Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Peptide self-cleavage at canaline residue: application to a solubilizing tag system for native chemical ligation

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Contents

1. General Information

a. Materials

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich Co. LLC. (St. Louis, MO). ***Notice**: During handling Can-peptides, fresh reagents and solvents should be used to avoid undesired oxime-based side product formation.

b. HPLC, MS and NMR

Preparative HPLC was carried out on a Shimadzu liquid chromatograph Model LC-8A (Kyoto, Japan) with a YMC-Pack ODS-A (30 x 250 mm) and the following solvent systems: 0.1% TFA in H_2O and 0.1% TFA in CH_3CN at a flow rate of 20 mL min^{-1} with detection at 220 nm. Analytical HPLC was performed on a Shimadzu liquid chromatograph Model LC-10A (Kyoto, Japan) with a DAISO-PAK SP-120-5-ODS-BIO (4.6 x 150 mm) or a YMC-Pack ODS-A (4.6 x 150 mm) and the following solvent systems: 0.1% TFA in H_2O and 0.1% TFA in CH_3CN at a flow rate of 1 mL min^{-1} (40 °C) with detection at 220 nm. Low resolution mass spectra (LRMS) were observed with an Agilent G1956B LC/MSD detector using an Agilent 1100 series HPLC system; observed masses (most abundant masses) were derived from the experimental m/z values for each protonation states of a target peptide. ^1H NMR spectrum was recorded on a JEOL-ECX400 spectrometer (Tokyo, Japan) in DMSO-d_6 with the solvent residual peak as an internal reference.

c. SPPS

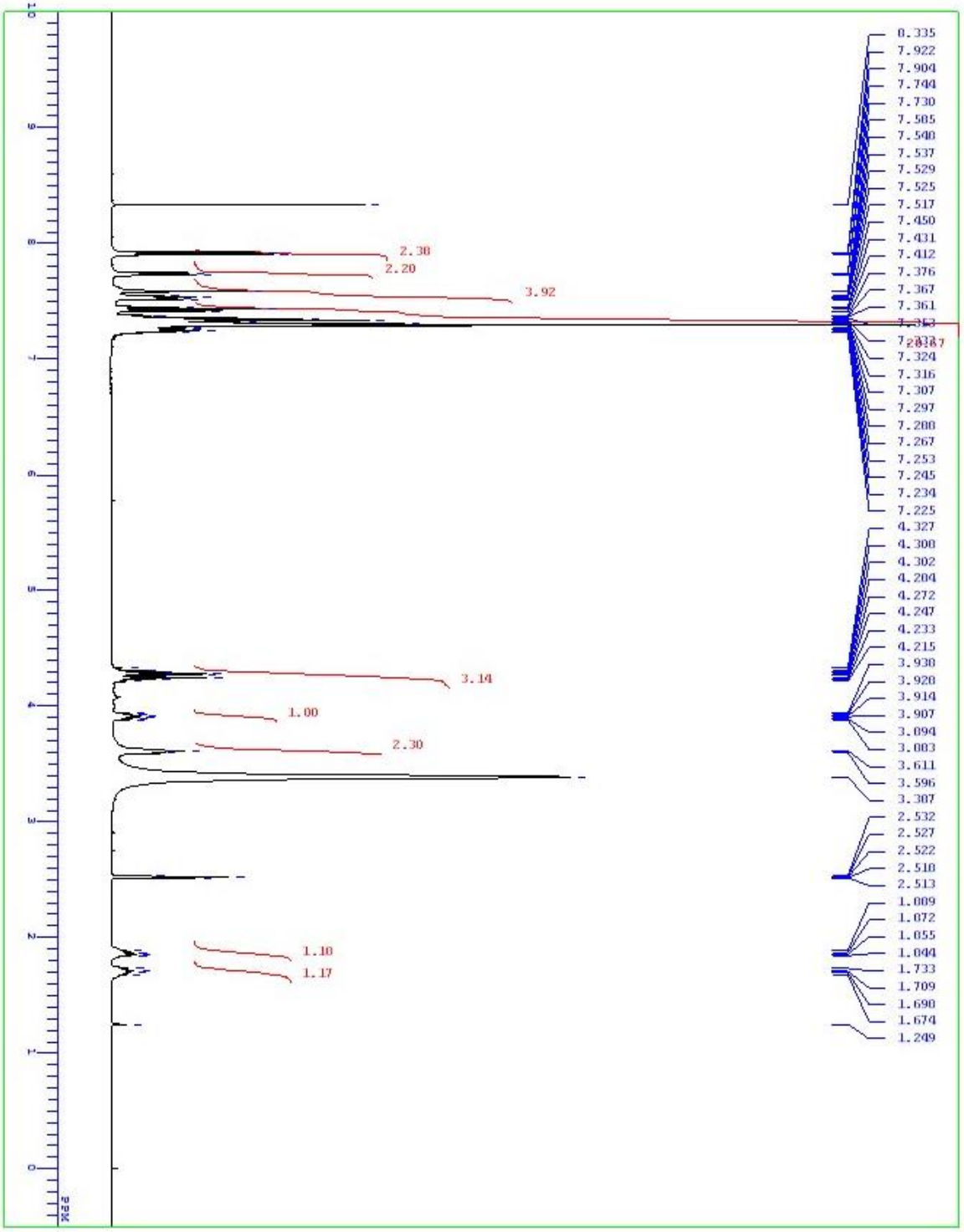
Automated peptide synthesis by Boc SPPS was performed on an ABI 433A (Applied Biosystems, USA) peptide synthesizer. The peptide chain was elongated using *in situ* neutralization protocols.[1] Automated peptide synthesis by Fmoc SPPS was performed on an ABI 433A peptide synthesizer. The peptide chain was elongated using the coupling protocol of Fmoc-amino acid/DIC/OxymaPure.[2] The acetyl capping was performed using acetic anhydride/NMP in the presence of DIEA after each coupling step. The following side-chain-protected amino acids were employed: Arg(Pbf), Asn(Trt), Asp(O*t*Bu), Can(2-Cl-Trt), Cys(Trt), Glu(O*t*Bu), Gln(Trt), His(Trt), Lys(Aloc), Lys(Boc), Orn(Boc), Ser(*t*Bu), Trp(Boc), Tyr(*t*Bu). Boc-amino acids were selected as N-terminal amino acids, when using MeNbz linker and/or Lys(Aloc). Fmoc-Can(2-Cl-Trt) (3 equiv) was condensed using DIC/OxymaPure (3/3 equiv) in NMP for 14-20 h without using the

automated peptide synthesizer.

2. Experimental Section

a. Synthesis of Fmoc-Can(2-Cl-Trt)-OH

Fmoc-Can(2-Cl-Trt)-OH was synthesized as previously reported.[3] The spectral data were in agreement with the reported values. ^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.90-7.23 (22H, m), 4.27 (3H, m), 3.91 (1H, m), 3.61 (2H, *t*, *J* = 6.4 Hz), 1.78 (2H, m).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1.690
1.674
1.249
2.38
2.20
3.92
3.14
1.00
2.30
1.10
1.17
ppm

b. Synthesis of peptide segments using SPPS

Arg-Leu-Tyr-Can-Ala-Tyr-Arg-Ala-Asn-NH_2 ***(1)***

The peptide was assembled on a Rink Amide resin (0.10 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv). The subsequent deprotection of the resin was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **1** (57 mg, 50%). Analytical HPLC: t_R = 10.8 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{50}H_{81}N_{18}O_{13}$ 1141.6, found 1141.5.

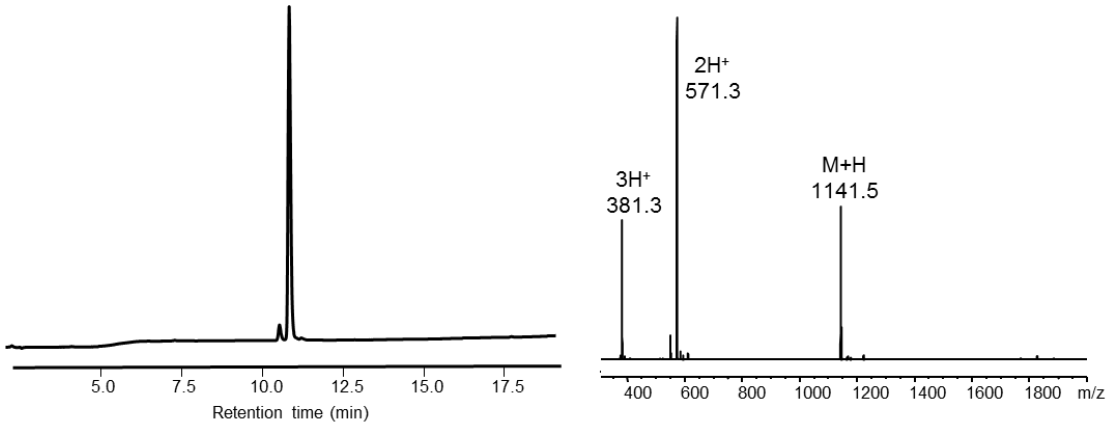


Figure S1. Analytical HPLC chromatogram and ESI-MS spectrum of compound **1**.

Arg-Leu-Tyr-Orn-Ala-Tyr-Arg-Ala-Asn-NH_2 ***(1')***

The peptide was assembled on a Rink Amide resin (0.25 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 4 equiv). The subsequent deprotection of the resin was carried out by TFA/TIS/H_2O (v/v, 95/2.5/2.5) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **1'** (121 mg, 42%). Analytical HPLC: t_R = 10.7 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{51}H_{83}N_{18}O_{12}$ 1139.6, found 1139.5.

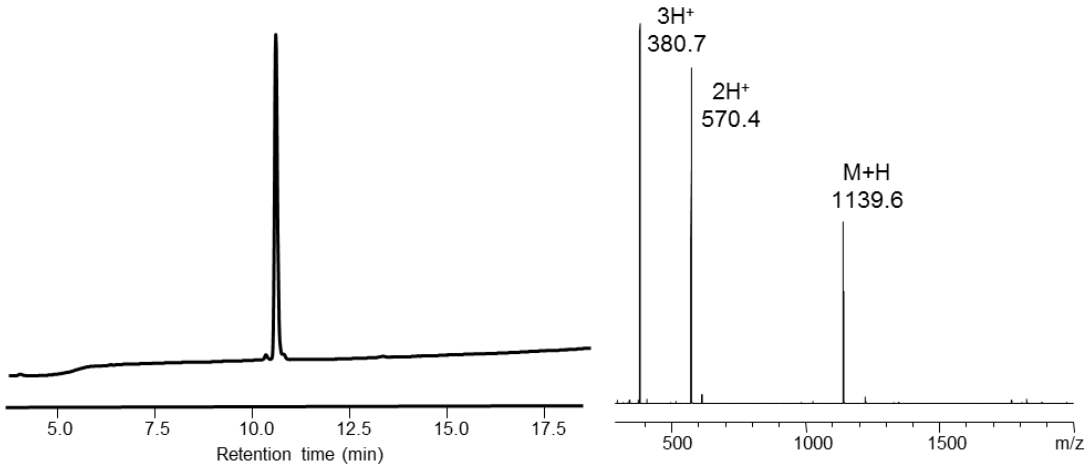


Figure S2. Analytical HPLC chromatogram and ESI-MS spectrum of compound **1'**.

*Cys-Ser-Pro-**Lys**(Lys-Lys-Lys-Can)-Gly-Tyr-Ser-NH_2* ***(4)***

The peptide was assembled on a Rink Amide resin (0.10 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Boc-Cys(Trt) as N-terminal Xaa). Fmoc-Lys(Aloc) was incorporated at an internal Lys shown in bold in the sequence above. Then, under Ar atmosphere, the Aloc-protected peptide resin was treated with $Pd(PPh_3)_4$ (23 mg, 0.020 mmol) and phenylsilane (0.49 mL, 4.0 mmol) in CH_2Cl_2 (20 mL) for 1.5 h. The subsequent peptide assembly on a side chain of the free Lys was conducted using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv). The final deprotection was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **4** (89 mg, 69%). Analytical HPLC: t_R = 8.7 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{53}H_{94}N_{17}O_{15}S_1$ 1240.7, found 1240.6.

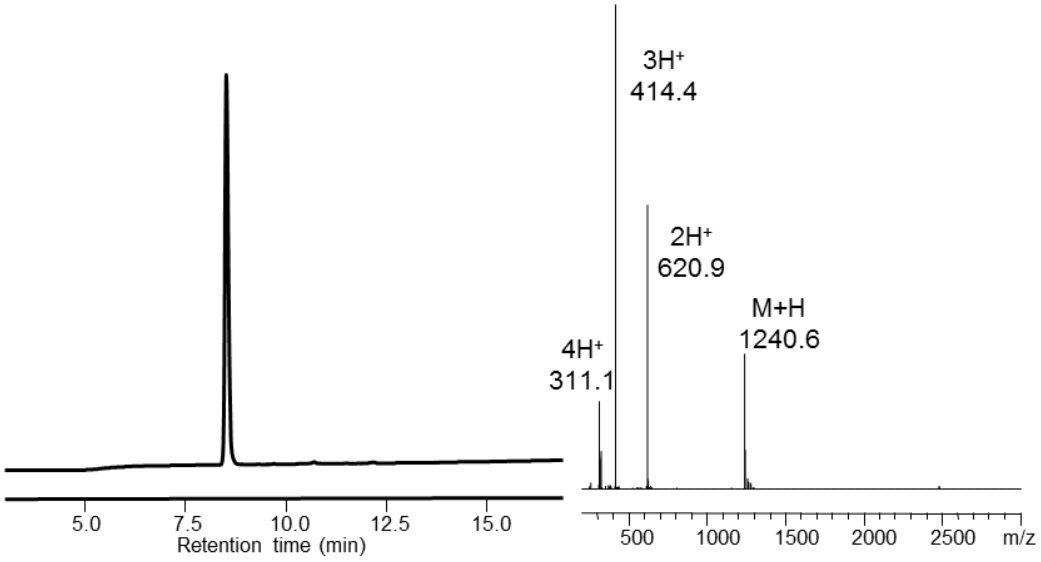


Figure S3. Analytical HPLC chromatogram and ESI-MS spectrum of compound **4**.

Thz-Tyr-Arg-Ala-Asn-Gly-MeNbz-Leu-NH$_2$ ***(5)***

The peptide was assembled on an Fmoc-Gly-MeDbz-Leu-Rink Amide resin (0.20 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Boc-Thz as N-terminal Xaa). The obtained peptide resin was activated by the method reported by Dawson *et al*.[4] The final deprotection was carried out by TFA/TIS/H_2O (v/v, 95/2.5/2.5) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **5** (71 mg, 36%). Analytical HPLC: t_R = 16.2 min (1–45% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{43}H_{61}N_{14}O_{11}S_1$ 981.4, found 981.4.

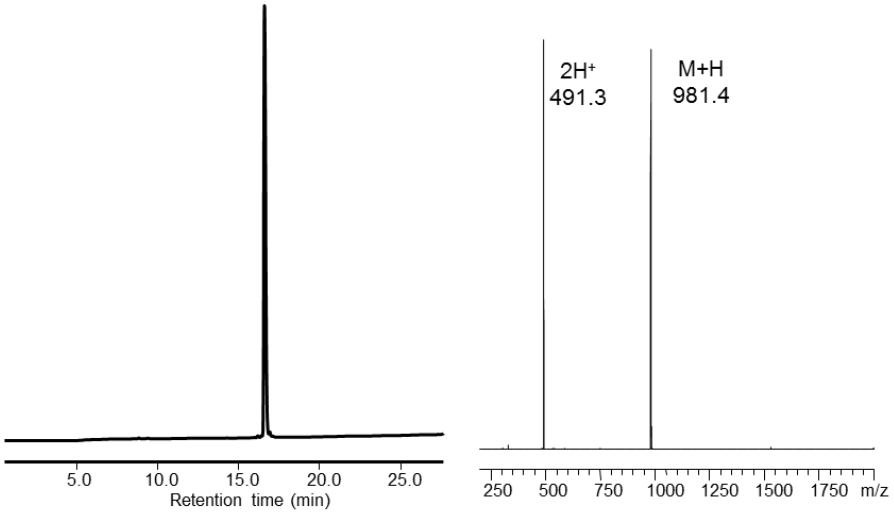


Figure S4. Analytical HPLC chromatogram and ESI-MS spectrum of compound **5**.

*Ac-Ala-Leu-Phe-SCH_2CH_2CO-Leu-NH_2 (**7**)*

The peptide was assembled on a Boc-Phe-SCH_2CH_2CO-Leu-MBHA resin (0.25 mmol) using automated Boc SPPS procedure as described in general information (Boc-Xaa: 4 equiv). The peptide resin was treated with HF/*p*-cresol (v/v, 90/10) at −2 °C to −5 ºC for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound **7** (31 mg, 21%). Analytical HPLC: t_R = 24.8 min (1–45% CH_3CN/0.1% TFA for 25 min); LRMS (ESI+) calcd for $C_{29}H_{46}N_5O_6S_1$ 592.3, found 592.3.

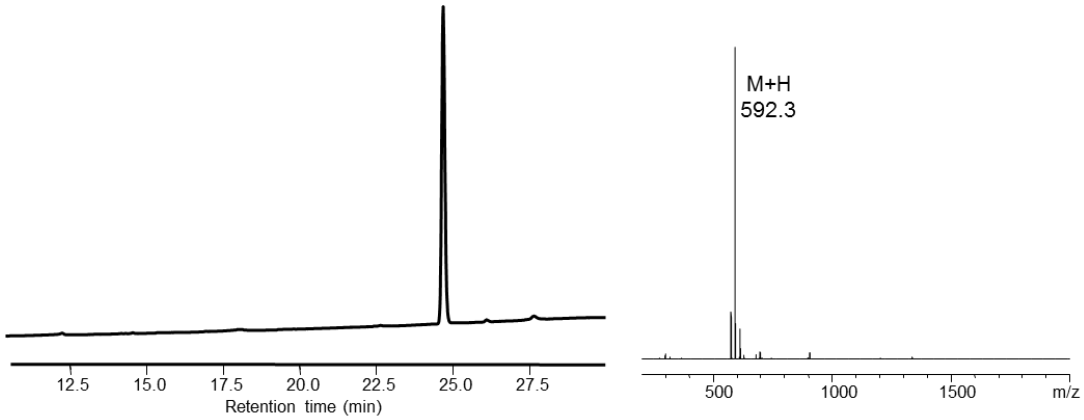


Figure S5. Analytical HPLC chromatogram and ESI-MS spectrum of compound **7**.

*Gly-Lys-Lys-Lys-Can-Cys-Leu-Tyr-Arg-Ala-Asn-Ala-N_2H_3 (**9**)*

The peptide was assembled on a hydrazine-Trt(2-Cl)-Resin (0.20 mmol) using automated Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv). The final deprotection was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **9** (59 mg, 43%). Analytical HPLC: t_R = 9.9 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{58}H_{105}N_{22}O_{15}S_1$ 1381.8, found 1381.7.

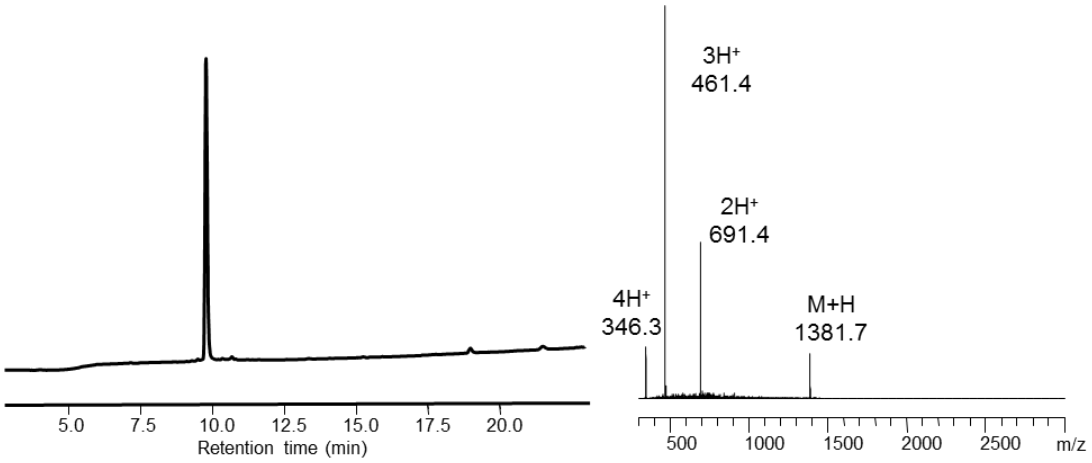


Figure S6. Analytical HPLC chromatogram and ESI-MS spectrum of compound **9**.

Gly-Arg-Arg-Arg-Can-Leu-Val-Tyr-Arg-Ala-Asn-Ala-MeNbz-Phe-NH_2 ***(10)***

The peptide was assembled on an Fmoc-Ala-MeDbz-Phe-Rink Amide resin (0.1 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv, Boc-Gly as N-terminal Xaa). The obtained peptide resin was activated by the method reported by Dawson *et al*..[4] The final deprotection was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **10** (86 mg, 49%). Analytical HPLC: t_R = 14.9 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{78}H_{122}N_{30}O_{18}$ 1767.0, found 1767.6.

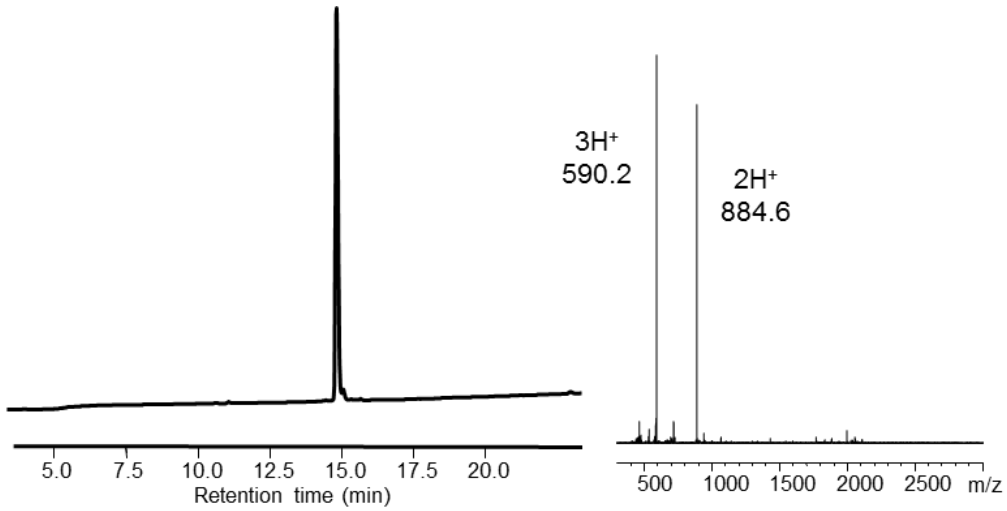


Figure S7. Analytical HPLC chromatogram and ESI-MS spectrum of compound **10**.

Cys-Leu-Tyr-Arg-Ala-Asn-Leu-p-F-PhCB-MeDbz-Arg-Arg-Arg-NH_2 ***(11)***

The peptide was assembled on an Fmoc-Leu-MeDbz-[Arg(Pbf)]$_3$-Rink Amide resin (0.05 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Boc-Cys(Trt) as N-terminal Xaa). The obtained peptide resin was treated by 4-fluorophenyl chloroformate (65 μL, 0.5 mmol) in CH_2Cl_2 (6 mL) for 1 h. The final deprotection was carried out by TFA/TIS/H_2O (v/v, 95/2.5/2.5) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **11** (30 mg, 37%). Analytical HPLC: t_R = 12.5 min (10-60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{70}H_{109}F_1N_{26}O_{15}S_1$ 1604.8, found 1605.4.

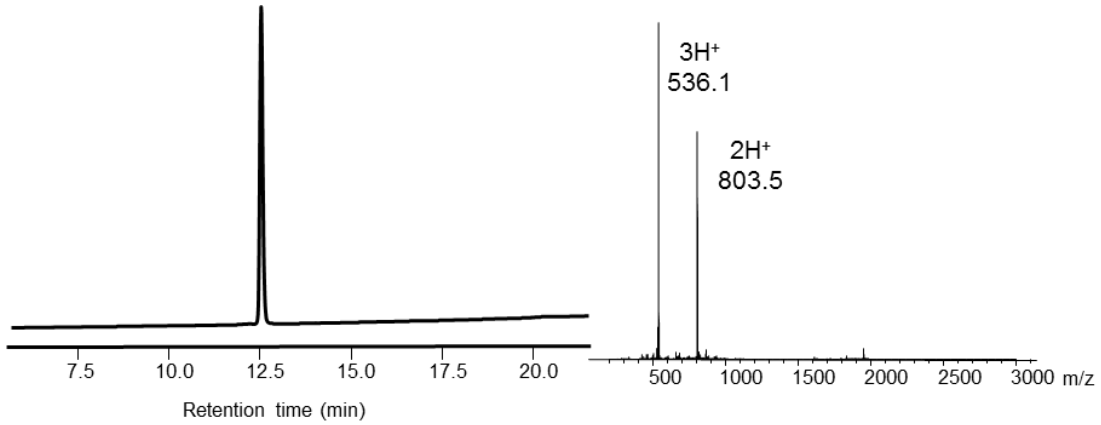


Figure S8. Analytical HPLC chromatogram and ESI-MS spectrum of compound **11**.

Cys-Ser-Pro-Gly-Tyr-Ser-NH_2 (***12***)

Synthesis of **12** was reported in ref. 3

M^1KKDIHPKYEEITASCSCGNVMKIRSTVGHDLNLDVCSL^{39}-MeNbz-RRR-NH_2 ***(14)***

The peptide was assembled on an Fmoc-Leu-MeDbz-[Arg(Pbf)$_3$]-TentaGel S RAM resin (0.10 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 10 equiv, Boc-Met as N-terminal Xaa). The obtained peptide resin was activated by the method reported by Dawson *et al.*[4] The final deprotection was carried out by TFA/TIS/H_2O/PhSH (v/v, 92.5/2.5/2.5/2.5) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **14** (8.4 mg, 1.7%). Analytical HPLC: t_R = 16.0 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{210}H_{347}N_{67}O_{63}S_5$ 4977.5, found 4977.9.

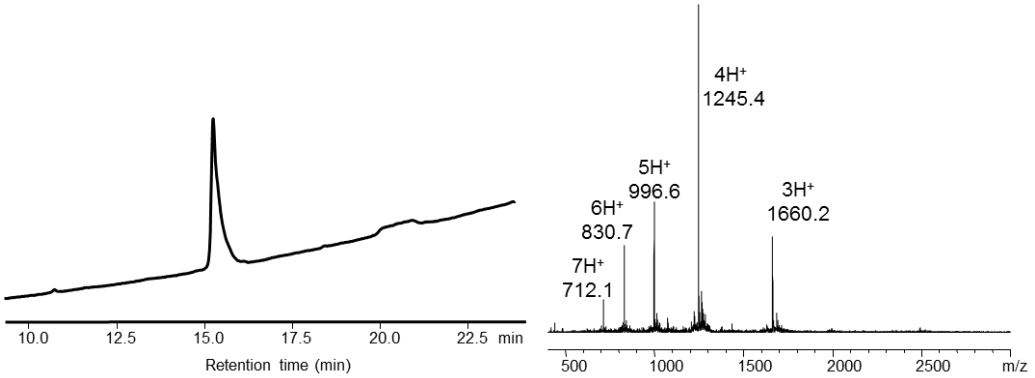


Figure S9. Analytical HPLC chromatogram and ESI-MS spectrum of compound **14**.

*C[40]HPFFTGKQRDVATGGRVDRFN**K[62]**RFNIPGSK[70]-NH$_2$* ***(15)***

The peptide was assembled on a TentaGel S RAM (0.20 mmol) using automated Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Boc-Cys(Trt) as N-teminal Xaa). Fmoc-Lys(Aloc) was incorporated at an internal Lys^{62} shown in bold in the sequence above. Then, under Ar atmosphere, the Aloc-protected peptide resin was treated with $Pd(PPh_3)_4$ (70 mg, 0.060 mmol) and phenylsilane (1.48 mL, 12 mmol) in CH_2Cl_2 (40 mL) for 1.5 h. The subsequent peptide assembly on a side chain of the free Lys was conducted using Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv). The final deprotection was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound **15** (121 mg, 15%). Analytical HPLC: t_R = 10.8 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{180}H_{291}N_{61}O_{47}S_1$ 4092.2, found 4093.2.

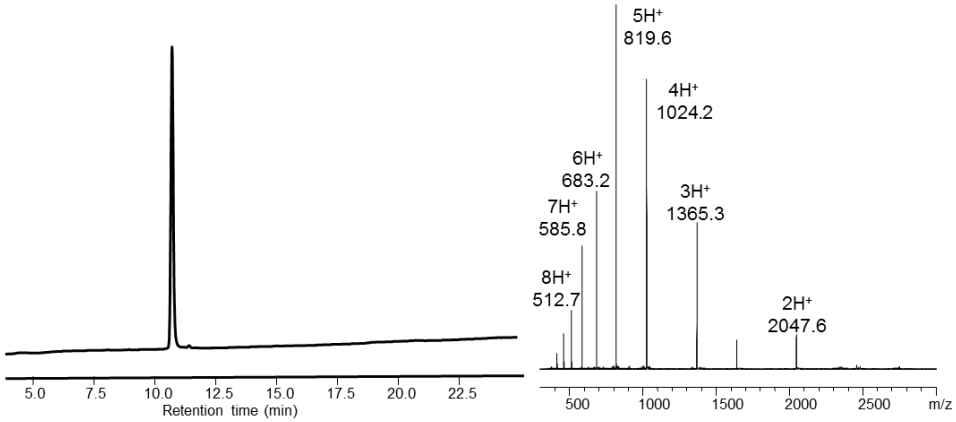


Figure S10. Analytical HPLC chromatogram and ESI-MS spectrum of compound **15**.

*Leu-Tyr-Arg-**Lys**(Gly-Lys-Lys-Lys-Can)-Ala-Asn-Phe-MeNbz-Leu-NH_2*

The peptide was assembled on an Fmoc-Phe-MeDbz-Leu-Rink Amide resin (0.20 mmol) using Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Boc-Leu as N-terminal Xaa). Fmoc-Lys(Aloc) was incorporated at an internal Lys shown in bold in the sequence above. Then, under Ar atmosphere, the Aloc-protected peptide resin was treated with $Pd(PPh_3)_4$ (47 mg, 0.040 mmol) and phenylsilane (0.49 mL, 4.0 mmol) in CH_2Cl_2 (30 mL) for 1.5 h. The subsequent peptide assembly on a side chain of the free Lys was conducted using Fmoc SPPS as described in general information (Fmoc-Xaa: 5 equiv, Fmoc-Can(2-Cl-Trt): 3 equiv). The obtained peptide resin (0.10 mmol) was activated by the method reported by Dawson *et al*.[4] The final deprotection was carried out by TFA/TIS/H_2O/EDT (v/v, 92.5/2.5/2.5/2.5) in the presence of $MeONH_2$·HCl (10 equiv) for 1.5 h to give a crude product, which was purified by preparative HPLC to yield the title compound (101 mg, 57%). Analytical HPLC: t_R = 15.6 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{82}H_{132}N_{25}O_{18}$ 1755.0, found 1754.9.

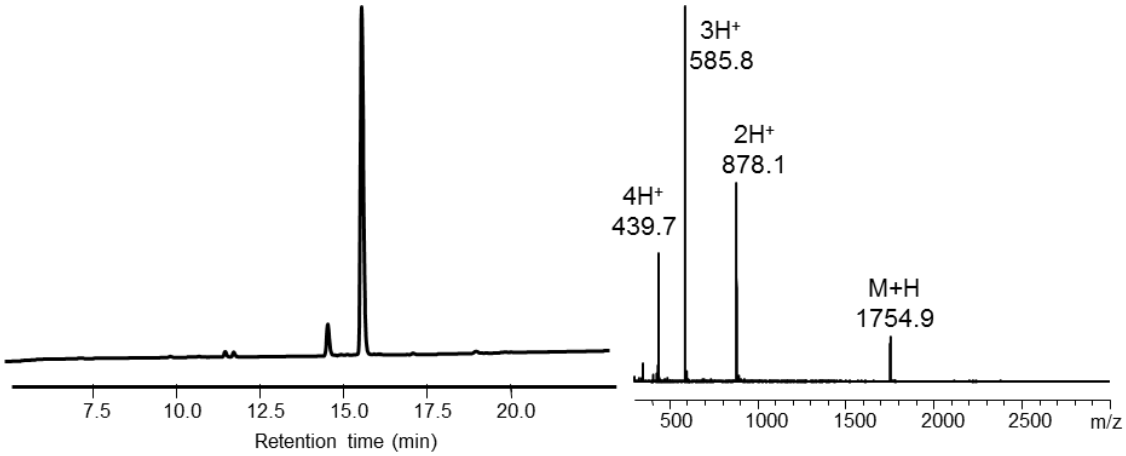


Figure S11. Analytical HPLC chromatogram and ESI-MS spectrum of the title compound.

c. Amide bond cleavage reaction using Can

Investigation of cleavage reaction of Orn-containing peptide (negative control)

RLY**<u>Orn</u>**AYRAN-NH_2 **1'** (0.8 mg) was dissolved in 0.2 M $AcONH_4$ buffers (0.30 mL) at 40 °C for 24 h. The reaction mixture was quenched by 3% TFA aq., and then the solutions were monitored by analytical HPLC. Analytical HPLC (1–60% CH_3CN/0.1% TFA for 25 min).

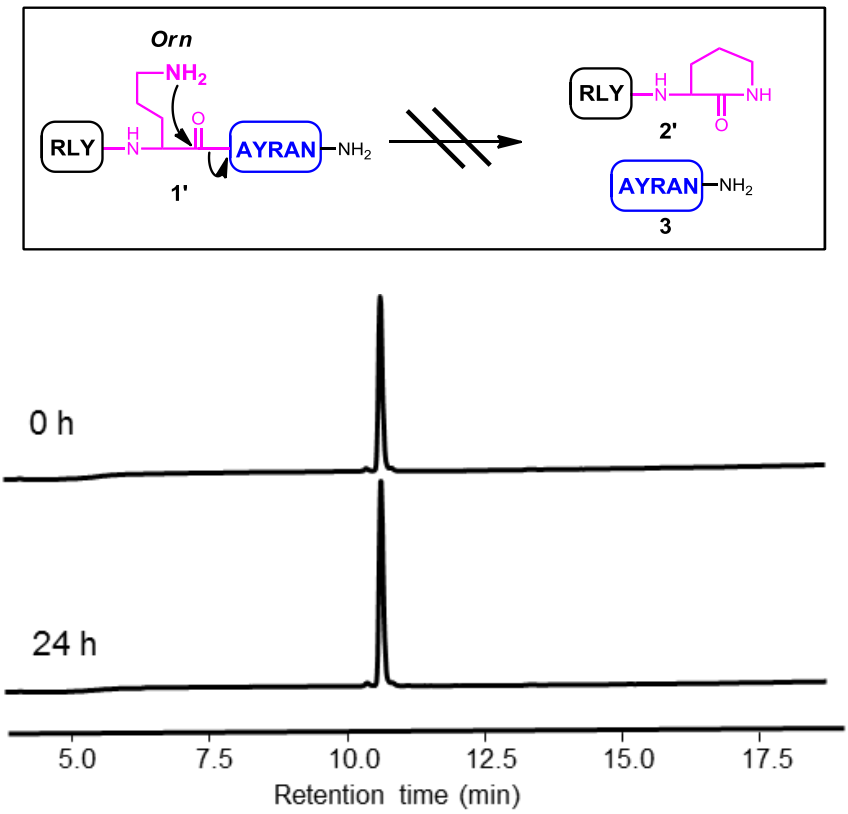


Figure S12. Analytical HPLC chromatograms of cleavage reaction of **1'**. Cleavage did not take place even after 24 h.

Investigation of cleavage reaction under other conditions.

RLYCanAYRAN-NH_2 **1** (0.8 mg) was dissolved in other solvents (0.30 mL). The reaction mixtures were quenched by 3% TFA aq., and then the solutions were monitored by analytical HPLC. Analytical HPLC (1–60% CH_3CN/0.1% TFA for 25 min).

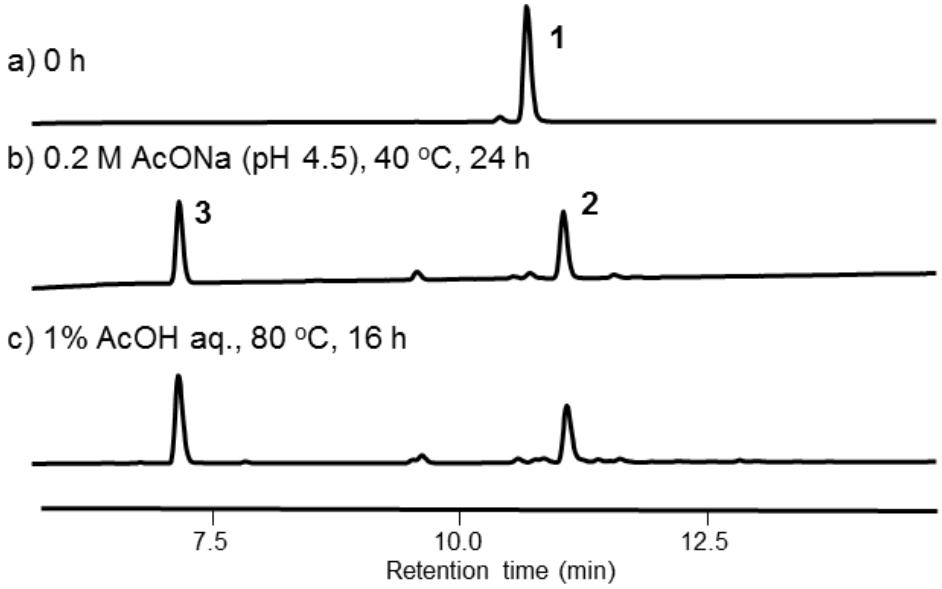


Figure S13. Analytical HPLC chromatograms of amide bond cleavage reaction: a) *t* = 0 h, b) 0.2 M AcONa (pH 4.5, 40 °C, *t* = 24 h), c) 1% AcOH aq. (80 °C, *t* = 16 h).

Investigation of cleavage reaction under various pH conditions.

RLYCanAYRAN-NH_2 **1** (0.8 mg) was dissolved in 0.2 M $AcONH_4$ buffers (0.30 mL) under various pH conditions (b-e) at 40 °C for 24 h. The reaction mixtures were quenched by 3% TFA aq., and then the solutions were monitored by analytical HPLC. Analytical HPLC (1–60% CH_3CN/0.1% TFA for 25 min).

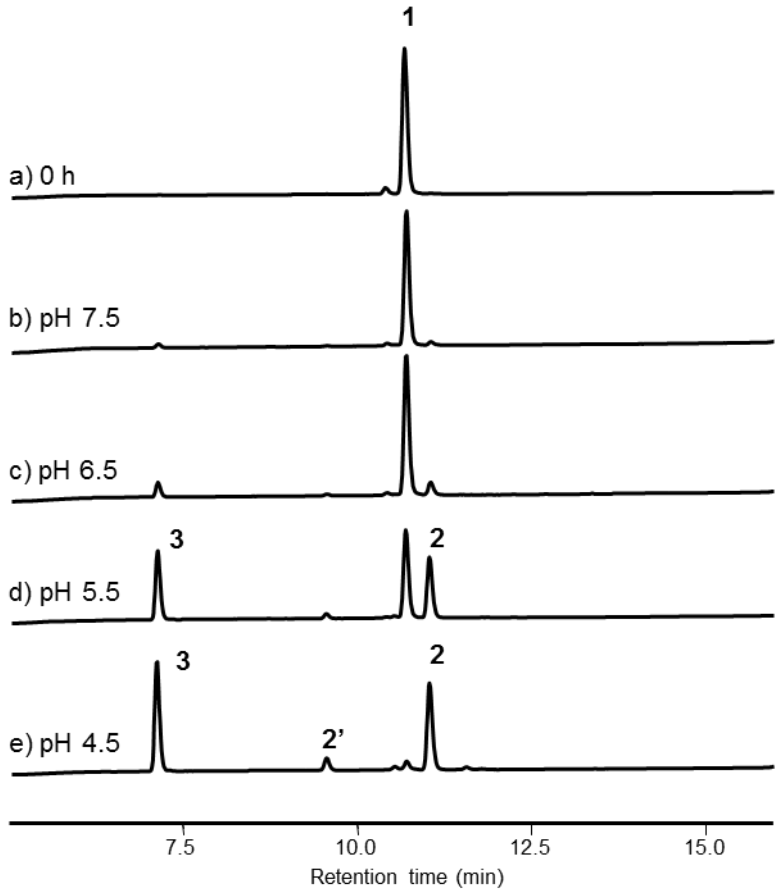
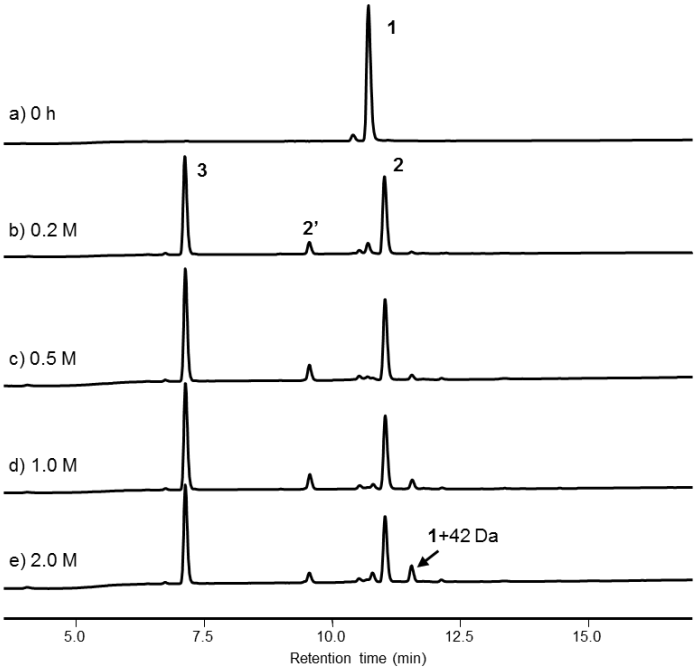


Figure S14. Analytical HPLC chromatograms of amide bond cleavage reaction under various pH conditions: a) 0 time, b) pH 7.5, c) pH 6.5, d) pH 5.5, e) pH 4.5.

Investigation of cleavage reaction at various concentration of $AcONH_4$.

RLYCanAYRAN-NH_2 **1** (0.8 mg) was dissolved in 0.2, 0.5, 1.0, and 2.0 M $AcONH_4$ buffers (0.30 mL) at 40 °C for 24 h. The reaction mixtures were quenched by 3% TFA aq., and then the solutions were monitored by analytical HPLC. Analytical HPLC (1–60% CH_3CN/0.1% TFA for 25 min).



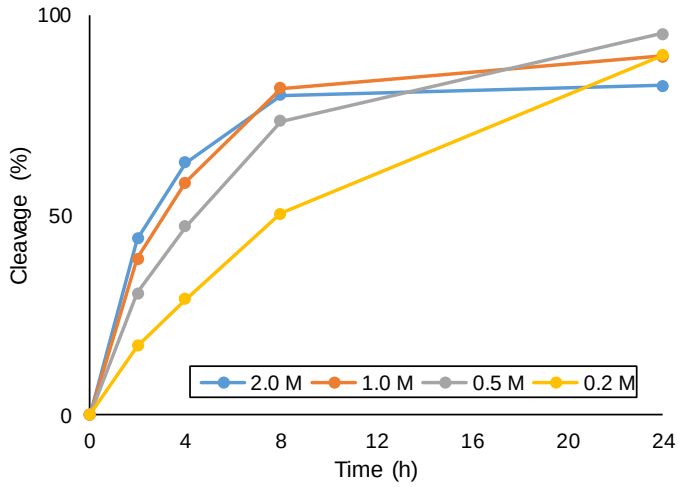


Figure S15. Analytical HPLC chromatograms of amide bond cleavage reaction at various concentrations of $AcONH_4$ (top): a) 0 time, b) 0.2 M, c) 0.5 M, d) 1.0 M, e) 2.0 M, and cleavage rates (bottom). Cleavage efficiency was determined by {**3**/[**3**+**1**+(**1**+42 Da)] ×100 (%), 220 nm}.

Cleavage reaction under the optimum condition.

Ala-Tyr-Arg-Ala-Asn-NH_2 ***(3)***

RLYCanAYRAN-NH_2 **1** (17 mg, 14.9 μmol) was dissolved in 0.51 mL of a buffer (0.5 M $AcONH_4$, pH 4.5) at 40 °C for 24 h. The reaction mixture was quenched by TFA, and directly subjected to preparative HPLC to yield the title compound **3** (5.4 mg, 61%). Analytical HPLC: t_R = 7.2 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{25}H_{41}N_{10}O_7$ 593.3, found 593.3.

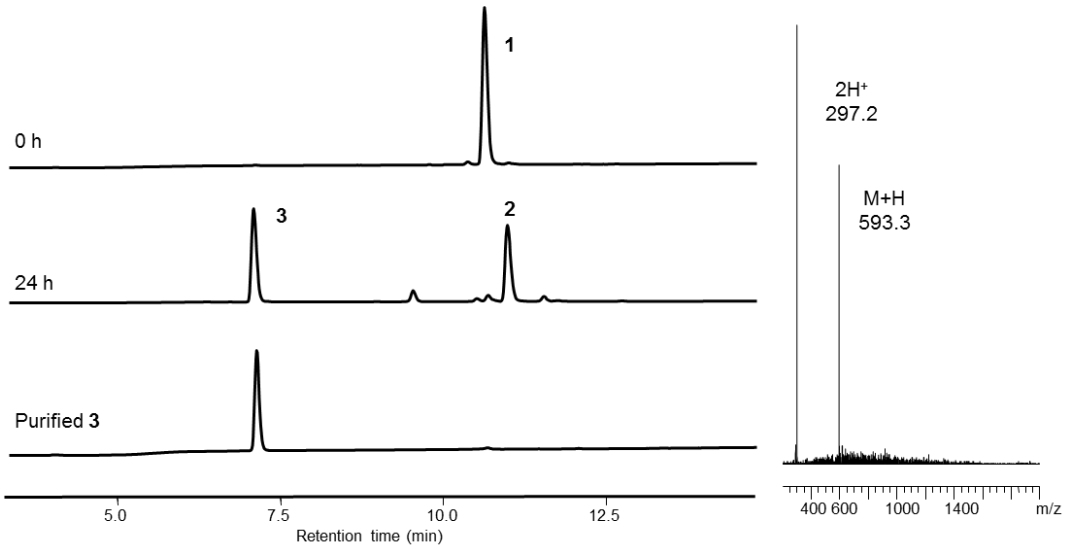


Figure S16. Analytical HPLC chromatograms of cleavage reaction and ESI-MS spectrum of Ala-Tyr-Arg-Ala-Asn-NH_2 (**3**).

d. Application of Can to a solubilizing tag

C-to-N NCL using Thz-mediated ligation.

*Thz-Tyr-Arg-Ala-Asn-Gly-Cys-Ser-Pro-**Lys**(Lys-Lys-Lys-Can)-Gly-Tyr-Ser-NH_2 (**6**)*

ThzYRANG-MeNbz-L-NH_2 **5** (26 mg, 26.9 μmol) was reacted with CSPK(K_3Can)GYS-NH_2 **4** (40 mg, 32.2 μmol) in 6.8 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM MPAA, 40 mM TCEP, pH 7.0) at 37 °C. After stirring for 2 h, the solution was quenched by 1 M HCl aq., and directly subjected to preparative HPLC to yield the title compound **6** (39 mg, 76%). Analytical HPLC: t_R = 11.7 min (1–45% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{81}H_{134}N_{27}O_{23}S_2$ 1917.0, found 1917.8.

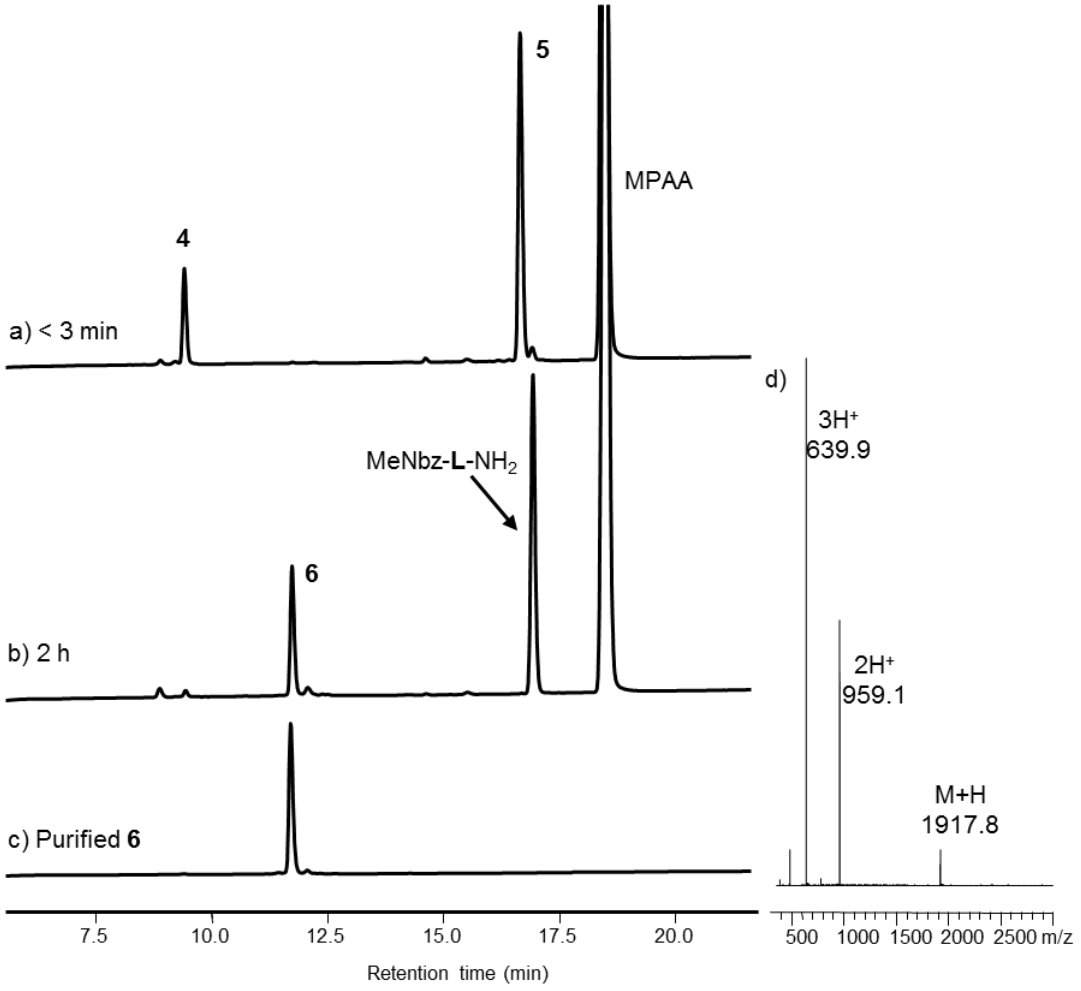


Figure S17. Analytical HPLC chromatograms and ESI-MS spectrum of **6**: a) NCL (t < 3 min), b) NCL (t = 2 h), c) purified product **6**, d) ESI-MS of **6**.

*Ac-Ala-Leu-Phe-Cys-Tyr-Arg-Ala-Asn-Gly-Cys-Ser-Pro-**Lys**(Lys-Lys-Lys-Can)-Gly-Tyr-Ser-NH$_2$ (**8**)*

ThzYRANGCSPK(K_3Can)GYS-NH_2 **6** (12 mg, 6.26 μmol) was dissolved in 0.8 mL of a buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 0.3 M $MeONH_2$·HCl, pH 4.0) at room temperature. After stirring for 4 h, to the reaction mixture were added Ac-ALF-MPA-L-NH_2 **7** (4.4 mg, 7.51 μmol) and a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM MPAA, 40 mM TCEP, pH 7.0, 0.8 mL). The solution (pH 7.0) adjusted by 1 N NaOH aq. was incubated at 37 °C. After stirring for 3 h, the solution was quenched by 1 M HCl aq., and directly subjected to preparative HPLC to yield the title compound **8** (6.2 mg, 43%). Analytical HPLC: t_R = 18.8 min (1–45% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{100}H_{161}N_{30}O_{27}S_2$ 2278.2, found 2278.3.

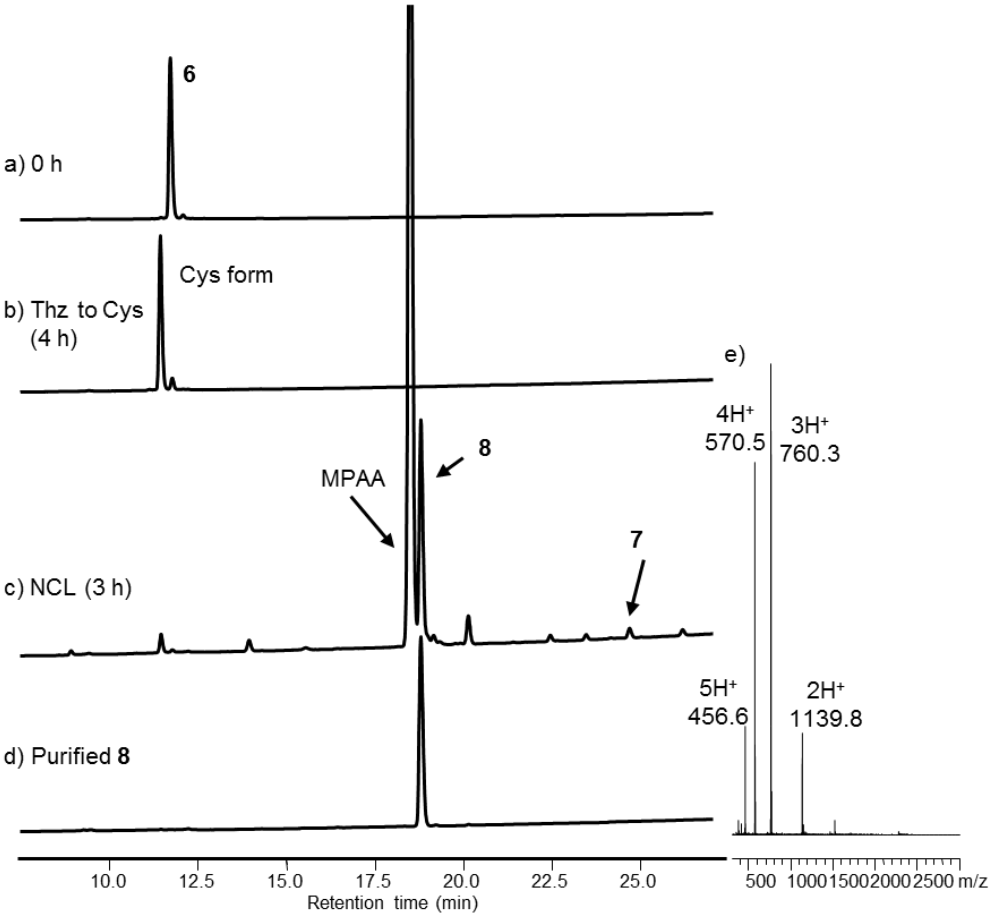


Figure S18. Analytical HPLC chromatograms and ESI-MS spectrum of **8**: a) Thz to Cys (t = 0 h), b) Thz to Cys (t = 4 h), c) NCL (t = 3 h), d) purified product **8**, e) ESI-MS of **8**.

*Ac-Ala-Leu-Phe-Cys-Tyr-Arg-Ala-Asn-Gly-Cys-Ser-Pro-**Lys**-Gly-Tyr-Ser-NH_2*

Ac-ALFLYRANGCSPK(K_3Can)GYS-NH_2 **8** (3.0 mg, 1.32 μmol) was dissolved in 300 μL of a buffer (0.5 M $AcONH_4$, pH 4.5) at 40 °C. After stirring for 18 h, the reaction mixture was quenched by TFA, and a tiny portion of TCEP·HCl was added to the solution. Then, the reaction mixture was directly subjected to preparative HPLC to yield the title compound (1.2 mg, 52%). Analytical HPLC: t_R = 20.1 min (1–45% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{78}H_{117}N_{22}O_{22}S_2$ 1777.8, found 1778.7.

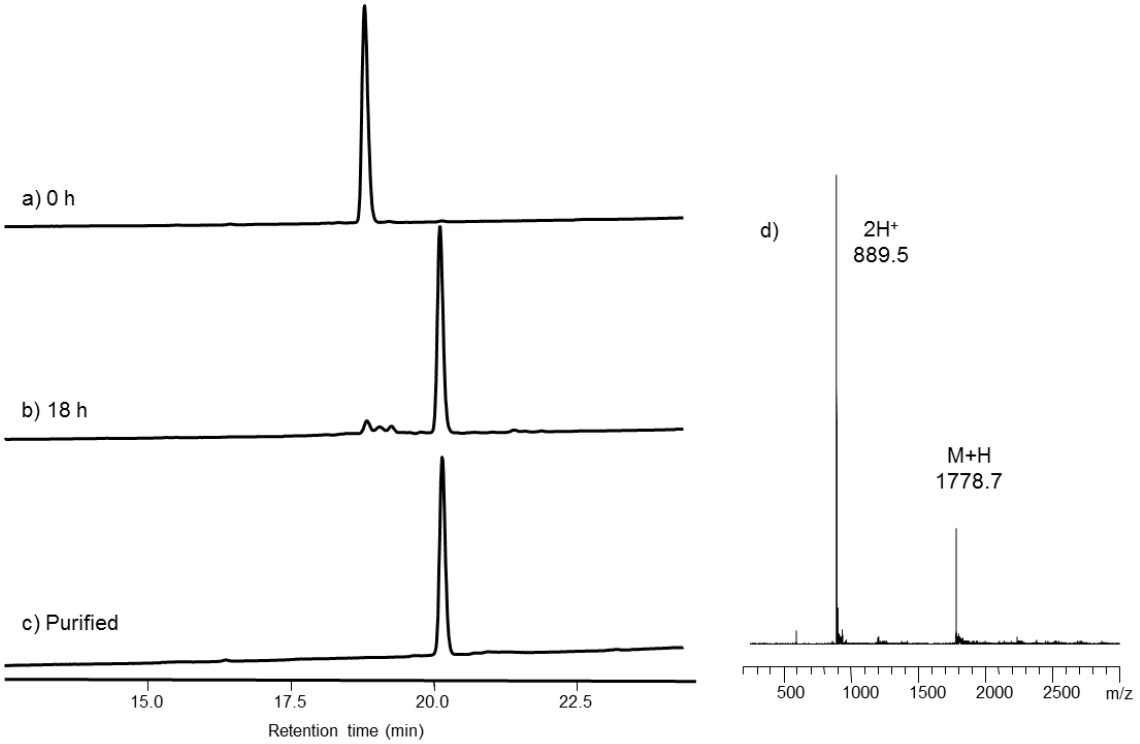


Figure S19. Analytical HPLC chromatograms and ESI-MS spectrum of the title compound: a) cleavage reaction (t = 0 h), b) cleavage reaction (t = 18 h), c) purified product, d) ESI-MS of the title compound.

Stability of a Can-containing peptide (6) under a $MeONH_4$-mediated condition. ThzYRANGCSPK(K_3Can)GYS-NH_2 **6** (1.2 mg) was dissolved in 80 μL of a buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 0.3 M $MeONH_2$·HCl, pH 4.0) at room temperature. Then, the solution was monitored by analytical HPLC (5–30% CH_3CN/0.1% TFA for 25 min).

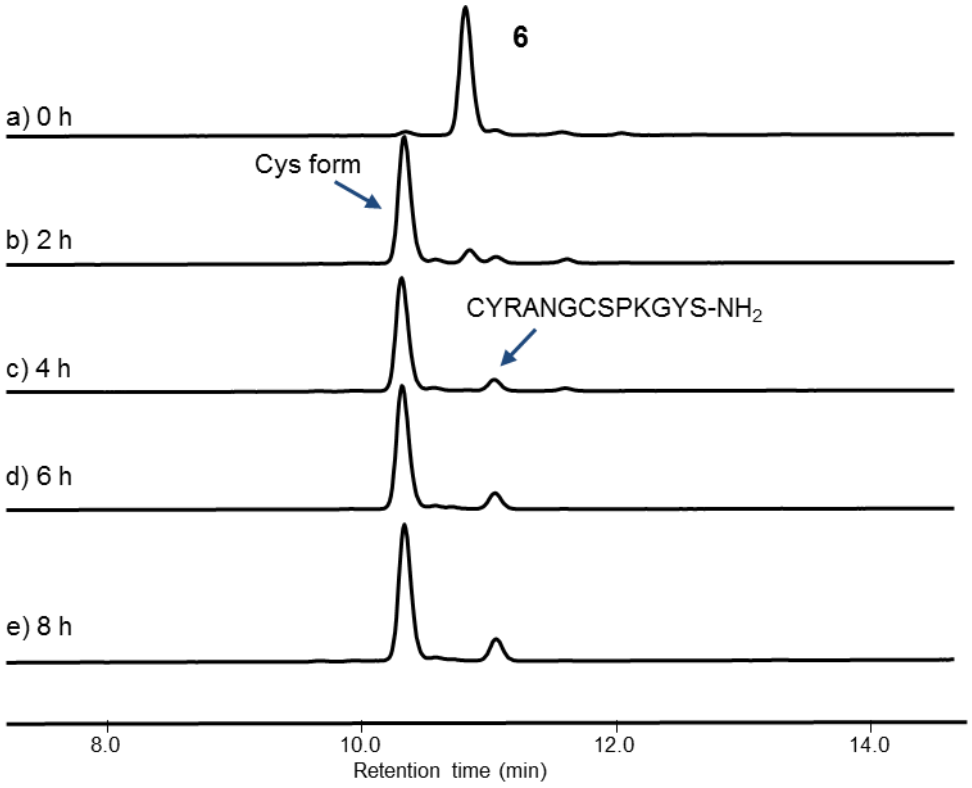


Figure S20. Analytical HPLC chromatograms of conversion of Thz form **6** to Cys form using a $MeONH_2$·HCl-containing buffer. a) t = 0 h, b) t = 2 h, c) t = 4 h, d) t = 6 h, e) t = 8 h.

Pd-mediated deprotection of Thz group of a Can-containing peptide (6).
ThzYRANGCSPK(K_3Can)GYS-NH_2 **6** (0.5 mg) was dissolved in 100 μL of a buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, pH 7.0), and allylpalladium(II) chloride dimer (1.4 mg) and $MgCl_2 \cdot H_2O$ (20 mg) were added. After stirring for 1 h at 40 °C, a tiny portion of DTT was added to the solution. The obtained suspension was diluted by 0.1%TFA and filtered. A tiny of $MeONH_2$·HCl was added to the acidified filtrate. Then the solution was immediately monitored by analytical HPLC (1–45% CH_3CN/0.1% TFA for 25 min).

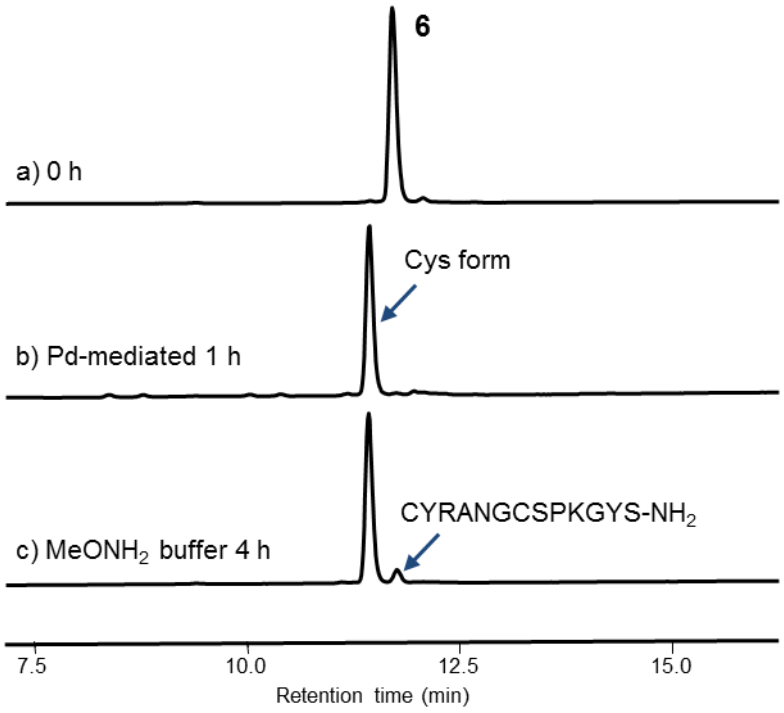


Figure S21. Analytical HPLC chromatograms of conversion of Thz form **6** to Cys form using a Pd-mediated method: a) *t* = 0 h, b) Pd-mediated method (*t* = 1 h), c) $MeONH_2$-mediated method (*t* = 4 h).

Stability of a Can-containing peptide (9) by $NaNO_2$-mediated activation.

Gly-Lys-Lys-Lys-Can-Cys-Leu-Tyr-Arg-Ala-Asn-Ala-MESNa

GKKKCanCLYRANA-N_2H_3 **9** (1.4 mg) was dissolved in 90 μL of a buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, pH 3.0) at -12 °C, and was activated using 1 M $NaNO_2$ in 10 μL of a buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, pH 3.0). After 15 min, to the reaction mixture was added a buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, pH 7.0, 100 μL) containing sodium 2-sulfanylethanesulfonate (10 mg) for 1 h. Then, the solution was monitored by analytical HPLC (5–45% CH_3CN/0.1% TFA for 25 min). However, MS value of the main peak (M+H: 1476.5) was not that of a desired product (M+H: 1431.7).

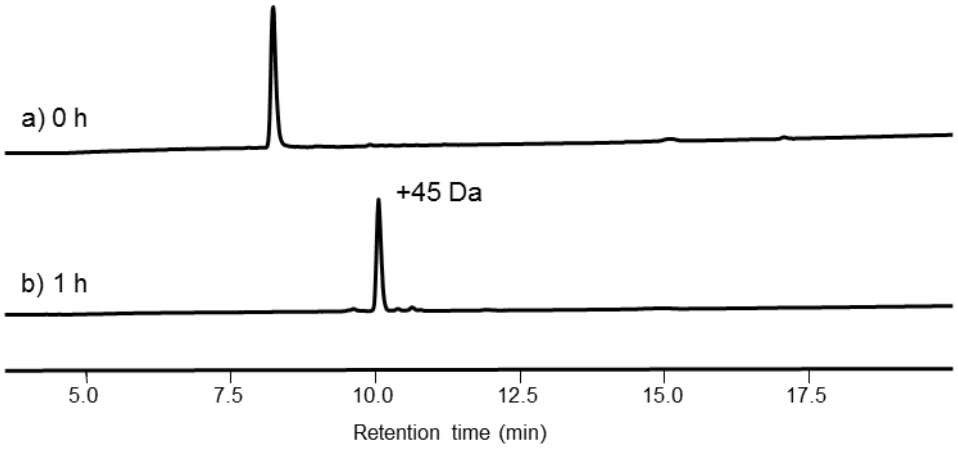


Figure S22. Analytical HPLC chromatograms: a) *t* = 0 h, b) conversion of GKKKCanCLYRANA-N_2H_3 (**9**) to MESNa thioester (*t* = 1 h).

N-to-C NCL using *p*-F-PhCB-MeDbz linker.

Gly-Arg-Arg-Arg-Can-Leu-Val-Tyr-Arg-Ala-Asn-Ala-Cys-Leu-Tyr-Arg-Ala-Asn-Leu-Cys-Ser-Pro-Gly-Tyr-Ser-NH_2 ***(13)***

GRRRCanLVYRANA-MeNbz-F-NH_2 **10** (6.4 mg, 3.6 μmol) was reacted with CLYRANL-*p*-F-PhCB-MeDbz-RRR-NH_2 **11** (4.8 mg, 3.0 μmol) in 0.6 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM MPAA, 30 mM TCEP, pH 6.8) at room temperature. After stirring for 3 h, to the reaction mixture was added CSPGYS-NH_2 **12** (2.7 mg, 4.5μmol), and pH was adjusted to 7.7 by 4 N NaOH aq.. The solution was incubated at 40 °C. After 6 h, the reaction mixture was treated with DTT for 15 min. Then the solution was quenched by 1 M HCl aq., and directly subjected to preparative HPLC to yield the title compound **13** (4.3 mg, 50%). Analytical HPLC: t_R = 18.6 min (7–35% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{122}H_{200}N_{44}O_{33}S_2$ 2874.5, found 2874.8.

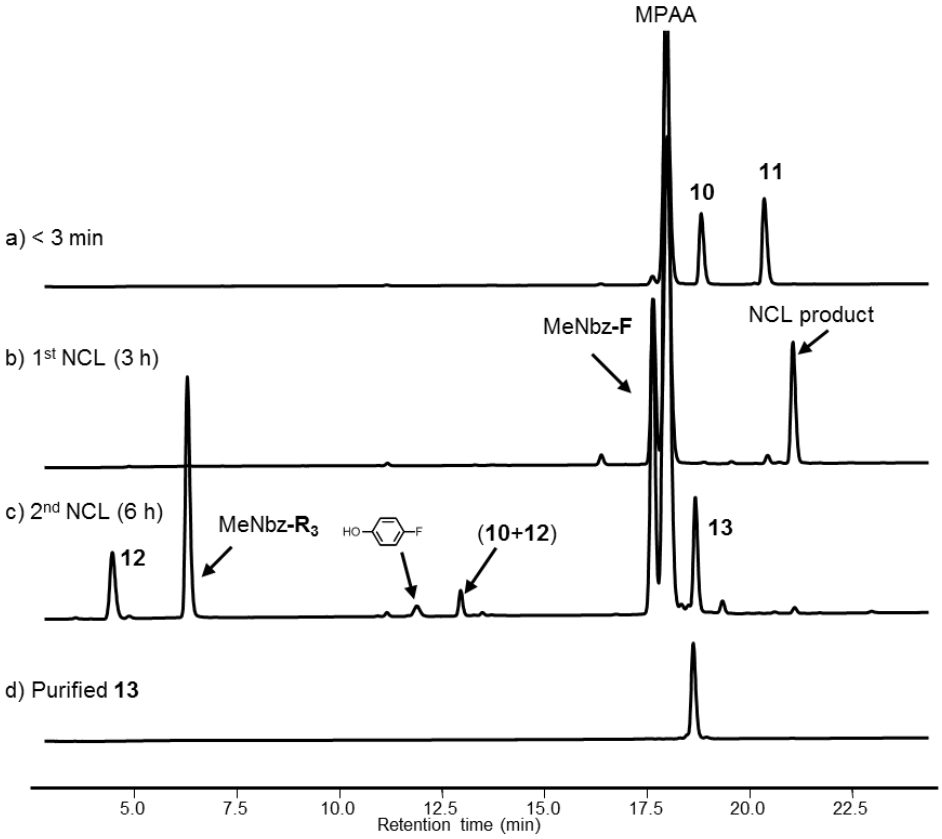


Figure S23. Analytical HPLC chromatograms and ESI-MS spectrum of **13**: a) 1st NCL (*t* < 3 min), b) 1st NCL (*t* = 3 h), c) 2nd NCL (*t* = 6 h), d) purified product **13**, e) ESI-MS of **13**.

Leu-Val-Tyr-Arg-Ala-Asn-Ala-Cys-Leu-Tyr-Arg-Ala-Asn-Leu-Cys-Ser-Pro-Gly-Tyr-Ser-NH_2

GRRRCanLVYRANACLYRANLCSPGYS-NH_2 **13** (3.0 mg, 1.04 μmol) was dissolved in 500 μL of a buffer (0.5 M $AcONH_4$, pH 4.5) at 40 °C. After stirring for 48 h, the reaction mixture was quenched by TFA, and a tiny portion of TCEP·HCl was added to the solution. Then, the reaction mixture was directly subjected to preparative HPLC to yield the title compound (1.1 mg, 45%). Analytical HPLC: t_R = 19.9 min (7–35% CH_3CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{98}H_{153}N_{29}O_{28}S_2$ 2233.1, found 2232.7.

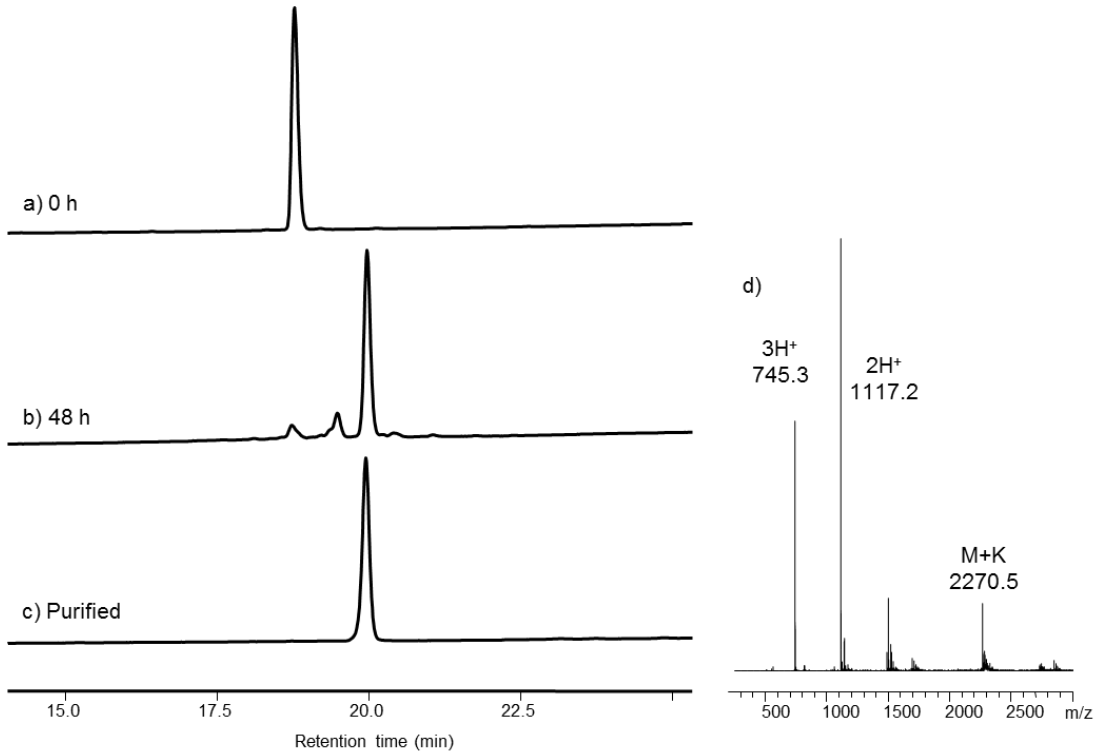


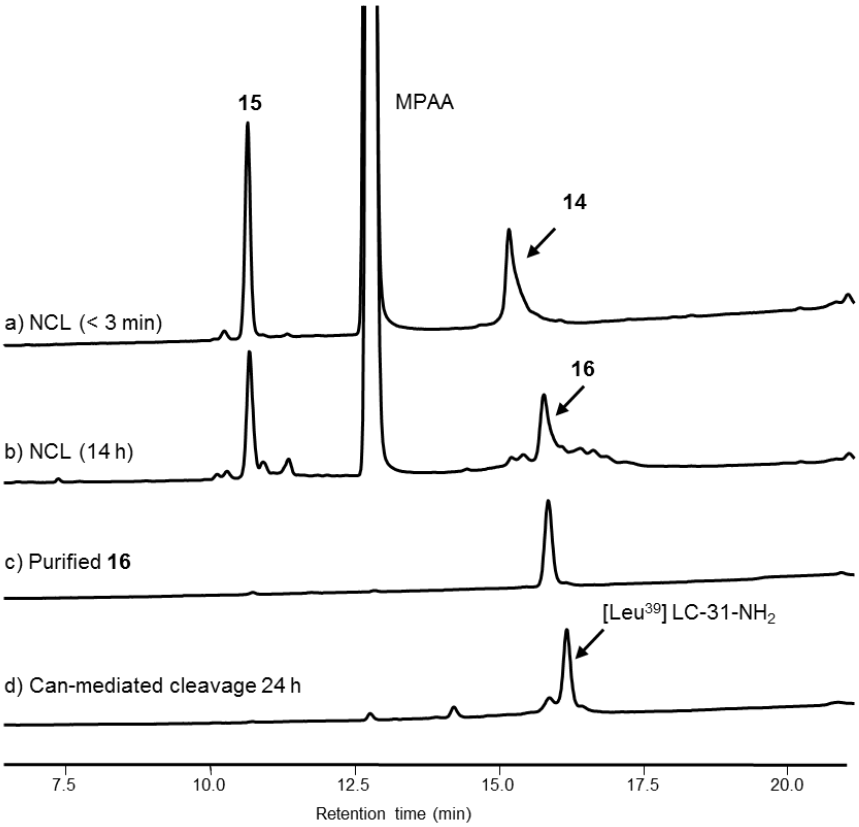
Figure S24. Analytical HPLC chromatograms and ESI-MS spectrum of a title compound: a) cleavage reaction (t = 0 h), b) cleavage reaction (t = 48 h), c) purified product, d) ESI-MS of a title compound.

Synthesis of a long peptide (70 AA) using Can-mediated amide bond cleavage.

[Leu^{39}]LC-31-NH$_2$

[Leu^{39}](1-39)-MeNbz-RRR-NH$_2$ (**14**) (3.0 mg, 0.60 μmol) was reacted with [Lys^{62}(GKKKCan)](40-70)-NH$_2$ (**15**) (3.9 mg, 0.96 μmol) in 0.3 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM MPAA, 40 mM TCEP, pH 7.2) at 37 °C. After stirring for 14 h, the reaction mixture was treated with DTT for 15 min, and directly subjected to preparative HPLC to yield [Leu^{39}, Lys^{62}(GKKKCan)]LC-31-NH$_2$ (**16**) (1.5 mg, 29%). Analytical HPLC: t_R = 16.0 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{363}H_{593}N_{113}O_{105}S_6$ 8410.3, found 8411.6.

Peptide **16** (1.0 mg, 0.12 μmol) was dissolved in 300 μL of a buffer (1.0 M $AcONH_4$, 0.2 M $MeONH_2$·HCl, pH 4.2) at 40 °C. After stirring for 24 h, the reaction mixture was quenched by TFA, and a tiny portion of TCEP·HCl was added to the solution. Then, the reaction mixture was directly subjected to preparative HPLC to yield the title compound (0.45 mg, 48%). Analytical HPLC: t_R = 16.3 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{339}H_{545}N_{104}O_{99}S_6$ 7851.9, found 7853.9.



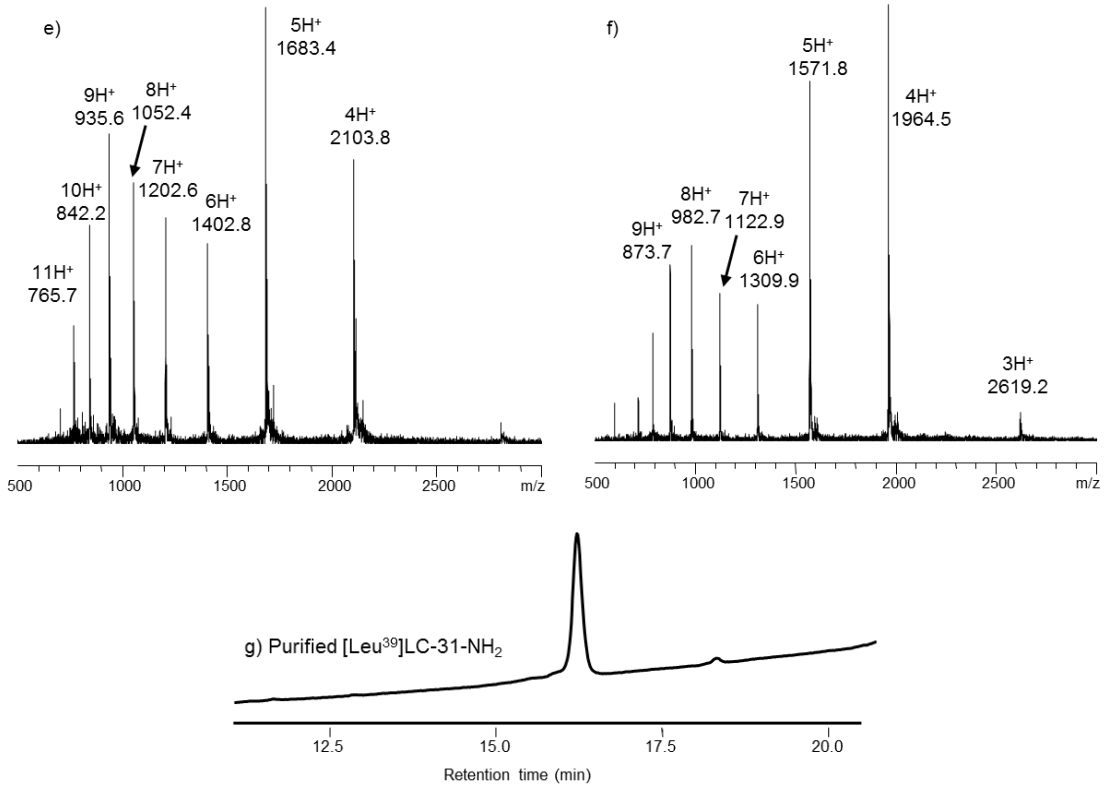


Figure S25. Analytical HPLC chromatograms and ESI-MS spectrum of [Leu^{39}]LC-31-NH_2 : a) NCL (t < 3 min), b) NCL (t = 14 h), c) purified product **16**, d) cleavage reaction (t = 24 h), e) ESI-MS of **16**, f) ESI-MS of [Leu^{39}]LC-31-NH_2, g) purified [Leu^{39}]LC-31-NH_2.

NCL between LYRK(GK$_3$Can)ANF-MeNbz-L-NH$_2$ and CSPGYS-NH$_2$ (12).

*Leu-Tyr-Arg-**Lys**(Gly-Lys-Lys-Lys-Can)-Ala-Asn-Phe-Cys-Ser-Pro-Gly-Tyr-Ser-NH$_2$* ***(17)***

LYRK(GK$_3$Can)ANF-MeNbz-L-NH$_2$ (40 mg, 22.8 μmol) was reacted with CSPGYS-NH$_2$ **12** (15 mg, 25.1 μmol) in 7.6 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na$_2$HPO$_4$, 100 mM MPAA, 50 mM TCEP, pH 7.0) at room temperature. After stirring for 3 h, the reaction mixture was directly subjected to preparative HPLC to yield the title compound **17** (19 mg, 40%). Analytical HPLC: t_R = 11.9 min (5–45% CH$_3$CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{92}H_{149}N_{28}O_{29}S_1$ 2062.1, found 2062.9.

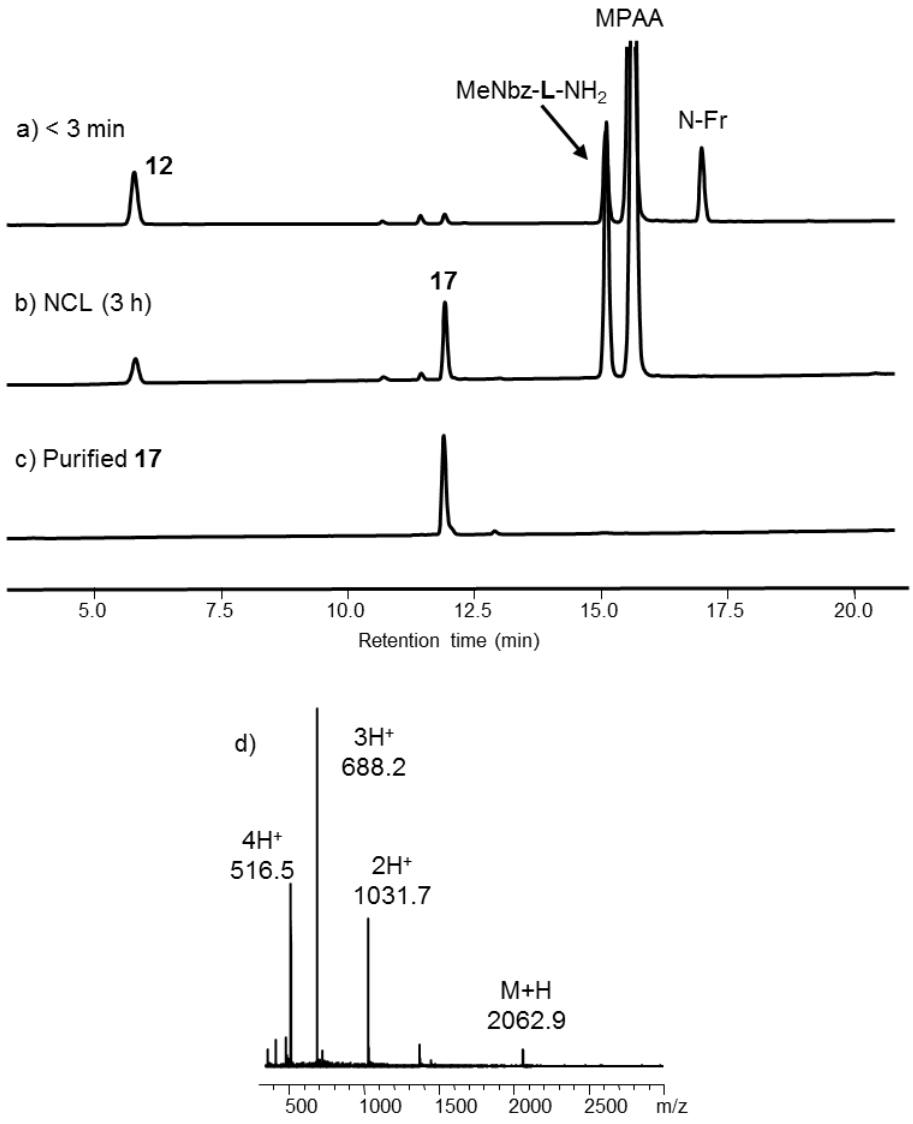


Figure S26. Analytical HPLC chromatograms and ESI-MS spectrum of **17**: a) NCL (t < 3 min), b) NCL (t = 3 h), c) purified product **17**, d) ESI-MS of **17**.

Amide bond cleavage reaction of LYRK(GK$_3$Can)ANFCSPGYS-NH$_2$ (17).

*Leu-Tyr-Arg-**Lys**-Ala-Asn-Phe-Cys-Ser-Pro-Gly-Tyr-Ser-NH$_2$*

LYRK(GK$_3$Can)ANFCSPGYS-NH$_2$ **17** (12 mg, 5.82 μmol) was dissolved in 0.6 mL of a buffer (0.5 M AcONH$_4$, pH 4.5) at 40 °C. After stirring for 20 h, the reaction mixture was quenched by TFA, and a tiny portion of TCEP·HCl was added to the solution. Then, the reaction mixture was directly subjected to preparative HPLC to yield the title compound (6.0 mg, 69%). Analytical HPLC: t_R = 13.0 min (5–45% CH$_3$CN/0.1% TFA for 25 min); LRMS (M+H) calcd for $C_{68}H_{102}N_{19}O_{18}S_1$ 1504.7, found 1504.6.

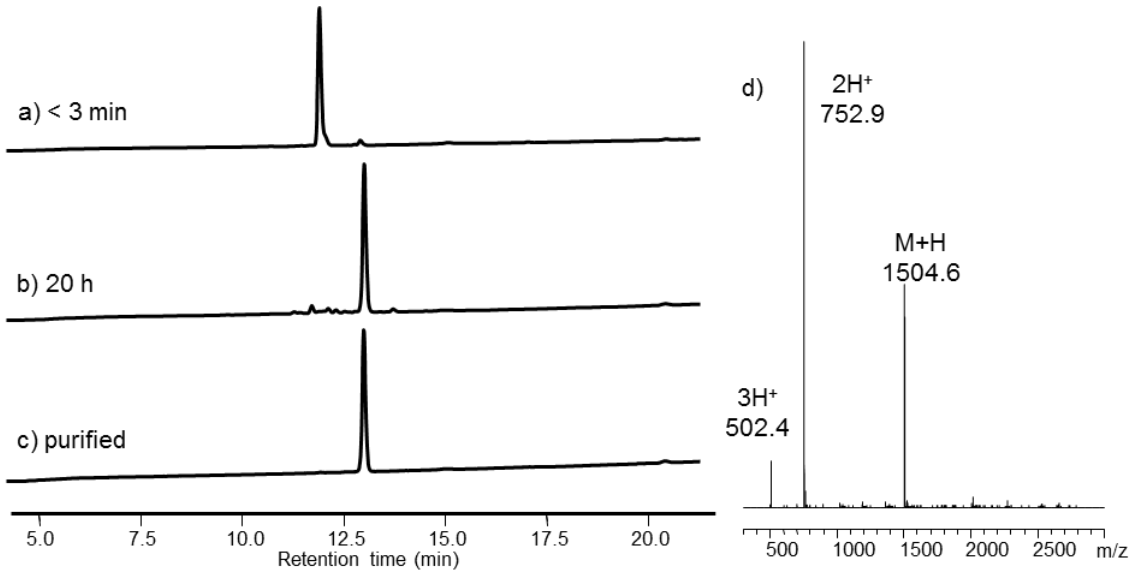


Figure S27. Analytical HPLC chromatograms and ESI-MS spectrum of the title compound: a) cleavage reaction (t < 3 min), b) cleavage reaction (t = 24 h), c) purified product, d) ESI-MS of the title compound.

Desulfurization of a Can-containing peptide (17).

LYRK(GK_3Can)ANFCSPGYS-NH_2 **17** (1.0 mg) was treated by conventional TCEP-VA-044-mediated method[5] and TCEP-$NaBH_4$-mediated method[6], respectively. Then, the solutions were monitored by analytical HPLC (5–45% CH_3CN/0.1% TFA for 25 min). However, majority of MS values of the observed peaks were not that of a desired product.

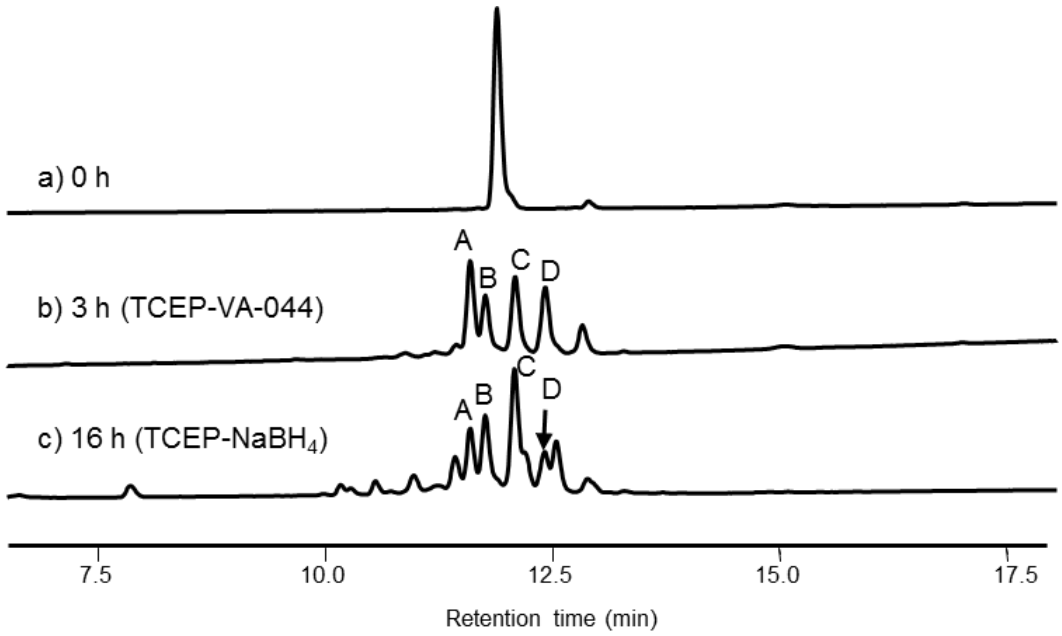


Figure S28. Analytical HPLC chromatograms of desulfurization: a) *t* = 0 h, b) TCEP-VA-044-mediated method (*t* = 3 h), c) TCEP-$NaBH_4$-mediated method (*t* = 16 h). Both desulfurization reactions did not proceed because of many side reactions. Observed MS value: A) M+H = 2015 (-15 Da: cleavage of N-O bond), B) 2248 (+218 Da), C) 2100 (+70 Da), D) 2056 (+26 Da), desired product (M+H: 2030).

3. References

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