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ORIGINAL ARTICLE

Chemical *O*-sulfation of *N*-sulfoheparosan: a route to rare *N*-sulfo-3-*O*-sulfoglucosamine and 2-*O*-sulfoglucuronic acid

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Abstract

Heparosan, the capsular polysaccharide of *E. coli* K5 is currently used as the starting material in the chemoenzymatic synthesis of heparan sulfate and the structurally related anticoagulant drug heparin. Base hydrolysis of *N*-acetyl groups and their subsequent *N*-sulfonation, are used to prepare *N*-sulfoheparosan an intermediate of biosynthesis. In the present study, when excess sulfonation reagent was used during *N*-sulfonation, some *O*-sulfation also took place in the *N*-sulfoheparosan product. After a nearly full digestion, a hexasaccharide fraction exhibited resistance to heparin lyase II. Excessive digestion by heparin lyase II and structural identification by NMR and mass spectroscopy indicated that the resistant hexasaccharide fraction has two structures, ΔUA-GlcNS-GlcA2S-GlcNS-GlcA-GlcNS and ΔUA-GlcNS-GlcA- GlcNS3S-GlcA-GlcNS in similar amounts. The 2-sulfated structure exhibited partial resistance to heparin lyase II; however the structure of ΔUA-GlcNS-GlcA-GlcNS3S was completely resistant to heparin lyase II.

Keywords Chemical *O*-sulfation · *N*-sulfoheparosan · 2-*O*-sulfoglucuronic acid · *N*-sulfo-3-*O*-sulfoglucosamine · Resistance to heparin lyase II

Introduction

Heparan sulfate and the structurally related heparin are biosynthesized in the Golgi compartments of animal cells [1]. Both of these glycosaminoglycans play critical biological roles in regulating cell growth and differentiation [2], as well as impacting infectious disease [3]. Heparin has an important pharmacological role as a frequently used clinical anticoagulant drug [4].

Heparosan, the capsular polysaccharide of *Escherichia coli* K5, has the repeating structure [→4) β-D-glucuronic acid (GlcA) (1 → 4) *N*-acetyl-α-D-glucosamine (GlcNAc) (1→] (Fig. 1a) and is currently used as the starting material in the chemoenzymatic synthesis of heparan sulfate and heparin [5].

N-sulfoheparosan (NSH) has the repeating structure [→4) β-D-GlcA (1 → 4) *N*-sulfo-α-D-GlcN (1→] (Fig. 1b) and is an early intermediate in the biosynthesis of heparan sulfate and heparin [6]. The heparosan polysaccharide (Fig. 1a) is modified through the action of *N*-deacetylase-*N*-sulfotransferases 1 and 2 [6]. These are relatively large bifunctional enzymes that have not been successfully prepared in bacterial expression systems [7]. Thus, chemical methods that rely on base hydrolysis of *N*-acetyl groups and their subsequent *N*-sulfonation are used to prepare NSH [8]. By controlling these reactions, hybrid structures, common to heparan sulfate and heparin can be prepared. Heparan sulfate and heparin primarily differ by their ratio of GlcNAc and GlcNS residues [9]. Moreover, heparan sulfate and heparin contain different contents of other frequent modifications, including epimerization of glucuronic acid to iduronic acid (IdoA) [10] and *O*-sulfation of the 2-position of IdoA residues and the 6-position of the GlcNAc or GlcNS residues [11]. In addition to these major modifications, there exist less frequent modifications such as 3-*O*-sulfation of the GlcNS residues and 2-*O*-sulfation of the GlcA residues. In biosynthesis or in vitro enzymatic synthesis, both of these rare modifications have been

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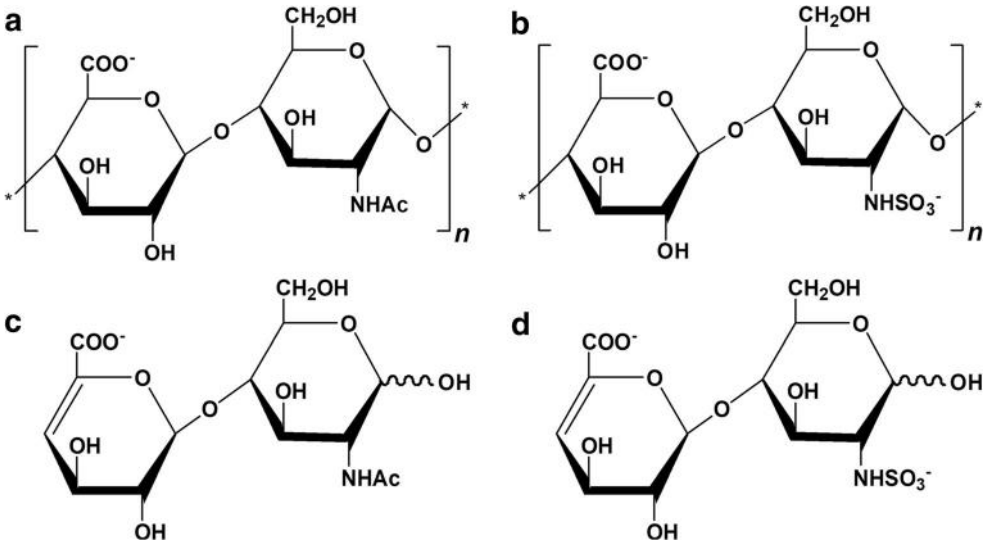

Fig. 1 Structure of (**a**) heparosan and (**b**) *N*-sulfoheparosan (NSH) as well as (**c**) heparosan disaccharide (ΔUA-GlcNAc) and (**d**) NSH disaccharide (ΔUA-GlcNS) digested by heparin lyases

made enzymatically by glucosamine 3-*O*-sulfotransferase or uronic acid 2-*O*-sulfotansferase, respectively [12, 13]. These rare modifications are critical for a number of biological and pharmacological activities of heparan sulfate and heparin. The *N*-sulfo-3-*O*-sulfoglucosamine (GlcNS3S) in heparin is the central residue of the heparin antithrombin III (AT) binding site [14]. While there are a number of structural variants of the AT-binding site in heparin [15], this is one of the most thoroughly studied and most specific glycan-binding site. The GlcNS3S residues in nerve tissue heparan sulfate are also important in the heparan sulfate co-receptor for herpes simplex virus in the brain [16]. A heparan sulfate rich in GlcNS3S residues is also important in ovulation and human fertility [17]. Less is known about the biological functions of the 2-*O*-sulfoglucuronic acid (GlcA2S) residues of heparan sulfate. This rare modification of 2-*O*-sulfation of a GlcA residue, discovered in nuclear heparan sulfate, was implicated in the growth state of hepatocytes [18]. Recent studies have shown that a heparan sulfate hexasaccharide with a GlcA2S residue could selectively activate heparin cofactor II, regulating the blood coagulation pathway [19]. The current study examines the selective introduction of GlcNS3S and GlcA2S residues into NSH using a simple chemical sulfation reaction to afford the rare structural features into a glycosaminoglycan chain.

Materials and methods

Materials

Porcine intestinal heparin (molecular weight (*M*w) = 16 kDa) was purchased from Celsus Laboratories (Cincinnati, OH). Unsaturated heparin/heparan sulfate disaccharide standards (ΔUA-GlcN (free amine); ΔUA-GlcNAc (0S); ΔUA-GlcNS (NS); ΔUA-GlcNAc6S (6S); ΔUA2S-GlcNAc (2S); ΔUA-GlcNS6S (NS6S); ΔUA2S-GlcNS (NS2S); ΔUA2S-GlcNAc6S (2S6S); ΔUA2S-GlcNS6S (TriS)) were purchased from Iduron (Manchester, UK). *E. coli* expression and purification of the recombinant *Flavobacterium heparinum* heparin lyase I, II, III (Enzyme Commission (EC) #s 4.2.2.7, 4.2.2.X, 4.2.2.8) were prepared in our laboratory as previously described [20].

Disaccharide analysis by HPLC

Samples (200 μg) were completely digested for 12 h using a heparinase I, II and III (10 mU each) mixture in 200 μL of 50 mM ammonium acetate buffer (pH 7.2) at 37 °C. The resulting disaccharides and tetrasaccharides were recovered using 3 K Da MWCO membrane centrifugal ultrafiltration units (Millipore, MA) and washed with deionized water. The ultrafiltrates containing disaccharides were collected and freeze-dried for disaccharide analysis. Disaccharide analysis was carried out with high-pressure liquid chromatography (HPLC)–ultraviolet spectrometry. The analyses were performed with a Shimadzu LC-20 AD pump, Shimadzu CBM-20A controller, Shimadzu SIL-20AHT auto-sampler, Shimadzu CTO-20 AC column oven and a Shimadzu SPD-20AV UV detector (Shimadzu, Kyoto, Japan). A Spherisorb-SAX chromatography column (4.0 × 250 mm, 5.0 μm, Waters) was equilibrated with 1.8 mM monobasic sodium phosphate (mobile phase A, pH 3.0) and followed with ingredient elution after injection using 1.8 mM monobasic sodium phosphate with sodium perchlorate (mobile phase B, pH 3.0). The weight/weight (*w*/w) percentage for each disaccharide was calculated. Peak areas were used for quantification.

High performance gel permeation chromatography (HPGPC) profiles of NSH oligosaccharides

HPGPC of digested supernatant was profiled on a column (10 × 300 mm) of Superdex Peptide 10/300 GL (GE Healthcare, Chicago, IL). Elution was performed with 0.2 M NH_4HCO_3 at a flow rate 0.4 mL/min, and was monitored with a refractive index detector.

Nuclear magnetic resonance (NMR) spectroscopy

Samples were dissolved in 500 μL of D_2O (99.9%) and lyophilized three times to substitute the exchangeable protons with deuterium, and then transferred to NMR microtubes after dissolving in 500 μL D_2O. ^1H, ^{13}C, ^1H-^1H COSY, ^1H-^1H TOCSY, ^1H-^{13}C HSQC NMR spectra were recorded on Bruker 600 spectrometer (Madison, WI, USA) with topspin 3.2 software at 298.15 K.

Mass spectroscopy

Samples were dissolved in HPLC grade water as 0.2–0.5 μg/μL and 5 μL were directly injected to the standard ESI source of LTQ-Orbitrap XL FTMS (Thermo Fisher Scientific, San-Jose, CA). LC parameter: mobile phase A was 5-mM ammonium acetate prepared with HPLC grade water, and mobile phase B was 5-mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. The flow was used 50% A and 50% B at a rate of 250 μL/min. The source parameters for FTMS were in the negative-ion mode, a spray voltage of 4.2 kV, a capillary voltage of −40 V, a tube lens voltage of −50 V, a capillary temperature of 275 °C, a sheath flow rate of 30 L/min and an auxiliary gas flow rate of 6 L/min. All FT mass spectra were acquired at a resolution 60,000 with 200–2000 Da mass range.

Results and discussion

Chemical preparation of NSH with excess sulfonation reagent

Heparosan, prepared by the fermentation of *E. coli* K5 on glucose was recovered in the supernatant [21]. After desalting and precipitation from isopropanol (50 vol%) the recovered heparosan had a *M*w of 41.0 kDa. Compositional analysis by treatment of heparosan with heparin lyase I, II & III followed by HPLC [22] afforded a single disaccharide ΔUA-GlcNAc (Fig. 1c, where ΔUA is a 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) (Table 1). Treatment with aqueous sodium hydroxide was used to hydrolyze 99.4% of the *N*-acetyl groups in heparosan to form completely de-*N*-acetylated heparosan. The resulting product showed a reduced *M*w of 13.3 kDa as a result of β-elimination [8]. Without isolation this intermediate was *N*-sulfated with sulfur trioxide trimethylamine complex. When a small stoichiometric excess of sulfonation reagent was used, pure NSH was formed with disaccharide compositional analysis showing only ΔUA-GlcNS (Fig. 1d) and ΔUA-GlcNAc. When a large excess of sulfonation reagent (1.5 g sulfur trioxide trimethylamine complex per g of heparosan) was used, some *O*-sulfation also took place as indicated by multiple disaccharide products (Table 1). Based on retention time, the minor disulfated disaccharide products appeared to result primarily from 2-*O*-sulfation of the GlcA residue of NSH. However, it is known that the glycosidic linkage in heparin and heparan sulfate downstream (towards the non-reducing end) of a 3-*O*-sulfoglucosamine residue is resistant to heparin lyases affording tetrasaccharides [23]. Since their resistant tetrasaccharides were not observable in disaccharide analysis and, thus are not reported in Table 1, we were unable to ascertain whether the 3-position in *N*-sulfoglucosamine residues were susceptible to chemical sulfonation. Thus, we undertook the analysis of heparin lyase resistant oligosaccharide formed after complete digestion.

Digestion of NSH by heparin lyase II

We examined the HPGPC profiles of digested NSH to examine the change of *M*w distribution from partial digestion to full digestion (Fig. 2). Heparin lyase II acts on heparan sulfate, heparin and related polysaccharides, such as biosynthetic intermediates like NSH, at the GlcNS-GlcA linkage [24] to afford a series of oligosaccharides with an even number of saccharide residues and a ΔUA residue at their non-reducing end were released during the digestion of NSH. Most of these oligosaccharides showed a side peak at the left of the main peak, indicating a mixture of higher *M*w compounds. These side peaks were produced and consumed during the course of enzymatic digestion. As the digestion was in its middle stage (the blue line in Fig. 2), we observed a build-up of a side peak along with the major octasaccharide (dp8). As the digestion completed, the side peak of dp8 disappeared and the side peak associated with a hexasaccharide (dp6) increased showing resistance to heparin lyase II at full digestion. This suggests that the dp6 side peak arises from the dp8 side peak. It appears that the dp6 side peak could be digested into a tetrasaccharide (dp4) side peak and a disaccharide (dp2) with exhaustive heparin lyase II treatment. The partial resistance of the dp6 side peak and the complete resistance of the dp4 side peak inform us of the selectivity of heparin lyase II for the NSH substrate.

Preparation and structure analysis of the dp6 side peak

We next scaled up complete digestion to prepare and separate the heparin lyase II resistant dp6 side peak by a gel

Table 1 Disaccharide analysis of glycosaminoglycans (mole percent)

Disaccharide varieties	Glycosaminoglycans			
	Heparosan	NSH	Heparan sulfate[a]	Heparin[b]
ΔUA-GlcN (free amine)	0	<0.01	<0.01	<0.01
ΔUA-GlcNAc (0S)	100.0	0.3	3.0–31.0	4.5
ΔUA-GlcNS (NS)	0	98.0	0.4–6.5	3.4
ΔUA-GlcNAc6S (6S)	0	0.0	0.2–2.6	3.5
ΔUA2S-GlcNAc (2S)	0	0.3	0–0.2	1.8
ΔUA-GlcNS6S (NS6S)	0	0.4	0.1–2.7	10.6
ΔUA2S-GlcNS (NS2S)	0	0.8	0.1–3.1	7.7
ΔUA2S-GlcNAc6S (2S6S)	0	0	0–0.02	1.4
ΔUA2S-GlcNS6S (TriS)	0	0	0–0.4	67.0

[a] Wide ranges of values are observed in human heparan sulfate for comparison

[b] Commercial anticoagulant heparin average value obtained for comparison

filtration chromatography on a 1.0 × 120 cm column (BIO-RAD, Moscow, Russian Federation) packed with Superdex 30 Prep Grade resin (GE Healthcare, MA, USA). The fraction corresponding to the dp6 side peak was analyzed by mass spectrometry (Fig. S1) and had an *M*w corresponding to NSH dp6 + SO_3. The position of the additional *O*-sulfation was next determined using ^{1}H-NMR spectroscopy. The normal dp2, dp4 and dp6 fractions from the partial digestion were also collected and analyzed for comparison.

The ^{1}H NMR spectra of NSH dp2 to dp6 having the structures ΔUA-(GlcNS-GlcA-)$_n$GlcNS, $n = 0$, 1 and 2, respectively, are shown in Fig. 3, and the signals of the H1, H3 and H4 of the ΔUA residue were easily assigned. The anomeric protons from all other residues were also easily distinguishable and assignable. The GlcNS residue at the reducing end of NSH dp2, dp4 and dp6 has a different anomeric chemical shift than the other GlcNS residues. The anomeric protons of the remaining GlcNS residues shared the same chemical shift. The GlcA residue closest to the reducing end showed a slightly downfield shifted chemical shift of anomeric proton than the central GlcA residues. On the basis of the above shift values, we were able to make a preliminary assignment for the position of the additional *O*-sulfation group on the dp6 + SO_3. The anomeric proton signals of the ^{1}H NMR spectrum of the central GlcNS residues were divided into four groups showing similar intensity. The significantly increased signal intensity at 4.64 ppm was assignable to the anomeric proton of a GlcA2S residue and the new signal at 4.34 ppm was assignable to the anomeric proton of a GlcNS3S or GlcNS6S residue [12]. Thus, we suggest that *O*-sulfation could be found on any one residue with the exception of the ΔUA residue at the non-reducing end and there are at least two structures of hexaose in dp6 + SO_3 containing a single *O*-sulfation group.

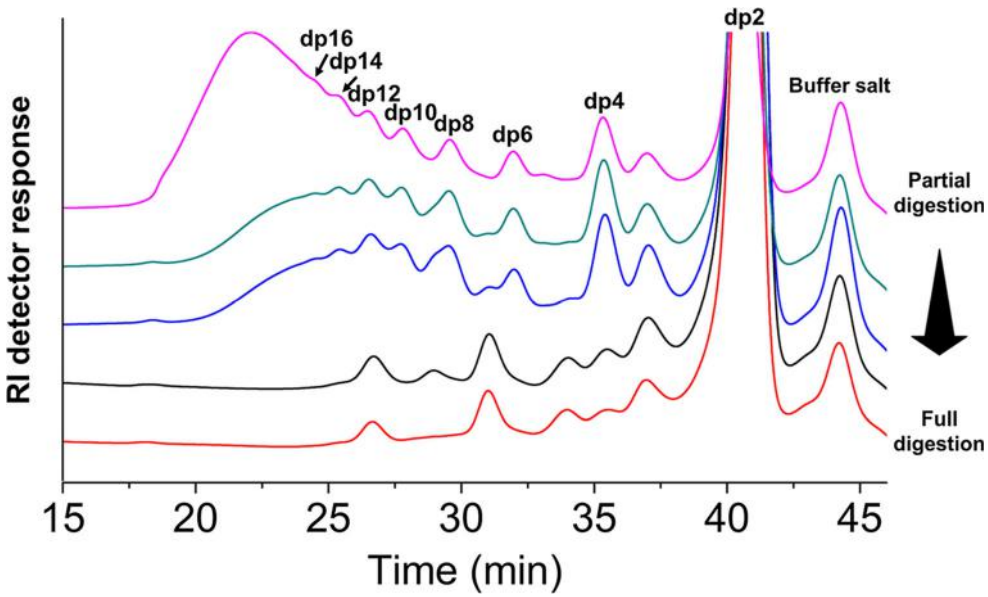

Fig. 2 HPGPC profiles of gradual digestion of NSH by heparin lyase II. NSH (5 mg) was dissolved in 500 μL digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride, pH 7.2), and then 0.1 U heparin lyase II was added. The reaction was incubated at 37 °C, and after every 30 min, a 100 μL aliquot of digestion solution was taken out and heated for deactivation and precipitation of the enzyme. The digested supernatant was profiled by HPGPC

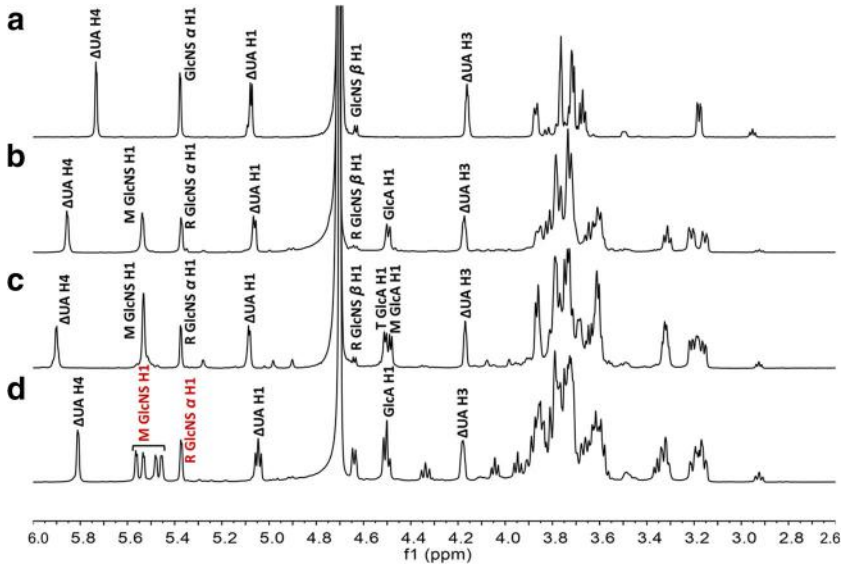

Fig. 3 ^{1}H NMR spectrum of (**a**) NSH dp2, (**b**) NSH dp4, (**c**) NSH dp6 and (**d**) NSH dp6 + SO_3. The dark red marked saccharide residues indicated an additional *O*-sulfation group on one of them. The NSH dp2, dp4 and dp6 were first verified by their mass spectra as shown in Fig. S2. For the example of dp6, as a structure of ΔUA-GlcNS-GlcA-GlcNS-GlcA-GlcNS, we marked the reducing end GlcNS residue, other GlcNS residues, the terminal GlcA residue (close to the reducing end) and central GlcA residue as 'R GlcNS', 'M GlcNS', 'T GlcA' and 'M GlcA', respectively. The reducing end GlcNS residue includes α- and β-configurations

Digestion again of the NSH dp6 + SO_3 fraction

We relied on extended digestion with heparin lyase I and II for the analysis of the structures present in the NSH dp6 + SO_3 fraction. Heparin lyase I could not cleave the hexasaccharides in the NSH dp6 + SO_3 fraction (Fig. 4), which may due to their insufficient level of *O*-sulfation or absence of IdoA [25]. In contrast, heparin lyase II cleaved the entire NSH dp6 + SO_3 fraction into smaller oligosaccharides. These smaller oligosaccharides were identified using mass spectrometry as NSH dp4 + SO_3, NSH dp2 + SO_3 and NSH dp2 (Fig. S3). In the HPGPC profile the area ratio of NSH dp4 + SO_3, NSH dp2 + SO_3 and NSH dp2 was approximately 2: 1: 3, similar to their molar ratio. Thus, we inferred that for about half of the NSH dp6 + SO_3, was digested to afford an NSH dp4 + SO_3 and an NSH dp2 molecule. The other half of the NSH dp6 + SO_3, was digested to afford an NSH dp2 + SO_3 and two NSH dp2 molecules.

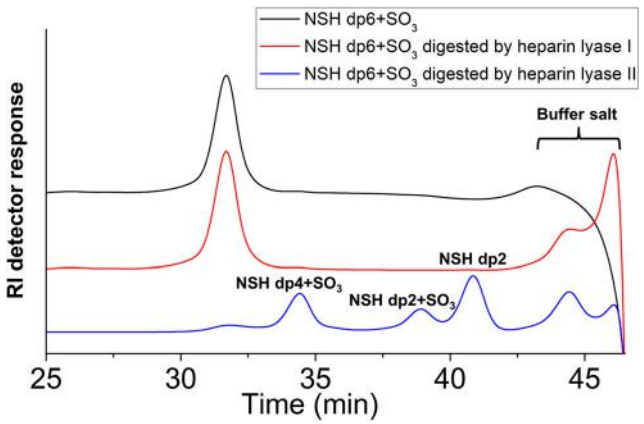

Fig. 4 HPGPC profiles of the NSH dp6 + SO_3, NSH dp6 + SO_3 that was digested by heparin lyase I and NSH dp6 + SO_3 that was digested by heparin lyase II. NSH dp6 + SO_3 fraction (1 mg) was dissolved in 100 μL digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride, pH 7.2), and then 0.1 U heparin lyase I or II was added. The reaction was incubated overnight at 37 °C. After the incubation, the digestion solution was heated to deactivate and precipitate the enzyme and the digested supernatant was profiled by HPGPC

Structural analysis of the NSH dp2 + SO_3 fraction and the NSH dp4 + SO_3 fraction

It is possible to determine the structure of the dp6 + SO_3 by solving the structures of the heparin lyase II derived dp2 + SO_3 fraction and dp4 + SO_3 fraction. First, we compared the ^{1}H NMR spectra of the NSH dp2 + SO_3 and dp4 + SO_3 with the spectra of NSH dp2 and dp4 shown in Fig. 5. Both NSH dp2 + SO_3 and dp4 + SO_3 fractions contained only a single structure (NSH dp2 + SO_3 contained a small amount of NSH dp2) based on the ^{1}H NMR and its mass spectrum (Fig. S3B). In the ^{1}H NMR spectrum of the NSH dp2 + SO_3, we found the chemical shifts of the GlcNS H1 were not altered but the chemical shifts of the ΔUA H1 and H4 were shifted downfield. This indicates that the additional *O*-sulfation is on the ΔUA residue. A new signal was also observed at about 4.47 ppm. In the ^{1}H NMR spectrum of the NSH dp4 + SO_3, we also found these same easily identifiable proton signals including a new signal at 4.43 ppm.

We next used 2D NMR spectra to analyze the structures of both NSH dp2 + SO_3 and NSH dp4 + SO_3. Based on ^{1}H-^{1}H

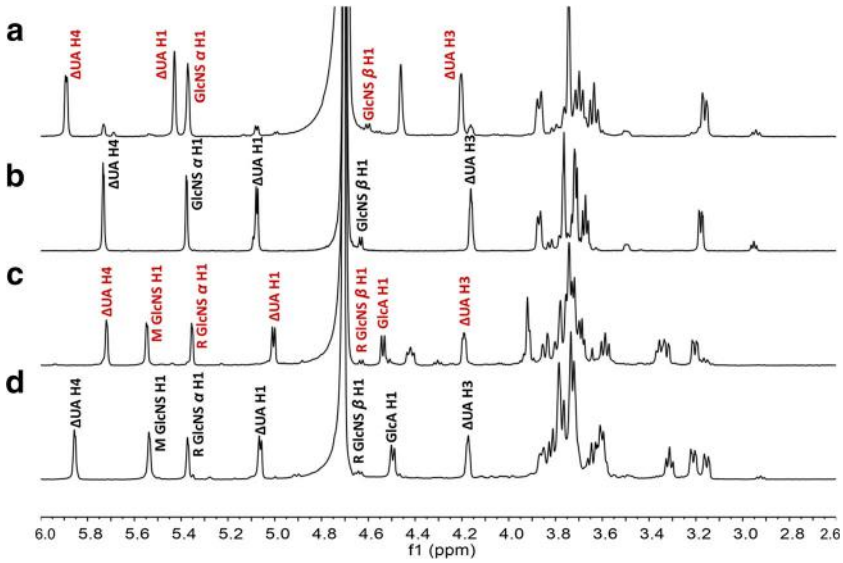

Fig. 5 ^{1}H NMR spectrum of (**a**) NSH dp2 + SO_3 fraction, (**b**) normal NSH dp2, (**c**) NSH dp4 + SO_3 fraction and (**d**) normal NSH dp4. The dark red marked saccharide residues indicated an additional *O*-sulfation group on one of them

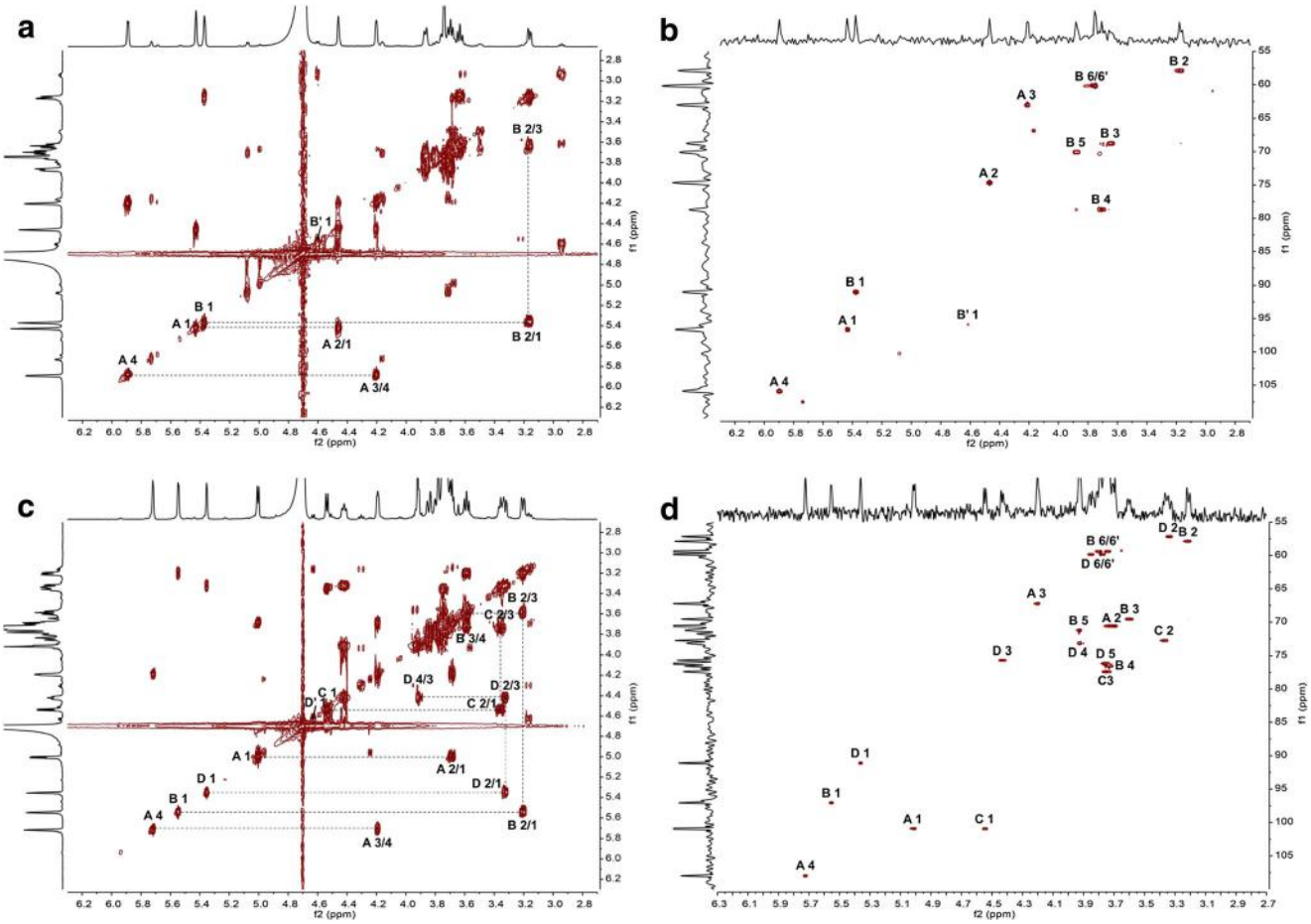

Fig. 6 (**a**) COSY spectrum of NSH dp2 + SO_3 (ΔUA2S-GlcNS), (**b**) HSQC spectrum of NSH dp2 + SO_3 (ΔUA2S-GlcNS), where A and B represent the residues of ΔUA2S and GlcNS, respectively. (**c**) COSY spectrum of NSH dp4 + SO_3 (ΔUA-GlcNS-GlcA-GlcNS3S), (**d**) HSQC spectrum of NSH dp4 + SO_3 (ΔUA-GlcNS-GlcA-GlcNS3S), where A, B, C and D represent the residues of ΔUA, central GlcNS, GlcA and reducing end GlcNS3S, respectively

correlation spectroscopy (COSY) of the NSH dp2 + SO_3 (Fig. 6a), the new signal at 4.47 ppm could be assigned to the ΔUA H2 and the ΔUA H3 signal was not shifted. Thus, NSH dp2 + SO_3 has the structure ΔUA2S-GlcNS, and this result is consistent with the disaccharide analysis in Table 1. The ^{1}H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) (Fig. 6b) compares the ^{1}H and ^{13}C chemical shifts for NSH dp2 + SO_3 (ΔUA2S-GlcNS) and NSH dp2 (ΔUA-GlcNS) (Table 2).

The ^{1}H-^{1}H-correlation spectroscopy (COSY) of the NSH dp4 + SO_3 (Fig. 6c) showed a new signal at 4.43 ppm corresponding to the reducing end GlcNS residue H3. Since the signal of reducing end GlcNS residue H3 differs from that of NSH dp4 based on the 3.65 ppm (Fig. S4C), we could conclude the additional *O*-sulfation was on the C3 of the reducing end GlcNS residue. Thus, the NSH dp4 + SO_3 has the structure of ΔUA-GlcNS-GlcA-GlcNS3S. These results are consistent with our previous publication [15] showing that tetrasaccharides with 3-*O*-sulfation at the reducing end had downstream linkages that are resistant to cleavage by heparin lyase II. The ^{1}H-^{13}C-heteronuclear single quantum coherence spectroscopy (HSQC) (Fig. 6d) afforded ^{1}H and ^{13}C chemical shifts for NSH dp4 + SO_3 (ΔUA-GlcNS-GlcA-GlcNS3S) as well as those for NSH dp4 (ΔUA-GlcNS-GlcA-GlcNS) (Table 2).

Determination the structure of NSH dp6 + SO_3 fraction

Approximately half of the NSH dp6 + SO_3 fraction contained a structure that was digested with exhaustive treatment by heparin lyase II into one disaccharide with the structure ΔUA2S-GlcNS and two disaccharides with the structure ΔUA-GlcNS. These disaccharides could be derived from either ΔUA-GlcNS-GlcA2S-GlcNS- GlcA-GlcNS or ΔUA-GlcNS-GlcA-GlcNS-GlcA2S-GlcNS. ^{1}H NMR spectroscopy of the NSH dp6 + SO_3 fraction (Fig. 3d), showed signals for the GlcA H1 at 4.50 ppm were comprised of a strong signal for the terminal GlcA H1 and a weak signal for the central GlcA H1. Thus, the signal of the GlcA2S H1 at 4.64 ppm was shifted from that of the central GlcA H1 as it was *O*-sulfated at C2. Thus, we confirmed that one of the NSH dp6 + SO_3 structures was ΔUA-GlcNS-GlcA2S-GlcNS-GlcA-GlcNS.

The second half of the NSH dp6 + SO_3 fraction showed a structure that afforded a tetrasaccharide of ΔUA-GlcNS-GlcA-GlcNS3S and a disaccharide of ΔUA-GlcNS when exhaustively digested with heparin lyase II. Thus, we could assign its structure as either ΔUA-GlcNS-GlcA-GlcNS-GlcA-GlcNS3S or ΔUA-GlcNS-GlcA-GlcNS3S- GlcA-GlcNS. The COSY spectrum of the NSH dp6 + SO_3 fraction (Fig. 7) showed a key signal at 4.34 ppm, corresponding to the GlcNS3S H3, which correlated to a signal of anomeric proton belonging to a central GlcNS residue rather than a reducing end GlcNS residue. Thus, we could confirm that the second structure of the NSH dp6 + SO_3 fraction could be assigned as ΔUA-GlcNS-GlcA-GlcNS3S-GlcA- GlcNS.

Conclusion

In the current study, a nearly fully heparin lyase II-digested NSH afforded an NSH dp6 fraction having one additional *O*-

Table 2 Chemical shifts (ppm) of the residues from NSH oligosaccharides [a]

NSH oligosaccharides	Residues	Chemical shifts (ppm)					
		H1α H1β (C1α) (C1β)	H2 (C2)	H3 (C3)	H4 (C4)	H5 (C5)	H6/6′ (C6)
NSH dp2 + SO_3 (ΔUA2S-GlcNS)	ΔUA2S	5.44 (96.7)	**4.47 (74.7)** [c]	4.21 (63.0)	5.90 (105.9)	/	/
	GlcNS	5.38 4.61 (91.0) (95.8)	3.18 (57.9)	3.66 (68.8)	3.71 (78.7)	3.88 (70.1)	3.80/3.76 (60.2)
NSH dp2 (ΔUA-GlcNS) [b]	ΔUA	5.09 (100.3)	3.74 (70.2)	4.17 (66.7)	5.78 (108.0)	/	/
	GlcNS	5.38 4.64 (91.0) (95.8)	3.20 (57.9)	3.68 (69.2)	3.73 (78.3)	3.88 (70.2)	3.82/3.77 (60.0)
NSH dp4 + SO_3 (ΔUA-GlcNS-GlcA-GlcNS3S)	ΔUA	5.01 (100.9)	3.71 (70.6)	4.20 (67.2)	5.72 (108.0)	/	/
	M GlcNS	5.55 (97.1)	3.22 (57.9)	3.60 (69.5)	3.73 (76.6)	3.93 (71.2)	3.81/3.75 (59.4)
	GlcA	4.55 (101.0)	3.37 (72.8)	3.75 (77.4)	ND [d]	ND [d]	/
	R GlcNS3S	5.36 4.63 (91.1) (96.7)	3.34 (57.2)	**4.43 (75.7)** [c]	3.93 (73.2)	3.76 (76.2)	3.84/3.79 (59.8)
NSH dp4 (ΔUA-GlcNS-GlcA-GlcNS) [b]	ΔUA	5.07 (100.5)	3.72 (70.7)	4.18 (66.7)	5.86 (109.2)	/	/
	M GlcNS	5.54 (97.3)	3.23 (57.8)	3.61 (69.3)	3.78 (75.9)	3.87 (70.0)	3.79/3.74 (59.4)
	GlcA	4.51 (102.1)	3.32 (72.7)	3.74 (77.5)	ND [d]	ND [d]	/
	R GlcNS	5.38 4.65 (90.9) (95.7)	3.17 (57.7)	3.65 (69.3)	3.74 (76.4)	3.83 (75.6)	3.85/3.79 (60.0)

[a] The chemical shifts of the reducing end in β-configuration were only shown H1 (C1), since others were too weak to find in the spectra

[b] The chemical shifts of the normal NSH dp2 and dp4 were from their 2D NMR spectra (Fig. S4)

[c] The chemical shifts highlighted in bold were shifted downfield, indicating the position of the additional *O*-sulfation

[d] ND, not detected

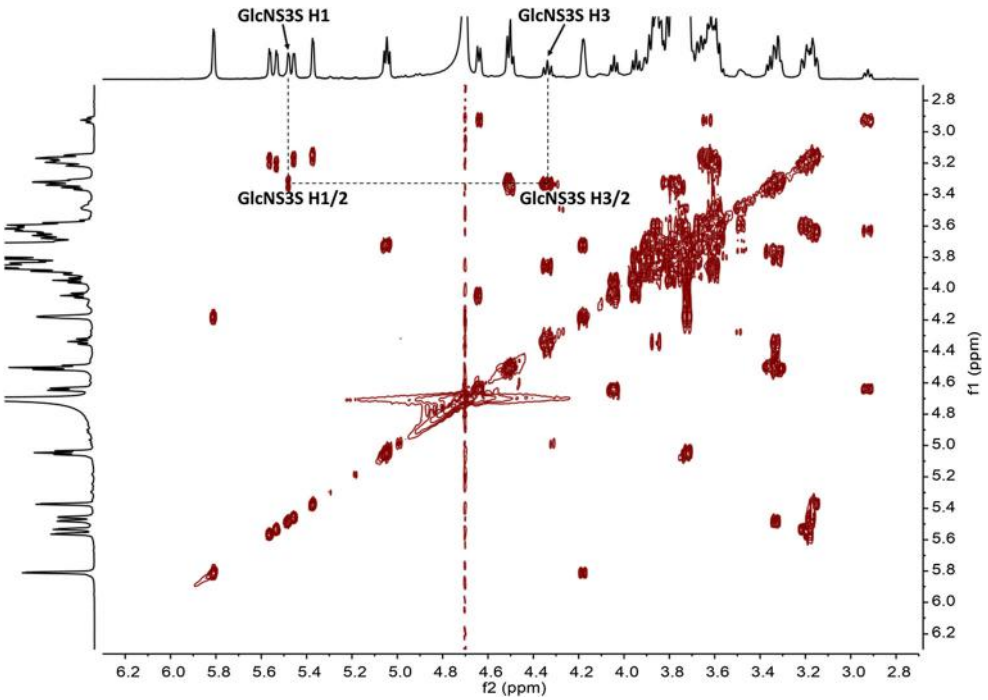

Fig. 7 COSY spectrum of the NSH dp6 + SO_3 fraction

sulfation group. After exhaustive digestion and analysis by mass and NMR spectroscopy, this fraction was found to contain two structures, ΔUA-GlcNS-GlcA2S-GlcNS-GlcA-GlcNS and ΔUA-GlcNS-GlcA-GlcNS3S-GlcA-GlcNS. Our study indicates that the important and rare modification of 3-*O*-sulfation on the GlcNS residues and 2-*O*-sulfation on the GlcA residues could be achieved during the chemical *N*-sulfation process. It provides the possibility for these two *O*-sulfation modifications as an easy chemical method during synthesizing heparan sulfate and heparin. We also found the preference of heparin lyase II on substrates. The linkages of GlcNS-GlcA2S and GlcNS-GlcA in ΔUA-GlcNS-GlcA2S-GlcNS-GlcA-GlcNS and the linkage of GlcNS3S-GlcA in ΔUA-GlcNS-GlcA-GlcNS3S-GlcA-GlcNS are partially resistant to the heparin lyase II and could only be cleaved after exhaustive treatment and in the presence of no additional cleavable substrate. The GlcNS-GlcA linkage in ΔUA-GlcNS-GlcA-GlcNS3S-GlcA-GlcNS is completely resistant to digestion with heparin lyase II.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations *NSH, N*-sulfoheparosan; *GlcA,* glucuronic acid; *GlcNAc, N*-acetyl-D-glucosamine; *IdoA,* iduronic acid; *GlcNS3S, N*-sulfo-3-*O*-sulfoglucosamine; *GlcA2S,* 2-*O*-sulfoglucuronic acid; *AT,* antithrombin III; *Mw,* molecular weight; *HPGPC,* high performance gel permeation chromatography; *NMR,* nuclear magnetic resonance

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