Supporting Information

Asparaginyl Endopeptidase-Mediated Peptide Ligation and Cyclization for Phage Display

Xiao-Cui Wan*, Yan-Ni Zhang*, Ying Chen, Zhi-Hui Cui, Hua Zhang, Wen-Jing Zhu, Ge-Min Fang*

*fanggm@ahu.edu.cn

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**General Information**

**Materials**
Amino acids for solid-phase peptide synthesis such as Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, et al., were ordered from GL BioChem (Shanghai, China). The Rinkamide AM resin for peptide synthesis was ordered from Nankaihecheng Ltd. N, N’-dimethylformamide (DMF), dichloromethane (DCM), TCEP (Tris(2-carboxyethyl)phosphine hydrochloride), cyano(hydroxyamino)acetate (oxyma Pure), N, N’-Diisopropyl-carbodiimide (DIC), N, N’-diisopropylethylamine (DIPEA), fluorescein isothiocyanate (FITC), N-hydroxysuccinimide (HOSu), D-biotin, glycolic acid and trifluoroacetic acid (TFA) (HPLC grade) were ordered from Energy Chemical Ltd. Formic acid (LC-MS grade) was ordered from Fisher Scientific. Acetonitrile (HPLC grade) for HPLC analysis and purification was purchased from J. T. Baker (Phillipsburg, NJ, USA). Yeast extract, agarose, agar, D-glucose, glycerin, 4S Red Plus nucleic acid stain, PBS (pH7.4), BSA, NaCl, kanamycin sulfate, ampicillin sodium salt, SanPrep column plasmid mini-preps kit, Klenow DNA polymerase and anhydrous ethanol were ordered from Sangon Biotech (Shanghai, China). NiSO₄, isopropyl-β-D-thiogalactopyranoside (IPTG), MgCl₂, PEG8000 and KCl were purchased from Shanghai Aladdin Biotech Ltd. Keypo enzyme, BsaI restriction enzyme and T4 ligase were ordered from Vazyme Biotech Co., Ltd. M13KE vector and ER2738 cells were ordered from NEB Ltd. TG1 cells and pCANTAB 5E vector were ordered from NB Biolab (Chengdu, China). DNA primers for PCR reaction were ordered from General Biol Ltd (Chuzhou, Anhui).

**HPLC, Mass Spectrometry (MS)**
HPLC experiments (reversed phase) were performed on Shimadzu Prominence HPLC systems (SPD-20A). For peptide analysis, Vydac C18 (4.6×250 mm) column was used, and the flow rate was set to be 1.0 mL/min. For peptide purification, Vydac C18 (10×250 mm) column was used, and the flow rate was set to be 3.0 mL/min. Water solution (**A solvent**) contains 1% acetonitrile and 0.1% TFA; acetonitrile solution (**B solvent**) contains 1% water and 0.1% TFA. For MS analysis of the peptide, LC-MS machine (Agilent 6520 Accurate-Mass Q-TOF ESI positive in high resolution mode) was used. The water solution for LC-MS contains 0.1% formic acid, and the acetonitrile solution for LC-MS contains 0.1% formic acid.

**General procedure for Peptide Synthesis**
All peptides mentioned in this study were prepared manually by using Fmoc-based solid-phase peptide synthesis. To a syringe filled with a filter was added rinkamide AM resin. The resin was swollen in DMF for 20 minutes at room temperature. Then, 20% piperidine in DMF was added to the resin for Fmoc deprotection. Wash the resin five times with DMF. The coupling of Fmoc-protected amino acid was conducted according to oxyma/DIC-based method: 4.5 equiv. Fmoc-protected amino acid (0.18 M in DMF), 4.5 equiv. DIC, and 4.5 equiv. oxyma. After 40 minutes at 55°C, the resin was washed five times with DMF. The unreacted amino group was capped by DMF/Ac₂O/2, 6-Lutidine (89/5/6). After 3 minutes at room temperature, the resin was washed five times with DMF. 20% piperidine in DMF was added to the resin for Fmoc deprotection. After assembly of peptide sequence, the resin was washed with DMF and DCM, and dried at room temperature. To the dried resin was added TFA cleavage cocktail (TFA/m-
cresol/water/TIPS, 88/5/5/2). After two hours at room temperature, pre-chilled ether (8~10 folds volume) was added to the combined TFA solution. After centrifugation, the crude peptide was obtained as a powder. The crude peptide was dissolved in a solvent, analyzed by HPLC, and confirmed by MS. After HPLC purification and lyophilization, the purified peptide was obtained as a powder.

**Experimental Section**

*Expression, purification and activation of OaAEP1[C247A]*

**Expression of SUMO-TEV-OaAEP1[C247A]**

DH5α *E. coli* containing SUMO-OaAEP1 plasmid (Addgene, plasmid#89482) was incubated with 3.0 mL LB medium containing kanamycin sulfate. After centrifugation, the cell pellet was obtained, and the desired SUMO-OaAEP1 plasmid was isolated from DH5α *E. coli*. To mutate 247Cys to 247Ala, four DNA primers were designed, as shown below.

- **Primer A:** 5′-TAATACGACTCACTATAGGG-3′
- **Primer B:** 5′-CGGACAATAATAAGGCCCCAGCTGCTTTTCGTTTGGTGGTGT-3′
- **Primer C:** 5′- ACCACCGAAAGCAGCTGGGCCCTATTATTTGCCCGGCAG-3′
- **Primer D:** 5′-GCTAGTTATTTGCTCAGCCG-3′

Starting from the template of SUMO-OaAEP1 plasmid, six 25 µL-scale PCR reactions were performed in the presence of Primer A, Primer B and PrimeSTAR. DNA Recovery Kit (Tiangen) was used to isolate the desired DNA product at a concentration of 32 ng/µL. At the same time, six 25 µL-scale PCR reactions were performed in the presence of Primer C, Primer D and PrimeSTAR. The desired DNA product was obtained by DNA Recovery Kit (Tiangen) at a concentration of 28 ng/µL. By combination of the two purified DNA samples, we conducted a third PCR reaction in the presence of Primer A, Primer D, and PrimeSTAR. Finally, the desired DNA mutant fragment was obtained at a concentration of 93.5 ng/µL.

The desired DNA mutant fragment (1.5 µg) was treated by BamHI and HindIII in 4×30 µL scale. At the same time, SUMO-OaAEP1 plasmid (2.0 µg) was treated by BamHI and HindIII in 2×50 µL scale. The SUMO-OaAEP1[C247A] fragment was obtained by DNA Recovery Kit (Tiangen) at a concentration of 10.2 ng/µL. The plasmid fragment was obtained by DNA Recovery Kit (Tiangen) at a concentration of 8.72 ng/µL.

The SUMO-OaAEP1[C247A] fragment was ligated with the plasmid fragment by T4 ligase at 22℃ for 2h. After heating at 65℃ for 10 min, the ligation product was transformed to DH5α *E. coli*. The desired DH5α *E. coil* containing SUMO-OaAEP1[C247A] was obtained after DNA sequencing of cell colonies. Then, the desired DH5α *E. coil* was incubated in 3.0 mL LB medium. After purification with DNA Recovery Kit (Sangon Biotech), we obtained the intact SUMO-OaAEP1[C247A] plasmid.

To insert a TEV sequence between SUMO and OaAEP1[C247A], we designed two DNA primers as shown below.

- **Primer E:** 5′-GCTGGATCCGAGGATCTGTACTTTTCGAGCGCTCGCGATGTTGATTATCT-3′
- **Primer F:** 5′-CGCAAGCTTACGGAATGCTCGCGCAG-3′

Starting from the template of SUMO-OaAEP1[C247A] plasmid, four 25 µL-scale PCR
reactions were performed in the presence of Primer E, Primer F and PrimeSTAR. With DNA Recovery Kit (Tiangen), we obtained the desired DNA product at a concentration of 108 ng/µL. The desired DNA product was treated by BamHI and HindIII in 2×30 µL scale. The desired DNA fragment was obtained by DNA Recovery Kit (Tiangen) at a concentration of 29.2 ng/µL. At the same time, SUMO-OaAEP1 plasmid was treated by BamHI and HindIII in 2×50 µL scale. After purification with DNA Recovery Kit (Tiangen), the plasmid fragment was obtained at a concentration of 22.1 ng/µL. The DNA fragment was ligated with the plasmid fragment by T4 ligase at 22°C for 2 hours. After heating at 65°C for 10 min, the ligation product was transformed to DH5α E. coil, and the desired DH5α E. Coil containing the plasmid of SUMO-TEV-OaAEP1[C247A] was obtained after DNA sequencing of cell colonies.

Expression and purification of OaAEP1[C247A]

The SUMO-TEV-OaAEP1[C247A] plasmid was transformed to BL21(DE3) E. coil. A colony from the BL21(DE3) cells was inoculated into 3.0 mL LB medium containing kanamycin sulfate. After incubation at 37°C for 10 h, 1.5 mL of the cells were inoculated in 500.0 mL LB medium containing kanamycin sulfate. After incubation at 37°C for 3 hours (OD600 = ~0.8), IPTG was added to the cell medium to give a final concentration of 0.3 mM. After incubation at 16°C for 20 h, the cells were centrifuged and washed with lysis buffer (20.0 mM Tris, 500.0 mM NaCl, 5% glycerol, pH 7.5). 50 mL of lysis buffer was added to the cells. After ultrasonic treatment, the supernatant was collected by 1-hour centrifugation (10000 rpm, 4°C). The SUMO-TEV-OaAEP1[C247A] was purified by Ni-NTA column. Change the protein buffer containing imidazole to lysis buffer (20.0 mM Tris, 500.0 mM NaCl, 5% glycerol, pH 7.5). After SDS-PAGE analysis, the purity of the protein is as high as 90%. The concentration of the protein was determined to be 18 mg/mL.

The concentration of SUMO-TEV-OaAEP1[C247A] was diluted to 1.0 mg/mL by adding lysis buffer. TEV protease was then added to the protein solution to give a final concentration of 0.1 mg/mL. After incubation at 25°C for 5 h, the intact OaAEP1[C247A] was obtained by Ni-NTA column. Note that, OaAEP1[C247A] was eluted by 20.0 mM imidazole in lysis buffer, and SUMO-TEV was eluted by 200.0 mM imidazole in lysis buffer. The purity of the eluted protein was analyzed by SDS-PAGE. High-quality protein was concentrated by ultracentrifugation. The protein was redissolved in lysis buffer without imidazole. The intact OaAEP1[C247A] was divided into several vials (216 µg/vial, in 12 µL lysis buffer) and stored at -80°C.

Activation of OaAEP1[C247A]

The intact OaAEP1[C247A] was activated under several conditions as shown below.

12.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 216.0 µg) was taken from a -80°C refrigerator. After melting at room temperature, OaAEP1[C247A] was added to 444.0 µL lysis buffer (20.0 mM Tris, 500.0 mM NaCl, 5% glycerol, pH 7.5) to give a final concentration of ~0.5 mg/mL. Then, the mixture was then transferred to a 3KD-scale dialysis membrane. The dialysis membrane was kept in pH4 buffer (100 mM NaOAc, 0.5 mM TCEP, 1.0 mM EDTA, pH4) at 4°C. After 8h and 20h, the supernatant was analyzed by SDS-PAGE, as shown in Figure S1. Note that, the activation of OaAEP1[C247A] was also performed at 4°C for 20 hours and 37°C for 5 hours. Under these conditions, the precipitation of OaAEP1[C247A] was observed, and the amount of the activated OaAEP1[C247A] in the supernatant is very low.
Figure S1: Activation of OaAEP1[C247A]. Band 1: Intact OaAEP1[C247A], Band 2: 8-hour activation at 4°C, Band 3: 20-hour activation at 4°C, Band 4: 20-hour activation at 4°C plus 5-hour activation at 37°C, Band 5: 5-hour activation at 37°C. Conditions: 100 mM NaOAc, 0.5 mM TCEP, 1.0 mM EDTA, pH4.

5.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 90.0 µg) was taken from a -80°C refrigerator. After melting at room temperature, OaAEP1[C247A] was added to 200.0 µL NaOAc buffer (50.0 mM NaOAc, 0.5 mM TCEP, 1.0 mM EDTA) to give a final concentration of 0.5 mg/mL. The mixture was kept at 37°C for 5 hours. Then, the supernatant was analyzed by SDS-PAGE. Note that, the activation of OaAEP1[C247A] was performed at pH6.0, 5.5, 5.0, 4.5 and 4.0. Under these conditions, the precipitation of OaAEP1[C247A] was observed, and the amount of the activated OaAEP1[C247A] in the supernatant is very low, as shown in Figure S2.

Figure S2: Activation of OaAEP1[C247A]. Band 1: 5-hour activation at pH6.5, Band 2: 5-hour activation at pH6.0, Band 3: 5-hour activation at pH5.5, Band 4: 5-hour activation at pH5.0, Band 5: 5-hour activation at pH4.5, Band 6: 5-hour activation at pH4.0, Band 7: the
precipitation from the activation sample at pH6.0.

*Optimization on the activation buffer*

i) 3.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125 µL NaOAc buffer (1.0 M NaOAc, 1.0 mM EDTA, 0.5 mM TCEP, pH4.0). The mixture was kept at 37°C for 5 hours.

ii) The pH value of 2.0 mL lysis buffer (20.0 mM Tris, 0.5 M NaCl, 5% glycerol, pH7.5) was adjusted to 4.0 by the addition of acetic acid/H₂O (1/1, v/v). Then, 24.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 432.0 µg) was added to the pH4 buffer. The mixture was kept at 37°C for 5 hours.

iii) The pH value of 2.0 mL TCEP in water (0.5 mM TCEP) was adjusted to 4.0 by the addition of acetic acid. Then, 3.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125 µL TCEP buffer (0.5 mM TCEP, pH4.0). The mixture was kept at 37°C for 5 hours.

iv) The pH value of 2.0 mL NaOAc in water (50.0 mM NaOAc) was adjusted to 4.0 by the addition of 6.0 M HCl. Then, 3.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5 hours.

The supernatant from the activation solution was analyzed by SDS-PAGE, as shown in Figure S3.

![Figure S3: Activation of OaAEP1[C247A]. Band 1: Intact OaAEP1[C247A], Band 2: 5-hour activation in NaOAc buffer (1.0 M NaOAc, 1.0 mM EDTA, 0.5 mM TCEP, pH4.0), Band 3: 5-hour activation in Tris buffer (20.0 mM Tris, 0.5 M NaCl, 5% glycerol, pH7.5), Band 4: 5-hour activation in TCEP buffer (0.5 mM TCEP, pH4.0), Band 5: 5-hour activation in NaOAc buffer (50.0 mM NaOAc, pH4.0).](image)

*Synthesis of*\(^{ClAc}\)*Asn or*\(^{Cab}\)*Lys or*\(^{Cab}\)*Gly or*\(^{Cab}\)*Asn-containing peptides*

(Note that, *\(^{ClAc}\)*Asn: N-terminal amino group of Asn is modified with chloroacetyl group; *\(^{Cab}\)*Lys: side-chain amino group of Lys is modified with 3,5-bis(2-chloroacetamido)benzoic acid; *\(^{Cab}\)*Gly: ...)
N-terminal amino group of Gly is modified with 3,5-bis(2-chloroacetamido)benzoic acid; 
\(\text{CabAsn}: \text{N-terminal amino group of Asn is modified with 3,5-bis(2-chloroacetamido)benzoic acid}\)

**Synthesis of 1**

1 was synthesized by Fmoc-based SPPS using 200 \(\mu\)mol scale of Rinkamide resin (loading of \(\sim 0.56 \text{ mmol/g}\)). After the assembly of the Asn-Ala-Leu-His sequence, the Fmoc-protecting group of the N-terminal Asn was deprotected with 20% piperidine in DMF. Then, a coupling mixture (4.5 equiv. chloroacetic acid, 4.5 equiv. DIC and 4.5 equiv. oxyma in 1.0 mL DMF) was added to the resin. After 60 min at 25°C, the resin was washed with DMF and DCM. After TFA cleavage, ether precipitation, preparative HPLC and lyophilization, 1 was obtained as a white powder (19.2 mg, 36.3 \(\mu\)mol, 18.2%).

Analytical HPLC: \(t_R = 19.2 \text{ min (2-90% B in 40 min)}\); \(m/z = 528.3\) \((C_{21}H_{33}N_8O_6Cl, \text{ calcd.: 528.2 g/mol})\)

Figure S4: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 1.

**Synthesis of 2**

2 was synthesized by Fmoc-based SPPS using 200 \(\mu\)mol scale of Rinkamide resin (loading of \(\sim 0.56 \text{ mmol/g}\)). After the assembly of the Asn-Gly-Leu-His sequence, the Fmoc-protecting group of the N-terminal Asn was deprotected with 20% piperidine in DMF. Then, a coupling mixture (4.5 equiv. chloroacetic acid, 4.5 equiv. DIC and 4.5 equiv. oxyma in 1.0 mL DMF)
was added to the resin. After 60 min at 25°C, the resin was washed with DMF and DCM. After TFA cleavage, ether precipitation, preparative HPLC and lyophilization, 2 was obtained as a white powder (20.1 mg, 39.0 µmol, 19.5%).

Analytical HPLC: t_R = 18.2 min (2-90% B in 40 min); m/z = 514.3 (C_{20}H_{31}N_{8}O_{6}Cl, calcd.: 514.2 g/mol).

Figure S5: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 2.

*Synthesis of 3*

3 was synthesized by Fmoc-based SPPS using 200 µmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After the coupling of glycolic acid, the resin was treated by 10% hydrazine in DMF for 30 min at room temperature. After washing the resin with DMF and DCM, a coupling mixture (5.0 equiv. Fmoc-Asn(Trt)-OH (0.59 g), 5.0 equiv. HOBt (135.1 mg), 140 µL DIC and 2.5 mg DMAP in 3 mL DCM/DMF(9/1, v/v)) was added to the resin. After 12h at 25°C, the resin was washed with DMF. After deprotection of Fmoc-protecting group, a coupling mixture (4.5 equiv. chloroacetic acid, 4.5 equiv. DIC, 4.5 equiv. oxyma in 1.0 mL DMF) was added to the resin. After 60 min at 25°C the resin was washed with DMF and DCM. After TFA cleavage, ether precipitation, preparative HPLC and lyophilization, 3 was obtained as a white powder (18.9 mg, 36.7 µmol, 18.3%).

Analytical HPLC: t_R = 19.8 min (2-90% B in 40 min); m/z = 515.3 (C_{20}H_{30}N_{7}O_{7}Cl, calcd.: 515.2 g/mol)
Figure S6: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 3.

*Synthesis of 6*

6 was synthesized in several steps, as shown below.

[Synthesis of 6a]

6a was synthesized by Fmoc-based SPPS using 200 µmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After TFA cleavage and ether precipitation, 6a was obtained as a white powder.

Analytical HPLC: $t_R = 20.3$ min (2-90% B in 40 min); m/z = 747.4 (C$_{32}$H$_{53}$N$_{13}$O$_{6}$S, calcd.: 747.4 g/mol).
Figure S7: (a) HPLC trace (210nm) and (b) MS spectrum of the crude 6a.

[Synthesis of 6b]

To 1.0 mL DMF were added Fmoc-Asn(Trt)-OH (38.0 mg, 80.0 µmol), HOSu (20.0 mg, 160.0 µmol) and DIC (32.0 µl, 160.0 µmol). After 6 h at room temperature, 10.0 µL DIPEA and 6a (7.4 mg, 10 µmol) was added. After 30 min at room temperature, the reaction was analyzed by HPLC. After preparative HPLC and lyophilization, 6b was obtained as a white powder (5.0 mg, 4.2 µmol, 42.0%).

Analytical HPLC: t_R = 27.5 min (2-90% B in 40 min); m/z = 1203.8 (C_{60}H_{81}N_{15}O_{10}S, calcd.: 1203.6 g/mol)

Figure S8: (a) HPLC trace (210nm) and (b) MS spectrum of the crude 6b.

[Synthesis of 6c and 6]
6b (5.0 mg) was dissolved in TFA/H₂O (4:1). After 1 hour at room temperature, the crude 6c was obtained as a white powder by ether precipitation. To 6c was added a coupling mixture (4.5 equiv. chloroacetic acid, 4.5 equiv. DIC, 4.5 equiv. oxyma in 1.0 mL DMF). After 60 minutes at 25°C, the reaction was analyzed by HPLC. After preparative HPLC and lyophilization, 6 was obtained as a white powder (3.0 mg, 56.1%).

Analytical HPLC of 6c: tᵣ = 17.4 min (2-90% B in 40 min); m/z = 862.0 (C₃₆H₅₉N₁₅O₈S, calcd.: 862.0 g/mol)

Analytical HPLC of 6: tᵣ = 22.2 min (2-90% B in 40 min); m/z = 937.6 (C₃₈H₆₀ClN₁₅O₉S, calcd.: 937.4 g/mol)
Figure S9: (a) HPLC trace (210nm) and (b) MS spectrum of 6c; (c) HPLC trace (210nm) and (d) MS spectrum of the purified 6.

**Synthesis of Biotin-1**

Biotin-1 was synthesized by Fmoc-based SPPS using 200 μmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After the coupling of glycolic acid, the resin was treated by 10% hydrazine in DMF for 30 min at room temperature. After washing the resin with DMF and DCM, a coupling mixture (5.0 equiv. Fmoc-Asn(Trt)-OH (0.59 g), 5.0 equiv. HOBt (135.1 mg), 140 μL DIC and 2.5 mg DMAP in 3 mL DCM/DMF(9/1, v/v)) was added to the resin. After 12 hours at 25°C, the resin was washed with DMF. After deprotection of Fmoc-protecting group, the coupling of Fmoc-Lys (Mtt)-OH and the coupling of Fmoc-Gly-OH were performed, respectively. Then, the Mtt-protecting group was deprotected with HFIP/DCM (1:4, v/v, 30 min×4), followed by the coupling of Biotin (119.0 mg of Biotin, 127.0 mg of oxyma, and 140.0 mg...
µL of DIC). After deprotection of Fmoc-protecting group of Gly, the coupling of chloroacetic acid was performed. After 60 minutes at 25°C, the resin was washed with DMF and DCM. After TFA cleavage, ether precipitation, preparative HPLC and lyophilization, **Biotin-1** was obtained as a white powder (20.9 mg, 22.6 µmol, 11.3%).

Analytical HPLC: $t_R = 21.7$ min (2-90% B in 40 min); m/z = 926.5 (C$_{38}$H$_{59}$ClN$_{12}$O$_{11}$S, calcd.: 926.4 g/mol)

![Figure S10: (a) HPLC trace (210nm) and (b) MS spectrum of Biotin-1.](image)

**Synthesis of 9**

9 was synthesized in several steps, as shown below.

[Synthesis of 9a]

9a was synthesized by Fmoc-based SPPS using 200 µmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After the coupling of glycolic acid, the resin was treated by 10% hydrazine in DMF for 30 min at room temperature. After washing the resin with DMF and...
DCM, a coupling mixture (5.0 equiv. Fmoc-Asn(Trt)-OH (0.59 g), 5.0 equiv. HOBt (135.1 mg), 140 μl DIC and 2.5 mg DMAP in 3 mL DCM/DMF (9/1, v/v)) was added to the resin. After 12 hours at 25℃, the resin was washed with DMF. After deprotection of Fmoc group, a coupling mixture (4.5 equiv. Fmoc-Lys (Boc)-OH, 4.5 equiv. DIC, 4.5 equiv. oxyma in 1.0 mL DMF) was added to the resin. After 40 minutes at 55℃, the resin was washed with DMF and DCM. After Fmoc deprotection, 2.0 mL of a capping solution (DMF: 2,6-lutidine: acetic anhydride=89:6:5, v/v) was added. 2 minutes later, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 9a was obtained as a white powder (18.9 mg, 36.7 μmol, 18.3%).

Analytical HPLC: t_R = 17.5 min (2-90% B in 40 min); m/z = 609.4 (C_{26}H_{43}N_{9}O_{8} calcd.: 609.3 g/mol).

Figure S11: (a) HPLC trace (210nm) and (b) MS spectrum of the crude 9a.

[Synthesis of 3,5-bis(2-chloroacetamido) benzoic acid]

3, 5-Diaminobenzoic acid (2.0 g, 20.0 mmol, 1.0 equiv.) was dissolved into 10.0 mL DMF. The mixture was stirred at 0℃ for 10 minutes. Then, 5.2 mL pyridine was added dropwise. Keep the reaction at 0℃ for 5 minutes. Then, 8.9 g acetic anhydride in 10.0 mL DMF was added dropwise. Keep the reaction at room temperature for 18 hours. Excessive DCM was added to the mixture until the precipitation of the desired product. The precipitation was washed with water and dried under a vacuum to give 3,5-bis(2-chloroacetamido) benzoic acid as a yellow powder (0.20 g, 3.3%).

Analytical HPLC: t_R = 22.6 min (2-90% B in 40 min); m/z = 304.1 (C_{11}H_{10}Cl_{2}N_{2}O_{4}, calcd.: 304.0 g/mol).
Figure S12: (a) HPLC trace (210nm) and (b) MS spectrum of the crude 3,5-bis(2-chloroacetamido) benzoic acid.

[Synthesis of 9]

To 0.8 mL DMF were added 3,5-bis(2-chloroacetamido) benzoic acid (15.2 mg, 50.0 µmol), N-hydroxysuccinimide (23.0 mg, 200.0 µmol) and DIC (31.2 µL). After 1 hour at room temperature, the mixture was quenched by 3.0 mL acetonitrile/water (1/1, v/v, 0.1% TFA). After preparative HPLC and lyophilization, the N-hydroxysuccinimide (NHS) ester of 3,5-bis(2-chloroacetamido) benzoic acid was obtained as a white powder.

To 0.2 mL DMF were added NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (10.0 mg, 25.0 µmol) and 9a (4.5 mg, 7.4 µmol). After the addition of 2.0 µL DIPEA, the reaction was stirred at room temperature for 60 minutes. 3.0 mL acetonitrile/water (1/1, v/v, 0.1% TFA) was added to quench the reaction. After HPLC purification and lyophilization, 9 was obtained as a white powder (3.8 mg, 4.2 µmol, 56.7%).

Analytical HPLC: $t_R = 22.7$ min (2-90% B in 40 min); m/z = 895.4 (C$_{37}$H$_{51}$Cl$_2$N$_{11}$O$_{11}$, calcd.: 895.3 g/mol).
**Synthesis of 10**

10a was prepared by Fmoc-based SPPS using 200 μmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After the coupling of glycolic acid, the resin was treated by 10% hydrazine in DMF for 30 min at room temperature. After washing the resin with DMF and DCM, a coupling mixture (5.0 equiv. Fmoc-Asn-Trt-OH (0.59 g), 5.0 equiv. HOBt (135.1 mg), 140 μL DIC and 2.5 mg DMAP in 3 mL DCM/DMF (9/1, v/v)) was added to the resin. After 12 hours at 25℃, the resin was washed with DMF. After deprotection of Fmoc group, a coupling mixture (4.5 equiv. Boc-Gly-OH, 4.5 equiv. DIC, 4.5 equiv. oxyma in 1.0 mL DMF) was added to the resin. After 40 minutes at 55℃, the resin was washed with DMF and DCM. The resin was washed with DMF and DCM. After TFA cleavage, ether precipitation, HPLC purification and lyophilization, 10a was obtained as a white powder.

To 0.2 mL DMF were added NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (10.0 mg, 25 μmol) and 10a (4.0 mg, 8.0 μmol). After the addition of 2.0 μL DIPEA, the reaction was stirred at room temperature for 60 minutes. 3.0 mL acetonitrile/water (1/1, v/v, 0.1% TFA) was added to quench the reaction. After HPLC purification and lyophilization, 10 was obtained as a white powder (3.2 mg, 4.0 μmol, 50.0%).

Analytical HPLC of 10a: t<sub>R</sub> = 17.1 min (2-90% B in 40 min); m/z = 496.4 (C<sub>20</sub>H<sub>32</sub>N<sub>8</sub>O<sub>7</sub>, calcd.: 496.2 g/mol)

Analytical HPLC of 10: t<sub>R</sub> = 21.7 min (2-90% B in 40 min); m/z = 783.8 (C<sub>31</sub>H<sub>40</sub>N<sub>10</sub>O<sub>10</sub>Cl<sub>2</sub>, calcd.: 783.6 g/mol)
Figure S14: (a) HPLC trace (210nm) and (b) MS spectrum of the crude 10a; (c) HPLC trace (210nm) and (d) MS spectrum of the purified 10.

**Synthesis of 11**

11a was assembled by Fmoc-based SPPS using 10 μmol scale of Rinkamide resin (loading of ~0.56 mmol/g). After the coupling of glycolic acid, the resin was treated by 10% hydrazine in DMF for 30 min at room temperature. After washing the resin with DMF and DCM, a coupling mixture (5.0 equiv. Fmoc-Asn(Trt)-OH (0.59 g), 5.0 equiv. HOBt (135.1 mg), 140 μL DIC and 2.5 mg DMAP in 3 mL DCM/DMF (9/1, v/v)) was added to the resin. After 12 hours at 25°C, the resin was washed with DMF, and the Fmoc group was deprotected with 20% piperidine in DMF. To 10 μmol of 11a-bound resin were added NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (20.0 mg, 40 μmol) and 2.0 μL of DIPEA. After 12 hours at room temperature, the peptide 11 was released from the resin by TFA cleavage. After HPLC
purification and lyophilization, 11 was obtained as a white powder (5.0 mg, 50.0%).
Analytical HPLC: $t_R = 20.8 \text{ min (2-90\% B in 40 min)}$; $m/z = 725.5$ (C$_{29}$H$_{37}$N$_9$O$_{10}$Cl$_2$, calcd: 725.2 g/mol).

Figure S15: HPLC trace (210 nm) and MS spectrum of the purified 11.

Synthesis of N-terminal Gly peptides

All N-terminal Gly peptides including 4, mutants of 4, and 8 are prepared by Fmoc-based SPPS using Rinkamide Resin.

4

H-Gly-Leu-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.7 \text{ min (2-90\% B in 40 min)}$; $m/z = 1826.8$ (C$_{81}$H$_{118}$N$_{24}$O$_{23}$S, calcd.: 1827.2 g/mol).

4[Leu2Glu]

H-Gly-Glu-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 20.7 \text{ min (2-90\% B in 40 min)}$; $m/z = 1842.7$ (C$_{80}$H$_{114}$N$_{23}$O$_{25}$S, calcd.: 1842.8 g/mol).

4[Leu2Lys]

H-Gly-Lys-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 20.4 \text{ min (2-90\% B in 40 min)}$; $m/z = 1841.8$ (C$_{81}$H$_{119}$N$_{23}$O$_{23}$S, calcd.: 1841.8 g/mol).

4[Leu2His]

H-Gly-His-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 20.4 \text{ min (2-90\% B in 40 min)}$; $m/z = 1850.8$ (C$_{81}$H$_{114}$N$_{20}$O$_{23}$S, calcd.: 1850.8 g/mol).

4[Leu2Asp]

H-Gly-Asp-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$
Analytical HPLC: $t_R = 20.6$ min (2-90% B in 40 min); $m/z = 1828.8$ (C$_{79}$H$_{112}$N$_{24}$O$_{22}$S, calcd.: 1828.8 g/mol).

4[Leu2Phe]
H-Gly-Phe-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.8$ min (2-90% B in 40 min); $m/z = 1860.6$ (C$_{84}$H$_{116}$N$_{24}$O$_{23}$S, calcd.: 1860.8 g/mol).

4[Leu2Ile]
H-Gly-Ile-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.6$ min (2-90% B in 40 min); $m/z = 1826.8$ (C$_{81}$H$_{118}$N$_{24}$O$_{23}$S, calcd.: 1826.8 g/mol).

4[Leu2Met]
H-Gly-Met-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.2$ min (2-90% B in 40 min); $m/z = 1844.8$ (C$_{80}$H$_{116}$N$_{24}$O$_{23}$S$_2$, calcd.: 1844.8 g/mol).

4[Leu2Val]
H-Gly-Val-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.2$ min (2-90% B in 40 min); $m/z = 1812.8$ (C$_{80}$H$_{116}$N$_{24}$O$_{23}$S, calcd.: 1812.8 g/mol).

4[Leu2Gly]
H-Gly-Gly-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 20.7$ min (2-90% B in 40 min); $m/z = 1770.8$ (C$_{78}$H$_{112}$N$_{24}$O$_{22}$S, calcd.: 1770.8 g/mol).

4[Leu2Pro]
H-Gly-Pro-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 21.1$ min (2-90% B in 40 min); $m/z = 1810.8$ (C$_{80}$H$_{114}$N$_{24}$O$_{23}$S, calcd.: 1810.8 g/mol).

4[Leu2Ala]
H-Gly-Ala-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 25.6$ min (2-90% B in 30 min); $m/z = 1784.8$ (C$_{79}$H$_{112}$N$_{24}$O$_{23}$S, calcd.: 1784.8 g/mol).

4[Leu2Thr]
H-Gly-Thr-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH$_2$

Analytical HPLC: $t_R = 20.4$ min (2-90% B in 40 min); $m/z = 1814.8$ (C$_{79}$H$_{114}$N$_{24}$O$_{23}$S, calcd.:
4[Leu2Trp]

H-Gly-Trp-Tyr-Asp-Pro-Ala-Asn-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH₂

Analytical HPLC: tᵣ = 22.1 min (2-90% B in 30 min); m/z = 1899.8 (C₁₈₆H₁₁₇N₂₅O₂₃S, calcd.: 1889.8 g/mol).

8

H-Gly-Leu-Tyr-Asp-Pro-Ala-Asn-Cys-Ile-His-Pro-Lys-Gly-Trp-Cys-Gly-Gly-Ser-Gly-NH₂

Analytical HPLC: tᵣ = 22.0 min (2-90% B in 40 min); m/z = 1929.8 (C₁₈₄H₁₂₃N₂₅O₂₄S₂, calcd.: 1929.8 g/mol).

OaAEP1[C247A]-mediated peptide monocyclization

Enzymatic Ligation between 1 and 4

[Case 1-HEPES buffer, pH7.7]

12 µL of the intact OaAEP1[C247A] (18 mg/mL, 216.0 µg) taken from a -80°C refrigerator was added to 500 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5 hours. Then, 1.0 mL of HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM CaCl₂, 1.0 mM TCEP, pH8.5) was added dropwise to the activation buffer. The pH value of the mixture was determined to be 7.7. To the pH7.7 buffer were added 6.0 µL of 1 (125 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 12.5 µL of 4 (12.5 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S16. Ligation yield: <20%.

Figure S16: (a) HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4; (b) MS spectrum of the ligation product 5.
[Case 2-HEPES buffer, pH6.6]
12 µL of the intact OaAEPI[C247A] (18 mg/mL, 216.0 µg) taken from a -80°C refrigerator was added to 500 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 1.0 mL of HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM CaCl₂, 1.0 mM TCEP, pH7.4) was added dropwise to the activation buffer. The pH value of the mixture was determined to be 6.6. To the pH6.6 buffer were added 6.0 µL of 1 (125 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 12.5 µL of 4 (12.5 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S17. Ligation yield: 38.3%.

Figure S17: HPLC trace (210 nm) of OaAEPI[C247A] mediated peptide ligation between 1 and 4 at pH6.6.

[Case 3-NaOAc buffer, pH3.5]
3.0 µL of the intact OaAEPI[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH3.5). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc, pH3.5) was added to the activation buffer. The pH value of the mixture was determined to be 3.5. To the pH3.5 buffer were added 3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S18. Ligation yield: 0%.
Figure S18: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 at pH3.5

[Case 4-NaOAc buffer, pH4.5]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.5). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc, pH4.5) was added to the activation buffer. The pH value of the mixture was determined to be 4.5. To the pH4.5 buffer were added 3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S19. Ligation yield: 45.9%.

Figure S19: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 at pH4.5

[Case 5-NaOAc buffer, pH5.5]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH5.5). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc, pH5.5) was added to the activation buffer. The pH value of the mixture was determined to be 5.5. To the pH5.5 buffer were added
3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S20. Ligation yield: <20%.

Figure S20: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 at pH5.5

[Case 6-NaOAc buffer, pH6.5]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH6.5). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc, pH6.5) was added to the activation buffer. The pH value of the mixture was determined to be 6.5. To the pH6.5 buffer were added 3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S21. Ligation yield: 0%.

Figure S21: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 at pH6.5
[Case 7-NaOAc buffer, activation of OaAEP1[C247A] at pH4.0 & ligation at pH5.0]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S22. Ligation yield: 86.7%.

![HPLC trace](image)

Figure S22: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 at pH5 (activation of OaAEP1[C247A] at pH4.0).

[Case 8-NH₄OAc buffer, activation of OaAEP1[C247A] at pH4.0 & ligation at pH5.0]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NH₄OAc buffer (50.0 mM NH₄OAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NH₄OAc buffer (50.0 mM NH₄OAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 1 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S23. Ligation yield: 87.1%.

![HPLC trace](image)
Figure S23: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 1 and 4 in NH₄OAc buffer.

*Enzymatic Ligation between 2 and 4*

![Enzymatic Ligation between 2 and 4](image)

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 2 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S24. Ligation yield: 67.0%.

![HPLC trace](image)

5 min

120 min

min

Figure S24: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 2 and 4.

*Enzymatic Ligation between 3 and 4*

![Enzymatic Ligation between 3 and 4](image)

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 3 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S24. Ligation yield: 67.0%.
mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 37°C, 25°C or 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S25-S27. Ligation yield: >95%.

Figure S25: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4 at 37°C in NaOAc buffer. Note that OaAEP1[C247A] precipitates after 30 min.

Figure S26: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4 at 25°C in NaOAc buffer. Note that OaAEP1[C247A] precipitates after 30 min.
3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NH₄OAc buffer (50.0 mM NH₄OAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NH₄OAc buffer (50.0 mM NH₄OAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 3 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 25°C or 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S28-S29. Ligation yield: >95%.

Figure S27: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4 at 4°C in NaOAc buffer. Note that OaAEP1[C247A] does not precipitate even after 3 hours.

Figure S28: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4 at 25°C in NH₄OAc buffer. Note that OaAEP1[C247A] does not precipitate after 15 minutes.
Figure S29: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4 at 4°C in NH₄OAc buffer. Note that OaAEP1[C247A] does not precipitate.

**Enzymatic Ligation between 6 and 4**

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 6 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S30. Ligation yield: >95%.
Enzymatic Ligation between 3 and 4[Leu2Ile]

3.0 µL of the intact *Oa*AEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NH₄OAc buffer (50.0 mM NH₄OAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NH₄OAc buffer (50.0 mM NH₄OAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 3 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4[Leu2Ile] (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S31. Ligation yield: 67.8%.
Figure S31: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4[Leu2Ile] at 4°C in NH₄OAc buffer and the MS spectrum of ligation product.

Enzymatic Ligation between 3 and 4[Leu2Val]

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NH₄OAc buffer (50.0 mM NH₄OAc, pH4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NH₄OAc buffer (50.0 mM NH₄OAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 3 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 4[Leu2Val] (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S32. Ligation yield: 37%.

![HPLC traces](image)

Figure S32: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4[Leu2Val] at 4°C in NH₄OAc buffer and the MS spectrum of ligation product.

Note that, other 12 mutant of 4 did not react with 3 in the presence of the activated OaAEP1[C247A].
Figure S33: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 3 and 4[Leu2Xaa] (Xaa=Glu, Lys, His, Asp, Phe, Gly, Met, Pro, Ala, Arg, Trp, Thr) at 4°C in NH₄OAc buffer.

Intramolecular cyclization of the ligation product 5

After the enzymatic ligation of 3 and 4, TCEP was added to the mixture to give a final concentration of 1.0 mM. Then, the pH value of the reaction mixture was adjusted to 8.0 by the addition of 6.0 M NaOH. Keep the mixture at 37°C (250 rpm) for 30 min. The reaction was quenched by 0.1% TFA in water, and analyzed by HPLC, as shown in Figure S34.
Figure S34: HPLC trace (210 nm) of the intramolecular cyclization of the ligation product 5 in pH8.0 buffer at 37℃.

*OaAEP1[C247A]-mediated peptide bicyclization*

*Enzymatic Ligation between 9 and 8*

3.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 54.0 µg) taken from a -80℃ refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37℃ for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 9 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 8 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4℃ (250 rpm). The reaction was monitored by HPLC, as shown in Figure S35. Ligation yield: >95%.

Figure S35: HPLC trace (210 nm) of *OaAEP1[C247A]* mediated peptide ligation between 9 and 8, and the MS spectrum of ligation product 12.
Figure S36: HPLC trace (210 nm) of peptide ligation between 9 and 8 after one-hour incubation at pH5.0 at 4°C, in absence of OaAEP1[C247A] (no ligation).

*Intramolecular cyclization of the ligation product 12*

After the enzymatic ligation of 9 and 8, TCEP was added to the mixture to give a final concentration of 1.0 mM. Then, the pH value of the reaction mixture was adjusted to 8.0 by the addition of 6.0 M NaOH. Keep the mixture at 37°C (250 rpm) for 30 min. The reaction was quenched by 0.1% TFA in water, and analyzed by HPLC, as shown in Figure S37.

Figure S37: HPLC trace (210 nm) of the intramolecular cyclization of the ligation product 12 in pH8.0 buffer at 37°C.

Note that we observed hydrolysis of the remaining 9 in the pH8.0 cyclization buffer. As a control, 9 was dissolved in the pH8.0 cyclization buffer, but in absence of OaAEP1[C247A] and 8. After 30-min incubation at 37°C (250 rpm), we observed the hydrolysis of 9 as shown in Figure S38b.
Figure S38: Hydrolysis of 9 in the cyclization buffer (pH 8.0). a) HPLC trace (210 nm) of the intramolecular cyclization of the ligation product 12 in pH 8.0 buffer at 37°C (9-i, 9-ii and 9-iii are the hydrolysis products from 9); b) HPLC trace (210 nm) of 9 in pH 8.0 buffer at 37°C for 30 minutes.

**Enzymatic Ligation between 10 and 8**

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH 4.0). The mixture was kept at 37°C for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH 5.0 buffer were added 3.0 µL of 10 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 8 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4°C (250 rpm). The reaction was monitored by HPLC, as shown in Figure S39. Ligation yield: >95%.

Figure S39: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 10
and 8, and the MS spectrum of ligation product 13.

**Intramolecular cyclization of the ligation product 13**

After the enzymatic ligation of 10 and 8, TCEP was added to the mixture to give a final concentration of 1.0 mM. Then, the pH value of the reaction mixture was adjusted to 8.0 by the addition of 6.0 M NaOH. Keep the mixture at 37°C (250 rpm) for 30 min. The reaction was quenched by 0.1% TFA in water, and analyzed by HPLC, as shown in Figure S40.

**Enzymatic ligation at pH4**

**Intramolecular cyclization at pH8**

Figure S40: HPLC trace (210 nm) of the intramolecular cyclization of the ligation product 13 in pH8.0 buffer at 37°C.

Note that we observed hydrolysis of the remaining 10 in the pH8.0 cyclization buffer. As a control, 10 was dissolved in the pH8.0 cyclization buffer, but in absence of OtaAEPI[C247A] and 8. After 30-min incubation at 37°C (250 rpm), we observed the hydrolysis of 10 as shown in Figure S41b.

**Figure S41: Hydrolysis of 9 in the cyclization buffer (pH8.0). a) HPLC trace (210 nm) of the**
intramolecular cyclization of the ligation product 13 in pH8.0 buffer at 37℃ (10-i, 10-ii and 10-iii are the hydrolysis products from 10); b) HPLC trace (210 nm) of 10 in pH8.0 buffer at 37℃ for 30 minutes.

*Enzymatic Ligation between 11 and 8*

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80℃ refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37℃ for 5h. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added 3.0 µL of 11 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture) and 3.0 µL of 8 (6.25 mM in a stock solution, a final concentration of 50.0 µM in the reaction mixture). The reaction was kept at 4℃ (250 rpm). The reaction was monitored by HPLC, as shown in Figure S42. Ligation yield: >95%.

Figure S42: HPLC trace (210 nm) of OaAEP1[C247A] mediated peptide ligation between 11 and 8, and the MS spectrum of ligation product 14.

*Intramolecular cyclization of the ligation product 14*

After the enzymatic ligation of 11 and 8, TCEP was added to the mixture to give a final concentration of 1.0 mM. Then, the pH value of the reaction mixture was adjusted to 8.0 by the addition of 6.0 M NaOH. Keep the mixture at 37℃ (250 rpm) for 30 min. The reaction was quenched by 0.1% TFA in water, and analyzed by HPLC, as shown in Figure S43.

S36
Figure S43: HPLC trace (210 nm) of the intramolecular cyclization of the ligation product 14 in pH8.0 buffer at 37℃.

**Construction of M13KE phage library**

A M13KE-GX12C phage library (a diversity of 10⁶) was taken from our previous study on phage displayed peptide library. For the enzymatic ligation on phage displayed library, a peptide sequence of GLSFYSHS was introduced to the N-terminus of the GX12 phage library. The phage vector was constructed by the use of BsaI restriction enzyme. Two DNA primers were ordered from Sangon Biotech Ltd (Shanghai), as shown below.

Primer G: 5’-TTGGTCTCGGCTTATCTTTCTATTCTCACTCTGGC-3’
Primer H: 5’-TTTGGTCTCAAAGCCAGAGTGAGAATAGAAAGGTACCAC-3’

Starting from the vector of M13KE-GX12C, PCR reaction was performed with the presence of Primer G, Primer H, and KeyPo DNA polymerase. The PCR product was purified by Universal DNA purification kit (Tiangen BioTech Beijing Ltd) and treated by BsaI restriction enzyme and DpnI restriction enzyme. After DNA ligation with T4 ligase, the constructed vector of M13KE-GLSFYSHSGX12C was electro transformed into *E. coli* ER2738. The quality of the library was evaluated by DNA sequencing of the phage colonies.

**Biotin-avidin pull-down on M13KE phage**

3.0 µL of the intact *OaAEP1[C247A]* (18 mg/mL, 54.0 µg) taken from a -80℃ refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37℃ for 5 hours. Then, 0.25 mL of NaOAc buffer (50.0 mM NaOAc in water) was added to the activation buffer. The pH value of the mixture was determined to be 5.0. To the pH5.0 buffer were added M13KE phage (2×10¹¹ pfu) and **Biotin-1** (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture). The reaction was kept at 4℃ (250 rpm) for 1 hour. Then, 72.0 µL of the polyethylene glycol solution (4% PEG8000, 0.5 M NaCl) was added to precipitate M13KE phage. The phage pellet was suspended in the cyclization buffer (50 mM NaOAc, 1.0 mM TCEP, pH8.0). After 30 min at 37℃ (250 rpm), the M13KE phage was precipitated with PEG8000. The phage was resuspended in TBS (50.0 mM Tris, 150.0 mM...
NaCl, pH7.5) and diluted to a concentration of 10^5 pfu/mL. 20.0 µL of the diluted phage solution were added two clean empty tubes, named A and B. 20.0 µL of PBS-prewashed streptavidin coated beads were resuspended in 50.0 µL of binding buffer (10.0 mM Tris, 150.0 mM NaCl, 10.0 mM MgCl2, 1.0 mM CaCl2, pH7.4) and 50.0 µL of blocking buffer (10.0 mM Tris, 150.0 mM NaCl, 10.0 mM MgCl2, 1.0 mM CaCl2, 0.3% Tween-20, 3% w/v BSA, pH7.4). After 1 hour at 37°C, the buffer was transferred to the A tube. After 30 min at room temperature, the beads in the A tube were captured on a magnetic separation device. Collect the supernatant with a clean empty tube. The beads were washed twice with 200.0 µL of washing buffer (10.0 mM Tris, 150.0 mM NaCl, 10.0 mM MgCl2, 1.0 mM CaCl2, 0.1% Tween-20, pH7.4). The phage in B tube was treated by the same procedure as described above. The titer of the collected phage in A tube and B tube was measured. The yield of the Biotin-1 modification on phage surface was calculated as follows: modified phage (%) = [(B titer - A titer)/ B titer] × 100%.

Figure S44: HPLC trace (210 nm) of OaAE1[C247A]-mediated peptide ligation between Biotin-1 and 4 (Biotin-1 overlaps with 4).

Figure S45: Phage titers after streptavidin magnetic bead pull-down of the sample B and the sample A.
Construction of pCANTAB 5E-GLX12C phage library

The phage displayed GLX12C peptide library was constructed by the use of pCANTAB 5E vector and M13KO7 helper phage (from Nanobody-biolab Ltd., Sichuan). In this phage displayed GLX12C peptide library, X is any L-amino acid encoded by NNK, N is A or T or C or G, K is G or T. The peptide library was inserted into the pCANTAB 5E vector by BsaI restriction enzyme. Four DNA strands were ordered from Sangon Biotech Ltd (Shanghai), as shown below.

Primer I: 5’-GAGCGTGTTGCTCGGTATCATTGCAGCAC-3’
Primer J: 5’-ACCGCGCGACCCACGCTCACCGGCTC-3’
Primer K: 5’-TTGGTCTCGGTGCGCCGATCCCGATCCCGCTG-3’
Primer L:
5’-TTTGGTCTCAGCCACCGGAGGCGTCGCGCGCATGGCCGGCTGGGCCGCATAGAAAGG-3’ (M is C or A)

Starting from the template of pCANTAB 5E vector, PCR reactions were performed in the presence of Primer I, Primer J and Keypo enzyme. DNA Recovery Kit (Tiangen) was used to isolate the desired DNA product. The DNA product was treated by DpnI restriction enzyme, and then was transformed into TG1 cells. After sequencing the transformed colonies, we obtained the desired pCANTAB 5E’ that does not contain the BsaI restriction site.

Starting from the template of pCANTAB 5E’ vector, PCR reactions were performed in the presence of Primer K, Primer L, and Keypo enzyme. After purification by DNA Recovery Kit (Tiangen), the DNA product was treated by BsaI restriction enzyme and DpnI restriction enzyme. The treated DNA was self-ligated by T4 ligase overnight. After purification, the engineered plasmid was electro transformed into E.coli TG1 cells. A small amount of the TG1 cells were taken for the titer determination. The titer of the transformed TG1 cells was determined to be $1.5 \times 10^9$, and the quality of the library was evaluated by randomly sequencing the transformed cell colonies. The remaining TG1 cells were resuspended in 2×YT, and inoculated into 2×YT plate containing Amp and glucose, and were overnight incubated at 28℃. The cells were taken out from the plates with 20% glycerin. The collected original TG1 library was stored in many vials at -80℃.

Influence of OaAEP1 ligation reactions on phage infectivity

To study the effect of OaAEP1 ligation reaction conditions on the phage infectivity, five portions of pCANTAB 5E-GLX12C phage library solvent (10.0 μL, $10^{12}$ pfu) were added to five different buffers, respectively. After 1 hour incubation at 4℃, each phage solution was diluted with LB medium. 10 μL of the diluted phage solution was mixed with 200.0 μL TG1 cells, and inoculated into 2×YT plate for the titer measurement. Buffer 1: PBS (pH 7.4); Buffer 2: 50.0 mM NaOAc, pH5.0; Buffer 3: 50.0 mM NaOAc, 50.0 μM peptide 3; Buffer 4: 50.0 mM NaOAc, 5.0 μM activated OaAEP1[C247A]; Buffer 5: 50.0 mM NaOAc, 50.0 μM peptide 3, 5.0 μM activated OaAEP1[C247A].
Construction of pCANTAB 5E-GLX6CX6C phage library

The phage displayed GLX6CX6C peptide library was constructed by the use of pCANTAB 5E vector and M13KO7 helper phage (from Nanobody-biolab Ltd. Sichuan). In this phage displayed GLX6CX6C peptide library, X is any L-amino acid encoded by NNK, N is A or T or C or G, K is G or T. The peptide library was inserted into the pCANTAB 5E vector by BsaI restriction enzyme. Two DNA strands were ordered from Sangon Biotech Ltd (Shanghai), as shown below.

Primer K: 5’-TTGGTCTCGGTGCAGCCGAGGGCCCGATCCGATCCGCTG-3’
Primer M:
5’-TTTGGTCTCAGCACCGCCAGAGCCGCCCATGGCCGGCTGGGCCGCATAGAAA
(M is C or A)
GG-3’

Starting from the template of pCANTAB 5E’ vector, PCR reactions were performed in the presence of Primer K, Primer M, and Keypo enzyme. After purification by DNA Recovery Kit (Tiangen), the DNA product was treated by BsaI restriction enzyme and DpnI restriction enzyme. The treated DNA was self-ligated by T4 ligase overnight. After purification, the engineered plasmid was electro transformed into E. coli TG1 cells. A small amount of the TG1 cells were taken for the titer determination. The titer of the transformed TG1 cells was determined to be 1.0×10⁹, and the quality of the library was evaluated by randomly sequencing the transformed cell colonies. The remaining TG1 cells were resuspended in 2×YT, and inoculated into 2×YT plate containing Amp and glucose, and were overnight incubated at 28°C. The cells were taken out from the plates with 20% glycerin. The collected original TG1 library was stored in many vials at -80°C.
Expression and purification of TEAD4

The SUMO-TEAD4 plasmid was provided by Prof. Zhaocai Zhou from Fudan University. E. coli BL21(DE) containing the desired plasmid was inoculated into 500.0 mL of LB medium (kanamycin sulfate). After several-hour incubation at 37℃, OD_{600} of the cell mixture reached 0.8. Then, IPTG was added to afford a final concentration of 0.3 mM. After 16-hour incubation at 16℃, the cell pellet was collected by centrifugation and suspended into HEPES buffer (20.0 mM HEPES, 500.0 mM NaCl, 5% glycerol, pH7.5). After crushing under ultrasonic conditions, the supernatant containing SUMO-TEAD4 was collected by centrifugation. The desired SUMO-TEAD4 protein was obtained by Ni-NTA purification.

The SUMO-TEAD4 protein was dissolved in 20.0 mM HEPES (20.0 mM HEPES, 500.0 mM NaCl, 5% glycerol, pH7.5) to give a final concentration of 1.0 mg/mL. Then, TEV enzyme was added to give a final concentration of 50.0 µg/mL. The mixture was incubated 3 hours at 25℃ and additional 6 hours at 4℃. The desired TEAD4 (without SUMO) was purified by Ni-NTA purification. The SUMO bearing His6-tag can be captured by Ni-NTA, and the desired TEAD4 was eluted by 20.0 mM imidazole solution. The eluted TEAD4 was concentrated to less than 5.0 mL, followed by purification by molecular sieve using the column of HiLoad 16/600 Superdex 200pg (GE Healthcare). The purified TEAD4 was stored at -80℃ in many vials.

Figure S47: SDS-PAGE of (a) SUMO-TEAD4 (Lane 1) and TEV-treated SUMO-TEAD4 (Lane 2) and (b) the purified TEAD4 (Lane 1).
Figure S48: amino acid sequence of SUMO-TEAD4 (SUMO colored with yellow, TEV site colored with green, and TEAD4 colored with purple, 210-434 of the intact TEAD4).

To 2.0 mL PBS (pH7.4) were added 600.0 µg TEAD4(26.53kDa, a final concentration of 10.0 µM, 1.0 equiv.), Biotin-PEG-NHS (1000g/mol, 20.0 equiv.). After 1-hour incubation at room temperature, the unreacted Biotin-PEG-NHS was removed by Zeba™ spin desalting column. The biotinylated TEAD4 was incubated with streptavidin magnetic beads. The binding of TEAD4 to streptavidin beads was verified by denaturing SDS-PAGE in the presence of 3.0 biotin and 8.0 M Urea. In SDS-PAGE analysis, the band of TEAD4 can be found in the biotinylated TEAD4-treated beads.

Selection of monocyclic peptides against TEAD4 by pCANTAB 5E-GLX12C phage library

A vial of TG1 cells containing pCANTAB 5E-GLX12C library was inoculated to 250.0 mL of 2×YT medium (100.0 µg/mL Amp, 2% glucose) to OD₆₀₀ of ~0.1. After ~2-hour incubation at 37°C (220 rpm), OD₆₀₀ of the cell mixture reaches 0.3~0.5. Then, M13KO17 helper phage was added to the cells with MOI of 20. Keep the cells at 37°C for one hour (slowly shaking the cells every 15 minutes). Then, the cells were centrifuged at 4°C (4000 rpm) for 15 minutes. The cell pellet was resuspended into 2×YT medium (100.0 µg/mL Amp, 50.0 µg/mL Kana) to afford OD₆₀₀ of 0.3. Incubate the cell mixture at 28°C (200 rpm) for 16 hours. Then, the cells were centrifuged at 4°C (8000 rpm) for 20 min. To the collected supernatant was added 1/5 volume of 5×PEG8000/NaCl solution (stored in 4°C). After 1-hour incubation at 0°C, the solution was centrifugated at 4°C (10000 rpm) for 20 min. The phage pellet was resuspended in 50.0~100.0 mL TBS, and re-precipitated with PEG8000. The final phage pellet was dissolved in 2.0~10.0 mL TBS buffer and stored in 4°C for the following biopanning. The titer of the phage was determined by gradient dilution.

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80°C refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37°C for 5 hours. Then, 0.25 mL of NaOAc buffer was added to the activation buffer. The pH value
was adjusted to 5.0. To the pH5.0 NaOAc buffer were added pCANTAB 5E-GLX12C phage library (2×10^{11} pfu in 10–20.0 µL TBS) and 3.0 µL of 3 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture). The reaction was kept at 4°C (250 rpm) for 1 hour. Then, 75.0 µL of PEG8000 solution (4% PEG8000, 0.5 M NaCl) was added to precipitate the phage. The phage pellet was suspended in the cyclization buffer (50 mM NaOAc, 1.0 mM TCEP, pH8.0). After 30 min at 37°C (250 rpm), the phage library was precipitated with PEG8000 and resuspended in to 1.5 mL of binding buffer and 750.0 µL of blocking buffer. The mixture was kept at room temperature for 30 min (shaking slowly). At the same time, 10.0 µL of streptavidin coated magnetic beads was washed with PBS buffer and resuspended in 376.0 µL of PBS buffer, followed by the 30-min incubation with 5.2 µL of biotinylated TEAD4 (10.0 µg). The magnetic beads were washed thoroughly three times with 1.0 mL PBS buffer and resuspended in 300.0 µL of binding buffer and 150.0 µL of blocking buffer. After 30-min incubation at room temperature, the treated phage library was mixed with the treated magnetic beads. After 30-min incubation at room temperature, the beads were washed thoroughly eight times with 1.0 mL washing buffer and two times with binding buffer. During the washing step, the tube for the beads was changed at least three times. The bound phages in the beads were eluted with 100.0 µL of elution buffer (pH2.2). The collected elution buffer was mixed with 50.0 µL of neutralization buffer (pH8.0). 10.0 µL of the eluted phage buffer was diluted for titer determination, and the rest were mixed with 20.0 mL of TG1 cells (OD_{600}=~0.4). After 1-hour incubation at 37°C. The phage-infected cells were collected by centrifugation and resuspended in 3.0 mL of LB medium. The LB medium was inoculated into 2×YT plates containing Amp and glucose. After overnight incubation at 28°C, the TG1 cells in the plates were collected by 2×YT medium. The collected TG1 cells were amplified as described above. The selection of monocyclic peptides against was continued as described above. Note that, the streptavidin-coated magnetic beads and the neutral avidin-coated magnetic beads were used alternately.

After three-round selection, we observed that the titer of the recovered phage was 111 times greater than that of the first-round selection. The titer is 10 times higher than that of the TEAD4-unbound beads. 10 colonies were randomly picked from 2×YT plate and amplified in 200.0 µL 2×YT medium (Amp, glucose) and sequenced by General Biol Ltd (Anhui).

Selection of bicyclic peptides against TEAD4 by pCANTAB 5E-GLX6CX6C phage library

A vial of TG1 cells containing pCANTAB 5E-GLX6CX6C library was inoculated to 250.0 mL of 2×YT medium (100.0 µg/mL Amp, 2% glucose) to OD_{600} of ~0.1. After ~2-hour incubation at 37°C (220 rpm), OD600 of the cell mixture reaches 0.3–0.5. Then, M13KO17 helper phage was added to the cells with MOI of 20. Keep the cells at 37°C for one hour (slowly shaking the cells every 15 minutes). Then, the cells were centrifugated at 4°C (4000 rpm) for 15 min. The cell pellet was resuspended into 2×YT medium (100.0 µg/mL Amp, 50.0 µg/mL Kana) to afford OD_{600} of 0.3. Incubate the cell mixture at 28°C (200 rpm) for 16 hours. Then, the cells were centrifugated at 4°C (8000 rpm) for 20 minutes. To the collected supernatant was added 1/5 volume of 5×PEG8000/NaCl solution (stored in 4°C). After 1-hour incubation at 0°C, the solution was centrifugated at 4°C (10000 rpm) for 20 minutes. The phage pellet was resuspended in 50.0–100.0 mL TBS, and re-precipitated with PEG8000. The final phage pellet
was dissolved in 2.0~10.0 mL TBS buffer and stored in 4℃ for the following biopanning. The titer of the phage was determined by gradient dilution.

3.0 µL of the intact OaAEP1[C247A] (18 mg/mL, 54.0 µg) taken from a -80℃ refrigerator was added to 125.0 µL NaOAc buffer (50.0 mM NaOAc, pH4.0). The mixture was kept at 37℃ for 5 hours. Then, 0.25 mL of NaOAc buffer was added to the activation buffer. The pH value was adjusted to 5.0. To the pH5.0 NaOAc buffer were added pCANTAB 5E-GLX6CX6C phage library (2×10\(^{11}\) pfu in 10~20.0 µL TBS) and 3.0 µL of 9 (62.5 mM in a stock solution, a final concentration of 500.0 µM in the reaction mixture). The reaction was kept at 4℃ (250 rpm) for 1 hour. Then, 75.0 µL of PEG8000 solution (4% PEG8000, 0.5 M NaCl) was added to precipitate the phage. The phage pellet was suspended in the cyclization buffer (50 mM NaOAc, 1.0 mM TCEP, pH8.0). After 30 min at 37℃ (250 rpm), the phage library was precipitated with PEG8000 and resuspended in to 1.5 mL of binding buffer and 750.0 µL of blocking buffer. The mixture was kept at room temperature for 30 min (shaking slowly). At the same time, 10.0 µL of streptavidin coated magnetic beads was washed with PBS buffer and resuspended in 376.0 µL of PBS buffer, followed by the 30-min incubation with 5.2 µL of biotinylated TEAD4 (10.0 µg). The magnetic beads were washed thoroughly three times with 1.0 mL PBS buffer and resuspended in 300.0 µL of binding buffer and 150.0 µL of blocking buffer. After 30-min incubation at room temperature, the treated phage library was mixed with the treated magnetic beads. After 30-min incubation at room temperature, the beads were washed thoroughly eight times with 1.0 mL washing buffer and two times with binding buffer. During the washing step, the tube for the beads was changed at least three times. The bound phages in the beads were eluted with 100.0 µL of elution buffer (pH2.2). The collected elution buffer was mixed with 50.0 µL of neutralization buffer (pH8.0). 10.0 µL of the eluted phage buffer was diluted for titer determination, and the rest were mixed with 20.0 mL of TG1 cells (OD\(_{600}\)=~0.4). After 1-hour incubation at 37℃. The phage-infected cells were collected by centrifugation and resuspended in 3.0 mL of LB medium. The LB medium was inoculated into 2×YT plates containing Amp and glucose. After overnight incubation at 28℃, the TG1 cells in the plates were collected by 2×YT medium. The collected TG1 cells were amplified as described above. The selection of monocyclic peptides against was continued as described above. Note that, the streptavidin-coated magnetic beads and the neutral avidin-coated magnetic beads were used alternately.

After three rounds of selection, we observed that the titer of the recovered phage was 1113 times greater than that of the first-round selection. The titer is 32 times higher than that of the TEAD4-unbound beads. 10 colonies were randomly picked from 2×YT plate and amplified in 200.0 µL 2×YT medium (Amp, glucose) and sequenced by General Biol Ltd (Anhui).

According to the abovementioned procedure, we also performed the screening of bicyclic peptides targeting TEAD4 by the treatment of the phage library with 10 and 11. 10-treated phage library gives a 3111-fold enrichment in the titer after three round selections. The titer is 168 times higher than that of the TEAD4-unbound beads. 10 colonies were randomly picked from 2×YT plate and amplified in 10.0 mL 2×YT medium (Amp, glucose). The collected plasmids were extracted from the cells and sequenced by General Biol Ltd (Anhui). 11-treated gives a 40-fold enrichment in the titer after three round selections. The titer is 6 times higher than that of the TEAD4-unbound beads. 20 colonies were randomly picked from 2×YT plate and amplified in 10.0 mL 2×YT medium (Amp, glucose). The collected plasmids were extracted from the cells and sequenced by General Biol Ltd (Anhui).
DNA sequencing of phage colonies after 3 rounds of biopanning

a) pCANTAB 5E-GLX12C phage library

\[
\text{PAMAGLXXXXXXXCGGSG}
\]

Leader sequence Peptide Linker

b)

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<th>Round</th>
<th>GLX12C sequence</th>
<th>Ratio</th>
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<tr>
<td>3</td>
<td>GLQTPLRRRTAQMKC</td>
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<td>3</td>
<td>GLEMSLHQKDRTHPC</td>
<td>1/10</td>
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<tr>
<td>3</td>
<td>GLILLVMEMPRRRGC</td>
<td>1/10</td>
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</table>

Figure S49: Sequencing of phage colonies after 3 rounds of the panning of pCANTABE-GLX12C phage library treated by 3.
Figure S50: Sequencing of phage colonies after 3 rounds of the panning of pCANTABE-GLX6CX6C phage library treated by 9.

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<td>GLYIYHSTCQLNSNTC</td>
<td>1/10</td>
</tr>
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<td>3</td>
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<tr>
<td>3</td>
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Figure S51: Sequencing of phage colonies after 3 rounds of the panning of pCANTABE-GLX6CX6C phage library treated by 10.

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<td>3</td>
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<td>1/10</td>
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<tr>
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<td>GLSWGP尽GCTVPRWC</td>
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**Figure S52:** Sequencing of phage colonies after 3 rounds of the panning of pCANTAB 5E-GLX6CX6C phage library treated by 11.

### a) pCANTAB 5E-GLX6CX6C phage library

```
PAMAGLXXXXXXXXXXXXXXXCGGSG
```

### b) Leader sequence       Peptide      Linker

<table>
<thead>
<tr>
<th>Round</th>
<th>GLX₈CX₈C sequence</th>
<th>Ratio</th>
</tr>
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<td>3</td>
<td>GLGWSFPGCIIQTHHC</td>
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Synthesis of macrocyclic peptides from biopanning

Synthesis of **18-FITC**

**18-FITC** (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine.

To 25.0 µmol of above-mentioned resin were added chloroacetic acid (4.5 equiv.), oxyma (4.5 equiv.), oxyma (4.5 equiv.), and DMF (1.0 mL). After TFA cleavage and ether precipitation, 8.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H$_2$O (1:1, pH of the mixture was adjusted by NH$_4$HCO$_3$ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (2.0 mg, 25.0%).

Analytical HPLC of **18-FITC**: $t_R = 23.5$ min (2-90% B in 40 min); m/z = 2524.8 ($C_{110}H_{166}N_{35}O_{30}S_{2}$, calcd.: 2524.2 g/mol).
18-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 18. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine. After TFA cleavage and ether precipitation, 15 mg of crude peptide was purified to give the desired peptide as a yellow powder (3.0 mg, 20.0%).

Analytical HPLC of 18-L: t_R = 22.9 min (2-90% B in 40 min); m/z = 2484.3 (C_{108}H_{169}N_{35}O_{29}S_{2}, calcd.: 2484.5 g/mol).
**Figure S54:** (a) HPLC trace (210nm) and (b) MS spectrum of the purified 18-L.

*Synthesis of 19-L*

19-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we
continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 μL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine, and the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. After Fmoc-deprotection by 20% piperidine, the α-amino group of Lys was capped by Ac₂O/DIPEA. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (2.0 mg, 20.0%).

Analytical HPLC of 19-L: t<sub>R</sub> = 25.3 min (2-90% B in 40 min); m/z = 2842.0 (C₁₃₀H₁₇₀N₃₀O₃₇S₅, calcd.: 2841.9 g/mol).

Figure S55: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 19-L.
**Synthesis of 19-FITC**

19-FITC (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 19-L. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine, and the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. After Fmoc-deprotection by 20% piperidine, the α-amino group of Lys was capped by Ac₂O/DIPEA. Then, NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (50.0 mg, 100 µmol, 4.0 equiv.) in 0.5 mL DMF, and 5.0 µL of DIPEA were added to the resin. After TFA cleavage and ether precipitation, 10.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the
mixture was adjusted by NH$_4$HCO$_3$ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (2.2 mg, 24.0%). Analytical HPLC of 19-FITC: $t_R = 26.7$ min (2-90% B in 40 min); m/z = 3055.8 (C$_{141}$H$_{176}$N$_{32}$O$_{40}$S$_{3}$, calcd.: 3055.9 g/mol).

Figure S56: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 19-FITC.

**Synthesis of 19**

![Diagram of synthetic pathway]

19 was prepared by Fmoc-based SPPS using 50 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 19-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys(Boc)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. The Fmoc-protecting group of Asn was deprotected by 20%...
piperidine, and the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyama-based method. After Fmoc-deprotection by 20% piperidine, the α-amino group of Lys was capped by Ac₂O/DIPEA. Then, NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (50.0 mg, 100 μmol, 4.0 equiv.) in 0.5 mL DMF, and 5.0 μL of DIPEA were added to the resin. After TFA cleavage and ether precipitation, 8.2 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (2.0 mg, 25.0%).

Analytical HPLC of 19: t_R = 25.9 min (2→90% B in 40 min); m/z = 2665.5 (C₁₂₀H₁₆₅N₃₁O₃₅S₂, calcd.: 2665.2 g/mol).

Figure S57: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 19.
Synthesis of 20-FITC

**20-FITC** (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 19-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33℃, the Fmoc-protecting group of Asn was deprotected by 20% piperidine, and the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. After Fmoc-deprotection by 20% piperidine, the α-amino group of Lys was capped by Ac₂O/DIPEA. Then, NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (50.0 mg, 100 µmol, 4.0 equiv.) in 0.5 mL DMF, and 5.0 µL of DIPEA were added to the resin. After TFA cleavage and ether precipitation, 9.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37℃. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.7 mg, 20.3%).
Analytical HPLC of 20-FITC: $t_R = 26.2$ min (2-90% B in 40 min); $m/z = 2787.6$ (C$_{120}$H$_{163}$N$_{31}$O$_{34}$S$_{3}$, calcd.: 2787.1 g/mol).

Figure S58: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 20-FITC.
**Synthesis of 21-FITC**

21-FITC (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 20-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 μL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33℃, the Fmoc-protecting group of Asn was deprotected by 20% piperidine, and the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. After Fmoc-deprotection by 20% piperidine, the α-amino group of Lys was capped by Ac₂O/DIPEA. Then, NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (50.0 mg, 100 μmol, 4.0 equiv.) in 0.5 mL DMF, and 5.0 μL of DIPEA were added to the resin. After TFA cleavage and ether precipitation, 9.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37℃. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.7 mg, 19.6%).
Analytical HPLC of **21-FITC**: $t_R = 26.3$ min (2-90% B in 40 min); m/z = 2886.6 ($\text{C}_{134}\text{H}_{172}\text{N}_{32}\text{O}_{35}\text{S}_3$, calcd.: 2886.2 g/mol).

Figure S59: (a) HPLC trace (210nm) and (b) MS spectrum of the purified **21-FITC**.
Synthesis of 22-L

22-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine, and the coupling of Fmoc-Gly-OH was performed using DIC/oxyma-based method. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (1.8 mg, 18.0%).

Analytical HPLC of 22-L: t_R = 24.3 min (2-90% B in 40 min); m/z = 2556.6 (C_{111}H_{61}N_{29}O_{35}S_{3}, calcd.: 2556.1 g/mol).
Figure S60: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 22-L.

**Synthesis of 22-FITC**

\[
\begin{align*}
\text{FmocNH-} & \xrightarrow{\text{Fmoc SPPS}} \text{FmocNH-Asn-Gly-Leu-Gln-Thr-Ser-Thr-Leu-Ser-Cys-His-Phe-Glu-Lys-Ser-Leu-Cys-Gly-Gly-Lys(Mtt)-CONH}_2 \\
& \xrightarrow{\text{HFIP/DCM(1:4)}} \text{FmocNH-Asn-Gly-Leu-Gln-Thr-Ser-Thr-Leu-Ser-Cys-His-Phe-Glu-Lys-Ser-Leu-Cys-Gly-Gly-Lys(Mtt)-CONH}_2 + \text{2NH}_2
\end{align*}
\]

- i) FITC, DIPEA
- ii) 20% piperidine
- iii) Cab-Gly-Osu
- iv) pH8.0
[Synthesis of Cab-Gly-OSu]

75.0 mg of glycine was dissolved in 0.5 mL of 50.0 mM NaOAc aqueous solution. The glycine solution was added to 4.0 mL of NaOAc/DMF mixture (3.6 mL of 50.0 mM NaOAc aqueous solution, and 0.4 mL of DMF), followed by the dropwise addition of 40.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid in 0.5 mL DMF. After 2-hour incubation at 25°C, the desired product Cab-Gly-OH was obtained by HPLC, and lyophilized to a white powder. 40.0 mg of the powder (75.0 µmol) and 25.0 mg of HOSu (150.0 µmol) were dissolved into 1.0 mL of DMF/NMP (4:1, v,v), followed by the addition of 30.0 µL of DIC. After 6-hour incubation at room temperature, the formed Cab-Gly-OSu was used directly for the on-resin coupling.

[Synthesis of 22-FITC]

22-FITC (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 25 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, the Fmoc-protecting group of Asn was deprotected by 20% piperidine. Then, 1.0 mL of Cab-Gly-OSu in DMF/NMP solution was added to the resin. After 12-hour incubation at 30°C, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 9.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.8 mg, 20.4%).

Analytical HPLC of 22-FITC: tᵣ = 26.1 min (2-90% B in 40 min); m/z = 2769.8 (C₁₁₂H₁₆₀N₃₁O₃₈S₃, calcd.: 2769.1 g/mol).
Figure S61: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 22-FITC.

**Synthesis of 23-L**

23-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 75.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 22-L. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyama-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine and the coupling of Fmoc-Gly-OH. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (2.2 mg, 22.2%).

Analytical HPLC of 23-L: \( t_R = 27.2 \text{ min} \) (2-90% B in 40 min); \( m/z = 2728.8 \) (C\(_{124}\)H\(_{159}\)N\(_{29}\)O\(_{36}\)S\(_{3}\), calcd.: 2728.1 g/mol).
Figure S62: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 23-L.

**Synthesis of 23-FITC**

40.0 mg of Cab-Gly-OH (75.0 μmol) and 25.0 mg of HOSu (150.0 μmol) were dissolved into 1.0 mL of DMF/NMP (4:1, v:v), followed by the addition of 30.0 μL of DIC. After 6-hour
incubation at room temperature, the formed Cab-Gly-OSu was used directly for the on-resin coupling. **23-FITC** (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 25 \( \mu \text{mol} \) scale of Rink Amide resin (loading of \(~0.56 \text{ mmol/g}\)). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 \( \mu \text{L} \)) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33\( ^\circ \)C, the Fmoc-protecting group of Asn was deprotected by 20\% piperidine. Then, 1.0 mL of Cab-Gly-OSu in DMF/NMP solution was added to the resin. After 12-hour incubation at 30\(^\circ\)C, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 8.5 mg of the crude peptide was dissolved in 2.0 mL DMF/H\(_2\)O (1:1, pH of the mixture was adjusted by NH\(_4\)HCO\(_3\) to 8.0). After 30-min incubation at 37\(^\circ\)C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (2.0 mg, 24.1\%). Analytical HPLC of **23-FITC**: \( t_R = 27.4 \text{ min} \) (2-90\% B in 40 min); \( m/z = 2942.6 \) (C\(_{135}H_{168}N_{31}O_{39}S_3\), calcld.: 2942.1 g/mol).

![Figure S63](image)

Figure S63: (a) HPLC trace (210nm) and (b) MS spectrum of the purified **23-FITC**.
40.0 mg of Cab-Gly-OH (75.0 µmol) and 25.0 mg of HOSu (150.0 µmol) were dissolved into 1.0 mL of DMF/NMP (4:1, v:v), followed by the addition of 30.0 µL of DIC. After 6-hour incubation at room temperature, the formed Cab-Gly-OSu was used directly for the on-resin coupling. 23 was prepared by Fmoc-based SPPS using 25 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. The Fmoc-protecting group of Asn was deprotected by 20% piperidine. Then, 1.0 mL of Cab-Gly-OSu in DMF/NMP solution was added to the resin. After 12-hour incubation at 30℃, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 8.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37℃. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.5 mg, 19.6%). Analytical HPLC of 23: tᵣ = 26.0 min (2-90% B in 40 min); m/z = 2553.2 (C₁₁₄H₁₅₄N₃₀O₃₄S₂, calcd.: 2553.1 g/mol).
Figure S64: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 23.

**Synthesis of 24-L**

Synthesis of 24-L was prepared by Fmoc-based SPPS using 75.0 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 23-L. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 μmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing,
FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine and the coupling of Fmoc-Gly-OH. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (2.5 mg, 25.0%). Analytical HPLC of 24-L: t_R = 26.8 min (2-90% B in 40 min); m/z = 2769.6 (C_{127}H_{169}N_{31}O_{34}S_3, calcd.: 2769.2 g/mol).

Figure S65: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 24-L.

**Synthesis of 24-FITC**

40.0 mg of Cab-Gly-OH (75.0 µmol) and 25.0 mg of HOSu (150.0 µmol) were dissolved
into 1.0 mL of DMF/NMP (4:1, v/v), followed by the addition of 30.0 µL of DIC. After 6-hour incubation at room temperature, the formed Cab-Gly-OSu was used directly for the on-resin coupling. **24-FITC** (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 25 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, Mtt-protecting group of Lys was deprotected by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33℃, the Fmoc-protecting group of Asn was deprotected by 20% piperidine. Then, 1.0 mL of Cab-Gly-OSu in DMF/NMP solution was added to the resin. After 12-hour incubation at 30℃, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 9.2 mg of the crude peptide was dissolved in 2.0 mL DMF/H2O (1:1, pH of the mixture was adjusted by NH4HCO3 to 8.0). After 30-min incubation at 37℃. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.5 mg, 16.8%). Analytical HPLC of **24-FITC**: \( t_R = 27.4 \text{ min} \) (2-90% B in 40 min); m/z = 2982.0 (C138H172N33O37S3, calcd.: 2982.2 g/mol).

Figure S66: (a) HPLC trace (210nm) and (b) MS spectrum of the purified **24-FITC**.
Synthesis of 24

40.0 mg of Cab-Gly-OH (75.0 µmol) and 25.0 mg of HOSu (150.0 µmol) were dissolved into 1.0 mL of DMF/NMP (4:1, v:v), followed by the addition of 30.0 µL of DIC. After 6-hour incubation at room temperature, the formed Cab-Gly-OSu was used directly for the on-resin coupling. 24 was prepared by Fmoc-based SPPS using 25 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g). After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. The Fmoc-protecting group of Asn was deprotected by 20% piperidine. Then, 1.0 mL of Cab-Gly-OSu in DMF/NMP solution was added to the resin. After 12-hour incubation at 30°C, the resin was washed with DMF and DCM. After TFA cleavage and ether precipitation, 8.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.8 mg, 23.1%).

Analytical HPLC of 24: tᵣ = 25.5 min (2-90% B in 40 min); m/z = 2594.2 (C₁₁₇H₁₆₄N₃₂O₃₂S₂, calcd.: 2594.2 g/mol).

Figure S67: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 24.
**Synthesis of 25-L**

25-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 24-L. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (2.3 mg, 23.0%).

Analytical HPLC of 25-L: t_R = 25.1 min (2-90% B in 40 min); m/z = 2499.9 (C_{114}H_{150}N_{30}O_{29}S_3, calcd.: 2500.0 g/mol).

**Figure S68:** (a) HPLC trace (210nm) and (b) MS spectrum of the purified 25-L.
Synthesis of 25-FITC

25-FITC (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 50.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 25-L. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine. Then, 50.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (Cab-OH) and DIPEA (5.0 µL) were added to the resin. The mixture was kept at room temperature for 12 hours. After TFA cleavage and ether precipitation, 8.4 mg of the crude peptide was dissolved in 2.0 mL DMF/H2O (1:1, pH of the mixture was adjusted by NH4HCO3 to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.2 mg, 14.7%).

Analytical HPLC of 25-FITC: t_R = 26.7 min (2-90% B in 40 min); m/z = 2715.0 (C_{125}H_{156}N_{32}O_{32}S_{3}, calcd.: 2715.0 g/mol).
Figure S69: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 25-FITC.

**Synthesis of 26-L**

26-L (FITC modification at C-terminal Lys residue) was prepared by Fmoc-based SPPS using 75.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 25-L. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine. After TFA cleavage and ether precipitation, 10 mg of crude peptide was purified to give the desired peptide as a yellow powder (2.1 mg, 21.0%).

Analytical HPLC of 26-L: $t_R = 25.6$ min (290% B in 40 min); $m/z = 2639.6$ (C$_{119}$H$_{164}$N$_{30}$O$_{33}$S$_3$, calcd.: 2639.6 g/mol).
Figure S70: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 26-L.

**Synthesis of 26-FITC**

26-FITC was prepared by Fmoc-based SPPS using 50.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 25-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine. Then, 50.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (Cab-OH) and DIPEA (5.0 µL) were added to the resin. The mixture was kept at room temperature for 12 hours. After TFA cleavage and ether precipitation, 8.5 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.8 mg, 21.0%).
Analytical HPLC of 26-FITC: \( t_R = 27.3 \) min (2-90% B in 40 min); m/z = 2851.8 (C\(_{130}\)H\(_{170}\)N\(_{32}\)O\(_{36}\)S\(_{3}\), calcd.: 2852.1 g/mol).

26 was prepared by Fmoc-based SPPS using 50.0 \( \mu \)mol scale of Rink Amide resin (loading of \( \sim 0.56 \) mmol/g) as 26-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. 25.0 \( \mu \)mol of the resin was taken for Fmoc-deprotection by 20% piperidine. Then, 50.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (Cab-OH) and DIPEA (5.0 \( \mu \)L) were added to the resin. The mixture was kept at room temperature for 12 hours. After TFA cleavage and ether precipitation, 8.5 mg of the crude peptide was dissolved in 2.0 mL DMF/H\(_2\)O (1:1, pH of the mixture was adjusted by NH\(_4\)HCO\(_3\) to 8.0). After 30-min incubation at 37\(^\circ\)C. The desired cyclized peptide was purified by HPLC and lyophilized to a white powder (1.7 mg, 23.0%).

Analytical HPLC of 26: \( t_R = 25.4 \) min (2-90% B in 40 min); m/z = 2463.0 (C\(_{109}\)H\(_{158}\)N\(_{31}\)O\(_{31}\)S\(_{2}\), calcd.: 2463.2 g/mol).

Figure S71: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 26-FITC.

Synthesis of 26

\[ \text{FmocNH} \xrightarrow{\text{Fmoc SPPS}} \text{FmocNH-Asn-Gly-Leu-Thr-Leu-Asp-Pro-Gln-Cys-} \]
\[ \text{Trp-Gln-Arg-Leu-Thr-Pro-Cys-Gly-Gly-Lys(Mtt)-CONH} \]

- i) 20% piperidine
- ii) NHS ester of Cab-OH
- iii) pH8.0
27-FITC was prepared by Fmoc-based SPPS using 50.0 μmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 26-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys(Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 μmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 μL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 μmol of the resin was taken for Fmoc-deprotection by 20% piperidine. Then, 50.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (Cab-OH) and DIPEA (5.0 μL) were added to the resin. The mixture was kept at room temperature for 12 hours. After TFA cleavage and ether precipitation, 8.1 mg of the crude peptide was dissolved in 2.0 mL DMF/H₂O (1:1, pH of the mixture was adjusted by NH₄HCO₃ to 8.0). After 30-min incubation at 37°C. The desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.9 mg,
Analytical HPLC of 27-FITC: $t_R = 25.6$ min (2-90% B in 40 min); m/z = 2617.6 (C$_{121}$H$_{148}$N$_{28}$O$_{33}$S$_{3}$, calcd.: 2618.0 g/mol).

Figure S73: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 27-FITC.

**Synthesis of 28-FITC**

28-FITC was prepared by Fmoc-based SPPS using 50.0 µmol scale of Rink Amide resin (loading of ~0.56 mmol/g) as 27-FITC. After Fmoc deprotection, the coupling of Fmoc-Lys (Mtt)-OH was performed using DIC/oxyma-based method. Then, we continued the coupling of amino acids until the N-terminal Asn. Then, 50.0 µmol of the resin was taken for Mtt-deprotection by HFIP/DCM (1:4, v/v). After DMF washing, FITC (58.4 mg, 3.0 equiv.) and DIPEA (26.0 µL) in 0.5 mL DMF were added to the resin. After 3-hour incubation at 33°C, 25.0 µmol of the resin was taken for Fmoc-deprotection by 20% piperidine. Then, 50.0 mg of NHS ester of 3,5-bis(2-chloroacetamido) benzoic acid (Cab-OH) and DIPEA (5.0 µL) were added to the resin. The mixture was kept at room temperature for 12 hours. After TFA cleavage and ether precipitation, 8.0 mg of the crude peptide was dissolved in 2.0 mL DMF/H$_2$O (1:1, pH of the mixture was adjusted by NH$_4$HCO$_3$ to 8.0). After 30-min incubation at 37°C. The
desired cyclized peptide was purified by HPLC and lyophilized to a yellow powder (1.7 mg, 22.1%).

Analytical HPLC of 28-FITC: $t_R = 25.6\text{ min}$ (2-90% B in 40 min); m/z = 2566.8 (C$_{118}$H$_{148}$N$_{28}$O$_{32}$S$_{3}$, calcd.: 2566.8 g/mol).

Figure S74: (a) HPLC trace (210nm) and (b) MS spectrum of the purified 28-FITC.

**Fluorescence polarization assay**

Binding affinity of macrocyclic peptides to TEAD4 was measured by fluorescence polarization assay. The peptides measured in the experiments contain a FITC moiety and were dissolved in DMF to give a stock solution with a concentration of 1.0 mM. The stock solution of the FITC-modified peptide (1.0 mM) was diluted to 22.22 nM by the addition of FP buffer (20.0 mM HEPES, 100.0 mM NaCl, 1.0 mg/mL chaps, pH7.5). Meanwhile, the recombinant SUMO-TEAD4 protein in lysis buffer was diluted a 1.0 mM stock solution by the addition of lysis buffer (20.0 mM HEPES, 500.0 mM NaCl, 5% glycerol, pH 7.5). The stock solution of the SUMO-TEAD4 solution (1.0 mM) was sequentially diluted by the addition of lysis buffer to give a different concentration: 0, 50.0 nM, 100.0 nM, 200.0 nM, 400.0 nM, 600.0 nM, 750.0 nM, 800.0 nM, 1.0 µM, 1.25 µM, 1.5 µM, 1.75 µM, 2.0 µM, 2.5 µM, 3.0 µM, 4.0 µM, 4.5 µM, 5.0 µM, 7.5 µM, 10.0 µM, 15.0 µM, 20.0 µM, et al. To an empty clean tube were added one of the abovementioned diluted SUMO-TEAD4 solution (20.0 µL) and 180.0 µL of the diluted peptide solution (22.22 nM, final concentration of 20.0 nM). Keep the mixture at 4°C for 2 hours. Then, 180.0 µL of the mixture was taken from the tube and transferred to a 96-well black polystyrene plate. The value of the polarization fluorescence signal was measured by the SpectraMax Paradigm multifunctional enzyme apparatus, with excitation wavelength set to be 485 nm, with emission wavelength set to be 535 nm. After subtracting the background signal, the binding affinity of the peptide to TEAD4 was calculated by using GraphPad Prism 9 with the method of Binding Saturation-one site specific Binding. Note that, each sample was repeated three times.
Figure S75: Binding affinity of the peptides to TEAD4 measured by fluorescence polarization assay.

The inhibitory effect of macrocyclic peptides on YAP·TEAD4 interaction was determined as follows. A stock SUMO-TEAD4 solution (1.0 mM in Lysis buffer) was diluted to 66.7 nM by the addition of FP buffer (20.0 mM HEPES, 100.0 mM NaCl, 1.0 mg/mL chaps, pH 7.5). 75.0 µL of DMF was added to 2.0 mg of peptide 19 to afford a stock solution (10.0 mM). 58.7 µL of DMF was added to 1.5 mg of peptide 23 to afford a stock solution (10.0 mM). 69.3 µL of DMF was added to 1.8 mg of peptide 24 to afford a stock solution (10.0 mM). 69.0 µL of DMF was added to 1.7 mg of peptide 26 to afford a stock solution (10.0 mM). The stock solution was diluted by the addition of DMF to give different series of concentrations (for peptide 19: 0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM, 2.5 µM, 5.0 µM, 7.5 µM, 10.0 µM, 15.0 µM; for peptide 23: 0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM, 2.5 µM, 5.0 µM, 7.5 µM, 10.0 µM, 15.0 µM; for peptide 24: 0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM, 2.5 µM, 5.0 µM, 7.5 µM, 10.0 µM, 15.0 µM; for peptide 26: 0.1 µM, 0.25 µM, 0.5 µM, 1.0 µM, 2.5 µM, 5.0 µM, 10.0 µM, 20.0 µM, 40.0 µM, 60.0 µM, 80.0 µM). To an empty clean tube were added 10.0 µL of peptide 19, 23, 24 or 26, and 180.0 µL of the diluted SUMO-TEAD4 solution (66.7 nM, a final concentration of 60.0 nM). Keep the mixture at 4°C for 2 hours. To 1.5 mg of YAP-FAM was added 304.8 µL of DMF to give a stock solution of 1.0 mM. Then, the stock YAP-FAM solution (1.0 mM) was diluted to 400.0 nM by the addition
of FP buffer. To the pre-incubated solution containing SUMO-TEAD4 and peptide 19, 23, 24 or 26 was added 10.0 µL of YAP-FAM (400.0 nM, a final concentration of 20.0 nM). Keep the mixture at 4°C for 20 minutes. Then, 180.0 µL from the mixture was taken and transferred to a 96-well black polystyrene plate. The value of the polarization fluorescence signal was measured by the SpectraMax Paradigm multifunctional enzyme apparatus, with excitation wavelength set to be 485 nm, with emission wavelength set to be 535 nm. After subtracting the background signal, the binding affinity of the peptide to TEAD4 was calculated by using GraphPad Prism 9 with the method of the dose-response suppression logarithm (inhibitor) and the response variable slope (four parameters). Note that, each sample was repeated three times.

Figure S76: Inhibition of TEAD4·YAP by (a) 19, (b) 23, (c) 24 and (d) 26 as assessed by fluorescence polarization assay. YAP-FAM: FAM-betaAla-Ser-Glu-Thr-Asp-Leu-Glu-Ala-Leu-Phe-Asn-Ala-Val-Nle-Asn-Pro-Lys-Thr-Ala-Asn-Val-Pro-Gln-Thr-Val-Pro-Nle-Arg-Leu-Arg-Lys-Leu-Pro-Asp-Ser-Phe-Phe-Lys-Pro-Pro-Glu-CO\textsubscript{NH}_2.