Electronic Supplementary Information for:

Inactivation of myostatin by photooxygenation using functionalized D-peptides

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General procedures

Myostatin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Aβ1–42 isopeptide, which contains an ester structure between the Gly25–Ser26 sequence, converts to intact Aβ1–42 *in situ* at neutral pH through an *O*-to-*N* intramolecular acyl migration ($t_{1/2}$ ~10 s),[S1,S2] was purchased from Peptide Institute, Inc. (Osaka, Japan). Methylene blue was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents and solvents were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Nacalai Tesque, Inc., Peptide Institute, Inc., Sigma-Aldrich Co. LLC (St Louis, MO, USA), Tokyo Chemical Industries Co., Ltd (Tokyo, Japan), Wako Pure Chemical Industries, Ltd, and Watanabe Chemical Industries, Ltd (Hiroshima, Japan). All chemicals were used as received. Analytical HPLC was carried out on a reverse-phase column (Nacalai Tesque COSMOSIL 5C18-AR-II, 4.6ID × 150 mm) using a linear gradient of CH_3CN (5–85%, 40 min) in 0.1% aqueous trifluoroacetic acid (TFA) with a flow rate of 1.0 mL min^{-1}, and detected at 230 or 200–800 nm. Preparative HPLC was carried out on a reverse-phase column (Waters SunFire Prep C18 OBD, 19ID × 150 mm) using a linear gradient of 0.1% TFA/CH_3CN in 0.1% aqueous TFA with a flow rate of 5.0 mL min^{-1}, and detected by absorbance at 230 or 700 nm. MALDI-TOF MS spectra were recorded on Shimadzu Biotech AXIMA Assurance using α-cyano-4-hydroxy cinnamic acid as a matrix. HRMS spectra were recorded on Waters MICRO MASS LCT premier. Absorbance measurements were performed using a Shimadzu UV-1700 UV-visible spectrophotometer with a rectangular quartz cell (10 mm path-length). CD measurements were performed using a Jasco J-1500CD spectrometer (JASCO, Japan) with a quartz cell (5 mm path-length).

Synthesis

Peptide **2**. Fmoc-based solid-phase peptide synthesis (SPPS) was performed with similar manner to that described by Takayama *et al.*[S3] On an Fmoc-NH SAL resin (108 mg, 0.04 mmol), Fmoc-amino acids (0.20 mmol each) were sequentially coupled using the *N,N'*-diisopropylcarbodiimide (DIPCI, 0.20 mmol)/1-hydroxybenzotriazole (HOBt, 0.20 mmol) method for 3 h in DMF after removing each Fmoc group with 20% piperidine/DMF for 10 × 2 min. The obtained protected peptide-bound resin was treated with TFA/*m*-cresol/thioanisole/1,2-ethanedithiol (100:2.5:2.5:1 v:v:v:v) for 2.5 h, filtered, concentrated, precipitated with diethyl ether, and purified by preparative HPLC in a 0.1% aqueous TFA–CH_3CN system to obtain peptide **2** as a TFA salt (white amorphous, 44 mg, 37%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2311.5 (calcd. for $C_{123}H_{192}N_{32}O_{18}$ 2311.5); purity >95% (analytical HPLC, t_R = 22.8 min).

Peptide **3**. To a solution of **8** (10 mM, 180 μL, 1 eq.), **13**[S4] (11 mM, 360 μL, 2.2 eq.) and L-

ascorbic acid (120 mM, 360 µL, 24 eq.) in DMF/MeOH (1:1 v:v, 1.8 mL), tetrakis(acetonitrile) copper(I) hexafluorophosphate (3.1 mg, 4.6 eq.) was added. The solution was stirred at room temperature (RT) for 20 min then purified by preparative HPLC in a 0.1% aqueous TFA–CH_3CN system to obtain peptide **3** as a TFA salt (dark blue amorphus, 2.3 mg, 36%).
HRMS *m/z* $[M+H]^+$ found 3062.6755 (calcd. for $C_{154}H_{223}N_{36}O_{20}BrBF_4$ 3062.6752); purity >95% (analytical HPLC, t_R = 26.3 min).

Peptide **4**. Following the same procedure used for peptide **3**, **9** (10 mM, 90 µL, 1 eq.) and **13** (11 mM, 90 µL, 1.1 eq.) was afforded **4** as a dark blue amorphous TFA salt (0.5 mg, 16%).
HRMS *m/z* $[M+H]^+$ found 2949.5952 (calcd. for $C_{148}H_{212}N_{35}O_{19}BrBF_4$ 2949.5911); purity >95% (analytical HPLC, t_R = 26.4 min).

Peptide **5**. Following the same procedure used for peptide **3**, **10** (10 mM, 90 µL, 1 eq.) and **13** (11 mM, 180 µL, 2.2 eq.) was afforded **5** as a dark blue amorphous TFA salt (0.9 mg, 26%).
HRMS *m/z* $[M+H]^+$ found 2921.5811 (calcd. for $C_{148}H_{212}N_{33}O_{19}BrBF_4$ 2921.5850); purity >95% (analytical HPLC, t_R = 26.6 min).

Peptide **6**. Following the same procedure used for peptide **3**, **11** (10 mM, 145 µL, 1 eq.) and **13** (11 mM, 290 µL, 2.7 eq.) was afforded **6** as a dark blue amorphous TFA salt (2.8 mg, 53%).
HRMS *m/z* $[M+H]^+$ found 2921.5886 (calcd. For $C_{148}H_{212}N_{33}O_{19}BrBF_4$ 2921.5850); purity >95% (analytical HPLC, t_R = 27.7 min).

Peptide **7**. Following the same procedure used for peptide **3**, **12** (10 mM, 90 µL, 1 eq.) and **13** (11 mM, 180 µL, 2.2 eq.) was afforded **7** as a dark blue amorphous TFA salt (0.5 mg, 14%).
HRMS *m/z* $[M+H]^+$ found 3077.6870 (calcd. for $C_{154}H_{224}N_{37}O_{20}BrBF_4$ 3077.6861); purity >95% (analytical HPLC, t_R = 26.3 min).

Peptide **8**. On an Fmoc-NH SAL resin (200 mg, 0.07 mmol), Fmoc-amino acids and pent-4-ynoic acid (0.22 mmol each) were sequentially coupled using the DIPCI (0.22 mmol)/HOBt (0.22 mmol) method for 1.5 h in DMF after removing each Fmoc group with 20% piperidine/DMF for 10 × 2 min. The obtained protected peptide-bound resin was treated with TFA/1,3-dimethoxybenzene[S5]/triisopropylsilane (92.5:5:2.5 v:v:v) for 90 min, filtered, concentrated, precipitated with diethyl ether, and purified by preparative HPLC in a 0.1%

aqueous TFA–CH_3CN system to obtain peptide **8** as a TFA salt (white amorphous, 33 mg, 29%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2392.6 (calcd. for $C_{123}H_{192}N_{32}O_{18}$ 2393.1); purity >95% (analytical HPLC, t_R = 18.7 min).

Peptide **9**. Following the same procedure used for peptide **8**, on an Fmoc-NH SAL resin (101 mg, 0.04 mmol), Fmoc-amino acids and 3,3-diphenylpropionicacid (0.20 mmol each) afforded **9** as a white amorphous TFA salt (2.3 mg, 11%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2279.7 (calcd for $C_{117}H_{181}N_{30}O_{17}$ 2279.9); purity >95% (analytical HPLC, t_R = 20.0 min).

Peptide **10**. Following the same procedure used for peptide **8**, on an Fmoc-NH SAL resin (103 mg, 0.05 mmol), Fmoc-amino acids and 3,3-diphenylpropionic acid (0.20 mmol each) afforded **10** as a white amorphous TFA salt (8.1 mg, 20%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2251.4 (calcd. for $C_{117}H_{181}N_{28}O_{17}$ 2251.9); purity >95% (analytical HPLC, t_R = 19.6 min).

Peptide **11**. Following the same procedure used for peptide **8**, on an Fmoc-NH SAL resin (99 mg, 0.04 mmol), Fmoc-amino acids and 3,3-diphenylpropionic acid (0.20 mmol each) afforded **11** as a white amorphous TFA salt (11 mg, 28%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2251.9 (calcd. for $C_{117}H_{181}N_{28}O_{17}$ 2252.6); purity >95% (analytical HPLC, t_R = 20.0 min).

Peptide **12**. Following the same procedure used for peptide **8**, on an Fmoc-NH SAL resin (104 mg, 0.05 mmol), Fmoc-amino acids and 3,3-diphenylpropionic acid (0.20 mmol each) afforded **12** as a white amorphous TFA salt (7.5 mg, 25%).
LRMS(MALDI) *m/z* $[M+H]^+$ found 2408.1 (calcd. for $C_{117}H_{181}N_{28}O_{17}$ 2408.6); purity >95% (analytical HPLC, t_R = 20.1 min).

Photooxygenation

The photooxygenation study was similar to that described by Okamoto *et al*.[S4,S6] A stock solution of myostatin (8 μM in 1 mM aqueous HCl) was diluted with 1 mM aqueous HCl and 20 mM phosphate buffer (pH 7.4) to produce 1 μM myostatin (10 mM phosphate, pH 7.4). A stock solution of Aβ1-42 isopeptide (250 μM in 0.1% aqueous TFA, ultracentrifuged)[S2] was diluted with water and 20 mM phosphate buffer (pH 7.4) to 20 μM Aβ1-42 (10 mM phosphate, pH 7.4). To each solution, functionalized peptide **3–7** or methylene blue (50 μM in

CH_3CN/DMSO = 9/1) was added to the final concentration (3 or 1 μM, respectively) for the oxygenation studies. The mixture was irradiated with LED ISLM-150 × 150-FF (λ_{max} = 730 nm, 14 mW: CCS Inc., Kyoto, Japan) at room temperature at approximately 3–5 cm from samples. The reaction mixtures were reduced with 1,4-dithiothreitol (50 mM) at 37 °C for 0.5 h, digested with endoproteinase Lys-C (1/20 of protein by weight) at 37 °C for 2.5 h, de-salted with ZipTip U-C18 (Millipore Co.), and analyzed using MALDI-TOF MS. For the comparison of oxygenation ratios based on the MS intensities, a comparative sample set was analyzed at one time, and the analytical conditions were standardized within the comparative sample set, for example, matrix substance, matrix/sample concentrations, solvent, laser power and laser-shot number. For cell-based assay, the 10 mM phosphate-buffered solution containing myostation (1 μM) or peptide **3–7** was diluted with serum-free DMEM (Dulbecco's modified Eagle's medium) to 0.6 nM myostatin, irradiated (λ_{max} = 730 nm, 30 min), and then added to the cell culture.

Luciferase-reporter assay

The cell-culture and luciferase-reporter assay were performed as described by Okamoto *et al.*[S4,S6] HEK293 cells were cultured in DMEM (10% FBS, x1 non-essential amino acids) at 37 °C under 5% CO_2. The cells were seeded at 2.0 × 10^4 cells per well in the 96-well plate (Greiner Bio-One GmbH). After 24 h, transfection of reporter (pGL4.48[luc2P/SBE/Hygro], Promega) and control (pGL4.74[hRluc/TK], Promega) vectors was carried out using FuGENE HD (Promega) and Opti-MEM (Thermo Fisher Scientific) for 24 h. After the culture, medium was exchanged to serum- and phenol red-free DMEM, the cells were incubated for 8 h. After the medium was exchanged to each sample solution (0.6 nM myostatin), the cells were incubated for 4 h and then washed with PBS. The preparation of cell lysates and the measurement of luciferase reporter activities were conducted according to manufacturer's protocol of a Dual-Luciferase Reporter Assay System (Promega). Each experiment was carried out in triplicate. Values represent means ± s.d. (n = 3). Statistical analyses were performed using Tukey's test.

Phototoxicity assay

The phototoxicity assay were performed as described by Okamoto *et al.*[S6] HEK293 cells, which were cultured in DMEM (10% FBS, x1 nonessential amino acids) at 37 °C under 5% CO_2, were seeded at 2.0 × 10^4 cells per well in the 96-well plate. After 48 h, the culture medium was exchanged with serum- and phenol red-free DMEM containing peptide **3–7** or methylene blue (3 μM). The cells were irradiated with LED (λ_{max} = 730 nm, 30 min), and then incubated for 24 h. Cell viability was determined using WST-1 (Roche Ltd). Statistical

analysis was performed using Student's *t*-test.

Stability assay with enzymes

Stability assay was performed with similar manner to that described by Takayama *et al.*[S7] Peptide **1** or **3** (100 μM) was incubated with chymotrypsin (5 μg/mL, from bovine pancreas, Thermo Fisher Scientific) or trypsin (5 μg/mL, from porcine pancreas, Wako Pure Chemical Industries, Ltd.) in Tris buffer (80 mM, pH 7.8) at 37 °C. After the incubation for 0, 1, or 3 h, 20 μL of the solution was subjected to analytical HPLC. The digested products were identified by MALDI-TOF MS analysis of the eluted solution in each peak. The residual ratio was calculated by comparing the corresponding peak areas of **1** or **3**.

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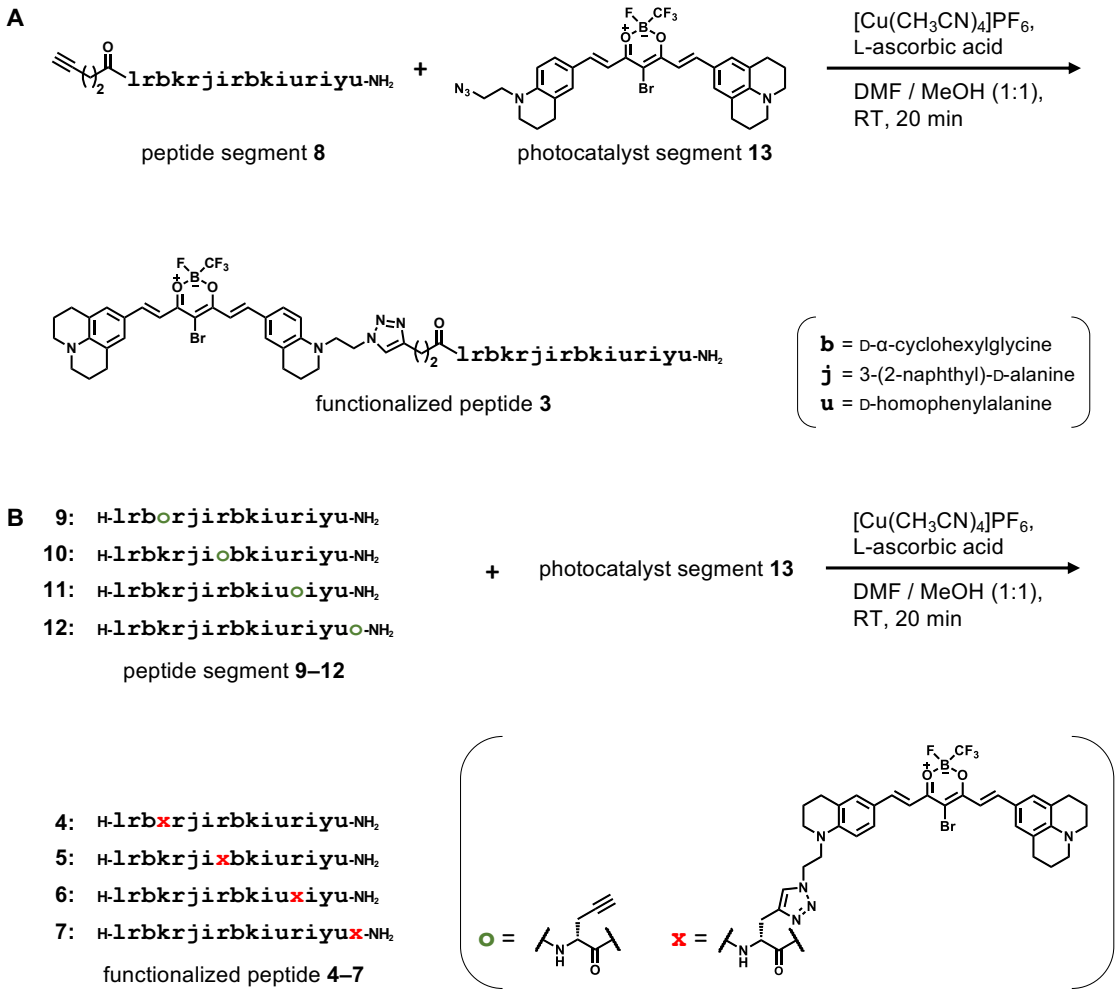


Scheme S1 Synthesis of functionalized D-peptides **3–7**.

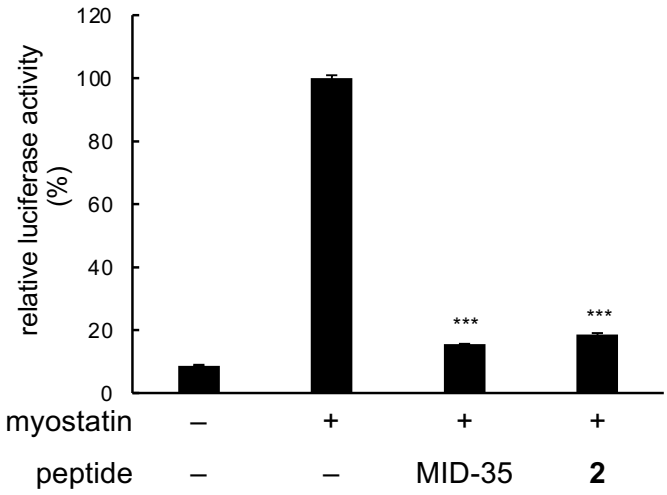


Fig. S1 Myostatin inhibitory activity of MID-35 and D-peptide **2**. Peptide: 300 nM, myostatin: 0.6 nM, n = 3, means ± s.d; ****p*<0.001 vs. myostatin alone by Tukey's test.

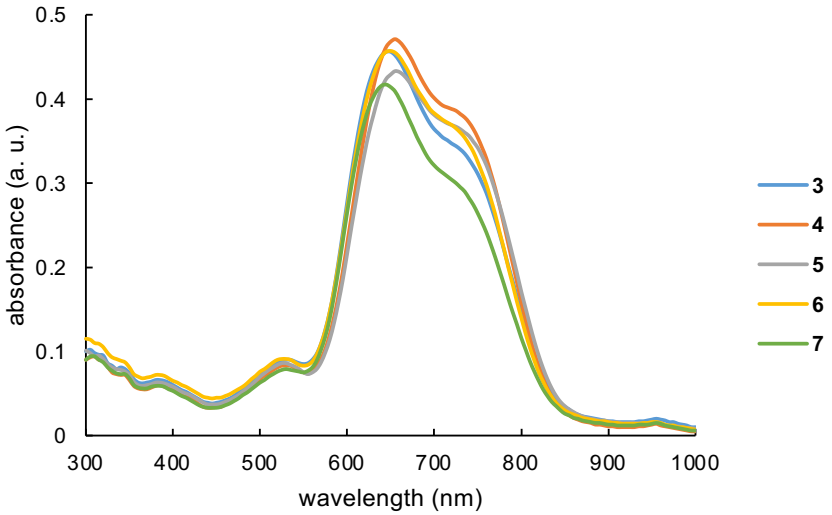
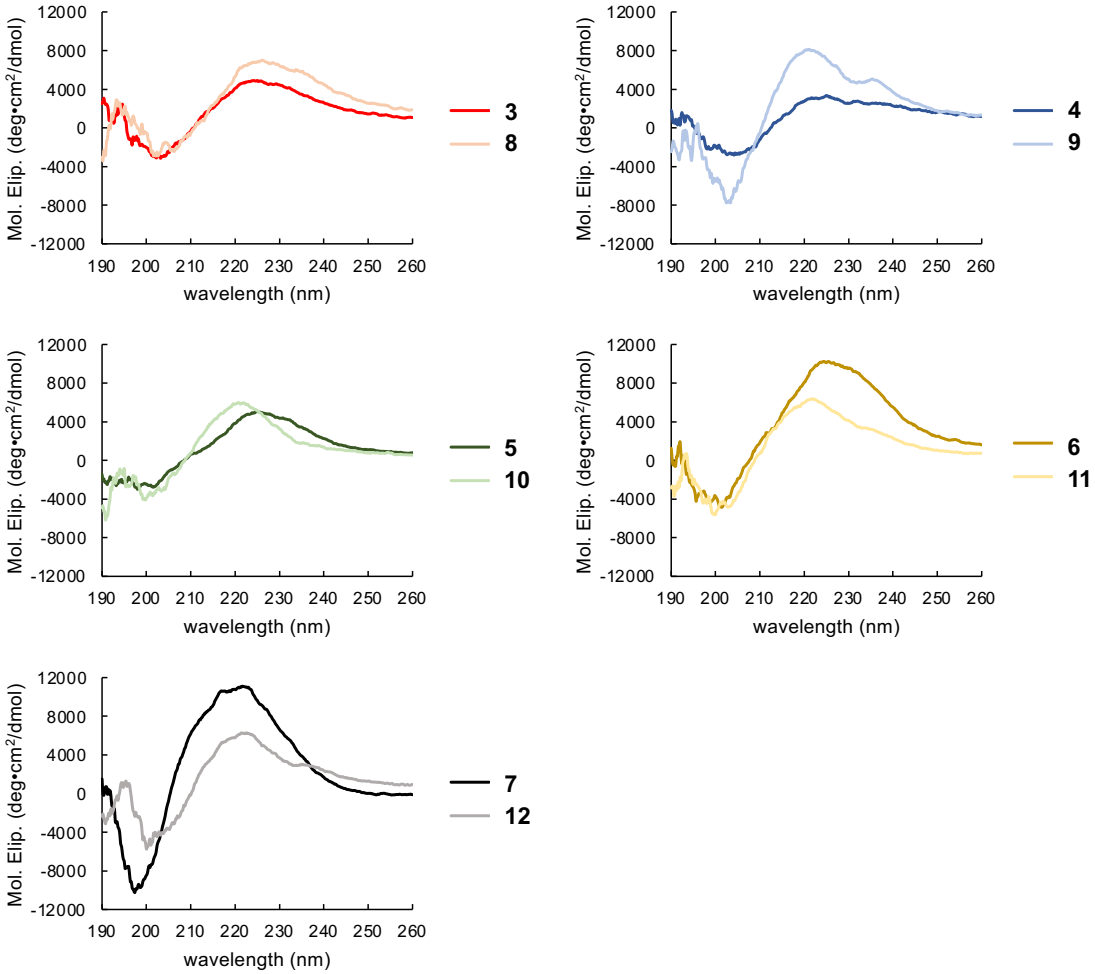


Fig. S2 Absorption spectra of functionalized D-peptides **3–7**. A phosphate-buffered solution (10 mM, pH 7.4, 1% DMSO) containing **3–7** (10 μM) was used for the measurements.

A



B

		functionalized peptide					peptide segment				
		3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
structural content (%)	α-helix	0	0	0	0	0	0	0	0	0	0
	β-strand	45	37	48	60	66	55	62	50	53	50
	turn	9	17	9	0	0	0	0	8	6	8
	random coil	46	46	43	40	34	45	38	41	40	42

Fig. S3 Secondary Structures of functionalized D-peptides **3–7** and peptide segments **8–12**. A) CD spectra and B) estimation of structural content (%) using Jasco secondary estimation software with Reed's reference set as reference spectra.[S8] A phosphate-buffered solution (10 mM, pH 7.4, 20% 2,2,2-trifluoroethanol) containing peptide (25 μM) was measured.

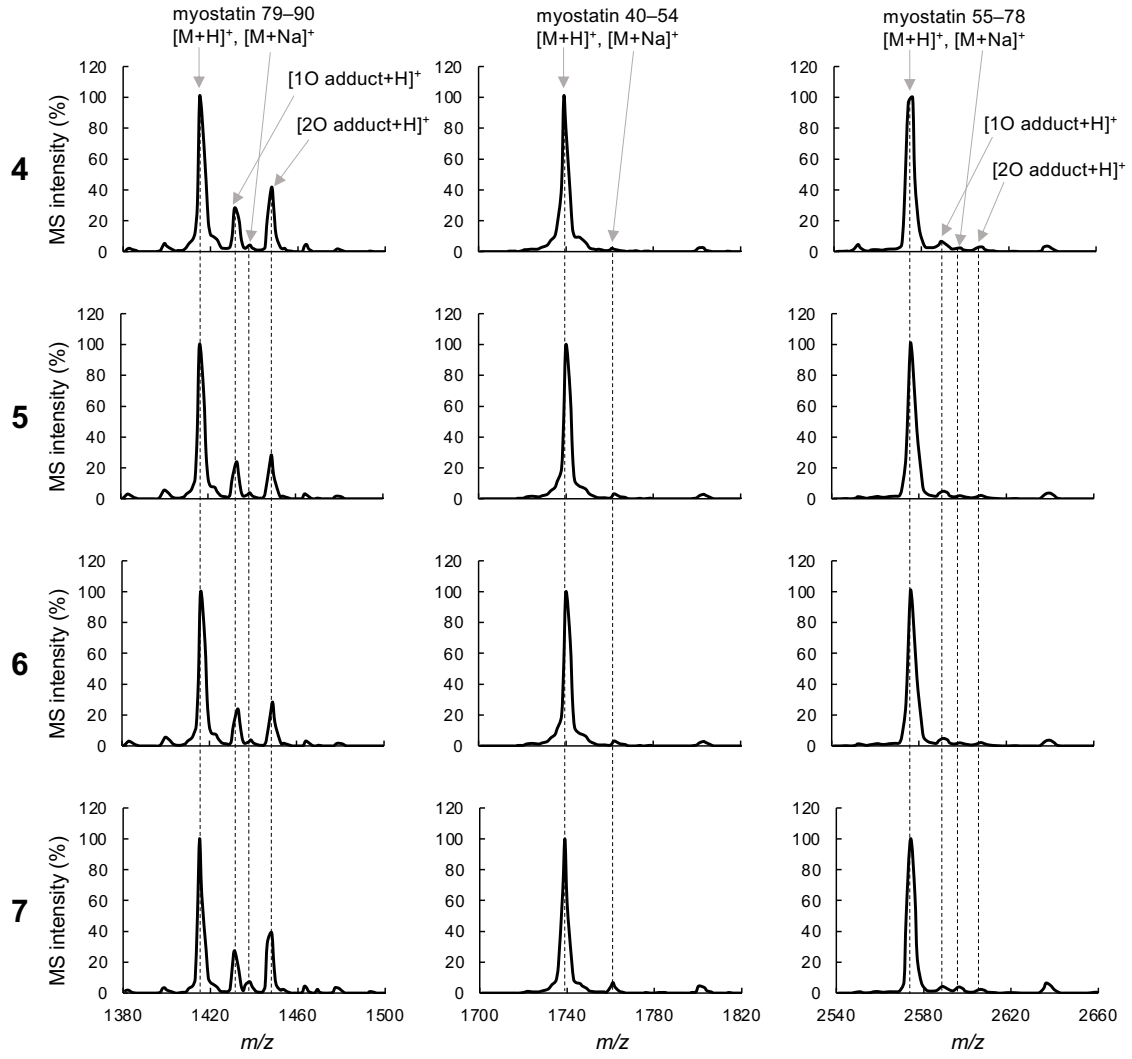


Fig. S4 Photooxygenation of myostatin using functionalized D-peptides **4–7**. MS spectra of myostatin fragments 79–90, 40–54 and 55–78 which were obtained from myostatin after oxygenation. A phosphate-buffered solution (10 mM, pH 7.4) containing myostatin (1 μM) and **4–7** (3 μM) was irradiated (λ_{max} = 730 nm) for 30 min, and then analyzed by MALDI-TOF MS following fragmentation of the myostatin.

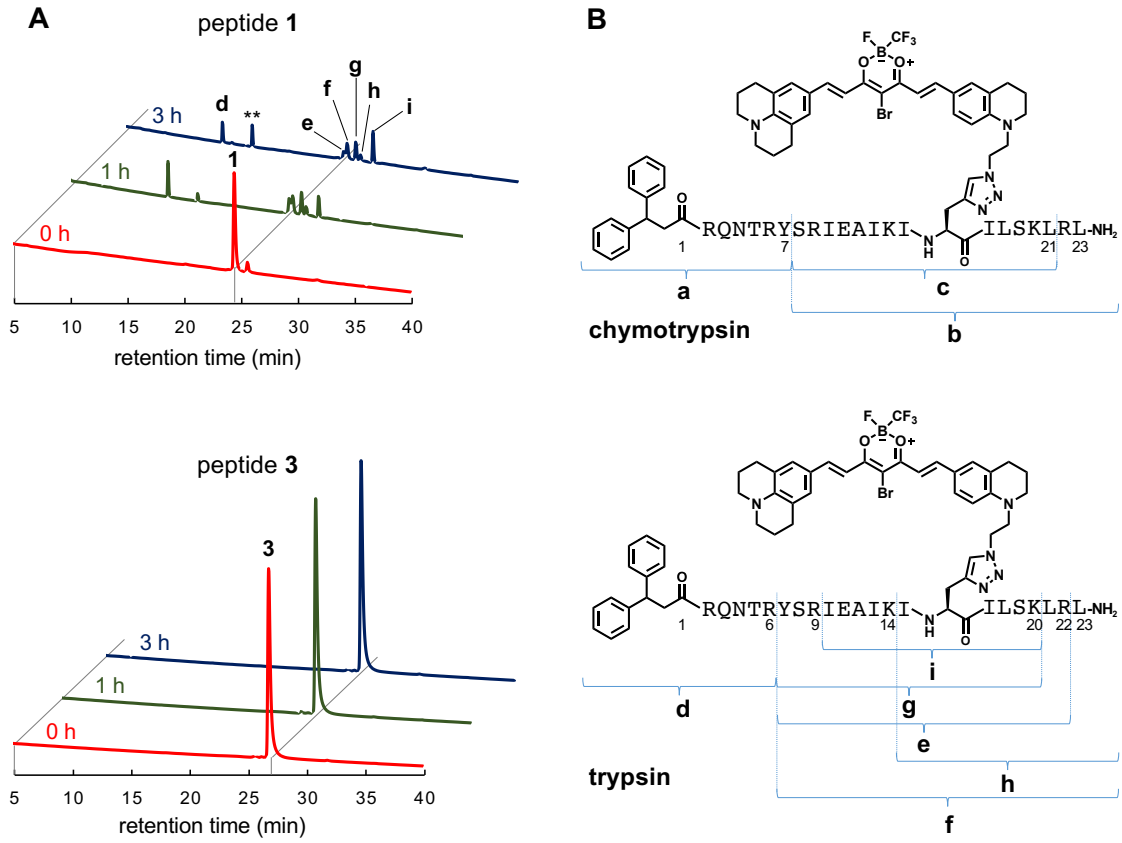


Fig. S5 Stabilities of peptides **1** and **3**. A) HPLC chromatograms of **1** and **3** treated with trypsin. Although the peak of **1** overlapped with that of **f**, the residual ratio of **1** was less than 18% after 1 h treatment with trypsin. B) The identified products by the digestion of **1** by chymotrypsin (upper, **a–c** in Fig. 6A) or trypsin (lower, **d–i** in Fig. S5A). **: unidentified. A Tris-buffered solution (80 mM, pH 7.8) containing **1** or **3** (100 μM) and chymotrypsin or trypsin (5 μg/ml) was incubated at 37 ˚C, and analyzed by HPLC.