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Supplementary Information for

A Parallel Permeability Assay of Peptides across Artificial Membrane and Cell Monolayers Using a Fluorogenic Reaction

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General remarks

Chemicals and solvents used in this study were purchased from commercial suppliers and used without further purification. MDCK-II cells from European Collection of Authenticated Cell Cultures were purchased from DS Pharma Biomedical. Preparative HPLC was performed on a Prominence HPLC system (Shimazu) with a 5C18-MS-II column (Nacalai tesque, 10 mm I.D.×150 mm, 34355-91). Analytical HPLC was performed on a Prominence HPLC system with a 5C18-AR-II column (Nacalai tesque, 4.6 mm I.D.×150 mm, 38144-31). All the HPLC were performed using two solvents (solvent A: H_2O containing 0.1% TFA; Solvent B: acetonitrile containing 0.1% TFA). ESI-MS data was obtained using micrOTOF II (Bruker Daltonics). NMR spectra were recorded using ECS-400 (JEOL). Fluorescence was measured using a plate reader, Infinite M200 PRO (TECAN).

Synthesis

Cyclo[D-Pro-D-Leu-Leu-Ala-D-Leu-Tyr(OMe)] (1) and cyclo[D-Pro-D-Leu-Leu-PGly-D-Leu-Tyr(OMe)] (2)

1 and **2** were manually synthesized on 2-chlorotrityl polystyrene resin (1.6 mmol/g). Resin (93 mg, 149 μmol) was first swelled in dichloromethane (DCM) in a 6 mL fritted syringe with continuous shaking. *N*,*N*-diisopropylethylamine (DIPEA) (586 μmol, 102 μL) and Fmoc-Leu-OH (103 mg, 291 μmol) was dissolved in 2.9 mL DCM and the solution was applied to the resin. The resin was incubated overnight at room temperature. After the reaction, resin was washed with DCM/methanol/DIPEA = 17/2/1 and DCM, three times each. The loading of the beads was quantitated according to the previous report.[1] The resin was applied to further peptide synthesis. Fmoc deprotection was performed by incubating the resin with 20% piperidine in DMF for 15 min. After the reaction, the resin was washed with DMF three times. Coupling reaction other than Fmoc-Tyr(OMe)-OH was performed using Fmoc-protected amino acid (4 equiv.), HATU (4 equiv.), HOAt (4 equiv.) and DIPEA (6 equiv.) in DMF (0.1 M with respect to Fmoc-protected amino acid) for 1–2.5 h. The coupling reaction of Fmoc-Tyr(OMe)-OH was performed using Fmoc-Tyr(OMe) (4 equiv.), Oxyma (4 equiv.) and *N,N'*-diisopropylcarbodiimide (DIC) (4 equiv.) in DMF (0.1 M with respect to Fmoc-Tyr(OMe)-OH). After the reaction, the resin was washed with DMF, three times each. The coupling and deprotection were repeated until the 6th residue. The *N*-terminal Fmoc was deprotected by incubating the resin with 20% piperidine/DMF for 3 min then, with fresh 20% piperidine/DMF, 12 min. The resin was washed with DMF three times. The synthesized peptides were cleaved from the resin by incubating the resin with 5% TFA in DCM for 60 min. The filtrate was collected in a recovery flask. The resin was incubated in 5% TFA in DCM again for 30 min and, after the filtrate was collected, washed with DCM. All the filtrates were combined and the solution was evaporated under a reduced pressure. Peptide was dissolved in 30% acetonitrile/water and purified using a reversed phase column. After lyophilization, peptide was cyclized by incubating with HATU (1.5 equiv.), 1-hydroxy-7-azabenzotriazole (HOAt) (1.5 equiv.) and DIPEA (6 equiv.)

in anhydrous DMF (5 mM with respect to peptide) for 21 h. The solution was evaporated under a reduced pressure. Peptide was dissolved in 30% acetonitrile/water and purified using a reversed phase column on HPLC. The product was identified by analytical HPLC (**Fig. S1**) and ESI-MS (**Table S1**).

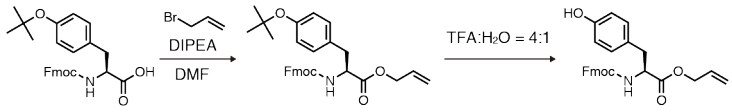
3-Azido-7-hydroxycoumarin

The compound was synthesized according to the previous report.[2] The final product was obtained in 7% overall yield. ^{1}H NMR (DMSO-d_6, 400 MHz): δ10.52 (s, 1H), 7.55 (s, 1H), 7.44 (d, J = 8.7 Hz, 1H), 6.77 (dd, J = 8.2, 2.3 Hz, 1H), 6.72 (d, J = 2.8 Hz, 1H). ^{13}C NMR (DMSO-d_6, 400 MHz): δ160.8, 157.8, 153.3, 129.6, 128.4, 121.7, 114.3, 111.9, 102.6. HRMS (ESI-TOF MS) *m/z*: $[M - H]^+$ Calcd for $C_9H_5N_3O_3^-$ 202.0253; Found 202.0266.

Cyclic peptide 2 conjugated with azidocoumarin (3)

187.5 µL of 40 mM azidocoumarin in dimethylsulfoxide (DMSO) was diluted with 82.5 µL of DMSO and mixed with 30 µL of 50 mM peptide **2** in DMSO. 780 µL of PBS, 60 µL of 10 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) in DMSO, 60 µL of 10 mM $CuSO_4$ in water, and 5.9 mg sodium ascorbate were added to the solution and the reaction mixture was stirred for 5.5 h in the dark. The reaction mixture was diluted with 30% acetonitrile/water (final composition is 19% DMSO and 22% acetonitrile in aqueous buffer) and filtered. The product was purified using a reversed phase column on HPLC. The product was identified by analytical HPLC (**Fig. S1**) and ESI-MS (**Table S1**).

Fmoc-Tyr-OAll



The compound was synthesized according to the previous report[3] with minor modifications. Fmoc-Tyr(tBu)-OH (2.50 g, 5.44 mmol) was dissolved in 5 mL of anhydrous *N*,*N*-dimethylformamide (DMF) and cooled on ice. *N*,*N*-diisopropylethylamine (DIPEA) (1.90 mL, 10.9 mmol) was added dropwise then allyl bromide (0.920 mL, 11.0 mmol) was added dropwise. The mixture was allowed to warm to RT and stirred for 16 h. The mixture was diluted with 50 mL of ethylacetate and washed with 50 mL of water five times. The organic phase was dried using sodium sulfate. The organic phase was evaporated under vacuum. The product was purified by silica gel column chromatography. 30 mL of a 4:1 mixture of trifluoroacetic acid and water was added dropwise to the product. The solution was stirred at RT for 2 h and evaporated under vacuum. The product was purified by column chromatography to give the objective compound. Yield 1877.7 mg (4.23 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.75 (d, J = 7.8 Hz, 2 H), 7.57-7.54 (m, 2 H), 7.39 (t, J = 7.3 Hz, 2 H), 7.30 (t, J = 7.3 Hz, 2 H), 6.95 (d, J = 8.2 Hz, 2 H), 6.71 (d, J = 8.2 Hz, 2 H), 5.87 (qd, J = 11.0, 6.4 Hz, 1 H),

5.33-5.19 (m, 4 H), 4.67-4.61 (m, 3 H), 4.38 (ddd, J = 28.9, 10.5, 7.3 Hz, 2 H), 4.19 (t, J = 7.3 Hz, 1 H), 3.10-3.00 (m, 2 H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 171.4, 155.3, 143.8, 141.4, 131.4, 130.6, 127.8, 127.1, 125.1, 120.1, 119.3, 115.6, 67.1, 66.2, 55.0, 47.2, 37.5 HRMS (ESI-TOF MS) *m/z*: $[M + Na]^+$ Calcd for $C_{27}H_{25}NO_5Na^+$ 466.1625; Found 466.1614.

Cyclo[D-Pro-D-Leu-Leu-PGly-D-Leu-Tyr] (4) and derivatives

Cyclic peptides were manually synthesized on 2-chlorotrityl polystyrene resin (1.6 mmol/g) according to the previous report.[4] Resin (191 mg, 306 μmol) was first swelled in 1 mL DCM in a 5 mL plastic tube with continuous shaking. The swelled resin was transferred to a round bottom flask and DIPEA (62 μmol, 108 μL) was added to the flask. Fmoc-Tyr-OAll (28 mg, 63 μmol) was dissolved in 3 mL DCM and the solution was applied to the resin. The resin was incubated for 4 h at room temperature under a nitrogen atmosphere. After the reaction, resin was transferred to a 10 mL fritted syringe and washed with DCM, DMF, and DCM, three times each. The loading of the beads was quantitated according to the previous report.[1] Resin loading of 0.1–0.3 mmol/g is desirable for preventing dimer formation at the later macrocyclization step. The resin was applied to further peptide synthesis. Fmoc deprotection was performed by incubating the resin with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene and 2% piperidine in DMF for 15 min. After the reaction, the resin was washed with DMF, DCM, and DMF, three times each. Coupling reaction was performed using 4 equiv. of Fmoc-protected amino acid, HATU (3.8 equiv.), and DIPEA (6 equiv.) in DMF (0.1 M with respect to Fmoc-protected amino acid) for 1–2.5 h. After the reaction, the resin was washed with DMF, DCM, and DMF, three times each. The coupling and deprotection were repeated until the *N*-terminal proline residue. The allyl group on the C-terminal Tyr residue was removed by incubating the resin with 1 equiv. of tetrakis(triphenylphosphine)palladium, 3 equiv. triphenylphosphine, 10 equv. of formic acid and 10 equiv. of diethylamine in anhydrous THF at room temperature for 3 h. The resin was washed with 5% sodium diethyldithiocarbamate and 5% DIPEA in DMF twice then with DMF, DCM, and DMF, three times each. The *N*-terminal Fmoc was deprotected by incubating the resin with 20% piperidine/DMF for 3 min then, with fresh 20% piperidine/DMF, 12 min. The resin was washed with DMF three times. Peptide was cyclized by incubating the resin with 3 eqiv. of (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 3 equiv. of HOAt, 6 equiv. of DIPEA in anhydrous DMF (0.02 M with respect to PyBOP) for 4 h. The resin was washed with DMF and DCM, three times each. The synthesized peptides were cleaved from resin by incubating the resin with 5% TFA in DCM for 15 min. The filtrate was collected in a recovery flask. The resin was incubated in 5% TFA in DCM for 30 min and, after the filtrate was collected, washed with DCM twice. For peptides bearing protecting groups, a solution of 95% TFA, 2.5% water, and 2.5% TIPS was used instead of 5% TFA in DCM to completely deprotect the peptides. All the filtrates were combined, and the solution was evaporated under a reduced pressure. Peptide was dissolved in 40% acetonitrile/water and

purified using a reversed phase column. The product was identified by analytical HPLC (**Fig. S3**) and ESI-MS (**Table S1**).

Assay

Measurement of permeability values of peptide 1–3 by PAMPA (Fig. 1c)

The assay was conducted using MultiScreen-IP Filter Plate, 0.45 μm (Merck) and MultiScreen 96-well Transport Receiver Plate (Merck). 5 μL of 1% lecithin/dodecane was dropped on each well of the donor plate to prepare an artificial lipid membrane. 150 μL of 100 μM peptide solution in 5% DMSO/PBS was added to each of the donor wells and 300 μL of 5% DMSO/PBS was added to each of the acceptor wells. The donor plate was docked on the acceptor well and the plates were incubated in a box containing a wet paper towel for 7 h at 25 °C. After the incubation, peptides in acceptor and donor wells were quantitated by HPLC. As an internal standard, propranolol was added to all the samples before HPLC analysis. The concentration of peptide in acceptor and donor wells were determined using standard curves that were generated from the HPLC analytical data of 6.25, 25 and 100 μM of each peptide. Using the determined concentrations of peptides in donor and acceptor wells, the permeability value (P_e) was calculated using the following equations:

$$P_e = -\frac{ln[1 - C_A(t) / C_{equilibrium}]}{A \times (1/V_D + 1/V_A) \times t}$$

$$C_{equilibrium} = \frac{C_D(t) \times V_D + C_A(t) \times V_A}{V_D + V_A}$$

Where:

A = filter area (0.3 cm^2)

V_D = donor well volume (0.15 mL)

V_A = acceptor well volume (0.3 mL)

t = incubation time (s) (7 h = 25,200 s)

$C_A(t)$ = compound concentration in acceptor well at time t

$C_D(t)$ = compound concentration in donor well at time t

Fluorescence spectra of peptide 4 and its conjugate with azidocoumarin (4-AC) (Fig. 2b)

400 μL of 50 μM peptide **4** in PBS was mixed with 1.84 mL of PBS (pH 7.2), 400 μL of 1 M phosphate buffer (pH 8.0), 40 μL of 500 mM sodium ascorbate in water, 80 μL of 10 mM $CuSO_4$ in water and 40 μL of 10 mM THPTA in water. 1.2 mL of 0.33 mM azidocoumarin in DMSO was applied to the mixture in the well to initiate the fluorogenic reaction. As a reference, the same reaction mixture without peptide **4** was also prepared. The solutions were incubated at room temperature for 1 h. 100 μL of the solution was diluted with 900 μL of 30% DMSO/PBS. The diluted solution was transferred to a quartz cell and fluorescence spectrum

of the solution was measured with an excitation wavelength of 400 nm using a spectrofluorophotometer (Shimazu, RF-6000) .

Visual image of azidocoumarin and peptide 4-AC (Fig. 2b, inset)

The reaction mixture described in the previous section was transferred to a quartz cell without dilution. The solution in the quartz cell was irradiated with UV (365 nm) (SLUV-4, AS ONE) in the dark and photographed.

CuAAC reaction of azidocoumarin with various concentrations of peptide 4 (Fig. 2c)

In a well on a 96-well black plate, 112 μL of PBS, 20 μL of 1 M phosphate buffer (pH 8.0), 4 μL of 10 mM $CuSO_4$ in water, 2 μL of THPTA in water, 2 μL of 500 mM sodium ascorbate in water were mixed. To the mixture, 54 μL of DMSO, 2 μL of 10 mM azidocoumarin, and 4 μL of 1,000, 500, 250, 50, 25, 5, or 2.5 μM peptide **4** in DMSO are added in this order. The reaction was performed for 1 h and the fluorescence from each well was measured using a plate reader.

Measurement of permeability values of peptide 4 and derivatives by PAMPA (Fig. 2d and Fig. S4)

The assay was conducted using MultiScreen-IP Filter Plate, 0.45 μm (Merck) and MultiScreen 96-well Transport Receiver Plate (Merck). 5 μL of 1% lecithin/dodecane was dropped on each well of the donor plate to prepare an artificial lipid membrane. 150 μL of peptide solution in 5% DMSO/PBS was added to each of the donor wells and 290 μL of 5% DMSO/PBS was added to each of the acceptor wells. Peptide concentration was set to 200 μM for all the tested compounds except for **4-L3F** which concentration was set to 130 μM due to the solubility limit. The donor plate was docked on the acceptor well and the plates were incubated in a box containing a wet paper towel for 18.5 h at 25 °C. After the incubation, peptides in acceptor and donor wells were determined by reacting with azidocoumarin under CuAAC reaction conditions or by HPLC.

When peptide was quantitated by the fluorogenic reaction, 112 μL of peptide solution in acceptor well and 112 μL of 10-fold diluted peptide solution in donor well after the permeability assay was transferred to a 96-well black plate. In each well, the peptide solution was mixed with 20 μL of 1 M phosphate buffer (pH 8.0), 2 μL of 500 mM sodium ascorbate in water, 4 μL of 10 mM $CuSO_4$ in water and 2 μL of 10 mM THPTA in water. 60 μL of 0.33 mM azidocoumarin in DMSO were mixed in a separate tube and the solution was applied to the mixture in the well to initiate the fluorogenic reaction. The reaction was performed for 1 h and the fluorescence from each well was measured using a plate reader with excitation and emission wavelengths of 400 nm and 480 nm, respectively. At the same time, three different concentrations of each peptide were also reacted with azidocoumarin under the same reaction conditions to generate a standard curve (**Fig. S4**). The standard curves were used to determine the concentrations of peptides in donor and acceptor

wells after PAMPA. From the concentrations, the effective permeability coefficient (P_e) of each peptide was calculated using the following equations:

$$P_e = -\frac{ln[1 - C_A(t) / C_{equilibrium}]}{A \times (1/V_D + 1/V_A) \times t}$$

$$C_{equilibrium} = \frac{C_D(t) \times V_D + C_A(t) \times V_A}{V_D + V_A}$$

Where:

A = filter area (0.3 cm^2)

V_D = donor well volume (0.15 mL)

V_A = acceptor well volume (0.29 mL)

t = incubation time (s) (18.5 h = 66,600 s)

$C_A(t)$ = compound concentration in acceptor well at time t

$C_D(t)$ = compound concentration in donor well at time t

($C_A(t)$ and $C_D(t)$ were determined using the standard curves shown in **Fig. S4**).

When peptide was quantitated by HPLC, the permeability value (P_e) was determined using a slightly modified equation as following where HPLC peak area ($A_A(t)$ and $A_D(t)$) is used as a measure of peptide concentration:

$$P_e = -\frac{ln[1 - A_A(t) / A_{equilibrium}]}{A \times (1/V_D + 1/V_A) \times t}$$

$$A_{equilibrium} = \frac{A_D(t) \times V_D + A_A(t) \times V_A}{V_D + V_A}$$

Where:

A = filter area (0.3 cm^2)

V_D = donor well volume (0.15 mL)

V_A = acceptor well volume (0.29 mL)

t = incubation time (s) (18.5 h = 66,600 s)

$A_A(t)$ = HPLC peak area of peptide from acceptor well at time t

$A_D(t)$ = HPLC peak area of peptide from donor well at time t

Measurement of permeability values of peptide 4 and derivatives by MDCK-II assay (Fig. 3 and Fig. S6)

The assay was conducted using Falcon 24-well TC-treated Cell Polystyrene Permeable Support Companion Plate (Corning) and Falcon Permeable Support for 24-well Plate with 1.0 μm Transparent PET Membrane (Corning). MDCK-II cells were cultured using E-MEM containing 5% FBS and 1% antibiotic and antimicrobial in a 5% CO_2 incubator at 37 °C. 250 μL of 5 x10^4 cells/mL was added to each apical chamber

and 750 μL of medium was added to each basolateral chamber and the plate was incubated in a 5% CO_2 incubator at 37 °C for 5 days. After the incubation, medium was removed using aspirator and an apical chamber and basolateral chamber was washed with 100 μL and 500 μL of HBSS buffer, respectively. 250 μL of 20 μM peptide solution in 0.2% DMSO/HBSS was added to an apical chamber and 750 μL of 0.2% DMSO/HBSS was added to a basolateral chamber. When the assay was conducted in the presence of FBS, E-MEM medium containing 10% FBS and 1% antibiotic-antimycotic was used instead of HBSS. The plate was incubated in 5% CO_2 incubator at 37 °C for 2 h. After the incubation, peptides in basolateral chambers were determined by reacting with azidocoumarin under CuAAC reaction conditions or by HPLC.

When the concentration of the peptide was quantitated by the fluorogenic reaction, after the 2 h incubation, peptides in basolateral chambers were reacted with azidocoumarin under CuAAC reaction conditions. For the assay without FBS, the CuAAC reaction was conducted as follows: 150 μL of peptide solution in basolateral chamber after the permeability assay was transferred to a 96-well black plate and the peptide solution was mixed with 2 μL of PBS (pH7.2), 20 μL of 1 M phosphate buffer (pH 8.0), 2 μL of 500 mM sodium ascorbate in water, 4 μL of 10 mM $CuSO_4$ in water and 2 μL of 10 mM THPTA in water then the mixture was further mixed with 18 μL of DMSO and 2 μL of 10 mM azidocoumarin to initiate the fluorogenic reaction. For the assay with FBS, the CuAAC reaction was conducted as follows: 144 μL of peptide solution in basolateral chamber after the permeability assay was transferred to a 96-well black plate and the peptide solution was mixed with 20 μL of 1 M phosphate buffer (pH 8.0), 2 μL of 500 mM sodium ascorbate in water, 12 μL of 10 mM $CuSO_4$ in water and 2 μL of 10 mM THPTA in water then the mixture was further mixed with 18 μL of DMSO and 2 μL of 10 mM azidocoumarin to initiate the fluorogenic reaction. In both cases, the CuAAC reaction was performed for 1 h and the fluorescence from each well was measured using a plate reader with excitation and emission wavelengths of 400 nm and 480 nm, respectively. As a reference, 20 μM of each peptide was also reacted with azidocoumarin. Fluorescent intensity from a blank sample that does not contain peptide was subtracted from the measured fluorescence and the peptide concentration in acceptor well was determined from the ratio of the fluorescence from the basolateral chamber and the fluorescence from the reference sample. From the concentration in basolateral chamber, the effective permeability coefficient (P_e) was calculated using the following equation where the steady-state flux from apical to basolateral is considered to be maintained, i.e. peptide concentration in basolateral chamber is less than 10% of that in apical chamber.[5]

$$P_{\mathrm{e}} = \frac{Q(t)}{t} \times \frac{1}{C_0} \times \frac{1}{A}$$

$$Q(t) = C_{\mathrm{B}}(t) \times V_{\mathrm{B}}$$

$$C_{\mathrm{B}}(t) = \frac{F_{\mathrm{s}} - F_{\mathrm{b}}}{F_{\mathrm{r}} - F_{\mathrm{b}}} \times C_{\mathrm{r}}$$

Where:

$Q(t)$ = quantity of peptide in basolateral chamber at time t

t = incubation time (s) (2 h = 7,200 s)

C_0 = initial concentration in apical chamber (20 μM)

A = filter area (cm^2)

$C_B(t)$ = peptide concentration in basolateral chamber at time t

V_B = basolateral well volume (0.75 mL)

F_s = fluorescent intensity from sample

F_r = fluorescent intensity from reference

F_b = fluorescent intensity from blank

C_r = Peptide concentration of reference

When peptide was quantitated by HPLC, concentration of peptide in basolateral chamber ($C_B(t)$) was determined from HPLC peak area that is compared with the peak area of 20 μM peptide.

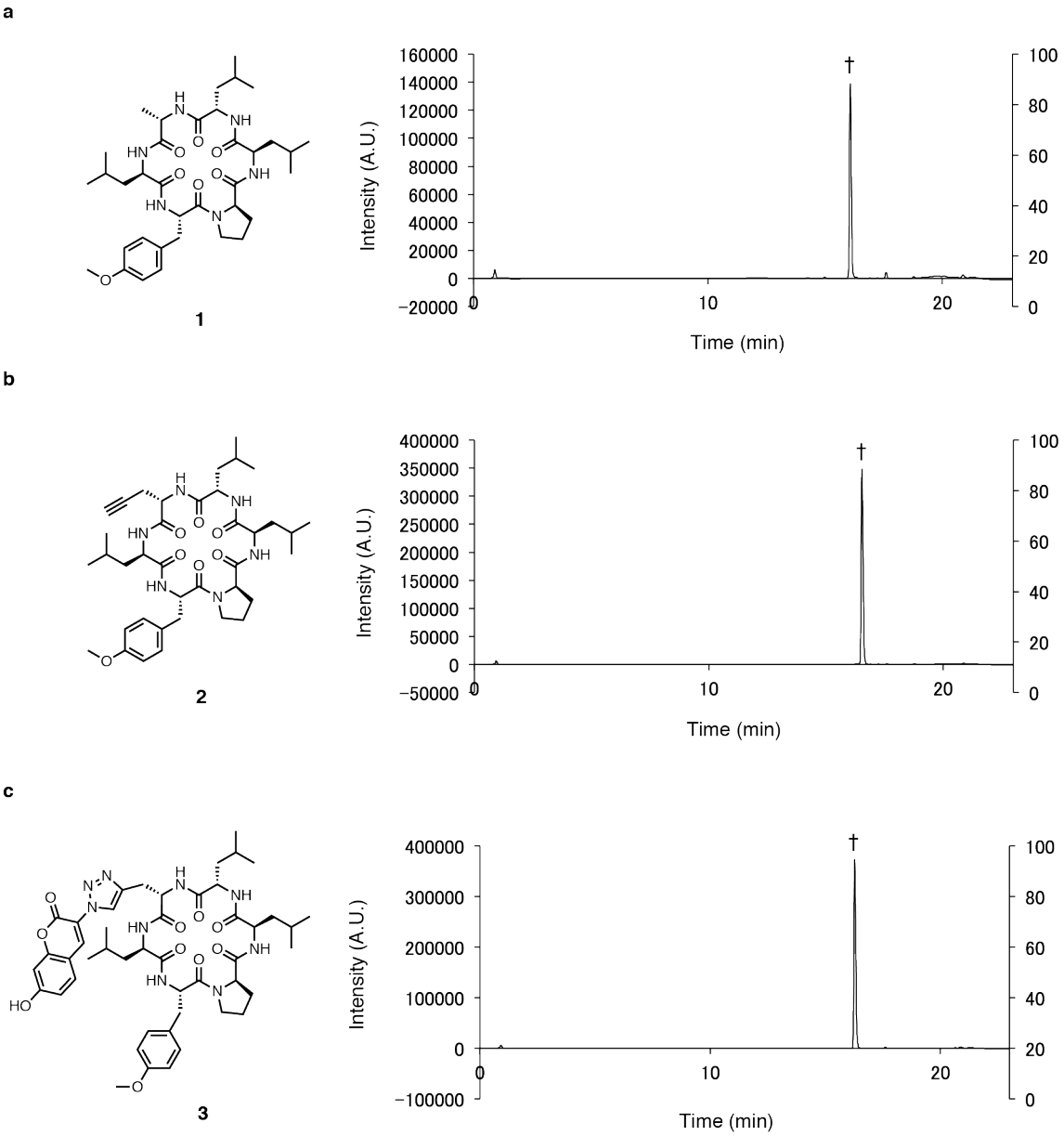


Fig. S1. HPLC chromatograms of cyclic peptides 1–3 after purification. Chromatograms of **(a) 1**, **(b) 2**, and **(c) 3**. The peak corresponding to the desired compound is indicated by †. Products were monitored at 280 nm. The column was first equilibrated with 20% solvent B for 3 min. Then the compound was eluted by a linear gradient of 20–99% solvent B over 8 min and the column was washed with 99% solvent B for 2 min. The solvent system was changed from 99% solvent B to 20% solvent B over 1 min and the column was equilibrated for 9 min at 20% solvent B.

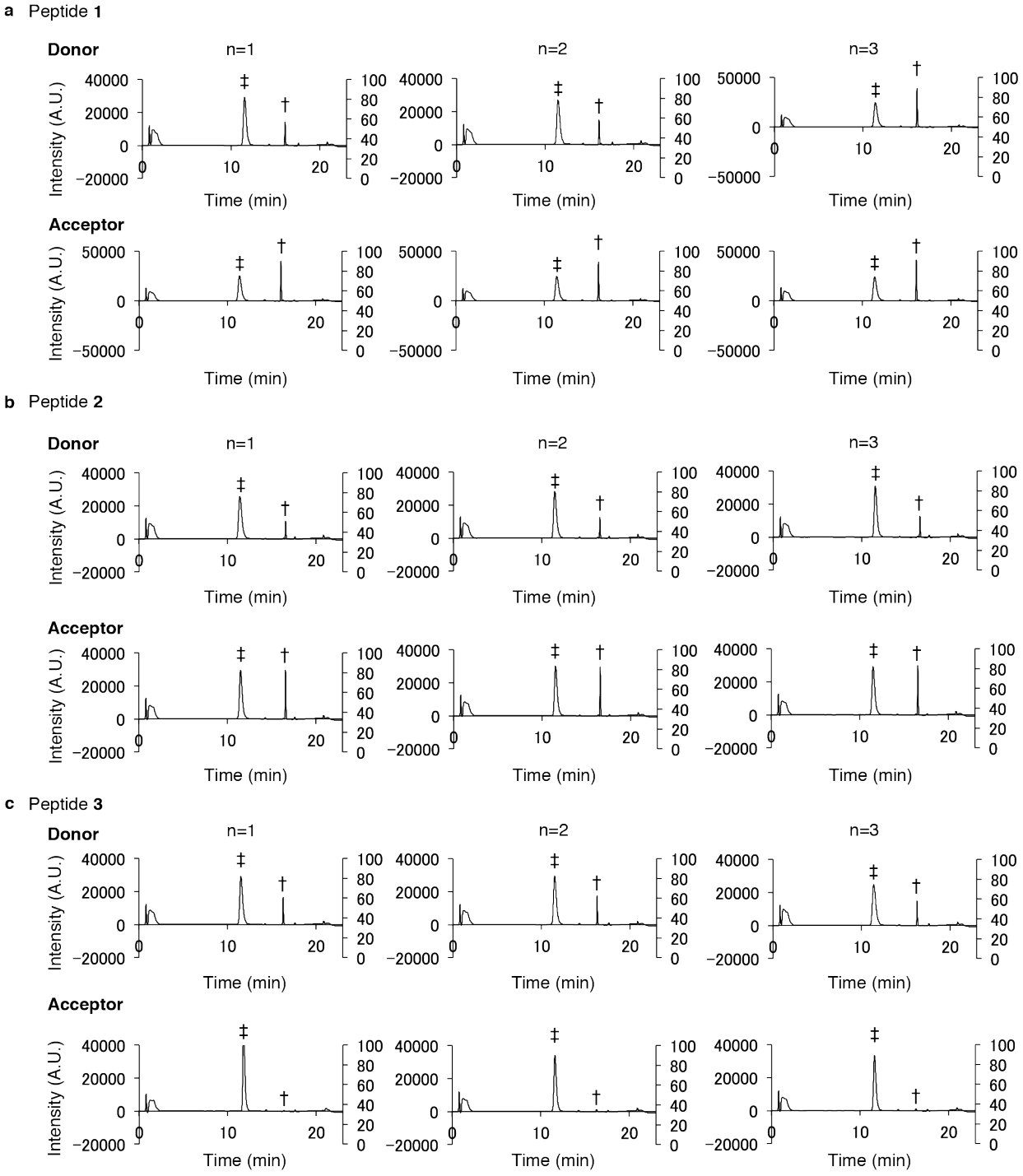


Fig. S2. HPLC chromatograms of peptides 1–3 after PAMPA. Chromatograms of **(a) 1**, **(b) 2**, and **(c) 3** from donor and acceptor wells after PAMPA. The peak corresponding to the desired compound is indicated by †. The peak corresponding to the internal standard (propranolol) is indicated by ‡. Products were monitored at 280 nm. The column was first equilibrated with 20% solvent B for 3 min. Then the compound was eluted by a linear gradient of 20–99% solvent B over 8 min and the column was washed with 99% solvent B for 2 min. The solvent system was changed from 99% solvent B to 20% solvent B over 1 min and the column was equilibrated for 9 min at 20% solvent B.

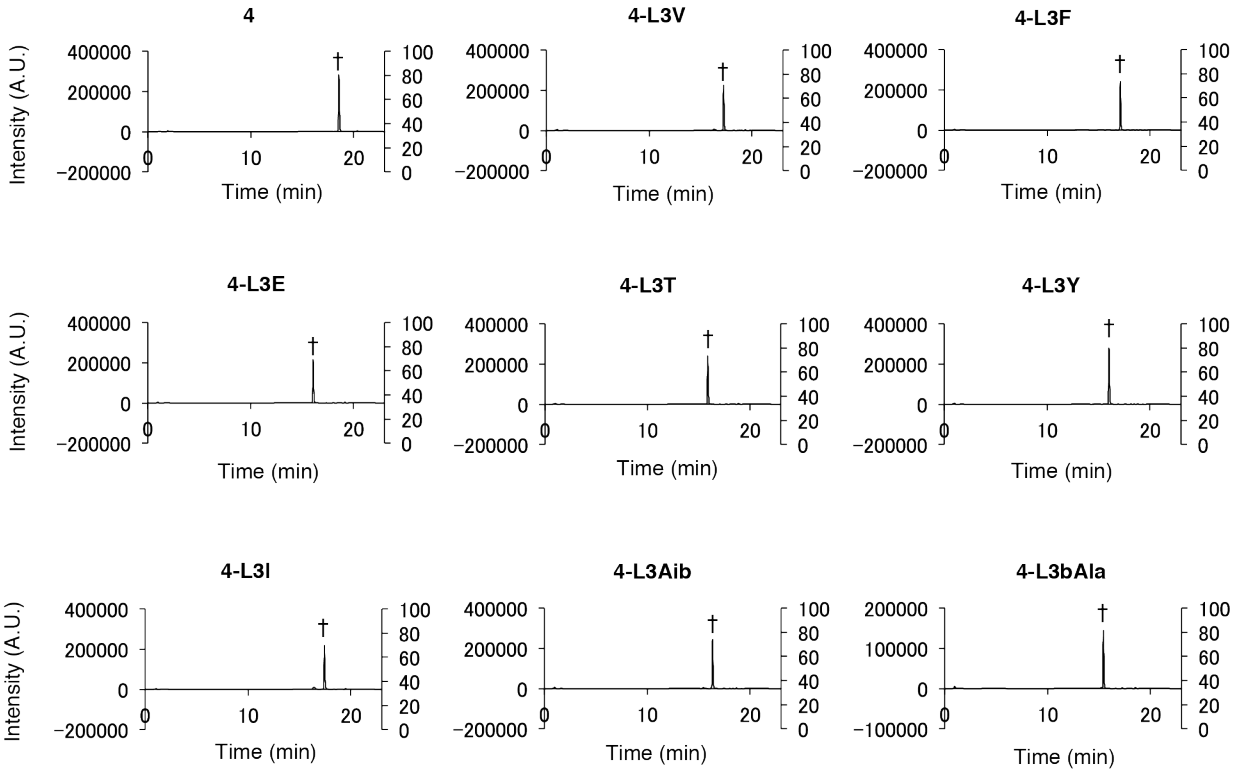


Fig. S3. HPLC chromatograms of peptide 4 and derivatives after purification. The peak corresponding to the desired compound is indicated by †. Products were monitored at 280 nm. The column was first equilibrated with 20% solvent B for 3 min. Then the compound was eluted by a linear gradient of 20–99% solvent B over 8 min and the column was washed with 99% solvent B for 2 min. The solvent system was changed from 99% solvent B to 20% solvent B over 1 min and the column was equilibrated for 9 min at 20% solvent B.

Table S1. ESI-MS data of synthesized peptides.

Compound	Peptide sequence	Observed ion	Calculated mass	Observed mass
1	cyclo[D-Pro-D-Leu-Leu-Ala-D-Leu-Tyr(OMe)]	$[M+Na]^+$	707.4103	707.4080
2	cyclo[D-Pro-D-Leu-Leu-Pgl-D-Leu-Tyr(OMe)]	$[M+Na]^+$	731.4103	731.4074
3	cyclo[D-Pro-D-Leu-Leu-Cmr-D-Leu-Tyr(OMe)]	$[M+Na]^+$	934.4434	934.4416
4	cyclo[D-Pro-D-Leu-Leu-Pgl-D-Leu-Tyr]	$[M+Na]^+$	717.3946	717.3857
4-L3l	cyclo[D-Pro-D-Leu-D-Leu-Pgl-D-Leu-Tyr]	$[M+Na]^+$	717.3946	717.3849
4-L3V	cyclo[D-Pro-D-Leu-Val-Pgl-D-Leu-Tyr]	$[M+Na]^+$	703.3790	703.3711
4-L3E	cyclo[D-Pro-D-Leu-Glu-Pgl-D-Leu-Tyr]	$[M+Na]^+$	733.3531	733.3475
4-L3F	cyclo[D-Pro-D-Leu-Phe-Pgl-D-Leu-Tyr]	$[M+Na]^+$	751.3790	751.3711
4-L3T	cyclo[D-Pro-D-Leu-Thr-Pgl-D-Leu-Tyr]	$[M+Na]^+$	705.3582	705.3510
4-L3Y	cyclo[D-Pro-D-Leu-Tyr-Pgl-D-Leu-Tyr]	$[M+Na]^+$	767.3739	767.3689
4-L3Aib	cyclo[D-Pro-D-Leu-Aib-Pgl-D-Leu-Tyr]	$[M+Na]^+$	689.3633	689.3551
4-L3bhA	cyclo[D-Pro-D-Leu-bhA-Pgl-D-Leu-Tyr]	$[M+Na]^+$	689.3633	689.3554

Pgl: Propargylglycine residue

Cmr: Propargylglycine resdiue conjugated with azidocoumarin

Aib: Aminoisobutyric acid residue

bhA: β-Homoalanine residue

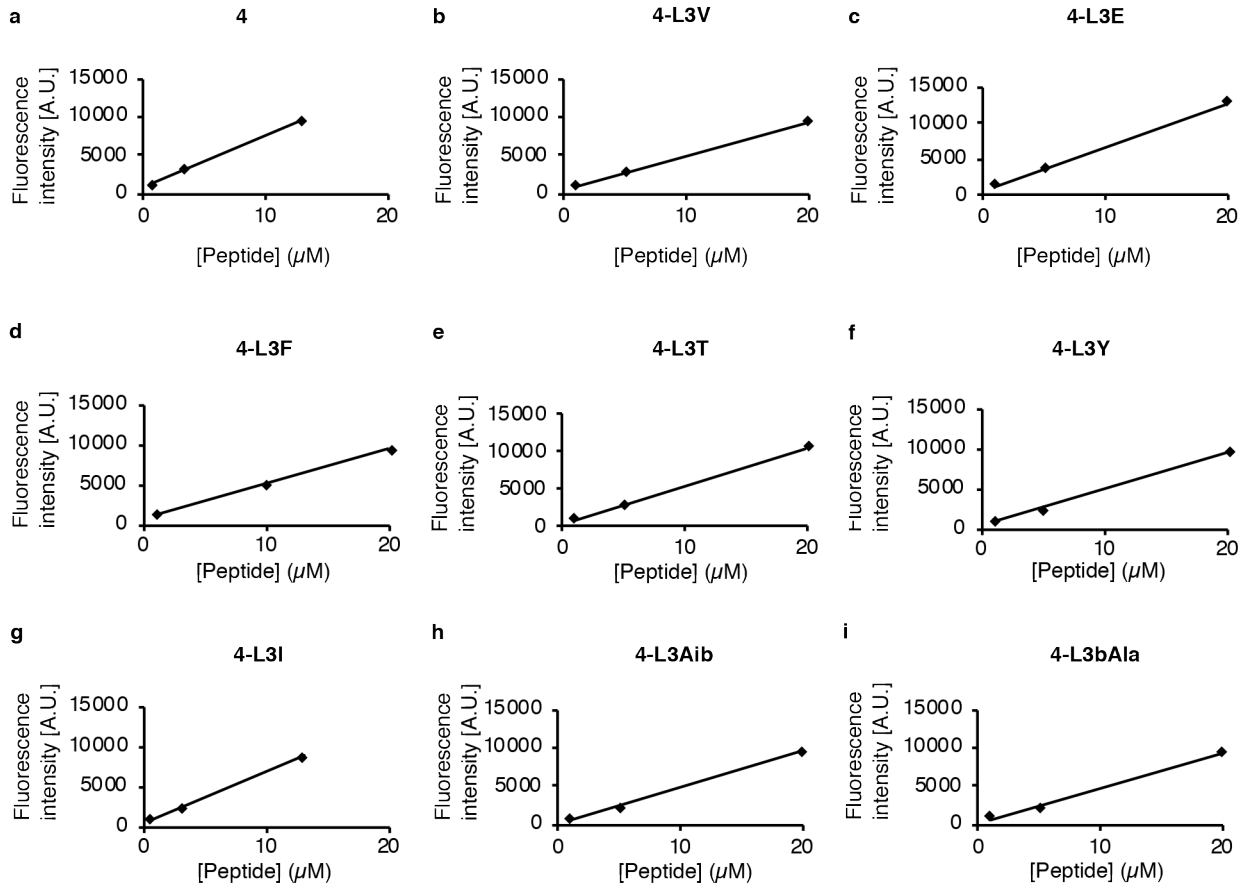


Fig. S4. Standard curves of peptide 4 and its derivatives for determination of peptide concentration from fluorescent intensity after PAMPA. Three different concentrations between 0.65–20 μM of peptides were reacted with azidocoumarin under the optimized CuAAC reaction conditions and the fluorescence intensities after 1 h were used for generating the standard curves.

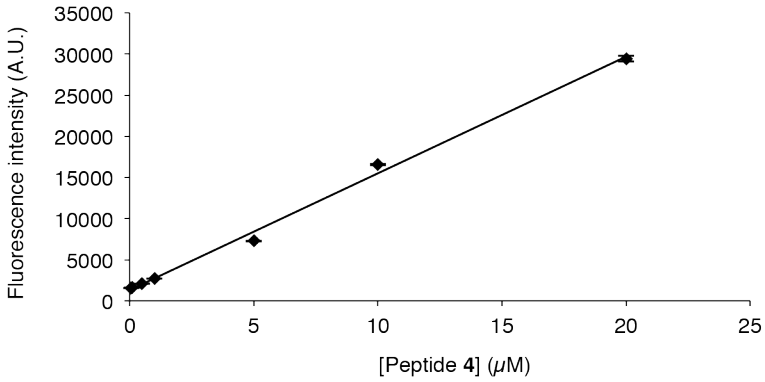


Fig. S5. Fluorescent intensity of coumarin conjugate of peptide 4 in HBSS buffer after 1 h CuAAC reaction. 0.05–20 μM of peptide **4** in HBSS buffer was reacted with 100 μM azidocoumarin for 1 h (200 μM $CuSO_4$, 5 mM sodium ascorbate, 100 μM THPTA in phosphate buffer (pH 8.0) containing 30% DMSO) and the fluorescence (Ex. 400 nm, Em. 480 nm) from the reaction mixture was measured on a plate reader. The error bars represent the standard deviations of triplicates.

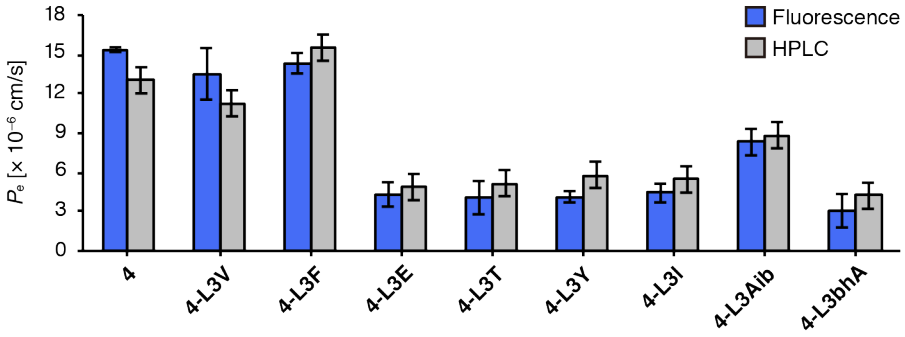


Fig. S6. MDCK-II assay quantitated by HPLC. MDCK-II assay was conducted in the same manner with **Fig. 3** and the concentration of peptide in the basolateral chamber was quantitated by HPLC. P_e values calculated from the peptide concentrations are shown with error bars representing standard deviations of triplicate (gray bars). As a reference, P_e values determined using the fluorogenic reaction shown in **Fig. 3** are also shown (blue bars).

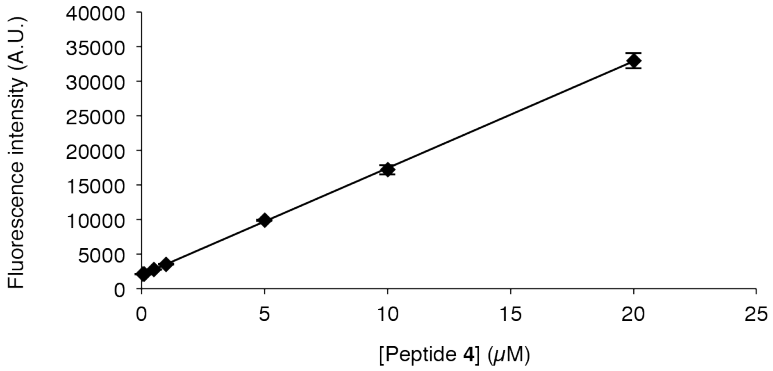


Fig. S7. Fluorescent intensity of coumarin conjugate of peptide 4 in 10% FBS/E-MEM after 1 h CuAAC reaction. 0.05–20 μM of peptide **4** in 10% FBS/E-MEM was reacted with 100 μM azidocoumarin for 1 h (600 μM $CuSO_4$, 5 mM sodium ascorbate, 100 μM THPTA in phosphate buffer (pH 8.0) containing 30% DMSO) and the fluorescence (Ex. 400 nm, Em. 480 nm) from the reaction mixture was measured on a plate reader. The error bars represent the standard deviations of triplicates.

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