Carbohydrate Research, 88 (1981) C1–C4
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Preliminary communication

The antithrombin-binding sequence of heparin studied by n.m.r. spectroscopy

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(Received October 20th, 1980; accepted for publication, November 3rd, 1980)

Heparin prevents the coagulation of blood by binding to, and thus activating, antithrombin[1]. Only a fraction of the molecules in heparin preparations bind with high affinity to this protein, and this fraction accounts for most of the anticoagulant activity[2–4].

In order to define the antithrombin-binding sequence of the active polysaccharide-molecules, oligosaccharides with high affinity for antithrombin were isolated from partially depolymerised (nitrite-degraded) heparin by affinity chromatography on immobilised antithrombin[5]. The oligosaccharides were fractionated further according to molecular weight by gel chromatography on Sephadex G-50; the smallest, high-affinity fragment isolated was an octasaccharide[6]. Structural studies revealed a major sequence, α-L-IdA*p*-(1→4)-α-D-GlcNAc*p*-(1→4)-β-D-GlcA*p*-(1→4)-α-D-GlcNSO$_3^-$*p*-(1→4)-α-L-IdA*p*-2-OSO$_3^-$-(1→4)-α-D-GlcNSO$_3^-$*p*-(1→4)-α-L-IdA*p*-2-OSO$_3^-$-(1→4)-2,5-anhydro-α-D-mannose, in which most of the 2-amino-2-deoxy-D-glucose residues were 6-sulfated[5,6]. In addition, recent evidence suggested that the *N*-sulfated 2-amino-2-deoxy-D-glucose residue substituted at C-4 with the D-glucuronic acid residue carries a unique 3-sulfate group[7]. We now report the characterisation of a similar oligosaccharide by means of n.m.r. spectroscopy. Due to insufficient yields of the octasaccharide, a decasaccharide (*A*) was used in the investigation.

Earlier studies by ^{1}H- and ^{13}C-n.m.r. spectroscopy demonstrated that heparins from different sources consist mainly of (1→4)-linked α-L-idopyranosyluronic acid 2-sulfate (**5**) and 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate (**1**) residues[8–10]. Small

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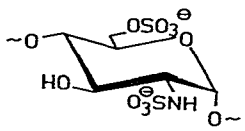
amounts of β-D-glucopyranosyluronic acid (**6**) residues, non-sulfated α-L-idopyranosyluronic acid (**4**) residues, and 2-acetamido-2-deoxy-D-glucosyl (**3**) residues have also been demonstrated[8]. The ^{13}C-n.m.r. spectrum obtained from native, pig-mucosal heparin (Fig. 1, *B*) is in good agreement with these previous results. By contrast, the spectrum produced by the high-affinity decasaccharide (*A*), isolated from the same heparin preparation, is markedly different (Fig. 1, *A*) and resembles that of the oligosaccharide isolated by fractionation of commercial (Choay), pig-mucosal heparin[11]. Signals assigned to residues **1** and **5**, the major components of the intact polysaccharide chain, are clearly seen but, in addition, there are prominent signals ascribed to residues **3**, **4**, and **6**, which are all minor constituents of the heparin molecule. Assignments of most of the signals in the ^{13}C-n.m.r. spectrum of *A* are made by reference to earlier studies[8—11]. Thus, the peaks at δ 95.35, 53.69, and 22.06 (not included in Fig. 1) are assigned to C-1, C-2, and the *N*-acetyl group of **3**, respectively. The signals for C-2 of **1** units appear at δ 57.34 and 57.65, reflecting attachment to different uronic acid residues (one is probably the non-sulfated L-iduronic acid which occurs at the non-reducing end of the octasaccharide sequence shown above). The signal for C-1 of 2-sulfated L-iduronic acid (**5**) appears at δ 99.23, the anomeric carbon atom of unsubstituted L-iduronic acid (**4**) appears at δ 100.96, and that of D-glucuronic acid (**6**) at δ 101.89. The carbonyl carbon atoms from carboxyl and *N*-acetyl groups appear at δ 174.6—175.1 (not included in Fig. 1). The signal at δ 56.54 is attributed to C-2 of the unique, 3-sulfated residue 2. This assignment is made by comparison with the corresponding signal from **1**, and adjustment for the β-shift effect on C-2 of an *O*-sulfate group at C-3. The shift effect was determined by comparing the spectra of methyl 2-amino-2-deoxy-α-D-glucopyranoside (δ 96.32, C-1; 54.28, C-2; 70.15, C-3; 69.78, C-4; 72.17, C-5; and 60.62, C-6) and its 3-sulfate[12] (δ 96.29, C-1; 53.33, C-2; 77.91, C-3; 67.96, C-4; 71.71, C-5; and 66.44, C-6). Further, in the ^{1}H-n.m.r. spectrum of *A*, H-3 of **2** exhibits a shift of δ 4.39 which is ~0.7 p.p.m. downfield of the corresponding signal for **1**.



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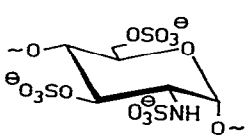

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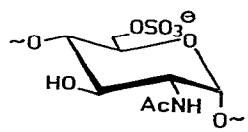

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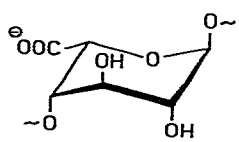

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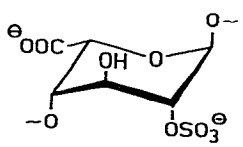

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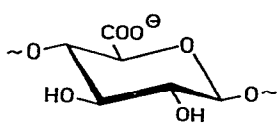

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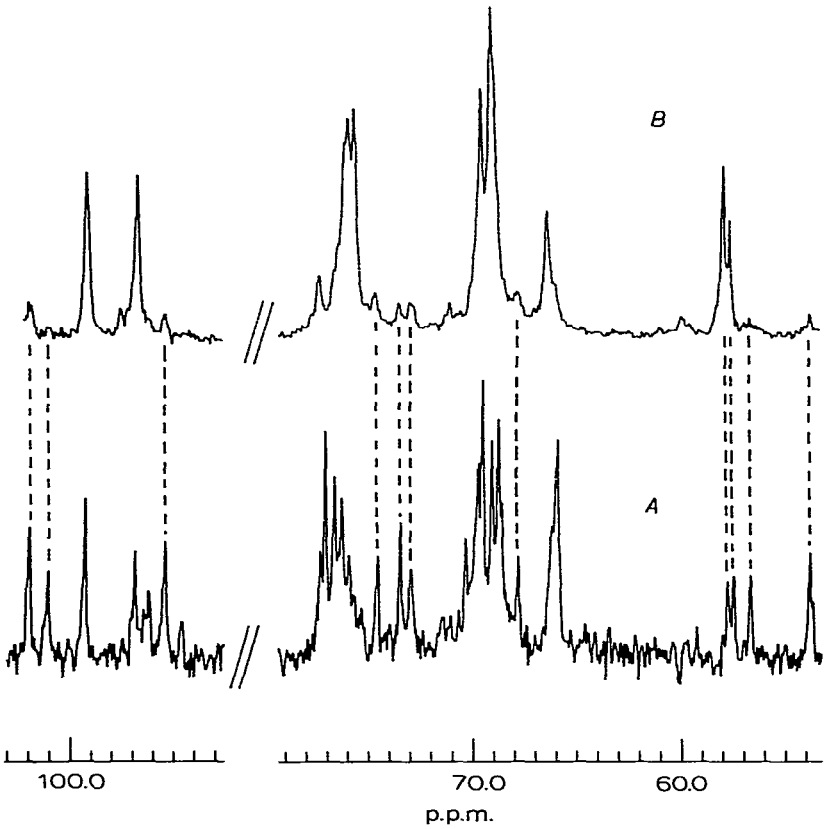

Fig. 1. Expansion of the ^{13}C-n.m.r. spectra (67.89 MHz; broad-band decoupled; reference, internal acetone, δ 30.5; 30°) of the antithrombin-binding oligosaccharide of heparin (*A*) and native heparin (*B*).

These results are in good agreement with the structure proposed for the antithrombin-binding sequence in heparin[7]. In particular, the postulated position of the unique sulfate group, at position 3 of a 2-amino-2-deoxy-D-glucosyl residue[7], is confirmed.

Most of the minor signals from the intact heparin chains (Fig. 1, *B*) correspond to major signals of the high-affinity decasaccharide (Fig. 1, *A*). The antithrombin-binding sequence thus shows a high proportion of sugar residues that are poorly represented in the heparin molecule as a whole (residues 2, 3, 4, and 6). The polymer regions outside the binding sequence should be largely composed of repeating disaccharide-units (residues 1 and 5).

ACKNOWLEDGMENTS

We thank Professor D. Leibfritz, Dr. M. Feigl, Professor M. Paulsen, and Dr. Sinwell for access to the 360- and 270-MHz n.m.r. instruments. The work was finan-

cially supported by KABI AB, the Swedish Board for Technical Development, and the Swedish Medical Research Council (2309).

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