Supplementary materials

Magainin 2-derived stapled peptides derived with the ability to deliver pDNA, mRNA, and siRNA into cells

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Contents

Materials and methods

1. Peptide synthesis

The peptides were synthesized by Fmoc-based solid-phase methods. A representative coupling and deprotection cycle are described as follows. Rink Amide ChemMatrix resin was soaked for 30 min in CH_2Cl_2. After the resin had been washed with DMF, the Fmoc protecting group was removed by treatment with 25% piperidine in DMF for 15 min at room temperature. Amino acids were coupled for 1.5 h using 4 equiv of Fmoc-protected amino acid, 4 equiv of (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholinocarbenium hexafluorophosphate (COMU) as the activating agent, and 8 equiv of diisopropylethylamine (DIPEA) in NMP.

Ring closing metathesis reactions were performed using 20 mol% 2nd generation Grubbs catalyst in 1,2-dichloroethane under bubbling N_2 gas. The resin was suspended in cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropylsilane) at room temperature for 3 h. TFA was evaporated to a small volume under a stream of N_2 and dripped into cold ether to precipitate the peptide. The peptides were dissolved in dimethyl sulfoxide and purified using reverse-phase HPLC (Waters) using a Discovery® BIO Wide Pore C18 column (Supelco, Bellefonte, PA, USA) (25 cm × 21.2 mm solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10.0 mL/min, gradient: 10-90% gradient of solvent B over 40 min). After purification, the peptide solutions were lyophilized, and peptide purity was assessed using UPLC (Waters) and a ACQUITY UPLC® BEH C18 1.7 μm column (2.1 × 50 mm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 0.5 mL/min, gradient: 10-90% gradient of solvent B over 4 min).

2. Preparation of peptide/pDNA complex

Each peptide and pDNA were dissolved separately in 10 mM HEPES buffer (pH 7.5). A twice excess amount of peptide solutions at various concentrations was added to the mRNA solution to form peptide/pDNA complexes of different compositions. The final concentration of pDNA was adjusted to 33.3 μg/mL and the complex solution was stored at room temperature for 15 min before use. The N/P ratio is defined as the molar ratio of the guanidino groups in the peptide to the phosphate group in the pDNA.

3. Transfection efficiency of peptide/pDNA

HeLa cells were seeded onto clear 24-well plates each well (100,000

cells/well) and incubated overnight at 37°C in 500 μL of Dullbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The peptide/pDNA complexes prepared at various NP ratios (NP ratio = 8, 16) were then added to 24-well plates at 30 μL/well. The amount of pDNA volume was adjusted to 1 μg/well. After a 24 h incubation, luciferase expression was evaluated based on photoluminescence intensity using the luciferase assay kit and a luminometer (ARVO^{tm} sx 1420 MULTILABEL COUNTER, PerkinElmer). The amount of protein in each well was concomitantly assessed using a BCA protein assay kit and Multiskan FC micro plate reader (Thermo Scientific). Results were presented as the mean and standard deviation of 3 samples.

The pDNA was prepared fluorescently labeled with Cy5 using the LabelIT Kit (Mirus, Madison, WI). HeLa cells were seeded onto clear 24-well plates each well (100,000 cells/well) and incubated overnight at 37°C in 500 μL of DMEM containing 10% FBS. The peptide/Cy5-labeled pDNA complexes prepared at 16 of NP ratio were then added to 24-well plates at 30 μL/well. The amount of Cy5-labeled pDNA volume was adjusted to 1 μg/well. After 24 h incubation, the medium was removed and the cells were washed with 20 units/mL heparin in PBS and treated with trypsin-EDTA. After addition of the medium containing 10% FBS, the cells were centrifuged at 3000 rpm for 5 min at 4°C and collected cell pelleted. The obtained cell pellets were suspended in PBS, centrifuged at 3000 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（BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences）. The results are presented as the mean and standard deviation obtained from 3 samples.

4. Cellular uptake of peptides

HeLa 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 medium was then replaced with fresh medium containing 10% FBS, and peptide solutions were added to each well at 5 μM. After 1 and 24 h incubation, the medium was removed and the cells were washed with 20 units/mL heparin in PBS and treated with trypsin-EDTA. After addition of the medium containing 10% FBS, the cells were centrifuged at 3000 rpm for 5 min at 4°C and collected cell pelleted. The obtained cell pellets were suspended in PBS, centrifuged at 3000 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（BD Accuri™ C6 Plus Flow Cytometer, BD

Biosciences）. The results are presented as the mean and standard deviation obtained from 3 samples.

5. Inhibition of intracellular uptake pathways

HeLa cells were seeded onto clear 24-well culture plates (100,000 cells/well) and incubated overnight at 37°C in 400 μL of DMEM containing 10% FBS. After the replacement of medium with fresh medium containing 10% FBS in the absence or presence of nystatin (25 μg/mL), nystatin (0.4 M), or amiloride (50 μM), cells were pre-incubated at 37°C or 4°C for 30min. Peptide solution was then added to each well. After 1 h incubation at 37°C or 4°C, the medium was removed and the cells were washed with 20 units/mL heparin in PBS and treated with trypsin-EDTA. After addition of the medium containing 10% FBS, the cells were centrifuged at 3000 rpm for 5 min at 4°C and collected cell pelleted. The obtained cell pellets were suspended in PBS, centrifuged at 3000 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 (BD AccuriTM C6 Plus Flow Cytometer, BD Biosciences). The results are presented as the mean and standard deviation obtained from 3 samples.

6. Fluorescence microscopy

HeLa cells were seeded onto 35 mm glass bottom dish (200,000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. The medium was then replaced with fresh medium containing 10% FBS, and peptide solution was applied to well at a concentration of 5 μM. After 2 h incubation at 37°C, the medium was removed, and the cells were washed 3 times with Hanks′ Balanced Salt solution (HBSS) supplemented with heparin (20 units/mL). The intracellular distribution of each peptide was observed by fluorescence microscopy after staining late endosomes/lysosomes with LysoTracker Red DND-99 and nuclei with Hoechst 33342. Fluorescence microscopy was performed using a BZ-X810 (Keyence, Osaka, Japan) equipped with a 100x objective lens, and further sectioning and Z-stacking were performed to acquire images.

Similarly, HeLa cells were seeded onto 35 mm glass bottom dishes (200,000 cells/well) and cultured overnight in 2 mL of DMEM containing 10% FBS. The medium was then replaced with fresh medium containing 10% FBS and 60 μL of the prepared Cy5-labeled pDNA/peptide complex (NP ratio = 16) was added. After 24 h

incubation at 37°C, the medium was removed, and the cells were washed 3 times with HBSS supplemented with heparin (20 units/mL). After staining lysosomes with LysoPrime Green-High Specificity and pH Resistance and nuclei with Hoechst 33342, observations were made as above.

7. Peptide cytotoxicity

HeLa cells were seeded onto clear 96-well plates (20,000 cells/well) and incubated overnight at 37°C in 100 μL of DMEM containing 10% FBS. Peptide solution was then added to each well at the appropriate concentration and incubated at 37°C for 24 hr. Cell counting kit-8 was used after the 24 h incubation according to the manufacturer's protocol. Cell viability was evaluated on the basis of the absorbance of formazan from each well, in which 100% cell viability was calculated from the wells without peptide. Results were presented as the mean and standard deviation of 4 samples.

8. Enzymatic resistance of peptides

Digestion tests were performed using trypsin (Sequencing Grade Modified Trypsin) as the digestive enzyme. Peptides and Trypsin were mixed in 50 mM NH_4HCO_3 buffer pH 7.8 to final concentrations of 500 μM and 0.2 unit/mL, respectively, and incubated at 37°C for any time. After each time incubation at 37°C, the enzymatic reaction was stopped by adding 20% MeCN/H_2O with 1% TFA. The Enzyme resistance of peptides was analyzed using UPLC (Waters) and a ACQUITY UPLC® BEH C18 1.7 μm column (2.1 × 50 mm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 0.5 mL/min, gradient: 0-90% gradient of solvent B over 5 min)

9. Agarose gel electrophoresis

1% agarose gel was prepared with PrimeGel Agarose LE 1-20K GAT using 1 × TAE buffer. Gel electrophoresis was conducted at 100 V for 45 min using 1% agarose gel.

10. Dynamic light scattering (DLS) Measurements

The size and size distribution of the peptide/pDNA complexes were evaluated by DLS using Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK) and a He–Ne ion laser (633 nm) as the incident beam. Light scattering data were obtained at a detection angle of 173° and a temperature of 25 °C and were subsequently analyzed by the

cumulant method to obtain the hydrodynamic diameters and PDI (μ/Γ^2) of the complexes. Results were presented as the mean and standard deviation obtained from 3 measurements.

11. Zeta-potential measurements

The zeta-potentials of peptide/pDNA complexes were evaluated by the electrophoretic light scattering method using Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK) with a He-Ne ion laser (633 nm). Zeta-potential measurements were carried out at 25 °C. A scattering angle of 173° was used in these measurements. Results were presented as the mean and standard deviation obtained from 3 measurements.

12. Preparation of peptide/mRNA complexes

Each peptide and mRNA were dissolved separately in 10 mM HEPES buffer (pH 7.5). A twice excess amount of peptide solutions at various concentrations was added to the mRNA solution to form peptide/mRNA complexes of different compositions. The final concentration of mRNA was adjusted to 33.3 μg/mL and the complex solution was stored at room temperature for 15 min before use. The NP ratio is defined as the molar ratio of the guanidino groups in the peptide to the phosphate group in the mRNA.

13. Transfection efficiency of peptide/mRNA

HeLa cells were seeded onto clear 96-well plates (5,000 cells/well) and incubated overnight at 37℃ in 100 μL of DMEM containing 10% FBS. The peptide/mRNA complex solutions were prepared at various NP ratios and then added to each well (250 ng of mRNA/well). After 48 h incubation, cells were lysed using a passive lysis buffer (Promega) and subjected to a luciferase assay using the Luciferase Assay System (Promega) and Lumat3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany).

14. Preparation of peptide/siRNA complexes

Each peptide and mRNA were dissolved separately in 10 mM HEPES buffer (pH 7.5). Peptide solutions at various concentrations was added to the siRNA solution to form peptide/siRNA complexes of different compositions. The final concentration of siRNA was adjusted to 4 μM and the complex solution was stored at room temperature for 15 min before use. The NP ratio is defined as the molar ratio of the

guanidino groups in the peptide to the phosphate group in the siRNA.

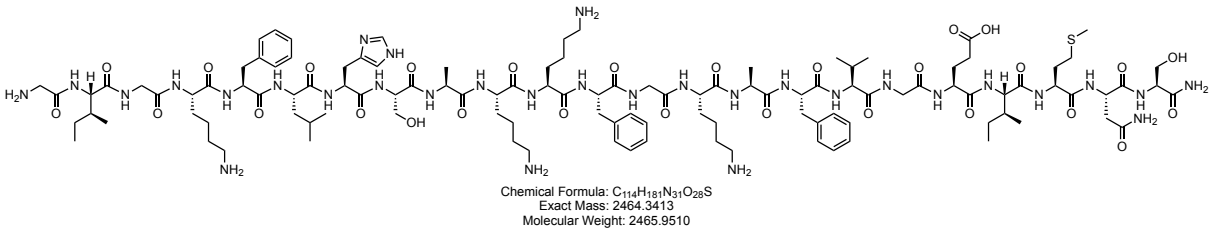
15. RNAi assay by luciferase assay

Luciferase-expressing human hepatoma cell line, Huh-7-Luc cells (JCRB Cell Bank, Osaka, Japan) were seeded onto 96-well culture plates (5,000 cells/well) and incubated overnight at 37°C in 100 μL of DMEM containing 10% FBS. The peptide/siRNA complex solutions were then added to each well and were incubated at 37°C. After 48 h incubation, cells were lysed using a passive lysis buffer (Promega) and the luciferase gene silencing was evaluated based on the intensity of photoluminescence using the luciferase assay kit (Promega) and Lumat3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Peptide data

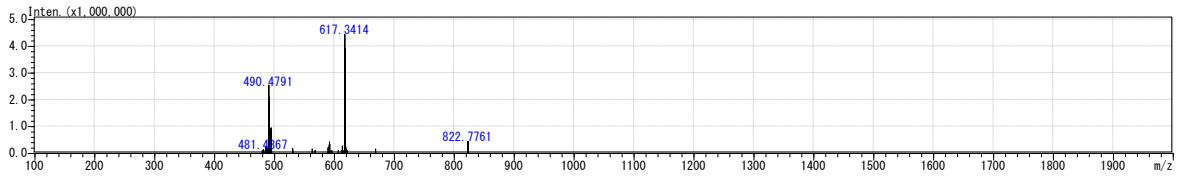
Magainin 2

H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-NH_2

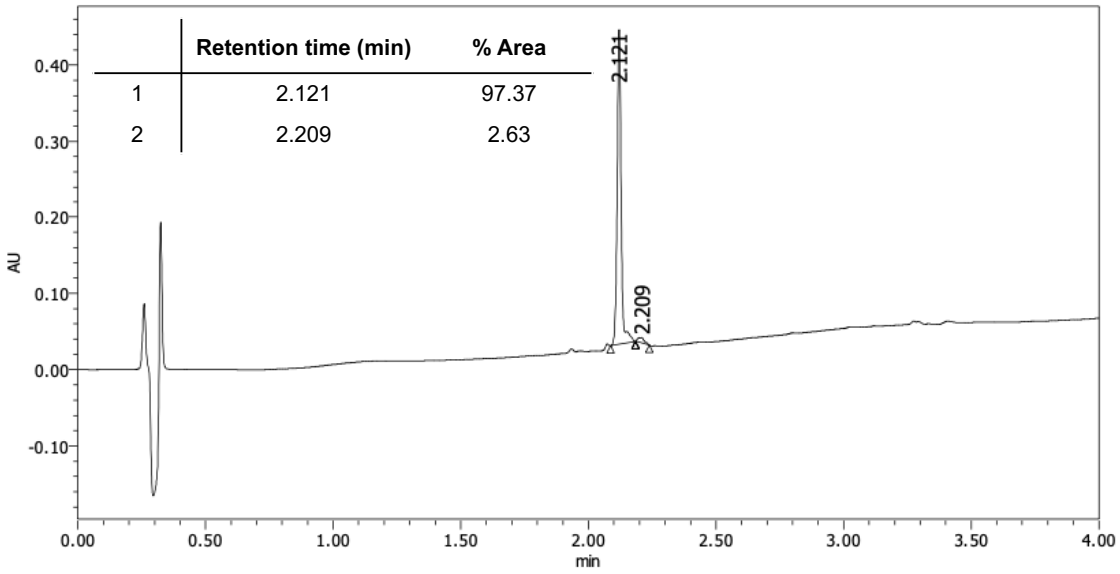


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 97.37%
- HRMS (ESI+) calcd for $C_{114}H_{181}N_{31}O_{28}S$ $[M+4H]^{4+}$: 617.0926; found: 617.3414

ESI-TOF-MS

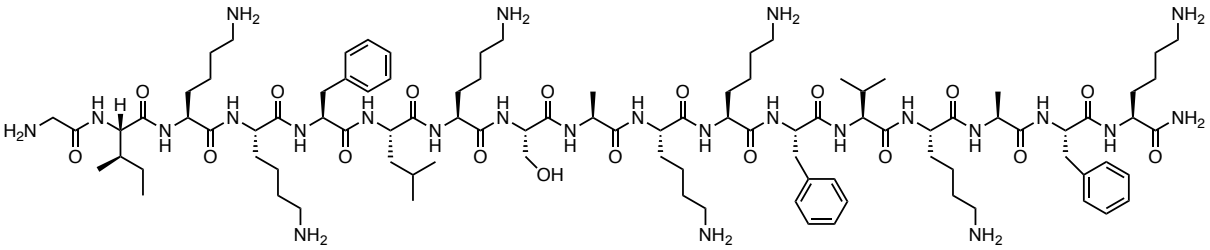


UPLC profile



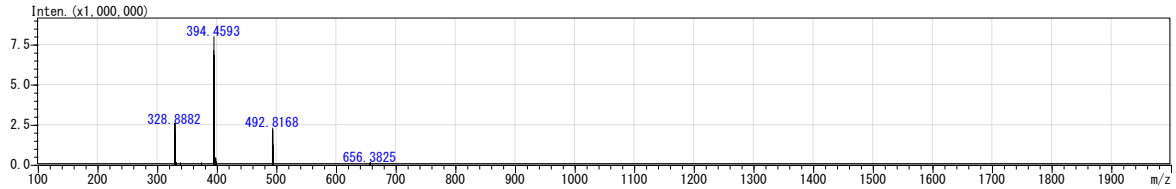
Pep-1

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-Ser-Ala-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH_2

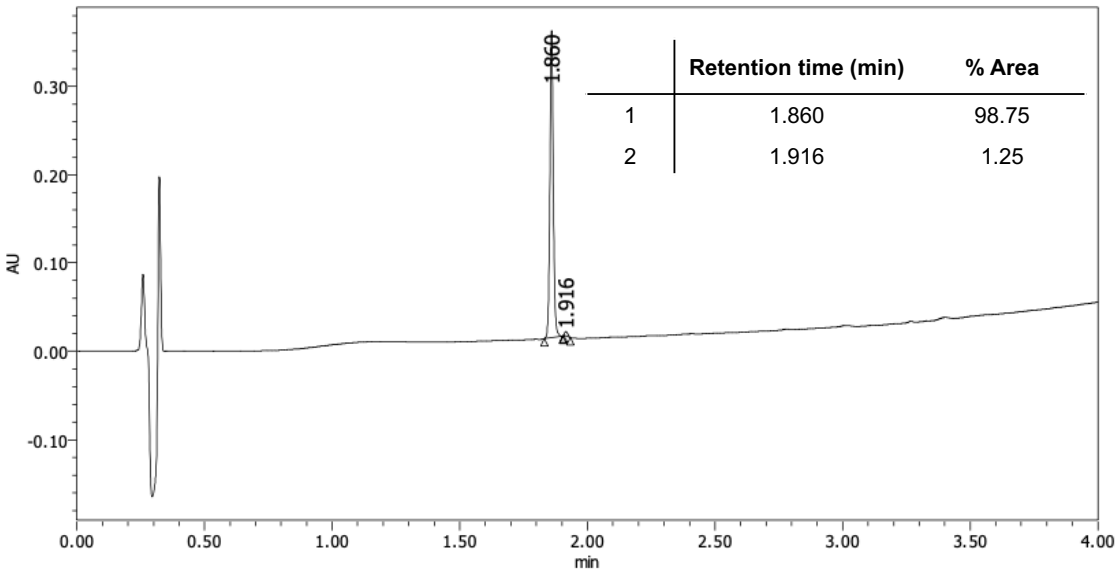


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 98.75%
- HRMS (ESI^+) calcd for $C_{97}H_{163}N_{25}O_{18}$ $[M+5H]^{5+}$: 394.2594; found: 394.4593

ESI-TOF-MS



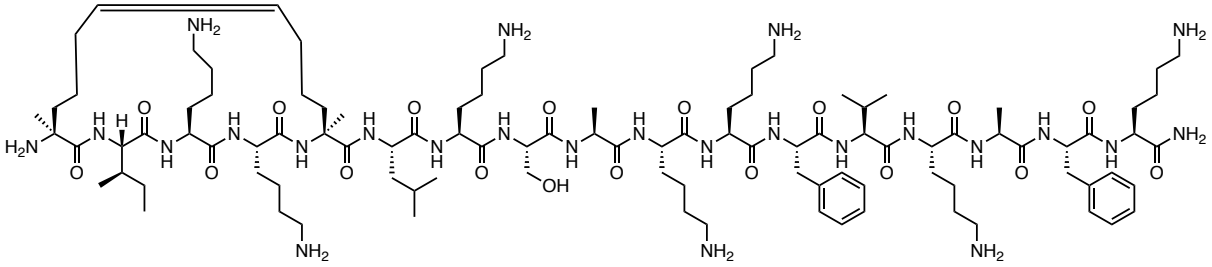
UPLC profile



	Retention time (min)	% Area
1	1.860	98.75
2	1.916	1.25

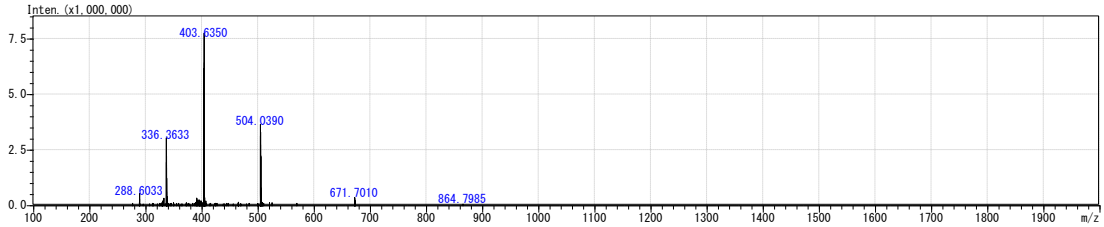
st4-1

H-S$_5$-Ile-Lys-Lys-S$_5$-Leu-Lys-Ser-Ala-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH$_2$

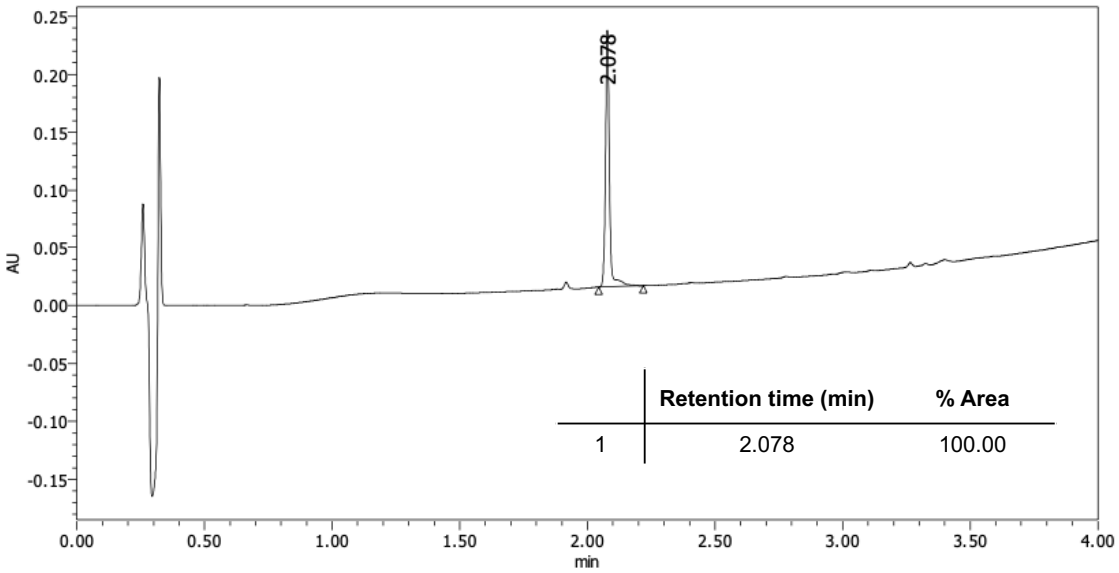


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{100}H_{173}N_{25}O_{18}$ $[M+5H]^{5+}$: 403.4751; found: 403.6350

ESI-TOF-MS

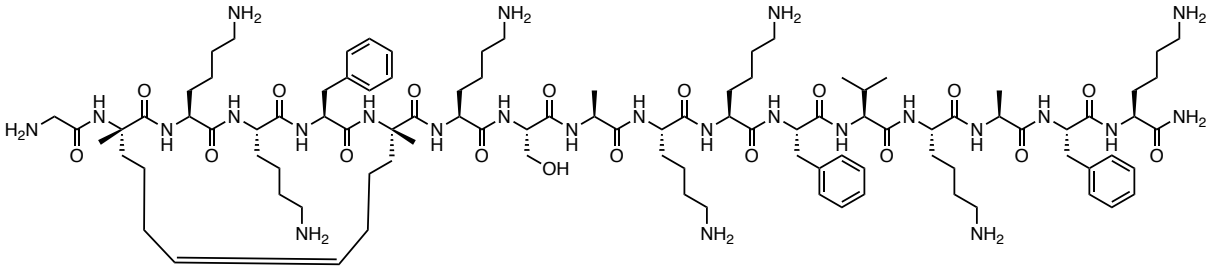


UPLC profile



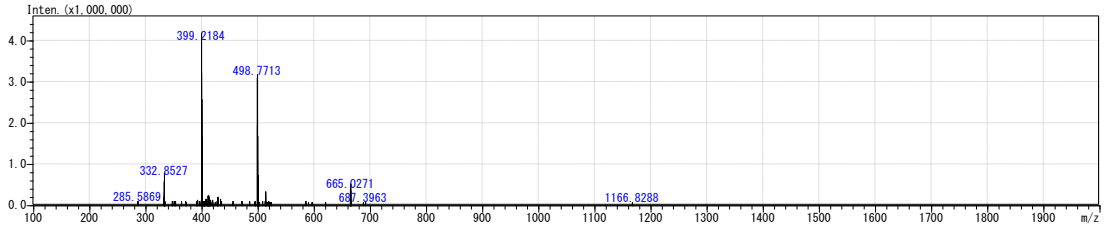
st4-2

H-Gly-S5-Lys-Lys-Phe-S5-Lys-Ser-Ala-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH_2

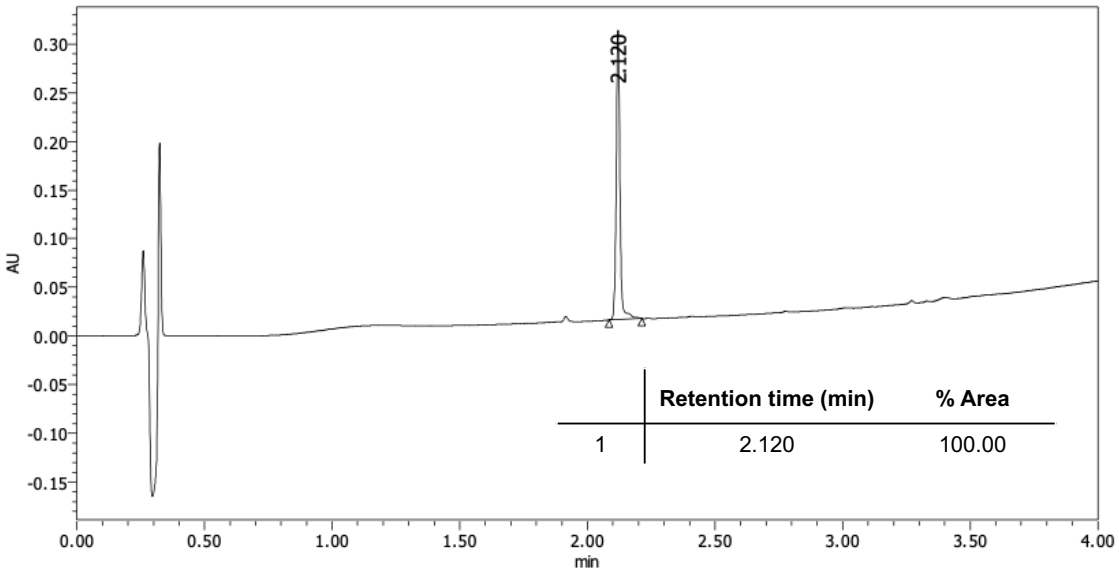


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI+) calcd for $C_{99}H_{163}N_{25}O_{18}$ $[M+5H]^{5+}$: 399.0594; found: 399.2184

ESI-TOF-MS

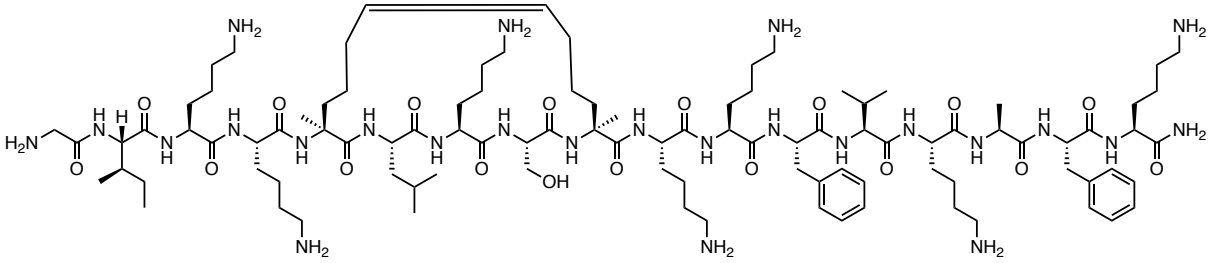


UPLC profile



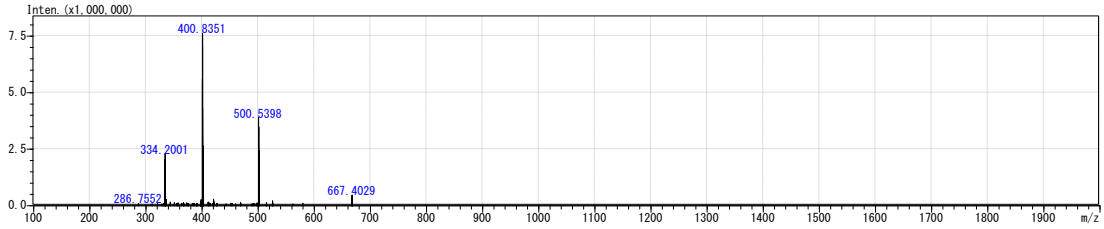
st4-3

H-Gly-Ile-Lys-Lys-S$_5$-Leu-Lys-Ser-S$_5$-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH$_2$

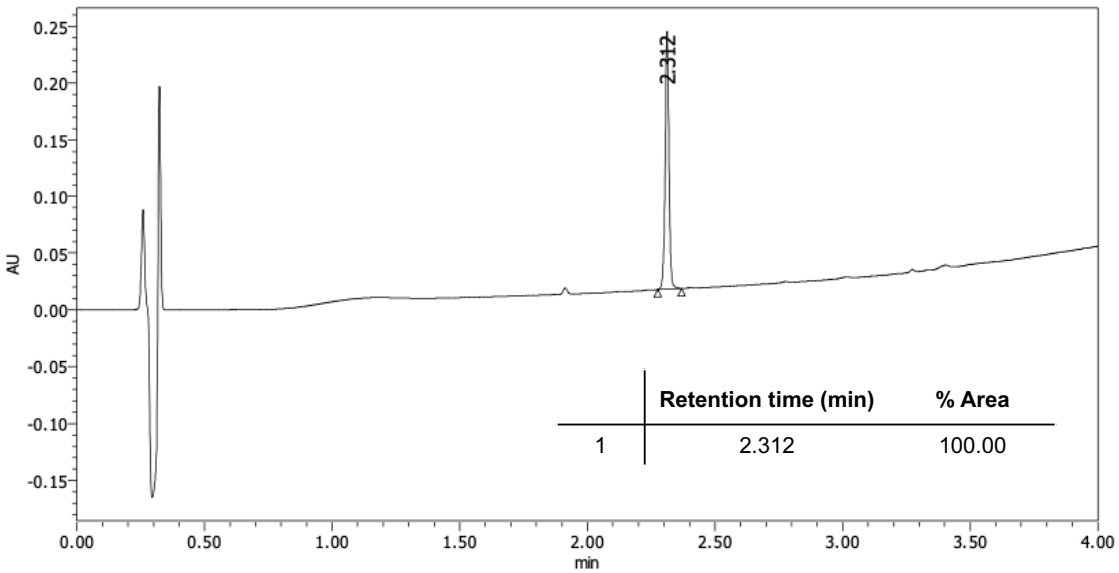


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{99}H_{171}N_{25}O_{18}$ [M+5H]$^{5+}$: 400.6720; found: 400.8351

ESI-TOF-MS

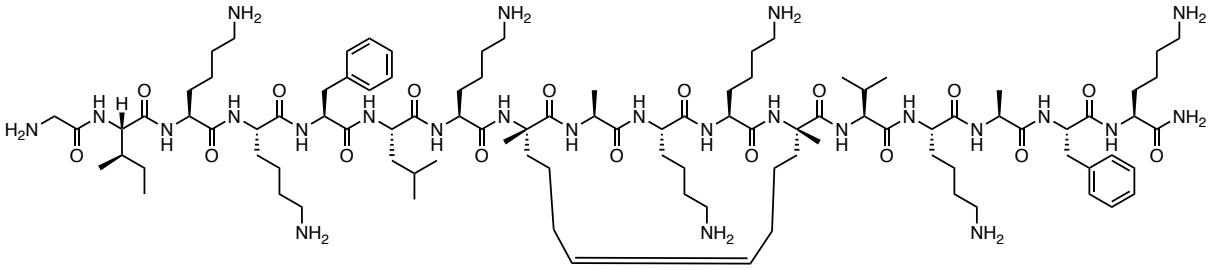


UPLC profile



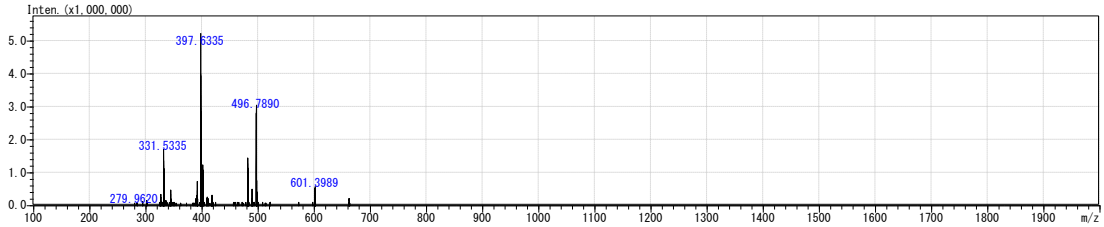
st4-4

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-S_5-Ala-Lys-Lys-S_5-Val-Lys-Ala-Phe-Lys-NH_2

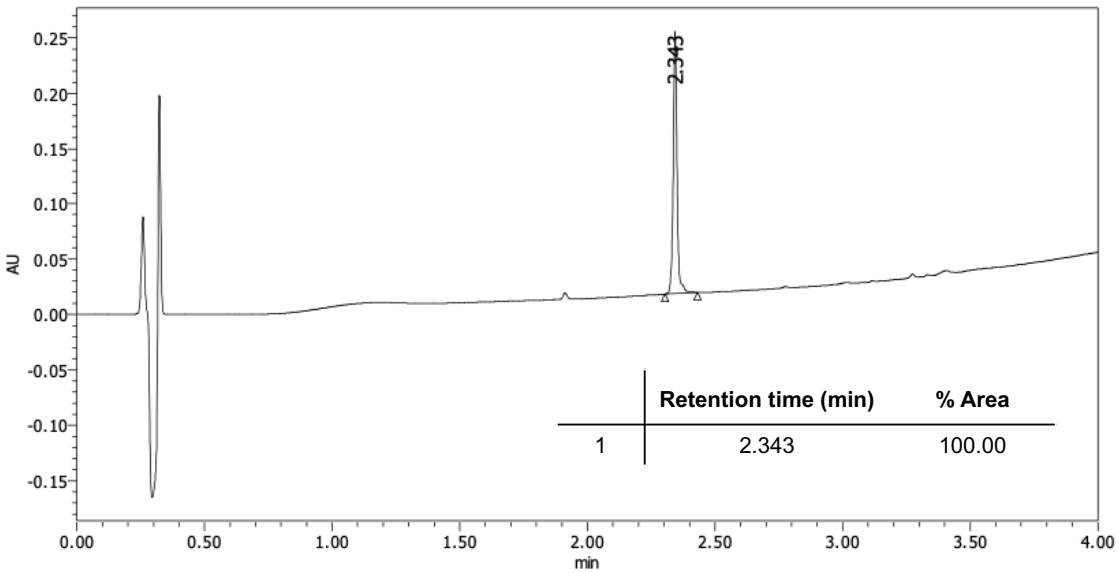


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{99}H_{171}N_{25}O_{17}$ $[M+5H]^{5+}$: 397.4730; found: 397.6335

ESI-TOF-MS

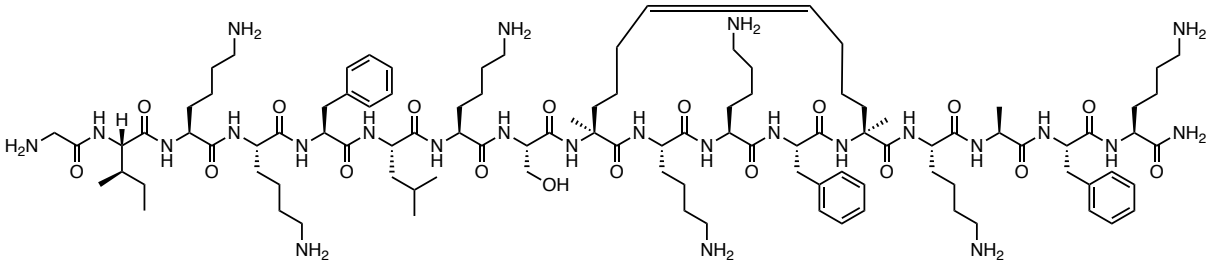


UPLC profile



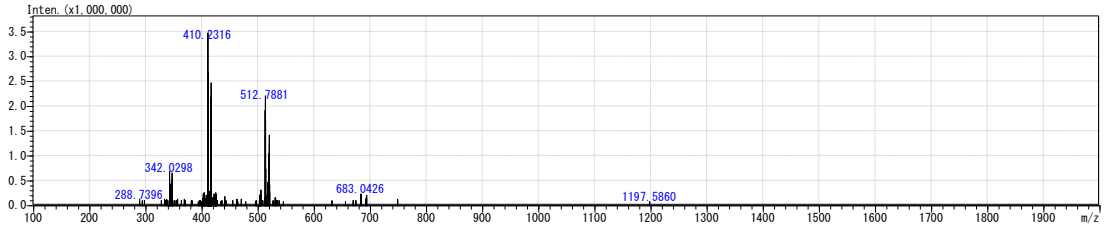
st4-5

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-Ser-S5-Lys-Lys-Phe-S5-Lys-Ala-Phe-Lys-NH_2

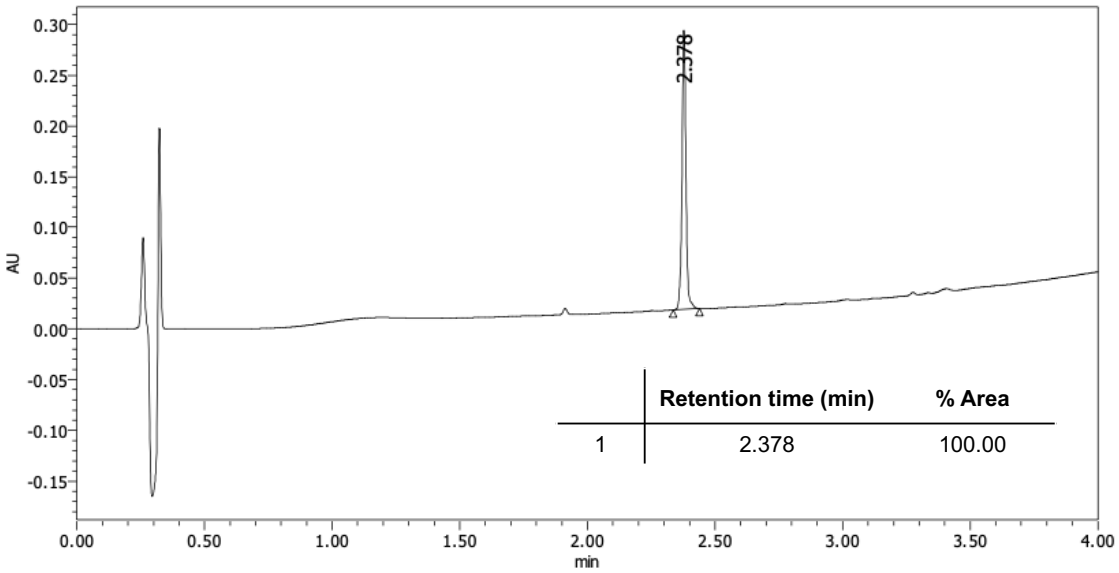


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI+) calcd for $C_{103}H_{171}N_{25}O_{18}$ $[M+5H]^{5+}$: 410.2720; found: 410.2316

ESI-TOF-MS

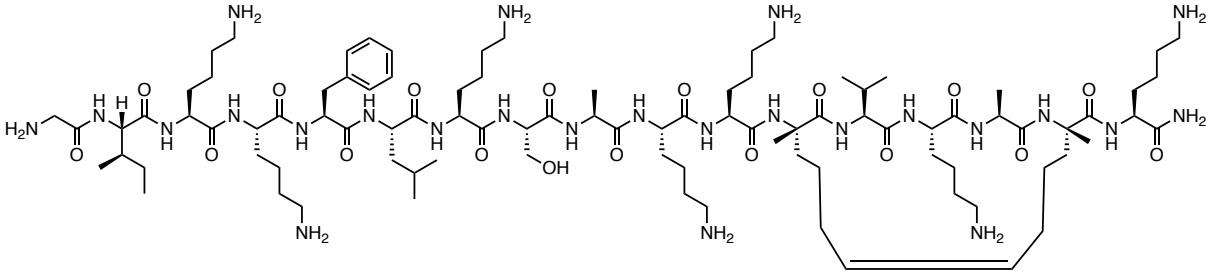


UPLC profile



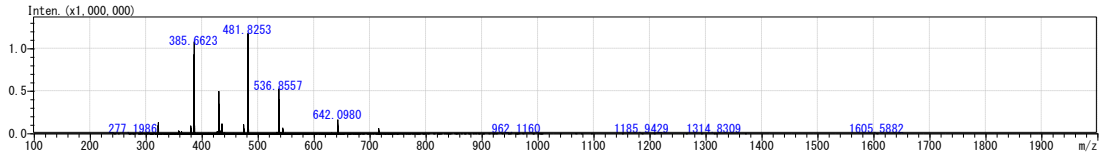
st4-6

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-Ser-Ala-Lys-LysS$_5$-Val-Lys-Ala-S$_5$-Lys-NH$_2$

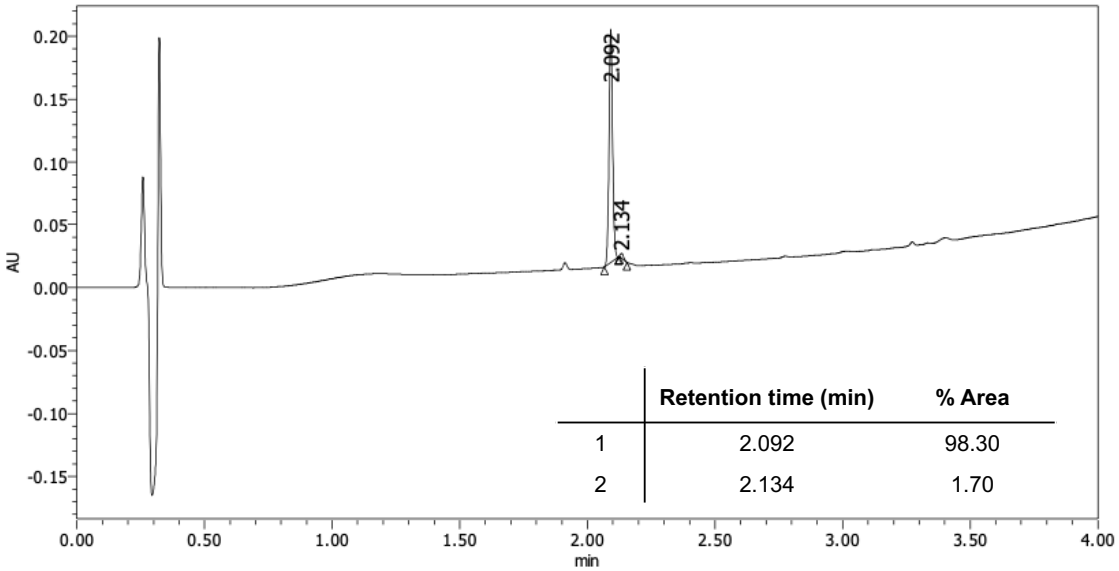


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 98.30%
- HRMS (ESI$^+$) calcd for $C_{93}H_{167}N_{25}O_{18}$ [M+4H]$^{4+}$: 481.5803; found: 481.8253

ESI-TOF-MS



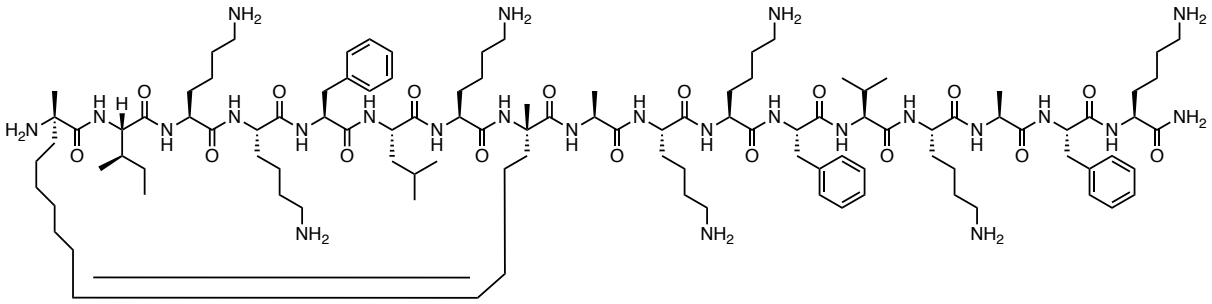
UPLC profile



	Retention time (min)	% Area
1	2.092	98.30
2	2.134	1.70

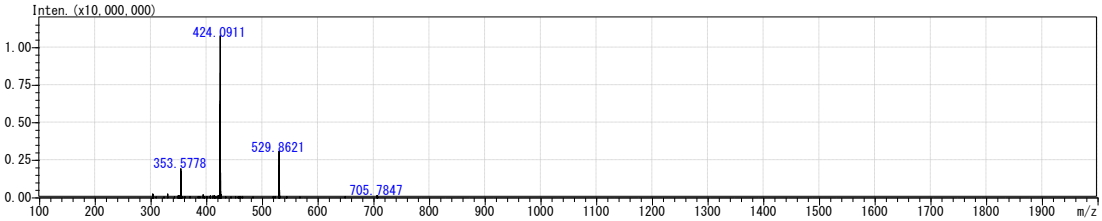
st7-1

H-R$_8$-Ile-Lys-Lys-Phe-Leu-Lys-S$_5$-Ala-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH$_2$

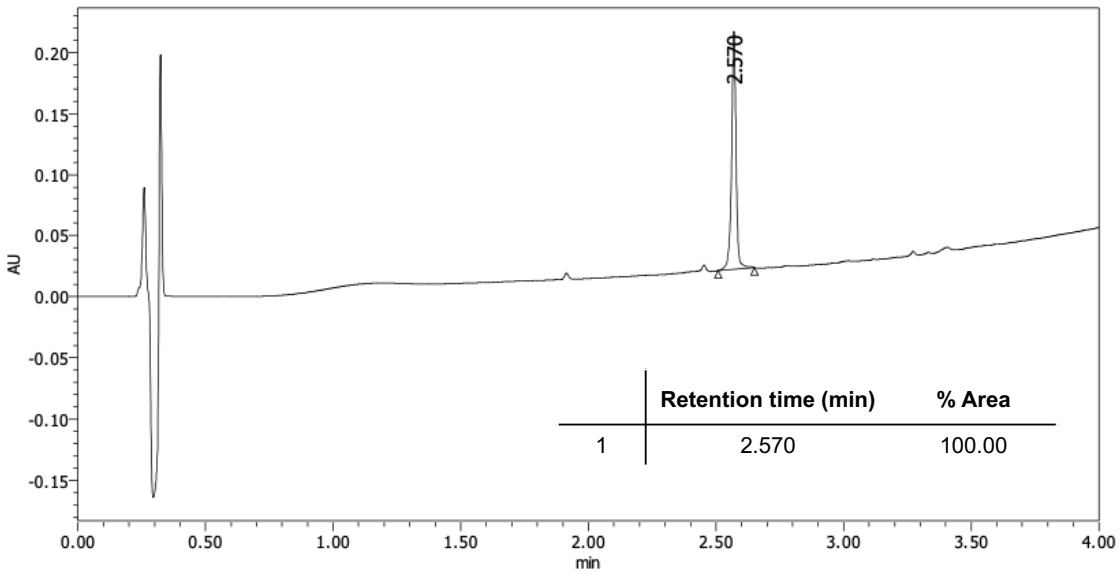


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{109}H_{183}N_{25}O_{17}$ [M+5H]$^{5+}$: 423.8918; found: 424.0911

ESI-TOF-MS

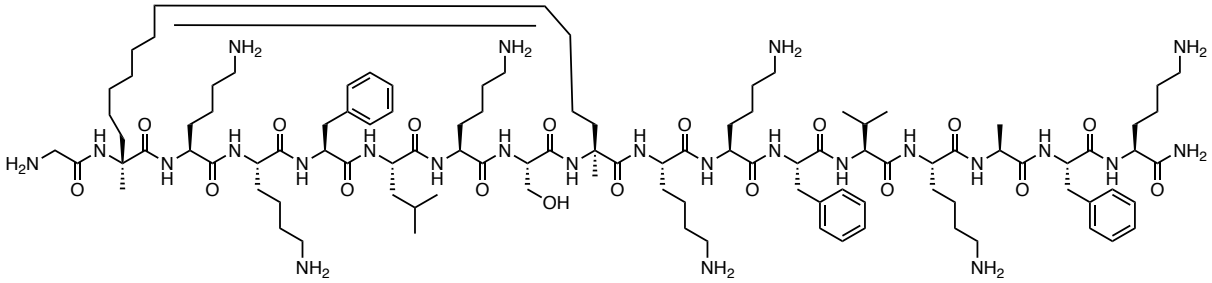


UPLC profile



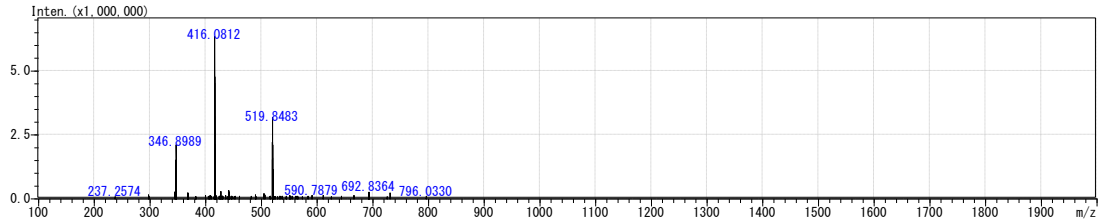
st7-2

H-Gly-R$_8$-Lys-Lys-Phe-Leu-Lys-Ser-S$_5$-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH$_2$

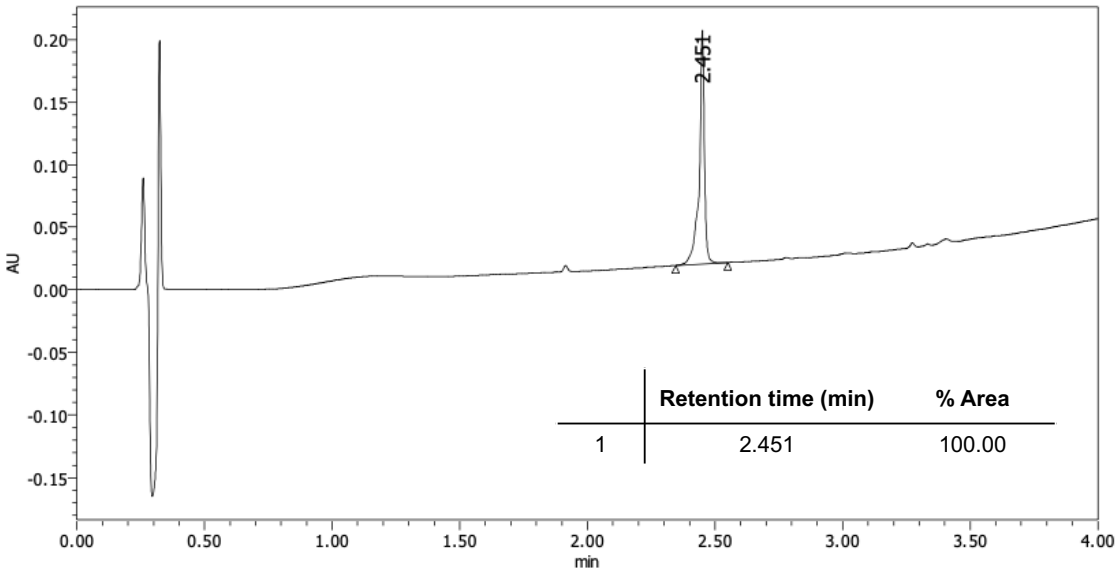


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{105}H_{175}N_{25}O_{18}$ [M+5H]$^{5+}$: 415.8782; found: 416.0812

ESI-TOF-MS

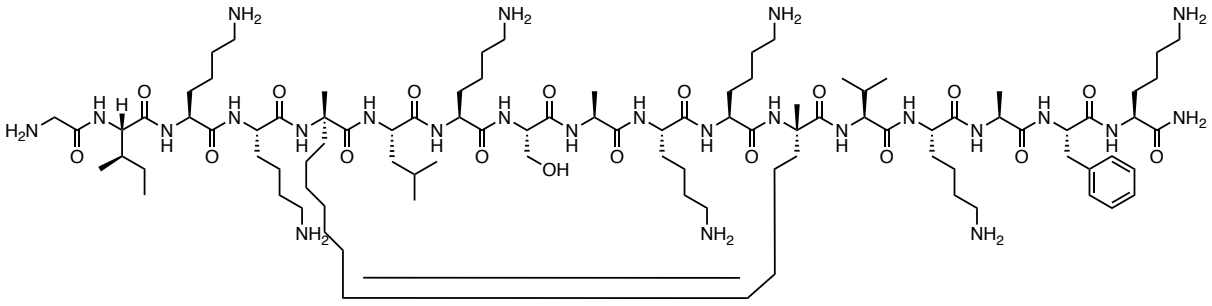


UPLC profile



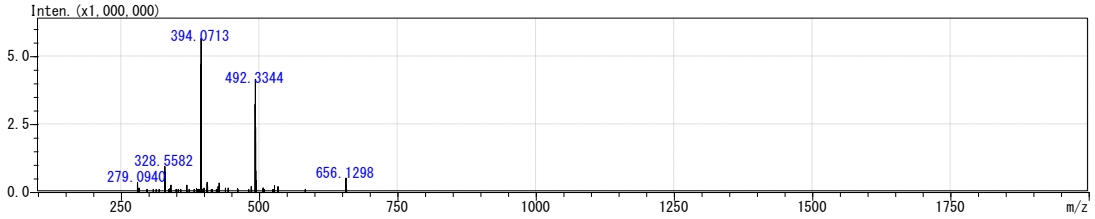
st7-3

H-Gly-Ile-Lys-Lys-R$_8$-Leu-Lys-Ser-Ala-Lys-Lys-S$_5$-Val-Lys-Ala-Phe-Lys-NH$_2$

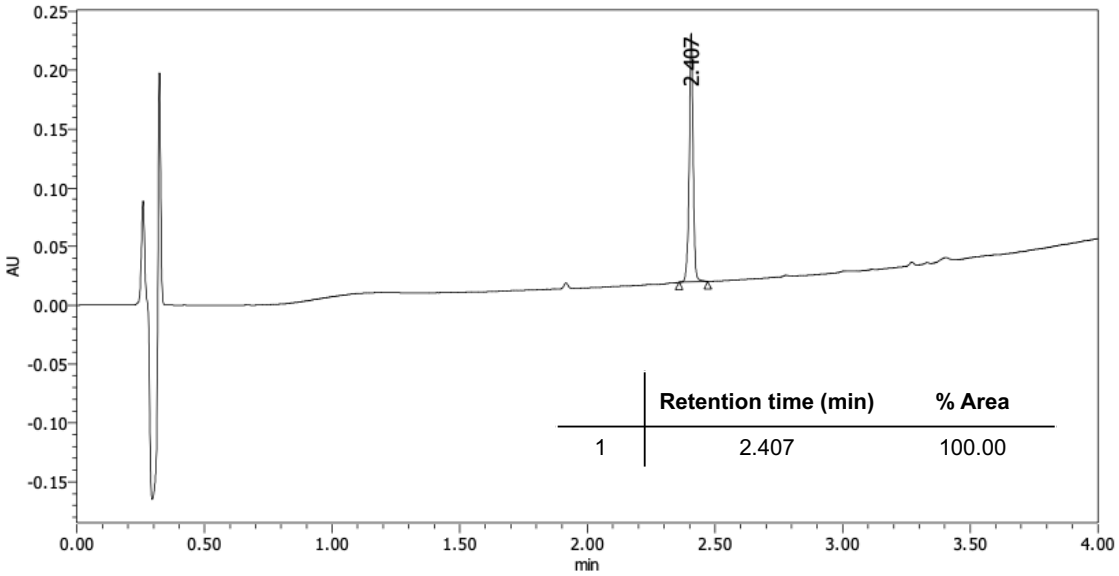


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{96}H_{173}N_{25}O_{18}$ $[M+5H]^{5+}$: 393.8751; found: 394.0713

ESI-TOF-MS

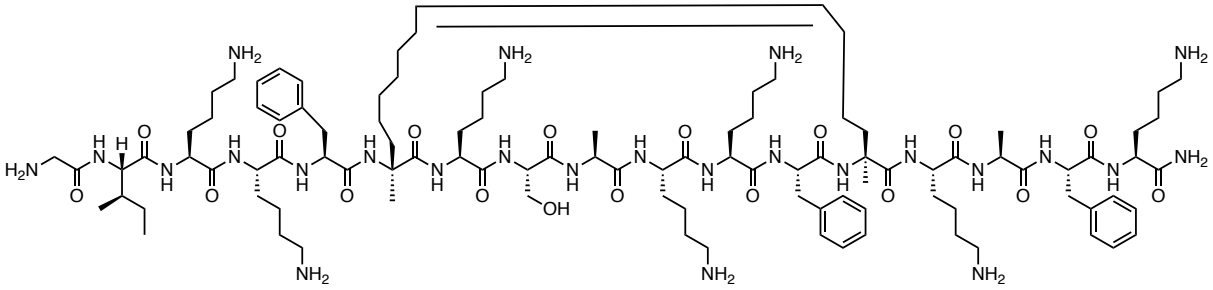


UPLC profile



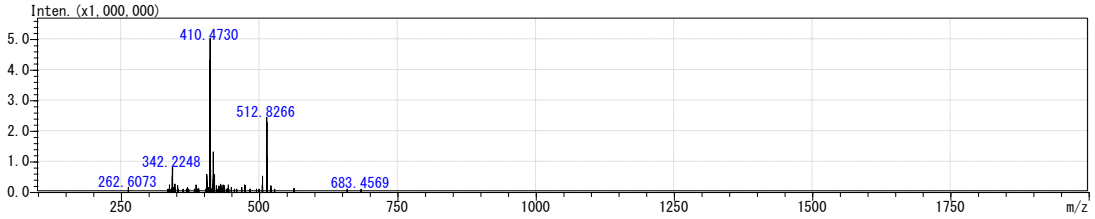
st7-4

H-Gly-Ile-Lys-Lys-Phe-R$_8$-Lys-Ser-Ala-Lys-Lys-Phe-S$_5$-Lys-Ala-Phe-Lys-NH$_2$

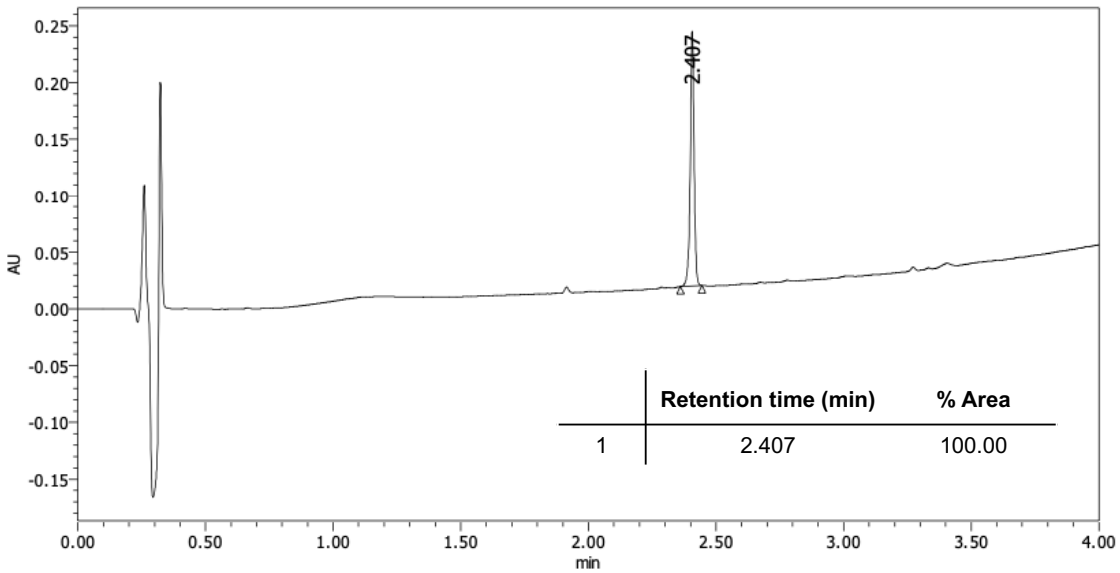


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{103}H_{171}N_{25}O_{18}$ [M+5H]$^{5+}$: 410.2720; found: 410.4730

ESI-TOF-MS

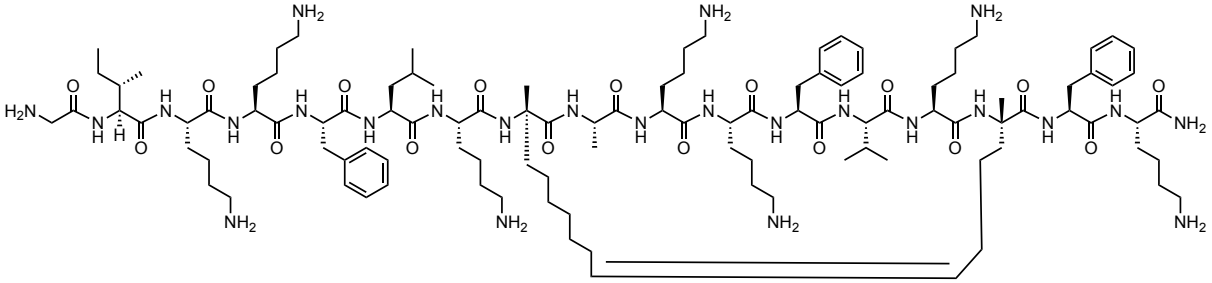


UPLC profile



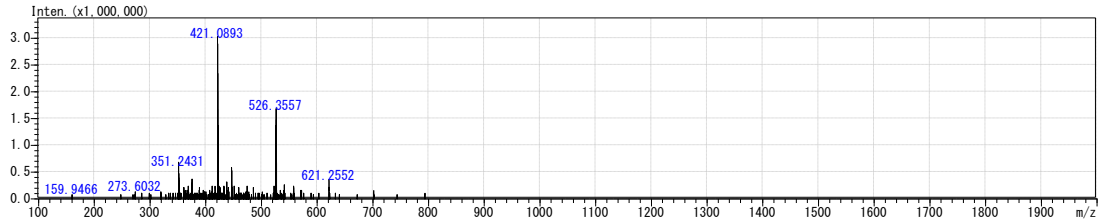
st7-5

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-R$_8$-Ala-Lys-Lys-Phe-Val-Lys-S$_5$-Phe-Lys-NH$_2$

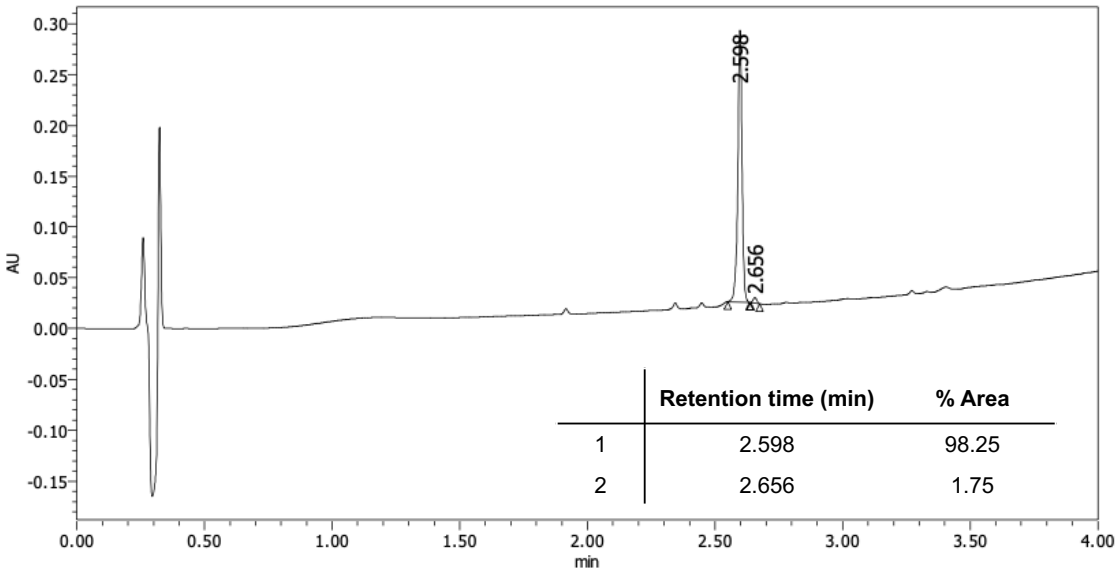


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 98.25%
- HRMS (ESI$^+$) calcd for $C_{108}H_{181}N_{25}O_{17}$ [M+5H]$^{5+}$: 421.0886; found: 421.0893

ESI-TOF-MS

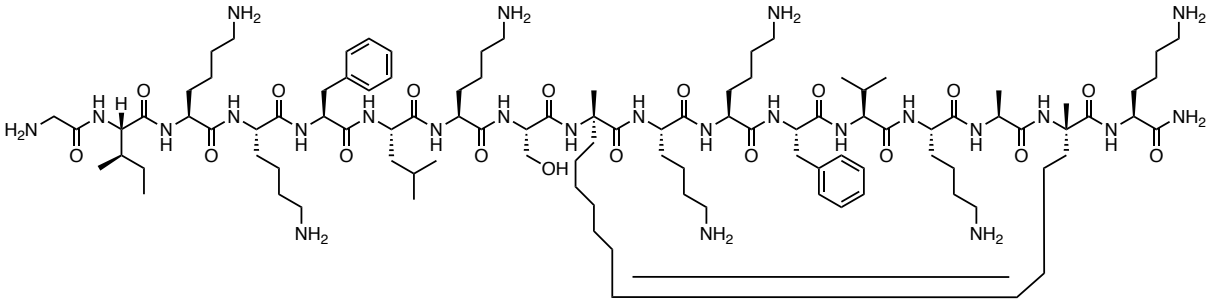


UPLC profile



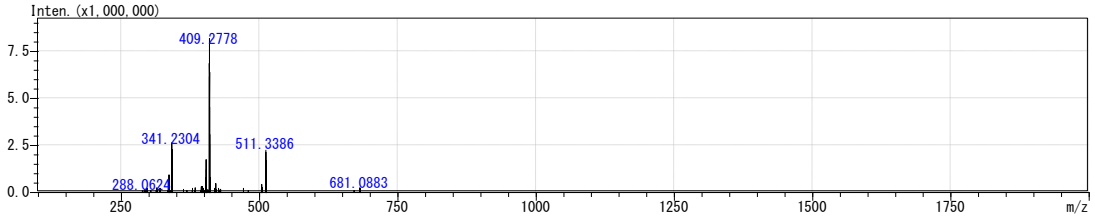
st7-6

H-Gly-Ile-Lys-Lys-Phe-Leu-Lys-Ser-R$_8$-Lys-Lys-Phe-Val-Lys-Ala-S$_5$-Lys-NH$_2$

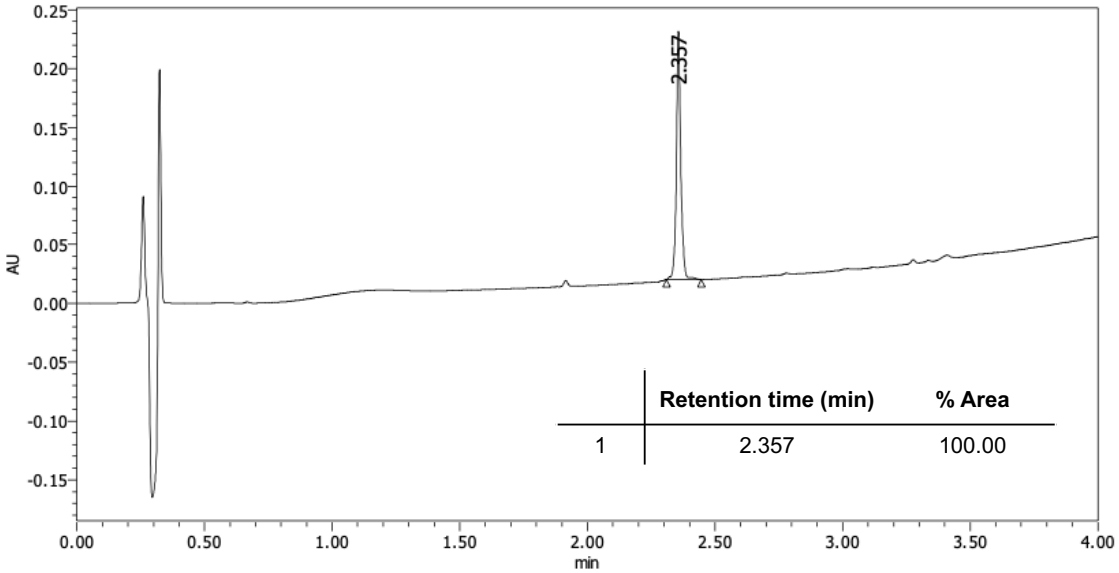


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{102}H_{177}N_{25}O_{18}$ [M+5H]$^{5+}$: 409.0813; found: 409.2778

ESI-TOF-MS

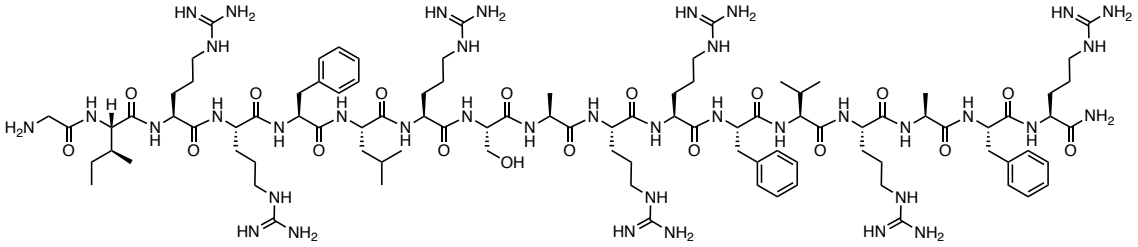


UPLC profile



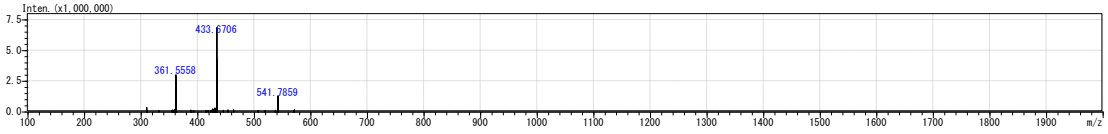
Pep-1_R

H-Gly-Ile-Arg-Arg-Phe-Leu-Arg-Ser-Ala-Arg-Arg-Phe-Val-Arg-Ala-Phe-Arg-NH_2

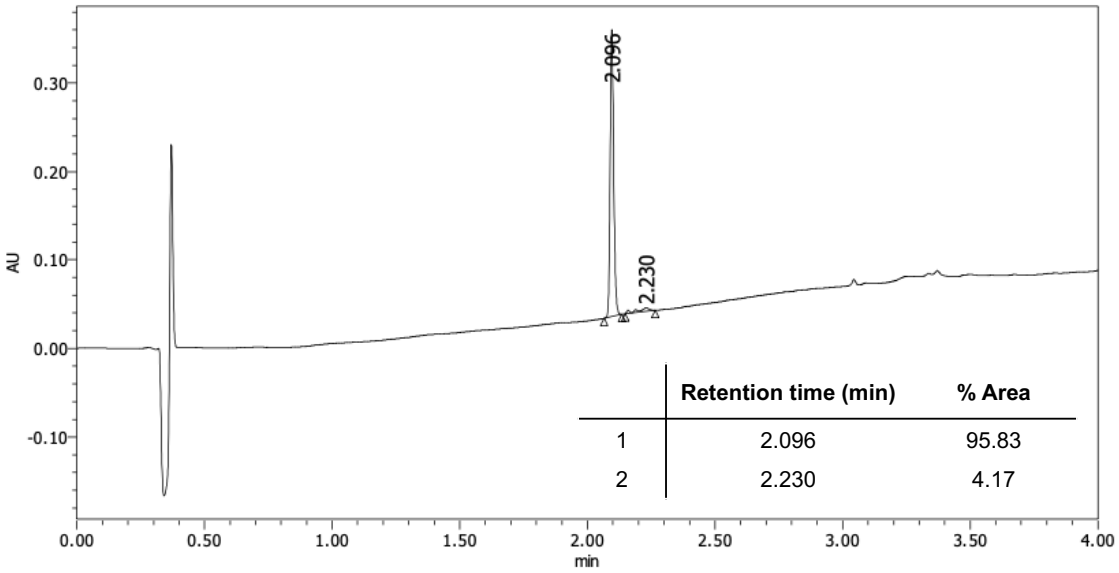


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 95.83%
- HRMS (ESI$^+$) calcd for $C_{97}H_{163}N_{39}O_{18}$ $[M+5H]^{5+}$: 433.4680; found: 433.6706

ESI-TOF-MS



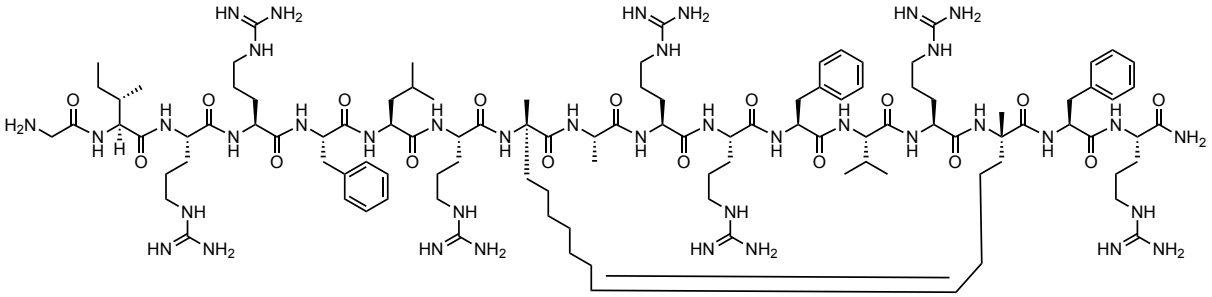
UPLC profile



	Retention time (min)	% Area
1	2.096	95.83
2	2.230	4.17

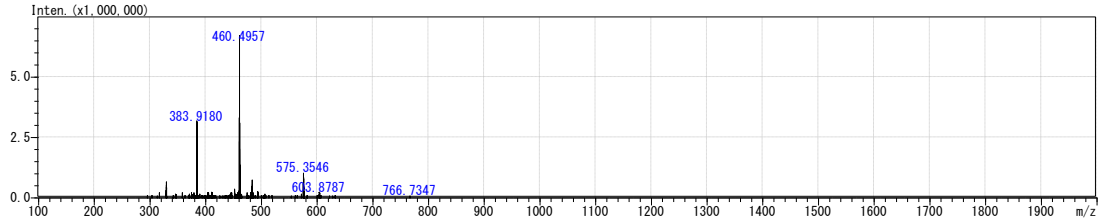
st7-5_R

H-Gly-Ile-Arg-Arg-Phe-Leu-Arg-R$_8$-Ala-Arg-Arg-Phe-Val-Arg-S$_5$-Phe-Arg-NH$_2$

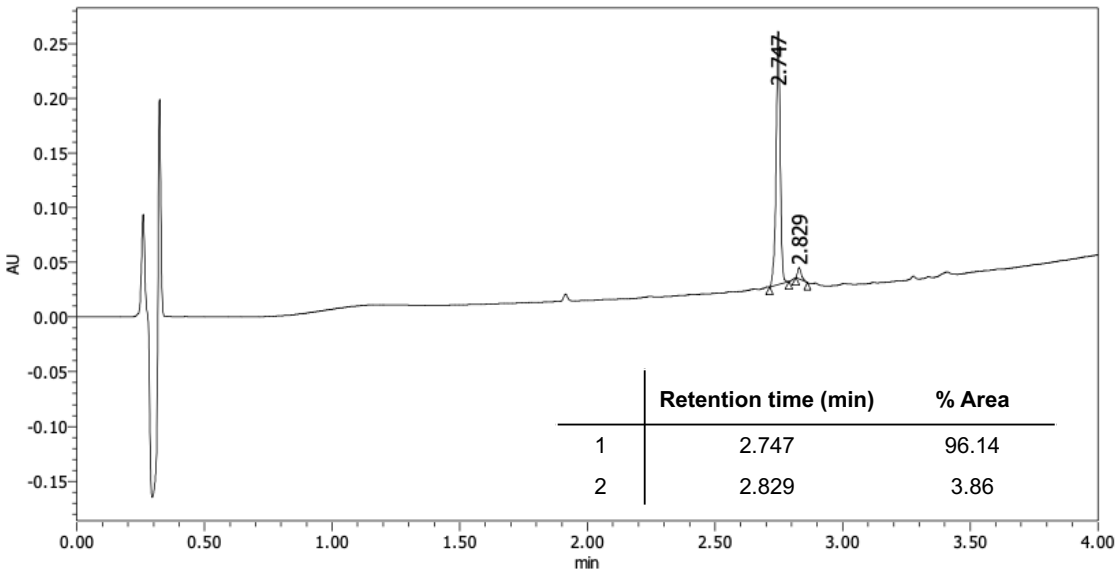


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 96.14%
- HRMS (ESI$^+$) calcd for $C_{108}H_{181}N_{39}O_{17}$ [M+5H]$^{5+}$: 460.2972; found: 460.4957

ESI-TOF-MS



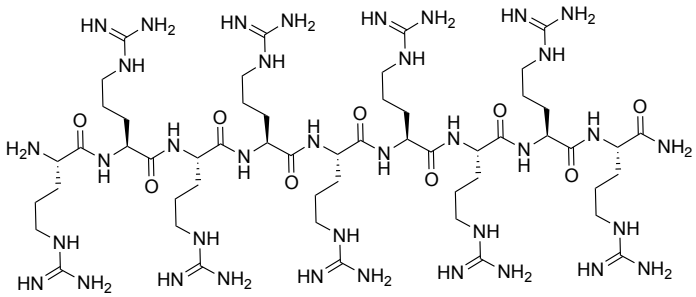
UPLC profile



	Retention time (min)	% Area
1	2.747	96.14
2	2.829	3.86

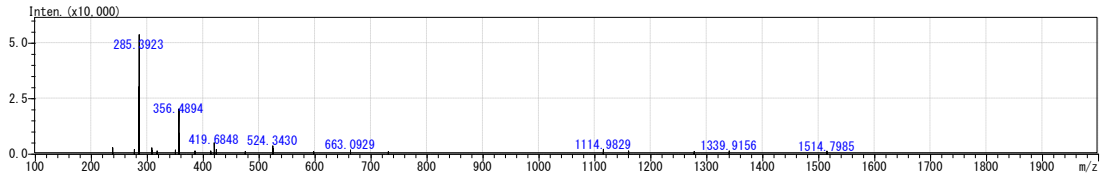
R9

H-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH_2

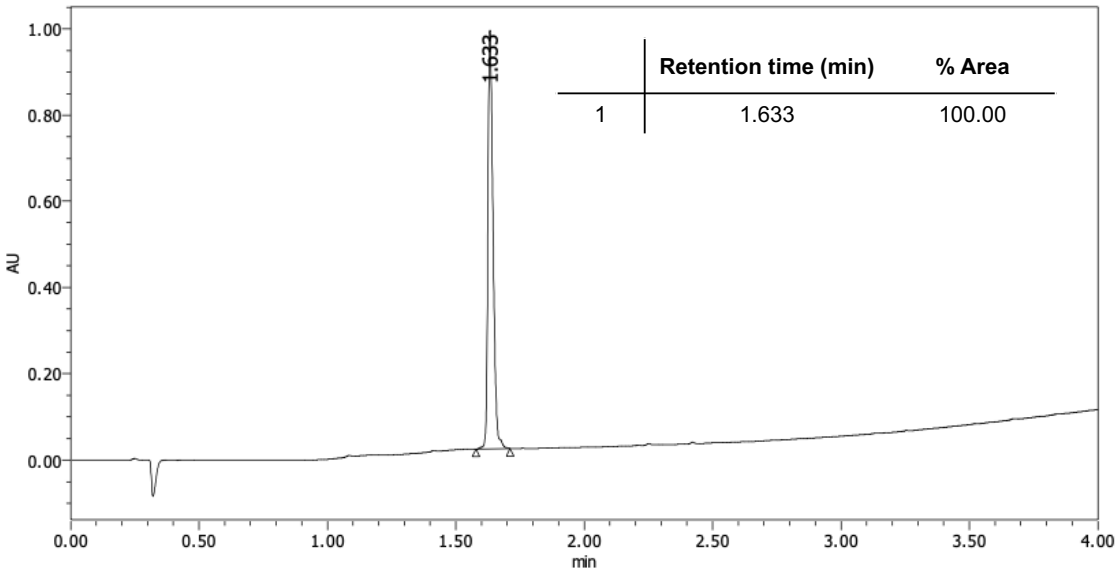


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 0-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI^+) calcd for $C_{54}H_{111}N_{37}O_9$ $[M+5H]^{5+}$: 285.3946; found: 285.3923

ESI-TOF-MS

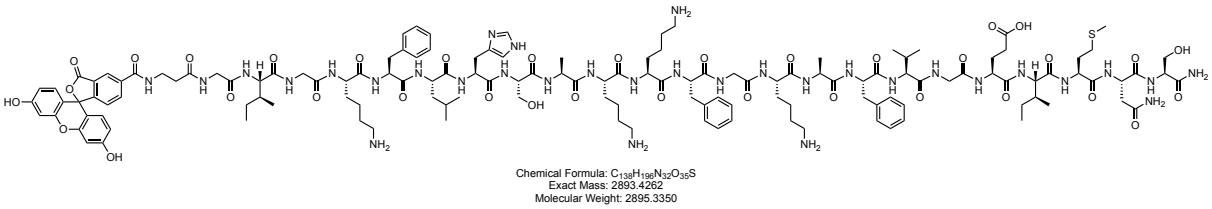


UPLC profile



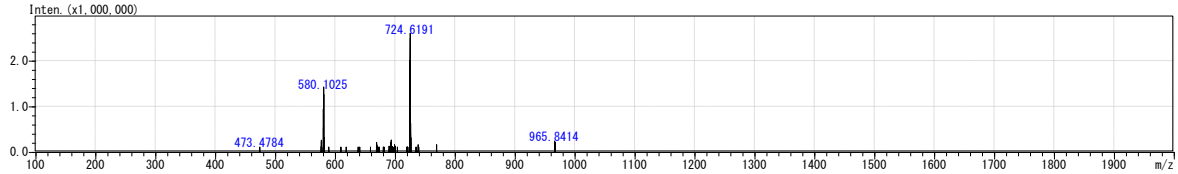
FAM-Magainin 2

FAM-βAla-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-NH_2

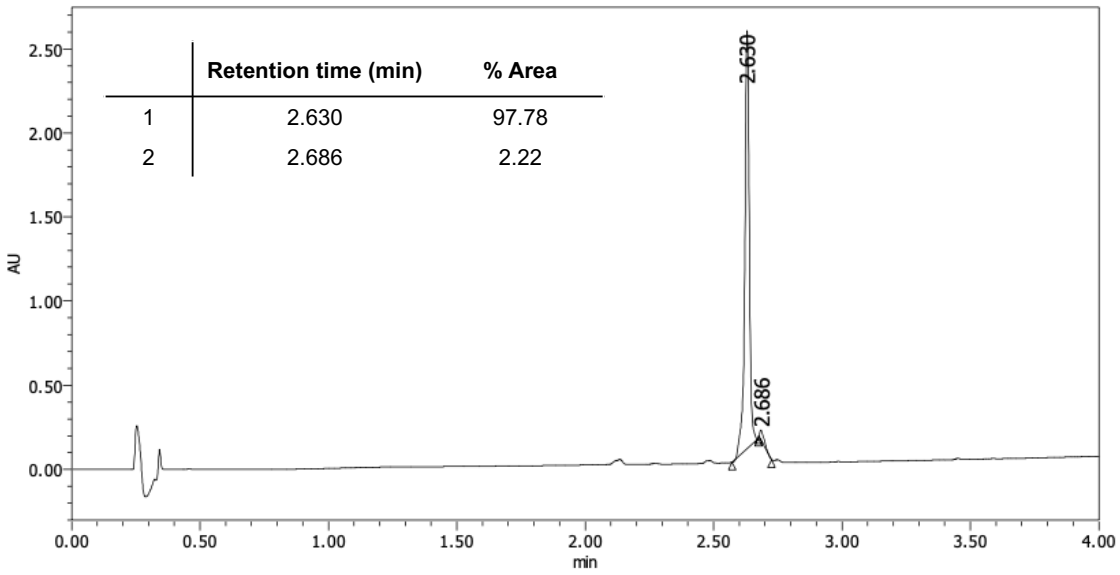


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 97.78%
- HRMS (ESI$^+$) calcd for $C_{138}H_{196}N_{32}O_{35}S$ $[M+4H]^{4+}$: 724.3638; found: 724.6191

ESI-TOF-MS

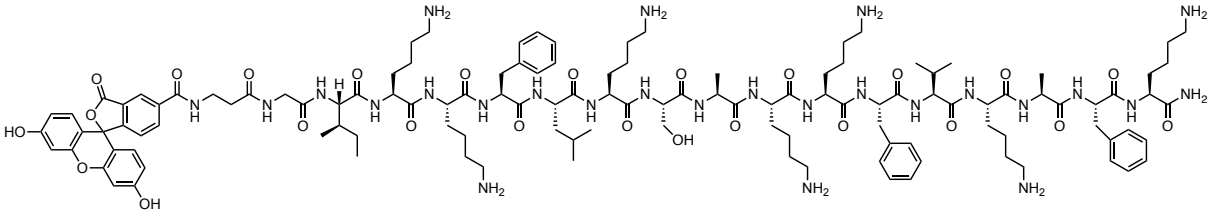


UPLC profile



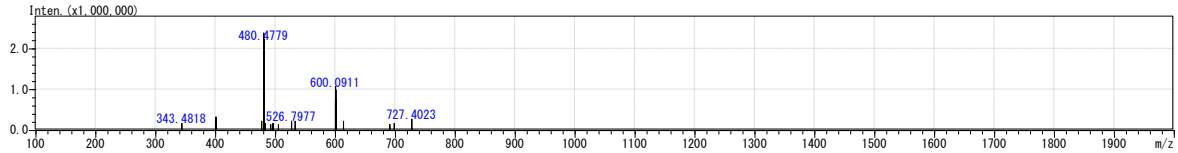
FAM-Pep-1

FAM-βAla-Gly-Ile-Lys-Lys-Phe-Leu-Lys-Ser-Ala-Lys-Lys-Phe-Val-Lys-Ala-Phe-Lys-NH_2

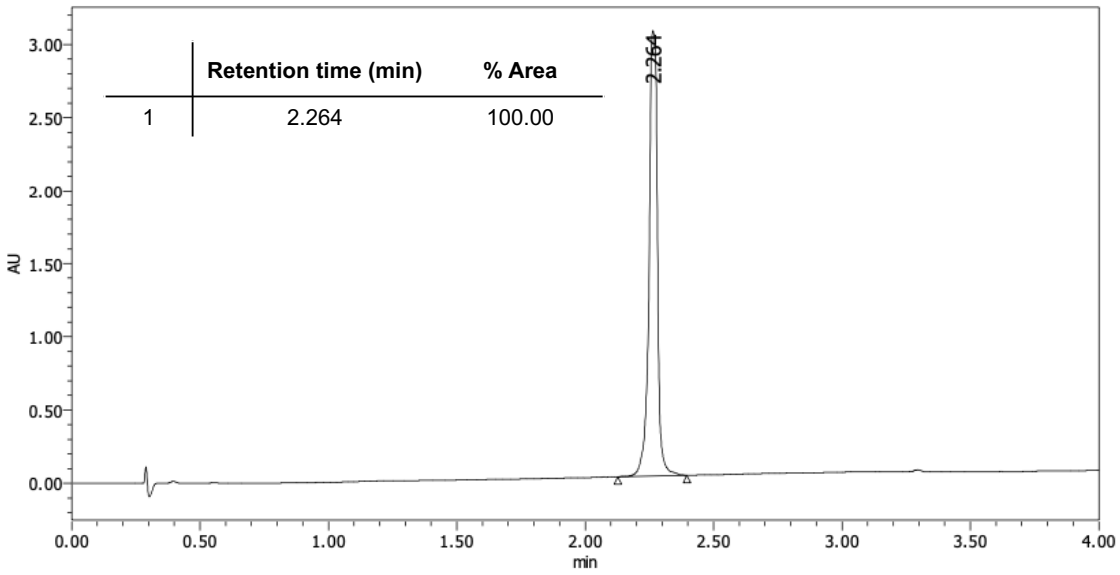


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{121}H_{178}N_{26}O_{25}$ $[M+5H]^{5+}$: 480.0764; found: 480.4779

ESI-TOF-MS

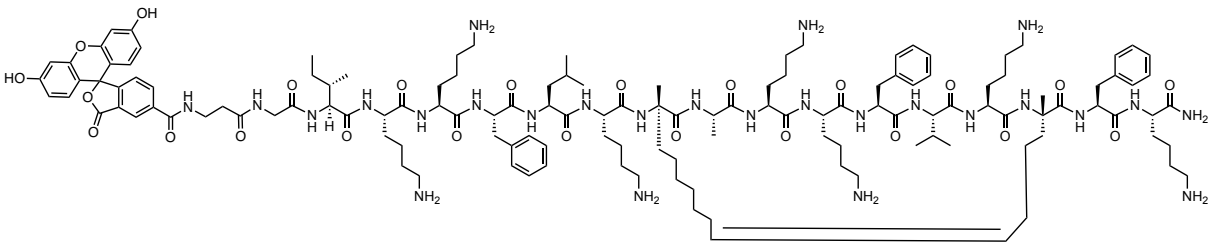


UPLC profile



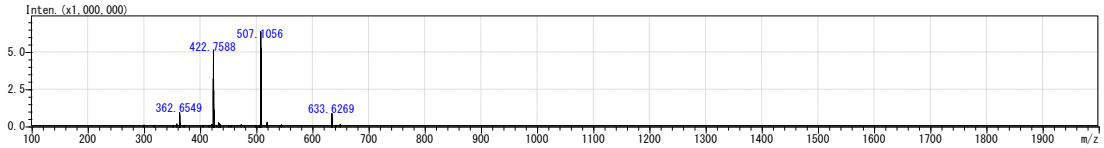
FAM-st7-5

FAM-βAla- Gly-Ile-Lys-Lys-Phe-Leu-Lys-R$_8$-Ala-Lys-Lys-Phe-Val-Lys-S$_5$-Phe-Lys-NH_2

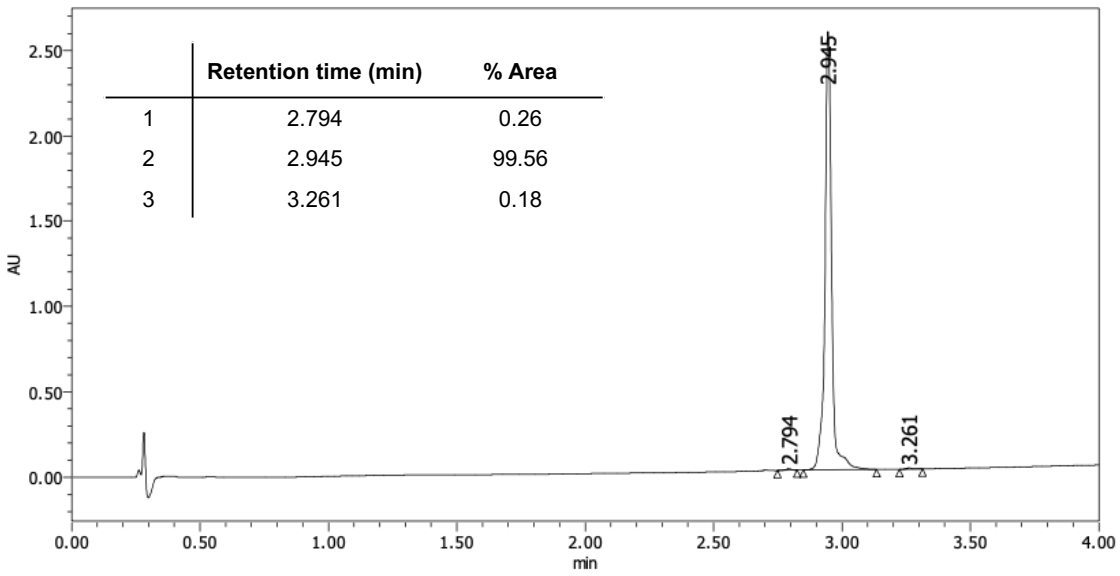


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: 99.56%
- HRMS (ESI$^+$) calcd for $C_{132}H_{196}N_{26}O_{24}$ [M+5H]$^{5+}$: 506.9056; found: 507.1056

ESI-TOF-MS

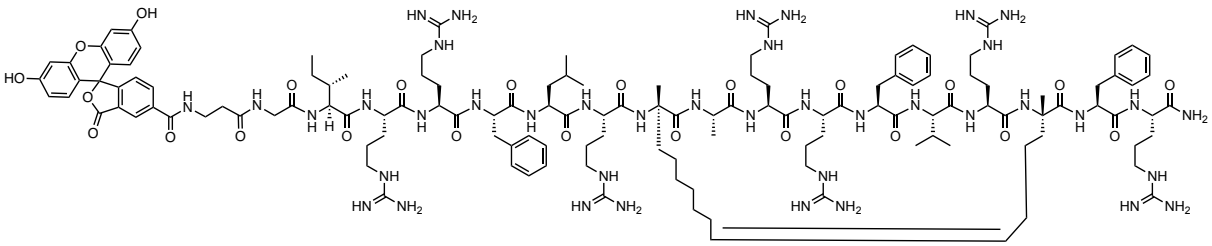


UPLC profile



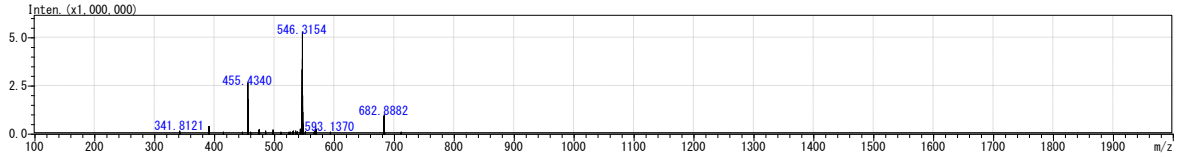
FAM-st7-5_R

FAM-βAla-Gly-Ile-Arg-Arg-Phe-Leu-Arg-R$_8$-Ala-Arg-Arg-Phe-Val-Arg-S$_5$-Phe-Arg-NH$_2$

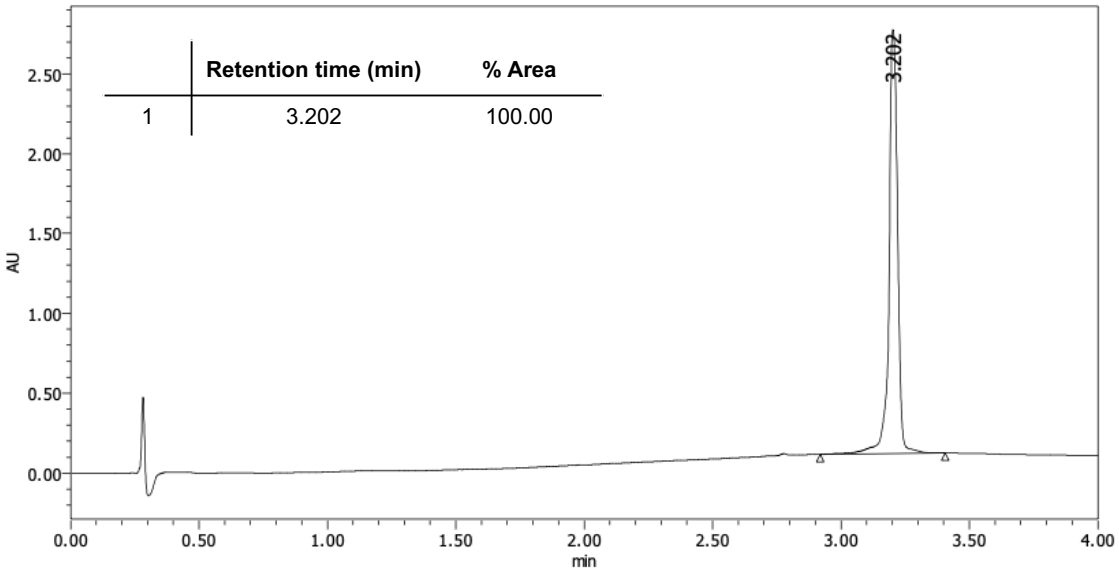


- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{132}H_{196}N_{40}O_{24}$ [M+5H]$^{5+}$: 546.1142; found: 546.3154

ESI-TOF-MS

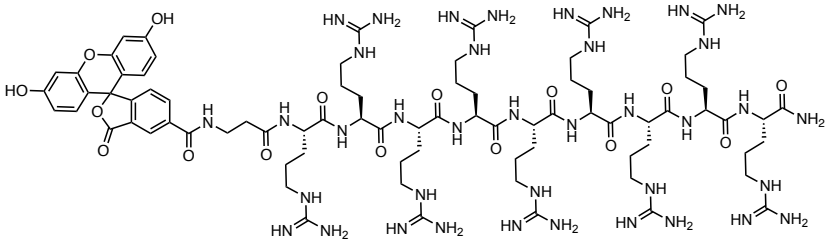


UPLC profile



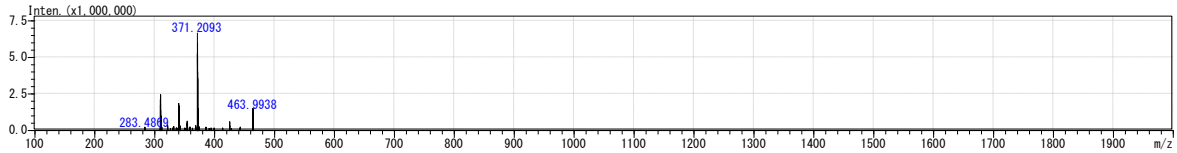
FAM-R9

FAM-βAla-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH_2



- UPLC: Conditions = solvent A 0.1% TFA/water, solvent B 0.1% TFA/MeCN, Gradient = 10-90% gradient of solvent B over 4 min
- Purity: >99%
- HRMS (ESI$^+$) calcd for $C_{78}H_{126}N_{38}O_{16}$ $[M+5H]^{5+}$: 371.2116; found: 371.2093

ESI-TOF-MS



HPLC profile

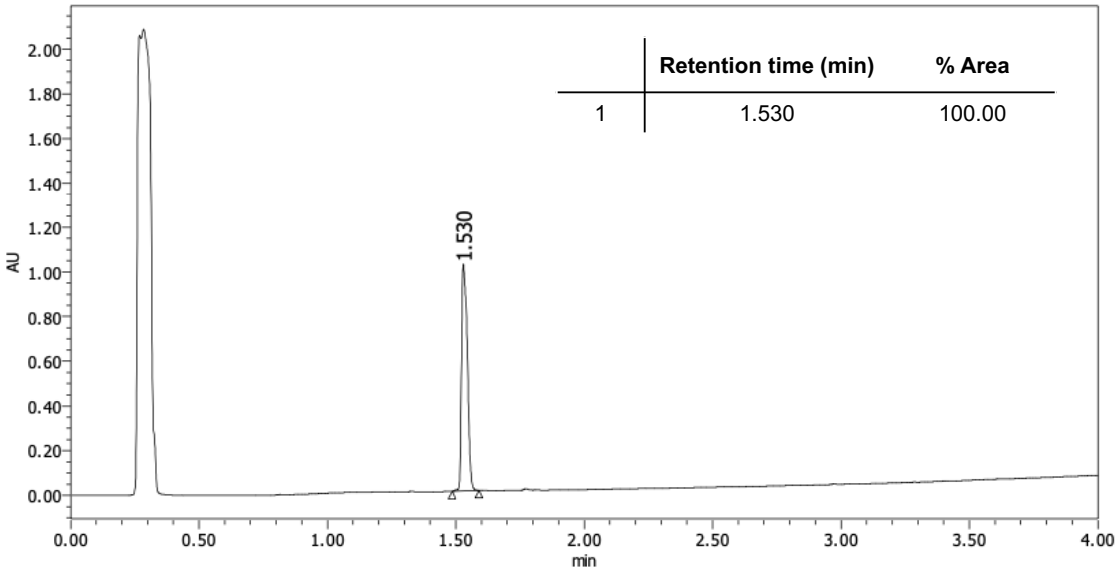


Table S1 Sequence and UPLC retention times of stapled magainin 2 derivative peptides.

Peptide	Sequence	Retention Time [min] (CH_3CN%)
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	2.12 (43.9)
Pep-1	GIKKFLKSAKKFVKAFK	1.86 (39.8)
st4-1	S_5IKKS_5LKSAKKFVKAFK	2.08 (43.2)
st4-2	GS_5KKFS_5KSAKKFVKAFK	2.12 (43.9)
st4-3	GIKKS_5LKSS_5KKFVKAFK	2.31 (47.0)
st4-4	GIKKFLKS_5AKKS_5VKAFK	2.34 (47.5)
st4-5	GIKKFLKSS_5KKFS_5KAFK	2.38 (48.0)
st4-6	GIKKFLKSAKKS_5VKAS_5K	2.09 (43.5)
st7-1	R_8IKKFLKS_5AKKFVKAFK	2.57 (51.1)
st7-2	GR_8KKFLKSS$_5$KKFVKAFK	2.45 (49.2)
st7-3	GIKKR_8LKSAKKS_5VKAFK	2.41 (48.5)
st7-4	GIKKFR_8KSAKKFS_5KAFK	2.41 (48.5)
st7-5	GIKKFLKR_8AKKFVKS_5FK	2.60 (51.6)
st7-6	GIKKFLKSR_8KKFVKAS_5K	2.36 (47.7)
Pep-1_R	GIRRFLRSARRFVRAFR	2.10 (41.9)
st7-5_R	GIRRFLRR_8ARRFVRS_5FR	2.75 (54.9)

Table S2 Size, polydispersity index (PDI), and zeta-potential of each peptide/(A) mRNA and (B)siRNA complex prepared at N/P ratios of 8 and 16.

(A)

Peptide	N/P ratio	Size (nm)	PDI (μ/Γ²)	Zeta-potential (mV)
R9	8	257.0 ± 2.5	0.28 ± 0.04	17.3 ± 2.2
	16	177.7 ± 28.8	0.48 ± 0.04	23.5 ± 3.4
st7-5	8	380.4 ± 16.2	0.35 ± 0.01	18.4 ± 0.6
	16	211.3 ± 3.2	0.39 ± 0.07	12.8 ± 1.8
st7-5_R	8	259.3 ± 13.1	0.39 ± 0.03	21.6 ± 1.3
	16	158.3 ± 47.4	0.50 ± 0.10	15.3 ± 1.6

(B)

Peptide	N/P ratio	Size (nm)	PDI (μ/Γ²)	Zeta-potential (mV)
R9	8	849.8 ± 6.5	0.35 ± 0.04	25.6 ± 0.3
	16	690.6 ± 8.7	0.20 ± 0.01	27.9 ± 0.7
st7-5	8	320.0 ± 4.7	0.34 ± 0.03	18.9 ± 0.5
	16	284.8 ± 21.1	0.43 ± 0.00	21.7 ± 1.1
st7-5_R	8	290.4 ± 6.7	0.33 ± 0.01	27.9 ± 1.1
	16	304.0 ± 5.7	0.42 ± 0.04	27.5 ± 2.1

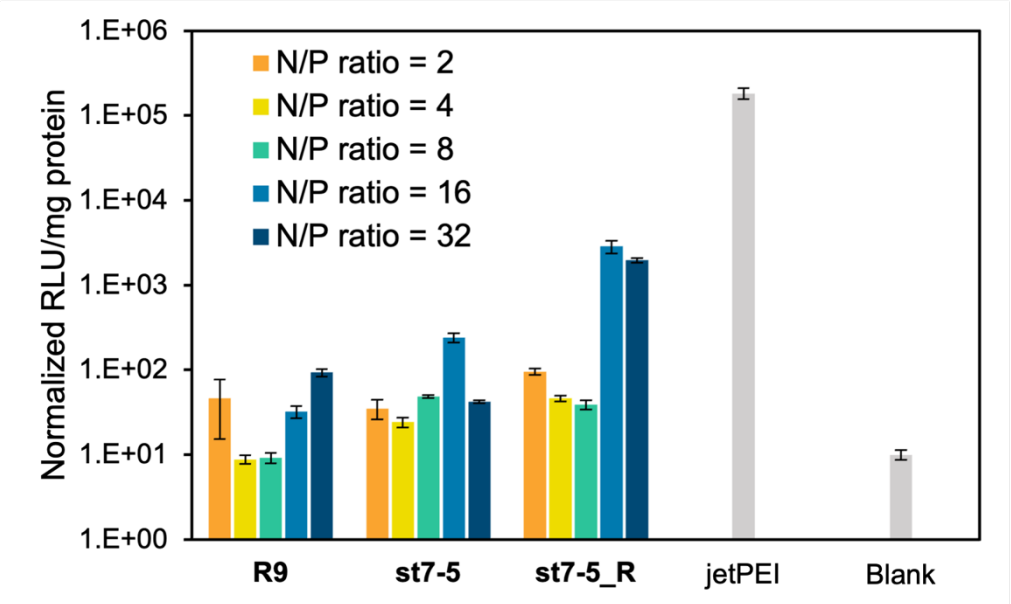


Fig. S1 Transfection efficiency of peptides/pDNA complexes with N/P ratios of 2-32. n = 3, mean ± standard deviation.

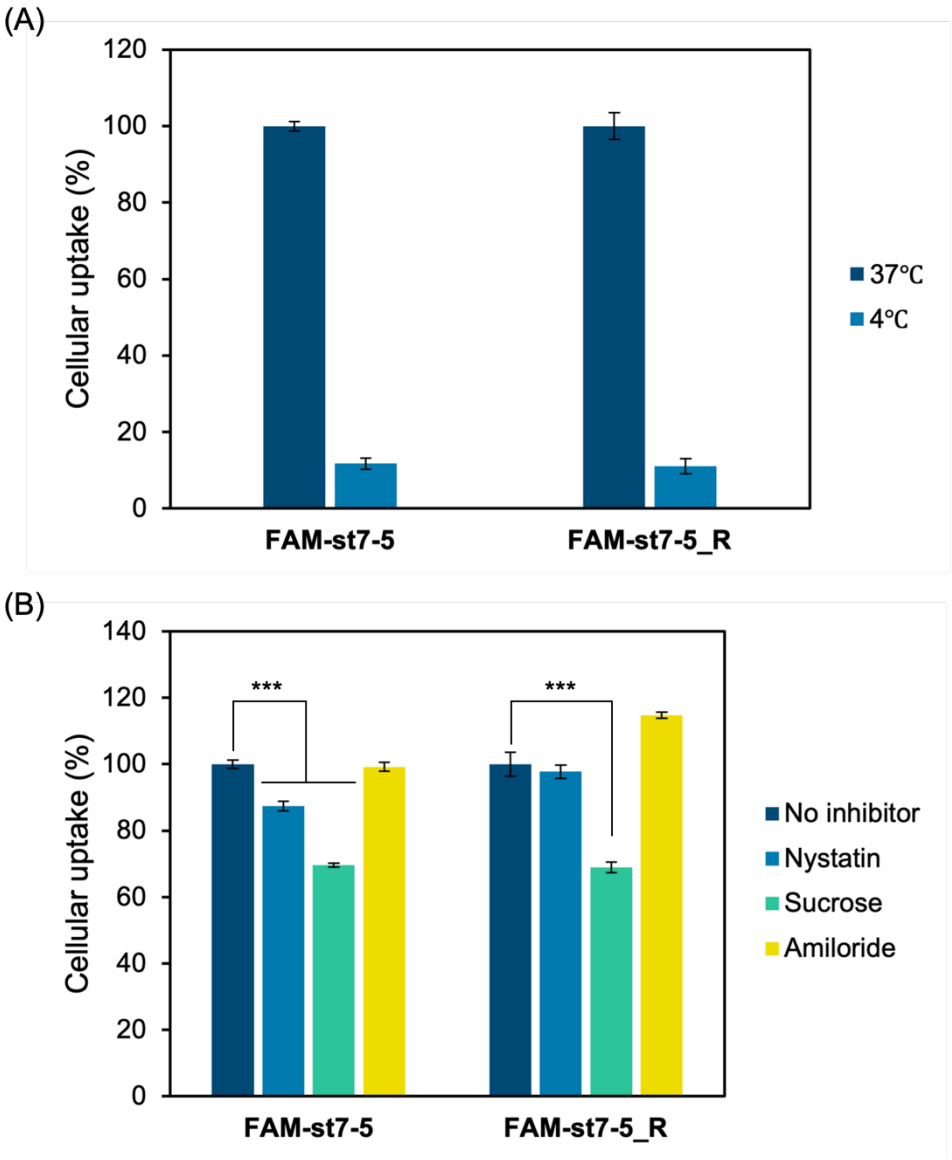


Fig. S2 Effects of (A) low temperature (4°C) and (B) endocytosis inhibitors (nystatin: a caveolae-mediated endocytosis inhibitor; sucrose: a clathrin-mediated endocytosis inhibitor; amiloride: a macropinocytosis inhibitor) on intracellular uptake of peptides on HeLa cells. n = 3, mean ± standard deviation. Symbols (*) indicate statistical significance (***$p < 0.001$).

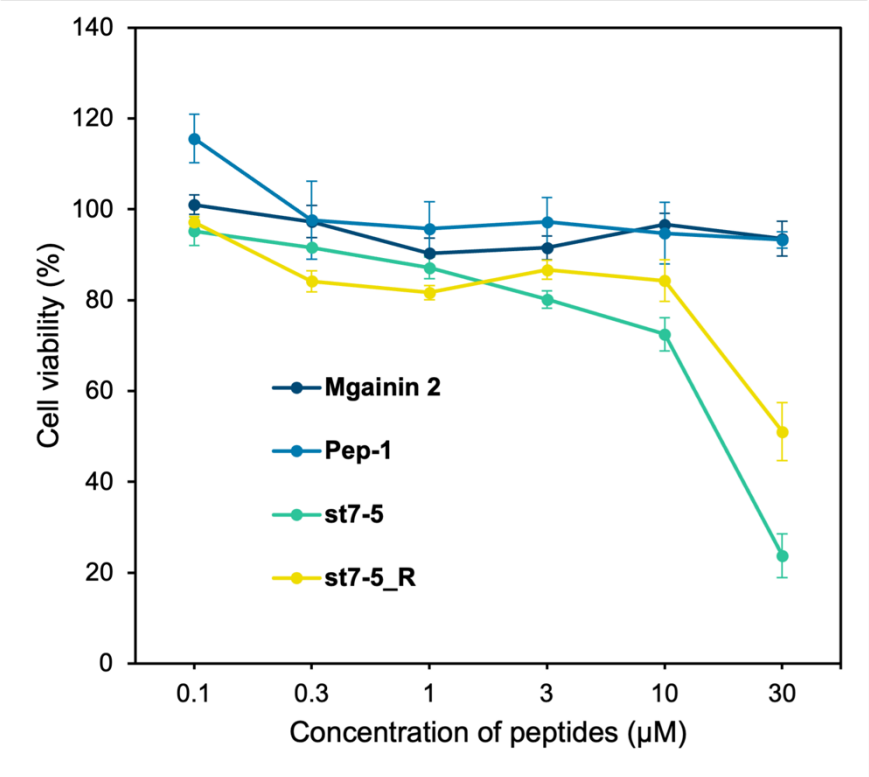


Fig. S3 Cytotoxicity of each peptide at various concentrations on HeLa cells. HeLa cells were added to the peptides and incubated for 24 hours, then cell viability was assessed using WST-8.

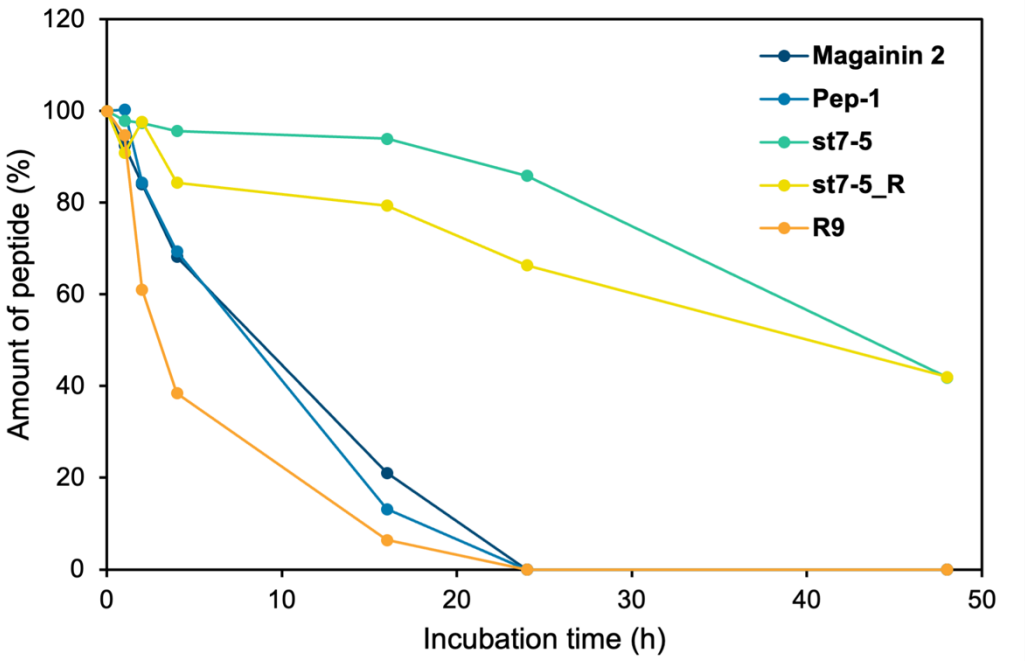


Fig. S4 Percentage of each peptide remaining in the presence of Trypsin. Trypsin was added to each peptide solution, incubated at 37°C for an arbitrary time, and analyzed using UPLC.

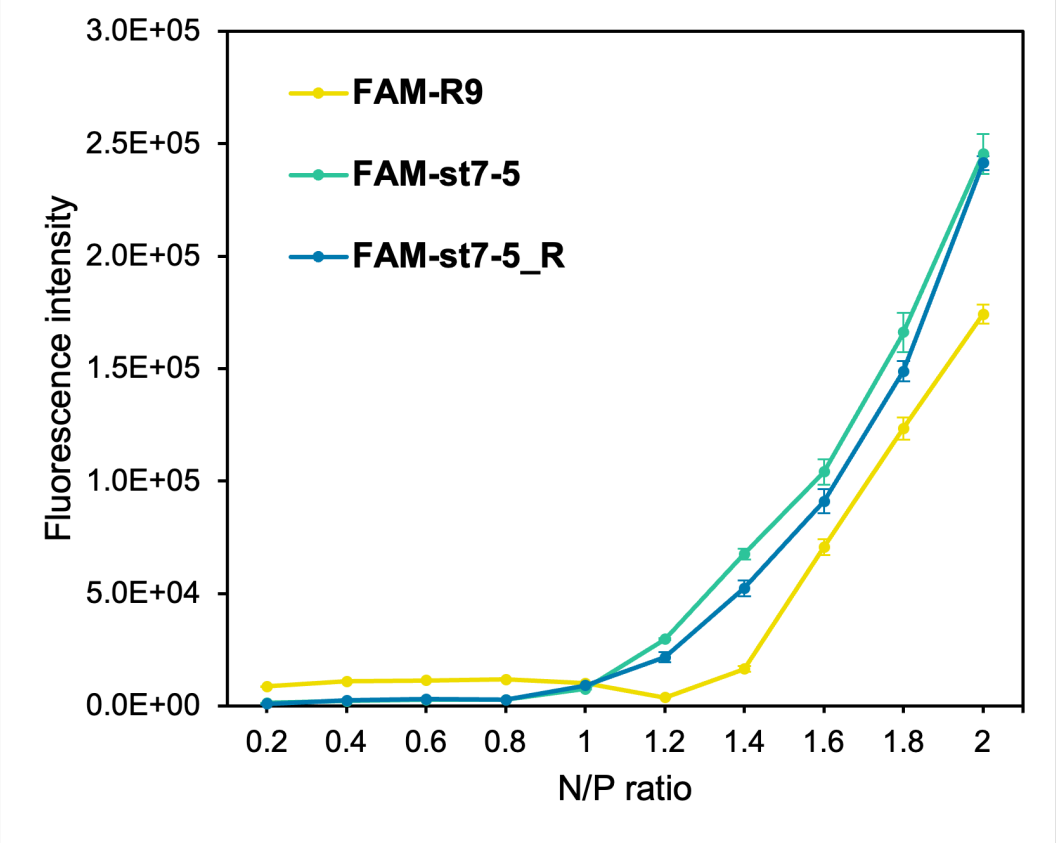
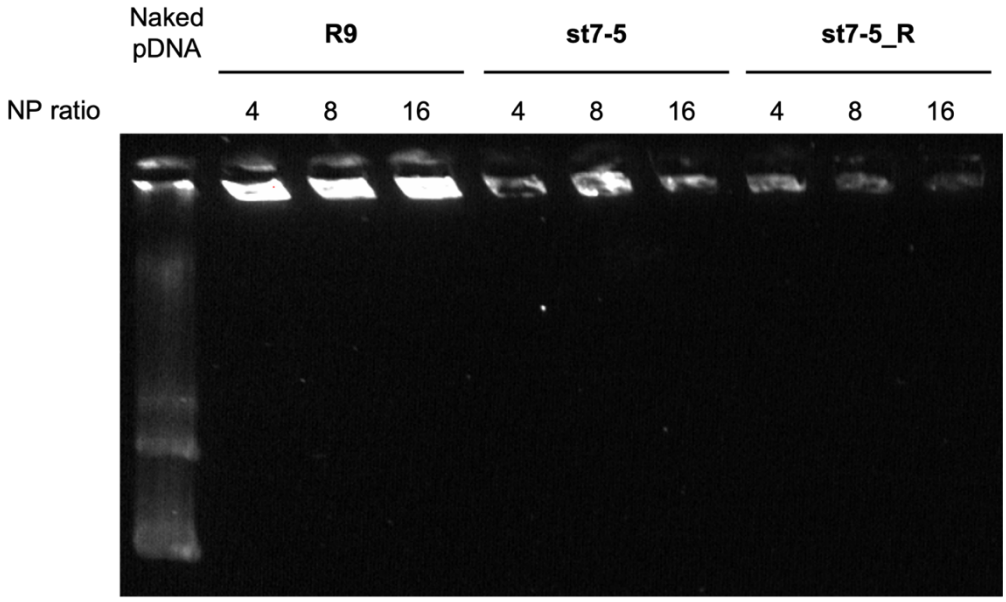
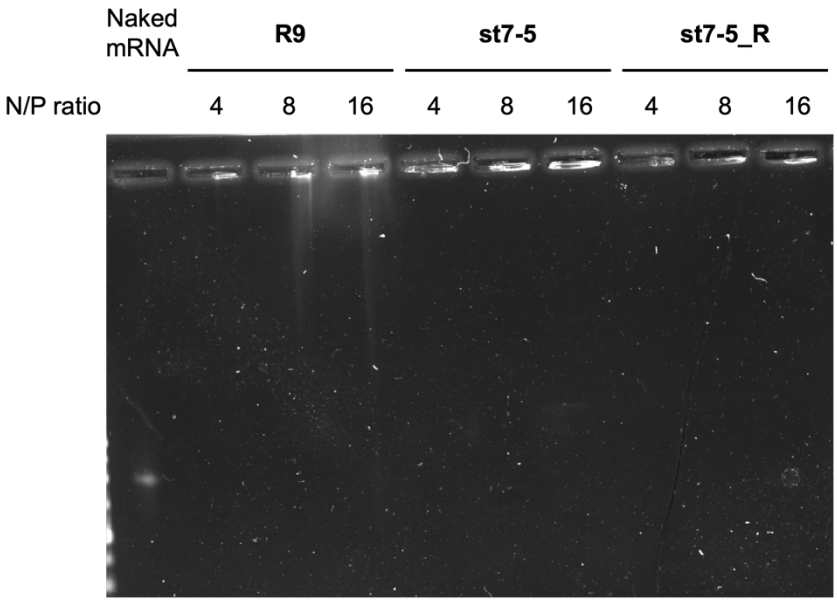


Fig. S5 Fluorescence intensities of each FAM-labeled peptide/pDNA complex solutions prepared at various N/P ratios. n = 3, mean ± standard deviation.

(A)


Naked pDNA
R9
st7-5
st7-5_R
NP ratio
4
8
16
4
8
16
4
8
16

(B)


Naked mRNA
R9
st7-5
st7-5_R
N/P ratio
4
8
16
4
8
16
4
8
16

(C)

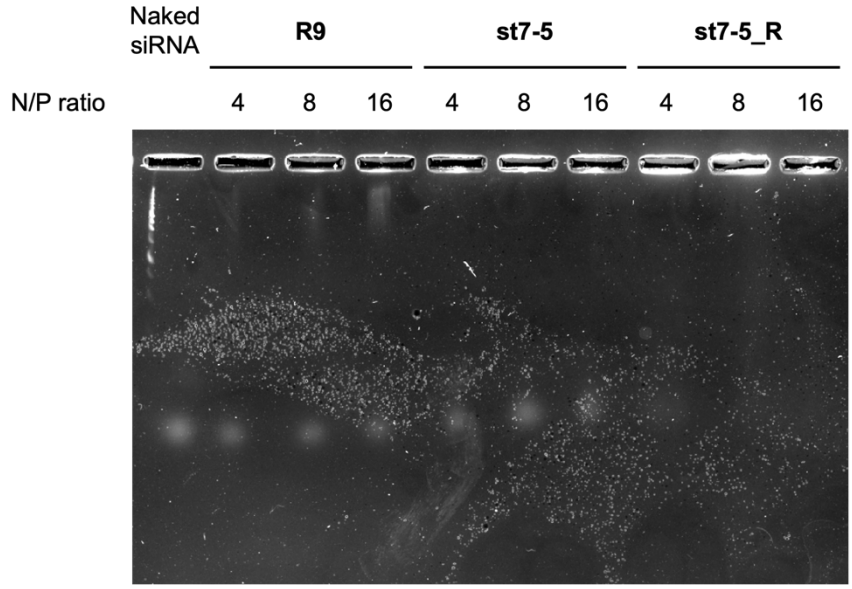
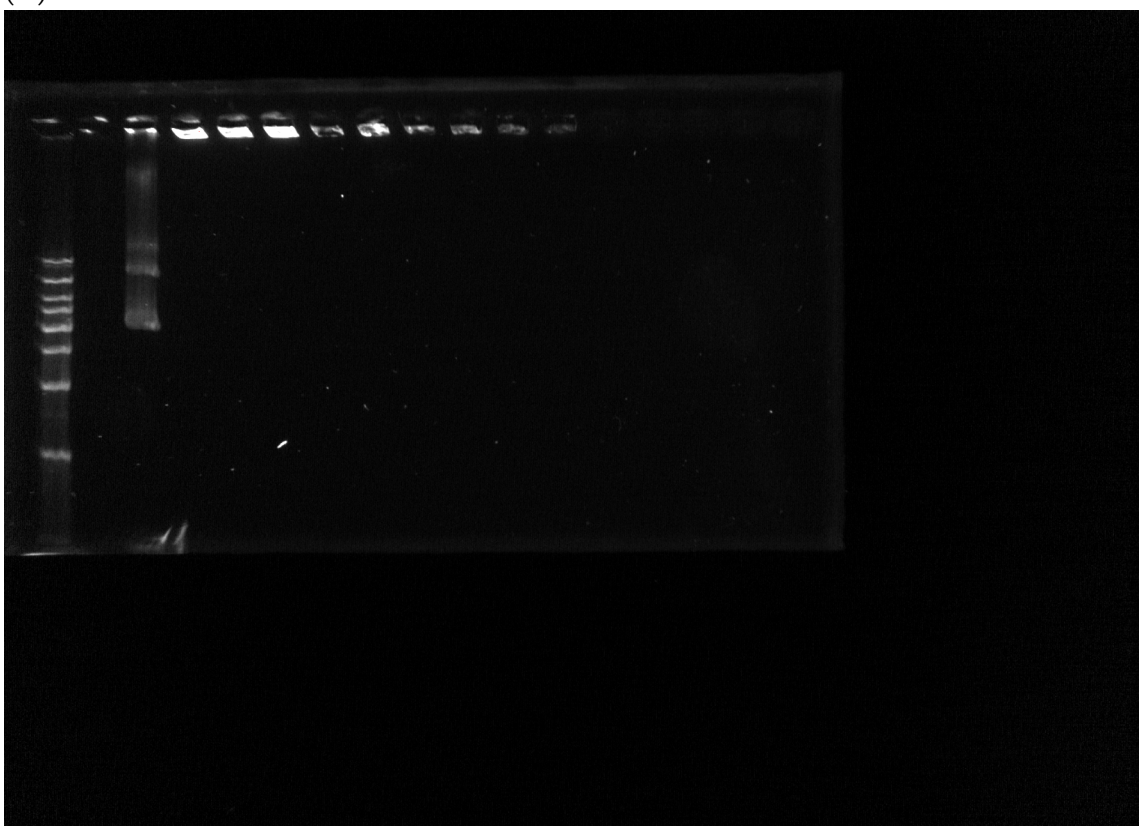
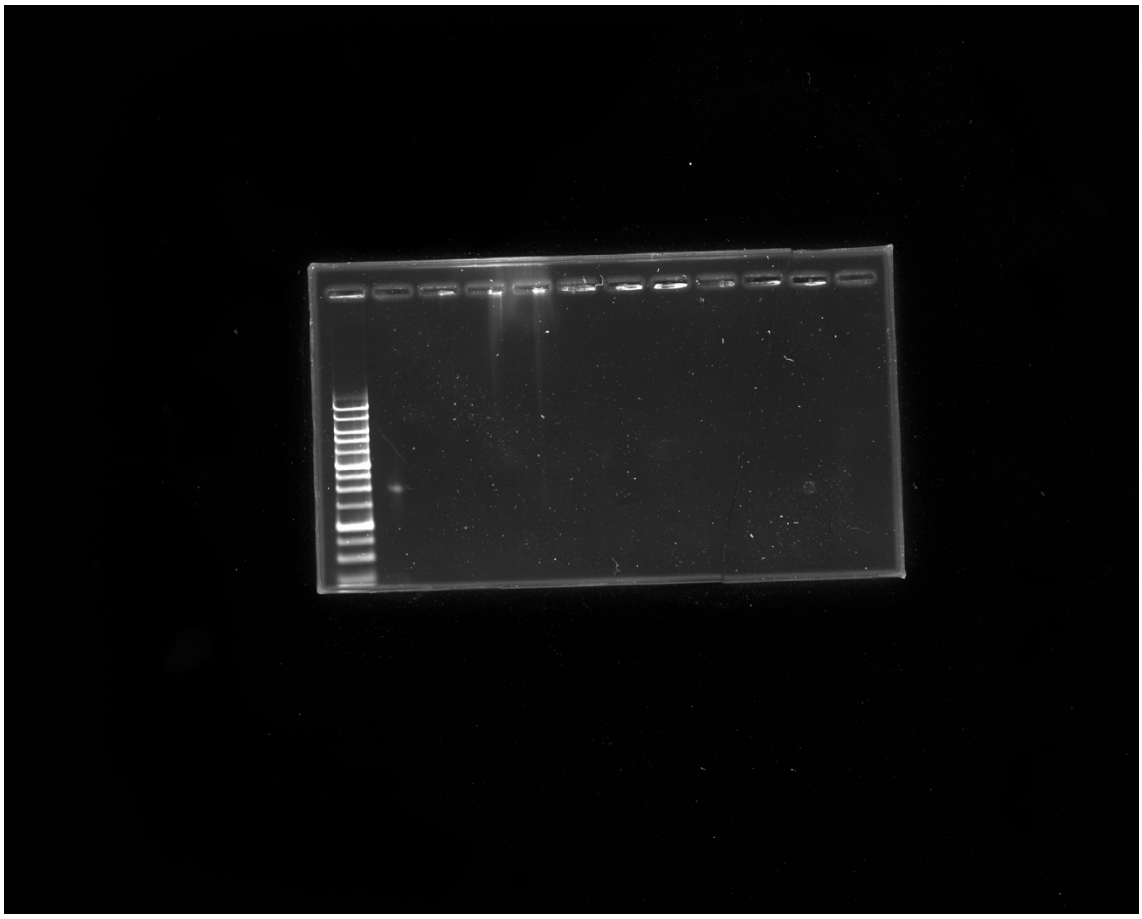


Fig. S6 Gel retardation assay of each peptide/(A) pDNA, (B) mRNA and (C) siRNA complex with NP ratio =4 and, 8, 16. Electrophoresis was performed using a 1% agarose gel for pDNA and mRNA, a 4% agarose for siRNA, and the gel was stained with Midori Green.

(A)



(B)



(C)

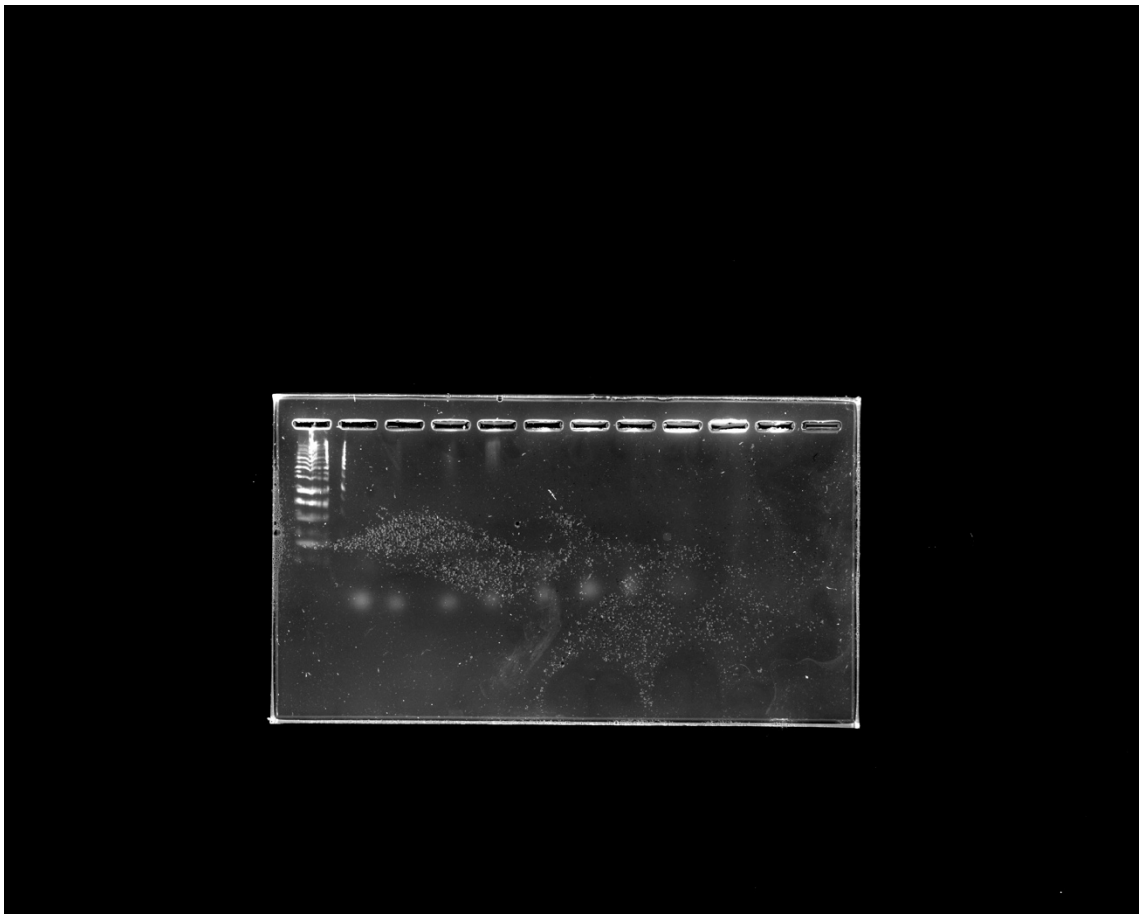
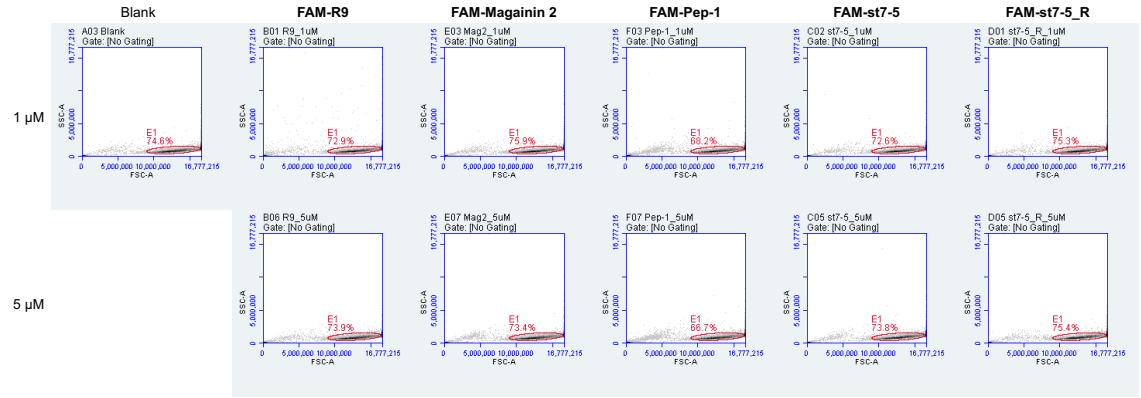


Fig. S7 Original image of gel retardation assay. Each peptides/(A) pDNA, (B) mRNA and (C) siRNA complex with NP ratio =4 and, 8, 16.

(A)



(B)

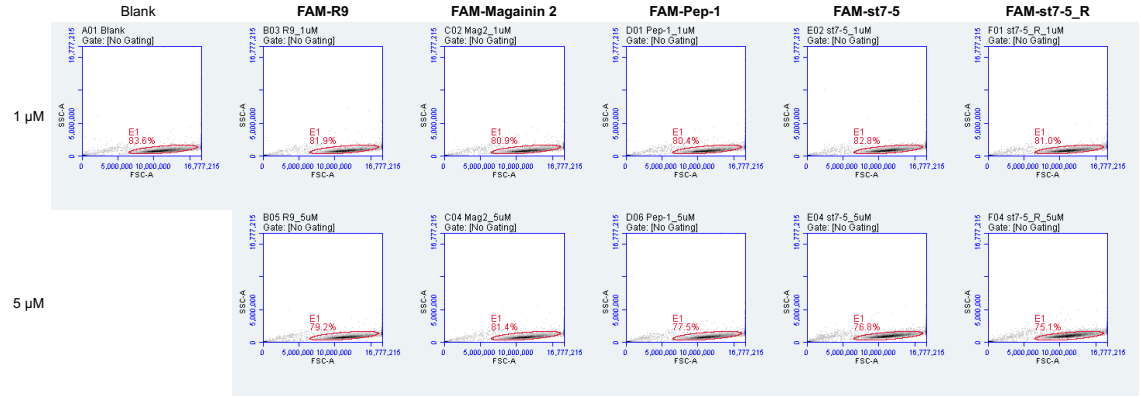
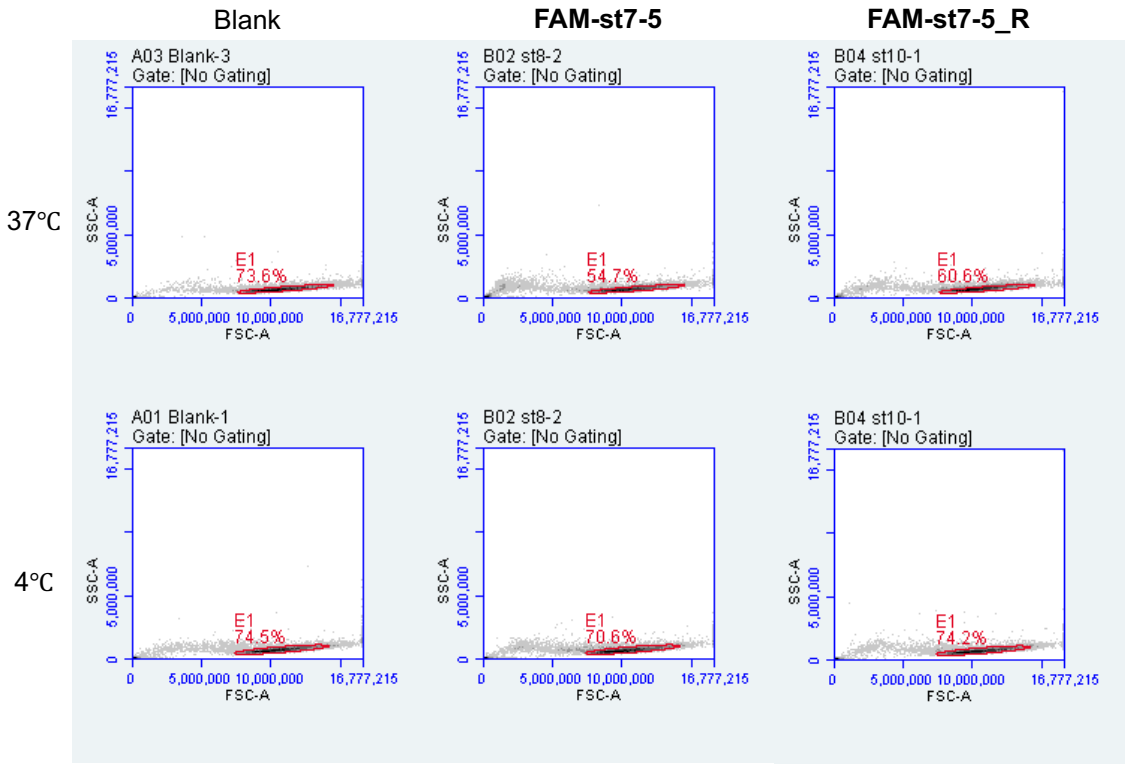


Fig. S8 Raw data of flow cytometry analysis for scatter plots with gating information in Fig. 3A. These data showed treatments of FAM-labelled peptides at 1 and 5 μM and incubation for (A) 1 h and (B) 24 h.

(A)


Blank
FAM-st7-5
FAM-st7-5_R
37℃
4℃
A03 Blank-3
Gate: [No Gating]
E1
73.6%
B02 st8-2
Gate: [No Gating]
E1
54.7%
B04 st10-1
Gate: [No Gating]
E1
60.6%
A01 Blank-1
Gate: [No Gating]
E1
74.5%
B02 st8-2
Gate: [No Gating]
E1
70.6%
B04 st10-1
Gate: [No Gating]
E1
74.2%
SSC-A
FSC-A
0
5,000,000
10,000,000
16,777,215

(B)

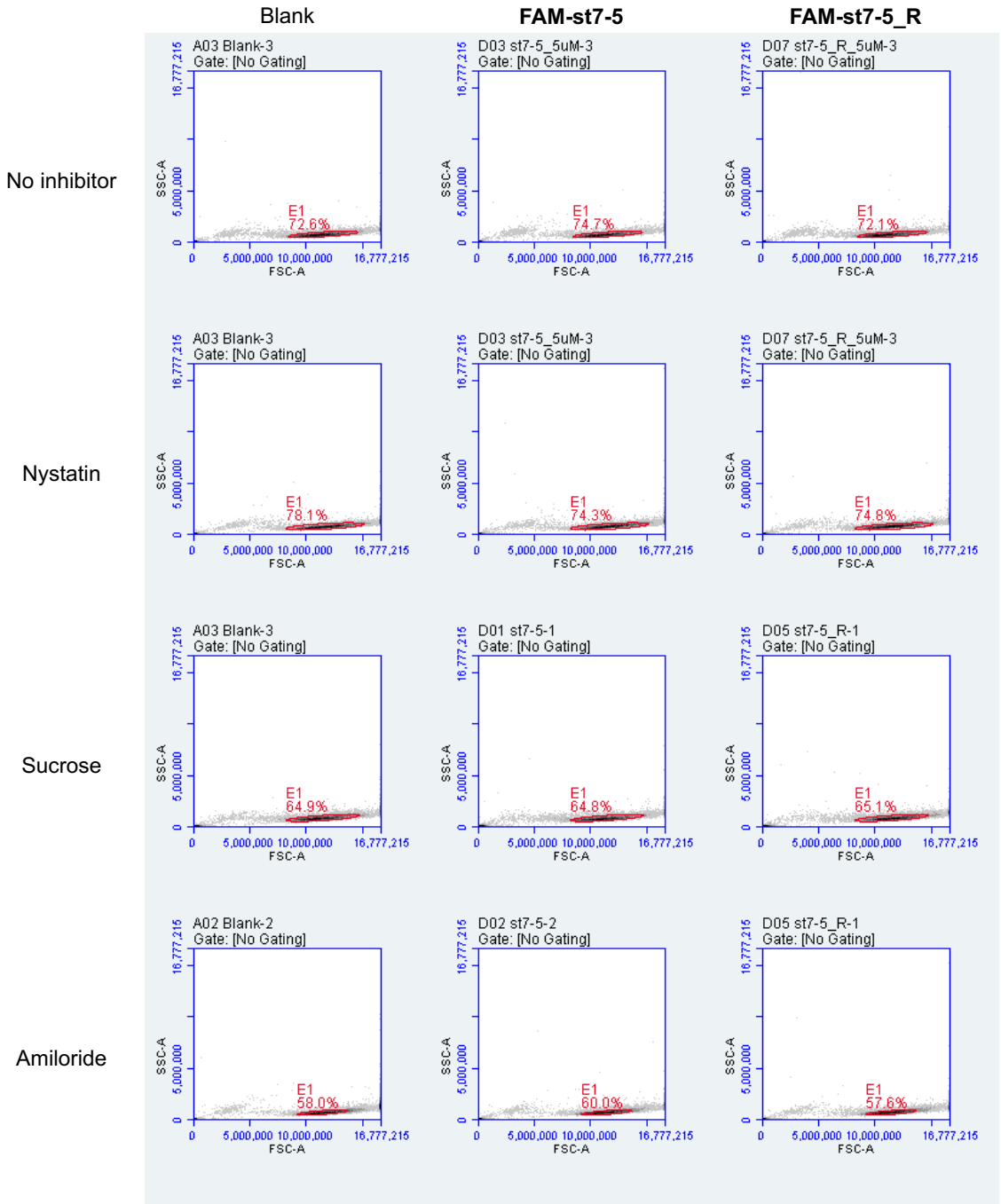
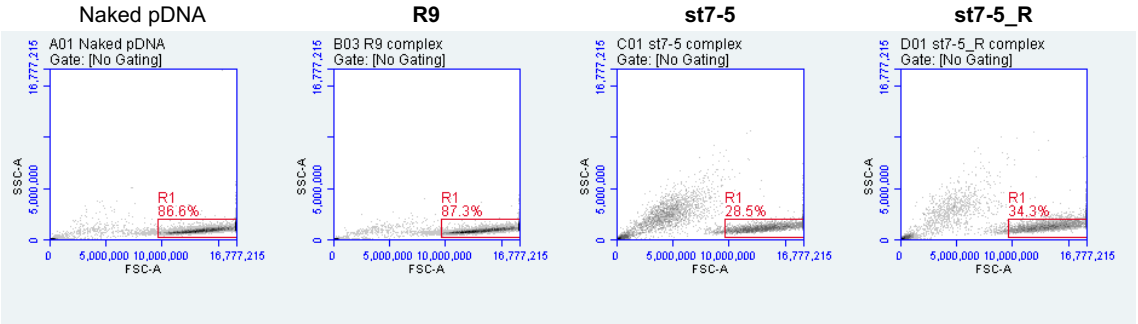


Fig. S9 Raw data of flow cytometry analysis for scatter plots with gating information in Fig. S2A and B. These data showed treatments of FAM-labelled peptides at 5 μM (A) under low temperature (4℃) or (B) with endocytosis inhibitor (nystatin: a caveolae-mediated endocytosis inhibitor; sucrose: a clathrin-mediated endocytosis inhibitor; amiloride: a macropinocytosis inhibitor).

(A)



(B)

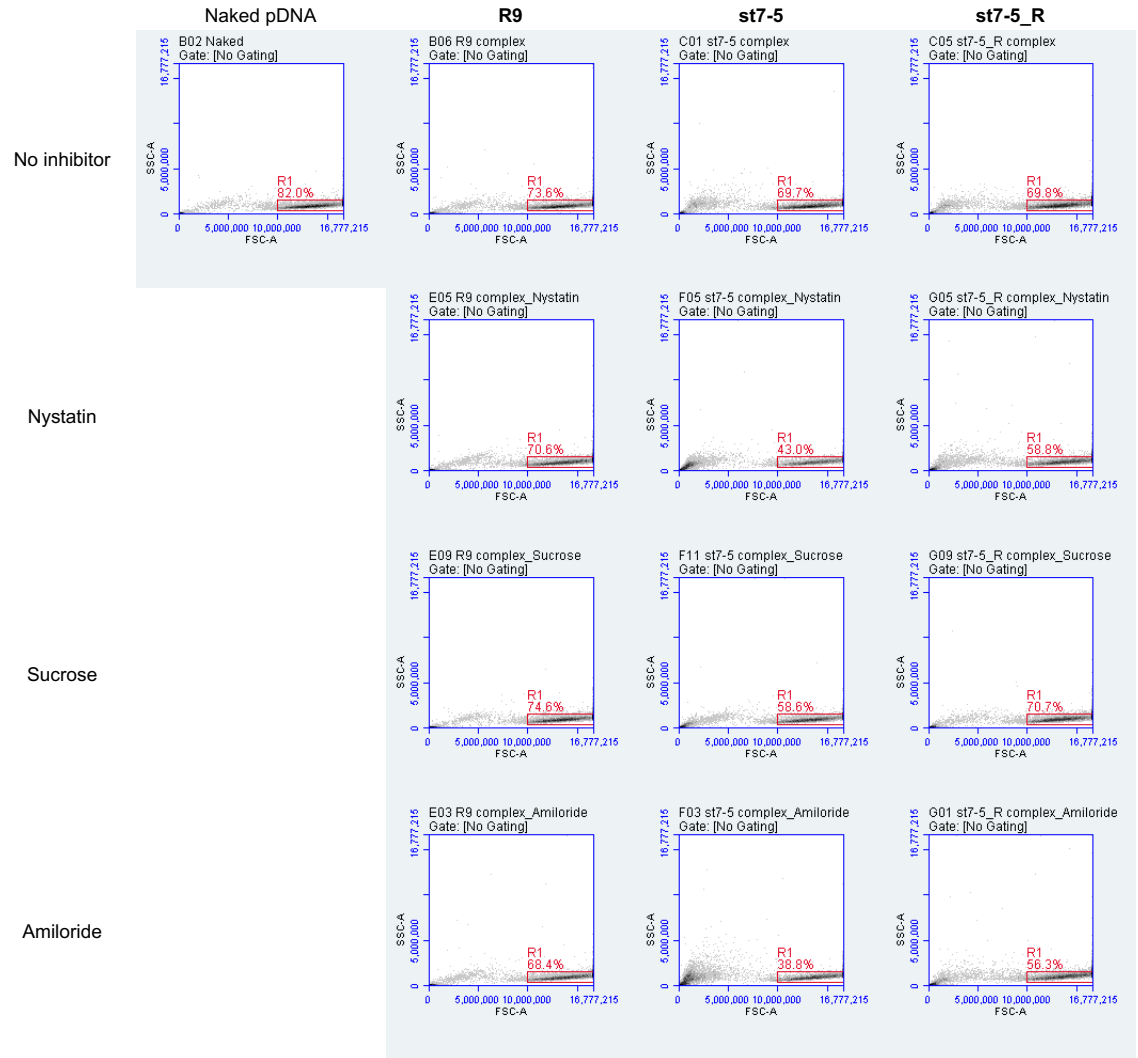


Fig. S10 Raw data of flow cytometry analysis for scatter plots with gating information in Fig. 4B and C. These data showed treatments of Cy5-labelled pDNA/peptide complex at 16 of N/P ratio (A) incubation for 24 h or (B) 1 h with endocytosis inhibitor (nystatin: a caveolae-mediated endocytosis inhibitor; sucrose: a

clathrin-mediated endocytosis inhibitor; amiloride: a macropinocytosis inhibitor).