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

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Abstract

A heparin oligosaccharide having a completely natural structure was successfully synthesized through a chemoenzymatic approach using an unnatural glycosyl acceptor, *p*-nitrophenyl glucuronide (GlcA-*p*NP). The use of an inexpensive and commercially available GlcA-*p*NP 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 pentasaccharide 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 polysaccharides¹. HS consists of repeating, 1→4-linked disaccharide units of alternating uronic acid residue, either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) and α-D-glucosamine (GlcN) residue²⁻⁴. 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 the 3-*O*- and 6-*O*-positions of the GlcN residue). Heparin is a unique form of HS, found within specialized granulated cells⁴, which has long highly sulfated domains⁵ consisting primarily of the trisulfated disaccharide repeating unit →4) IdoA2S (1→4) GlcNS6S (1→4).

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Highly sulfated heparin chains display a number of prominent biological activities including growth factor⁶⁻⁸ and chemokine⁹ signaling and anticoagulant activity¹⁰. The pentasaccharide sequence containing a $\rightarrow 4$ IdoA2S (1 \rightarrow 4) GlcNS6S (1 \rightarrow at its reducing end with a central GlcNS3S6S residue, having the structure, \rightarrow GlcNS6S \rightarrow GlcA \rightarrow GlcNS3S6S \rightarrow IdoA2S \rightarrow 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 the *O*-methylglycoside and is used clinically as an antithrombotic agent known as the ultra-low molecular weight heparin (ULMWH) fondaparinux (Arixtra®)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), low molecular weight heparins (LMWHs) can contain a repeating trisulfated disaccharide sequence ($\rightarrow 4$ IdoA2S (1 \rightarrow 4) GlcNS6S (1 \rightarrow)₆) flanking their AT pentasaccharide binding sites. This trisulfated disaccharide rich domain corresponds to heparins thrombin (FIIa) binding site and facilitates the assembly of the ternary heparin-AT-FIIa complex required for heparin's global anticoagulant activity¹⁴.

UFH and LMWH are polypharmacological agents, complex mixtures of molecules prepared from animal tissues¹⁵. The 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 US^{16, 17}. Another potential threat is that other bioactive entities, such as viruses or prions that 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®¹¹. 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-*p*NP)^{18, 19}, that is ultraviolet detectable, hydrophobic, easily binding 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 *p*NP from chemoenzymatically synthesized HS oligosaccharides using ceric ammonium nitrate²⁰.

In the current study we faced the challenge of removing GlcA-*p*NP from chemoenzymatically synthesized highly sulfated oligosaccharide 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 GlcA-*p*NP moiety from a more structurally complex heparin-like oligosaccharide using both Smith degradation and alkaline elimination^{22–24}. This approach has been previously used for 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 synthetic homogeneous low-molecular-weight heparins¹⁸, the chemoenzymatic synthesis of heparin-like oligosaccharide containing IdoA2S-GlcNS6S (where S is sulfate) repeating unit was initiated from the commercially available *p*-nitrophenyl glucuronide (GlcA-*p*NP, **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 TFA group on nitrogen atom followed by *N*-sulfation, C₅ 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 °C²⁹ for 3.5 h, furnishing the corresponding aldehyde **4**, which could be used to explore the cleavage of GlcA-*p*NP group using either alkaline elimination or Smith degradation method (Scheme 2). The periodate oxidation was terminated by the addition of ethylene glycol followed by dialysis (MWCO 100–500 Da) against distilled water and lyophilization. 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 to 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 spectrum (Fig. 2 and Supplementary data). 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 show that two GlcA residues have been successfully oxidatively cleaved (Fig. 2 I–III). The anomeric

signals appearing at 5.34 and 5.14 ppm confirmed the presence of GlcNS6S and IdoA2S residues, respectively (Fig. 2 II, III). In addition, according to H-H COSY spectrum of compound **5** and **7**, the chemical shifts of H-2, H-4 and H-5 on 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, V).

Acidic hydrolysis was used to remove the unnatural glycoside in oligosaccharides **5** and **7** to prepare natural heparin pentasaccharide (Scheme 3). Hydrolysis of compound **5** was easier since enol group was more acid labile. We found that either 0.5 M TFA or 0.05 M HCl aqueous could successfully produce target oligosaccharide **8** in good yield with <5% starting material left detected by LC-MS. However, removal of the unnatural glycoside from **7** turned out to be more challenging. Using the same conditions described above only recovered starting material. Finally, transformation of **7** to **8** was achieved in 0.5 M TFA solution at 45°C for 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 supports the success of this acid hydrolysis step (Figure 3). The anomeric signals at 5.34 and 5.14 ppm confirmed the presence of GlcNS6S and IdoA2S residues, respectively (Fig. 3 I). The selected anomeric and IdoA carbon region (64–104 ppm) in 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 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 residues²⁷, no selectivity in periodate cleavage was observed for unsulfated 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 4–5) 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 complete cleavage of GlcA residues and afforded oligosaccharides of chain lengths expected based on the spacing of these GlcA residues.

In conclusion, an unmodified heparin heptaasaccharide was successfully prepared through a chemoenzymatic synthesis using *p*-nitrophenyl glucuronide (GlcA-*p*NP) as a glycosylation acceptor, which greatly facilitates the synthesis. The selective removal of GlcA-*p*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 saccharide. To our knowledge, this is the first time to these two degradation methods has 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 for saccharide residues with vicinal

diols, *i.e.*, GlcA or IdoA or residues at the non-reducing end, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

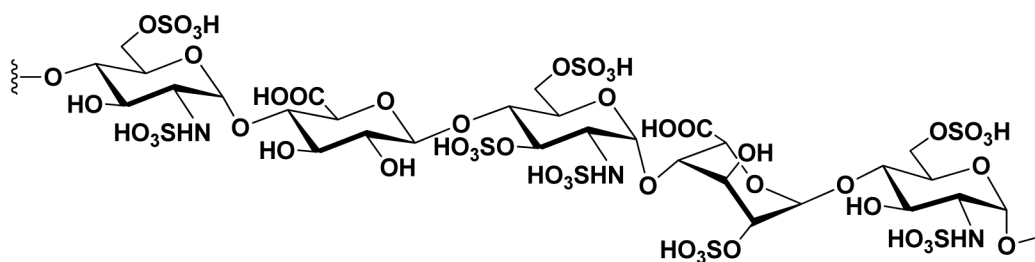


Figure 1.
The antithrombin III-binding pentasaccharide found in heparin.

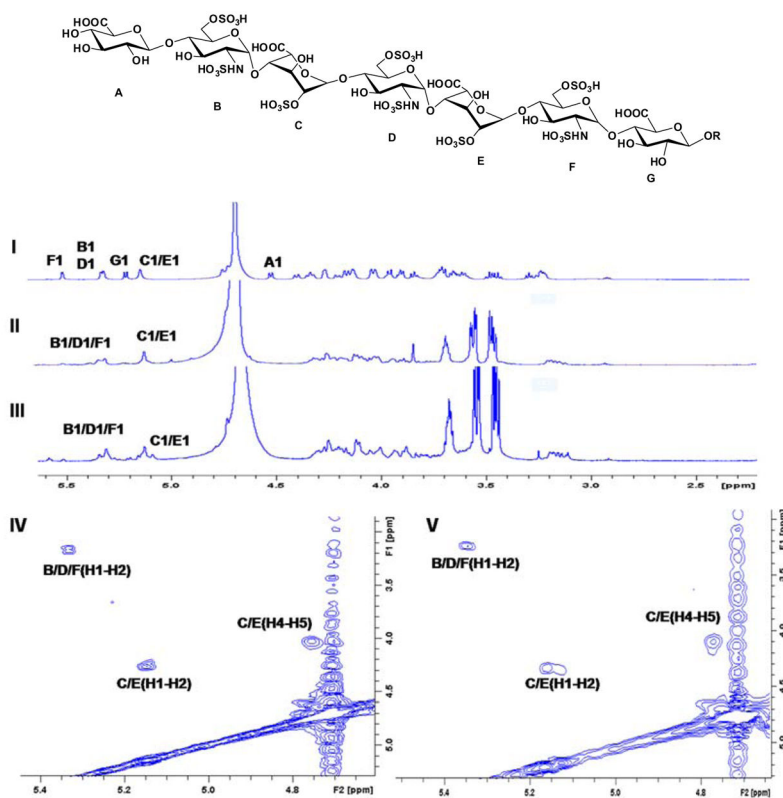


Figure 2. NMR characterization of degradation oligosaccharides ($R = pNP$). Panel I, II, III show the 1D 1H NMR spectra of compound **3**, **5** and **7**, respectively. Peaks corresponding to the anomeric protons of these compounds can be clearly identified. Panel IV, V show the selected H-H correlation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

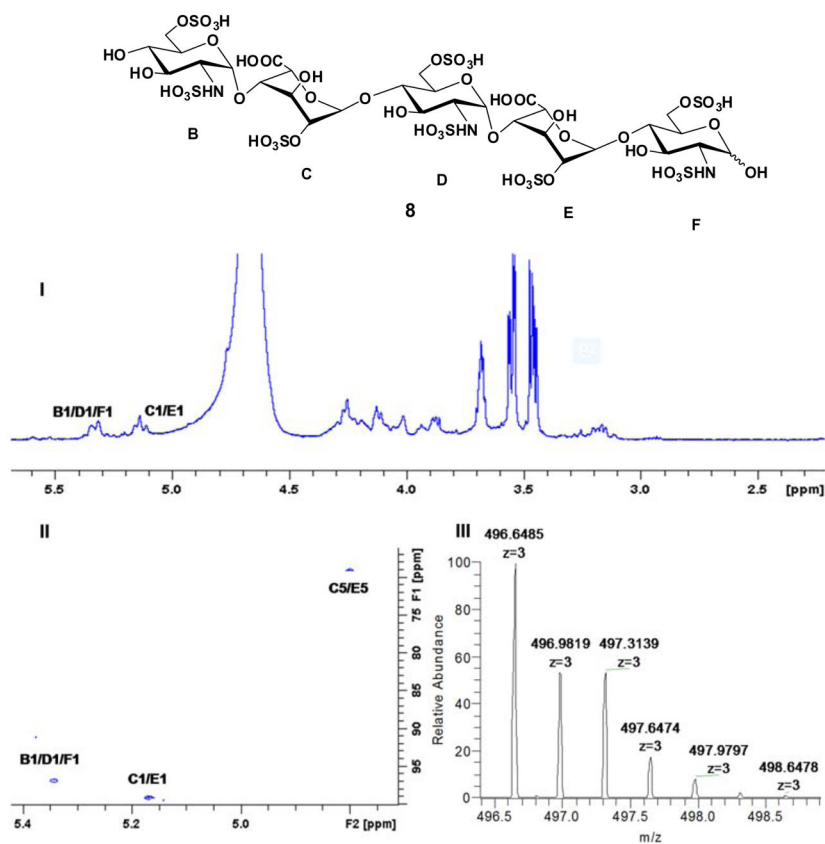
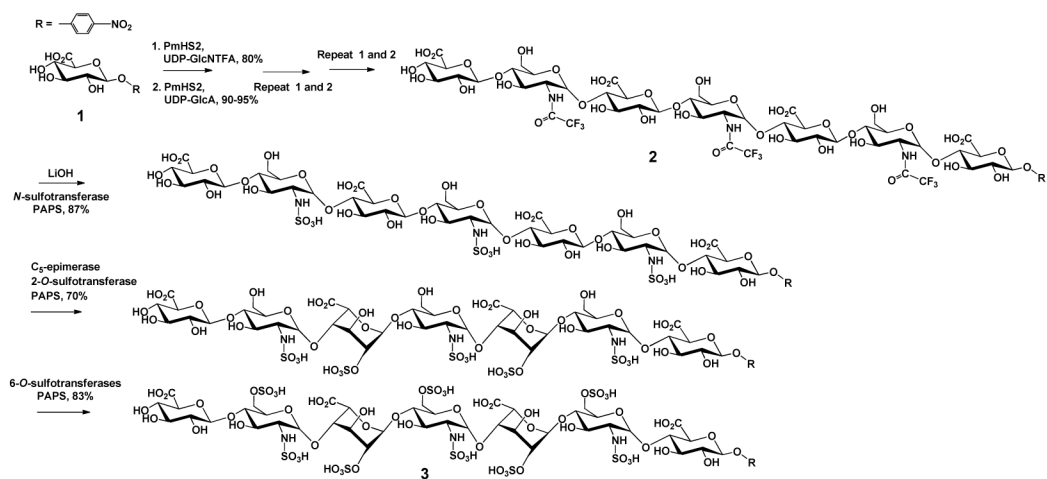
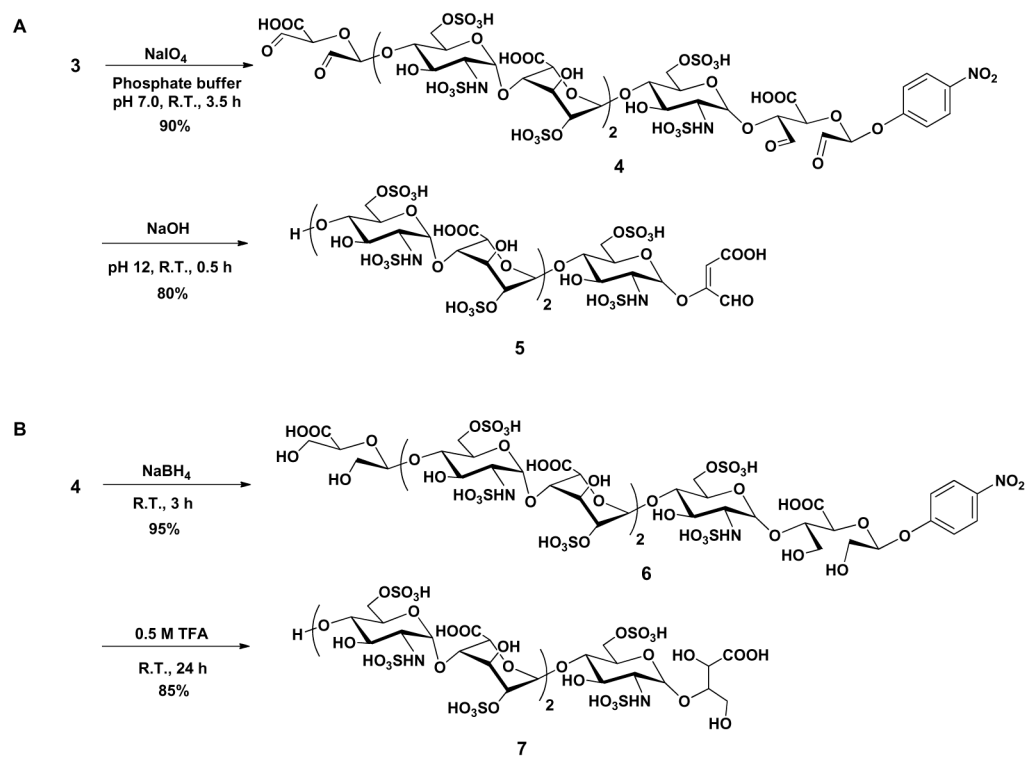


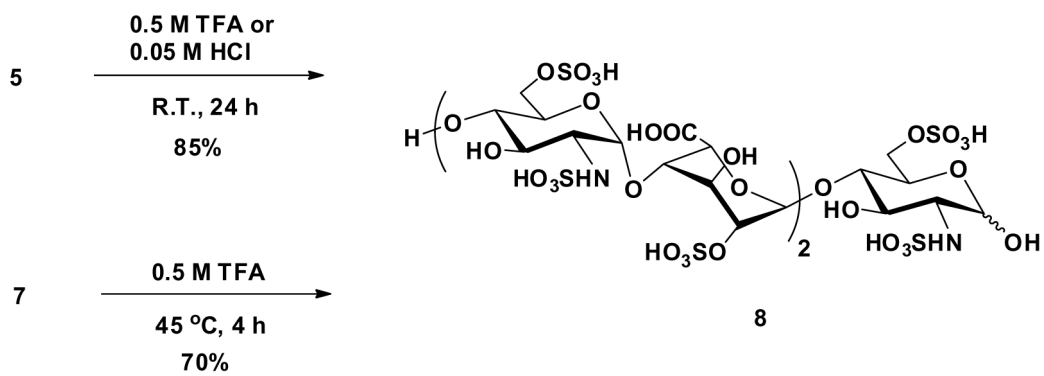
Figure 3. NMR and HRMS of natural heparin oligosaccharide **8**. Panel I shows the 1D ¹H NMR spectrum. Panel II shows the selected anomeric and IdoA2S carbon region (64–104 ppm) in 2D ¹H-¹³C HSQC spectrum. Panel III shows the HRMS (negative mode) of compound **8**.



Scheme 1.
Construction of heparin-like heptasaccharide (**3**)

**Scheme 2.**

GlcA-*p*NP cleavage via alkaline elimination (A) and Smith degradation (B).



Scheme 3.
Acidic hydrolysis of glycoside on degradation oligosaccharides.

Table 1

Alkaline Smith degradation of different synthetic oligosaccharides.

Entry	Substrates	Products ^a
1	GlcNAc-GlcA-(GlcNAc-GlcA) ₅ -GlcNAc-GlcA- <i>p</i> NP	Monosaccharide-R
2	GlcNS-GlcA-(GlcNS-GlcA) ₅ -GlcNS-GlcA- <i>p</i> NP	Monosaccharide-R
3	GlcNS6S-GlcA-(GlcNS6S-GlcA) ₅ -GlcNS6S-GlcA- <i>p</i> NP	Monosaccharide-R
4	GlcNS-GlcA-GlcNS-IdoA-GlcNS-GlcA- <i>p</i> NP	Monosaccharide-R
5	GlcNS6S-GlcA-GlcNS6S3S-IdoA-GlcNS6S-GlcA- <i>p</i> NP	Monosaccharide-R
6	GlcNS6S-GlcA-(GlcNS6S-IdoA2S) ₅ -GlcNS6S-GlcA- <i>p</i> NP	Undecassaccharide-R ^b
7	GlcNS6S-GlcA-(GlcNS6S-IdoA2S) ₇ -GlcNS6S-GlcA- <i>p</i> NP	Pentadecasaccharide-R ^b
8	GlcNS-GlcA-(GlcNS-IdoA2S) ₇ -GlcNS-GlcA- <i>p</i> NP	Pentadecassaccharide-R ^b

^aR=C₄H₃O₃ (see **5** in Scheme 2 for structure of aglycone).

^bConfirmed by LC-MS (see Supplemental Information).