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Note

Effect of substitution pattern on ^{1}H, ^{13}C NMR chemical shifts and $^{1}J_{CH}$ coupling constants in heparin derivatives

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Abstract

^{1}H, ^{13}C NMR chemical shifts and $^{1}J_{CH}$ coupling constants were measured for derivatives of heparin containing various sulfation patterns. ^{1}H and ^{13}C chemical shifts varied considerably after introducing electronegative sulfate groups. Chemical shifts of protons linked to carbons changed by up to 1 ppm on substitution with *O*- and *N*-sulfate or acetyl groups. Differences up to 10 ppm were detected for ^{13}C chemical shifts in substituted glucosamine, but a less clear dependence was found in iduronate. $^{1}J_{CH}$ values formed two groups, corresponding to either sulfation or non-sulfation at positions 2 and 3 of glucosamine. O-sulfation caused increases up to 6 Hz in $^{1}J_{CH}$ and N-sulfation decreases up to 4 Hz. N-acetylation gave similar $^{1}J_{CH}$ values to N-sulfation. At positions 2 and 3 of iduronate the trend was less marked; $^{1}J_{CH}$ for O-sulfated positions usually increasing. Introduction of sulfate groups influences chemical shift and $^{1}J_{CH}$ values at the position of substitution, but also at more remote positions. $^{1}J_{CH}$ at the glycosidic linkage positions varied between free-amino and N-sulfated compounds, by up to 9 Hz. These results and changes in chemical shift values suggest that iduronate residues and the glycosidic linkages are affected, indicating overall conformational change. This may have important implications for biological activities.

Keywords: Heparin; NMR; Chemical shifts; $^{1}J_{CH}$ coupling constants; Sulfated polysaccharides; Conformation

The sulfated (1→4)-linked linear polysaccharide heparin, which has found widespread therapeutic use as an anticoagulant, is composed of alternating α-D-glucosaminyl (glucosamine) and uronic acid units, either α-L-iduropyranosyluronate (iduronate) or β-D-glucopyranosyluronate (glucuronate) [1]. The glucosamine residues are usually N-sulfated, but N-acetylation also occurs and C-6 is predominantly O-sulfated. L-iduronate can be found unsubstituted, but is usually O-sul-

Abbreviations: A refers to amino sugar (glucosamine) residues and I refers to iduronate residues, A-N refers to the Nth hydrogen or carbon atom of the glucosamine residue while I-N refers to the Nth hydrogen or carbon of the iduronate residue.

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Table 1
^{13}C and ^{1}H chemical shift data (in ppm, relative to TSP) for compounds **1**–**4** [a]

Compound	A-1	A-2	A-3	A-4	A-5	A-6	I-1	I-2	I-3	I-4	I-5
1	93.7	56.6	71.6	78.7	71.7	68.2	103.6	69.3	75.0	73.7	70.6
	5.37	3.21	3.84	3.74	4.04	4.18–4.37	5.04	3.95	4.60	4.36	4.80
2	98.0	56.3	81.6	75.2	72.1	68.6	100.5	73.6	72.4	73.0	69.9
	5.22	3.21	4.57	4.06	4.09	4.36–4.42	5.32	4.57	4.77	4.43	5.08
3	99.6	59.3	82.9	76.8	72.1	68.7	100.8	73.6	72.9	73.3	69.8
	5.32	3.50	4.48	4.04	4.05	4.27–4.41	5.32	4.55	4.72	4.39	5.05
4 [b]	98.4	60.8	73.8	80.5	72.1	69.5	104.2	68.9	74.2	74.5	70.6
	5.34	3.27	3.74	3.76	4.00	4.21–4.32	5.10	4.02	4.72	4.28	4.98

[a] Chemical shift values not shown for carbonyl and methyl signals.
[b] For **4**, the following approximate ^{1}H chemical shift values were reported [3]; I-1 5.1, I-2 4.0, I-3 4.7, recorded at 338 K in D_2O which agree with the above assignments.

fated at C-2 while glucuronate is only rarely substituted. In addition, there are low levels of 2,3- and 2,3,6-O-sulfated glucosamine; some of the latter occurring in the pentasaccharide sequence that is essential for the binding of heparin to antithrombin and the subsequent inhibition of factor Xa. In recent years there has been increased interest in the chemical modification of heparin and related polysaccharides, not only with the aim of producing improved or alternative antithrombotic agents [2–4], but also as potential drugs resulting from the interaction of these polysaccharides, or fragments derived from them, with growth factors and selectins [5–9]. Many of these compounds contain sulfation patterns that have not been found in naturally occurring heparin. Chemically de-sulfated heparin samples are also used extensively to probe a wide range of biological activities. We have extended previous NMR studies of chemically modified heparin derivatives [10] to include a number containing unnatural substitution patterns and have undertaken investigations into other NMR parameters, particularly $^{1}J_{CH}$ coupling constants, which may be expected to vary with the nature of the substituent [11–19]. The effects of substitution on this parameter have been measured both at the site of substitution and at other positions. The analysis reveals both the utility and the limitations of $^{1}J_{CH}$ coupling constants as indicators of substitution pattern and illustrates their potential as indicators of overall conformational change.

1. Chemical shift values

The ^{13}C and ^{1}H chemical shifts of a number of heparin derivatives have already been reported [10,20] and Table 1 extends these to include those sulfated at I-3 and A-3 (Table 2). Table 3 shows the differences in ^{1}H and ^{13}C chemical shift values between derivatives bearing sulfate groups at I-3 and their non-sulfated counterparts (**1** and **6**, **4** and **5**) and those bearing I-3 and A-2 sulfation compared with their A-2 desulfated analogues (**4** and **1**, **3** and **2**).

Table 2
All of these compounds possess O-sulfate at A-6 with the exception of those marked * which contain unsubstituted hydroxyls at A-6 [a]

Compound	I-3	I-2	A-3	A-2	% Substitution
1	SO_3	H	H	H	90
2	SO_3	SO_3	SO_3	H	95
3	SO_3	SO_3	SO_3	SO_3	95
4	SO_3	H	H	SO_3	95
5	H	H	H	SO_3	80
6	H	H	H	H	95
7	SO_3	SO_3	SO_3	Ac	95
8*	H	H	H	SO_3	80
9	H	SO_3	H	H	80
10	H	SO_3	H	Ac	90
11	H	SO_3	H	SO_3	90
12*	H	H	H	H	80

[a] All the compounds were derived from polymeric heparin.

Table 3
Differences in ^{13}C and ^{1}H chemical shift values between various chemically modified heparins and their parent compounds [a]

	Position										
Compounds	A-1	A-2	A-3	A-4	A-5	A-6	I-1	I-2	I-3	I-4	I-5
6-1	4.2	1.0	2.3	0.9	1.0	0.8	1.2	3.6	−2.6	4.1	2.2
4-5	0.3	0.5	1.4	0.4	0.6	0.8	−0.4	−2.2	3.8	−2.7	−0.6
4-1	4.7	4.2	2.2	1.8	0.4	1.3	0.6	−0.4	−0.8	0.8	0
3-2	1.6	3.0	1.3	1.6	0	0.1	0.3	0	0.5	0.3	−0.1
^{1}H chemical shift differences between pairs of compounds differing by a single substituent. Changes are shown above.											
6-1	−0.08	−0.23	−0.12	−0.02	−0.08	0.05/−0.02	−0.09	−0.21	−0.53	−0.22	−0.06
4-5	−0.04	0.03	0.09	0.05	−0.02	−0.02/−0.04	0.06	0.24	0.60	0.20	0.14
4-1	−0.03	0.06	−0.10	0.02	−0.04	0.03/−0.05	0.06	0.07	0.12	−0.08	0.18
3-2	0.10	0.29	−0.09	−0.02	−0.04	−0.09/−0.01	0	−0.02	−0.05	−0.04	−0.03

[a] ^{13}C chemical shift differences between pairs of compounds differing by a single substituent. Changes are as follows: **6-1**; introduction of *O*-sulfate at I-3 to heparin already O-sulfated at A-6. **4-5**; introduction of an *O*-sulfate at I-3 to heparin already O-sulfated at A-6 and N-sulfated at A-2. **4-1**; removal of *N*-sulfate at A-2 from heparin O-sulfated at A-6 and I-3. **3-2**; removal of *N*-sulfate at A-2 from heparin O-sulfated at I-3, I-2 and A-3. A positive value indicates a downfield shift. The two values given for the A-6 position are for the two protons of the CH_2 group at that position.

Effects of sulfation at individual positions in the relevant reference compound

O-*sulfation*. The introduction of an *O*-sulfate group results in an increased chemical shift, typically between 2 and 6 ppm in the ^{13}C, and from 0.2 to 0.6 ppm in the ^{1}H spectra. At I-3, comparing **6** with **1** and **8** with **4**, the changes in ^{13}C chemical shifts are increases of 2.6 and 2.7 ppm and of 0.53 and 0.61 ppm in the ^{1}H spectra. At I-2, comparing **6** with **9** and **8** with **11**, the changes in ^{13}C chemical shifts are higher than at I-3, consisting of increases of 3.6 and 5.1 ppm. In the ^{1}H spectra the changes in chemical shifts are increases of 0.31 and 0.24 ppm. At A-3 no direct comparison can be made for compounds in which only O-sulfation at A-3 has been achieved, because this reaction is accompanied by the substitution of other free hydroxyl groups. However, **2**,**3** and **7** exhibit chemical shift values at A-3 higher than their non-sulfated counterparts, the shifts being of the order of 6–10 ppm in the ^{13}C and 0.5–0.8 ppm in the ^{1}H spectra.

N-sulfation. The introduction of an *N*-sulfate group into a free amino group, comparing **6** with **5**, **9** with **11**, **2** with **3** and **1** with **4** causes increases in the ^{13}C chemical shifts of 2.7, 2.8, 3.0 and 4.2 ppm. The same modifications cause increases in the ^{1}H chemical shifts of 1.26, 0.23, 0.29 and 0.06 ppm.

N-acetylation. Substituting an amino group with an acetyl group results in reduced ^{13}C chemical shifts (comparing **2** with **7** and **9** with **10**) of 0.9 and 1.4 ppm, while the same modifications cause increases in the ^{1}H chemical shifts of 1.05 and 1.0 ppm.

Sulfation at an individual position (I-3) and the influence of different substitution patterns elsewhere

I-3 sulfation in the presence of an O-*sulfate at A-6 and a free amino group at A-2*. When an *O*-sulfate group is introduced at I-3 in the absence of *N*-sulfate at A-2 (**1** compared with **6**) (Table 3), the most significant change occurs at I-3 in the ^{1}H spectrum consisting of an upfield shift of 0.53 ppm, as expected, followed by moderate upfield shifts in I-2 and I-4 of 0.21 and 0.22 ppm, respectively, as well as an upfield shift in the signal of A-2 of 0.23 ppm. These result from the proximity of an *O*-sulfate group at I-3 and could be due to the formation of a hydrogen bond between I-3 and A-2. In the ^{13}C spectrum, a downfield shift is observed at I-3 of 2.6 ppm with downfield shifts at positions I-2 and I-4 of 3.6 and 4.1 ppm. The largest downfield shift is observed at A-1 of 4.2 ppm. Downfield shifts are also observed at I-5 and A-3 of 2.2 and 2.3 ppm.

I-3 O-*sulfation in the presence of an* O-*sulfate at A-6 and* N-*sulfate group at A-2*. If this substitution is made in the presence of an

N-sulfate group at A-2, a different pattern of chemical shift changes is seen (**4** and **5**). In the ^{1}H spectrum of iduronate, a large downfield shift of 0.60 ppm at I-3 is accompanied by moderate downfield shifts at I-2 and I-4 of 0.24 and 0.20 ppm. The most significant change in glucosamine occurs at A-3, a downfield shift of 0.09 ppm. In the ^{13}C spectra, the biggest changes in iduronate occur at I-3, then I-4 and I-2; one down- and two upfield shifts of 3.8, 2.7 and 2.2 ppm. The largest shift in glucosamine occurs at A-3, a downfield shift of 1.4 ppm.

A-2 sulfation in the presence of A-6 and I-3 O-*sulfate groups*. In the ^{1}H spectra, the largest changes are observed at I-3 and A-3, consisting of down- and upfield shifts of 0.12 and 0.10 ppm (**1** and **4**). In the ^{13}C spectra, there are significant changes at A-1 and A-2 (downfield shifts of 4.7 and 4.2 ppm, respectively). Downfield shifts of 2.2 and 1.8 ppm are observed in A-3 and A-4.

A-2 sulfation in the presence of A-6, I-2 and I-3 O-*sulfate groups*. When I-2, I-3, A-3 and A-6 all carry *O*-sulfate groups and position A-2 is converted from free amine to *N*-sulfate (**2** and **3**), the most significant change that occurs in the ^{1}H spectra is at A-2, which experiences a downfield shift of 0.29 ppm, while A-1 exhibits a downfield shift of 0.1 ppm. In the ^{13}C spectra, A-2 exhibits a downfield shift of 3.0 ppm while A-1, A-3 and A-4 exhibit downfield shifts of 1.6, 1.3 and 1.6 ppm.

It can be seen that substitution with *N*- or *O*-sulfate groups gives rise to a variety of chemical shift changes. The best indicator of these substitutions in iduronate, appears to be ^{1}H chemical shifts while for N-sulfation in glucosamine, it is the ^{13}C chemical shifts. At A-3, the situation is more complicated because substitution is accompanied by O-sulfation elsewhere. Both ^{1}H and ^{13}C chemical shift values at A-3 fall into two groups [10], Table 1; 3.5–4.0 ppm and 70–74 ppm in the unsubstituted case and 4.4–4.7 and 79–83 ppm in the substituted case. However, it has been shown that certain patterns of sulfation can cause unexpected changes, for example, **11** and **9** [10] and one aim of these investigations was to discover whether the measurement of other spectral parameters in addition to chemical shifts would be a useful aid to the deduction of substitution pattern.

Table 4
$^{1}J_{CH}$ coupling constants at exo-cyclic substitution sites (values in Hz) for selected compounds [a]

Compound	Substitution site			
	I-3	I-2	A-3	A-2
1	148	147	nm	143
2	156	156	153	146
3	156	151	154	141
4	155	nm	151	138
5	150	150	150	139
6	150	147	149	142
7	153	152	155	140
8	149	147	150	138
9	151	151	150	148
10	151	154	144	140
11	149	148	148	139
12	145	150	147	148

[a] nm indicates that these values could not be measured.

2. Coupling constants

The value of $^{1}J_{CH}$ coupling constant is affected by changes in the electron distribution in the C–H bond. Factors which can influence this include conformation and the proximity of electronegative substituents. In the absence of major conformational change, the introduction of a sulfate group might be expected to alter $^{1}J_{CH}$ values at that position. This is observed in the glucosamine residues, presence of *O*-sulfate correlating well with $^{1}J_{CH}$. However, in the more flexible [20] iduronate residues the relationship between sulfation and $^{1}J_{CH}$ is less well-defined; values of $^{3}J_{I\text{-}1,I\text{-}2}$ and $^{3}J_{I\text{-}4,I\text{-}5}$ indicate a change in the population of ring conformers as the sulfation pattern is altered.

The $^{1}J_{CH}$ coupling constants for **1**–**12** for the exo-cyclic substitution positions I-3, I-2, A-3 and A-2 are shown in Table 4. Table 5 shows the $^{1}J_{CH}$ coupling constant values at glycosidic linkage positions (A-1, I-4, I-1 and A-4) for selected compounds. O-sulfation increases $^{1}J_{CH}$ at A-3 (Fig. 1(c and g)) and

Table 5
$^1J_{CH}$ coupling constants (values in Hz) at the glycosidic linkage positions

Compound	Position			
	A-1	I-4	I-1	A-4
1	176	152	172	150
4	175	153	177	156
5	176	150	173	156
6	172	150	170	147
9	179	153	176	147
11	173	149	173	149

together with ^{1}H and ^{13}C chemical shifts allows two groups of compounds, sulfated and non-sulfated, to be distinguished. The effect of sulfate is evident at A-2 as well but acetylated derivatives have smaller couplings, comparable with those of N-sulfated compounds. However, if ^{13}C chemical shifts are taken together with couplings, the effect of acetyl substitution at A-2 is clear (Fig. 1(d and h)). In iduronate, a less clear pattern emerges; in both I-3 and I-2 (Fig. 1(a,e and b,f)), the data are broadly separated into two groups corresponding to the nature of the substituent. In the case of I-3, differences were observed in $^1J_{CH}$ except for **1**, in which $^1J_{CH}$ was 148 Hz. This datum belongs to an I-3 sulfated compound lacking sulfate groups at either positions A-2 or A-3 in glucosamine. There is a dramatic effect on the value of the $^1J_{CH}$ coupling constant which is substantially lower than the next lowest value in the group. This could be due to an interaction between glucosamine and the I-3 sulfate group, possibly a hydrogen bond between I-3 sulfate and free-

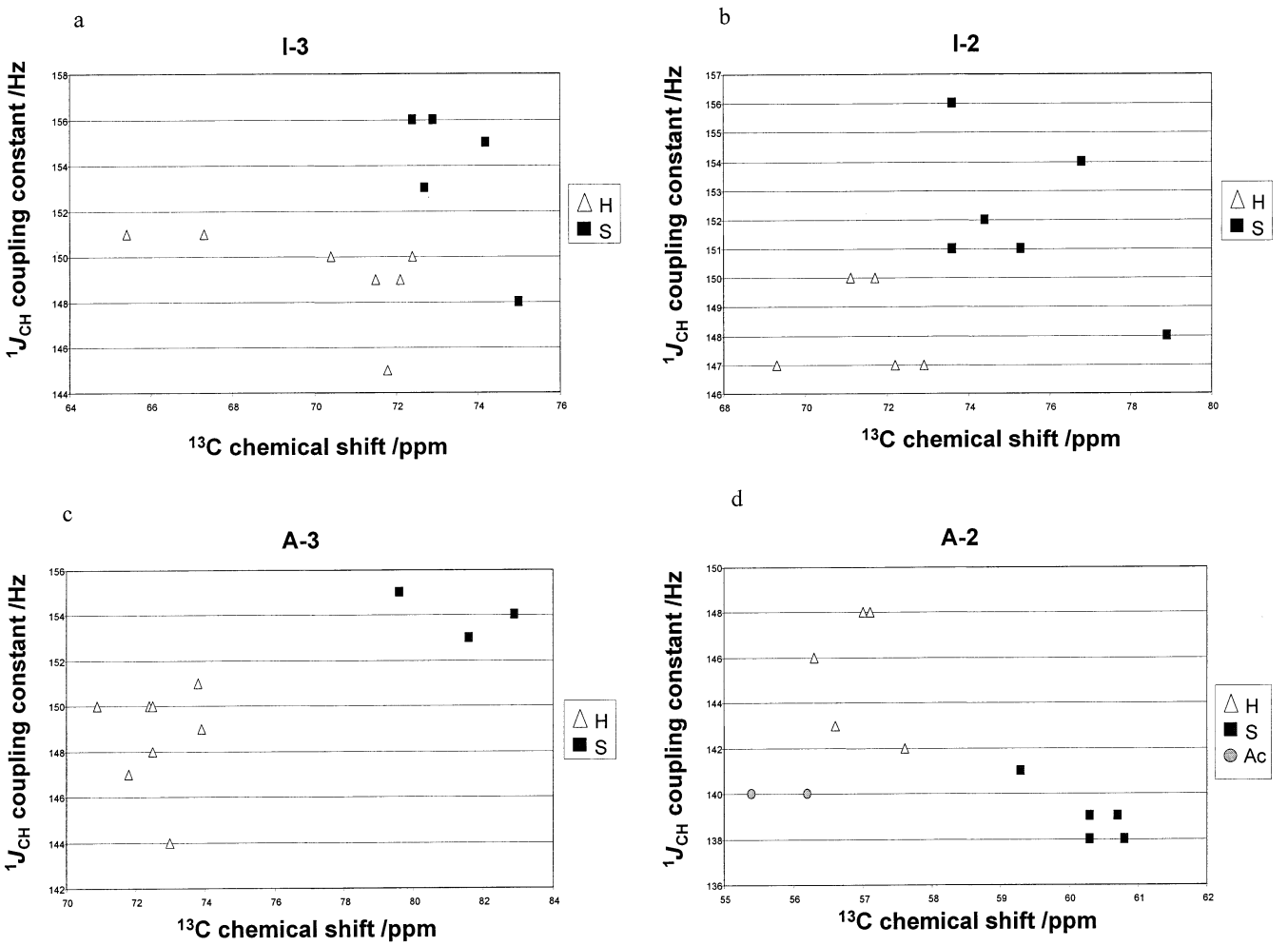

Fig. 1. Plots of $^1J_{CH}$ coupling constants (**1**–**12**) against ^{13}C for; (a) I-3, (b) I-2, (c) A-3 and (d) A-2 and ^{1}H chemical shifts for positions; (e) I-3 (f) I-2 (g) A-3 (h) A-2.

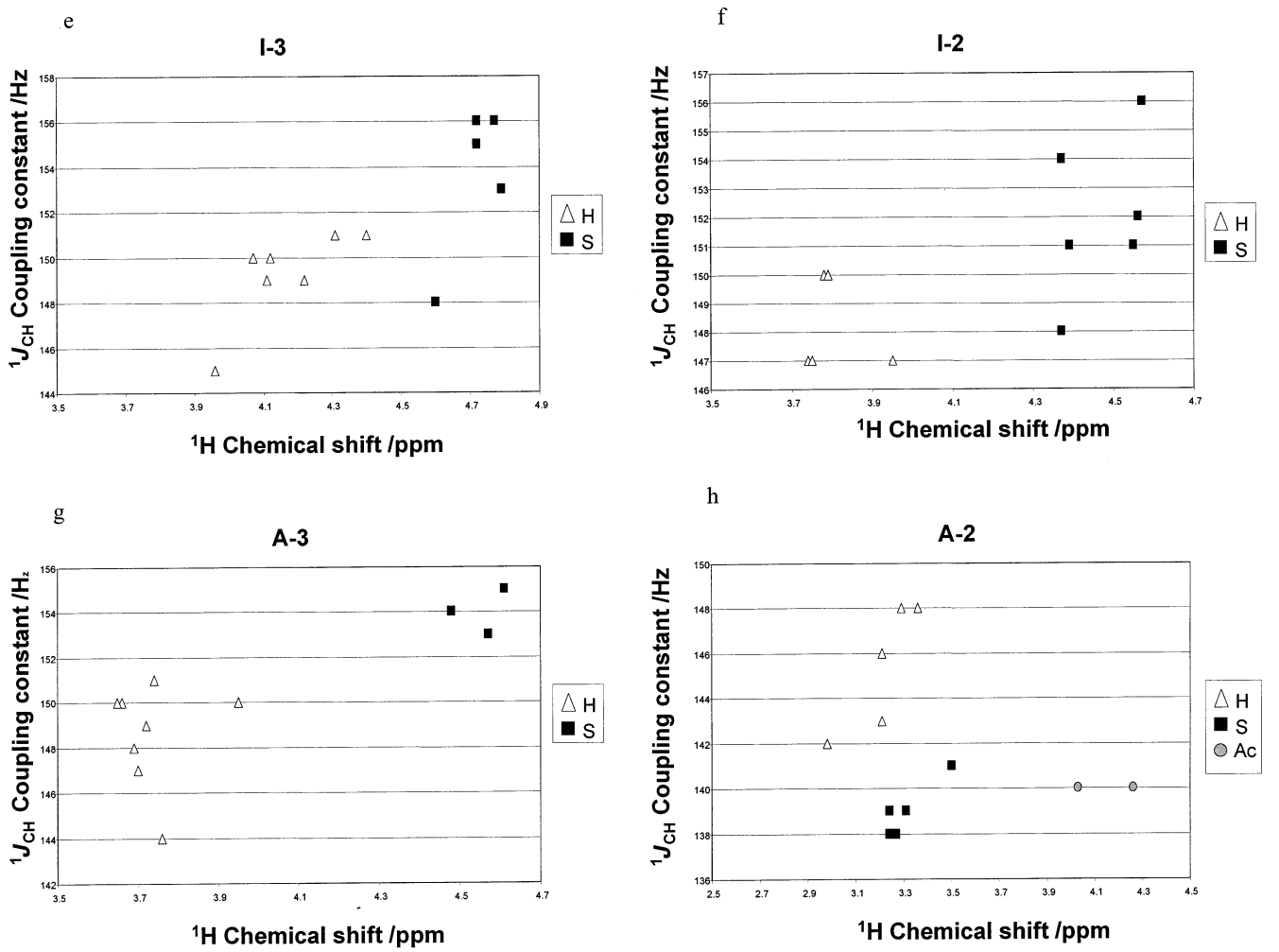

Fig. 1. (*Continued*)

Table 6
$^1J_{CH}$ changes (in Hz) for atoms at the glycosidic linkage positions upon the introduction of sulfate groups in selected positions (I-3 and A-2)

Compounds	**6** to **1**	**6** to **5**	**1** to **4**	**5** to **4**	**9** to **11**	**5** to **11**	**6** to **9**
I-1	2	3	5	4	3	0	6
A-4	3	9	6	0	2	7	0
A-1	4	4	1	1	6	3	7
I-4	2	0	1	3	4	1	3

amino at A-2 in glucosamine. Comparing **11** (intact heparin) with its de N-sulfated counterpart **9**, suggests that a factor contributing to the lack of correlation between changes in some chemical shifts with $^1J_{CH}$ coupling constants apparent (from Fig. 1(b,f, and a,e)) may be the effect of interactions between residues. In this case, the effect of de N-sulfation at A-2 results in large changes in ^{13}C chemical shifts at position I-3 (72.1–65.4 ppm), significant changes in ^{1}H shifts (4.22–4.40 ppm), but little change in the value of the $^1J_{CH}$ coupling constant (149–151 Hz, Fig. 1(a and e)).

Comparison of the $^1J_{CH}$ values at the anomeric positions (Tables 5 and 6) revealed their sensitivity towards certain substitution patterns [13]. In particular, N-sulfation (comparing **6** with **5**, and **1** with **4**) caused changes

in the $^1J_{CH}$ coupling constants at I-1 and A-4, but also to a lesser extent in A-1 and I-4. The majority of the compounds used in this study were O-sulfated at position A-6, but we also measured two compounds, **8** and **12**, possessing free hydroxyl groups at A-6 (Table 4). Compound **8** showed little variation in the $^1J_{CH}$ couplings for positions I-3, I-2, A-3 and A-2 when compared with its A-6 O-sulfated analogue **5**, whereas the values for **12** showed marked differences, especially at I-3 (5 Hz) and A-2 (6 Hz) when compared with its A-6 free hydroxyl derivative **6**. These observations indicate that A-6 sulfation can also have a similar marked influence on the $^1J_{CH}$ coupling constant value at remote positions.

Such a strong influence of negatively charged groups at distant nuclei (e.g., changes to $^1J_{C\text{-}4\text{–}H\text{-}4}$ in the substituted unit, but also in the values of $^1J_{C\text{-}1\text{–}H\text{-}1}$ in the neighbouring unit) is rather surprising and a possible explanation of such effects is variation in the glycosidic linkage conformation [13,14,16,19]. This also applies to **9** and **11** where free amino is replaced by *N*-sulfate in the case of I-3 sulfation (**6** and **1**) with the largest change in $^1J_{CH}$ values occurring in the glucosamine residues. Theoretical analysis of $^1J_{CH}$ in oligosaccharides showed that these values depend upon the dihedral angles ϕ and ψ. The difference in magnitudes were up to 10 Hz, depending on the type of linkage. The variation of $^1J_{CH}$ is the consequence of stereoelectronic effects and therefore depends on C–H bond lengths [14–16]. Similar effects have also been observed in monosaccharides where $^1J_{CH}$ varied as a function of furanose ring conformation [17–19].

Furthermore, the conformations of the iduronate residues of a number of derivatives were investigated by measurement of $^3J_{I\text{-}1,I\text{-}2}$ and $^3J_{I\text{-}4,I\text{-}5}$ coupling constants. These are shown in Table 7 together with an estimation of the corresponding conformations. These indicate different populations of chair and skew- boat conformers in various derivatives [20]. Comparisons of **5** with **11** and **9** with **11** reveal changes in the population of iduronate conformers as a result of different substitution patterns which, together with chemical shift [10] and $^1J_{CH}$ changes consistent with altered glycosidic linkage geometry indicate complex conformational effects.

It is clear that the addition or removal of *O*- and *N*-sulfate groups from certain positions within the heparin molecule can have considerable effects, which are apparent from changes in NMR spectral parameters [10,21,22], not only at these positions but also at other more remote positions. These effects seem to be complex, but are consistent with conformational changes in both the iduronate ring and in the glycosidic linkages. Such changes could have implications for the structure–activity relationships of heparin and related molecules.

Table 7
$^3J_{HH}$ coupling constants (values in Hz) in iduronate for selected compounds

Compound	$^3J_{H\text{-}1\text{–}H\text{-}2}$	$^3J_{H\text{-}4\text{–}H\text{-}5}$	Conformer population ($^1C_4$:$^2S_0$)
3	<2	<2	95:5
4	<2	<2	95:5
5	2.5	2.5	85:15
8	1.9	2.4	95:5
9	2.5	2.9	85:15
11	3.0	3.6	75:25

3. Experimental

The preparation of **5** and **6** to **12** have been described elsewhere [10].

1 *I-3 and A-6* O-*sulfated heparin*.—De O/N-sulfated heparin was prepared according to the method described in Ref. [23]. Typically, 100 mg of this product was converted to the acid form on cation (Dowex 50 W, H^+ form) exchange resin, neutralised with a solution of tetrabutylammonium hydroxide and dried. This was dissolved in DMF (5 mL) to which was added pyridine sulfur trioxide complex (165 mg, 1.04 mmol) and the reaction was maintained at 55 °C for 16 h. At the end of this period, the reaction was cooled and adjusted to pH 9 with NaOH (2 M aq solution) and the polysaccharide products were recovered and purified as described.

2 *I-2, I-3, A-3 and A-6* O-*sulfated heparin*.—De O/N-sulfated, re-N-sulfated heparin (100 mg) was converted into the tetrabutylammonium salt, dried and dissolved in DMF (2.5 mL). Pyridine sulfur trioxide complex (35 mg, 12 equiv per disaccharide unit) were added and the solution stirred (55 °C, 18 h). The reaction was cooled and adjusted (pH 9) with NaOH (2 M aq. solution) and the polysaccharide products recovered and purified as described.

3 *I-2, I-3, A-3 and A-6* O-*sulfated, N-sulfated heparin*.—Compound **2**, (which can also be prepared by an alternative procedure [4] although this leads to some depolymerisation) was prepared as follows: De N/O-sulfated heparin [24] (typically 200 mg, Na^+ salt form) was subjected to re-N-sulfation essentially as described [24], but with the precaution of stirring (45 °C, 18 h) in order to avoid a ring closure reaction previously described in Ref. [20], which also occurs in basic conditions under strong heating. The polysaccharide products were recovered and purified as described.

4 *I-3 and A-6* O-*sulfated, N-sulfated heparin*.—Compound **1**, (typically 200 mg, Na^+ salt form) was subjected to re-N-sulfation essentially as described [24]. After the reaction period was complete, the polysaccharide products were recovered and purified as described.

Purification and preparation of compounds for NMR experiments.—In order to simplify the analysis, experiments were carried out on samples in which D-glucuronate was minimal and complicating factors such as variable temperature, the presence of divalent cations and multiple monovalent cations were minimised by standardised sample preparation and experimental conditions [10].

NMR experimental procedure.—The ^1H (COSY and HSQC) spectra were obtained at 500 MHz with a Bruker AMX500 spectrometer equipped with a 5-mm $^1H/X$ inverse probe. ^{13}C spectra were obtained at 100 MHz employing a Bruker AM400 instrument equipped with a 10-mm broad band probe. Chemical shift values were recorded downfield from trimethylsilyl propionate sodium salt (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 proton decoupling. $^3J_{HH}$ coupling constants were determined from ^1H spectra with a precision of 0.2 Hz. Based on these values, the conformer population in the iduronate ring were determined using a program applying the approach previously described [20]. Proton spectra were assigned with the use of double quantum filtered COSY spectra. Data were acquired using 8 scans per series in $1K \geq 512W$ data points with zero filling in F1 and a squared cosine function was applied before Fourier transformation. $^1H/^{13}C$ chemical shift correlations (HSQC) were obtained with and without decoupling during the acquisition period in phase sensitivity-enhanced pure-absorption mode [25]. The matrix size $1K \times 256$ data points was zero filled to $4K \times 2K$ by application of a squared cosine function prior to Fourier transformation. Heteronuclear coupling constants were determined from the HSQC spectra (± 1 Hz).

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