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Preparation and application of a "clickable" acceptor for enzymatic synthesis of heparin oligosaccharides

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Abstract

A "clickable" disaccharide was prepared by treating the aldehyde precursor with hydroxylamine, followed by the catalytic hydrogenation and diazotransfer reaction. This disaccharide was successfully applied to the elongation of the backbone construction of ultralow molecular weight (ULMW) heparins using two bacterial glycosyl transferases, *N*-acetyl glucosaminyl transferase from *Escherichia coli* K5 (KfiA) and heparosan synthase-2 (pmHS2) from *Pasteurella multocida*.

Keywords

Heparan sulfate; Heparin; Heparosan; Depolymerization; Azido-clickable acceptor; Enzymatic glycosylation

1. Introduction

Heparan sulfate (HS) and heparin are linear, highly sulfated, anionic polysaccharides that belong to the glycosaminoglycan (GAG) family.[1] These GAGs are composed of a repeating disaccharide motif of a glucuronic acid (GlcA) or an iduronic acid (IdoA) residue and a glucosamine residue, including *N*-acetylglucosamine (GlcNAc), *N*-sulfoglucosamine (GlcNS) or *N*-unsubstituted glucosamine ($GlcNH_2$).[2] HS is an abundant GAG on the surface of mammalian cells and in the extracellular matrix. It plays regulatory roles in several

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Supplementary data
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pathophysiological processes, such as development, angiogenesis, blood coagulation, and tumor metastasis.[3,4,5]

Preparation of pure GAG oligosaccharides is a major challenge for carbohydrate chemists[6,7,8] because traditional chemical synthesis of structurally defined GAGs relies on the efficient introduction of protecting groups, epimerization of GlcA to IdoA, stereoselective glycosylation, the efficient removal of protecting groups and sulfonation, requiring a large number of synthetic steps to furnish the product.[9,10,11] The biosynthesis of GAGs in the Golgi of eukaryotic cells suggests a more efficient enzymatic route, which includes the building of the polysaccharide backbone, as well as introducing sulfo groups and IdoA residues.[12,13] Employing a chemoenzymatic approach in our previous work, we successfully synthesized the ULMW heparin heptasaccharides with 10 and 12 steps in 45% and 37% overall yields based on the disaccharide acceptor degraded from heparosan (Figure 1), which showed very similar binding affinities and *in vitro* anti-Xa activities comparing with the commercial drug Arixtra.[14,15]

The chemoenzymatic synthesis of structurally defined heparin oligosaccharide glycoconjugates or immobilized heparin oligosaccharides might be accomplished through the introduction of a reactive site into the heparin oligosaccharide. A heparin oligosaccharide backbone can be elongated on a disaccharide acceptor to achieve this goal.[16] We report the synthesis of a disaccharide acceptor with an azido group as a reactive site at the reducing end (Figure 1). This disaccharide acceptor can be applied to a “click” reaction or released as a free amine group for diverse conjugation. This modified disaccharide is quite suitable for use as an extendable acceptor for the backbone elongation for the construction of ultralow molecular weight (ULMW) heparins.

2. Results and discussion

Heparosan (→4) GlcA (1→4) GlcNAc (1→)$_n$[17] has been prepared from the *Escherichia coli* K5 strain[18] and isolated at kilogram scale and was employed as starting material for our disaccharide acceptor target (Scheme 1). Heparosan was dissolved in 2 M NaOH solution under 60°C for 24 h to obtain the *N*-deacetylated heparosan polysaccharide according previously described methods.[19,20] The *N*-deacetylated heparosan polysaccharide was deaminated with nitrous acid at low pH (~ 4.5), and neutralized with 2 M NaOH to furnish disaccharide **4** (GlcA (1→4) anhydromannose (AnMan)).[21,22] We found that the free aldehyde group at the reducing end of disaccharide **4** was primarily obtained as the hydrate form, hemiacetal **5.** Hemiacetal **5** was identified by its ^{1}H NMR and mass spectrum, which showed the disappearance of the characteristic aldehyde peak, at ~9 ppm, and displayed the hydrate form $[M+H_2O]^-$. The β-configuration of the glycosidic linkage was confirmed on the basis of the 7.8 Hz $J_{1,2}$ coupling constant, which indicates that the H-1^{II} and H-2^{II} atoms are in a trans relationship to one another.[23] Reductive amination of **5** was initially attempted with sodium cyanoborohydride, however, low yields were achieved using both aliphatic and aromatic amines. Treatment of **5** with hydroxylamine in aqueous sodium acetate solution gave significantly improved yields (~90%), resulting in the formation of oxime **6** as a mixture of the *E* and *Z* isomers.[24] After the size exclusion chromatography, the ^{1}H NMR spectrum of oxime **6** showed doublets at 7.44 (J = 3.78 Hz) and 6.84 (J = 6.88 Hz) corresponding to the presence of the C*H*=N group in an (*E*)-oxime to (*Z*)-oxime ratio of 7:2.

Reduction of oxime **6** was carried out smoothly employing hydrogen with $Pd(OH)_2$/C catalyst affording the corresponding amino derivative **7** in 91% yield. The structure of **7** was confirmed by ^{1}H and 2D NMR spectroscopy as it showed the disappearance of the C*H*=N proton and the appearance of a multiplet at ~3.6 ppm, attributable to a newly formed CH_2 group. Conversion of the amino group to an azido group was subsequently accomplished by

treating **7** with sodium azide, Tf_2O and catalytic $CuSO_4$ in a combined CH_2Cl_2/MeOH/H_2O solution, furnishing compound **1** in 88% yield.[25] The ^{13}C NMR spectrum of compound **1** showed that C-1^I shifted to 51.4 ppm, corresponding to a CH_2 group after introducing an azido group at the reducing end. Compound **1**, an azido-functional acceptor, could be employed to construct heparin oligosaccharide backbones. However, this disaccharide acceptor always contained some impurities that were difficult to remove by the size exclusion chromatography when performing large-scale reactions. Consequently, we protected the carboxyl group with a methyl ester in 95% yield under acidic conditions and the free hydroxyl groups as acetyl esters in quantitative yield to obtain compound **8,** which was easily purified on a silica column. Compound **8** was then quantitatively deprotected by treating with 2M NaOH and desalted to obtain pure disaccharide acceptor **1** (Scheme 2).

Next, the heparin oligosaccharide backbone was prepared by enzymatic glycosylation of disaccharide acceptor **1**. Disaccharide **1** (GlcA-AnMan-N_3) was incubated overnight with UDP-GlcNTFA and KfiA at room temperature to furnish the trisaccharide product. Reverse-phase ion-pairing HPLC (RPIP-HPLC) was employed to monitor the generation of uridine diphosphate (UDP). Here, we employed an unnatural UDP-GlcNTFA donor, as it can be readily converted to an *N*-sulfoglucosamine residue in a subsequent step.[14] Afterwards, one equivalent of UDP-GlcA was added into the above mixture and incubated for 4–5 h in the presence of pmHS2, followed by addition of a second equivalent of UDP-GlcUA and additional pmHS2 to ensure reaction completion. The resulting tetrasaccharide **9** was recovered by size exclusion chromatography. The structures of the trisaccharide intermediate and tetrasaccharide **9** were both confirmed by HR ESI-MS and NMR spectroscopy.

3. Conclusions

We have developed a practical route including acidic depolymerization, oximation, hydrogenation and diazotransfer reaction, towards the large scale preparation of the N_3-containing “clickable” acceptor, which was also successfully applied on the backbone construction of ultralow molecular weight (ULMW) heparin oligosaccharide employing the enzymatic glycosylation. Further elongation and sulfonation of this tetrasaccharide acceptor is ongoing.

4. Experimental

4.1. General Methods

^1H and ^{13}C NMR spectra were recorded at 600 MHz for ^1H NMR, 150 MHz for ^{13}C NMR or 800 MHz for ^1H NMR, 200 MHz for ^{13}C NMR with Topsin 2.1 software. Mass data were acquired by high-resolution ESI-MS. Thin-layer chromatography (TLC) was carried out using plates of silica gel 60 with fluorescent indicator and revealed with UV light (254 nm) when possible and Von’s reagent or ninhydrin solution in ethanol. Flash chromatography was performed using silica gel 230–400 mesh. Yields are given after purification, unless otherwise noted. When reactions were performed under anhydrous conditions, the mixtures were maintained under argon.

4.2. N-Deacetylation of Heparosan (3)

Heparosan **2** (3.3 g, 0.22 mmol) dissolved in 2 M NaOH (165 mL) was heated at 60 °C until the solution is clear. After stirring for another 24 h under an argon atmosphere at 60 °C, the solution was dialyzed with 1000 molecular weight cutoff (MWCO) dialysis membrane against double-distilled water for 24 h. Then, the dialysate was lyophilized to give the *N*-

deactylated heparosan (**3**) as yellow powder. Spectra were in agreement with reported data.[20]

4.3. β-D-glucopyranosiduronate-(1→4)-2,5-Anhydro-D-mannose (4)

A 50 mL solution of 2:5 (1 M H_2SO_4: 5.5 M $NaNO_2$) was added to dry *N*-deacetylated heparosan **3** (2.0 g, 0.13 mmol), and vigorously stirred under ice bath for 30 min. After the pH was adjusted to neutral with 30 mL of 3:5:5 (1 M Na_2CO_3: dd H_2O: 1 M $NaHCO_3$), the solution was dialyzed using 1000 molecular weight cutoff (MWCO) dialysis membrane against double-distilled water for 4 h and the dialysate was lyophilized to give the crude product **4** as off-white powder (BuOH/HCOOH/H_2O = 4:8:1, R_f = 0.44). ^{1}H NMR (600 MHz, D_2O): δ 4.95 (d, 1 H, J = 5.5 Hz, H-2^I), 4.36 (d, 1 H, J = 7.8 Hz, H-1^{II}), 4.23 (t, 1 H, J = 5.0 Hz, H-3^I), 3.99 (m, 1 H, H-5^I), 3.97 (t, 1 H, J = 5.6 Hz, H-4^I), 3.58-3.64 (m, 3 H, H-5^{II}, H-6b^I, H-6a^I), 3.36–3.40 (m, 2 H, H-3^{II}), 3.20 (t, 1 H, J = 6.5 Hz, H-2^{II}). Selected ^{13}C NMR (150 MHz, D_2O): δ 102.0 (C-1^{II}), 89.2 (C-2^I), 85.7 (C-5^I), 84.6 (C-4^I), 82.0 (C-2^I), 76.5 (C-3^I), 75.1 (C-4^{II}), 74.8 (C-5^{II}), 72.8 (C-2^{II}), 71.6 (C-3^{II}); HRMS-FAB: $[M-H]^-$ *m/z* calcd for $C_{12}H_{20}O_{12}$: 355.0877; found: 355.0882.

4.3. β-D-glucopyranosiduronate-(1→4)-2,5-Anhydro-D-mannose oxime (6)

To a solution of **4** (450 mg, 1.33 mmol) in water hydroxylamine hydrochloride (0.11 g, 1.59 mmol) and sodium acetate (0.16 g, 1.91 mmol) were successively added. The solution was stirred overnight at room temperature. The solvent was removed under reduced pressure and the resulting syrup was purified by a Biogel P-2 column (1.5 × 200 cm) at a flow rate of 15 ml/h to give the crude product **6** (420 mg, 89.5%) as off-white powder (BuOH/HCOOH/H_2O = 4:8:1, R_f = 0.51). ^{1}H NMR (600 MHz, D_2O): δ 7.44 (d, 1 H, J = 3.78 Hz, C*H*=N(*E*)), 6.84 (d, 1H, J = 6.88 Hz, C*H*=N(*Z*)), 4.38 (d, 1 H, J = 7.8 Hz, H-1^{II}), 4.28 (m, 1 H, H-3^I), 4.05-4.04 (m, 2 H, H-4^I, H-5^I), 3.96 (m, 1 H, H-2^I), 3.61–3.64 (m, 2 H, H-5^{II}, H-6b^I), 3.57 (m, 2 H, H-6a^I)), 3.37 (m, 1 H, H-3^{II}, H-4^{II}), 3.20 (t, 1 H, J = 6.5 Hz, H-2^{II}). Selected ^{13}C NMR (150 MHz, D_2O): δ 102.0 (C-1^{II}), 85.1 (C-5^{II}), 82.1 (C-3^I), 79.4 (C-4^I), 77.8(C-2^I), 75.1 (C-5^I), 72.8 (C-2^{II}), 71.6 (C-3^{II}), 60.3 (C-6^I); HRMS-FAB: $[M-H]^-$ *m/z* calcd for $C_{12}H_{18}NO_{11}$ 352.0880; found, 352.0883.

4.4. β-D-glucopyranosiduronate-(1→4)-1-amino-2,5-anhydro-1-deoxy-D-mannitol (7)

$Pd(OH)_2$-C 10% (0.19 g) was added to a solution of oxime **6** (240 mg, 0.68 mmol) in water and several drops of acetic acid were subsequently added. The suspension was stirred under H_2 at atmospheric pressure and room temperature for 2 h. The solvent was removed under reduced pressure and the resulting syrup was purified by a Biogel P-2 column (1.5 × 200 cm) at a flow rate of 15 ml/h to obtain compound **7** (210 mg, 0.62 mmol, 91%) as white powder (BuOH/HCOOH/H_2O = 4:8:1, R_f = 0.28). ^{1}H NMR (600 MHz, D_2O): δ 4.39 (d, 1 H, J = 7.6 Hz, H-1^{II}), 4.04–4.08 (m, 2 H), 3.95 (s, 1 H), 3.91 (s, 1 H), 3.77–3.84 (m, 2 H), 3.70–3.75 (m, 1 H), 3.60–3.64 (m, 2 H), 3.55–3.59 (m, 1H), 3.41 (t, 1 H, J = 8.4 Hz), 3.36 (m, 1 H). Selected ^{13} C NMR (150 MHz, D_2O): δ 103.1 (C-1^{II}), 80.3, 76.6, 75.2 (C-5^{II}), 72.8 (C-2^{II}), 71.6 (C-3^{II}), 71.0, 69.4, 68.8, 61.9, 61.1(C-6^I); HRMS-FAB: $[M-H]^-$ *m/z* calcd for $C_{12}H_{20}NO_{10}$: 338.1087; found: 338.1088.

4.5. β-D-glucopyranosiduronate-(1→4)-2,5-anhydro-1-azido-1-deoxy-D-mannitol (1)

A solution of NaN_3 (992 mg, 15.2 mmol) in 2.5 mL of H_2O was cooled to ~0 °C in an ice bath and subsequently added to 5 mL of CH_2Cl_2. A biphasic mixture generally formed that was stirred vigorously and Tf_2O (872 mg, 3.08 mmol) was slowly added drop-wise over a period of 10 min. The reaction was then stirred under ice bath for 2 h, and the organic phase was separated and aqueous phase was extracted with CH_2Cl_2 (2 × 2.5 mL). Then combined

organic phase was extracted with saturated Na_2CO_3 solution and used without further purification. The final concentration of TfN_3 was 0.2 M in 10 mL solvent.

Substrate **7** (120 mg, 0.35 mmol) was dissolved in 5 mL of MeOH: H_2O (1: 1). $CuSO_4$ (2.8 mg, 0.018 mmol) and triethylamine (97 μL, 0.70 mmol, 2 equiv per amine substrate) were added to the solution of substrate **7** with stirring. The mixture was cooled in an ice bath for 15 min, and 0.2 M CH_2Cl_2 solution of triflyl azide (2.1 mL, 1.2 equiv per amino group based on the amount of triflic anhydride used in the preparation of TfN_3) was added into the above reaction solution dropwise. The reaction mixture was allowed to warm to room temperature and a homogeneous solution was obtained after the addition. The reaction was finished in 6 h as determined by monitoring triflyl azide by TLC ($BuOH/HCOOH/H_2O$ = 4:8:1, R_f = 0.56). The solvent was removed under reduced pressure and the resulting syrup was purified by chromatography on a Biogel P-2 column (1.5 × 200 cm) eluted with water at a flow rate of 15 ml/h. The fractions of the crude product **6** (412 mg, 88%) as off-white powder were then collected and subjected to NMR and ESI-MS analysis. ^{1}H NMR (600 MHz, D_2O): δ 4.38 (d, 1 H, J = 7.8 Hz, H-1^{II}), 4.13 (t, 1 H, J = 5.7 Hz, H-3^{I}), 3.99 (m, 2 H, H-5^{I}, H-4^{I}), 3.91 (s, 1 H, H-2^{I}), 3.64 (d, 1 H, J = 12.0 Hz, H-6b^{I}), 3.63 (s, 1 H, H-5^{II}), 3.59 (dd, 1 H, J = 4.7, 12.4 Hz, H-6a^{I}), 3.52 (d, 1 H, J = 13.0 Hz, H-1b^{I}), 3.40-3.35 (m, 3 H, H-1a^{I}, H-4^{II}, H-3^{II}), 3.22 (t, 1 H, J = 7.92 Hz, H-2^{II}). ^{13}C NMR (150 MHz, D_2O): δ 175.6 (C-6^{II}), 101.9 (C-1^{II}), 85.4 (C-5^{I}), 81.5 (C-4^{I}), 80.9 (C-2^{I}), 76.5 (C-3^{I}), 75.3 (C-4^{II}), 74.8 (C-5^{II}), 72.8 (C-2^{II}), 71.5 (C-3^{II}), 60.9 (C-6^{I}), 51.4 (C-1^{I}); HRMS-FAB: $[M-H]^-$ *m/z* calcd for $C_{12}H_{18}N_3O_{10}$, 364.0992; found: 364.1000.

4.6. Methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate-(1→4)-3,6-di-O-acetyl-2,5-anhydro-1-azido-1-deoxy-D-mannitol (8)

The crude compound **1** (45 mg, 0.12 mmol) was dissolved in 3 mL methanol, and 90 mg Amberlite 120 (H^+) resin was added into the solution. The mixture was vigorously stirred for 12 h at room temperature. The resin was filtered to obtain the organic solvent, which was concentrated under vacuum. The resulting residue was dissolved in 2 mL pyridine and 1 mL Ac_2O and was stirred for 12 h at room temperature. The organic solvent was concentrated under reduced pressure, and the residue was purified by flash silica column chromatography (EtOAc–Hexanes 1:2) giving compound **8** (67 mg, 95%) as syrupy. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.19 (t, 1 H, J = 9.5 Hz, H-4^{II}), 5.16 (d, 1 H, J = 9.0 Hz, H-3^{II}), 5.14 (t, 1 H, J = 3.5 Hz, H-3^{I}), 4.95 (t, 1 H, J = 8.5 Hz, H-2^{II}), 4.63 (d, 1 H, J = 7.5 Hz, H-1^{II}), 4.14–4.16 (m, 1 H, H-4^{I}), 4.13 (t, 1 H, J = 3.5 Hz, H-2^{I}), 4.09–4.11 (m, 1 H, H-6b^{I}), 4.03–4.09 (m, 2 H, H-5^{I}, H-6a^{I}), 3.99 (d, 1 H, J = 9.5 Hz, H-5^{II}), 3.68 (s, 3 H, O*Me*), 3.46 (dd, 1 H, J = 7.5, 13.0 Hz, H-1b^{I}), 3.39 (dd, 1 H, J = 4.5, 13.0 Hz, H-1a^{I}), 2.05 (s, 3 H, *Ac*), 2.01 (s, 3H, *Ac*), 1.99 (s, 3 H, *Ac*), 1.96 (s, 2 × 3 H, *Ac*). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.5 (C-6^{II}), 166.9 (*Ac*), 100.6 (C-1^{II}), 84.6 (C-5^{I}), 82.8 (C-2^{I}), 80.2 (C- 4^{I}), 79.3 (C-3^{I}), 72.6 (C-5^{II}), 71.8 (C-4^{II}), 70.8 (C-2^{II}), 69.1 (C-3^{II}), 63.0 (C-6^{I}), 53.0 (O*Me*), 51.9 (C-1^{I}); HRMS-FAB: $[M+H]^+$ *m/z* calcd for $C_{23}H_{32}N_3O_{15}$, 590.1833; found, 590.1840.

4.7. β-D-glucopyranosiduronate-(1→4)- α-D-2-trifluroacetamido-2-deoxy-glucopyranosyl-(1→4)- β-D-glucopyranosiduronate-(1→4)-2,5-anhydro-1-azido-1-deoxy-D-mannitol (9)

To synthesize oligosaccharide backbone, disaccharide (GlcA-AnMan-N_3, **1**) (3.6 mg, 10 μmol) was incubated with UDP-GlcNTFA (8.5 mg, 12 μmol) and KfiA (0.1 mg) in 10 ml of buffer containing 25 mM Tris-HCl (pH 7.2) and 10 mM $MgCl_2$. The reaction was incubated at room temperature overnight, and the reaction mixture was analyzed by a polyamine-based HPLC column to ensure that 95% of UDP-GlcNTFA was converted to UDP. Then 10-μL of reaction solution was passed through a 3000 MWCO spin-column and subjected to mass spectral analysis. HRMS-FAB: $[M-H]^-$ *m/z* calcd for $C_{20}H_{28}F_3N_4O_{15}$, 621.1503; found: 621.1509. Upon the complete consumption of UDP-GlcNTFA, pmHS2 (0.1 mg) and UDP-

GlcA (7.5 mg, 12 μmol) were added into the reaction mixture for additional 4–5 h at room temperature. Another portion of pmHS2 (0.1 mg) and UDP-GlcA (12 μmol) was added to drive the transfer of GlcUA unit to completion. The product was purified using Biogel P-2 chromatography on a column (1.5 × 200 cm) that was equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 15 ml/h. The fraction containing the product was lyophilized to give **9** (6.4 mg, 80%) and subjected to NMR and ESI-MS analysis. ^{1}H NMR (600 MHz, D_2O): δ 5.25–5.31 (m, 1 H), 4.38 (d, 1 H, J = 9.6 Hz), 4.35 (d, 1 H, J = 9.6 Hz), 4.29 (s, 1 H), 4.14 (t, 1 H, J = 5.4 Hz), 4.03 (t, 1 H, J = 5.4 Hz), 3.98 (dd, 1 H, J = 5.4, 10.2 Hz), 3.93–3.95 (m, 1 H), 3.90 (dd, 1 H, J = 5.4, 10.2 Hz), 3.84–3.87 (m, 1 H), 3.69–3.75 (m, 2 H), 3.57–3.69 (m, 4 H), 3.52–3.56 (m, 1 H), 3.44–3.50 (m, 2 H), 3.34–3.39 (m, 3 H), 3.22 (t, 2 H, J = 8.2 Hz). HRMS-FAB: $[M-H]^-$ m/z calcd for $C_{26}H_{36}F_3N_4O_{21}$, 797.1824; found: 797.1832.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A disaccharide was prepared by chemical treatment of heparosan
- An azido group was introduced into this heparosan disaccharide
- The azido heparosan disaccharide was used as a glycosylation acceptor
- A heparin tetrasaccharide was enzymatically synthesized

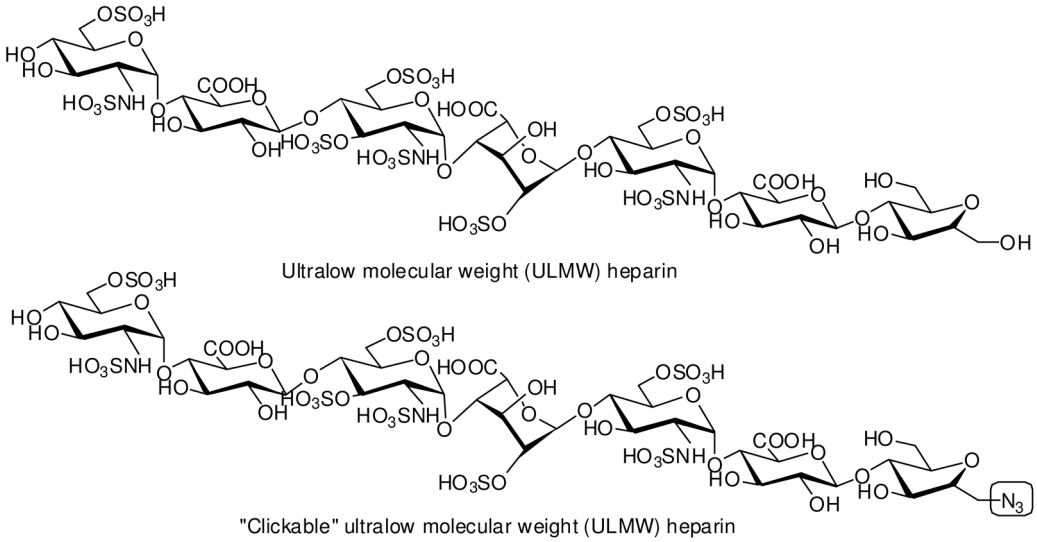
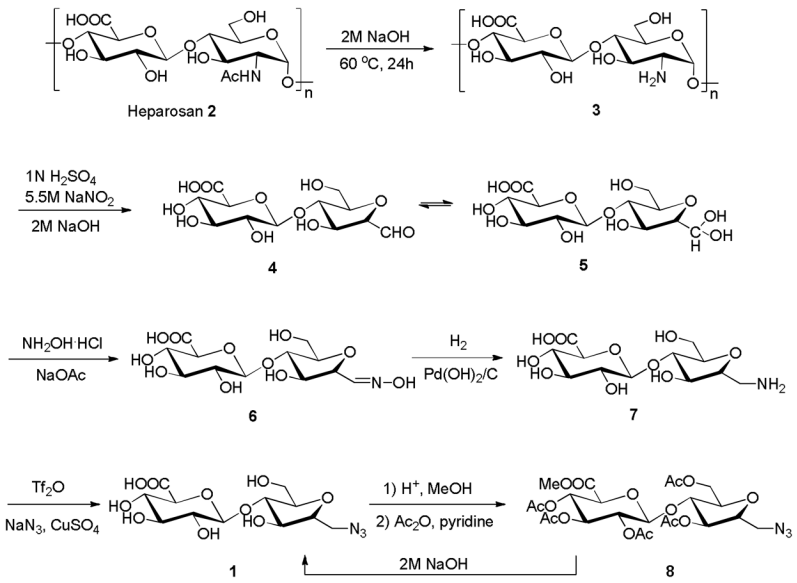
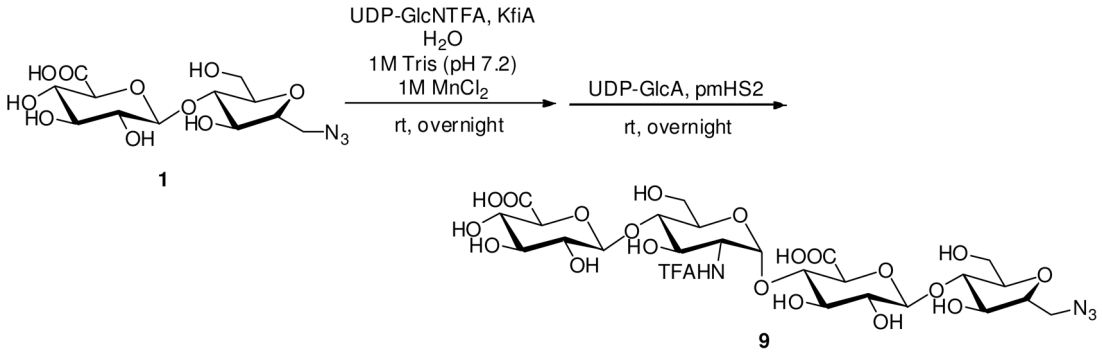


Figure 1.
Structures of targeted ULMW heparin oligosaccharides



Scheme 1.
Synthesis of “clickable” disaccharide acceptor **1**



Scheme 2.
Chemoenzymatic synthesis of HS tetrasaccharide