Carbohydrate Research, 138 (1985) 199–206
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

N.M.R. STUDIES OF THE DISULPHATED DISACCHARIDE OBTAINED BY DEGRADATION OF BOVINE LUNG HEPARIN WITH NITROUS ACID*

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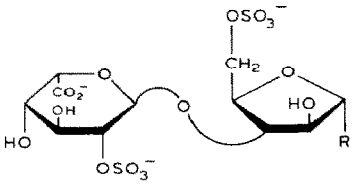
(Received July 2nd, 1984; accepted for publication, September 17th, 1984)

ABSTRACT

The disulphated disaccharide IdoA($2SO_3$)–anManOH($6SO_3$) was prepared from bovine lung heparin by treatment with nitrous acid followed by borohydride reduction. The ^{1}H- (400 MHz) and ^{13}C-n.m.r. (100 MHz) spectra of this disaccharide derivative have been assigned completely using homonuclear spin-decoupling experiments, ^{13}C–^{1}H correlations, and a COSY-45 two-dimensional homonuclear correlation experiment. The $^3J_{H,H}$ values show that the IdoA($2SO_3$) residue exists in a single conformation throughout the temperature range 20–90°.

INTRODUCTION

The ^{13}C-n.m.r. spectra of heparins from bovine lung typically contain eleven major resonances in the range of chemical shifts δ 50–110, which may be assigned[1] to the carbon atoms (excluding the carbonyl) of the major IdoA($2SO_3$)–GlcNSO_3($6SO_3$) disaccharide repeating-unit. The small resonances observed throughout the spectrum indicate the presence of other minor but important com-



1 R = CH_2OH
2 R = CHO

*Presented at the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1–7 July, 1984.
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0008-6215/85/$ 03.30

ponents in the polymer. It has been assumed from n.m.r. investigations[2] and from X-ray studies[3] that the conformation of the sulphated α-L-iduronate residue is $^1C_4$, possibly slightly distorted; the *N*-sulphated 2-amino-2-deoxy-D-glucosyl residue is in the $^4C_1$ chair form.

We now report a ^{1}H- and ^{13}C-n.m.r. study of the disulphated disaccharide derivative produced by degradation of bovine lung heparin with nitrous acid followed by reduction with borohydride.

EXPERIMENTAL

Materials. — Heparin from bovine lung was supplied by Dr. W. E. Lewis (formerly of Glaxo Operations, Runcorn, Cheshire). Sephadex G-50 (superfine grade, Pharmacia), Amberlite MB-3 resin (B.D.H.), DE-52 resin (Whatman), ^2H_2O (>99.8 atom % ^{2}H) for routine n.m.r. use (Nuclear Magnetic Resonance Ltd., High Wycombe, Bucks.), and ^2H_2O (100.0 atom % ^{2}H) for high-field ^{1}H-n.m.r. studies (Aldrich) were commercial products.

Degradation of heparin. — To a solution of bovine lung heparin (5 g) in water (50 mL) were added M citric acid (200 mL) and 2M $NaNO_2$ (25 mL). All solutions were maintained at 10°. The solution was stirred for 4 min, and the reaction was then stopped by the addition of aqueous ammonium sulphamate (0.14 g/mL; 50 mL). Terminal anhydromannose residues were reduced overnight at pH ~8 with KB^3H_4 (12 mCi), followed by a further overnight treatment with excess of $NaBH_4$.

The resulting mixture of reduced oligosaccharides was recovered by the addition of ethanol (4 vol.) followed by centrifugation. The supernatant solution was decanted off, leaving a viscous uronic acid-containing layer which was applied to a column (38 × 4.3 cm) of DE-52 resin pre-equilibrated with 0.05M ammonium acetate, and eluted with 0.05M ammonium acetate (3 column volumes) followed by 2M ammonium acetate (3 column volumes), at 48 mL/h. The fractions containing uronic acid-type material that bound to the resin in 0.05M ammonium acetate, and which eluted in 2M ammonium acetate, were concentrated and then applied to a column (169 × 2.6 cm) of Sephadex G-50 which was eluted with 0.2M ammonium hydrogencarbonate at 15 mL/h. The fractions containing the material eluted in the range K_{av} 0.25–0.78 were combined and freeze-dried. The resulting mixture of reduced oligosaccharides was fractionated in 6 aliquots on the same column. Disaccharide derivatives, which were eluted in the range K_{av} 0.79–0.88, were recovered. Bovine serum albumin and Cl^- were used as V_0 and V_t markers, respectively.

N.m.r. spectroscopy. — Preliminary ^{13}C-n.m.r. spectra (25.05 MHz, 60°) were obtained with a JEOL FX-100 spectrometer equipped with a 10-mm variable-temperature probe, and high-field spectra (^{13}C, 100 MHz, 60°; ^{1}H, 400.13 MHz, 20°, 60°, and 90°) were determined with a Bruker WH 400 instrument, using 5-mm variable-temperature probes.

The sample (~50 mg) was converted into the sodium form by passage through a column of Amberlite MB-3 (10 mL) at 7°, followed by titration to pH 6–7 with M NaOH. The solution was then buffered to pH 7 with phosphate and exchanged several times with ^2H_2O; the product was finally dissolved in ^2H_2O (0.5 mL for 5-mm probes; 1.1 mL for the 10-mm probe), using sodium 3-trimethylsilyl[$^2H_4$]propionate (TSP-d_4) as internal reference[4] for both ^{13}C and ^1H.

RESULTS

A 100-MHz ^{13}C-n.m.r. spectrum for the disaccharide **1** has already been published[5] and provides clear evidence that it is disulphated. There was one resonance for $CH_2OSO_3^-$ at δ 70.85 [anManOH(6SO_3) C-6] and one for CH_2OH at δ 63.95 [anManOH(6SO_3) C-1]. A ring-carbon resonance at δ 78.18 may be assigned to C-2 in IdoA(2SO_3) (see below). It is apparent that the reaction conditions for the degradation with nitrous acid did not cause significant hydrolysis of *O*-sulphate and that the subsequent borohydride reduction of the anhydromannose derivative **2** to **1** proceeded to completion. The homogeneity of **1** was further demonstrated by the fact that it migrated as a single peak in electrophoresis.

Partial assignments of the resonances in the ^{13}C-n.m.r. spectrum of **2** have been published[6,7], but a detailed analysis of the resonances for the sulphated iduronate moiety and definitive assignments for **1** have not been reported.

The ^{13}C resonances at δ 102.11 and 178.17 for **1** are readily assigned to C-1 and C=O, respectively, of the IdoA(2SO_3) moiety. The remainder of the ^{13}C signals can be assigned by means of ^{13}C–^1H correlations, in conjunction with homonuclear spin-decoupling experiments and a COSY-45 two-dimensional homonuclear correlation experiment[8]. Single-frequency, off-resonance decoupled, ^{13}C-n.m.r. spectra can be employed to determine accurately the chemical shifts of the signals of the attached protons[9]. When the decoupler position is offset relative to that of a specific proton, a residual multiplet of magnitude J_r (Hz) is observed at the appropriate carbon resonance position (d for CH, t for CH_2). Although there is an approximately linear relationship between J_r and the offset frequency, this deviates from a simple form at higher separations as J_r approaches the full value for $J_{C,H}$. If, however, a plot of ν against $J_r/(J^2 - J_r^2)^{1/2}$ is made, where ν represents the position of the ^1H resonance decoupling-frequency, J is $^1J_{C,H}$ (174 Hz for C-1, 148 Hz for other sites), and J_r is the residual splitting (in Hz) of the ^{13}C-n.m.r. spectra, then the y-axis intercept (*i.e.*, where J_r is 0) represents the ^1H frequency at which a specific ^{13}C resonance is fully decoupled, and the linear relationship permits the ^1H shift to be calculated with high accuracy by means of a least-squares fitting routine.

The ^1H decoupler frequency was moved in steps of 100 or 200 Hz across twelve positions covering the main region of ^1H resonances; an ABm ($J_{1,1'}$ −12.4, $J_{1,2}$ 3.6, $J_{1',2}$ 5.8 Hz) corresponding to the non-equivalent protons from the CH_2OH group in anManOH(6SO_3) at δ 3.77–3.705 was excluded from the range.

The connections between individual ^{13}C resonances and ^1H decoupling sites

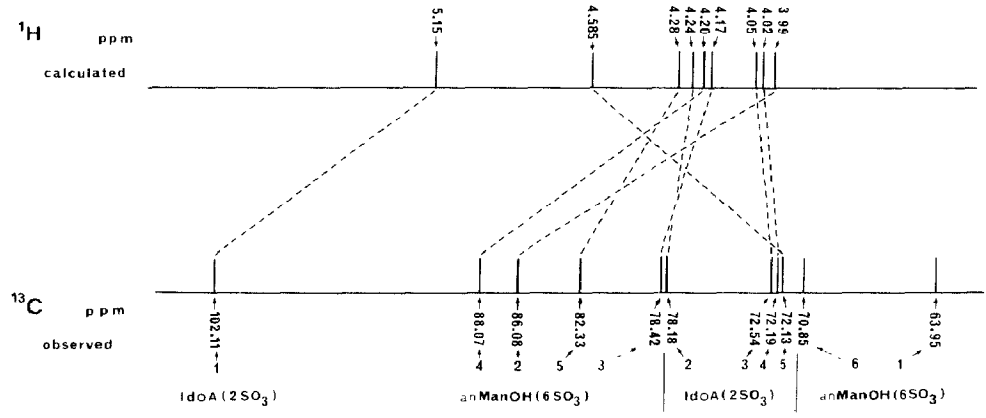

Fig. 1. Correlations between ^{13}C and ^{1}H chemical shift values for **1**. The ^{1}H values are calculated positions as described in the text; the signals for H-6,6′ (centred at δ ~4.21) and H-1,1′ (δ 3.77 and 3.705) have been omitted for clarity. Shifts for both nuclei are given from internal TSP-d_4 at 60°

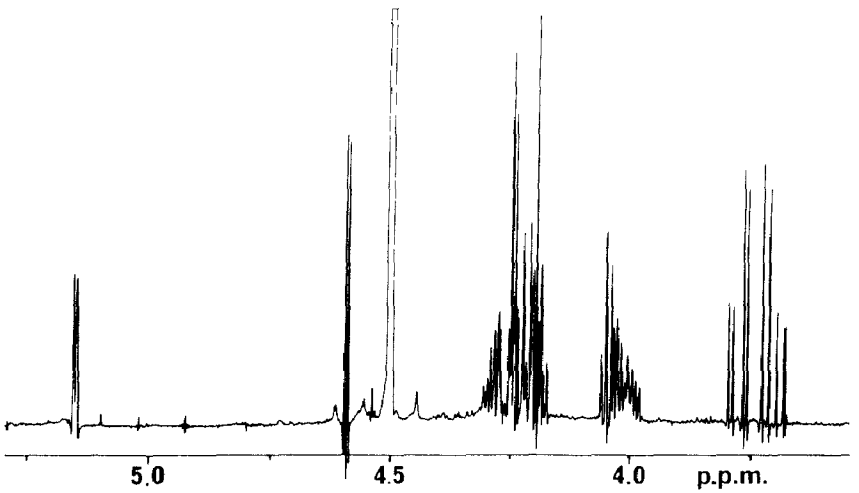

Fig. 2 ^{1}H-N.m.r. spectrum (400 MHz) for **1** at 60° The large resonance at δ ~4.5 arises from residual HOD.

are shown in Fig. 1; the ^{1}H chemical shifts are the values calculated *via* the least-squares fitting routine, and all correspond to CH protons. The triplet for the methylene carbon at δ 70.85 shows an apparent collapse corresponding with a ^{1}H position of δ 4.21. This would again be expected to possess an AB structure, and the shift represents the mean of the H-6,6′ signal positions for the $CH_2OSO_3^-$ group in anManOH(6SO$_3$). The 400-MHz ^{1}H-n.m.r. spectrum (Fig. 2) is too complex for these signals to be identified clearly, as four other proton shifts also fall within a range of 0.11 p.p.m. at this point.

Before these correlations can lead to definitive assignments for specific

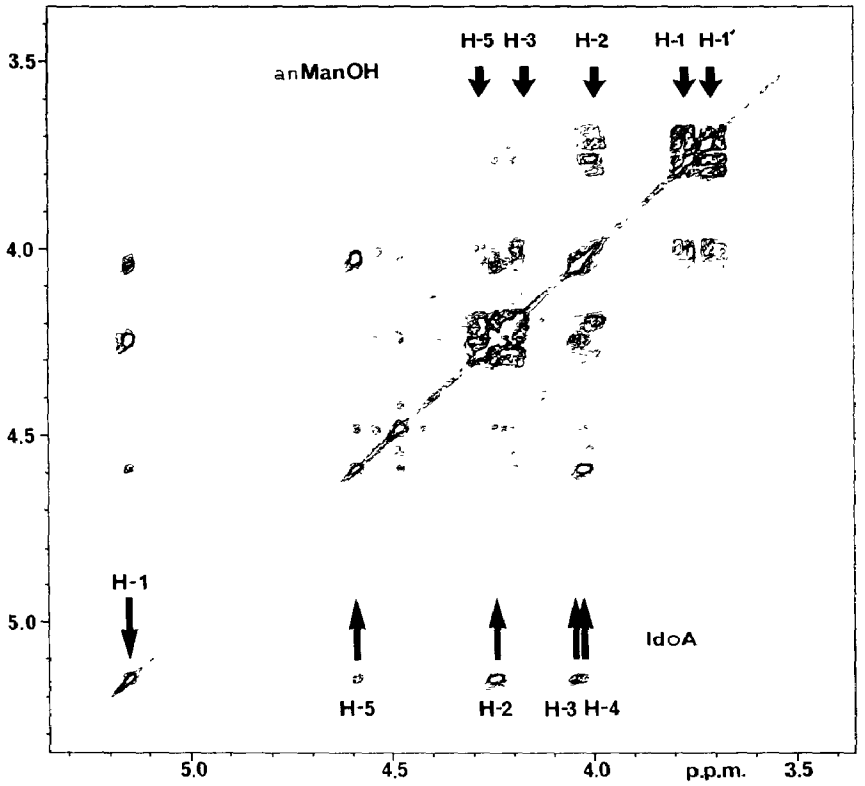

Fig. 3. COSY-45 correlation plot for **1** at 60°. The shift positions of the signals for H-4 (δ 4.20) and H-6,6′ (centred at δ ~4.21) of anManOH(6SO_3) have been omitted for clarity. Squares drawn through the off-diagonal elements indicate the connections between spin-coupled nuclei. Minor spurious correlations arose from the overlap of "tails" running out from major resonances (particularly the HOD at δ ~4.48).

carbon atoms, the ^{1}H resonances must be identified. The resonances of the sulphated iduronate moiety may be characterised by means of specific decoupling experiments. There are two clearly separated resonances at the low-field end of the ^{1}H-n.m.r. spectrum. The signal at δ 5.148 may be assigned to H-1, whereas that at δ 4.586 arises from H-5. These resonances are seen as major signals displaced downfield in the ^{1}H-n.m.r. spectrum for heparin, and in that[10] of **2**. Irradiation at δ 5.148 simplified the spectrum at δ 4.24; conversely, irradiation at δ 4.24 removed the splitting (d, J ~2.6 Hz) from the signal for H-1. The resonance at δ 4.24 may therefore be assigned to H-2 in the iduronate moiety. Decoupling at δ 4.586 (H-5) simplified the complex set of lines at δ 4.027, which therefore represented H-4 in the iduronate moiety. The resonance at δ 4.042 had a marked second-order structure because of the close proximity of the signal for H-4 at δ 4.027 and was simplified when the H-2 position was irradiated. It is therefore assigned to H-3 of the iduronate moiety. A long-range coupling ($J_{1,3}$ ~0.7 Hz) disappeared when H-1 was irradiated.

The assignments for the iduronate moiety were confirmed by a COSY-45 two-dimensional n.m.r. experiment[8] shown in Fig. 3, which also demonstrated clearly the long-range couplings from H-1 to all the other proton sites around the iduronate ring (seen as off-diagonal elements in Fig. 3).

Therefore, it is possible to identify, *via* the correlation scheme in Fig. 1, the chemical shifts for all the carbon atoms of the iduronate moiety as follows: C-1,2,3,4,5 at δ 102.11, 78.18, 72.54, 72.19, and 72.13, respectively. The C-2 resonance was at a position similar to that observed for C-2 in **2** and was displaced downfield due to the presence of the sulphate ester.

For the anManOH(6SO_3) moiety, the signals for the CH_2OH and $CH_2OSO_3^-$ groups have already been assigned and the signals for C-1,6 were at δ 63.95 and 70.85, respectively. Identification of the C-5 resonance at δ 82.33 was facilitated by the observation that it was shifted downfield by ~2.3 p.p.m. due to the loss of a β-substituent effect when the adjacent C-6 methylene group was unsulphated, as seen in the spectra of heparan sulphate tetrasaccharides[11] and sulphated oligosaccharides from heparin[7], whereas the resonances of the other ring-carbon atoms were little affected by this change.

Further examination of the COSY-45 data assisted in the assignment of the other resonances of the anhydromannitol moiety. Although a spin-decoupling experiment involving H-1,1′ was not practicable, the strong off-diagonal components clearly define the resonance at δ 3.99 as arising from H-2. This in turn connects with the H-3 resonance at δ 4.17. The two ^{13}C signals at δ 86.08 and 88.07, by comparison with assignments for anhydromannose residues[7], represent C-2 and C-4, respectively, in the anhydromannitol residue; these assignments were confirmed by the COSY-45 data. The signal for C-3 was at δ 74.82, and the assignment of the signal for C-5 at δ 82.33 was confirmed by the ^{13}C–^1H correlation scheme (Fig. 1).

It is possible to identify sufficient individual transitions on a resolution-

TABLE I

BEST-FIT PARAMETERS FOR δ AND J VALUES FOR THE SULPHATED IDURONATE RESIDUE OF **1** AT 60°

Atom	*Shift, δ (p.p.m.)*	*Atoms*	*Coupling constant, J (Hz)*
H-1	5.148	H-1,H-2	2.62 ±0.03
H-2	4.243	H-1,H-3	0.75 ±0.04
H-3	4.042	H-1,H-4	0.62 ±0 05
H-4	4.027[a]	H-1,H-5	0.62 ±0.03
H-5	4.586	H-2,H-3	4.42 ±0.04
		H-2,H-4	0.49 ±0.05
		H-2,H-5	<0.2[b]
		H-3,H-4	4.19 ±0.06
		H-3,H-5	<0.2[b]
		H-4,H-5	2.67 ±0.05

[a]Calculated from ^{13}C–^1H correlations. [b]Excluded from the iterations; splittings not observable.

enhanced ^{1}H-n.m.r. spectrum for an iterative fit between observed line positions and calculated values to be performed for the sulphated iduronate residue, using the computer programme LAME. The calculated best-fit parameters for ^{1}H shifts and ^{1}H–^{1}H coupling constants are given in Table I. The shift for the signal for H-4 was excluded from the iteration process because it was not possible to identify lines for this resonance, as these are overlaid by a signal from the anhydromannitol residue. The chemical shift was therefore assumed for calculation purposes to lie at the position predicted from the ^{13}C–^{1}H correlation data. The couplings between H-4,3 and H-4,5 are adequately defined by line positions within the other signals. No attempt was made to refine the data further by investigation of sign changes for the long-range couplings, or by changes in the assumed shift value for the signal for H-4.

DISCUSSION

The assignments given above for the IdoA($2SO_3$) moiety in **1** are in general agreement with those described by Gatti *et al.*[1,2] for this residue in heparin. A full comparison is not pertinent, since this residue in heparin is glycosidically linked at C-4. Thus, for example, the chemical shift for the signal for C-4 in heparin is displaced considerably downfield relative to the position observed for the same carbon atom in **1** because of an α-substituent effect. It is of interest to compare the $J_{H,H}$ values for **1** with those reported[2] for the iduronate residue in heparin.

It is possible, using the empirical parameters derived by Altona and Haasnoot[12], to predict the main vicinal proton–proton spin–spin couplings for iduronate, by taking into account the orientations of the attached electronegative oxygen atoms relative to the pairs of coupled protons. These data, for the $^1C_4$ conformation, are summarised in Table II, together with the observed values for heparin[2] and for **1**. Predictive data for a CO_2^- substituent are not available, but examination of experimental values for $J_{4,5}$ in sulphated iduronate residues suggests that a magnitude comparable with the $J_{1,2}$ value is reasonable.

Both for heparin and **1**, there are deviations from the predicted values for a $^1C_4$ conformation of the uronate residue. Gatti *et al.*[2] were aware of the notably high $J_{2,3}$ value and postulated that the $^1C_4$ chair would be slightly distorted. These

TABLE II

PREDICTED AND OBSERVED VICINAL PROTON–PROTON $^3J_{H,H}$ VALUES (Hz) FOR SULPHATED IDURONATE RESIDUES

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
$^1C_4$	2.4	3.6	3.6	(2.4)
Heparin	2.6	5.9	3.4	3.1
1	2.62	4.42	4.19	2.67

observations could be explained if it were assumed that the H-2,3 dihedral angle was reduced from 60°, which would increase the magnitude of the coupling, whereas the H-3,4 dihedral angle was slightly increased. In **1**, the influence of the mass from the C-4 substituent is no longer present, and there are fewer conformational constraints. This would permit the distortion from the predicted $^1C_4$ conformer, which is observed for the sulphated iduronate residues in heparin, to become more evenly distributed over the C-2,3,4 region in **1**, as evidenced by deviations in the two couplings.

^{1}H-N.m.r. spectra for **1** have also been recorded at 20° and at 90°. At 20°, the resonances are significantly broader because of increased solvent viscosity and more substantial hydrogen-bonding. However, there are only minor changes in the magnitudes of spin–spin coupling constants across this temperature range. The value of $J_{1,2}$ is, for example, 2.38 Hz at 20°, 2.62 Hz at 60°, and 2.79 Hz at 90°. The $J_{2,3}$ and $J_{3,4}$ couplings remain similar in magnitude, both relative to each other and in absolute terms. Therefore, it appears that the iduronate moiety in **1** exists in a *single conformation* which is little perturbed across the whole of the accessible temperature range. This situation does not always pertain for an *unsulphated*, non-reducing, terminal iduronate residue[11].

ACKNOWLEDGMENTS

We thank the S.E.R.C. for a research assistantship, Glaxo Operations Ltd. for a C.A.S.E. studentship (to P.N.S.), the S.E.R.C. for use of their 400-MHz n.m.r. facility, and Drs. B. E. Mann and C. Spencer (Sheffield University) for assistance and valuable discussions.

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