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Fluorous-Assisted Chemoenzymatic Synthesis of Heparan Sulfate Oligosaccharides

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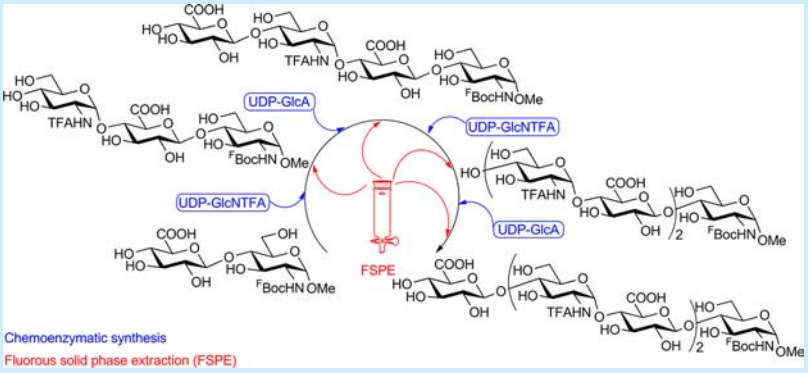
Supporting Information



ABSTRACT: The chemoenzymatic synthesis of heparan sulfate tetrasaccharide (**1**) and hexasaccharide (**2**) with a fluorous tag attached at the reducing end is reported. The fluorous *tert*-butyl dicarbonate (^{F}Boc) tag did not interfere with enzymatic recognition for both elongation and specific sulfation, and flash purification was performed by standard fluorous solid-phase extraction (FSPE). Based on an ^{F}Boc attached disaccharide as acceptor, a series of partial *N*-sulfated, 6-*O*-sulfated heparan sulfate oligosaccharides were successfully synthesized employing fluorous techniques.

Glycosaminoglycans (GAGs) are anionic polysaccharides found in all animal cells that are composed of repeating disaccharide units of hexuronic acid and hexosamine.[1] Of all the classes of GAGs, the heparan sulfate (HS) and heparin family of GAGs are the most attractive therapeutic targets[2] as they are known to regulate a wide range of physiological processes[3] through their interactions with biologically important proteins, such as growth factors.[4] However, even with recent advances in synthetic carbohydrate chemistry,[5] the preparation of HS and heparin oligosaccharides remains a major challenge due to their structural complexity and heterogeneity. Therefore, chemoenzymatic approaches, relying on biosynthetic enzymes for the synthesis of highly sulfated GAG oligosaccharides, represent powerful and efficient alternatives to traditional methods.[6]

Fluorous chemistry emerged as a new tool for solution-phase high-throughput organic synthesis in the late 1990s.[7] Fluorous separation techniques rely on the high affinity of perfluoroalkyl chains toward fluorous surfaces and solvents. Fluorous tag-facilitated chemical synthesis has been developed extensively by Curran[8] over the past decade and applied to proteomics,[9] peptide synthesis,[10] and carbohydrate microarrays.[11] In contrast to the streptavidin−biotin system, fluorous tags bind to a fluorous surface through fluorous solid-phase extraction (FSPE) and can be easily released through fluorophilic elution. Reversible binding, ease of purification, broad reaction scope, and an ability to be automated all make fluorous tagging especially suitable for high-throughput combinatorial synthesis.[12]

In recent years, fluorous techniques have been applied to oligosaccharide synthesis and have significantly facilitated purification. In their automated solid-phase oligosaccharide synthesis, Seeberger and co-workers used a TIPS-like fluorous linker to "cap" unreacted sugar residues and to remove unwanted deletion sequences from the glycosylation mixture.[13] Huang and co-workers reported a similar strategy, where the fluorous "cap" is applied to the product, and synthesized linear and branched oligosaccharides in a one-pot manner.[14] Pohl and co-workers attached a fluorous linker at the reducing end of a mannoside to prepare linear and branched mannose oligosaccharides[15] and immobilized these onto fluorocarbon-coated glass slides to study their binding with concanavalin A.[16]

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Boons and co-workers reported a modular synthesis of heparan sulfate tetrasaccharide with a fluorous tagged aminopentyl linker at the reducing end of glucosamine substrates.[17]

Although recent advances in chemoenzymatic synthesis of heparan sulfate oligosaccharides have included one-pot synthesis[6d] and the synthesis at the hundreds of milligram scale,[6c] development of more efficient and less time-consuming methodology is still necessary to speed up the purification process.[18] The application of fluorous techniques in enzymatic reactions is still not well developed. In this paper, we report a fluorous-assisted chemoenzymatic synthesis of heparan sulfate oligosaccharides from a chemically synthesized disaccharide acceptor, with a fluorous Boc (^{F}Boc)[19] on the glucosamine nitrogen at the reducing end. Liu and co-workers synthesized a similar heparan sulfate oligosaccharide with a fluorous linker but with an unnatural anhydromannitol residue at the reducing end.[20] The newly designed disaccharide acceptor in this study has an α-configured *O*-methyl glycoside at the reducing end, which can serve as a potential starting point for chemoenzymatic synthesis of the heparin pentasaccharide anticoagulant drug Arixtra (fondaparinux).

Our retrosynthetic analysis of the heparan sulfate oligosaccharides (**1** and **2**) with fluorous-assisted methodology is shown in Figure 1. These would be prepared from the fluorous

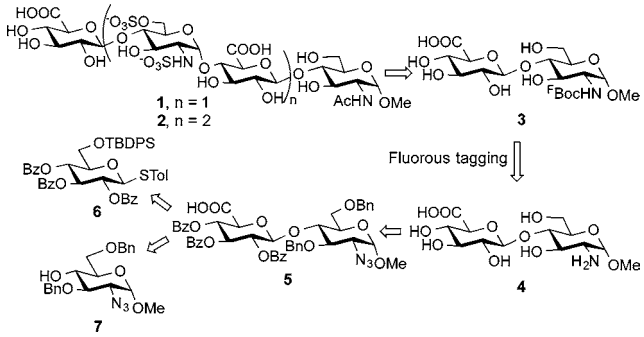
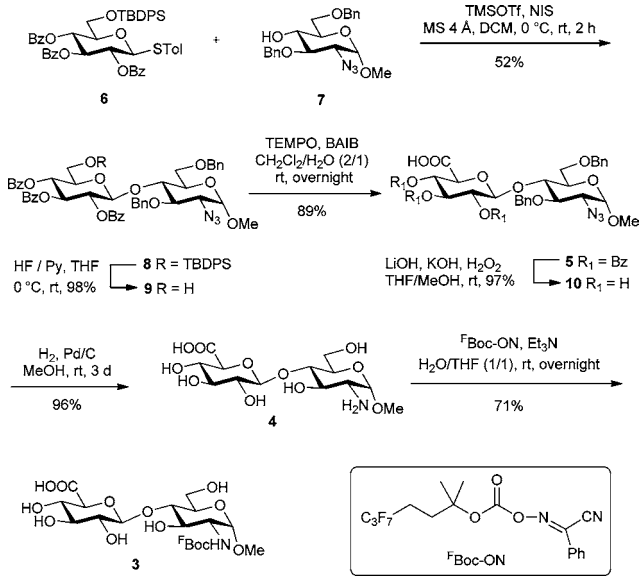
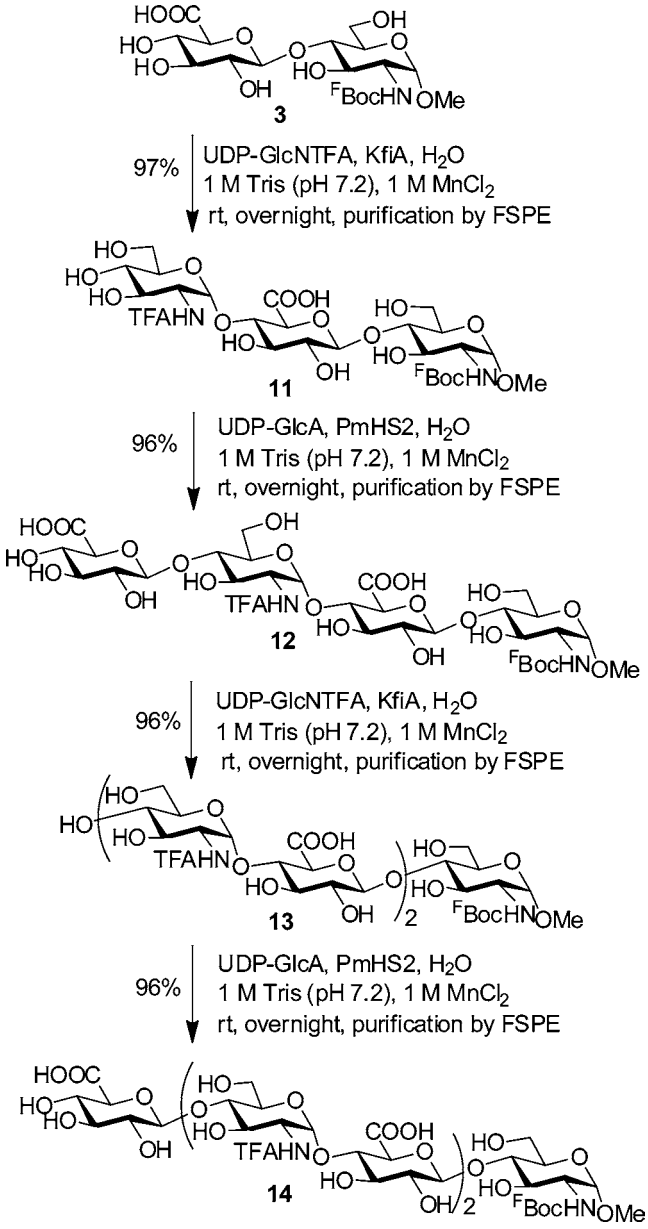


Figure 1. Retrosynthetic analysis of heparan sulfate tetrasaccharide (**1**) and hexasaccharide (**2**).

Scheme 1. Synthesis of Fluorous-Tagged Disaccharide 3



Scheme 2. Synthesis of Heparosan Hexasaccharide Analogue 14



acceptor **3** through a repetition of enzymatic backbone elongation followed by *N*-sulfation, 6-*O*-sulfation, and deprotection of the ^{F}Boc tag. Fluorous disaccharide **3** would be chemically synthesized through introduction of the ^{F}Boc tag to the free amino group on the reducing end of disaccharide **4**, which would be obtained through a chemical glycosylation of acceptor **7** with donor **6** and global deprotection and hydrogenation of the fully protected disaccharide **5**.

The synthesis of donor **6** and acceptor **7** followed a conventional synthetic route described in the Supporting Information (Schemes SI1 and SI2). As shown in Scheme 1, the glycosylation of **6** with **7** afforded disaccharide **8** in moderate yield. With the fully protected disaccharide in hand, the TBDPS group was removed initially by treatment with HF·Py[21] to afford disaccharide **9**. The unprotected C6-hydroxyl group was then oxidized to a carboxylic acid using TEMPO-BAIB[22] to afford **5**. The Bz groups were removed by strong

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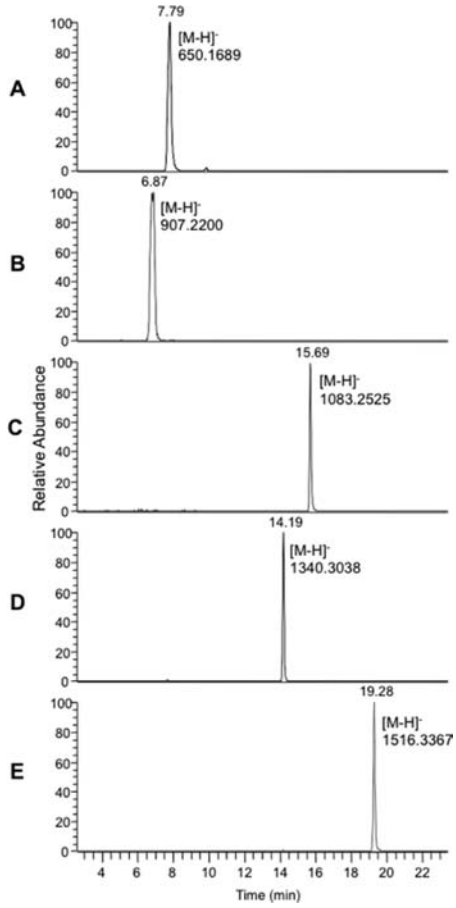


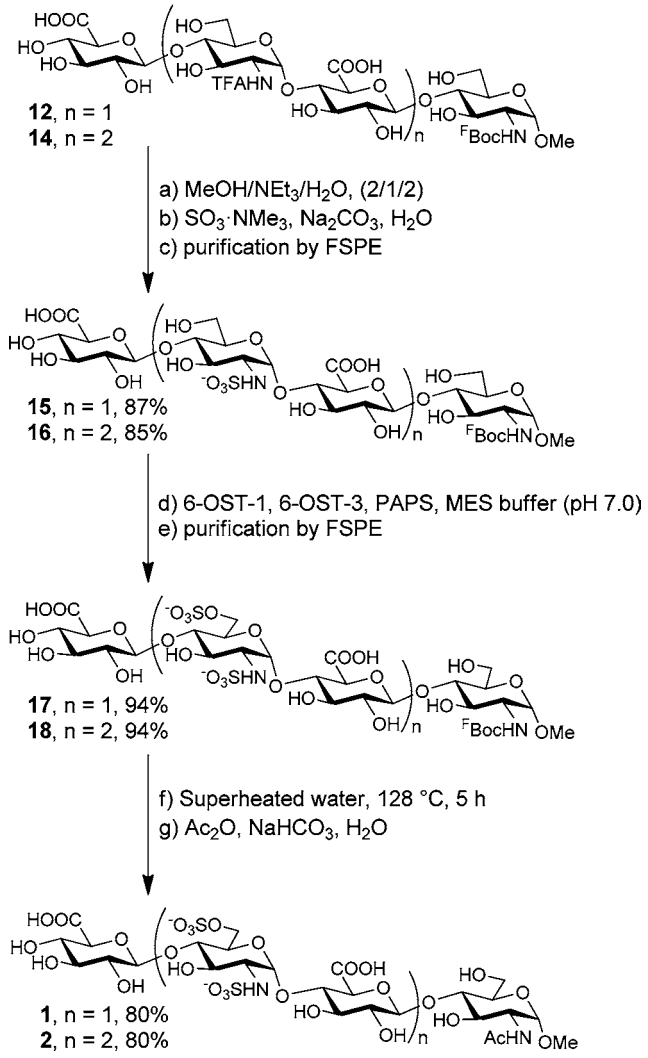
Figure 2. Illustration of extracted ion chromatograms (EIC) corresponding to the HS backbone oligosaccharides indicated: (A) disaccharide (**3**); (B) trisaccharide (**11**); (C) tetrasaccharide (**12**); (D) pentasaccharide (**13**); and (E) hexasaccharide (**14**).

base treatment followed by hydrogenation for 3 d using Pd/C as the catalyst. All deprotection steps proceeded smoothly to give the disaccharide **4** in high yields (Scheme 1).

Fluorous-tagged disaccharide acceptor **3** was next elongated with *Escherichia coli* glycosyltransferase KfiA and uracil diphosphate-*N*-trifluoroacetylglucosamine (UDP-GlcNTFA) to construct the trisaccharide **11** (Scheme 2). After flash elution through FSPE with water and methanol, respectively, the pure trisaccharide **11** was collected in methanol and identified by LC−MS and NMR spectroscopy. One cycle of fluorous-assisted purification generally takes <0.5 h and affords a relatively pure product. The GlcNTFA residue is an unnatural analogue of GlcNAc and should allow the selective introduction of *N*-sulfate groups in the future synthesis of HS oligosaccharides. Following the same protocol as above, *Pasteurella multocida* heparan synthase (PmHS2) and uracil diphosphate−glucuronic acid (UDP−GlcA) were employed to construct the tetrasaccharide (**12**). These steps were repeated one more time to afford the pentasaccharide (**13**) and hexasaccharide (**14**) with the GlcA−GlcNTFA repeating unit. LC−MS data analysis of unsulfated HS backbone from disaccharide to hexasaccharide is shown in Figure 2.

With the HS backbone constructed and in hand, we subsequently sulfated these substrates to check their performance with FSPE separation. Base-catalyzed ($MeOH/NEt_3/H_2O$, 2/1/2) deprotection of the trifluoroacetamide group was followed by the chemical *N*-sulfation with $SO_3{\cdot}MeN_3$ and

Scheme 3. Chemoenzymatic Synthesis of Heparan Sulfate Tetrasaccharide and Hexasaccharide



Na_2CO_3 to form *N*-sulfated tetrasaccharide (**15**) (Scheme 3). A high-field shift of 0.5 ppm for H-2 was observed in the ^1H and 2D COSY NMR (^2H_2O, 600 MHz) in the glucosamine residue that had been *N*-sulfated. The heparan sulfate 6-*O*-sulfotransferase isoforms -1 and -3 (6-OST-1 and 6-OST-3) were incubated together with **15** and PAPS to obtain the 6-*O*-sulfate group containing tetrasaccharide **17**, and excess PAPS and buffer salts were easily removed by FSPE. ^1H NMR (^2H_2O, 600 MHz) showed that the peaks at 3.67 and 3.78 ppm, corresponding to the protons on the C6 of the internal glucosamine residue in **15**, shifted to 4.09 and 4.37 ppm in the product **17**. 2D COSY and HMQC NMR spectroscopy also confirmed the formation of **17**. The glucosamine residue at the reducing end of **15** was not 6-*O*-sulfated, suggesting that additional studies are required to more fully understand the specificity of the 6-*O*-sulfotransferases.[23] Subsequently, hexasaccharide **18** was obtained using the same protocol, including *N*-sulfation, 6-*O*-sulfation, and FSPE steps. We found that some product was retained on the FSPE column after four sulfate groups had been added on the HS chain, but it could be easily released by employing trifluoroethanol as a cosolvent. Deprotection of ^{F}Boc was initially attempted, based on a literature method,[19] using either 50% aqueous TFA at room

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temperature or 3 N aqueous HCl at 60 °C for 2 h. We observed that while 3 N HCl removed the fluorous tag from tetrasaccharide (**17**), it also resulted in complete sulfate loss. Treatment of the model substrate **3** with 50% aqueous TFA only afforded a 10% yield even for 24 h. Finally, superheated water (liquid water between 100 and 374 °C),[24] a green solvent, was successfully employed to remove ^{F}Boc tag from substrates **17** and **18**, and the HS tetrasaccharide (**1**) and hexasaccharide (**2**) were obtained after *N*-acetylation.

In conclusion, we have successfully applied the ^{F}Boc linker and FSPE technique in our chemoenzymatic synthesis of heparan sulfate oligosaccharides. The fluorous-linked disaccharide was extended with glycosyltransferases KifA and PmHS2 respectively, and recognized by 6-OSTs, revealing that the ^{F}Boc linker does not interfere with the action of these enzymes. The FSPE technique was suitable for the purification of heparan sulfate oligosaccharides as well. Currently, additional biosynthetic enzymes, including C5-epimerase, 2-OST and 3-OST, are under investigation to regioselectively add additional sulfate groups on these HS backbones.[6c]

■ ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMQC NMR, and ESI MS spectra and experimental procedures for the preparation of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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