Supporting Information

Intramolecular methionine alkylation constructs sulfonium tethered peptides for protein conjugation

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Abbreviations

HATU, 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-Tetramethyluronium Hexafluorophosphate; DIPEA, N,N-Diisopropylethylamine; TFA, Trifluoroacetic Acid; TIPS, Triisopropylsilane; FAM, 5-Carboxyfluorescein; HPLC, High-Performance Liquid Chromatography; MS, Mass Spectrometry; OD, Optical Density; PBS, Phosphate buffer saline; SPPS, Solid-Phase peptide synthesis; Fmoc,9-flurenylmethyloxycarbonyl.

Reagents and materials

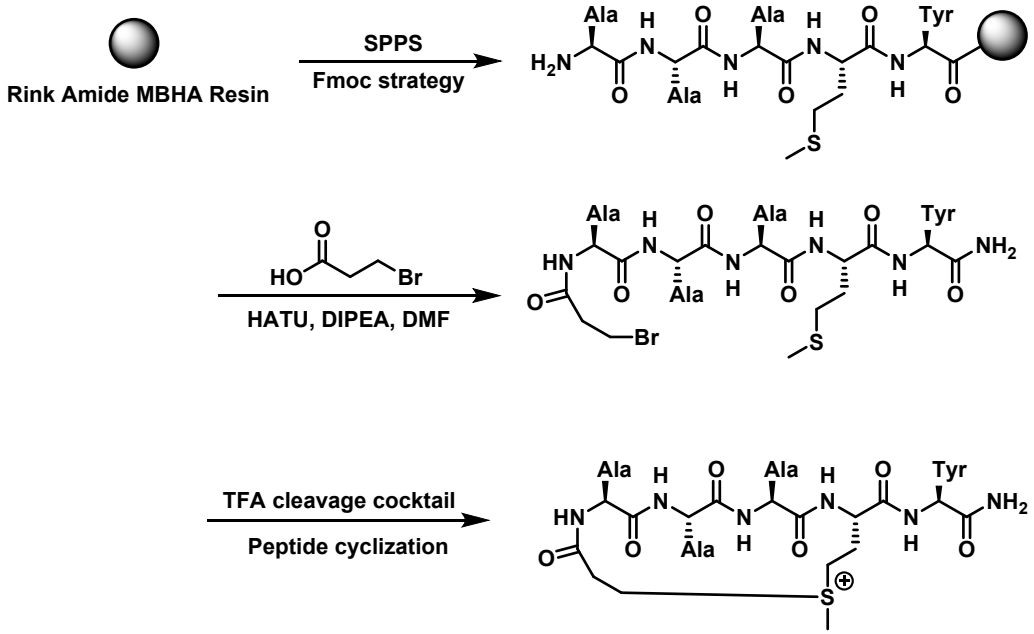
All amino acids and resins used for peptide synthesis were purchased from GL Biochem (Shanghai), Shanghai Hanhong, Aladdin or Energy Chemical and used without further purifications. All solvents were purchased from Cantotech Chemicals, Ltd. Anhydrous solvents were purchased from J&K Scientific and used without purification. The reagents used for biological assays were purchased from Sigma Aldrich and Thermo Fisher. Cells were purchased through ATCC and cultured according to ATCC guidelines.

Experimental Procedures

Peptide synthesis and purification

Peptides were synthesized on Rink Amide MBHA resin or Fmoc-Val-Wang resin by standard manual Fmoc solid--phase synthesis (SPPS). Rink-amide resin was pre-swelled with DMF for 30 min, filtered, the Fmoc (9-fluorenylmethyloxycarbonyl) group was removed with 50% (vol/vol) morpholine for 30min*2; the resin was sequentially washed with DCM and DMF for three times. For natural amino acids coupling, Fmoc-protected amino acids (5.0 equiv) and HATU (5.0 equiv) were dissolved in DMF, followed by DIPEA (10.0 equiv) . The mixture was pre-activated for 1 min and added to the resin for 1hour with N_2 bubbling. The resin was washed sequentially with DCM, DMF for three times, then dried under a stream of nitrogen for next step. The N-terminus was acetylated with Ac_2O/DIPEA/DMF (1:4:20) for 2*30min. FAM （5-Carboxyfluorescein, 7 equiv labeling was performed on the resin mixed with PYBOP (Benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate,3eq), HOBT (1-Hydroxybenzotriazole, 3eq) and NMM (4-Methylmorpholinein, 6eq) in DMF for 12 hours. For cleavage of resin, the final resin were treated with TFA/DCM/TIS/water (90:5:2.5:2.5) at room temperature for 3 h and concentrated

under a stream of nitrogen. The crude peptides were precipitated and washed with cold hexane/diehyl ether (1:2, v/v) at 4°C, redissolved in 50% acetonitrile in water. Crude peptides were purified by RP-HPLC. The linear peptide was assembled on the solid support as described in the general procedure. The mono-halogenated linker (2.0 equiv.) was added with DIPEA (4.0 equiv.) and HATU (4.0 equiv.) in DMF and was bubbled for 3 hours at room temperature with N_2. The peptide was cleaved from resin treated with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. Peptides were synthesized, purified and characterized by HPLC and LC-MS.



Circular dichroism spectroscopy

As for the secondary structure of protein conjugate, the protein-peptide mixture was desalted with ddH_2O avoiding the PBS interfere with the Circular dichroism(CD) spectra below 200nm. Circular dichroism(CD) spectra was obtained on a CD spectrometer at 20°C using a 1 mm path length, scanning between 260 and 180nm at 1nm/s with a bandwidth of 1.0 nm and response time of 1 s. Each spectrum is the average of twice scans. The data was fit to a two state folding model using Origin Pro 9.0.After replacing the PBS buffer, the protein conjugate was diluted with ddH_2O to the final concentration of 20μM and was detected the CD spectrometer as the same as peptides did.

NMR spectroscopy

NMR data were recorded on a Bruker AVANCE III 400 (or 500) MHz spectrometer. DMSO-d6 was used for ^{1}H NMR to characterize the peptides. NMR data were processed using MestReNova 3.0.

Dealkylation of the cyclic peptide

The cyclic peptides equipped with different linkers (1mM) were dissolved in 10mM nucleophile (2-mercaptopyridine, thiourea, GSH) in PBS (pH=7.4) and incubated at 37 ^{o}C for 0h, 2h, 4h, 8h, 12h,24h and 48h, an aliquot of each reaction solution was removed and monitored by LC-MS. The cyclic peptides can be gradually reduced to the linear products. The coupled linkers had different reduction rate towards the reducing reagents.

Tumor cell lines and culture

Human kidney cells, 293T cells (CRL-11268, ATCC) were cultured in DMEM with 10% (v/v) FBS and 100×penicillin/streptomycin (100 g/mL). Human ovarian cancer cells, A2780 cells, a gift from professor Fei Lu (Peking University Shenzhen Graduate School) were cultured in RPMI-1640 with 10% (v/v) FBS and 100×penicillin/streptomycin (100 g/mL). All these cells were maintained in a humidified incubator containing 5 % CO_2 at 37 °C.

Flow cytometry analysis

HeLa cells were seeded in a 24-well dishes (50000 cells/well) for 24 hours in a humidified incubator containing 5 % CO_2 at 37 °C. The plates were incubated with 10μM FAM-labeled peptides in 5% FBS medium for 4h at 37°C. After washing with media, the cells were exposed to 0.25% trypsin digestion for 1 min at 37°C, washed with PBS for 3 times. Then the cells were harvested and incubated with 0.05% Trypan Blue for 3 min before FACS analysis.

Confocal microscopy imaging

Cell viability for 293T cell line and Hela cell line were measured by MTT (3-(4, 5-dimethylthiazol-

2-yl)-2, 5-diphenylt-etrazolium bromide, from Sigma) assays. The cells were seeded in a 96-wells plate for 24 hours with growth medium. Then the media was removed followed by adding peptides in medium with 5% FBS (fetal bovine serum, v/v) for 24 hours' incubation. MTT (5 mg/mL, 20 μL) in PBS was added and the cells were incubated for 4 h at 37 °C with 5% CO_2. Then DMSO (100 μL, Sigma) was added to solubilize the precipitate with 10 min of gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The coverslips were mounted onto slides and visualized by a confocal laser scanning microscope (Nikon A1R).

Protein Expression and Purification

The primers used in this study were listed in **table S1**. $PDZ^{\Delta RGS3}$ fragment(a gift from Prof. Jiang Xia's group, Hongkong Chinese University) and their mutants($PDZ^{\Delta RGS3C33S\ C34S}$ and $PDZ^{\Delta RGS3C73S}$, created by PCR-based site directed mutagenesis) were transformed into E. coli BL21 (DE3) cells and were grown overnight at 37ºC in LB media supplemented with 100 μg/mL ampicillin.[1] The culture was then used to inoculate 1L LB media supplemented with 100 μg/mL ampicillin. When the cell culture was grown at 37ºC to reach OD_{600}~0.6, the cell culture was removed to 16ºC with 1 mM IPTG to induce protein expression for 8 hours. The cells were harvested and resuspended in 50 mL of lysis buffer (containing 20 mM tris, 500 mM NaCl, 3 mM DTT, 0.1 mM PMSF, pH 7.5), sonicated, and centrifugated at 14,000 g for 1 hour to obtain the supernatant and remove cell debris. Recombinant proteins were extracted from cleared lysates by nickel nitrilotriacetic acid-agarose (Qiagen) followed by washes with PBS containing 10 mM imidazole. The fusion protein was eluted with 250 mM imidazole and dialyzed against PBS. The other proteins such as BCL_2, MgrA and Sortase A were gifts from Li group.

Fluorescence polarization

Fluorescence polarization experiments were performed in 96-well plates (Perkin Elmer Optiplate-96F) on plate reader (Perkin Elmer, Envision, 2104 multilabel reader) at 25 ºC with excitation at 485 nm and emission at 520 nm. The FAM labeled peptides (5nM) were incubated with increasing concentration of purified $PDZ^{\Delta RGS3}$ in PBS (pH7.4) in the dark for 1 hours. Then the mixture was measured at 25 ºC using a plate reader (PerkinElmer, Envision) and the binding affinity (Kd) values were determined by fitting the experiment data to nonlinear regression analysis by Origin 8.0 or Prism 6.

In vitro protein-peptide covalent conjugation assay

For peptide specificity of covalent protein conjugation, the purified $PDZ^{\triangle RGS3}$ (20μM) were reacted with peptides(100μM) in pH 7.4 PBS buffer for 1 hours. Then the reaction mixture was analyzed with 16% tricine gels. As for the site specificity of protein conjugation, the protein mutants (20μM) were constructed and reacted with peptide PDC-1-C3(100μM) in pH 7.4 PBS buffer for 12 hours at 37 ˚C. Then the reaction mixture was analyzed with 16% tricine gels. As for the reaction kinetics of the covalent conjugation, $PDZ^{\Delta RGS3}$ (1.0 equal, 15μM) was incubated with peptide PDC-1-C3 (5 eq, 75μM) in PBS (pH 7.4, 37 ˚C) for 0min, 1min, 10min, 60min, 240min, 480min and 720min, respectively. Then the reaction were analyzed with 16% tricine gels. To examine the specificity of the conjugation reaction, the purified protein $PDZ^{\Delta RGS3}$, BCL_2, MgrA and SrtA(15μM) were reacted with peptide PDC-1-C3(75μM) in PBS (pH 7.4, 37 ˚C)for 12 hours, then the reaction mixture were analyzed with 16% tricine gels. To measure the conjugation reactions at different protein/peptide ratios, different peptide concentrations (0μM, 7.5μM, 15μM, 75μM, 150μM) were incubated with protein in PBS (pH 7.4, 37 ˚C) for 4 hours and then analyzed through 16% tricine gels. For the competition assays, the linear peptide L(200μM or 400μM) was added to $PDZ^{\Delta RGS3}$（20μM） containing peptide PDC-1-C3(100μM) in PBS (pH 7.4, 37 ˚C) for 0min,10min, 30min, 1h, 2h and 4h, 8h and 12h, respectively. In order to judge the method of macro cyclization method via the bis-alkylation between methionine and cysteine, a control experiment was also performed.

Protein thermal shift assays *in vitro*

The in vitro thermal shift assays were conducted in the CFX96 Real-Time PCR Detection System (Bio-Rad). The fluorescent dye SYPRO orange (Sigma) was used to monitor the unfolding of $PDZ^{\Delta RGS3}$. As for the protein only system, 25μL of mixtures containing 5μL of 100× SYPRO Orange, 10μL of 20μM $PDZ^{\Delta RGS3}$ and 10μL PBS were mixed in a 8-well PCR tube, and fluorescence was measured from 25 °C to 100 °C with a heating rate of 0.5 °C/10 s (excitation, 450-490 nm; detection, 560-580 nm). To investigate the Tm of protein-conjugate, the protein (20μM) and peptide PDC-1-C3 (100μM) was incubated at 37 °C for 8 hours and detected by protein gel to ensure the successful conjugation. Then, 25 μL of mixtures containing 5 μL of 100× SYPRO Orange, 10μL of 20μM protein-peptide conjugate and 10μL PBS were mixed in a 8-well PCR tube, then fluorescence was measured from 25 °C to 100 °C with a heating rate of 0.5 °C/10 s.

Protein-peptide covalent conjugation in cell lysates

To assess the protein-peptide conjugation in a complex proteome environment, 293T cell lysates (100μg) were spiked with $PDZ^{\Delta RGS3}$ (10μg) and then treated with FAM-labeled peptide PDC-1-C3-

FAM (50μM) for 24 hours at 37˚C.Then the reaction mixture was incubated with PBS-washed Ni-NTA Agarose Beads with rotation for 4 hours at 4˚C. The beads were centrifuged at 2000g, 2min, and washed three times with NP-40 lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.5], 120 mM NaCl), once with PBS and eluted by boiling in SDS loading buffer and analyzed with 16% tricine gels for fluorescence detection and western blot analysis. The concentration of total cellular lysate protein was determined using Bicinchoninic Acid (BCA) Kit (Sigma-Aldrich). 10^7 Hela cells were washed with PBS and harvested using the 1mL lysis buffer containing 1% protease inhibitor mixture. The 25 μL of each standard or unknown sample and 200 μL of the working reagent (50:1, Reagent A:B) were added into a microplate well. Subsequently, the plate was covered and incubated at 37°C for 30minutes. And the absorbance was measured at or near 562 nm on a plate reader. The concentration was 4.43 mg / mL .

Protein-peptide conjugation in HA- PDZ$^{\Delta RGS3}$ transfected cell lysates

293T cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin, and transfections performed with pCAG plasmid containing HA-PDZ using lipo2000 (Invitrogen) for 4-6 hours. Then the cells were replaced by 10% FBS medium for another 24 hours. The cells were washed with PBS and harvested using the lysis buffer (0.5% NP40, 50 mM Tris [pH 7.5], 120 mM NaCl, 5mM DTT). The extracted protein concentrations were measured by BCA.

In-gel trypsin digestion

The protein was digested by trypsin using standard in-gel trypsin digestion protocols.[1] The protein bands of interest from 16% tricine gel were excised after electrophoresis and cut bands into pieces for destaining. Then the gels were destained by treating the gel pieces with a solution of ammonium bicarbonate:acetonitrile and incubated for 30min at 37 ˚C. Digested protein fragments were further extracted from gel with 0.5% formic acid/50% acetonitrile and incubated for 15 min and combined with trypsin digestion supernatant and dried for 5-10 minutes. The treated samples were used for MS/MS analysis.

Supplementary Figures

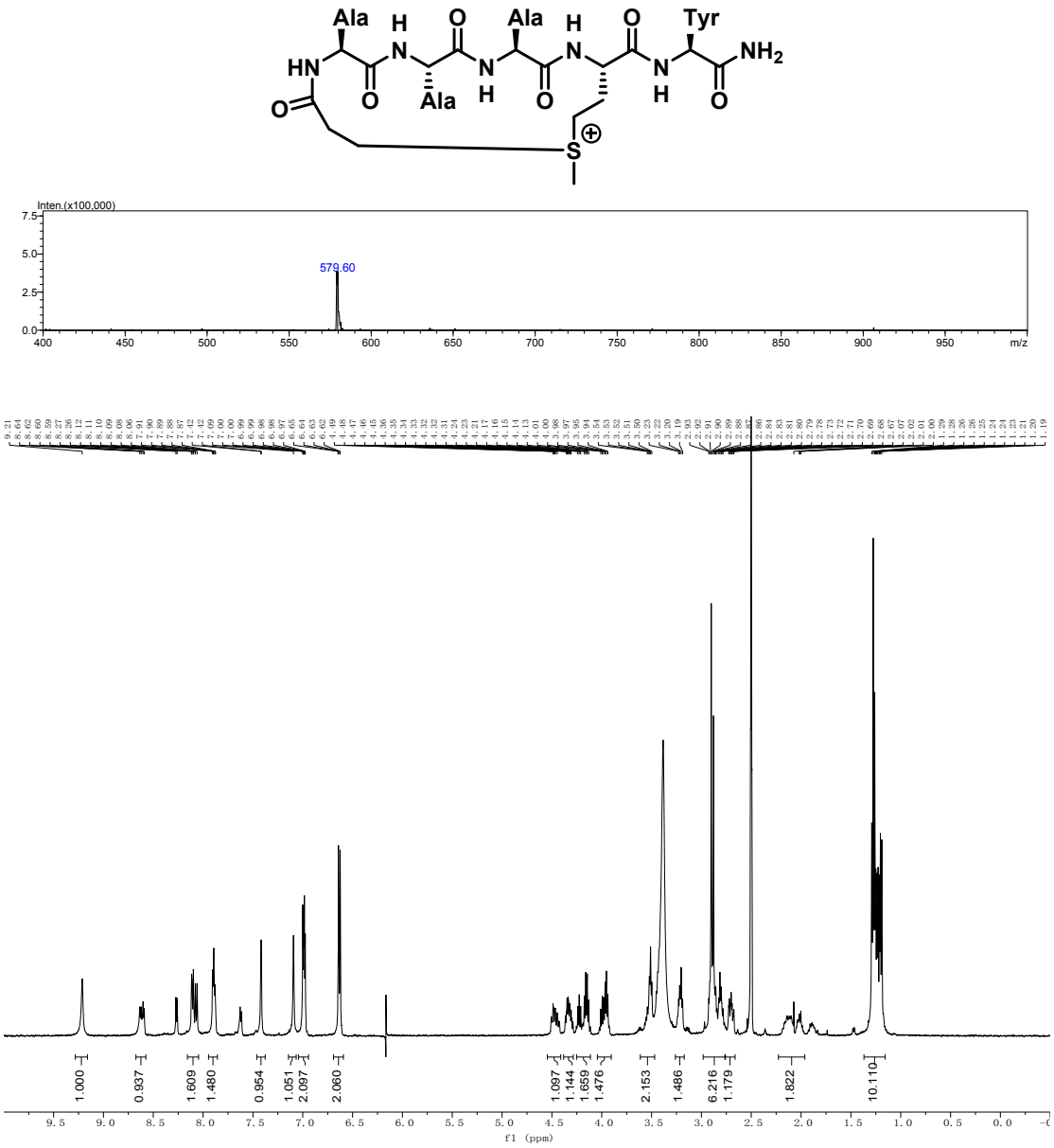
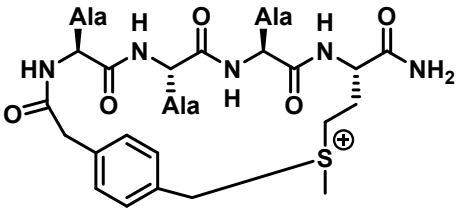


Figure S1-1. ^{1}H NMR (500 MHz, DMSO-d6) δ 9.21 (s, 1H), 8.61 (dd, J = 15.9, 6.6 Hz, 1H), 8.16 – 8.05 (m, 2H), 7.89 (dd, J = 7.5, 5.0 Hz, 1H), 7.42 (d, J = 2.1 Hz, 1H), 7.09 (s, 1H), 7.04 – 6.94 (m, 2H), 6.69 – 6.59 (m, 2H), 4.55 – 4.41 (m, 1H), 4.33 (dtd, J = 15.8, 8.4, 4.9 Hz, 1H), 4.25 – 4.11 (m, 2H), 3.97 (tt, J = 14.7, 7.5 Hz, 1H), 3.53 (dt, J = 17.8, 5.8 Hz, 2H), 3.21 (q, J = 7.3, 6.5 Hz, 1H), 2.98 – 2.76 (m, 6H), 2.70 (ddd, J = 13.5, 8.8, 3.3 Hz, 1H), 2.23 – 1.96 (m, 2H), 1.37 – 1.16 (m, 10H).



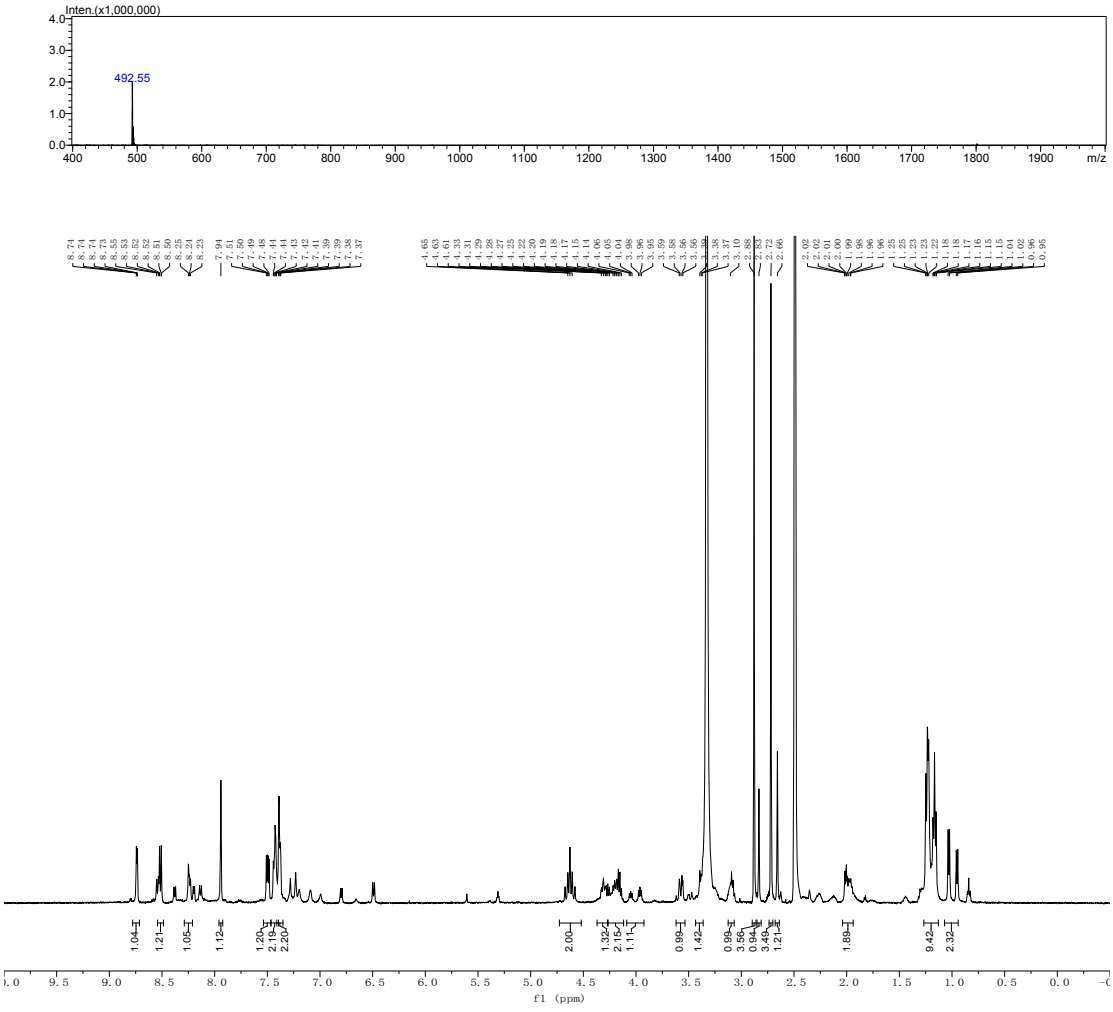


Figure S1-2. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.74 (dd, J = 4.4, 1.4 Hz, 1H), 8.51 (dd, J = 8.4, 1.5 Hz, 1H), 8.29 – 8.21 (m, 1H), 7.94 (s, 1H), 7.50 (dd, J = 8.4, 4.4 Hz, 1H), 7.43 (dd, J = 8.0, 3.8 Hz, 2H), 7.41 – 7.35 (m, 2H), 4.63 (t, J = 10.5 Hz, 2H), 4.37 – 4.26 (m, 1H), 4.27 – 4.12 (m, 2H), 4.01 (dt, J = 44.0, 6.6 Hz, 1H), 3.57 (dd, J = 13.0, 4.2 Hz, 1H), 3.39 (d, J = 6.8 Hz, 1H), 3.10 (s, 1H), 2.88 (s, 4H), 2.83 (s, 1H), 2.72 (s, 3H), 2.66 (s, 1H), 2.04 – 1.94 (m, 2H), 1.27 – 1.13 (m, 9H), 1.07 – 0.94 (m, 2H).

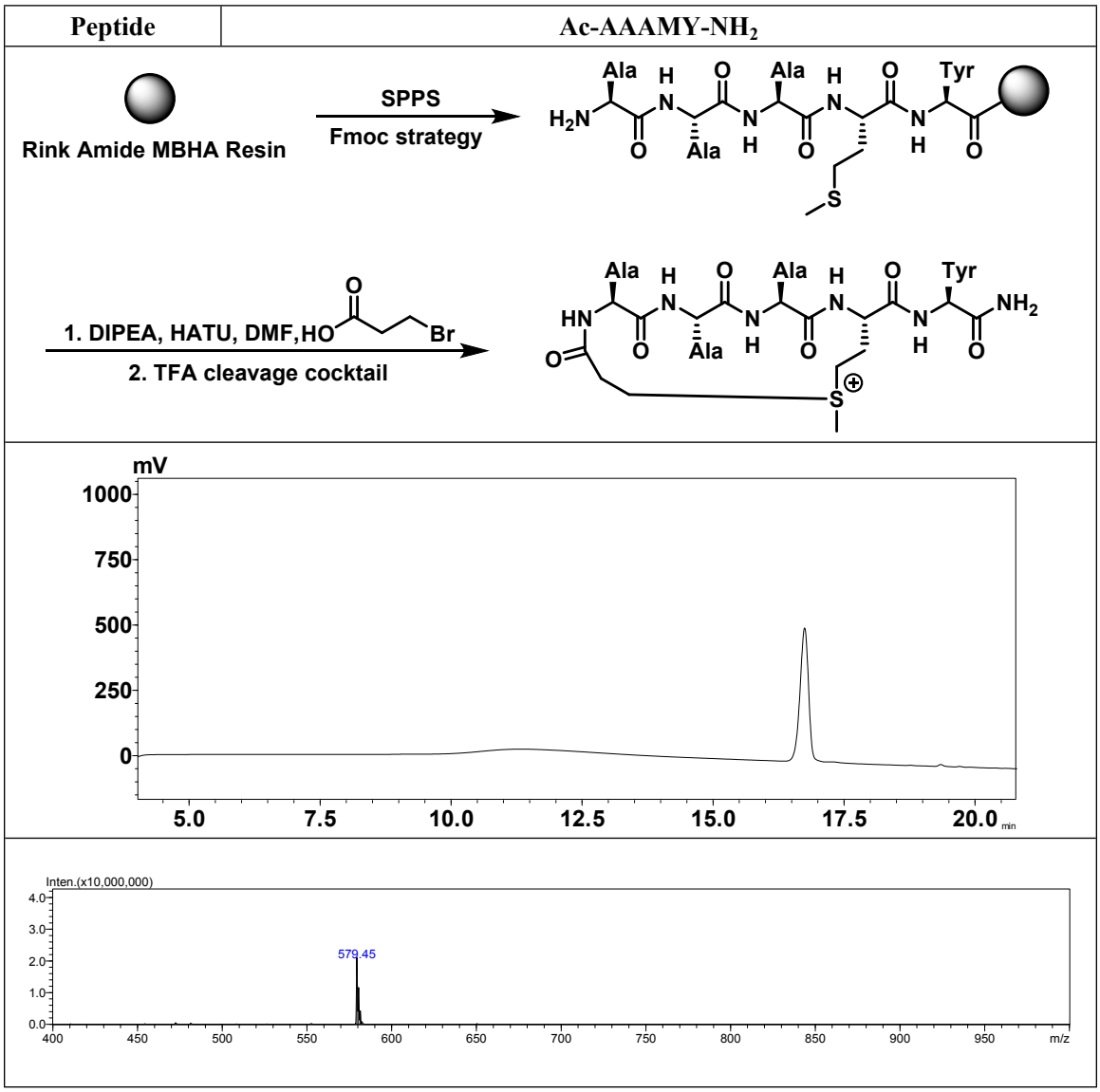
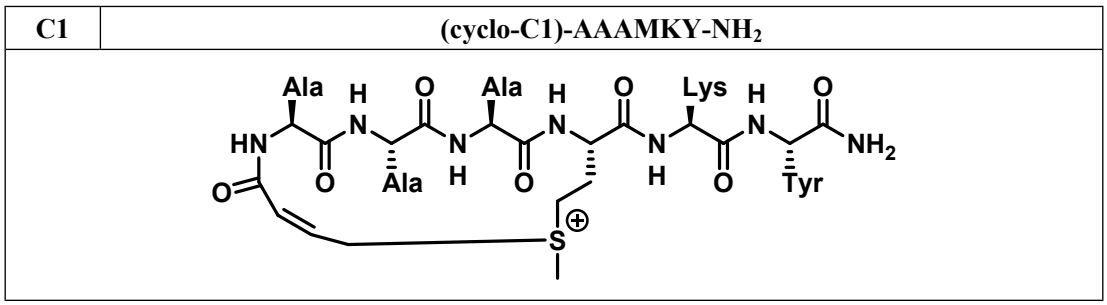
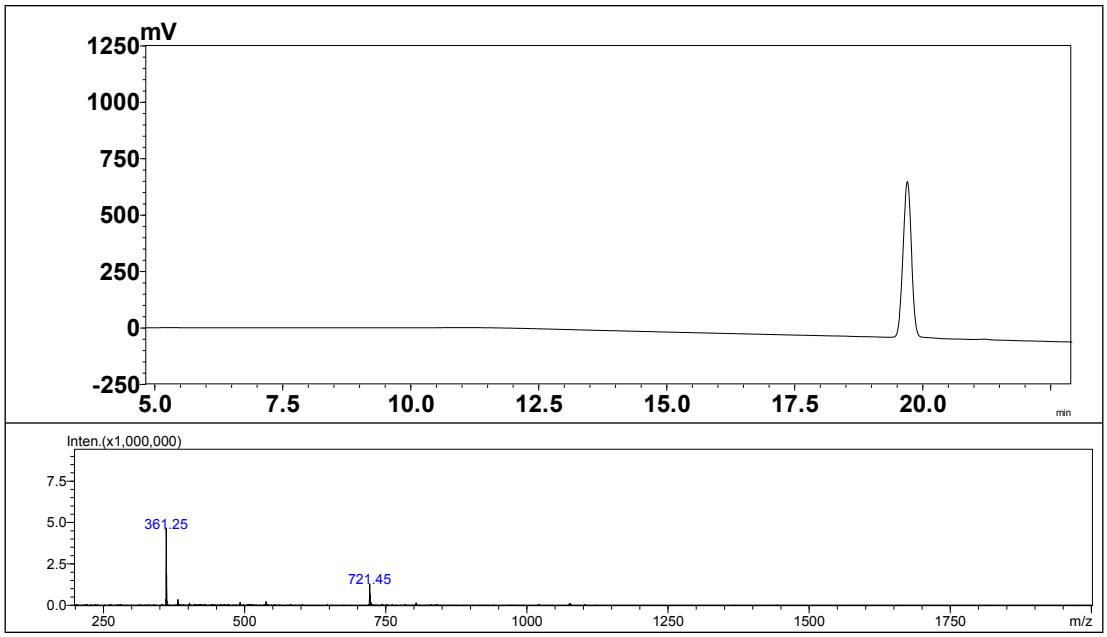
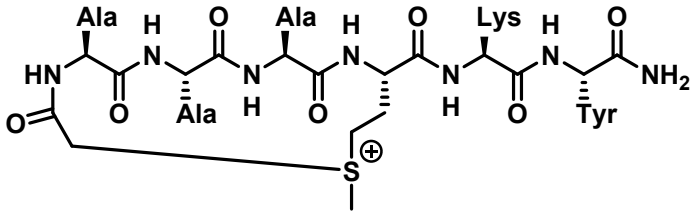


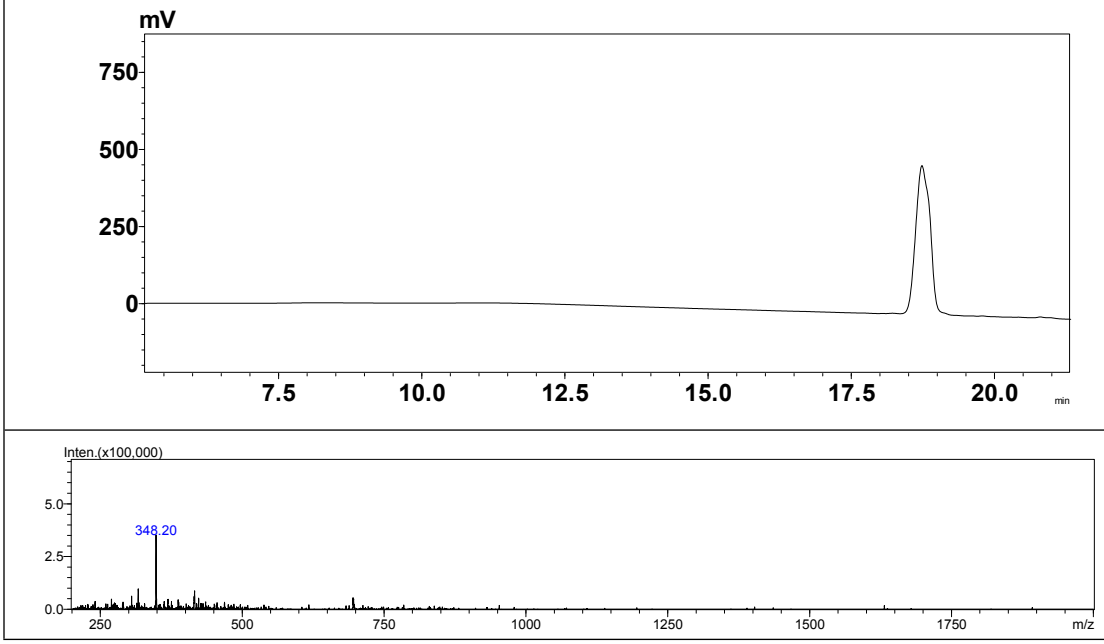
Figure S2. The peptide cyclization by intramolecular methionine alkylation. The MS data indicated the product.



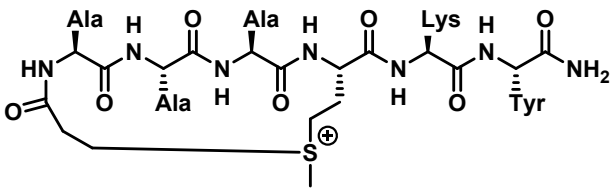


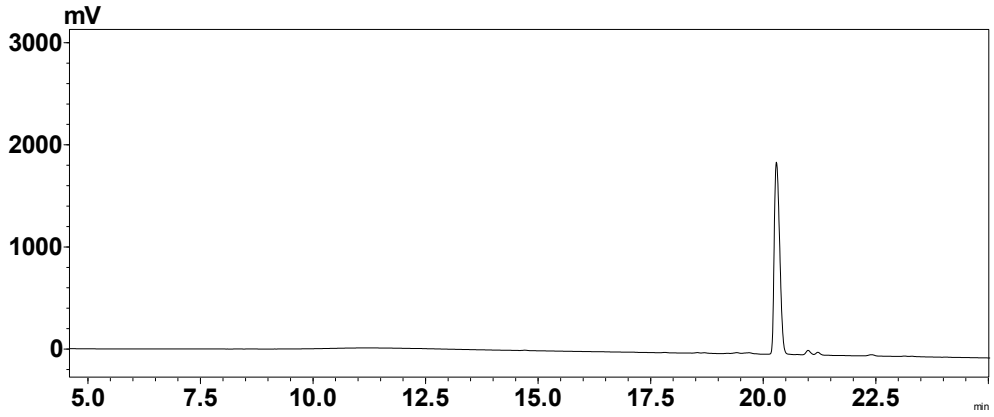
C2	(cyclo-C2)-AAAMKY-NH_2

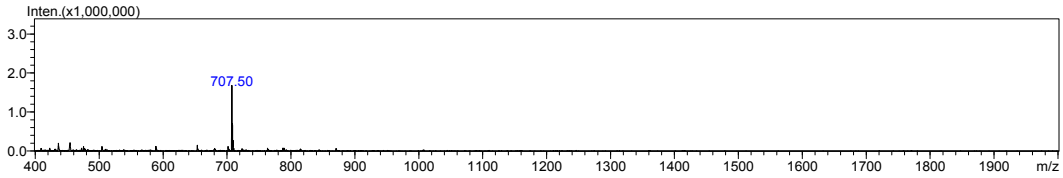




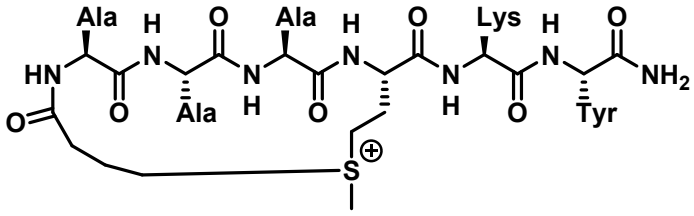
C3	(cyclo-C3)-AAAMKY-NH_2

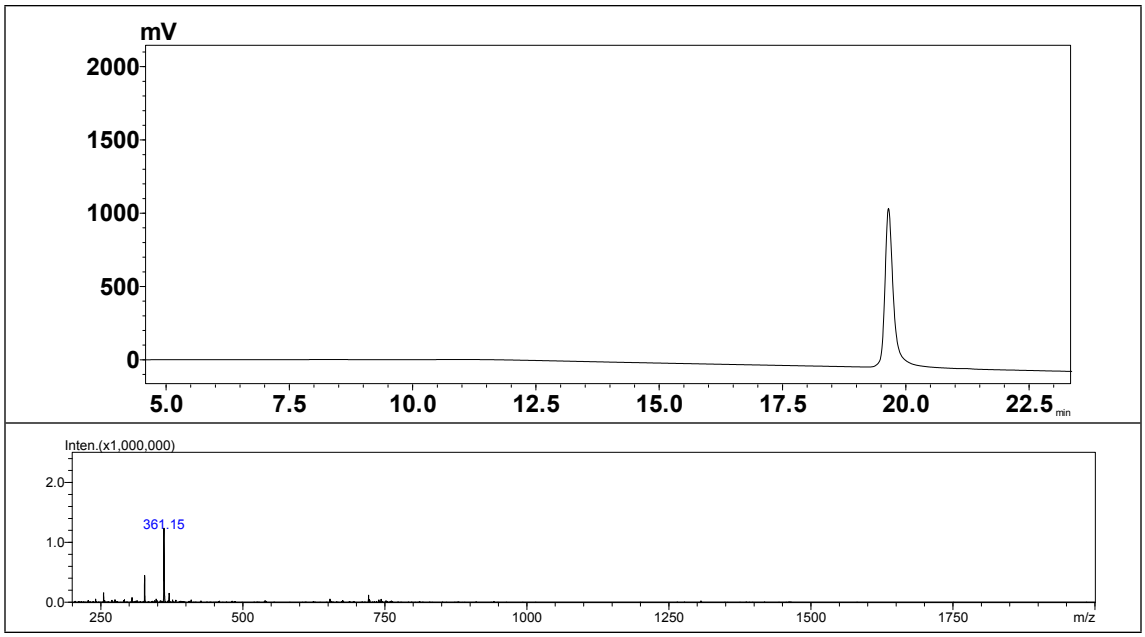






C4	(cyclo-C4)-AAAMKY-NH_2





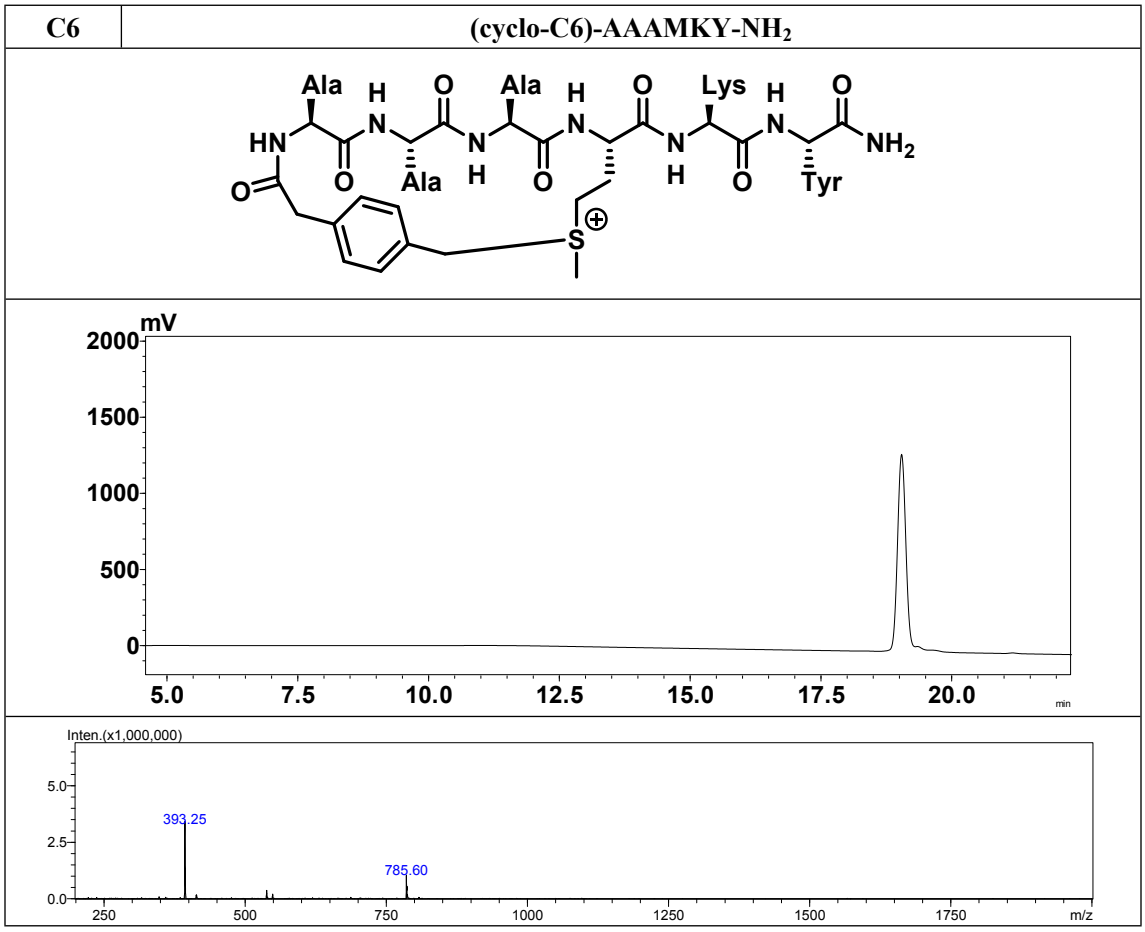


Figure S3. HPLC separation spectra of peptides **C3 to C4**. (Column: C18 analytic column - Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 µm.Flow rate: 1.0 mL/min)

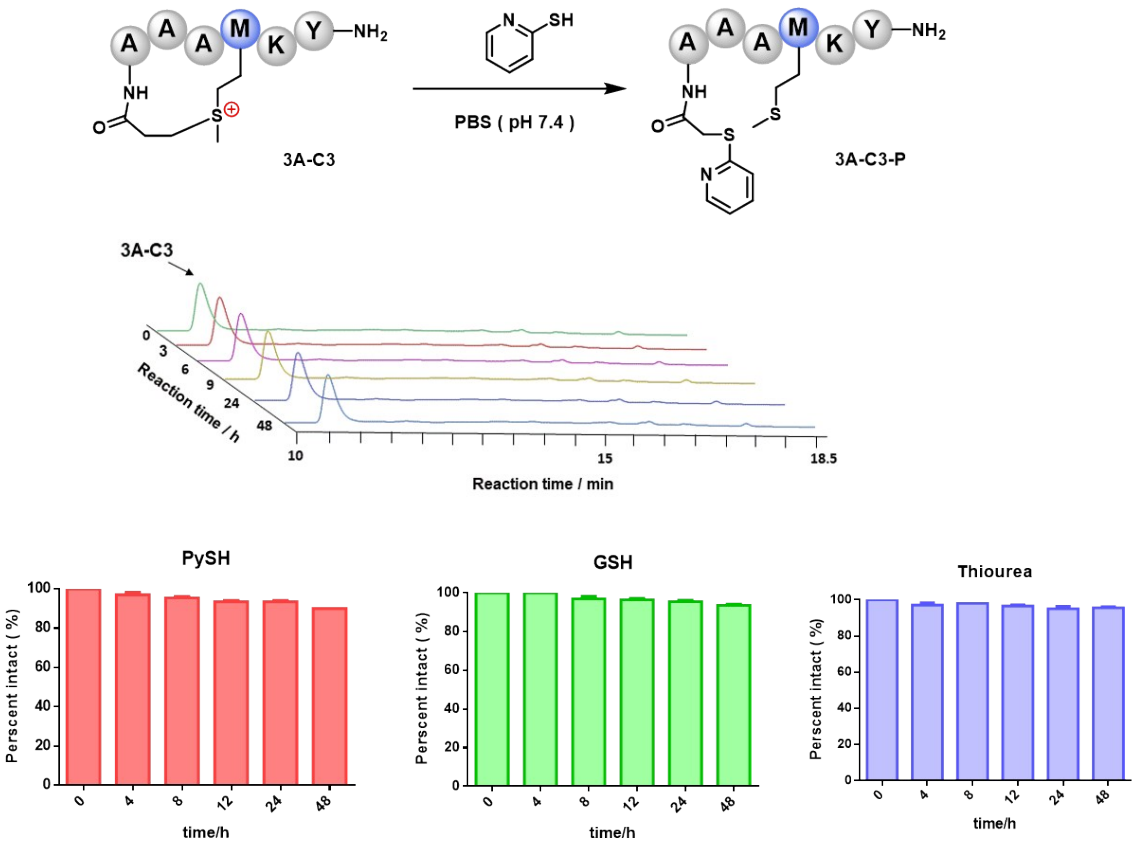


Figure S4. The kinetics of peptide dealkylation reactions equipped with different cross linkers. Peptides(1mM) were incubated with PyS (10mM) in PBS (pH=7.4) at 37°C for 48 hours. It is difficult to detect the formation of products through LC-MS.

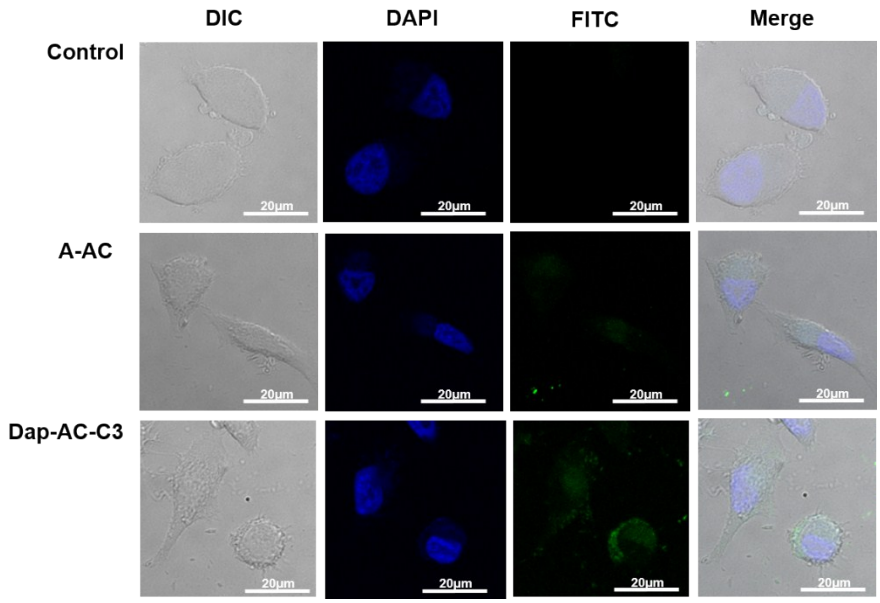


Figure S5. Confocal microscopy images of HeLa cells treated with 10 μM FAM-labelled peptides with different linkers at 37°C for 4 hours.

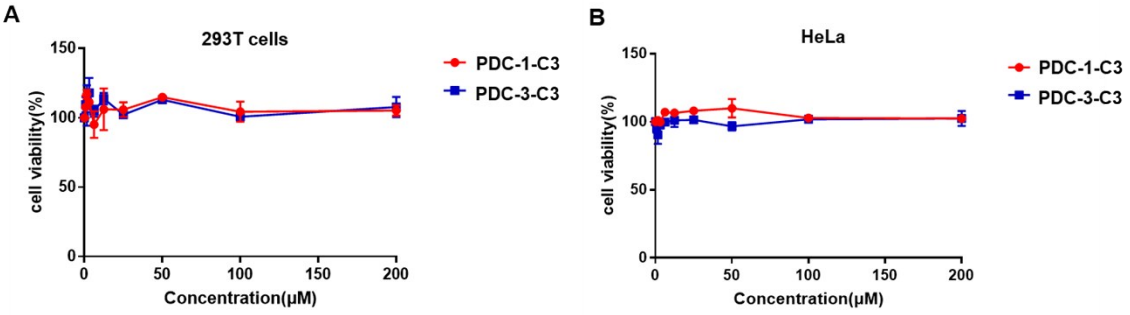


Figure S6. As shown in the two picture. These peptides were used to treat different cell lines at different concentration for 24h. The negligible effect on the 293T and Hela cell lines indicated the low nonspecific toxicity of these peptides.

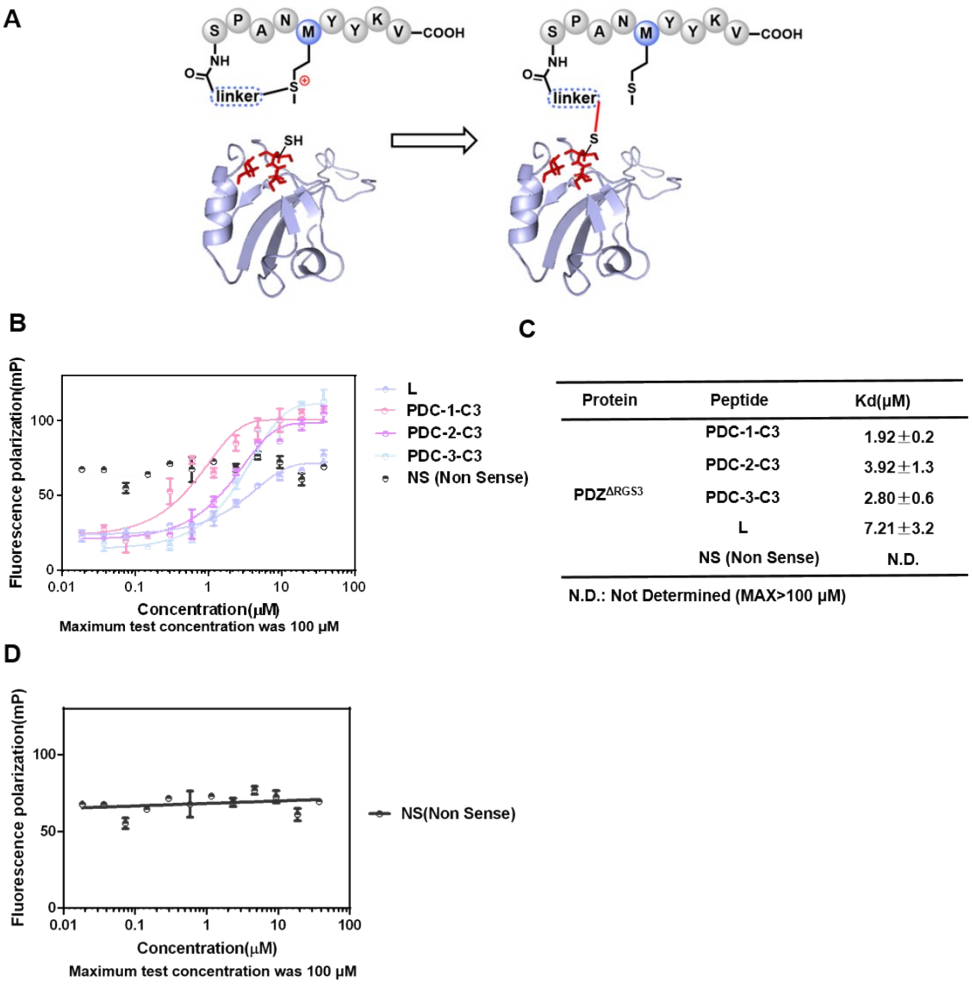


Figure S7. Fluorescence polarization assays of FAM labeled peptides binding to PDZ$^{\Delta RGS3}$ and their mutants. mP, mean ± s. d. and n>3.(A) Structure based design of peptide sequences for selective protein conjugation.(B) Binding affinity of FAM-labelled peptides to PDZ$^{\Delta RGS3}$.(C) Data conclusion from Figure B. (D) The Kd of NS peptide was N.D. (>100 μM).

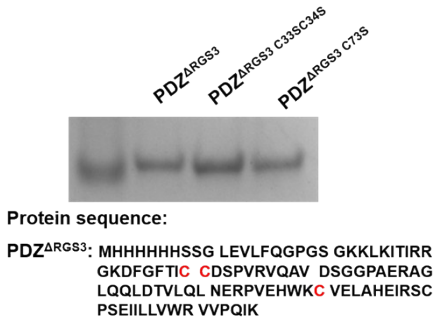


Figure S8: Protein expression and SDS-PAGE gel for PDZ$^{\Delta RGS3}$ (lane1), PDZ$^{\Delta RGS3C33SC34S}$ (lane2), PDZ$^{\Delta RGS3C73S}$(lane3).

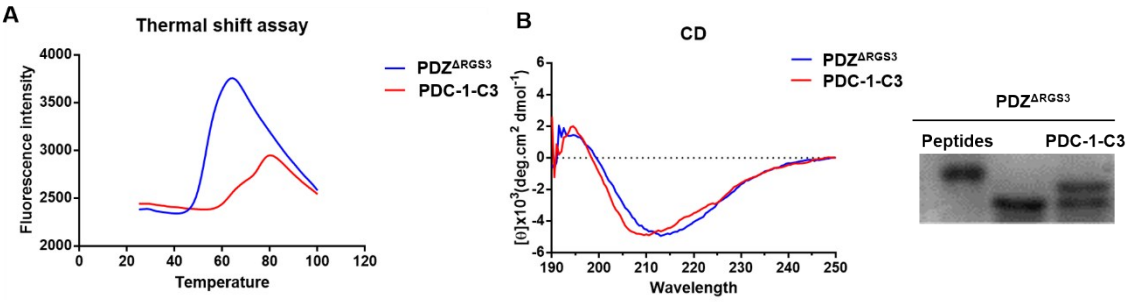


Figure S9. The structure analysis of protein-peptide conjugation. (A) Wild type PDZ$^{\Delta RGS3}$ protein and the protein-peptide conjugate was examined by the circular dichroism(CD). (B) The thermal shift assay curve was performed to test the thermo stability of wild type protein and protein-peptide conjugates. PDZ$^{\Delta RGS3}$ (20μM) was incubated with peptide PDC-1-C3(100μM) at 37^{o}C for 8 hours and the relevant protein samples were further confirmed by protein gel to ensure the successful conjugation.

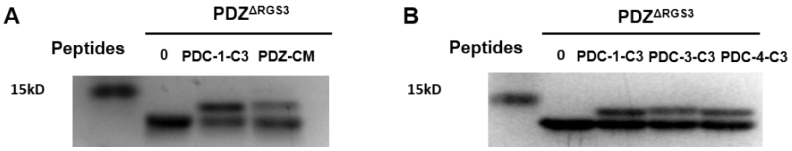


Figure S10. (A) In order to judge the method of macro cyclization method via the bis-alkylation between methionine and cysteine, a control experiment was also performed. (B) Compare PDC-1-C3, PDC-3-C3 and PDC-4-C3 peptides for peptide-protein conjugation. We found the best results for PDC-1-C3.

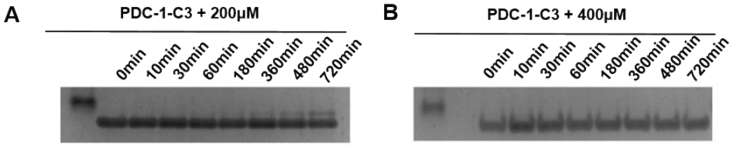


Figure S11. The competition of linear peptide L for peptide-protein conjugation. The mixture of peptide PDC-1-C3(100μM), linear peptide L and PDZ$^{\Delta RGS3}$ (20μM) was incubated for different time points (pH 7.4, 37^{o}C). The excess of linear peptide could influence the peptide-protein conjugation.

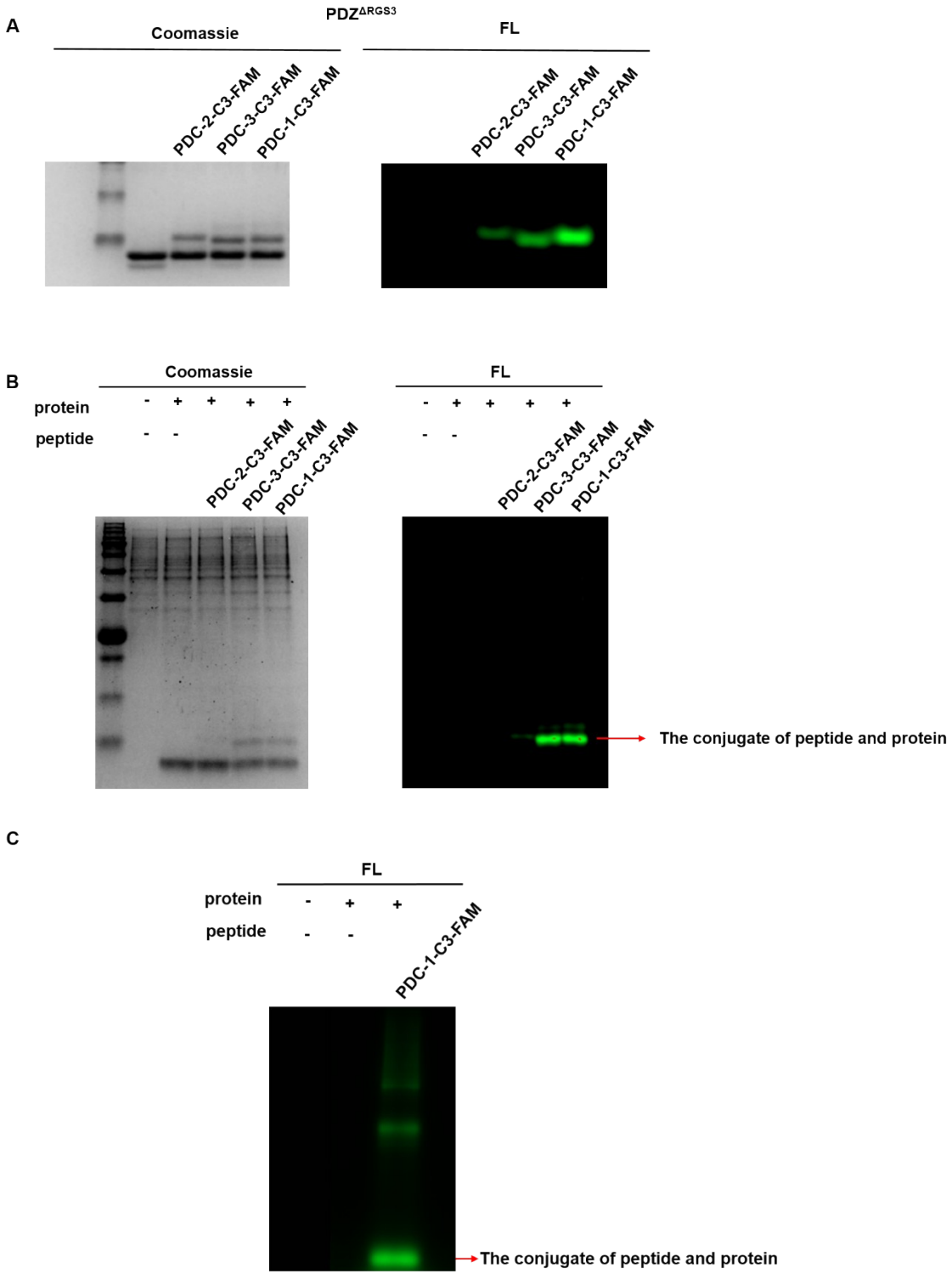


Figure S12. The cyclic peptide showed selectivity with its target protein ($PDZ^{\Delta RGS3}$). The selectivity efficiency increases as the protein concentration increases. (A) FAM-labelled peptides (PDC-1-C3-FAM, 100μM) were incubated with $PDZ^{\Delta RGS3}$ (20μM) (B) FAM labelled peptides

(PDC-1-C3-FAM, 50 μM) and PDZ$^{\Delta RGS3}$ (10 μg) were incubated with 293T cell lysates (100μg) for 24 hours. FL, in-gel fluorescence scanning.(C) FAM labelled peptides (PDC-1-C3-FAM, 50 μM) and PDZ$^{\Delta RGS3}$ (10 μg) were incubated with 293T cell lysates (400 μg) for 24 hours. FL, in-gel fluorescence scanning.

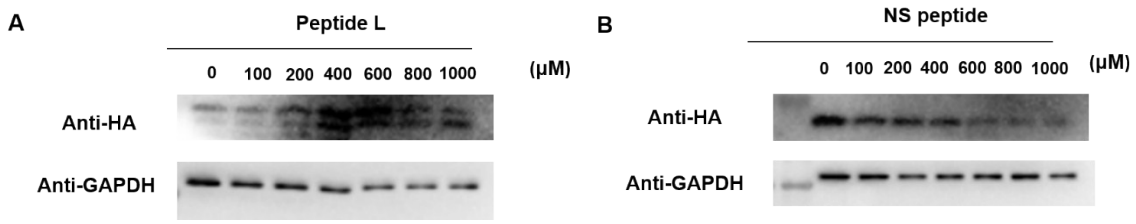


Figure S13. The covalent reaction in HA-PDZ$^{\Delta RGS3}$ transfected cell lysates. (A) The high concentration of linear peptide competitively blocked the labeling of peptide PDC-1-C3(100μM) to PDZ$^{\Delta RGS3}$ in cell lysates in dose-dependent manner. (B) Covalent bonding of peptide NS to PDZ$^{\Delta RGS3}$ after 8h incubation at room temperature with PDZ$^{\Delta RGS3}$ transfected cell lysates.

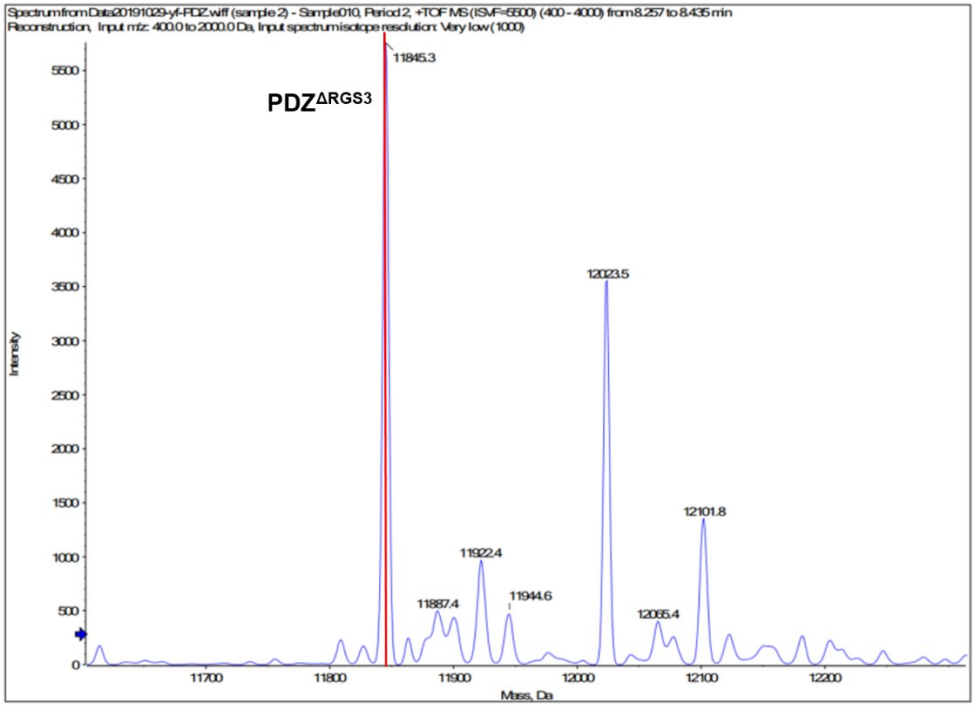


Figure S14. ESI MS analysis of protein PDZ$^{\Delta RGS3\ C33SC34S}$

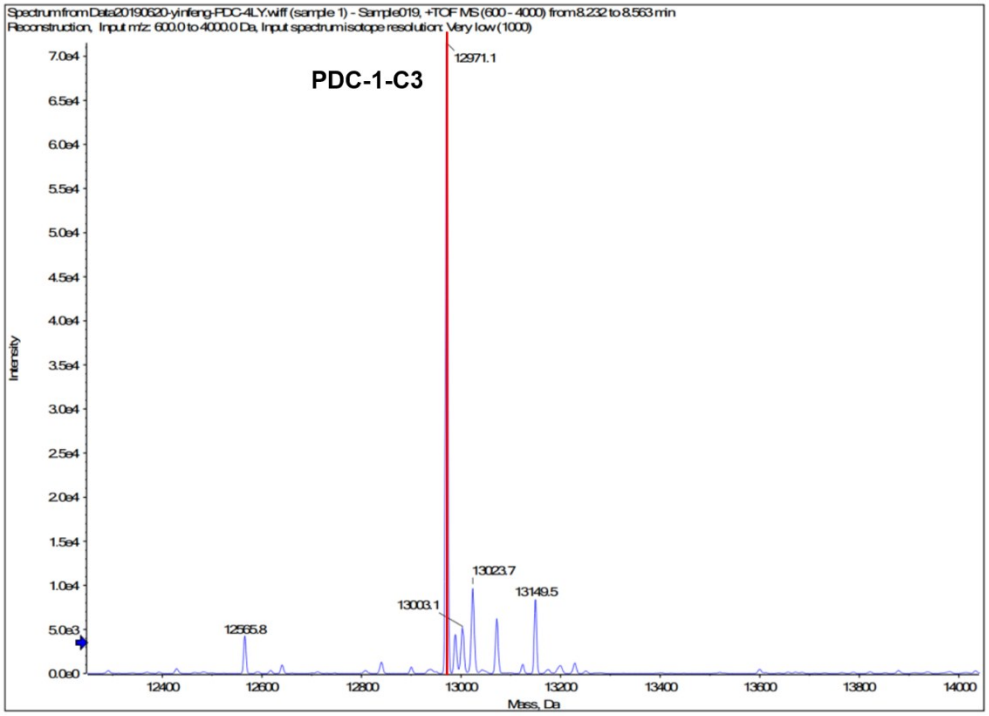


Figure S15. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PDC-1-C3 conjugation.

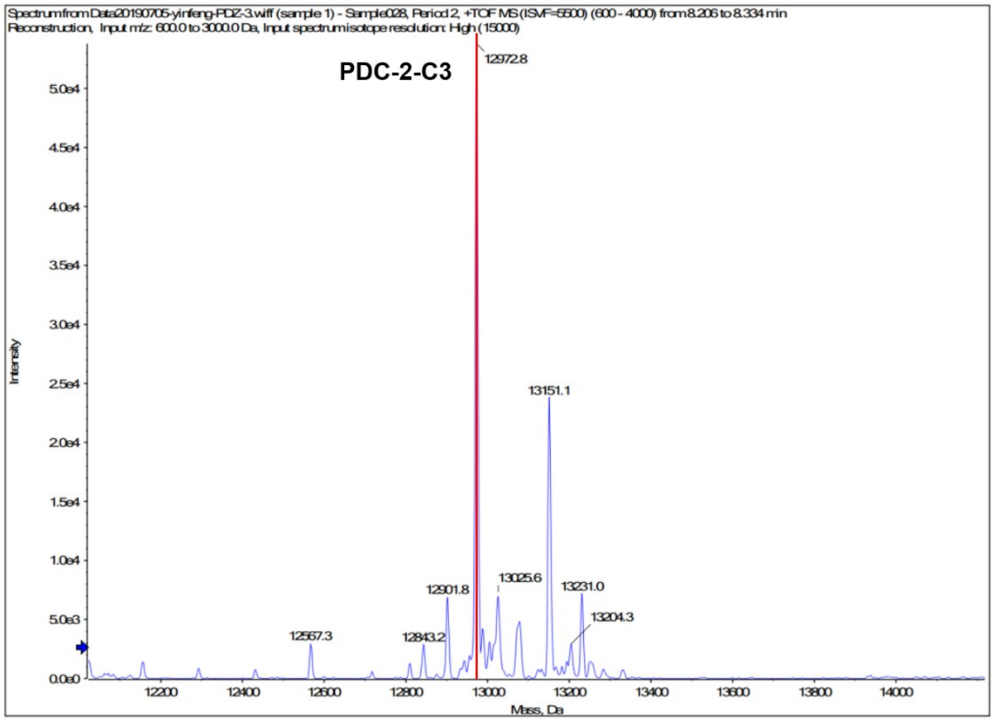


Figure S16. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PDC-2-C3 conjugation.

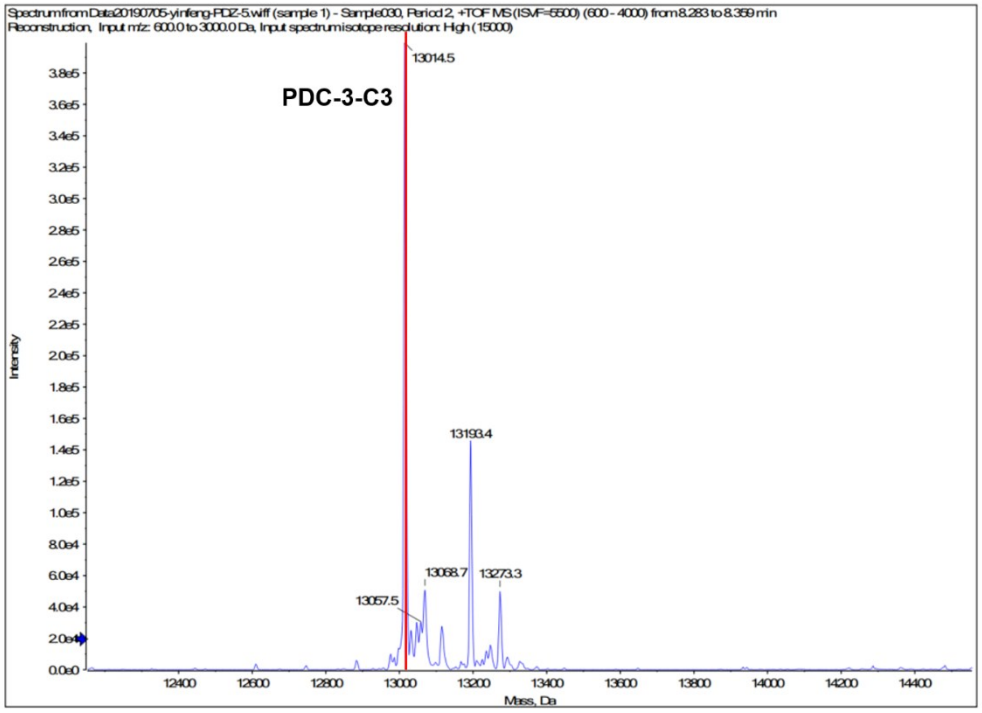


Figure S17. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PDC-3-C3 conjugation.

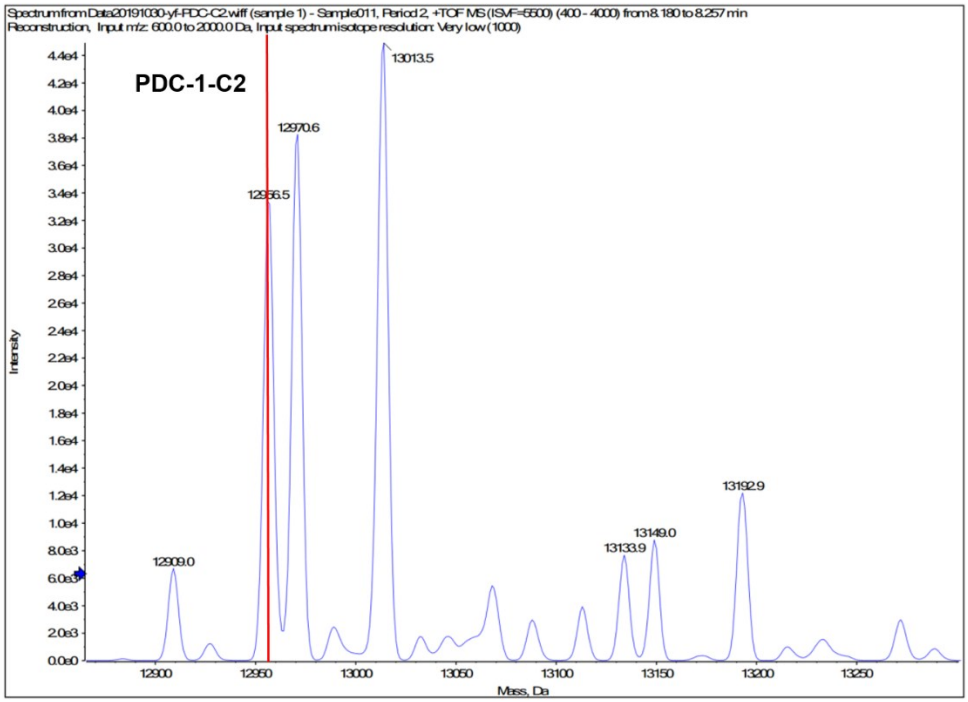


Figure S18. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PDC-1-C2 conjugation.

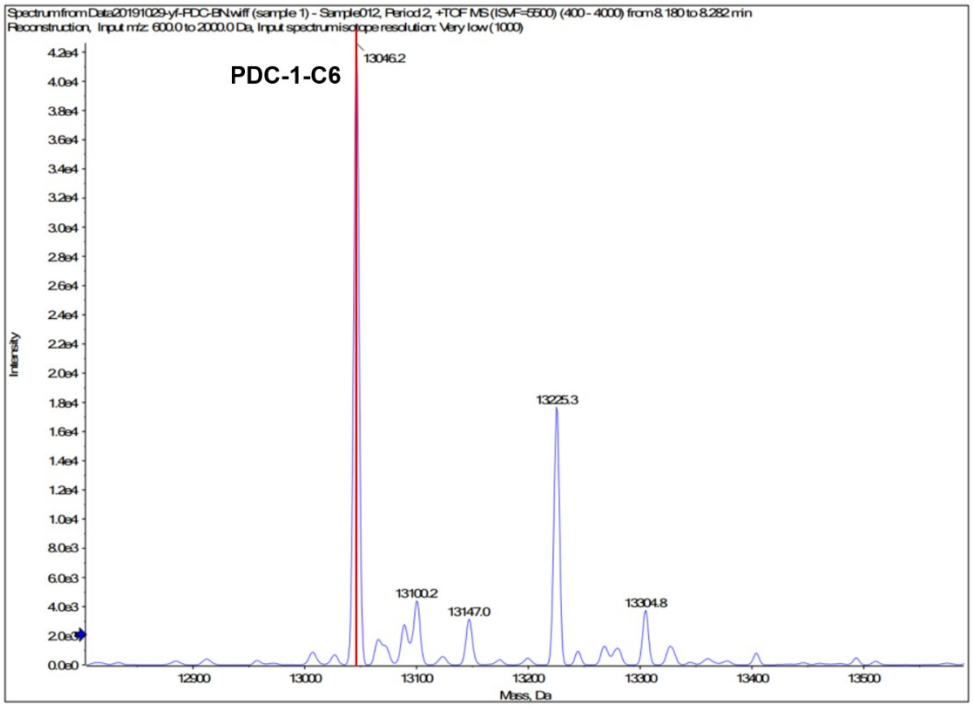


Figure S19. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PDC-1-C6 conjugation.

Supplementary Tables

Table S1. Primers using in this work.

Protein name	Oritentation	Primers
PDZ$^{\Delta RGS3}$ C33SC34S	Forward	TTCACCATCAGTAGCGACTCTCCGGTC
	Reverse	GAGTCGCTACTGATGGTGAAGCCAA
PDZ$^{\Delta RGS3}$ C73S	Forward	CACTGGAAAAGTGTGGAGCTGGCACATG
	Reverse	CTCCACACTTTTCCAGTGCTCCACGG

Table S2. Mass statistics data for the peptides and structure of unnatural amino acids. Calculated and Founded m/z are presented as $[M+1H]^+/[M/2+H]^+$

Peptide	Sequence	Calculated mass	Observerd mass
1	(cyclo-C3)- AAAMY-NH_2	579.26	579.45
2	(cyclo-C6)- AAAM-NH_2	492.23	492.55

3	(cyclo-C1)- AAAMKY-NH_2	719.35	361.25/721.45
4	(cyclo-C2)- AAAMKY-NH_2	693.34	348.20
5	(cyclo-C3)- AAAMKY-NH_2	707.36	707.50
6	(cyclo-C4)- AAAMKY-NH_2	721.37	361.15
7	(cyclo-C6)- AAAMKY-NH_2	783.39	393.25/785.60
8	(cyclo-C3)- AAAKYM-NH_2	707.36	354.05/707.40
9	(cyclo-C3)- AAAKMY-NH_2	707.36	354.20/707.40
10	(cyclo-C3)- AAMAKY-NH_2	707.36	354.20/707.35
11	(cyclo-C3)- AMAAKY-NH_2	707.36	707.50
12	(cyclo-C3)- MAAAKY-NH_2	707.36	354.25/707.50
A-FMOC	FMOC-ARRRMK(FAM)Y-NH_2	1558.68	521.15/780.75
DAP-FMOC	FMOC-(cyclo-C3)-DapRRRMK(FAM)Y-NH2	1628.71	408.15/544.10/815.35
A-AC	AC-ARRRMK(FAM)Y-NH_2	1378.62	461.05/690.60
DAP-AC	AC-(cyclo-C3)-DapRRRMK(FAM)Y-NH_2	1448.65	483.90
PDC-1-C3	(cyclo-C3)- SPAMIYYKV-OH	1124.58	563.55/1125.85
PDC-2-C3	(cyclo-C3)-SPANMYYKV-OH	1126.52	564.15/1126.50
PDC-3-C3	(cyclo-C3)- SPMNIYYKV-OH	1168.57	585.15/1168.55
PDC-4-C3	(cyclo-C3)- AMSNIYYKV-OH	1142.56	572.15/1142.50
PDC-1-C2	(cyclo-C2)- SPAMIYYKV-OH	1111.51	556.65
PDC-1-C6	(cyclo-C6)- SPAMIYYKV-OH	1201.60	601.70
L	AC-SPANIYYKV-OH	1095.56	548.75/1096.60
NS-C3	(cyclo-C3)-AAAWMAQLTS-NH_2	1104.55	552.75
PDC-1-C3-FAM	(cyclo-C3)-(FAM)K-SPAMIYYKV-OH	1525.63	509.00/763.05
PDC-2-C3-FAM	(cyclo-C3)-(FAM)K-SPANMYYKV-OH	1524.68	509.30/763.55
PDC-3-C3-FAM	(cyclo-C3)-(FAM)K-SPMNIYYKV-OH	1567.68	523.3/784.55/1568.40

Table S3. Protein sequence

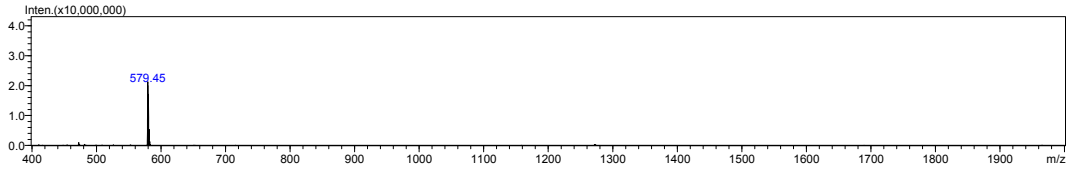
Protein	**Protein sequences**
$PDZ^{\Delta RGS3}$	**MHHHHHHSSG LEVLFQGPGS GKKLKITIRR GKDGFGFTIC CDSPVRVQAV DSGGPAERAG LQQLDTVLQL NERPVEHWKC VELAHEIRSC PSEIILLVWR VVPQIK**
$PDZ^{\Delta RGS3C33SC34S}$	**MHHHHHHSSG LEVLFQGPGS GKKLKITIRR GKDGFGFTIS SDSPVRVQAV DSGGPAERAG LQQLDTVLQL NERPVEHWKC VELAHEIRSC PSEIILLVWR VVPQIK**

PDZ$^{\Delta RGS3C73S}$	**MHHHHHHSSG LEVLFQGPGS GKKLKITIRR GKDGFGFTIC CDSPVRVQAV DSGGPAERAG LQQLDTVLQL NERPVEHWKS VELAHEIRSC PSEIILLVWR VVPQIK**
BCL2	**MAHAGRSGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGA APAPGFFSSQ PGHTPHPAASRDPVARTSPLQTPAAPGAAAGPALSPVPPVVHLT LRQAGDDFSRRYRRDFAEMSSQLHLTPFTARGCFATVVEELFR DGVNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYL NRHLHTWIQNGGWDAFVELYGPSMR**
SortaseA	**MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSKPHIDNYL HDKDKDEKIEQYDKNVKEQASKDKKQQAKPQIPKDKSKVAGYI EIPDADIKEPVYPGPATPEQLNRGVSFAEENESLDDQNISIAGHTF IDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRDVKPTD VGVLDEQKGKDKQLTLITCDDYNEKTGVWEKRKIFVATEVK**
MgrA	**MGSSHHHHHHSSGLVPRGSHM MSDQHNLKEQLCFSLYNAQRQVNRYYSNKVFKKYNLTYPQFLV LTILWDESPVNVKKVVTELALDTGTVSPLL KRMEQVDLIKRERSEVDQREVFIHLTDKSETIRPELSNASDKVAS ASSLSQDEVKELNRLLGKVIHAFDETKEK**

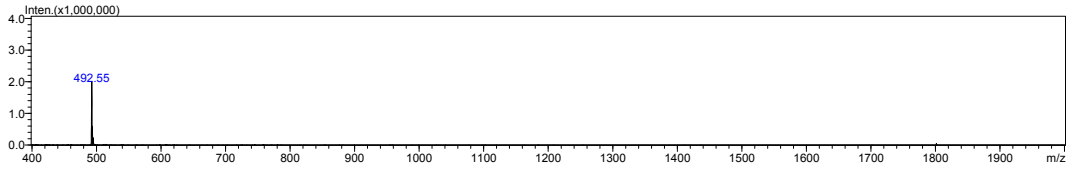
Appendix

LC-MS spectra of peptides used in the manuscript.

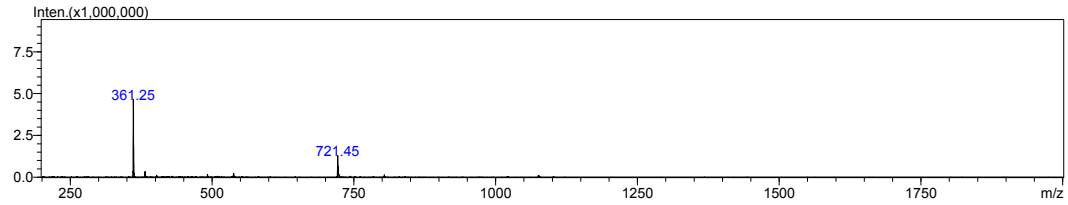
1. (cyclo-C3)- AAAMY-NH_2



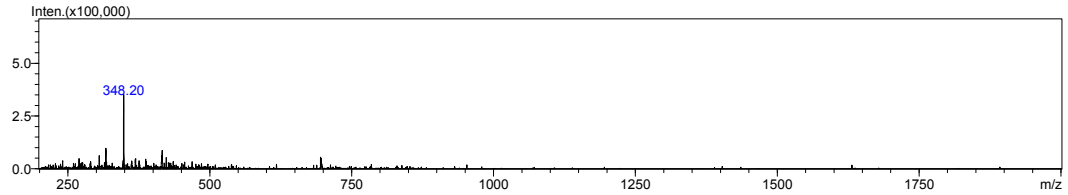
2. (cyclo-C6)- AAAM-NH_2



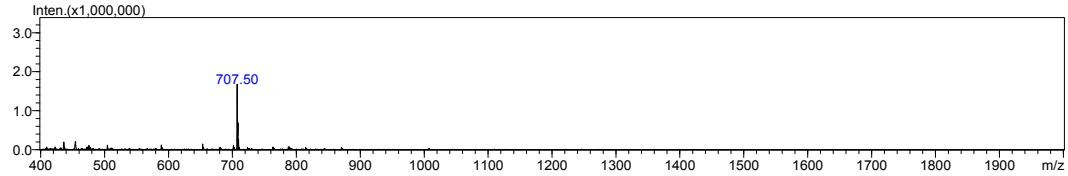
3. (cyclo-C1)- AAAMKY-NH_2



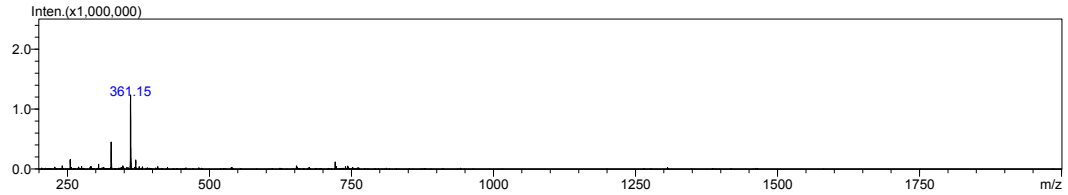
4. (cyclo-C2)- AAAMKY-NH_2



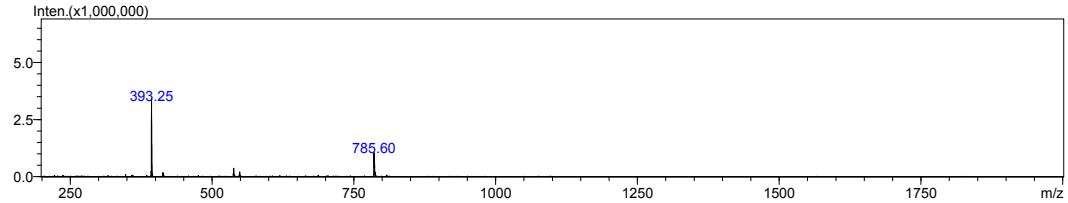
5. (cyclo-C3)- AAAMKY-NH_2



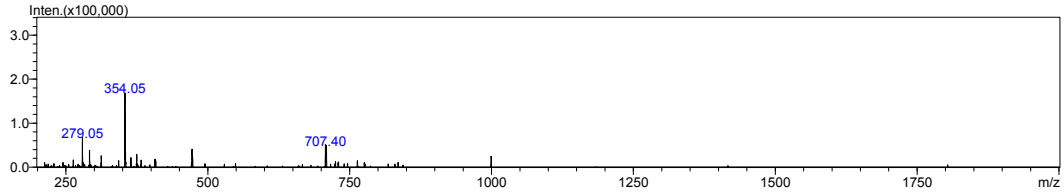
6. (cyclo-C4)- AAAMKY-NH_2



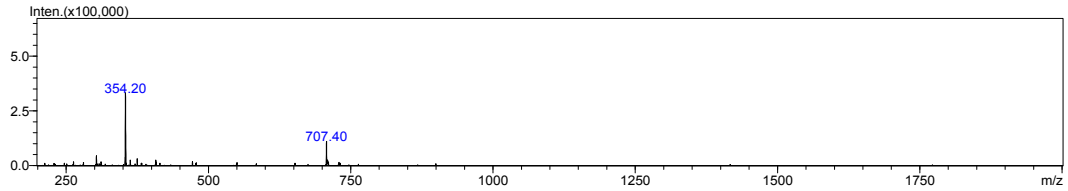
7. (cyclo-C6)- AAAMKY-NH_2



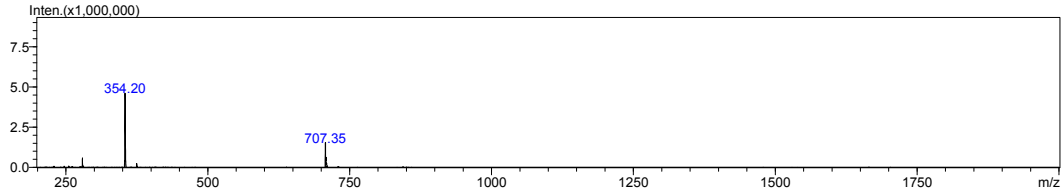
8. (cyclo-C3)- AAAKYM-NH_2



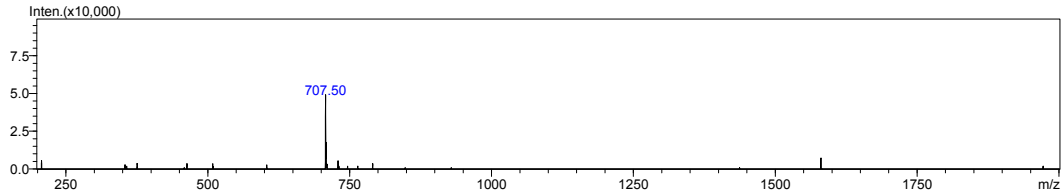
9. (cyclo-C3)- AAAKMY-NH_2



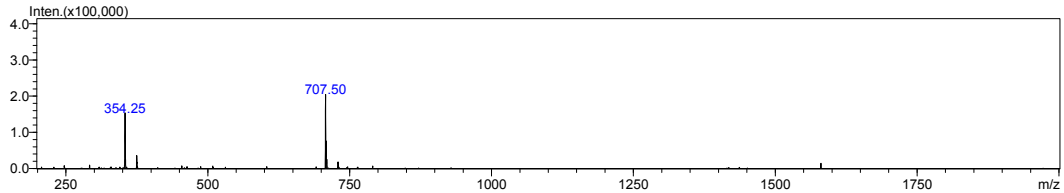
10. (cyclo-C3)- AAMAKY-NH_2



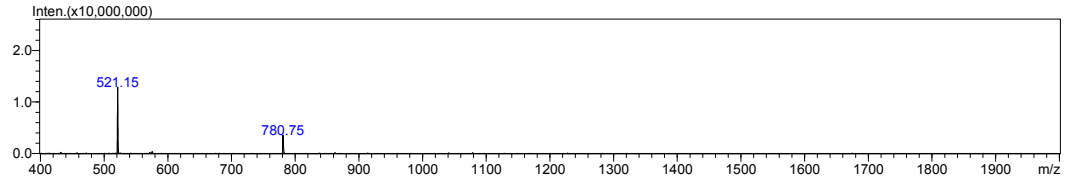
11. (cyclo-C3)- AMAAKY-NH_2



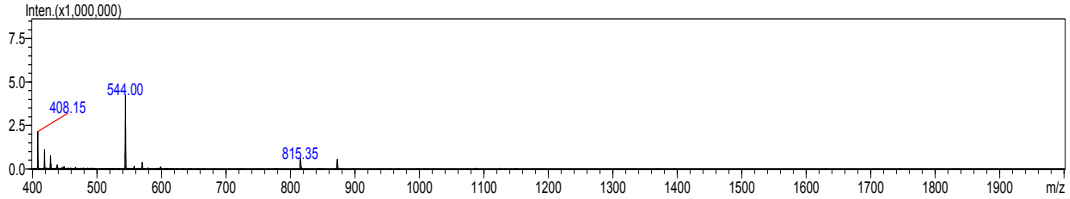
12. (cyclo-C3)- MAAAKY-NH_2



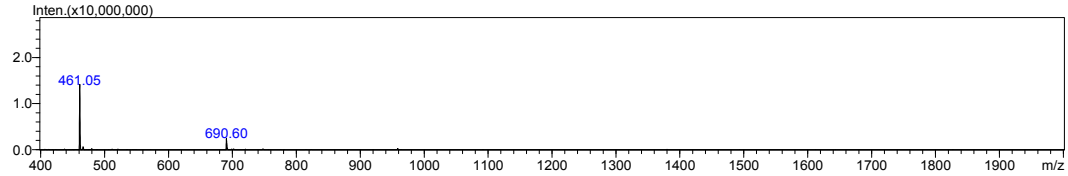
13. FMOC-RRRMK(FAM)Y-NH_2



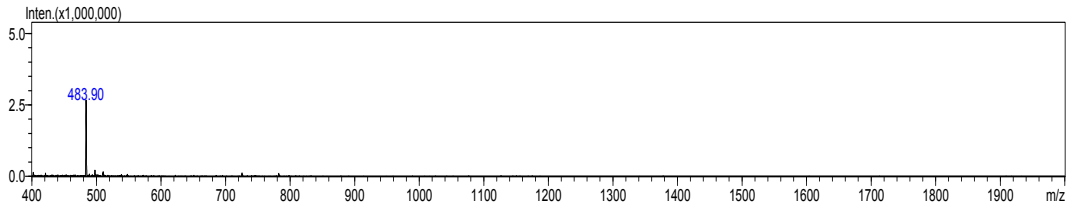
14. FMOC-(cyclo-C3)-RRRMK(FAM)Y-NH_2



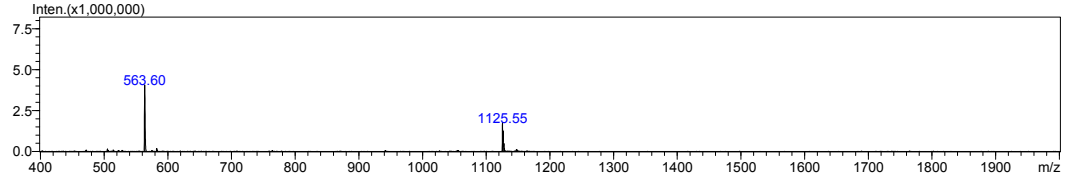
15. AC-RRRMK(FAM)Y-NH_2



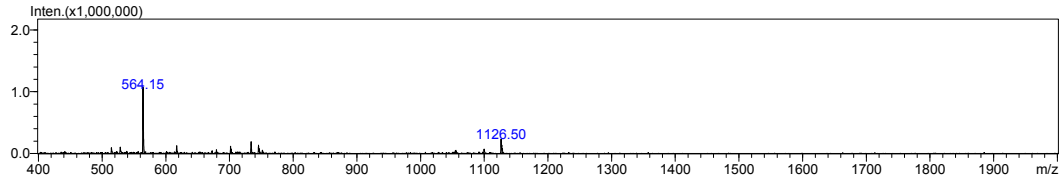
16. AC-(cyclo-C3)-RRRMK(FAM)Y-NH_2



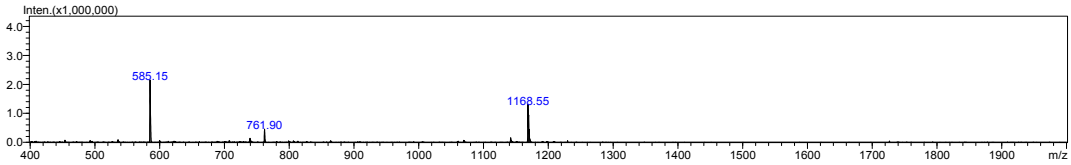
17. (cyclo-C3)- SPAMIYYKV-OH



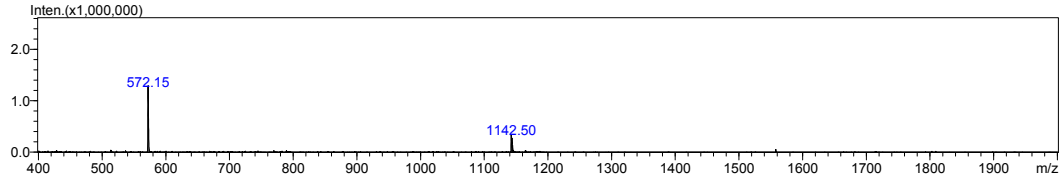
18. (cyclo-C3)-SPANMYYKV-OH



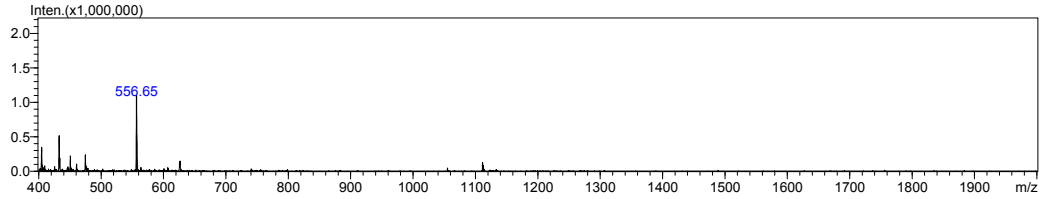
19. (cyclo-C3)- SPMNIYYKV-OH



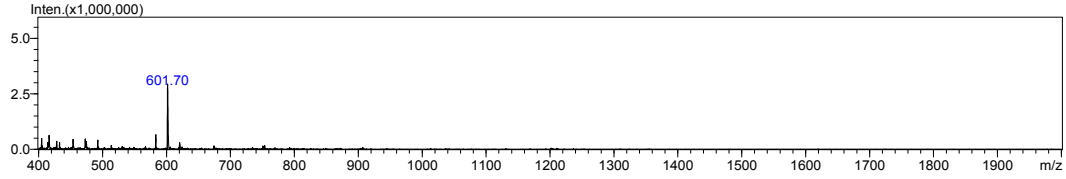
20. (cyclo-C3)- AMSNIYYKV-OH



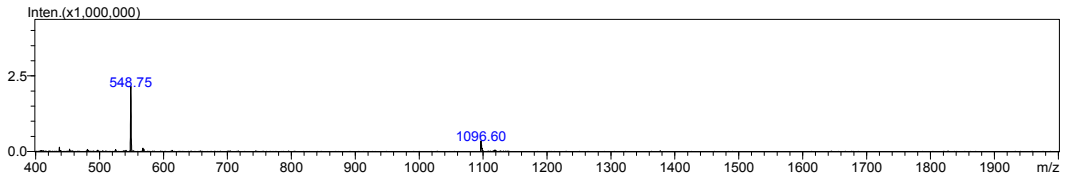
21. (cyclo-C2)- SPAMIYYKV-OH



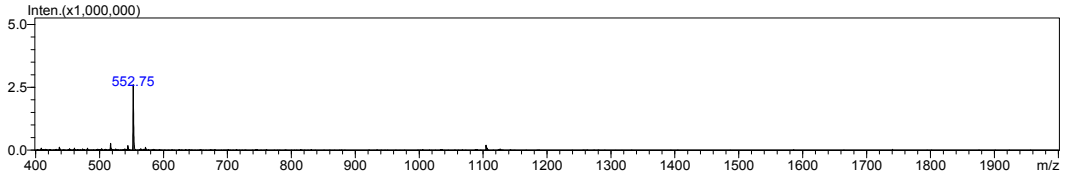
22. (cyclo-C6)- SPAMIYYKV-OH



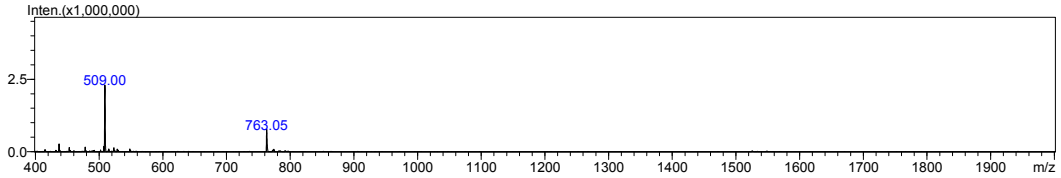
23. AC-SPANIYYKV-OH



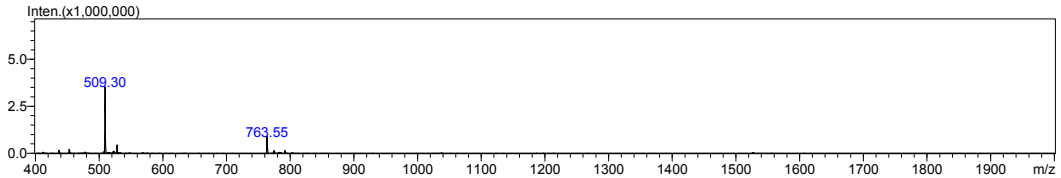
24. (cyclo-C3)-AAAWMAQLTS-NH_2



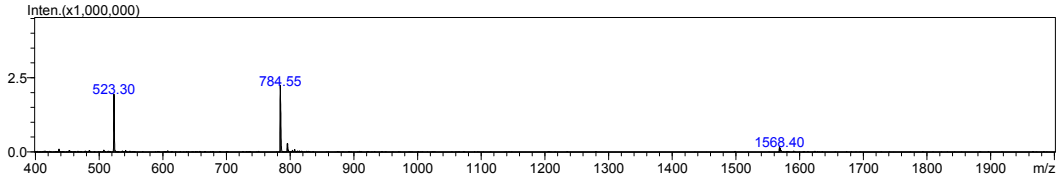
25. (cyclo-C3)-(FAM)K-SPAMIYYKV-OH



26. (cyclo-C3)-(FAM)K-SPANMYYKV-OH



27. (cyclo-C3)-(FAM)K-SPMNIYYKV-OH



References

1. D. Wang, M. Yu, N. Liu, C. Lian, Z. Hou, R. Wang, R. Zhao, W. Li, Y. Jiang, X. Shi, S. Li, F. Yin and Z. Li, *Chem Sci*, 2019, **10**, 4966-4972.