



Chemoenzymatic synthesis of heparan sulfate tetrasaccharide from a *N*-acetyl- α -D-glucosamine-*O*-methylglycoside acceptor

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ABSTRACT

Heparan sulfate tetrasaccharide **2** was efficiently prepared in seven steps through chemoenzymatic synthesis. A monosaccharide **5**, *N*-acetyl- α -D-glucosamine-*O*-methylglycoside (GlcNAc-OME), was successfully used as an acceptor in a heparosan synthase (pmHS2)-catalyzed glycosylation reaction. This avoided the multi-step synthesis of a more complex disaccharide acceptor **3**, greatly simplifying the route to tetrasaccharide target **2**. This approach provides a critical tetrasaccharide intermediate for subsequent chemoenzymatic transformation to fondaparinux and its analogues.

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Heparan sulfate (HS) is a linear sulfated polysaccharide, consisting of repeating 1 \rightarrow 4 linked disaccharide units of α -D-glucosamine (GlcN) and alternating uronic acid residues, either β -D-glucuronic acid (GlcA) or α -L-iduronic acid (IdoA), \rightarrow GlcN \rightarrow GlcA/IdoA \rightarrow [1,2]. These HS disaccharide units can be substituted with *N*-sulfo (S) or -acetyl (Ac) groups and *O*-S groups at the 3- and/or 6-positions of the GlcN residues and *O*-S groups at the 2-position of the uronic acid residues [2]. HS is widely found on the surface of animal cell and in the extracellular matrix and HS exhibits many diverse biological functions, controlling angiogenesis, inflammatory responses, lipid metabolism, tumor metastasis, and blood coagulation [3,4]. Heparin is a highly sulfated HS, displaying prominent anticoagulant activity. A pentasaccharide sequence, \rightarrow GlcNS6S \rightarrow GlcA \rightarrow GlcNS3S6S \rightarrow IdoA2S \rightarrow GlcNS6S \rightarrow (Fig. 1) within heparin, is responsible for much of heparin's anticoagulant activity and its specific binding to the serine protease inhibitor (serpin) antithrombin III [5].

There are three forms of clinically used heparins, unfractionated heparin (UFH, MW_{avg} 18,000 Da), low molecular weight heparin (LMWH, MW_{avg} 3500–6000 Da) and ultralow molecular weight heparin (ULMWH, MW < 2000 Da) [6]. UFH and LMWH are polypharmacological agents comprised of complex mixtures of

oligosaccharide and polysaccharide chains that are generally extracted from animal tissues. Due to a poorly regulated supply chain as well as the presence of other associated bioactive entities from animal extracts, such as viruses or prions, pharmaceutical UFH and LMWH products present unique safety concerns [7,8]. Thus, an approach for the efficient synthesis of structurally defined heparin oligosaccharides (ULMWH) from non-animal sources is highly desirable. Fondaparinux (Arixtra[®]), a synthetic ULMWH, with α -*O*-methylglycoside at the reducing end of its pentasaccharide-binding site, is used worldwide as a clinical antithrombotic agent (Fig. 1) [9]. Thus, a diverse collection of fondaparinux analogs containing an *O*-methylglycoside at their reducing end could represent a very important resource in the development of new therapeutic agents and result in an improved understanding of the biology of blood coagulation [10].

The preparation of homogeneous HS oligosaccharides using traditional chemical synthesis is very challenging because of the need of repetitive steps of protection, activation, coupling, deprotection often requiring purification steps to remove undesirable isomers [9,11,12]. For example, it requires as many as 60 chemical steps to prepare fondaparinux in a reported overall yield of 0.1% [9,13]. Chemoenzymatic synthesis, relies on combined chemical and enzymatic methods, mimicking the biosynthetic pathway of heparin, and represents a promising strategy to address many of these synthetic challenges [2,14]. Enzymatic synthesis, usually performed under mild conditions (20–37 °C) using aqueous “green” solvents, involves glycosylation reactions conducted with exquisite

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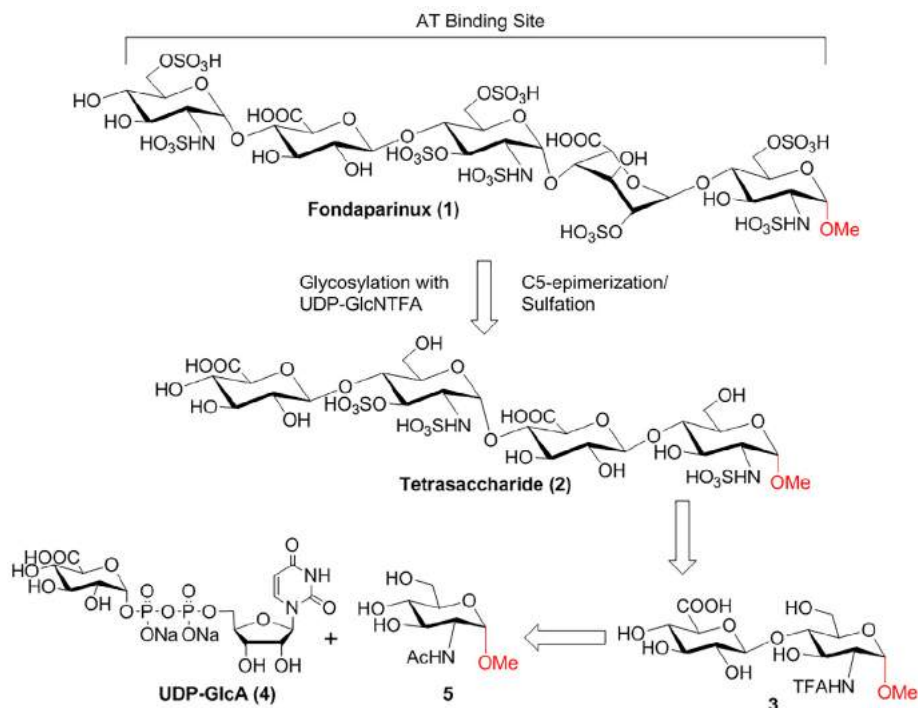


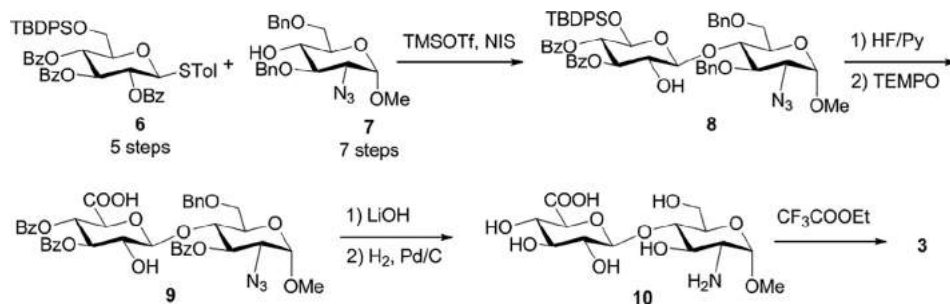
Fig. 1. Structure of fondaparinux and retrosynthetic analysis.

stereoselectivity in the formation of α - or β -glycosidic linkages. A number of reports on the chemoenzymatic synthesis of structurally defined oligosaccharides have demonstrated the feasibility of preparing larger oligosaccharides that have been difficult to synthesize by traditional organic approaches [15–19]. We have also generated a series of diverse HS oligosaccharides through chemoenzymatic synthesis [8,17–19]. One limitation of our current chemoenzymatic synthetic approach is that we begin with an unnatural acceptor, *p*-nitrophenyl glucuronide (GlcA-*p*NP) [5,17,19], so there is a *p*NP modified GlcA residue at the acceptor's reducing end that carries through to the oligosaccharide target, which is very different from the reducing end in the FDA approved anticoagulant drug fondaparinux. Herein, we report the efficient chemoenzymatic preparation of a heparan sulfate tetrasaccharide intermediate containing *O*-methylglycoside at its reducing end. GlcNAc-OMe monosaccharide serves as an acceptor in an enzymatic glycosylation, representing an approach that will also be generally useful for preparing fondaparinux analogs.

A retrosynthetic analysis of fondaparinux is shown in Fig. 1. We initially envisioned the synthesis of fondaparinux **1** could ultimately be achieved by enzymatic glycosylation between uridine diphosphate *N*-trifluoroacetyl glucosamine (UDP-GlcNTFA) and a

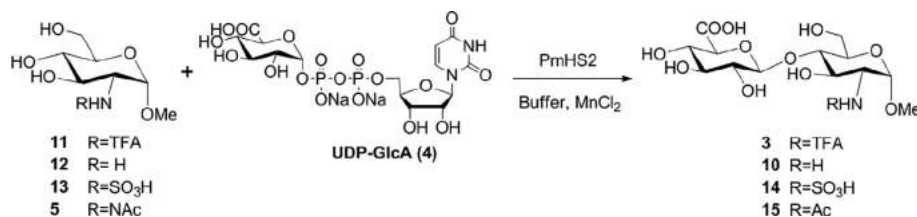
chemically synthesized *N*-sulfated tetrasaccharide **2** followed by C5 epimerization [8,20,21] and *O*-sulfation. This key tetrasaccharide intermediate **2** would be prepared from the disaccharide acceptor **3** through enzymatic backbone elongation and deprotection of the *N*-TFA groups and *N*-sulfation. Using a traditional chemical synthesis to prepare disaccharide intermediate **3** [10] (Scheme 1), followed by two glycosyltransferase-catalyzed couplings (Fig. 1), we anticipated that the synthesis and purification of the target tetrasaccharide **2** would be possible.

As shown in Scheme 1, disaccharide **3** was chemically synthesized from two monosaccharides **6** and **7** [10,22], however, this approach took five to seven steps for the preparation of each building block. The subsequent coupling of **6** with **7** followed by the deprotection of the TBS group, oxidation, hydrolysis and hydrogenation, afforded disaccharide **10** with a free amino group in another 5 steps. Finally, trifluoroacetylation generated disaccharide acceptor **3** and, thus, the entire chemical synthesis by this route took 13 linear steps affording an overall yield of ~2%. This chemical synthesis also required many separation steps needed to remove undesirable isomers as well as repetitive steps for protection, activation, coupling and deprotection, adding to costs and decreasing overall yields.



Scheme 1. Previously reported synthesis of disaccharide acceptor **3** [10].

Table 1
Optimization of disaccharide GlcA-GlcNHR-OMe preparation.^a



| Entry | Acceptor | Buffer | pH | T (°C) | Product | Yield (%) ^b |
|-------|----------------------|------------------|-----|--------|-----------|------------------------|
| 1 | 11 | TRIS | 7.5 | 25 | 3 | 2 |
| 2 | 12 | TRIS | 7.5 | 25 | 10 | ND ^c |
| 3 | 13 | TRIS | 7.5 | 25 | 14 | ND ^c |
| 4 | 5 | TRIS | 7.5 | 25 | 15 | 10 |
| 5 | 5 (1.5 equiv) | TRIS | 7.5 | 25 | 15 | 10 |
| 6 | 5 | MES | 7.5 | 25 | 15 | 5 |
| 7 | 5 | HEPES | 7.5 | 25 | 15 | 3 |
| 8 | 5 | sodium phosphate | 7.5 | 25 | 15 | ND ^c |
| 9 | 5 | TRIS | 7.5 | 37 | 15 | 12 |
| 10 | 5 | TRIS | 7.2 | 37 | 15 | 15 |
| 11 | 5 | TRIS | 8.0 | 37 | 15 | 9 |
| 12 | 5 | TRIS | 8.5 | 37 | 15 | 3 |

^a Unless otherwise noted, donor UDP-GlcA and acceptor were 1.0 equiv in each enzymatic reaction.

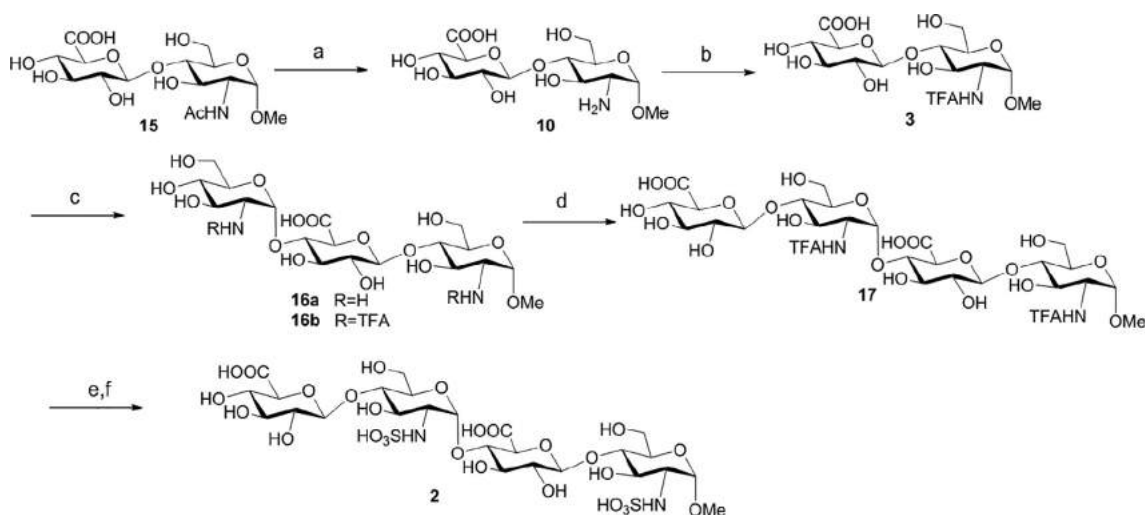
^b Yield was obtained by high-resolution electrospray ionization mass spectrometry (ESI-MS) to approximately quantify the disaccharide product and starting materials present in the crude reaction mixture.

^c No desired product.

In the current work, we focused on using an entirely chemoenzymatic approach, envisioning the facile assembly of the disaccharide intermediate through enzyme-catalyzed glycosylation. The study began by examining the glycosylation of the GlcNTFA-OMe acceptor **11** with UDP-GlcA donor. Unfortunately, using reported conditions for heparosan synthase 2 from *Pasteurella multocida* (pmHS2) [8,23], tris (hydroxymethyl) amino methane (TRIS) buffer pH 7.5 and MnCl₂, only trace amounts of disaccharide product were obtained (<1% yield) even after prolonged incubation times of up to 48 h and starting material **11** was recovered (Table 1, entry 1). Since the efficiency enzymatic catalysis can be impacted by even a slight modification of reaction conditions or substrate structure, additional investigations were conducted. Monosaccharide **12**, containing a free amino group, and *N*-sulfo monosaccharide **13** were also tested as acceptors in enzyme-catalyzed glycosylation but failed to generate the desired disaccharides (entries 2–3), as this enzyme is selective for substrate structure [2,8] and the structures of these two methyl glycoside monosaccharides were not accepted by glycosyltransferase pmHS2. However, enzyme-catalyzed glycosylation was achieved with GlcNAc-OMe **5** (1.0 equiv) and UDP-GlcA **4** (1.0 equiv), stereo- and regio- selectively affording disaccharide **15** with a β -configuration and 1 \rightarrow 4 linkage in 10% yield (entry 4). The use of excess acceptor **5** (1.5 equiv) and/or prolonged reaction time (48 h) did not increase the percent conversion (entry 5). Since acceptor GlcNAc-OMe could be recognized by pmHS2, subsequent studies were focused on the optimization of enzymatic conditions by varying the reaction temperature and time, buffer type and the pH within the effective buffering range of each respective buffer (entries 6–12). Several different common buffers, such as, 2-(*N*-morpholino) ethane sulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) and sodium phosphate were tested but did not afford higher yields than TRIS buffer (entries 6–8), thus, TRIS was selected as buffer in this enzymatic reaction. By increasing the reaction temperature to 37 °C the yield of disaccharide **15** could be increased to 12% (entry 9). Performing the enzymatic reaction at pH 7.2 increased product yield to 15%, but higher pH values (pH 8.0 or 8.5)

decreased the yield (entries 10–12). Thus, the optimized coupling reaction was established as follows: acceptor **5** (1.0 equiv) incubated with pmHS2 (20 μ g mL⁻¹) in buffer containing TRIS (25 mmol, pH 7.2), MnCl₂ (8 mmol) and UDP-GlcA (1.0 equiv) at 37 °C for 24 h.

With disaccharide **15** in hand, de-*N*-acetylation was first attempted using Ba(OH)₂·8H₂O in the methanol/water system [24]. However, these reaction conditions were too harsh and resulted in cleavage of the glycosidic bond. Hydrazine [25] was subsequently used and successfully afforded disaccharide **10** having a free amino group, in 85% yield. Trifluoroacetylation of the disaccharide **10** proceeded in methanol with triethylamine and ethyl trifluoroacetate at room temperature and generate acceptor **3** in 95% yield [26]. *N*-TFA-protected disaccharide **3**, acceptor, was then subjected to an enzyme-catalyzed glycosylation catalyzed by *N*-acetyl glucosaminyltransferase (KfiA) [23,27], using UDP-*N*-trifluoroacetylglucosamine (UDP-GlcNTFA) as donor, under the same conditions used for the pmHS2 extension (Scheme 2). However, the two *N*-TFA groups were cleaved after Bio-gel P-2 purification, affording trisaccharide, **16a**, based on LC-MS analysis. The loss of these TFA groups was entirely unexpected as desalting using P-2 polyacrylamide resin is generally regarded as extremely mild (room temperature in water). Thus, full structural analysis of **16a** was undertaken using ¹H NMR and 2D ¹H–¹H COSY NMR (Fig. 2B and C), further confirming the loss of the two *N*-TFA groups, resulting in the H-2 chemical shifts for these glucosamine residues of 3.22 and 3.24 ppm, characteristic of free amino groups and not the strongly electron-withdrawing *N*-TFA groups. We speculate that the polyacrylamide of the P-2 gel resin might be sufficiently basic to cleave these labile *N*-TFA groups. To eliminate this concern, Sephadex G-10 polysaccharide-based (cross-linked dextran) resin was used in place of P-2 gel resin to isolate desired trisaccharide **16b** in 75% yield. Subsequent ¹H–¹H COSY NMR showed chemical shifts of 3.92 and 3.95 ppm for the H-2 of both glucosamine residues, consistent with the presence of *N*-TFA groups (Fig. 2D). The ¹H NMR spectrum of trisaccharide **16b** (Fig. 2A), showed three anomeric proton signals, at 5.41 and 4.76 ppm for the GlcNTFA



Scheme 2. Synthesis of HS tetrasaccharide **2**. (a) Hydrazine, hydrazine sulfate, 90 °C, 4 h, 85%; (b) CF_3COOEt , Et_3N , MeOH, room temperature, 15 h, 95%; (c) KfA, TRIS, MnCl_2 , UDP-GlcNTFA, room temperature, 24 h, 75%; (d) PmHS2, TRIS, MnCl_2 , UDP-GlcA, 37 °C, 24 h, 87%; (e) Et_3N , H_2O , MeOH, room temperature, 15 h, 99%; (f) $\text{SO}_3\text{-Py}$, sodium carbonate, H_2O , room temperature, 5 h, 91%.

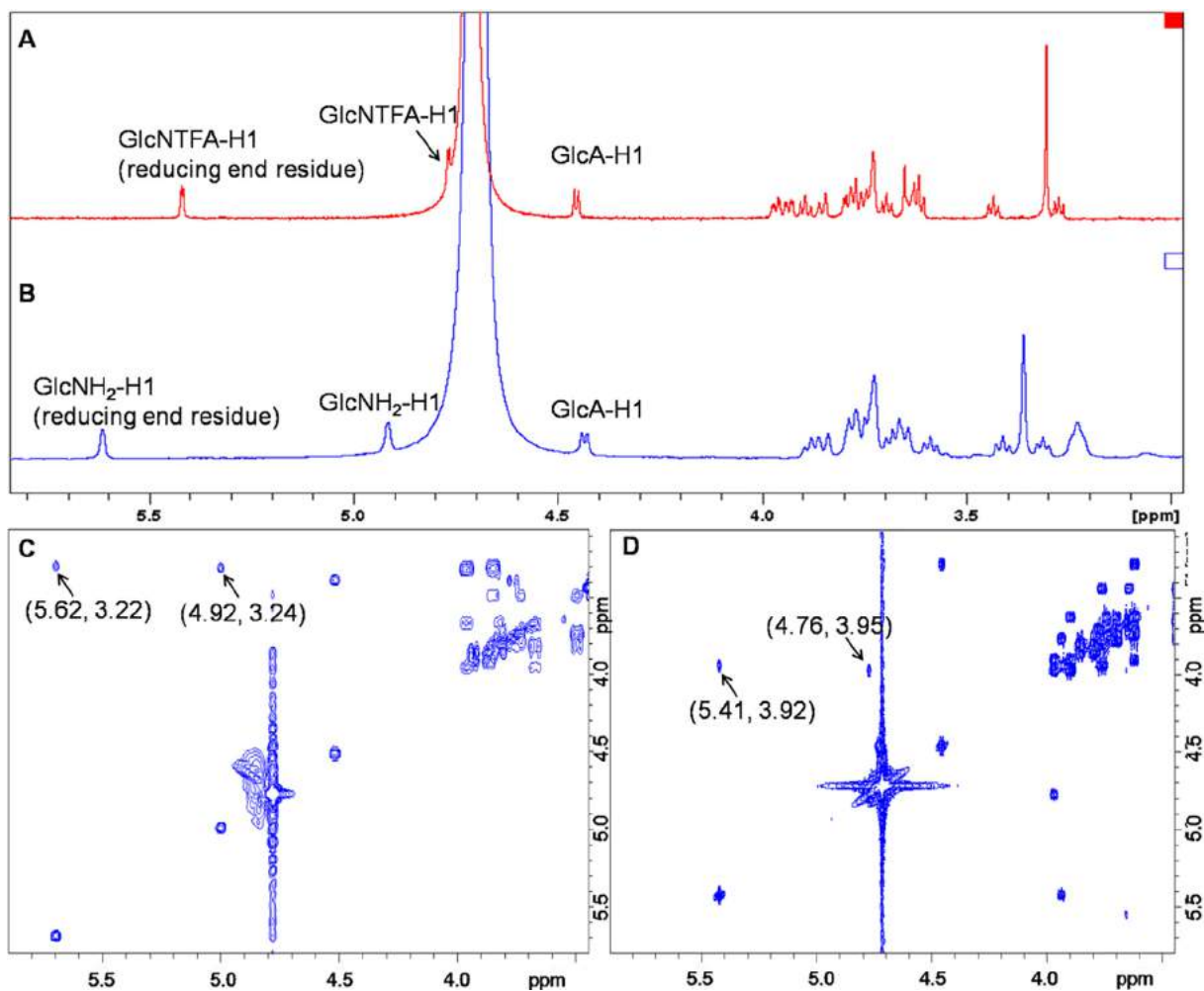


Fig. 2. NMR characterization to distinguish free amino group-containing trisaccharide **16a** and *N*-TFA-protected trisaccharide **16b**. Panel A and B show the 1D ^1H NMR spectra of **16b** and **16a**, respectively. Peaks corresponding to the anomeric protons of the two compounds can be clearly identified. Panel C and D show the 2D ^1H - ^1H COSY spectra of **16a** and **16b**, respectively. Cross-peaks of H-1 with H-2 from glucosamine residues are identified.

residues and 4.45 ppm for the GlcA residue, consistent with the structure of trisaccharide **16b**. The 3J ^1H – ^1H coupling constant of GlcA is 7.89 Hz, confirmed its β -linkage, while the coupling constants of 3.48 and 3.14 Hz for the two GlcNTFA residues were consistent with α -glycosidic bond and α -methyl glycoside.

Trisaccharide **16b** was next elongated using pmHS2 with donor UDP-GlcA to form tetrasaccharide **17** in 87% yield. It is noteworthy that the glycosylation yield increases as the elongation of the carbohydrate chain proceeds. This is probably because the elongated acceptors have a closer structural similarity to natural substare structures, compared to the smaller monosaccharide substrate, GlcNAc-OMe **5**. Removal of *N*-TFA groups under mild basic conditions in 99% yield was followed by selective *N*-sulfonation, affording the target heparan sulfate tetrasaccharide **2**.

In conclusion, we report the first use of GlcNAc-OMe as an acceptor in pmHS2-catalyzed glycosylation and efficiently prepared a HS tetrasaccharide **2** in seven steps with overall 7.1% yield. Future studies will examine protein engineering of the pmHS2 biocatalyst to improve its yield when acting on this monosaccharide acceptor. We also found that P-2 polyacrylamide resin in water could unexpectedly cleave *N*-TFA protecting groups from glucosamine residues. Our chemoenzymatic approach for the synthesis of very small oligosaccharides supplements our recent success of the chemoenzymatic method for the synthesis of larger oligosaccharides and also demonstrates its utility in preparing fondaparinux analogs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tetlet.2019.02.036>.

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