

**Chemoenzymatic synthesis of unmodified heparin oligosaccharides:
Cleavage of *p*-nitrophenyl glucuronide by alkaline and Smith
degradation**

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General materials and methods

All reagents were purchased from commercial corporations. Unless noted otherwise, commercially available materials were used without further purification. Yields are given after purification, unless otherwise noted. NMR spectrum were recorded on Bruker 600 spectrometer with topspin 2.1 software at 298 K. Mass data were acquired by low- or high-resolution ESI-MS (Thermo LTQ XL Orbitrap, Bremen, Germany).

Expression of HS biosynthetic enzymes

A total of seven enzymes were used for the synthesis, including NST, C₅-epi, 2-OST, 6-OST-1, 6-OST-3, KfiA and PmHS2. All enzymes were expressed in *E. coli* and purified by appropriate affinity chromatography as described previously¹.

Preparation of enzyme cofactors

Preparation of UDP-GlcNTFA was completed using GlcNH₂-1-phosphate (Sigma) and glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridylyltransferase (GlmU) as described previously². Briefly, GlcNH₂-1-phosphate was converted to GlcNTFA using *S*-ethyl trifluorothioacetate. The resultant GlcNTFA-1-phosphate was then converted to UDP-GlcNTFA using GlmU in a buffer containing 50 mM Tris-HCl (pH 7.0), 5 mM MgCl₂, 200 μM dithiothreitol, 2.5 mM UTP and 0.012 U/L inorganic pyrophosphatase. A sulfo donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), was prepared using adenosine phosphokinase and ATP-sulfurylase.

General procedure to introduce GlcNTFA residue for elongation

Acceptor substrate (1.2 mmol) was incubated with KfiA (20 ug mL⁻¹) in buffer containing Tris (25 mmol, pH 7.2), MnCl₂ (15 mmol) and UDP-GlcNTFA (1.5 mmol), at room temperature overnight. The product was purified using a C₁₈ column and the identity was confirmed by ESI-MS.

General procedure to introduce GlcA residue for elongation

Acceptor substrate (1.2 mmol) was incubated with PmHS2 (20 $\mu\text{g mL}^{-1}$) in buffer containing Tris (25 mmol, pH 7.2), MnCl_2 (15 mmol) and UDP-GlcA (1.5 mmol), at room temperature overnight. The product was purified using a C_{18} column and the identity was confirmed by ESI-MS.

Prodecure to introduce *N*-sulfate groups on the oligosaccharide backbone

The oligosaccharide was suspended at 10 mg mL^{-1} in 0.1 M LiOH and then the reaction mixture was incubated on ice for 2 h. Upon the completion of detrifluoroacetylation, the pH of the reaction mixture decreased to 7.0 and incubated with 50 mM MES (pH 7.0), 10 $\mu\text{g mL}^{-1}$ NST and 1 mM PAPS at 37 °C overnight. *N*-sulfated product was purified by Q-Sepharose (GE Health Care), and the identity was confirmed by ESI-MS.

Procedure for C_5 -epimerization/2-*O*-sulfation

The Oligosaccharide was dissolved in 50 mM MES (pH 7.0), 2 mM CaCl_2 , C_5 -epi 10 $\mu\text{g mL}^{-1}$, 2-OST 10 $\mu\text{g mL}^{-1}$ and 0.2 mM PAPS in 800 mL volume at 37°C overnight. The reaction mixture was then purified by Q-Sepharose.

Procedure to introduce 6-*O*-sulfate groups on the oligosaccharide backbone

Oligosaccharide backbone was dissolved in 50 mM MES (pH 7.0), 1.5 mM PAPS, 0.2 mg mL^{-1} 6-OST-1 and 0.2 mg mL^{-1} 6-OST-3 in 30 mL overnight at 37°C. The extent reaction was monitored by DEAE-HPLC to ensure the desired product reached to the highest level and the substrate was consumed. The reaction mixture was then purified by Q-Sepharose to obtain compound **3**. ^1H NMR (600 MHz, D_2O) δ : 8.19 (d, $J = 8.96$, 2H), 7.17 (d, $J = 8.96$, 2H), 5.53 (d, $J = 3.60$, 1H, F1), 5.36-5.32 (m, 2H, B1/D1), 5.23 (d, $J = 8.14$, 1H, G1), 5.16 (br, 2H, C1/E1), 4.76-4.72 (m, 2H), 4.53 ($J = 8.05$, 1H, A1), 4.40 ($J = 11.46$, 1H), 4.36-4.31 (m, 2H), 4.29-4.25 (m, 2H), 4.23-4.10 (m, 5H), 4.06-3.99 (m, 3H), 3.98-3.93 (m, 2H), 3.92-3.87 (m, 2H), 3.86-3.80 (m, 2H), 3.75-3.67 (m,

3H), 3.67-3.56 (m, 4H), 3.50-3.41 (m, 2H), 3.28 (t, $J = 8.75$, 1H), 3.26-3.18 (m, 3H). ^{13}C NMR (200 MHz, D_2O) δ : 101.7, 99.2, 99.1, 97.4, 96.4, 77.1, 77.0, 76.7, 75.9, 75.7, 75.4, 74.8, 72.7, 72.2, 71.7, 69.5, 69.3, 69.0, 68.9, 68.8, 66.2, 65.9, 58.1, 57.8. MS (ESI) m/z calcd for $(\text{M}-3\text{H})^3-$ 654.3459, found 654.3333.

Procedure for periodate oxidation

Oligosaccharide **3** (2 mg) was dissolved in 0.4 mL NaH_2PO_4 buffer (pH 7.0), 20 equiv sodium periodate (NaIO_4) was added at 37°C in the dark for 3.5 h. The reaction was quenched by the addition of ethylene glycol (20 equiv) followed by dialysis (MWCO 100-500 Da) against distilled water and lyophilization to produce aldehyde **4** in 90% yield.

Procedure for alkaline elimination

Oligosaccharide aldehyde **4** (1.8 mg) was dissolved in 0.3 mL NaH_2PO_4 buffer (pH 7.0) and then adjusted to pH 12.0 with 0.5 M NaOH aqueous. After standing at room temperature for 30 min, the solution was neutralized with 0.5 M acetic acid. The resulting mixture was dialyzed (MWCO 100-500 Da) against distilled water and lyophilized to obtain the elimination compound **5** in 80% yield. ^1H NMR (600 MHz, D_2O) δ : 5.35-5.29 (m, 3H, B1/D1/F1), 5.14-5.09 (br, 2H, C1/E1), 4.74-4.72 (m, 2H, C5/E5), 4.34-4.14 (m, 8H), 4.13-4.06 (m, 2H), 4.03-3.97 (m, 2H), 3.96-3.91 (m, 2H), 3.91-3.88 (m, 1H), 3.81-3.73 (m, 3H), 3.58-3.52 (m, 3H), 3.21-3.11 (m, 3H). ^{13}C NMR (200 MHz, D_2O) δ : 99.1, 99.0, 96.5, 96.4, 96.1, 76.2, 75.6, 75.5, 70.9, 69.8, 69.5, 69.2, 69.0, 66.4, 66.2, 66.1, 61.2, 57.8. MS (ESI) m/z calcd for $(\text{M}-3\text{H})^3-$ 529.3042, found 529.3040. Three set peaks between 3.4-3.7 ppm in ^1H NMR spectrum (Fig. 2 in the manuscript) was from glycerol.

Procedure for Smith degradation

Oligosaccharide aldehyde **4** (1.2 mg) was treated with sodium borohydride (NaBH_4) in distilled water for 3 h at room temperature and then excess borohydride was quenched with 1 M acetic acid. After dialysis against distilled water and lyophilization, the

oxidized-reduced oligosaccharide was dissolved in 0.5 M trifluoroacetic acid (TFA) solution at room temperature for 24 h. The reaction was terminated by the addition of 0.5 M NaOH to pH 7.0 and dialyzed to afford the degradation product **7** (81% yield in two steps). ¹H NMR (600 MHz, D₂O) δ : 5.34-5.25 (m, 3H, B1/D1/F1), 5.16-5.10 (m, 2H, C1/E1), 4.74-4.72 (m, 2H, C5/E5), 4.34-4.13 (m, 9H), 4.13-4.07 (m, 2H), 4.06-3.97 (m, 2H), 3.96-3.90 (m, 1H), 3.89-3.84 (m, 2H), 3.83-3.76 (m, 2H), 3.75-3.71 (m, 1H), 3.62-3.51 (m, 5H), 3.21-3.16 (m, 1H), 3.16-3.12 (m, 1H), 3.12-3.09 (m, 1H). ¹³C NMR (200 MHz, D₂O) δ : 99.1, 99.0, 96.5, 96.4, 96.0, 77.5, 75.8, 75.7, 75.3, 71.8, 70.7, 69.7, 69.3, 69.1, 68.8, 66.3, 66.2, 66.1, 61.2, 57.9. MS (ESI) m/z calcd for (M-3H)³⁻ 535.9796, found 535.9796. Three set peaks between 3.4-3.7 ppm in ¹H NMR spectrum (Fig. 2 in the manuscript) was from glycerol.

Procedure for acidic hydrolysis of compound 5

Compound **5** (0.9 mg) was dissolved 0.5 M TFA or 0.05 M HCl in 0.4 mL volume at room temperature for 24 h. The reaction was terminated by the addition of 0.5 M NaOH to pH 7.0 and dialyzed to afford the degradation product **8** in 85% yield.

Procedure for acidic hydrolysis of compound 7

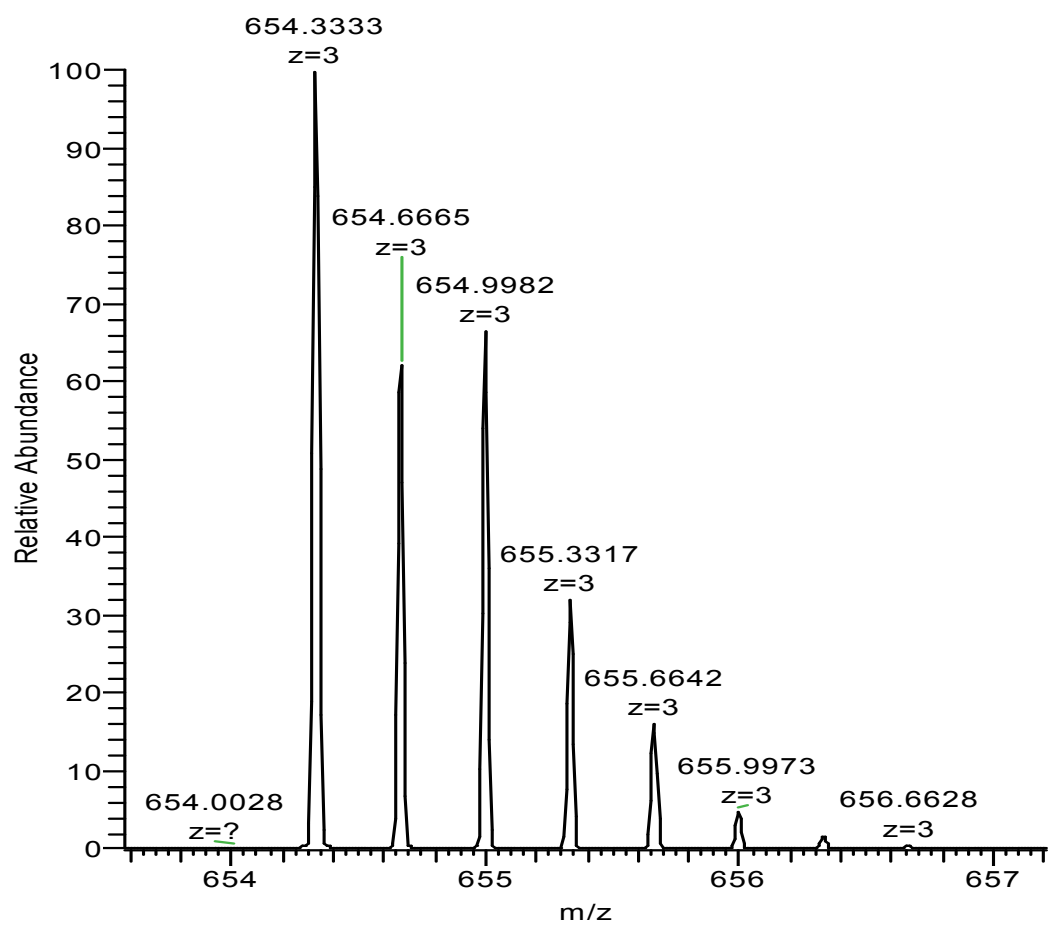
Compound **7** (0.9 mg) was dissolved 0.5 M TFA in 0.4 mL volume at 45°C for 4 h. The reaction was terminated by the addition of 0.5 M NaOH to pH 7.0 and dialyzed to afford the degradation product **8** in 70% yield. ¹H NMR (600 MHz, D₂O) δ : 5.37-5.27 (m, 3H, B1/D1/F1), 5.18-5.10 (m, 2H, C1/E1), 4.81-4.78 (m, 2H, C5/E5), 4.36-4.16 (m, 8H), 4.15-4.00 (m, 5H), 3.97-3.85 (m, 3H), 3.73-3.69 (m, 2H), 3.63-3.57 (m, 3H), 3.23-3.12 (m, 3H). ¹³C NMR (200 MHz, D₂O) δ : 99.2, 98.8, 96.6, 96.5, 96.3, 75.5, 75.3, 71.8, 71.7, 70.7, 69.8, 69.5, 69.0, 68.6, 66.2, 66.1, 62.2, 57.7. MS (ESI) m/z calcd for (M-3H)³⁻ 496.6374, found 496.6377.

References

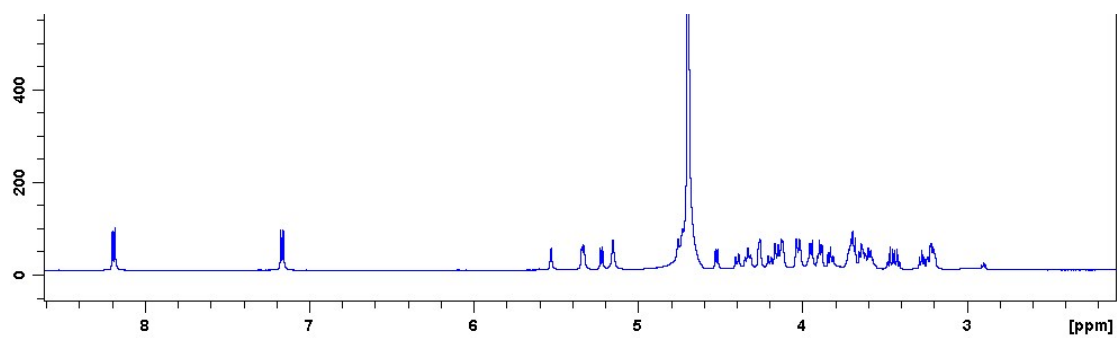
1. R. Liu, Y. Xu, M. Chen, M. Weiwer, X. Zhou, A. S. Bridges, P. L. DeAngelis, Q. Zhang, R.

- J. Linhardt and J. Liu, *J. Biol. Chem.*, 2010, **285**, 34240.
2. S. Masuko, S. Bera, D. E. Green, M. Weïwer, J. Liu, P. L. DeAngelis and R. J. Linhardt, *J. Org. Chem.*, 2012, **77**, 1449.

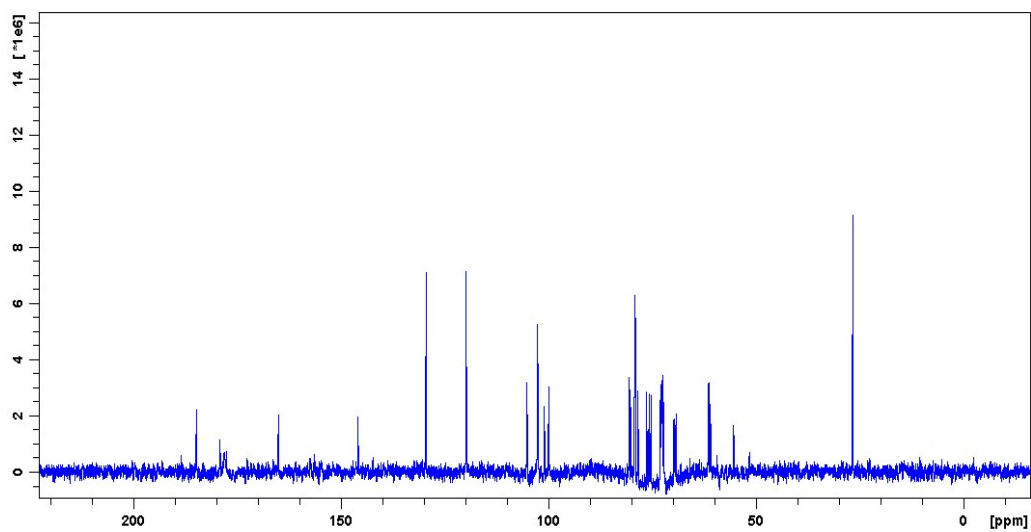
NMR and MS spectra



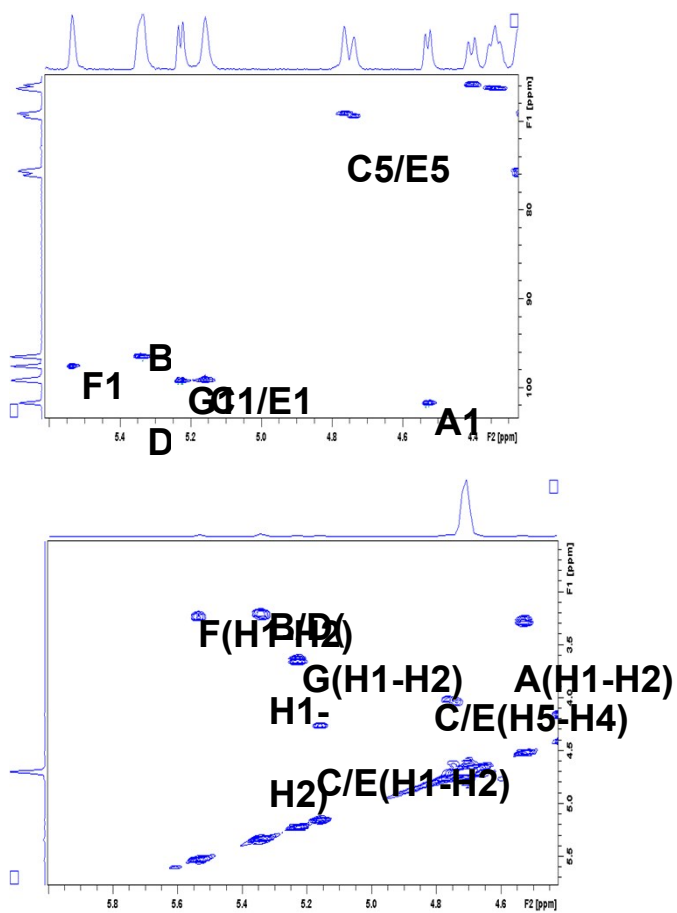
HRMS of compound 3



¹H NMR spectrum of compound 3

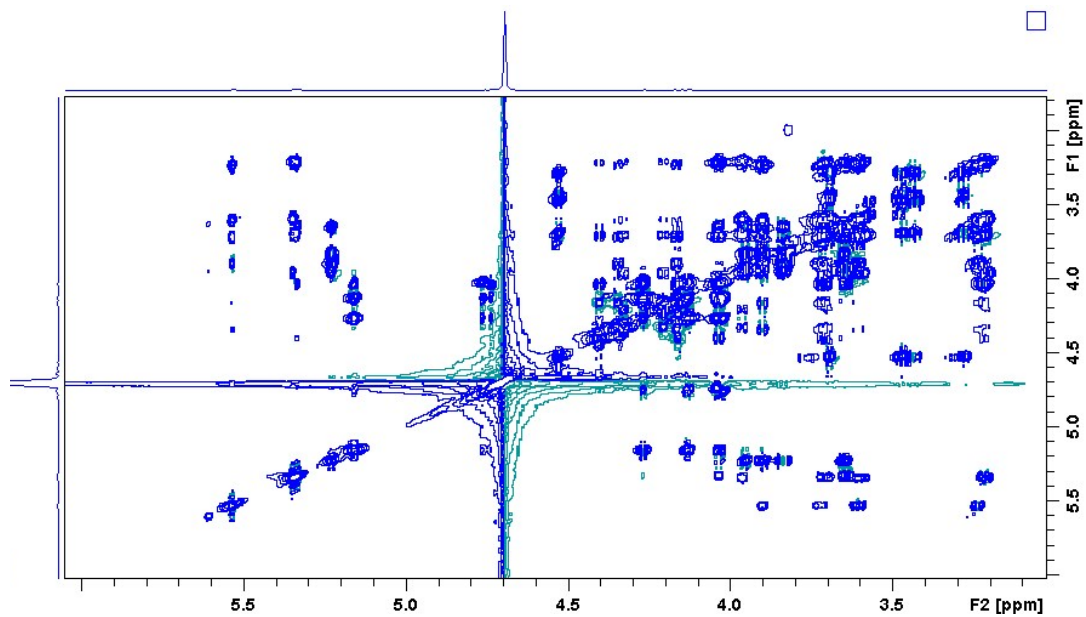


^{13}C NMR spectrum of compound 3

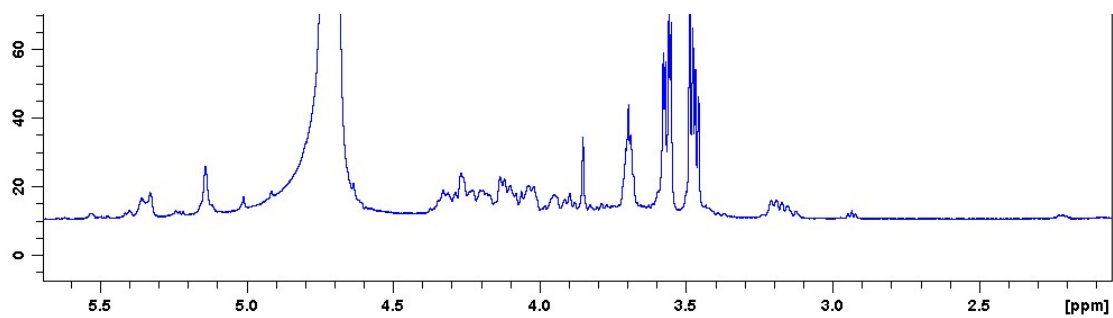


HSQC spectra of compound 3

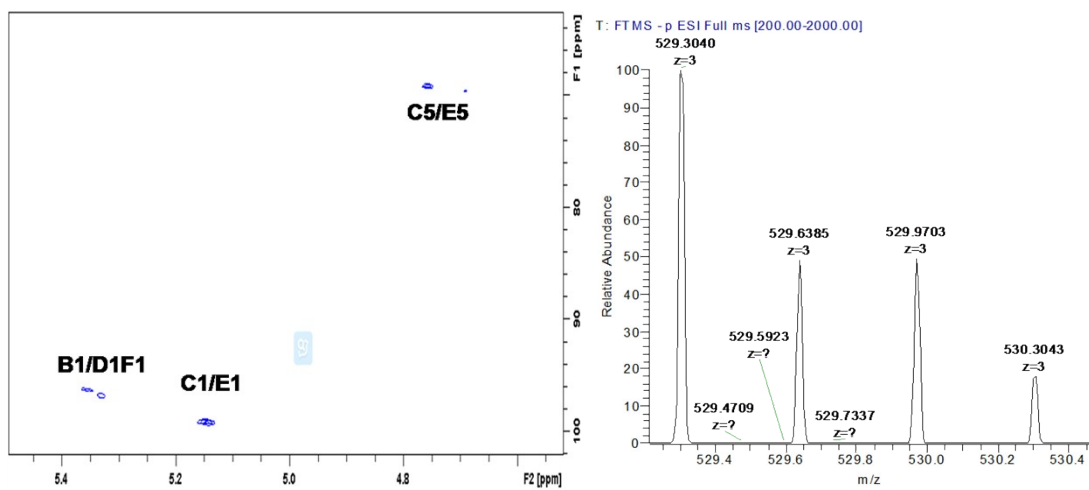
H-H COSY spectra of compound 3



H-H TOCSY spectrum of compound 3

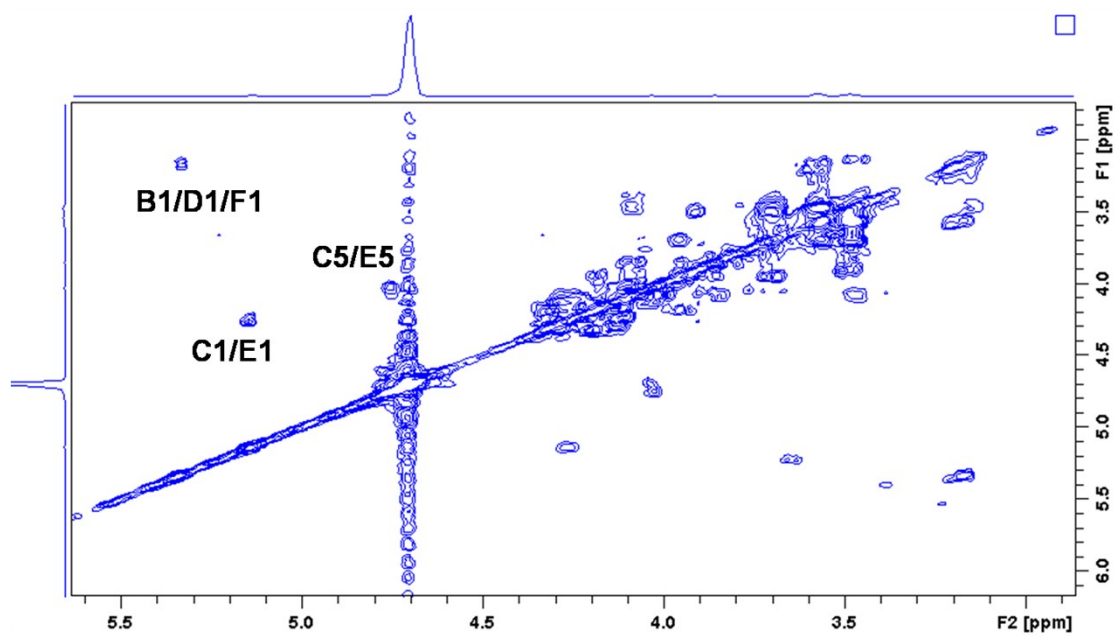


¹H NMR spectrum of compound 5

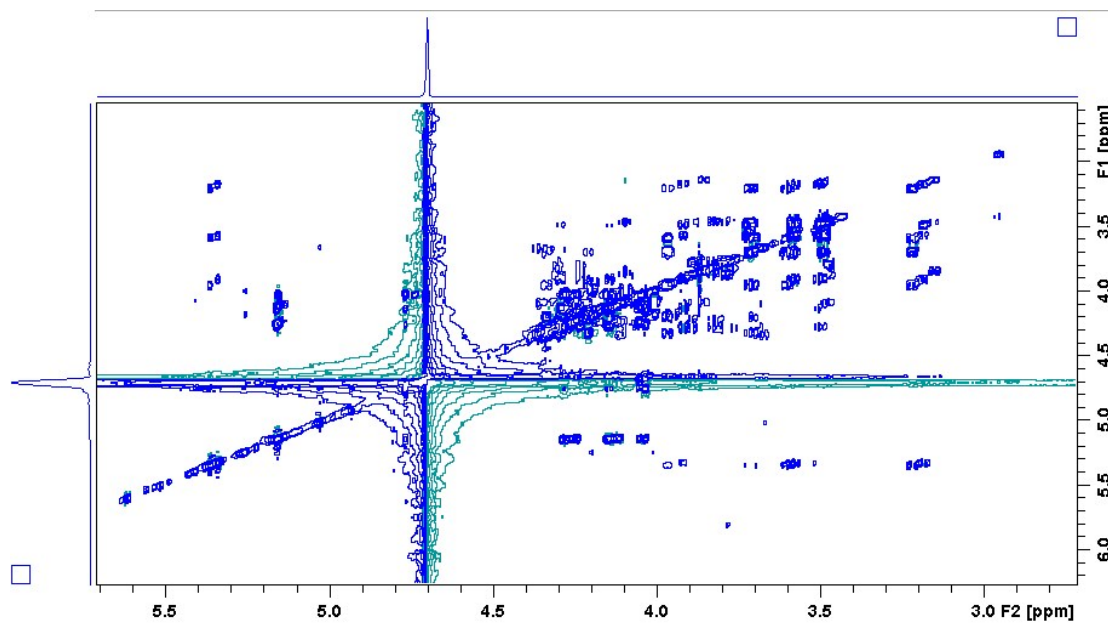


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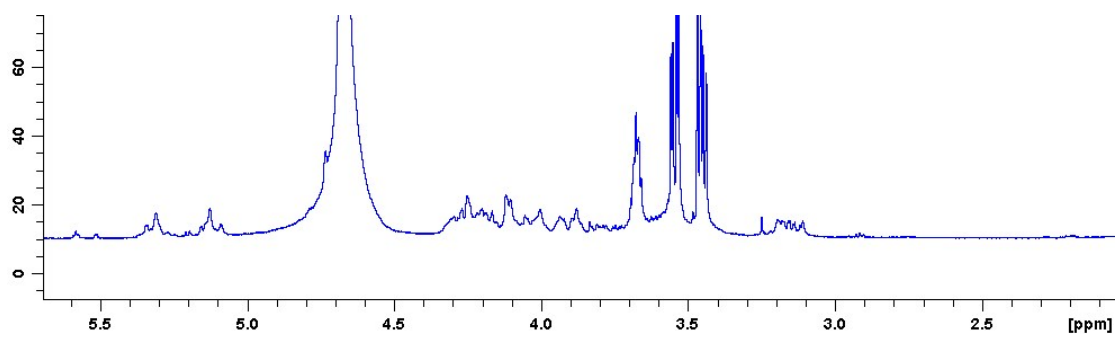
HRMS of compound 5



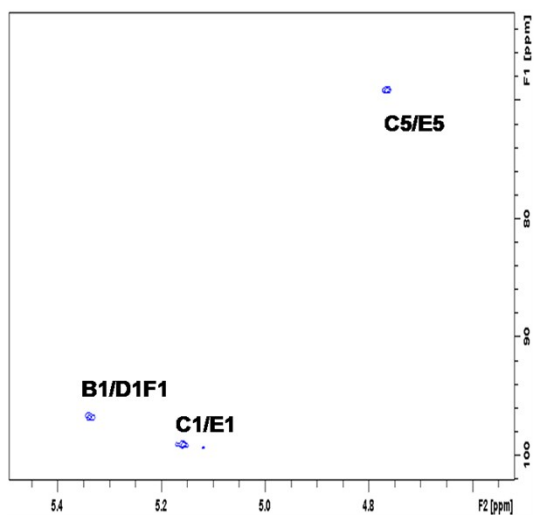
H-H COSY spectrum of compound 5



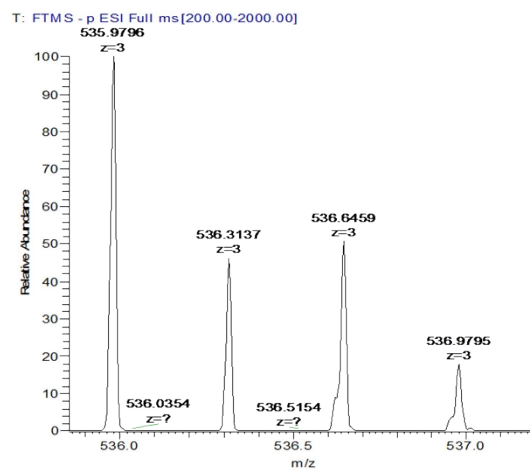
H-H TOCSY spectrum of compound **5**



^1H NMR spectrum of compound **7**



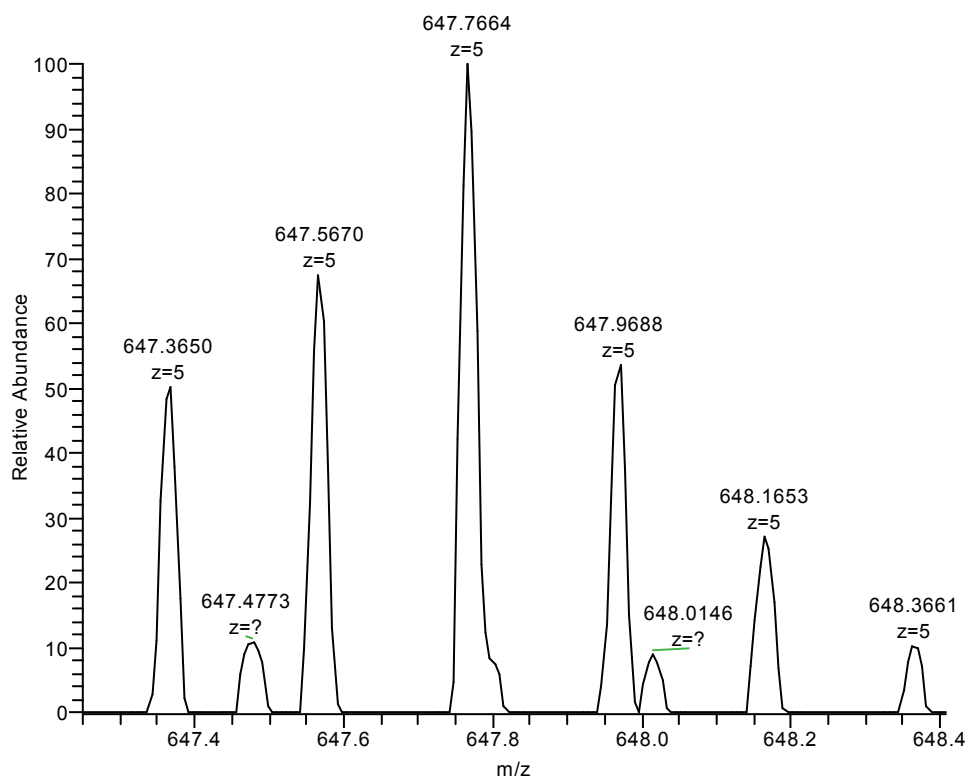
HSQC spectrum of compound **7**



HRMS of compound **7**

Alkaline Degradation Product (GlcNS6S-IdoA2S)₅-GlcNS6S-OC₄H₃O₃ from
GlcNS6S-GlcA-(GlcNS6S-IdoA2S)₅-GlcNS6S-GlcA-*p*NP:

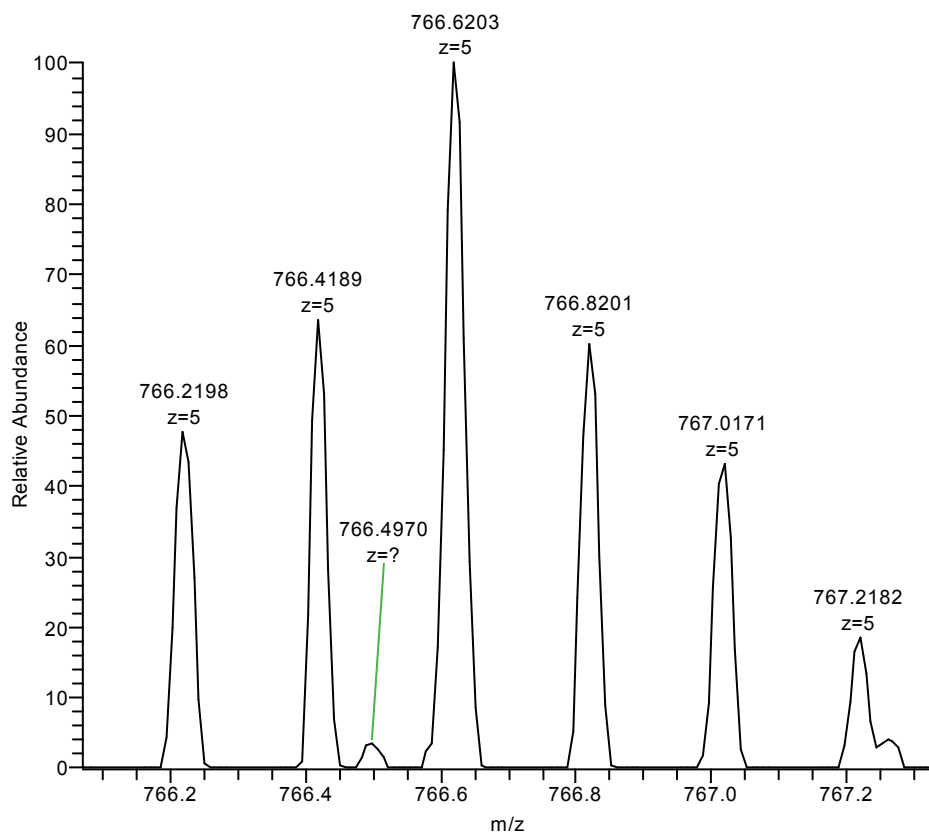
MS (ESI) *m/z* calcd for (M-5H)⁵⁻ 647.3708, found 647.3650.



HRMS of (GlcNS6S-IdoA2S)₅-GlcNS6S-OC₄H₃O₃

Alkaline Degradation Product (GlcNS-IdoA2S)₇-GlcNS-OC₄H₃O₃ from GlcNS-GlcA-(GlcNS-IdoA2S)₇-GlcNS-GlcA-*p*NP:

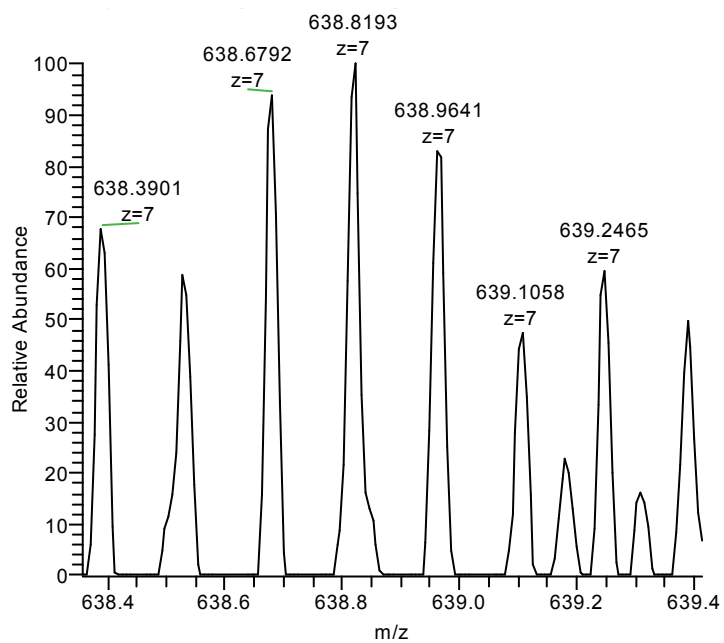
MS (ESI) *m/z* calcd for (M-5H)⁵⁻ 766.2198, found 766.2198.



HRMS of (GlcNS-IdoA2S)₇-GlcNS-OC₄H₃O₃

Alkaline Degradation Product (GlcNS6S-IdoA2S)₇-GlcNS6S-OC₄H₃O₃ from
GlcNS6S-GlcA-(GlcNS6S-IdoA2S)₇-GlcNS6S-GlcA-*p*NP:

MS (ESI) m/z calcd for (M-7H)⁷⁻ 638.3911, found 638.3901.



HRMS of (GlcNS6S-IdoA2S)₇-GlcNS6S-OC₄H₃O₃