.; Fowler, L. J. *Biochem. Pharmacol.* **1971**, *20*, 637–648.
22. Casu, B.; Guerrini, M.; Naggi, A.; Torri, G.; De Ambrosi, L.; Boveri, G.; Gonella, S.; Cedro, A.; Ferro, L.; Lanzarotti, E.; Paternò, M.; Attolini, M.; Valle, M. G. *Arzneim.-Forsch. (Drug Res.)* **1996**, *46*, 472–477.