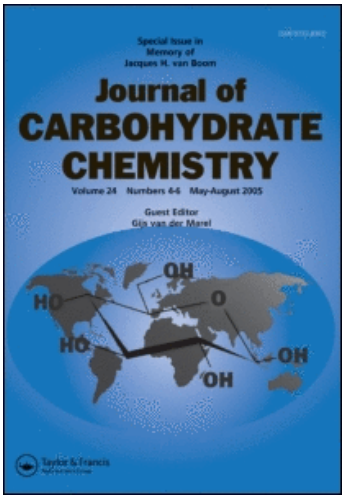

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C-2 EPIMERIZATION OF *N*-ACETYLGLUCOSAMINE IN AN OLIGOSACCHARIDE DERIVED FROM HEPARAN SULFATE

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ABSTRACT

The isolation of a heparan sulfate derived tetrasaccharide containing *N*-acetylmannosamine is described. The *N*-acetylmannosamine residue at the reducing terminus of heparin lyase derived tetrasaccharide is formed by base catalyzed C-2 epimerization of *N*-acetylglucosamine. This C-2 epimerization takes place under very mild conditions suggesting that this residue may be formed in small amounts under physiologic conditions.

INTRODUCTION

Glycosaminoglycans (GAGs) are a family of polydisperse, structurally heterogeneous, linear, anionic polysaccharides found in most animal tissues.[1,2] There are two major classes of GAGs: (1) glucosaminoglycans, heparin, heparan sulfate, keratan sulfate and hyaluronic acid and (2) galactosaminoglycans, chondroitin sulfates and dermatan sulfate. Except for hyaluronic acid, all GAGs are biosynthesized as proteoglycans (PGs) comprised of a core protein to which one or more GAG chain is attached. Some PGs, such as heparin, are biochemically processed into GAGs through the action of proteinases and

endo-β-glucuronidases.[3] Thus, both PGs and GAGs are a natural component of most animal tissues.

Glycosaminoglycans are commercially prepared from animal tissues. Heparin, for example, is produced in metric ton quantities yearly for use as a pharmaceutical anticoagulant.[4] Typically, a tissue (or defatted tissue) obtained from a slaughter house is treated with non-specific proteases.[5] After filtration, the soluble GAG containing fraction is further purified by: (1) strong anion exchange chromatography; (2) ethanol precipitation,[6] and/or (3) precipitation as the water insoluble cetylpyridinium salt.[7] Treatment with base or controlled bleaching with oxidizing agents removes residual protein and peptide components.[8,9] Despite the harsh treatment used in their preparation, GAGs are believed to be recovered structurally unaltered from tissue.[10,11] Analyses of GAGs prepared in such manner from a wide variety of tissues suggest that their structure closely reflects the structure of the GAG chains found in the native PG.[5,10]

The stability of glycosaminoglycans has only recently been examined in detail.[11,12] Heparin decomposes by treatment under strongly acidic (1 M HCl), strongly basic (1 M NaOH) conditions or at neutral pH under elevated temperatures (>100 °C).[12] Harsh, oxidative conditions are often used for the controlled partial depolymerization of heparin to prepare low molecular weight (LMW) heparins.[8,11,13] No decomposition has been reported under the mild basic (pH~9) and mild oxidative (peracetic acid at low temperature) conditions used in the preparation of pharmaceutical heparin.[8,9]

It is widely known that 2-acetamido-2-deoxy-D-glucose (GlcNAc) and 2-acetamido-2-deoxy-D-mannose (ManNAc) are reversibly epimerized in the presence of base.[14,15] This manuscript reports the serendipitous discovery that such processes may take place during the commercial preparation of GAGs and GAG-derived oligosaccharides.

RESULTS AND DISCUSSION

Preparation of Heparan Sulfate and Heparan Sulfate Oligosaccharides. Recently a commercial scale preparation of heparan sulfate GAG was reported by our laboratory.[16] The isolation of multigram quantities of purified heparan sulfate facilitated the preparation, purification and structural characterization of heparan sulfate derived oligosaccharides. These oligosaccharides were prepared by treatment of heparan sulfate with purified heparin lyases obtained from *Flavobacterium heparinum*.[17] Exhaustive enzymatic treatment with each of these enzymes[18] afforded mixtures of oligosaccharides of sizes ranging from disaccharide to larger than decasaccharide. Gel permeation chromatography on Sephadex G-50 afforded various sized fractions.[19] These sized fractions were then further fractionated using strong-anion exchange (SAX) high

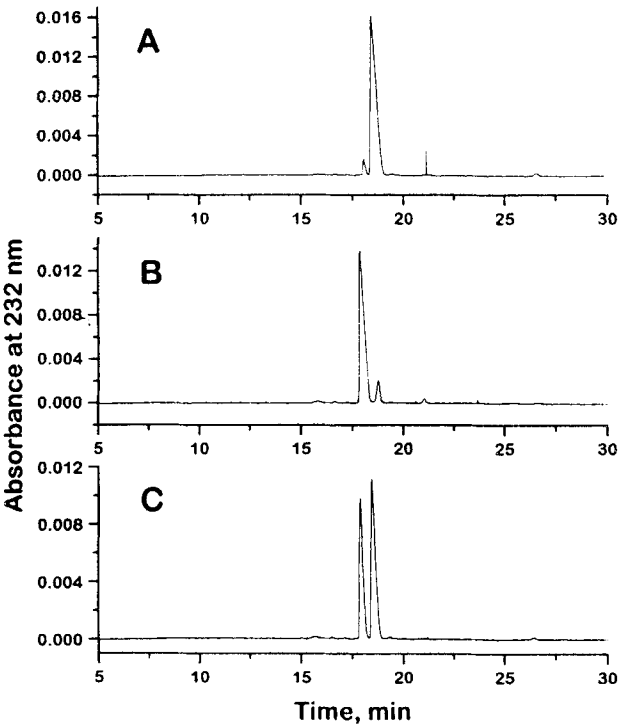

Figure 1. CE analysis of tetrasaccharides purified from porcine intestinal mucosa heparan sulfate. (a) tetrasaccharide **1**, (b) tetrasaccharide **2**, (c) mixture of tetrasaccharide **1** and **2**.

performance liquid chromatography (HPLC) to obtain tetrasaccharides for spectral characterization.[19] Prior to structural determination, the homogeneity of each size and charge fractionated tetrasaccharide was assessed by capillary electrophoresis (CE).[20]

Initial Isolation and Tentative Identification of an Unusual Heparan Sulfate-derived Tetrasaccharide. While purifying tetrasaccharides obtained by heparin lyase II treatment of heparan sulfate, a well resolved, symmetrical peak was obtained on semi-preparative SAX-HPLC. The peak isolated by SAX-HPLC was desalted on a Biogel P-2 column and lyophilized for analysis by CE. Surprisingly, on CE analysis this tetrasaccharide appeared to contain a 10% impurity (Figure 1A). Re-examination of this tetrasaccharide on the same SAX-HPLC column using a very shallow gradient system (from 0 to 0.5 M sodium chloride for 120 min) showed two peaks having very different retention times. The major peak corresponded to the original peak isolated on SAX-HPLC and the newly observed minor (~10%) peak eluted earlier. These results suggested that a

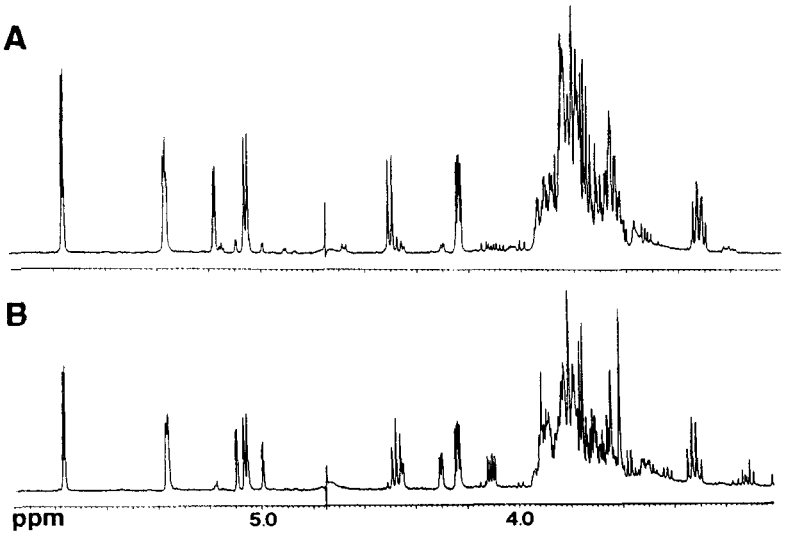

Figure 2. ^{1}H NMR spectra of tetrasaccharides. A. Spectrum of tetrasaccharide **1**; and B. Spectrum of tetrasaccharide **2**.

decomposition process was taking place under the relatively mild conditions used in oligosaccharide fractionation and/or analyses. One dimensional ^{1}H NMR spectroscopy (Figure 2A) clearly established the structure of the major tetrasaccharide (**1**) as ΔUA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-(1→4)-D-GlcNAc (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid and GlcA is glucopyranosyluronic acid) and suggested the presence of a minor contaminant containing a ManNAc residue based on the characteristic signals at 5.094 and 4.993 ppm and their very small $^3J_{1,2}$ coupling constants.

Purification and Structural Characterization of a ManNAc Containing Tetrasaccharide Derived from Heparan Sulfate. Tetrasaccharide mixtures prepared by gel permeation chromatography of heparin lyase I, II and III treated heparan sulfate were examined by SAX-HPLC for the presence of a peak having a retention time corresponding to the minor ManNAc containing tetrasaccharide. A peak corresponding to this retention time was observed in a well resolved area of the SAX-HPLC chromatogram of a tetrasaccharide mixture obtained from heparan sulfate treated with heparin III lyase. This peak was recovered, desalted, lyophilized and analyzed by CE (Figure 1B). The major component in electropherogram 1B now corresponded to the minor component in 1A and the minor component in 1B to the major component in 1A (see Figure 1). Simple mixing of both samples followed by CE analysis proved that each sample contained both

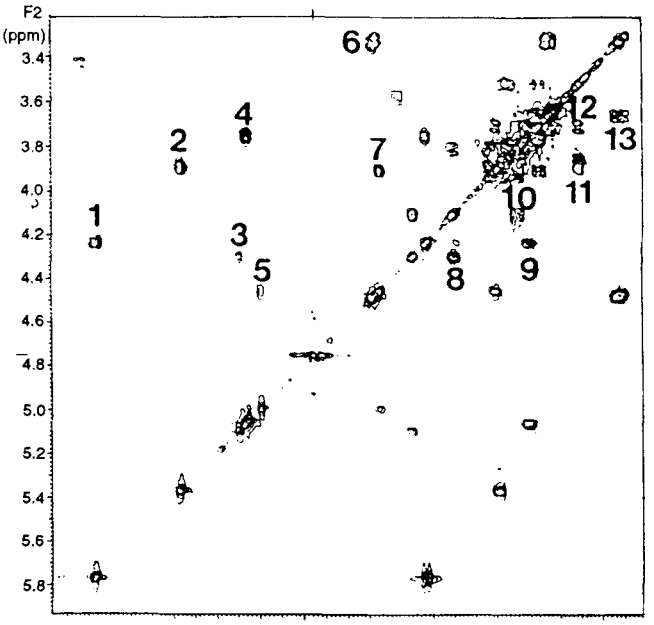

Figure 3. 2D-COSY of tetrasaccharide **2**. *Cross peaks:* 1, H-3/H-4 of ΔUA; 2, H-1/H-2 of GlcNAc; 3, H-1/H-2 of ManNAcα; 4, H-1/H-2 of ΔUA; 5, H-1/H-2 of ManNAcβ; 6, H-1/H-2 of GlcA; 7, H-2/H-3 of ManNAcβ; 8, H-2/H-3 of ManNAcα; 9, H-3/H-4 of ΔUA; 10, H-3/H-4 of ΔUA; 11, H-5/H-6 (2H) of ManNAcβ; 12, H-4/H-5 of ManNAcβ; 13, H-2/H-3 of GlcA.

tetrasaccharide components (Figure 1C). One and two dimensional ^{1}H NMR (Figures 2B and 3) confirmed the structure of this unusual tetrasaccharide (**2**) to be ΔUA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-(1→4)-D-ManNAc (Table). Peaks at 5.094 and 4.993 ppm show the signals from α and β anomeric protons of ManNAc at the reducing end. Both coupling constants of α and β-anomeric protons of ManNAc are characteristically similar, because the dihedral angles between anomeric protons and H-2 of α and β ManNAc are about 60°. The anomeric proton signals of the β-GlcA attached to the ManNAc were also split into a double of doublets (at 4.483 and 4.468 ppm) by the α and β-ManNAc at the reducing end.

Base-catalyzed Conversion of Tetrasaccharide 1 to Tetrasaccharide 2. Tetrasaccharide (**1**) was dissolved in water at 1 mM and 0.25% of ammonium hydroxide was added. The sample was incubated at 30 °C and monitored periodically using CE. These conditions were chosen based on the literature reports of the conversion of GlcNAc to ManNAc.[14] The concentration of tetrasaccharide **1** and **2** were determined and plotted as a function of time (Figure 4). The observed kinetic profile clearly demonstrated the conversion of **1** to **2** reaching a final equilibrium containing a 6:4 molar ratio.

Table. Chemical shifts and coupling constants of tetrasaccharides 1 and 2

Tetrasaccharide 1				**Tetrasaccharide 2**						
	ΔUA	GlcNAc	GlcA	GlcNAc		ΔUA	GlcNAc	GlcA	ManNAc	
				α	β				α	β
H-1	5.058	5.371, 5.364	4.504	5.178	4.681	5.059	5.369, 5.365	4.468, 4.483	5.094	4.993
J 1,2	6.5	3.7, 2.2	7.9	3.1	7.9	6.3	1.5, 1.4	7.8, 7.8	1.4	1.7
H-2	3.741	3.894	3.328	3.829	3.656	3.758	3.921	3.331, 3.315	4.302	4.456
J 2,3	4.9	7.4	8.1	6.4	6.8	4.6	6.8	8.9, 8.8	6.3	6.3
H-3	4.239	3.81	3.661	3.946	3.789	4.236	3.806	3.688	4.107	3.921
J 3,4	3.5	-	7.6	7.6	7.8	3.4	7.4	7.4	7.1	7.1
H-4	5.764	3.85	3.806	3.806	3.792	5.765	3.752	3.801	3.897	3.712
J 4,5		7.8	7.9	7.9	7.6		7.6	8.1	8.1	8.1
H-5	-	3.844	3.842	3.927	3.561	-	3.854	3.838	3.955	3.527
J 5,6a		6.1		5.1	5.4		6.4		5.6	5.8
H-6a	-	3.755	-	3.81		-	3.75	-	3.81	
J 6a,6b		10.4		10.1	11.1		10.1		11.1	10.5
H-6b	-	3.826	-	3.85		-	3.82	-	3.84	
J 5,6b		3.4		3.2	2.8		3.2		3.1	2.6
NAc-methyl	-	2.019	-	2.007		-	2.044	-	2.024	2.083

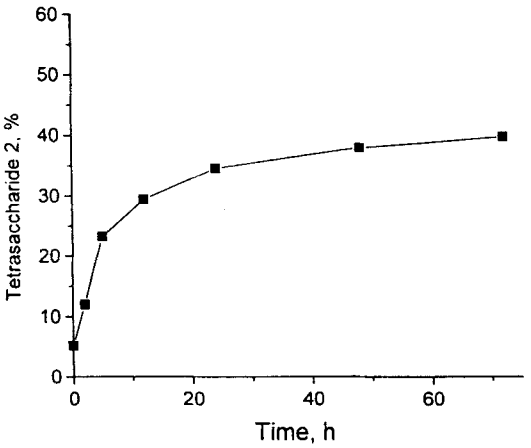

Figure 4. Kinetic profile of base catalyzed C-2 epimerization of GlcNAc residue in **1** to ManNAc residue in **2**.

Proposed Mechanism for the Conversion of Tetrasaccharide 1 to 2. The mechanism for C-2 epimerization of GlcNAc containing tetrasaccharide **1** to ManNAc containing tetrasaccharide **2** is presented in Figure 5. This mechanism is consistent with the isolation of a tetrasaccharide containing a ManNAc residue at the reducing terminal. The pyranose form is in equilibrium with the ring opened aldehyde form. Treatment of this base sensitive molecule even with diluted ammonium hydroxide solution leads to abstraction of the acidic proton at C-2. Furthermore, the fate of the generated C-2 carbanion depends on the substituent at C-3: (1) If it is a poor leaving group such as OH, a simple C-2 epimerization takes place where the *gluco*- and *manno*-epimers are in dynamic equilibrium. (2) It is a better leaving group, C-2/C-3 β-elimination will first occur through an E1cB mechanism, followed by further degradation of the elimination products.

Biologically, GlcNAc and ManNAc are reversibly epimerized by a C-2 epimerase found in hog kidney; and GlcNAc 6-phosphate and ManNAc 6-phosphate are reversibly epimerized by a C-2 epimerase found in bacteria. In addition, UDP-GlcNAc is acted upon enzymatically by UDP-GlcNAc C-2 epimerase to afford ManNAc and UDP; and by UDP-GlcNAc C-2 epimerase to afford UDP-ManNAc. The mechanisms for these enzymatic reactions are all recognized to involve C-2 epimerization of 2-acetamido sugars. Thus, the observed conversion of GlcNAc to ManNAc represents a unifying type of mechanism involving a tautomerization reaction between the proton on the C-2 carbon of a 2-acetamido sugar and the neighboring C-1 carbonyl group. Such processes may take place during the commercial preparation of GAGs and GAG-derived oligosaccharides.

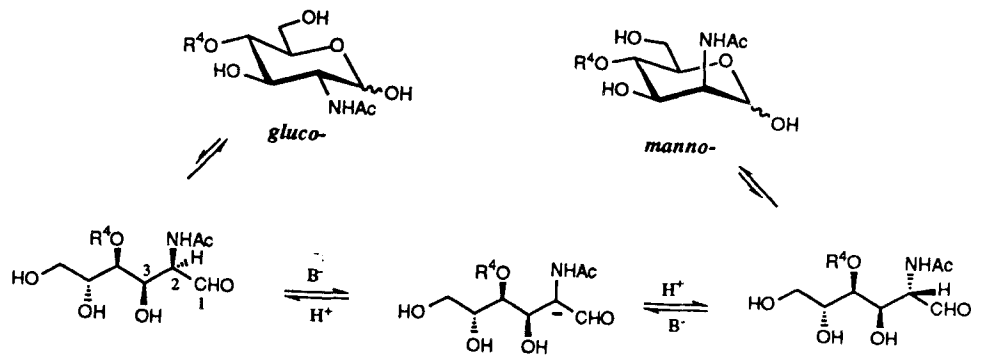

Figure 5. Proposed mechanism of C-2 base catalyzed epimerization of reducing terminal GlcNAc. R^4 represents an oligosaccharide moiety.

EXPERIMENTAL

Materials. Porcine intestinal mucosa heparan sulfate[16] (MWavg 14,800) was from Celusus, Cincinnati, OH, USA. Heparin lyase I (heparinase I, EC 4.2.2.7) was from IBEX Technologies (Montreal, Canada). Heparin lyase II (no EC assigned) and heparin lyase III (EC 4.2.2.8) were prepared from *Flavobacterium heparinum* and purified to homogeneity.[17] Heparin and heparan sulfate disaccharide standards were used to calibrate the CE separations. The disaccharide standard ΔUA2S-(1→4)-D-GlcNS6S (where S is sulfate) was prepared from heparin and characterized. Disaccharides ΔUA2S-(1→4)-D-GlcNS and ΔUA-(1→4)-D-GlcNAc6S were from Sigma Chemical Co. (St. Louis, MO). Disaccharides ΔUA-(1→4)-D-GlcNS, ΔUA2S-(1→4)-D-GlcNAc6S, ΔUA-(1→4)-D-GlcNAc6S and ΔUA2S-(1→4)-D-GlcNAc and ΔUA-(1→4)-D-GlcNAc were from Grampian Enzymes (Aberdeen, Scotland).

Preparation of Heparan Sulfate-derived Tetrasaccharides. Complete depolymerization of 2.5 g samples of heparan sulfate with heparin lyase III and heparin lyase II were carried out, under the optimum conditions as previously described.[16,17,18] The heparan sulfate oligosaccharides obtained from heparin lyase III and II digestions were independently fractionated on a Sephadex G-50 (superfine) column (4.8 x 100 cm) eluted with 200 mM sodium chloride at a flow rate of 2 mL/min. Oligosaccharide mixtures were dissolved in 10 mL of de-ionized distilled water and applied to this column and the tetrasaccharide fractions were collected, desalted and lyophilized.[19] Next, the tetrasaccharide fractions obtained from heparan sulfate were applied to preparative SAX-HPLC using a linear gradient of sodium chloride at pH 3.5, as described before.[19] The

major peaks were pooled, freeze-dried, and desalted on a Bio-Gel P-2 column.[19] The purified major tetrasaccharide from each heparin lyase digestion was lyophilized and stored in -60 °C.

^{1}H NMR Spectroscopy. For ^{1}H NMR spectroscopy, approximately 1 mg of each sample was dried three times with 0.5 mL portions of ^2H_2O (99.96%, Sigma, St. Louis, MO), by desiccation over P_2O_5 *in vacuo* to exchange the labile protons with deuterium. The thoroughly dried sample was re-dissolved in 0.7 mL of ^2H_2O (99.96%), and transferred to an NMR tube. All spectra were performed using a UNITY-500 spectrometer at the operating frequency of 500 MHz on a Varian Instrument equipped with a VXR 5000 computer system, with a process controller and an array processor. The operation conditions for one-dimensional (1D) spectra were as follows: frequency, 500 MHz; sweep width, 6 kHz; flip angle, 90 (11.1 μs); sampling point, 48k; accumulation, 256 pulses; temperature, 298 K. Chemical shifts were indicated by ppm from the signal of 3-trimethylsilyl[$^2H_4$] propionic acid sodium salt as an internal standard.

All two-dimensional (2D) experiments were recorded in phase-sensitive mode using the time-proportional phase incrementation (TPPI) method. Conventional pulse sequences were used for homonuclear correlated spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY). The mixing time in the NOESY experiment was set to 300 and 500 ms. Homonuclear Hartman-Hahn total correlation spectroscopy (TOCSY) spectra were acquired using the standard MLEV17 spin-locking sequence and 90 ms mixing time. The spectra were recorded at 298 K using a spectral width of 3,200 Hz in using the phase-sensitive mode. The size of the acquisition data matrix was 2048 x 512 words in f_2 and f_1, respectively, and zero filling up to 2k in f_1 was made prior to Fourier transformation. Shifted sine-bell or square sine-bell window functions, with the corresponding shift optimized for every spectrum, were used for resolution enhancement and baseline correction was applied in both dimensions. Water suppression was carried out by selective pre-saturation placing the carrier on the solvent resonance.

Capillary Electrophoresis. The experiments were performed on a Dionex capillary electrophoresis system (Sunnyvale, CA) equipped with a variable wavelength ultraviolet detector. System operation and data handling were fully controlled using version 3.1 A1-450 chromatography software on a IBM-compatible PC. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and running using 20 mM phosphoric acid adjusted to pH 3.5 with 1 M dibasic sodium phosphate as previously described.[20] The capillary (75 μm i.d., 375 μm o.d, 68 cm long) was manually washed before use with 0.5 mL of 0.5 M sodium hydroxide followed by 0.5 mL distilled water, then 0.5 mL running buffer. Samples were applied using gravity

injection (12 s) by hydrostatic pressure (45 mm) resulting in a sample volume of 5.5 nL. Each experiment was conducted at a constant 18 kV. Data collection was at 232 nm.

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