Supplementary information

Ribosomal Incorporation of *N*-Acetyl-3,5-Bis(Chloromethyl)benzylthio-L-Alanine for Developing an mRNA-Displayed Bicyclic Peptide Library Cyclized via 1,3,5-Tris(methyl)benzene

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Table of Contents

Methods and materials

Abbreviations

The abbreviations used here are as follows: TBMB, 1,3,5-tris(bromomethyl)benzene; Ac-BCBT-^{L}Ala, *N*-acetyl-3,5-bis(chloromethyl)benzylthio-L-alanine; MeCN, acetonitrile; NaHCO3, sodium bicarbonate; EtOAc, ethyl acetate; CME, cyanomethyl ester; DMF, dimethylformamide; TLC, thin layer chromatography; HCl, hydrogen chloride; Na_2SO_4, sodium sulfate; DMSO, dimethyl sulfoxide; NaOAc, sodium acetate; $Mg(OAc)_2$, magnesium acetate; KOH, potassium hydroxide; dNTPs, deoxynucleotide triphosphates; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; EtOH, ethanol; EtBr, ethidium bromide; PBST, Phosphate Buffered Saline with Tween-20; SPPS，standard Fmoc solid-phase peptide synthesis； DCM, dichloromethane; HOBT, 1-Hydroxybenzotriazole; EDT, ethanedithiol; DIC, N, *N*-diisopropylcarbodiimide; TFA, trifluoroacetic acid; TIS, triisopropylsilane; HPLC, high-performance liquid chromatography; TEA, triethylamine.

Procedure for synthesis of Ac-BCBT-^{L}Ala-CME

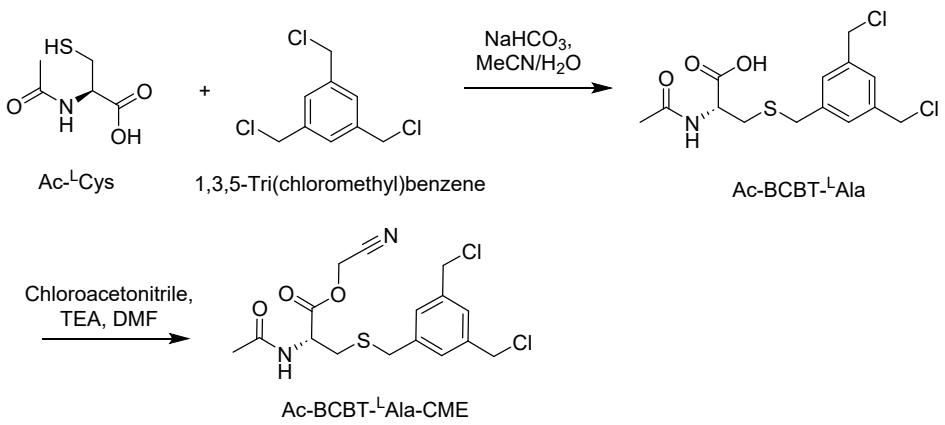


Figure S1. Synthesis of Ac-BCBT-^{L}Ala-CME

N-acetyl-3,5-bis(chloromethyl)benzylthio-L-alanine (Ac-BCBT-^{L}Ala) was first synthesized. Briefly, a total of 890 mg of *N*-acetyl-L-cysteine (Ac-^{L}Cys) was dissolved in a 20 mL mixture of acetonitrile (MeCN) and saturated aqueous sodium bicarbonate ($NaHCO_3$) solution (1:1 in volume). The prepared solution was then added dropwise to the flask containing 1,3,5-tri(chloromethyl)benzene (1.6 g) in 10 mL of MeCN while maintaining the temperature at 0℃. The reaction mixture was then stirred at room temperature for 3 h. Following this, the pH was adjusted to 3–4 using 5% HCl. The mixture was subjected to extraction three times with ethyl acetate (EtOAc), and the combined organic layers were washed with brine and dried with anhydrous Na_2SO4. The solvent was removed, yielding 1.88 g of crude product, which was subsequently purified via silica gel chromatography to obtain 920 mg of the desired product. ESI-MS (m/z): calculated for $[M+H]^+$ of $C_{14}H_{17}Cl_2NO_3S$: 350.04, 352.04, 354.03; found:

350.00, 352.00, 354.00.

The carboxyl group of the synthesized compound was subsequently activated by introducing a cyanomethyl ester (CME) moiety. Specifically, 50 mg of Ac-BCBT-^{L}Ala was dissolved in 2 mL of DMF, followed by the addition of 25.3 μL of TEA and 100 μL of chloroacetonitrile. The mixture was stirred overnight at room temperature using magnetic stirring. The following day, the completion of the reaction was confirmed by thin-layer chromatography (TLC). Upon completion, the reaction mixture was transferred to a separatory funnel using ethyl acetate added in small portions to ensure complete transfer. The organic phase was washed three times with 1 M HCl, and the aqueous layers were discarded. The organic phase was then sequentially washed three times with saturated $NaHCO_3$ solution and saturated NaCl solution. The ethyl acetate phase containing the crude product was dried over anhydrous Na_2SO_4. The mixture was filtered to remove Na_2SO_4, and the filtrate was concentrated under reduced pressure using a rotary evaporator to yield the crude product, Ac-BCBT-^{L}Ala-CME. The crude product was further purified by high-performance liquid chromatography (HPLC), using water and acetonitrile as the mobile phases. The purified product was lyophilized to remove residual water and acetonitrile, yielding Ac-BCBT-^{L}Ala-CME as a white powder. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.53 (d, 1H), 7.40 (t, 1H), 7.36 (d, 2H), 5.01 (s, 2H), 4.76 (s, 4H), 4.52-4.56 (m, 1H), 3.81 (s, 2H), 2.69-2.82 (m, 2H), 1.88 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.43, 170.06, 139.60, 138.79, 129.66, 128.29, 116.12, 51.99, 50.03, 46.11, 40.52, 40.39, 35.28, 32.34, 22.66. ESI-MS (m/z): calculated for $[M+H]^+$ of $C_{16}H_{18}Cl_2N_2O_3S$: 389.05, 391.05, 393.04; found: 389.05, 391.05, 393.07.

Evaluation of aminoacylation efficiency of Ac-BCBT-^{L}Ala-CME

The reaction mixture was prepared by combining 4 μL of H_2O, 1 μL of 500 μM HEPES-KOH buffer (pH 7.5), 1 μL of 250 mM Microhelix RNA, and 1 μL of 250 mM eFx, resulting in a total volume of 6 μL. The mixture was gently pipetted, followed by incubation at 95 °C for 2 min. The sample was then cooled to room temperature for 5 min. Subsequently, 1 μL of 3 M $MgCl_2$ was added and the mixture was incubated for an additional 5 min. It was then placed on ice for 3 min before 2 μL of 25 mM Ac-BCBT-^{L}Ala-CME was added. The reaction proceeded on ice for 2 h to facilitate the aminoacylation of Ac-BCBT-^{L}Ala-CME to the Microhelix RNA. Positive and negative control groups were prepared using the same protocol. In the positive control, Ac-BCBT-^{L}Ala-CME was replaced with 2 μL of 25 mM ClAc-^{L}Tyr-CME. In the negative control, 2 μL of DMSO was used instead of Ac-BCBT-^{L}Ala-CME. After the 2-hour incubation, samples from all groups were collected. Each sample was recovered by adding 40 μL of 0.3 M sodium acetate (NaOAc, pH 5.2) and 100 μL of ethanol, followed by vortexing and centrifugation at 13,000 rpm for 15 min. After removing the supernatant, the pellet was washed with 50 μL of 70% ethanol containing 0.1 M NaOAc (pH 5.2), centrifuged again at 13,000 rpm for 5 min, and allowed to air-dry for 5 min. The aminoacylation efficiency was evaluated using the acid-PAGE. Initial efforts to aminoacylate Ac-BCBT-^{L}Ala-CME onto Microhelix RNA using standard HEPES-KOH buffer (pH 7.5) conditions yielded minimal results. Consequently, adjustments were made to the pH of the reaction buffer and the incubation time to enhance

aminoacylation efficiency.

RaPID selections against Trop2 and VEGF165 using the tris(methyl)benzene-bridged bicyclic peptide library

For the first round of selection, the mRNA template library (AUG-$(NNK)_{5-7}$-UGC-$(NNK)_{5-6}$-UGC-$(GGC-AGC)_3$-UAG was ligated to a puromycin linker using T4 RNA ligase, generating the mRNA-Pu library. A total of 1.2 μM of the mRNA-Pu library was added to a reconstituted FIT system, in which both Release Factor 1 (RF1) and methionine (Met) were omitted. The system included 50 μM Ac-BCBT-^{L}Ala-$tRNA^{fMet}_{CAU}$. Translation was carried out at 37°C for 120 min. After translation, the mixture was incubated at 25°C for 15 min, followed by the addition of 5 μL of 100 mM EDTA (pH 8.0) and further incubation at 37°C for 30 min. A reverse-transcription solution was then prepared by combining the reaction mixture with RNase inhibitor. The solution contained 25 mM Tris-HCl (pH 8.3), 15 mM $Mg(OAc)_2$, 10 mM KOH, 0.25 mM dNTPs, 2 μM CGS3an13.R39, and 50 U M-MLV reverse transcriptase (RNase H Minus). The mixture was then incubated at 42°C for 1 h. The resulting mixture was combined in a 1:1 volume ratio with blocking buffer (0.2% acetylated BSA in 2×PBST). This was followed by incubation (positive selection) with Dynabeads™ M-280 streptavidin magnetic beads pre-bound to Trop2 or VEGF165 (Biotinylated human Trop2 Protein, His, Avitag™; or Biotinylated human VEGF165 Protein, His, Avitag™; from ACROBiosytems, Beijing, China) with 200 nM as the final concentration. Following incubation, the supernatant was discarded and the beads were washed three times with 1×PBST (10 mM phosphate buffer, pH 7.4; 137 mM NaCl; 2.7 mM KCl; 0.05% Tween-20). Bound cDNA was then eluted by heating the beads at 95°C for 5 min using 1×PCR mix (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 μM T7g10M.F46, 0.25 μM CGS3an13.R22). The eluted cDNA was quantified using a QuantStudio™ real-time PCR system (Applied Biosystems), followed by amplification using Phusion® High-Fidelity DNA polymerase. The amplified DNA was transcribed *in vitro* to regenerate an mRNA library enriched in peptide binders for the next selection round. From the second round onward, 6 more negative selection were introduced before each positive selection step to remove non-specifically binding molecules. Finally, cDNA recovered after round 4 or 5 of selection was subjected to high-throughput sequencing using the NovaSeq 6000 system (Illumina) for binder identification.

Chemical synthesis of the selected 1,3,5-tri(methyl)benzene-bridged bicyclic peptides

Peptides were synthesized using standard Fmoc solid-phase peptide synthesis (SPPS) techniques on Rink amide resin. Rink amide resin (0.5 g; 0.18 mmol) was swollen in dichloromethane (DCM) for 30 minutes. The resin was then treated with 20% piperidine in DMF (3-fold volume of the resin) for 20 min under nitrogen bubbling to remove the Fmoc group. The resin was washed three times with DMF and once with DCM. Fmoc-Gly-OH (0.3 mmol) and HOBt (0.3 mmol) were dissolved in 8 mL DMF with 0.5 mL DIC, mixed thoroughly, and added to the resin. The coupling was carried out with nitrogen bubbling for 1.5 h. The resin was washed three times with DMF.

Fmoc groups were removed using 20% piperidine in DMF (3-fold resin volume) for 20 min with nitrogen bubbling, followed by thorough washing. Sequential amino acid couplings were performed by dissolving 0.9 mmol of the corresponding Fmoc-protected amino acid and 0.9 mmol HOBt in 10 mL DMF, adding 1 mL DIC, and transferring the activated mixture to the resin. The coupling was performed for 1 h under nitrogen bubbling and repeated iteratively to elongate the peptide chain according to the desired sequence. The *N*-terminal amino group was acetylated by reacting with 0.9 mmol of acetic anhydride in the presence of 0.9 mmol of TEA in 10 mL DMF under nitrogen bubbling, with the reaction carried out for 1 h. The resin was washed and then rinsed three times with DCM. Afterward, the resin was dried under vacuum. Cleavage cocktail was prepared using 95 mL trifluoroacetic acid (TFA), 1 mL water, 2 mL ethanedithiol (EDT), and 2 mL triisopropylsilane (TIS). The dried resin was treated with the cleavage cocktail for 2 h. The cleaved peptide was precipitated with cold ether, washed with cold ether for six times, and dried. The crude peptide was dissolved in DMSO again and adjusted over pH 8.0 using triethylamine. The mixture was rotated for 1 h at room temperature to allow intramolecular cyclization with 0.2 mmol 1,3,5-tri(bromomenthyl)benzene (TBMB) in the presence of TCEP. The crude cyclized peptide was purified by high-performance liquid chromatography (HPLC). Solvent A consisted of water with 0.1% TFA, and Solvent B was acetonitrile with 0.1% TFA. Peptides with >95% purity were obtained and confirmed by electrospray ionization mass spectrometry (ESI-MS).

Surface plasmon resonance (SPR) analysis of 1,3,5-tri(methyl)benzene-bridged bicyclic peptides

The binding affinities of 1,3,5-tri(methyl)benzene-bridged bicyclic peptides targeting Trop2 and VEGF165 were measured using surface plasmon resonance (SPR) on a Biacore T200 system. For peptides targeting Trop2, a CM5 sensor chip was used, and multi-cycle kinetic (MCK) mode was employed, in which each injection cycle involved a single peptide concentration. Approximately 2100 response units (RU) of Trop2 protein were immobilized onto the chip surface via standard amine coupling. For peptides targeting VEGF165, a Series S Sensor Chip CAP was utilized. Single-cycle kinetic (SCK) mode was applied, where a single binding cycle involved sequential injections of increasing peptide concentrations. Approximately 1500 RU of VEGF165 protein was captured onto the chip. All measurements were performed at 25°C using HBS-EP$^+$ buffer (10 mM HEPES, 150 mM NaCl, 50 μM EDTA, 0.05% Tween 20, pH 7.4) containing 0.1% DMSO as running buffer. The data were fitted using a 1:1 binding model to obtain kinetic parameters and equilibrium dissociation constants (K_D).

Serum stability assay of 1,3,5-tri(methyl)benzene-bridged bicyclic peptides

To evaluate the metabolic stability of the 1,3,5-tri(methyl)benzene-bridged bicyclic peptides in serum, both Trop2-targeting and VEGF165-targeting peptides were incubated with mouse serum. Prior to use, mouse serum was thawed at room temperature, centrifuged at 13,000 rpm for 10 min to remove precipitates, and equilibrated at 37°C for 15 min. A mixture containing 100 μL of 500 μM peptide solution and 300 μL of mouse serum was incubated at 37°C. At each designated time

point (0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24h), 50 μL of the incubation mixture was taken and mixed with 0.36 μL of 17.5 mM acetaminophen (internal standard). Then, 50 μL of acetonitrile containing 0.3% TFA was added, vortexed, and incubated at 4°C for 5 min. Afterward, 100 μL of 20% trichloroacetic acid was added and allowed to stand for another 5 min before centrifugation at 14,000 rpm for 5 min. The supernatant was filtered through a 0.45 μm filter membrane and analyzed by HPLC. HPLC analysis was performed using a mobile phase consisting of solvent A (water with 0.1% TFA) and solvent B (acetonitrile with 0.1% TFA). The gradient program was as follows: 10% B for 0–10 min, 10%–100% B for 10–40 min, and then 10% B for 40–50 min. The peptide concentration at each time point was calculated based on the peak area, and the average of three replicates was used to determine the percentage of remaining peptide over time. Stability curves were plotted accordingly.

Neutralization Inhibition of Bicyclic Peptides Targeting VEGF165

VEGFR2-His (from ACROBiosytems, Beijing, China) was introduced into 96-well plates at a final concentration of 2 μg/ml (100 μl per well) and left to incubate overnight at 4°C. After incubation, the solution was carefully removed, and the wells were blocked using 2% BSA (in 1×PBS) for a minimum of 1 h. In the meantime, varying concentrations of peptides (0, 0.01, 0.02, 0.03, 0.05, 0.2, 0.4, 0.5, 1.25, 2.5, 5, 10, and 20 μM; final concentration) along with 50 ng/ml (final concentration) of VEGF165 were prepared by mixing and incubated at 25°C for 1 h. This mixture was added to the wells and incubated at 25°C for another one hour. Horseradish Peroxidase-Labeled Streptavidin (Streptavidin-HRP) was diluted to 1:2000, added to the wells, and allowed to incubate for 30 min at 25°C. Subsequently, a 3,3',5,5'-Tetramethylbenzidine (TMB) Single-Component Substrate solution was added and incubated for 3–5 min, after which the reaction was stopped by introducing 1 M H_2SO_4. The absorbance at 450 nm was determined using the Tecan SPARK microplate reader, and the data were analyzed with GraphPad Prism 8.

Supplementary Data

The HPLC and NMR spectra of Ac-BCBT-^{L}Ala-CME

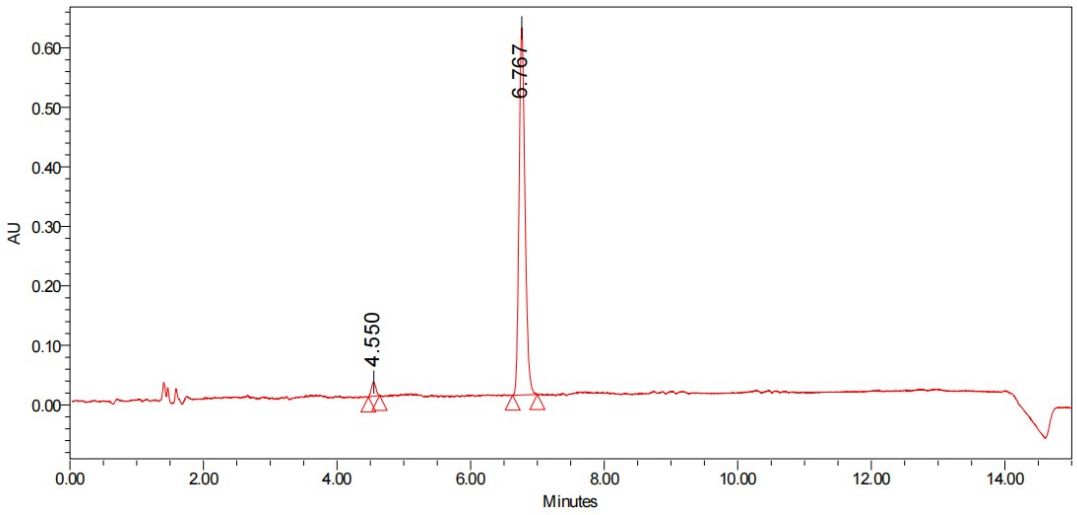


Figure S2. The HPLC spectra of Ac-BCBT-^{L}Ala-CME

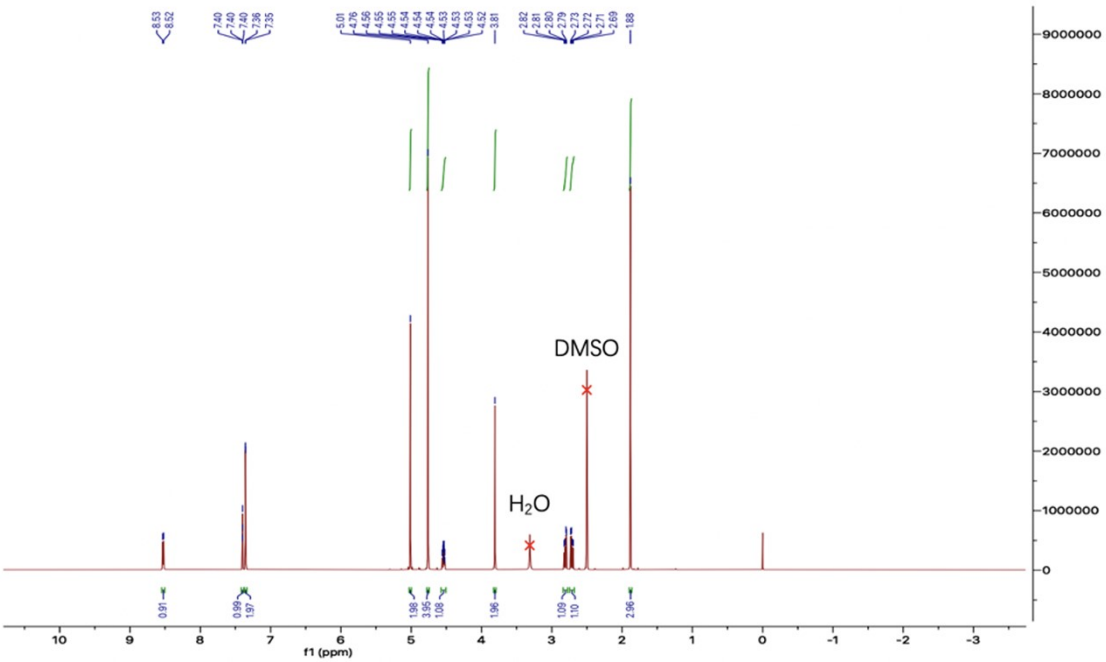


Figure S3. The ^{1}H NMR spectrum of Ac-BCBT-^{L}Ala-CME

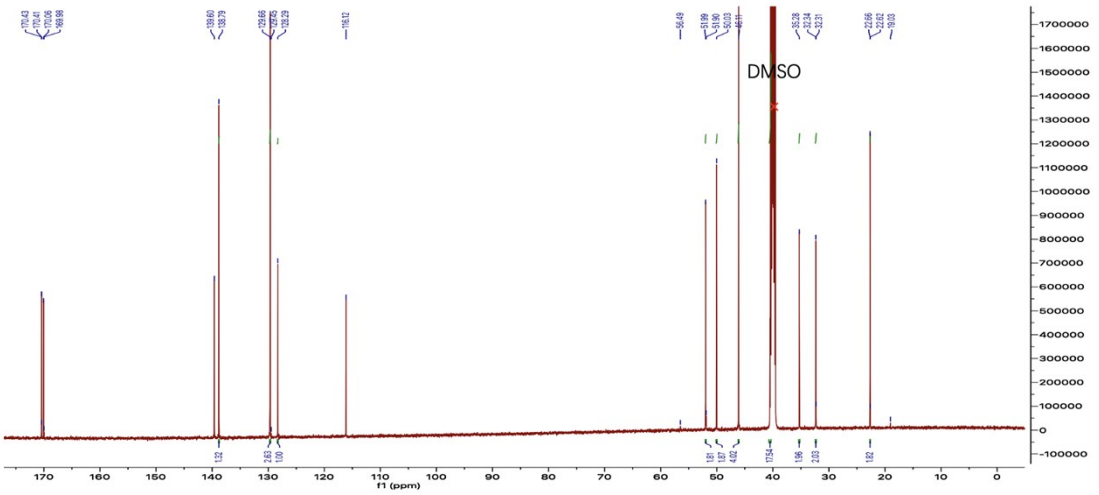
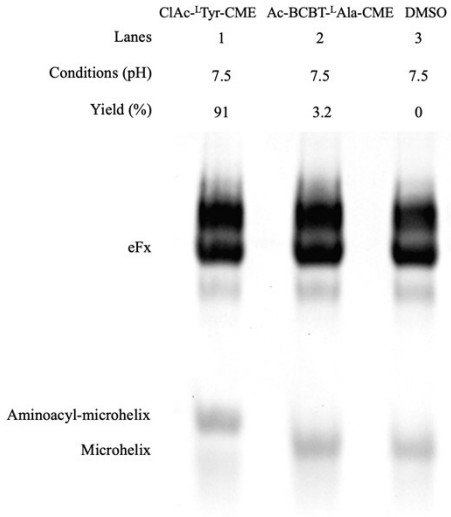


Figure S4. The ^{13}C NMR spectrum of Ac-BCBT-^{L}Ala-CME

The aminoacylation efficiency of Ac-BCBT-^{L}Ala-CME under different



conditions

Figure S5. The aminoacylation efficiency of Ac-BCBT-^{L}Ala-CME onto microhelix RNA was evaluated by incubating the reaction mixture at 37°C for 2 hours in HEPES-KOH buffer (pH 7.5). The efficiency was assessed via acid PAGE, where three lanes were loaded: the left lane represents the positive control using ClAc-^{L}Tyr-CME, the middle lane contains the Ac-BCBT-^{L}Ala-CME reaction, and the right lane is the negative control using DMSO.

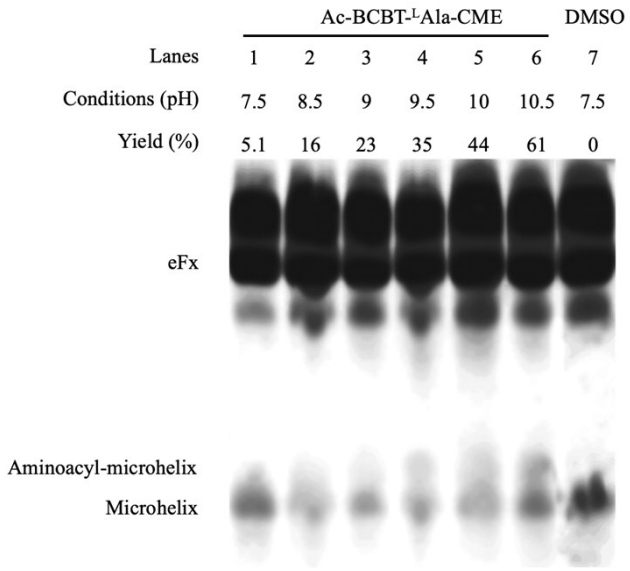


Figure S6. To investigate the influence of pH on the aminoacylation efficiency of Ac-BCBT-^{L}Ala-CME, reactions were carried out in HEPES-KOH buffer at various pH values (7.5, 8.5, 9.0, 9.5, 10.0, and 10.5). The reactions were incubated on ice for 2 hours and subsequently analyzed by acid PAGE. As shown in the gel, detectable aminoacylation products begin to appear at pH values above 8.5. The intensity of the

product bands increases progressively with higher pH, indicating enhanced aminoacylation efficiency at alkaline conditions.

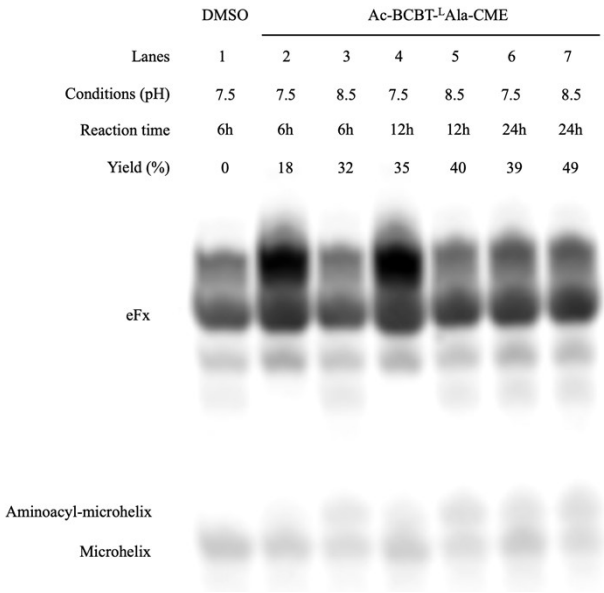


Figure S7. To evaluate the effect of both pH and incubation time on the aminoacylation efficiency of Ac-BCBT-^{L}Ala-CME, reactions were carried out in HEPES-KOH buffer at pH 7.5 and 8.5. Each reaction was incubated on ice for 6, 12, and 24 hours, respectively, and analyzed by acid PAGE. From left to right, the gel lanes correspond to pH 7.5 (6 h), pH 8.5 (6 h), pH 7.5 (12 h), pH 8.5 (12 h), pH 7.5 (24 h), and pH 8.5 (24 h). The band intensity increased with both higher pH and longer incubation time. The highest aminoacylation efficiency was observed at pH 8.5 after 24 hours, reaching 49.3%. This condition was subsequently selected for aminoacylation of $tRNA^{fMet}_{CAU}$.

RaPID salectious against Trop2 and VEGF165 using bicyclic peptide library

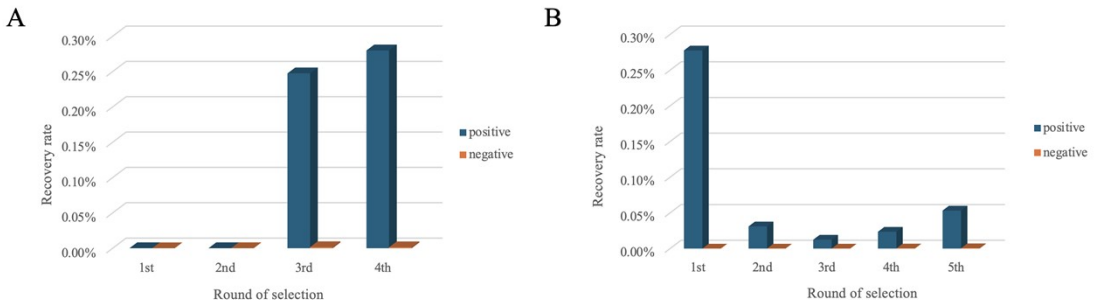


Figure S8. *De novo* selections of bicyclic peptides targeting Trop2 and VEGF165. (A) Selection against Trop2 protein. The y-axis shows the recovery rate of cDNA, and the x-axis represents the number of selection rounds. (B) Selection against VEGF165 protein.

Results of bicyclic peptide library against Trop2 and VEGF165

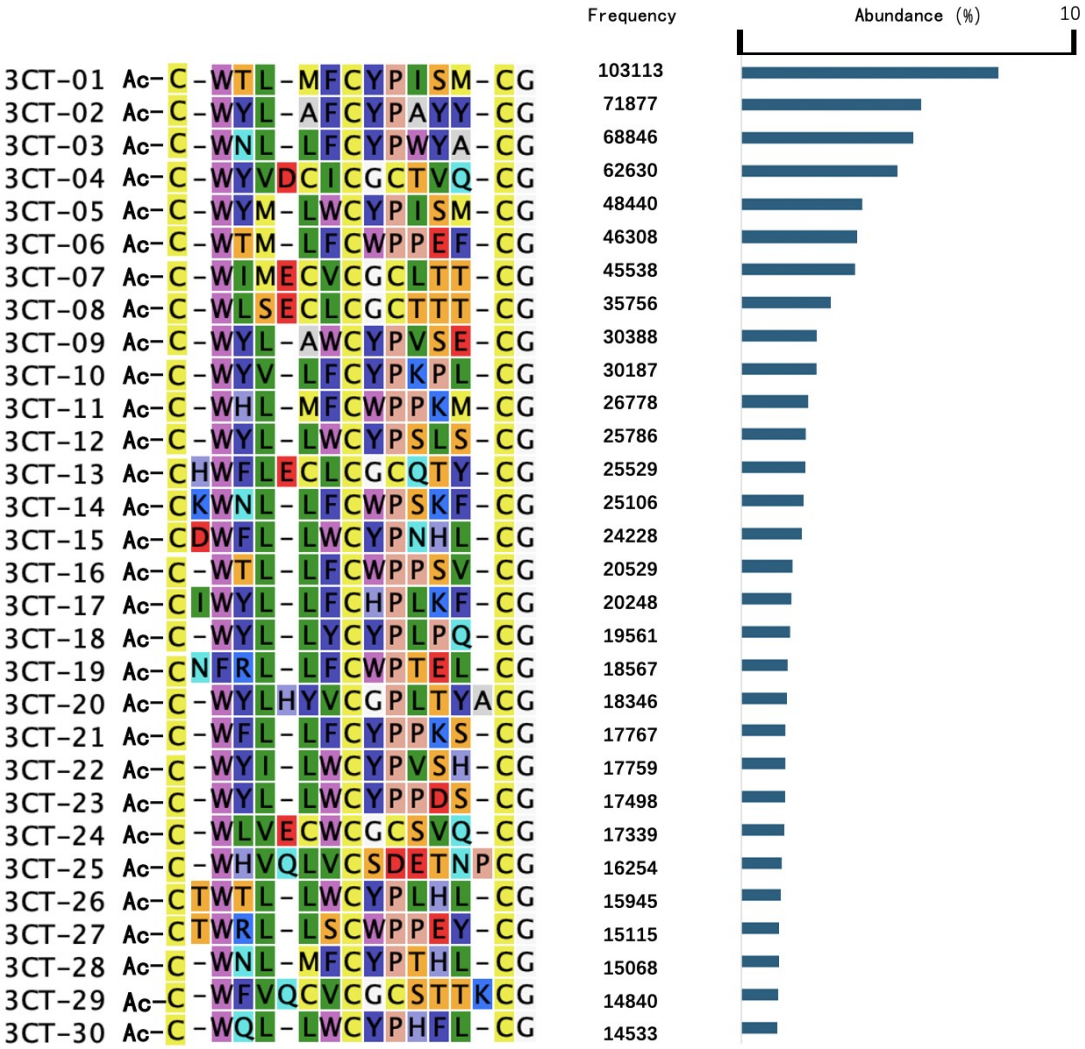


Figure S9. Top 30 sequences of bicyclic peptides targeting Trop2 protein. “M” can be replaced with “Ile” due to misincorporation.

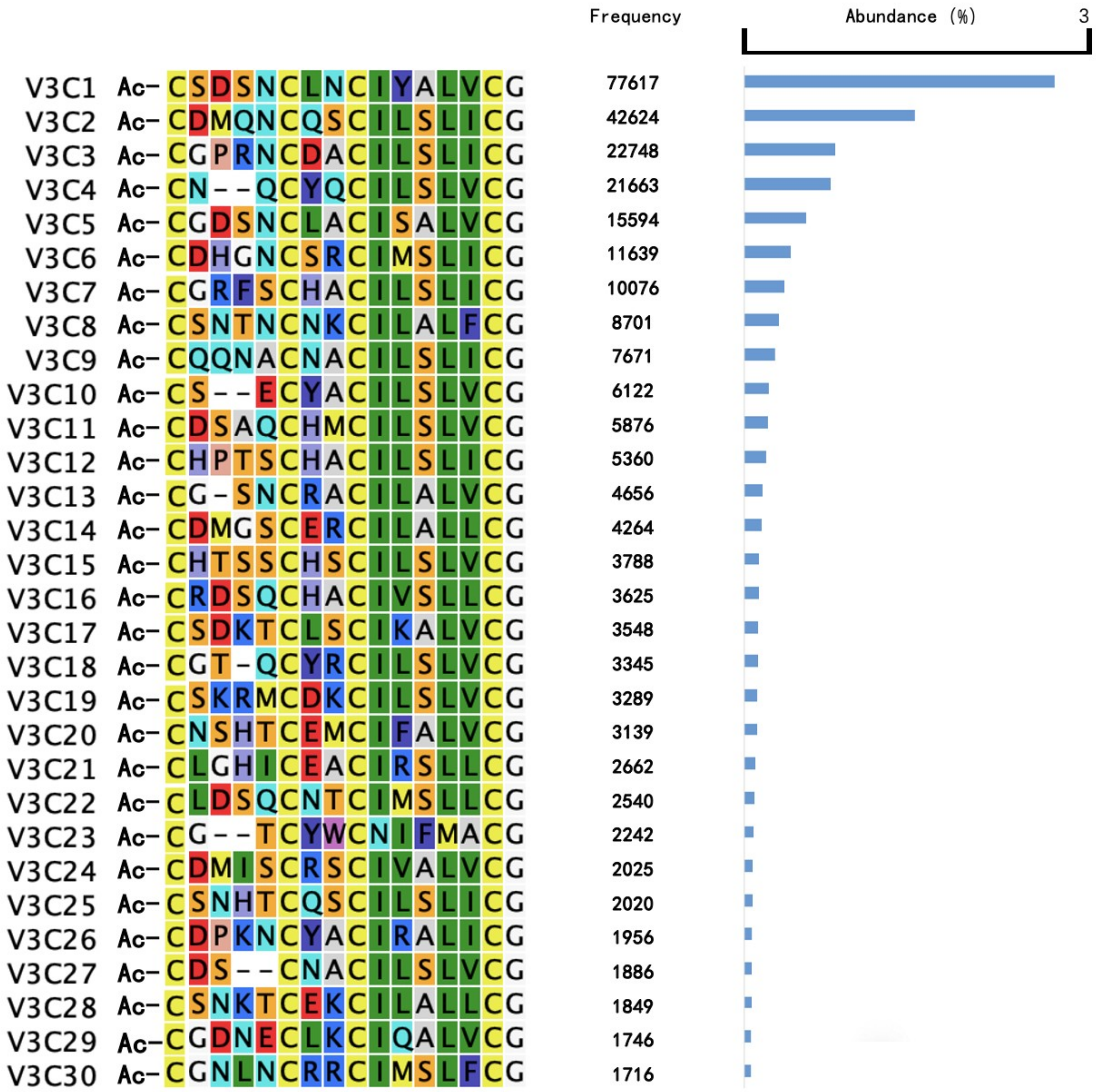


Figure S10. Top 30 sequences of bicyclic peptides targeting VEGF165 protein. “M” can be replaced with “Ile” due to misincorporation.

Structure and Binding Affinity Result of Bicyclic Peptide Targeting Trop2

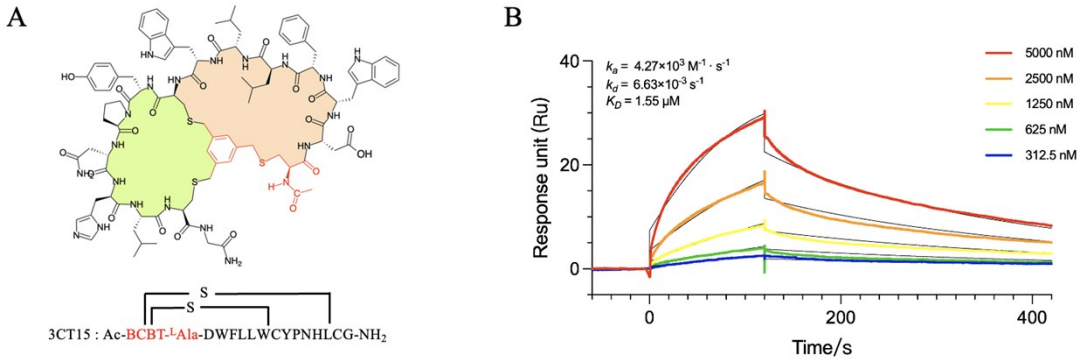


Figure S11. Chemical structure and binding affinity of bicyclic peptide 3CT-15 targeting Trop2. (A) The chemical structure of the 3CT-15. (B) SPR sensorgram of 3CT-15 targeting Trop2. Five different concentrations were injected to measure the kinetic constants: 312.5, 625, 1250, 2500, and 5000 nM.

Structures and Binding Affinity Results of Bicyclic Peptides Targeting VEGF165

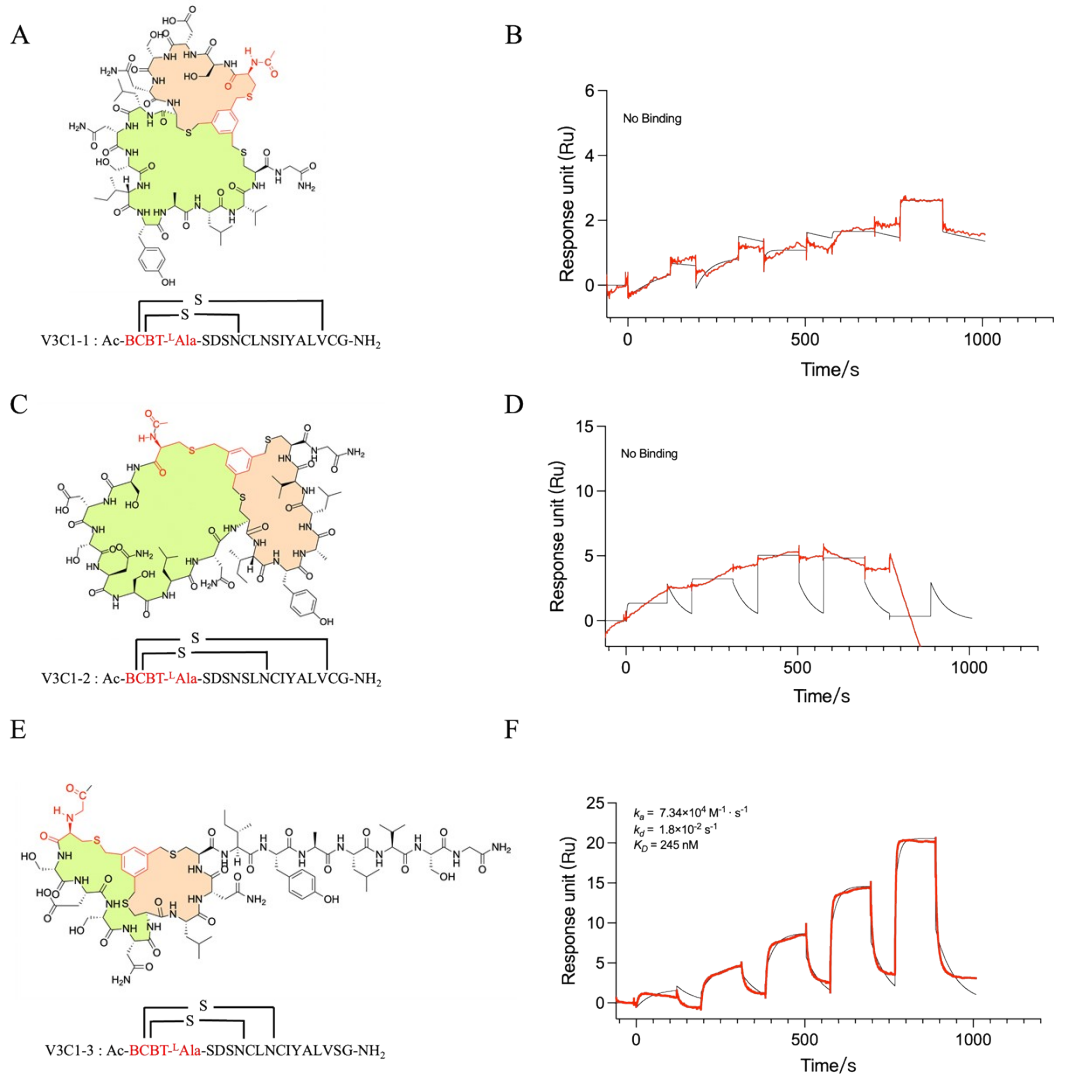


Figure S12. Chemical structure and binding affinity of the bicyclic peptides targeting VEGF165. (A) The chemical structure of the V3C1-1. (B) SPR sensorgram of V3C1-1. Five different concentrations were injected to measure the kinetic constants: 62.5, 125, 250, 500, and 1000 nM. (C) The chemical structure of the V3C1-2. (D) SPR sensorgram of V3C1-2. Five different concentrations were injected to measure the kinetic constants: 62.5, 125, 250, 500, and 1000 nM. (E) The chemical structure of the V3C1-3. (F) SPR sensorgram of V3C1-3. Five different concentrations were injected to measure the kinetic constants: 62.5, 125, 250, 500, and 1000 nM.

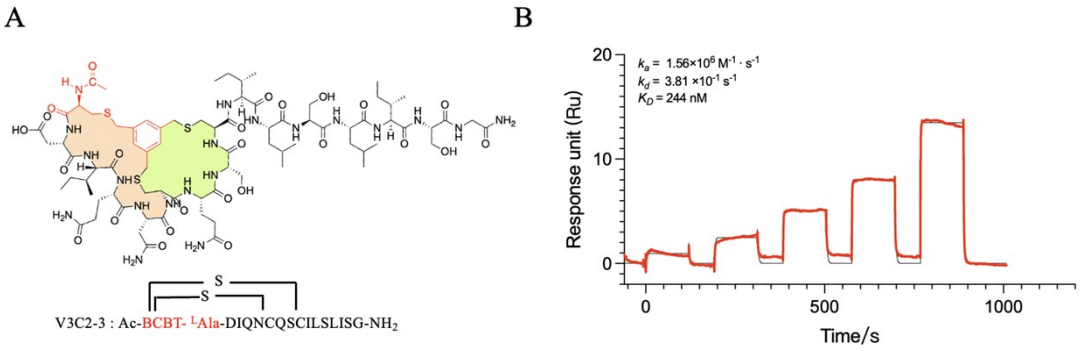


Figure S13. Chemical structure and binding affinity of the bicyclic peptide targeting VEGF165. (A) The chemical structure of the V3C2-3. (B) SPR sensorgram of V3C2-3. Five different concentrations were injected to measure the kinetic constants: 62.5, 125, 250, 500, and 1000 nM.

Neutralization Inhibition of Bicyclic Peptides Targeting VEGF165

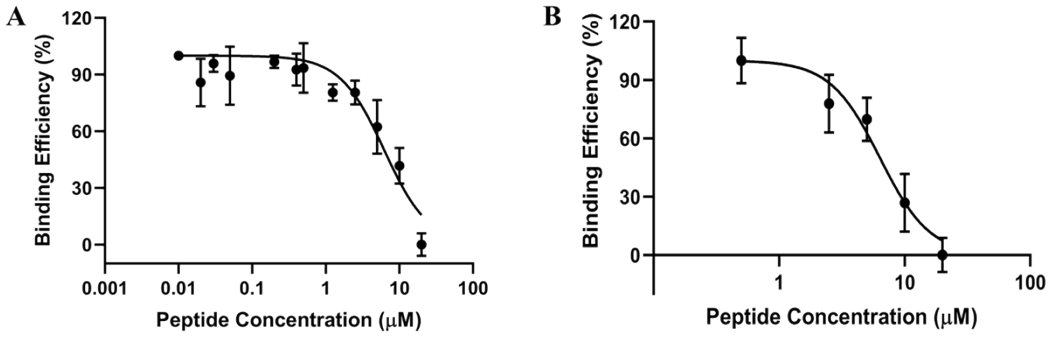
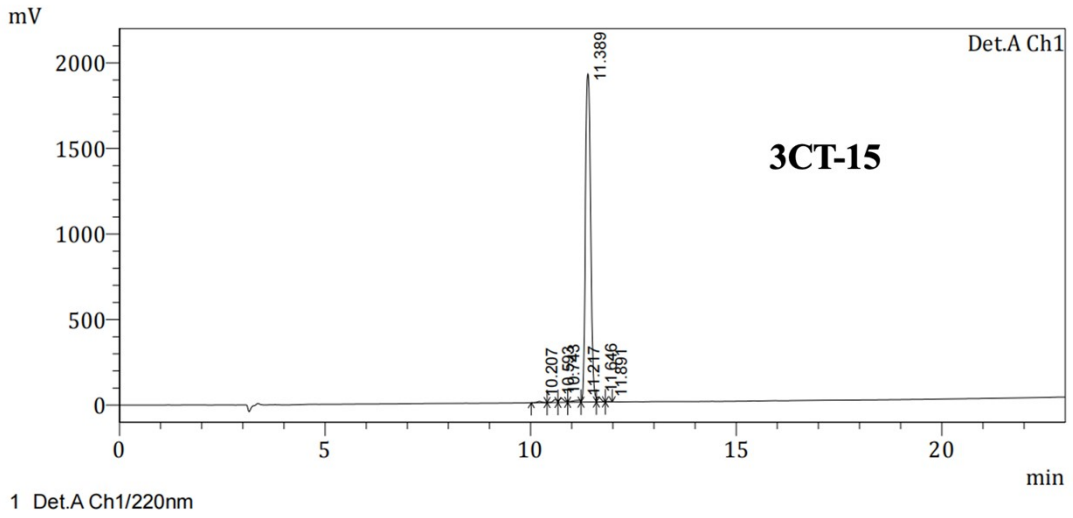
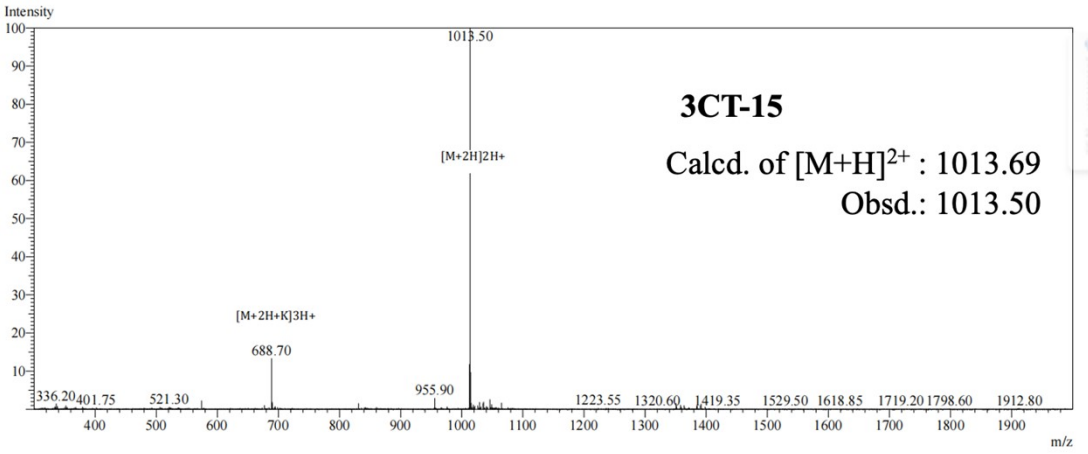
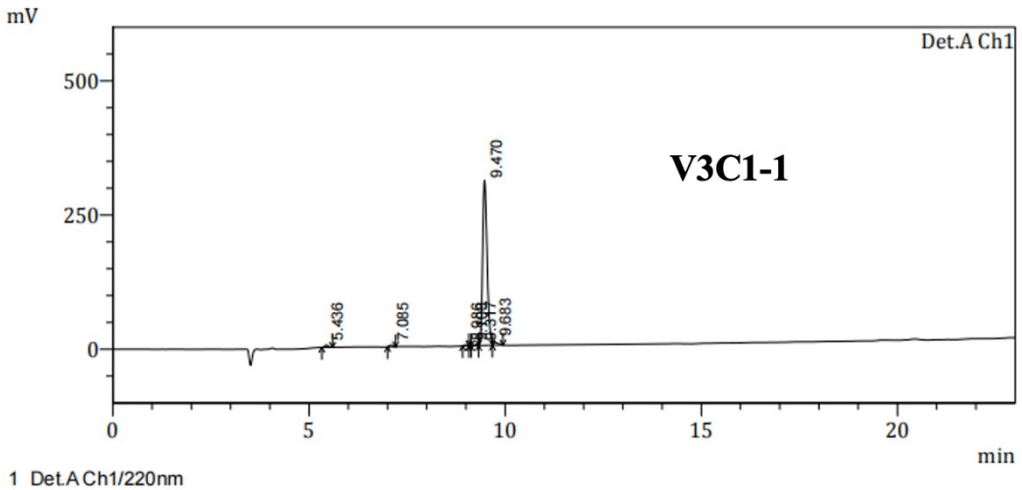


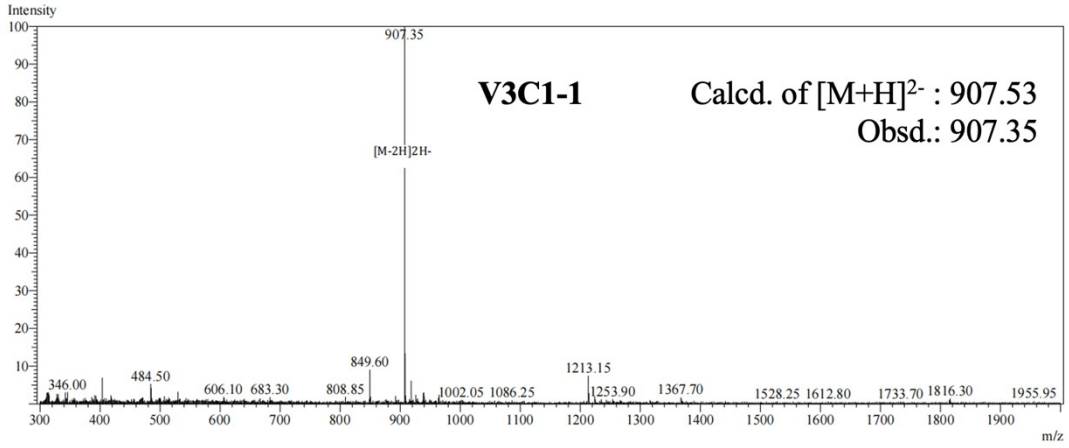
Figure S14. Neutralization inhibition activities of the two bicyclic peptides. (A) V3C1-3; (B) V3C2-3.

Characterization of synthesized bicyclic peptides by ESI-MS and HPLC





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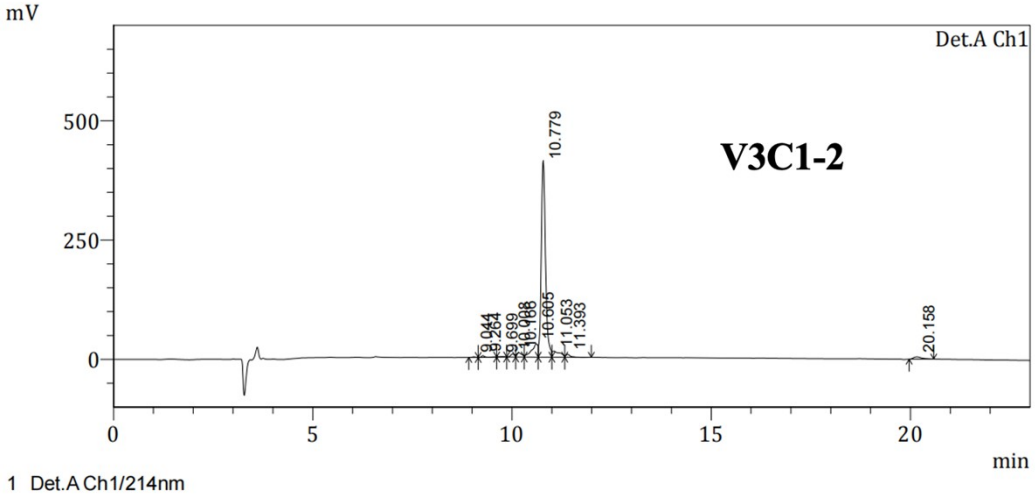
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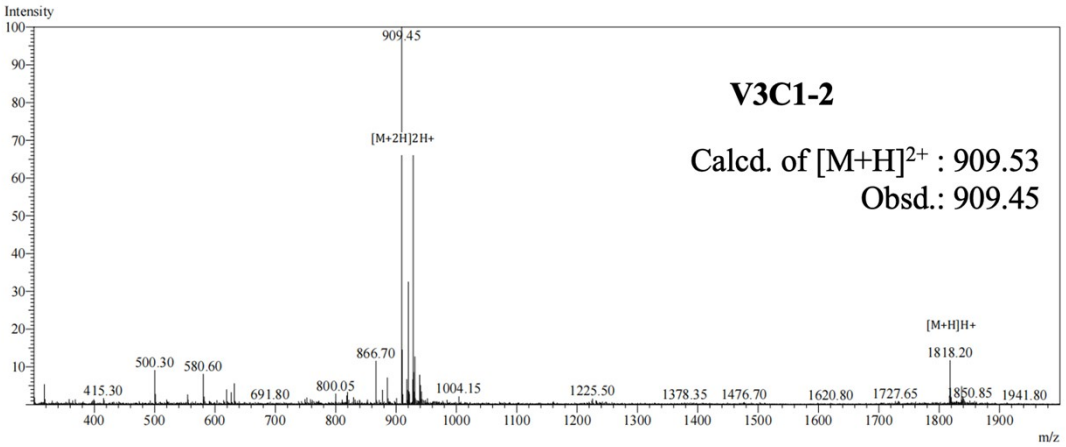
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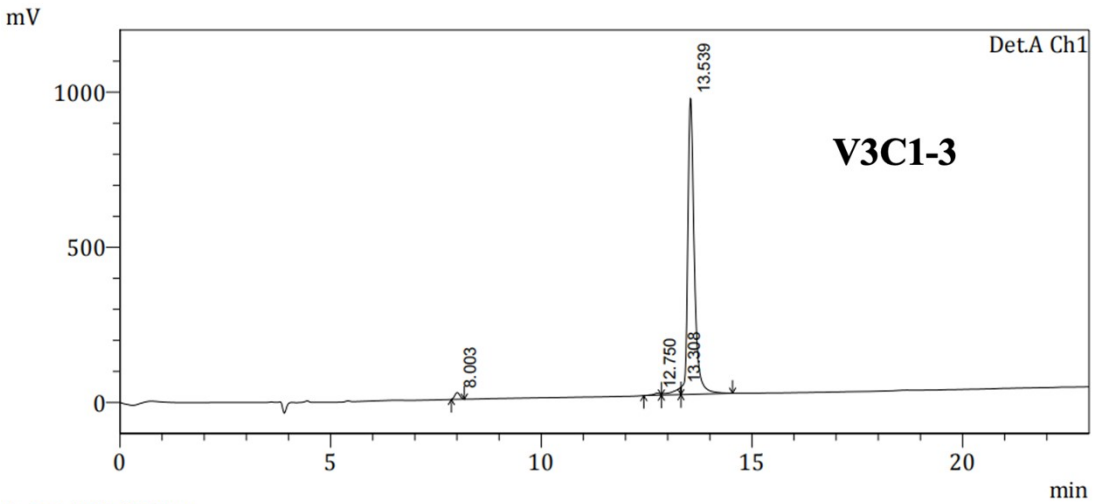
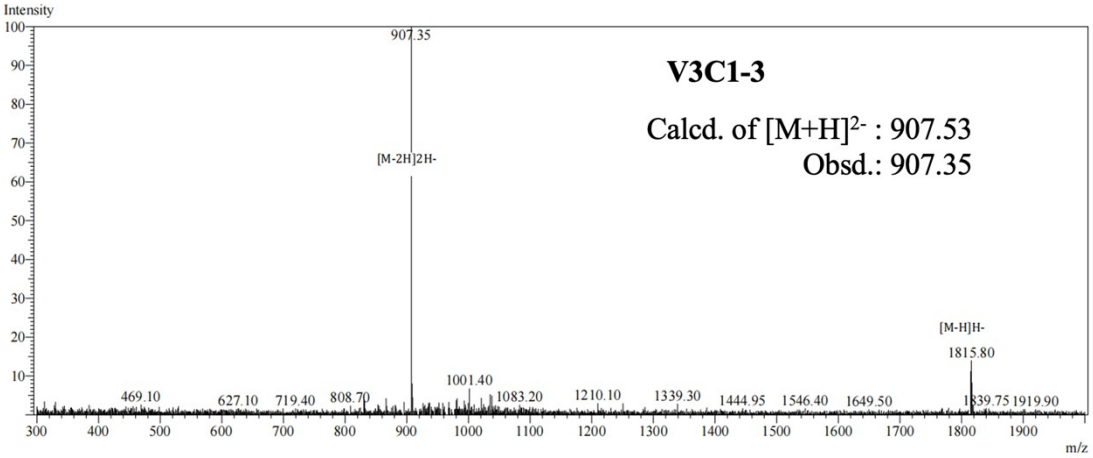
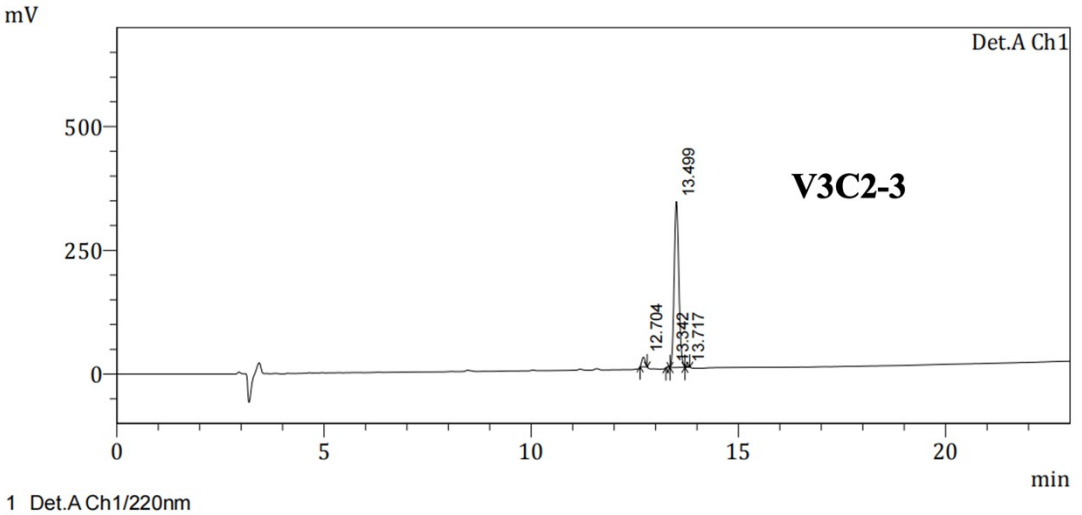
[M+2H]2H+
V3C1-2
Calcd. of [M+H]2+ : 909.53
Obsd.: 909.45
[M+H]H+
1818.20
866.70
500.30
580.60
415.30
691.80
800.05
1004.15
1225.50
1378.35
1476.70
1620.80
1727.65
1850.85
1941.80
m/z

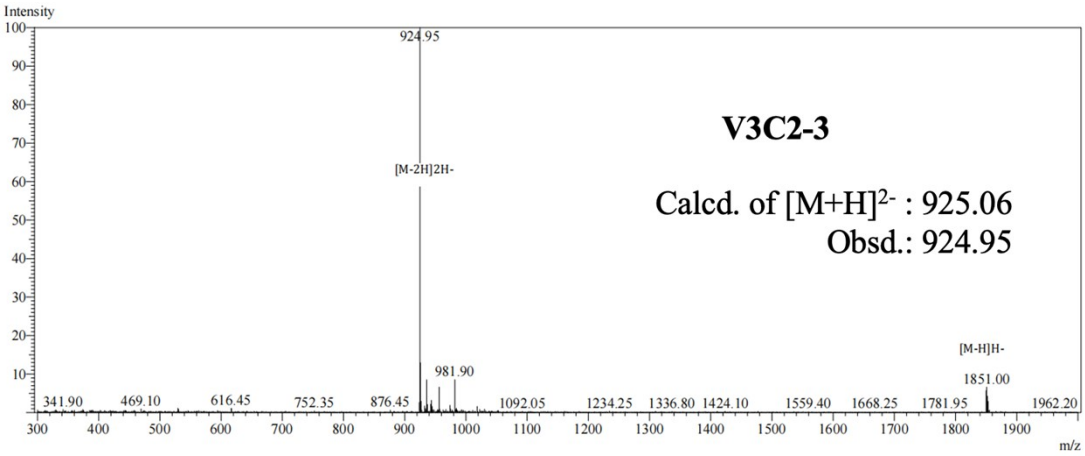


1 Det.A Ch1/220nm





1 Det.A Ch1/220nm


Intensity
924.95
[M-2H]2H-
V3C2-3
Calcd. of [M+H]2- : 925.06
Obsd.: 924.95
981.90
[M-H]H-
1851.00
341.90
469.10
616.45
752.35
876.45
1092.05
1234.25
1336.80
1424.10
1559.40
1668.25
1781.95
1962.20
m/z