Supporting Information

A sulfonium tethered peptide ligand rapidly and selectively modifies protein cysteine in vicinity

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Contents

Reagents and materials

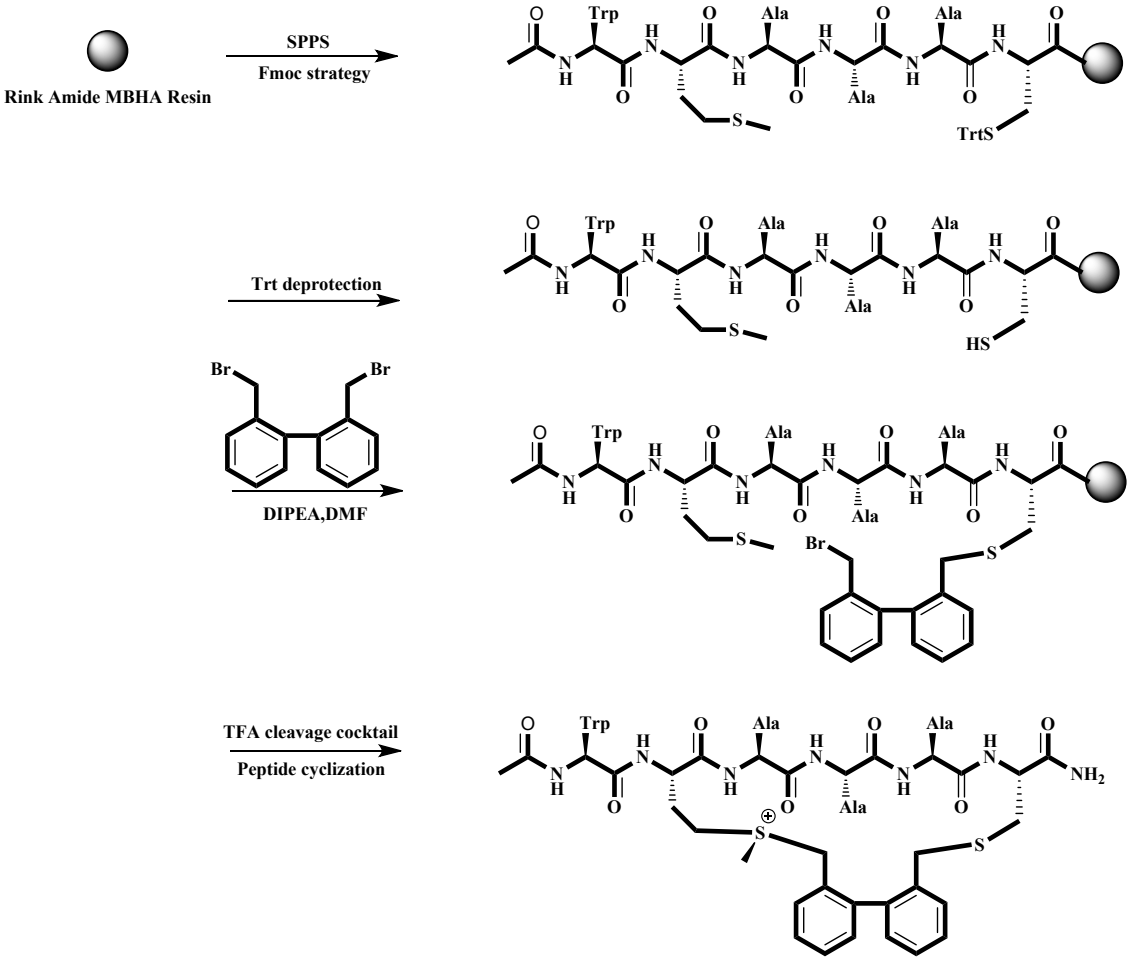
All reagents including amino acids and resins were purchased from GL Biochem (Shanghai), Shanghai Hanhong, Aladdin or Energy Chemical and used without further purifications. All solvents were purchased from Cantotech Chemicals, Ltd. Anhydrous solvents were purchased from J&K Scientific and used without purification. Antibodies against 6*His tag was obtained from Proteintech. 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 (4.9 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 TRT(trityl) protecting group of cysteine were removed by treating the resin with a solution of TFA/TIS/H2O (3:5:92) for 5*10 min, and washed with DCM. On-resin cysteine alkylation was conducted with linkers (2 eq) and DIPEA (4 eq) in DMF for 3h with N2 bubbling. The peptide was cleaved from resin treated with TFA/TIS/H2O (95:2.5:2.5) for 2 h. Peptides were

synthesized, purified and characterized by HPLC and LC-MS.



Circular dichroism spectroscopy

Peptides were dissolved in deionized water or 20% TFE buffer to final concentrations of 10-100 μM. 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. Final concentrations of the peptides were determined by 280 nm absorption of Tyr or Trp.

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. 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 1H NMR to characterize the peptides. NMR data were processed using MestReNova 3.0.

Dealkyation 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.

***In vitro* serum stability**

Peptides were incubated with 25% fresh mouse serum (v/v) in PBS to a final concentration of 100 μM at 37°C. Aliquots (50 μL) were taken periodically at 0, 0.5, 1.5h, 3h, 6h, 9h,12h,16h and 24h, 48h and then 100 μl 12% trichloroacetic acid in H2O/CH3CN (1:3) was added and cooled to 4 °C for 30 min to precipitate serum proteins. Then all the samples were removed by centrifugation at 14,000 rpm for 10 min. The supernatant was analyzed by LC/MS. Peptide serum half-life was calculated according to the HPLC integral area versus time using linear regression by Prism.

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

Human HEK 293T or A2780 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

A2780 cells were cultured with RPMI-1640 with 10% FBS (v/v) in imaging dishes (50000 cells/well) in a humidified incubator containing 5 % CO2 at 37 °C. Then, cells were incubated with 10μM FAM-labeled peptides in 5% FBS medium for 4h at 37°C. After that, Then the cells were washed 3 times with phosphate buffered saline (PBS) and then fixed with 4% (wt/vol) formaldehyde (Alfa Aesar, MA) in PBS for 10 min. They were then washed 3 times with PBS and stained with DAPI (4', 6-diamidino-2-phenylindole) (Invitrogen, CA) for 10 min. 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 °C with excitation at 485 nm and emission at 520 nm. The FAM labeled peptides (5nM) were incubated with increasing concentration of purified $PDZ^{\Delta RGS3}$ or their mutants ($PDZ^{\Delta RGS3C33SC34S}$,$PDZ^{\Delta RGS3C33SC34S}$) in PBS (pH7.4) in the dark for 1 hours. Then the mixture was measured at 25 °C 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 PD3-I(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 PD3-I (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 PD3-I(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 (0, 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 PD3-I(100μM) in PBS (pH 7.4, 37 ˚C) for 0min,10min, 30min, 1h, 2h and 4h, 8h and 12h, respectively. As for the stability of the protein conjugate, the GSH with different concentrations was added to the protein-peptide conjugate and incubated for 12 hours (pH 7.4, 37 ˚C). To test the pH tolerance of this conjugation reaction, the peptide and protein were incubated at different pH for 12 hours at 37 ˚C.

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 T_m of protein-conjugate, the protein (20μM) and peptide PD3-I (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 (300μg) were spiked with PDZ$^{\Delta RGS3}$ (10μg) and then treated with FAM-labeled peptide PD3-I(50μM) for 24 hours at 37˚C.Then the reaction mixture was incubated with PBS-washed Ni-NTA Agarose Beads with rotationfor 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.[2]

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

293T cells were maintained in DMEM containing 10% FBS and penicillin/streptomycin, and transfections performed with 15μg pCAG-F plasmid containing HA- PDZ$^{\Delta RGS3}$ using lipo2000 (Invitrogen). For lysate experiments, cells were trypsinized after 48 hour post-transfection, washed with PBS, and lysed by incubation with 0.5% NP40 lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.5], 120 mM NaCl, 5mM DTT). Protein concentration of the soluble fraction was measured using nanodrop. Then different peptides with indicated concentration was added to 300μg cell lysates and incubated at RT for 8 hours. Samples were then boiled in SDS buffer and subjected to western analysis using 1:300 dilutions of HA (Proteintech) and 1:1000 dilutions of GAPDH (Proteintech) antibodies.

In-gel trypsin digestion

The protein was digested by trypsin using standard in-gel trypsin digestion protocols[3]. 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

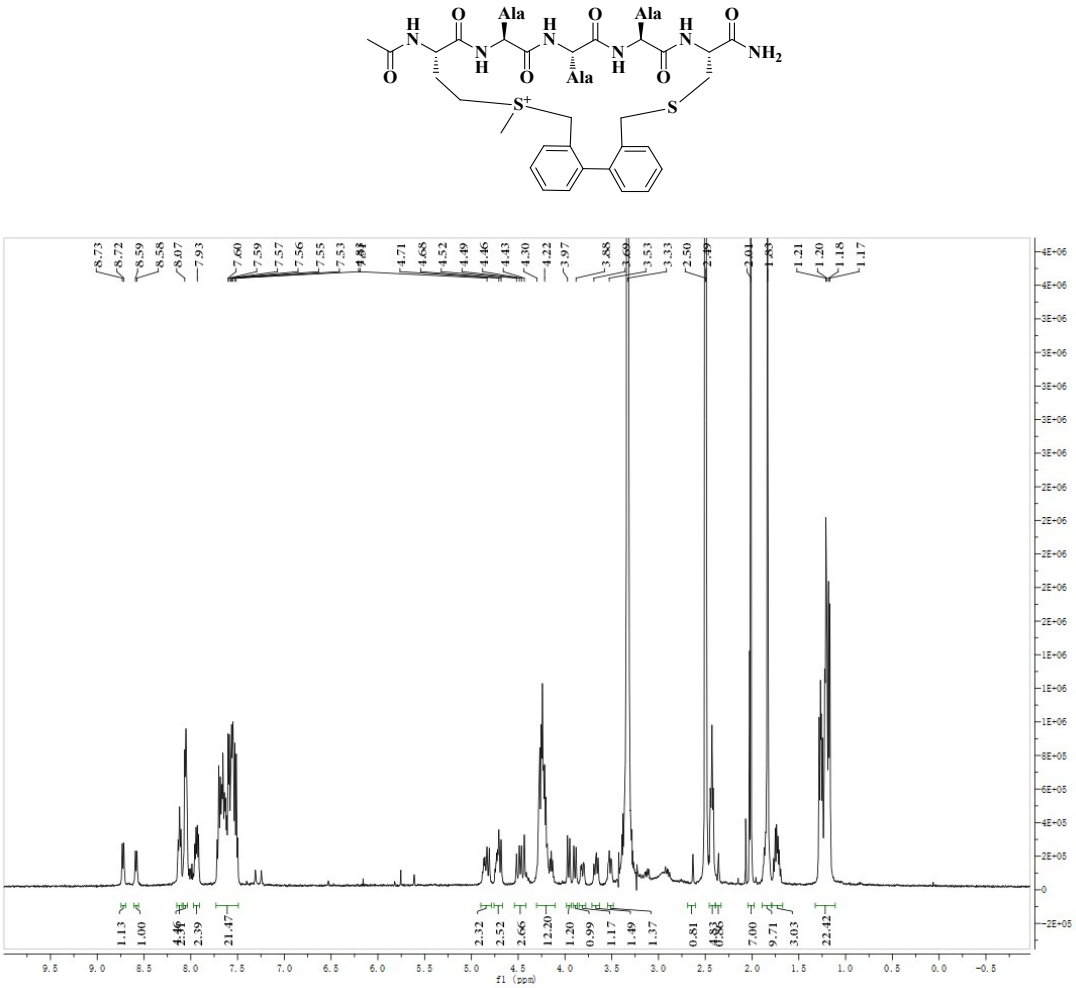


Figure S1. 1H NMR (500 MHz, DMSO) δ 8.72 (d, J = 8.2 Hz, 1H), 8.58 (d, J = 7.9 Hz, 1H), 8.11 (d, J = 6.5 Hz, 2H), 8.06 (d, J = 7.0 Hz, 4H), 7.94 (d, J = 6.6 Hz, 2H), 7.73 – 7.49 (m, 21H), 3.89 (d, J = 12.2 Hz, 1H), 3.81 (d, J = 8.8 Hz, 1H), 4.84 (m, 2H), 4.71 (m, 3H), 4.48 (m, 3H), 4.26 (m, 12H), 3.96 (d, J = 12.2 Hz, 1H), 3.89 (d, J = 12.2 Hz, 1H), 3.81 (m, 1H), 3.71 – 3.63 (m, 1H), 3.54 – 3.48 (m, 1H), 2.63 (s, 1H), 2.46 – 2.39 (m, 5H), 2.36 (m, 1H), 2.01 (s, 7H), 1.89 – 1.79 (m, 10H), 1.79 – 1.67 (m, 3H), 1.33 – 1.11 (m, 22H). **1H NMR (500 MHz, DMSO-d6) δ 8.72 (d, J = 8.2 Hz, ~1H), N-terminal NH, 8.58 (d, J = 7.9 Hz, 1H), N-terminal NH, 8.12-7.93 (m, ~8H), CONH, 7.70-7.51(m, ~20H), biphenyl and C-terminal NH2.**

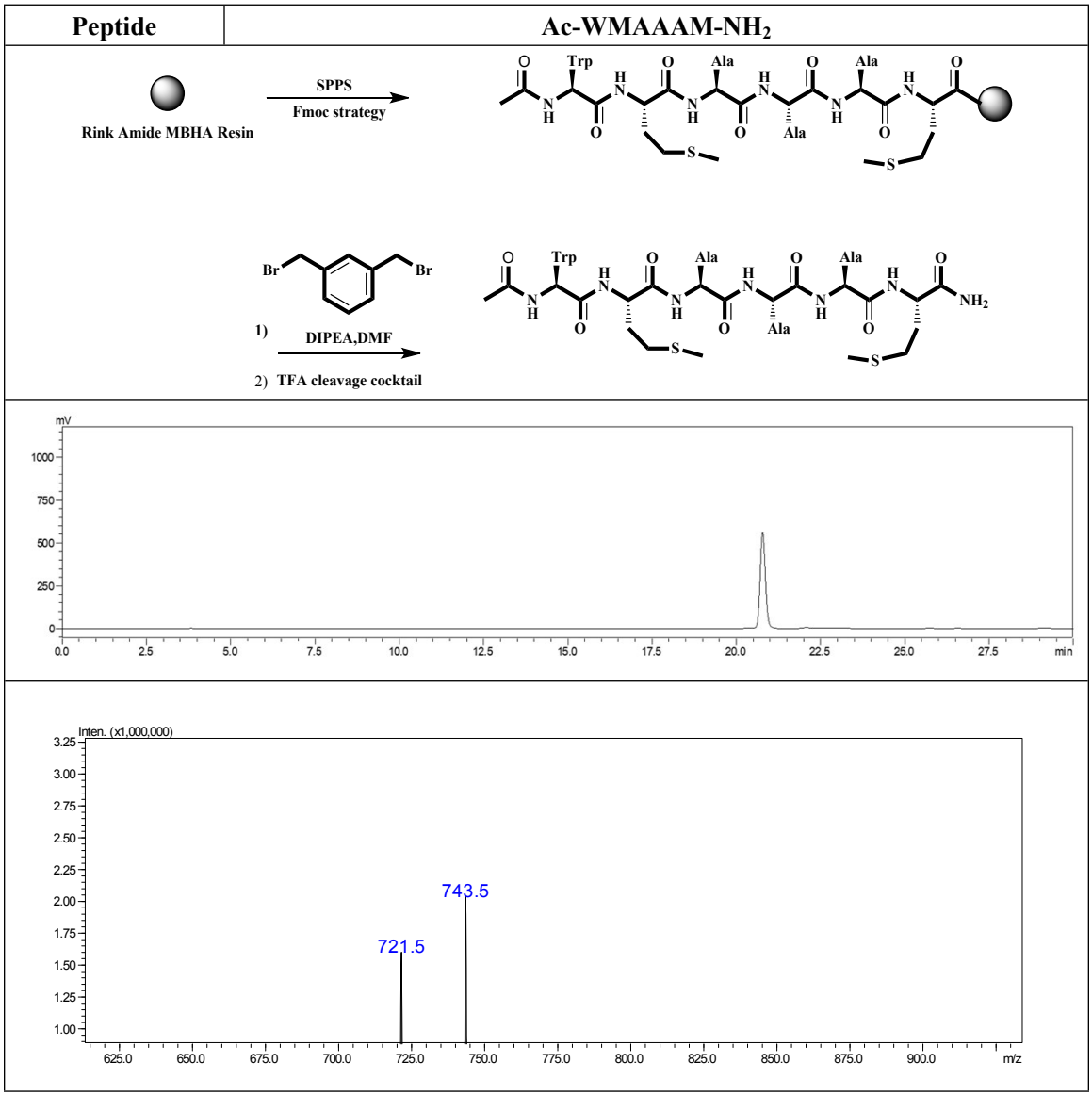
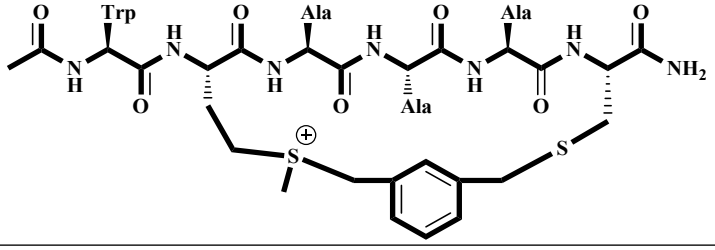
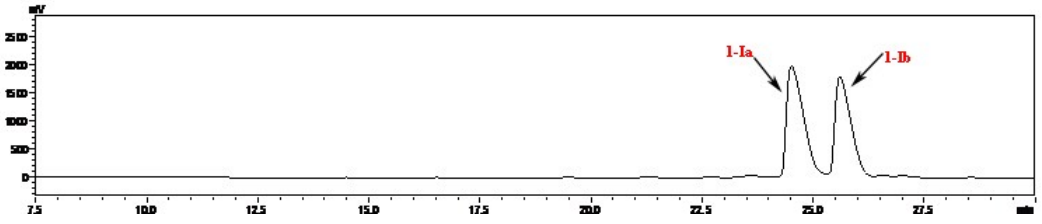
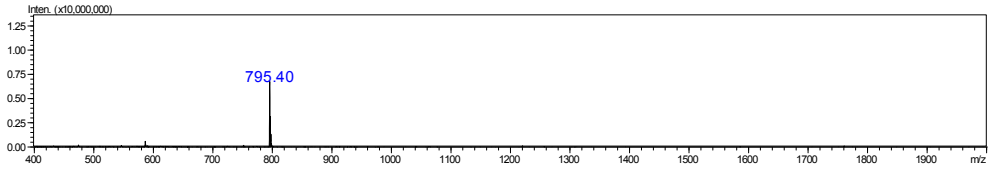


Figure S2. The peptide cyclization between Met and Met. The MS data indicated that the cyclization couldn't happen under DIPEA/DMF condition.

1-I	Ac-(cyclo-I)-WMAAAC-NH_2







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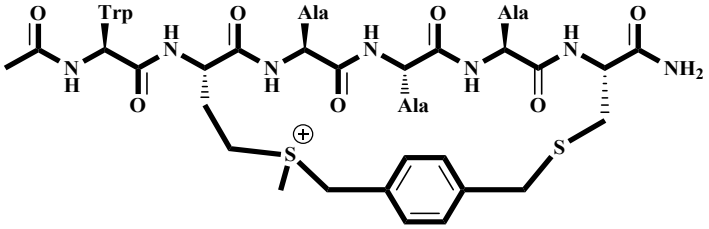

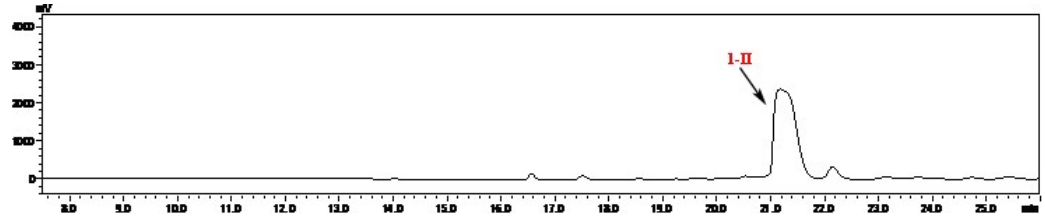

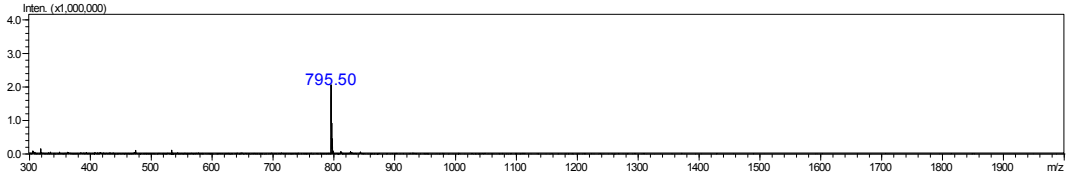

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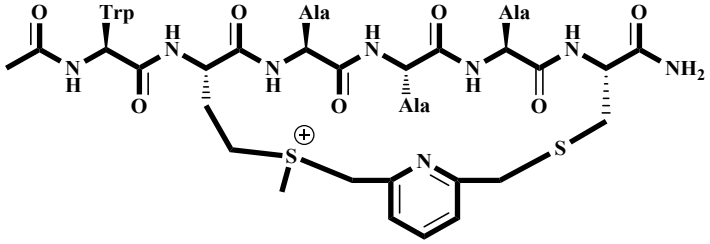

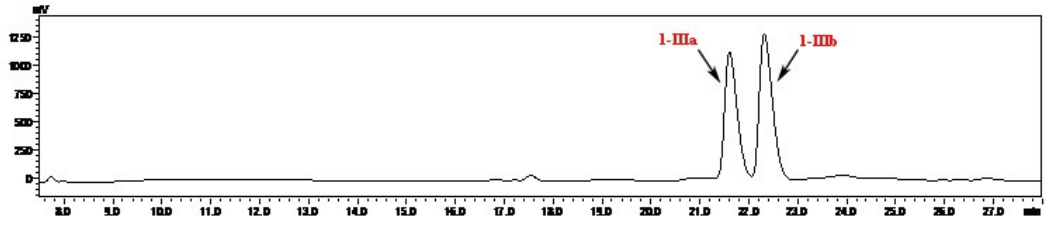

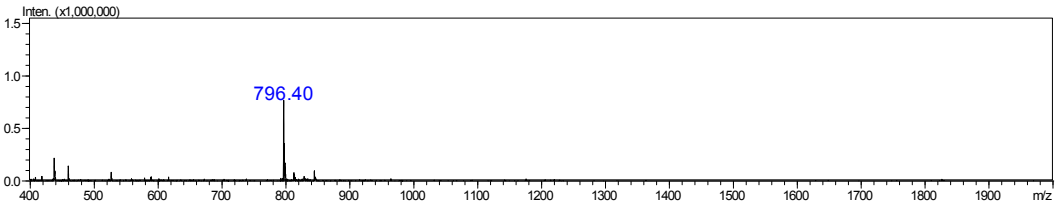

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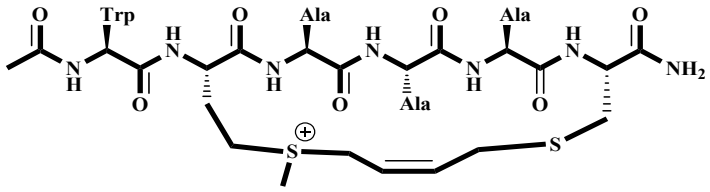
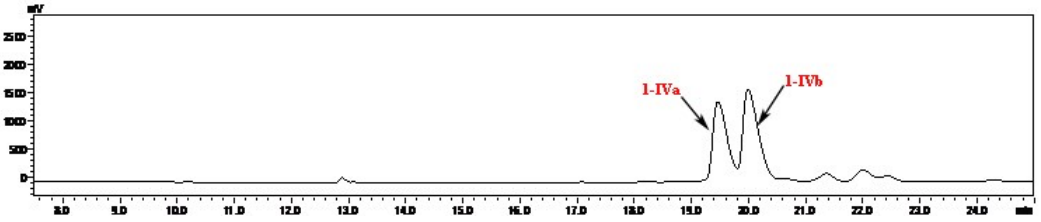
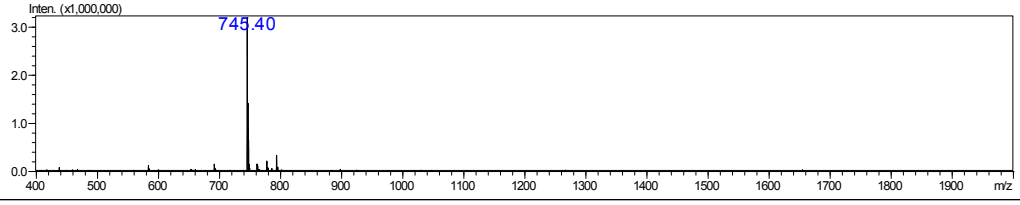
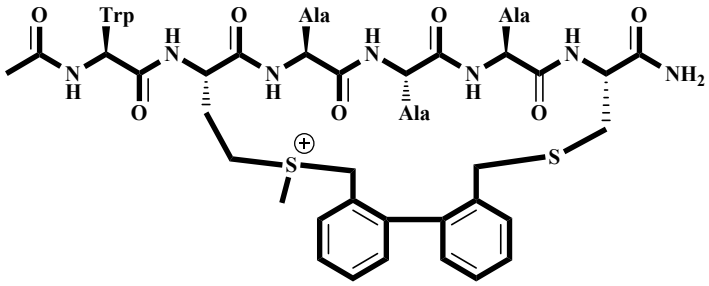
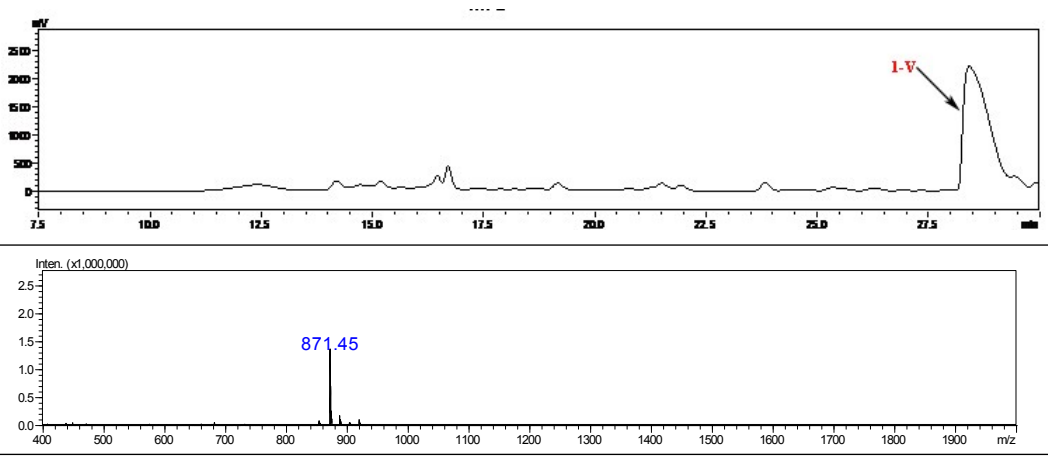




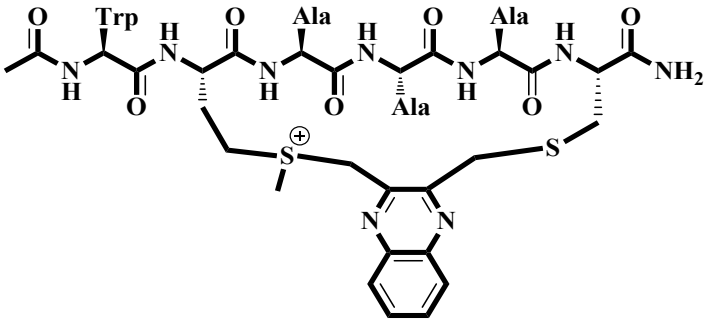


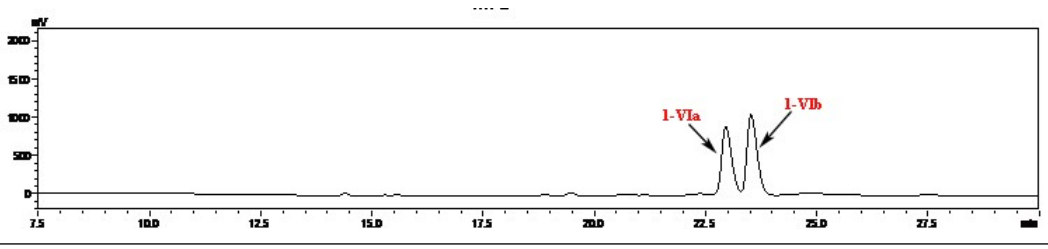
1-V	Ac-(cyclo-V)-WMAAAC-NH_2





1-VI	Ac-(cyclo-VI)-WMAAAC-NH_2





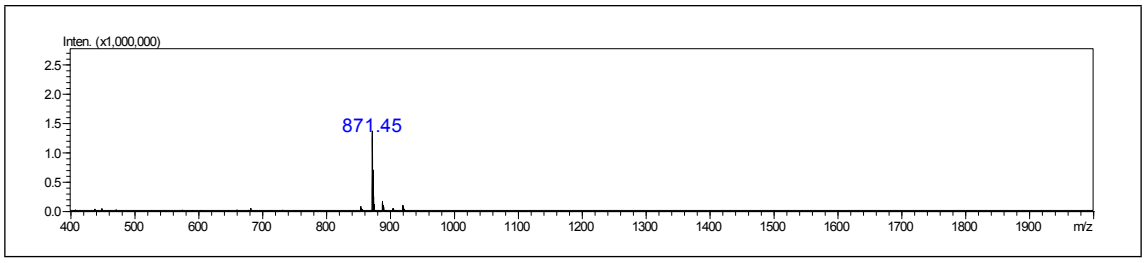


Figure S3. HPLC separation spectra of peptides **1-I to 1-VI**. (Column: C18 analytic column - Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm.Flow rate: 1.0 mL/min)

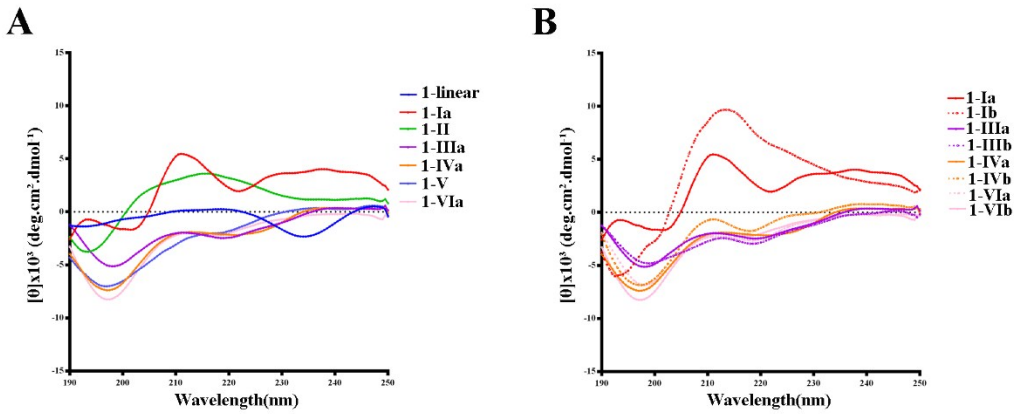
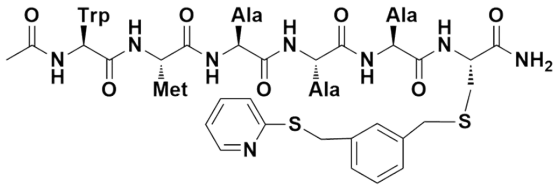
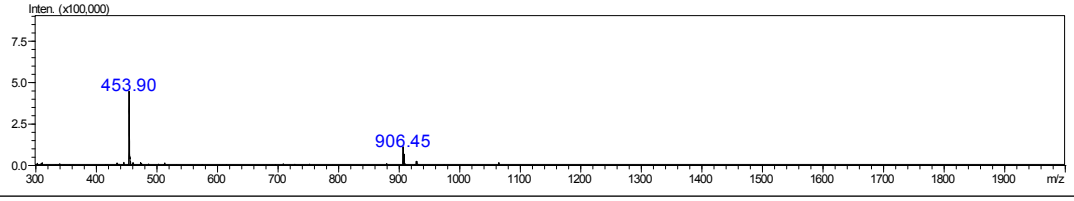


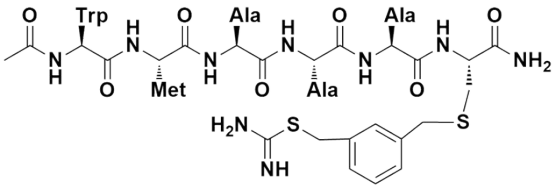
Figure S4. Circular Dichroism Spectra (CD). (A)The CD spectra of peptide **1** with linkers **I**, **II**, **III**, **IV**, **V**,**VI** and the linear peptides in ddH_2O.(B) The CD spectra of peptides with different isomers in ddH_2O.

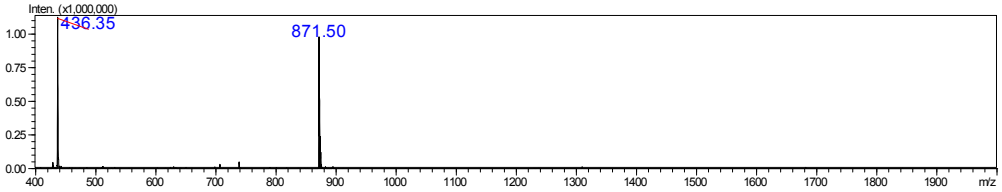
1. Dealkylated product of peptide 1-I by Pys



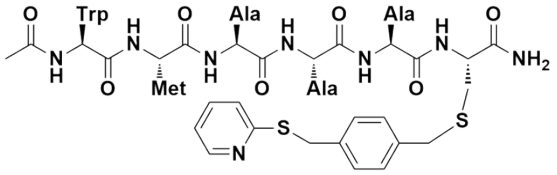


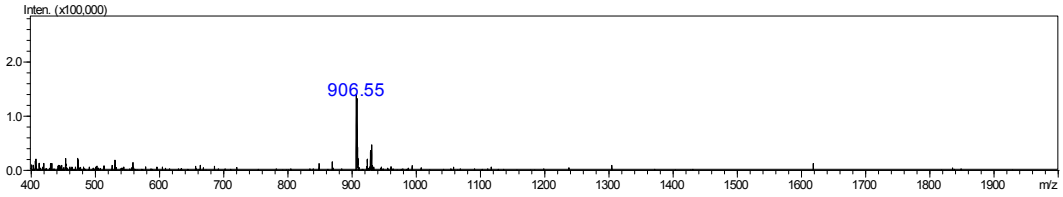
2. Dealkylated product of peptide 1-I by thiourea



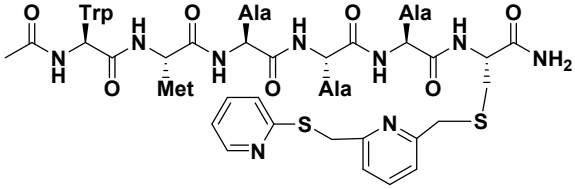


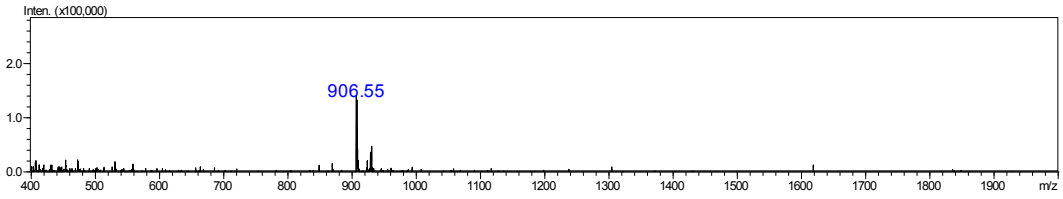
3. Dealkylated product of peptide 1-II by Pys



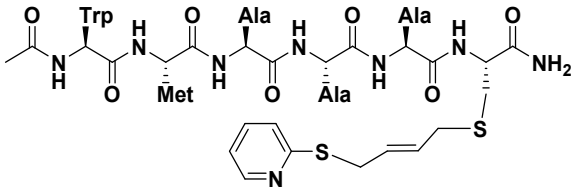


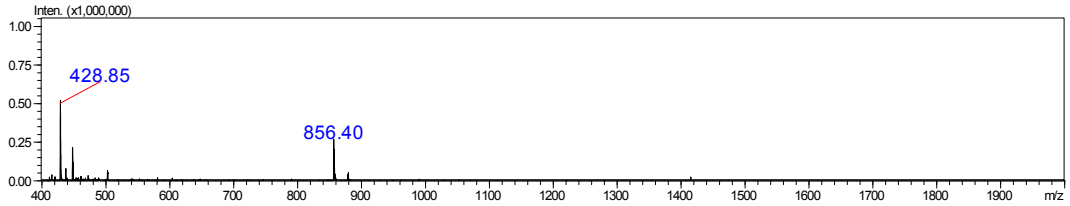
4. Dealkylated product of peptide 1-III by Pys



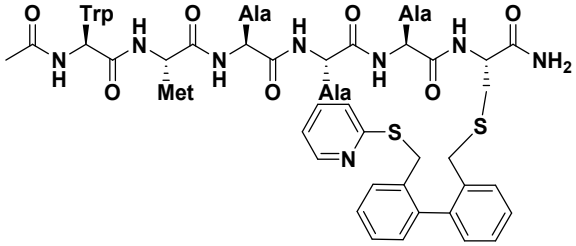


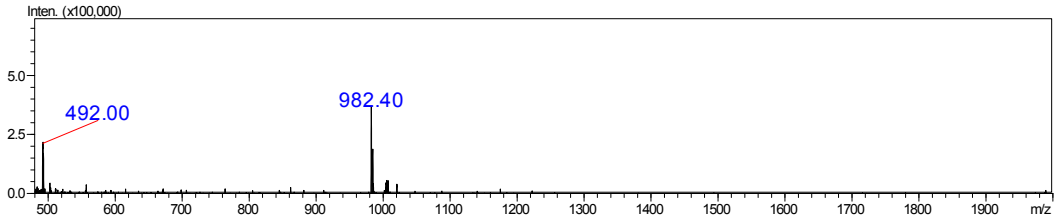
5. Dealkylated product of peptide 1-IV by Pys



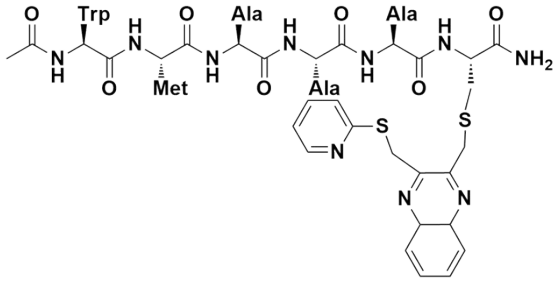


6. Dealkylated product of peptide 1-V by Pys





7. Dealkylated product of peptide 1-VI by Pys



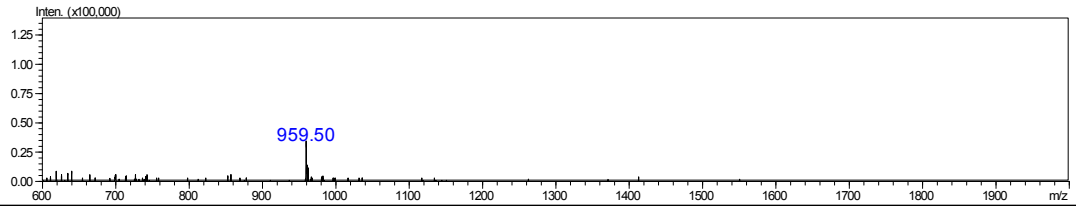


Figure S5. LC-Ms analysis of Dealkylated product of cyclic peptide Ac--WMAAAC-NH_2 with different linkers in the presence of PyS and thiocarbamide.

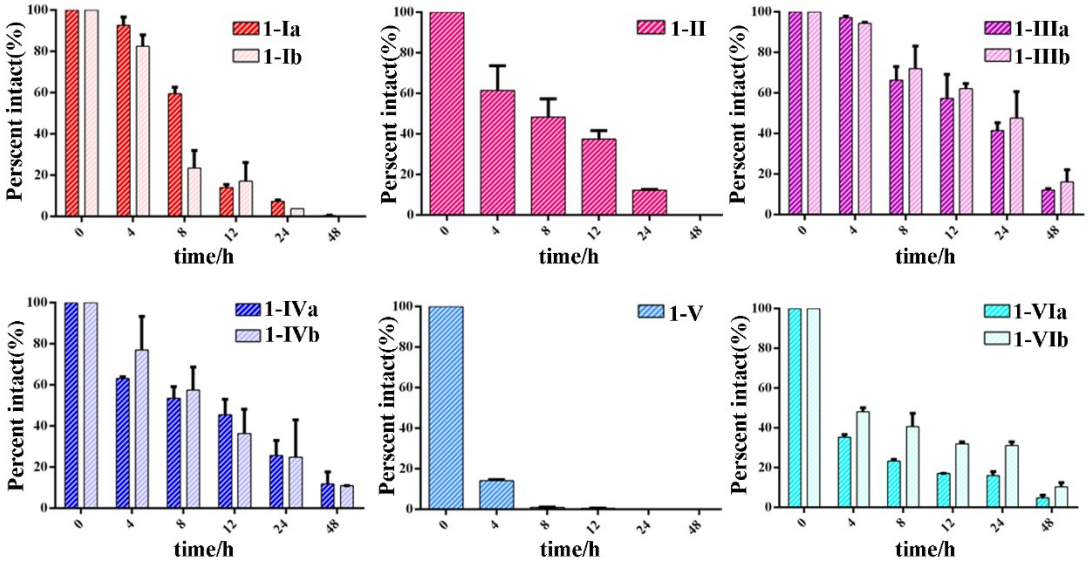


Figure S6. The kinetics of peptide dealkylation reactions equipped with different cross linkers. Peptides(1mM) were incubated with PyS (10mM) in PBS (pH=7.4) at 37°C for 48 hours.

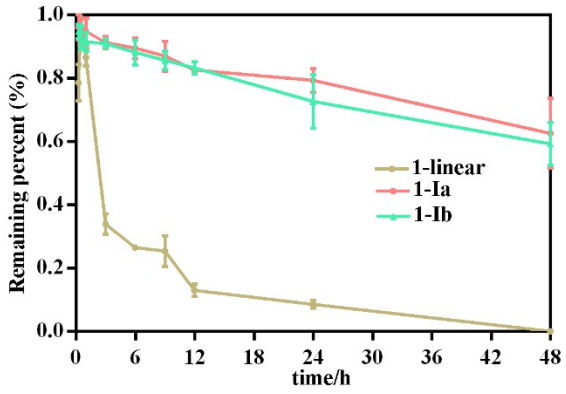


Figure S7. In vitro serum digestion assay of 1-linear, 1-Ia, and 1-Ib peptides. Peptides were incubated with 25% fresh mouse serum (v/v) in PBS to a final concentration of 100 μM at 37°C.

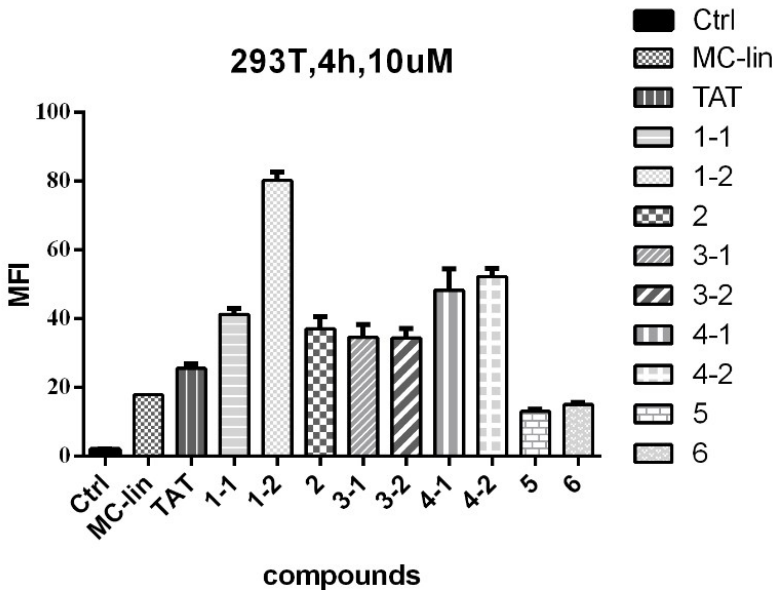


Figure S8. FACS analysis of 293T cells treated with 10μM FAM-labelled peptides at 37°C for 4 hours.

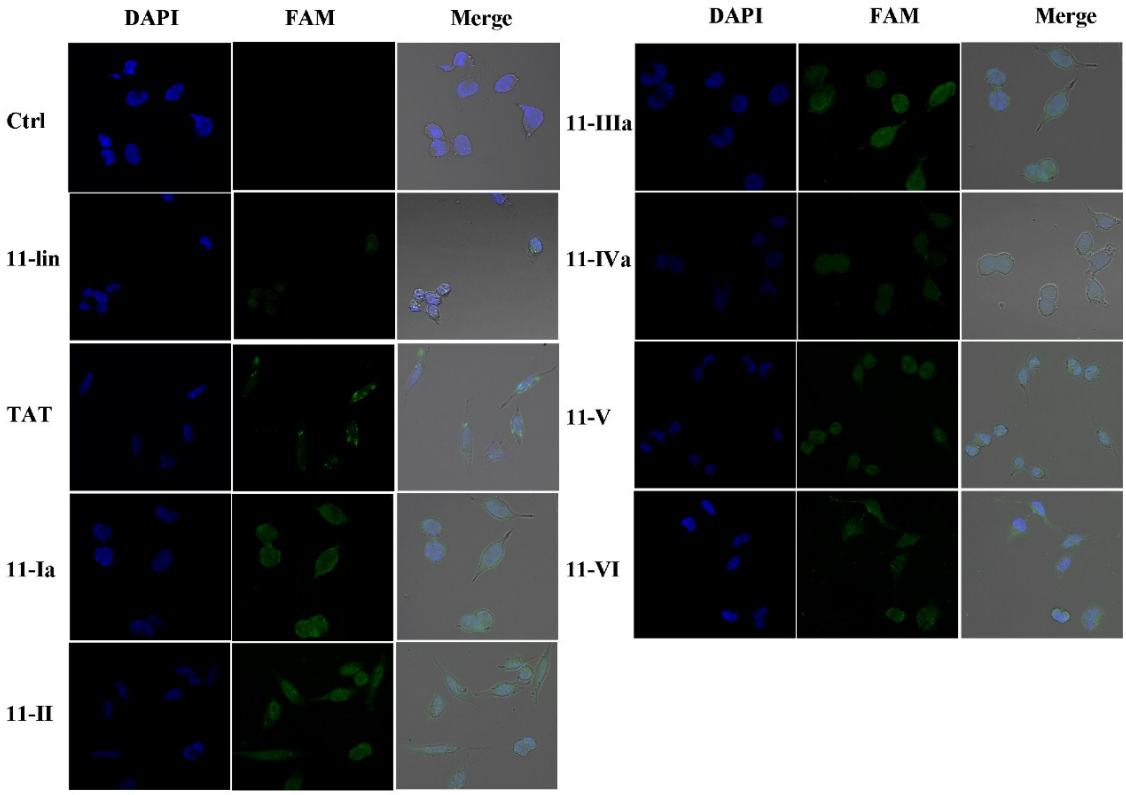
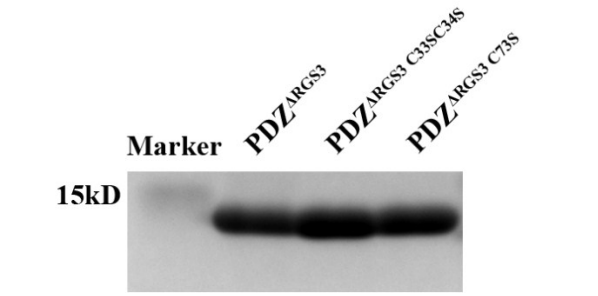


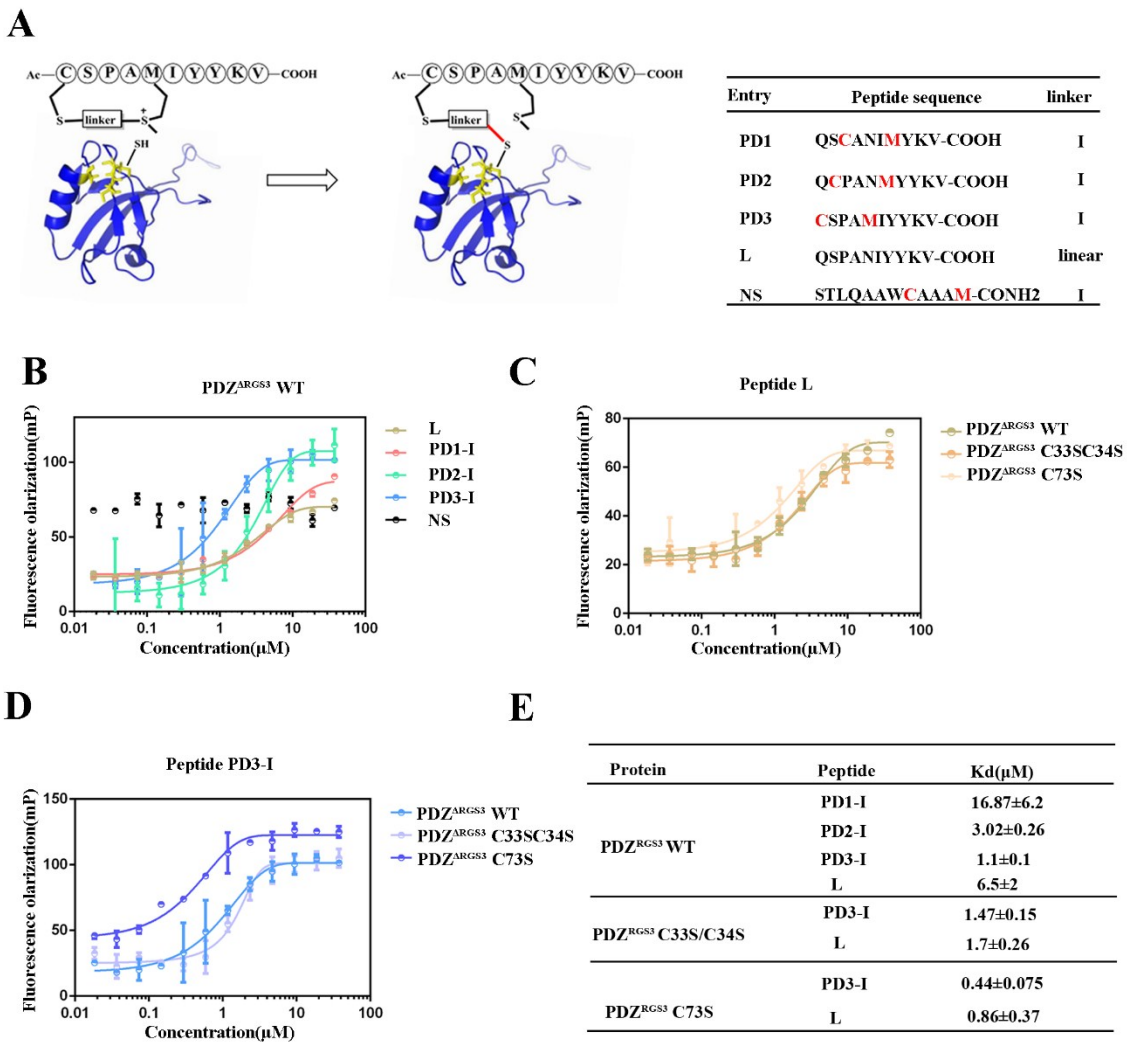
Figure S9. Confocal microscopy images of A2780 cells treated with 10 μM FAM-labelled peptides with different linkers at 37°C for 4 hours.



Protein sequence:

PDZ$^{\Delta RGS3}$: MHHHHHHSSG LEVLFQGPGS GKKLKITIRR GKDGFGFTIC CDSPVRVQAV DSGGPAERAG LQQLDTVLQL NERPVEHWKC VELAHEIRSC PSEIILLVWR VVPQIK

Figure S10: Protein expression and SDS-PAGE gel for PDZ$^{\Delta RGS3}$ (lane1), PDZ$^{\Delta RGS3C33SC34S}$ (lane2), PDZ$^{\Delta RGS3C73S}$(lane3).



Entry	Peptide sequence	linker
PD1	QSCANIMYKV-COOH	I
PD2	QCPANMYYKV-COOH	I
PD3	CSPAMIYYKV-COOH	I
L	QSPANIYYKV-COOH	linear
NS	STLQAAWCAAAM-CONH2	I

Protein	Peptide	Kd(μM)
PDZ^{RGS3} WT	PD1-I	16.87±6.2
	PD2-I	3.02±0.26
	PD3-I	1.1±0.1
	L	6.5±2
PDZ^{RGS3} C33S/C34S	PD3-I	1.47±0.15
	L	1.7±0.26
PDZ^{RGS3} C73S	PD3-I	0.44±0.075
	L	0.86±0.37

Figure S11. 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) Binding affinity of peptide L to wild type PDZ$^{\Delta RGS3}$ and their mutants.(D) Binding affinity of peptide PD3-I to wild type PDZ$^{\Delta RGS3}$ and their mutants.(E) Data conclusion from Figure A-D.

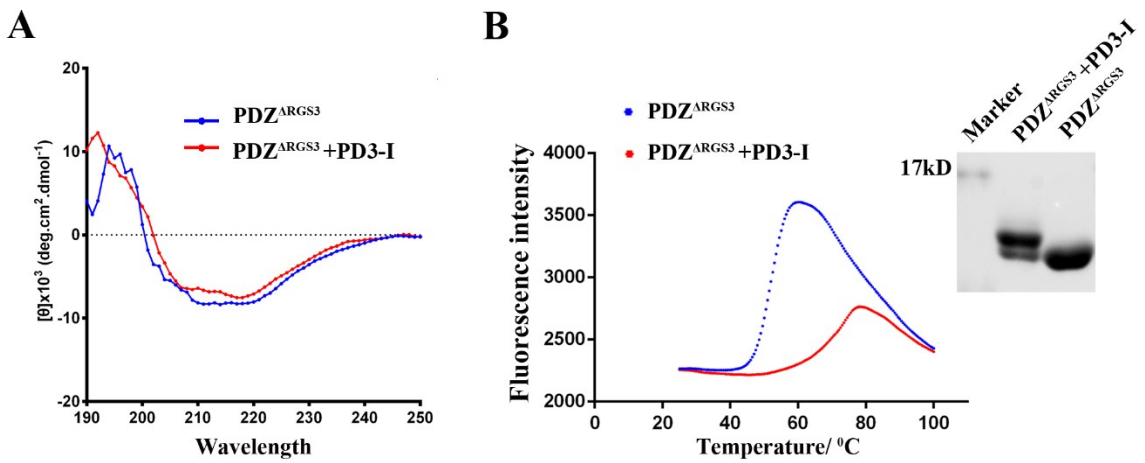


Figure S12. The structure analysis of protein-peptide conjugation. (A) Wild type PDZ$^{\Delta RGS3}$ protein and the protein-peptide conjugate was examined by the circular dichroism(CD). (B) The thermal shift assay curve was performed to test the thermo stability of wild type protein and protein-peptide conjugates. PDZ$^{\Delta RGS3}$ (20μM) was incubated with peptide PD3-I(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 S13. The competition of linear peptide L for peptide-protein conjugation. The mixture of peptide PD3-1(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.

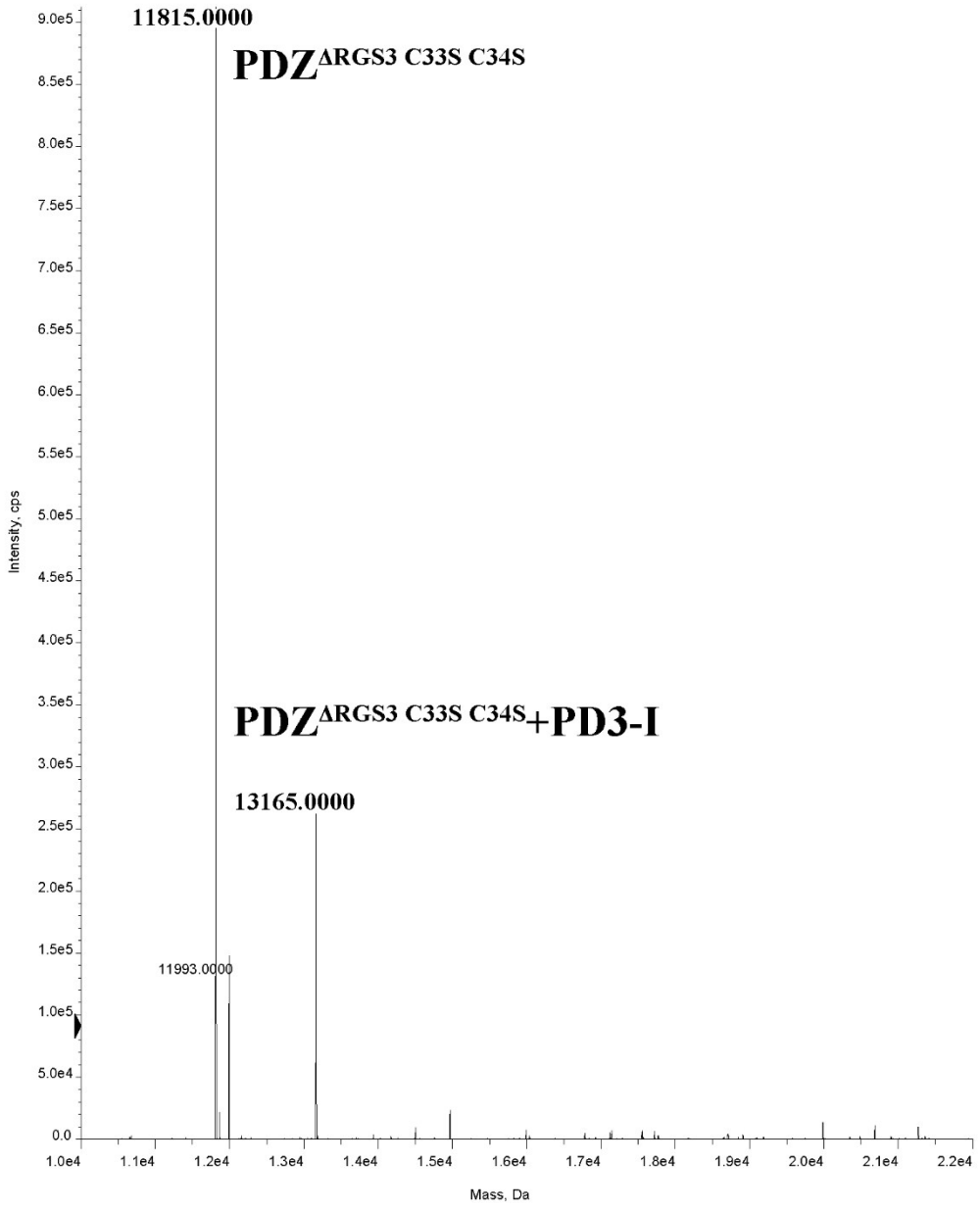


Figure S14. ESI MS analysis of protein PDZ$^{\Delta RGS3\ C33SC34S}$ and peptide conjugation.

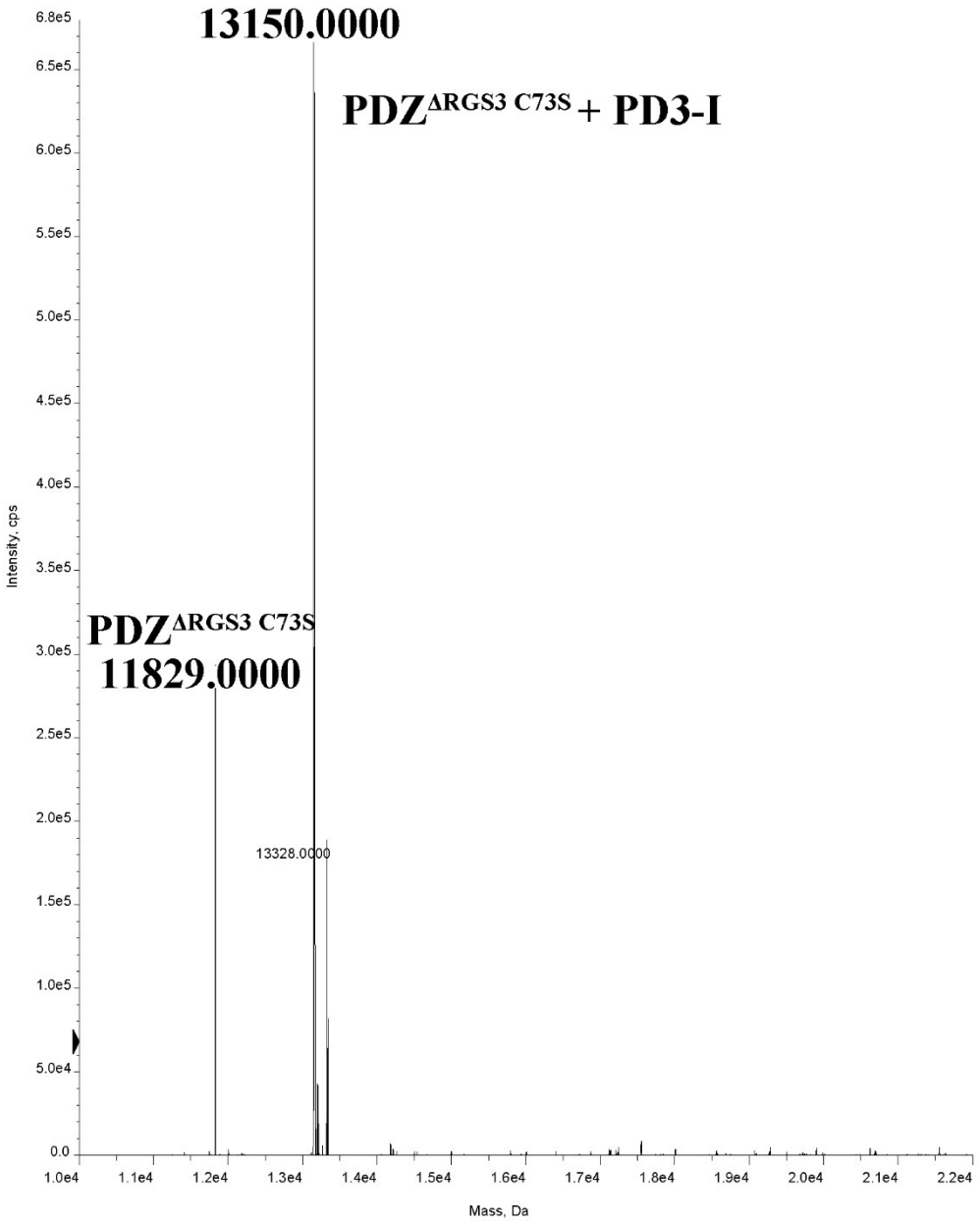


Figure S15. ESI MS analysis of protein mutants PDZ$^{\Delta RGS3\ C73S}$ and peptide conjugation.

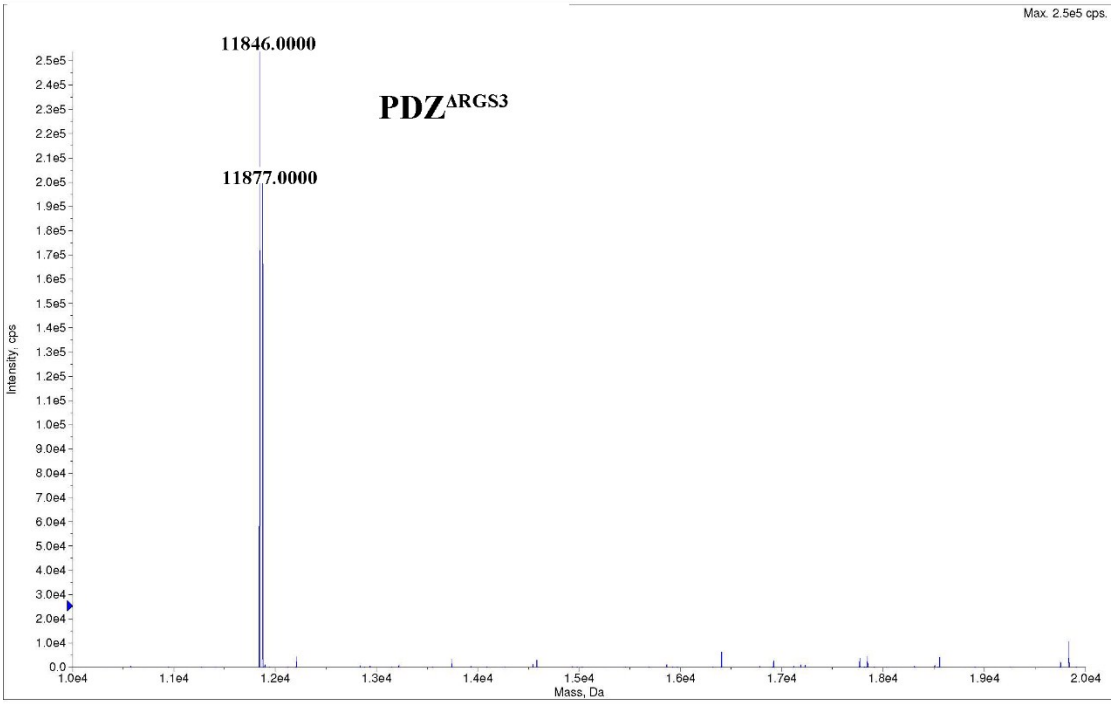


Figure S16. ESI MS analysis of protein PDZ$^{\Delta RGS3}$

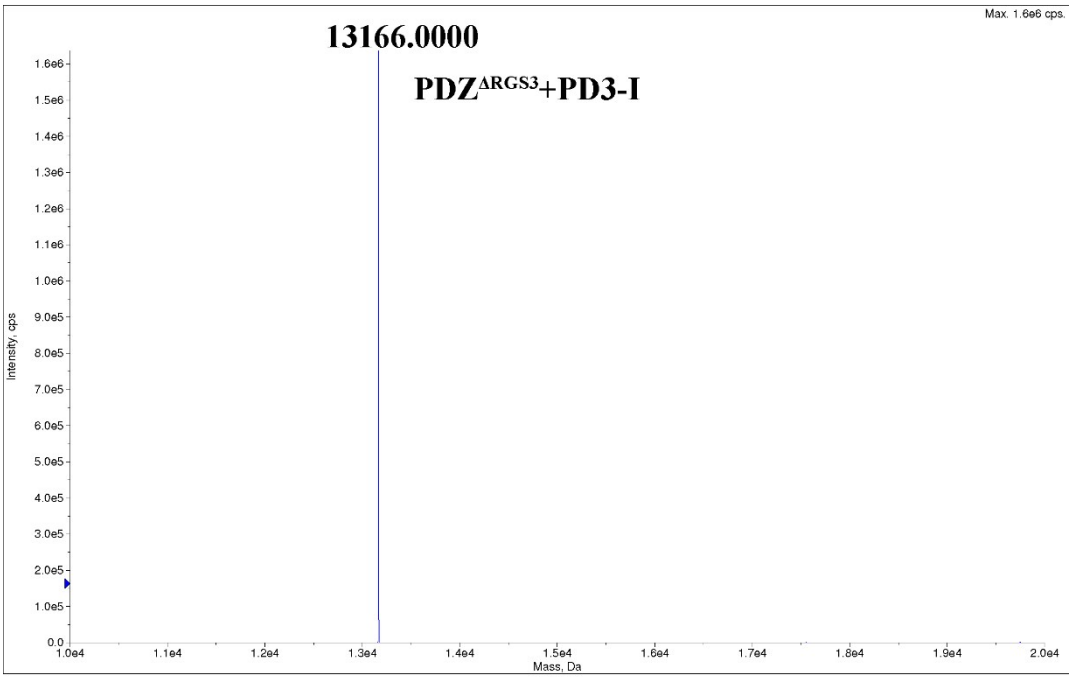


Figure S17. ESI MS analysis of protein $PDZ^{\Delta RGS3}$ and peptide PD3-I conjugation.

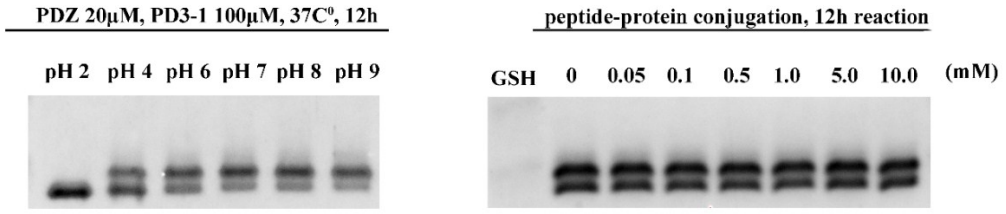


Figure S18.The conjugation efficiency at different pH conditions. The peptide PD3-1(100μM) was incubated with $PDZ^{\Delta RGS3}$ (20μM) for 12 hours at 37°C. The stability of the protein conjugates under GSH conditions for 12 hours. The peptide PD3-1(100μM) was incubated with $PDZ^{\Delta RGS3}$ (20μM) for 12 hours, then the mixture was incubated with GSH at different concentrations for another 12 hours.

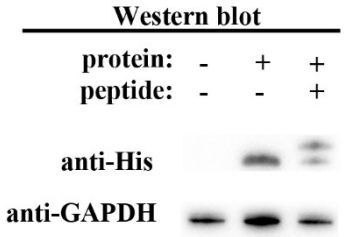


Figure S19. FAM-labelled peptides (**PD3-I**, 10μg) were incubated with 293T cell lysates(300μg) for 24 hours. His beads pull-down and western blot assays were further performed to confirm the selective protein modification.

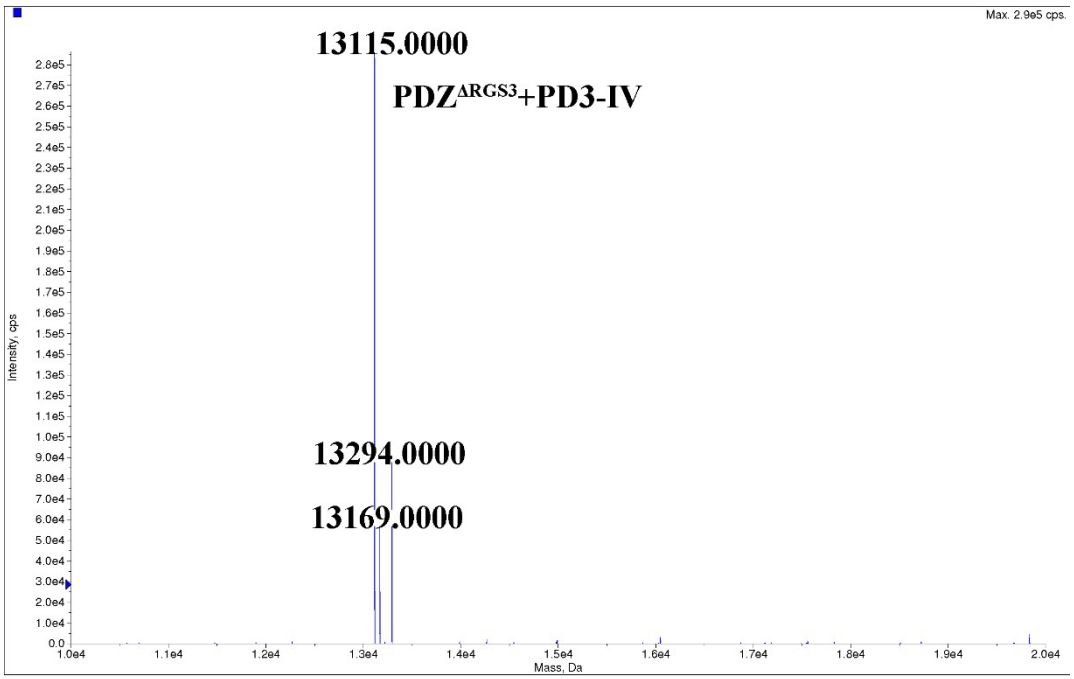


Figure S20. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PD3-IV conjugation.

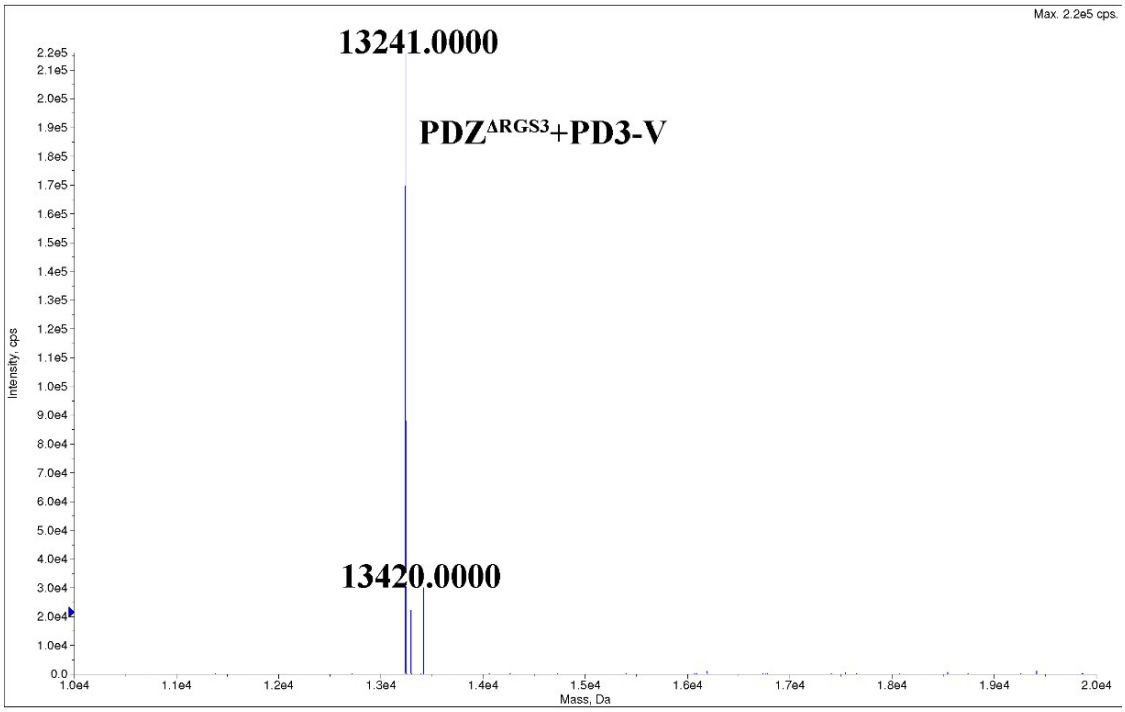


Figure S21. ESI MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PD3-V conjugation.

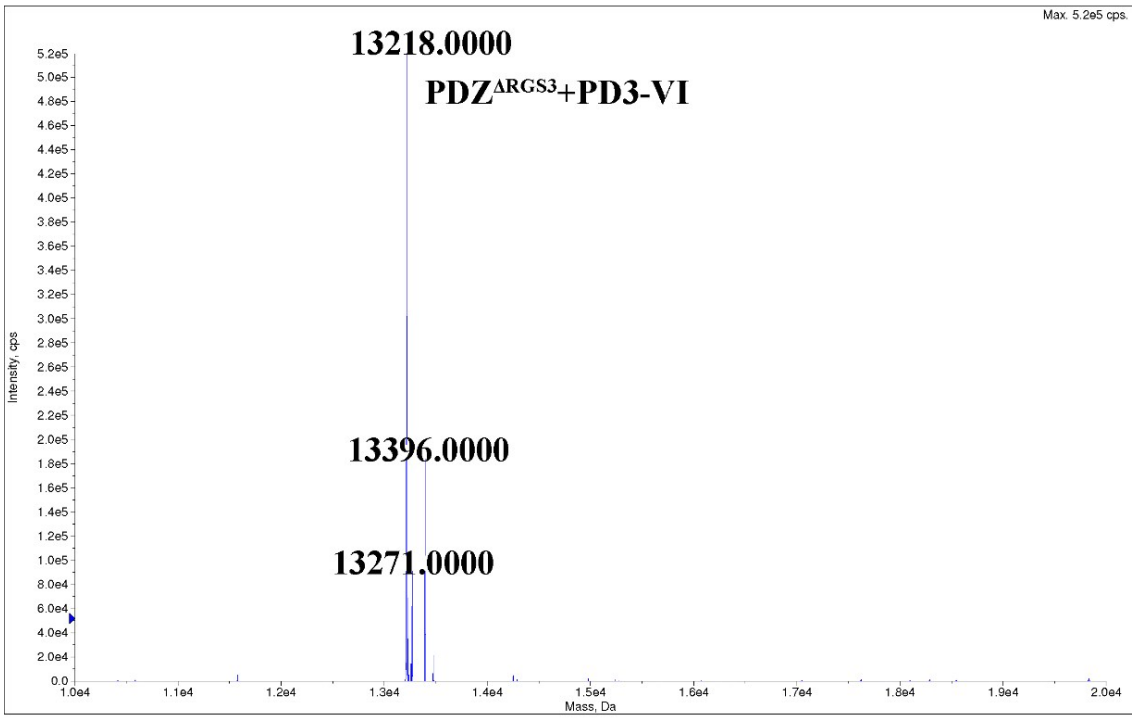


Figure S22. ESI MS analysis of protein $PDZ^{\Delta RGS3}$ and peptide PD3-VI conjugation.

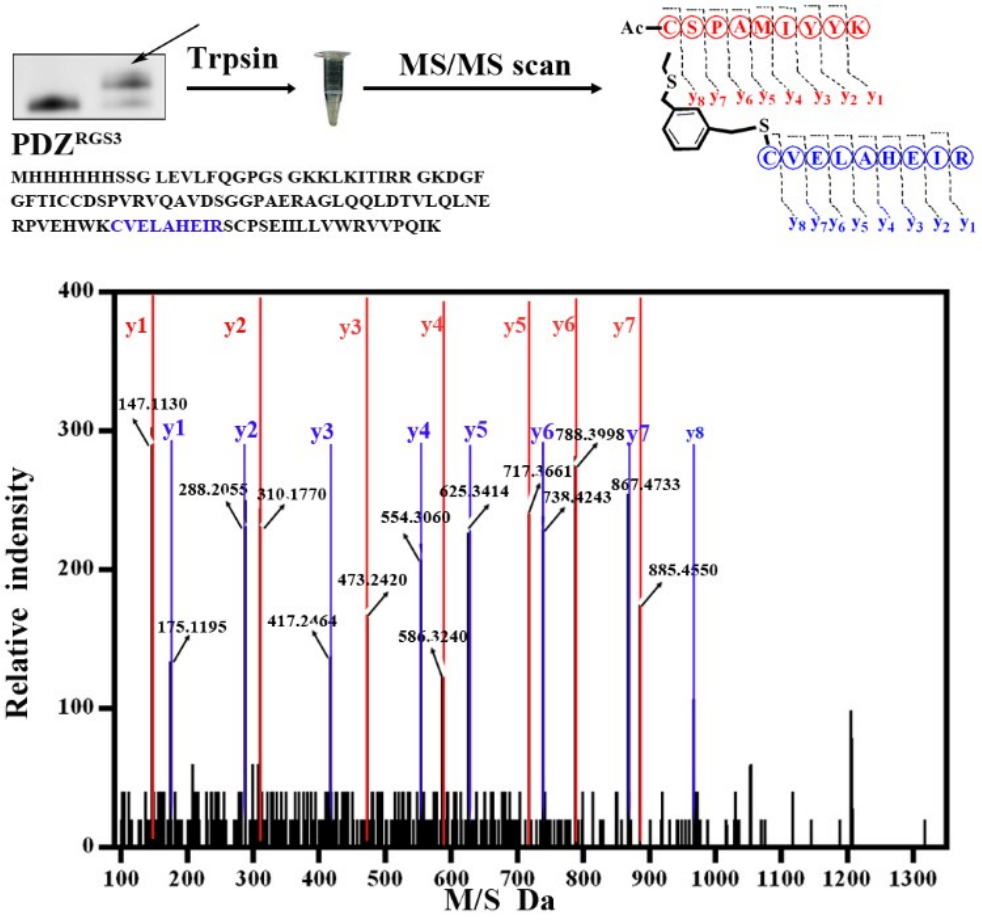


Figure S23. MS/MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide PD3-I conjugation at C73 site.

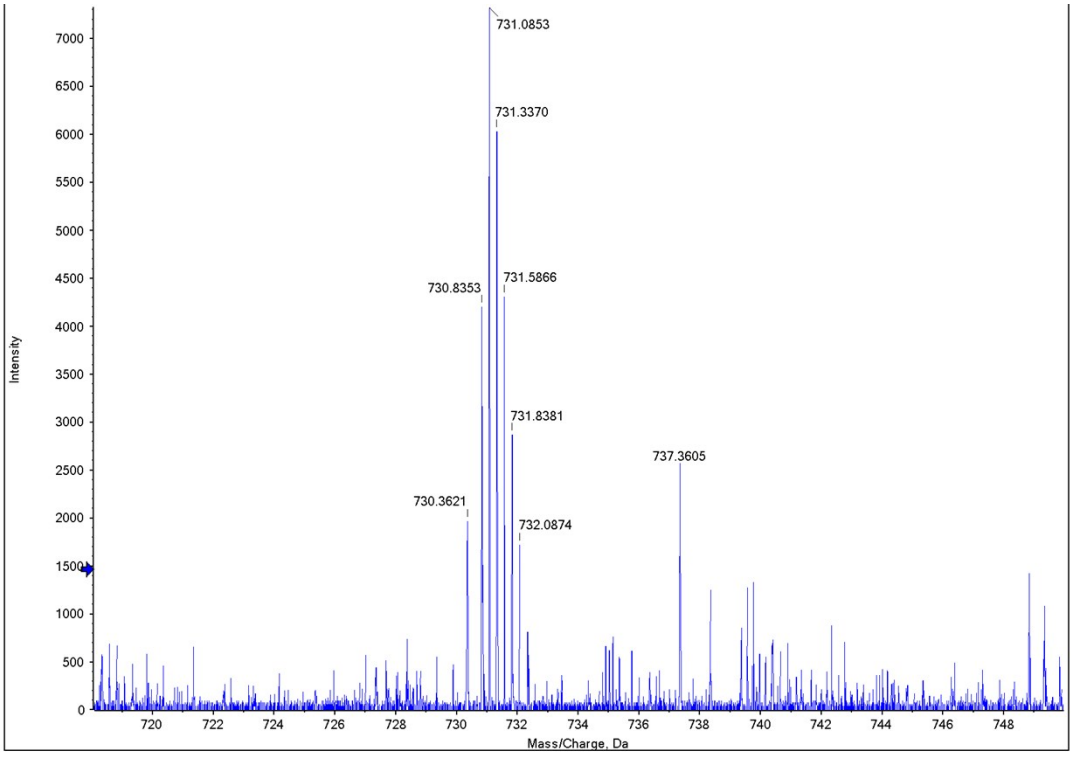


Figure S24. ESI MS/MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide conjugation at C33 or C34 (MS 2919.307296) from (730.834100, 4^{+}).

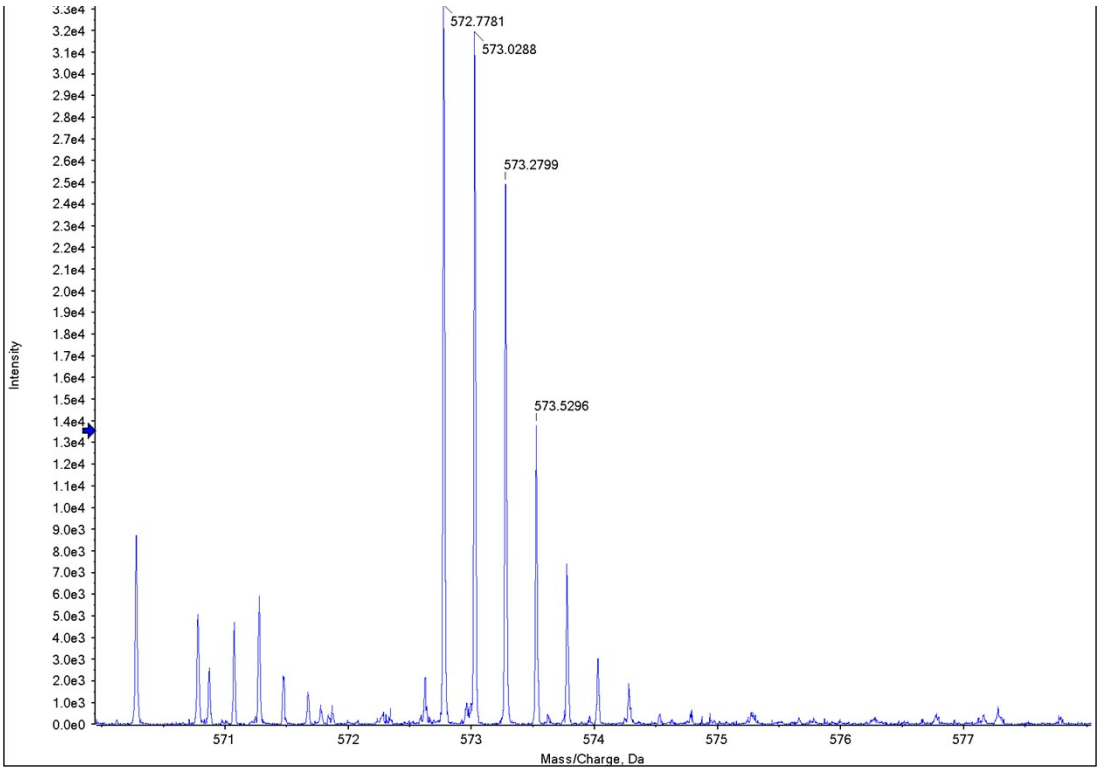


Figure S25. ESI MS/MS analysis of protein PDZ$^{\Delta RGS3}$ and peptide conjugation at C73 (MS 2287.080896) from (572.777500, 4^{+}).

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-linear	Ac-WMAAAC-NH_2	692.28	693.40
1-I	Ac-(cyclo-I)-WMAAAC-NH2	795.33	795.40

1-II	Ac-(cyclo-II)-WMAAAC-NH2	795.33	795.50
1-III	Ac-(cyclo-III)-WMAAAC-NH2	796.33	796.40
1-IV	Ac-(cyclo-IV)-WMAAAC-NH2	745.32	745.40
1-V	Ac-(cyclo-V)-WMAAAC-NH2	871.36	871.45
1-VI	Ac-(cyclo-VI)-WMAAAC-NH2	847.34	847.40
2-linear	Ac-CAAAM-NH2	506.20	507.35
2-I	Ac-(cyclo-I)-CAAAM-NH2	609.25	609.40
3-linear	Ac-MAEAC-NH2	564.20	565.25
3- I	Ac-(cyclo- I)-MAEAC-NH2	667.26	667.40
4-linear	Ac-MAKAC-NH2	563.26	563.45
4- I	Ac-(cyclo- I)-MAKAC-NH2	666.31	666.45
5-linear	Ac-MAQAC-NH2	634.26	635.40
5- I	Ac-(cyclo- I)-MAQAC-NH2	737.31	738.35
6-linear	Ac-MADAC-NH2	550.19	551.25
6- I	Ac-(cyclo- I)-MADAC-NH2	653.24	653.30
7-linear	Ac-MATAC-NH2	607.25	608.15
7- I	Ac-(cyclo- I)-MATAC-NH2	710.30	710.40
8-linear	Ac-MANAC-NH2	549.20	550.30
8- I	Ac-(cyclo- I)-MANAC-NH2	652.26	652.35
9-linear	Ac-MRESC-NH2	736.30	736.05
9- I	Ac-(cyclo- I)-MRESC-NH2	839.35	839.50
10-linear	Ac-MHYWC-NH2	779.29	780.40
10- I	Ac-(cyclo- I)-MHYWC-NH2	882.34	882.40
11-linear	FAM-βA-MRRRC-NH2	1150.48	575.30/384.10
11- I	FAM-βA-(cyclo- I)-MRRRC-NH2	1253.53	626.45/418.20
11- II	FAM-βA-(cyclo- II)-MRRRC-NH2	1253.53	626.55/418.10
11-III	FAM-βA -(cyclo-III)-MRRRC-NH2	1254.53	627.40/418.80
11-IV	FAM-βA -(cyclo-IV)-MRRRC-NH2	1203.52	601/401
11-V	FAM-βA -(cyclo-V)-MRRRC-NH2	1329.57	664.60/443.55
11-VI	FAM-βA -(cyclo-VI)-MRRRC-NH2	1305.54	652.60/435.45
TAT	FAM -βA -YGRKKRRNRRR- NH2	1989.07	664.40/498.65/ 399.10/332.80
L1	Ac-QSPANIYYKV-OH	1224.36	1225.50/613.25
PD1-I	Ac-(cyclo-I)-QSCANIMYKV-OH	1301.6	650.95
PD2-I	Ac-(cyclo-I)-QCPANMYYKV-OH	1361.6	681.10
PD3-I	Ac-(cyclo-I)-CSPAMIYYKV-OH	1320.6	660.05
PD3-IV	Ac-(cyclo-IV)-CSPAMIYYKV-OH	1269.6	635.10
PD3-V	Ac-(cyclo-V)-CSPAMIYYKV-OH	1395.7	698.25
PD3-VI	Ac-(cyclo-VI)-CSPAMIYYKV-OH	1371.6	685.95
NS	Ac-(cyclo-I)-CAAAMWAAQLTS- NH2	1367.61	684.15

FAM-PD1-I	FAM-BA- (cyclo-I)-QSCANIMYKV- OH	1690.70	845.30/564.05
FAM-PD2-I	FAM-BA- (cyclo-I)-QCPANMYYKV-OH	1750.70	874.75/583.65
FAM-PD3-I	FAM-BA- (cyclo-I)-CSPAMIYYKV- OH	1708.7	857,80/572.25
FAM-NS	FAM-BA- (cyclo-I)-CAAAMWAAQLTS-NH2	1756.73	877.60

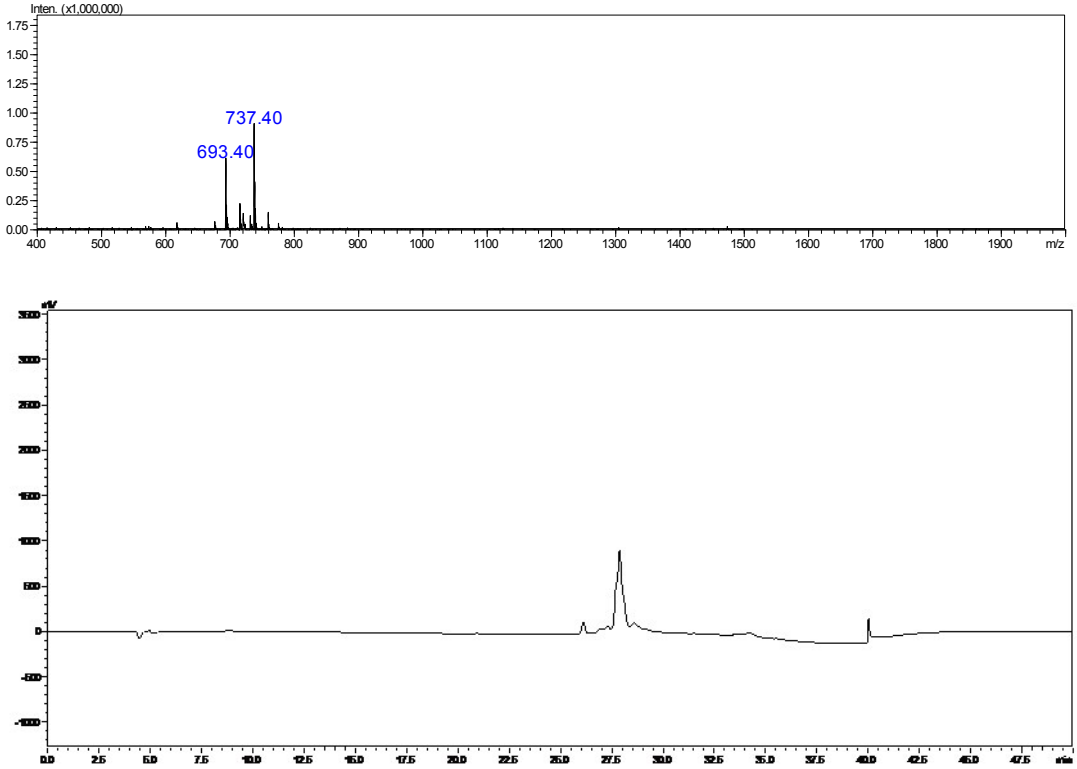
Table S3. Protein sequence

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**

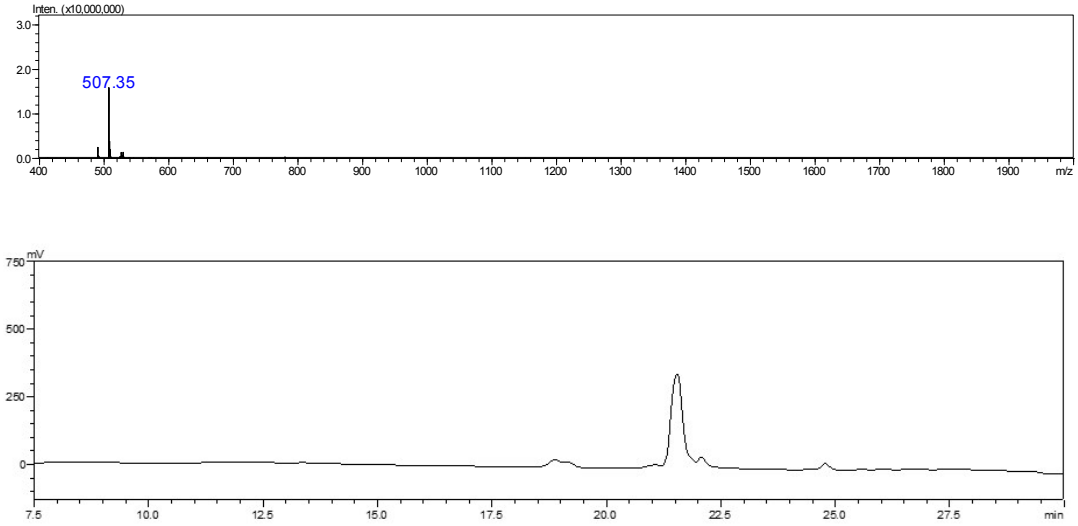
Appendix

LC-MS spectra of peptides used in the manuscript.

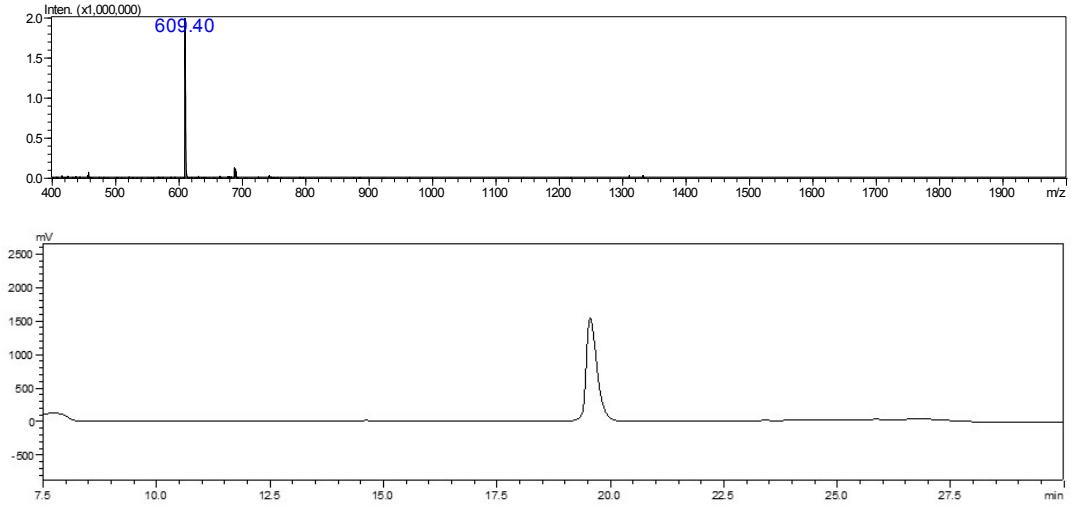
1. Ac-WMAAAC-NH_2(1-linear)



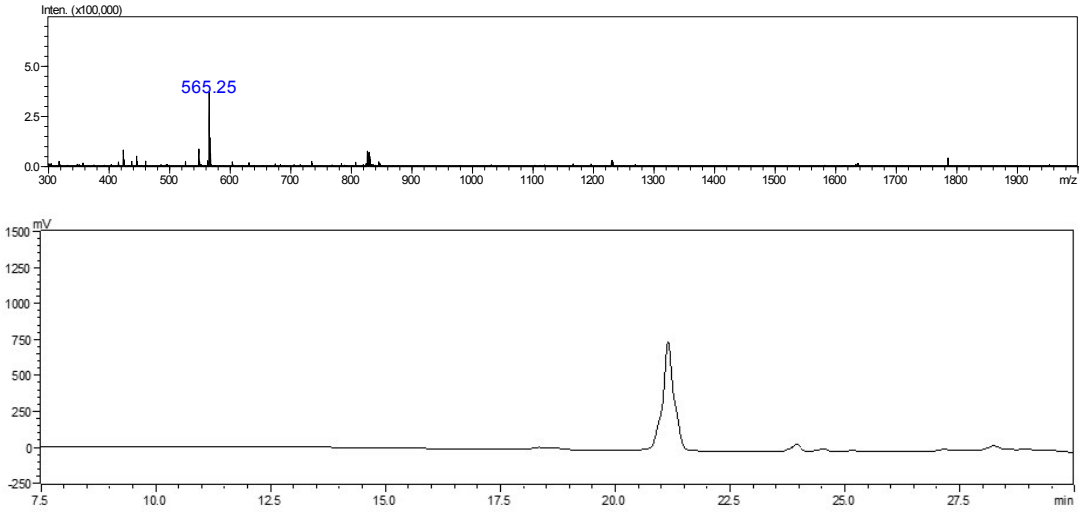
2. Ac-CAAAM-NH_2(2-linear)



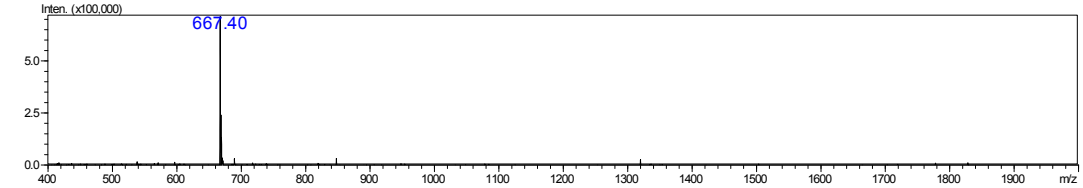
3. Ac-(cyclo-I)-CAAAM-NH_2 (2-I)



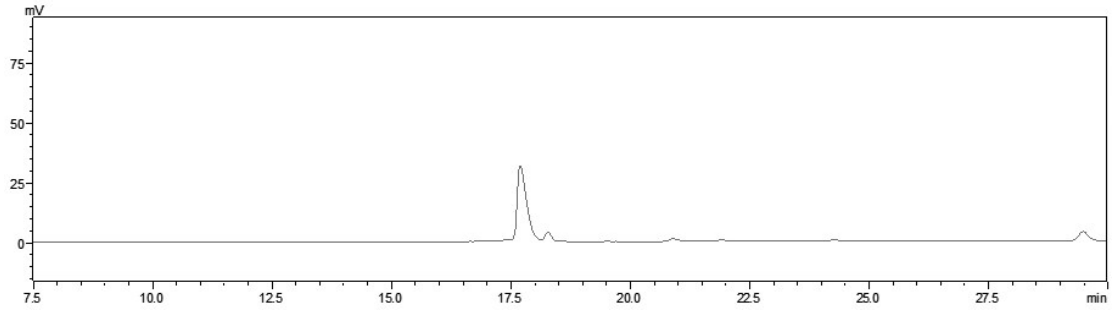
4. Ac-MAEAC-NH_2(3-linear)



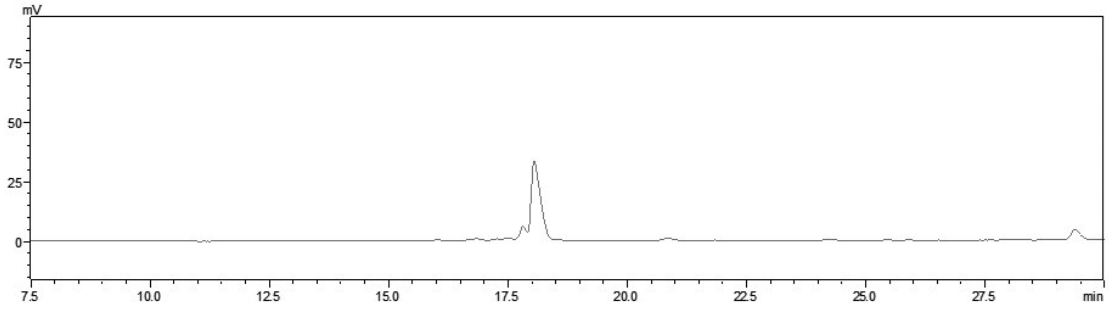
5. Ac-MAEAC-NH_2(3-I)



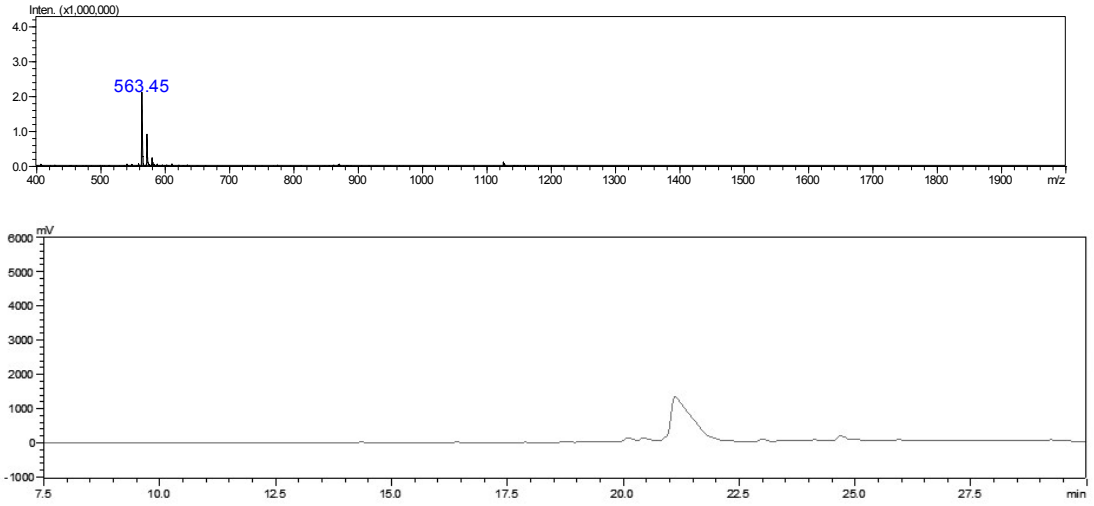
3-Ia



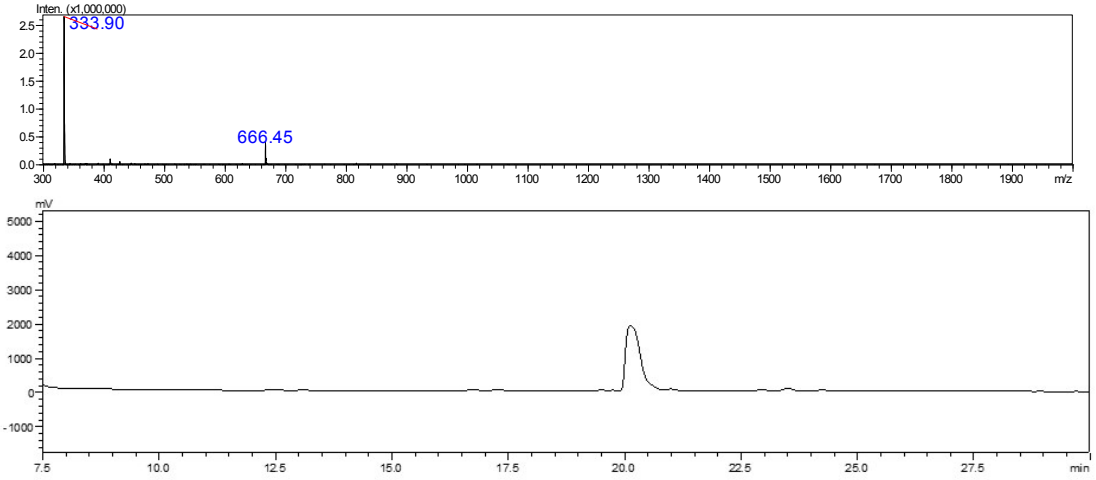
3-Ib



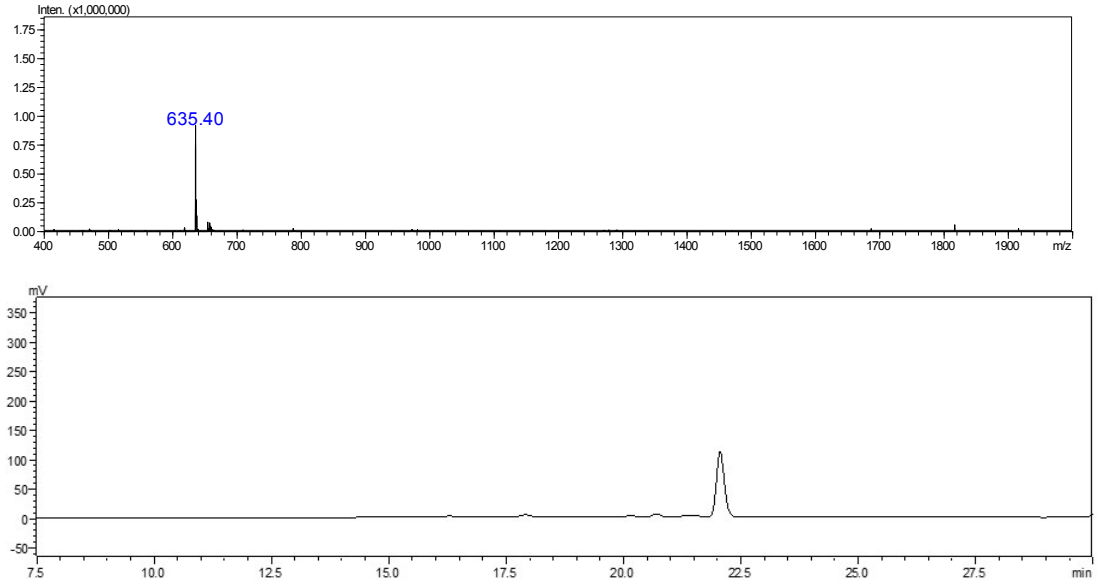
6. Ac-MAKAC-NH_2(4-linear)



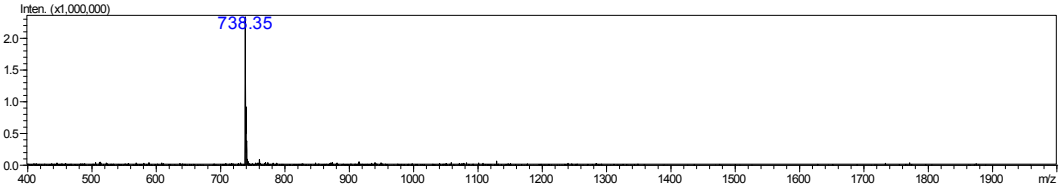
7. Ac-(cyclo-I)-MAKAC-NH_2(4-I)

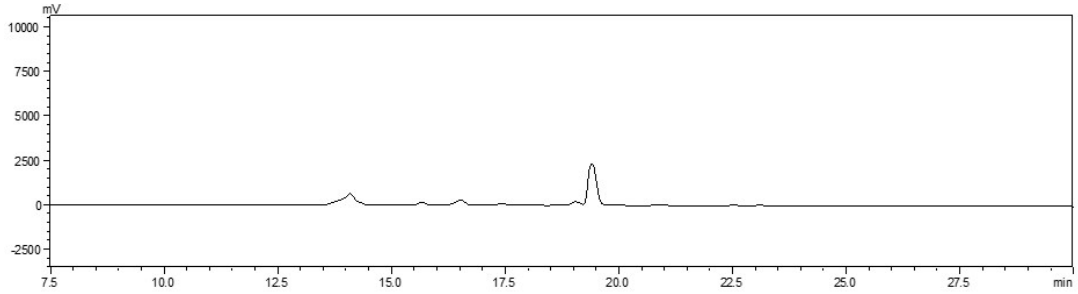


8. Ac-MAQAC-NH_2(5-linear)

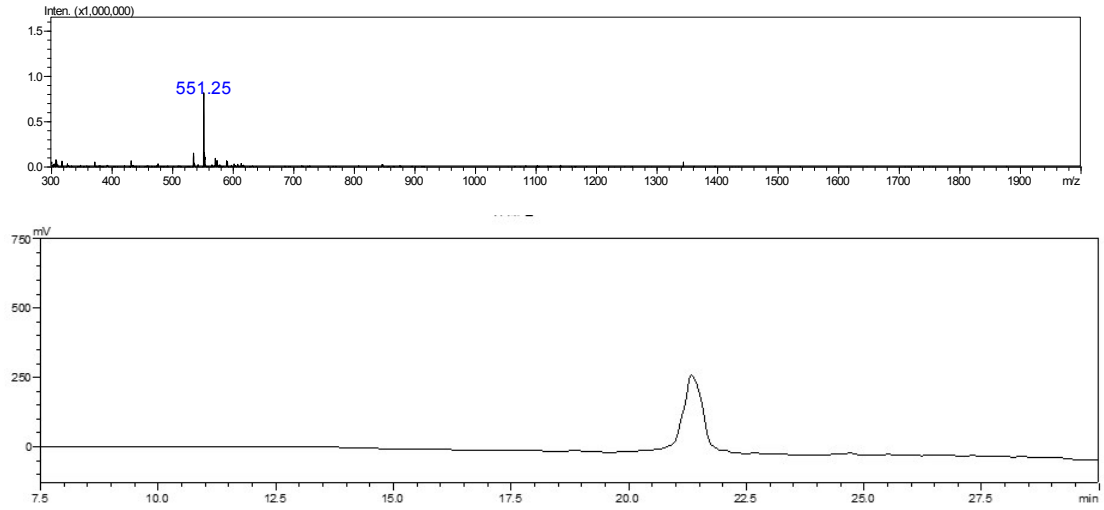


9. Ac-(cyclo-I)-MAQAC-NH_2(5-I)

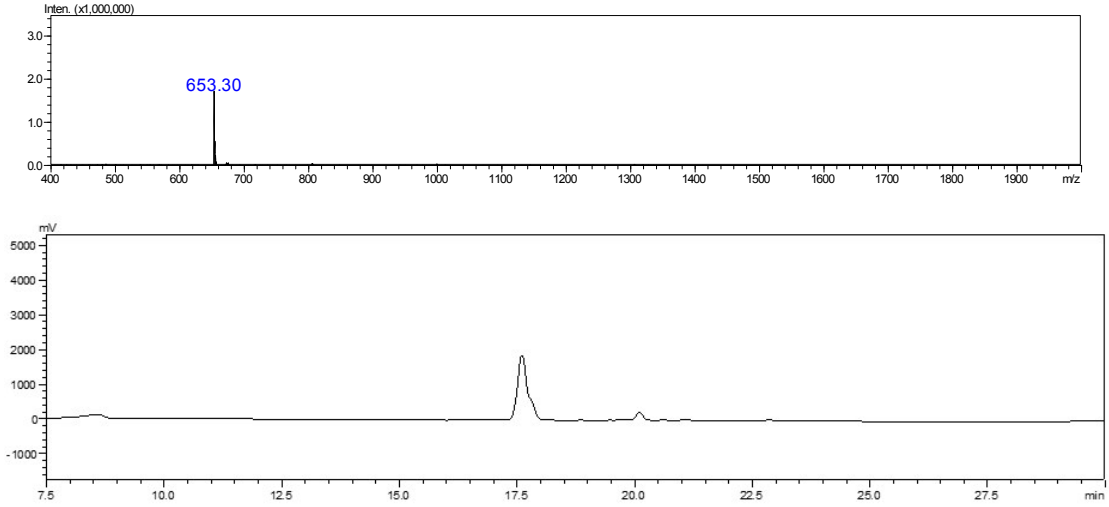




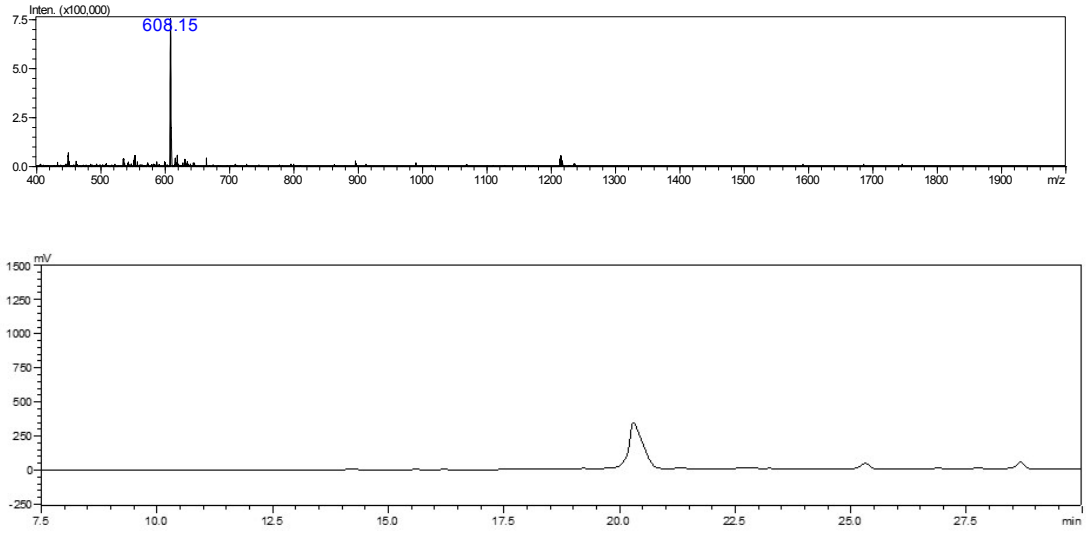
10. Ac-MADAC-NH_2(6-linear)



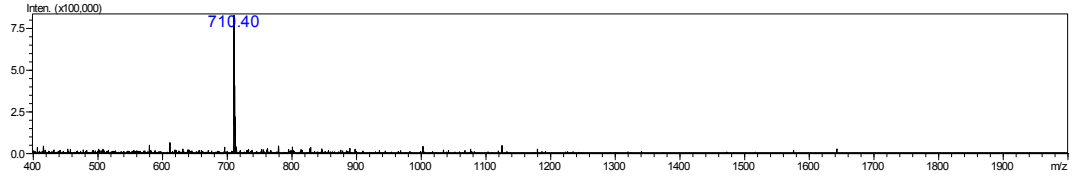
11. Ac-(cyclo-I)-MADAC-NH_2(6-I)



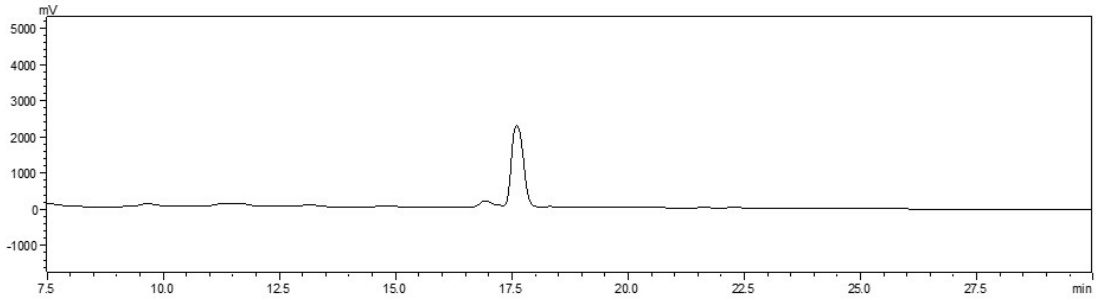
12. Ac-MATAC-NH_2(7-linear)



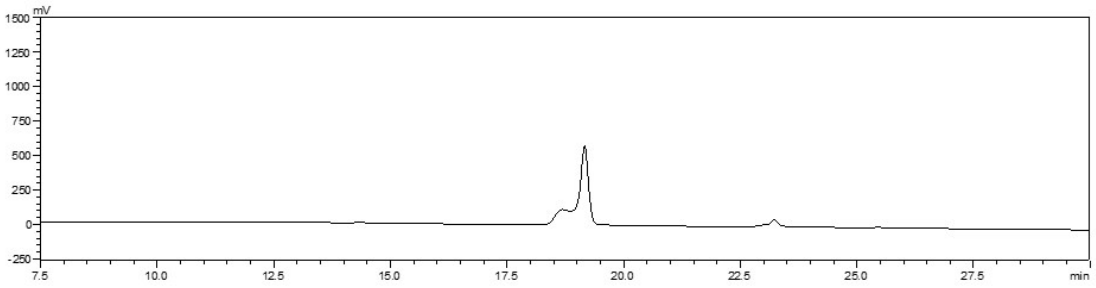
13. Ac-(cyclo-I)-MATAC-NH_2 (7-I)



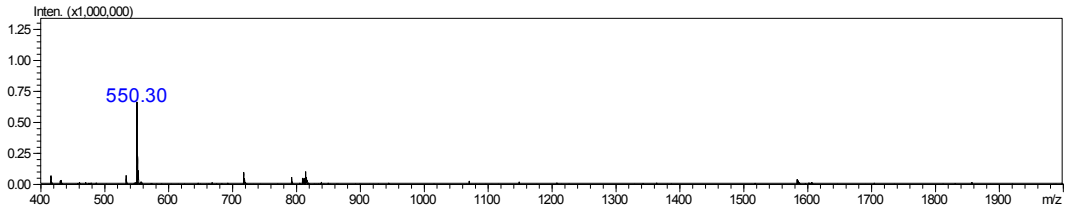
7-Ia

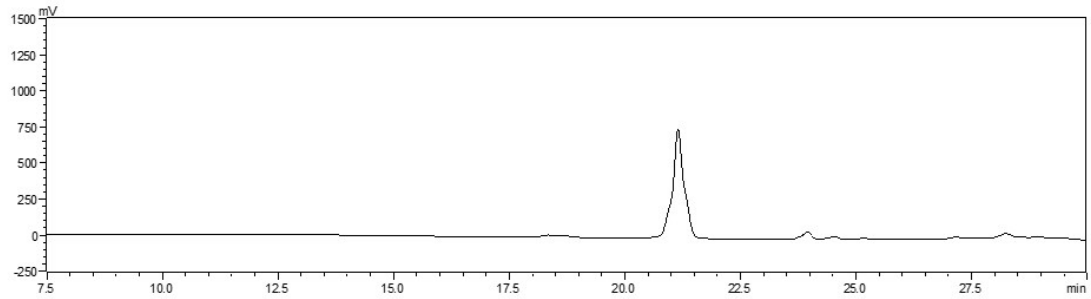


7-Ib

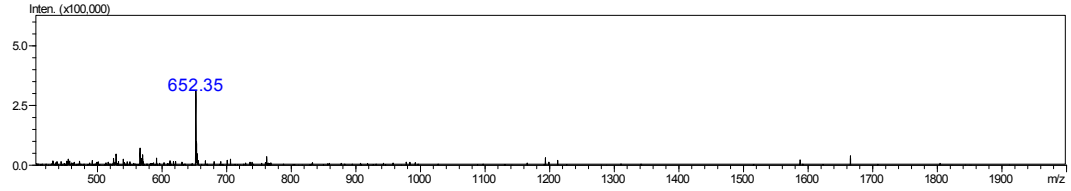


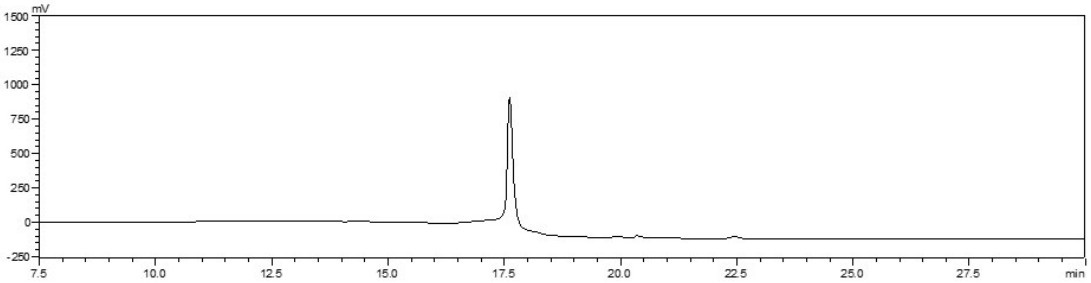
14. Ac-MANAC-NH_2(8-linear)



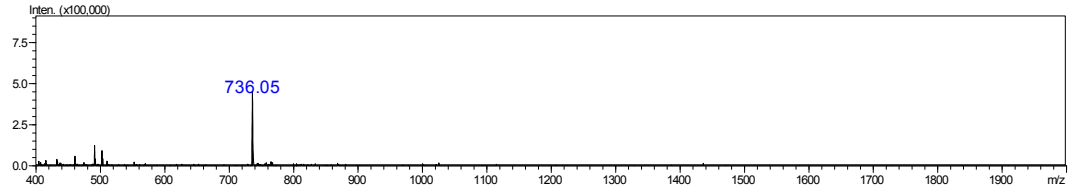


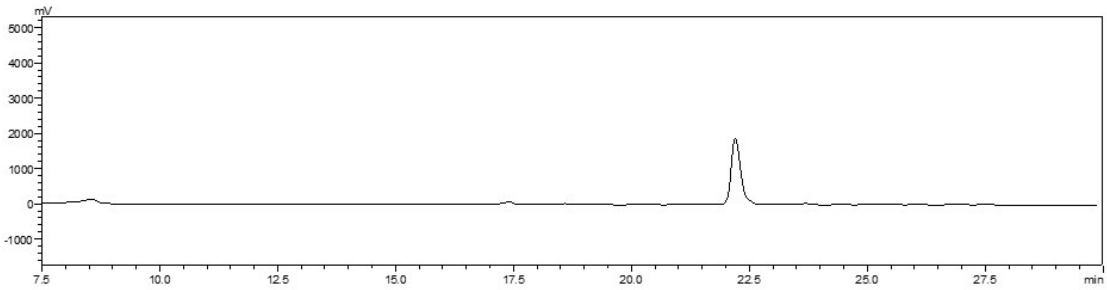
15. Ac-(cyclo-I)-MANAC-NH_2 (8-I)



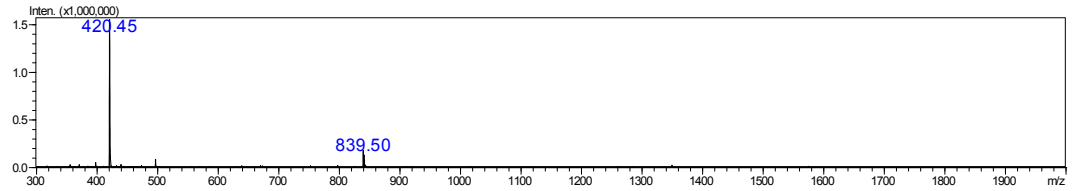


16. Ac-MRESC-NH_2 (9-linear)

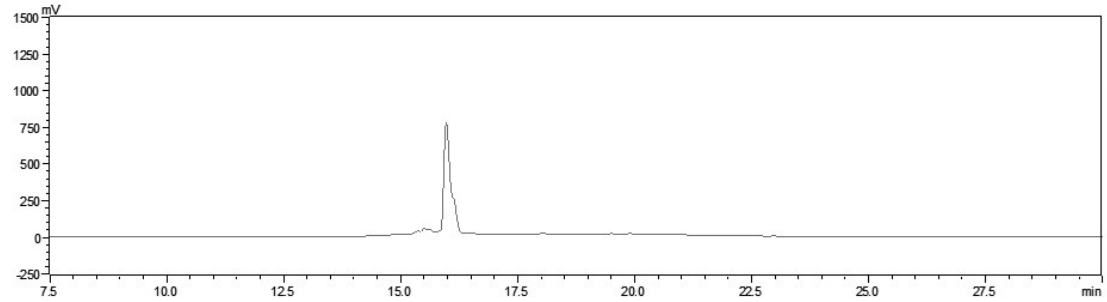




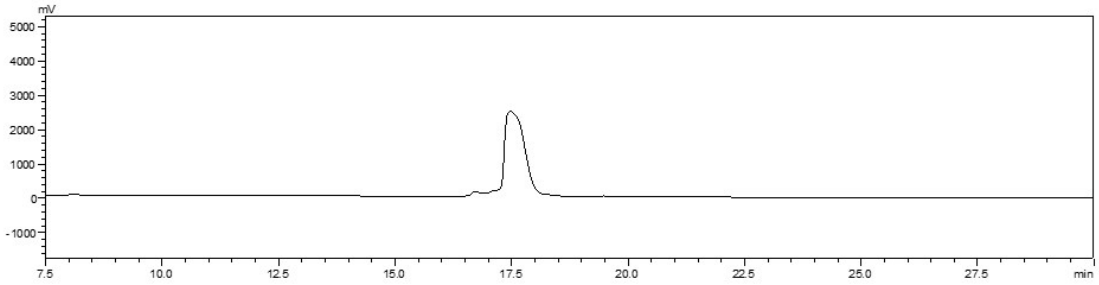
17. Ac-(cyclo-I)- MRESC-NH_2 (9-I)



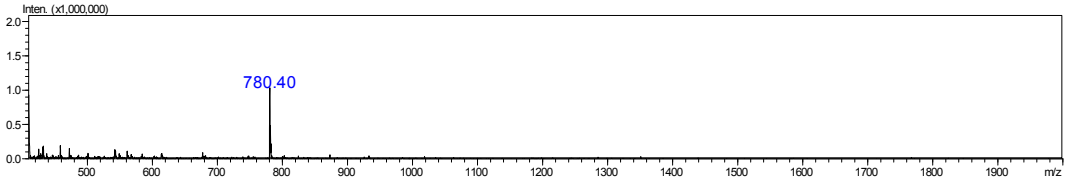
9-Ia

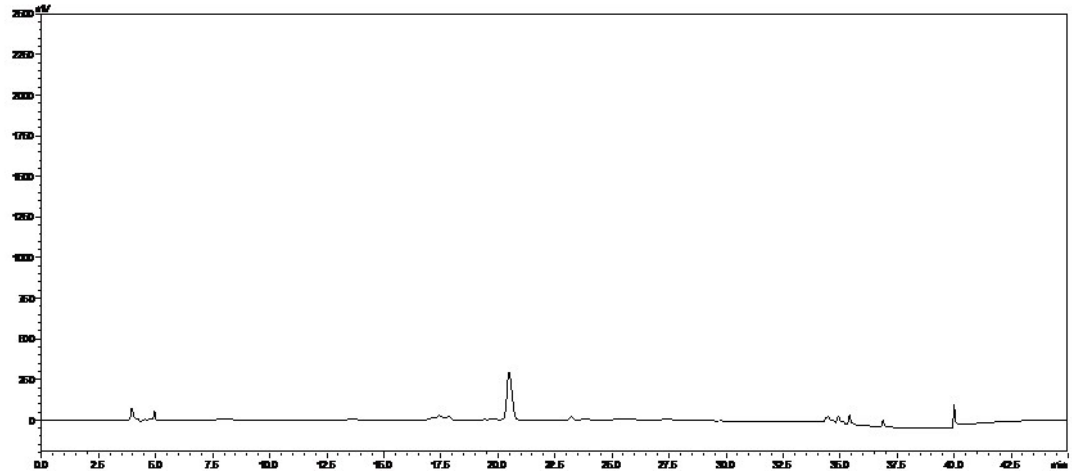


9-Ib

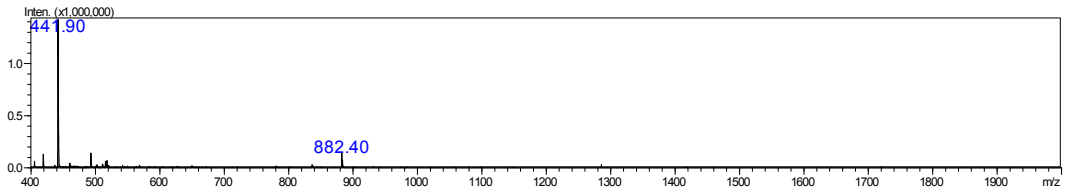


18. Ac-MHYWC-NH_2 (10-linear)

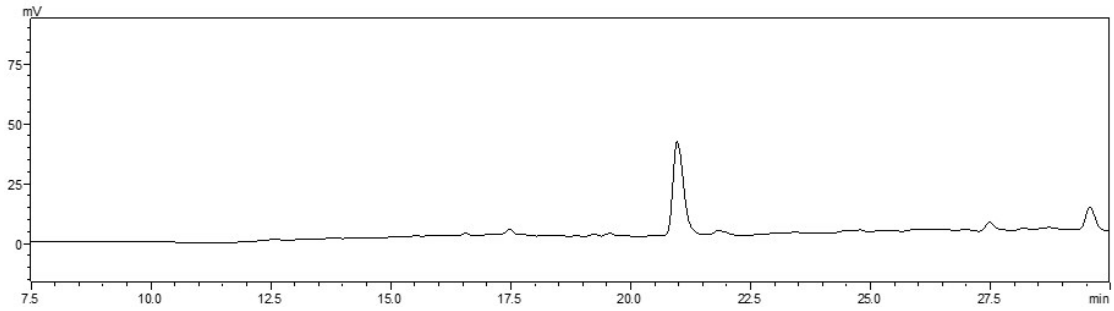




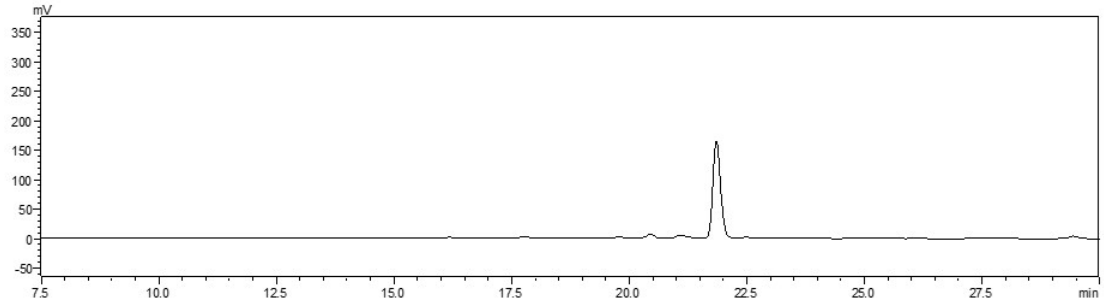
19. Ac-(cyclo-I)-MHYWC-NH_2 (10-I)



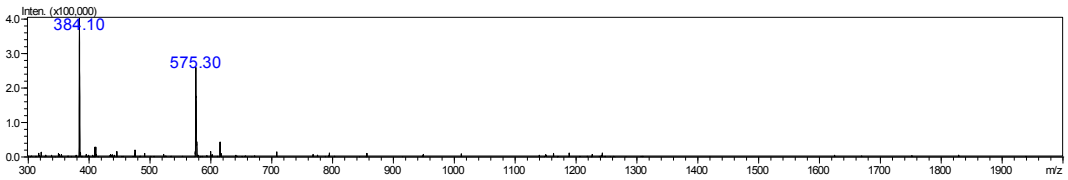
10-Ia

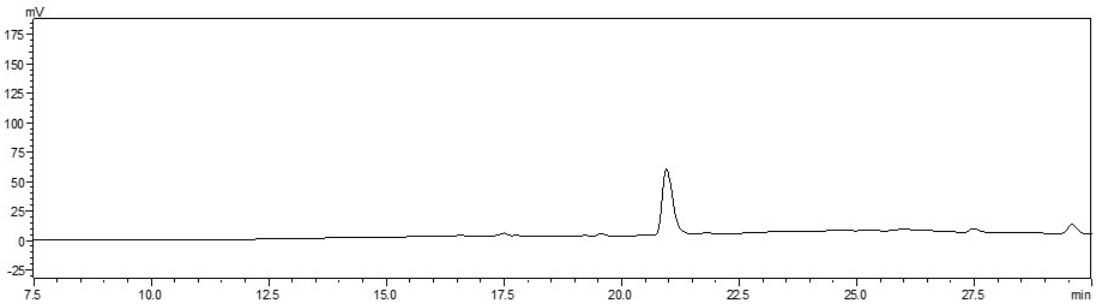


10-Ib

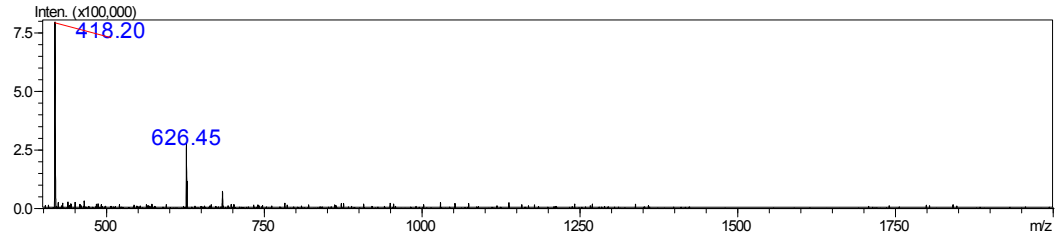


20. FAM-βA-MRRRC-NH_2 (11-linear)

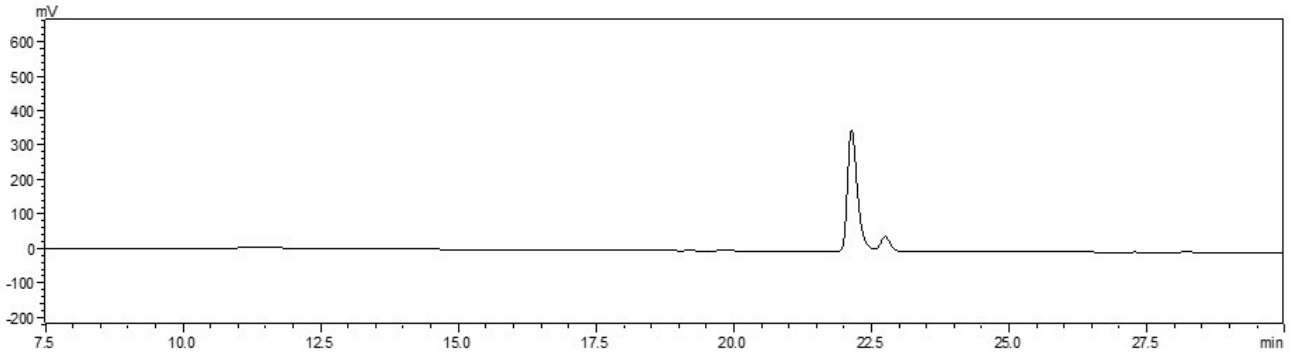




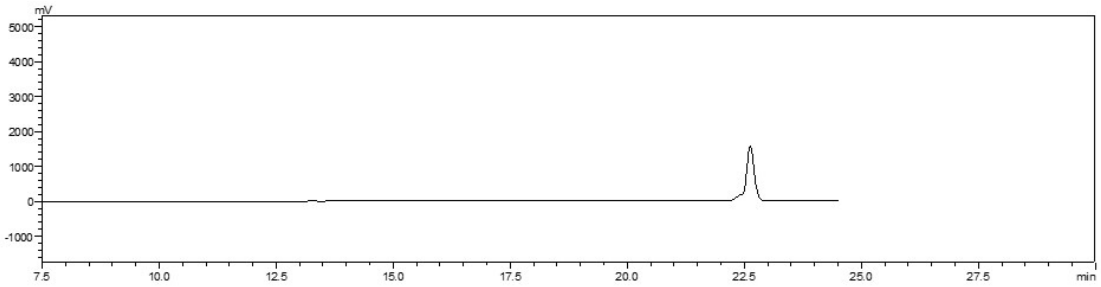
21. FAM-βA-(cyclo-I)-MRRRC-NH_2 (11-I)



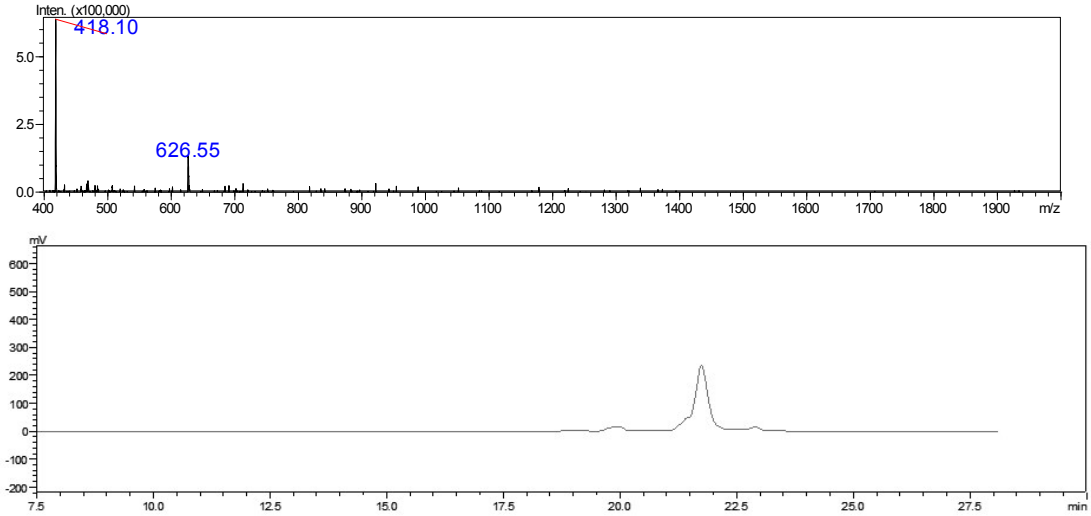
11-Ia



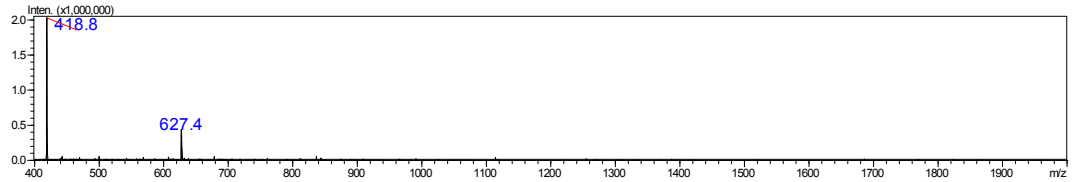
11-Ib



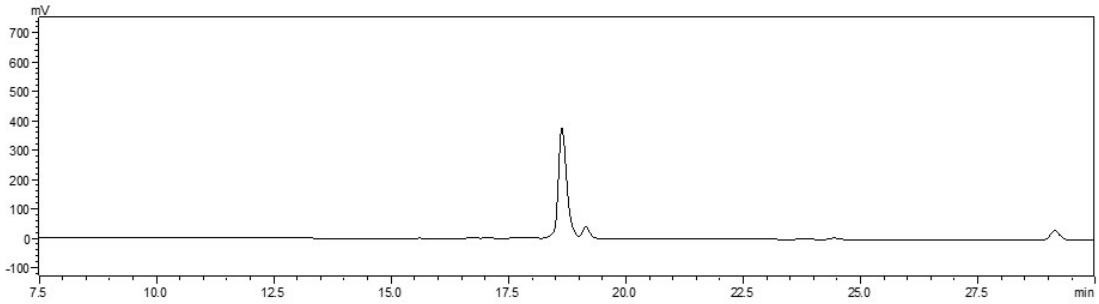
22. FAM-βA-(cyclo-II)-MRRRC-NH_2 (11-II)



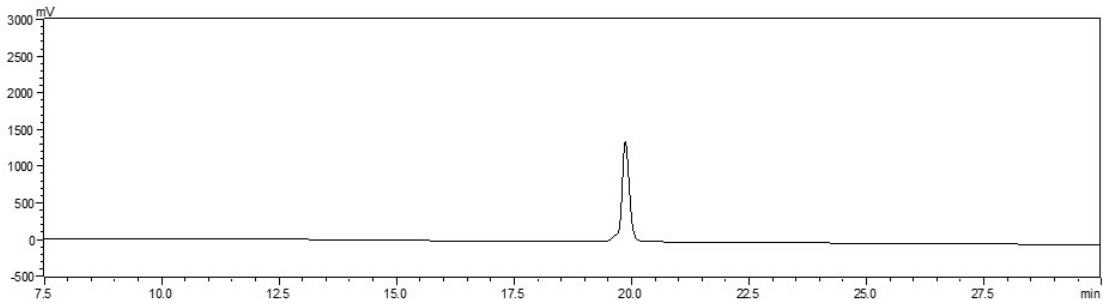
23. FAM-βA-(cyclo-III)-MRRRC-NH_2 (11-III)



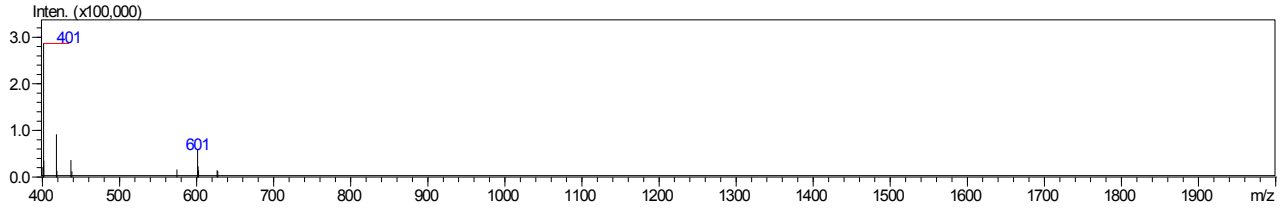
11-IIIa



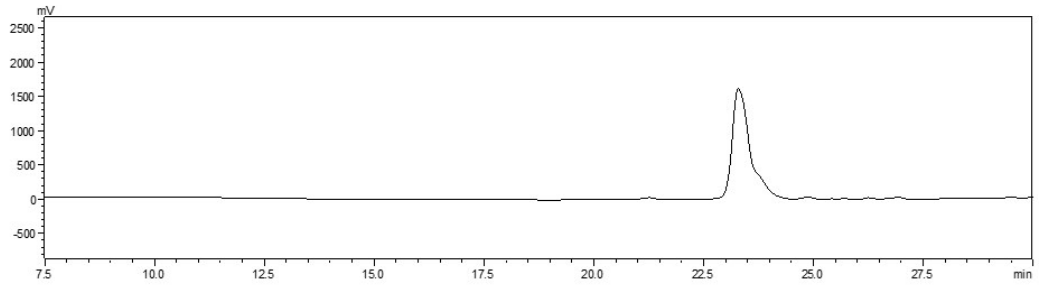
11-IIIb



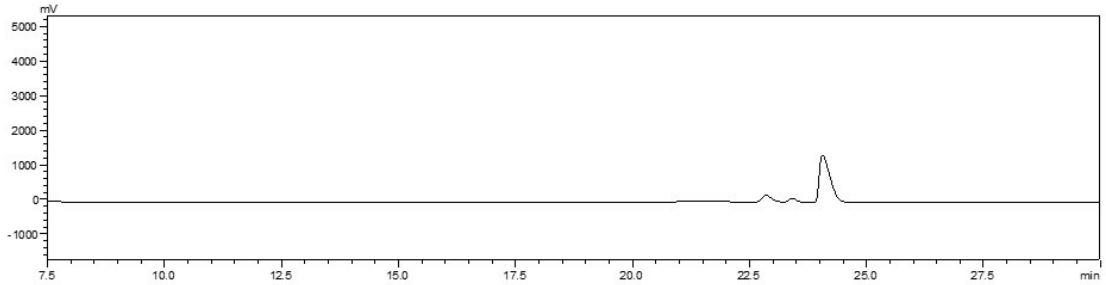
24. FAM-βA-(cyclo-IV)-MRRRC-NH_2 (11-IV)



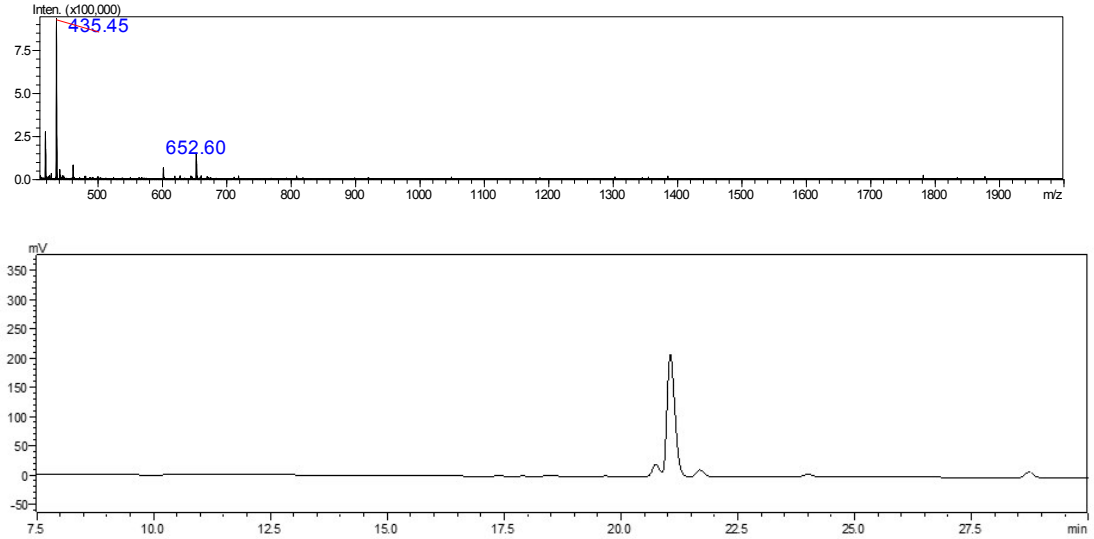
11-IVa



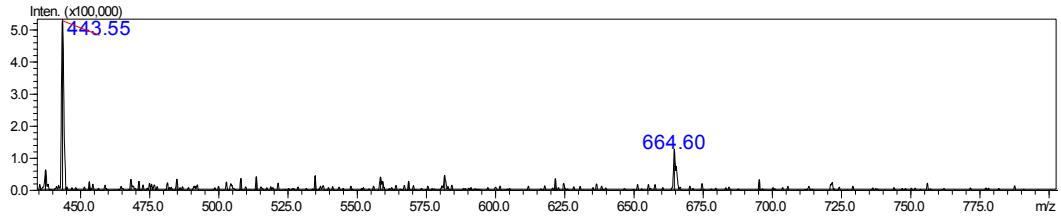
11-IVb

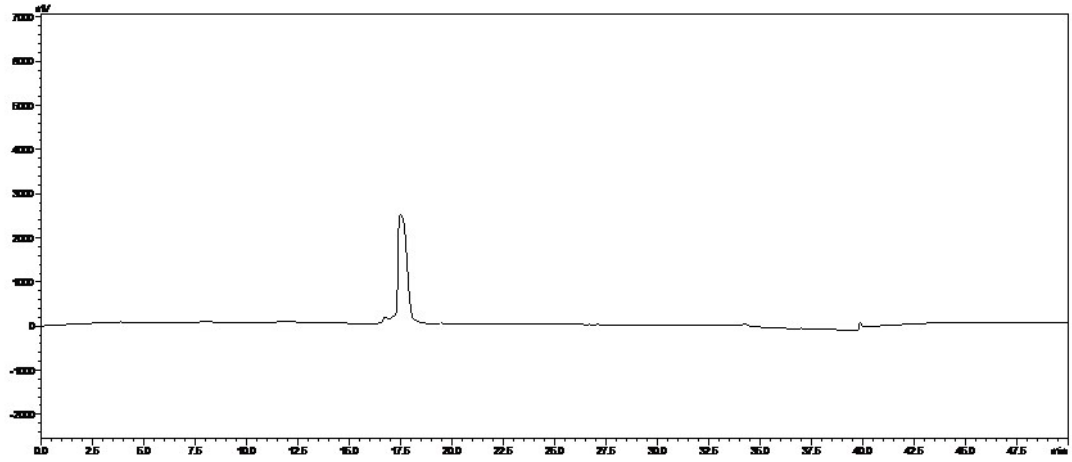


25. FAM-βA-(cyclo-V)-MRRRC-NH_2 (11-V)

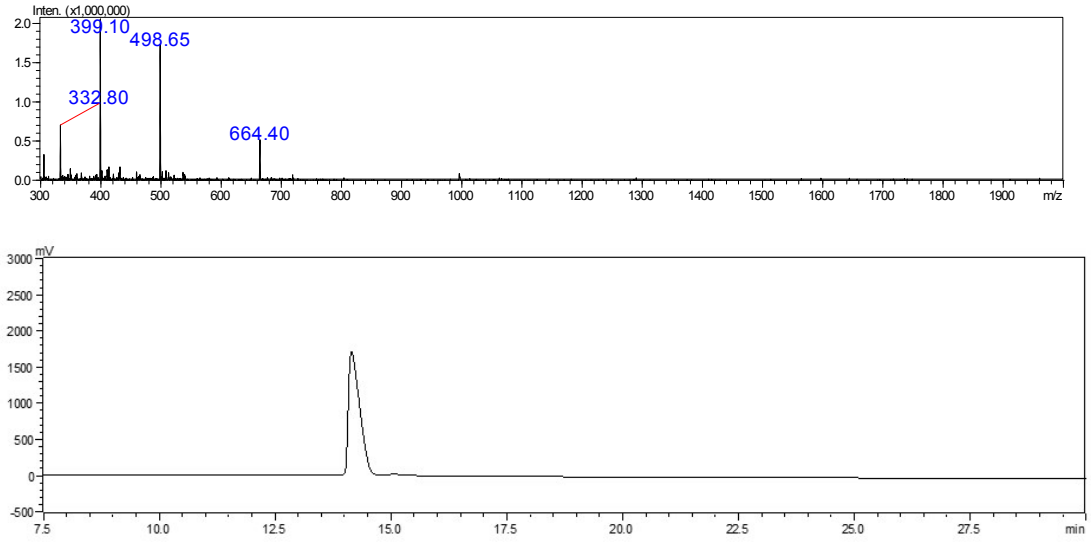


26. FAM-βA-(cyclo-VI)-MRRRC-NH_2 (11-VI)

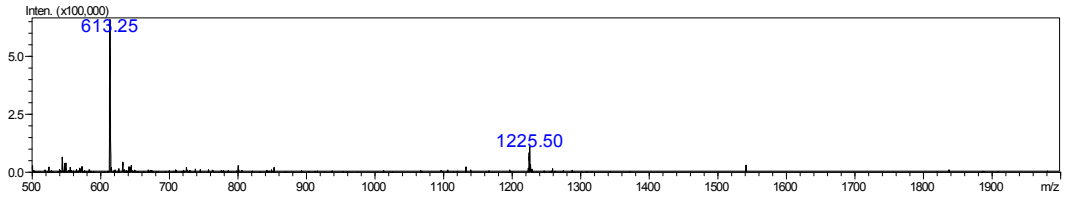


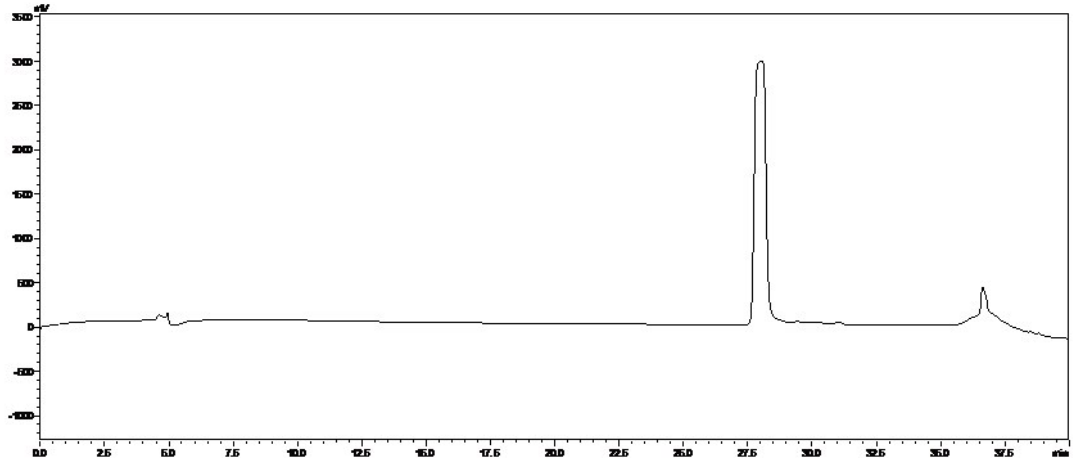


27.TAT

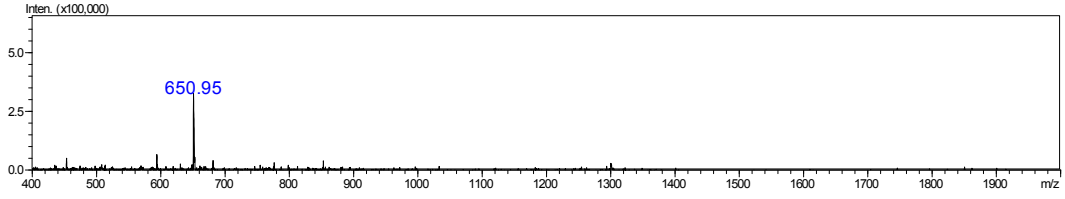


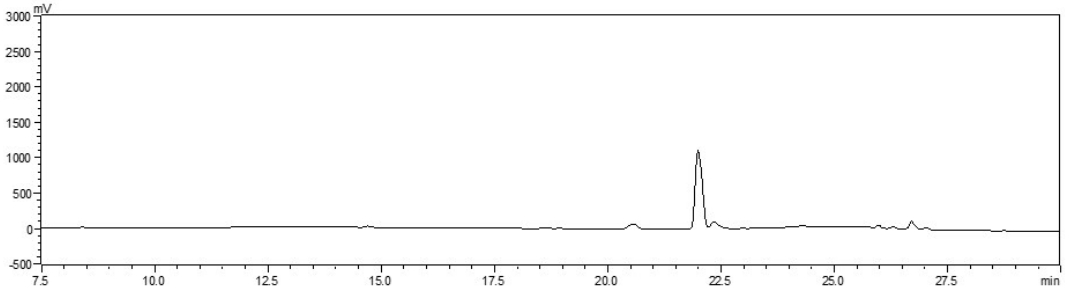
28. Ac-QSPANIYYKV-NH_2(L1)



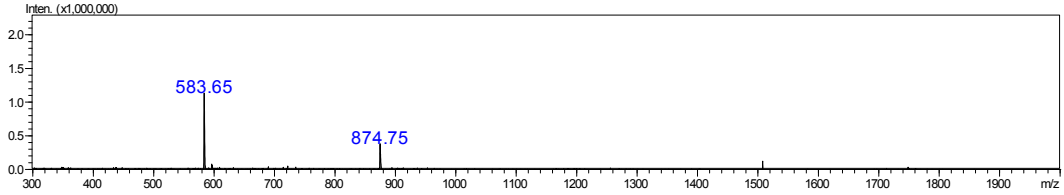


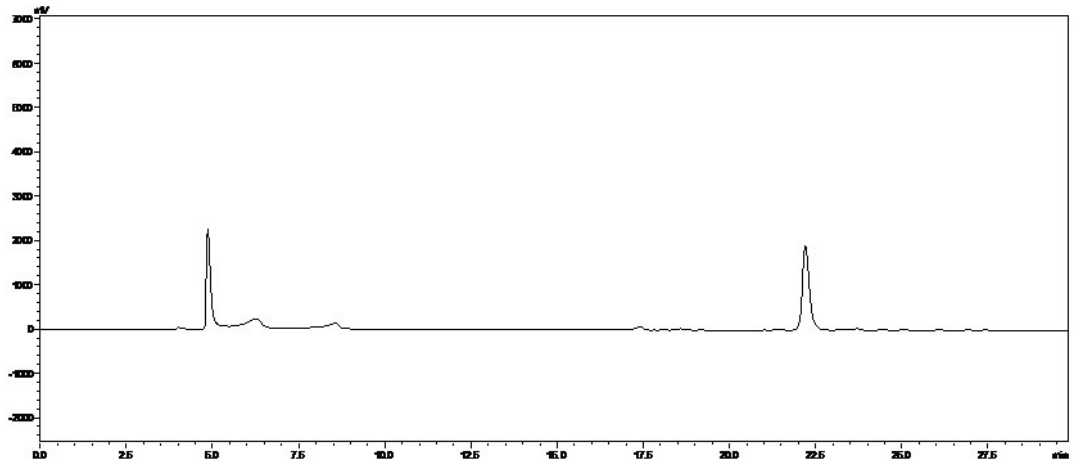
29. Ac-(cyclo-I)-QSCANIMYKV-NH_2(PD1-I)



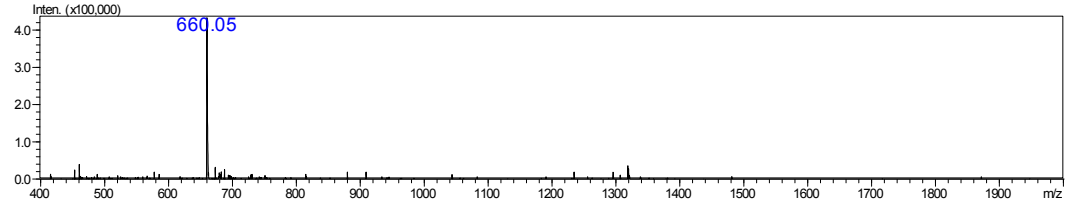


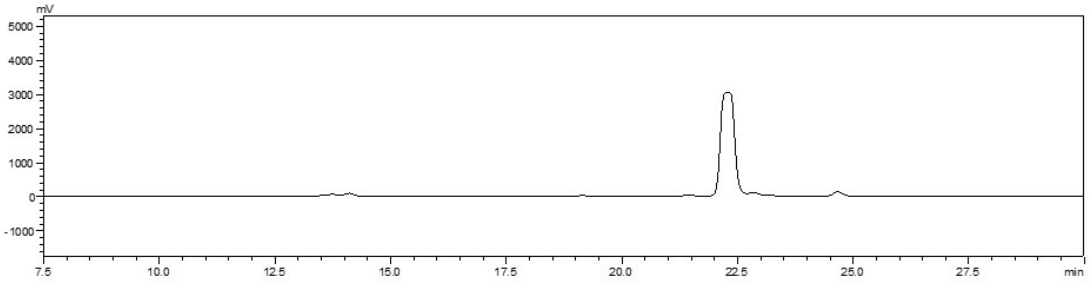
30. Ac-(cyclo-I)-QCPANMYYKV-NH_2 (PD2-I)



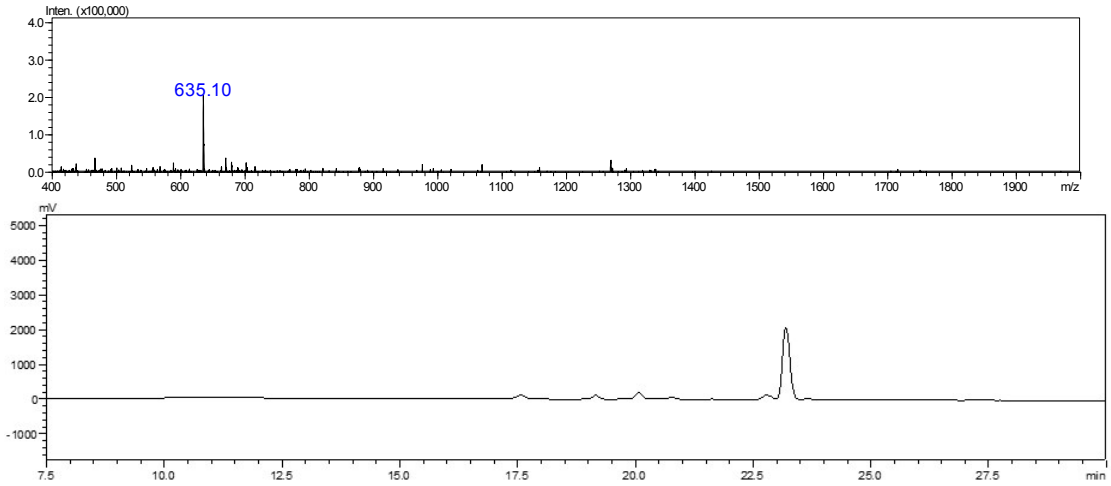


31. Ac-(cyclo-I)-CSPAMIYYKV-NH2 (PD3-I)

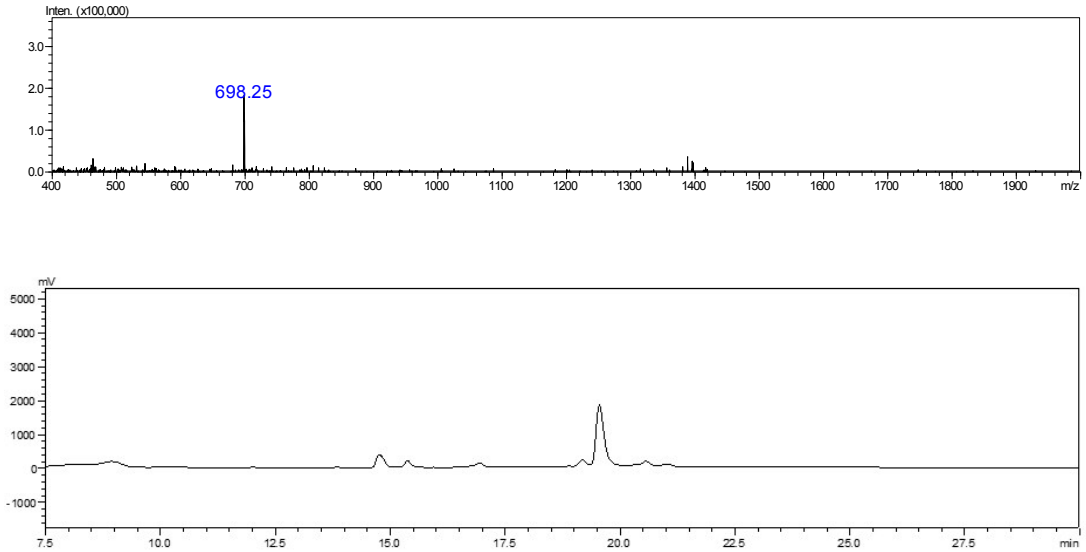




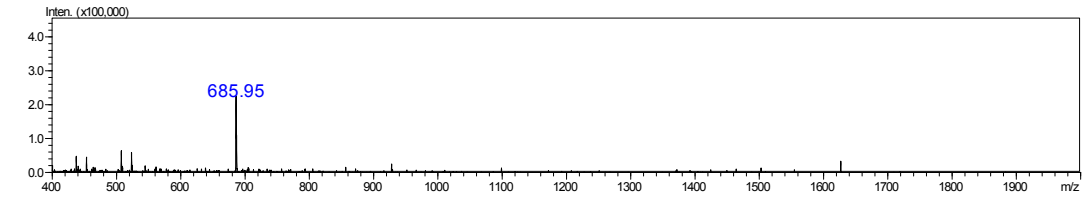
32. Ac-(cyclo-IV)-CSPAMIYYKV-NH2 (PD3-IV)

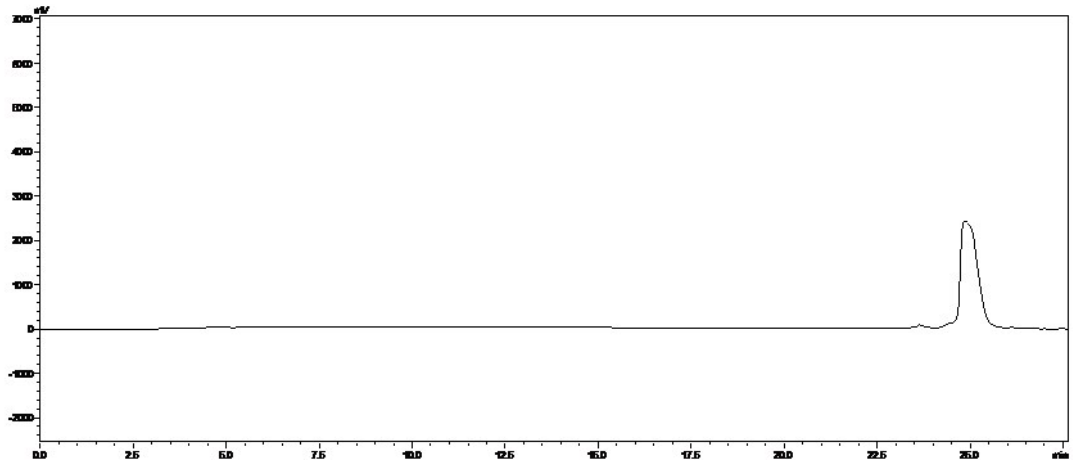


33. Ac-(cyclo-V)-CSPAMIYYKV-NH2 (PD3-V)

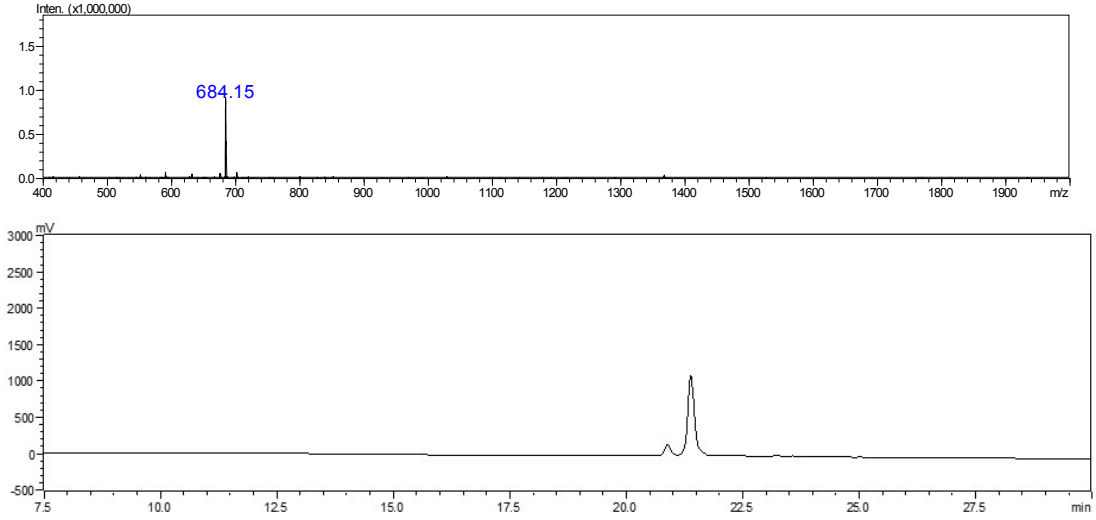


34. Ac-(cyclo-VI)-CSPAMIYYKV-NH2 (PD3-VI)

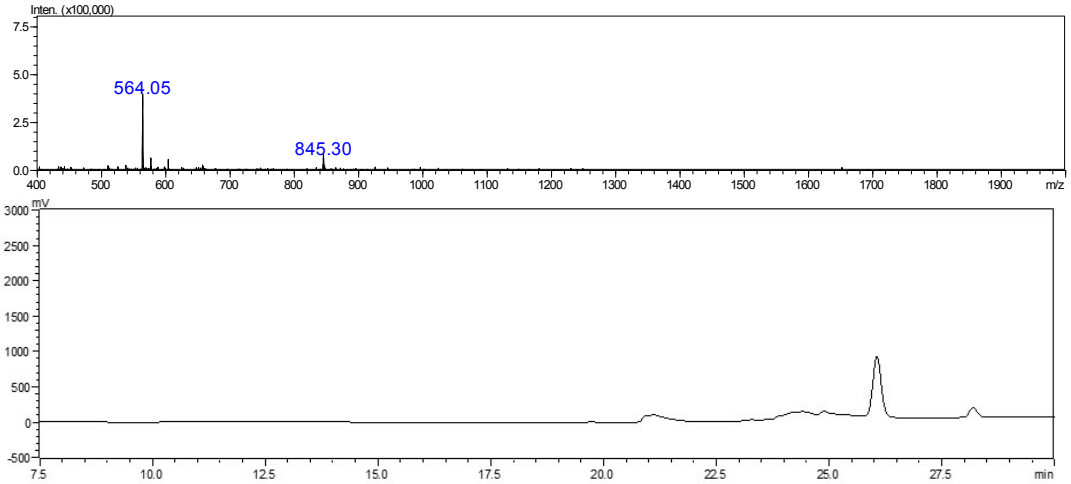




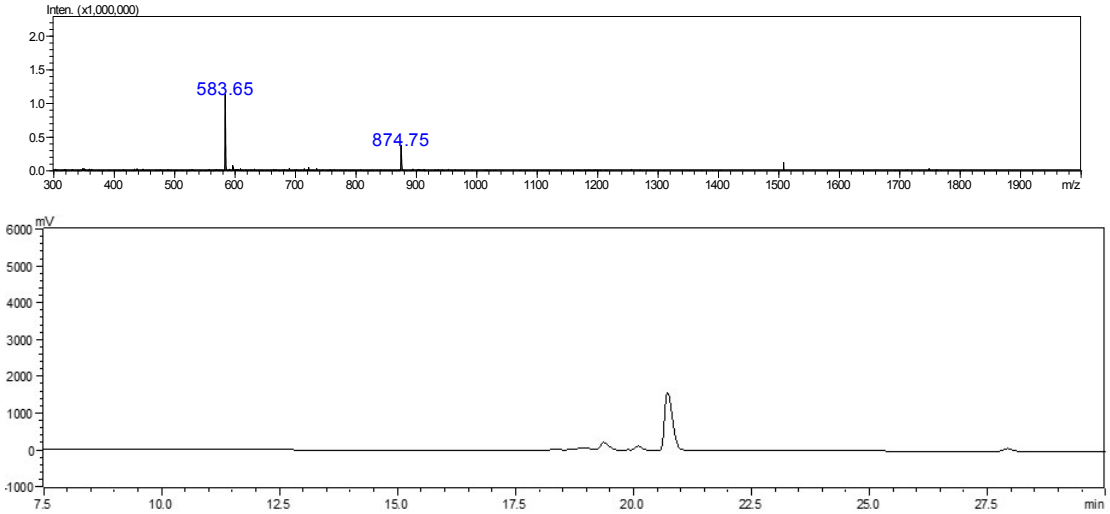
35. Ac-(cyclo-I)-CAAAMWAAQLTS-NH2 (NS)



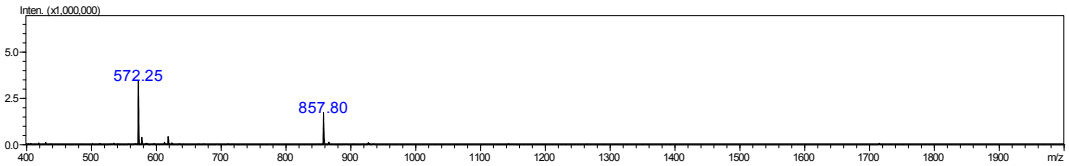
36. FAM-βA- (cyclo-I)-QSCANIMYKV-NH_2 (FAM-PD1-I)

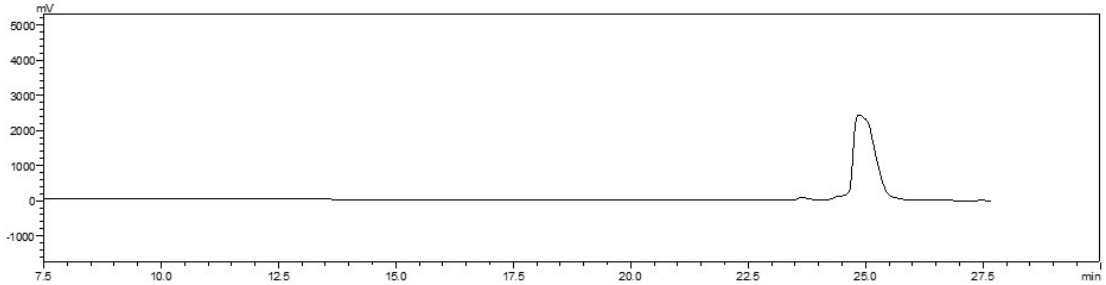


37. FAM-βA- (cyclo-I)- QCPANMYYKV-NH_2 (FAM-PD2-I)

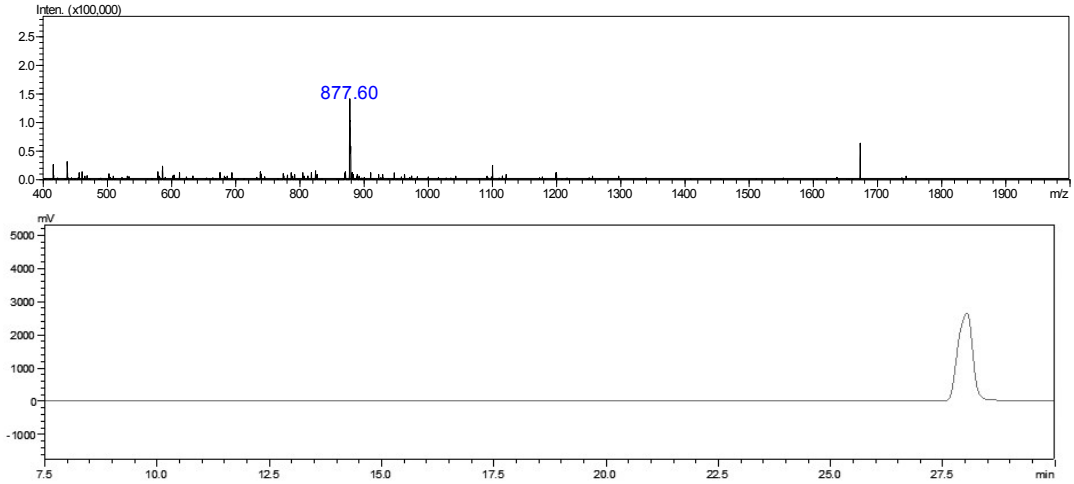


38. FAM-βA- (cyclo-I)- CSPAMIYYKV-NH_2 (FAM-PD3-I)





39. FAM-βA- (cyclo-I)- CAAAMWAAQLTS -NH_2 (FAM-NS)



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