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Physicochemically and Pharmacokinetically Stable Nonapeptide KISS1 Receptor Agonists with Highly Potent Testosterone-Suppressive Activity

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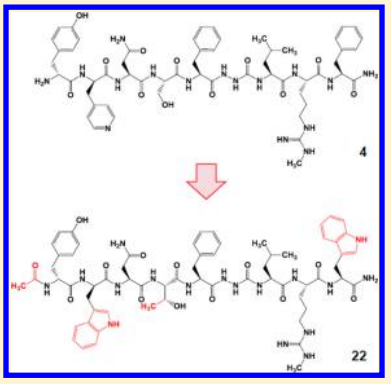
Supporting Information

ABSTRACT: Modifications of metastin(45−54) produced peptide analogues with higher metabolic stability than metastin(45−54). N-terminally truncated nonapeptide **4** ([D-Tyr^{46},D-Pya(4)^{47},azaGly^{51},Arg(Me)^{53}]metastin(46−54)) is a representative compound with both potent agonistic activity and metabolic stability. Although **4** had more potent testosterone-suppressant activity than metastin, it possessed physicochemical instability at pH 7 and insufficient in vivo activity. Instability at pH 7 was dependent upon Asn^{48} and Ser^{49}; substitution of Ser^{49} with Thr^{49} reduced this instability and maintained KISS1 receptor agonistic activity. Furthermore, [D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Arg-(Me)^{53},Trp^{54}]metastin(46−54) (**14**) showed 2-fold greater $[Ca^{2+}]_i$-mobilizing activity than metastin(45−54) and an apparent increase in physicochemical stability. N-terminal acetylation of **14** resulted in the most potent analogue, **22** (Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46−54)). With continuous administration, **22** possessed 10−50-fold more potent testosterone-suppressive activity in rats than **4**. These results suggested that a controlled release of short-length KISS1 receptor agonists can suppress the hypothalamic−pituitary−gonadal axis and reduce testosterone levels. Compound **22** was selected for further preclinical evaluation for hormone-dependent diseases.



INTRODUCTION

Metastin/kisspeptin, a 54-amino acid peptide, is a ligand of the orphan G-protein-coupled receptor hOT7T175, also known as AXOR12, and GPR54, currently renamed the KISS1 receptor, KISS1R.[1−6] KISS1R is most closely related to the galanin receptor family with approximately 45% homology; however, it binds neither galanin nor galanin-like peptide.[7] Several neuropeptides, including the RFamide and RWamide families, have been identified as ligands for KISS1R.[8] Metastin neurons have been mapped in several species, including fish, rodents, and humans. The strongest KISS1R expression has been observed in hypothalamic tissues, with little expression in other brain areas.[4−11]

Although first described in the context of cancer metastasis,[1,2] metastin−KISS1R signaling has subsequently been shown to play a vital role in reproduction via regulation of the HPG axis. In 2003, two independent studies reported that inactivation mutations of KISS1R led to idiopathic hypogonadotropic hypogonadism in humans and mice.[9,10] Further research suggests that metastin potently elicits GnRH and gonadotropin secretion in mammals and non-mammalian species.[4,12]

In preclinical studies, single administration of metastin markedly stimulated FSH/LH release in rat models,[13] and continuous administration reduced testosterone levels.[14] The molecular mechanisms for KISS1R signaling suggest that appropriate dosing of KISS1R agonists should activate or suppress the HPG axis, thereby potentially preventing or treating a number of sex-hormone-dependent diseases.

An N-terminally truncated metastin(45−54) analogue has demonstrated biological activity in vitro.[1] This fragment peptide was 3−10 times more potent than metastin, whereas the truncated metastin(46−54) was less potent than full-length metastin,[5] indicating that the core residues for biological activity are located in C-terminal short peptides.

In our previous studies, rational modification of metastin(45−54) resulted in [D-Tyr^{45},Arg(Me)^{53}]metastin(45−54) (**2**), which demonstrated higher metabolic stability than metastin(45−54).[15,16] Substitutions of D-Tyr at position 45 and N^{ω}-methylarginine [Arg(Me)] at position 53 eliminated N-terminal and C-terminal degradation, respectively, and retained

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KISS1R activity. Metabolic stability was further improved by azaGly[51] substitution; deletion of Asn[46] and substitution of Trp[47] produced highly potent and long-acting KISS1R agonists, e.g., [D-Tyr[46],D-Pya(4)[47],azaGly[51],Arg(Me)[53]]metastin(46−54), compound **4**.[17]

Compound **4** was the first short-length metastin analogue to show testosterone suppression activity in vivo, as evidenced by reduced plasma testosterone levels in male rats upon continuous administration of **4**.[17] This result suggested that metabolically stable metastin analogues could form the basis for the development of therapeutic candidates for hormone-dependent diseases such as prostate cancer.

The chemical stability of **4**, containing Asn−Ser residues, was investigated using a range of analytical techniques.[18] Hydrolysis was monitored by changes in mass, size, charge, hydrophobicity, and UV absorption. Compound **4** was incubated at 37 °C in aqueous buffer solutions at four different pH levels. Analyses by reversed-phase HPLC showed the deamidation of Asn[48] of **4** with the formation of the two aspartyl derivatives [D-Tyr[46],D-Pya(4)[47],Asp[48],azaGly[51],Arg(Me)[53]]metastin(46−54) and [D-Tyr[46],D-Pya(4)[47],β-Asp[48],azaGly[51],Arg(Me)[53]]metastin-(46−54). Residual compound **4** was quantified by UV absorption via HPLC chromatography. **4** was stable in buffer solutions of pH 3 and 5; however, deamidation was observed at pH 1.2 and 7, and the rate of deamination was temperature-dependent. The decomposition products obtained at pH 7 were assumed to be Asp[48] and β-Asp[48] derivatives by LC−MS molecular weight and HPLC retention time compared with those of known compounds.[19,20] These results suggested that physicochemically more stable analogues would be needed for the development of useful therapeutic candidates.

In this study, we designed and synthesized metastin analogues that show greater agonistic activities and physicochemically and pharmacokinetically more stable profiles than those of compound **4**.

RESULTS

Chemistry. All peptides were synthesized by standard Fmoc-based solid-phase synthetic methods. The obtained crude peptides were purified to homogeneity using preparative HPLC. The purity of each peptide was ascertained by analytical HPLC, and the structure assignment was performed by MALDI-TOF MS.

Biological Activity. Synthesized analogues were characterized in two assays of human KISS1R (hKISS1R) receptor activity using an intracellular calcium mobilization FLIPR assay and a cell membrane binding assay. The FLIPR assay used CHO cells stably expressing recombinant hKISS1R, and the binding assay used membrane fractions isolated from hKISS1R-expressing CHO cells.

The maximum response observed in the presence of compound was corrected for baseline measurements and expressed as a percentage of the corrected response to a maximal concentration of control metastin(45−54). Nonlinear regression of normalized data was performed to estimate the potency (EC_{50}) of selected compounds.

Design and Synthesis of Nonapeptide Metastin Analogues Substituted at Positions 48 and 49. The irreversible, spontaneous, and nonenzymatic deamidation of Asn residues is one of the major chemical degradation pathways for peptides and proteins. This occurs frequently during synthesis, purification, sequencing, and storage[21,22] and introduces undesired heterogeneity into peptide and protein preparations. Intramolecular formation of an Asu peptide intermediate is known to be the first step in these reactions, as shown in Figure 1. The mechanism of succinimide formation

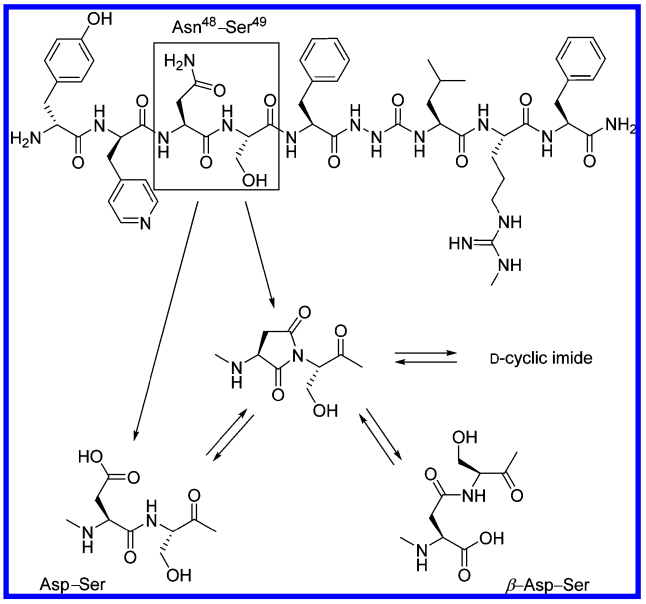

Figure 1. Scheme of the deamidation reaction of Asn-containing metastin analogue **4** in aqueous solution.

involves deprotonation of the backbone amide of Ser[49] followed by attack of the anionic nitrogen on the carbonyl group of Asn[48]. Neighboring amino acid residues that allow for chain flexibility and hydrogen-bonding interactions, such as Gly or Ser, facilitate the formation of the Asu intermediate. The subsequent hydrolysis of the Asu intermediate occurs on either side of the imide nitrogen, leading to the formation of α-aspartyl (Asp) or β-aspartyl (or isoaspartic acid, isoAsp). The rate of this degradation process is dependent upon the primary sequence of the peptide, temperature, buffer pH, and concentration.[23−28]

To initiate this research, we synthesized compound **5** where Phe[54] of compound **4** was substituted with Trp[54]. In our previous metastin research, Trp[54] analogue **1** had shown the highest receptor affinity (data not shown). The substitution of Phe[54] with Trp[54] resulted in 3-fold greater in vitro human activity of **5** compared with **4**, as shown in Table 1. On the other hand, in vitro rat activities of **4** and **5** were comparable (Table 1). However, plasma testosterone concentrations were below the lower limit of detection (0.04 ng/mL) in two of five rats when **5** was administered at a dose of 0.3 nmol/h (zero of five rats after dosing of **4**) (Table 2). It is supposed that the higher in vivo activity of **5** than **4** was dependent on its pharmacokinetic profiles, such as CL_{total}.

To reduce deamination, further chemical modifications of **5** comprising substitutions of Ser[49] with β-branched amino acids such as Thr (**6**), Val (**7**), Phg (**8**), and Tle (**9**) were carried out. Trp[49] analogue **10** was also synthesized as a representative compound with a bulky side chain. Substitution at position 49 with cyclic amino acids such as Pro (**11**) and *trans*-4-Hyp (**12**) was expected to avoid cyclic imide formation completely.

All amino acid replacements at position 49 of **5** showed excellent $[Ca^{2+}]_i$-mobilizing activities for hKISS1R (Table 1). Among compounds with β-branches or a cyclic amino acid at position 49, the Thr (**6**), Val (**7**), and Pro (**11**) derivatives had

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Table 1. Biological Activities of Nonapeptide Metastin Analogues H-AA45-AA46-AA47-AA48-AA49-Phe-AA51-Leu-AA53-AA54-NH_2 Substituted between Positions 47 and 54

									agonist activity, EC_{50},[a] nM (95% CI)		affinity, K_i,[b] nM (95% CI)	
compd	AA45	AA46	AA47	AA48	AA49	AA51	AA53	AA54	human	rat	human	rat
metastin(45−54)	Tyr	Asn	Trp	Asn	Ser	Gly	Arg	Phe	0.96 (0.55−1.7)	4.2 (3.0−6.1)	0.035 (0.029−0.044)	0.088 (0.073−0.11)
1	Tyr	Asn	Trp	Asn	Ser	Gly	Arg	Trp	0.44 (0.25−0.77)	1.3 (0.88−1.9)	0.026 (0.020−0.033)	ND[c]
4		D-Tyr	D-Pya(4)	Asn	Ser	azaGly	Arg(Me)	Phe	2.6 (1.9−3.5)	10 (8.0−12)	0.11 (0.092−0.12)	0.14 (0.12−0.17)
5		D-Tyr	D-Pya(4)	Asn	Ser	azaGly	Arg(Me)	Trp	0.87 (0.64−1.2)	6.0 (5.6−6.5)	0.074 (0.059−0.093)	0.13 (0.10−0.17)
6		D-Tyr	D-Pya(4)	Asn	Thr	azaGly	Arg(Me)	Trp	1.6 (1.3−1.9)	5.7 (4.4−7.2)	0.086 (0.069−0.11)	ND[c]
7		D-Tyr	D-Pya(4)	Asn	Val	azaGly	Arg(Me)	Trp	1.8 (1.1−2.7)	12 (7.5−18)	0.052 (0.039−0.069)	ND[c]
8		D-Tyr	D-Pya(4)	Asn	Phg	azaGly	Arg(Me)	Trp	4.4 (3.7−5.2)	110 (85−130)	ND[c]	ND[c]
9		D-Tyr	D-Pya(4)	Asn	Tle	azaGly	Arg(Me)	Trp	1.5 (1.2−1.9)	22 (17−27)	ND[c]	ND[c]
10		D-Tyr	D-Pya(4)	Asn	Trp	azaGly	Arg(Me)	Trp	0.59 (0.39−0.88)	7.4 (5.9−9.2)	ND[c]	ND[c]
11		D-Tyr	D-Pya(4)	Asn	Pro	azaGly	Arg(Me)	Trp	1.2 (0.89−1.6)	13 (9.2−17)	0.096 (0.080−0.12)	ND[c]
12		D-Tyr	D-Pya(4)	Asn	Hyp	azaGly	Arg(Me)	Trp	2.8 (2.1−3.9)	95 (68−130)	ND[c]	ND[c]
13		D-Tyr	D-Trp	Asn	Ser	azaGly	Arg(Me)	Trp	0.51 (0.43−0.62)	2.3 (1.2−4.4)	0.037 (0.030−0.045)	0.077 (0.062−0.095)
14		D-Tyr	D-Trp	Asn	Thr	azaGly	Arg(Me)	Trp	1.0 (0.77−1.3)	4.1 (2.9−5.8)	0.036 (0.029−0.046)	ND[c]
15		D-Tyr	D-Trp	Asn	Val	azaGly	Arg(Me)	Trp	0.31 (0.17−0.56)	2.2 (0.56−8.7)	0.028 (0.022−0.035)	ND[c]
16		D-Tyr	D-Pya(4)	Ala	Ser	azaGly	Arg(Me)	Trp	0.70 (0.48−1.0)	9.3 (7.3−12)	0.20 (0.17−0.25)	3.6 (2.7−4.6)
17		D-Tyr	D-Pya(4)	Thr	Ser	azaGly	Arg(Me)	Trp	1.3 (1.0−1.8)	32 (19−54)	ND[c]	ND[c]
18		D-Tyr	D-Trp	Ala	Ser	azaGly	Arg(Me)	Trp	0.49 (0.28−0.85)	6.8 (5.1−9.2)	ND[c]	ND[c]
19		D-Tyr	D-Trp	Ala	Thr	azaGly	Arg(Me)	Trp	0.63 (0.40−1.0)	13 (8.5−19)	ND[c]	ND[c]
20		D-Tyr	D-Trp	Abu	Ser	azaGly	Arg(Me)	Trp	0.64 (0.37−1.1)	2.8 (1.8−4.2)	0.12 (0.10−0.13)	0.83 (0.64−1.1)

[a] EC_{50} values of agonist activities were determined as the concentrations of peptide analogues that gave half-maximum $[Ca^{2+}]$-mobilizing activities. [b] K_i values of peptide analogues were the concentrations required to displace radiolabel binding by 50%. [c] Not determined.

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Table 2. Testosterone-Suppressive Activities of Nonapeptide Metastin Analogues H-D-Tyr-AA47-AA48-AA49-Phe-azaGly-Leu-Arg(Me)-AA54-NH_2 Substituted between Positions 47 and 54

					testosterone-suppressive activity,[a] rats with testosterone levels of <0.04 ng/mL [*n* (%)]			
compd	AA47	AA48	AA49	AA54	2 nmol/h	0.5 nmol/h	0.3 nmol/h	0.1 nmol/h
4	D-Pya(4)	Asn	Ser	Phe	5 (100)	3 (60)	0	0
5	D-Pya(4)	Asn	Ser	Trp	4 (80)	3 (60)	2 (40)	0
7	D-Pya(4)	Asn	Val	Trp	4 (80)	0	ND[b]	ND[b]
11	D-Pya(4)	Asn	Pro	Trp	ND[b]	1 (20)	ND[b]	ND[b]
13	D-Trp	Asn	Ser	Trp	ND[b]	4 (80)	2 (40)	0
14	D-Trp	Asn	Thr	Trp	5 (100)	3 (60)	3 (60)	0
15	D-Trp	Asn	Val	Trp	ND[b]	0	ND[b]	ND[b]
16	D-Pya(4)	Ala	Ser	Trp	0	ND[b]	ND[b]	ND[b]
20	D-Trp	Abu	Ser	Trp	0	ND[b]	ND[b]	ND[b]

[a]Plasma testosterone-suppressive activities of peptide analogues at 2, 0.5, 0.3, or 0.1 nmol/h. Peptides were dissolved in distilled water to prepare 2, 0.5, 0.3, or 0.1 mM solutions used in ALZET osmotic pumps implanted subcutaneously in five Crl:CD(SD) male rats. After 6 days, plasma testosterone levels were measured using RIA. Testosterone suppression was expressed as the number of rats with plasma testosterone levels below the limit of detection (0.04 ng/mL). [b]Not determined.

better agonistic activities in the FLIPR hKISS1R assay than the other peptides. The in vivo activity of selected compounds was evaluated by testosterone-suppressive activity in rats (Table 2). Six days of continuous administration of compound **7** at 2 nmol/h attenuated the plasma testosterone concentration in rats; however, at 0.5 nmol/h, **7** demonstrated less activity than **5**. Three D-Trp^{47} analogues (**13**, **14**, **15**) with Ser^{49}, Thr^{49}, and Val^{49}, respectively, were expected to improve KISS1R agonistic activities due to the hydrophobic interaction at the side chain of D-Trp^{47} with KISS1R. All derivatives exhibited high functional activities of both human and rat receptors (Table 1). [D-Trp^{47},Thr^{49}] analogue **14** possessed higher agonistic activity than **5**. Moreover, **14** reduced plasma testosterone at a dose of 0.3 nmol/h. On the other hand, Val^{49} derivative **15** dosed at 0.5 nmol/h did not reduce the testosterone concentration in rats.

Since the Asn^{48} residue caused the decomposition of **4**, the Ala (**16**) and Thr (**17**) analogues of **5** were synthesized to avoid the deamidation reaction. Ala analogue **16** retained agonistic activity similar to that of **5** for both the human and rat receptor. However, 2 nmol/h continuous administration of **16** was not associated with reduced testosterone concentration in rats. Combined amino acid replacements at positions 47 and 48 yielded three analogues, [D-Trp^{47},Ala^{48},Ser^{49}] (**18**), [D-Trp^{47},Ala^{48},Thr^{49}] (**19**), and [D-Trp^{47},Abu^{48},Ser^{49}] (**20**), which showed excellent agonistic activity. However, 2 nmol/h continuous administration of **20**, which possessed the highest in vitro activity of these three analogues in rats, did not suppress plasma testosterone in vivo. On the basis of the results of compounds **16** and **20**, the Asn^{48} residue was proposed to be essential for testosterone-suppressive activity in vivo.

We concluded that the combination of D-Trp^{47} and Thr^{49} in **14** gave an optimal core structure in terms of both stability in solution and biological activity.

Physicochemical Properties of Metastin Analogues 13 and 14. The effect of Thr^{49} substitution on the deamination of metastin analogues was evaluated by comparing the stability of Ser^{49} analogue **13** in solution with that of Thr^{49} analogue **14**. Compounds **13** and **14** were incubated at 37 °C in aqueous solution at four different pH values, 1.2, 3, 5, and 7.

The residual amount of **13** and **14** was quantified respectively by UV absorption using HPLC chromatography. Although **13** and **14** were stable in buffer solution at pH 3 and 5, deamination of **13** was observed in solution at pH 1.2 and 7 (Table 3). The deamination rate of **13** at pH 7 was as fast as that of **4**, with 28.0% of the product remaining after a 2-week incubation period. In contrast, 72.8% of **14** remained after a 2-week incubation at pH 7. Thr^{49} replacement was therefore considered to contribute to decreased succinimide formation and improved solution stability in neutral buffer.

Table 3. Stability Studies of Ser^{49} Analogues 4 and 13, and Thr^{49} Analogue 14 in Aqueous Solution

		residual ratio[a] (%)			
compd	time	pH 1.2 (JP1)	pH 3	pH 5	pH 7
4	initial	100.0	100.0	100.0	100.0
	1 week	60.3	99.4	94.8	54.9
	2 week	32.8	100.5	91.2	28.0
13	initial	100.0	100.0	100.0	100.0
	1 week	60.8	98.2	96.0	56.5
	2 week	33.4	97.1	90.8	28.0
14	initial	100.0	100.0	100.0	100.0
	1 week	64.9	98.7	98.8	84.9
	2 week	39.1	98.2	96.5	72.8

[a]Aqueous solutions of peptides at pH 1.2 (JP1), 3, 5, or 7 were incubated at 37 °C for up to 2 weeks. The amount of peptide in the buffer solutions was determined from the area under the HPLC peaks. The percentage of a given compound recovered from the buffer solutions after incubation (stability index) was calculated relative to the amount of peptide at time 0 taken as 100%.

Design and Synthesis of Nonapeptide Metastin Analogues Modified at the N-Terminus. Peptide metabolism and excretion are associated with α-amino acid residues with a free amino group at the N-terminus. As such, N-terminal modifications were planned in an attempt to further improve the testosterone-suppressive activity of **14**. For N-terminal modification, deaminated analogue **21** and acylated analogues **22**–**25** of **14** were synthesized. Acetylated **22**, butylated **23**, cyclopropylcarbonylated **24**, and benzoylated analogue **25** were selected as compounds with representative acyl moieties, i.e., aliphatic, alicyclic, and aromatic moieties. The in vivo activities of these peptides were evaluated using 50% DMSO as the primary solvent due to the low water solubility of several of these peptides.

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All analogues possessed excellent agonistic activities, and the $[Ca^{2+}]_i$-mobilizing activities were more potent than that of metastin(45−54) (Table 4). Among these analogues, N-terminal acetylated analogue **22** showed a significant increase in testosterone-suppressive activities (Table 5). Plasma testosterone concentrations in rats given 0.05 nmol/h of **22** were below the limit of detection (0.04 ng/mL) in RIA. Moreover, the 0.03 nmol/h dose of **22** attenuated the testosterone levels, with more than 20-fold higher activity than **4**. This improvement was thought to result from N-terminal acetylation. The pharmacokinetic profiles of **22** are discussed below.

Although other N-terminal acylated analogues, **23**−**25**, retained high in vitro activities, testosterone-suppressive activities were weaker compared with that of the acetylated analogue **22**. Increased hydrophobicity of N-terminal acylated analogues **23**−**25** may result in their lower BA after subcutaneous administration. In addition, the in vivo activity of N-terminal deaminated analogue **21** was less than that of **22**, although **21** had more potent agonistic activity than **22**.

N-terminal acetylation was considered the most effective approach for improving in vivo activity in this series of peptides.[29] The N-terminal acetamido moiety of **22** was thought to contribute to not only the avoidance of N-terminal cleavage but also the improved penetration into blood. The N-terminal acetylated Pya(4)[47] (**26**) and Phe[54] (**27**) analogues also attenuated the plasma testosterone concentration in all rats tested at the 0.05 nmol/h dose.

Physicochemical Profiles of Metastin Analogues 22, 26, and 27. The physicochemical stability of the highly potent analogues **22**, **26**, and **27** was evaluated using the same method as for **13** and **14** described above. All peptides maintained >95% purity after 2 weeks in pH 3 and 5 buffer solutions (Table 6). The deamination rates of **22**, **26**, and **27** at pH 7 were lower than that of **4**, as shown in Table 6, with 66.4%, 69.8%, and 67.6%, respectively, of product remaining after 2-week incubations. Thr[49] replacement contributed to decreased succinimide formation and improved solution stability in neutral buffer solution.

Pharmacokinetic Profiles of Metastin Analogues 14, 22, 26, and 27. The pharmacokinetic profiles of the prototype key compound **14** and potent analogues **22**, **26**, and **27** were evaluated using Crl:CD(SD) male rats. These peptides were administered intravenously (Table 7) or subcutaneously (Table 8) at a dose of 1 mg/kg. Although the plasma stability of **4** was high in vitro, 2 nmol/h continuous administration of **4** in Copenhagen rats was very low (1.1 ± 0.3 ng/mL).[30] The renal excretion of compound **4** via the urine was likely due to the hydrophilic nature of **4**. On the other hand, the value of V_{dSS} of **22** was 3-fold lower than that of **14**. **22** showed better phamacokinetic parameters compared with **14** after intravenous administration. The N-terminal acetylation effectively improved the AUC values when administered subcutaneously. The hydrophilic substitution of D-4-pyridylalanine [D-Pya(4)][47] (**26**) with D-Trp[47] (**22**) resulted in a small improvement in the BA value in subcutaneous administration. Another amino acid replacement with a smaller side chain, Phe[54] (**27**), produced higher BA values than **22**, although this replacement was not useful in terms of V_{dSS} and CL_{total} values.

Continuous Administration of Metastin Analogues 22, 26, and 27 in Male Rats. Three selected analogues, **22**, **26**, and **27**, were tested in an intact male rat assay (Figure 2). The purpose of this assay was to examine the steady-state

Table 4. Biological Activities of Nonapeptide Metastin Analogues R-AA[46]-AA[47]-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-AA[54]-NH_2 Substituted at the N-Terminus, Positions 46, 47, 49, and 54

compd	R	AA[46]	AA[47]	AA[49]	AA[54]	agonist activity, EC_{50},[a] nM (95% CI)		affinity, K_i,[b] nM (95% CI)	
						human	rat	human	rat
metastin(45−54)						0.96 (0.55−1.7)	4.2 (3.0−6.1)	0.035 (0.029−0.044)	0.088 (0.073−0.11)
21		deaminoTyr	D-Trp	Thr	Trp	0.30 (0.18−0.50)	1.1 (0.66−1.9)	0.039 (0.027−0.055)	ND[c]
22	Ac	D-Tyr	D-Trp	Thr	Trp	0.33 (0.25−0.44)	1.3 (1.1−1.6)	0.036 (0.026−0.051)	0.069 (0.056−0.084)
23	butyryl	D-Tyr	D-Trp	Thr	Trp	0.80 (0.53−1.2)	3.7 (1.7−8.1)	ND[c]	0.11 (0.090−0.14)
24	cyclopropylcarbonyl	D-Tyr	D-Trp	Thr	Trp	0.82 (0.61−1.1)	4.2 (2.3−7.5)	0.034 (0.025−0.046)	0.087 (0.071−0.11)
25	benzoyl	D-Tyr	D-Trp	Thr	Trp	0.44 (0.30−0.63)	1.7 (1.2−2.6)	0.027 (0.018−0.040)	ND[c]
26	Ac	D-Tyr	D-Pya(4)	Thr	Trp	0.75 (0.54−1.0)	4.1 (2.8−6.1)	0.089 (0.070−0.11)	0.17 (0.13−0.23)
27	Ac	D-Tyr	D-Trp	Thr	Phe	0.94 (0.72−1.2)	3.9 (2.5−6.2)	0.038 (0.030−0.049)	0.087 (0.069−0.11)

[a] EC_{50} values of agonist activities were determined as the concentrations of peptide analogues that gave half-maximum $[Ca^{2+}]$-mobilizing activities. [b] K_i values of peptide analogues were the concentrations required to displace radiolabel binding by 50%. [c] Not determined.

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Table 5. Testosterone-Suppressive Activities of Nonapeptide Metastin Analogues R-AA^{46}-AA^{47}-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-AA^{54}-NH$_2$ Substituted at the N-Terminus, Positions 46, 47, and 54

					testosterone-suppressive activity,[a] number of rats with testosterone levels of <0.04 ng/mL (max 5)			
compd	R	AA^{46}	AA^{47}	AA^{54}	0.1 nmol/h	0.05 mol/h	0.03 mol/h	0.01 mol/h
4		D-Tyr	D-Pya(4)	Phe	0	ND[b]	ND[b]	ND[b]
14		D-Tyr	D-Trp	Trp	0	ND[b]	ND[b]	ND[b]
21		deaminoTyr	D-Trp	Trp	5	ND[b]	ND[b]	0
22	Ac	D-Tyr	D-Trp	Trp	ND[b]	5	4	0
23	butyryl	D-Tyr	D-Trp	Trp	3	ND[b]	0	ND[b]
24	cyclopropylcarbonyl	D-Tyr	D-Trp	Trp	1	ND[b]	ND[b]	ND[b]
25	benzoyl	D-Tyr	D-Trp	Trp	0	ND[b]	ND[b]	ND[b]
26	Ac	D-Tyr	D-Pya(4)	Trp	ND[b]	5	ND[b]	2
27	Ac	D-Tyr	D-Trp	Phe	ND[b]	5	4	0

[a]Plasma testosterone-suppressive activities of peptide analogues at 2, 0.5, 0.3, or 0.1 nmol/h. Peptides were dissolved in 50% DMSO to prepare 2, 0.5, 0.3, or 0.1 mM solutions used in ALZET osmotic pumps implanted subcutaneously in five Crl:CD(SD) male rats. After 6 days, plasma testosterone levels were measured using RIA. Testosterone suppression was expressed as the number of rats with plasma testosterone levels below the limit of detection (0.04 ng/mL). [b]Not determined.

Table 6. Stability of Metastin Analogues 22, 26, and 27 in Aqueous Solutions

		residual ratio[a] (%)			
compd	time	pH 1.2 (JP1)	pH 3	pH 5	pH 7
22	initial	100.0	100.0	100.0	100.0
	1 week	55.6	97.7	95.9	79.7
	2 week	28.8	95.6	93.6	66.4
26	initial	100.0	100.0	100.0	100.0
	1 week	58.3	98.7	98.5	82.6
	2 week	33.0	96.8	97.0	69.8
27	initial	100.0	100.0	100.0	100.0
	1 week	50.6	97.5	98.1	84.5
	2 week	22.3	95.0	96.3	67.6

[a]Aqueous solutions of peptides at pH 1.2 (JP1), 3, 5, or 7 were incubated at 37 °C for up to 2 weeks. The amount of peptide in the buffer solutions was determined from the area under the HPLC peaks. The percentage of a given compound recovered from the buffer solutions after incubation (stability index) was calculated relative to the amount of peptide at time 0 taken as 100%.

concentration of plasma testosterone after 1 and 4 weeks of continuous administration of metastin analogues. These results provide a direct measure of the in vivo ability to suppress testosterone in an intact animal model. All infused analogues produced undetectable steady-state concentrations of testosterone for up to 1 week. Values below the limit of detection (0.04 ng/mL) in RIA were treated as 0.04.

Continuous administration of **26** and **27** at 30 or 100 pmol/h for 1 week suppressed testosterone in all five rats. Four weeks later, one of five rats dosed with 30 nmol/h of **26** or 100 nmol/h of **27** did not decrease plasma testosterone to undetectable levels. In contrast, all rats had decreased plasma testosterone to undetectable levels when **22** was administered at 30 and 100 pmol/h. These results suggested that **22** was the most potent of the three peptides. The minimum effective dose of **22** was 30 pmol/h in this study.

Single Administration of Compound 22 in Male Rats. The effect of single subcutaneous administration of **22**, metastin, or metastin(45−54) on plasma LH in male rats was examined at various concentrations from 0.01 to 1000 nmol/kg (Figure 3). Comparing AUC data for **22** and metastin, **22** was approximately 100-fold more potent than metastin in stimulating the release of LH.

Plasma testosterone levels were also elevated following single subcutaneous administration of **22**, metastin, or metastin(45−54) (Figure 4). At 0.01 nmol/kg **22** and 1 nmol/kg metastin or metastin(45−54), plasma testosterone levels were sensitive to changes in plasma LH levels. Thus, **22** had a potent effect on LH and testosterone release in male rats, most likely due to its improved agonistic activity against KISS1R and physicochemical stability in vivo. Moreover, there were no significant issues in preclinical toxicity studies in rats and dogs (data not shown). Safety margins based on the 4-week continuous dosing studies in rats and dogs were more than 1000-fold.

Table 7. Pharmacokinetic Parameters of Metastin Analogues after Intravenous Administration in Rats[a]

compd	**4**	**14**	**22**	**26**	**27**
C_{5min} (ng/mL)	969 ± 88	1443 ± 86	2509 ± 287	2072 ± 142	1449 ± 171
AUC_{0-24h} [(ng·h)/mL]	239 ± 9	503 ± 39	1225 ± 79	1262 ± 73	497 ± 98
MRT (h)	0.38 ± 0	0.73 ± 0.1	0.69 ± 0	0.69 ± 0.1	0.44 ± 0.1
V_{dss} (mL/kg)	1577 ± 110	1455 ± 227	562 ± 37	547 ± 37	896 ± 33
CL_{total} (mL/h/kg)	4196 ± 164	1997 ± 157	819 ± 52	794 ± 45	2067 ± 423

[a]Peptides were administered intravenously to SD rats at a dose of 1 mg/kg. Blood samples were collected at 5, 10, 15, and 30 min and 1, 2, 4, 8, and 24 h after injection. Plasma was collected by removing plasma proteins using 0.2% formic acid/methanol (1:4, v/v) and acetonitrile, followed by centrifugation at 15 000 rpm for 5 min. The obtained supernatant was treated with 0.4% aqueous formic acid and centrifuged at 15 000 rpm for 5 min. The plasma concentration of peptides was determined by LC−MS. C_{5min}, AUC_{0-24h}, MRT, V_{dss}, and CL_{total} were obtained for each rat after intravenous administration and are expressed as the mean ± standard deviation (n = 3).

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Table 8. Pharmacokinetic Parameters of Metastin Analogues after Subcutaneous Administration in Rats[a]

compd	4	14	22	26	27
C_{max} (ng/mL)	145 ± 20	47 ± 5	100 ± 44	90 ± 19	114 ± 13
T_{max} (h)	0.14 ± 0.1	0.19 ± 0.05	0.19 ± 0.10	0.42 ± 0.14	0.42 ± 0.1
AUC_{0-24h} [(ng·h)/mL]	96 ± 11	46 ± 9	115 ± 44	152 ± 36	144 ± 41
MRT (h)	0.61 ± 0.1	0.88 ± 0.1	0.86 ± 0.2	1.07 ± 0.1	0.88 ± 0.2
BA (%)	40.4 ± 4.9	9.2 ± 2.0	9.4 ± 3.6	12.1 ± 3.0	28.9 ± 10.1

[a]Peptides were administered subcutaneously to SD rats at a dose of 1 mg/kg. Blood samples were collected at 5, 10, 15, and 30 min and 1, 2, 4, 8, and 24 h after injection. Plasma was collected by removing plasma proteins using 0.2% formic acid/methanol (1:4, v/v) and acetonitrile, followed by centrifugation at 15 000 rpm for 5 min. The obtained supernatant was treated with 0.4% aqueous formic acid and centrifuged at 15 000 rpm for 5 min. The plasma concentration of peptides was determined by LC–MS. C_{max}, T_{max}, AUC_{0-24h}, MRT, and BA were obtained for each rat after subcutaneous administration and are expressed as the mean ± standard deviation (n = 3).

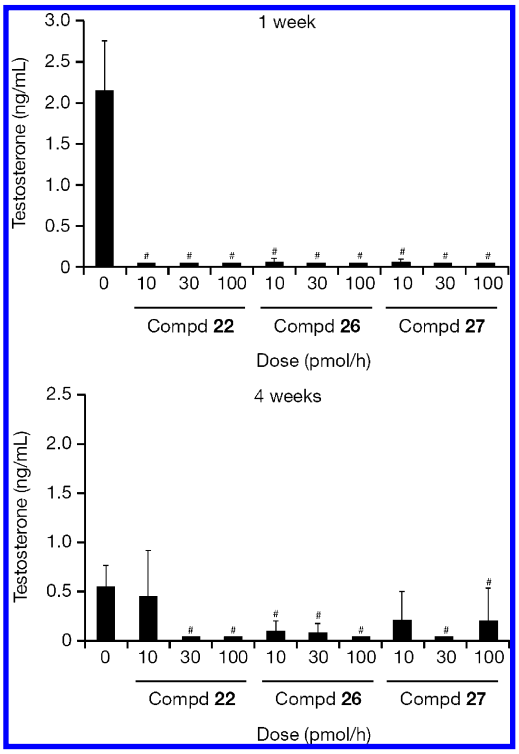

Figure 2. Plasma testosterone-suppressive activities by continuous administration of compound **22**, **26**, or **27** in male rats at 0, 10, 30, or 100 pmol/h. Compound **22**, **26**, or **27** dissolved in 50% DMSO was diluted in distilled water to prepare 10, 30, or 100 μM peptide solutions added to ALZET osmotic pumps which were implanted subcutaneously in five Crl:CD(SD) male rats. After 1 or 4 weeks, the plasma testosterone level of each rat was measured using RIA. The value below the limit of detection (0.04 ng/mL) in RIA was treated as 0.04. Each bar represents the mean ± standard deviation of plasma testosterone from five rats. A pound sign indicates $p \leq 0.025$ (one-tailed Shirley–Williams test) vs vehicle (0 pmol/h) group.

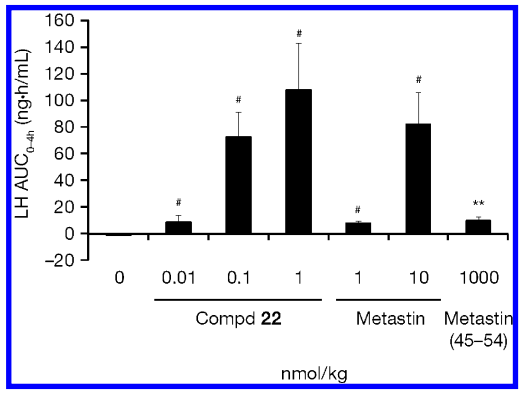

Figure 3. Effects of single subcutaneous administration of **22**, metastin, or metastin(45–54) on plasma LH in male rats at 0, 0.01, 0.1, 1, 10, or 1000 nmol/kg. Compound **22**, metastin, or metastin(45–54) was dissolved in 50% DMSO and administered subcutaneously in five Crl:CD(SD) male rats. LH AUC_{0-4h} was measured using blood samples (0.4 mL) obtained from the tail vein of **22**-, metastin-, or metastin(45–54)-treated animals before administration (0 h) and 0.5, 1, 2, 4, and 8 h after administration. Data are expressed as the mean ± standard deviation (n = 6). A pound sign and two asterisks indicate $p \leq 0.025$ (one-tailed Shirley–Williams test) and $p \leq 0.01$ (Aspin–Welch t test) vs vehicle (0 nmol/kg) group, respectively.

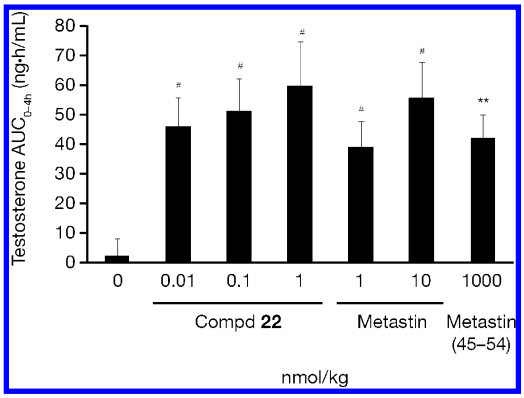

Figure 4. Effects of single subcutaneous administration of **22**, metastin, or metastin(45–54) on plasma LH in male rats at 0, 0.01, 0.1, 1, 10, or 1000 nmol/kg. Compound **22**, metastin, or metastin(45–54) was dissolved in 50% DMSO and administered subcutaneously in five Crl:CD(SD) male rats. Testosterone AUC_{0-4h} was measured using blood samples (0.4 mL) obtained from the tail vein of **22**-, metastin-, or metastin(45–54)-treated animals before administration (0 h) and 0.5, 1, 2, 4, and 8 h after administration. Each bar represents the mean ± standard deviation (n = 6). A pound sign and two asterisks indicate $p \leq 0.025$ (one-tailed Shirley–Williams test) and $p \leq 0.01$ (Student's t test) vs vehicle (0 nmol/kg) group, respectively.

■ CONCLUSION

This study of metastin(45–54) analogues, designed to improve their pharmacokinetic and physicochemical properties, led to the identification of a novel reduced-sized metastin analogue, **22**. Compound **22** was 10–50-fold more potent than **4** in vivo. Three key replacements of N-terminal acetyl, D-Trp^{47}, and Trp^{54} in **22** were conducive to the production of a long-acting analogue with low CL_{total}. These replacements significantly contributed to both improved metabolic stability and inherent potency in increasing testosterone suppression in vivo. Continued administration of KISS1R agonists suppressed

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testosterone levels and led us to select **22** as an investigational agent for the treatment of sex-hormone-dependent diseases.

EXPERIMENTAL SECTION

Instruments and Materials. Peptide synthesis was carried out with an ABI 433A and/or manual shaker using Fmoc chemistry. N^{α}-Fmoc-Rink amide MBHA resin was purchased from Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan) and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). PAL resin was purchased from Advanced Chemtech (Louisville, KY). N^{α}-Fmoc-(and side-chain)-protected amino acids were obtained from Peptide Institute, Inc. (Osaka, Japan), Kokusan Chemical Co., Ltd. (Tokyo, Japan), Watanabe Chemical Industries, Ltd., and Calbiochem-Novabiochem Japan, Ltd. Side-chain protections were as follows: Arg(Pbf), Asn(Trt), Orn(Mtt), Ser(*t*-Bu), Tyr(*t*-Bu), Trp(Boc). Solvents and other reagents were reagent grade and used without further purification unless otherwise noted. DIPCDI, HOBt, and TFA were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan). Piperidine, PyBOP, PyBrop, and TIS were purchased from Watanabe Chemical Industries, Ltd. PyAOP was purchased from PerSeptive Biosystems Inc. (Hamburg, Germany). The substitution of the resins was determined by spectrophotometric analysis (Shimadzu UV-160A UV/vis spectrophotometer) at 290 nm for the dibenzofulvene−piperidine adduct formed upon deprotection of the amino-terminal Fmoc group.

The crude peptides were purified to homogeneity by RP-HPLC. HPLC conditions: YMC C18 column (20 × 150 mm), YMC C18 column (20 × 250 mm), or YMC C18 column (30 × 250 mm); solvent gradient A (0.1% TFA in water), B (0.1% TFA in acetonitrile) with the gradient indicated below; flow rate 8 mL/min for C18 column (20 × 150 mm, 20 × 250 mm), 20 mL/min for C18 column (30 × 250 mm); UV detector 220/254 nm.

The purity of the products was characterized by analytical HPLC. Reversed-phase and ion-exchange HPLC analyses were performed with a Shimadzu gradient system using a MERCK Chromolith FastGradient RP-18 end-capped column (2.0 × 50 mm) and Phenomenex Luna 5 μm SCX 100 Å column (4.6 × 100 mm). Prior to injection, a 1 mM DMSO solution of each peptide was diluted with water to give a 100 μM solution; 10 and 20 μL of each solution were injected into the HPLC system, respectively. For reversed-phase HPLC analyses, all peptides were eluted with a linear gradient of 0−50% acetonitrile in water containing 0.1% TFA at a flow rate of 0.5 mL/min, and the purity was >95% at wavelengths of 210 and 254 nm. The peptide purity was also checked by ion-exchange HPLC at a flow rate of 1.0 mL/min using a linear gradient of 0−0.5 M NaCl in 20 mM sodium phosphate buffer (pH 7)/acetonitrile (75/25) at 210 and 254 nm. The molecular weights of the peptides were confirmed by MALDI−TOF MS on a Voyager DE-PRO system (Applied Biosystems, Foster City, CA).

General Procedure for Metastin Analogue Synthesis. All peptides were synthesized by standard Fmoc-based solid-phase methods. The obtained crude peptides were purified to homogeneity with preparative HPLC. The purity of each peptide was ascertained by analytical HPLC, and the structure assignment was performed by MALDI-TOF MS. Structures, HPLC retention times, and MALDI-TOF MS data of all peptides are shown in Table S1 of the Supporting Information. The general procedure is described below as the synthesis of Ac-[D-Tyr^{46},D-Trp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46−54) (**22**).

After 5 g (0.4 mmol/g) of commercially available Rink amide MBHA resin was swollen in DMF, the resin was treated with 50 mL of 20% piperidine/DMF solution for 20 min to remove the Fmoc group. The resin was washed with DMF, and Trp(Boc) was introduced by treating the resin with 4.213 g (8 mmol) of Fmoc-Trp(Boc)-OH, 1.272 mL (8 mmol) of DIPCDI, and 16 mL (8 mmol) of 0.5 M HOAt/DMF solution at room temperature for 90 min. In a similar manner, Orn(Mtt) was introduced to give 2 mmol of Fmoc-Orn(Mtt)-Trp(Boc)-Rink amide MBHA resin. The resin obtained was washed with DCM, and after swelling, 50 mL of TFA/TIS/TFE/DCM (1/5/19/75) was added. The mixture was shaken for 10 min, and the solution was distilled off. This procedure was repeated until yellow coloration, caused by the free Mtt group in the TFA/TIS/TFE/DCM (1/5/19/75) mixture, disappeared when the solution was added, indicating that the Mtt group had been removed.

The resulting Fmoc-Orn-Trp(Boc)-Rink amide MBHA resin was neutralized with 5% DIEA/DCM solution. After washing with DCM, 25 mL of DCM/TFE (4:1) and 1.946 g (6 mmol) of *N*-methyl-*N*,*N*′-bis-Boc-1-guanylpyrazole were added to it. DIEA was added to the mixture to adjust the pH of the solution to 10, and the mixture was shaken for 15 h to give Fmoc-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin. Fmoc-Leu was introduced into the resin as described above. The Fmoc group was removed from the subsequent Fmoc-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin (2 mmol) to give H-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin (2 mmol).

Separately, 2.326 g (8 mmol) of Fmoc-NHNH$_2$·HCl was suspended in 20 mL of DMF. Under ice cooling, a suspension of 297 mg (8 mmol) of CDI in 20 mL of THF and 2.787 mL (16 mmol) of DIEA was added to the first suspension, followed by stirring at room temperature for 1 h. The resulting reaction solution was added to the H-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin described above. The mixture was stirred for 15 h at room temperature. The resultant resin was washed and dried to give 6.394 g of Fmoc-azaGly-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin.

After 3.197 g (1 mmol) of the resin obtained was swollen in DMF, the resin was treated with 30 mL of 20% piperidine/DMF solution for 20 min to remove the Fmoc group. The resin obtained was washed with DMF and treated with 1.806 g (4 mmol) of Trt-Phe-OH·0.5AcOEt, 2.086 g (4 mmol) of PyAOP, 8 mL (4 mmol) of 0.5 M HOAt/DMF, and 2.787 mL (16 mmol) of DIEA for 90 min at room temperature to give Trt-Phe-azaGly-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin. The resin obtained was washed with DCM, and after swelling, 30 mL of TFA/TIS/TFE/DCM (1/5/19/75) was added. The mixture was shaken for 10 min, and the solution was distilled off. This procedure was repeated until yellow coloration caused by the free Trt group in the TFA/TIS/TFE/DCM (1/5/19/75) mixture disappeared when the solution was added, indicating that the Trt group had been removed. The H-Phe-azaGly-Leu-Arg-(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin obtained was neutralized with 5% DIEA/DMF solution and washed with DMF. Thereafter, the resin was treated with 1.590 g (4 mmol) of Fmoc-Thr(*t*-Bu)-OH, 0.636 mL (4 mmol) of DIPCDI, and 8 mL (4 mmol) of 0.5 M HOAt/DMF for 90 min at room temperature to introduce Thr(*t*-Bu). Subsequently, Fmoc deprotection was performed by treatment with 30 mL of 20% piperidine/DMF solution for 20 min and condensation by the DIPCDI/HOAt method, similar to the introduction of Thr(*t*Bu); this was repeated so that Asn(Trt), D-Trp(Boc), and D-Tyr(*t*-Bu) were introduced to give Fmoc-D-Tyr(*t*-Bu)-D-Trp(Boc)-Asn(Trt)-Thr(*t*-Bu)-Phe-azaGly-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin. The resin obtained was treated with 30 mL of 20% piperidine/DMF solution for 20 min to remove the Fmoc group. The resin obtained was washed to give H-D-Tyr(*t*-Bu)-D-Trp(Boc)-Asn(Trt)-Thr(*t*-Bu)-Phe-azaGly-Leu-Arg(Boc$_2$,Me)-Trp(Boc)-Rink amide MBHA resin. The resin obtained was treated with 188.7 μL (2 mmol) of Ac$_2$O and 348.4 μL (2 mmol) of DIEA in 20 mL of DMF for 30 min at room temperature to acetylate the N-terminus. The resin was then washed and dried to give 4.168 g of Ac-D-Tyr(*t*-Bu)-D-Trp(Boc)-Asn(Trt)-Thr(*t*-Bu)-Phe-azaGly-Leu-Arg(Boc$_2$,Me)-Trp-(Boc)-Rink amide MBHA resin.

A mixture of half (2.111 g) of the resin obtained and 15 mL of TFA/PhSMe/*m*-cresol/H$_2$O/TIS/EDT (80/5/5/5/2.5/2.5) was stirred for 90 min. 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 extract was filtered to remove the resin and lyophilized to give crude peptide powders. Deprotection was performed on the remaining 2.111 g of the resin under the same conditions to give about 650 mg of crude peptide powders in total. Approximately 50 mg each of the crude peptide obtained was purified by sequential application (60 min) at a flow rate

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of 15 mL/min with eluent A/eluent B = 71/29 to 61/39 (eluent A is 0.1% TFA in water, and eluent B is 0.1% TFA in acetonitrile) on preparative HPLC using a YMC Pack R&D-ODS-5-B S-5, 120A column (30 × 250 mm). The fractions containing the product were collected and lyophilized to give 255.5 mg of white powders as the purified sample; mass spectrum: MALDI-TOF (α-cyano-4-hydroxycinnaminic acid, monoisotopic) $C_{64}H_{83}N_{17}O_{13}$ $[M + H]^+$ 1298.82 (calcd 1298.64). Elution time on RP-HPLC: 11.92 min. Elution conditions: Merck Chromolith FastGradient RP-18 end-capped column (2.0 × 50 mm), linear density gradient elution with eluent A/eluent B = 95/5 to 25/75 (14 min), using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile as eluent B; flow rate 0.5 mL/min. Elution time on IE-HPLC: 7.27 min. Elution conditions: Phenomenex Luna 5 μm SCX 100 Å column (4.6 × 100 mm), linear density gradient elution with eluent A/eluent B = 100/0 to 0/100 (20 min) using 20 mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluent A and 0.5 M NaCl in 20 mM sodium phosphate buffer (pH 2.1)/acetonitrile (75/25) as eluent B; flow rate 1.0 mL/min.

Calcium Mobilization Assay. Intracellular calcium flux was measured as the increase in fluorescence emitted by the calcium-binding fluorophore, fluo-3. Human KISS1R-transfected CHO cells (hKISS1R) were seeded into 96-well plates (Corning International, Tokyo, Japan) at 15 000 cells/mL and cultured for 24 h until they were used in the functional FLIPR assays. The wells were then loaded with 100 μL of HANKS/HBSS−probenecid−FBS solution containing fluo-3 (0.5 μg, Molecular Probes), DMSO (2.1 μL), and pluronic acid (2.1 μL) for 1 h at 37 °C (5% CO_2).

HANKS/HBSS−probenecid−FBS solution was prepared by mixing HANKS/HBSS (21 mL, prepared by mixing HANKS (9.8 g) and sodium bicarbonate (0.35 g) with 1 M HEPES (20 mL) and adjusting the solution to pH 7.4 with 1 N NaOH, followed by sterilization) with 250 mM probenecid (210 μL) and FBS (210 μL). The cells were washed four times with washing buffer (HANKS/HBSS containing 2.5 mM probenecid). After the final wash, 100 μL of residual volume remained in each well of the cell plate. Peptides were dissolved in DMSO to form 1 mM stock solutions and diluted in buffer (HANKS/HBSS containing 2.5 mM probenecid, 0.2% BSA, and 0.1% CHAPS) in 96-well sample plates (V-Bottom plate type 3363, Corning International, Tokyo, Japan). The FLIPR (Molecular Devices) was programmed to transfer 50 μL from each well of the sample microplate to each well of the cell plate and to record fluorescence for 3 min in 1 s intervals during the first minute and 3 s intervals during the last 2 min. Peak fluorescence counts from the 0 to 180 s time points were used to determine agonist activity. The instrument software normalized the fluorescent reading to give equivalent initial readings at time zero.

Pharmacokinetics of Metastin Analogues in Rats. The pharmacokinetics of the metastin analogues was evaluated using 10 groups of Crl:CD(SD) rats (3 rats/group; Charles River Laboratories Japan, Inc.). Compound **4**, **14**, **22**, **26**, or **27** was given intravenously or subcutaneously to rats at a dose of 1 mg/kg. Blood samples (100 μL) were collected 5, 10, 15, and 30 min, and 1, 2, 4, 8, and 24 h after injection to determine the concentration of each compound. The plasma was obtained by removing plasma proteins with 0.2% formic acid/methanol (1:4, v/v) and acetonitrile, followed by centrifugation at 15 000 rpm for 5 min. The obtained supernatant was treated with 0.4% aqueous formic acid and centrifuged at 15 000 rpm for 5 min. The plasma concentration of compound **4**, **14**, **22**, **26**, or **27** was determined by LC−MS. The pharmacokinetic parameters associated with each group were assessed using noncompartmental analysis. The C_{5min}, AUC_{0-24h}, MRT, V_{dss}, and CL_{total} were obtained for each rat after intravenous administration. C_{max}, T_{max}, AUC_{0-24h}, MRT, and BA were also determined for each rat after subcutaneous administration.

Chemical Stability of Metastin Analogues in Aqueous Solutions. Aqueous solutions of compound **1**, **13**, **14**, **22**, **26**, or **27** at the desired pH, pH 1.2 (JP1, first fluid for the disintegration test;[31] containing 10% acetonitrile), 3, 5, or 7, were stored in the dark at 37 °C. At preselected times the reaction mixtures were analyzed by HPLC. The chromatograms were achieved using CAPCELLPAK C18 MG (250 × 4.6 mm) eluted with a 10−50% linear gradient of solvent B over 20 min at a flow rate of 1.0 mL/min. Solvent A comprised H_2O/0.05 mol/L $AcONH_4$(aq)/acetonitrile (8:1:1), and solvent B was 0.05 mol/L $AcONH_4$(aq)/acetonitrile (1:9). Peptides were detected spectrophotometrically at 230−280 nm. The peptide mixtures were incubated at 37 °C for up to 2 weeks, depending on the experiment. The quantity of peptide in the buffer solutions was determined from the area under the HPLC peaks. The percentage of a given compound **1**, **13**, **14**, **22**, **26**, or **27** recovered from the buffer solutions after incubation (stability index) was calculated relative to the amount of peptide at time 0 (100%).

Evaluation of the Effect of Metastin Peptide Derivatives on Blood Testosterone Levels in Mature Male Rats. A metastin peptide derivative (hereinafter peptide) was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc.) or 50% DMSO to prepare 2, 1, 0.5, 0.3, 0.1, 0.05, 0.03, or 0.01 mM peptide solutions and loaded into 5 ALZET osmotic pumps (0.2 mL volume, release rate 0.001 mL/h, model 2001, DURECT Corp., Cupertino, CA). The filled ALZET pumps were implanted subcutaneously into the back of 9-week old male Crl:CD(SD) rats (n = 5; Charles River Laboratories Japan, Inc.) under ether anesthesia, one pump per animal. For negative controls, distilled water (Otsuka Pharmaceutical Factory, Inc.) was used in five ALZET osmotic pumps, which were similarly implanted into five rats. These pump-implanted rats were fed for 6 days under normal feeding conditions. After weighing, the animals were decapitated, and blood samples were collected. After 0.03 mL of aprotinin solution (Trasylol, Bayer) containing 0.1 mg/mL EDTA·2Na was added to each milliliter of blood, the mixture was centrifuged at 1800g for 25 min to recover the plasma. From the obtained plasma, 0.05 mL was applied to RIA (Siemens Healthcare Diagnostics Inc., Deerfield, IL) to measure the plasma testosterone level of each rat. Values less than the lower limit of detection (0.04 ng/mL) in RIA were treated as 0.04. Mean values of the testosterone levels from five rats receiving peptide treatment were calculated as a percentage of the control values.

Single Administration of 22, a Metastin Agonist, Induced Marked LH and Testosterone Release in Male Rats. *Animals.* This study was performed on two consecutive days. In each experiment, nine male Crl:CD(SD) rats (Charles River Laboratories Japan, Inc.) were randomly divided into seven groups (Group 1: **22**, 1 nmol/kg; Group 2: **22**, 0.1 nmol/kg; Group 3: **22**, 0.01 nmol/kg; Group 4: metastin, 10 nmol/kg; Group 5: metastin, 1 nmol/kg; Group 6: metastin(45−54), 1000 nmol/kg; group 7, vehicle control; n = 3) based on body weight 1 day before administration. Drug solutions were administered subcutaneously, and blood samples (0.4 mL) were obtained via the tail vein before administration (0 h) and 0.5, 1, 2, 4, and 8 h after administration. Plasma samples were prepared by collecting the blood samples in plastic tubes containing 4 IU of sodium heparin (Ajinomoto Pharmaceuticals Co., Ltd. Tokyo, Japan) and centrifugation. Data obtained from two independent experiments were combined. Animal experiments were approved by the Takeda Experimental Animal Care and Use Committee, in accordance with NIH standards.

Chemicals. Metastin(45−54) (code no. 4389-v, lot no. 560213) was purchased from Peptide Institute, Inc., Kyoto, Japan. The vehicle was 1:4 (v/v) 20% DMSO (Sigma-Aldrich, St. Louis, MO)/saline (Otsuka Pharmaceutical Factory, Inc.). Compound **22**, metastin, or metastin(45−54) solutions were prepared using this vehicle.

Endocrine Measurements. Plasma LH and total testosterone were determined using a commercially available RIA kit by T.N. Technos, Ltd. (LH: Rat LH [^{125}I] RIA System, GE Healthcare; total testosterone: DPC Total Testosterone Kit, Diagnostic Products Corp.). The value of plasma LH below the detection limit (0.78 ng/mL) was presumed to be 0.78 ng/mL. The detection limit of plasma testosterone was 0.04 ng/mL.

Statistics. After baseline (0 h) LH or testosterone levels were subtracted, the effect of LH or testosterone release was expressed as the area under the curve between 0 and 4 h, i.e., LH AUC_{0-4h} or testosterone AUC_{0-4h}, after administration. LH AUC_{0-4h} was analyzed by the one-tailed Shirley−Williams test (**22**- or metastin-treated group vs vehicle-treated group) or Aspin−Welch t test (metastin[45−54]-

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treated group vs vehicle-treated group). Testosterone AUC_{0-4h} was analyzed by one-tailed Williams's test (**22**- or metastin-treated group vs vehicle-treated group) or Student's *t* test (metastin[45−54]-treated group vs vehicle-treated group). The differences were considered significant for $p \leq 0.05$ (Student's and Aspin−Welch *t* test) or $p \leq 0.025$ (Williams's test or Shirley−Williams test).

■ ASSOCIATED CONTENT

Supporting Information

Table S1 lists the chemical data for decapeptide and nonapeptide metastin analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED[32]

$[Ca^{2+}]_i$, calcium ion concentration; Abu, 2-aminobutyric acid; AcOEt, ethyl acetate; Arg(Boc$_2$,Me), $N^{\omega,\omega'}$-bis(*tert*-butoxycarbonyl)-N^{ω}-methylarginine; Arg(Me), N^{ω}-methylarginine; Asu, aminosuccinimidyl; azaGly, azaglycine; BA, bioavailability; CDI, 1,1′-carbonyldiimidazole; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; C_{5min}, plasma concentration at 5 min after administration; CL_{total}, total body clearance; C_{max}, maximum plasma concentration; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, diisopropylcarbodiimide; EDT, 1,2-ethanedithiol; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; HANKS/HBSS, Hank's balanced salt solution; HCl, hydrochloric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hKISS1R, human KISS1R-expressing CHO cells; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, *N*-hydroxybenzotriazole; HPG, hypothalamic−pituitary−gonadal; Hyp, hydroxyproline; KISS1R, KISS1 receptor; MBHA resin, 4-methylbenzhydrylamine hydrochloride salt resin; MRT, mean residence time; NaOH, sodium hydroxide; Orn, ornithine; PAL resin, peptide amide linker resin; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Phg, phenylglycine; PhSMe, thioanisole; Pya(4), 4-pyridylalanine; PyAOP, ((7-azabenzotriazol-1-yl)oxy)tripyrrolidinophosphonium hexafuorophosphate; PyBOP, (1*H*-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; PyBrop, bromotripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; TFE, 2,2,2-trifluoroethanol; TIS, triisopropylsilane; Tle, *tert*-leucine; T_{max}, time to maximum plasma concentration; Trt, triphenylmethyl (trityl); V_{dSS}, volume of distribution at steady state

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