Palindromic Peptide Foldamers : A Strategy for Structural Stability and Cellular Uptake

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Peptide synthesis

The peptides were synthesized using microwave-assisted Fmoc-based solid-phase methods on a Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM corp., NC). A representative coupling and deprotection cycle are described as follows. Rink Amide Protide (LL) resin was soaked in CH_2Cl_2. After the resin had been washed with DMF, Fmoc-amino acid (5 equiv.), Oxyma Pure (10 equiv.) and DIC (10 equiv.) dissolved in a solution of DMF, were added to the resin. For additional coupling, the same condition or Fmoc-amino acid (6 equiv.), Oxyma Pure (6 equiv.) and DIC (6 equiv.) dissolved in a solution of DMF, were added to the resin. Fmoc protective groups were deprotected using 20% piperidine in DMF. Ring-closing metathesis reactions were performed using 20 mol% 2nd generation Grubbs catalyst in 1,2-dichloroethane for 15 min at 60 °C by MARS 6 (CEM corp.). This cycle was performed three times. FAM (5(6)-carboxyfluorescein) was introduced into the side chain of lysine. The ivDde protecting group of lysine was deprotected with 2% hydrazine in DMF. After deprotection, FAM (4 equiv.), HBTU (4 equiv.), HOBt•H_2O (4 equiv.) and DIPEA (8 equiv.) dissolved in DMF were added to the resin. The resins were suspended in cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropylsilane) and cleaved at 42 °C for 30 min on Razor (CEM corp.). TFA was evaporated to a small volume under a stream of N_2 and dripped into cold ether to precipitate the peptide. The crude peptide was purified by using a JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD (19 × 250 mm, 5 μm, Waters). Mobile phases A and B were 0.1% TFA in water and 0.1% TFA in MeCN, respectively. The fractions containing the target product were collected, and lyophilized to obtain the desired peptides. The purified peptides were analyzed by a JASCO analytical HPLC system equipped with a J-Pak Core C18 column (2.7 μm HP, 4.6×100 mm, JASCO Engineering). Mobile phases A and B were 0.1% TFA in water and 0.1% TFA in MeCN, respectively. MS identification was performed by a Shimadzu Q-TOF MS equipped with an electrospray ionization source.

CD spectra measurements

The synthesized peptides (Peptide stock ACN : H_2O=1 : 1) were dissolved in 10 mM HEPES (pH 7.5) at a concentration of 50 μM. Peptides were denatured by dissolving the peptides in 10 mM HEPES (pH 7.5) containing 1 M guanidine hydrochloride. CD spectra were measured on a CD spectrometer J-1100 (JASCO). The data were acquired in the condition of measurement (Wavelength 190-260 nm, Response 1 s, Bandwidth 1 nm, Step resolution 0.1 nm, Scan speed 20 nm/min, Accumulations 3).

Computational methods

Computational studies were performed on MOE 2024.06 software (CCD, Canada). Peptide models were constructed using the initial secondary structure as an α-helix. Peptide conformation minimization was carried on by molecular dynamics (low-mode MD) using AMBER-10 EHT force field and Born solvation conditions with tethering peptide main-chain. Obtained minimized structures were solvated under periodic boundary condition and were neutralized by adding a counter ion (Cl^-). Energy minimization of whole systems was performed by gradually releasing the restraints. Temperature-raising MD simulations was carried out under AMBER-10 EHT force field, R-Field solvation, particle mesh Ewald method with following time evolution; equilibration 0–10 ps at 10 K, heating 10–210 ps at 10–310 K, production 210–500 ps at 310 K. Temperature-raising MD simulations was performed on NAMD creating the batch file on MOE 2024.06 software. RMSD was calculated from peptide main-chain.

Cellular uptake of peptides

MCF-7 cells were seeded on clear 24-well plates (100,000 cells/well) and incubated overnight at 37 °C in 500 μL of DMEM containing 10% FBS. The peptide solutions were added to each well at 3 μM. After 24 h or 72 h incubation, the medium was removed and the cells were washed with PBS and treated with trypsin. After addition of the medium containing 10% FBS, the cells were centrifuged at 1500 rpm for 5 min at 4 °C and collected cell pellets were subjected to flow cytometer and LC-MS/MS. For flow cytometer analysis, the obtained cell pellets were suspended in PBS, centrifuged at 1500 rpm for 5 min at 4°C, and then the collected cells were suspended in 500 μL of 2% FBS in PBS. The mean fluorescence intensity in cells was measured using flow cytometer (SA3800 Cell Analyzer (Sony Corp., Tokyo)). The results are presented as the mean and standard deviation of repeated measurements. The fluorescence intensity was calculated based the ratio of quantum yield among peptides.

Inhibition of intracellular uptake pathways

MCF-7 cells were seeded onto clear 24-well culture plates (100,000 cells/well) and incubated overnight at 37°C in 500 μL of DMEM containing 10% FBS. After the cells were pre-incubated at 37°C or 4 °C for 30 minutes, peptide solution was added to each well. After 2 h incubation at 37 °C or 4 °C, the medium was removed and the cells were treated with trypsin. After addition of the medium containing 10% FBS, the cells were centrifuged at 1500 rpm for 5 min at 4 °C and collected cell pelleted. The obtained cell pellets were suspended in PBS, centrifuged at 1500 rpm for 5 min at 4 °C, and then the collected cells were suspended in 500 μL of 2% FBS in PBS. The mean fluorescence intensity in cells was measured using flow cytometer (SA3800 Cell Analyzer (Sony Corp., Tokyo)). The results are presented as the mean and standard deviation of repeated measurements.

LC-MS/MS analysis

For quantification of palindromic peptides in cells by LC-MS/MS, cell pellets were mixed with 200 μL of methanol containing 1% formic acid to extract peptides. After removal of protein precipitant by centrifugation, the extracts were filtered and subjected to LC-MS/MS analysis using Ultimate3000 UHPLC with TSQ-Quantiva (Thermo Fisher Scientific, Waltham, MA, USA). A Triart Bio C18 column (3 μm, 2.1 mm × 100 mm) (YMC, Kyoto, Japan) was used for LC at 55 °C. Mobile phase A consisted of water containing 0.2% formic acid, and mobile phase B consisted of methanol containing 0.2% formic acid. The flow rate was 0.3 mL/min, and the injected sample volume was 1 μL. The gradient program of the mobile phase was initiated by 40% B and increased to 100% B at 2.5 min, followed by maintaining 100% B for 1.5 min, and immediate decrease to 40% B and increase to 100% B every 0.1 min for 0.5 min), and equilibrated at 40% B for next run. The mass spectrometer was operated in the heated electrospray ionization (ESI) mode with following ion source parameter: Spray voltage: 3500V, Sheath Gas: 40 Arb, Aux Gas: 10 Arb, Sweep Gas: 1 Arb, Transfer Temp: 350 °C, Vaporizer Temp: 200 °C. The data were acquired in the selective reaction monitoring mode (Collision Energy: 30 V for 1, and 27.5V for **Nst-1** and **Cst-1**; Collision Gas: Ar, 1.5 mTorr; Mass Transition (m/z): 767.0/762.5 for 1, and 784.0/779.5 for **Nst-1** and **Cst-1**; Q1/Q3 resolution (FWHM): 2/2). Along with sample analysis, calibration standards (1, 3, 10 μM of individual peptides in methanol containing 1% formic acid) were also analyzed. TraceFinder 4.1 (Thermo Fisher Scientific) was used for peak detection and smoothening. Following the quantification of the peak area, the calibration curve was analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) and calculated peptide concentrations in cells.

Table S1. Peptide sequences and purity.

Peptide	Sequence	Purity (%)
1	Ac-LRLLRLLRLL-K(FAM)-LLRLLRLLRL-NH_2	>99
Nst-1	Ac-LR-R_8*-LRLLRLL-S_5*-K(FAM)-LLRLLRLLRL-NH_2	>99
Cst-1	Ac-LRLLRLLRLL-K(FAM)-R_8*-LRLLRL-S_5*-RL-NH_2	>99

FAM = 5(6)-carboxyfluorescein; X = β-alanine. The cyclization between *i* and *i* + 7 of (*S*)-4-pentenylalanine (S_5) and (*R*)-7-octenylalanine (R_8) is indicated by an asterisk.

Method

HPLC: Conditions = solvent A 0.1% TFA in water, solvent B 0.1% TFA in MeCN

Gradient = 10-90% gradient of solvent B for 10 min

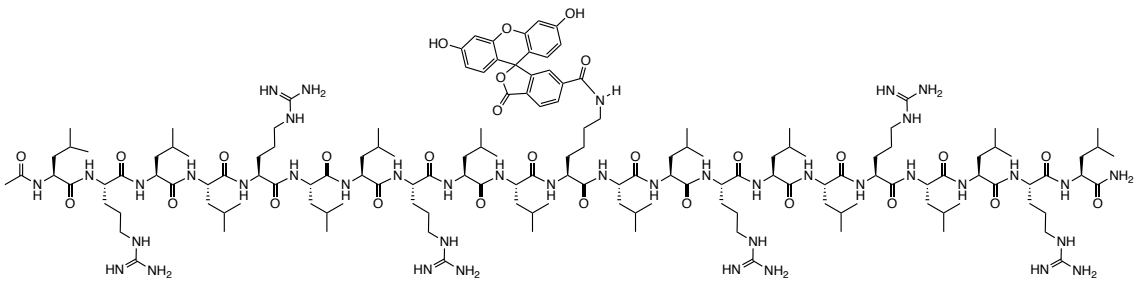
Column: J-Pak Core C18 column (2.7 μm HP, 4.6×100 mm)

Flow rate: 1.2 mL/min

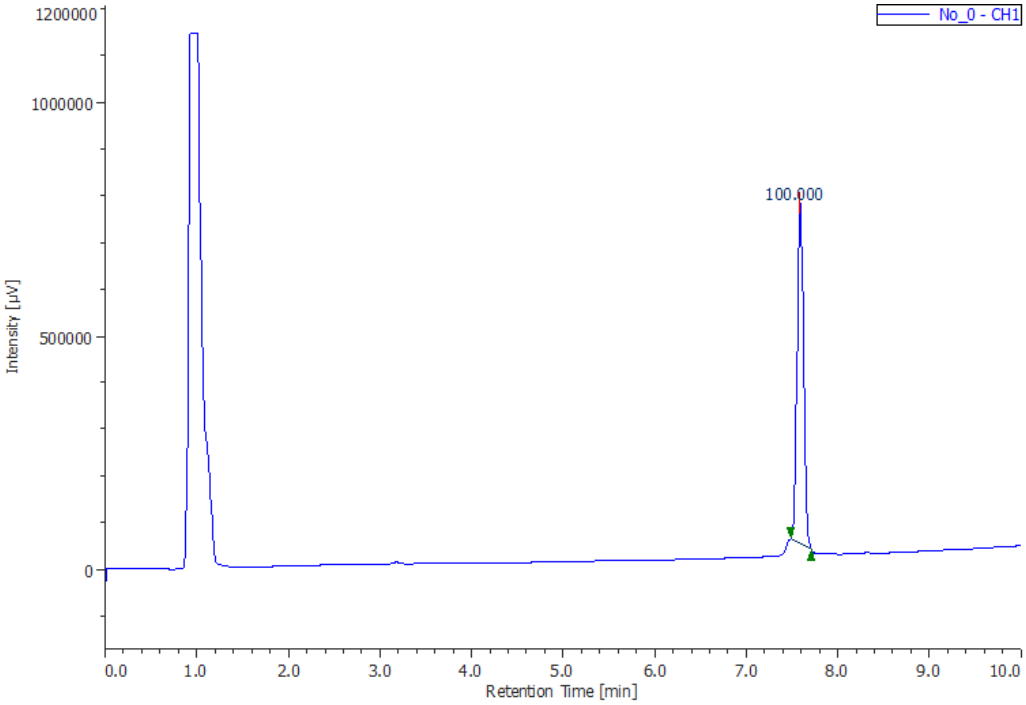
Temperature: 40 °C

Detection: 220 nm

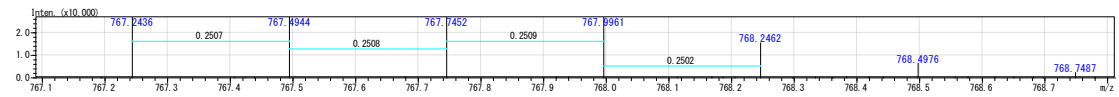
Peptide **1**



Analytical HPLC of Peptide **1**

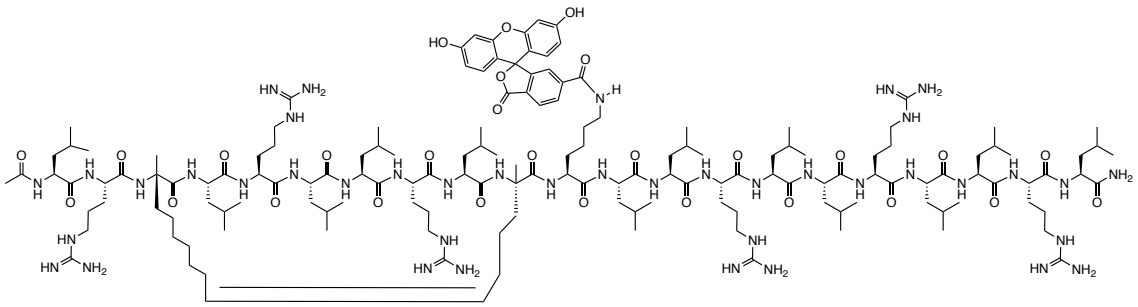


LC-MS analysis of Peptide **1**

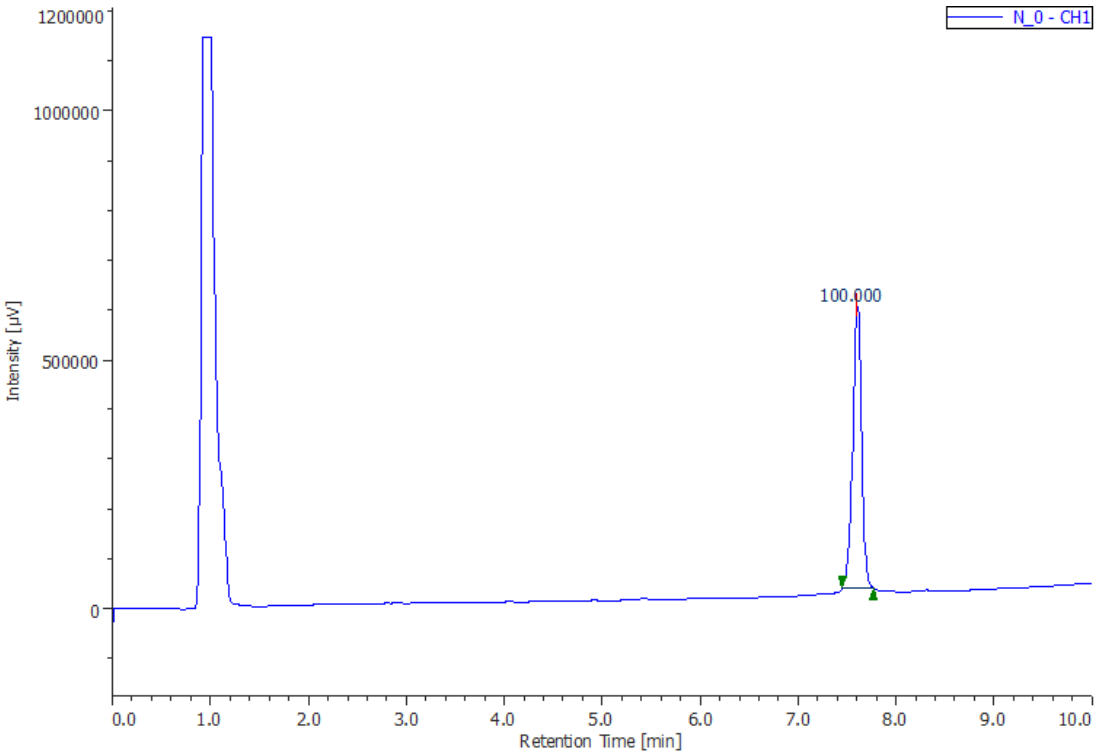


Calculated Mass $(M+4H)^{4+}$: 767.4990, Found Mass $(M+4H)^{4+}$: 767.2436

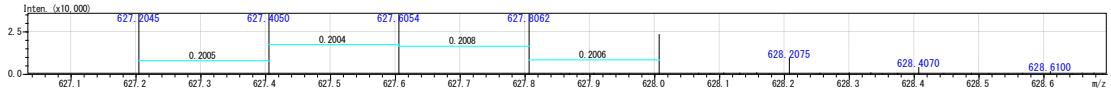
Peptide **Nst-1**



Analytical HPLC of Peptide **Nst-1**

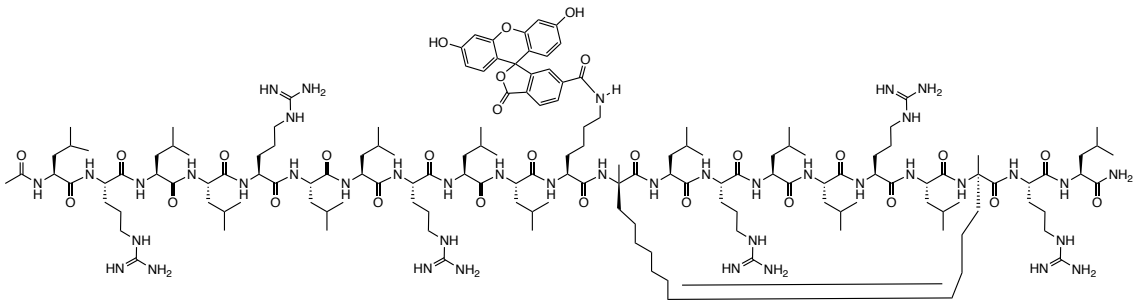


LC-MS analysis of Peptide **Nst-1**

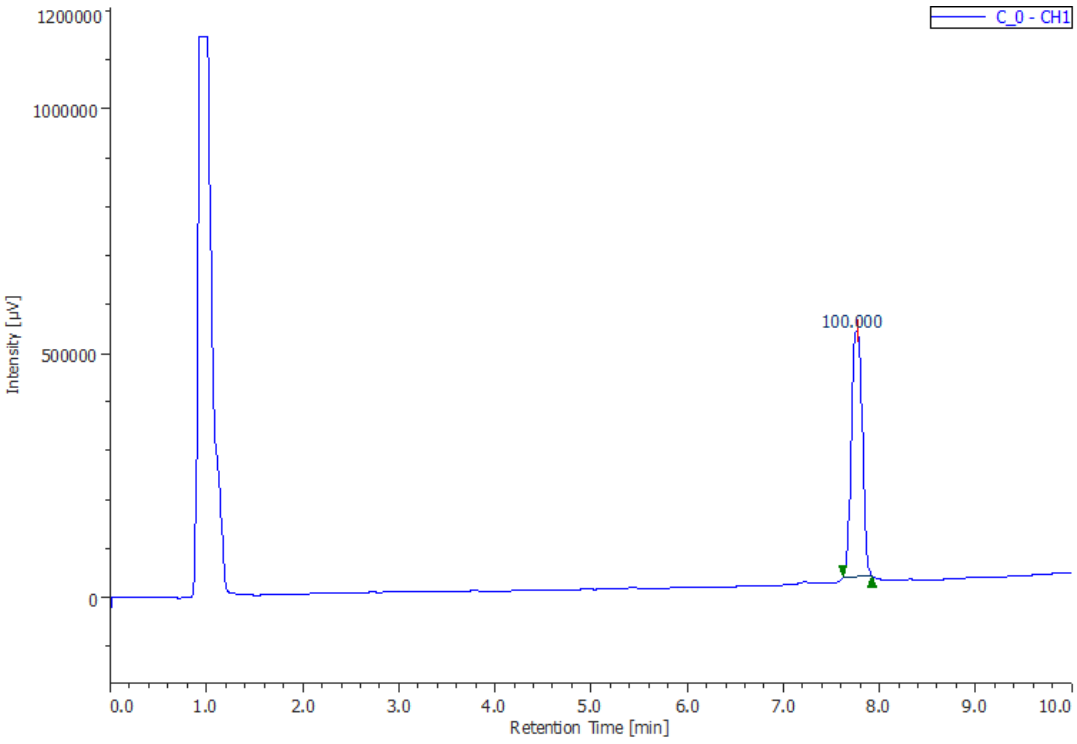


Calculated Mass $(M+5H)^{5+}$: 627.4100, Found Mass $(M+5H)^{5+}$: 627.2045

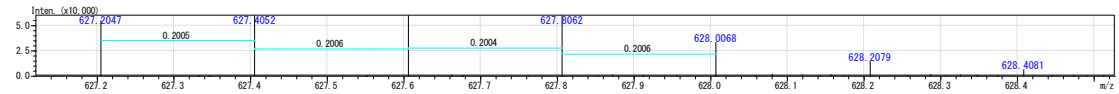
Peptide **Cst-1**



Analytical HPLC of Peptide **Cst-1**



LC-MS analysis of Peptide **Cst-1**



Calculated Mass $(M+5H)^{5+}$: 627.4100, Found Mass $(M+5H)^{5+}$: 627.2047

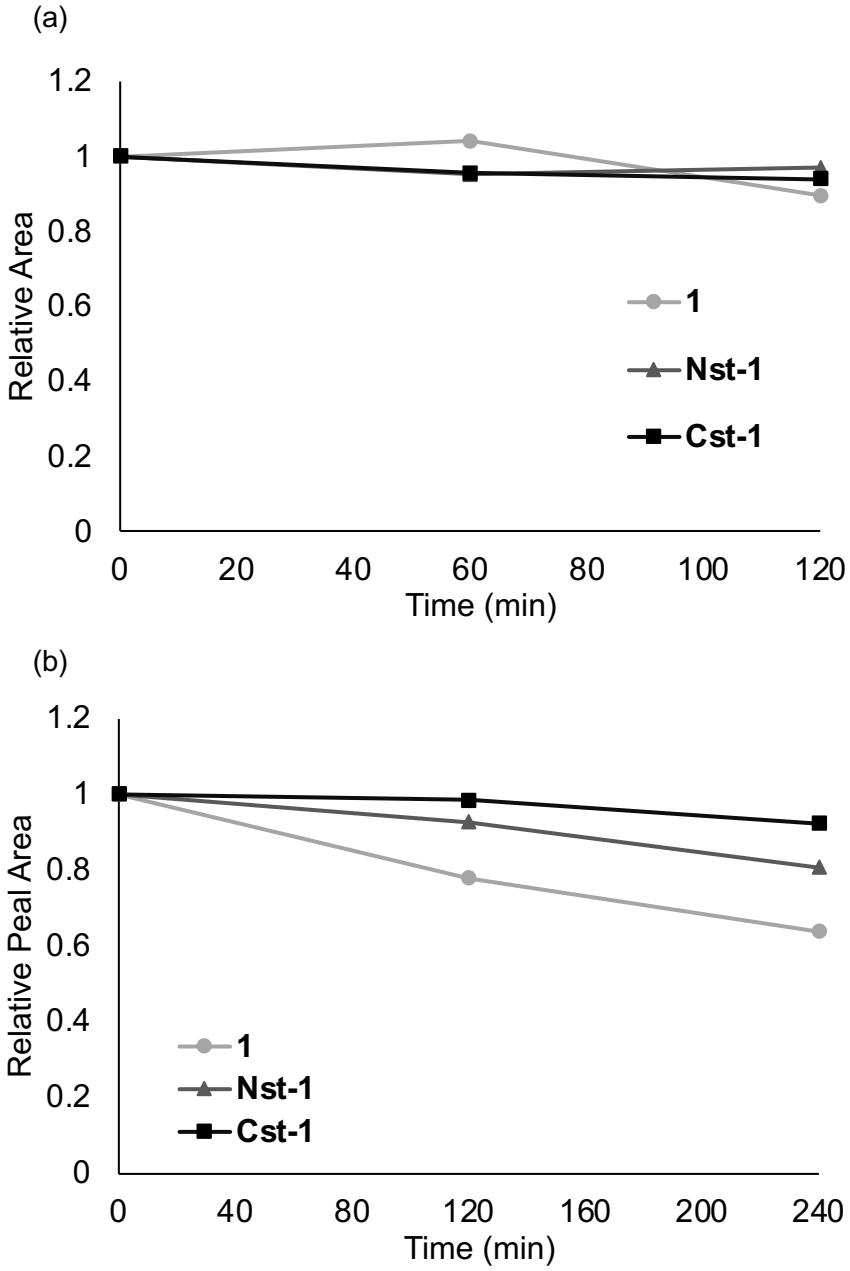
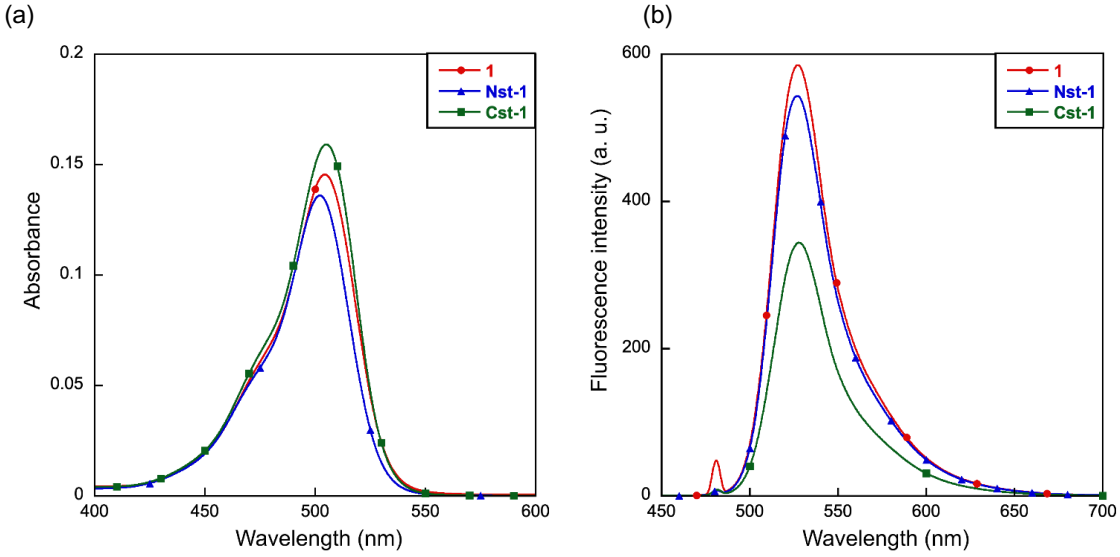


Figure S1. Evaluation of proteolytic stability of peptides after incubation with (a) trypsin at 37°C for 0, 60, and 120 min and (b) proteinase K at 37°C for 0, 120, and 240 min.



(c)

Peptide	(%)
1	100
Nst-1	98.56
Cst-1	55.09

Figure S2. Photophysical properties of peptides. (a) Absorption spectra of peptides. (b) Fluorescence spectra of peptides. Excitation wavelength was 480 nm. (c) The ratio of quantum yield of peptides relative to peptide **1**.