Enabling *N-to-C* Ser/Thr Ligation for Convergent Protein Synthesis via Combining Chemical Ligation Approaches

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Supplementary Information

I. General Information

A. Materials and methods and abbreviations

All commercial materials (Aldrich, Chemimpex, Fluka and GL Biochem) were used without further purification. All solvents were reagent grade or HPLC grade (RCI or DUKSAN). Dry dichloromethane (CH_2Cl_2) was distilled from calcium hydride (CaH_2). All reversed-phase (RP) high-performance liquid chromatography (HPLC) separations involved a mobile phase of 0.1% trifluoroacetic acid (TFA) (*v*/*v*) in acetonitrile (CH_3CN)/0.1% TFA (*v*/*v*) in water (H_2O) were performed with a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Vydac 214TP^{TM} C4 column (5 μm, 300 Å, 4.6 x 250 mm) at a flow rate of 0.6 mL/min for analytical HPLC and Vydac 214TP^{TM} C4 column (10 μm, 300 Å, 22 x 250 mm) or Vydac 218TP^{TM} C18 column (10 μm, 300 Å, 22 x 250 mm) at a flow rate of 10 mL/min for preparative HPLC. Low-resolution mass spectral (MS) analyses were performed with a Waters 3100 mass spectrometer using electrospray ionization (ESI, in positive mode unless otherwise specified). The results were analyzed with Waters Empower software. Calculated masses were based upon the most abundant isotope of a given ion. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and visualized under UV light (254 nm) or by staining with ninhydrin or 5 % sulfuric acid in methanol. Silica flash column chromatography was performed on E. Merck 230-400 mesh silica gel 60. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 298 K on Bruker Avance DRX 300 FT-NMR Spectrometer at 75 MHz for ^{13}C NMR or Bruker Avance DRX 400 FT-NMR spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR or Bruker Avance DRX 600 FT-NMR spectrometer at 150 MHz for ^{13}C NMR. Chemical shifts are reported in parts per million (ppm) and are referenced to solvent residual signals: $CDCl_3$ (δ 7.26 [1H]). 1H NMR data is reported as chemical shift (δ), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, td = triplet of doublets), coupling constant (J Hz). LCMS = Liquid chromatography mass-spectrometry; PG = protecting groups; SAL = salicylaldehyde; DMF = dimethylformamide; TIPS = triisopropylsilane.

II. General Experimental Procedures

A. Fmoc-based Solid-phase Peptide Synthesis (SPPS)

The solid phase peptide synthesis of peptides/glycopeptides was carried out manually using 2-chlorotrityl resin (GL Biochem, loading: ~0.5 mmol/g) unless otherwise specified. 2-chloro-trityl chloride resin was swollen in dry CH_2Cl_2 for 30 min then washed with CH_2Cl_2 (5 × 3 mL). A solution of FmocHN-Xaa-COOH (4.0 equiv. relative to resin capacity) and DIEA (8.0 equiv. relative to resin capacity) in CH_2Cl_2 was added and the resin shaken at room temperature (r. t.) for 2 h. The resin was washed with DMF (5 × 3 mL) and CH_2Cl_2 (5 × 3 mL). The resin was treated with a solution of CH_2Cl_2/CH_3OH/DIEA (17:2:1, *v*/*v*/*v*, 3 mL) for 1 h and washed with DMF (5 × 3mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS). The following Fmoc amino acids and Boc amino acids from GL Biochem were employed: FmocHN-Ala-COOH, FmocHN-Cys(Trt)-COOH, FmocHN-Cys(S^{t}Bu)-COOH, FmocHN-Asp(O^{t}Bu)-COOH, FmocHN-Glu(O^{t}Bu)-COOH, FmocHN-Phe-COOH, FmocHN-Gly-COOH, FmocHN-His(Trt)-COOH, FmocHN-Ile-COOH, FmocHN-Lys(Boc)-COOH, FmocHN-Leu-COOH, FmocHN-Met-COOH, FmocHN-Asn(Trt)-COOH, FmocHN-Pro-COOH, FmocHN-Gln(Trt)-COOH, FmocHN-Arg(Pbf)-COOH, FmocHN-Ser(^{t}Bu)-COOH, FmocHN-Thr(^{t}Bu)-COOH, FmocHN-Val-COOH, FmocHN-Trp(Boc)-COOH, FmocHN-Tyr(^{t}Bu)-COOH, BocHN-Ser(^{t}Bu)-COOH, BocHN-Thr(^{t}Bu)-COOH, BocHN-Tyr(^{t}Bu)-COOH and BocN-Thz-COOH. The following pseudoproline dipeptides from Chemimpex were used: FmocHN-Gln-Thr($\Psi^{Me,Me}$Pro)-COOH and FmocHN-Asn-Ser($\Psi^{Me,Me}$Pro)-COOH. The glycosyl amino acid FmocHN-Asn($GlcNAc_4$)-COOH was obtained by synthesis. The removal of Fmoc group was executed using a deblock solution of 20% piperidine in DMF at room temperature for 20 min. The resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). For the coupling step, a solution of Fmoc protected amino acid or Boc protected amino acid (2.0 equiv. according to the resin capacity), HATU (2.0 equiv.) and DIEA (5.0 equiv.) in DMF was gently agitated with the resin at room temperature for 40 min. The resin was washed with DMF (5 × 3

mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). This procedure was repeated twice for coupling each amino acid.

B. Cleavage of crude protected peptide bearing the free carboxylic acid at the C-terminus from resin with Cocktail A

The on-resin fully protected peptidyl acid, obtained as described in the previous section, was subjected to mild acidic cleavage cocktail (5 - 10 mL) of CH_2Cl_2/AcOH/trifluoroethanol (8/1/1, *v*/*v*/*v*), 3 times for 60 min each. Following filtration, the resulting cleavage solutions were combined and concentrated to give crude protected peptide bearing the free carboxylic acid at the C-terminus.

C. Synthesis of C-terminus '1' L-Amino salicylaldehyde semicarbazone ester hydrochloride (HCl·*H_2N*-Xaa-*CO*-SAL^{off})

To a solution of fully protected Boc amino acid, *BocHN*-Xaa(PG)-*COOH*, (1.0 equiv.) in CH_2Cl_2 at room temperature, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) (3.0 equiv.) and 4-Dimethylaminopyridine (DMAP) (0.1 equiv.) were added, followed by salicylaldehyde semicarbazone (1.0 equiv.). The reaction mixture was stirred overnight at room temperature and concentrated *in vacuo*. Purification by silica gel chromatography (CH_2Cl_2/EtOAc, 2:1) gave *BocHN*-Xaa(PG)-*CO*-SAL^{off} as a white solid. This compound was treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H_2N*-Xaa-*CO*-SAL^{off} as a white solid. Without purification, this salt was subjected to the n+1 reaction.

D. Synthesis of *C*-terminus Peptide SAL^{off} esters using n+1 strategy

The fully protected peptidyl acid (1.0 equiv.), obtained as described in the previous section A and B, was dissolved in $CHCl_3$/trifluoroethanol (15 mM, 3/1, *v*/*v*), and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) (3.0 equiv.) and Hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) (3.0 equiv.) were then

added. After 5 min, the corresponding amino L-Amino salicylaldehyde semicarbazone ester hydrochloride (HCl·H_2N-Xaa-CO-SAL^{off})(3.0 equiv.), obtained as described in the previous section, was added, and the reaction mixture was stirred for 3 h to form the crude protected C-terminal peptide SAL^{off} ester.

E. Preparation of Native chemical ligation buffer

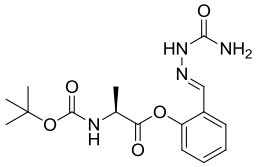
Native chemical ligation (NCL) buffer was freshly prepared prior to the reaction by dissolving Na_2HPO_4 (56.6 mg, 0.2 M) and guanidine (Gn)·HCl (1.146 g, 6 M) in water. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) (10.8 mg, 0.02 M) was then added, and the mixture was diluted to 2.0 mL. After complete solubilization, the pH was brought to ~7.0 by the addition of a NaOH solution (5.0 M). 4-mercaptophenylacetic acid (MPAA) (67 mg, 0.2 M) was then added and the pH of the mixture was adjusted to 6.8–7.2 with NaOH (5.0 M). The solution was sonicated and sparged with Ar for 30 min before use.

F. Preparation of hydrazine 2-chlorotrityl chloride resin

2-chlorotrityl chloride resin (1 g, loading = ~0.5 mmol/g) was swelled in 10 mL CH_2Cl_2/DMF (1/1, *v*/*v*). Then 10 mL $NH_2NH_2·H_2O$/DMF (1/20, *v*/*v*) were added. The reaction was conducted for 30 min. 10 mL of methanol/DMF (1/20, *v*/*v*) was added to quench the remaining 2-chlorotrityl chloride resin. After 30 min, the resin was washed with DMF and CH_2Cl_2 and kept under high vacuum for 2 h and ready for iterative peptide assembly (Fmoc-SPPS).

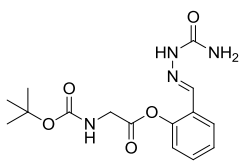
III. Synthesis of C-terminus '1' L-Amino salicylaldehyde semicarbazone ester hydrochloride (HCl·*H₂N*-Xaa-*CO*-SAL^{off}) for n+1 reaction

_N_-(*tert*-Butoxycarbonyl)-L-Alanine salicylaldehyde semicarbazone ester (*BocHN*-Ala-*CO*-SAL^{off})



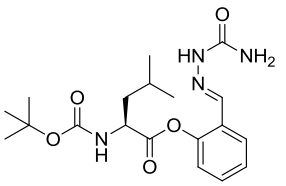
***BocHN*-Ala-*CO*-SAL^{off}** (157.2 mg, 80 % yield) was obtained according to the general procedure C with the use of *BocHN*-Ala-*COOH* (106.1 mg, 561.4 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H₂N*-Ala-*CO*-SAL^{off} (A) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ10.15 (1H, s), 7.99 (1H, s), 7.87 (1H, d, *J* = 7.6 Hz), 7.37 (1H, t, *J* = 7.2 Hz), 7.25 (1H, t, *J* = 7.5 Hz), 7.08 (1H, d, *J* = 7.9 Hz), 6.15 (2H, br), 5.55 (1H, br), 4.60 (1H, br), 1.57 (3H, d, *J* = 7.2 Hz), 1.46 (9H, s) ^{13}C NMR (100 MHz, $CDCl_3$) δ172.5, 158.5, 155.9, 149.4, 137.1, 130.8, 126.7, 126.5, 122.8, 80.8, 49.8, 28.7, 18.3 ESI-MS calcd. for $C_{16}H_{23}N_4O_5$ $[M+H]^+$ *m*/*z*=351.17, found 351.22.

_N_-(*tert*-Butoxycarbonyl)-L-Glycine salicylaldehyde semicarbazone ester (*BocHN*-Gly-*CO*-SAL^{off})



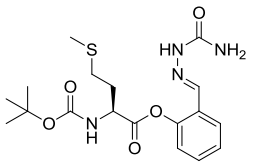
***BocHN*-Gly-*CO*-SAL^{off}** (130.5 mg, 58% yield) was obtained according to the general procedure C with the use of *BocHN*-Gly-*COOH* (117.2 mg, 669.7 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H_2N*-Gly-*CO*-SAL^{off} (G) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (1H, s), 7.95 (1H, s), 7.83 (1H, d, *J* = 7.6 Hz), 7.36 (1H, t, *J* = 7.6 Hz), 7.24 (1H, t, *J* = 7.5 Hz), 7.10 (1H, d, *J* = 8.1 Hz), 6.18 (2H, br), 5.68 (1H, s), 4.22 (2H, m), 1.44 (9H, s) ^{13}C NMR (75 MHz, $CDCl_3$) δ169.7, 158.2, 156.4, 149.3, 137.4, 131.0, 126.9, 126.6, 122.9, 80.9, 42.9, 28.7 ESI-MS calcd. for $C_{15}H_{21}N_4O_5$ $[M+H]^+$ *m*/*z*=337.15, found 337.19.

***N*-(*tert*-Butoxycarbonyl)-L-Leucine salicylaldehyde semicarbazone ester (*BocHN*-Leu-*CO*-SAL^{off})**



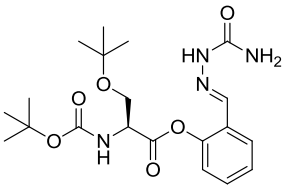
***BocHN*-Leu-*CO*-SAL^{off}** (107.5 mg, 64% yield) was obtained according to the general procedure C with the use of *BocHN*-Leu-*COOH* (99.0 mg, 428.6 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H_2N*-Leu-*CO*-SAL^{off} (L) as a white solid. Without any purification, this salt was subjected to the n+1 reaction.^{1}H NMR (400 MHz, $CDCl_3$) δ 9.31 (1H, s), 8.09 (1H, s), 7.89 (1H, dd, *J* = 7.8, 1.2 Hz), 7.41 (1H, td, *J* = 8.0, 1.6 Hz), 7.29 (1H, t, *J* = 7.6 Hz), 7.08 (1H, d, *J* = 8.1 Hz), 6.17 (2H, br), 5.14 (1H, d, *J* = 7.7 Hz), 4.51 (1H, m), 1.83 (1H, m), 1.69 (1H, m), 1.45 (9H, s), 1.03 (6H, m) ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6, 157.6, 156.2, 149.6, 137.4, 130.9, 126.8, 126.4, 122.7, 80.9, 52.8, 41.2, 28.7, 25.3, 23.3, 22.1 ESI-MS calcd. for $C_{19}H_{29}N_4O_5$ $[M+H]^+$ *m*/*z*=393.21, found 393.37

***N*-(*tert*-Butoxycarbonyl)-L-Methionine salicylaldehyde semicarbazone ester (*BocHN*-Met-*CO*-SAL^{off})**



***BocHN*-Met-*CO*-SAL^{off}** (84.9 mg, 48% yield) was obtained according to the general procedure C with the use of *BocHN*-Met-*COOH* (107.5 mg, 431.7 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H*$_2$*N*-Met-*CO*-SAL^{off} (M) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.96 (1H, s), 8.02 (1H, s), 7.88 (1H, d, *J* = 7.7 Hz), 7.40 (1H, t, *J* = 8.2 Hz), 7.29 (1H, t, *J* = 7.6 Hz), 7.11 (1H, d, *J* = 8.0 Hz), 6.23 (2H, br), 5.51 (1H, d, *J* = 7.8 Hz), 4.70 (1H, m), 2.68 (2H, m), 2.34 (1H, m), 2.17 (4H, m), 1.46 (9H, s) ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 157.2, 155.8, 149.1, 136.7, 130.6, 126.6, 126.3, 126.1, 122.4, 80.8, 53.1, 31.2, 30.2, 28.4, 15.6 ESI-MS calcd. for $C_{18}H_{27}N_4O_5S$ $[M+H]^+$ *m*/*z*=411.17, found 411.30.

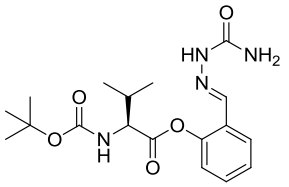
***N*-(*tert*-Butoxycarbonyl)-*O*-*tert*-butyl-L-Serine salicylaldehyde semicarbazone ester (*BocHN*-Ser(O^{t}Bu)-*CO*-SAL^{off})**



***BocHN*-Ser(O^{t}Bu)-*CO*-SAL^{off}** (66.8 mg, 40% yield) was obtained according to the general procedure C with the use of *BocHN*-Ser(O^{t}Bu)-*COOH* (103.3 mg, 395.7 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford

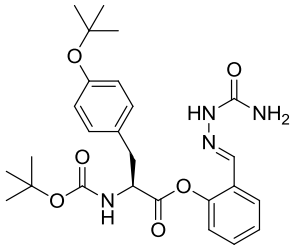
HCl·*H*$_2$*N*-Ser-*CO*-SAL^{off} (S) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.73 (1H, s), 8.03 (1H, s), 7.87 (1H, d, *J* = 7.7 Hz), 7.41 (1H, td, *J* = 7.9, 1.5 Hz), 7.28 (1H, t, *J* = 7.4 Hz), 7.08 (1H, d, *J* = 8.1 Hz), 6.31 (2H, br), 5.61 (1H, d, *J* = 8.6 Hz), 4.66 (1H, d, *J* = 8.2 Hz), 4.02 (1H, dd, *J* = 9.0, 2.8 Hz), 3.70 (1H, dd, J = 9.1, 3.0 Hz), 1.46 (9H, s), 1.22 (9H, s) ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2, 157.7,156.3, 149.6, 137.3,130.8, 126.7, 126.5, 122.9, 80.8, 74.1, 62.5, 54.9, 28.7, 27.7 ESI-MS calcd. for $C_{20}H_{31}N_4O_6$ $[M+H]^+$ *m*/*z*=423.22, found 423.30.

_N_-(_tert_-Butoxycarbonyl)-L-Valine salicylaldehyde semicarbazone ester (_BocHN_-Val-_CO_-SAL^{off})



***BocHN*-Val-*CO*-SAL^{off}** (45.2 mg, 24% yield) was obtained according to the general procedure C with the use of *BocHN*-Val-*COOH* (108.1 mg, 498.2 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H*$_2$*N*-Val-*CO*-SAL^{off} (V) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.45 (1H, s), 8.09 (1H, s), 7.89 (1H, dd, *J* = 7.8, 1.1 Hz), 7.42 (1H, td, *J* = 7.9, 1.5 Hz), 7.29 (1H, t, *J* = 7.6 Hz), 7.06 (1H, d, *J* = 8.5 Hz), 6.20 (2H, br), 5.10 (1H, d, *J* = 8.2 Hz), 4.40 (1H, dd, J = 8.1, 5.3 Hz), 2.32 (1H, m), 1.46 (9H, s), 1.12 (3H, d, *J* = 6.8 Hz), 1.06 (3H, d, *J* = 6.8 Hz) ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 158.2, 156.4, 149.4, 137.2, 130.7, 126.8, 126.7, 126.5, 122.7, 80.7, 59.5, 31.2, 28.7, 19.6, 18.0 ESI-MS calcd. for $C_{18}H_{27}N_4O_5$ $[M+H]^+$ *m*/*z*=379.43, found 379.27.

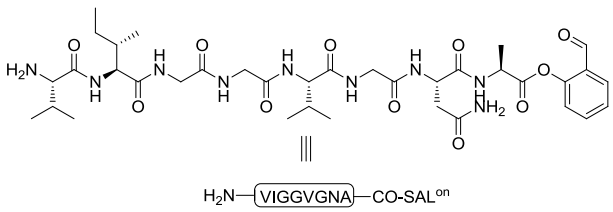
_N_-(_tert_-Butoxycarbonyl)-_O_-_tert_-butyl-L-Tyrosine salicylaldehyde semicarbazone ester (_BocHN_-Tyr(O^{t}Bu)-_CO_-SAL^{off})



***BocHN*-Tyr(O^{t}Bu)-*CO*-SAL^{off}** (115.1 mg, 68% yield) was obtained according to the general procedure C with the use of *BocHN*-Tyr(O^{t}Bu)-*COOH* (114.5 mg, 339.8 μmol). This compound was then treated with a solution of HCl/dioxane (4 M) and stirred for 2 h. The solvent was removed by blowing a stream of condensed air and the residue triturated with diethyl ether (× 2) and dried under vacuum to afford HCl·*H$_2$N*-Tyr-*CO*-SAL^{off} (Y) as a white solid. Without any purification, this salt was subjected to the n+1 reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.44 (1H, s), 7.85 (1H,s), 7.36 (1H, t, *J* = 7.5 Hz), 7.26 (1H, m), 7.17 (1H, d, *J* = 8.3 Hz), 6.98 (2H, d, *J* = 8.0 Hz), 6.89 (1H, d, *J* = 8.0 Hz), 6.09 (2H, br), 5.25 (1H, d, *J* = 7.1 Hz), 4.76 (1H, m), 3.18 (2H, m), 1.42 (9H, s), 1.35 (9H, s) ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 157.2, 155.9, 155.0, 149.4, 137.2, 130.8, 130.3, 126.7, 126.6, 126.3, 124.7, 122.7, 80.9, 79.0, 55.3, 37.6, 29.2, 28.7 ESI-MS calcd. for $C_{26}H_{35}N_4O_6$ $[M+H]^+$ *m*/*z*=499.26, found 499.21.

IV. Synthesis of model peptide SAL^{on} and SAL^{off} ester by n+1 strategy

Peptide 1a: *H$_2$N*-VIGGVGNA-*CO*-SAL^{on} ester



Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (30.3 mg, 31.7 μmol) was dissolved in $CHCl_3$/trifluoroethanol (2.0 mL) and reacted with

HCl·H_2N-Ala-*CO*-SAL^{off} (A) (27.2 mg, 95.1 µmol) in the presence of EDC (14.7 mg, 95.1 µmol) and HOOBt (15.5 mg, 95.1 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (223.3 µL, 3.2 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1a** (22.0 mg, 88% yield) as a white powder.

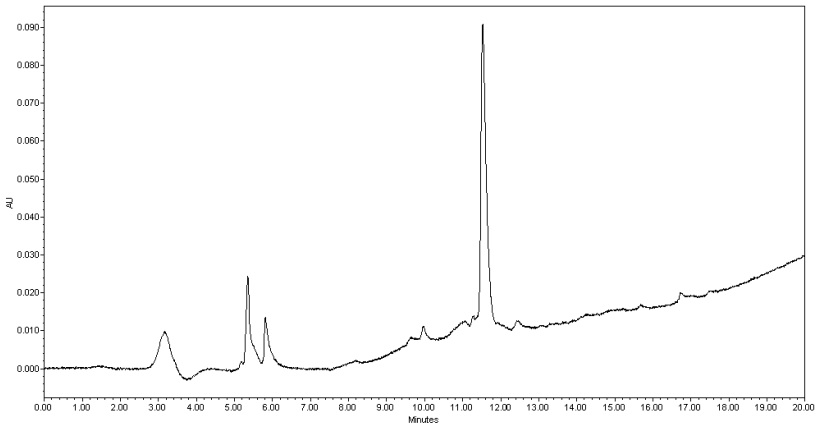


Figure S1. UV trace from analytical LC-MS analysis of purified peptide **1a**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

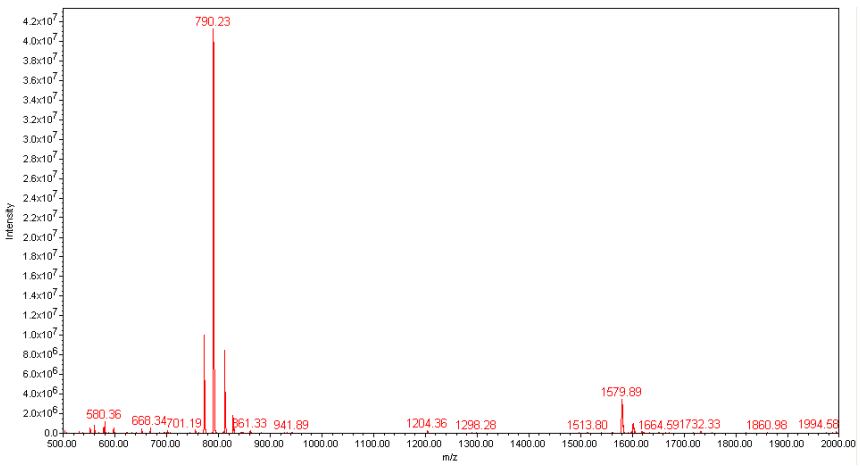
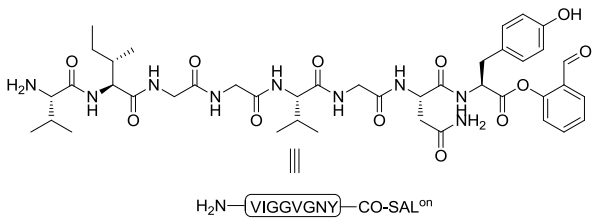


Figure S2. ESI-MS calcd. for $C_{36}H_{56}N_9O_{11}$ $[M+H]^+$ *m*/*z* = 790.41, found 790.23; $[2M+H]^+$ *m*/*z* = 1579.81, found 1579.89.

Peptide 1b: H_2N-VIGGVGNY-*CO*-SAL^{on} ester



Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (27.2 mg, 28.5 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.9 mL) and reacted with **HCl·*H*$_2$*N*-Tyr-*CO*-SAL^{off} (Y)** (32.3 mg, 85.4 µmol) in the presence of EDC (13.2 mg, 85.4 µmol) and HOOBt (13.9 mg, 85.4 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (200.4 µL, 2.9 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1b** (17.3 mg, 69% yield) as a white powder.

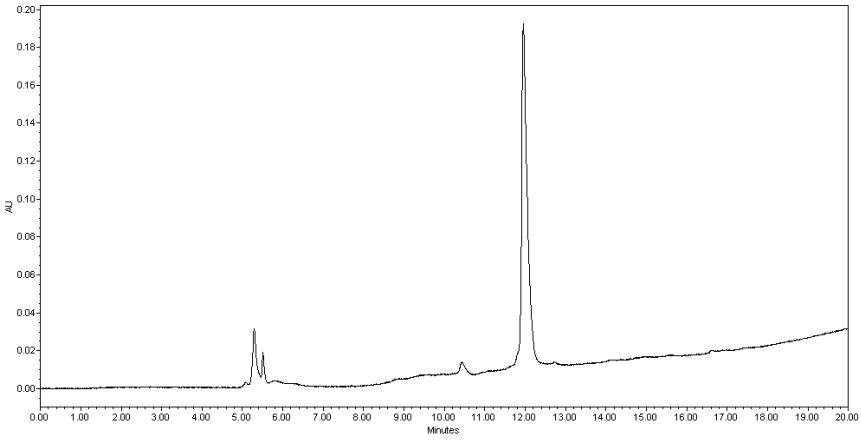


Figure S3. UV trace from analytical LC-MS analysis of purified peptide **1b**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

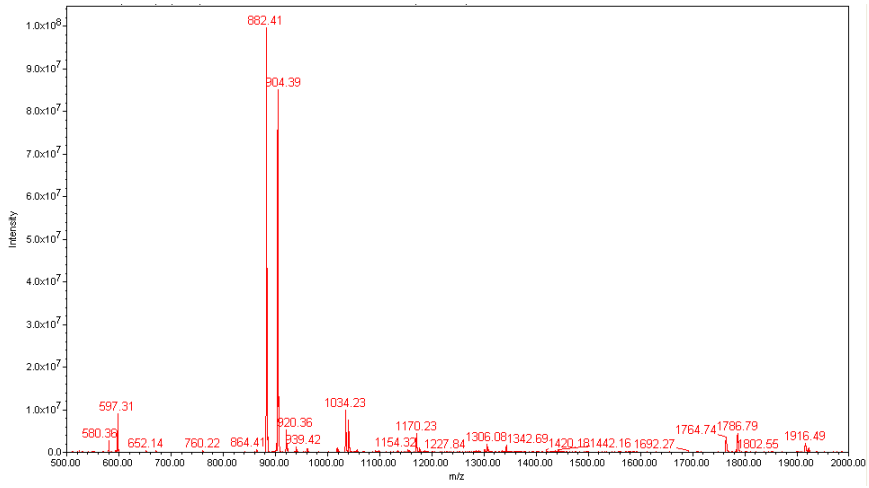
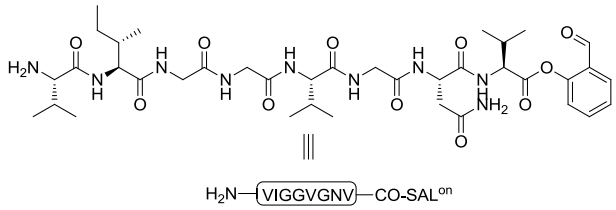


Figure S4. ESI-MS calcd. for $C_{42}H_{60}N_9O_{12}$ $[M+H]^+$ *m*/*z* =882.44, found 882.41; $[M+Na]^+$ *m*/*z* = 904.42, found 904.39.

Peptide 1c: *H₂N*-VIGGVGNV-*CO*-SAL^{on} ester



Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (34.7 mg, 36.3 µmol) was dissolved in $CHCl_3$/trifluoroethanol (2.4 mL) and reacted with **HCl·*H₂N*-Val-*CO*-SAL^{off} (V)**(34.2 mg, 108.8 µmol) in the presence of EDC (16.9 mg, 108.8 µmol) and HOOBt (17.7 mg, 108.8 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (255.7 µL, 3.6 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1c** (20.8 mg, 70% yield) as a white powder.

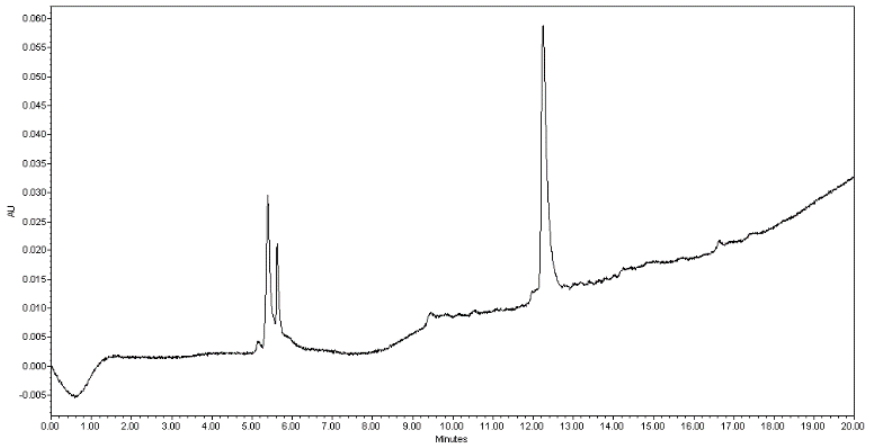


Figure S5. UV trace from analytical LC-MS analysis of purified peptide **1c**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

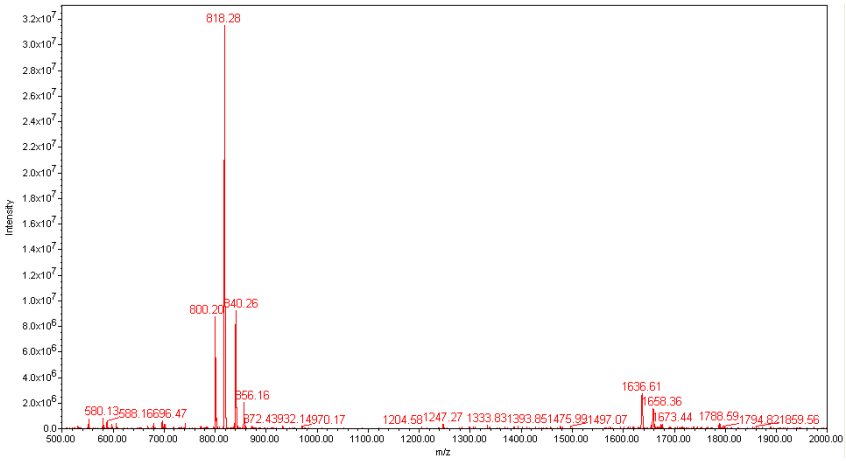
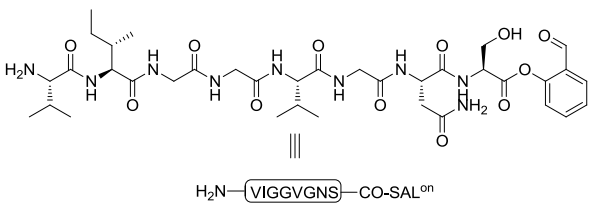


Figure S6. ESI-MS calcd. for $C_{38}H_{60}N_9O_{11}$ $[M+H]^+$ *m/z* =818.44, found 818.28; $[M+Na]^+$ *m/z* =840.42, found 840.26; $[2M+H]^+$ *m/z* =1635.87, found 1636.61; $[2M+Na]^+$ *m/z* =1657.86, found 1658.36

Peptide 1d: *H₂N*-VIGGVGNS-*CO*-SAL^{on} ester



Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (28.5 mg, 29.8 µmol) was dissolved in $CHCl_3$/trifluoroethanol (2.0 mL) and reacted with **HCl·*H*$_2$*N*-Ser-*CO*-SAL^{off} (S)**(27 mg, 89.4 µmol) in the presence of EDC (13.9 mg, 89.4 µmol) and HOOBt (14.6 mg, 89.4 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (210.0 µL, 3.0 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1d** (19.9 mg, 83% yield) as a white powder.

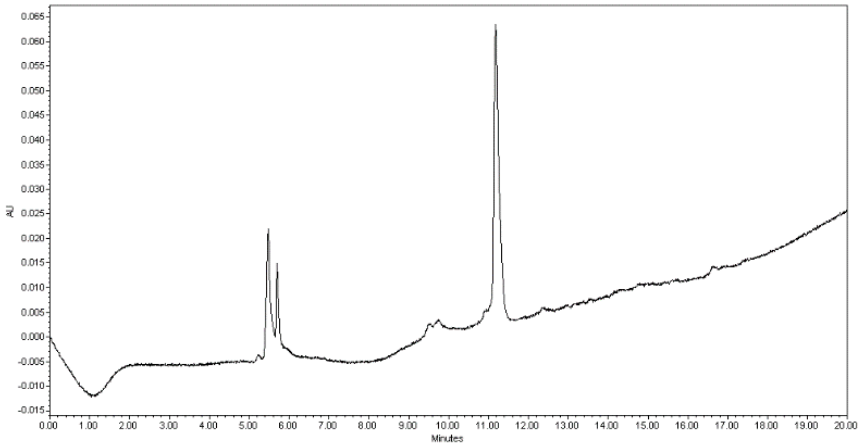


Figure S7. UV trace from analytical LC-MS analysis of purified peptide **1d**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

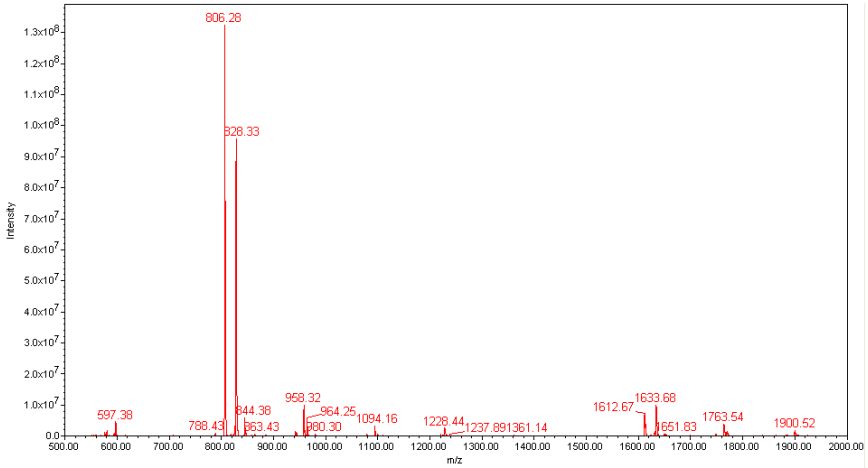
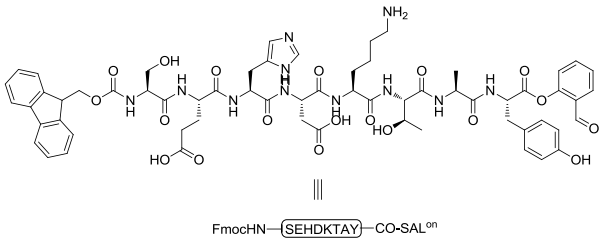


Figure S8. ESI-MS calcd. for $C_{36}H_{56}N_9O_{12}$ $[M+H]^+$ *m*/*z* =806.40, found 806.28; $[M+Na]^+$ *m*/*z* =828.37, found 828.33; $[2M+H]^+$ *m*/*z* =1611.80, found 1612.67; $[2M+Na]^+$ *m*/*z* =1633.78, found 1633.68.

Peptide 1e: *FmocHN*-SEHDKTAY-*CO*-SAL^{on} ester



Crude protected peptide F*mocHN*-Ser(^{t}Bu)-Glu(O^{t}Bu)-His(Trt)-Asp(O^{t}Bu)-Lys(Boc)-Thr(^{t}Bu)-Ala-*COOH* prepared according to general experimental procedure A and B) (248.0 mg, 157.5 µmol) was dissolved in $CHCl_3$/trifluoroethanol (10.5 mL) and reacted with **HCl·*H*$_2$*N*-Tyr-*CO*-SAL^{off} (Y)** (178.6 mg, 472.4 µmol) in the presence of EDC (73.2 mg, 472.4 µmol) and HOOBt (77.0 mg, 472.4 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 7.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (1109.3 µL, 15.7 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1e** (78.4 mg, 39% yield) as a white powder.

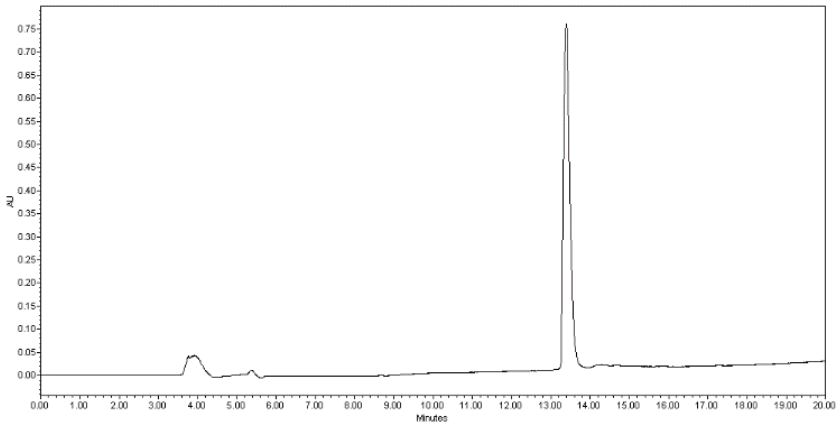


Figure S9. UV trace from analytical LC-MS analysis of purified peptide **1e**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

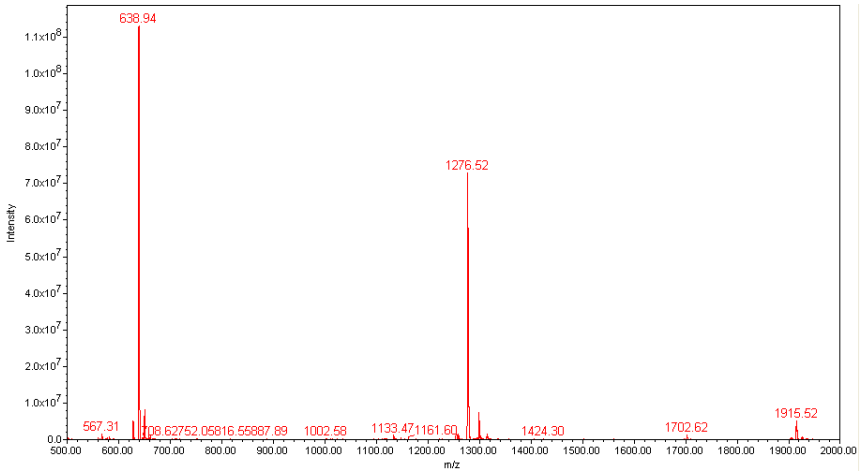
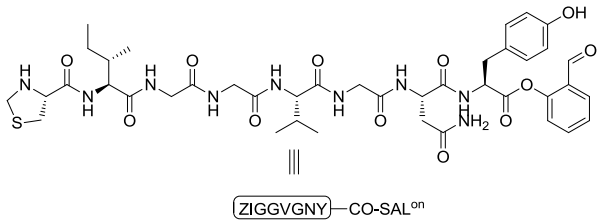


Figure S10. ESI-MS calcd. for $C_{62}H_{74}N_{11}O_{19}$ $[M+H]^+$ *m/z*= 1276.52, found 1276.52; $[M+2H]^{2+}$ *m/z*= 638.76, found 638.94;

Peptide 1f: ZIGGVGNY-*CO*-SAL^{on} ester



Crude protected peptide *BocN*-Thz-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (71.1 mg, 73.1 µmol) was dissolved in $CHCl_3$/trifluoroethanol (4.8 mL) and reacted with **HCl·*H*$_2$*N*-Tyr-*CO*-SAL^{off} (Y)** (83.0 mg, 219.4 µmol) in the presence of EDC (34.0 mg, 219.4 µmol) and HOOBt (35.8 mg, 219.4 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (515.3 µL, 7.3 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1f** (16.4 mg, 25% yield) as a white powder.

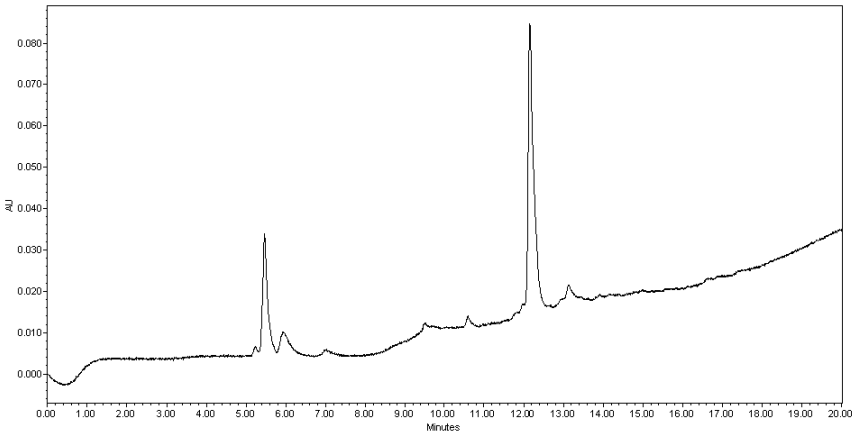


Figure S11. UV trace from analytical LC-MS analysis of purified peptide **1f**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

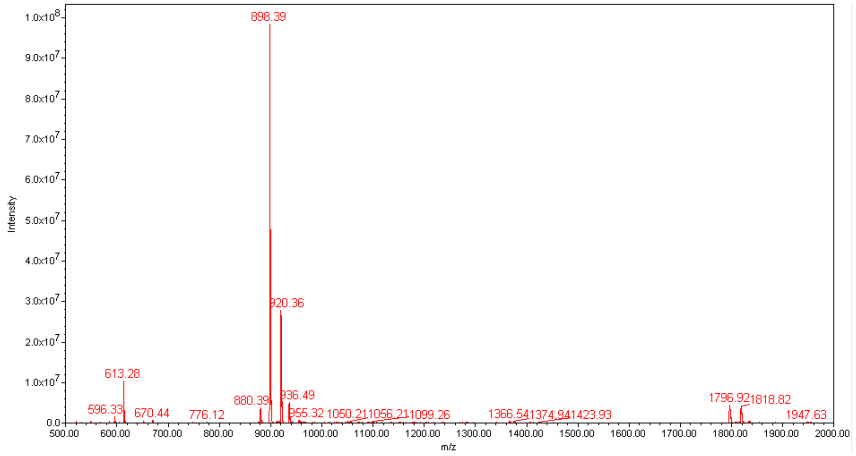
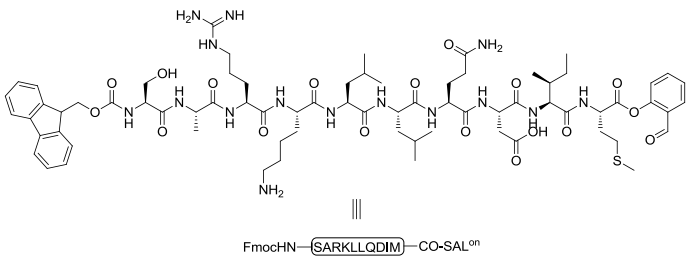


Figure S12. ESI-MS calcd. for $C_{41}H_{56}N_9O_{12}S$ $[M+H]^+$ *m/z* = 898.38, found 898.39; $[M+Na]^+$ *m/z* =920.37, found 920.36; $[2M+H]^+$ *m/z* =1795.75, found 1796.92; $[2M+Na]^+$ *m/z* =1817.73, found 1818.82.

Peptide 1g: *FmocHN*-SARKLLQDIM-*CO*-SAL^{on} ester



Crude protected peptide *FmocHN*-Ser(^{t}Bu)-Ala-Arg(Pbf)-Lys(Boc)-Leu-Leu-Gln(Trt)-Asp(O^{t}Bu)-Ile-OH (prepared according to general experimental procedure A and B) (100.1 mg, 50.8 µmol) was dissolved in $CHCl_3$/trifluoroethanol (3.4 mL) and reacted with **HCl·*H*$_2$*N*-Met-*CO*-SAL^{off} (M)** (52.7 mg, 152.4 µmol) in the presence of EDC (23.6 mg, 152.4 µmol) and HOOBt (24.8 mg, 152.4 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (357.8 µL, 5.1 mmol) for 3 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **1g** (23.6 mg, 31% yield) as a white powder.

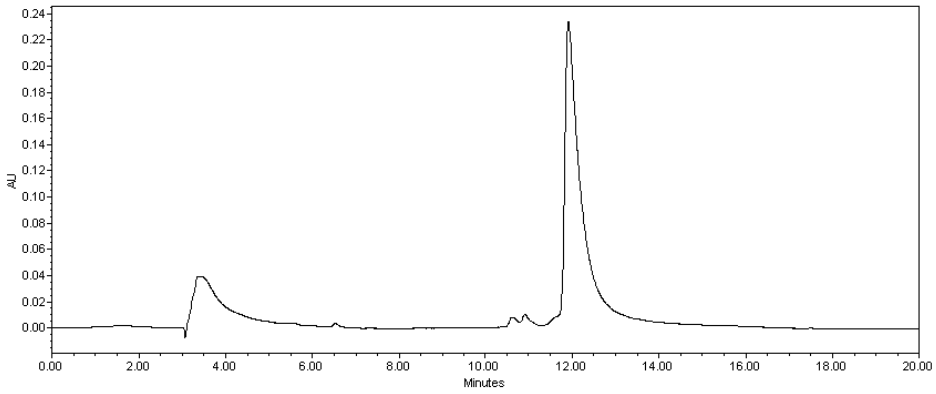


Figure S13. UV trace from analytical LC-MS analysis of purified peptide **1g**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

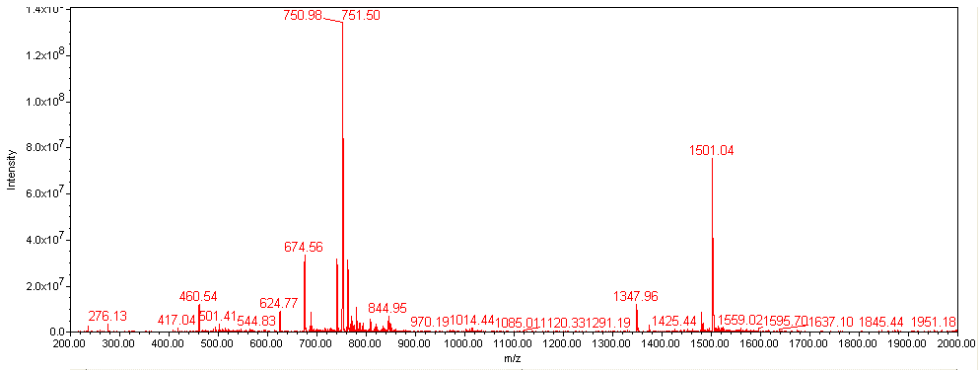
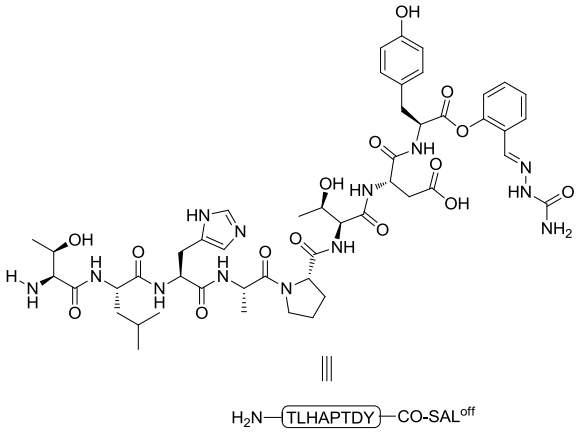


Figure S14. ESI-MS calcd. for $C_{72}H_{105}N_{15}O_{18}S$ $[M+H]^+$ *m*/*z* = 1500.75, found 1501.04; $[M+2H]^{2+}$ *m*/*z* = 750.88, found 750.98.

Peptide 2a: *H*$_2$*N*-TLHAPTDY-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Thr(ᵗBu)-Leu-His(Trt)-Ala-Pro-Thr(ᵗBu)-Asp(OᵗBu)-*COOH* (prepared according to general experimental procedure A and B) (32.9 mg, 26.0 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.7 mL) and reacted with **HCl·*H₂N*-Tyr-*CO*-SAL^{off} (Y)**(29.5 mg, 78.1 µmol) in the presence of EDC (12.1 mg, 78.1 µmol) and HOOBt (12.7 mg, 78.1 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **2a** (17.1 mg, 61% yield) as a white powder.

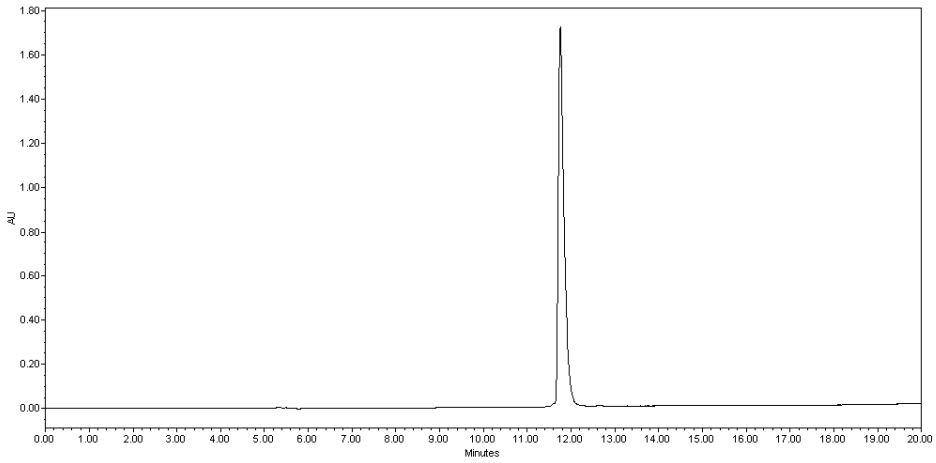


Figure S15. UV trace from analytical LC-MS analysis of purified peptide **2a**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

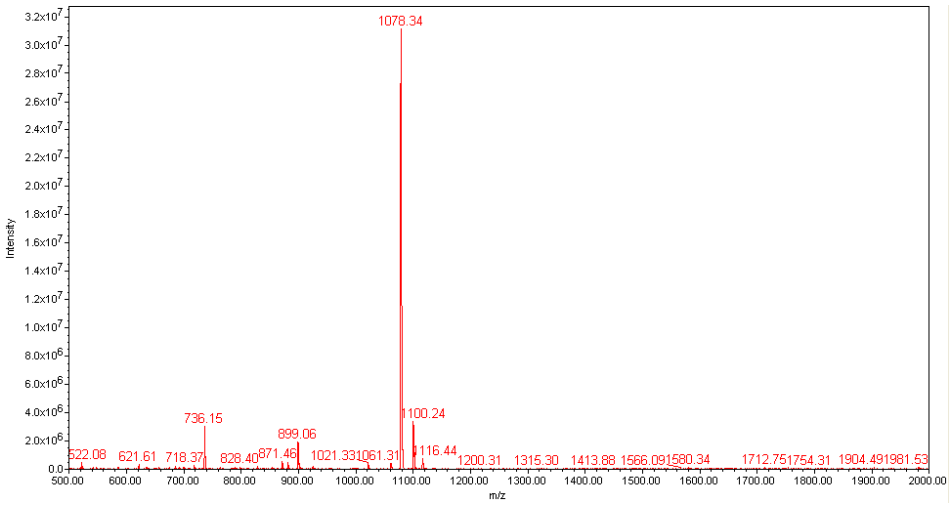
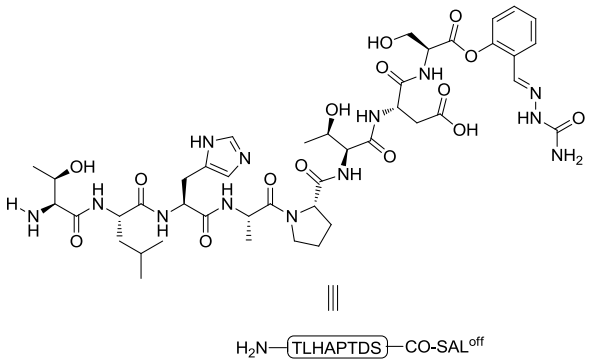


Figure S16. ESI-MS calcd. for $C_{49}H_{68}N_{13}O_{15}$ $[M+H]^+$ *m/z* =1078.50, found 1078.34; $[M+Na]^+$ *m/z* =1100.47, found 1100.24.

Peptide 2b: *H_2N*-TLHAPTDS-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Thr(^{t}Bu)-Leu-His(Trt)-Ala-Pro-Thr(^{t}Bu)-Asp(O^{t}Bu)-*COOH* (prepared according to general experimental procedure A and B) (31.1 mg, 24.6 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.6 mL) and reacted with **HCl·*H_2N*-Ser-*CO*-SAL^{off} (S)**(22.3 mg, 73.8 µmol) in the presence of EDC (11.4 mg, 73.8 µmol) and HOOBt (12.0 mg, 73.8 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **2b** (17.8 mg, 72% yield) as a white powder.

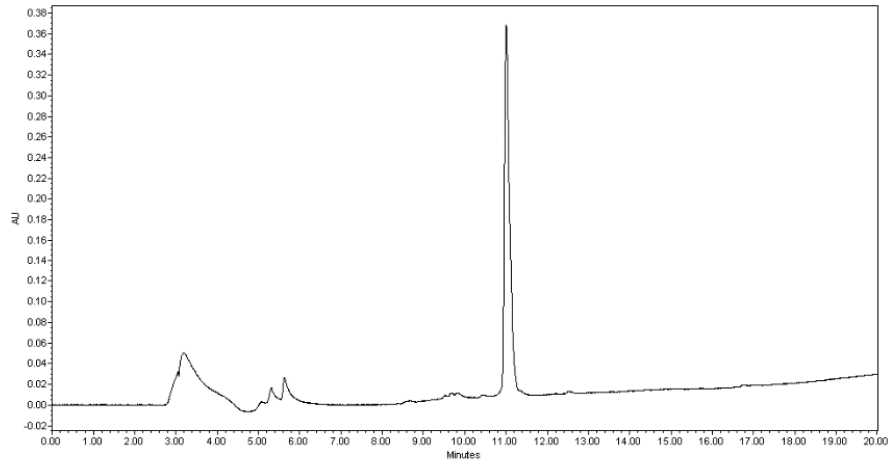


Figure S17. UV trace from analytical LC-MS analysis of purified peptide **2b**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

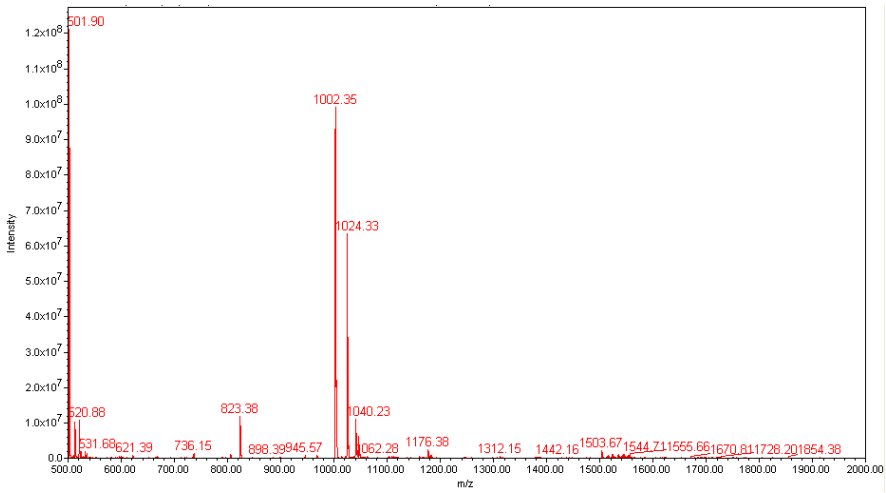
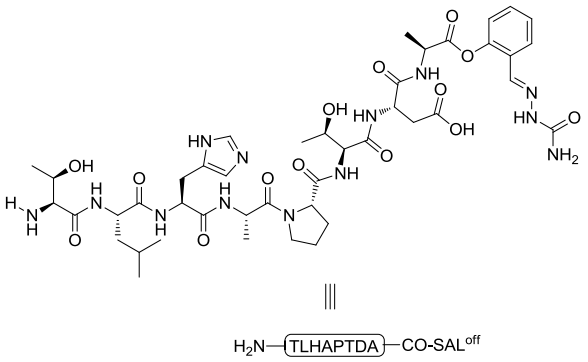


Figure S18. ESI-MS calcd. for $C_{43}H_{64}N_{13}O_{15}$ $[M+H]^+$ m/z = 1002.46, found 1002.35; $[M+Na]^+$ m/z = 1024.45, found 1024.33; $[M+2H]^{2+}$ m/z = 501.74, found 501.90.

Peptide 2c: *H₂N*-TLHAPTDA-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Thr(ᵗBu)-Leu-His(Trt)-Ala-Pro-Thr(ᵗBu)-Asp(Oᵗ Bu)-*COOH* (prepared according to general experimental procedure A and B) (30.8 mg, 24.4 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.6 mL) and reacted with **HCl·*H₂N*-Ala-*CO*-SAL^{off} (A)**(20.9 mg, 73.1 µmol) in the presence of EDC (11.3 mg, 73.1 µmol) and HOOBt (11.9 mg, 73.1 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **2c** (15.9 mg, 66% yield) as a white powder.

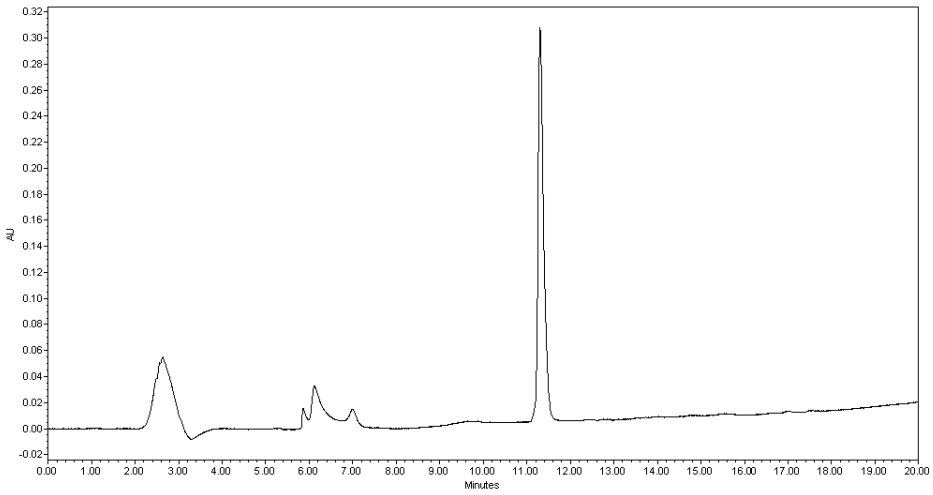


Figure S19. UV trace from analytical LC-MS analysis of purified peptide **2c**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

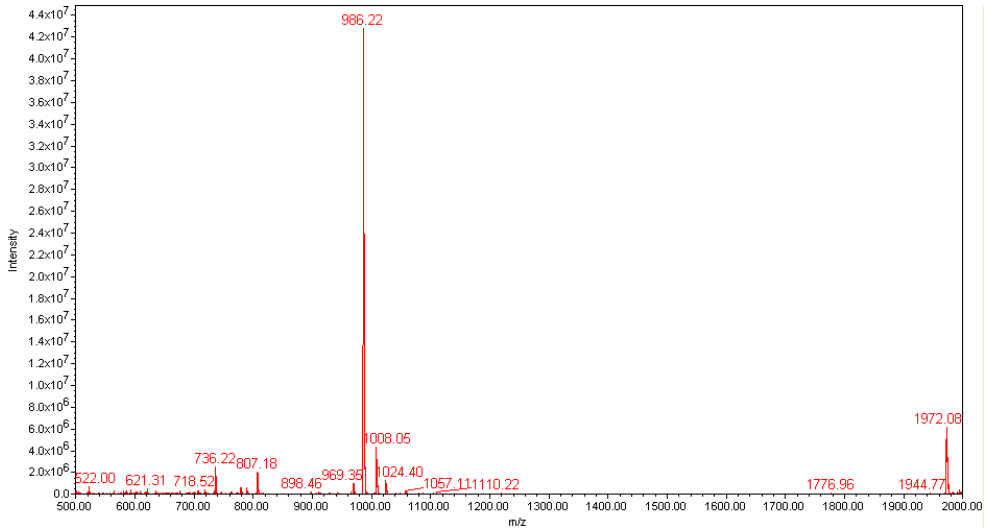
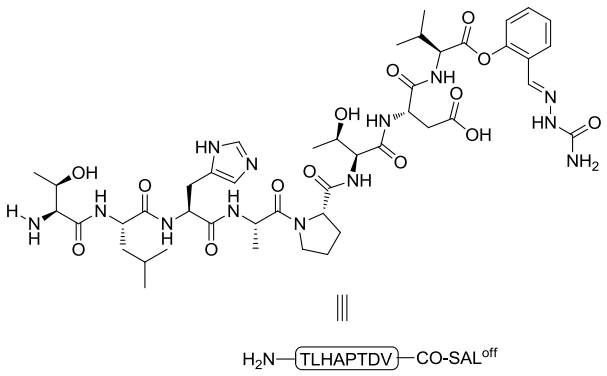


Figure S20. ESI-MS calcd. for $C_{43}H_{64}N_{13}O_{14}$ $[M+H]^+$ *m/z* = 986.47, found 986.22; $[M+Na]^+$ *m/z* = 1008.45, found 1008.05; $[2M+H]^+$ *m/z* = 1971.93, found 1972.08.

Peptide 2d: *H₂N*-TLHAPTDV-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Thr(^{t}Bu)-Leu-His(Trt)-Ala-Pro-Thr(^{t}Bu)-Asp(O^{t}Bu)-*COOH* (prepared according to general experimental procedure A and B) (32.3 mg, 25.6 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.7 mL) and reacted with **HCl·*H₂N*-Val-*CO*-SAL^{off} (V)** (24.1 mg, 76.7 µmol) in the presence of EDC (11.9 mg, 76.7 µmol) and HOOBt (12.5 mg, 76.7 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **2d** (17.9 mg, 69% yield) as a white powder.

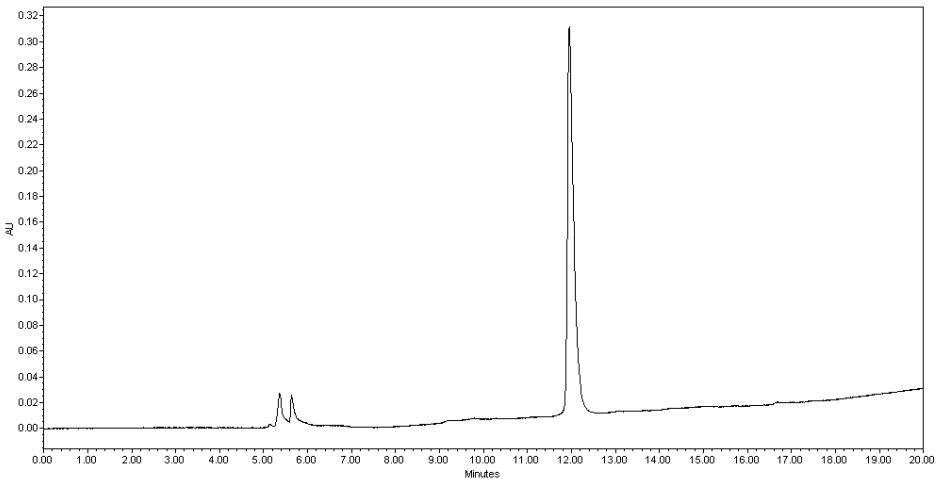


Figure S21. UV trace from analytical LC-MS analysis of purified peptide **2d**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

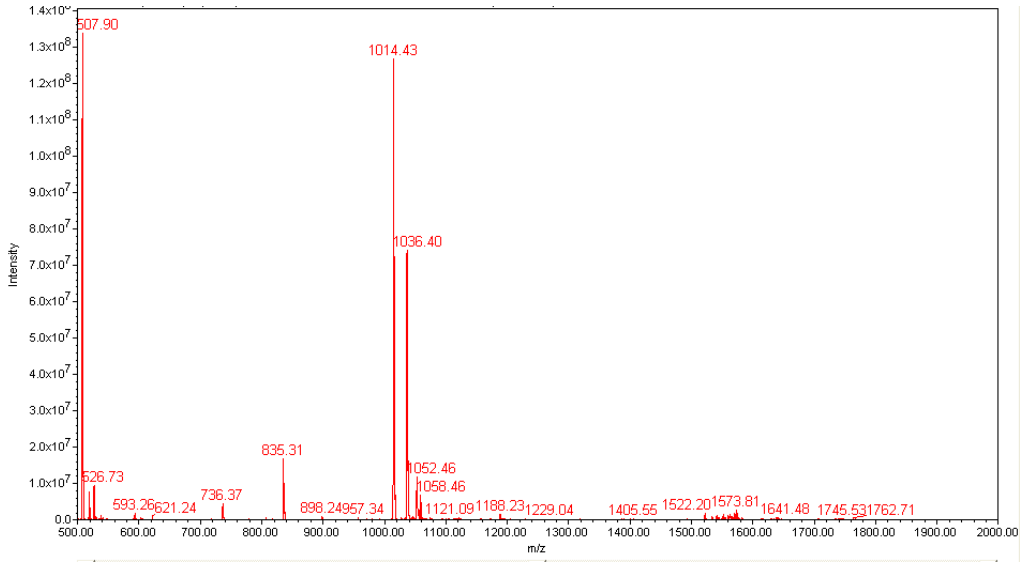
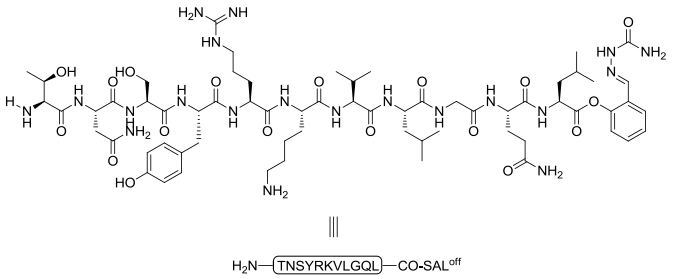


Figure S22. ESI-MS calcd. for $C_{45}H_{68}N_{13}O_{14}$ $[M+H]^+$ m/z = 1014.50, found 1014.43; $[M+Na]^+$ m/z = 1036.48, found 1036.40; $[M+2H]^{2+}$ m/z = 507.75, found 507.90.

Peptide 2e: *H_2N*-TNSYRKVLGQL-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Thr(ᵗBu)-Asn(Trt)-Ser(ᵗBu)-Tyr(ᵗBu)-Arg(Pbf)-Lys(Boc)-Val-Leu-Gly-Gln(Trt)-*COOH* (prepared according to general experimental procedure A and B) (50.0 mg, 22.6 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.5 mL) and reacted with **HCl·*H₂N*-Leu-*CO*-SAL^{off} (L)** (22.2 mg, 67.8 µmol) in the presence of EDC (10.5 mg, 67.8 µmol) and HOOBt (11.0 mg, 67.8 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **2e** (15.9 mg, 49% yield) as a white powder.

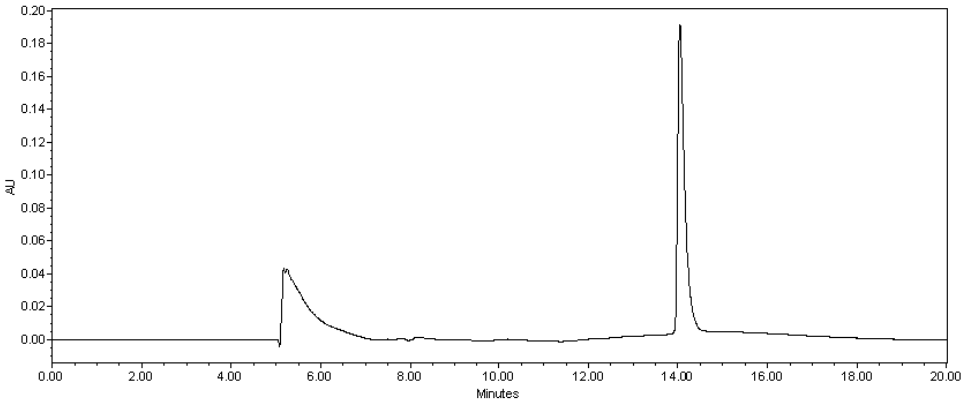


Figure S23. UV trace from analytical LC-MS analysis of purified peptide **2e**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

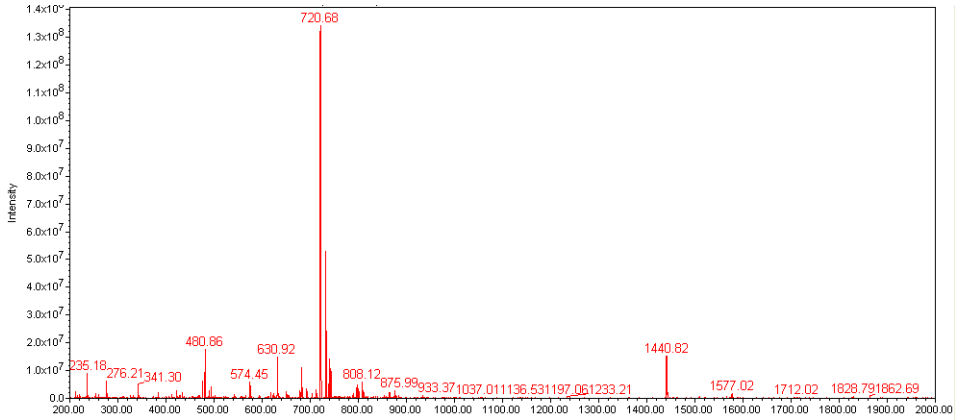
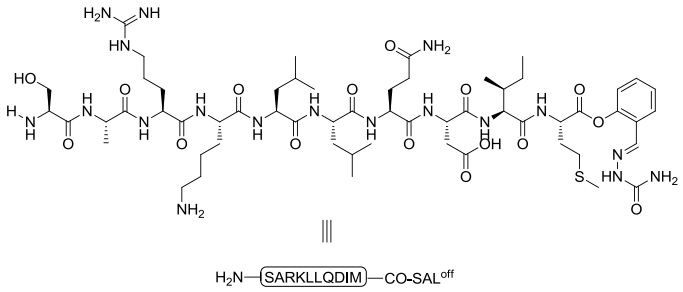


Figure S24. ESI-MS calcd for $C_{64}H_{102}N_{20}O_{18}$ $[M+H]^+$ *m*/*z* = 1439.77, found 1440.82; $[M+2H]^{2+}$ *m*/*z* = 720.39, found 720.68

Peptide 2f: *H₂N*-SARKLLQDIM-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Ser(ᵗBu)-Ala-Arg(Pbf)-Lys(Boc)-Leu-Leu-Gln(Trt)-Asp(Oᵗ Bu)-Ile-*COOH* (prepared according to general experimental procedure A and B) (50.3 mg, 27.2 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.8 mL) and reacted with **HCl·*H₂N*-Met-*CO*-SAL^{off} (M)**(28.3 mg, 81.6 µmol) in the presence of EDC (12.6 mg, 81.6 µmol) and HOOBt (13.3 mg, 81.6 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 2 h. Preparative HPLC purification (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **2f** (17.1 mg, 47% yield) as a white powder.

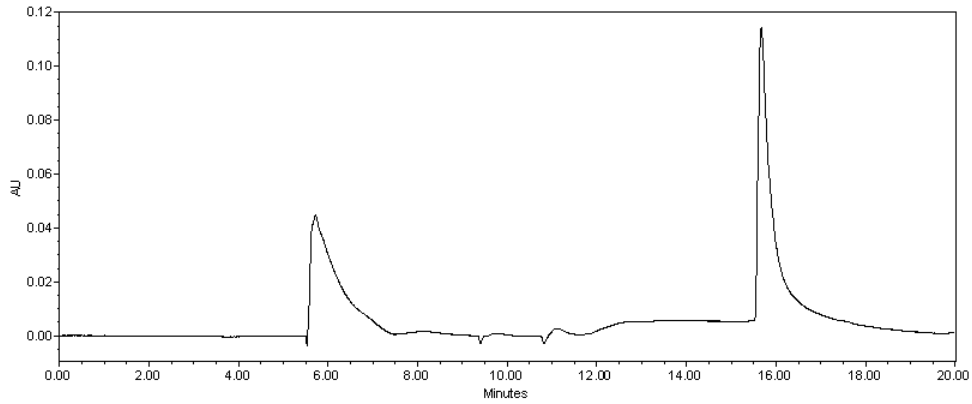


Figure S25. UV trace from analytical LC-MS analysis of purified peptide **2f**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

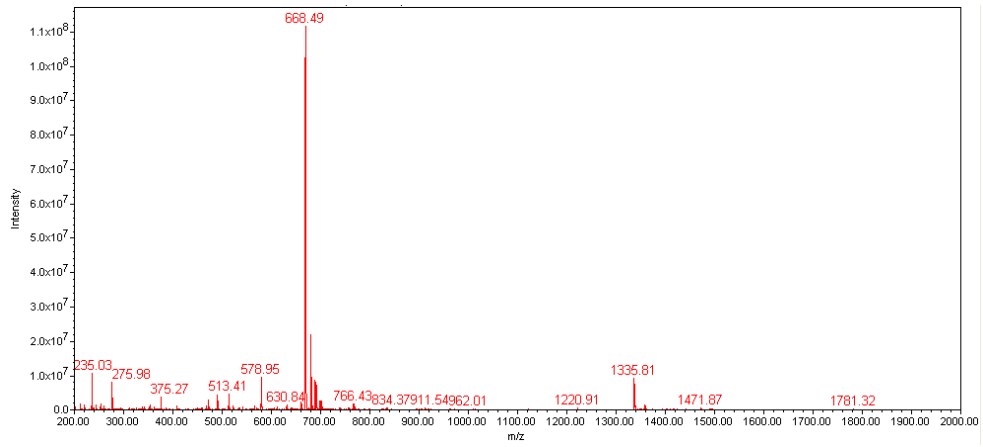
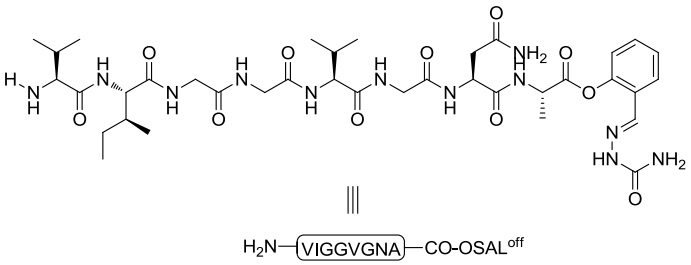


Figure S26. ESI-MS calcd. for $C_{58}H_{99}N_{18}O_{16}S$ $[M+H]^+$ *m/z* = 1335.72, found 1335.81; $[M+2H]^{2+}$ *m/z* = 668.36, found 668.49.

V. Epimerization study of peptide ester generated by n+1 strategy

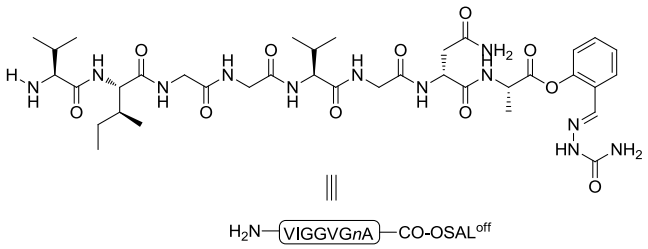
Both epimeric *H₂N*-VIGGVGNA-*CO*-SAL^{off} ester **(2g)** and *H₂N*-VIGGVG*n*A-*CO*-SAL^{off} ester **(2h)** were synthesized by n+1 strategy to demonstrate that no epimerization occurred at the C-terminal Asn residues.

Crude peptide 2g: *H₂N*-VIGGVGNA-*CO*-SAL^{off} ester



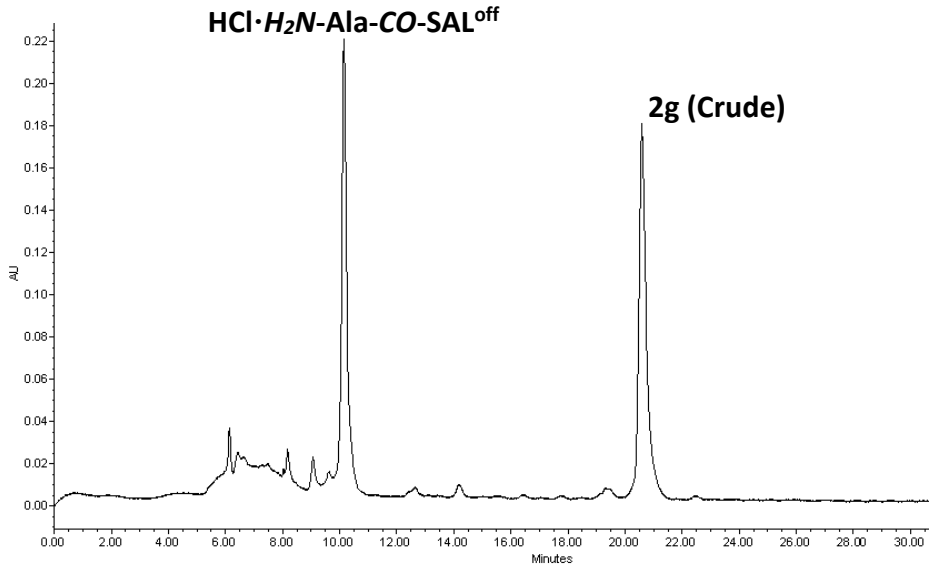
Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-Asn(Trt)-*COOH* (prepared according to general experimental procedure A and B) (20.2 mg, 21.1 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.4 mL) and reacted with **HCl·*H₂N*-Ala-*CO*-SAL^{off} (A)**(18.1 mg, 63.3 µmol) in the presence of EDC (9.8 mg, 63.3 µmol) and HOOBt (10.3 mg, 63.3 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h to obtain crude peptide **2g**: *H₂N*-VIGGVGNA-*CO*-SAL^{off}

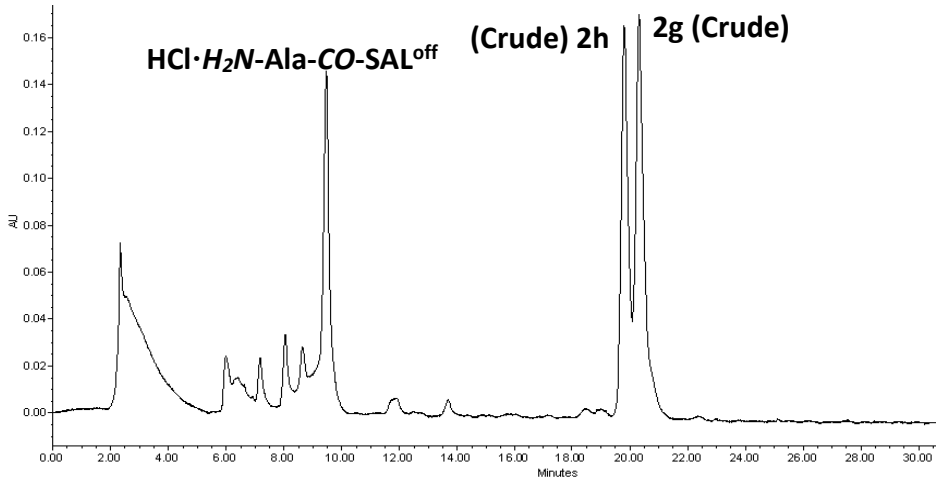
Crude peptide 2h: *H₂N*-VIGGVG*n*A-*CO*-SAL^{off} ester



Crude protected peptide *BocHN*-Val-Ile-Gly-Gly-Val-Gly-*asn*(Trt)-*COOH* (prepared according to general experimental procedure A and B) (20.5 mg, 21.4 µmol) was dissolved in $CHCl_3$/trifluoroethanol (1.4 mL) and reacted with **HCl·*H₂N*-Ala-*CO*-SAL^{off} (A)**(18.4 mg, 64.3 µmol) in the presence of EDC (10.0 mg, 64.3 µmol) and HOOBt (10.5 mg, 64.3 µmol), as described in general procedure D. After stirring for 3 h, the reaction mixture was concentrated and subjected to 5.0 mL of TFA/H_2O (95/5, *v*/*v*) for 1 h to obtain crude peptide **2h**: *H₂N*-VIGGVG*n*A-*CO*-SAL^{off}

Crude peptide **2g** and **2h** and a co-injection of a mixture of crude peptide **2g** and **2h** were analyzed separately by LC-MS. No epimerization was observed for n+1 strategy.





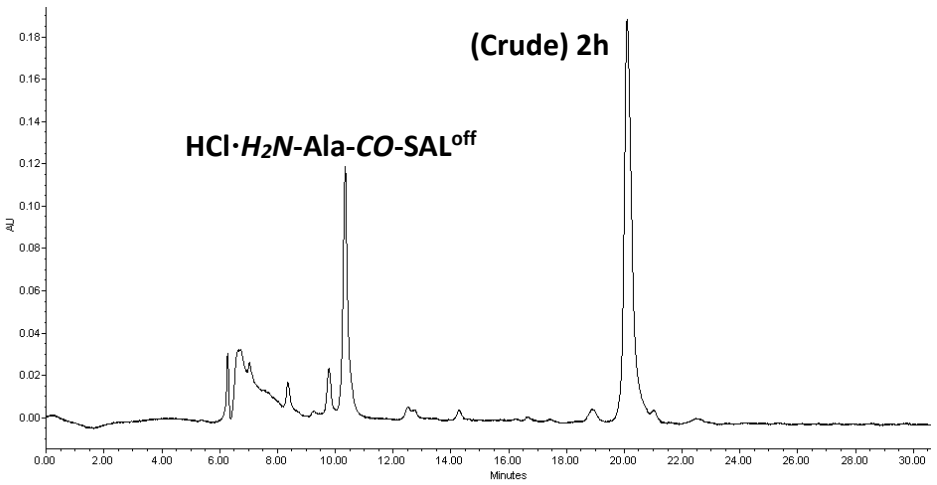
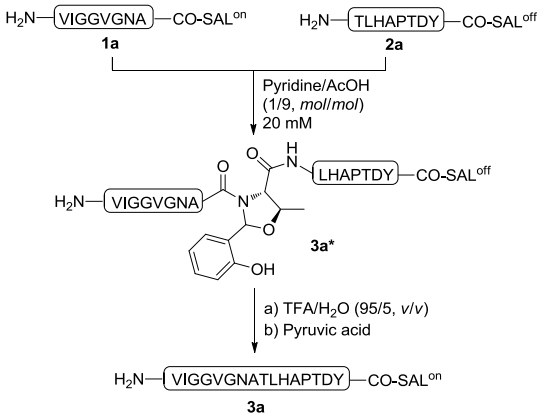


Figure S27. UV trace (190-400 nm) from LC-MS analysis of crude **2g**, a mixture of crude **2g** and **2h** and crude **2h** for epimerization study: gradient 20-30% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

VI. Model *N-to-C* STL study between peptide SAL^{on} ester and peptide SAL^{off} ester to generate ligated peptide SAL^{on} ester through the use of pyruvic acid

Peptide 3a: *H_2N*-VIGGVGNATLHAPTDY-*CO*-SAL^{on} ester



Scheme S1. Model *N-to-C* STL study between peptide **1a** and **2a**.

Peptide **1a** (5.0 mg, 6.3 µmol) and peptide **2a** (10.2 mg, 9.5 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 9 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (4.5 µL, 63.4 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 4.2 mg (39 % yield) of peptide **3a** as a white powder.

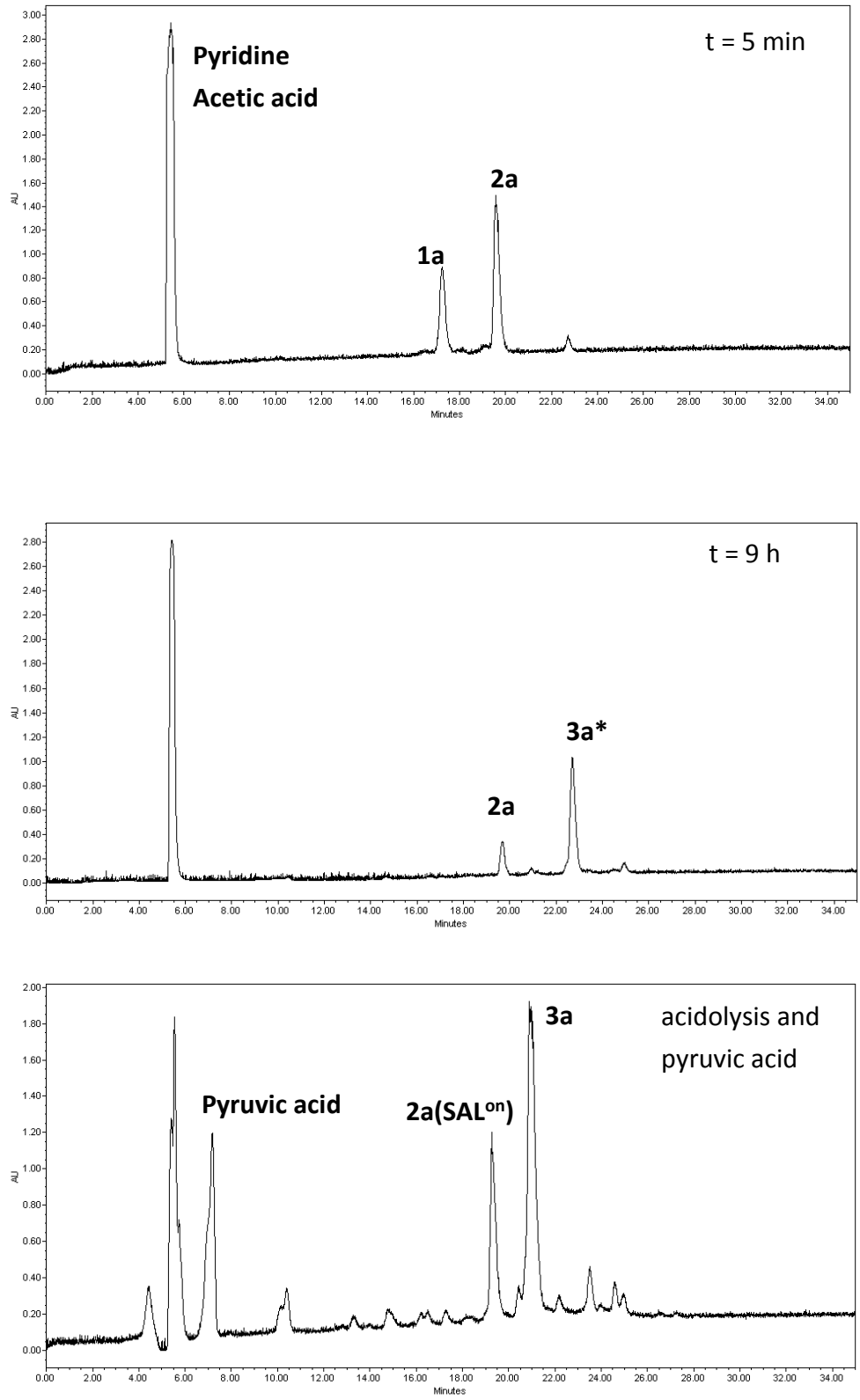


Figure S28. UV trace (190-400 nm) from LC-MS analysis of STL between **1a** and **2a** at 5 min, 9 h to generate **3a*** and after *in situ* acidolysis and pyruvic acid to give **3a**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

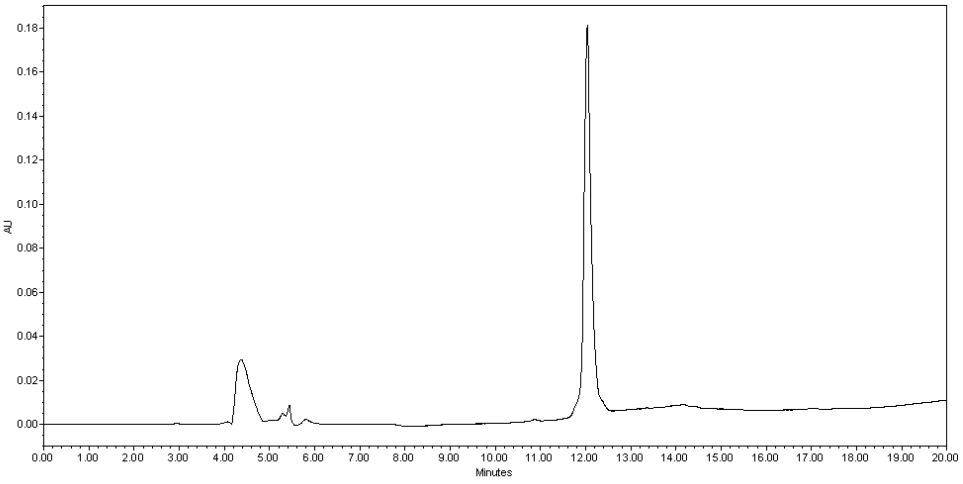


Figure S29. UV trace from analytical LC-MS analysis of purified peptide **3a**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

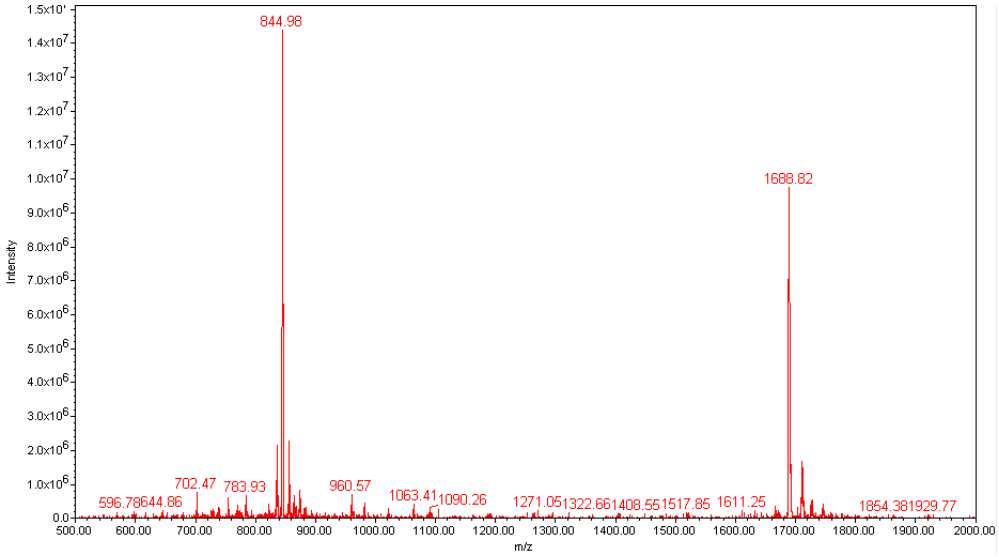
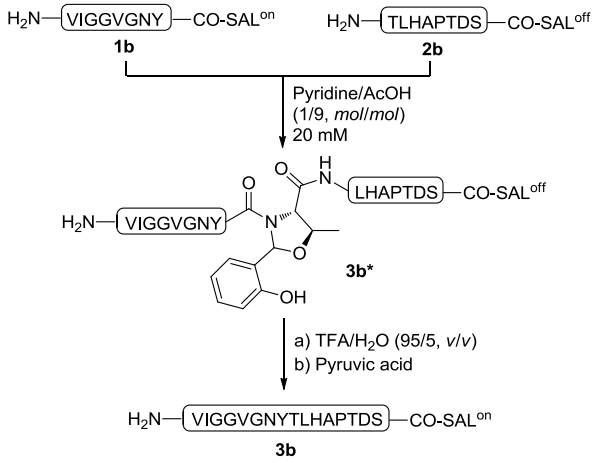


Figure S30. ESI-MS calcd. for $C_{77}H_{114}N_{19}O_{24}$ $[M+H]^+$ *m/z* = 1688.83, found 1688.82; $[M+2H]^{2+}$ *m/z* = 844.92, found 844.98

Peptide 3b: *H₂N*-VIGGVGNYTLHAPTDS-*CO*-SAL^{on} ester



Scheme S2. Model *N-to-C* STL study between peptide **1b** and **2b**.

Peptide **1b** (5.0 mg, 5.7 µmol) and peptide **2b** (8.5 mg, 8.5 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 3 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (4.0 µL, 56.8 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 2.9 mg (30 % yield) of peptide **3b** as a white powder.

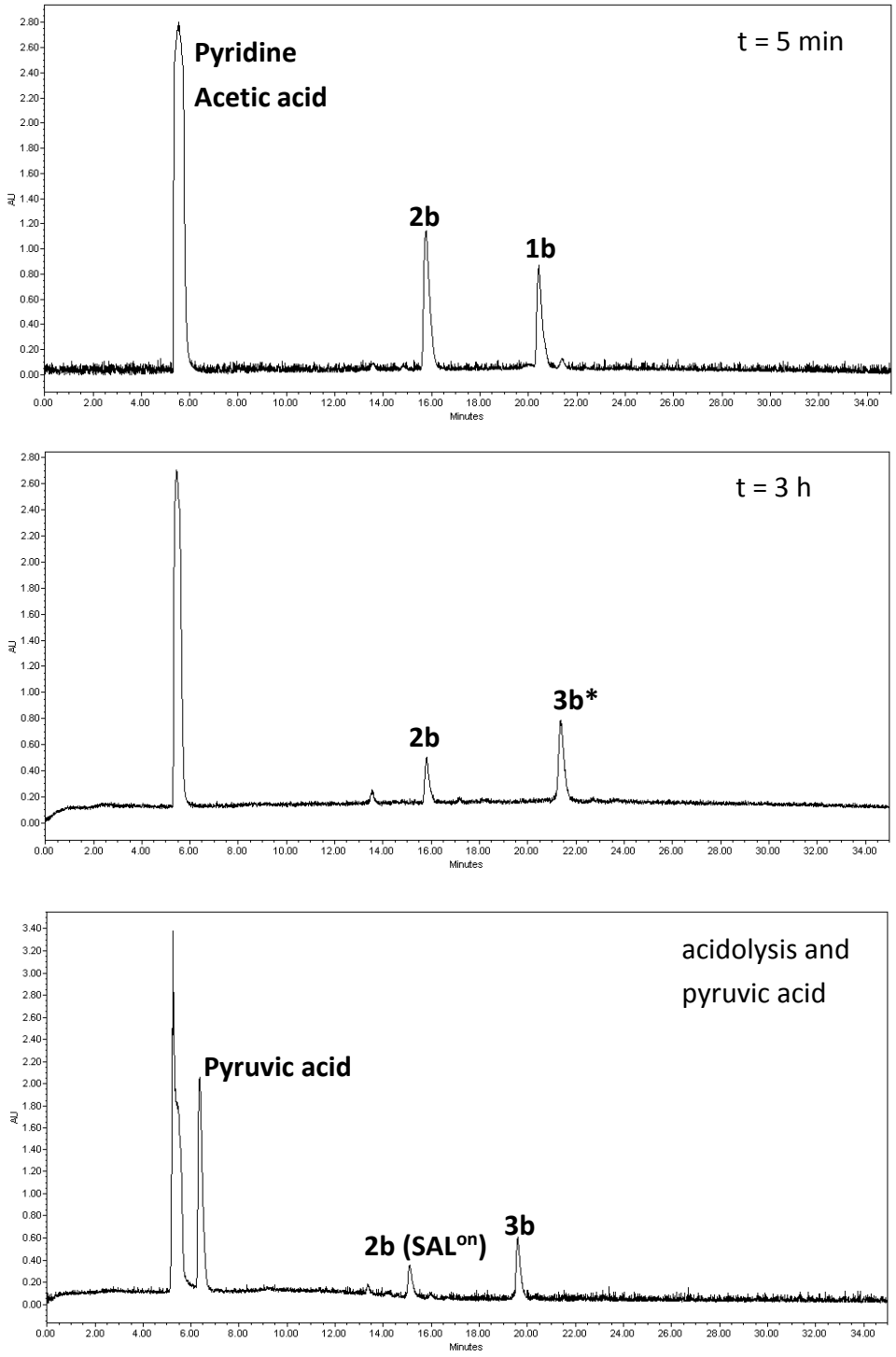


Figure S31. UV trace (190-400 nm) from LC-MS analysis of STL between **1b** and **2b** at 5 min, 3 h to generate **3b*** and after *in situ* acidolysis and pyruvic acid to give **3b**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

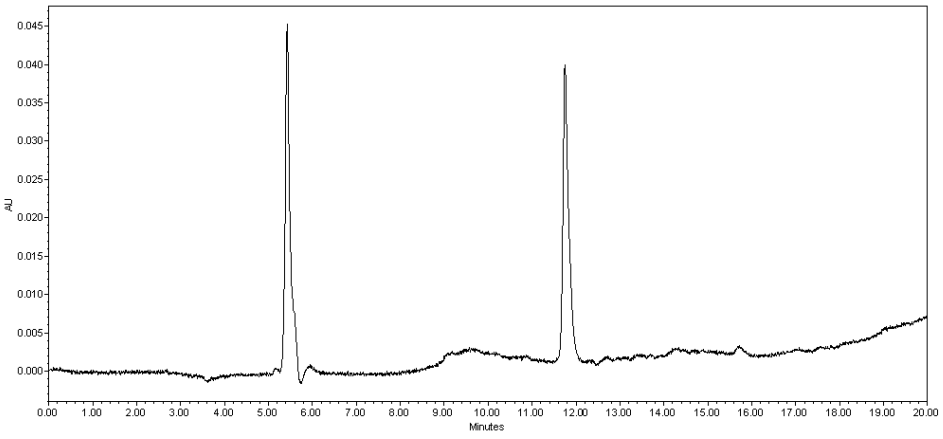


Figure S32. UV trace from analytical LC-MS analysis of purified peptide **3b**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

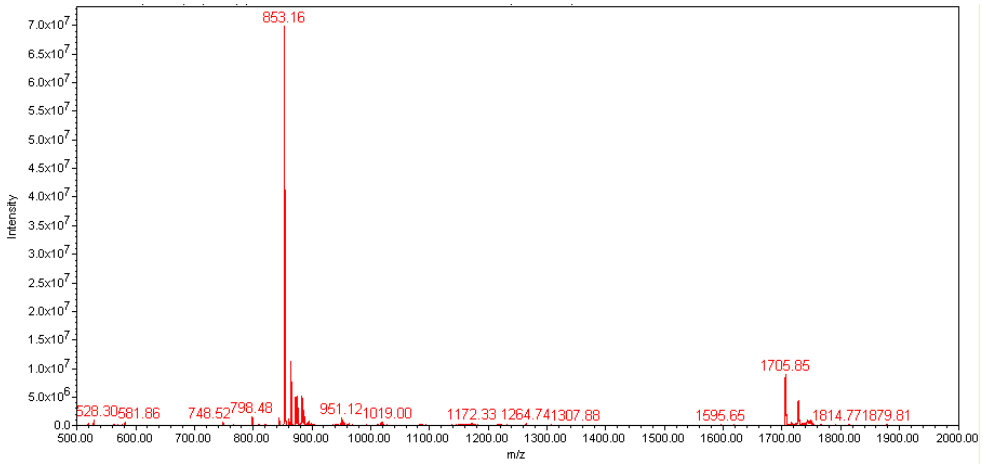
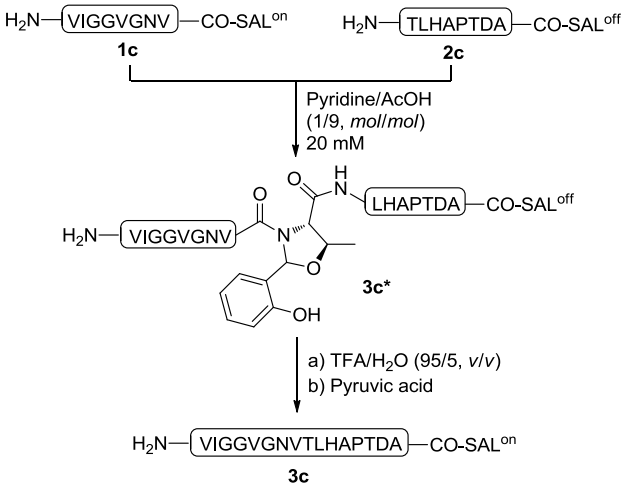


Figure S33. ESI-MS calcd. for $C_{77}H_{114}N_{19}O_{25}$ $[M+H]^+$ m/z = 1705.84, found 1705.85; $[M+2H]^{2+}$ m/z = 853.42, found 853.16.

Peptide 3c: *H₂N*-VIGGVGNVTLHAPTDA-*CO*-SAL^{on} ester



Scheme S3. Model *N-to-C* STL study between peptide **1c** and **2c**.

Peptide **1c** (5.0 mg, 6.1 µmol) and peptide **2c** (9.0 mg, 9.2 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 9 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (4.2 µL, 60.2 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 3.4 mg (34 % yield) of peptide **3a** as a white powder.

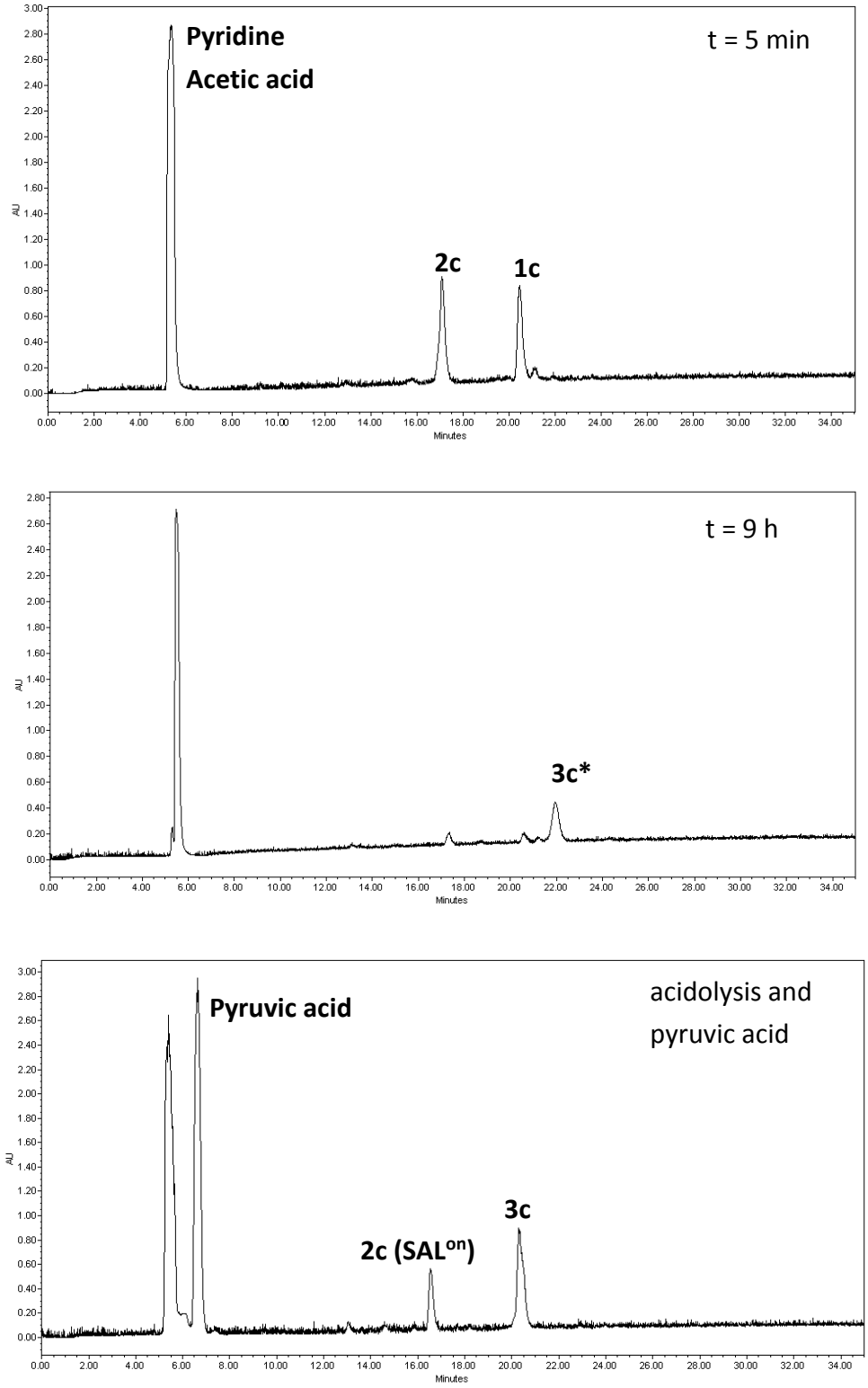


Figure S34. UV trace (190-400 nm) from LC-MS analysis of STL between **1c** and **2c** at 5 min, 9 h to generate **3c*** and after *in situ* acidolysis and pyruvic acid to give **3c**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

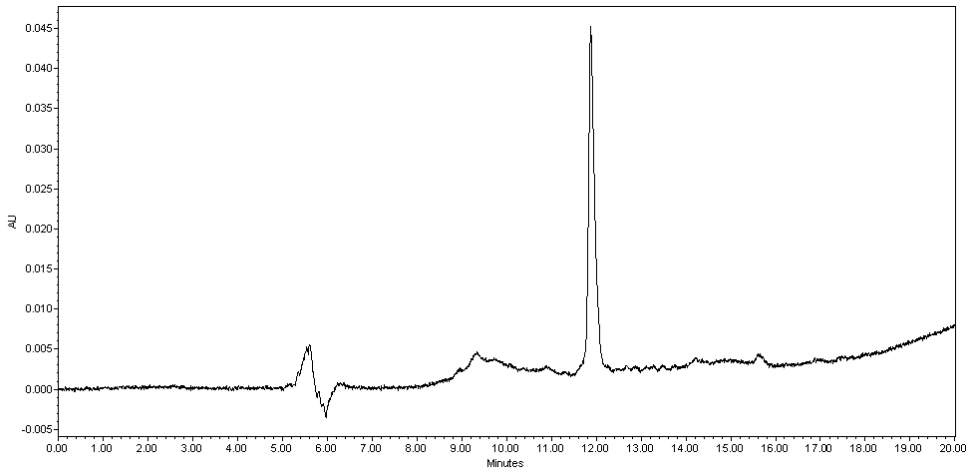


Figure S35. UV trace from analytical LC-MS analysis of purified peptide **3c**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

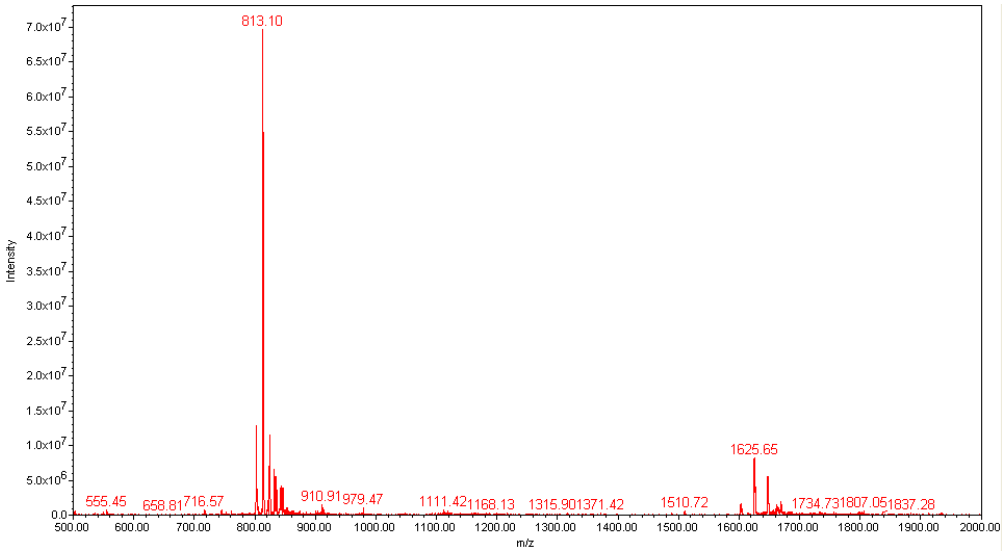
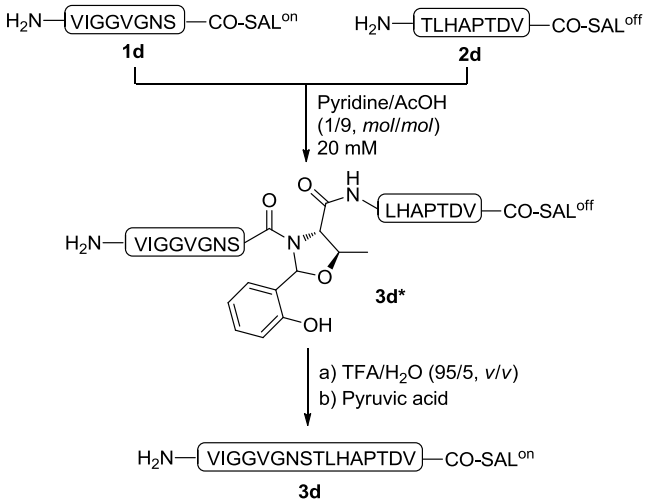


Figure S36. ESI-MS calcd. for $C_{73}H_{114}N_{19}O_{23}$ $[M+H]^+$ m/z = 1625.80, found 1625.65; $[M+2H]^{2+}$ m/z = 813.40, found 813.10.

Peptide 3d: *H₂N*-VIGGVGNSTLHAPTDV-*CO*-SAL^{on} ester



Scheme S4. Model *N-to-C* STL study between peptide **1d** and **2d**.

Peptide **1d** (5.0 mg, 6.2 µmol) and peptide **2d** (9.4 mg, 9.3 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (4.4 µL, 60.2 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 4.3 mg (42 % yield) of peptide **3d** as a white powder.

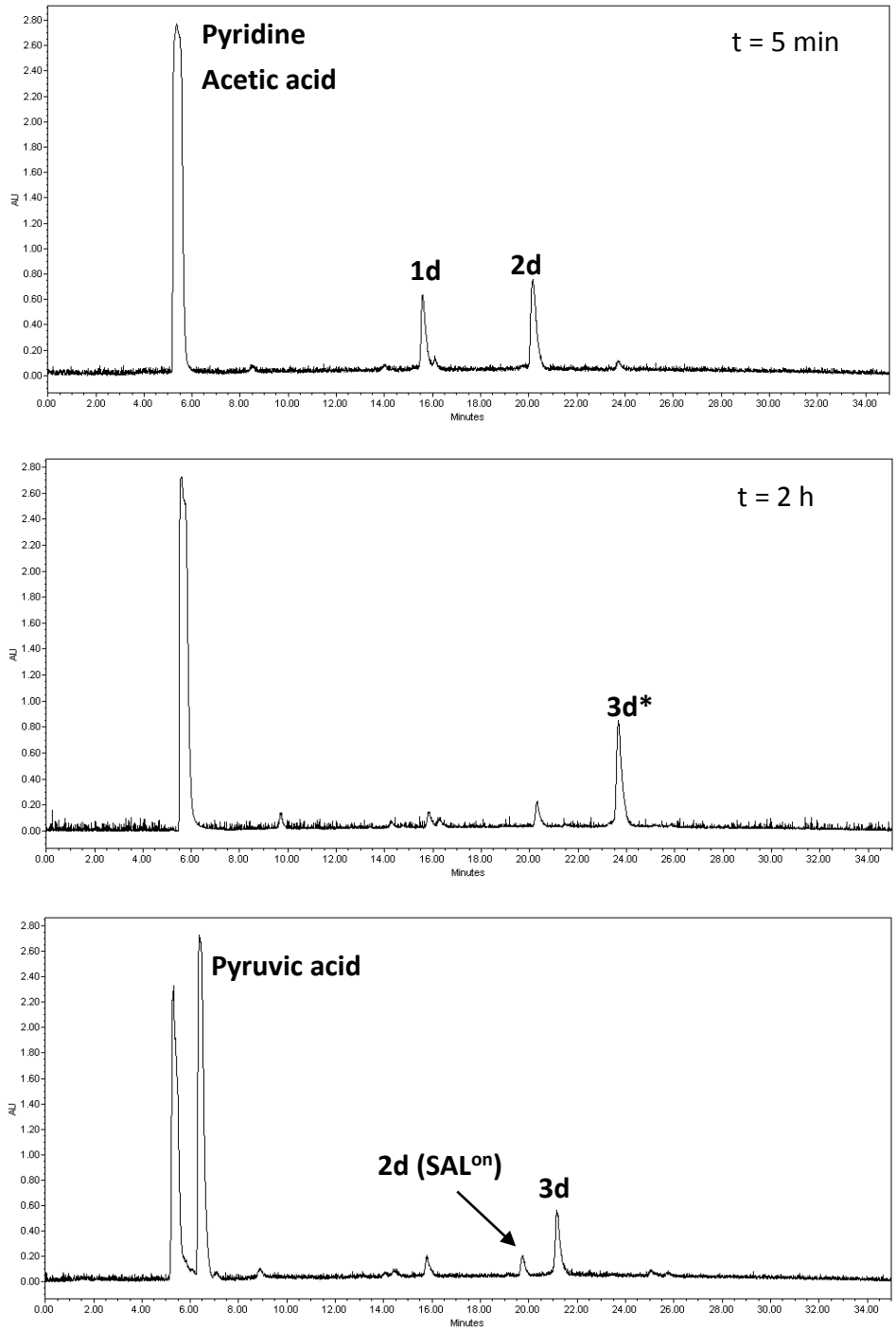


Figure S37. UV trace (190-400 nm) from LC-MS analysis of STL between **1d** and **2d** at 5 min, 2 h to generate **3d*** and after *in situ* acidolysis and pyruvic acid to give **3d**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

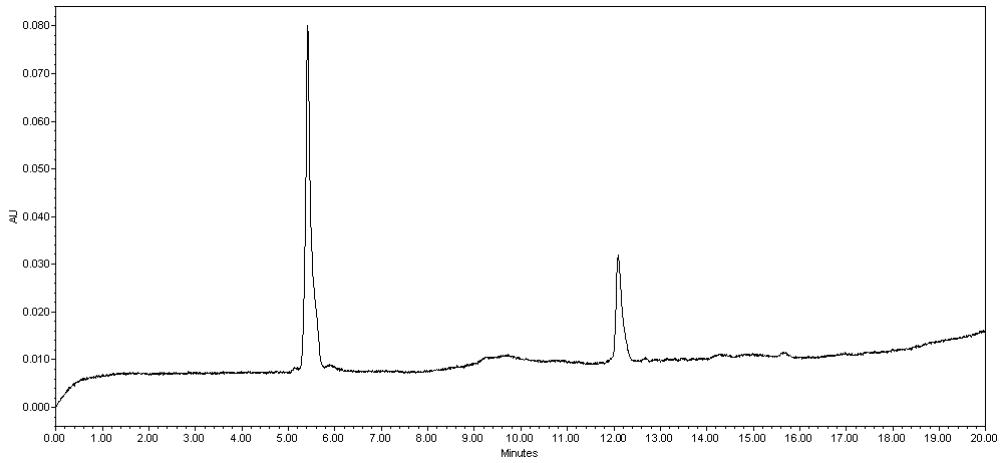


Figure S38. UV trace from analytical LC-MS analysis of purified peptide **3d**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

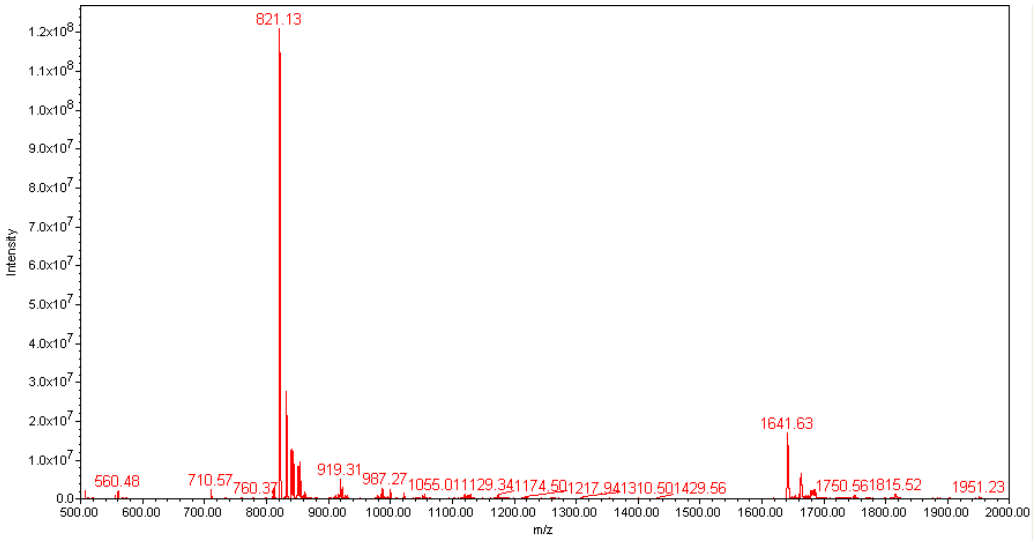
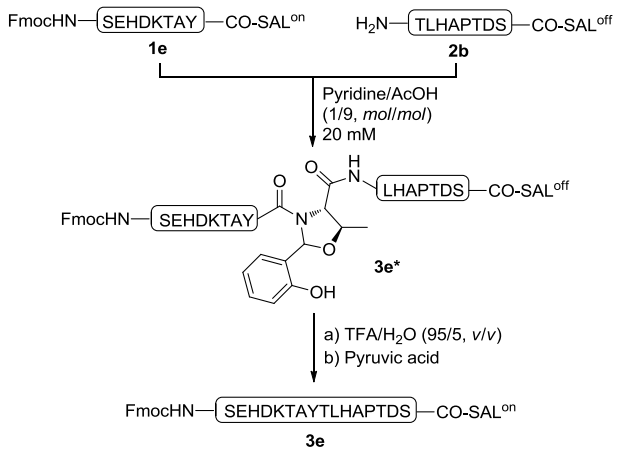


Figure S39. ESI-MS calcd. for $C_{73}H_{114}N_{19}O_{24}$ $[M+H]^+$ m/z = 1641.80, found 1641.63; $[M+2H]^{2+}$ m/z = 821.40, found 821.13.

Peptide 3e: *FmocHN*-SEHDKTAYTLHAPTDS-*CO*-SAL^{on} ester



Scheme S5. Model *N-to-C* STL study between peptide **1e** and **2b**.

Peptide **1e** (5.0 mg, 3.9 µmol) and peptide **2b** (5.9 mg, 5.9 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 3 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (2.8 µL, 39.1 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 5.1 mg (62 % yield) of peptide **3e** as a white powder.

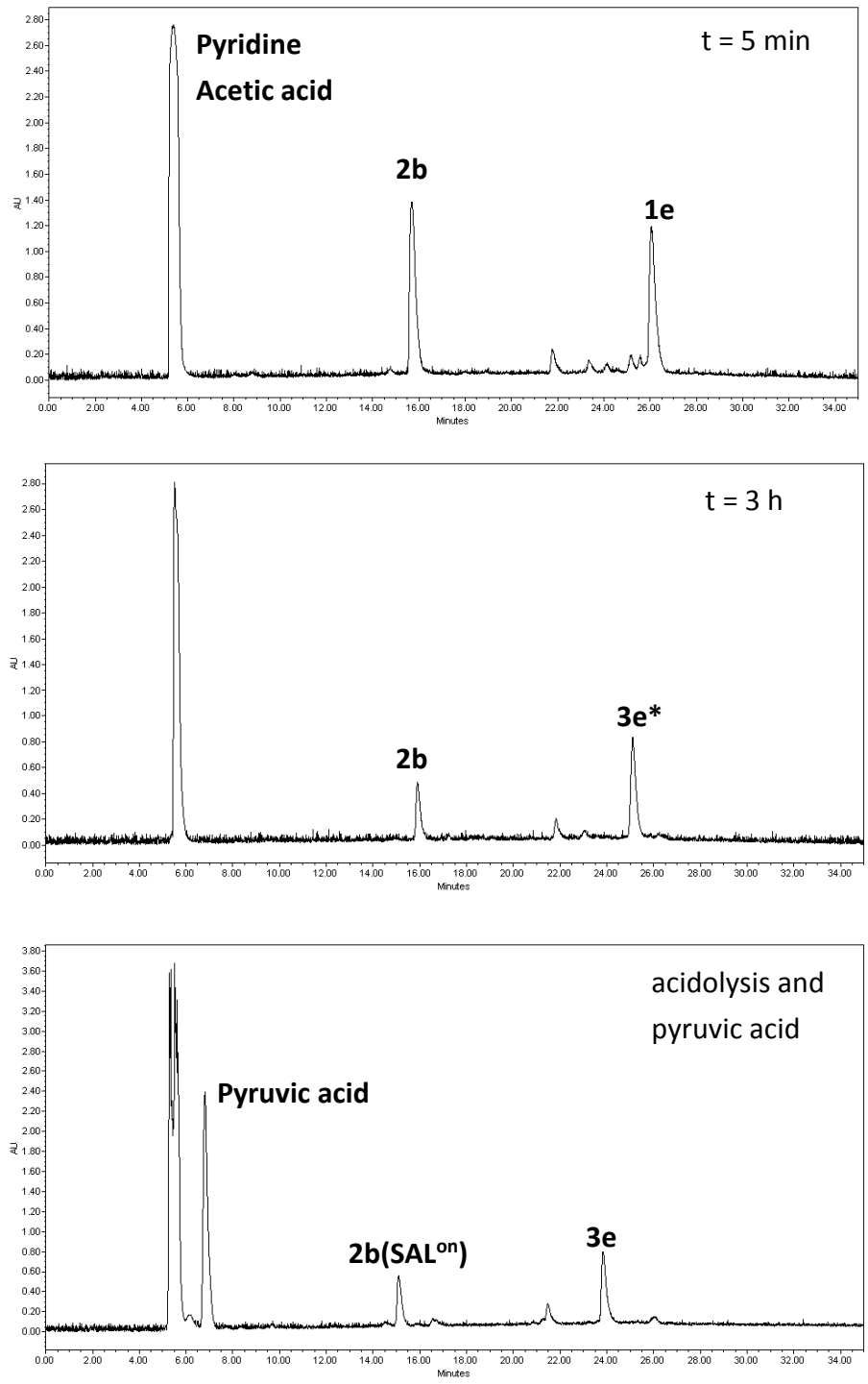


Figure S40. UV trace (190-400 nm) from LC-MS analysis of STL between **1e** and **2b** at 5 min, 3 h to generate **3e*** and after *in situ* acidolysis and pyruvic acid to give **3e**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

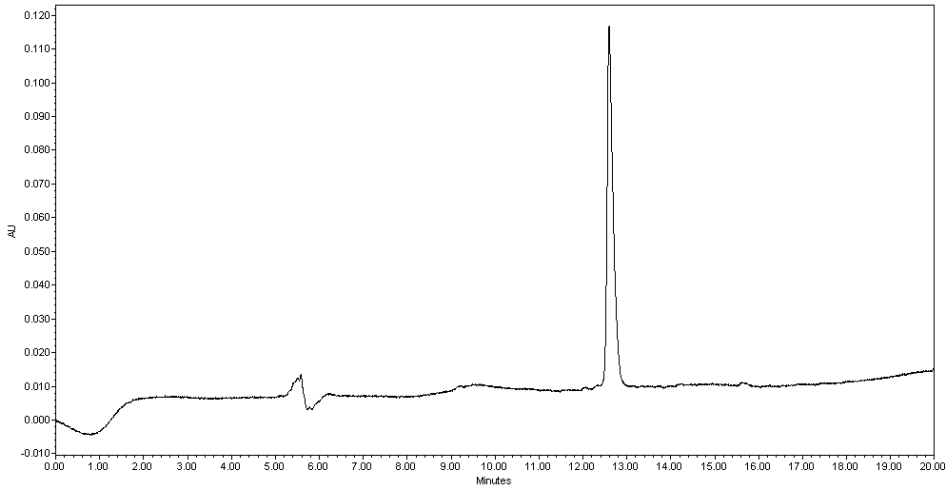


Figure S41. UV trace from analytical LC-MS analysis of purified peptide **3e**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

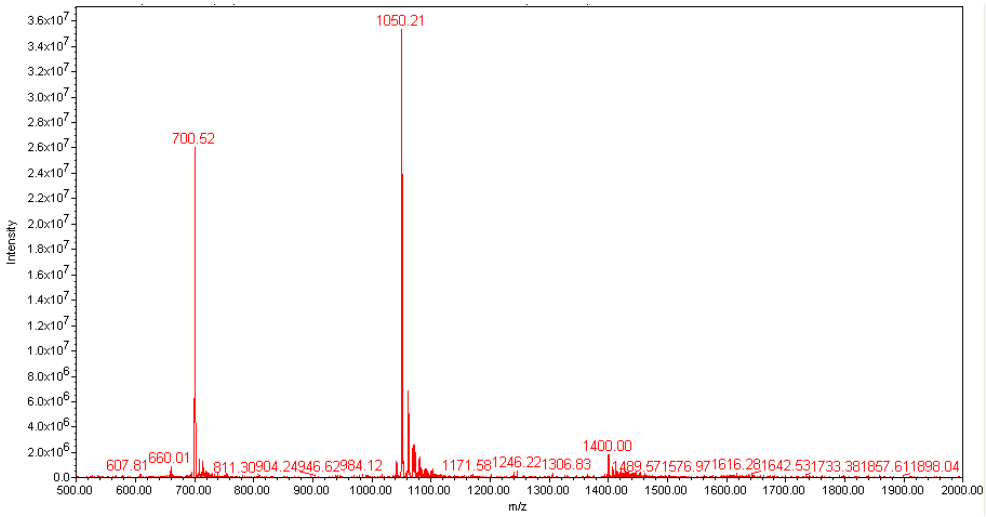
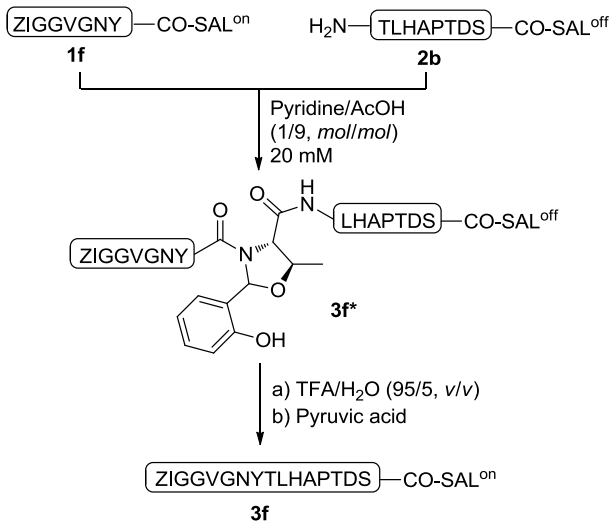


Figure S42. ESI-MS calcd. for $C_{97}H_{127}N_{21}O_{32}$, 2099.17, $[M+2H]^{2+}$ m/z = 1050.59, found 1050.21; $[M+3H]^{3+}$ m/z = 700.73, found 700.52.

Peptide 3f: ZIGGVGNYTLHAPTDS-*CO*-SAL^{on} ester



Scheme S6. Model *N-to-C* STL study between peptide **1f** and **2b**.

Peptide **1f** (5.0 mg, 5.6 µmol) and peptide **2b** (8.4 mg, 8.4 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 5 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (3.9 µL, 55.7 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 4.5 mg (47 % yield) of peptide **3f** as a white powder.

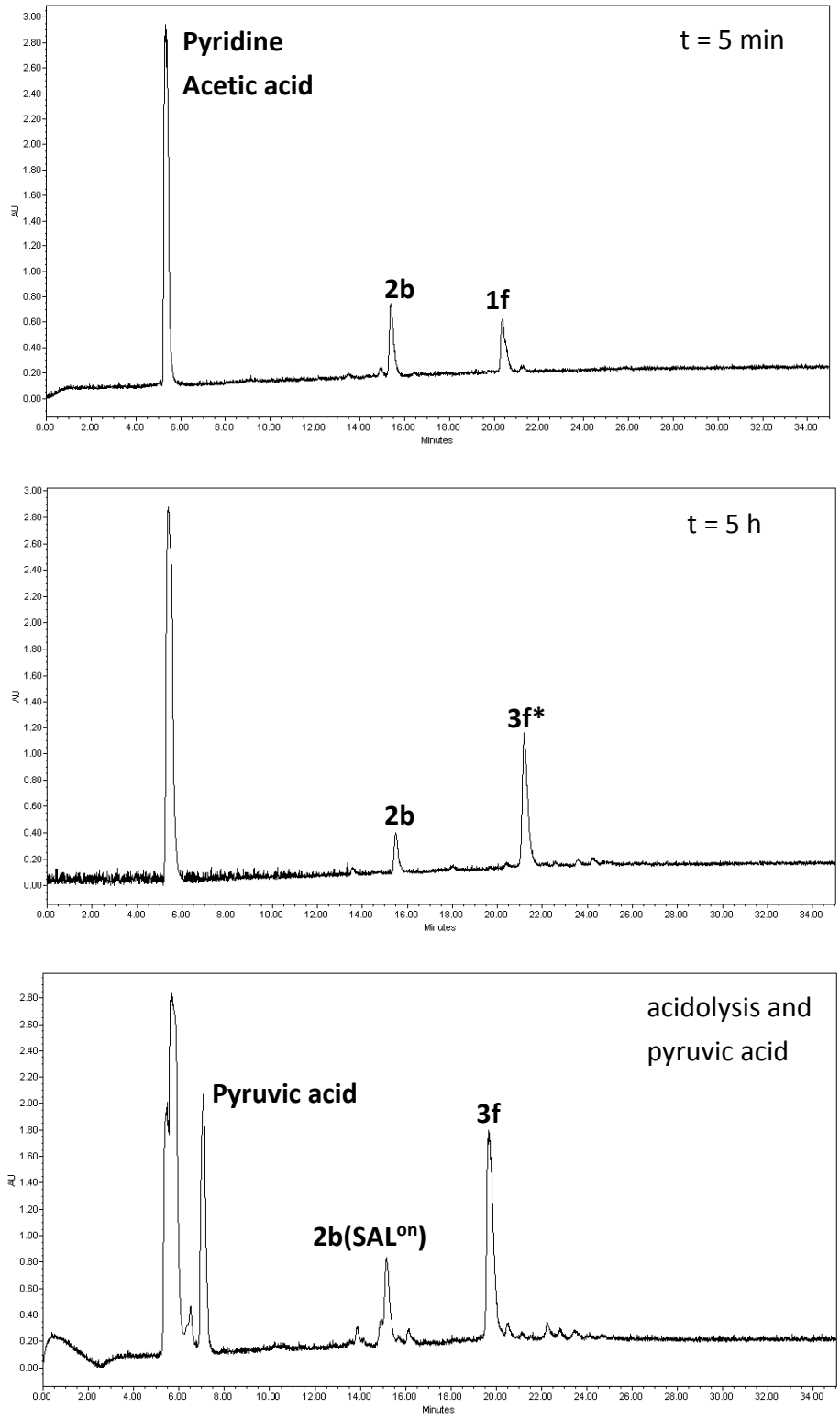


Figure S43. UV trace (190-400 nm) from LC-MS analysis of STL between **1f** and **2b** at 5 min, 5 h to generate **3f*** and after *in situ* acidolysis and pyruvic acid to give **3f**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

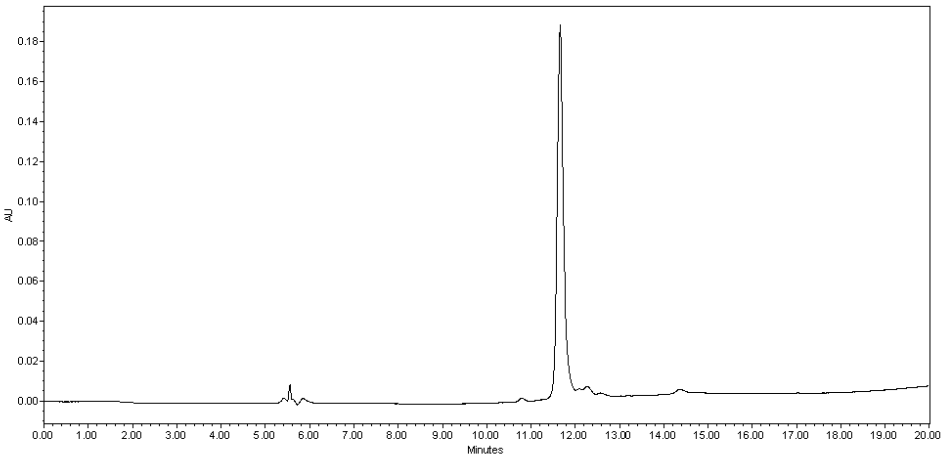


Figure S44. UV trace from analytical LC-MS analysis of purified peptide **3f**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

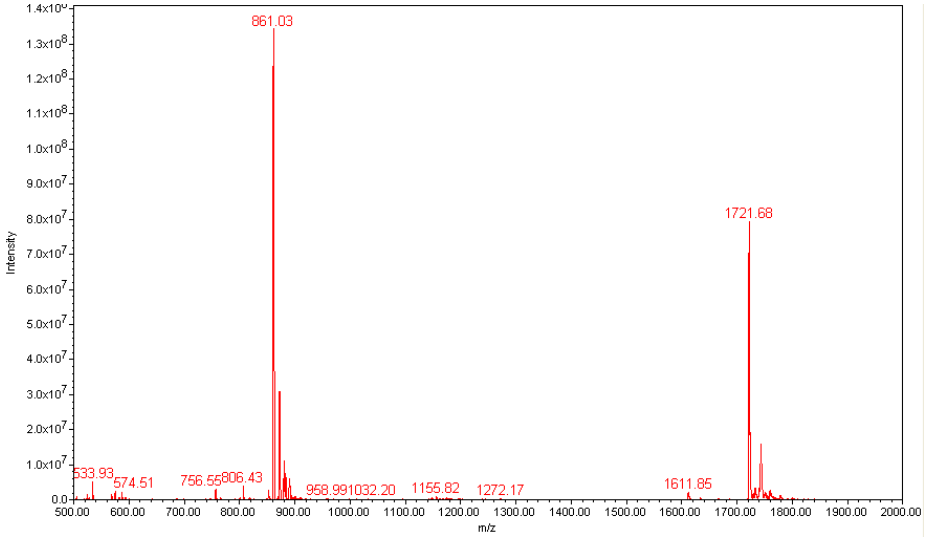
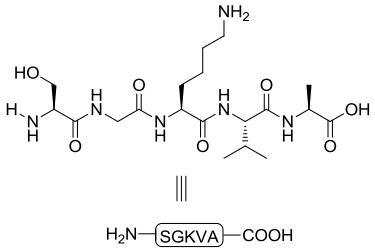


Figure S45. ESI-MS calcd. for $C_{76}H_{110}N_{19}O_{25}S$ $[M+H]^+$ m/z = 1721.86, found 1721.68; $[M+2H]^{2+}$ m/z = 861.44, found 861.03.

VII. Synthesis of peptide 5, 7 and 8

Peptide 5: *H_2N*-SGKVA-*COOH*



Peptide **5** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure A. The cleavage and global deprotection mixture was a mixture of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*). Preparative HPLC purification (0–50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **5** as a white powder.

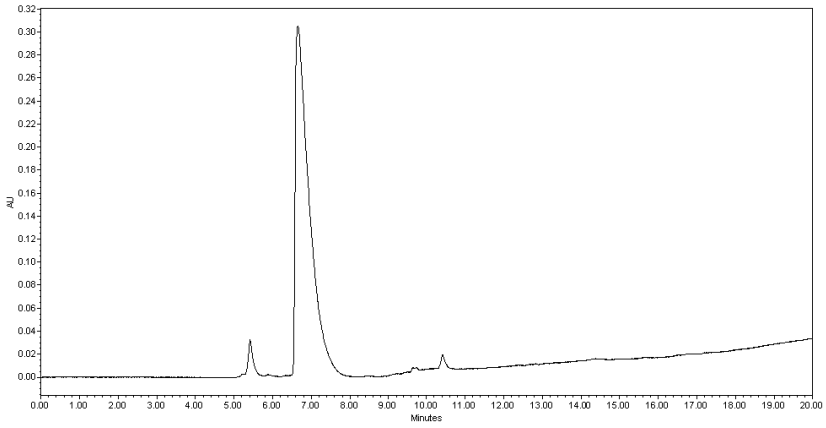


Figure S46. UV trace from analytical LC-MS analysis of purified peptide **5**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

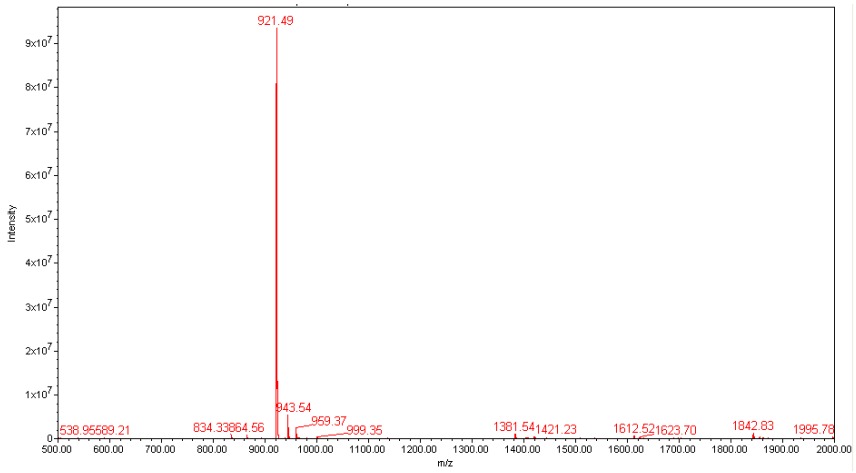
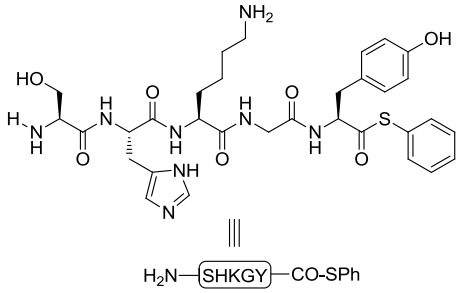


Figure S47. ESI-MS calcd. for $C_{38}H_{73}N_{12}O_{14}$ $[2M+H]^+$ *m*/*z* = 921.54, found 921.49; $[2M+Na]^+$ *m*/*z* = 943.51, found 943.54.

Peptide 7: *H₂N*-SHKGY-*CO*-*SPh*[1]



Crude protected peptide *BocHN*-Ser(*t*Bu)-His(Trt)-Lys(Boc)-Gly-*COOH* (prepared according to general experimental procedure A and B) (102.3 mg, 110.6 µmol) was dissolved in $CHCl_3$/trifluoroethanol (7.4 mL), and EDC (51.4 mg, 331.8 µmol) and HOOBt (54.1 mg, 331.8 µmol) were then added. After 5 min, **HCl·*H₂N*-Tyr-*SPh*** (102.5 mg, 331.8 µmol) was added, and the reaction mixture was stirred for 3 h to form the crude protected C-terminal peptide thioester. The reaction mixture was then concentrated and subjected to 7.0 mL of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) for 1 h. Preparative HPLC purification (10-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **7** (24.1 mg, 32% yield) as a white powder.

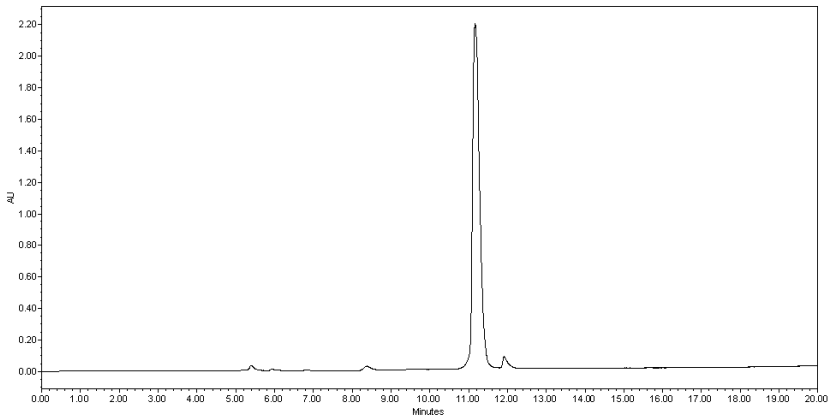


Figure S48. UV trace from analytical LC-MS analysis of purified peptide **5**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

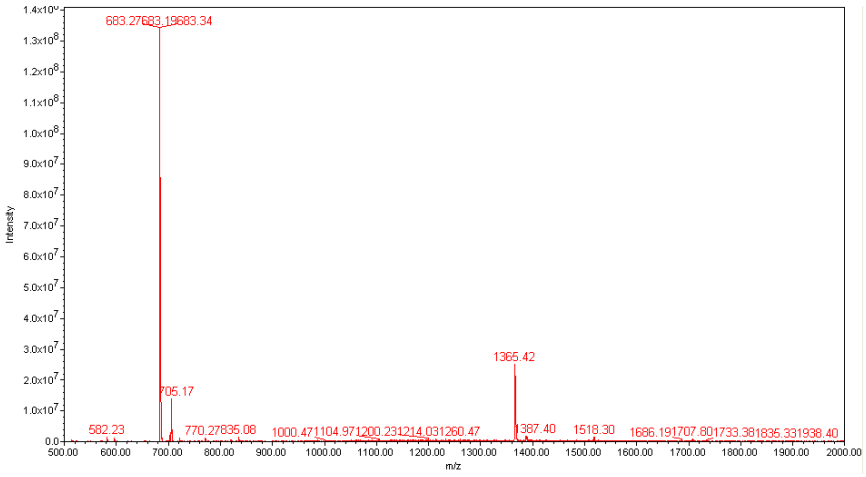
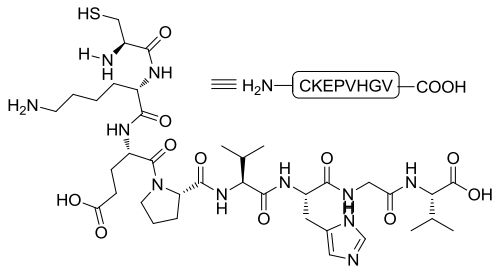


Figure S49. ESI-MS calcd. for $C_{32}H_{43}N_8O_7S$ $[M+H]^+$ *m/z* = 683.29, found 683.34; $[2M+H]^+$ *m/z* = 1365.59, found 1365.42.

Peptide 8: *H_2N*-CKEPVHGV-*COOH*



Peptide **8** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure A. The cleavage and global deprotection mixture was a mixture of

TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*). Preparative HPLC purification (10–50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **8** as a white powder.

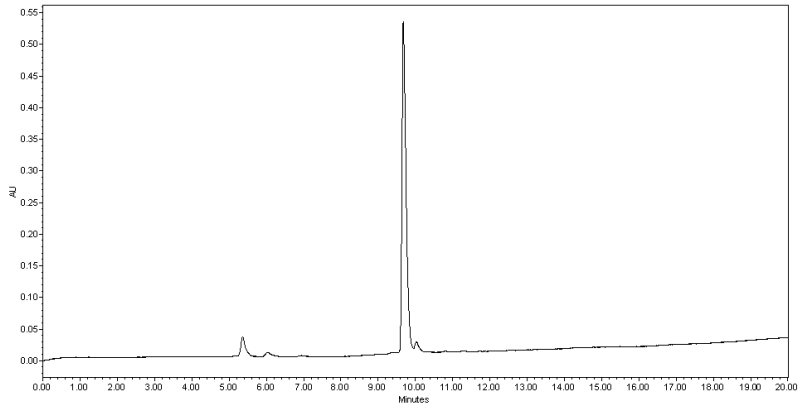


Figure S50. UV trace from analytical LC-MS analysis of purified peptide **8**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

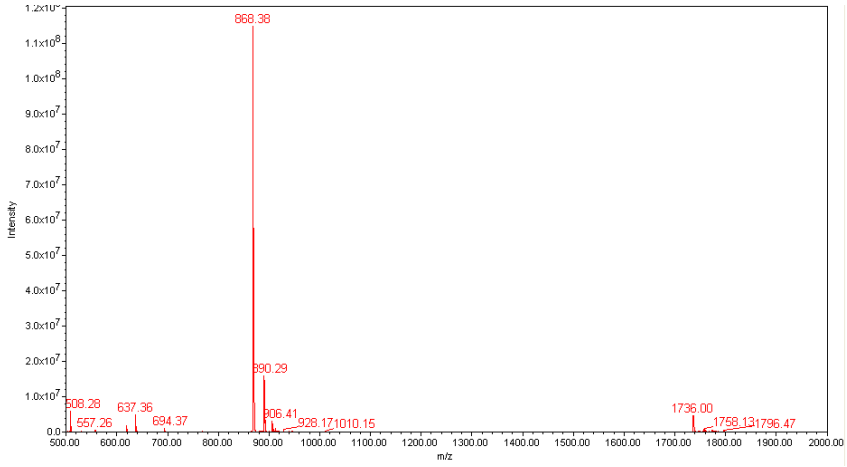
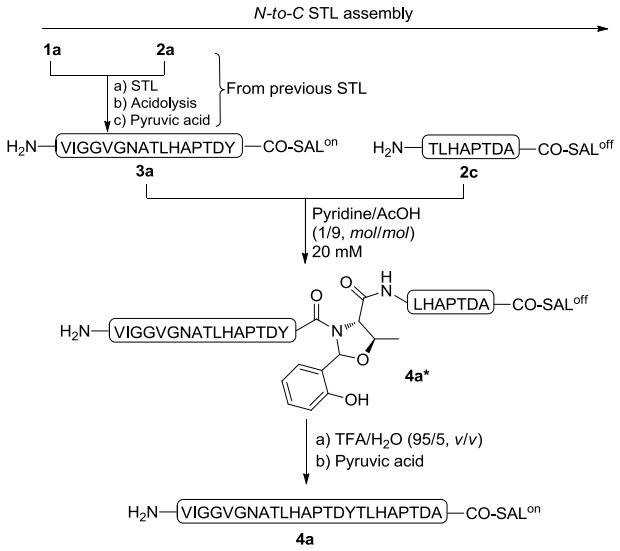


Figure S51. ESI-MS calcd. for $C_{37}H_{62}N_{11}O_{11}S$ $[M+H]^+$ *m*/*z* = 868.43, found 868.38; $[M+Na]^+$ *m*/*z* = 890.41, found 890.29; $[2M+H]^+$ *m*/*z* = 1735.86, found 1736.00.

VIII. Application of *N-to-C* strategy on convergent STL

A. Synthesis of a model C-terminal peptide SAL^{on} ester to demonstrate the feasibility on tandem STL in the *N-to-C* direction

Peptide 4a: *H$_2$N*-VIGGVGNATLHAPTDYTLHAPTDA-*CO*-SAL^{on} ester



Scheme S7. Model *N-to-C* STL study between peptide **3a** and **2c**.

Peptide **3a** (3.0 mg, 1.8 µmol) (obtained previously from *N-to-C* ligation using **1a** and **2a**) and peptide **2c** (1.6 mg, 1.8 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 4 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and pyruvic acid (1.3 µL, 17.8 µmol). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 2.3 mg (51 % yield) of peptide **3f** as a white powder.

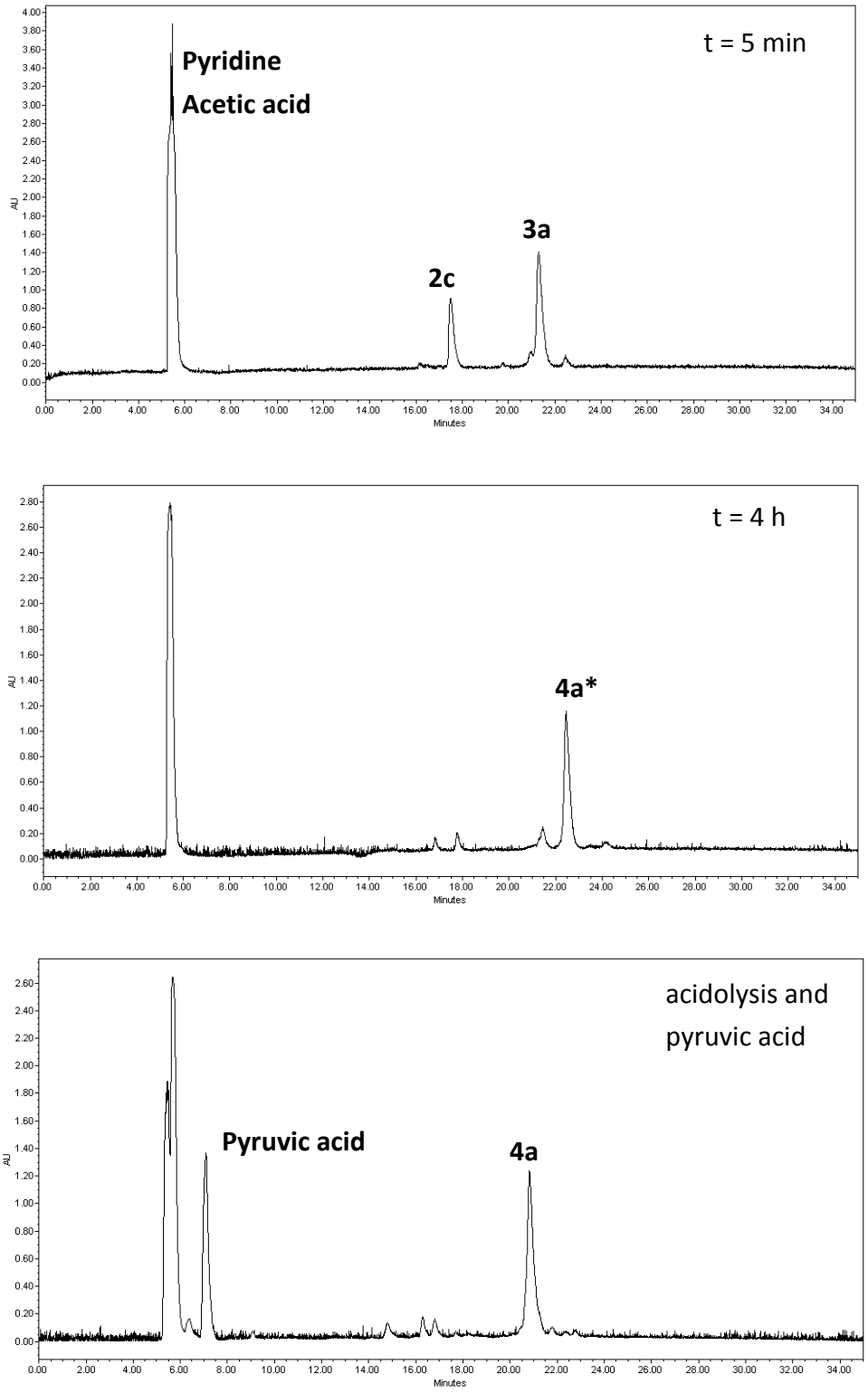


Figure S52. UV trace (190-400 nm) from LC-MS analysis of STL between **3a** and **2c** at 5 min, 4 h to generate **4a*** and after *in situ* acidolysis and pyruvic acid to give **4a**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

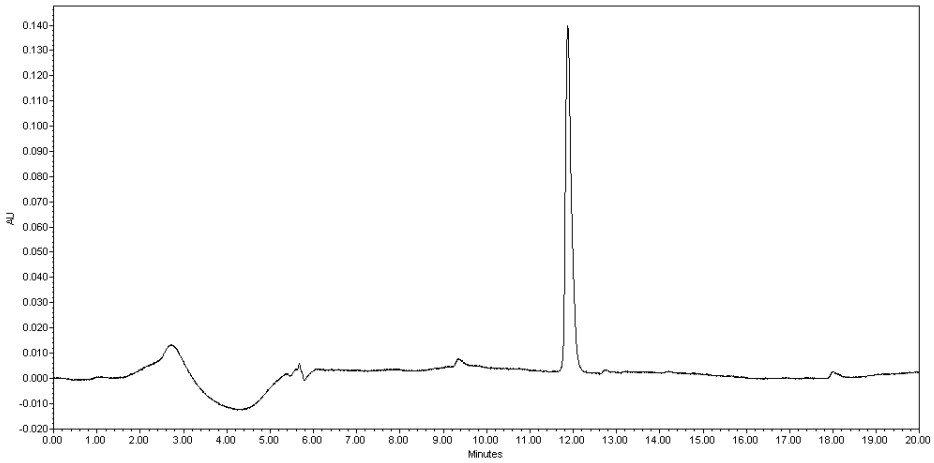


Figure S53. UV trace from analytical LC-MS analysis of purified peptide **4a**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

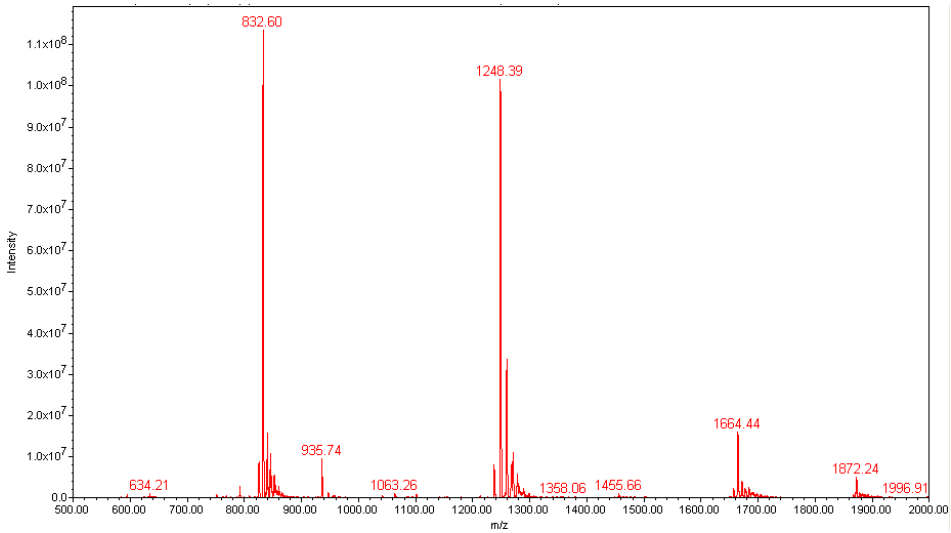
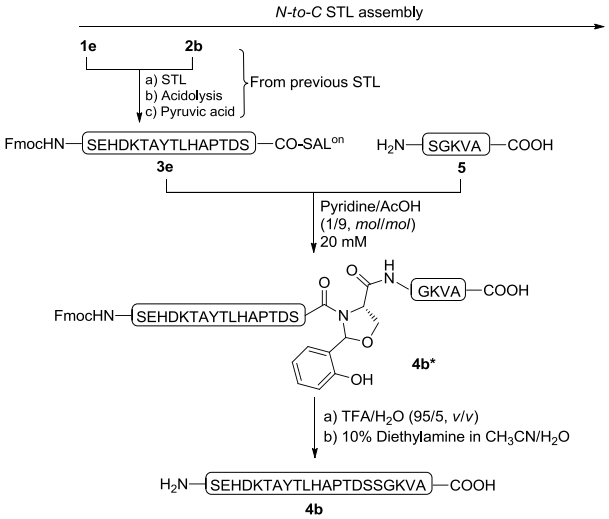


Figure S54. ESI-MS calcd. for $C_{112}H_{167}N_{29}O_{36}$, 2495.70, $[2M+3H]^{3+}$ m/z =1664.81, found 1664.44; $[M+2H]^{2+}$ m/z = 1248.86, found 1248.39; $[M+3H]^{3+}$ m/z = 832.91, found 832.60.

B. Synthesis of a model N-terminal Serine peptide using STL in novel *N-to-C* direction rather than traditional *C-to-N* direction

Peptide 4b: *H_2N*-SEHDKTAYTLHAPTDSSGKVA-*COOH*



Scheme S8. Model *N-to-C* STL study between peptide **3e** and **5.**

Peptide **3e** (3.0 mg, 1.4 µmol) (obtained previously from *N-to-C* ligation using **1e** and **2b**) and peptide **5** (1.3 mg, 2.9 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 3 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) and followed by 0.5 mL of diethylamine/CH_3CN/H_2O (1/4.5/4.5, *v*/*v*/*v*) for 1 h at room temperature to remove terminal Fmoc protecting group. Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.3 mg (40 % yield) of peptide **4b** as a white powder.

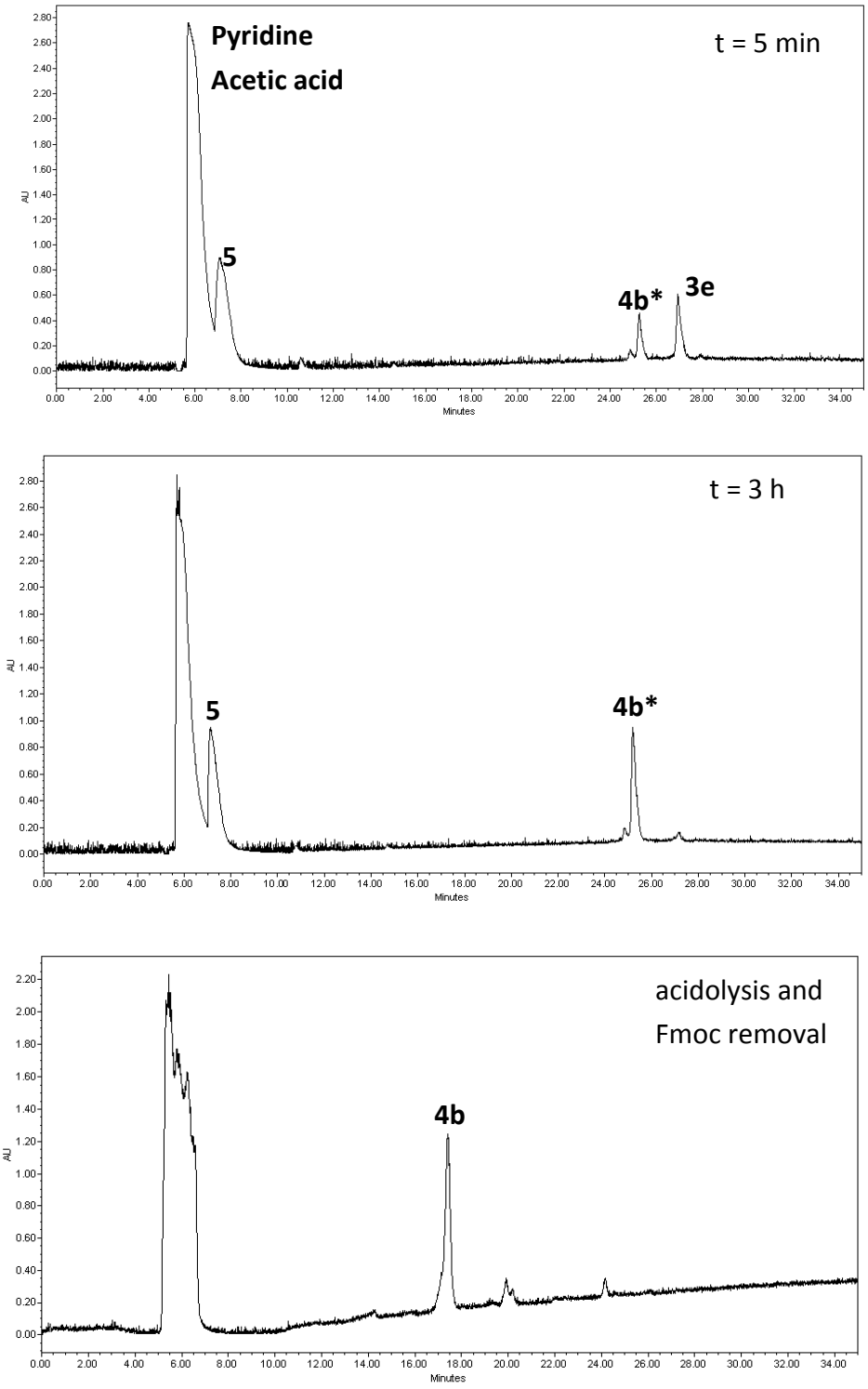


Figure S55. UV trace (190-400 nm) from LC-MS analysis of STL between **3e** and **5** at 5 min, 3 h to generate **4b*** and after acidolysis and Fmoc removal to give **4b**: gradient 0-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

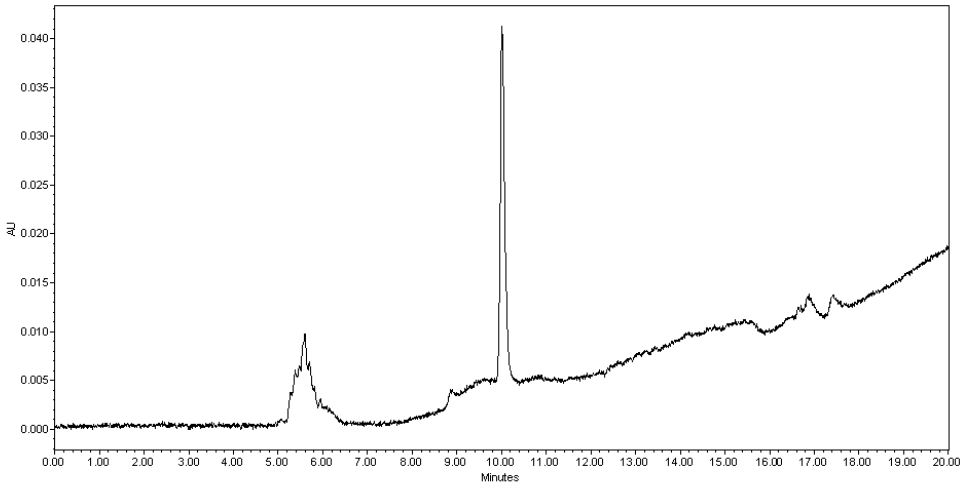


Figure S56. UV trace from analytical LC-MS analysis of purified peptide **4b**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

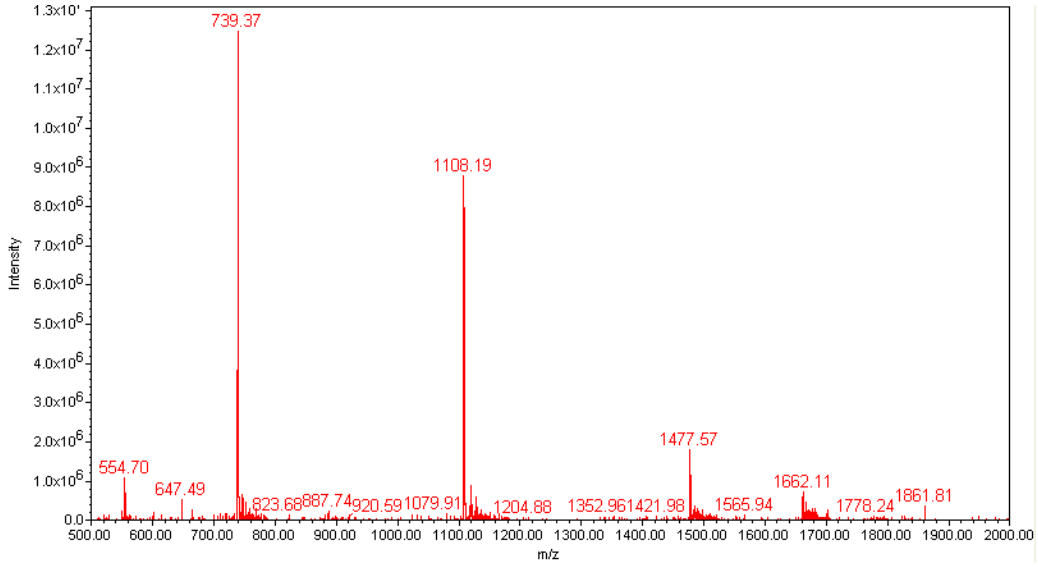
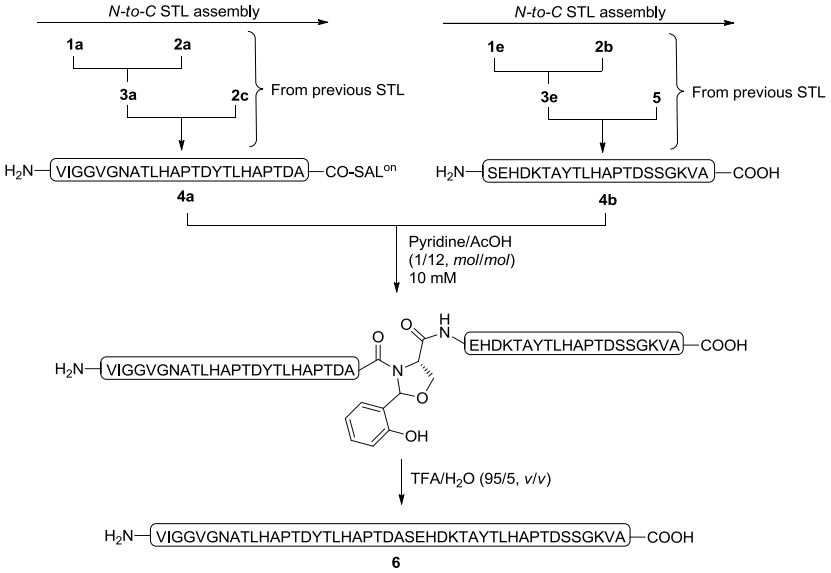


Figure S57. ESI-MS calcd. for $C_{94}H_{147}N_{27}O_{35}$, 2215.33, $[2M+3H]^{3+}$ *m/z* =1477.90, found 1477.57; $[M+2H]^{2+}$ *m/z* = 1108.67, found 1108.19; $[M+3H]^{3+}$ *m/z* = 739.45, found 739.37.

C. Realization of Convergent STL

Peptide 6:

H_2N*-VIGGVGNATLHAPTDYTLHAPTDASEHDKTAYTLHAPTDSSGKVA-*COOH



Scheme S9. Model *N-to-C* STL study between peptide **4a** and **4b.**

Peptide **4a** (1.0 mg, 0.4 µmol) (obtained previously from *N-to-C* ligation using **3a** and **2c**) and peptide **4b** (1.3 mg, 0.6 µmol) (obtained previously from *N-to-C* ligation using **3e** and **5**) were dissolved in pyridine/acetic acid (1/12, *mol*/*mol*) buffer at a concentration of 10 mM at room temperature. The reaction mixture was stirred at room temperature for 6 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*). Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.1 mg (59 % yield) of peptide **6** as a white powder.

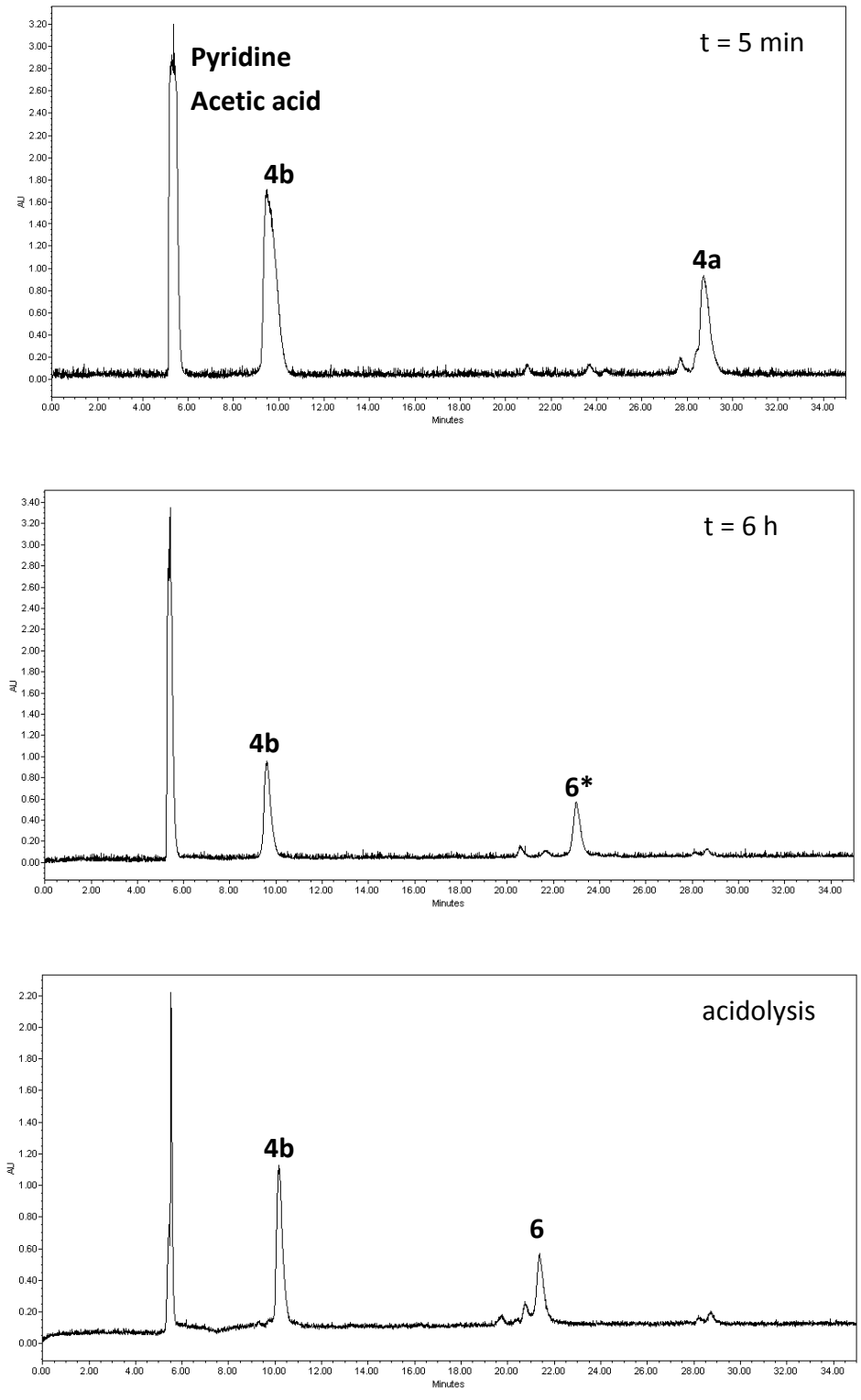


Figure S58. UV trace (190-400 nm) from LC-MS analysis of STL between **4a** and **4b** at 5 min, 6 h to generate **6*** and after acidolysis to give **6**: gradient 15-30% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

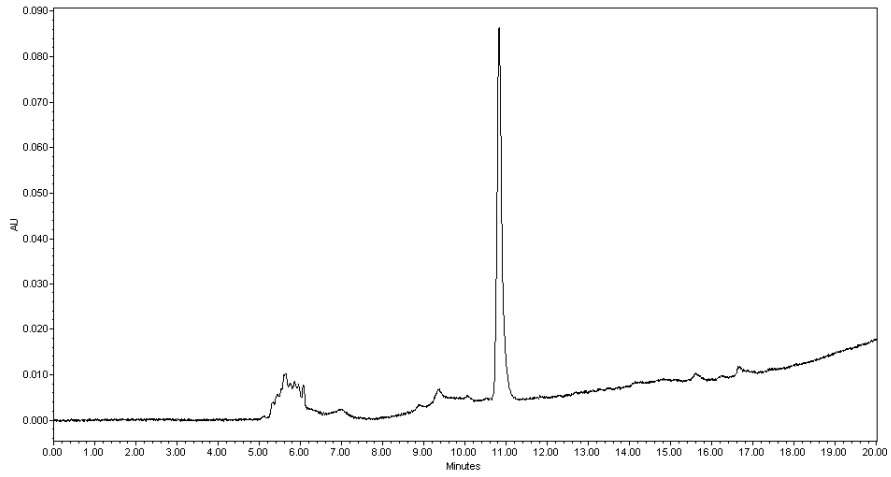


Figure S59. UV trace from analytical LC-MS analysis of purified peptide **6**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

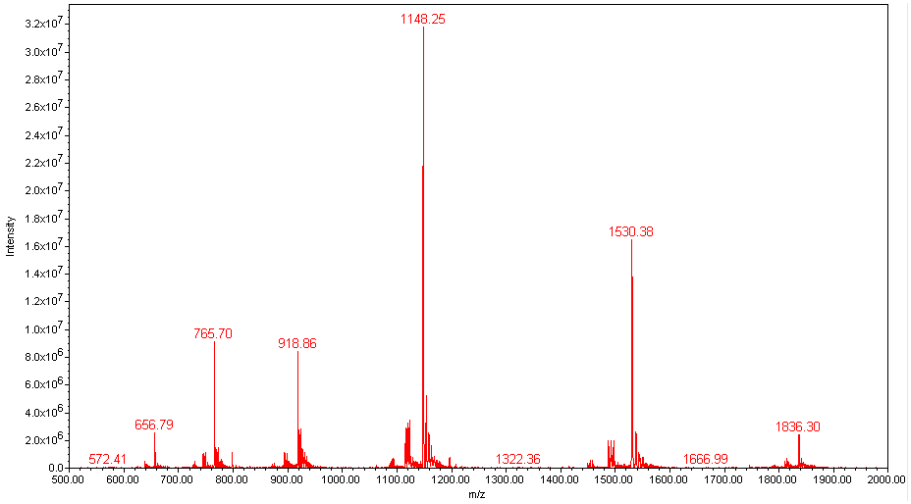
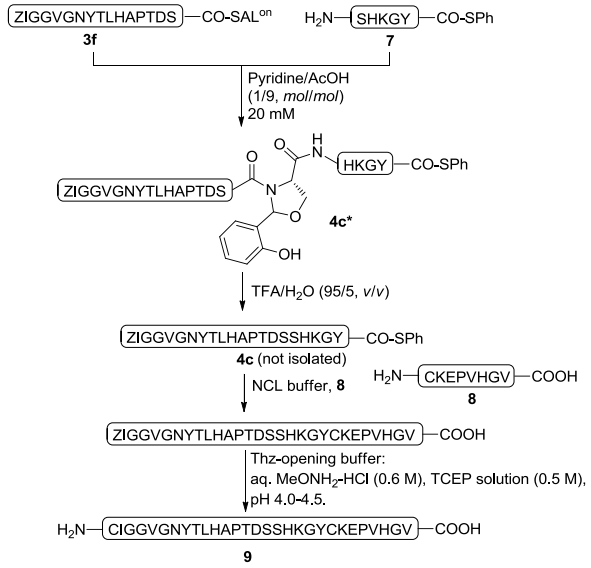


Figure S60. ESI-MS calcd. for $C_{199}H_{308}N_{56}O_{69}$, 4588.91, $[2M+5H]^{5+}$ *m*/*z* = 1836.57, found 1836.30; $[M+3H]^{3+}$ *m*/*z* = 1530.64, found 1530.38; $[M+4H]^{4+}$ *m*/*z* = 1148.23, found 1148.25; $[M+5H]^{5+}$ *m*/*z* = 918.79, found 918.86; $[M+6H]^{6+}$ *m*/*z* = 765.83, found 765.70; $[M+7H]^{7+}$ *m*/*z* = 656.57, found 656.79.

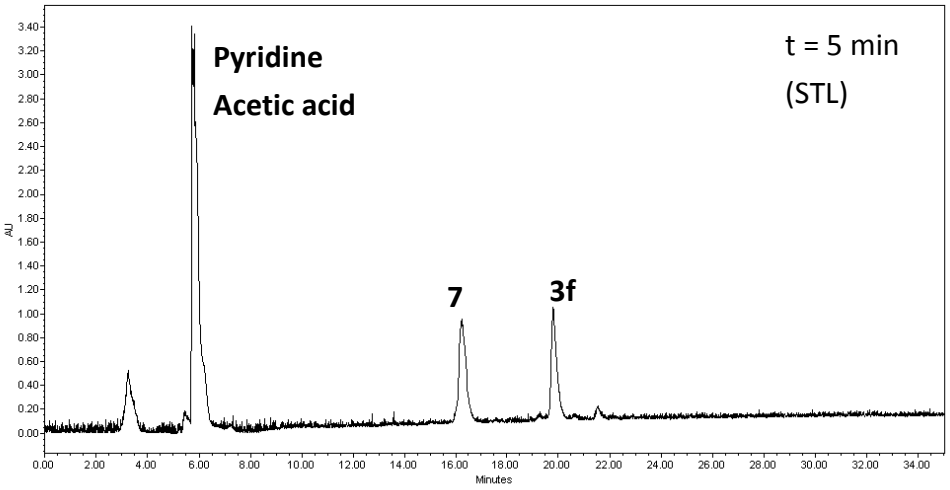
IX. One-pot Three-segment STL and NCL

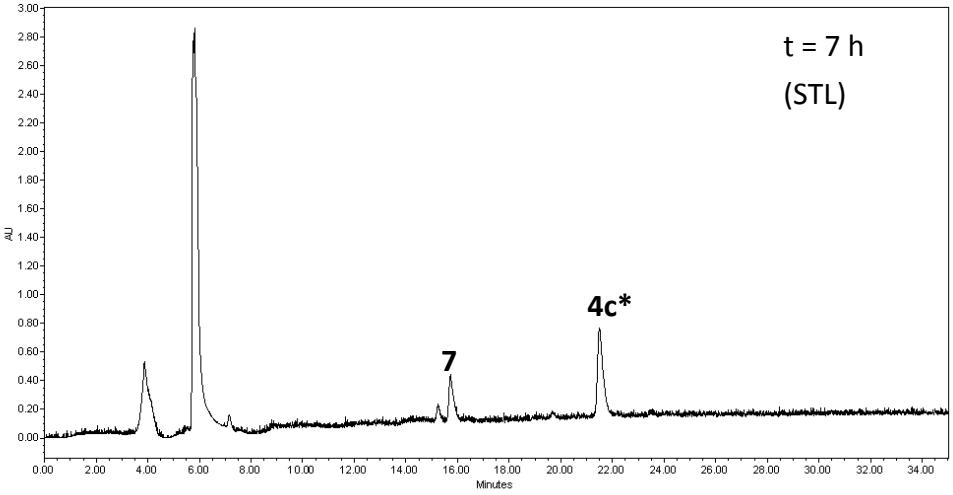
Peptide 9: *H_2N*-CIGGVGNYTLHAPTDSSHKGYCKEPVHGV-*COOH*

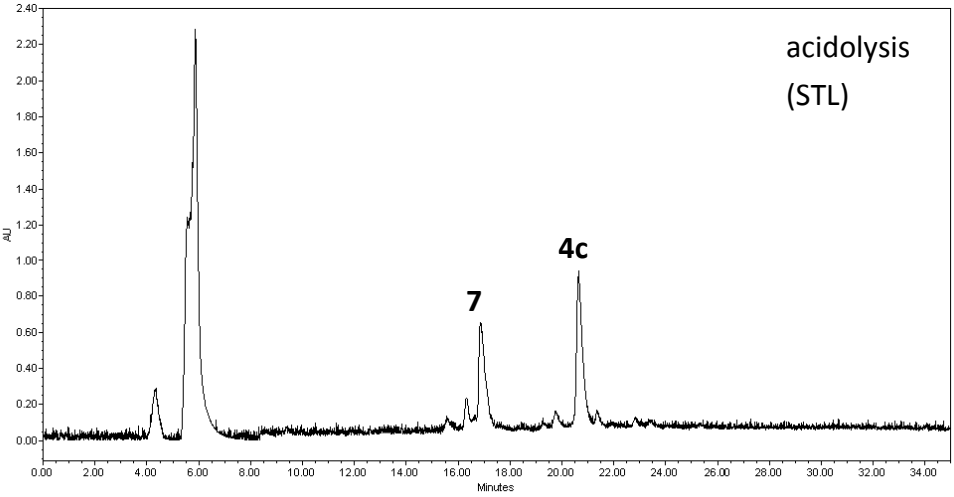


Scheme S10. One-pot Three-segment STL and NCL study between peptide **3f**, **7** and **8**.

Peptide **3f** (3.0 mg, 1.7 µmol) and peptide **7** (1.8 mg, 2.6 µmol) were dissolved in pyridine/acetic acid (1/9, *mol*/*mol*) buffer at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 7 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) to afford crude peptide thioester **4c**. TFA was blown off under a stream of condensed air. Without any purification, peptide **8** (4.5 mg, 5.2 µmol) in NCL buffer (prepared according to general experimental procedure E) was added into crude peptide thioester **4c** (final concentration = 2 mM). The reaction mixture was stirred for 1 h, and after completion of the ligation as assessed by HPLC, an aqueous solution of $MeONH_2$·HCl (29 µL, 0.6 M) was added followed by TCEP solution (35 µL, 0.5 M). The pH was adjusted to ~4.0–4.5 by addition of 2 M HCl solution and the turbid mixture was stirred for 18 h. Preparative HPLC purification (10-50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.4 mg (27 % yield) of peptide **9** as a white powder.


Pyridine
Acetic acid
t = 5 min
(STL)
7
3f
AU
Minutes


t = 7 h
(STL)
4c*
7
AU
Minutes


acidolysis
(STL)
4c
7
AU
Minutes

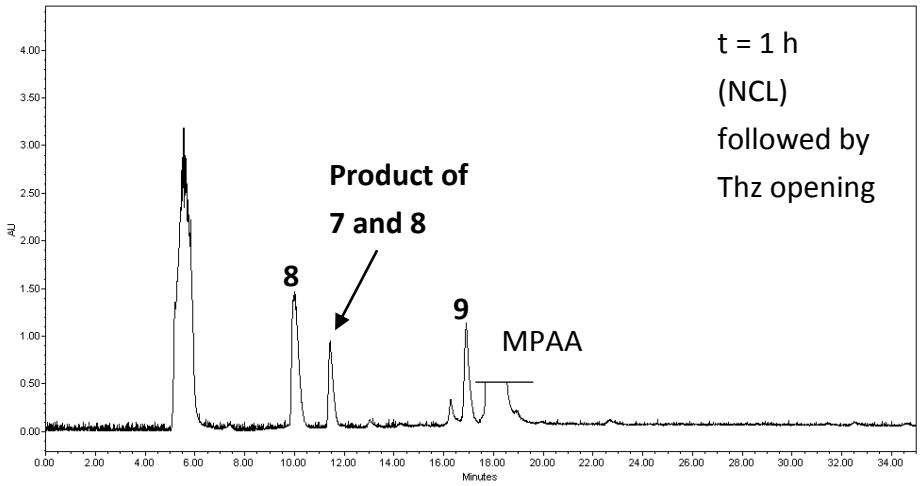


Figure S61. UV trace (190-400 nm) from LC-MS analysis of STL between **3f** and **7** at 5 min, 7 h and acidolysis to give **4c** and NCL between crude **4c** and **8** at 1 h followed by Thz opening to generate **9**: gradient 10-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

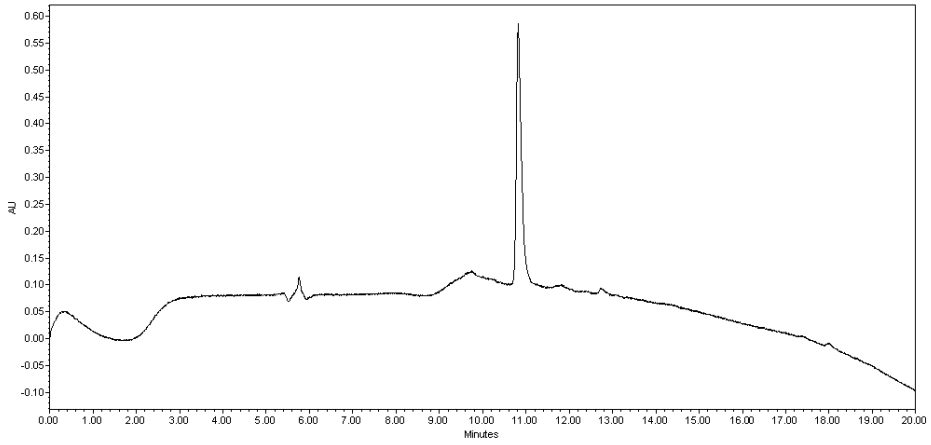


Figure S62. UV trace from analytical LC-MS analysis of purified peptide **9**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

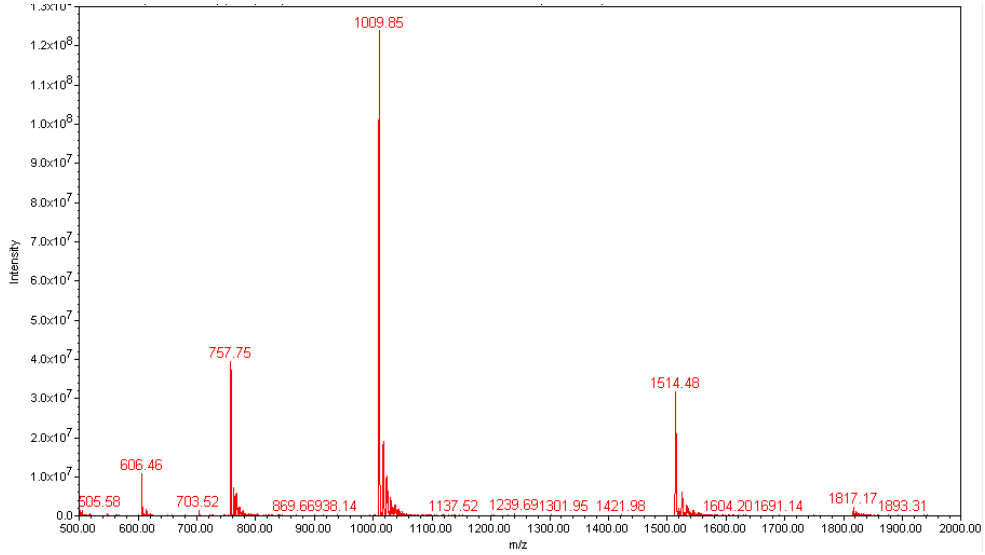
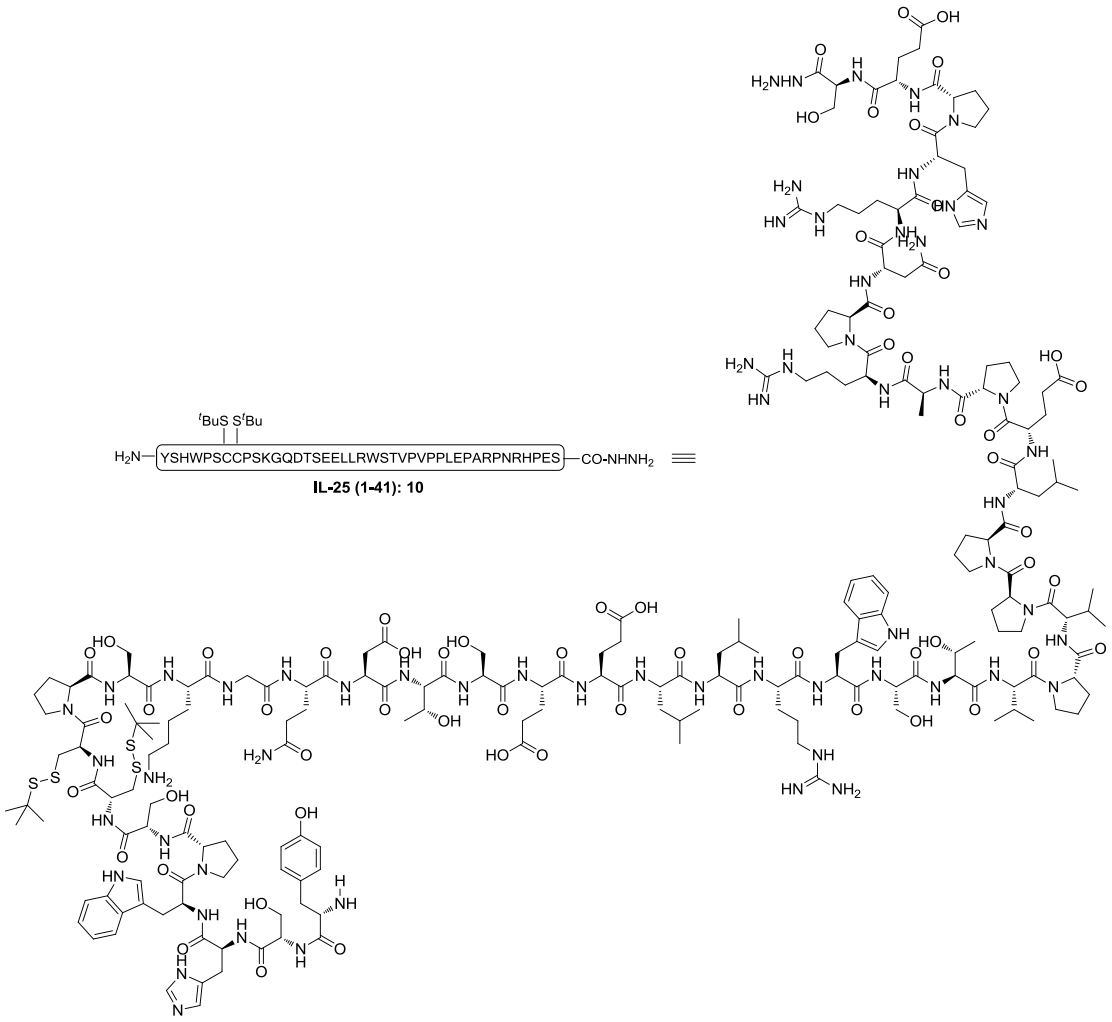


Figure S63. ESI-MS calcd. for $C_{131}H_{200}N_{38}O_{41}S_2$, 3027.35, $[M+2H]^{2+}$ *m*/*z* = 1514.68, found 1514.48; $[M+3H]^{3+}$ *m*/*z* = 1010.12, found 1009.85; $[M+4H]^{4+}$ *m*/*z* = 757.84, found 757.75; $[M+5H]^{5+}$ *m*/*z* = 606.48, found 606.46.

X. Total synthesis of glycosylated IL-25 by *N-to-C* STL strategy and One-pot Three-segment STL and NCL

A. Preparation of IL-25 glycopeptide/peptide fragments

Preparation of peptide hydrazide 10: IL-25 (1-41) [2]



IL-25 (1-41) **(10)** was synthesized according to the general procedure A using hydrazine 2-chlorotrityl chloride resin (0.5 g) (prepared according to general experimental procedure F). Upon completion of synthesis, the peptide resin was subjected to a cleavage cocktail of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) for 2 h. The resin was filtered and the combined filtrates were blown off under a stream of condensed air. The crude product was triturated with cold diethyl ether to give a white

suspension for centrifuge. After decanting diethyl ether, the remaining solid was subjected to preparative HPLC purification (30-40% CH_3CN/H_2O over 30 min) and lyophilization to give **10** (85.6 mg, 7 %) as a white powder.

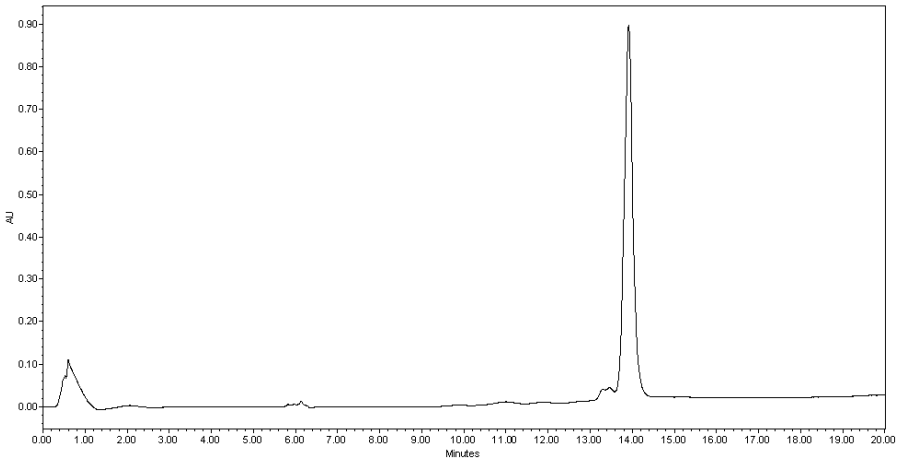


Figure S64. UV trace from LC-MS analysis of purified **10**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

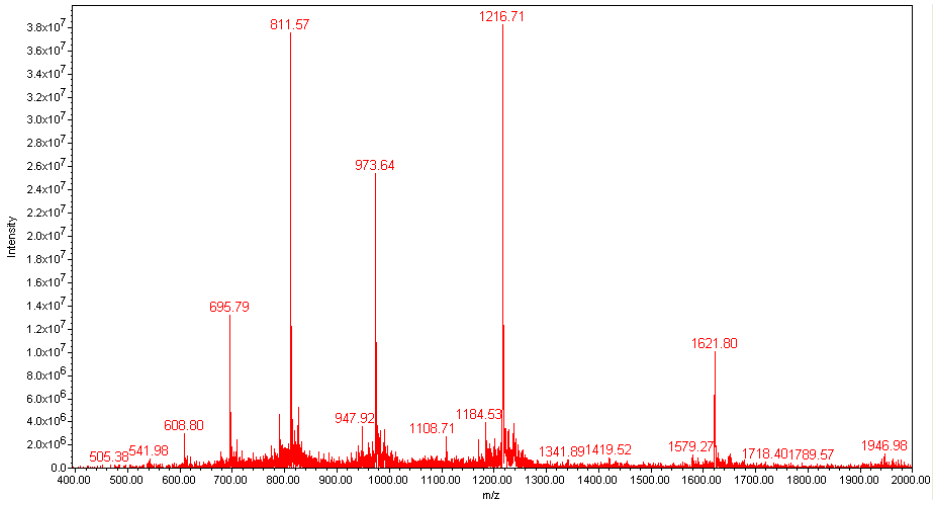
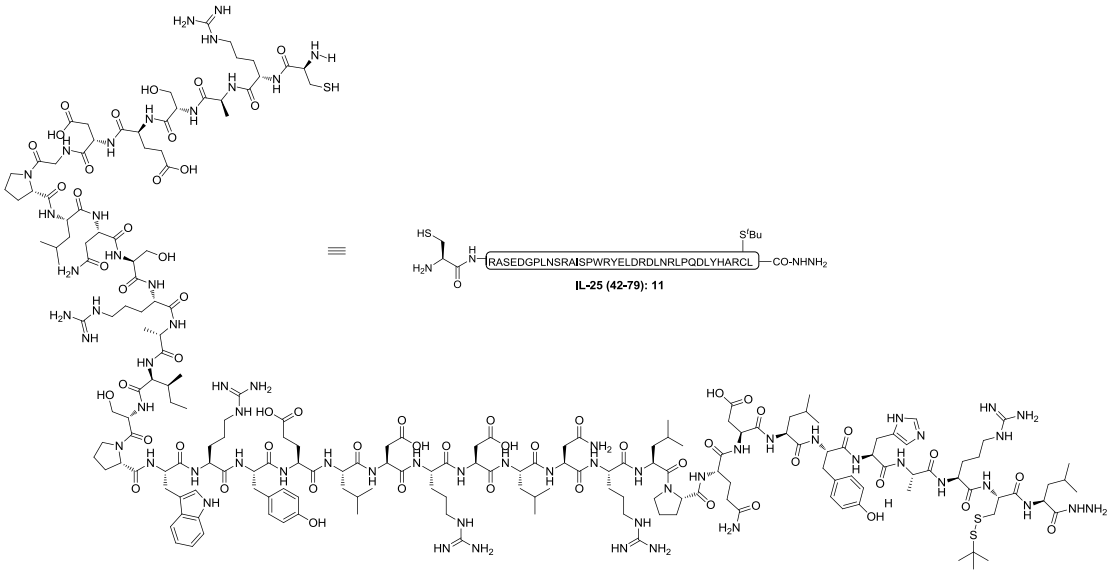


Figure S65. ESI-MS calcd. for $C_{213}H_{327}N_{61}O_{62}S_4$, 4862.51 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1621.67, found 1621.80; $[M+4H]^{4+}$ *m/z* = 1216.63, found 1216.71; $[M+5H]^{5+}$ *m/z* = 973.50, found 973.64; $[M+6H]^{6+}$ *m/z* = 811.42, found 811.57; $[M+7H]^{7+}$ *m/z* = 695.64, found 695.79; $[M+8H]^{8+}$ *m/z* = 608.81, found 608.80.

Preparation of peptide hydrazide 11: IL-25 (42-79) [2]



Peptide **11** was synthesized according to the general procedure A using hydrazine 2-chlorotrityl chloride resin (0.5 g) (prepared according to general experimental procedure F). Upon completion of synthesis, the peptide resin was subjected to a cleavage cocktail of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) for 2 h. The resin was filtered and the combined filtrates were blown off under a stream of condensed air. The crude product was triturated with cold diethyl ether to give a white suspension for centrifuge. After decanting diethyl ether, the remaining solid was subjected to preparative HPLC purification (25-40% CH_3CN/H_2O over 30 min) and lyophilization to give **11** (102.6 mg, 9%) as a white powder.

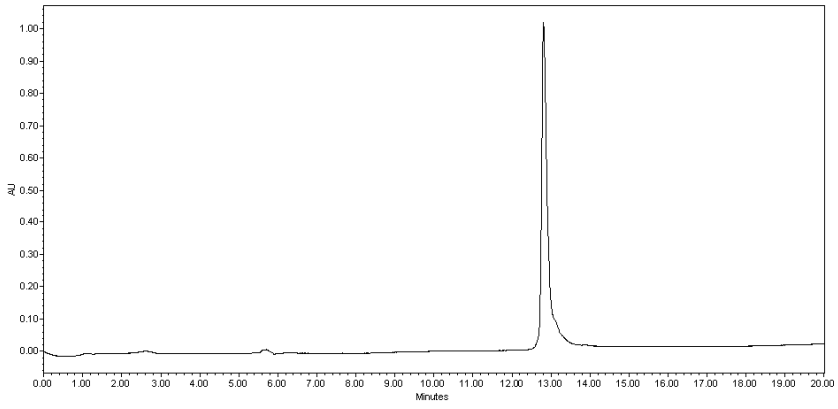


Figure S66. UV trace from LC-MS analysis of purified **11**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

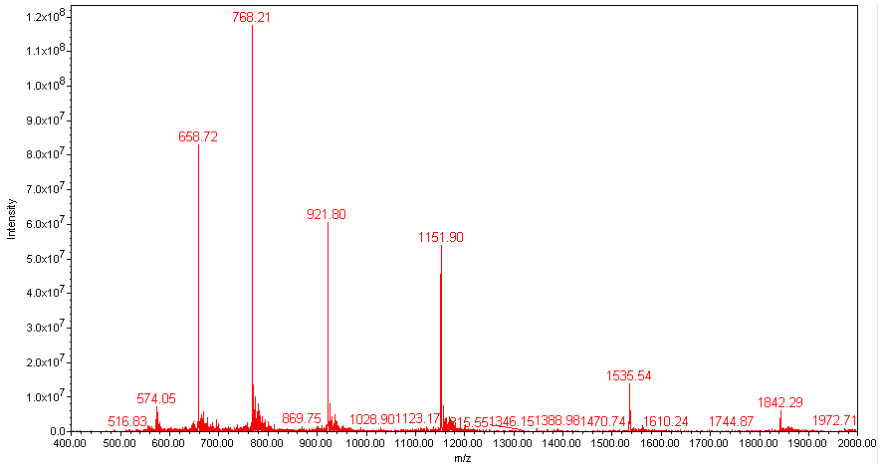
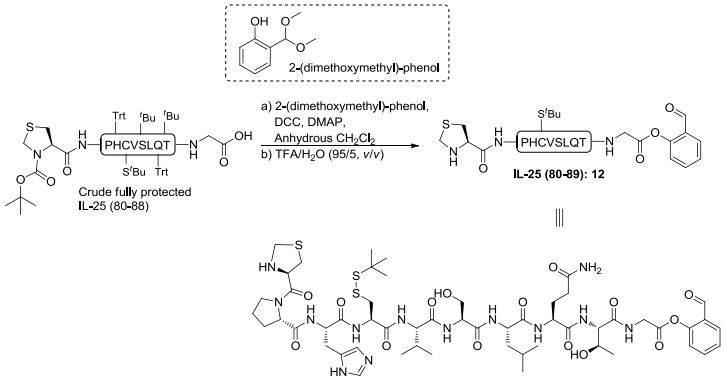


Figure S67. ESI-MS calcd. for $C_{197}H_{314}N_{64}O_{58}S_3$, 4603.19 Da (average isotopes), $[2M+5H]^{5+}$ m/z = 1842.28, found 1842.29; $[M+3H]^{3+}$ m/z = 1535.40, found 1535.54; $[M+4H]^{4+}$ m/z = 1151.80, found 1151.90; $[M+5H]^{5+}$ m/z = 921.64, found 921.80; $[M+6H]^{6+}$ m/z = 768.21, found 768.2; $[M+7H]^{7+}$ m/z = 658.60, found 658.72.

Preparation of C-terminal peptide SAL^{on} ester 12: IL-25 (80-89)



Scheme S11. Synthesis of **(12)**.

Crude fully protected IL-25 (80-88) bearing C-terminus free carboxylic acid was firstly synthesized according to the general procedure A and B using 2-chlorotrityl chloride resin. The fully protected peptidyl acid (100.0 mg, 54.4 μmol) was coupled with 2-(dimethoxymethyl)-phenol (182.7 mg, 1087.5 μmol), *N*,*N'*-dicyclohexylcarbodiimide (DCC) (56.1 mg, 271.9 μmol) and 4-dimethylaminopyridine (DMAP) (33.2 mg, 271.9 μmol) in anhydrous CH_2Cl_2 overnight. Upon completion, the reaction mixture was concentrated and subjected to the treatment with TFA/H_2O (95/5, *v*/*v*) for 2 h. The crude peptide salicylaldehyde

ester was precipitated out by diethyl ether. Preparative HPLC purification (25-45% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **12** (67.9 mg, 31% yield) as a white powder.

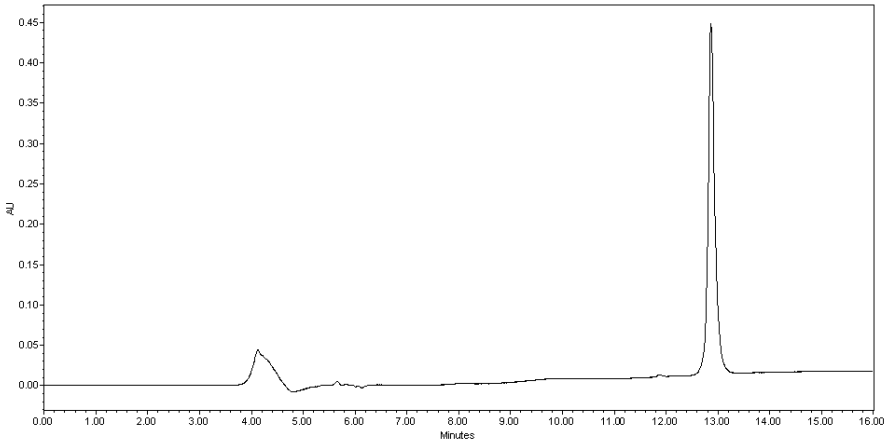


Figure S68. UV trace (190-400 nm) from LC-MS analysis of purified **12**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

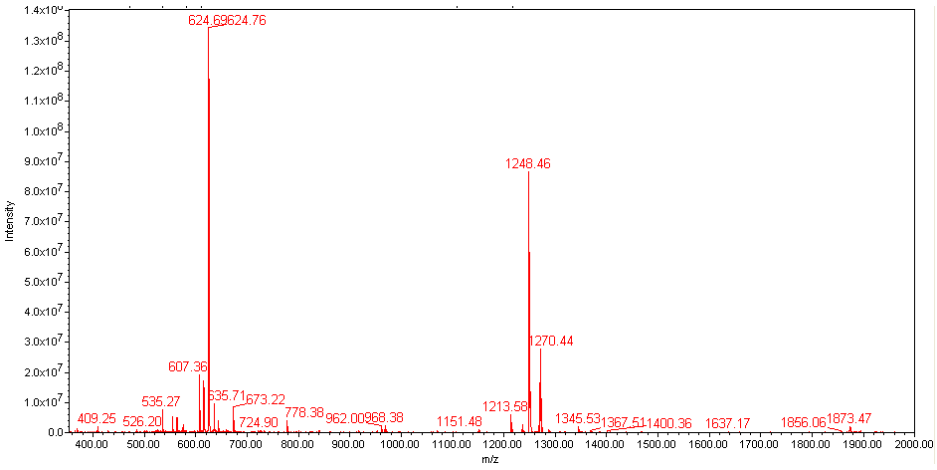
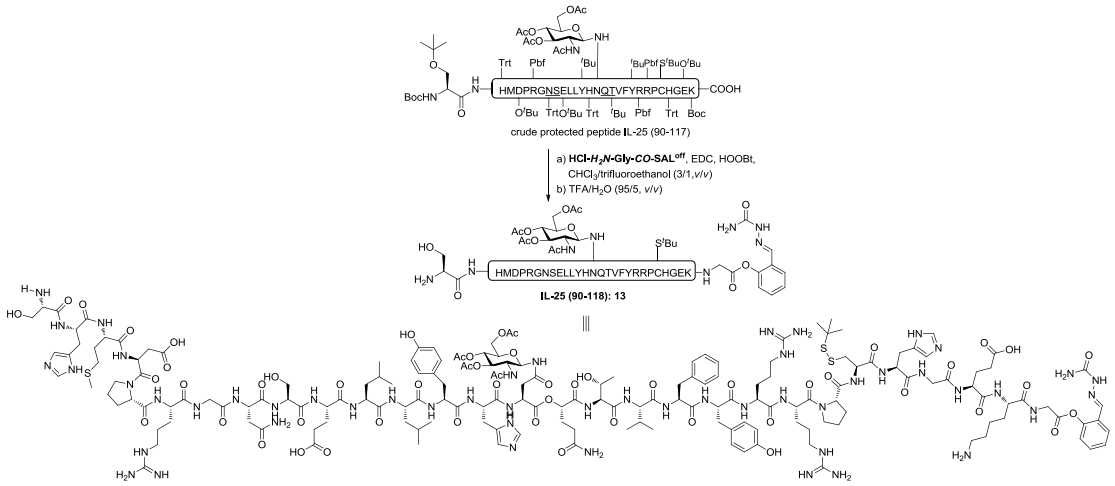


Figure S69. ESI-MS calcd. for $C_{54}H_{82}N_{13}O_{15}S_3$, $[M+H]^+$ *m/z* = 1248.52, found 1248.46; $[M+Na]^+$ *m/z* = 1270.50, found 1270.44; $[M+2H]^{2+}$ *m/z* = 624.76, found 624.76.

Synthesis of C-terminal glycopeptide SAL^{off} esters 13: IL-25 (90-118)



Scheme S12. Synthesis of **(13)** by n+1 strategy. Pseudoproline dipeptides are involved during SPPS (underlined).

Crude fully protected IL-25 (90-117) bearing C-terminus free carboxylic acid was firstly synthesized according to the general procedure A and B using 2-chlorotrityl chloride resin. The fully protected peptidyl acid (150.5 mg, 23.5 μmol) was dissolved in $CHCl_3$/trifluoroethanol (1.6 mL), and EDC (10.9 mg, 70.5 μmol) and HOOBt (11.5 mg, 70.5 μmol) were then added followed by **HCl·*H*$_2$*N*-Gly-*CO*-SAL^{off}** (19.2 mg ,70.5 μmol), as described in general procedure D. The reaction mixture stirred for 3 h to form the crude protected C-terminal peptide SAL^{off} ester. Upon completion, the reaction mixture was concentrated and subjected to treatment with TFA/H_2O (95/5, *v*/*v*). After stirring for 2 h, the solution was evaporated under a stream of nitrogen to half of the initial volume. The residue was treated with ice-cold diethyl ether (45 mL), and the resulting suspension was centrifuged to give a white pellet. The supernant was decanted and the pellet was triturated with ice-cold diethyl ether (45 mL). This process was repeated three times in total. Preparative HPLC purification (23-30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded peptide **13** (26.4 mg, 28% yield) as a white powder.

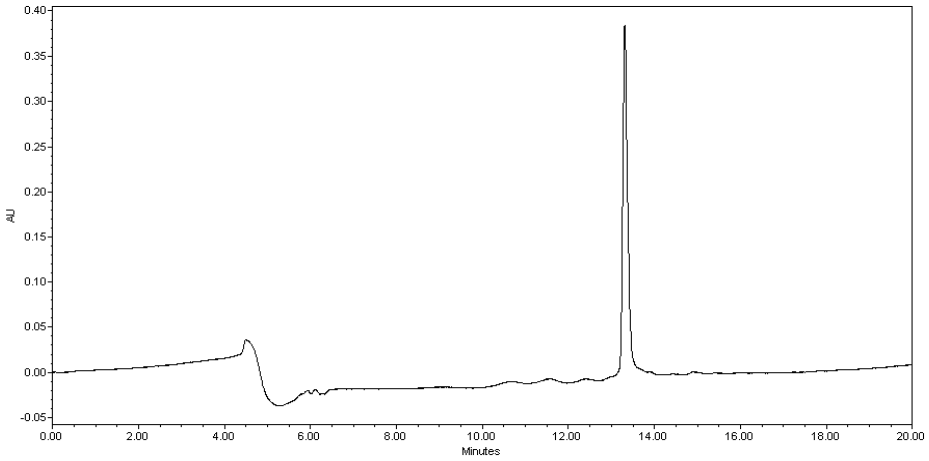


Figure S70. UV trace (190-400 nm) from LC-MS analysis of purified **13**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

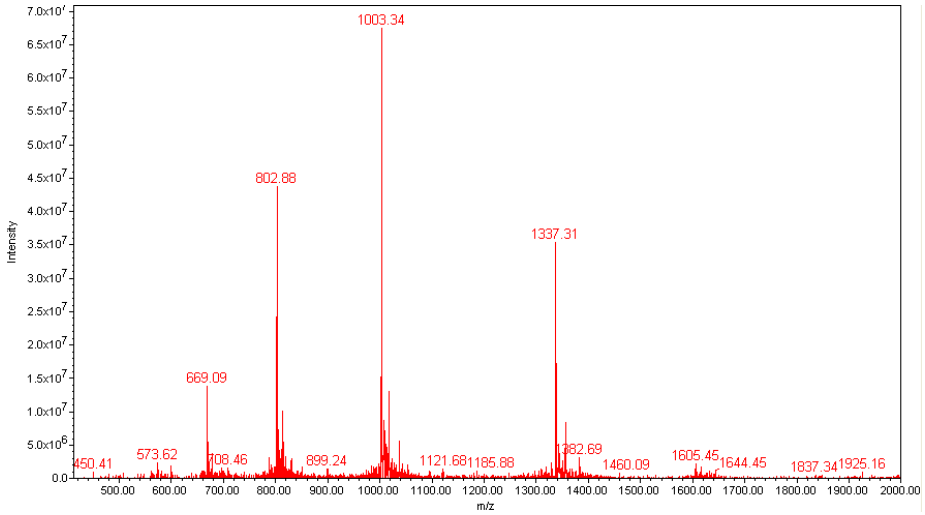
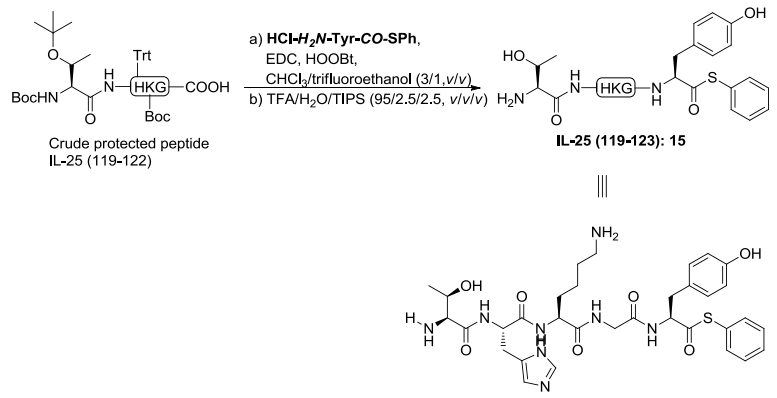


Figure S71. ESI-MS calcd. for $C_{173}H_{256}N_{52}O_{53}S_3$, 4008.40 Da (average isotopes) $[M+3H]^{3+}$ m/z = 1337.13, found 1337.31; $[M+4H]^{4+}$ m/z = 1003.10, found 1003.34; $[M+5H]^{5+}$ m/z = 802.68, found 802.88; $[M+6H]^{6+}$ m/z = 669.07, found 669.09.

Synthesis of C-terminal peptide thioesters 15: IL-25 (119-123) [1]



Scheme S13. Synthesis of **(15)** by n+1 strategy.

Crude protected peptide *BocHN*-Ser(ᵗBu)-His(Trt)-Lys(Boc)-Gly-*COOH* (prepared according to general experimental procedure A and B) (55.3 mg, 73.4 µmol) was dissolved in $CHCl_3$/trifluoroethanol (7.4 mL), and EDC (34.1 mg, 220.1 µmol) and HOOBt (35.9 mg, 220.1 µmol) were then added. After 5 min, **HCl·*H₂N*-Tyr-*SPh*** (70.0 mg, 220.1 µmol) was added, and the reaction mixture was stirred for 3 h to form the crude protected C-terminal peptide thioester. The reaction mixture was then concentrated and subjected to 5.0 mL of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*). After stirring for 2 h, the solution was evaporated under a stream of nitrogen to half of the initial volume. The residue was treated with ice-cold diethyl ether (45 mL), and the resulting suspension was centrifuged to give a white pellet. The supernant was decanted and the pellet was triturated with ice-cold diethyl ether (45 mL). Preparative HPLC purification (20-30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization to give **15** (17.4 mg, 34% yield) as a white powder.

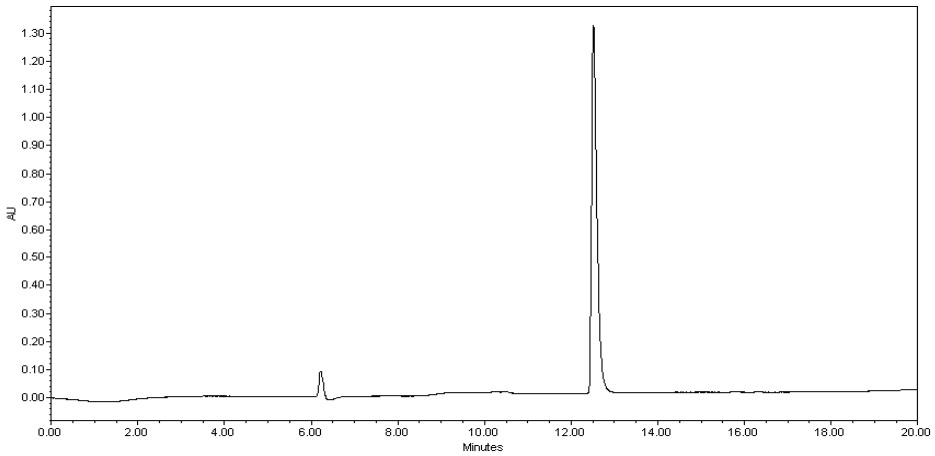


Figure S72. UV trace (190-400 nm) from LC-MS analysis of purified **15**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

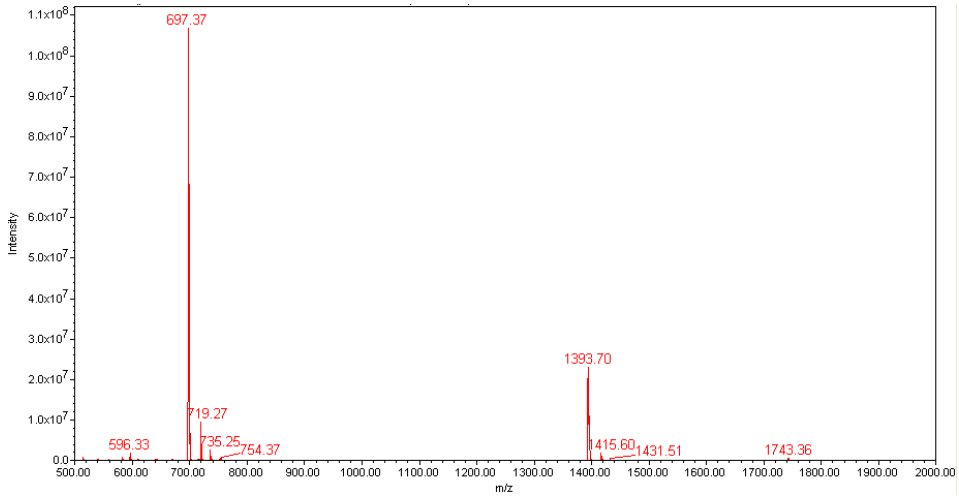
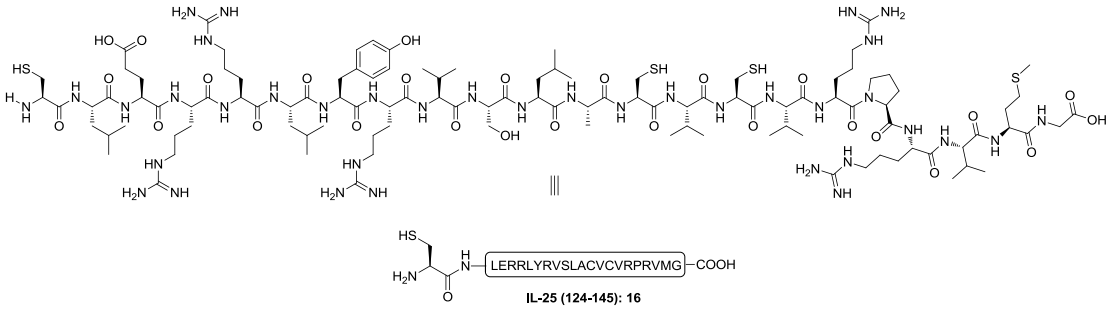


Figure S73. ESI-MS calcd. for $C_{33}H_{45}N_8O_7S$ $[M+H]^+$ *m*/*z* = 697.31, found 697.37; $[M+Na]^+$ *m*/*z* = 719.29, found 719.27 $[2M+H]^+$ *m*/*z* = 1393.62, found 1393.70.

Synthesis of peptide 16: IL-25 (124-145)



Peptide **16** was synthesized according to the general procedure A using 2-chlorotrityl chloride resin (0.5 g). Upon completion of synthesis, the peptide resin was subjected to a cleavage of TFA/H_2O/TIPS (95/2.5/2.5, *v*/*v*/*v*) for 2 h. The resin was filtered and the combined filtrates were blown off under a stream of condensed air. The crude product was triturated with cold diethyl ether to give a white suspension for centrifuge. After decanting diethyl ether, the remaining solid was subjected to preparative HPLC purification (20-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization to afford **16** (22.5 mg, 4%) as a white powder.

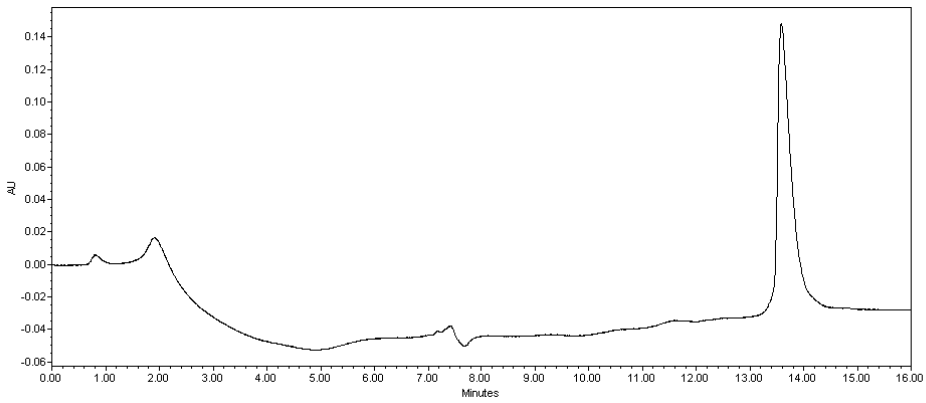


Figure S74. UV trace (190-400 nm) from LC-MS analysis of purified **16**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

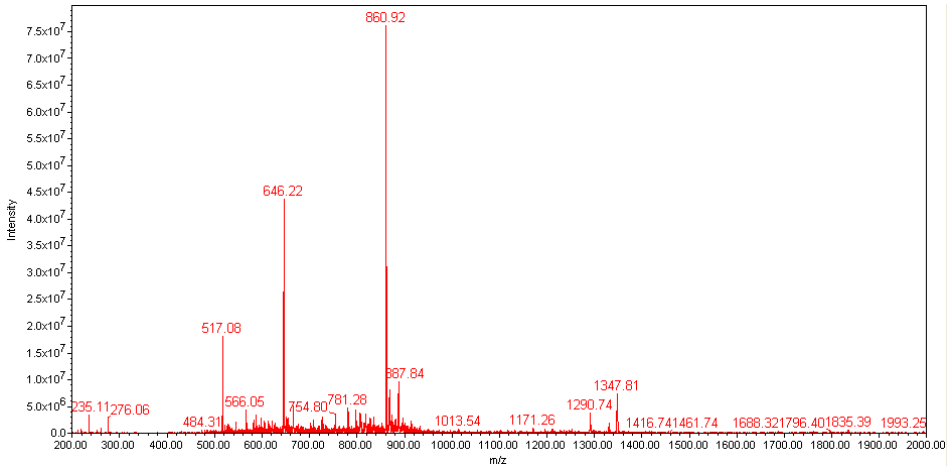
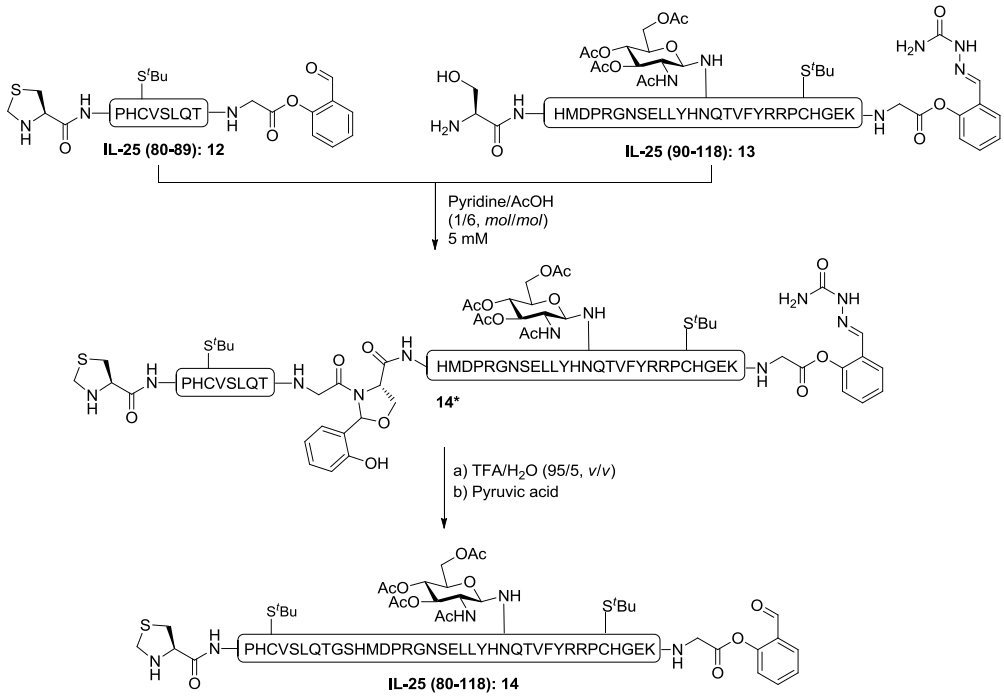


Figure S75. ESI-MS calcd. for $C_{109}H_{191}N_{37}O_{27}S_4$, 2580.17 Da (average isotopes), $[M+2H]^{2+}$ *m/z* = 1291.09, found 1290.74; $[M+3H]^{3+}$ *m/z* = 861.07, found 860.92; $[M+4H]^{4+}$ *m/z* = 646.05, found 646.22; $[M+5H]^{5+}$ *m/z* = 517.04, found 517.08.

B. Assembly of IL-25 glycopeptide/peptide fragments

Peptide fragment 14: IL-25 (80-118)



Scheme S14. Serine Ligation between **(12)** and **(13)** to generate **(14)** in *N-to-C* direction.

12 (12.3 mg, 9.9 µmol) and **13** (26.4 mg, 6.6 µmol) were dissolved in pyridine/acetic acid (1/6, mole/mole) buffer at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for 6 h, and the solvent was then blown off under a stream of condensed air. The residue was then treated with 1.0 mL of TFA/H_2O (95/5, *v*/*v*) for 5 min followed by pyruvic acid (4.6 µL, 65.9 µmol) for 1 h. Preparative HPLC purification (25-30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 13.4 mg (40% yield) of **14** as a white solid.

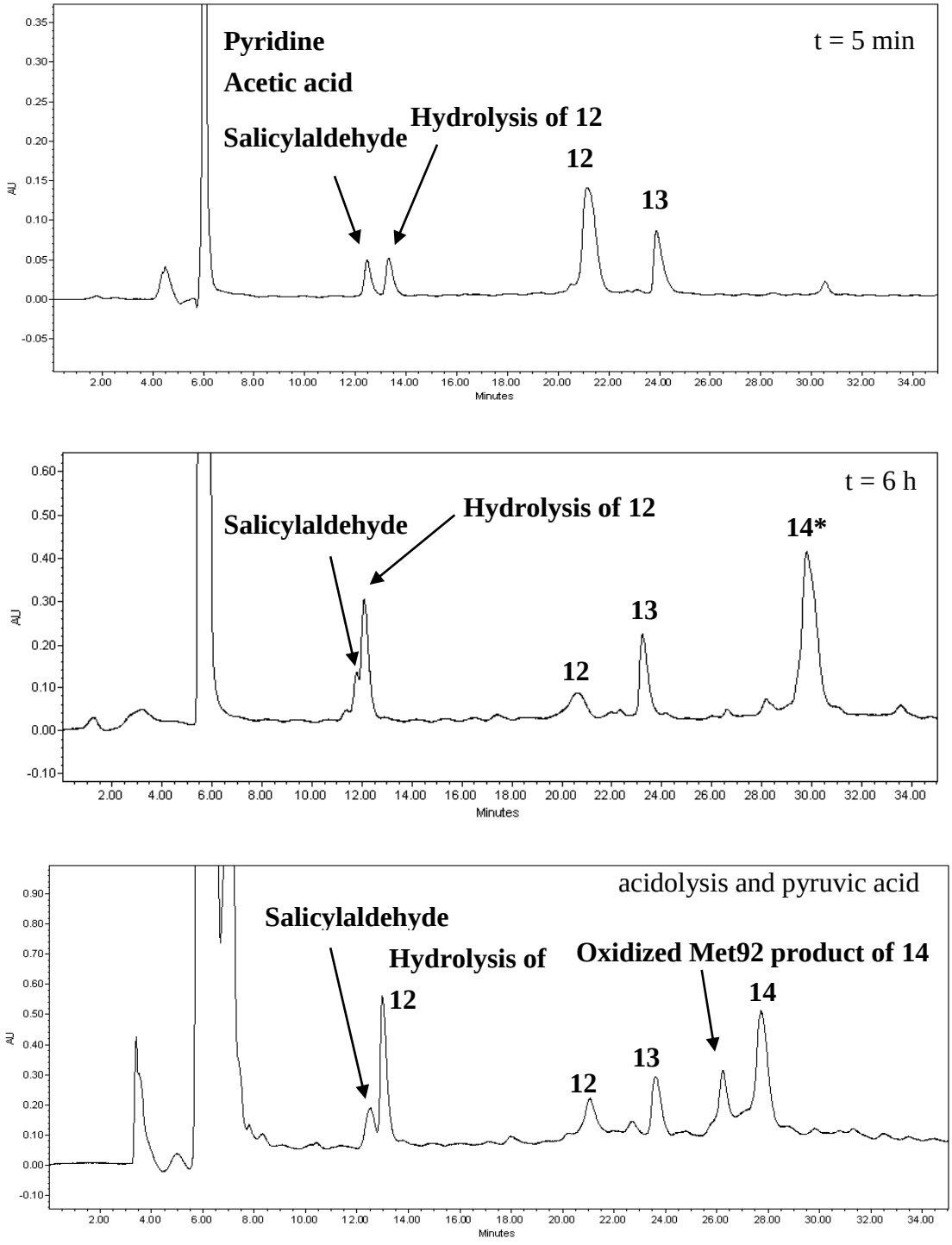


Figure S76. UV trace (190-400 nm) from LC-MS analysis of STL between **12** and **13** to give **14*** at 5 min, 6 h and after *in situ* acidolysis and pyruvic acid to produce **14**: gradient 25-35% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

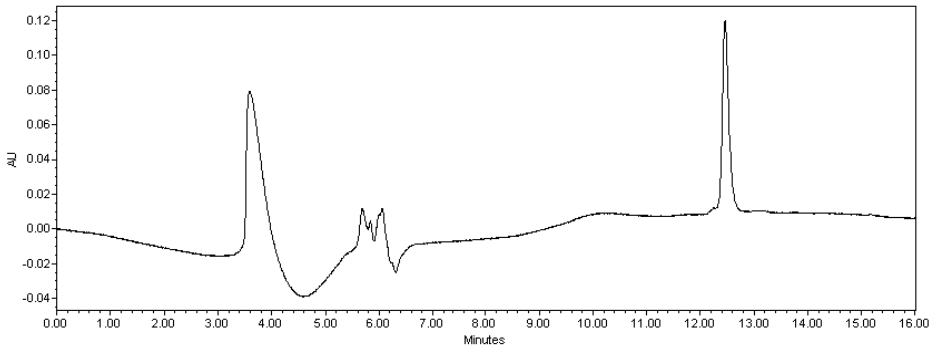


Figure S77. UV trace (190-400 nm) from LC-MS analysis of purified **14**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

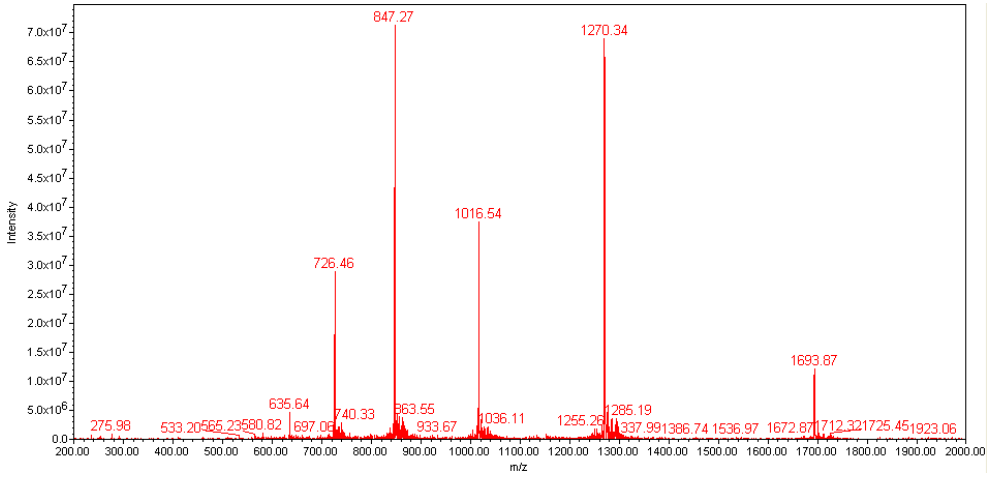
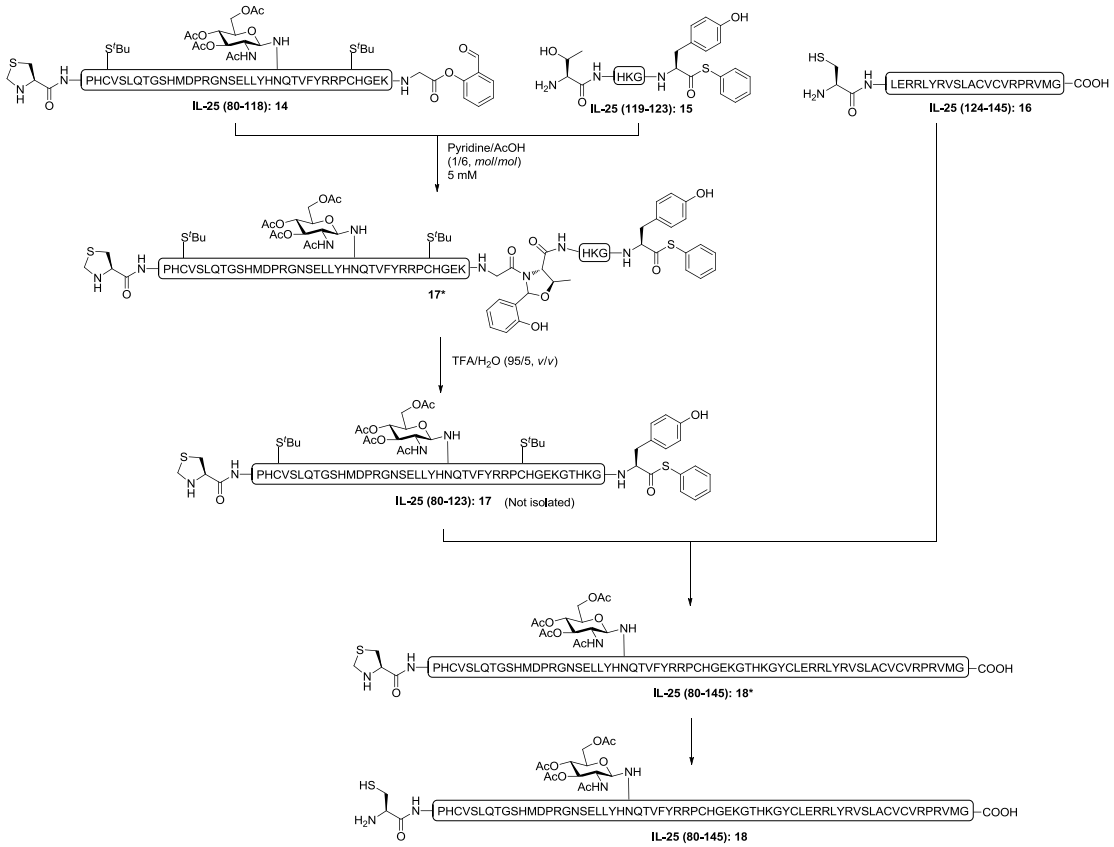


Figure S78. ESI-MS calcd. for $C_{219}H_{328}N_{62}O_{66}S_6$, 5077.71 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1693.57, found 1693.87; $[M+4H]^{4+}$ *m/z* = 1270.43, found 1270.34; $[M+5H]^{5+}$ *m/z* = 1016.54, found 1016.54; $[M+6H]^{6+}$ *m/z* = 847.29, found 847.27; $[M+7H]^{7+}$ *m/z* = 726.39, found 726.46; $[M+8H]^{8+}$ *m/z* = 635.71, found 635.64.

Glycopeptide fragment 18: IL-25 (80-145)



Scheme S15. One-pot Three-segment STL and NCL to produce **(18)**.

14 (11.4 mg, 2.2 μmol) and **15** (2.4 mg, 3.4 μmol) were dissolved in pyridine/acetic acid (1/6, mole/mole) buffer at a concentration of 5 mM at room temperature. The reaction mixture was stirred at room temperature for 4 h to give **17***, and the solvent was then blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (95/5, *v*/*v*) for 5 min to obtain crude **17**. Without purification, crude **17** and **16** (17.3 mg, 6.7 μmol) were dissolved in NCL buffer (2 mM), prepared as described in general procedure E. The reaction mixture was stirred for 2 h, and after completion of the ligation as assessed by HPLC, an aqueous solution of $MeONH_2$·HCl (74.8 μL, 0.6 M) was added followed by TCEP solution (62.4 μL, 0.5 M). The pH was adjusted to ~4.0–4.5 by addition of 2 M HCl solution and the turbid mixture was stirred for 18 h. After this time, the contents were purified by preparative HPLC (15-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 3.6 mg (20 % yield) of **18** as a white solid.

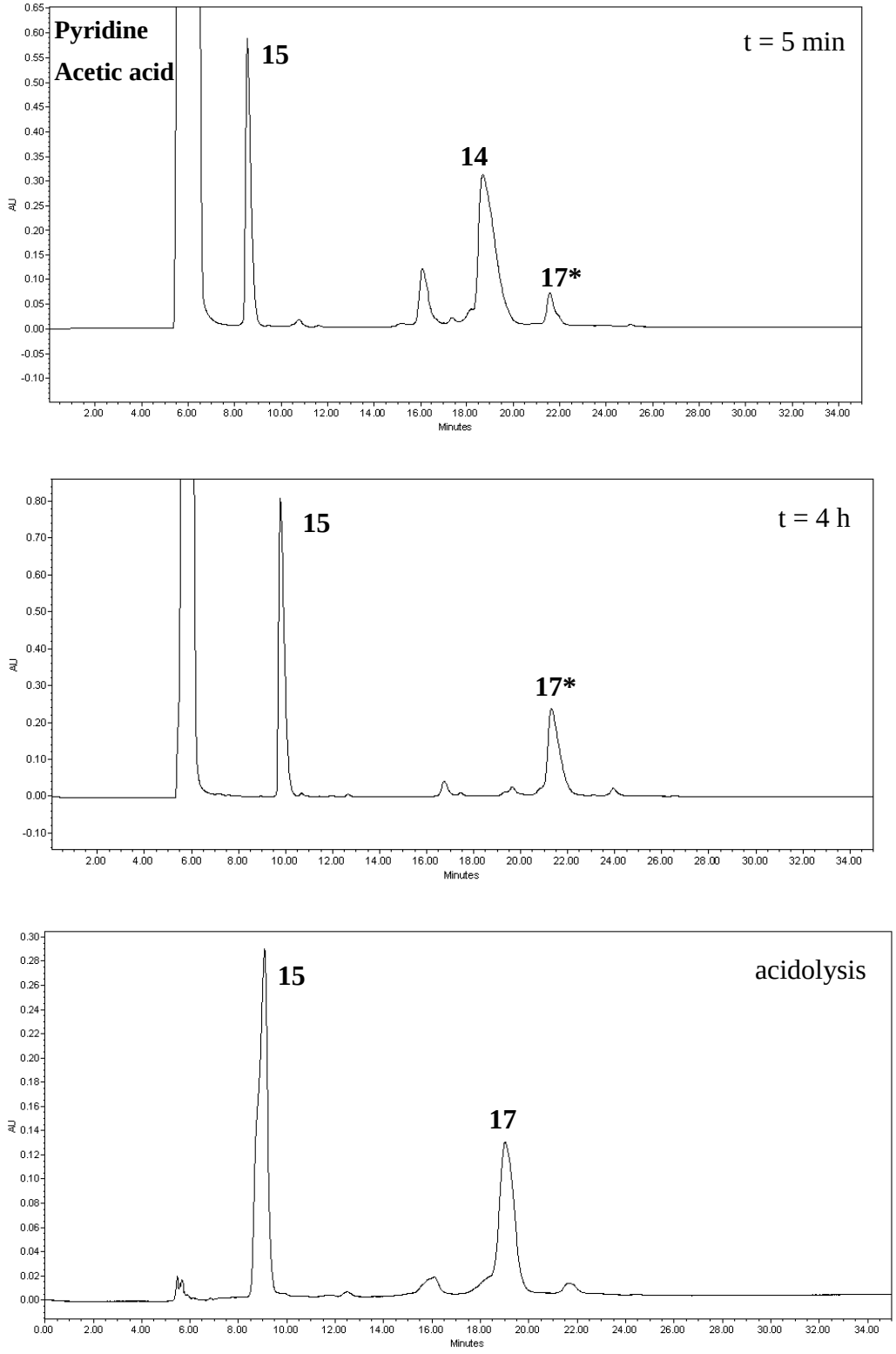


Figure S79. UV trace (190-400 nm) from LC-MS analysis of STL between **14** and **15** to generate **17*** at t = 5 min, 4 h and after acidolysis to generate crude **17**: gradient 25-40% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

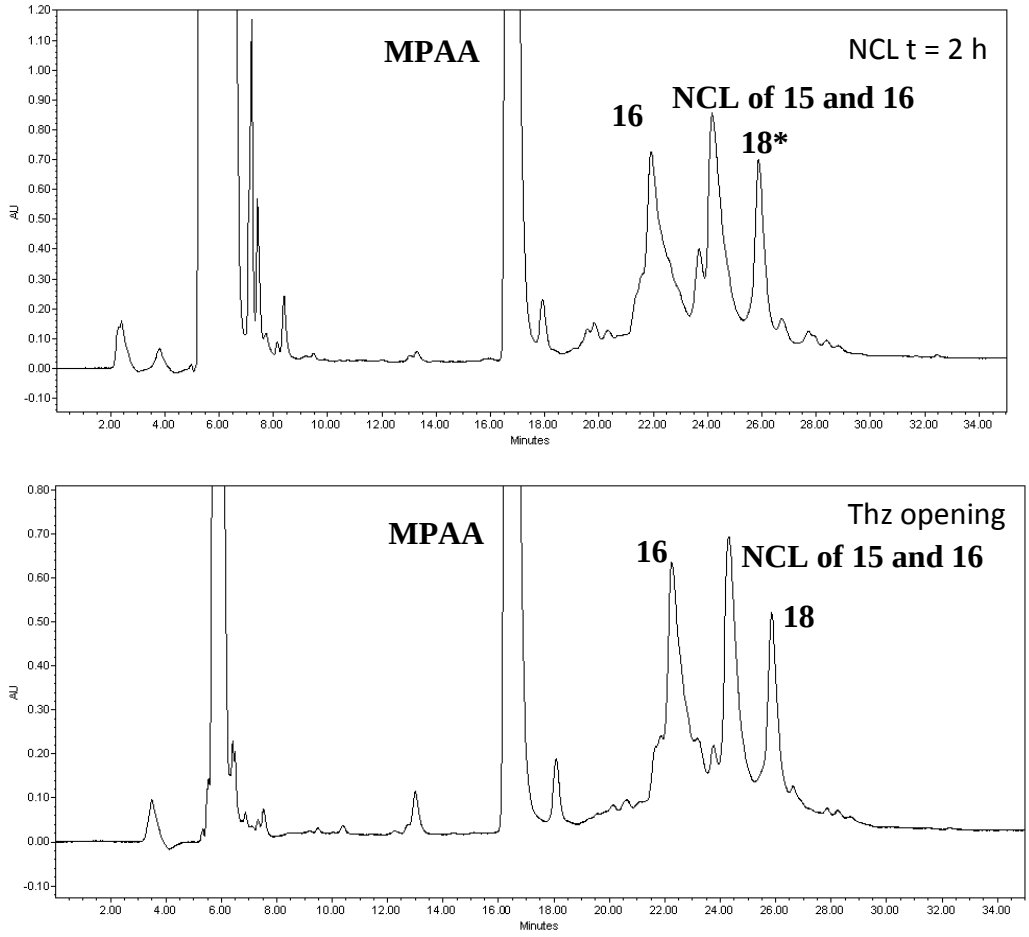


Figure S80. UV trace (190-400 nm) from LC-MS analysis of NCL between **16** and crude **17** to generate **18*** at 2 h and the conversion of **18*** to **18** (Thz opening): gradient 20-40% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

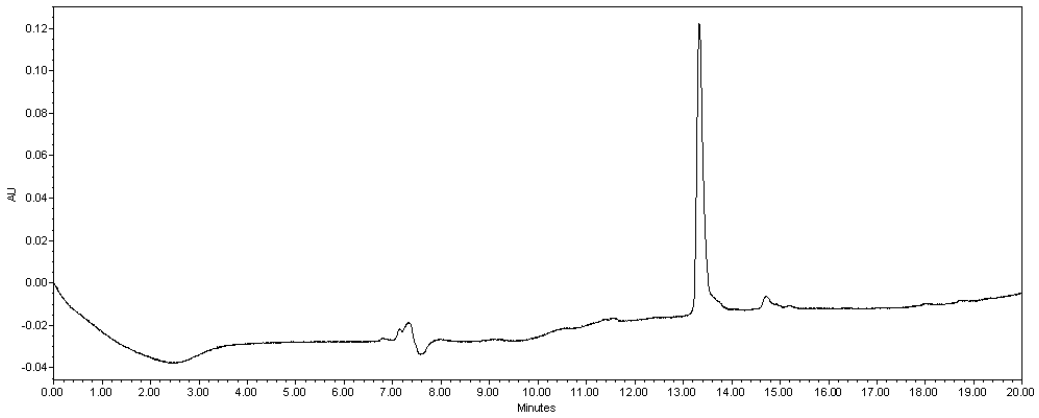


Figure S81. UV trace (190-400 nm) from LC-MS analysis of purified **18**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

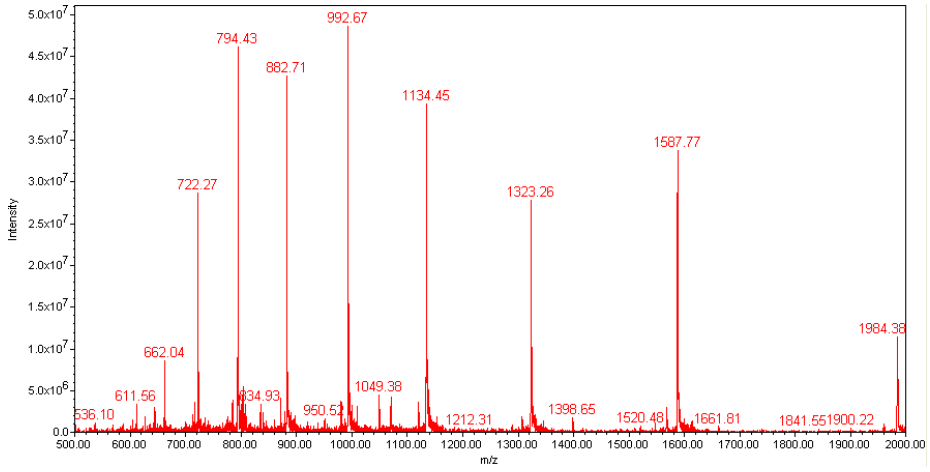
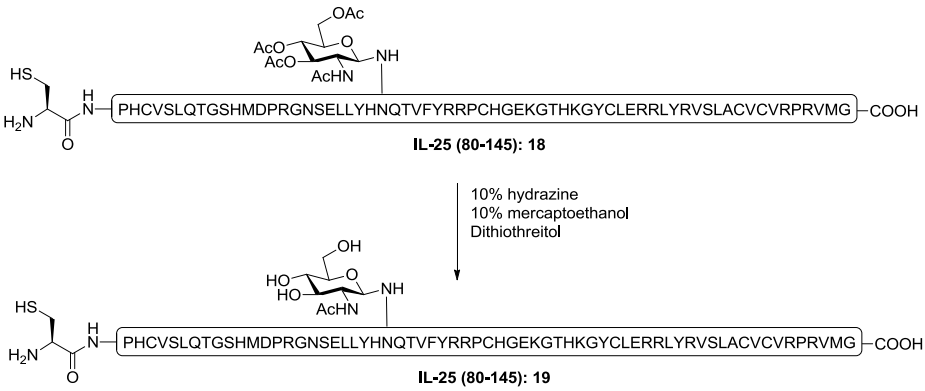


Figure S82. ESI-MS calcd. for $C_{339}H_{535}N_{107}O_{98}S_8$, 7934.05 Da (average isotopes), $[M+4H]^{4+}$ *m/z* = 1984.51, found 1984.38; $[M+5H]^{5+}$ *m/z* = 1587.81, found 1587.77; $[M+6H]^{6+}$ *m/z* = 1323.34, found 1323.26; $[M+7H]^{7+}$ *m/z* = 1134.44, found 1134.45; $[M+8H]^{8+}$ *m/z* = 992.77, found 992.67; $[M+9H]^{9+}$ *m/z* = 882.56, found 882.71; $[M+10H]^{10+}$ *m/z* = 794.41, found 794.43; $[M+11H]^{11+}$ *m/z* = 722.28, found 722.27; $[M+12H]^{12+}$ *m/z* = 662.17, found 662.04.

Glycopeptide fragment 19: IL-25 (80-145)



18 (3.6 mg, 0.4 μmol) was treated with 10% aqueous hydrazine, 10% β-mercaptoethanol, and dithiothreitol (3.5 mg, 22.7 μmol) (0.5 mL, using hydrazine solution 64% wt in H_2O), and the solution was stirred for 2 h. The reaction solution was quenched by aqueous tris(2-carboxyethyl)phosphine (TCEP) (10 mM) containing 6 M Gn·HCl (pH = 4.0) and the mixture was subjected to HPLC purification (20-35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.8 mg (51% yield) of **19** as a white solid.

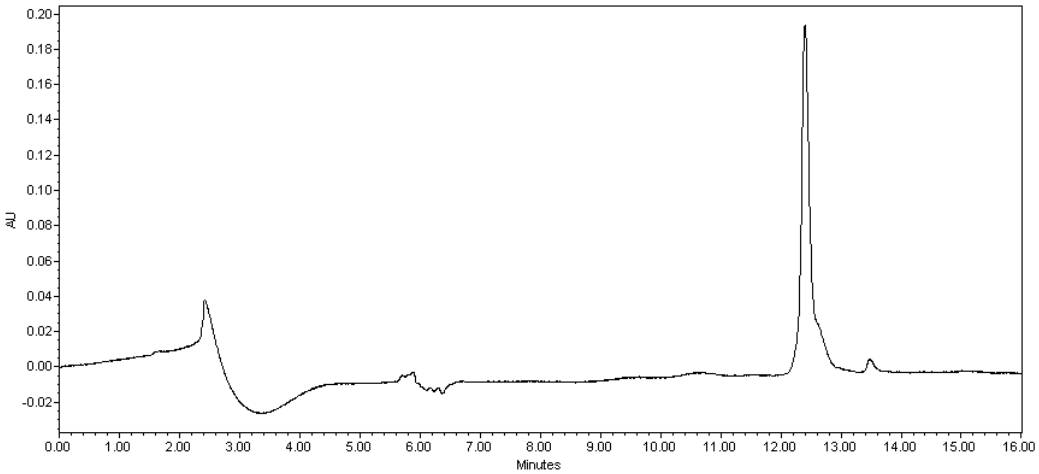


Figure S83. UV trace (190-400 nm) from LC-MS analysis of purified **19**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

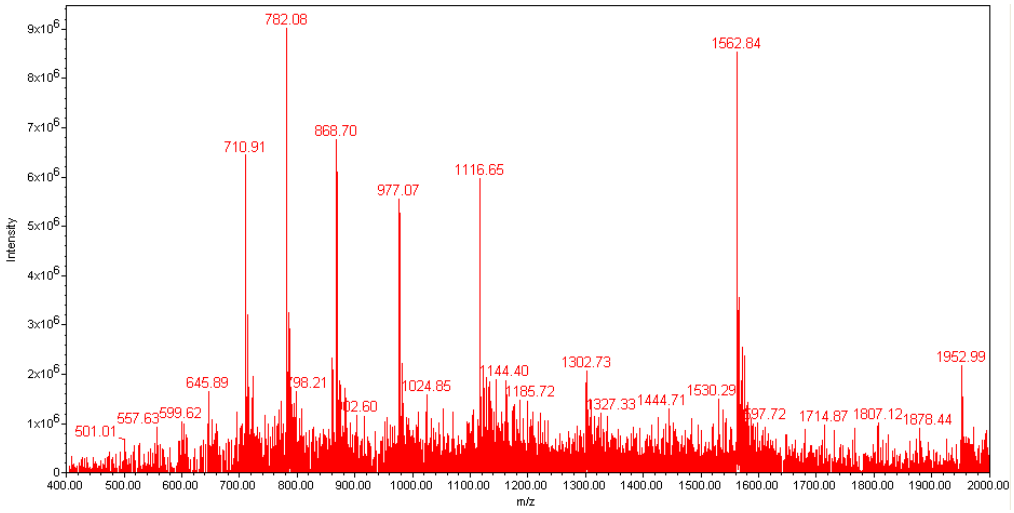
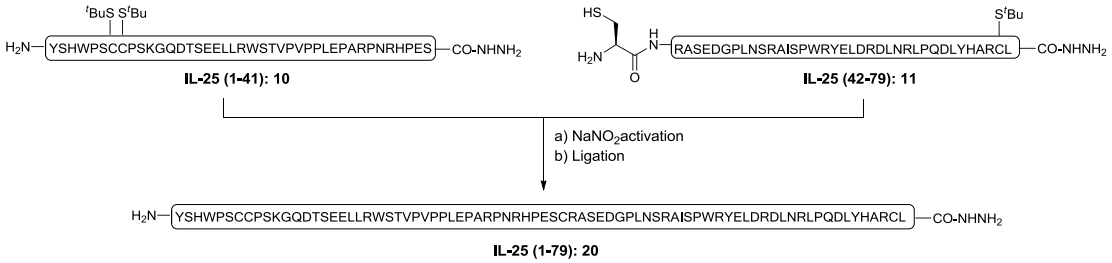


Figure S84. ESI-MS calcd. for $C_{333}H_{529}N_{107}O_{95}S_8$, 7807.94 Da (average isotopes), $[M+4H]^{4+}$ *m*/*z* = 1952.99, found 1952.99; $[M+5H]^{5+}$ *m*/*z* = 1562.56, found 1562.84; $[M+6H]^{6+}$ *m*/*z* = 1302.72, found 1302.73; $[M+7H]^{7+}$ *m*/*z* = 1116.42, found 1116.65; $[M+8H]^{8+}$ *m*/*z* = 976.99, found 977.07; $[M+9H]^{9+}$ *m*/*z* = 868.55, found 868.70; $[M+10H]^{10+}$ *m*/*z* = 781.79, found 782.08; $[M+11H]^{11+}$ *m*/*z* = 710.81, found 710.91.

Peptide fragment 20: IL-25 (1-79) [2]



Scheme S16. Production of **(20)** by the hydrazide method.

Peptide **10** (15.1 mg, 3.1 μmol) was dissolved in an aqueous buffer containing 6 M Gn·HCl and 0.2 M NaH_2PO_4 (pH = 3.0), and cooled to approximately -11°C in an ice-salt bath. A solution of $NaNO_2$ (2.14 mg, 31.0 μmol) in the same buffer (pH = 3.0) was then added dropwise to activate **10**. After stirred at -11°C for 15 min, 4-mercaptophenylacetic acid (MPAA, 26.0 mg, 154.8 μmol) was dissolved in 0.2 M NaH_2PO_4 solution containing 6 M Gn·HCl (pH 7.0) was added into the mixture. The reaction was then taken out of the ice-salt bath and stirred at room temperature for 2 min. Subsequently, peptide **11** (9.5 mg, 2.1 μmol, final concentration 1 mM) was added into the reaction mixture. The pH value of reaction mixture was then adjusted to 7.0 slowly with aqueous NaOH solution (1 M) to initiate the NCL at room temperature. The reaction solution was reduced by aqueous Tris(2-carboxyethyl)phosphine (TCEP) (10 mM) containing 6 M Gn·HCl (pH = 4.0) with a quick operation before reaction analysis or product isolation. The reaction process was monitored by analytic RP-HPLC. After 6 h, the ligation between **10** and **11** was completed. The product was purified by preparative HPLC (20-40% CH_3CN/H_2O over 30 min,) followed by concentration at reduced pressure and lyophilization afforded 7.0 mg (37% yield) of **20** as a white solid.

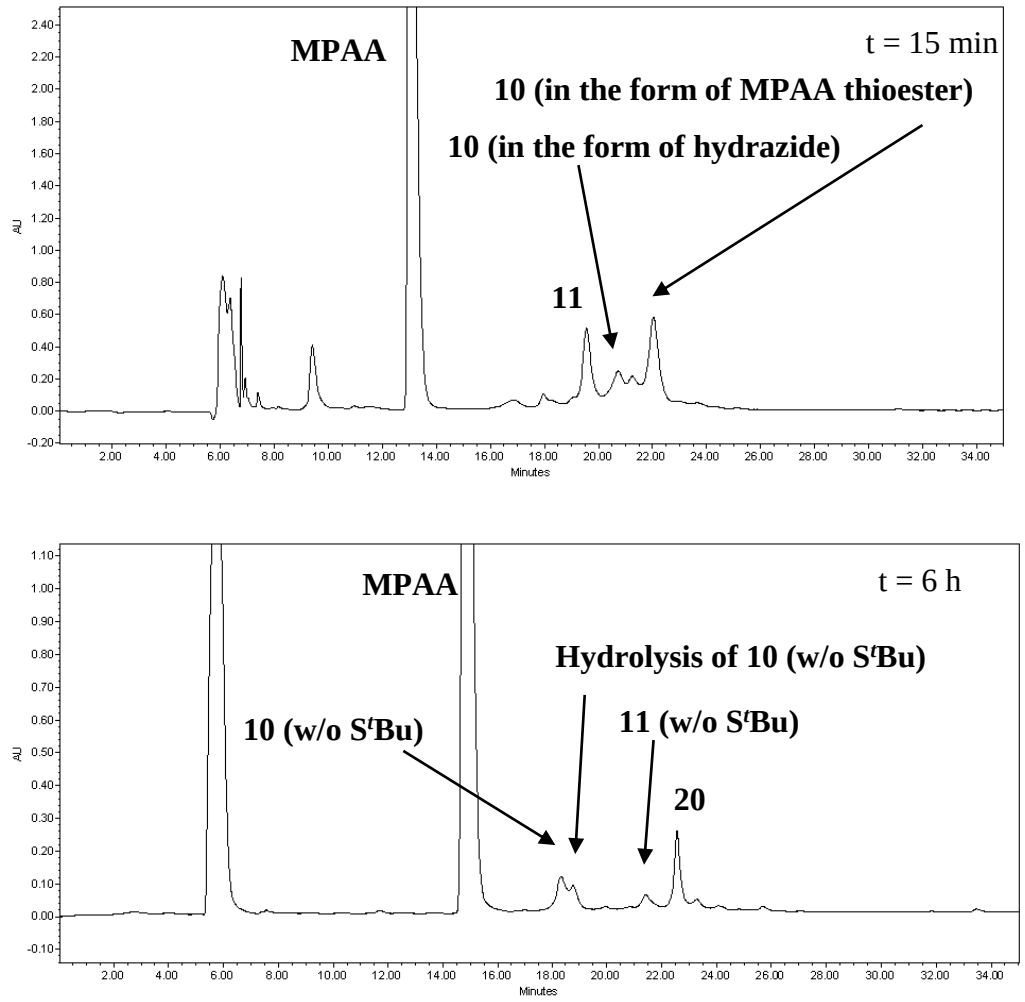


Figure S85. UV trace (190-400 nm) from LC-MS analysis of NCL between **10** and **11** to generate **20** at t = 5 min and 6 h: gradient 25-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

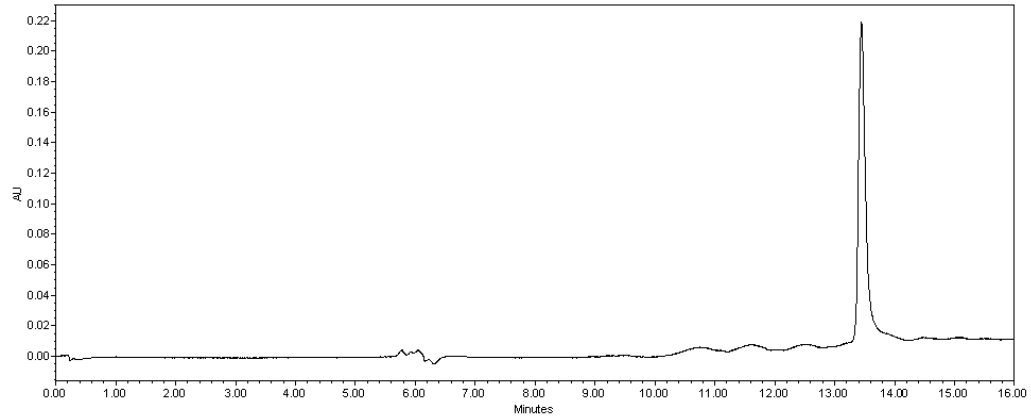


Figure S86. UV trace (190-400 nm) from LC-MS analysis of purified **20**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

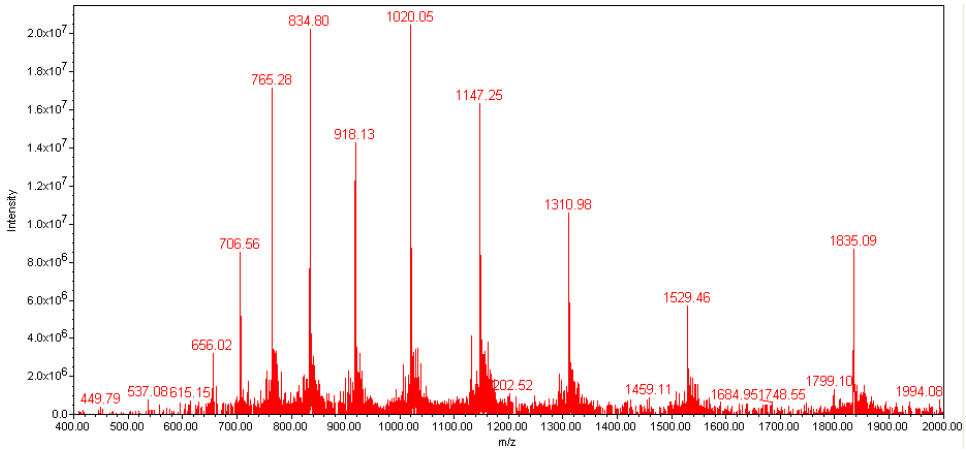
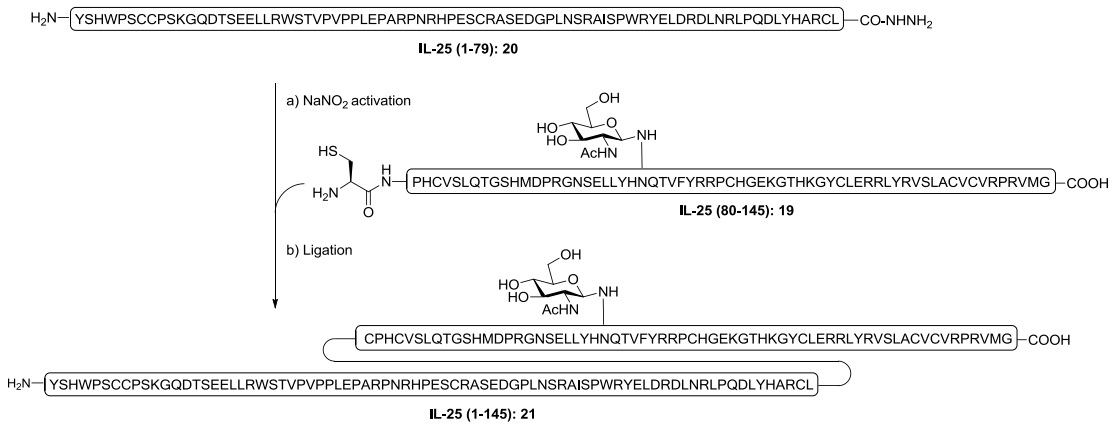


Figure S87. ESI-MS calcd. for $C_{398}H_{613}N_{123}O_{120}S_4$, 9169.14 Da (average isotopes) $[M+5H]^{5+}$ *m*/*z* = 1834.83, found 1835.09; $[M+6H]^{6+}$ *m*/*z* = 1529.19, found 1529.46; $[M+7H]^{7+}$ *m*/*z* = 1310.88, found 1310.98; $[M+8H]^{8+}$ *m*/*z* = 1147.14, found 1147.25; $[M+9H]^{9+}$ *m*/*z* = 1019.79, found 1020.05; $[M+10H]^{10+}$ *m*/*z* = 917.91, found 918.13; $[M+11H]^{11+}$ *m*/*z* = 834.56, found 834.80; $[M+12H]^{12+}$ *m*/*z* = 765.10, found 765.28; $[M+13H]^{13+}$ *m*/*z* = 706.32, found 706.56; $[M+14H]^{14+}$ *m*/*z* = 655.94, found 656.02.

21: IL-25 (1-145) [2]



Scheme S17. Production of **(21)** by the hydrazide method.

Peptide **20** (2.1 mg, 0.2 μmol, final concentration 1 mM) was dissolved in an aqueous buffer containing 6 M Gn·HCl and 0.2 M NaH_2PO_4 (pH = 3.0), and cooled to approximately -11 °C in an ice-salt bath. A solution of $NaNO_2$ (15.8 μg, 2.3 μmol) in the same buffer (pH = 3.0) was then added dropwise to activate **20**. After stirred at -11°C for 15 min, 4-mercaptophenylacetic acid (2.9 mg, 17.1 μmol) was dissolved in 0.2 M Na_2HPO_4 solution containing 6 M Gn·HCl (pH 7.0) was added into the mixture. The reaction was then taken out of the ice-salt bath and stirred at room temperature for 2 min. Subsequently, peptide **19** (1.8 mg, 0.2 μmol) was added into the reaction mixture. The pH value of reaction mixture was then adjusted to 7.2-7.4 slowly with aqueous NaOH solution (1 M) to initiate the NCL at room temperature for 16 h. The reaction solution was reduced by aqueous tris(2-carboxyethyl)phosphine (TCEP) (10 mM) containing 6 M Gn·HCl (pH = 4.0) with a quick operation before reaction analysis or product isolation. The reaction process was monitored by analytic RP-HPLC. After 16 h, the ligation between **19** and **20** was completed. The product was purified by preparative HPLC (25-40% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.3 mg (32% yield) of **21** as a white solid.

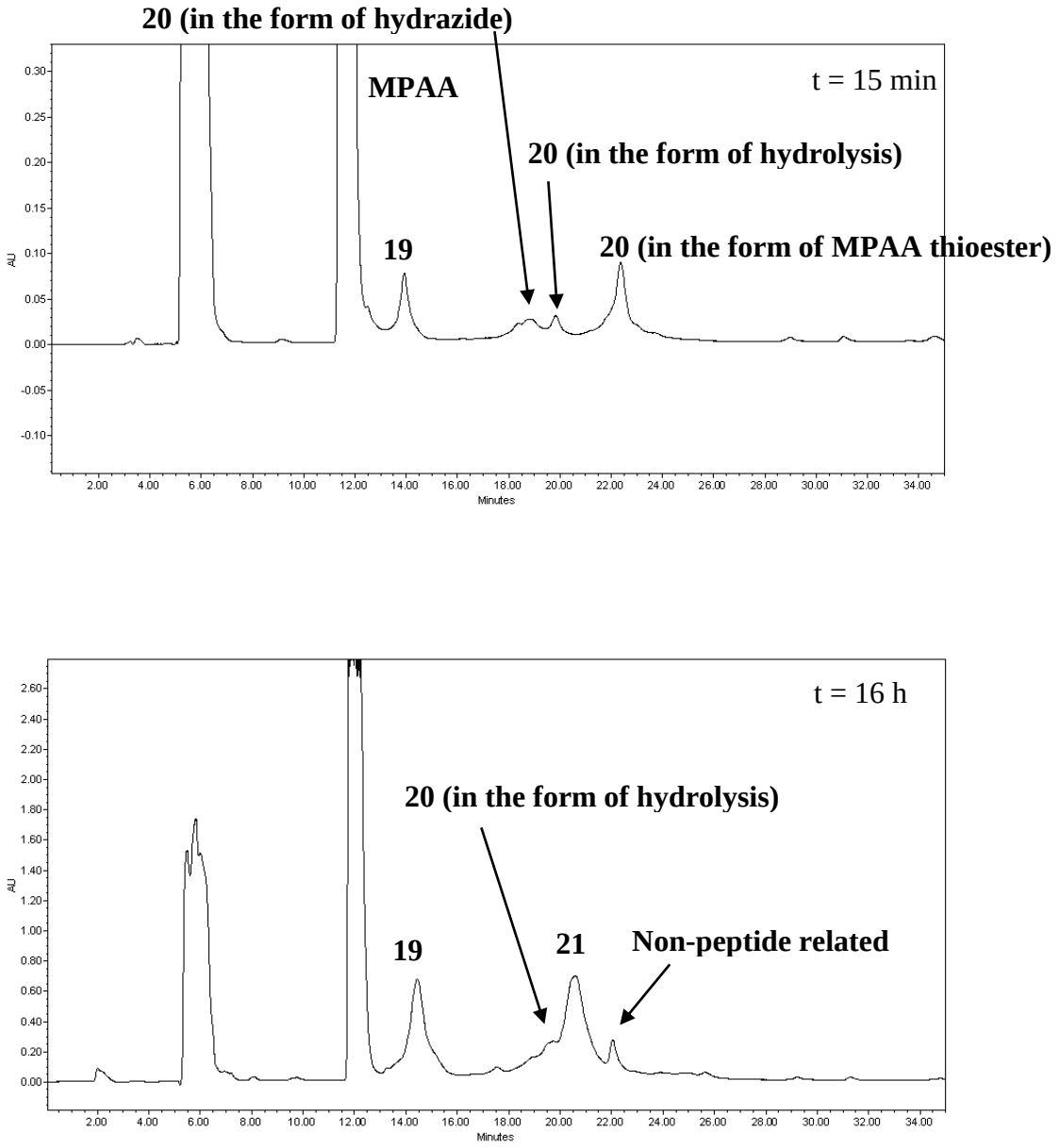


Figure S88. UV trace (190-400 nm) from LC-MS analysis of NCL between **19** and **20** to generate **21** at t = 5 min and 16 h: gradient 30-50% CH_3CN/H_2O containing 0.1% TFA over 30 min at a flow rate of 0.6 mL/min.

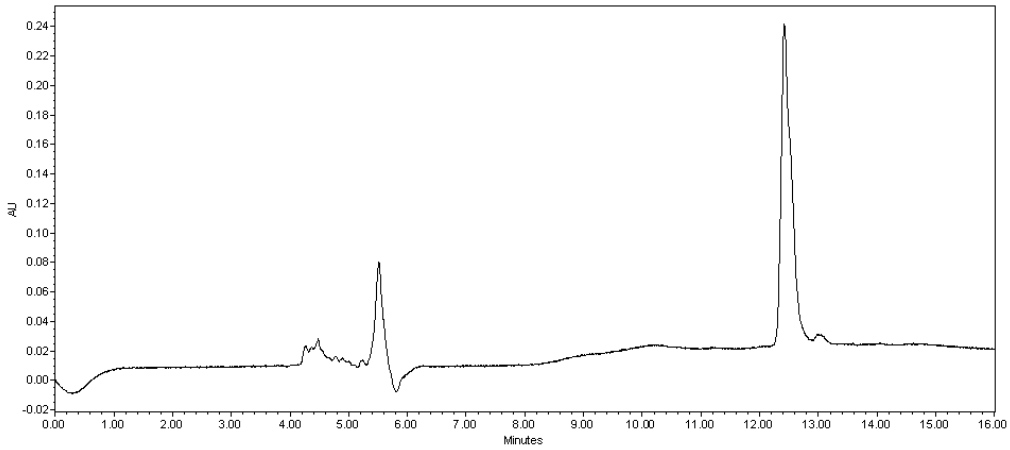


Figure S89. UV trace (190-400 nm) from LC-MS analysis of purified **21**: gradient 5-95% CH_3CN/H_2O containing 0.1% TFA over 15 min at a flow rate of 0.6 mL/min.

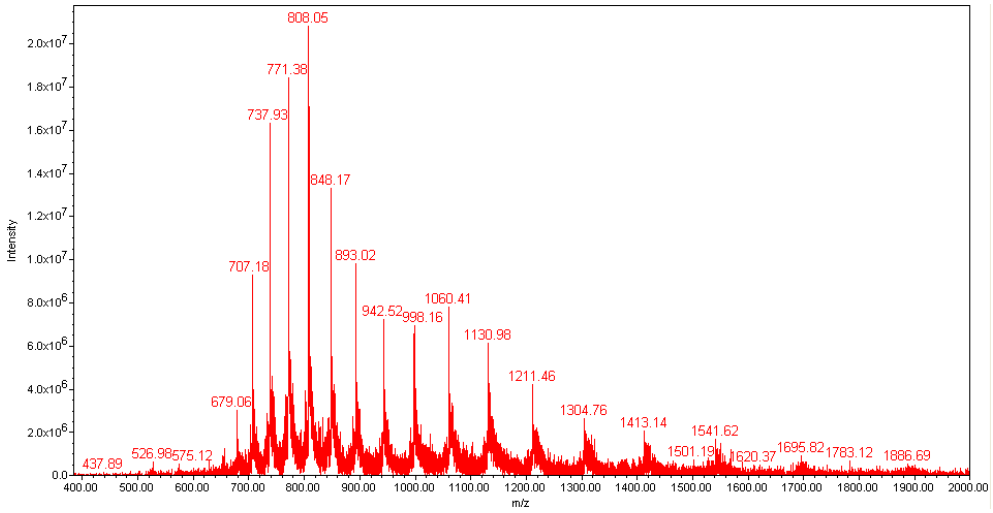


Figure S90. ESI-MS calcd. for $C_{731}H_{1138}N_{228}O_{215}S_{12}$, 16945.04 Da (average isotopes), $[M+10H]^{10+}$ *m/z* = 1695.50, found 1695.82; $[M+11H]^{11+}$ *m/z* = 1541.46, found 1541.62; $[M+12H]^{12+}$ *m/z* = 1413.09, found 1413.14; $[M+13H]^{13+}$ *m/z* = 1304.47, found 1304.76; $[M+14H]^{14+}$ *m/z* = 1211.36, found 1211.46; $[M+15H]^{15+}$ *m/z* = 1130.67, found 1130.98; $[M+16H]^{16+}$ *m/z* = 1060.07, found 1060.41; $[M+17H]^{17+}$ *m/z* = 997.77, found 998.16; $[M+18H]^{18+}$ *m/z* = 942.39, found 942.52; $[M+19H]^{19+}$ *m/z* = 892.84, found 893.02; $[M+20H]^{20+}$ *m/z* = 848.25, found 848.17; $[M+21H]^{21+}$ *m/z* = 807.91, found 808.05; $[M+22H]^{22+}$ *m/z* = 771.23, found 771.38; $[M+23H]^{23+}$ *m/z* = 737.74, found 737.93; $[M+24H]^{24+}$ *m/z* = 707.04, found 707.18; $[M+25H]^{25+}$ *m/z* = 678.80, found 679.06.

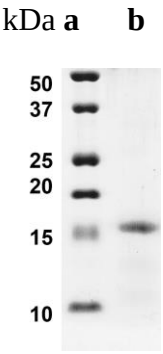


Figure S91. SDS-PAGE analysis of **21**. Lane a: marker; lane b: IL-25 (1-145) **21**.

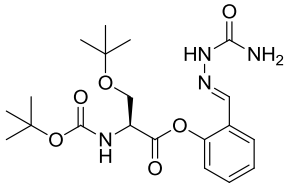
C. Folding of IL-25 glycopeptide (21)

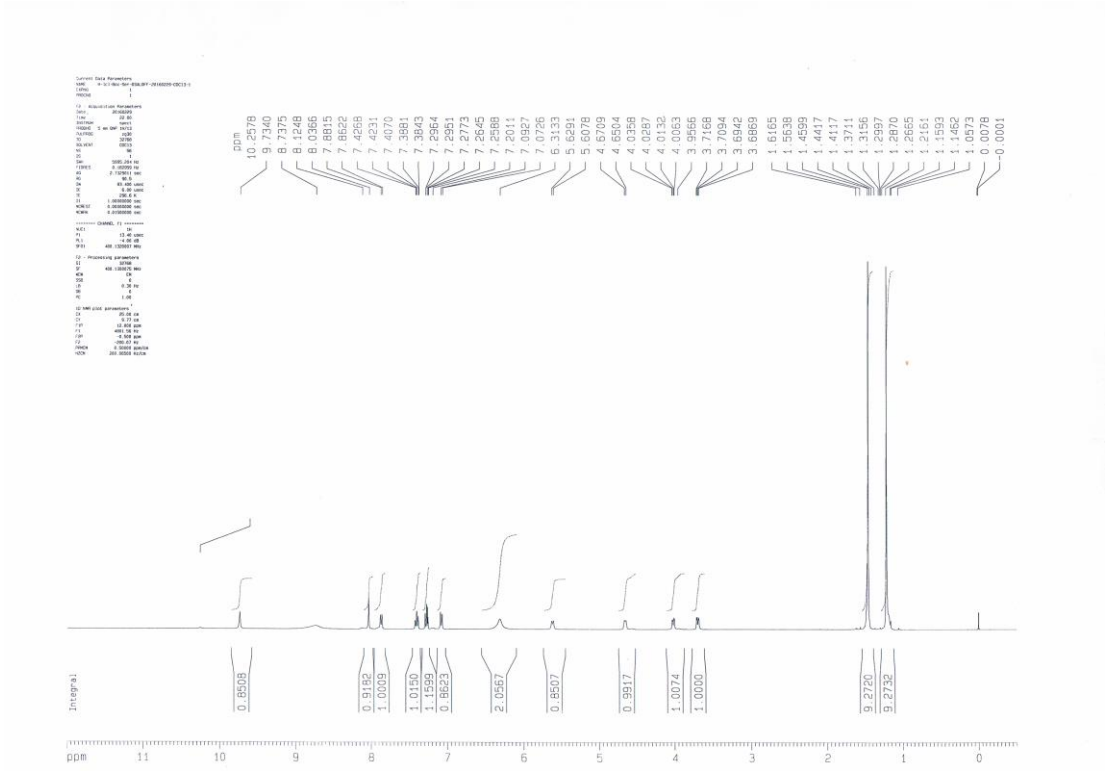
Several folding conditions were tried to perform oxidative folding of **21**.
1) Oxidative folding of **21** was carried out, by following Rao's condition[3], at 4ºC for 6 hours in the folding buffer of 25 mM Tris·HCl, 200 mM NaCl, 10 % glycerol, 1 mM GSH, 10 mM GSSG, 0.5 M arginine and 2 mM EDTA at a concentration of 1 mg/mL at pH 7.2. LCMS analysis showed that no desired folded protein was formed. 2) By using Unverzagt's condition[4], **21** was then incubated in buffer of 0.1 M Tris, 2 mM EDTA, 0.3 mM GSSG at pH 7.7 for 4 days. However, this did not work in our case as suggested by LCMS. 3) Following another procedure published by Unverzagt's group[5], **21** was then dissolved in buffer containing 6 M Gn·HCl, 0.1 M Na_2HPO_4, 0.1 M MPAA, 5 mM TCEP at pH 7. The buffer was then diluted to refolding buffer of 0.5 M arginine, 150 mM NaCl, 50 mM Na_2HPO_4, pH 7.4. It was oxidized in the open tube for 4 days. However, LCMS analysis showed no desired folded product. 4) According to Liu's folding condition[6], **21** was subjected to Tris·HCl buffer containing 1 M Gn·HCl, 10% (*v*/*v*) DMSO at pH 8.5 for 24 hours. However, the result for **21** folding was failed. 5) Finally, dialysis was adopted following Kajihara's condition[7]. **21** was dissolved in a Tris·HCl buffer (100 mM, pH 7.5) containing 6 M Gn·HCl at concentration of 0.1 mg/mL. This solution was poured into the dialysis tubing (MWCO at 3,000) and then dialyzed against the first folding buffer (3 M Gn-HCl, 100 mM Tris HCl, pH 8.5) containing 4 μM GSH and 0.5 μM GSSG for redox system and left for 12 hours at 4ºC. Then, the external buffer solution was replaced the second

folding buffer solution (1 M Gn·HCl, 100 mM Tris·HCl, pH 8.0) and dialysis was performed for 8 hours. Finally, the external buffer was discarded and changed again to the third folding buffer solution (10 mM Tris HCl, pH 7.0) and dialysis was performed for 24 hours. Nonetheless, there is no good results as precipitate was formed. LCMS suggested no correctly folded IL-25 was observed.

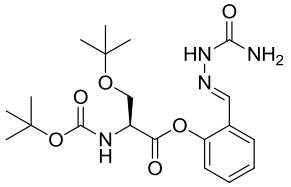
XI. ^{1}H and ^{13}C NMR spectra

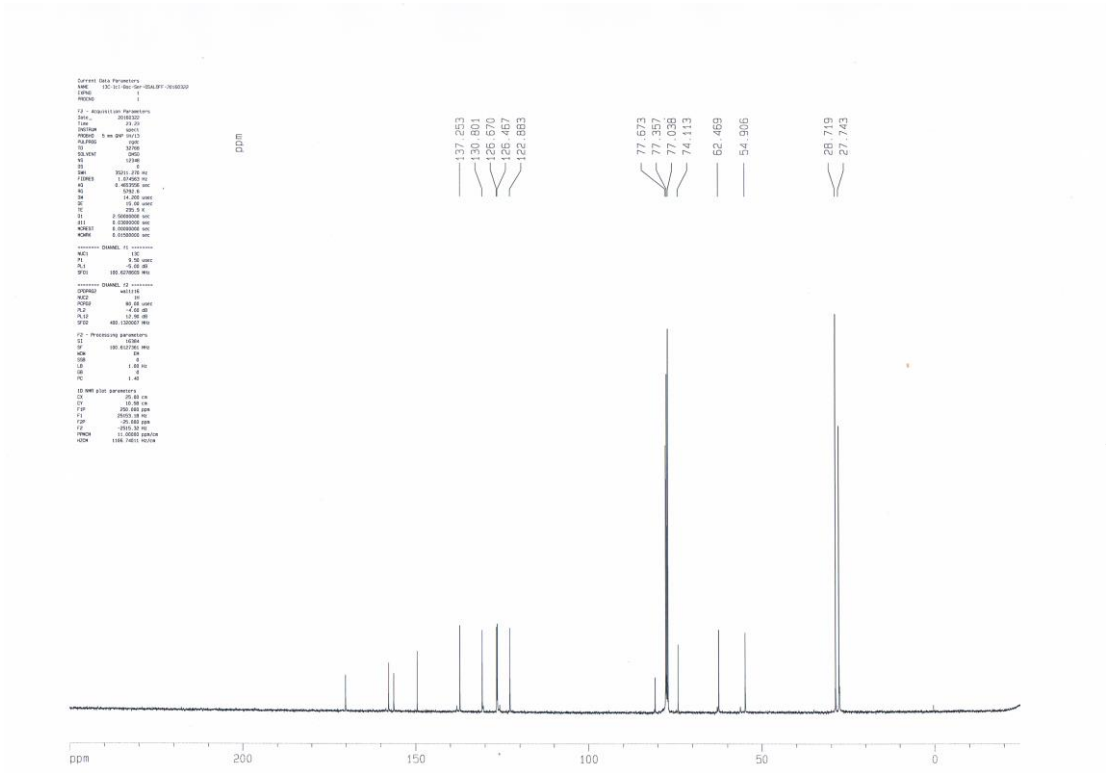
^{1}H spectrum of *BocHN*-Ser(O^{t}Bu)-*CO*-SAL^{off}



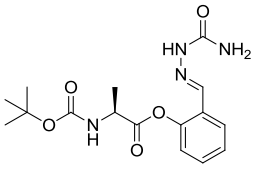


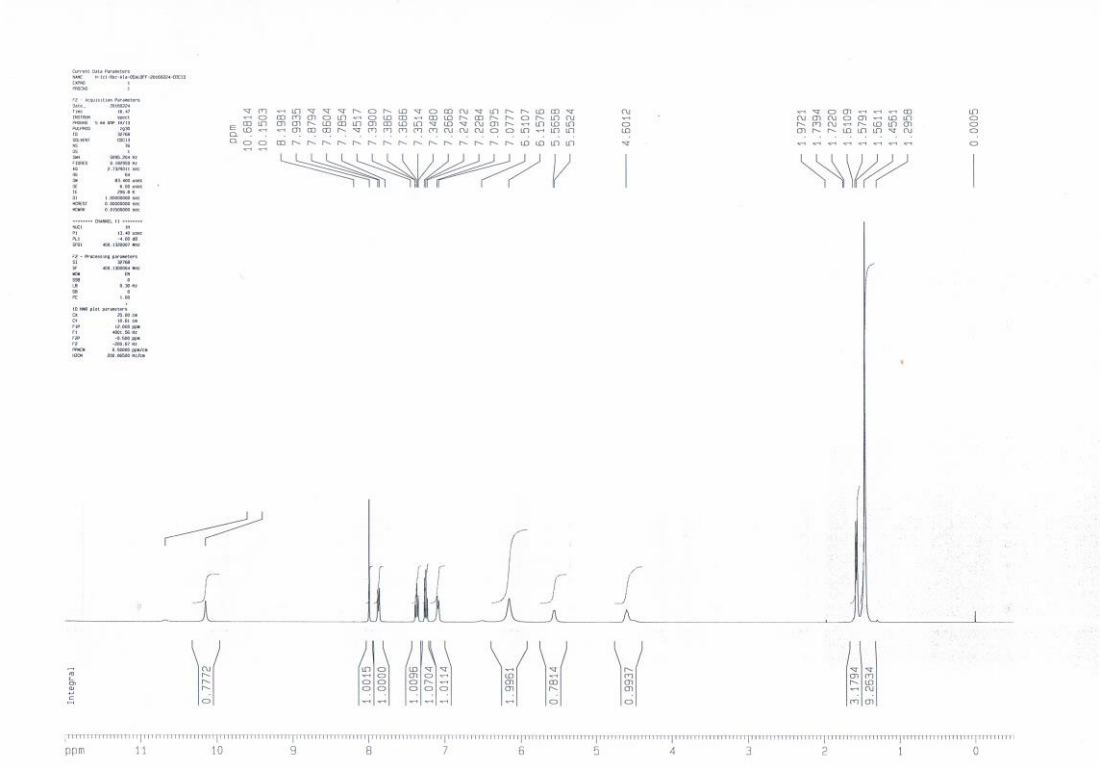
^{13}C spectrum of *BocHN*-Ser(O^{t}Bu)-*CO*-SAL^{off}



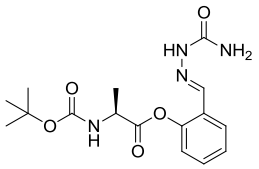


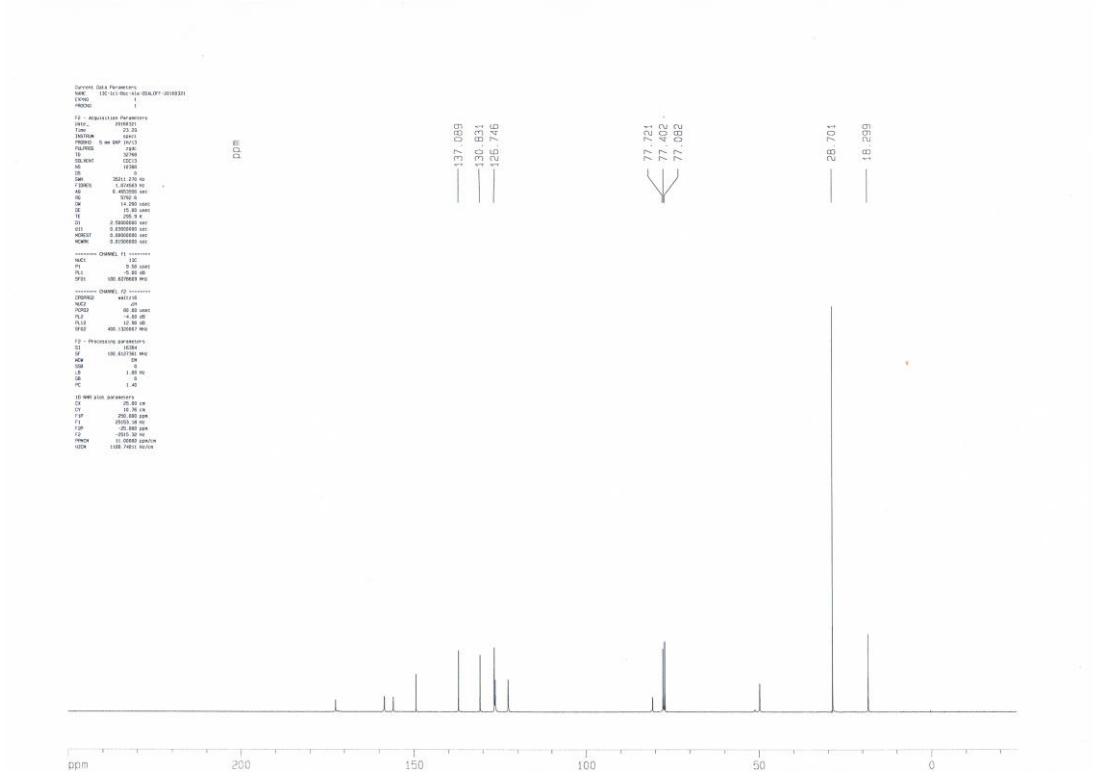
^{1}H spectrum of *BocHN*-Ala-*CO*-SAL^{off}



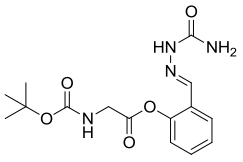


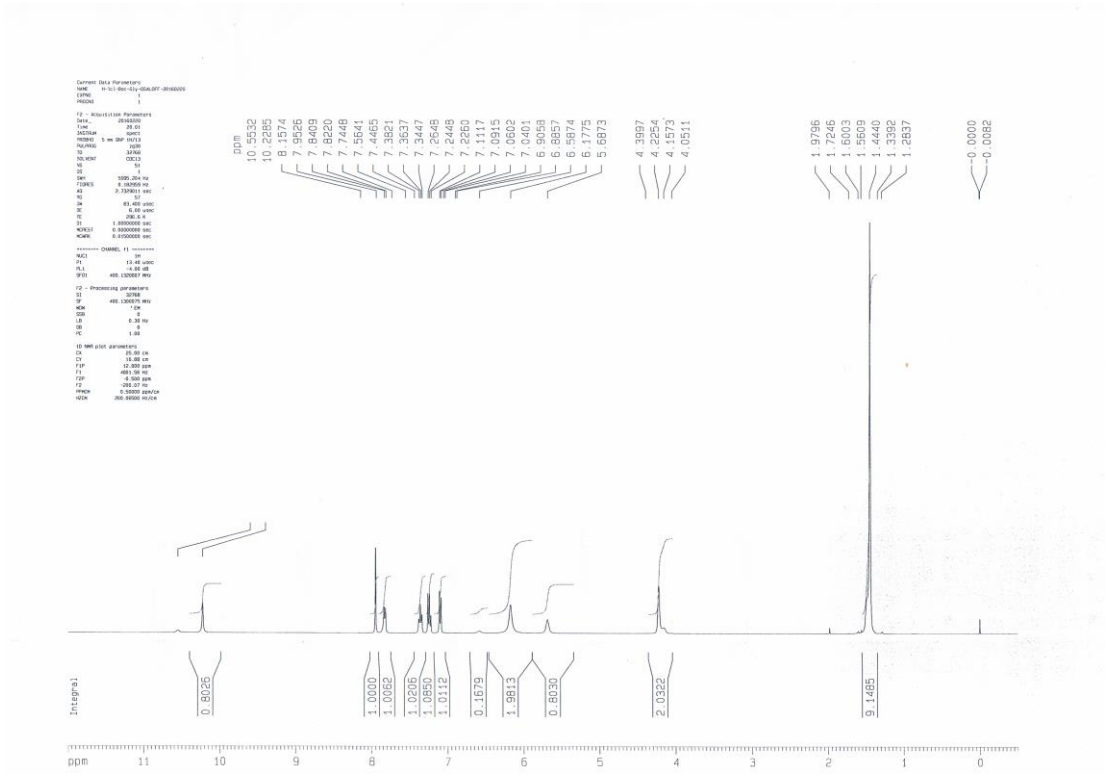
^{13}C spectrum of *BocHN*-Ala-*CO*-SAL^{off}



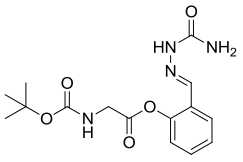


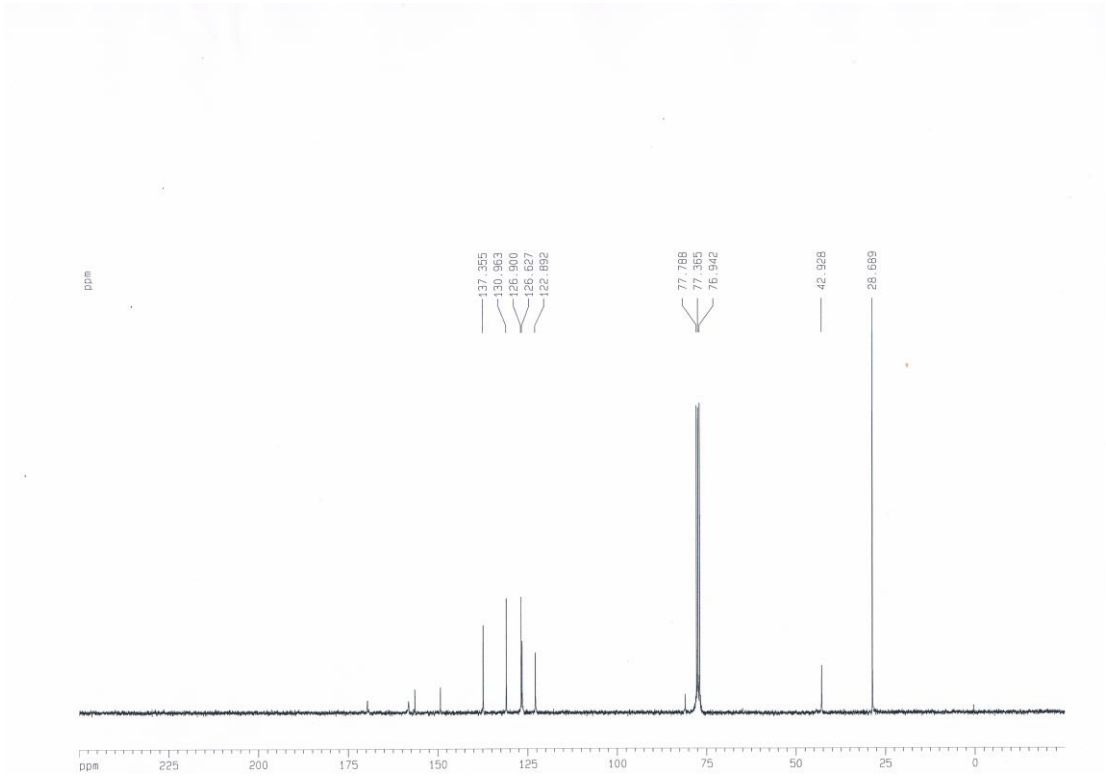
^{1}H spectrum of *BocHN*-Gly-*CO*-SAL^{off}



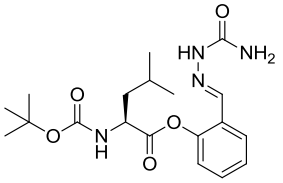


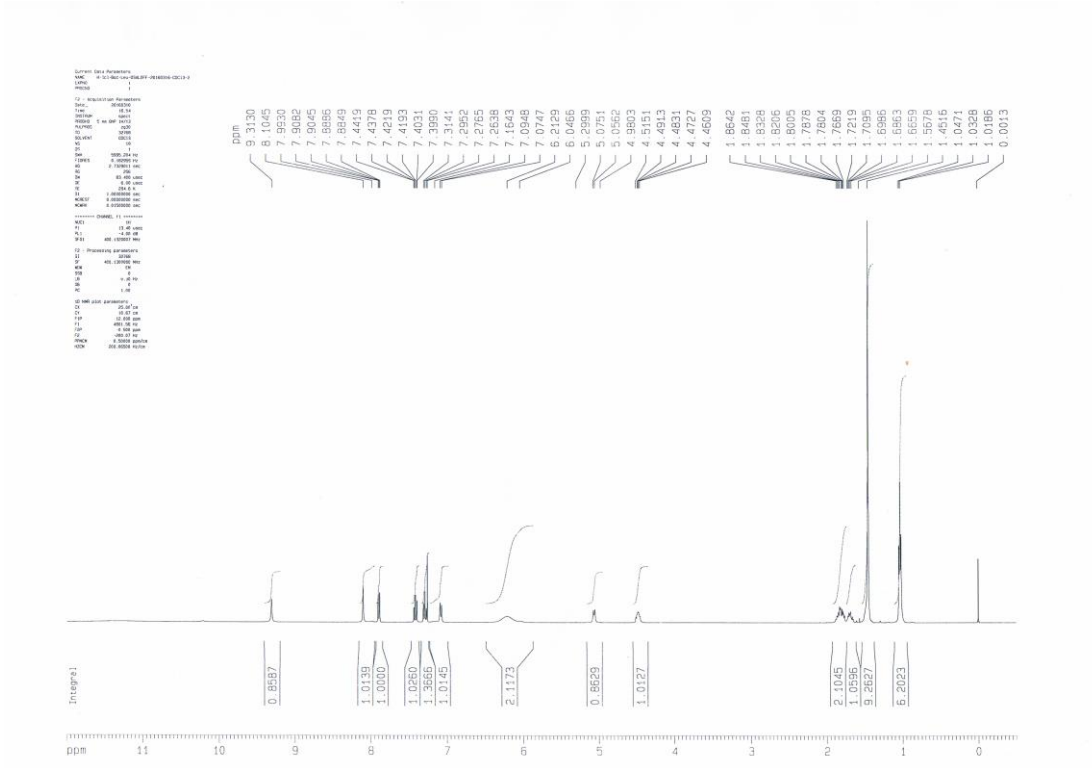
^{13}C spectrum of *BocHN*-Gly-*CO*-SAL^{off}



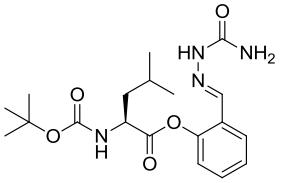


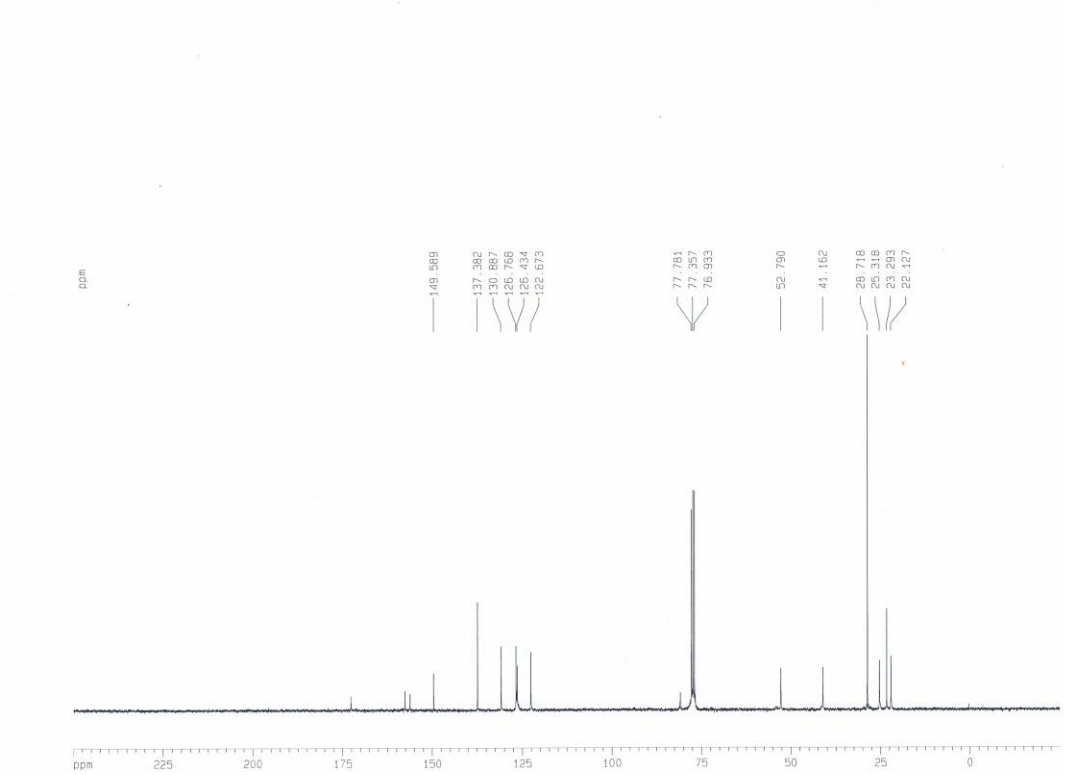
^{1}H spectrum of *BocHN*-Leu-*CO*-SAL^{off}



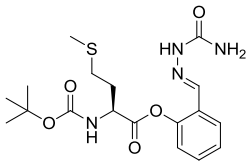


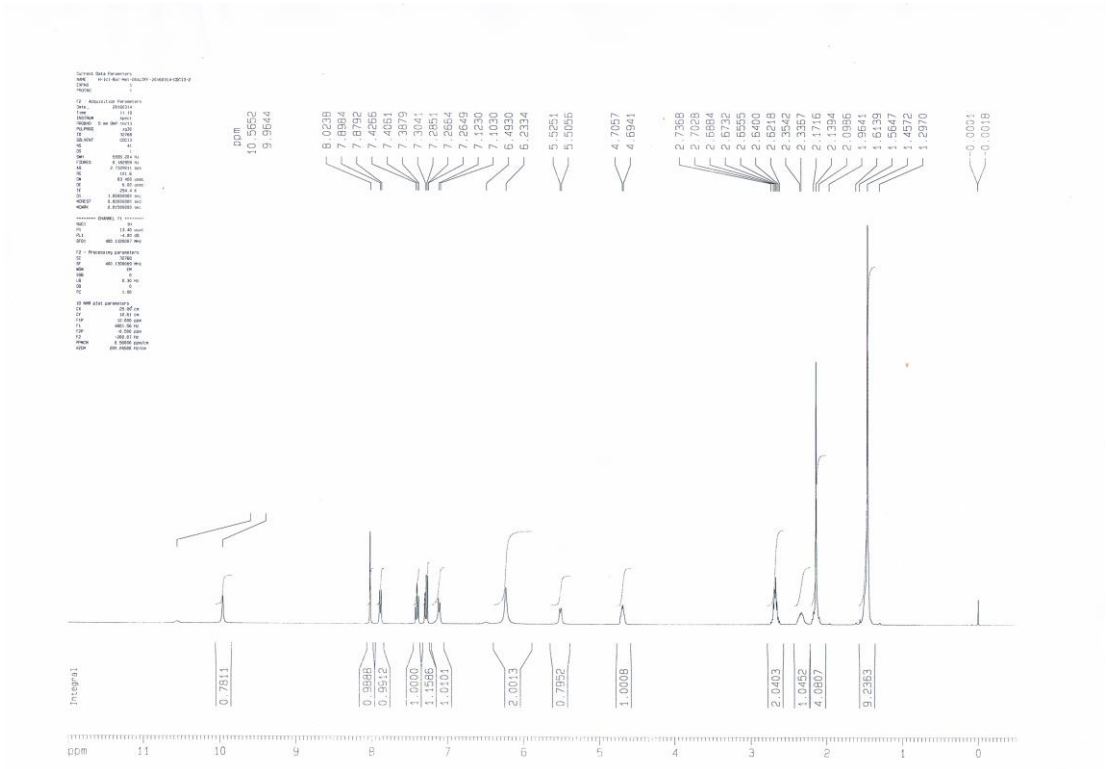
^{13}C spectra of *BocHN*-Leu-*CO*-SAL^{off}



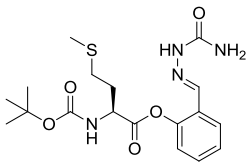


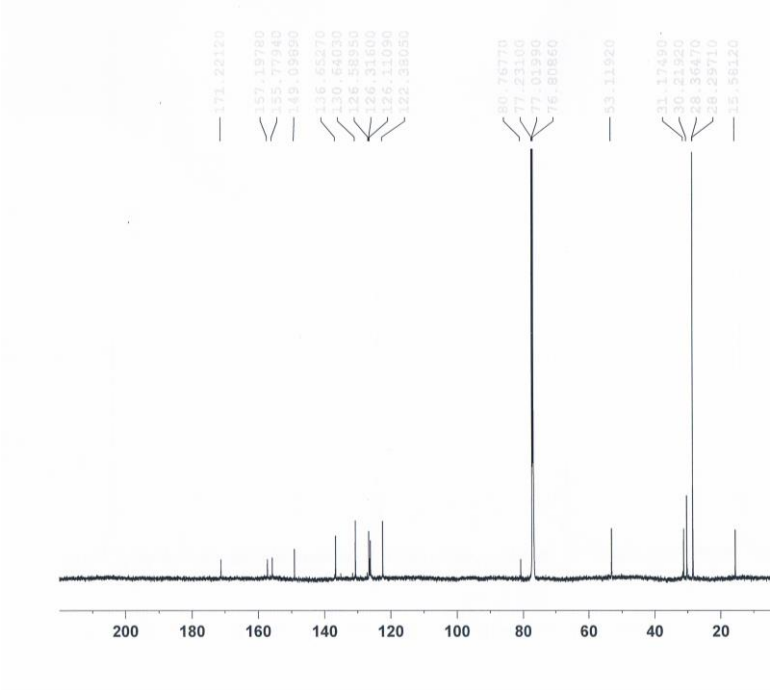
^{1}H spectrum of *BocHN*-Met-*CO*-SAL^{off}



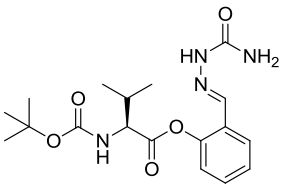


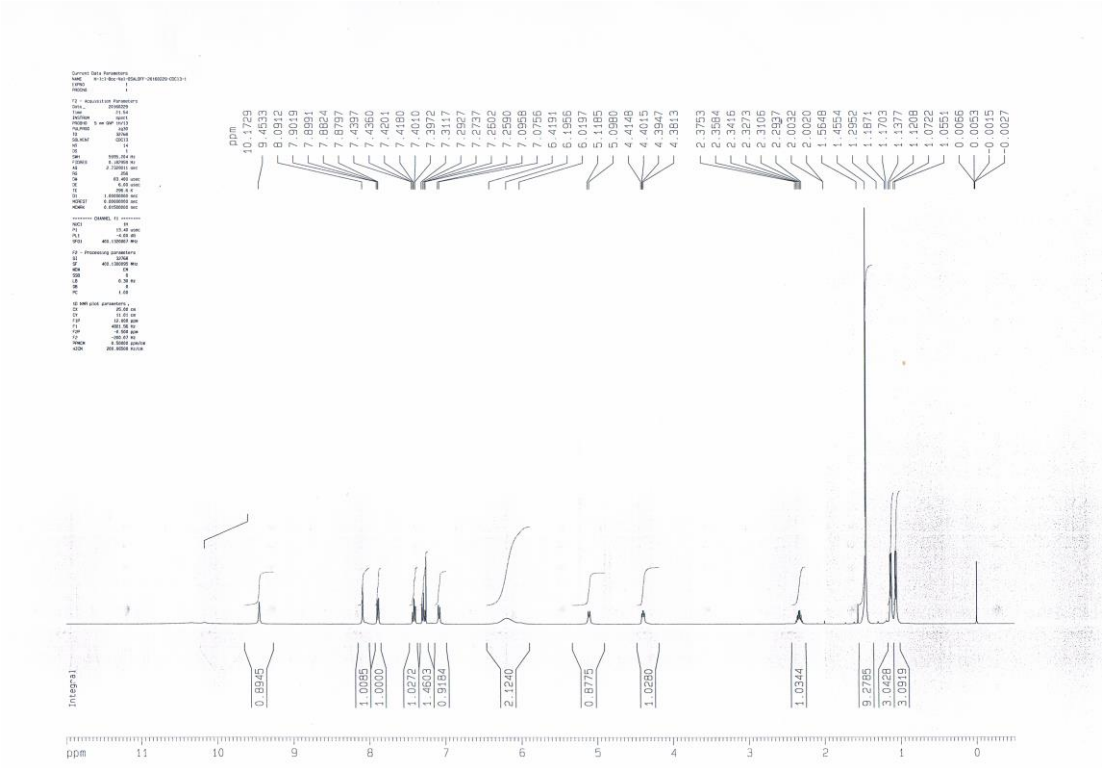
^{13}C spectrum of *BocHN*-Met-*CO*-SAL^{off}



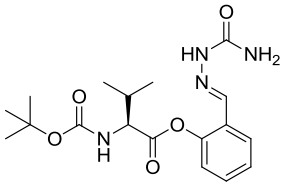


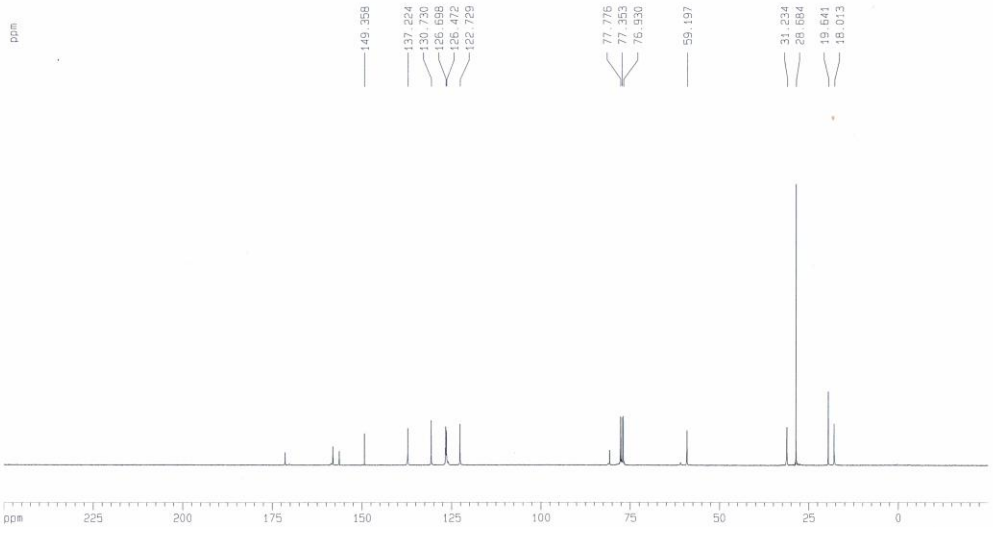
^{1}H spectrum of *BocHN*-Val-*CO*-SAL^{off}



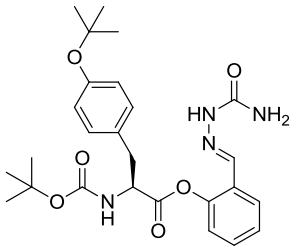


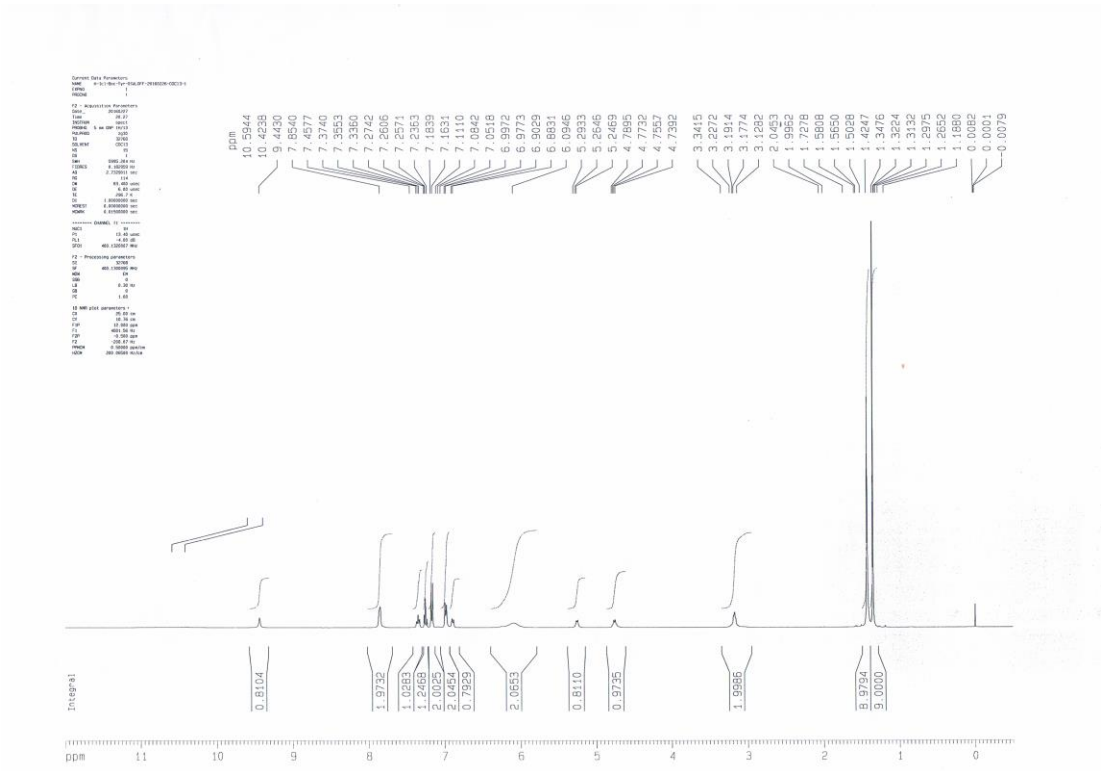
^{13}C spectrum of *BocHN*-Val-*CO*-SAL^{off}



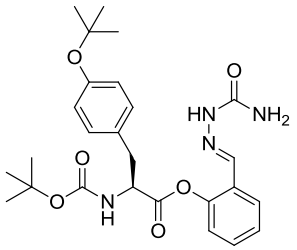


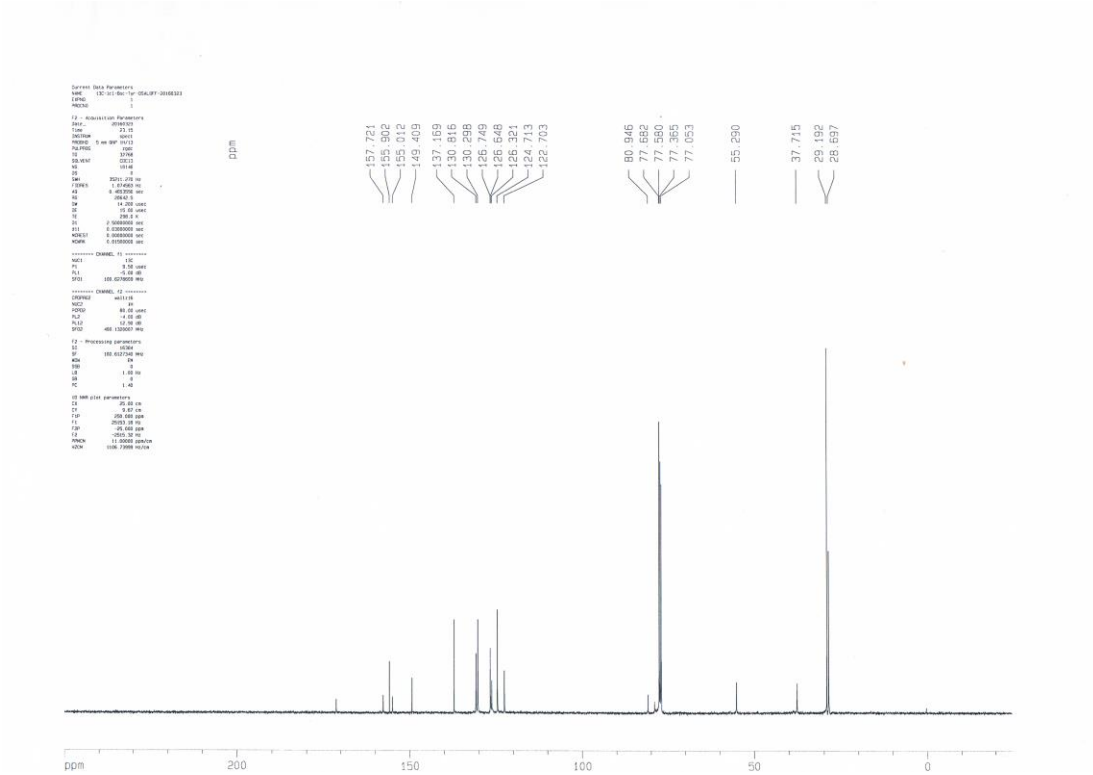
^{1}H spectrum of *BocHN*-Tyr(O^{t}Bu)-*CO*-SAL^{off}





^{13}C spectrum of *BocHN*-Tyr(O^{t}Bu)-*CO*-SAL^{off}





XII. References

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