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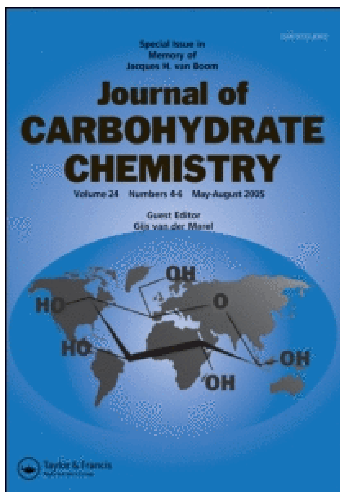

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SEMI-SYNTHETIC HEPARINS WITH 2-DEOXY-2-SULFAMINO-α-L-IDURONIC ACID RESIDUES: CHEMICAL REACTIVITY AND BIOLOGICAL ACTIVITY [1]

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ABSTRACT

Ammonolysis of the epoxide rings of 2,3-anhydro-α-L-guluronic acid residues, generated in alkaline medium from 2-*O*-sulfated α-L-iduronic acid residues of heparin, quantitatively afforded 2-amino-2-deoxy-α-L-iduronic acid residues. *N*-sulfation of these residues by TMA·SO_3 complex led to a formal replacement of the original 2-*O*-sulfate groups of heparin with *N*-sulfates, without configurational changes. These modified uronic acid residues (no longer amenable to alkaline epoxidation) can be easily *N*-desulfated. The presence of negative or positive charges at position 2 of the newly generated 2-amino-2-deoxy-α-L-iduronic acid residues influences the *in vivo* antithrombotic activity and haemorrhagic effects in different ways. A free amino group mainly decreases the haemorrhagic properties of heparin, while a negatively charged *N*-sulfate group decreases the coagulation parameters.

INTRODUCTION

The glycosaminoglycan heparin and its semi-synthetic derivatives, are widely used for therapy of thrombosis, but they display other pharmacological activities, such as inhibition of smooth muscle cells (SMC) proliferation after intraarterial injury, inhibition of tumor heparanase, and anticoagulant properties.[2] Anticoagulant and haemorrhagic properties of heparin are undesirable side-effects for its use in the treatment of vascular diseases.

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The implication of glycosaminoglycans in various biological activities is probably associated with different structural features in the heterogeneous sequences of these polysaccharides. Among the structural determinants of glycosaminoglycans that define a structure-activity relationship, *N*- and *O*-sulfate groups, carboxyl groups, *N*-acetyl groups and molecular size have been extensively investigated,[3] and chemical modifications of heparin (e.g. *N*-desulfation[3] and periodate oxidation[4]) have been used to decrease or nearly eliminate the anticoagulant properties.

However, the contribution to the anticoagulant activity of 2-*O*-sulfate groups on α-L-iduronic acid residues has been only recently evaluated on heparins selectively 2-*O*-desulfated at the level of **IdoA2SO$_3$** units.[5]

To evaluate the contribution of substitutions on the 2-position of α-L-iduronic acid residues to biological activities, we synthesized heparin derivatives containing 2-substituted α-L-iduronic acid residues, without chemical change in other regions of the polymer, by exploiting the epoxide reactivity to nucleophiles.

Our interest was also to modify the ratio of anticoagulant to antithrombotic activity of glucosaminoglycans, to obtain new heparin or heparan sulfate derivatives with reduced haemorrhagic potential.

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RESULTS AND DISCUSSION

Two modified forms of heparin have been prepared, one containing residues of 2-amino-2-deoxy-α-L-idopyranosyluronic acid (**IdoANH$_2$**) and the other having residues of 2-deoxy-2-sulfamino-α-L-idopyranosyluronic acid (**IdoANSO$_3$**) instead of the normal α-L-idopyranosyluronic acid 2-sulfate (**IdoA2SO$_3$**), without any other changes (Scheme 2).

The axial leaving group 2-*O*-sulfate of the **IdoA2SO$_3$** unit of heparin undergoes alkali-catalyzed displacement by oxygen in position 3 leading to a 2,3-anhydro-α-L-guluronic acid. The polymer containing 2,3-epoxigulonic acid residues is stable in neutral or acidic conditions at room temperature, and may be easily isolated (reaction a in Scheme 1).[5-9]

In aqueous alkaline solution, the epoxide ring undergoes hydrolysis, leading to 2-*O*-desulfated uronic acid residues (**IdoA**), while in aqueous neutral solution, at high temperature, galacturonic acid residues (**GalA**) are formed (reactions b and c in Scheme 1). Both reactions take place *via* an intermediate epoxide (reaction a).[5-9]

The oxirane ring of the epoxide may undergo selective nucleophilic addition, with formation of 2-deoxy-2-substituted-α-L-iduronic acid; its regioselective opening at room

Heparin

[a]

Heparin "epoxide"

[b] [c]

2-*O*-desulfated Heparin

Galacturonylglucosaminoglycan sulfate

Scheme 1

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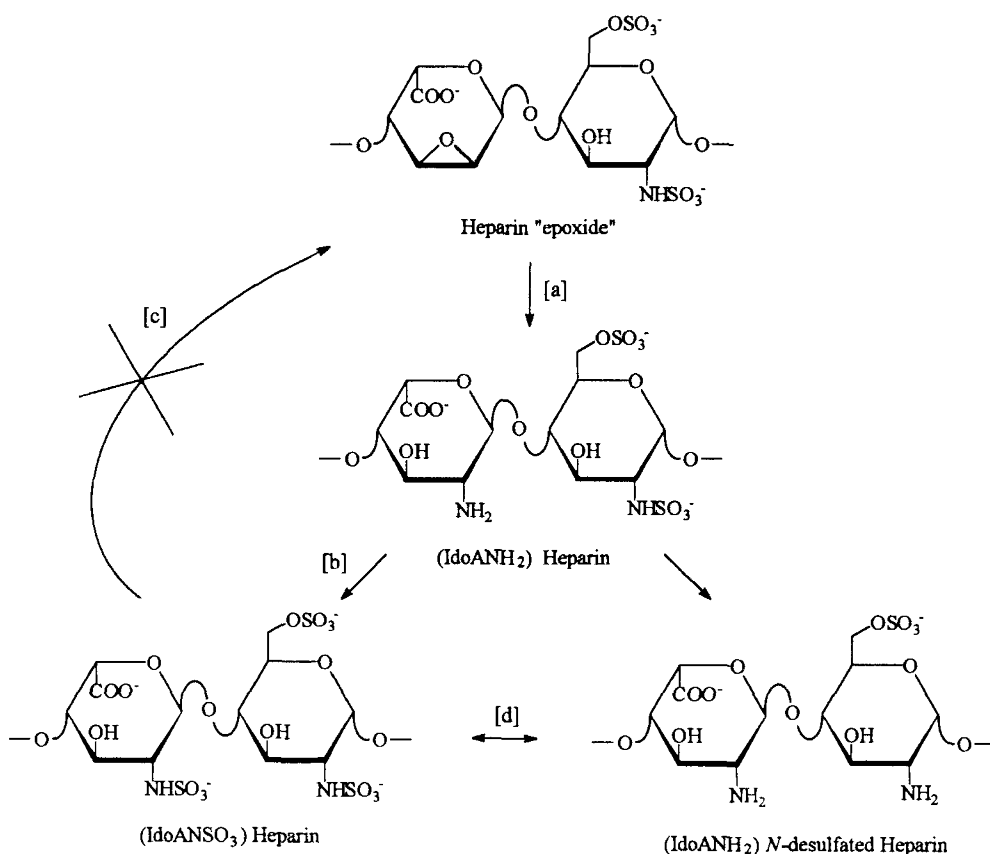
temperature and in the presence of ammonia introduces an amino group at position 2, with the 2-amino-2-deoxy uronic acid in *ido* configuration (reaction a in Scheme 2).[6] Under usual conditions, this amino group undergoes selective *N*-sulfation, leading to a formal substitution of 2-*O*-sulfate group with *N*-sulfate on the original 2-*O*-sulfated α-L-iduronic acid unit (reaction b in Scheme 2).

Physico-chemical data for some of these heparin derivatives are reported in Table 1. Their structures were defined by ^{13}C NMR spectroscopy; the spectra of a pig mucosal heparin, its "epoxide", and the corresponding ammonolysis and subsequent sulfation derivatives are reported in Figure 1.

Analytical and structural data are in good agreement with previously reported kinetic studies,[9] indicating that practically all the **IdoA2SO$_3$** residues (about 75% of total uronic acid units in present heparin) are involved in epoxidation, while the **IdoA** and **GlcA** residues (25%) are unaffected.



Scheme 2

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The epoxide conversion to 2-amino derivative and sulfation of the latter intermediate are complete, as indicated by sulfate/carboxyl ratio and *N*-sulfate molar fraction values.

The ^{13}C NMR spectra are also compatible with the structures shown in Scheme 2. In fact, in the spectra of the 2-amino derivative (Figure 1c) the appearance of peak at 54.3 ppm for *C*-2 of $\mathbf{IdoANH_2}$ and the disappearance of characteristic peaks at 53.3 and 54.3 ppm of *C*-2 and *C*-3 of the epoxide indicate a quantitative ammonolysis.

The peaks at 57.1 and 58.1 ppm of *C*-2 of *N*-sulfated uronic acid ($\mathbf{IdoANSO_3}$), and the disappearance of the *C*-2 peak of $\mathbf{IdoANH_2}$ (54.3 ppm), in Figure 1d, also demonstrate a quantitative sulfation to the newly introduced amino group on uronic acid unit. The peak splitting of *C*-2 of *N*-sulfated uronic acid probably reflects a sequence effect, e.g. due to adjacent 6-*O*-sulfated or nonsulfated hexosamine residues. In fact, the spectra of $\mathbf{IdoANSO_3}$-containing derivatives, obtained from heparins having different

Table 1. Physico-chemical data of heparin and heparin derivatives: pig mucosal heparin [**H**]; *N*-desulfated heparin [***N*-des H**]; epoxide [**Epox**]; *N*-desulfated epoxide [***N*-des Epox**]; galacturonyl derivative [**GalGGS**]; 2-*O*-desulfated heparin [**2-*O*-des H**]; **IdoANH$_2$**-containing [**2-NH$_2$ H**] and **IdoANSO$_3$**-containing [**2-NS H**] derivatives.

Compound	R (SO_3^-/COO^-)	S%	P.M. (average)	NSO_3^- (mol. fr.)	$[\alpha]^{25}$ (at 546 nm)
H	2.1	11.6	12,000	0.94	+59°
N-des H	1.2	8.9	10,560	0.05	+68°
Epox	1.4	9.2	10,800	0.94	+107°
N-des Epox	0.5	6.7	9,400	0.03	+108°
GalGGS	1.3	8.8	10,680	0.90	+18°
2-*O*-des H	1.4	8.6	10,570	0.91	+21°
2-NH$_2$ H	1.3	8.4	10,540	0.93	+62°
2-NS H	2.1	10.4	11,480	1.74	+56°

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degrees of sulfation at position 6 of hexosamine residues, reflect this effect (as measured from the area ratios of peaks at 69.2 and 62.6 ppm of *C*-6 of 6-*O*-sulfated and 6-*O*-desulfated glucosamine, respectively) (data not shown).

The *N*-sulfate group on hexosamine residues is also unaffected during all the reactions reported in Scheme 2, as suggested by unaffected peaks at 60.8 of *C*-2 of **GlcNSO$_3$** units.

The 2-deoxy-2-sulfamino uronic acid unit was unaffected by alkaline treatment leading to *O*-desulfation of **IdoA2SO$_3$** residues of heparin and heparan sulfates. This stability is probably because the sulfamino group is not a good leaving group in intramolecular substitution reactions (reaction c in Scheme 2). On the other hand, this non-natural *N*-sulfate group on the uronic acid residue undergoes either solvolytic or acid-induced *N*-desulfation, similarly to the *N*-sulfate group on the hexosamine unit in heparin and heparin "epoxide" (reactions d in Scheme 2). A variety of heparin-like polymers

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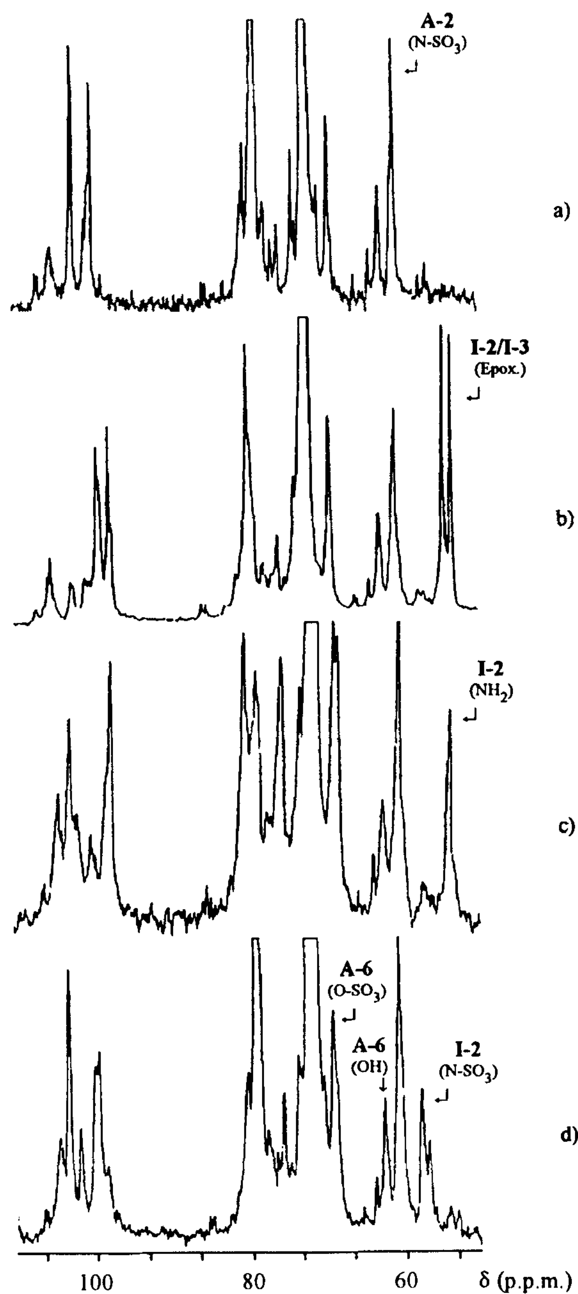

Figure 1. ^{13}C NMR spectra of: a) pig mucosal heparin; b) epoxide derivative; c) **IdoANH$_2$**-containing derivative; d) **IdoANSO$_3$**-containing derivative.

Table 2. Biological data of heparin and its semi-synthetic derivatives.

Compound [a]	Antithrombotic activity	Anticoagulant activity	
	Loss thrombus weight ED_{50} (mg/kg/i.v.)	APTT (2t) µg/ml	Bleeding time (2t) mg/kg/i.v.
H	0.2	2	0.5
LMWH [b]	0.4	20	0.7
Epox	0.5	12	0.8
2-*O*-des H	1.1	60	2.2
GalGGS	1.3	50	1.3
N-des H	> 4	> 500	> 4
2-NH_2 H	0.6	40	1.5
2-NS H	0.4	10	0.7

a - for abbrevations see Table 1.
b - Low Molecular Weight Heparin.

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having free amino groups or *N*-sulfate groups on both the iduronic and aminosugar residues can be obtained by proper control of reaction conditions, and these modified heparins can be used to evaluate the contribution to the biological activity of substitution on position 2 of iduronic acid residues.

As shown in Table 2, both the 2-amino-α-L-iduronic acid-containing derivative and its *N*-sulfated analogue show significant antithrombotic activity, expressed as a decrease in thrombus weight, with ED_{50} 0.6 and 0.4 mg/kg/i.v. respectively. These activities are similar to that of the intermediate epoxide (ED_{50} 0.5) and Low Molecular Weight Heparin (ED_{50} 0.4), and only somewhat less than for unmodified heparin (ED_{50} 0.2). The 2-*O*-desulfated heparin (ED_{50} 1.1) and the galacturonic acid-containing derivative (ED_{50} 1.3) are less active. Quantitatively *N*-desulfated heparin (ED_{50} >4) is devoid of antithrombotic activity.

The "*in vivo*" haemorrhagic potency of compounds (expressed in Table 2 as bleeding time) is not correlated with the antithrombotic activity. Haemorrhagic doubling time for epoxide, LMWH and **IdoANSO$_3$**-containing derivative is at a dose two-fold higher (0.7-0.8 mg/kg/i.v.) than for the parent heparin (0.5), while 2-*O*-desulfated heparin, the galacturonyl derivative and the **IdoANH$_2$**-containing polymer all require a dose three or four-fold higher (respectively 2.2, 1.3 and 1.5). Completely *N*-desulfated heparin shows the same effects at much higher dose (more than 4 mg/kg/i.v.).

The antithrombotic and anticoagulant activity ratio of the present heparin derivatives are especially noteworthy for the **IdoANH$_2$** derivative, which has a long bleeding time while still retaining good antithrombotic properties.

A similar trend is shown by APTT (Activated Partial Thromboplastin Time) which represents an "*in vitro*" evaluation of anticoagulant potency.

The introduction at position 2 on iduronic acid residues of an amino group (positively charged at physiological pH) influences the haemorrhagic properties of the glucosaminoglycan, probably as a result of interaction with the sulfate groups on the hexosamine unit, which may influence conformational features of the macromolecule. The conformation of **IdoA2SO$_3$** is especially sensitive to substitution and sequence effects.[10]

Theoretical (force-field) calculations and rationalization of ^{1}H NMR coupling constants indicated that the conformation of these residues in heparin in aqueous solution can be represented by an equilibrium between the chair $^1C_4$ and the skew-boat ^2S_O form, the former being prevalent (~ 60%).[11]

In a helical model based on X-ray diffraction data obtained from heparin "fibers" and assuming a $^1C_4$ conformation of the **IdoA2SO$_3$** residues, the 2-OSO$_3$ groups are located close to N-SO$_3$ groups in adjacent hexosamine residues.[12] Interaction between these two groups may stabilize the 2-*O*-sulfate group of uronic acid residues to solvolysis. Similar interactions may occur between the positive or negative charge introduced on the uronic acid units and the negative charges on the adjacent hexosamine, which could, in turn, influence the expression of some biological properties of heparin derivatives.

Other substituents at position 2 are possible, and structure-activity relationship[13] and conformational studies[14-16] of a number of new heparin derivatives are in progress.

CONCLUSIONS

A selective chemical modification in position 2 of the iduronic acid residues affects both the antithrombotic and the haemorrhagic properties of heparin. A free amino group mainly decreases the bleeding time parameter, while the antithrombotic activity is largely retained. The formal substitution of the 2-*O*-sulfate group on uronic acid units of heparin

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with a negatively charged *N*-sulfate group, obtained by sulfation of free amino groups, has a negative effect on the coagulation parameter.

These different biological effects probably depend on interaction betweei. a positive charge on uronic acid and the negative charges on the adjacent hexosamine residues, and consequent altered secondary structure. These heparin analogs with mutated primary and secondary structure must have modified interaction with their target proteins.

EXPERIMENTAL

Materials. Pig mucosa intestinal heparin (sodium salt; 166 UI/mg) and Low Molecular Weight Heparin (sodium salt; M_w 4500 D; 82.8 U-AXa/mg against 1st LMWH International Standard), obtained by peroxide radical depolymerization,[17] were from Opocrin (Modena, Italy). Ammonia, trimethylamine•SO_3 complex, sodium carbonate, sodium hydroxide and acetic acid were from Carlo Erba, Aldrich and Fluka and used without further purification.

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General Methods. Optical rotations were recorded at 25 °C with an Optical Activity Ltd. polarimeter, at 1% concentration in H_2O. The ^{13}C NMR spectra were recorded with a Varian Gemini 300 spectrometer at room temperature, operating at 75 MHz in D_2O solutions. Chemicals shifts are referenced to the internal standard 2,2,3,3-d_4-3-trimethylsilylpropionic acid, sodium salt. The final concentration of sample for spectroscopic analysis was about 20% (w/w). The sulfate/carboxyl molar ratios and sulfur percentages were obtained by conventional potentiometric titration, with a Mettler DL 25 titrator. The average molecular weights were determined with HPLC-GPC analysis with Waters Model 510 HPLC instrument, using a Waters Model R 401 refraction index detector. *N*-sulfate molar fractions were obtained by quantitative ^{13}C NMR spectroscopy analysis. Anticoagulant activity expressed as the activated partial thromboplastin time (APTT) was determined according to Larrieu.[18] Each compound was dissolved in fasting rat plasma and diluted to reach appropriate concentrations. Ten assays were performed for each compound and the activity was expressed as the concentration (μg/mL) that doubles the APTT time (2t). The bleeding time determinations were carried out in rats according to Dejana et al.[19] and the data were expressed as the compound dose (mg/kg/i.v.) that doubles the bleeding time. The antithrombotic activity was assessed by the test of stasis venous thrombosis according to Reyers et al.[20]

Epoxidation Reaction. — Pig mucosa intestinal heparin (1.0 g) was dissolved in 1M NaOH (25 mL), the solution was stirred at 60 °C for 210 minutes, diluted with cold water (40 mL), and its pH adjusted to neutrality with acetic acid. Following dialysis against running distilled water for 16 h (cut off 1.5 kD), the glycosaminoglycan was

recovered by lyophilization (quantitative yield). The product was characterized by ^{13}C NMR spectroscopy (see Figure 1b) and optical rotation value measurements.[9]

Epoxide Hydrolysis. - Reactions and isolation of products were performed essentially as described by Jaseja et al.[5]

Ammonolysis. - Reaction and isolation of product were performed essentially as described by Rej et al.[6] To the epoxide (1.0 g) a concentrated ammonia solution (52 mL) was added; the reaction was stirred at room temperature for 48 h and the excess ammonia evaporated under reduced pressure. Following dialysis against running distilled water for 16 h (cut off 1.5 kD), the glycosaminoglycan was recovered by lyophilization. The product was characterized by ^{13}C NMR spectroscopy (see Figure 1c) and its analytical data are reported in Table 1.

***N*-Sulfation.** - To a solution of the ammonolysis product (0.6 g) in water (45 mL) sodium carbonate (0.6 g) and commercial trimethylamine·SO_3 complex (0.3 g) were added. The mixture was stirred at 55 °C for 1 h, additional sulfation agent (0.3 g) was added and the solution stirred again for 23 h, at the same temperature. The reaction was stopped at 10 °C by cold water addition (75 mL). Following dialysis against running distilled water for 16 h (cut off 1.5 kD), the glycosaminoglycan was quantitatively recovered by lyophilization. The product was characterized by ^{13}C NMR spectroscopy (see Figure 1d) and its analytical data are reported in Table 1.

***N*-Desulfation.** - The reaction was carried out either by solvolysis according to Inoue and Nagasawa,[21] or by dilute acid treatment.[22]

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