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Controlled Chemoenzymatic Synthesis of Heparan Sulfate Oligosaccharides

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Abstract

A chemoenzymatic approach has been developed for the preparation of diverse libraries of heparan sulfate (HS) oligosaccharides. It employs chemically synthesized oligosaccharides having a chemical entity at a GlcN residue, which in unanticipated manners influences the site of

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Conflict of interest

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modification by NST, C5-Epi/2-OST and 6-OST₁/6-OST₃, thus resulting in oligosaccharides differing in N/O-sulfation and epimerization pattern. The enzymatic transformations defined fine substrate requirements of NST, C5-Epi, 2-OST, and 6-OST.

Keywords

enzymes; epimers; heparan; oligosaccharides; synthetic methods

Heparan sulfates (HSs) are highly sulfated linear polysaccharides that occur on the cell surface and in the extracellular matrix of all animal cells.^[1] The interaction between HSs and proteins is critical for many biological processes, and it has been suggested that by regulating the expression of the HS biosynthetic enzymes, cells can create unique epitopes for the recruitment of specific HS-binding proteins.^[2]

The biosynthesis of HS occurs in the Golgi where a protein-linked heparosan polymer is assembled and composed of alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) moieties.^[3] Discrete regions of the polymer are then modified by *N*-deacetylase/*N*-sulfotransferases (HSN₂Ac/NST), C5 epimerases (C5-Epi), uronosyl 2-*O*-sulfotransferase (2-OST), glucosaminyl 6-*O*-sulfotransferases (6-OST), and 3-*O*-sulfotransferases (3-OST). These modifications are often incomplete, thus creating considerable structural diversity.

Although it is widely accepted that HS is an information-rich polymer, ligand requirements for only a few proteins have been determined.^[4] This deficiency can be addressed by preparing large collections of well-defined HS oligosaccharides for structure–activity relationship studies. Several laboratories have reported elegant chemical syntheses of HS oligosaccharides,^[5] however these approaches are mainly focused on one-compound at the time of preparation. To prepare more diverse libraries of HS oligosaccharides, we have introduced a modular synthetic approach whereby a set of disaccharides, which resemble the differently sulfated disaccharides found in HSs, are used for oligosaccharide synthesis.^[6] The end game, which involves the selective introduction of *O*- and *N*-sulfates, requires a relatively large number of steps, thus hampering the preparation of large collections of compounds. Enzyme-mediated synthesis of HS oligosaccharides requires significantly fewer steps, but because of the promiscuity of the biosynthetic enzymes, it is difficult to control the site of modification.^[7] This deficiency is being addressed by genetically engineering sulfotransferases with alternate substrate specificities,^[8] but despite these new enzymes, mainly highly sulfated compounds with repetitive IdoA-GlcN moieties are obtained.

Herein we describe a chemoenzymatic approach that addresses deficiencies associated with chemical and enzymatic HS synthesis. The premise of the approach is the chemical synthesis of heparosan oligosaccharides in which a given site is modified by a chemical entity that blocks specific enzymatic transformations. In a proof of principle study, we found that methylation of the C6 hydroxy group of a glucosamine residue of a heparosan oligosaccharide (e.g. compounds **2** and **3** in Figure 1) is tolerated by many HS biosynthetic enzymes. Obviously, the methyl ether prevented sulfation of the methylated hydroxy group. Surprisingly, it also blocked epimerization of the GlcA moiety at the +1 site, whereas GlcA at the –1 site could be readily transformed into IdoA. Furthermore, the compounds **1–3**

could be chemically desulfated, and resulfation with NST resulted in di-*N*-sulfated compounds in which the reducing GlcN moiety is unmodified. It was also found that a GlcNS moiety at the reducing end of a hexasaccharide cannot be modified by 6-OST. These observations offer unique possibilities to control the sites of sulfation and epimerization.

The targeted hexasaccharides **1–3** were assembled from the disaccharide building blocks **4**, **5**, and **6** (Scheme 1).^[6] The disaccharide **5** is modified by a levulinoyl ester (Lev) at C6 of GlcN, which at a late stage of the synthesis can be removed for introduction of a methyl ether. We opted for this strategy because previous studies had shown that an ester at C6 of GlcN, in compounds such as in **5** and **6**, is critical for high α -anomeric selectivity.^[6a] Triflic acid catalyzed glycosylations of the glycosyl donors **5** and **6** with the acceptor **4** gave the tetrasaccharides **7** and **8**, respectively, which were treated with triethylamine to remove the Fmoc-protecting group. The resulting glycosyl acceptors **9** and **10** were coupled with **6** and **5**, respectively, to afford the corresponding hexasaccharides **11** and **12**. The Lev ester of **11** and **12** was removed by treatment with hydrazine acetate and the corresponding alcohols **13** and **14** were methylated with TMSiCHN₂/HBF₄ to afford **15** and **16**, respectively. The latter compounds were treated with LiOH/H₂O₂ to remove the base-sensitive protecting groups, with subsequent reduction of the azido functions using PMe₃ in THF/H₂O to give **18** and **19**. In addition, **13** was subjected to base treatment and reduction with PMe₃ to provide the unmethylated hexasaccharide **17**. The amines of **17**, **18**, and **19** were sulfated with pyridinium sulfate, which was followed by catalytic hydrogenation over Pd(OH)₂/C to give the hexasaccharides **20**, **21**, and **22**, respectively. The anomeric aminopentyl moieties of **20–22** were protected as a carboxybenzyl (Cbz) moieties by reaction with benzyl chloroformate to give the corresponding fully *N*-sulfated target compounds **1–3**.

Next, attention was focused on the chemoenzymatic diversification of **1–3**. The compounds **23–25**, having GlcNH₂ moieties, were prepared by hydrolysis of the sulfonamides of **1–3** by conversion of the *N*-sulfates into pyridinium salts followed by solvolysis in a mixture of DMSO and H₂O (Scheme 2).^[9] Treatment of these compounds with NST in the presence of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) gave the di-*N*-sulfated derivatives **26–28**. Mass spectrometry and two-dimensional NMR experiments by COSY and HSQC confirmed the identity of the compounds, and specifically, C2 of the reducing-end GlcN (A-residue, Figure 2) was substantially shifted up-field compared to that of GlcNS residues of the C and E residues. These results highlight that NST is not affected by methylation of a C6 hydroxy group in GlcN, and furthermore a GlcA residue at the +1 site of GlcN is critical for *N*-sulfation. The compounds **26–28** were chemically *N*-acetylated with *N*-acetyl succinimide (AcOSu) in PBS buffer to give **29–31**, respectively, after purification by BioRad P4 size exclusion column chromatography.

C5-Epi is an enzyme that establishes an equilibrium between GlcA and IdoA for residues within sequences containing GlcNS-GlcA-GlcNS/Ac.^[10] By performing the epimerization in the presence of 2-OST, this transformation can be driven to completion because an IdoA2S residue is not recognized by the epimerase, and thus the reverse reaction will not take place.^[7b] Thus, tri-*N*-sulfated compounds **1** and **2**, which have two GlcA moieties flanked by GlcNS, were treated with PAPS and C5-Epi/2-OST to give compounds **32** and

33, respectively in which only one GlcA had been epimerized (Scheme 3). ^1H and HSQC spectra of **33** demonstrated a significant chemical shift change of H1^{E} ($\delta=5.48$ to 5.18 ppm), thus indicating the epimerization had occurred at the central GlcA moiety (Figure 3). Furthermore, $^2\text{DNOESY}$ experiments showed crosstalk between H1^{E} and $\text{H4}^{\text{D}}/\text{H3}^{\text{D}}$, thereby unambiguously confirming the structure of the product. These results highlight that GlcA at the +2 position is critical for enzyme recognition. Interestingly, the presence of a 6-*O*-methyl ether of GlcN at the +1 site of GlcA, as in **2**, does not interfere with the epimerization and 2-*O*-sulfation. In contrast, methylation of GlcN at the -1 site, as in **3**, resulted in a complete block of epimerization and 2-*O*-sulfation. As expected, treatment of **29** and **30** with PAPS and C5-Epi/2-OST gave **34** and **35**, respectively, while **31** remained unmodified because of the presence of a 6-OMe at the -1 site of GlcA (Scheme 3).

Next, sulfation of the oligosaccharides by 6-OST, which are enzymes that require *N*-sulfation of the targeted GlcN moiety, was explored.^[11] Treatment of **1** with a mixture of 6-OST₁/6-OST₃ in the presence of PAPS gave the di-6-*O*-sulfated derivative **36** (Scheme 4). Interestingly, the GlcNS at the reducing end had remained unmodified as confirmed by diagnostic fragments such as B₄, Y₂, B₅, Y₁, C₄, C₅, and $^{2,4}\text{A}_6$ in the MS₂ spectrum and a 2D TOCSY NMR experiments (see Figures S6 and S7). These results indicate that these enzymes require, in addition to GlcNS, a GlcA residue at the +1 site. This observation is in agreement with a recently reported cocrystal structure of 6-OST₃ with an octosaccharide and it showed that the majority of the interactions were made with the acceptor GlcNS and the adjacent disaccharide GlcA-GlcNS on the reducing side.^[12] Next, **3**, **31**, **2**, and **33** were subjected to 6-OST and PAPS, and as expected the derivatives **37–40**, having a single 6-*O*-sulfate at different positions, were readily obtained.

In summary, enzymatic modifications of three chemically synthesized hexasaccharides, with or without a 6-*O*-methyl ether on a GlcN residue, with NST, C5-Epi/2-OST, and 6-OST₁/6-OST₃ provided a library of 21 hexasaccharides that differ in *N/O*-sulfation and epimerization pattern. It is known that consecutive GlcA moieties that are flanked by GlcNS residues are randomly modified by C5-Epi and even in the presence of 2-OST, only a mixture of partially epimerized products is obtained.^[13] Therefore, current enzymatic approaches can only provide HS sequences with repetitive IdoA moieties,^[7c] and therapeutically valuable targets with discrete epimerization patterns are out of reach.^[13] Our discovery that a C6 masking group at GlcN allows epimerization of only one of the neighboring GlcA residues, is laying the foundation for development of next-generation chemoenzymatic synthesis of HS oligosaccharides with control over epimerization and sulfation. In this respect, it is known that the enzyme PmHS2 can employ UDP-GlcNAc derivatives in which C6 of GlcNAc is modified and thus can provide heparanose oligosaccharides with modified GlcN residues.^[14] In this study, a methyl ether was chosen to mask C6 of a GlcN residue. Future studies will focus on the identification of chemical modifications which can be removed to achieve even greater synthetic flexibility.

Supplementary Material

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Acknowledgments

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Figure 1.
The hexasaccharides **1–3**.

Figure 2.
HSQC and COSY experiments for structure elucidation of **28**.

Figure 3.
 ^1D proton (a) and ^2D NOESY (b) experiments for structure elucidation of **33**.

Scheme 1.

Chemical synthesis of the HS backbones **1–3**. Reagents and conditions: a) TfOH, CH₂Cl₂, –20°C, 51% for **7**, 50% for **8**, 57% for **11**, 60% for **12**; b) Et₃N, CH₂Cl₂; c) N₂H₄, AcOH, MeOH/CH₂Cl₂ (1:1); d) TMSCHN₂, HBF₄, CH₂Cl₂, 60%; e) LiOH, H₂O₂, NaOH; f) PMe₃, NaOH, THF; g) SO₃-Py, Et₃N, NaOH, CH₃OH, H₂O; h) H₂, Pd(OH)₂, H₂O, 67% for **21**, 33% for **22**, 50% for **20** in 4 steps; i) CbzCl, K₂CO₃, H₂O, 48% for **1**, 80% for **2**, 91% for **3**. Py=pyridine, Tf=trifluoromethanesulfonyl, THF=tetrahydrofuran.

Scheme 2.

Enzymatic *N*-sulfation and chemical acetylation of hexasaccharides. Reagents and conditions: a) 1. AG 50W-X8 resin (pyridinium form); 2. DMSO/H₂O (95:5), 60% for **23**, 80% for **24**, 72% for **25**; b) PAPS, NST, MES (50 mM, pH 7.1); c) AcOSu, PBS (pH 7.4), 65% for **29**, 45% for **30**, 100% for **31**.

Scheme 3.

Selective epimerization/2-*O*-sulfation. Reagents and conditions: a) PAPS, C5-epi/2-OST, Tris (25 mM, pH 7.5).

Scheme 4.

Selective enzymatic 6-*O*-sulfation. Reagents and conditions: a) PAPS, 6-OST, MES (50 mM, pH 7.1).