Supporting Information

Prior disulfide bond-mediated Ser/Thr ligation (PD-STL)

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1 Experimental section

1.1 General experimental details

All commercially available materials were used without further purification. Unless otherwise specified, amino acids, coupling reagents, and resins were obtained from GL Biochem, CSBio, and Chem-Impex. All solvents were reagent grade or HPLC grade (RCI or DUKSAN). Dry dichloromethane (DCM) was distilled from calcium hydride (CaH_2). Anhydrous DMF was purchased from Scharlau. All analytical reversed-phase high-performance liquid chromatography (RP-HPLC) separations involving a mobile phase of 0.1% trifluoroacetic acid (TFA) (*v*/*v*) in acetonitrile (Solvent A) and 0.1% TFA (*v*/*v*) in water (Solvent B) were performed on a Waters UPLC H-class system equipped with an ACQUITY UPLC photodiode array detector and a Waters SQ Detector 2 mass spectrometer using a Waters ACQUITY BEH C18 column (1.7 μm, 130 Å, 2.1 x 50 mm) at a flow rate of 0.4 mL/min. Preparative HPLC separations involving a mobile phase of 0.1% trifluoroacetic acid (TFA) (*v*/*v*) in acetonitrile (Solvent A) and 0.1% TFA (*v*/*v*) in water (Solvent B) were performed on a Waters HPLC system equipped with a quaternary pump (Waters 2545) and a UV/Vis detector (Waters 2489) using a Grace Vydac 218TP^{TM} C18 column (10 μm, 300 Å, 22 x 250 mm) at a flow rate of 10 mL/min, or YMC-Pack ODS-A-HG Preparative Column (20μm, 300 Å, 30 x 250mm) at a flow rate of 20 mL/min. Analytical TLC was performed on E. Merck silica gel 60-F254 plates and visualized under UV light (254 nm) or by staining with ninhydrin. The flash column chromatography was performed on E. Merck 230-400 mesh silica gel 60.

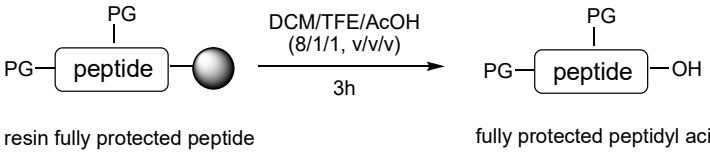
1.2 General procedures

1.2.1 Fmoc-based solid-phase peptide synthesis (SPPS)

The 2-chlorotrityl chloride resin (100 mg, loading: ~ 0.5 mmol/g) was swollen in dry DCM for 20 min in a 5 mL disposable vessel (TORIVQ) equipped with a porous polypropylene disc at the bottom. A solution of Fmoc amino acid (4.0 equiv) and DIEA (8.0 equiv) in DCM was added, and the reaction

vessel was shaken on the vortex at room temperature for 1 h. The resin was washed with DMF (5 × 3 mL) followed by a solution of DCM/MeOH/DIEA (17:2:1, *v*/*v*/*v*, 3 mL) for 20 min and washed with DMF (5 × 3 mL). Subsequently, the resin was submitted to iterative peptide assembly via the standard Fmoc-SPPS protocol. The Fmoc deprotection was carried out using 3 mL of 20% piperidine in DMF at room temperature for 20 min. The resin was then washed with DMF (5 × 3 mL). For the coupling step other than Fmoc- Cys(Trt)-OH, Fmoc amino acid (4 equiv), HATU (4 equiv), and DIEA (8 equiv) were fully dissolved in DMF and mixed for 30 s before adding to the resin. The solution with the resin was gently agitated on the vortex at room temperature for 1 h. The resin was then washed with DMF (5 × 3 mL). Fmoc-Cys(Trt)-OH (4 equiv), DIC (4 equiv), and HOAt (4 equiv) were dissolved in DMF and mixed for 30 s before adding to the resin. The solution with the resin was gently agitated on the vortex at room temperature for 1 h. The resin was then washed with DMF (5 × 3 mL).

1.2.2 Cleavage of fully protected crude peptide from resin



The fully protected peptide on resin was treated with a cocktail of DCM/AcOH/TFE=8:1:1 (*v*/*v*/*v*, 5-10 mL) cocktail, 2 times and 1.5 h for each time. After cleavage, the resulting cleavage solutions were combined and concentrated to give crude protected peptide bearing the free carboxylic acid at the C-terminus.

1.2.3 Preparation of peptide salicylaldehyde ester with S-SPyr

For the formation of S-SPyr, a crude peptide with free -SH group was first dissolved in DCM (1 equiv, around 25 mM); if not fully dissolved, DIEA (2 equiv) was added. An equal volume of DCM was used to dissolve 2,2'-dithiodipyridine (15 equiv). Then the crude peptide solution was added dropwise to the stirring solution of 2,2'-dithiodipyridine. The solution was allowed to react for a further 30 min after the addition.

For salicylaldehyde ester formation by the direct coupling method (for Gly/Pro as C-terminal amino acid of the crude peptide), the reaction mixture after the formation of S-SPyr was cooled to 0 ºC, then

salicylaldehyde dimethyl acetal (15 equiv) or HCl·Xaa-salicylaldehyde semicarbazone (Xaa is an amino acid, 3 equiv), DMAP (0.5 equiv) were added, followed by EDCI (5 equiv). The reaction was allowed to react at 0 °C for 1 h and 15 h at RT. Then the solvent was removed *in vacuo*.

For salicylaldehyde ester formation by the n+1 method (for amino acids other than Gly/Pro as C-terminal amino acid of the crude peptide), DCM in the reaction mixture after the formation of S-SPyr was removed in vacuo, then chloroform was added and the reaction mixture was cooled to 0 °C. After that, HCl • Xaa-salicylaldehyde semicarbazone (Xaa is an amino acid, 3 equiv), DIEA (2 equiv, for neutralizing HCl salt), HOOBt (3 equiv) were added, followed by EDC (3 equiv). The reaction was allowed to react at 0 °C for 1 h and 15 h at RT. Then the solvent was removed *in vacuo*.

For the global deprotection of crude peptide side-chain protecting groups and salicylaldehyde semicarbazone, after the solvent was removed, a deprotection cocktail of TFA/Phenol/H_2O (95/2.5/2.5, *v*/*v*/*v*) cocktail was added, and the solution was allowed to react for 2 h. If using the n+1 strategy, after 2 h, pyruvic acid (30 equiv) was added to the deprotection cocktail. The reaction was stirred for another 1 h (this step was omitted for the direct coupling method). Then, the reaction was concentrated under a stream of compressed air and then added dropwise to cold diethyl ether. The precipitate was washed with cold diethyl ether for two times. The precipitate was further vacuum dried before HPLC purification.

1.2.4 Disulfide-bond assisted STL

The formation of the disulfide bond between the peptide SAL ester (1 equiv) and N-terminal Ser/Thr peptide (1.3-1.8 equiv) was performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) and monitored by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (Spyr). The peptide pellet was allowed to air dry for 10 min before the next step or stored at -20°C (stable for at least 1 week). Alternatively, 50% acetonitrile in H_2O could be used as a solvent for the disulfide bond formation. After the reaction was finished, the solvent was removed by lyophilization. Then, the dried powder was washed twice with diethyl ether to remove the SPyr.

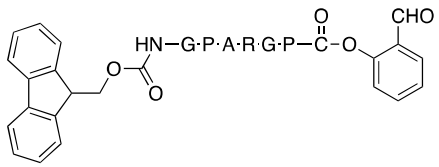
After that, the disulfide SAL ester was first dissolved in DMF or DMSO (10 mM concentration) and then was added to ligation buffer (pyridine/AcOH, typically in a ratio of 1/1, *v*/*v*, unless otherwise specified) to a final concentration of 0.25 mM or lower. After the ligation finished (the disappearance of the disulfide SAL ester peak as monitored by LC-MS), the Pyridine/AcOH was blown off under a stream of compressed air or removed by the rotary evaporator, and the ligation intermediate was then ready for acidolysis.

For acidolysis, a solution of TFA/TIPS/H_2O (95/2.5/2.5, *v*/*v*/*v*) cocktail was added to the ligation intermediate for 5-30 min (as monitored by LC-MS). After the acidolysis finished, the TFA solution was concentrated by a stream of compressed air or rotary evaporator. The concentrated mixture was washed with cold diethyl ether for two times to precipitate the product out. The precipitate was allowed to air dry or vacuum dry for 10 min. Then the product was ready for disulfide bond reduction and desulfurization, if necessary.

The resulting residues were dissolved in 20 mM TCEP in H_2O or H_2O/ACN (1:1) with 0.1% TFA for disulfide reduction. After the disulfide bond reduction finished (5-30 min, monitored by LC-MS), the mixture was diluted by H_2O/ACN and was ready for HPLC purification.

2 Synthesis of model C-terminal peptide salicylaldehyde esters

2.1 Fmoc*NH*-GPARGP-*CO*-SAL ester (1a)



Fmoc*NH*-GPARGP-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1** The crude product was purified by HPLC using a gradient of 25-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

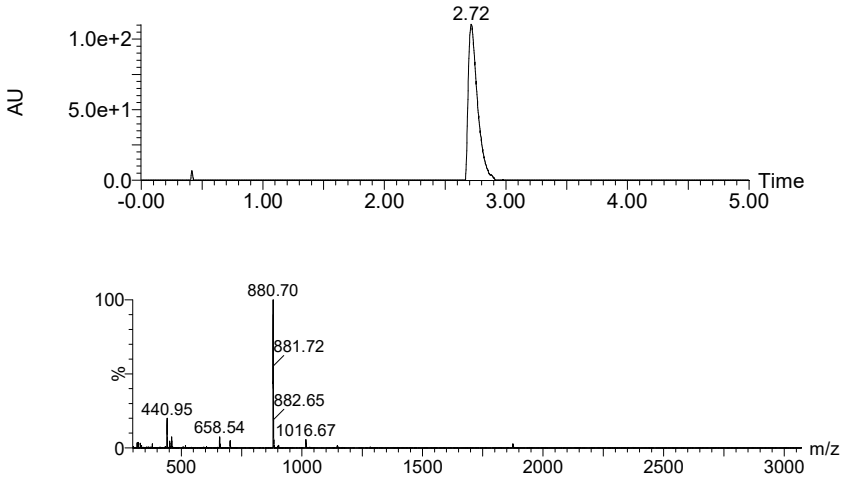
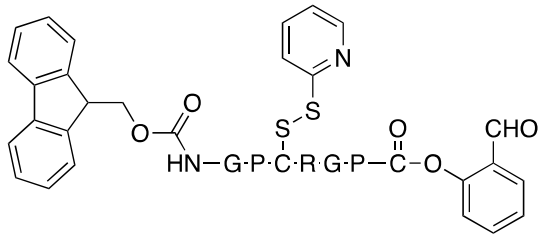


Fig. S1 Analytical HPLC analysis UV trace and ESI-MS of peptide Fmoc*NH*-GPARGP-*CO*-SAL ester. Gradient: 30-70% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{45}H_{53}N_9O_{10}$ $[M+H]^+$ *m/z:* 880.96; found: 880.70; $[M+2H]^{2+}$ *m/z:* 440.99; found: 440.95.

2.2 Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b)



Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 30-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

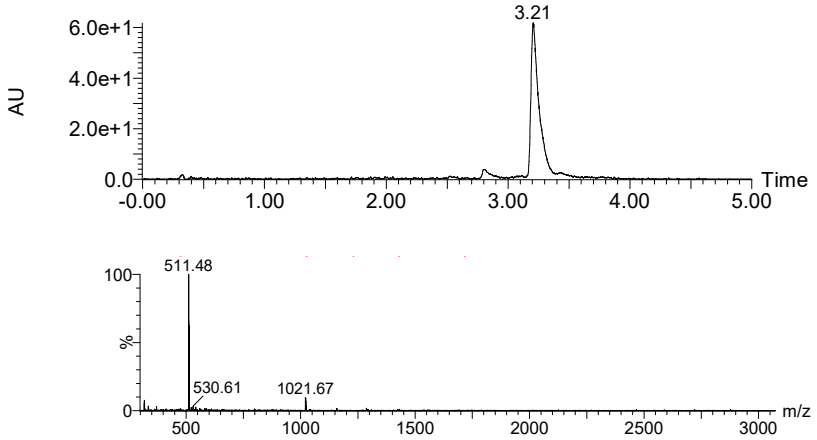
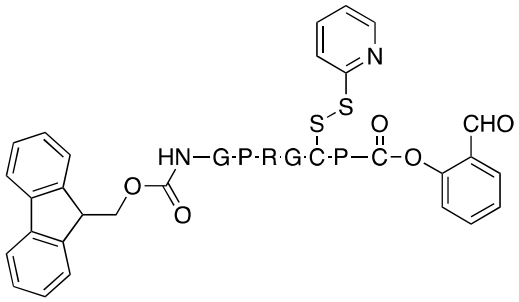


Fig. S2 Analytical HPLC analysis UV trace and ESI-MS of peptide Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester. Gradient: 30-70% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{50}H_{56}N_{10}O_{10}S_2$ $[M+H]^+$ *m/z:*1022.18; found:1021.67; $[M+2H]^{2+}$ *m/z:* 511.59; found: 511.48.

2.3 Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester (1c)



Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 30-60% ACN containing 0.1% TFA/ H_2O containing 0.1% TFA over 30 min.

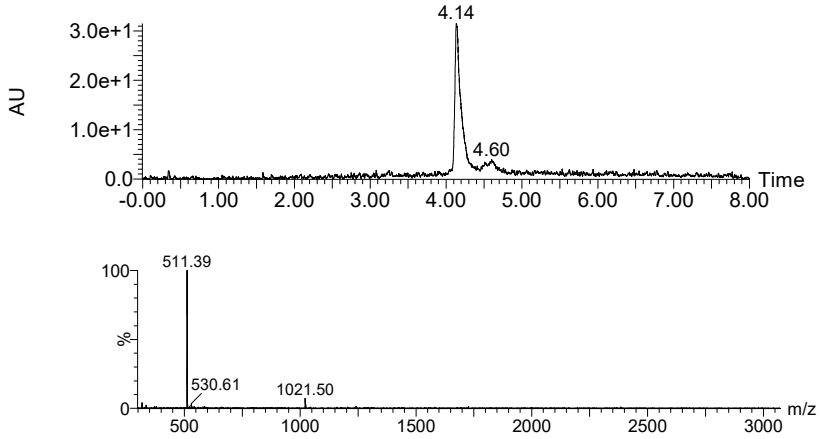
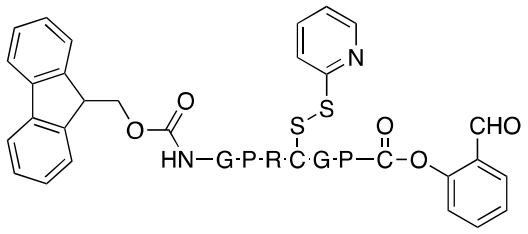


Fig. S3 Analytical HPLC analysis UV trace and ESI-MS of peptide Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester. Gradient: 30-70% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{50}H_{56}N_{10}O_{10}S_2$ $[M+H]^+$ *m/z:*1022.18; found:1021.50; $[M+2H]^{2+}$ *m/z:* 511.59; found: 511.39.

2.4 Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester (1d)



Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 30-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

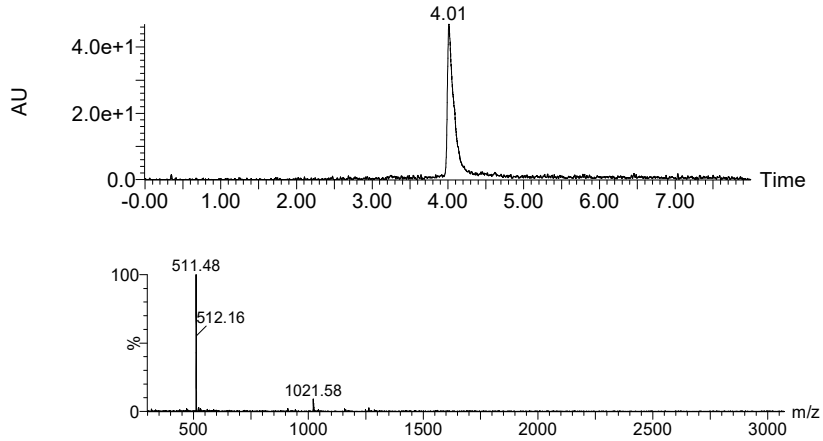
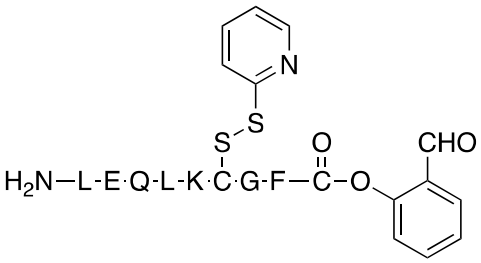


Fig. S4 Analytical HPLC analysis UV trace and ESI-MS of peptide Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester. Gradient: 30-70% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{50}H_{56}N_{10}O_{10}S_2$ $[M+H]^+$ *m/z:*1022.18; found:1021.58; $[M+2H]^{2+}$ *m/z:* 511.59; found: 511.48.

2.5 *H_2N*-LEQLKC(SSPyr)GF-*CO*-SAL ester (1e)



H_2N-LEQLKC(SSPyr)GF-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 25-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

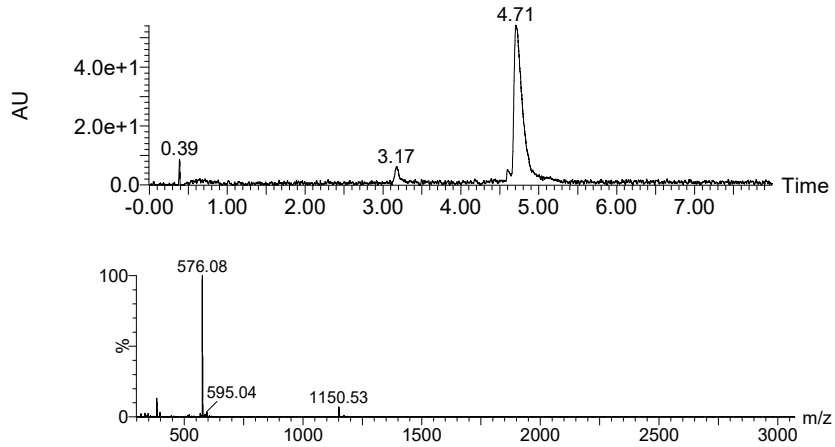
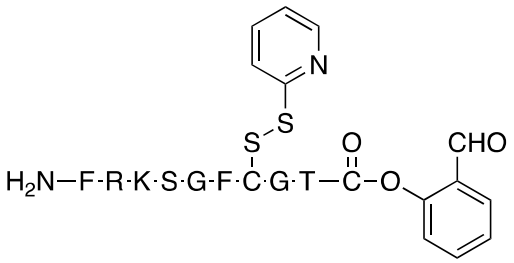


Fig. S5 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-LEQLKC(SSPyr)GF-*CO*-SAL ester. Gradient: 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{54}H_{75}N_{11}O_{13}S_2$ $[M+H]^+$ *m/z:*1151.38; found:1150.53; $[M+2H]^{2+}$ *m/z:* 576.19; found: 576.08.

2.6 *H_2N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester (1f)



H_2N-FRKSGFC(SSPyr)GT-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

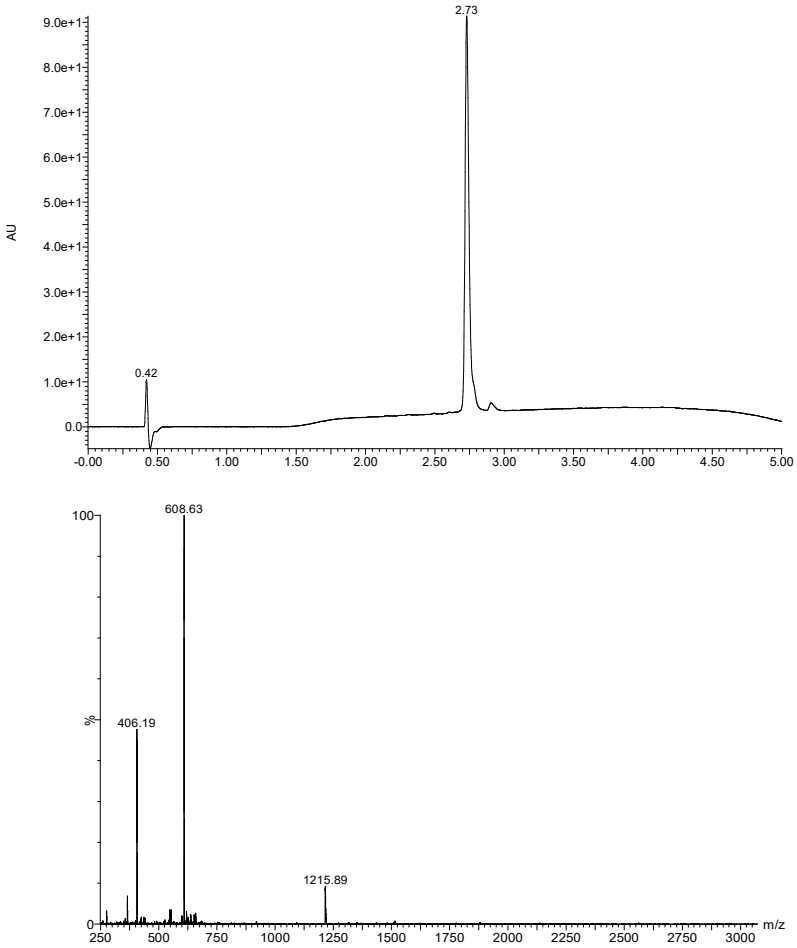
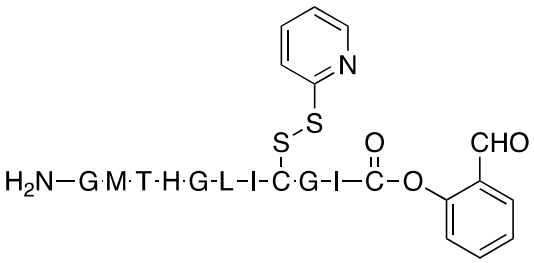


Fig. S6 Analytical HPLC analysis UV trace and ESI-MS of peptide *H₂N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{56}H_{74}N_{14}O_{13}S_2$ $[M+H]^+$ *m/z:*1216.41; found: 1215.89; $[M+2H]^{2+}$ *m/z:* 608.71; found: 608.63; $[M+3H]^{3+}$ *m/z:*406.13; found: 406.19.

2.7 *H₂N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester (1g)



H₂N-GMTHGLIC(SSPyr)GI-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 25-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

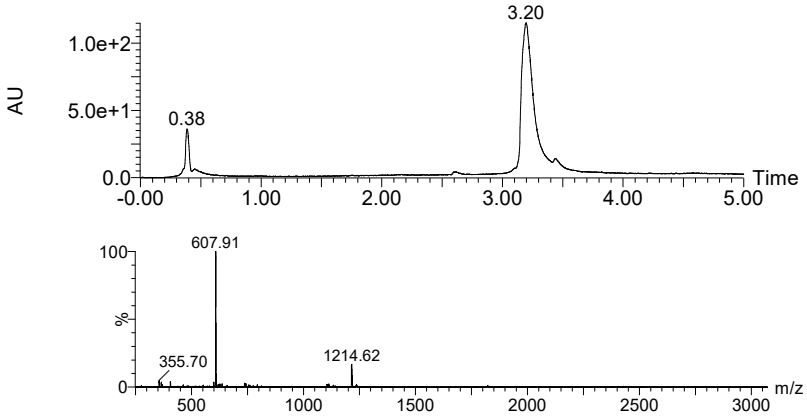
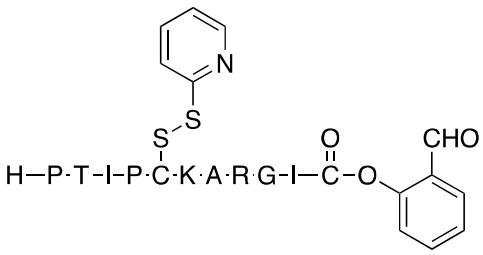


Fig. S7 Analytical HPLC analysis UV trace and ESI-MS of peptide *H₂N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester. Gradient: 20-70% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{54}H_{79}N_{13}O_{13}S_3$ $[M+H]^+$ *m/z:*1215.49; found: 1214.62; $[M+2H]^{2+}$ *m/z:* 608.25; found: 607.91.

2.8 *H₂N*-PTIPC(SSPyr)KARGI-*CO*-SAL ester (1h)



H-PTIPC(SSPyr)KARGI-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

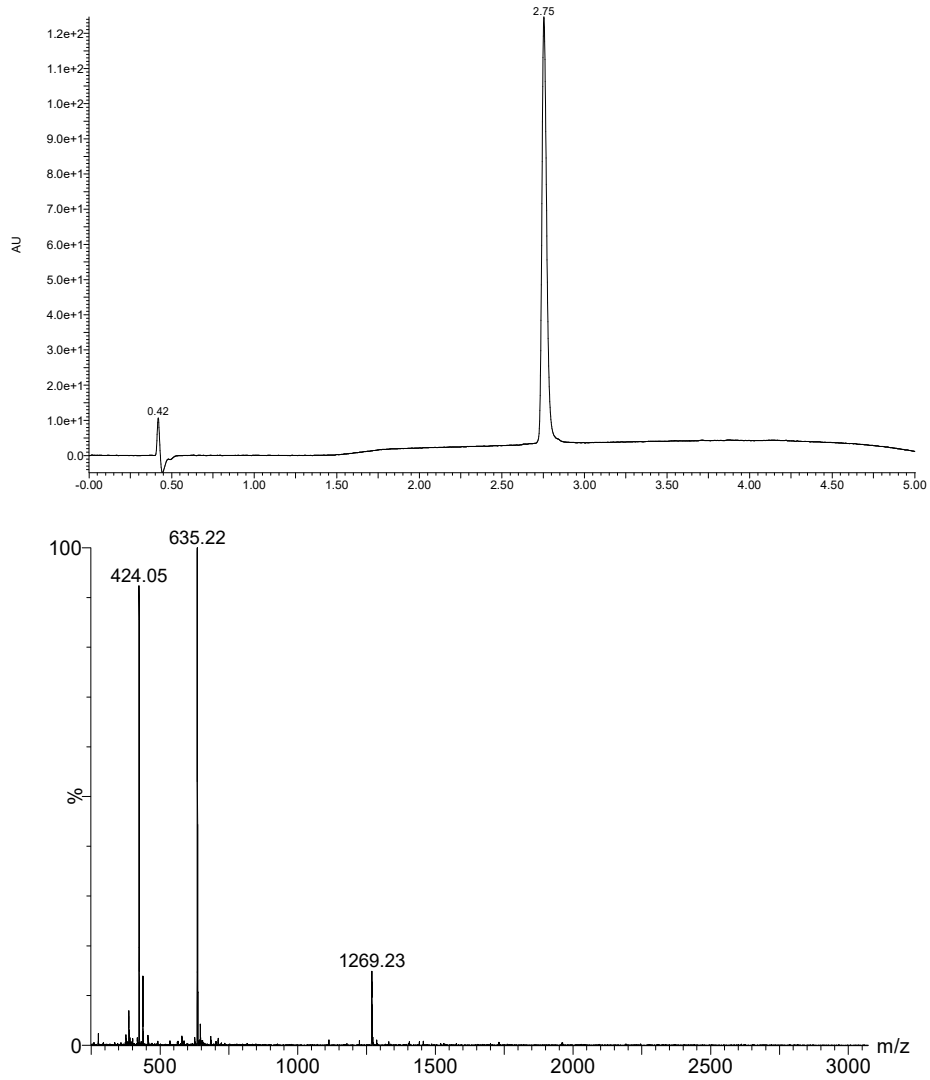
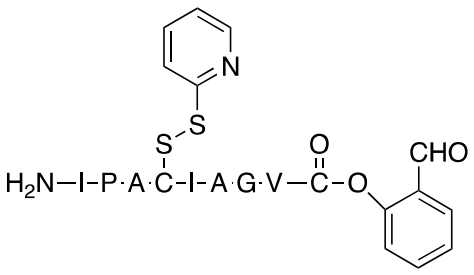


Fig. S8 Analytical HPLC analysis UV trace and ESI-MS of peptide *H*-PTIPC(SSPyr)KARGI-*CO*-SAL ester. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{58}H_{89}N_{15}O_{13}S_2$ $[M+H]^+$ *m/z:*1269.56; found: 1269.23; $[M+2H]^{2+}$ *m/z:* 635.28; found: 635.22; $[M+3H]^{3+}$ *m/z:* 423.86; found: 424.05.

2.9 *H_2N*-IPAC(SSPyr)IAGV-*CO*-SAL ester (1i)



H_2N-IPAC(SSPyr)IAGV-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 25-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

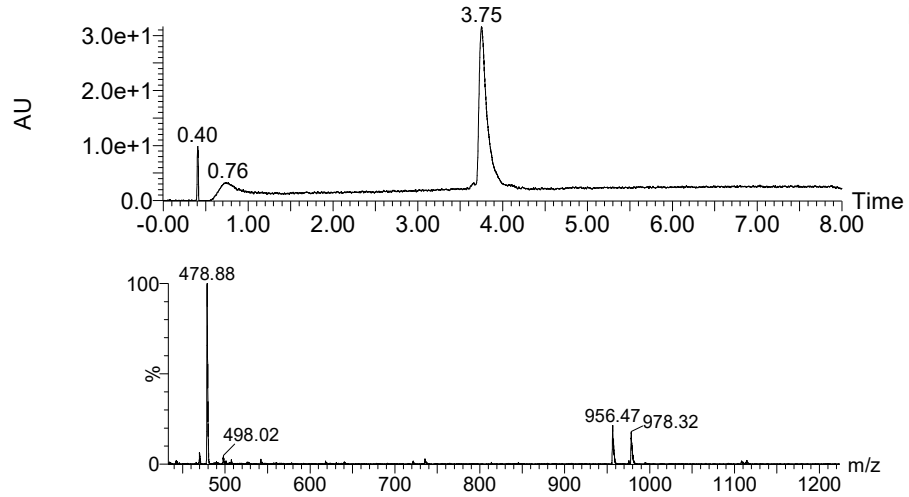
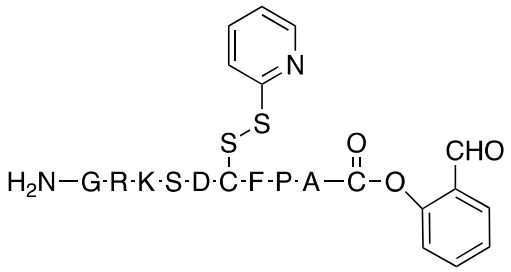


Fig. S9 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-IPAC(SSPyr)IAGV-*CO*-SAL ester. Gradient: 25-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{45}H_{65}N_9O_{10}S_2$ $[M+Na]^+$ *m/z:*979.17; found: 978.32; $[M+H]^+$ *m/z:* 957.19; found: 956.47; $[M+2H]^{2+}$ *m/z:* 479.10; found: 478.88.

2.10 *H_2N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester (1j)



H_2N-GRKSDC(SSPyr)FPA-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 15-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

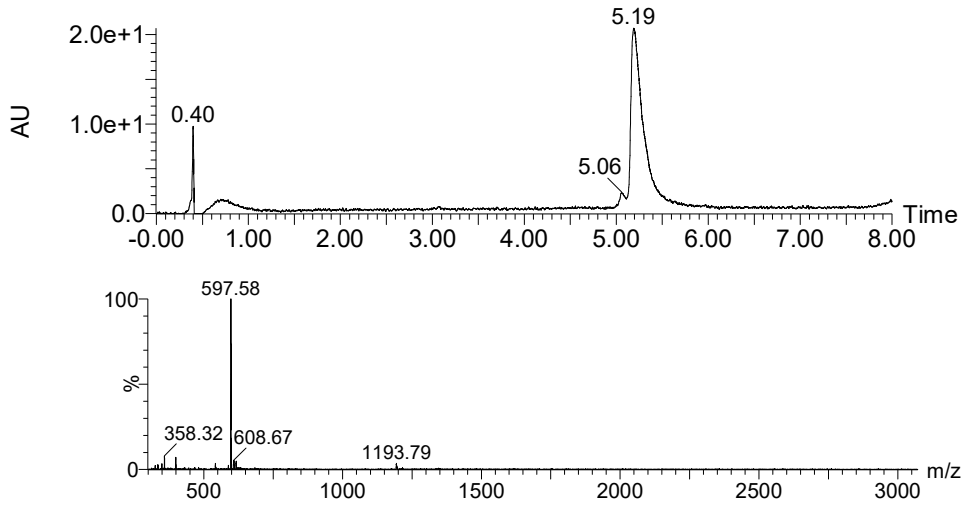
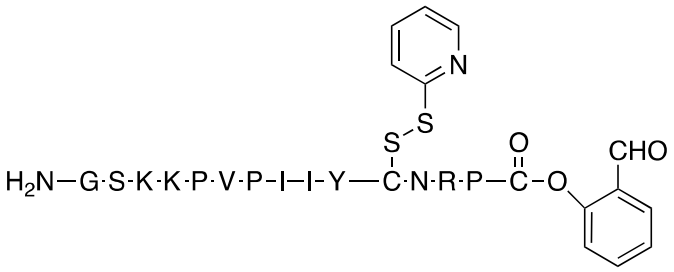


Fig. S10 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester. Gradient: 15-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{53}H_{72}N_{14}O_{14}S_2$ $[M+H]^+$ *m/z:* 1193.49; found: 1193.79; $[M+2H]^{2+}$ *m/z:* 597.25; found: 597.58.

2.11 *H_2N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester (1k)



H_2N-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

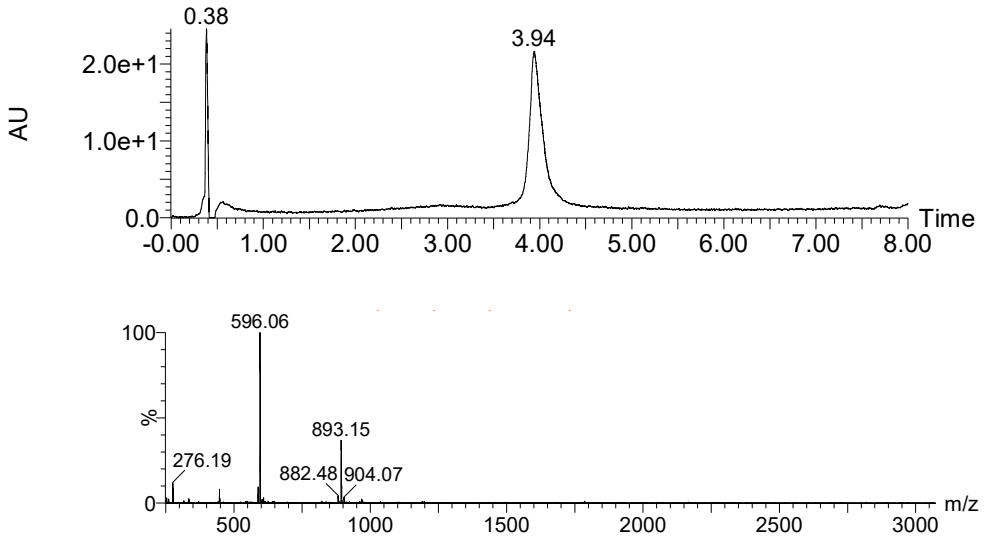
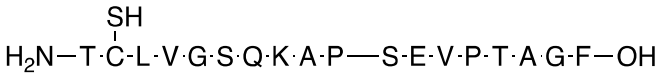


Fig. S11 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester. Gradient: 20-40% ACN containing 0.1% TFA/ H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{83}H_{125}N_{21}O_{19}S_2$ $[M+2H]^{2+}$ *m/z:* 893.58; found: 893.15; $[M+3H]^{3+}$ *m/z:* 596.05; found: 596.06.

3 Synthesis of model N-terminal peptides

3.1 *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a)



H_2N-TCLVGSQKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

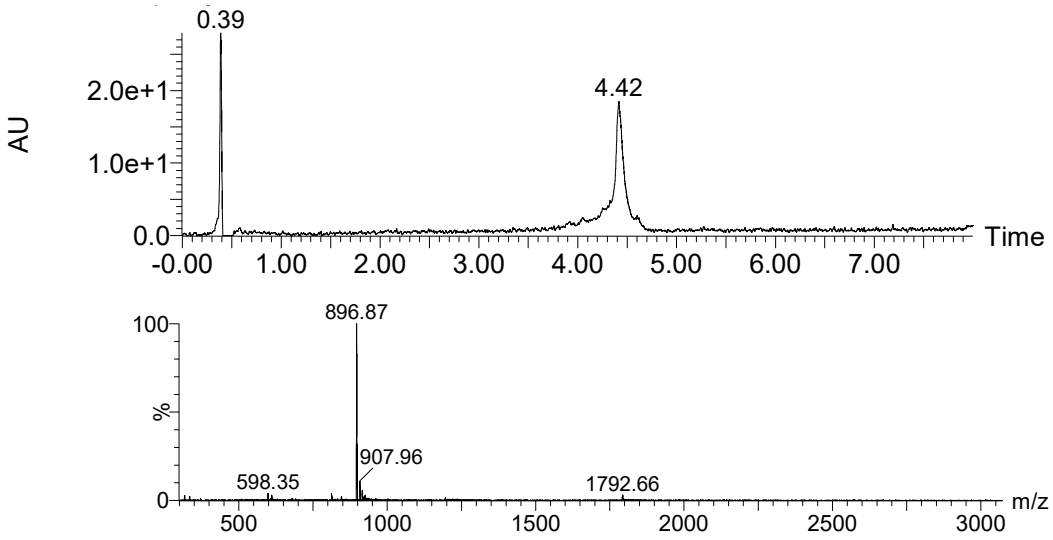
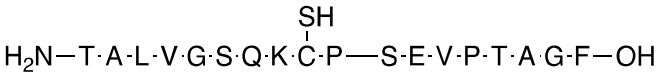


Fig. S12 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{78}H_{126}N_{20}O_{26}S$ $[M+H]^+$ *m/z:* 1793.03; found: 1792.66; $[M+2H]^{2+}$ *m/z:* 897.02; found: 896.87.

3.2 *H_2N*-TALVGSQKCPSEVPTAGF-*COOH* (2b)



H_2N-TALVGSQKCPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

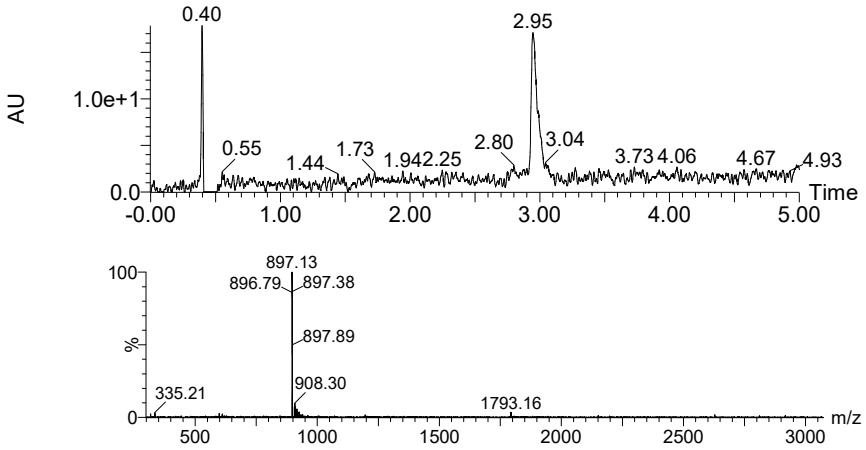
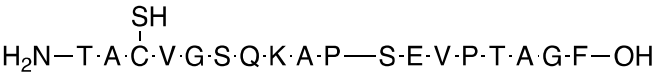


Fig. S13 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALVGSQKCPSEVPTAGF-*COOH*. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow

rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{78}H_{126}N_{20}O_{26}S$ $[M+H]^+$ *m/z:* 1793.03; found: 1793.16; $[M+2H]^{2+}$ *m/z:* 897.02; found: 897.13.

3.3 *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* (2c)



H₂N-TACVGSQKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

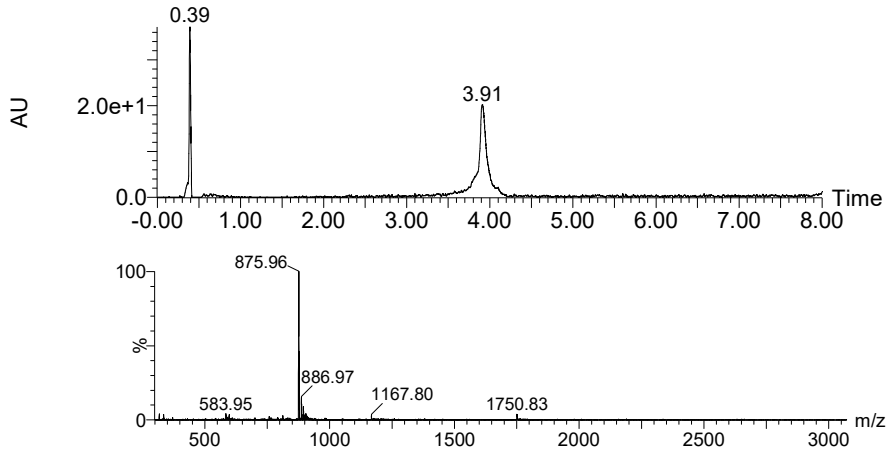
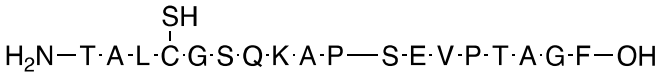


Fig. S14 Analytical HPLC analysis UV trace and ESI-MS of peptide *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{75}H_{120}N_{20}O_{26}S$ $[M+H]^+$ *m/z:* 1750.95; found: 1750.83; $[M+2H]^{2+}$ *m/z:* 875.98; found: 875.96.

3.4 *H₂N*-TALCGSQKAPSEVPTAGF-*COOH* (2d)



H₂N-TALCGSQKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

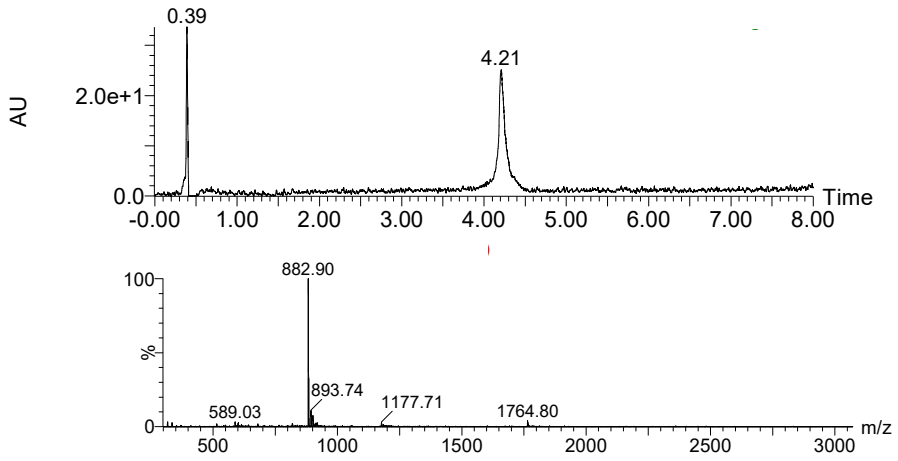
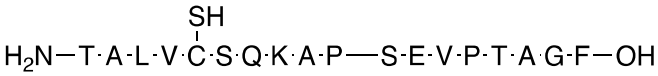


Fig. S15 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALCGSQKAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{76}H_{122}N_{20}O_{26}S$ $[M+H]^+$ *m/z:* 1764.97; found: 1764.80; $[M+2H]^{2+}$ *m/z:* 882.99; found: 882.90.

3.5 *H_2N*-TALVCSQKAPSEVPTAGF-*COOH* (2e)



H_2N-TALVCSQKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chlride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

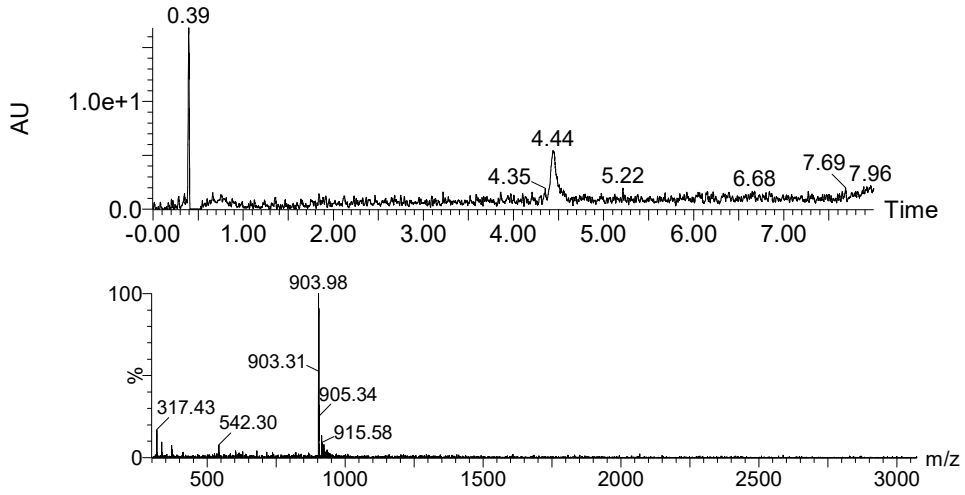
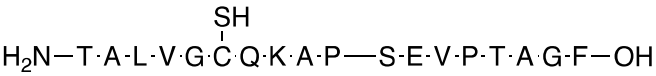


Fig. S16 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALVCSQKAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{79}H_{128}N_{20}O_{26}S$ $[M+2H]^{2+}$ *m/z:* 904.03; found: 903.98.

3.6 *H_2N*-TALVGCQKAPSEVPTAGF-*COOH* (2f)



H_2N-TALVGCQKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

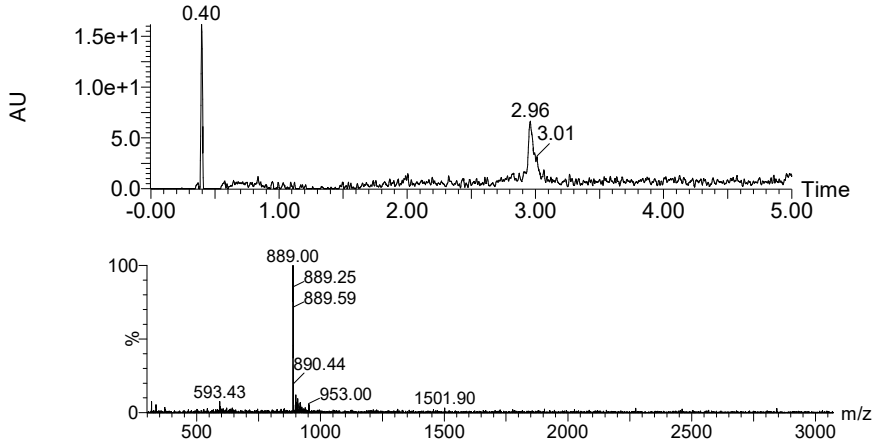
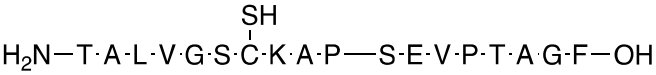


Fig. S17 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALVGCQKAPSEVPTAGF-*COOH*. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{79}H_{128}N_{20}O_{26}S$ $[M+2H]^{2+}$ *m/z:* 889.01; found: 889.00.

3.7 *H_2N*-TALVGSCKAPSEVPTAGF-*COOH* (2g)



H_2N-TALVGSCKAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

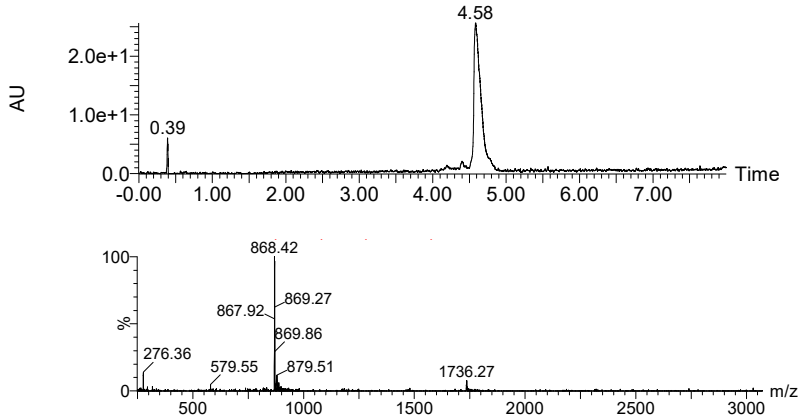
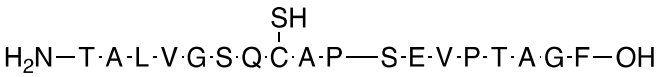


Fig. S18 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALVGSCKAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{76}H_{123}N_{19}O_{25}S$ $[M+H]^+$ *m/z:* 1735.98; found: 1736.27; $[M+2H]^{2+}$ *m/z:* 868.49; found: 868.42.

3.8 *H_2N*-TALVGSQCAPSEVPTAGF-*COOH* (2h)



H_2N-TALVGSQCAPSEVPTAGF-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

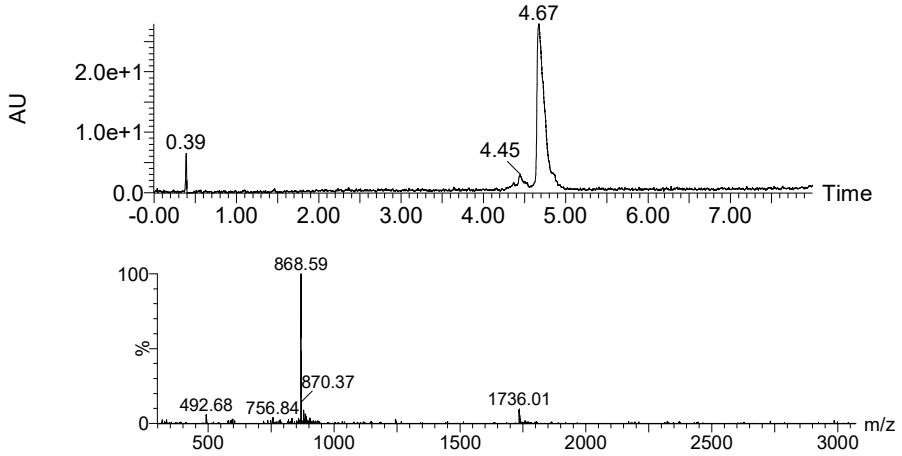
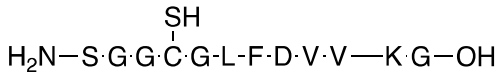


Fig. S19 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TALVGSQCAPSEVPTAGF-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{75}H_{119}N_{19}O_{26}S$ $[M+H]^+$ *m/z:* 1735.93; found: 1736.01; $[M+2H]^{2+}$ *m/z:* 868.47; found: 868.59.

3.9 *H_2N*-SGGCGLFDVVKG-*COOH* (2i)



H_2N-SGGCGLFDVVKG-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

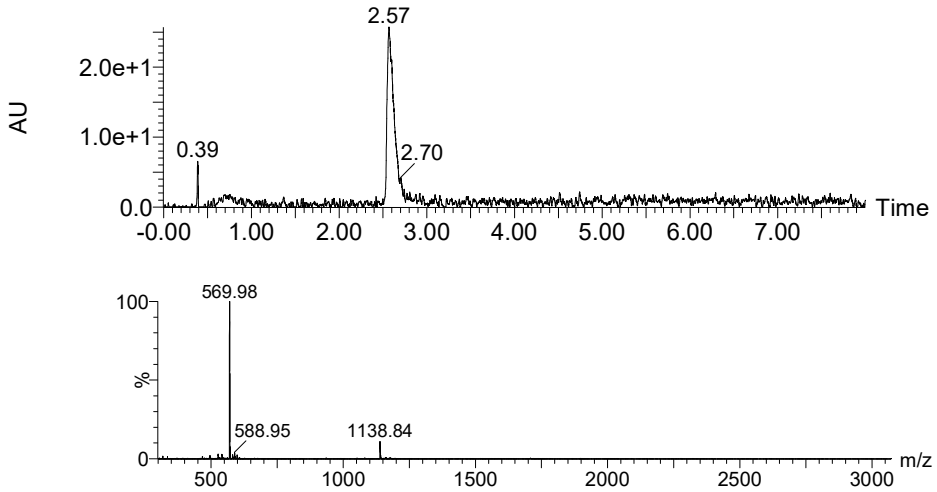
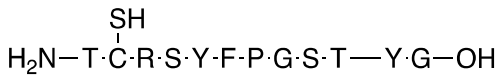


Fig. S20 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-SGGCGLFDVVKG-*COOH*. Gradient: 20-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{49}H_{79}N_{13}O_{16}S$ $[M+H]^+$ *m/z:* 1139.30; found: 1138.84; $[M+2H]^{2+}$ *m/z:* 570.16; found: 569.98.

3.10 *H_2N*-TCRSYFPGSTYG-*COOH* (2j)



H_2N-TCRSYFPGSTYG-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-28% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

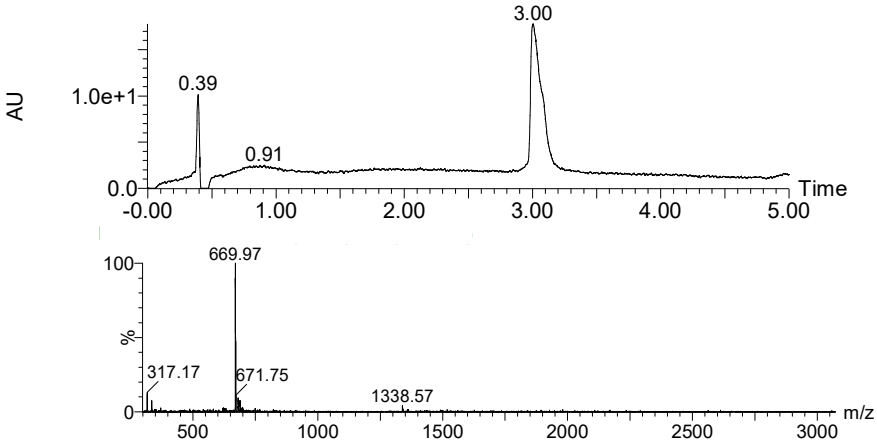
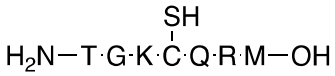


Fig. S21 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TCRSYFPGSTYG-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI$^+$) calcd. for $C_{59}H_{83}N_{15}O_{19}S$ $[M+H]^+$ *m/z:* 1338.58; found: 1338.57; $[M+2H]^{2+}$ *m/z:* 669.79; found: 669.97.

3.11 *H_2N*-TGKCQRM-*COOH* (2k)



H_2N-TGKCQRM-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 5-12% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

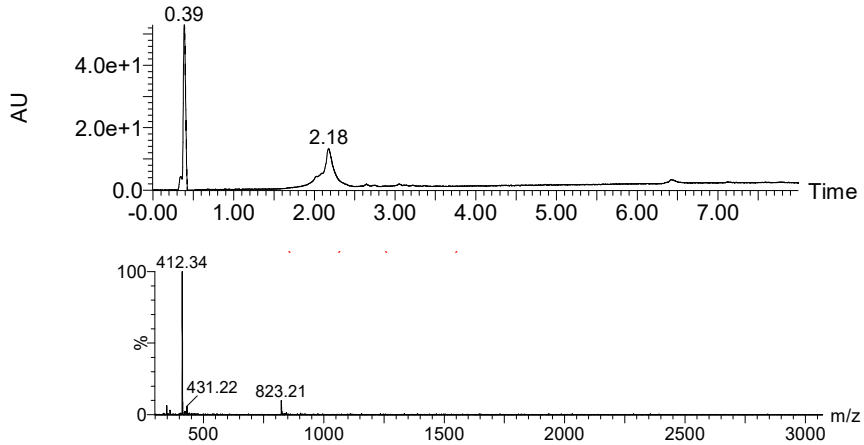
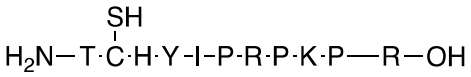


Fig. S22 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TGKCQRM-*COOH*. Gradient: 5-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI$^+$) calcd. for $C_{31}H_{58}N_{12}O_{10}S_2$ $[M+H]^+$ *m/z:* 823.39; found: 823.21; $[M+2H]^{2+}$ *m/z:* 412.20; found: 412.34.

3.12 *H_2N*-TCHYIPRPKPR-*COOH* (2l)



H_2N-TCHYIPRPKPR-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 8-28% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

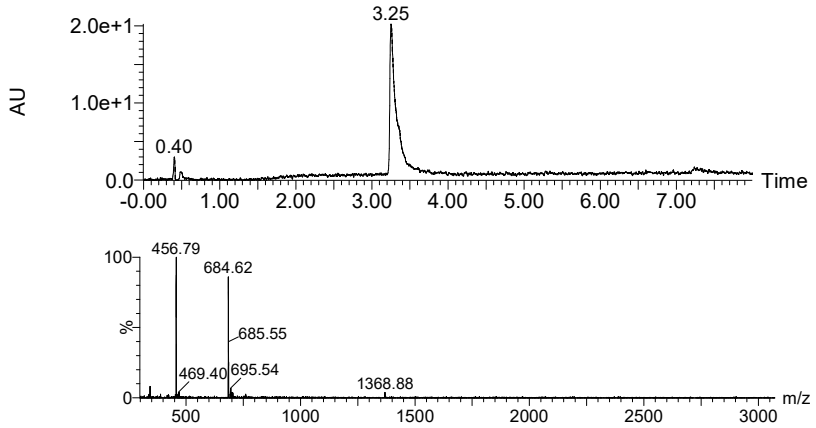
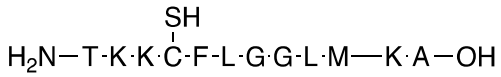


Fig. S23 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TCHYIPRPKPR-*COOH*. Gradient: 5-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{61}H_{98}N_{20}O_{14}S$ $[M+H]^+$ *m/z:* 1368.63; found: 1368.63; $[M+2H]^{2+}$ *m/z:* 684.82; found: 684.62; $[M+3H]^{3+}$ *m/z:* 456.88; found: 456.79.

3.13 *H_2N*-TKKCFLGGLMKA-*COOH* (2m)



H_2N-TKKCFLGGLMKA-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 20-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

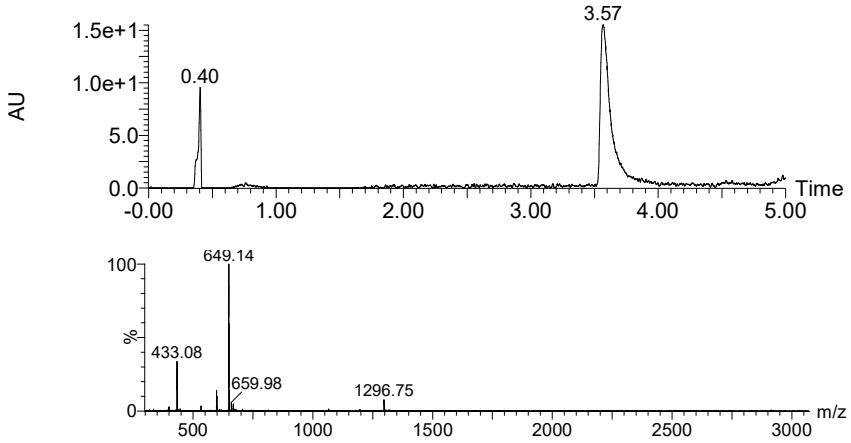
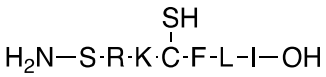


Fig. S24 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-TKKCFLGGLMKA-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{58}H_{101}N_{15}O_{14}S_2$ $[M+H]^+$ *m/z:* 1296.72; found: 1296.75; $[M+2H]^{2+}$ *m/z:* 648.86; found: 649.14; $[M+3H]^{3+}$ *m/z:* 432.91; found: 433.08.

3.14 *H_2N*-SRKCFLI-*COOH* (2n)



H_2N-SRKCFLI-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 12-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

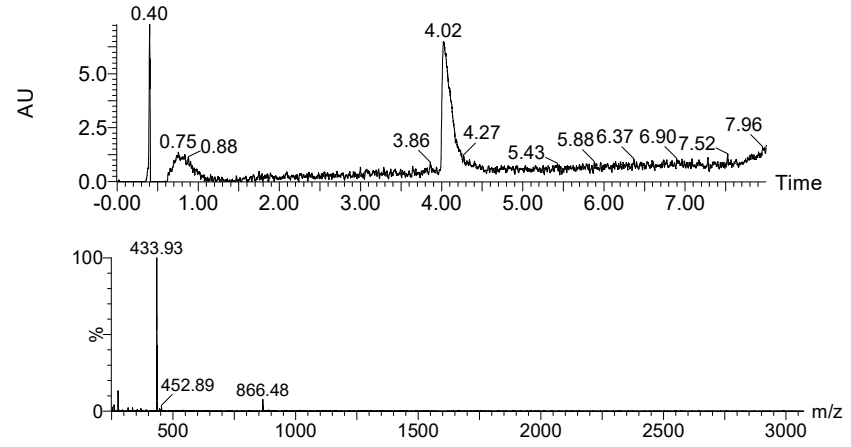
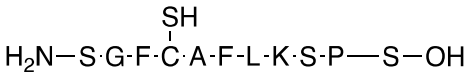


Fig. S25 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-SRKCFLI-*COOH*. Gradient: 15-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{39}H_{67}N_{11}O_9S$ $[M+H]^+$ *m/z:* 866.49; found: 866.48; $[M+2H]^{2+}$ *m/z:* 433.75; found:433.93.

3.15 *H_2N*-SGFCAFLKSPS-*COOH* (2o)



H_2N-SGFCAFLKSPS-*COOH* was synthesized using 2-chlorotrityl chloride resin. The crude product was purified by HPLC using a gradient of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min.

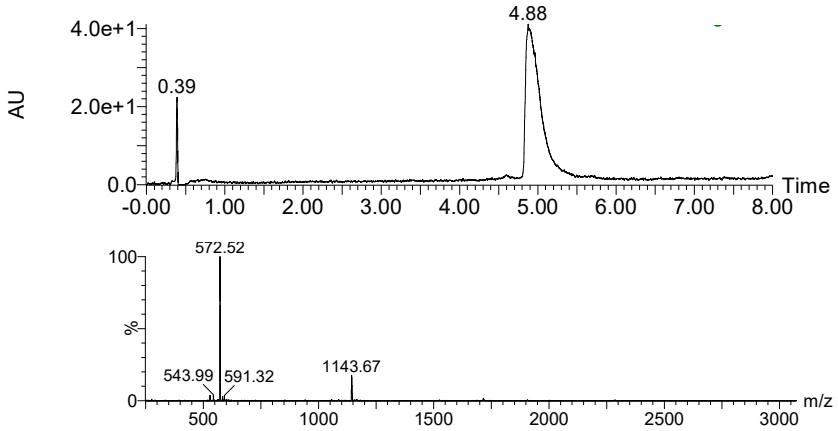


Fig. S26 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-SGFCAFLKSPS-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{52}H_{78}N_{12}O_{15}S$ $[M+H]^+$ *m/z:* 1143.55; found: 1143.67; $[M+2H]^{2+}$ *m/z:* 572.28; found:572.52.

4 Serine/threonine ligation without disulfide bond as the control experiment

4.1 Serine/threonine ligation between Fmoc*NH*-GPARGP-*CO*-SAL ester (1a) and *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a) at a concentration of 0.25 mM

0.50 mg (0.57 μmol, 1 equiv) of Fmoc*NH*-GPARGP-*CO*-SAL ester and 1.64 mg (0.86 μmol, 1.5 equiv) of *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h as the control experiment. After that, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added. After 15 min, TFA was

removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether.

After that, 57 μL of DMF was added to dissolve the solid, and the DMF was added to 2.2 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash.

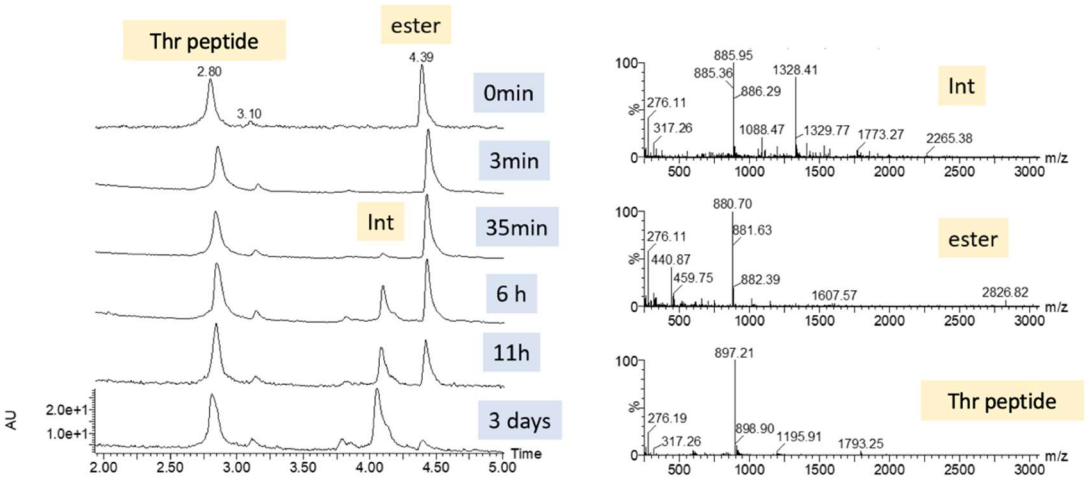


Fig. S27 Ligation progress of Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* at a concentration of 0.25 mM over 0 min, 3 min, 35 min, 6 h, 11 h and 3 days. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate.

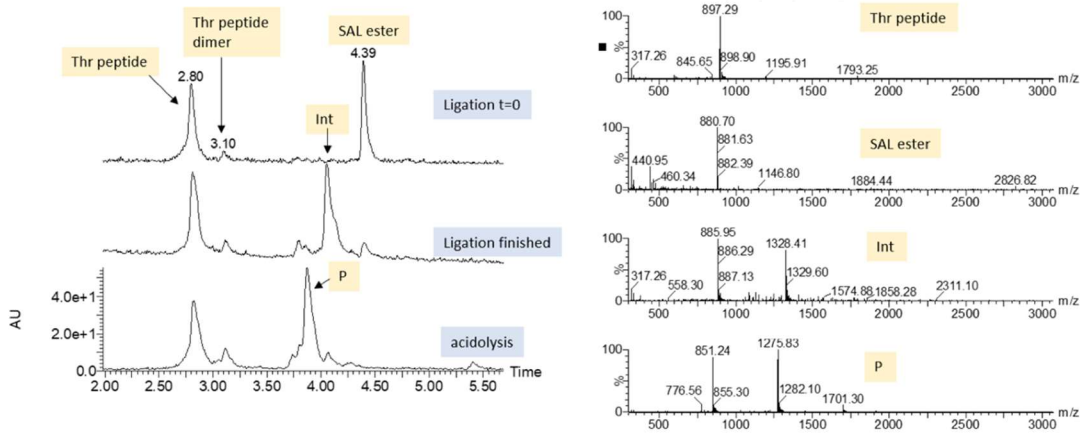


Fig. S28 LC-MS after ligation and acidolysis between Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* at a concentration of 0.25 mM. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate. P: product.

4.2 Serine/threonine ligation between Fmoc*NH*-GPARGP-*CO*-SAL ester (1a) and *H₂N*-TALVGSQKAPSEVPTAGF-*COOH* (2b) at a concentration of 0.25 mM

0.50 mg (0.57 μmol, 1 equiv) of Fmoc*NH*-GPARGP-*CO*-SAL ester and 1.64 mg (0.86 μmol, 1.5 equiv) of *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h as the control experiment. After that, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether.

After that, 57 μL of DMF was added to dissolve the solid, and the DMF was added to 2.2 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash.

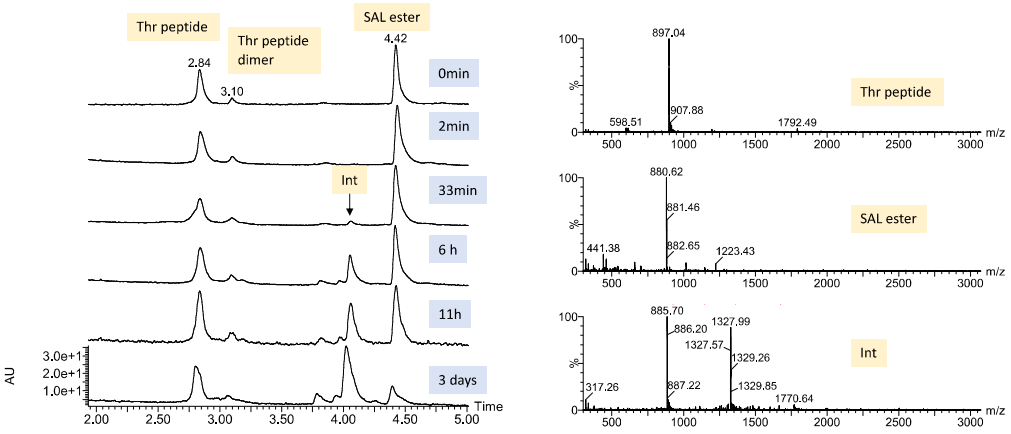


Fig. S29 Ligation progress of Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* at a concentration of 0.25 mM over 0 min, 2 min, 33 min, 6 h, 11 h and 3 days. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a

flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TALVGSQKCPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate.

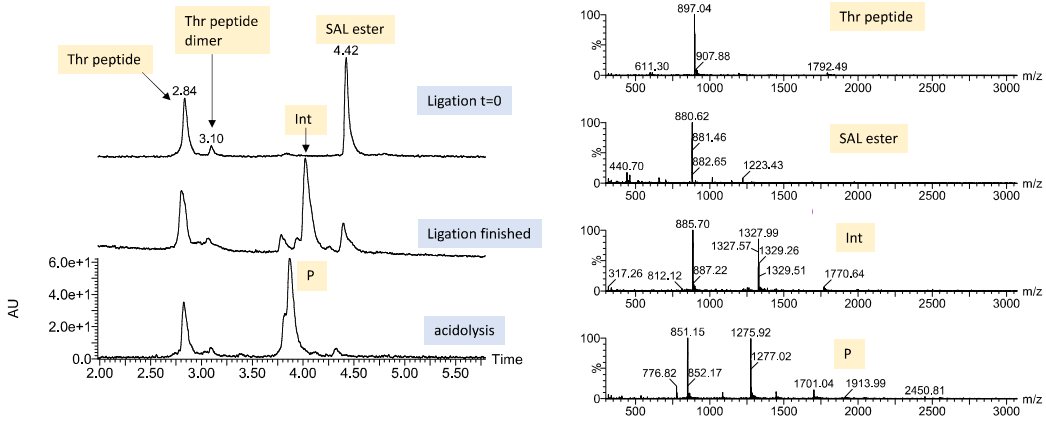


Fig. S30 LC-MS after ligation and acidolysis between Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* at a concentration of 0.25 mM. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TALVGSQKCPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate. P: product.

4.3 Serine/threonine ligation between Fmoc*NH*-GPARGP-*CO*-SAL ester (1a) and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a) at a concentration of 10 mM

0.50 mg (0.57 μmol, 1equiv) of Fmoc*NH*-GPARGP-*CO*-SAL ester and 1.64 mg (0.86 μmol, 1.5 equiv) of *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h as the control experiment. After that, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether.

After that, 57 μL of pyridine/AcOH (1:1, *v*/*v*) was added to dissolve the solid with a final concentration of 10 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash.

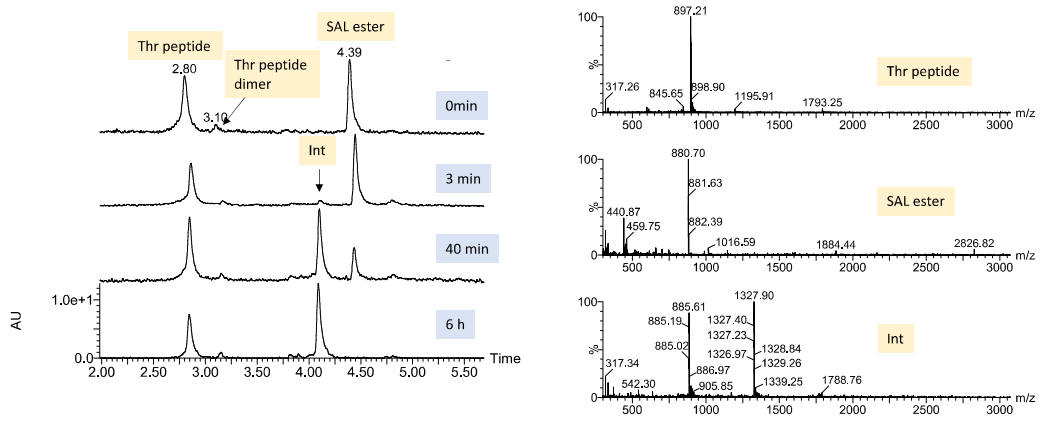


Fig. S31 Ligation progress of Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* at a concentration of 10 mM over 0 min, 3 min, 40 min and 6 h. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate.

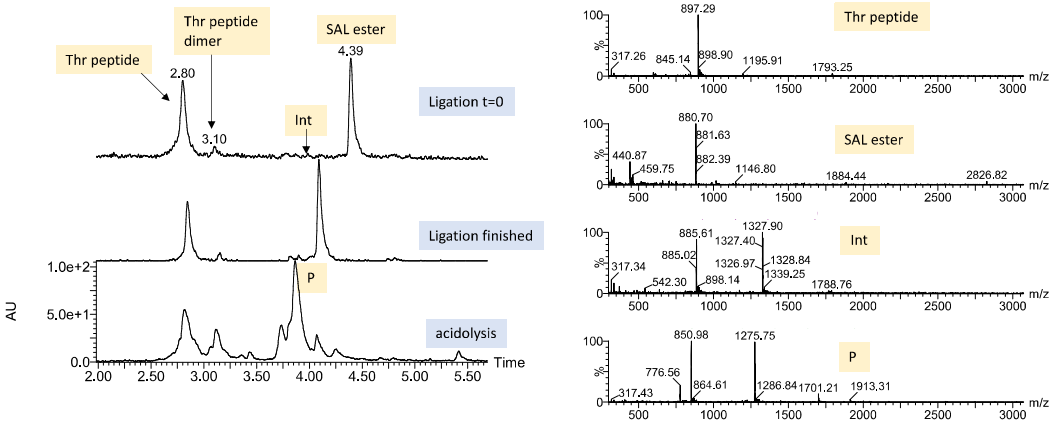


Fig. S32 LC-MS after ligation and acidolysis between Fmoc*NH*-GPARGP-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* at a concentration of 10 mM. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate. P: product.

4.4 Serine/threonine ligation between Fmoc*NH*-GPARGP-*CO*-SAL ester (1a) and *H₂N*-TALVGSQKAPSEVPTAGF-*COOH* (2b) at a concentration of 10 mM

0.50 mg (0.57 μmol, 1 equiv) of Fmoc*NH*-GPARGP-*CO*-SAL ester and 1.64 mg (0.86 μmol, 1.5 equiv) of *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a

concentration of 10 mM (concerning the limiting SAL ester) for 4 h as the control experiment. After that, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether.

After that, 57 µL of pyridine/AcOH (1:1, *v*/*v*) was added to dissolve the solid with a final concentration of 10 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash.

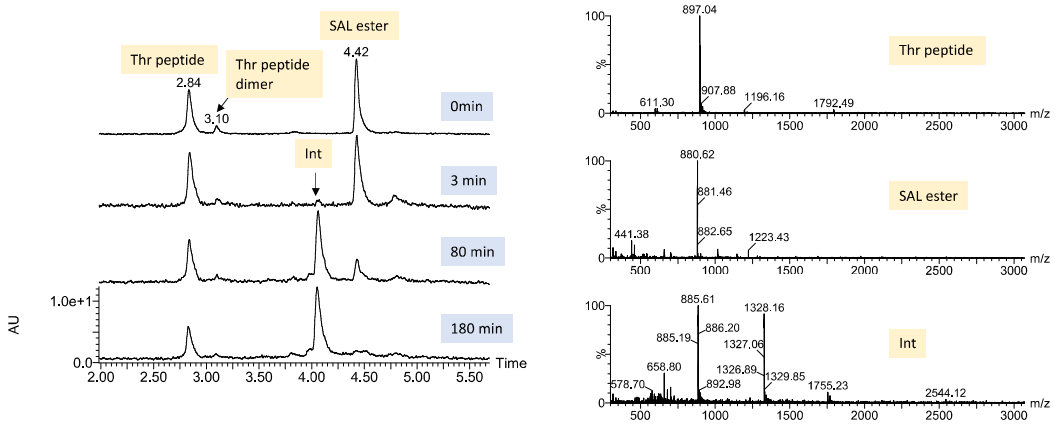


Fig. S33 Ligation progress of Fmoc*NH*-GPARGP-*CO*-SAL ester and H_2N-TALVGSQKCPSEVPTAGF-*COOH* at a concentration of 10 mM over 0 min, 3 min, 80 min, and 180 min. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: H_2N-TALVGSQKCPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate.

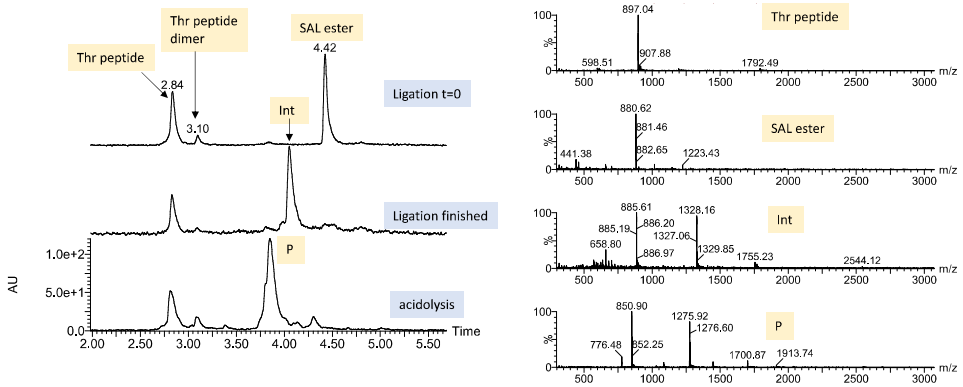


Fig. S34 LC-MS after ligation and acidolysis between Fmoc*NH*-GPARGP-*CO*-SAL ester and H_2N-TALVGSQKCPSEVPTAGF-*COOH* at 10 mM. Gradient: 10-90% ACN containing 0.1% TFA/H_2O

containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H_2N*-TALVGSQKCPSEVPTAGF-*COOH*. Ester: Fmoc*NH*-GPARGP-*CO*-SAL ester. Int: ligation intermediate. P: product.

5 Comparison of the rate of Prior disulfide bond-mediated Ser/Thr ligation with different numbers of amino acids between two designated cysteine residues

5.1 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester (1c) and *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a) (Table 2, Entry 2)

0.45 mg (0.44 μmol, 1 equiv) of Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and 1.18 mg (0.66 μmol, 1.5 equiv) of *H_2N*-TCLVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 40 μL of DMF was added to dissolve the solid, and the DMF was added to 1.7 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

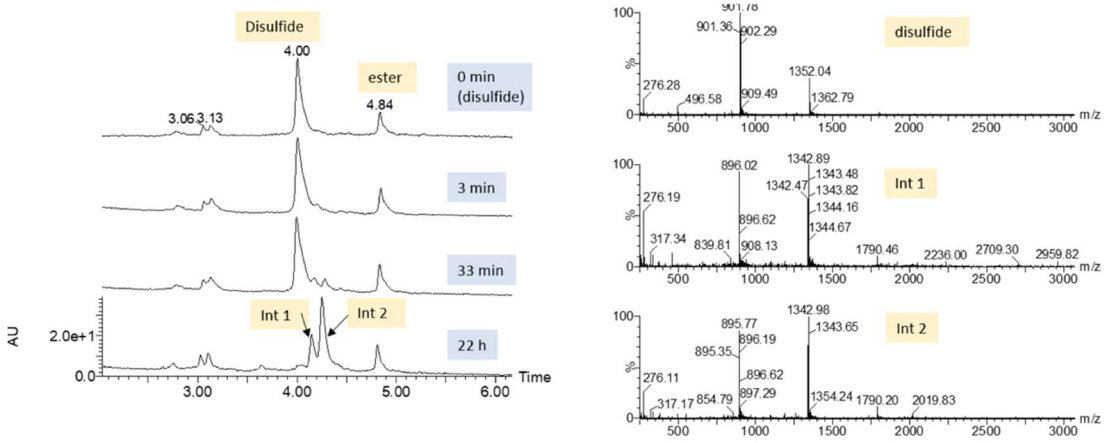


Fig. S35 Disulfide-bridged ligation progress of Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, 33 min, and 22 h. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. The appearance of the two intermediate peaks may be due to the two different conformers with the rigid proline at the ligation site. Int 1: ligation intermediate 1. Int 2: ligation intermediate 2. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Ester: Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester.

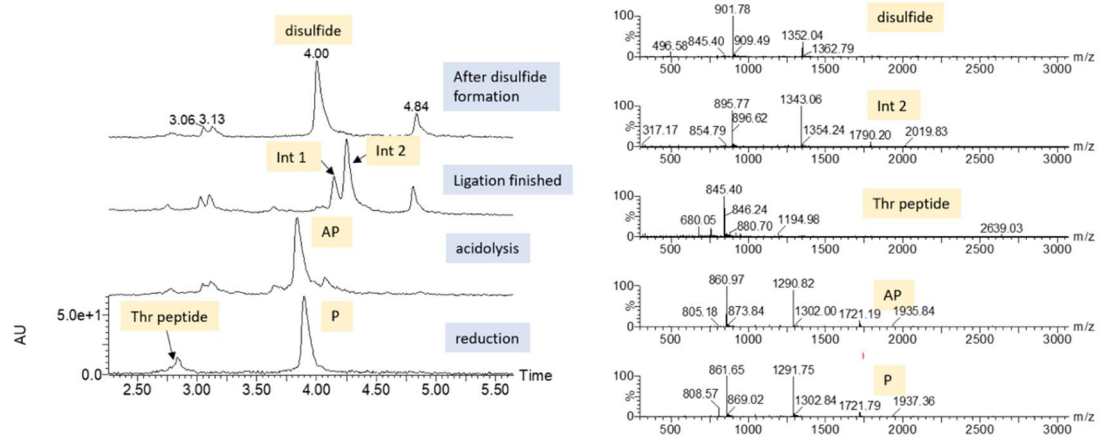


Fig. S36 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int 1: ligation intermediate 1. Int 2: ligation intermediate 2. AP: acidolysis product. Thr Peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. P: product.

5.2 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester (1c) and *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* (2c) (Table 2, Entry 3)

0.45 mg (0.44 μmol, 1 equiv) of Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and 1.16 mg (0.66 μmol, 1.5 equiv) of *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (Spyr).

After that, 44 μL of DMF was added to dissolve the solid, and the DMF was added to 1.7 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 2 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

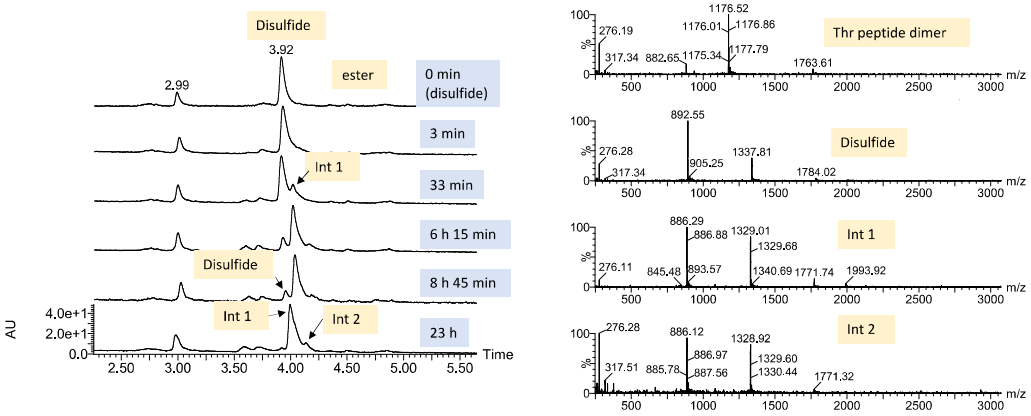


Fig. S37 Disulfide-bridged ligation progress of Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, 33 mins, 6 h 15 min, 8 h 45 min, and 23 h. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. The appearance of the two intermediate peaks may be due to the two different conformers with the rigid proline at the ligation site. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Ester: Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester. Int 1: ligation intermediate 1. Int 2: ligation intermediate 2.

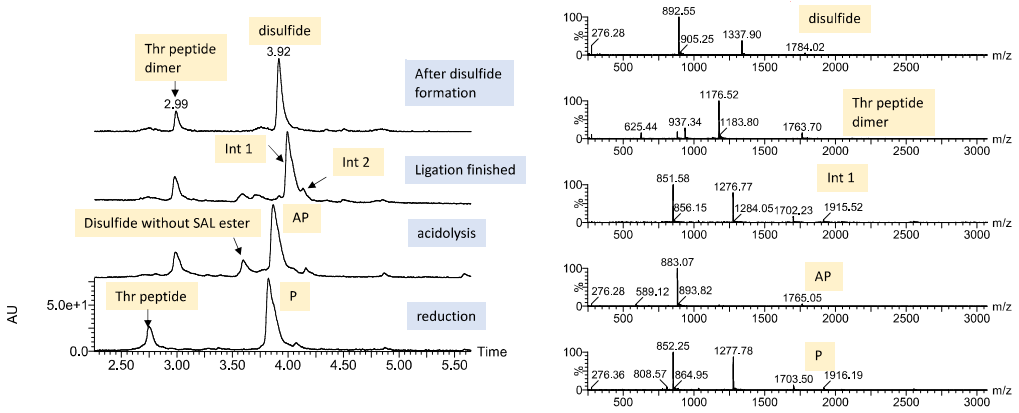


Fig. S38 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPRGC(SSPyr)P-*CO*-SAL ester and *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Disulfide without SAL ester: N-terminal Thr peptide and hydrolysis of peptide SAL ester linked with disulfide. Int 1: ligation intermediate 1. Int 2: ligation intermediate 2. AP: acidolysis product. Thr Peptide: *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. P: product.

5.3 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester (1d) and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a) (Table 2, Entry 4)

0.39 mg (0.38 μmol) of Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester and 1.04 mg (0.57 μmol) of *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 105 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (Spyr).

After that, 37 μL of DMF was added to dissolve the solid, and the DMF was added to 1.5 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

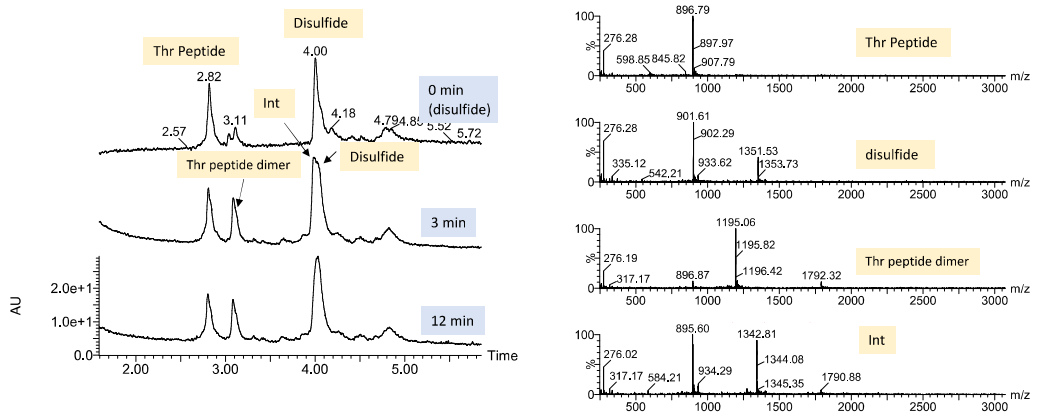


Fig. S39 Disulfide-bridged ligation progress of Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester and *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, and 12 min. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

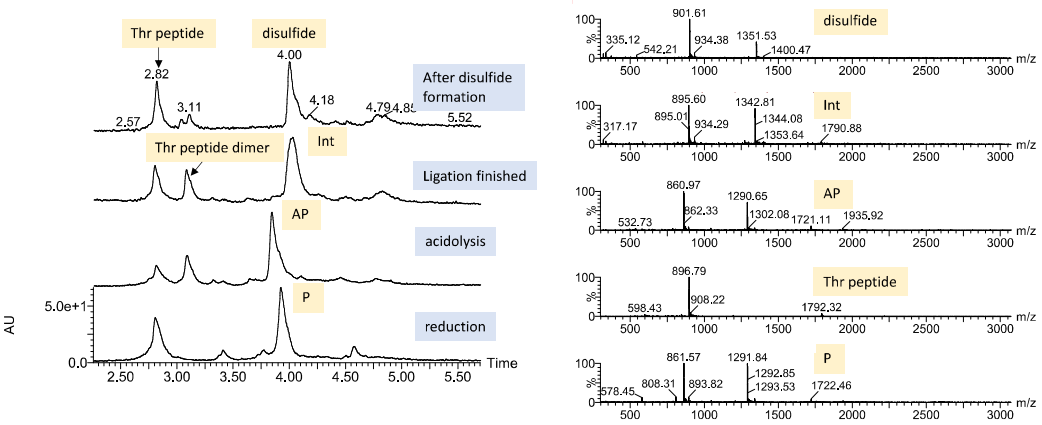


Fig. S40 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPRC(SSPyr)GP-*CO*-SAL ester and *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. Thr Peptide: *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH*. P: product.

5.4 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* (2a) (Table 2, Entry 5)

0.39 mg (0.38 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 1.04 mg (0.57 μmol, 1.5 equiv) of *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 210 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (Spyr).

After that, 37 μL of DMF was added to dissolve the solid, and the DMF was added to 1.5 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

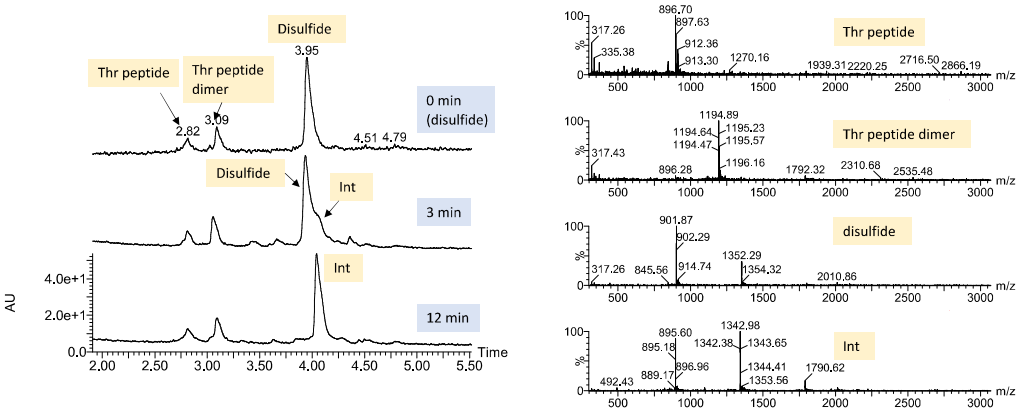


Fig. S41 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, and 12 min. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TCLVGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

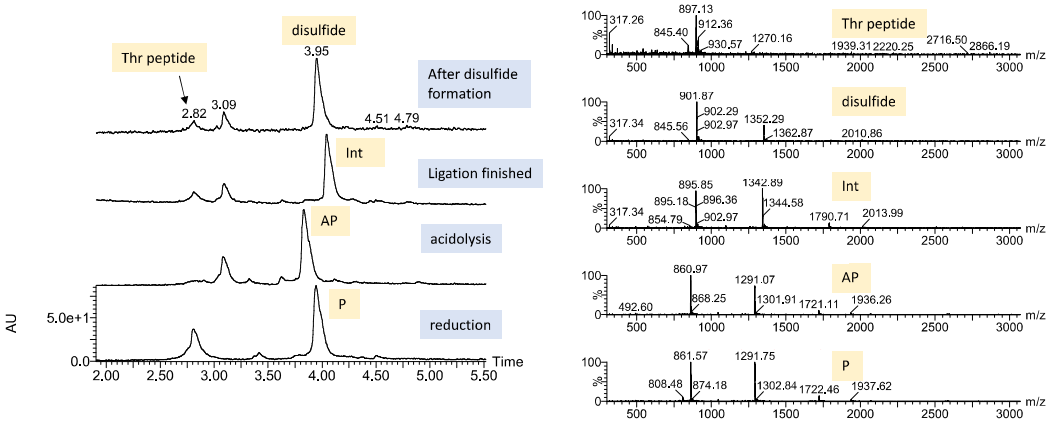


Fig. S42 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H$_2$O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TCLVGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.5 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H*$_2$*N*-TACVGSQKAPSEVPTAGF-*COOH* (2c) (Table 2, Entry 6)

0.35 mg (0.34 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 0.97 mg (0.55 μmol, 1.5 equiv) of *H*$_2$*N*-TACVGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 4 h and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 34 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

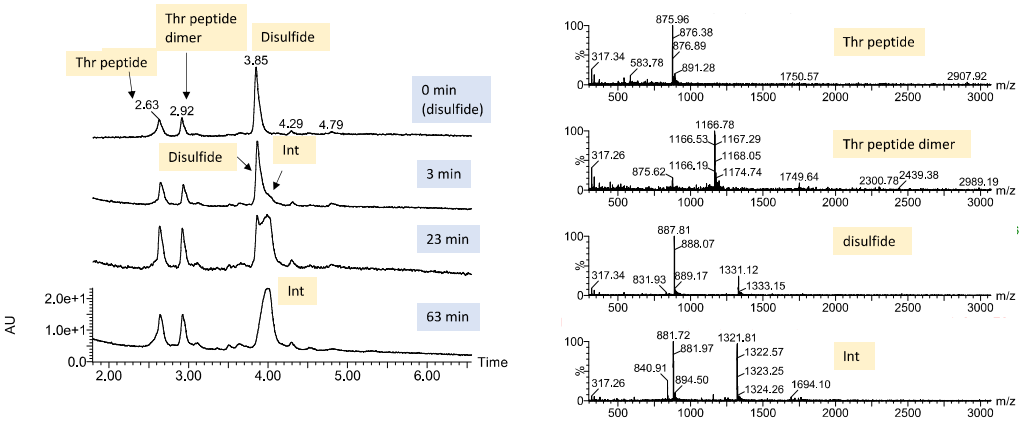


Fig. S43 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TACVGSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, 23 min, and 63 min. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

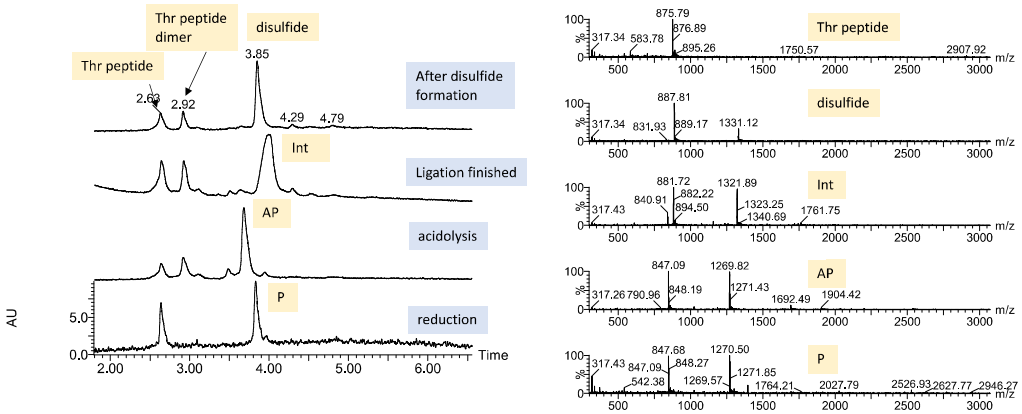


Fig. S44 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TACVGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.6 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H₂N*-TALCGSQKAPSEVPTAGF-*COOH* (2d) (Table 2, Entry 7)

0.35 mg (0.34 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 0.98 mg (0.55 μmol, 1.5 equiv) of *H₂N*-TALCGSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 60 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 34 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

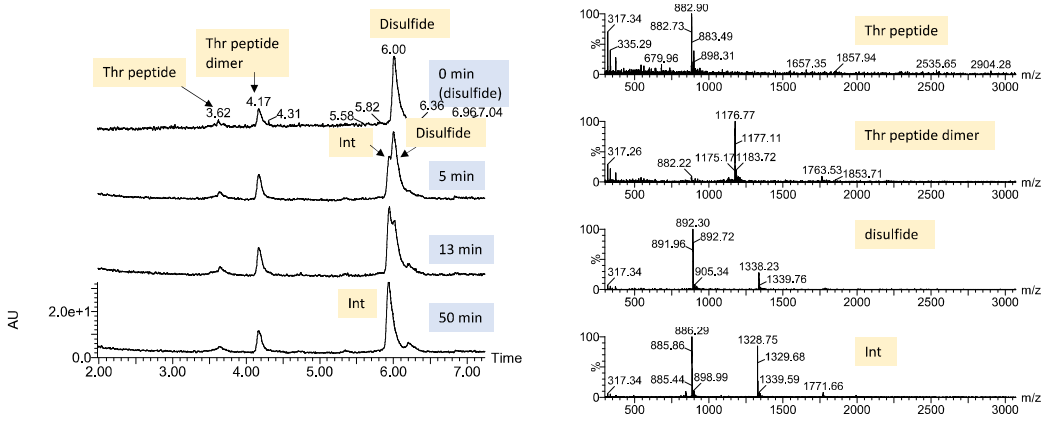


Fig. S45 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALCGSQKAPSEVPTAGF-*COOH* over 0 min, 5 min, 13 min, and 50 min. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALCGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

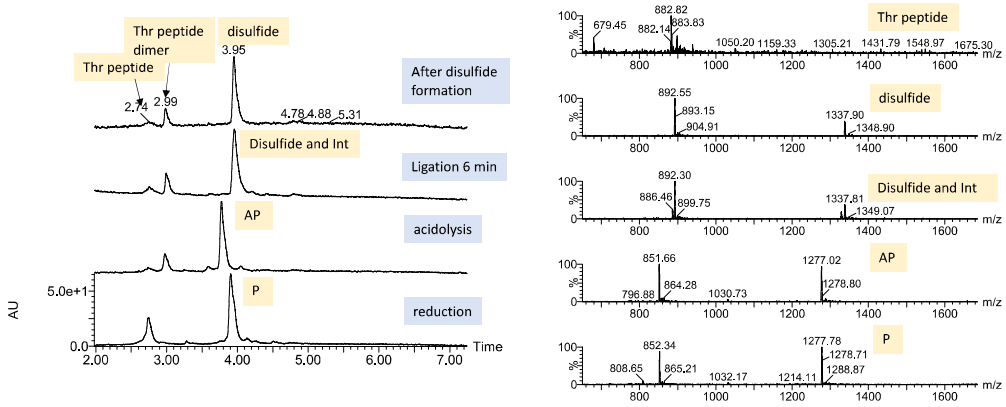


Fig. S46 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TALCGSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TALCGSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.7 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH* (2e) (Table 2, Entry 8)

0.35 mg (0.34 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 1.00 mg (0.55 μmol, 1.5 equiv) of *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 45 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 34 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

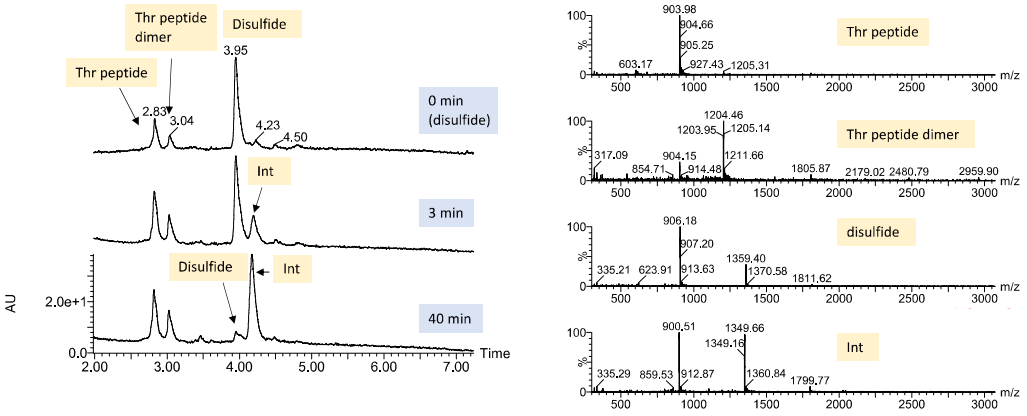


Fig. S47 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH* over 0 min, 3 min, and 40 min. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

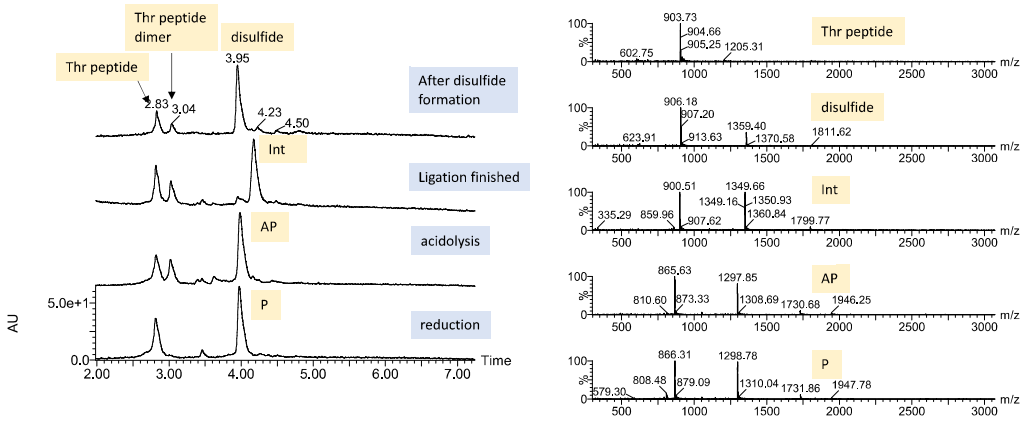


Fig. S48 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TALVCSQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.8 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H₂N*-TALVGCQKAPSEVPTAGF-*COOH* (2f) (Table 2, Entry 9)

0.36 mg (0.35 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 1.00 mg (0.56 μmol, 1.5 equiv) of *H₂N*-TALVGCQKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 60 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 35 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

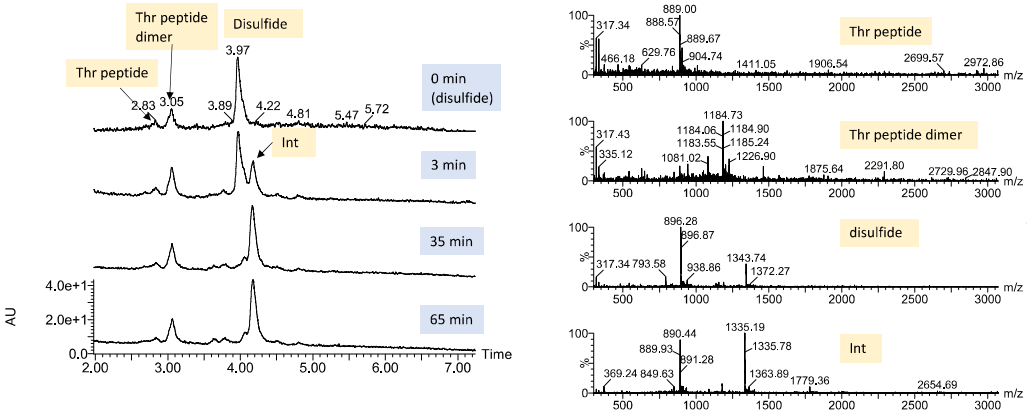


Fig. S49 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALVGCQKAPSEVPTAGF-*COOH* over 0 min, 5 min, 35 min, and 65 min. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALVGCQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

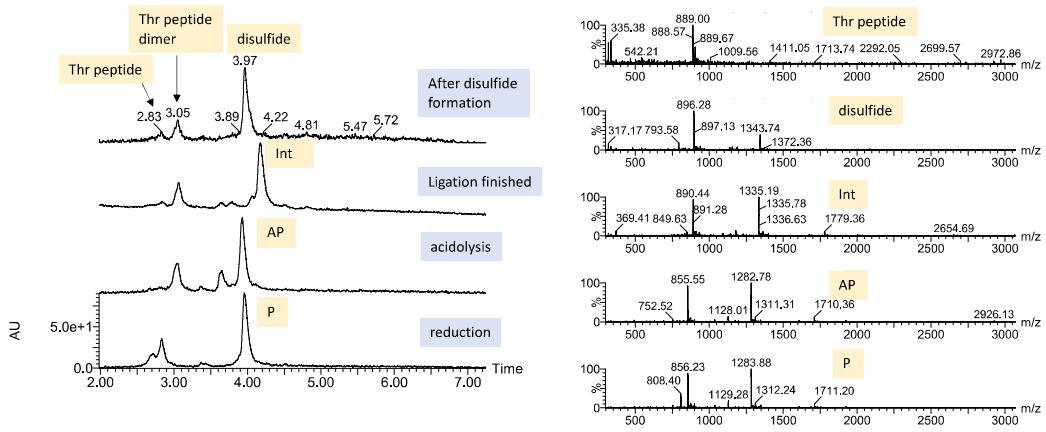


Fig. S50 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and H_2N-TALVGCQKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: H_2N-TALVGCQKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.9 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and H_2N-TALVGSCKAPSEVPTAGF-*COOH* (2g) (Table 2, Entry 10)

0.36 mg (0.35 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 0.97 mg (0.56 μmol, 1.5 equiv) of H_2N-TALVGSCKAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 60 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 35 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

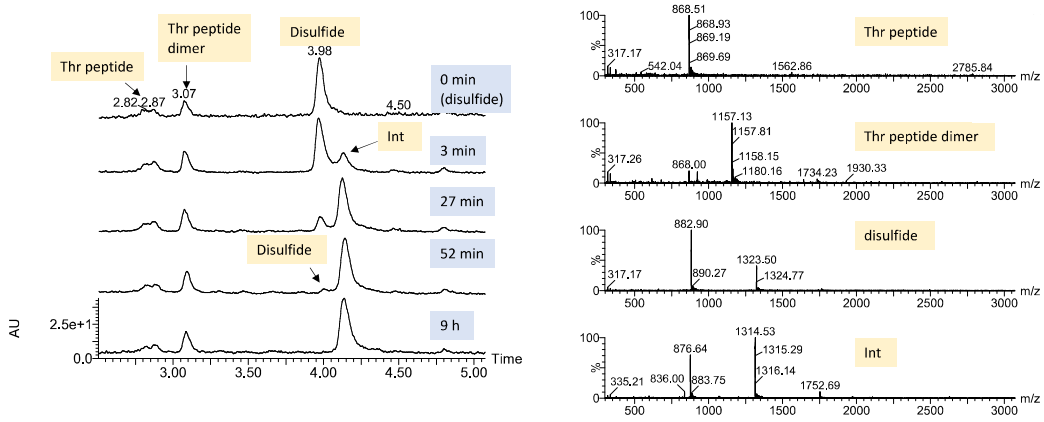


Fig. S51 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALVGSCKAPSEVPTAGF-*COOH* over 0 min, 3 min, 27 min, 52 min and 9 h. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALVGSCKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

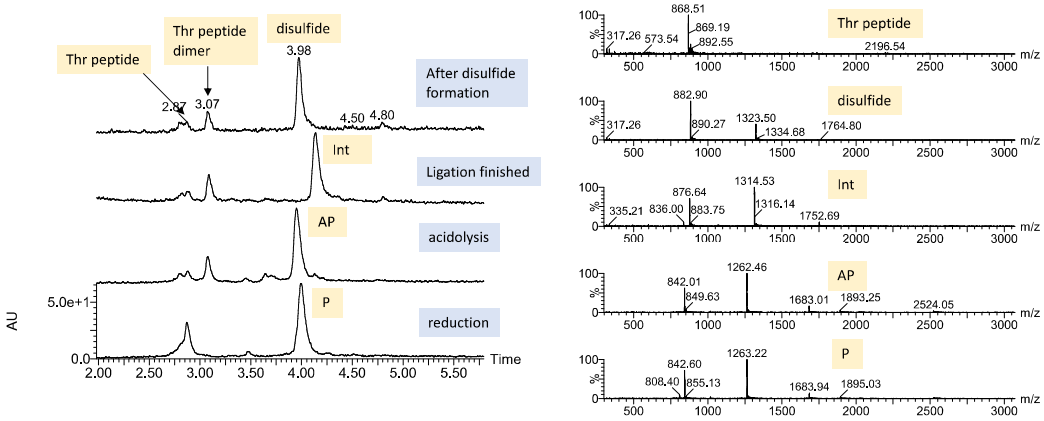


Fig. S52 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALVGSCKAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALVGSCKAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.10 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H₂N*-TALVGSQCAPSEVPTAGF-*COOH* (2h) (Table 2, Entry 11)

0.36 mg (0.35 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 0.97 mg (0.56 μmol, 1.5 equiv) of *H₂N*-TALVGSQCAPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 90 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 35 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a stream of compressed nitrogen gas, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

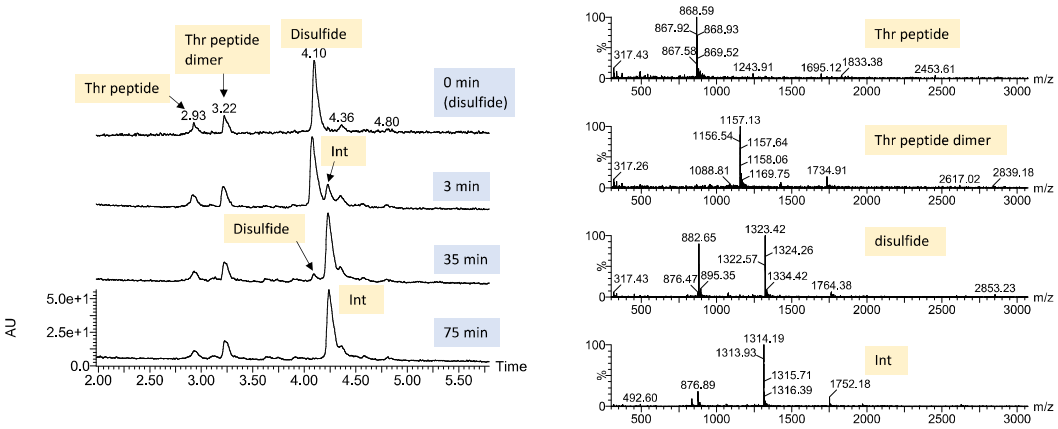


Fig. S53 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALVGSQCAPSEVPTAGF-*COOH* over 0 min, 3 min, 35 min, and 75 min. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALVGSQCAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

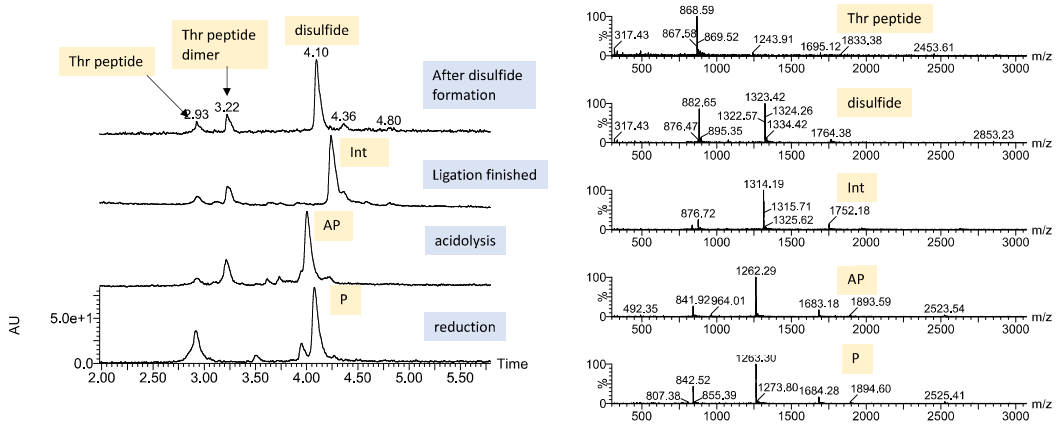


Fig. S54 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H₂N*-TALVGSQCAPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TALVGSQCAPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

5.11 Disulfide-bridged serine/threonine ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester (1b) and *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* (2b) (Table 2, Entry 12)

0.36 mg (0.35 μmol, 1 equiv) of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and 1.01 mg (0.56 μmol, 1.5 equiv) of *H₂N*-TALVGSQKCPSEVPTAGF-*COOH* were performed in 0.1% TFA in MeOH with a concentration of 10 mM (concerning the limiting SAL ester) for 90 min and monitor by LC-MS. After the reaction was finished, MeOH was blown off under a stream of nitrogen. Then, neat TFA was added to remove the protecting group installed on the SAL ester during disulfide bond formation. After 15 min, TFA was removed by blowing off under a stream of nitrogen. The peptide was then washed twice with diethyl ether to remove the 2-thiopyridone (SPyr).

After that, 35 μL of DMF was added to dissolve the solid, and the DMF was added to 1.3 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine and acetic acid were removed by a rotary evaporator. Then, acidolysis was carried out using 1.5 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP.

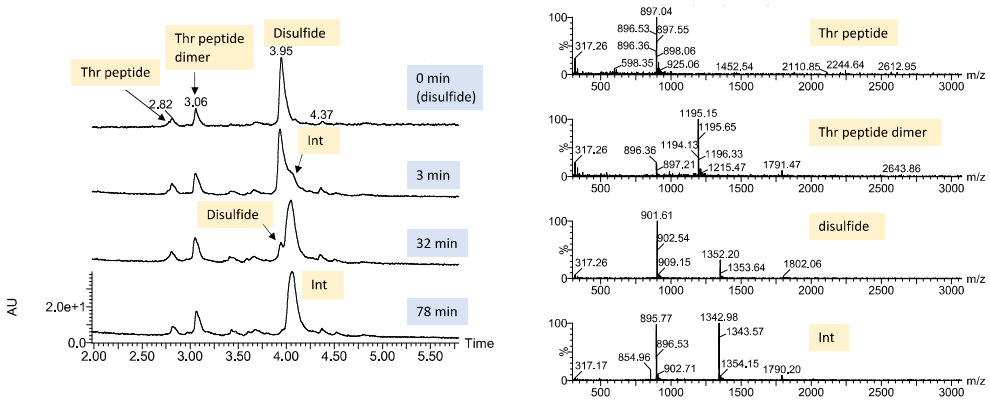


Fig. S55 Disulfide-bridged ligation progress of Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TALVGSQKCPSEVPTAGF-*COOH* over 0 min, 3 min, 32 min, and 78 min. Gradient: 10-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TALVGSQKCPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

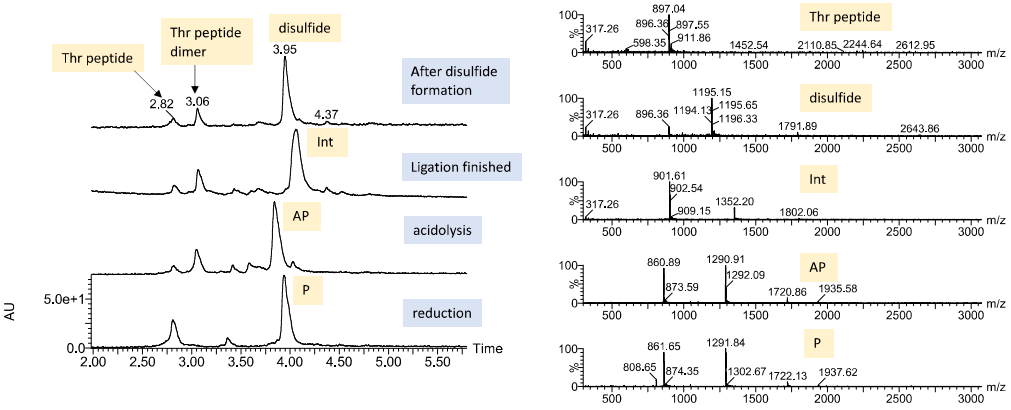


Fig. S56 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between Fmoc*NH*-GPC(SSPyr)RGP-*CO*-SAL ester and *H*$_2$*N*-TALVGSQKCPSEVPTAGF-*COOH*. Gradient: 10-90% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H*$_2$*N*-TALVGSQKCPSEVPTAGF-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

6 Prior disulfide bond-mediated Ser/Thr ligation using different C-terminal amino acids

6.1 Disulfide-bridged serine/threonine ligation between *H₂N*-LEQLKC(SSPyr)GF-*CO*-SAL ester (1e) and *H₂N*-SGGCGLFDVVKG-*COOH* (2i) (Table 3, Entry 1)

Synthesis of *H₂N*-LEQLKCGF SGGCGLFDVVKG-*COOH* followed the **General Procedure 1.2.4**. Briefly, 5.19 mg of *H₂N*-LEQLKC(SSPyr)GF-*CO*-SAL ester and 6.7 mg of *H₂N*-SGGCGLFDVVKG-*COOH* were dissolved in 450 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for three times. After air drying, 100 μL of DMF was added to dissolve the solid. The DMF was added to 18 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 20-45% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min and yielded *H₂N*-LEQLKCGF SGGCGLFDVVKG-*COOH* (2.74 mg, 30%).

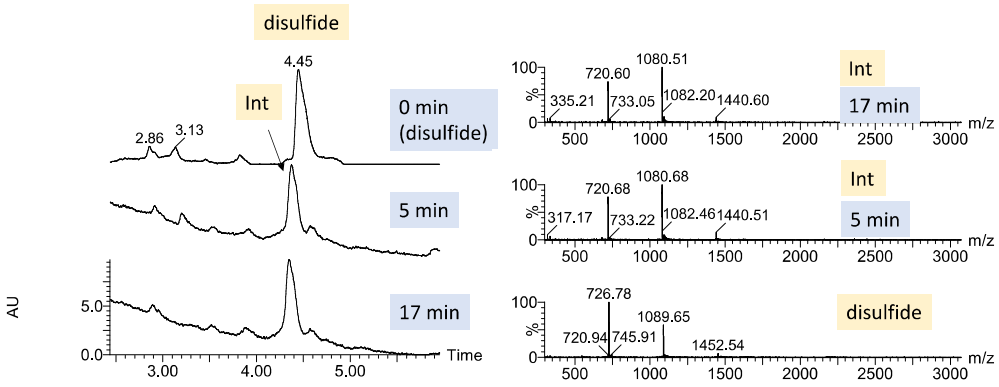


Fig. S57 Disulfide-bridged ligation progress of *H₂N*-LEQLKC(SSPyr)GF-*CO*-SAL ester and *H₂N*-SGGCGLFDVVKG-*COOH* over 0 min, 5 min, and 17 min. Gradient: 20-50% ACN containing 0.1%

TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

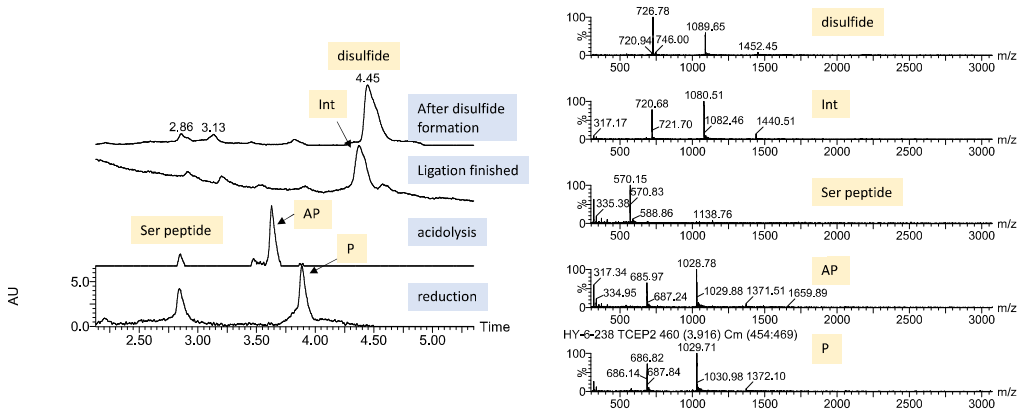


Fig. S58 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H_2N*-LEQLKC(SSPyr)GF-*CO*-SAL ester and *H_2N*-SGGCGLFDVVKG-*COOH*. Gradient: 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. Ser Peptide: *H_2N*-SGGCGLFDVVKG-*COOH*. AP: acidolysis product. P: product.

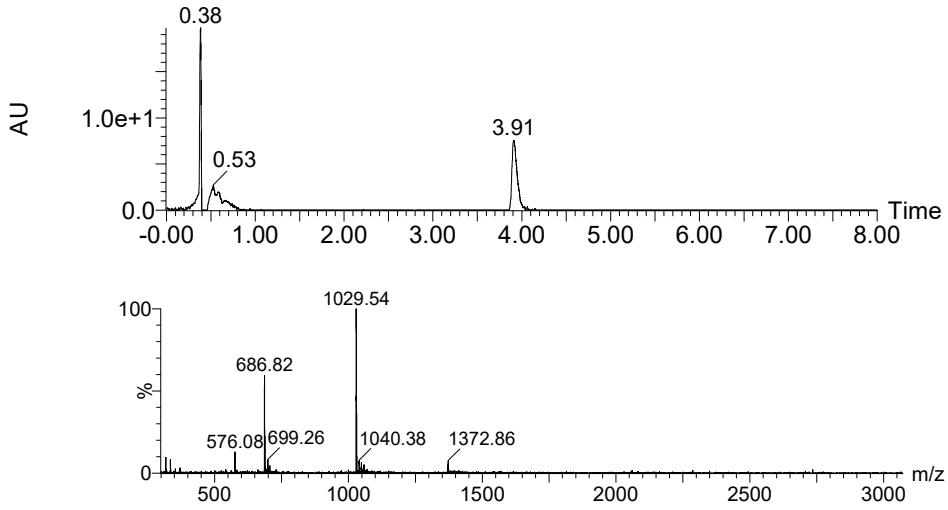


Fig. S59 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-LEQLKCGF SGGCGLFDVVKG-*COOH*. Gradient: 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{91}H_{145}N_{23}O_{27}S_2$ $[M+2H]^{2+}$ *m/z:* 1029.01; found: 1029.54; $[M+3H]^{3+}$ *m/z:* 686.34; found:686.82.

6.2 Disulfide-bridged serine/threonine ligation between *H₂N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester (1f) and *H₂N*-TCRSYFPGSTYG-*COOH* (2j) (Table 3, Entry 2)

Synthesis of *H₂N*-FRKSGFCGT TCRSYFPGSTYG-*COOH* followed the **General Procedure 1.2.4**. Briefly, 5.14 mg of *H₂N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester and 8.48 mg of *H₂N*-TCRSYFPGSTYG-*COOH* were dissolved in 425 mL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for three times. After air drying, 300 mL of DMF was added to dissolve the solid. The DMF was added to 17 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 10-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min and yielded *H₂N*-FRKSGFCGT TCRSYFPGSTYG-*COOH* (4.90 mg, 50%).

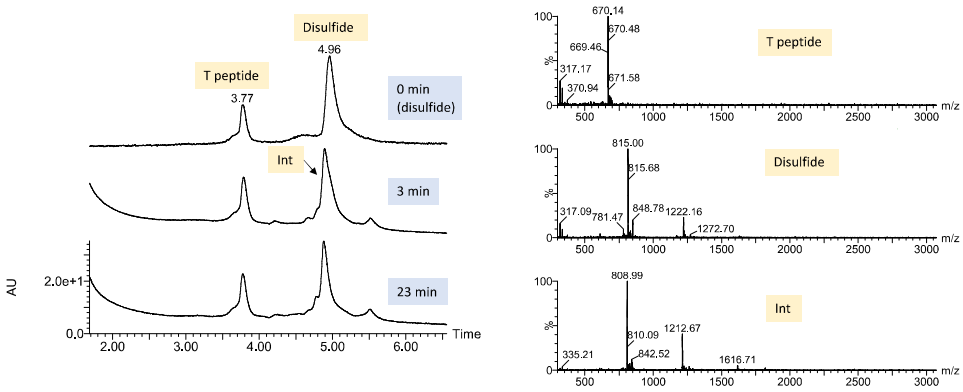


Fig. S60 Disulfide-bridged ligation progress of *H₂N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester and *H₂N*-TCRSYFPGSTYG-*COOH* over 0 min, 3 min, and 23 min. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. T peptide: *H₂N*-TCRSYFPGSTYG-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

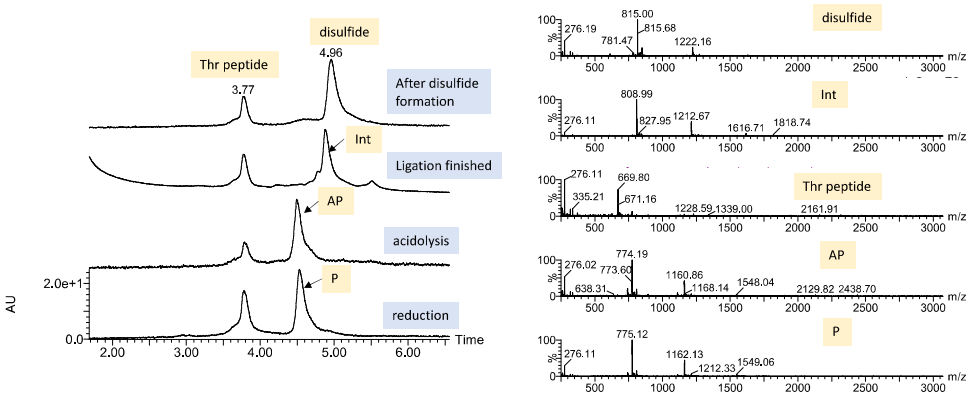


Fig. S61 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H₂N*-FRKSGFC(SSPyr)GT-*CO*-SAL ester and *H₂N*-TCRSYFPGSTYG-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TCRSYFPGSTYG-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

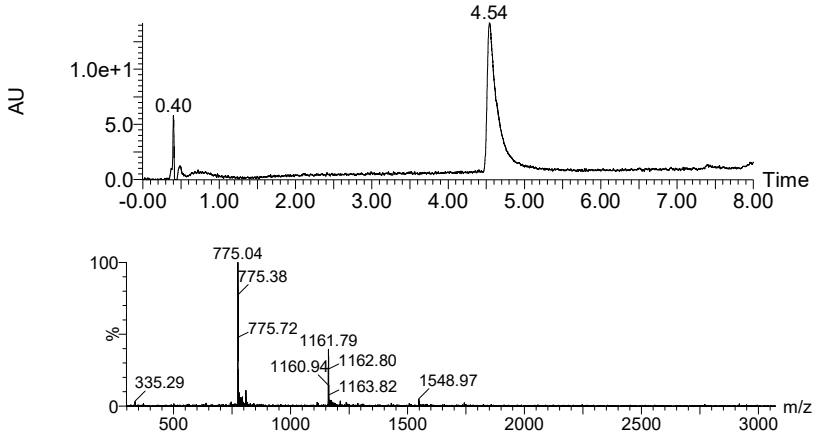


Fig. S62 Analytical HPLC analysis UV trace and ESI-MS of peptide *H₂N*-FRKSGFCGT TCRSYFPGSTYG-*COOH*. Gradient: 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{103}H_{148}N_{28}O_{30}S_2$ $[M+2H]^{2+}$ *m/z:* 1161.52; found: 1162.79; $[M+3H]^{3+}$ *m/z:* 774.69; found:775.04.

6.3 Disulfide-bridged serine/threonine ligation between *H₂N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester (1g) and *H₂N*-TGKCQRM-*COOH* (2k) (Table 3, Entry 3)

Synthesis of *H₂N*-GMTHGLICGI TGKCQRM-*COOH* followed the **General Procedure 1.2.4**. Briefly, 3.03 mg of *H₂N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester and 3.68 mg of *H₂N*-TGKCQRM-*COOH* were dissolved in 250 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation

was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for three times. After air drying, 175 μL of DMF was added to dissolve the solid. The DMF was added to 10 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 15-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min and yielded *H_2N*-GMTHGLICGI TGKCQRM-*COOH* (1.30 mg, 29%).

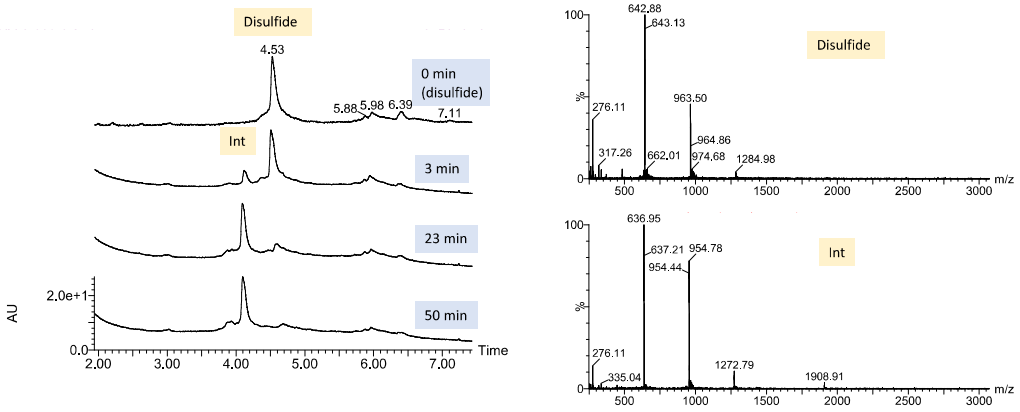


Fig. S63 Disulfide-bridged ligation progress of *H_2N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester and *H_2N*-TGKCQRM-*COOH* over 0 min, 3 min, 23 min, and 50 min. Gradient: 5-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

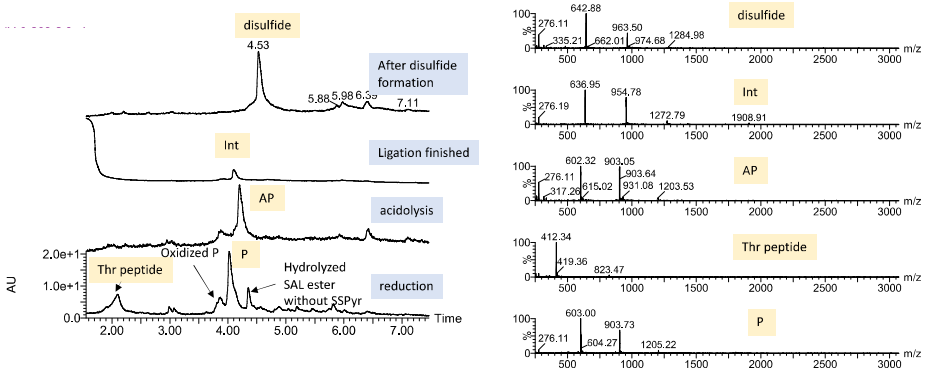


Fig. S64 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H_2N*-GMTHGLIC(SSPyr)GI-*CO*-SAL ester and *H_2N*-TGKCQRM-*COOH*. Gradient:

5-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H_2N*-TGKCQRM-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product. Oxidized P: oxidized product. Hydrolyzed SAL ester without SSPyr: *H_2N*-GMTHGLICGI-*COOH*.

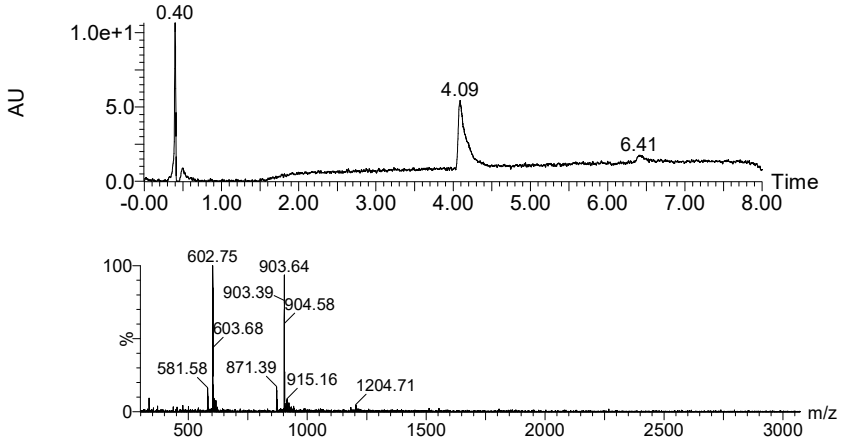


Fig. S65 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-GMTHGLICGI TGKCQRM-*COOH*. Gradient: ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{73}H_{128}N_{24}O_{21}S_4$ $[M+2H]^{2+}$ *m/z:* 903.44; found: 903.64; $[M+3H]^{3+}$ *m/z:* 602.63; found: 602.75.

6.4 Disulfide-bridged serine/threonine ligation between *H_2N*-PTIPC(SSPyr)KARGI-*CO*-SAL ester (1h) and *H_2N*-TCHYIPRPKPR-*COOH* (2l) (Table 3, Entry 4)

Synthesis of *H_2N*-PTIPCKARGI TCHYIPRPKPR-*COOH* followed the **General Procedure 1.2.4**. Briefly, 4.83 mg of *H_2N*-PTIPC(SSPyr)KARGI-*CO*-SAL ester and 7.98 mg of *H_2N*-TCHYIPRPKPR-*COOH* were dissolved in 386 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for three times. After air drying, 270 μL of DMF was added to dissolve the solid. The DMF was added to 15.4 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient

of 10-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min and yielded *H_2N*-PTIPCKARGI TCHYIPRPKPR-*COOH* (5.32 mg, 58%).

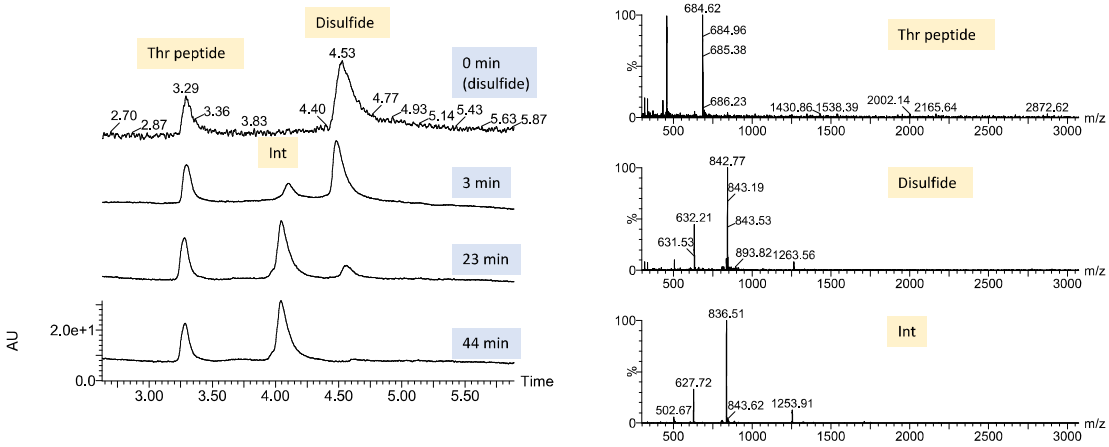


Fig. S66 Disulfide-bridged ligation progress of *H_2N*-PTIPC(SSPyr)KARGI-*CO*-SAL ester and *H_2N*-TCHYIPRPKPR-*COOH* over 0 min, 3 min, 23 min, and 44 min. Gradient: 5-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H_2N*-TCHYIPRPKPR-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

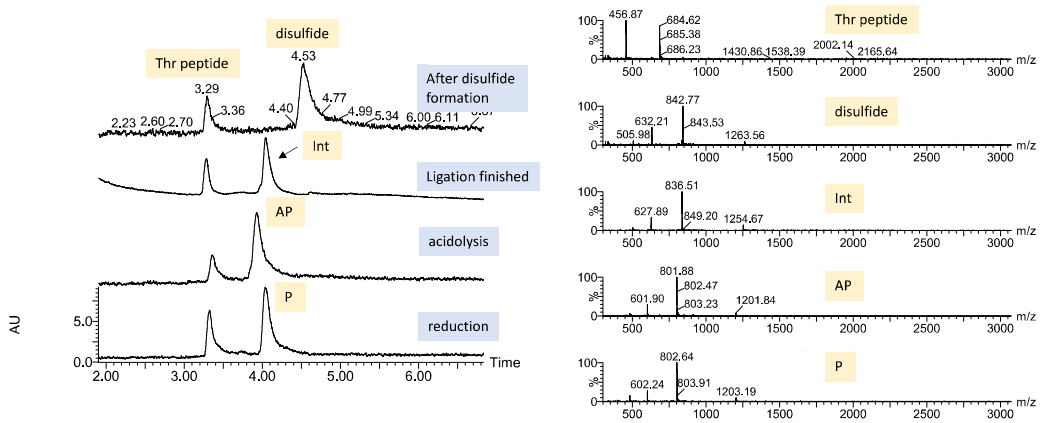


Fig. S67 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H_2N*-PTIPC(SSPyr)KARGI-*CO*-SAL ester and *H_2N*-TCHYIPRPKPR-*COOH*. Gradient: 5-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H_2N*- TCHYIPRPKPR-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

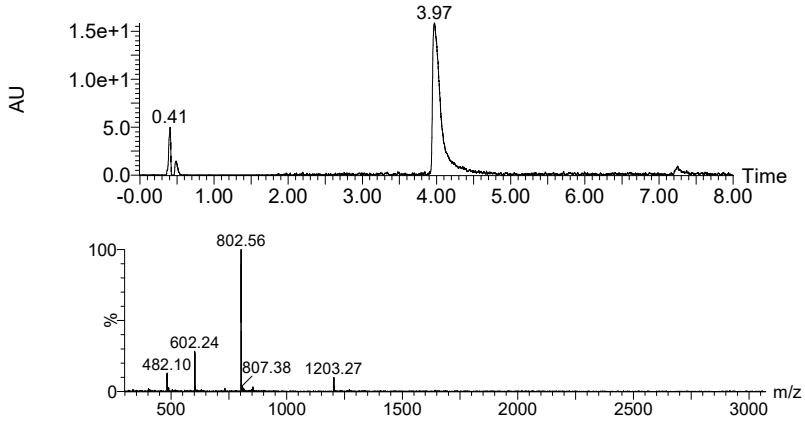


Fig. S68 Analytical HPLC analysis UV trace and ESI-MS of peptide *H₂N*-PTIPCKARGI TCHYIPRPKPR-*COOH*. Gradient: 5-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{107}H_{178}N_{34}O_{25}S_2$ $[M+2H]^{2+}$ *m/z:* 1202.66; found: 1203.27; $[M+3H]^{3+}$ *m/z:* 802.11; found: 802.56; $[M+4H]^{4+}$ *m/z:* 601.84; found: 602.24; $[M+5H]^{5+}$ *m/z:* 481.67; found: 482.10.

6.5 Disulfide-bridged serine/threonine ligation between *H₂N*-IPAC(SSPyr)IAGV-*CO*-SAL ester (1i) and *H₂N*-TKKCFLGGLMKA-*COOH* (2m) (Table 3, Entry 5)

Synthesis of *H₂N*-IPACIAGV TKKCFLGGLMKA-*COOH* followed the **General Procedure 1.2.4**. Briefly, 4.57 mg of *H₂N*-IPAC(SSPyr)IAGV-*CO*-SAL ester and 9.54 mg of *H₂N*-TKKCFLGGLMKA-*COOH* were dissolved in 480 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for three times. After air drying, 335 μL of DMF was added to dissolve the solid. The DMF was added to 19.1 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 20-50% ACN containing 0.1% TFA/ H_2O containing 0.1% TFA over 30 min and yielded *H₂N*-IPACIAGV TKKCFLGGLMKA-*COOH* (4.99 mg, 52%).

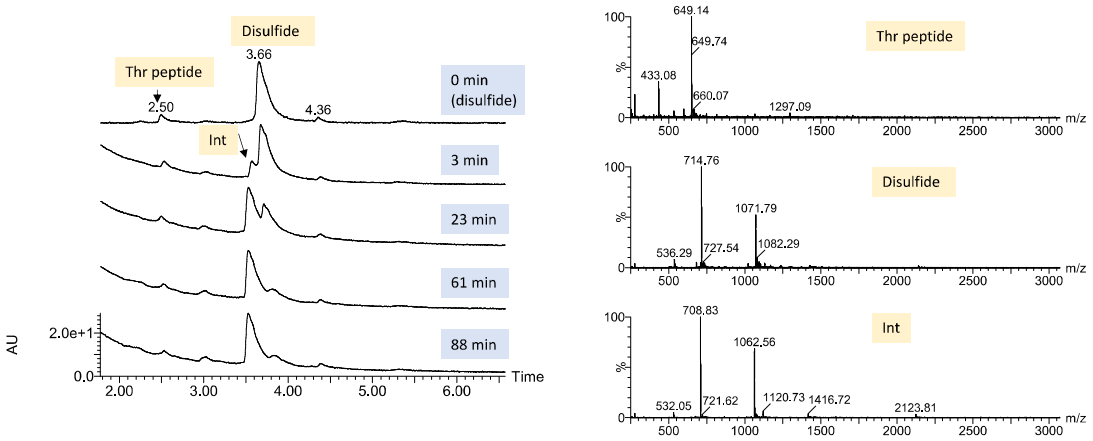


Fig. S69 Disulfide-bridged ligation progress of *H₂N*-IPAC(SSPyr)IAGV-*CO*-SAL ester and *H₂N*-TKKCFLGGLMKA-*COOH* over 0 min, 3 min, 23 min, and 44 min. Gradient: 20-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr peptide: *H₂N*-TKKCFLGGLMKA-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

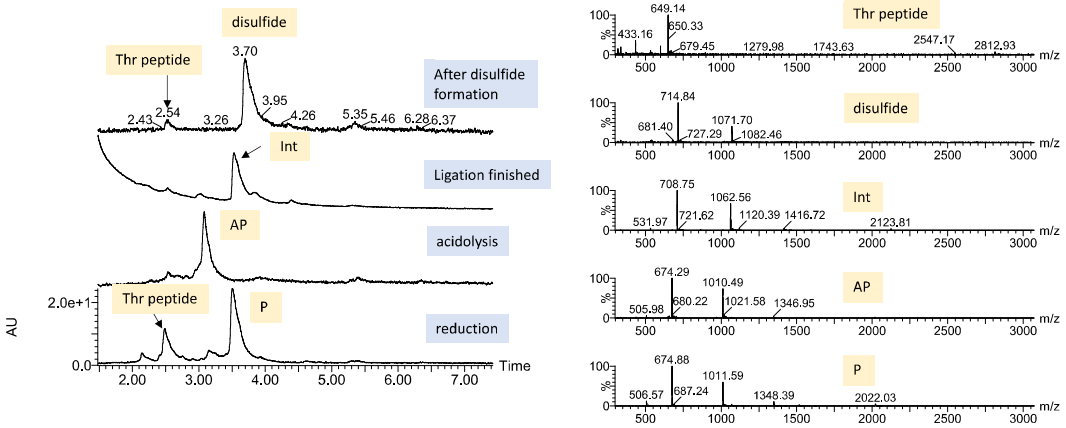


Fig. S70 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H₂N*-IPAC(SSPyr)IAGV-*CO*-SAL ester and *H₂N*-TKKCFLGGLMKA-*COOH*. Gradient: 20-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Thr Peptide: *H₂N*-TKKCFLGGLMKA-*COOH*. Disulfide: N-terminal Thr peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

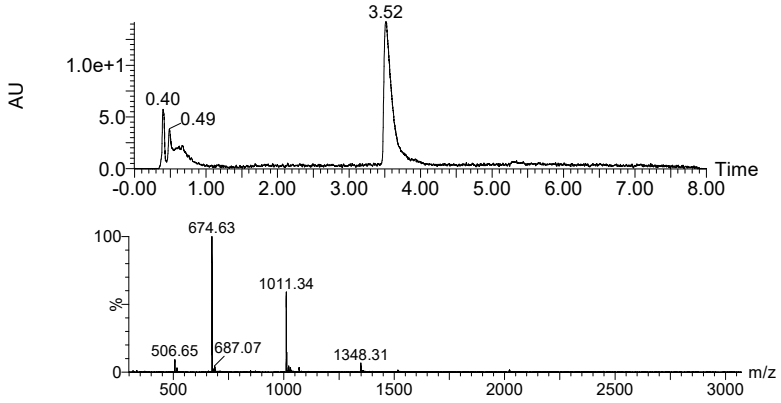


Fig. S71 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-IPACIAGV TKKCFLGGLMKA-*COOH*. Gradient: 20-60% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{91}H_{157}N_{23}O_{22}S_3$ $[M+2H]^{2+}$ *m/z:* 1011.06; found: 1011.34; $[M+3H]^{3+}$ *m/z:* 674.38; found: 674.63; $[M+4H]^{4+}$ *m/z:* 506.03; found: 506.65.

6.6 Disulfide-bridged serine/threonine ligation between *H_2N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester (1j) and *H_2N*-SRKCFLI-*COOH* (2n) (Table 3, Entry 6)

Synthesis of *H_2N*-GRKSDCFPASRKCFLI-*COOH* followed the **General Procedure 1.2.4**. Briefly, 5.09 mg of *H_2N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester and 5.79 mg of *H_2N*-SRKCFLI-*COOH* were dissolved in 430 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for 3 times. After air drying, 300 μL of DMF was added to dissolve the solid. The DMF was added to 17 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 15-30% ACN containing 0.1% TFA/ H_2O containing 0.1% TFA over 30 min and yielded *H_2N*-GRKSDCFPA SRKCFLI-*COOH* (4.90 mg, 63%).

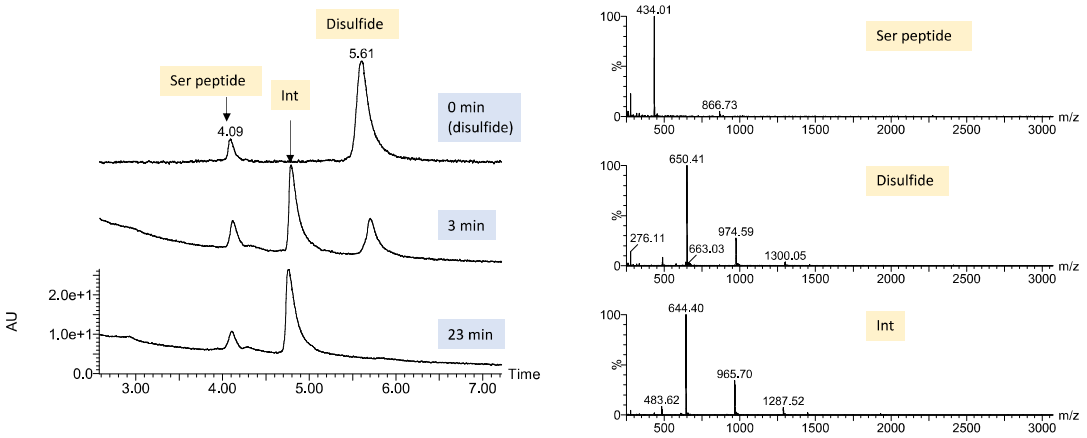


Fig. S72 Disulfide-bridged ligation progress of *H₂N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester and *H₂N*-SRKCFLI-*COOH* over 0 min, 3 min, and 23 min. Gradient: 15-40% ACN containing 0.1% TFA/ H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Ser peptide: *H₂N*-SRKCFLI-*COOH*. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

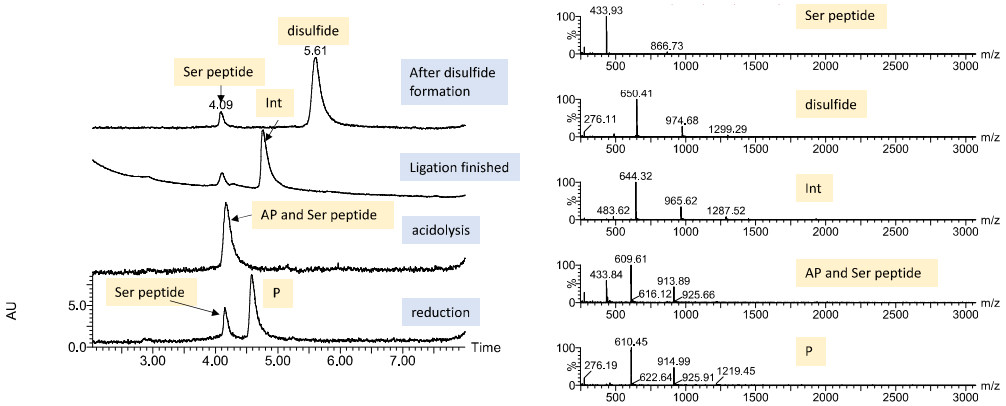


Fig. S73 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H₂N*-GRKSDC(SSPyr)FPA-*CO*-SAL ester and *H₂N*-SRKCFLI-*COOH*. Gradient: 15-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Ser Peptide: *H₂N*-SRKCFLI-*COOH*. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. P: product.

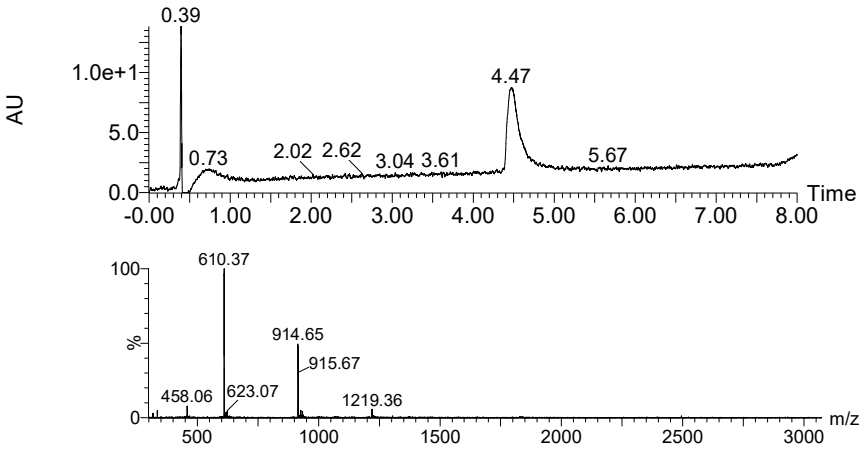


Fig. S74 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-GRKSDCFPASRKCFLI-*COOH*. Gradient: 15-40% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{80}H_{130}N_{24}O_{21}S_2$ $[M+2H]^{2+}$ *m/z:* 914.47; found: 914.65; $[M+3H]^{3+}$ *m/z:* 609.98; found: 610.37; $[M+4H]^{4+}$ *m/z:* 457.73; found: 458.06.

6.7 Disulfide-bridged serine/threonine ligation between *H_2N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester (1k) and *H_2N*-SGFCAFLKSPS-*COOH* (2o) (Table 3, Entry 7)

Synthesis of *H_2N*-GSKKPVPIIYCNRPSGFCAFLKSPS-*COOH* followed the **General Procedure 1.2.4**. Briefly, 4.37 mg of *H_2N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester and 4.25 mg of *H_2N*-SGFCAFLKSPS-*COOH* were dissolved in 244 μL H_2O/ACN (1:1, *v*/*v*) with 0.1% TFA. After the disulfide bond formation was finished, the reaction was freeze-dried to remove the solvent. Then the solid was washed with diethyl ether for 3 times. After air drying, 171 μL of DMF was added to dissolve the solid. The DMF was added to 9.8 mL pyridine/AcOH (1:1, *v*/*v*) with a final concentration of 0.25 mM, concerning the limiting peptide. After the reaction was finished, the pyridine/AcOH was removed by a rotary evaporator. Then, acidolysis was carried out using 4 mL of the TFA cocktail. After the acidolysis, the reaction mixture was concentrated under a compressed air stream, followed by diethyl ether wash. The precipitate formed was allowed to air dry and subject to disulfide reduction using 1 mL of 20 mM TCEP in ACN/H_2O (1/1, *v*/*v*). The reaction was then diluted and subject to HPLC purification utilizing a gradient of 20-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min and yielded *H_2N*-GSKKPVPIIYCNRP SGFCAFLKSPS-*COOH* (3.24 mg, 49%).

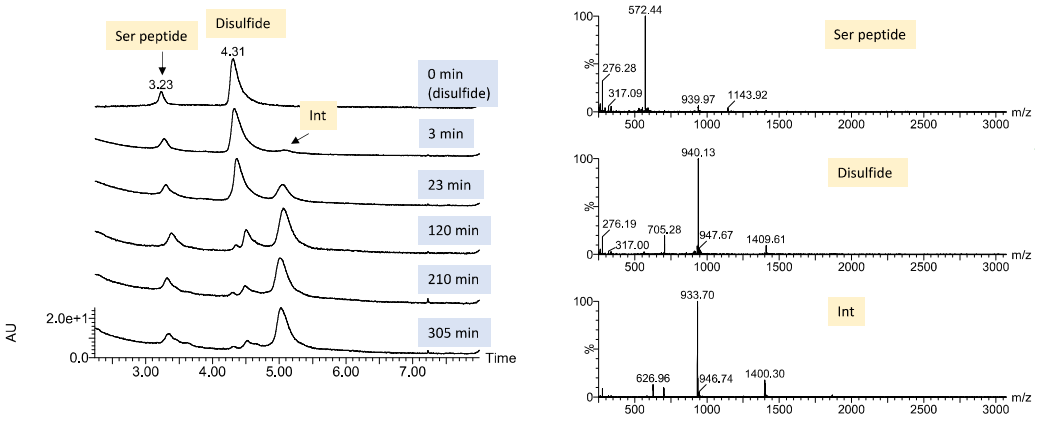


Fig. S75 Disulfide-bridged ligation progress of *H₂N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester and *H₂N*-SGFCAFLKSPS-*COOH* over 0 min, 3 min, 23 min, 120 min, 210 min and 305 min. Gradient: 20-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Ser peptide: *H₂N*-SGFCAFLKSPS-*COOH*. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate.

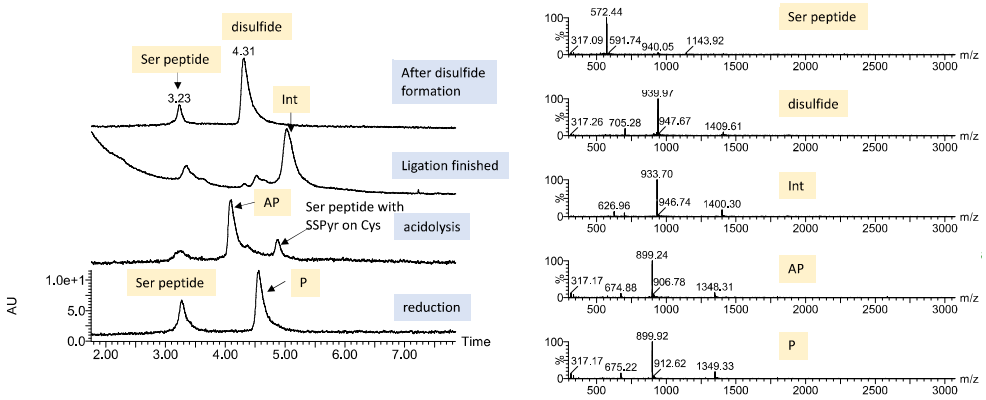


Fig. S76 LC-MS after disulfide formation, ligation, acidolysis, and reduction of disulfide-bridged ligation between *H₂N*-GSKKPVPIIYC(SSPyr)NRP-*CO*-SAL ester and *H₂N*-SGFCAFLKSPS-*COOH*. Gradient: 20-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. Ser Peptide: *H₂N*-SGFCAFLKSPS-*COOH*. Disulfide: N-terminal Ser peptide and peptide SAL ester linked with disulfide. Int: ligation intermediate. AP: acidolysis product. Ser peptide with SSPyr on Cys: *H₂N*-SGFC(SSPyr)AFLKSPS-*COOH*. P: product.

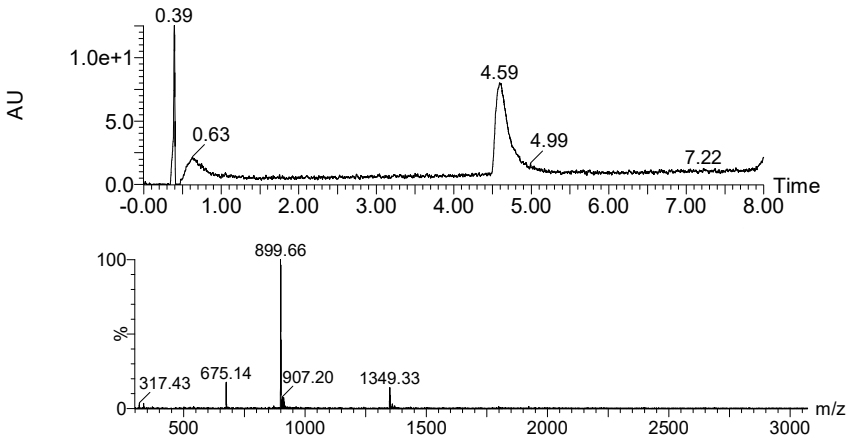
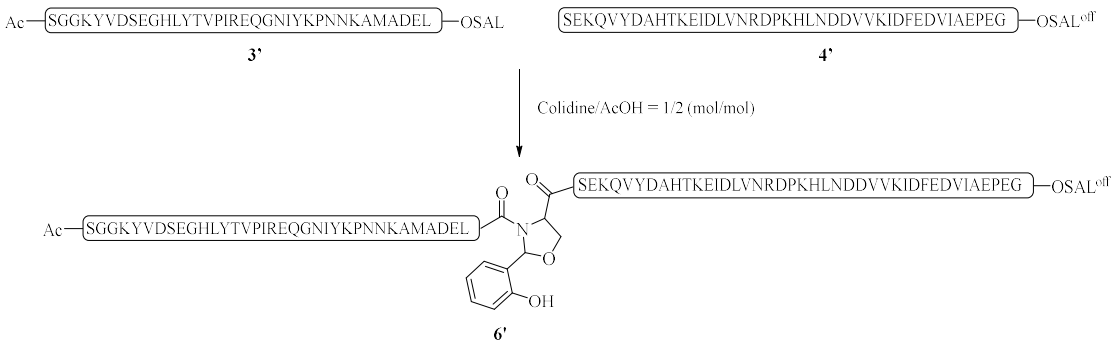


Fig. S77 Analytical HPLC analysis UV trace and ESI-MS of peptide *H_2N*-GSKKPVPIIYCNRP SGFCAFLKSPS-*COOH*. Gradient: 20-35% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{123}H_{194}N_{32}O_{32}S_2$ $[M+2H]^{2+}$ *m/z:* 1349.60; found: 1349.33; $[M+3H]^{3+}$ *m/z:* 900.07; found: 899.66; $[M+4H]^{4+}$ *m/z:* 675.30; found: 675.14.

7 Synthesis of Caveolin-1 (2-104)

7.1 Control experiment



7.1.1 Synthesis of 3'

3'

0.02 mmol peptide salicylaldehyde ester **3'** was synthesized by SPPS and salicylaldehyde ester formation via n+1. The crude product was purified by HPLC using a gradient of 20-50% ACN containing

0.1% TFA/H_2O containing 0.1% TFA over 30 min. After lyophilization, 26.37 mg peptide salicylaldehyde ester **3'** was synthesized in an isolated yield of 33%.

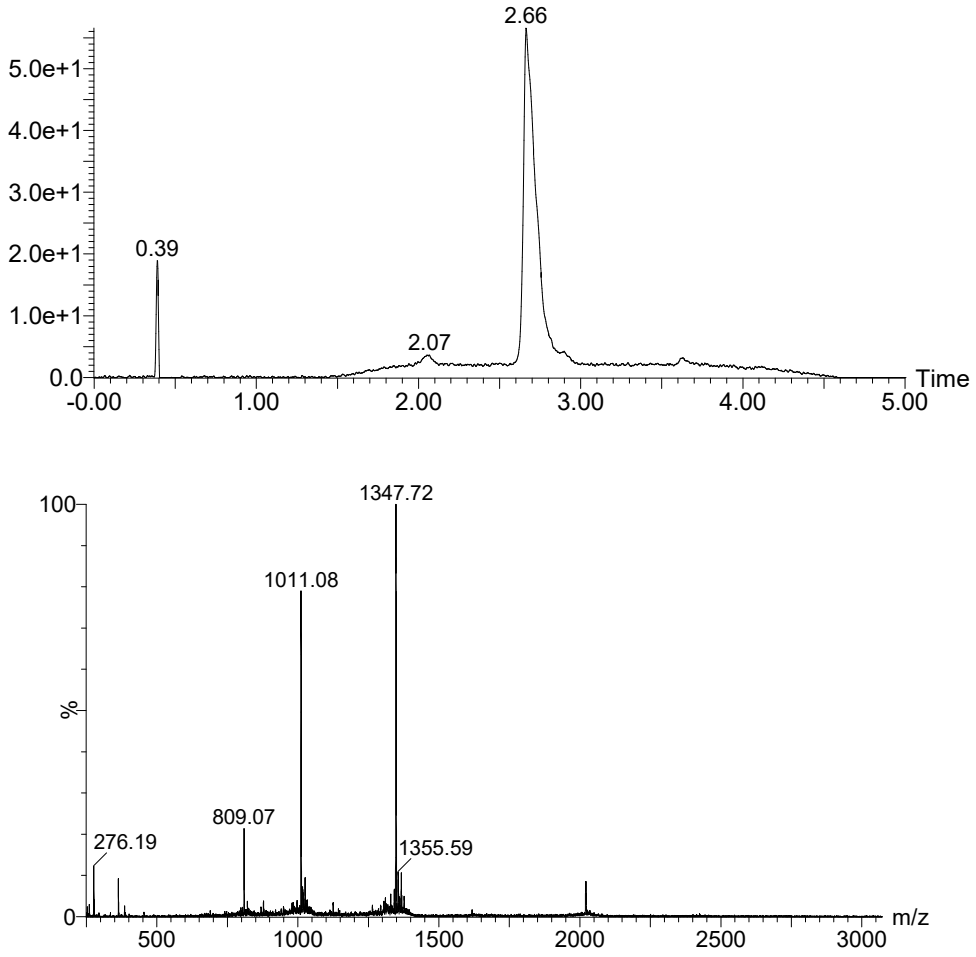


Fig. S78 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **3'**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI$^+$) calcd. for $C_{179}H_{271}N_{47}O_{58}S$ $[M+2H]^{2+}$ *m/z:* 2021.73; found: 2020.85; $[M+3H]^{3+}$ *m/z:* 1348.16; found: 1347.72; $[M+4H]^{4+}$ *m/z:* 1011.37; found: 1011.08; $[M+5H]^{5+}$ *m/z:* 809.29; found: 809.07.

7.1.2 Synthesis of 4'

SEKQVYDAHTKEIDLVNRDPKHLNDDVVKIDFEDVIAEPEG—OSAL^{off}

4'

0.02 mmol peptide salicylaldehyde ester **4'** was synthesized by SPPS and directly coupling followed by treating with 3 equiv semicarbazide·HCl for 15 min to turn off the SAL^{on} ester. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O containing 0.1%

TFA over 30 min. After lyophilization, 34.82 mg peptide salicylaldehyde ester **4'** was synthesized in an isolated yield of 36%.

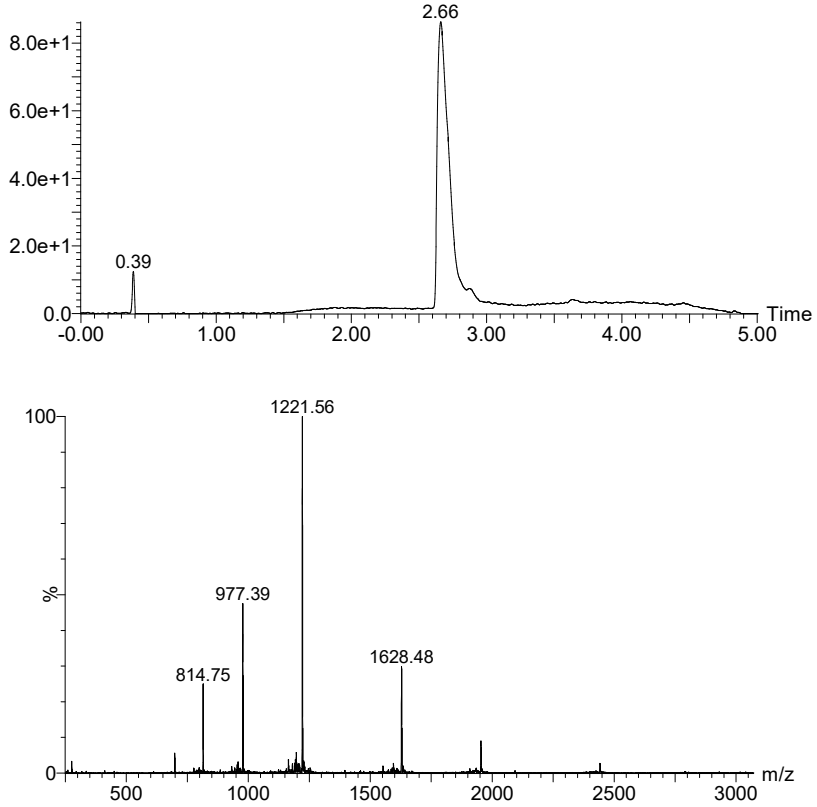


Fig. S79 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **4'**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{214}H_{330}N_{58}O_{73}$ $[M+2H]^{2+}$ *m/z:* 2441.19; found: 2441.96; $[M+3H]^{3+}$ *m/z:* 1627.80; found: 1628.48; $[M+4H]^{4+}$ *m/z:* 1221.10; found: 1221.56; $[M+5H]^{5+}$ *m/z:* 977.08; found: 977.39; $[M+6H]^{6+}$ *m/z:* 814.40; found: 814.75; $[M+7H]^{7+}$ *m/z:* 698.20; found: 698.59.

7.1.3 Serine/threonine ligation between 3' and 4' at a concentration of 5 mM

0.5 mg (0.12 μmol, 1 equiv) **3'** and 0.6 mg (0.12 μmol, 1 equiv) **4'** were performed in collidine/AcOH (1/2, *mol*/*mol*) with a concentration of 5 mM at 37 ºC for overnight and monitor by LC-MS.

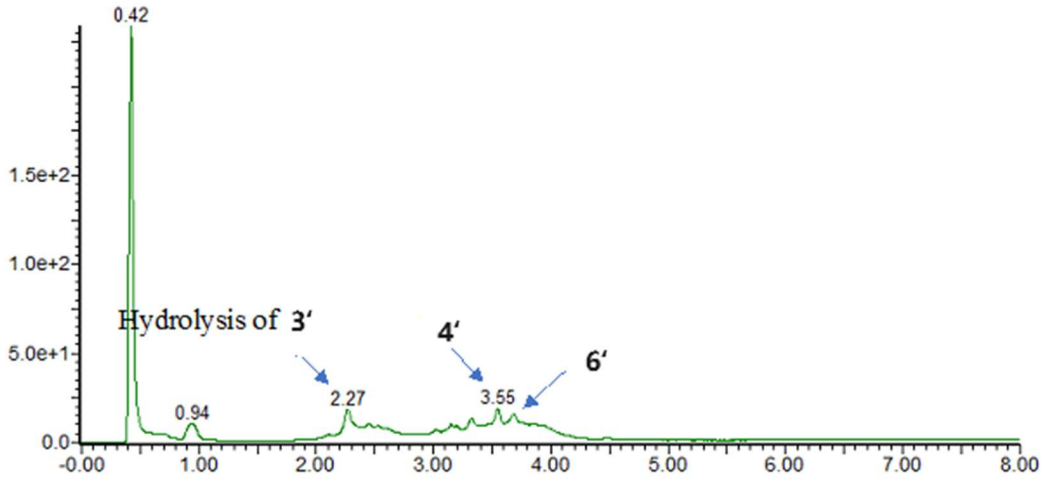


Fig. S80 UV trace from analytical RP-UPLC of ligation between **3'** and **4'** at a concentration of 5 mM. Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

7.1.4 Serine/threonine ligation between 3' and 4' at a concentration of 1 mM

0.5 mg (0.12 μmol, 1 equiv) **3'** and 0.6 mg (0.12 μmol, 1 equiv) **4'** were performed in collidine/AcOH (1/2, *mol*/*mol*) with a concentration of 1 mM at 37 °C for overnight and monitor by LC-MS.

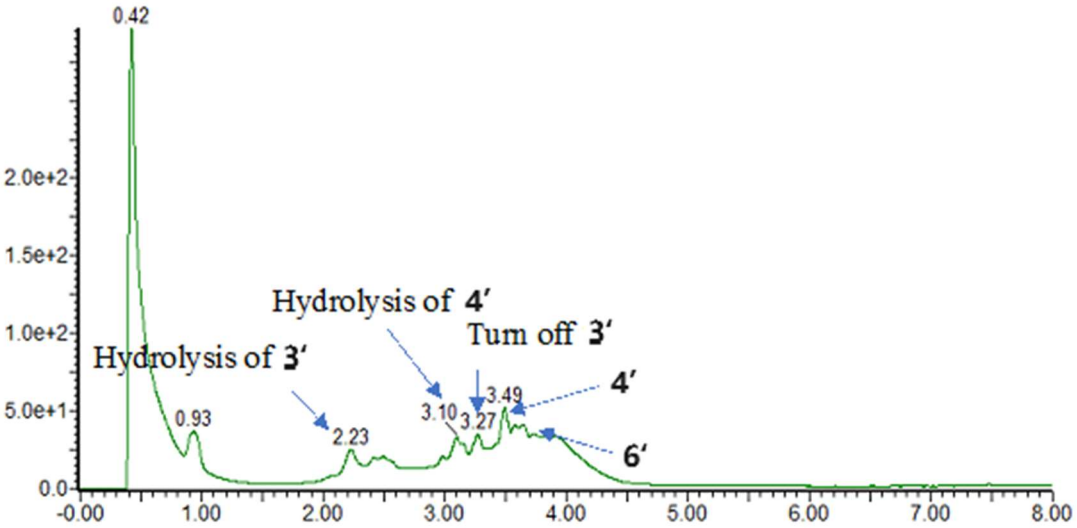
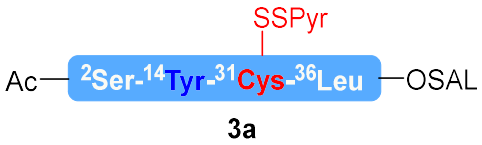


Fig. S81 UV trace from analytical RP-UPLC of ligation between **3'** and **4'** at a concentration of 1 mM. Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

7.2 Prior disulfide bond-mediated STL to synthesize CAV-1 (2-104)

7.2.1 Synthesis of 3a



3a

0.04 mmol peptide salicylaldehyde ester **3a** was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min. After lyophilization, 79.17 mg peptide salicylaldehyde ester **3a** was synthesized in an isolated yield of 47%.

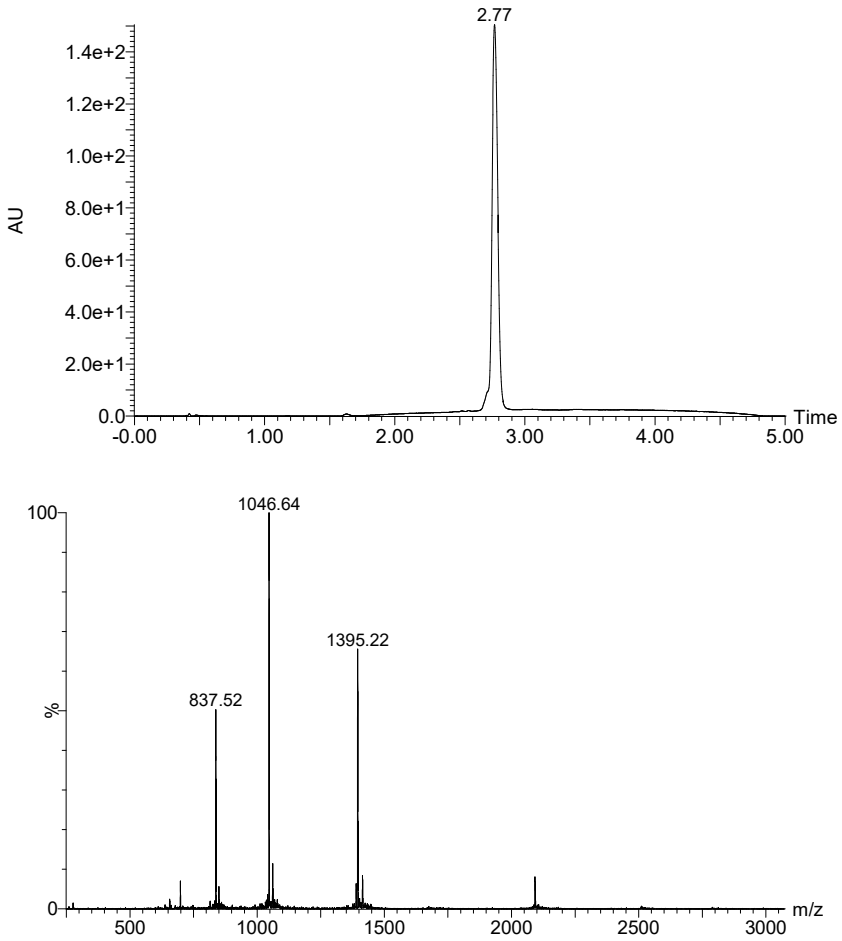
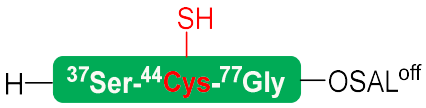


Fig. S82 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **3a**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min.

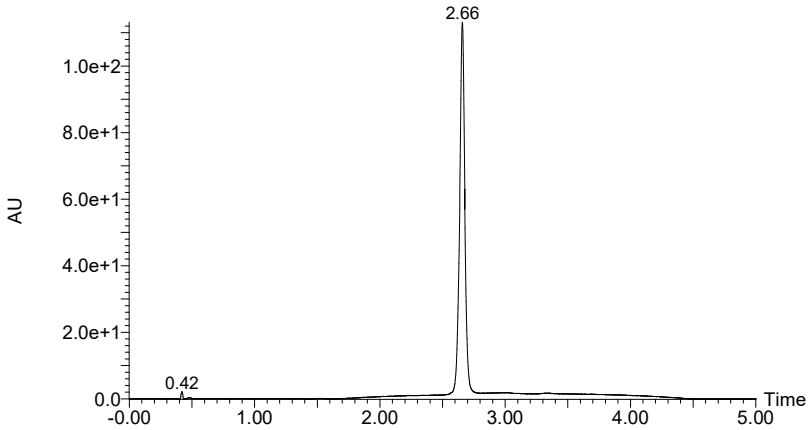
MS (ESI^{+}) calcd. for $C_{184}H_{274}N_{48}O_{58}S_3$ $[M+2H]^{2+}$ *m/z:* 2092.33; found: 2092.82; $[M+3H]^{3+}$ *m/z:* 1395.20; found: 1395.22; $[M+4H]^{4+}$ *m/z:* 1046.67; found: 1046.64; $[M+5H]^{5+}$ *m/z:* 837.53; found: 837.52.

7.2.2 Synthesis of 4



4

0.04 mmol peptide salicylaldehyde ester **4** was synthesized according to **General Procedure 1.2.1** and treated with 3 equiv semicarbazide·HCl for 15 min to turn off the SAL^{on} ester. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 30 min. After lyophilization, 53.92 mg peptide salicylaldehyde ester **4** was synthesized in an isolated yield of 27%.



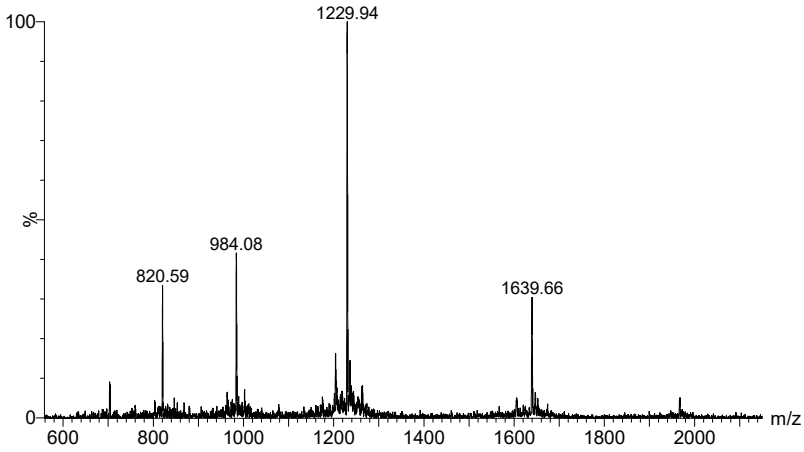
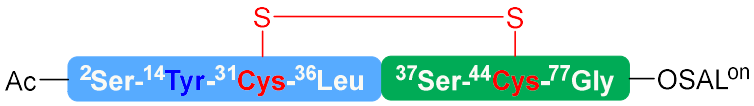


Fig. S83 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **4**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{214}H_{330}N_{58}O_{73}S$ $[M+3H]^{3+}$ *m/z:* 1639.46; found:1639.66; $[M+4H]^{4+}$ *m/z:* 1229.85; found: 1229.94; $[M+5H]^{5+}$ *m/z:* 984.06; found: 984.08; $[M+6H]^{5+}$ *m/z:* 820.22; found: 820.59; $[M+7H]^{6+}$ *m/z:* 703.20; found: 703.50.

7.2.3 Synthesis of 6



6a

3a (20 mg, 4.8 μmol) and **4** (23.6 mg, 4.8 μmol) were dissolved in ACN/H_2O (3/1, *v*/*v*) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature overnight to give **5a**. Then, the solvent was removed under freeze-drying. The crude product was then dissolved in 4.8 mL collidine/AcOH (1/2, *mol*/*mol*) containing 50% DMSO at the concentration of 1 mM and reacted at 37 ºC for overnight, giving ligated intermediate **6a'**. After that, the reaction mixture was precipitated with diethyl ether, washed twice, and dried under vacuum. The resulting residues were treated with 4 mL TFA/H_2O/DMS (90/5/5, *v*/*v*/*v*) cocktail for 30 min and then added 0.2 mL pyruvic acid, reacting for another 3h. Then, the solvent was removed under condensed nitrogen gas, washed with diethyl ether, and centrifuged. The product was purified by preparative reverse-phase HPLC using the gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 14.09 mg **6a** was obtained in an isolated yield of 34%.

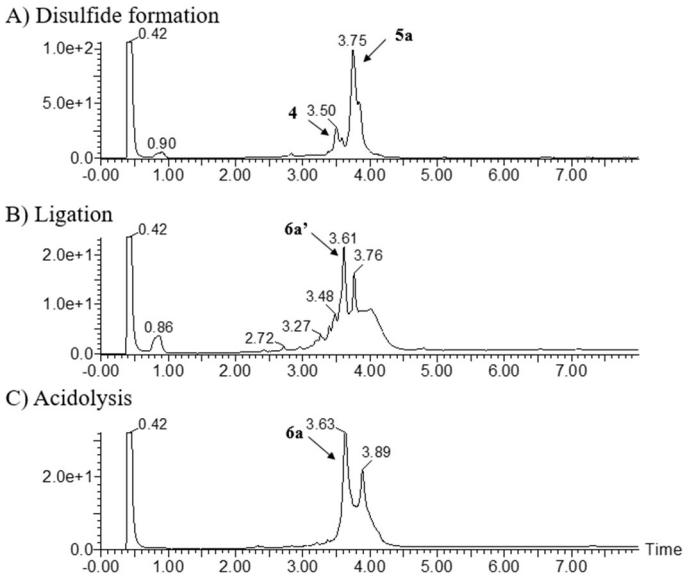
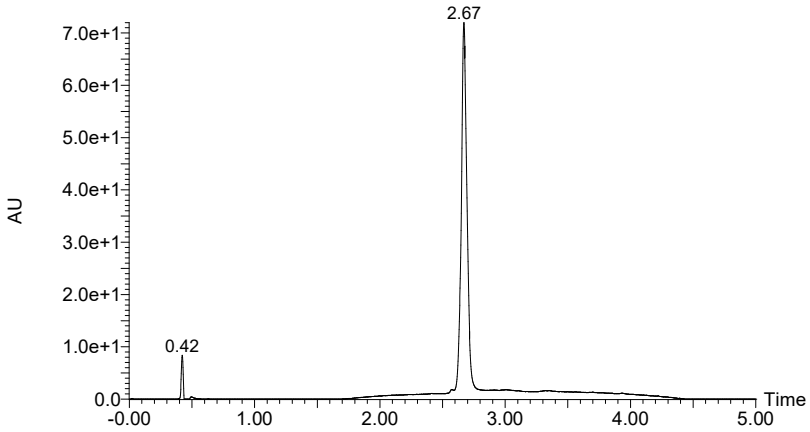


Fig. S84 UV trace from analytical RP-UPLC of disulfide formation, ligation, and acidolysis between **3a** and **4**. (A) Disulfide formation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Acidolysis at 3.5 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.



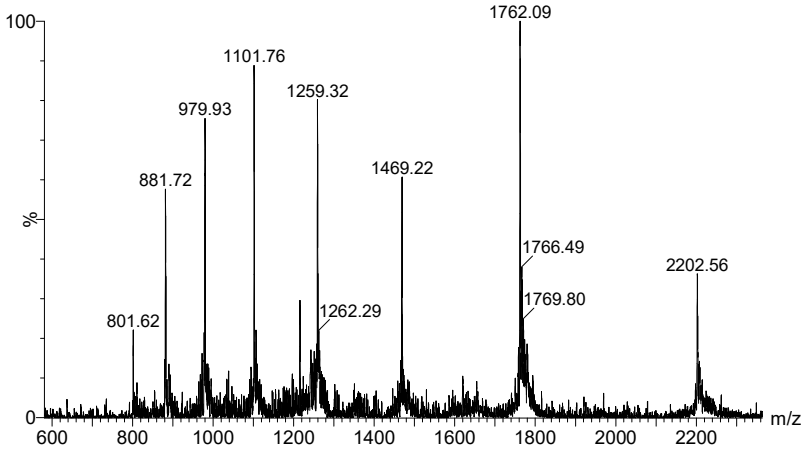
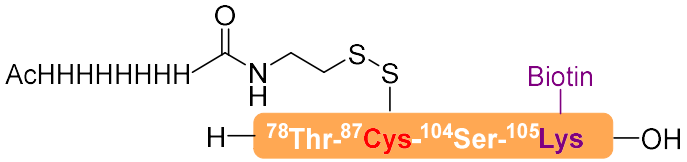


Fig. S85 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **6a**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{385}H_{591}N_{102}O_{129}S_3$ $[M+4H]^{4+}$ *m/z:* 2203.15; found: 2202.56; $[M+5H]^{5+}$ *m/z:* 1762.72; found: 1762.09; $[M+6H]^{6+}$ *m/z:* 1469.10; found: 1469.22; $[M+7H]^{7+}$ *m/z:* 1259.37; found: 1259.32; $[M+8H]^{8+}$ *m/z:* 1102.07; found: 1101.76; $[M+9H]^{9+}$ *m/z:* 979.73; found: 979.93; $[M+10H]^{10+}$ *m/z:* 881.86; found: 881.72; $[M+11H]^{11+}$ *m/z:* 801.78; found: 801.62.

7.2.4 Synthesis of 7



7

0.02 mol peptide **7** was assembled by Fmoc-SPPS general procedure and the reducible solubilizing tags strategy[35] to attach the H_8 tag. After Fmoc-SPPS, 10 mL TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) cocktail was added to cleavage the peptide from the resin and globally deprotect for 2 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC using a 20% ACN/H_2O gradient to 50% ACN/H_2O over 30 min. After lyophilization, 29.49 mg **7** was obtained in an isolated yield of 30%.

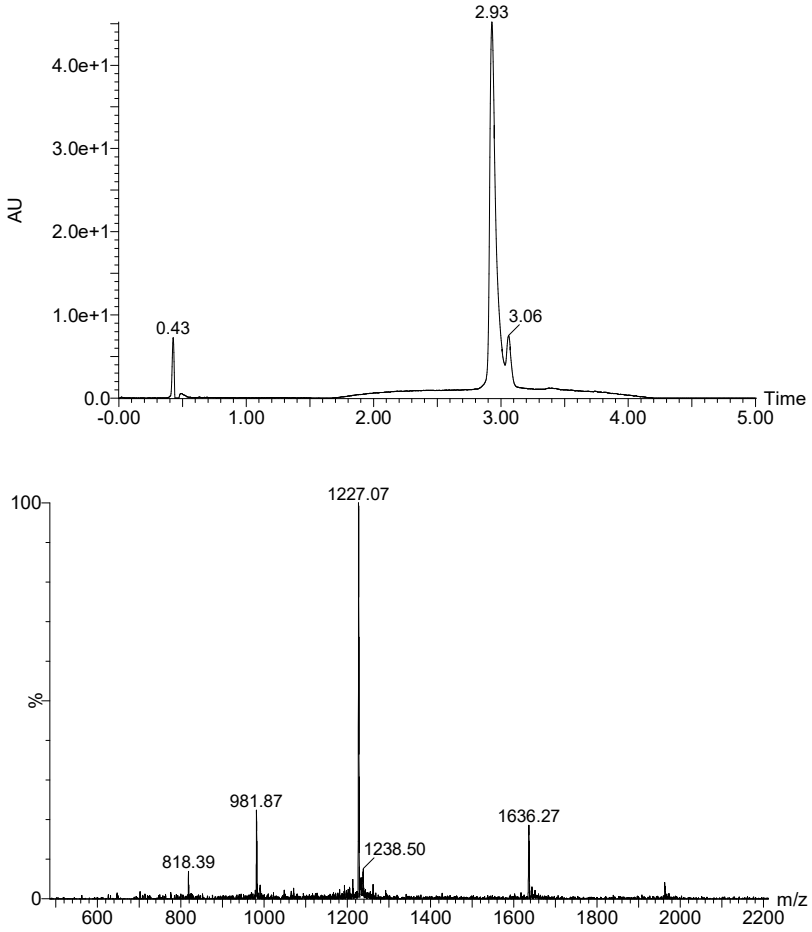


Fig. S86 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **7**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI$^+$) calcd. for $C_{229}H_{313}N_{65}O_{52}S_3$ $[M+3H]^{3+}$ *m/z:* 1635.87; found: 1635.27; $[M+4H]^{4+}$ *m/z:* 1227.15; found: 1227.07; $[M+5H]^{5+}$ *m/z:* 981.92; found: 981.87; $[M+6H]^{6+}$ *m/z:* 818.44; found: 818.39.

7.2.5 Synthesis of 9a

9a

6a (10 mg, 1.1 μmol) and **7** (5.57 mg, 1.1 μmol) were dissolved in 450 μL 2-picoline/AcOH (1/2, *mol*/*mol*) containing 50% DMSO at the concentration of 2.5 mM, and reacted at 37 °C for overnight, giving ligation intermediate **8a'**. After that, the reaction mixture was precipitated with diethyl ether, washed twice, and dried under vacuum. The product was purified by preparative reverse-phase HPLC

using the gradient of 30% ACN/H_2O to 60% ACN/H_2O over 30 min. After lyophilization, 2.65 mg **8a'** was obtained in an isolated yield of 17.04%. **8a'** was treated with 0.2 mL TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) cocktail for 30 min to afford **8a**. Then, TFA was blown off by the condensed nitrogen gas, and the residues was precipitated with diethyl ether, washed twice, and dried under vacuum. The residue was dissolved in 300 μL phosphate buffer (pH 7), 300 μL TCEP solution 1 M in phosphate buffer, pH 7), 30 μL *t*BuSH, and 300 μL VA-044 solution (0.1 M in phosphate buffer, pH 7) were added. The reaction mixture was stirred at 37 ºC for overnight. Finally, the product was purified by preparative reverse-phase HPLC using a gradient of 30% ACN/H_2O to 60% ACN/H_2O over 30 min. After lyophilization, 1.23 mg **9a** was obtained in an isolated yield of 52%.

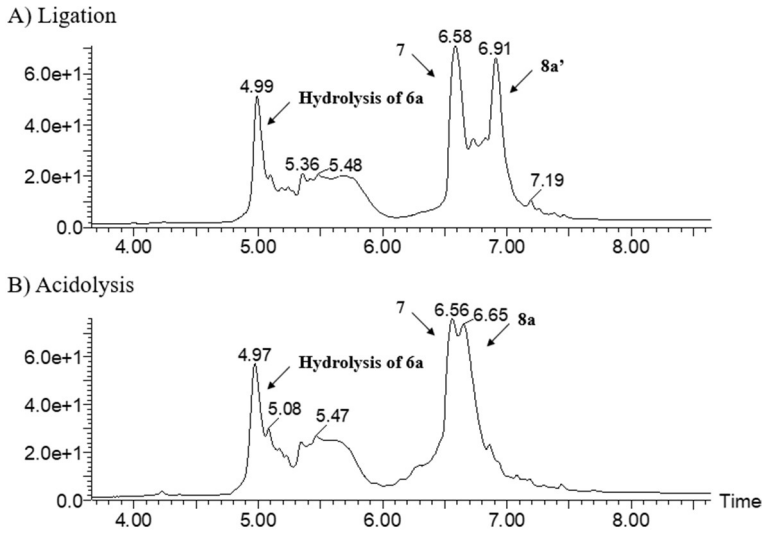
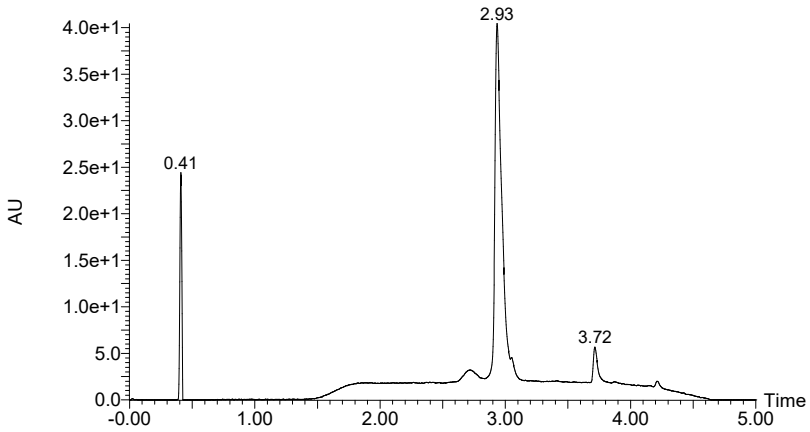


Fig. S87 UV trace from analytical RP-UPLC of ligation between **6a** and **7**. Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.



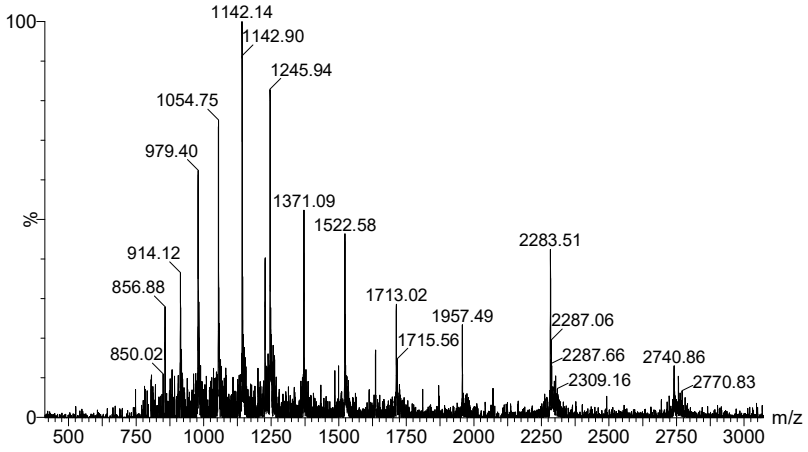


Fig. S88 Analytical HPLC analysis UV trace and ESI-MS of **8a'**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{614}H_{902}N_{167}O_{180}S_6$ $[M+6H]^{6+}$ *m/z:* 2283.55; found: 2283.51; $[M+7H]^{7+}$ *m/z:* 1957.47; found: 1957.49; $[M+8H]^{8+}$ *m/z:* 1712.91; found: 1713.02; $[M+9H]^{9+}$ *m/z:* 1522.70; found: 1522.58; $[M+10H]^{10+}$ *m/z:* 1370.53; found: 1371.09; $[M+11H]^{11+}$ *m/z:* 1246.03; found: 1245.94; $[M+12H]^{12+}$ *m/z:* 1142.28; found: 1142.14; $[M+13H]^{13+}$ *m/z:* 1054.49; found: 1054.75; $[M+14H]^{14+}$ *m/z:* 979.24; found: 979.40; $[M+15H]^{15+}$ *m/z:* 914.02; found: 914.12; $[M+16H]^{16+}$ *m/z:* 856.96; found: 856.88.

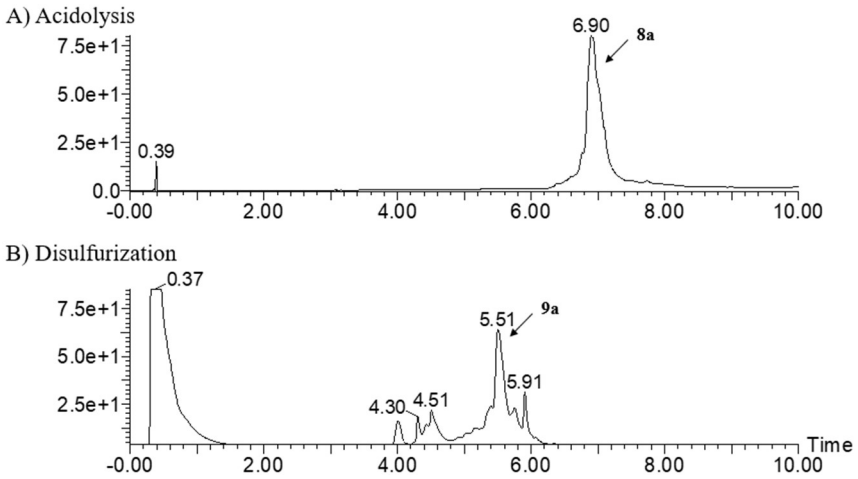


Fig. S89 UV trace from analytical RP-UPLC of acidolysis of **8a'** and desulfurization of **8a**. (A) Acidolysis at 30 min. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min. (B) Desulfurization at 12 h. Gradient: 30-60% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

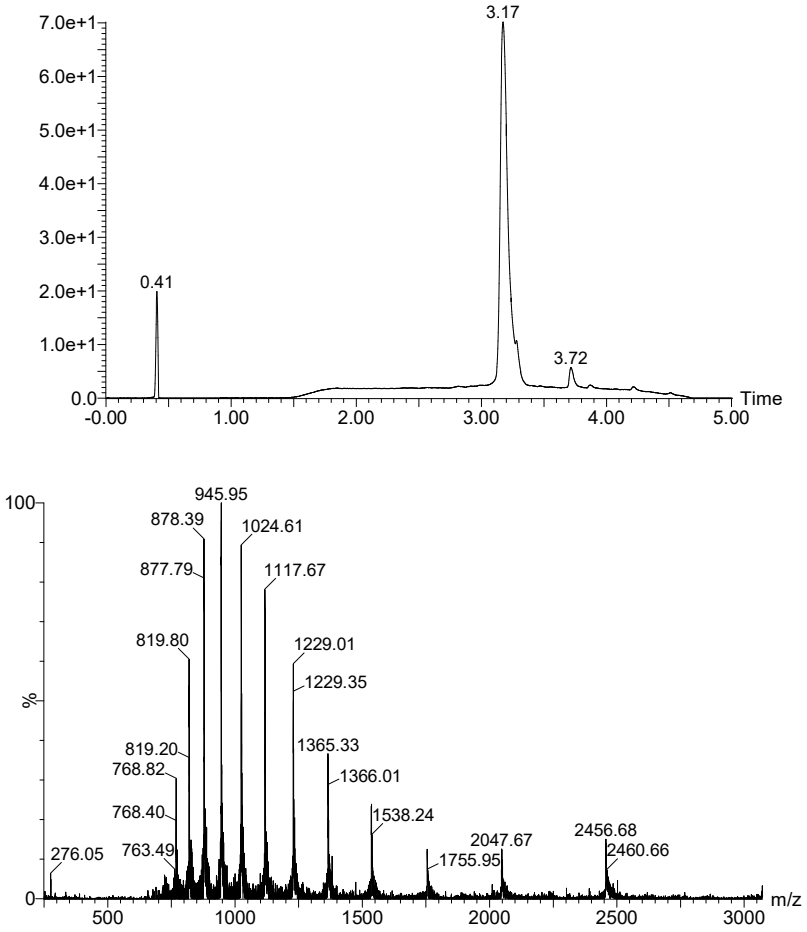


Fig. S90 Analytical HPLC analysis UV trace and ESI-MS of **9a**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{555}H_{836}N_{142}O_{170}S_2$ $[M+5H]^{5+}$ *m/z:* 2457.33; found: 2456.68; $[M+6H]^{6+}$ *m/z:* 2047.92; found: 2047.67; $[M+7H]^{7+}$ *m/z:* 1755.51; found: 1755.95; $[M+8H]^{8+}$ *m/z:* 1536.19; found: 1538.24; $[M+9H]^{9+}$ *m/z:* 1365.62; found: 1365.33; $[M+10H]^{10+}$ *m/z:* 1229.25; found: 1229.01; $[M+11H]^{11+}$ *m/z:* 1117.59; found: 1117.67; $[M+12H]^{12+}$ *m/z:* 1024.54; found: 1024.61; $[M+13H]^{13+}$ *m/z:* 945.81; found: 945.95; $[M+14H]^{14+}$ *m/z:* 878.32; found: 878.39; $[M+15H]^{15+}$ *m/z:* 819.83; found: 819.80; $[M+16H]^{16+}$ *m/z:* 768.66; found: 768.82.

7.2.6 CD spectra of 9a

9a was dissolved in 400 μL aqueous buffer containing 10 mM H_3BO_3-KOH, 50 mM NaCl, and 20 μM DTT at pH 9.0. The solution was poured into Slide-A-Lyzer MINI dialysis device (MW cut-off at 3000, Thermo Fisher Scientific, USA), then dialyzed against 15 mL Tris buffer (20 mM Tris-HCl, 2 mM $MgCl_2$,

100 mM NaCl at pH 7.5) for 12 h at 4 ºC, twice. The folded Cav-1 was concentrated by ultrafiltration, using Millipore tube (MW cut-off at 3000). After that, the protein solutions were subjected to buffer exchange (10 mM H_3BO_3-KOH, 50 mM NaCl, and 20 μM DTT at pH 9.0) by ultrafiltration for five times. The final concentration was 0.277 mg/mL (The concentration was determined by Thermo Fisher Scientific NanoDrop UV-Vis spectrophotometers). The CD spectrum was measured by a J-815 circular dichroism spectrometer (JASCO, Inc., Tokyo, Japan), sample was scanned for 1 time at room temperature using H_3BO_3-KOH buffer as blank in a 0.1 cm cell. The CD result was shown that our synthetic Cav-1 N-terminal cytoplasmic domain has similar structure with recombinant one, reported by Evgeni Yu. Zernii et al.[1]

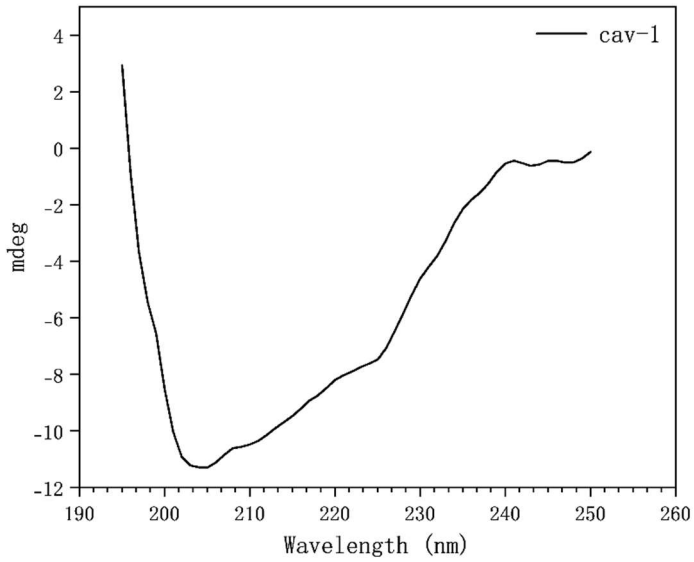
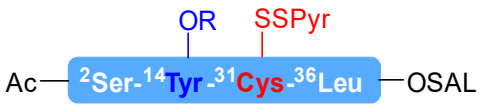


Fig. S91 CD of Synthetic Cav-1 N-terminal cytoplasmic domain (2-104).

7.3 Prior disulfide bond-mediated STL to synthesize CAV-1 (2-104) with pY14

7.3.1 Synthesis of 3b



3b (R = PO_3^{2-})

0.04 mmol peptide salicylaldehyde ester **3b** was synthesized according to **General Procedure 1.2.1**. The crude product was purified by HPLC using a gradient of 20-50% ACN containing 0.1% TFA/H_2O

containing 0.1% TFA over 30 min. After lyophilization, 57.44 mg peptide salicylaldehyde ester **3b** was synthesized in an isolated yield of 34%.

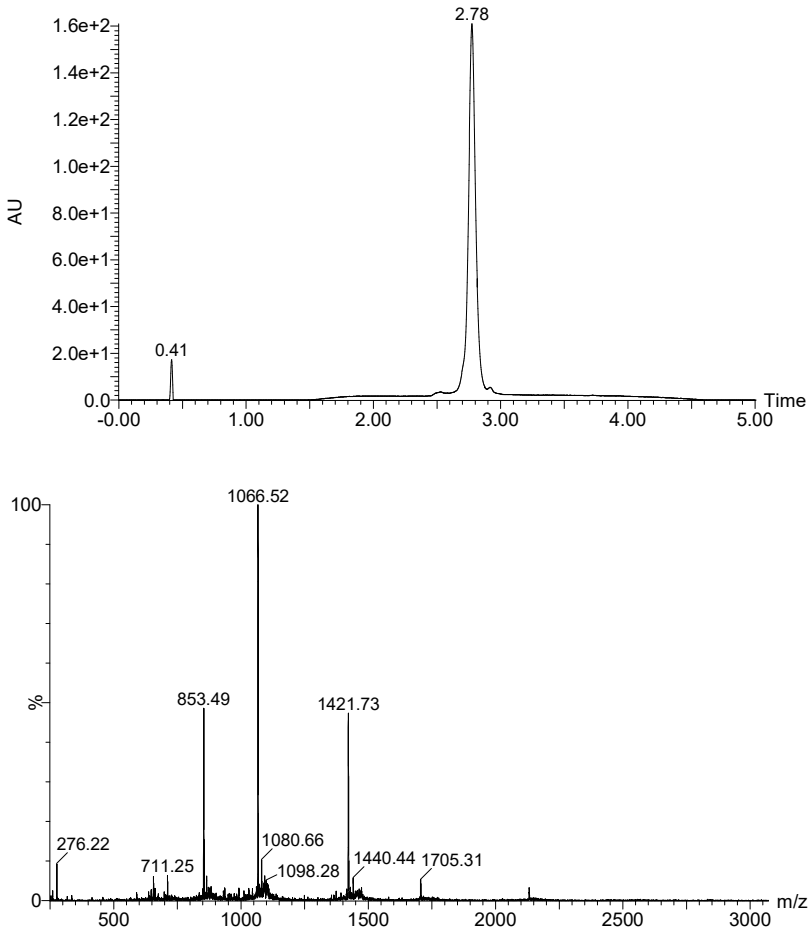
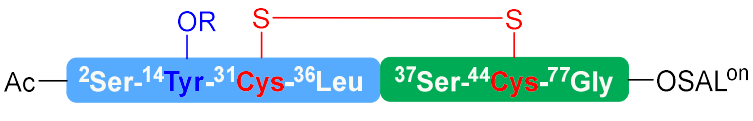


Fig. S92 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **3b**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{184}H_{275}N_{48}O_{61}PS_3$ $[M+3H]^{3+}$ *m/z:* 1421.88; found: 1421.73; $[M+4H]^{4+}$ *m/z:* 1066.66; found: 1066.52; $[M+5H]^{5+}$ *m/z:* 853.53; found: 853.49.

7.3.2 Synthesis of 6b



6b (R = PO_3^{2-})

3b (20 mg, 4.7 μmol) and **4** (23.1 mg, 4.7 μmol) were dissolved in ACN/H_2O (3/1, *v*/*v*) at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature

overnight to give **5b**. Then, the solvent was removed under freeze-drying. The crude product was then dissolved in 4.7 mL 2-picoline/AcOH (1/2, *mol*/*mol*) containing 50% DMSO at the concentration of 1.25 mM and reacted at 37 °C for overnight, giving ligation intermediate **6b'**. After that, the reaction mixture was precipitated with diethyl ether, washed twice, and dried under vacuum. The resulting residues were treated with 4 mL TFA/H_2O/DMS (90/5/5, *v*/*v*/*v*) cocktail for 30 min and then added 0.2 mL pyruvic acid, reacting for another 3 h. Then, the solvent was removed under condensed nitrogen gas, washed with diethyl ether, and centrifuged. The product was purified by preparative reverse-phase HPLC using the gradient of 20% ACN/H_2O to 50% ACN/H_2O over 30 min. After lyophilization, 9.8 mg **6b** was obtained in an isolated yield of 24%.

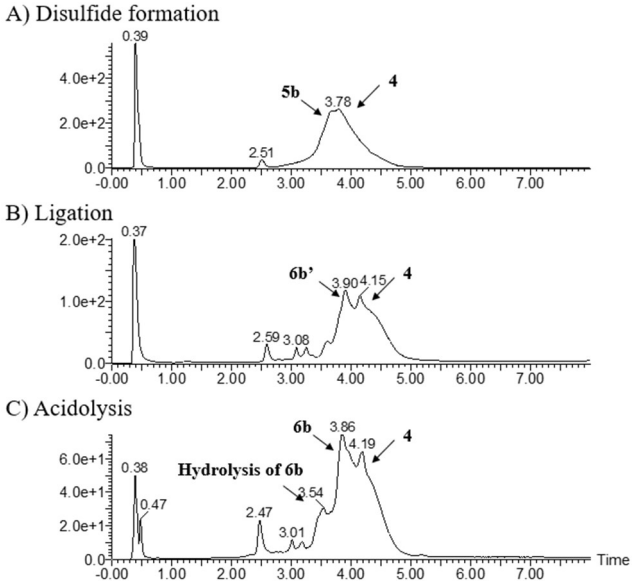


Fig. S93 UV trace from analytical RP-UPLC of disulfide formation, ligation, and acidolysis between **3b** and **4**. (A) Disulfide formation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (B) Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. (C) Acidolysis at 3.5 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.

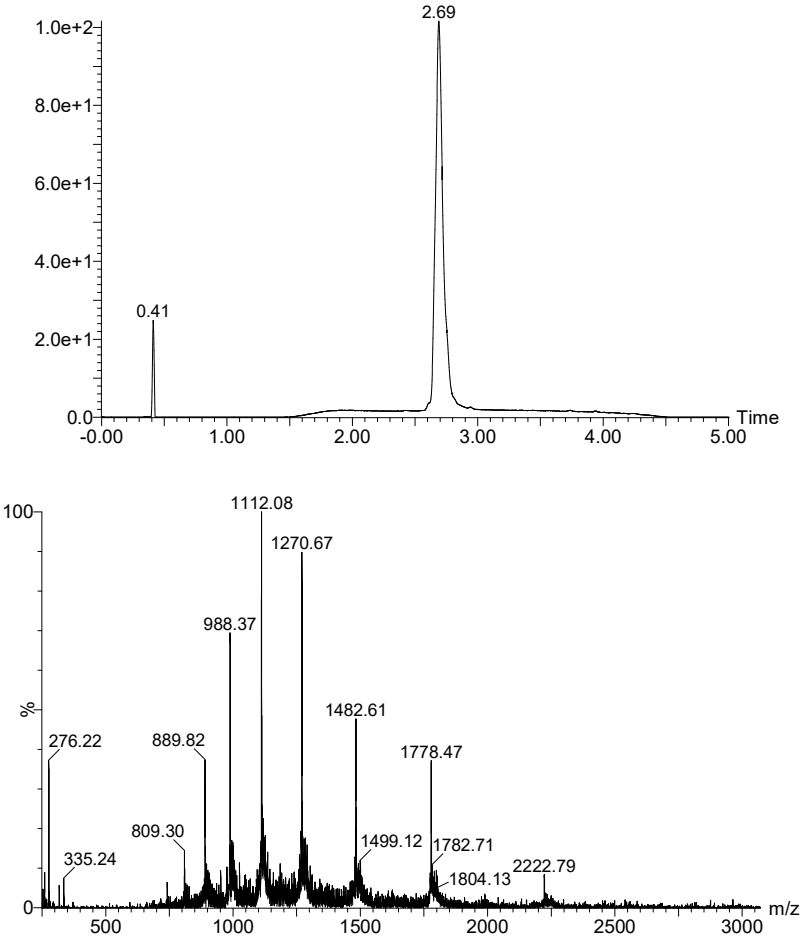
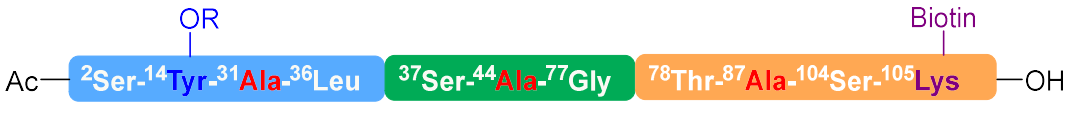


Fig. S94 Analytical HPLC analysis UV trace and ESI-MS of peptide salicylaldehyde ester **6b**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{385}H_{591}N_{102}O_{132}PS_3$ $[M+4H]^{4+}$ *m/z:* 2222.92; found: 2222.79; $[M+5H]^{5+}$ *m/z:* 1778.54; found: 1778.47; $[M+6H]^{6+}$ *m/z:* 1482.28; found: 1482.61; $[M+7H]^{7+}$ *m/z:* 1270.67; found: 1270.67; $[M+8H]^{8+}$ *m/z:* 1111.96; found: 1112.08; $[M+9H]^{9+}$ *m/z:* 988.52; found: 988.37; $[M+10H]^{10+}$ *m/z:* 889.77; found: 889.82; $[M+11H]^{11+}$ *m/z:* 808.97; found: 809.30.

7.3.3 Synthesis of 9b



9b (R = PO_3^{2-})

6b (10 mg, 1.1 μmol) and **7** (5.57 mg, 1.1 μmol) were dissolved in 450 μL 2-picoline/AcOH (1/2, *mol*/*mol*) containing 50% DMSO at the concentration of 2.5 mM, and reacted at 37 °C for overnight,

giving ligation intermediate **8b'**. After that, the reaction mixture was precipitated with diethyl ether, washed twice, and dried under vacuum. The product was purified by preparative reverse-phase HPLC using the gradient of 30% ACN/H_2O to 60% ACN/H_2O over 30 min. After lyophilization, 1.36 mg **8b'** was obtained in an isolated yield of 9.1%. **8b'** was treated with 0.2 mL TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) cocktail for 30 min to afford **8b**. Then, TFA was blown off by the condensed nitrogen gas, and the residues was precipitated with diethyl ether, washed twice, and dried under vacuum. The residue was dissolved in 130 μL phosphate buffer (pH 7), 130 μL TCEP solution 1 M in phosphate buffer, pH 7), 15 μL *t*BuSH, and 130 μL VA-044 solution (0.1 M in phosphate buffer, pH 7) were added. The reaction mixture was stirred at 37 °C for overnight. Finally, the product was purified by preparative reverse-phase HPLC using a gradient of 30% ACN/H_2O to 60% ACN/H_2O over 30 min. After lyophilization, 0.62 mg **9b** was obtained in an isolated yield of 50 %.

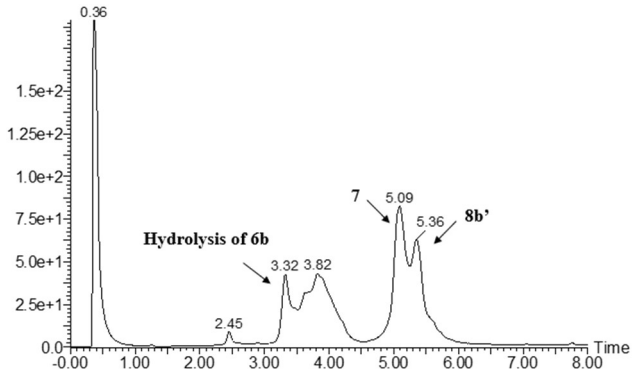
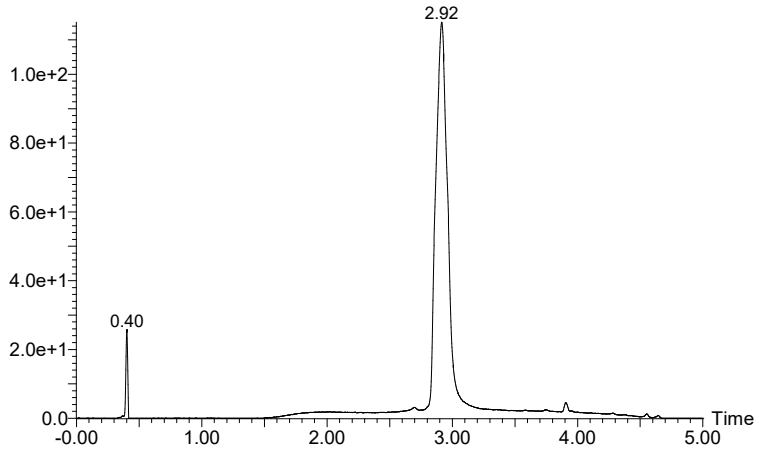


Fig. S95 UV trace from analytical RP-UPLC of ligation between **6b** and **7**. Ligation at 12 h. Gradient: 20-50% ACN/H_2O containing 0.1% TFA over 8 min at a flow rate of 0.4 mL/min.



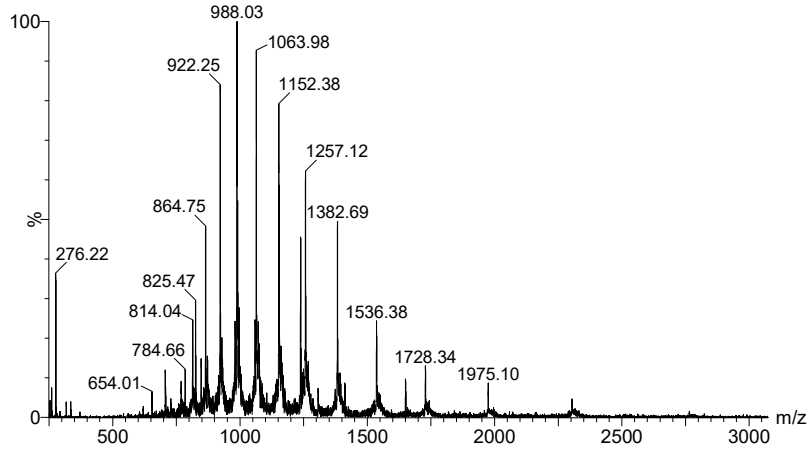


Fig. S96 Analytical HPLC analysis UV trace and ESI-MS of **8b'**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{615}H_{902}N_{167}O_{185}PS_6$ $[M+7H]^{7+}$ *m/z:* 1973.78; found: 1975.10; $[M+8H]^{8+}$ *m/z:* 1727.18; found: 1728.34; $[M+9H]^{9+}$ *m/z:* 1535.38; found: 1536.38; $[M+10H]^{10+}$ *m/z:* 1381.94; found: 1382.69; $[M+11H]^{11+}$ *m/z:* 1256.40; found: 1257.12; $[M+12H]^{12+}$ *m/z:* 1151.79; found: 1152.38; $[M+13H]^{13+}$ *m/z:* 1063.26; found: 1063.98; $[M+14H]^{14+}$ *m/z:* 987.39; found: 988.03; $[M+15H]^{15+}$ *m/z:* 921.63; found: 922.25; $[M+16H]^{16+}$ *m/z:* 864.09; found: 864.75; $[M+17H]^{17+}$ *m/z:* 813.32; found: 814.04.

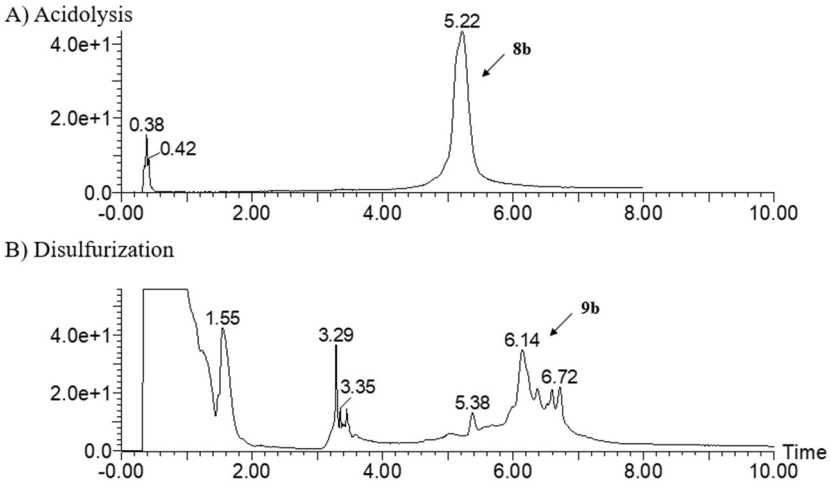


Fig. S97 UV trace from analytical RP-UPLC of acidolysis of **8b'** and desulfurization of **8b**. Desulfurization at 12 h. Gradient: 30-60% ACN/H_2O containing 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

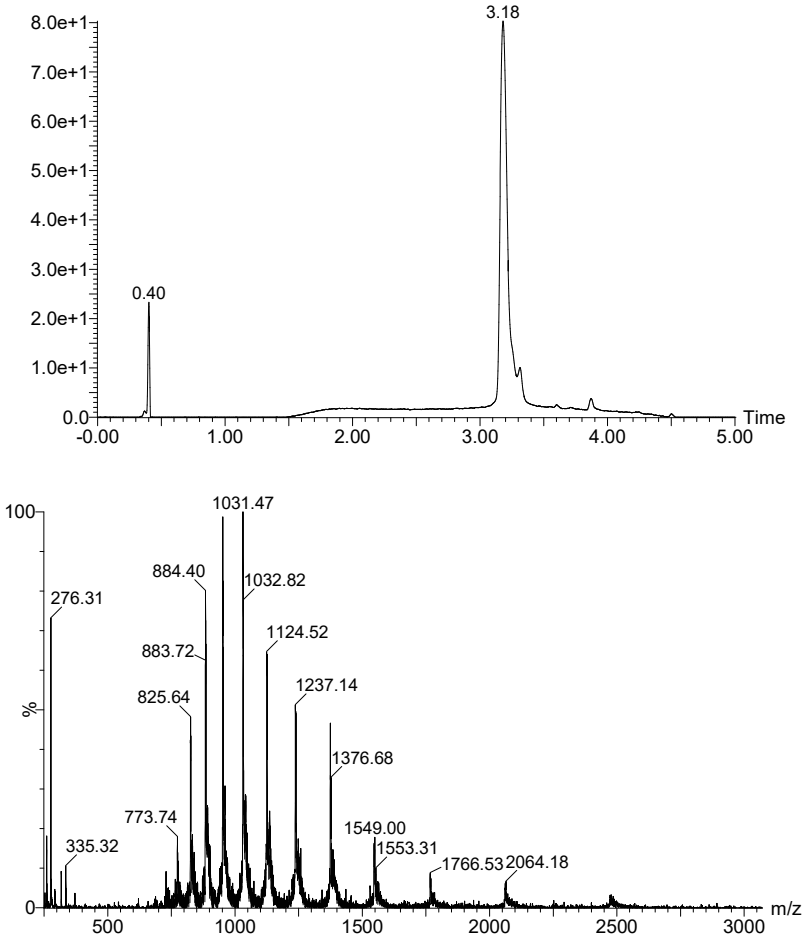


Fig. S98 Analytical HPLC analysis UV trace and ESI-MS of **9b**. Gradient: 5-95% ACN containing 0.1% TFA/H_2O containing 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. MS (ESI^+) calcd. for $C_{555}H_{837}N_{142}O_{173}PS_2$ $[M+6H]^{6+}$ *m/z:* 2061.28; found: 2064.18; $[M+7H]^{7+}$ *m/z:* 1766.96; found: 1766.93; $[M+8H]^{8+}$ *m/z:* 1546.22; found: 1549.00; $[M+9H]^{9+}$ *m/z:* 1374.53; found: 1374.65; $[M+10H]^{10+}$ *m/z:* 1237.18; found: 1237.14; $[M+11H]^{11+}$ *m/z:* 1124.80; found: 1124.52; $[M+12H]^{12+}$ *m/z:* 1031.15; found: 1031.47; $[M+13H]^{13+}$ *m/z:* 951.91; found: 952.22; $[M+14H]^{14+}$ *m/z:* 883.99; found: 883.72; $[M+15H]^{15+}$ *m/z:* 825.12; found: 825.64; $[M+16H]^{16+}$ *m/z:* 773.61; found: 773.74.

References

1. V. I. Vladimirov, M. P. Shchannikova, A. V. Baldin, A. S. Kazakov, M. P. Shevelyova, A. A. Nazipova, V. E. Baksheeva, E. L. Nemashkalova, A. S. Frolova, N. K. Tikhomirova, P. P. Philippov, A. A. Zamyatnin, Jr., S. E. Permyakov, D. V. Zinchenko and E. Y. Zernii, *Biomolecules*, 2022, **12**, 1698.