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Chemoenzymatic Design of Heparan Sulfate Oligosaccharides*[S]

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Heparan sulfate is a sulfated glycan that exhibits essential physiological functions. Interrogation of the specificity of heparan sulfate-mediated activities demands a library of structurally defined oligosaccharides. Chemical synthesis of large heparan sulfate oligosaccharides remains challenging. We report the synthesis of oligosaccharides with different sulfation patterns and sizes from a disaccharide building block using glycosyltransferases, heparan sulfate C_5-epimerase, and sulfotransferases. This method offers a generic approach to prepare heparan sulfate oligosaccharides possessing predictable structures.

Heparan sulfate (HS)[3] is a unique class of macromolecular natural product that is present in large quantities on the mammalian cell surface and in the extracellular matrix. HS participates in regulating blood coagulation, embryonic development, and the inflammatory response and assists viral/bacterial infections. It consists of a repeating disaccharide unit of glucuronic acid (GlcUA) or iduronic acid (IdoUA) and glucosamine, both capable of carrying sulfo groups (1). The sulfation pattern of HS dictates its biological activity (2, 3). Heparin, a widely used anticoagulant drug, is a specialized form of highly sulfated HS. The diverse biological functions present considerable opportunities for exploiting HS or HS-protein conjugates for developing new classes of anticancer (4), antiviral (5), and improved anticoagulant drugs (6). Furthermore, a recent worldwide outbreak of contaminated heparin underscores the needs for synthetic heparins to replace those isolated from animal tissues (7). Chemical synthesis is a powerful tool to obtain structurally defined heparin/HS oligosaccharides. The most successful example is the total synthesis of an antithrombin-binding pentasaccharide (8). This pentasaccharide is marketed under the trade name Arixtra for the treatment of venous thromboembolic disorders. However, the chemical synthesis of oligosaccharides larger than an octasaccharide is extremely difficult, especially when multiple target structures are required for biological evaluation (8). An enzyme-based method offers a promising alternative approach to synthesize HS.

The HS biosynthetic pathway involves multiple enzymes, including HS polymerase, epimerase, and sulfotransferases (Fig. 1). HS polymerase is responsible for building the polysaccharide backbone, containing the repeating unit of -GlcUA-GlcNAc-. The backbone is then modified by *N*-deacetylase/*N*-sulfotransferase (having two separate domains exhibiting the activity of *N*-deacetylase and *N*-sulfotransferase, respectively), C_5-epimerase (C_5-epi, converting GlcUA to IdoUA), 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST) and 3-*O*-sulfotransferase (3-OST) to produce the fully elaborated HS. With the exception of HS polymerase, all of these biosynthetic enzymes have been expressed at high levels in *Escherichia coli* (1), permitting easy access to an abundance of enzymes. Using HS sulfotransferases and C_5-epi, we previously developed a method to synthesize HS from a bacteria capsular polysaccharide with biological activities (6, 9, 10).

The synthetic HS products are a mixture of polysaccharides with different sizes and sulfated monosaccharide sequences. A mixture of polysaccharides can sometimes confound structure and activity relationship studies. Methods for the synthesis of structurally defined oligosaccharides have been reported (11, 12). These methods describe synthetic strategies that targeted a single product from a specialized oligosaccharide starting material. These approaches clearly lack the ability for rapid synthesis of HS oligosaccharides differing in sizes and structures due to the difficulty in obtaining the starting materials. Therefore, a technique combining the oligosaccharide backbone synthesis with saccharide modifications, using HS biosynthetic enzymes, should expand the capability of HS oligosaccharide synthesis. In this paper, we utilize bacterial glycosyltransferases and an unnatural UDP-monosaccharide donor to build an oligosaccharide backbone from a disaccharide. These backbone oligosaccharides can be then selectively modified with different HS sulfotransferases and C_5-epi to prepare structurally defined products with desired sulfation patterns. Further, this method was employed to identify a novel HS structure that binds to antithrombin.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6 and Tables 1 and 2.

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[3] The abbreviations used are: HS, heparan sulfate; AT, antithrombin; C_5-epi, C_5-epimerase; ESI, electrospray ionization; GlcNTFA, trifluoroacetylglucosamine; MS/MS, tandem MS; NST, *N*-sulfotransferase; OST, *O*-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

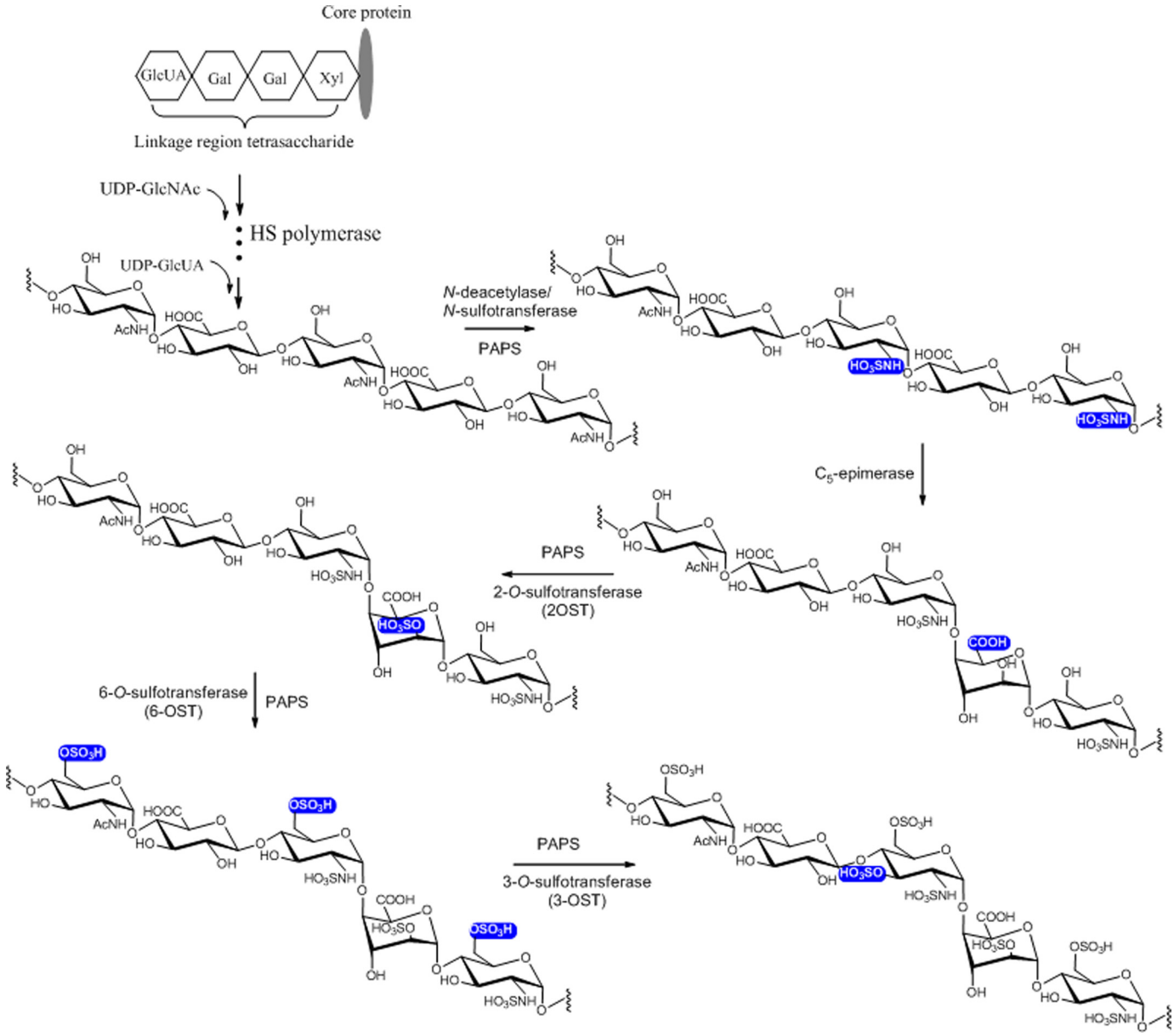

FIGURE 1. **Biosynthetic pathway of HS.** The biosynthetic pathway includes the biosynthesis of polysaccharide backbone as well as the modification steps. The synthesis is initiated with a tetrasaccharide linkage region that contains xylose-galactose-galactose-glucuronic acid. The backbone is synthesized by HS polymerase. The backbone polysaccharide is then modified via five enzymatic modification steps. The modification site at each step is *highlighted* in a *blue box*.

EXPERIMENTAL PROCEDURES

Expression of HS Biosynthetic Enzymes—A total of nine enzymes were used for the synthesis of HS oligosaccharides, including HS sulfotransferases and glycosyl transferases. *N*-sulfotransferase (NST), C_5-epi, 2-OST, 6-OST-1, 6-OST-3, 3-OST-1, 3-OST-5, and *N*-acetyl-D-glucosaminyl transferase of *E. coli* K5 strain (KfiA) were expressed and purified as previously described (6, 13–16). PmHS2 was expressed as an *N*-terminal fusion to His_6 using a PET-15b vector (Novagen) (17). The expression of pmHS2 was carried out in BL21 star (DE3) cells (Invitrogen) coexpressing bacteria chaperone proteins, GroEL and GroES.

Preparation of Disaccharide (GlcUA-AnMan, **14***) and Oligosaccharide Backbones*—The disaccharide **14** was prepared from nitrous acid-degraded heparosan as previously described (16). The resultant disaccharide **14** was dialyzed against water using 1000 MWCO membrane (Spectrum).

To synthesize oligosaccharide backbone, disaccharide (GlcUA-AnMan, **14**) (4.5 μmol) was incubated with UDP-GlcNTFA (3.9 μmol) and KfiA (0.1 mg) in a 1 ml of buffer containing 25 mM Tris-HCl (pH 7.2) and 10 mM $MgCl_2$. The reaction was incubated at room temperature overnight. An aliquot of reaction mixture was analyzed by a polyamine-based HPLC column (from Waters) to ensure that >95% of UDP-GlcNTFA was converted to UDP. Upon the complete consumption of UDP-GlcNTFA, pmHS2 (0.5 mg) and UDP-GlcUA (4 μmol) were added into the reaction mixture for additional 4–5 h at room temperature. Another aliquot of pmHS2 (0.5 mg) and UDP-GlcUA (4 μmol) was added to drive the transfer of GlcUA unit to completion. It is important to note that pmHS2 has both

activities in transferring GlcNAc (or GlcNTFA) and GlcUA. Without removal of UDP-GlcNTFA, pmHS2 led to polymerization or uncontrollable oligomerization of the saccharide, resulting in low yield of the tetrasaccharide product. The reaction mixture was resolved on a Bio-Gel P-10 column (0.75 × 200 cm), which was equilibrated with 20 mM Tris (pH 7.5) and 1 M NaCl at a flow rate of 4 ml/h. The elution position of the tetrasaccharide was determined by a ^{3}H-labeled tetrasaccharide. The product was dialyzed against water using 1000 MWCO membrane. The reaction cycle was repeated four and five times to prepare the decasaccharide **15**, undecasaccharide **16**, and dodecasaccharide **17**.

Synthesis of Fluorous-tagged Disaccharide (GlcUA-AnMan-Rf, **13***) and Tagged Oligosaccharide Backbones*—A disaccharide (GlcUA-AnMannose) was also prepared from heparosan followed a procedure very similar to that described above, omitting the $NaBH_4$ reduction step. To synthesize fluorous-tagged disaccharide **13**, the disaccharide (GlcUA-AnMannose) was incubated with 2 equivalent 4-(1H, 1H, 2H, 2H-perfluoropentyl) benzylamine hydrochloride (Fluorous Technologies) and $NaBH_3CN$ (10 eq) in MeOH overnight at room temperature. The resulting tagged disaccharide **13** was purified by a FluoroFlash column, further purified using paper chromatography by Whatman 3MM chromatography paper (Fisher) developed with 100% acetonitrile. Disaccharide **13** was finally purified by a C_{18} column (0.46 × 25 cm; Thermo Fisher Scientific) under reverse phase HPLC conditions. The column was eluted with a linear gradient from 90% solution A (0.1% trifluoroacetic acid in water) to 50% solution A for 40 min at a flow rate of 0.5 ml/min, then followed by an additional wash for 20 min with 100% solution B (0.1% trifluoroacetic acid in acetonitrile) at a flow rate of 0.5 ml/min. The product was confirmed by electrospray ionization (ESI) mass spectrometry.

To prepare the fluorous-tagged octasaccharides (**1**-**4**), the synthesis was started with a fluorous-tagged disaccharide GlcUA-AnMan-Rf **13**. In each monosaccharide incorporation step, we supplied the reaction mixture with either KfiA or pmHS2 and appropriate UDP-monosaccharide donor. As a result, only one sugar residue was transferred into the backbone. The reaction mixture was assembled as for the unlabeled oligosaccharide backbone synthesis as described above. A FluoroFlash column was used to separate the tagged oligosaccharides from unreacted UDP-monosaccharides and enzymes. Briefly, Fluorous silica gel (40 μm; Fluorous Technologies) was washed with water and eluted with methanol. The reaction cycle was repeated three times to prepare octasaccharide backbones.

Preparation of UDP-GlcNTFA—UDP-GlcNTFA was synthesized using a chemoenzymatic approach, involved in preparing a GlcNTFA 1-phosphate and coupling it with UDP. Briefly, 11 mg of $GlcNH_2$ 1-phosphate (Sigma-Aldrich) was dissolved in 200 μl of anhydrous methanol and mixed with 60 μl of $(C_2H_5)_3N$ and 130 μl of *S*-ethyl trifluorothioacetate (Sigma-Aldrich). The reaction was incubated at room temperature for 24 h. The resultant GlcNTFA 1-phosphate was then converted to UDP-GlcNTFA using glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU) in a buffer containing 46 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 200 μM dithiothreitol, 2.5 mM UTP, and 0.012 units/μl of inorganic pyrophosphatase (Sigma-Aldrich). Recombinant GlmU was expressed in *E. coli* and purified by a Ni-agarose column (16). The UDP-GlcNTFA was purified by removing proteins using centrifugal filters (10,000 MWCO; Millipore) followed by the dialysis against water using 1000 MWCO membrane for 4 h. The product was confirmed by MS analysis. The concentration was determined by a quantitative analysis with PMAN-HPLC using UDP-GlcNAc as a standard.

Selective De-N-trifluoroacetylation of Oligosaccharides Carrying GlcNTFA Units—Various amounts of oligosaccharides (100–200 μg) were dried and resuspended in a solution (200 μl) containing CH_3OH, H_2O, and $(C_2H_5)_3N$ (v/v/v = 2:2:1). The reaction was incubated at room temperature (or at 37 °C for fluorous-tagged octasaccharides) overnight. The samples were dried and reconstituted in H_2O to recover de-*N*-trifluoroacetylated oligosaccharides.

Preparation of Sulfated Oligosaccharide—*N*-Sulfation of oligosaccharide was carried out by incubating the de-*N*-trifluoroacetylated oligosaccharide substrates with NST and 3′-phosphoadenosine 5′-phosphosulfate. The reaction mixture typically contained 6 μg de-*N*-trifluoroacetylated decasaccharide, undecasaccharide, and dodecasaccharide, 80 μM PAPS, 50 mM MES, pH 7.0, 1% Triton X-100 (v/v), and 4 μg of NST in a total volume of 300 μl. The reaction mixture was incubated at 37 °C overnight.

The oligosaccharides were purified by a DEAE column. The reaction mixture (300 μl) was mixed with 1 ml of 0.01% Triton X-100 buffer at pH 5.0 containing 150 mM NaCl, 50 mM NaOAc, 3 M urea, 1 mM EDTA, then followed by four washes with the same buffer, each time 1 ml, and was eluted with 1 M NaCl in 0.001% Triton X-100 buffer. The purified oligosaccharides were dialyzed using 2500 MWCO 3500 membrane and dried. The final product was further purified by a DEAE-NPR HPLC column (0.46 × 7.5 cm; Tosohaas). For tagged octasaccharide (**1**–**4**), heptasaccharide **5**, *N*-sulfo 6-*O*-sulfo decasaccharide **9**, and dodecasaccharide **10**, and *N*-sulfo 6-*O*-sulfo 3-*O*-sulfo decasaccharide **11** and dodecasaccharide **12**, the procedures were very similar to those for *N*-sulfo oligosaccharides (**6**-**8**) as described above using appropriate enzymes.

HPLC Analysis—HPLC analysis of oligosaccharides followed the procedures as previously described (6, 9).

Determination of the Binding Affinity of Oligosaccharides to Antithrombin (AT)—The dissociation constant (K_d) of each sample and AT was determined using affinity coelectrophoresis (18).

Microdialysis of Oligosaccharides—The synthesized oligosaccharides were subjected to microdialysis prior to the MS analysis. The dialysis was carried out using hollow fiber dialysis tubing (13,000 MWCO; Spectrum) against 20 mM ammonium acetate.

Liquid Chromatography-linked Mass Spectrometry (LC-MS) Analysis and Mass Spectrometry (MS) Analysis—LC-MS analyses were performed on an Agilent 1100 HPLC-MSD-Trap. Nonsulfated backbone oligosaccharides were injected onto an Aquasil C_{18} column (3 μm 2.1 × 50 mm; Thermo Fisher). A gradient of acetonitrile with a flow rate of 0.4 ml/min was directed into the ion trap mass spectrometer. The gradient con-

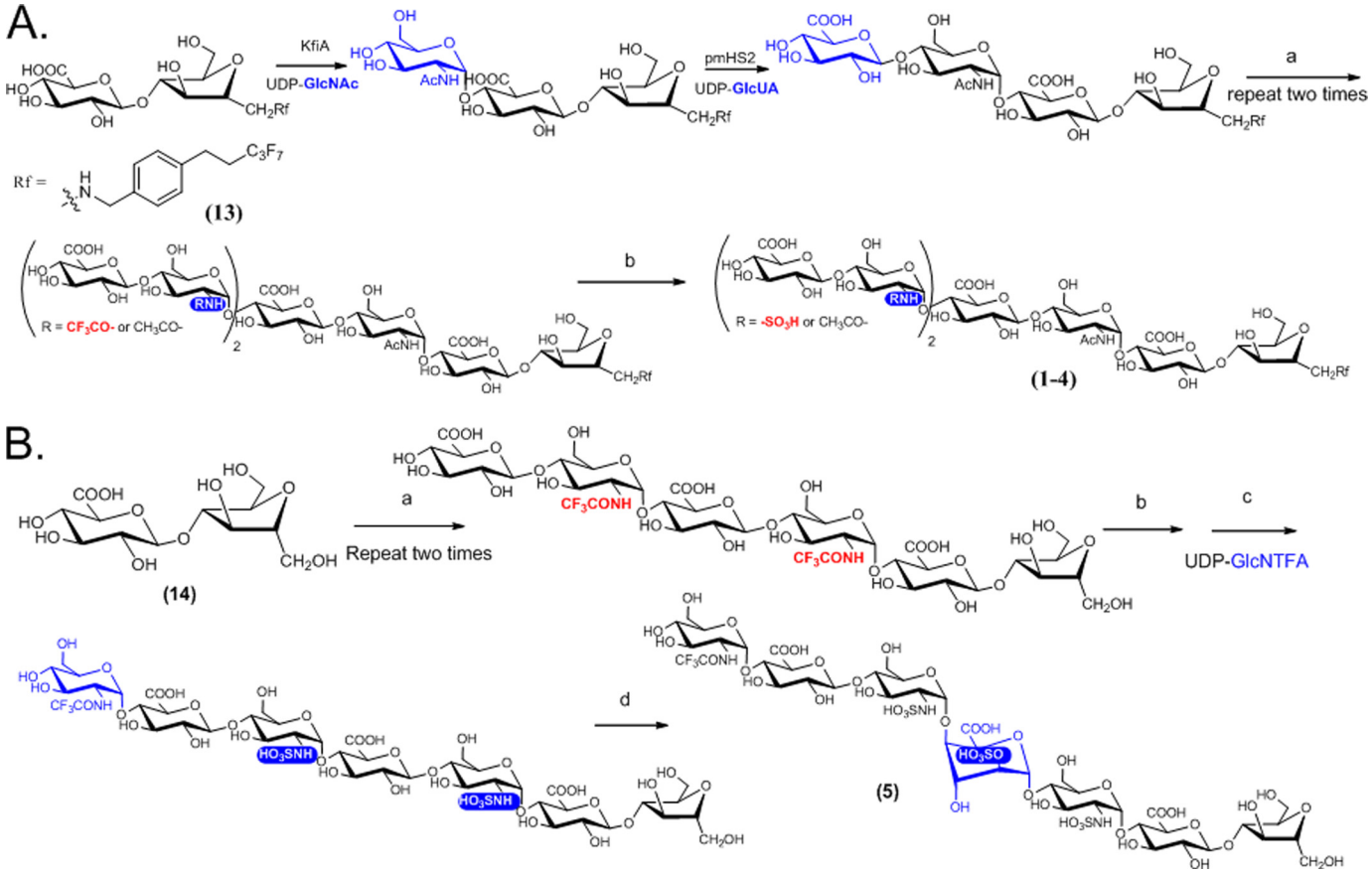

FIGURE 2. **Scheme for the synthesis of *N*-sulfo octasaccharides and a heptasaccharide carrying an IdoUA2S.** *A*, steps involved in the synthesis of *N*-sulfo octasaccharide library (**1**-**4**). The individual structure of **2**, **1**, **3**, and **4** is also shown in **Fig. 3*C***, **supplemental Fig. 1**, and **supplemental Fig. 2**, ***B*** and ***D***, respectively. *B*, steps involved in the synthesis of hepatasaccharide **5**. The modification sites are either *colored* in *blue* or *highlighted* in *filled boxes*. The residue that is epimerized is *colored* in *blue*. Reagents and recovery yield of each step are as follows: *a*, KfiA, UDP-GlcNAc (or UPD-GlcNTFA), pmHS2, and UDP-GlcUA. The purification yield by fluorous column was 80%, whereas the purification yield without fluorous column (*B*) was about 40%. *b*, methanol/triethylamine/water (2:1:2), NST, PAPS. Recovery yield was 40–50%. *c*, KfiA and UDP-GlcNTFA. Recovery yield was 50%. *d*, C_5-epi/2-OST, PAPS. Recovery yield was 40%.

sisted of an initial 5-min hold at 90% aqueous (0.1% formic acid in water), a change to 90% organic (0.1% formic acid in acetonitrile) over 2 min, a 2-min hold at 90% organic, a change to 90% aqueous over 2 min, and a 4-min reequilibration at 90% aqueous. Experiments were performed in positive ionization mode for untagged backbone oligosaccharide. Alternatively, the analyses were performed in negative ionization mode for tagged backbone oligosaccharides. Under both conditions, the electrospray source set to 3000 V and 350 °C, and the compound stability was set to 30%. Nitrogen was used for both nebulizer (8 liters/min) and drying gas (45 p.s.i.). Helium was used for collision-induced dissociation. The MS and tandem MS (MS/MS) data were acquired and processed using Bruker Trap software 4.1. All product ions in MS/MS data were labeled according to the Domon-Costello nomenclature (19).

Oligosaccharides with sulfo groups were dissolved in 70% acetonitrile and 10 μM imidazole. A syringe pump (Harvard Apparatus) was used to introduce the sample via direct infusion (10 μl/min) into an Agilent 1100 MSD Trap. Experiments were performed in negative ionization mode with the electrospray source set to 3000 V and 200 °C and the compound stability set to 30%. Nitrogen was used for both nebulizer (5 liters/min) and drying gas (15 p.s.i.). The procedures for MS/MS analysis are described above.

RESULTS

Enzymatic Synthesis of N-sulfo Oligosaccharides—A scheme for the synthesis of octasaccharides (**1**–**4**) from a disaccharide is shown in Fig. 2*A*, involving the use of glycosyltransferases, UDP-monosaccharide donors, and NST. Elongation from the disaccharide to the octasaccharide was achieved by two bacterial glycosyltransferases: *N*-Acetylglucosaminyl transferase (KfiA) from *E. coli* K5 (16) and heparosan synthase-2 (pmHS2) from *Pasteurella multocida* (17). A fluorous affinity tag, 4-(1H, 1H, 2H, 2H-perfluoropentyl) benzylamine (Rf), at the reducing end was introduced for the product purification. The fluorous tag allowed easy isolation of the product with FluoroFlash affinity chromatography and has absorbance at 260 nm that facilitated HPLC analysis during the preparation.

We designed a unique chemoenzymatic approach to build the *N*-sulfo oligosaccharides using KfiA and pmHS2. An initial attempt to transfer an *N*-unsubstituted glucosamine ($GlcNH_2$) residue from UPD-$GlcNH_2$ failed because UDP-$GlcNH_2$ was not a substrate for KfiA. An unnatural monosaccharide donor, UDP-GlcNTFA, was next used in the synthesis. We found that UDP-GlcNTFA served as an excellent donor substrate for KfiA, being efficiently incorporated. The product could be further extended by pmHS2 following the backbone synthesis as shown

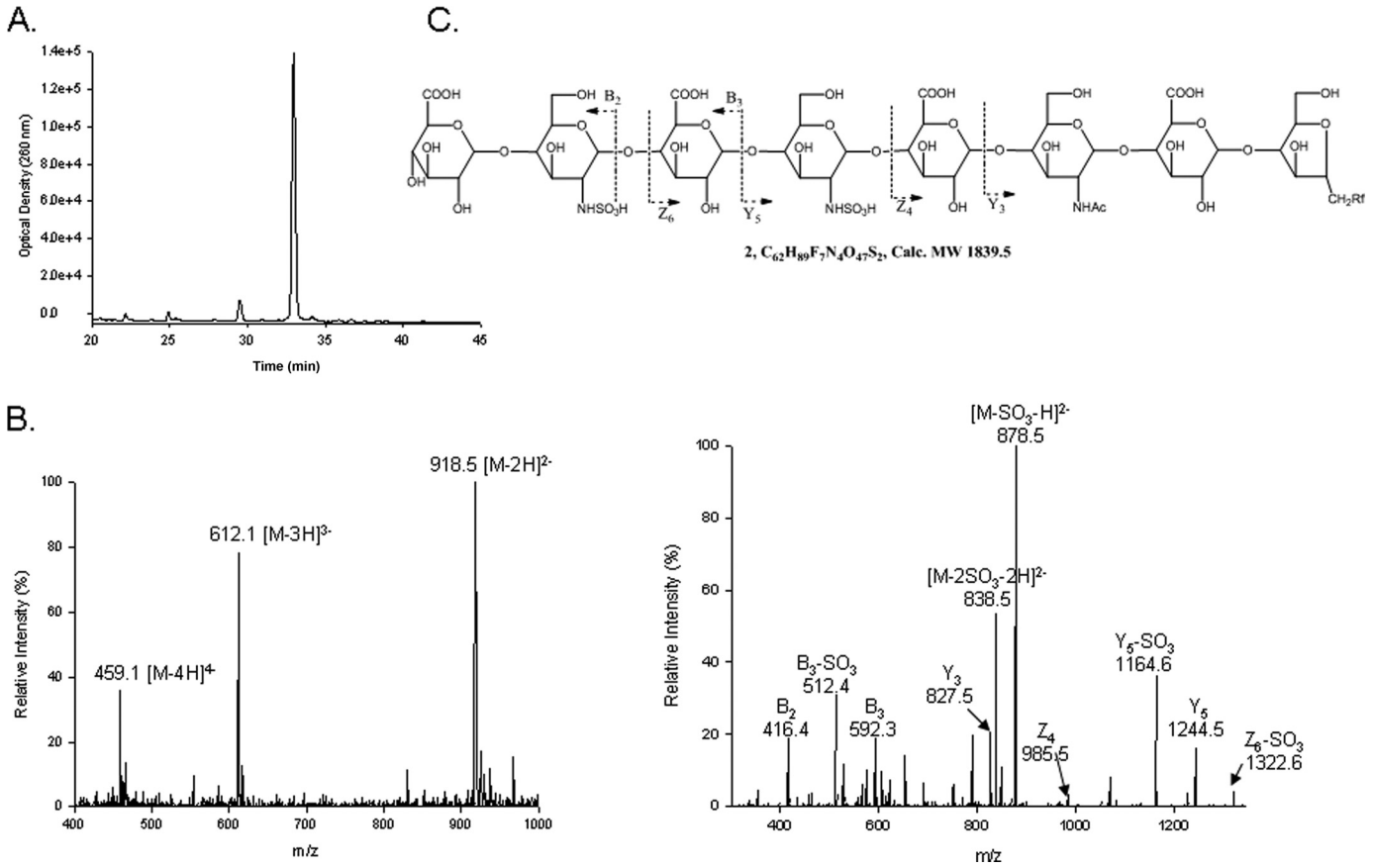

FIGURE 3. **Structural characterization of octasaccharide 2.** *A*, HPLC of octasaccharide **2** using a C_{18} column under reverse-phase condition. *B*, MS of octasaccharide **2**. *C*, MS/MS of octasaccharide **2** (precursor ion selection at *m/z* 459.1). The fragmentation pattern is depicted in the *top*. The product ions in MS/MS data were labeled according to the Domon-Costello nomenclature (19).

in Fig. 2*A*. The GlcNTFA residue was selectively deprotected under mild basic conditions, yielding $GlcNH_2$ unit in the presence of GlcNAc residues. The resulting $GlcNH_2$ residue was converted to GlcNS using NST in the presence of PAPS, *step b* Fig. 2*A*).

The efforts resulted in four different octasaccharide products that differed by the location and the number of GlcNS residue: octasaccharide **1** (no GlcNS, supplemental Fig. 1), octasaccharide **2** (two GlcNS residues, Fig. 3) and **3** and **4** (single GlcNS at different positions, supplemental Fig. 2). The structures of the octasaccharides were determined by MS. For example, octasaccharide **2** was resolved as a symmetric peak by HPLC using a C_{18} column (Fig. 3*A*). ESI-MS analysis revealed its molecular mass to be 1839.6 Da in close agreement to the calculated mass of 1839.5 Da (Fig. 3*B*). MS/MS analysis confirmed the position of the GlcNS residues in **2** (Fig. 3*C*) from the two characteristic daughter ions, Y_5 (*m/z*, 1244.5) and B_3 (*m/z*, 592.3), products of the cleavage of an internal glycosidic linkage (see supplemental Figs. 1 and 2for structural analysis of **1**, **3** and **4**).

Synthesis of an Oligosaccharide Carrying an IdoUA2S Unit—The IdoUA2S residue is a critical structural motif involved in binding to fibroblast growth factor to confer the cell proliferation activity of HS (6). Heptasaccharide **5** carrying one IdoUA2S residue in its center was synthesized from a disaccharide (Fig. 2*B*). The enzymatic synthesis of IdoUA2S residue involves the concerted action of C_5-epi and 2-OST, where C_5-epi transforms a GlcUA residue to an IdoUA residue, and 2-OST sulfates the 2-OH position of the IdoUA residue (*step d*, Fig. 2*B*). It should be noted that it was essential to introduce a GlcNTFA residue at the nonreducing terminus of the heptasaccharide. This GlcNTFA residue blocks the action of C_5-epi on the immediately adjacent GlcUA. C_5-epi is known to act only on GlcUA residues flanked by two GlcNS residues as in the sequence, -GlcNS-GlcUA-GlcNS-, but does not act on the GlcUA present in the sequence, -GlcNAc-GlcUA-GlcNS- (20).

The purity analysis and MS spectrum of heptasaccharide **5** are shown in Fig. 4. A ^{35}S-labeled heptasaccharide was initially synthesized by incubating with ^{35}S-labeled PAPS, and the product was resolved as a symmetric peak at the expected retention time on anion exchange HPLC, suggesting that it was >90% pure (Fig. 4*A*). Unlabeled heptasaccharide was then synthesized under identical conditions and purified by the HPLC. ESI-MS demonstrated that heptasaccharide **5** had a molecular mass of 1511.0 Da (calculated 1512.2 Da). These data are consistent with a heptasaccharide carrying three sulfo groups and one GlcNTFA residue.

The position of the IdoUA2S residue in heptasaccharide **5** was confirmed by disaccharide analyses using ^{35}S site-specifically labeling techniques (supplemental Fig. 3, *A* and *B*). Only IdoUA2S-AnMan disaccharide was observed from the nitrous acid-degraded 2-*O*-[^{35}S]sulfated heptasaccharide, suggesting that the 2-*O*-sulfo group was present only at the IdoUA2S, and

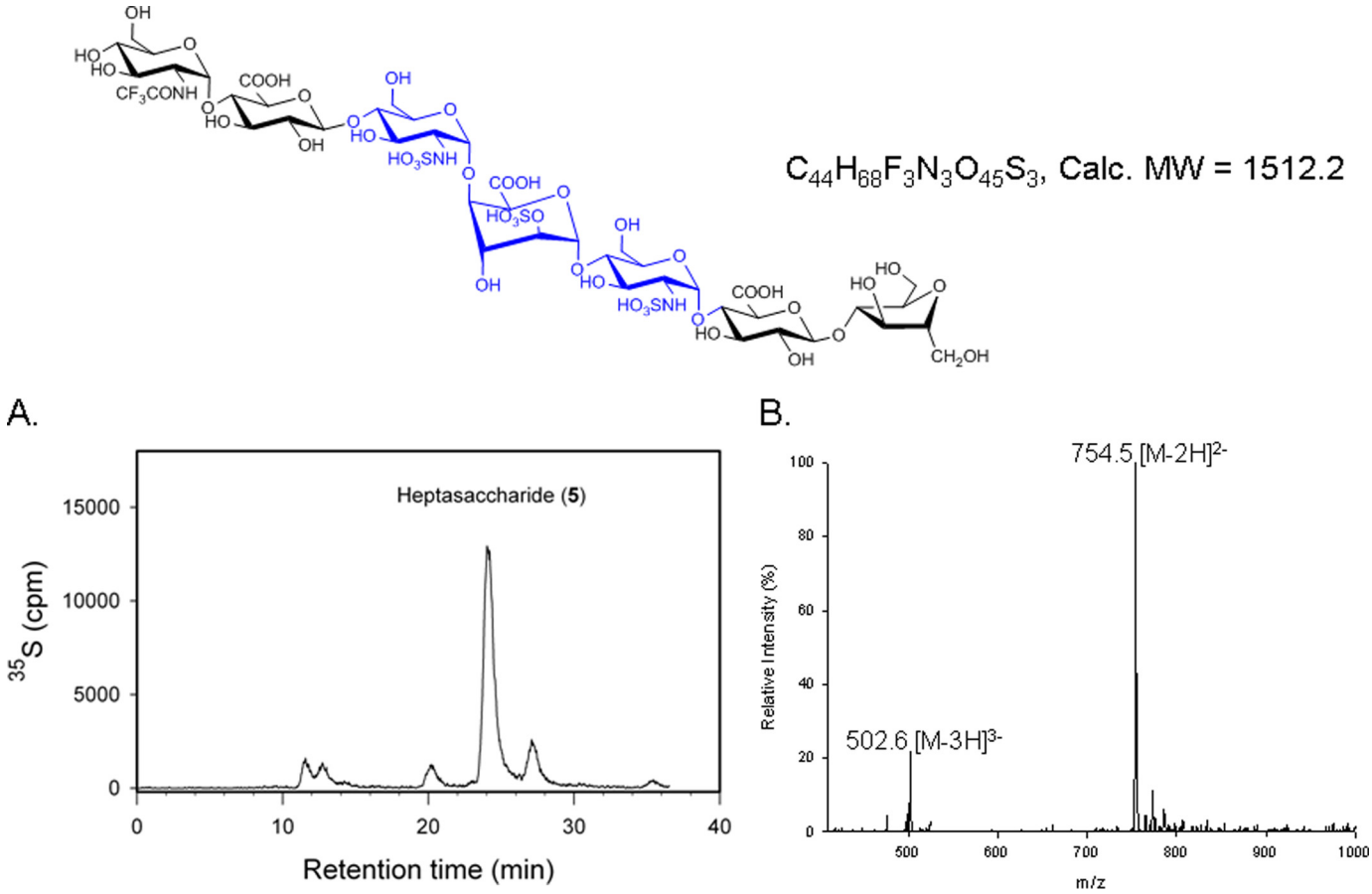

FIGURE 4. **Structural characterization of heptasaccharide 5.** *A*, elution profile of heptasaccharide **5** on polyamine-based HPLC. *B*, ESI-MS spectrum of purified heptasaccharide **5**. Chemical structure of heptasaccharide **5** and the calculated molecular mass of the heptasaccharide are presented above *A* and B.

it was flanked by two GlcNS residues (supplemental Fig. 3*A*). Two disaccharides of ΔUA-GlcNS and ΔUA2S-GlcNS were observed from the *N*-[^{35}S]sulfated heptasaccharide that was degraded with heparin lyases (supplemental Fig. 3*B*). Further, the ratio of the resultant disaccharide (ΔUA-GlcNS/ΔUA2S-GlcNS) was determined to be 1.0:0.8, very close to the theoretical value of 1:1 expected for heparin lyase-degraded heptasaccharide **5**. These data clearly demonstrate that the IdoUA2S residue is located at the center of the heptasaccharide **5**.

Synthesis of Oligosaccharides Carrying 6-O-Sulfo and 3-O-Sulfo Groups—We next examined the introduction of 6-*O*-sulfo and 3-*O*-sulfo groups into the HS oligosaccharides using *O*-sulfotransferases (Fig. 5). To that end, oligosaccharides with *N*-sulfo groups (**6**–**8**, Fig. 5) needed to be first synthesized. Disaccharide acceptor **14**, a nontagged disaccharide primer, was extended to decasaccharide, undecasaccharide, and dodecasaccharide, respectively, using UDP-GlcNTFA and UDP-GlcUA donors. The structures of the backbone oligosaccharides were confirmed by LC-MS (supplemental Table 1). In eight enzymatic steps at the milligram scale, a disaccharide was converted to decasaccharide **15**. Further extension to undecasaccharide **16** and dodecasaccharide **17** was also highly effective. Although some unexpected partial detrifluoroacetylation occurred during product purification, this did not impact the synthesis of *N*-sulfated oligosaccharides because the next step required complete detrifluoroacetylation.

Conversion of the remaining GlcNTFA residues in decasaccharide, undecasaccharide, and dodecasaccharide to GlcNS proceeded through detrifluoroacetylation and treatment with NST using [^{35}S]PAPS to afford *N*-[^{35}S]sulfated products. The product afforded a single prominent peak at the expected retention time on high resolution DEAE-HPLC, suggesting that the products were of high purity (supplemental Fig. 4, *A*, *C*, and *E*). The nonradioactive oligosaccharides (**6**-**8**) were next resynthesized under identical conditions using NST and unlabeled PAPS and purified by DEAE-HPLC. The ESI-MS analyses of the oligosaccharides (supplemental Fig. 4, *B*, *D*, and *F*) confirm that all three compounds are fully *N*-sulfated with the structures shown in Fig. 5.

Next, we introduced 6-*O*-sulfo groups using a mixture of 6-OST isoform 1 (6-OST-1) and 6-OST isoform 3 (6-OST-3) affording the *N*-sulfo-6-*O*-sulfo decasaccharide **9** and *N*-sulfo-6-*O*-sulfo dodecasaccharide **10** (Fig. 5). DEAE-HPLC analysis of the ^{35}S-labeled oligosaccharide displayed a prominent symmetric ^{35}S peak at the expected retention time, suggesting that the preparation was pure (supplemental Fig. 5, *A* and *C*). Nonradioactive decasaccharide **9** and dodecasaccharide **10** were next resynthesized under identical conditions using PAPS, purified by DEAE-HPLC, and subjected to MS analysis. ESI-MS analysis of each product revealed molecular masses of 2329.9 Da and 2828.1 Da, consistent with the structure of decasaccharide **9** having 8 sulfates and dodecasaccharide **10** having 10

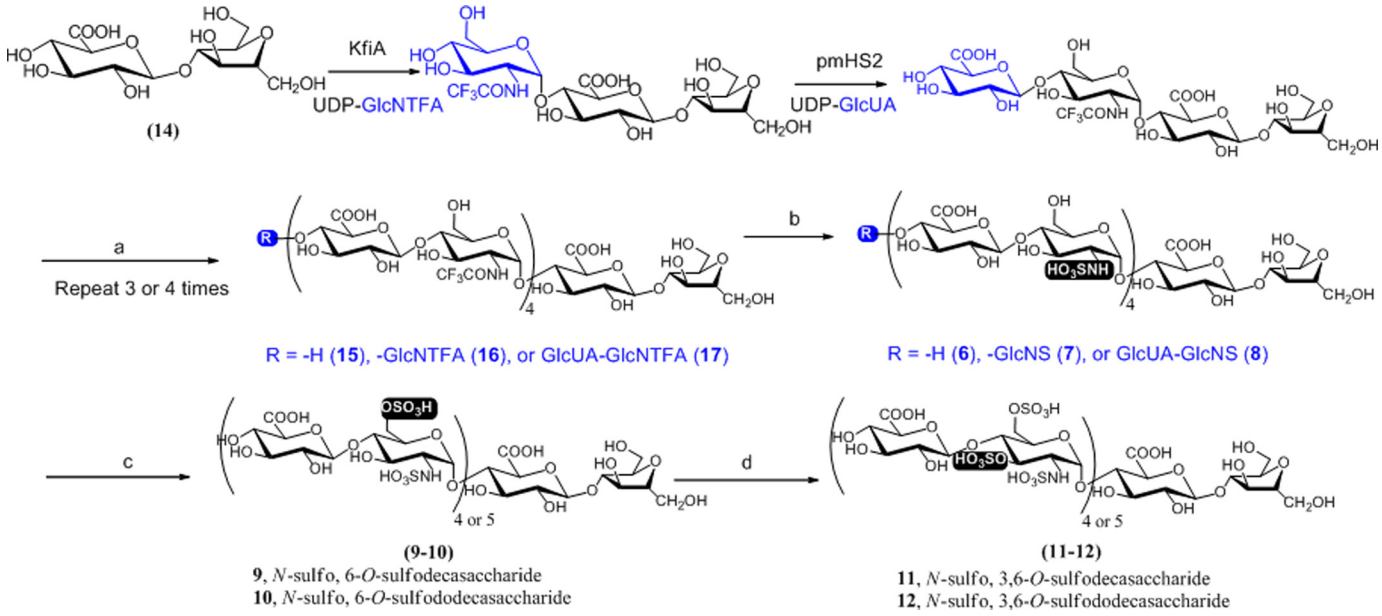

FIGURE 5. **Scheme for the synthesis of deca-, undeca-, and dodecasaccharides.** The reaction sites are either *colored* in *blue* or *highlighted* in *filled boxes*. Different sizes of oligosaccharides are represented *R*, where *R* = H (decasaccharide), *R* = -GlcNTFA/NS (undecasaccharide), and *R* = GlcUA-GlcNTFA/NS (dodecasaccharide). Reagents and recovery yield of each step are as follows: *a*, KfiA, UDP-GlcNAc (or UPD-GlcNTFA), pmHS2, and UDP-GlcUA. The purification yield by was at 35% on average in the first four cycles. When the oligosaccharide was extended beyond to decasaccharide, the recovery from the Bio-Gel P-10 reached 75–80%. *b*, methanol/triethylamine/water (2:1:2), NST, PAPS. Recovery yield was 25–30%. *d*, 6-OST-1/6-OST-3, PAPS. Recovery yield was 30%. *e*, 3-OST-1/3-OST-5, PAPS. Recovery yield was 10%. Oligosaccharide **15** represents a *N*-detrifluoroacetylated decasaccharide, **16** represents an *N*-detrifluoroacetylated undecasaccharide, and **17** represents a *N*-detrifluoroacetylated dodecasaccharide. Oligosaccharides **6**, **7**, and **8** represent *N*-sulfo decasaccharide, *N*-sulfo undecasaccharide, and *N*-sulfo dodecasaccharide, respectively. Only decasaccharide and dodecasaccharide proceeded to make *N*-sulfo and 6-*O*-sulfo oligosaccharides (**9** and **10**).

sulfates (calculated 2329.9 Da and 2827.3 Da). Disaccharide analyses of the labeled products, carrying 6-*O*-[^{35}S]sulfo groups, confirmed the structures of decasaccharide **9** and dodecasaccharide **10** (supplemental Table 2). Disaccharide analysis is a commonly used approach to analyze the structures of HS oligosaccharides (21, 22).

Incubation of decasaccharide **9** and dodecasaccharide **10** with both 3-OST isoform 1 (3-OST-1) and 3-OST isoform 5 (3-OST-5) afforded decasaccharide **11** and dodecasaccharide **12**, respectively (Fig. 5). In each case, a mixture of two major products was obtained as analyzed by DEAE-HPLC (Fig. 6*A* and supplemental Fig. 6*A*), leading to a reduced yield for the target products. Consequently, we could not obtain sufficient amount of these products for MS analysis. Instead, to assign structure we decided to use the site-specific [^{35}S]sulfo-labeled technique followed by disaccharide analysis as illustrated in Fig. 6. The 6-*O*-[^{35}S]sulfated decasaccharide **9** was first prepared. The 3-*O*-sulfo groups were then introduced using 3-OST-1 and 3-OST-5 with unlabeled PAPS to afford 6-*O*-[^{35}S]sulfo-labeled decasaccharide **11** (Fig. 6*D*). The two ^{35}S-labeled products, labeled as Peak I and Peak II, were separated by the DEAE-HPLC (Fig. 6*A*). Nitrous acid resulted in disaccharides that were analyzed by RPIP-HPLC (Fig. 6, *B* and *C*). Nitrous acid treatment of Peak I (Fig. 6*E*) afforded two ^{35}S-labeled disaccharides assigned to GlcUA-AnMan6S and GlcUA-AnMan3S6S in the ratio of 1:3.4, suggesting that Peak I carried three 3-*O*-sulfo groups. Nitrous acid-treated Peak II (Fig. 6*F*) afforded a single type of ^{35}S-labeled disaccharide, assignable to GlcUA-AnMan3S6S, suggesting that Peak II was the anticipated fully 3-*O*-sulfo decasaccharide shown in Fig. 5. Using the same analytical approach, we also demonstrated the completion of the synthesis of dodecasaccharide **12** (Fig. 5 and supplemental Fig. 6).

It should be noted that a domain consisting of multiple repeats of GlcUA-GlcNS3S6S in **11** and **12** has not been identified in the HS isolated from natural sources. A recent finding of high abundance of 3-*O*-sulfated glucosamine residue of the HS isolated from human follicular fluid raised the possibility that this particular HS contains domain structures similar to those found in **11** and **12** (23). Unlike HS, **11** and **12** do not contain the IdoUA2S-GlcNS motif. It is known that 3-OST-1 acts on GlcUA (or IdoUA)-GlcNS6S sequences, but not on IdoUA2S-GlcNS sequences (15, 24, 25). It is possible that the presence of IdoUA2S plays a role in down-regulating the level of 3-*O*-sulfation.

Determination of AT-binding Affinity of O-Sulfooligosaccharides—We next measured the binding affinity of the synthesized oligosaccharides to AT. The AT binding correlates to HS anticoagulant activity. We previously demonstrated that an AT-binding HS does not require the presence of iduronic acid or 2-*O*-sulfoiduronic acid residues (6), which greatly simplifies the synthesis of anticoagulant HS. However, the minimum length and the precise structure of this novel AT-binding domain are not known. We hypothesized that decasaccharide **11** and dodecasaccharide **12** would provide insights on the structural requirement for this AT-binding site.

The AT-binding affinities of oligosaccharides **10, 11,** and **12** were determined (Table 1). Dodecasaccharide **10**, having no 3-*O*-sulfo groups, exhibited a K_d of >100 μM. Dodecasaccharide **12,** having 3-*O*-sulfo groups, showed the K_d to be 145 nM, close to that of a full-length HS polysaccharide (57 nM) (6). The size dependence of this interaction was clearly demonstrated by

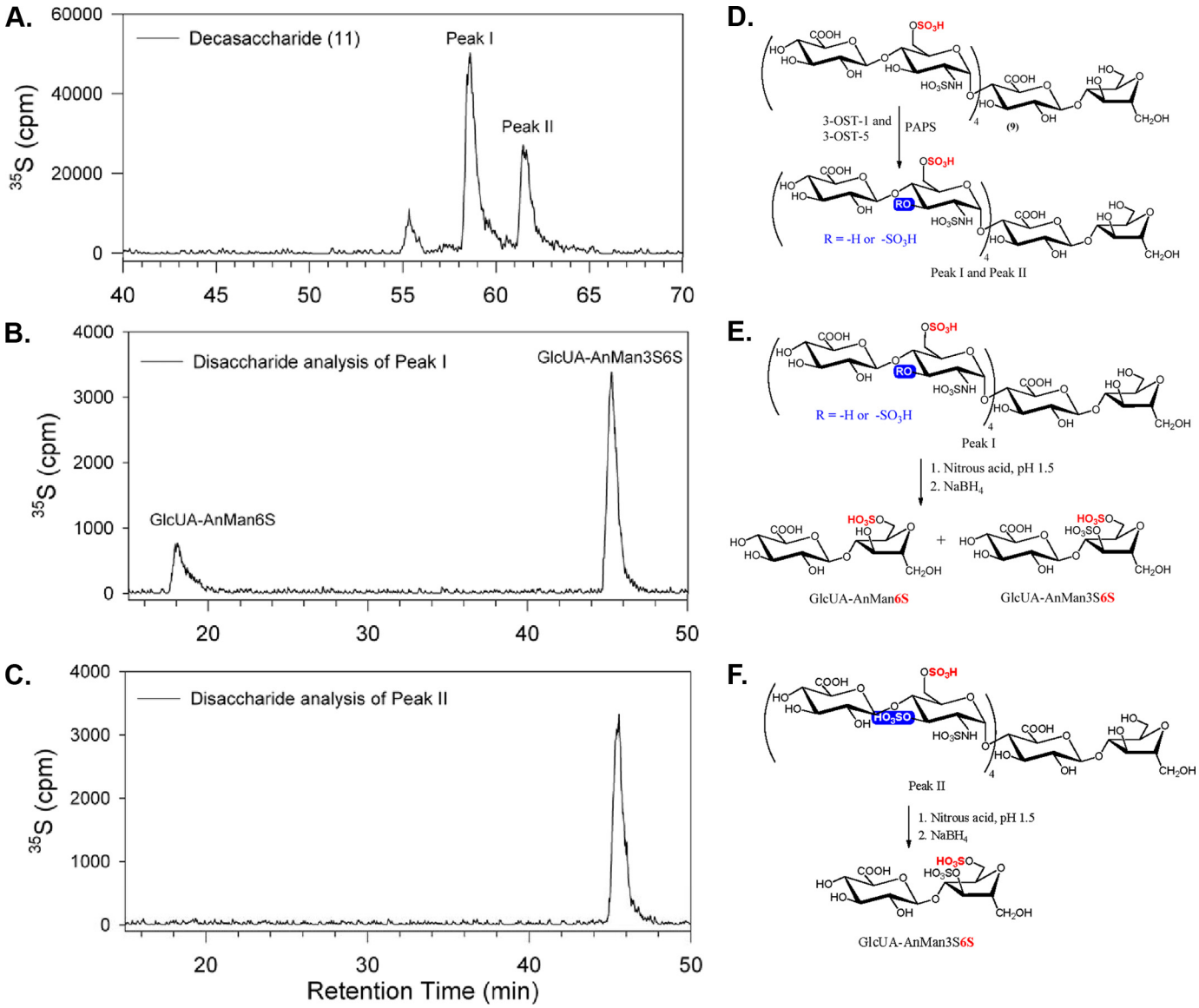

FIGURE 6. **Determination of the structures of 3-*O*-sulfo decasaccharide (11).** *A*, HPLC of 6-*O*-[^{35}S]sulfo-labeled decasaccharide **11** using a DEAE-HPLC column. The decasaccharide was prepared by incubating decasaccharide **9** with 3-OST-1 and 3-OST-5 as depicted in *D*. Two major products, designated as Peak I and Peak II, were observed. Both components were purified and subjected to disaccharide analysis. *B*, HPLC of disaccharide analysis of Peak I using a C_{18} column under RPIP-HPLC conditions. The chemical reaction involved in the disaccharide analysis of Peak I is shown in *E*. *C*, HPLC of the disaccharide analysis of Peak II using a C_{18} column under RPIP-HPLC conditions. *F*, reaction involved in the disaccharide analysis of Peak II. The radioactively labeled 6-*O*-sulfo group is *colored* in *red*. The potential modification sites modified by 3-OSTs are *highlighted* in *blue boxes*. Only the disaccharide products carrying 6-*O*-[^{35}S]sulfo groups were detected from nitrous acid degradation and are shown in *E* and *F*.

TABLE 1

Binding affinity of oligosaccharides to AT

The binding affinity of the oligosaccharides to AT was determined using affinity coelectrophoresis.

Substrates	Proposed structure	K_d
		nM
11	$(GlcUA\text{-}GlcNS3S6S)_4$-GlcUA-AnMan	515 ± 40
12	$(GlcUA\text{-}GlcNS3S6S)_5$-GlcUA-AnMan	145 ± 24
10	$(GlcUA\text{-}GlcNS6S)_5$-GlcUA-AnMan	>100,000
Arixtra[a]	GlcNS6S-GlcUA-GlcNS3S6S-IdoUA2S-GlcNS6S-OMe	33
Recomparin[b]	Polysaccharide, no defined structure	57

[a] The ^{35}S-labeled Arixtra was prepared as described in a paper published previously (33).
[b] The binding affinity of Recomparin to AT was taken from our previous publication (6).

decasaccharide **11** having a K_d of 515 nM, representing considerably weaker AT-binding affinity. These results both confirm the critical role of 3-*O*-sulfo groups for AT-binding and the size dependence of this interaction (26). Dodecasaccharide **12** binds AT with lower affinity than the commercial pentasaccharide drug, Arixtra. It is possible that a lower binding affinity to AT is because an IdoUA2S residue is absent in the structure.

DISCUSSION

In this paper, we demonstrated the feasibility of synthesizing structurally defined oligosaccharides from a simple starting material. The critical advance is the ability to be able to convert a disaccharide to oligosaccharides carrying *N*-sulfo groups. The placement of the GlcNS residue is believed to be critical in modulating the susceptibilities to the subsequent *O*-sulfation and epimerization (27). Although there are numerous ways to prepare the oligosaccharide with the repeating unit of (-GlcUA-GlcNAc-) (16, 28), the product cannot be used

directly for the subsequent enzymatic modifications to prepare HS due to the lack of *N*-sulfoglucosamine (GlcNS) residue. *In vivo*, the GlcNS residue is synthesized by *N*-deacetylase/*N*-sulfotransferase (29). However, it is unclear how to selectively convert a GlcNAc residue to a GlcNS residue when multiple GlcNAc residues are present in an oligosaccharide using *N*-deacetylase/*N*-sulfotransferase *in vitro*. Therefore, synthesis of oligosaccharides having defined *N*-sulfation positions is a critical step in developing a route for the controlled enzymatic synthesis of HS oligosaccharides and polysaccharides. As an alternative approach to the NST-PAPS method, *N*-sulfation can be also carried out with a chemical approach using SO_3·pyridine. We observe that NST-PAPS system affords complete *N*-sulfation with absolute selectivity for the amino group. Furthermore, the costs associated with enzymatic *N*- and *O*-sulfation might be substantially reduced through the enzymatic synthesis of PAPS.

We demonstrate a convincing example for utilizing an unnatural UDP-monosaccharide to expand the capability of using glycosyltransferase to synthesize the specialized oligosaccharides. A review by Boons and colleagues suggests that the scope of the use of glycosyltransferases for the synthesis of oligosaccharide is limited due to the high donor and acceptor substrate specificities (30). We demonstrate for the first time that KfiA is capable of using UDP-GlcNTFA as a donor substrate in high efficiency. The use of the unnatural monosaccharide donor empowers the placement of a GlcNS residue at any desired position in the oligosaccharide backbone. We also demonstrate that pmHS-2 can catalyze the extension of an oligosaccharide that has a GlcNTFA unit at its nonreducing end. We have developed an effective approach to introduce *N*-sulfoglucosamine units into specific locations within a given octasaccharide. Furthermore, we demonstrate the synthesis of defined heparan sulfate oligosaccharides from these *N*-sulfo oligosaccharides. Although the use of UDP-GlcNTFA and KfiA for the synthesis of HS is novel, it should be noted that the demonstration of the synthesis of UDP-GlcNTFA and use as a donor substrate for the core-2 GlcNAc transferase was previously reported by Sala *et al*. (31). Our method can also be used to conduct parallel synthesis through the introduction of a fluorous affinity tag. The fluorous affinity-tagged technique has been previously employed for preparing tagged monosaccharide conjugates for carbohydrate-based microarray to determine the structural specificities of the interactions of proteins and carbohydrates (32).

Whether our method is able to control all sulfation types remains to be investigated. The control of the *N*-sulfation can be readily achieved. The method also appears to be capable of placing an IdoUA2S residue based on the distribution of GlcNS residue. Although we demonstrated that 6-*O*- and 3-*O*-sulfation can proceed to completion in a given oligosaccharide, it is unclear how to place a single 6-*O*- or 3-*O*-sulfo group in the context of repeating unit of -GlcUA-GlcNS-. We suspect that the presence of IdoUA2S influences the placement of 3-*O*-sulfo group. This may ultimately be controlled by relying on the substrate specificities of different 3-*O*-sulfotransferase isoforms. Each isoform is believed to exhibit unique substrate specificities to recognize the saccharide structures around the modification site (15, 33). Unlike 3-*O*-sulfotransferase, 6-*O*-sulfotransferase isoforms appear to have same substrate specificities (34), suggesting than an additional strategy will be needed to introduce a 6-*O*-sulfo group in a specific position.

The synthetic scale of our method is ultimately determined by the amount of enzymes and substrates. A large scale enzymatic synthesis of a targeted oligosaccharide is underway. We have observed no limitation in the scale-up of the enzymes and substrates, particularly in the laboratory scale synthesis. Furthermore, the conversion at each enzymatic step is nearly quantitative, and the loss of the sample largely occurs in the purification steps. Thus, the yield will undoubtedly be improved by combining several sulfotransferases in one pot to reduce the number of purification steps.

The structural analysis of HS oligosaccharides still remains a major roadblock for the complex HS synthesis. We demonstrated the use of MS/MS to conduct the sequence analysis of *N*-sulfo octasaccharides. However, this method is not sufficiently reliable in pinpointing the sulfo groups in an oligosaccharide having a complex sulfation pattern. The analysis of HS oligosaccharides by MS is further complicated as desulfation occurs often during analysis (35, 36). It should be noted that the lack of structurally defined oligosaccharide standards also hinders the efforts for developing the techniques for analyzing HS.

In summary, the current study demonstrates the feasibility of total synthesis of structurally defined HS oligosaccharides using a chemoenzymatic approach. This method is capable of synthesizing oligosaccharides with different sulfation patterns and sizes by transferring UDP-sugars onto a readily available disaccharide acceptor. A key advance involves the utilization of an unnatural UDP-monosaccharide donor that allows the controlled placement of GlcNS and GlcNAc residues throughout the oligosaccharide backbone. A recent report describes 12 HS tetrasaccharides prepared using a modular chemical synthesis (37). It is somewhat difficult to compare the efficiency of chemical synthesis and chemoenzymatic synthesis quantitatively because each method targeted to different oligosaccharide products. However, it is noteworthy that the chemical synthesis of IdoUA2S requires at least eight synthetic steps (37). Only a single step is required utilizing C_5-epi and 2-OST with about 40% yield (*step d*, Fig. 2*B*). Moreover, the targets synthesized in the current study are considerably larger than those synthesized chemically. However, chemical synthesis offers the benefits of the preparation of unnatural structural motif, which cannot be accomplished by enzymes due to the restrictions in the substrate specificity. Therefore, an effective combination of chemical and chemoenzymatic methods will further optimize the synthesis. Structurally defined HS will be employed to interrogate structure and activity relationship studies in the new field of HS glycomics. These results also open up the possibility of discovering novel HS-based anticoagulant drugs as well as other HS-based therapeutic agents.

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