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ENZYMATIC PREPARATION OF HEPARIN DISACCHARIDES AS BUILDING BLOCKS IN GLYCOSAMINOGLYCAN SYNTHESIS

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ABSTRACT

Pharmaceutical heparin and heparan sulfate, isolated from a side-stream of a commercial heparin manufacturing process, have been enzymatically depolymerzed with heparin lyases obtained from *Flavobacterium heparinun*. Heparin afforded a trisulfated disaccharide product that was recovered from the reaction mixture using gel permeation chromatography. Heparan sulfate afforded unsulfated disaccharide that was conveniently recovered from the product mixture by ion exchange chromatography. Both disaccharides were obtained in gram amounts at 90% or higher purity.

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Both enzymatically prepared disaccharides were chemically protected to prepare building blocks required for the future chemical synthesis of therapeutically valuable heparin oligosaccharides.

INTRODUCTION

Heparin **1** is a linear, highly sulfated polysaccharide (Figure 1) that has been used clinically as an anticoagulant for over 60 years.[1] Heparin and the related glycosaminoglycan, heparan sulfate **2** also exhibit a number of additional biological activities[2] mediated through their specific interaction with many different heparin binding proteins.[3,4]

Pharmaceutical heparin is prepared from animal tissues, primarily porcine intestinal mucosa, in quantities of over 30 metric tons per year.[1] During the past decade, low molecular weight (LMW) heparins have been successfully introduced as new anticoagulant, anti-thrombotic drugs.[5] LMW heparins (Mr (avg.) 4,000-8,000) are prepared through the controlled, partial depolymerization of pharmaceutical heparin (Mr (avg.) 12,000) using chemical or enzymatic methods. Heparin and LMW heparin are polydisperse ($M_W/M_N > 1$) and microheterogeneous (having sequence heterogeneity) mixtures.[1,2] There has been a growing interest in preparing chemically defined heparins with improved pharmacological properties by either chemical or enzymatic methods. A heparin pentasaccharide, which binds the serine protease inhibitor antithrombin III (ATIII), has been prepared from monosaccharide building blocks in an elegant but complex multistep chemical synthesis.[6]

The high number of synthetic steps required and the low product yields makes the therapeutic exploitation of this chemically synthesized defined heparin pentasaccharide problematic. Recently, our laboratory prepared an octasaccharide, containing the ATIII pentasaccharide binding site sequence, directly from pharmaceutical heparin using a single enzymatic step relying on a heparin lyase.[7,8] The low yield and tedious purification steps required for the isolation of this octasaccharide limit its utility as a pharmaceutical agent.

Our laboratory has proposed a chemoenzymatic method to synthesize therapeutically useful heparin oligosaccharides.[9] In this approach, disaccharide building blocks would be prepared enzymatically from heparin or modified heparins.[10] These disaccharides would then be chemically protected and coupled to each other to obtain heparin oligosaccharides having therapeutic utility. This manuscript reports the gram-scale enzymatic preparation of two heparin disaccharides and the chemical protection of their free hydroxyl groups, for use as building blocks in the chemical synthesis of heparin oligosaccharides.

EXPERIMENTAL

Materials

Porcine intestinal mucosal heparin (180 U/mg) and heparan sulfate (< 4 U/mg)[11] were purchased from Celsius Laboratories (Franklin, OH). Heparin lyases I (EC 4.2.2.7) and III (EC 4.2.2.8) from *Flavobacterium heparinum* were kindly provided from IBEX (Montreal, Canada). Dowex macroporous resin (MSA-1, H) and bovine serum albumin were from Sigma (St. Louis, MO).

Gel permeation chromatography was performed on Bio-Gel P-2 (fine) and Bio-Gel P-10 from Bio-Rad (Richmond, CA). Disaccharide standards (ΔUA2S(1$\rightarrow$4)-D-GlcNS6Sα **3** and ΔUA2S(1$\rightarrow$4)-D-GlcNAα,β **4**) were from Grampian Enzymes (Aberdeen, Scotland). D_20 (99.996 atm%) and $CDCl_3$ were from Aldrich Chemical Co. (Milwaukee, WI).

All other reagents used were analytical grade.

SAX-HPLC was performed on 5 µm Spherisorb columns from Phenomenex (Torrells, CA) of dimensions 2.5 x 25 cm and 0.46 × 25 cm using dual-face programmable LC-7A titanium-based pumps from Shimadzu (Kyoto, Japan). This system was equipped with a Rheodyne (Cotati, CA) titanium injector and a Pharmacia LKB variable-wavelength UV detector (Piscataway, NJ) and with a Shimadzu chromatopac C-R2A integrating recorder.

CE was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, model I, equipped with high-voltage power supply capable of constant or gradient voltage control using a fused silica capillary, 50 µm i.d., 375 µm o.d., 75 cm in long, 70 cm effective length, from Dionex Corporation (Sunnyvale, CA). UV spectrometry was performed on a HP 8453 spectrometer equipped with a thermostated cell.

Enzymatic Preparation of Heparin Disaccharide 3

Heparin (5 gm) was depolymerized with 5 U of heparin lyase I (EC 4.2.2.7) in 50 mL of 50 mM sodium phosphate pH 7.0 buffer containing 2 mg/mL bovine serum albumin for 3.5 days. At various time points, 10 µL aliquots were removed, added to 990 µL of 30 mM HCl, the absorbance at 232 nm was measured and the absorbance was determined until absorbance was constant (complete digestion). The mixture was boiled for 5 min, cooled and loaded in either 1 or 2 gm portions onto a 2.5 x 100 cm. Bio-Gel P-10 column monitored by measuring absorbance at 232 nm was eluted with 0.1 M sodium chloride at a flow rate of 36 mL/h. The heparin disaccharide **3** (ΔUA2S(1$\rightarrow$4)-D-GlcNS6Sα), elut-

ing last as the largest peak between 380 and 445 mL, was freeze-dried, dissolved in 5 mL of water and desalted using a 5 x 50 cm Bio-Gel P-2 column and freeze-dried to afford a total of 1 gm disaccharide **3**.

Preparation of Heparan Sulfate Disaccharide 4a,b

Heparan sulfate (2 gm) was depolymerized with 400 mU of heparin lyase III (EC 4.2.2.8) in 20 ml of 25 mM sodium phosphate pH 7.0 buffer containing150 mM NaCl for 3 days. At various time points, aliquots were removed, added to 30 mM HCl the absorbance at 232 nm was measured and digestion was continued until the absorbance was constant (complete digestion). The mixture was boiled for 5 min to stop the reaction. The pH of the mixture was adjusted to 1.3 and applied on the column (2.5 x 20 cm) packed with Dowex macroporous resin (MSA-1) equilibrated with 0.01 M HCl at a flow rate of 100 mL/h. The heparan sulfate disaccharide **4a,b** ΔUA (1→4)-D-GlcNAcα,β, was eluted with five column volumes of 0.01 N HCl. The non-interacting fraction was collected and neutralized with 0.1 N NaOH. and freeze dried to afford 1.2 gm of disaccharide **4a,b**.

CE Analysis of Heparin and Heparan Sulfate Disaccharides

The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with 1 M dibasic sodium phosphate as described previously.[12] The capillary (50 μm inner diameter, 375 μm outer diameter, 75 cm long, 70 cm effective length) was manually washed before use with 0.5 mL of 0.5 M sodium hydroxide followed by 0.5 mL of distilled water and then 0.5 ml running buffer. Samples were applied using pressure injection (5 psi, 2 or 3 sec) resulting in a sample volume of 70 or 105 nL. Each experiment was conducted at a constant 18 k V. Data collection was at 232 nm.

MS Analysis

Negative-ion and positive-ion fast atom bombardment (FAB) mass spectra were obtained on a VG Analytical ZAB-HF instrument in the using a Xenon beam for ionization. Triethanolamine was used as matrix for the sodium salts of the sulfated oligosaccharides.[13] The negative-ion mass electrospray ionization (ESI) spectra were also obtained by using a Micromass Autospec instrument equipped with an electrospray interface. Samples dissolved in 1:1 water/acetonitrile with 0.05% NH_4OH were injected at a flow rate of 20 μL/min.[14]

NMR Spectroscopy

For ^{1}H-NMR spectroscopy in D_2O, each sample was exchanged three times with 0.5 mL portions of D_2O (99.996%, Sigma), followed by *in vacuo* desiccation over P_2O_5. The thoroughly dried sample was re-dissolved in 0.7 mL of ^2H_2O (99.90%). Spectra were obtained using a UNITY- Varian 500 MHz, a Bruker AMX 600 MZ and a Bruker WM 360 MHz spectrometers.

Two-dimensional (2D) spectra were obtained on the unity Varian 500 spectrometer. 2D double quantum-filtered COSY and TOCSY spectra were recorded using the phase-sensitive mode. All 2D spectra were recorded using the phase-sensitive mode. All two-dimensional spectra were recorded with 512 x 2048 data points and a spectral width of 3200 Hz. The water resonance was suppressed by selective irradiation during the relaxation delay. A total of 128-256 scans were accumulated for each t_1, with a relaxation delay of 2 s. The digital resolution was 1.6 Hz/point in both dimensions with zero-filling in the t_1 and t_2 dimensions in the case of double quantum-filtered COSY, and a Lorentz-Gauss function was applied in all other cases. The inverse heteronuclear correlation (HETCOR) experiment was performed using standard Varian software with 128 t_1 increments, each acquired with 104 scans.

2-O-Sulfo-α-L-threo-hex-4-enopyranosyluronic Acid-(1→4)-2-Deoxy-2-sulfamido-6-O-sulfo-α-D-glucopyranose, Tetrasodium Salt (3)

FABMS (-ve) *m/z* 642, 620 and 598 [M-4H+3Na]$^-$[M-3H+2Na]$^-$and [M-2H+Na];^{1}H NMR (600 MHz, D_2O) δ3.29 (dd,1H, $J_{1,2}$ 3.5 Hz, $J_{2,3}$ 10.3 Hz, H-2), 3.77 (t, 1H, $J_{3,4}$ 10.3 Hz, H-3), 3.88 (t, 1H, $J_{4,5}$ 9.7 Hz, H-4), 4.17 (m, 1H, H-5), 4.23 (dd, 1H, $J_{2',3'}$ 4.0Hz, $J_{3',4'}$ 4.4 Hz, H-3'), 4.29 (dd, 1H, $J_{5,6a}$ 3.8 Hz, $J_{6a,b}$ 11 Hz, H-6a), 4.37 (dd, 1H, $J_{5,6b}$ 2.8 Hz, H-6b), 4.57 (dd, 1H, H-2'), 5.46 (d, 1H, H-1), 5.52 (d, 1H, $J_{1',2'}$ 3.6 Hz, H-1'), 5.95 (d, 1H, H-4').^{13}C NMR (90.56 MHz, D_2O) 60.4 (C-2), 65.9 (C-3'), 69.4 (C-6),70.7 (C-5), 72.1 (C-3), 77.6 (C-2'), 81.2 (C-4), 93.9 (C-1), 99.7 (C-1'), 109.4 (C-4') 147.3 (C-5),171.8 (CO_2H).

4-Deoxy-α-L-threo-hex-4-enopyranosyluronic Acid-(1→4)-2-Acetamido-2-deoxy-α-D-gluçopyranose (4a) and 4-Deoxy-α-L-threo-hex-4-enopyranosyluronic Acid-(1→4)-2-Acetamido-2-deoxy-β-D-glucopyranose (4b)

FABMS (-ve) *m/z* 400 [M-2H+Na]$^-$; **4a**: ^{1}H NMR (500 MHz, D_2O), δ 2.00 (s, 3 H, NAc), 3.77 (m, 1H, H-4), 3.81 (dd, 1 H, $J_{1',2'}$ 5.8 Hz, $J_{2',3'}$ 5.7 Hz, H-2'), 3.85-3.88 (m, 2 H, H-6a and H-6b), 3.89 (m, 2 H, H-2 and H-3), 3.96 (m, 1 H, H-5), 4.23 (dd, 1 H, $J_{3',4'}$ 2.9 Hz, H-3'), 5.15 (d, 1 H, H-1'), 5.21 (d, 1 H, $J_{1,2}$ < 1.0

Hz, H-1), 5.82 (d, 1 H, H-4'). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 25.92 (OCH_3), 56.83 (C-2), 69.41 (C-3' and C-6), 72.17 (C-3), 73.00 (C-2'), 73.44 (C-5), 81.32 (C-4), 93.65 (C-1), 103.55 (C-1'), 110.85 (C-4'), 147.56 (C-5'), 172.15 (CO_2H), 177. 69 (CO). **4 b**: ^{1}H NMR (500 MHz, D_2O), δ 2.00 (s, 3 H, NAc), 3.58 (m, 1 H, H-5), 3.70-3.71 (m, 2 H, H-2 and H-3), 3.78 (m, 1 H, H-4), 3.81 (dd, 1 H, $J_{1',2'}$ 5.8 Hz, $J_{2',3'}$ 5.7 Hz, H-2'), 3,82-3.91 (m, 2 H, H-6a and H-6b), 4.23 (dd, 1 H, $J_{3',4'}$ 2.9 Hz, H-3'), 4.70 (d, 1 H, $J_{1,2}$ 7.7 Hz H-1), 5.15 (d, 1 H, H-1'), 5.82 (d, 1 H, H-4'). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 25.92 (OCH_3), 59.44 (C-2), 69.41 (C-3' and C-6), 75.07 (C-3), 73.00 (C-2'), 77.72 (C-5), 81.60 (C-4), 97.69 (C-1), 103.55 (C-1'), 110.85 (C-4'), 147.56 (C-5'), 172.15 (CO_2H), 178. 12 (CO).

3-*O*-Acetyl-4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosylvronic Acid-(1→4)-1,3-di-*O*-Acetyl-2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyran-ose, Tetrasodium Salt (5)

Compound **1** (tetrasodium salt) (46.8 mg, 70.4 μmol) was dissolved in 1 mL of dry formamide under nitrogen. To this solution, 1 mL of dry pyridine containing dimethylaminopyridine catalyst was injected. At 0°C with stirring, acetic anhydride (120 μL, 1.26 mmol) was injected. After 30 min the ice bath was removed and the mixture warmed to room temperature. After 9 h the reaction flask was placed in an ice bath and quenched with 2 mL of water and stirred at room temperature for 2 h. The crude solution was evaporated under high vacuum, followed by addition of water, to give 2 mL. The mixture was purified using SAX-HPLC to give as the major product which was **5**.[15] Desalting on Bio-Gel P-2 with water eluent, concentration under high vacuum, passing through Dowex 50 (Na^+), and lyophilization gave 25 of **5** in mg, 45% as an amorphous solid.[16] FABMS (-ve) *m/z* 790, 768, 746 [M-5H+4Na]$^-$, [M-4H+3Na]$^-$[M-3H+2Na]$^-$, 688, 666, 644^{1}H NMR (360 MHz, D_2O): δ 2.11, 2.13, 2.22 (3s, 9H, CO_2CH_3), 3.70 (dd, 1 H, $J_{2,3}$ 11Hz, H-2), 4.17-4.31 (m, 4H, H-4,5,6a6b) (confirmed by decoupling experiments), 4.62 (dd, 1 H, H-2'), 5.19 (dd, 1 H, H-3), 5.33 (dd, 1 H, $J_{3',4'}$5.1 Hz, H-3'), 5.53 (d, 1 H, $J_{1',2'}$ 1.7 Hz, H-1'), 5.93 (d, H, H-4'), 6.25 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1).

1,3,6,2',3'-Penta-*O*-acetyl-4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic Acid-(1→4)-2-Acetamido-2-deoxy-α-D-glucopyranose (6)

Compounds **4a** and **4b** (50 mg, 0.13 mmol) in solution in anhydrous pyridine (5 mL) were reacted with acetic anhydride (0.1 mL) and dimethylaminopyridine (catalytic amount) at room temperature and under nitrogen. After 48 h, the reaction mixture was quenched by addition of methanol (5 mL) and the solvent

were evaporated. The residue was purified by chromatography on silica gel ($CHCl_3$-CH_3OH, v/v 1:3) to yield **6** in 90 % yield. ^1H NMR (500 MHz, D_2O), δ 1.90-2.20 (6 s, 3 H each, NAc and OAc), 3.96 (d, 1 H, $J_{6a,b}$ 12.2 Hz, H-6b), 4.15 (t, 1 H, $J_{3,4}$ 9.5 Hz, $J_{4,5}$ 9.8 Hz, H-4), 4.36 (dd, 1 H, $J_{5,6a}$ 2.9 Hz, H-6a), 4.37 (t, 1 H, $J_{2,3}$ 10.1 Hz, H-2), 4.42 (m, 1 H, H-5), 4.93 (bs, 1 H, $J_{1',2'}$ < 1.0 Hz, H-2'), 5.17 (bs, 1 H, $J_{2',3'}$ < 1.0 Hz, H-3'), 5.22 (t, 1 H, H-3), 5.30 (bs, 1 H, H-1'), 5.73 (d, 1 H, $J_{NH,2}$ 8.9 Hz, NH), 6.12 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 6.16 (bs, 1 H, H-1').

Methyl 1,3,6,2',3'-Penta-*O*-acetyl-4-Deoxy-α-L-*threo*-hex-4-enopyranosyl-uronate (1→4)-2-Acetamido-2-deoxy-α-D-glucopyranose) (7)

Compound **6** (71 mg, 0.13 mmol) in solution in anhydrous DMF (2 mL) was esterified with CH_3 (41 µL, 0.65 mmol) and $KHCO_3$ (65 mg, 0.65 mmol) at room temperature and under nitrogen. After 18 h, the reaction mixture was filtered and concentrated under vacuum. Purification of the residue by chromatography on silica gel afforded **7** in 76 % yield. ^1H NMR (500 MHz, $CDCl_3$), δ 1.90-2.20 (6 s, 3 H each, NAc and OAc), 3.83 (s, 3 H, CO_2CH_3), 3.95 (m, 1 H, H-5), 4.07 (t, 1 H, $J_{3,4}$ 9.7 Hz, $J_{4,5}$ 9.7 Hz, H-4), 4.29 (d, 1 H, $J_{6a,b}$ 12.3 Hz, H-6b), 4.37 (m, 1 H, $J_{1,2}$ 3.7 Hz, $J_{2,3}$ 9.2 Hz, H-2), 4.40 (d, 1 H, H-6a), 4.92 (bs, 1 H, $J_{1',2'}$ < 1.0 Hz, H-1'), 5.21 (t, 1 H, H-3), 5.17 (bs, 1 H, $J_{2',3'}$ < 1.0 Hz, H-3'), 5.31 (bs, 1 H, H-2'), 5.63 (d, 1 H, $J_{NH,2}$ 9.1 Hz, NH), 6.12 (d, 1 H, H-1), 6.21 (bs, 1 H, H-4').

RESULTS AND DISCUSSION

Porcine intestinal mucosal heparin **1** and heparan sulfate **2** (Figure 1) commercially obtained in 100 gm quantities, were analyzed on small (100 µg) scale for disaccharide composition.[12] Capillary electrophoresis (CE) analysis of the product mixtures demonstrated that exhaustive heparin lyase I treatment of heparin yielded 30 wt. % of the desired trisulfated disaccharide **3**, ΔUA2S(1→4)-D-GlcNS6Sα. The remaining 67 wt % of product was primarily higher oligosaccharides (tetra-, hexa-, and octasaccharides) with a small amount (<3 wt. %) of contaminating disulfated disaccharide, ΔUA2S(1→4)-D-GlcNS6Sα.

Similarly, exhaustive treatment of heparan sulfate with heparin lyase III yielded 60 wt % unsulfated disaccharide **4a,b**, ΔUA(1→4)-D-GlcNAcα, β, with 30 wt. % higher oligosaccharides. Significant quantities of monosulfated disaccharides, ΔUA(1→4)-D-GlcNAc6Sα,β (2%) and ΔUA2S(1→4)-D-GlcNSα (8%), are also formed on enzymatic depolymerization.

The large scale depolymerization of both heparin **1** and heparan sulfate **2** with heparin lyase I and III, respectively, proceeded as expected based on the small scale reaction (Fig.1). To conserve enzyme the reaction was run over a

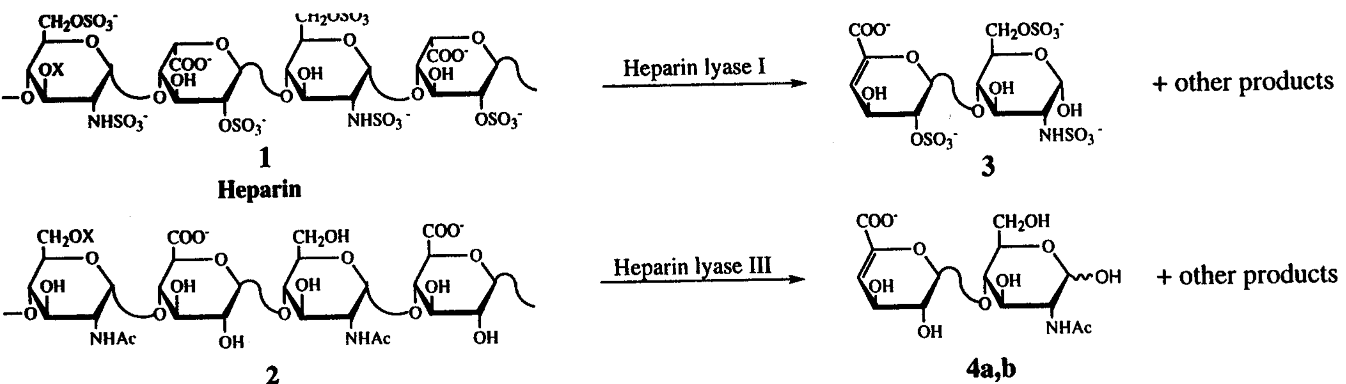

Figure 1. Structure of heparin **1** and heparan sulfate **2** and their enzymatic depolymerization into disaccharides **3** and **4a,b**.

period of 3 days. Longer reaction times were examined but these resulted in microbial contamination, even when care was taken to filter sterilize both enzyme and substrate prior to digestion. At reaction completion both reactions were stopped by thermally inactivating the enzyme.

Different strategies were used to recover the two desired disaccharide products **3** and **4a,b** from the reaction mixtures. In the case of the heparin reaction mixture, the separation was primarily between the desired, trisulfated, heparin disaccharide **3** and sulfated higher oligosaccharides. Gel permeation chromatography on Bio-Gel P-10 was used successfully in this separation (Figure 2) affording trisulfated disaccharide **3** recovered in the final peak in 20 wt % yield from heparin at a purity of 90%. By collecting the final two-thirds of this peak, a reduced yield of 15% could be obtained with purity of > 99%. The recovery of the desired unsulfated disaccharide **4a,b** from the heparin lyase III depolymerized heparan sulfate was considerably simpler. Low pressure anion-exchange chromatography was used, since disaccharide **4a,b** was not sulfated and all the remaining disaccharides and higher oligosaccharides in the reaction mixture were sulfated.

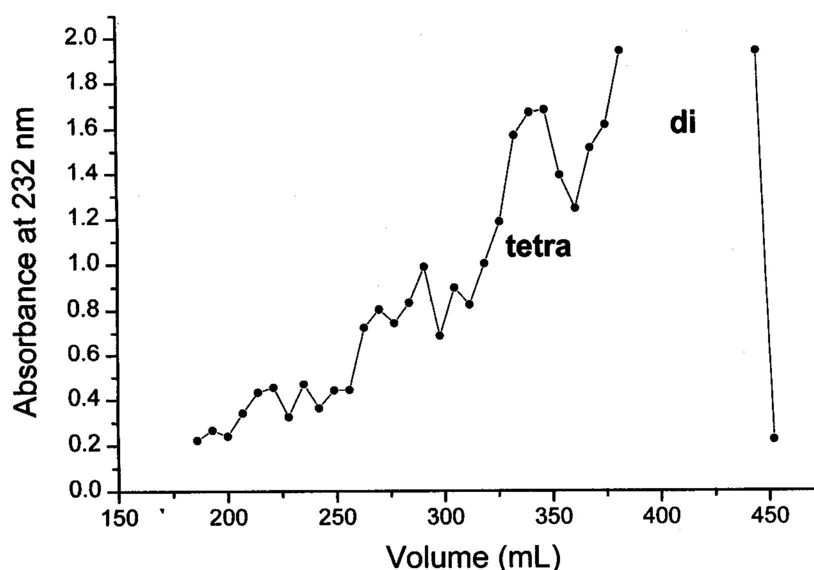

Figure 2. Gel permeation chromatography of heparin lyase I depolymerized heparin. The peaks corresponding to the disaccharide (di, 375–450 mL) and the tetrasaccharide (tetra, 320–360 mL) are labeled.

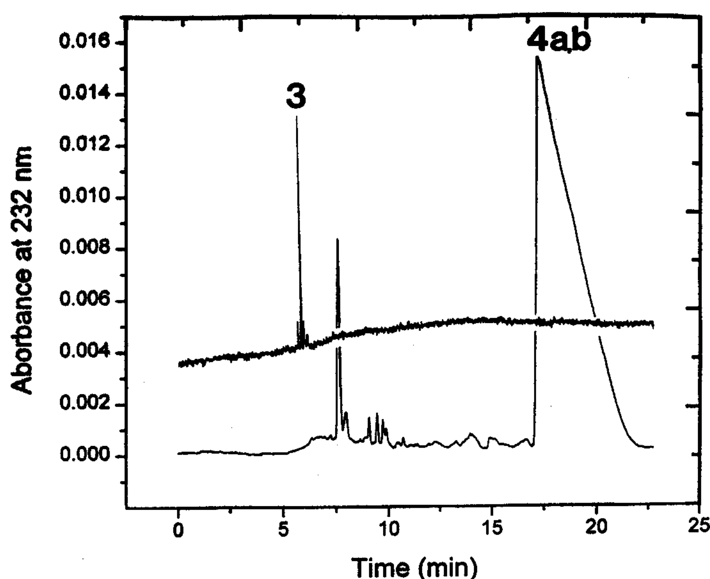

Figure 3. CE analysis of the heparin trisulfated disaccharide **3** and the heparan sulfate unsulfated disaccharide **4a,b**.

Previously, our laboratory has shown that the pKa of the carboxyl group on unsaturated uronides was ~3.0[17] while the pKa of *N*- and *O*-sulfo groups are < 1.5.[18] At a pH 2, disaccharide **4a,b** is uncharged, while all the contaminating sulfated disaccharides and oligosaccharides are negatively charged. This permitted the simple recovery of purified disaccharide **4a,b** in the column run-through with a yield of 55 wt. % and a purity of 91%.

Purity was determined by analysis of the recovered disaccharides using CE (Fig. 3). FAB and ESI mass spectrometry were used to confirm the molecular weight of each disaccharide through the observation of the molecular-ions present in their spectra. The NMR spectra of both disaccharides, were assigned with the assistance of various 2D-NMR methods and were consistent with their chemical structure.

Next, each enzymatically prepared disaccharide was chemically protected to prepare building blocks commonly used for the synthesis of higher oligosaccharides. The trisulfated disaccharide **3** obtained from heparin was converted to the tetramethylammonium salt and peracetylated with acetic anhydride in formamide-pyridine to give protected disaccharide **5** in good yield (Fig. 4). The sodium salt of unsulfated disaccharide **4a,b** was directly peracetylated to afford **6**, which was then converted to the peracetylated methyl ester **7** in high yield.

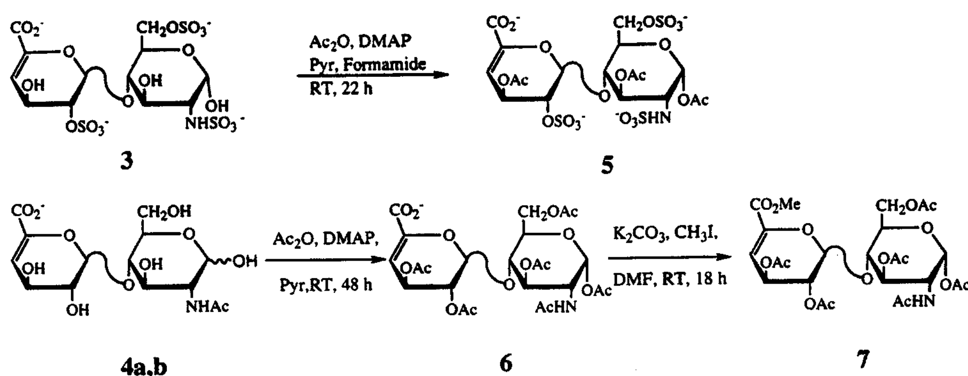

Figure 4. Chemical synthesis of peracetylated trisulfated disaccharide **5** and peracetylated and methyl esterified unsulfated disaccharides **6** and **7**.

These protected disaccharides can be prepared in a single enzymatic and one or two chemical steps in high yields and in gm quantities. Disaccharides **5-7** represent the building blocks for the future chemical synthesis of larger heparin and heparan sulfate oligosaccharides. Recently, we reported the selective conversion of various Δ 4,5 unsaturated uronate model compounds into iduronic and glucuronic acid derivatives.[19] Such derivatives contain a single unprotected hydroxyl group at the 4-position, facilitating their conversion to higher oligosaccharides using standard glycosylation chemistry.[9] Work is currently in progress to explore this chemistry on the disaccharides prepared in this study.

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