Supplementary Information

Chemoenzymatic synthesis of sulfated *O*-glycopeptides from human CD34

Yinyu Jiang,[†ab] Qi Du,[†c] Fengzhuo Wu,[b] Wei Zhang,[b] Yuan Ma,[a] Zhuojia Xu,[a] Zhonghua Li,*[c] Wenjing Ma*[ad] and Tiehai Li*[ab]

[a]State Key Laboratory of Chemical Biology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China.

[b]School of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing 210023, China

[c]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, NHC Key Laboratory of Glycoconjugates Research, and Department of Hepatobiliary Oncology, Zhongshan Hospital, Fudan University, Shanghai 200032, China.

[d]School of Pharmacy, Xi'an Jiaotong University, Shaanxi 710061, China

E-mail: zhonghua_@fudan.edu.cn; mwj@xjtu.edu.cn; tiehaili@simm.ac.cn

[†]These authors contributed equally to this work.

1. General materials and methods

Low-resolution mass spectrometry (LRMS) was measured on ESI apparatus using a Shimadzu LC-MS2020. High-resolution mass spectrometry (HRMS) was measured on ESI apparatus using Agilent 1290 G6460A Q-TOF. Compound analysis was recorded on Shimadzu LC-MS2020-ESI with XBrigde® Amide 3.5 μm, 2.1 mm x 150 mm column (Waters). Compound purification was performed on a C18 column (Bio-Rad Laboratories, 1.5 x 20 cm, 73711522) packed with C18 silica gel (Waters, WAT035672).

Fmoc-Ser(*t*Bu)-Wang Resin was purchased from GL Biochem (shanghai) Ltd. (GLS240319-41701). Chemical reagents were purchased from *J&K* Scientific Ltd. and TCI Shanghai, China. Cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac), uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), guanosine 5′-diphospho-β-L-fucose (GDP-Fuc), uridine 5′-diphosphogalactose (UDP-Gal) and PAPS were purchased from GLYCOGENE. Calf intestine alkaline phosphatase (CIAP) were purchased from BioLabs® Inc. Biotinylated lectins SNA, MAL-II, AAL was purchased from Vector Labs. Recombinant human L-selectin–Fc chimera was purchased from Abclonal. Recombinant human E-selectin–Fc chimera and recombinant human P-selectin–Fc chimera were purchased from Acro Biosystems. Biotinylated goat anti-Human IgG Fc Antibody and Streptavidin-AlexaFluor® 635 were purchased from Thermo-Fisher Scientific.

2. Glycosyltransferases expression and purification

Bacterial *Photobacterium phosphoreum* α-2,3-sialyltransferase (*Pp*α-2,3SiaT) was expressed in *E. coli* system and purified as described previously.[1] The construct was transformed into *E. coli* BL21 (DE3) cells, and protein expression was induced in LB medium containing 100 μg/mL kanamycin with agitation at 37 ºC with 200 rpm until the OD value reached 0.4-0.6, followed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to reach a final concentration of 0.4-0.6 mM. After growth at 20 ºC with agitation for 16 h, cells were harvested by

centrifugation (8000 g, 10 min, 4 ºC) and resuspended in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, pH 7.5). Next, cells in the lysis buffer was processed using a high-pressure crusher (Union-Biotech (Shanghai) Co., Ltd.) at 10,000 psi, and the lysate was clarified by centrifugation (8000 g, 30 min, 4 ºC). The lysate was centrifuged to remove the precipitate, and the resulting crude enzyme solution was purified as described previously.[1]

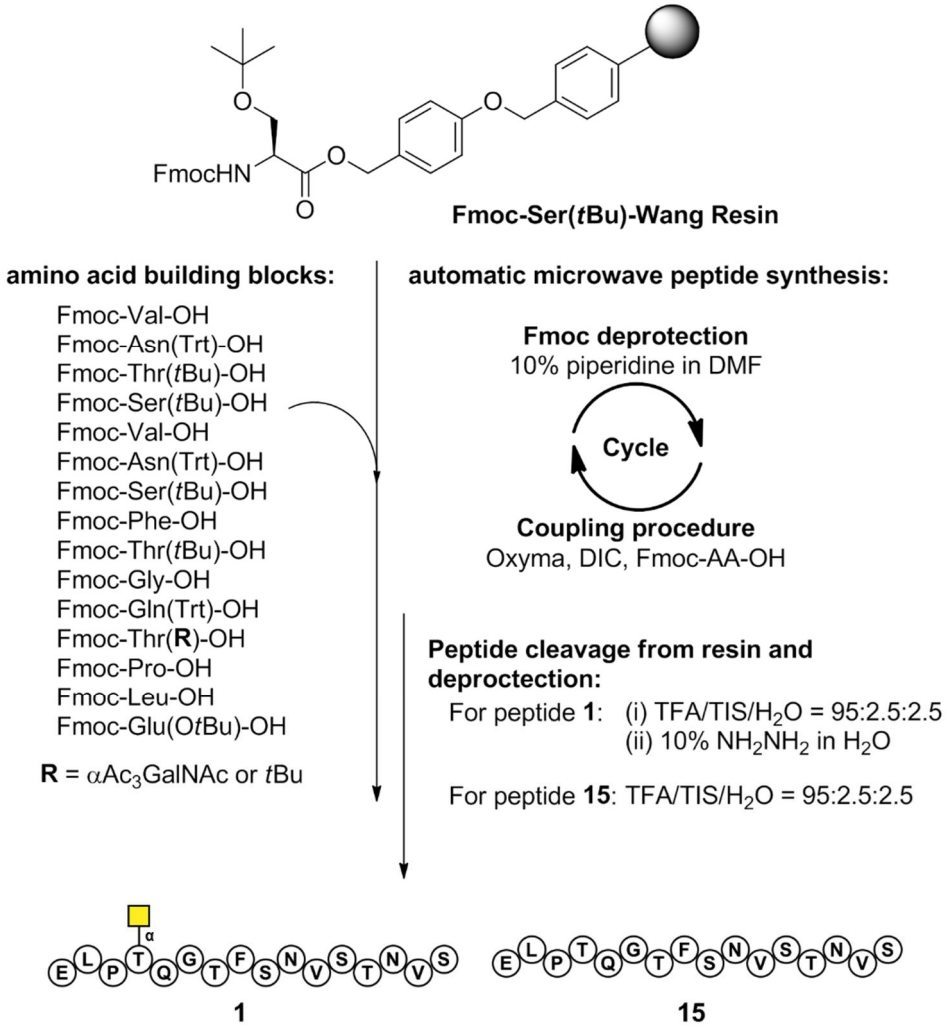
Human glycosyltransferases C1GalT1, GCNT1, B4GalT1, ST6Gal1, ST3Gal4, CHST2, and FUT6 were expressed and purified according to the methods described in the literature.[2-7] In this study, the catalytic domains of human glycosyltransferases were expressed as soluble secretory fusion proteins by transient transfection of suspension-cultured HEK293 cells. His-tagged proteins from the supernatant of HEK293 cells and the lysate of *E. coli* BL21 (DE3) cells were purified by Ni^{2+}-NTA affinity chromatography. The Ni^{2+}-NTA column was pre-equilibrated with five column volumes of binding buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM imidazole, pH 7.5). The column was washed with washing buffer (50 mM Tris-HCl, 250 mM NaCl, 40 mM imidazole, pH 7.5). The target proteins were eluted with elution buffer (50 mM Tris-HCl, 250 mM NaCl, 200 mM imidazole, pH 7.5). Peak fractions were pooled and concentrated to ~1 mg/mL using an ultrafiltration pressure cell membrane (Millipore, Billerica, MA) with a 10 kDa molecular weight cut off.

3. The synthesis of the target peptides 1-15

The synthesis of the target peptides 1 and 15

The synthesis of peptide **1** and **15** was carried out using the automatic microwave peptide synthesizer Liberty Blue 2.0 (Scheme S1). The glycosyl amino acid Fmoc-Thr(αAc_3GalNAc)-OH was synthesized as described in our previous study.[8] The procedure includes: 1) deprotection with 10 % piperidine in DMF for 2 min; 2) coupling with Oxyma (1 mmol, 5 equiv) in DMF and DIC (2 mmol, 10 equiv) in DMF with Fmoc-AA-OH (1.2 mmol, 6.0 equiv) for 2 min. For the synthesis of glycopeptide **1**, Fmoc-Thr(αAc_3GalNAc) building block (2.5 equiv.) was used. The peptide chain was cleaved from the resin with trifluoroacetic acid (TFA) (10 mL, containing 2.5% TIS and 2.5% H_2O) at room temperature for 3 h, followed by

filtration through a polytetrafluoroethylene (PTFE) filter. Isopropyl ether was added to make the product as precipitate, which was then collected and deacetylated with a 10% hydrazine hydrate solution for 10 min and purified on a C18 column to obtain the pure peptide (eluent: H_2O to 30% MeCN; 100.0 mg for compound **1** that can serve as a Gal acceptor for subsequent enzymatic elongation, 26% yield, 0.2 mmol scale; 57.3 mg for compound **15**, 34% yield, 0.1 mmol scale).



Scheme S1. Chemical synthesis of compounds **1** and **15**

Enzymatic synthesis of diverse *O*-glycopeptides 2-14

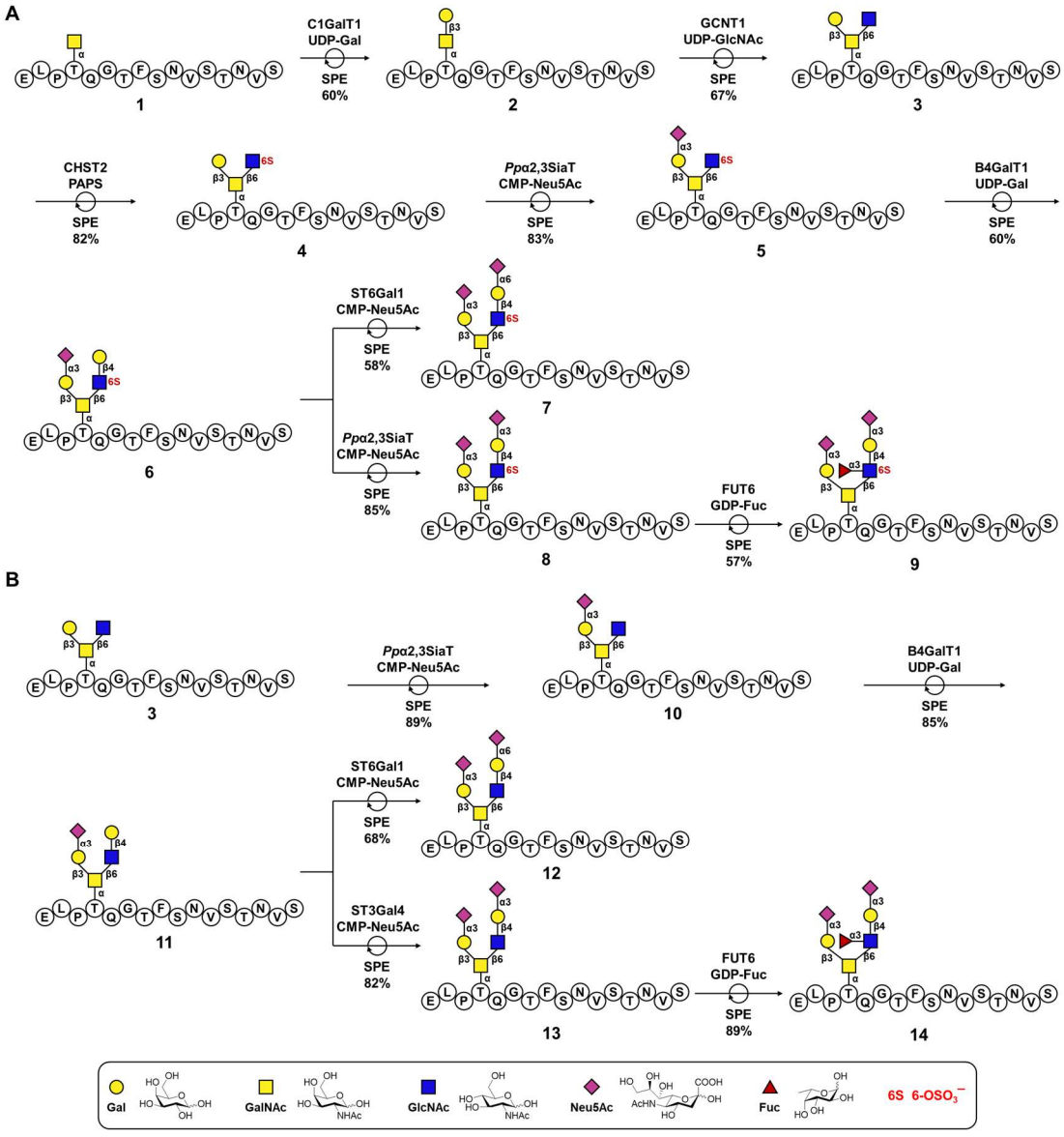


Figure S1. Synthesis of *O*-glycopeptides **2-14**

General procedure for the installation of β1,3-Gal by C1GalT1

To a solution of glycopeptides (2-20 mM), UDP-Gal (3-30 mM, 1.5 equiv), and $MnCl_2$ (10 mM) in Tris HCl buffer (100 mM, pH ≈ 7.0) was added C1GalT1 (350 μg/mL) and calf intestine alkaline phosphatase (CIAP, 10 U/mL), and the mixture was incubated at 37 °C for 16 h. Subsequently, ESI-MS analysis was performed to monitor the reaction every 8 h, and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN).

Product containing fractions were combined and lyophilized to give the β1,3-galactosylated glycopeptides as a white amorphous solid.

General procedure for the installation of β1,6-GlcNAc by GCNT1

To a solution of glycopeptides (2-20 mM), UDP-GlcNAc (3-30 mM, 1.5 equiv), and $MnCl_2$ (10 mM) in Tris-HCl buffer (50 mM, pH ≈ 7.5) was added GCNT1 (110 μg /mL) and calf intestine alkaline phosphatase (CIAP, 10U/mL), and the mixture was incubated at 37 °C for 12 h. Subsequently, ESI-MS analysis was performed to monitor the reaction every 4 h, and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the β1,6-GlcNAc branched glycopeptides as a white amorphous solid.

General procedure for the installation of β1,4-Gal by B4GalT1

To a solution of glycopeptides (2-20 mM), UDP-Gal (3-30 mM, 1.5 equiv), and $MnCl_2$ (10 mM) in Tris-HCl buffer (50 mM, pH ≈ 7.5) was added B4GalT1 (150 μg /mL) and calf intestine alkaline phosphatase (CIAP, 10U/mL), and the mixture was incubated at 37 °C for 12 h. Subsequently, ESI-MS analysis was performed to monitor the reaction every 6 h, and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the β1,4-galactosylated glycopeptides as a white amorphous solid.

General procedure for the installation of α2,6-Neu5Ac by ST6Gal1

To a solution of glycopeptides (2-20 mM) and CMP-Neu5Ac (3-30 mM, 1.5 equiv) in Tris-HCl buffer (100 mM, pH ≈ 7.2) was added ST6Gal1 (300 μg/mL) and calf intestine alkaline phosphatase (CIAP, 10 U/mL), and the mixture was incubated for 16 h at 37 °C. Subsequently, ESI-MS analysis was performed to monitor the reaction every 6 h, and the additional enzymes and sugar nucleotide were added until no

starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the α2,6-sialylated glycopeptides as a white amorphous solid.

General procedure for the installation of α2,3-Neu5Ac by ST3Gal4

To a solution of glycopeptides (2-20 mM) and CMP-Neu5Ac (3-30 mM, 1.5 equiv) in Tris-HCl buffer (100 mM, pH ≈ 7.2) was added ST3Gal4 (300 μg/mL) and calf intestine alkaline phosphatase (CIAP, 10 U/mL), and the mixture was incubated for 18 h at 37 °C. Subsequently, ESI-MS analysis was performed to monitor the reaction every 8 h, and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the α2,3-sialylated glycopeptides as a white amorphous solid.

General procedure for the installation of GDP-Fuc by FUT6

To a solution of glycopeptides (2-20 mM) and GDP-Fuc (3-30 mM, 1.5 equiv) and $MnCl_2$ (10 mM) in Tris-HCl buffer (50 mM, pH ≈ 7.5) was added FUT6 (200 μg/mL) and calf intestine alkaline phosphatase (CIAP, 10 U/mL), and the mixture was incubated for 18 h at 37 °C. Subsequently, ESI-MS analysis was performed to monitor the reaction every 8 h, and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the fucosylated glycopeptides as a white amorphous solid.

General procedure for the installation of PAPS by CHST2

To a solution of glycopeptides (2-20 mM) and PAPS (3-30 mM, 1.5 equiv) in Tris-HCl buffer (50 mM, pH ≈ 7.5) was added CHST2 (1000 μg/mL) and the mixture was incubated for 12 h at 37 °C. Subsequently, ESI-MS analysis was performed to

monitor the reaction every 4 h, and the additional enzymes and PAPS were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the sulfated glycopeptides as a white amorphous solid.

General procedure for the installation of α2,3-Neu5Ac by *Ppα*-2,3SiaT

To a solution of glycopeptides (2-20 mM) and CMP-Neu5Ac (3-30 mM, 1.5 equiv) in Tris-HCl buffer (100 mM, pH ≈ 8.0) was added *Pp*α-2,3SiaT (300 μg/mL) and the mixture was incubated for 6 h at 37 ^{o}C. Subsequently, ESI-MS analysis was performed to monitor the reaction every 3 h and the additional enzymes and sugar nucleotide were added until no starting material was detected. The reaction mixture was centrifuged, and the resulting supernatant was loaded on a C18 cartridge (eluent: H_2O to 30% MeCN). Product containing fractions were combined and lyophilized to give the α2,3-sialylated glycopeptides as a white amorphous solid.

4. LC-MS characterizations of compounds 1-15

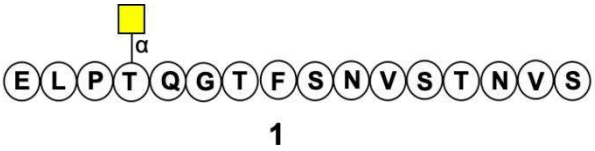
4.1 The procedure for analysis of compounds 1-15

Table S1. The procedure for analysis of compounds **1-15**

Time(min)	A%
0	30
20	50
23	60
26	30
30	30

Analysis was performed on Shimadzu LC-MS2020 with XBridge® Amide 3.5 μm, 2.1 mm x 150 mm column (Waters) at a flow rate of 0.20 mL/min using ESI-MS for compound detection. Mobile phase A consisted of 10 mM ammonium formate adjusted to pH ≈ 3.5 using formic acid; mobile phase B consisted of 100% acetonitrile.

4.2 LC-MS data for compounds 1-15



1

ESI-MS: $[M-2H]^{2-}$calcd for $C_{79}H_{124}N_{20}O_{33}^{2-}$, 940.44; found 940.80, (100.0 mg, 26% overall yield).

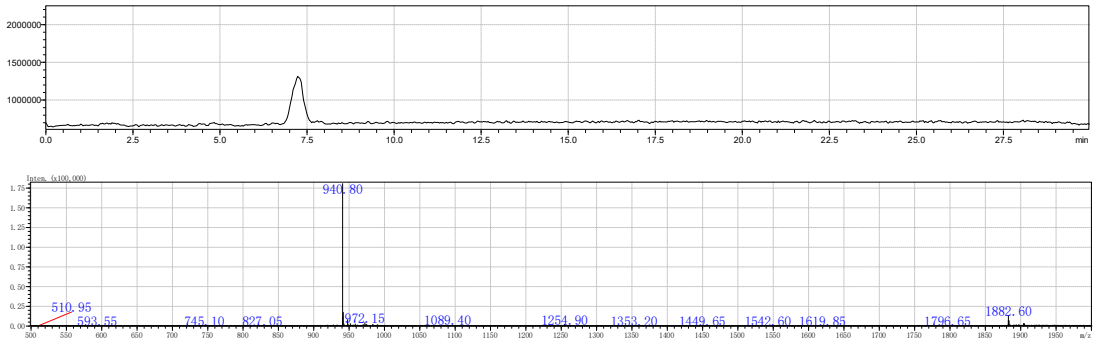


Figure S2. LC-MS profile and focused ESI-MS of compound **1**, eluting at 7.23 min

2

ESI-MS: $[M-2H]^{2-}$calcd for $C_{85}H_{134}N_{20}O_{38}^{2-}$, 1021.47; found 1021.75, (13.0 mg, 60% yield).

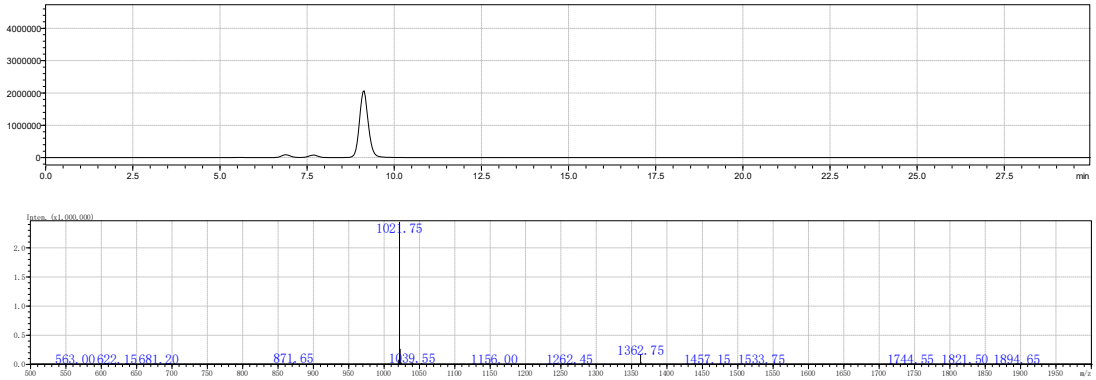
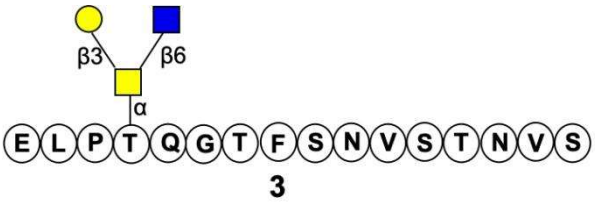


Figure S3. LC-MS profile and focused ESI-MS of compound **2**, eluting at 9.13 min



3

ESI-MS: $[M-2H]^{2-}$calcd for $C_{93}H_{147}N_{21}O_{43}^{2-}$, 1123.01; found 1123.30, (9.0 mg, 67% yield).

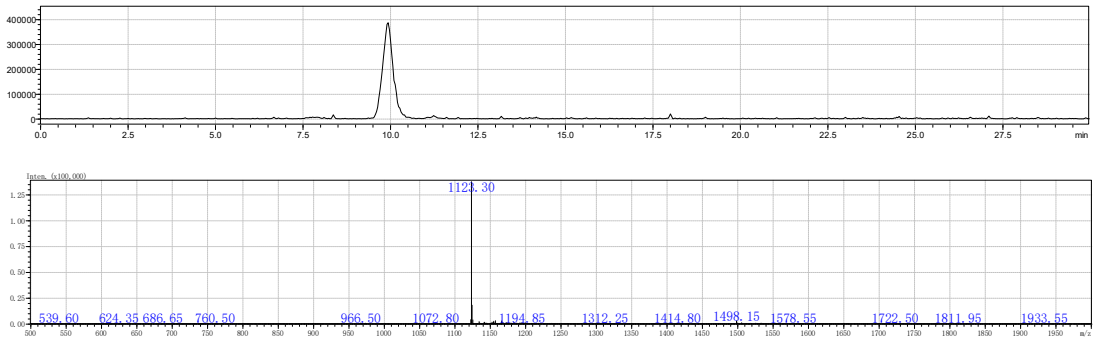
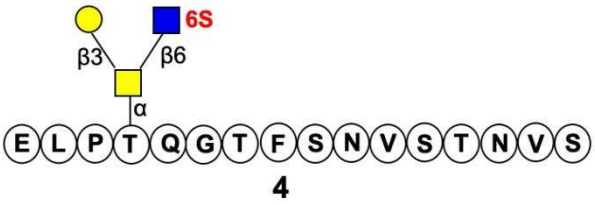


Figure S4. LC-MS profile and focused ESI-MS of compound **3**, eluting at 9.93 min



4

ESI-MS: $[M-2H]^{2-}$calcd for $C_{93}H_{147}N_{21}O_{46}S^{2-}$, 1162.98; found 1163.30, (8.4 mg, 82% yield).

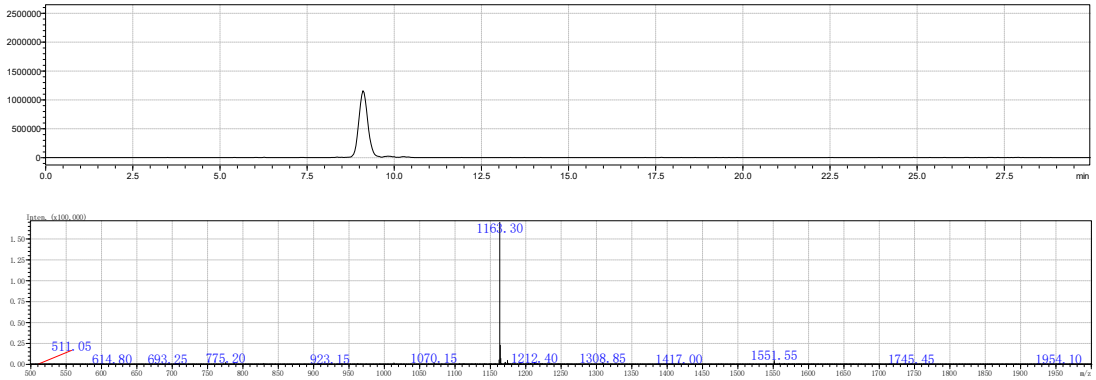
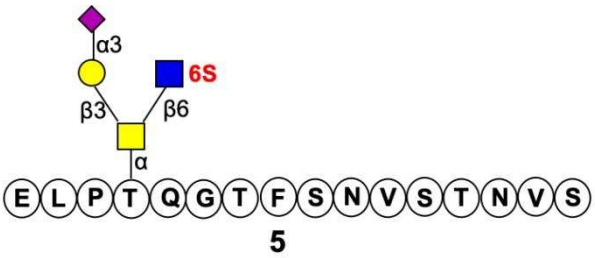


Figure S5. LC-MS profile and focused ESI-MS of compound **4**, eluting at 9.10 min



5

ESI-MS: $[M-2H]^{2-}$calcd for $C_{104}H_{164}N_{22}O_{54}S^{2-}$, 1308.53; found 1308.90, (5.2 mg, 83% yield).

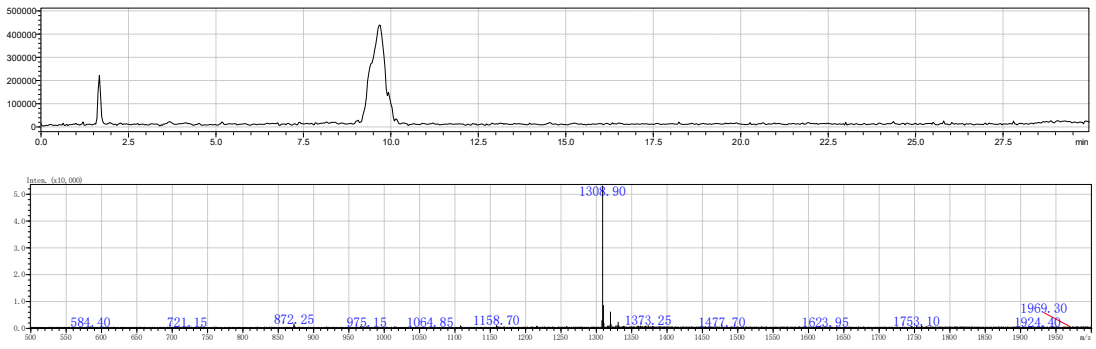
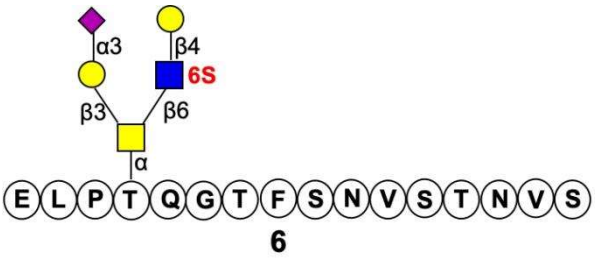


Figure S6. LC-MS profile and focused ESI-MS of compound **5**, eluting at 9.66 min



6

ESI-MS: $[M-2H]^{2-}$calcd for $C_{110}H_{174}N_{22}O_{59}S^{2-}$, 1389.56; found 1390.00, (4.4 mg, 60% yield).

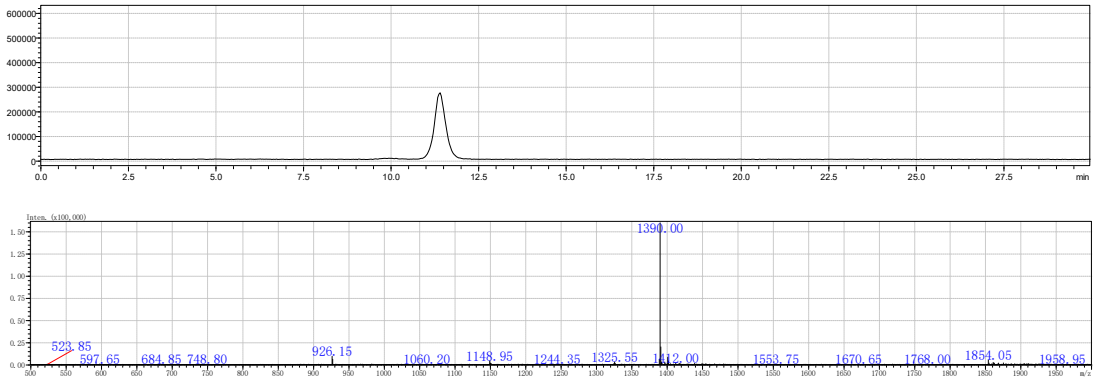
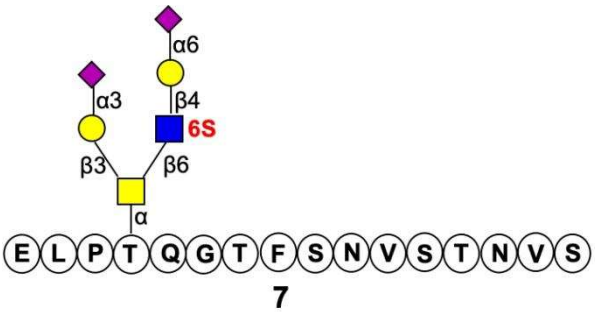


Figure S7. LC-MS profile and focused ESI-MS of compound **6**, eluting at 11.40 min



7

ESI-MS: $[M-2H]^{2-}$ calcd for $C_{121}H_{191}N_{23}O_{67}S^{2-}$, 1535.11; found 1535.65; $[M-3H]^{3-}$ calcd for $C_{121}H_{190}N_{23}O_{67}S^{3-}$, 1023.07; found 1023.45, (1.2 mg, 58% yield).

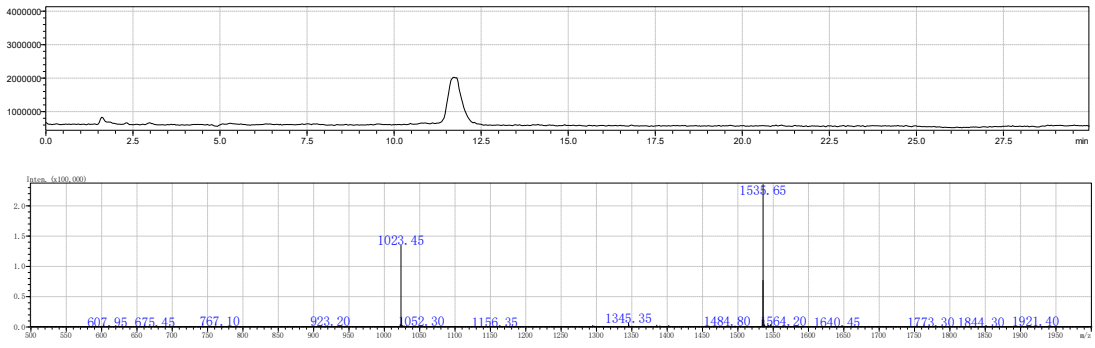
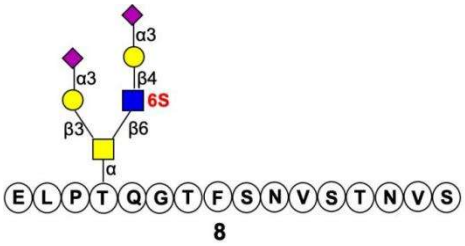


Figure S8. LC-MS profile and focused ESI-MS of compound **7**, eluting at 12.10 min



8

ESI-MS: $[M-2H]^{2-}$ calcd for $C_{121}H_{191}N_{23}O_{67}S^{2-}$, 1535.11; found 1535.60; $[M-3H]^{3-}$ calcd for $C_{121}H_{190}N_{23}O_{67}S^{3-}$, 1023.07; found 1023.40, (1.7 mg, 85% yield).

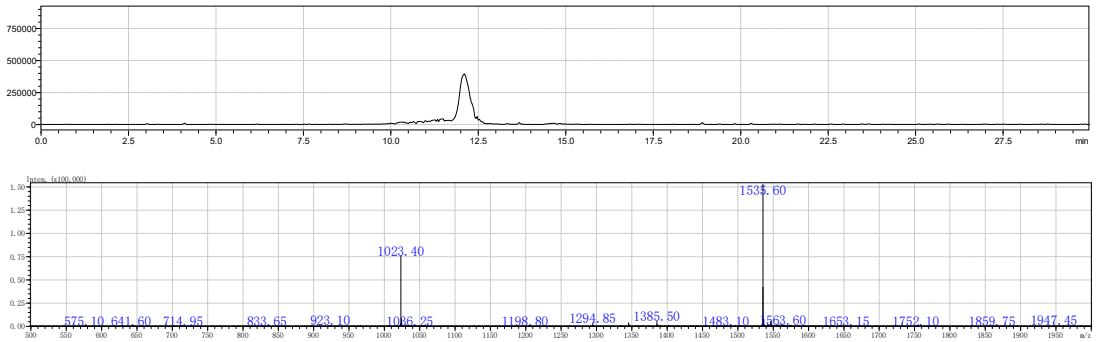
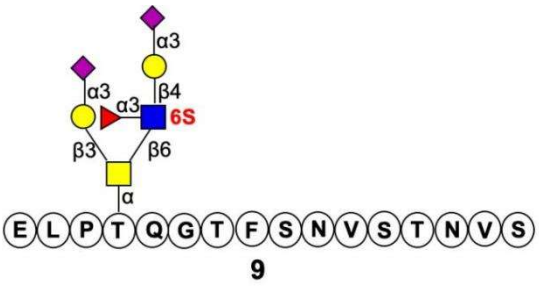


Figure S9. LC-MS profile and focused ESI-MS of compound **8**, eluting at 11.73 min



9

ESI-MS: $[M-2H]^{2-}$ calcd for $C_{127}H_{201}N_{23}O_{71}S^{2-}$, 1608.13; found 1608.65; $[M-3H]^{3-}$ calcd for $C_{127}H_{200}N_{23}O_{71}S^{3-}$,1071.76; found 1072.10, (0.9 mg, 57% yield).

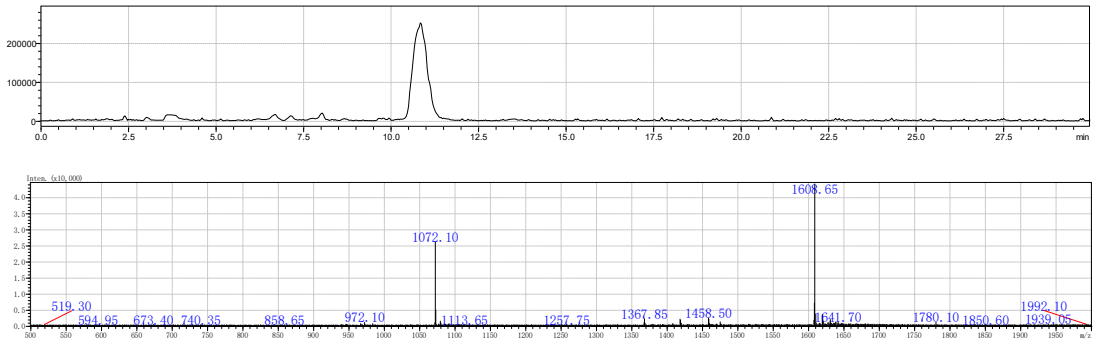
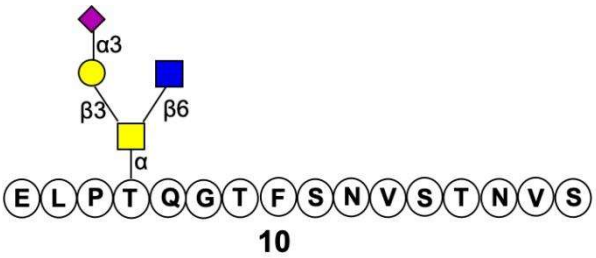


Figure S10. LC-MS profile and focused ESI-MS of compound **9**, eluting at 10.83 min



10

ESI-MS: $[M-2H]^{2-}$ calcd for $C_{104}H_{164}N_{22}O_{51}^{2-}$, 1268.55; found 1268.90, (6.3 mg, 89% yield).

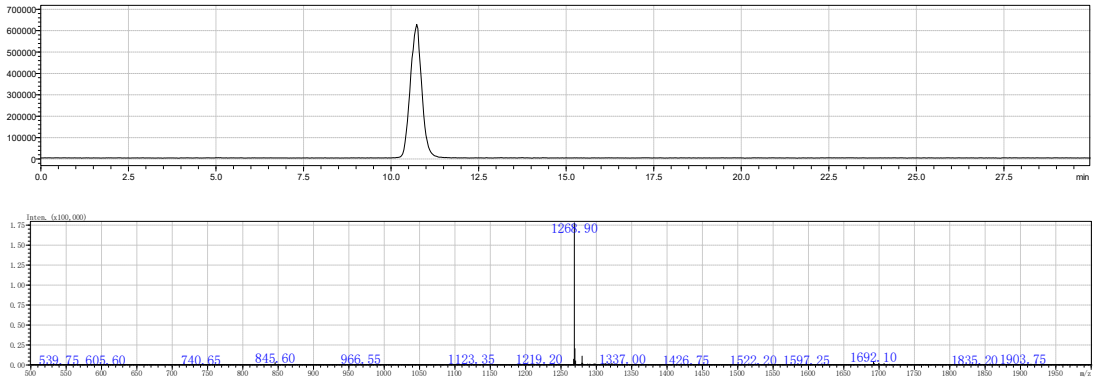
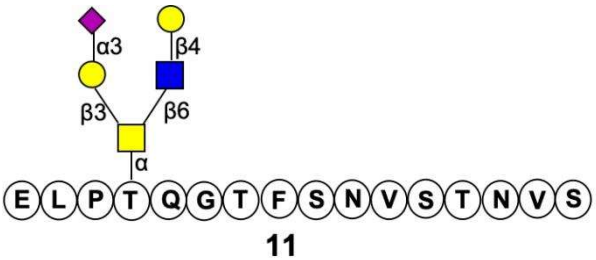


Figure S11. LC-MS profile and focused ESI-MS of compound **10**, eluting at 10.73 min



11

ESI-MS: $[M-2H]^{2-}$calcd for $C_{110}H_{174}N_{22}O_{56}^{2-}$, 1349.58; found 1349.90, (5.8 mg, 85% yield).

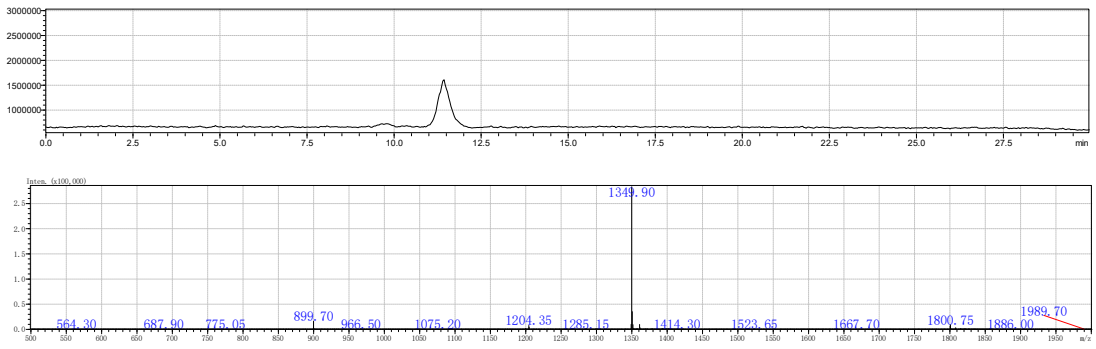
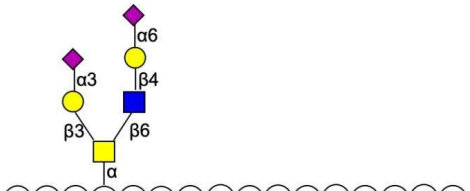


Figure S12. LC-MS profile and focused ESI-MS of compound **11**, eluting at 11.43 min



12

ESI-MS: $[M-2H]^{2-}$calcd for $C_{121}H_{191}N_{23}O_{64}^{2-}$, 1495.13; found 1495.55; $[M-3H]^{3-}$calcd for $C_{121}H_{190}N_{23}O_{64}^{3-}$, 996.42; found 996.75, (1.0 mg, 68% yield).

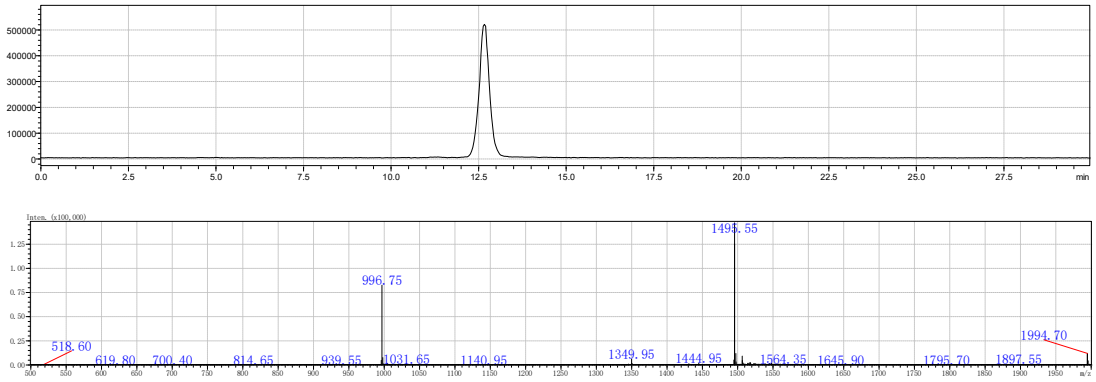
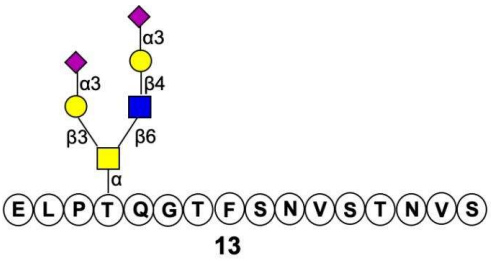


Figure S13. LC-MS profile and focused ESI-MS of compound **12**, eluting at 12.66 min



13

ESI-MS: $[M-2H]^{2-}$calcd for $C_{121}H_{191}N_{23}O_{64}^{2-}$, 1495.13; found 1495.55; $[M-3H]^{3-}$calcd for $C_{121}H_{190}N_{23}O_{64}^{3-}$, 996.42; found 996.75, (2.0 mg, 82% yield)

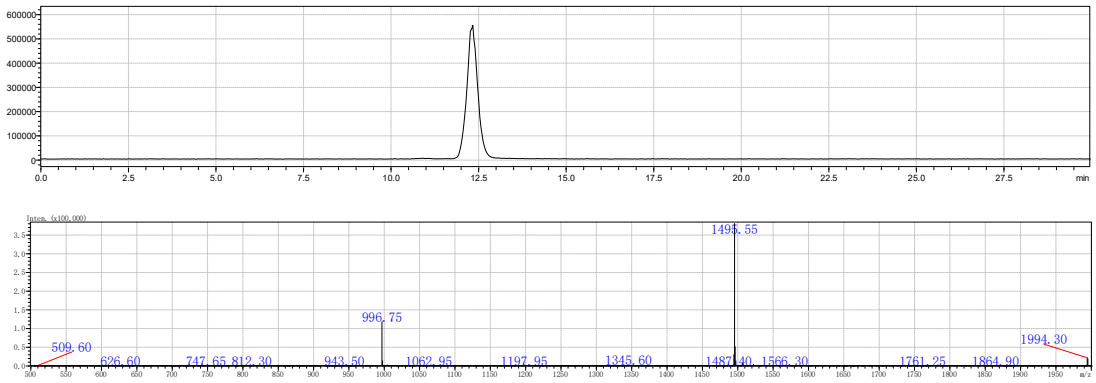
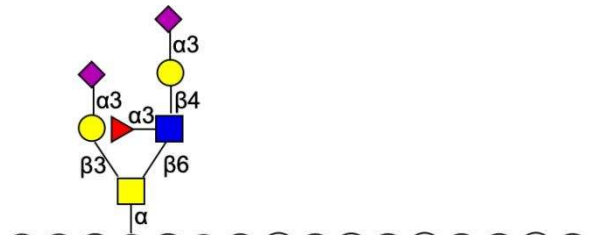


Figure S14. LC-MS profile and focused ESI-MS of compound **13**, eluting at 12.33 min



14

ESI-MS: $[M-2H]^{2-}$calcd for $C_{127}H_{201}N_{23}O_{68}^{2-}$, 1568.16; found 1568.70; $[M-3H]^{3-}$calcd for $C_{127}H_{200}N_{23}O_{68}^{3-}$, 1045.10; found 1045.50, (1.4 mg, 89% yield)

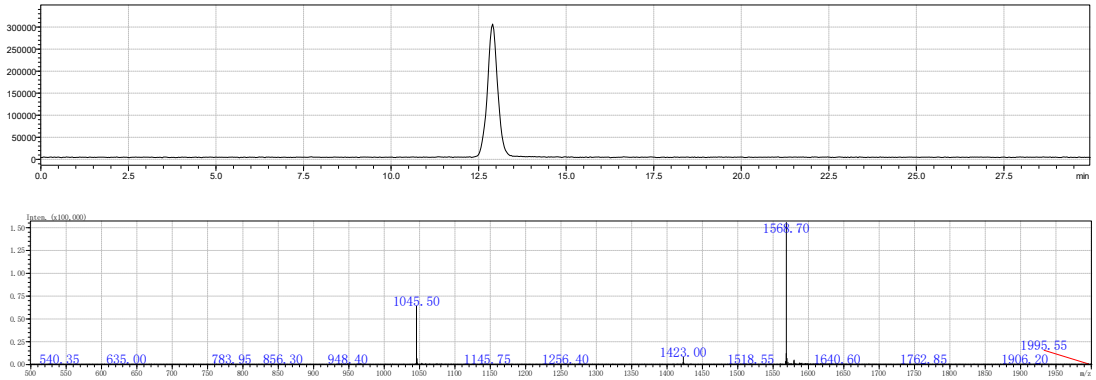


Figure S15. LC-MS profile and focused ESI-MS of compound **14**, eluting at 12.90 min

15

ESI-MS: $[M-2H]^{2-}$calcd for $C_{71}H_{111}N_{19}O_{28}^{-}$, 839.90; found 839.15. (57.3 mg, 34% yield)

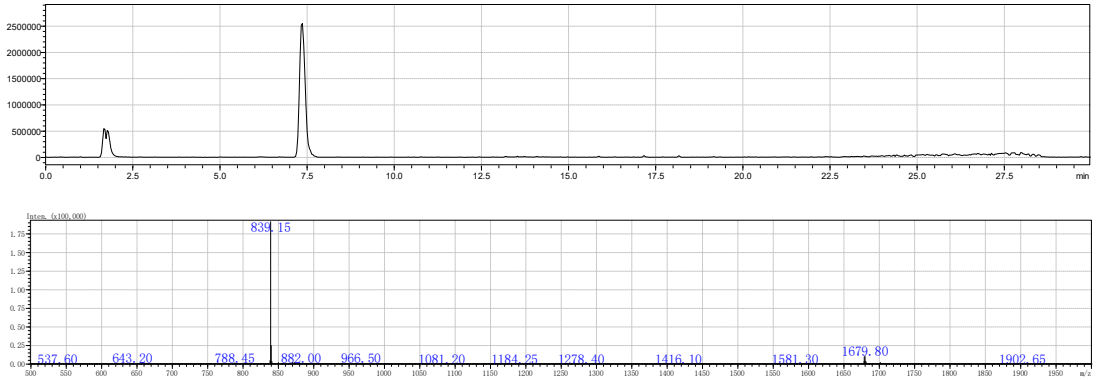


Figure S16. LC-MS profile and focused ESI-MS of compound **15**, eluting at 7.36 min

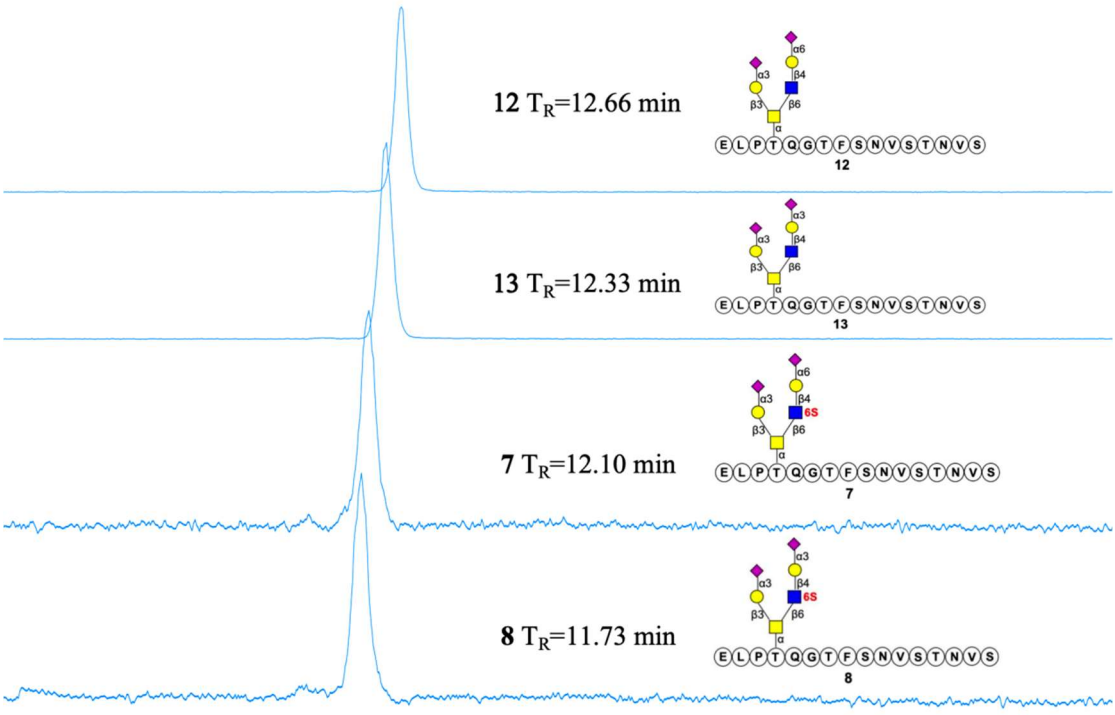


Figure S17. LC-MS profiles of different sialylated glycopeptides **12**, **13**, **7** and **8** exhibit distinct retention times to clear differentiation of each compound

5. Glycan microarray experiment

Glycan microarray printing

The synthesized glycopeptides (100 μM) were dissolved in sodium phosphate buffer solution (pH 8.5, 250 mM) and printed on NHS-activated glass slides (Nexterion Slide H, Schott Inc.) using a Scienion sciCLEAN 8 non-contact microarray printer at a relative humidity of 55% in replicates of six with spot volume ~400 pL. The slides were incubated overnight in a saturated NaCl chamber (providing enough humidity environment), after which were quenched with 50 mM ethanolamine in a Tris buffer (pH 9.0, 100 mM). The blocked slides were then rinsed with DI water, dried by centrifugation, and kept in a desiccator at room temperature for future use.

Screening procedure

Plant lectins： Subarrays were incubated with 100 μL biotinylated proteins at specific concentrations (AAL: 10 μg/mL; SNA: 10 μg/mL; MALII: 10 μg/mL) in TSM binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween, 1% BSA) for 1 h at room temperature. Then the solution was removed

from each chamber, followed by four repeating washes with 200 μL TSM washing buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween), four repeating washes with 200 μL TSM buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$), and two repeating washes with 200 μL DI water. Subarrays were then incubated with 100 μL Streptavidin-AlexaFluor® 635 (5 μg/mL) in TSM binding buffer for 1 h at room temperature, followed by the washing procedure as described above.

Selectins: Subarrays were incubated with 100 μL recombinant human E-, P-, L-Selectin (Fc tag) protein (50 μg/mL) in TSM binding buffer (100 μL) for 1 h at room temperature, followed by a washing procedure as described above. Next, the subarray was incubated with biotinylated goat anti-Human IgG Fc Antibody (5 μg/mL, 100 μL) for 1 h at room temperature and washed as previous procedure. Finally, Streptavidin-AlexaFluor® 635 (5 μg/mL, 100 μL) was added for 1 h incubation, followed by washing procedure as described above.

Data analysis

The pre-prepared slides were scanned for fluorescence on an InnoScan 710 Microarray Scanner. The data were processed with Mapix software and further analyzed using IBM SPSS. Relative fluorescence values (RFU) were calculated by averaging four independent replicates (corrected for mean background) on the glycan array after removal of the highest and lowest signals. The error bars indicate the standard deviation (SD) of RFU. Data were graphed using OriginPro2024. Bar graphs represent the mean ± SD for each compound.

6. HR-MS data and spectra

Table S2. Analysis of glycopeptides by HR-MS

Entry	Glycopeptide	Formula	Theoretical mass	Charge	Observed m/z	Diff(ppm)
1	**1**	$C_{79}H_{126}N_{20}O_{33}$	1882.8796	2	942.4472	-0.11
2	**2**	$C_{85}H_{136}N_{20}O_{38}$	2044.9324	2	1023.4742	-0.66
3	**3**	$C_{93}H_{149}N_{21}O_{43}$	2248.0118	2	1125.013	0.19
4	**4**	$C_{93}H_{149}N_{21}O_{46}S$	2327.9686	2	1164.9919	-0.28
5	**5**	$C_{104}H_{166}N_{22}O_{54}S$	2619.0640	2	1310.5394	-0.11
6	**6**	$C_{110}H_{176}N_{22}O_{59}S$	2781.1169	2	1391.5648	0.63
7	**7**	$C_{121}H_{193}N_{23}O_{67}S$	3072.2123	2	1537.1142	-0.53
8	**8**	$C_{121}H_{193}N_{23}O_{67}S$	3072.2123	2	1537.1129	0.31
9	**9**	$C_{127}H_{203}N_{23}O_{71}S$	3218.2702	2	1610.1425	-0.06
10	**10**	$C_{104}H_{166}N_{22}O_{51}$	2539.1072	2	1270.5608	0.04
11	**11**	$C_{110}H_{176}N_{22}O_{56}$	2701.1601	2	1351.5878	-0.39
12	**12**	$C_{121}H_{193}N_{23}O_{64}$	2992.2555	2	1497.1345	0.35
13	**13**	$C_{121}H_{193}N_{23}O_{64}$	2992.2555	2	1497.1343	0.5
14	**14**	$C_{127}H_{203}N_{23}O_{68}$	3138.3134	2	1570.1646	-0.42
15	**15**	$C_{71}H_{113}N_{19}O_{28}$	1679.8002	2	840.9079	-0.58

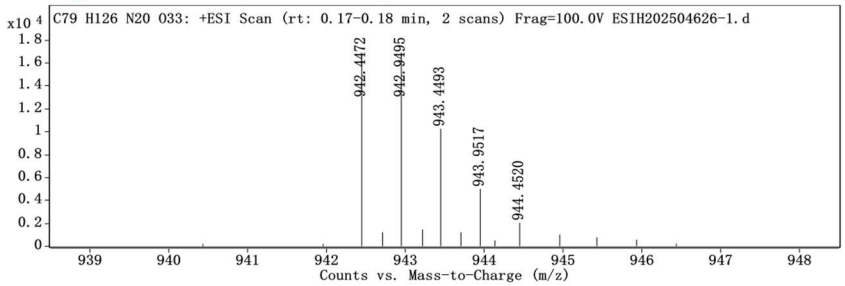
Qualitative Analysis Report

Data Filename	ESIH202504626-1.d	**Sample Name**	G0-O-1
Sample ID		**Position**	P1-A2
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 3:43:35 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		

1

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
942.4472	942.4471	-0.11	-0.11	C79 H128 N20 O33	(M+2H)+2

--- End Of Report ---

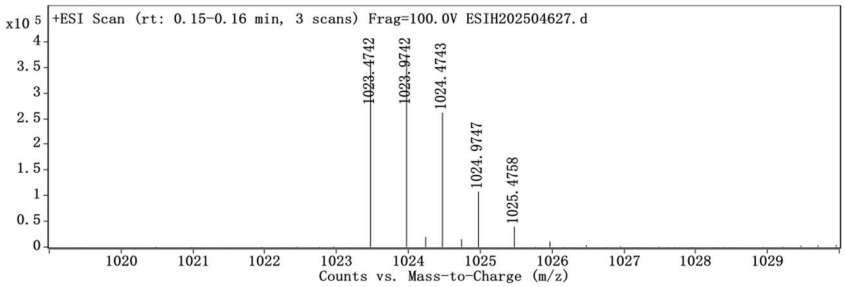
Qualitative Analysis Report

Data Filename	ESIH202504627.d	**Sample Name**	G0-O-2
Sample ID		**Position**	P1-A3
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:32:37 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		

2

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1023.4742	1023.4735	-0.68	-0.66	C85 H138 N20 O38	(M+2H)+2

--- End Of Report ---

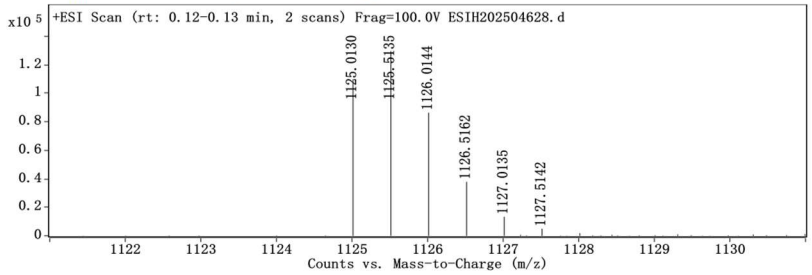
Qualitative Analysis Report

Data Filename	ESIH202504628.d	**Sample Name**	G0-O-3
Sample ID		**Position**	P1-A4
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:33:58 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		

3

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1125.013	1125.0132	0.21	0.19	C93 H151 N21 O43	(M+2H)+2

--- End Of Report ---

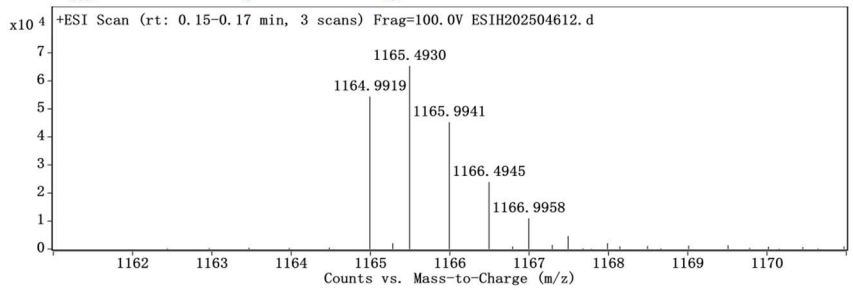
Qualitative Analysis Report

Data Filename	ESIH202504612.d	**Sample Name**	G0-S1
Sample ID		**Position**	P1-A6
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/24/2025 1:39:53 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		

4

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1164.9919	1164.9916	-0.33	-0.28	C93 H151 N21 O46 S	(M+2H)+2

--- End Of Report ---

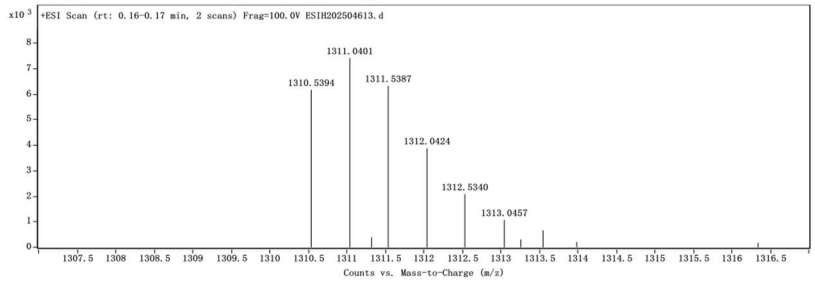
Qualitative Analysis Report

Data Filename	ESIH202504613.d	**Sample Name**	G0-S2
Sample ID		**Position**	P1-A7
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/24/2025 1:41:10 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		

5

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



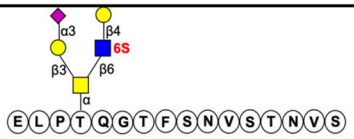
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1310.5394	1310.5393	-0.14	-0.11	C104 H168 N22 O54 S	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

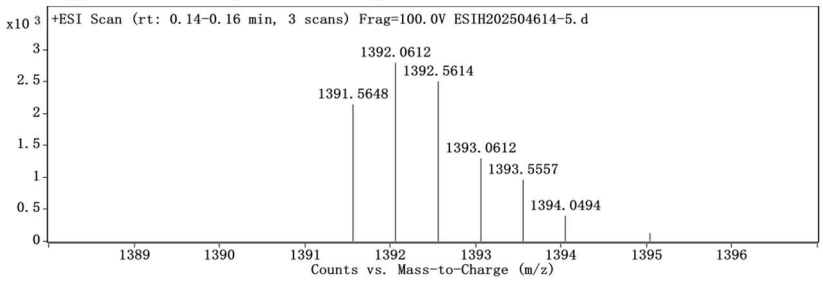
Data Filename	ESIH202504614-5.d	**Sample Name**	G0-S3
Sample ID		**Position**	P1-A8
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/24/2025 4:24:20 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



6

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



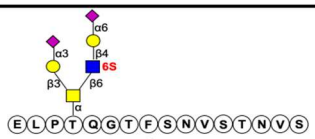
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1391.5648	1391.5657	0.87	0.63	C110 H178 N22 O59 S	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

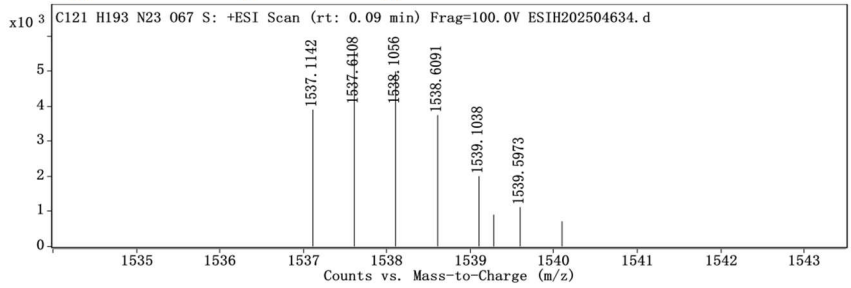
Data Filename	ESIH202504634.d	**Sample Name**	G0-S6
Sample ID		**Position**	P1-B1
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:41:42 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



7

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



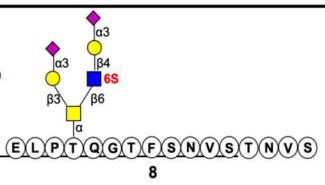
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1537.1142	1537.1134	-0.82	-0.53	C121 H195 N23 O67 S	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

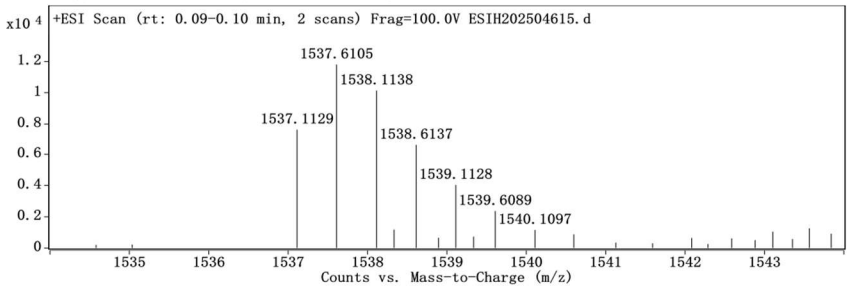
Data Filename	ESIH202504615.d	**Sample Name**	G0-S4
Sample ID		**Position**	P1-A9
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/24/2025 1:43:46 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



8

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



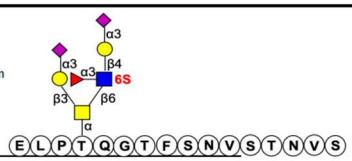
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1537.1129	1537.1134	0.47	0.31	C121 H195 N23 O67 S	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

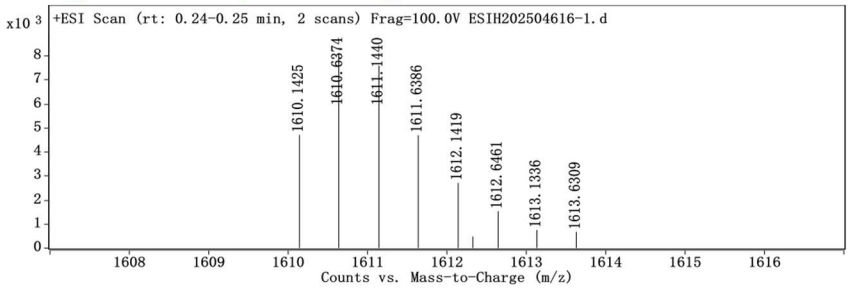
Data Filename	ESIH202504616-1.d	**Sample Name**	G0-S5
Sample ID		**Position**	P1-B1
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/24/2025 2:29:53 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



9

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



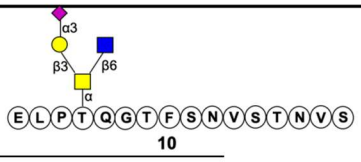
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1610.1425	1610.1424	-0.09	-0.06	C127 H205 N23 O71 S	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

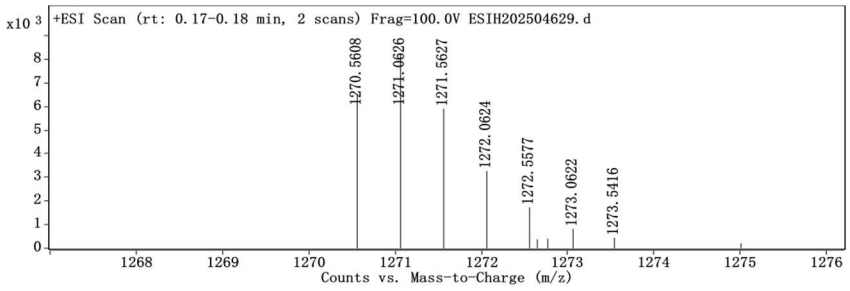
Data Filename	ESIH202504629.d	**Sample Name**	G0-O-4
Sample ID		**Position**	P1-A5
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:35:14 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



10

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



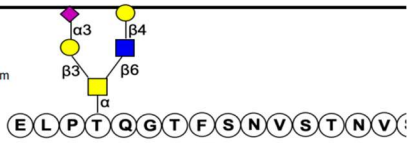
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1270.5608	1270.5609	0.05	0.04	C104 H168 N22 O51	(M+2H)+2

--- End Of Report ---

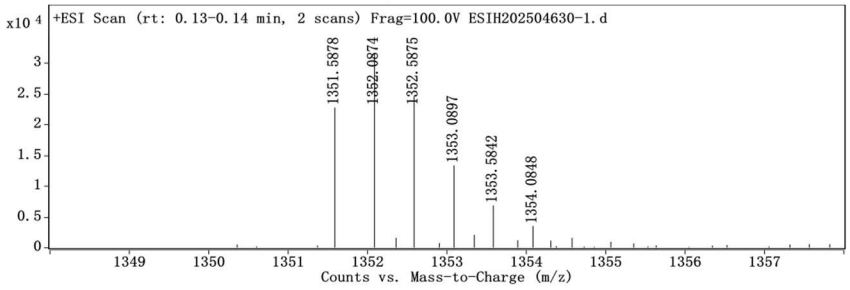
Qualitative Analysis Report

Data Filename	ESIH202504630-1.d	**Sample Name**	G0-O-5
Sample ID		**Position**	P1-A6
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 4:04:44 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



User Spectra **11**

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



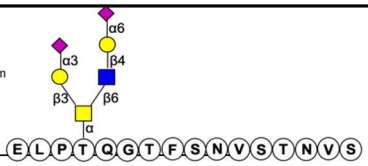
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1351.5878	1351.5873	-0.53	-0.39	C110 H178 N22 O56	(M+2H)+2

--- End Of Report ---

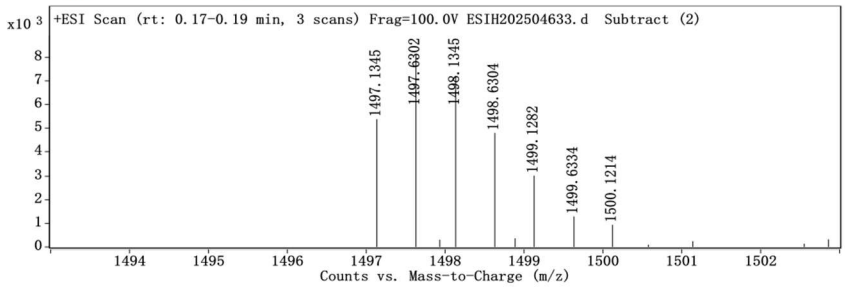
Qualitative Analysis Report

Data Filename	ESIH202504633.d	**Sample Name**	G0-O-8
Sample ID		**Position**	P1-A9
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:40:25 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



User Spectra **12**

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI



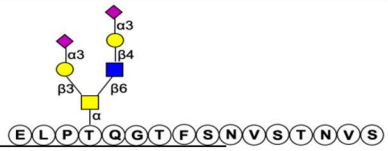
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1497.1345	1497.135	0.52	0.35	C121 H195 N23 O64	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

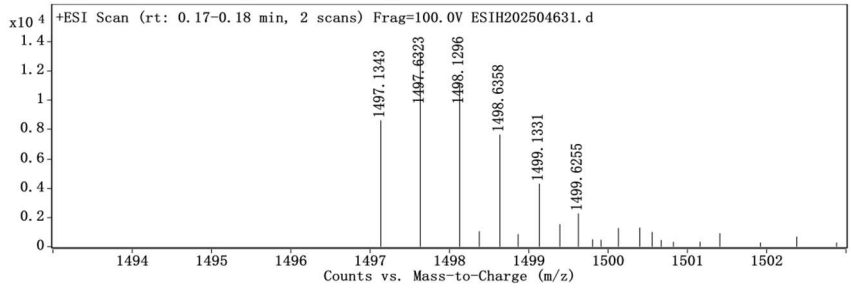
Data Filename	ESIH202504631.d	**Sample Name**	G0-O-6
Sample ID		**Position**	P1-A7
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:37:50 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI

13



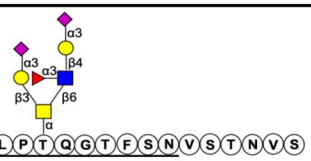
Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1497.1343	1497.135	0.75	0.5	C121 H195 N23 O64	(M+2H)+2

--- End Of Report ---

Qualitative Analysis Report

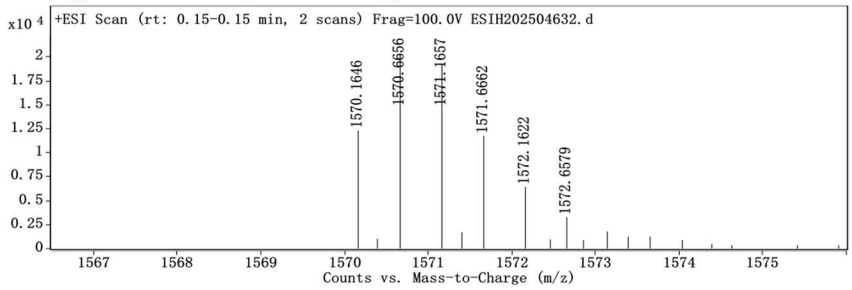
Data Filename	ESIH202504632.d	**Sample Name**	G0-O-7
Sample ID		**Position**	P1-A8
Instrument Name	Agilent 6520 Q-TOF	**Acq Method**	20160322_MS_ESIH_POS_1min.m
Acquired Time	9/26/2025 2:39:06 PM	**DA Method**	ESI-HR-20231114.m
Comment	ESIH by huangqiongping		



User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
100	0	ESI

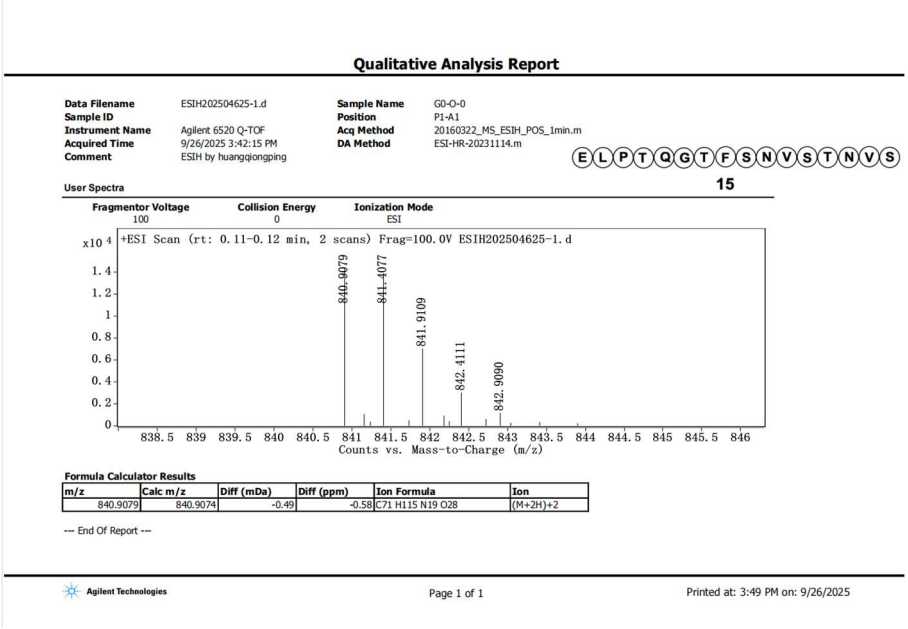
14



Formula Calculator Results

m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
1570.1646	1570.164	-0.67	-0.42	C127 H205 N23 O68	(M+2H)+2

--- End Of Report ---



7. HPLC spectra of compounds 1-15

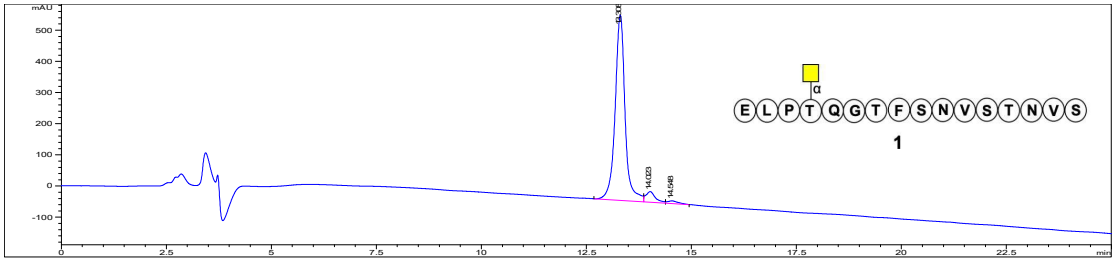


Figure S18. HPLC spectra of glycopeptide **1**. Purity: 93.2%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 μm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

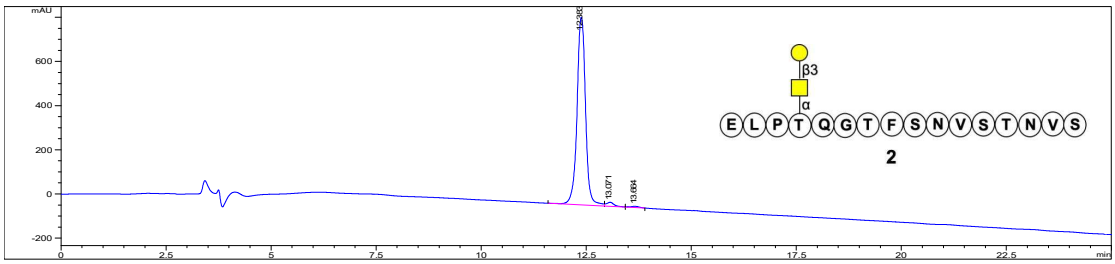


Figure S19. HPLC spectra of glycopeptide **2**. Purity: 97.4%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 μm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

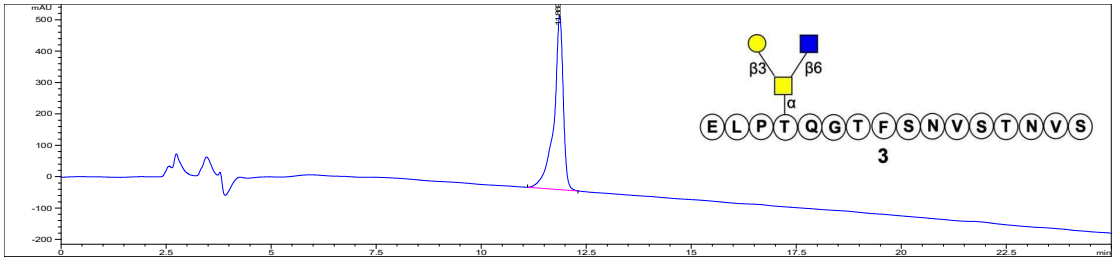


Figure S20. HPLC spectra of glycopeptide **3**. Purity: 99.2%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

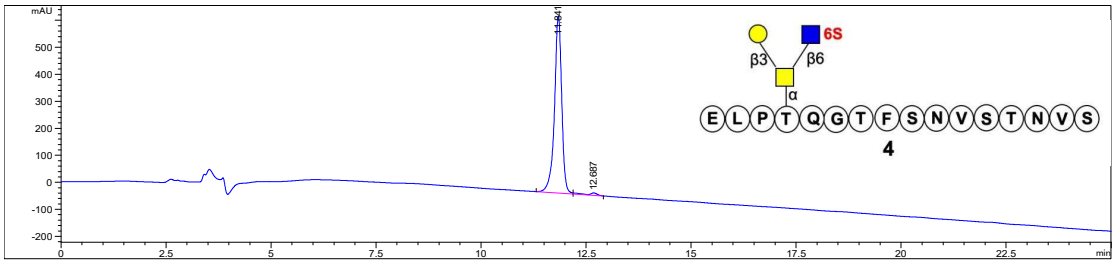


Figure S21. HPLC spectra of glycopeptide **4**. Purity: 97.9%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

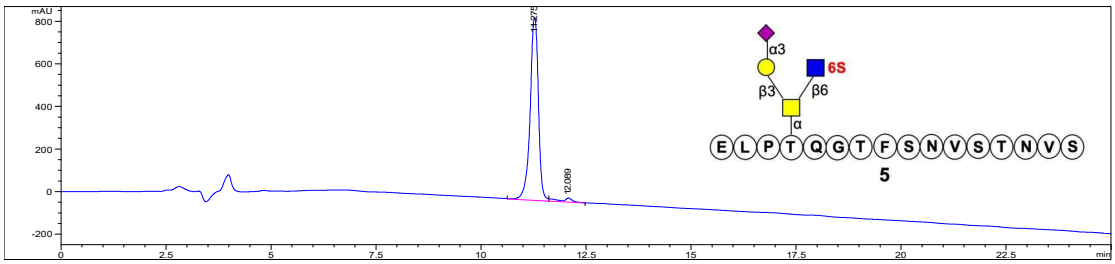


Figure S22. HPLC spectra of glycopeptide **5**. Purity: 97.0%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

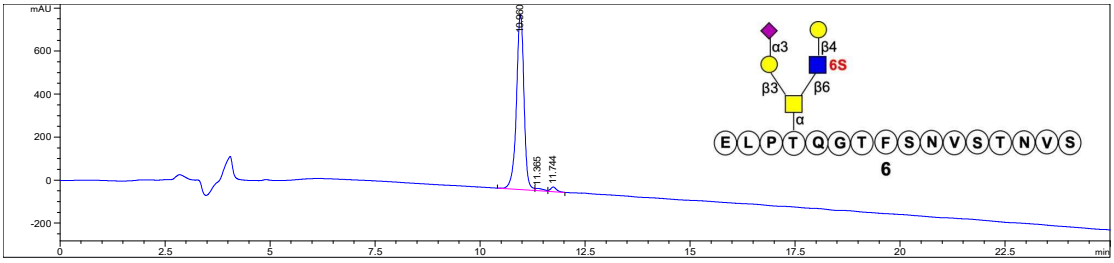


Figure S23. HPLC spectra of glycopeptide **6**. Purity: 96.5%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

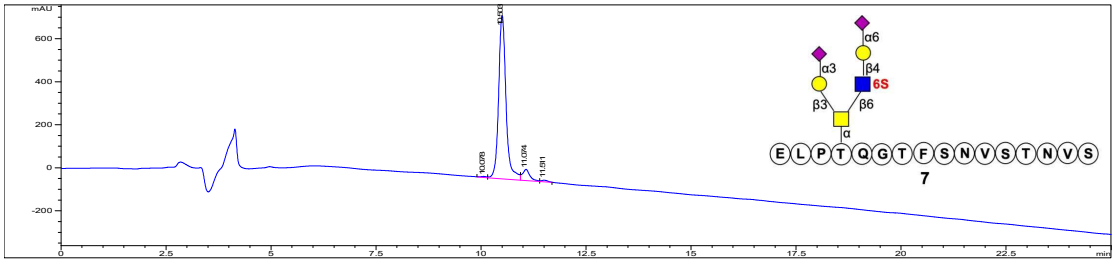


Figure S24. HPLC spectra of glycopeptide **7**. Purity: 92.4%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

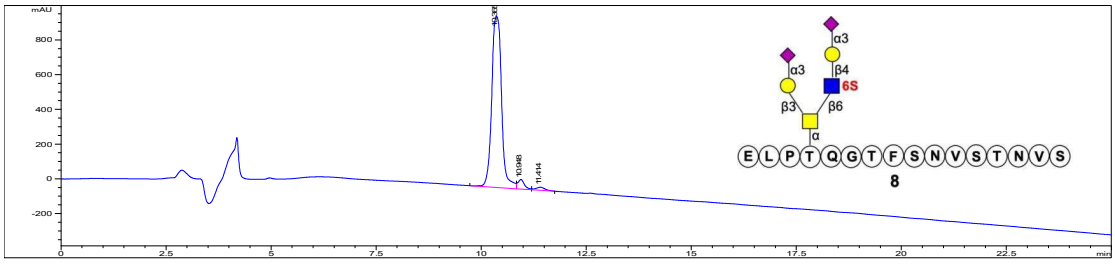


Figure S25. HPLC spectra of glycopeptide **8**. Purity: 94.8%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

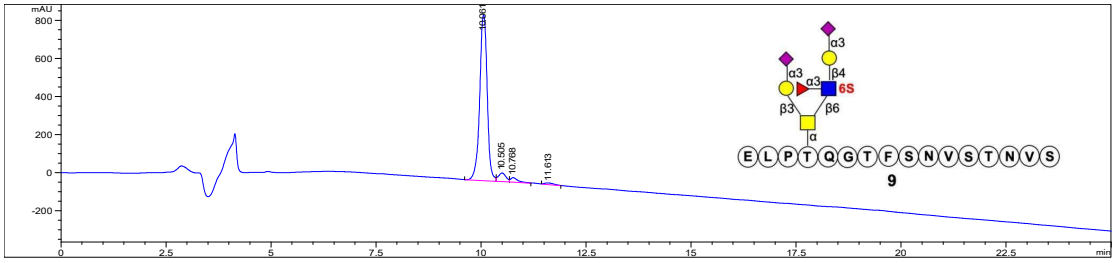


Figure S26. HPLC spectra of glycopeptide **9**. Purity: 91.5%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

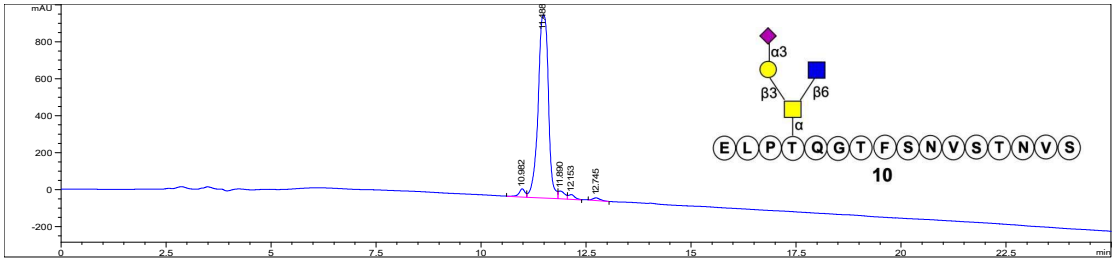


Figure S27. HPLC spectra of glycopeptide **10**. Purity: 92.5%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

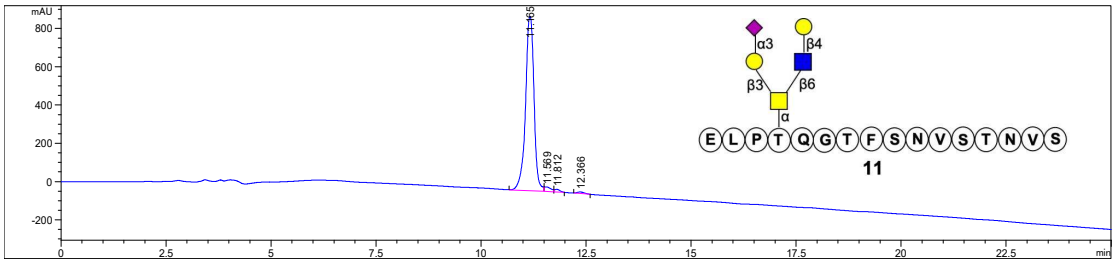


Figure S28. HPLC spectra of glycopeptide **11**. Purity: 96.3%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

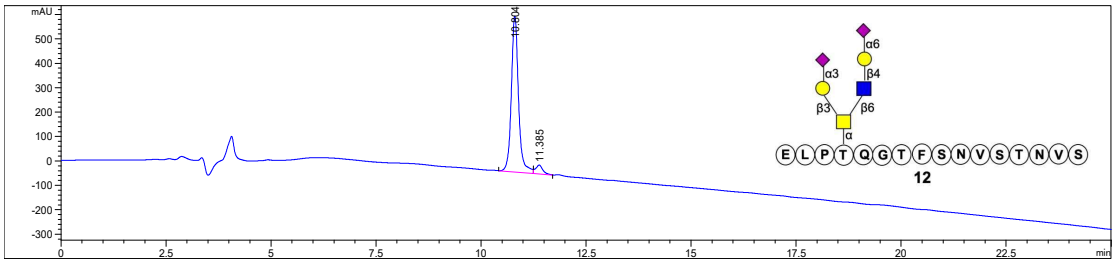


Figure S29. HPLC spectra of glycopeptide **12**. Purity: 94.5%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

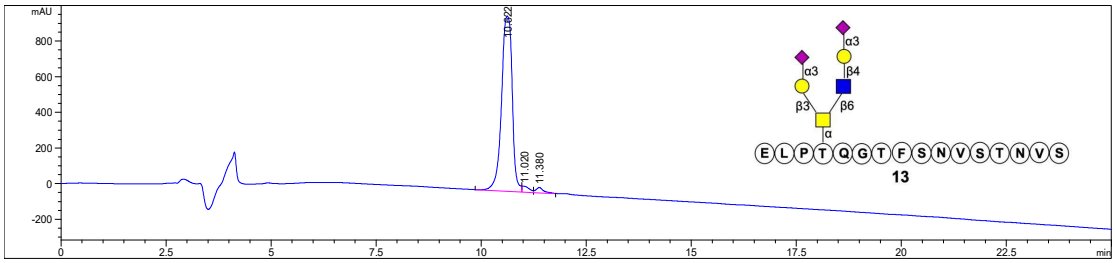


Figure S30. HPLC spectra of glycopeptide **13**. Purity: 95.6%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

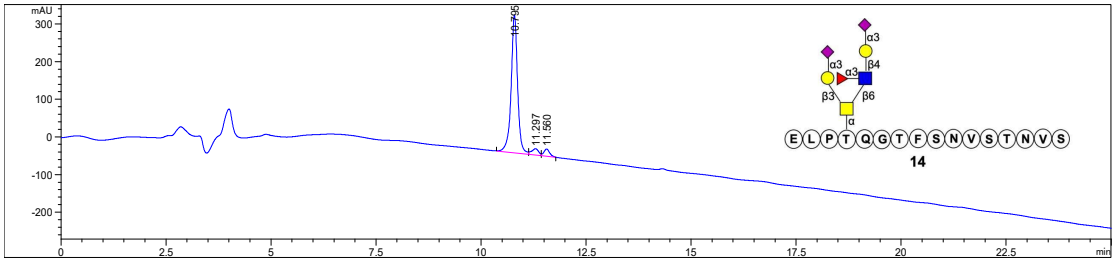


Figure S31. HPLC spectra of glycopeptide **14**. Purity: 91.2%; HPLC instrument: Aglient Technologies 1260 Infinity equipped with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 µm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

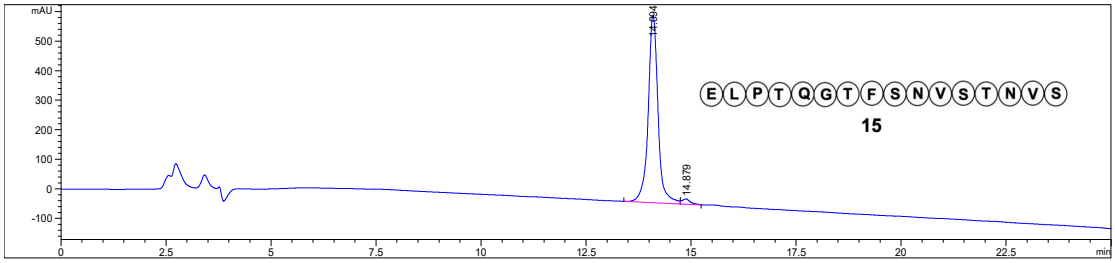


Figure S32. HPLC spectra of glycopeptide **15**. Purity: 97.7% HPLC instrument: Aglient Technologies 1260 Infinity coupled with UV detector; Detection: UV absorption at 210 nm; Column: SB-C18, 5 μm, 4.6 x 150 mm. Mobile Phase: A, 10 mM ammonium formate (pH 3.5); B, Acetonitrile. Gradient elution: B from 10% to 45% in 25 min. Flow rate: 0.5 mL/min.

8. Reference

[1] Mertsch A, He N, Yi D, Kickstein M, Fessner WD. An α2,3-Sialyltransferase from *Photobacterium phosphoreum* with Broad Substrate Scope: Controlling Hydrolytic Activity by Directed Evolution. *Chem. Eur. J.* **2020**, *26*, 11614-11624.

[2] Mehta AY, Veeraiah RKH, Dutta S, et al. Parallel Glyco-SPOT Synthesis of Glycopeptide Libraries. *Cell Chem. Biol*. **2020**, *27*, 1207-1219.

[3] Prudden AR, Liu L, Capicciotti CJ, et al. Synthesis of asymmetrical multiantennary human milk oligosaccharides. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 6954-6959.

[4] Moremen KW, Ramiah A, Stuart M, et al. Expression system for structural and functional studies of human glycosylation enzymes. *Nat. Chem. Biol.* **2018**, *14*, 156-162.

[5] Blixt O, Allin K, Pereira L, Datta A, Paulson JC. Efficient chemoenzymatic synthesis of O-linked sialyl oligosaccharides. *J. Am. Chem. Soc.* **2002**, *124*, 5739-5746.

[6] Ma W, Deng Y, Xu Z, et al. Integrated Chemoenzymatic Approach to Streamline the Assembly of Complex Glycopeptides in the Liquid Phase. *J. Am. Chem. Soc.* **2022**, *144*, 9057-9065.

[7] Wu Y, Bosman GP, Chapla D, et al. A Biomimetic Synthetic Strategy Can Provide Keratan Sulfate I and II Oligosaccharides with Diverse Fucosylation and Sulfation Patterns. *J. Am. Chem. Soc.* **2024**, *146*, 9230-9240.

[8] Ma W, Luo J, Liu H, et al. Chemoenzymatic Synthesis of Highly *O*-Glycosylated MUC7 Glycopeptides for Probing Inhibitory Activity against *Pseudomonas aeruginosa* Biofilm Formation. *Angew. Chem. Int. Ed.* **2025**, *64*, e202424312.