

Supporting Information

**Convergent Chemoenzymatic Synthesis and Biological Evaluation of a Heparan Sulfate Proteoglycan Syndecan-1 Mimetic**

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## Supporting Information

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## Materials and Methods

**Materials.** Sortase A-expressing BL21 cells were obtained from Prof. Xue-long Sun (Cleveland State University, OH). Gibco LB broth and LB agar were purchased from Thermo Fischer Scientific (Waltham, MA). Nickel columns and Nickel resins were purchased from Bio-rad (Hercules, California). SDS-PAGE gels and 10x Tris/Glycine/SDS electrophoresis buffer were purchased from Bio-rad (Hercules, California). Tris-HCl buffer was purchased from MilliporeSigma (St. Louis, MO). Sephadex G-15 and G-25 were purchased from MilliporeSigma (St. Louis, MO). EZ-Link™ Sulfo-NHS-LC-Biotin was purchased from Thermo Fischer Scientific (Waltham, MA). Recombinant human integrin  $\alpha\text{v}\beta\text{3}$  was purchased from R&D Systems (Minneapolis, MN). Heparin sodium salt was purchased from MilliporeSigma (St. Louis, MO). MDA-MB-231 breast carcinoma cells were obtained from Prof. Kathy Gallo (Michigan State University, MI). DMEM (Dulbecco's Modified Eagle Medium) was purchased from MilliporeSigma (St. Louis, MO). Fetal Bovine Serum was purchased from Thermo Fischer Scientific (Waltham, MA). Human EGF was purchased from Alomone labs Ltd. (Jerusalem, Israel). Human vitronectin protein was purchased from R&D Systems (Minneapolis, MN).

**Synthesis of Oligosaccharide 4.** The starting compound **6** was dissolved in H<sub>2</sub>O (5 mg/ml), to which Pd/C (10 mg/ml) was added. The mixture was then placed under a hydrogen balloon and stirred at room temperature for 1 h. After completion of the reaction, the mixture was filtered through a PTFE syringe filter (0.2 mm, 13 mm). The filtrate was concentrated, and the desired product **7** was subjected to a Sephadex G-10 column, after which it was dissolved in aqueous solution of NaHCO<sub>3</sub> at pH 8.5. 1.5 equivalents of 6-azidohexanoic acid NHS ester **10** in anhydrous *N,N*-dimethylformamide (DMF) was added. The reaction was then stirred at room temperature for 6 hours. Upon completion, the reaction mixture was directly loaded onto a Sephadex G-10 column for purification.

**General Procedure for Automated Solid-Phase Peptide Synthesis.** All the peptides reported were synthesized on a Liberty Blue™ Automated Microwave Peptide Synthesizer following standard Fmoc-based solid-phase peptide synthesis protocol. The 2-chlorotrityl resins with or without Fmoc-amino acid loaded were purchased from Chem-Impex. The Liberty Blue software from CEM Corporation was used to program the synthesis, including resin swelling, amino acid loading, couplings and Fmoc- removals. Commercially available DMF from Fischer Chemical was supplied to the synthesis module as reaction and washing solvent. Peptide synthesis was enabled by sequential couplings of Fmoc-amino acid (purchased from Chem-Impex) which was

preactivated by *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), *N,N*-diisopropylethylamine (DIPEA), at 50 °C for 10 min, and deprotections with 20% piperidine in DMF at 60 °C for 4 min. In-between each coupling/deprotection step, resin-bound peptide was thoroughly washed with DMF. For the synthesis of Fmoc-Gly5-OH peptide, Fmoc-glycine was preactivated by 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and DIPEA instead. Resin-bound peptides were cleaved off the solid support with a cocktail solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (TFA/TIPS/H<sub>2</sub>O, 95:2.5:2.5). The crude peptides were then purified with reverse-phase C18 preparative HPLC. Compound purity was each confirmed with chromatograms from C18 analytical HPLC.

**High-Performance Liquid Chromatography.** LC-8A Solvent Pumps, DGU-14A Degasser, SPD-10A UV-Vis Detector, SCL-10A System Controller (Shimadzu Corporation, JP) and Vydac 218TP 10 μm C18 Preparative HPLC column (HICHROM Limited, VWR, UK) or 20RBAX 300SB-C18 Analytical HPLC column (Agilent Technologies, CA) were installed for HPLC purifications with HPLC-grade acetonitrile (EMD Millipore Corporation, MA) and Milli-Q water (EMD Millipore Corporation, MA). A variety of eluting gradients were set up on LabSolutions software. Dual-wavelength UV detector was set to 220 nm and 254 nm for monitoring the absorbance from amide and Fmoc respectively. The eluted compounds were analyzed with ESI-MS to confirm their identities. The aqueous solutions of purified compounds were then lyophilized to obtain the dry solid.

**Sortase A Expression, Purification and Quantification.** Sortase A-expressing BL21 competent cells (2 μL) was transferred onto a kanamycin/chloramphenicol containing petri dish and incubated at 37 °C overnight. One colony of BL21 cells was picked and inoculated into 10 mL starter culture, which contained kanamycin (35 mg/L). The cell culture was incubated at 37 °C for 12-16 hours until OD<sub>600</sub> value reached 0.85, and transferred into autoclaved culture medium (1L, 35 mg/L kanamycin). After roughly 5 hours, the OD<sub>600</sub> reached 0.85. IPTG (0.5 mM) was added to induce protein expression. The cells were further incubated for another 4 hours at 37 °C, and centrifuged at 4 °C, 5000 rpm for 10 min. The cells were suspended into the lysis buffer (40 mL, 20 mM Tris, 250 mM NaCl, pH 8.0), then lysed through sonication. The lysate was centrifuged at 20,000 g for 20 min. The supernatant was purified by Nickel column (a. washing buffer: 20 mM Tris, 0.5 M NaCl and 250 mM imidazole; b. eluting buffer: 20 mM Tris, 0.5 M NaCl and 250 mM imidazole). Dialysis in buffer (20 mM Tris, 150 mM NaCl, pH 8.0) was performed to harvest the protein solution. Protein purity was confirmed with SDS-

PAGE gel electrophoresis and standard Bradford assay was used to determine the concentration and the expression yield of sortase A.

**General Procedure for Sortase A-Mediated Ligation.** The pH of the 10X reaction buffer (500 mM Tris-HCl, 1.5 M NaCl, 50 mM CaCl<sub>2</sub>, 5 mM mercaptoethanol and 2mM Ni(II) sulfate) was adjusted to 8.5 with the addition of NaOH or HCl. Ligation substrates (250-500  $\mu$ M) were dissolved and added into Tris-HCl reaction buffer, followed by the addition of sortase A. The reaction vessel was kept at 25 °C until reaction completion, with the reaction progress monitored with LC-MS. After the reaction, the enzyme was deactivated and precipitated out with addition of ethanol. Clear aqueous solutions from centrifuge were loaded onto G-15/G-25 size exclusion column for purification.

**Size-Exclusion Purification of HS Glycopeptide.** Samples were prepared in distilled water (500  $\mu$ L) and then slowly transferred onto a G-15 or G-25 size-exclusion column. Fractions of 1 mL elute were collected. Fractions containing desired compounds were identified by ESI-MS analysis. The purified compounds were lyophilized to obtain the dry solid.

**BLI Binding Experiment.** BLI Octet K2 instrument (ForteBio, Molecular Devices, CA) was installed for binding experiments. Polypropylene black 96-well plates (Greiner Bio-one, Austria) and streptavidin (SA) sensor chips (ForteBio, Molecular Devices, CA) were used to assist sample preparations and detections of binding activities. Assay buffer was phosphate buffered saline (PBS) unless otherwise noted. Integrin  $\alpha$ v $\beta$ 3 protein solutions were prepared according to the assay design. To prepare biotinylated analytes, 1 mM of each amine-containing ligand compound and EZ-Link™ Sulfo-NHS-LC-Biotin (1.2 equiv.) were added to 0.1 M NaHCO<sub>3</sub> solution (pH 8.5). The reaction was allowed to proceed overnight. Upon completion, reaction mixture was passed through G-10 column to remove unconjugated biotin reactant. Sensors were then loaded with biotin-labeled compounds. The binding activity (including association and dissociation) between ligand and protein was measured under the monitoring via BLI. Biotin was used as negative control for all BLI assays. The assay results were then processed through the licensed software. Various concentrations of protein were tested against each ligand to obtain the kinetic data. The curve fitting was achieved using a 2:1 heterogenous ligand binding model provided by the data-processing software.

**Wound-Healing Assay Procedure.** MDA-MB-231 breast carcinoma cells were cultivated in the 6-well plate until 90% confluency was reached. After 24-hour starvation with serum-free medium, wounds were created by scratching the monolayer by sterile P200 pipet tips. This process was done carefully to make sure that all wounds were similar in size. Microscopic

images (taken with Zeiss Axionvert 200 Pred Axio Observer microscopy, Boston Industries, Inc.) were collected right after the wounding process ( $T=0$ ). Then, starving serum-free medium was replaced by the growth medium that contains certain concentration of the analyte (growth medium without analytes was treated as control group). And human EGF was added to stimulate cancer cell migrations. After 20-hour ( $T = 20$ ) incubation at 37 °C, microscopic images were collected with same microscopy instrument. Images at  $T=0$  and  $T=20$  of the same site were processed with GraphPad Prism Version 5.0c to obtain the cell migration results.

**Identification of Ligand Binding Sites.** To initiate the search for ligand binding sites on the integrin  $\alpha\beta3$  protein surface, synstatin peptide SSTN<sub>92-119</sub> model was constructed *de novo* using an open computation platform developed by Tuffery group.<sup>1</sup> Integrin protein (PDB:4G1M) was used as the receptor reference to facilitate the model construction and improve the subsequent docking simulations. 200 rounds of independent model simulations with sOPEP force field were applied to get good-quality peptide conformation predictions. The best candidate models were selected for the ligand-receptor molecular docking simulations.

Hot spot on the protein surface for synstatin binding was identified through examining the docking results. For the heparan sulfate binding simulations, a heparan sulfate tetrasaccharide structure was utilized to identify potential HS binding sites on integrin  $\alpha\beta3$ . After uploading the integrin coordinate file to ClusPro docking platform, binding simulations were initiated under the built-in ‘Heparin Ligand’ mode.<sup>2</sup> Simulation results were then visualized and processed with UCSF Chimera software to pinpoint potential HS binding sites.<sup>3</sup>

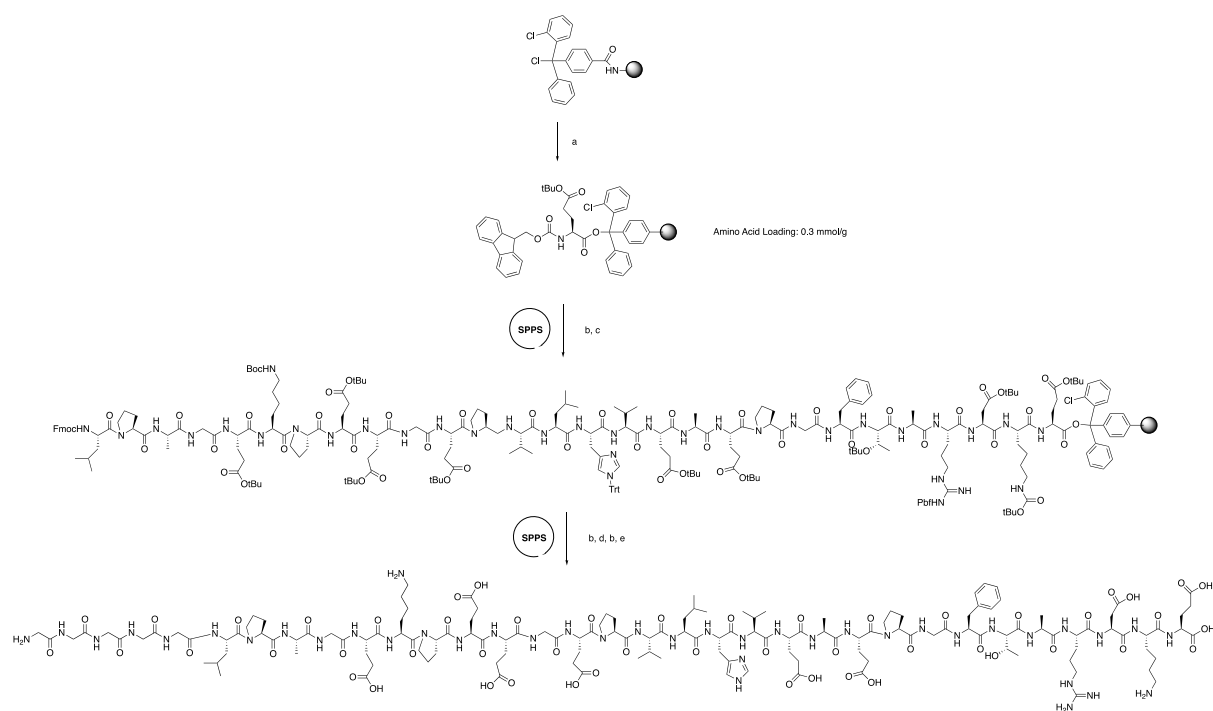
**Biomolecule Visualization.** The construction of syndecan-1 mimetic started with the heparan sulfate octasaccharide moiety. Its structure was prepared through ‘GAG Builder’ program at GLYCAM-Web.<sup>4</sup> Counter ions were added to the negatively charged sulfate groups and the HS octasaccharide was solvated into a cube of water molecules. Structural optimization was accomplished with GLYCAM force field. The generated PDB file of HS octasaccharide was later used to construct the glycopeptide.

*Ab initio* modeling of the peptide backbone was achieved with QUARK program.<sup>5</sup> Top model that adopted a more extended conformation was selected for the syndecan-1 mimetic construction.

The structure coordinates of HS octasaccharide and peptide backbone were input into Maestro software.<sup>6</sup> The artificial linkage connecting HS and peptide backbone was manually created. The resulted syndecan-1 mimetic structure was then optimized using all-atom minimization function to approximate its conformation.

The dimensions of syndecan-1 mimetic and integrin  $\alpha\beta3$  were measured with UCSF Chimera to provide a rough estimation of their sizes.

## Solid-Phase Synthesis of Synstatin Peptide 3



**Scheme S1.** Solid-phase synthesis of Gly<sub>5</sub>SSTN<sub>92-117</sub> peptide **3**. Reagents and conditions: (a) Amino acid loading: Fmoc-Glu(O-tBu)-OH, DIPEA, DMF; (b) Fmoc- cleavage: 20% piperidine/DMF, 50 °C, 2 min, microwave; (c) Amino acid coupling: 5 eq. Fmoc-AA-OH, HBTU, HOBT, DIPEA, DMF, 50 °C, 10 min, microwave; (d) Oligopeptide coupling: 5 eq. Fmoc-Gly<sub>5</sub>-OH **6**, HATU, DIPEA, DMF, 50 °C, 10 min, microwave; (e) Resin cleavage: TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5, v/v/v), 24 % overall.



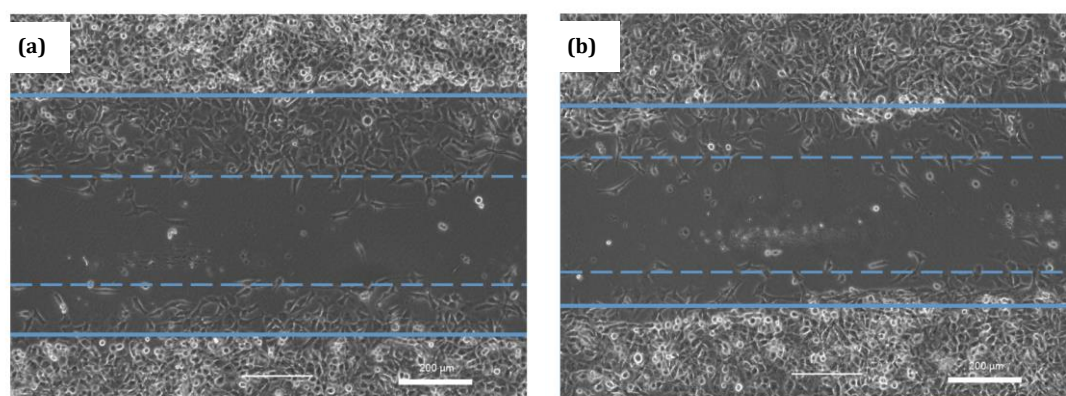
## Wound Healing Assay Result

Gly <sub>5</sub> SSTN						
Concentration ( $\mu\text{M}$ )	Relative Migration Area (Artificial Unit)					
6	103.92	99.61	100.60	107.91	98.96	96.18
2	102.01	102.77	99.73	97.85	122.89	109.22
1	111.56	109.67	118.42	115.84	115.74	113.54
0	100.70	98.40	94.45	99.88	103.13	100.35

Heparin						
Concentration ( $\mu\text{M}$ )	Relative Migration Area (Artificial Unit)					
6	82.62	95.3	83.28	80.77	73.85	81.61
2	88.3	83.49	85.63	83.17	82.23	79.42
1	97.95	98.89	88.91	83.87	88	83.28
0	100.70	98.40	94.45	99.88	103.13	100.35

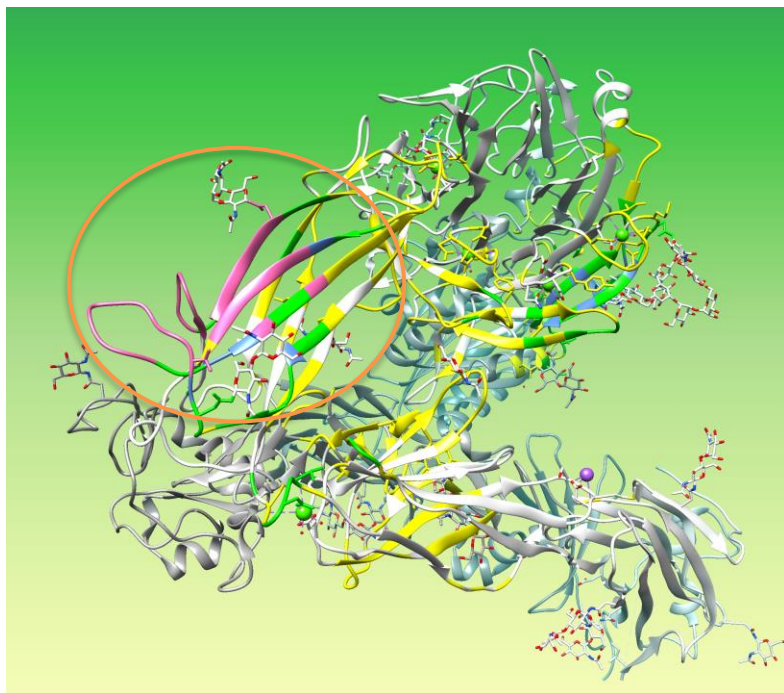
Syndecan-1 Mimetic						
Concentration ( $\mu\text{M}$ )	Relative Migration Area (Artificial Unit)					
6	65.04	65.37	80.11	52.96	65.27	71.59
2	82.81	84.61	92.50	81.69	82.47	80.88
1	82.26	85.77	103.42	94.08	106.81	84.19
0	100.70	98.40	94.45	99.88	103.13	100.35

**Table S1.** Summary of wound-healing assay results. The results presented were from 6 biological replicates.

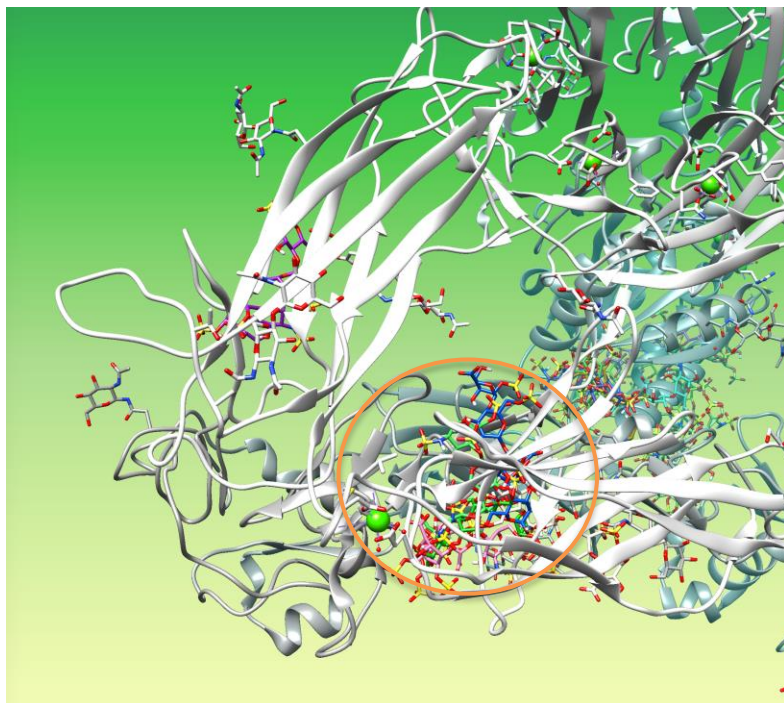


**Figure S1.** Representative microscopy images of MDA-MB-231 treated with (a) PBS as control and (b) glyco-polypeptide mimetic **1** ( $6 \mu\text{M}$ ) after 20-hour incubation (solid lines for cell frontiers at  $T=0$  and dashed lines for  $T=20$  hours; 10X magnification; scale bar,  $200 \mu\text{m}$ ). The addition of glyco-polypeptide mimetic **1** significantly reduced the migratory abilities of the cancer cells, leaving larger relative wound areas.

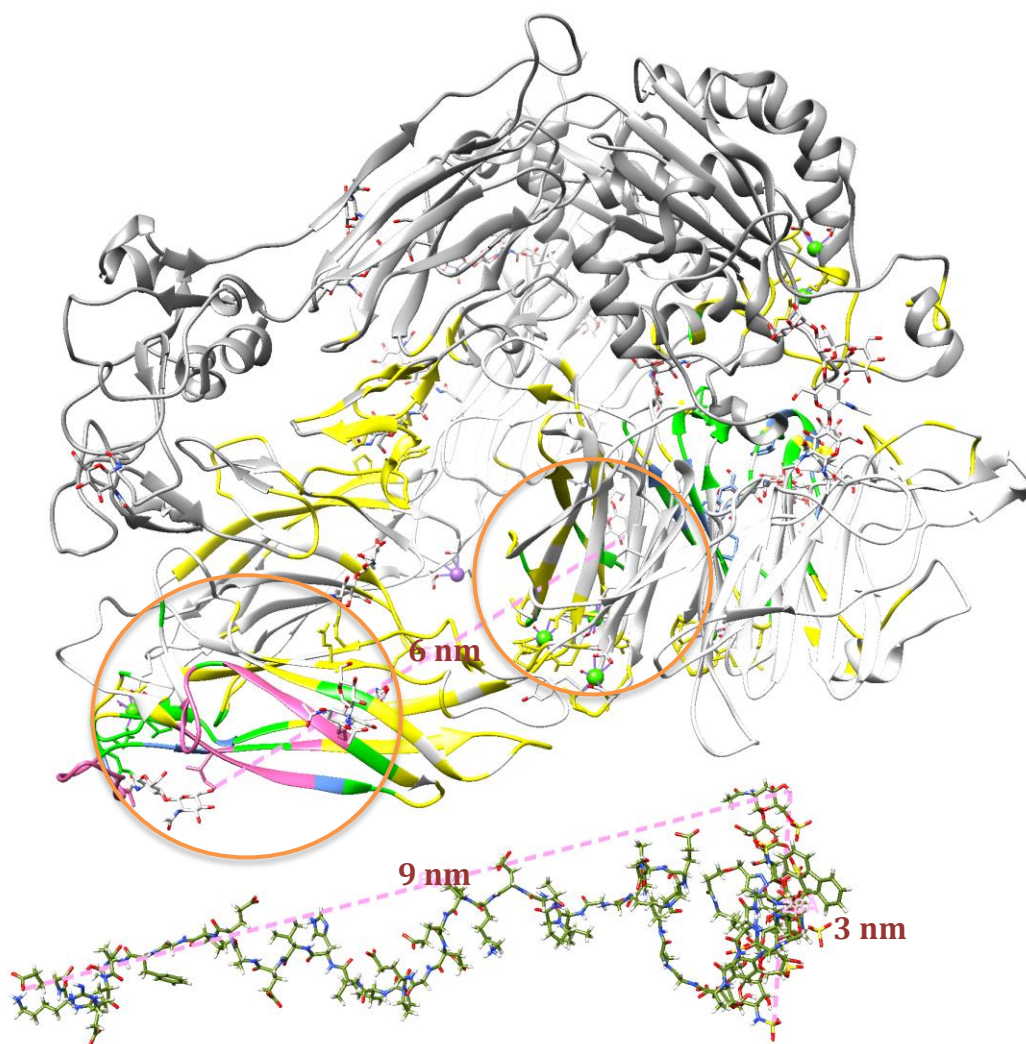
## Computer Docking Simulation Result and Biomolecule Visualization



**Figure S2.** Identified synstatin peptide binding site (as circled) on the surface of integrin  $\alpha\beta 3$  (PDB: 4G1M).



**Figure S3.** One of the identified heparan sulfate binding sites (as circled) on the surface of integrin  $\alpha\beta 3$  (PDB: 4G1M).



**Figure S4.** Biomolecule visualization and approximate size comparison of syndecan-1 mimetic 1 and integrin  $\alpha\text{v}\beta\text{3}$  (PDB: 4G1M). Predicted binding areas of synstatin peptide and heparan sulfate are highlighted with orange circles.

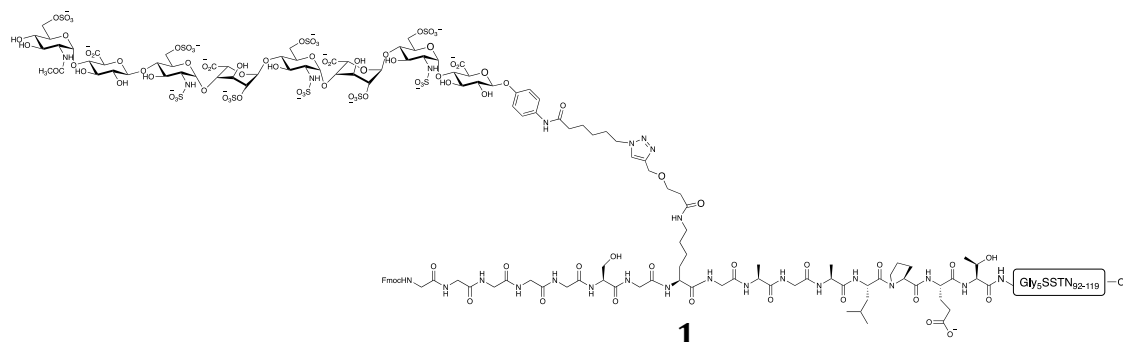
	<b>Estimated Center-to-Center Distance</b>
<b>The Spotted Binding Sites</b>	<b>6 nm</b>

**Table S2.** Measured estimated distance of the spotted synstatin and heparan sulfate binding sites

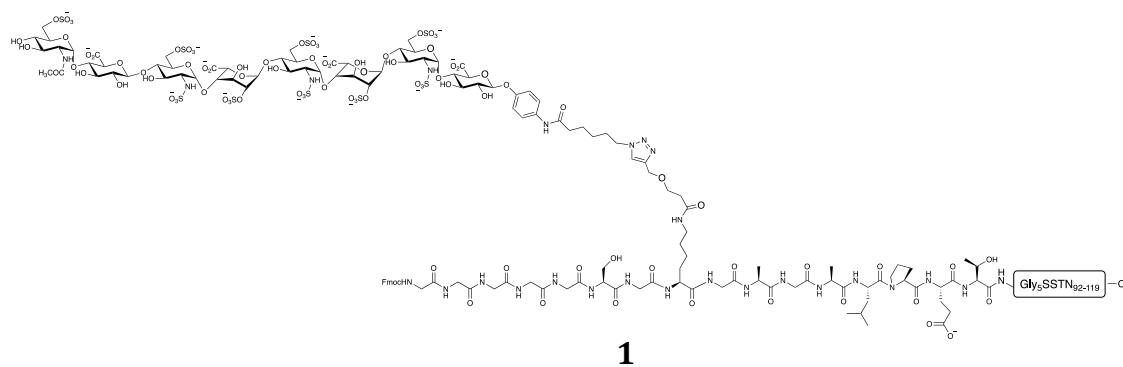
	<b>Longitudinal</b>	<b>Transversal</b>
<b>Syndecan-1 Mimetics</b>	<b>9 nm</b>	<b>3 nm</b>

**Table S3.** Measured approximate dimensions of syndecan-1 mimetic.

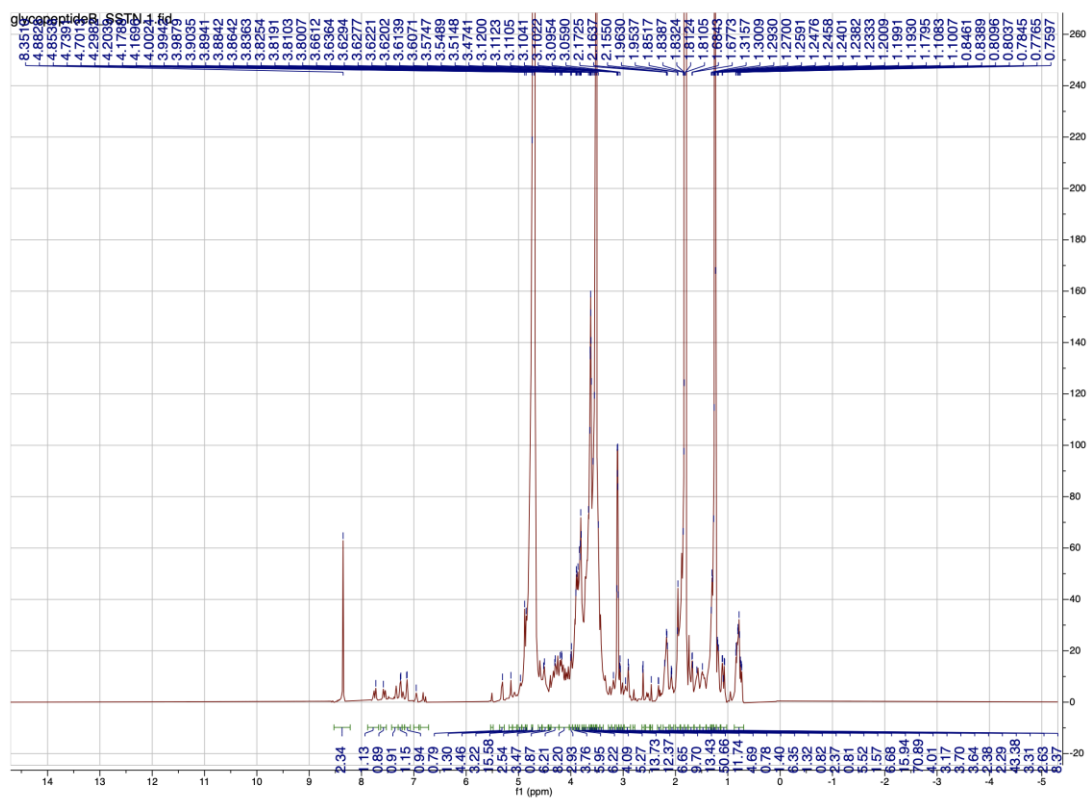
## Product Characterization Data and Spectra

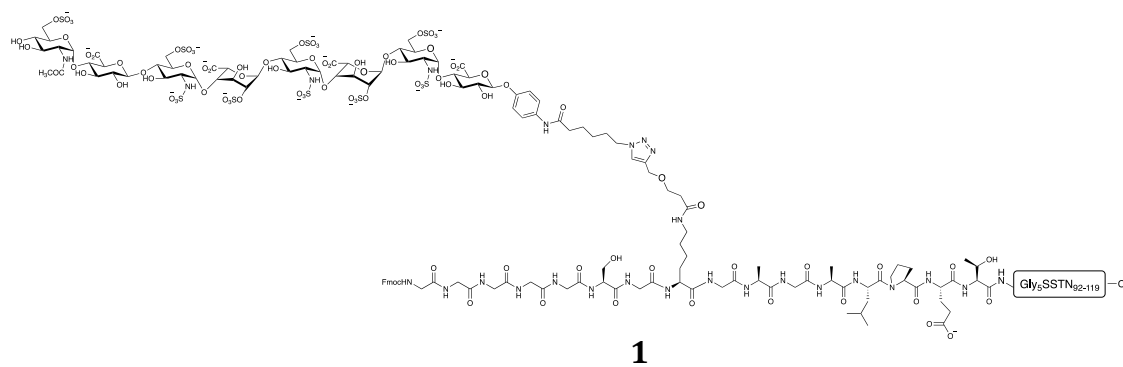


The purity of glycopeptide **1** was verified with analytical C-18 HPLC (5-100% acetonitrile/water; 0.1% trifluoroacetic acid). <sup>1</sup>H-NMR (900 MHz, D<sub>2</sub>O), δ 0.70 – 0.88 (8 H, m), 1.02 – 1.13 (3 H, m), 1.14-1.22 (3 H, m), 1.22 – 1.27 (43 H, m), 1.27-1.28 (2 H, m), 1.31 – 1.33 (2 H, m), 1.33-1.42 (4 H, m), 1.42-1.54 (4H, m), 1.54-1.64 (3H, m), 1.65-1.76 (4 H, m), 1.76-1.81 (71 H, m), 1.83 – 1.90 (16 H, m), 1.91 – 2.00 (7 H, m), 2.03-2.10 (2H, m), 2.13-2.24 (6 H, m), 2.28-2.35 (1H, m), 2.49-2.57 (1H, m), 2.59-2.64 (1 H, m), 2.86-2.98 (2H, m), 2.99-3.04 (1 H, m), 3.04-3.08 (1H, m), 3.09 – 3.13 (6 H, m), 3.16-3.22 (1 H, m), 3.22-3.28 (1H, m), 3.38-3.44 (5 H, m), 3.44-3.50 (12H, m), 3.51-3.53 (51 H, m), 3.53 – 3.56 (13 H, m), 3.56-3.59 (10 H, m), 3.59-3.61 (7 H, m), 3.61 – 3.65 (12 H, m), 3.65-3.72 (14H, m), 3.72-3.75 (5H, m), 3.76-3.78 (4H, m), 3.83-3.87 (6 H, m), 3.88-3.92 (6H, m), 3.92-3.97 (4H, m), 3.97-4.02 (3H, m), 4.02-4.22 (8H, m), 4.22-4.39 (6H, m), 4.39-4.41 (1H, m), 4.42-4.55 (3H, m), 4.55-4.61 (3H, m), 4.77-4.82 (16 H, m), 4.82-4.86 (3 H, m), 4.88-4.92 (4 H, m), 4.94-5.01 (1H, m), 5.03-5.11 (1H, m), 5.11-5.18 (1H, m), 5.27-5.37 (1H, m), 7.07-7.17 (1 H, m), 7.23 – 7.30 (1 H, m), 7.30-7.42 (1 H, m), 7.52 – 7.64 (1 H, m), 7.67 – 7.88 (1 H, m), 8.30-8.40 (2 H, m). <sup>13</sup>C-NMR (225 MHz, D<sub>2</sub>O), δ 12.1, 12.2, 12.3, 12.8, 12.9, 13.3, 15.7, 16.4, 16.5, 16.5, 16.6, 16.7, 17.2, 17.7, 17.8, 17.9, 18.0, 18.2, 18.2, 18.4, 18.6, 18.7, 18.8, 19.3, 20.0, 20.6, 20.9, 21.4, 21.7, 21.9, 22.0, 22.0, 22.4, 22.4, 22.7, 23.0, 23.3, 24.0, 24.1, 24.2, 24.3, 24.4, 24.6, 24.7, 26.2, 27.1, 28.6, 28.9, 29.2, 29.3, 30.0, 30.2, 30.5, 33.4, 33.5, 33.7, 33.8, 34.0, 34.4, 35.9, 36.0, 37.0, 37.1, 38.6, 38.7, 38.9, 38.9, 39.0, 39.1, 39.8, 39.9, 40.4, 42.3, 42.4, 42.6, 43.1, 43.2, 43.2, 43.6, 43.6, 44.4, 44.5, 46.7, 46.8, 47.4, 47.9, 49.5, 49.6, 49.8, 50.1, 50.2, 50.3, 51.6, 51.7, 51.9, 52.0, 53.3, 53.4, 53.5, 53.6, 53.7, 53.8, 54.2, 54.3, 54.4, 55.0, 55.1, 55.2, 55.7, 55.8, 57.5, 57.7, 58.0, 58.2, 58.3, 58.5, 58.8, 59.0, 59.1, 59.2, 59.2, 59.4, 59.6, 60.3, 60.4, 60.5, 61.1, 61.2, 62.0, 62.4, 62.5, 62.8, 62.9, 64.0, 64.3, 65.4, 65.6, 66.0, 66.1, 66.2, 66.5, 66.8, 67.1, 67.2, 67.3, 67.4, 67.5, 68.6, 68.8, 68.9, 69.0, 69.1, 69.4, 69.5, 69.6, 69.8, 70.1, 70.2, 70.7, 71.1, 71.3, 71.4, 71.7, 71.8, 72.1, 72.5, 73.2, 73.3, 73.4, 74.0, 75.1, 75.3, 75.4, 75.7, 75.8, 75.9, 76.2, 76.4, 76.5, 79.9, 93.1, 95.2, 95.3, 95.6, 96.4, 96.5, 96.9, 97.6, 98.3, 98.4, 99.0, 100.1, 100.2, 101.6, 101.6, 116.8, 120.2, 123.4, 123.5, 125.0, 127.1, 127.2, 127.4, 128.0, 128.5, 128.6, 128.6, 129.1, 145.9, 146.5, 146.8, 146.9, 147.0, 147.1, 147.2, 147.3, 147.5, 147.6, 147.7, 148.0, 152.2, 152.3, 152.3. ESI-MS: C<sub>277</sub>H<sub>411</sub>N<sub>65</sub>O<sub>145</sub>S<sub>9</sub> [M+10H]<sup>4+</sup> calcd: 1451.0859, obsd: 1451.0818 (2.84 ppm).

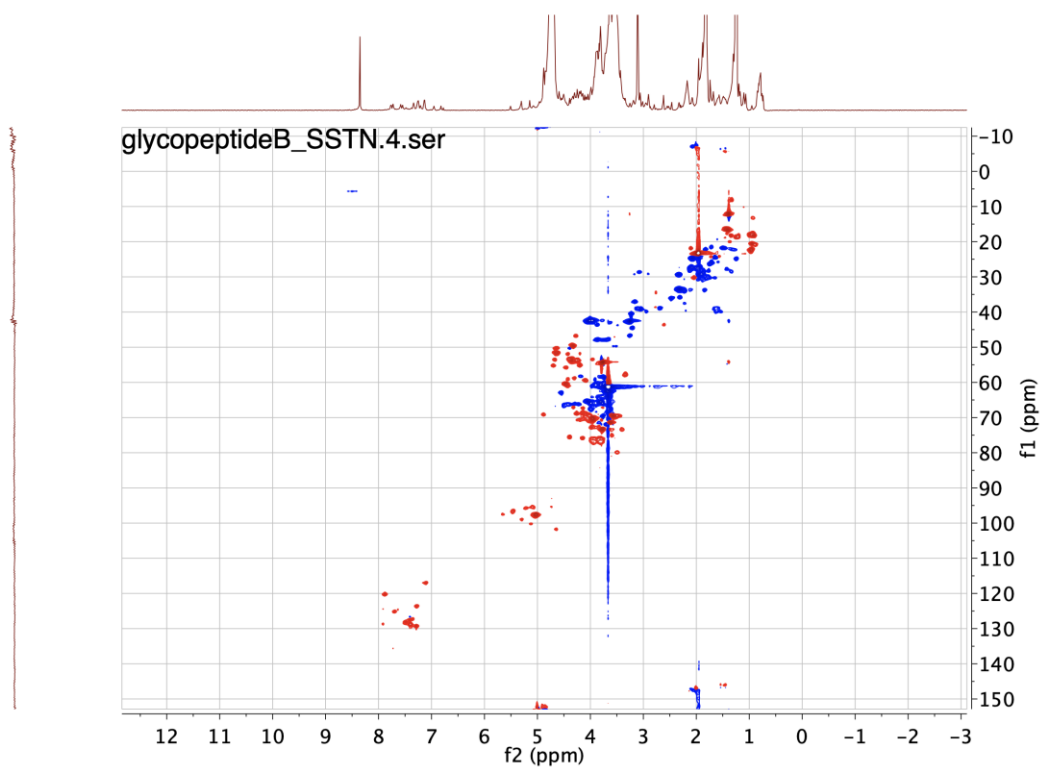


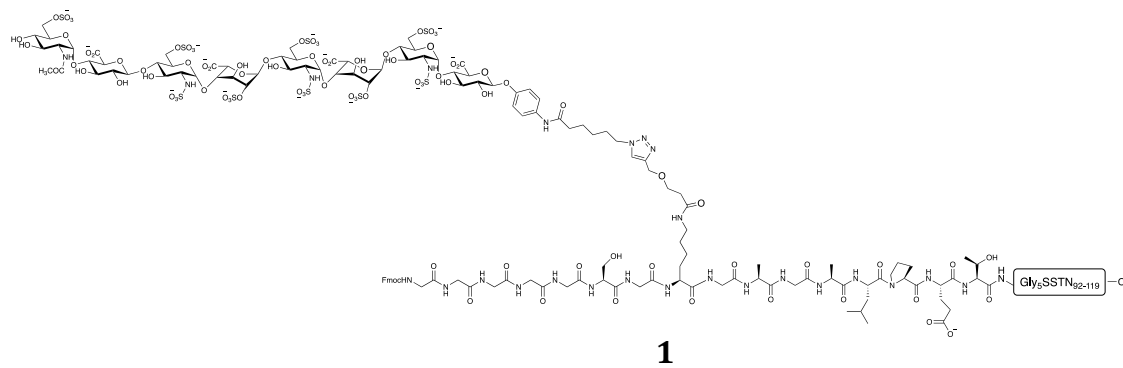
<sup>1</sup>H-NMR of **1** (900 MHz D<sub>2</sub>O)



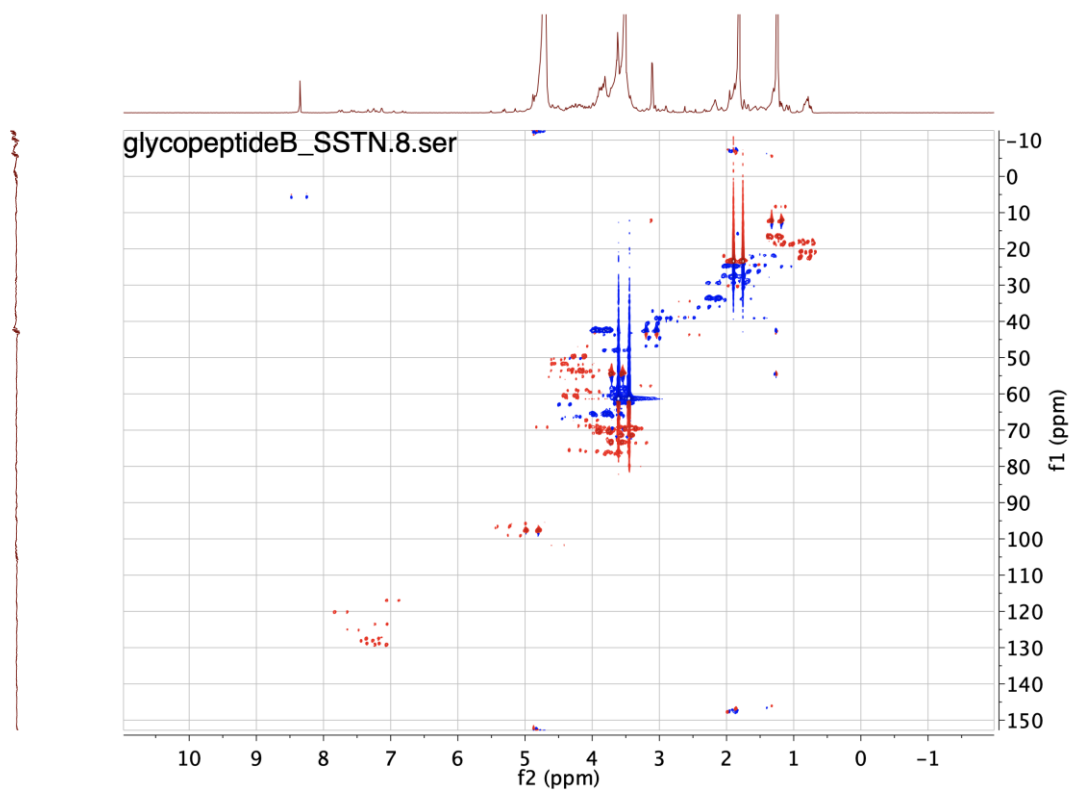


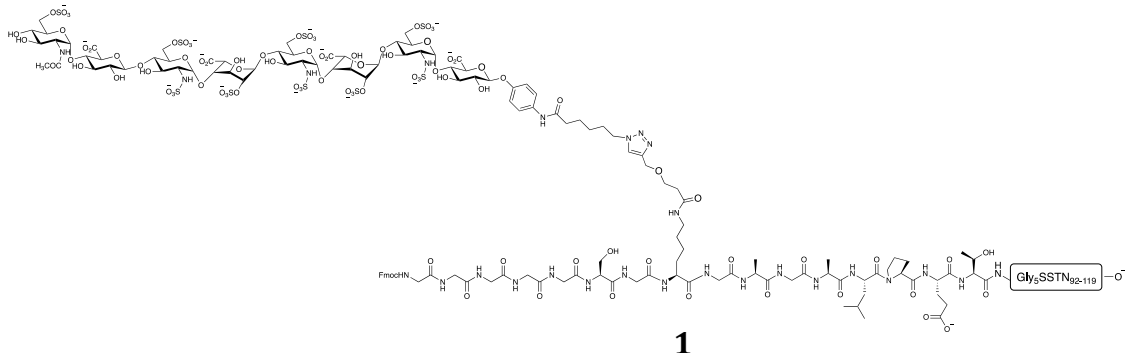
$^1\text{H}$ - $^{13}\text{C}$  gHSQCAD of **1** (900 MHz  $\text{D}_2\text{O}$ )





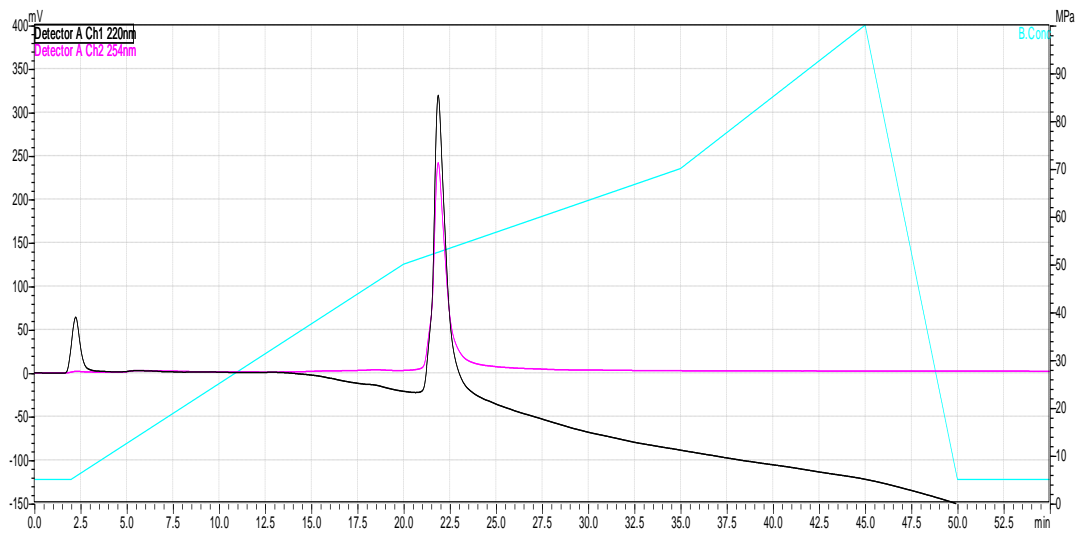
$^1\text{H}$ - $^{13}\text{C}$  Coupled gHSQCAD of **1** (900 MHz  $\text{D}_2\text{O}$ )



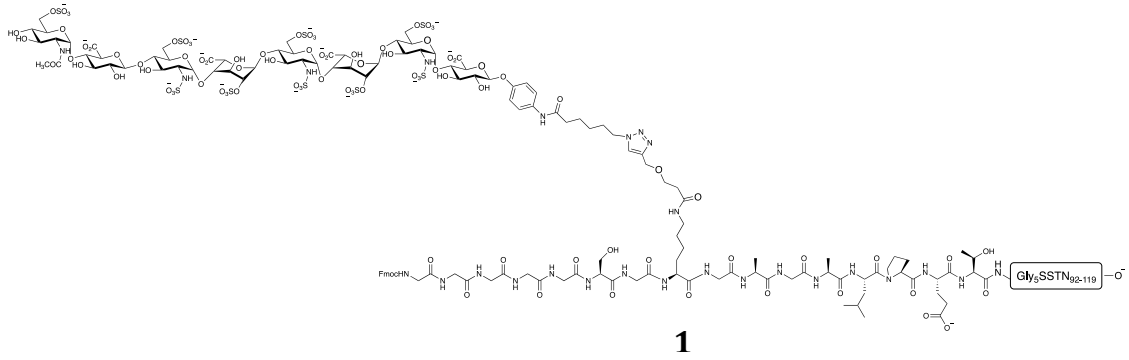


## HPLC Chromatogram of 1

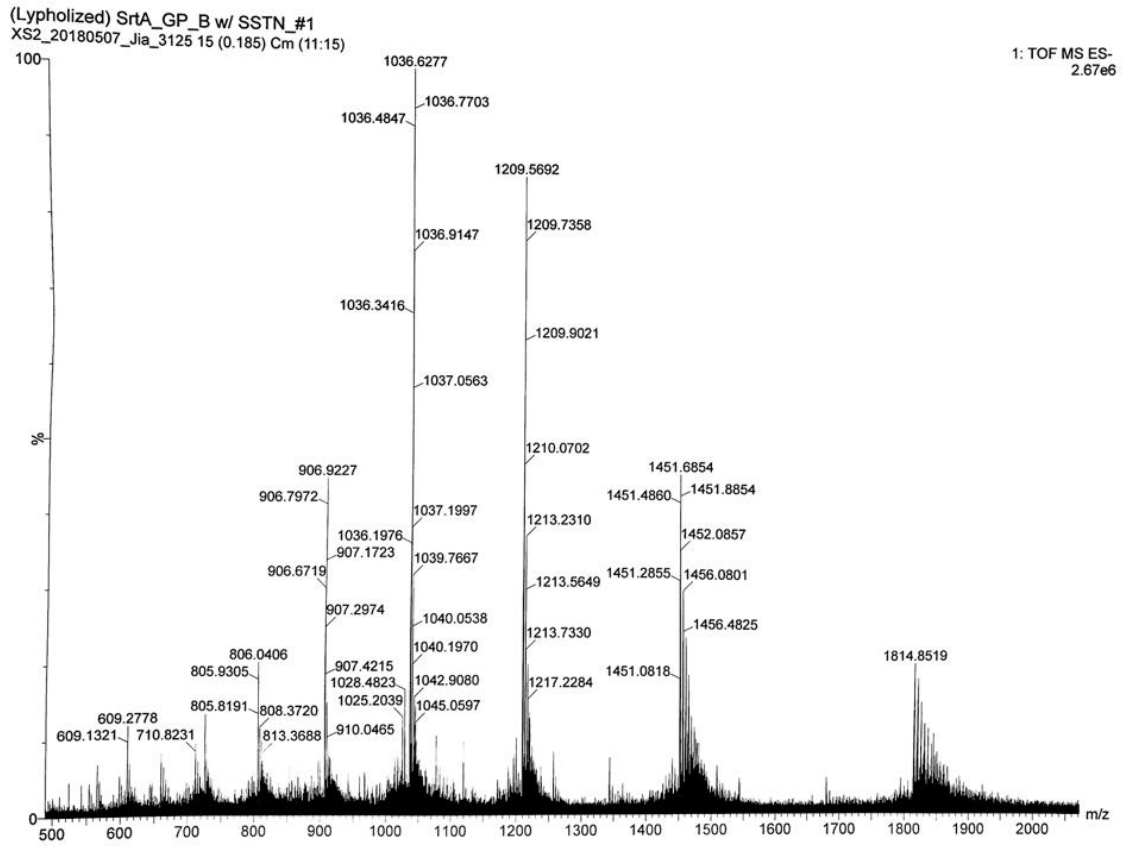
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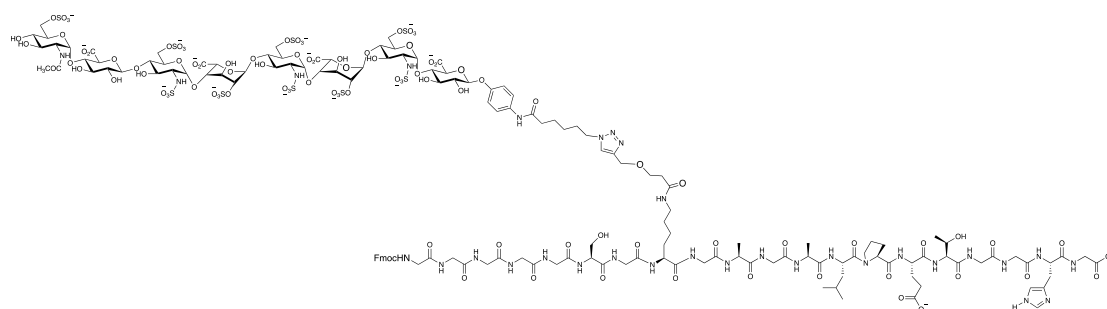






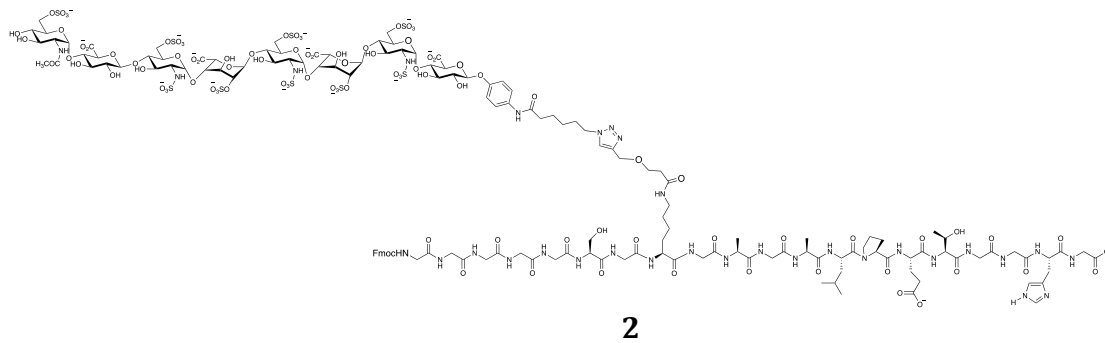
### ESI-MS of 1



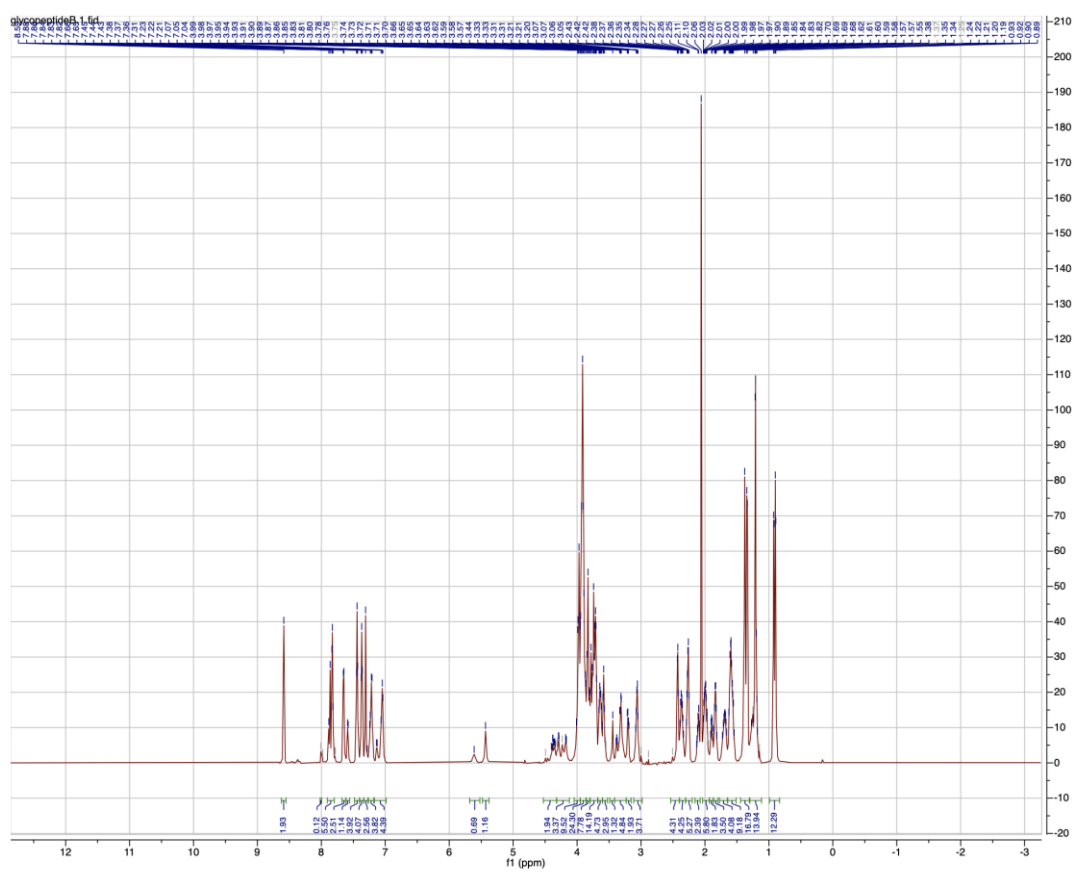


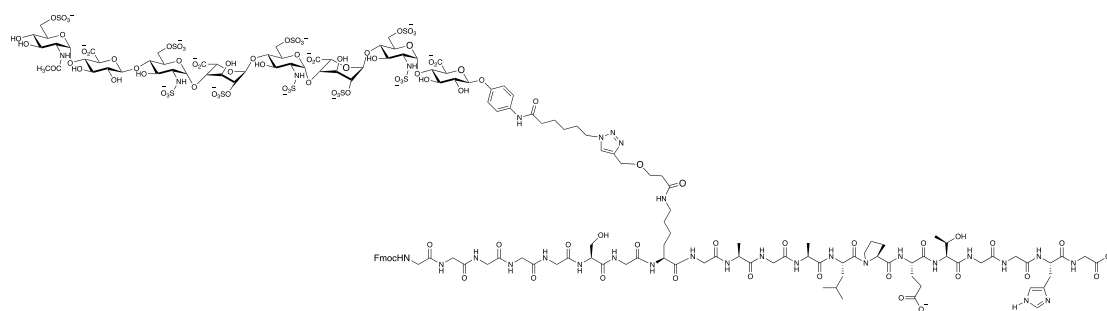
## 2

The purity of glycopeptide **2** was verified with analytical C-18 HPLC (5-100% acetonitrile/water; 0.1% trifluoroacetic acid). <sup>1</sup>H-NMR (900 MHz, D<sub>2</sub>O), δ 0.91 (12 H, m), 1.12 – 1.30 (14 H, m), 1.30 – 1.44 (17 H, m), 1.51 – 1.64 (9 H, m), 1.65 – 1.77 (4 H, m), 1.84 (3 H, m), 1.89 (2 H, m), 2.00 (6 H, m), 2.11 (2 H, m), 2.27 (5 H, m), 2.36 (4 H, m), 2.40 – 2.53 (4 H, m), 3.06 (4 H, m), 3.20 (2 H, m), 3.23 – 3.41 (5 H, m), 3.44 (1 H, m), 3.58 (3 H, m), 3.64 (5 H, m), 3.67 – 3.80 (14 H, m), 3.84 (8 H, m), 3.92 (24 H, m), 3.95 – 4.05 (10 H, m), 4.12 – 4.32 (3 H, m), 4.37 (2 H, m), 5.43 (1 H, m), 5.61 (1 H, m), 6.99 – 7.17 (4 H, m), 7.23 (4 H, m), 7.31 (3 H, m), 7.37 (4 H, m), 7.44 (4 H, m), 7.59 (1 H, m), 7.65 (3 H, m), 7.80 – 7.91 (6 H, m), 8.59 (2 H, m). <sup>13</sup>C-NMR (225 MHz, D<sub>2</sub>O), δ 16.3, 16.6, 18.7, 20.5, 21.9, 22.3, 22.4, 24.3, 24.4, 24.6, 24.8, 26.6, 26.9, 27.7, 28.8, 29.2, 30.3, 32.3, 35.8, 36.0, 38.9, 39.0, 42.3, 42.3, 42.5, 43.2, 43.6, 46.7, 47.7, 49.3, 49.9, 50.1, 50.2, 52.2, 53.5, 53.6, 53.9, 55.8, 57.5, 57.9, 58.0, 59.0, 60.4, 61.0, 62.8, 65.8, 66.1, 66.2, 66.8, 67.0, 68.8, 69.0, 69.3, 70.0, 70.5, 72.4, 73.4, 75.8, 76.2, 76.3, 77.0, 77.3, 97.0, 97.6, 100.2, 101.8, 116.9, 117.5, 120.1, 123.4, 124.5, 124.7, 125.0, 127.4, 128.0, 128.1, 131.8, 133.5, 140.8, 143.6, 143.8, 153.8, 170.8, 171.1, 171.3, 171.8, 172.7, 174.0, 174.2, 174.6. ESI-MS: C<sub>146</sub>H<sub>207</sub>N<sub>31</sub>O<sub>99</sub>S<sub>9</sub> [M+11H]<sup>4+</sup> calcd: 1066.4901, obsd: 1066.4874 (2.51 ppm).



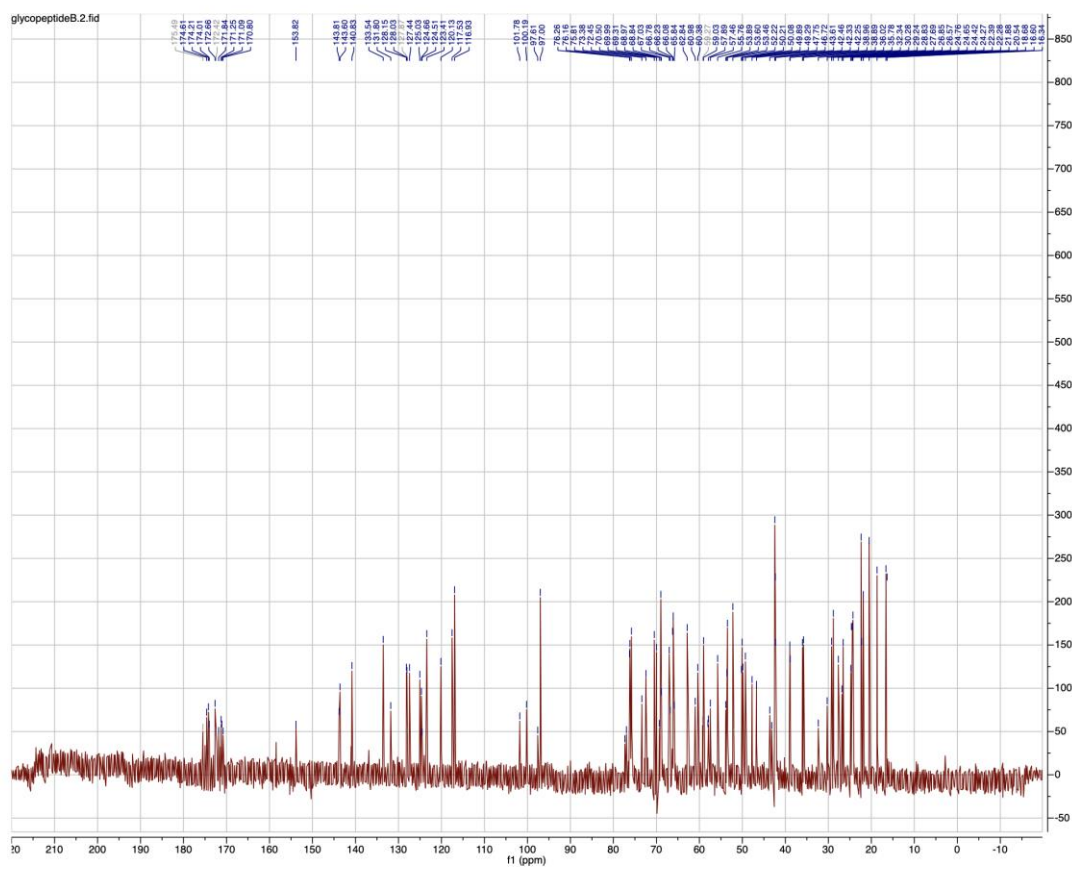
<sup>1</sup>H-NMR of **2** (900 MHz D<sub>2</sub>O)



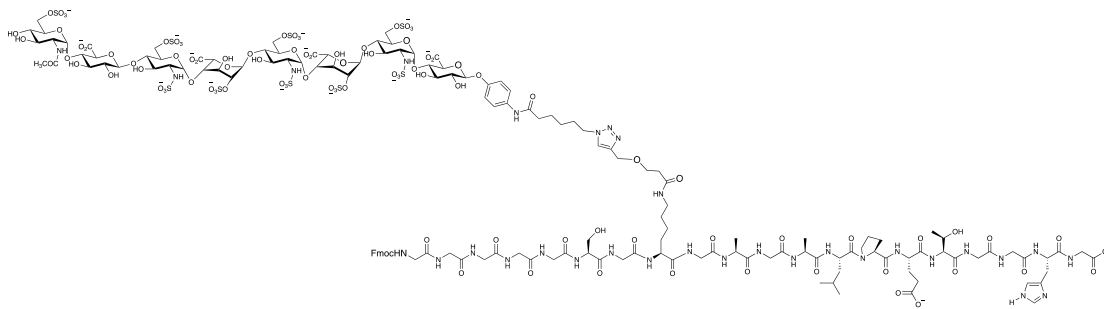


**2**

$^{13}\text{C}$ -NMR of **2** (225 MHz  $\text{D}_2\text{O}$ )

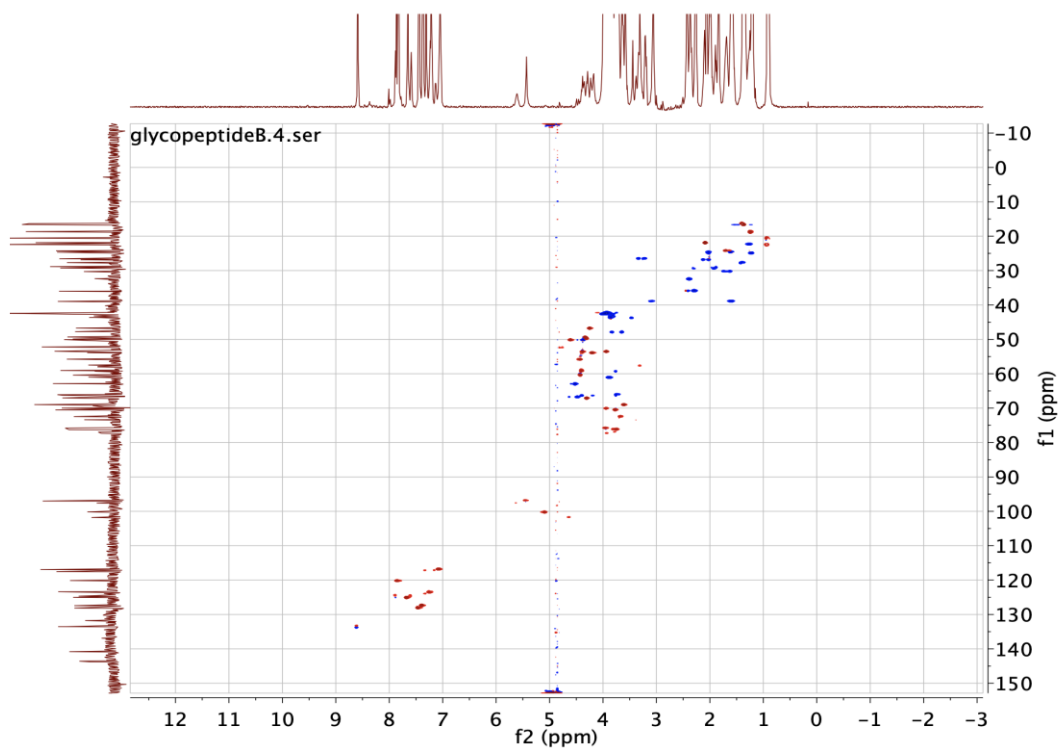




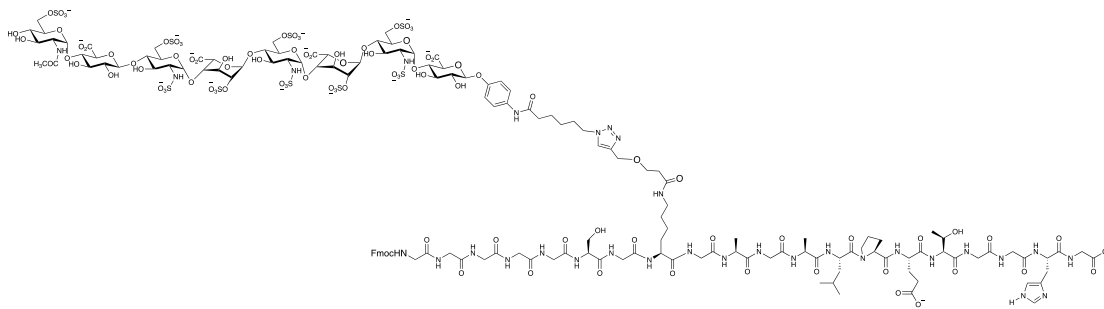


**2**

$^1\text{H}$ - $^{13}\text{C}$  gHSQCAD of **2** (900 MHz  $\text{D}_2\text{O}$ )

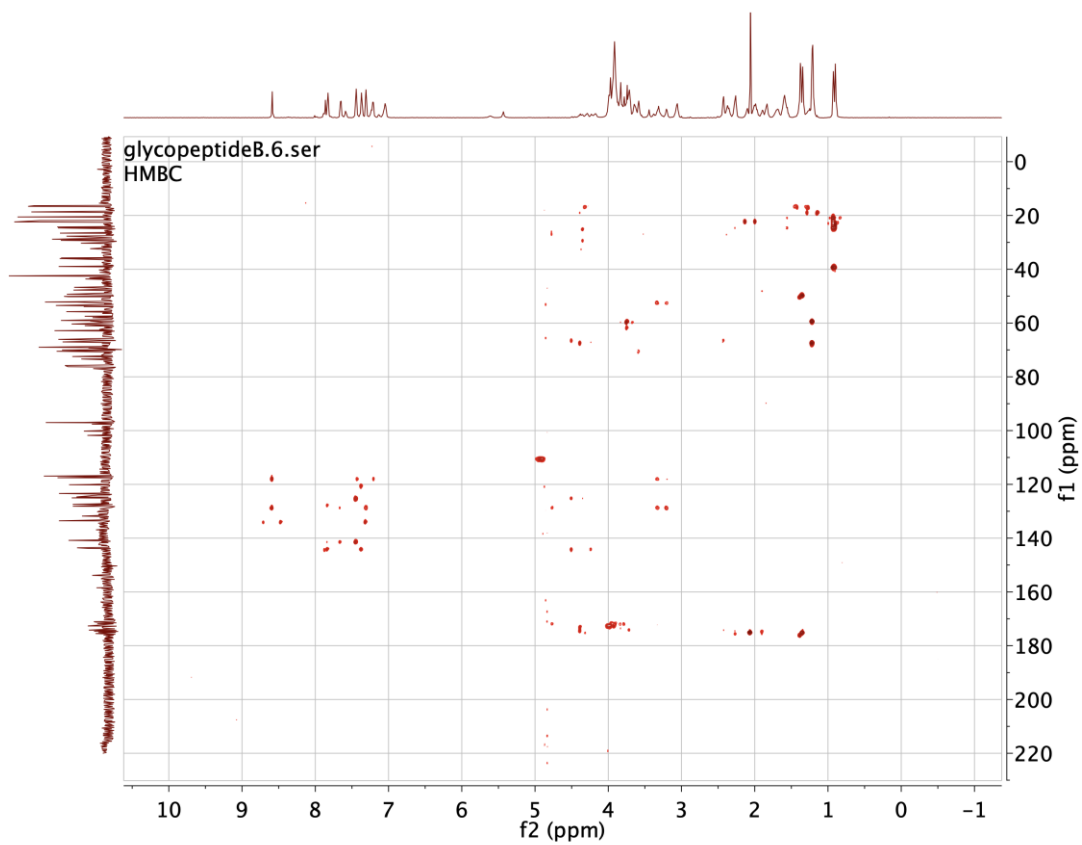




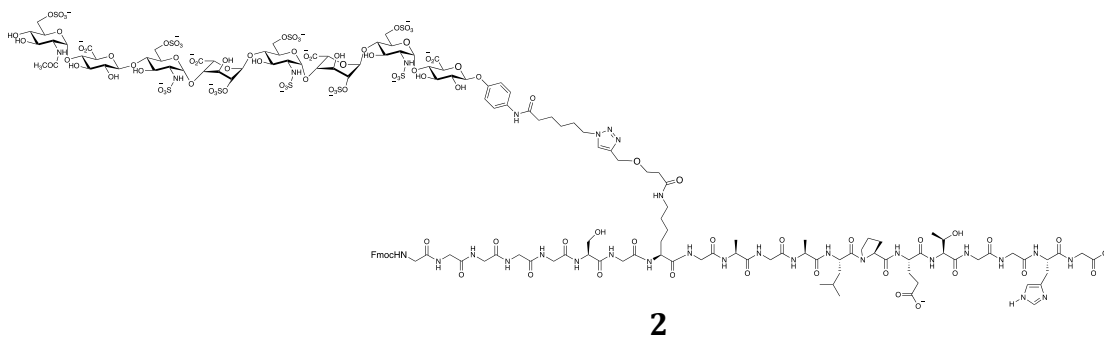


2

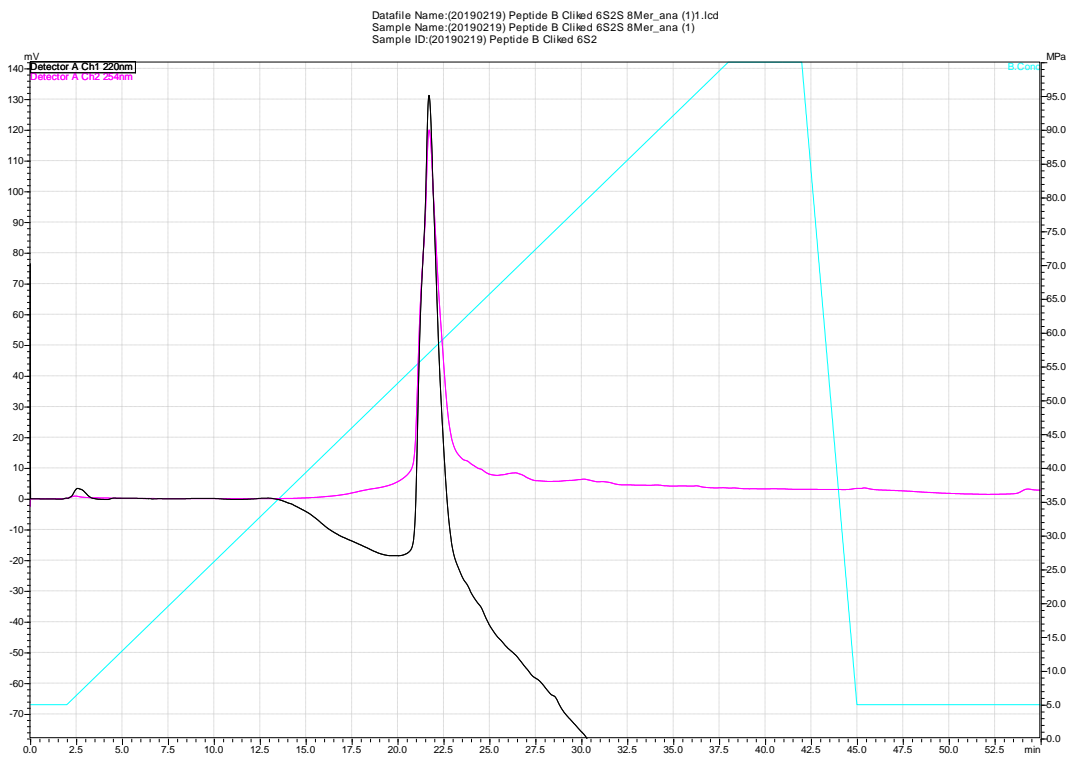
$^1\text{H}$ - $^{13}\text{C}$  gHMBC of 2 (900 MHz  $\text{D}_2\text{O}$ )

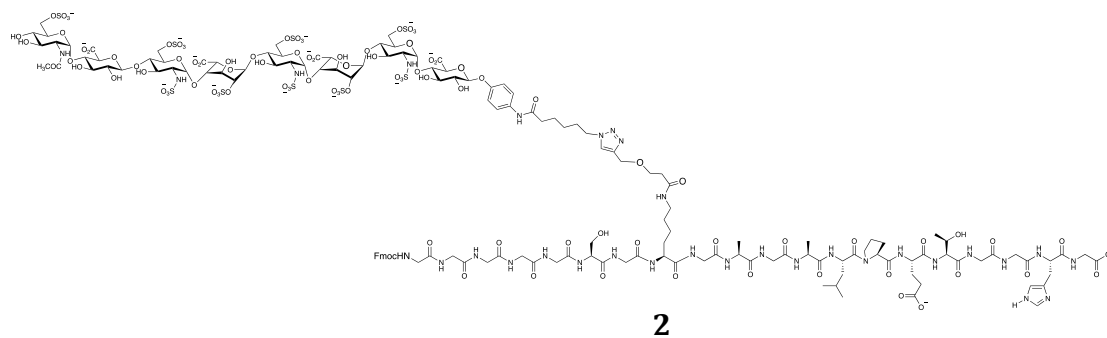




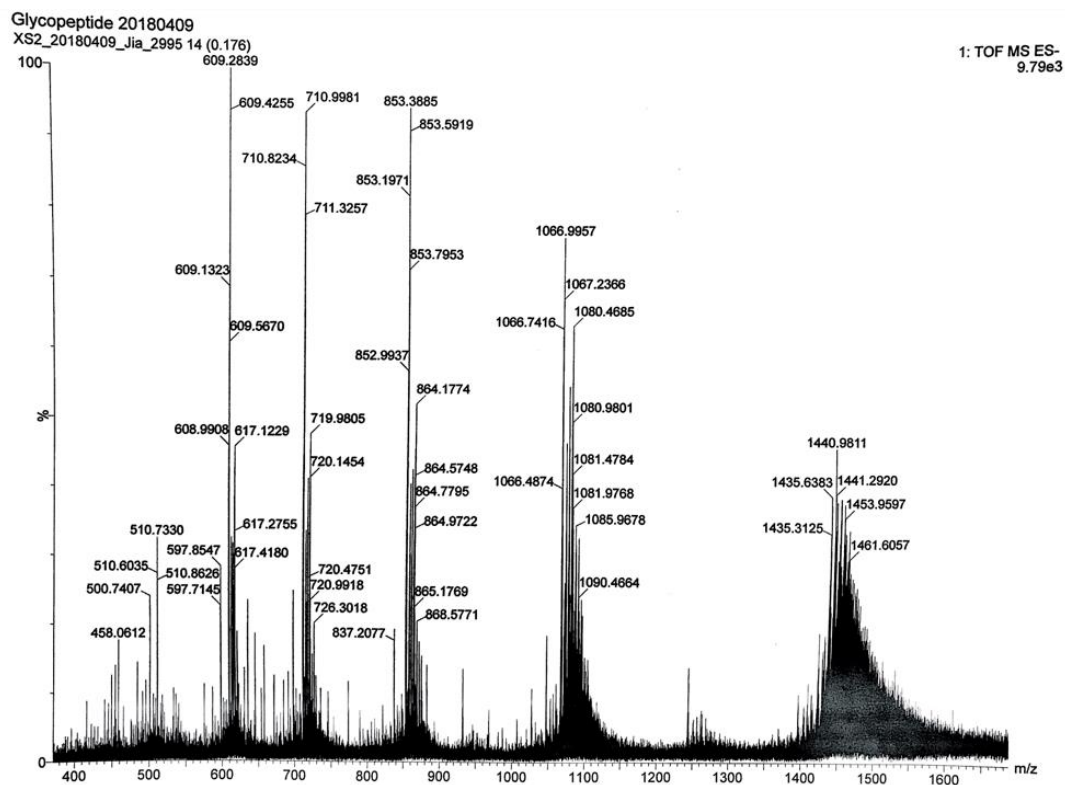


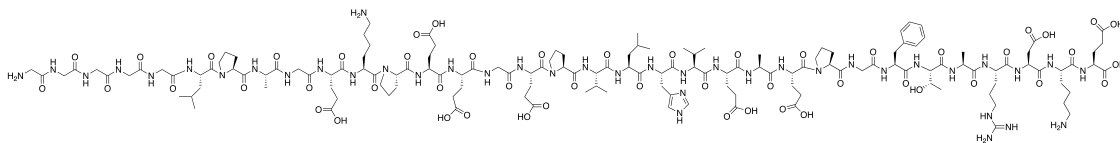
### HPLC Chromatogram of 2





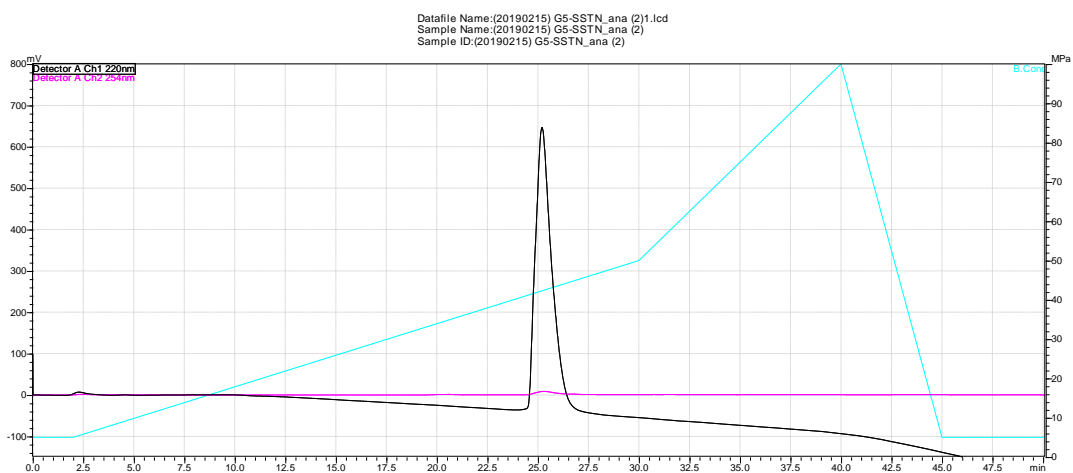
ESI-MS of 2



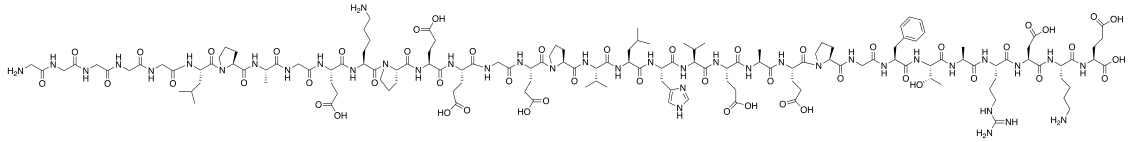


**3**

### HPLC Chromatogram of 3

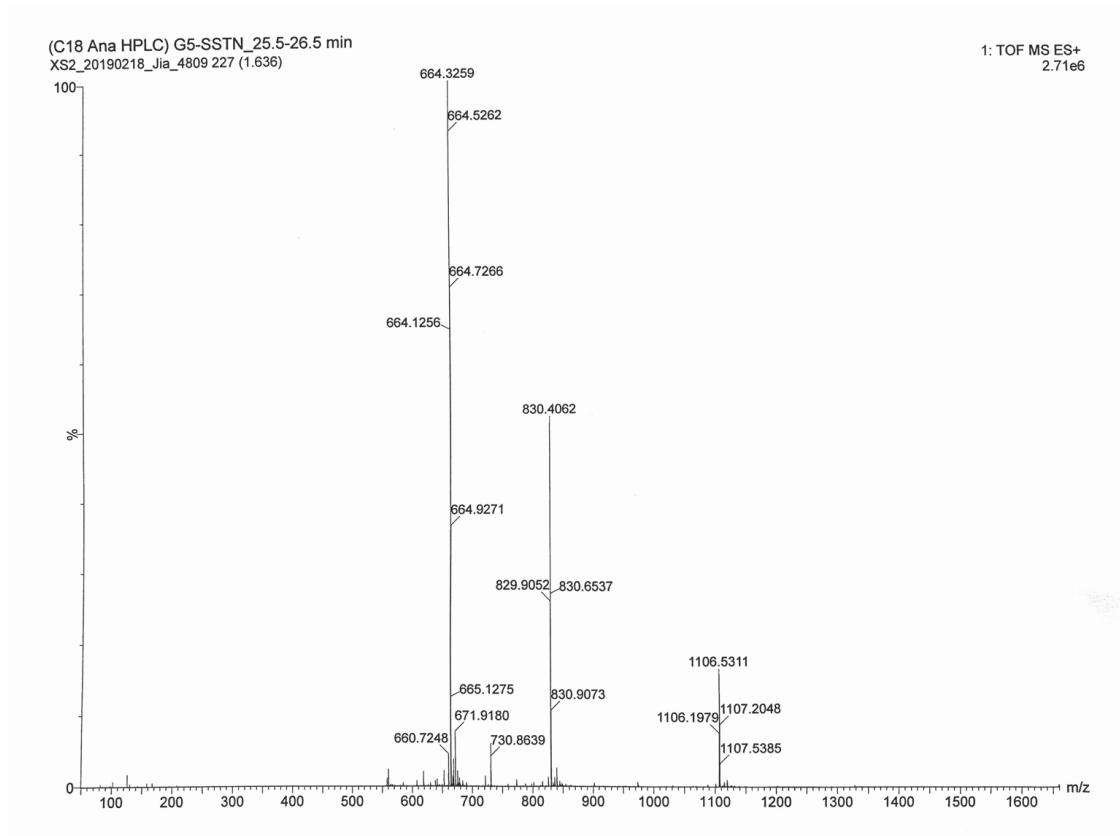


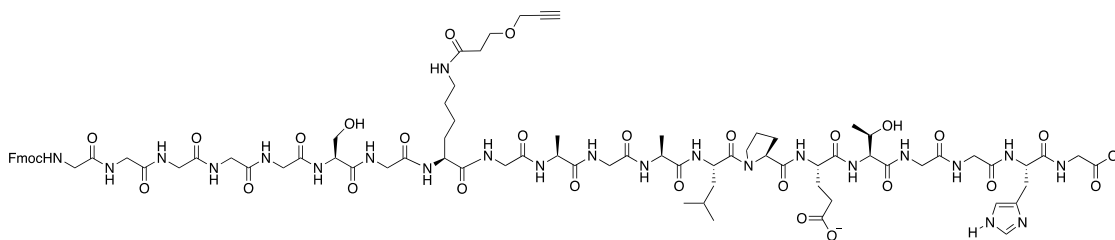
The purity of peptide **3** was verified with analytical C-18 HPLC (5-100% acetonitrile/water; 0.1% trifluoroacetic acid). ESI-MS:  $C_{143}H_{226}N_{40}O_{51}$   $[M+4H]^{4+}$  calcd: 829.9075, obsd: 829.9052 (2.77 ppm).



3

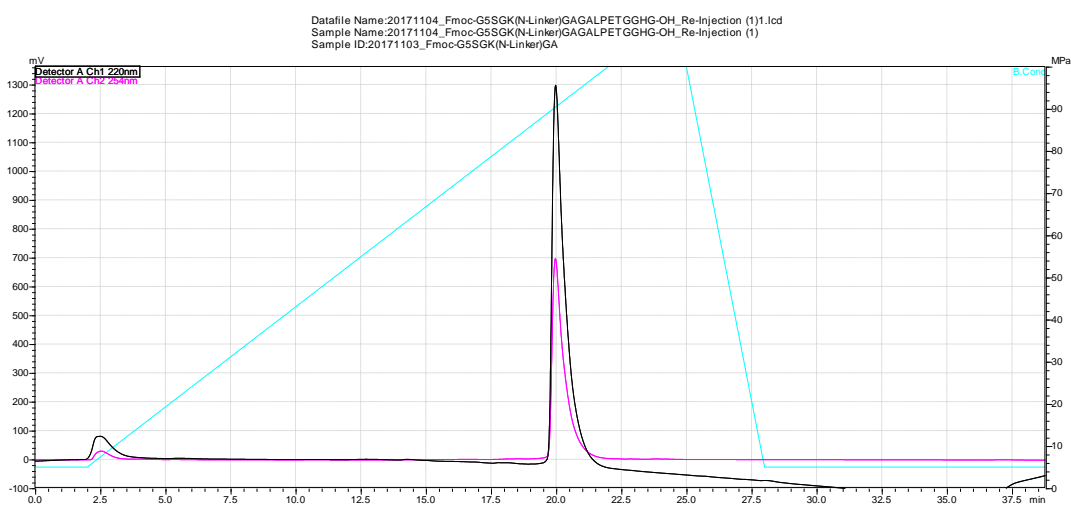
ESI-MS of 3



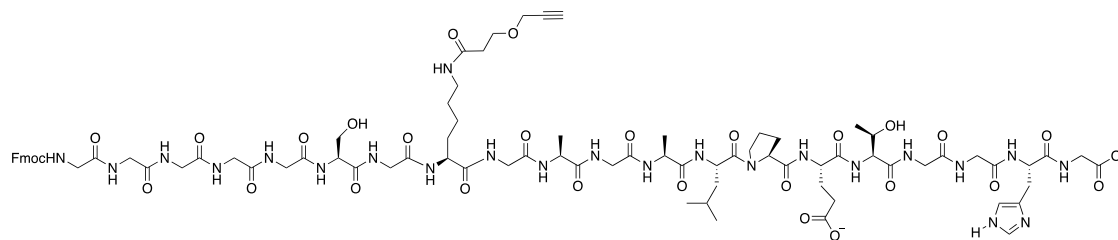


5

### HPLC Chromatogram of 5

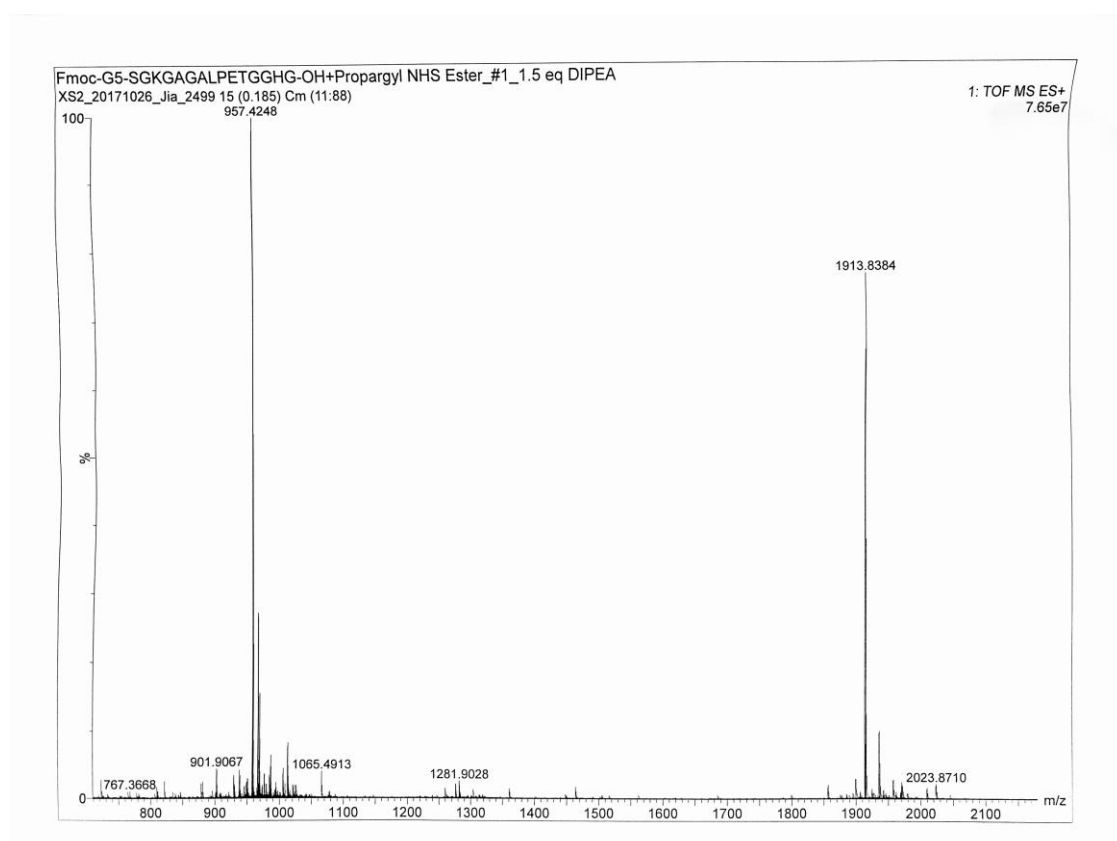


The purity of peptide **5** was verified with analytical C-18 HPLC (5-100% acetonitrile/water; 0.1% trifluoroacetic acid). ESI-MS:  $C_{84}H_{118}N_{23}O_{29}$   $[M+4H]^{4+}$  calcd: 1912.8461, obsd: 1912.8388 (3.82ppm).



5

ESI-MS of 5



## References

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