

Electronic Supplementary Information

A Catalytic One-step Synthesis of Peptide Thioacids: The Synthesis of Leuprorelin via Iterative Peptide-fragment Coupling Reactions

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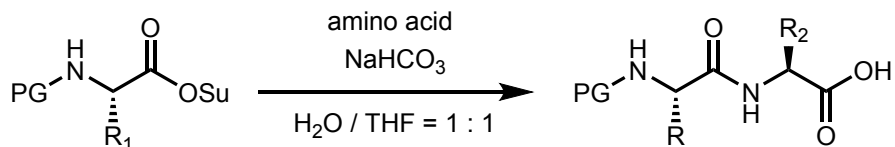
Materials & Methods

1. General Method

¹H NMR spectra were recorded on a JEOL ECS400 (400 MHz for ¹H NMR and 100 MHz for ¹³C NMR) spectrometer. Chemical shifts were reported downfield from TMS (δ = 0 ppm) for ¹H NMR. For ¹³C NMR, chemical shifts were reported in the scale relative to the solvent used as an internal reference. Infrared (IR) spectra were recorded on a JASCO FT/IR 410 Fourier transform infrared spectrophotometer. Electrospray ionization (ESI) mass spectra were measured on a Waters ZQ4000 spectrometer and Shimadzu LCMS-2020 spectrometer (for LRMS), and a JEOL JMS-T100LC AccuTOF spectrometer (for HRMS). Column chromatographies were performed with silica gel 60 (spherical) 40-50 μ m (Kanto Chemicals), or by using Yamazen EPCLC-W-Prep 2XY A type (for peptide thioacids). Analytical HPLC charts were obtained by using a JASCO HPLC system equipped with a UV-2075 spectrometer, PU-2080 pumps, a DG-2080-54 degasser, and an MX-2080-32 mixer. Preparative HPLC was conducted by using a Shimadzu HPLC system equipped with a SPD-20A spectrometer, and LC-6AD pumps. LC/MS (ESI) analyses were conducted using Shimadzu LCMS system equipped with a LCMS-2020, and a LC-2030C. All non-commercially available compounds were prepared and characterized as described in Section 2. Other reagents were purchased from Sigma Aldrich, Tokyo Chemical Industry Co., Ltd. (TCI), Kanto Chemical Co., Inc., FUJIFILM Wako Pure Chemical Co., Peptides Institute, Inc., Watanabe Chemical Industries, Ltd., and Nacalai Tesque, Inc. and used without further purification. Water was purified using a Merck Millipore Milli-Q water purification system.

2. Synthesis and Analytical Data for Starting Materials (Peptides)

2-1. General Protocol for Dipeptide Synthesis (**1a–1t**)



To a solution of amino acid and NaHCO₃ in H₂O with stirring was added dropwise a solution of *N*-hydroxysuccinimide (HOSu) ester in THF. The reaction mixture was stirred at room temperature for 3 h and concentrated in vacuo. The mixture was diluted with ethyl acetate and 1 N HCl aq, and the compounds were extracted with ethyl acetate three times. The combined organic layer was washed with 1 N HCl aq and brine, and dried over Na₂SO₄. After filtration, the organic layer was concentrated in vacuo to afford the crude dipeptide, which was purified with flash column chromatography (SiO₂, eluent = hexane/EtOAc/acetic acid or CH₂Cl₂/MeOH/acetic acid).

2-2. General Solid-Phase Protocol for Longer Peptide Synthesis (**1u, 1v, 1y, epi-1y**)

Solid-phase peptide synthesis (SPPS) were performed manually in 0.3 mmol scale using chlorotrityl chloride resin. Fmoc-protected amino acids were sequentially coupled using a 2.5-fold excess (0.75 mmol) using a DIC-HOBt method (60 min) after removal of each Fmoc group with 20% piperidine-DMF (10 min) to obtain a peptide-resin.

For side-chain unprotected peptide: Treating the obtained peptide-resin with TFA-triisopropylsilane (TIS)-water (95:2.5:2.5) for 180 min at room temperature, concentrated in vacuo, and precipitation with diethyl ether produced the crude peptide.

For side-chain protected peptide: The obtained peptide-resin was treated with 0.1 % TFA in CH₂Cl₂ 10 times, and the filtrate was added into 10 % pyridine in MeOH. Resulting solution was concentrated in vacuo and precipitated with water to produce the crude peptide.

The crude peptide was purified using a preparative HPLC with 0.1% aqueous TFA-acetonitrile system. Freeze-drying of the collected fractions produced the desired peptide as a white solid.

2-3. Analytical HPLC

Peptide compositions were evaluated by analytical reverse phase HPLC using a gradient of acetonitrile versus 0.1% trifluoroacetic acid (TFA) in water. Analytical HPLC was carried out as follows unless otherwise noted: YMC-Triart-C₁₈ (4.6 mm I.D. × 150 mm) column using a linear gradient of 0–100% acetonitrile in 0.1% aqueous TFA over 40 min at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 230 nm (for non-Fmoc-peptides) or 301 nm (for Fmoc-peptides).

2-4. Preparative HPLC

Larger amount of peptides were purified by preparative reverse phase HPLC using a gradient of acetonitrile versus 0.1% TFA in water. Preparative HPLC was carried out as follows: YMC-Triart C₁₈ (20 mm I.D. × 250 mm) column using a linear gradient of 0-100% acetonitrile in 0.1% aqueous TFA over 100 min at 40 °C with a flow rate of 3.0 mL min⁻¹. The eluent was monitored by absorbance at 230 nm (for non-Fmoc-peptides) or 301 nm (for Fmoc-peptides).

Cbz-Phe-Phe-OH (1a). MS (ESI): m/z 445.05 (calcd [M-H]⁻ = 445.50).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 28.6 min.

Cbz-Phe-Val-OH (1b). MS (ESI): m/z 399.10 (calcd [M+H]⁺ = 399.46).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 26.6 min.

Boc-Phe-Phe-OH (1c). MS (ESI): m/z 411.05 (calcd [M-H]⁻ = 411.48).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 28.3 min.

Ac-Phe-Phe-OH (1d). MS (ESI): m/z 353.10 (calcd [M-H]⁻ = 353.41).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 22.8 min.

Fmoc-Phe-Phe-OH (1e). MS (ESI): m/z 533.10 (calcd [M-H]⁻ = 533.61).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 32.3 min.

Fmoc-Ala-Gly-OH (1f). MS (ESI): m/z 391.00 (calcd [M+Na]⁺ = 391.38).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 24.5 min.

Fmoc-Ala-Val-OH (1g). MS (ESI): m/z 433.05 (calcd [M+Na]⁺ = 433.46).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 27.7 min.

Fmoc-Ala-Ile-OH (1h). MS (ESI): m/z 447.05 (calcd [M+Na]⁺ = 447.49).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 28.9 min.

Fmoc-Ala-Pro-OH (1i). MS (ESI): m/z 431.00 (calcd [M+Na]⁺ = 431.44).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 26.3 min.

Fmoc-Ala-Cys(Trt)-OH (1j). MS (ESI): m/z 679.10 (calcd [M+H]⁺ = 679.79).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 35.5 min.

Fmoc-Ala-Arg(Pbf)-OH (1k). MS (ESI): m/z 720.10 (calcd [M+H]⁺ = 719.85).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 30.7 min.

Fmoc-Ala-Lys(Boc)-OH (1l). MS (ESI): m/z 562.05 (calcd [M+Na]⁺ = 562.62).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 29.4 min.

Fmoc-Ala-Asp(^tBu)-OH (1m). MS (ESI): m/z 505.00 (calcd [M+Na]⁺ = 505.52).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 29.2 min.

Fmoc-Ala-His(Trt)-OH (1n). MS (ESI): m/z 691.10 (calcd [M+H]⁺ = 691.81).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 30.4 min.

Fmoc-Ala-Tyr(^tBu)-OH (1o). MS (ESI): m/z 553.00 (calcd [M+Na]⁺ = 553.61).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 31.7 min.

Fmoc-Ala-Trp-OH (1p). MS (ESI): m/z 520.00 (calcd $[M+Na]^+ = 520.54$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 28.9 min.

Fmoc-Ala-Ser(Trt)-OH (1q). MS (ESI): m/z 663.10 (calcd $[M+H]^+ = 663.73$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 36.0 min.

Fmoc-Ala-Asn(Trt)-OH (1r). MS (ESI): m/z 668.10 (calcd $[M+H]^+ = 668.76$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 33.8 min.

Fmoc-Ala-Leu-OH (1s). MS (ESI): m/z 447.10 (calcd $[M+Na]^+ = 447.49$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 28.9 min.

Fmoc-Ala-Aib-OH (1t). MS (ESI): m/z 419.00 (calcd $[M+Na]^+ = 419.43$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 26.3 min.

Fmoc-Gly-Ala-Gly-Ala-OH (1u). This compound was obtained by SPPS in 53% yield. MS (ESI): m/z 519.05 (calcd $[M+H]^+ = 519.51$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 23.0 min.

Fmoc-Gly-Ala-Gly-Ala-Gly-Ala-OH (1v). This compound was obtained by SPPS in 24% yield. MS (ESI): m/z 647.05 (calcd $[M+H]^+ = 647.46$).

Purity: >95% (HPLC analysis at 301 nm). Retention time: 22.1 min.

pGlu-His(Trt)-Trp(Boc)-OH (1w). This compound was obtained by SPPS in 34% yield. MS (ESI): m/z 795.5 (calcd $[M+H]^+ = 795.51$).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H_2O 2 min. then 0-100% MeCN / H_2O over 80 min.). Retention time 47.8 min.

DNS-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH (1x). This compound was obtained by capping at *N*-terminal amino acids with 2,4-dinitrobenzenesulfonyl chloride (DNS-Cl) according to Suchek's SPPS procedure² in 6.9% yield. MS (ESI): m/z 724.6 (calcd $[M+H]^+ = 724.80$).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H_2O 2 min. then 0-100% MeCN / H_2O over 40 min.). Retention time: 34.2 min.

H-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH (1x'). This compound was obtained by SPPS in 71% yield. MS (ESI): m/z 494.6 (calcd $[M+H]^+ = 494.65$).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H_2O 2 min. then 0-100% MeCN / H_2O over 80 min.). Retention time 22.8 min.

pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH (1y). Authentic compound was obtained by SPPS in 47% yield. MS (ESI): m/z 1269.1 (calcd $[M-H]^- = 1269.51$).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H_2O 2 min. then 0-100% MeCN / H_2O over 80 min.). Retention time 59.7 min.

pGlu-His(Trt)-D-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH (epi-1y). Authentic compound was obtained by SPPS in 40% yield. MS (ESI): m/z 1265.9 (calcd $[M-H]^- = 1269.51$).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H₂O 2 min. then 0-100% MeCN / H₂O over 80 min.). Retention time 62.8 min.

DNS-Leu-Arg(Pbf)-Pro-NHEt (3). Fmoc-Arg(Pbf)-Pro-NHEt (308 mg, 0.4 mmol) was added into ice-cold 50% (v/v) Et₂NH in DMF (2 mL). After stirring at the same temperature for 10 min, AcOEt (ca. 40 mL) and brine (ca. 40 mL) were added. The separated organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated. The residue was triturated with hexane to give crude H-Arg(Pbf)-Pro-NHEt (90 mg) as white powder, which was used in the next step without further purification. To a stirred ice-cold solution of H-Arg(Pbf)-Pro-NHEt (90 mg of crude) and 'Pr₂NEt (524 μL, 3 mmol), DNS-Leu-Cl (1 mmol) in CH₂Cl₂ (5 mL), prepared from DNS-Leu-OH (361 mg, 1 mmol), SOCl₂ (145 μL, 2 mmol) and pyridine (81 μL, 1 mmol) according to Schwarzer's procedure³, was added dropwise over 10 min. After stirring at the same temperature for 3 h, AcOH (570 μL, 10 mmol) was added, then all volatiles were removed under reduced pressure. The residue was purified with column chromatography (SiO₂, AcOEt / MeOH = 100 / 0 → 98 / 2 → 95 / 5) to give **3** as pale yellow powder in 19% yield (67 mg).

Purity: >95% (HPLC analysis at 230 nm). HPLC (H₂O 2 min. then 0-100% MeCN / H₂O over 80 min.) Retention time: 55.5 min. MS (ESI): *m/z* 894.5 (calcd [M+H]⁺ = 895.05).

Fmoc-Arg(Pbf)-Pro-NHEt was synthesized from commercially available Fmoc-Arg(Pbf)-OH and H-Pro-NHEt (prepared from deprotection of Boc-Pro-NHEt), by using WSCI-HCl and Oxyma in solution phase. MS (ESI): *m/z* 773.8 (calcd [M+Na]⁺ = 772.96).

DNS-Leu-OH was synthesized according to Schwarzer's procedure³. MS (ESI): *m/z* 384.3 (calcd [M+Na]⁺ = 384.31).

pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu--Leu-Arg(Pbf)-Pro-NHEt (Protected Leuprorelin). MS (ESI): *m/z* 1912 (calcd [M+Na]⁺ = 1917.40).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 67.5 min.

pGlu-His-Trp-Ser-Tyr-Leu-D-Leu-Arg-Pro-NHEt (Leuprorelin). This compound was purchased from TCI Co., Ltd. Retention time: 30.3 min.

pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-Leu--Leu-Arg(Pbf)-Pro-NHEt (Protected *epi*-Leuprorelin). MS (ESI): *m/z* 1917 (calcd [M+H]⁺ = 1917.40).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 65.2 min.

pGlu-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-NHEt (*epi*-Leuprorelin). MS (ESI): *m/z* 1210 (calcd [M+Na]⁺ = 1210.42).

Purity: >95% (HPLC analysis at 230 nm). Retention time: 30.1 min.

3. Synthesis and Characterization of Peptide Thioacids

3-1. Preparation of Calibration Curves for Peptide Thioacids

An peptide thioacid (5 μmol) was dissolved in DMF (500 μL) to make a 10 mM of thioacid solution. This solution was diluted to 8 mM, 5 mM, 2 mM and 1 mM, then these solutions were analyzed with analytical HPLC by absorbance at 230 nm (for non-Fmoc-peptides) or 301 nm (for Fmoc-peptides). For each solution, the peak areas corresponding to the peptide thioacid (monomer) and peptide diacyldisulfide (dimer) were measured and added together, and the calibration curve was prepared from these values.

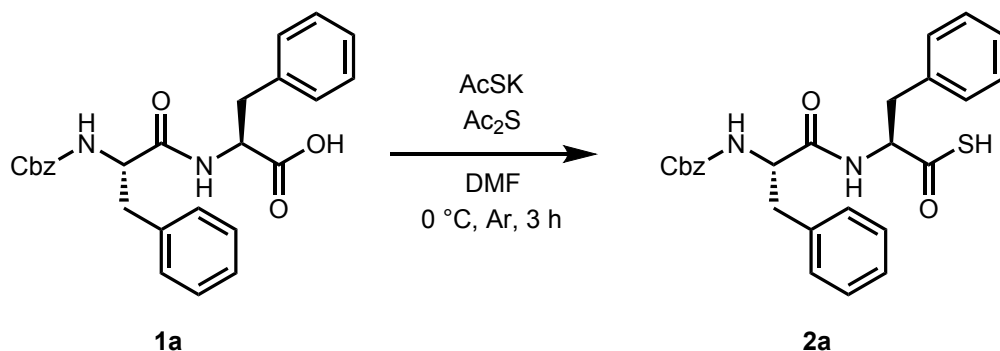
3-2. Protocol for Calculation of HPLC Yield for Peptide Thioacids

After completion of peptide thioacid synthesis, TFA (the equal molar amount to the used potassium thioacetate) was added to quench the reaction. DMF was added to adjust the concentration of solution to 10 mM, then the solution was analyzed by analytical HPLC without purification. The peak areas corresponding to the peptide thioacid (monomer) and the peptide diacyldisulfide (dimer) were added together, and the yield was determined from this value using the calibration curve (see Section 3-2). For Fmoc-peptide thioacids, the calibration curve of Fmoc-Phe-Phe-SH was used. For non-Fmoc-peptide thioacids, the calibration curve of each authentic thioacid was used.

3-3. Analytical HPLC

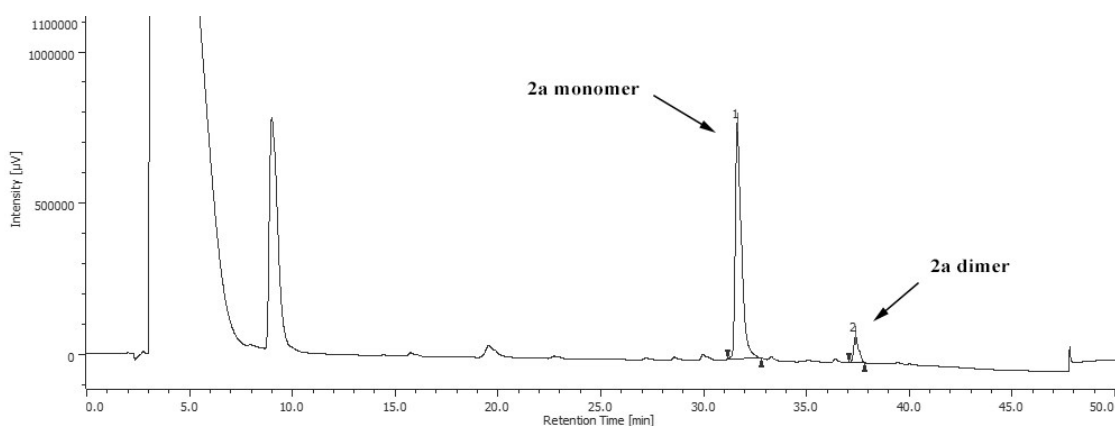
Executed reactions were evaluated by analytical reverse phase HPLC using a gradient of acetonitrile versus 0.1% trifluoroacetic acid (TFA) in water. Analytical HPLC was carried out as follows unless otherwise noted: YMC-Triart-C₁₈ (4.6 mm I.D. \times 150 mm) column using a linear gradient of 0–100% acetonitrile in 0.1% aqueous TFA over 40 min at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 230 nm (for non-Fmoc-peptides) or 301 nm (for Fmoc-peptides).

Cbz-Phe-Phe-SH (2a)

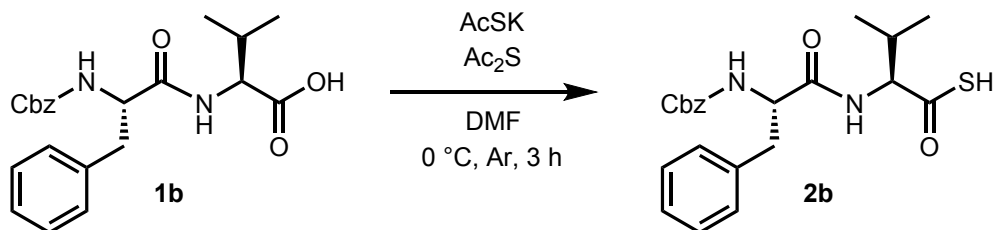


1a (13.4 mg, 30 μ mol), AcSK (10.3 mg, 90 μ mol) and DMF (200 μ L) were mixed under argon atmosphere in a test tube. To the solution a DMF solution of Ac₂S (60 mM, 100 μ L, 6 μ mol) was added, and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-2, the yield was determined as 97%.

2a: MS (ESI): m/z 460.75 (calcd $[M-H]^- = 461.56$). Retention time: 31.6 min.

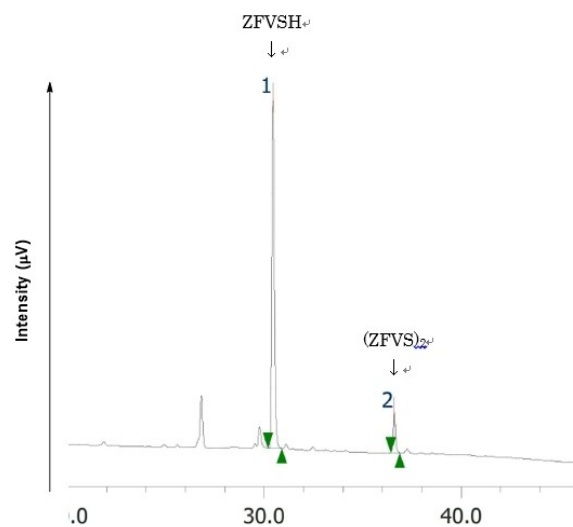


Cbz-Phe-Val-SH (2b)

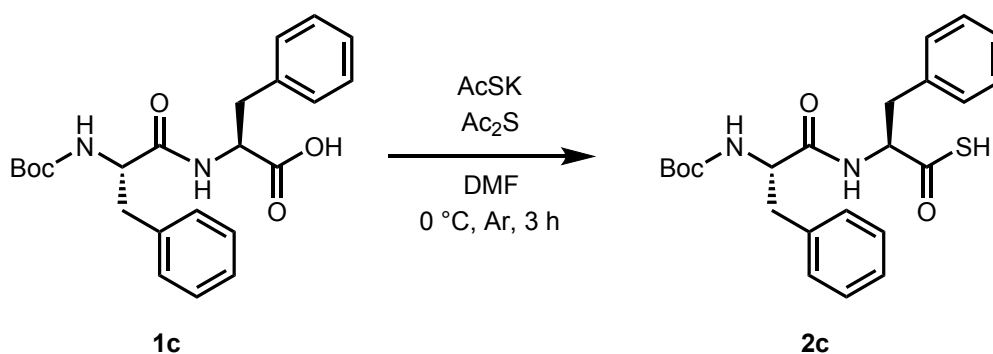


1b (7.96 mg, 20 μ mol), AcSK (22.8 mg, 200 μ mol) and DMF (198 μ L) were mixed under argon atmosphere. To the solution was added a DMF solution of Ac₂S (2 M, 2 μ L, 4.0 μ mol), and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-2, the yield was determined as 74%.

2b: MS (ESI): m/z 437 (calcd $[M+Na]^+ = 437.15$). Retention time: 30.5 min.

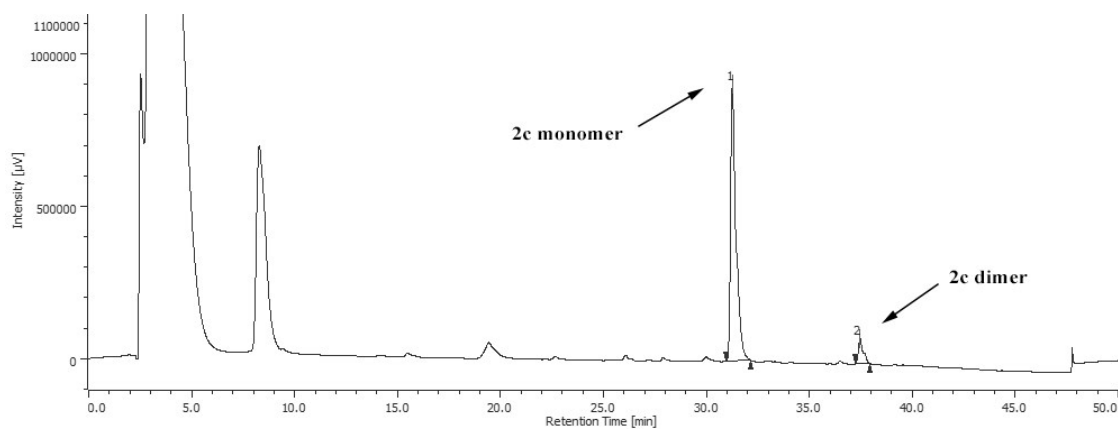


Boc-Phe-Phe-SH (2c)

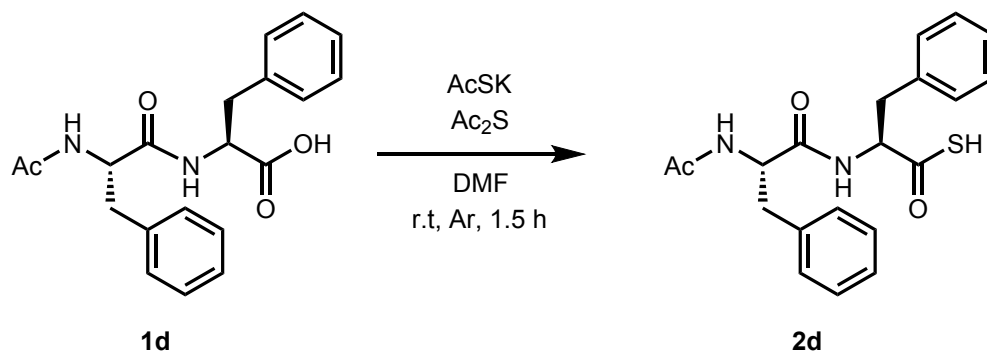


1c (12.4 mg, 30 μmol), AcSK (10.3 mg, 90 μmol) and DMF (200 μL) were mixed under argon atmosphere in a test tube. To the solution was added a DMF solution of Ac₂S (60 mM, 100 μL , 6 μmol), and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-2, the yield was determined as 100%.

2c: MS (ESI): m/z 427.05 (calcd $[\text{M-H}]^- = 427.55$). Retention time: 31.2 min.

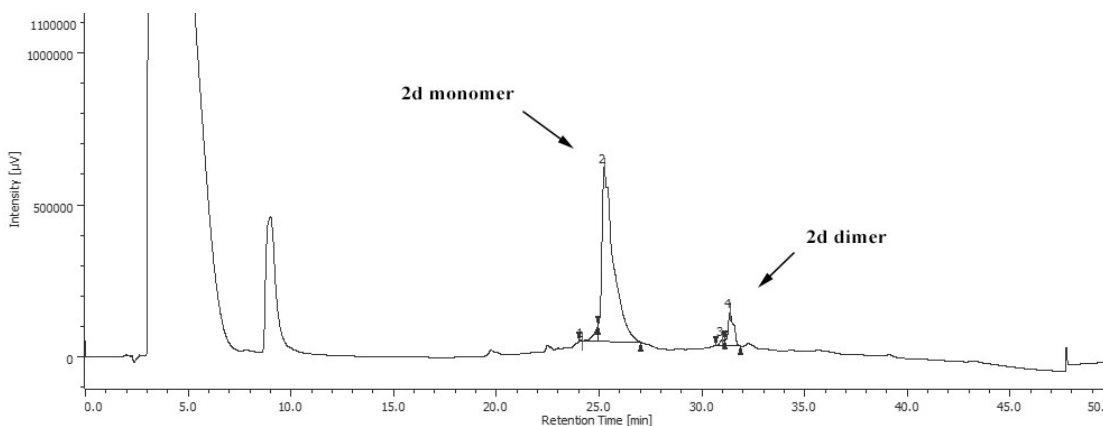


Ac-Phe-Phe-SH (2d)

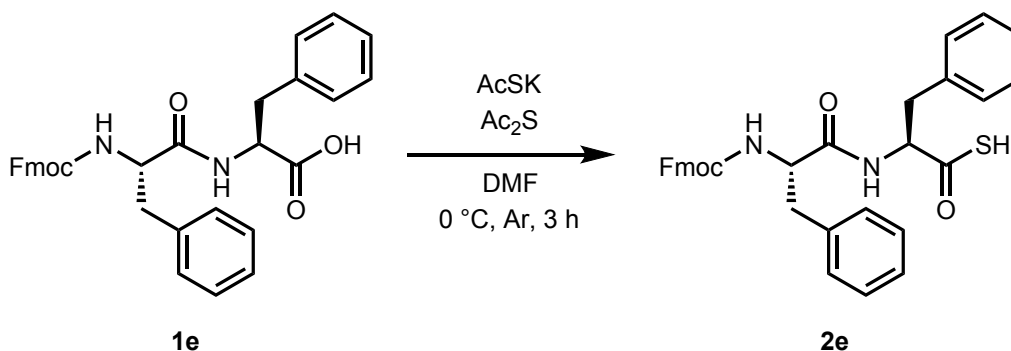


1d (7.1 mg, 30 μ mol), AcSK (6.9 mg, 90 μ mol) and DMF (500 μ L) were mixed under argon atmosphere in a test tube. To the solution was added a DMF solution of Ac₂S (60 mM, 100 μ L, 6 μ mol) and the mixture was stirred for 1.5 h at room temperature. According to the protocol described in Section 3-2, the yield was determined as 100%.

2d: MS (ESI): m/z 369.00 (calcd [M-H]⁻ = 369.47). Retention time: 25.2 min.



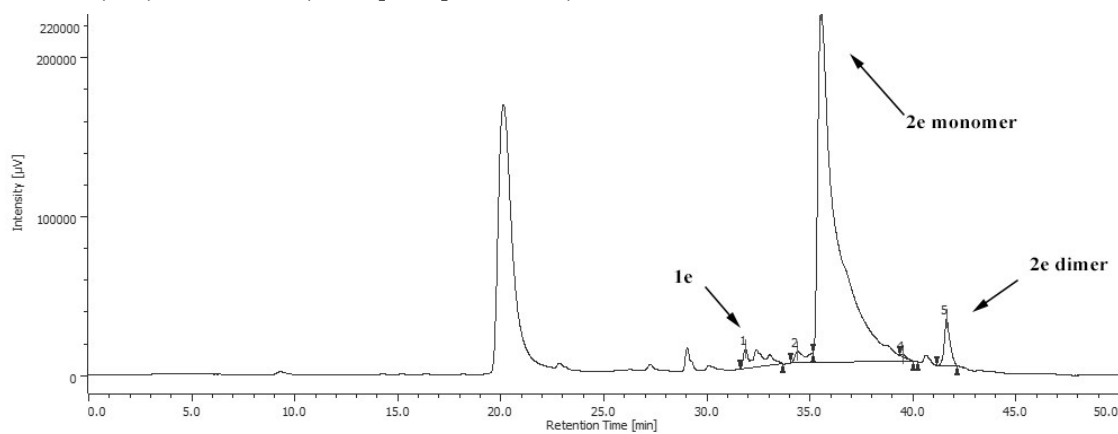
Fmoc-Phe-Phe-SH (2e)



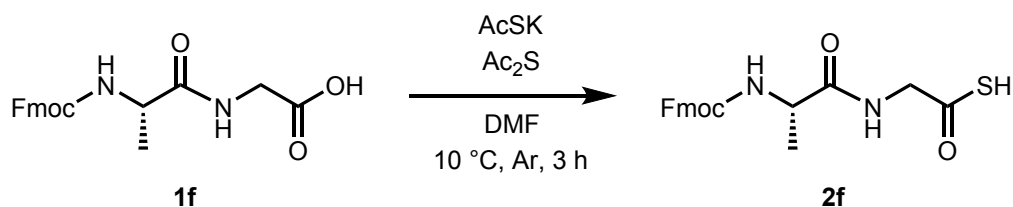
1e (16.0 mg, 30 μ mol), AcSK (10.3 mg, 90 μ mol) and DMF (200 μ L) were mixed under argon atmosphere in a test tube. To the solution was added a DMF solution of Ac₂S (60 mM, 100 μ L, 6 μ mol), and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-

2, the yield was determined as 87%.

2e: MS (ESI): m/z 549.15 (calcd $[M-H]^- = 549.67$). Retention time: 35.5 min.

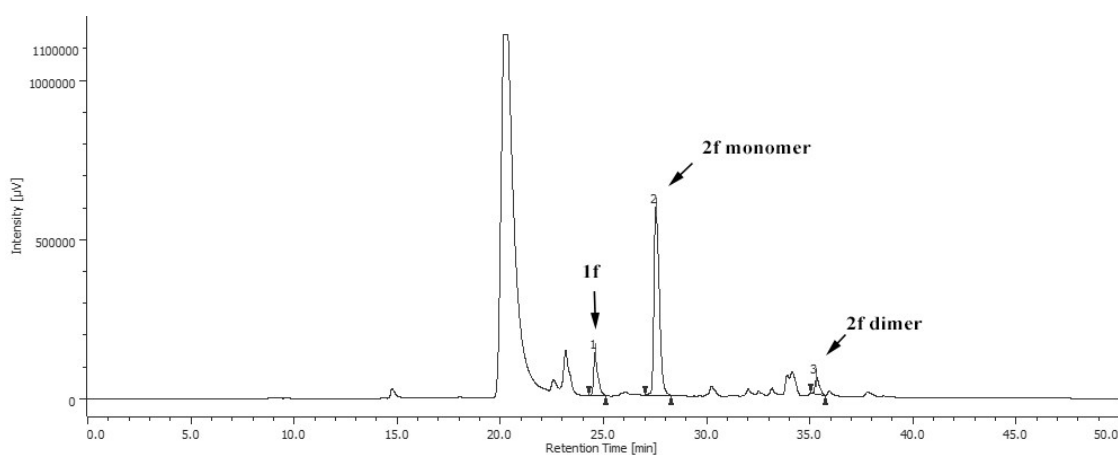


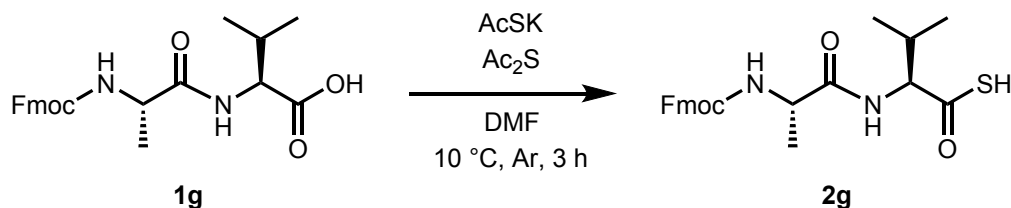
Fmoc-Ala-Gly-SH (2f)



1f (3.68 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac_2S (100 mM, 50 μL , 5 μmol), and the mixture was stirred for 3 h at 10 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 69%.

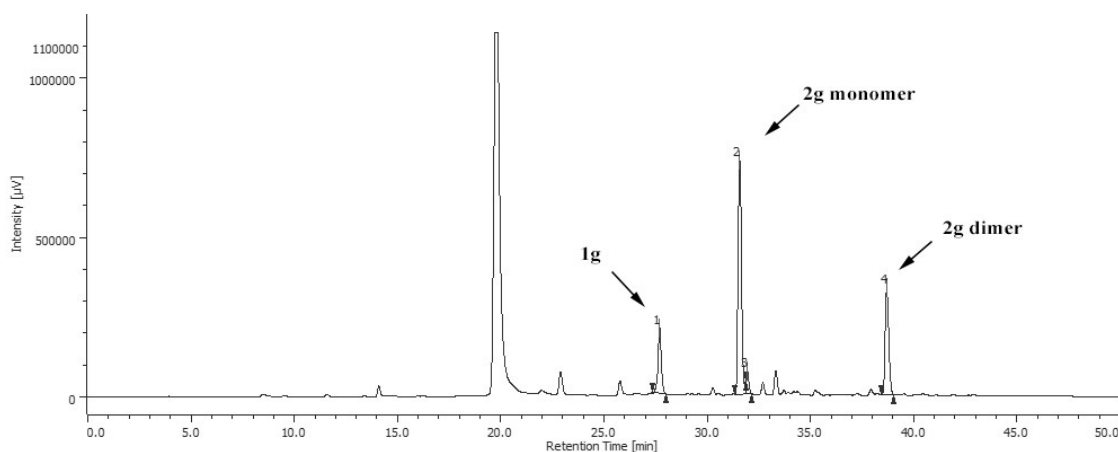
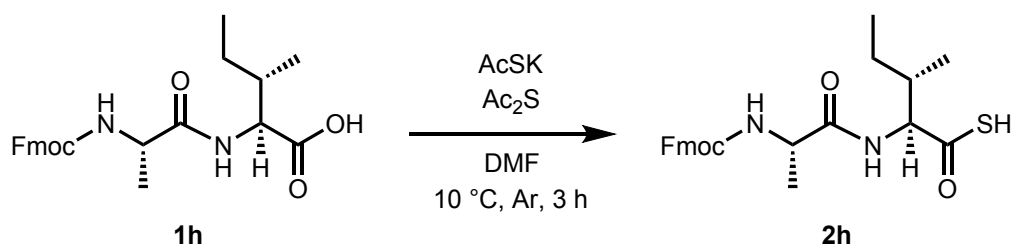
2f: MS (ESI): m/z 406.95 (calcd $[M+\text{Na}]^+ = 407.44$). Retention time: 27.5 min.



Fmoc-Ala-Val-SH (2g)

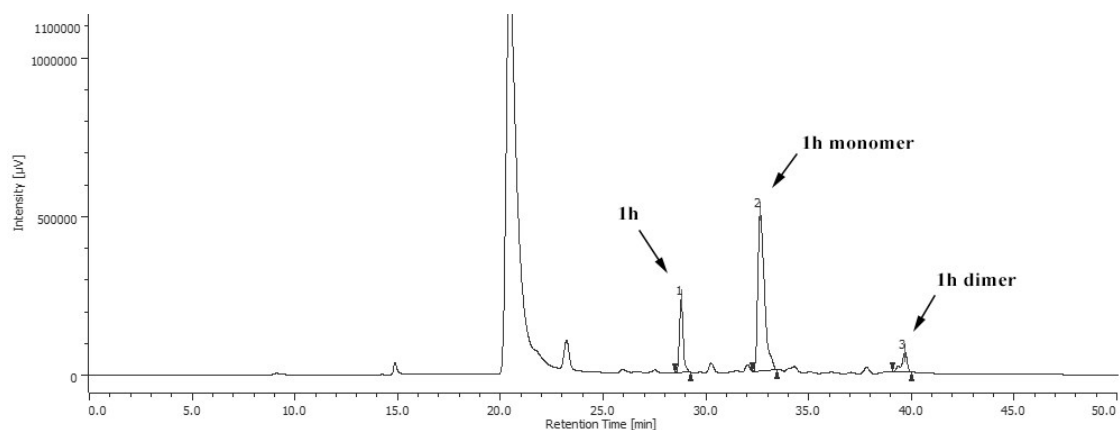
1g (4.10 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL, 10 μmol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 3-2, the yield was determined as 76%.

2g: MS (ESI): *m/z* 425.05 (calcd [M-H]⁻ = 425.05). Retention time: 31.4 min.

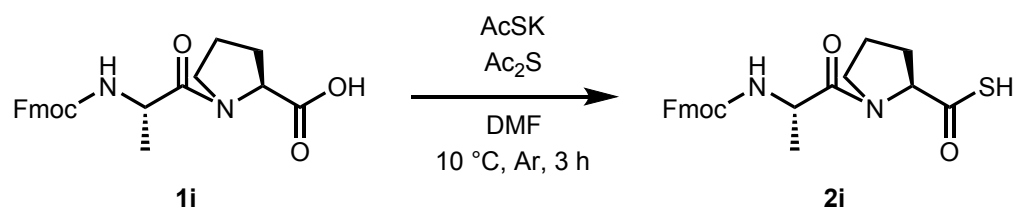
**Fmoc-Ala-Ile-SH (2h)**

1h (4.25 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL, 10 μmol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 3-2, the yield was determined as 76%.

2h: MS (ESI): *m/z* 463.00 (calcd [M+Na]⁺ = 463.55). Retention time: 32.6 min.

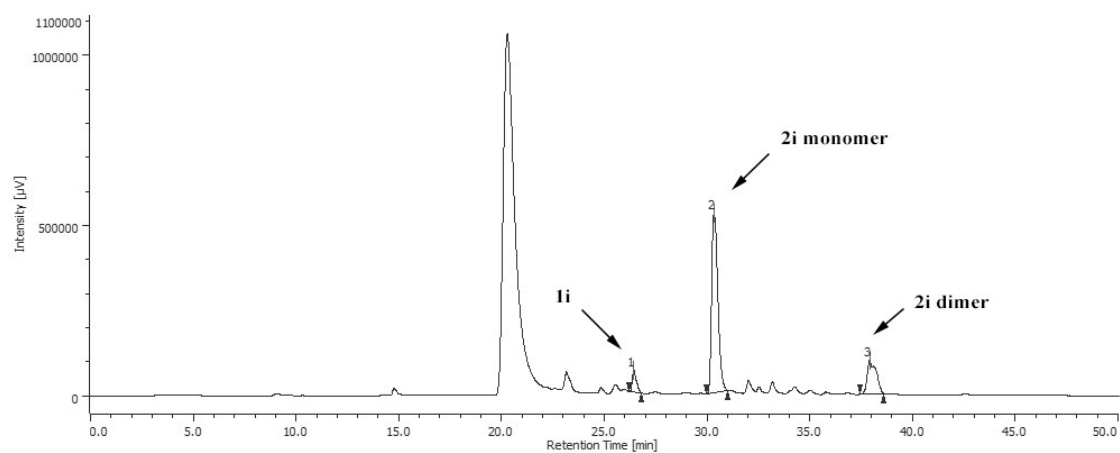


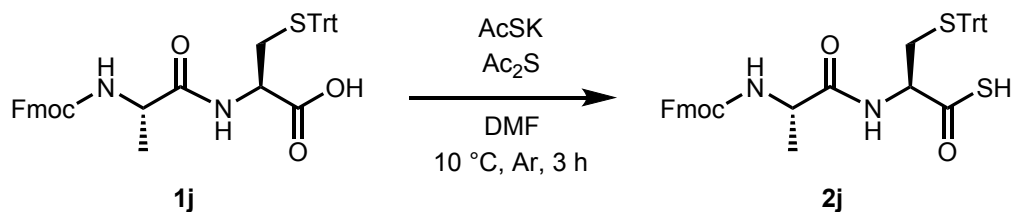
Fmoc-Ala-Pro-SH (2i)



1i (4.08 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL, 10 μmol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 3-2, the yield was determined as 85%.

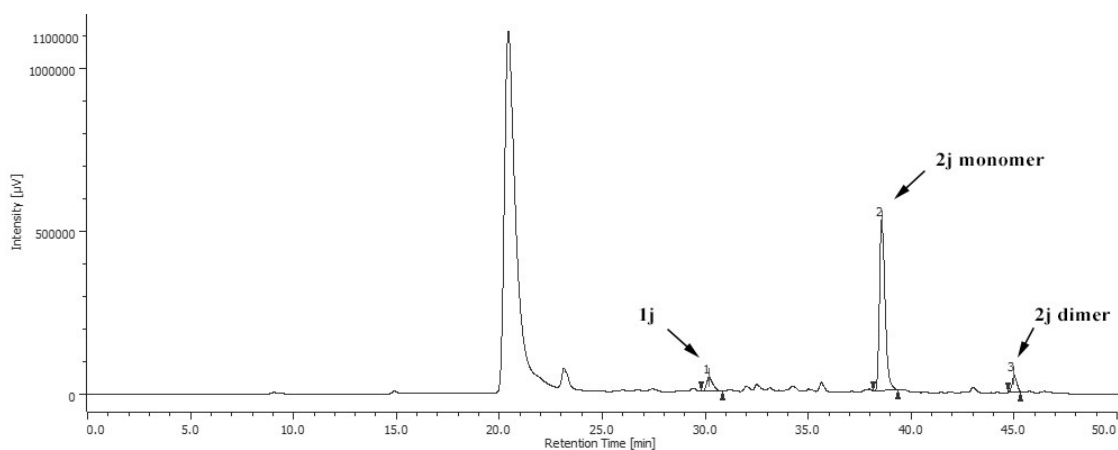
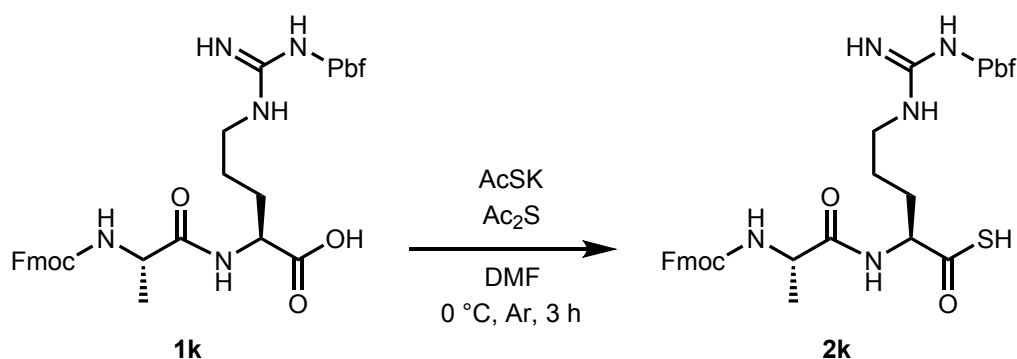
2i: MS (ESI): m/z 425.52 (calcd $[M+H]^+ = 424.95$). Retention time: 30.3 min.



Fmoc-Ala-Cys(Trt)-SH (2j)

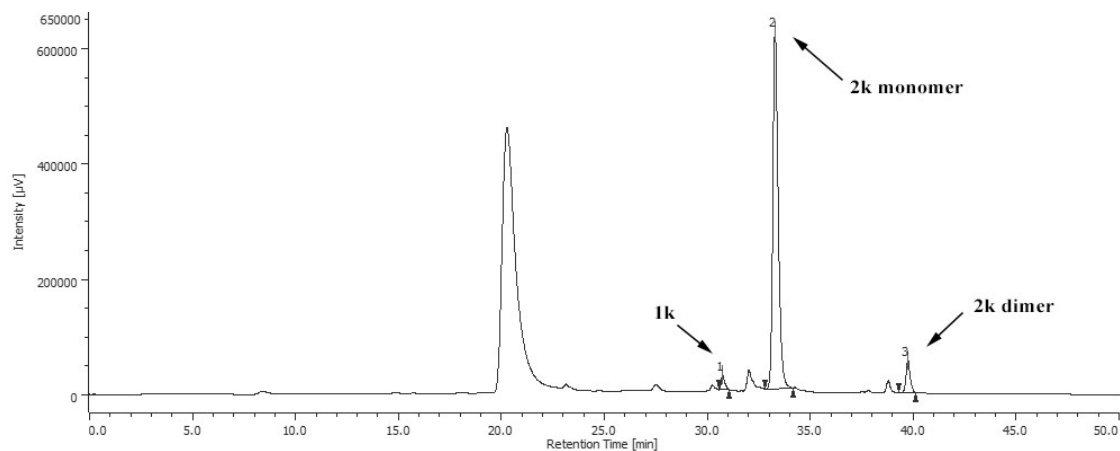
1j (6.57 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL , 10 μmol), and the mixture was stirred for 3 h at 10 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 66%.

2j: MS (ESI): m/z 659.85 (calcd $[\text{M}+\text{Na}]^+ = 694.95$). Retention time: 39.1 min.

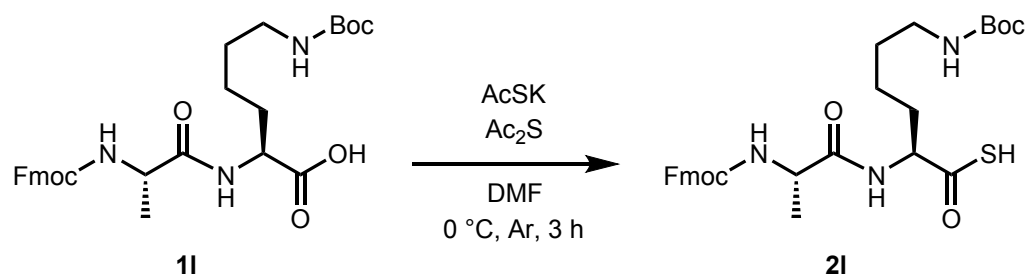
**Fmoc-Ala-Arg(Pbf)-SH (2k)**

1k (7.20 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL , 5 μmol), and the mixture was stirred for 3 h at 0 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 73%.

2k: MS (ESI): m/z 736.15 (calcd $[\text{M}+\text{H}]^+ = 736.92$). Retention time: 33.3 min.

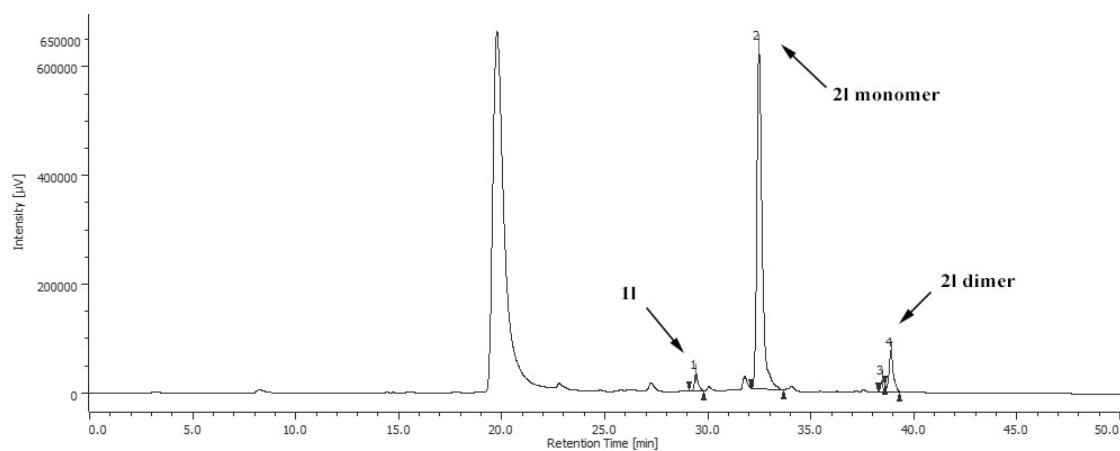


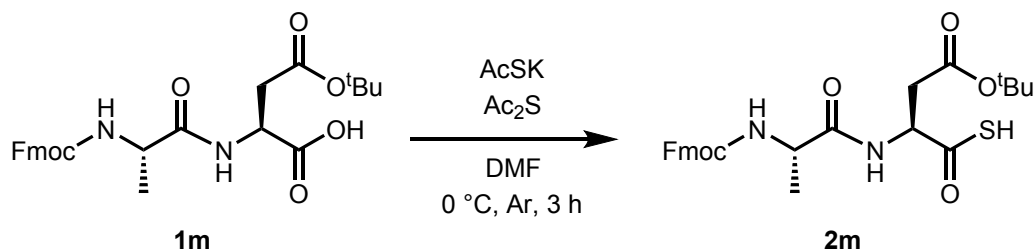
Fmoc-Ala-Lys(Boc)-SH (**2l**)



1l (5.40 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac_2S (100 mM, 50 μL , 5 μmol), and the mixture was stirred for 3 h at 0 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 75%.

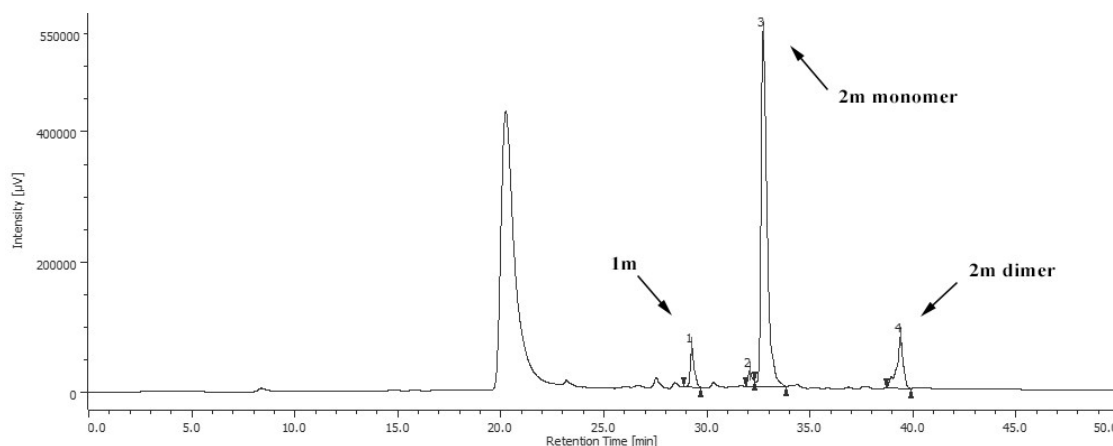
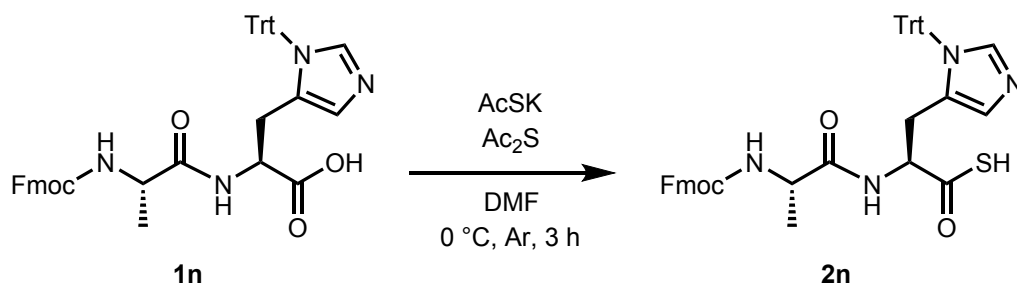
2l: MS (ESI): m/z 556.25 (calcd $[\text{M}+\text{H}]^+ = 556.69$). Retention time: 32.4 min.



Fmoc-Ala-Asp(^tBu)-SH (2m)

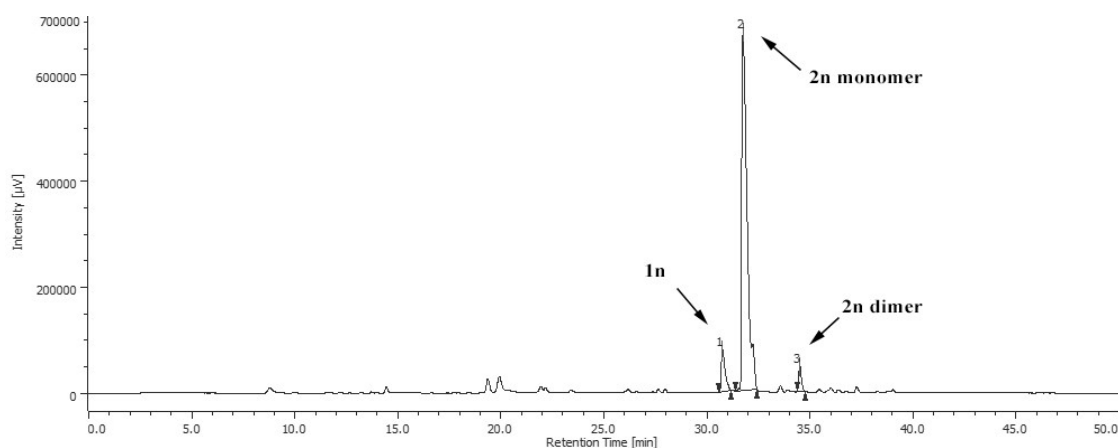
1m (4.83 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (100 mM, 50 μ L, 5 μ mol), and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-2, the yield was determined as 77%.

2m: MS (ESI): m/z 499.59 (calcd $[M+H]^+ = 499.10$). Retention time: 32.7 min.

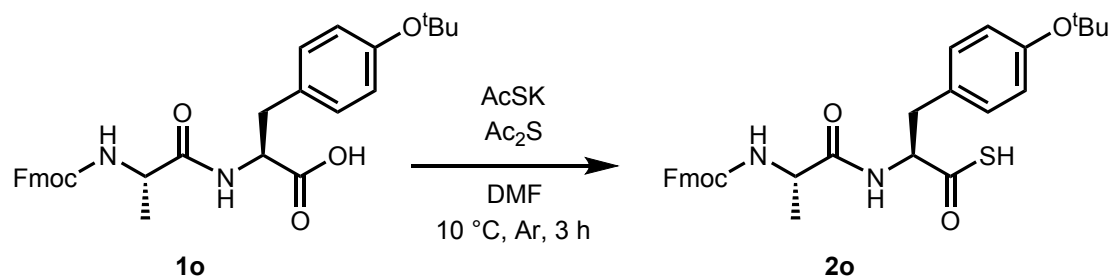
**Fmoc-Ala-His(Trt)-SH (2n)**

1n (6.91 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (40 mM, 50 μ L, 2 μ mol), and the mixture was stirred for 3 h at 0 °C. According to the protocol described in Section 3-2, the yield was determined as 77%.

2n: MS (ESI): m/z 707.35 (calcd $[M+H]^+ = 707.86$). Retention time: 31.7 min.

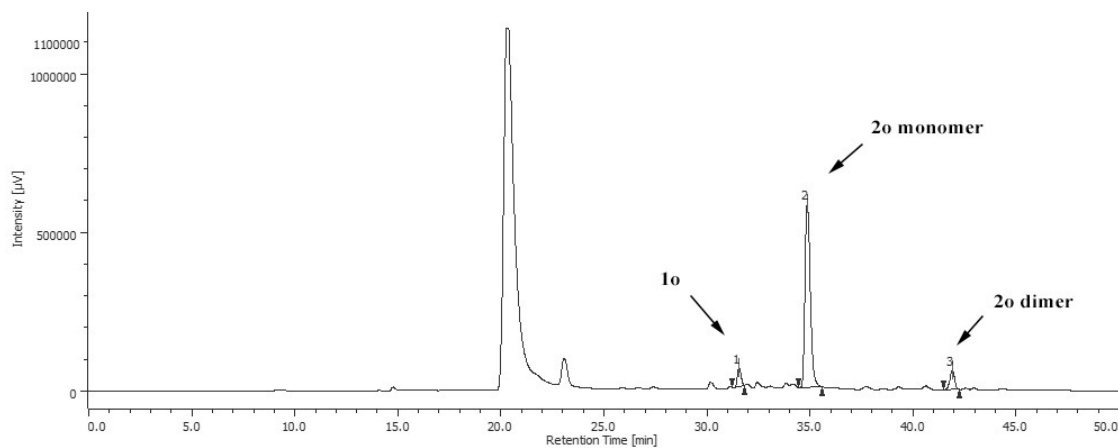


Fmoc-Ala-Tyr(^tBu)-SH (**2o**)

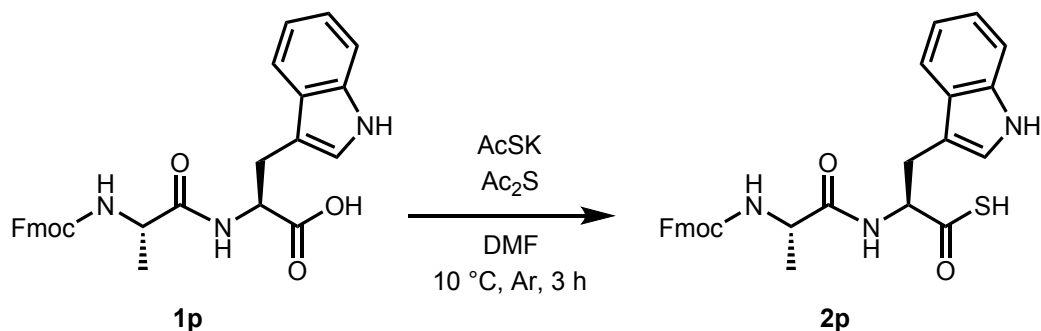


1o (5.81 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL , 10 μmol), and the mixture was stirred for 3 h at 10 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 69%.

2o: MS (ESI): m/z 547.20 (calcd $[\text{M}+\text{H}]^+ = 547.68$). Retention time: 34.8 min.

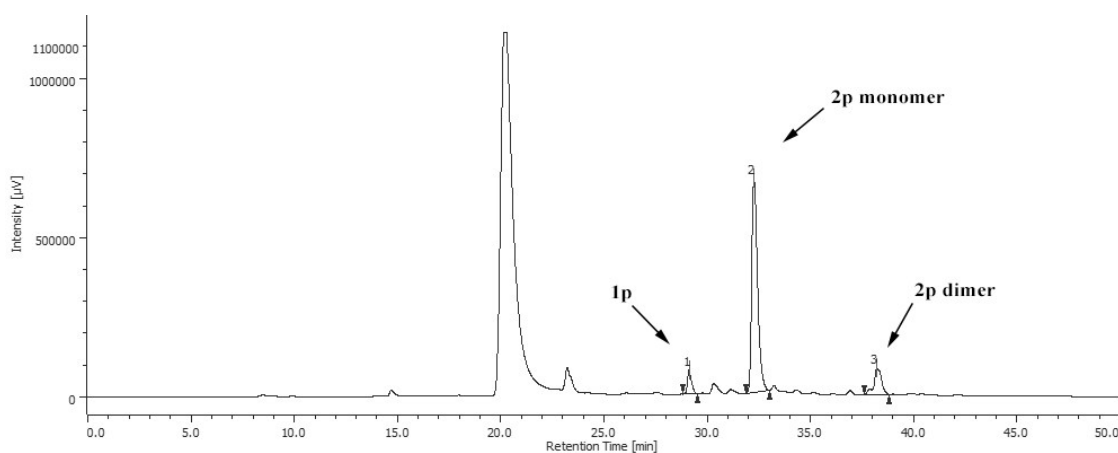


Fmoc-Ala-Trp-SH (2p)

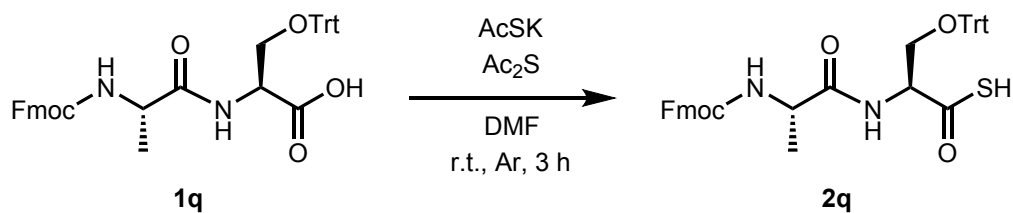


1p (4.98 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μ L, 10 μ mol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 1-7, the yield was determined as 93%.

2p: MS (ESI): m/z 514.10 (calcd $[M+H]^+ = 514.61$). Retention time: 32.2 min.

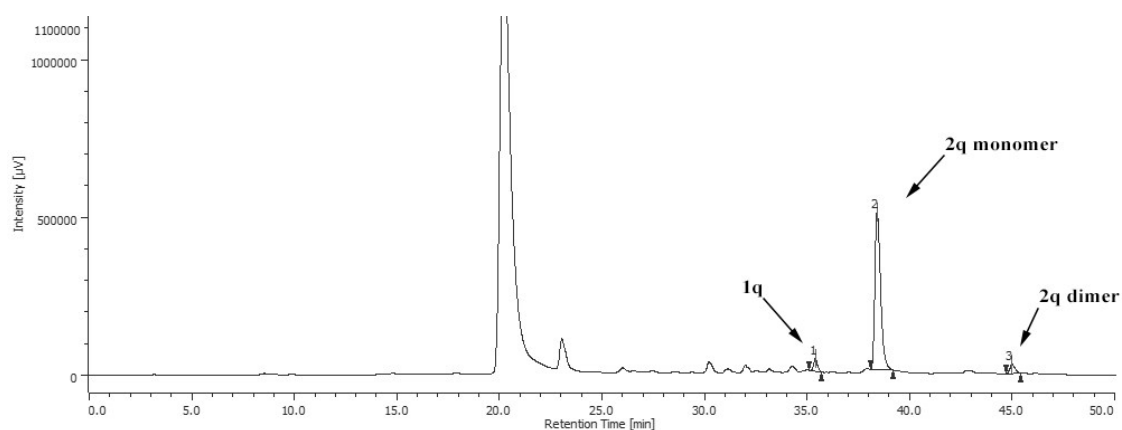


Fmoc-Ala-Ser(Trt)-SH (2q)

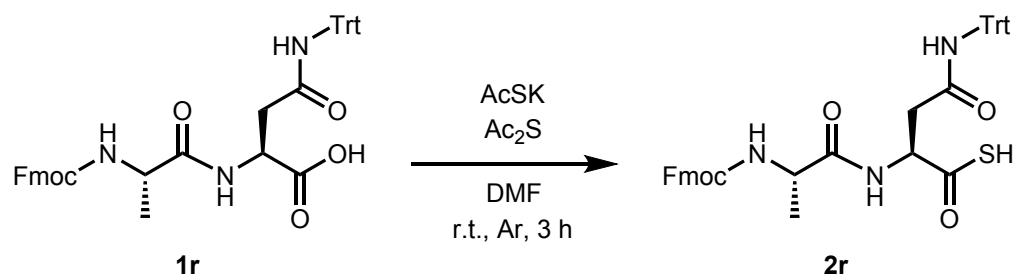


1q (6.41 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μ L, 10 μ mol), and the mixture was stirred for 3 h at room temperature. According to the protocol described in Section 3-2, the yield was determined as 62%.

2q: MS (ESI): m/z 655.10 (calcd $[M-H]^- = 655.80$). Retention time: 38.4 min.

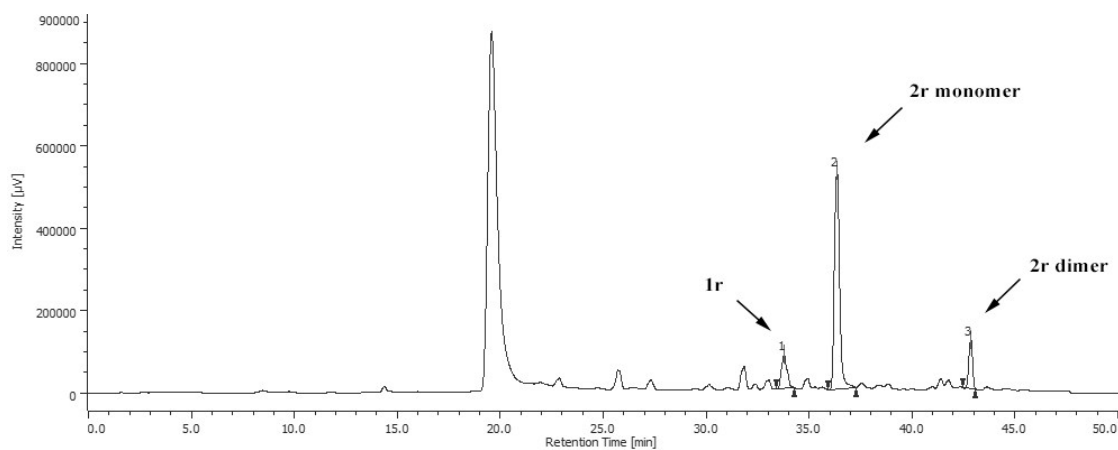


Fmoc-Ala-Asn(Trt)-SH (2r)

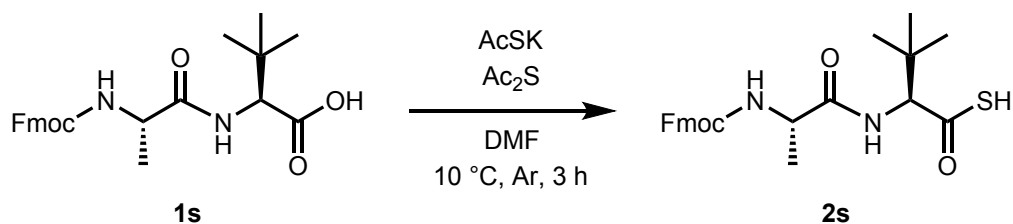


1r (6.68 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL , 10 μmol), and the mixture was stirred for 3 h at room temperature. According to the protocol described in Section 3-2, the yield was determined as 66%.

2r: MS (ESI): m/z 684.10 (calcd $[\text{M}+\text{H}]^+ = 684.82$). Retention time: 36.4 min.

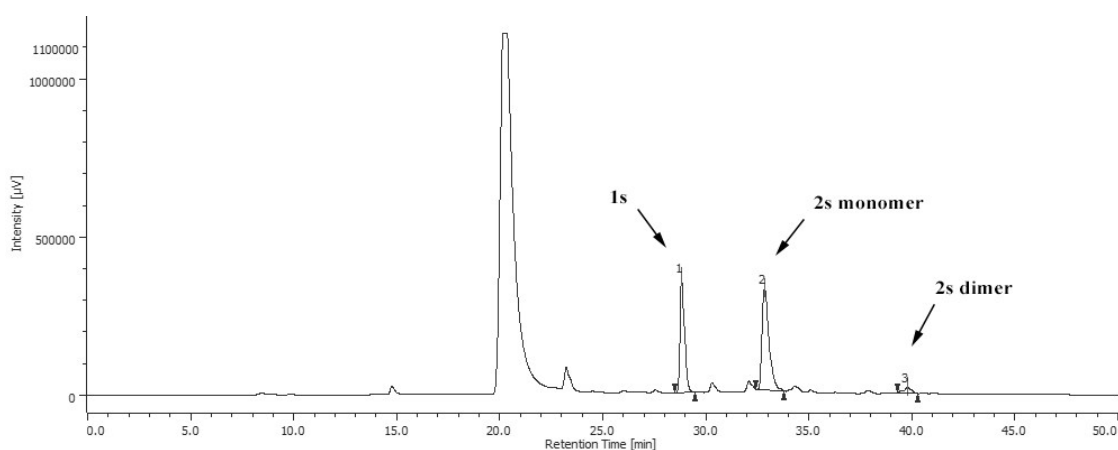


Fmoc-Ala-^tLeu-SH (2s)

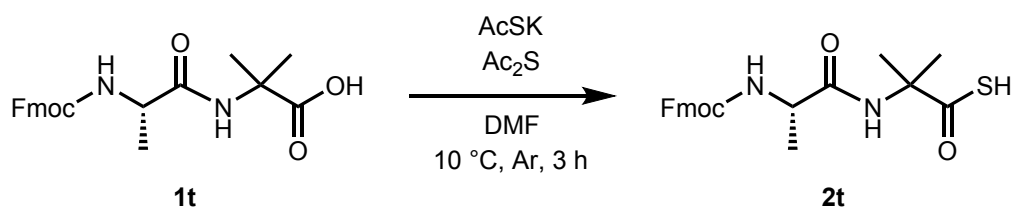


1s (4.25 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μ L, 10 μ mol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 3-2, the yield was determined as 49%.

2s: MS (ESI): m/z 441.10 (calcd $[M+H]^+ = 441.56$). Retention time: 32.8 min.

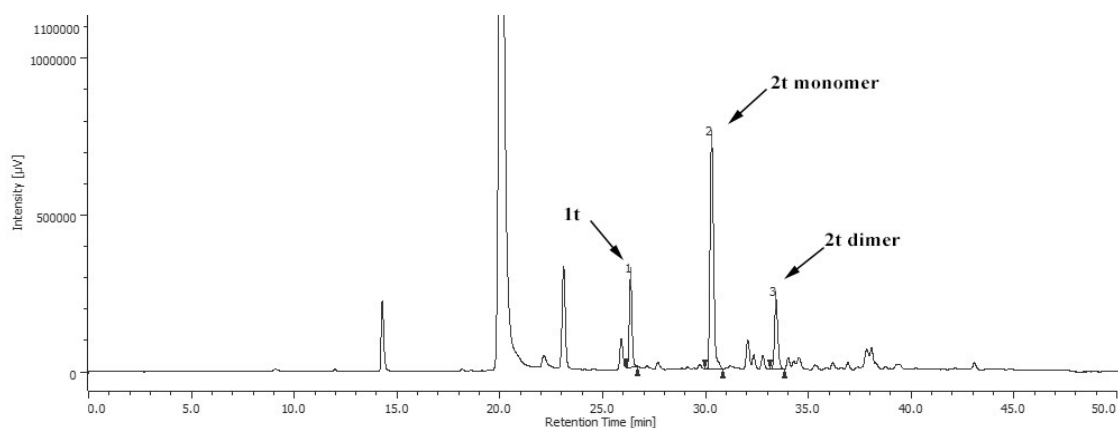


Fmoc-Ala-Aib-SH (2t)

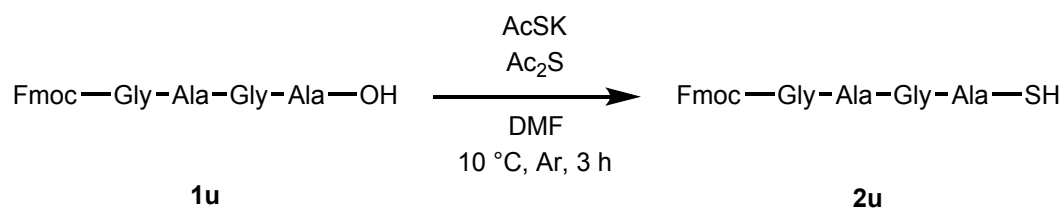


1t (3.96 mg, 10 μ mol), AcSK (11.4 mg, 100 μ mol) and DMF (50 μ L) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μ L, 10 μ mol), and the mixture was stirred for 3 h at 10 °C. According to the protocol described in Section 3-2, the yield was determined as 69%.

2t: MS (ESI): m/z 413.05 (calcd $[M+H]^+ = 413.50$). Retention time: 26.4 min.

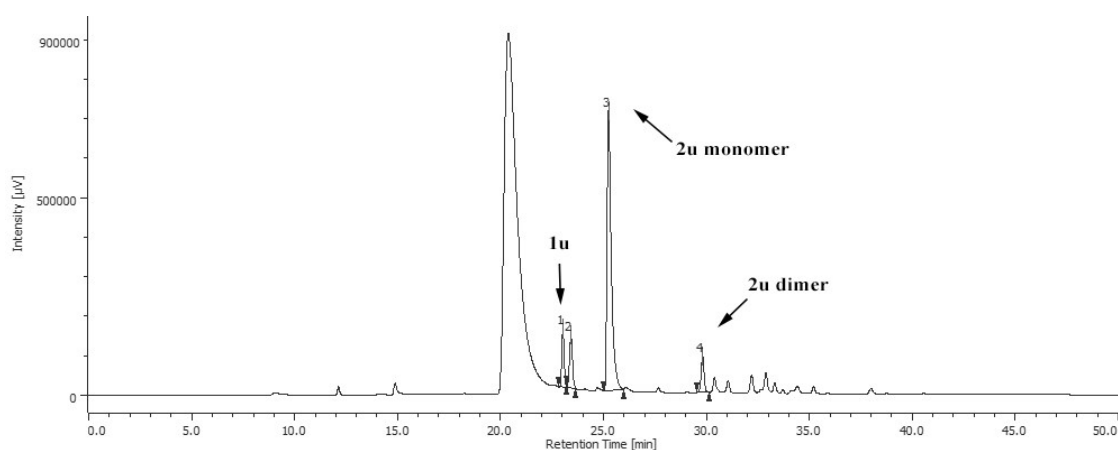


Fmoc-Gly-Ala-Gly-Ala-SH (2u)

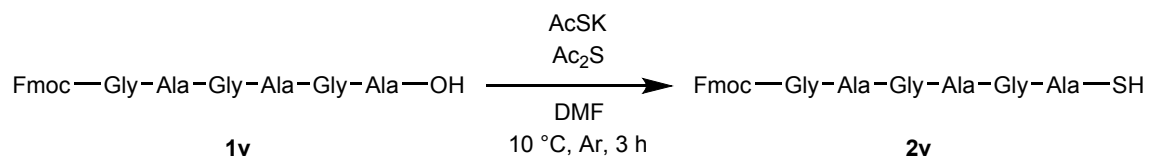


1u (4.97 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac_2S (200 mM, 50 μL , 10 μmol), and the mixture was stirred for 3 h at 10 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 67%.

2u: MS (ESI): m/z 511.10 (calcd $[\text{M-H}]^- = 511.58$). Retention time: 25.2 min.

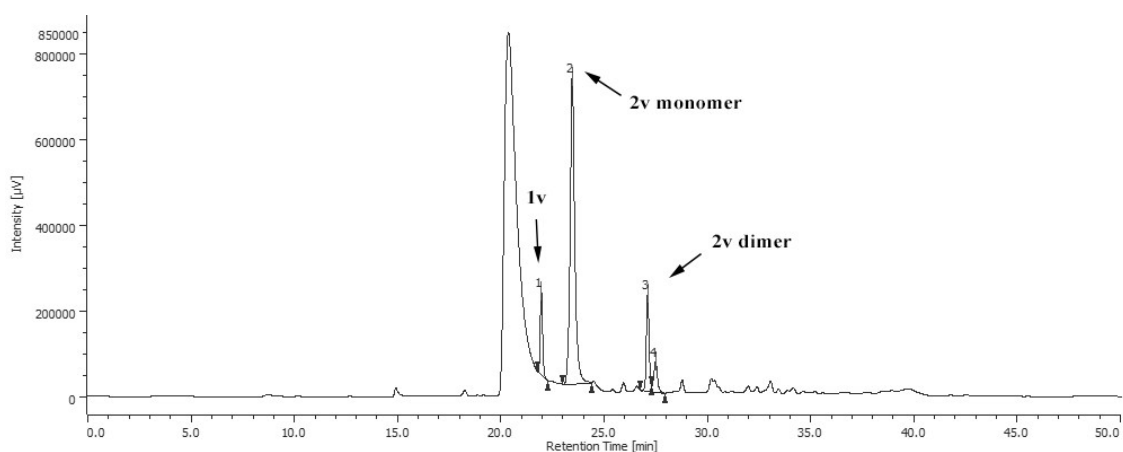


Fmoc-Gly-Ala-Gly-Ala-Gly-Ala-SH (2v)



1v (7.37 mg, 10 μmol), AcSK (11.4 mg, 100 μmol) and DMF (50 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (200 mM, 50 μL , 10 μmol), and the mixture was stirred for 3 h at 10 $^\circ\text{C}$. According to the protocol described in Section 3-2, the yield was determined as 81%.

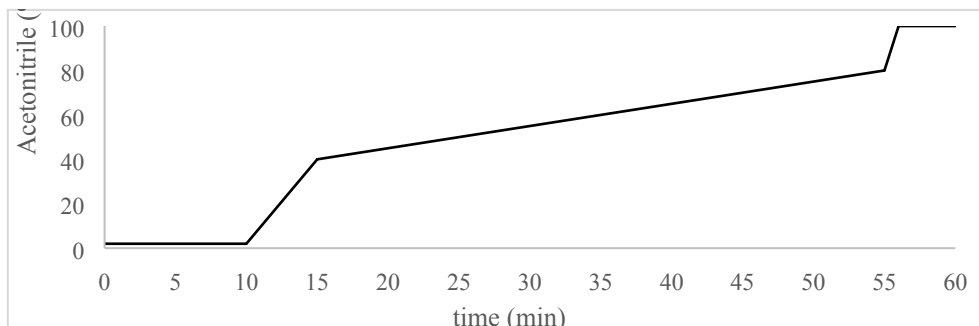
2v: MS (ESI): m/z 623.15 (calcd $[\text{M-H}]^- = 623.65$). Retention time: 23.4 min.



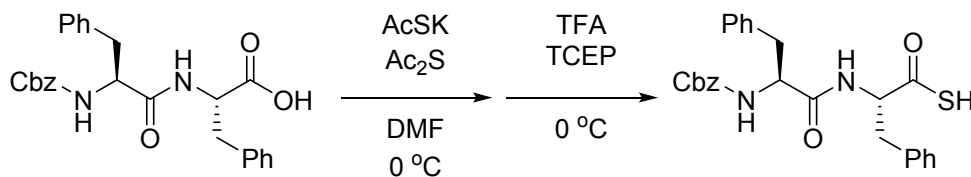
4. Gram-Scale Synthesis

Purification

Peptide thioacids **2a** and **2b** were purified by Yamazen automated column chromatography system using a gradient eluent system of acetonitrile versus 0.1% TFA in water. The chromatography was carried out as follows: Yamazen HI-FLASH COLUMN (L 26 mm I.D \times 100, ODS-SM 50 μ m) using the gradient eluent system shown below over 60 min with a flow rate of 20.0 mL min⁻¹. The eluent was monitored by absorbance at 230 nm.



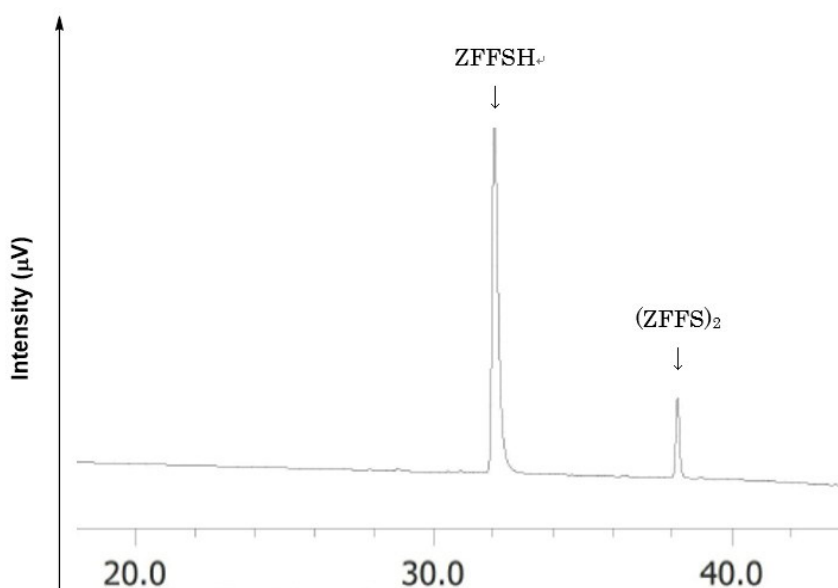
Cbz-Phe-Phe-SH (**2a**)



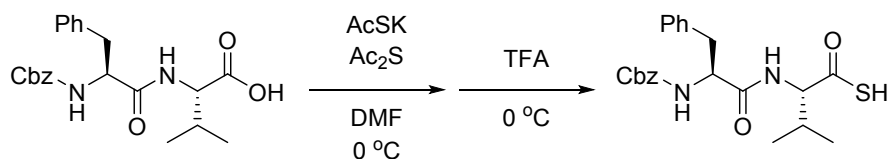
To a flame-dried flask under argon atmosphere, **1a** (1.12 g, 2.5 mmol), AcSK (857 mg, 7.5 mmol), Ac₂S (53.7 μ L, 0.50 mmol) and DMF (25 mL) were added. The resulting mixture was stirred for 3 h at 0 °C. The reaction mixture was treated with TFA (574 μ L, 7.5 mmol) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, 717 mg, 0.50 mmol). After stirring for another 1 h at 0 °C, H₂O and 1 N HCl aq was added to adjust the pH to 2. The mixture was extracted with EtOAc, and the combined organic layer was washed with HCl aq (pH 2) and brine, then dried over Na₂SO₄. After the desiccant was filtered off, the filtrate was concentrated under reduced pressure. The residue was dissolved in MeCN and freeze-dried. Resulting solid was purified by the protocol described in Purification section to give light orange solid (705 mg, 61%), comprising monomer **2a** : dimer (**Cbz-Phe-Phe-S**)₂ = 89:11.

2a: ¹H NMR (CDCl₃): δ = 3.00 (m, 2H+2H), 4.35 (dd, J =13.8, 6.3 Hz, 2H), 4.81 (dd, J =13.5, 7.2 Hz, 1H), 5.04 (m, 2H+1H), 6.29 (d, J = 5.0 Hz, 1H), 7.00 (d, J = 7.4 Hz, 2H), 7.13 (d, J = 6.3 Hz, 2H), 7.27 (m, 11H) ; ¹³C NMR* (DMSO-*d*₆): δ = 195.0, 172.5, 155.7, 137.8, 136.9, 136.4, 129.24, 129.18, 128.29, 128.21, 128.12, 18.03, 127.71, 127.49, 127.41, 126.7, 126.4, 65.3, 60.5, 56.2, 37.0, 36.3; IR (KBr): 3283, 3029, 2924, 2568, 1685, 1658, 1533, 1287, 1241, 736, 698 cm⁻¹; LRMS (ESI): m/z 485 [M+Na]⁺; HRMS (ESI): m/z calcd for C₂₆H₂₆N₂NaO₄S [M+Na]⁺ 485.1505, found 485.1525.

RP-HPLC analysis



Cbz-Phe-Val-SH (2b)

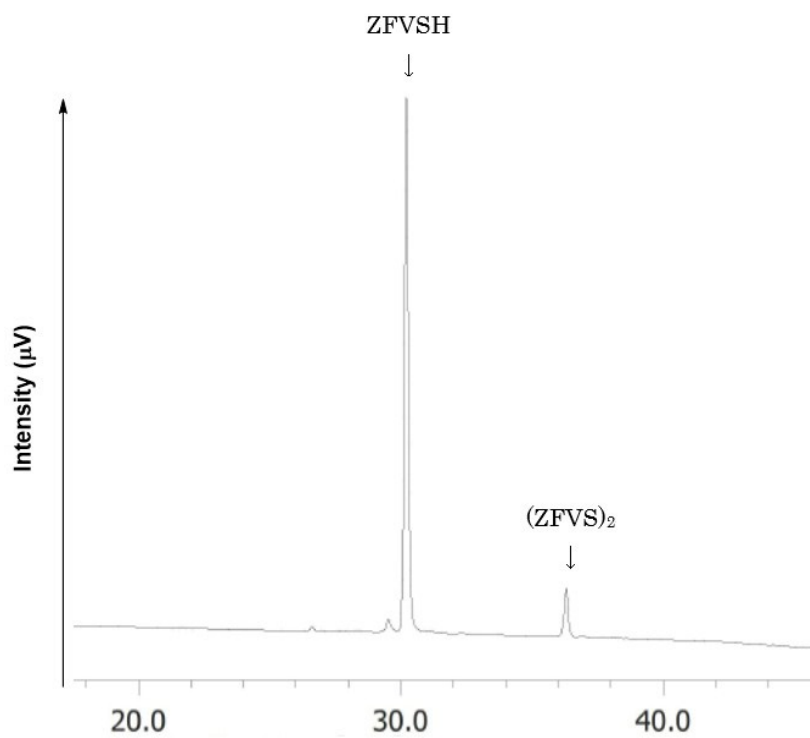


To a flame-dried flask under argon atmosphere, **2a** (800 mg, 2.0 mmol), AcSK (2.28 g, 20 mmol), Ac₂S (42 μ L, 0.40 mmol) and DMF (20 mL) were added. The resulting mixture was stirred for 3 h at 0 °C. The reaction mixture was treated with TFA (1.53 mL, 20 mmol), H₂O, and 1 N HCl aq to adjust the pH to 2. The mixture was extracted with hexane/EtOAc (4/1), and the combined organic layer was washed with HCl aq (pH 2) and brine, then dried over Na₂SO₄. After the desiccant was filtered off, the filtrate was concentrated under reduced pressure. The residue was dissolved in MeCN and freeze-dried. Resulting solid was purified by protocol described in Purification section to give orange solid (433 mg, 52%), comprising monomer **2b** : dimer (**Cbz-Phe-Val-S**)₂ = 91.6 : 8.4.

2b: ¹H NMR (CD₃OD): δ = 0.94 (dd, J = 12.3, 6.6 Hz, 6H), 2.20 (m, 1H), 2.84 (dd, J = 14.0, 9.4 Hz, 1H), 3.17 (dd, J = 13.7, 5.2 Hz, 1H), 4.42 (m, 1H), 4.48 (m, 1H), 5.01 (s, 2H), 7.26 (m, 10+1H), 8.31 (d, J = 6.0 Hz, 1H); ¹³C NMR* (CD₃OD): δ = 174.4, 158.2, 138.46, 138.13, 130.4, 129.45, 129.41, 128.90, 128.67, 127.73, 67.5, 66.8, 57.7, 38.8, 32.2, 19.8, 17.8 ; IR (KBr): 3297, 3062, 2965, 2549, 1700, 1660, 1540, 1261, 1029, 740, 697 cm⁻¹; LRMS (ESI): m/z 437 [M+Na]⁺; HRMS (ESI): m/z calcd for C₂₂H₂₆N₂NaO₄S [M+Na]⁺ 437.1505, found 437.1515.

*Thioacid easily dimerizes during ^{13}C -NMR measurement in $\text{DMSO-}d_6$. In CD_3OD , dimerization did not occur, but -COSH carbon was difficult to detect.¹

RP-HPLC analysis



5. Epimerization check

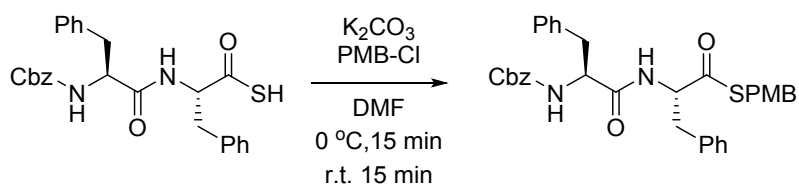
General

Epimerization levels of the synthesized thioacids **2** were calculated after conversion into *p*-methoxybenzyl thioester (peptide-SPMB) by treatment with *p*-methoxybenzyl chloride. The reaction mixture containing thioester was analyzed by normal-phase chiral HPLC. The peak areas corresponding to the LL / LD isomers of the thioester were measured and the epimerization level was calculated based on the following equation.

$$\text{epimerization level [\%]} = \text{peak area (LD)} / (\text{peak area (LD)} + \text{peak area (LL)}) \times 100$$

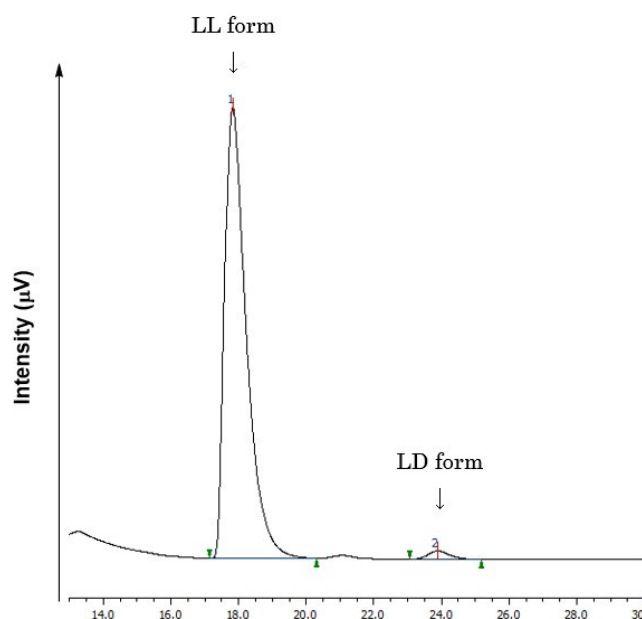
Thioacids were not suitable for epimerization check because the LL / LD isomers were difficult to separate by analytical HPLC. Peptide-PMB esters were more easily separable by normal phase chiral HPLC.

Epimerization check for **2a**

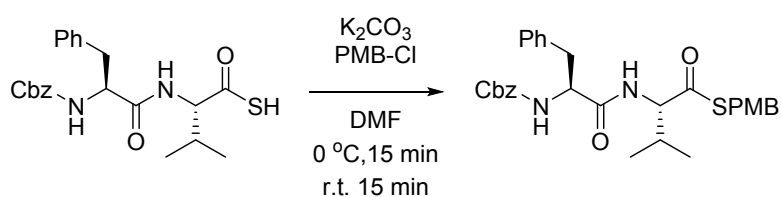


To a micro tube, **2a** (2.31 mg, 5.0 μmol, synthesized by following the gram-scale protocol described in Section 4), K₂CO₃ (0.69 mg, 5.0 μmol), and DMF (50 μL) were added under argon atmosphere. *p*-Methoxybenzyl chloride (2.04 μL, 6.0 μmol) was added to the solution. The resulting mixture was stirred at 0 °C for 15 min followed by room temperature for 15 min. The resulting solution containing **Cbz-Phe-Phe-SPMB** was analyzed by chiral HPLC. The epimerization level was calculated as 1.8 %. LRMS (ESI): *m/z* 605.05 (calcd [M+Na]⁺ = 605.21).

HPLC conditions: DAICEL CHIRALPAK IB (4.6 mm I.D. × 250 mm) column and isocratic solvent system of hexane : ethanol = 47 : 3 at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 254 nm. Retention time: 18.0 min (LL form), 23.8 min (LD form).

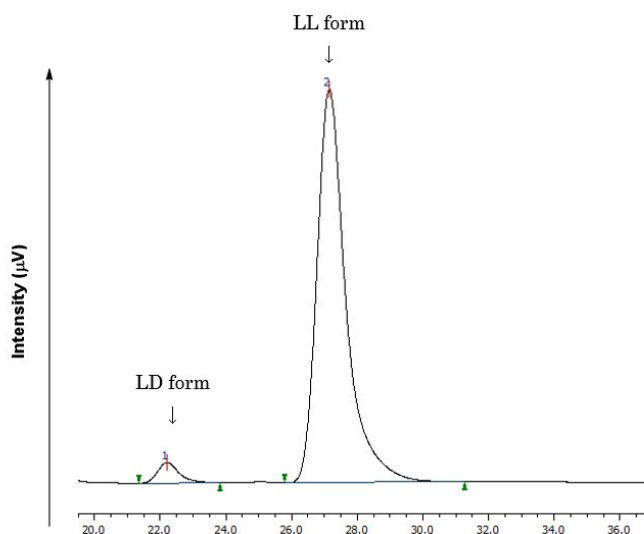


Epimerization check for **2b**

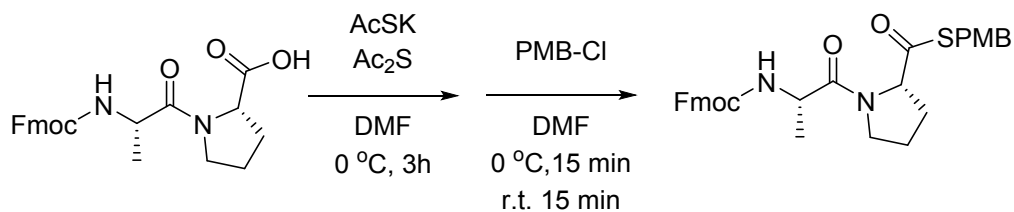


To a micro tube, **2b** (2.07 mg, 5.0 μmol , synthesized by following the gram-scale protocol described in Section 4), K_2CO_3 (0.69 mg, 5.0 μmol), and DMF (50 μL) were added under argon atmosphere. *p*-Methoxybenzyl chloride (2.04 μL , 6.0 μmol) was added to the solution. The resulting mixture was stirred at 0 $^\circ\text{C}$ for 15 min followed by room temperature for 15 min. The resulting solution including **Cbz-Phe-Val-SPMB** was analyzed by chiral HPLC. The epimerization level was calculated as 3.8 %. LRMS (ESI): m/z 557.00 (calcd $[\text{M}+\text{Na}]^+ = 557.20$).

HPLC conditions: DAICEL CHIRALPAK IC-3 (4.6 mm I.D. \times 250 mm) column and isocratic solvent system of hexane : ethanol = 47 : 3 at room temperature with a flow rate of 1 mL min^{-1} . The eluent was monitored by absorbance at 254 nm. Retention time: 27.3 min (LL form), 22.2 min (LD form).



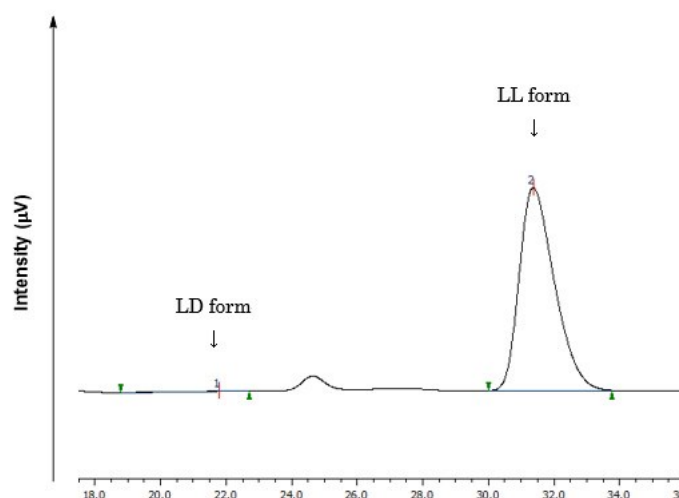
Epimerization check for **2i**



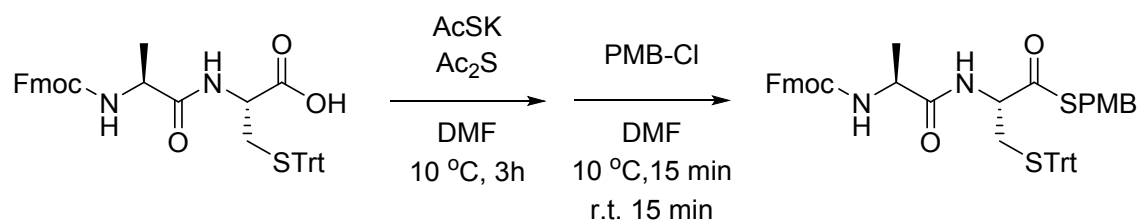
1i (2.04 mg, 5 μmol), AcSK (5.7 mg, 50 μmol) and DMF (45 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (5.0 μL , 2.5 μmol), and the mixture was stirred for 3 h at 10 $^{\circ}\text{C}$. *p*-Methoxybenzyl chloride (8.14 μL , 60 μmol) was added to the solution. The resulting mixture was stirred at 10 $^{\circ}\text{C}$ for 15 min, then at room temperature for 15 min. The resulting solution including **Fmoc-Ala-Pro-SPMB** was analyzed by chiral HPLC. The epimerization level was calculated as 0.1 %.

LRMS (ESI): m/z 567.05 (calcd $[\text{M}+\text{Na}]^+ = 567.19$).

HPLC conditions: DAICEL CHIRALPAK IB (4.6 mm I.D. \times 250 mm) column and isocratic solvent system of hexane : ethanol = 17 : 3 at room temperature with a flow rate of 1 mL min^{-1} . The eluent was monitored by absorbance at 301 nm. Retention time: 32.4 min (LL form), 21.7 min (LD form).



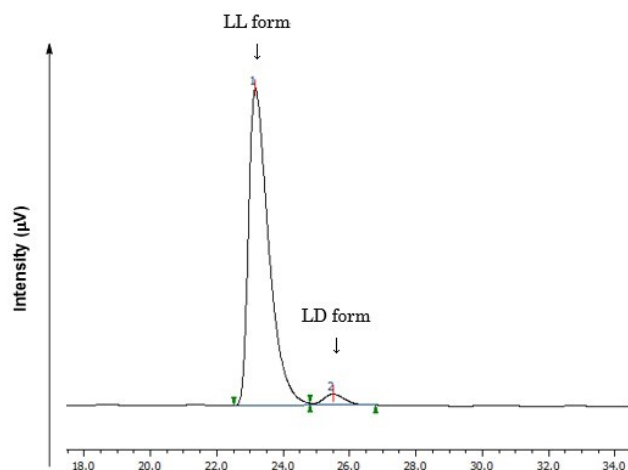
Epimerization check for **2j**



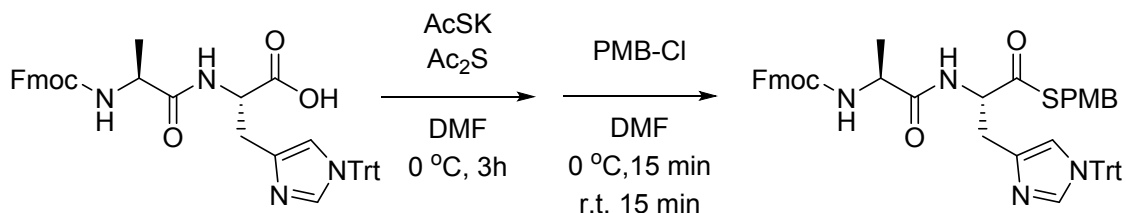
1j (3.28 mg, 5.0 μmol), AcSK (5.7 mg, 50 μmol) and DMF (45 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S (5.0 μL , 5.0 μmol), and the mixture was stirred for 3 h at 10 °C. *p*-Methoxybenzyl chloride (8.14 μL , 60 μmol) was added to the solution. The resulting mixture was stirred at 10 °C for 15 min, then at room temperature for 15 min. The resulting solution including **Fmoc-Ala-Cys(Trt)-SPMB** was analyzed by chiral HPLC. The epimerization level was calculated as 3.7 %.

LRMS (ESI): m/z 815.05 (calcd $[\text{M}+\text{Na}]^+ = 815.26$).

HPLC conditions: DAICEL CHIRALPAK IA-3 (4.6 mm I.D. \times 250 mm) column and isocratic solvent system of hexane : ethanol = 47 : 3 at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 301 nm. Retention time: 23.2 min (LL form), 25.4 min (LD form).



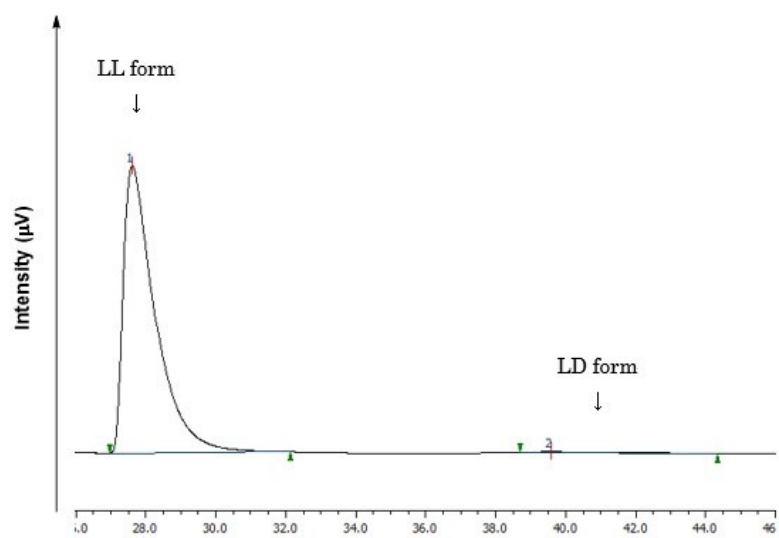
Epimerization check for **2n**



1n (3.46 mg, 5 μmol), AcSK (5.7 mg, 50 mmol) and DMF (45 μL) were mixed under argon atmosphere in a micro tube. To the solution was added a DMF solution of Ac₂S 5.0 μL (1.0 μmol), and the mixture was stirred for 3 h at 0 $^{\circ}\text{C}$. Then, *p*-methoxybenzyl chloride (8.14 μL , 60 μmol) was added to the solution. The resulting mixture was stirred at 0 $^{\circ}\text{C}$ for 15 min, then at room temperature for 15 min. The resulting solution including **Fmoc-Ala-His(Trt)-SPMB** was analyzed by chiral HPLC. The epimerization level was calculated as 0.7 %.

LRMS (ESI): m/z 827.05 (calcd $[\text{M}+\text{H}]^+ = 827.33$).

HPLC conditions: DAICEL CHIRALPAK IB (4.6 mm I.D. \times 250 mm) column and a isocratic solvent system of hexane : ethanol = 47 : 3 at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 301 nm. Retention time: 27.6 min (LL form), 39.6 min (LD form).

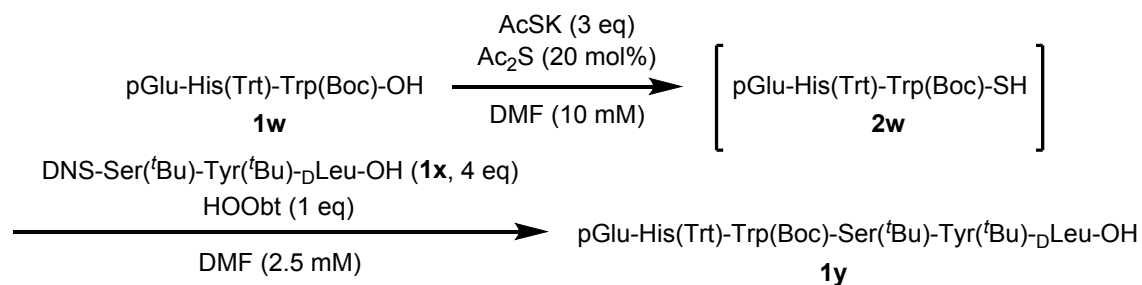


6. Synthesis of Leuprorelin

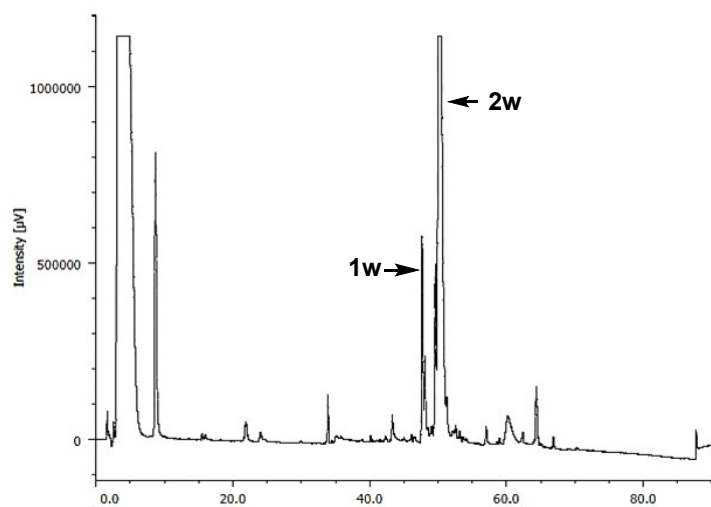
6-1. Protocol for Fragment Coupling

Reactions were carried out in dry solvents in polypropylene Eppendorf tube under air. To a stirred solution of the *N*-terminal fragment peptide (10 μ L, 25 mM in DMF, 0.25 μ mol) and AcSK (15 μ L, 50 mM in DMF, 0.75 μ mol), Ac₂S (1 μ L, 50 mM in DMF, 0.05 μ mol) was added at room temperature. After stirred at the same temperature for 3 h, the mixture was added into a solution of *C*-terminal fragment DNS peptide (50 μ L, 20 mM in DMF, 1.0 μ mol) and HOOBT (25 μ L, 40 mM in DMF, 1.0 μ mol). After stirred at room temperature for 3 h, the crude mixture was directly injected into HPLC systems for analysis. The yield was calculated based on a calibration curve, prepared using authentic samples as described in Section 3-1. Analytical HPLC was carried out as follows unless otherwise noted: YMC-Triart-C₁₈ (4.6 mm I.D. \times 150 mm) column using a linear gradient of 0–100% acetonitrile in 0.1% aqueous TFA over 40 min at room temperature with a flow rate of 1 mL min⁻¹. The eluent was monitored by absorbance at 230 nm.

6-2. The First Fragment Coupling



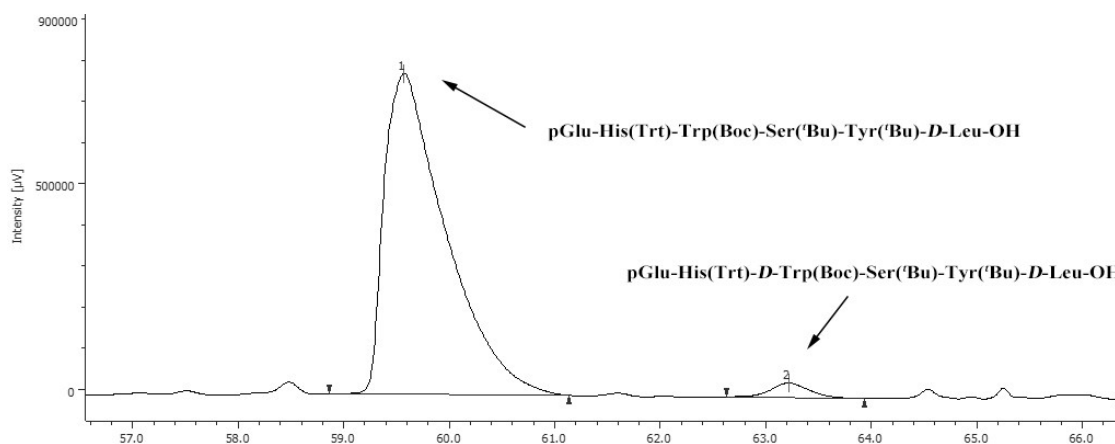
HPLC trace of thioacid formation



pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH (**1y**)

According to the protocol described in Section 6-1, this compound was obtained. The yield was determined as 85% and the epimerization level was determined as 2.7%.

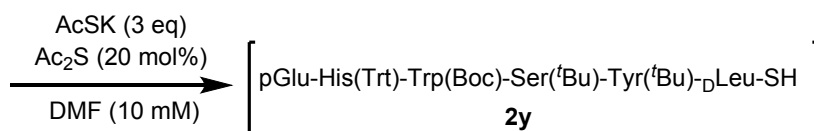
HPLC (H₂O 2 min. then 0-100% MeCN / H₂O over 80 min.). Retention time: 59.7 min (**1y**), 62.8 min (*epi-1y*). MS (ESI): m/z 1269.1 (calcd [M+H]⁺ = 1269.51).



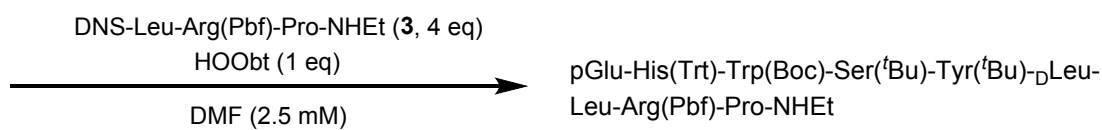
6-3. The Second Fragment Coupling

pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu-OH

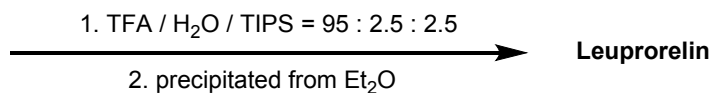
1y



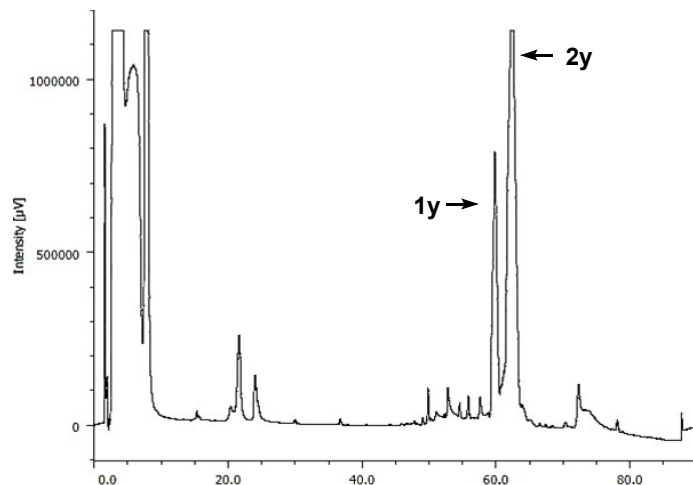
2y



Protected Leuporelin

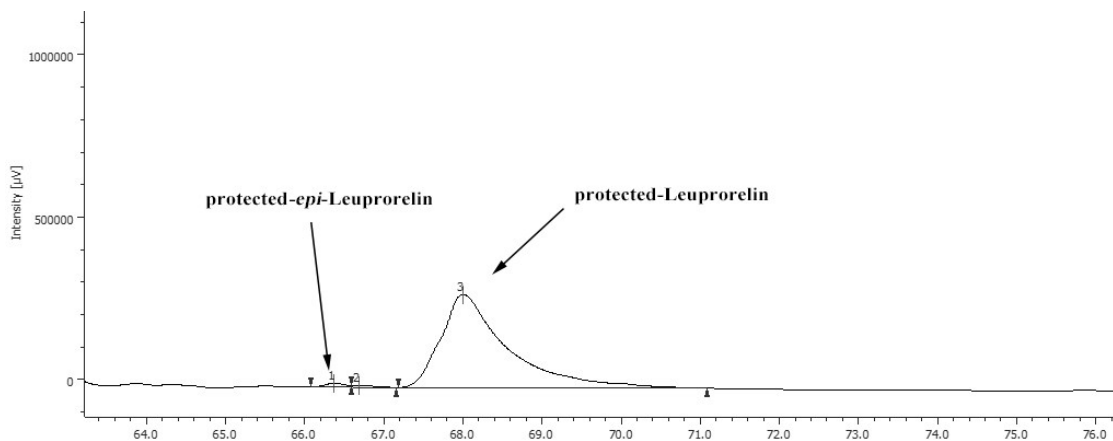


HPLC trace of thioacid formation



pGlu-His(Trt)-Trp(Boc)-Ser(^tBu)-Tyr(^tBu)-D-Leu--Leu-Arg(Pbf)-Pro-NHEt (Protected Leuprorelin)

According to the protocol described in Section 6-1, this compound was obtained and used for the next step without purification. The yield was determined as 78% and the epimerization level was less than 1%.

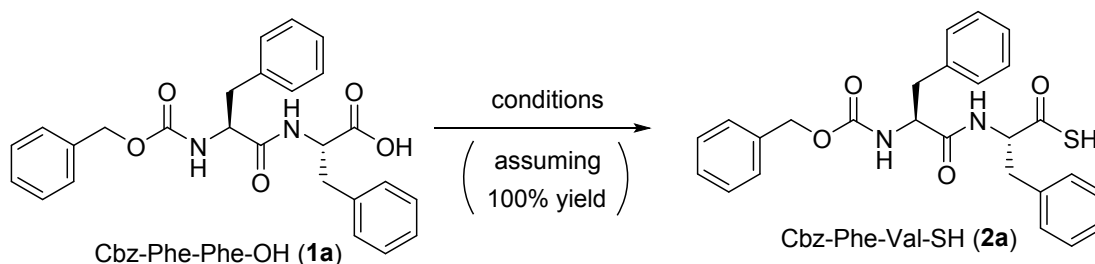


pGlu-His-Trp-Ser-Tyr-Leu-D-Leu-Arg-Pro-NHEt (Leuprorelin)

To a stirred solution of **Protected Leuprorelin** (100 μ L) and H₂O (25 μ L) in TFA (950 μ L), Et₃SiH (25 μ L, 300 μ mol) was added at room temperature. After concentration under reduced pressure at 60 °C, the thus-obtained mixture (ca. 300 μ L) was diluted with diethyl ether (1.4 mL) to precipitate the peptide out. The precipitate was filtered, washed with diethyl ether and dried under reduced pressure to give **Leuprorelin** as a white solid in 55% yield.

7. Comparison of E-Factor

E-factors⁴ of various conditions suitable for peptide thioacids synthesis are calculated as follows. We did not take into consideration of used solvents and excess reagents because any optimization study of the same reaction pattern focusing on improving e-factor has never been reported to date.



conditions	reference	e-factor
Ac₂S (0.2 eq), AcSK (3 eq)	This work	0.76
1. HATU (5 eq), DIPEA (4 eq) 2,4,6-trimethoxybenzylthiol (5 eq) 2. TFA, PhOH (30 mg), TIPS (0.07 mL) for 0.02 mmol SM	<i>J. Am. Chem. Soc.</i> 2010 , 132, 17045.	17.6 (discounting TFA)
1. TrtSH (1 eq), DMAP (0.2 eq) EDCI-HCl (1.2 eq) 2. TFA, Et ₃ SiH (100 μL) for 0.11 mmol SM	<i>Angew. Chem. Int. Ed.</i> 2009 , 48, 7591.	2.6 (discounting TFA)
1. (9-fluorenyl)CH ₂ SH (25 eq), HOBT (5 eq) EDCI-HCl (5 eq) 2. 50% piperidine for 0.01 mmol SM	<i>Angew. Chem. Int. Ed.</i> 2009 , 48, 2355.	15.2 (discounting piperidine)

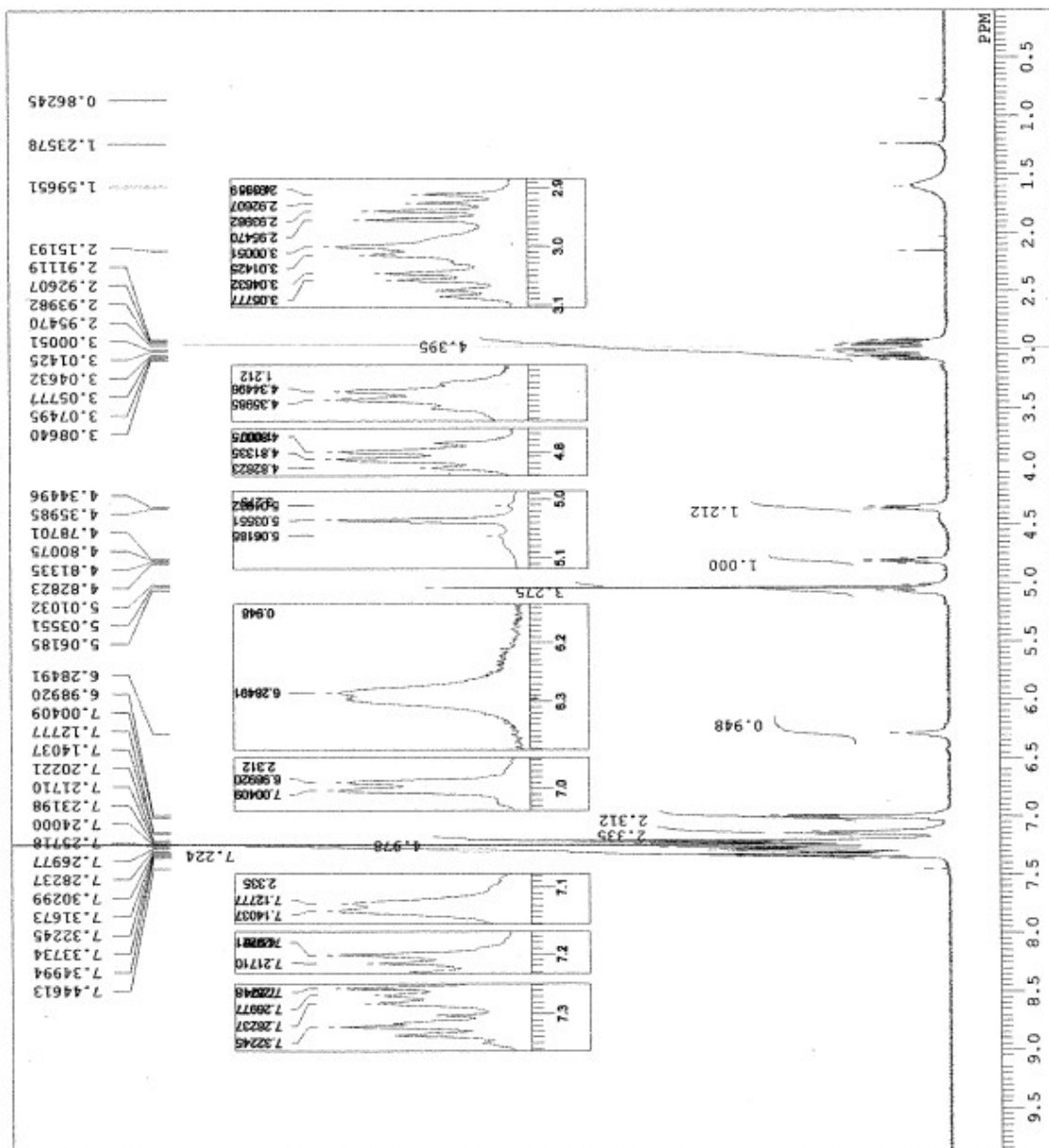
References

- ¹ P. Wang, S. J. Danishefsky, *J. Am. Chem. Soc.* **2010**, 132, 17045.
- ² P. Karmakar, R. S. Talan, S. J. Sucheck, *Org. Lett.* **2011**, 13, 5298.
- ³ A. Dose, J. O. Jost, A. C. Spieß, P. Henklein, M. Beyermann, D. Schwarzer, *Chem. Commun.* **2012**, 48, 9525.
- ⁴ (a) R. A. Sheldon, *Green Chem.* **2007**, 9, 1273. (b) R. A. Sheldon, *Chem. Commun.* **2008**, 3352.

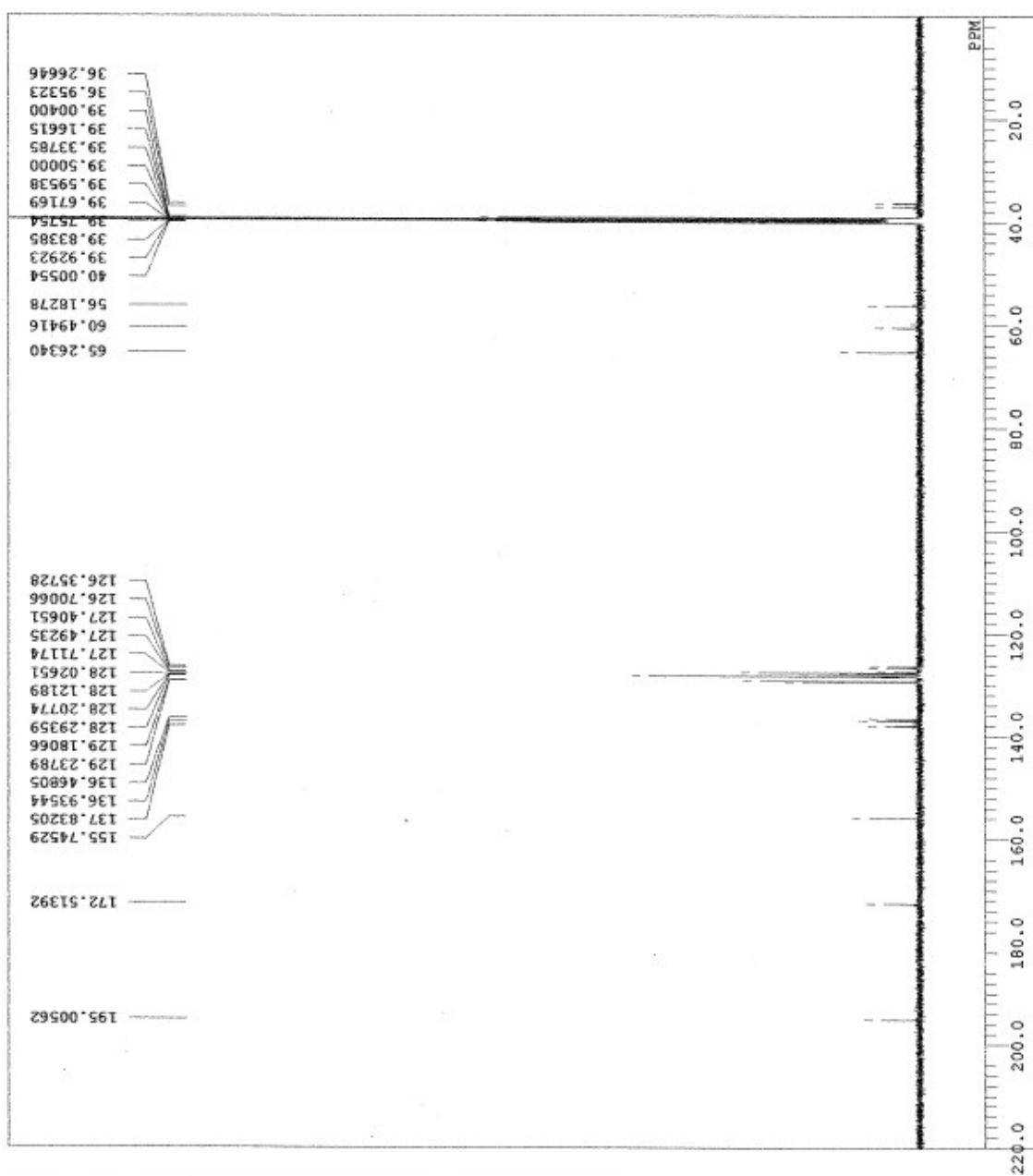
NMR Spectra

Cbz-Phe-Phe-SH (2a)

^1H NMR (CDCl_3)

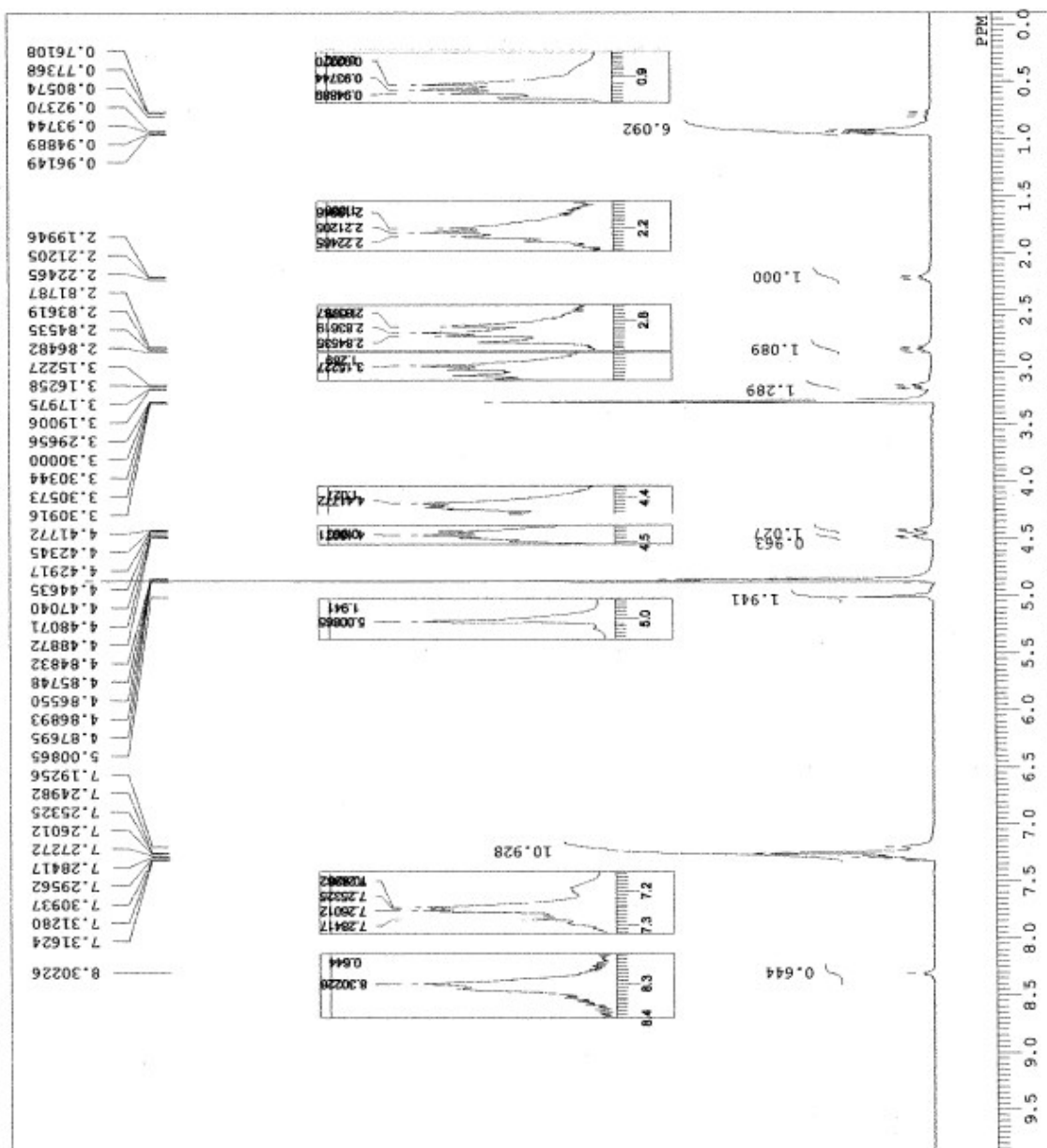


^{13}C NMR (DMSO- d_6)



Cbz-Phe-Val-SH (2b)

¹H NMR (CD₃OD)



^{13}C NMR (CD_3OD)

