MONO- AND BIDIMENSIONAL 500 MHz ^{1}H-NMR SPECTRA OF A SYNTHETIC PENTASACCHARIDE CORRESPONDING TO THE BINDING SEQUENCE OF HEPARIN TO ANTITHROMBIN-III : EVIDENCE FOR CONFORMATIONAL PECULIARITY OF THE SULFATED IDURONATE RESIDUE

G. TORRI[a], B. CASU[a], G. GATTI[b], M. PETITOU[c], J. CHOAY[c], J.C. JACQUINET[d] and P. SINAŸ[d]

[a]Istituto di Chimica e Biochimica "G. Ronzoni",
via G. Colombo 81, 20133 Milano, Italy

[b]Bruker Spectrospin, via G. Pascoli, 70/3, Milano, Italy

[c]Institut Choay, 46 avenue Théophile Gautier, 75016 Paris, France

[d]Laboratoire de Biochimie Structurale, ERA 739,
Université d'Orléans, 45046 Orléans, France

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SUMMARY : ^{1}H-NMR spectra of the synthetic pentasaccharide (N-sulfate-6-O-sulfate-α-D-glucosamine) 1→4 (β-D-glucuronic acid) 1→4 (N-sulfate-3,6-di-O-sulfate-α-D-glucosamine) 1→4 (2-O-sulfate-α-L-iduronic acid) 1→4 (N-sulfate-6-O-sulfate-α-D-glucosamine) , corresponding to the active site of heparin for antithrombin (AT-III), have been resolved at 500 MHz and assigned by mono- and bidimensional techniques. Vicinal proton coupling constants of the D-glucosamine residues are similar to those in the regular sequences of heparin, indicating that the $^4C_1$ conformation of the ring, and preference for the g,g conformation of the sulfated hydroxymethyl groups of these residues are neither affected by the unique 3-O-sulfo group nor by sequence effects. By contrast, an unusually large coupling between H-2 and H-3 of the sulfated L-iduronic acid residue suggests a greater departure from the $^1C_4$ conformation of this residue when present in the binding sequence to AT-III than in the regular sequences. Such a departure, leading to different orientation and spacing of essential sulfate groups, may have implications for high-affinity binding to AT-III.

Heparin inhibits a number of procoagulant proteases mainly by binding to antithrombin-III (AT-III) and enhancing the effects of this inhibitor(1). Depending on the length of the heparin molecules, the anticoagulant activity is directed against one only, or two types of coagulation factors (2). ^{13}C-NMR analysis of heparin species having affinity for AT-III revealed the presence of peculiar signals (3,4) that were subsequently related to a 3-O-sulfate group present in one of the glucosamine units (5). It was

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IUPAC nomenclature : O-(2-deoxy-2-sulfamido-6-O-sulfo-α -D-glucopyranosyl)-(1→4)-O-(β-D-glucopyranosyluronic acid)-(1→4)-O-(2-deoxy-2-sulfamido-3,6-di-O -sulfo-α-D-glucopyranosyl)-(1→4)-O-(2-O-sulfo-α-L-idopyranosyluronic acid)-(1→4) 2-deoxy-2-sulfamido-6-O-sulfo-D-glucopyranose decasodium salt.

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suggested that a critical pentasaccharide sequence is a sine qua non condition for high-affinity binding to AT-III (6-8). Such a sequence has been chemically synthesized (9). It contains all the structural features required for binding to AT-III, and binds to this inhibitor with an association constant similar to high affinity heparin. It also elicits a very potent anti-factor Xa activity in plasma (10). Figure 1 shows the structure of the regular sequences that account for the most part of heparin molecules (**1**) and of the irregular active synthetic pentasaccharide (**2**).

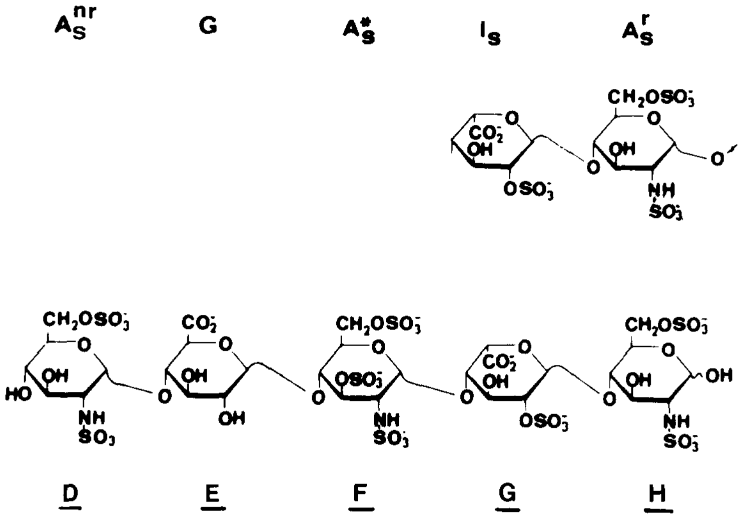
The 270 MHz ^{1}H-NMR spectra and the 67.9 MHz ^{13}C-NMR spectra of heparin have provided information on the conformation of monosaccharide residues in the regular part of this polymer (11,12). With the availability of the "active site" of heparin through organic synthesis, we have the unique opportunity of unraveling some conformational features of this important segment of the irregular region, with special regards to the influence of the 3-O-sulfo group on the local conformation of the trisulfated-D-glucosamine residue, as well as on the conformation of the adjacent L-iduronic acid residue. These features might be relevant to the expression of the biological activity of the molecule. This note reports the assignment of ^{1}H-NMR signals of the active pentasaccharide (Figure 1, **2**), as derived from mono- and bidimensional (1-D and 2-D) spectra obtained at 500 MHz, and a preliminary discussion of some of the conformation-dependent NMR parameters.

MATERIALS AND METHODS

The pentasaccharide **2** (figure 1) was synthesized as outlined in reference 9. NMR measurements were performed on a 10 % (w|v) solution in D_2O, following several exchanges with the same solvent (99,9 %, Merck, Darmstadt). The spectra were recorded at 500 MHz, on a Bruker WH-500 spectrometer, at 20°C. Chemical shifts were measured with reference to internal TSP. Coupling constants for the aminosugar residues were calculated from spectra simulated with the PANIC.84 version of the LAOCOON computer program.

RESULTS AND DISCUSSION

Figure 2A shows the monodimensional ^{1}H-NMR spectrum of pentasaccharide **2**. Signal assignments were made by homonuclear spin-decoupling (starting from signals of the anomeric protons, assigned by comparison of a number of synthetic di- and oligosaccharides (13), and confirmed through J-resolved and chemical shift correlated 2-D spectra. Figure 2B shows a representative 2-D ^{1}H-correlated spectrum of pentasaccharide **2**. Tables 1 and 2 respectively contain ^{1}H chemical shifts and ^{3}J interproton coupling constants. The values obtained (11,12) for the regular disaccharide sequence of heparin (Figure 1, **1**) are also reported in these tables.



1

2

Figure 1 : Structure of heparin sequences. 1 : trisulfated disaccharide of the regular regions ; 2 : pentasaccharide corresponding to the binding site for AT-III. (A_S : N-sulfate-6-O-sulfate-α-D-glucosamine ; G : β-D-glucuronic acid ; A_S^* : N-sulfate-3,6-di-O-sulfate-α-D-glucosamine ; I_S : 2-O-sulfate-α-L-iduronic acid) ; D, E, F, G, H designate individual monosaccharide residues according to a nomenclature used in sequence analysis (6).

Major differences of the ^1H chemical shifts (Table 1) of the pentasaccharide with respect to the regular sequences of heparin can be rationalized essentially in terms of direct effects of different residues (i.e., D-glucuronic acid instead of sulfated L-iduronic acid), or new electronegative groups (such as the 3-O-sulfo group of residue A_S^*). Second-order differences are attributable to sequence (next-neighbour) effects. Thus, sulfation at O-3 of A_S^* causes the expected downfield shifts for the protons of this unit. Sequence effects are reflected on shifts associated with end residues A_S^{nr} (non reducing end) and A_S^r (reducing end), as well as on A_S^{nr} and A_S^* for their being adjacent to a glucuronic acid (G) instead of a sulfated iduronate (I_S) residue.

More relevant to conformational aspect (Figure 3) is the comparison of interproton coupling constants. It is thus clearly indicated (Table 2) that the aminosugar residues (A_S^{nr}, A_S^*, A^r) are essentially in the same $^4C_1$ (D) conformation as in the regular sequences of heparin. Also coupling across the C_5H-C_6H fragments of the foregoing residues are similar to those of the regular sequences, and compatible also with a preferred g,g local conformation of the sulfated hydroxymethylene groups. Overall, the foregoing results indicate that neither the unique 3-O-sulfo group nor the adjacency of different uronic acids substantially modify the local conformation of the aminosugars. Also the conformation of the glucuronic acid residue (G) is the normal $^4C_1$ (D), as found for other glycosaminoglycans (14).

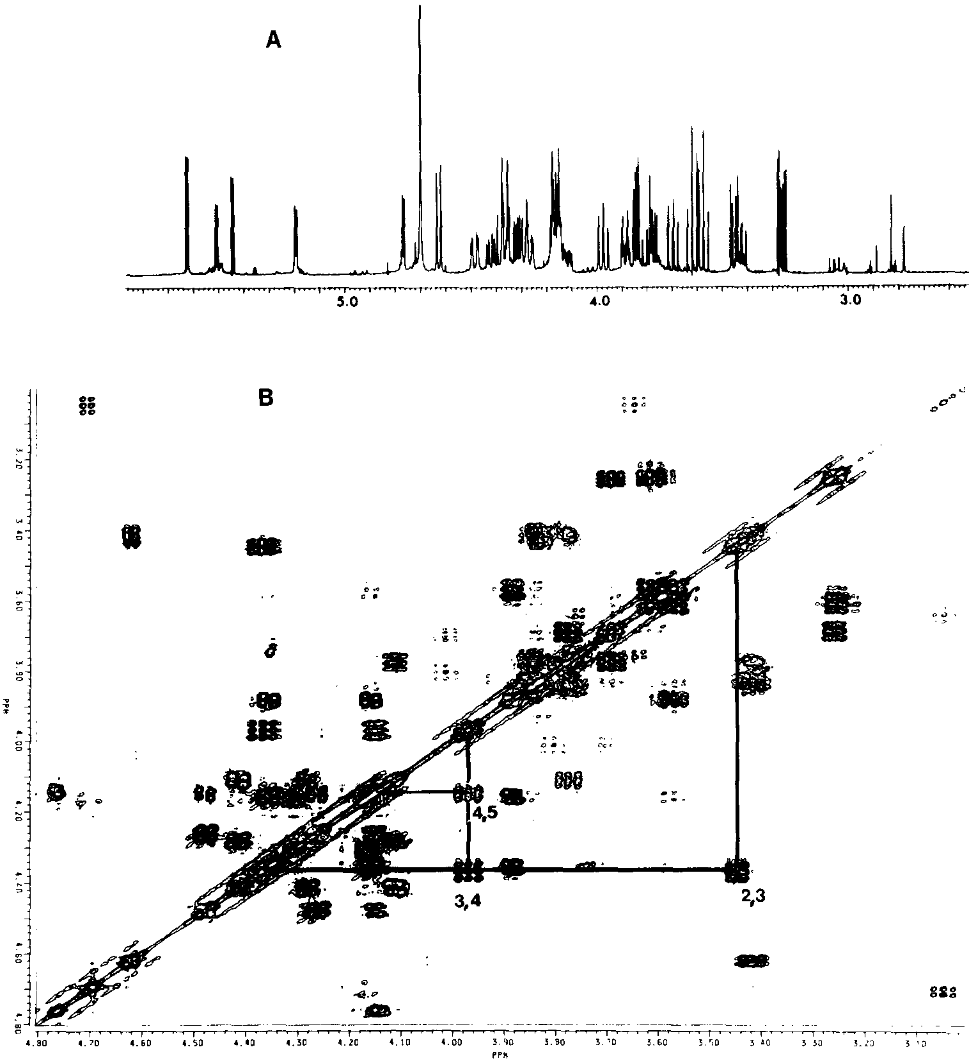


Figure 2 : ^{1}H-NMR spectra (500 MHz) of pentasaccharide 2. A) 1-D spectrum. B) 2-D ^{1}H-correlated partial spectrum, with connectivities between resonances of the A_s^* residue.

By contrast, at least one ^3J value ($J_{2,3}$ = 7,5 Hz) of the sulfated iduronate residue (I_s) in pentasaccharide 2 is consistently higher than in the regular sequences of heparin ($J_{2,3}$ = 5.9 Hz (11,12)). The latter value is itself larger than expected for a perfect $^1C_4$ (L) chair. This large coupling observed in the pentasaccharide suggests either a substantial distorsion of a $^1C_4$ chair (as depicted by arrows in Figure 3), or a contribution of a different conformation, possibly a $^2S_0$ form (13).

Preliminary NMR and force-field calculation studies (13,15) indeed indicate that iduronate ring can be present in more than one conformation, its

Table 1 : ^{1}H-NMR chemical shifts (δ, ppm) of sequences 1 and 2 (see figure 1)

	Sequence 2[a]					Sequence 1 (ref. 11)	
	A_s^{nr}	G	A_s^*	I_s	A_s^r	A_s	I_s
H-1	5.624	4.625	5.508	5.192	5.441	5.40	5.22
H-2	3.260	3.420	3.450	4.319	3.265	3.28	4.35
H-3	3.621	3.840	4.374	4.150	3.695	3.67	4.20
H-4	3.575	3.845	3.975	4.140	3.780	3.77	4.11
H-5	3.885	3.770	4.150	4.762	4.115	4.03	4.82
H-6a	4.363	-	4.483	-	4.420	4.41	-
H-6b	4.17	-	4.261	-	4.28	4.28	-

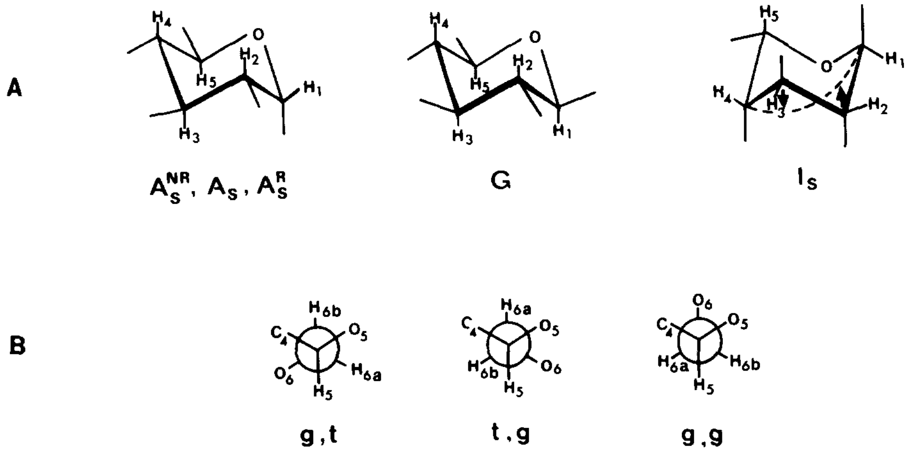
[a] Notations A_s^{nr}, A_s^* and A_s^r refer to non reducing end, central, and reducing end residues of glucosamine respectively.

shape being largely determined by the type of neighbouring residues. The data obtained for the pentasaccharide representing the binding site of heparin to AT-III indicate the largest departure from the $^1C_4$ conformation yet observed for L-iduronate residues in solution. This behaviour is most probably due to the adjacent trisulfated glucosamine unit, an important role is thus suggested for the unique 3-O-sulfate group in changing the conformation of the adjacent

Table 2 : Vicinal proton coupling constants (^3J, Hz) for sequences 1 and 2 (see figure 1)

	Sequence 1[a]					Sequence 2 (ref. 12)	
	A_s^{nr}	G	A_s^*	I_s	A_s^r	A_s	I_s
$J_{1,2}$	3.73	8.0	3.51	3.5	3.54	3.66	2.64
$J_{2,3}$	10.34	9.5	10.73	7.5	10.47	9.98	5.90
$J_{3,4}$	9.50	nd	9.11	nd	8.93	9.09	3.44
$J_{4,5}$	10.21	nd	10.00	3.0	10.12	9.23	3.09
$J_{5,6a}$	2.47	-	1.91	-	3.96	2.15	-
$J_{5,6b}$	2.31	-	1.33	-	2.36	2.15	-
$J_{6a,6b}$	-10.95	-	-11.65	-	-11.12	-11.23	-

[a] Notations A_s^{nr}, A_s^* and A_s^r refer to non reducing end, central, and reducing end residues of glucosamine respectively ; data for aminosugar residues are refined ; nd not determined.



A_S^{NR}, A_S, A_S^R G I_S

g,t t,g g,g

Figure 3 : A) Conformation of hexopyranose rings in pentasaccharide **2**. The arrows indicate possible deformation of the iduronate ring (I_S) which would explain the abnormally high coupling constant ($J_{2,3}$) observed for this residue in pentasaccharide **2** compared to **1**.
B) Newman projection of possible conformers of the C_5H-C_6H fragments of aminosugar residues.

iduronate residue, and consequently the relative orientation of other sulfate groups considered as essential (7,16) for high-affinity binding to AT-III.

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