

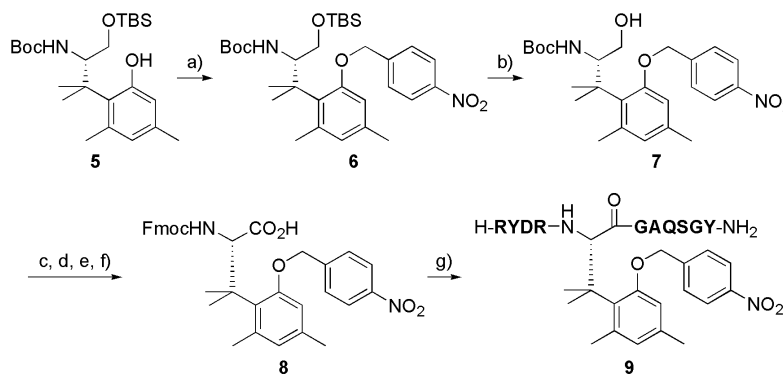
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General Methods

All reactions were carried out under a positive pressure of argon. For column chromatography, silica gel (KANTO KAGAKU N-60) was employed. Exact mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ or a Bruker Esquire200T. NMR spectra were recorded using a JEOL GSX400 spectrometer or a Bruker AV400N at 400 MHz frequency for ¹H and 100 MHz frequency for ¹³C in CDCl₃. Chemical shifts are calibrated to the solvent signal. For HPLC separations, a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min) or a Cosmosil 5C₁₈-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA in H₂O (solvent A) and 0.1% TFA (v/v) in MeCN (solvent B) was used for HPLC elution. An Olympus IX70 was used to obtain microscopic images.

Synthesis of reduction-responsive amino acid **8** and its incorporation into model peptide **9**



Scheme S1. Reagents and conditions. a) *p*-Nitrobenzyl bromide, K₂CO₃, DMF, 99%; b) AcOH aq., THF, quant.; c) PDC, DMF; d) NaClO₂, 2-methyl-2-butene, NaH₂PO₄, acetone, H₂O; e) HCl, AcOEt; f) FmocOSu, Na₂CO₃ aq., MeCN, 97% (4 steps); g) Fmoc SPPS. (A: alanine; D: aspartic acid; G: glycine; Q: glutamine; R: arginine; S: serine; Y: tyrosine)

(*S*)-2-*tert*-Butoxycarbonylamino-3,3-dimethyl-3-{2,4-dimethyl-6-(4-nitrobenzyloxy)phenyl}propanol *tert*-butyldimethylsilyl ether (**6**)

To a stirred solution of phenol **5**^[S1] (0.500 g, 1.14 mmol) in DMF (5.00 mL) were added K₂CO₃ (0.378 g, 2.73 mmol) and *p*-nitrobenzyl bromide (0.296 g, 1.37 mmol), and the resulting suspension was stirred overnight. The reaction mixture was quenched by the addition of saturated aqueous solution of NH₄Cl. After being stirred for 30 min, the mixture was extracted with Et₂O. The combined organic layer was washed with water (×2) followed by brine, dried over MgSO₄ and concentrated in vacuo. The crude product was purified by column chromatography (hexane/AcOEt = 20/1 (v/v)) and 0.648 g of *p*-nitrobenzyl ether **6** (1.13 mmol, 99%) was obtained as a colorless oil: [α]_D²¹ -52.1 (c 0.83, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ = -0.01 (3H, s), 0.00 (3H, s), 0.87 (9H, s), 1.45 (9H, s), 1.54 (3H, s), 1.58 (3H, s), 2.25 (3H, s), 2.58 (3H, s), 3.54 (1H, dd, *J* = 10.6 and 4.4 Hz), 3.60 (1H, dd, *J* = 10.6 and 4.4 Hz), 4.74 (1H, dt, *J* = 10.4 and 4.4 Hz), 4.88 (1H, d, *J* = 10.4 Hz),

5.19 (1H, d, $J = 13.4$ Hz), 5.28 (1H, d, $J = 13.4$ Hz), 6.61 (1H, s), 6.63 (1H, s), 7.77 (2H, d, $J = 8.8$ Hz), 8.27 (2H, d, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) $\delta = -5.6$ (CH_3), -5.5 (CH_3), 18.2 (C), 20.7 (CH_3), 25.9 (CH_3), 26.0 (CH_3), 27.4 (CH_3), 28.4 (CH_3), 29.1 (CH_3), 45.0 (C), 56.0 (CH), 63.7 (CH_2), 70.0 (CH_2), 78.6 (C), 112.7 (CH), 123.8 (CH), 128.0 (CH), 126.2 (CH), 131.3 (C), 136.3 (C), 138.7 (C), 144.9 (C), 147.5 (C), 156.1 (C), 158.2 (C); HRMS (ESI-TOF) m/z calcd for $\text{C}_{31}\text{H}_{48}\text{N}_2\text{NaO}_6\text{Si}$ ($[\text{M} + \text{Na}]^+$) 595.3179, found 595.3157.

(S)-2-tert-Butoxycarbonylamino-3,3-dimethyl-3-{2,4-dimethyl-6-(4-nitrobenzyloxy)phenyl}-propanol (7)

Glacial acetic acid (8.49 mL) and water (2.84 mL) were added to a solution of silyl ether **6** (0.648 g, 1.13 mmol) in THF (2.84 mL). The reaction mixture was stirred overnight and was diluted with water. After extraction with AcOEt, the organic phase was washed with saturated aqueous solution of NaHCO_3 and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography (hexane/AcOEt = 4/1 (v/v)) and 0.519 g of alcohol **7** (1.13 mmol, quant.) was obtained as a white amorphousness: $[\alpha]_D^{21} -35.1$ (c 1.01, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) $\delta = 1.40$ (9H, s), 1.51 (3H, s), 1.53 (3H, s), 2.22 (3H, s), 2.53 (3H, s), 3.52 (1H, dd, $J = 11.0$ and 8.0 Hz), 3.63 (1H, dd, $J = 10.5$ and 3.2 Hz), 4.70 (1H, br m), 4.99 (1H, br m), 5.19 (1H, d, $J = 13.6$ Hz), 5.26 (1H, d, $J = 13.6$ Hz), 6.61 (2H, br s), 7.71 (2H, d, $J = 8.2$ Hz), 8.25 (2H, d, $J = 8.2$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) $\delta = 20.7$ (CH_3), 26.0 (CH_3), 27.4 (CH_3), 28.3 (CH_3), 28.6 (CH_3), 44.0 (C), 58.6 (CH), 64.0 (CH_2), 70.2 (CH_2), 79.3 (C), 113.0 (CH), 123.9 (CH), 128.0 (CH), 128.4 (CH), 130.5 (C), 136.7 (C), 138.3 (C), 144.8 (C), 147.5 (C), 157.2 (C), 158.2 (C); HRMS (ESI-TOF) m/z calcd for $\text{C}_{25}\text{H}_{34}\text{KN}_2\text{O}_6$ ($[\text{M} + \text{K}]^+$) 497.2054, found 497.2058.

(S)-3,3-Dimethyl-3-{2,4-dimethyl-6-(4-nitrobenzyloxy)phenyl}-2-(9-fluorenylmethylcarbonylamino)propionic acid (8)

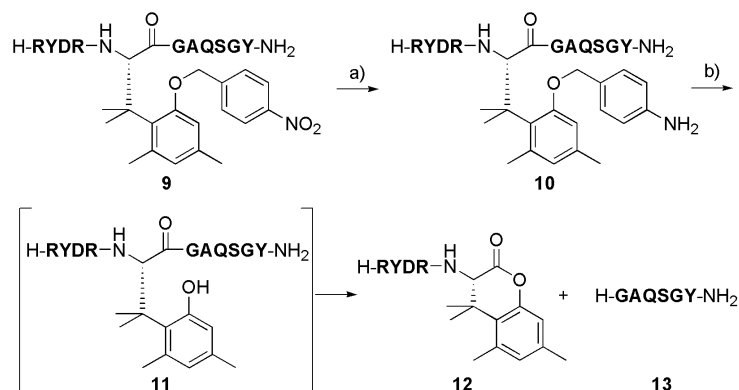
Pyridinium dichromate (2.20 g, 4.80 mmol) was added to a solution of alcohol **7** (0.536 g, 1.17 mmol) in DMF (5.90 mL). The reaction mixture was stirred overnight. After addition of 5% (w/v) aqueous solution of KHSO_4 , the obtained mixture was extracted with diethyl ether. The combined organic layer was washed with brine, dried over Na_2SO_4 , and concentrated in vacuo. The obtained crude material was subjected to subsequent reactions without purification. To a solution of the crude material in acetone (21.9 mL)/*tert*-BuOH (14.6 mL)/ H_2O (3.65 mL) were added 2-methyl-2-butene (0.837 mL, 7.90 mmol), NaH_2PO_4 (0.211 g, 1.76 mmol) and NaClO_2 (0.556 g, 6.14 mmol), and the resulting mixture was stirred for 2.5 h. After addition of saturated aqueous solution of NH_4Cl , the reaction mixture was extracted with diethyl ether. The organic phase was dried over Na_2SO_4 and was concentrated in vacuo. Hydrogen chloride in AcOEt (4 M, 17.8 mL) was added to the crude product, and the resulting mixture was stirred for 1 h. After concentration in vacuo, the obtained residue was

dissolved in MeCN (10.5 mL)/10% (w/v) aqueous solution of Na₂CO₃ (10.5 mL). To the resulting solution was added FmocOSu (0.414 g, 1.23 mmol), and the reaction mixture was stirred for 2 h. After acidification with 5% (w/v) aqueous solution of KHSO₄, the obtained mixture was extracted with diethyl ether. The organic phase was washed with brine and concentrated in vacuo. The obtained crude product was purified by column chromatography (CHCl₃, then CHCl₃/MeOH = 30/1 (v/v)) and 0.558 g of Fmoc derivative **8** (1.13 mmol, 97% (4 steps)) was obtained as a white powder: $[\alpha]^{19}_D$ -23.9 (*c* 0.73, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ = 1.59 (3H, s), 1.70 (3H, s), 2.20 (3H, s), 2.55 (3H, s), 4.15 (1H, t, *J* = 7.4 Hz), 4.25 (1H, dd, *J* = 10.4 and 7.4 Hz), 4.40 (1H, dd, *J* = 10.4 and 7.4 Hz), 5.08-5.30 (2H, m), 5.58 (1H, d, *J* = 9.6 Hz), 5.64 (1H, d, *J* = 9.6 Hz), 6.59 (1H, s), 6.60 (1H, s), 7.32 (2H, t, *J* = 7.4 Hz), 7.42 (2H, t, *J* = 7.4 Hz), 7.50 (1H, d, *J* = 7.4 Hz), 7.57 (1H, d, *J* = 7.4 Hz), 7.69 (2H, d, *J* = 8.2 Hz), 7.78 (2H, d, *J* = 7.4 Hz), 8.23 (2H, d, *J* = 8.2 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ = 20.8 (CH₃), 25.8 (CH₃), 27.8 (CH₃), 28.2 (CH₃), 44.5 (C), 47.1 (CH), 59.6 (CH), 67.0 (CH₂), 69.9 (CH₂), 112.6 (CH), 120.0 (CH), 120.0 (CH), 123.9 (CH), 125.0 (CH), 125.1 (CH), 127.1 (CH), 127.1 (CH), 127.7 (CH), 127.9 (CH), 128.4 (CH), 128.6 (C), 137.1 (C), 138.1 (C), 141.3 (C), 141.3 (C), 143.8 (C), 144.8 (C), 147.5 (C), 156.1 (C), 158.2 (C), 177.0 (C); HRMS (ESI-TOF) *m/z* calcd for C₃₅H₃₄N₂NaO₇ ([M + Na]⁺) 617.2264, found 617.2245.

Model peptide **9**

Peptide **9** was synthesized on NovaSyn TGR resin (0.25 mmol amine/g) using Fmoc SPPS according to a previous report.^[S2] Briefly, on the resin was coupled Fmoc protected naturally occurring amino acid derivatives (4 eq., a protective group of a side chain: *tert*-Bu for tyrosine, serine, and aspartic acid; Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) for arginine; Trt for glutamine) or Fmoc protected reduction-responsive amino acid **8** (2 eq.) in the presence of *N,N'*-diisopropylcarbodiimide (DIC, 4 or 2 eq.) and 1-hydroxybenzotriazole hydrate (HOBT·H₂O, 4 or 2 eq.) in DMF/DMSO = 3/1 (v/v) (8.0 μ L/1.0 μ mol resin) for 1 h or overnight, respectively. The resulting completed resin was treated with TFA/thioanisole/*m*-cresol/H₂O/Et₃SiH (80/5/5/5/2.5 (v/v)) at room temperature for 1.5 h. After the resin was filtered off, cooled diethyl ether was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with diethyl ether and purified by preparative HPLC to give model peptide **9** as a white lyophilized powder. Analytical HPLC conditions: linear gradient of solvent B in solvent A, 10 to 60% over 30 min. Retention time = 17.6 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. LRMS (ESI-Ion Trap) *m/z* calcd for [M + 2H]²⁺ 763.4, found 763.5.

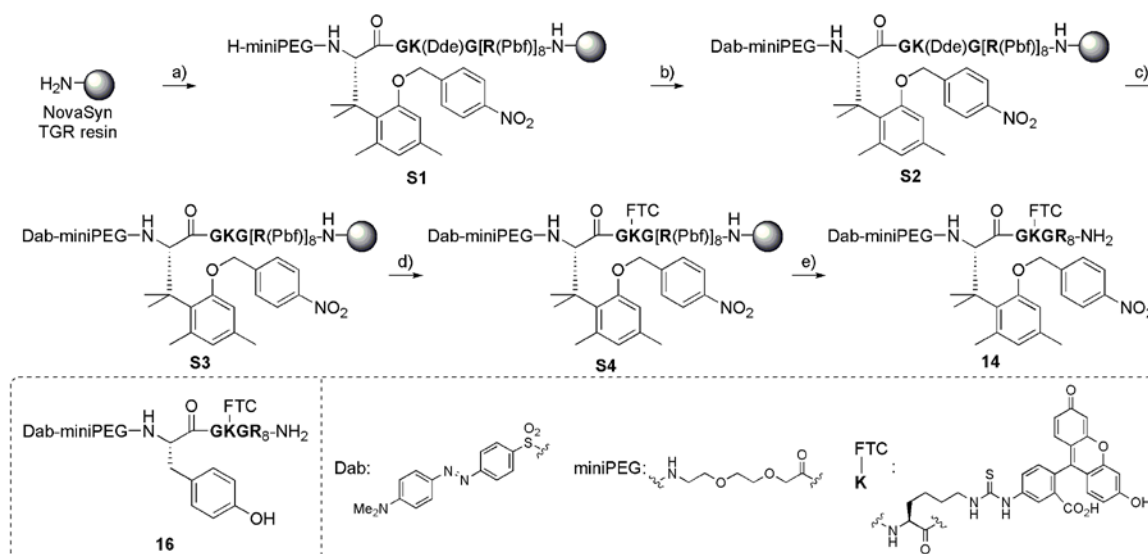
Chemical reduction and peptide bond cleavage of model peptide **9**



Scheme S2. Reagents and conditions. a) Zn, NH₄Cl aq., 37 °C; b) Sodium phosphate buffer (pH 7.4 or 6.0, 20 mM), 37 °C.

To a solution of peptide **9** in H₂O (1.0 mM, 4.5 μL) were added 10% (w/v) NH₄Cl aq. (15 μL, final concentration: 2.5% (w/v)), water (40.5 μL), and Zn (1.3 mg), and the reaction mixture was incubated at 37 °C for 30 min. After filtration followed by HPLC purification and lyophilization, obtained peptide **10** was dissolved in a sodium phosphate buffer (pH 7.4 or 6.0, 20 mM, 550 μL). In the case of kinetic studies, 0.0001% (v/v) *m*-cresol was added as an internal standard. The reaction mixture was incubated at 37 °C. Progress of the reaction was monitored by HPLC and the peptides were characterized by ESI-MS. Percentage of remaining **10** was estimated based on HPLC peak area. **10**: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 10 to 60% over 30 min. Retention time = 13.5 min; 1 to 50% over 30 min. Retention time = 19.3 min. LRMS (ESI-Ion Trap) *m/z* calcd for [M + 2H]²⁺ 748.4, found 748.5. **12**: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 1 to 50% over 30 min. Retention time = 22.2 min. LRMS (ESI-Ion Trap) *m/z* calcd for [M + H]⁺ 810.4, found 810.5. **13**: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 1 to 50% over 30 min. Retention time = 10.0 min. LRMS (ESI-Ion Trap) *m/z* calcd for [M + H]⁺ 581.3, found 581.3. *m*-Cresol: Analytical HPLC conditions: linear gradient of solvent B in solvent A, 1 to 50% over 30 min. Retention time = 26.7 min.

Preparation of hypoxia-responsive peptide **14** and reference peptide **16**



Scheme S3. Reagents and conditions. a) Fmoc SPPS; b) Dabsyl chloride, Et₃N, DMF; c) 2% (v/v) Hydrazine hydrate in DMF; d) FITC, DIEA, DMF; e) TFA/thioanisole/H₂O = 95/2.5/2.5 (v/v).

Preparation of hypoxia-responsive peptide **14**

Peptide on NovaSyn TGR resin **S1** was synthesized according to the synthesis of peptide **9**. Obtained resin **S1** was reacted with dabsyl chloride (3 eq.) and Et₃N (6 eq.) in DMF (16 μL/1.0 μmol resin) overnight twice. After washing with DMF, CH₂Cl₂, DMF, 20% (v/v) piperidine in DMF, and subsequent DMF, resin **S2** was treated twice with 2% (v/v) hydrazine hydrate in DMF for 5 min to remove Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) group. Then the resin was washed with DMF. Fluorescein isomer I (FITC, 2.4 eq.) and *N,N*-diisopropylethylamine (DIEA, 2.5 eq.) in DMF (8.0 μL/1.0 μmol resin) were reacted with obtained resin **S3** for 2 d. Resulting completed resin **S4** was treated with TFA/thioanisole/H₂O (95/2.5/2.5 (v/v)) at room temperature for 2 h. After the resin was filtered off, cooled diethyl ether was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with diethyl ether and purified by preparative HPLC to give hypoxia-responsive peptide **14** as a red lyophilized powder. Analytical HPLC conditions: linear gradient of solvent B in solvent A, 10 to 60% over 30 min. Retention time = 24.8 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 25 to 35% over 30 min. LRMS (ESI-TOF) *m/z* calcd for [M + 3H]³⁺ 895.4, [M + 4H]⁴⁺ 671.8, [M + 5H]⁵⁺ 537.7, found 895.8, 671.9, 537.7.

Preparation of reference peptide **16**

Peptide **16** was synthesized as similar to that of peptide **14**. Analytical HPLC conditions: linear gradient of solvent B in solvent A, 10 to 60% over 30 min. Retention time = 21.9 min. Preparative

HPLC conditions: linear gradient of solvent B in solvent A, 28 to 38% over 30 min. LRMS (ESI-TOF) m/z calcd for $[M + 4TFA + 3H]^{3+}$ 983.7, $[M + 4TFA + 4H]^{4+}$ 738.1, $[M + 3TFA + 4H]^{4+}$ 709.6, $[M + 3TFA + 5H]^{5+}$ 567.8, found 983.7, 738.2, 709.5, 567.8.

Hypoxia-responsive peptide bond cleavage in living cells

Caco-2 cells were cultured in DMEM with 10% FBS for 37 °C under aerobic (5% CO₂ in air) or hypoxic (5% CO₂ and 1% O₂ in N₂) conditions (1×10^4 cells/mL, 0.5 mL). Then aqueous solution of peptide **14** or **16** was added to the cells (final concentration: 300 nM), and the resulting mixture was incubated under the aerobic or the hypoxic conditions for additional 15 h. After sucking medium off, the cells were washed with DMEM with 10% FBS followed by D-PBS(-) twice. Finally, to the obtained cells was added D-PBS(-), and microscopic images were collected using Olympus IX70 inverted microscope equipped with a filter set (U-MWIB2) (Fluorescence images: $\lambda_{ex} = 460-490$ nm, $\lambda_{em} = 510$ nm, exposure time = 10 s) and MetaMorph software. Before use, DMEM with 10% FBS and D-PBS(-) were kept under 5% CO₂ in air or 5% CO₂ and 1% O₂ in N₂ for the aerobic or the hypoxic experiment, respectively. Then, fluorescence intensity per cell area was estimated based on the microscopic images. Results depicted in Figure 3 were average and standard deviation of 4 runs (10 cells/run), and Dunnett's test was used to statistical analysis.

References

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- [S2] A. Shigenaga, D. Tsuji, N. Nishioka, S. Tsuda, K. Itoh, A. Otaka, *ChemBioChem* **2007**, *8*, 1929-1931.