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## Automated Solid Phase Assisted Synthesis of a Heparan Sulfate Disaccharide Library

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### Abstract

Heparan sulfate (HS) regulates a wide range of biological events, including blood coagulation, cancer development, cell differentiation, and viral infections. It is generally recognized that structures of HS can critically impact its biological functions. However, with complex structures of naturally existing HS, systematic investigations into the structure-activity relationship (SAR) of HS and efforts to unlock their “sulfation code” have been largely limited due to the challenges in preparing diverse HS oligosaccharide sequences. Herein, we report an automated machine-aided solid-phase strategy that significantly expedited the assembly of HS disaccharides. The key strategically protected advanced disaccharide intermediates were immobilized onto Synphase lanterns. Divergent deprotections and sulfations of the disaccharides were achieved on the lanterns in high yields. In addition, the full synthetic process was automated, enabling the reproducible production of HS disaccharides. A library of 16 HS disaccharides with diverse sulfation patterns was prepared via this method. Compared to the traditional HS synthesis, this new strategy led to a reduction of 50% of the number of synthetic steps and over 80% of the number of column purification steps needed from the disaccharide intermediates, significantly improving the overall synthetic efficiency. The potential utility of the method was highlighted in a microarray study

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Conflicts of Interests

JL is a founder for Glycan Therapeutics. GS is an employee of Glycan Therapeutics. The authors declare no other conflicts of interests.

using the synthetic HS disaccharide library with fibroblast growth factor-2 (FGF-2), which yielded insights into the SAR of HS/FGF-2 interactions.

### Keywords

Automated synthesis; fibroblast growth factor 2; heparan sulfate; library preparation; Synphase lanterns

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### Introduction:

Heparan sulfate (HS), a member of the glycosaminoglycan (GAG) family, is known to play important roles in a wide range of biological processes, including cancer development, inflammation, viral infections, and Alzheimer's disease development.<sup>1-4</sup> HS chains are composed of repeating disaccharide units of glucosamine (GlcN)- $\alpha$ -1 $\rightarrow$ 4 linked to a uronic acid (either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)). The sulfation may occur at O-2 of uronic acid and O-3, O-6 of GlcN, and the amine of GlcN can be sulfated, acetylated or unmodified. It is well known that the specific structure of HS can dictate their functions.<sup>5, 6</sup> Sulfation patterns in naturally existing HS are highly heterogeneous, rendering it challenging to obtain sufficient amounts of pure HS from natural sources for detailed study of structure-activity relationship (SAR).<sup>7-9</sup> The availability of HS oligosaccharides with diverse well-defined structures is critical to advance the knowledge on the SAR of HS.

During the last two decades, tremendous advances have been achieved in synthesis of HS oligosaccharides.<sup>1, 10-18</sup> Chemical synthesis can enable access to well-defined HS molecules.<sup>19-21</sup> However, the majority of the strategies were designed for target-oriented total synthesis with a relatively small number of HS structures generated in each study.<sup>1, 12, 13</sup> One significant bottleneck for HS library preparation is the number of synthetic and purification steps required as it typically takes 6 – 8 synthetic steps to transform a protected oligosaccharide backbone to a fully deprotected sulfated HS glycan. Following each synthetic transformation, chromatography purifications need to be performed, which is tedious and challenging with the polar nature of sulfated glycan intermediates. Thus, the number of synthetic and purification steps needed for a library preparation presents a significant hurdle. With tremendous efforts, the Hung<sup>22, 23</sup> and Wei<sup>24</sup> groups have developed elegant synthesis to produce libraries of HS disaccharides. Boons and coworkers reported the synthesis of the largest HS tetrasaccharide library (47 compounds) to date based on a modular approach.<sup>25</sup> Innovative new methods are needed to further accelerate HS synthesis.

Solid-phase strategies can offer an attractive approach to expedite synthesis. Although significant advances have been achieved in solid phase-supported glycosylation during the last two decades,<sup>26-31</sup> a large excess (5 – 20 eq) of high value glycosyl donors is typically needed to obtain high yields, which presents significant roadblocks for library preparation. Furthermore, it has been challenging to synthesize sulfated glycans on solid phase. For example, although an automated synthesis of keratan sulfate tetrasaccharides was reported,<sup>28</sup> extending the method to other classes of sulfated glycans has met challenges as suggested from the lack of fully deprotected chondroitin sulfate oligosaccharide products in

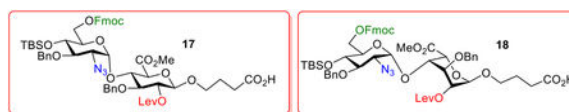
the solid-phase synthesis.<sup>29</sup> Recently, an on resin sulfation protocol was reported for several linear and branched glycans, which did not include HS oligosaccharides.<sup>32</sup> While protected HS precursors have been assembled on solid phase,<sup>33, 34</sup> to the best of our knowledge, no automated synthesis of sulfated HS oligosaccharides has been published.

To expedite HS assembly, herein, we report a new solid phase-based HS synthesis strategy to prepare a library of 16 HS disaccharides **1–16** (Figure 1). Our method integrates key disaccharide intermediates prepared from solution-phase synthesis with protective group removal and sulfation performed on solid phase assisted by microwave irradiation. Furthermore, the synthesis was automated on the commercially available Liberty Blue peptide synthesizer, which significantly enhanced the overall synthetic efficiency.

## Results and discussion

### Design of the synthesis

To access the 16 disaccharides, we designed two advanced common disaccharide building blocks, i.e., **17** and **18**, for HS disaccharides containing IdoA and GlcA in the backbone respectively. Disaccharides **17** and **18** bear three strategically placed protective groups, i.e., fluorenylmethoxycarbonyl (Fmoc), levulinoyl (Lev), and *tert*-butyl-dimethylsilyl (TBS) as well as the azide moiety to mask the amino group of GlcN. Each of these protective groups may be orthogonally removed without affecting others for divergent synthesis of the HS library.<sup>17</sup>



To further enhance the overall synthetic efficiency, we envision the key disaccharides can be immobilized on solid phase support followed by divergent deprotection and sulfation (Scheme 1a). With the ability to use an excess amount of reagent to drive the solid phase-supported reactions to completion without the need for intermediate purification, the overall production time may be significantly shortened.

We selected Synphase lanterns as the solid-phase support, which have a cylindrical shape (5 mm diameter and 5–17 mm length).<sup>35</sup> Unlike the more common micron sized polymer beads, the lanterns can be easily transferred from flask to flask using a pair of tweezers without losing samples during the transfer. The lanterns have an unreactive rigid base polymer as the inner core, providing a robust and convenient framework, which is coated with a low cross-linked uniform polymer graft 50  $\mu\text{m}$  thick as the outer layer where reactions take place. The unique “Lantern” shape provides high surface area, and enables free flow-through of reactants and rapid drainage of solutions. Furthermore, the lanterns can be coated with a hydrophilic polyamide surface<sup>36–38</sup> suitable for HS modifications with high loading and reaction rates. Synphase lanterns can combine the advantages of solution-phase and solid-phase chemistry in one system, overcoming some of the limitations of either system alone.

In order to perform solid-phase synthesis, the linker used to immobilize the glycan is critical. This linker needs to be stable under the typical synthetic transformations encountered during HS synthesis including base treatment, oxidation, and sulfation. Yet, it should be readily cleavable under a mild condition without affecting the labile *O*- and *N*-sulfate moieties. After screening multiple structures, we selected the Hycron linker<sup>39</sup> to attach the glycan. We originally chose an amino-oxy moiety to immobilize the glycan with the Hycron linker (Figure S1). However, the N-O bond of the oxime was cleaved during the final hydrogenolysis. We also investigated the usage of an ester linkage, which turned out to be too labile during the typical reactions employing base for HS functionalization (Figure S1). Finally, we designed the allyl carbamate linkage for attaching the glycan to the lantern through the Hycron linker (Scheme 1b). The three methylene units at the reducing end of the glycan (highlighted in cyan color oval in scheme 1b) turned out to be important as a shorter glycolic amide linker led to premature cleavage of the glycan by base (Figure S1). The multiple ethylene glycol units in linker **20** can increase the hydrophilicity of the lantern surface and facilitate the formation and reactions of highly polar sulfated glycans attached on the lantern. Furthermore, the glycan may be released from the lantern through the treatment of a mild Pd(0) catalyst<sup>39</sup> to cleave the Hycron linker to afford the desired HS disaccharide.

### Synthesis of GlcN-GlcA Building Block 17, GlcN-IdoA Building Block 18, and the Hycron linker 20.

Our synthesis started with preparation of the disaccharide **17** by the glycosylation reaction between donor **21**<sup>40</sup> (1.1 eq) and thioglycoside acceptor **22**<sup>41</sup> (1 eq) in the presence of a bulky base tri-*t*-butyl-pyrimidine (TTBP)<sup>42</sup> (Scheme 2) following the pre-activation based glycosylation using the *p*-TolSCI/AgOTf promoter.<sup>43, 44</sup> Disaccharide **23** was obtained in 70% yield as the desired  $\alpha$ -anomer ( $^1J(C_1, H_1) = 173.9$  Hz suggesting  $\alpha$ -linkage)<sup>45</sup> without the need of resorting to 5–20 eq of the donor typically required for solid-phase glycosylation reactions. Disaccharide **23** underwent protective group manipulation to give *tert*-butyl-diphenylsilyl (TBDPS) protected disaccharide **24**. Coupling between **24** and 4-pentene-1-ol gave disaccharide **25** in 82% yield as the  $\beta$ -isomer exclusively ( $^1J(C_1, H_1) = 160.5$  Hz confirming  $\beta$ -linkage). It is important to switch the 6-*O*-PMB in **23** to 6-*O*-TBDPS, as direct usage of **23** as the thioglycoside donor gave the 1,6-anhydrosugar as a side product presumably due to participation of the 6-*O*-PMB moiety.<sup>41</sup> The pentenyl glycoside **25** underwent silyl deprotection with HF/pyridine complex, and the resulting diol was subjected to selective oxidation,<sup>46</sup> methyl esterification followed by TBS protection to give disaccharide **26** in 52.1% yield over 4 steps. Protective group manipulation on **26** installed a Fmoc group on 6-*O* of the GlcN and a Lev on 2-*O* of GlcA for future selective deprotection. Subsequent ozonolysis followed by Pinnick oxidation<sup>47, 48</sup> of the resulting aldehyde afforded the desired disaccharide acid **17**.

For GlcN-IdoA building block **18**, the synthesis started with glycosylation between idose donor **28**<sup>44</sup> and 4-pentene-1-ol acceptor with the *p*-TolSCI/AgOTf promoter system, which gave the pentenyl glycoside **29** in 88% yield (Scheme 3). The newly formed  $\alpha$ -linkage was confirmed by NMR analysis ( $^1J(C_1, H_1) = 170.3$  Hz). Subsequent treatment of **29** with *p*-toluene sulfonic acid (*p*-TSA) followed by oxidation and esterification afforded acceptor

**30** in 77.4% yield over 3 steps. The coupling between donor **21** and acceptor **30** went smoothly and gave disaccharide **31** in 78% yield exclusively as the  $\alpha$ -isomer ( $^1J_{(C_1,H_1)}=170.3$  Hz).<sup>45</sup> Similar sequence of protective group adjustments and oxidation of the pentenyl moiety of **31** as in the synthesis of disaccharide acid **17** led to the desired GlcN-IdoA disaccharide building block **18**.

After the synthesis of disaccharides **17** and **18**, we turned to the preparation of the Hycron linker **20** (Scheme 4).<sup>39, 49–51</sup> Coupling between **32** and the mono Fmoc-protected diamine **33**, followed by acid treatment to deprotect the <sup>t</sup>Bu ester gave the Hycron linker **20** in 77% yield.

### Lantern-supported automated synthesis of disaccharide 16

With all components in hands, we functionalized the amine bearing Synphase lanterns **35** with the Hycron linker **20** (Scheme 5). The coupling of Hycron linker **20** onto the lantern was promoted by 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) under microwave irradiation at 50 °C on a CEM Liberty Blue automated peptide synthesizer (Scheme 5). To quantify the loading of Hycron linker on the lanterns, the Fmoc moiety was deprotected by piperidine. Based on the UV-vis absorbance of the cleavage product dibenzofulvene, the loading level of Hycron linker was determined to be 6.4  $\mu$ mol per lantern. Microwave ( $\mu$ W) irradiation significantly shortened the reaction time from 20 h with a traditional oil bath to 3 h, presumably due to better heating and agitation of the reaction mixture. Subsequently, disaccharides **17** and **18** (0.7 equiv. to amine on the lantern) were loaded respectively onto the lantern promoted by HOAt with microwave irradiation at 50 °C. 4.2  $\mu$ mol of disaccharide **18** per lantern was immobilized corresponding to over 90% of the disaccharide added to the reaction, suggesting a high efficiency of the immobilization protocol. Following a similar procedure, 3.2  $\mu$ mol of the disaccharide **17** was loaded per lantern.

With the disaccharide successfully immobilized, synthetic transformations of the disaccharide were carried out on the lanterns. The sequence of reactions was optimized, and reagent compatibility with the Liberty Blue synthesizer was investigated. As each lantern is composed of 9 rings of polypropylene, half a ring of the lantern could be cut off easily with a razor blade. The attached glycans (~ 100  $\mu$ g) could be released with a Pd(0) catalyst, which was sufficient for mass spectrometry (MS) analysis to enable monitoring reaction progress. For Lev deprotection, the reaction was performed using  $\mu$ W at 50 °C for 3h for 2 times, which was sufficient for complete Lev-cleavage as indicated by MS analysis. *O*-Sulfation was carried out using  $SO_3 \cdot Et_3N$  complex with  $Et_3N$  in DMF using  $\mu$ W at 60 °C for 6h for 2 times. *N*-sulfation was performed in MeOH three times with the addition of NaOH (2M) to adjust pH to be around 8.5. Azide reduction was performed using the Staudinger reaction using  $\mu$ W at 60 °C for 30 minutes in 3 rounds to ensure its completion. Hydrolysis of the methyl ester was accomplished in two cycles using freshly prepared LiOH solutions. For TBS removal, while HF pyridine was efficient in promoting the reaction, it was corrosive to the tubing and the reaction vessel of the synthesizer. As a result, tetrabutylammonium fluoride (TBAF) was utilized to cleave TBS, the rate of which was expedited by microwave irradiation enabled by the synthesizer. As sulfates can

be labile, we investigated the stability of sulfates during lantern supported deprotections. *O*-Sulfates were found to be stable to Lev, TBAF, and Staudinger reduction conditions on the lanterns. However, some *N*-sulfates were cleaved during the TBAF reaction. Thus, the reaction sequence was designed that the *N*-sulfate was to be installed at late stage of the synthesis. Throughout these transformations, no disaccharide products were observed to be prematurely cleaved suggesting the compatibility of the Hycron linker with protective group manipulation and sulfation reaction conditions typically encountered in HS synthesis.

Besides the possibility of using microwave irradiation to shorten the reaction time, the reagent addition and washing steps can be programmed and automated on the CEM Liberty Blue system. As shown in Figure 3, the HS disaccharide **18** functionalized lantern **38** (**18@lantern**) was subjected to Fmoc cleavage (for quantification), *O*-acetylation, Lev-deprotection, *O*-sulfation, azide reduction, hydrolysis, TBS deprotection, and *N*-sulfation. The synthesizer was programmed to carry out the 7 synthetic steps from **38** in an automated fashion in 96 hours (Figure 2). Upon completion of the synthesis, the disaccharide product **39** was released from the lantern by Pd(PPh<sub>3</sub>)<sub>4</sub> and borane, and purified by LH-20 size exclusion chromatography. It should be emphasized that from **38** to **39**, only one chromatography purification is needed. The overall yield of **39** from **38** was an excellent 60% for the seven steps representing an average of 92% yield for each synthetic transformation. The high yield for the lantern supported synthesis could be due to 1) with the easy removal of unconsumed reagent by washing the lanterns, excess reagent was used to drive the reaction to completion; 2) the hydrophilic PA surface of the lantern enabling facile glycan sulfation on the solid phase; and 3) the avoidance of product loss during multiple purification steps as required in solution phase synthesis. Following hydrogenolysis to remove the benzyl protective groups from **39**, the tri-sulfated HS disaccharide **16** was obtained in good purity as indicated by <sup>1</sup>H-NMR analysis (Figure 3).

### Synthesis of the 16-membered HS disaccharide library

As disaccharide **18** bears orthogonally removable protective groups at the strategic positions, the deprotection and sulfation reaction sequence can be varied to divergently create multiple HS disaccharide. As an example, starting from **38** (**18@lantern**), skipping the Lev deprotection step while keeping the rest of the reactions in the synthesis of **16** produced disaccharide **14** in 41.5% overall yield.

The lantern supported automated synthesis process is robust. Without the need for further optimization, simply by adjusting the sequence of the reactions, a panel of 8 disaccharides **9**–**16** were produced in good yields with each synthesis taking about 90 hours on the synthesizer starting from **18@lantern** (Table 1). Similarly, the GlcN-GlcA disaccharide bearing **37** (**17@lantern**) was converted to 8 disaccharides **1**–**8** with the GlcN-GlcA backbone in good overall yields (Table 1).

Each of the 16 disaccharides (**1**–**16**) was prepared on 1–3 mg scales with >95% purity. Thus, the overall operation for library preparation was significantly expedited by Synphase lantern aided synthesis using the CEM microwave synthesizer, which reduced the number of purification steps by over 80% for each member of the library with the synthesis automated.



## The disaccharide library yielded insights on structural features important for fibroblast growth factor 2 (FGF-2) binding

To demonstrate the utility of the disaccharides, we investigated the binding of the disaccharides with a representative member of the fibroblast growth factor (FGF) family, the FGF-2. FGFs play crucial roles in cellular development, which can bind with endogenous HS facilitating the complex formation with FGF receptors to induce signal transduction and promote cell proliferation and growth. A better understanding of the structural requirements of HS for FGF-2 interactions can enhance our knowledge and facilitate the development of therapeutics in areas including wound healing, angiogenesis, and cancer therapy.<sup>52, 53</sup>

To investigate HS disaccharide binding to FGF-2, the disaccharides **1–16** were immobilized onto an *N*-hydroxysuccinimide (NHS) ester functionalized glass slide through amide linkages at three different concentrations (0.25 mM, 0.5 mM, and 1 mM) to fabricate the disaccharide microarray. Fluorescently labeled FGF-2 was incubated with the microarray. Upon washing off unbound protein, the microarray was scanned by a fluorescence slide reader to semi-quantify the amounts of FGF-2 remaining bound.

HS disaccharides exhibited structure dependent binding with FGF-2. As shown in Figure 4, compound **12** (GlcNSIdoA2S) bound the strongest with FGF-2, with the corresponding GlcA disaccharide **4** bearing the same sulfation patterns also binding well when printed at 1 mM suggesting the backbone structure in this context does not drastically impact FGF-2 binding. Interestingly, addition of the 6-*O* sulfate to GlcN (compound **16** GlcNS6SIdoA2S) decreased the binding by about 50% indicating higher sulfation density on the disaccharide could negatively influence the FGF-2 interaction. Compounds **12** (GlcNSIdoA2S) and **13** (GlcNS6SIdoA) both have two sulfates in their structures. Yet, **12** exhibited significantly stronger binding than **13** suggesting the positions of the sulfates can be important for binding. The *N*-sulfation was important as GlcNAcIdoA2S **11** and GlcNAc6SIdoA2S **15** are much weaker binders than the corresponding *N*-sulfated disaccharides GlcNSIdoA2S **12** and GlcNS6SIdoA2S **16**. The importance of 2-*O* sulfation and *N*-sulfation is consistent with literature reports on HS-FGF-2 interactions obtained using HS oligosaccharides including HS disaccharides,<sup>54–58</sup> suggesting the HS disaccharide library prepared via the current automated synthesis can be used to probe HS interactions.

### Conclusion:

We report the establishment of a new method to rapidly access a library of HS disaccharides. The two key strategically protected intermediates were efficiently assembled in solution without the need for 5–20 equiv. of high-valued glycosyl donors typical for solid-phase synthesis. These advanced disaccharides could be divergently deprotected to enable production of a library of HS disaccharides. To expedite the library preparation, the two advanced disaccharides were immobilized onto Synphase lanterns through an allyl carbamate bearing Hycron linker, which was found to be compatible with a wide range of typical reactions encountered in HS synthesis, including acid, base, Staudinger reduction, and silyl removal. Multiple sulfates could be installed on the disaccharides immobilized on the lanterns in high yields. In addition, the synthetic process was automated on a microwave-assisted peptide synthesizer, enabling the expedient production of the 16-membered HS

disaccharide library. Compared to the traditional HS synthesis, this new strategy led to a reduction of 50% of the number of synthetic steps and over 80% of the number of column purification steps needed from the disaccharide intermediates, thus significantly enhancing the overall synthetic efficiency. The disaccharide library obtained was transformed into a glycan microarray and screened against FGF-2, which yielded insights into the SAR of HS/FGF-2 interactions. This is the first time that Synphase lanterns and the microwave-assisted peptide synthesizer have been utilized for HS synthesis, presenting an attractive platform for automated glycan synthesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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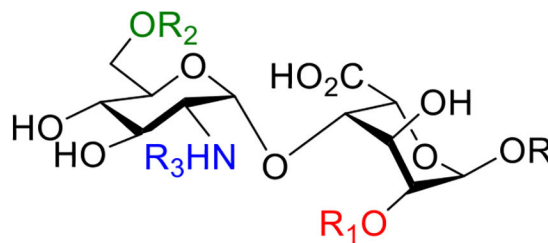
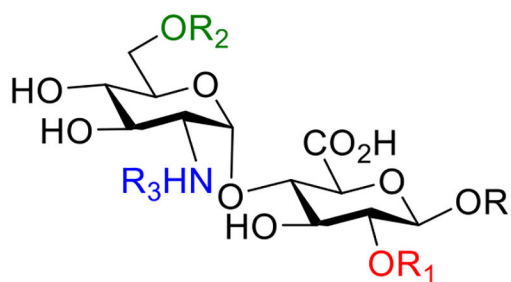
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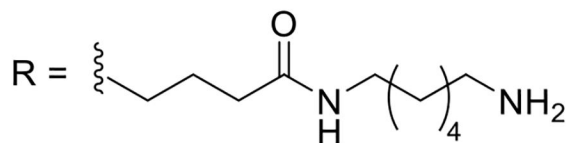
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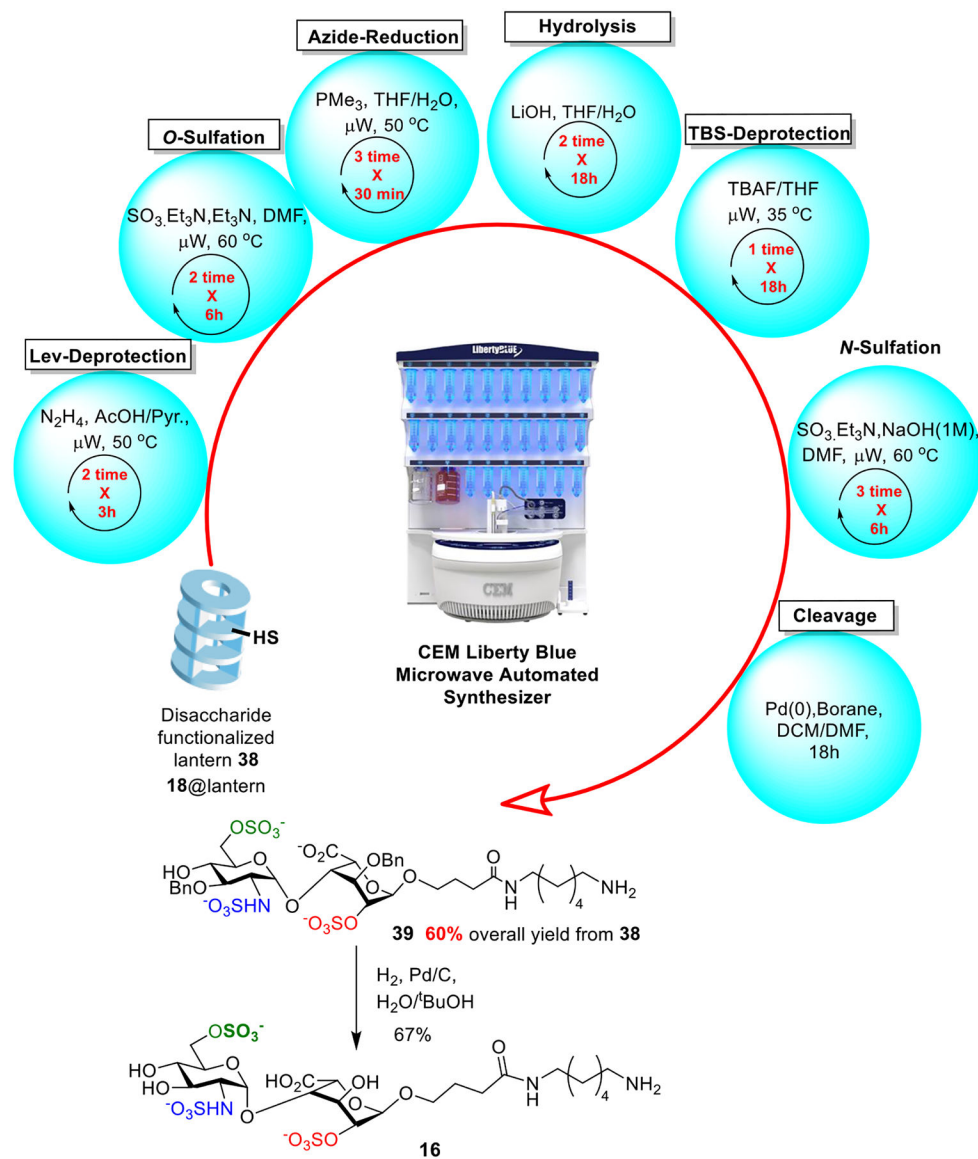


- 1:**  $R_1=R_2=H, R_3=Ac$   
**2:**  $R_1=R_2=H, R_3=SO_3^-$   
**3:**  $R_1=SO_3^-, R_2=H, R_3=Ac$   
**4:**  $R_1=R_3=SO_3^-, R_2=H$   
**5:**  $R_1=H, R_2=SO_3^-, R_3=Ac$   
**6:**  $R_1=H, R_2=R_3=SO_3^-$   
**7:**  $R_1=R_2=SO_3^-, R_3=Ac$   
**8:**  $R_1=R_2=R_3=SO_3^-$

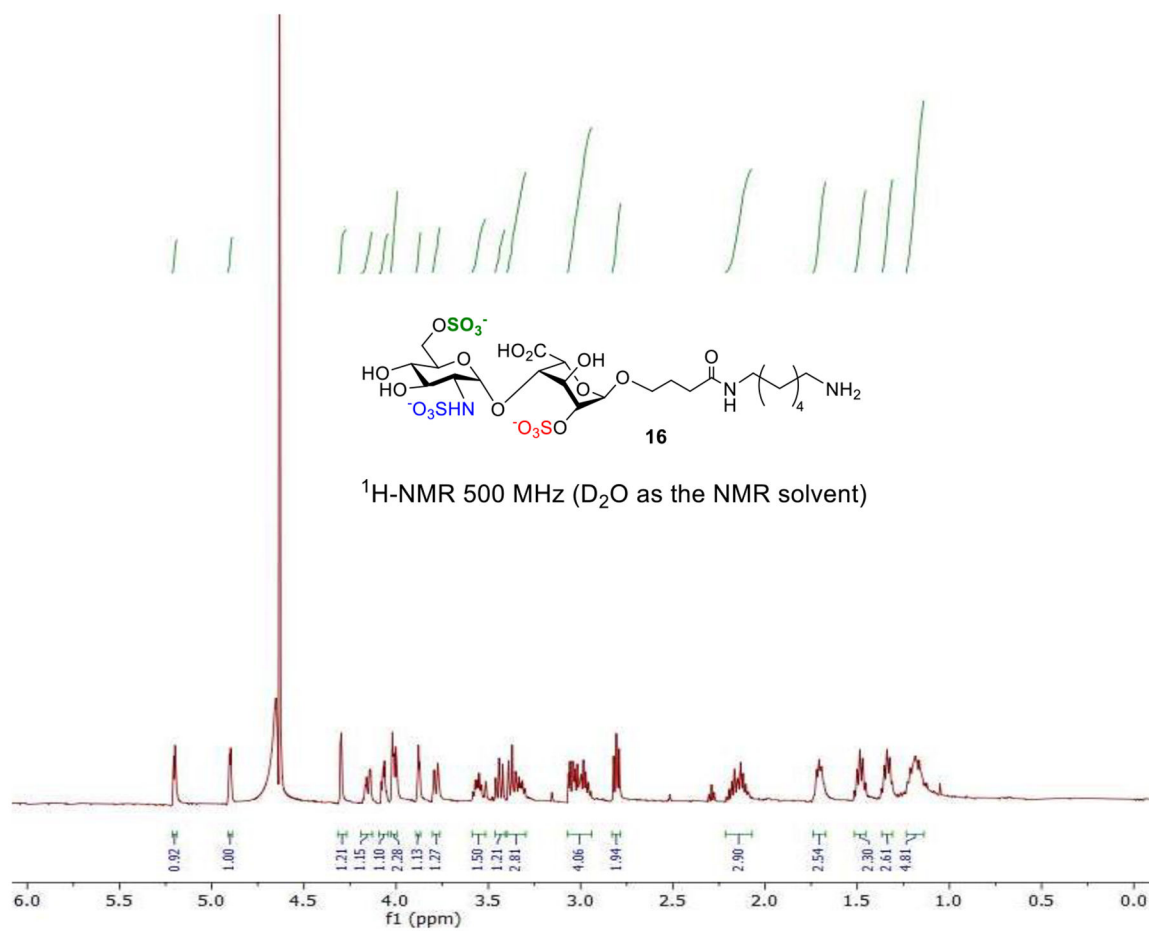
- 9:**  $R_1=R_2=H, R_3=Ac$   
**10:**  $R_1=R_2=H, R_3=SO_3^-$   
**11:**  $R_1=SO_3^-, R_2=H, R_3=Ac$   
**12:**  $R_1=R_3=SO_3^-, R_2=H$   
**13:**  $R_1=H, R_2=SO_3^-, R_3=Ac$   
**14:**  $R_1=H, R_2=R_3=SO_3^-$   
**15:**  $R_1=R_2=SO_3^-, R_3=Ac$   
**16:**  $R_1=R_2=R_3=SO_3^-$



**Figure 1:**  
Structures of 16 HS disaccharides **1–16**.

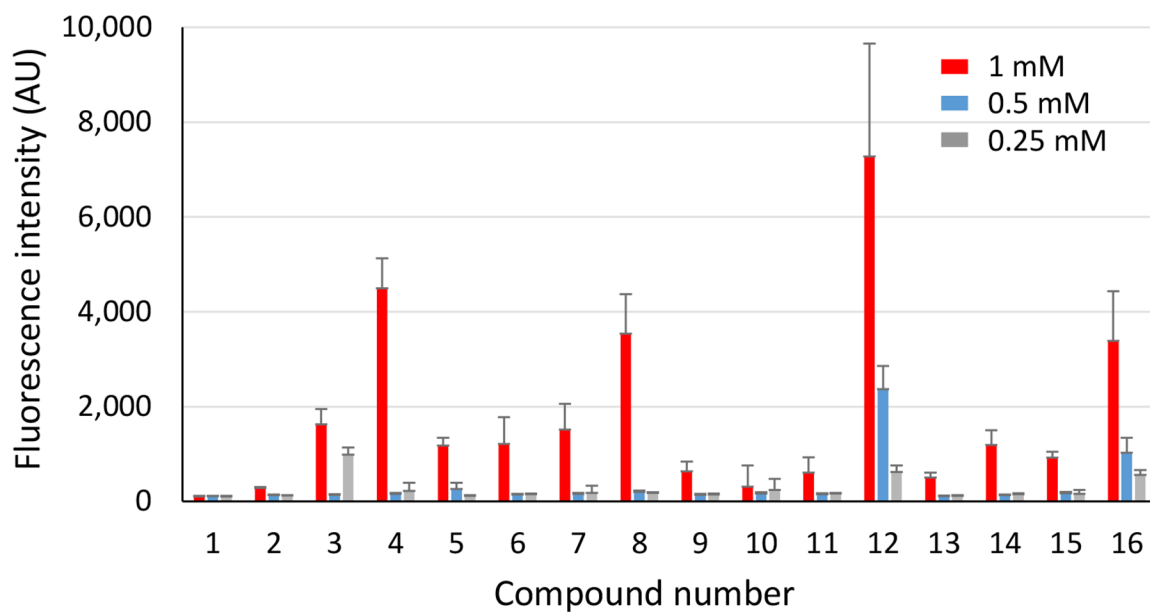


**Figure 2.** Automated synthesis of the trisulfated HS disaccharide **16** from disaccharide loaded lantern **38** (18@lantern) on the Liberty Blue system.

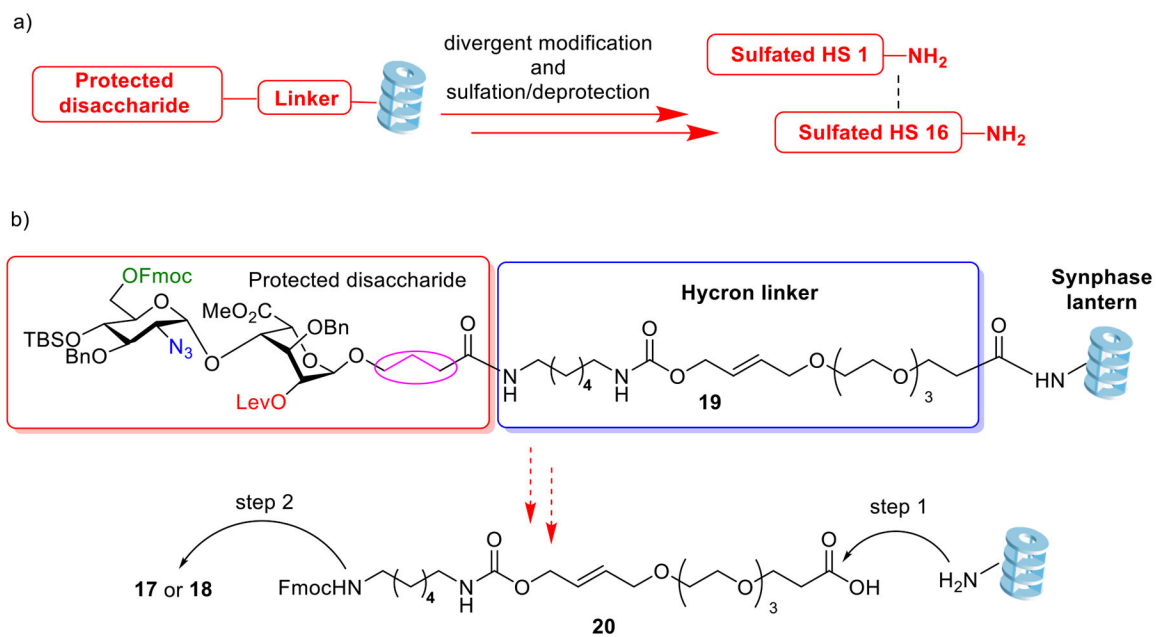


**Figure 3.**  
<sup>1</sup>H-NMR spectrum of compound **16**.



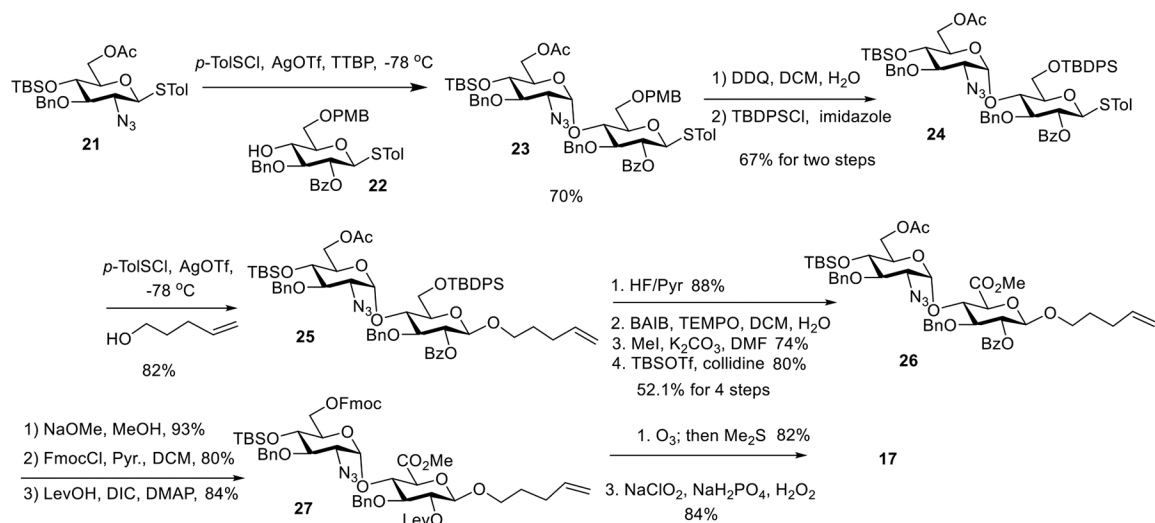


**Figure 4.** Fluorescence signal intensities observed for each arrayed HS disaccharide binding to FGF-2. Three different concentrations of each disaccharide, ranging from 1 mM to 0.25 mM from left to right were printed on the microarray. All samples were printed in replicates of sixteen.

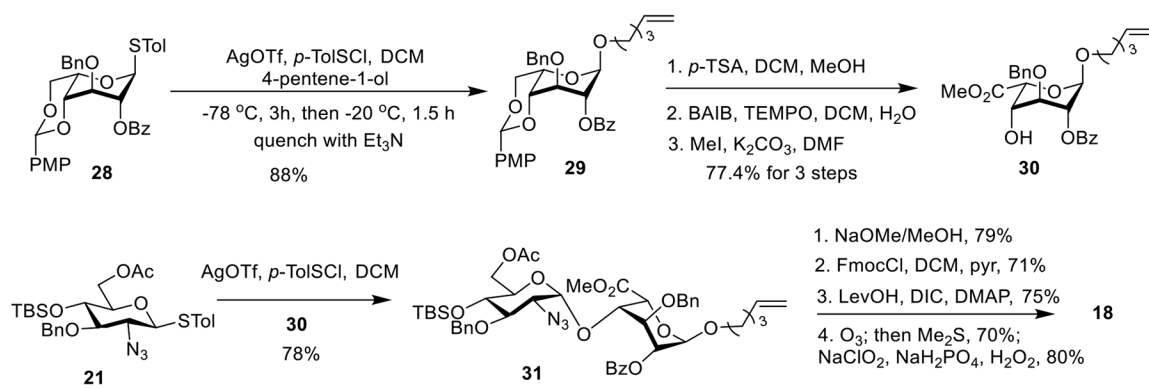


**Scheme 1.**

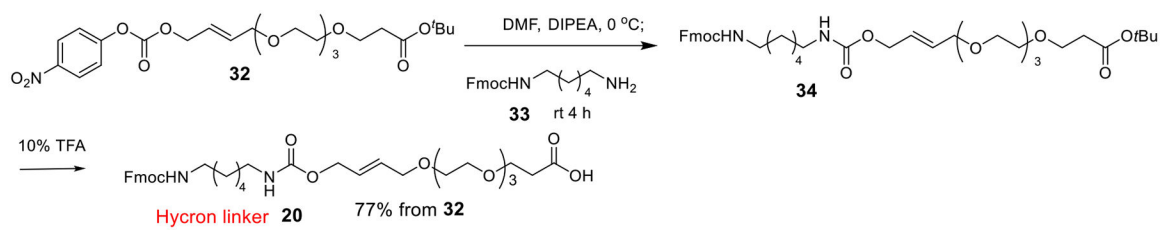
a) Schematic demonstration of the synthesis of sulfated HS disaccharides on Synphase lanterns; b) Retrosynthetic analysis of the HS synthesis on Synphase lanterns with the protected disaccharide linked through the Hycron linker.



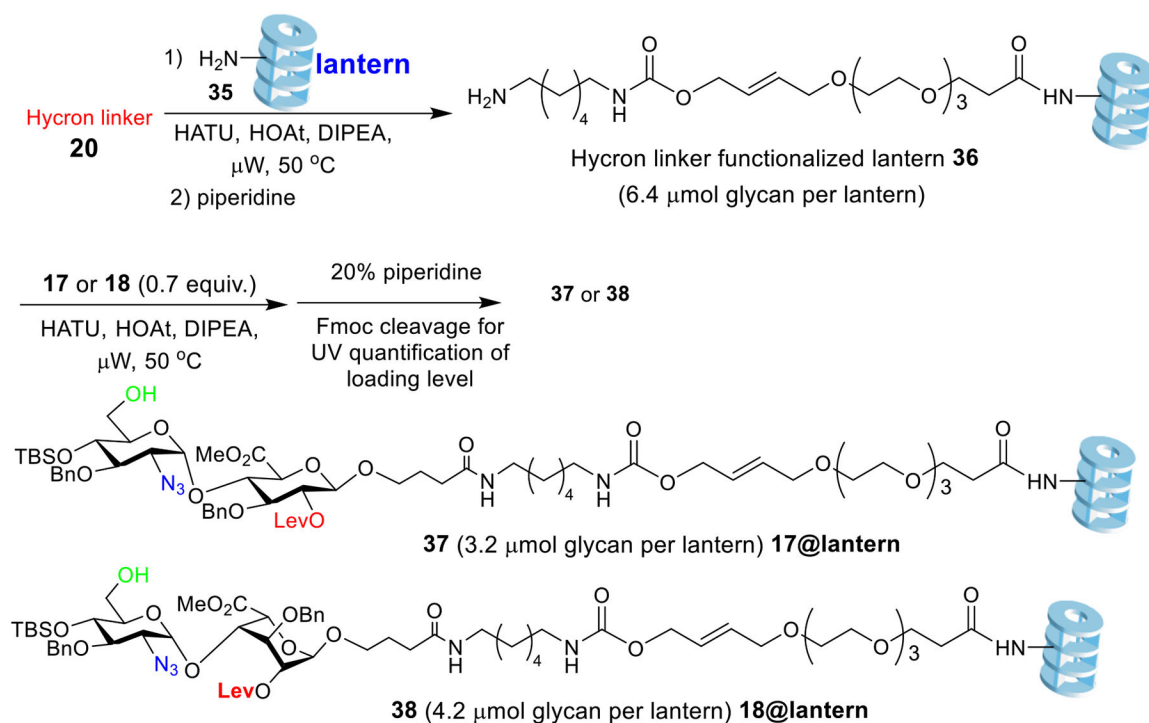
**Scheme 2.**  
Synthesis of the GlcN-GlcA Building Block 17.



**Scheme 3.**  
Synthesis of GlcN-IdoA Building Block **18**.



**Scheme 4:**  
Synthesis of the Hycron Linker **20**.

**Scheme 5.**

Disaccharides **17** and **18** can be efficiently loaded onto amine functionalized lantern **35** through the Hycron linker **20**.



**Table 1.**

Overall yields for the multi-step synthesis of **1–16** from **37** (for compounds **1–8**) and **38** (for compounds **9–16**) respectively.

Compound number	Overall yields from 37 (17@lantern) %	Compound number	Overall yields from 38 (18@lantern) %
1	57.7	9	38.5
2	57	10	39
3	38.9	11	49
4	33.6	12	24
5	33	13	37.5
6	41	14	41.5
7	60	15	47.6
8	42	16	40.2

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