Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Cell-Penetrating, Amphipathic Cyclic Peptoids as Molecular Transporters for Cargo Delivery

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Supplementary Information

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1. Materials and General Methods.

All commercial reagents were purchased from commercial suppliers (Sigma-Aldrich, TCI, Alfa Aesar, and Thermo Fisher Scientific) and used without further purification. Rink amide MBHA resin (0.45 mmol/g) and Fmoc-protected amino acids were purchased from BeadTech. Analytical HPLC and LC/MS characterization were performed on an Agilent system with a C18 reversed phase HPLC column (Agilent, 3.5 μm, 4.6 mm x 150 mm). A gradient elution of 10% to 100% B in 7 min (keep 100% B till 13 min) was used at flow rate of 0.7 mL/min (solvent A : distilled water, 0.01% trifluoroacetic acid (TFA); B : acetonitrile, 0.01% TFA). With a C18 reversed-phase column (Agilent, 5 μm, 21.2 mm x 150 mm) changing solvent composition with a linear gradient of 100% A in 5 min followed by 100 % B in 65 min. Matrix-assisted Laser Desorption-Time of Flight (MALDI-TOF) mass spectrometry (MS) was performed on 4700 Proteomics Analyzer (Applied Biosystems) and Autoflex speed LRF (Bruker) using α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid as matrices. Flow cytometric analysis was performed by BD LSR Fortessa (BD Biosciences) flow cytometer and BD FACSDiva Software.

2. Synthesis and Purification.

2.1 Synthesis of fluorescently-labeled linear peptoids (L1 – L4)

Rink amide MBHA resins (100 mg, 45 μmol) were swollen with DMF (2 mL) in a 5 mL fritted syringe overnight. The Fmoc protecting group was removed by treatment with 20% piperidine in DMF (2 × 10 min). On the resin, peptoid residues were added by a standard submonomer route. In short, amino functionalized with NH_2, were treated with 1 M bromoacetic acid (BAA) (20 equiv.) and 1 M *N*,*N*-diisopropylcarbodiimide (DIC) (20 equiv.) in DMF. After shaking for 20 min, the reaction mixture was drained and the resin was washed with DMF (3×), MeOH (2×), CH_2Cl_2 (2×), and DMF (3×). Unless otherwise noted, this washing cycle was used for each reaction step. Then the bead was treated with a

primary amine (2 M in DMF) for 2 hr for amine displacement reaction. This process was repeated until desired sequences of peptoids were obtained. The N-terminus of peptoids was coupled with aminobutanoic acid (Abu) linker by standard Fmoc chemistry. After Fmoc deprotection, NH_2 group of the Abu was coupled with 5,6-carboxyfluorescein (5 equiv.) using the peptide coupling conditions. For cleavage, the beads were treated with 1 mL of a cleavage cocktail (95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% water) for 2 hr. Then the crude products were purified by HPLC. Their purity and identity were analyzed by LC/MS and MALDI-TOF MS.

2.2 Synthesis of fluorescently-labeled cyclic peptoids (C1-C15)

Cyclic peptoids were synthesized by the similar procedure described above except for the introduction of cysteine and triazine moiety for cyclization reaction. In this case, prior to peptoid synthesis, cysteine residue was first loaded on Rink amide MBHA resins under the peptide coupling conditions. After Fmoc deprotection followed by peptoid synthesis, the N-termini of the peptoids were capped with triazine by treating with cyanuric chloride (5 equiv.) and DIPEA (10 equiv.) in THF for 3 hr at rt. Next, the Mmt group on the cysteine was removed by treatment with 2% TFA and 5% TIS in CH_2Cl_2 for 30 min. For macrocyclization, the beads were treated with 2 M DIPEA (20 equiv.) in DMF overnight at rt. The remaining chloride group on cyclic peptoids was replaced with diamine group by treating with diaminobutane (20 equiv.) and DIPEA (20 equiv.) in N-methyl-2-pyrrolidone at 60 ℃ overnight.

The NH_2 group of the diamine was coupled with 5,6-carboxyfluorescein (5 equiv.) using peptide coupling conditions. For cleavage, the beads were treated with 1 mL of a cleavage cocktail (95% TFA, 2.5% TIS, and 2.5% water) for 2 hr. Then the crude products were purified by HPLC. Their purity and identity were analyzed by LC/MS and MALDI-TOF MS.

2.3 Synthesis of PIP-Box peptide-linker

Rink amide MBHA resins (50 mg, 22.5 μmol) were placed in a 5 mL fritted syringe and swollen in DMF overnight at 4 ℃. After the removal of Fmoc protection group with 20% piperidine in DMF (2 x 10 min), the beads were treated with a Fmoc protected amino acid (5 equiv.) in the presence of HBTU (5 equiv.), HOBt (5 equiv.), and DIPEA (10 equiv.) in DMF (500 μL) at rt for 2 hr. After peptide coupling reaction, the reaction mixture was drained, and the resins were washed with DMF (3×), MeOH (3×), CH_2Cl_2 (3×), and DMF (3×). This process was repeated to afford the desired 16-residue peptide. 3-(2-pyridyldithio)butyric acid linker was prepared as previously reported[1] and coupled to the N-terminal of the 16-mer peptide (25 mg, 11.25 22.5 μmol) under the standard peptide coupling condition to synthesize PIP-Box peptide-linker. Then the 16-mer peptide and 16-mer peptide-linker were cleaved from resins by treating with cleavage cocktail (95% TFA, 2.5% TIPS, and 2.5% water) for 2 hr at rt and purified by reverse-phase HPLC.

2.4 Synthesis of disulfide-bridged PIP-Box peptide-transporter conjugates (PIP-Box peptide-R8 conjugate and PIP-Box peptide-C15 conjugate)

C-R8 was prepared under the standard peptide coupling condition. The **HS-C15** was synthesized using a similar procedure described in section 2.2. After cyclization, the remaining chloride on cyclic peptoid was replaced with cysteamine by reacting with S-(4-methoxytrityl)-2-aminoethanethiol (20 equiv.) and DIPEA (20 equiv.) in N-methyl-2-pyrrolidone at 60 ℃ overnight. S-(4-methoxytrityl)-2-aminoethanethiol was prepared as previously reported.[2] For cleavage, the beads were treated with cleavage cocktail (95% TFA, 2.5% TIS, and 2.5% water) for 2 hr. Crude product was then purified by reverse-phase HPLC. **C-R8** or **HS-C15** (1 equiv.) and PIP-box peptide-linker (1 equiv.) were dissolved in 500 μL of anhydrous DMF and stirred at rt overnight. Crude products of PIP-Box peptide-**R8** conjugate and PIP-Box peptide-**C15** conjugate were purified by HPLC. The purity and identity were analyzed by LC/MS and MALDI-TOF MS.

2.5 Synthesis of TAMRA-labeled disulfide-bridged PIP-Box peptide-transporter conjugates (TAMRA-PIP-Box peptide-R8 conjugate and TAMRA-PIP-Box peptide-C15 conjugate)

TAMRA-labeled PIP-Box peptide were synthesized using the procedure using peptide coupling reaction. In this case, Fmoc-Lys(Alloc)-OH residue was first loaded on Rink amide MBHA resins under the peptide coupling conditions. Then the 16-mer PIP-Box peptide conjugated with 3-(2-pyridyldithio)butyric acid linker was prepared as described in section 2.3. Alloc protecting group was deprotected by treating with $Pd(PPh_3)_4$ (0.2 equiv.) and $PhSiH_3$ (10 equiv.) in anhydrous CH_2Cl_2 (1 mL) for 2 hr. The NH_2 group on Lys was coupled with 5(6)-carboxytetramethylrhodamine (TAMRA, 5 equiv.) using the peptide coupling condition. Then the peptides were cleaved from resins by treating with cleavage cocktail (95% TFA, 2.5% TIPS, and 2.5% water) for 2 hr at rt and purified by reverse-phase HPLC.

To synthesize TAMRA-labeled PIP-Box peptide-**R8** conjugate and TAMRA-labeled PIP-Box peptide-**C15** conjugate, **C-R8** or **HS-C15** (1 equiv.), synthesized in section 2.4, and TAMRA-labeled PIP-box peptide-linker (1 equiv.) were dissolved in 500 μL of anhydrous DMF and stirred at rt overnight. Crude products were purified by HPLC. The purity and identity were analyzed by LC/MS and MALDI-TOF MS.

3. Cell Culture and Flow Cytometry Analysis

A549 cells were maintained in medium consisting of RPMI, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37 ℃. HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ℃. 1.0×10^5 cells/well were placed in a 24-well plate and incubated overnight. Cells were then incubated with 500 μL of 3 μM or 10 μM compound solution in Opti-MEM. After 1 hr incubation at 37 ℃, cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) buffer (1 mL × 3). For the cellular uptake studies at ATP depletion condition, the cells

were incubated for 30 min at 4 ℃ in Opti-MEM, or in the presence of 0.25% sodium azide in Opti-MEM at 37 ℃ prior to the addition of the compounds. After collecting by trypsinization, cells were resuspended in cold DPBS, stained with propidium iodide (PI) (final concentration: 0.5 μg/mL) to exclude dead cells from analysis, and analyzed immediately by flow cytometer. The data presented are the median fluorescence intensity for 10,000 cells collected.

4. Confocal Microscopy

1×10^4 A549 cells/well were seeded in a Lab-Tek™ chambered coverglass (8 well). After incubation for 24 hr, cells were treated with 300 μL of peptoid solution (10 μM in Opti-MEM). After incubation at 37 ℃ for 1 hr, the cells were washed with DPBS twice. Then the nuclei were stained with Hoechst 33342 (10 μg/mL) in Opti-MEM for 20 min at 37 ℃. The cells were washed with DPBS twice. Then the mitochondria were stained with MitoTracker™ Red CMSRos (4 μM) in Opti-MEM for 20 min. Cells were then imaged using confocal microscope.

5. Proteolysis Assay

Compound solutions were incubated in at 37 ℃. 100μL of samples were collected at various time point. After addition of 100 μL of DPBS, the sample was filtrated and analyzed with HPLC containing a C18 column. The remaining peptide was determined by integrating area of the compound peak in chromatogram (monitored in 490 nm). The area was normalized with that of the control sample (without trypsin incubation).

6. Cell Viability Assay

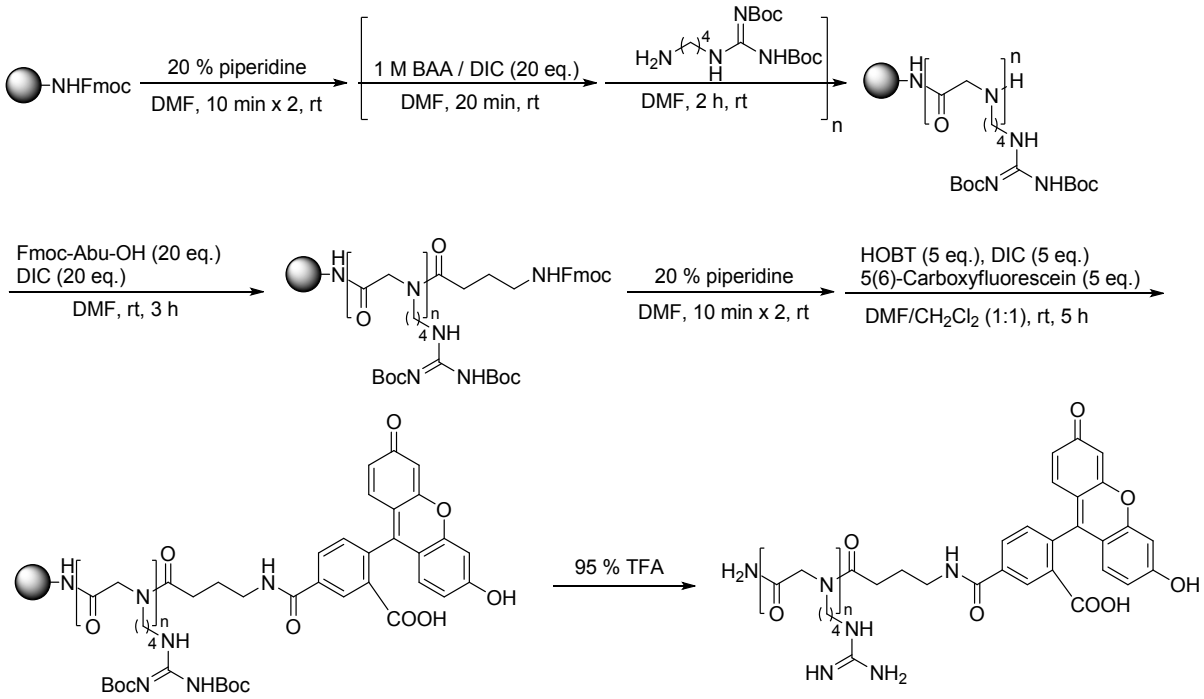
For cell viability assays, 1×10^4 HeLa cells were plated in each well of 96-well plates for 24 hr, washed

with DPBS twice, then treated with various concentrations of compounds or DMSO in Opti-MEM for 24 hr. Cell viability was measured by Cyto X Cell Viability Assay kit (LPS solution) according to the manufacturer's instruction.

7. Annexin-V Apoptosis Assay

1×10^5 HeLa cells were seeded in 24-well plate for 24 hr and washed with DPBS twice. Then, cells were treated with 10 μM of **C15**, PIP-Box peptide, PIP-Box peptide-**C15** conjugate, or DMSO for 24 hr in Opti-MEM medium. After incubation, cells were collected by centrifugation and washed with cold DPBS twice. Washed cells were resuspended in cold DPBS. Apoptosis activity was measured using Ezway AnnexinV-FITC apoptosis kit (KOMABIOTECH) according to the manufacturer's instructions. Flow cytometry was conducted using 10,000 live cells by LSR Foretessa 4 Laser (BD Biosciences).

Scheme S1. Synthesis of fluorescently-labeled linear peptoids.



Scheme S2. Synthesis of fluorescently-labeled cyclic peptoids.

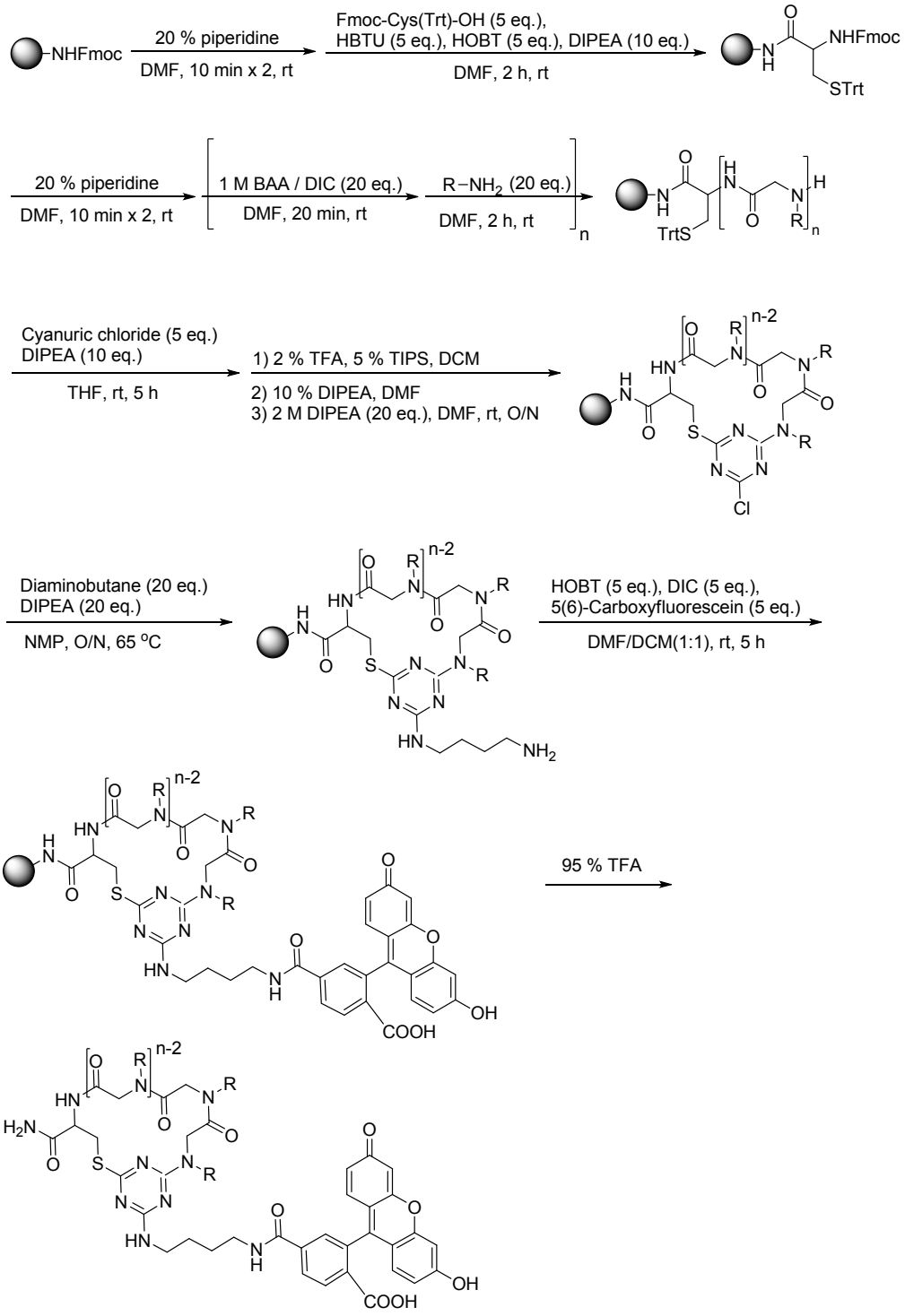
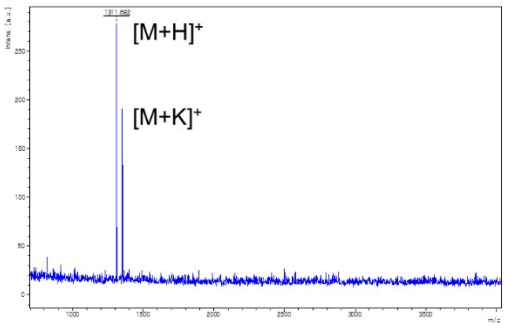
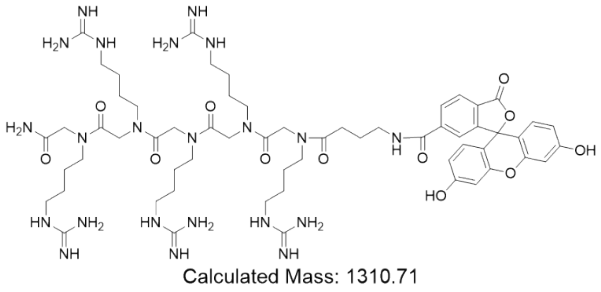
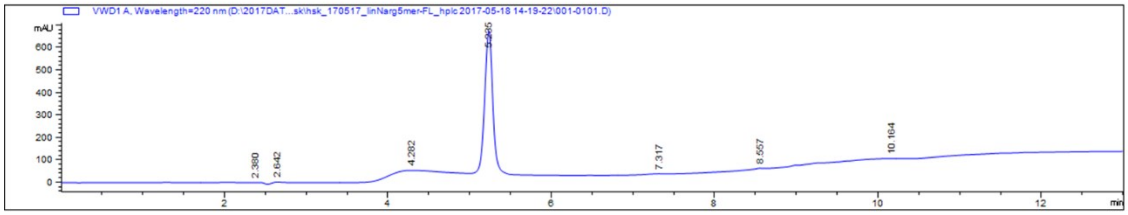


Fig. S1. LC and MS spectra of purified fluorescently-labeled linear peptoids (**L1-L4**) and cyclic peptoids (**C1-15**).

L1







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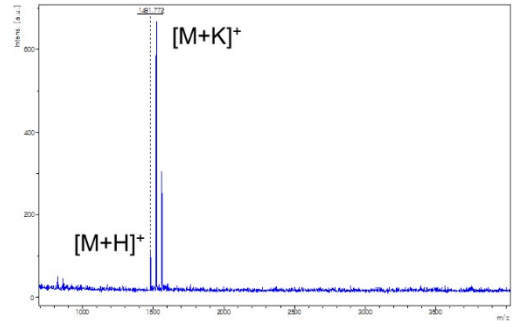

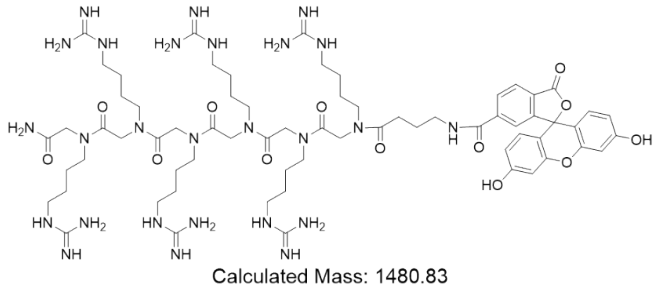

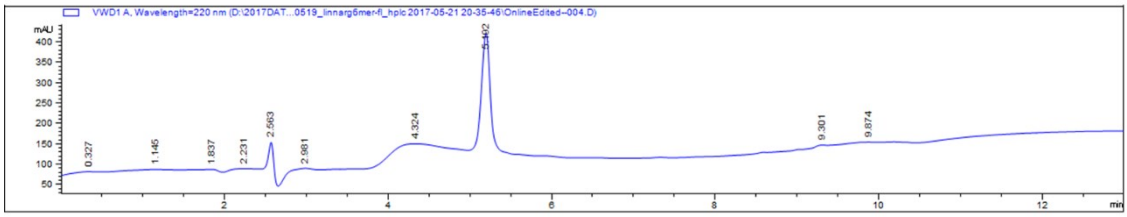

Fig. S1. (Cont'd)

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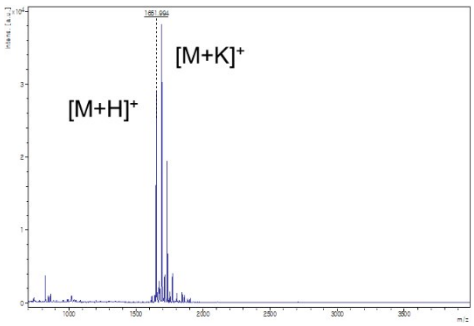

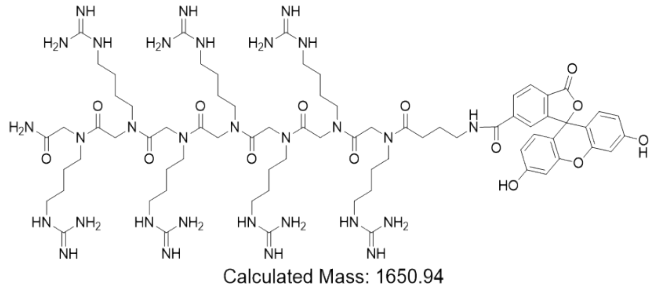

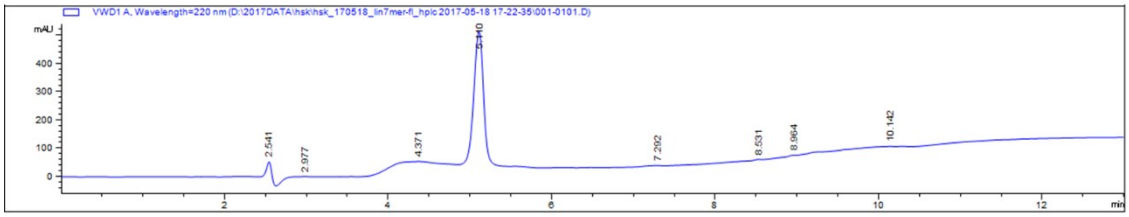

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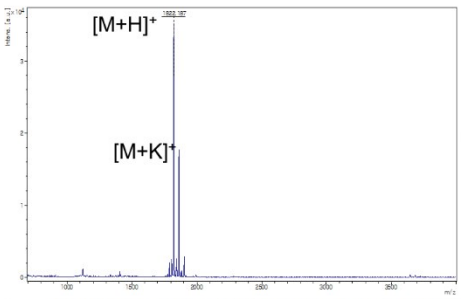

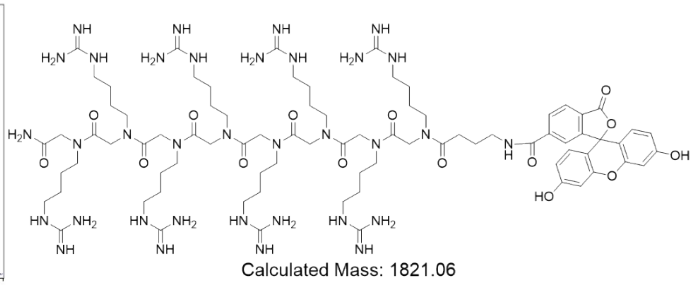

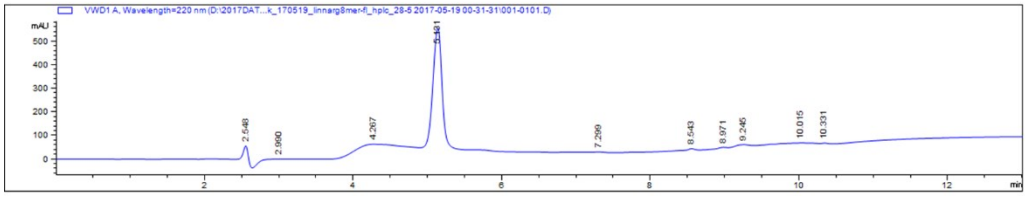

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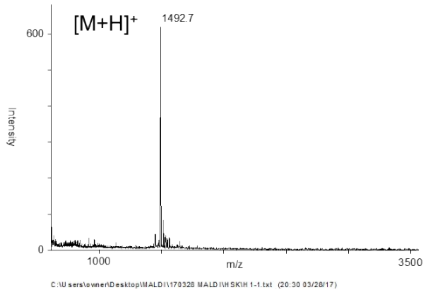

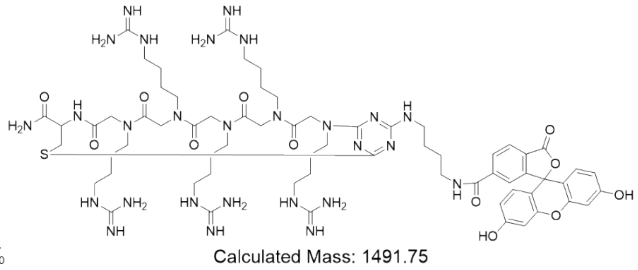

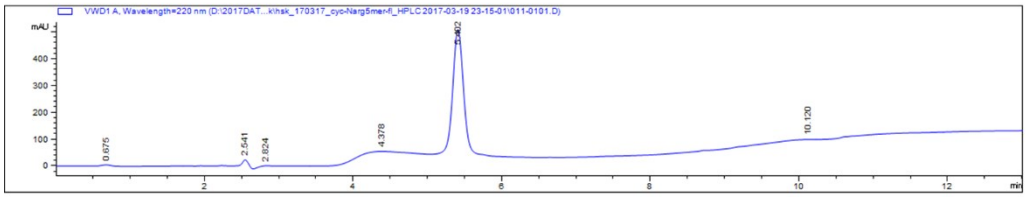

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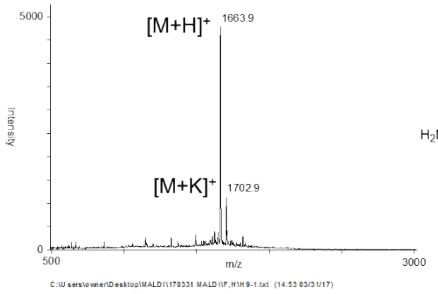

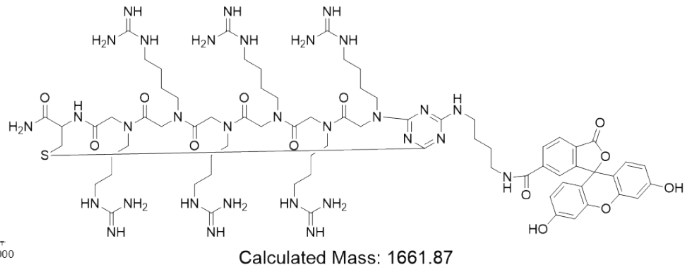

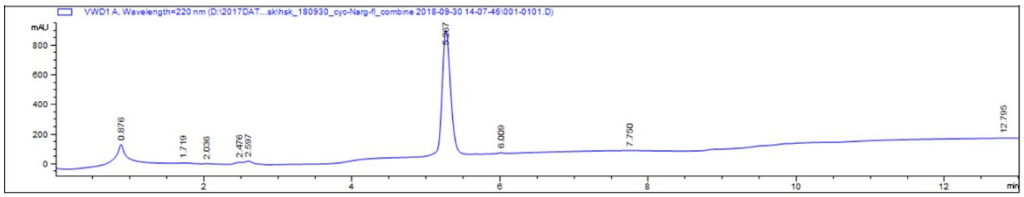

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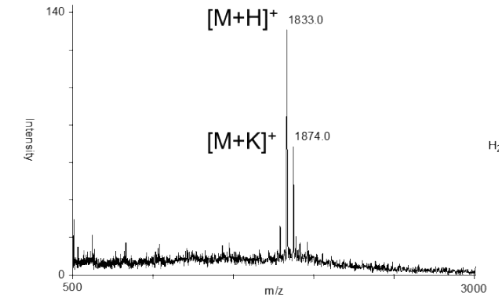

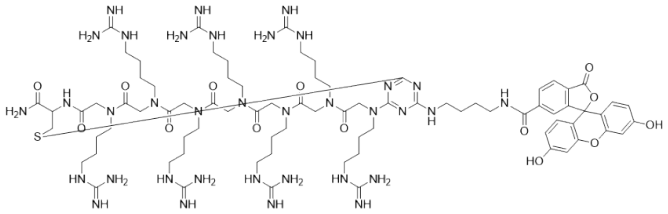

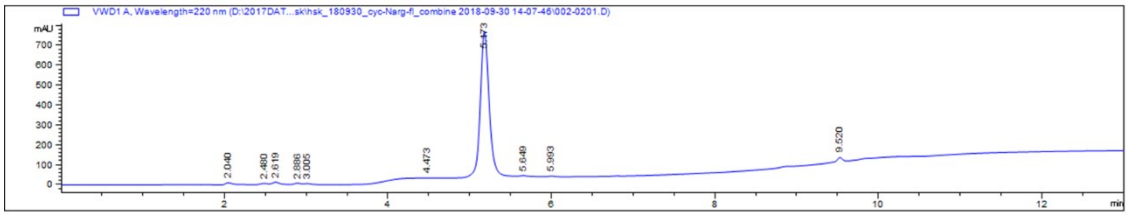

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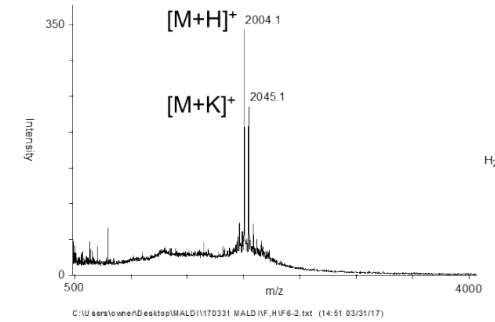

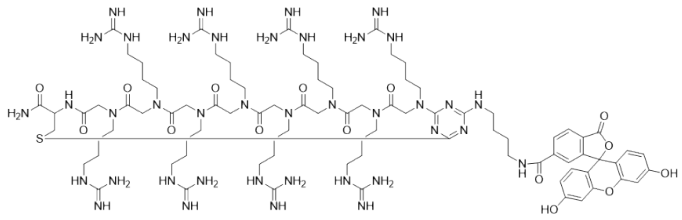

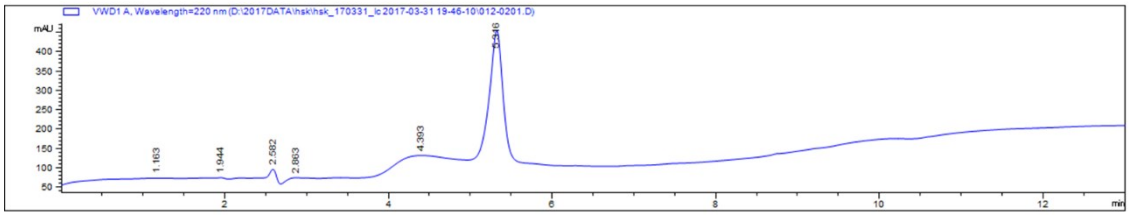

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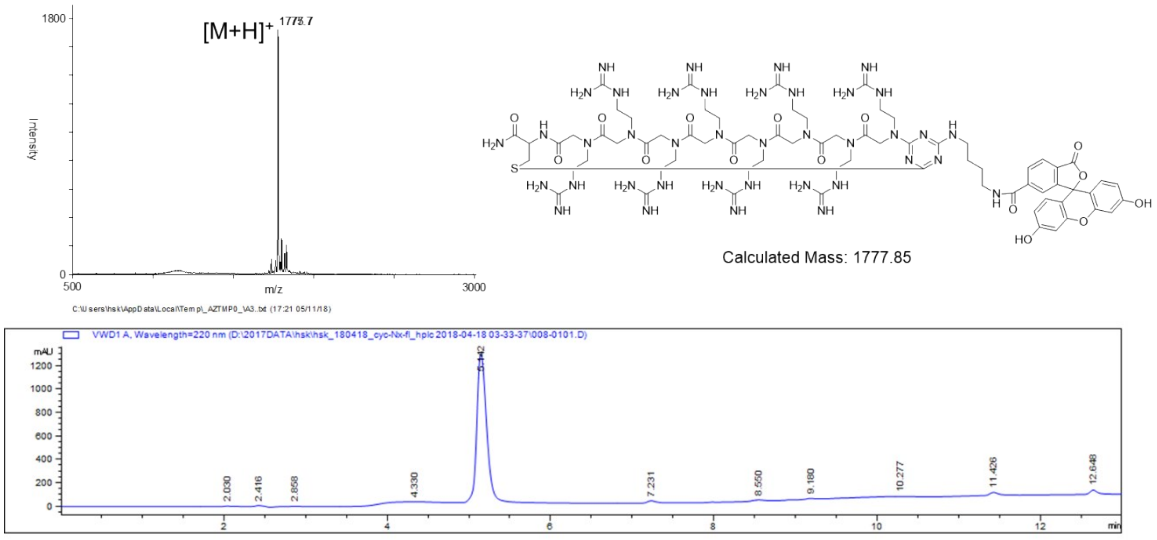

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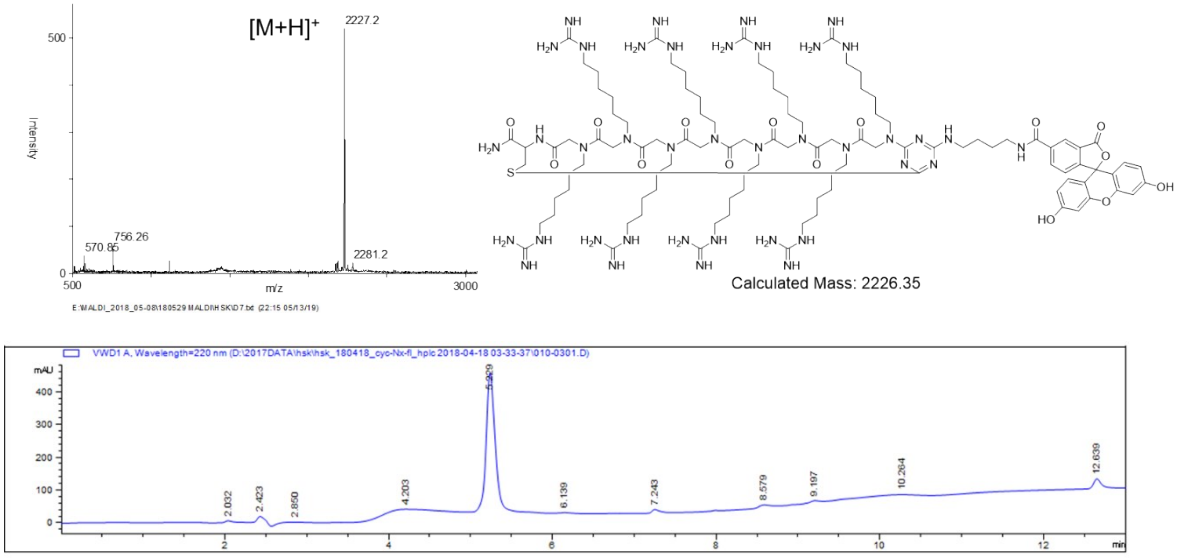

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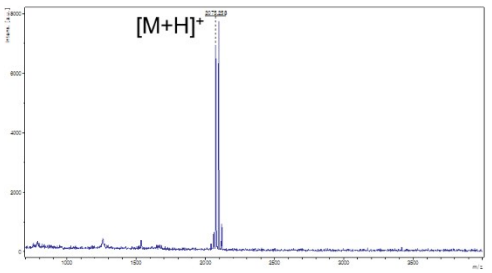

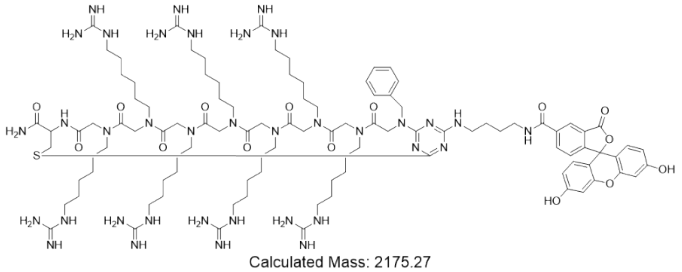

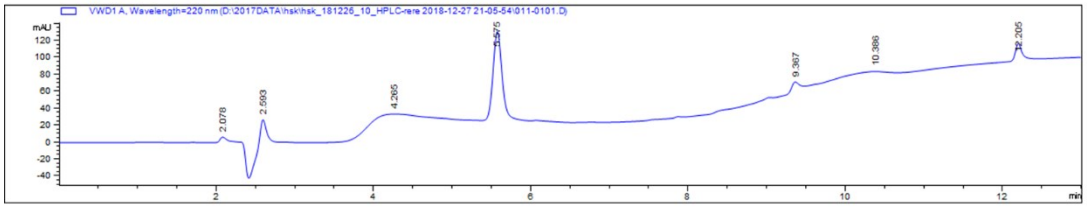

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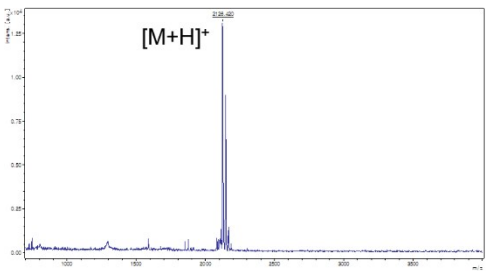

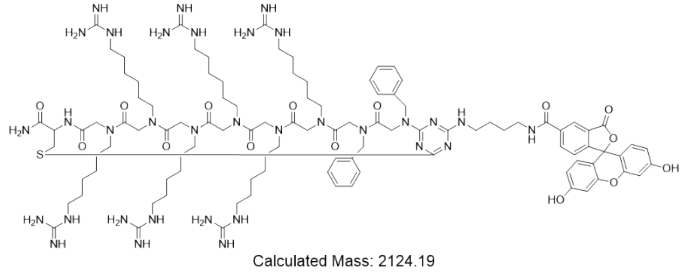

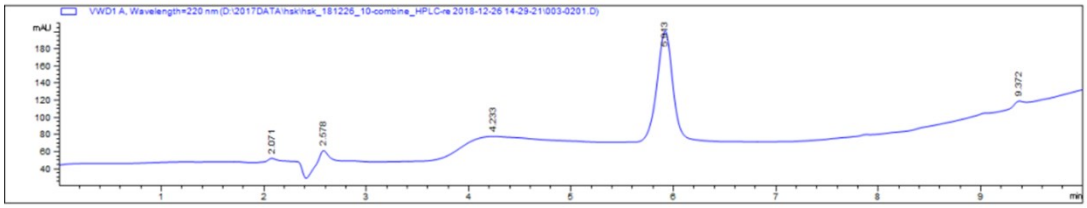

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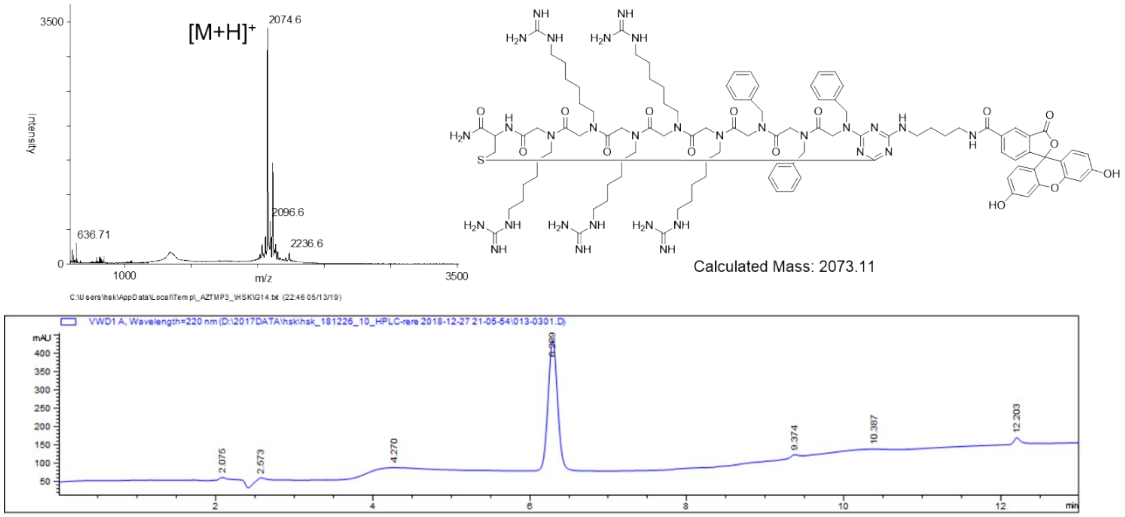

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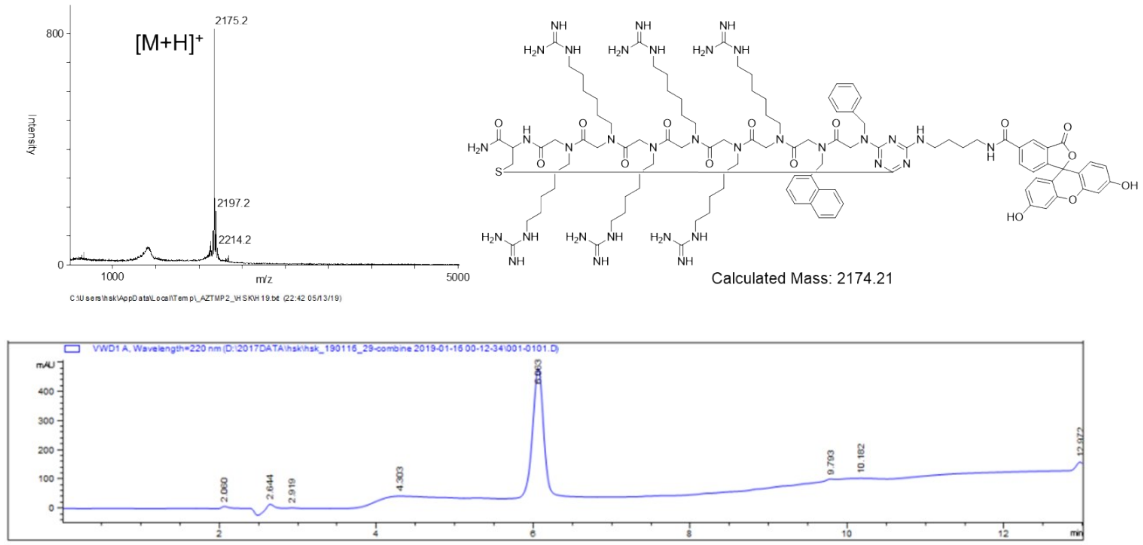

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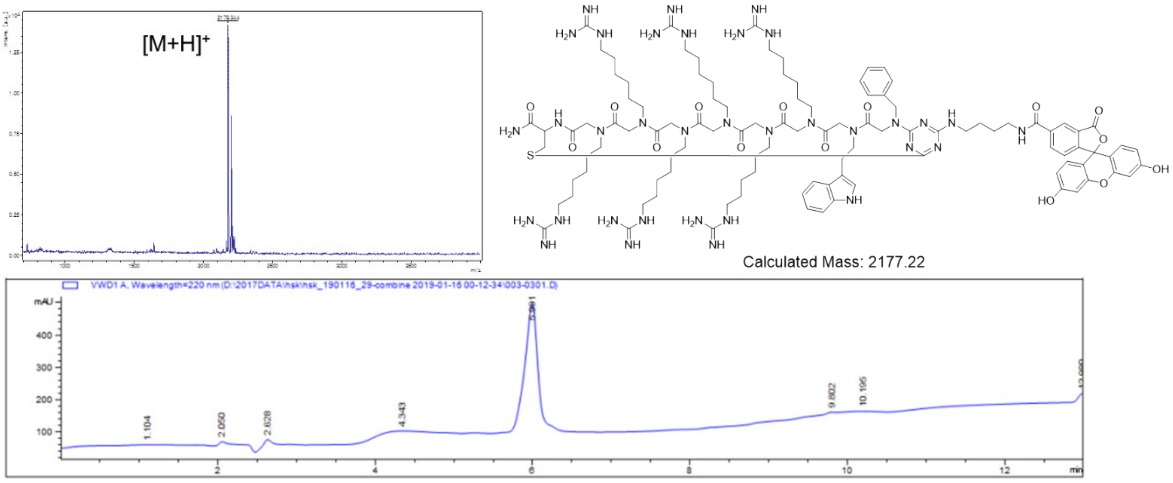


C12

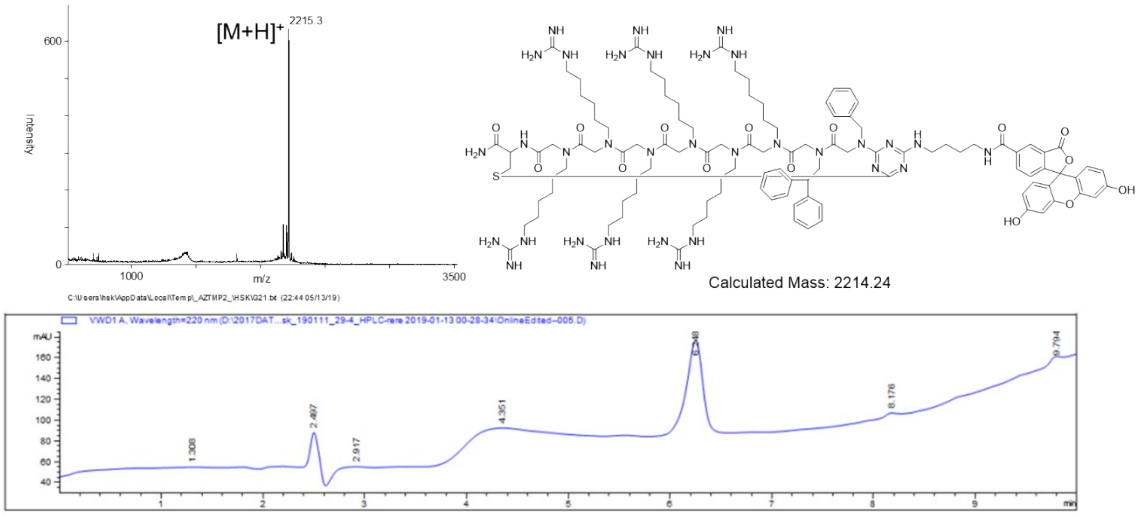
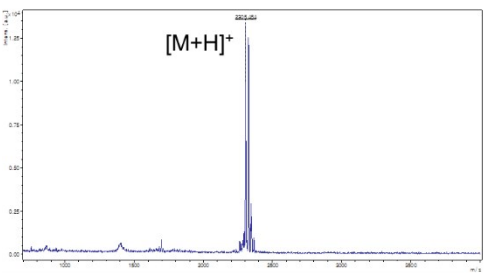
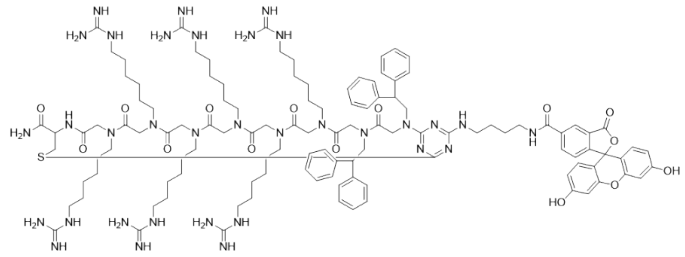


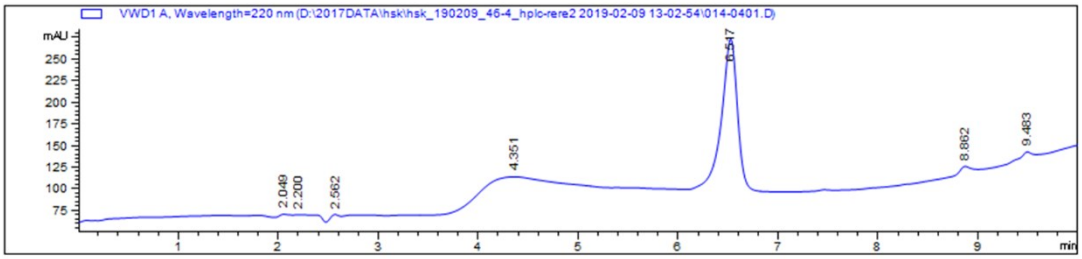
Fig. S1. (Cont'd)

C13

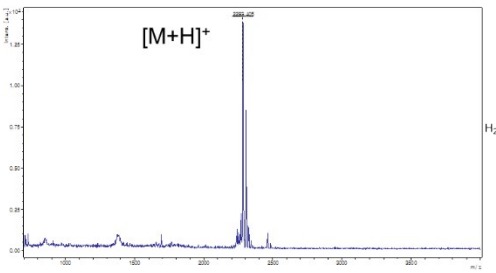


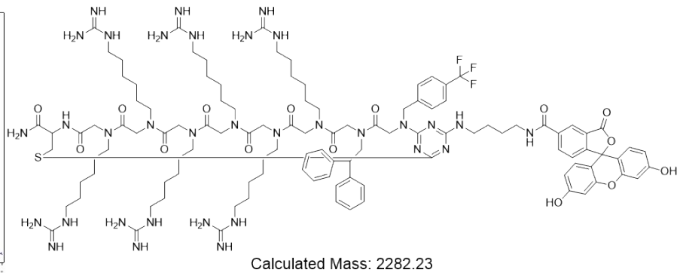


Calculated Mass: 2304.29



C14





Calculated Mass: 2282.23

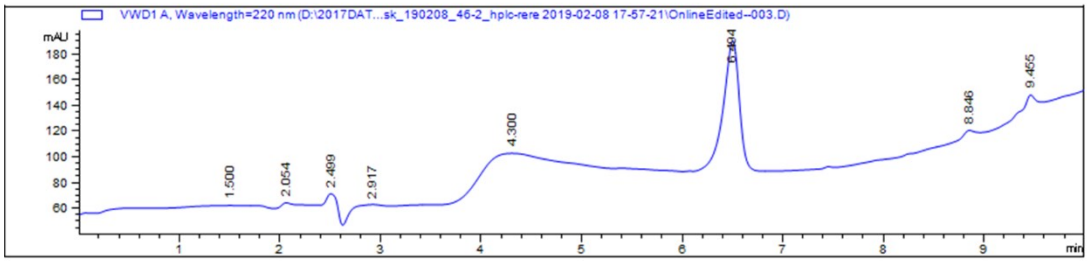
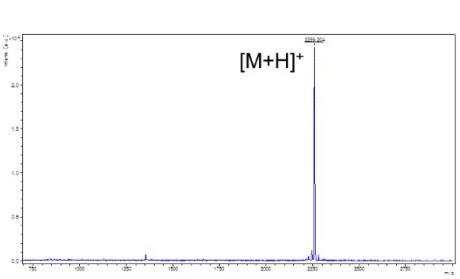
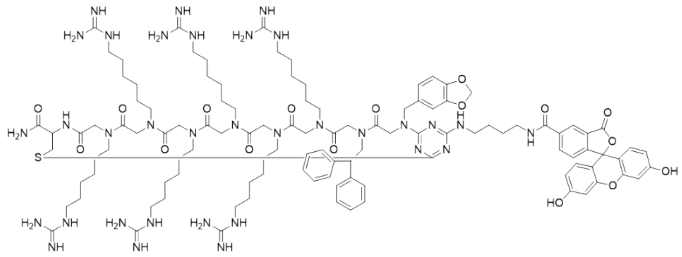


Fig. S1. (Cont'd)

C15





Calculated Mass: 2258.23

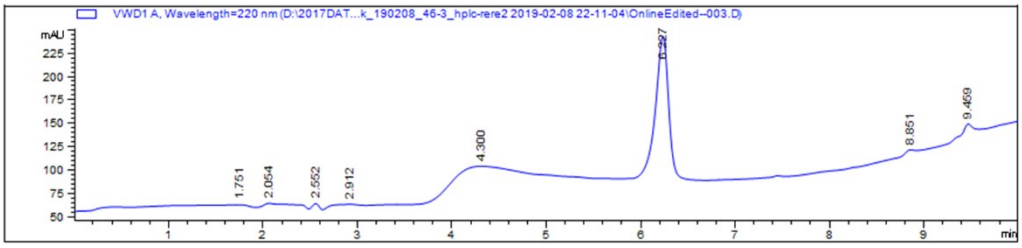


Fig. S2. Confocal microscopy images of A549 treated with 10 μM of **C6** and **C15**. Scale bars represent 20 μm.

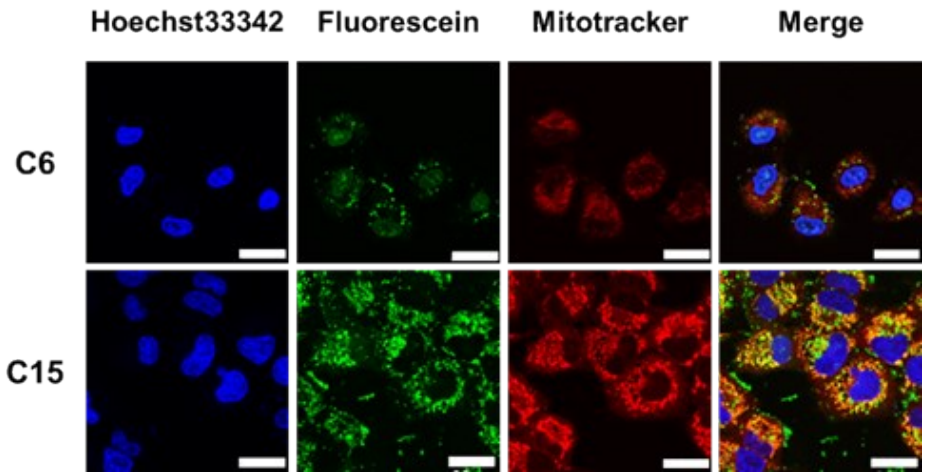
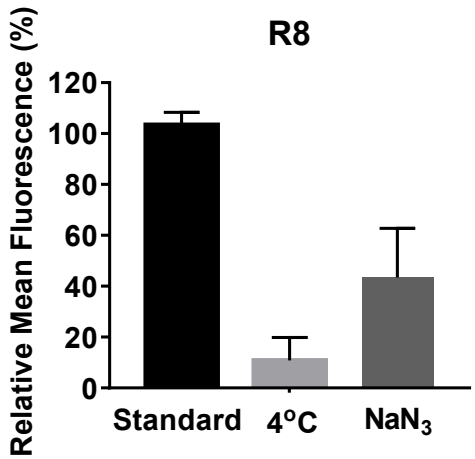


Fig. S3. Cellular uptake of (A) **R8** and (B) **C15** in A549 cells at 4 ℃ or in the presence of sodium azide. The cells were analyzed by flow cytometry. The fluorescence values were normalized to cellular uptake at 37 ℃ in the absence of sodium azide (standard condition).

A



B

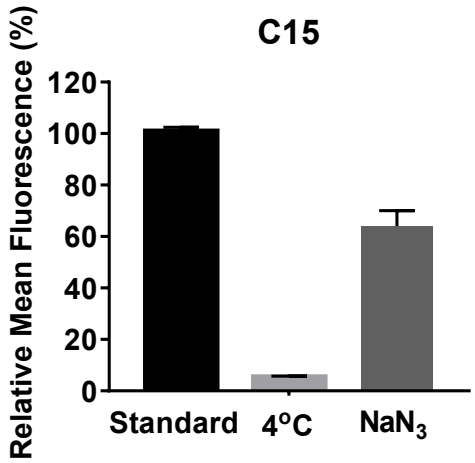


Fig. S4. Proteolytic stability of **C15** and **R8**. Each compound (10 μM) was incubated with trypsin (0.1 μg mL^{-1}) at 37 ℃. Aliquots were withdrawn at indicated time points, quenched by the addition of 50% ACN, and analyzed by HPLC.

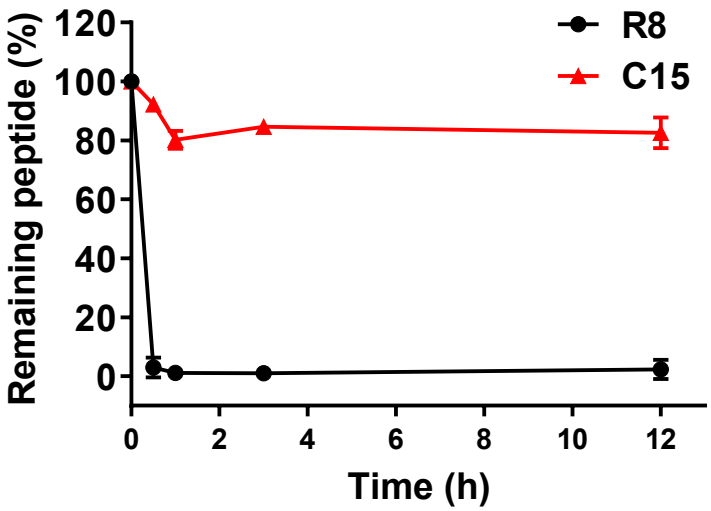
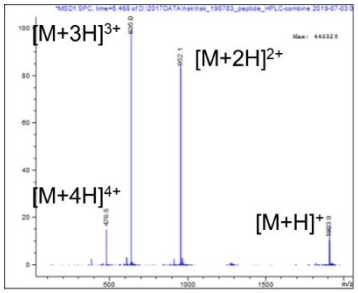
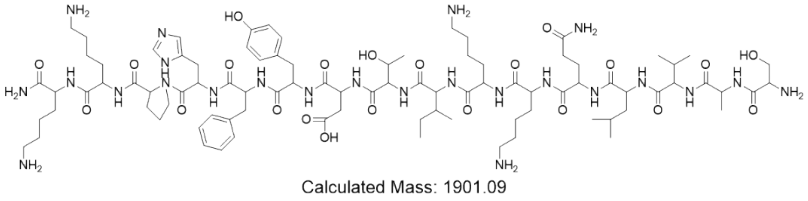
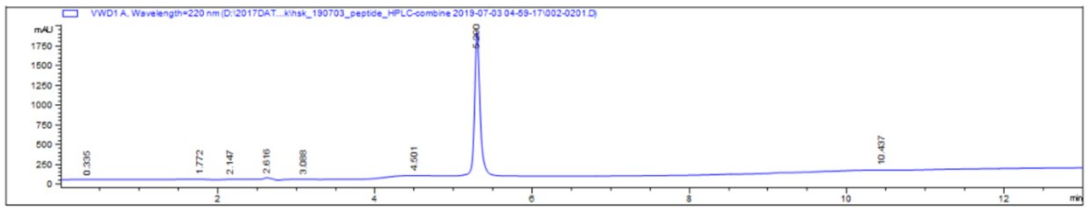


Fig. S5. LC and MS spectra of purified PIP-Box peptide, PIP-Box peptide-linker, **HS-C15**, PIP-Box peptide-**R8** conjugate, and PIP-Box peptide-**C15** conjugate.

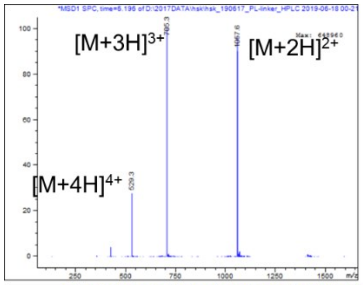
PIP-Box peptide (SAVLQKKITDYFHPKK)

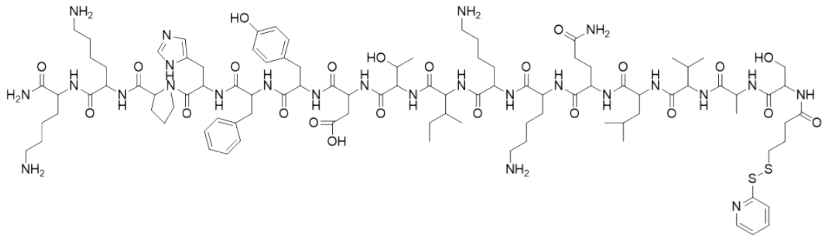






PIP-Box peptide-linker





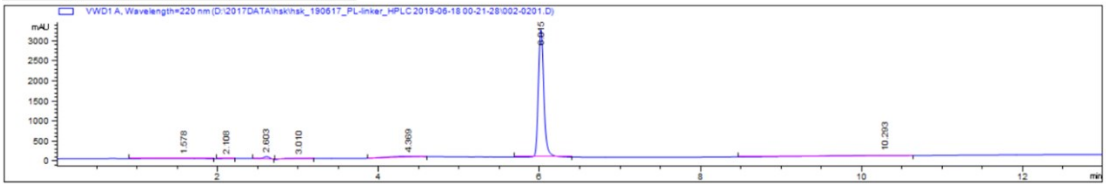
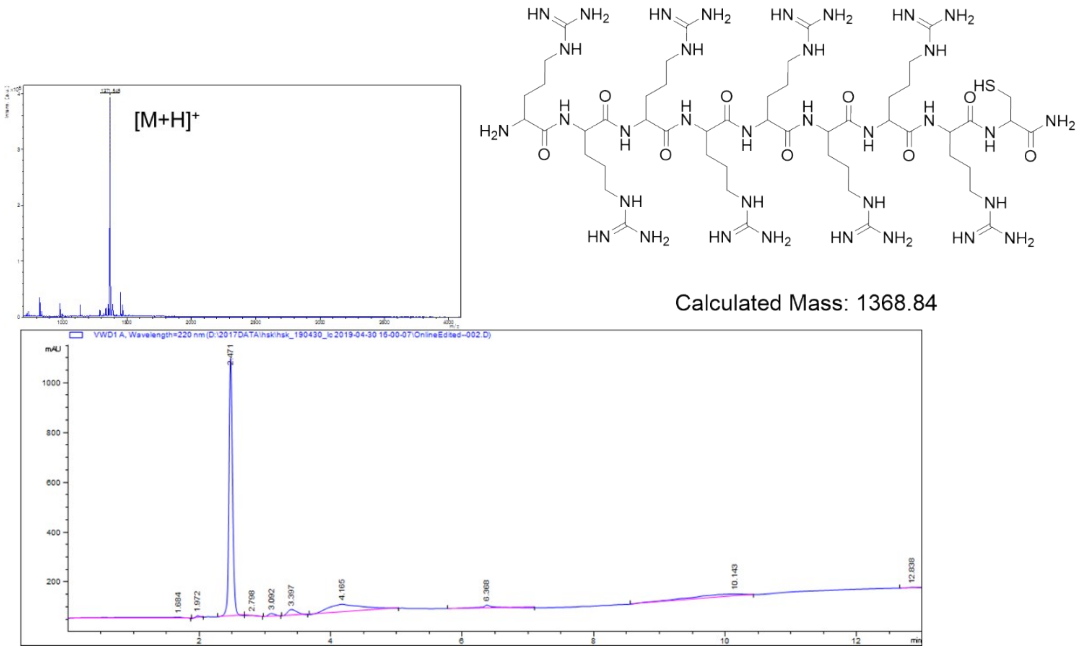


Fig. S5. (Cont'd)

C-R8



HS-C15

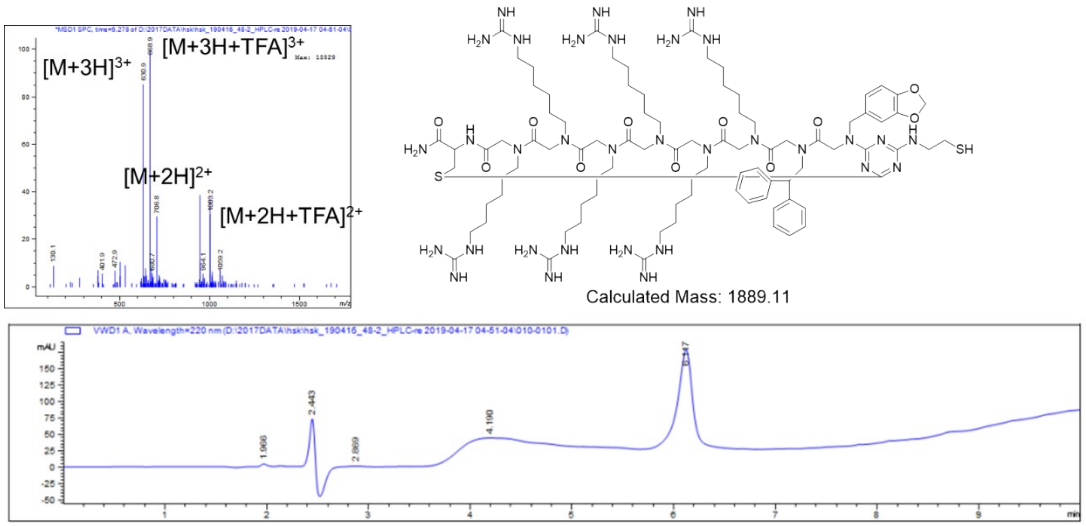
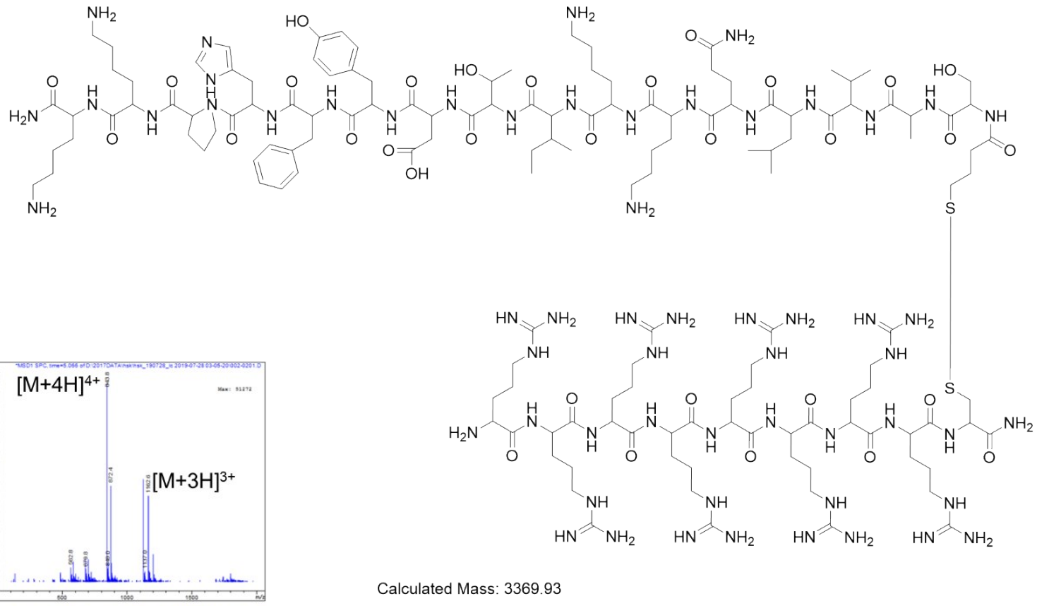


Fig. S5. (Cont'd)

PIP-box peptide-**R8** conjugate



Calculated Mass: 3369.93

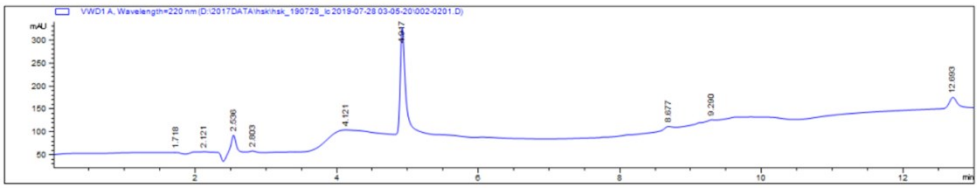
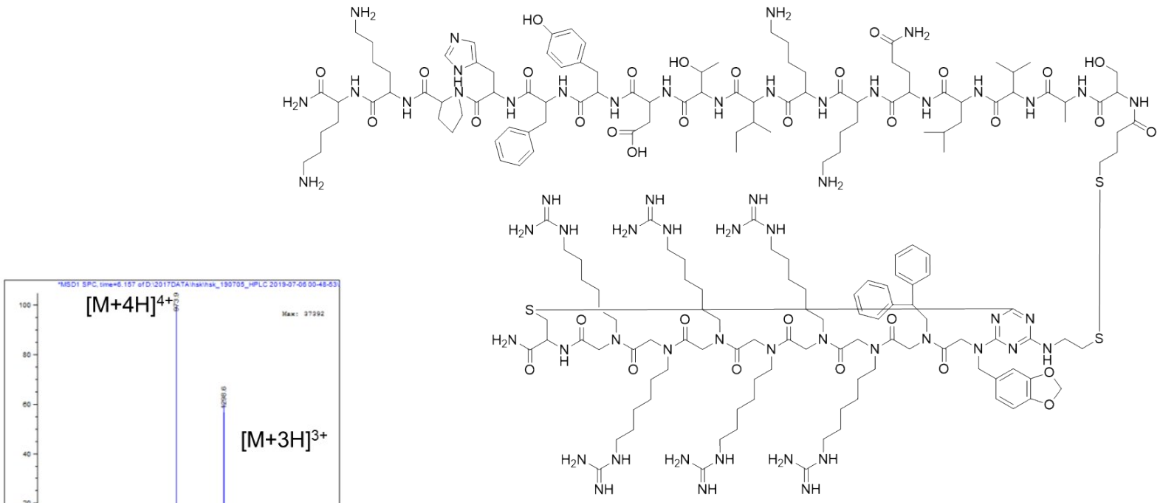


Fig. S5. (Cont'd)

PIP-Box peptide-**C15** conjugate



Calculated Mass: 3890.20

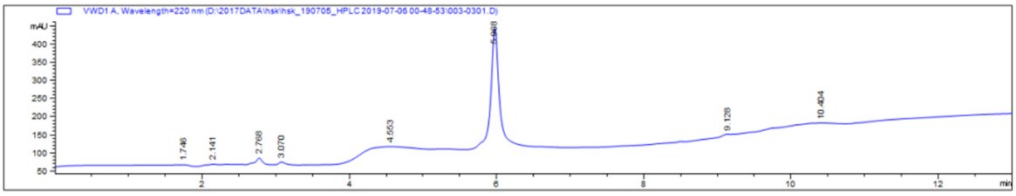


Fig. S6. Effects of PIP-Box peptide-cyclic peptoid conjugate on A549 cell viability as measured by the WST-8 assay. Error bars represent standard deviation from three independent experiments.

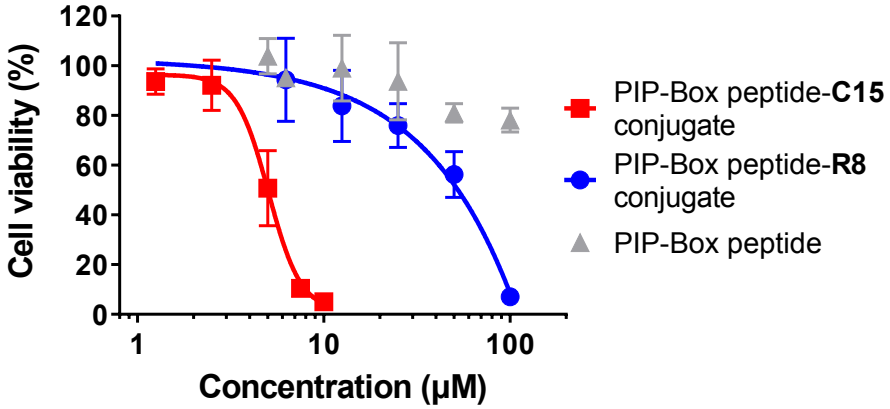
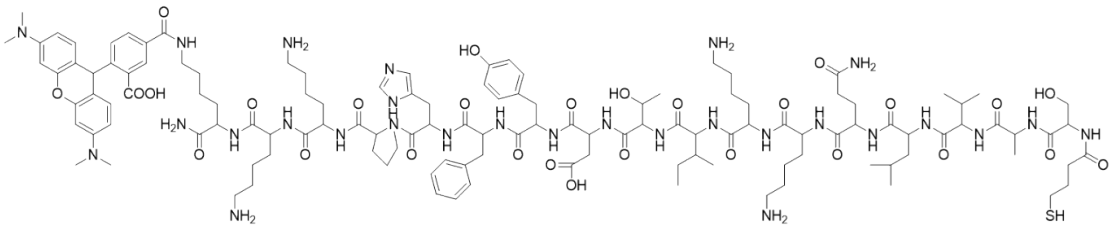
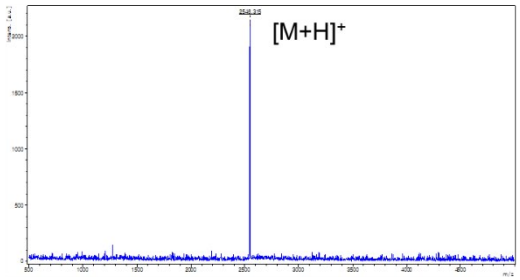


Fig. S7. LC and MS spectra of purified TAMRA-labeled PIP-Box peptide, PIP-Box peptide-**R8** conjugate, and PIP-Box peptide-**C15** conjugate.

TAMRA-PIP-Box peptide



Calculated Mass: 2545.36



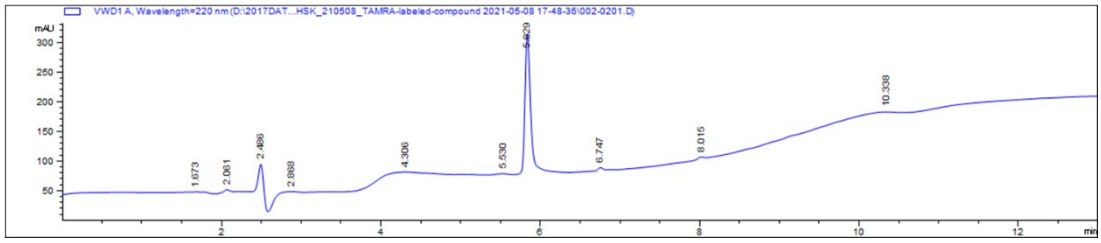


Fig. S7. (Cont'd)

TAMRA-PIP-Box peptide-**R8** conjugate

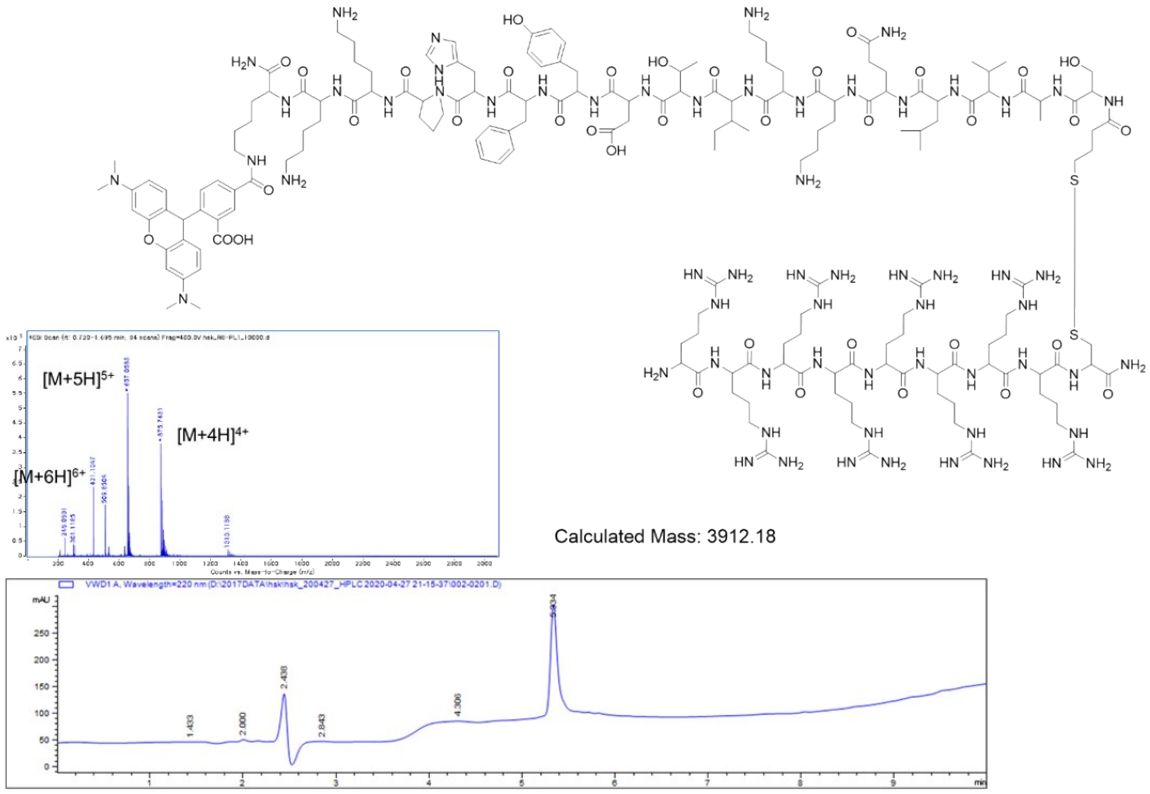


Fig. S7. (Cont'd)

TAMRA-PIP-Box peptide-**C15** conjugate

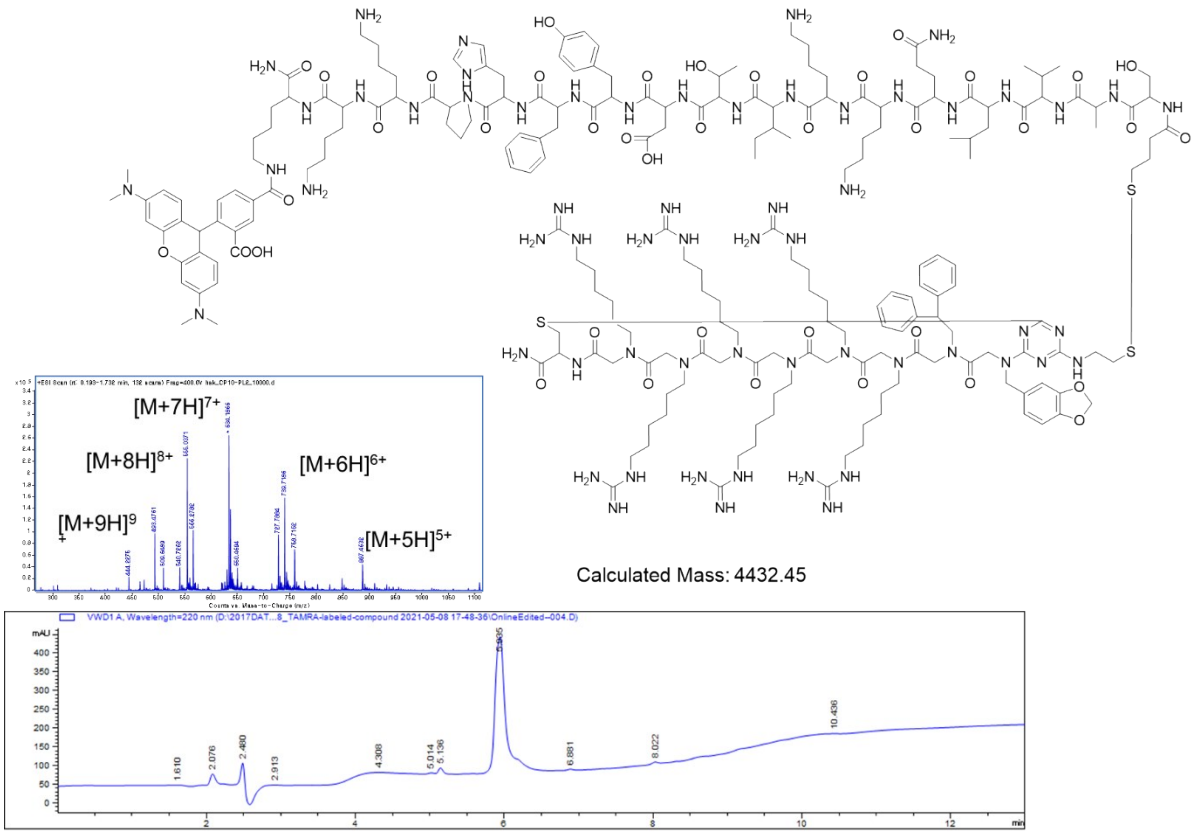


Fig. S8. Cellular uptake of TAMRA, TAMRA-PIP-Box peptide, TAMRA-PIP-Box peptide-**R8** conjugate, and TAMRA-PIP-Box peptide-**C15** conjugate. HeLa cells were incubated with 3 μM of the compounds for 1 hr at 37 ℃ and analyzed by flow cytometry. TMR = TAMRA. Error bars represent standard deviation from three independent experiments.

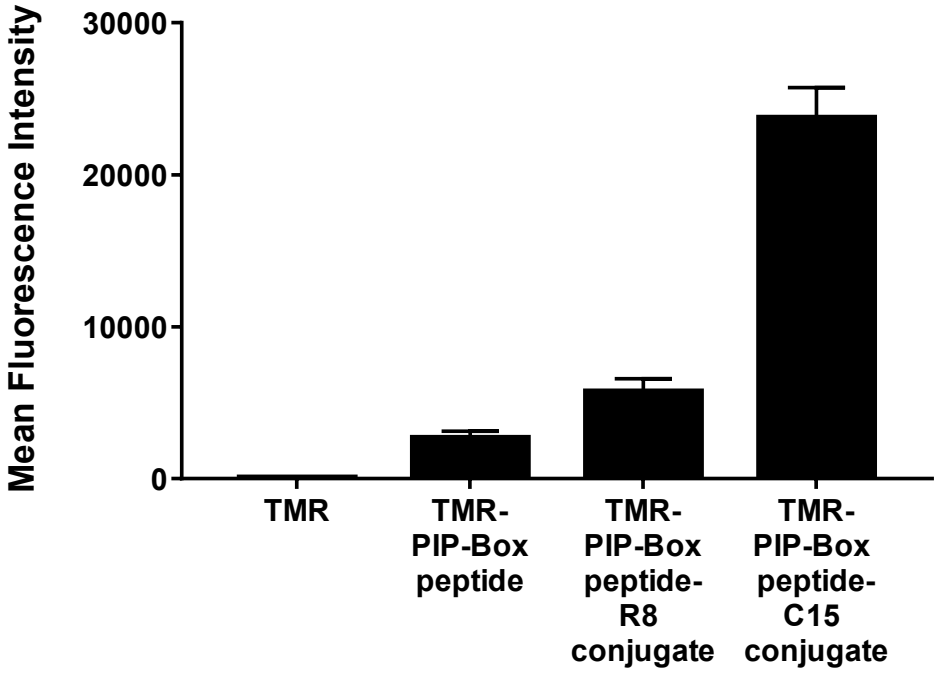
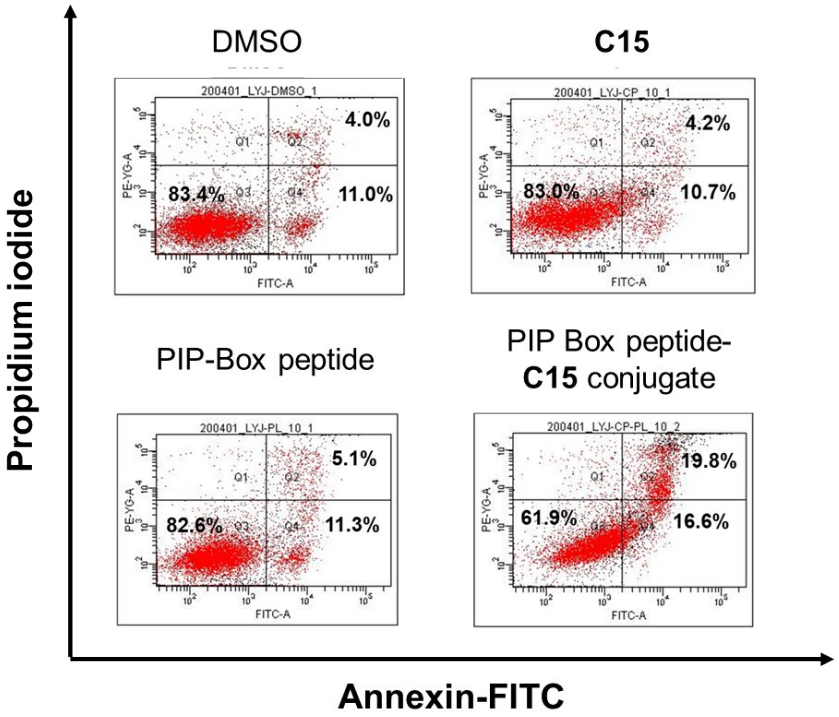


Fig. S9. Flow cytometric analysis of Annexin V/PI-stained, apoptotic HeLa cells after treatment of DMSO, PIP-Box peptide (10 μM), **C15** (10 μM), or PIP Box peptide-**C15** conjugate (10 μM).



Reference

1. E. A. Dubikovskaya, S. H. Thorne, T. H. Pillow, C. H. Contag and P. A. Wender, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12128-12133.
2. A. B. Mahon and P. S. Arora, *Chem. Commun.*, 2012, **48**, 1416-1418.