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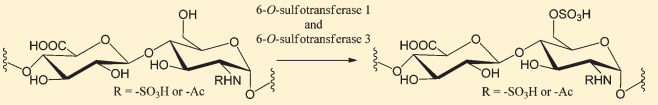
Enzymatic Placement of 6-*O*-Sulfo Groups in Heparan Sulfate

Renpeng Liu and Jian Liu*

Division of Medicinal Chemistry and Natural Products, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Heparan sulfate is a highly sulfated polysaccharide that exhibits important physiological and pathological functions. The glucosamine residue of heparan sulfate can carry sulfo groups at the 2-*N*, 3-*O*, and 6-*O* positions, leading to diverse polysaccharide structures. 6-*O*-Sulfation at the glucosamine residue contributes to a wide range of biological functions. Here, we report a method for controlling the positioning of 6-*O*-sulfo groups in oligosaccharides. This was achieved by synthesizing oligosaccharide backbones from a disaccharide building block utilizing glycosyltransferases followed by modifications using heparan sulfate *N*-sulfotransferase and 6-*O*-sulfotransferases. This method offers a viable approach for preparing heparan sulfate oligosaccharides with precisely located 6-*O*-sulfo groups.



Heparan sulfate (HS) is a linear polysaccharide that is present on mammalian cell surfaces and in the extracellular matrix. HS is involved in numerous biological processes, including blood coagulation, wound healing, embryonic development, and regulation of tumor growth, as well as in assisting viral and bacterial infections.[1] The wide range of biological functions of HS attracts considerable interest in exploiting HS-based anticoagulant,[2,3] antiviral,[4] and anticancer drugs.[5] Heparin, a commonly used anticoagulant, is a highly sulfated form of HS. HS consists of repeating disaccharide units of glucosamine and glucuronic acid (GlcUA) or iduronic acid (IdoUA), both capable of carrying sulfo groups (Figure 1A). The positions of the sulfo groups and IdoUA dictate the functions of HS.[6,7]

The biosynthesis of HS is accomplished by a complex pathway involving backbone elongation and multiple modification steps. HS polymerase is responsible for building the polysaccharide backbone, which contains repeating units of -GlcUA-GlcNAc-. The backbone is then modified by *N*-deacetylase/*N*-sulfotransferase (which has two separate domains that exhibit *N*-deacetylation and *N*-sulfation, respectively), C_5-epimerase (which converts GlcUA to IdoUA), 2-*O*-sulfotransferase, 6-*O*-sulfotransferase (6-OST), and 3-*O*-sulfotransferase to produce the fully elaborated HS. Understanding this biosynthetic mechanism has improved our ability to synthesize HS.[8] Using HS sulfotransferases and C_5-epimerase, we developed a method for synthesizing HS polysaccharides[2,9,10] and oligosaccharides with defined structures.[11] Previously, a method for positioning *N*-sulfo glucosamine and IdoUA2S residues was developed.[11] It is still unknown whether placing a 6-*O*-sulfo group in a given oligosaccharide is possible.

The 6-*O*-sulfo glucosamine unit is a common monosaccharide in HS. It has been found that the 6-*O*-sulfo glucosamine residue plays a critical role in numerous biological functions. For example, heparin carrying 6-*O*-sulfo groups exhibits anti-inflammatory effects by blocking the binding of HS to L- and P-selectins.[12] The 6-*O*-sulfated glucosamine residue was also found to regulate the fibroblast growth factor-mediated signaling pathway by binding to the fibroblast growth factor receptor.[13,14] 6-OST-1 knockout mice were shown to exhibit numerous defects. Most of the mice died at the embryonic and perinatal stages. Those that survived were significantly smaller in size than the wild-type mice.[15] These observations underscore the essential role of HS 6-OSTs in developmental physiology. In *Drosophila*, the removal of 6-OST genes has no obvious phenotype, suggesting the complex roles of 6-*O*-sulfation in different organisms. Mutant strains of *Drosophila* have elevated levels of 2-*O*-sulfation in response to the loss of 6-OST.[16]

Little is known about how 6-*O*-sulfation patterns are regulated. HS 6-OSTs, which are present in three isoforms (6-OST-1, -2, and -3[17]), catalyze the transfer of a sulfo group to the C6 position of a glucosamine residue (GlcN) to form 6-*O*-sulfo glucosamine (Figure 1B). Furthermore, HS 6-*O*-sulfatases remove 6-*O*-sulfo groups from HS in vivo and remodel HS structures.[18,19] 6-*O*-Sulfation occurs predominantly at *N*-sulfo glucosamine (GlcNS) residues. However, in some cases, it can also occur at the GlcNAc residues. Unlike different 3-*O*-sulfotransferase isoforms, 6-OST isoforms appear to recognize the same substrates,[20] suggesting that a different strategy will be needed to introduce a 6-*O*-sulfo group at a specific position.

In this work, we report the placement of a 6-*O*-sulfo group at specific oligosaccharides using an enzymatic approach. We demonstrate the feasibility of selectively introducing a 6-*O*-sulfo group using two distinct methods. First, by controlling the reaction time, we prepared partially 6-*O*-sulfated hexasaccharides, although a mixture of two products was obtained. Second, we elongated the oligosaccharides already carrying 6-*O*-sulfo groups using glycosyltransferases. Our method provided a

Received: January 17, 2011
Revised: March 25, 2011
Published: April 20, 2011

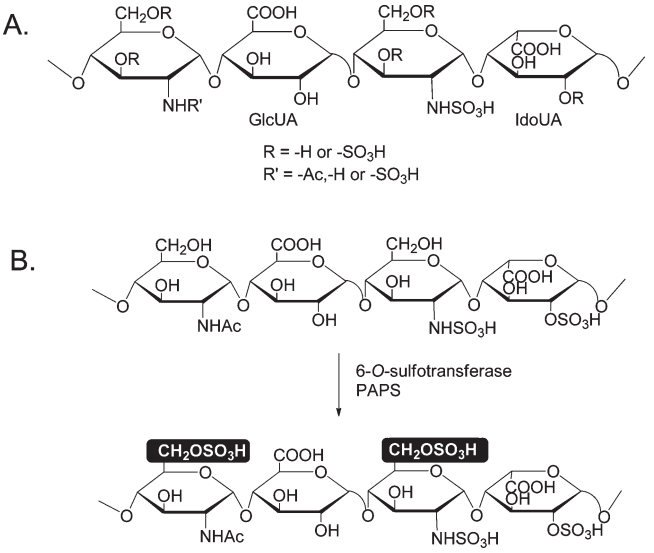


Figure 1. Structure of HS and 6-*O*-sulfation of HS. (A) Chemical structure of heparan sulfate. The structures of glucuronic acid (GlcUA) and iduronic acid (IdoUA) are indicated. (B) Enzymatic reaction catalyzed by 6-OST, where PAPS is a sulfo donor. The modification site is highlighted.

generic tool for synthesizing HS oligosaccharides with defined 6-*O*-sulfation patterns with which to elucidate the structure—activity relationship of HS. The results also aid our understanding of the substrate specificity of 6-OSTs.

EXPERIMENTAL PROCEDURES

Expression and Purification of HS Biosynthetic Enzymes. A total of five enzymes were employed for the synthesis of oligosaccharide substrates and for 6-*O*-sulfation, including *N*-acetyl D-glucosaminyl transferase from the *Escherichia coli* K5 strain (KfiA), *N*-sulfotransferase (NST), 6-*O*-sulfotransferase 1 (6-OST-1), 6-*O*-sulfotransferase 3 (6-OST-3), and heparosan synthase-2 (PmHS2) from *Pasteurella multocida*. All of these enzymes were expressed in *E. coli* using different fusion proteins as described previously.[2,21,22] For 6-OST-1 and 6-OST-3, maltose-binding fusion proteins were used, and the fusion proteins were purified with an amylose column (New England Biolabs). For NST, a glutathione *S*-transferase fusion protein was prepared and purified with a glutathione Sepharose column (GE Healthcare).[21] PmHS2 was expressed as an N-terminal fusion protein with a six-His tag using a PET-15b vector (Novagen).[11] The expression of KfiA was conducted in BL21 star (DE3) cells (Invitrogen) coexpressing the bacterial chaperone proteins, GroEL and GroES.

Synthesis of a Fluorous-Tagged Disaccharide (GlcUA-AnMan-Rf) and Tagged Oligosaccharide Substrates. A disaccharide (GlcUA-AnMannose) was prepared from nitrous acid-degraded heparosan as previously described omitting the $NaBH_4$ reduction step.[22] The resultant disaccharide was dialyzed against water using a 1000 molecular weight cutoff (MWCO) membrane (Spectrum). To introduce a fluorous tag, the disaccharide was incubated with 2 equiv of 4-(1*H*,1*H*,2*H*,2*H*-perfluoropentyl) benzylamine hydrochloride (Fluorous Technologies) and $NaBH_3CN$ (10 equiv) in MeOH overnight at room temperature. The resulting tagged disaccharide was purified with a FluoroFlash column. Then, it was further purified via paper chromatography using Whatman 3 MM chromatography paper (Fisher) that was developed with 100% acetonitrile. Finally, the fluorous-tagged disaccharide (GlcUA-AnMan-Rf) was purified using a C_{18} column (0.46 cm × 25 cm, Thermo Scientific) under reverse phase high-performance liquid chromatography (RP-HPLC) conditions. The column was eluted with a linear gradient from 90% solution A (0.1% TFA in water) to 50% solution A for 40 min at a flow rate of 0.5 mL/min, followed by an additional wash for 20 min with 100% solution B (0.1% TFA in acetonitrile) at a flow rate of 0.5 mL/min. The product was confirmed by electrospray ionization (ESI) mass spectrometry.

The fluorous-tagged hexasaccharides were synthesized from a fluorous-tagged disaccharide GlcUA-AnMan-Rf. To incorporate additional monosaccharides, we supplemented the reaction mixture with either KfiA or PmHS2 and the appropriate UDP-monosaccharide donor. As a result, only one sugar residue was transferred to the backbone as described previously.[11] A FluoroFlash column was used to separate the tagged oligosaccharides from the 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 to prepare hexasaccharide substrates.

Preparation of UDP-GlcNTFA. UDP-GlcNTFA was synthesized using a chemoenzymatic approach as described previously.[11] 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 mixture 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 unit/μL inorganic pyrophosphatase (Sigma-Aldrich). Recombinant GlmU was expressed in *E. coli* and purified with a Ni-agarose column.[22] The UDP-GlcNTFA was purified via removal of proteins using centrifugal filters (10000 MWCO, Millipore) followed by dialysis against water using a 1000 MWCO membrane for 4 h. The product was confirmed by MS analysis. The concentration was determined by a quantitative analysis via PAMN-HPLC using UDP-GlcNAc as a standard.

Removal of the *N*-Trifluoro Group from GlcNTFA Units. Various amounts of oligosaccharides (50—100 μg) were dried and resuspended in a solution (500 μL) containing CH_3OH, H_2O, and $(C_2H_5)_3N$ (2:2:1, v/v/v). The reaction mixture was incubated at 37 °C overnight. The samples were dried and reconstituted in H_2O to recover de-*N*-trifluoroacetylated oligosaccharides.

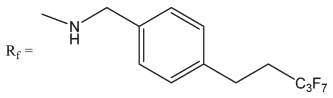
Enzymatic Preparation of *N*-Sulfated and 6-*O*-Sulfated Oligosaccharides. For the *N*-sulfation step, the oligosaccharide substrate (20—30 μg) was incubated with NST (80 μg) and PAPS (2 equiv) in 500 μL of buffer containing 50 mM MES (pH 7.0) and 1% Triton X-100 overnight at 37 °C. After the reaction mixture had been incubated for 12 h, the sulfated oligosaccharide was purified with a C_{18} column (0.46 cm × 25 cm, Thermo Scientific) under RP-HPLC conditions as described above. To prepare *N*-[^{35}S]sulfated oligosaccharides, [^{35}S]PAPS (1—5 × 10^6 cpm) was included in the reaction mixture. To prepare 6-*O*-sulfated oligosaccharides, a similar procedure was followed except for the replacement of NST with 6-OST-1 and 6-OST-3. The product was confirmed by ESI-mass spectrometry whenever sufficient amounts of samples were prepared.

HPLC Analysis. HPLC analyses of the oligosaccharides were conducted as previously described.[2,9,11]

Table 1. List of Oligosaccharide Substrates and Products

compound	structure	calcd molecular mass (Da)	observed molecular mass (Da)
trisaccharide **1**[a]	GlcNAc-GlcUA-AnMan-Rf	828.7	828.6
tetrasaccharide **2**	GlcUA-GlcNAc-GlcUA-AnMan-Rf	1004.8	1004.8
hexasaccharide **3**[b]	GlcUA-GlcNS-GlcUA-GlcNAc-GlcUA-AnMan-Rf	1422.1	1422.5
hexasaccharide **4**[b]	GlcUA-GlcNAc-GlcUA-GlcNS-GlcUA-AnMan-Rf	1422.1	1422.4
trisaccharide **5**	GlcNAc6S-GlcUA-AnMan-Rf	908.7	908.6
tetrasaccharide **6**	GlcUA-GlcNAc6S-GlcUA-AnMan-Rf	1084.2	1084.4
hexasaccharide **7**	GlcUA-GlcNS6S-GlcUA-GlcNAc6S-GlcUA-AnMan-Rf	1582.3	1582.2
hexasaccharide **8**	GlcUA-GlcNAc-GlcUA-GlcNAc6S-GlcUA-AnMan-Rf	1464.2	1463.4
hexasaccharide **9**	GlcUA-GlcNS-GlcUA-GlcNAc6S-GlcUA-AnMan-Rf	1502.2	1501.8

[a] Rf represents the fluorous tag.



[b] Both hexasaccharides **3** and **4** have identical molecular masses. The location of the *N*-sulfo group in each hexasaccharide was determined by a tandem MS method as shown in Figure 2.

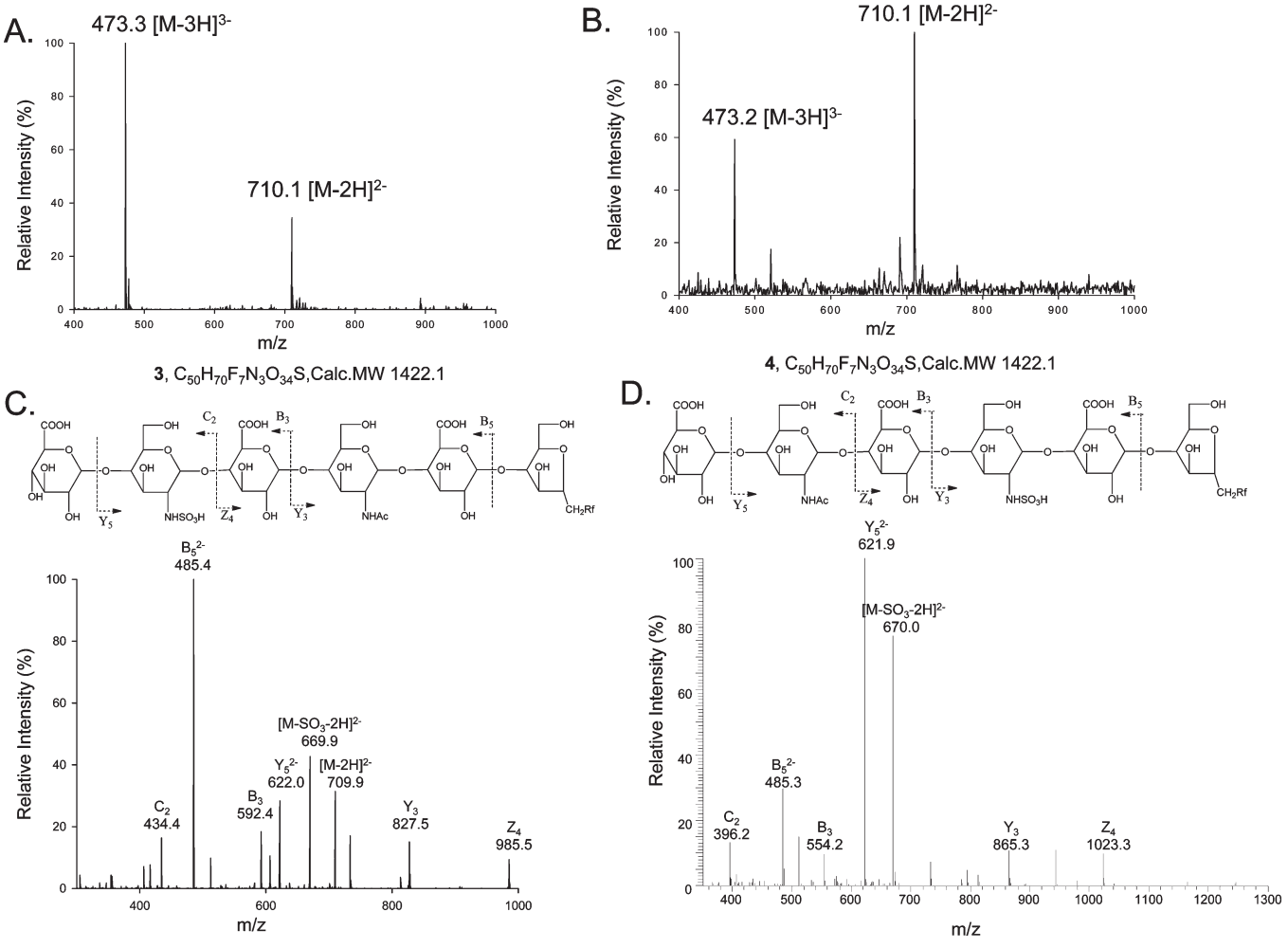


Figure 2. Structural characterization of *N*-sulfo hexasaccharide **3** and hexasaccharide **4**. (A) Mass spectrometry spectrum of hexasaccharide **3**. (B) Mass spectrometry spectrum of hexasaccharide **4**. (C) MS/MS spectrum of hexasaccharide **3** (precursor ion selection at *m*/*z* 710.1). The fragmentation pattern is depicted at the top. (D) MS/MS spectrum of hexasaccharide **4** (precursor ion selection at *m*/*z* 710.1). The fragmentation pattern is depicted at the top. The product ions in the MS/MS data were labeled according to Domon−Costello nomenclature.[26]

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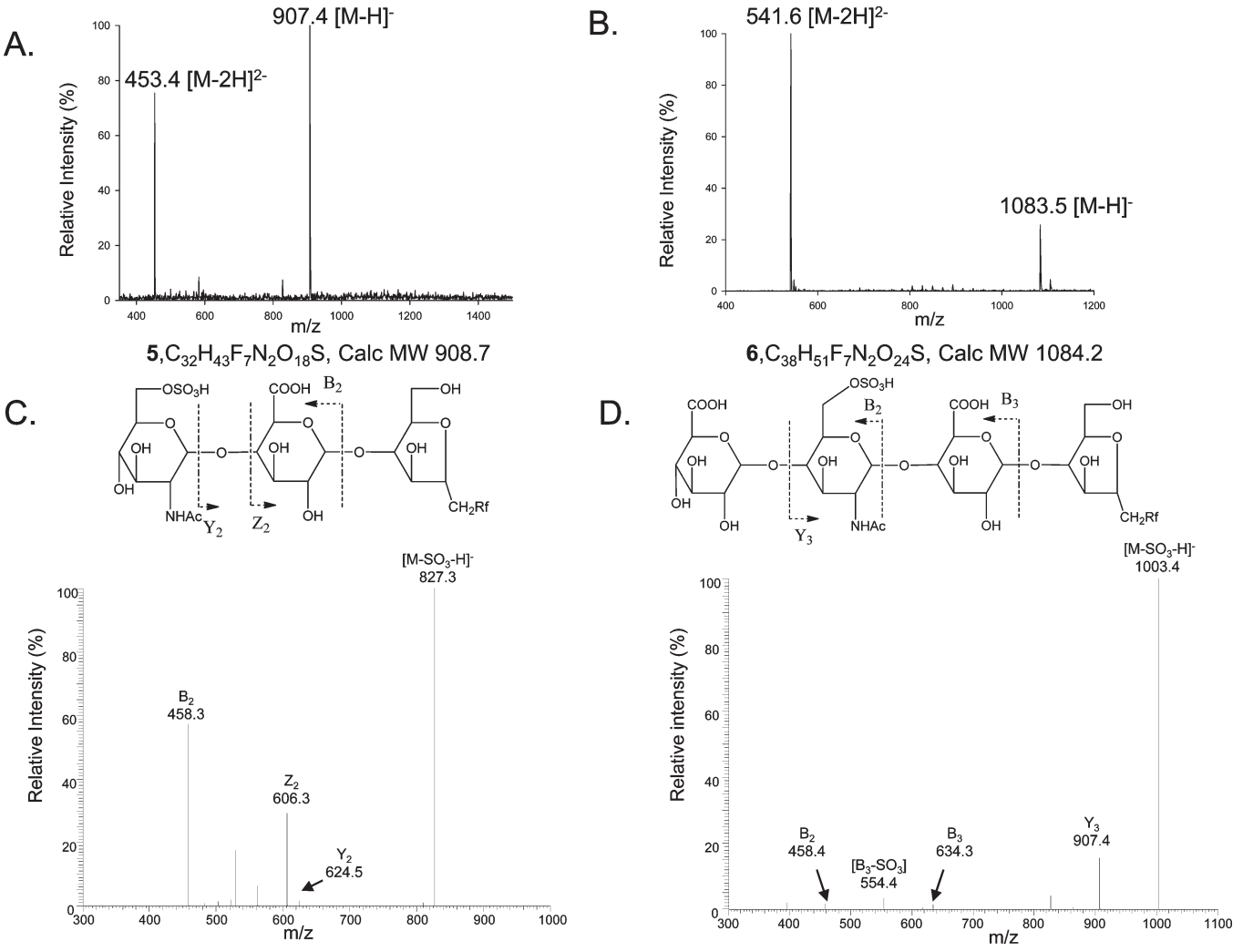

Figure 3. Structural characterization of 6-*O*-sulfo trisaccharide **5** and 6-*O*-sulfo tetrasaccharide **6**. (A) MS spectrum of 6-*O*-sulfo trisaccharide **5**. (B) MS spectrum of 6-*O*-sulfo tetrasaccharide **6**. (C) MS/MS spectrum of 6-*O*-sulfo trisaccharide **5** (precursor ion selection at *m*/*z* 907.4). The fragmentation pattern is depicted at the top. (D) MS/MS spectrum of 6-*O*-sulfo tetrasaccharide **6** (precursor ion selection at *m*/*z* 1083.5). The fragmentation pattern is depicted at the top. The product ions in the MS/MS data were labeled according to Domon−Costello nomenclature.[26]

Disaccharide Analysis. The oligosaccharides were digested with a mixture of heparin lyases to yield disaccharides. The oligosaccharide (100000 cpm) was incubated with a mixture of heparin lyase I, II, and III (each at ~20 μg) in 200 μL of 50 mM Na_2HPO_3 (pH 7.0) at 37 °C for 48 h. Additional aliquots of heparin lyase I, II, and III were added after 24 h. The recombinant heparin lyases were expressed and purified as described previously.[23] The resultant disaccharides were analyzed using a reverse phase ion pairing HPLC (RPIP-HPLC) method as described previously.[24] The identities of the disaccharides were determined by coelution with HS disaccharide standards (Seikagaku America).

Mass Spectrometry (MS) Analysis. MS analyses were performed on a Thermo LCQ-Deca instrument. Oligosaccharides were dissolved in a 1:1 MeOH/H_2O mixture in 10 mM ammonium hydroxide. A syringe pump (Harvard Apparatus) was used to introduce the sample via direct infusion (30 μL/min) into the instrument. Experiments were performed in negative ionization mode with a spray voltage of 3.8 kV and a capillary temperature of 200 °C.[25] The automatic gain control was set to 1×10^7 for full scan MS and 2×10^7 for MS/MS experiments. For MS/MS experiments, the selection of each precursor ion was achieved using an isolation width of 3 Da and the activation energy was 45% of the normalized collision energy. The product ions in the MS/MS data were labeled according to Domon−Costello nomenclature.[26] The MS and MS/MS data were acquired and processed using Xcalibur version 1.3.

RESULTS

Preparation of Oligosaccharide Substrates. Four oligosaccharide substrates **1**−**4** were synthesized for this study (Table 1). These oligosaccharides were prepared from a fluorous-tagged disaccharide precursor using bacterial glycosyltransferases, KfiA and PmHS2, as described in Experimental Procedures. The fluorous tag allowed us to purify the products in a single step using a fluorous flush column. The structures of both trisaccharide **1** and tetrasaccharide **2** were confirmed by ESI-MS (Table 1). To prepare the *N*-sulfated hexasaccharide **3** and **4**, we utilized UDP-GlcNTFA as a monosaccharide donor followed by base treatment and *N*-sulfotransferase modification as described in our prior publication.[11] The ESI-MS analysis of both hexasaccharides **3** and **4** demonstrated that the hexasaccharides have

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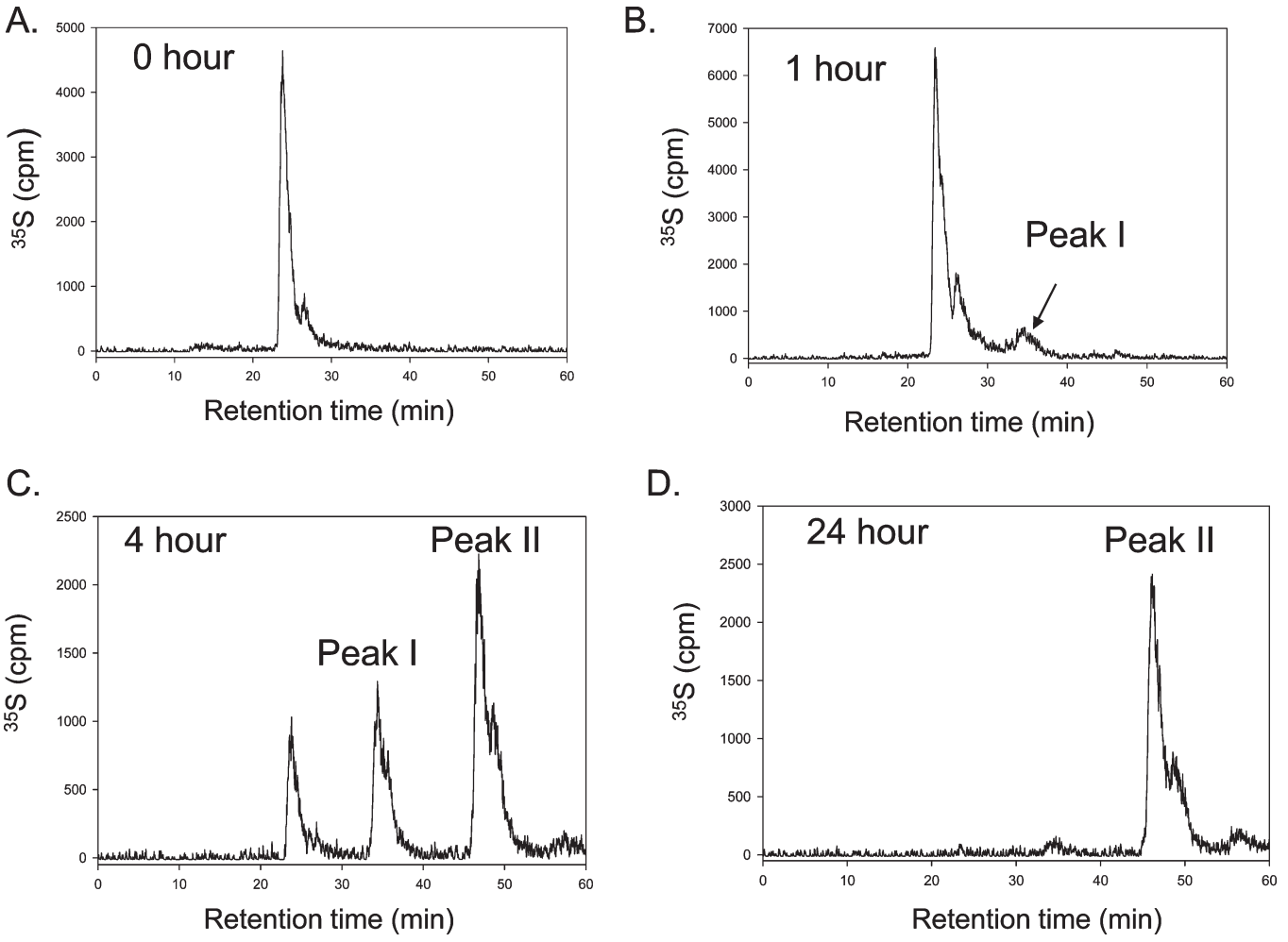

Figure 4. Preparation of 6-*O*-sulfo hexasaccharides with different reaction times. (A) HPLC chromatogram of [*N*-^{35}S]sulfo-labeled hexasaccharide **3** using a DEAE-HPLC column. (B) HPLC chromatogram of [*N*-^{35}S]sulfo-labeled hexasaccharide **3** using a DEAE-HPLC column after a 1 h reaction time. (C) HPLC chromatogram of [*N*-^{35}S]sulfo-labeled hexasaccharide **3** using a DEAE-HPLC column after a 4 h reaction time. (D) HPLC chromatogram of [*N*-^{35}S]sulfo-labeled hexasaccharide **3** using a DEAE-HPLC column after a 24 h reaction time.

molecular masses of 1422.5 and 1422.4 Da, respectively. These molecular masses are very close to the calculated value of 1422.1 Da, suggesting that both **3** and **4** have six saccharide units and carry a single sulfo group (Figure 2A,B).

The locations of the sulfo group in each hexasaccharide were determined using tandem MS (MS/MS) (Figure 2C,D). For hexasaccharide **3**, a daughter ion with an *m*/*z* value of 827.5 was detected, which confirmed that the GlcNAc residue is the third residue from the reducing end (Figure 2C, Y_3 fragment). We observed a daughter ion with an *m*/*z* value of 622.0, demonstrating that the GlcNS residue is the fifth residue from the reducing end (Figure 2D, Y_5^{2-} fragment). Likewise, for hexasaccharide **4**, a daughter ion with an *m*/*z* value of 865.3 was observed, which confirmed that the GlcNS residue is the third residue from the reducing end (Figure 2D, Y_3 fragment).

Synthesis of 6-*O*-Sulfated Trisaccharide and Tetrasaccharide. Incubation of trisaccharide **1** with a mixture of 6-OST-1 and 6-OST-3 yielded 6-*O*-sulfated trisaccharide **5**. Both ESI-MS analysis and tandem MS analysis confirmed the structure of the anticipated product (Figure 3A,C). The 6-*O*-sulfated tetrasacccharide **6** was prepared by incubating tetrasaccharide **2** with 6-OST-1 and 6-OST-3. Mass spectrometry analysis revealed the molecular mass of the 6-*O*-sulfated tetrasaccharide **6** to be 1084.8, which is close to the calculated mass of 1084.2 (Figure 3B). Tandem MS analysis confirmed the position of the GlcNAc6S residues in **6** (Figure 3D) from two characteristic daughter ions, Y_3 (*m*/*z* 907.4) and B_2 (*m*/*z* 458.4), which are products of the cleavage of internal glycosidic linkages. Our results demonstrated that the mixture of 6-OST-1 and -3 can sulfate substrates as short as a trisaccharide. It should be noted that only ~40% of the trisaccharide was converted to the 6-*O*-sulfated trisaccharide product even after extensive incubation, suggesting that the sulfation efficiency for a trisaccharide is low.

Synthesis of 6-*O*-Sulfated Hexasaccharide. We tested whether a mixture of 6-OST-1 and -3 preferably sulfates GlcNS residues using a hexasaccharide model substrate [hexasaccharide **3** (Table 1)]. Exhaustive incubation of the substrate with 6-OSTs resulted in a major product, hexasaccharide **7**. Analysis of the product by ESI-MS revealed its molecular mass to be 1582.2 Da, which is close to the calculated molecular mass (1582.3 Da) for a trisulfated hexasaccharide (Figure 1 of the Supporting Information), suggesting that the product carried two 6-*O*-sulfo groups. The structure of this 6-*O*-sulfated hexasaccharide was further confirmed by a disaccharide analysis as described below.

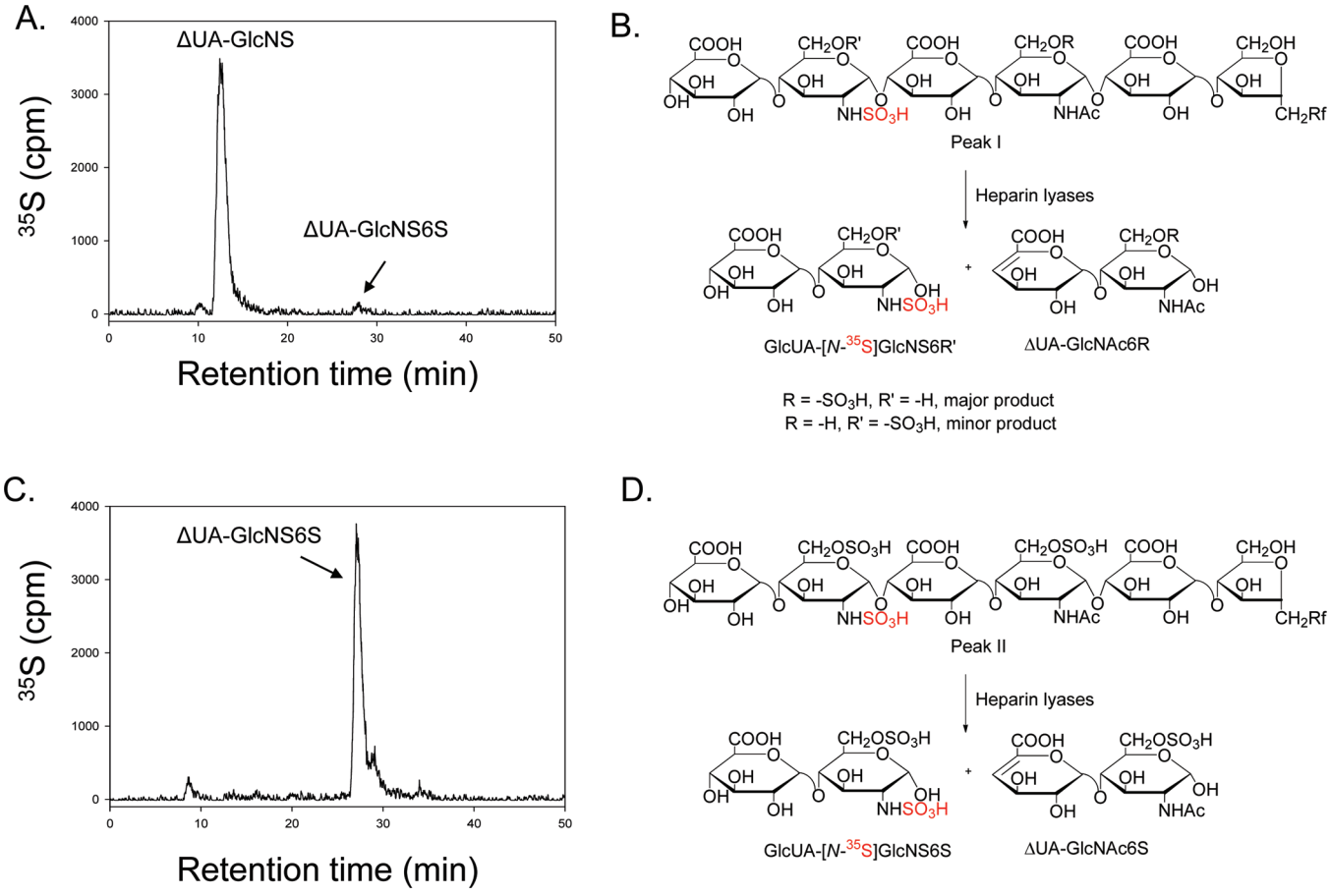

Figure 5. Determination of the structures of 6-*O*-sulfo hexasaccharides. (A) HPLC chromatogram of the disaccharide analysis of peak I. The enzymatic reaction involved in the disaccharide analysis of peak I is shown in panel B. (C) HPLC chromatogram of the disaccharide analysis of peak II. (D) Reaction involved in the disaccharide analysis of peak II. The radioactively labeled *N*-sulfo group is colored red. The disaccharide products from heparin lyase degradation are shown in panels B and D. The disaccharide analysis was performed on a C_{18}-HPLC column under RPIP-HPLC conditions. The eluent was monitored by an online radioactive detector. Under these conditions, the nonradioactively labeled disaccharide (ΔUA-GlcNAc6R shown in panels B and D) could not be detected.

Our results suggest that the enzymes do not distinguish GlcNAc and GlcNS residues with exhaustive incubation. To compare whether 6-OST-1 alone and a mixture of 6-OST-1 and -3 produce similar sulfation products, we incubated the enzymes with hexasaccharide **3** under two different concentrations of the sulfo donor PAPS (Figure 2 of the Supporting Information). At a low concentration of PAPS (0.7 μM), both 6-OST-1 and the mixture of 6-OST-1 and -3 yielded one major ^{35}S-labeled hexsaccharide that eluted at 35 min via DEAE-HPLC that is consistent with a singly 6-*O*-sulfated product; likewise, at a high concentration of PAPS (80 μM), the enzymes yielded a major ^{35}S-labeled hexasaccharide that eluted at 48 min via DEAE-HPLC that is consistent with the structure of hexasaccharide **7**. Taken together, our data suggest that the substrate specificities of 6-OST-1 and the mixture of 6-OST-1 and -3 are indistinguishable. Thus, a mixture of 6-OST-1 and -3 was used to sulfate the oligosaccharides throughout the study unless otherwise specified.

Next, we examined if selective 6-*O*-sulfation can be achieved under partial or limited sulfation conditions. To facilitate the detection, an *N*-^{35}S-sulfated hexasaccharide **3** was prepared, in which a GlcNS residue is located closer to the nonreducing end. The ^{35}S-labeled hexasaccharide was then modified by the mixture of 6-OST-1 and -3 for various amounts of time. The 6-*O*-sulfated hexasaccharide products were then analyzed with a DEAE-HPLC column (Figure 4). The starting *N*-sulfated hexasaccharide **3** had a retention time of 24 min (Figure 4A). After a 1 h reaction time, a small but detectable ^{35}S-labeled peak (peak I) was observed at 35 min, suggesting that a 6-*O*-sulfated product was formed (Figure 4B). After reaction for 4 h (Figure 4C), the intensity of peak I increased, and another ^{35}S-labeled peak (peak II) emerged at 48 min, suggesting that the products contained two hexasaccharides carrying one and two 6-*O*-sulfo groups, respectively. After reaction for 24 h (Figure 4D), only peak II was observed, suggesting that the reaction was completed.

The structures of peak I and peak II were confirmed by disaccharide analysis. The result of the disaccharide analysis of peak I revealed a single ^{35}S-labeled disaccharide with a structure of ΔUA-[^{35}S]GlcNS and a small but detectable amount of ΔUA-[*N*-^{35}S]GlcNS6S (Figure 5A,B). This result suggests that the 6-*O*-sulfo group is predominantly absent on the GlcNS residue that is located closer to the nonreducing end, resulting in a nonradioactively labeled disaccharide, ΔUA-GlcNAc6S. The results of the disaccharide analysis also suggest that peak I is a mixture of two hexasaccharides carrying a single 6-*O*-sulfo group, and the 6-*O*-sulfo group is mainly on the residue that is closer to the reducing end, i.e., on the GlcNAc residue. As expected, the

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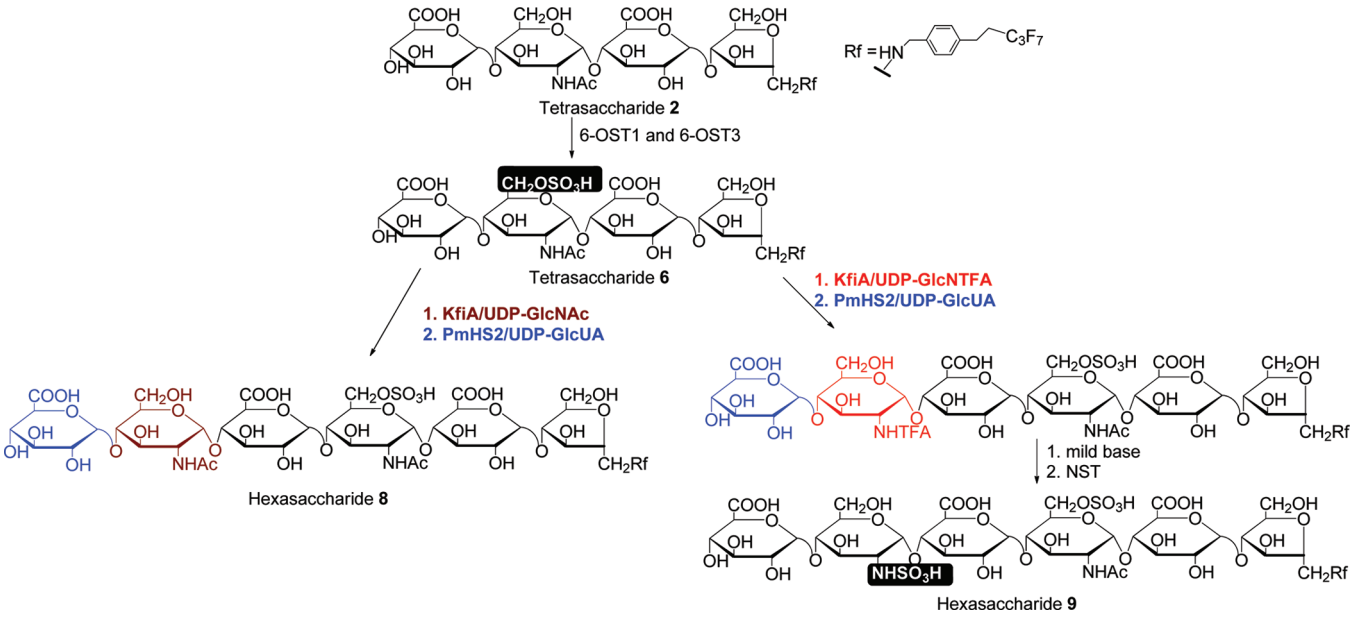

Figure 6. Scheme for the synthesis of monosulfated hexasaccharides **8** and **9**. Tetrasaccharide **2** was first 6-*O*-sulfated to yield the 6-*O*-sulfated tetrasaccharide. The tetrasaccharide was then elongated by KfiA and PmHS2 using different monosaccharide donors, UDP-GlcNAc and UDP-trifluoroacetylglucosamine (UDP-GlcNTFA). To convert the GlcNTFA to a GlcNS in hexasaccharide **9**, we subjected the hexasaccharide to mild base treatment followed by *N*-sulfation with NST.

disaccharide analysis of peak II (or hexasaccharide **7**) confirmed the presence of a ΔUA-[*N*-^{35}S]GlcNS6S product, consistent with our conclusion that peak II carried two 6-*O*-sulfo groups (Figure 5C,D). Our data suggest that 6-OST-1 and 6-OST-3 kinetically prefer to sulfate the reducing end especially when a GlcNAc residue is located closer to the reducing end. We also conducted a disaccharide analysis of the ^{35}S-labeled peak that eluted at 35 min that resulted from the partial 6-*O*-sulfation of hexasaccharide **3** with 6-OST-1 alone (as shown in Figure 2A of the Supporting Information). As when using a mixture of 6-OST-1 and -3, the 6-*O*-sulfo group was determined to be at the GlcNAc residue (data not shown).

To examine whether partial 6-*O*-sulfation occurs at a GlcNS residue located closer to the reducing end, we employed another hexasaccharide substrate (hexasaccharide **4**). Unlike hexasaccharide **3**, this substrate carries a GlcNS residue that is closer to the reducing end, and it was ^{35}S-labeled at the *N*-sulfo position. Again, two ^{35}S-labeled peaks, designated as peaks III and IV, were observed at 35 and 48 min after incubation for 4 h with a mixture of 6-OST-1 and -3 (Figure 3A of the Supporting Information). The disaccharide analysis of peak III revealed the presence of two disaccharides, ΔUA-[*N*-^{35}S]GlcNS and ΔUA-[*N*-^{35}S]GlcNS6S, at a ratio of 1:2 (Figure 3C of the Supporting Information). Our results suggest that peak III is a mixture of two hexasaccharides: one hexasaccharide (the major product) carried the 6-*O*-sulfo group at the GlcNS residue, and another hexasaccharide (the minor product) carried the 6-*O*-sulfo group at the GlcNAc residue. Peak IV was expected to carry two 6-*O*-sulfo groups because it was eluted at ~48 min via DEAE-HPLC. The results of using hexasaccharide **4** as a substrate suggest that the mixture of 6-OST-1 and -3 displayed a modest to minimal preference for the GlcNS residue when it is located closer to the reducing end.

A Strategy for Preparing Selectively 6-*O*-Sulfated Hexasaccharides. It is difficult to prepare a hexasaccharide that carries a single 6-*O*-sulfo group by partial sulfation using a mixture of 6-OST-1 and -3 because a mixture of 6-*O*-sulfated products is obtained. We attempted to use an alternative strategy to achieve this goal. Taking advantage of the fact that the mixture of 6-OST-1 and -3 sulfates short oligosaccharide substrates, we exploited the possibility of elongating oligosaccharides carrying 6-*O*-sulfo groups with KfiA and PmHS2. We started with 6-*O*-sulfated tetrasaccharide **6** and elongated it to hexasaccharides using bacterial glycosyltransferases (Figure 6). To demonstrate the utility of this approach, we synthesized two hexasaccharides (hexasaccharides **8** and **9**) that differ at the glucosamine residue closer to the nonreducing end: hexasaccharide **8** has a GlcNAc, and hexasaccharide **9** has a GlcNS residue. The final structures of hexasaccharides **8** and **9** were determined by mass spectrometry. For example, ESI-MS analysis revealed the molecular mass of hexasaccharide **9** to be 1502.2 Da, equal to the calculated molecular mass (1502.2 Da) (Figure 7B). MS/MS analysis confirmed the position of the GlcNS residues in **9** (Figure 7D) from the two characteristic daughter ions, Y_3 (m/z 907.3) and B_3 (m/z 592.2), which are products of the cleavage of an internal glycosidic linkage. Taken together, our results suggest that the elongation approach allowed us to prepare partially 6-*O*-sulfated hexasaccharides.

We noted that significant desulfation occurred for hexasaccharide **9** while it underwent ESI-MS analysis. To eliminate the possibility that hexasaccharide **9** is a mixture, we synthesized an *N*-^{35}S-labeled hexasaccharide **9** following an identical procedure. The ^{35}S-labeled hexasaccharide **9** was eluted at 35 min on a DEAE-HPLC column (Figure 4 of the Supporting Information). This retention time is consistent with a hexasaccharide carrying an *N*-sulfo group and a 6-*O*-sulfo group.

DISCUSSION

The long-term goal of our research is to develop an enzyme-based method to synthesize heparin and structurally defined HS

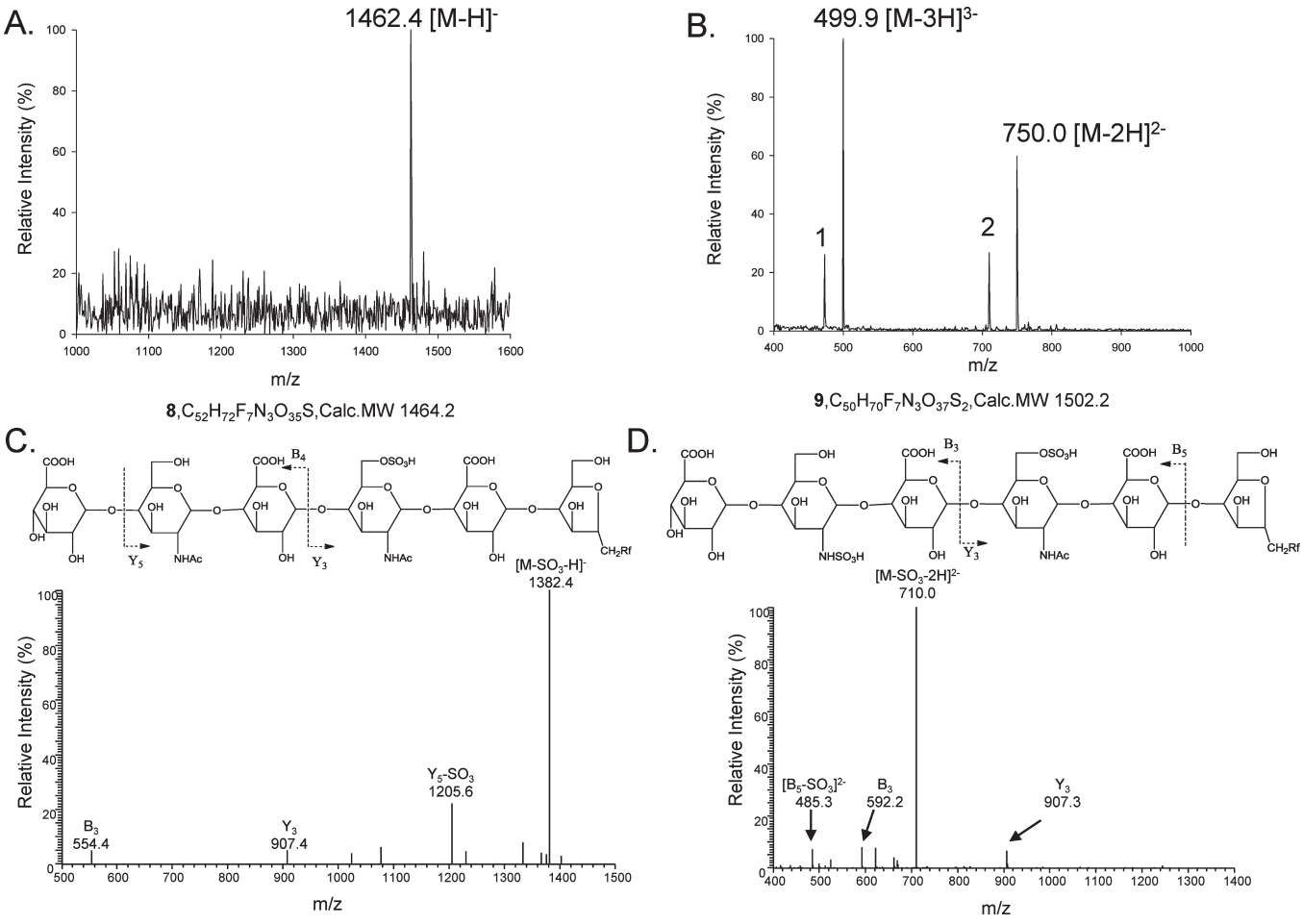

Figure 7. Structural characterization of 6-*O*-sulfo hexasaccharides **8** and **9**. (A) Mass spectrometry spectrum of hexasaccharide **8**. (B) Mass spectrometry spectrum of hexasaccharide **9**. Additional molecular ions are labeled as 1 and 2, where 1 is at m/z 473.2 ($[M - SO_3 - 3H]^{3-}$) and 2 is at m/z 710.0 ($[M - SO_3 - 2H]^{2-}$). (C) MS/MS spectrum of hexasaccharide **8** (precursor ion selection at m/z 1462.4). The fragmentation pattern is depicted at the top. (D) MS/MS spectrum of hexasaccharide **9** (precursor ion selection at m/z 750.0). The fragmentation pattern is depicted at the top. The product ions in the MS/MS data were labeled according to Domon−Costello nomenclature.[26]

oligosaccharides. A method for selectively placing a 6-*O*-sulfo group represents an essential part of this initiative. 6-OST is present in three different isoforms,[17,27] and we have obtained purified recombinant 6-OST-1 and 6-OST-3 in large quantities using *E. coli* expression. Previously, Smeds and colleagues concluded that the three isoforms have no distinguishable substrate specificities.[20] Although preparing fully sulfated oligosaccharides using a mixture of 6-OST-1 and -3 has been accomplished by our lab,[11] placing a 6-*O*-sulfo group in a specific position remained a challenge. In this study, we demonstrated the feasibility of placing a 6-*O*-sulfo group within an oligosaccharide using a combination of bacterial glycosyl transferases and 6-OSTs. In our approach, we preassemble the 6-*O*-sulfo group on a tetrasaccharide. The 6-*O*-sulfated tetrasaccharide can then be elongated to a hexasaccharide with high efficiency. In our study, we used a mixture of 6-OST-1 and -3 to conduct most of the experiments because we did not detect any difference in substrate specificity between 6-OST-1 alone and a mixture of 6-OST-1 and -3, although the mixture may provide stronger 6-*O*-sulfation capacity to avoid incomplete 6-*O*-sulfation. Nevertheless, we cannot rule out the possibility that subtle differences in substrate specificities among 6-OST isoforms exist at the polysaccharide level.

The results from our study also provide insights into the substrate specificities of 6-OSTs using synthetic oligosaccharide model substrates. As in previous reports, the enzymes were found to sulfate both GlcNAc and GlcNS residues.[20] We observed that a trisaccharide is sufficiently long to serve as a sulfo group receptor. Furthermore, we observed that it is very difficult to obtain a hexasaccharide carrying a single 6-*O*-sulfo group simply by shortening the reaction time. Our results found that the combination of 6-OST-1 and -3 has a clear preference for sulfating the *N*-acetylated glucosamine residue closer to the reducing end of a hexasaccharide substrate. However, this preference was significantly reduced when this residue was replaced with a GlcNS residue. A report by Jemth and colleagues also demonstrated that 6-OSTs prefer to sulfate an internal glucosamine residue that is closer the reducing end using different oligosaccharide substrates.[28]

In summary, we developed an enzyme-based method that can prepare oligosaccharides carrying fully 6-*O*-sulfo groups. Our method is also capable of introducing a single 6-*O*-sulfo group followed by elongation of the oligosaccharides using bacterial glycosyl transferases. By doing so, we are able to introduce a 6-*O*-sulfo group to a glucosamine located in the middle of a target

oligosaccharide. Although the 6-*O*-sulfated tetrasaccharide has been extended to only hexasaccharides in this study, it is possible to further extend it to larger oligosaccharides.[22] However, our method is unable to introduce a 6-*O*-sulfo group at the glucosamine residue located closer to the nonreducing end when the reducing end glucosamine residues are devoid of 6-*O*-sulfation. It should be noted that HS 6-*O*-sulfatases remove the 6-*O*-sulfo group from HS in vivo.[18,19] It could be possible to modify fully 6-*O*-sulfated HS substrates by 6-*O*-sulfatases so that a 6-*O*-sulfo group remains at the glucosamine residue located at the nonreducing end. Given the important role of 6-*O*-sulfation for the function of HS, our method should provide a powerful tool for synthesizing structurally defined oligosaccharides to probe the structure and activity relationship of HS.

■ ASSOCIATED CONTENT

Supporting Information. Additional data for structural characterization of hexasaccharide **7**, peak III, and peak IV and purity determination of hexasaccharide **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Room 303, Beard Hall, University of North Carolina, Chapel Hill, NC 27599. Telephone: (919) 843-6511. Fax: (919) 843-5432. E-mail: jian_liu@unc.edu.

Funding Sources

This work is supported in part by National Institutes of Health Grants AI50050, HL094463, and AI074775.

■ ACKNOWLEDGMENT

We thank Dr. Yongmei Xu for comments on the HPLC analyses and disaccharide analyses and for providing the *E. coli*-expressed and purified 6-OST-1 and 6-OST-3. We also thank Elizabeth Pempe and Courtney Jones for critically reading the manuscript.

■ ABBREVIATIONS

HS, heparan sulfate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GlcNTFA, UDP-*N*-trifluoroacetylglucosamine; UDP-GlcUA, UDP-*N*-glucuronic acid; GlcN-1-P, glucosamine 1-phosphate; GlmU, glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

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