Supporting Information

Traceless β-Mercaptan-Assisted Activation of Valinyl Benzimidazolinones in Peptide Ligations

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I. General Information

Materials and Methods

All commercial materials (Sigma Aldrich, Fisher, Acros, TCI, Adamas, J&K, GL Biochem, Energy, etc.) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher, Sigma, Acros, Oceanpak). Anhydrous tetrahydrofuran, dichloromethane, diethyl ether, toluene, and \( N,N \)-dimethyl formamide were purified and dried by PURE SOLV\textsuperscript{®} solvent purification system (Innovative Technology, Inc.). Analytical thin layer chromatography was performed using Merck TLC silica gel 60-F254 glass plates. Flash chromatography was performed using 200-300 mesh silica gel (Qingdao Haiyang Chemical Co., Ltd.). Filtration for crude peptide was performed using a Bulk GHP Acrodisc\textsuperscript{®} 13 mm syringe filter with 0.22 \( \mu \)m GHP membrane. Ultra-pure argon (\( \geq 99.999\% \)) was used in all ligation and desulfurization reactions. Yields refer to chromatographically and spectroscopically pure materials unless otherwise stated.

\(^1\)H NMR spectra were recorded on Bruker Avance III 400 MHz at ambient temperature using CDCl\(_3\) as solvent unless otherwise stated, referenced to TMS or residual solvent. \(^{13}\)C NMR spectra were recorded at 100.0 MHz at ambient temperature using CDCl\(_3\) as solvent unless otherwise stated. Chemical shifts are reported in parts per million relative to CDCl\(_3\) (\( ^1\)H, \( \delta \) 7.26; \(^{13}\)C, \( \delta \) 77.0). Data for \(^1\)H NMR are reported as follows: chemical shift, integration, multiplicity (ovrlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constants (\( J \) Hz). All \(^{13}\)C NMR spectra were recorded as chemical shift (\( \delta \)). Infrared spectra were recorded on a Nicolet Nexus 670 FT-IR spectrophotometer. High-resolution mass spectra were obtained in the Chemical Instrumentation Center, Peking University Health Science Center using a Waters Q-TOF mass spectrometer (Xevo G2 Q-TOF). Low-resolution mass spectra analyses were performed with a Waters SQD mass spectrometer (Alliance e2695-SQD). Melting points were recorded on a melting point-M560 (Buchi). Optical rotations were recorded on an AUTOPOL III digital polarimeter at 589 nm and are recorded as \([\alpha]_D^{22}\)
(concentration in grams/100 mL solvent).

All HPLC separations involved a mobile phase of 0.05% (v/v) TFA in water (solvent A) and 0.04% (v/v) TFA in acetonitrile (solvent B). Analytical LC-MS analyses were performed using a Waters Alliance e2695 Separations Module and a Waters 2489 UV/Visible (UV/Vis) Detector equipped with an Agilent C18 column (5.0 μm, 4.6 × 150 mm) at a flow rate of 0.4 mL/min, a Beim Brueckle C4 column (5.0 μm, 4.6 × 150 mm) at a flow rate of 0.4 mL/min, or a Higgins Analytical PROTO-300 C4 (5.0 μm, 2.1 × 150 mm) at a flow rate of 0.2 mL/min. The wavelengths of UV-detector were set to 210 nm and 220 nm.

Analytical HPLC separations were performed using an Agilent Technologies 1260 Infinity LC system.

Preparative HPLC separations were performed using a Hanbon Sci. & Tech. NP7005C solvent delivery system and a Hanbon Sci. & Tech. NU3010C UV detector equipped with an Agilent Eclipse XDB-C18 column (7.0 μm, 21.2 × 250 mm), a Beim Brueckle C4 column (10.0 μm, 20 × 250 mm), or a Proto 300 C4 column (10.0 μm, 20 × 250 mm) at a flow rate of 16 mL/min. The wavelengths of UV-detector were set to 210 nm and 220 nm.
II. Synthesis and Characterization of Penicillamine Derivative

Synthesis of the N-9-fluorenylmethoxycarbonyl-methylthio-L-penicillamine (2)

To a solution of penicillamine (300 mg, 2.0 mmol) in 15 mL of degassed EtOH was added methyl methanethiosulfonate (MMTS, 0.2 mL, 2.0 mmol) dropwise. The mixture was stirred at room temperature for 4 h. Upon the full consumption of penicillamine, the reaction was concentrated in vacuo to afford compound S1 without further purification.

To a solution of crude S1 and 9-fluorenylmethyl chloroformate (Fmoc-Cl, 624 mg, 2.4 mmol) in 15mL DCM, triethylamine (TEA, 0.67 mL, 4.8 mmol) was added dropwise at 0 °C. The reaction was stirred at room temperature under an argon atmosphere overnight, then quenched with 1 M aqueous HCl at 0 °C. The mixture was extracted three times with EtOAc, and the combined extracts were washed with H2O and brine, dried over magnesium sulfate, and concentrated in vacuo. The crude residue was purified using silica gel column chromatography eluting with petroleum ether/EtOAc (5:1) containing 1% of HOAc to afford compound 2 as a white solid (656 mg, 78% two steps). IR (thin film): ν_{max} 2970, 1719, 1513, 1450, 1417, 1368, 1340, 1226, 1152, 1119, 1048, 1008, 912 cm⁻¹; [α]_{D}^{20} = + 9.7° (c 0.01, CHCl₃); m.p. 74-75 °C; ^1H NMR (400 MHz, CDCl₃): δ 7.76 (d, J = 7.4 Hz, 2H), 7.64-7.57 (m, 2H), 7.44-7.36 (m, 2H), 7.36-7.28 (m, 2H), 5.64 (d, J = 8.8 Hz, 1H), 4.57-4.30 (m, 3H), 4.24 (t, J = 6.8 Hz, 1H), 2.40 (s, 3H), 1.53-1.31 (m, 6H); ^13C NMR (100 MHz, CDCl₃): δ 174.9, 156.0, 143.7, 143.6, 141.3 (two carbons overlap), 127.7 (two carbons overlap), 127.1 (two carbons overlap), 125.1 (two carbons overlap), 120.0 (two carbons overlap), 67.3, 60.9, 52.1, 47.1, 26.3, 25.1, 24.5; HRMS-ESI (m/z): calcd for C_{21}H_{23}NO_{2}S_{2} [M+H]^+: 418.1141, [M+Na]^+:
Compounds S2 was synthesized following literature reported procedures.\textsuperscript{S1} To the solution of methanesulfonyl chloride (77 \( \mu \)L, 1.0 equiv) and 2-methyl-2-propanethiol (0.11 mL, 1.0 equiv) in Et\(_2\)O (1.0 mL) was added TEA (0.14 mL, 1.2 equiv) dropwise at 0 \( ^\circ \)C. The reaction was stirred at 0 \( ^\circ \)C for 30 min and then quenched with 1N HCl and water at 0 \( ^\circ \)C. The resulting mixture was extracted with ethyl acetate (\( \times 3 \)), and the combined extracts were washed with water and brine, dried over MgSO\(_4\) and concentrated in vacuo. The crude material was purified using silica gel column chromatography (petroleum ether: CH\(_2\)Cl\(_2\) = 5:1) to afford compound S2 as a colorless oil (150 mg, 90%).

A solution of compound S3\textsuperscript{S2} (160 mg, 1.0 equiv) in methanol (3.7 mL) was treated with 1N NaOH solution (5.5 ml, 5.0 equiv) at 0\(^\circ\)C for 10 min. The reaction mixture was carefully neutralized using 1N HCl and water at 0\(^\circ\)C. The resulting mixture was extracted with ethyl acetate (\( \times 3 \)). The combined extracts were washed with water and brine, dried with MgSO\(_4\) and concentrated in vacuo. The obtained crude intermediate was used directly in next step without purification, which was mixed with S2 (270 mg,

\textsuperscript{S1} H. T. Pham, N.-L. T. Nguyen, F. Duus, T. X. T. Luu, \textit{Phosphorus, Sulfur, and Silicon and the Related Elements} \textbf{2015}, \textit{190}, 1934-1941.

\textsuperscript{S2} K. K. Pasunooti, R. Yang, B. Banerjee, T. Yap, C. F. Liu, \textit{Org. Lett.} \textbf{2016}, \textit{18}, 2696-2699.
3.5 equiv) in anhydrous CH$_2$Cl$_2$ (3 mL), followed by the addition of TEA (80 µL, 1.2 equiv) at 0 °C. The reaction mixture was stirred at room temperature overnight, then quenched with 1N HCl and water at 0°C. The resulting mixture was extracted with ethyl acetate ($\times$3). The combined extracts were washed with water and brine, dried with MgSO$_4$ and concentrated in vacuo to afford crude compound S4, which was treated with TFA/DCM = 9:1 (v/v) solution at room temperature for 2 hours. The solvent was removed under a nitrogen atmosphere. The residue was co-evaporated with toluene, and dissolved in 3 mL dioxane/H$_2$O (1:1, v/v), followed by the addition of Boc$_2$O (216 µL, 2.0 equiv) and TEA (80 µL, 1.3 equiv) at 0 °C. After stirring at room temperature for 24 hours, the reaction was quenched with 1N HCl and water at 0 °C. The resulting mixture was extracted with ethyl acetate ($\times$3). The combined extracts were washed with water and brine, dried with MgSO$_4$ and concentrated in vacuo. The crude residue was purified using silica gel column chromatography eluting with petroleum ether/EtOAc (6:1) containing 1% of HOAc to afford compound S5 as a light yellow syrup (77 mg, 50% in 4 steps). IR (thin film): $\nu_{\text{max}}$ 2969, 2931, 1716, 1506, 1455, 1164, 1058 cm$^{-1}$; [$\alpha$]$^24.9_D$ = - 63.2° (c 1.0, MeOH); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.13 (d, $J$=7.6 Hz 1H), 4.43-4.26 (m, 1H), 2.98-2.85 (m, 1H), 2.65-2.47 (m, 1H), 2.41-2.28 (m, 1H), 1.45 (s, 9H), 1.32 (s, 9H), 1.09 (d, $J$=6.3 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 176.1, 155.6, 80.3, 57.3, 47.9, 43.8, 36.5, 29.9 (three carbons overlap), 28.3 (three carbons overlap),15.6; HRMS-ESI ($m/z$): calcd for C$_{14}$H$_{26}$NO$_4$S$_2$ [M-H]: 336.1303; found, 336.1297.
NMR Spectra of amino acids derivatives

Compound 2 – ^1^H NMR Spectrum - CDCl3, 400 MHz

Compound 2 – ^13^C NMR Spectrum - CDCl3, 100 MHz
Compound S5 - $^1$H NMR Spectrum - CDCl$_3$, 400 MHz

Compound S5 – $^{13}$C NMR Spectrum - CDCl$_3$, 100 MHz
III. General Procedures for Peptide Synthesis

3.1 Preparation of amino acid pre-loaded resin and determination of resin loading

**Pre-load an amino acid to 2-chlorotritylchloride resin**

The first Fmoc-amino acid residue was loaded to 2-chlorotritylchloride resin before Fmoc-SPPS following the general procedure below.

To a mixture of Fmoc-amino acid (1.0 equiv) and 2-chlorotritylchloride resin was added dry DCM (approx. 10 mL per gram of resin) and DIEA (4.0 equiv). The reaction was agitated for 2 hours. The resin was collected and washed with 17/2/1 (v/v/v) of DCM/MeOH/DIEA (×3), DCM (×3), DMF (×2), DCM (×3), and dried in vacuo for 12 hours before the loading test.

**Pre-load Fmoc-Dbz to Rink-MBHA resin**

The Rink-MBHA resin (1.0 equiv) was deprotected with 2% DBU and 2% piperidine in DMF for 5 min (×2) to remove the Fmoc group. The synthesized Fmoc-Dbz (4.0 equiv), HATU (4.0 equiv) in DMF (approx. 10 mL per gram of resin) was added to the resin. DIEA (8.0 equiv) was added dropwise to the reaction mixture. The reaction was agitated for 1 hour. The resin was collected and washed with DMF (×2), DCM (×3), DMF (×2), DCM (×3), and dried in vacuo for 24 hours before the loading test.

**Determination of resin loading**

Dry Fmoc amino-acid resin (approx. 5 μmol with respect to Fmoc) was weighted into a clean test tube, followed by the addition of 2 mL of 2% DBU in DMF. The mixture was agitated gently for 30 min, and then diluted to 10 mL with CH$_3$CN. 2 mL of the resulting solution was taken out and diluted to 25 mL in a 50 mL centrifuge tube as the test solution. A reference solution was prepared in the same manner without the addition of resin.

Two matched silica UV cells were filled with reference solution to blank the U.V. spectrophotometer. The solution in one of the silica UV cells was changed to the test

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$^{53}$ J. B. Blanco-Canosa, P. E. Dawson, *Angew. Chem. Int. Ed.* 2008, 47, 6851-6855.

$^{54}$ Peptide Synthesis, 2010/2011 Catalog, Merck.
solution after washing with the test solution for three times. The optical density at 304 nm was recorded for three times and the average value was calculated as Abs<sub>sample</sub>. The Fmoc loading of resin could be calculated using the equation below:

\[
\text{Fmoc loading: mmol/g = Abs}_{\text{sample}} \times 16.4/(\text{mg of resin})
\]

### 3.2 Automated solid-phase peptide synthesis

Automated peptide synthesis was performed on a Pioneer peptide synthesis system (GEN600611) or a CS Bio peptide synthesizer (CX136XT).

**Pioneer peptide synthesizer** Peptides were synthesized under standard automated Fmoc protocols using DMF as solvent, degrading for 5 min in piperidine/DBU/DMF (2:2:96, v/v/v), coupling for 25 min (‘standard cycle’), or 55 min (‘extended cycle’) for amino acids after steric hindered residues such as prolines, valines, threonines, isoleucines and arginines using HATU as coupling reagent.

**CS Bio peptide synthesizer** Peptide synthesis was performed following the general protocol using DMF as solvent, degrading (5 min × 2) in piperidine/DMF (20:80, v/v) containing Oxyma (0.1 M), couple for 25 min using HATU/HOBt (1:1) as coupling agent, for amino acids after steric hindered residues, the coupling cycle was repeated as needed.

The following "N-Fmoc or "N-Boc-protected amino acids and pseudoproline dipeptides from Novabiochem, GL Biochem or CS Bio were employed in SPPS: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O'Bu)-OH, Fmoc-Glu(O'Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser('Bu)-OH, Fmoc-Thr('Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr-OH, Fmoc-Val-OH, Fmoc-Nle-OH, Fmoc-Asn(Trt)-Ser(ψ<sup>Me,Me</sup>Pro)-OH, Boc-Glu(O'Bu)-OH, Boc-Cys(S'Bu)-OH, Boc-Cys(Trt)-OH, Boc-Ser('Bu)-OH.

The employed 2-chlorotritylchloride resin (1.147 mmol/g) employed in SPPS was purchased from GL Biochem, and Rink MBHA resin (0.42 mmol/g) was purchased from CS Bio.
3.3 Preparation of peptidyl acids and peptidyl amides

Upon completion of the automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide synthesis vessel using DCM. Resin cleavage and global deprotection was performed under the treatment of TFA/H$_2$O/TIPS (95:2.5:2.5, v/v/v) solution for 2 hours. The resin was then removed by filtration, and the filtrate was concentrated under a nitrogen atmosphere. The residue was washed with cold diethyl ether to give a white solid, which was then dissolved in a mixture of acetonitrile and water containing 5% of acetic acid. The resulting solution was ready for HPLC purification after filtration. Utilization of 2-chlorotrityl resin and Rink- MBHA resin afforded the peptidyl acids and peptidyl amides respectively.

For side-chain protected peptide, the resin after SPPS was treated with DCM/TFE/AcOH (3:1:1, v/v/v) solution 40 min ($\times$3), the combined cleavage solution was concentrated under reduced pressure, and lyophilized to remove the residual acid.

3.4 Preparation of C-terminal Pen-Nbz containing peptides

Fmoc-Pen(SMe)-OH (2) (5.0 equiv) was coupled to pre-loaded Dbz-Rink-MBHA resin (1.0 equiv, loading = 0.307 g/mol) manually using DIC (5.0 equiv) and oxyma pure (5.0 eq.) in DMF for 8 hours. The resulting resin was washed with DMF ($\times$2), DCM ($\times$3), DMF ($\times$2) and DCM ($\times$3), followed by the automated synthesis on a 0.02-0.05 mmol scale, where the last residue was introduced as Boc-Xaa.

After peptide elongation, the resin was washed with DCM and treated with p-nitrophenylchloroformate solution (50 mM in dry DCM) for 40 min at room temperature, unless otherwise stated. Then it was washed with DCM ($\times$3), DCM ($\times$2), treated with DIEA (0.5 M in DMF) for 15 min to convert Dbz to Nbz.$^{33}$ Finally, the resin was washed thoroughly using DMF/DCM. Resin cleavage and global deprotection was performed under the treatment of TFA/H$_2$O/TIPS (95:2.5:2.5, v/v/v) solution for 2 hours. The resin was then removed by filtration, and the filtrate was concentrated under a nitrogen atmosphere. The residue was washed with cold diethyl ether to give a white solid, which was then dissolved in a mixture of acetonitrile and water containing 5% of acetic acid. The resulting solution was ready for HPLC
puriﬁcation after ﬁltration.

3.5 Native Chemical Ligation
To a mixture of the west-side peptide (1.0 equiv), and east-side peptide containing thiol-amino acid at the N-terminus (1.2 equiv), was added appropriate volume of ligation buffer (6 M Gn·HCl, 200 mM Na₂HPO₄, 20 mM TCEP·HCl, pH 7.0) under an argon atmosphere, the concentration of the west-side peptides is approximately 3 mM, and the resulting solution was stirred at room temperature (25 °C) and monitored using LC-MS. The reaction was quenched with H₂O/MeCN/AcOH (90:5:5, v/v/v) and puriﬁed using HPLC.

3.6 Metal-free Desulfurization
To a solution of the thiol-containing peptide (3 mM) in appropriate volume of degassed buffer (6 M Gn•HCl, 200 mM Na₂HPO₄, pH 7.0) was added 0.5 M Bond-breaker® TCEP solution (Pierce), 20 μL of 2-methyl-2-propanethiol and 100 μL of radical initiator VA-044 (0.1 M in degassed water). The reaction mixture was stirred at 37 °C and monitored by LC-MS. Upon completion, the reaction was quenched by adding H₂O/MeCN/AcOH (90:5:5, v/v/v) and further puriﬁed using HPLC.

3.7 One-pot Ligation-Desulfurization
Upon completion of the ligation as indicated by LC-MS analysis, to the reaction vessel was added proper volume of 0.5 M Bond-breaker® TCEP solution (Pierce), 20 μL of 2-methyl-2-propanethiol and 100 μL of radical initiator VA-044 (0.1 M in degassed water). The reaction mixture was stirred at 37 °C and monitored by LC-MS. Upon completion, the reaction was quenched by adding H₂O/MeCN/ AcOH (90:5:5, v/v/v) and further puriﬁed using HPLC.
IV. Preparation and Characterization of Peptide Segments

**Peptidyl Pen-Nbz 7a**

Peptide 7a (mixture of 3-/4-substituted Nbz isomers) was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.03 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 7a as a white solid after lyophilization (11.1 mg, 45%). Analytical HPLC: t<sub>R</sub> = 18.1, 18.8 min (10 to 40% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C<sub>34</sub>H<sub>52</sub>N<sub>10</sub>O<sub>10</sub>S<sub>2</sub>: 825.0 Da (average isotopes), (m/z) [M+H]⁺: 825.3; found [M+H]⁺: 825.2.

![UV trace from LC-MS analysis of peptide 7a](image)

![ESI-MS data of peptide 7a](image)

**Figure S1.** Left: UV trace from LC-MS analysis of peptide 7a; Right: ESI-MS data of peptide 7a.

**Peptidyl Pen-Nbz 7b**

Peptide 7b was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (15 to 35% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 7b as a white solid after lyophilization (9.5 mg, 60%). Analytical HPLC: t<sub>R</sub> = 22.0 min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for
C$_{34}$H$_{55}$N$_{11}$O$_{5}$S$_{2}$: 825.0 Da (average isotopes), ($m/z$) [M+H]$^+$: 858.4; found [M+H]$^+$: 858.3.

**Figure S2.** Left: UV trace from LC-MS analysis of peptide 7b; Right: ESI-MS data of peptide 7b.

**Peptidyl Pen-Nbz 7c**

Peptide 7c (mixture of 3-/4-substituted Nbz isomers) was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.03 mmol scale. Purification of the crude peptide using preparative HPLC (15 to 40% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 7c as a white solid after lyophilization (10.2 mg, 35%). Analytical HPLC: t$_R$ = 20.6, 21.5 min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C$_{42}$H$_{54}$N$_{12}$O$_{11}$S$_{2}$: 967.1 Da (average isotopes), ($m/z$) [M+H]$^+$: 967.4; found [M+H]$^+$: 967.4.

**Figure S3.** Left: UV trace from LC-MS analysis of peptide 7c; Right: ESI-MS data of peptide 7c.
**Peptidyl Pen-Nbz 7d**

Peptide 7d (mixture of 3-/4-substituted Nbz isomers) was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 7d as a white solid after lyophilization (10.9 mg, 59%). Analytical HPLC: $t_R = 16.3$, 16.9 min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C$_{39}$H$_{61}$N$_{11}$O$_{11}$S$_2$: 924.1 Da (average isotopes), ($m/z$) [M+H]$^+$: 924.4, [M+2H]$^{2+}$: 462.7; found [M+H]$^+$: 924.4, [M+2H]$^{2+}$: 462.9.

![UV trace from LC-MS analysis of peptide 7d](image)

**Figure S4.** Left: UV trace from LC-MS analysis of peptide 7d; Right: ESI-MS data of peptide 7d.

**peptidyl Val-Nbz 10**

Peptide 10 (mixture of 3-/4-substituted Nbz isomers) was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 10 as a white solid after lyophilization (13.6 mg, 36%). Analytical HPLC: $t_R = 17.0$, 17.7 min (10 to 30% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C$_{33}$H$_{50}$N$_{10}$O$_{10}$: 746.8 Da (average isotopes), ($m/z$) [M+H]$^+$: 747.4; found [M+H]$^+$: 747.2.
Peptidyl Val-Nbz S6

Peptide S6 was prepared according to General Procedure 3.4 using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide S6 as a white solid after lyophilization (15.5 mg, 36%). Analytical HPLC: $t_R = 21.8$ min (10 to 30% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for $C_{37}H_{58}N_{10}O_{10}S_2$: 867.1 Da (average isotopes), ($m/z$) [M+H]$^+$: 867.4; found [M+H]$^+$: 867.4.

Peptidyl Cys-Nbz S7

Peptide S7 (mixture of 3-/4-substituted Nbz isomers) was prepared according to General
Procedure 3.4 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (15 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide S7 as a white solid after lyophilization (4.2 mg, 24%). Analytical HPLC: $t_R = 19.8, 20.3$ min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for $C_{37}H_{50}N_8O_8S_2$: 839.0 Da (average isotopes), $(m/z) [M+H]^+: 839.4$; found [M+H]$^+$: 839.3.

![Figure S7](image)

**Figure S7.** Left: UV trace from LC-MS analysis of peptide S7; Right: ESI-MS data of peptide S7.

**Peptide 8a**

Peptide 8a was prepared according to General Procedure 3.3 using CS Bio peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8a as a white solid after lyophilization (27.1 mg, 66%). Analytical HPLC: $t_R = 19.6$ min (10 to 40% solventB over 30min, Agilent C18 column); ESI-MS: calcd for $C_{34}H_{65}N_{11}O_8S_2$: 820.1 Da (average isotopes), $(m/z) [M+H]^+: 820.5$; found [M+H]$^+$: 820.6.
**Figure S8.** Left: UV trace from LC-MS analysis of peptide 8a; Right: ESI-MS data of peptide 8a. (* Denotes the peaks of fragmentation during ionization in mass spectrometer)

**Peptide 8b**

Peptide 8b was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide 8b as a white solid after lyophilization (13.6 mg, 36%). Analytical HPLC: t_R = 18.1 min (10 to 30% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C_{26}H_{51}N_{11}O_{6}S_{2}: 677.9 Da (average isotopes), (m/z) [M+H]^+: 678.4; found [M+H]^+: 678.2.

**Figure S9.** Left: UV trace from LC-MS analysis of peptide 8b; Right: ESI-MS data of peptide 8b.

**Peptide 8c**

Peptide 8c was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide 8c as a white solid after lyophilization (9.7 mg, 21%). Analytical HPLC: t_R = 17.2 min (10 to 30% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C_{24}H_{49}N_{10}O_{6}S_{2}: 675.3 Da (average isotopes), (m/z) [M+H]^+: 675.3; found [M+H]^+: 675.3.
synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8c as a white solid after lyophilization (11.2 mg, 41%). Analytical HPLC: t_R = 13.8 min (20 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C_{23}H_{42}N_{6}O_{5}S_{2}: 546.7 Da (average isotopes), (m/z) [M+H]^+: 547.3; found [M+H]^+: 547.1.

**Figure S10.** Left: UV trace from LC-MS analysis of peptide 8c; Right: ESI-MS data of peptide 8c.

**Peptide 8d**

Peptide 8d was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8d as a white solid after lyophilization (6.1 mg, 11%). Analytical HPLC: t_R = 19.3 min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C_{46}H_{71}N_{9}O_{18}S_{2}: 1102.2 Da (average isotopes), (m/z) [M+H]^+: 1102.4; found [M+H]^+: 1102.3.
Peptide 8e was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8e as a white solid after lyophilization (13.6 mg, 36%). Analytical HPLC: \( t_R = 17.4 \) min (10 to 30% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for \( \text{C}_{31}\text{H}_{57}\text{N}_{11}\text{O}_{10}\text{S} \): 775.9 Da (average isotopes), \( m/z \) [M+H]^+: 776.4; found [M+H]^+: 776.2.
**Peptide 8f**

Peptide 8f was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.02 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8f as a white solid after lyophilization (4.5 mg, 28%). Analytical HPLC: t_R = 18.6 min (10 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C_{33}H_{63}N_{11}O_{8}S: 806.1 Da (average isotopes), (m/z) [M+H]^+: 806.4; found [M+H]^+: 806.3.

![Figure S13](image_url)

Figure S13. Left: UV trace from LC-MS analysis of peptide 8f; Right: ESI-MS data of peptide 8f. (* Denotes the peaks of fragmentation during ionization in mass spectrometer)

**Peptide 8g**

Peptide 8g was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30min, Agilent Eclipse XDB-C18 column) afforded peptide 8g as a white solid after lyophilization (10.5 mg, 26%). Analytical HPLC: t_R = 9.3 min (20 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C_{33}H_{63}N_{11}O_{8}S: 806.1 Da (average isotopes), (m/z) [M+H]^+: 806.4; found [M+H]^+: 806.3.
Figure S14. Left: UV trace from LC-MS analysis of peptide 8g; Right: ESI-MS data of peptide 8g. (* Denotes the peaks of fragmentation during ionization in mass spectrometer)

**Peptide S8**

Peptide S8 was prepared according to General Procedure 3.3 using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 30% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide S8 as a white solid after lyophilization (10.5 mg, 30%). Analytical HPLC: t_R = 15.4 min (5 to 50% B over solvent 30 min, Agilent C18 column); ESI-MS: calc'd for C_{30}H_{57}N_{11}O_{8}: 699.9 Da (average isotopes), (m/z) [M+H]^+: 700.5; found [M+H]^+: 700.2.

Figure S15. Left: UV trace from LC-MS analysis of peptide S8; Right: ESI-MS data of peptide S8. (* Denotes the peaks of fragmentation during ionization in mass spectrometer)

**Peptide S9**
Peptide S9 was prepared following our previous work.\textsuperscript{55} Analytical HPLC: $t_R = 13.7$ min (5 to 40\% B over solvent 30min, Agilent C18 column); ESI-MS: calcd for C\textsubscript{26}H\textsubscript{42}N\textsubscript{8}O\textsubscript{7}S\textsubscript{2}: 642.8 Da (average isotopes), (m/z) [M+H]+: 643.3; found [M+H]+: 643.2.

**Figure S16.** Left: UV trace from LC-MS analysis of peptide S9; Right: ESI-MS data of peptide S9.

**Peptidyl Pen-Nbz 7h**

Peptide 7h was prepared according to General Procedure 3.4 using CS Bio peptide synthesizer on a 0.03 mmol scale. Purification of the crude peptide using preparative HPLC (25 to 35\% solvent B over 30min, Proto-300 C4 column) afforded peptide 7h as a white solid after lyophilization (18.4 mg, 17\%). Analytical HPLC: $t_R = 20.1$ min (20 to 40\% solvent B over 30min, Proto-300 C4 column); ESI-MS: calcd for C\textsubscript{148}H\textsubscript{230}N\textsubscript{46}O\textsubscript{55}S\textsubscript{2}: 3597.9 Da (average isotopes), (m/z) [M+2H]\textsuperscript{2+}: 1799.3, [M+3H]\textsuperscript{3+}: 1199.9; found [M+2H]\textsuperscript{2+}: 1799.7, [M+3H]\textsuperscript{3+}:1200.1.

\textsuperscript{55} Y. Gui, L. Qiu, Y. Li, H. Li and S. Dong, *J. Am. Chem. Soc.*, 2016, \textbf{138}, 4890-4899.
**Peptide 8h**

Peptide 8h was prepared according to General Procedure 3.3 using CS Bio peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (15 to 30% solvent B over 30min, Beim Brueckle C4 column) afforded peptide 8h as a white solid after lyophilization (64.4 mg, 65%). Analytical HPLC: \( t_R = 9.7 \) min (20 to 40% solvent B over 30min, Beim Brueckle C4 column); ESI-MS: calcld for \( \text{C}_{125}\text{H}_{187}\text{N}_{35}\text{O}_{36}\text{S}_3\): 2852.3 Da (average isotopes), \((m/z)\) \([\text{M}+2\text{H}]^{2+}\): 1426.7, \([\text{M}+3\text{H}]^{3+}\): 951.4, \([\text{M}+4\text{H}]^{4+}\): 713.8; found: \([\text{M}+2\text{H}]^{2+}\): 1426.7, \([\text{M}+3\text{H}]^{3+}\): 951.5, \([\text{M}+4\text{H}]^{4+}\): 713.9.

**Figure S18.** Left: UV trace from LC-MS analysis of peptide 8h; Right: ESI-MS data of peptide 8h.
Side-chain protected peptide Cys^{34}-Pro^{71} with Boc-protection at the N-terminus was synthesized using CS Bio peptide synthesizer on a 0.05 mmol scale. The resin was cleaved using DCM/TFE/AcOH (3:1:1, v/v/v) for three times (7 mL, 7 mL and 6 mL). The filtrate was concentrated under an argon atmosphere, and lyophilized to remove the residual acid. To the obtained powder (1.0 equiv) was added leucine thioester (2.0 equiv) and HATU (2.0 equiv), and the mixture was dissolved in anhydrous DCM, followed by the addition of DIEA (4.0 equiv) at 0 °C. The reaction mixture was stirred at room temperature for 30 min. After removal of the solvent under a nitrogen atmosphere, the resulting residue was treated with a solution of TFA/H₂O/TIPS (95:2.5:2.5, v/v/v) for 2 hours, and then concentrated under a nitrogen atmosphere. The residue was washed with cold diethyl ether to afford a white solid, which was then dissolved in a mixture of acetonitrile and water containing 5% of acetic acid. The resulting solution was ready for HPLC purification after filtration. Purification of the crude peptide using preparative HPLC (15 to 30% solvent B over 30min, Proto-300 C4 column) afforded peptide 13 as a white solid after lyophilization (23.6 mg, 10%). Analytical HPLC: t_R = 19.6 min (20 to 40% solvent B over 30min, Proto-300 C4 column); ESI-MS: calcd for C_{212}H_{325}N_{57}O_{61}S_{2}: 4744.5 Da (average isotopes), (m/z) [M+3H]^{3+}: 1582.1, [M+4H]^{4+}: 1186.8, [M+5H]^{5+}: 949.7; found: [M+3H]^{3+}: 1582.3, [M+4H]^{4+}: 1186.9, [M+5H]^{5+}: 949.9.
Peptide 15b was prepared according to General Procedure 3.3 using CS Bio peptide synthesizer on a 0.05 mmol scale, and the N-terminal thio-valine derivative was coupled manually using Boc-Val(‘SSMe)-OH$^2$ (1.5 equiv) and HATU (1.5 equiv) for 30 min ($\times 2$). Purification of the crude peptide using preparative HPLC (15 to 30% solvent B over 30min, Beim Brueckle C4 column) afforded peptide 15b as a white solid after lyophilization (35.4mg, 59%). Analytical HPLC: t$_R$ = 18.5 min (15 to 45% solvent B over 30min, Beim Brueckle C4 column); ESI-MS: calcd for C$_{91}$H$_{151}$N$_{23}$O$_{24}$S$_{2}$: 2015.5 Da (average isotopes), (m/z) [M+2H]$^{2+}$: 1008.0, [M+3H]$^{3+}$: 672.3; found: [M+2H]$^{2+}$: 1008.1, [M+3H]$^{3+}$: 672.6.

Figure S19. Left: UV trace from LC-MS analysis of peptide 13; Right: ESI-MS data of peptide 13.

Figure S20. Left: UV trace from LC-MS analysis of peptide 15b; Right: ESI-MS data of peptide 15b.
Peptidyl Pen-Nbz 18

Peptide 18 was prepared according to General Procedure 3.4 using CS Bio peptide synthesizer on a 0.05 mmol scale. Asn^{16}Ser^{17} was installed manually using Fmoc-Asn(Trt)-Ser(ψ^{Me,Me}Pro)-OH as the building block. After peptide elongation, the resin was treated with a p-nitrophenylechloroformate solution (50 mM in dry DCM) for 2 hours at room temperature. Purification of the crude peptide using preparative HPLC (30 to 40% solvent B over 30min, Proto-300 C4 column) afforded peptide 18 as a white solid after lyophilization (21.5 mg, 13%). Analytical HPLC: t_R = 28.6 min (20 to 40% solvent B over 30min, Beim Brueckle C4 column); ESI-MS: calcd for C_{197}H_{311}N_{59}O_{53}S_{2}: 4418.1 Da (average isotopes), (m/z) [M+3H]^3+: 1473.4, [M+4H]^4+: 1105.3, [M+5H]^5+: 884.5; found: [M+3H]^3+: 1473.3, [M+4H]^4+: 1105.2, [M+5H]^5+: 884.4.

Figure S21. Left: UV trace from LC-MS analysis of peptide 18; Right: ESI-MS data of peptide 18.
**Peptidyl thioester 19**

Side-chain protected peptide Cys\(^{36}\)-Leu\(^{57}\) with Boc-protection at the N-terminus was synthesized using CS Bio peptide synthesizer on a 0.1 mmol scale. The resin was cleaved using DCM/TFE/AcOH (3:1:1, v/v/v) for three times (7 mL, 7 mL and 6 mL). The filtrate was concentrated under a nitrogen atmosphere, and lyophilized to remove the residual acid. To a solution of the obtained powder (1.0 equiv) and 6-Chlorobenzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyClock, 3.0 equiv) in anhydrous DMF, ethyl 3-mercaptopropionate (30 equiv) was added followed by the addition of DIEA (5.0 equiv) at -20 °C. The reaction mixture was stirred at the same temperature for 2 hours. After removal of the solvent under lyophilization, the resulting residue was treated with a solution of TFA/H\(_2\)O/TIPS (95:2.5:2.5, v/v/v) for 2 hours, and then concentrated under a nitrogen atmosphere. The residue was washed with cold diethyl ether to afford a white solid, which was then dissolved in a mixture of acetonitrile and water containing 5% of acetic acid. The resulting solution was ready for HPLC purification after filtration. Purification of the crude peptide using preparative HPLC (20 to 35% solvent B over 30min, Proto-300 C4 column) afforded peptide 19 as a white solid after lyophilization (61.5 mg, 22%). Analytical HPLC: \(t_R = 20.9\) min (20 to 40% solvent B over 30min, Proto-300 C4 column); ESI-MS: calcd for C\(_{118}\)H\(_{203}\)N\(_{37}\)O\(_{35}\)S\(_{3}\): 2796.3 Da (average isotopes), (m/z) \([M+2H]^{2+}\): 1398.7, \([M+3H]^{3+}\): 932.8, \([M+4H]^{4+}\): 699.9; found: \([M+2H]^{2+}\): 1398.9, \([M+3H]^{3+}\): 932.7, \([M+4H]^{4+}\): 699.9.

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\(^{56}\) Y. Kajihara, A. Yoshihara, K. Hirano, N. Yamamoto, *Carbohydr. Res.* **2006**, *341*, 1333-1340.
Peptide 21 was prepared according to General Procedure 3.3 using CS Bio peptide synthesizer on a 0.05 mmol scale, and the N-terminal thio-valine derivative was coupled manually using S5 (1.5 equiv) for 30 min (×2). Purification of the crude peptide using preparative HPLC (15 to 30% solvent B over 30min, Proto-300 C4 column) afforded peptide 21 as a white solid after lyophilization (16.4mg, 13%). Analytical HPLC: t_R = 16.9 min (15 to 45% solvent B over 30min, Proto-300 C4 column); ESI-MS: calcd for C_{117}H_{190}N_{33}O_{42}S_{2}: 2804.2 Da (average isotopes), (m/z) [M+2H]^{2+}: 1402.7, [M+3H]^{3+}: 935.5, [M+4H]^{4+}: 701.9; found: [M+2H]^{2+}: 1402.8, [M+3H]^{3+}: 935.5, [M+4H]^{4+}: 701.9.
V. Ligation Reactions Probing the Mechanistic Insight

**Ligation reactions between S6 and 8a**

Peptidyl Val-Nbz S6 (1.0 equiv) and cysteinyl peptide 8a (1.2 equiv) were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred at room temperature for 8 hours, and the progress was monitored with LC-MS. As shown in Figure S24, a complex mixture was generated with low conversion to the ligation product after 8 h.

![Figure S24](image)

Figure S24. UV trace of the ligation reaction between peptides S6 and 8a.

**Ligation reactions between S7 and 8a**

Peptidyl Cys-Nbz S7 (1.0 equiv) and cysteinyl peptide 8a (1.2 equiv) were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred at room temperature for 2 hours, and the progress was monitored with LC-MS. As shown in Figure S25, large amount of S7 decomposed after 2 hours, and only trace amount of ligation product was generated.

![Figure S25](image)

Figure S25. UV trace of the ligation reaction between peptides S7 and 8a.
**Ligation reactions between 7a and S8**

Peptidyl Pen-Nbz 7a (1.0 equiv) and alanyl peptide S8 (1.2 equiv) were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred at room temperature for 8 hours, and the progress was monitored with LC-MS. As shown in Figure S26, large amount of byproduct was generated and trace amount of ligation product was observed.

![UV trace of the ligation reaction between peptides 7a and S8.](image1)

**Figure S26.** UV trace of the ligation reaction between peptides 7a and S8.

**7a in ligation buffer**

Peptidyl Pen-Nbz 7a (0.4 mg) was dissolved in 160 μL of ligation buffer, and monitored with LC-MS (5 to 30% B over solvent 30min, Agilent C18 column). As shown in Figure S27, the intermediate 7a' gradually decomposes and hydrolizes over time if not intercepted with other reactants.

![UV trace of peptide 7a dissolved in ligation buffer.](image2)

**Figure S27.** UV trace of peptide 7a dissolved in ligation buffer.
VI. One-Pot Ligation and Desulfurization Reactions

*One-pot ligation and desulfurization reactions between 7a and 8a*

0.70 mg of peptidyl Nbz 7a and 0.81 mg of cysteiny1 peptide 8a were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bondbreaker® TCEP solution, tBuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12a (0.72 mg, 65%) as a white solid. Analytical HPLC for 12a: t_R = 19.0 min (5 to 50% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C₅₅H₁₀₀N₁₈O₁₆: 1269.5 Da (average isotopes), (m/z) [M+H]^+: 1269.8, [M+2H]^2+: 635.4; found: [M+H]^+: 1269.5, [M+2H]^2+: 635.4.

![UV traces](image)

**Figure S28.** Top left: UV trace of ligation reaction between peptides 7a and 8a; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12a; Bottom right: ESI-MS data of purified 12a.
One-pot ligation and desulfurization reactions between 7b and 8b

0.70 mg of peptidyl Nbz 7b and 0.60 mg of cysteiny1 peptide 8b were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, 'BuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12b (0.65 mg, 62%) as a white solid. Analytical HPLC for 12b: tᵣ = 17.0 min (10 to 50% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C₅₁H₈₉N₁₉O₁₂: 1160.4 Da (average isotopes), (m/z) [M+H]+: 1160.7, [M+2H]²⁺: 580.9; found: [M+H]+: 1160.4, [M+2H]²⁺: 581.1.

Figure S29. Top left: UV trace of ligation reaction between peptides 7b and 8b; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12b; Bottom right: ESI-MS data of purified 12b.
**One-pot ligation and desulfurization reactions between 7c and 8c**

0.95 mg of peptidyl Nbz 7c and 0.73 mg of cysteinyl peptide 8c were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, 'BuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12c (0.63 mg, 50%) as a white solid. Analytical HPLC for 12c: t_R = 18.4 min (10 to 50% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C_{52}H_{79}N_{15}O_{14}: 1138.3 Da (average isotopes), (m/z) [M+H]^+ : 1138.6; found: [M+H]^+ : 1138.6.

**Figure S30.** Top left: UV trace of ligation reaction between peptides 7c and 8c; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12c; Bottom right: ESI-MS data of purified 12c. (* Denotes the peaks of fragmentation during ionization in mass spectrometer)
One-pot ligation and desulfurization reactions between 7d and 8d

0.42 mg of peptidyl Nbz 7d and 1.37 mg of cysteinyl peptide 8d were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, tBuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12d (0.45 mg, 60%) as a white solid. Analytical HPLC for 12d: t_R = 13.6 min (10 to 50% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C_{72}H_{115}N_{17}O_{27}: 1650.8 Da (average isotopes), (m/z) [M+H]^+: 1650.8, [M+2H]^2+: 825.9; found: [M+H]^+: 1651.8, [M+2H]^2+: 826.3.

Figure S31. Top left: UV trace of ligation reaction between peptides 7d and 8d; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12d; Bottom right: ESI-MS data of purified 12d.
One-pot ligation and desulfurization reactions between 7a and 8e

0.60 mg of peptidyl Nbz 7a and 0.64 mg of cysteiny1 peptide 8e were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bondbreaker® TCEP solution, 'BuSH and ACVA following General Procedure 3.7. The reaction was stirred for another 5 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12e (0.47 mg, 50%) as a white solid. Analytical HPLC for 12e: t_R = 15.5 min (10 to 50% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for C₅₆H₁₀₀N₁₈O₁₈: 1313.5 Da (average isotopes), (m/z) [M+H]⁺: 1313.8, [M+2H]²⁺: 657.4; found: [M+H]⁺: 1313.8, [M+2H]²⁺: 657.5.

Figure S32. Top left: UV trace of ligation reaction between peptides 7a and 8e; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12e; Bottom right: ESI-MS data of purified 12e.
One-pot ligation and desulfurization reactions between 7a and 8f

0.68 mg of peptidyl Nbz 7a and 0.9 mg of cysteinyl peptide 8f were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, 'BuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12f (0.36 mg, 34%) as a white solid. Analytical HPLC for 12f: tR = 19.5 min (10 to 40% solvent B over 30min, Agilent C18 column); ESI-MS: calcd for C₅₇H₁₀₄N₁₈O₁₆: 1297.6 Da (average isotopes), (m/z) [M+H]+: 1297.8, [M+2H]²⁺: 649.4; found: [M+H]+: 1297.7, [M+2H]²⁺: 649.7.

Figure S33. Top left: UV trace of ligation reaction between peptides 7a and 8f; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12f; Bottom right: ESI-MS data of purified 12f.
One-pot ligation and desulfurization reactions between 7a and 8g

0.50 mg of peptidyl Nbz 7a and 0.48 mg of cysteinyl peptide 8g were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 2 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, tBuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (10-30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide was concentrated affording 12f (0.14 mg, 18%) as a white solid.

Figure S34. Left: UV trace of ligation reaction between peptides 7a and 8g; Right: UV trace of one-pot desulfurization following ligation.

One-pot ligation and desulfurization reactions between 7d and S9

0.83 mg of peptidyl Nbz 7d and 0.70 mg of cysteinyl peptide S9 were subjected to the ligation conditions following General Procedure 3.5 as described previously. The reaction was stirred for 8 h at room temperature, followed by the addition of Bond-breaker® TCEP solution, tBuSH and VA-044 following General Procedure 3.7. The
reaction was stirred for another 3 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. As shown in Figure S35, only trace amount of ligation and desulfurized products were observed, which failed to afford isolable amount of product after preparative HPLC purification.

**Figure S35.** Left: UV trace of ligation reaction between peptides 7d and S9; Right: UV trace of one-pot desulfurization following ligation.

**One-pot ligation and desulfurization reactions between 7h and 8h**

3.9 mg of peptidyl Nbz 7h (1.0 equiv) and 4.7 mg of cysteinyl peptide 8h (1.5 equiv) were subjected to the ligation buffer (6 M Gn·HCl, 200 mM Na₂HPO₄, 50 mM TCEP·HCl, pH 6.9) under an argon atmosphere, the concentration of the 7h is approximately 5 mM, and the resulting solution was stirred at room temperature for 8 hours, followed by the addition of Bond-breaker® TCEP solution, tBuSH and VA-044 following General Procedure 3.7. The reaction was stirred for another 8 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (15-30% solvent B over 30 min, Beim Brueckle C4 column). The fractions containing pure peptide was concentrated affording 12h (3.5 mg, 55%) as a white solid. Analytical HPLC for 12h: t_R = 24.7 min (15 to 35% solvent B over 30min, Beim Brueckle C4 column); ESI-MS: calcd for C_{260}H_{400}N_{78}O_{89}S: 6073.9 Da (average isotopes), (m/z) [M+4H]^{4+}: 1519.2, [M+5H]^{5+}: 1215.6, [M+6H]^{6+}: 1013.2, [M+7H]^{7+}:
868.6; found: [M+4H]^{4+}: 1519.3, [M+5H]^{5+}: 1215.7, [M+6H]^{6+}: 1013.2, [M+7H]^{7+}: 868.7.

**Figure S36.** Top left: UV trace of ligation reaction between peptides 7h and 8h; Bottom left: UV trace of one-pot desulfurization following ligation; Top right: UV trace of purified 12h; Bottom right: ESI-MS data of purified 12h.
VII. Synthesis of [Nle\textsuperscript{45,63}]\(\beta\)-LPH (17) and [Nle\textsuperscript{8,18}]hPTH (23)

**One-pot three segment ligation for the preparation of peptide 16**

Peptidyl Pen-Nbz 7h (2.6 mg, 1.0 equiv) and peptidyl thioester 13 (4.0 mg, 1.2 equiv) were subjected to the ligation buffer (6 M Gn·HCl, 100 mM Na\textsubscript{2}HPO\textsubscript{4}, 100 mM sodium ascorbate, 50 mM TCEP·HCl, pH 7.5) under an argon atmosphere, the concentration of the 7h is approximately 5 mM, and the resulting solution was stirred for 8 h at room temperature. To the reaction mixture was then added a solution of 15b (2.2 mg, 1.5 equiv) in buffer containing MPAA. The reaction was stirred for another 8 hours at room temperature, and quenched with 1.0 mL of CH\textsubscript{3}CN/H\textsubscript{2}O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (25-33% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide was concentrated affording 16 as a white solid. Analytical HPLC for 16:

\[ t_R = 25.6 \text{ min} \] (20 to 40% solvent B over 30 min, Proto-300 C4 column); ESI-MS: calcd for C\textsubscript{432}H\textsubscript{677}N\textsubscript{123}O\textsubscript{136}S\textsubscript{3}: 9866.1 Da (average isotopes), \((m/z)\) [M+5H]\textsuperscript{5+}: 1974.0, [M+6H]\textsuperscript{6+}: 1645.2, [M+7H]\textsuperscript{7+}: 1410.3, [M+8H]\textsuperscript{8+}: 1234.1, [M+9H]\textsuperscript{9+}: 1097.1, [M+10H]\textsuperscript{10+}: 987.5, [M+11H]\textsuperscript{11+}: 897.8, [M+12H]\textsuperscript{12+}: 823.1; found: [M+5H]\textsuperscript{5+}: 1974.0, [M+6H]\textsuperscript{6+}: 1645.2, [M+7H]\textsuperscript{7+}: 1410.4, [M+8H]\textsuperscript{8+}: 1234.1, [M+9H]\textsuperscript{9+}: 1096.9, [M+10H]\textsuperscript{10+}: 987.5, [M+11H]\textsuperscript{11+}: 897.9, [M+12H]\textsuperscript{12+}: 823.1
Desulfurization of 16

Purified peptide 16 was subjected to desulfurization conditions following General Procedure 3.6 as described previously. The reaction mixture was allowed to stirred at 37 °C for 8 hours, and quenched with 1.0 mL of CH$_3$CN/H$_2$O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (25-33% solvent B over 30 min, Proto 300-C4 column). The fractions containing pure peptide was concentrated affording 17 (2.2 mg, 31%, 3 steps) as a white solid. Analytical HPLC for 17: $t_R = 25.6$ min (20 to 40% solvent B over 30min, Proto-300 C4 column); ESI-MS: calc'd for C$_{432}$H$_{777}$N$_{123}$O$_{136}$: 9769.9 Da (average isotopes), (m/z) [M+5H]$^{5+}$: 1954.8, [M+6H]$^{6+}$: 1629.2, [M+7H]$^{7+}$: 1396.6, [M+8H]$^{8+}$: 1222.1, [M+9H]$^{9+}$: 1086.5, [M+10H]$^{10+}$: 977.9, [M+11H]$^{11+}$: 889.1, [M+12H]$^{12+}$: 815.1; found: [M+5H]$^{5+}$: 1954.8, [M+6H]$^{6+}$: 1629.3, [M+7H]$^{7+}$: 1396.7, [M+8H]$^{8+}$: 1222.0, [M+9H]$^{9+}$: 1086.2, [M+10H]$^{10+}$: 978.0, [M+11H]$^{11+}$: 889.1, [M+12H]$^{12+}$: 815.0.
Peptidyl Pen-Nbz 18 (6.8 mg, 1.0 equiv) and peptidyl thioester 19 (5.0 mg, 1.2 equiv) were subjected to the ligation buffer (6 M Gn·HCl, 100 mM Na₂HPO₄, 100 mM sodium ascorbate, 50 mM TCEP·HCl, pH 7.5) under an argon atmosphere, and the resulting solution was stirred overnight at room temperature. To the reaction mixture was then added a solution of 21 (5.9 mg, 1.4 equiv) in buffer containing MPAA. The reaction was stirred for another 8 hours at room temperature, and quenched with 1.0 mL of CH₃CN/H₂O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (27-38% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide was concentrated affording 22.
as a white solid with small amount of inseparable byproduct that could be removed in the next step. Analytical HPLC for 22: t_R = 23.7 min (27 to 38% solvent B over 30 min, Proto-300 C4 column); ESI-MS: calcd for C_{410}H_{678}N_{126}O_{126}S_{3}: 9484.9 Da (average isotopes), (m/z) [M+5H]^5+: 1897.6, [M+6H]^6+: 1581.5, [M+7H]^7+: 1355.7, [M+8H]^8+: 1186.4, [M+9H]^9+: 1054.7, [M+10H]^10+: 949.3, [M+11H]^11+: 863.1, [M+12H]^12+: 791.3, [M+13H]^13+: 730.5; found: [M+5H]^5+: 1898.0, [M+6H]^6+: 1581.8, [M+7H]^7+: 1365.1, [M+8H]^8+: 1186.8, [M+9H]^9+: 1054.9, [M+10H]^10+: 949.5, [M+11H]^11+: 863.3, [M+12H]^12+: 791.5, [M+13H]^13+: 730.7.

![Figure S39](image-url) Top left: UV trace of ligation reaction between peptides 18 and 19; Bottom left: UV trace of one-pot ligation between 20 and 21; Top right: UV trace of purified 22; Bottom right: ESI-MS data of purified 22.

**Desulfurization of 22**

Peptide 22 was subjected to desulfurization conditions following General Procedure 3.6 as described previously. The reaction mixture was allowed to stirred at 37 °C for 8 hours, and quenched with 1.0 mL of CH\textsubscript{3}CN/H\textsubscript{2}O/AcOH (5/90/5) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (27-38% solvent B over 30 min, Proto 300-C4 column). The fractions containing pure peptide was concentrated affording 23 (5.0 mg, 35%, 3 steps) as a white solid. Analytical HPLC for 23: t_R = 22.4 min (20 to 40% solvent B over 30 min, Proto 300-
C4 column); ESI-MS: calcd for C_{410}H_{678}N_{126}O_{126}: 9388.7 Da (average isotopes), (m/z) [M+5H]^5+: 1878.4, [M+6H]^6+: 1565.5, [M+7H]^7+: 1342.0, [M+8H]^8+: 1174.4, [M+9H]^9+: 1044.9, [M+10H]^10+: 939.7, [M+11H]^11+: 854.4, [M+12H]^12+: 783.3, [M+13H]^13+: 723.1; found: [M+5H]^5+: 1879.1, [M+6H]^6+: 1566.0, [M+7H]^7+: 1342.3, [M+8H]^8+: 1174.7, [M+9H]^9+: 1044.4, [M+10H]^10+: 940.0, [M+11H]^11+: 854.6, [M+12H]^12+: 783.5, [M+13H]^13+: 723.3.

**Figure S40.** Top: UV trace of desulfurization of 22; Bottom left: UV trace of purified 23; Bottom right: ESI-MS data of purified 23.