Supporting Information

Chain-shortened myostatin inhibitory peptides improve grip strength in mice

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1. Materials

Reagents and solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan), and Sigma-Aldrich (St. Louis, MO), Watanabe Chemical Industries (Hiroshima, Japan), and Tokyo Chemical Industries (Tokyo, Japan). All were used as received. Sterile Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Nacalai Tesque (Kyoto, Japan) and Nichirei Bioscience Inc. (Tokyo, Japan), 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 (Franklin Lakes, 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

In the synthesis of all peptides, protected peptide-bound resins were automatically prepared using Prelude (Gyros Protein Technologies, Inc., Tucson, AZ). Fmoc-NH-SAL resin (54 mg, 0.02 mmol) and Fmoc-amino acids (0.2 mmol) were sequentially coupled using the *O*-(7-Aza-1*H*-benzotriazol-1-yl)-*N,N,N′,N′*-tetra-methyluronium hexafluorophosphate (HATU, 0.2 mmol)-1-hydroxy-7-azabenzotriazole (HOAt, 0.2 mmol)-*N,N*-diisopropylethylamine (DIEA) method for 30 min in *N,N*-dimethylformamide (DMF, 2.0 mL) after removing Fmoc group with 20% piperidine-DMF (2.5 mL, 20 min) in each step. The resins were treated with TFA-*m*-cresol-thioanisole-EDT (4.3 mL, 40:1:1:1, v:v:v:v) for 150 min at rt to obtain crude peptides. Purified peptides as a TFA salt were obtained by preparative RP-HPLC in a 0.1% aqueous TFA-CH_3CN system, and solubilized in DMSO as 3 mM stock solutions for the reporter assay. The purity of synthesized peptides was >95% in RP-HPLC analysis using a C18 reverse-phase column [4.6 x 150 mm; COSMOSIL 5C18-AR-II (column 1), COSMOSIL 5C4-AR-300 (column 2), or SunFire C18 5 μm (column 3)] with a binary solvent system: a linear gradient of CH_3CN (20-40%, 40 min; 30-50%, 40 min for peptides **6**, **7b**, **7d**, **7e**, **E31K**, and **E31R**; 5-65%, 30 min for peptide **4b**; 25-45%, 40 min for peptides **8a** and **8c**; 20-50%, 30 min for peptide **8b**) 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.

peptide **4a**: Yield of 18%; HRMS *m*/*z* $[M+H]^+$ found 1995.2606 (calcd. for $C_{93}H_{164}N_{27}O_{21}$ 1995.2595); HPLC purity 99.8% (t_R = 22.77 min) (column 1).

peptide **4b**: Yield of 29%; HRMS *m*/*z* $[M+H]^+$ found 2068.2524 (calcd. for $C_{98}H_{163}N_{28}O_{21}$ 2068.2548); HPLC purity 100% (t_R = 17.14 min) (column 2).

peptide **5**: Yield of 20%; HRMS *m*/*z* $[M+H]^+$ found 1986.2250 (calcd. for $C_{96}H_{161}N_{24}O_{21}$ 1986.2268); HPLC purity 100% (t_R = 23.90 min) (column 3).

peptide **6**: Yield of 13%; HRMS *m*/*z* $[M+3H]^{3+}/3$ found 725.4232 (calcd. for $C_{109}H_{164}N_{26}O_{21}$ 725.4266); HPLC purity 99.1% (t_R = 13.47 min) (column 3).

E31K: Yield of 16%; HRMS *m/z* $[M+H]^+$ found 2173.3159 (calcd. for $C_{110}H_{170}N_{27}O_{19}$ 2173.3166); HPLC purity 99.5% (t_R = 8.64 min) (column 1).

E31R: Yield of 12%; HRMS *m/z* $[M+H]^+$ found 2201.3240 (calcd. for $C_{110}H_{170}N_{29}O_{19}$ 2201.3228); HPLC purity 99.4% (t_R = 9.04 min) (column 1).

E31N: Yield of 5.2%; HRMS *m/z* $[M+H]^+$ found 2159.2668 (calcd. for $C_{108}H_{164}N_{27}O_{20}$ 2159.2646); HPLC purity 97.7% (t_R = 32.97 min) (column 1).

E31Q: Yield of 8.7%; HRMS *m/z* $[M+H]^+$ found 2173.2837 (calcd. for $C_{109}H_{166}N_{27}O_{20}$ 2173.2802); HPLC purity 96.9% (t_R = 34.16 min) (column 1).

peptide **7a**: Yield of 4.3%; HRMS *m/z* $[M+H]^+$ found 2227.3347 (calcd. for $C_{112}H_{172}N_{29}O_{19}$ 2227.3384); HPLC purity 98.5% (t_R = 30.81 min) (column 1).

peptide **7b**: Yield of 4.3%; HRMS *m/z* $[M+H]^+$ found 2227.3345 (calcd. for $C_{112}H_{172}N_{29}O_{19}$ 2227.3384); HPLC purity 100% (t_R = 10.25 min) (column 1).

peptide **7c**: Yield of 4.3%; HRMS *m/z* $[M+H]^+$ found 2253.3604 (calcd. for $C_{114}H_{174}N_{29}O_{19}$ 2253.3541); HPLC purity 98.5% (t_R = 30.81 min) (column 1).

peptide **7d**: Yield of 10%; HRMS *m/z* $[M+H]^+$ found 2235.3057 (calcd. for $C_{113}H_{168}N_{29}O_{19}$ 2235.3071); HPLC purity 99.7% (t_R = 10.03 min) (column 1).

peptide **7e**: Yield of 17%; HRMS *m/z* $[M+H]^+$ found 2235.3086 (calcd. for $C_{113}H_{168}N_{29}O_{19}$ 2235.3071); HPLC purity 100% (t_R = 9.01 min) (column 1).

peptide **8a**: Yield of 8.5%; HRMS *m/z* $[M+H]^+$ found 2253.3518 (calcd. for $C_{114}H_{174}N_{29}O_{19}$ 2253.3541); HPLC purity 95.4% (t_R = 23.26 min) (column 1).

peptide **8b**: Yield of 12%; HRMS *m/z* $[M+H]^+$ found 2253.3506 (calcd. for $C_{114}H_{174}N_{29}O_{19}$ 2253.3541); HPLC purity 97.7% (t_R = 18.26 min) (column 2).

peptide **8c**: Yield of 7.4%; HRMS *m/z* $[M+H]^+$ found 2253.3503 (calcd. for $C_{114}H_{174}N_{29}O_{19}$ 2253.3541); HPLC purity 98.8% (t_R = 21.79 min) (column 1).

I30Chg: Yield of 21%; HRMS *m/z* $[M+5H]^{5+}/5$ found 582.1447 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 582.1454); HPLC purity 100.0% (t_R = 21.16 min) (column 3).

I33Chg: Yield of 22%; HRMS *m/z* $[M+H]^+$ found 2910.7319 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 2910.7270); HPLC purity 97.6% (t_R = 19.86 min) (column 3).

I35Chg: Yield of 21%; HRMS *m/z* $[M+H]^+$ found 2910.7297 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 2910.7270); HPLC purity 99.2% (t_R = 18.84 min) (column 3).

I37Chg: Yield of 11%; HRMS *m/z* $[M+H]^+$ found 2910.7236 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 2910.7270); HPLC purity 97.9% (t_R = 20.56 min) (column 3).

I30Phg: Yield of 23%; HRMS *m/z* $[M+H]^+$ found 2904.6721 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 97.6% (t_R = 17.62 min) (column 3).

I33Phg: Yield of 17%; HRMS *m/z* $[M+H]^+$ found 2904.6746 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 96.3% (t_R = 17.22 min) (column 3).

I35Phg: Yield of 22%; HRMS *m/z* $[M+H]^+$ found 2904.6794 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 97.3% (t_R = 17.58 min) (column 3).

I37Phg: Yield of 11%; HRMS *m/z* $[M+H]^+$ found 2904.6709 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 100% (t_R = 15.70 min) (column 3).

L38Chg: Yield of 9.5%; HRMS *m/z* $[M+H]^+$ found 2910.7280 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 2910.7270); HPLC purity 96.4% (t_R = 19.68 min) (column 3).

L41Chg: Yield of 5.7%; HRMS *m/z* $[M+H]^+$ found 2910.7285 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 2910.7270); HPLC purity 98.3% (t_R = 20.35 min) (column 3).

L43Chg: Yield of 3.2%; HRMS *m/z* $[M+4H]^{4+}/4$ found 728.6867 (calcd. for $C_{132}H_{225}N_{42}O_{32}$ 728.6818); HPLC purity 99.0% (t_R = 20.80 min) (column 3).

L38Phg: Yield of 5.4%; HRMS *m/z* $[M+H]^+$ found 2904.6799 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 95.6% (t_R = 17.60 min) (column 3).

L41Phg: Yield of 2.5%; HRMS *m/z* $[M+H]^+$ found 2904.6768 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 98.0% (t_R = 17.06 min) (column 3).

L43Phg: Yield of 4.8%; HRMS *m/z* $[M+H]^+$ found 2904.6829 (calcd. for $C_{132}H_{219}N_{42}O_{32}$ 2904.6801); HPLC purity 98.2% (t_R = 19.07 min) (column 3).

S28s: Yield of 28%; HRMS *m/z* $[M+H]^+$ found 2884.7090 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.8% (t_R = 18.20 min) (column 3).

R29r: Yield of 21%; HRMS *m/z* $[M+H]^+$ found 2884.7119 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.8% (t_R = 18.35 min) (column 3).

I30i: Yield of 18%; HRMS *m/z* $[M+H]^+$ found 2884.7126 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.7% (t_R = 16.20 min) (column 3).

E31e: Yield of 21%; HRMS *m/z* $[M+H]^+$ found 2884.7109 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 97.2% (t_R = 17.95 min) (column 3).

A32a: Yield of 30%; HRMS *m/z* $[M+H]^+$ found 2884.7129 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.6% (t_R = 16.68 min) (column 3).

I33i: Yield of 26%; HRMS *m/z* $[M+H]^+$ found 2884.7122 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.1% (t_R = 15.14 min) (column 3).

K34k: Yield of 27%; HRMS *m/z* $[M+H]^+$ found 2884.7085 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.4% (t_R = 14.15 min) (column 3).

I35i: Yield of 17%; HRMS *m/z* $[M+H]^+$ found 2884.7114 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.7% (t_R = 15.04 min) (column 3).

Q36q: Yield of 42%; HRMS *m*/*z* $[M+H]^+$ found 2884.7065 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 100% (t_R = 18.32 min) (column 3).

I37i: Yield of 28%; HRMS *m*/*z* $[M+H]^+$ found 2884.7124 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.3% (t_R = 16.14 min) (column 3).

L38l: Yield of 45%; HRMS *m*/*z* $[M+H]^+$ found 2884.7112 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.9% (t_R = 22.60 min) (column 1).

S39s: Yield of 39%; HRMS *m*/*z* $[M+H]^+$ found 2884.7117 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.6% (t_R = 15.98 min) (column 3).

K40k: Yield of 31%; HRMS *m*/*z* $[M+H]^+$ found 2884.7114 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 96.9% (t_R = 15.24 min) (column 3).

L41l: Yield of 34%; HRMS *m*/*z* $[M+H]^+$ found 2884.7129 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 99.6% (t_R = 14.71 min) (column 3).

R42r: Yield of 18%; HRMS *m*/*z* $[M+H]^+$ found 2884.7092 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 98.6% (t_R = 21.23 min) (column 3).

L43l: Yield of 42%; HRMS *m*/*z* $[M+H]^+$ found 2884.7090 (calcd. for $C_{130}H_{223}N_{42}O_{32}$ 2884.7114); HPLC purity 97.6% (t_R = 19.48 min) (column 3).

3. Luciferase reporter assay

As previously reported,[19–21] HEK293 cells were subcultured in DMEM containing 10% FBS and nonessential 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 plates. After incubation for 24 h, transfection of reporter (pGL4.48[luc2P/SBE/Hygro]) and control (pGL4.74[hRluc/-TK]) plasmids was carried out using FuGENE HD. After 24 h of transfection, the culture medium was exchanged to serum-free DMEM for 8 h starvation. Respective synthesized peptides were dissolved in H_2O, diluted by addition of DMEM containing recombinant human/mouse/rat myostatin to a final concentration of 8 ng/mL (0.32 nM)], and incubated for 20 min at rt. Cells were treated with a peptide solution and incubated for 4 h at 37 °C under 5% CO_2, and then washed with PBS. The preparation of cell lysates and the measurement of the luciferase reporter activities were conducted according to manufacturer's protocol of a Dual-Luciferase Reporter Assay System (Promega). Mouse myostatin-derived recombinant propeptide (prodomain) was used as a positive control and underwent the same manipulation arriving at a final concentration of 10 nM. Each experiment was carried out in triplicate. Values represent means ± SD (n= 3). To determine IC_{50} values, the peptide derivatives were dissolved at concentrations of 0.016–2 μM and the inhibitory activities of peptides were determined in triplicate at each concentration. Curve fitting was performed using KaleidaGraph 4.5.

4. Measurement of circular dichroic spectra

As previously reported,[18,24] circular dichroic (CD) spectra of peptides 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. Briefly, peptides with a final concentration of 5 μM were prepared in 20 mM sodium phosphate buffer (pH 7.4) containing 10% 2,2,2-trifluoroethanol. All 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. 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•cm^2/dmol) and plotted as functions of wavelength. Obtained CD spectra were analyzed by using Jasco secondary estimation software with Reed's reference set as reference spectra.

5. Intramuscular administration of peptides into anterior tibialis of mdx mice

Animal studies were approved by the Animal Research Committee of Tokyo University of Pharmacy and Life Sciences. The peptide solution (0.75 mM peptide **E31R** or **8a** in saline, 40 μL) and a saline (control, 40 μL) were intramuscularly injected into left and right tibialis anterior or gastrocnemius muscle of 5-week-old *mdx* or C57BL/6 mice, respectively (at day 0). At day 14), the treatment was repeated for the same muscle. Then at day 42, the muscles were collected and weighed. Statistical analysis was performed using a Student's *t*-test.

6. Histological analysis

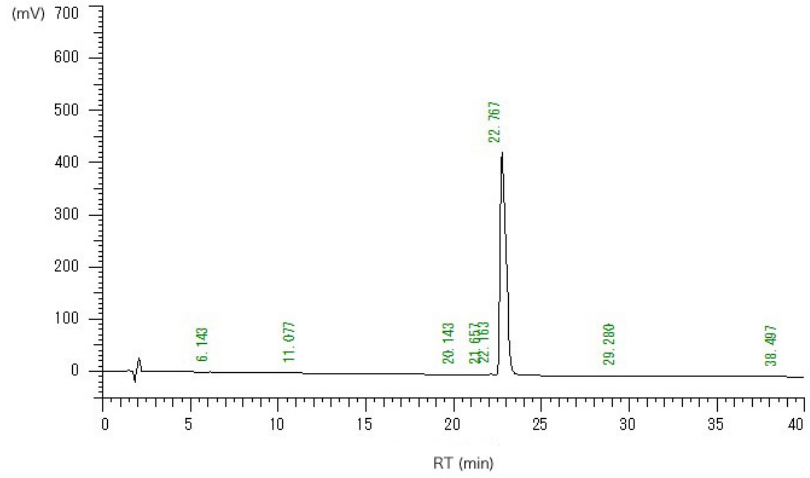
The treated gastrocnemius muscles were dissected 28 days after the 2nd injection of **E31R** or at day 14 for the saline-treated animals. The frozen tissue sections were prepared transversely (6 μm) using a cryostat, and then hematoxylin and eosin staining was performed on each section. Fiber sizes were determined by measuring the area of each myofiber in a fixed area. Two hundred cross-sectioned myofibers were randomly selected from 3 fields of each tissue section.

7. Measurement of grip strength in mdx mice

Animal studies were approved by the Animal Research Committee of Tokyo University of Pharmacy and Life Sciences. The peptide solution in saline (0.75 mM peptide **8a**, 40 μL) were intramuscularly injected into respective four points per leg in 5-week-old *mdx* mice (at day 0) (n = 4). After two weeks (at day 14), the treatment was repeated. As a control, saline (40 μL) was similarly treated in another group of *mdx* mice (n = 4). Then four weeks after the last treatment (at day 42), the grip strength of mice treated with peptide **8a** or with saline were measured by a grip strength meter MK-380M (Muromachi, Tokyo, Japan). Data normalized with body weight were presented as mean values ± SD. Statistical analysis was performed using a Student's *t*-test.

Analytical HPLC chromatograms

peptide **4a**:



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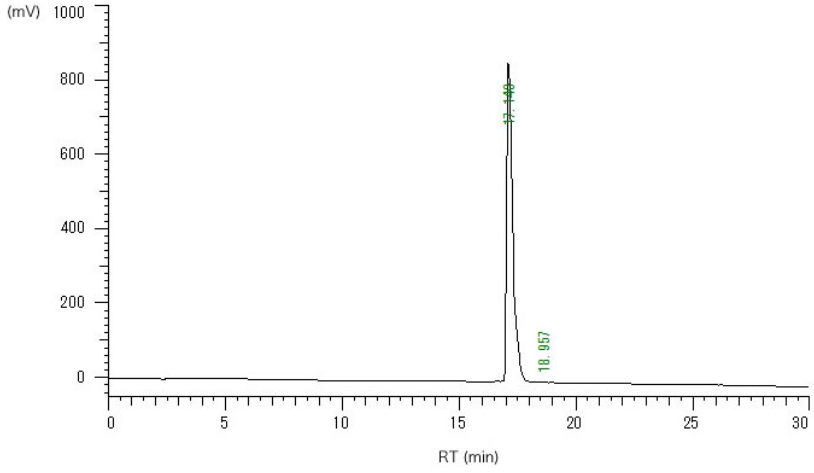

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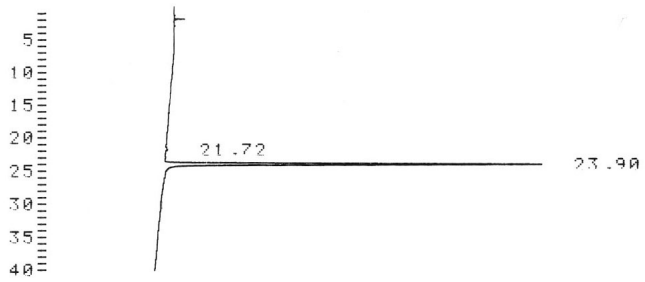


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D-2500

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                     1838911    100.000
PEAK REJ :          10000
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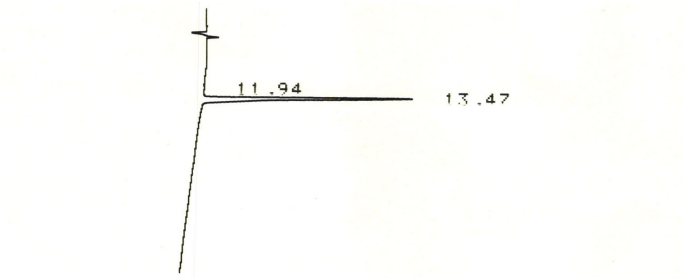


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D-2500

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TOTAL
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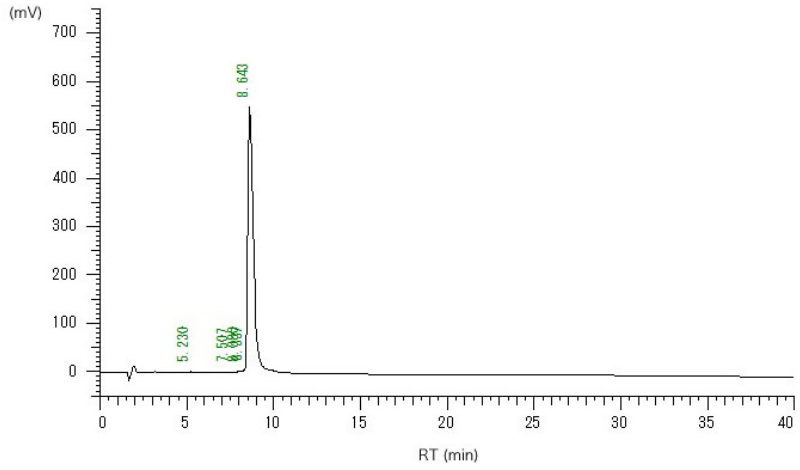

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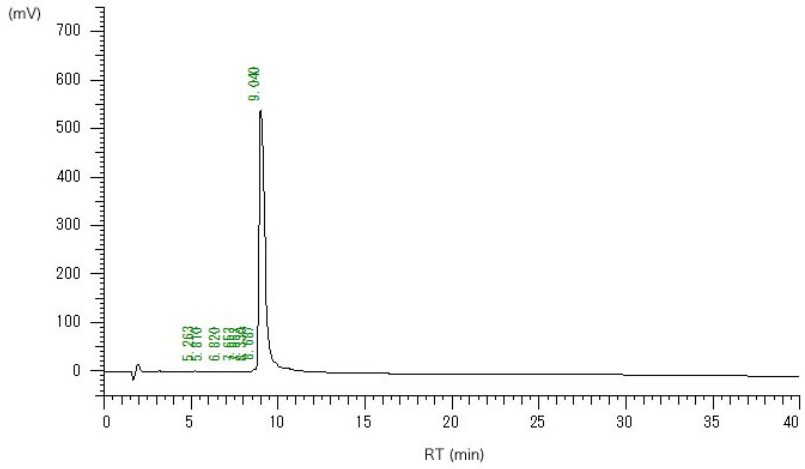

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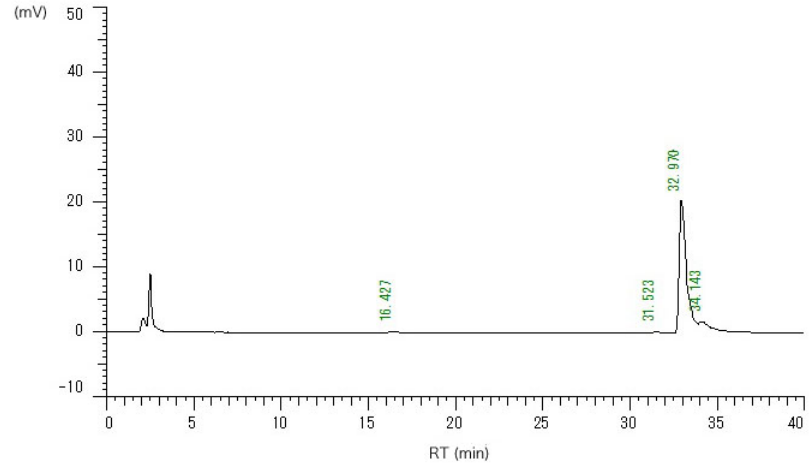

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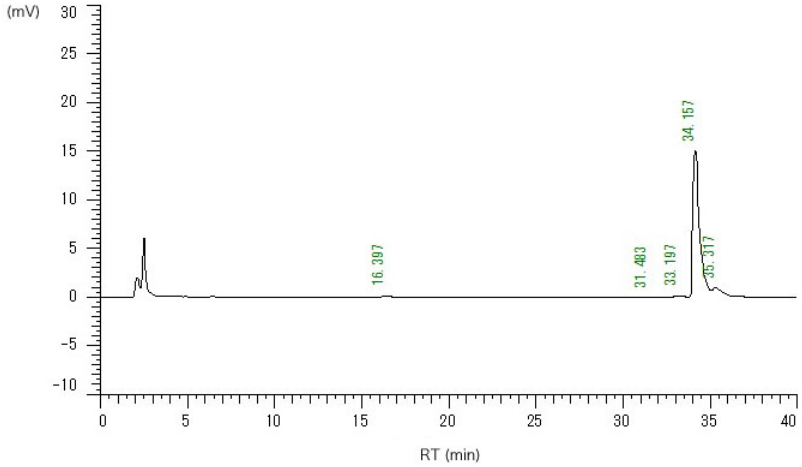

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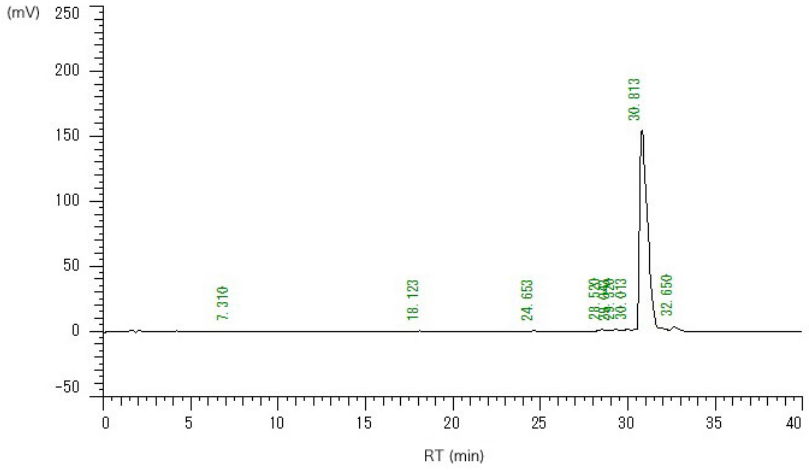

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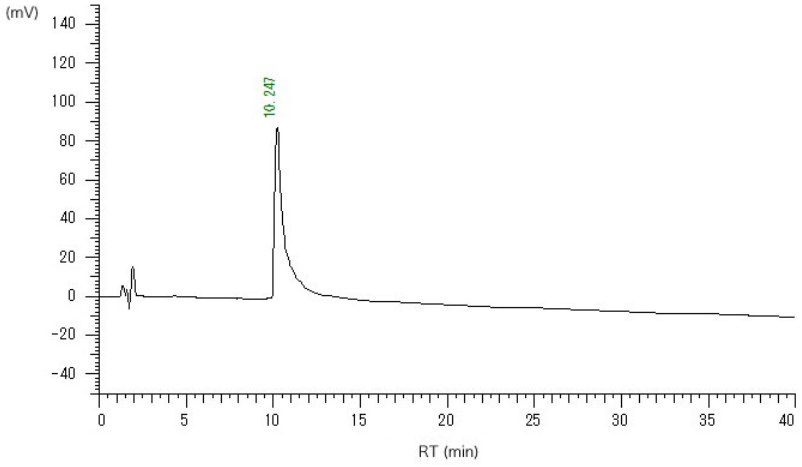

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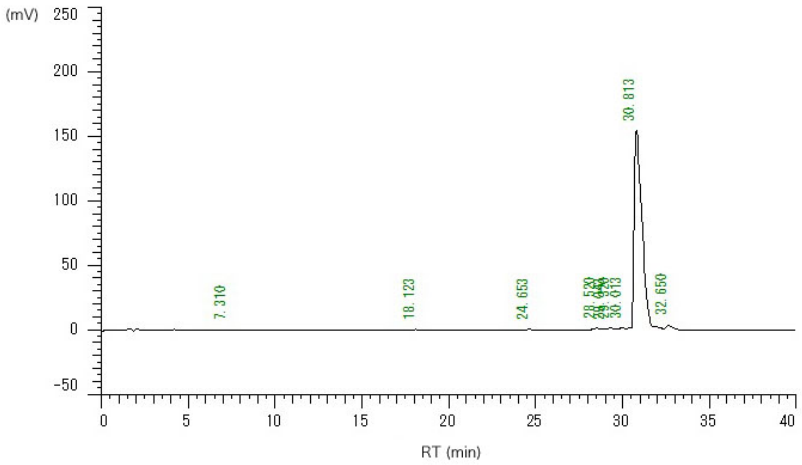
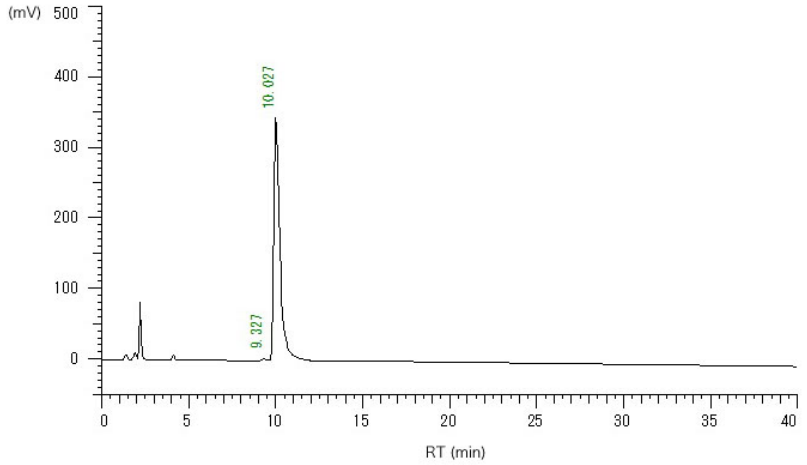
peptide **7c**:



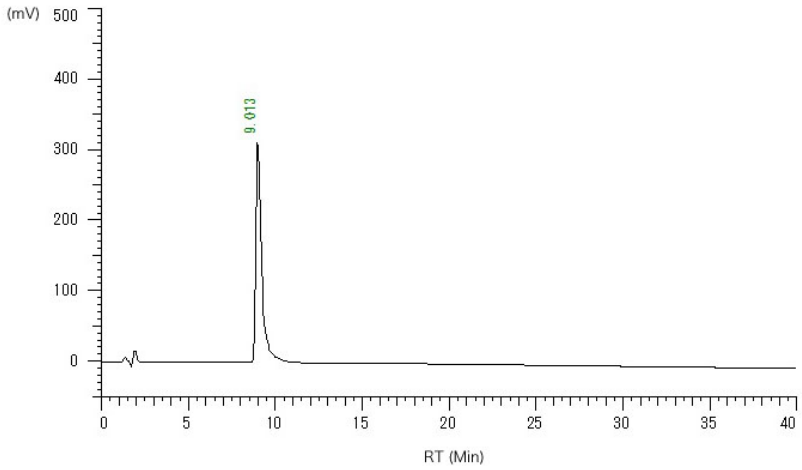
NO	RT	AREA	CONC	BC
1	7.31	1772	0.043	BB
2	18.123	3212	0.077	BB
3	24.653	4817	0.116	BB
4	28.52	8691	0.209	BB
5	29.043	2121	0.051	BB
6	29.32	3730	0.09	BB
7	30.013	5475	0.132	BB
8	30.813	4096758	98.514	BB
9	32.65	31966	0.769	BB
		4158542	100	

peptide **7d**:



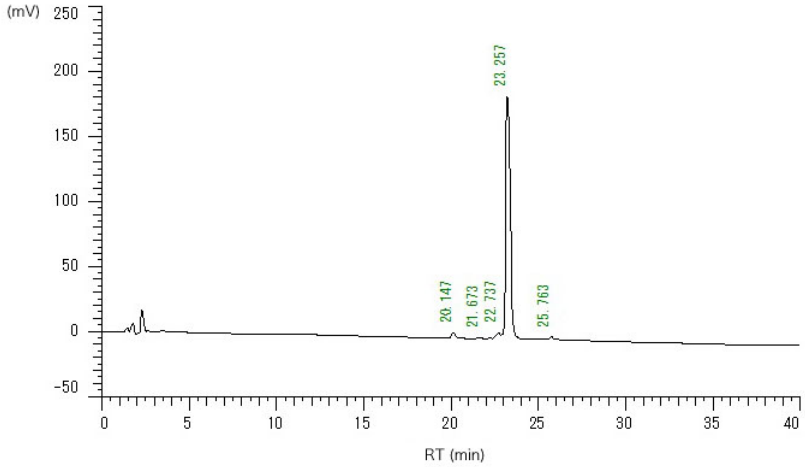
NO	RT	AREA	CONC	BC
1	9.327	24822	0.333	BB
2	10.027	7421475	99.667	BB
		7446297	100	

peptide **7e**:



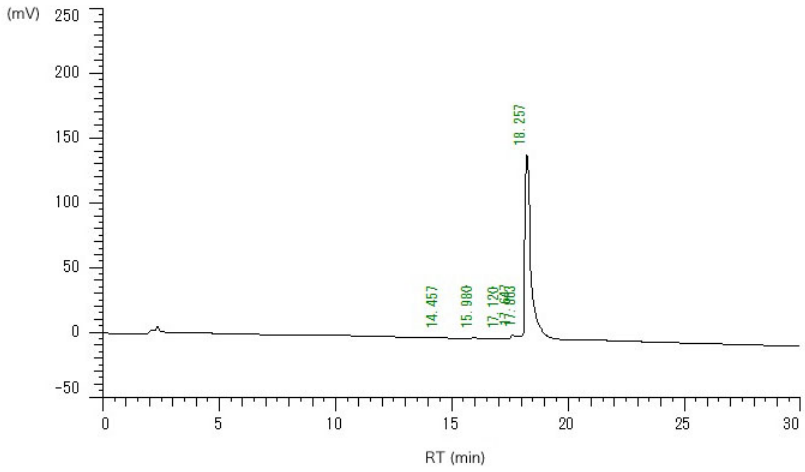
NO	RT	AREA	CONC	BC
1	9.013	6365911	100	BB
		6365911	100	

peptide **8a**:



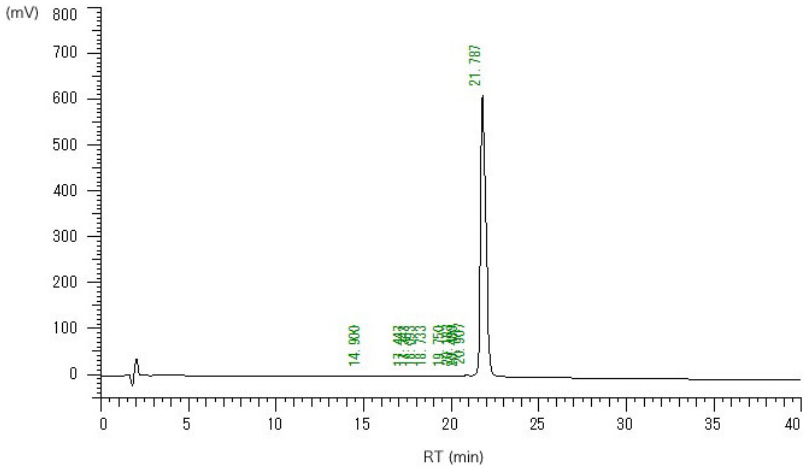
NO	RT	AREA	CONC	BC
1	20.147	46521	1.611	BB
2	21.673	1769	0.061	BB
3	22.737	53356	1.847	BV
4	23.257	2755097	95.398	VB
5	25.763	31271	1.083	BB
		2888014	100	

peptide **8b**:



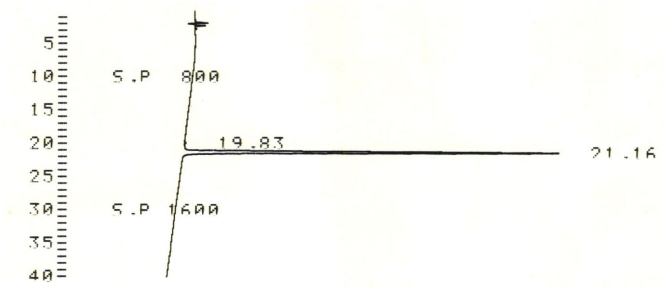
NO	RT	AREA	CONC	BC
1	14.457	1063	0.05	BB
2	15.98	2633	0.124	BB
3	17.12	5696	0.268	BB
4	17.647	20520	0.966	BV
5	17.863	18917	0.891	VV
6	18.257	2074787	97.701	VB
		2123616	100	

peptide **8c**:



NO	RT	AREA	CONC	BC
1	14.9	3022	0.026	BB
2	17.447	1465	0.013	BB
3	17.743	1718	0.015	BB
4	18.093	2251	0.019	BB
5	18.733	6200	0.053	BB
6	19.75	16183	0.138	BV
7	20.183	29914	0.255	VV
8	20.48	10418	0.089	VV
9	20.907	69622	0.594	VV
10	21.787	11585305	98.799	VB
		11726098	100	

I30Chg:



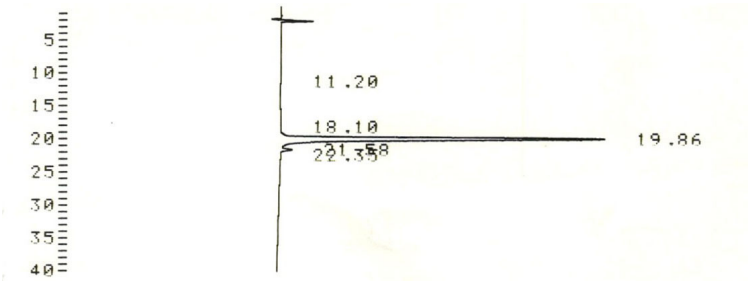
D-2500

METHOD: TAG: 546 CH: 1

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

NO.	RT	AREA	CONC	BC
2	21.16	1914512	100.000	BB
TOTAL				
		1914512	100.000	
PEAK REJ :		10000		

I33Chg:



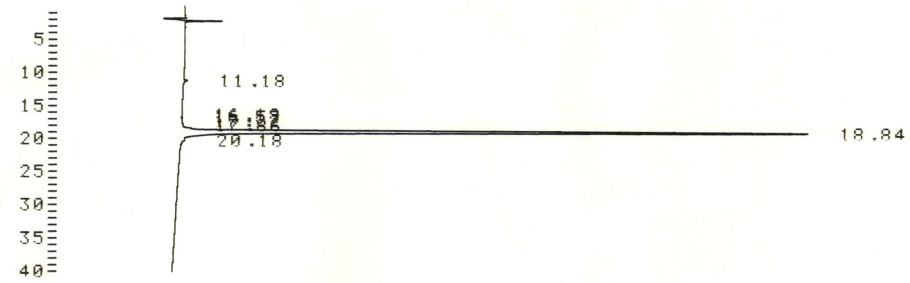
D-2500

METHOD: TAG: 1031 CH: 1

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

NO.	RT	AREA	CONC	BC
6	11.20	24521	0.200	BB
8	18.10	15762	0.129	BU
9	19.86	11940908	97.560	UU
10	21.58	237600	1.941	TBB
11	22.35	20732	0.169	TBB
TOTAL				
		12239523	100.000	
PEAK REJ :		10000		

I35Chg:



D-2500

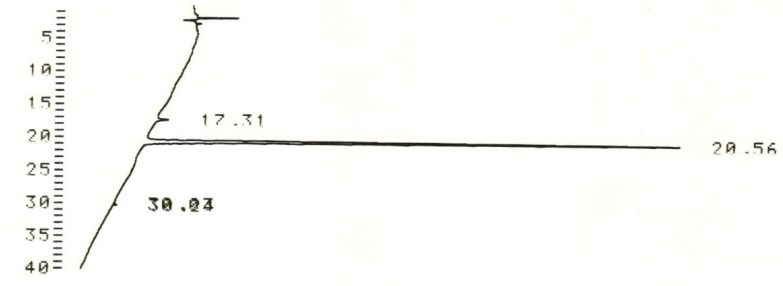
METHOD: TAG: 796 CH: 1

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

NO.	RT	AREA	CONC	BC
5	11.18	39355	0.431	BB
8	17.22	11268	0.123	VV
9	17.86	25669	0.281	VV
10	18.84	9064325	99.165	VV
TOTAL		9140617	100.000	

PEAK REJ : 10000

I37Chg:



D-2500

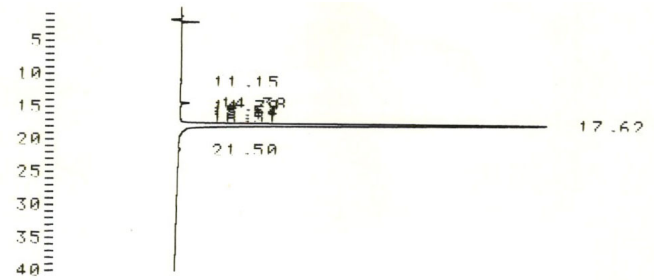
METHOD: TAG: 807 CH: 1

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

NO.	RT	AREA	CONC	BC
1	17.31	14852	2.128	BB
2	20.56	683182	97.872	BB
TOTAL		698034	100.000	

PEAK REJ : 10000

I30Phg:



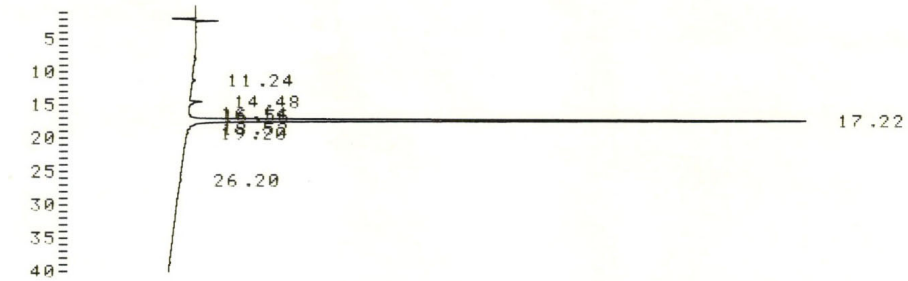
D-2500

METHOD: TAG: 794 CH: 1

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

NO.	RT	AREA	CONC	BC
6	11.15	27354	0.244	BB
7	14.38	167171	1.491	BV
8	14.79	10946	0.098	TBB
11	16.51	20138	0.180	VV
12	17.62	10948278	97.639	VV
13	21.50	39152	0.349	TBB
TOTAL				
		11213039	100.000	
PEAK REJ :		10000		

I33Phg:



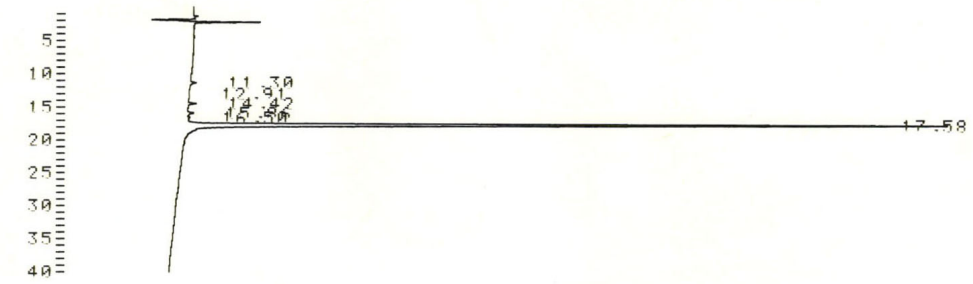
D-2500

METHOD: TAG: 805 CH: 1

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

NO.	RT	AREA	CONC	BC
5	11.24	30964	0.891	VV
6	14.48	75068	2.160	VV
8	16.56	14053	0.404	VV
9	17.22	3345573	96.251	VV
12	26.20	10212	0.294	BB
TOTAL				
		3475870	100.000	
PEAK REJ :		10000		

I35Phg:



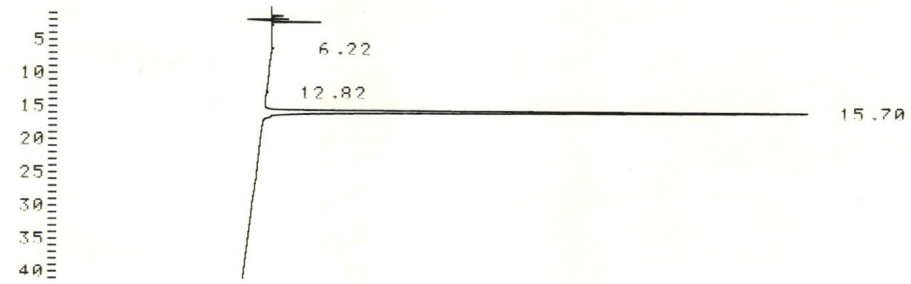
D-2500

METHOD: TAG: 771 CH: 1

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

NO.	RT	AREA	CONC	BC
6	11.30	32355	0.660	BB
8	14.42	41568	0.849	VV
9	15.86	44308	0.904	VV
10	16.50	16469	0.336	VV
11	17.58	4764149	97.250	VB
TOTAL		4898849	100.000	
PEAK REJ :		10000		

I37Phg:



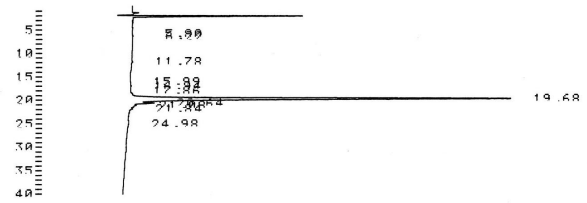
D-2500

METHOD: TAG: 1001 CH: 1

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

NO.	RT	AREA	CONC	BC
3	15.70	3145378	100.000	VB
TOTAL		3145378	100.000	
PEAK REJ :		10000		

L38Chg:



D-2500

METHOD: TAG: 18 CH: 1

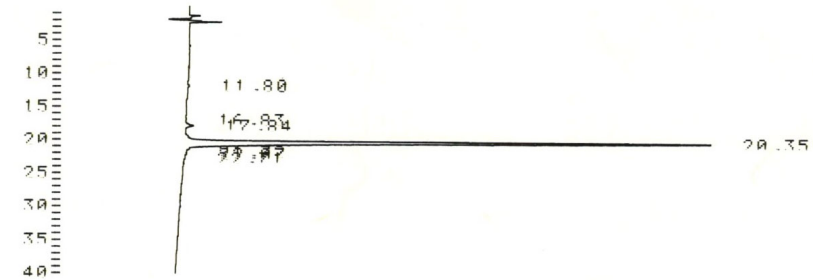
FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
2	6.22	25403	0.267	VV
3	11.78	27257	0.286	VB
5	16.94	27988	0.294	VV
6	17.86	11029	0.116	VV
7	19.68	9184139	96.428	VV
8	20.64	155041	1.628	TBB
9	21.08	35572	0.373	TBB
10	21.84	20252	0.213	TBB
11	24.98	37700	0.396	TBB

TOTAL 9524381 100.000

PEAK RET : 10000

L41Chg:



D-2500

METHOD: TAG: 20 CH: 1

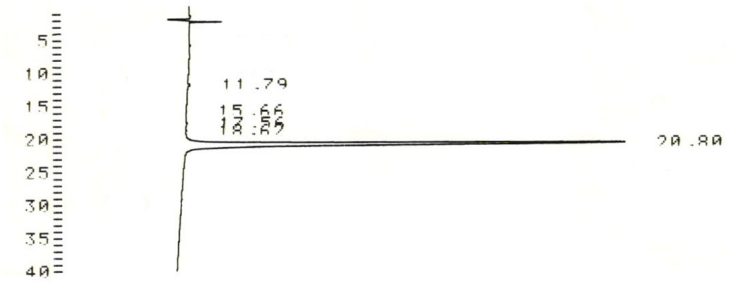
FILE: 0 CALC-METHOD: AREA% TABLE: 0 CONC: AREA

NO.	RT	AREA	CONC	BC
6	11.80	26192	0.313	BB
8	17.84	119397	1.429	BV
9	20.35	8212149	98.258	VV

TOTAL 8357738 100.000

PEAK RET : 10000

L43Chg:



D-2500

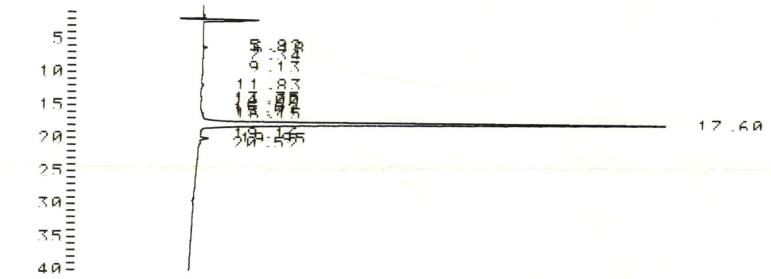
METHOD: TAG: 28 CH: 1

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

NO.	RT	AREA	CONC	BC
5	11.79	34537	0.463	BB
7	17.56	24581	0.329	BU
8	18.62	11881	0.159	UU
9	20.80	7389585	99.048	UU
TOTAL		7460584	100.000	

PEAK RET : 10000

L38Phg:



D-2500

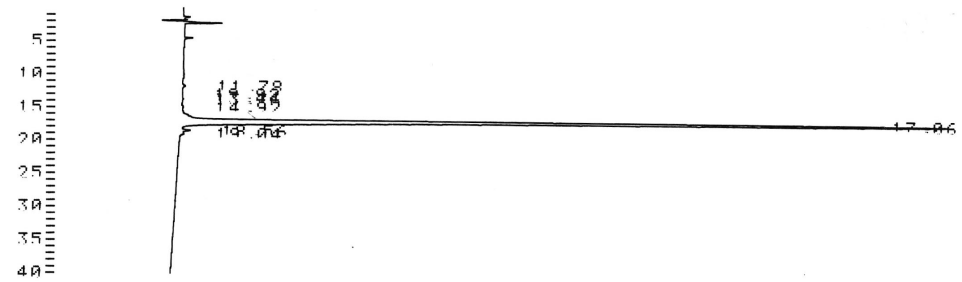
METHOD: TAG: 19 CH: 1

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

NO.	RT	AREA	CONC	BC
2	6.18	61621	0.845	UU
5	11.83	34365	0.471	UB
7	14.00	18012	0.247	UU
9	15.71	19490	0.267	UU
10	16.15	50348	0.690	UU
11	17.60	6975954	95.634	UU
13	19.95	109772	1.505	TBU
14	20.52	24893	0.341	TUB
TOTAL		7294455	100.000	

PEAK RET : 10000

L41Phg:



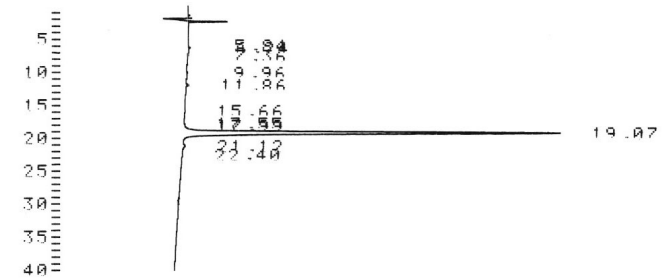
D-2500

METHOD: TAG: 26 CH: 1

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

NO.	RT	AREA	CONC	BC
4	11.78	52153	0.408	BU
7	13.76	24239	0.189	UU
8	14.92	41853	0.327	UU
9	17.06	12534396	97.976	UU
10	18.56	97944	0.766	TBU
11	19.04	42753	0.334	TUB
TOTAL		12793338	100.000	
PEAK RET :		10000		

L43Phg:



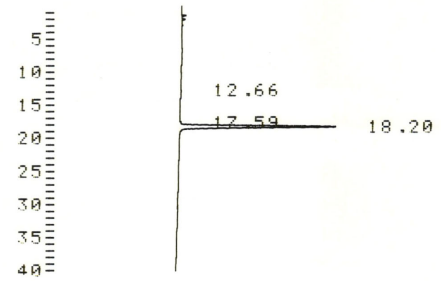
D-2500

METHOD: TAG: 29 CH: 1

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

NO.	RT	AREA	CONC	BC
2	6.24	21914	0.405	IIII
5	11.86	24108	0.445	BB
8	17.99	17672	0.326	IIII
9	19.07	5319002	98.241	IIII
10	21.12	31567	0.583	TBB
TOTAL		5414263	100.000	
PEAK RET :		10000		

S28s:



D-2500

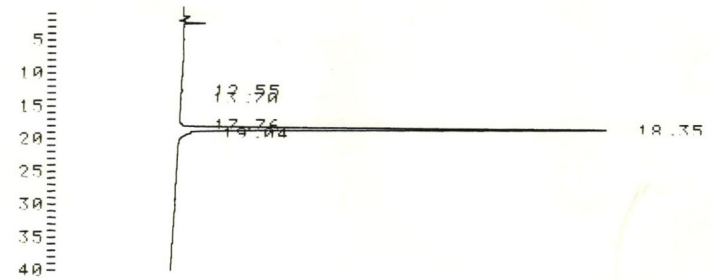
METHOD: TAG: 177 CH: 1

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

NO.	RT	AREA	CONC	BC
3	12.66	25926	0.621	BV
4	17.59	25344	0.607	VV
5	18.20	4125004	98.772	VB
TOTAL		4176274	100.000	

PEAK REJ : 10000

R29r:



D-2500

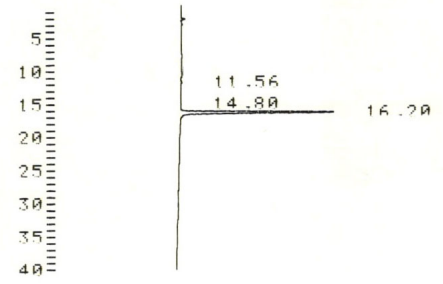
METHOD: TAG: 186 CH: 1

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

NO.	RT	AREA	CONC	BC
5	12.55	14298	0.224	VB
6	13.70	21152	0.331	BV
7	17.76	40817	0.639	VV
8	18.35	6306471	98.805	VV
TOTAL		6382738	100.000	

PEAK REJ : 10000

I30i:



D-2500

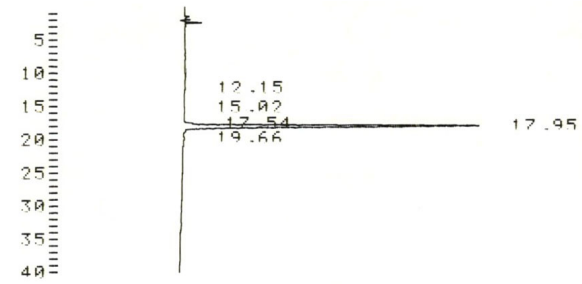
METHOD: TAG: 254 CH: 1

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

NO.	RT	AREA	CONC	BC
2	11.56	11646	0.336	BB
4	16.20	3458055	99.664	VB
TOTAL		3469701	100.000	

PEAK REJ : 10000

E31e:



D-2500

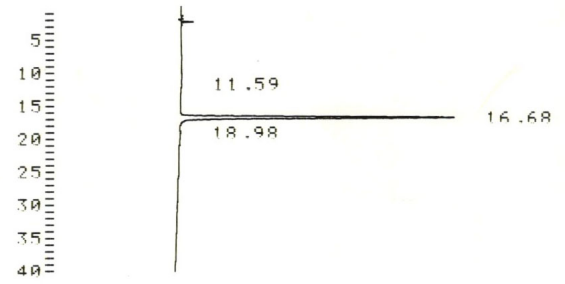
METHOD: TAG: 255 CH: 1

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

NO.	RT	AREA	CONC	BC
5	12.15	21930	0.293	BB
7	17.54	154383	2.061	BV
8	17.95	7284248	97.242	VV
9	19.66	30293	0.404	TBB
TOTAL		7490854	100.000	

PEAK REJ : 10000

A32a:



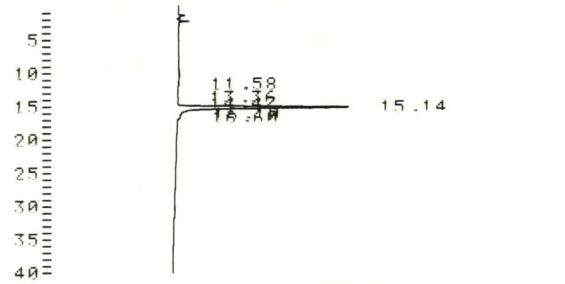
D-2500

METHOD: TAG: 136 CH: 1

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

NO.	RT	AREA	CONC	BC
2	11.59	10009	0.143	BB
3	16.68	6964051	99.624	BV
4	18.98	16256	0.233	TBB
TOTAL		6990316	100.000	
PEAK REJ :		10000		

I33i:



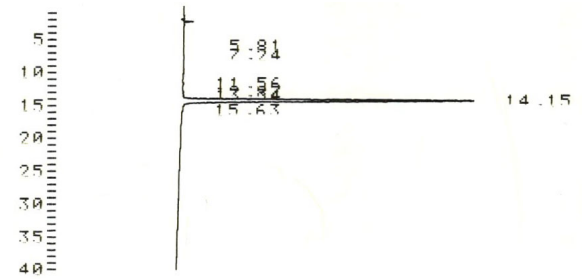
D-2500

METHOD: TAG: 146 CH: 1

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

NO.	RT	AREA	CONC	BC
3	11.58	11328	0.235	BB
5	14.47	15137	0.315	BV
6	15.14	4770543	99.133	VV
8	16.60	15240	0.317	TBB
TOTAL		4812248	100.000	
PEAK REJ :		10000		

K34k:



D-2500

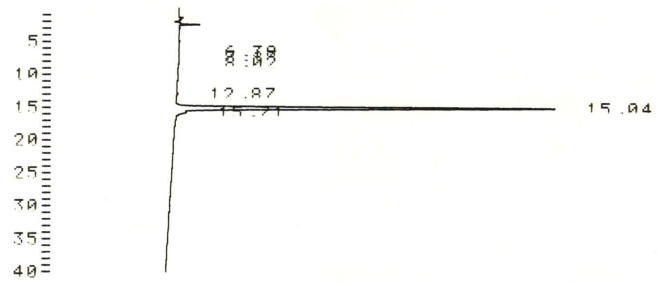
METHOD: TAG: 147 CH: 1

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

NO.	RT	AREA	CONC	BC
2	7.24	30136	0.407	VV
3	11.56	29452	0.398	VV
4	12.87	28288	0.382	VV
5	13.34	33899	0.458	VV
6	14.15	7281137	98.355	VV
TOTAL		7402912	100.000	

PEAK RET : 10000

I35i:



D-2500

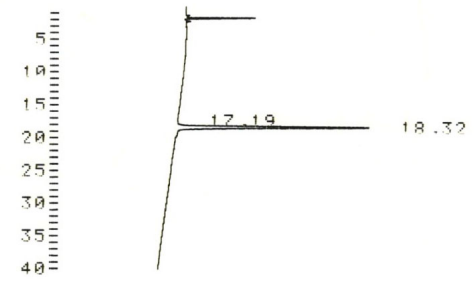
METHOD: TAG: 181 CH: 1

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

NO.	RT	AREA	CONC	BC
3	8.02	14591	0.289	VB
4	12.87	14302	0.283	BB
5	15.04	4991129	98.731	BV
6	15.71	35253	0.697	TBB
TOTAL		5055275	100.000	

PEAK RET : 10000

Q36q:



D-2500

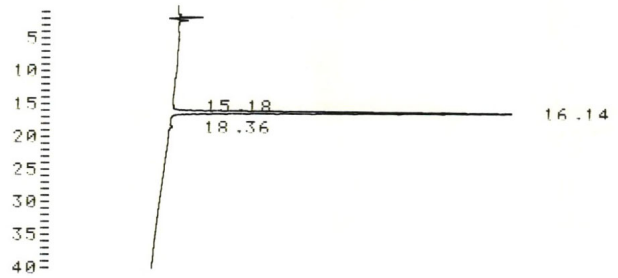
METHOD: TAG: 118 CH: 1

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

NO.	RT	AREA	CONC	BC
2	18.32	1156864	100.000	VB
TOTAL				
		1156864	100.000	

PEAK RET : 10000

I37i:



D-2500

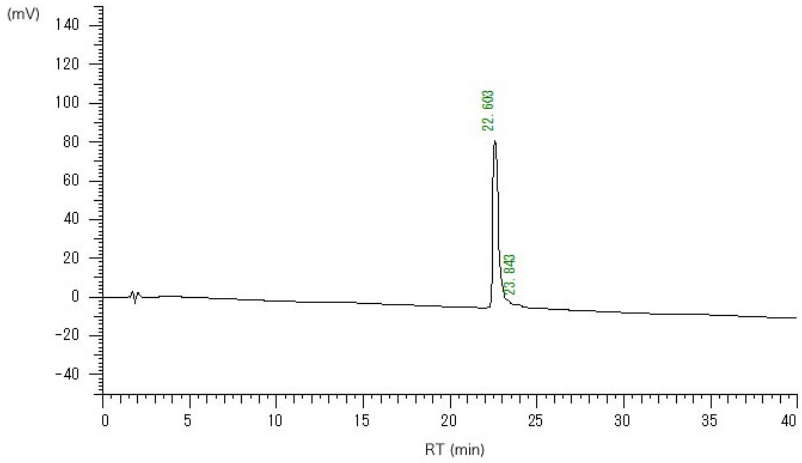
METHOD: TAG: 119 CH: 1

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

NO.	RT	AREA	CONC	BC
2	16.14	1815669	99.273	VB
3	18.36	13288	0.727	BB
TOTAL				
		1828957	100.000	

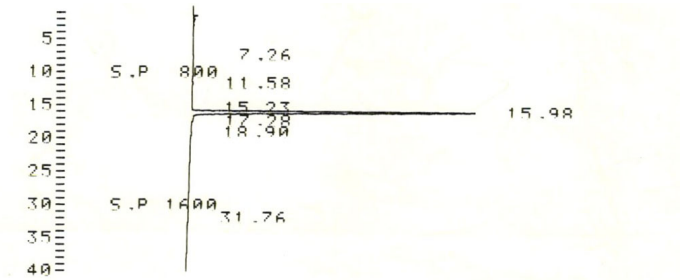
PEAK RET : 10000

L38l:



NO	RT	AREA	CONC	BC
1	22.603	1660835	99.907	BB
2	23.843	1543	0.093	BB
		1662378	100	

S39s:



D-2500

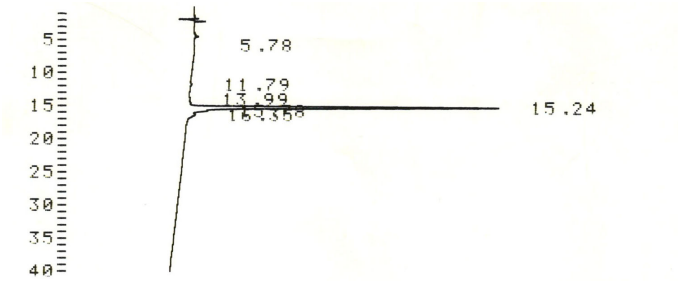
METHOD: TAG: 253 CH: 1

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

NO.	RT	AREA	CONC	BC
2	11.58	13699	0.212	BB
4	15.98	6436206	99.621	VV
7	31.76	10770	0.167	BB
TOTAL		6460675	100.000	

PEAK REJ : 10000

K40k:



D-2500

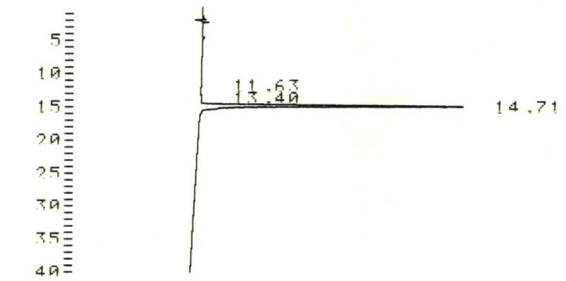
METHOD: TAG: 352 CH: 1

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

NO.	RT	AREA	CONC	BC
1	5.78	20605	1.094	BB
4	15.24	1824859	96.891	VV
5	15.78	12772	0.678	TBB
6	16.35	25173	1.337	TBB
TOTAL		1883409	100.000	

PEAK REJ : 10000

L41l:



D-2500

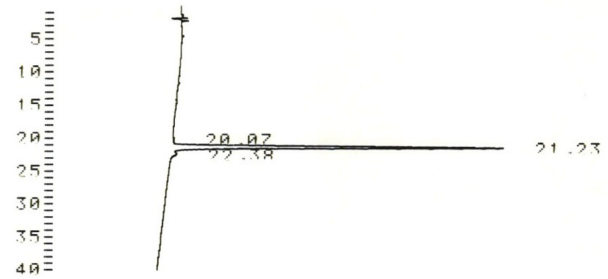
METHOD: TAG: 357 CH: 1

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

NO.	RT	AREA	CONC	BC
2	13.40	10826	0.358	BV
3	14.71	3009602	99.642	VB
TOTAL		3020428	100.000	

PEAK REJ : 10000

R42r:



D-2500

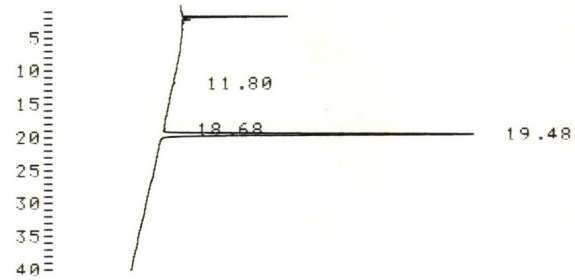
METHOD: TAG: 358 CH: 1

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

NO.	RT	AREA	CONC	BC
3	20.07	11684	0.491	BV
4	21.23	2344060	98.550	VV
5	22.38	22794	0.958	TBB
TOTAL		2378538	100.000	

PEAK REJ : 10000

L43l:



D-2500

METHOD: TAG: 366 CH: 1

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

NO.	RT	AREA	CONC	BC
1	11.80	20720	2.362	BV
3	19.48	856530	97.638	VB
TOTAL		877250	100.000	

PEAK REJ : 10000

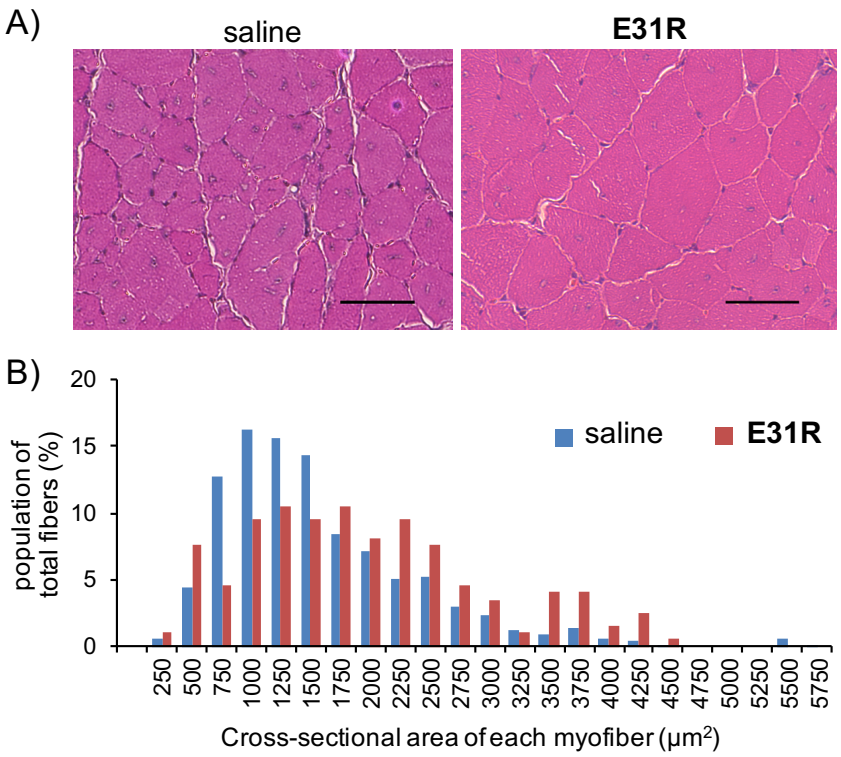


Figure S1. Induction of GAS muscle hypertrophy by **E31R**-treatment in Duchenne muscular dystrophic model *mdx* mice. (A) Hematoxylin and eosin staining of GAS muscles of *mdx* mice (n = 3) at day 42 after treatment with **E31R** or saline at days 0 and 14. Scale bars = 50 μm. (B) Cross-sectional areas and distributions of myofiber sizes in **E31R** or saline treated GAS muscles.

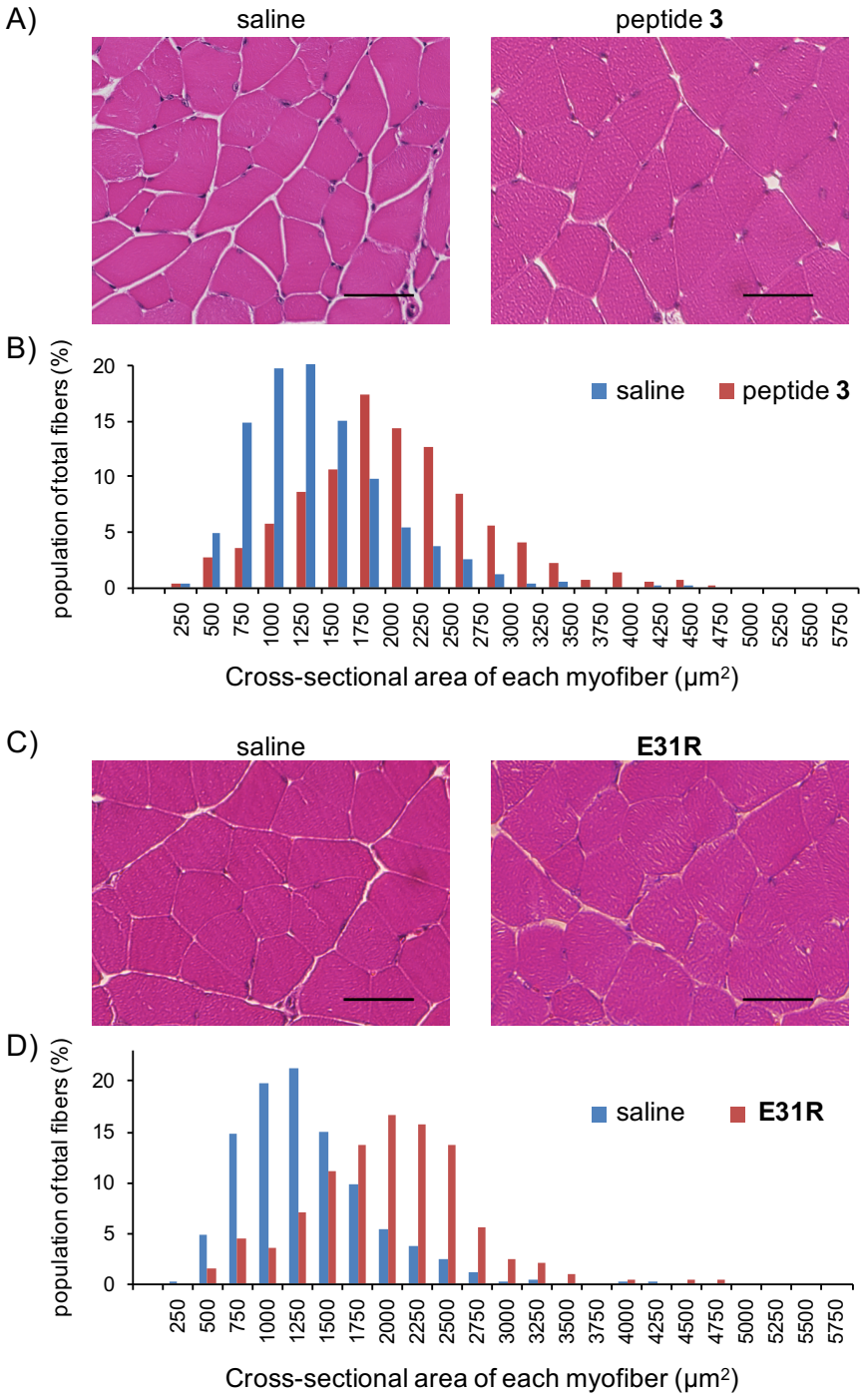


Figure S2. Induction of GAS muscle hypertrophy by peptide **3**- or **E31R**-treatment in wild-type mice. (A, C) Hematoxylin and eosin staining of GAS muscles of C57BL/6 mice (n = 3) at day 42 after treatment with peptide **3** (A), **E31R** (C) or saline (A, C) at days 0 and 14. Scale bars = 50 μm. (B, D) Cross-sectional areas and distributions of myofiber sizes in peptide **3** (B), **E31R** (D) or saline (B, D) treated GAS muscles.

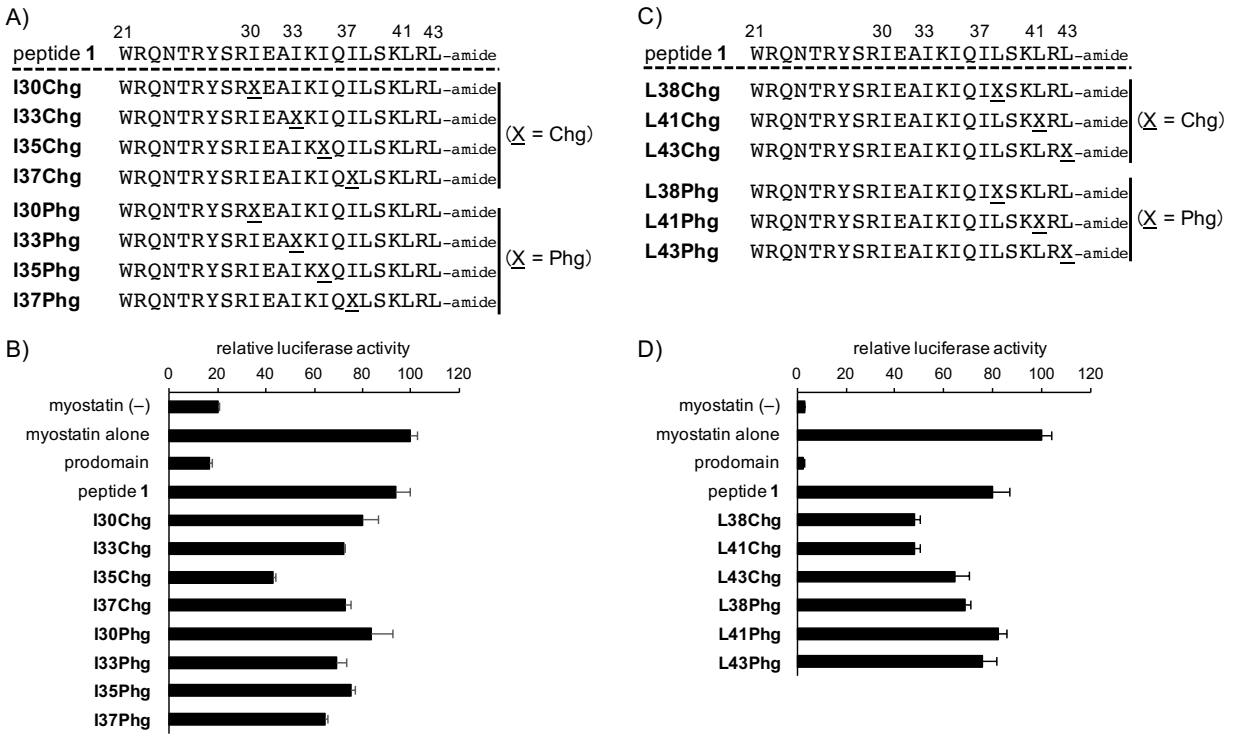


Figure S3. The aliphatic residue-directed SAR studies of peptide **1** using either chyclohexylglycine (Chg) or phenylglycine (Phg) residue. (A, C) Sequences of the Ile-substituted (A) and Leu-substituted (C) peptides. The numbers above each amino acid indicate its position in the prodomain sequence of mouse myostatin. (B, D) The luciferase reporter assay determined the activities of the Ile-substituted (B) and Leu-substituted (D) peptides toward myostatin inhibition relative to peptide **1**. Peptide concentration: 1 μM. Results are presented as mean values ± SD (n = 3).

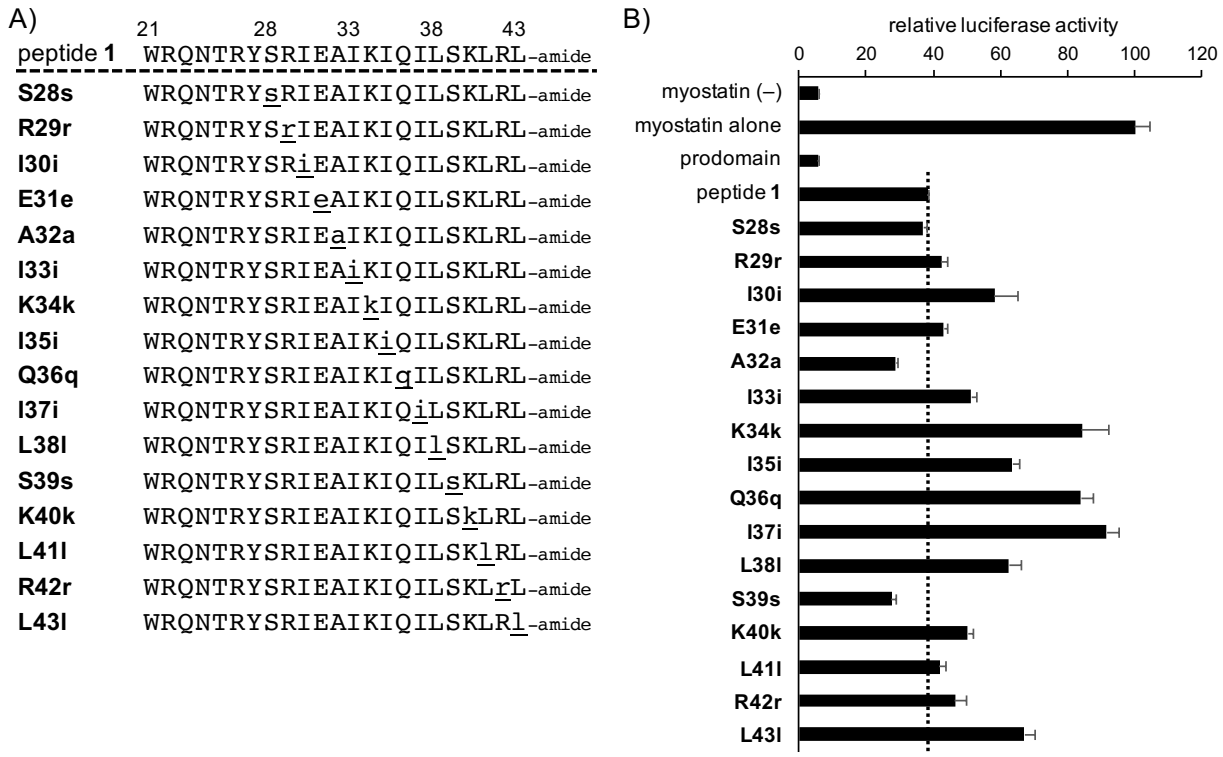


Figure S4. D-form scanning in peptide **1**. (A) Sequences of a series of peptides substituted with D-form amino acid. The numbers above each amino acid indicate its position in the prodomain sequence of mouse myostatin. Lower case letter = D-form of the amino acid. (B) The luciferase reporter assay determined the activities of a series of peptides substituted with D-form amino acid toward myostatin inhibition relative to peptide **1**. Peptide concentration: 3 μM. Results are presented as mean values ± SD (n = 3).

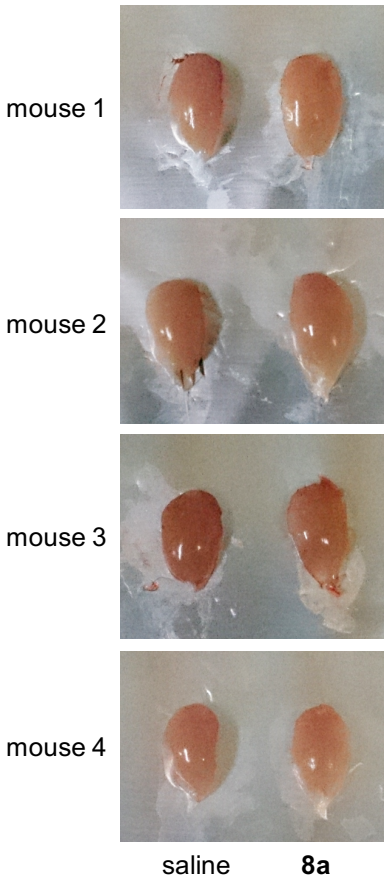


Figure S5. Investigation of morphological change to peptide treatment. Appearance of tibialis anterior (TA) muscles of *mdx* mice (mouse 1–4, n = 4) at day 42 after treatment with peptide **8a** (left TA) or saline (saline, right TA).