Supporting Information

Development of potent myostatin inhibitory peptides through hydrophobic residue-directed structural modification

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1. Materials

Reagents and solvents, which were used as recieved, were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), Watanabe Chemical Industries (Hiroshima, Japan), and Tokyo Chemical Industries (Tokyo, Japan). Sterile Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Nacalai Tesque (Kyoto, Japan) and Life Technologies (Carlsbad, CA), respectively. Sterile 100-mm dishes, 96-well clear-wall poly-D-Lys-coated plates and 96-well white-wall plates were purchased from BD Biosciences (Flanklin Lake, NJ), Thermo Fisher Scientific (Waltham, MA) and Corning (Cambridge, MA), respectively. Plasmids, FuGENE HD and Dual-Luciferase Reporter Assay System for cell-based assay were purchased from Promega (Madison, WI). Recombinant human/mouse/rat myostatin and mouse myostatin-derived recombinant prodomain protein were purchased from Merck Millipore (Billerica, MA) and R&D Systems (Minneapolis, MN), respectively.

2. Synthesis of Peptide Derivatives

Fmoc-amino acids/R-COOH (0.141 mmol) were sequentially coupled to a Fmoc-NH-SAL Resin (100 mg, 0.047 mmol) using the DIPCI (0.141 mmol)-HOBt (0.141 mmol) method. Coupling steps were performed for 2 h in DMF (1.0 mL) after removal of each Fmoc group with 20% piperidine-DMF (1.5 mL, 20 min) to obtain resin-bound peptide. Cleavage from the resins was achieved by treatment with TFA-*m*-cresol-thioanisole-EDT (4.0 mL, 40:1:1:1 v:v:v:v) for 150–180 min at room temperature, followed by preparative RP-HPLC purification in a 0.1% aqueous TFA-CH_3CN system to obtain peptide agonists as TFA salts. The purity of synthesized peptides was > 95% in RP-HPLC analysis using a C18 reverse-phase column [4.6 x 150 mm; Waters SunFire C18 5μm] with a binary solvent system: a linear gradient of CH_3CN (20-40%, 40 min) in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, detected at UV 230 nm. Yields of all products obtained as a white powder were calculated as TFA salts. HR-MS (TOF MS ES+) was recorded on a micromass LCT. Analytical data of synthetic peptide derivatives are shown below.

I(30,33)L: Yield of 23%; HRMS *m*/*z* $[M+H]^+$ found 2884.7117 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 100.0% (t_R = 19.47 min).

I(33,35)L: Yield of 27%; HRMS *m*/*z* $[M+H]^+$ found 2884.7070 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 97.7% (t_R = 17.70 min).

I(35,37)L: Yield of 27%; HRMS *m*/*z* $[M+H]^+$ found 2884.7090 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 97.7% (t_R = 18.71 min).

L(38,41)I: Yield of 11%; HRMS *m*/*z* $[M+H]^+$ found 2884.7214 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.3% (t_R = 17.07 min).

L(41,43)I: Yield of 7.5%; HRMS *m*/*z* $[M+H]^+$ found 2884.7200 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.8% (t_R = 17.67 min).

L38I: Yield of 14%; HRMS *m*/*z* $[M+H]^+$ found 2884.7158 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 100.0% (t_R = 18.20 min).

L41I: Yield of 17%; HRMS *m/z* $[M+H]^+$ found 2884.7124 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.8% (t_R = 18.18 min).

L43I: Yield of 13%; HRMS *m/z* $[M+H]^+$ found 2884.7166 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 100.0% (t_R = 18.59 min).

A32V: Yield of 14%; HRMS *m/z* $[M+H]^+$ found 2912.7471 (calcd. for $C_{132}H_{227}N_{42}O_{32}$ 2912.7427); HPLC purity 98.6% (t_R = 18.44 min).

A32L: Yield of 28%; HRMS *m/z* $[M+H]^+$ found 2926.7581 (calcd. for $C_{133}H_{229}N_{42}O_{32}$ 2926.7583); HPLC purity 97.5% (t_R = 19.24 min).

A32M: Yield of 26%; HRMS *m/z* $[M+H]^+$ found 2944.7114 (calcd. for $C_{132}H_{227}N_{42}O_{32}S$ 2944.7147); HPLC purity 100.0% (t_R = 18.50 min).

A32F: Yield of 23%; HRMS *m/z* $[M+H]^+$ found 2960.7427 (calcd. for $C_{136}H_{227}N_{42}O_{32}$ 2960.7400); HPLC purity 99.5% (t_R = 19.22 min).

A32W: Yield of 29%; HRMS *m/z* $[M+H]^+$ found 2999.7593 (calcd. for $C_{138}H_{228}N_{43}O_{32}$ 2999.7536); HPLC purity 100.0% (t_R = 20.23 min).

A32Y: Yield of 21%; HRMS *m/z* $[M+H]^+$ found 2976.7376 (calcd. for $C_{136}H_{227}N_{42}O_{33}$ 2976.7417); HPLC purity 98.9% (t_R = 18.90 min).

A32H: Yield of 20%; HRMS *m/z* $[M+H]^+$ found 2950.7312 (calcd. for $C_{133}H_{225}N_{44}O_{32}$ 2950.7332); HPLC purity 98.5% (t_R = 15.72 min).

A32K: Yield of 26%; HRMS *m/z* $[M+4H]^{4+}/4$ found 736.2019 (calcd. for $C_{133}H_{233}N_{43}O_{32}$ 736.1982); HPLC purity 100.0% (t_R = 19.75 min).

A32R: Yield of 23%; HRMS *m/z* $[M+4H]^{4+}/4$ found 743.1982 (calcd. for $C_{133}H_{233}N_{45}O_{32}$ 743.1997); HPLC purity 100.0% (t_R = 18.06 min).

A32E: Yield of 27%; HRMS *m/z* $[M+4H]^{4+}/4$ found 736.6876 (calcd. for $C_{132}H_{228}N_{42}O_{34}$ 736.4351); HPLC purity 100.0% (t_R = 19.59 min).

A32Q: Yield of 25%; HRMS *m/z* $[M+4H]^{4+}/4$ found 736.1920 (calcd. for $C_{132}H_{229}N_{43}O_{33}$ 736.1891); HPLC purity 100.0% (t_R = 19.84 min).

3a: Yield of 25%; HRMS *m/z* $[M+4H]^{4+}/4$ found 750.7043 (calcd. for $C_{138}H_{231}N_{43}O_{32}$ 750.6942); HPLC purity 100.0% (t_R = 25.26 min).

3b: Yield of 32%; HRMS *m/z* $[M+4H]^{4+}/4$ found 721.4141 (calcd. for $C_{131}H_{224}N_{40}O_{33}$ 721.4270); HPLC purity 100.0% (t_R = 26.94 min).

3c: Yield of 39%; HRMS *m/z* $[M+4H]^{4+}/4$ found 750.1732 (calcd. for $C_{139}H_{229}N_{41}O_{33}$ 750.1875); HPLC purity 96.8% (t_R = 29.15 min).

3d: Yield of 29%; HRMS *m/z* $[M+4H]^{4+}/4$ found 750.1732 (calcd. for $C_{139}H_{229}N_{41}O_{33}$ 750.1875; HPLC purity 100.0% (t_R = 28.63 min).

L38F: Yield of 34%; HRMS *m/z* $[M+4H]^{4+}/4$ found 730.4254 (calcd. for $C_{133}H_{224}N_{42}O_{32}$ 730.4298); HPLC purity 99.1% (t_R = 19.60 min).

L38W: Yield of 35%; HRMS *m/z* $[M+4H]^{4+}/4$ found 740.1840 (calcd. for $C_{135}H_{225}N_{43}O_{32}$ 740.1825); HPLC purity 99.4% (t_R = 20.51 min).

I30V: Yield of 12%; HRMS *m/z* $[M+H]^+$ found 2870.6912 (calcd. for $C_{129}H_{221}N_{42}O_{32}$ 2870.6957); HPLC purity 99.1% (t_R = 17.35 min).

I33V: Yield of 17%; HRMS *m/z* $[M+H]^+$ found 2870.6987 (calcd. for $C_{129}H_{221}N_{42}O_{32}$ 2870.6957); HPLC purity 98.3% (t_R = 17.43 min).

I35V: Yield of 15%; HRMS *m/z* $[M+H]^+$ found 2870.6982 (calcd. for $C_{129}H_{221}N_{42}O_{32}$ 2870.6957); HPLC purity 98.6% (t_R = 18.87 min).

I37V: Yield of 15%; HRMS *m/z* $[M+4H]^{4+}/4$ found 718.4222 (calcd. for $C_{129}H_{224}N_{42}O_{32}$ 718.4298); HPLC purity 99.1% (t_R = 17.98 min).

I30F: Yield of 18%; HRMS *m/z* $[M+H]^+$ found 2918.6953 (calcd. for $C_{133}H_{221}N_{42}O_{32}$ 2918.6957); HPLC purity 99.0% (t_R = 19.07 min).

I33F: Yield of 33%; HRMS *m/z* $[M+H]^+$ found 2918.6973 (calcd. for $C_{133}H_{221}N_{42}O_{32}$ 2918.6957); HPLC purity 100.0% (t_R = 18.87 min).

I35F: Yield of 40%; HRMS *m/z* $[M+H]^+$ found 2918.6968 (calcd. for $C_{133}H_{221}N_{42}O_{32}$ 2918.6957); HPLC purity 96.0% (t_R = 19.02 min).

I37F: Yield of 21%; HRMS *m/z* $[M+H]^+$ found 2918.6978 (calcd. for $C_{133}H_{221}N_{42}O_{32}$ 2918.6957); HPLC purity 100.0% (t_R = 17.78 min).

3. Cell-based assay

HEK293 cells were subcultured in DMEM containing 10% FBS and nonessential amino acids. The cells were seeded at 2.0×10^4 cells per well in the 96-well plates the day before transfection of reporter (pGL4.48[luc2P/SBE/Hygro]) and control (pGL4.74[hRluc/TK]) vectors using FuGENE HD. After 24 h of transfection, the medium was exchanged to serum-free DMEM and the cells were incubated for 8 h at 37 °C under 5% CO_2. Each synthesized peptide was dissolved with H_2O, diluted by adding DMEM containing recombinant human/mouse/rat myostatin [final concentration; 8 ng/mL (0.32 nM)], and incubated for 20 min. Cells were treated with a peptide solution and incubated at 37 °C under 5% CO_2. After 4 h, cells were washed with PBS. The lysates were prepared, and the luciferase activities were measured using a Dual-Luciferase Reporter Assay System according to manufacturer's protocol (Promega). Mouse myostatin-derived recombinant propeptide (prodomain) was used as a positive control and underwent the same manipulation (final concentration, 10 nM). Each experiment was carried out in triplicate. Values represent means ± SD (n= 3). Non-linear regressions are performed using GraphPad Prism software, using the integrated log(inhibitor) vs. response – Variable slope (four parameters). Data was normalized based on negative and positive controls using first and last data points of each series as 0 and 100% plateaus, and outliers (ROUT coefficient of 1% was

used to discriminate legitimate data points from outliers) were not included in curve fitting calculations. All the curve fitting parameters details can be found in Table S1.

4. Measurement of the circular dichroic (CD) spectra

CD spectra of peptides **1** and **3d** were obtained at 25 ^{o}C using a Jasco J-1500 CD spectrometer (JASCO, Japan) in a quartz cell with a 0.5-cm path length. Spectra were collected between 190–250 nm with a scan speed of 100 nm/min, a response time of 1 s, and a bandwidth of 1 nm. Peptide samples with a final concentration of 5 μM were prepared in 20 mM sodium phosphate buffer (pH 7.4) containing 10% 2,2,2-trifluoroethanol. The baseline scan, which was acquired by measuring the buffer alone, was subtracted from the experimental readings. CD data, which were collected every 0.1 nm, were the average of nine scans. The normalized CD data was expressed in the mean residue ellipticity (deg cm2 dmol-1) and plotted as functions of wavelength.

5. Intramuscular administration of peptide 3d

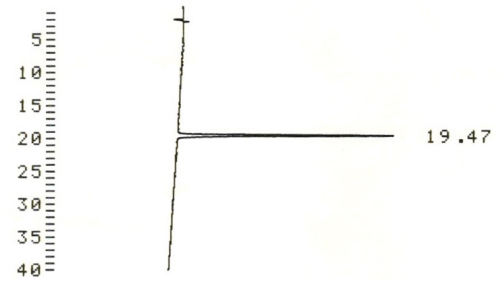
Animal studies were approved by the Animal Research Committee of Tokyo University of Pharmacy and Life Sciences. Forty microliter of the peptide solution (0.75 mM peptide **3d** in saline) and saline (control) were intramuscularly injected into left and right tibialis anterior or gastrocnemius muscle of 5-week-old *mdx* or ICR mice, respectively. After two weeks, the treatment was repeated for the same muscle. Then four weeks after the last treatment, the muscles were collected and weighed.

6. Histological analysis

The treated GAS muscles were dissected 28 days after the 2nd injection of **3d** or saline at day 14. The frozen tissue sections were prepared transversely (6 μm) using a cryostat. Each section was stained with hematoxylin and eosin and fiber sizes were determined by measuring the area of each myofiber in a fixed area. Two hundred cross-sectioned myofibers were randomly selected from 3 fields of tissue sections from each tissue sample.

Analytical HPLC chromatograms

I(30,33)L:

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D-2500

METHOD:                TAG:  1033  CH: 1

FILE: 0  CALC-METHOD: AREA%    TABLE:   0  CONC: AREA

  NO.      RT         AREA       CONC  BC
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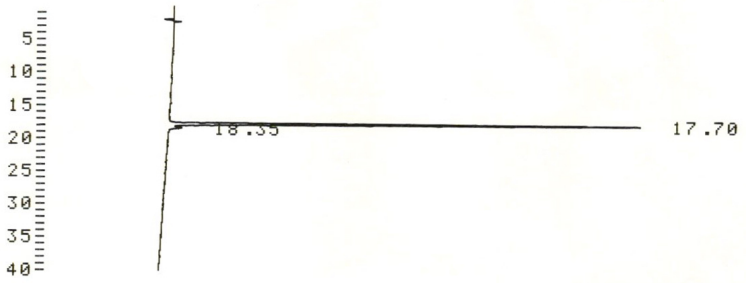
TOTAL
                   1958411    100.000
PEAK REJ :        10000
```

I(33,35)L:

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D-2500

METHOD:                TAG:  1034  CH: 1

FILE: 0  CALC-METHOD: AREA%    TABLE:   0  CONC: AREA

  NO.      RT         AREA       CONC  BC
    1   17.70      3566123     97.732  BV
    2   18.35        82742      2.268 TBB
TOTAL
                   3648865    100.000
PEAK REJ :        10000
```
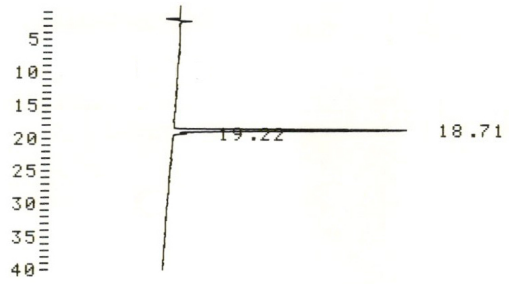

I(35,37)L:



D-2500

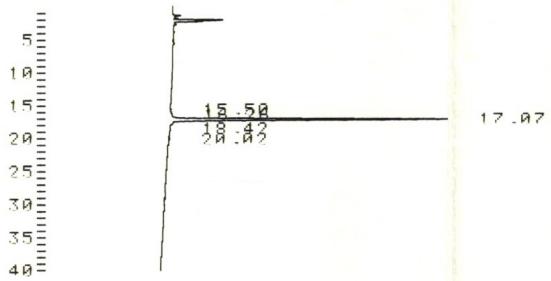
METHOD: TAG: 1036 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.71	1962018	97.668	BV
2	19.22	46844	2.332	TBB
TOTAL		2008862	100.000	

PEAK REJ : 10000

L(38,41)I:



D-2500

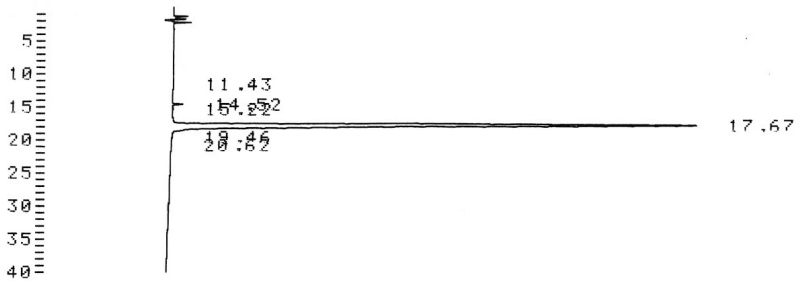
METHOD: TAG: 76 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
5	16.26	35959	1.297	VV
6	17.07	2723655	98.250	VV
7	18.42	12553	0.453	TBB
TOTAL		2772167	100.000	

PEAK REJ : 10000

L(41,43)I:



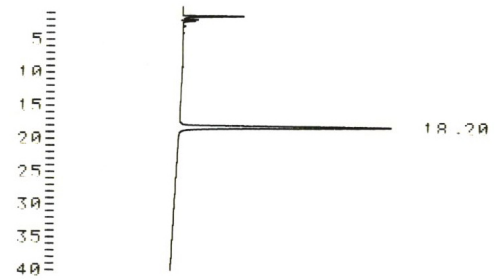
D-2500

METHOD: TAG: 352 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	11.43	10199	0.058	BB
2	14.52	149527	0.849	BV
3	15.22	27688	0.157	VV
4	17.67	17400985	98.835	VV
5	19.46	17677	0.100	TBB
TOTAL		17606076	100.000	
PEAK REJ :		10000		

L38I:



D-2500

METHOD: TAG: 184 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
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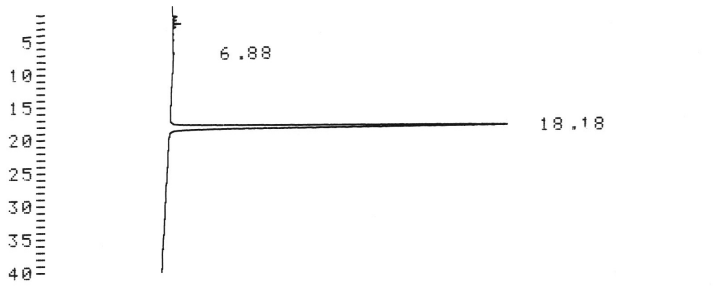
TOTAL		2918253	100.000	
PEAK REJ :		10000		

L41I:



D-2500

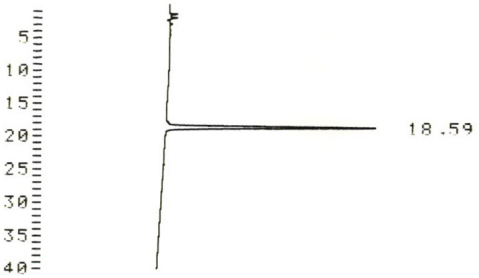
METHOD: TAG: 187 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	6.88	11349	0.243	BB
2	18.18	4653264	99.757	BB
TOTAL		4664613	100.000	

PEAK REJ : 10000

L43I:



D-2500

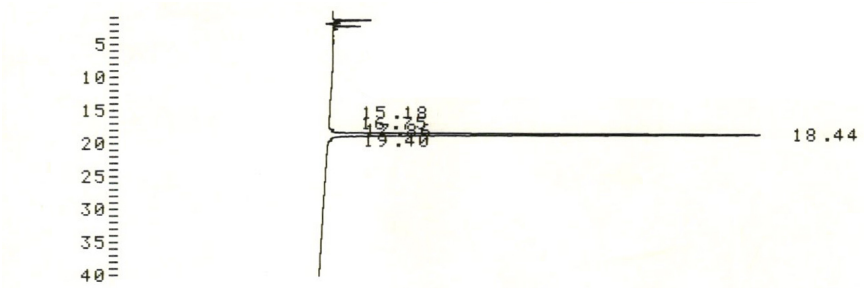
METHOD: TAG: 188 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.59	2735800	100.000	BB
TOTAL		2735800	100.000	

PEAK REJ : 10000

A32V:



D-2500

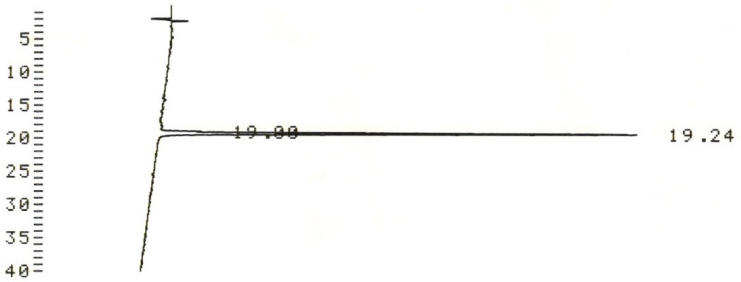
METHOD: TAG: 387 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
5	15.18	12612	0.284	VV
7	17.86	49324	1.112	VV
8	18.44	4372155	98.603	VV
TOTAL		4434091	100.000	

PEAK REJ : 10000

A32L:



D-2500

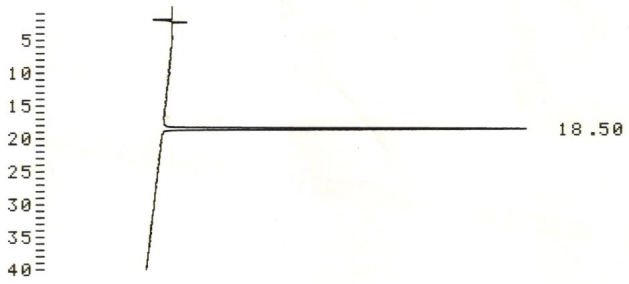
METHOD: TAG: 11 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	19.00	45018	2.459	BV
2	19.24	1785421	97.541	VB
TOTAL		1830439	100.000	

PEAK REJ : 10000

A32M:



D-2500

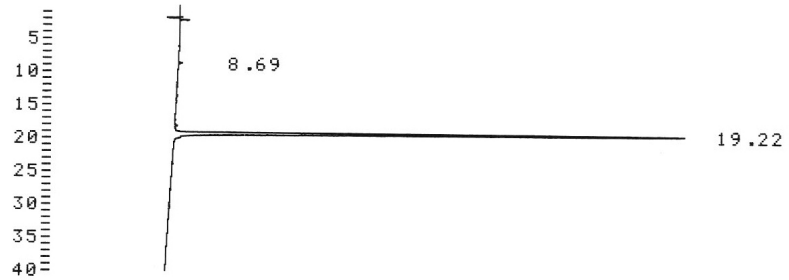
METHOD: TAG: 1194 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.50	1588924	100.000	BB
TOTAL				
		1588924	100.000	

PEAK REJ : 10000

A32F:



D-2500

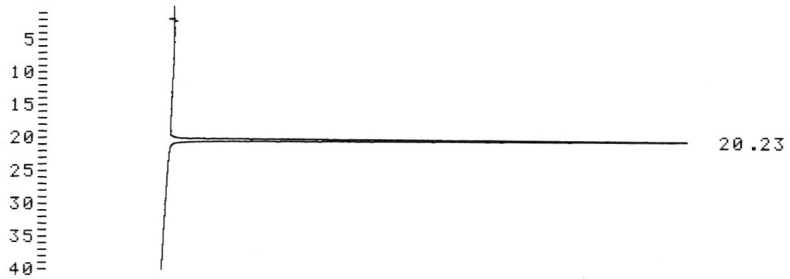
METHOD: TAG: 636 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	8.69	23918	0.491	BB
2	19.22	4850562	99.509	BB
TOTAL				
		4874480	100.000	

PEAK REJ : 10000

A32W:



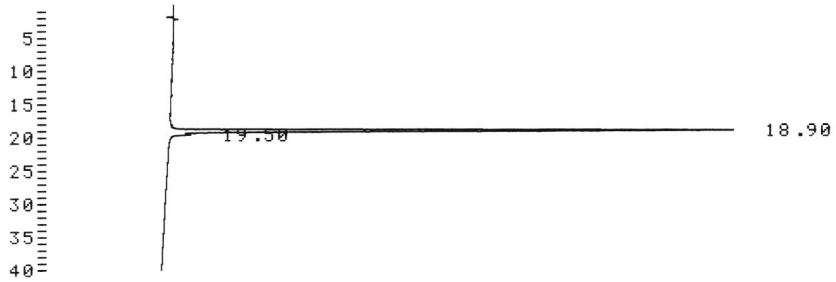
D-2500

METHOD: TAG: 639 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	20.23	4599092	100.000	BB
TOTAL				
		4599092	100.000	
PEAK REJ :		10000		

A32Y:



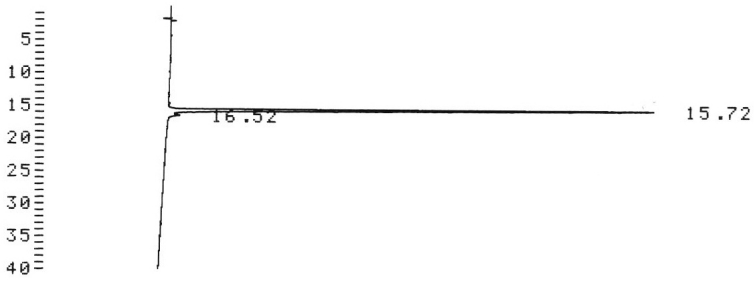
D-2500

METHOD: TAG: 637 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.90	5812614	98.905	BV
2	19.50	64349	1.095	TBB
TOTAL				
		5876963	100.000	
PEAK REJ :		10000		

A32H:



D-2500

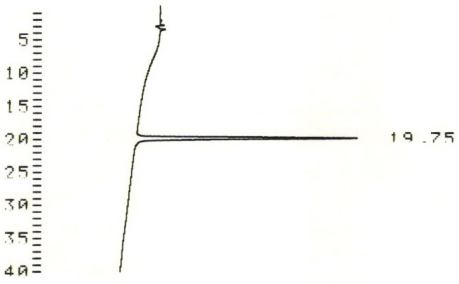
METHOD: TAG: 638 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	15.72	4548524	98.495	BV
2	16.52	69480	1.505	TBB
TOTAL		4618004	100.000	

PEAK REJ : 10000

A32K:



D-2500

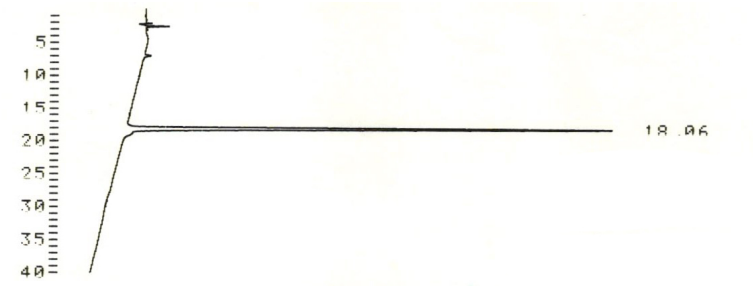
METHOD: TAG: 618 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	19.75	1424655	100.000	BB
TOTAL		1424655	100.000	

PEAK REJ : 10000

A32R:



D-2500

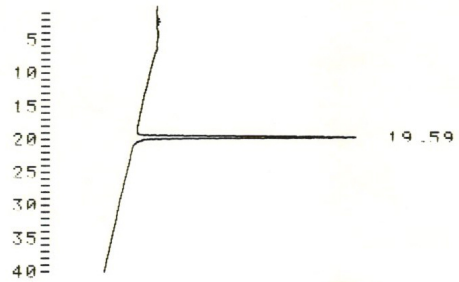
METHOD: TAG: 637 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.06	1452292	100.000	BB
TOTAL		1452292	100.000	

PEAK REJ : 10000

A32E:



D-2500

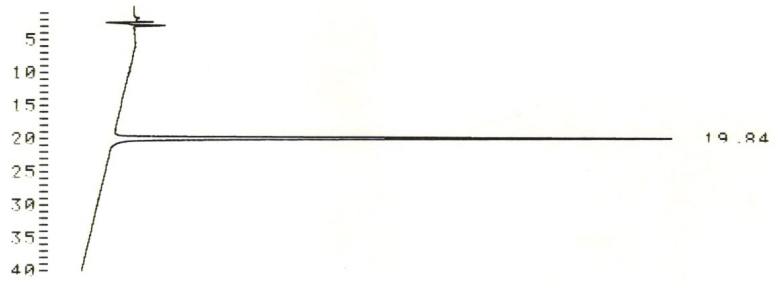
METHOD: TAG: 621 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	19.59	666646	100.000	BB
TOTAL		666646	100.000	

PEAK REJ : 10000

A32Q:



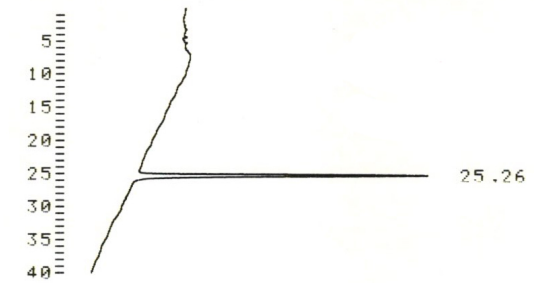

```
D-2500

METHOD:                TAG:    626  CH: 1

FILE: 0  CALC-METHOD: AREA%     TABLE:    0  CONC: AREA

  NO.      RT         AREA       CONC  BC
    1   19.84      1732657    100.000  BB
TOTAL
                   1732657    100.000
PEAK REJ :        10000
```

3a:



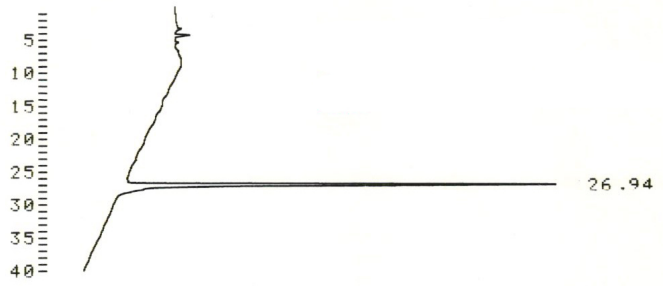

```
D-2500

METHOD:                TAG:      4  CH: 1

FILE: 0  CALC-METHOD: AREA%     TABLE:    0  CONC: AREA

  NO.      RT         AREA       CONC  BC
    1   25.26       471954    100.000  BB
TOTAL
                    471954    100.000
PEAK REJ :        10000
```

3b:



D-2500

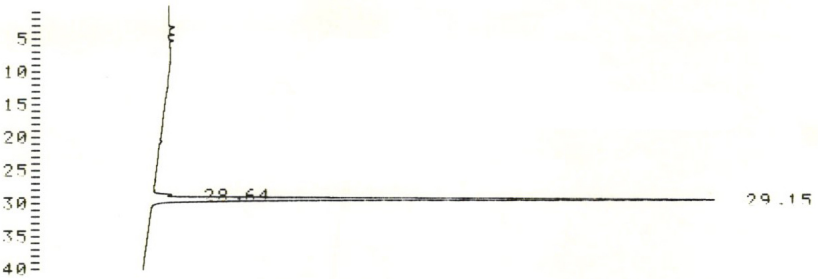
METHOD: TAG: 7 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	26.94	730808	100.000	BB
TOTAL		730808	100.000	

PEAK REJ : 10000

3c:



D-2500

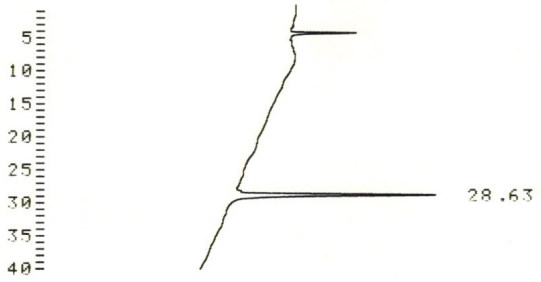
METHOD: TAG: 12 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	28.64	126204	3.177	BV
3	29.15	3846398	96.823	VB
TOTAL		3972602	100.000	

PEAK REJ : 10000

3d:



D-2500

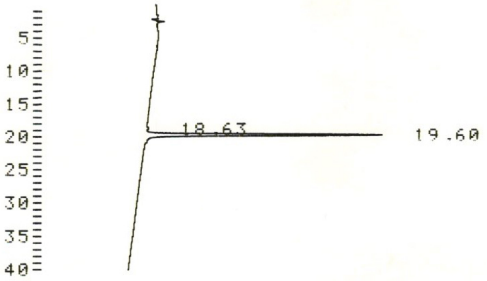
METHOD: TAG: 18 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	28.63	376544	100.000	BB
TOTAL		376544	100.000	

PEAK REJ : 10000

L38F:



D-2500

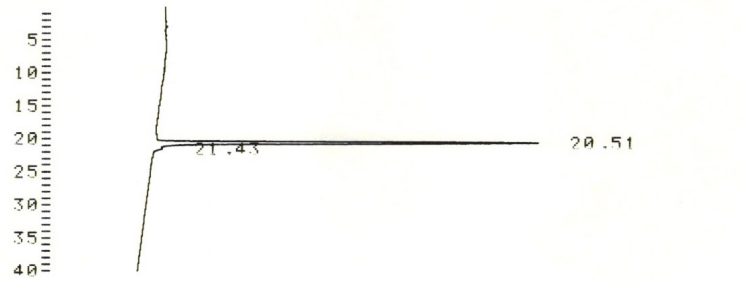
METHOD: TAG: 603 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	18.63	13839	0.949	BV
2	19.60	1443886	99.051	VB
TOTAL		1457725	100.000	

PEAK REJ : 10000

L38W:



D-2500

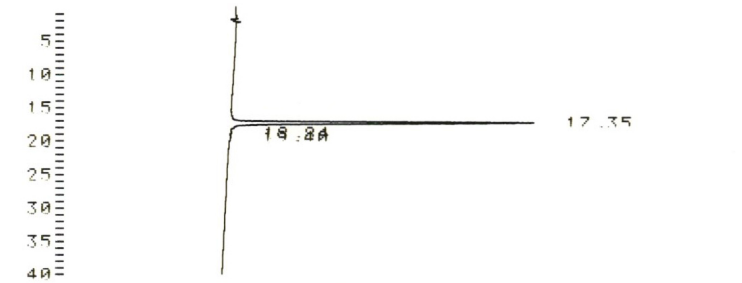
METHOD: TAG: 604 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	20.51	2494714	99.385	BV
2	21.43	15431	0.615	TBB
TOTAL		2510145	100.000	

PEAK REJ : 10000

I30V:



D-2500

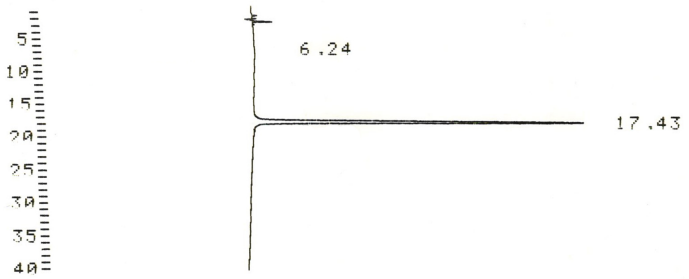
METHOD: TAG: 184 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	17.35	3517792	99.135	BV
3	18.84	14054	0.396	TBB
4	19.40	16650	0.469	TBB
TOTAL		3548496	100.000	

PEAK REJ : 10000

I33V:



D-2500

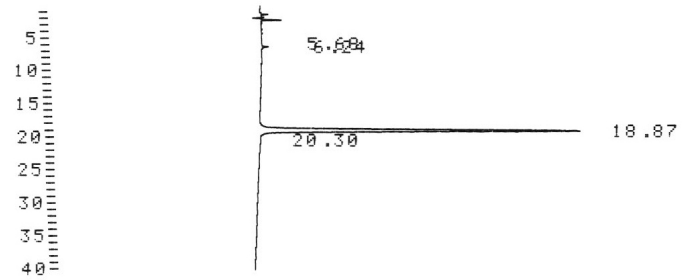
METHOD: TAG: 136 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	6.24	72098	1.673	BV
2	17.43	4236269	98.327	VV
TOTAL		4308367	100.000	

PEAK RET : 10000

I35V:



D-2500

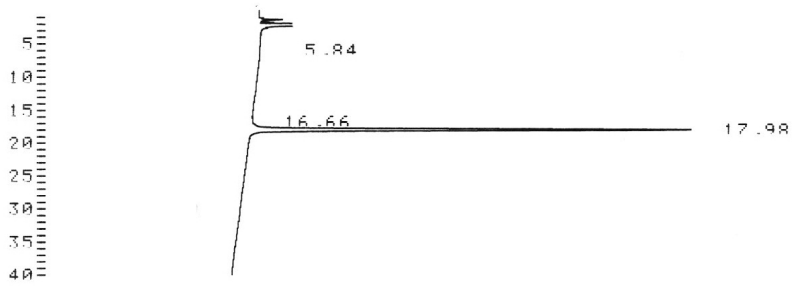
METHOD: TAG: 135 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	6.24	47950	1.191	BB
3	18.87	3969178	98.555	BV
4	20.30	10226	0.254	TBB
TOTAL		4027354	100.000	

PEAK RET : 10000

I37V:



D-2500

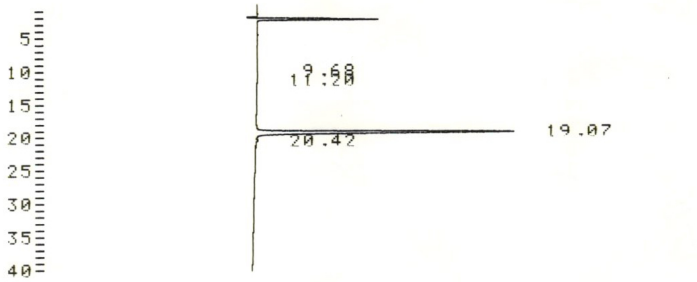
METHOD: TAG: 129 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	5.84	26659	0.947	BB
3	17.98	2788903	99.053	VB
TOTAL		2815562	100.000	

PEAK REJ : 10000

I30F:



D-2500

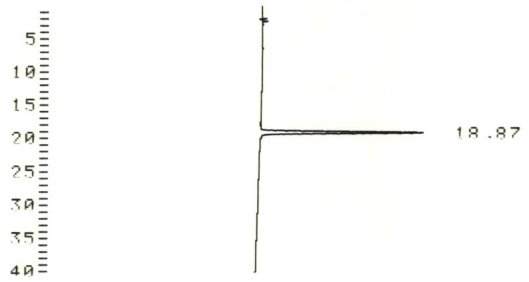
METHOD: TAG: 1032 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
5	11.20	38727	0.546	VV
6	19.07	7023679	98.981	VV
7	20.42	33578	0.473	TBB
TOTAL		7095984	100.000	

PEAK REJ : 10000

I33F:



D-2500

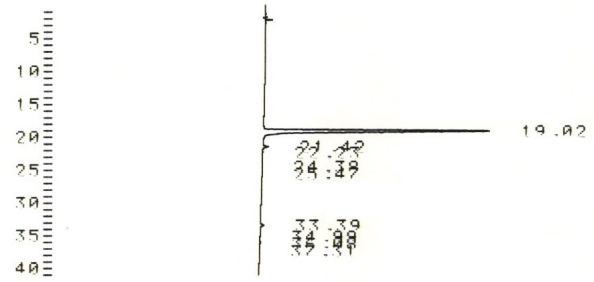
METHOD: TAG: 1003 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	18.87	3932032	100.000	BV
TOTAL				
		3932032	100.000	

PEAK REJ : 10000

I35F:



D-2500

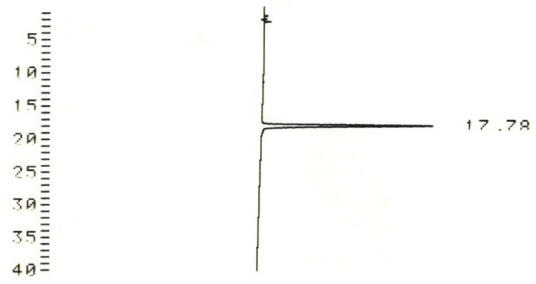
METHOD: TAG: 1002 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
1	19.02	5861079	96.023	BV
2	21.42	108257	1.774	TBB
5	25.47	22456	0.368	VV
10	33.39	81176	1.330	BV
12	36.08	14398	0.236	VV
13	37.31	16460	0.270	VB
TOTAL				
		6103826	100.000	

PEAK REJ : 10000

I37F:



D-2500

METHOD: TAG: 995 CH: 1

FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
4	17.78	4015991	100.000	BB
TOTAL				
		4015991	100.000	

PEAK REJ : 10000

Table S1. GraphPad Prism parameters obtained for the 4-parametric non-linear regression of dose-response curves of peptides **1** and **3d**.

	Peptide **1**	Peptide **3d**
Best-fit values		
Bottom	-0.1120	-0.9718
Top	96.86	104.2
$LogIC_{50}$	-5.448	-6.492
HillSlope	-3.721	-1.866
IC_{50}	3.562e-006	3.220e-007
Span	96.97	105.2
Std. Error		
Bottom	1.915	2.777
Top	1.910	3.689
$LogIC_{50}$	0.01569	0.03293
HillSlope	0.3512	0.2513
Span	2.838	5.271
95% Confidence Intervals		
Bottom	-4.221 to 3.997	-6.765 to 4.821
Top	92.76 to 101.0	96.48 to 111.9
$LogIC_{50}$	-5.482 to -5.415	-6.561 to -6.423
HillSlope	-4.475 to -2.968	-2.390 to -1.342
IC_{50}	3.297e-006 to 3.849e-006	2.749e-007 to 3.772e-007
Span	90.88 to 103.1	94.16 to 116.1
Goodness of Fit		
Degrees of Freedom	14	20
R square	0.9924	0.9826
Absolute Sum of Squares	241.4	711.5
Sy.x	4.153	5.964

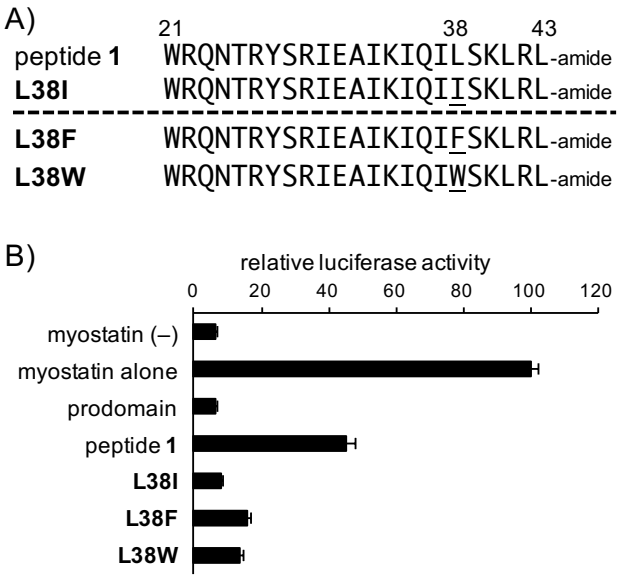


Figure S1. (A) Structures of the Leu-substituted peptides at position 38. The numbers above each amino acid indicate its position in the prodomain sequence of mouse myostatin. (B) The luciferase reporter assay determined the activities of the Leu-substituted peptides toward myostatin inhibition relative to peptide **1**. Peptide concentration: 3 μM. Results are presented as mean values ± SD (n = 3).

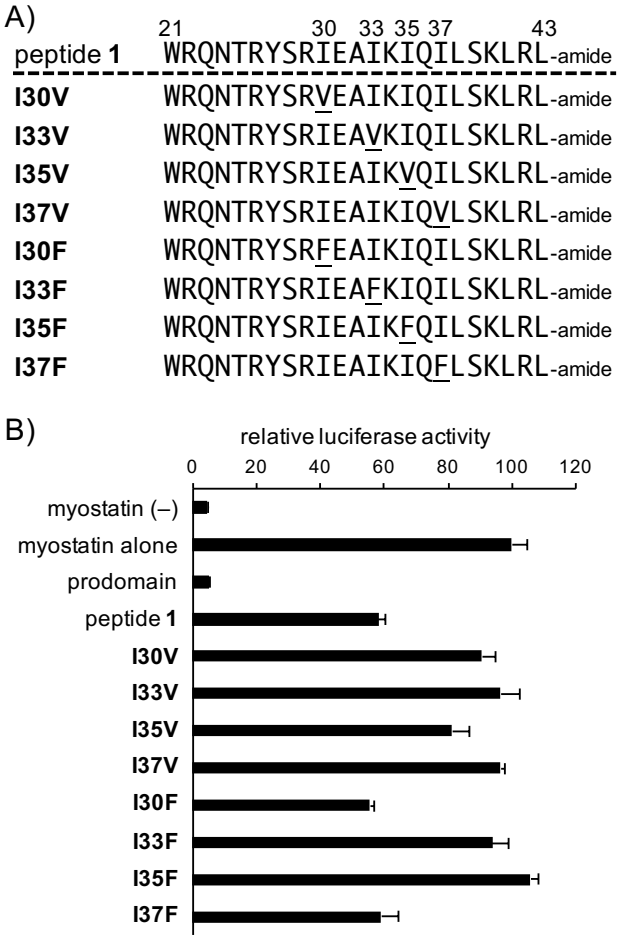


Figure S2. (A) Structures of the Ile-substituted peptides. The numbers above each amino acid indicate its position in the prodomain sequence of mouse myostatin. (B) The luciferase reporter assay determined the activities of the Ile-substituted peptides toward myostatin inhibition relative to peptide **1**. Peptide concentration: 3 μM. Results are presented as mean values ± SD (n = 3).

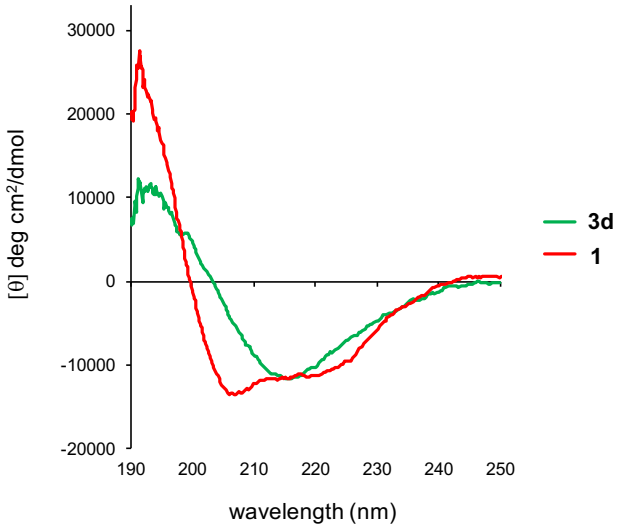


Figure S3. CD spectra of peptides **1** and **3d** in 20 mM sodium phosphate buffer (pH 7.4) containing 10% 2,2,2-trifluoroethanol; peptide concentration, 5 μM.

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