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Note

Chemical modification of glycosaminoglycans Selective 2-sulfation of D-glucuronic acid units in heparan sulfate

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Abstract

O-Sulfation of heparan sulfate was performed in conditions that minimise *N*-desulfation. NMR spectra and methylation analysis of sulfated heparan sulfate confirmed the selective substitution at O-2 of glucuronic acid units. © 1997 Elsevier Science Ltd.

Keywords: Sulfation; Heparan sulfate; D-Glucuronic acid

Sulfated glycosaminoglycans (GAGs) are complex polysaccharides composed of alternating units of hexuronic acid and hexosamine. Both heparin (HEP) and heparan sulfates (HS) have $1 \rightarrow 4$ linkages and 2-amino-2-deoxy-D-glucose, but while 2-sulfated L-iduronic acid is the major uronic acid in HEP, D-glucuronic acid is more abundant in HS. Sulfate groups are disposed allowing the polysaccharide to bind the plasma proteins such as antithrombin III (AT III), heparin cofactor II (HC II), lipoprotein lipase (LPL), and low-density lipoproteins (LDL), and therefore the sulfation pattern is closely related to the biological activity of the polymer.

An increasing charge density of GAGs is usually associated with stronger protein binding. Consequently, a number of studies have been performed in order to modify the sulfate content of HEP and HS. Nagasawa et al. [1] prepared modified heparins for the first time. They found conditions to desulfate HEP, and they studied the products obtained by re-sulfation of completely desulfated HEP. These authors claimed that sulfation of non-sulfated L-iduronic acid units led selectively to the 2-sulfated residues.

On the other hand, Perlin et al. [2] performed 2-desulfation of L-iduronic acid units reacting HEP in alkaline medium. Sulfation of the obtained partially desulfated HEP led exclusively to L-iduronic acid residues sulfated at C-3, as seen from spectroscopic evidence. As glucuronic acid is a minor component of HEP, no report on the sulfation behaviour of this uronic acid has been made.

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The natural abundance and the wide distribution of HS make this polysaccharide a suitable starting material for the synthesis of modified GAGs. Heparan sulfates have been sulfated previously in order to improve their anticoagulant activity [3]. However, little or no structural characterisation of the products can be found in the literature. Our previous studies on chemical modification of GAGs include salicylic acid [4], carboxymethyl [5], and sulfated derivatives of previously periodate-oxidised HS [6]. In this paper, we report on the structural features resulting from sulfation of HS. Sulfation of glucuronic acid residues may allow the study of the biological activity of these derivatives that have been suggested to be involved in the control of cell growth [7,8] and contribute to the elucidation of their actual functional role.

Sulfation [2] of HS with a sulfur trioxide–triethylamine complex was assayed using different experimental conditions. Partial *N*-desulfation was observed by the appearance of a resonance in the ^{13}C NMR spectrum at δ 55.6, assigned to C-2 of $GlcNH_2$. A high degree of *N*-desulfation occurred when methyl sulfoxide or *N,N*-dimethylformamide solutions of the pyridinium or triethylammonium salt of HS were employed. Occasional *N*-desulfation has been reported previously [9,10]. In our experience, anhydrous conditions for reactions in *N,N*-dimethylformamide with the tributylammonium salt of the polysaccharide minimise this side reaction.

Two different heparan sulfates (**HS I** and **HS II**) from bovine mucosa with different iduronic/glucuronic acid ratios were used for the sulfation experiments in dry *N,N*-dimethylformamide. Homogeneity of **HS I** and **HS II** preparation as well as of the corresponding sulfated derivatives was analysed by agarose gel electrophoresis. Sulfated derivatives showed a slightly increased mobility due to their higher anionic charge. Band broadening, which would appear as a consequence of either chain depolymerisation or inhomogeneous charge distribution, was not observed.

HS I, a heparan sulfate of high iduronic acid content (Table 1), was converted into its tributylammonium salt and sulfated (5-fold excess $SO_3 \cdot Et_3N$, DMF, room temperature). 2-Sulfated iduronic acid units were converted to 2,3-disulfated residues, as seen from the NMR spectra (^1H and ^{13}C) of the product. The signal corresponding to H-1 of IdoA was shifted from δ 5.21 to 5.36, while the C-1 resonance of IdoA appears at δ 100.5 in the sulfated product. In contrast, non-sulfated GlcA residues were transformed into the 2-sulfated counterparts, although ^{13}C NMR evidence was not sufficiently conclusive to rule out completely substitution at C-3 due to overlapping with signals corresponding to the anomeric carbon of the more abundant IdoA residues.

Therefore, we performed the same sequence of experiments on **HS II**, composed mainly of GlcA (IdoA/GlcA 30:70, Table 1, Fig. 1 top). The ^{13}C NMR analysis of the sulfated product (Fig. 1 bottom) indicated that only sulfation at C-2 had occurred. The C-1 resonance of GlcA was shifted from δ 104.2 to 103.0, while sulfation at C-3 should shift this signal in less than 1 ppm [9,11]. The signal at δ 103.0 was assigned to C-1 of 2-sulfated GlcA in comparison with the ^{13}C NMR of a natural rat liver HS containing 2-sulfated GlcA as a major component [12]. Methylation analysis of sulfated **HS II** by GLC–MS confirmed the selective substitution at O-2 of GlcA. Alditol acetates, arising from a 3-sulfated or 2,3-disulfated D-glucuronic acid units, could not be detected.

The selectivity observed in the polymer should be a consequence of the polysaccharide conformation due to hydrogen-bond interactions between different residues. 2-Sulfated GlcA residues in HS are major components in the naturally highly sulfated HS from rat liver tissues [12] that do not exhibit anticoagulant properties in spite of their high degree of sulfation [13]. On the other hand, it has been suggested [7,8] that HS oligosaccharides with a high content of sulfated GlcA units are accumulated in the nuclei of cultured rat hepatocytes and may be involved in cell growth proliferation. Therefore, preparation of heparan sulfates selectively 2-sulfated at glucuronic acid

Table 1
Comparative data for **HS I** and **HS II**

	SO_3^- (%)	COO^- (%)	SO_3^-/COO^-	IdoA/GlcA
HS I	16.8	7.44	1.26	50:50
Sulfated **HS I**	28.3	5.42	2.94	nd[a]
HS II	11.1	7.04	0.87	30:70
Sulfated **HS II**	26.8	6.60	1.80	nd
Heparin	32.8	7.19	2.51	75:25

[a] nd: Not determined.

[2] The term sulfation is used in the sense that sulfate groups are formed. More exactly, the *sulfonation* of a hydroxyl group occurs to give a sulfate (or *O*-sulfo) substituent.

residues under the conditions described here may aid in the elucidation of the biological significance of these compounds.

1. Experimental

Materials.—Heparin and heparan sulfate **HS II** were kindly given by Syntex Argentina S.A. Heparan sulfate **HS I** was fractionated from commercial heparin by-products as previously reported [13]. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ACE 200 instrument. ^{1}H NMR of **HS I** (D_2O): δ 5.30 (H-1 GlcNS), 5.21 (H-1 IdoA), 5.06 (H-1 GlcNAc), 4.87 (H-1 GlcA, H-5 IdoA), 3.40–3.20 (H-2 GlcN), 2.01 (Ac). ^{13}C NMR of **HS I** (1:1 D_2O–H_2O): δ 175.7 (CO), 104.4 (C-1 GlcA), 101.7 (C-1 IdoA), 99.9–98.8 (C-1 GlcN), 67.4 (C-6 OS GlcN), 62.4

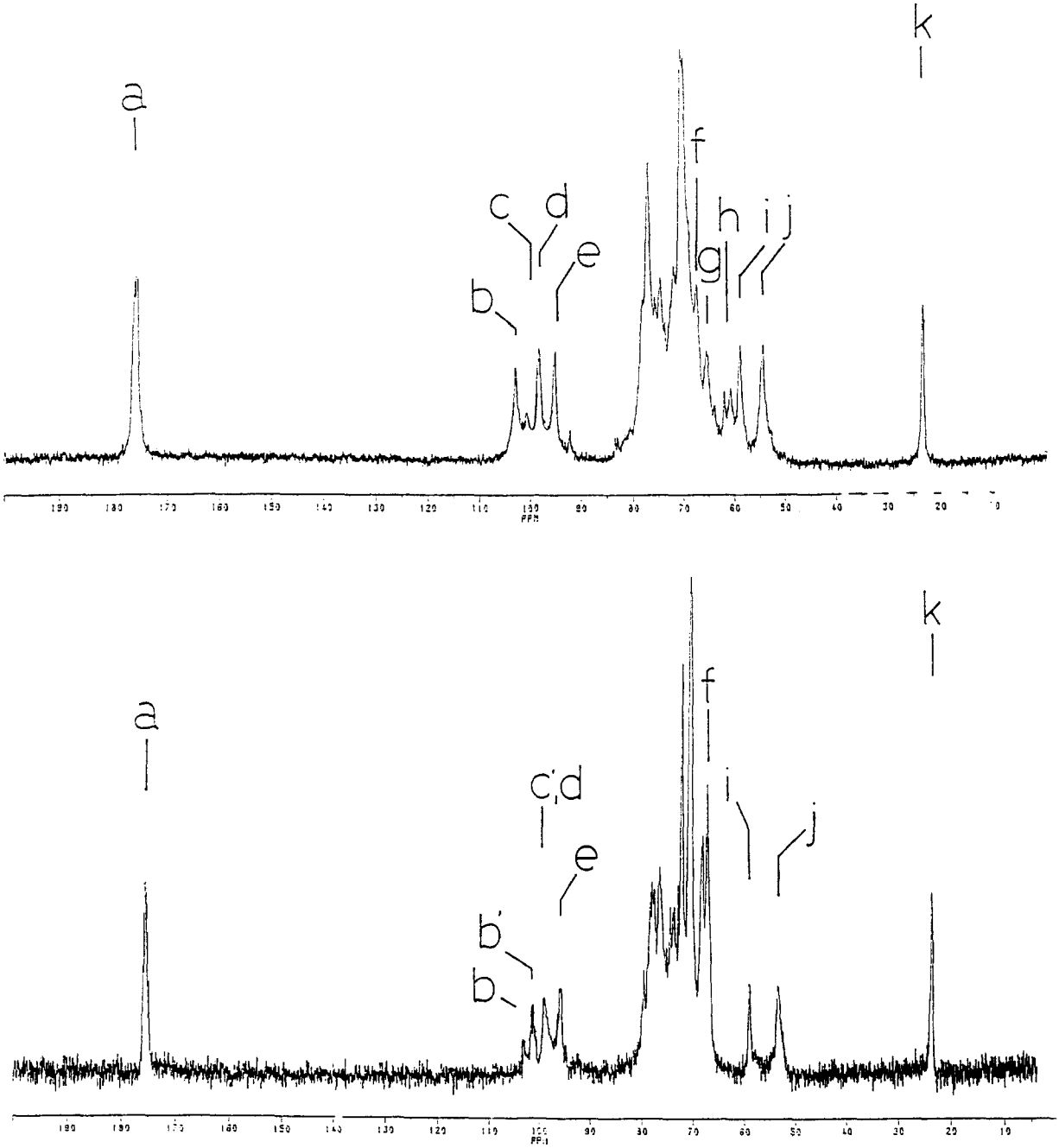

Fig. 1. 50-Mhz ^{13}C NMR of heparan sulfate **HS II** (top) and the sulfated derivative (bottom); a: C-6 GlcA, C-6 IdoA, acetyl; b: C-1 GlcA; b′: C-1 GlcA 2-*O*-SO_3^-; c: C-1 IdoA 2-*O*-SO_3^-; c′: C-1 IdoA 2,3-di-*O*-SO_3^-; d: C-1 $GlcNSO_3^-$; e: C-1 GlcNAc; f: C-6 GlcN 6-*O*-SO_3^-; g: C-3 GlcN; h: C-6 GlcNAc; i: C-2 $GlcNSO_3^-$; j: C-2 GlcNAc; k: acetyl.

(C-6 OH), 59.0 (C-2 GlcNS), 54.6 (C-2 GlcNAc), 23.5 (Ac). Similar spectra were obtained for **HS II** (Fig. 1 top), with changes in signal intensities. Sulfur trioxide–triethylamine complex was prepared according to Nair and Bernstein [14].

Analytical methods.—Sulfate and carboxyl groups were determined by conductimetry [15]. Uronic acid estimations by carbazole-borate and orcinol methods and agarose gel electrophoresis in 0.04 M barium acetate were performed as described previously [13], as well as methylation analysis of sulfated **HS-II** [16]. Uronic acids were analysed as the corresponding methyl aldonates.

Sulfation of HS.—Method (a). **HS I** (150 mg) was converted into the triethylammonium salt and dissolved in dry DMF (15 mL). Sulfur trioxide–triethylamine complex (1.5 g) was added. After leaving it overnight at room temperature, cold water (12 mL) was added, and the pH of the mixture was adjusted to 8.0 with M NaOH. Ethanol (50 mL) was added and the soln was satd with NaOAc. After 24 h at 4 °C, the mixture was centrifuged. The precipitate obtained was dissolved in water and dialysed. Evaporation under reduced pressure yielded 165 mg of the sulfated product. ^{1}H NMR (D_2O): δ 5.36 (H-1 IdoA), 5.26 (H-1 GlcNS), 5.12 (H-1 GlcA, H-1 GlcN), 3.40–3.20 (H-2 GlcN), 2.05 (Ac). ^{13}C NMR (1:1 D_2O–H_2O): δ 175.8 (CO), 103.0 (C-1 GlcA), 100.5 (C-1 IdoA), 99.9–98.3 (C-1 GlcN), 67.4 (C-6 GlcN 6-*O*-SO_3^-), 58.8 (C-2 GlcNS), 55.6 (C-2 $GlcNH_2$), 54.5 (C -2 GlcNAc), 23.3 (Ac).

Method (b). **HS I** (80 mg) was converted into the pyridinium salt and dissolved in dry Me_2SO (8 mL). Sulfur trioxide – triethylamine complex (0.8 g) was added. After workup as in method (a), 62 mg of the sulfated product were obtained. The ^{13}C NMR presented the same resonances as in method (a), the *N*-desulfation being more important, as seen from the increased signal intensity corresponding to $GlcNH_2$ at δ 55.6.

Method (c). **HS I** (100 mg) was converted into the tributylammonium salt and dissolved in dry DMF (10 mL). Sulfur trioxide–triethylamine complex (1 g) was added. After workup, 118 mg of the sulfated product were obtained. The ^{13}C NMR presented the same signals as in (a), but in this case the resonance at δ 55.6 was not observed. For analytical data, see Table 1.

Sulfation of **HS II** was performed by method (c). The product showed similar spectroscopic resonances in the ^{13}C NMR spectrum (Fig. 1 bottom), although the intensity of the signal at δ 103.0 appeared to have increased according to the higher proportion of GlcA residues. For analytical data, see Table 1.

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