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Note

NMR studies of a tetrasaccharide from hyaluronic acid

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Hyaluronic acid (hyaluronan) is a polydisaccharide composed of β-D-glucopyranosyluronic acid and 2-acetamido-2-deoxy-β-D-glucopyranosyl residues linked (1 → 3) and (1 → 4), respectively[1]. It is widely distributed in connective tissues and is also found in bacteria[2]. It is unique among the connective tissue glycosaminoglycans in that it is not sulfated and is not covalently attached to a protein core[3]. Its biosynthesis also differs from that of other glycosaminoglycans[4]. Protein-binding sites for high-molecular-weight hyaluronan have been identified, and putative functions for hyaluronan have been advanced which hinge on its specific binding properties[5]. To further our understanding of this widely distributed and important biopolymer, especially its roles as a "space filler" and lubricant[5], information bearing on its solution conformation is needed.

Welti et al.[6] noted anomalous effects in the 300-MHz NMR spectra of hyaluronan on changing the solution from neutral to one in 0.4 M NaOH. Their results suggested that the drastically alkaline conditions, which were known from previous studies[7,8] to cause a decrease in the stiffness of the polymer, disrupted an interresidue hydrogen-bonding network. X-ray diffraction studies[9,10] shed light on the problem of hyaluronan conformation. Hyaluronan may exist in several polymorphic forms in the solid state, resulting from different conditions such as humidity and temperature used in sample preparation. Extended (*h* 9.5 Å) two-, three-, and four-fold helical forms are known, as are "compact", (*h* 8.5 Å) four-fold helical forms. In addition, the manner in which the helical strands pack three-dimensionally is quite variable and may involve considerable interstrand contacts. The most common forms of sodium hyaluronate are a fully extended, three-fold helix, and a compressed four-fold helix. In these forms, interresidue hydrogen bonds presumably stabilize the glycosidic linkages toward geometric

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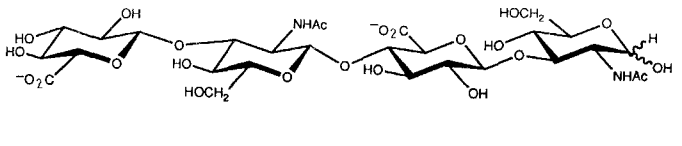

GlcA-4 GlcNAc-3 GlcA-2 GlcNAc-1

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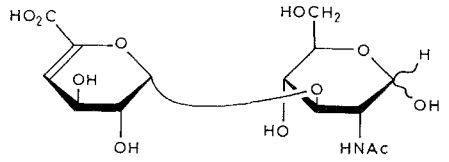

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distortion. It was, therefore, not unreasonable to propose that such hydrogen bonds are preserved in solution and that the effect of alkali was to disrupt these hydrogen bonds. Intermolecular association of short segments of hyaluronan in 0.15 M NaCl solution have been reported[11]. Circular dichroism studies on hyaluronan suggested structure in aqueous solution[12], and a double-stranded helical structure in solutions of low-dielectric constants[13].

Scott and coworkers focused their attention on the NH proton NMR signals to determine interresidue hydrogen-bond network in fragments of hyaluronan; in order to detect protons that are exchangeable, Me_2SO was used as the solvent in earlier studies[14–16]. The question of possible solvent-dependent conformational effects has been probed by Cowman[17] and by Scott et al.[15] by examining the NH protons of hyaluronan in water. The orientation of the acetamido group was found to change from a Me_2SO to a water solution.

As part of a continuing interest on the characterization of proteoglycans[18,19], we have initiated a study of the hyaluranon fragments, and report herein a study of the tetrasaccharide β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-GlcNAc (**1**), derived from hyaluronan by digestion with bovine testicular hyaluronidase. The primary focus of this investigation was the sequence-specific assignment of ^1H and ^{13}C resonances from the individual sugar units of this oligomer in aqueous solution.

Assignment of nonexchangeable protons.—Of crucial importance in the determination of the structure of an unknown oligosaccharide are the signals due to the anomeric protons and other "reporter groups", which fall in regions of the spectrum free from interference from other proton resonances[20]. In the present case, however, the chemical structure of the tetrasaccharide is not in doubt. Therefore, the anomeric region was expected to be straightforward, a doublet for the α anomer of GlcNAc-*1*, one for the β anomer, and one each for GlcA-*2*,

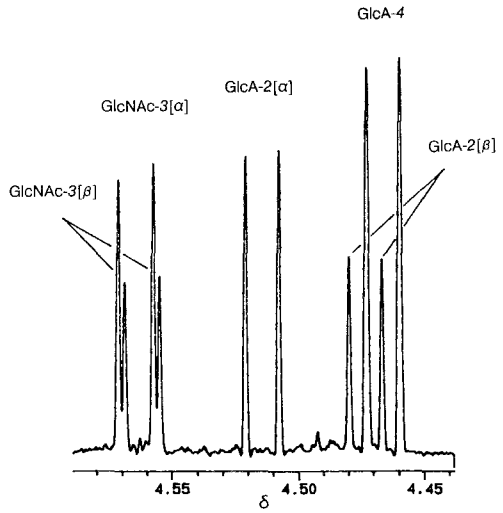


Fig. 1. Anomeric region in the ^{1}H NMR spectrum of tetrasaccharide **1** (4.0 mM in D_2O at 600 MHz). The doublets due to GlcNAC-*1α* at δ 5.155 (*J* 3.50 Hz), and GlcNAc-*1β*, at δ 4.715 (*J* 8.46 Hz), are omitted. The bracketed α or β indicate the anomeric configuration of the reducing GlcNAc-*1* residue.

GlcNAc-*3*, and GlcA-*4*. However, the anomeric region consists of seven doublets (five of which are shown in Fig. 1). The assignments from Fig. 1 were made from a consideration of the COSY experiment and by noting that the α-to-β ratio of 1.6:1 for GlcNAc-*1* (the literature value[21] for GlcNAc is 2.1 at 40°C) is approximately repeated in some pairs of doublets. It is particularly striking that the configuration at C-1 of the reducing end residue influences markedly the chemical shift of H-1 of the third sugar residue, eleven bonds away. In fact, if the spectrum is very strongly resolution-enhanced, shoulders on the GlcA-*4* doublet may be seen, indicating that the orientation of H-1 of GlcNAc-*1* is being "sensed" 15 bonds away at H-1 of GlcA-*4*. The effect appears to fall off with distance; the chemical shift difference at H-1 is 0.440, 0.041, 0.002, and 0 ppm for GlcNAc-*1*, GlcA-*2*, GlcNAc-*3*, and GlcA-*4*, respectively. Table I lists the chemical shifts and coupling constants for compound **1**. Despite differences in the sample pH and temperature, our assignments for the two central residues are in good agreement with those reported by Welti et al.[6] for low-molecular-weight hyaluronan.

It is seen that configuration at C-1 of the reducing end residue measurably affects protons as far away as H-2 of GlcNAc-*3*. Vliegenthart et al.[20] reported NMR data of several oligosaccharides in which the remote chemical shift effects of the configuration at C-1 of the GlcNAc reducing end residue α-to-β ratio could be observed. For example, in α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-GlcNAc, H-2 of the terminal D-mannosyl group shows Δδ, 0.004 ppm, but H-1 of this residue is not affected. Remarkably, in di-, tri-, and tetra-antennary oligosaccharides[20], the residues of the (1 → 3) branch were more shift-sensitive to the α-to-β ratio of the

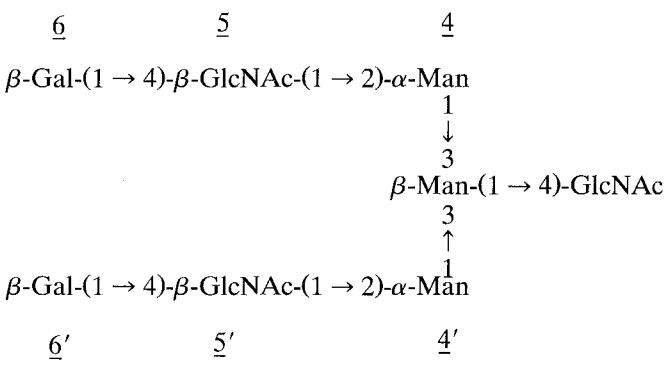
TABLE I

^{1}H NMR spectrum of tetrasaccharide **1** [a]

Unit	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6a}$)	H-6a ($J_{6a,6b}$)	H-6b ($J_{5,6b}$)
GlcNAc-*1α*	5.154 (3.50)	4.044 (10.60)	3.900 (8.86)	3.560 (10.03)	3.488 [b] (2.21)	3.922 (12.51)	3.780 (4.02)
GlcNAc-*1β*	4.715 (8.44)	3.814 (10.42)	3.721 [b] (8.25)	3.526 (9.94)	3.474 (2.26)	3.890 (11.04)	3.751 (4.41)
ClcA-*2*[α]	4.540 (7.90)	3.368 [c] (9.62)	3.609 (8.42)	3.772 (9.71)	3.803 [c]		
GlcA-*2*[β]	4.495 (7.88)		3.603 (8.16)	3.768 (9.78)			
GlcNAc-*3*[α]	4.567 (8.50)	3.857 (10.2)					
GlcNA3-*3*[β]	4.564 (8.52)	3.854 (10.4)	3.721 [b,c] (8.25)	3.560 [c] (10.03)	3.488 [b,c] (2.21)	3.922 [b,c] (12.51)	3.780 [b,c] (4.02)
GlcA-*4*	4.495 [b] (7.88)	3.332 (9.30)	3.52 [d]	3.52 [d]	3.829 (9.74)		

[a] For a 2.0 mM solution in D_2O at 600 MHz; chemical shifts in δ values and coupling constants in Hz. [b] Signals within a column labeled with a superscript b were exactly coincident. [c] Separate signals corresponding to GlcNAc-*1α* and GlcNAc-*1β* could not be discerned. [d] Complex multiplet.

reducing end than were the corresponding residues on the (1 → 6) branch. For example, in **3**, H-1 of Gal-*6* showed $\Delta\delta$ 0 and H-1 of Gal-*6′* $\Delta\delta$ 0.002 ppm.



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Apparently, the chemical-shirt sensitivity of a particular proton to a remote α-to-β anomer ratio is not related in any obvious way to the "distance" between the proton and the anomeric center, if one takes "distance" to mean the number of intervening bonds. Whereas previous reports have dealt with reporter groups only, the present study has found anomer-dependent chemical shift effects in the remaining protons as well, as shown by COSY, COSY-45, relayed COSY, and double-relay COSY experiments (Table I).

The acetyl methyl proton signals of **1** are observed as a series of four singlets at δ 2.033, 2.031, 2.019, and 2.017 (Fig. 2). The ratio of intensity of the larger to that

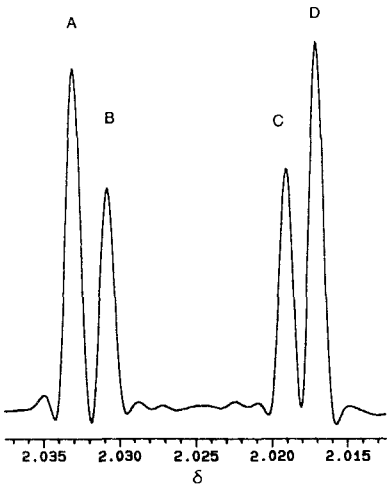

Fig. 2. Acetyl methyl protons in the ^{1}H NMR spectrum of tetrasaccharide **1** (4.0 mM in D_2O at 289 K).

of the smaller peaks is essentially the same as the α-to-β ratio. For GlcNAc itself (61 mM in D_2O), two methyl proton singlets are observed at δ 2.054 and 2.052. The magnitude of this separation (0.002 ppm) suggested that in **1**, A and B (Fig. 2) arose from one GlcNAc and C and D from the other. The chemical shifts of the *N*-acetyl methyl protons of β-Gal-(1 → 3)-β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc (δ 2.025)[21], of α-NeuAc-(2 → 3)-β-Gal-(1 → 3)-β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc (δ2.030)[22], and β-Gal-(1 → 3)-β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc (δ2.029)[23], all containing (1 → 3)-linked GlcNAc, suggested that the δ 2.033/2.031 signals of **1** should be assigned to GlcNAc-*3* (and by default δ 2.019/2.017 signals to GlcNAc-*1*). Although not exactly analogous, the observation that the *N*-acetyl methyl proton resonance (at δ 2.039) of the reducing terminal GlcNAc is upfield of the *N*-acetyl methyl resonance (δ 2.074)[24] of the internal GlcNAc of **4** lends support to our assignment. In fact, a very strong piece of evidence in favor of our assignment is our finding that the *N*-acetyl methyl proton signals of **2**, a compound which accurately models GlcNAc-*1* of **1**, fall at δ 2.066 (smaller) and 2.062 (larger).

$$\begin{array}{c} \alpha\text{-Man} \\ 1 \\ \downarrow \\ 6 \\ \alpha\text{-Man-(1} \rightarrow 3)\text{-}\beta\text{-Man-(1} \rightarrow 4)\text{-}\beta\text{-GlcNAc-(1} \rightarrow 4)\text{-GlcNAc} \\ 2 \\ \uparrow \\ 1 \\ \beta\text{-Xyl} \end{array}$$

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The D-glucuronic acid H-2 signals of **1** were well separated from the bulk of proton signals (see Fig. 3). The multiplet centered at δ 3.366 is an unremarkable

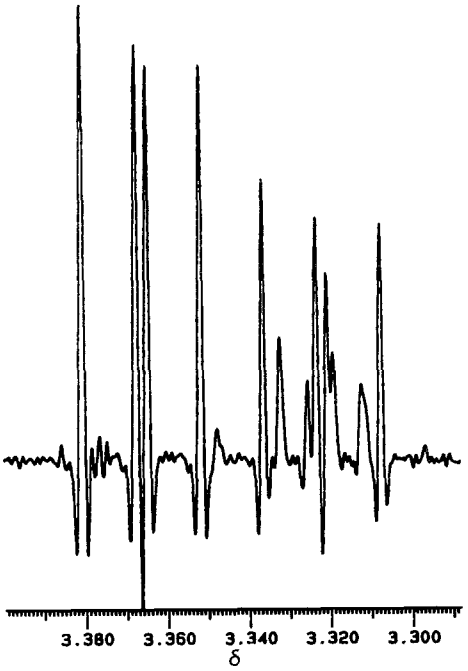

Fig. 3. Glucuronic acid H-2 region in the ^{1}H NMR spectrum of tetrasaccharide **1** (4.0 mM in D_2O at 600 MHz).

doublet of doublets ($J_{1,2}$ 7.87, $J_{2,3}$ 9.52 Hz) due to the H-2 of GlcA-*2* in the $^4C_1$(D) conformation. By contrast, the H-2 of GlcA-*4* gave a somewhat more complicated spectrum. It appeared as two doublets of doublets, both centered at δ 3.322. The major component had $J_{1,2}$ 7.88 and $J_{2,3}$ 9.42 Hz and, thus, is presumably similar in conformation to GlcA-*2*, viz., $^4C_1$(D). The minor component had J 7.23 and $\sim$6.5 Hz. In the spectrum of the sample at a concentration of 4.0 mM, the minor component had J 8.02 and 3.97 Hz (data not shown). Furthermore, GlcA-*4* H-2 shows two crosspeaks in the COSY map, labelled X and Y in Fig. 4. This experiment included extra-fixed delays of 0.25 s to aid in the detection of long-range couplings[25]. Crosspeak Y moved as a function of concentration, appearing at δ 3.83 for the sample at 2.0 mM and at δ 3.71 for the sample at 4.0 mM. Crosspeak X connects H-2 to H-3. Crosspeak Y may be due to long-range H-2–H-5 coupling. Long-range couplings are known to be fairly common in rigid cyclic systems such as carbohydrates. For example, a five-bond coupling, $J_{2,5}$, of 0.5 Hz has been reported[26] for 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose. The major conformer of GlcA-*4* is $^4C_1$(D) as it is for Glc-*2*. The minor conformer of GlcA-*4* might conceivably be the twist-boat, by analogy to recent investigations which implicate this form for L-iduronic acid[27–32]. However, since the minor form is introduced only when GlcA is a nonreducing terminal group, it will ultimately play no role in determining the shape of intact hyaluronan.

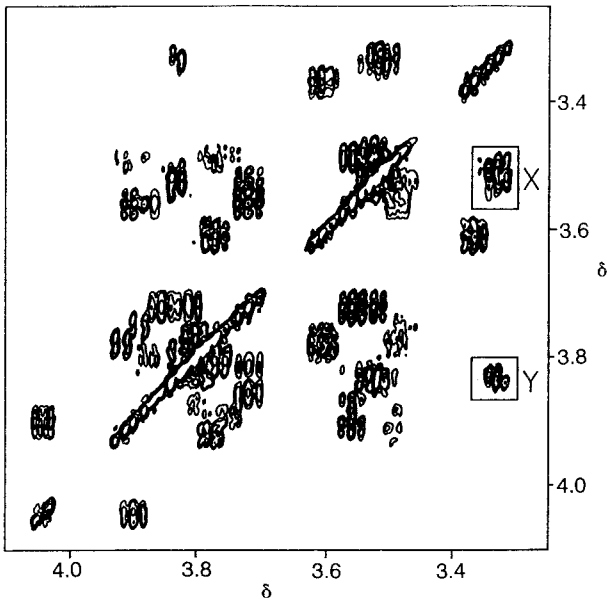

Fig. 4. Partial COSY-45 spectrum (unsymmetrized) of tetrasaccharide **1** (2.0 mM in D_2O at 600 MHz); anomeric protons are omitted. Crosspeaks X and Y are discussed in the text.

TABLE II

^{13}C NMR spectrum of tetrasaccharide **1** [a]

Chemical shift (δ)	Assignment (atom and unit)
175.12, 174.79, 174.10, 173.22, and 173.16	C=O of GlcNAc-*1* and GlcNAc-*3*; and CO_2^- of GlcA-*2* and GlcA-*4*
103.32	C-1 and GlcA-*2*[β]
103.12 and 103.07	C-1 of GlcA-*4*, GlcA-*2*[α], and GlcNAc-*3*[β]
101.09	C-1 of GlcNAc-*3*[α]
95.01	C-1 of GlcNAc-*1*[β]
91.32	C-1 of GlcNAc-*1*α
83.04	C-3 of GlcNAc-*3*
82.81	C-3 of GlcNAc-*1*β
80.38 and 80.29	C-4 and GlcA-*2* and C-3 of GlcNAc-*1*α
75.75, 75.64, 75.43, 75.36, and 75.26	C-5 and GlcNAc-*1*α, GlcNAc-*1*β, and GlcNAc-*3*; and C-3 and GlcA-*4*
73.93	C-3 of GlcA-*2*
72.83	C-2 of GlcA-*4*
72.58	C-2 of GlcA-*2*
71.69 and 71.56	C-4 of GlcA-*4*
68.74 and 68.65	C-4 of GlcNAc-*1*α and GlcNAc-*3*
60.96 and 60.76	C-6 of GlcNAc-*1* and GlcNAc-*3*
55.81	C-2 of GlcNAc-*1*β
54.48	C-2 of GlcNAc-*3*
53.16	C-2 of GlcNAc-*1*α
22.69, 22.49, and 22.24	CH_3 of GlcNAc-*1* and GlcNAc-*3*

[a] For a 5.4 mM solution in D_2O.

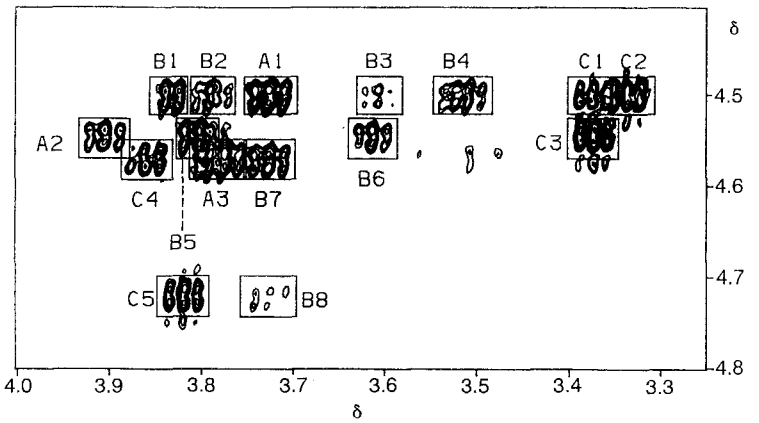

Fig. 5. Magnitude-mode, 600-MHz NOESY spectrum of the anomeric protons of tetrasaccharide **1**. Mixing time was 500 ms. Crosspeaks in this region are as follows: interresidue crosspeaks are indicated with the letter A, intraresidue crosspeaks by the letter B, and COSY-type peaks by the letter C. Specifically: A1, GlcA-*4* H-1–GlcNAc-*3* H-3; A2. GlcA-*2*[α] H-1–GlcNAc-*1*[α] H-3; A3, GlcNAc-*3* H-1–GlcA-*2* H-4; B1, GlcA-*4* H-1–GlcA-*4* H-5; B2, unidentified; B3, GlcA-*2*[β] H-1–GlcA-*2*[β] H-3; B4; GlcA-*4* H-1–GlcA-*4* H-3 + H-4; B5, GlcA-*2*[α] H-1–GlcA-*2*[α] H-5; B6, GlcA-*2*[α] H-1–GlcA-*2*[α] H-3; B7, GlcNAc-*3* H-1–GlcNAc-*3* H-3; C1, GlcA-*2*[β] H-1–GlcA-*2*[β] H-2; C2, GlcA-*4* H-1–GlcA-*4* H-2; C3, GlcA-*2*[α] H-1–GlcA-*2*[α] H-2; C4, GlcNAc-*3* H-1–GlcNAc-*3* H-2; and C5, GlcNAc-*1*[β] H-1–GlcNAc-*1*[β] H-2

^{13}C Spectra at 150 MHz with composite-pulse decoupling exhibited narrow lines and a very good resolution. Assignments result from a ^{13}C–^{1}H correlation experiment and are shown in Table II. It was not possible to unambiguously assign all ^{13}C peaks owing to resonance overlap. In those instances where the proton resonances overlapped (e.g., GlcA-*2*[β] and GlcA-*4* in Table I), the respective ^{13}C resonances were identified on the basis of intensities of the correlation peaks.

In order to confirm the order of connection of the sugar residues in this molecule, a NOESY experiment was performed. Since all linkages are either (1 → 3) or (1 → 4), examination of the anomeric protons NOEs should provide connectivity information. A partial NOESY spectrum (Fig. 5) confirmed the assignments given in Table 1. In addition to confirming proton assignments, the NOESY experiment showed crosspeaks corresponding to all protons spanning glycosydic linkages, viz. GlcA-*4* H-1–GlcNAc-*3* H-3, GlcNAc-*3* H-1–GlcA-*2* H-4 and GlcA-*2* H-1–GlcNAc-*1* H-3. This implies that the geometry of each linkage is approximately as depicted in **1**, namely in accord with the exo-anomeric effect.

Assignment of NH protons.—The NH proton signals were observed with a 2.0 mM solution of tetrasaccharide in 1 : 1 (v/v) H_2O–D_2O (see Fig. 6). Assignments resulted from 1D selective decoupling experiments, so-called "underwater decoupling"[33]. The NH–CH coupling constants range from 9.4–9.7 Hz (Table III) indicated an average H–N–C–H dihedral angle of near 180° according to an equation relating $J_{NH,CH}$ to a dihedral angle[34].

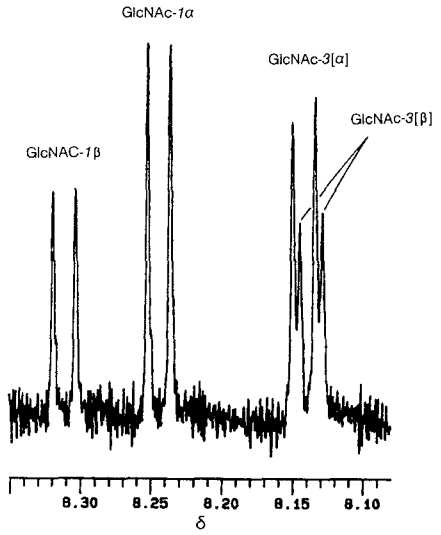

Fig. 6. NH resonance in the ^1H NMR spectrum of tetrasaccharide **1** [2.0 mM in 1:1 (v/v) H_2O–D_2O]. The bracketed α or β indicate the anomeric configuration of the reducing GlcNAc-*1* residue.

EXPERIMENTAL

D-Glucuronic acid was obtained from J.T. Baker Chemical Co., (Phillipsburg, NJ), and 2-acetamido-2-deoxy-D-glucose from Pfanstiehl Laboratories, Inc. (Waukegan, IL). The disaccharide *O*-(4-deoxy-D-*threo*-hex-4-enopyranosyluronic acid)-(1 → 3)-D-glucose (**2**) was purchased from Seikagaku America, Inc. (Rockville, MD). All the commercial products were used as received. The tetrasaccharide **1** was prepared as previously described[35]. Compound **1** was dissolved in D_2O and then lyophilized. This process was repeated several times, and finally the solid was dissolved in 99.96 atom% D_2O (MSD Isotopes). To observe the acetamido NH signal, a sample in 1:1 (v/v) H_2O–D_2O was also prepared. The solution pH was 3.27. In all spectra, acetone was included as a chemical shift standard (δ 2.225)[36]. ^1H NMR spectra were obtained with a Bruker WH-400 (with an Aspect 3000 computer) and a Bruker AM-600 NMR spectrometer operating at proton frequencies of 400.135 and 600.138 MHz, respectively. ^{13}C Spectra were obtained with the

TABLE III

NH resonances in the ^1H spectrum of tetrasaccharide **1** [a]

Unit	Chemical shift (δ)	$J_{NH,CH}$ (Hz)
GlcNAc-*1α*	8.244	9.43
GlcNAc-*1β*	8.311	9.62
GlcNAc-*3α*	8.142	9.71
GlcNAc-*3β*	8.137	9.69

[a] For a 2.0 mM solution in 1:1 H_2O–D_2O at 600 MHz.

AM-600 spectrometer operating at 150.905 MHz and were referenced to external 1,4-dioxane (set to δ 67.4). Concentration of tetrasaccharide **1** was 5.4 mM for ^{13}C experiments. Two sample concentrations were used for ^{1}H NMR spectroscopy, viz., 2.0 and 4.0 mM. For 1D ^{1}H spectra at 400 MHz, 32K points were acquired with a sweepwidth of 1887 Hz, then zero-filled to 128K after application of a Gaussian-weighting function. Digital resolution was 0.029 Hz/point. The same procedure applied at 600 MHz resulted in a digital resolution of 0.040 Hz/point. 1D ^{13}C spectra, obtained with a composite pulse decoupling, utilized 16K points over a sweepwidth of 41667 Hz, giving a digital resolution of 5.1 Hz/point. COSY[37] and relayed-COSY[38] experiments used 2K points in the t_2 domain and 512 t_1 increments. The data matrix was zero-filled to 4K × 2K and processed on a microVAX II computer using the FTNMR software (Hare Research, Inc., Woodinville, WA). In general, sinebell weighting functions were applied in both dimensions. Because of the large chemical shift dispersion available at 600 MHz, multiplets in each proton resonance could, in the majority of cases, be discerned clearly in the 1D spectra (guided by the COSY results), and chemical shifts and coupling constants were taken directly. For relay COSY and double-relay COSY experiments, delay times of 31.25 and 39.06 ms, respectively, were used. NOESY[39] experiments utilized a mixing time of 500 msec and were run both in magnitude mode and in phase-sensitive mode (TPPI)[40]. Solvent suppression in all cases was achieved through application of a presaturation pulse.

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