Enzymatic Synthesis

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Tailored Design and Synthesis of Heparan Sulfate Oligosaccharide Analogues Using Sequential One-Pot Multienzyme Systems**

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Heparan sulfate (HS) and heparin are linear sulfated heteropolysaccharides that consist of alternating α1–4-linked D-glucosamines (GlcN) and 1–4-linked uronic acids, with an α-linkage for L-iduronic acid (IdoA) and a β-linkage for D-glucuronic acid (GlcA). Possible modifications include 2-*O*-sulfation on the uronic acid residues, and one or more modifications on the glucosamine residues, including *N*-sulfation, *N*-acetylation, 6-*O*-sulfation, and 3-*O*-sulfation. Heparin and low-molecular-weight heparin (LMWH) are the most commonly used anticoagulants or antithrombotic drugs. Compared to HS, heparin has a higher level of sulfation and a higher IdoA content.[1] Heparin is mostly produced by mast cells, and heparan sulfates are produced by different cell types in animals.[2] They are attractive synthetic targets because of the therapeutic application of heparin, and the important roles of HS and heparin in regulating cancer growth, blood coagulation, inflammation, assisting against viral and bacterial infections, signal transduction, lipid metabolism, and cell differentiation.[3]

Synthetic heparins can eliminate the side effects caused by inherently heterogeneous heparins purified from natural sources. Their syntheses, however, present great synthetic challenges owing to their structural complexity. Although much progress has been made over the last decade in terms of synthesis, analysis, and understanding of complex HS and heparin, the mechanisms for the formation and regulation of HS/heparin and the structure–function relationship of complex HS/heparin are still not fully understood.[2] A tailor-made synthetic process is still lacking.

Early chemical syntheses of heparin fragments and analogues[4] required many protection and deprotection steps, making the synthesis of even relatively small oligosaccharides time-consuming and rather inefficient. More recently, various chemical synthetic approaches[5] including target-oriented,[6] modular,[7] combinatorial,[8] one-pot,[9] and solid-phase[10] syntheses, have been developed and used to produce HS/heparin oligosaccharides that range from di- to octasaccharides of different sequences and sulfation patterns. Glycan microarrays have been developed to study heparin/HS–protein interactions.[11] Nevertheless, the synthesis of HS oligosaccharides of non-repetitive sequences is much more challenging than that of oligosaccharides with repetitive sequences. Synthetic efficiency decreases dramatically as the length of the target molecule increases. Furthermore, variations in the structure and length of the target molecules can completely change the whole synthetic design.

Traditional methods of purifying oligosaccharides after enzymatic digestion of GAG chains have provided useful quantities of HS/heparin oligosaccharides for early structure–activity relationship studies.[12] Cloning and characterization of HS biosynthetic enzymes have allowed chemoenzymatic syntheses of various HS/heparin derivatives.[13] Bioactive HS structures that bind to antithrombin, fibroblast growth factor FGF2, or herpes simplex virus glycoprotein D (HSV gD) were enzymatically synthesized on milligram scales from completely desulfated and *N*-sulfated heparin by multiple *O*-sulfation reactions using immobilized *O*-sulfotransferases with the regeneration of the activated sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS).[13c] Heparin-like polysaccharides with anticoagulant activity were obtained from purified *Escherichia coli* K5 capsular polysaccharide, followed by epimerization of the C5 position of the uronic acid residues, chemical persulfation, and selective desulfonation.[13b] A heparin polysaccharide library with different sulfation patterns was obtained using different HS biosynthetic enzymes.[13d] Recently, two homogeneous heparin oligosaccharides were chemoenzymatically synthesized on a milligram scale, and showed comparable activity as Arixtra (fondaparinux sodium), a synthetic anticoagulant heparin pentasaccharide.[14] Owing to the complex nature of the HS biosynthesis and the involvement of multiple isoenzymes with overlapping substrate preferences, the existing methods do not provide precise control for the syntheses of many desired structures.

An efficient approach to synthesize carbohydrates that contain post-glycosylational modifications (PGMs),[15] includ-

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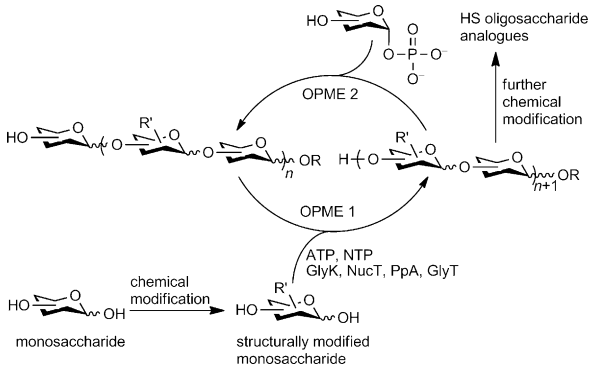
ing HS oligosaccharides and analogues, with precise control starts from the corresponding structurally modified monosaccharides, and uses sequential one-pot multienzyme (OPME) glycosylation reactions (Scheme 1). The simplest

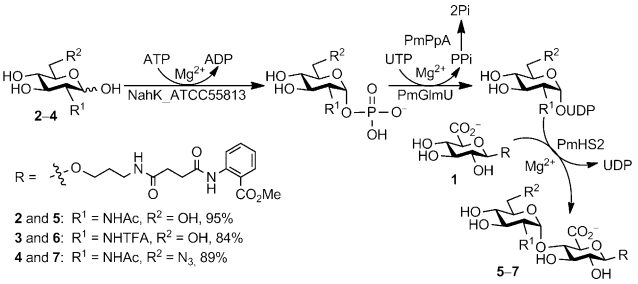


Scheme 1. Sequential one-pot multienzyme (OPME) chemoenzymatic synthesis of heparan sulfate (HS) oligosaccharide analogues. GlyK = glycokinase, GlyT = glycosyltransferase, NucT = monosaccharide-1-phosphate nucleotidyltransferase, PpA = inorganic pyrophosphatase.

OPME system consists of a monosaccharide kinase (GlyK), a nucleotidyltransferase (NucT), and a glycosyltransferase (GlyT) to allow the formation of the desired glycosidic bonds in a regio- and stereospecific manner. An inorganic pyrophosphatase (PpA) can also be used to remove the pyrophosphate by-product formed to drive the reaction towards the formation of the sugar nucleotide. Further chemical modifications can be performed to introduce additional functionality after the formation of the oligosaccharides. Herein, we describe such a method for the efficient synthesis of HS oligosaccharide analogues with desired sulfation patterns.

PmHS2 from the Gram-negative bacterium *Pasteurella multocida* is a well-studied heparosan synthase.[16] It is a bifunctional enzyme that acts as both an α1-4-*N*-acetylglucosaminyltransferase (α1-4GlcNAcT) and a β1-4-glucuronyltransferase (β1-4GlcAT). PmHS2 cloned from the *Pasteurella multocida* strain P-1059 (ATCC#15742, type A strain) has a good expression level in *Escherichia coli* (ca. 17 mg L^{-1} cell culture). The α1-4GlcNAcT activity of PmHS2 is more promiscuous than that of *Escherichia coli* K5 α1-4GlcNAcT (KfiA) when using UDP-GlcNAc derivatives as donor substrates.[17]

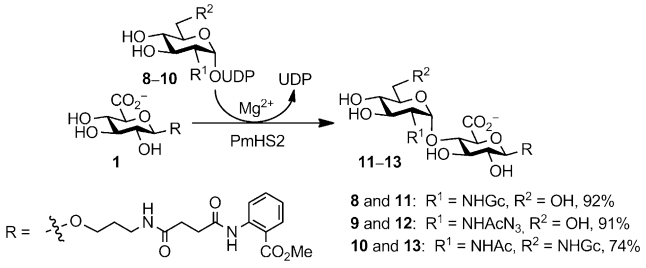
As shown in Scheme 2, using glucuronide GlcAβ2AA (**1**) with a fluorescent label as the acceptor substrate and taking advantage of the substrate promiscuities of several enzymes involved in GlcNAc activation and transfer, disaccharide GlcNAcα1-4GlcAβ2AA (**5**) and its derivatives GlcNTFAα1-4GlcAβ2AA (**6**) and GlcNAc6N_3α1-4GlcAβ2AA (**7**) were synthesized in high yields (84–95%) from *N*-acetylglucosamine (GlcNAc, **2**), *N*-trifluoroacetylglucosamine (GlcNTFA, **3**), and 6-azido-6-deoxy-*N*-acetylglucosamine (GlcNAc6N_3, **4**), respectively, using a one-pot four-enzyme reaction containing NahK_ATCC55813,[18] PmGlmU,[19] PmPpA,[20] and PmHS2.[17] It was found that the cleavage of the *N*-TFA (TFA = trifluoroacetyl) group in **3** and **6** occurred



Scheme 2. One-pot four-enzyme GlcNAc activation and transfer system for the synthesis of disaccharide GlcNAcα1-4GlcAβ2AA and derivatives. Tris-HCl buffer (100 mM, pH 7.5) was used for the synthesis of disaccharides **5** and **7**, and MES buffer (100 mM, pH 6.5) was used for the synthesis of disaccharide **6**. Ac = acetyl, NahK_ATCC-55813 = *Bifidobacterium longum* ATCC55813 *N*-acetylhexosamine 1-kinase, PmGlmU = *Pasteurella multocida* strain P-1059 (ATCC15742) *N*-acetylglucosamine-1-phosphate uridylyltransferase, PmHS2 = *Pasteurella multocida* strain P-1059 heparosan synthase 2, PmPpA = *Pasteurella multocida* strain P-1059 inorganic pyrophosphatase, TFA = trifluoroacetyl.

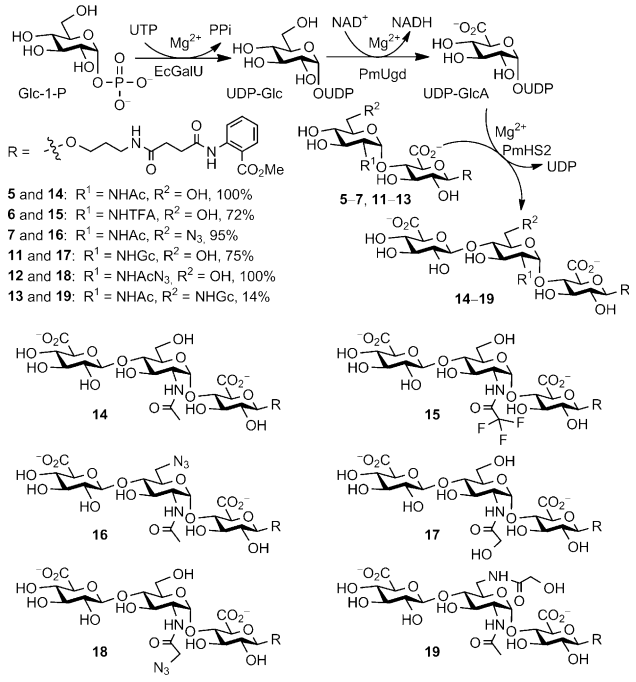
in significant amounts at pH 7.5, but this could be reduced by lowering the pH to 6.5 and by shortening the reaction time.

In addition, using three previously synthesized UDP-GlcNAc derivatives, including UDP-*N*-glycolylglucosamine (UDP-GlcNGc, **8**), UDP-*N*-azidoacetylglucosamine (UDP-GlcNAcN_3, **9**), and UDP-6-deoxy-6-glycolylamido-*N*-acetylglucosamine (UDP-GlcNAc6NGc, **10**),[19] a PmHS2-catalyzed GlcNAc transfer reaction produced disaccharides GlcNGcα1-4GlcAβ2AA (**11**), GlcNAcN_3α1-4GlcAβ2AA (**12**), and GlcNAc6NGcα1-4GlcAβ2AA (**13**) in good to excellent yields (74–92%) (Scheme 3).



Scheme 3. PmHS2-catalyzed synthesis of GlcNAcα1-4GlcAβ2AA disaccharide derivatives **11**–**13** in Tris-HCl buffer (100 mM, pH 7.5). Gc = glycolyl.

The obtained disaccharides **5**–**7** and **11**–**13** were used in a one-pot three-enzyme GlcA activation and transfer system (Scheme 4) to test the acceptor–substrate specificity of the β1-4GlcAT activity of PmHS2. In this system, a glucose-1-phosphate uridylyltransferase cloned from *Escherichia coli* K-12 (EcGalU)[21] was used to catalyze the formation of UDP-Glc from Glc-1-P and UTP. A UDP-glucose dehydrogenase, which was cloned from the *Pasteurella multocida* strain P-1059 (PmUgd; see the Supporting Information for cloning, purification, and DNA and protein sequences), was used in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD^+) to oxidize the C6 position of glucose

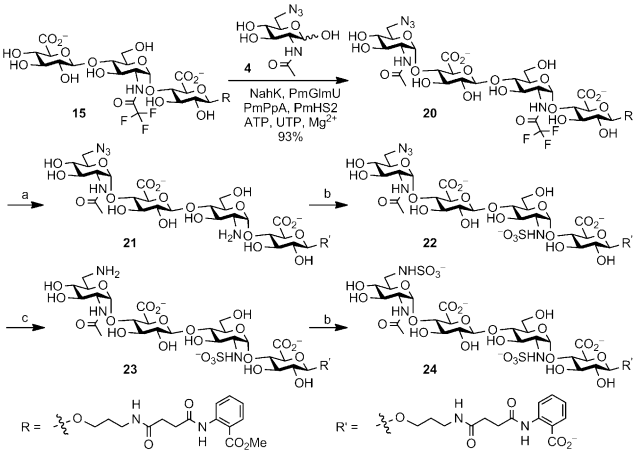


Scheme 4. One-pot three-enzyme GlcA activation and transfer system for the synthesis of trisaccharide GlcAβ1-4GlcNAcα1-4GlcAβ2AA and derivatives. EcGalU = *Escherichia coli* K-12 glucose-1-phosphate uridylyltransferase, PmUgd = *Pasteurella multocida* strain P-1059 UDP-glucose dehydrogenase.

(Glc) in UDP-Glc to form UDP-glucuronic acid (UDP-GlcA); in combination with the β1-4GlcAT activity of PmHS2, this was used to form trisaccharides. The reactions were analyzed by a high-performance liquid chromatography (HPLC) system equipped with a fluorescence detector. As shown in Scheme 4, all disaccharides (**5**–**7** and **11**–**13**) tested were suitable acceptor substrates for the β1-4GlcAT activity of PmHS2. Trisaccharides GlcAβ1-4GlcNAcα1-4GlcAβ2AA (**14**), GlcAβ1-4GlcNAc6N_3α1-4GlcAβ2AA (**16**), and GlcAβ1-4GlcNAcN_3α1-4GlcAβ2AA (**18**) were formed in quantitative or near quantitative yields (95–100%). GlcAβ1-4GlcNTFAα1-4GlcAβ2AA (**15**) was formed in a lower yield (72%), mainly because of the loss of the *N*-TFA-group to form another trisaccharide, GlcAβ1-4GlcNH_2α1-4GlcAβ2AA. Disaccharide GlcNGcα1-4GlcAβ2AA (**11**), which posses an *N*-glycolyl group at the C2 position of GlcN, was a good acceptor for the β1-4GlcAT activity of PmHS2 to form GlcAβ1-4GlcNGcα1-4GlcAβ2AA (**17**) in 75% yield. In comparison, disaccharide GlcNAc6NGcα1-4GlcAβ2AA (**13**) with the same *N*-glycolyl group at the C6 position of the GlcNAc moiety was a poorer acceptor, and trisaccharide GlcAβ1-4GlcNAc6NGcα1-4GlcAβ2AA (**19**) was formed in only 14% yield.

Taken together, these results indicate that aside from the previously identified donor–substrate promiscuity of the α1-4GlcNAcT activity of PmHS2,[16b,17] the β1-4GlcAT activity of PmHS2 also shows a broader acceptor–substrate promiscuity than previously described.[22] These substrate promiscuities allowed the synthesis of GlcNAcα1-4GlcAβ2AA disaccharide derivatives containing modified GlcNAc residues, which were further used for the formation of diverse trisaccharide derivatives. In principle, the same strategy can be repeated for the production of longer oligosaccharides and derivatives. The oligosaccharide derivatives can be used as important probes for investigating the acceptor-binding pocket of PmHS2, which may bind to oligosaccharide acceptors that are longer than monosaccharides or disaccharides, as discussed previously.[22]

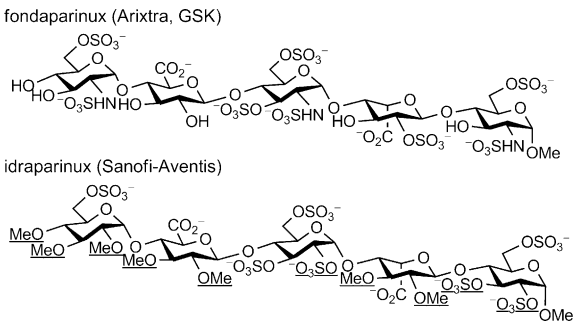
A strategy for the synthesis of HS oligosaccharide analogues on a preparative scale with controllable sulfation patterns was also explored. Trisaccharide GlcAβ1-4GlcNTFAα1-4GlcAβ2AA (**15**; >50 mg) was synthesized using the one-pot three-enzyme GlcA activation and transfer system shown in Scheme 4. The loss of the *N*-TFA group was minimized by carrying out the reaction at pH 7.0 instead of pH 7.5, and the yield of the reaction was thus increased to 87%. As shown in Scheme 5, the obtained trisaccharide **15**



Scheme 5. One-pot four-enzyme synthesis of tetrasaccharide **20** and subsequent chemical derivatization reactions for the formation of tetrasaccharides **21**–**24**. Reagents and conditions: a) K_2CO_3, H_2O, RT, overnight, 81%; b) pyridine–SO_3 complex, NaOH (2 M), H_2O, 3 days, 70%; c) H_2, Pd/C, MeOH, H_2O, 1 h, quantitative.

was used as a substrate in the one-pot four-enzyme GlcNAc activation and transfer system (the same one as shown in Scheme 2) for the synthesis of tetrasaccharide GlcNAc6N_3α1-4GlcAβ1-4GlcNTFAα1-4GlcAβ2AA (**20**) in high yield (93%). The *N*-TFA group and the N_3 group were individually converted into free amine groups, as shown for tetrasaccharides **21** and **23**, allowing sequential *N*-sulfation to generate HS tetrasaccharide analogues **22**–**24**. More specifically, the *N*-TFA group at the C2 position of the internal GlcNTFA residue in tetrasaccharide **20** was removed under mildly basic conditions to produce tetrasaccharide GlcNAc6N_3α1-4GlcAβ1-4GlcNH_2α1-4GlcAβ2AA′ (**21**) in 81% yield. As the removal of the *N*-TFA group was accompanied by the hydrolysis of the methyl carboxylate ester, tetrasaccharide **21** contains a free carboxylate in the 2AA′ motif instead of the carboxylic ester in the 2AA moiety of tetrasaccharide **20**. Sulfation of tetrasaccharide **21** for the formation of tetrasaccharide **22** required a larger excess of sulfation reagent (60 equiv) and a prolonged reaction time (3 days) to achieve

a reasonable 70 % yield. Catalytic hydrogenation of the azido group at the C6 position of the non-reduced GlcNAc6N_3 end in tetrasaccharide **22** produced tetrasaccharide **23**. Tetrasaccharide **24** was subsequently synthesized by *N*-sulfation. These *N*-sulfated oligosaccharide analogues mimic naturally occurring HS oligosaccharides and may have improved therapeutic potentials, as they will most likely be more resistant to the activities of HS/heparin lyases and hydrolases. Indeed, idraparinux, a synthetic fully *O*-sulfated and *O*-methylated analogue of heparin pentasaccharide, has a higher anti-Xa activity and a longer duration of action that fondaparinux (or Arixtra), an FDA-approved synthetic compound, which contains an unmodified antithrombin-binding heparin pentasaccharide for the prevention and treatment of venous thromboembolism (Scheme 6).[23] The *N*-sulfated analogues of HS oligosaccharides may also have unexpected or improved functions.



Scheme 6. Fondaparinux (Arixtra) and idraparinux; differences are underlined.

In conclusion, we have developed efficient one-pot multienzyme (OPME) systems for the activation and transfer of GlcNAc and GlcA, which have been successfully used for the synthesis of *N*-sulfated analogues of HS oligosaccharides. We have demonstrated that PmHS2, a bifunctional heparosan synthase from *Pasteurella multocida* responsible for the production of *N*-acetylheparosan, has promiscuous donor and acceptor substrate activities. The method of synthesizing *N*-sulfated analogues of HS oligosaccharides in a tailor-designed fashion will be explored further for the synthesis of longer oligosaccharides with more diverse sulfation patterns. The resulting compounds may compete efficiently with naturally occurring HS and heparin for binding to their target proteins and are potential therapeutics.

Experimental Section

One-pot four-enzyme synthesis of disaccharides **5**–**7**: GlcAβ2AA **1** (5–30 mg, 1 equiv), GlcNAc or derivatives (1.5 equiv), ATP (1.8 equiv), and UTP (1.8 equiv) were dissolved in water in a 15 mL centrifuge tube containing $MgCl_2$ (10 mM) in a Tris-HCl (100 mM, pH 7.5) or MES (100 mM, pH 6.5) buffer. After adding NanK_ATCC55813 (0.5–2.1 mg), PmGlmU (1–3 mg), PmPpA (0.5–1.5 mg), and PmHS2 (1–6 mg), water was added to bring the concentration of GlcAβ2AA **1** to 5 mM. The reaction mixture was incubated for 12–36 h at 37 °C with gentle shaking.

PmHS2-catalyzed synthesis of disaccharides **11**–**13**: GlcAβ2AA **1** (5–10 mg, 1 equiv) and UDP-GlcNAc derivatives (1.2 equiv) were dissolved in water in a 15 mL centrifuge tube containing $MgCl_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 7.5). After adding PmHS2 (1–2 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction mixture was incubated for 12–36 h at 37 °C with gentle shaking.

Product formation was monitored by thin layer chromatography (EtOAc/MeOH/H_2O = 4:2:1, v/v), using a *p*-anisaldehyde sugar staining solution. The reaction was stopped by adding the same volume of ice-cold ethanol and incubating at 4 °C for 30 min. The mixture was concentrated and passed through a BioGel P-2 gel filtration column to obtain the desired product. Purification by column chromatography on silica gel (EtOAc/MeOH/H_2O = 5:2:1) was used when necessary to achieve further purification.

Small-scale one-pot three-enzyme synthesis of trisaccharides **14**–**19**: Typical reactions were performed in a total volume of 20 μL in Tris-HCl buffer (100 mM, pH 7.5) containing $MgCl_2$ (10 mM), UTP (7.5 mM), disaccharides (5 mM), Glc-1-P (6 mM), NAD^+ (12 mM), GalU (2.5 μg), PmUgd (8 μg), and PmHS2 (11.5 μg). Reactions were allowed to proceed for 12 h at 37 °C and quenched by adding ice-cold ethanol (20 μL) and water (1.96 mL) to achieve 100-fold dilution.

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