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^{1}H and ^{13}C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives

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Abstract

The complete ^{1}H and ^{13}C NMR spectral assignments are described for the most prevalent patterns of sulfation and acetylation which can be found in polymeric heparin or can be obtained by standard chemical modifications. These include a number of novel structures containing unsubstituted or acetylated amino groups and the first complete NMR assignments of many of the other derivatives. Beef lung heparin was chosen as a model system and studies were carried out using conditions to control the influences on the chemical shift positions in heparin samples of divalent cations and variations in pH and temperature. © 1996 Elsevier Science Ltd.

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1. Introduction

Heparin, a polysaccharide which has found widespread use as an anticoagulant and antithrombotic agent, structurally consists of a linear carbohydrate backbone made up of

Abbreviations: In general, A refers to aminosugar (glucosamine) residues and I refers to iduronic acid residues, more specifically the following abbreviations are used; A_{6OH}, glucosamine; A_{6S}, glucosamine 6-O-sulfate; I_{2S}, iduronic acid 2-O-sulfate; I_{2OH}, iduronic acid; A-*N*, the *N*th proton or carbon of the glucosamine residue; I-*N*, the *N*th proton or carbon of the iduronic acid residue

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alternating 1,4-linked β-D-GlcA or α-L-IdoA and α-D-glucosamine residues. Most of the constituent monosaccharides bear one or several substituents at different positions, distributed along the chains. The most highly represented disaccharide unit is → 4)-α-L-IdoA 2-*O*-sulfate-α-(1 → 4)-D-glucosamine *N*-sulfate with extensive sulfation at position 6 and with some of the aminosugar residues *N*-acetylated instead of *N*-sulfated.

Recently, several attempts have been made to modify chemically heparin [1,2] and other naturally occurring polysaccharides, particularly with the aim of producing antithrombotic drugs [3,4]. A number of other interactions between sulfated glycosaminoglycans or related compounds and, for example, growth factors and selectins have also stimulated interest [5–8].

Reports of chemically modified heparins which have been characterised by NMR and some of which also include associated biological activities have referred to some of the more accessible modifications of heparin, for example, totally desulfated heparin, selectively de *N*-sulfated and *N*-acetylated heparin [1,2], chemically re-*O*-sulfated heparins [3] as well as data on heparin oligosaccharides [9]. Hitherto, there has been no systematic study made of the NMR chemical shifts for many other derivatives. The substitution patterns which are obtained in many chemical modifications can only be tentatively ascribed with the limited NMR analyses available to many practitioners. For a definitive structural characterisation, more complex procedures may be required, especially in light of the observation made below that, in some cases, conventional chemical shift arguments alone may be inadequate to explain the observed shifts. This could lead to difficulties of assignment and/or structural characterisation, especially in cases where only one-dimensional NMR spectra are available for the analysis of chemical modifications or other treatments. In addition, low levels of structural heterogeneities, such as the presence of *N*-acetylglucosamine or *N*-acetylglucosamine 6-*O*-sulfate which are also usually prevalent in heparan sulfate preparations, can occur in some heparin samples [8,10], and these are not easy to analyse, not only as a result of their low abundance but also because of the high degree of signal overlap in one-dimensional spectra.

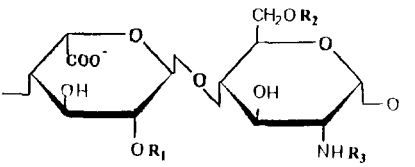
Here, we present the complete ^{1}H and ^{13}C NMR spectral assignments for all possible patterns of sulfation at I-2, A-2, A-6, and acetylation at A-2 (see Abbreviations) including those patterns which can be found in polymeric heparin or can be obtained by standard chemical modifications at these positions. These include a number of novel structures containing unsubstituted or *N*-acetylated amino groups **7**, **8**, and **10** and the first complete NMR assignments of many of the other derivatives. Recently there has been increased interest in compounds containing free amino groups which were previously overlooked as being of little biological relevance but have now been found to exhibit interesting interactions, for example, with selectins [6].

Beef lung heparin was chosen as a model system for study because it contains fewer complicating heterogeneities, such as D-GlcA, when compared to pig mucosal heparin. It has been confirmed that in heparin samples, the chemical shift positions can be influenced by the presence of divalent cations [11], variations in pH, and to some extent by temperature [12,13], and we therefore undertook this study taking precautions to minimise these problems, particularly the presence of unwanted cations by including treatments with cation-exchange resin.

2. Results

The complete ^{1}H and ^{13}C assignments for the 12 possible patterns (see Fig. 1) of sulfation and acetylation mentioned above are given in Table 1.

The most complete data with which to compare our results are those of Mulloy et al. [1,2] in which ^{1}H and ^{13}C data are reported for compounds corresponding to **2**, **5**, **6**, **9**, and **12**. For some of these compounds, however, a number of ^{1}H resonances are reported as overlapping, and therefore only approximate chemical shift positions are reported. In general our data are in good agreement, and any small discrepancies probably arise as a consequence of the different temperatures (70 °C by Mulloy et al. and 40 °C in this study). The only notable difference is observed in the case of the ^{13}C spectrum of **5** in which there appear to be systematic differences throughout the spectrum. It seems unlikely that the difference is caused solely by temperature differences because we would expect larger discrepancies in the iduronate resonances (particularly in the ^{1}H spectrum), which are known to be more sensitive to temperature changes [12,13] and, furthermore, all the signals in the spectrum, including those which can normally be



Compound	R1	R2	R3
1	H	H	H
2	H	H	SO_3
3	H	SO_3	SO_3
4	SO_3	SO_3	H
5	SO_3	SO_3	$COCH_3$
6	SO_3	H	SO_3
7	H	SO_3	H
8	H	SO_3	$COCH_3$
9	H	H	$COCH_3$
10	SO_3	*H/SO_3	H
11	SO_3	*H/SO_3	$COCH_3$
12	SO_3	SO_3	SO_3

* In order to investigate the feasibility of resolving signals arising from subtley different environments (i.e. different neighbouring residues) compounds (**10**) and (**11**) were prepared with a ratio of sulphation to free hydroxyl groups at A-6 of approximately 2/3 as determined by ^{13}C signal integration.

Fig. 1. Predominant disaccharide repeats in compounds **1–12**.

Table 1
^{13}C and ^{1}H chemical shift data [a] (in ppm) for compounds **1–12**

Compound	A-1	A-2	A-3	A-4	A-5	A-6	I-1	I-2	I-3	I-4	I-5
1	95.8	57.0	71.8	79.0	74.3	61.9	104.5	71.7	71.8	77.4	71.8
	5.40	3.29	3.70	3.70	3.91	3.84	4.89	3.79	3.96	4.14	4.72
						3.90					
2	98.2	60.5	72.5	80.2	73.5	62.4	104.3	72.2	71.5	77.8	72.2
	5.39	3.26	3.67	3.72	3.87	3.84	4.95	3.74	4.11	4.08	4.77
						3.88					
3	98.1	60.3	72.4	80.1	71.5	68.7	104.6	71.1	70.4	77.2	71.2
	5.38	3.24	3.65	3.71	4.02	4.36	5.04	3.78	4.12	4.08	4.84
						4.23					
4	93.7	57.1	70.9	78.2	72.0	68.8	101.3	75.3	65.4	73.0	69.8
	5.40	3.36	3.95	3.79	4.03	4.30	5.24	4.39	4.40	4.18	4.95
						4.40					
5	96.6	56.2	73.0	79.3	72.3	69.6	102.2	76.8	67.3	74.2	70.8
	5.15	4.03	3.76	3.78	4.04	4.31	5.20	4.37	4.31	4.08	4.91
						4.37					
6	100.0	60.8	72.4	80.5	73.8	62.6	102.0	77.6	70.7	78.7	71.4
	5.31	3.27	3.71	3.70	3.89	3.86	5.26	4.35	4.25	4.06	4.84
						3.88					
7	97.9	57.6	73.9	79.6	72.7	69.0	104.8	72.9	72.4	77.8	72.8
	5.29	2.98	3.72	3.72	3.96	4.35	4.95	3.74	4.07	4.14	4.74
						4.23					
8	97.1	56.2	72.5	79.6	71.8	68.8	104.6	72.0	71.4	77.0	71.9
	5.18	4.00	3.78	3.79	4.08	4.37	5.01	3.75	3.42	4.10	4.78
						4.26					
9	97.1	56.2	72.3	79.6	73.7	62.3	104.3	72.5	72.2	77.3	72.6
	5.18	3.97	3.76	3.74	3.89	3.88	4.92	3.69	3.89	4.07	4.73
						3.85					
10	97.2	58.0	73.5	80.1	74.2	62.8	101.9	76.0	67.0	74.4	70.1
	5.22	3.04	3.76	3.72	3.88	3.88	5.22	4.38	4.30	4.14	4.93
						3.90					
11	96.8	56.6	72.9	80.6	74.2	62.9	102.3	76.6	67.1	74.1	70.6
	5.14	4.03	3.79	3.76	3.91	3.92	5.26	4.37	4.28	4.07	4.91
						3.87					
12	99.5	60.7	72.5	78.8	72.0	69.2	102.1	78.9	72.1	79.0	72.3
	5.42	3.31	3.69	3.79	4.05	4.42	5.23	4.37	4.22	4.14	4.82
						4.30					

[a] The ^{1}H chemical shift values quoted for A-6 resonances are intervals. I-6 and acetyl CH_3 signals are not shown.

considered as relatively invariant also exhibit the same systematic variation. For example, the acetyl signal for **5** is reported at 23.2 ppm whereas in the other compounds it is at, or near, 24.8 ppm, and this difference of 1.6 ppm corresponds with the average difference between the data of Mulloy et al. for **5** and the data in this report which is also 1.6 ppm in the same direction.

The data of Jaseja et al. [14], which are reported to one decimal place covering the ^{1}H and ^{13}C spectra of compounds **3** and **12**, are in close agreement with our data. Small

discrepancies seem most likely to be caused by different reference compounds and the slightly different temperatures used in the two studies. There are also a number of other reports containing partial assignments for heparin derivatives [11,12,15–17].

The structures containing the predominant repeating disaccharides: → 4)-α-L-IdoA-2-*O*-sulfate-α-(1 → 4) *N*-acetylated D-glucosamine 6-*O*-sulfate-(1 → and → 4)-α-L-IdoA-2-*O*-sulfate-α-(1 → 4) *N*-acetylated D-glucosamine-(1 →, correspond to the most common heterogeneities in the glucosamine residues, and their assignments are shown in Table 1 as **5** and **11**, respectively. The anomeric signals (of both A-1 and I-1) for these compounds may prove useful for the rapid identification and quantification (by integration of the proton signal compared to the total integrated area of anomeric signals) of these heterogeneities. In the ^{13}C spectra, the signals for A-1 resonate at 96.6 and 96.8 ppm and in the ^{1}H spectra at 5.15 and 5.14 ppm, respectively. The I-1 signals appear at 102.2 and 102.3 in the ^{13}C spectra and at 5.20 and 5.26 ppm, respectively, in the ^{1}H spectra.

Effects of sulfation and acetylation at individual positions on the unsubstituted polysaccharide backbone.—(i) A-6 O-*sulfation.* The addition of an *O*-linked sulfate group to the unsubstituted polysaccharide backbone causes the expected large downfield shift at the carbon to which the sulfate group is attached. Comparing **7** with **1** we observe a large downfield shift from 61.9 to 69.0 ppm (7.1 ppm) in the ^{13}C signal due to A-6 and from 3.84–3.90 to 4.23–4.35 ppm (0.42 ppm) in the ^{1}H spectra. The signal due to A-5 is also subject to a smaller ^{13}C upfield shift from 74.3 to 72.7 ppm (1.6 ppm). Other changes occur in the spectra, the most significant among these being the downfield shift in the ^{13}C signal of A-1 from 95.8 to 97.9 ppm (2.1 ppm) and the upfield shift of the ^{1}H signal from 5.40 to 5.29 ppm (0.11 ppm).

(ii) I-2 O-*sulfation.* Considering the effects of *O*-sulfation at position I-2 we can compare **10** and **1**. In the ^{13}C spectrum, the signal for I-2 undergoes a downfield shift from 71.7 to 76.0 ppm (4.3 ppm) while those of I-1, I-3, I-4, and I-5 are also quite strongly affected, experiencing upfield shifts of 2.6, 4.8, 3.0, and 1.7 ppm, respectively. In the ^{1}H spectrum, I-2 experiences a downfield shift of 0.59 ppm whereas I-1, I-3, and I-5 show downfield shifts of 0.33, 0.34, and 0.21 ppm, respectively. The most strongly affected signal in the ^{13}C spectrum of the glucosamine residue is that of A-1 which exhibits a downfield shift of 1.4 ppm, but other small changes are evident throughout the glucosamine signals. In the ^{1}H spectrum, the most notable changes occur in A-1 and A-2 signals which show upfield shifts of 0.18 and 0.25 ppm, respectively.

(iii) A-2 N-*sulfation.* The effect of *N*-sulfation on the otherwise unsubstituted polysaccharide can be observed by comparing **2** with **1**. The signal due to A-2 undergoes a downfield shift from 57.0 to 60.5 ppm (3.5 ppm) in the ^{13}C spectra and an upfield shift from 3.29 to 3.26 ppm (0.03 ppm) in the ^{1}H spectra. The signal due to A-1 also experiences a downfield shift from 95.8 to 98.2 ppm (2.4 ppm), and other changes are observed throughout the spectrum.

(iv) A-2 N-*acetylation.* Comparing **9** and **1**, the effect of *N*-acetylation can be observed. The signal due to A-2 experiences an upfield shift from 57.0 to 56.2 ppm (0.8 ppm) in the ^{13}C spectra and a downfield shift from 3.29 to 3.97 ppm (0.68 ppm) in the ^{1}H spectra. A-1 experiences a downfield shift from 95.8 to 97.1 ppm (1.3 ppm) in the ^{13}C spectra and an upfield shift from 5.40 to 5.18 ppm (0.22 ppm) in the ^{1}H signals.

Table 2
Differences in ^{13}C and ^{1}H chemical shift values [a] between intact heparin **12** and selectively modified heparins [b]

Compound	(^{13}C NMR)										
	A-1	A-2	A-3	A-4	A-5	A-6	I-1	I-2	I-3	I-4	I-5
3	1.4	0.4	0.1	−1.3	0.5	0.5	−2.5	7.8	1.7	1.8	1.1
6	−0.5	−0.1	0.1	−1.7	−1.8	6.6	0.1	1.3	1.4	0.3	0.9
4	5.8	3.6	1.6	0.6	0	0.4	0.8	3.6	6.7	6.0	2.5
5	2.9	4.5	−0.5	−0.5	−0.3	−0.4	−0.1	2.1	4.8	4.8	1.5
2	1.3	0.2	0	−1.4	−1.5	6.8	−2.2	6.7	0.6	1.2	0.1
1	3.7	3.7	0.7	−0.2	−2.3	7.3	−2.4	7.2	0.3	1.6	0.5
Compound	(^{1}H NMR)										
	A-1	A-2	A-3	A-4	A-5	A-6	I-1	I-2	I-3	I-4	I-5
3	0.04	0.07	0.04	0.08	0.03	0.07	0.19	0.59	0.10	0.06	−0.02
6	0.11	0.04	−0.02	0.09	0.16	0.49	−0.03	0.02	−0.03	0.08	−0.02
4	0.02	−0.05	−0.26	0	0.02	0.01	−0.01	−0.02	−0.18	−0.04	−0.13
5	0.27	−0.72	−0.07	0.01	0.01	0.02	0.03	0	−0.09	0.06	−0.09
2	0.03	0.05	0.02	0.07	0.18	0.50	0.28	0.63	0.11	0.06	0.05
1	0.02	0.02	−0.01	0.09	0.14	0.49	0.34	0.58	0.26	0	0.10

[a] A positive value indicates an upfield shift, a negative value a downfield shift.
[b] Modifications are as follows: (**3**) I-2 de *O*-sulfated. (**6**) A-6 de *O*-sulfated. (**4**) A-2 de *N*-sulfated. (**5**) A-2 de *N*-sulfated, *N*-acetylated. (**2**) totally de *O*/*N*-sulfated, *N*-sulfated heparin. (**1**) totally de *O*/*N*-sulfated heparin.

Effects of the O/N-*sulfations and* N-*acetylation on intact heparin* **12**.—We can also consider the effect of the removal of one sulfate group from either I-2, A-6, or A-2, the substitution of an *N*-sulfate group by an *N*-acetyl group, or combinations of these modifications by comparison to intact heparin **12**. All of the changes which are evident in both the ^{13}C and ^{1}H spectra for the six modifications detailed below are shown in Table 2.

(i) I-2 de O-*sulfation.* Comparing **12** with **3**: Upon I-2 de *O*-sulfation, the most significant change is observed in the signal of I-2 which moves upfield by 7.8 ppm in the ^{13}C spectrum and upfield by 0.59 ppm in the ^{1}H spectrum. I-1 is subject to a downfield shift of 2.5 ppm in the ^{13}C spectrum and an upfield shift of 0.19 ppm in the ^{1}H spectrum. Other changes which are significant in the ^{13}C spectra include moderate upfield shifts in A-1, I-3, I-4, and I-5.

(ii) A-6 de O-*sulfation.* Comparing **12** and **6**: The largest chemical shift changes observed upon de *O*-sulfation at A-6 are those of the signals for A-6 itself. An upfield shift of 6.6 ppm is observed in the ^{13}C spectrum, and of 0.49 ppm in the ^{1}H spectrum. There are also moderate changes in the position of the signals of A-4, A-5, I-2, and I-3 in the ^{13}C spectra of 1.7 and 1.8 ppm downfield and of 1.3 and 1.4 ppm upfield, respectively. In the ^{1}H spectrum there are moderate upfield changes in the positions of the signals for A-1, A-4, and A-5 of 0.11, 0.09, and 0.16 ppm, respectively.

(iii) A-2 de N-*sulfation.* Comparing compounds **12** and **4**: Upon de *N*-sulfation at position A-2, there are considerable changes in the ^{13}C spectrum, including upfield shifts of 5.8, 3.6, and 1.6 ppm in the ^{13}C signals of A-1, A-2, and A-3, respectively, while a

downfield shift of 0.26 ppm in ^{1}H signal of A-3 is the most prominent change in the signals of the glucosamine residue.

In addition, there are also very prominent changes in the ^{13}C spectra of the IdoA signals, the most significant being the upfield shift of 6.7 and 6.0 ppm experienced by I-3 and I-4, but there are also upfield shifts of 3.6 and 2.5 ppm in the signals of I-2 and I-5. In the ^{1}H spectrum, the largest changes are the downfield shifts observed in I-3 and I-5 of 0.18 and 0.13 ppm, respectively.

(iv) Substitution of N-*sulfate with* N-*acetyl at A-2.* Comparing **12** and **5**: When an *N*-sulfate group is substituted with an *N*-acetyl group, many changes are evident in the signals of both the glucosamine and the iduronic acid residues. In the ^{13}C spectra, A-1 and A-2 experience upfield shifts of 2.9 and 4.5 ppm and an upfield shift of 0.27 and a downfield shift of 0.72 ppm, respectively, in the ^{1}H spectra. However, the largest changes occur in the signals of iduronic acid: the ^{13}C signals of I-3 and I-4 both experience a large upfield shift of 4.8 ppm, but less significant changes are evident in the ^{1}H spectra for these signals. The signals due to I-2 and I-5 are also subjected to significant changes in the ^{13}C spectra involving upfield shifts of 2.1 and 1.5 ppm, respectively.

(v) I-2 and A-6 de O-*sulfation.* Comparing **12** and **2**: De *O*-sulfation at I-2 and A-6 causes changes in the spectra of a magnitude very similar to the largest changes evident when the modifications are made individually at I-2 and A-6. In the ^{13}C spectra, a large upfield shift is observed in the signal for A-6 of 6.8 ppm. Moderate downfield shifts are observed in the signals of A-4 and A-5 of 1.4 and 1.5 ppm, and an upfield shift is observed for the A-1 signal. In the ^{1}H spectra, the most significant changes are a considerable upfield shift in A-6 of 0.50 ppm and a moderate upfield shift of 0.18 ppm in the signal of A-5. In the iduronic acid residue, the most significant change is seen in the I-2 signal; in the ^{13}C spectra, it undergoes an upfield shift of 6.7 ppm, accompanied by a smaller upfield shift of 1.2 ppm for the I-4 signal and a downfield shift of 2.2 ppm for the I-1 signal. In the ^{1}H spectra, the largest change is an upfield shift of 0.63 ppm experienced by I-2 and then of 0.28 by the I-1 signal.

(vi) I-2, A-6 de O-*sulfation and A-2 de* N-*sulfation.* Comparing **12** and **1**: When heparin is de *O*-sulfated at I-2 and A-6 and also de *N*-sulfated at A-2, the changes in ^{13}C chemical shift patterns are most significant for the A-6 signal which experiences an upfield shift of 7.3 ppm, I-2 which undergoes an upfield shift of 7.2 ppm, and A-1 and A-2 which both experience upfield shifts of 3.7 ppm. Other significant changes are observed in the signals of A-5, I-1, and I-4 which experience downfield shifts of 2.3 and 2.4 ppm and an upfield shift of 1.6 ppm, respectively. In the ^{1}H spectra, the most notable changes are observed in the signals of A-6 and I-2 which show upfield changes of 0.49 and 0.58 ppm, respectively. Other changes in the signals of A-5, I-1, and I-3 consisting of upfield shifts of 0.14, 0.34, and 0.26 ppm are also observed.

3. Discussion

Apart from the expected large changes in the ^{13}C and ^{1}H shifts at the positions of substitution, one of the most noticeable chemical shift changes that occurs in this series

of compounds is observed in ^{13}C spectra of derivatives possessing an *O*-sulfate group at I-2 but which have been subjected to de *N*-sulfation at A-2 or de *N*-sulfation followed by *N*-acetylation (for example, comparing **6** with **10** or **11**, or comparing **12** with **4** or **5**). In all of these cases, there is a marked upfield shift in the ^{13}C signals of I-3 and I-4 upon de *N*-sulfation or de *N*-sulfation followed by *N*-acetylation. However, it is apparent that when these modifications are made at position A-2 in the absence of an *O*-sulfate group at I-2, the same upfield shifts in I-3 and I-4 signals are not observed. These observations are consistent with a conformational change in the residues and possible changes in the chain conformation, or changes in the population of different conformers of the iduronate residue upon sulfation at I-2 [18]. As has been noted [1,2], a change in the ^{3}J proton–proton coupling constants is observed in the case of de *N*-sulfated, *N*-acetylated heparin (corresponding to **5**) ($^{3}J_{I\text{-}1,2} < 2$, $^{3}J_{I\text{-}4,5}$ 2.9 Hz) when compared to intact heparin (**12**) ($^{3}J_{I\text{-}1,2}$ 3.0, $^{3}J_{I\text{-}4,5}$ 3.6 Hz), and a low molecular weight sample of de *N*-sulfated heparin (**4**) showed similar coupling constants to those found for de *N*-sulfated, *N*-acetylated heparin ($^{3}J_{I\text{-}1,2}$ 1.8, $^{3}J_{I\text{-}4,5}$ 2.9 Hz). Upon removal of the *N*-sulfate group from **12** and its substitution with either a proton or acetyl group, as in **4** and **5**, respectively, the large deshielding effect of the sulfate group is removed allowing the signals for I-3 and I-4 to resonate further upfield. Such large shifts could not have been easily predicted nor could their signals have been assigned with mono-dimensional NMR spectra alone. The presence of the I-3 signal in the relatively uncluttered region (65–67 ppm) of the ^{13}C spectrum, which has in the past been mistakenly assigned as

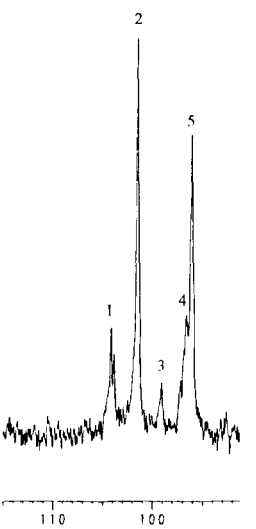


Fig. 2. Details of the anomeric region of the ^{13}C spectrum of **11** showing the following signals: (1) Low levels of I-1 signals corresponding to IdoA (I_{2OH}). (2) Principal I-1 signal of iduronic acid 2-*O*-sulfate (I_{2S}). (3) Low level of A-1 signal corresponding to glucosamine *N*-sulfate. (4) A-1 signals of GlcNAc 6-*O*-sulfate (A_{6S}) and GlcNAc (A_{6S}) and GlcNAc (A_{6OH}) adjacent to IdoA (I_{2OH}). (5) Principal A-1 signal corresponding to GlcNAc 6-*O*-sulfate (A_{6S}) and GlcNAc (A_{6OH}) residues adjacent to IdoA 2-*O*-sulfate (I_{2S}).

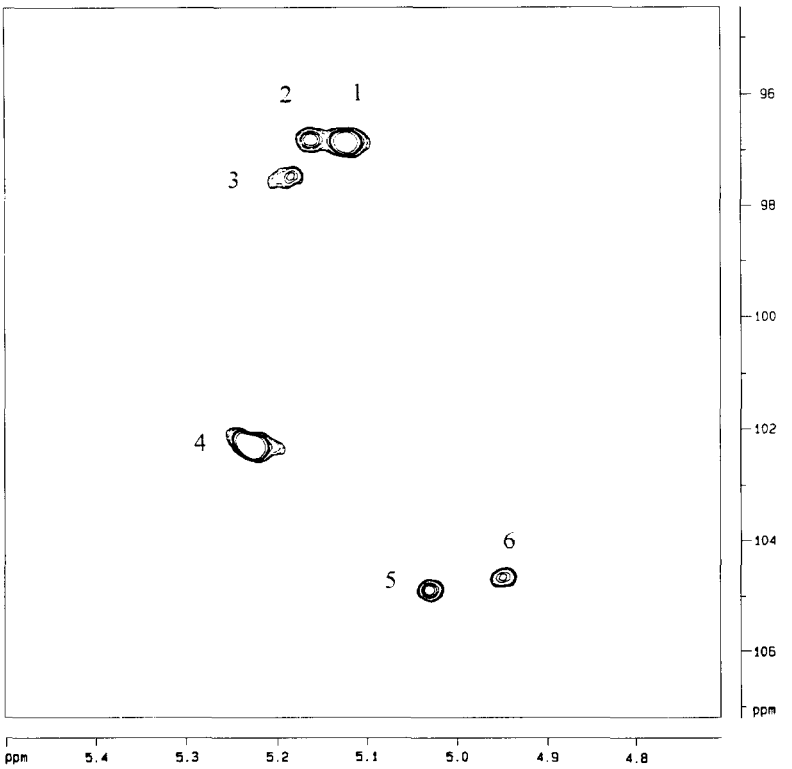


Fig. 3. $^{1}H/^{13}C$ spectrum (HMQC) of anomeric region of **11** showing principal anomeric signals and lower levels of structural heterogeneity. (1) A-1 of GlcNAc linked to IdoA 2-*O*-sulfate. (2) A-1 of GlcNAc 6-*O*-sulfate linked to IdoA 2-*O*-sulfate. (3) A-1 of GlcNAc linked to IdoA. (4) I-1 of IdoA 2-*O*-sulfate linked to GlcNAc and GlcNAc 6-*O*-sulfate. (5) I-1 of IdoA linked to GlcNAc 6-*O*-sulfate. (6) I-1 of IdoA linked to GlcNAc.

A-3 [17], serves as an additional indicator of the presence of de *N*-sulfation in heparin preparations.

In order to investigate the feasibility of resolving signals which arise from subtly different environments (i.e. chemically distinct nearest neighbours), **10** and **11** (see Fig. 2) were prepared with a ratio of sulfate to hydroxyl at position A-6 of about 2:3. A statistical distribution of sequences for iduronic acid 2-*O*-sulfate (I_{2S}), glucosamine 6-*O*-sulfate (A_{6S}), and glucosamine (A_{6OH}) would therefore be expected with the following combinations of nearest neighbours for the glucosamine signals: $I_{2S}\,A_{6S}\,I_{2S}$ and $I_{2S}\,A_{6OH}\,I_{2S}$, and for the iduronic acid signals: $A_{6OH}\,I_{2S}\,A_{6OH}$, $A_{6S}\,I_{2S}\,A_{6OH}$, $A_{6OH}\,I_{2S}\,A_{6S}$, and $A_{6S}\,I_{2S}\,A_{6S}$. In the standard HMQC spectrum of **11** (Fig. 3) for example, the signals for A-1 arising from *N*-acetylated glucosamine in the two forms mentioned above are resolved, as is the smaller signal arising from 6-*O*-sulfated *N*-acetylglucosamine adjacent to non-sulfated iduronic acid, but the signals for I-1 arising from the four distinct situations of iduronic acid are not clearly resolvable (even using higher digital resolution), although the irregular shape of the I-1 signal suggests a

number of close but distinct signals and higher field experiments may be able to resolve these signals.

The data presented in this report will be useful for the identification of regions of heterogeneity which are sometimes observed in heparins of different biological origin and as the first step towards understanding sequence effects in heparin and heparan sulfate chains which will require studies at higher resolution than is currently routinely available. The data should also be of use to workers in the field who require rapid verification of the effects of a chemical or other treatment on heparin polysaccharide samples monitored by mono-dimensional ^{1}H and ^{13}C NMR experiments.

4. Experimental

Beef lung heparin (**12**) (MW 16 kDa) was obtained from Crinos (Villaguardia, CO, Italy).

Preparation of compounds **1** *to* **11**.—(**1**) *Totally de* N- *and* O-*sulfated heparin.* Totally de *N*- and *O*-sulfated heparin was prepared essentially as described [19]. Typically, heparin (1 g, pyridinium salt) was heated in a solution of Me_2SO containing 10% MeOH (25 mL) at 105 °C for 24 h. After this period, the pH was adjusted to 9.0 with dilute aq NaOH, the solution was cooled, and polysaccharide products were precipitated and purified as described below to yield **1**.

(**2**) *De* O-*sulfated, re*-N-*sulfated heparin.* Totally de *N*- and *O*-sulfated heparin **1** was re-*N*-sulfated by a modification of the method described [20]. Typically, a solution of **1** (200 mg, sodium salt) was dissolved in distilled water (15 mL) and the pH adjusted to 9.0 with saturated sodium bicarbonate solution. To this solution was added trimethylamine sulfur trioxide complex (200 mg), the pH maintained at 9.0, and the solution was heated at 55 °C for 6 h. Another addition of trimethylamine sulfur trioxide (200 mg) was made after 3 h, again maintaining pH 9.0. After completion of the reaction, the solution was cooled and polysaccharide products were precipitated and purified as described below to yield **2**.

(**3**) *De* 2-O-*sulfated heparin (at I-2).* Selectively de *O*-sulfated (at I-2) heparin was prepared essentially as described [14]. Typically, heparin **12** (700 mg, sodium salt) was dissolved in 0.1 M aq NaOH, frozen, and then lyophilised. The dried product was then re-dissolved in a small quantity of distilled water and the pH was adjusted to 7.0 with dilute hydrochloric acid. The polysaccharide products were then retrieved and purified as described below to yield **3**.

(**4**) *De* N-*sulfated heparin.* Heparin **12** was de *N*-sulfated essentially according to the procedure described [21]. Heparin (100 mg, pyridinium salt) was stirred with Me_2SO containing 10% MeOH (15 mL) at 55 °C for 2 h, isolated, and purified as described below to yield **4**.

(**5**) *De* N-*sulfated,* N-*acetylated heparin.* De *N*-sulfated heparin **4** was *N*-acetylated essentially as described [22]. Typically, **4** (100 mg) was dissolved in saturated sodium bicarbonate solution (1.0 mL), and acetic anhydride (1.0 mL) was added at 0 °C, further additions of saturated sodium bicarbonate being made to maintain pH 9 or above. The

solution was stirred at 0 °C for 2 h, after which the products were precipitated and purified as described below to yield **5**.

(6) *De 6-O-sulfated heparin (at A-6).* Heparin was selectively de *O*-sulfated at A-6 and concomitantly de *N*-sulfated at A-2 by a slight modification of the method described [17], in which conditions of temperature and reaction time were optimised (25 °C, 72 h) to give de A-6 sulfation while leaving I-2 sulfates intact, the products being isolated and purified as described below to yield **6**.

(7) *De O-sulfated (at I-2) and de N-sulfated heparin.* I-2 de *O*-sulfated heparin **3** was de *N*-sulfated essentially as described above for **4**, isolated and purified as described below to yield **7**.

(8) *De O-sulfated (at I-2), de N-sulfated and N-acetylated heparin.* **7** was *N*-acetylated essentially as described above for **5**, isolated and purified as described below to yield **8**.

(9) *De O- and N-sulfated and N-acetylated heparin.* **1** was *N*-acetylated essentially as described above for **5**, isolated and purified as described below to yield **9**.

(10) *De O-sulfated (60% at A-6), de N-sulfated heparin.* Heparin (pyridinium salt, typically 100 mg) was selectively de *O*-sulfated (60% de *O*-sulfated at A-6) and concomitantly de *N*-sulfated by stirring in Me_2SO containing 10% MeOH (10 ml) for 24 h at 50 °C, isolated and purified as described below to yield **10**.

(11) *De O-sulfated (60% at A-6), de N-sulfated, N-acetylated heparin.* **10** was *N*-acetylated essentially as described above for **5**, isolated and purified as described below to yield **11**.

(12) *Unmodified heparin.* **12** was purified as described below.

Purification and preparation of compounds for NMR experiments.—After the above reactions had been completed (and in those cases where reactions were carried out in Me_2SO–MeOH mixtures after the pH was adjusted to 9.0 with 1.0 N NaOH) the polysaccharide components were precipitated with a large volume of EtOH saturated with NaOAc and left to stand overnight at 4 °C. The precipitates were retrieved by filtration, washed with cold EtOH, the filtrate dissolved in a small volume of distilled water and dialysed (6–8 kDa cut-off) against distilled water. After dialysis, the solution was concentrated to a small volume by rotary evaporation and subjected to cation exchange on a column of Dowex 50 W (H^+ form) followed by careful neutralisation with dilute NaOH and then purified by gel chromatography on a Sephadex G-25 column (125 × 1.25 cm) eluting with an EtOH–degassed distilled water mixture (1:10 v/v). Fractions were collected monitoring for the presence of polysaccharide material or salts by their absorbance at 215 nm. The samples containing polysaccharide materials were then again subjected to cation exchange on a column of Dowex 50 W resin (H^+ form), carefully neutralised with dilute NaOH, and dried by rotary evaporation, exchanged three times with D_2O and finally dissolved in 0.5 mL of D_2O in a 5-mm tube (or in 2.0 mL of D_2O in a 10-mm tube for the mono-dimensional ^{13}C spectra) and used for NMR analyses. In all cases the pD of the solutions were carefully adjusted to 8.0 with NaOD. Typically, 15–20 mg of purified polysaccharide was employed in the COSY and HMQC experiments and 100–150 mg was used for the mono-dimensional ^{13}C spectra. The effects of chemical modifications were monitored by comparing the integrated areas of their respective signals (Table 1) and were at least 90% complete in each case except for

10 and **11** which contained significant proportions of hydroxyl and sulfate groups at A-6 (see footnote to Fig. 1). In all NMR experiments, high quality D_2O containing low paramagnetic impurities was employed.

NMR Experimental procedure.—The ^1H (COSY and HMQC) spectra were obtained at 500 MHz with a Bruker AMX500 spectrometer equipped with a 5-mm $^1H/X$ inverse probe, and the ^{13}C spectra were obtained at 100 MHz using a Bruker AM400 instrument equipped with a 10-mm broad band probe. Chemical shift values were recorded downfield from external trimethylsilyl propionate (TSP) as standard at 40 °C. Mono-dimensional ^1H spectra were obtained with presaturation of the HDO signal with digitalisation of 0.1 Hz/point, and mono-dimensional ^{13}C spectra were recorded using the standard Bruker sequence with powergated proton decoupling, utilising signal enhancement by the nuclear Overhauser effect. Proton spectra were assigned with the use of double quantum filtered COSY spectra with gradient enhancement [23] and were recorded employing a Bruker BGU (unit-z) with a maximum strength of 50 $G \cdot cm^{-1}$. The z-gradient was a square wave of 1-ms duration and with a maximum amplitude of 5, 5, and 15 $G \cdot cm^{-1}$. These gradient strengths were determined empirically to be the minimum required to completely eliminate to the HDO signal in the sample. Data were acquired using 8 scans per series in 1 K × 512 W data points with zero filling in F1, and a squared cosine function was applied before Fourier transformation. ^1H–^{13}C chemical shift correlations were obtained with ^1H detection via gradient enhanced heteronuclear multiple-quantum coherence spectra [24] which employed a square wave with a maximum amplitude of 15, 20, and 15 $G \cdot cm^{-1}$. The first value was optimised to obtain a good presaturation of the HDO signal in the sample. Sixteen scans were collected for each series in 1 K × 256 W data points before processing and were zero filled to 2 K × 512 W by application of a squared cosine function prior to Fourier transformation.

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References

[1] B. Mulloy, M.J. Forster, C. Jones, and D.B. Davies, *Biochem. J.*, 293 (1993) 849–858.
[2] B. Mulloy, M.J. Forster, C. Jones, A.F. Drake, E.A. Johnson, and D.B. Davies, *Carbohydr. Res.*, 255 (1994) 1–26.
[3] R.N. Rej, K.G. Ludwig-Baxter, and A.S. Perlin, *Carbohydr. Res.*, 210 (1991) 299–310.
[4] B. Casu, G. Grazioli, N. Razi, M. Guerrini, A. Naggi, G. Torri, P. Oreste, F. Tursi, G. Zoppetti, and U. Lindahl, *Carbohydr. Res.*, 263 (1994) 271–284.
[5] D. Aviezer, E. Levy, M. Safran, C. Svahn, E. Buddecke, A. Schmidt, G. David, I. Vlodavsky, and A. Yayon, *J. Biol. Chem.*, 269 (1994) 114–121.
[6] K.E. Norgard-Sumnicht and N.M. Varki, *Science*, 261 (1993) 279–283.
[7] M.R. Bartlett, W.B. Cowden, and C.R. Parish, *J. Leukocyte Biology*, 57 (1995) 207–213.
[8] U. Lindahl, K. Lidholt, D. Spillmann, and L. Kjellèn, *Thromb. Res.*, 75 (1994) 1–32.
[9] W. Chai, E.F. Hounsell, C.J. Bauer, and A.M. Lawson, *Carbohydr. Res.*, 269 (1995) 139–156.

[10] B. Casu, *Adv. Carb. Chem. Biochem.*, 43 (1985) 51–134.
[11] B. Mulloy and E.A. Johnson, *Carbohydr. Res.*, 170 (1987) 151–165.
[12] G. Gatti, B. Casu, G.K. Hamer, and A.S. Perlin, *Macromolecules*, 12 (1979) 1001–1007.
[13] D.R. Ferro, A. Provasoli, M. Ragazzi, G. Torri, B. Casu, G. Gatti, J.-C. Jacquinet, P. Sinaÿ, M. Petitou, J. Choay, *J. Am. Chem. Soc.*, 108 (1986) 6775–6778.
[14] M. Jaseja, R.N. Rej, F. Sauriol, and A.S. Perlin, *Can. J. Chem.*, 67 (1989) 1449–1456.
[15] E.F. Hounsell, *Prog. Nucl. Mag. Res. Spectr.*, 27 (1995) 445–474.
[16] L.A. Gransson, T.N. Huckerby, and I.A. Nieduszynski, *Biochem. J.*, 175 (1978) 299–309.
[17] L. Ayotte and A.S. Perlin, *Carbohydr. Res.*, 145 (1986) 267–277.
[18] C.A.A. Van Boeckel, H. Lucas, S.F. Van Aelst, M.W.P. van den Niewenhof, G.N. Wagenaans, and J.-R. Melema, *Recl. Trav. Chim. Pays-Bas*, 106 (1987) 581–59.
[19] K. Nagasawa and Y. Inoue, *Methods Carbohydr. Chem.*, 8 (1980) 287–289.
[20] A.G. Lloyd, G. Embrey, and L.J. Fowler, *Biochem. Pharmacol.*, 20 (1971) 637–648.
[21] K. Nagasawa and Y. Inoue, *Carbohydr. Res.*, 46 (1976) 87–95.
[22] I. Danishevsky, *Carbohydr. Res.*, 5 (1965) 407–409.
[23] A.G. Palmer II, J. Cavanagh, P.E. Wright, and M. Rance, *J. Magn. Reson.*, 93 (1991) 151–170.
[24] L.E. Kay, P. Keifer, and T. Saaringen, *J. Am. Chem. Soc.*, 114 (1992) 10663–10665.