Supporting Information for:

Investigation of the Amide Proton Solvent Exchange Properties of Glycosaminoglycan Oligosaccharides

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Contents

1 Preparation of Oligosaccharides

1.1 Partial Digestion of CS-A with Chondroitinase ABC[1]

A 0.90 g sample of bovine trachea chondroitin sulfate A (*bt*CS-A) sodium salt was dissolved in 35 mL of 50 mM Tris buffer at pH 8.0 with 150 mM sodium acetate and 5 mg of BSA was added. Chondroitinase ABC from *Proteus vulgaris* equivalent to 2.5 IU was added to the room temperature solution which was carefully mixed. An aliquot of 524 µL taken from the digestion mixture was combined with 60 µL of D_2O and 16 µL of a 100 mM DSS-d_6 solution and transferred to a 5 mm NMR tube to monitor digestion progress. Both samples were incubated at 37 °C for 2 h 50 min at which point both solutions were combined and the reaction quenched by placing the solution in boiling water for 15 min.

1.2 Digestion of HA with Hyaluronidase[2-4]

Two batches of hyaluronic acid (HA) oligosaccharides were prepared at different times using the same amounts, concentrations, and proportions of the starting reagents. Vials of Hyaluronidase from *Streptomyces hyalurolyticus* were received at different times and came from different lots. The digestions were carried out using a 50 mg sample of sodium HA dissolved in 15 mL of 200 mM sodium acetate buffer at pH 5.1 with 0.15 M NaCl stored overnight in a refrigerator at 4 °C. Hyaluronidase equivalent to 900 IU was dissolved in 1 mL of 200 mM acetate buffer at pH 5.1 containing 0.15 M NaCl and added to the HA solution pre-equilibrated to 37 °C. The resulting digestion mixture was carefully but thoroughly agitated and an aliquot of 540 µL was combined with 60 µL D_2O and 10 µL of 100 mM DSS-d_6 solution and transferred to a 5 mm NMR for reaction progress monitoring. Both samples were incubated for 10 h 5 min at 37 °C after which the samples were recombined, and the reaction quenched by placing the solution in boiling water for 10 min.

1.3 SEC of HA Digested Oligosaccharides[3]

Batch #1. A mixture of HA oligosaccharides prepared by enzymatic depolymerization was separated by size-exclusion chromatography (SEC) using a 50 mM ammonium bicarbonate solution at an average flow rate of 0.124 mL/min using a 3.0 x 200 cm atmospheric pressure chromatographic column packed with Bio-Rad Bio-Gel P-10 fine polyacrylamide resin. Eluted oligosaccharides were collected in separate 3.5 mL

fractions. The separation was monitored offline at 232 nm using a Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer. The resulting SEC chromatogram is shown in Figure S1a.

An unstable flow rate and poor column packing resulted in suboptimal resolution of different sized HA oligosaccharides in the chromatogram in Figure S1a. Screening of isolated fractions by ESI-MS revealed the presence of odd-numbered oligosaccharides, which was inconsistent with the expected specificity of the enzyme used in this digestion. Major products of enzymatic depolymerization of HA by Hyaluronidase from *Streptomyces hyalurolyticus* are expected to be even-numbered oligosaccharides.[4] The fractions constituting the chromatographic peak labeled #1 in Figure S1a were pooled and lyophilized before being subjected to an additional round of SEC using the same mobile phase at a flow rate of 0.15 mL/min on a 1.6 x 70 cm atmospheric pressure column packed with Bio-Rad Bio-Gel P-6 fine polyacrylamide resin (Bio-Rad Laboratories Hercules, CA). Eluents were collected in separate 0.85 mL tubes and monitored offline at 232 nm by using a Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer. A SEC chromatogram of this separation is shown in Figure S1b, where it can be observed that peak #1 was indeed composed of two unresolved tri- and tetrasaccharides (labeled as dp3 and dp4, respectively). A portion of peak #2 was further resolved on a 1.6 x 70 cm size-exclusion chromatography column, yielding the penta- and hexasaccharides labeled dp5 and dp6, respectively, shown in Figure S1c.

Because of the poor separation achieved in Figure S1a, the 3.0 x 200 cm atmospheric pressure chromatographic column was repacked with Bio-Rad Bio-Gel P-10 fine polyacrylamide resin and the collected fractions including the second part of peak #2, were pooled, lyophilized and reconstituted in 15 mL of 50 mM ammonium bicarbonate buffer and the size-exclusion separation carried out at a stable flow rate of 0.08 mL/min. The result of this separation is shown in Figure S1d. As before, the isolated fractions were identified by ESI-MS, which clearly indicated that the digestion mixture was composed of even- and odd-numbered oligosaccharides. Fractions constituting each peak were combined, lyophilized, and stored at -80 °C for future use. Batch #2. A second batch of HA oligosaccharides were prepared by enzymatic depolymerization with Hyaluronidase (from a different lot) and separated by size using a 50 mM ammonium bicarbonate solution at a flow rate of 0.07 mL/min on a 3.0 x 200 cm atmospheric pressure chromatographic column packed with Bio-Rad Bio-Gel P-10 fine polyacrylamide resin. The eluted oligosaccharides were

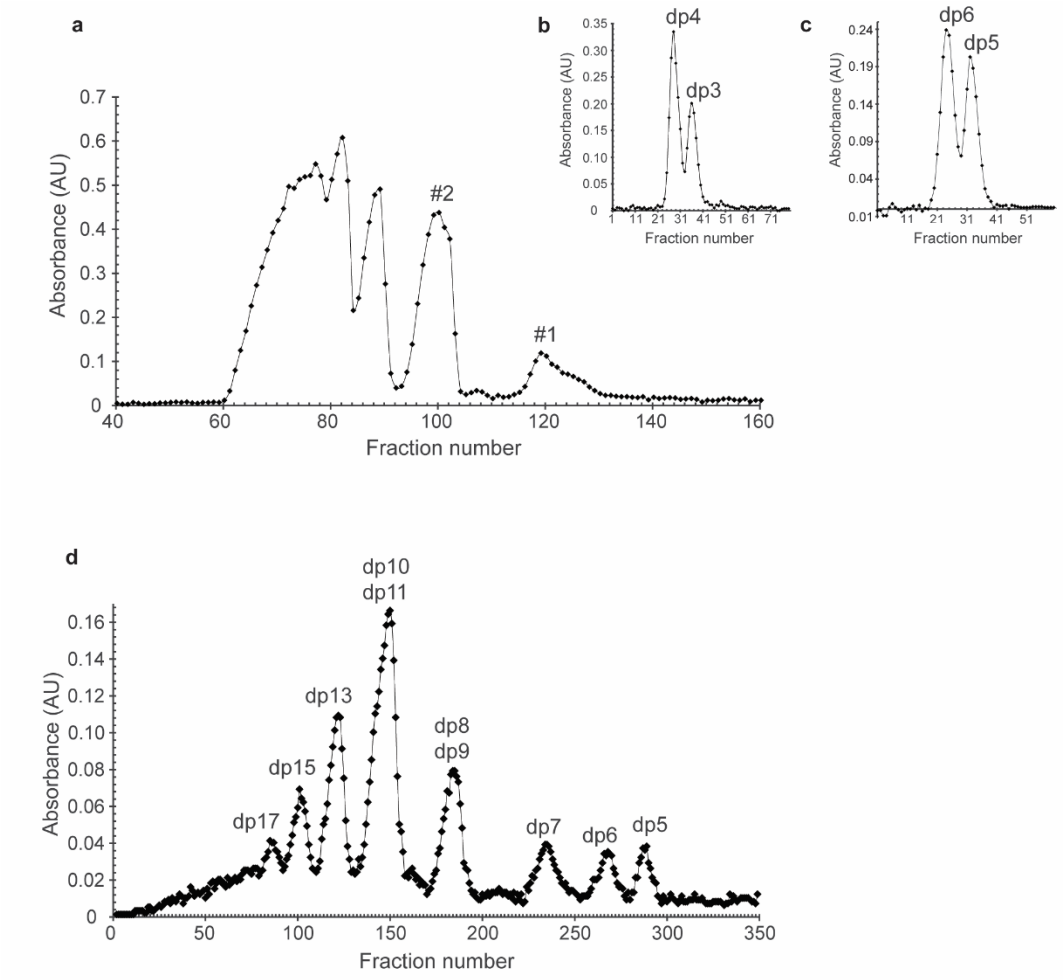

Figure S1. SEC chromatograms of HA digested by Hyaluronidase from *Streptomyces hyalurolyticus* in batch #1. (a) The poor separation of the mixture of HA oligosaccharides was due to column performance. Peaks labeled #1 and #2 were further separated at atmospheric pressure on a 1.6 x 70 cm column packed with Bio-Rad Bio-Gel P-6 fine polyacrylamide resin. (b) Peak #1 contains tri- (dp3) and tetrasaccharides (dp4). (c) Peak #2 contains penta- (dp5) and hexasaccharides (dp6). (d) Unresolved peaks from (a) were combined and separated again on a 3.0 x 200 cm column repacked with Bio-Gel P-10 fine resin which yielded improved chromatographic resolution.

collected in separate 3.5 mL fractions with the separation monitored offline at 232 nm by using a Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer. The chromatogram for this separation is shown in Figure S2a and revealed good resolution of the depolymerization products. This digestion with Hyaluronidase from *Streptomyces hyalurolyticus* yielded even-numbered oligosaccharides, as expected. Size-uniform fractions were pooled, lyophilized, desalted using deionized water on a 1.6 x 70 cm column packed with Sephadex G10 resin (GE Healthcare, Pittsburgh, PA), lyophilized again and stored at -80 °C for future use.

1.4 SEC of CS-A Digested Oligosaccharides[1]

The mixture of chondroitin sulfate A (CS-A) oligosaccharides prepared by enzymatic depolymerization was separated by size-exclusion chromatography (SEC) using a mobile phase of 1 M NaCl with 10% of ethanol at a flow rate of 0.07 mL/min and a 3.0 x 200 cm atmospheric pressure chromatographic column packed with Bio-Rad Bio-Gel P-10 fine polyacrylamide resin (Bio-Rad Laboratories Hercules, CA). The eluted oligosaccharides were collected in separate 4.5 mL fractions and the separation monitored offline at 232 nm using a Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer (Wilmington, DE). A representative SEC chromatogram is shown in Figure S2b. The size-uniform fractions were pooled, lyophilized and desalted using deionized water on a 1.6 x 70 cm column packed with Sephadex G10 resin (GE Healthcare, Pittsburgh, PA). After desalting, the isolated oligosaccharide samples were lyophilized and stored at -80 °C for future use.

1.5 SAX HPLC Separation of the Reduced CS-A Oligosaccharides

The fractions of the *bt*CS-A digestion labeled dp4 (tetrasaccharide) and dp6 (hexasaccharide) in the SEC shown Figure S2b were reduced according to the procedure described in the following section and separated by strong anion-exchange high-performance liquid chromatography (SAX HPLC) using a Waters Spherisorb 5 µm (Milford, MA) semi-preparative column (10 x 250 mm) and a Dionex 500 ion chromatography system equipped with a GP40 gradient pump and AD20 UV-Vis detector. The SEC fractions of the reduced CS-A dp4 (CS dp4-ol) and reduced CS-A dp6 (CS dp6-ol) were individually dissolved in degassed deionized water and introduced onto the SAX column in 500 µL injections at a flow rate of 3.0 mL/min. The oligosaccharides were separated using a 20 mM CH_3COONa buffer at pH 3.9 as

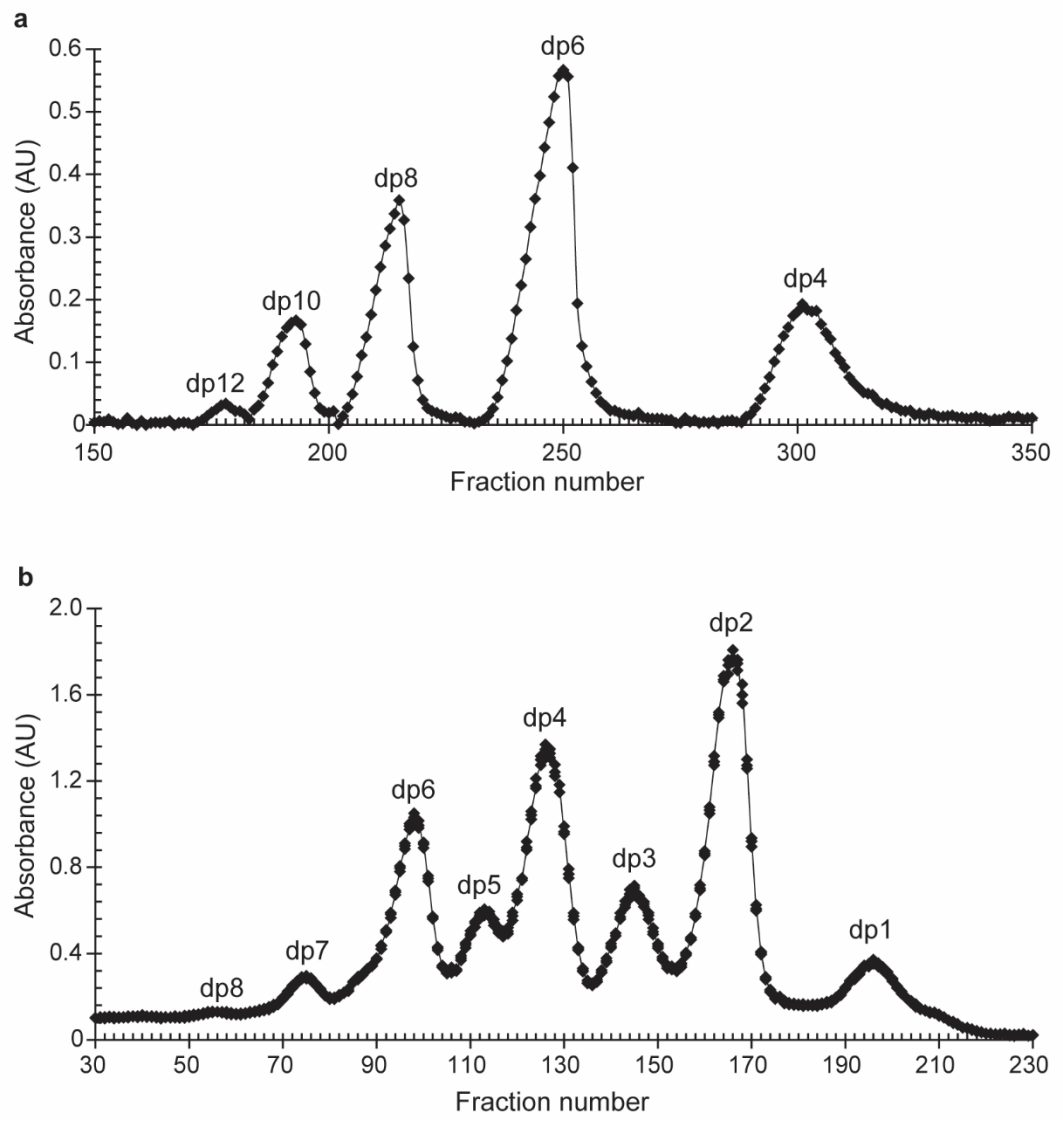

Figure S2. SEC of digests of (a) HA and (b) CS-A. Each data point represents individual 3.5 mL (a) or 4.5 mL (b) size-uniform fractions collected for HA or CS-A, respectively. The peak labeling corresponds to oligosaccharide size: mono- (dp1), di- (dp2), tri- (dp3), tetra-(dp4), penta-(dp5), hexa-(dp6), hepta-(dp7), octa-(dp8), deca-(dp10), and dodecasaccharides (dp12) where dp refers to depolymerization product.

mobile phase A, and 1.0 M NaCl in a 20 mM CH_3COONa buffer at pH 3.9, as mobile phase B. The following gradient profile was employed to separate the tetrasaccharides: 0 min, 100% A and 0% B; 1 min, 89% A and 11% B; and 23 min, 70% A and 30% B. This gradient profile was modified for the hexasaccharide separation: 0 min, 100% A and 0% B; 3 min, 85% A and 15% B; and 27 min, 65% A and 35% B. Each run was followed by a washing step of 100% B for 10 min and column equilibration with 100% A for 20 min. The eluted chromatographic peaks were collected separately over multiple consecutive injections. Each sample was lyophilized and reconstituted in the minimum amount of degassed deionized water for desalting on a 1.6 x 70 cm column packed with Sephadex G10 resin. Deionized water sonicated for 25 min was used as the mobile phase at a flow rate of 0.14 mL/min with the eluent collected in 1.0 mL fractions. The collected fractions were monitored offline using a NanoDrop 2000 UV-Vis spectrophotometer at 232 nm. Fractions containing the desalted oligosaccharides were combined, lyophilized, and stored at -80 °C for future use.

1.6 Reduction of Oligosaccharides with Sodium Borohydride.[5]

SEC isolated fractions of CS-A dp4 and CS-A dp6 were reduced with sodium borohydride ($NaBH_4$). Approximately 77.0 mg of CS-A dp4 (ΔUA-GalNAc4S-GlcA-GalNAc4S) isolated from the enzymatic depolymerization reaction was dissolved in 1.5 mL of solution containing 1M $NaBH_4$ in 100 mM NaOH. The resultant mixture was incubated overnight in an Eppendorf ThermoMixer *C* (Thermo Fisher Scientific, Asheville, NC) at 50 °C. The reaction was quenched through the careful and slow addition of 40 µL of glacial acetic acid to avoid excessive effervescence from decomposing borohydride. The excess of borohydride and any reaction by-products were removed by cation exchange chromatography. The column was prepared by packing Dowex 50WX8 cation exchange resin into a 1 cc plastic cartridge and converting it to the hydrogen form with 3 mL of 1M HCl followed by conditioning with 3 mL of methanol and 5 mL of deionized water. The sample was introduced onto the cartridge and the tetrasaccharide was eluted by washing three times with 1 mL aliquots of deionized water. The collected effluent was neutralized with 1M NaOH solution and lyophilized. The final purification step was performed by dissolving the lyophilized solid in 400 µL of deionized water and passing it through a 1.6 x 70 cm desalting column packed with Sephadex G-10 superfine resin using degassed deionized water as a mobile phase at a flow rate of 0.15 mL/min. The collected fractions containing the reduced tetrasaccharide (CS dp4-ol) were combined and lyophilized,

yielding 64.5 mg of lyophilized product. Similarly, 74.4 mg of the isolated CS-A dp6 (ΔUA-GalNAc4S-GlcA-GalNAc4S-GlcA-GalNAc4S) was reduced by this procedure to yield 68.5 mg of lyophilized product. Additionally, HA dp6 (ΔUA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc) was converted to the alditol by similar reduction reaction with $NaBH_4$. A 2.0 mg sample of the HA dp6 was dissolved in 0.1 mL of solution containing 1M $NaBH_4$ in 100 mM NaOH. However, due to the problems with reduction of unsulfated HA dp6 leading to oligosaccharide degradation, all steps of the HA dp6 reduction were performed in presence of 30 mM phosphate buffer. After desalting, fractions containing reduced HA hexasaccharides (HA dp6-ol) were combined and buffered with 10 mM phosphate followed by lyophilization to yielded 1.7 mg of product. Freeze dried samples then were stored at -80 °C for future use.

1.7 Preparation of *N*-Acetylated Arixtra and 3-*O*-Sulfated Glucosamine

The *N*-acetylated Arixtra was prepared in two steps: (1) Arixtra was de-*N*-sulfated followed by (2) chemoselective *N*-acetylation. (1) The de-*N*-sulfated Arixtra was prepared by a selective solvolytic de-*N*-sulfation reaction.[6-7] All operations were performed in a cold room at 4 °C with materials pre-equilibrated to this temperature. A cation exchange column was prepared by packing Dowex 50 WX8 cation exchange resin into a 1 cc plastic cartridge. The resin was converted to the hydrogen form with 3 mL of 1M HCl followed by conditioning with 3 mL of methanol and 5 mL of deionized water. About 6.0 mg of Arixtra sodium salt was dissolved in 200 µL of deionized water and introduced onto the cartridge. The protonated form of Arixtra was eluted by washing three times with 1 mL aliquots of deionized water. The effluent was then neutralized with pyridine and freeze dried. The resultant light-brown Arixtra pyridinium salt was reconstituted in 400 µL of DMSO-d_6 containing 5% deionized water and incubated at 50°C and 350 rpm for 90 min in Eppendorf ThermoMixer *C*. The reaction was quenched by addition of 400 µL of deionized water followed by pH adjustment to 9.3 with 0.1 M NaOH. Volatile organic components of the mixture were removed by speed vacuum at medium heat for 3 h using a Speedvac Savant SC 110 equipped with a refrigerated vapor trap RVT400 (Thermo Fisher Scientific, Asheville, NC). The residual solution was diluted with 300 µL of deionized water and speed vacuumed for an additional 3 h and the remaining solution was lyophilized. The sample was purified by dissolving the solid in 400 µL of deionized water and passing it through a 1.6 x 70 cm desalting column packed with Sephadex G-10 superfine resin using degassed deionized water as a

mobile phase at a flow rate of 0.15 mL/min. About 2.7 mg of de-*N*-sulfated Arixtra sodium salt was recovered as a white powder, a 45% yield.

(2) About 2.0 mg of de-*N*-sulfated Arixtra was dissolved in 0.2 mL of 10% CH_3COOH, the pH of the solution was adjusted to 4.6 with 1M NaOH solution. Aliquots of 1.0 mL of methanol and 14 µL of 99.5% $(CH_3CO)_2O$ were added into the solution. The resultant solution mixture was agitated, followed by overnight incubation at room temperature. The next morning, the mixture was placed into a speed vacuum at medium heat for 3 h to remove volatile components of the mixture. The residual solution after speed vacuum was diluted with 0.1 mL of deionized water and lyophilized. The product was dissolved in 400 µL of deionized water and purified by passing the solution through a 1.6 x 70 cm desalting column packed with Sephadex G-10 superfine resin using degassed deionized water as the mobile phase at a flow rate of 0.15 mL/min. Finally, the collected fractions containing *N*-acetylated Arixtra (NAcA) were combined and lyophilized. About 1.8 mg of white dry powder was recovered, a 90% yield. Using the same method *N*-acetylated 3-*O*-sulfated glucosamine (GlcNAc3S) was also prepared from 1.75 mg of 3-*O*-sulfated glucosamine (GlcN3S).

2 Structure Elucidation of Prepared Oligosaccharides

2.1 ESI-MS Measurements

About 1.0 mg of each desalted oligosaccharide: de-*N*-sulfated Arixtra, NAcA, CS dp4-ol and CS dp6-ol after SAX chromatography, and SEC separated HA dp3, dp4, dp5, and dp6 was dissolved in separate 500 µL aliquots of deionized water. An aliquot of 30 µL of each solution was transferred to a 1.5 mL Eppendorf safe-lock tube and diluted with 200 µL of 30% CH_3OH and 70% H_2O. These samples were infused into a Micromass/Waters ESI quadrupole time-of-flight (Q-Tof micro) mass spectrometer (Waters Corporation, Millford, MA) at a flow rate of 10 µL/min. Data acquisition was performed using Masslynx 4.1. The mass spectrum was obtained in negative mode using the following instrumental parameters: capillary voltage, 2800-3000 kV, cone voltage, 25-35 V, extractor voltage, 1-3 V, source temperature, 120 °C, desolvation temperature, 275 °C, interscan delay, 0.1 s, and m/z range, 200 – 1500. Representative mass spectra for de-*N*-sulfated Arixtra and NAcA with assigned ion peaks are shown in Figure S3a and S4b, respectively.

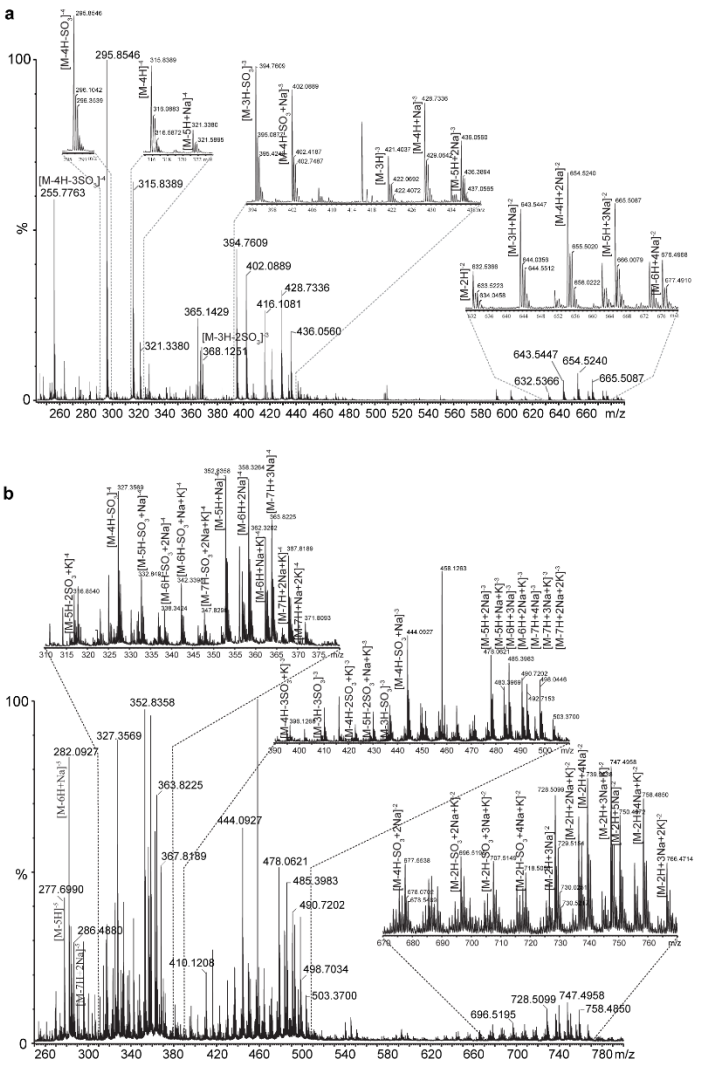

Figure S3. (a) Mass spectrum of de-*N*-sulfated Arixtra. The loss of sulfate and the presence of sodium adducts is common in analysis of GAG oligosaccharides by ESI-MS. The expected mass of de-*N*-sulfated Arixtra in 1267.08 U was confirmed. (b) Mass spectrum of NAcA. The expected mass of NAcA, 1393.11 U, was confirmed. In contrast to the results for de-*N*-sulfated Arixtra, the NAcA molecular ions were also accompanied by potassium adducts introduced during pH measurement due to a leaky pH electrode. Even though sample was desalted it still retained potassium ions. The spectrum also indicates that the sample contains other trace impurities possibly arising from unreacted or partially reacted reaction mixture components.

2.2 NMR Measurements: Carbon-Bound Proton Resonance Assignments

About 1.1 mg of CS dp4-ol and 2.0 mg CS dp6-ol were dissolved in 700 µL of 10 mM phosphate buffer, pH 7.40, containing 0.5 mM DSS-d_6 and lyophilized. To reduce the intensity of the residual HOD peak, the freeze-dried samples were dissolved in 300 µL of 99.9% D_2O, lyophilized again, and reconstituted in 700 µL of 99.9% D_2O. The CS dp4-ol solution was adjusted to pD 9.10 and the CS dp6-ol solution was adjusted to pD 9.00 using NaOD and DCl prepared from concentrated solutions by dilution with the appropriate volume of 99.9% D_2O). Solution pD values were calculated from pH meter readings measured in deuterated solvents (pH^*) using eq 1[8-9] ($pD = pH^* + 0.4$).

$$pD = pH^* + 0.40 \quad (1)$$

Both solutions were lyophilized and stored at -80 °C. Prior to NMR measurements, the solutions were reconstituted in 700 µL of 99.9% D_2O and 600 µL aliquots transferred to individual 5 mm NMR tubes.

About 2.6 mg of de-*N*-sulfated Arixtra was dissolved in 1.5 mL of 10 mM phosphate buffer containing 6 µL of 100 mM DSS-d_6 as a chemical shift reference (0.00 ppm) and lyophilized. To reduce the residual HOD peak, the sample was twice re-dissolved in 300 µL of 99.9% D_2O and lyophilized. The resultant solid was dissolved in 1.5 mL of 99.9% D_2O for NMR measurements. A 300 µL aliquot was transferred to a 1.5 mL Eppendorf tube and the pD of the solution was adjusted to 7.50 with 10 mM and 100 mM solutions of NaOD and DCl prior to transferring the solution to a NE-H5/3-Br NMR tube.

About 1.9 mg of NAcA was dissolved in 300 µL of 10 mM phosphate buffer, pH 7.40, containing 0.5 mM DSS-d_6 and lyophilized. The sample was reconstituted in 300 µL of 99.9% D_2O, adjusted to pD 7.80 with NaOD and DCl, and lyophilized again. The solid sample was stored at -80 °C and reconstituted for NMR measurements in 300 µL of 99.9% D_2O before transferring 200 µL to a NE-H5/3-Br NMR tube.

The 1D ^1H, and 2D COSY, TOCSY, and ROESY spectra of the CS dp4-ol and CS dp6-ol were acquired at 316.2 K using a Bruker Avance 600 MHz spectrometer operating at 599.58 MHz and equipped with a BBI probe. The 90° pulse lengths ranged between 9.25 - 9.27 µs with solvent suppression of the HOD resonance provided by presaturation at a power of 59 dB during the 2.0 s relaxation delay. All spectra were

referenced to the ^{1}H resonance of DSS-d_6 (0.00 ppm). The 1D ^{1}H NMR spectra were acquired using a spectral window of 7002 Hz and 256 scans coadded into 42,014 complex points. Spectra were zero-filled to 65,536 points and apodized using an exponential multiplication window function equivalent to 0.3 Hz line broadening. The phase-sensitive 2D COSY, TOCSY, and ROESY spectra were acquired using States-TPPI with a spectral window of 7002 Hz in both dimensions. COSY spectra were acquired into 2048 complex points with 64 coadded scans and 416 t_1 increments. TOCSY spectra were acquired into 2048 complex points using 48 coadded scans and 416 t_1 increments using a mixing time of 120 ms with a 41 µs spinlock pulse at a power of 8.20 dB. Finally, ROESY spectra were acquired into 2048 complex points using 48 coadded scans, and 512 t_1 increments using a 350 ms spinlock pulse at a power of 59 dB.

The 1D ^{1}H, and 2D COSY, TOCSY, and ROESY spectra of the de-*N*-sulfated Arixtra was acquired using a Bruker Avance 600 MHz spectrometer operating at 599.52 MHz and equipped with a BBI probe at 298.2 K. All spectra were acquired using a 90° pulse of 9.17 µs with solvent suppression of the residual HOD resonance provided by presaturation at a power of 59 dB during the relaxation delay of 2.0 s, and were referenced to the ^{1}H resonance of DSS-d_6 (0.00 ppm). The one-dimensional ^{1}H NMR spectrum was acquired using a spectral window of 6361 Hz and 112 scans coadded into 42,014 complex points. This spectrum was processed in 65,536 points using an exponential multiplication window function equivalent to 0.4 Hz line broadening. Phase-sensitive two-dimensional COSY, TOCSY, and ROESY spectra were acquired in States-TPPI with a spectral window of 3597 Hz in both dimensions. The COSY spectrum was acquired into 2048 complex points with 64 coadded scans at each of 416 t_1 increments. The TOCSY spectrum was acquired using a mixing time of 120 ms with a 36 µs spinlock pulse at a power of 8.20 dB into 2048 complex points with 56 coadded scans and 416 t_1 increments. The ROESY spectrum was acquired into 2048 complex points with 48 coadded scans and 512 t_1 increments.

The 1D ^{1}H, COSY, TOCSY, and ROESY spectra of NAcA were acquired using a Bruker Avance 600 MHz spectrometer operating at 599.88 MHz and equipped with a TBI probe at 298.2 K. A 90° pulse length of 10.12 µs was used with solvent suppression of the HOD resonance provided by presaturation at a power of 59 dB during the 2.0 s relaxation delay. The 1D ^{1}H NMR spectrum was acquired using a spectral window of 6361 Hz with 120 scans coadded into 42,014 complex points. The spectrum was processed by zero

filling to 65,536 points and apodized with an exponential multiplication window function equivalent to 0.3 Hz line broadening. Phase-sensitive 2D COSY, TOCSY, and ROESY spectra were acquired using States-TPPI with a spectral window of 7002 Hz in both dimensions. The COSY and TOCSY spectra were acquired into 2048 complex points using 40 coadded scans at each of the 416 t_1 increments. The TOCSY spectrum was measured using a 120 ms mixing time with a 35 µs spinlock pulse at a power of 8.20 dB. Finally, the ROESY spectrum was acquired into 2048 complex points using a 350 ms spinlock pulse at a power of 53 dB with 48 coadded scans for each of the 512 t_1 increments.

All two-dimensional spectra were processed using Bruker TopSpin by zero filling to 4096 × 1024 data points and $\cos^2$ apodization. Chemical shifts assignments for the CS dp4-ol and CS dp6-ol are provided in Tables S1 and S2, respectively. The de-*N*-sulfated Arixtra and NAcA carbon-bound proton assignments are provided in Table S3.

Table S1. Assignment of the carbon-bound proton of the reduced chondroitin sulfate tetrasaccharide (CS dp4-ol) at pD 9.10 in 10 mM phosphate buffer acquired at 316.2 K.

Residue	Proton	^{1}H Chemical shift, ppm pD 9.1
ΔUA (I)	1	5.26
	2	3.83
	3	3.94
	4	5.95
GalNAc4S (II)	1	4.66
	2	4.07
	3	4.15
	4	4.62
	5	3.86
	6	3.76
	6’	3.80
	amide -CH_3	2.01
GlcA (III)	1	4.62
	2	3.46
	3	3.632
	4	3.86
	5	3.77
GalNAc4S-ol (IV-ol)	1	3.69
	1’	3.70
	2	4.27
	3	4.24
	4	4.48
	5	4.13
	6	3.68
	6’	3.70
	amide -CH_3	2.09

Table S2. Assignments of the carbon-bound protons of the reduced chondroitin sulfate hexasaccharide (CS dp6-ol) at pD 9.0 in 10 mM phosphate buffer acquired at 316.2 K.

Residue	Proton	^{1}H Chemical shift, ppm pD 9.0
ΔUA (I)	1	5.25
	2	3.82
	3	3.94
	4	5.95
GalNAc4S (II)	1	4.64
	2	4.07
	3	4.14
	4	4.62
	5	3.85
	6	3.74
	6’	3.76
	amide -CH_3	2.00
GlcA (III)	1	4.47
	2	3.38
	3	3.57
	4	3.80
	5	3.70
GalNAc4S (IV)	1	4.74
	2	4.02
	3	4.04
	4	4.60
	5	3.99
	6	3.78
	6’	3.80
	amide -CH_3	2.03
GlcA (V)	1	4.62
	2	3.45
	3	3.63
	4	3.86
	5	3.77
GalNAc4S-ol (VI-ol)	1	3.67
	1’	3.71
	2	4.27
	3	4.24
	4	4.50
	5	4.12
	6	3.67
	6’	3.69
	amide -CH_3	2.09

Table S3. Chemical shifts of carbon-bound protons measured at 298.2 K for de-*N*-sulfated Arixtra (pD 7.5), and NAcA (pD 7.8)

Residue	Proton	^{1}H Chemical shift, ppm	
		de-*N*-sulfated Arixtra	NAcA
GlcN6S (I)/	1	5.692	5.423
GlcNAc6S (I)	2	3.287	3.90
	3	3.832	3.56
	4	3.583	3.72
	5	3.90	3.72
	6	4.18	4.36
	6’	4.38	4.73
	amide -CH_3	-	2.023
GlcA (II)	1	4.595	4.602
	2	3.422	3.363
	3	3.736	3.690
	4	3.878	3.79
	5	3.794	3.85
GlcN3S6S (III)/	1	5.433	5.184
GlcNAc6S (III)	2	3.618	4.212
	3	4.556	4.403
	4	4.013	3.983
	5	4.13	4.12
	6	4.27	4.27
	6’	4.507	4.486
	amide -CH_3	-	2.047
IdoA2S (IV)	1	5.250	5.196
	2	4.33	4.30
	3	4.33	4.25
	4	4.18	4.06
	5	4.869	4.846
GlcN6S (V)/	1	4.971	4.75
GlcNAc6S (V)	2	3.305	3.954
	3	3.935	3.81
	4	3.756	3.73
	5	4.05	4.012
	6	4.28	4.30
	6’	4.37	4.36
	-CH_3	3.447	3.38
	amide -CH_3	-	2.023

2.3 NMR Measurements: Amide Proton Resonance Assignments

Following completion of the carbon-bound proton resonance assignments, the samples of the CS dp4-ol and CS dp6-ol were lyophilized, reconstituted in 630 µL of H_2O and adjusted to pH 7.40 with NaOH and HCl solutions. After pH adjustment, 70 µL of D_2O was added and a 600 µL aliquot transferred to individual 5 mm NMR tubes. Similarly, the lyophilized NAcA sample was reconstituted in 270 µL of H_2O and adjusted to pH 7.40 before adding 30 µL of D_2O. A 200 µL aliquot was transferred to the NE-H5/3-Br NMR tube. The assignment of the carbon-bound protons and the amide protons of the GlcNAc3S monosaccharide was performed using the solution prepared for variable temperature experiments.

The 1D ^{1}H, COSY and TOCSY spectra of the CS dp4-ol and CS dp6-ol solutions were acquired at 316.2 K and 308.2 K, respectively, using a Bruker Avance spectrometer operating at 599.58 MHz and equipped with a 5 mm BBI probe. A 90° pulse length of 8.73 µs (tetrasaccharide) or 8.82 µs (hexasaccharide) were used at a power level of -4.00 dB with solvent suppression provided by excitation sculpting. The one-dimensional ^{1}H NMR spectrum was recorded using a spectral window of 6887 Hz and a relaxation delay of 2.0 s with 280 scans coadded into 32,768 complex points. Free induction decays (FIDs) were zero-filled to 65,536 points and apodized by multiplication with an exponential function equivalent to 0.3 Hz line broadening prior to Fourier transformation. The phase-sensitive COSY and TOCSY spectra were acquired into 2048 complex points over 256 increments using States-TPPI with a 2.0 s relaxation delay and a spectral window of 7003 Hz in both dimensions. The COSY spectra were recorded by coaddition of 96 and 160 scans for the tetra- and hexasaccharide, respectively, while 72 (tetrasaccharide) and 96 scans (hexasaccharide) were coadded for the TOCSY spectra. The TOCSY spectra were acquired using a mixing time of 120 ms with a 43 µs spinlock pulse. The COSY and TOCSY spectra were zero-filled to 4096 × 1024 data points and apodized using a $\cos^2$ window function.

^{1}H, COSY, and TOCSY spectra of GlcNAc3S and NAcA were acquired using a Bruker Avance spectrometer operating at 599.88 MHz equipped with a 5 mm TBI probe. Spectra were acquired at 298.2 K using a 90° pulse of 10.4 µs (GlcNAc3S) or 9.83 µs (NAcA) at a power level of -4.00 dB with solvent suppression provided by excitation sculpting. The 1D ^{1}H NMR spectra were recorded using a spectral window of 6887 Hz and a relaxation delay of 2.0 s with 128 scans coadded into 32,768 complex points. FIDs were zero

filled to 65,536 points and apodized by multiplication with an exponential function equivalent to 0.3 Hz line broadening prior to Fourier transformation.

Phase-sensitive COSY and TOCSY spectra were acquired using States-TPPI into 2048 complex points over 256 increments using a 2.0 s relaxation delay and a spectral window of 6614 Hz in both dimensions. The COSY spectrum was recorded by coaddition of 48 scans while 40 scans were coadded for the TOCSY spectrum. The TOCSY spectrum was acquired using a mixing time of 120 ms with a 41 µs (GlcNAc3S) or a 39 µs (NAcA) spinlock pulse. The COSY and TOCSY spectra were zero filled to 4096 × 1024 data points and apodized using a $\cos^2$ window function.

Amide protons assignments for the CS dp4-ol, CS dp6-ol, NAcA, and GlcNAc3S are shown in Table S4. Carbon-bound proton chemical shift assignments for GlcNAc3S is shown in Table S5.

Table S4. Amide proton chemical shifts assignments. (a) CS dp4-ol, (b) CS dp6-ol, (c) NAcA, (d) GlcNAc3S.

a

Residue	^{1}H Chemical shift, ppm	Solution conditions	
		pH	T, K
GalNAc4S (II)	7.652	7.4	316.2
GalNAc4s-ol (IV-ol)	8.008		

b

Residue	^{1}H Chemical shift, ppm	Solution conditions	
		pH	T, K
GalNAc4S (II)	7.702		
GalNAc4S (IV)	7.926	7.4	308.2
GalNAc4s-ol (VI-ol)	8.042		

c

Residue	^{1}H Chemical shift, ppm	Solution conditions	
		pH	T, K
GlcNAc6S (I)	8.289		
GlcNAc3S6S (III)	7.912	7.4	298.2
GlcNAc6S (V)	8.085		

d

Residue	^{1}H Chemical shift, ppm	Solution conditions	
		pH	T, K
GlcNAc3S (α)	7.825	7.4	289.2
GlcNAc3S (β)	8.135		

Table S5. *N*-acetylated GlcN3S (GlcNAc3S) carbon-bound proton chemical shifts assignments acquired in 90% H_2O/10% D_2O solution at 298.2K.

Residue	Proton	^{1}H Chemical shift, ppm pH 7.4
GlcNAc3S (α)	1	5.235
	2	4.044
	3	4.494
	4	3.662
	5	3.92*
	6	4.74**
	6'	3.83**
	amide -CH_3	2.029
GlcNAc3S (β)	1	4.84*
	2	3.78*
	3	4.350
	4	3.628
	5	3.92*
	6	4.74**
	6'	3.53**
	amide -CH_3	2.016

* from the COSY spectrum
** from the TOCSY spectrum

3 Variable Temperature Experiments

3.1 Actual Saccharide Weights for Each Solution

Sample masses used for these experiments were: 1.75 mg GlcNAc3S, 0.77 mg GalNAc, 0.67 mg GlcNAc, 1.02 mg GlcNAc6S, 1.0 mg ΔUA-GlcNAc, 1.0 mg ΔUA-GlcNAc6S, 1.0 mg ΔUA2S-GlcNAc, 1.33 mg ΔUA2S-GlcNAc6S, 1.0 mg ΔUA-GalNAc, 1.14 mg ΔUA-GalNAc4S, 1.0 mg ΔUA-GalNAc6S, 0.97 mg unsaturated reduced chondroitin sulfate A hexasaccharide (CS dp6-ol), 1.80 mg unsaturated reduced chondroitin sulfate A tetrasaccharide (CS dp4-ol), 1.90 mg *N*-acetylated Arixtra (NAcA), 1.2 mg unsaturated reduced hyaluronic acid hexasaccharide (HA dp6-ol), 0.90 mg unsaturated hyaluronic acid (HA dp3), 1.05 mg unsaturated hyaluronic acid pentasaccharide (HA dp5), 1.26 mg unsaturated hyaluronic acid tetrasaccharide (HA dp4), 3.0 mg unsaturated hyaluronic acid hexasaccharide (HA dp6), and 1.12 mg chitin disaccharide (GlcNAc-GlcNAc).

Solutions of GlcNAc and GalNAc were also prepared containing various concentrations of the monosaccharides and buffers. For instance, 6.55 mg GlcNAc and 6.40 mg GalNAc were dissolved in 300 µL of 10 mM phosphate buffer pH 7.48 containing 0.5 mM DSS-d_6. Alternatively, 0.74 mg GlcNAc and 0.62 mg GalNAc were dissolved in 300 µL of 50 mM phosphate buffer at pH 7.48 containing 0.5 mM DSS-d_6. The pH of each solution was adjusted to pH 7.40 with NaOH and HCl, followed by lyophilization. Prior to NMR experiments, the solid samples were dissolved in 270 µL of H_2O and the pH, if needed, was re-adjusted to 7.40. Then, 30 µL of D_2O was added and 200 µL aliquots were transferred to NE-H5/3-Br NMR tubes.

3.2 Determination of Amide Proton Temperature Coefficients

An example of the graph used to calculate amide proton temperature coefficients for GlcNAc monosaccharide is shown in Figure S4. The temperature coefficient is calculated from the slope of the line obtained by plotting chemical shift vs the calibrated temperature. Values of all temperature coefficients can be found in Table S6.

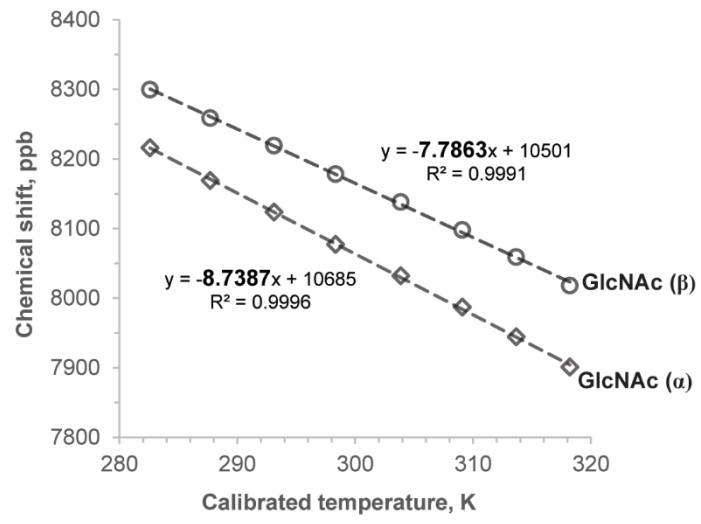

Figure S4. Representative graph for GlcNAc at pH 7.40 for the determination of the amide proton temperature coefficient, $\Delta\delta/\Delta T$ (in ppb/K).

Table S6. Absolute values of the amide proton temperature coefficients, Δδ/ΔT, in ppb/K measured at pH 7.40. To facilitate the interpretation of the relative differences, temperature coefficients are discussed throughout in terms of their absolute values.

Saccharide name	Residue number					
	I	II	III	IV	V	VI
GlcNAc		8.7(α) / 7.8(β)				
GlcNAc6S		8.6(α) / 7.6(β)				
GlcNAc3S		7.1(α) / 8.1(β)				
GalNAc		8.6(α) / 7.5(β)				
ΔUA-GlcNAc		8.7(α) / 7.6(β)				
GlcNAc-GlcNAc	8.3	9.0(α) / 7.8(β)				
ΔUA-GlcNAc6S		8.4(α) / 7.5(β)				
ΔUA2S-GlcNAc		8.3(α) / 7.4(β)				
ΔUA2S-GlcNAc6S		8.3(α) / 7.5(β)				
ΔUA-GalNAc		8.1(α) / 7.2(β)				
ΔUA-GalNAc4S		7.4(α) / 7.2(β)				
ΔUA-GalNAc6S		8.2(α) / 7.4(β)				
HA dp3		6.7				
HA dp4		6.7		8.9(α) / 7.6(β)		
CS dp4-ol		6.1		5.8		
HA dp5		6.6		6.7		
NAcA	7.8		7.4		7.8	
HA dp6		6.5		6.6		8.9(α) / 7.5(β)
HA dp6-ol		6.7		6.1		8.4
CS dp6-ol		6.1		6.1		6.2

3.3 Determination of Activation Energy Barriers Associated with Amide Proton Solvent Exchange

An example of determination of activation energy barriers, $\Delta G^{\ddagger}$ (in kcal/mol), associated with amide proton solvent exchange for the GlcNAc α-anomer is shown in Figure S5. Experimentally measured spectrum at 293.2K (shown in black) was fitted into a simulated Lorentzian peak shape (shown in blue) using MestReNova-12.0.1 (Figure S5a). The extracted line width at half-height of fitted spectrum (shown in purple) with residuals shown in red, were consequently plotted as a function of the calibrated temperature and fitted into the Eyring-Polanyi equation (Figure S5b) using Mathematica 11.0. Each red data point in this graph corresponds to amide proton resonance line width measured at half height as function of calibrated temperature, while the blue line represents an exponential fit of the data to the Eyring-Polanyi equation. In Figure S5c, is also shown residual line widths (difference between measured and fitted line widths) for plot in Figure S5b. Results with determined activation energy barriers for all amide protons in experimental oligosaccharides can be found in Table S7.

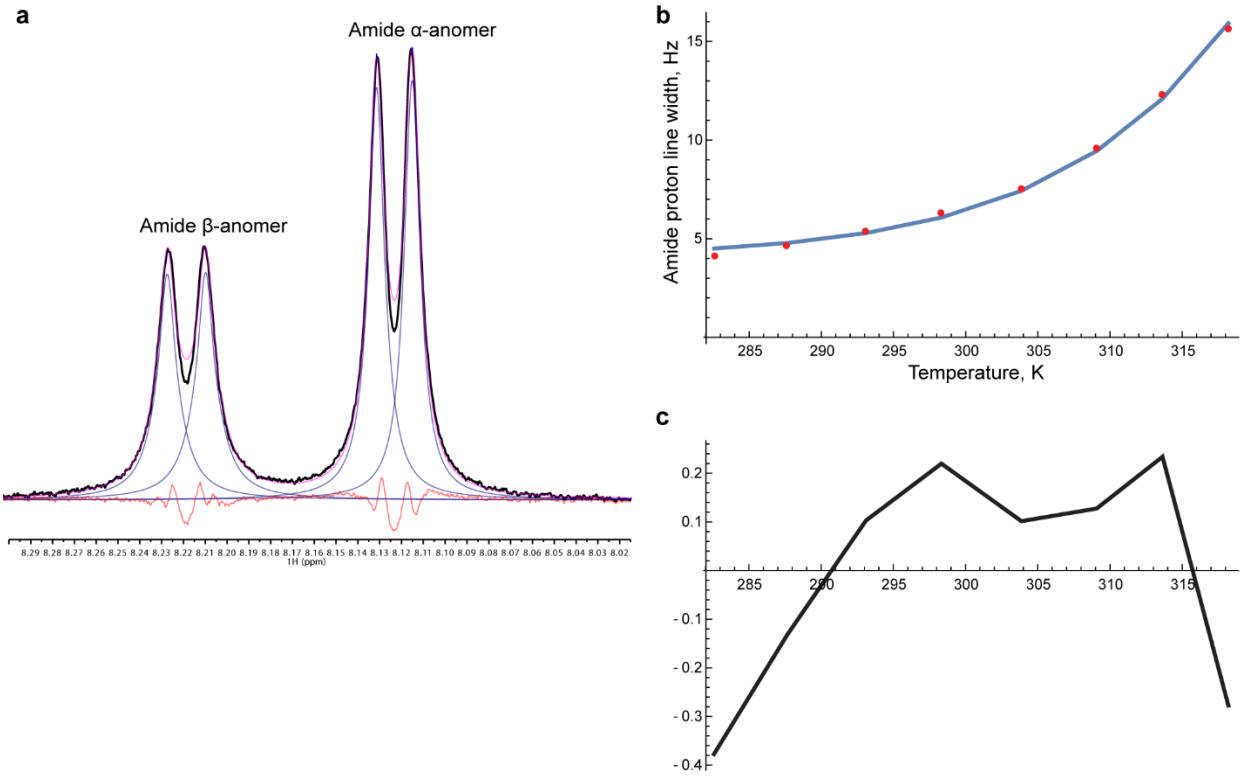

Figure S5. Amide proton activation energy barriers calculations for GlcNAc α-anomer. (a) Amide proton resonance peak fitting to extract the line widths at half height for the GlcNAc α and β anomers measured at 293.2 K using MestReNova-12.0.1 software. (b) Example plot of the determination of activation energy barriers, $\Delta G^{\ddagger}$, for GlcNAc α anomer. (c) Residual line widths (the difference between measured and fitted line widths) for the plot shown in (b)

Table S7. Calculated activation energy barriers, $\Delta G^{\ddagger}$ (in kcal/mol) associated with amide proton solvent exchange. Errors were calculated through a numerical estimate of the covariance matrix and corresponded to ± 2.48 times the standard error for each parameter in this two-parameter fit. To compensate for systematic errors, the calculated errors for each measurement were rounded up to ± 0.1 kcal/mol.

Saccharide name	Residue number					
	I	II	III	IV	V	VI
GlcNAc		16.4(α) / 15.8(β)				
GlcNAc6S		16.2(α) / 15.7(β)				
GlcNAc3S		17.8(α) / 17.7(β)				
GalNAc		16.5(α) / 15.9(β)				
ΔUA-GlcNAc		16.5(α) / 15.5(β)				
GlcNAc-GlcNAc	15.8	16.2(α) / 15.6(β)				
ΔUA-GlcNAc6S		16.5(α) / 15.7(β)				
ΔUA2S-GlcNAc		16.4(α) / 15.9(β)				
ΔUA2S-GlcNAc6S		16.6(α) / 16.0(β)				
ΔUA-GalNAc		16.8(α) / 16.0(β)				
ΔUA-GalNAc4S		17.7(α) / 16.2(β)				
ΔUA-GalNAc6S		16.9(α) / 15.9(β)				
HA dp3		17.6				
HA dp4		17.5		16.6(α) / 16.0(β)		
CS dp4-ol		17.6		17.7		
HA dp5		17.8		18.0		
NAcA	17.7		19.5		17.4	
HA dp6		17.6		17.7		16.9(α) / 16.0(β)
HA dp6-ol		17.6		17.7		16.9
CS dp6-ol		17.6		17.8		18.4

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