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Preactivation-Based, One-Pot Combinatorial Synthesis of Heparin-like Hexasaccharides for the Analysis of Heparin–Protein Interactions

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Abstract: Heparin (HP) and heparan sulfate (HS) play important roles in many biological events. Increasing evidence has shown that the biological functions of HP and HS can be critically dependent upon their precise structures, including the position of the iduronic acids and sulfation patterns. However, unraveling the HP code has been extremely challenging due to the enormous structural variations. To overcome this hurdle, we investigated the possibility of assembling a library of HP/HS oligosaccharides using a preactivation-based, one-pot glycosylation method. A major challenge in HP/HS oligosaccharide synthesis is stereoselectivity in the formation of the *cis*-1,4-linkages between glucosamine and the uronic acid. Through screening, suitable protective groups were identified on the matching glycosyl donor and acceptor, leading to stereospecific formation of both the *cis*-1,4- and *trans*-1,4-linkages present in HP. The protective group chemistry designed was also very flexible. From two advanced thioglycosyl disaccharide intermediates, all of the required disaccharide modules for library preparation could be generated in a divergent manner, which greatly simplified building-block preparation. Furthermore, the reactivity-independent nature of the preactivation-based, one-pot approach enabled us to mix the building blocks. This allowed rapid assembly of twelve HP/HS hexasaccharides with systematically varied and precisely controlled backbone structures in a combinatorial fashion. The speed and the high yields achieved in glycoassembly without the need to use a large excess of building blocks highlighted the advantages of our approach, which can be of general use to facilitate the study of HP/HS biology. As a proof of principle, this panel of hexasaccharides was used to probe the effect of backbone sequence on binding with the fibroblast growth factor-2 (FGF-2). A trisaccharide sequence of 2-O-sulfated iduronic acid flanked by N-sulfated glucosamines was identified to be the minimum binding motif and N-sulfation was found to be critical. This provides useful information for further development of more potent compounds towards FGF-2 binding, which can have potential applications in wound healing and anticancer therapy.

Keywords: carbohydrates · combinatorial chemistry · growth factors · heparin · oligosaccharides

Introduction

Heparin (HP) and heparan sulfate (HS), a member of the glycosaminoglycan (GAG) family, are structurally related linear polyanionic polysaccharides.[1] The backbones of HP and HS are composed of disaccharide subunits with D-glucosamine (GlcN) and uronic acid (either L-iduronic (IdoA) or D-glucuronic acid (GlcA)) joined by alternating α-1,4 and β-1,4 linkages. The variation in uronic acid structures, the modification of the amino group in GlcN (N-acetylation and sulfation) and differential sulfations of hydroxyl groups (2-O of the uronic acid, 3-O and 6-O of the GlcN) lead to tremendous structural diversity in naturally existing HP/HS.

Through interactions with a large number of polypeptides and proteins,[1–3] HP/HS play crucial roles in numerous physiological processes such as viral infection, blood coagulation, inflammatory response, cell adhesion, cell growth regulation, and tumor metastasis.[1,4–9] The binding between HP/HS and their receptors can be critically dependent on the saccharide structures, as exemplified by the high-affinity pentasacchar-

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ide sequence for antithrombin III (ATIII) interaction,[10,11] with the removal of a single 3-O-sulfate in the sequence reducing its activity by about 20000-fold. This knowledge led to the development of Arixtra, a fully synthetic HP/HS pentasaccharide, which is approved for the treatment of deep vein thrombosis.[10,12] However, despite this success, in many cases, it has been difficult to unravel the biological activities encoded in the HP/HS structures, since it is extremely challenging to obtain homogeneous HP/HS oligosaccharides from natural sources. Furthermore, pharmaceutical HP is isolated from pig and bovine organs, which has the potential risks of pathogen contamination and adulteration.[13,14] Synthesis, therefore, provides a powerful means to access these complex molecules for the establishment of detailed structure–activity relationships and analysis of HP/HS–protein interactions.[10,15–17]

Nature synthesizes HP and HS by modifying a homopolymer of heparosan GlcNAc-α-1,4-GlcA through the actions of many enzymes, including N-deacetylase/N-sulfotransferase, C_5-epimerase, and O-sulfotransferases.[9,18] Most of these reactions are incomplete, thus explaining the structural heterogeneity of native HP/HS. Attempts to harness the power of enzymes for HP/HS synthesis have been reported.[19–24] However, the substrate specificities of most of these enzymes are not yet well characterized, which hinders the application of enzymatic approaches to the assembly of a diverse array of HP/HS oligosaccharides.

As an alternative to using enzymes, chemical syntheses of HP/HS oligosaccharides have been actively pursued,[15–17] which can provide compounds with any sequences and sulfation patterns as well as unnatural analogues. However, there are several challenges associated with chemical synthesis: 1) with the rich functionalities in the HP/HS oligosaccharides, selection of appropriate protective groups is crucial for backbone extension and oligosaccharide functionalization. Even groups distant from the reactive sites can have a profound effect on the outcome of glycosylation reactions.[25] 2) Stereochemical control is crucial for HP/HS oligosaccharide synthesis, especially in the formation of the *cis*-1,4-linkage between GlcN and uronic acid. Although, in general, the *cis* linkage is preferred due to the anomeric effect, the stereoselectivity can vary drastically with the building-block structures as well as reaction conditions[26–29] and the separation of anomers can be tedious and at times impossible.[28] Thus, it is highly desirable that conditions can be established for stereospecific formation of the *cis*-1,4-linkage.

The aforementioned challenges notwithstanding, the groups of van Boeckel, Petitou, and Sinaÿ accomplished the first total synthesis of the ATIII binding HP pentasaccharide.[30–32] This landmark achievement required about 60 synthetic steps, which served to highlight the power of synthetic carbohydrate chemistry for saccharide-based drug development.[10] To minimize the number of steps required for protective group manipulation and aglycon leaving group adjustment on intermediate oligosaccharides, strategies such as anomeric-reactivity-based, one-pot synthesis[33] and selective activation[34] have been applied for the synthesis of the ATIII-binding pentasaccharide. Several other targeted syntheses of HP/HS pentasaccharides or longer have also been accomplished.[29,35–46] Despite these significant progresses in the field, the target-oriented approaches required a new total synthesis for each HP/HS structure. This is not efficient for structure–activity relationship (SAR) studies, in which a number of HP/HS structures would be required to decipher the HP code.

For SAR studies, a diversity-oriented combinatorial approach needs to be developed to access a wide range of HP/HS structures. This can be accomplished by using modular building blocks, in which a set of properly protected building blocks can be converted to multiple glycosyl donors and acceptors.[29,34,43,47,48] Recently, this strategy has been applied to the preparation of HP/HS tetrasaccharides, which were subsequently used to elucidate structural requirements for inhibition of β-secretase involved in Alzheimer's disease development.[35] To apply the modular strategy to higher oligosaccharides, the availability of robust glycosylation chemistry is crucial, which must give high yields in reactions of a variety of building blocks without the need for time-consuming individual optimization.

Herein, we report the establishment of a modular assembly strategy for a panel of HP/HS hexasaccharides using the preactivation-based, one-pot method.[49–53] This method granted much more freedom in protective group selection, enabling us to achieve high-yielding stereospecific glycosylations. Furthermore, the integration of several glycosylation reactions into a single synthetic operation (one-pot) significantly expedited the glyco-assembly processes, allowing the rapid construction of the hexasaccharide library in a combinatorial fashion. The availability of these HP/HS hexasaccharides with well-defined sulfation patterns and systematically varied backbone structures facilitated the SAR studies towards binding with the fibroblast growth factor-2 (FGF-2), an important protein involved in angiogenesis, cell proliferation, and embryonic development.[54,55]

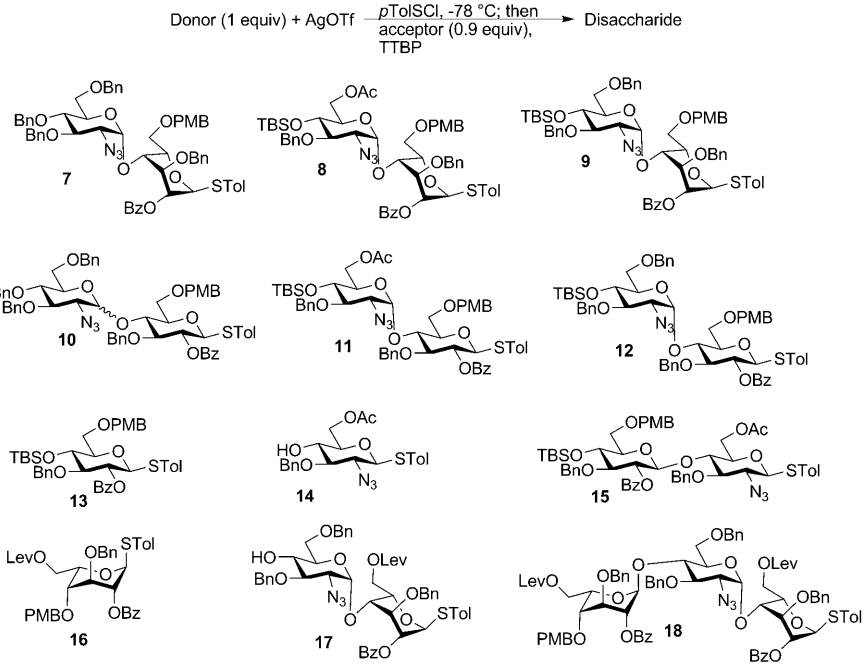
Results and Discussion

Building-block design: There are two general routes to synthesize HP/HS oligosaccharides through uronic acids or the corresponding hexose building blocks. Although uronic acid donors have been used directly in GAG synthesis,[34,56,57] the presence of electron-withdrawing carboxylate moieties may lower glycosylation yields.[58] Furthermore, the possibility of epimerization limits the synthetic operations that can be performed on these compounds. Therefore, we decided to explore glucoside and idoside as building blocks, which would be converted to the corresponding uronic acids after oligosaccharide backbone formation.

Instead of using the popular trichloroacetimidate donors,[35,38,42] we chose thioglycosyl building blocks because they give superior stability in storage and yet are readily activated with thiophilic promoters.[59] We designed glucoside **1** and idoside **2**, containing benzyl (Bn), *p*-methoxybenzyl

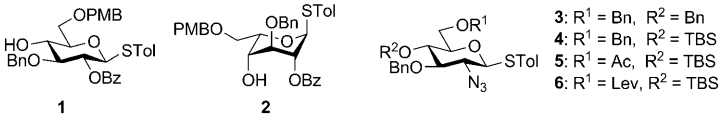
(PMB), and benzoyl (Bz) protecting groups, as suitable building blocks. The 2-*O*-Bz group can direct the formation of 1,2-*trans*-glycosyl linkage through neighboring group participation and be selectively removed to expose the hydroxyl group for future sulfation. The PMB group on the 6-O position can be transformed for late-stage oxidation-state adjustments. For the GlcN donors, we examined the azido moiety as a nonparticipatory group (donors **3**–**6**). Previously, Kerns and co-workers have discovered that the use of an oxazolidinone moiety to simultaneously protect the C2-nitrogen and C3-OH of a GlcN donor led to the exclusive formation of α-glycoside.[60] However, later studies showed that the oxazolidinone could cause side reactions due to its nucleophilicity, thus limiting its wide usage in HP/HS oligosaccharide preparation.[26,61] Instead, the azido group can not only facilitate the formation of α-linkages,[62] but also be selectively reduced to amines in the presence of other protective groups for subsequent N-sulfation or N-acetylation. However, the stereochemical outcome of using donors with the azido group is dependent upon donor and acceptor structures as well as reaction conditions.[26–28] Thus suitable protective group chemistry needs to be established for the construction of the *cis*-1,4-linkages prior to oligosaccharide synthesis.

Table 1. Evaluation of glycosyl donors.[a]



Entry	Donor	Acceptor	Disaccharide	Yield [%]
1	**3**	**2**	**7**	84 (α only)
2	**5**	**2**	**8**	84 (α only)
3	**4**	**2**	**9**	79 (α only)
4	**3**	**1**	**10**	73 (α/β = 2.5:1)
5	**5**	**1**	**11**	89 (α only)
6	**4**	**1**	**12**	75 (α only)
7	**13**	**14**	**15**	53
8	**16**	**17**	**18**	64

[a] TTBP = 2,4,6-tri-*tert*butyl pyrimidine, *p*TolSCl = *p*-toluenesulfenyl chloride.



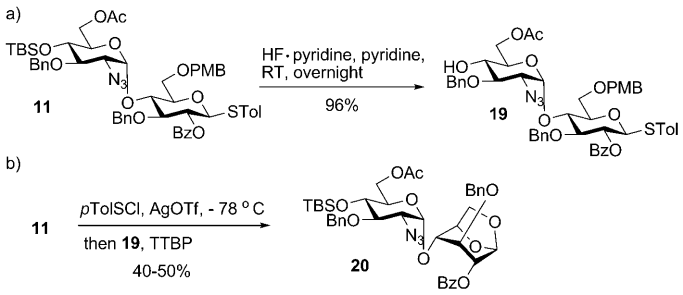
Evaluation of building blocks for disaccharide formation: The formation of a *cis*-1,4-linkage using GlcN donors was evaluated first (Table 1). The donor **3** was preactivated[53,63] in the absence of any acceptors with the powerful promoter *p*TolSOTf (1 equiv), which was formed in situ by reaction of *p*TolSCl with AgOTf at −78 °C. Upon the completion of donor activation, as determined by TLC analysis, the glycosyl acceptor **2** was added together with a sterically hindered base, TTBP,[64] leading to the formation of disaccharide product **7** in an excellent 84% yield with no β-linked *trans*-disaccharide isolated (Table 1, entry 1). The newly formed α-1,4-linkage was confirmed by NMR spectroscopy analysis (^1J(C1′,H1′) = 172.1 Hz).[65] Examination of GlcN donors with different protective groups (**4** and **5**) demonstrated that this chemistry was robust because they all gave complete *cis* selectivities (Table 1, entries 2 and 3).

It has been reported that acceptors with the gluco-configuration tend to produce stereoisomeric mixtures in reactions with 2-azido GlcN donors.[29] Indeed, the reaction of the tribenzylated GlcN **3** with glucoside acceptor **1** led to the formation of both α and β anomers of disaccharide **10** (α/β ≈ 2.5:1) (Table 1, entry 4). The differential stereochemical outcome of using donor **3** to glycosylate gluco-configured acceptor **1** versus the idoside acceptor **2** could be attributed to double stereodifferentiation.[66] Seeberger and co-workers developed a clever approach that by locking the glucoside acceptor into the $^1C_4$ conformation, thus converting the 4-OH into the idose-like axial position, high α selectivity was obtained.[29] However, several additional synthetic steps were necessary to modify the disaccharide products with suitable protective groups and aglycon leaving groups for further chain elongation. Instead of changing the glycosyl acceptor, we explored the effect of donor protective groups

on stereoselectivities. Interestingly, GlcN donor **5** led to the exclusive formation of the *cis*-linked disaccharide (^1J-(C1′,H1′) = 173.9 Hz) with no corresponding *trans*-glycoside isolated (Table 1, entry 5). The levulinoyl acid (Lev)-protected donor **6** also gave exclusively α anomer, but the glycosylation yield was lower (data not shown). With the 6-OAc moiety on donor **5**, it is possible that the acetate might facilitate the formation of the *cis*-glycosidic linkage through the remote neighboring group participation by the ester carbonyl moiety.[67,68] To test this possibility, the 6-OAc was substituted with 6-OBn (donor **4**). The reaction of donor **4** with acceptor **1** maintained the exclusive *cis* selectivity with good yield (Table 1, entry 6), thus demonstrating 6-OAc was not responsible for the observed high stereoselectivity. Based on these observations, the *tert*-butyldimethyl silyl (TBS) group is not only useful as a temporary protective group, masking the 4-OH of GlcN for backbone elongation, but also plays an important role for facilitating the stereospecific formation of the *cis*-1,4 glycosidic bond. Furthermore, the possibility of having either Bn or Ac on the 6-O of GlcN without affecting stereoselectivities bestows great flexibility for installing 6-O-sulfation in the future.

Next, we examined the formation of the *trans*-1,4-linkage using the hexose donors. The glycosylation of glucoside donor **13** with acceptor **14** led to the formation of disaccharide product **15** in 53% yield with some acceptor (40%) recovered (Table 1, entry 7). In a similar manner, idose donor **16** was preactivated and reacted with disaccharide **17**, producing trisaccharide **18** in 64% yield (Table 1, entry 8). Donor **16** was obtained as a side product during preparation of idoside **2**, thus making full use of all available building blocks.

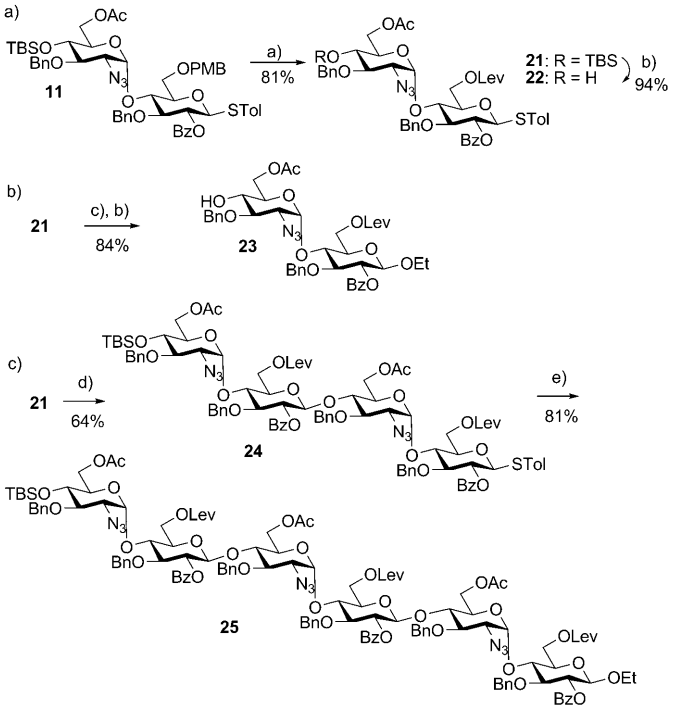
Stepwise and one-pot synthesis of HP/HS hexasaccharides: With the successful establishment of stereospecificity in disaccharide formation, we moved on to oligosaccharide assembly. In the absence of neighboring group participation, the size of the glycosylating agent and the nature of the nucleophile can have unpredictable effects on the stereo-selectivity.[28,50,69] For example, although a 2-azido GlcN donor reacted with an IdoA monosaccharide acceptor to form a *cis*-1,4-linked HP trisaccharide exclusively, reaction between a tetrasaccharide donor and disaccharide acceptor with similar protective groups led to an inseparable anomeric mixture of hexasaccharides.[28] Therefore, to avoid this potential complication in stereochemistry, for oligosaccharide chain extensions, we chose disaccharide donors (e.g., **9** and **11**) with the idose or glucose at the reducing end and participating acyl groups at the 2-O-positions. The TBS group in **11** was removed to generate acceptor disaccharide **19** (Scheme 1a). Interestingly, although donor **13** successfully reacted with acceptor **14**, the glycosylation of disaccharide **11** by **19** failed to yield the desired tetrasaccharide. Instead, the major side product isolated was the anhydro compound **20** (40–50% yield) (Scheme 1b). This side product is presumably formed through the participation of electron-rich 6-OPMB to stabilize the oxa-carbenium ion followed by cleavage of the



Scheme 1. Synthesis of disaccharide acceptor **19** and evaluation of tetrasaccharide formation.

PMB moiety.[52] The differential outcome in glycosylations with monosaccharide versus disaccharide building blocks despite similar protective groups used is probably due to the lower reactivities of the disaccharides resulting from the presence of additional glycosyl units.[70] The reduction in glycosylation rate could favor the competing side reaction from remote participation of the OPMB group.

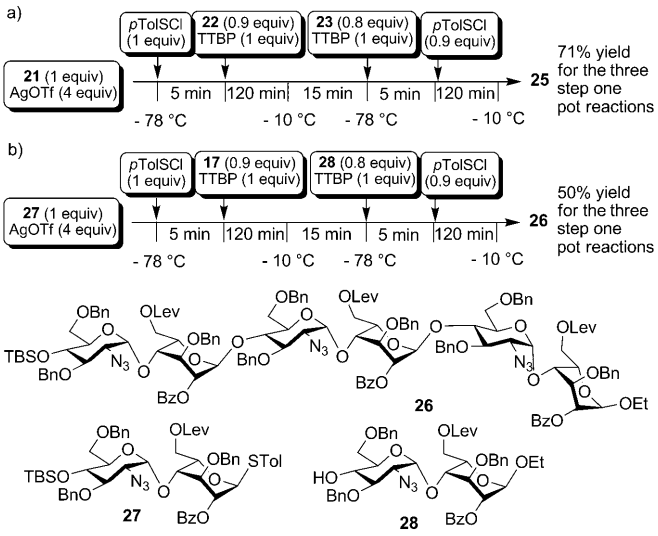
To overcome this difficulty, the 6-OPMB group in disaccharide **11** was replaced with OLev (donor **21**), from which two disaccharide acceptors **22** and **23** were generated in high yields (Scheme 2a, b). Gratifyingly, glycosylation of **22** by **21** proceeded smoothly to afford the desired tetrasaccharide **24**



Scheme 2. Synthesis of disaccharide building blocks **21**–**23** and hexasaccharide **25**. Reagents and conditions: a) 2,3-dichloro-5,6-dicyano *p*-benzoquinone (DDQ), CH_2Cl_2/H_2O, RT, 3 h; LevOH, *N*′-(3-diemthylaminopropyl)-*N*-ethylcarbodiimide (EDC), 4-dimethylaminopyridine (DMAP), CH_2Cl_2, RT, 2 h; b) HF in pyridine, pyridine, RT, overnight; c) *p*TolSCl/AgOTf, CH_2Cl_2/Et_2O, EtOH, −78 to −10 °C; d) *p*TolSCl/AgOTf, CH_2Cl_2/Et_2O, then **22**, −78 to −10 °C; e) *p*TolSCl/AgOTf, **23**, CH_2Cl_2/Et_2O, −78 to −10 °C.

in 64% isolated yield with exclusive β selectivity (Scheme 2c). Subsequent reaction of **24** with disaccharide **23** produced hexasaccharide **25** in 81% yield. The stereochemistry of **25** was confirmed by NMR spectroscopy[65] and MS analysis with three α-glycosyl linkages (^1J(C1,H1) = 174.9, 173.9, 173.9 Hz) and three β-linkages (^1J(C1,H1) = 161.9, 161.9, 159.6 Hz).

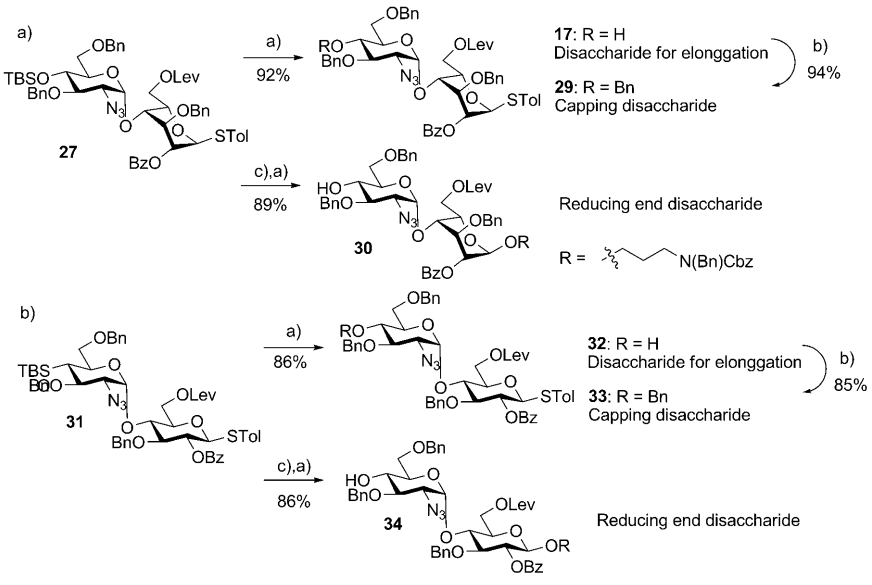
Encouraged by the results of stepwise glycosylations, we performed the one-pot assembly of **25** (Scheme 3a).[59] Preactivation of the disaccharide donor **21** (1 equiv) at −78 °C with *p*TolSCl/AgOTf was followed by the addition of the first disaccharide acceptor **22** (0.9 equiv). The reaction temperature was raised to −10 °C to expedite glycosylation and the acceptor was **22** completely consumed in 2 h based on TLC analysis. The reaction temperature was cooled back to −78 °C, followed by addition of the second disaccharide acceptor **23** (0.8 equiv) and *p*TolSCl/AgOTf. The fully protected hexasaccharide **25** was obtained in 71% yield in one pot in 5 h and its structure was identical with that from the stepwise reactions. The higher yield from the one-pot reaction compared with the stepwise synthesis was presumably due to less product loss during workup and purification. The one-pot reaction condition were general, which were extended without any optimization to the one-pot assembly of hexasaccharide **26** by sequential glycosylations of three idose-containing disaccharides **27**, **17**, and **28** in 50% yield (Scheme 3b). The presence of six α-linkages (^1J(C1,H1) = 172.1 (×3), 171.6, 171.1 Hz (×2)) unequivocally confirmed the stereochemistry of compound **26**. The TBS moieties at the nonreducing end of **25** and **26** can be selectively removed to generate hexasaccharide acceptors for future synthesis of longer oligosaccharides.



Scheme 3. One-pot synthesis of HP-like hexasaccharides **25** and **26**.

The speed and high overall yield using only close to stoichiometric amounts of building blocks in the assembly of hexasaccharides **25** and **26** highlighted the advantages of our approach. In addition, the building blocks used have the same protective group patterns, thus possessing similar anomeric reactivities.[70,71] For the popular reactivity-based armed–disarmed strategy, time-consuming protective group manipulations of these building blocks would be required to render the glycosyl donor much more reactive than the acceptor for selective donor activation.[70–72] As the preactivation-based protocol can be performed independent of the anomeric reactivities, it allowed us to use the building blocks directly without the need to fine-tune their anomeric reactivities, thus simplifying building-block preparation. This will be particularly advantageous in oligosaccharide library preparation, in which a large number of structures need to be prepared.

One-pot modular synthesis of an HP-like hexasaccharide library for studying HP/HS FGF-2 interactions: The fibroblast growth factors (FGFs) are involved in many important developmental and physiological processes.[54,55] FGFs bind with endogenous HP/HS, which is essential for high-affinity interactions with the membrane-bound FGF receptors sub-



Scheme 4. Divergent functionalization of disaccharide building blocks from common intermediates. Reagents and conditions: a) HF in pyridine, pyridine, RT, overnight; b) BnBr, Ag_2O, molecular sieves, RT, 5 h; c) *p*TolSCl/AgOTf, CH_2Cl_2/Et_2O, *N*-(benzyl)benzyloxycarbonyl 3-amino propanol, −78 to −10 °C.

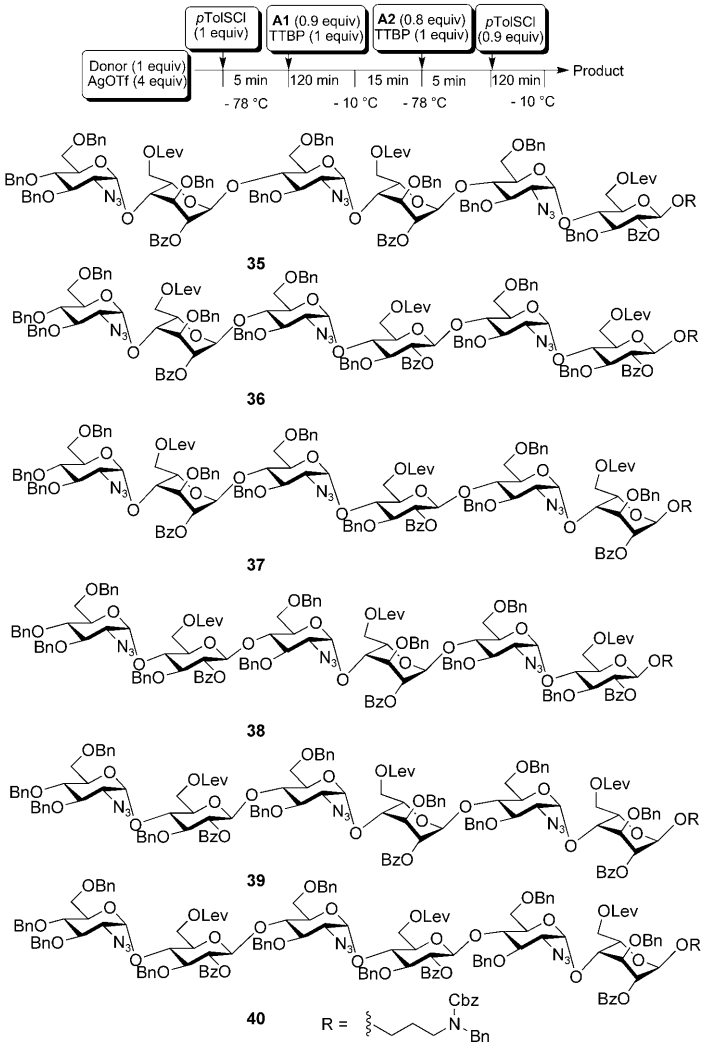
sequently resulting in signal transduction for cell proliferation and growth.[73–76] Therefore, the identification of strong-binding HP/HS oligosaccharides can lead to the development of agents mediating the biological functions of FGFs, which can have important therapeutic applications in areas including wound healing, angiogenesis, and cancer therapy.[77,78]

The basic fibroblast growth factor (FGF-2) is a member of the FGF family. SAR studies on FGF-2 binding with HP/HS have shown that the 3-O- and 6-O-sulfates on the GlcN of HP/HS are not essential, whereas 2-O-sulfation of the uronic acid is important.[24,38,79,80] The conversion of N-sulfation to acetamide significantly reduced the activity.[38] However, the activity lost by N-acylation could be partially regained by O-sulfation.[80] Whereas much study has been devoted to examine the effects of sulfation on FGF-2 binding, the information regarding backbone sequence preference is not very well established.[38,46] The majority of studies performed to date have used HP/HS oligosaccharides mainly containing of IdoA.[38,46,81,82] Nevertheless, GlcA-containing oligosaccharides have been shown to bind and activate FGF-2.[83] Therefore, a systematic investigation on the effects of backbone structures can further advance our understanding of the FGF-2 activities.

Since HP/HS hexasaccharides have been shown to be biologically active,[78] we decided to focus on the preparation of a library of hexasaccharides. As 3-O- and 6-O-sulfation of GlcN are not essential for FGF-2 binding,[6,38,84] this library of 12 hexasaccharides contains systematically varied backbone sequences and 2-O-sulfated uronic acids (i.e., IdoA2S and GlcA2S) to probe the effects of the number and location of IdoA as well as N-sulfation.

To acquire this library efficiently, we developed a divergent approach to access all of the necessary modules from two common thioglycosyl disaccharides **27** and **31**. Specifically, the 4-OTBS group in disaccharide **27** was selectively removed by treatment with HF in pyridine to give the disaccharide acceptor **17** in 92% yield as the disaccharide module for backbone elongation. The newly liberated hydroxyl group in **17** was benzylated as promoted by silver oxide leading to the capping disaccharide **29** in 94% yield. It should be emphasized that the mild basic condition employed for benzylation did not affect the base-sensitive Lev and Bz groups in the molecule. Glycosylation of disaccharide **27** with *N*-(benzyl)benzyloxycarbonyl 3-amino propanol followed by TBS removal gave the reducing end module **30** in 89% yield with an exclusive *trans* selectivity due to neighboring group participation by the 2-OBz moiety (Scheme 4a). In a similar manner, three glucose-containing disaccharides **32**, **33**, and **34** were prepared divergently from disaccharide **31** (Scheme 4b).

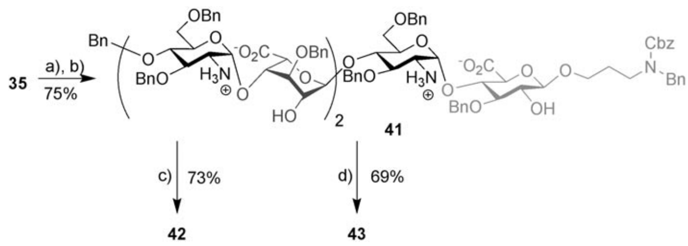
Table 2. One-pot synthesis of HP/HS hexasaccharides.



Entry	Donor	A1	A2	Product	Yield [%]
1	**29**	**17**	**34**	**35**	54
2	**29**	**32**	**34**	**36**	59
3	**29**	**32**	**30**	**37**	58
4	**33**	**17**	**34**	**38**	62
5	**33**	**17**	**30**	**39**	57
6	**33**	**32**	**30**	**40**	50

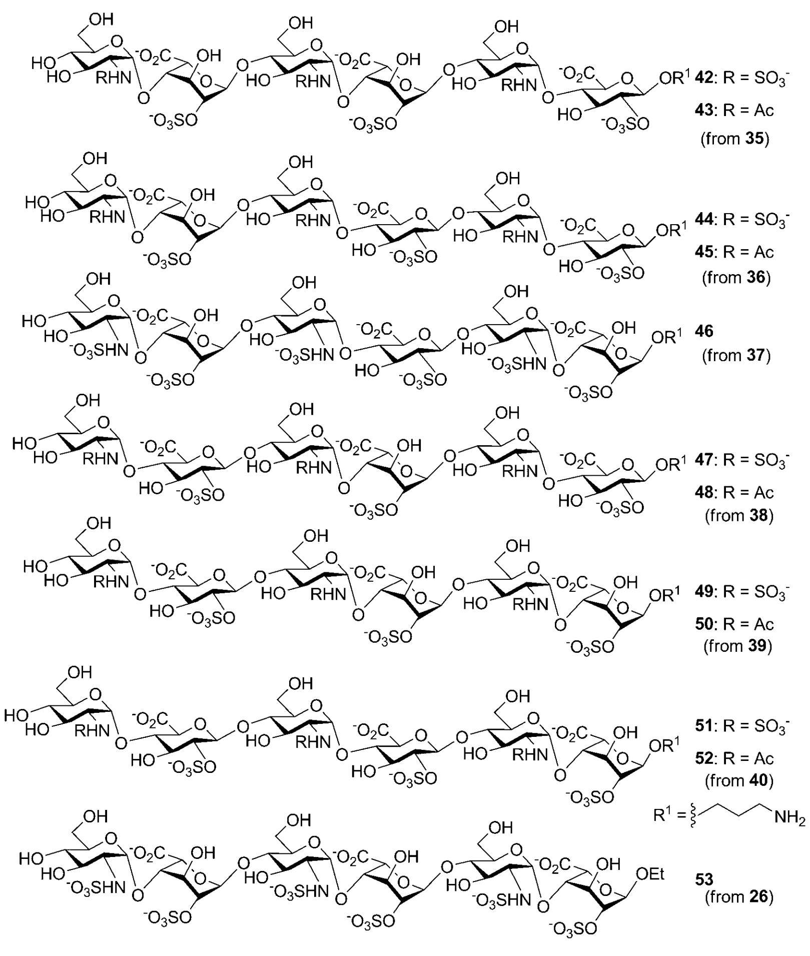
With the disaccharide building blocks in hand, we explored the construction of the hexasaccharide library. Previously, Bonnaffé and co-workers developed an interesting approach, in which three HP/HS tetrasaccharides have been obtained by subjecting three glycosyl acceptors to simultaneous glycosylation in a mixture synthesis.[85] However, the subsequent demixing required time-consuming HPLC separation of the three differentially sulfated glycoside products. Instead of relying on mixture synthesis, we adopted a parallel reaction approach, in which a single desired product was to be obtained from each reaction. Since the preactivation method was independent of the anomeric reactivities, it allowed us to mix the available building blocks rapidly to create sequence diversity. For example, sequential glycosylations using three disaccharide modules **29**, **17**, and **34** following the one-pot procedure gave hexasaccharide **35** with the idose, idose, and glucose backbone structure in 54% yield within just a few hours (Table 2, entry 1). Simple substitution of **17** by **32** in the one-pot synthesis generated hexasaccharide **36** with the idose, glucose, and glucose backbone structure (Table 2, entry 2). In a similar manner, hexasaccharides **37–40** were produced in good yields with potential N- and 2-O-sulfation sites and precisely positioned idose moieties (Table 2, entries 3 to 6). Combined with hexasaccharides **25** and **26**, these compounds covered all of the possible hexasaccharide backbone sequences with GlcN at the nonreducing end. Although the glycosylation outcome can often be highly dependent on building-block structures, it is remarkable that all of the one-pot reactions tested here proceeded well without individual reaction optimization. This highlighted the robustness of our protective group chemistry and glycosylation technology, which should be applicable for future preparation of larger HP/HS oligosaccharide library.

Deprotection of the hexasaccharide **35** was performed by first selectively removing the three Lev protective groups with hydrazine, followed by oxidation employing 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO)/NaOCl and acid protection to facilitate product characterization (Scheme 5).[42,52,86] Saponification of the hexasaccharide by LiOH, H_2O_2, and KOH, followed by Staudinger reduction of the azido group provided compound **41** in 75% yield for



Scheme 5. Deprotection and selective N-derivatization led to two HP/HS hexasaccharides (**42**, **43**) divergently from **35**. Reagents and conditions: a) NH_2NH_2, acetic acid, pyridine, 0°C, TEMPO, NaOCl, KBr, Bu_4NBr, $NaHCO_3$, CH_2Cl_2/H_2O, 0°C to RT, $NaClO_2$, then $PhCHN_2$; b) LiOH, H_2O_2, THF, RT, then NaOH, Me_3P, NaOH, THF, H_2O; c) SO_3•Et_3N, DMF, 55°C, SO_3•Py, Et_3N/pyridine, H_2, $Pd(OH)_2$; d) Ac_2O, MeOH, Et_3N, RT; SO_3•Et_3N, DMF, 55°C, H_2, $Pd(OH)_2$.

the four steps. Sulfation of the free hydroxyls and amines in **41** with subsequent catalytic hydrogenation over the Pearlman catalyst produced HP-like hexasaccharide **42**. The aminopropyl group at the reducing end should not interfere with FGF-2 binding;[38] it can be useful for future development of multivalent constructs[87] as well as microarrays.[38] Alternatively, the structurally related hexasaccharide **43** was obtained by selective amine acetylation of **41** followed by O-sulfation and catalytic hydrogenation.[33,42] Hexasacchar-



ides **44** to **53** were prepared analogously, which contain systematically varied backbones, the 2-O-sulfates on the uronic acid units and the amino groups are either sulfated or acetylated.

The binding between the synthetic HP/HS hexasaccharides and FGF-2 was examined through a competition assay[24] with radiolabeled HS polysaccharides.[24] ^{35}S-labeled HS was harvested from Chinese hamster ovary cells that were grown in the medium containing sodium [^{35}S]sulfate. The complex formed between [^{35}S]HS and FGF-2 could be captured on a nitrocellulose membrane. For the binding assay, the synthetic hexasaccharides or unlabeled HS were incubated with [^{35}S]HS and FGF-2. The FGF-2 binding compounds would compete with [^{35}S]HS, resulting in its dissociation from FGF-2 and consequently lower radioactivity on the membrane.

The panel of hexasaccharides was assayed and found to exhibit differential potencies in FGF-2 binding (Figure 1). Although compound **51** contained the same number and location of sulfates, it was a much weaker binder than other 2-O- and N-sulfated hexasaccharides (compounds **42**, **44**, **46**, **47**, **49**, and **53**). This suggests that FGF-2 binding is not a sole reflection of charge density; rather the specific HS sequence is important. From the SAR analysis, it is clear that the trisaccharide domain N-sulfated GlcN (GlcNS)-IdoA2S-GlcNS represents a minimum FGF-2 binding motif, which can form a kink in polysaccharide chains as induced by FGF binding.[88] The absence of the GlcNS unit at the reducing end of IdoA2S as in hexasaccharide **51** significantly reduced the FGF-2 affinity. The IdoA2S structure is also important because the corresponding GlcNS-GlcA2S-GlcNS was not effective in binding. This is presumably due to the conformational plurality of the IdoA ring.[89] Furthermore, the replacement of GlcNS with *N*-acetylated GlcN (hexasaccharides **43**, **45**, **48**, **50**, and **52**) led to the complete loss of FGF-2 binding, which was consistent with previous reports albeit on different backbone structures.[38] 2-O-Sulfation of the uronic acids could not compensate for the loss of N-sulfates in these hexasaccharides. With the large groovelike binding site on FGF-2 to accommodate the polysaccharide,[74] the potency of the oligosaccharides can be further enhanced by elongating their length.

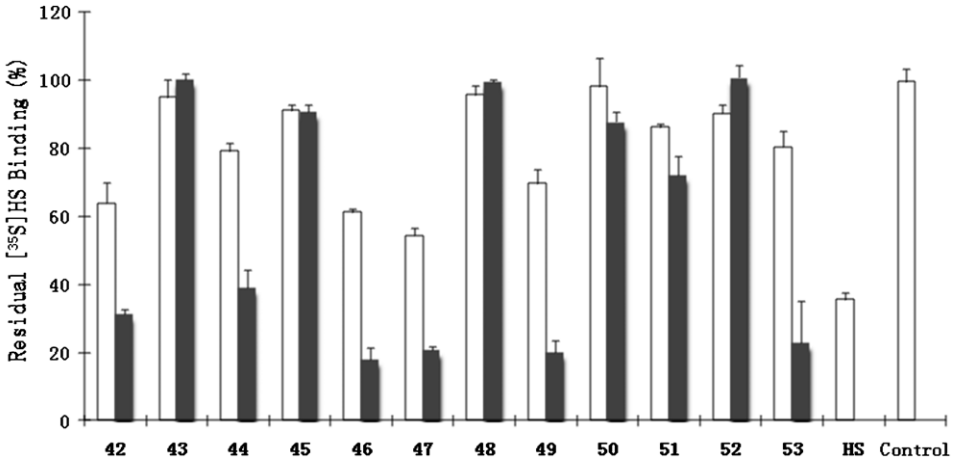


Figure 1. FGF2 (1 μg) was incubated with 10 (empty bar) or 40 μg (filled bar) unlabeled hexasaccharides **42**–**53** or HS (1.6 μg) in PBS buffer (200 μL) at room temperature for 30 min. Then, [^{35}S]HS (4500 cpm) was added to the mixture followed by incubation at 37 °C for 90 min. The data represent the average of four or more experiments with the error bars showing standard deviations. The control was the sample without any hexasaccharides or unlabeled HS. The percentage of residual [^{35}S]HS binding was calculated by dividing the residual ^{35}S counts on the membrane with sample incubation by control counts. For more details, see the Experimental Section.

Conclusion

We have successfully developed a methodology to assemble a library of HP/HS oligosaccharides. Matched donor and acceptor pairs were identified to allow stereospecific formation of the disaccharide building blocks, including those containing the challenging *cis*-1,4-linkages. Preactivation-based, one-pot sequential glycosylations using the disaccharides led to the rapid construction of hexasaccharides in high yields. To test the generality of the methodology, the synthesis of an HP/HS hexasaccharide library was then explored. As our reaction protocol does not require the glycosyl donor to have higher anomeric reactivities than the acceptor, building blocks with similar protective groups, hence similar anomeric reactivities, can be employed without the need for time-consuming protective group adjustment to achieve exact anomeric reactivities. Thus, mixing six disaccharide modules divergently derived from two common intermediate disaccharides allowed combinatorial synthesis of 12 hexasaccharides with systematically varied backbone, precisely positioned IdoA units, and well-defined amine functionalization. The availability of such a library allowed the establishment of important structural features for HS interactions with the growth factor FGF-2. It was found that N-sulfation and trisaccharide motif GlcNS-IdoA2S-GlcNS is important for FGF-2 binding and GlcA2S cannot substitute IdoA2S effectively. This knowledge can provide valuable leads for further development of novel HP/HS-based therapeutics targeting FGF-2.

The successful synthesis of the hexasaccharide library and the high synthetic efficiency achieved herein showcased the power of our synthetic methodology. With further development, this can provide a promising option for the rapid assembly of HP/HS oligosaccharides. With their well-defined structures, the synthetic oligosaccharides can greatly aid in

the analysis of many HP–protein interactions and deciphering the structural information encoded in HP sequences.

Experimental Section

General procedure for preactivation-based single-step glycosylation: A solution of donor (60 μmol) and freshly activated 4 Å molecular sieves (200 mg) in CH_2Cl_2 (2 mL) was stirred at room temperature for 30 min, and cooled to −78 °C, which was followed by the addition of AgOTf (47 mg, 180 μmol) dissolved in Et_2O (1 mL) without touching the wall of the flask. After 5 min, orange-colored *p*TolSCl (9.5 μL, 60 μmol) was added to the solution through a microsyringe. Since the reaction temperature was lower than the freezing point of *p*TolSCl, *p*TolSCl was added directly into the reaction mixture to prevent it from freezing on the flask wall. The characteristic yellow color of *p*TolSCl in the reaction solution dissipated within a few seconds, indicating depletion of *p*TolSCl. After the donor was completely consumed, according to TLC analysis (about 5 min at −78 °C), a solution of acceptor (54 μmol) in CH_2Cl_2 (0.2 mL) was slowly added dropwise by using a syringe (one equivalent of TTBP was added with acceptor if the donor or acceptor contained the PMB protecting group). The reaction mixture was warmed to −20 °C under stirring over 2 h. Then, the mixture was diluted with CH_2Cl_2 (20 mL) and filtered over Celite. The Celite was further washed with CH_2Cl_2 until no organic compounds were observed in the filtrate by TLC analysis. All solutions in CH_2Cl_2 were combined and washed twice with a saturated aqueous solution of $NaHCO_3$ (20 mL) and twice with water (10 mL). The organic layer was collected and dried over Na_2SO_4. After removal of the solvent, the desired oligosaccharide was purified from the reaction mixture by silica gel flash chromatography.

General procedure for preactivation-based three-component one-pot glycosylation: A solution of donor (60 μmol) and freshly activated 4 Å molecular sieves (200 mg) in CH_2Cl_2 (2 mL) was stirred at room temperature for 30 min, and cooled to −78 °C, which was followed by addition of AgOTf (47 mg, 180 μmol) dissolved in Et_2O (1 mL) without touching the wall of the flask. After 5 min, orange-colored *p*TolSCl (9.5 μL, 60 μmol) was added to the solution through a microsyringe. Since the reaction temperature was lower than the freezing point of *p*TolSCl, *p*TolSCl was added directly into the reaction mixture to prevent it from freezing on the flask wall. The characteristic yellow color of *p*TolSCl in the reaction solution dissipated within a few seconds, indicating depletion of *p*TolSCl. After the donor was completely consumed, according to TLC analysis (about 5 min at −78 °C), a solution of acceptor (54 μmol) in CH_2Cl_2 (0.2 mL) was slowly added dropwise by using a syringe. The reaction mixture was warmed to −20 °C under stirring in 2 h and then the mixture was cooled to −78 °C, followed by sequential additions of AgOTf (16 mg, 60 μmol) in Et_2O (1 mL), the second acceptor (48 μmol) in CH_2Cl_2 (1 mL). The mixture was stirred for 5 min at −78 °C and then *p*TolSCl (7.6 μL, 48 μmol) was added to the solution. The reaction mixture was warmed to −20 °C under stirring in 2 h. Then the mixture was diluted with CH_2Cl_2 (20 mL) and filtered over Celite. The Celite was further washed with CH_2Cl_2 until no organic compounds were observed in the filtrate by TLC analysis. All solutions in CH_2Cl_2 were combined and washed twice with a saturated aqueous solution of $NaHCO_3$ (20 mL) and twice with water (10 mL). The organic layer was collected and dried over Na_2SO_4. After removal of the solvent, the desired oligosaccharide was purified from the reaction mixture by silica gel flash chromatography.

General procedure for deprotection of PMB: The PMB-protected compound (1.0 equiv) was dissolved in a mixture of CH_2Cl_2/H_2O (for 0.5 g of compound, 9 mL/1 mL) and the solution was cooled to 0 °C. DDQ (1.1 equiv) was added to the solution and the mixture was stirred at room temperature for 3 h. The mixture was filtered, diluted with CH_2Cl_2 (50 mL), and the organic phase was washed with H_2O until the solution became colorless. The solvent was concentrated in vacuo and the compound was purified by silica gel column chromatography.

General procedure for protection of 6-OH with Lev: The compound containing 6-OH (1 equiv) was dissolved in CH_2Cl_2 (for 0.5 g of compound, 5 mL), followed by addition of Lev (1.4 equiv), EDC·HCl (1.6 equiv) and DMAP (0.1 equiv). The mixture was stirred at room temperature overnight and then was diluted with CH_2Cl_2 (100 mL). The organic phase was washed with saturated $NaHCO_3$ and then dried over Na_2SO_4. The solvent was concentrated in vacuo and the compound was purified by silica gel column chromatography.

General procedure for deprotection of TBS: The TBS-protected compound was dissolved in pyridine (for 100 mg of compound, 1.5 mL). The mixture was cooled to 4 °C, followed by addition of HF in pyridine (0.75 mL, 65–70 % in pyridine). The mixture was stirred at room temperature overnight and then the residue was diluted in EtOAc (100 mL), washed with saturated $NaHCO_3$, and then the organic layer was dried over Na_2SO_4. The solvent was concentrated in vacuo and the compound was purified by silica gel column chromatography.

General procedure for deprotection of Lev: The Lev-protected compound (1 equiv) was dissolved in pyridine (for 150 mg of compound, 2.4 mL) and acetic acid (1.6 mL). The mixture was cooled to 0 °C, followed by addition of hydrazine monohydrate (5 equiv for each Lev). The mixture was stirred at 0 °C for 2 h and then was quenched with acetone (0.28 mL). The mixture was stirred at room temperature for 1 h and the acetone was evaporated under vacuum. The residue was diluted with EtOAc (50 mL) and washed with saturated $NaHCO_3$, 10 % HCl, and water, and the organic phase was dried over Na_2SO_4. The solvent was concentrated in vacuo and the compound was purified by silica gel column chromatography.

General procedure for 6-OH oxidation to carboxylic acid and benzyl ester formation: H_2O (2 mL), 1 M KBr (1.5 equiv per OH), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (1 equiv for each OH), 0.5 M $NaHCO_3$ (10 equiv per OH) and Bu_4NBr (10 equiv per OH) were consecutively added to a solution of 6-OH-containing compound (for 100 mg of compound, 1 equiv) in CH_2Cl_2 (2 mL) at room temperature. The mixture was cooled to 0 °C, followed by slow addition of NaOCl (100 equiv per OH). The pH value of the mixture was calibrated with 0.5 M NaOH to maintain at pH 10 and the resulting solution was warmed slowly to room temperature (maintain at pH 10). After stirring for 3 h, the CH_2Cl_2 was evaporated under vacuum and the residue was diluted with EtOAc (50 mL) and washed with 10 % HCl, saturated $NaHCO_3$, and water. The organic phase was dried over Na_2SO_4, filtered, and the solvents were removed in vacco. Without separation, the resulting residue was dissolved in CH_2Cl_2 (5 mL), followed by addition of phenyldiazomethane in diethyl ether until the color turned red. The mixture was stirred at room temperature for 1 h and then was diluted with CH_2Cl_2 (50 mL), and the organic phase was washed with saturated $NaHCO_3$ and dried over Na_2SO_4. The solvent was concentrated in vacuo and the compound was purified by silica gel column chromatography.

General procedure for saponification: The mixture of compound (for 100 mg of compound, 1 equiv), THF (2.5 mL), and 1 M LiOH (13 equiv per COOBn) was cooled to −5 °C, followed by addition of H_2O_2 (150 equiv per COOBn, 30 %). The mixture was stirred at room temperature for 16 h and then MeOH (6 mL) and 3 M KOH (80 equiv per COOBn) were added to the solution. The mixture was stirred for another 24 h and then was acidified with 10 % HCl, concentrated to dryness. The resulting residue was purified by quickly passing through a short silica gel column (4:1, CH_2Cl_2-MeOH).

General procedure for azide reduction: 1 M PMe_3 solution in THF (5 equiv per N_3), 0.1 M aqueous solution of NaOH (2.6 equiv per N_3), and H_2O (2 mL) were added consecutively to a solution of azide-containing compound (for 50 mg of compound, 1 equiv) in THF (7 mL). The mixture was stirred at room temperature overnight and neutralized with 0.1 M HCl until pH 7. The mixture was concentrated to dryness and the resulting residue was purified by quickly passing through a short silica gel column (4:1, CH_2Cl_2/MeOH).

General procedure for O-sulfation: The mixture of OH-containing compound (for 20 mg of compound, 1 equiv), DMF (1 mL), and SO_3·NEt_3 (5 equiv per OH) was stirred at 55 °C for 24 h. The mixture was quenched by adding Et_3N (0.2 mL) and then diluted with CH_2Cl_2/MeOH (1 mL/1 mL). The resulting solution was layered on the top of a Sephadex LH-20 chromatography column that was eluted with CH_2Cl_2/MeOH (1/1, v/

v). The solvent was evaporated to dryness under vacuo without further purification.

General procedure for N-sulfation: A mixture of NH_2-containing compound (for 20 mg of compound, 1 equiv), pyridine (1 mL), Et_3N (0.2 mL), and SO_3·Pyridine (5 equiv per NH_2) was stirred at room temperature for 3 h. The mixture was diluted with CH_2Cl_2/MeOH (1 mL/1 mL) and the resulting solution was layered on the top of a Sephadex LH-20 chromatography column that was eluted with CH_2Cl_2/MeOH (1/1, v/v). The solvent was evaporated to dryness under vacuo without further purification.

General procedure for selective N-acetylation: A mixture of OH, NH_2-containing compound (for 12 mg of compound, 1 equiv), Ac_2O (5 equiv per NH_2), Et_3N (15 μL), and MeOH (1 mL) was stirred at room temperature for 5 h. The mixture was diluted with CH_2Cl_2/MeOH (1 mL/1 mL) and the resulting solution was layered on the top of a Sephadex LH-20 chromatography column that was eluted with CH_2Cl_2/MeOH (1/1, v/v). The solvent was evaporated to dryness under vacuo without further purification.

General procedure for global debenzylation: A mixture of the Bn-containing compound (for 12 mg of compound, 1 equiv), MeOH/H_2O (4 mL/2 mL), and $Pd(OH)_2$ (100 mg) was stirred under H_2 at room temperature overnight and then filtered. The filtrate was concentrated to dryness under vacuum and then diluted with H_2O (15 mL). The aqueous phase was further washed with CH_2Cl_2 (3×5 mL) and EtOAc (3×5 mL) and then the aqueous phase was dried under vacuum. The crude product was further purified by size exclusion chromatography (G-15) and then eluted from a column of Dowex 50WX4-Na^+ to convert the compound into the sodium salt form.

Determination of the binding of [^{35}S]HS and FGF-2: The binding of [^{35}S]HS and FGF-2 was carried out using a "filter-trapping" assay.[24] Briefly, FGF-2 (1 μg) was incubated with HS polysaccharide (1.6 μg) or various amounts of hexasaccharides in PBS buffer (200 μL) at room temperature for 30 min. Then, [^{35}S]HS (4500 cpm) was added into the reaction mixture followed by incubation at 37 °C for 90 min. The mixture was then spotted onto nitrocellulose membrane. The membrane was thoroughly washed with PBS and a PBS-based buffer containing 280 mM NaCl. The spotted membrane was mixed with scintillation fluid to measure ^{35}S-counts to determine the percentage of residual [^{35}S]HS bound to FGF-2.

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