Expedient Synthesis of a Library of Heparan Sulfate Like "Head to Tail" Linked Multimers for Structure and Activity Relationship Studies

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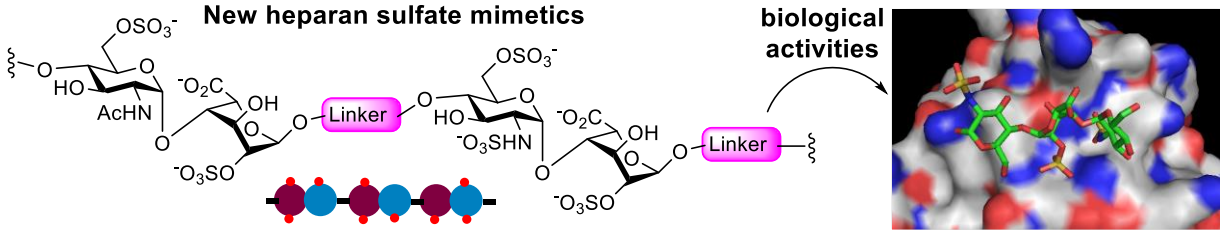
Abstract

Heparan sulfate (HS) plays significant roles in various biological processes such as inflammation, cell proliferation, and bacterial and viral infection. The inherent complexity of naturally existing HS has severely hindered the thorough understanding of the relationship between their diverse structures and biological functions. While HS syntheses have advanced significantly in recent years, preparation of HS libraries remains a tremendous challenge due to the difficulties in achieving high yields in glycosylation and sulfation reactions especially with longer glycans and the need to prepare multiple compounds. A new strategy to synthesize a library of HS-like pseudo-hexasaccharides has been developed to expedite library preparation. HS disaccharides were linked in a "head-to-tail" fashion from the reducing end of a module to the non-reducing end of a neighboring module to mimic native HS. Three differentially sulfated HS disaccharides were designed and prepared from a common intermediate. Conjugation of these modules using amide chemistry bypassed the need for challenging glycosylation reactions to extend the HS backbone. Combinatorial syntheses of 27 HS-like pseudo-hexasaccharides were achieved using these three HS modules. This new class of compounds mimicked well the native HS with their binding to fibroblast growth factor 2 (FGF-2) exhibiting similar structure-activity relationship trends as HS hexasaccharides. The ease of synthesis and the ability to mimic natural HS suggest the new head-to-tail linked pseudo-hexasaccharides could be an exciting tool to facilitate the understanding of HS biology.

TOC figure



Introduction

Heparan sulfate (HS) is a highly sulfated complex linear polysaccharide that belongs to the family of glycosaminoglycans. It is composed of disaccharide repeating units D-glucosamine (GlcN) α-(1-4)-linked to a uronic acid (L-iduronic acid (IdoA) or D-glucuronic acid (GlcA)).[1] In nature, multiple hydroxyl groups within the disaccharide units of HS can be *O*-sulfated and these can include the 2-OH of the uronic acid residue, 6-OH and 3-OH of GlcN, respectively. Furthermore, the GlcN unit can be *N*-acetylated (GlcNAc) or *N*-sulfated (GlcNS). Due to its diverse structures in nature, HS can interact with a wide variety of proteins including fibroblast growth factors, serine protease inhibitor antithrombin III, and amyloid β, explaining its multifaceted role in important biological events such as cell proliferation, anti-coagulation, cancer and Alzheimer's disease development.[2-3]

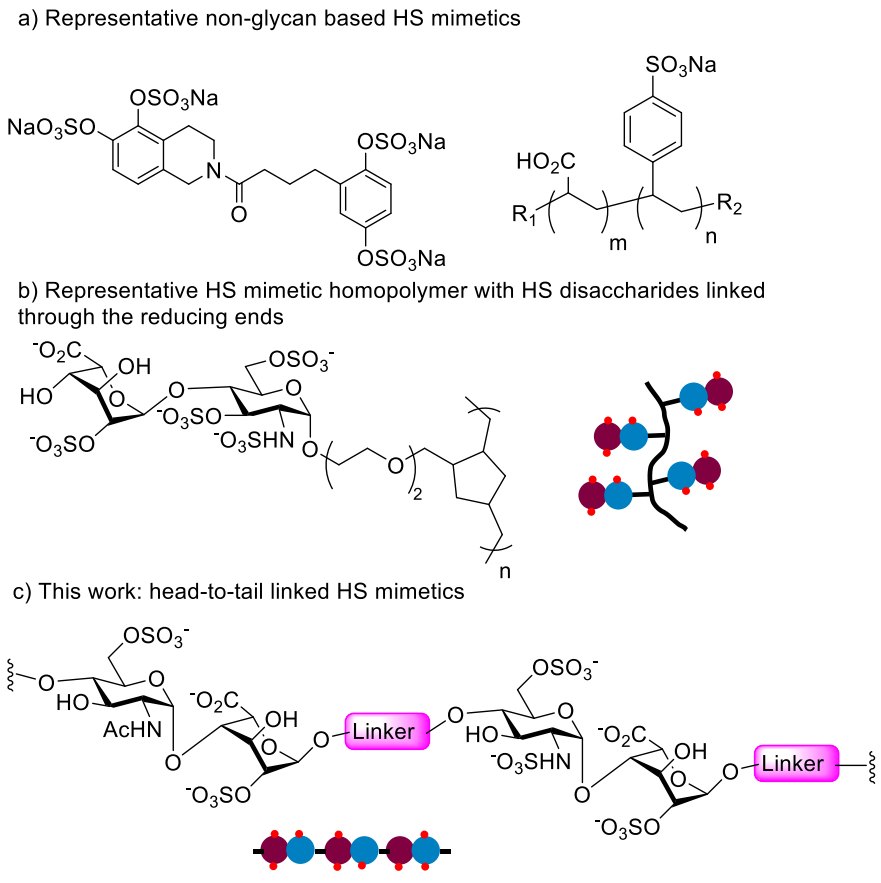
The establishment of detailed structure-activity relationship (SAR) of HS has been stymied by the complexity of HS structures. It is critical that diverse structures of HS can be readily accessed to better understand the SAR of HS.[4-6] While significant advances in HS synthesis have been made in the past two decades, with some of the targets prepared approaching the full length of HS polysaccharides,[7-8] the synthesis of HS oligosaccharides remains a challenging task.[9-11] Glycosylation reactions to form the backbone of HS often results in low yields and low stereoselectivities. Extensive efforts on synthetic method screening and optimization are often needed. It is also possible that the reaction conditions/protective groups established for the synthesis of shorter oligosaccharides fail in the formation of longer glycans.[12] In addition, with traditional synthetic designs more geared toward specific HS structures, it is tedious to generate a large number of oligosaccharides, as it is not straightforward to adjust the sulfation patterns of the oligosaccharide post glycan-assembly.

One strategy to address the challenges of accessing HS oligosaccharides is to prepare glycomimetics,[13-14] which include non-glycan-based HS mimics such as sulfated phenols and phenolic polymers (**Figure 1a**).[15-17] While they can be more easily prepared, it is challenging to closely mimic the structural subtlety of HS through this approach. As a parallel strategy, glycopolymers have been constructed with HS disaccharides connected through the reducing ends (**Figure 1b**).[18-20] Although these glycopolymers can be biologically active, the

disaccharides are linked through the reducing end only in these constructs form branches off the backbone, which are not representative of the linear structure of HS. In addition, it is difficult to precisely control the polymer sequence if more than one type of disaccharide monomer is used for polymerization to mimic the diverse sulfation patterns within a naturally occurring HS.

In this work, we report an alternative strategy for the design of HS like mimetics with HS disaccharides connected in a more native-like linear "head-to-tail" fashion with a module connected from its reducing end to the non-reducing end of a neighboring unit (**Figure 1c**). A significant advantage of this approach is that mimetics with diverse sulfation patterns can be readily prepared with precise control of the structures. A library of 27 HS pseudo-hexasaccharides was designed and synthesized using this method. The biological activities of these new mimetics were studied in fibroblast growth factor-2 (FGF-2) binding, which were shown to exhibit SAR similar to that of native HS hexasaccharides.

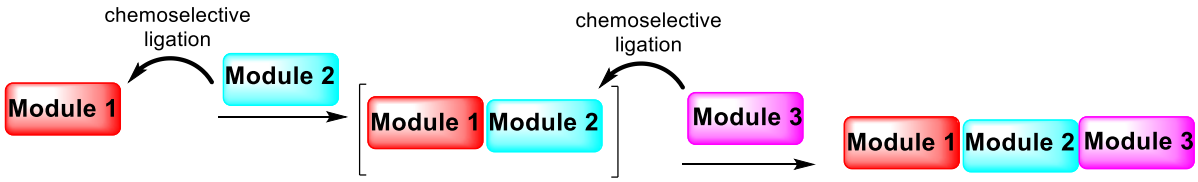
Figure 1. a) Representative non-glycan-based HS mimetics; b) Representative HS-based branched homopolymer with disaccharide units linked through the reducing ends; c) Schematic demonstration of head-to-tail linked linear HS mimetics reported in this work.



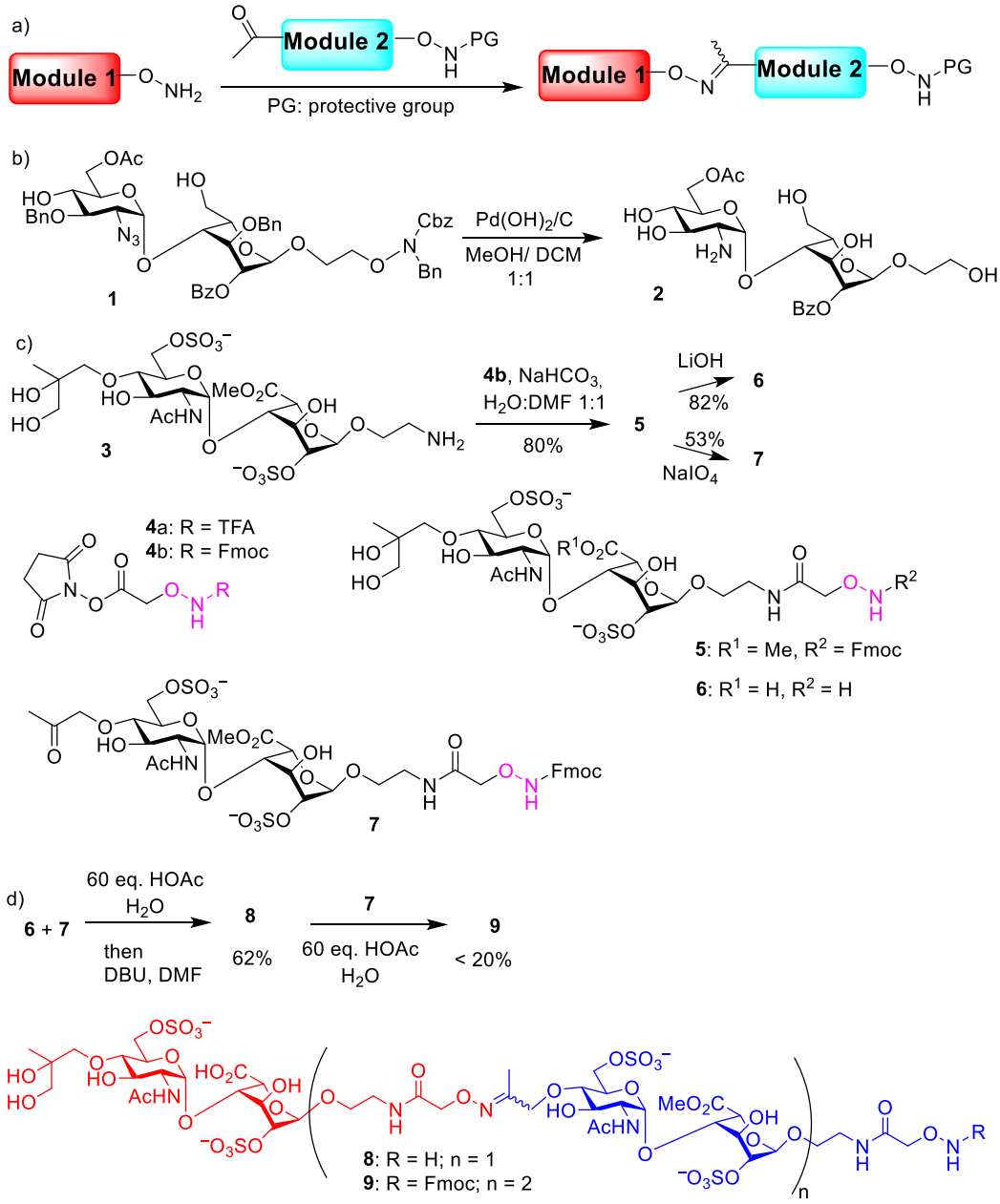
Results and Discussion

Synthetic design of the head-to-tail HS multimers and probing suitable ligation chemistry

Our HS mimetics library is based on iterative ligation of disaccharide modules in the head-to-tail fashion (**Scheme 1**). To accomplish this, we first explored oxime ligation between a disaccharide bearing an alkoxyamine at the reducing end with another disaccharide having a carbonyl functionalized linker at the non-reducing end (**Scheme 2a**). Oxime formation is a powerful method for chemoselective ligation due to the high nucleophilicity of the alkoxyamine, which has been applied for conjugation of biomolecules including carbohydrates in aqueous media.[21-24] Initially, we installed a protected alkoxyamine directly to the reducing end of the glycan (e.g., compound **1**). However, in preparation of the alkoxyamine containing module, we found that the alkoxyamine moiety underwent N-O cleavage under the Pd/C catalyzed hydrogenolysis reaction in methanol, which was typically utilized to remove benzyl protective groups (**Scheme 2b**). Performing the hydrogenolysis reaction with additives such as DMSO and triethylamine,[25] a condition reported for syntheses of hydroxylamine containing compounds, also led to significant O-N bond cleavage in our study.



Scheme 1. Schematic demonstration of iterative ligation to form the head-to-tail multimer HS mimetics.



Scheme 2. Attempts to form the head-to-tail multimers through oxime bond formation.

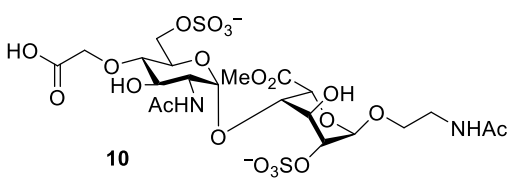
We next installed the alkoxyamine through the amidation reaction by coupling disaccharide **3** with a free amine at the reducing end to the *N*-hydroxysuccinimide (NHS) ester of alkoxyamine **4a** protected as a trifluoro-*N*-alkoxyacetamide (TFA) to overcome the N-O cleavage problem during hydrogenolysis. While the amidation went smoothly, surprisingly, the TFA group could not be removed in high yields through basic hydrolysis or by $NaBH_4$ reduction.[26] The major byproduct obtained was the free amine **3** as the newly formed amide was

presumably more labile than the TFA group. We explored the 9-fluorenylmethoxycarbonyl (Fmoc) as the protective group by conjugating Fmoc protected alkoxyamine **4b** with disaccharide **3** (**Scheme 2c**) facilitating the deprotection of the alkoxyamine. The Fmoc moiety in the resulting disaccharide product **5** was readily removed to afford the desired disaccharide **6**. The vicinal diol at the non-reducing end of disaccharide **5** was oxidized to ketone with sodium periodate yielding ketone **7**, which was coupled with **6** followed by Fmoc deprotection to give the oxime linked pseudo-tetrasaccharide **8** (**Scheme 2d**). However, chain elongation of **8** with the disaccharide ketone **7** led to a low yield (<20%) of the desired pseudo-hexasaccharide **9**. Instead, several pseudo-tetrasaccharides were isolated, which were formed due to scrambling of the oxime bond. We attempted to reduce the oxime in the pseudo-tetrasaccharide **8** to eliminate oxime reversibility. Neither $NaBH_4$ nor $NaCNBH_3$ reduction afforded the desired reduced alkoxyamine products.

Since the oxime formation presented a significant hurdle for pseudo-hexasaccharide synthesis, we next explored the alternative of ligating disaccharide amine **6** with the keto disaccharide **7** through reductive amination.[27] Unfortunately, under a variety of conditions including $NaBH_4$, $NaCNBH_3$ or $NaBH(OAc)_3$,[28] none of the desired pseudo-tetrasaccharides were obtained.

Design and synthesis of disaccharide modules with various sulfation patterns to form the head-to-tail HS mimetics through amide chemistry

The difficulties associated with oxime ligation prompted us to shift our focus to explore amide bonds to link the disaccharide modules. A glycolic acid moiety was installed first to the non-reducing end of the disaccharide module (**10**). However, when **10** was subjected to amidation with amine **3**, little desired pseudo-tetrasaccharide was obtained. Rather, the six-membered lactone **11** was formed through intramolecular cyclization as the major product under the amide coupling condition.



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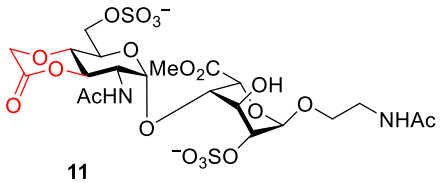

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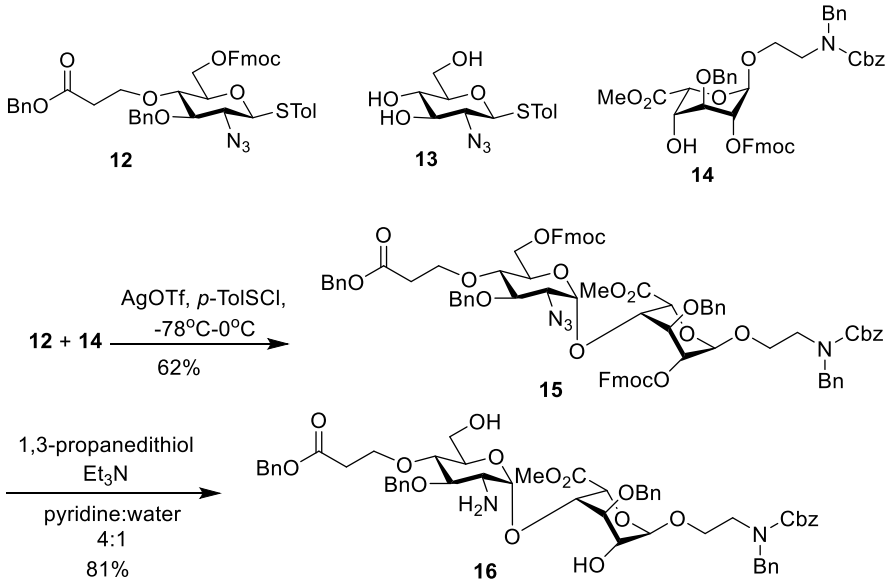
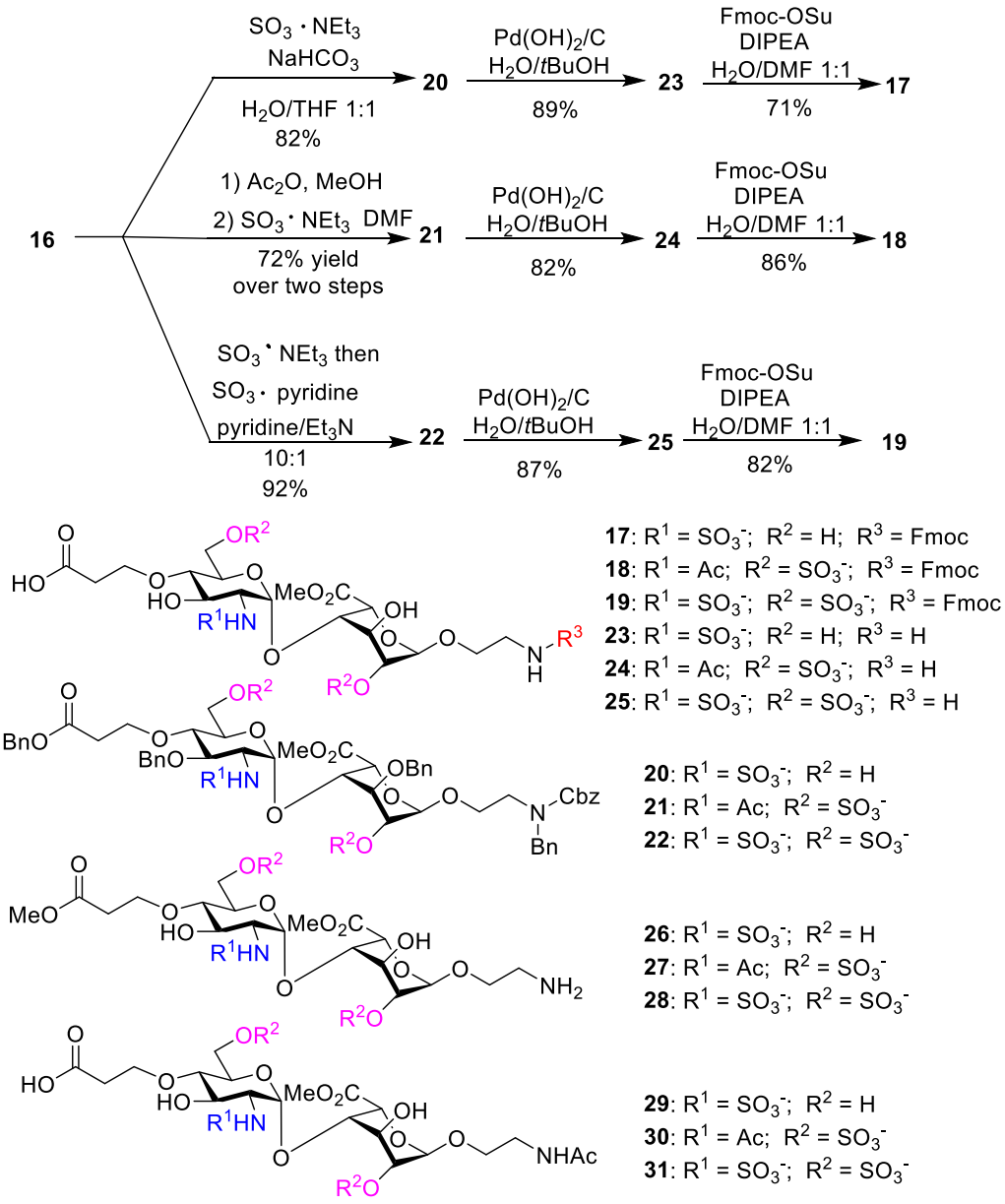
We envisioned that if the carboxylic acid linker was extended by one additional methylene unit, it would slow down the lactonization due to the formation of the seven-membered ring, and thus, favor the desired intermolecular amide coupling. Based on this consideration, glucosamine building block **12** was designed and synthesized from the glucosamine thioglycoside **13**[29] (**Scheme S1**). The glycosylation of IdoA acceptor **14** by donor **12** was carried out with the promoter of *p*-TolSCl and AgOTf[30] (**Scheme 3**). The presence of the non-participating azide group at C2 of GlcN donor **12** assisted the formation of the desired

1,2-*cis* disaccharide **15** ($^1J_{C1H1}$ = 173 Hz for the newly formed glycosidic linkage[31]). Treatment of **15** with 1,3-propanedithiol in Et_3N[32] furnished **16** with azide reduction and Fmoc removal in 81% yield.



Scheme 3. Synthesis of key disaccharide building block **16**.

With disaccharide **16** in hand, it was divergently modified to generate disaccharide modules **17**-**19** having diverse sulfation patterns (**Scheme 4**). *N*-sulfation of **16** was performed with $SO_3{\cdot}Et_3N$ in a water and THF cosolvent system leading to disaccharide **20**. The amine in **16** was converted to acetamide followed by *O*-sulfation using $SO_3{\cdot}Et_3N$ at 55 °C in DMF to produce disaccharide **21**. In parallel, $SO_3{\cdot}$pyridine complex in pyridine was tested to sulfate all *N*-, 6-*O* and 2-*O* positions of compound **16** in one pot. However, incomplete sulfation was observed even with extended reaction times and excess sulfation reagents. Two-step sulfation was performed next with *O*-sulfation using $SO_3{\cdot}Et_3N$ followed by *N*-sulfation using $SO_3{\cdot}$pyridine under basic conditions to furnish **22** in 92% yield. The Bn and Cbz protecting groups of **20**-**22** were removed by hydrogenolysis with Pd/C, followed by Fmoc protection[33] of the free amine affording compounds **17**-**19** (**Scheme 4**). Compounds **17**-**19**, having free carboxylic acids at their non-reducing ends and protected amines at their reducing ends, are bifunctional affording an elongation module for modular synthesis of the mimetics. The non-reducing end modules **26**-**28** were prepared by methylating the free carboxylic acids in **17**-**19** followed by Fmoc deprotection (**Scheme S2a**). Disaccharides **29**-**31** were prepared by acetylating the amine moieties of **23**-**25** and were used as reducing end modules (**Scheme S2b**).

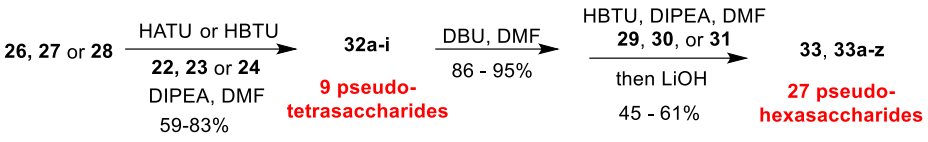


Scheme 4. Divergent syntheses of building blocks **17**-**19** from disaccharide **16**.

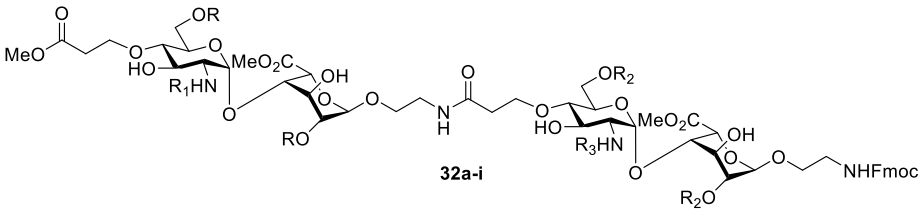
Synthesis of a library of 27 pseudo-hexasaccharide heparin mimetics

With three sets of disaccharide modules available having different sulfation patterns, including *N*-sulfation only, *O*-sulfation only and *N*- and *O*- sulfation, a library of HS mimetics could be rapidly built up. Amide coupling of the non-reducing end modules **26**-**28** with the elongation modules **17**-**19** produced 9 pseudo-tetrasaccharides (**32a-i**) in 59-83% yields (**Scheme 5**). These results suggested elongation of the carboxylic acid linker by one methylene moiety as

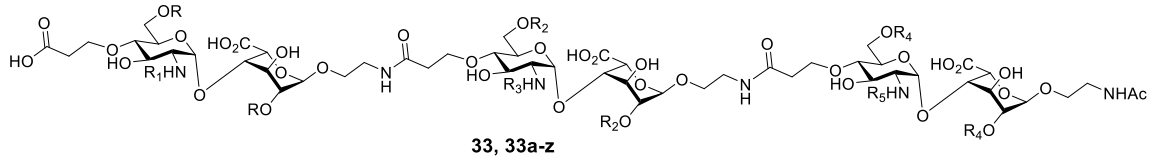
in **17-19** can effectively reduce the lactone formation following the activation of the carboxylic acid. The pseudo-tetrasaccharides (**32a-i**) were then treated with DBU to deprotect the *N*-Fmoc groups followed by a second round of amide coupling reactions with the reducing end disaccharide modules **29-31**, respectively. Saponification of the methyl esters afforded the fully deprotected 27 pseudo-hexasaccharides (**33, 33a-z**).



Scheme 5. Formation of a library of 27 HS like pseudo-hexasaccharides **33, 33a-z**.



32a: R, R_1, R_2, R_3 = SO_3^-
32b: R, R_1, R_3 = SO_3^-; R_2 = H
32c: R, R_1, R_2 = SO_3^-; R_3 = Ac
32d: R = H; R_1, R_2, R_3 = SO_3^-
32e: R, R_2 = H; R_1, R_3 = SO_3^-
32f: R = H; R_1, R_2 = SO_3^-; R_3 = Ac
32g: R, R_2, R_3 = SO_3^-; R_1 = Ac
32h: R, R_3 = SO_3^-; R_1 = Ac; R_2 = H
32i: R, R_2 = SO_3^-; R_1, R_3 = Ac



33: R, R_1, R_2, R_3 = SO_3^-; R_4, R_5 = SO_3^-
33a: R, R_1, R_2, R_3 = SO_3^-; R_4 = H; R_5 = SO_3^-
33b: R, R_1, R_2, R_3 = SO_3^-; R_4 = SO_3^-; R_5 = Ac
33c: R, R_1, R_3 = SO_3^-; R_2 = H; R_4, R_5 = SO_3^-
33d: R, R_1, R_3 = SO_3^-; R_2 = H; R_4 = H; R_5 = SO_3^-
33e: R, R_1, R_3 = SO_3^-; R_2 = H; R_4 = SO_3^-; R_5 = Ac
33f: R, R_1, R_2 = SO_3^-; R_3 = Ac; R_4, R_5 = SO_3^-
33g: R, R_1, R_2 = SO_3^-; R_3 = Ac; R_4 = H; R_5 = SO_3^-
33h: R, R_1, R_2 = SO_3^-; R_3 = Ac; R_4 = SO_3^-; R_5 = Ac
33i: R = H; R_1, R_2, R_3 = SO_3^-; R_4, R_5 = SO_3^-
33j: R = H; R_1, R_2, R_3 = SO_3^-; R_4 = H; R_5 = SO_3^-
33k: R = H; R_1, R_2, R_3 = SO_3^-; R_4 = SO_3^-; R_5 = Ac
33l: R, R_2 = H; R_1, R_3 = SO_3^-; R_4, R_5 = SO_3^-
33m: R, R_2 = H; R_1, R_3 = SO_3^-; R_4 = H; R_5 = SO_3^-
33n: R, R_2 = H; R_1, R_3 = SO_3^-; R_4 = SO_3^-; R_5 = Ac
33o: R = H; R_1, R_2 = SO_3^-; R_3 = Ac; R_4, R_5 = SO_3^-
33p: R = H; R_1, R_2 = SO_3^-; R_3 = Ac; R_4 = H; R_5 = SO_3^-
33q: R = H; R_1, R_2 = SO_3^-; R_3 = Ac; R_4 = SO_3^-; R_5 = Ac
33r: R, R_2, R_3 = SO_3^-; R_1 = Ac; R_4, R_5 = SO_3^-
33s: R, R_2, R_3 = SO_3^-; R_1 = Ac; R_4 = H; R_5 = SO_3^-
33t: R, R_2, R_3 = SO_3^-; R_1 = Ac; R_4 = SO_3^-; R_5 = Ac
33u: R, R_3 = SO_3^-; R_1 = Ac; R_2 = H; R_4, R_5 = SO_3^-
33v: R, R_3 = SO_3^-; R_1 = Ac; R_2 = H; R_4 = H; R_5 = SO_3^-
33w: R, R_3 = SO_3^-; R_1 = Ac; R_2 = H; R_4 = SO_3^-; R_5 = Ac
33x: R, R_2 = SO_3^-; R_1, R_3 = Ac; R_4, R_5 = SO_3^-
33y: R, R_2 = SO_3^-; R_1, R_3 = Ac; R_4 = H; R_5 = SO_3^-
33z: R, R_2 = SO_3^-; R_1, R_3 = Ac; R_4 = SO_3^-; R_5 = Ac

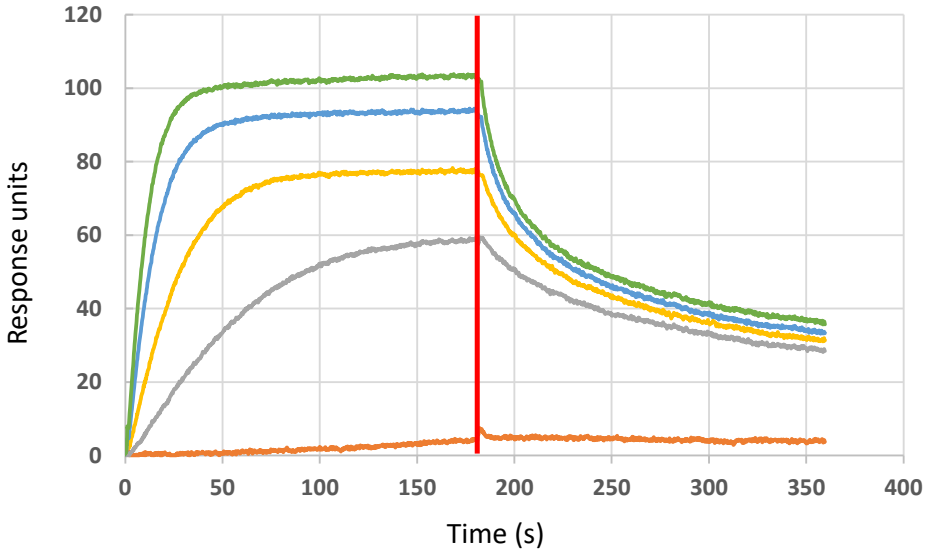
Binding studies on pseudo-hexasaccharides and fibroblast growth factor-2 (FGF-2) using surface plasmon resonance (SPR)

With the successful preparation of a library of HS-like pseudo-hexasaccharides, we explored whether these pseudo-hexasaccharides could mimic natural HS glycans. We examined binding with FGF-2, which is essential for normal physiology and cancer biology.[34] FGF-2 is one of the most extensively studied heparin-binding proteins, which can mediate cell growth, differentiation, survival, and patterning.[35]

The direct binding of FGF-2 to the HS mimetics was measured by surface plasmon resonance (SPR). FGF-2 was immobilized on a carboxymethyl dextran high capacity (CDH) sensor chip surface through carbodiimide mediated coupling. *O*-/*N*-Sulfated (**33**), *O*-sulfated (**33z**) and *N*-sulfated (**33m**) pseudo-hexasaccharides were selected as analytes in addition to the heparin polysaccharide (MW: 17.2 kDa). The dissociation constant (K_D) of heparin binding to FGF-2 was measured to be 6.8 nM, which is comparable with the reported value.[36] The *O*-/*N*-sulfated **33** exhibited strong binding to FGF-2 with a K_D value of 19.6 nM (**Figure 2**). In comparison, *O*-sulfated **33z** had a weaker affinity than **33** (K_D = 940 nM) (**Figure S1a**), while **33m** bearing only *N*-sulfate was the weakest binder with little binding at 3 μM (**Figure S1b**). Seeberger and coworkers studied the binding of HS hexasaccharides with FGF-2 through a powerful glycan microarray technology.[37] The *O*-/*N*- sulfated hexasaccharide **34** exhibited ~60% binding to FGF-2 at the glycan concentration of 16 μM as compared to heparin polysaccharide. The *N*-acetylated, *O*-sulfated hexasaccharide **35** gave binding of ~20% of that of heparin, while the hexasaccharide **36** having three *N*-sulfates only gave the lowest signal (< 2%) binding to FGF-2. The relative trend of decreasing FGF-2 affinity from *N*- and *O*- sulfated, to *O*-sulfated only, to *N*-sulfated only sequences of HS hexasaccharides (**34**, **35**, **36**) correlates well with those of the pseudo-hexasaccharides **33**, **33z**, and **33m**. In addition, the pseudo-hexasaccharide **33** exhibits much stronger FGF-2 binding affinity than HS disaccharide **37** (K_D = 17.5 μM) and tetrasaccharide **38** (K_D = 1.2 μM) bearing similar sulfation patterns.[4-5]

A competition assay was performed to establish the SAR between the full panel of 27 HS-like pseudo-hexasaccharides and FGF-2 (**Figure 3**). Biotinylated heparin was immobilized on the biosensor, and the pseudo-hexasaccharides (10 μM) were mixed with FGF-2 (50 nM) individually. As expected, the fully sulfated pseudo-hexasaccharide (**33**) showed the most potent inhibition of FGF-2 binding to immobilized heparin. Among 27 pseudo-hexasaccharides, the best inhibitors (**33, 33a, 33b, 33i, 33r**), which had 50-65% inhibition of binding between FGF-2 and heparin at 10 μM concentration, contain successive disaccharide modules with *N*- and *O*-sulfations. The importance of *O*- and *N*-sulfates is consistent with literature reports on HS-FGF-2 interactions obtained using HS oligosaccharides with native glycosidic linkages.[5, 37-39]

Figure 2. The SPR sensorgram of FGF-2 binding with compound **33**. The concentrations of **33** from top to bottom curves were 750 nM, 500 nM, 250 nM, 100 nM, 10 nM, and 1 nM. Each experiment was repeated at least three times with the representative data shown. The red vertical line indicates the time the dissociation process started.



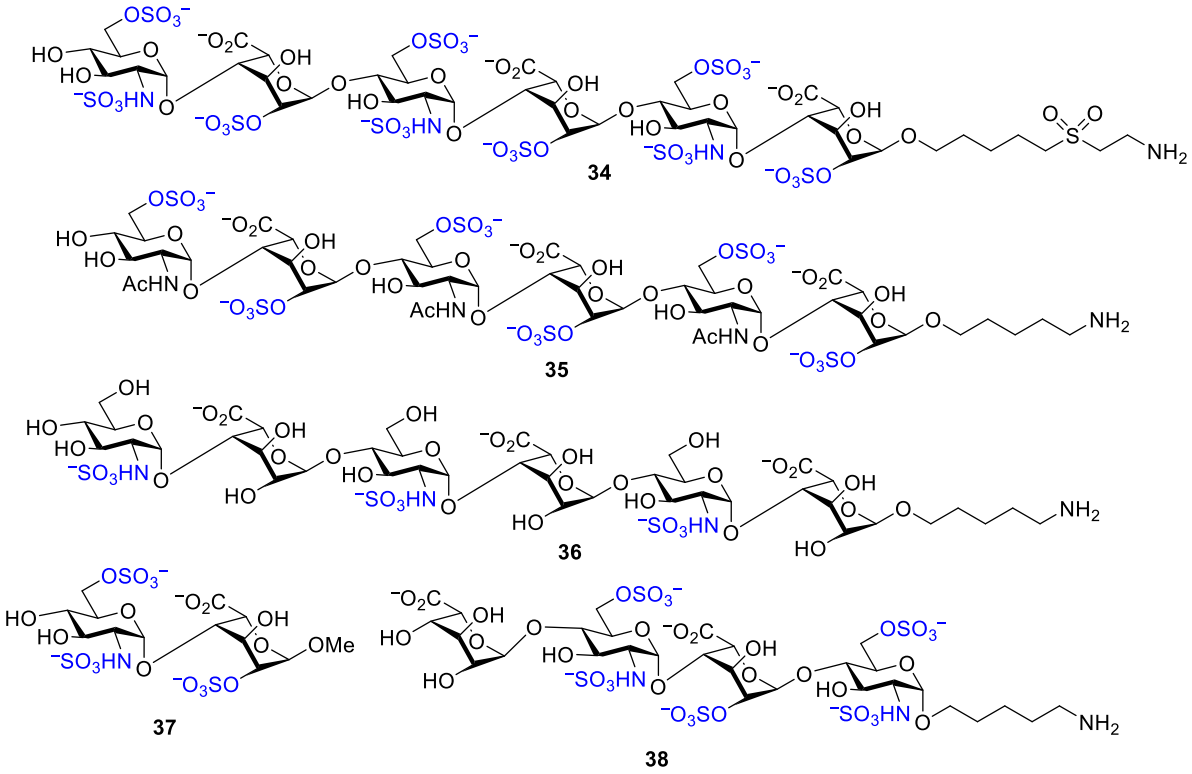
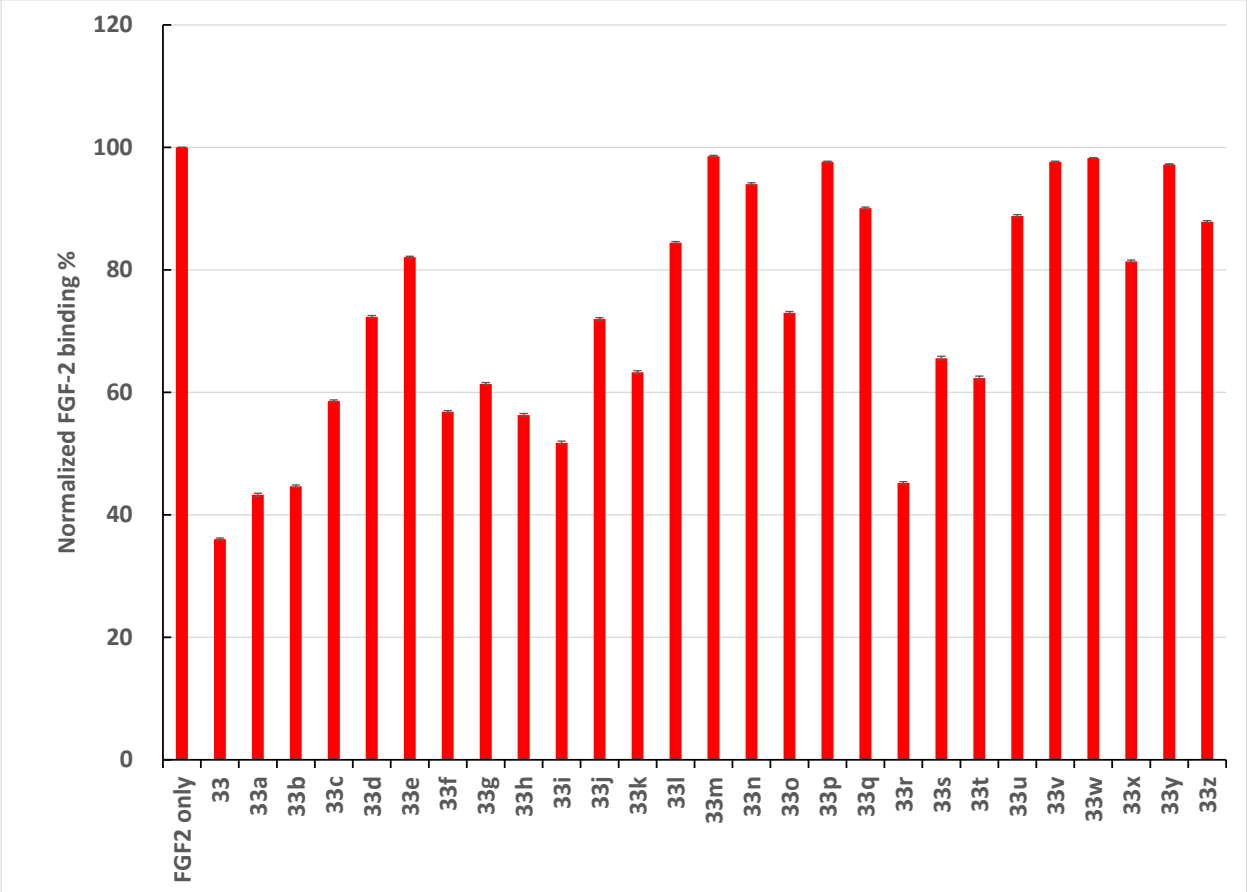


Figure 3. Inhibition of 27 pseudo-hexasaccharides (**33**, **33a-z**) on FGF-2 interaction with heparin through a competition SPR assay. Biotinylated heparin was immobilized on the SPR biosensor. For the control well, FGF-2 (50 nM) was flown over the sensor and the intensity of the signal due to FGF-2 binding was set as a reference (100%). The pseudo-hexasaccharides (1 μM) were mixed with FGF-2 (50 nM) individually, and each solution was flown over the sensor respectively. Pseudo-hexasaccharide capable of binding with FGF-2 would compete with heparin for FGF-2 and reduce the signal intensity. Normalized FGF-2 binding % was calculated based on the following formula: (signal intensity of FGF-2/pseudohexasaccharide)/(signal intensity of FGF-2 only) x 100%. Each binding experiment was performed three times with the standard deviations (all < 0.4%) shown.



Conclusions

The availability of well-defined HS structures is critical to aid in the understanding of their important biological functions with oligosaccharides longer than disaccharides often needed to better recapitulate the biological activities of HS. Synthesis of HS is challenging and time-consuming, especially for glycosylation and sulfation reactions on longer sequences. We

prepared a new class of HS mimetics with HS disaccharide modules linked in a more native-like "head-to-tail" linear configuration to expedite biological studies of HS. Among many potential chemoselective ligation methods, amide chemistry was the most effective, leading to the facile formation of 27 pseudo-hexasaccharide HS mimetics bearing precisely controlled and extensively varied sulfation patterns. The deprotection and sulfation reactions were applied on disaccharide modules instead of on longer oligosaccharides, significantly reducing the complexity of the synthetic operations. Binding experiments with FGF-2 showed the synthetic pseudo-hexasaccharides could mimic the native HS hexasaccharides, and compounds with successive *N*-/*O*- sulfated disaccharide modules exhibited the most potent binding to FGF-2.

It should be pointed out that in parallel to our work, Niu and coworkers recently submitted the synthesis of head to tail HS mimetics through the alkyne azide [3+2] cycloaddition reaction.[40] Sulfated monosaccharide serine conjugate has been utilized to form glyco-amino acid oligomers to mimic HS with only homo-oligomers prepared without varying sulfation patterns within the molecule.[41] These reports combined with our work suggest linear HS mimetics strategies provide an exciting approach to expedite the synthesis of HS-like compounds and faciliate the understanding of the fascinating biological functions of HS.

Competing Interests

JL is a founder and chief scientific officer for Glycan Therapeutics. He has equity of the company and serves as a paid consultant.

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