

Electronic Supplementary Information for:

Use of solid-supported 4-fluorophenyl 3-nitro-2-pyridinesulfenate in construction of disulfide-linked hybrid molecules

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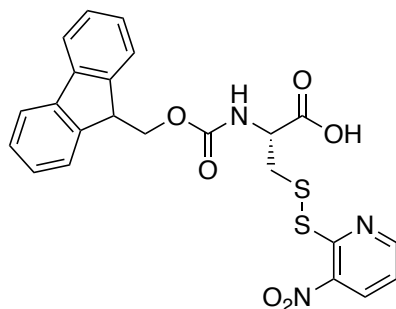
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1. General information

Solvents and reagents were purchased from Kanto Chemical Co., Inc., Kokusan Chemical Co., Ltd., NACALAI TESQUE, INC., Tokyo Chemical Industry Co., Ltd., Wako Pure Chemical Industries, Ltd., and Watanabe Chemical Industries, Ltd. All reaction mixtures were stirred magnetically. ^1H NMR spectra were measured in MeOD or D_2O solutions using a Bruker AVANCE-III (400 MHz) spectrophotometer and referenced to TMS (0.00 ppm) and 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid, sodium salt (TSP- d_4 , 0.00 ppm). ^{13}C NMR spectra were measured in CDCl_3 solution using Bruker AVANCE-III (400 MHz) spectrophotometer and CDCl_3 (77.16 ppm) as a reference. Peak multiplicities are reported with the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra (TOF MS ES^+) were obtained on Waters MICRO MASS LCT-premier. Column chromatography was performed on silica gel 60N (spherical, neutral) (40-50 μm), thin layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merk Kieselgel 60F₂₅₄), and compounds were visualized with UV light, and phosphomolybdic acid and ninhydrin stains. Preparative thin layer chromatography was performed on precoated plates (2 mm, PLC Silica gel 60 F₂₅₄). Preparative HPLC was performed using a C18 reversed-phase column (19 x 150 mm; SunFireTM Prep C18 OBDTM 5 μm) with a binary solvent system. Analytical HPLC was performed using a C18 reversed-phase column (4.6ID x 150 mm; COSMOSIL Packed Column 5C₁₈-AR-II) with a binary solvent system.

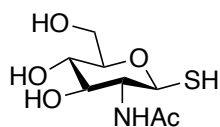
2. Synthesis of Fmoc-Cys(Npys)-OH and compound 9

N-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-((3-nitropyridin-2-yl)thio)-*L*-cysteine,
Fmoc-Cys(Npys)-OH



4-Fluorophenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*F), 40.0 mg, 0.15 mmol, 1.2 equiv.) was added to a solution of Fmoc-Cys(*t*-Bu)-OH (50.0 mg, 0.13 mmol, 1.0 equiv.) in 90% aqueous HCOOH (4 mL) and the mixture was stirred at 0 °C for 30 min. The solvent was removed by lyophilization and the residue was purified by silica gel preparative layer chromatography with CHCl₃:MeOH = 3:1 to give the desired product (59.7 mg, 0.12 mmol, 96% yield) as a yellow solid. ¹H NMR (MeOD, 400 MHz) δ 8.71 (d, *J* = 4.14 Hz, 1H), 8.53 (d, *J* = 8.16 Hz, 1H); 7.78 (d, *J* = 7.47 Hz, 2H), 7.67 (d, *J* = 5.77 Hz, 2H), 7.37 (t, *J* = 14.24 Hz, 3H), 7.30-7.29 (m, 2H), 4.43-4.39 (m, 1H), 4.33 (t, *J* = 16.56 Hz, 2H), 4.22 (d, *J* = 13.12 Hz, 1H), 3.37-3.34 (m, 1H), 3.29-3.17 (m, 1H); ¹³C NMR (CDCl₃, 100 MHz) δ 173.46, 157.34, 156.46, 153.57, 143.72 (2 carbons), 142.99, 141.47, 141.43, 134.43, 127.93 (2 carbons), 127.32, 127.28, 125.08, 125.03, 121.62, 120.16 (2 carbons), 66.97, 53.45, 47.34, 41.38; HRMS (ESI) *m/z* calcd for C₂₃H₁₉N₃O₆NaS₂ [M+Na]⁺ 520.0613, found 520.0615.

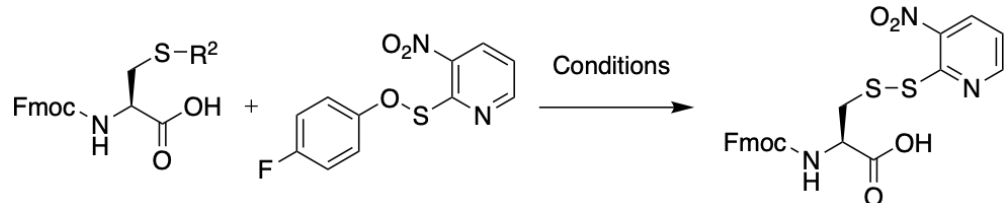
1-Thio-2-acetamido-2-deoxy- β -D-glucopyranose (**9**)



Compound **9** was synthesized by the previously reported method^[1,2] with minor modifications. NaOMe (26.66 mg, 0.494 mmol) was added to a solution of 1-thioacetyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-acetamido- β -D-glucopyranoside (50 mg, 0.123 mmol) in MeOH. After stirring overnight at rt, the reaction was quenched with Amberlyst[®] 15 ion-exchange resin, acidifying it to pH 2-3. After the filtration to remove the resin, the solvent was removed *in vacuo*. Compound **9** was obtained as a white solid (25.69 mg, 0.434 mmol, 88% yield) and was not further purified. ¹H NMR (400 MHz, D₂O) δ 4.70-4.67 (m, 1H), 3.91-3.88 (m, 1H), 3.78-3.73 (m, 2H), 3.50-3.48 (m, 3H), 2.06 (s, 3H).

3. Reactivity of Npys-OPh(*p*F) with thiols and other thiol-protecting groups

Table S1. Conversion of conventional thiol-protecting groups into Npys



Entry	R ²	Conditions ^[a]		Isolated yield (%)
		Solvent		
1 ^[b]	<i>t</i> -Bu	90% HCOOH aq.		96
2	Acm	90% HCOOH aq.		95
3	4-MeOBn	90% HCOOH aq.		98
4	Trt	90% HCOOH aq.: CH ₂ Cl ₂ = 1 : 1		50
5	Bn	90% HCOOH aq.: CH ₂ Cl ₂ = 5 : 1		30
6	<i>S</i> <i>t</i> -Bu	90% HCOOH aq.		N.O. ^[c]
7	H	90% HCOOH aq.		26 ^[d]

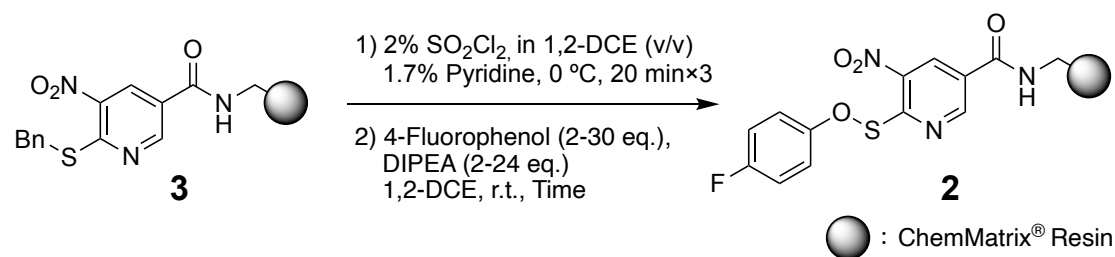
[a] Reaction conditions: at 0 °C for 30 min.

[b] Same as Table 1, Entry 7 in main text.

[c] N.O.: not obtained.

[d] (Fmoc-Cys-OH)₂ was also isolated with a yield of 18%.

4. Synthesis of Npys-OPh(*p*F) Resin (2)



ChemMatrix[®] resin with 3-nitro-2-benzylthiopyridine-5-carboamine resin **3** was synthesized by the previously reported method.^[3] Resin **3** (1 eq.) was added to a solution of 2% (v/v) SO₂Cl₂ in 1,2-DCE in the presence of 1.7 % pyridine (v/v) at 0 °C. After vortex stirring for 20 min at 0 °C, the solution was filtered under a gentle stream of nitrogen. This operation was repeated thrice. Then the resin was sequentially washed with ice-chilled CH₂Cl₂ (x 5) and ice-chilled 1,2-DCE (x 3) under a gentle stream of nitrogen to give the Npys-Cl resin. Next, the Npys-Cl resin was immediately mixed with 4-fluorophenol (2-30 eq.) and *N,N*-diisopropylethylamine (DIPEA, 2-24 eq.) in the presence of 1,2-DCE and stirred by vortex mixing for 10 min or 1 h followed by filtration. The resin was sequentially washed with CH₂Cl₂ (x 5) and MeOH (x 3). The resin was dried *in vacuo* to obtain the resin (**2**). The yield was calculated from the content of fluorine atom content in resin **2** by elemental analysis. The yields are shown below in Table S2.

Table S2. Evaluation of fluorine content in synthesized Npys-OPh(*p*F) resin

Entry	DIPEA (eq.)	4-Fluorophenol (eq.)	Time	Yield (%) ^[a]
1	2	2	10 min	34
2	8	10	10 min	75
3	24	30	10 min	64
4	8	10	1 h	54

[a] Yields were calculated from the content of fluorine atom on resin **2** by elemental analysis.

In a typical experiment (Table S2, Entry 2), resin **3** (30.0 mg, 15.9 μmol) was added to a solution of 2% SO_2Cl_2 in 1,2-DCE (736 μL) in the presence of pyridine (12.98 μL , 160.7 μmol). This operation was repeated thrice. After obtaining the Npys-Cl resin, it was immediately mixed with 4-fluorophenol (17.82 mg, 160.0 μmol) and DIPEA (22.16 μL , 127.2 μmol) in the presence of 1,2-DCE (1.8 mL). The product was then dried *in vacuo* to obtain the resin (**2**) (28.42 mg, 11.3 μmol , 75% yield). Elemental analysis of resin **2** indicated that the formula was $\text{C}_{196.62}\text{H}_{354.44}\text{N}_6\text{O}_{91.11}\text{S}_2\text{F}_2$ (Calcd: C, 54.08; H, 8.20; N, 1.93; S, 1.47; F, 0.87; Found: C, 52.12; H, 7.82; N, 2.19; S, 1.11; F, 0.65).

5. Synthesis of the peptide fragment

5-1. H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH₂ (**4**)

Peptide **4** was prepared in the same manner as previously reported³ using Fmoc-Rink amide resin (500 mg, 0.29 mmol). The crude was purified by reversed-phase HPLC to give peptide **4** (53 mg, 78.9 μmol, 27% yield). HRMS (ESI) *m/z* calcd for C₂₄H₄₄N₇O₆S [M+H]⁺ 558.3074, found 558.3076.

5-2. Fmoc-Cys-Tyr-Ile-Gln-OH (**5**)

Peptide **5** was prepared in the same manner as previously reported³ using Fmoc-Gln(Trt)-Wang-resin (500 mg, 0.355 mmol). The crude was purified by reversed-phase HPLC to give peptide **5** (50 mg, 66.8 μmol, 19% yield). HRMS (ESI) *m/z* calcd for C₃₈H₄₆N₅O₉S [M+H]⁺ 748.3016, found 748.3016.

5-3. Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH₂ (**8**)

A 20% piperidine/DMF solvent was added to the Fmoc-Rink amide resin (300 mg, 0.135 mmol). After removing the 20% piperidine/DMF solution, the peptide chains were elongated by treatment with Fmoc-amino acid (3 eq.), *N,N'*-diisopropylcarbodiimide (DIPCI, 3 eq.), and 1-hydroxybenzotriazole (HOBT, 3 eq.) for 2-3 h. These reactions were repeated to lengthen the desired peptide. Then, acetic anhydride (Ac₂O, 5 eq.) with DIPEA (5 eq.) in DMF was added to the resins and reacted for 30 min at rt. The synthesized resins were treated with TFA:1,3-dimethoxybenzene (DMB):H₂O (40:1:2, 8 mL) for 3 h. The crude products were precipitated with Et₂O and washed twice. After drying, the residual solids were purified by reversed-phase HPLC to give the peptide (**8**) (36.3 mg, 0.052 mmol, 39%). HRMS (ESI) *m/z* calcd for C₃₄H₄₉N₆O₈S [M+H]⁺ 701.3333, found 701.3334.

6. Solid-phase disulfide ligation

6-1. Synthesis of intermediate disulfide peptide (7) of oxytocin

A solution of H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH₂ (**4**) (0.95 mg, 1.42 μmol, 1.0 eq.) and 0.4 M lithium chloride (LiCl, 4.8 mg) in AcOH (284 μL) was added to Npys-OPh(*p*F) resin **2** (9.9 mg, 5.25 μmol, 3.7 eq.) at rt. After vortex stirring for 1 h at rt, the reaction mixture was filtered. Resulting peptide-resin **6** was washed with H₂O ten times.

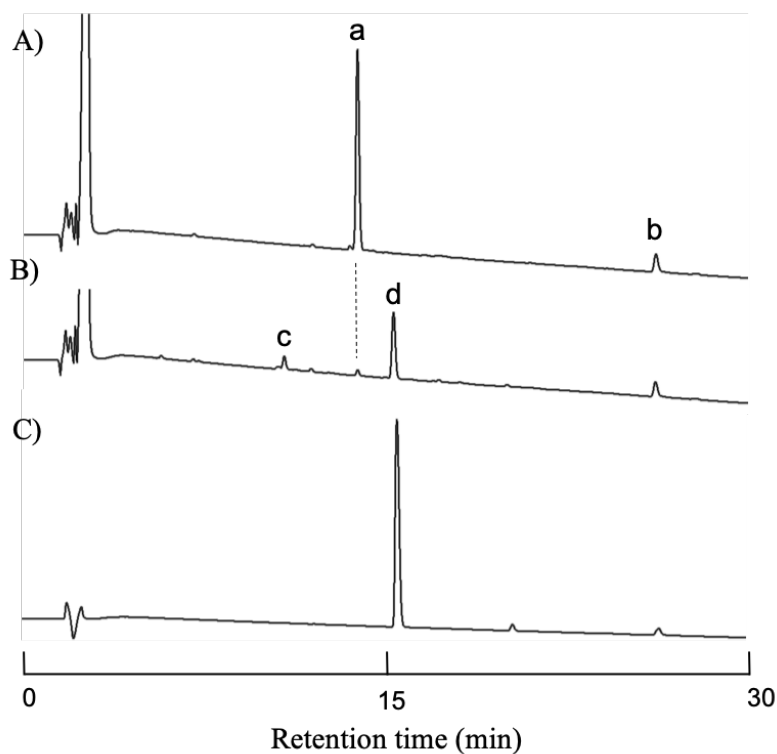
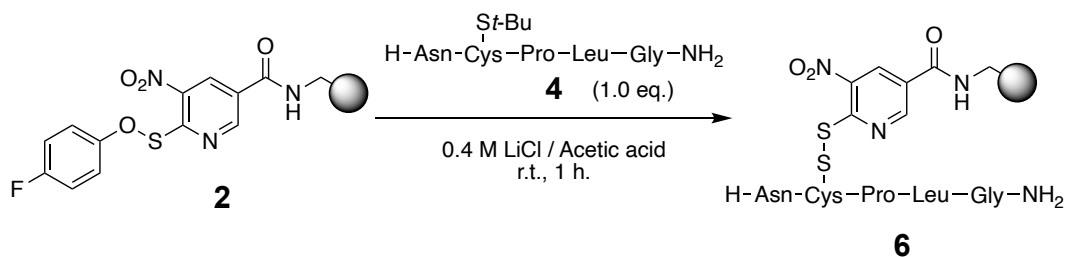


Fig. S1 HPLC analysis of the reaction mixture. A) 0 h, B) 1 h, C) 4-fluorophenol, a: peptide **4**, b: original impurity peak, c: H-Asn-Cys-Pro-Leu-Gly-NH₂, d: 4-fluorophenol. HPLC conditions are a linear gradient starting from 5% CH₃CN in 0.1% aqueous TFA to 65% CH₃CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 230 nm.

Then, a solution of Fmoc-Cys-Tyr-Ile-Gln-OH (**5**) (0.88 mg, 1.18 μmol , 0.83 eq.) in DMF/H₂O (2:1, 787 μL) was added to the peptide-resin (**6**) at rt. After vortex stirring for 30 min at rt, the mixture was filtered and the resin was washed with DMF five times. The filtrate and DMF used for the wash were collected and condensed *in vacuo* to give disulfide peptide (**7**) (1.07 mg, 0.86 μmol , 73% yield, HPLC purity: 94%). HRMS (ESI) *m/z* calcd for C₅₈H₇₉N₁₂O₁₅S₂ [M+H]⁺ 1247.5229, found 1247.5226.

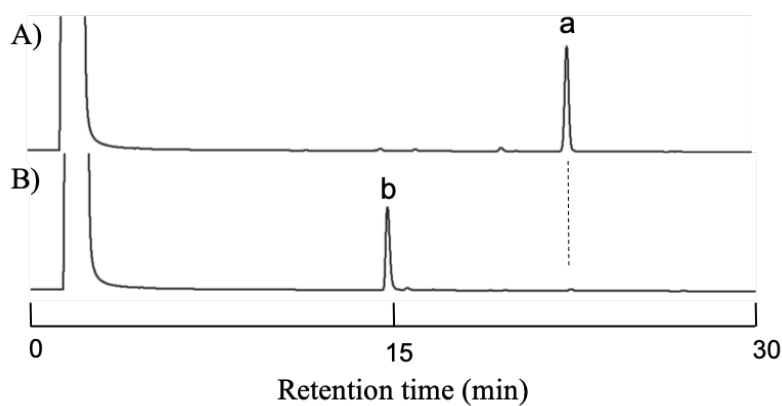
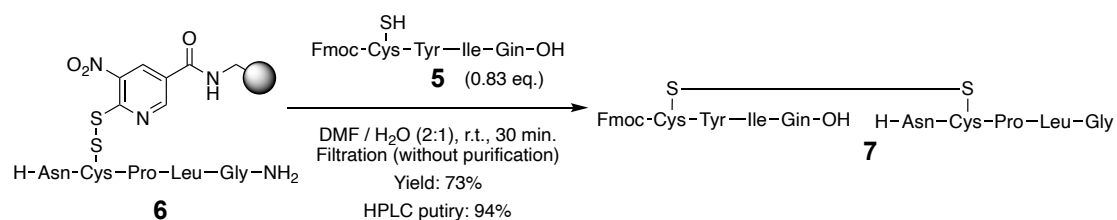


Fig. S2 HPLC analysis of the reaction mixture. A) 0 h, B) 30 min, a: peptide **5**, b: peptide **7**. HPLC conditions are a linear gradient starting from 25% CH₃CN in 0.1% aqueous TFA to 55% CH₃CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL / min and detection at 230 nm.

6-2. Synthesis of disulfide linked glycoconjugate 10

Glycoconjugate **10** was prepared in the same manner as described for the disulfide peptide (**7**) using Npys-OPh(*p*F) resin **2**. Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH₂ (**8**) (1.53 mg, 2.18 μmol, 1.0 eq.) in 0.4 M LiCl (7.4 mg)/AcOH (436 μL) was added to Npys-OPh(*p*F) resin (20 mg, 8.07 μmol, 3.7 eq.). The resulting peptide-resin was washed with H₂O ten times.

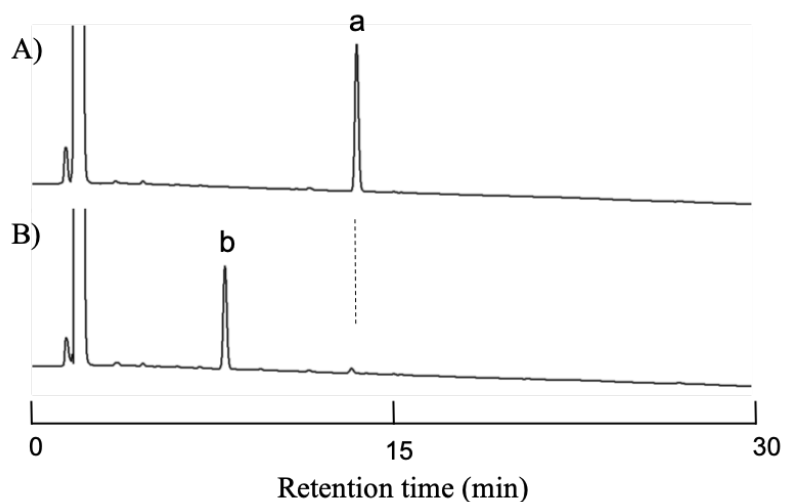
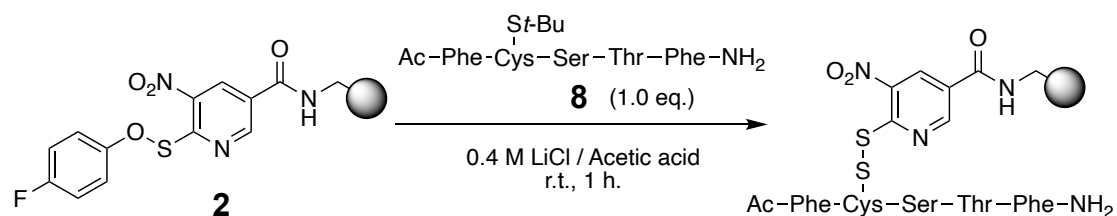


Fig. S3 HPLC analysis of the reaction mixture. A) 0 h, B) 1 h, a: peptide **8**, b: 4-fluorophenol. HPLC conditions are a linear gradient starting from 25% CH₃CN in 0.1% aqueous TFA to 55% CH₃CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 230 nm.

Then, a solution of **9** (0.43 mg, 1.81 μmol , 0.83 eq.) in CH_3CN : 50 mM sodium acetate buffer (2:3, pH = 4.5, 1.2 mL) was added to the peptide-resin. After vortex stirring for 30 min at room temperature, the end point of the reaction was confirmed by thin layer chromatography. HPLC purification was used to collect the purity and condensed *in vacuo* to give disulfide linked glycoconjugate **10**. (0.9 mg, 0.85 μmol , 47% yield). HRMS (ESI) m/z calcd for $\text{C}_{38}\text{H}_{52}\text{N}_7\text{O}_{13}\text{S}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ 902.3040, found 902.3038.

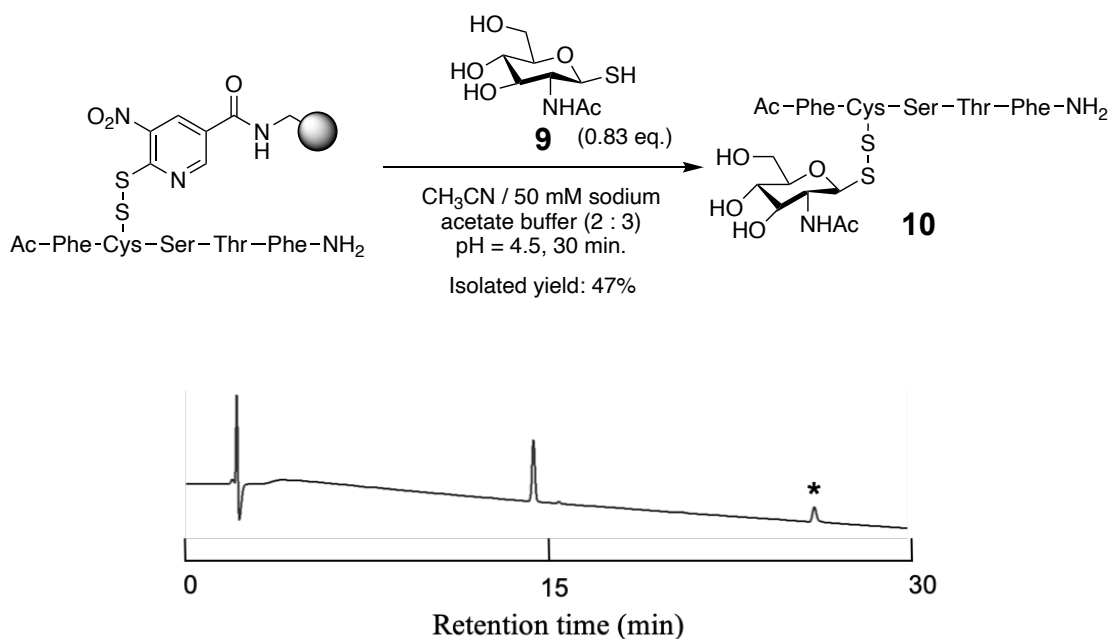


Fig. S4 HPLC analysis of purified glycoconjugate (**10**). *original impurity peak. HPLC purity: 96%. HPLC conditions are a linear gradient starting from 25% CH_3CN in 0.1% aqueous TFA to 55% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 230 nm.

7. Stability of Npys-Cl resin 1 and Npys-OPh(*p*F) resin 2

The Npys-Cl resin **1** was prepared in the same manner described for synthesis of resin **2**. Then, Npys-Cl resin **1** and Npys-OPh(*p*F) resin **2** were stored separately at rt for 1, 3 and 7 days, at 4 °C for 30 days and at -20 °C for 1, 30 and 100 days. The stability of resins was assessed by loading of peptide **4** onto the resin. A solution of H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH₂ (**4**) in 0.4 M LiCl/AcOH was added to the stored resin **1** or **2** at rt. After vortex stirring for 1 h at rt, the peptide **4** in the reaction mixture was analyzed by HPLC. The loading yield of peptide **4** is shown below in Table S3.

Table S3. Stability of Npys-Cl resin **1** and Npys-OPh(*p*F) resin **2**

Entry	Storage condition	Time	Loading yield of peptide 4 (%) ^[a]	
			Npys-Cl Resin 1	Npys-OPh(<i>p</i> F) Resin 2
1	Room temperature	1 day	0	97
2		3 days	0	63
3		7 days	0	22
4	4 °C	30 days	0	36
5	-20 °C	1 day	0	98
6		30 days	0	99
7		100 days	0	98

[a] Loading yield (%) = [1-(HPLC peak area of residual peptide **4**/HPLC peak area of original peptide **4**)] x 100.

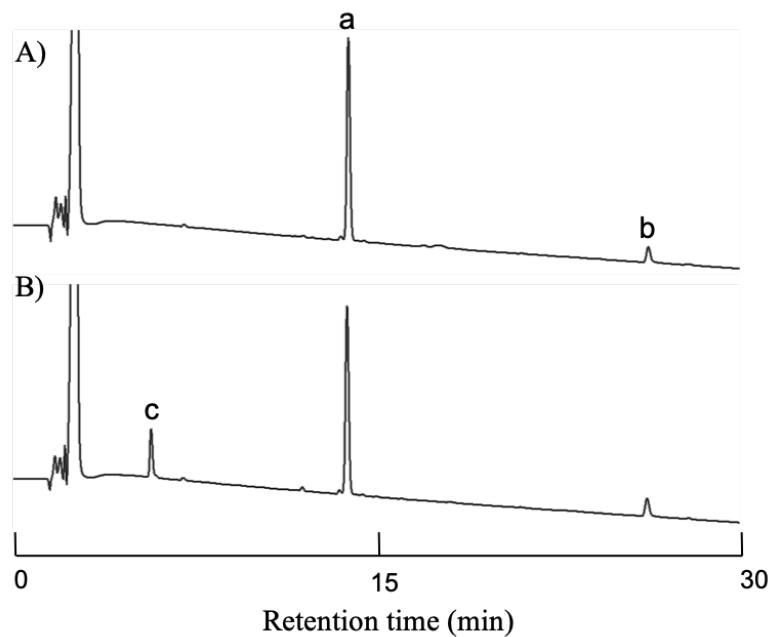


Fig. S5 HPLC analysis of the reaction mixture (Entry 1 in Table S2). A) 0 h, B) 1 h, a: peptide **4**, b: original impurity peak, c: non-peptide peak. HPLC conditions are a linear gradient starting from 5% CH₃CN in 0.1% aqueous TFA to 65% CH₃CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL/min and detection at 230 nm.

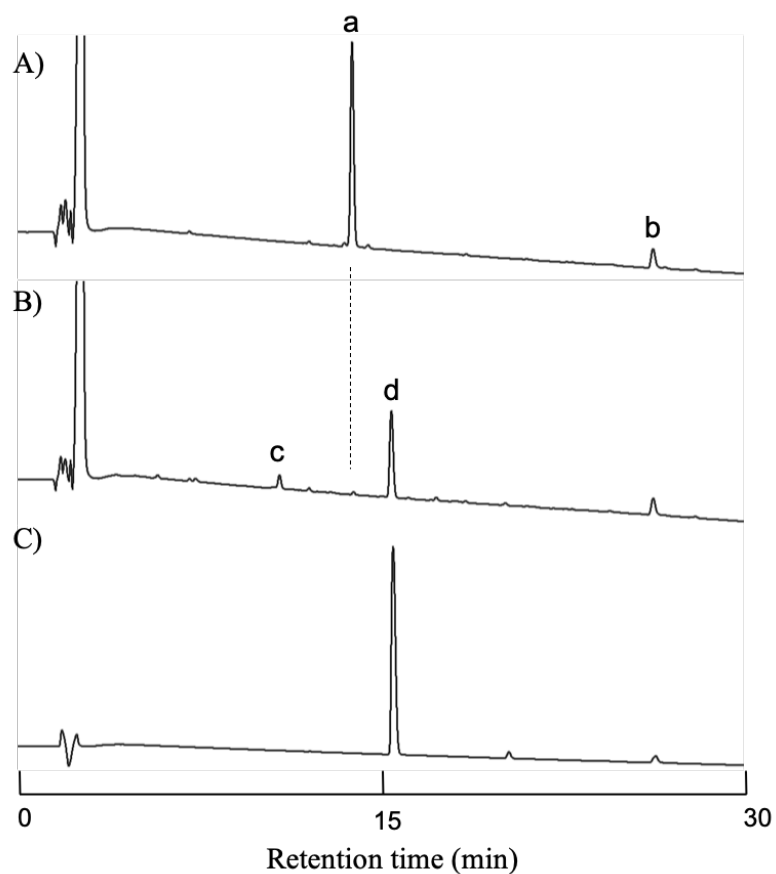
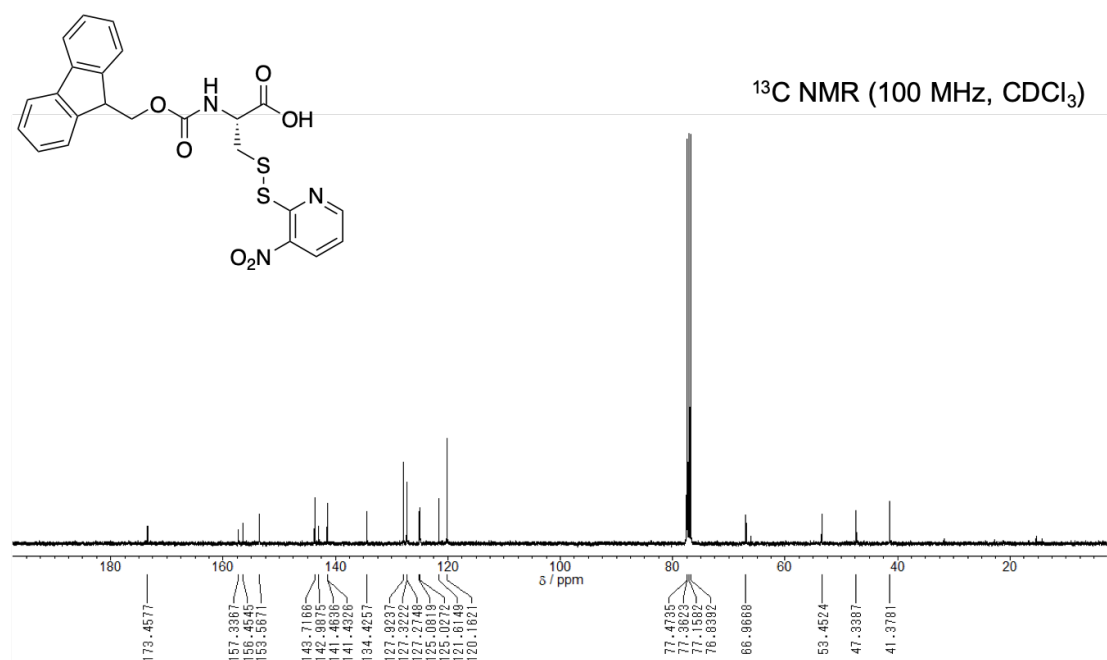
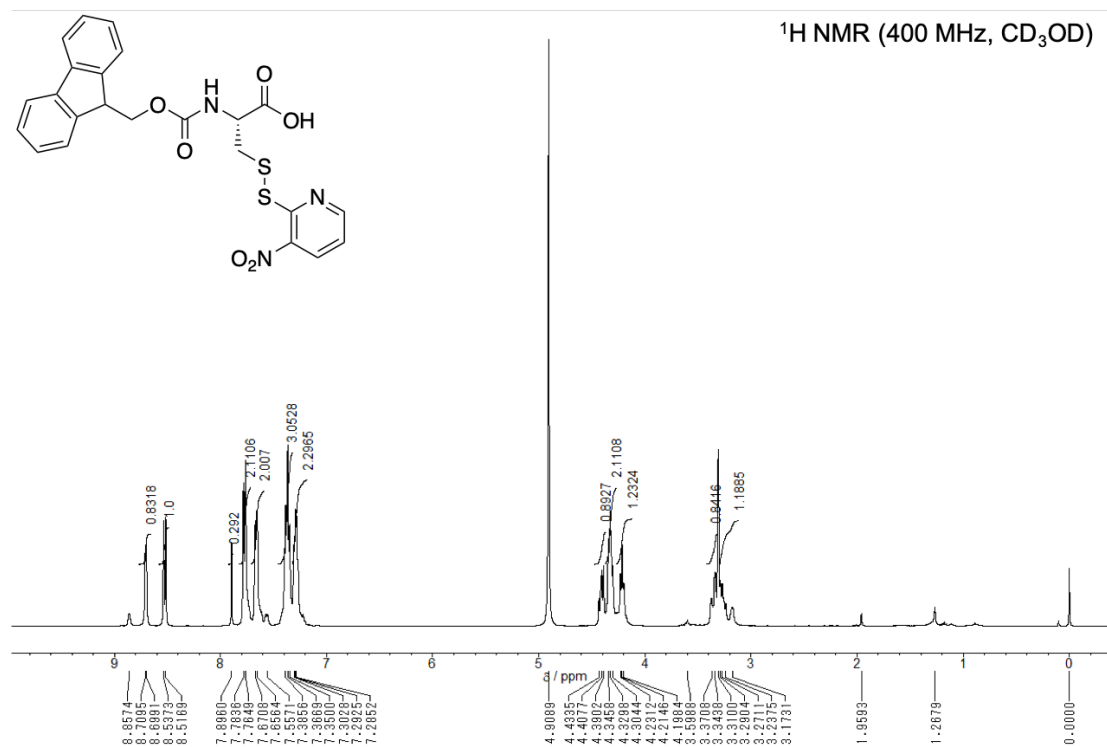


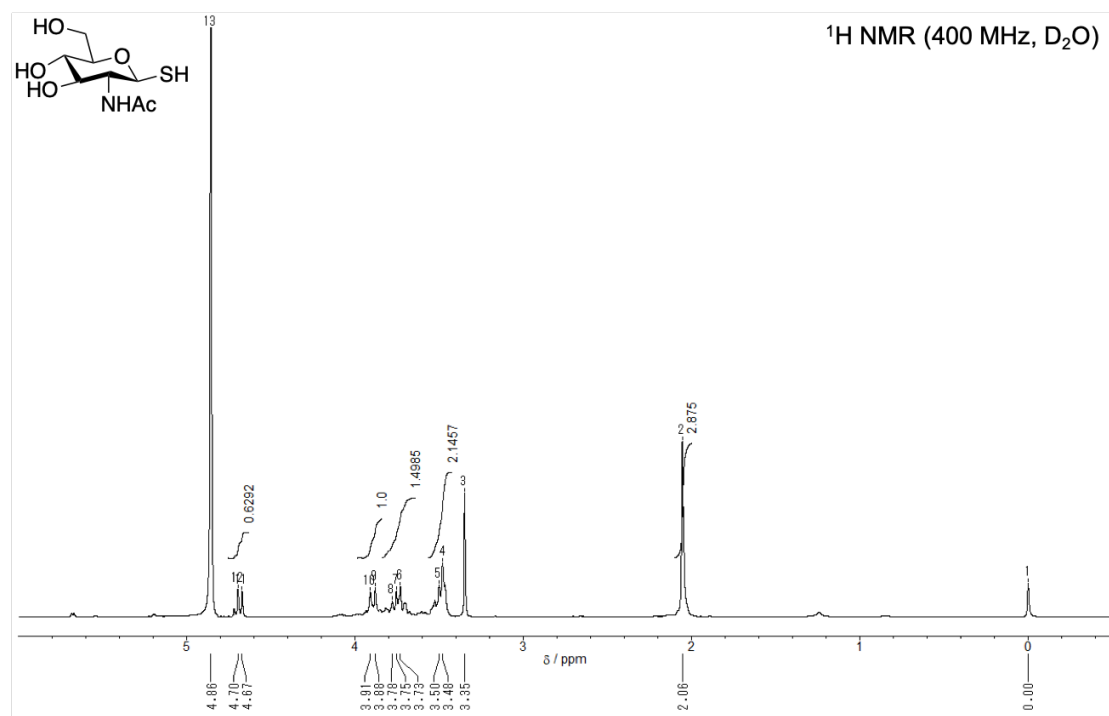
Fig. S6 HPLC analysis of the reaction mixture (Entry 7 in Table S2). A) 0 h, B) 1 h, C) 4-fluorophenol, a: peptide **4**, b: original impurity peak, c: H-Asn-Cys-Pro-Leu-Gly-NH₂, d: 4-fluorophenol. HPLC conditions are a linear gradient starting from 5% CH₃CN in 0.1% aqueous TFA to 65% CH₃CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL/min and detection at 230 nm.

8. NMR spectra

1. Fmoc-Cys(Npys)-OH

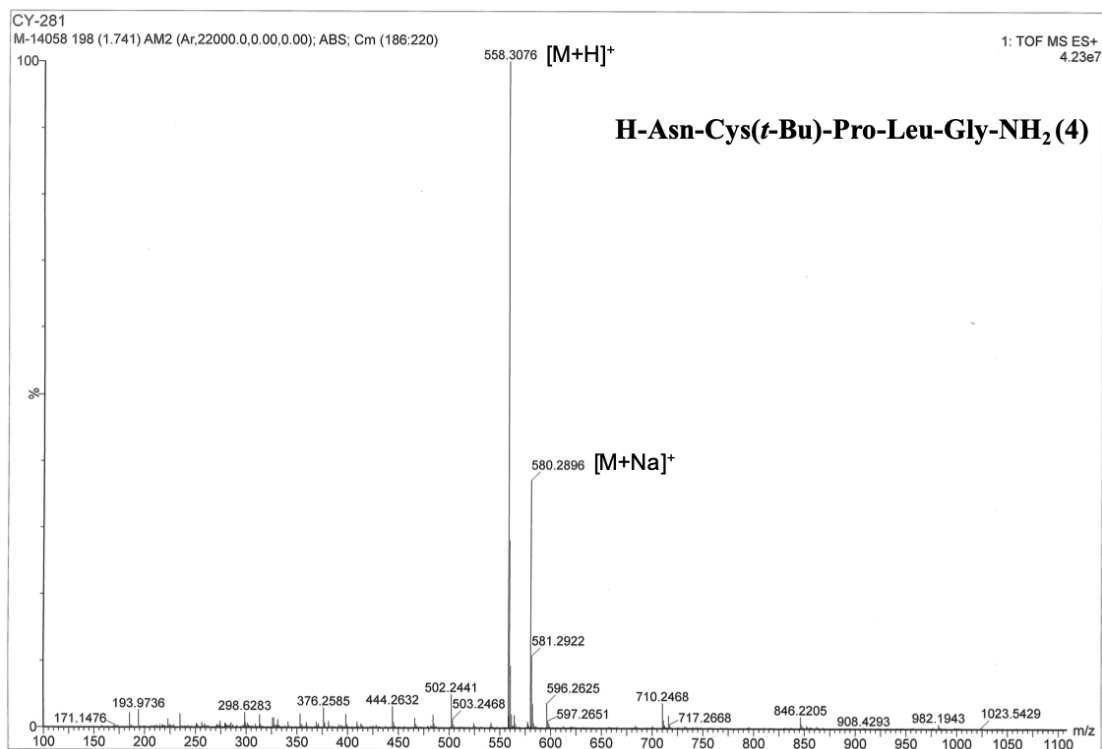


2. Compound 9

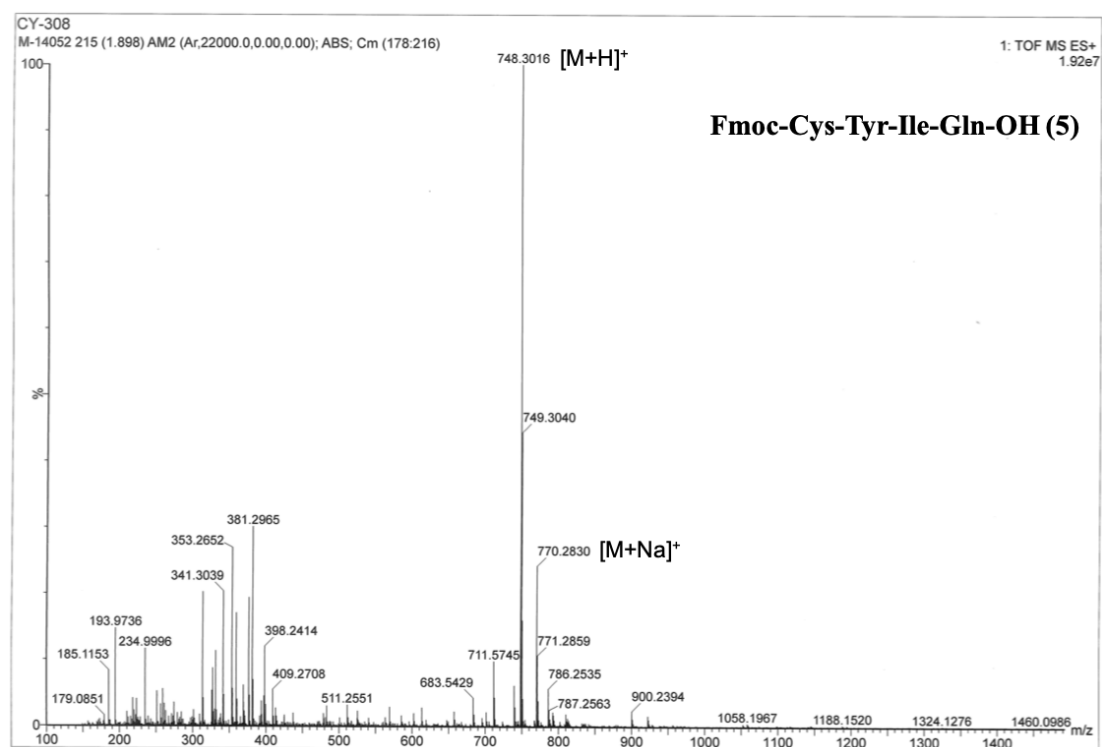


9. Mass spectra

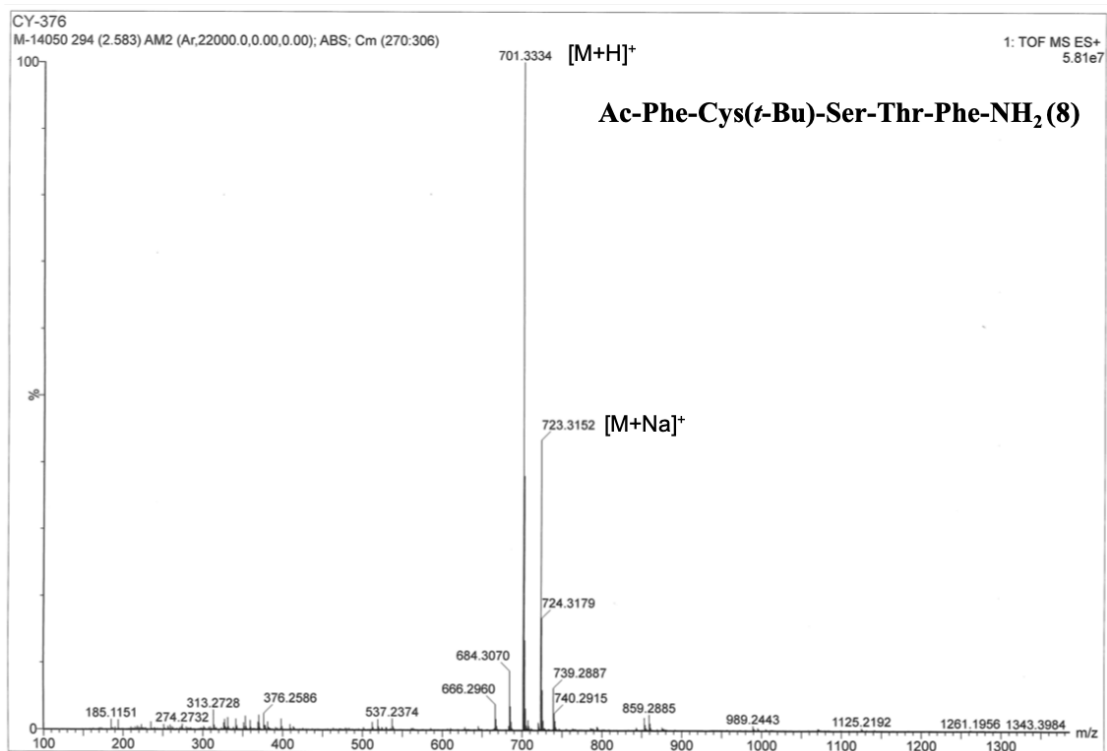
1. Compound 4



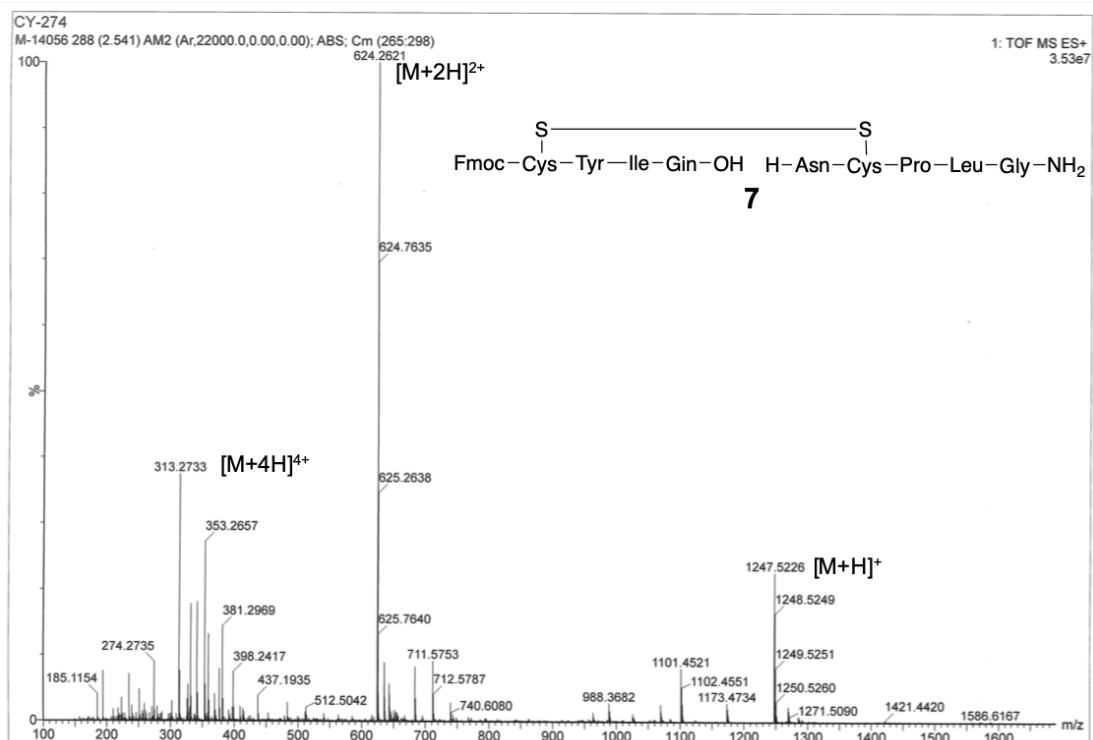
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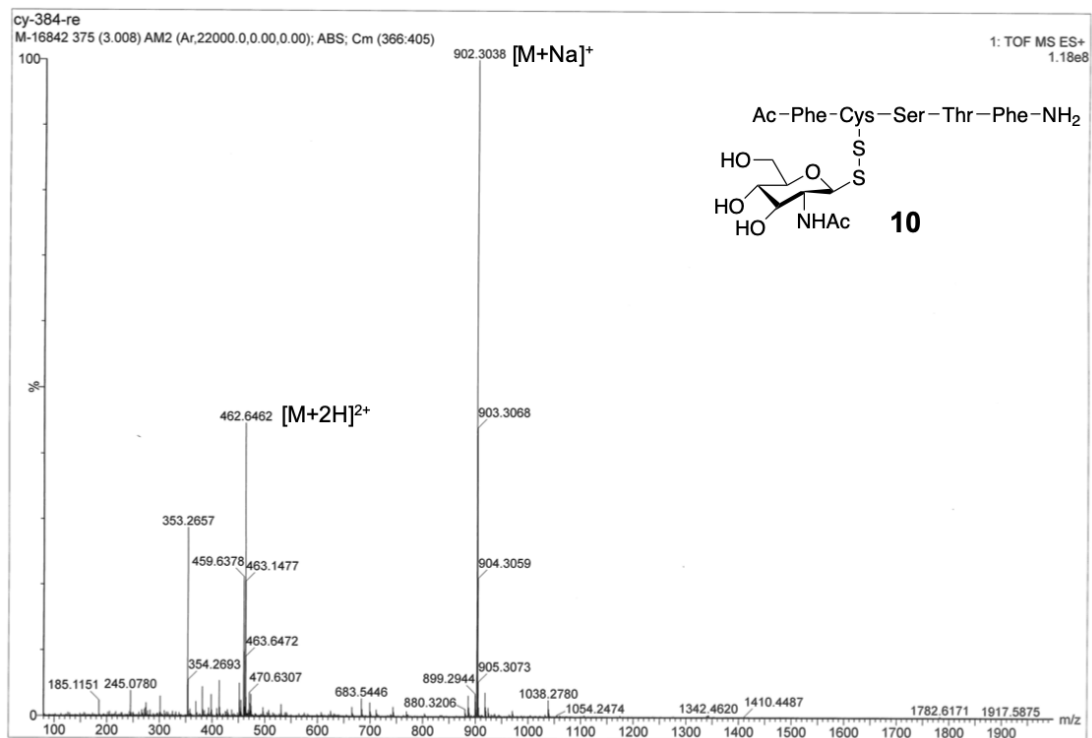
3. Compound 8



4. Compound 7



5. Compound 10



10. References

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