Supporting Information

Design and Synthesis of an Investigational Nonapeptide KISS1 receptor (KISS1R), Agonist Ac-D-Tyr-Hydroxyproline (Hyp)-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2 (TAK-448) with Highly Potent Testosterone-Suppressive Activity and Excellent Water Solubility

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Table of Contents

1. General procedure for synthesis of metastin analogues.....S2

2. Table S1. Chemical data for nonapeptide metastin analogues.....S6

3. Calcium mobilization assay.....S7

4. Molecular formula strings and the associated biochemical and biological data.....S10

5. Figure S1. Far-UV circular dichroism spectra of metastin analogues.....S11

1. General procedure for synthesis of metastin analogues

All peptides were synthesized in the same manner as the following synthesis procedure for Ac-[D-Tyr^{46},Hyp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54) **24**. To five grams (2 mmol) of Rink Amide MBHA resin (0.4 mmol/g), Fmoc-Trp(Boc)-OH and Fmoc-Orn(Mtt)-OH were sequentially introduced using the standard Fmoc SPPS method to form a 2-mmol resin of Fmoc-Orn(Mtt)-Trp(Boc)-NH-Rink Amide MBHA. After the resin was washed and swollen with dichloromethane (DCM), 50 mL of TFA/triisopropylsilane (TIS)/DCM (1/5/94) was added and the resin was stirred for ten minutes. The solution was washed out by filtration, and this procedure was repeated until the yellow color caused by the free Mtt group in a TFA/TIS/DCM (1/5/94) solution disappeared when the solution was added, indicating that the Mtt group was completely removed. The resulting Fmoc-Orn-Trp(Boc)-NH-Rink Amide MBHA resin was neutralized with 5% DIEA/DCM solution. The resin was washed with DCM, and then 25 mL of DCM-trifluoroethanol (4/1) and 1.946 g (6 mmol) of *N*-methyl-*N*, *N′*-bis-Boc-1-guanylpyrazole were added to the resin. *N,N′*-diisopropylethylamine (DIEA) was added to the mixture to adjust the pH to 10. The mixture was stirred for 15 hours, and then the resin was washed with DMF and methanol (MeOH). The resin was dried in vacuo to form a 6.195-g resin of Fmoc-Arg(Boc$_2$,Me)-Trp(Boc)-NH-Rink Amide MBHA. To this resin, Fmoc-Leu-OH was introduced using the standard Fmoc SPPS method and the N-terminal Fmoc group was removed to form a 2-mmol resin of H-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-NH-Rink Amide MBHA. Separately, 1.745 g (6

mmol) of Fmoc-$NHNH_2$ HCl was suspended in 20 mL of DMF-THF (4/1). Under ice cooling, 973 mg of *N,N′*-carbonyldiimidazole (CDI) and 2.09 mL of DIEA were added to the suspension, followed by stirring at room temperature for one hour. The resulting reaction solution was added to the H-Leu-Arg(Boc_2,Me)-Trp(Boc)-NH-Rink Amide MBHA resin described above, and the mixture was stirred at room temperature for 15 hours. Once the reaction was complete, the resin was washed and dried to form a 6.394-g resin of Fmoc-azaGly-Leu-Arg(Boc_2,Me)-Trp(Boc)-NH-Rink Amide MBHA. 800 mg (0.3 mmol) of the Fmoc-azaGly-Leu-Arg(Boc_2,Me)-Trp-NH-Rink Amide MBHA resin, was used as a starting material, and the starting material was reacted with the peptide synthesizer ABI 433A (as per the Fmoc/DCC/HOBt 0.25 mmol protocol) to introduce Phe, Thr(*t*Bu), Asn(Trt), Hyp(*t*Bu), and D-Tyr(*t*Bu) in this order to form the H-D-Tyr(*t*Bu)-Hyp(*t*Bu)-Asn(Trt)-Thr(*t*Bu)-Phe-azaGly-Leu-Arg(Boc_2,Me)-Trp-NH-Rink Amide MBHA resin. Subsequently, the resin was treated for 20 min in DMF with 94.4 μL (1 mmol) of acetic anhydride and 174.2 μL (1 mmol) of DIEA for the N-terminal acetylation to form a 1.049-g resin of Ac-D-Tyr(*t*Bu)-Hyp(*t*Bu)-Asn(Trt)-Thr(*t*Bu)-Phe-azaGly-Leu-Arg(Boc_2,Me)-Trp-NH-Rink Amide MBHA. The same procedure was repeated to form 1.035 g of the resin. To each resin, 8 mL of a mixed solution of TFA-*m*-cresol-thioanisole-H_2O-TIS-ethandithiol (80/5/5/5/2.5/2.5) was added, followed by stirring for 90 minutes. Diethyl ether was added to the reaction solution, the resulting precipitate was centrifuged, and the supernatant was removed. This

procedure was repeated twice for washing. The residue was extracted with an aqueous acetic acid solution and the resin was removed by filtration. The solution of the crude peptide was purified by preparative HPLC using eluent A (0.1% TFA-H_2O) and eluent B (0.1% TFA-acetonitrile) on YMC pack R&D-ODS-5-B S-5, 120 A column (30 × 250 mm) and lyophilized to form a white powder of purified peptide. The white powders obtained, 159.5 mg, were dissolved in 200 mL of water, and 550 μL of BioRAD AG 1×8 acetate ion exchange resin (0.66 mmol) was added to the solution. The solution was stayed for one hour and occasionally gently shaken. The solution was filtered through a 0.45 μm pore membrane filter to remove the resin, leaving 134.5 mg of white powder as the purified sample; mass spectrum: MALDI-TOF (α-cyano-4-hydroxycinnaminic acid, monoisotopic) $C_{64}H_{83}N_{17}O_{13}$ $[M+H]^+$ 1225.79 (Calcd 1225.61). Elution time on RP-HPLC: 11.05 min. Elution conditions: MERCK Chromolith® FastGradient RP-18 end-capped column (2.0 × 50 mm), linear density gradient elution with eluents A/B = 95/5−25/75 (14 min), using 0.1% TFA in water as eluent A and 0.1% TFA-containing acetonitrile as eluent B; flow rate: 0.5 mL/min. Elution time on IE-HPLC: 7.48 min. Elution conditions: Phenomenex Luna® 5-μm SCX 100 Å column (4.6 × 100 mm), linear density gradient elution with eluents A/B = 100/0−0/100 (20 min) using 20-mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluent A and 0.5-M NaCl containing a 20-mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluent B; flow rate: 1.0 mL/min. Abbreviations: Arg(Boc_2,Me), $N^{\omega,\omega'}$-bis-*tert*-butoxycarbonyl-N^{ω}-methylarginine; Boc,

tert-butyloxycarbonyl; *t*Bu, *tert*-butyl; THF, tetrahydrofuran; Trt, triphenylmethyl (trityl).

Table S1. Chemical data for nonapeptide metastin analogues

compound		RP-HPLC[a]		RP-HPLC[b]		IE-HPLC[c]		IE-HPLC[d]		mass (Da)[e]	
		t_{ret} (min)	Purity (%)	t_{ret} (min)	Purity (%)	t_{ret} (min)	Purity (%)	t_{ret} (min)	Purity (%)	Calculated	Theoretical
2	Ac-[D-Tyr^{46},D-Ala^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.06	99.4	11.06	100.0	7.63	100.0	7.63	100.0	1183.74	1183.60
3	Ac-[D-Tyr^{46},D-Leu^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.76	97.2	11.75	97.2	7.50	100.0	7.50	100.0	1225.77	1225.65
4	Ac-[D-Tyr^{46},D-Lys^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.65	98.6	10.66	98.0	13.14	100.0	13.14	100.0	1240.56	1240.66
5	Ac-[D-Tyr^{46},D-Glu^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.99	97.9	10.99	98.0	7.16	100.0	7.16	97.3	1241.35	1241.61
6	Ac-[D-Tyr^{46},Trp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.08	96.8	12.09	100.0	7.53	100.0	7.53	100.0	1298.38	1298.64
7	Ac-[D-Tyr^{46},Lys^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.70	99.3	10.71	100.0	13.30	98.2	13.30	98.0	1240.50	1240.66
8	Ac-[D-Tyr^{46},Glu^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.01	99.6	11.01	100.0	7.18	100.0	7.18	100.0	1241.80	1241.61
9	Ac-[D-Tyr^{46},Gly^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.63	99.0	11.64	98.3	7.41	100.0	7.41	95.2	1169.69	1169.59
10	Ac-[D-Tyr^{46},Ala^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.06	99.3	11.16	100.0	7.51	100.0	7.51	100.0	1183.81	1183.60
11	Ac-[D-Tyr^{46},Aib^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.63	97.7	11.63	99.3	7.48	100.0	7.48	100.0	1197.62	1197.62
12	Ac-[D-Tyr^{46},MeAla^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.23	98.6	11.23	100.0	7.66	98.8	7.66	100.0	1197.82	1197.62
13	Ac-[D-Tyr^{46},D-MeAla^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.61	98.6	11.61	96.5	7.65	98.4	7.66	100.0	1197.79	1197.62
14	Ac-[D-Tyr^{46},Sar^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.08	98.2	11.08	96.8	7.66	100.0	7.66	100.0	1183.46	1183.60
15	Ac-[D-Tyr^{46},β-Ala^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.99	95.0	10.90	96.4	7.70	100.0	7.70	100.0	1183.77	1183.60
16	Ac-[D-Tyr^{46},Abz(2)^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.62	100.0	11.62	100.0	7.77	96.9	7.77	95.1	1231.66	1231.60
17	Ac-[D-Tyr^{46},Pro^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.40	100.0	11.40	100.0	7.81	98.1	7.81	98.6	1209.71	1209.62
18	Ac-[D-Tyr^{46},D-Pro^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.07	98.7	11.07	96.1	7.88	100.0	7.88	100.0	1209.25	1209.62
19	Ac-[D-Tyr^{46},Aze(2)^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.13	99.8	11.13	100.0	7.72	100.0	7.73	99.0	1195.62	1195.60
20	Ac-[D-Tyr^{46},Pic(2)^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.42	97.8	11.42	95.8	7.68	100.0	7.68	98.0	1223.78	1223.63
21	Ac-[D-Tyr^{46},Aze(3)^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.91	100.0	10.91	100.0	7.87	100.0	7.88	98.5	1195.49	1195.60
22	Ac-[D-Tyr^{46},Pic(3)^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.17	96.5	11.17	95.5	7.92	100.0	7.93	100.0	1223.83	1223.63
23	Ac-[D-Tyr^{46},Tic^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.95	100.0	11.95	100.0	7.89	100.0	7.90	100.0	1271.39	1271.63
24	Ac-[D-Tyr^{46},Hyp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.05	99.3	11.05	98.3	7.48	100.0	7.48	100.0	1225.79	1225.61
25	Ac-[D-Tyr^{46},Thz^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.43	97.3	11.43	95.6	7.73	100.0	7.74	100.0	1227.73	1227.57
26	Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Phe(2F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.05	100.0	12.05	100.0	7.50	100.0	7.51	100.0	1316.72	1316.63
27	Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.11	96.0	12.11	97.5	7.51	98.6	7.51	97.3	1316.67	1316.63
28	Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Phe(4F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.12	100.0	12.12	100.0	7.49	100.0	7.49	100.0	1316.16	1316.63
29	Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Phe(4Cl)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.45	100.0	12.45	100.0	7.64	100.0	7.65	100.0	1332.73	1332.60
30	Ac-[D-Tyr^{46},Trp^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	12.25	98.5	12.25	97.4	7.44	100.0	7.45	100.0	1316.96	1316.63
31	Ac-[D-Tyr^{46},Lys^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	10.88	96.4	10.88	97.5	12.95	99.3	12.95	100.0	1258.45	1258.65
32	Ac-[D-Tyr^{46},Glu^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.21	98.5	11.21	96.9	7.18	97.7	7.19	95.0	1259.88	1259.60
33	Ac-[D-Tyr^{46},Gly^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.19	97.5	11.19	97.5	7.60	95.1	7.60	95.0	1187.29	1187.58
34	Ac-[D-Tyr^{46},Aib^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.82	100.0	11.82	97.7	7.41	100.0	7.40	97.4	1215.55	1215.61
35	Ac-[D-Tyr^{46},Hyp^{47},Thr^{49},azaGly^{51},Phe(3F)^{50},Arg(Me)^{53},Trp^{54}]metastin(46-54)	11.23	99.1	11.23	100.0	7.45	97.3	7.46	98.2	1243.82	1243.60

[a] Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped column (2.0 × 50 mm) at a wavelength of 210 nm. Peptides were eluted with a linear gradient 5-75% acetonitrile in water containing 0.1% TFA over 14 min (0.5 mL/min). [b] Retention times and purities of peptides were characterized by reversed phase HPLC analyses at a wavelength of 254 nm. [c] Retention times and purities of peptides were characterized by ion exchange HPLC analyses performed with a Shimadzu gradient system using Phenomenex Luna® 5 μm SCX 100 Å column (4.6 × 100 mm) at a wavelength of 210 nm. Peptides were eluted with a linear gradient with eluants A/B = 100/0–0/100 (20 min) using 20 mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluant A and 0.5 M NaCl-containing 20 mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluant B at a flow rate of 1.0 mL/min. [d] Retention times and purities of peptides were characterized by ion exchange HPLC analyses at a wavelength of 254 nm. [e] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

3. Calcium Mobilization Assay

The stable expression cell line hOT7T175 was obtained by transfection of the expression plasmid pAK-rO7T175 for animal cells into CHO/dhfr cells, using a CellPhect Transfection Kit (Amersham Pharmacia Biotech, Inc.) First, 240 μL of Buffer A (included in the CellPhect Transfection Kit) was added to 9.6 μg of plasmid DNA dissolved in 240 μL of distilled water followed by stirring. After the mixture was settled for 10 minutes, 480 μL of Buffer B (included in the CellPhect Transfection Kit) was added to the mixture, which was vigorously stirred to form liposomes containing the DNA. Then, 4 × 105 CHO/dhfr- cells (obtained from ATCC) were inoculated on a 60-mm Petri dish. After culturing the cells in Ham's F-12 medium (Nissui Seiyaku Co., Ltd) supplemented with 10% fetal bovine serum (BIO WHITTAKER, Inc.) at 37°C for two days in 5% CO_2 gas, 480 μL of the liposomes were added drop-wise to the cells on the Petri dish. After culturing the cells at 37°C for six hours in 5% CO_2 gas, the cells were washed twice with serum-free Ham's F-12 medium and 3 mL of 15% glycerol was added to the cells on the Petri dish followed by treatment for two minutes. The cells were again washed twice with serum-free Ham's F-12 medium followed by incubation in Ham's F-12 medium supplemented with 10% fetal bovine serum at 37°C for 15 hours in 5% CO_2 gas. The cells were dispersed by trypsin treatment for recovery from the Petri dish. The recovered cells were inoculated on a six-well plate in 1.25 × 104 cells/well and incubation was initiated at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Nissui Seiyaku Co., Ltd.) containing 10% dialyzed fetal bovine serum (hereinafter abbreviated as dFBS)

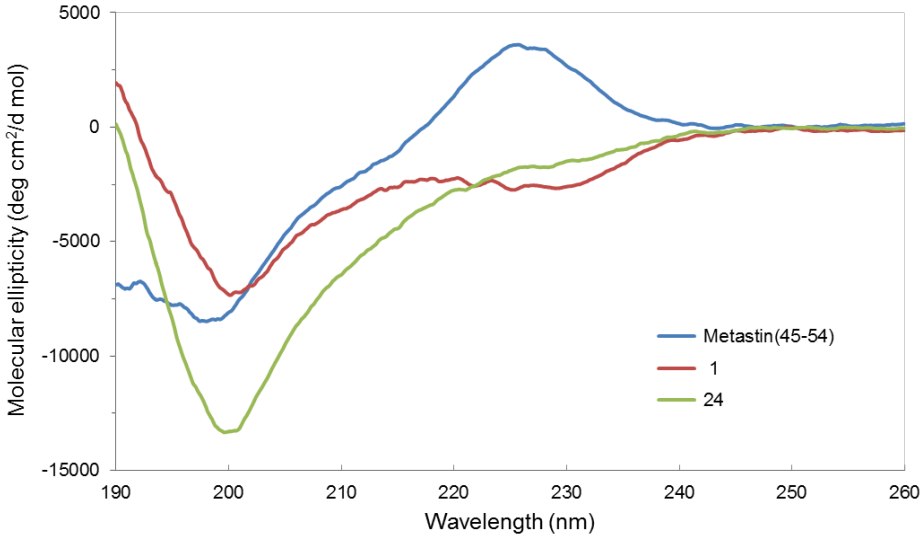
(JRH BIOSCIENCES, Inc.) in 5% CO_2 gas. The plasmid-transfected CHO transformants grew in the medium, but the non-transfected cells gradually died, accordingly the medium was exchanged on day 1 and 2 after initiation of incubation to remove the dead cells. Approximately 20 colonies of CHO transformants that kept growing on days 8 to 10 after incubation were isolated. From the cells in these colonies, cells showing high reactivity with the ligand peptide metastin (hereinafter merely referred to as hOT7T175/CHO) were selected for the following experiment.

The intracellular Ca ion level-increasing activity of the synthetic peptide in hOT7T175/CHO was determined using FLIPR (Molecular Devices, Inc.). hOT7T175/CHO was subcultured in DMEM supplemented with 10% dFBS (10% dFBS/DMEM) for the experiment. The hOT7T175/CHO was suspended in 10% dFBS/DMEM with 15×10^4 cells/mL. The suspension was inoculated on a 96-well plate for FLIPR (Black Plate Clear Bottom, Coster, Inc.) at 200 μL/well (3.0 × 104 cells/200 μL/well), followed by incubation at 37°C overnight in a 5% CO_2 incubator (hereinafter referred to as the cell plate). Then, 21 mL of HANKS/HBSS (9.8 g of HANKS, 0.35 g of sodium hydroxide, 20 mL of 1M HEPES, after adjusting the pH to 7.4 with 1N sodium hydroxide, the mixture was sterilized by filtration), 210 μL of 250-mM Pobenecid and 210 μL of FBS (HANKS/HBSS-Probenecid-FBS) were mixed.

Furthermore, two vials of Fluo 3-AM (50 μg/vial) were dissolved in 21 μL of dimethylsulfoxide and 21 μL of 20% pluronic acid. The resulting solution was added to and mixed with 10 mL of the HANKS/HBSS-Probenecid-FBS described above.

After the culture medium was removed, the mixture was dispensed onto the cell plate at 100 μL each/well, followed by incubation at 37°C for one hour in a 5% CO_2 incubator (pigment loading). The peptide was dissolved in dimethylsulfoxide at 1×10^4 M. The peptide solution was diluted with HANKS/HBSS containing 2.5-mM probenecid, 0.2% BSA and 0.1% CHAPS. The dilution was then transferred to a 96-well plate for FLIPR (V-Bottom plate, Coster, Inc.) (hereinafter referred to as the sample plate). After completion of pigment loading onto the cell plate, the cell plate was washed four times with wash buffer, which was prepared by adding 2.5-mM probenecid to HANKS/HBSS, using a plate washer to leave 100 μL of the wash buffer after the washing. The cell plate and the sample plate were set in FLIPR and 0.05 mL of a sample from the sample plate was automatically transferred to the cell plate with the FLIPR device to promote the cell response. A change in the intracellular calcium ion level for 180 seconds was measured against the passage of time. The EC_{50} values of intracellular Ca ion-increasing activity of metastin (45-54) and samples are shown in Table 1.

4. Molecular formula strings and the associated biochemical and biological data

Compound_ID	SMILES	agonist activities EC50 nM (95% CI), human	agonist activities EC50 nM (95% CI), rat
1	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O)=O	0.33 (0.25-0.44)	1.3 (1.1-1.6)
2	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](C)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	2.7 (0.92-8.1)	16 (7.9-33)
3	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC(C)C)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	2.0 (0.71-5.9)	12 (5.7-26)
4	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CCCCN)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	3.6 (1.1-11)	18 (6.5-50)
5	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CCC(O)=O)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	22 (9.7-52)	40 (21-79)
6	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	0.61 (0.25-1.5)	4.8 (2.5-9.3)
7	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCCCN)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	1.6 (1.1-2.3)	6.8 (3.3-14)
8	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCC(O)=O)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	2.8 (2.1-3.6)	11 (5.9-21)
9	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(CNC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	5.2 (2.3-12)	46 (10-200)
10	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](C)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	1.2 (0.76-2.0)	6.0 (3.2-11)
11	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(C(C)C)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	2.4 (1.6-3.6)	17 (6.4-47)
12	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](C)N(C)C)[C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	4.4 (1.5-13)	43 (15-130)
13	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](C)N(C)C)[C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	1.8 (1.4-2.4)	7.3 (5.9-9.0)
14	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(CN(C)C)[C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O	4.9 (3.0-8.1)	20 (15-30)
15	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(CCNC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	3.3 (2.0-5.4)	11 (6.4-18)
16	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(C4=C(C=CC=C4)NC([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O)=O	5.2 (2.5-11)	47 (11-200)
17	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCC4)N4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O	0.48 (0.20-1.2)	9.3 (7.6-11)
18	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCC4)N4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O	5.8 (3.0-11)	110 (96-130)
19	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CC4)N4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O	1.9 (1.1-3.4)	16 (9.7-26)
20	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCCC4)N4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O)=O	2.8 (1.5-5.3)	17 (11-28)
21	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(C(C4)CN4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O)=O	2.3 (1.3-4.3)	15 (10-21)
22	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H]4CN(CCC4)C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O)=O	5.5 (4.3-7.0)	45 (23-85)
23	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CC(C=CC=C4)=C4C5)N5C([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	0.49 (0.20-1.2)	7.0 (4.0-12)
24	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H]4N(C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)C[C@H](O)C4)=O)=O)=O)=O)=O	5.2 (2.3-12)	36 (15-81)
25	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CSC4)N4C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)=O)=O)=O)=O	2.3 (1.3-4.3)	23 (10-54)
26	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC=C3)P)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)N(C)[C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	0.65 (0.31-1.4)	1.8 (0.91-3.6)
27	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC(F)=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	1.4 (1.1-1.8)	4.8 (3.3-7.1)
28	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=C(F)C=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	1.9 (0.62-6.0)	4.7 (1.8-13)
29	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=C(Cl)C=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	0.56 (0.27-1.1)	2.8 (1.7-4.8)
30	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC(F)=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@@H](CC4=CNC5=C4C=CC=C5)NC([C@@H](CC6=CC=C(O)C=C6)NC(C)=O)=O)=O)=O)=O)=O	0.073 (0.043-0.12)	1.2 (0.80-1.9)
31	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC(F)=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCCCN)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	0.43 (0.16-1.1)	7.1 (5.8-8.6)
32	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC(F)=CC=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H](CCC(O)=O)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	0.47 (0.27-0.84)	20 (14-28)
33	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC(F)=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(CNC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	1.1 (0.81-1.6)	7.4 (7.0-8.0)
34	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC(F)=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC(C(C)(C)NC([C@@H](CC4=CC=C(O)C=C4)NC(C)=O)=O)=O)=O)=O)=O	1.2 (0.81-1.7)	7.0 (4.7-11)
35	O=C(N)[C@H](CC1=CNC2=C1C=CC=C2)NC([C@H](CCCNC(NC)=N)NC([C@H](CC(C)C)NC(NNC([C@H](CC3=CC=CC(F)=C3)NC([C@H]([C@@H](C)O)NC([C@H](CC(N)=O)NC([C@H]4N(C([C@@H](CC5=CC=C(O)C=C5)NC(C)=O)=O)C[C@H](O)C4)=O)=O)=O)=O)=O	0.75 (0.36-1.6)	8.5 (6.5-11)

5. Figure S1. Far-UV circular dichroism spectra of metastin analogues, metastin (45-54) (blue), **1** (red) and **24** (green) in distilled water at a concentration of 0.1 mg/mL. The CD spectra of metastin, **1** and **24** were obtained with a JASCO J-820 CD spectropolarimeter. Peptide solutions were prepared in distilled water at a concentration of 0.1 mg/mL and their spectra were measured at 20°C in a 10-mm-path-length cuvette. Molar ellipticities were recorded between wavelengths of 190 and 260 nm by changing the distance in 0.1 nm decrements. The scan speed, bandwidth and response time were 100 nm/min, 1.0 nm and 1 sec, respectively. Each spectrum for each peptide was an average of ten acquisitions. All CD data is reported as mean residue ellipticity, [θ] (deg cm^2/d mol), and analyzed by JWSSE-480 Protein Secondary Structure Estimation Program (JASCO) to obtain the proportion of α-helices, β-sheets, turns, and random conformations using Yang's and Reed's references. Analysis of the spectrum of **1** using the Spectra Manager software indicated 2.3% α-helices, 34.8% ß-sheets, 8.4% turns and 54.6% random conformations. In contrast, the dichroism analysis of **24** showed a slight decrease in β-sheets content (21.9%).