Supplementary Information

Stapling of Unprotected Helical Peptides via Photo-Induced Intramolecular Thiol-Yne Hydrothiolation

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General Information

1. Abbreviations

Fmoc, 9-fluorenylmethyloxycarbonyl; HCTU, 2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, diisopropylethylamine; DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; MBHA, 4-methyl-benzylhydrylamine; RP-HPLC, reserved-phase high performance liquid chromatography; RT, room temperature; SPPS, solid-phase peptide synthesis; tBu, tert-butyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIS, triisopropylsilane; Trt, triphenylmethyl; Et_2O, diethyl ether; EDT, 1, 2-ethanedithiol; LC-MS, liquid chromatography–mass spectrometry; NMR, Nuclear magnetic resonance; S-AA, stapling amino acid; HPLC, high-performance liquid chromatography; DMPA, 2, 2-dimethoxy-2-phenylacetophenone; IHT-PI 659, 2-hydroxy-1-[4-(2-hydroxyethoxy)-phenyl]-2-methyl-1-propanone;

2. Materials

All solvents and reagents used for solid phase peptide synthesis were purchased from commercial suppliers including GL Biochem (Shanghai) Ltd., Shanghai Hanhong Chemical Co., J&K Co. Ltd., Shenzhen Tenglong Logistics Co. or Energy Chemical Co. and were used without further purification unless otherwise stated.

3. HPLC and Mass spectrometry

Peptides were analyzed and purified by HPLC (SHIMAZU Prominence LC-20AT) using a C18 analytic column (Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min) and a C18 semi-preparative column (Agilent Eclipse XDB-C18, 9.4 × 250 mm, 5 μm, flow rate 5 mL/min). H_2O (containing 0.1% TFA) and pure acetonitrile (containing 0.1% TFA) were used as solvents in linear gradient mixtures. LC-MS spectra were carried out on SHIMAZU LC-MS 8030 (ESI-MS).

4. Peptide Synthesis and Characterization

Peptide synthesis was performed manually on Rink Amide MBHA resin (loading capacity: 0.54 mmol/g) (GL Biochem Ltd.) by standard Fmoc-based solid-phase peptide synthesis. Generally, Rink amide AM resin was pre-swelled with DCM/NMP (1/1) for 30 min. Fmoc deprotection was performed with morpholine (50% in NMP) for 30min × 2. Then the resin was washed with NMP (5 times), DCM (5 times) and NMP (5 times). Fmoc-protected amino acids (6.0 equiv according to initial loading of the resin) and HCTU (5.9 equiv) were dissolved in NMP, followed by DIPEA (12.0 equiv). The mixture was pre-activated for 1 min and added to the resin for 1-2 h, then the resin was washed with NMP (5 times), DCM (5 times) and NMP(5 times). Upon completion of peptide assembly, peptides were N-terminally acetylated with a solution of acetic anhydride and DIPEA in NMP (1:1: 8 in volume) for 1h. Peptides were cleaved from the resin with a mixture of TFA/H_2O/EDT/TIS (94:2.5:2.5:1) for 2 h and concentrated under a stream of nitrogen. The crude peptides were then precipitated with Hexane/Et_2O (1:1 in volume) at -20 ℃, isolated by centrifugation then dissolved in water/acetonitrile, purified by semi-preparative HPLC and analyzed by LC-MS. For FAM labeled peptides, purified peptides were incubated with 5-carboxyfluorescein succinimidyl ester in a mixture of 50mM borate buffer, pH 8.5 and DMF (3:1) at room temperature overnight and then purified by semi-preparative HPLC. For synthesis of all-hydrocarbon stapled peptides, unnatural olefinic amino acids were incorporated in the peptides followed by ring closing metathesis (RCM) with 4 mg/mL Grubbs 1st generation in dichloroethane (DCE) for 2 h twice. Analytical data are shown in Supporting Table S1. Acetylated

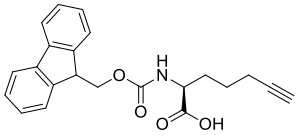
peptides were quantified by their UV absorbance. FAM labeled peptides were quantified by absorbance of 494nm with an extinction coefficient of 77,000 M^{-1} cm^{-1}.

5. NMR Spectroscopy

NMR spectra were recorded on Bruker Avance III 400MHz spectrometer in $CDCl_3$ or DMSO-d_6 or Bruker Avance III 600 MHz spectrometer with a TXI probe in phosphate buffer (pH 5.0) with 10% D_2O at 298K. Data are reported in the following order: chemical shifts are given (δ); multiplicities are indicated as br (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and app (apparent).

Experimental section

1. Synthesis of Unnatural Amino Acids

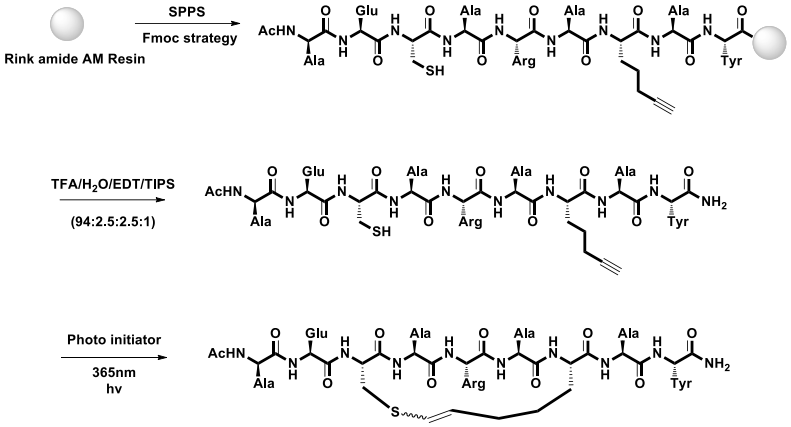


2S-{[(9H-Fluoren-9-ylmethoxy)carbonyl]amino}-6-heptynoic Acid

M_5

Alkyne bearing amino acid M_5 was synthesized according to a well-established protocol as previously reported by Papini, A. M. et al[1]. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 12.58 (br. s, COOH), 7.88 (d, J = 7.6 Hz, 2H, fluorenyl 4-H and 5-H), 7.72 (d, J = 7.2 Hz, 2H, fluorenyl 1-H and 8-H), 7.66 (d, 1H, NH), 7.41 (t, 2H, J = 7.2 Hz, fluorenyl 3-H and 6-H), 7.32 (t, 2H, J = 7.2 Hz, fluorenyl 2-H and 7-H), 4.33–4.20 (m, 3H, CH_2-O and fluorenyl 9-H), 3.91-3.97 (m, 1H, α-H), 2.78 (pseudo d, 1 H, HC-C), 2.18–2.14 (m, 2H), 1.86–1.77 (m, 1H), 1.71–1.61 (m, 1H), 1.54–1.46 (m, 2H, CH_2) ppm. ^{13}C-NMR (100 MHz, DMSO-d_6):δ = 175.71 (COOH), 155.22 (CONH), 144.26, 141.13 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 128.05, 127.50 and 125.74 (fluorenyl C-2 to C-7), 120.52 (fluorenyl C-1 and C-8), 84.66 (HC-C), 71.80 (CH_2-O), 65.67 (HC-C), 58.46 (C- α), 47.14 (fluorenyl C-9), 36.30 (C- β), 23.07 (C- γ), 18.35 (C- δ) ppm. ESI-MS: calcd for $C_{22}H_{21}NO_4$ $[M+Na]^+$ 386.14; found 386.5

2. Represent Example for Synthesis of Vinyl Sulfide Stapled Peptide 2d



SPPS was performed by standard protocol mentioned in general information. After cleavage and precipitation, the peptide was isolated by centrifugation. The resulting residue was then dried in vacuo and dissolved in degassed DMF to reach a concentration of 0.5 mM (based on resin substitution). Photo initiator (0.5 eq) was added and the solution was degassed with N_2 and subsequently irradiated by *UV* light at room temperature for 0.5-1h without agitation. DMF was then evaporated and the crude residue was dissolved in water followed by adding ether to dissolve organic byproducts then the crude peptide solution was purified by RP-HPLC mentioned above with a linear gradient of 5 to 45% acetonitrile in 50 min. Fractions containing the desired peptide were combined and lyophilized. The overall isolated yield is ~28% (based on resin substitution), which is comparable with KD lactam cross-link via macrolactamization reported by Fairlie *et al*.. All *UV* irradiations were carried out in LZC-EDU (110V/60 Hz) Luzchem Photoreactor with 10 Luzchem LZC-UVA 350nm lamps.

3. Circular Dichroism Spectroscopy (CD)

CD spectra were obtained using a Chirascan Plus Circular Dichroism Spectrometer at 20 ˚C. Peptides were dissolved in 10 mM sodium phosphate buffer (pH 7.4) at concentrations of 50 μM. Parameters used in the experiment are as followed: wavelengths from 250 to 190 nm were measured with resolution of 0.5 nm, response of 1 s, bandwidth of 1 nm, scanning speed of 20 nm/min. Each spectrum represents the average of two scans and smoothed using Pro-Data Viewer by Applied Photophysics with smooth window of 10. CD data are presented as mean residual elipticity [θ] in deg cm^2 $dmol^{-1}$. Concentrations were determined as mentioned in general information. Percent helicity was calculated based on the equation described by Arora[2]: Helicity% = $[\theta]_{222}/[\theta]_{max} \times 100$, where $[\theta]max = (-44\,000 + 250T)(1 - k/n)$ for k = 4.0 and n = number of amino acid residues in the peptide, T = 20 ˚C.

4. 2D NMR Experiments

Sample was prepared by dissolving dried peptide in phosphate buffer (pH 5.0) containing 10% D_2O at 298K. 2D NMR data were collected on a Bruker Avance III 600 MHz spectrometer with a TXI probe. Watergate pulse sequence with gradients were used for water suppression in 1D and 2D ^1H spectrum. 2D ^1H-^1H TOCSY and ROESY spectra were acquired with mixing time of 80 ms and 350 ms, respectively. The TOCSY, ROESY and COSY spectra were acquired with a 10 ppm spectra width and 2048×256 complex points. 2D ^1H-^{13}C HSQC and HMBC spectra were acquired with ^1H spectra width of 10 ppm and ^{13}C spectra width of 100 ppm, and size of 1024×400 complex points. All the 2D NMR spectra were processed by TopSpin® to final 2048×1024 complex points, and analyzed by CCPNMR software[3]. ^3J(NH-Hα) couplings were measured from 1D ^1H spectrum. Temperature dependence for amide NH chemical shifts were measured form 2D TOCSY spectra recorded at temperature ranges from 288K to 313K with a 5K interval. At each temperature, the sample were allowed to equilibrate for 15 min, and the chemical shifts were calibrated with standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

5. Molecular Dynamics Simulation

All simulations were performed by Gromacs 4.5.4[4] using the AMBER 99SB force field[5] and tip3p water model.[6] Parameters for the bonded interactions of the linker in the stapled peptide system as well as the carbon-carbon triple bond of M_5 in the linear peptide system are not available in the AMBER 99SB force field, and we thus take these parameters from the general amber force field.[7] Moreover, the partial charges of the linker were obtained from the RESP fitting to a HF/6-31G* electrostatic potential.[8] Both the initial structures of linear and stapled peptides were modeled as alpha helix by Modeller.[9] The initial structures were first solvated in a cubic box of 50×50×50 $Å^3$, and we then performed an energy minimization using steepest descent method, which was followed equilibrated by 500 ps position restrained simulations in the NPT ensemble. Finally, five production NPT simulations starting from different initial velocities were run for 100 ns each. In all these simulations, the temperature was kept at 310 K by v-rescale thermostat[10] with coupling time constant of 0.1 ps and pressure was coupled by Parrinello-Rahman method[11] at 1 bar with coupling time constant of 2 ps. The integration time-step was selected to be 2 fs. All bonds were constrained by the LINCS algorithm[12]. The long range electrostatic potentials were calculated by using the PME method[13] with the short-range cutoff at 12 Å. The cutoff of van der Waals potential was set to 11 Å. The secondary structure of the peptide was calculated by DSSP method [14].

6. Protein Expression and Purification

Plasmids were constructed by cloning DNA encoding human ER-α LBD (301-553) and ER-β LBD (259-498 with mutations C334S C369S C481S) into pET23b *via* NdeI and XhoI and overexpressed by high-density culture of *E. coli*. BL 21 (DE3) host cell without IPTG. Cultures were grown in 2YT medium and incubated at 37 ˚C till an OD600 of 0.8 and cultures were transferred to 20 ˚C for another 18 hr. Cells were harvested by centrifugation and lysed by sonication in a buffer containing 100 mM Tris-Cl pH 8.1, 300 mM KCl, 5 mM EDTA, 4 mM DTT and 1 mmol/L PMSF. Cell debris was removed by centrifugation and the supernatant was purified by flowing through a pre-equilibrated 1 mL estradiol affinity column (PDI technology) and washed with 100 μM estradiol in 20 mM Tris pH 8.1, 0.25 M NaSCN. High molecular weight species and excess salts were removed on a Superdex 200 column equilibrated in 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM DTT. Finally, the eluted ERα LBD (301-553) and ER-β LBD (259-498 with mutations C334S C369S C481S) were concentrated to 1-5 mg/ml.

7. Fluorescence Polarization Assay (FP)

Fluorescence polarization experiments were performed in 96-well plates on plate reader (Perkin Elmer, Envision, 2104 multilabel reader) at 25 ˚C with excitation at 485 nm and emission at 520 nm. Briefly, FAM-labeled peptides were mixed with increasing concentration of purified ER-α LBD (301-553) or ER-β LBD (259-498 with mutations C334S C369S C481S) in assay buffer (10 μM 17-β-estradiol, 20 mM Tris-HCl pH 8.0, 25 mM NaCl, 10% glycerol, 10 μM beta-estradiol and 1 mM TCEP) to a final peptide concentration of 10 nM. The mixture were then incubated at 4 ˚C for 1h in the dark. The binding affinity (K_d) values were determined by fitting the experiment data to log (inhibitor) vs. response - variable slope (four parameters) model in Prism.

8. Stability Assays

A. Chemical stability: Stability under acidic or basic condition was carried out by incubation of peptides in either 3% TFA or 0.1 mM NaOH aqueous buffer at room temperature. Aliquots (20 μL) were taken periodically at 0 to 24 h and was analyzed by LC-MS (Agilent ZORBAX SB-Aq: 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min, 220 nm).

B. Serum stability: Stability in rabbit serum was carried out by incubation of peptides at a final concentration of 100 μM (25% serum) at 37 ˚C. Aliquots (5 μL) were taken periodically at 0 to 12 h, and then 100 μl 12% trichloroacetic acid in H_2O/CH_3CN (1:3) was added and cooled to 4 ℃ for 30 min to precipitate serum proteins. Samples were then centrifuged at 14000 rpm for 10 min. The supernatant was analyzed by reversed-phase HPLC (Agilent ZORBAX SB-Aq: 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min, 495 nm). Peptide serum half-life was determined by plotting the ln %intact from the HPLC integral area versus time using linear regression by GraphPad Prism.

9. Hemolysis and LDH Release Assay

A. Hemolytic activity: Fresh mouse red blood cells were collected and centrifuged at 8600 rpm for 10 s. The erythrocytes were washed and resuspended in 0.9% NaCl to 10^8 /mL. A serial dilution of peptides were added and incubate at 37 ℃. After 1h incubation, erythrocytes were centrifuged and the release of hemoglobin was monitored by measuring the absorbance of supernatant at 570 nm by NanoDrop 2000c. 0.1% Triton X-100 and 0.9% NaCl were employed as positive and negative controls. Percent hemolysis was calculated according to the following equation[15]: %Hemolysis = [(O.D. 576 nm of sample - O.D. 576 nm of negative control) / (O.D. 576 nm of positive control - O.D.576 nm of negative control)]×100.

B. LDH release: LDH release was performed by using Cytotoxicity LDH Assay Kit-WST®. Briefly, 100 μL of 5 × 10^5/mL cell suspension was added to each well of the 96 well culture plate and allowed to grow overnight. The cells were then incubated with 50μL serial dilution of peptides at 37 ℃ for 2h. Lysis Buffer was added as positive control at 37 ℃ for 30min. 100μL Working Solution was then added to each well for 30min at room temperature followed by adding 50μL Stop Solution. The absorbance at 490 nm was measured by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader).

10. Flow Cytometry Analysis

HEK293T cells were seeded in 24 well culture plate. The plates were incubated with FAM-labeled peptides at concentration of either 1μM or 5μM at 37 ℃ in fetal bovine serum (FBS)-free DMEM media in the presence of 5% CO_2. After 1h incubation, peptide containing media was removed followed by washing with phosphate buffered saline (PBS). The cells were then digested with 0.25% trypsin for 10 min to remove surface binding peptides. Then the cells were washed and re-suspended in PBS buffer and analyzed by flow cytometry (FACSCalibur™). A minimum of 10,000 gated events were acquired and analyzed. Experiments were performed in triplicate.

11. Confocal Microscopy Imaging

HEK293T cells stably expressed wild-type transmembrane glycoprotein CD38 were seeded on the 0.001 % Polylysine (PLL) treated coverslip at 37 ℃ in the presence of 5% CO_2. Then cells were then incubated with FAM-labeled peptides for 3 hours. After that, peptide containing media was removed followed by washing with phosphate buffered saline (PBS) twice. Then the cells were stained with anti-CD38 polyclonal antibody (1:500) at 4 ℃ for 30 mins. The cells were then incubated with Alexa Fluor 647-conjugated donkey anti-rabbit IgG (1:1000) for 30 mins on ice to monitor the cell membrane and fixed with 4% paraformaldehyde for 15 mins. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min then washed with PBS. The coverslips were mounted onto slides and visualized by confocal laser scanning microscope.

12. Immunofluorescence

MCF-7 cells were seeded in 12 well culture plate on coverslips. The cells were grown for 48h in DMEM supplemented with 10% fetal bovine serum (FBS) until experiment. The cells were then incubated with 10 μM FAM-labeled peptide for 2h at 37 ℃ in the presence of 5% CO_2 followed by washing with PBS and fixed in 4% paraformaldehyde in PBS for 25 min at room temperature. After fixation the cells were treated with 0.25% TritonX-100 for 10 min and were blocked in 5% BSA/PBS for 1 h at room temperature followed by washing with PBS. Then the cells were incubated with Anti-estrogen receptor-α antibody (sigma) at a dilution rate of 1:100 in 3% BSA/PBS solution on water bath at 4℃ overnight. The cells were then washed with PBS and were incubated with Cy5-conjugated goat anti-rabbit IgG antibody (Santa Cruz) at a dilution rate of 1:300 for 1 h at 37 ℃. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min then washed with PBS. After that, cells were washed with PBS three times and the coverslips were mounted onto slides and visualized by confocal laser scanning microscope.

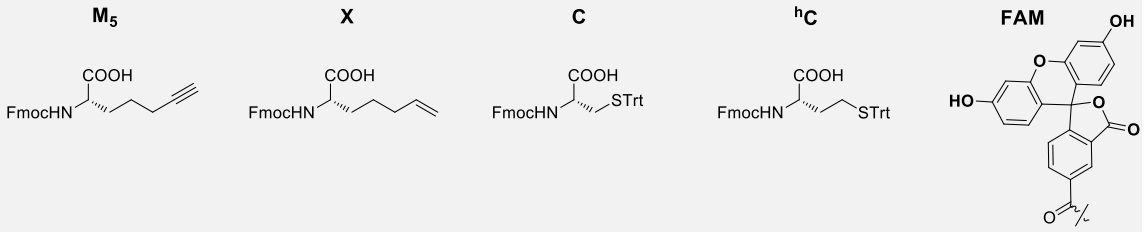
13. Cell Viability by MTT Assay

100μL of 5×10^5/mL cell suspension was placed in each well of the 96 well culture plate and allowed to grow in DMEM supplemented with 10% FBS overnight. The cells were incubated with serial dilution of peptides at 37 ℃ in FBS-free media for 4 h and then 5% FBS containing media for 20 h supplied with 5% CO_2. At the end of the peptide exposure, 20 μL of MTT reagent was added and incubated at 37 ℃ for 4 h. The absorbance of formazan product was measured at 490 nm by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader). Cells without peptide were treated as control.

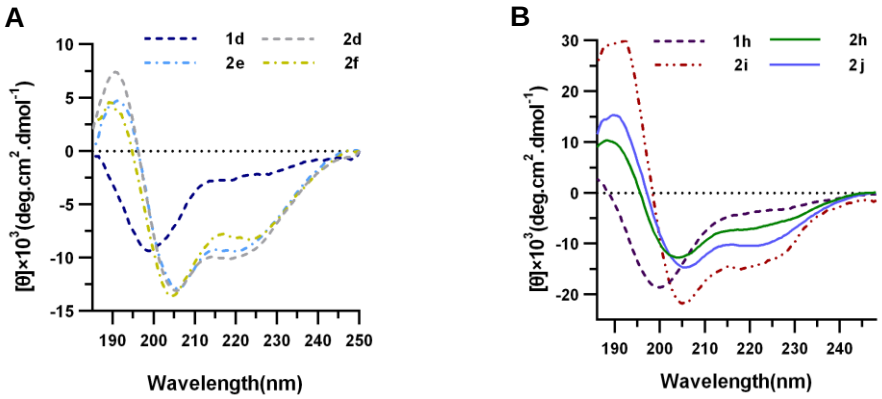
Tables and Figures

Supporting Table S1: Peptides Characterization. Calculated and Found m/z are presented as $[M+1H]^{1+}$ / $[M/2+H]^{+}$ / $[M/3+H]^{+}$

Peptide	Sequence	Chemical Formula	Calculated m/z	Found m/z
1a	Ac-AE^{h}CARAM$_5$AY-NH$_2$	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1032.5/517.0
1b	Ac-AEM$_5$ARA^{h}CAY-NH$_2$	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1033.0/516.6
1c	Ac-AEM$_5$ARACAY-NH$_2$	$C_{44}H_{67}N_{13}O_{13}S$	1018.5/509.7	1018.4/509.7
1d	Ac-AECARAM$_5$AY-NH$_2$	$C_{44}H_{67}N_{13}O_{13}S$	1018.5/509.7	1018.6/509.9
1e	Ac-EACLRQM$_5$AY-NH$_2$	$C_{49}H_{76}N_{14}O_{14}S$	1117.5/559.2	1117.5/559.4
1f	Ac-QDCAHRM$_5$SEAY-NH$_2$	$C_{56}H_{82}N_{18}O_{19}S$	1342.6/672.3	1343.7/672.3
1h	Ac-WKRAILRALLQE-NH$_2$	$C_{71}H_{120}N_{22}O_{16}$	1537.9/769.4/513.3	769.7/513.5
2a-A	Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$ -A	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1032.6/517.0
2a-B	Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$ -B	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1032.6/517.1
2b-A	Ac-(cyclo-3, 7)-[AEM$_5$ARA^{h}CAY]-NH$_2$ -A	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1032.6/517.0
2b-B	Ac-(cyclo-3, 7)-[AEM$_5$ARA^{h}CAY]-NH$_2$ -B	$C_{45}H_{69}N_{13}O_{13}S$	1032.5/516.7	1032.6
2d	Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$ -B	$C_{44}H_{67}N_{13}O_{13}S$	1018.5/509.7	1018.6/510.0
2e	Ac-(cyclo-3, 7)-[AECLRQM$_5$AY]-NH$_2$	$C_{49}H_{76}N_{14}O_{14}S$	1117.5/559.2	1117.6
2f	Ac-(cyclo-3, 7)-[QDCAHRM$_5$SEAY]-NH$_2$	$C_{56}H_{82}N_{18}O_{19}S$	1342.6/672.3	1343.3/672.6
2h	Ac-(cyclo-4, 8)-[WKRCILRM$_5$LLQE]-NH$_2$	$C_{75}H_{124}4N_{22}O_{16}S$	1621.9/811.4	811.7
2i	Ac-(cyclo-4, 8)-[WKRXILRXLLQE]-NH$_2$	$C_{77}H_{128}N_{22}O_{16}$	1617.9/809.4	809.9
2j	Ac-(cyclo-3, 7)-[WKCRILM$_5$RLLQE]-NH$_2$	$C_{75}H_{124}N_{22}O_{16}S$	1621.9/811.4	811.6
1g-FAM	Ac-WK(FAM)RSILRM$_5$LLQE-NH$_2$	$C_{75}H_{124}N_{22}O_{17}$	1962.9/982.5/655.3	982.9/655.6
1h-FAM	Ac-WK(FAM)RAILRALLQE-NH$_2$	$C_{92}H_{130}N_{22}O_{22}$	1894.9/947.9/632.3	948.8/633.0
2h-FAM	Ac-(cyclo-4, 8)-[WK(FAM)RCILRM$_5$LLQE]-NH$_2$	$C_{96}H_{134}N_{22}O_{22}S$	1979.9/990.4/660.6	990.8/660.8



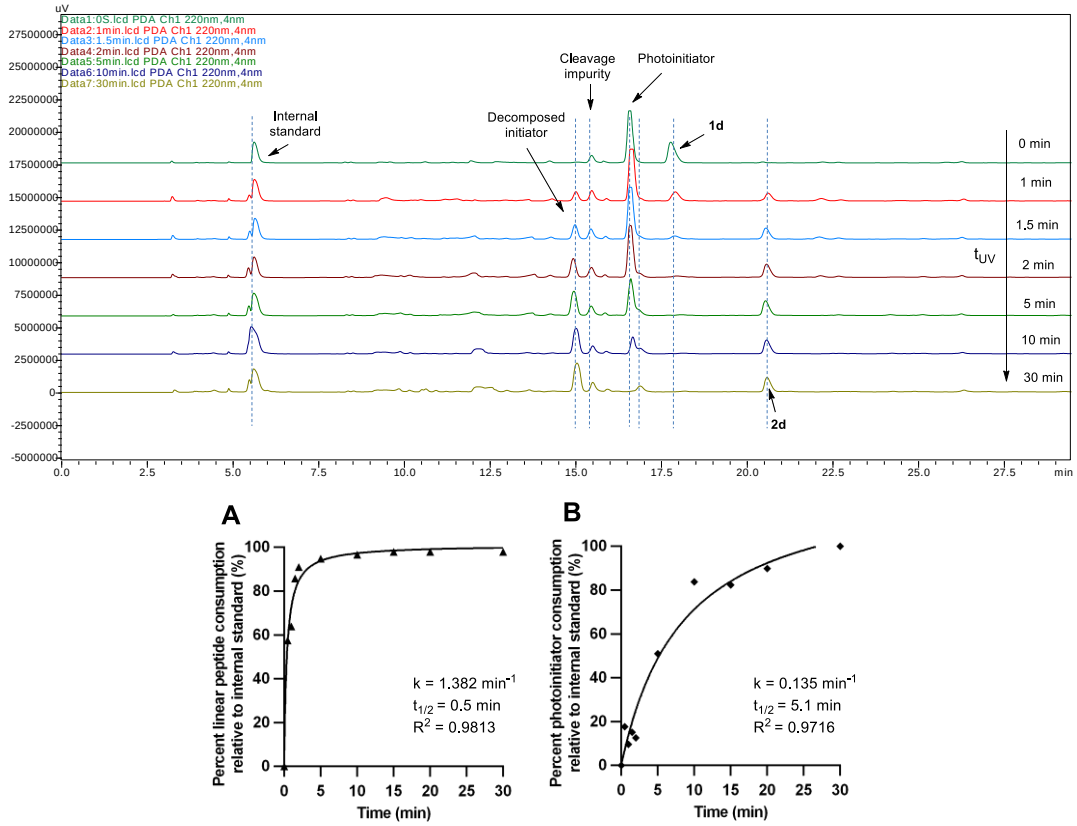
Circular Dichroism Spectra (CD)



Peptide	Sequence	Helicity (%)
1d	Ac-AECARAM$_5$AY-NH$_2$	15
2d	Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$	46
2e	Ac-(cyclo-3, 7)-[AECLRQM$_5$AY]-NH$_2$	44
2f	Ac-(cyclo-3, 7)-[QDCAHRM$_5$SEAY]-NH$_2$	39
1h	Ac-WKRAILRALLQE-NH$_2$	17
2h	Ac-(cyclo-4, 8)-[WKRCILRM$_5$LLQE]-NH$_2$	30
2i	Ac-(cyclo-4, 8)-[WKRXILRXLLQE]-NH$_2$	68
2j	Ac-(cyclo-3, 7)-[WKCRILM$_5$RLLQE]-NH$_2$	44

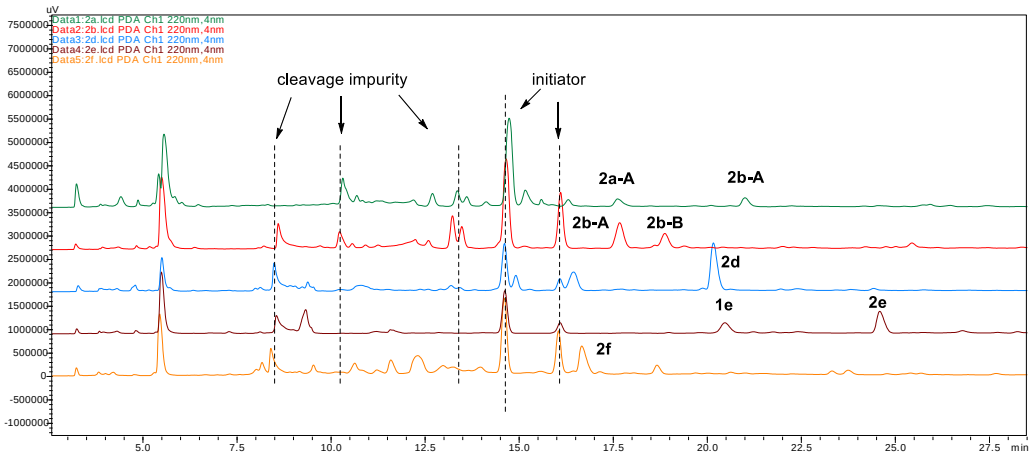
Supporting Figure S1: Circular Dichroism Spectra (CD). A) CD spectra of peptide **1d**, **2d-2f**; B) CD spectra of peptide**s** targeting estrogen receptor. All hydrocarbon stapled peptide **2i** displayed better helical induction than vinyl sulfide stapled peptides (in 10mM phosphate buffer, pH 7.4, 20 ℃). Percent helicity was calculated as mentioned in the experimental section.

Reaction Kinetics



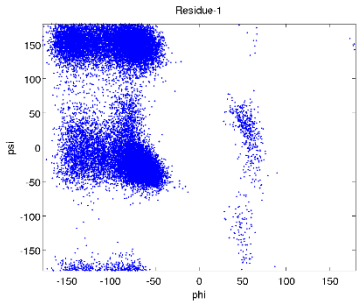
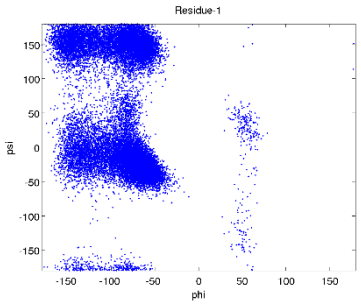
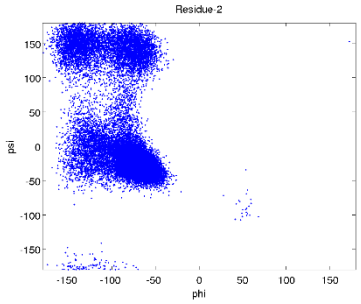
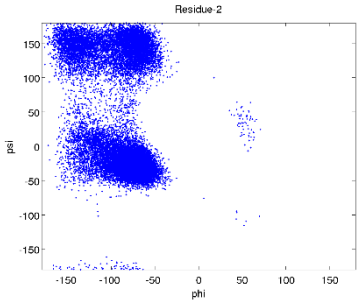
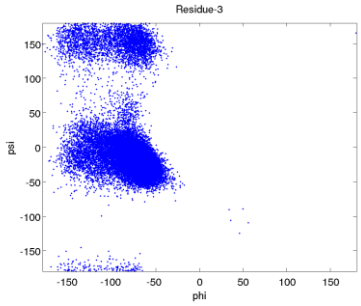
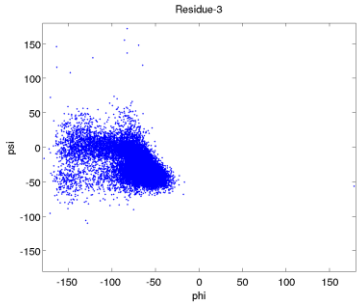
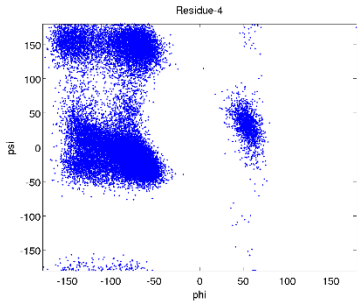
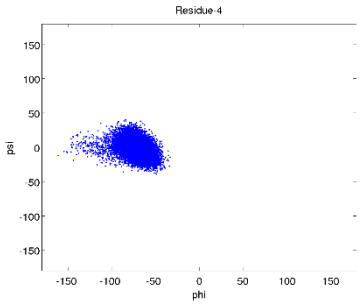
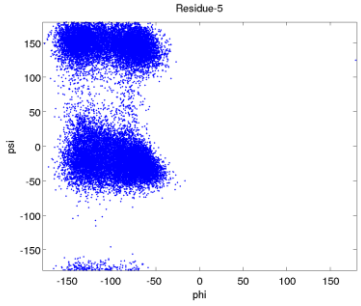
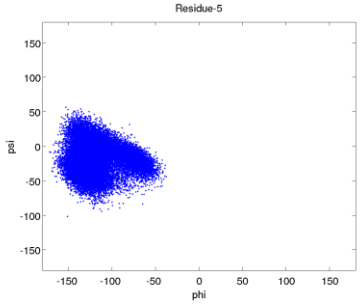
Supporting Figure S2: HPLC trace of crude reaction mixture of 2d after *UV* irradiation at different time interval. A) Percent linear peptide consumption as a function of time for intramolecular thiol-yne reaction of **1d**; B) Percent photoinitiator IHT-PI 659 consumption as a function of time. Reactant concentration was 0.5mM and 0.5eq photoinitiator (Calculated based on initial resin loading capacity). Reaction rate constant and half-life was determined by fitting curve with one-phase decay equation in GraphPad Prism.

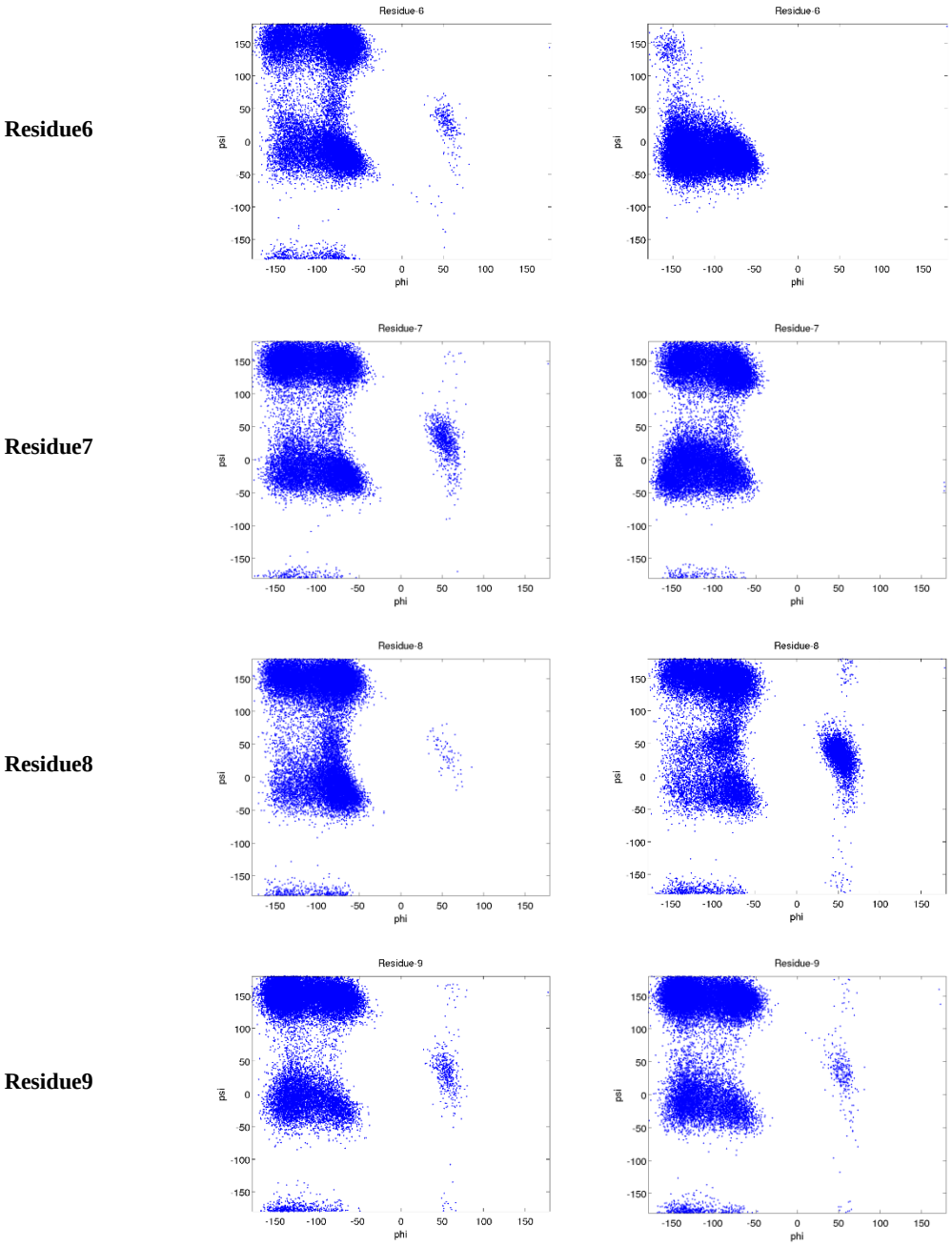
HPLC Traces of Crude Reaction Mixtures



Supporting Figure S3: HPLC traces of crude reaction mixture of 2a, 2b, 2d, 2e, 2f after *UV* irradiation. (*UV* irradiation was performed directly after cleavage and precipitation without further purification in the presence of 0.5eq photoinitiator IHT-PI 659 (Calculated based on initial resin loading capacity). Despite polar impurities associated with cleavage, photoinitiator or decomposed photoinitiator, the cyclized products with longer retention times were easily purified.)

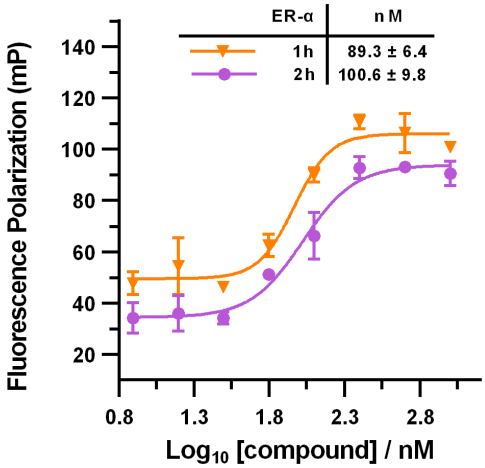
Molecular Dynamics Simulation

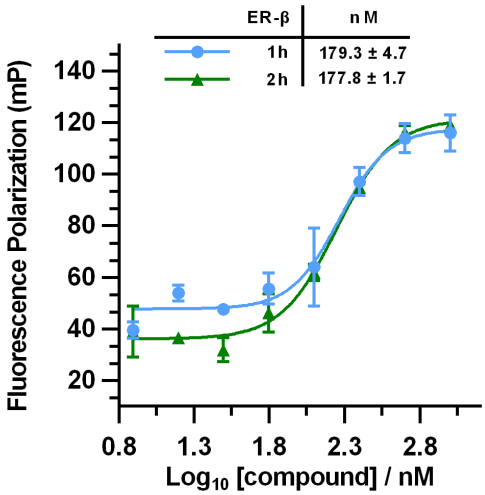
Peptide	1d	2d
Residue1		
Residue2		
Residue3		
Residue4		
Residue5		



Supporting Figure S4: Comparison of Ramachandran plots for each residue of peptide 1d and 2d derived from molecular dynamics simulation.

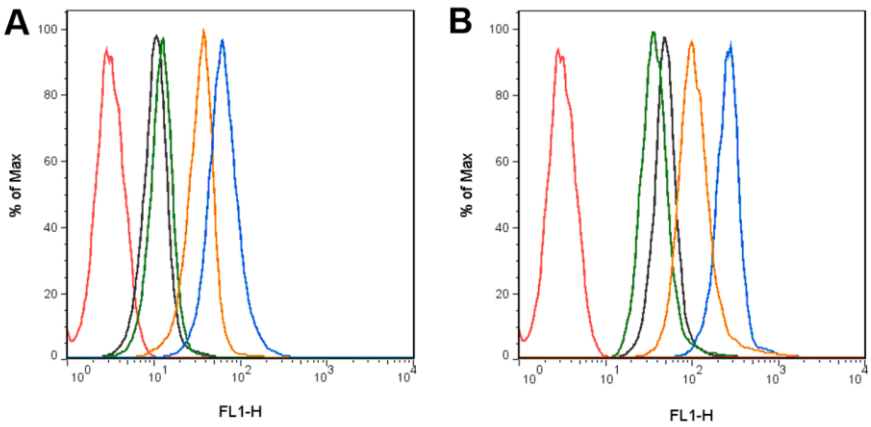
Fluorescence Polarization Assay (FP)



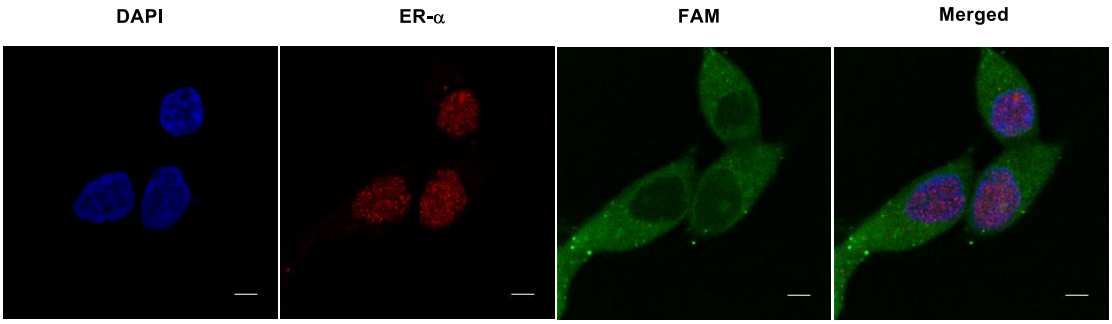


Supporting Figure S5: Fluorescence Polarization Assay (FP). Binding affinity of FAM-labeled peptide derivatives with ER-α/ER-β, respectively. mP, mean ± s. d., n=2. Measurements were performed in triplicates.

Flow cytometry analysis and immunofluorescence

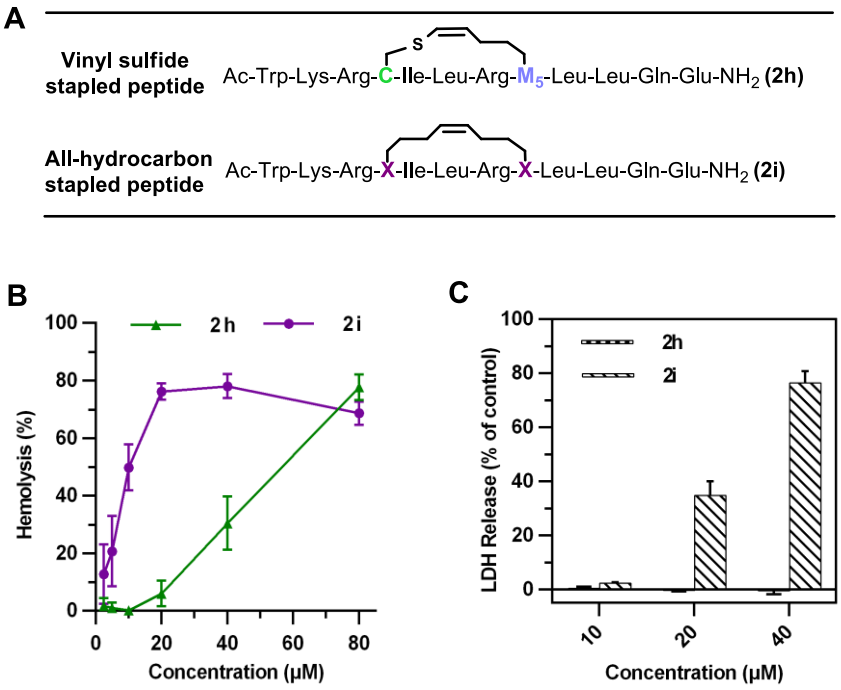


Supporting Figure S6: Flow cytometry analysis. Flow cytometry analysis of HEK293T cells treated with FAM-labelled peptides 1μM (A) and 5 μM (B) at 37 ℃ for 1h (**blank**-red, **1h-FAM**-black, **1g-FAM**-green, **TAT**-yellow, **2h-FAM**-blue). TAT: FITC labelled protein transduction domain of the HIV Tat protein (GRKKRRQRRR).



Supporting Figure S7: Immunofluorescence. Immunofluorescence of ER-α in MCF-7 cells treated with 10μM FAM-labelled peptide **2h-FAM** at 37 ℃ for 2h (in green). Cells were incubated with Anti-estrogen receptor-α antibody followed by Cy5-conjugated goat anti-rabbit IgG antibody (in red). Nuclei were labeled with DAPI (in blue). Scale bar, 10 μm.

Hemolysis and LDH release assay



Supporting Figure S8: Hemolysis and LDH release assay. A) Peptides used in this study. X refers to olefinic amino acid (see Table S1 for chemical structure); B) Hemolysis of all hydrocarbon stapled peptide **2i** and vinyl sulfide stapled peptide **2h**. Peptide **2i** displayed strong hemolytic activity. Percent hemolysis, mean ± s. d., n=2; C) LDH release from Hela cells for 2 h. Peptide **2i** displayed strong membrane toxicity. Percent LDH release, mean ± s. d., n=2.

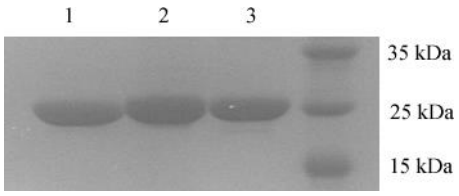
SDS-PAGE

ER-alpha-NdeI-301:GTGTACACATATGtctaagaagaacagcctggccttgt

ER-alpha-XhoI-553:Ccctcgagttaagtgggcgcatgtaggcggt

ER-beta-NdeI-259:GTGTACACATATGctgctggacgccctga

ER-beta-XhoI-498:Ccctcgagttagtgggcattcagcatct



1: ER-beta(27.05 kDa); 2,3: ER-alpha (28.86 kDa)

Supporting Figure S9: Plasmid constructs and SDS-PAGE gel for ER-α LBD and ER-β LBD.

2D NMR

Supporting Table S2: ^{1}H-NMR chemical shifts (δ, ppm) and $^{3}J_{NH\text{-}\alpha H}$ (Hz) for peptide 1d and 2d in phosphate buffer (pH 5.0) with 10% D_2O at 298K

Peptide **1d**

No.	Residue	NH	Hα	Hβ	Hγ	Hδ
1	Ala	8.14	-	1.34	-	-
2	Glu	8.49	4.24	1.96, 2.03	2.30	-
3	Cys	8.26	4.60	3.18, 2.95	-	-
4	Ala	8.21	4.41	1.34	-	-
5	Arg	8.13	4.22	1.79, 1.73	1.59	3.14
6	Ala	8.27	4.25	1.34	-	-
7	M5	8.06	4.17	1.49	2.17	1.74
8	Ala	8.03	4.19	1.23	-	-
9	Tyr	7.88	4.45	3.02, 2.89	-	-

Peptide **2d**

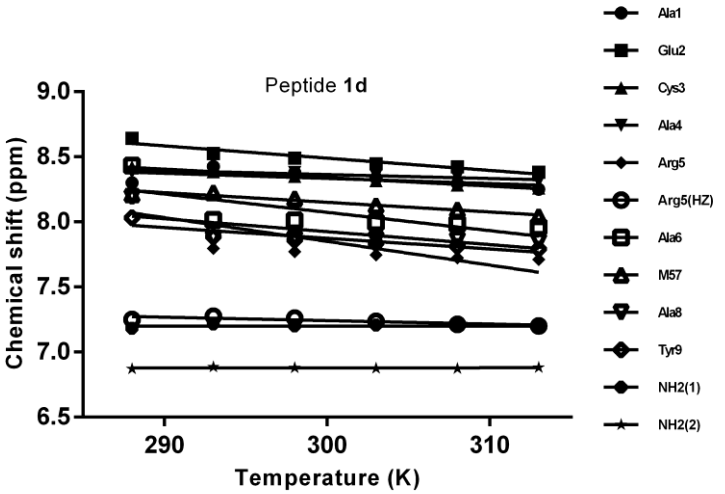
No.	Residue	NH	Hα	Hβ	Hγ	Hδ
1	Ala	8.29	4.17	1.36	-	-
2	Glu	8.39	4.19	1.98	2.38	-
3	Cys	8.26	4.33	3.03, 3.10	-	-
4	Ala	8.29	4.07	-	-	-
5	Arg	7.68	4.18	1.78	1.57	3.15
6	Ala	7.91	4.19	1.36	-	-
7	M5	8.08	4.11	1.33	2.03, 2.10	1.68, 1.59
8	Ala	7.78	4.17	1.25	-	-
9	Tyr	7.77	4.44	3.05, 2.91	-	7.11

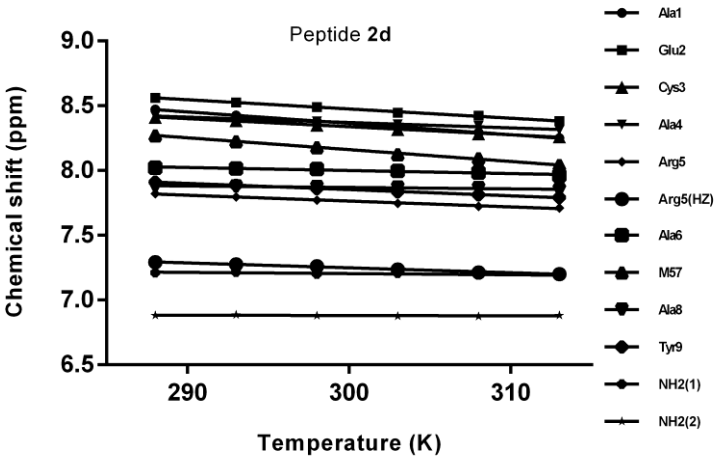
$^{3}J_{NH\text{-}\alpha H}$ (Hz)	Ala1	Glu2	Cys3	Ala4	Arg5	Ala6	$M_5$7	Ala8	Tyr9
1d	*	6.6	*	7.2	*	*	6.0	9.6	7.8
2d	4.2	6.6	4.8	4.2	6.0	5.4	6.6	*	*

*Not determined due to peak overlap

Supporting Table S3 and Figure S10: Temperature dependence for amide NH chemical shifts

Δδ/T(ppb/K)	Ala1	Glu2	Cys3	Ala4	Arg5	Ala6	$M_5$7	Ala8	Tyr9
1d	-3.9	-9.4	-6.5	-3.0	-18.2	-14.0	-7.4	-9.9	-8.2
2d	-8.8	-7.1	-6.2	-4.1	-4.5	-2.3	-9.1	-1.0	-4.8



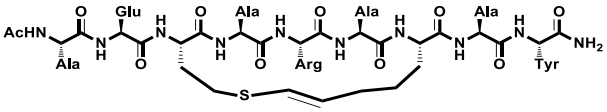


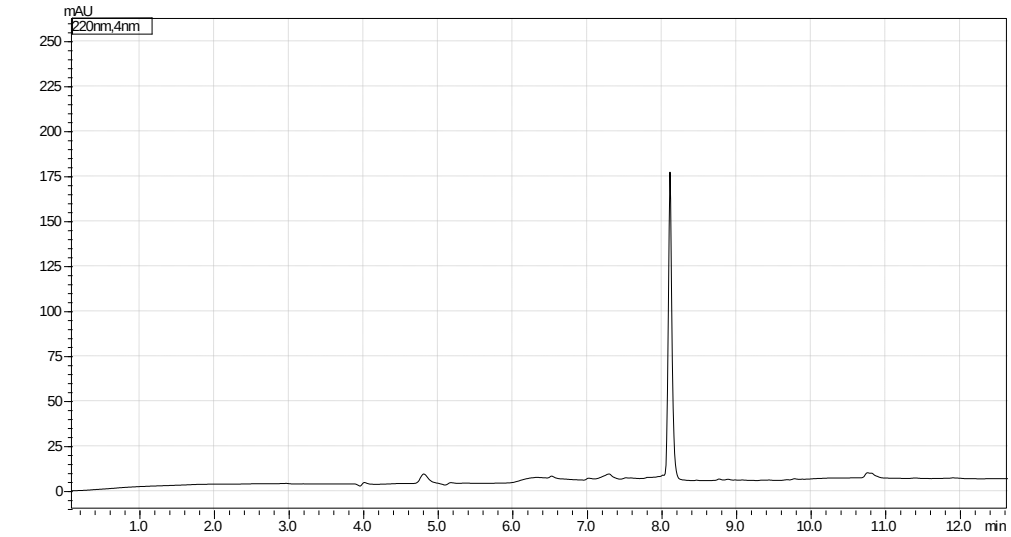
Appendix

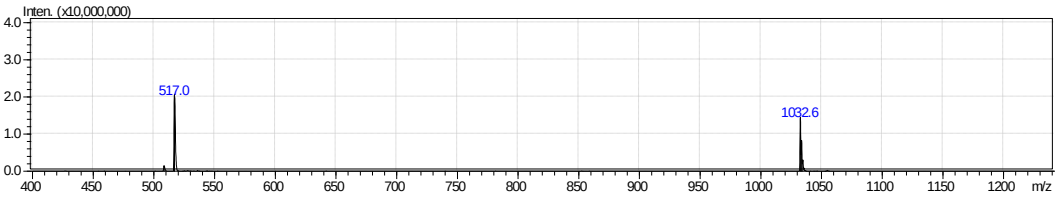
HPLC Traces and MS Spectra

2a-A

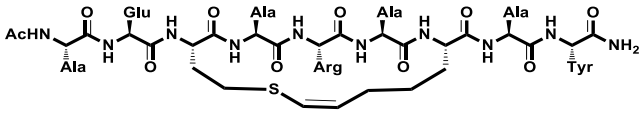
Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$ -A

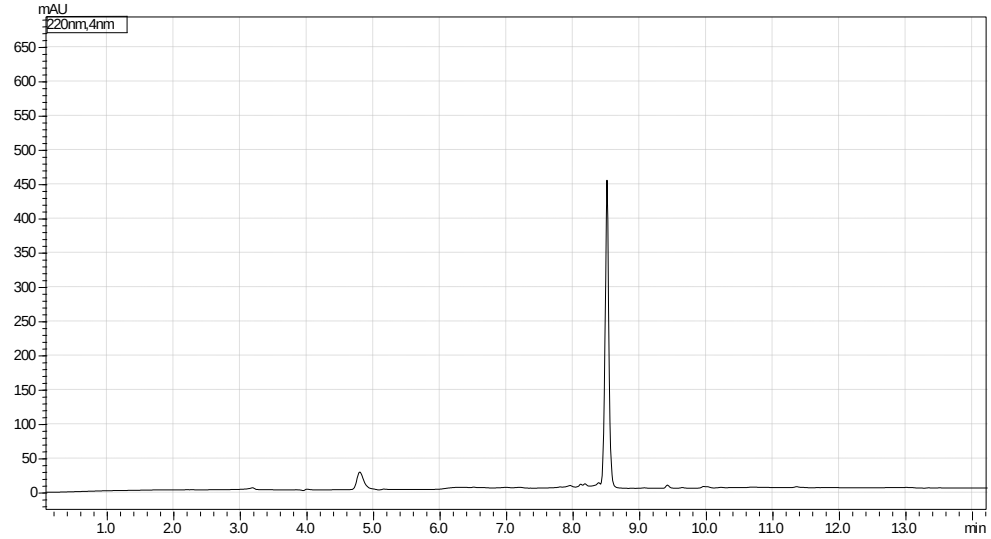


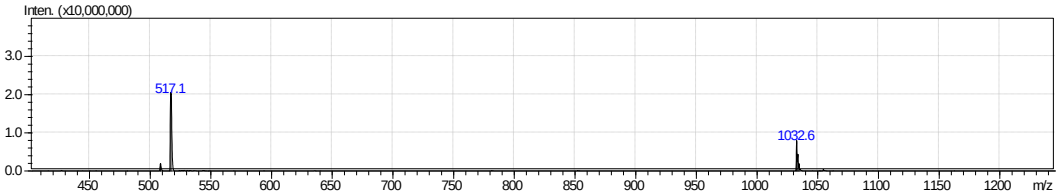




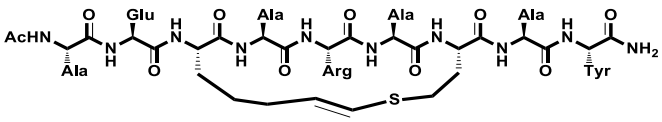
2a-B **Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$ -B**

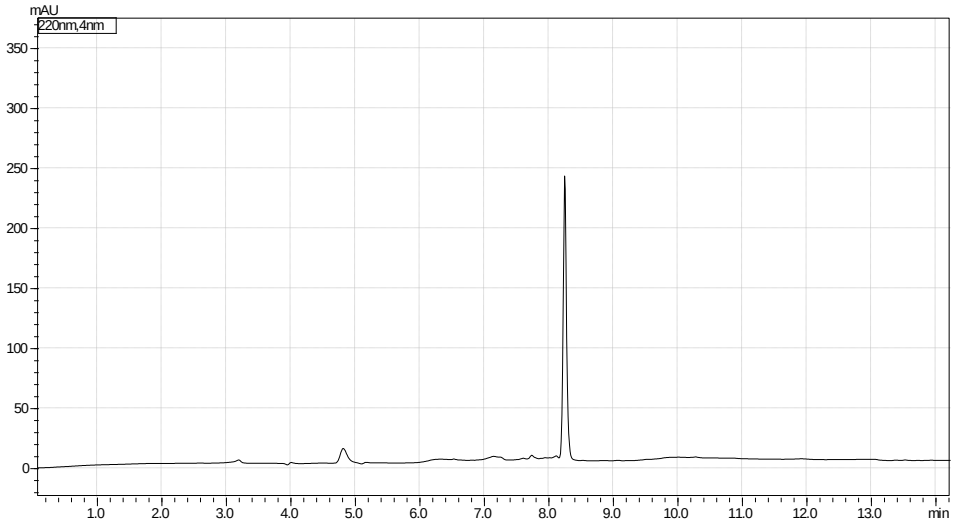


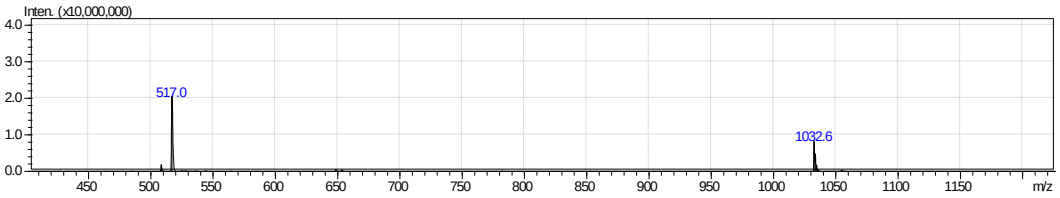




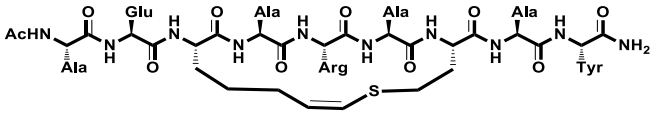
2b-A **Ac-(cyclo-3, 7)-[AEM$_5$ARA^{h}CAY]-NH$_2$ -A**

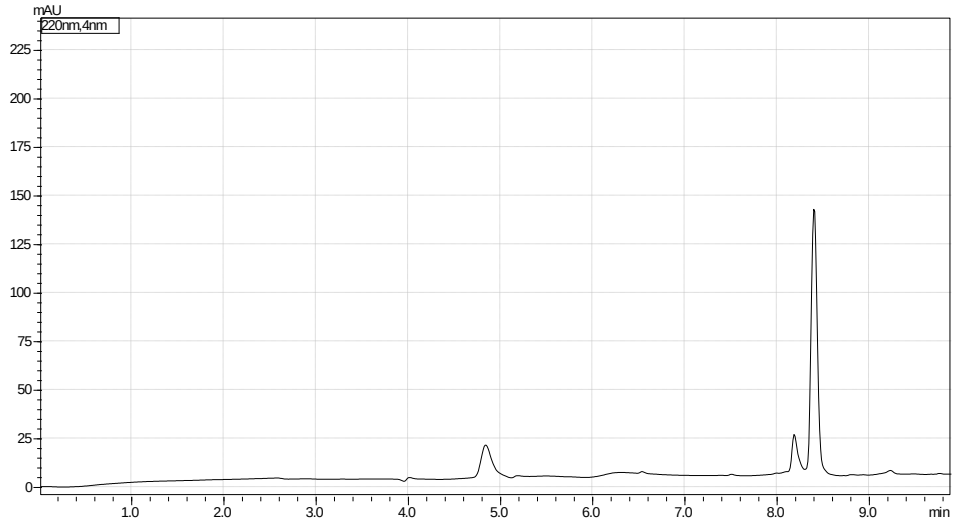


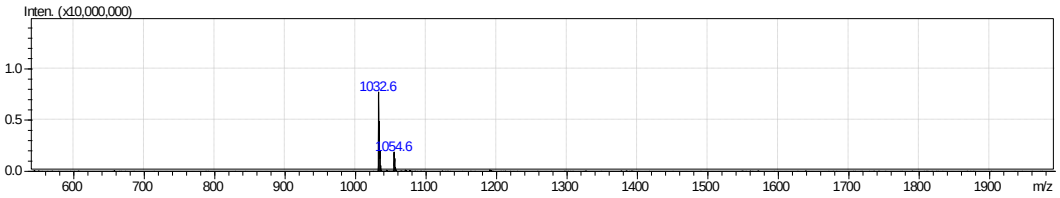




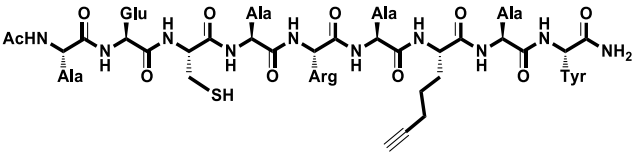
2b-B **Ac-(cyclo-3, 7)-[AEM$_5$ARA^{h}CAY]-NH$_2$ -B**

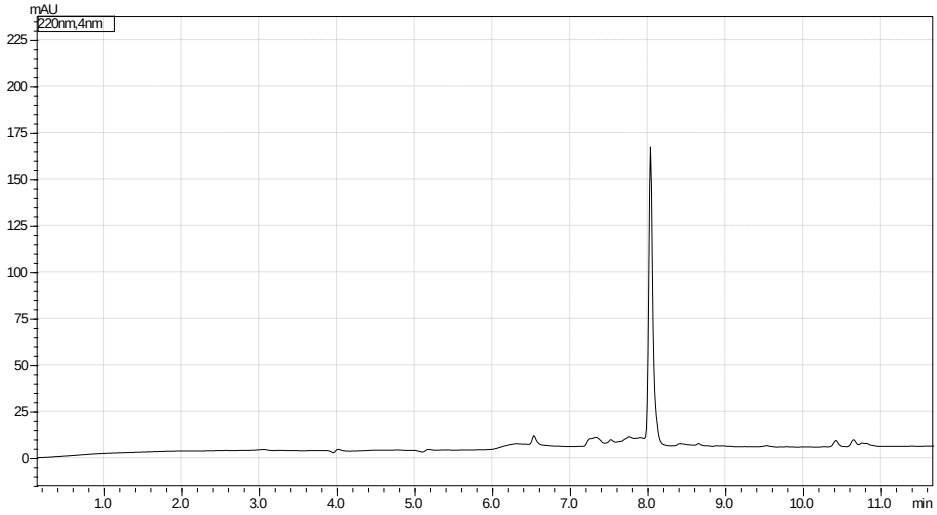


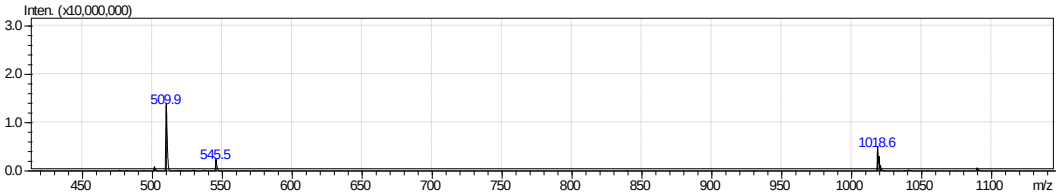




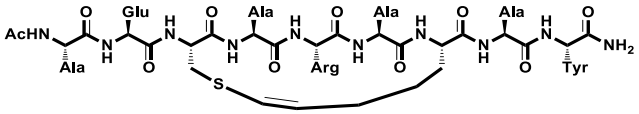
1d **Ac-AECARAM$_5$AY-NH$_2$**

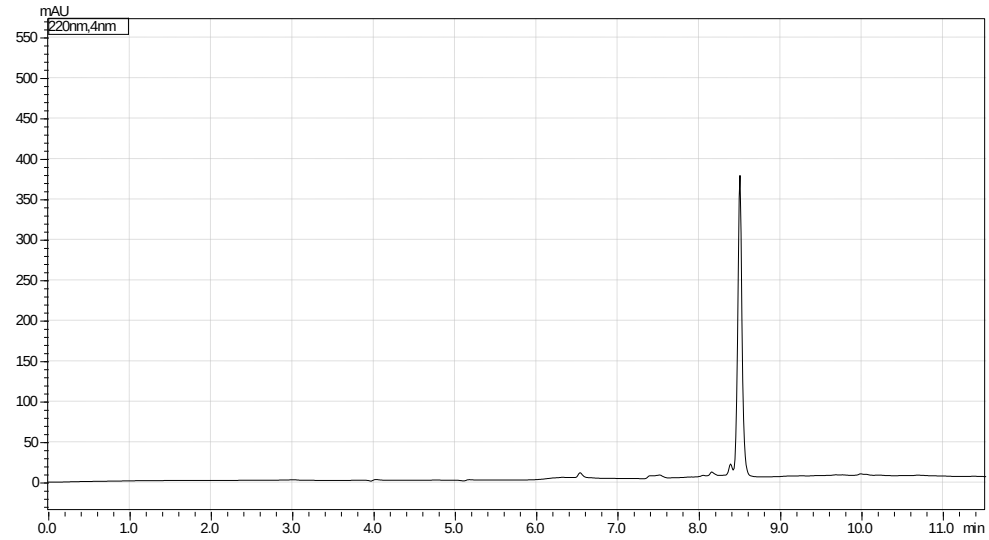


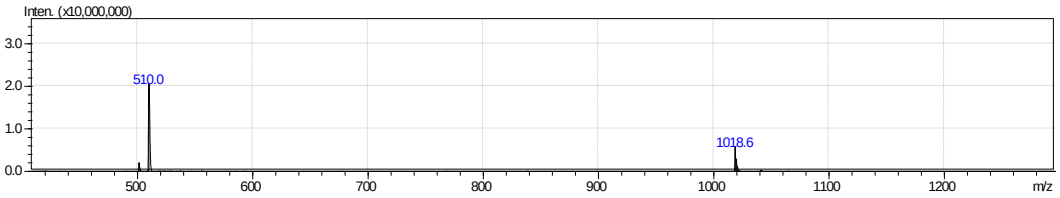




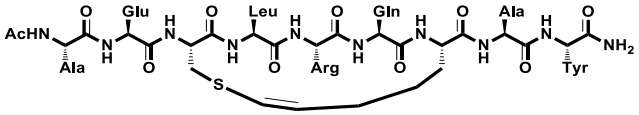
2d **Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$**

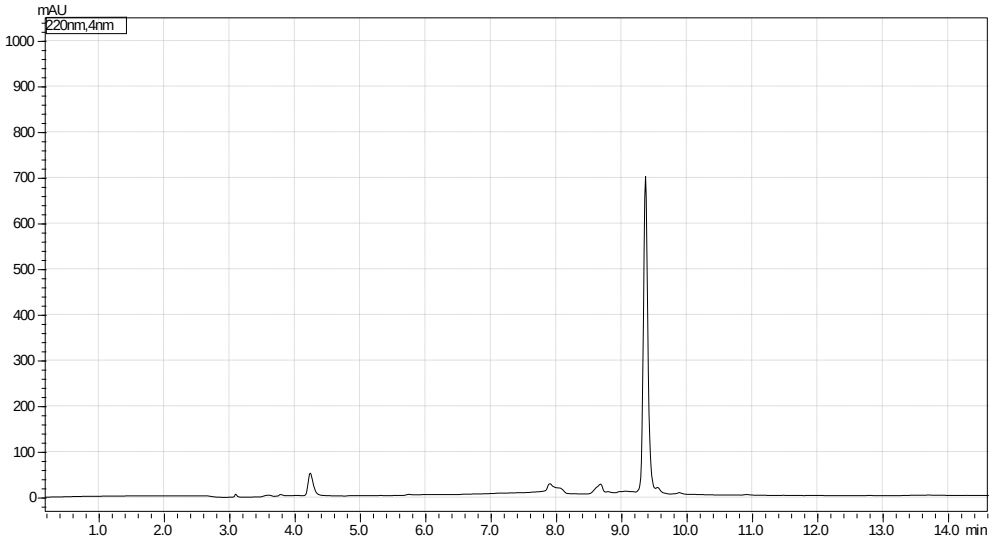


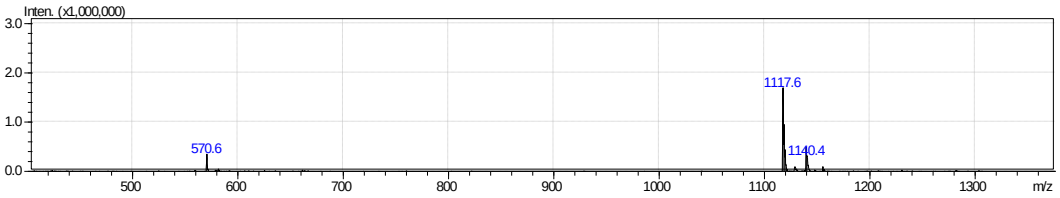




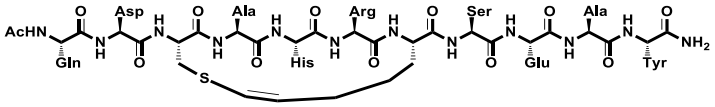
2e **Ac-(cyclo-3, 7)-[EACLRQM$_5$AY]-NH$_2$**

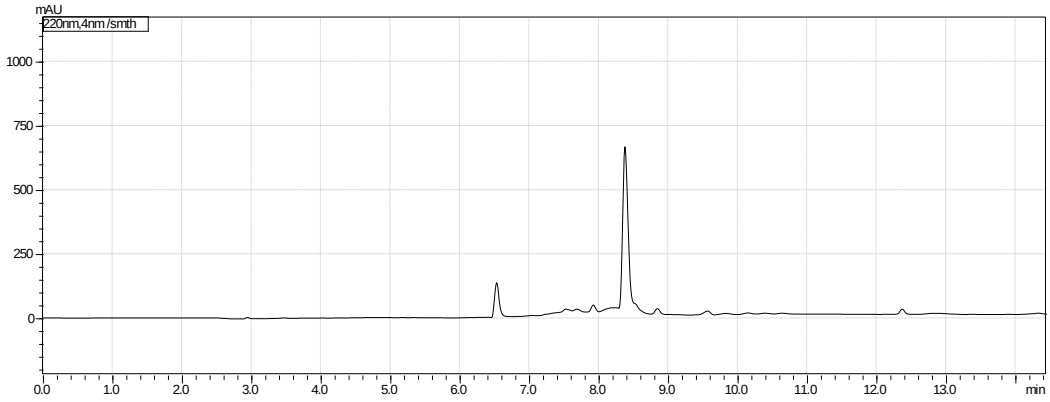


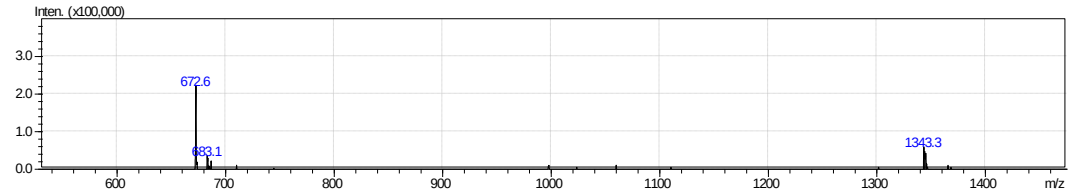




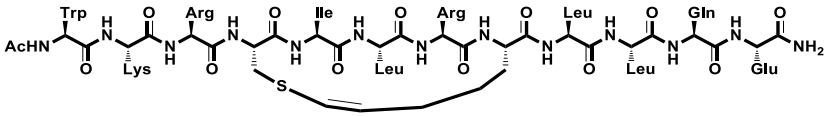
2f **Ac-(cyclo-3, 7)-[QDCAHRM$_5$SEAY]-NH$_2$**

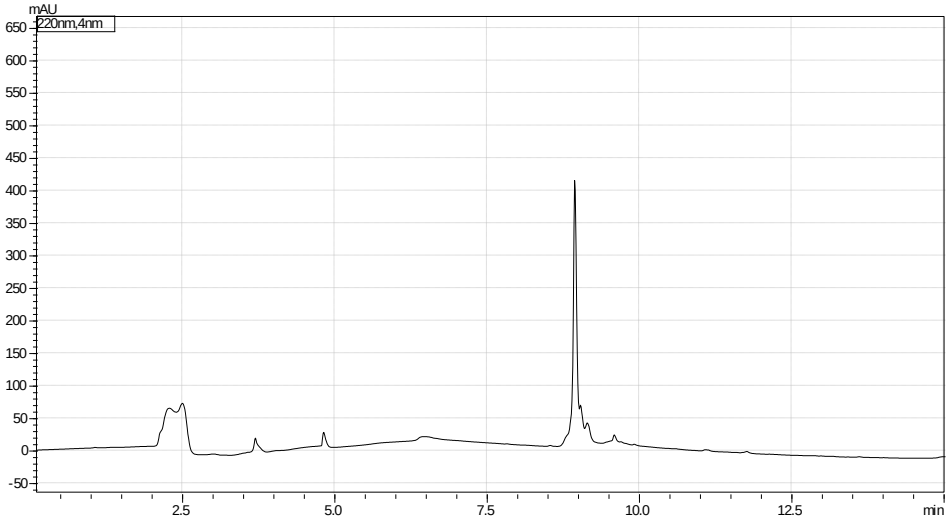


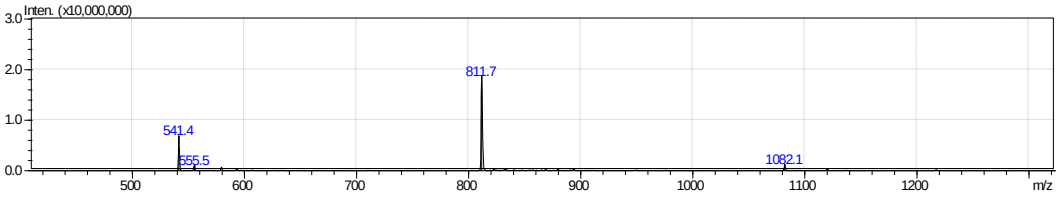




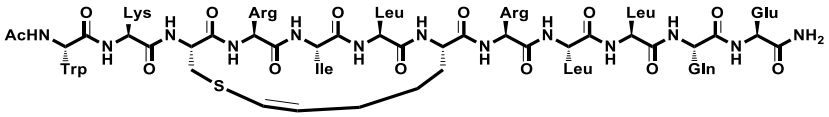
2h **Ac-(cyclo-4, 8)-[WKRCILRM$_5$LLQE]-NH$_2$**

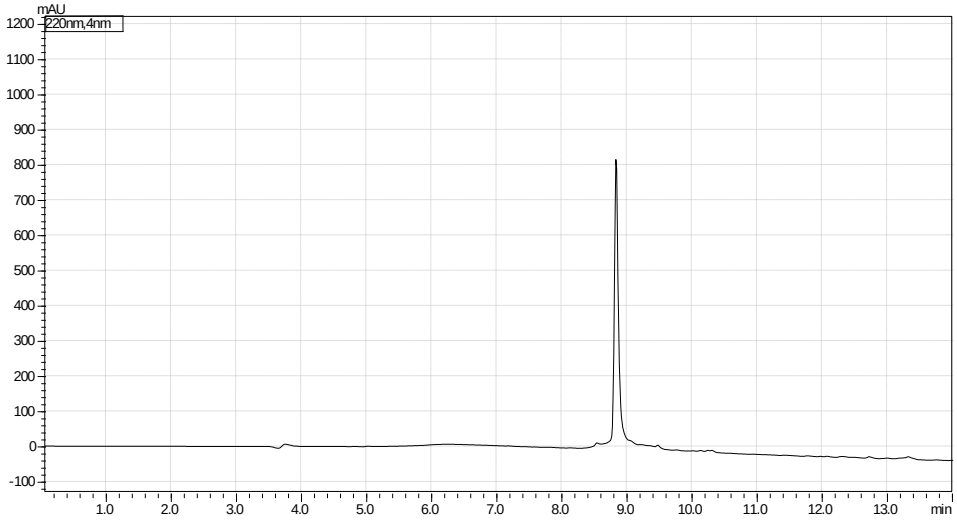


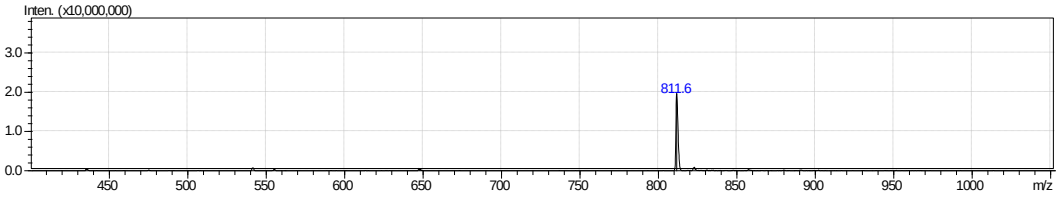




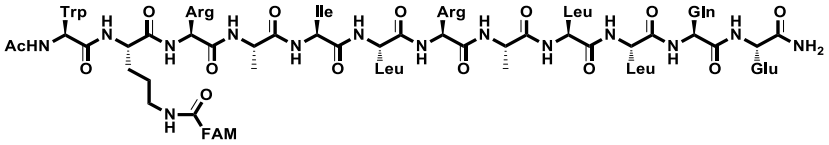
2j **Ac-(cyclo-3, 7)-[WKCRILM$_5$RLLQE]-NH$_2$**

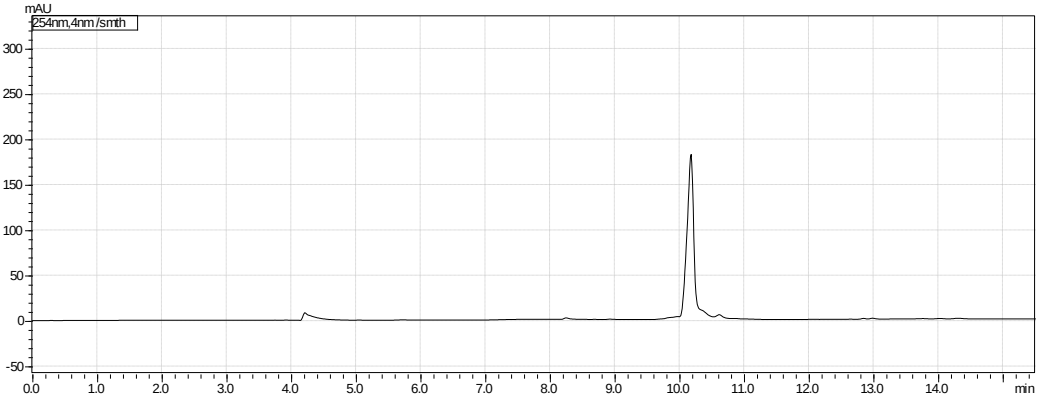


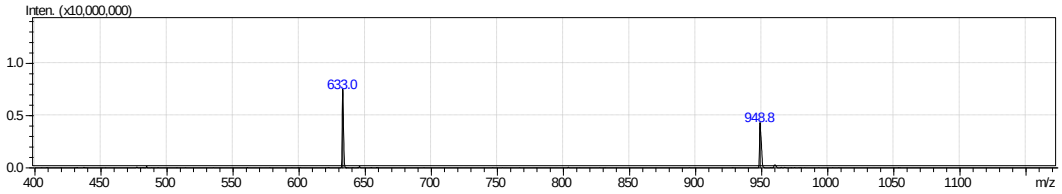




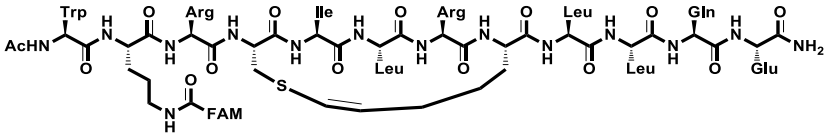
1h-FAM **Ac-WK(FAM)ARILARLLQE-NH_2**

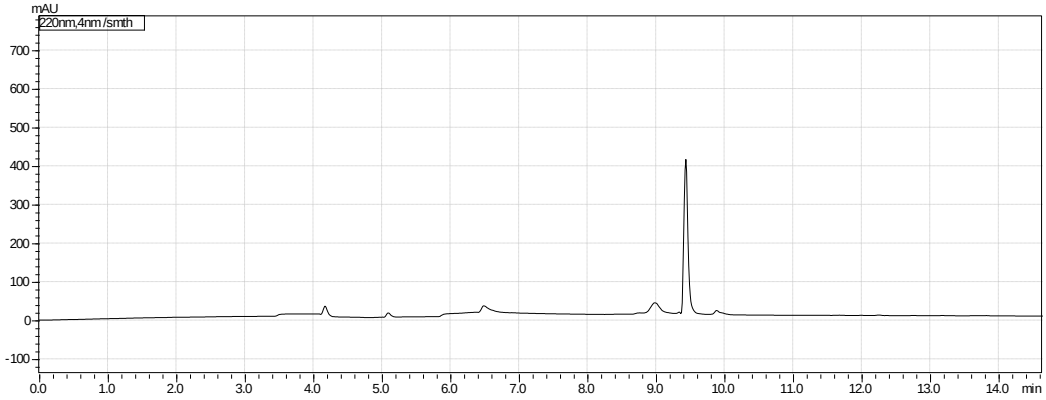


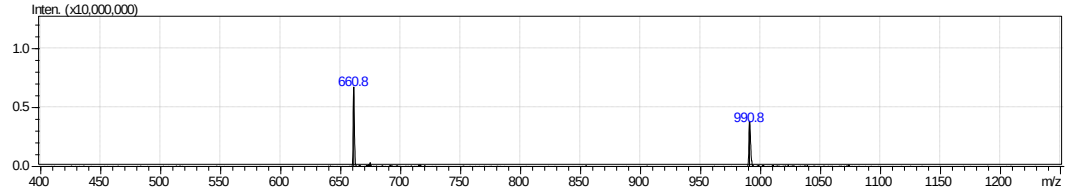




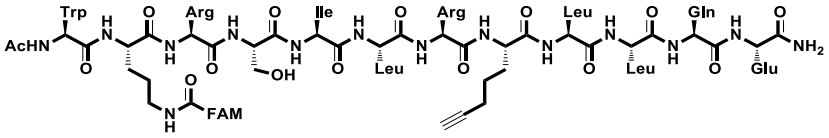
2h-FAM **Ac-(cyclo-4, 8)-[WK(FAM)RCILRM$_5$LLQE]-NH$_2$**

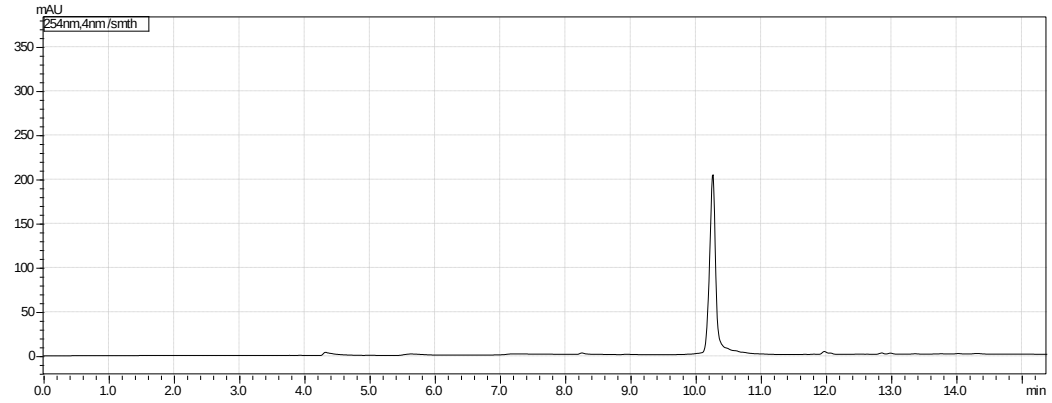


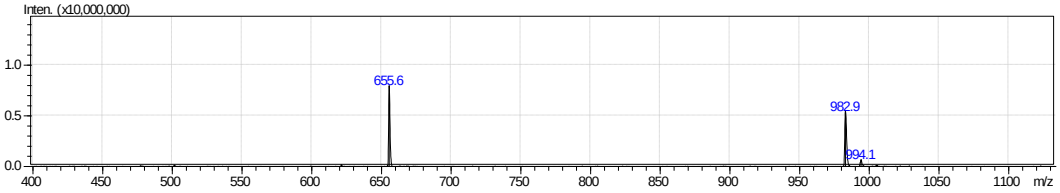




1g-FAM **Ac-WK(FAM)RSILRM$_5$LLQE-NH$_2$**

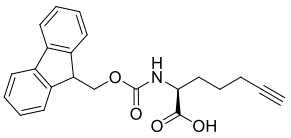


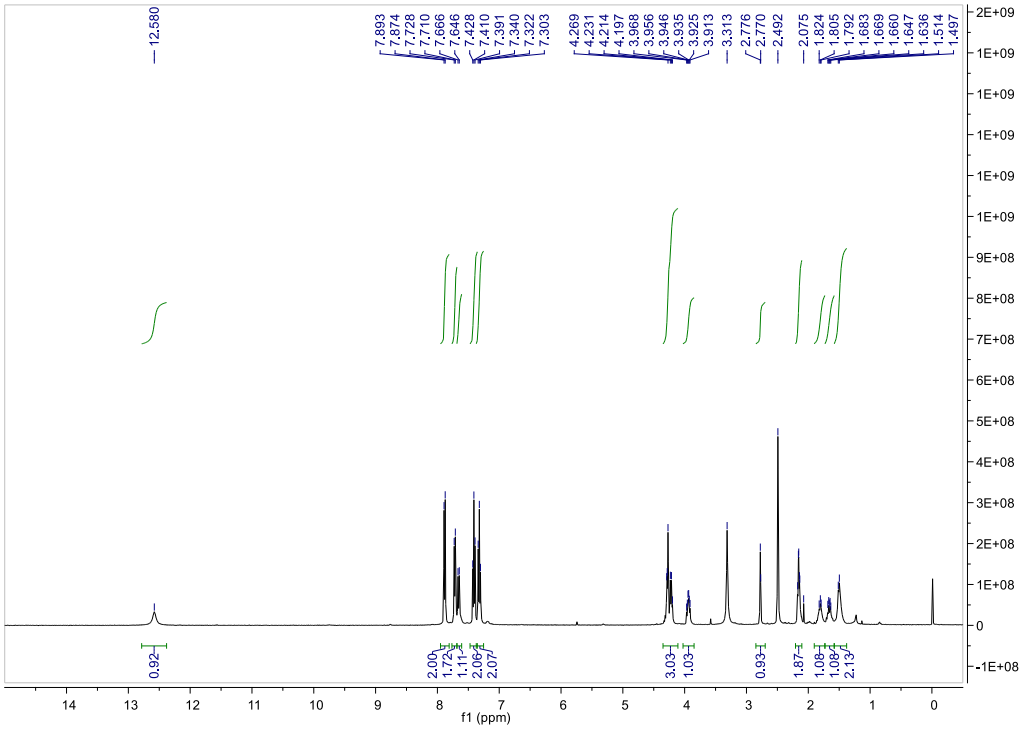


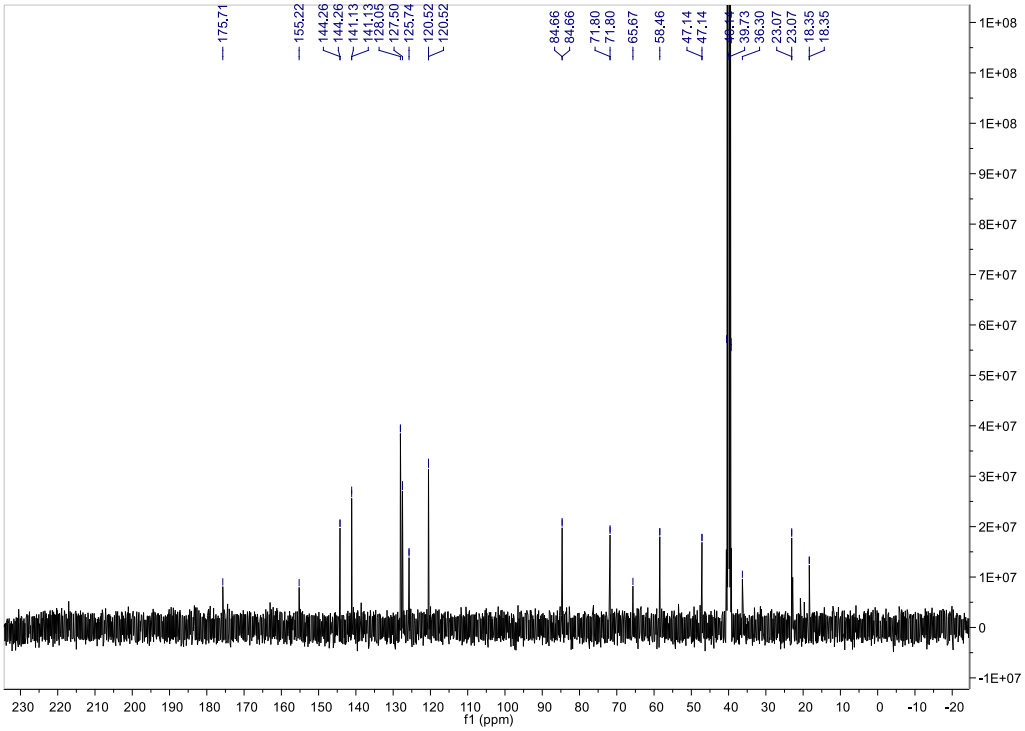


NMR Spectra

M_5 **2S-{[(9H-Fluoren-9-ylmethoxy)carbonyl]amino}-6-heptynoic Acid**

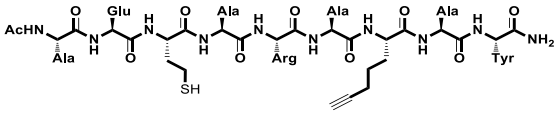


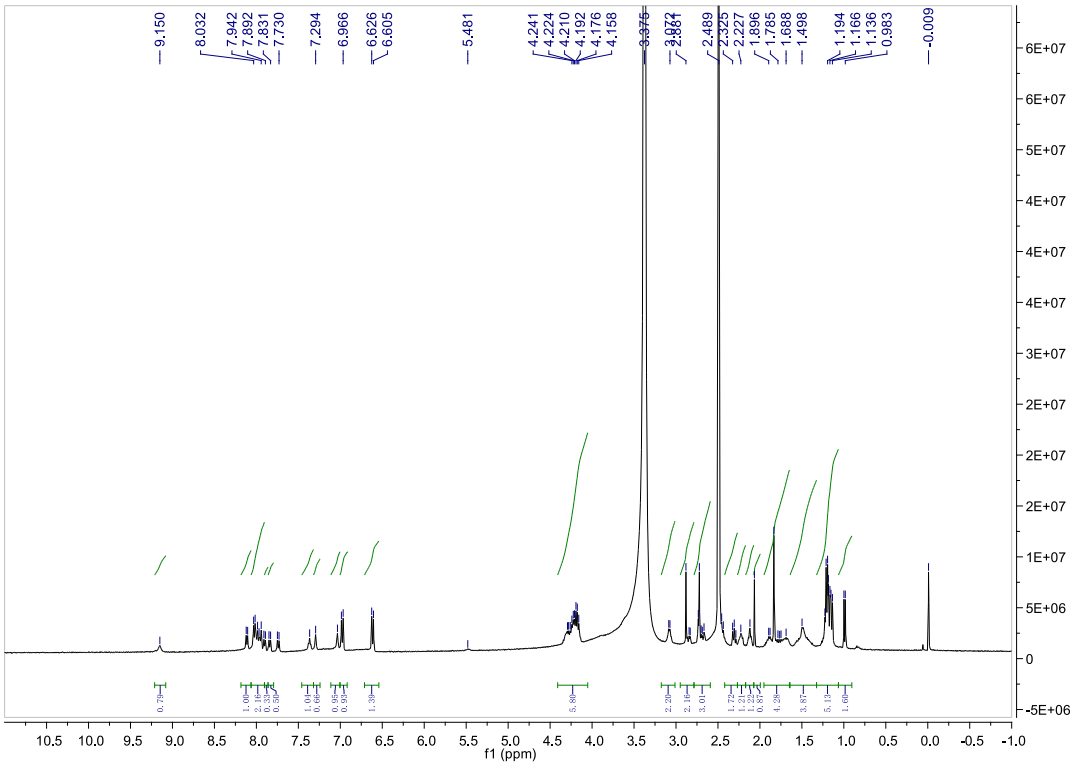




^{1}H-NMR and ^{13}C-NMR spectrum of **M_5** (at 400 MHz in DMSO-d_6)

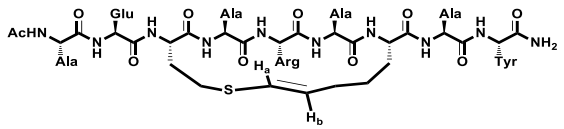
1a **Ac-AE^{h}CARAM$_5$AY-NH$_2$**

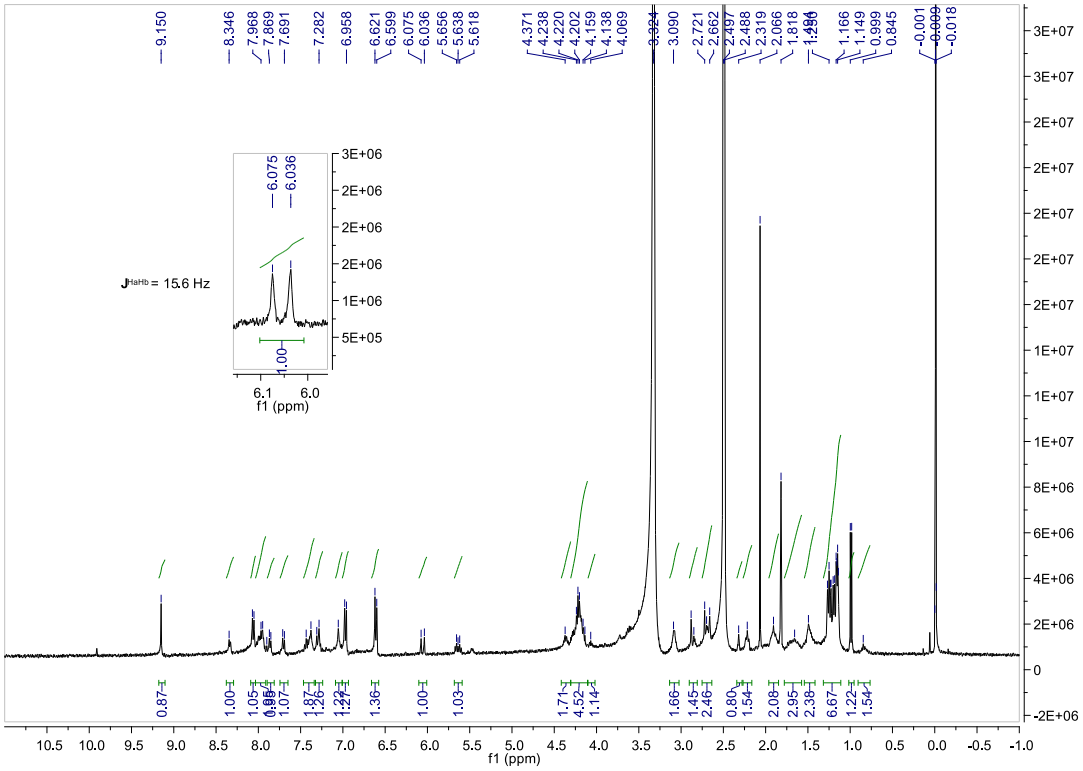




^{1}H-NMR spectrum of **1a** (at 400 MHz in DMSO-d$_6$)

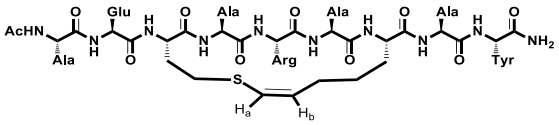
2a-A **Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$**

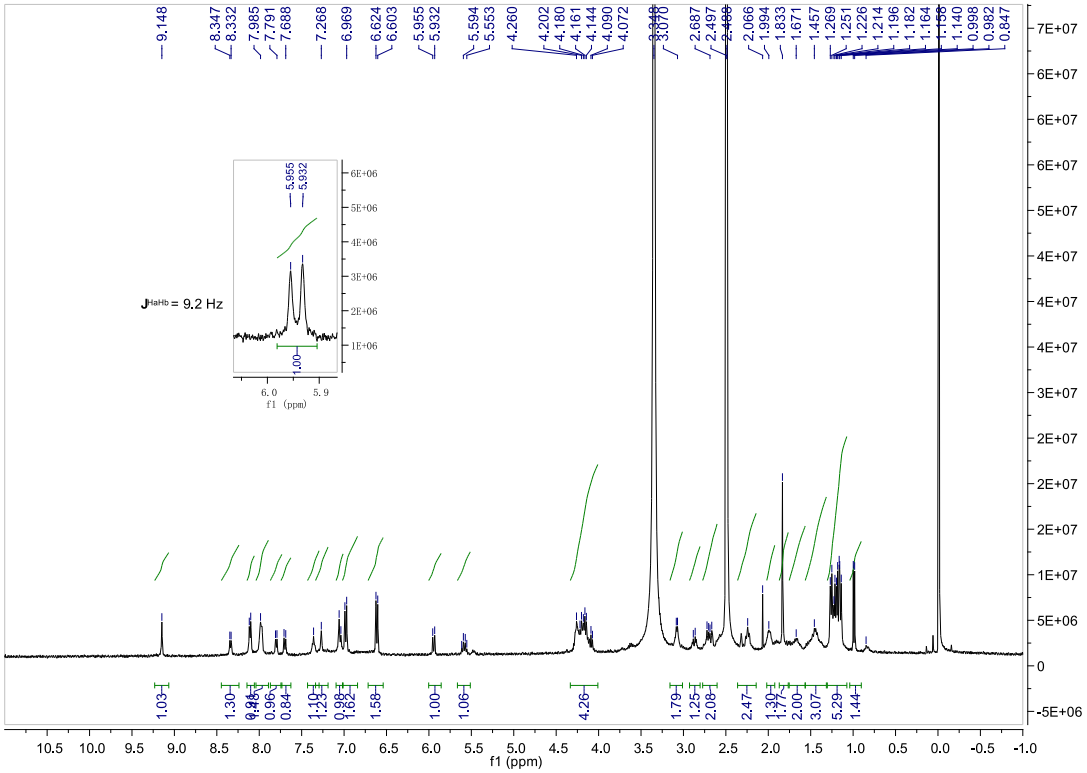




^{1}H-NMR spectrum of **2a-A** (at 400 MHz in DMSO-d$_6$)

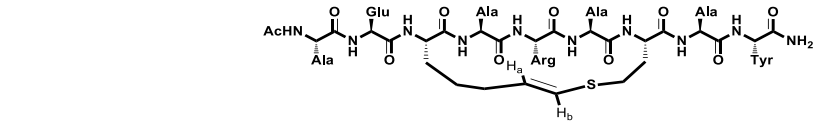
2a-B **Ac-(cyclo-3, 7)-[AE^{h}CARAM$_5$AY]-NH$_2$**

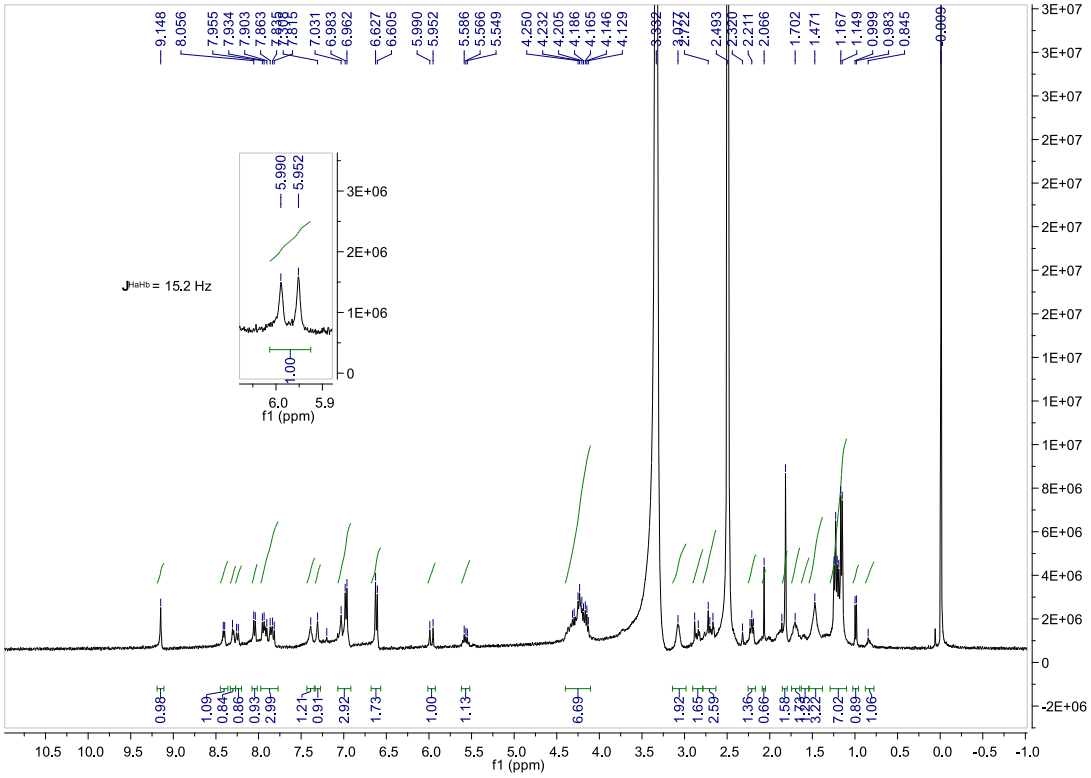




^{1}H-NMR spectrum of **2a-B** (at 400 MHz in DMSO-d$_6$)

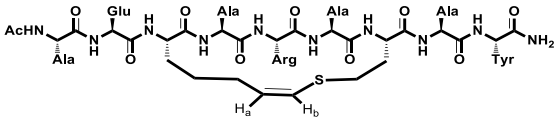
2b-A **Ac-(cyclo-3, 7)-[AE M$_5$ARA^{h}CAY]-NH$_2$**

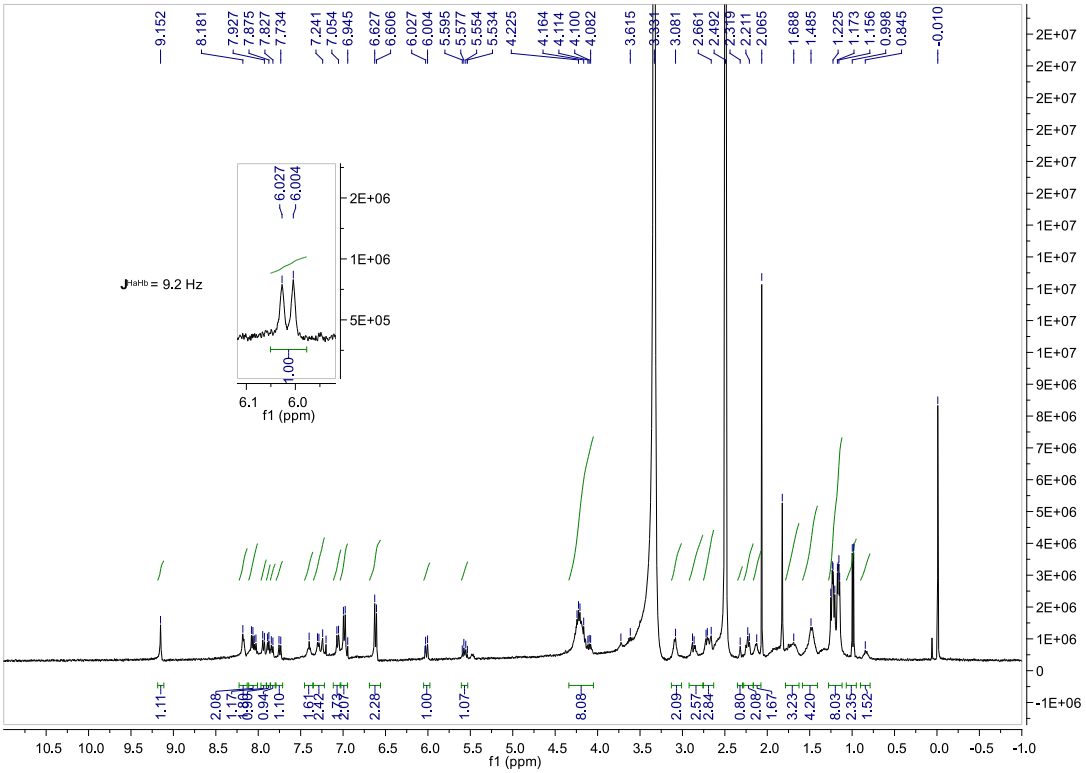




^{1}H-NMR spectrum of **2b-A** (at 400 MHz in DMSO-d$_6$)

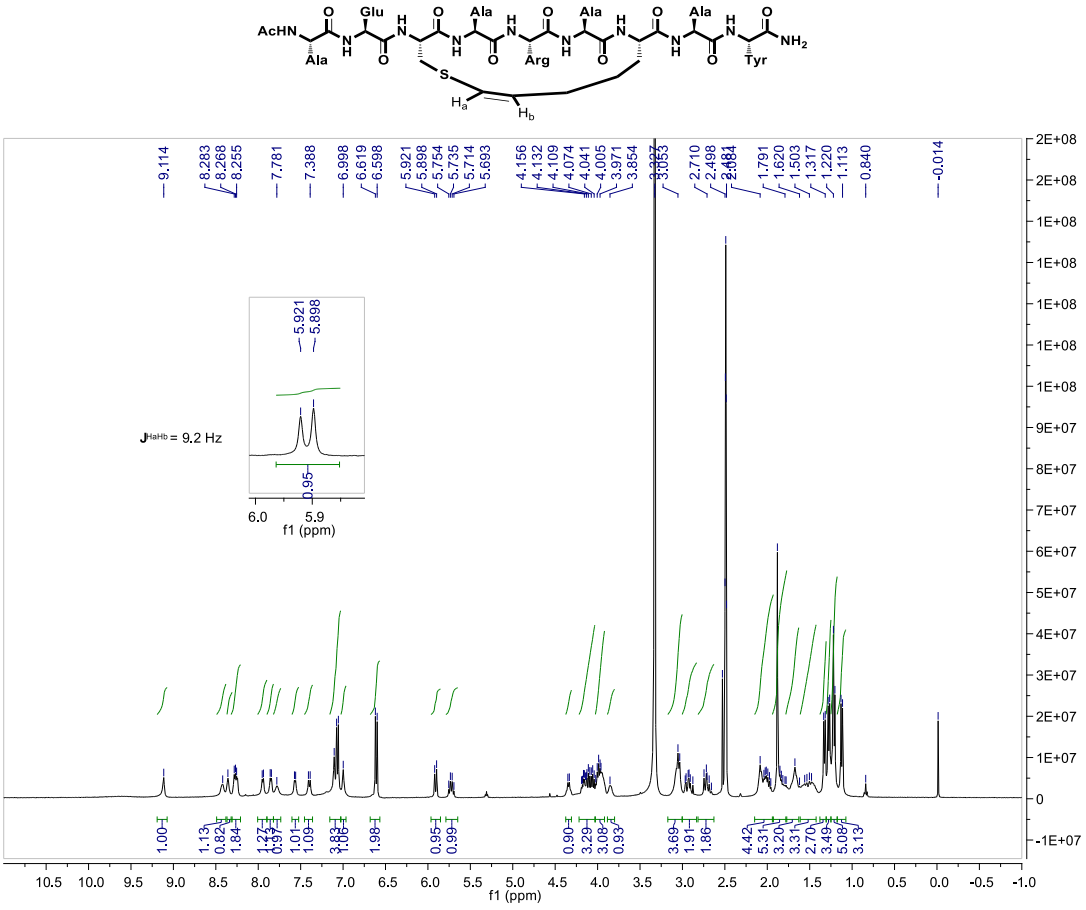
2b-B **Ac-(cyclo-3, 7)-[AE M$_5$ARA^{h}CAY]-NH$_2$**





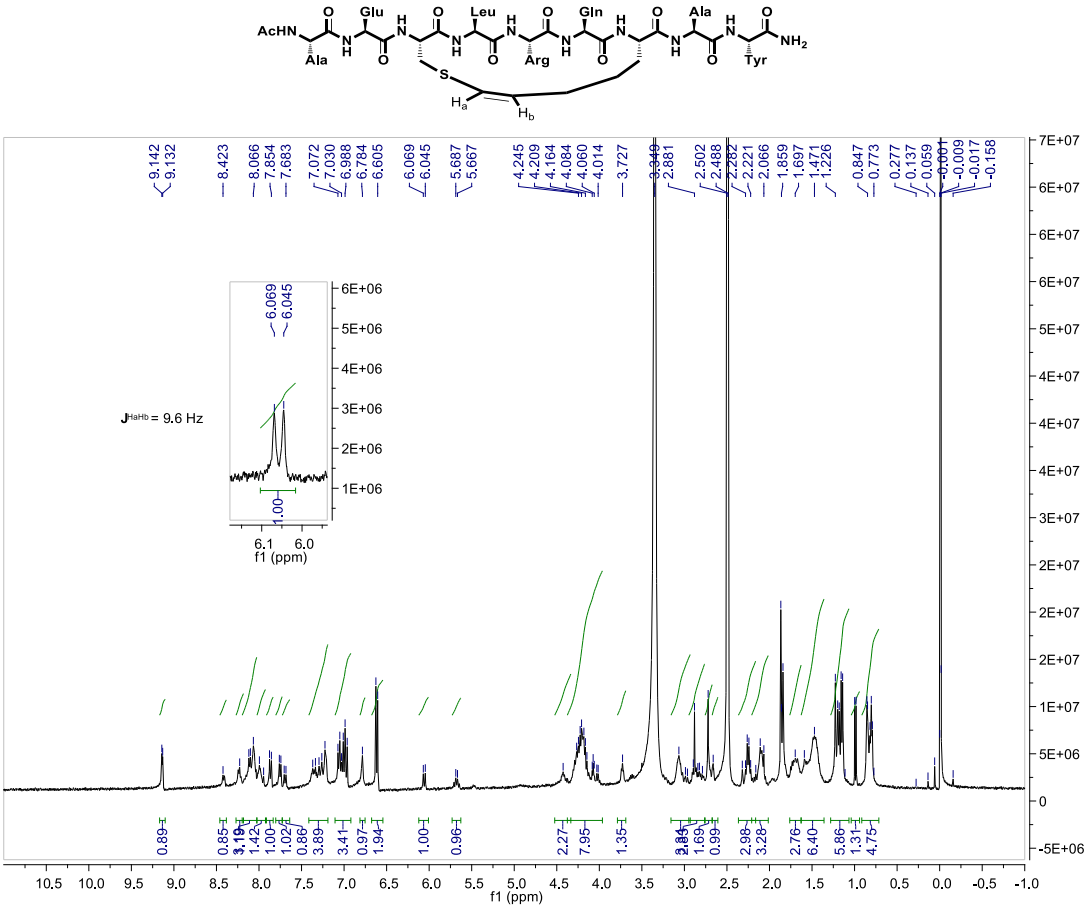
^{1}H-NMR spectrum of **2b-B** (at 400 MHz in DMSO-d$_6$)

2d **Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$**



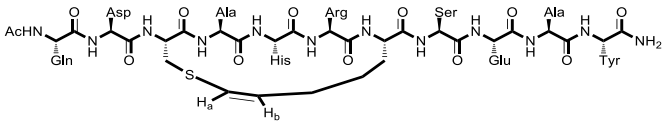
^{1}H-NMR spectrum of **2d** (at 400 MHz in DMSO-d$_6$)

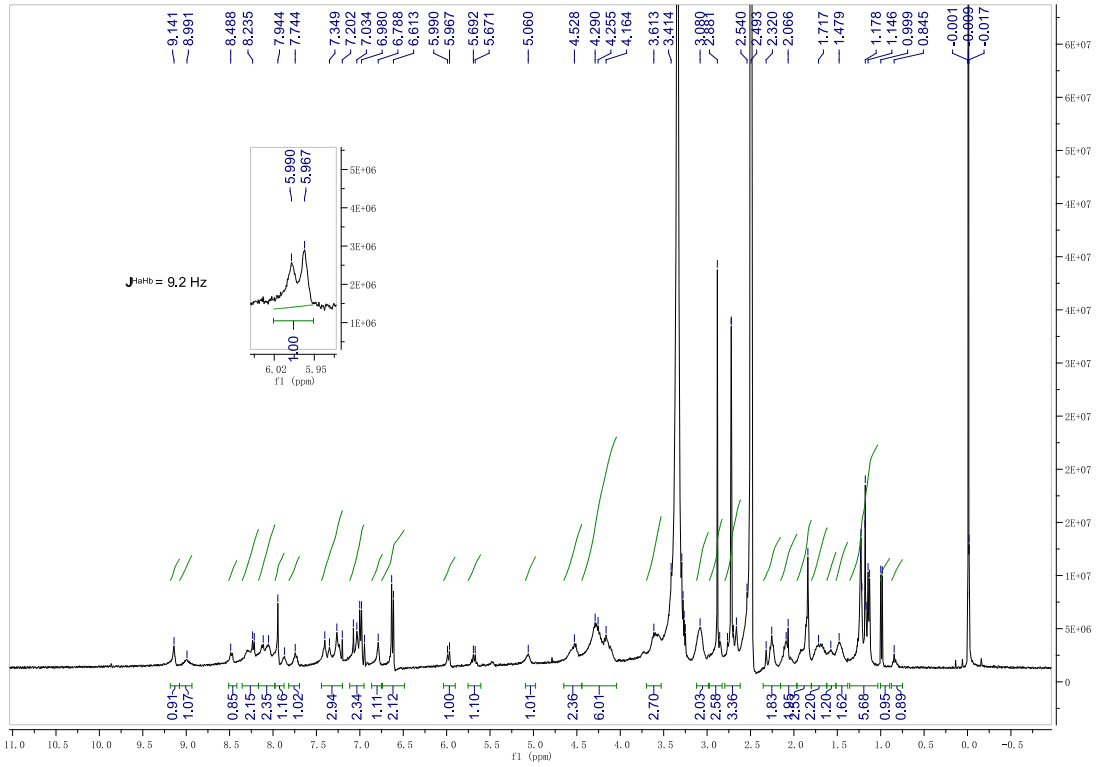
2e **Ac-(cyclo-3, 7)-[AECLRQM$_5$AY]-NH$_2$**



^{1}H-NMR spectrum of **2e** (at 400 MHz in DMSO-d$_6$)

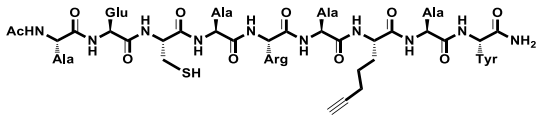
2f **Ac-(cyclo-3, 7)-[QDCAHRM$_5$SEAY]-NH$_2$**

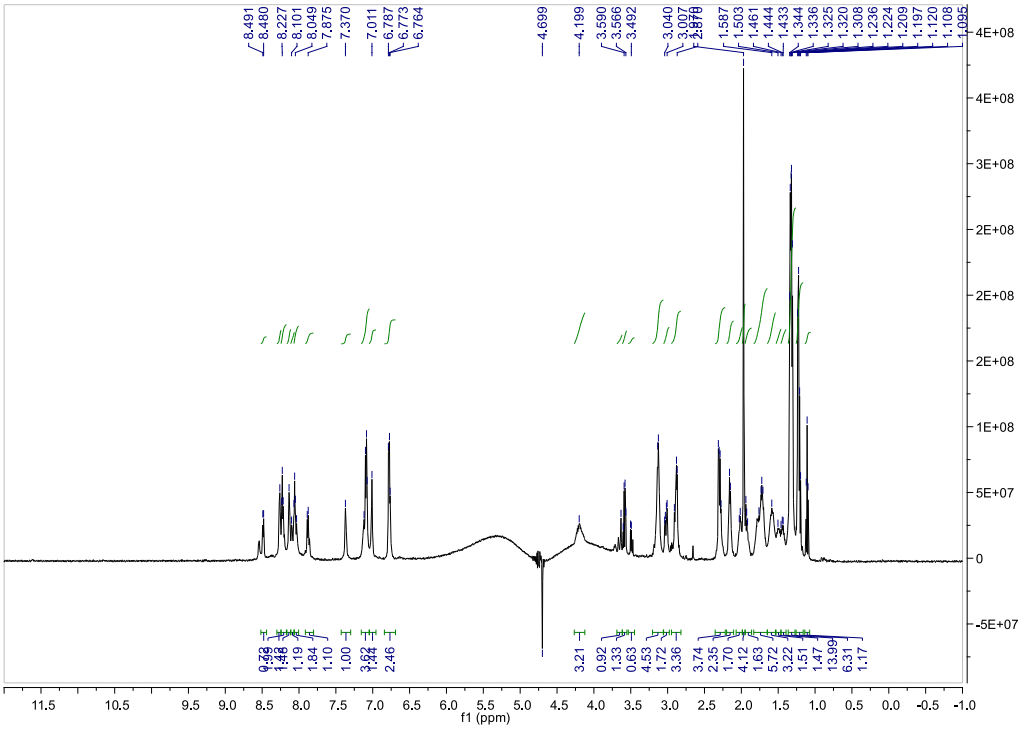


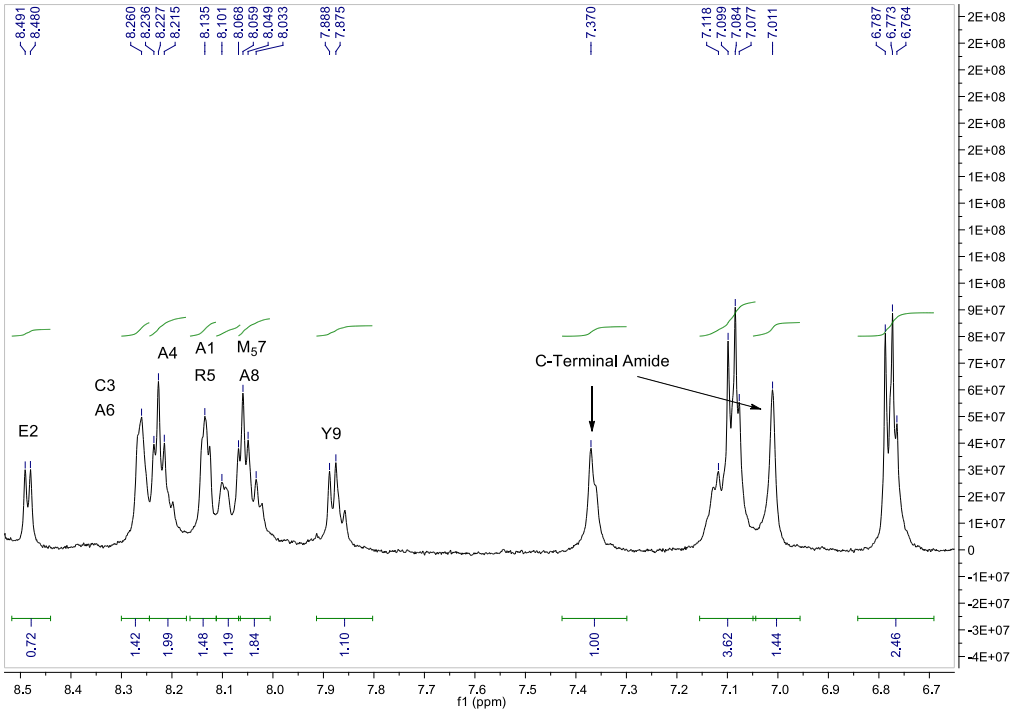


^{1}H-NMR spectrum of **2f** (at 400 MHz in DMSO-d$_6$)

1d **Ac-AECARAM$_5$AY-NH$_2$**

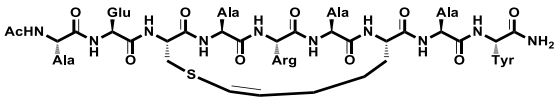


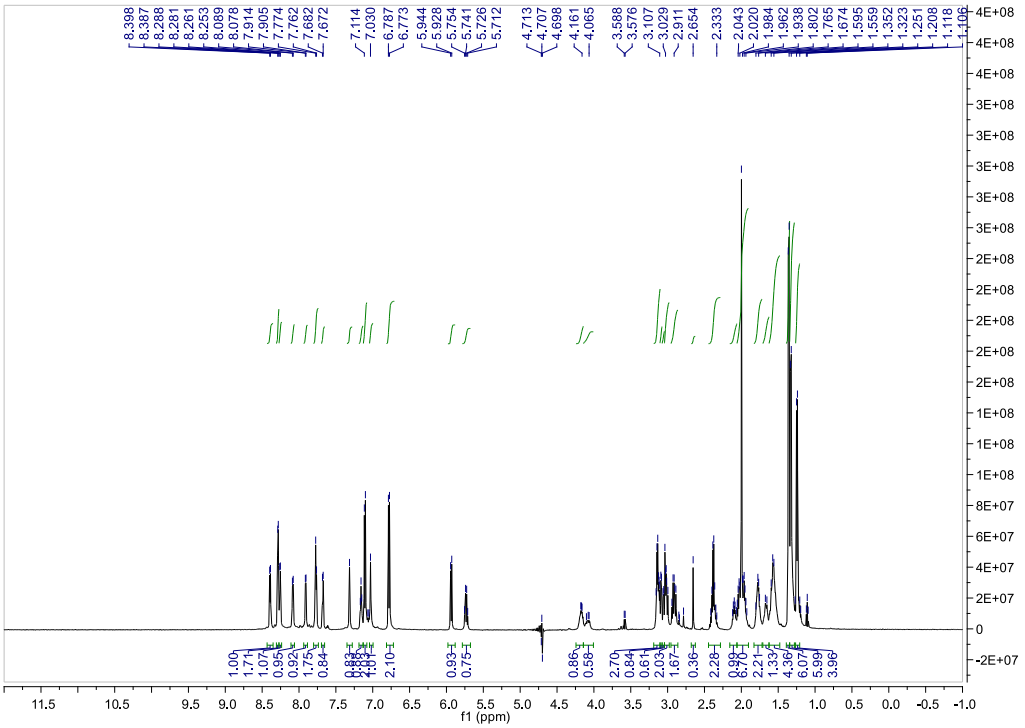


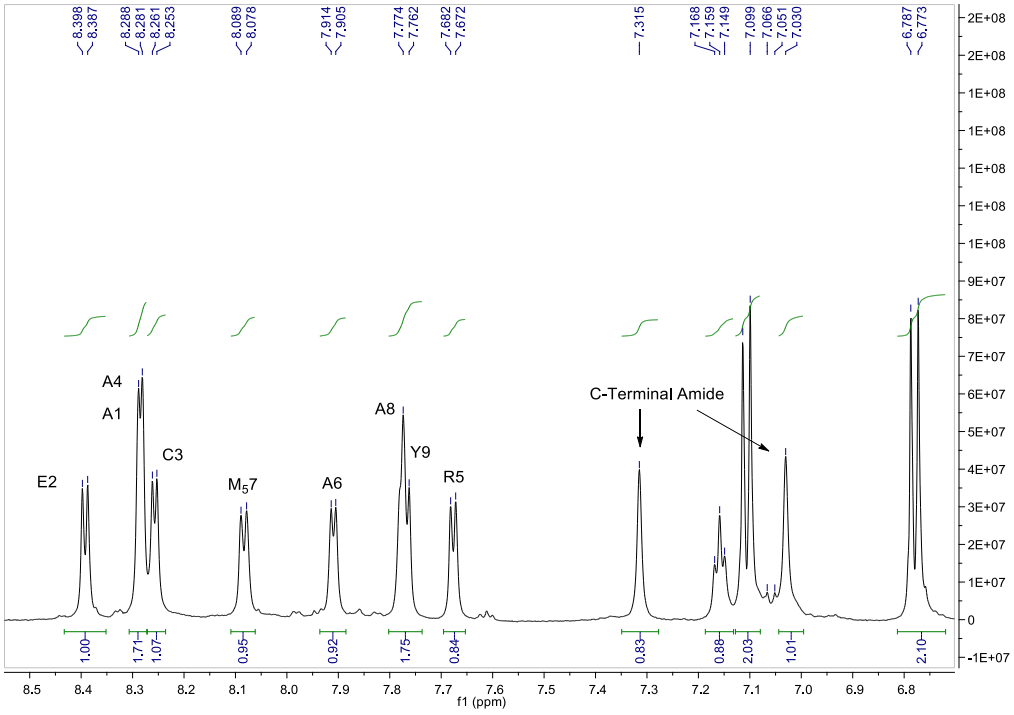


^{1}H-NMR spectrum of **1d** (at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K)

2d **Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$**

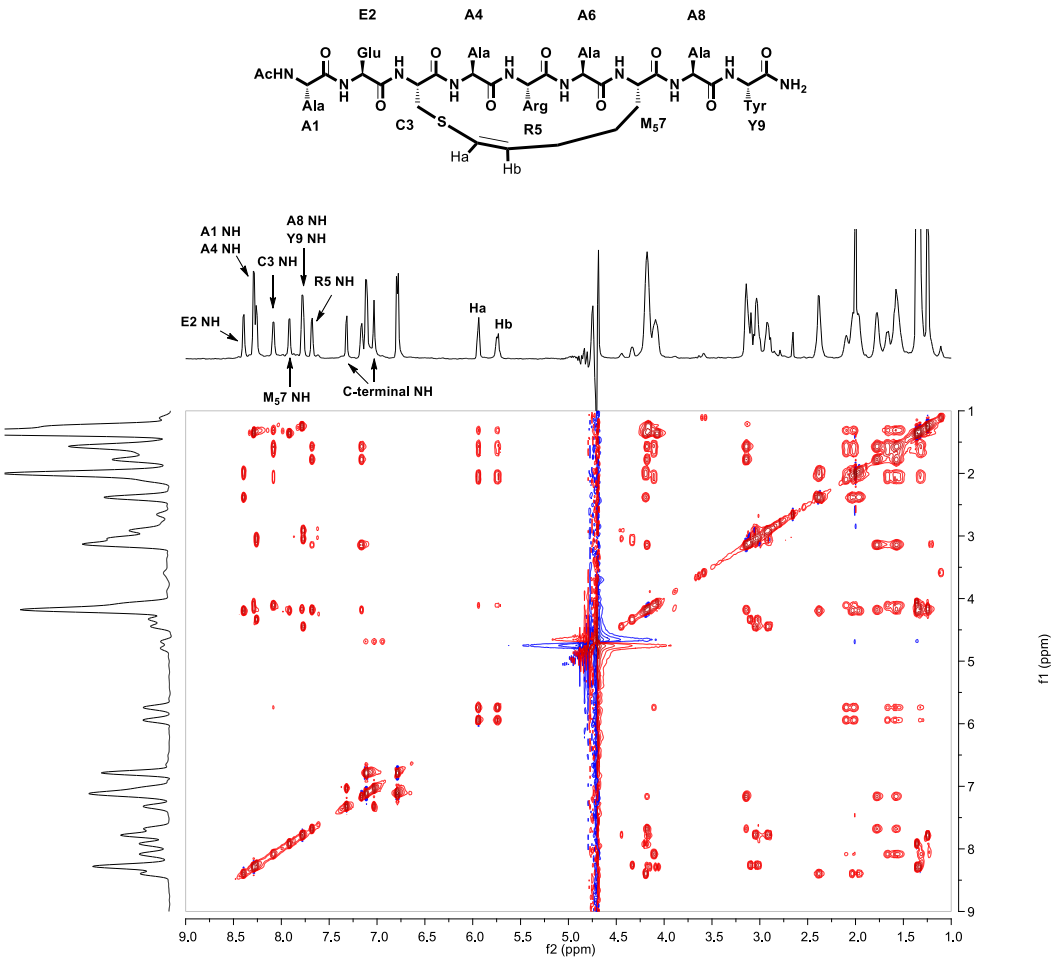




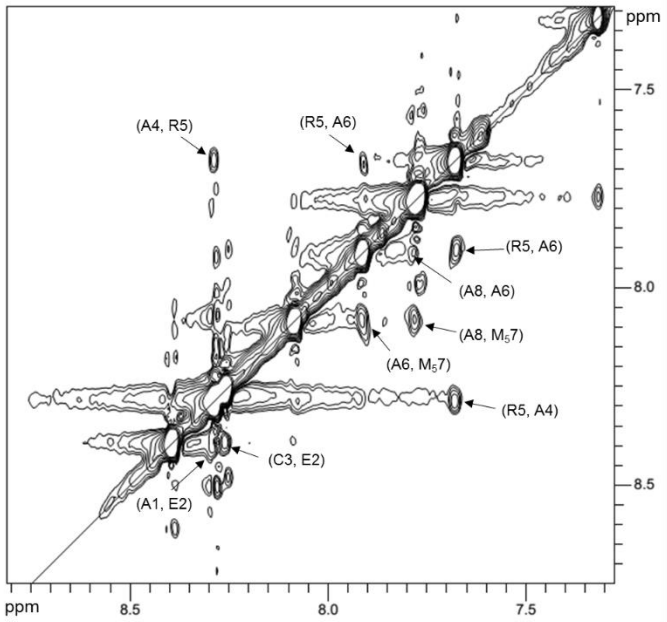


^{1}H-NMR spectrum of **2d** (at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K)

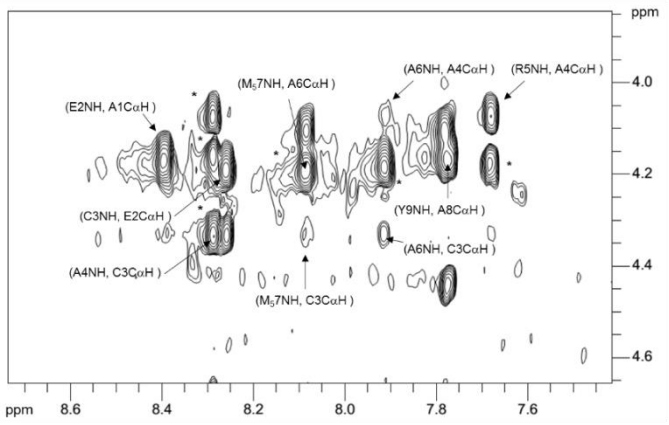
2d **Ac-(cyclo-3, 7)-[AECARAM$_5$AY]-NH$_2$**



TOCSY spectrum of **2d** (at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K)

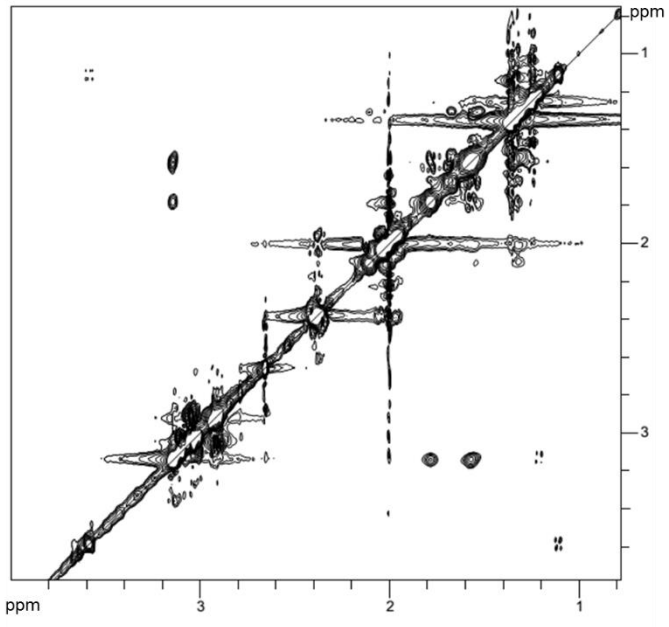


NH region of ROESY spectrum of **2d** (at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K)



NH-αCH region of 2D-ROESY spectrum of **2d**

(at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K) Asterisk indicates peak overlap.



Aliphatic region of 2D-ROESY spectrum of **2d**

(at 600 MHz in phosphate buffer (pH 5.0) with 10% D_2O at 298K)

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