Supporting Information

Chemical Synthesis of *Torenia* Plant Pollen Tube Attractant Proteins by KAHA Ligation

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1. General Methods
1.1. Reagents and solvents and abbreviations

Fmoc-amino acids with suitable side-chain protecting groups, HCTU and HATU were purchased from Merck KGaA (Darmstadt, Germany). HPLC grade CH$_3$CN from Kanto Chemical Co., Inc. (Tokyo, Japan) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) was used for analytical and preparative HPLC purification. DMF from FUJIFILM Wako Pure Chemical Corporation was directly used without further purification for solid phase peptide synthesis. Fmoc-protected-Phe-α-ketoacid[1] Boc-(S)-5-oxaproline[2] were prepared according to reported procedures. Other commercially available reagents and solvents were purchased from Merck KGaA (Darmstadt, Germany), Kanto Chemical Co., Inc. (Tokyo, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used without further purification.

List of the different building blocks previously reported by our group and other building blocks used in this work:
**List of abbreviations used:**

- **DMSO:** dimethylsulfoxide
- **NMP:** $N$-methyl-2-pyrrolidone
- **DMF:** $N,N$-dimethylformamide
- **TFA:** trifluoroacetic acid
- **NMM:** $N$-methylmorpholine
- **HATU:** $O$-(7-azabenzotriazol-1-yl)-$N,N,N',N'$-tetramethyluronium hexafluorophosphate
- **HCTU:** $O$-(1H-6-chlorobenzotriazol-1-yl)-$N,N,N',N'$-tetramethyluronium hexafluorophosphate
- **HOBt:** hydroxybenzotriazole
- **DIC:** $N,N'$-diisopropylcarbodiimide
- **DODT:** 2,2′-(ethylenedioxy)diethanethiol
- **TIPS:** triisopropylsilane
- **DTT:** dithiothreitol
- **Gdn•HCl:** guanidine hydrochloride
- **Fmoc:** 9-fluorenylmethyloxycarbonyl
- **Pbf:** 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl
- **Boc:** tert-butoxycarbonyl
- **Trt:** trityl
- **Acm:** acetamidomethyl
- **MALDI-TOF-MS:** Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
- **SPPS:** solid phase peptide synthesis
- **HMPB:** 4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid
1.2. Peptide synthesis

a) Automated Solid Phase Peptide Synthesis (SPPS)
Peptides were synthesized on a CS Bio 136X synthesizer using Fmoc SPPS chemistry. The following Fmoc amino acids with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH. SPPS was performed on aminomethyl polystyrene resin or HMPB-ChemMatrix resin. Manual loading of the first amino acid residue onto the resin and subsequent Fmoc-SPPS followed established standard protocols. A brief summary of the utilized synthesis protocols: Fmoc-deprotections were performed with 20% (v/v) piperidine in DMF (8 min × 2). Couplings were performed with Fmoc-amino acid (4.0 equiv relative to resin substitution), HCTU (3.8 equiv) and NMM (8.0 equiv) in DMF for 60 min. If required, the coupling step was repeated (double coupling) and LiCl washes (0.8 M LiCl in DMF) were performed before Fmoc-deprotection and coupling. After coupling, unreacted free amine was capped by treatment with 20% (v/v) acetic anhydride and 10% (v/v) NMM in DMF for 10 min. Amino acid residues prone to epimerization such as cysteine were coupled using preformed HOBt esters. In a typical procedure, Fmoc-Cys(Acm)-OH (4.0 equiv relative to resin loading) was dissolved in DMF, and HOBt (4.0 equiv) and DIC (4.0 equiv) were added. The mixture was added to the resin and allowed to react for 2 h.

b) Manual coupling of special amino acids
Valuable non-standard monomers (e.g. Boc-5-oxaproline: Boc-Opr) were coupled manually. The monomer (1.5 equiv) was dissolved in a minimal amount of anhydrous DMF (the minimal concentration of the monomer was 0.1 M), HATU (1.5 equiv) and NMM (3.0 equiv) were added. After a brief period of preactivation (2 min), the solution was added to the resin and allowed to react for 2 h. If required, the coupling was repeated with 1.0 equiv of monomer, 1.0 equiv of HATU, and 2.0 equiv of NMM.
1.3. General HPLC analysis and purification
Peptides and proteins were analyzed and purified by reverse phase high performance liquid chromatography (RP-HPLC) on JASCO analytical and preparative instruments equipped with dual pumps, a mixer, an in-line degasser, and a variable wavelength UV detector (simultaneous monitoring of the eluent at 220 nm, 254 nm and 301 nm) or on a Gilson preparative instrument fitted with a 10 mL injection loop. If required, the columns were heated using a column heater or a water bath. The mobile phase for RP-HPLC were Milli-Q water containing 0.1% TFA and HPLC grade CH₃CN containing 0.1% TFA. In the described HPLC analysis and purifications, TFA was always used as solvent modifier.

**Analytical RP-HPLC:** Analytical HPLC was performed on a Shiseido Capcell Pak C18 MG-II (5 µm, 120 Å pore size, 4.6 mm I.D. × 250 mm), on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 4.6 mm I.D. × 250 mm), or on a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 4.6 mm I.D. × 250 mm) at a flow rate of 1 mL/min.

**Preparative RP-HPLC:** Preparative HPLC was performed on a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm), on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm), on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm), on a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm), or on a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm). The following type of method was used: the column was pre-equilibrated at starting solvent composition for typically 10 min. After injection of the sample, the solvent composition was run to the final solvent composition (e.g., 50% CH₃CN). After the gradient run time, the solvent composition was changed to 95% CH₃CN within 1 min and the column was flushed for 5–7 min. Within 1 min, the solvent composition was changed to 10% CH₃CN and the run ended. For the sake of simplicity, only the gradient time, the starting and end composition of the eluent will be stated at the individual experiments, although all experiments included the full cycle as described above.

1.4. Characterization
MALDI-TOF-MS data were obtained on a Bruker Microflex MALDI-TOF spectrometer using 4-hydroxy-α-cyanocinnamic acid as matrix. High-resolution mass spectra were recorded by the
Molecular Structure Center at ITbM, Nagoya University on a Thermo Scientific™ Exactive™ Plus Orbitrap Mass Spectrometer. SDS-PAGE was carried out on 16.5% acrylamide gels using the Mini-PROTEAN electrophoresis system (Bio-Rad Laboratories) on Precision Plus Protein™ Dual Xtra Prestained Protein Standards with Coomassie Brilliant Blue R250 stain.
2. General Experimental Procedures

2.1. General Experimental Procedure for the Synthesis of $\alpha$-Ketoacid Segment 1

The $\alpha$-ketoacid segment 1 were synthesized on aminomethyl polystyrene resin preloaded with Fmoc-protected-Phe $\alpha$-ketoacid (0.30–0.35 mmol/g loading) by automated Fmoc SPPS. To the resin placed in a glass vial, a mixture of 95:2.5:2.5 TFA:DODT:H$_2$O (20 mL/g resin) was added and the suspension was shaken at room temperature. After 2 h, the crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et$_2$O (ca. 15 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice. The crude peptide was dried and dissolved in a suitable solvent (aqueous CH$_3$CN or aqueous CH$_3$CN/DMF or AcOH with 0.1% TFA) for RP-HPLC purification.

2.2. General Experimental Procedure for the Synthesis of 5-Oxaproline Segment 2:

5-Oxaproline segment 2 was prepared on HMPB-ChemMatrix resin preloaded with Fmoc-Lys(Boc)-OH (0.25–0.30 mmol/g loading). After automated Fmoc SPPS, Boc-Opr (1.5 equiv) was coupled at N-terminus using HATU (1.4 equiv) and NMM (3.0 equiv) in DMF for 4 h at room temperature to complete the SPPS. The resin was washed several times with DMF followed by CH$_2$Cl$_2$ and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H$_2$O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et$_2$O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice. The crude peptide was dried and dissolved in aqueous CH$_3$CN with 0.1% TFA for RP-HPLC purification.
2.3. Optimization of the KAHA Ligation Conditions using 1a and 2a.

Various reaction conditions were examined using 2a (1.0 equiv, 20 mM) and 1a (1.2 equiv, 24 mM) in 0.1 M oxalic acid solution (Table 1). The reactions were analyzed by analytical RP-HPLC.

| Entry | solvent     | temp. | time | Yield of 3a[^a] |
|-------|-------------|-------|------|----------------|
| 1     | NMP/H2O (5:1) | 50 °C | 7 h  | 8%             |
| 2     | NMP/H2O (5:1) | 55 °C | 7 h  | 0%[^b]         |
| 3     | NMP/H2O (5:1) | 60 °C | 7 h  | 0%[^b]         |
| 4     | NMP/H2O (1:1) | 50 °C | 72 h | 42%            |
| 5     | NMP/H2O (1:1) | 55 °C | 6.5 h | 18%           |
| 6     | NMP/H2O (1:1) | 60 °C | 4.5 h | 10%           |
| 7     | DMSO/H2O (5:1) | 50 °C | 12 h | 27%            |
| 8     | DMSO/H2O (5:1) | 55 °C | 12 h | 32%            |
| 9     | DMSO/H2O (5:1) | 60 °C | 10 h | 40%            |
| 10    | DMSO/H2O (1:1) | 50 °C | 24 h | 37%            |
| 11    | DMSO/H2O (1:1) | 55 °C | 24 h | 51%            |
| 12    | DMSO/H2O (1:1) | 60 °C | 24 h | 64%            |

[^a] ligation product conversion determined by analytical RP-HPLC.  
[^b] decomposition of α-ketoacid segment 1a was observed during KAHA ligation.

Table S1: Optimization of the KAHA Ligation using 1a and 2a

In the case of NMP/H2O as solvent, the C-terminal α-ketoacid of 1a decomposed into carboxylic acid. The formal decarbonylation was observed at 55 °C and was reduced at 60 °C (entries 2 and 3), however we did not observe complete conversion for the ligated depsi peptide 3a, even longer reaction time (entry 4). To improve the yield, we switched to DMSO/water solvent system with changing the ratio (entries 7–12). We found that 50% aqueous DMSO and 60 °C were suitable for the KAHA ligation to produce the desired depsi peptide 3a (entry 12).

2.4. General Experimental Procedure for the KAHA Ligation and Rearrangement:

5-Oxaproline segment 2 (1.0 equiv) and α-ketoacid Segment 1 (1.2 equiv) were weighed into a glass vial and dissolved in a mixture of 1:1 DMSO/H2O (20 mM concentration of 2) with 0.1 M oxalic acid. The mixture was heated to 60 °C for 24 h. After 24 h, the crude depsi-peptide was subjected to O-to-N acyl shift (rearrangement) by dilution to 10-fold volume with 6 M Gdn•HCl.
solution set to pH 9.6, and the mixture was stirred at room temperature for 2 h. The reaction mixture was purified by preparative RP-HPLC.

2.5. General Experimental Procedure for the Cysteine Acm-deprotection:
The cysteine-Acm protected protein (10 mg) was dissolved in 2.5 mL of 50% (v/v) aq. acetic acid containing 1% AgOAc (w/v) and the mixture was stirred at 50 °C for 2 h. The reaction was quenched with 4.0 mL of 10% DTT (x/v) in 50% aq. acetic acid, and precipitation was separated by centrifugation. The precipitate was repeatedly washed with 50% (v/v) aq. acetic acid solution and the combined supernatant was purified by preparative RP-HPLC.

2.6. General Experimental Procedure for Protein Folding:
The deprotected, reduced linear protein (1 mg) was dissolved in denaturing buffer containing 6 M Gdn•HCl and 0.3 M Tris•HCl buffer, pH 7.0 (0.5 mM peptide concentration) stirred at room temperature to open air. After 1 h, the mixture was diluted with 8-fold volume of folding buffer containing 5 mM of reduced glutathione and 2.5 mM oxidized glutathione and adjusted to pH 8.2. The reaction was incubated in a shaker with slow movement at 4 °C for 24 h. The folding progress was monitored by analytical RP-HPLC. The resulting solution was acidified with aqueous HCl adjust to pH 4–5 and purified by preparative HPLC.
3. Chemical Synthesis of TfLURE by KAHA Ligation

3.1. Synthesis of Cys(Acm) Protected α-Ketoacid Peptide 1a

Peptide 1a was synthesized according to General Procedure 2.1. The crude peptide was purified by preparative RP-HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 15–65% CH3CN (with 0.1% TFA) in 20 min, flow rate 40 mL/min and re-purified using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm) at room temperature with a gradient of 20–60% CH3CN (with 0.1% TFA) in 20 min, flow rate 20 mL/min. The fractions containing the desired product were pooled and lyophilized to give 83 mg of peptide 1a (obtained from 1 g of dried resin after SPPS). The purity and identity of 1a was confirmed using analytical HPLC and ESI-HRMS. m/z calculated for 1a C_{120}H_{171}N_{27}O_{37}S [M+2H]^2+: 1307.1019 Da, measured: 1307.0998 Da.
3.2. Synthesis of Cys(Acm) Protected 5-Oxaproline Peptide 2a

Peptide 2a was synthesized according to General Procedure 2.2. The crude peptide was purified by preparative RP-HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 15–55% CH₃CN (with 0.1% TFA) in 20 min, flow rate 40 mL/min and re-purified by a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature with a gradient of 15–50% CH₃CN (with 0.1% TFA) in 20 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give 97 mg of desired 5-oxaproline peptide 2a (obtained from 1 g of dried resin after SPPS). The purity and identity of 2a was confirmed using analytical HPLC and ESI-HRMS. m/z calculated for peptide 2a C₂₁₂H₃₂₇N₇O₆₆S₅[M+4H]⁴⁺: 1221.8141 Da; measured 1221.8126 Da.
3.3. Synthesis of Cys(Acm) Protected Linear Protein 4a by KAHA ligation

Linear protein 4a was synthesized according to General Procedure 2.4 using peptide 2a (30 mg, 6.5 μmol, 1.0 equiv) and peptide 1a (42 mg, 7.8 μmol, 1.2 equiv). The reaction mixture was purified by preparative RP-HPLC using a Phenomenex Jupiter C18 (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm), heated to 60 °C; with a gradient of 10–60% CH$_3$CN with 0.1% TFA in 30 min, flow rate 20 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure 4a (29 mg, 64% yield). The purity and identity of 4a was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 4a C$_{33}$H$_{497}$N$_{84}$O$_{101}$S$_6$ [M+5H]$^{5+}$: 1491.4932 Da; measured 1491.4903 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) 2 h after addition of 6M Gdn•HCl solution pH 9.6; (d) purified 4a.
3.4. Synthesis of Linear Protein 5a

Reduced linear protein 5a was synthesized according to General Procedure 2.5 with Cys(Acm) protected protein 4a (10 mg, 14 µmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm), heated to 60 °C, with a gradient of 30–80% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure 5a (6.6 mg, 70% yield). The purity and identity of 5a was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 5a C$_{313}$H$_{469}$N$_{78}$O$_{95}$S$_{6}$[M+7H]$^{7+}$: 1004.7512 Da; measured 1004.7509 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 2$ h; (c) purified 5a
3.5. Synthesis of Folded TfLURE Protein 6a

Folded TfLURE protein 6a was synthesized according to General Procedure 2.6 with 1 mg of reduced linear protein 5a. The resulting solution was purified by preparative HPLC using a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure folded TfLURE 6a (0.32 mg, 32% yield). The purity and identity of 6a was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for folded TfLURE protein 6a C$_{313}$H$_{457}$N$_{78}$O$_{95}$S$_6$ [M+5H]$^{5+}$: 1405.0393 Da; measured 1405.0375 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) purified 6a.

Folded TfLURE protein 6a was dissolved in 25 mM of potassium phosphate buffer (pH = 7.0) and the CD spectrum was measured by a J-1500 spectropolarimeter (JASCO, Tokyo) at room temperature using 0.1 cm cell.
4. Chemical Synthesis of Sulforhodamine-B TfLURE by KAHA Ligation

4.1. Synthesis of Sulforhodamine-B Cys(Acm) Protected $\alpha$-Ketoacid Peptide 1a´

Sulforhodamine-B $\alpha$-ketoacid peptide 1a´ was synthesized according to General Procedure 2.1 on 0.30 mmol scale. After automated Fmoc SPPS, functionalized sulforhodamine-B carboxylic acid (1.1 equiv, 0.33 mmol) in anhydrous DMF was manually coupled with HATU (1.1 equiv, 0.33 mmol), NMM (2.2 equiv, 0.66 mmol) for 2 h at room temperature. The crude peptide was obtained after TFA cleavage described in General Procedure 2.1. The crude peptide was purified by preparative HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 15–65% CH$_3$CN in 20 min, flow rate 40 mL/min and re-purified using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm) at room temperature
with a gradient of 20–60% CH₃CN (with 10% DMF and 0.1% TFA) in 20 min, flow rate 20 mL/min. The fractions containing the desired product were pooled and lyophilized to give 33 mg of sulforhodamine B peptide 1a' (obtained from 1 g of dried resin after SPPS). The purity and identity of 1a' was confirmed using analytical HPLC and ESI-HRMS. m/z calculated for 1a' C₁₅₉H₂₁₀N₃₀O₄₄S₃ [M+2H]²⁺: 1633.7134 Da, Found: 1633.7108 Da.

4.2. Synthesis of Sulforhodamine-B Cys(Acm) Protected Protein 4a by KAHA Ligation

Peptide 4b was synthesized according to General Procedure 2.4 using peptide 2a (30 mg, 6.1 μmol, 1.0 equiv) and sulforhodamine-B peptide 1a' (24 mg, 7.4 μmol, 1.2 equiv). The reaction mixture was purified by preparative RP-HPLC using on a Phenomenex Jupiter C4 (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) at room temperature with a gradient of 10–60% CH₃CN with 0.1% TFA in 30 min, flow rate 20 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure 4b (27 mg, 54% yield). The purity and identity of 4b was confirmed using...
analytical HPLC and ESI-HRMS. The m/z calculated for 4b C\(_{364}H_{537}N_{87}O_{108}S_{8}\) [M+6H]\(^{6+}\): 1351.9494 Da; measured 1351.9446 Da.

(a) reaction time t = 0 h; (b) reaction time t = 24 h; (c) 2 h after addition of 6M Gdn•HCl solution pH 9.6; (d) purified 4b.
4.3. Synthesis of Sulforhodamine-B Linear Protein 5b

Sulforhodamine-B reduced linear protein 5b was synthesized according to General Procedure 2.5 with Cys(Acm) protected protein 4b (10 mg, 13 µmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm), with a gradient of 10–60% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure reduced protein 5b (5.7 mg, 60% yield). The purity and identity of 5b was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 5b C$_{346}$H$_{507}$N$_{81}$O$_{102}$S$_{8}$ [M+6H]$^{6+}$: 1280.7451 Da; measured 1280.7416 Da.

(a) reaction time t = 0 h; (b) reaction time t = 2 h; (c) purified 5b.
4.4. Synthesis of Folded Sulforhodamine B TfLURE Protein 6b

Folded Sulforhodamine B TfLURE protein 6b was synthesized according to General Procedure 2.6 with 1 mg of reduced protein 5b. The resulting solution was purified by preparative HPLC using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature with a gradient of 10–60% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure folded protein 6b (0.36 mg, 36% yield). The purity and identity of 6b was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 6b C$_{346}$H$_{500}$N$_{81}$O$_{102}$S$_{8}$ [M+5H]$^{5+}$: 1535.6839 Da; measured 1535.6811 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) purified 6b.
5. Chemical Synthesis of TcLURE Protein by KAHA Ligation

5.1. Synthesis of Cys(Acm) Protected α-Ketoacid Peptide 1b

![Peptide 1b structure](image)

Peptide 1b was synthesized according to General Procedure 2.1. The crude peptide was purified by preparative HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 15–65% CH$_3$CN (with 0.1% TFA) in 20 min, flow rate 40 mL/min and re-purified using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature with a gradient of 20–60% CH$_3$CN (with 0.1% TFA) in 20 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give 71 mg of peptide 1b (obtained from 1 g of dried resin after SPPS). The purity and mass of the peptide was confirmed using analytical HPLC and MALDI respectively. m/z calculated for 1b C$_{117}$H$_{168}$N$_{26}$O$_{35}$S [M+2H]$^{2+}$: 1264.5938 Da; measured 1264.5902 Da.
5.2. Synthesis of Cys(Acm) Protected 5-Oxaproline Peptide 2b

Peptide 2b was synthesized according to General Procedure 2.2. The crude peptide was purified by preparative HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with 10–65% CH$_3$CN (with 0.1% TFA) in 20 min, flow rate 40 mL/min and re-purified by a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature with 10–65% CH$_3$CN (with 0.1% TFA) in 20 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give 106 mg of desired peptide 2b (obtained from 1 g of dried resin after SPPS). The purity and identity of 2b was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 2b C$_{213}$H$_{343}$N$_{62}$O$_{67}$S$_{5}$ [M+7H]$^{7+}$: 714.4843 Da; measured 714.4816 Da.
5.3. Synthesis of Cys(Acm) Protected Linear Protein 4c by KAHA Ligation

Linear protein 4c was synthesized according to General Procedure 2.4 using peptide 2b (20 mg, 5 μmol, 1.0 equiv) and peptide 1b (30 mg, 6.0 μmol, 1.2 equiv). The reaction mixture was purified by preparative RP-HPLC on a Phenomenex Jupiter C4 (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm), heated to 60 °C, with a gradient of 10–60% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure Acm protected protein 4c (33 mg, 72% yield). The purity and identity of 4c was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 4c C₃₂₉H₅₁₀N₈₈O₁₀₀S₆ [M+8H]⁸⁺: 935.8230 Da; measured 935.8219 Da.
(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) 2 h after addition of 6M Gdn.HCl solution pH 9.6; (d) purified 4c.
5.4. Synthesis of Reduced Linear Protein 5c

Reduced linear protein 5c was synthesized according to General Procedure 2.5 with Cys(Acm) protected protein 4c (10 mg, 1.3 µmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature with a gradient of 30–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure 5c (6.1 mg, 65% yield). The purity and identity of 5c was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 5c C₃₁₁H₄₇₉N₈₂O₉₄S₆[M+7H]⁷⁺: 1008.3385 Da; measured 1008.3353 Da.

(a) reaction time t = 0 h; (b) reaction time t = 2 h; (c) purified 5c.
5.5. Synthesis of Folded TcLURE Protein 6c

Folded TcLURE protein 6c was synthesized according to General Procedure 2.6 with 1.0 mg of reduced linear protein 5c. The resulting solution was purified by preparative HPLC using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 30–80% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure folded TcLURE 6c (0.24 mg, 24% yield). The purity and identity of 6c was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 6c $C_{311}H_{474}N_{82}O_{94}S_{6}$ [M+8H]$^8^+$: 881.7892 Da; measured 881.7881 Da.

![Graphs showing retention time and CD spectra](image)

(a) reaction time t = 0 h; (b) reaction time t = 24 h; (c) purified 6c.

Folded TcLURE protein 6c was dissolved in 25 mM of potassium phosphate buffer (pH = 7.0) and the CD spectrum was measured by a J-1500 spectropolarimeter (JASCO, Tokyo) at room temperature using 0.1 cm cell.
6. Chemical Synthesis of Analogues of LURE Proteins by KAHA Ligation

6.1. Synthesis of Cys(Acm) Protected Linear Protein 4d by KAHA ligation

Linear protein 4d was synthesized according to General Procedure 2.4 using peptide 2b (30 mg, 6.0 µmol, 1.0 equiv) and peptide 1a (9.0 mg, 7.2 µmol, 1.2 equiv). The reaction mixture was purified by preparative RP-HPLC on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm), at room temperature; with a gradient of 10–60% CH₃CN with 0.1% TFA in 30 min, flow rate 10mL/min. The fractions containing the desired product were pooled and lyophilized to give pure Acm protected protein 4d (31 mg, 67% yield over two steps). The purity and identity of 4d was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 4d C₃₃₂H₅₁₃N₈₉O₁₀₂S₆ [M+8H]⁸⁺: 946.4500 Da; measured 946.4469 Da.
(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) 2 h after addition of 6M Gdn.HCl solution pH 9.6; (d) purified 4d.
6.2. Synthesis of Linear Protein 5d

Reduced linear protein 5d was synthesized according to General Procedure 2.5 with Cys(Acm) protected protein 4d (10 mg, 1.32 µmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at 25 °C, with a gradient of 30–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure 5d (6.4 mg, 68% yield). The purity and identity of 5d was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 5d C314H482N83O96S6[M+7H]7+: 1020.6243 Da; measured 1020.6245 Da.

(a) reaction time t = 0 h; (b) reaction time t = 2 h; (c) purified 5d.
6.3. Synthesis of Folded TfTcLURE Protein 6d

Folded TfTcLURE protein 6d was synthesized according to General Procedure 2.6 with 1.0 mg of reduced linear protein 5d. The resulting solution was purified by preparative HPLC using a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure folded TfTcLURE 6d (0.27 mg, 27% yield). The purity and identity of 6d was confirmed using analytical HPLC and ESI-HRMS. m/z calculated for 6d C₃₁₄H₄₇₇N₈₃O₉₆S₆ [M+8H]⁺: 892.4163 Da; measured 892.4154 Da. 

![Graph](image_url)

(a) reaction time t = 0 h; (b) reaction time t = 24 h; (c) purified 6d. 

Folded TfTcLURE protein 6d was dissolved in 25 mM of potassium phosphate buffer (pH = 7.0) and the CD spectrum was measured by a J-1500 spectropolarimeter (JASCO, Tokyo) at room temperature using 0.1 cm cell.
6.4. Synthesis of Cys(Acm) Protected Linear Protein 4e by KAHA ligation

Linear protein 4e was synthesized according to General Procedure 2.4 using peptide 2a (30 mg, 6.2 μmol, 1.0 equiv) and peptide 1b (19 mg, 7.4 μmol, 1.2 equiv). The reaction mixture was purified by preparative RP-HPLC on a Phenomenex Jupiter C4 (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) at room temperature with a gradient of 10–70% CH$_3$CN with 0.1% TFA in 30 min, flow rate 20 mL/min. The fractions containing the desired product were pooled and lyophilized to give pure Acm protected protein 4e (28 mg, 60% yield over two steps). The purity and identity of 4e was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 4e $\text{C}_{328}\text{H}_{497}\text{N}_{83}\text{O}_{99}\text{S}_6$ [M+8H]$^{8+}$: 921.9340 Da; measured 921.9304 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) 2 h after addition of 6M Gdn•HCl solution pH 9.6; (d) purified 4e.
6.5. Synthesis of Linear Protein 5e

Reduced linear protein 5e was synthesized according to General Procedure 2.5 with Cys(Acm) protected protein 4e (10 mg, 1.35 μmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter C18 (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) at room temperature, with a gradient of 30–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product was pooled and lyophilized to give pure 5e (6.8 mg, 72% yield). The purity and identity of 5e was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for 5e C₃₁₀H₆₆N₇₇O₉₃S₆ [M+7H]⁷⁺: 992.6060 Da; measured 992.6057 Da.

(a) reaction time t = 0 h; (b) reaction time t = 2 h; (c) purified 5e.
6.6 Synthesis of Folded TcTfLURE Protein 6e

Folded TcTfLURE protein 6e was synthesized according to General Procedure 2.6 with 1.0 mg of reduced linear protein 5e. The resulting solution was purified by preparative HPLC using a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 10–70% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired product was pooled and lyophilized to give pure folded TcTfLURE 6e (0.30 mg, 30% yield). The purity and identity of 6e was confirmed using analytical HPLC and ESI-HRMS. m/z calculated for 6e $C_{310}H_{454}N_{77}O_{95}S_{6}$ [M+7H]$^7^+$: 991.7421 Da; measured 991.7405 Da.

(a) reaction time $t = 0$ h; (b) reaction time $t = 24$ h; (c) purified 6e

Folded TcTfLURE protein 6e was dissolved in 25 mM of potassium phosphate buffer (pH = 7.0) and the CD spectrum was measured by a J-1500 spectropolarimeter (JASCO, Tokyo) at room temperature using 0.1 cm cell.
7. Analysis of LURE Proteins by SDS-Page
analyzed by SDS-PAGE (100 V, BIO-RAD mini-PROTEAN Tris-Tricine precast gel).
8. References

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