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Organic & Biomolecular Chemistry

COMMUNICATION

Facile Chemoenzymatic Synthesis of Biotinylated Heparosan Hexasaccharide

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A biotinylated heparosan hexasaccharide was synthesized using one-pot multi-enzyme strategy, *in situ* activation and transfer of *N*-trifluoroacetylglucosamine (GlcNTFA) to heparin backbone significantly improved the synthetic efficiency. The biotinylated hexasaccharide could serve as a flexible core to diversify its conversion into heparan sulfate isoforms with potential biological applications and therapeutics

Heparosan is a linear polysaccharide copolymer consisting of α-1,4-linked D-glucosamine (GlcN) and β-1,4-D-glucuronioc acid (GlcA) disaccharide repeating unit (GlcN-α-1,4-GlcA-β-1,4). It belongs to the glycosaminoglycans (GAGs) family and is found in the capsule polysaccharide of certain pathogentic bacteria. Heparosan also serves as the biosynthetic precursor of heparan sulfates (HSs), which are formed by extensive modifications of the heparosan backbone, namely, sulfations (*O*-sufations and *N*-sufations) and epimerization (C5 epimerization of GlcA to L-iduronic acid).[1] Heparin and HS bind to a variety of protein ligands and regulate a wide variety of important biological activities, including blood coagulation, bacterial and viral infection, inflammation, growth factor regulation, cell adhesion, cell growth, tumor metastasis, lipid metabolism and diseases of the nervous system.[2]

Heparin and HS have been widely used as anticoagulant drugs for a century due to their capacity of strong binding to antithrombin.[3] Synthetic homogenous heparins can eliminate detrimental effects caused by contamination of heterogeneous heparins purified from natural sources heparins.[4] FDA-approved synthetic pentasaccharide (Fondaparinux or Arixtra®) exhibits antithrombin III mediated anti-factor Xa activity for prevention and treatment of venous thrombolytic disorders[5] (Figure 1). To date, there is still no efficient antidote available to neutralize the anticoagulant effect of fondaparinux in the case of hemorrhage due to overdose.[6] Extensive efforts have been focused on the development of neutralizable strategy for heparin-based antithrombotic treatment.[7] It was proposed that stable protein-small molecule complexes, which are

formed by strong binding of non-toxic avidin with biotin moiety of biotinylated HS drug molecules, facilitate the drug's fast clearance from circulation.[4a,8] Therefore, the biotinylated-fondaparinux conjugate can be neutralized by avidin to reverse the anticoagulant effect of fondaparinux.
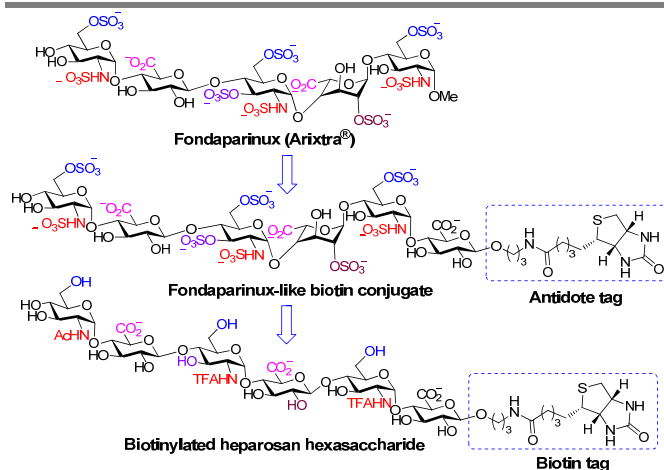


Figure 1. Fondaparinux (Arixtra®) and its analogues

Although much progress has been made in the chemical synthesis of heparin and HS，there are still significant challenges to synthetic chemists.[9] These challenges include: 1) formation of the challenging 1,2-*cis* linkage from the GlcN donor; 2) tedious and non-productive protection/deprotection manipulations resulting in lengthy steps and low overall yields; 3) difficult access to L-iduronic acid and L-idose; 4) the necessity of choosing a set of orthogonal protecting groups for selective sulfations at desired positions, which often lead to unexpected difficulties.[10] During the last decade, numerous methodologies, including the different protection strategies utilized for GlcN donors and acceptors (e.g., the late stage oxidations of glucose into GlcA after glycosylation), have been developed to address such limitations.[11] However, large scale chemical synthesis of heparin and HSs in a pure and fully

deprotected form is still not a routine practice. Alternatively, recent advances in identification and understanding of heparin biosynthetic enzymes' substrate specificity in assembly of heparin and HS have made *in vitro* enzymatic heparin reconstitution practical and realistic.[12] In this communication, we present a highly efficient one-pot multi-enzyme system to chemo-enzymatically construct a biotinylated heparosan hexasaccharide in a straightforward means, which can be used for multiple later-stage enzymatic modifications to furnish a library of HS-like isoforms bearing a highly valuable biotin moiety that could allow wide varieties of biological applications. For example, biotin could serve as an anchoring tip to immobilize such a oligosaccharide library onto the streptavidin-coated microarray surface for identification of previously unknown glycan-binding proteins (GBPs) at the molecular-level, providing insight into the potential relationship between sulfation/epimerization patterns and protein binding specificities.[13] Another important factor of designing such biotin conjugate arises from the fact that a fondaparinux-like biotin conjugate could be constructed by subsequently selective enzymatic epimerization, *N*-, *2*-O-, *6*-O-, *3*-O- sulfations (Figure 1).[12g] This will provide an opportunity for efficient regulation and management of its anti-coagulation treatment to prevent hemorrhage by avidin neutralization.[14]

To date, several enzyme systems for heparosan synthesis have been identified. In *Escherichia coli* (*E. coli*), a pair of glycosyltransferases KfiA and KfiC are used to transfer hexosamine and glucuronic acid UDP precursors respectively to the non-reducing end of the growing chain.[15] While in *Pasteurella multocida* (*P. multocida*), bifunctional glycosytransferase PmHS1 or PmHS2 is used to execute consecutive incorporation of hexosamine and glucuronic acid.[16] Interestingly, the heparosan synthases from *P. multocida* (PmHS1 and PmHS2) possess both UDP-GlcN and UDP-GlcA transfering sites in the same peptide chain, and employ both inverting and retaining polymerization mechanisms. Recently, KfiA from *E. coli* K5 and PmHS2 from *P. multocida* have been used in the synthesis of heparosan with a disaccharide as chain initiating scaffold, which is derived from degradation of heparosan by fermentation.[12g, 17] However, the use of chemically modified biotinylated GlcA as a chain initiating scaffold, and *in situ* activation and transfer of *N*-trifluoroacetylglucosamine (GlcNTFA) to heparosan backbone in one-pot multi-enzyme system have not been reported.

Prior to initiating heparin synthesis, the first issue needed to be addressed is whether the GlcA-biotin conjugate **5** could be tolerated and recognized by either KfiA or PmHS2 to form GlcNTFA-α-1-4-GlcA-biotin disaccharide using the corresponding sugar nucleotide donors. To this end, GlcA-biotin conjugate **5** was chemically synthesized.[18] As outlined in Scheme 1, the synthesis started from glucose pentaacetate **1**. Lewis acid-mediated glycosylation of **1** with 3-azidopropanol gave azido-glucoside **2** in 60% yield, which then went through Zemplén deacetylation, selective TEMPO-mediated oxidation of primary hydroxyl group of glucose, azide reduction by NaBH[4] in the presence of palladium catalyst, and final conjugation with activated biotin succinimide ester (Biotin-OSu) **4**. After purification by silica gel column chromatography, GlcA-biotin **5** was provided with 40-45% overall yield (over 4 steps) from compound **2**.

Remarkably, the synthesis of GlcA-biotin **5** starting from compound **1** required only two silica gel column chromatographic purification steps, dramatically facilitating its synthetic efficacy. In fact, conjugate **5** was easily synthesized in grams scale in one batch.
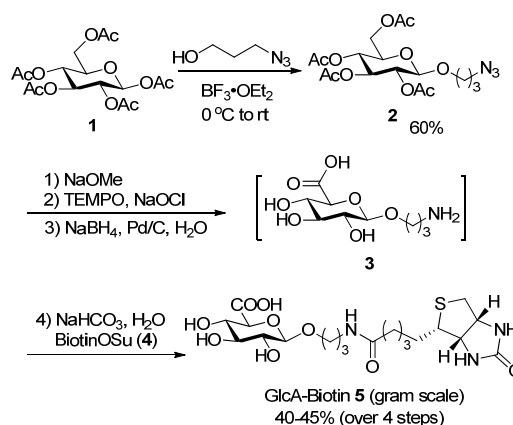
Scheme 1. Synthesis of GlcA-biotin conjugate **5**

With the substrate **5** in hand, KfiA from *E. coli* K5 and PmHS2 from *P. multocida* P-1059 were overexpressed by *E. coli* BL21 (DE3) expression system and purified, respectively. Specifically, the KfiA gene from *E. coli* K5 was cloned and expressed as a fusion enzyme with maltose-binding protein (MBP) at the *N*-terminal and histidine[6X]-tag at the C-terminal. PmHS2 was cloned and expressed as an *N*-terminal histidine[6X]-tagged recombinant protein. In this work, the bi-functional PmHS2 was chosen as the sole chain elongation enzyme owing to its easier over-expression compared to KfiA in *E. coli* BL21 (DE3) expression system. Initially, one-pot three-enzyme system was used to convert GlcA-biotin conjugate **5** to disaccharide **6**. The reaction mixture (pH 7.0) containing GlcA-β-biotin conjugate **5** (1.0 eq.), GlcNTFA (1.2 eq.), ATP (1.2 eq.), UTP (1.2 eq.), MgCl[2], *N*-acetyl-hexosamine 1-kinase (NahK)[19], *N*-acetylglucosamine-1-phosphate uridyltransferase (PmGlmU)[20] and PmHS2 were incubated in one-pot fashion at 37 °C for 2 days to afford disaccharide **6** in 90% yield (Schem 2, step 1). It's worth mentioning that UDP-GlcNTFA donor is *in situ* generated using NahK and PmGlmU in the presence of GlcNTFA, ATP and UTP. The use of UDP-GlcNTFA rather than UDP-GlcNAc as donor substrate is based on the consideration of later stage *N*-sulfation for future preparation of heparin-like oligosaccharide, since trifluoroacetyl (TFA) group could be removed under mild basic conditions. Subsequently, enzymatic glycosylation reaction of **6** (1.0 eq.) with UDP-GlcA (1.0 eq.) using bi-functional PmHS2 in the presence of MnCl[2] and Tris-HCl buffer (pH 7.0) at 37°C overnight to afford trisaccharide **7** in 88% yield (Scheme 2, step 2). Encouraged by the results of synthesis of disaccharide **6**, we attempted to integrate UDP-GlcNTFA preparation with chain elongation in a one-pot three-enzyme system using simple GlcNTFA as starting substrate to synthesize tetrasaccharide **8** (repeat step 1). As anticipated, the overall reaction rate was dramatically accelerated and the reaction completed within 24 h, providing far more straightforward stoichiometric control of the reaction than the stepwise version. As depicted in Scheme 2, the whole chain elongation was performed by repeating step 1 and step 2

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alternatively. The progress of each chain elongation step was conveniently monitored by thin layer chromatography (TLC), in which the reactants and products could well be resolved using the combination of ethyl acetate, methanol, water and acetic acid as developing solvent system (typical TLC in Supporting Information S5). The corresponding products were purified by Biogel@ P2 size-exclusion chromatographic column. Interestingly, the enzymatic reaction normally proceeded at a faster rate as the chain became longer, indicating the preference and dependence of PmHS2's activity on the acceptor length. In addition, the oligosaccharide acceptors in each elongation step could be cleanly and completely converted by PmHS2 to form the desired products. In last step of hexasaccharide preparation, non-reducing end terminal was capped with GlcNAc residue instead of GlcNTFA to provide a heparosan backbone potentially compatible for future selective epimerization of internal GlcA residue flanked by two GlcNTFA units into corresponding iduronic acid (IdoA) isoform after N-sulfation.[12g] Remarkably, by capitalizing on our improved one-pot three-enzyme approach and convenient monitoring of the reaction progress by a quick TLC analysis, one run of the whole synthetic scheme starting from 60 mg of GlcA-biotin conjugate **5** yielded 65 mg of the final biotinylated hexasaccharide **10** with 33% overall yield (averaging 80% yield of each step) in two weeks.
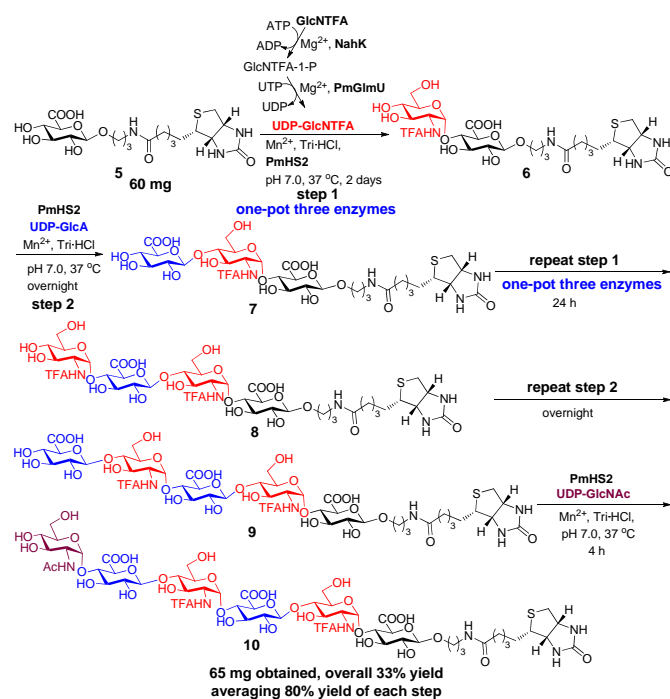
Scheme 2. Sequential one-pot multi-enzyme synthesis of biotinylated heparosan hexasaccharide **10**

In summary, we have developed a streamlined, preparative scale and HPLC-free chemoenzymatic synthesis of biotinylated heparosan hexasaccharide. The synthesis features integration of *in situ* UDP-GlcNTFA generation with heparin backbone elongation in one-pot fashion. As a result of such technical reformation, the overall synthetic efficacy is significantly increased. The flexible core hexasaccharide backbone allows multiple later-stage enzymatic modifications to furnish a library of HS as highly useful molecular

probes to dissect the heparin/heparin binding proteins' interactions in biological settings, owing to that biotin moiety could be fished out by streptavidin. Efforts towards the final construction of fondaparinux-like biotin conjugate, which is formed via consecutive modifications of **10** by *N*-sulfation, selective epimerization of internal GlcA into iduronic acid (IdoA) and 2-*O*-, 6-*O*-, 3-*O*-sulfations, are currently underway.

Notes and references

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Electronic Supplementary Information (ESI) is available, including detailed experimental procedure and characterization of compounds. See DOI: 10.1039/c000000x/

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