Chemoenzymatic synthesis of unmodified heparin oligosaccharides: cleavage of p-nitrophenyl glucuronide by alkaline and Smith degradation†

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A heparin oligosaccharide having a completely natural structure was successfully synthesized through a chemoenzymatic approach using an unnatural glycosyl acceptor, p-nitrophenyl glucuronide (GlcA-pNP).

The use of an inexpensive and commercially available GlcA-pNP acceptor facilitates oligosaccharide recovery and purification on C-18 resin during chemoenzymatic synthesis. Oligosaccharide chain extension and modification afforded a heptasaccharide with gluconic acid residues at its reducing and non-reducing ends. Treatment with periodate oxidation followed by Smith degradation or alkaline elimination resulted in the selective cleavage of vicinal diol-containing glucuronic acid residues affording highly sulfated heparin pentasaccharides having a completely natural structure. This methodology should facilitate the chemoenzymatic synthesis of a family of highly sulfated heparin oligosaccharides with unmodified structures for biological evaluation.

Heparin is the most highly sulfated member of the heparan sulfate (HS) family of linear, highly anionic polysaccharides.\(^1\) HS consists of repeating units of alternating uronic acid residue, either \(\beta\)-n-glucuronic acid (GlcA) or \(\alpha\)-l-iduronic acid (IdoA) and \(\alpha\)-n-glucosamine (GlcN) residue.\(^2\)\(^\text{-}^4\) Both saccharide units in the HS disaccharide repeating units can be substituted with \(N\)-sulfo (S) or \(N\)-acetyl (Ac) groups and \(O\)-sulfo (S) groups (at the 2-O-position of the uronic acid residues and at the 3-O and 6-O-positions of the GlcN residue). Heparin is a unique form of HS, found within specialized granulated cells,\(^4\) which has long highly sulfated domains\(^5\) consisting primarily of the trisulfated disaccharide repeating unit \(\alpha\rightarrow 4\) IdoA2S \((\alpha\rightarrow 4)\) GlcNS6S \((\alpha\rightarrow 4)\).

Highly sulfated heparin chains display a number of prominent biological activities including growth factor\(^6\)\(^-^8\) and chemokine\(^9\) signaling and anticoagulant activities.\(^10\) The pentasaccharide sequence containing \(\alpha\rightarrow 4\) IdoA2S \((\alpha\rightarrow 4)\) GlcNS6S \((\alpha\rightarrow 4)\) at its reducing end with a central GlcNS3S6S residue, having the structure, \(\rightarrow\text{GlcNS6S} \rightarrow \text{GlcA} \rightarrow \text{GlcNS3S6S} \rightarrow \text{IdoA2S} \rightarrow \text{GlcNS6S} \rightarrow\) (Fig. 1), is responsible for its specific binding to the serine protease inhibitor (serpin) antithrombin III (AT). This AT pentasaccharide binding site has been chemically synthesized as \(O\)-methylglucosido and is used clinically as an antithrombotic agent known as ultra-low molecular weight heparin (ULMWH) fondaparinux (Arixtra\textsuperscript{®}).\(^11\) Fondaparinux is a specific anti-factor Xa (FXa) agent and acts by binding to an allosteric site on AT making it a potent FXa inhibitor.\(^12\)\(^,\)\(^13\) Unfractionated heparins (UFHs) and low molecular weight heparins (LMWHs) can contain a repeating trisulfated disaccharide sequence \((\alpha\rightarrow 4)\) IdoA2S \((\alpha\rightarrow 4)\) GlcNS6S \((\alpha\rightarrow 1)\) flanking their AT pentasaccharide binding sites. This trisulfated disaccharide rich domain corresponds to the heparin thrombin (FIIa) binding site and facilitates the assembly of the ternary heparin–AT–FIIa complex required for heparin's global anticoagulant activity.\(^14\)

UFH and LMWH are polypharmacological agents, complex mixtures of molecules prepared from animal tissues.\(^15\) The

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**Fig. 1** The antithrombin III-binding pentasaccharide found in heparin.
supply chain of pharmaceutical heparin has been poorly regulated and this presented safety concerns. A worldwide distribution of contaminated heparin in 2007, caused by its adulteration with a semi-synthetic oversulfated chondroitin sulfate contaminant, adversely affected the purity and safety of animal-sourced UFH and LMWH, and was associated with over 200 deaths in the USA. 15,16 Another potential threat is that other bioactive entities, such as viruses or prions might remain associated with the HS chains in animal extracts. Thus, the cost-effective preparation of structurally defined heparin oligosaccharides (ULMWH) from non-animal sources is highly desirable.

There are two ways to prepare homogeneous heparin oligosaccharides, one uses a purely synthetic chemical method, based on repetitive steps of protection, activation, coupling and de-protection, and is very challenging for molecules larger than the pentasaccharide Arixtra®. 17 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. Our previous studies 18,19 have demonstrated that chemoenzymatic synthesis is capable of generating a series of heparin oligosaccharides in good overall yield and possessed excellent anticoagulant activity. One limitation of our current chemoenzymatic synthetic approach is that we begin with an unnatural inexpensive commercially available acceptor, p-nitrophenyl glucuronide (GlcA-pNP). 18,19 that is ultraviolet detectable, hydrophobic, and easily binds to reversed phase chromatography resins, enabling the detection recovery, and purification of the resulting oligosaccharides using a C-18 resin. However, the resulting target molecules contain an unnatural structural feature. We have previously investigated the removal of pNP from chemoenzymatically synthesized HS oligosaccharides using ceric ammonium nitrate. 20

In the current study we faced the challenge of removing GlcA-pNP from chemoenzymatically synthesized highly sulfated oligosaccharides with domains consisting of repeating trisulfated disaccharide units. These heparin oligosaccharides represent potential precursors in the chemoenzymatic synthesis of Arixtra® and also display interesting biological activities. 18,21 We report the selective removal of the GlcA-pNP moiety from a structurally more complex heparin-like oligosaccharide using both Smith degradation and alkaline elimination. 22-24 This approach has been previously used for the micro scale analysis of polysaccharides relying on mass spectrometry (MS) and leaves unnatural moieties at the reducing end of the resulting degradation products. 25-28 We have modified this method to prepare a natural heparin pentasaccharide rich in trisulfated disaccharide units.

Based on our previous effort to synthesize homogeneous low-molecular-weight heparins, 18 the chemoenzymatic synthesis of heparin-like oligosaccharides containing a IdoA2S-GlcNS6S (where S is sulfate) repeating unit was initiated from the commercially available p-nitrophenyl glucuronide (GlcA-pNP, 1) (Scheme 1). The monosaccharide acceptor 1 was repetitively elongated with PmHS2 (heparosan synthase 2 from Pasteurella multocida), UDP-N-trifluoroacetyl glucosamine (GlcNTFA) donor and UDP-glucuronic acid (GlcA), to form heptasaccharide 2. Selective removal of the TFA group on the nitrogen atom followed by N-sulfation, C4 epimerization/2-O-sulfation and 6-O-sulfation produced structure 3 for further modification. During this chemoenzymatic synthesis, the strong UV absorbance and high C-18 binding affinity of the p-NP group facilitate the detection and purification of the intermediates. The sulfated oligosaccharides were purified by Q-Sepharose. A total of 20 mg of 3 was prepared.

With substrate 3 in hand, a selective oxidative-cleavage of vicinal diol was carried out with sodium periodate (NaIO4) in neutral sodium phosphate buffer at 37 °C (ref. 29) for 3.5 h, furnishing the corresponding aldehyde 4, which could be used to explore the cleavage of the GlcA-pNP group using either the alkaline elimination or Smith degradation method (Scheme 2). The periodate oxidation was terminated by the addition of ethylene glycol followed by dialysis (MWCO

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**Scheme 1  Construction of heparin-like heptasaccharide (3).**
100–500 Da) against distilled water and lyophilization. The alkaline elimination method was first investigated (Scheme 2A). A solution of oligosaccharide aldehyde 4 was adjusted to pH 12 with 0.5 M NaOH aqueous. After standing at room temperature for 30 min, the solution was neutralized with 0.5 M acetic acid. The resulting mixture was dialyzed (MWCO 100–500 Da) against distilled water and lyophilized to obtain the elimination compound 5 with an unnatural enol glycoside at the reducing end. Scheme 2B shows the Smith degradation of oligosaccharide aldehyde 4. Next, 4 was treated with sodium borohydride (NaBH₄) in distilled water for 3 h at room temperature and then excess borohydride was quenched with 1 M acetic acid. After dialysis against distilled water and lyophilization, the oxidized–reduced oligosaccharide was dissolved in 0.5 M trifluoroacetic acid (TFA) solution at room temperature for 24 h. The reaction was terminated by the addition of 0.5 M NaOH at pH 7 and dialyzed to afford the degradation product 7. Gratifyingly, no glycosidic bond cleavage or de-sulfation was detected throughout this reaction scheme. Moreover, detection by LC-MS showed a yield of less than 5% of the desired target compound 8. This discovery suggested that the glycoside could be selectively cleaved while leaving the other glycosidic bonds within the oligosaccharide intact. Both the structures of 5 and 7 were clearly supported by LC-MS, 1D and 2D NMR spectra (Fig. 2 and ESI†). In the ¹H spectra of alkaline elimination product 5 and Smith degradation product 7, the disappearance of signals at 4.52 and 5.23 ppm shows that two GlcA residues have been successfully oxidatively cleaved (Fig. 2 I–III). The anomic signals appearing at 5.34 and 5.14 ppm confirmed the presence of GlcNS6S and IdoA2S residues, respectively (Fig. 2 II and III). In addition, according to the H–H COSY spectrum of compounds 5 and 7, the chemical shifts of H-2, H-4 and H-5 on the IdoA residue appear at 4.26, 4.02 and 4.76 ppm, respectively, indicating that 2-O-sulfo groups were still present on the IdoA residues (Fig. 2 IV and V).

Acidic hydrolysis was used to remove the unnatural glycoside in oligosaccharides 5 and 7 to prepare natural heparin pentasaccharides (Scheme 3). The hydrolysis of compound 5 was easier since the enol group was more acid labile. We found that both 0.5 M TFA and 0.05 M aqueous HCl solutions could successfully produce the target oligosaccharide 8 in good yield with <5% starting material detected by LC-MS. However, the removal of the unnatural glycoside from 7 turned out to be more challenging. Using the same conditions described above, only the starting material was recovered. Finally, the transformation of 7 to 8 was achieved in 0.5 M TFA solution at 45 °C in 4 h. These reaction conditions need to be strictly controlled, as higher temperatures or longer reaction times result in significant decomposition. The NMR spectra and LC-MS of the target compound 8 clearly support the success of this acid hydrolysis step (Fig. 3). The anomic signals at 5.34 and 5.14 ppm confirmed the presence of GlcNS6S and IdoA2S residues, respectively (Fig. 3 I). The selected anomic and IdoA carbon regions (64–104 ppm) in the 2D ¹H–¹³C HSQC spectrum also confirm the structure (Fig. 3 II). The structure was also characterized by LC-MS with m/z 496.6377 in the negative mode (Fig. 3 III).
We next expanded the oligosaccharide substrates subjected to our alkaline degradation method to investigate the reaction scope (Table 1). These synthetic oligosaccharides contained both GlcA and IdoA residues and possessed different chain lengths, sulfation levels, and flanking residues. The first step, periodate oxidation forming the intermediate aldehyde, is critical to the entire degradation process. Although it was reported that the neighbouring environment might influence the reactivity of uronic acid residues, no selectivity in periodate cleavage was observed for unsulfured GlcA or IdoA residues within the oligosaccharides tested. Oligosaccharides having different residues flanking GlcA (entries 1–3) were fully cleaved to monosaccharides at 37 °C and pH 7.0. Oligosaccharides containing both unsulfated GlcA and IdoA (entries 4 and 5) were also fully cleaved to monosaccharide products, thus, no selectivity between GlcA and IdoA was observed. Oligosaccharides containing GlcA residues and of different chain lengths and sulfation levels (entries 6–8) afforded the complete cleavage of GlcA residues and afforded oligosaccharides of the expected chain lengths based on the spacing of these GlcA residues.

In conclusion, an unmodified heparin heptasaccharide was successfully prepared through chemoenzymatic synthesis using p-nitrophenyl glucuronide (GlcA-pNP) as a glycosylation

Fig. 2  NMR characterization of degradation oligosaccharides (R = pNP). Panels I, II, and III show the 1D 1H NMR spectra of compounds 3, 5 and 7, respectively. Peaks corresponding to the anomeric protons of these compounds can be clearly identified. Panels IV and V show the selected H–H correlation in the 2D H–H COSY spectrum of compounds 5 and 7, respectively. Three sets of peaks between 3.4–3.7 ppm in the 1H NMR spectrum were from glycerol that came from the dialysis membrane.

Scheme 3  Acidic hydrolysis of glycoside on degradation oligosaccharides.
acceptor, which greatly facilitates the synthesis. The selective removal of the GlcA-\(\beta\)NP moiety from the reducing end of the molecule and GlcA from its non-reducing end, using both Smith degradation and alkaline elimination, afforded the target pentasaccharide. To our knowledge, this is the first time that these two degradation methods have been applied to the preparative synthesis of an oligosaccharide. Most importantly, no de-sulfation was detected during the synthesis. Additional oligosaccharide substrates were investigated and demonstrated that this degradation method was specific to saccharide residues with vicinal diols, i.e., GlcA or IdoA or to residues at the non-reducing end, and could be applied to diverse oligosaccharides containing different chain lengths, sulfation levels, and flanking residues. This strategy should be useful in the construction of more structurally complex heparin oligosaccharides. Since these targets are all natural and with a GlcNS6S residue at their reducing end, they could be used as precursors in the chemoenzymatic synthesis of Arixtra® or other oligosaccharides with therapeutic anticoagulant and antithrombotic activities.

Fig. 3  NMR and HRMS of natural heparin oligosaccharide 8. Panel I shows the 1D \(^{1}H\) NMR spectrum. Panel II shows the selected anomeric and IdoA2S carbon regions (64–104 ppm) in the 2D \(^{1}H–^{13}C\) HSQC spectrum. Panel III shows the HRMS (negative mode) of compound 8.

### Table 1  Alkaline Smith degradation of different synthetic oligosaccharides

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrates</th>
<th>Products(^a)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>GlcNAc-GlcA-(GlcNAc-GlcA)-GlcNAc-GlcA-(\beta)NP</td>
<td>Monosaccharide-R</td>
</tr>
<tr>
<td>2</td>
<td>GlcNS-GlcA-(GlcNS-GlcA)-GlcNS-GlcA-(\beta)NP</td>
<td>Monosaccharide-R</td>
</tr>
<tr>
<td>3</td>
<td>GlcNS6S-GlcA-(GlcNS6S-GlcA)-GlcNS6S-GlcA-(\beta)NP</td>
<td>Monosaccharide-R</td>
</tr>
<tr>
<td>4</td>
<td>GlcNS-GlcA-GlcNS-IdoA-GlcNS-GlcA-(\beta)NP</td>
<td>Monosaccharide-R</td>
</tr>
<tr>
<td>5</td>
<td>GlcNS6S-GlcA-GlcNS6S3S-IdoA-GlcNS6S-GlcA-(\beta)NP</td>
<td>Monosaccharide-R</td>
</tr>
<tr>
<td>6</td>
<td>GlcNS6S-GlcA-(GlcNS6S-IdoA2S)-GlcNS6S-GlcA-(\beta)NP</td>
<td>Pentadecasaccharide-R(^b)</td>
</tr>
<tr>
<td>7</td>
<td>GlcNS6S-GlcA-(GlcNS6S-IdoA2S)-GlcNS6S-GlcA-(\beta)NP</td>
<td>Pentadecasaccharide-R(^b)</td>
</tr>
<tr>
<td>8</td>
<td>GlcNS-GlcA-(GlcNS-IdoA2S)-GlcNS-GlcA-(\beta)NP</td>
<td>Pentadecasaccharide-R(^b)</td>
</tr>
</tbody>
</table>

\(^a\) \(r = C_rH_{2r}O_r\) (see 5 in Scheme 2 for structure of aglycone). \(^b\) Confirmed by LC-MS (see the ESI).
Acknowledgements

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Notes and references

29 Elevated temperature (37 °C) and pH ≥ 7.0 are required to fully cleave all the vicinal diols.