Using a 3-*O*-sulfated Heparin Octasaccharide to Inhibit the Entry of Herpes Simplex Virus 1

(Supplementary Materials)

Ronald Copeland[1], Arun Balasubramaniam[3], Vaibhav Tiwari[3], Fuming Zhang[4], Arleen Bridges[2], Robert J Linhardt[4], Deepak Shukla[3], and Jian Liu[1]*

1. Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599
2. Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599
3. Departments of Ophthalmology & Visual Sciences and Microbiology & Immunology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612
4. Departments of Chemistry and Chemical Biology, Biology and Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York, 12180

The 3-OH octasaccharide contained four glucosamine residues, thereby giving it four potential sites for 3-*O*-sulfation by 3-OST-3 as shown in Suppl Fig 1. These four positions include residue G2, G4, G6, and G8. Here we describe the results that served to identify which residue carried the 3-*O*-[^{35}S]sulfo group. The structural characterization was accomplished using a combination of chemical and enzymatic degradations from both non-reducing and reducing ends. Non-reducing end analysis permitted the identification of whether the 3-*O*-[^{35}S]sulfo group was present on either residue G2 or G4. Reducing end analysis permitted the identification of whether residue G6 or G8 carried the 3-*O*-[^{35}S]sulfo group.

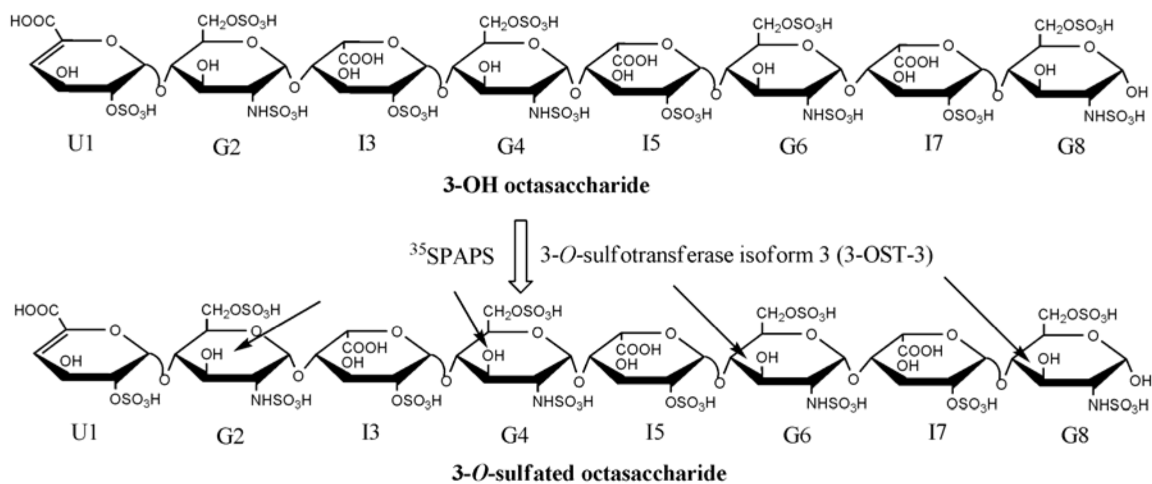
Reducing end analysis (disaccharide portion)-The results of reducing end analysis for the tetrasaccharide analysis have been presented in the main text. The 2-AB labeled 3-*O*-sulfated octasaccharide was digested with heparin lyases. The products were analyzed by PANM-HPLC, and yielded a ^{35}S-labeled component that was eluted at 55 min. We believed that this component is a disaccharide (Supplementary Fig 2A). We found that the ^{35}S-labeled component coeluted with ΔUA2S-[3-^{35}S]GlcNS3S6S, but not with ΔUA2S-[3-^{35}S]GlcNS3S6S on PAMN-HPLC (Supplementary Fig 2). Thus, our results demonstrated that the ^{35}S-labeled component that was eluted at 55 min is ΔUA2S-GlcNS3S6S. Our results also confirmed that the 3-O-sulfo group is not present at G8 residue.

Non-reducing end analysis-The non-reducing end analysis takes advantage of the substrate specificity of $\Delta^{4,5}$ glycuronate-2-sulfatase (2ase), an exolytic sulfatase. This enzyme is known to specifically remove the 2-*O*-sulfo group from ΔUA2S, which is present at the non-reducing end of the 3-*O*-[^{35}S]sulfated octasaccharide (Suppl Fig 3,

U1). By subjecting the 3-*O*-[^{35}S]sulfated octasaccharide to heparin lyases digestion with or without pretreatment with 2ase permitted the determination of whether the 3-*O*-[^{35}S]sulfo group was on residue 2 as illustrated in Suppl Fig 2.

The treatment of 3-*O*-sulfated octasaccharide with 2ase was completed as determined by the elution profile by DEAE-NPR-HPLC (Suppl Fig 4B). Before the treatment, 3-*O*-sulfated octasaccharide was eluted as a major peak at 63 min (Suppl Fig 4A). The treatment with 2ase resulted in the shift of the major ^{35}S-peak to 57 min, consistent with the loss of a negative charge due to the action of sulfates (Suppl Fig 4B). We estimated that the 2ase reaction of the 3-*O*-[^{35}S]sulfated octasaccharide was close to being 95% complete.

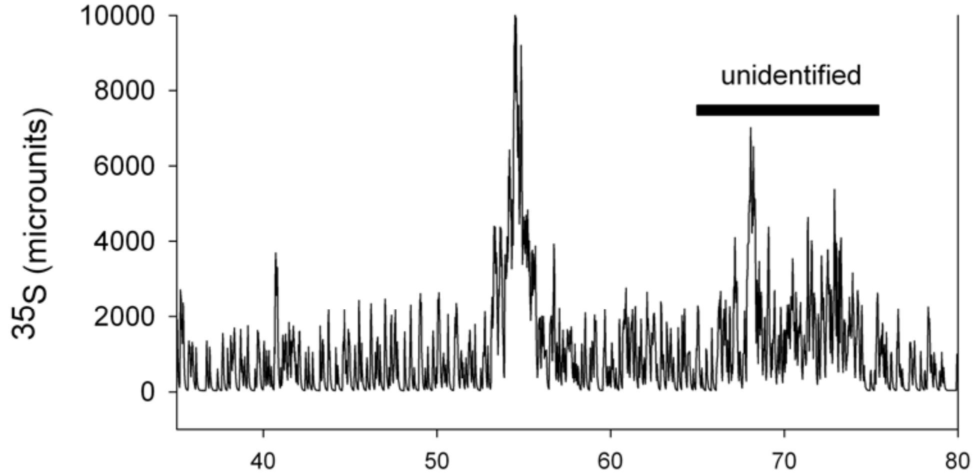
We then digested the 3-*O*-[^{35}S]sulfated octasaccharide and 2ase pretreated octasaccharide with a mixture of heparin lyases, including heparin lyase I, II and III. The resultant disaccharides were analyzed by PAMN-HPLC. As shown in Suppl. Fig 4C and 4D, both preparations gave an identical ^{35}S-labeled disaccharide, coeluting with the disaccharide standard, ΔUA2S-GlcNS3S6S. To this point, we concluded that the 3-*O*-[^{35}S]sulfo group was not present at residue 2. Although whether or not residue 4 carried the 3-*O*-[^{35}S]sulfo group could be proved by conducting partial digestion of the 3-*O*-[^{35}S]sulfated octasaccharide, we decided not to pursue those experiments as the data from reducing end analysis had concluded that the 3-*O*-sulfo group is on G6 residue as described in the main text.



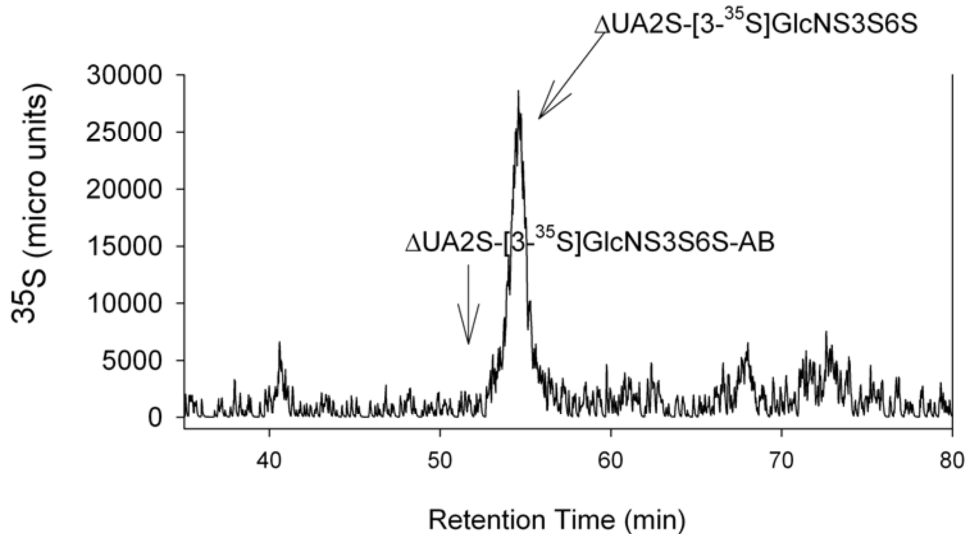
Supplementary Figure 1. Potential 3-*O*-sulfation sites in the 3-*O*-sulfated octasaccharide. There are four N-sulfoglucosamine 6-*O*-sulfate residues can accept the 3-*O*-sulfo group. These sites are indicated by arrows.

Supplementary Figure 2

A. Heparin lyases digested 2-AB-labeled 3-*O*-sulfated octasaccharide

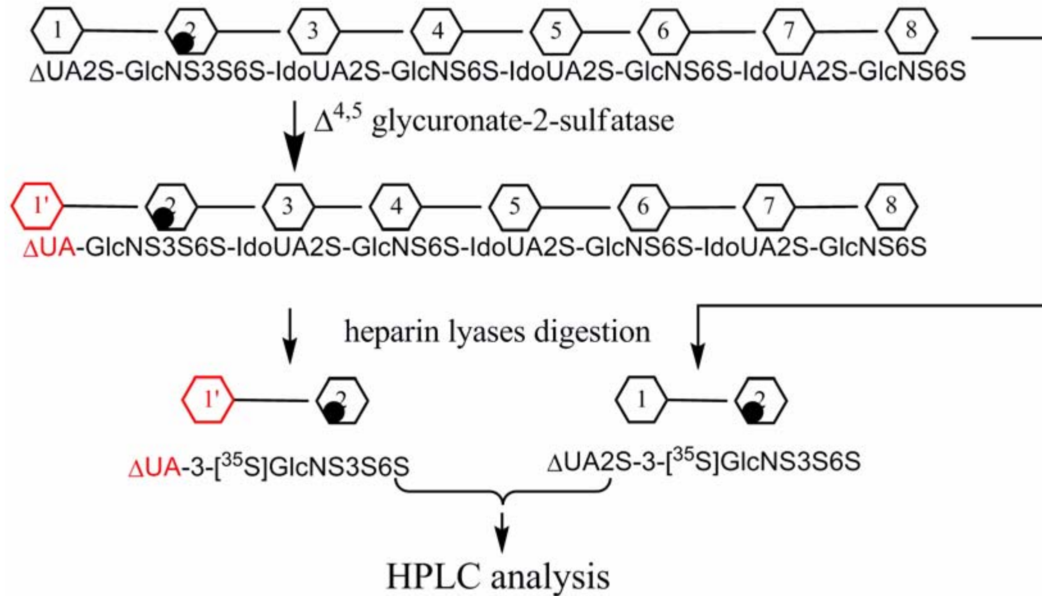


B. Coinjection of ΔUA2S-[3-^{35}S]GlcNS3S6S with the digested octasaccharide



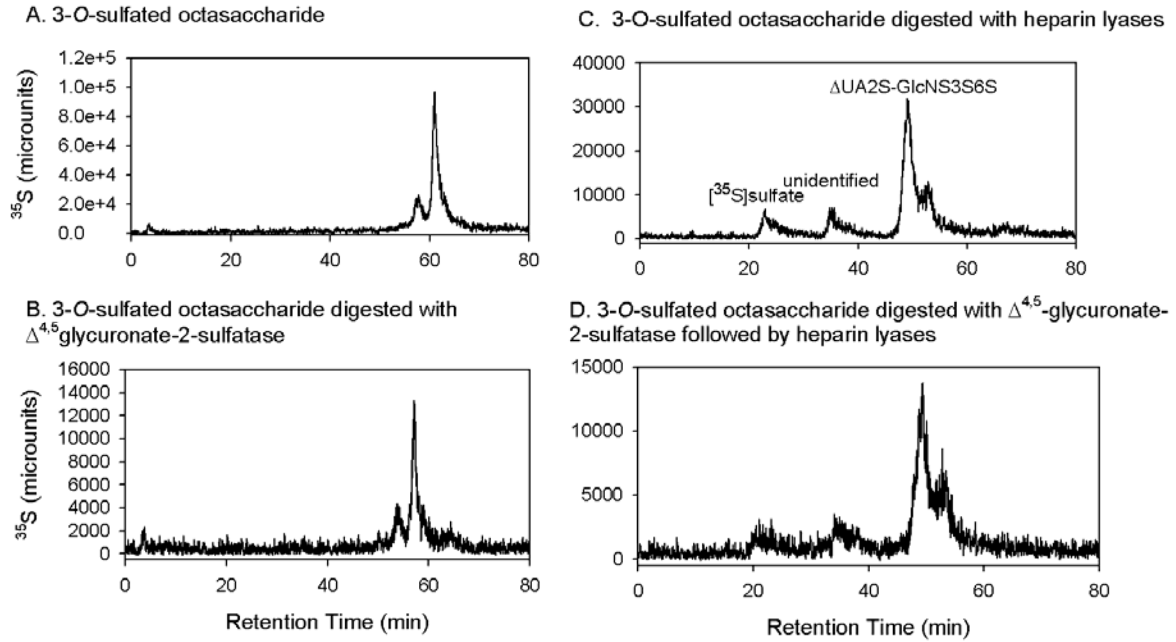
Supplementary Figure 2. Reducing end sequence analysis of 3-*O*-sulfated octasaccharide (disaccharide portion only). Purified 3-*O*-sulfated octasaccharide was digested with heparin lyases, including lyase I, II and III. The products were analyzed by PAMN-HPLC. The identities of the digested products were confirmed by coinjecting with appropriate disaccharide standards. *Panel A* shows the digestion of the 2-AB-labeled 3-*O*-sulfated octasaccharide alone in the region where trisulfated disaccharides are eluted. *Panel B* shows the digested octasaccharide coinjected with the ΔUA2S-[3-^{35}S]GlcNS6S3S standard.

Supplementary Figure 3



Supplementary Figure 3. Strategy for nonreducing end analysis of the 3-*O*-sulfated octasaccharide. The octasaccharide was digested with $\Delta^{4,5}$glycuronate-2-sulfatase, which selectively removes the 2-*O*-sulfo group from Residue 1. The resultant octasaccharide was then digested to a disaccharide by a mixture of heparin lyases. The structure of the disaccharide was then compared to the disaccharide from heparin lyases digested 3-*O*-sulfated octasaccharide that was not treated with $\Delta^{4,5}$-glycuronate-2-sulfatase. The structural comparison was carried out by polyamine-based anion exchange (PAMN)-HPLC. *Black circle* represents potential location of 3-*O*-[^{35}S]sulfo group on residue 2.

Supplementary Figure 4



Supplementary Figure 4. HPLC chromatograms of the nonreducing end analysis of 3-*O*-[^{35}S]sulfated octasaccharide. *Panels A* and *B* show the chromatograms of 3-*O*-[^{35}S]sulfated octasaccharide and $\Delta^{4,5}$-glycuronate-2-sulfatase digested 3-*O*-[^{35}S]sulfated octasaccharide on DEAE-NPR-HPLC, respectively. *Panels C* and *D* show the chromatograms of heparin lyases-digested 3-*O*-[^{35}S]sulfated octasaccharide and 3-*O*-[^{35}S]sulfated octasaccharide pretreated with $\Delta^{4,5}$glycuronate-2-sulfatase on polyamine-based anion exchange (PAMN)-HPLC. The conditions for the enzyme digestions and HPLC elution conditions are described under "Supplementary Methods".