Supporting Information

Development of 2-aminobutyric acid (Aib)-rich cell-penetrating foldamers for efficient siRNA delivery

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1. Experimental section

1.1 General

All coupling reagents were obtained from Watanabe Chemical Industries, LTD. and were used as supplied without further purification. Fmoc-protected amino acids were obtained from Tokyo Chemical Industry Co. LTD.. The purified peptides were characterized using SHIMADZU LCMS-IT-TOF spectrometer and 4800 Plus MALDI TOF/TOFTM Analyzer (Applied Biosystem/MDS SCIEX), JASCO J-720W and J-1000, and Fluorescent intensity was recorded with BD accuri C6 Plus (Becton Dickinson Co., Ltd.).

1.2 Chemistry

Synthesis and characterization of N-terminal-protected amino acids and peptides. The peptides were synthesized using solid-phase methods on NovaPEG Rink amide resin following the standard Fmoc chemistry. The following describes a representative coupling and deprotection cycle at a 50 μmol scale. First, 100 mg NovaPEG Rink amide resin (loading: 0.5 mmol/g) was soaked for 1 hr in CH_2Cl_2. After the resin had been washed with *N*,*N*-dimethylformamide (DMF), Fmoc-amino acid (4 Eq), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) (4 Eq) and 1-hydroxy-7-azabenzotriazole (HOAt) (4 Eq) dissolved in 2 mL *N*-methyl-2-pyrrolidone (NMP) were added to the resin. Then, *N*,*N*-diisopropylethylamine (8 Eq) was added for the coupling reaction and the resulting mixture was shaken for 1 hr at room temperature. Fmoc-deprotection was carried out by 2 mL of 20% piperidine in DMF (2 mL) for 15 min at room temperature. After the peptide elongation, the resin was suspended in cleavage cocktail [1.9 mL trifluoroacetic acid (TFA), 50 μL water, 50 μL triisopropylsilane; final concentration: 95% TFA, 2.5% water, 2.5% triisopropylsilane] for 3 h at room temperature. The TFA solution was evaporated to a small volume under a stream of N_2 and dripped into cold ether to precipitate the peptides. The dried crude peptides were dissolved in 1.3 mL of 50% acetonitrile in water and then purified by reversed-phase HPLC using a Discovery® BIO Wide Pore C18 column (25 cm x 21.2 mm). After being purified, the peptide solutions were lyophilized. Peptide purity was assessed using analytical HPLC and a Discovery® BIO Wide Pore C18 column (25 cm x 4.6 mm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.0 $mL \bullet mL^{-1}$, gradient: 10-100% gradient of solvent B over 30 min). The peptides were characterized by liquid chromatography–mass spectrometry-ion trap-time-of-flight spectroscopy. Detailed experimental procedures, HPLC charts, and mass spectrometric data of each peptide were shown in Supplementary data.

CD spectrometry. CD spectra were recorded using a 1.0 mm path length cell. The data are expressed in terms of [θ]; i.e., total molar ellipticity (deg cm^2 $dmol^{-1}$). 20 mM phosphate buffer (pH = 5.0) were used as solvents. Peptide concentration; 50 μM.

1.3 Biology

Materials. Tissue culture plastics were purchased form Greiner Bio-One. Fetal bovine serum (FBS) was purchased form Equitech-Bio. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium , and Kanamycin were purchased form Sigma-Aldrich. Opti-MEM Medium without serum was purchased form Life Technologies. All of cell lines were purchased from ATCC. Antibodies were

purchased from Cell Signaling, Sigma, or Santa Cruz (see below). siRNAs were purchased from Life Technologies or FASMAC (see below).

Cell culture. Human breast carcinoma MCF-7 cells were maintained in RPMI 1640 medium containing 10% FBS and 100 μg/mL kanamycin. Human cervical carcinoma HeLa cells, bronchioalveolar carcinoma NCI-H358 cells, and human hepatocellular carcinoma HepG2 cells were maintained in DMEM containing 10% FBS and 100 μg/mL kanamycin.

Western blotting. Cells were lysed with SDS lysis buffer (0.1 M Tris-HCl at pH 8.0, 10% glycerol, 1% SDS) and immediately boiled for 10 min to obtain clear lysates. Protein concentration was measured by the BCA method (Pierce); lysates containing equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (purchased from Millipore) for western blot analysis using the appropriate antibodies. Immunoreactive proteins were visualized using the Immobilon Western chemiluminescent HRP substrate (purchased from Millipore) or Clarity Western ECL substrate (Bio-Rad); light emission intensity was quantified using an LAS-3000 lumino-image analyzer equipped with Image Gauge v2.3 software (Fuji). The antibodies used in this study were: anti-ERα rabbit monoclonal antibody (mAb) (Cell Signaling Technology, 8644), anti-β-actin mouse mAb (Sigma, A5316), anti-XIAP rabbit pAb (Cell Signaling Technology, 2042), anti-K-Ras mouse mAb (Santa Cruz, sc-30), anti-NOTCH1 rabbit mAb (Cell Signaling Technology, 3608), anti-AhR rabbit mAb (Cell Signaling Technology, 13790).

siRNA transfection by Block peptides. The indicated dose of Block peptides and siRNA were mixed in 250 μl Opti-MEM Medium without serum (Life Technologies) in 12 well of cell culture plate. After 10-20 minutes at room temperature, cells in complete growth medium were added into the plate and they were incubated for the indicated periods at 37 ˚C in a humidified atmosphere of 5% CO_2. The stealth RNAi siRNA (Life Technologies) sequences used in this study were: human XIAP-1 (5'-ACACUGGCACGAGCAGGGUUUCUUU-3'); XIAP-2 (5'-GAAGGAGAUACCGUGCGGUGCUUUA-3'); XIAP-3 (5'-CCAGAAUGGUCAGUACAAAGUUGAA-3'); ERα-1 (5'-CGACAUGCUGCUGGCUACAUCAUCU-3'); ERα-2 (5'-UCACAGACACUUUGAUCCACCUGAU-3'); and ERα-3 (5'-GACCGAAGAGGAGGGAGAAUGUUGA-3'). The siRNA (FASMAC) sequences used in this study were: human NOTCH1-1 (5'-GCAACAGCUCCUUCCACUUtt-3'); NOTCH1-2 (5'-GCAUGGUGCCGAACCAAUAtt-3'); AhR-1 (5'-GCUCUGAAUGGCUUUGUAUtt-3'); AhR-2 (5'-GCUACCACAUCCACUCUAAtt-3'); and AhR-3 (5'-CCUGUAAUCAGCCUGUAUUtt-3').

Cell proliferation assay. Cell viability was determined using water-soluble tetrazolium WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) for the spectrophotometric assay according to the manufacturer's instructions (purchased from Dojindo). Cells treated with compounds were incubated with WST-8 reagent for 0.5 h at 37 ˚C in a humidified atmosphere of 5% CO_2. The absorbance at 450 nm of the medium was measured using an EnVision Multilabel Plate Reader (PerkinElmer).

Cellular uptake of peptides. MCF-7 cells were seeded in 12-well dishes at a density of 4.0 x 10^6 cells/well and cultured in RPMI-1640 (MCF-7) for 24 h, respectively. The cells were treated with each peptide (peptide concentration; 10 μM) and each ROX-siRNA 100 pmol, and incubated for 24 h. Then, the cells were washed three times with phosphate buffer (PBS) supplemented with heparin (20 units/mL) and detached by treatment of trypsin-EDTA. The collected cells were pelleted by centrifugation at 3000 rpm for 5 min and the supernatant was removed. The cells were washed twice with PBS buffer. Then, the collected cells were suspended in 500 μL of PBS buffer and mean fluorescence intensity in cells was measured by flow cytometer. The results are presented as the mean and standard deviation obtained from 3 samples.

Inhibition of endocytosis. The cells were seeded onto 6-well culture plates (400,000 cells/well) and incubated overnight in 2 mL of RPMI-1640 containing 10% FBS. After the medium had been replaced with fresh medium containing 10% FBS in the absence or presence of amiloride (25 μM), sucrose (0.4 M), or nystatin (25 μg/mL), the cells were pre-incubated at 37 °C for 30 min. Peptide solution was applied to each well at a concentration of 10 μM. After the cells had been incubated for 1 h, the medium was removed, and the cells were washed 3 times with PBS supplemented with heparin (20 units/mL) and detached by treatment of trypsin-EDTA. Then, fluorescence intensity in the cells was measured as above. The results are presented as the mean and standard error values obtained from 3 samples.

Flow cytometry analysis. The cells were seeded onto 6-well culture plates (400,000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. After the medium had been replaced with fresh medium containing 10% FBS and peptide solution was applied to each well at a concentration of 1 μM. After the cells had been incubated for 1 hr at 37 °C, the medium was removed, and the cells were washed 3 times with PBS supplemented with heparin (20 units/mL) and detached by treatment of trypsin-EDTA. Then, fluorescence intensity in the cells was measured as above.

Gel shift assay. 4 pmol of ROX-siRNA (FASMAC) and **Block3**, **4** in Opti-MEM were incubated for 30 min at room temperature, and the mixture was added the loading buffer and loaded to agarose gel

(4% agarose 21). Subsequently electrophoresis at 100 V for 45 min was performed, and the resulting gel was recorded using Gel imager (typhoon scanner).

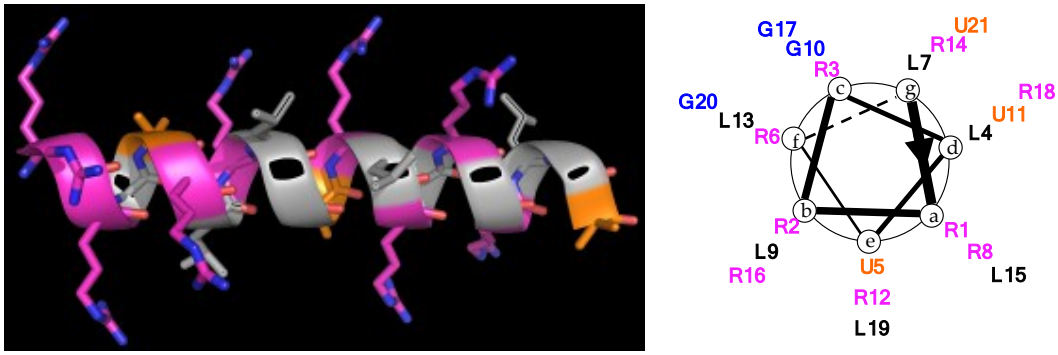
Stability analysis. 7.5 μg of R9 and **Block3** peptides were incubated at 37 ˚C in RPMI medium containing 10% FBS. The peptides degradations were detected using HPLC.

Size and zeta-potential measurements. The size and size distribution of **Block3**/siRNA and **Block4**/siRNA in 10 mM HEPES buffer (pH 7.3) were evaluated by dynamic light scattering using Nano ZS (ZEN3600, Malvern Instruments, Ltd.) with an incident light (633 nm). Measurements were carried out with a detection angle of 173º and temperature of 25 ºC, and data were subsequently analyzed by the cumulant method. The zeta-potential was evaluated using the same apparatus by the laser-Doppler electrophoresis method. Results were presented as a mean and standard deviation of three measurements.

1. Supplementary Results

Block3 FAM-βAla-LLULLULLUGGGRRRRRRRRR-NH_2

Block4 FAM-βAla-RRRLURLRLGURLRLRGRLGU-NH_2



Block6 FAM-βAla-RLLRRUGRLLRRUGRLLRRUG-NH_2

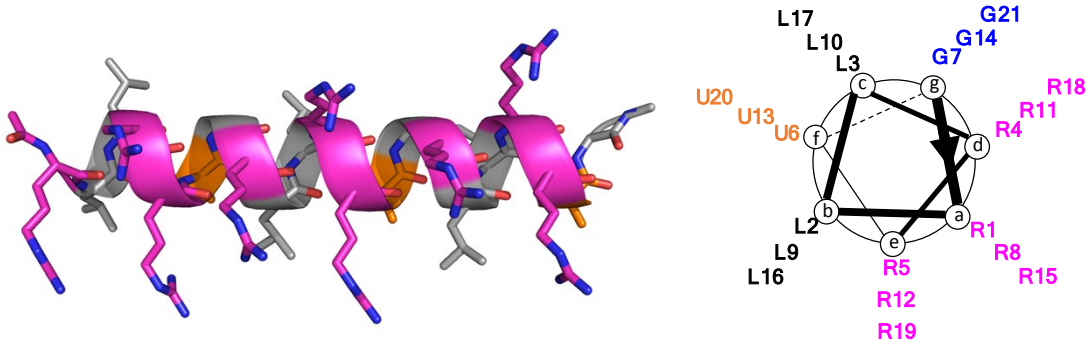


Figure S1. (a) Chemical structure and (b) helical wheel diagram of Block3, **Block4** and **Block6**. Magenta and tan represents Arg and Aib residue, respectively.

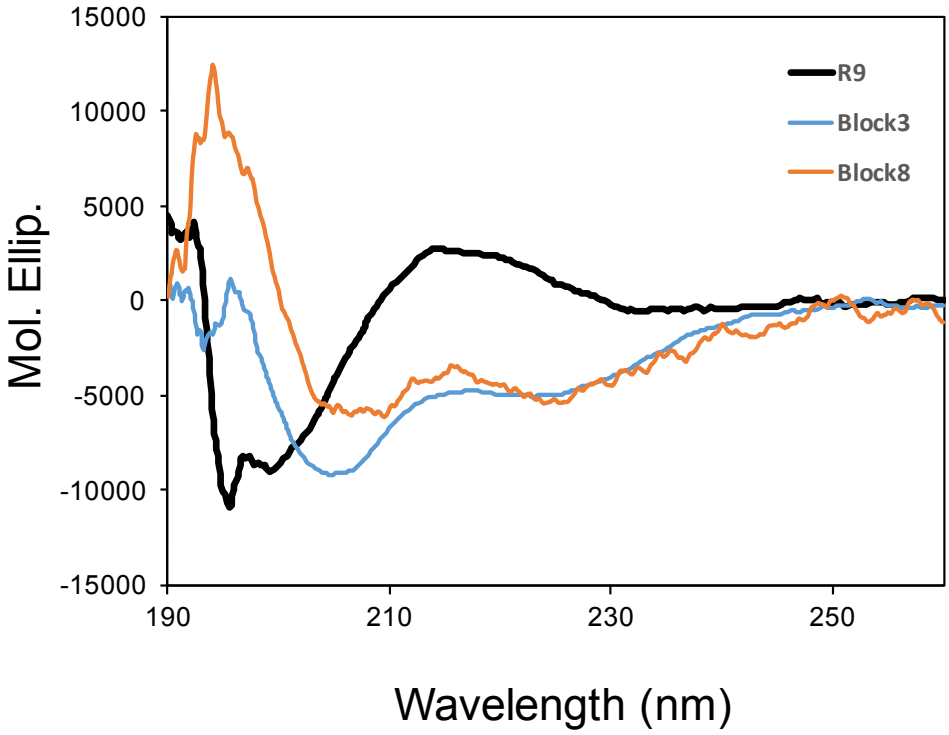


Figure S2. The preferred secondary structure of R9, **Block3**, and **Block8** by CD spectra in 20 mM PBS (pH = 5.0), Peptide concentration = 50 μM.

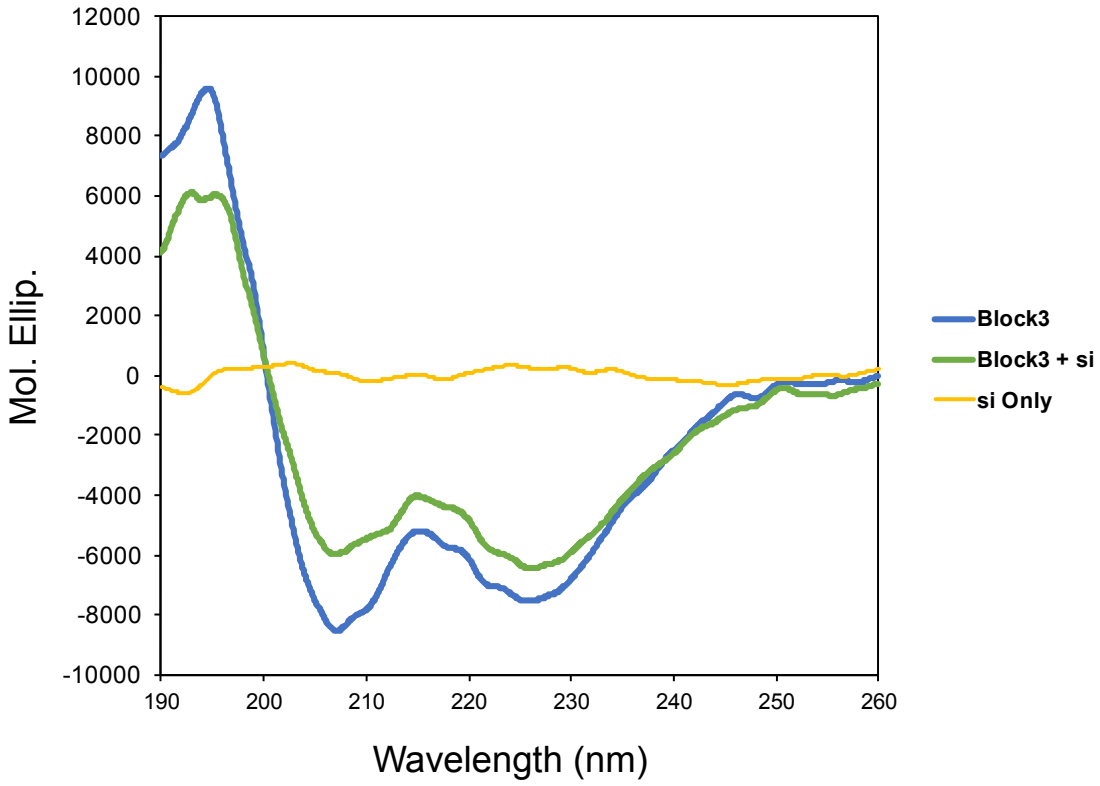


Figure S3. Effects of siRNA (100 pmol) treatment on preferred secondary structure of **Block3** by CD spectra in 20 mM PBS (pH = 5.0), Peptide concentration = 50 μM.

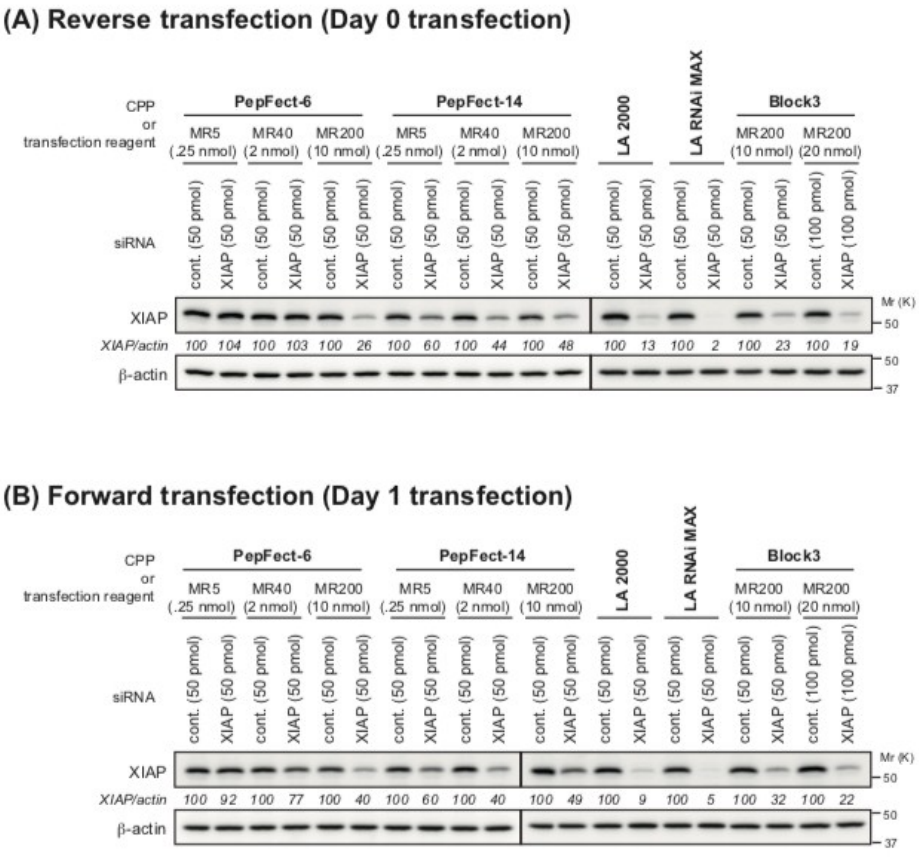


Figure S4. The Comparison of siRNA delivery efficiency between **Block3** and commercially available carrier peptides or transfection reagent. (A) The indicated siRNAs were mixed with CPPs (or transfection reagents) in Opti-MEM of 12-well plates at the indicated molar ratios (MRs). Complexes were formed for 20 min or 30 min at room temperature. Thereafter, MCF-7 cells (2.0 x 10^5 cells) were added to each well. (B) MCF-7 cells (1.0 x 10^5 cells) were seeded 24 h prior to transfections into 12-well plates. The indicated siRNAs and CPPs (or transfection reagents) were respectively diluted with Opti-MEM at the indicated molar ratios (MRs), and mixed. Complexes were formed for 20 min or 30 min at room temperature. Thereafter, complexes were added to each well. (A, B) When using Lipofectamine 2000™ (LA2000), Lipofectamine RNAiMAX™, and PepFects, the complexes were prepared according to the manufacture's protocol or the protocol indicated in previous papers[8]. After 72 h, whole-cell lysates were analyzed by Western blotting with the indicated antibodies.

PepFect-6 and PepFect-14 were purchased from SYNTIDES®.

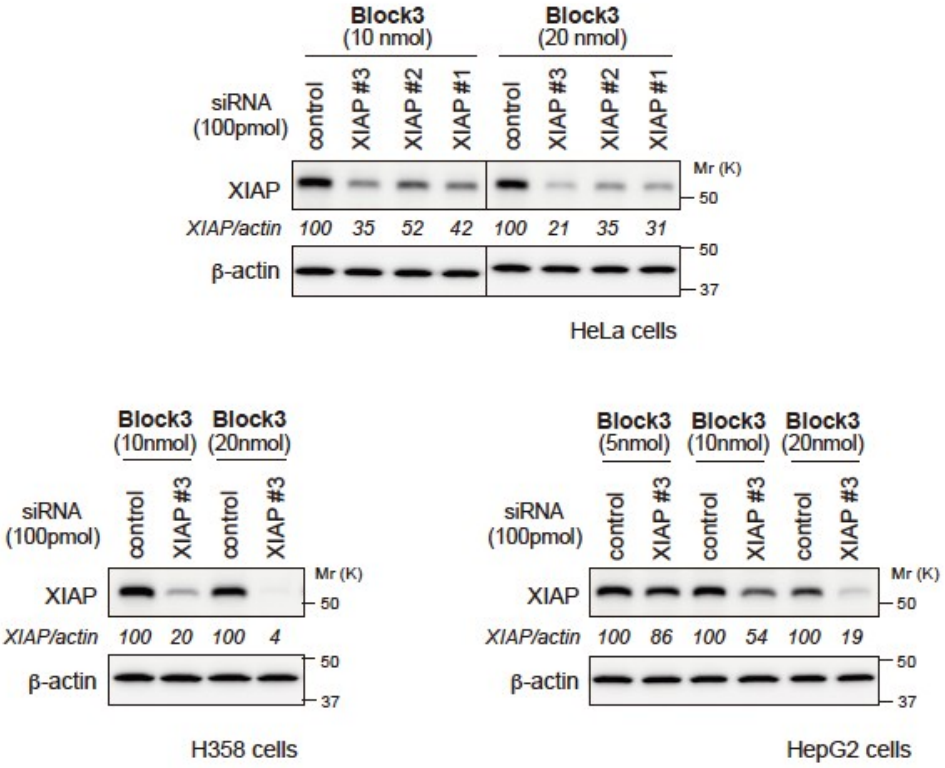


Figure S5. The **Block3**-mediated siRNA delivery in HeLa, NCI-H358, and HepG2 cells. Cells were treated with the indicated dose of siRNA and **Block3** for 72 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. Numbers below the XIAP panels represent XIAP/actin ratios, normalized by designating the expression from the control siRNA-treated condition as 100%.

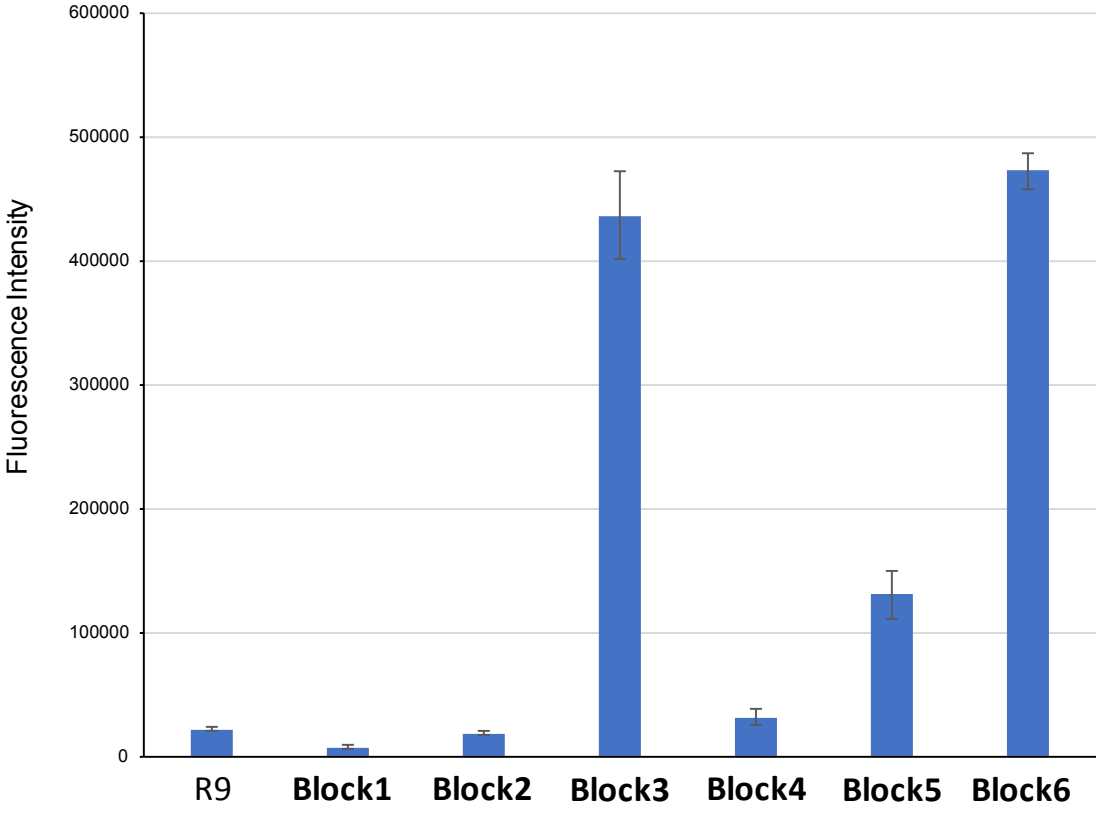


Figure S6. Intracellular uptakes of R9 and **Block1-6** at 1 μM treatment were detected by fluorescein with flow cytometry using HeLa cells.

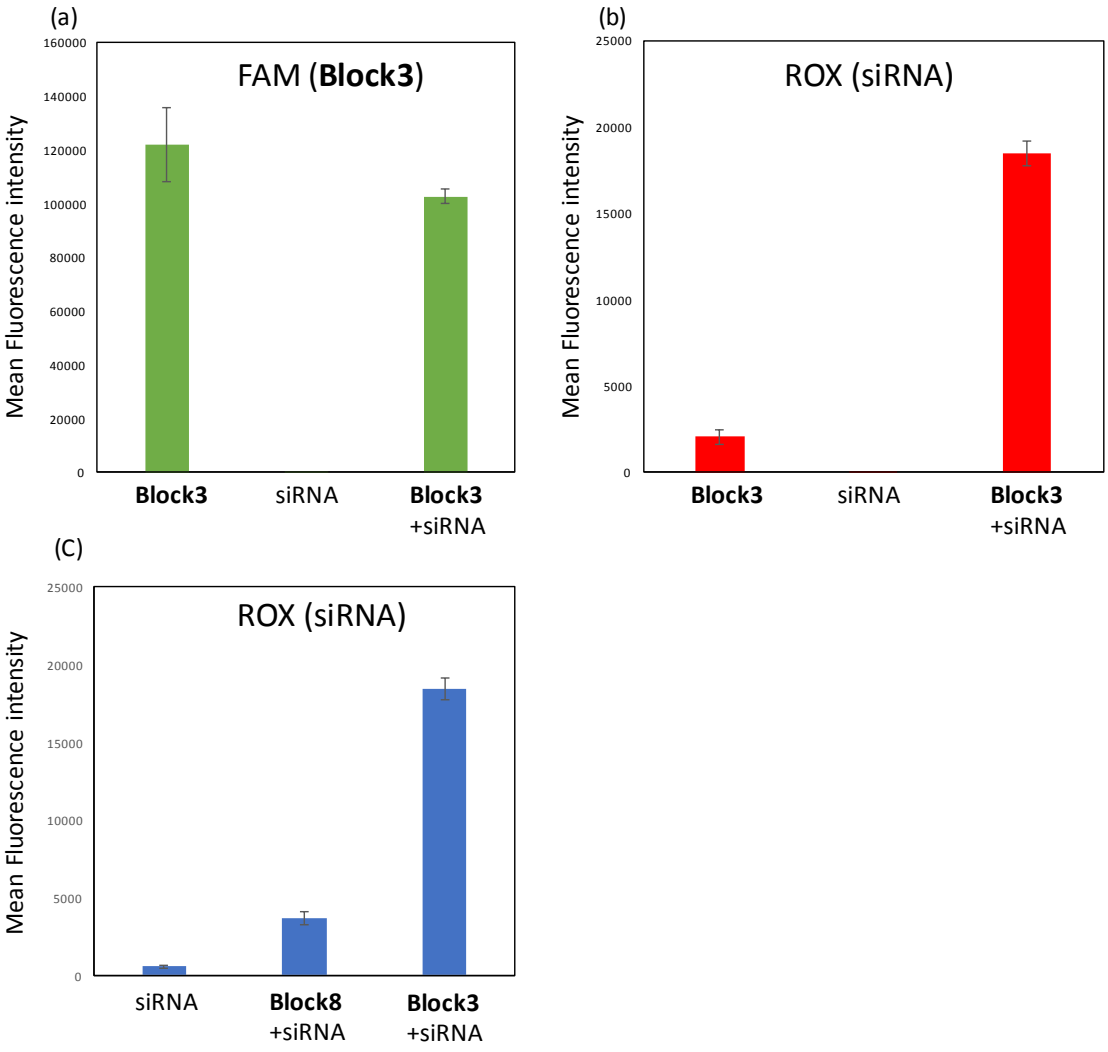


Figure S7. Intracellular uptakes of **Block3** and siRNA were detected by fluorescein (a) and ROX (b) using flow cytometry using MCF-7 cells. Lane 1: **Block3** (10 nmol) only, Lane 2: ROX-siRNA (100 pmol) only, Lane 3: **Block3** and ROX-siRNA. (c) Comparison of intracellular uptake of ROX-siRNA (100 pmol) between **Block3** and **Block8**. Lane 1: ROX-siRNA (100 pmol) only Lane 2: **Block8** and ROX-siRNA, Lane 3: **Block3** and ROX-siRNA.

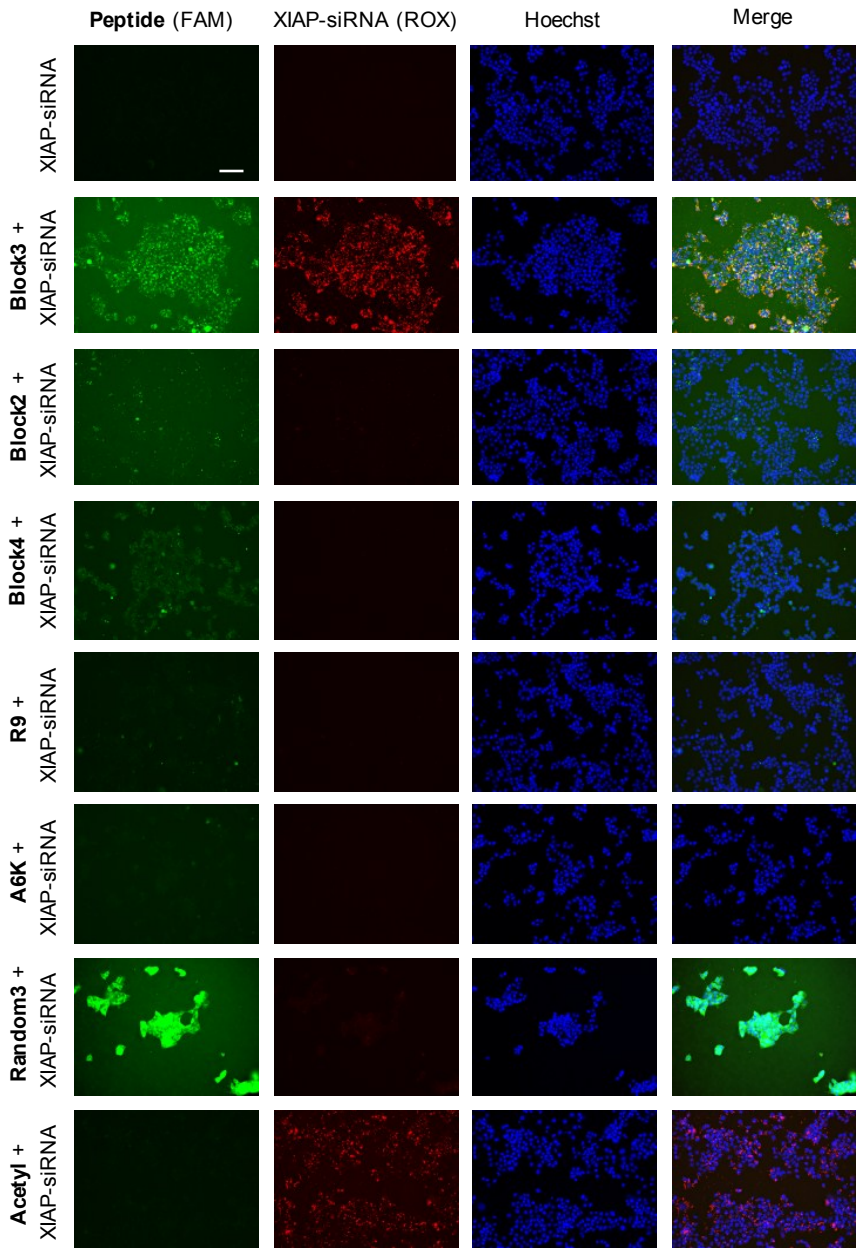


Figure S8. Enlarged scale of fluorescent microscopy analysis. Intracellular uptake of siRNA by **Block2**, **Block3**, **Block4**, **Block5**, and **Block6**. MCF-7 cells were treated with 10 nmol **Block3**, **Block4**, **Block8** in the presence of 100 pmol ROX-labeled XIAP-siRNA for 24 h. The nuclei were stained with 10 mg/ml Hoechst 33342. Live cell images were obtained by fluorescent microscopy. *Scale Bars*: 100 μm.

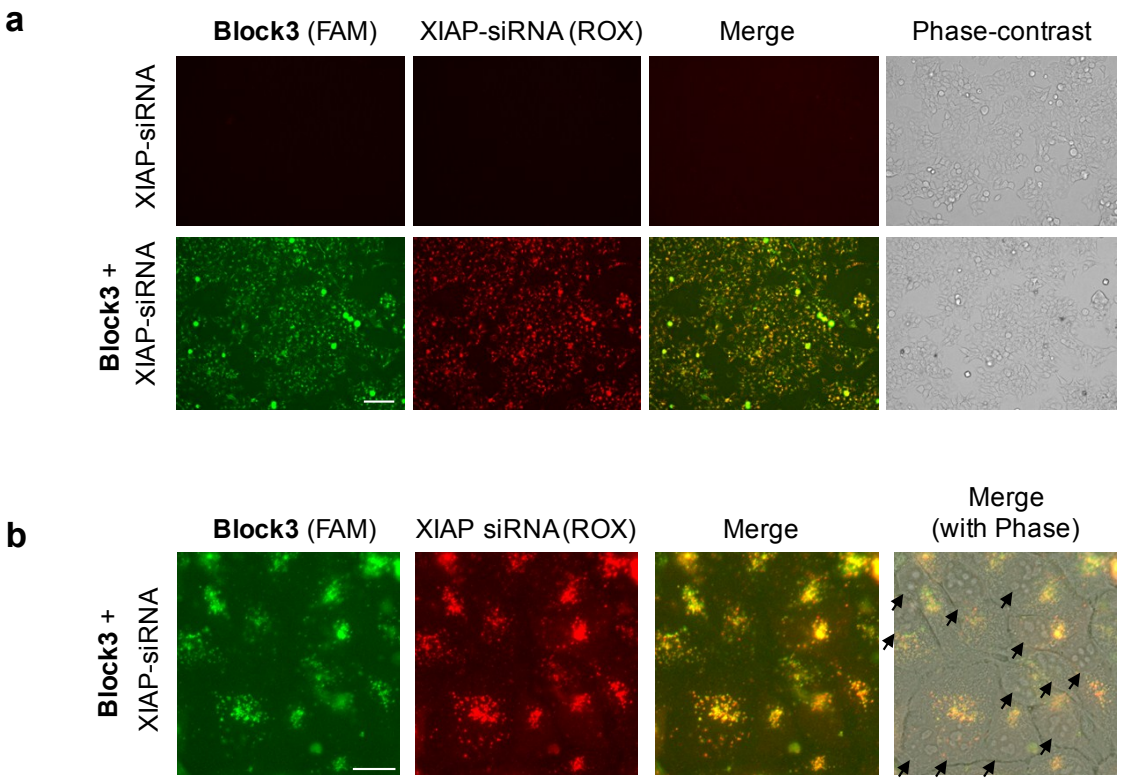


Figure S9 Intracellular uptake of siRNA by **Block3**. MCF-7 cells were treated with10 nmol **Block3** in the presence or absence of 200 pmol ROX-labeled XIAP-siRNA for 24 h. Live cell images were obtained by fluorescent microscopy. Merged images represent **Block3** and siRNA with or without phase-contrast. (a) *Scale Bars*: 100 μm. (b) *Scale Bars*: 20 μm. Arrows indicate nucleus.

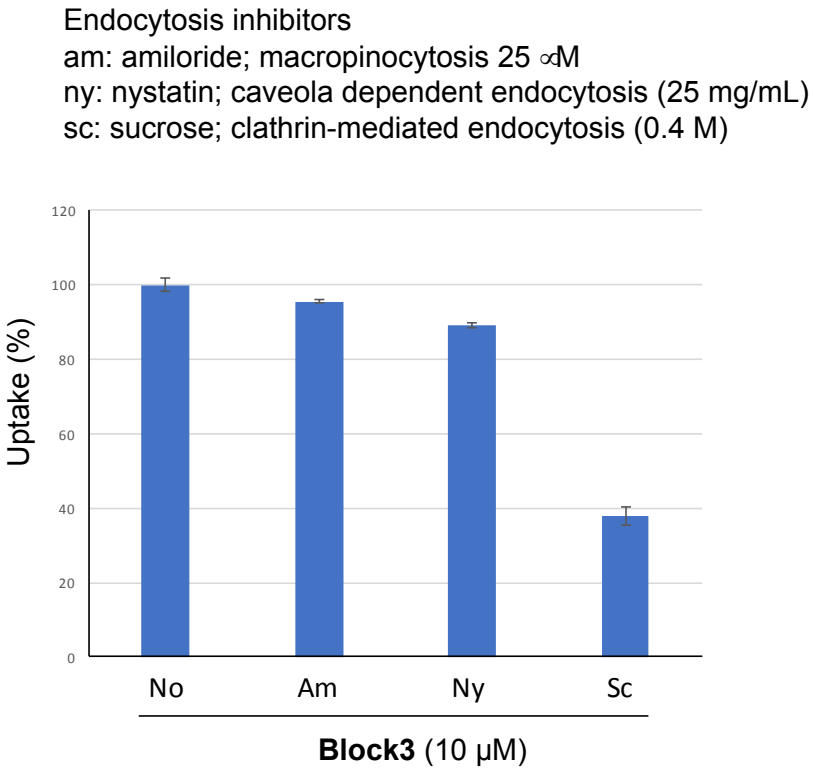


Figure S10. Effects of inhibitors of endocytosis. Hela cells were pre-treated with amirolide, nystatin, and sucrose and subsequently treated with 10 μM **Block3**. The intracellular uptake was measured using flow cytometry.

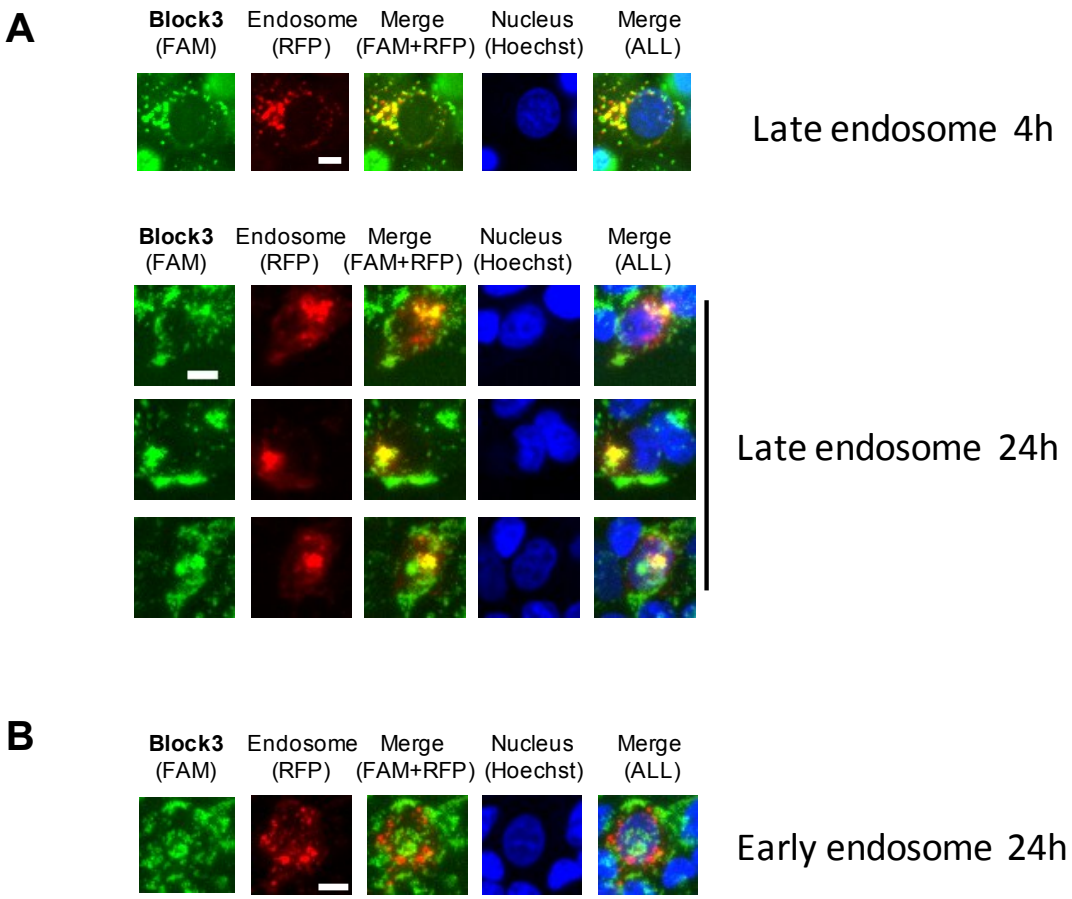


Figure S11. Endosomal escape of **Block3**. MCF-7 cells were treated with 10 nmol **Block3** in the presence of 100 pmol XIAP-siRNA for 4 or 24 h. The late endosomes or early endosomes were stained with CellLight® Late Endosome-RFP, BacMan 2.0 (A) or CellLight® Early Endosome-RFP, BacMan 2.0 (B), and the nuclei were stained with 10 mg/ml Hoechst 33342. Live cell images were obtained by fluorescent microscopy. *Scale Bars*: 10 μm.

(a) Size and size distribution

Peptide	Mixing ratio	Size (nm)	PDI (μ/G^2)
Block3	200 : 1	163.9±18.7	0.289±0.008
	100 : 1	773.2±118.1	0.324±0.017
	50 : 1	1853.7±155.1	0.221±0.068
Block4	200 : 1	425.8±47.2	0.222±0.020
	100 : 1	491.6±71.9	0.158±0.055
	50 : 1	513.7±32.7	0.158±0.079

(b) Zeta-potential

Peptide	Mixing ratio	Zeta-potential (mV)
Block3	200 : 1	+20.37±0.42
Block4	200 : 1	–0.08±0.25

(c) Electron microscopic observation

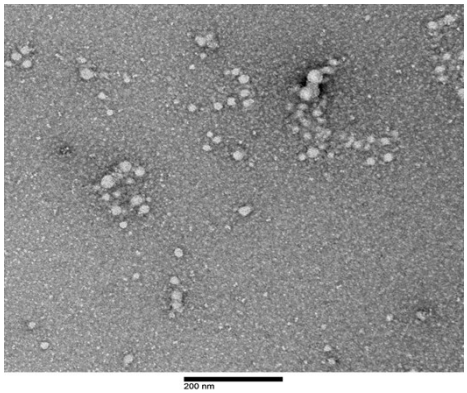


Figure S12. (a) Size and size distribution, and (b) zeta-potential of **Block3**/siRNA and **Block4**/siRNA prepared at various mixing ratios. (c) Electron microscopic observation of complex formation. **Block3** and siRNA were incubated (mixing ratio = 100:1) in opti-MEM, and the complex formation were observed.

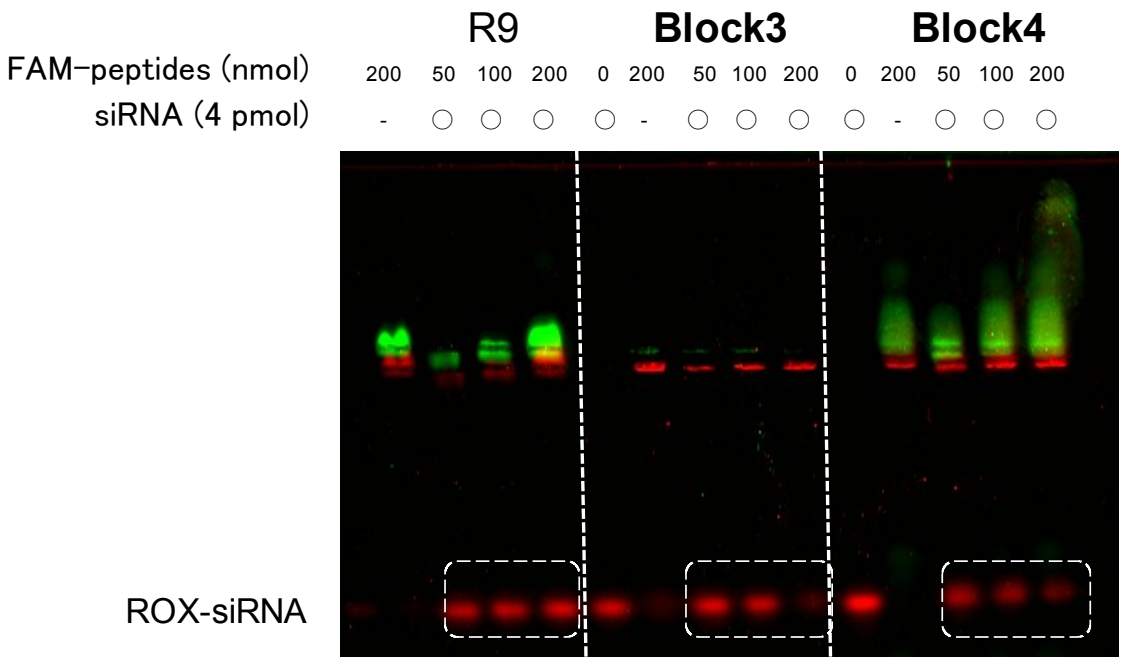


Figure S13. Gel shift assay to investigate the electrostatic interaction between siRNA and peptides (R9, **Block3**, and **Block4**). ROX-siRNA (4 pmol) and each peptide was incubated in opti-MEM, and the mixture loaded to agarose gel (agarose 21). The fluorescence was detected using Gel imager (Typhoon). Red and green represents ROX and FAM moiety, respectively.

(a) (b)

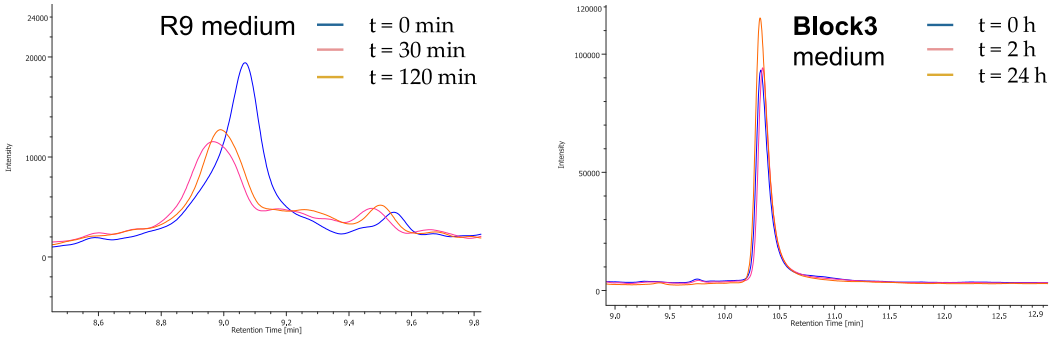


Figure S14. The digestion assay to determined the stability of peptides in medium containing 10% FBS (a,b) and containing trypsin. The peptides were incubated in medium for indicated time and each sample was analized using HPLC. We confirmed that the R9 was largely digested in medium for 30 min. (a) R9, (b)**Block3**.

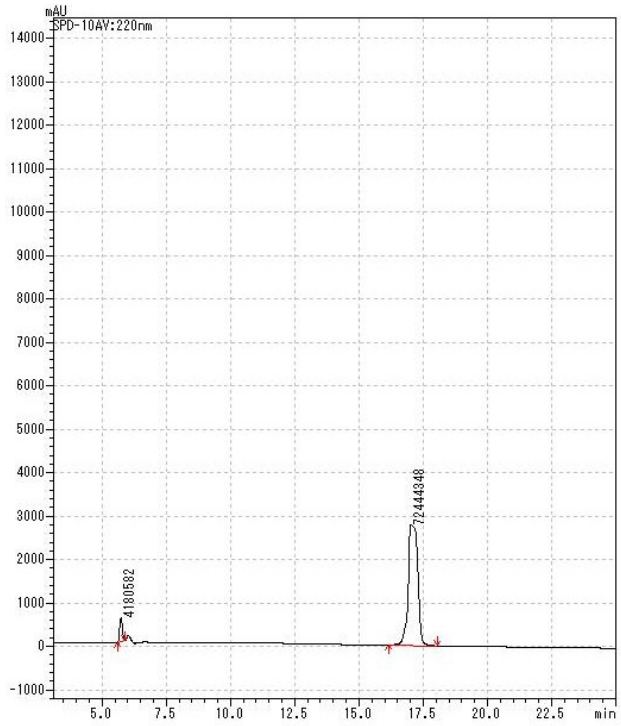
2. HPLC data of the synthesized peptides

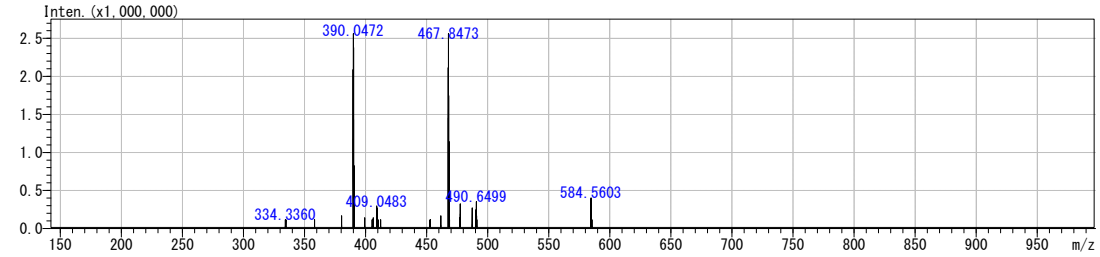
HPLC data of the synthesized peptides

Block1: FAM-βAla-(Leu-Leu-Aib)$_1$-(Gly)$_3$-(Arg)$_9$-NH_2 (purity > 99%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

[HR-ESI(+)]: Calculated pentaisotopic $[M+5H]^{5+}$ = 467.6686, observed pentaisotopic $[M+H]^{+}$ = 467.6696

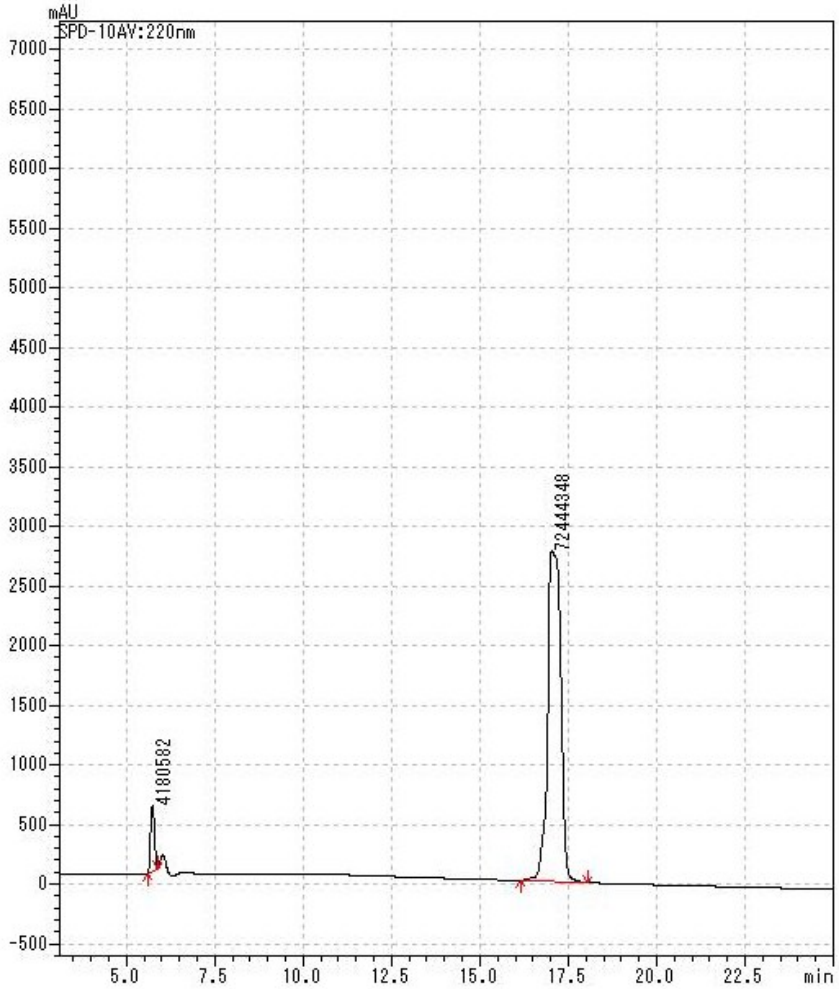


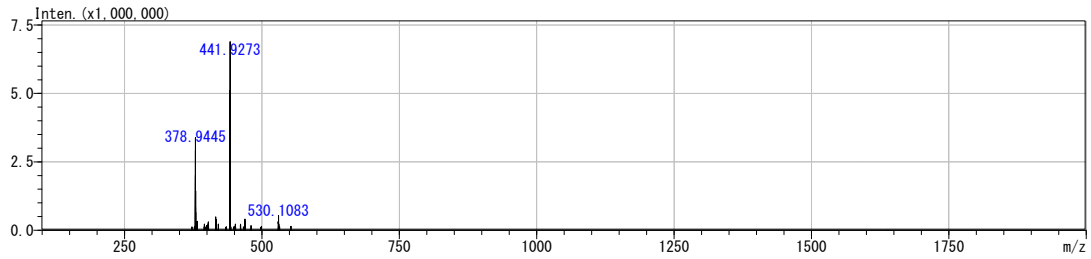


Block2: FAM-βAla-(Leu-Leu-Aib)$_2$-(Gly)$_3$-(Arg)$_9$-NH$_2$ (purity > 99%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

[HR-ESI(+)]: Calculated hexaisotopic $[M+6H]^{6+}$ = 441.7619, observed hexaisotopic $[M+6H]^{6+}$ = 441.7424

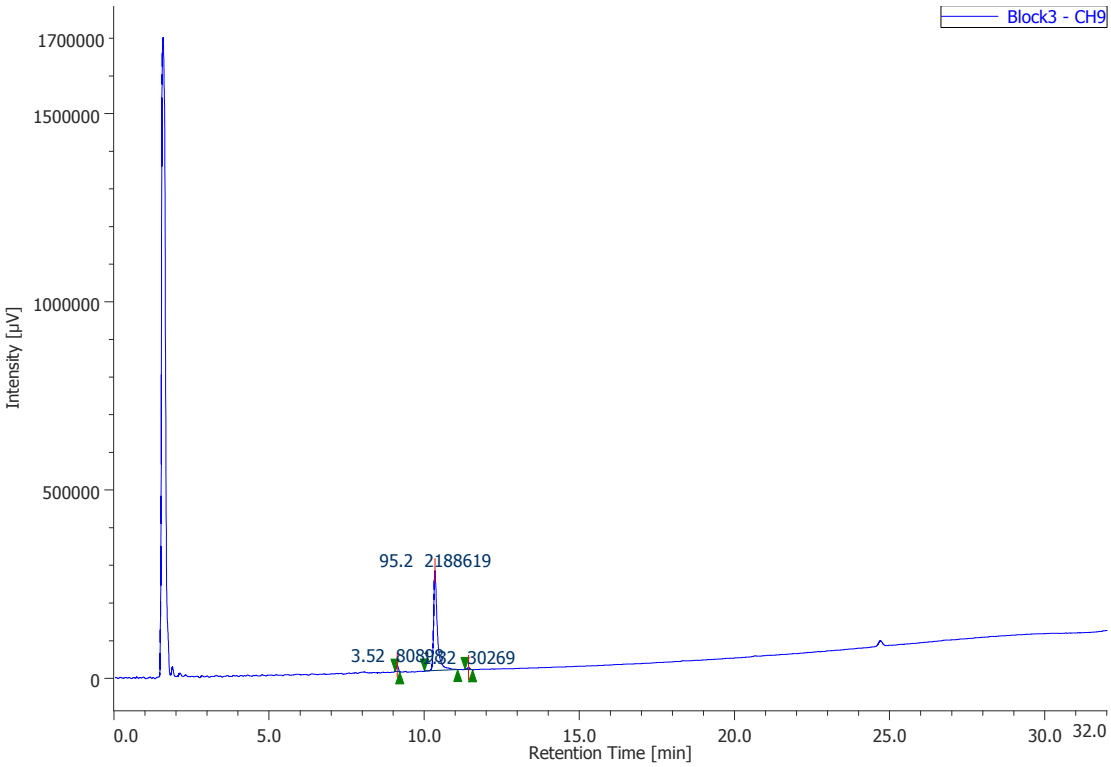


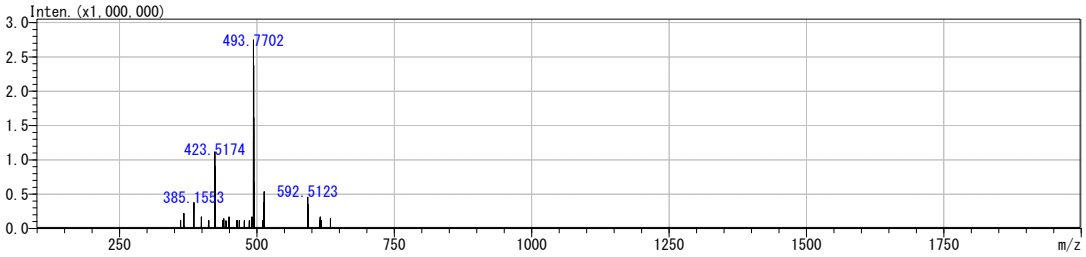


Block3: FAM-βAla-(Leu-Leu-Aib)$_3$-(Gly)$_3$-(Arg)$_9$-NH_2 (purity: 95.2%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

[HR-ESI(+)]: Calculated hexaisotopic $[M+6H]^{6+}$ = 493.6258, observed hexaisotopic $[M+6H]^{6+}$ = 493.6220





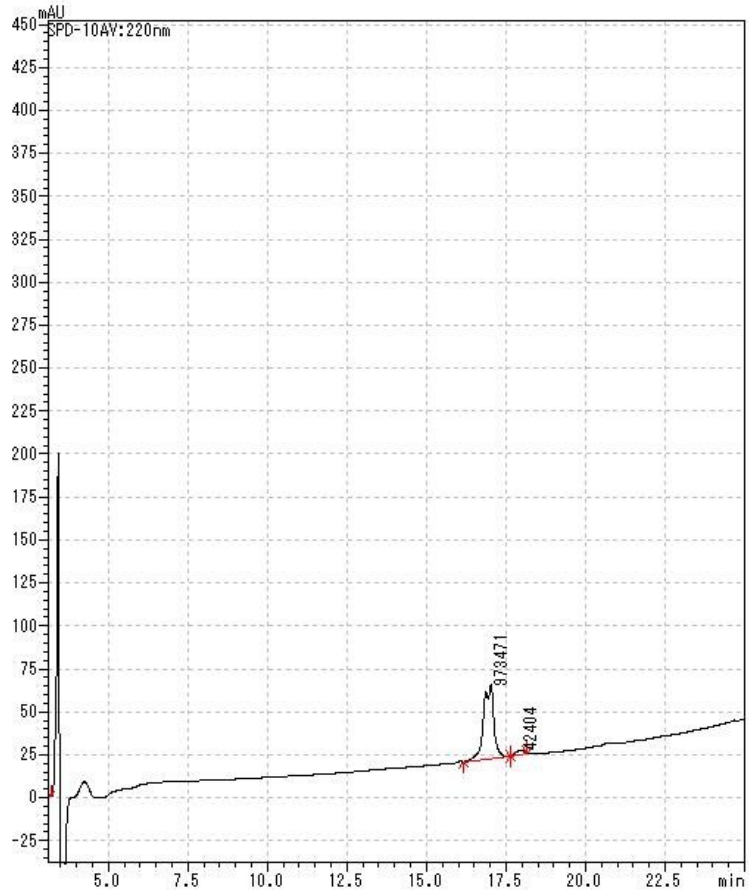
Block4: FAM-βAla-Arg-Arg-Arg-Leu-Aib-Arg-Leu-Arg-Leu-Gly-Aib-Arg-Leu-Arg-Leu-Arg-Gly-Arg-Leu-Gly-Aib-NH_2 (purity: 95.3%)

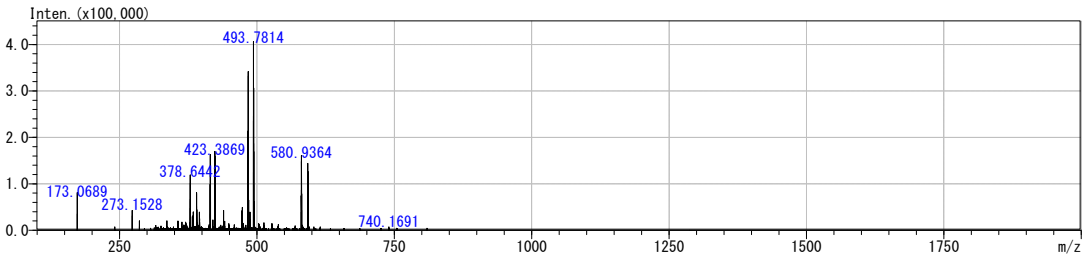
HPLC: Gradient = 10 – 90% solvent B over 30 minutes

[HR-ESI(+)]: Calculated hexaisotopic $[M+6H]^{6+}$ = 493.6258, observed hexaisotopic

$[M+6H]^{6+}$ = 493.6236

5- and 6- FAM isomers were detected.



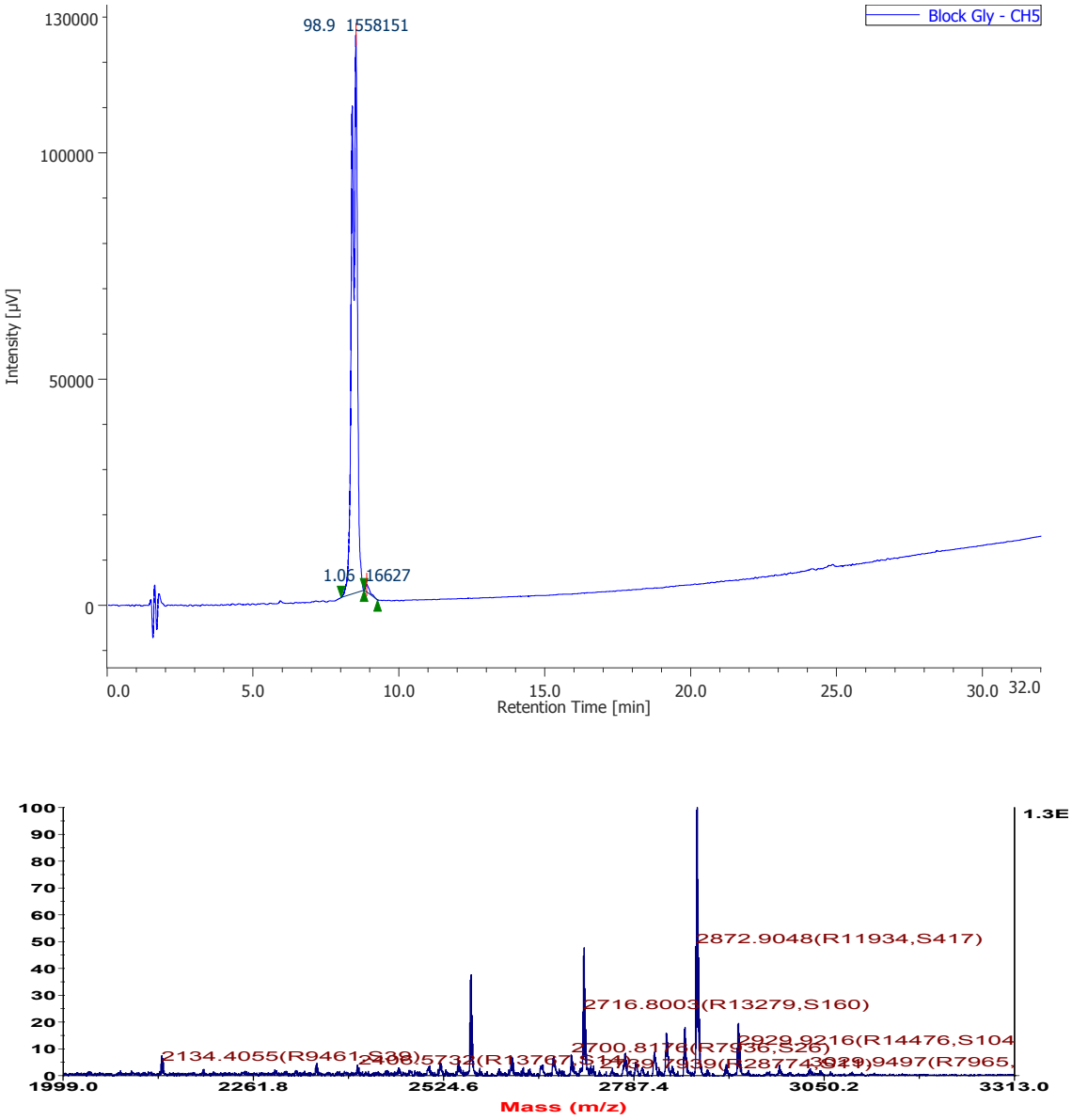


Block5: FAM-βAla-$(Leu\text{-}Leu\text{-}Gly)_3$-$(Gly)_3$-$(Arg)_9$-$NH_2$ (purity: 98.9%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 2872.66, observed monoisotopic $[M+H]^+$ = 2872.90

5- and 6- FAM isomers were detected.

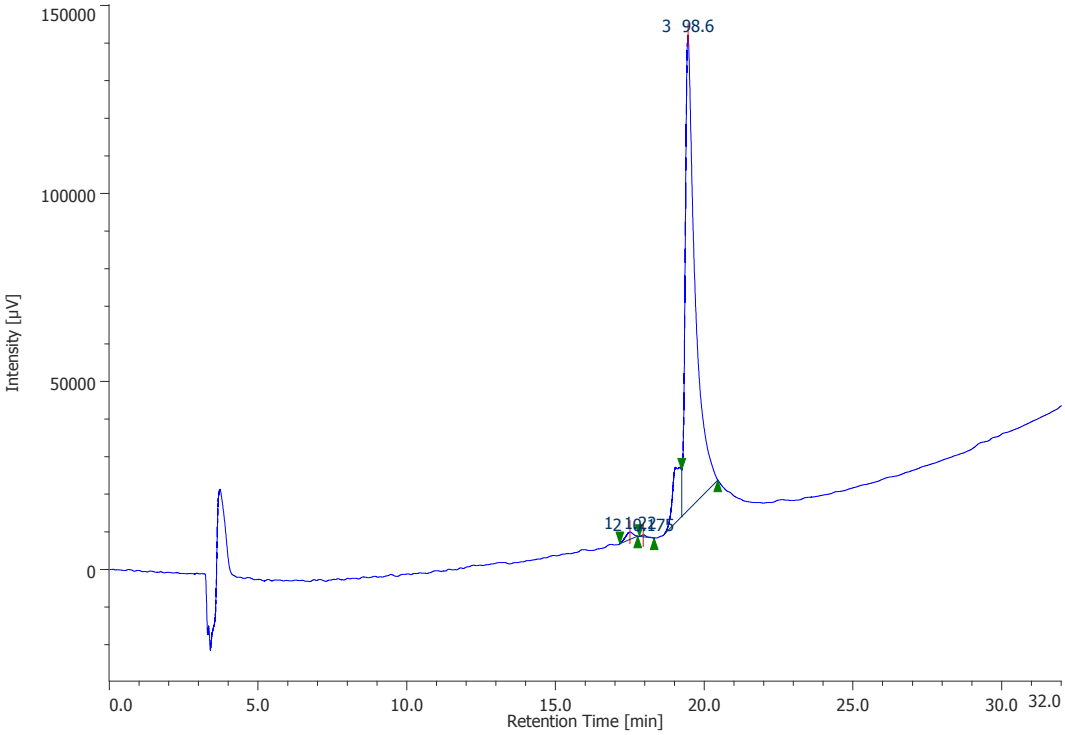


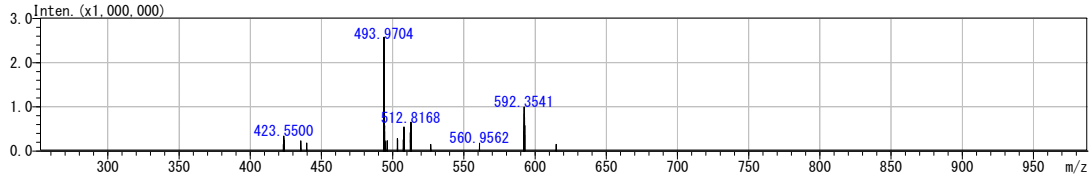
Block6: FAM-βAla-Arg-Leu-Leu-Arg-Arg-Aib-Gly-Arg-Leu-Leu-Arg-Arg-Aib-Gly-Arg-Leu-Leu-Arg-Arg-Aib-Gly -NH_2 (purity: 98.6%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

MALDI-TOF: Calculated pentaisotopic $[M+5H]^{5+}$ = 592.158, observed hexaisotopic $[M+5H]^{5+}$ = 592.155

5- and 6- FAM isomers were detected.



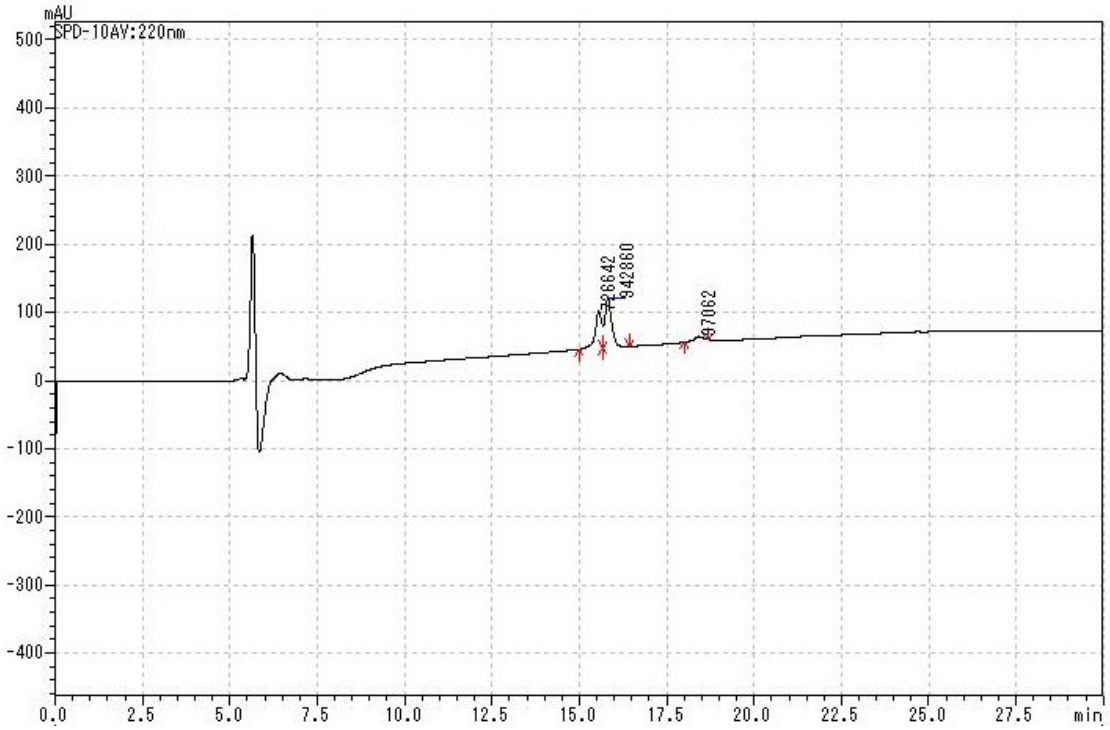


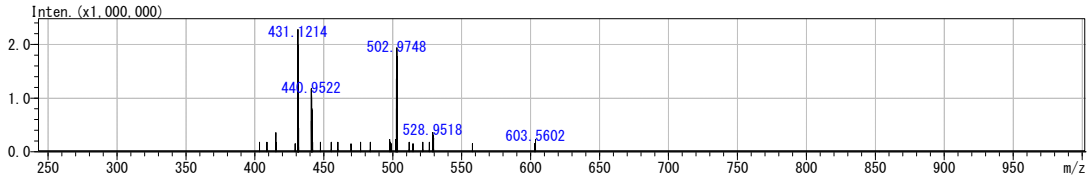
Block7: TAMRA-βAla-(Leu-Leu-Aib)$_3$-(Gly)$_3$-(Arg)$_9$-NH$_2$ (purity: 94.6%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

MALDI-TOF: Calculated hexaisotopic $[M+6H]^{6+}$ = 502.648, observed hexaisotopic $[M+6H]^{6+}$ = 502.637

5- and 6- FAM isomers were detected.

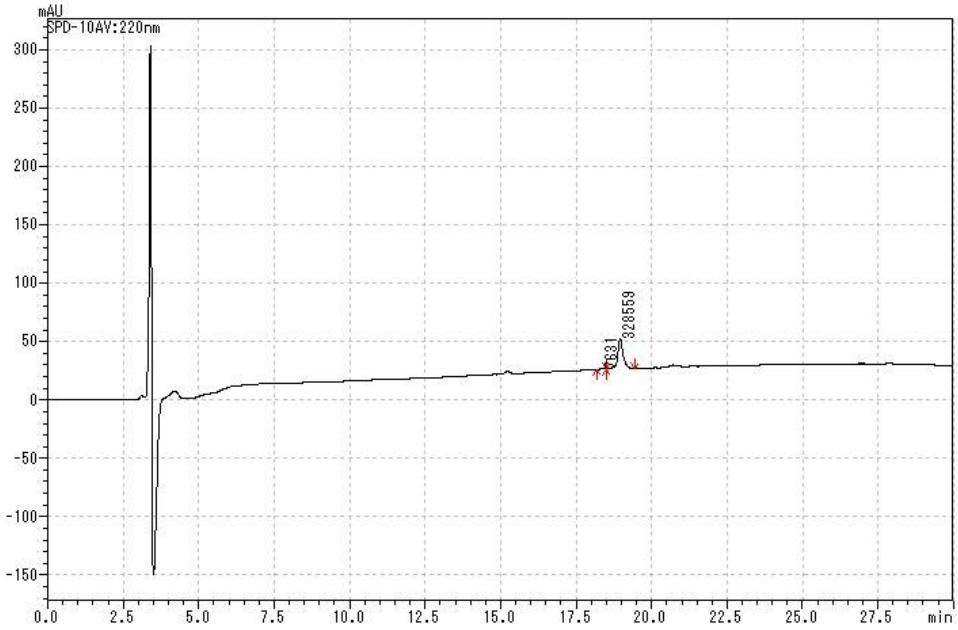


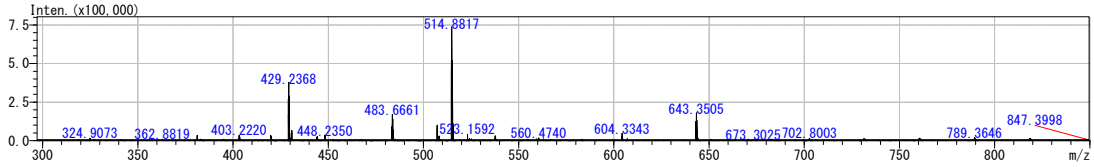


Block8: Ac-βAla-(Leu-Leu-Aib)$_3$-(Gly)$_3$-(Arg)$_9$-NH$_2$ (purity: 99.0%)

HPLC: Gradient = 10 – 90% solvent B over 30 minutes

MALDI-TOF: Calculated pentaisotopic $[M+5H]^{5+}$ = 514.7426, observed pentaisotopic $[M+5H]^{5+}$ = 514.6819


mAU
SPD-10AV:220nm
328559
min


Inten. (x100,000)
514.8817
429.2368
483.6661
643.3505
324.9073
362.8819
403.2220
448.2350
523.1592
560.4740
604.3343
673.3025
702.8003
789.3646
847.3998
m/z