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Design and Synthesis of an Investigational Nonapeptide KISS1 Receptor (KISS1R) Agonist Ac-D-Tyr-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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ABSTRACT

Metastin/kisspeptin is an endogenous ligand of KISS1R. Metastin and KISS1R are suggested to play crucial roles in regulating the secretion of GnRH and continuous administration of metastin derivatives attenuated the plasma testosterone levels in male rats. Our optimization studies of metastin derivatives led to the discovery of **1** (Ac-D-Tyr-D-Trp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2, TAK-683), which suppressed plasma testosterone in rats at lower doses than those of leuprolide. Although **1** possessed an extremely potent pharmacological activity, 20-mg/mL aqueous solution of **1** has a gel formation property. In order to improve this

physicochemical property, we substituted D-Trp at position 47 with a variety of amino acids; we identified that substitution with cyclic amino acids, which could change peptide conformation, retained its potency. Especially, Hyp[47] analogue **24** (TAK-448) showed not only superior pharmacological activity to **1**, but also excellent water solubility. Furthermore, 20-mg/mL aqueous solution of **24** did not show a gel formation up to five days.

INTRODUCTION

Metastin/kisspeptin is a peptide with 54 amino acids isolated from human placental tissues, as an endogenous ligand of an orphan G protein-coupled receptor GPR54, also known as hOT7T175, and AXOR12, currently termed KISS1 receptor (KISS1R).[1-6] The mRNA of the KISS1 gene, which encodes metastin, is expressed in the rodent and human placenta and brain. The expression of metastin and KISS1R mRNA shows similar pattern in various regions of the rat and human nervous systems. KISS1R is expressed mainly in hypothalamic tissues; the expression levels in other brain areas are low.[4−11]

At first, metastin and KISS1R were thought to correlate with cancer metastasis;[1,2] however, subsequent studies clarified that metastin-KISS1R signaling plays a key role in reproduction and regulation of the hypothalamic-pituitary-gonadal (HPG) axis. The relationship between inactivating mutations of KISS1R and idiopathic hypogonadotropic hypogonadism in humans and mice was reported by two independent groups in 2003.[9,10] Following studies suggested that metastin significantly

induced gonadotropin-releasing hormone (GnRH) release in mammals and other species.[4,12] Single administration of metastin markedly elevated the plasma levels of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in adult male Wistar rats.[13] In contrast, chronic administration of metastin analogues was found to increase both plasma LH and testosterone levels transiently, followed by a profound decrease below the detectable level in male rats.[14-16] Our immunohistochemical analysis of rat brain tissue suggested the suppression of intrinsic GnRH pulses and downstream pituitary-gonadal functions by the chronic administration of metastin analogues. This may be due to the attenuation of the responsiveness of GnRH neurons to endogenous metastin stimulation and the stimulation of GnRH neurons to continuously release low levels of GnRH.[14] The central roles of kisspeptin/KISS1R signaling suggest that activation or suppression of the HPG axis by appropriate dosing of KISS1R agonists should potentially prevent or treat a variety of sex-hormone-dependent diseases.

Native metastin is the 54-amino-acid peptide, but metastin (45-54), which is an *N*-terminally truncated analogue, shows full in vitro activities. Furthermore, the short-length peptide displays higher potency (3-10 times) than native metastin,[1] which indicates that the *C*-terminal region of metastin contains the active core of biological activity. Generally, peptides, which are thought to be poor drug candidates, are fragile molecules rapidly degraded by various exogenous proteases in blood circulation. Several groups and we have reported that rational modification of metastin (45-54) led to increased biological stability and enhanced the therapeutic potential; however, few

reports demonstrated in vivo activity.[15-24] Our previous optimization studies of *N*-terminal truncated metastin derivatives led to the discovery of a nonapeptide **1** (Ac-D-Tyr-D-Trp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2, TAK-683) as a novel class of anti-prostate cancer drug.[16,25] Compound **1** suppressed plasma testosterone in rats at lower doses than a GnRH super agonist, leuprolide, with four-week sustained administration. However, the aqueous solubility of **1** was much lower than that of leuprolide, and 20-mg/mL aqueous solution of **1** has a gel formation property. Therefore, we performed synthetic studies to improve these physicochemical properties of **1**.

RESULTS

Chemistry

We synthesized all peptides by use of the standard Fmoc-based solid phase synthetic method. Following purification of the obtained crude peptides using preparative HPLC led to homogeneity. The purity of each peptide was verified by analytical reversed-phase and ion-exchange HPLC, and the structure was assigned using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

Biological Activities

All synthesized peptides were evaluated for their agonistic activities as first screening by fluorometric imaging plate reader (FLIPR) assay, which determined the

ligand-induced elevation of Ca^{2+} in Chinese hamster ovary (CHO) cells expressing human KISS1R. We observed the maximum response in the presence of peptide and expressed the response as a percent of the corrected one to a maximal concentration of the control peptide, metastin (45-54). Nonlinear regression of smoothed data determined the potency (EC_{50}) of peptides. Peptides with good agonistic activities were further evaluated for their testosterone suppressive activities in rats with a six-day sustained subcutaneous administration study (0.1 nmol/h, i.e., 50 nmol/kg/week) as second screening. In addition, low-dose administrations of the selected compounds, which showed good activities at the second screening, were carried out to evaluate their minimum effective doses. Results of in vivo screening are shown in Table 1. The minimum effective doses were defined as the minimum dosing rate to suppress the testosterone level below the measurement limit in all individual rats (testosterone < 0.04 ng/mL of plasma level, n=5).

Design and syntheses of comprehensive nonapeptide metastin analogues

In order to improve the physicochemical properties of **1**, some of the component amino acids should be replaced with appropriate amino acids. First, we performed sequential replacement studies at each residue of **1** with a variety of amino acids; small side chain (Ala), branched (Leu), aromatic (Phe), acidic (Asp and Glu) and basic (Lys) amino acids, then determined no possibility to improve physicochemical properties by substitution between positions 48 and 54 (data not shown). On the other hand, substitutions at the *N*-terminus and position 47 retained capabilities to improve

the physicochemical characteristics. Especially, amino acid replacements of D-Trp at position 47 with small side chain amino acid D-Ala **2**, aliphatic D-Leu **3**, basic D-Lys **4**, acidic D-Glu **5** and stereoisomer Trp **6** retained in vitro/in vivo activities (Table 1). These results suggested that the side chain at position 47 was not important for the interaction with KISS1R, and might be useful to improve physicochemical characteristics of **1**. Thus, we focused on analogues substituted at position 47.

Design and syntheses of nonapeptide metastin analogues substituted at position 47

There were two different ways to increase peptide solubility. One possible way was to replace D-Trp^{47} with a hydrophilic amino acid such as an acidic (e.g., Asp and Glu), basic (e.g., Lys and Arg) or neutral amino acid (e.g., Ser and Thr). The second approach was to replace D-Trp^{47} with L-amino acids, *N*-substituted amino acids (e.g., *N*-methylalanine (MeAla)), β-amino acids (e.g., β-alanine (β-Ala)), cyclic amino acids (e.g., Pro) and achiral amino acids (i.e. Gly and 2-aminoisobutyric acid (Aib)), which could change the peptide conformation.

As described above, a broad spectrum of amino acids were tolerable in position 47, and especially L-Trp substitution **6** was superior to D-Trp **1** in terms of testosterone suppressive activity (Table 1). Generally, D-amino acid substitutions enhance metabolic stability of the scissile peptide bond. D-Amino acids at position 47 had been thought to contribute to the increased stability of metastin analogues in circulating blood. However, Trp^{47} analogue **6** was found to show enhanced in vivo activity, which

led us to synthesize L-amino acid substitutions as well as D-amino acid analogues in order to obtain more potent and water soluble analogues than those of **1**.

On the basis of these results, we prepared Lys^{47} **7** and Glu^{47} **8** analogues, which enhanced not only agonistic activities but also in vivo activities. The activities of **7** and **8** were more potent than those of their corresponding D-amino-acid analogues **4** and **5**, respectively. Compounds **7** and **8** showed comparable or more potent activity with respect to that of **1** in a six-day sustained subcutaneous administration study in rats. It was expected that the polar substitution of Glu or Lys at position 47 would enhance the water-solubility of each compound.

Substitutions at position 47 with small side chain amino acids, *N*-substituted amino acids and achiral amino acids were also expected to improve physicochemical properties by changing the peptide conformation. Similar to the results for Trp and D-Trp analogues, Ala analogue **10** demonstrated more potent activity than that of D-Ala analogue **2**. On the other hand, replacement with *N*-substituted amino acids (i.e., MeAla **12**, D-MeAla **13** and sarcosine (Sar) **14**) reduced in vitro/in vivo activities. Interestingly, replacement with achiral amino acids (i.e., Gly **9** and Aib **11**) retained the pharmacological activities, which were comparable or more potent than those of **1**. Gly **9** and Aib **11** analogues were predicted to have better water solubility because of the removal of hydrophobic indole moiety of **1**.

Replacements of Trp^{47} **6** with β-amino acids, β-Ala **15**, 2-aminobenzoic acid (Abz(2)) **16** azetidine-3-carboxylic acid (Aze(3)) **21** and piperidine-3-carboxylic acid (Pic(3)) **22** did not provide good results for biological activities. $[Ca^{2+}]_i$–mobilizing

activities of **15**, **16**, **21** and **22** for human KISS1R decreased more than three-fold compared to **6**. Testosterone suppressive activities of **15**, **16** and **22** were not observed with a 0.03-nmol/h dose in rats. Compound **23** with 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), which is a constrained Phe analogue, had good in vitro activities; however, significant testosterone suppressive activity was not demonstrated at 0.01 nmol/h. On the other hand, **17**, which has L-form cyclic α-amino acid Pro at position 47, showed higher pharmacological activities in rats than those of **1**, although in vitro activities of **17** were lower than those of **1**. These results led us to consider that the analogues replaced with cyclic amino acids at position 47 might induce an increase in both testosterone suppressive activity and water solubility. Therefore, we carried out further studies on replacements with other cyclic α-amino acids.

As a result, we discovered the following:

(1) Four-membered cyclic α-amino acid (azetidine-2-carboxylic acid (Aze(2)) **19** and cyclic β-amino acid (azetidine-3-carboxylic acid (Aze(3)) **21** showed comparable agonistic activities; on the other hand, as for six-membered cyclic amino acids, cyclic α-amino acid (piperidine-2-carboxylic acid (Pic(2)) **20**) was more favorable than cyclic β-amino acid (Pic(3) **22**).

(2) When comparing cyclic α-amino acids, pyrrolidine ring **17** was preferable to other rings (**19** and **20**).

(3) Insertion of a sulfur atom to the ring (thiazolidine-2-carboxylic acid (Thz) **25**) proved ineffective for in vivo activity. Consequently, *trans*-4-hydroxyproline (Hyp) **24**

was the most suitable amino acid at this position among the cyclic amino acids examined in this study. Hyp^{47} analogue **24** completely suppressed the blood testosterone level in a sustained administration study of rats at a dose rate of 0.01 nmol/h. Moreover, plasma testosterone in four-fifths of individuals was suppressed at a dose rate of 0.003 nmol/h. The most striking aspect of the present results is that the effect of **24** was approximately three-fold higher than that of **1**.

Design and syntheses of nonapeptide metastin analogues with combined replacements at positions 47 and 50

It has been reported that halogenation of peptides may change the physicochemical properties and enhance tissue distribution and pharmacokinetic stability.[26-30] We thought introduction of a halogen atom into the benzene ring of Phe at position 50 might cause an increase in pharmacological activity, thus we examined further modifications of the position 47 substituted analogues.

Firstly, Phe at position 50 of compound **1** was replaced by 2-fluorophenylalanine (Phe(2F)), 3-fluorophenylalanine (Phe(3F)), 4-fluorophenylalanine (Phe(4F)) and 4-chlorophenylalanine (Phe(4Cl)). Among these peptides, Phe(3F)^{50} analogue **27** had the highest potency in rat pharmacological studies (Table 1). Moreover, we performed similar modifications on the analogues with Trp^{47}, Lys^{47}, Glu^{47}, Gly^{47}, Aib^{47} and Hyp^{47}, respectively. As a result, Trp^{47} analogue **30** represented decreased testosterone suppressive activities in rats. On the other hand, a combination of Lys^{47} and Phe(3F)^{50} **31** resulted in equivalent activities to those of the corresponding parent (Phe(3F)^{50})

compound **27**; and **32** (Glu[47] and Phe(3F)[50]) demonstrated a slight increase in activity. Among all halogenated analogues, Glu analogue **32** and Hyp analogue **35** suppressed plasma testosterone in all rats at a dose of 0.01 nmol/h; however, none of the peptides including Gly **33** and Aib **34** analogues had significant testosterone suppressive activity at the lower dose, 0.003 nmol/h.

Although in vitro agonistic activity against rat KISS1R of **32** was lower than those of **1** and **27**, **32** showed good pharmacological activities with in vivo studies in rats, and such data suggests that the pharmacokinetic parameters of **32** were improved from those of **1** and **27**. Since the agonistic activity of **32** on human KISS1R was higher than that on rat KISS1R, testosterone suppressive activity of **32** in humans might be more potent than that of **1**.

Continuous administration of compounds 1, 24 and 32 in male rats

In vivo efficacy of the two selected derivatives were evaluated in terms of steady-state concentration of plasma testosterone after one week and four weeks of continuous administration of peptides in intact male rats. Approximately steady-state testosterone concentrations by the infusion of all peptides were produced up to one week. We treated the value below the detection limit (0.04 ng/mL of plasma level) in radioimmunoassay as 0.04.

As is the case with **1**,[16] continuous administration of **24** or **32** at a dose of 30 pmol/h for one week inhibited plasma testosterone in all rats (Figure 1, Table 2). In a one-week study, 10 pmol/h dosing of **1** did not attenuate testosterone concentrations

completely in all rats. On the other hand, the plasma testosterone levels in all rats decreased below the limit of measurement in radioimmunoassay when **24** or **32** was dosed at 10 pmol/h. Furthermore, plasma testosterone for all rats administered at 10 pmol/h of **24** was suppressed for one week. As for the four-week studies, testosterone suppressive activities of **24** and **32** were more potent than those of **1**; approximately one-third dose of **24** showed similar efficacy to that of **1**.

Pharmacokinetic parameters of compounds 24 and 32 in rats

The pharmacokinetic parameters of **1** and highly potent analogues, **24** and **32,** were examined by use of IGS/SD rats. All peptides were injected intravenously and subcutaneously at 1 mg/kg. Mean pharmacokinetic variable estimates of **1** were as follows: C_{5min}, 2509 ng/mL; V_{dSS}, 1225 mL/kg; and CL_{total}, 819 mL/h/kg (Table 3). Area under the plasma concentration-time curve ($AUC_{0\text{-}24h}$) values after intravenous injections improved slightly with the replacement of D-Trp[47] in **1** with Hyp **24** or Glu **32**. The V_{dSS} values for **24** and **32** were 0.3-fold lower than that of **1,** probably because of the reduced hydrophobicity. Reduced metastin analogues are supposed to be excreted by the kidneys and urine; therefore, the decrease in the V_{dSS} values attenuated the CL_{total} values of **24** and **32** after administration. The bioavailability (BA) values of **24** and **32** increased dramatically from 9.4 for **1** to 43.9 and 94.5, respectively. The hydrophilic substitution at position 47 is thought to cause the improvement in the absorption rates across subcutaneous tissues. These replacements are extremely effective in terms of improving the AUC values in subcutaneous dosing.

Water solubility of nonapeptide metastin analogues substituted at position 47

On the basis of the above-mentioned results, we selected seven compounds, including **24** and **32**, which had different amino acids at position 47, and determined their solubility in water at a concentration of 20 mg/mL (Table 4). Gel-forming ability of these compounds was also evaluated visually up to five days after dissolution. Initially **1** was dissolved completely; however, the clear solution turned into gel within three hours at room temperature. Among the six compounds synthesized in this study, Aib^{47} **11**, Hyp^{47} **24** and Lys^{47} **31** analogues showed excellent water solubility at the same concentrations, and gelation was not observed up to five days. Furthermore, **24** was completely dissolved in water up to the concentration of 500 mg/mL. Meanwhile, the Trp^{47} **6** and Glu^{47} **32** analogues exhibited poor solubility and the solution turned into gel as with **1**. The remarkable difference in water solubility between **1** and **24** with only one amino acid substitution was assumed to be due to the difference in total hydrophobicity and global conformation of these peptides. Preliminary circular dichroism (CD) analysis of **1** and **24** in water showed a slight decrease in β-sheets content (**1**: 34.8%, **24**: 21.9%) (Figure S1). Further investigation of the physicochemical analysis of **2** and **24** would clarify the effect of the amino acid substitution to improve water solubility and avoid gelation.

CONCLUSION

In this study, we performed synthetic studies to improve the physicochemical properties of an aqueous solution of **1**. As a result, we determined that replacement at position 47 of **1** can modify the physicochemical characteristics without any loss in pharmacological activity. By replacing D-Trp[47] with Lys, Aib and Hyp, 20-mg/mL aqueous solution of the compounds acquired no gelation properties. Among these peptides, the Hyp[47]-substituted analogue **24** was completely soluble in water at a concentration of 500 mg/mL. The water solubility of **24** was dramatically improved compared with that of **1**. Testosterone suppressive activities of **24** in rats were approximately three times more potent than of **1**. The minimum effective doses of **1** and **24** for decreasing plasma testosterone to levels similar to surgical castration were 100 and 30 pmol/h, respectively. The extremely high pharmacological activity and the excellent solubility of **24** are expected to promote development of sustained-release devices for a novel class of drugs for sex hormone-dependent diseases, e.g., prostate cancer. These results of in vitro and in vivo studies strongly suggested that a controlled release of KISS1R agonists suppress the HPG axis to achieve suppression of testosterone concentrations and prompted us to select **24** (Ac-D-Tyr-Hyp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2, TAK-448) as a clinical candidate of metastin analogue.[31-35]

EXPERIMENTAL SECTION

Instruments and Materials

Solid phase syntheses were carried out by manual Fmoc SPPS consisting of

Fmoc cleavage with 20% piperidine/DMF (20 min) and Fmoc amino acid condensation reaction using DIPCDI/HOAt (4 eq.), or by automated peptide synthesizer ABI 433A (as per the Fmoc/DCC/HOBt 0.25-mmol protocol). All final compounds were purified to homogeneity of ≥95% in RP-HPLC analysis with UV detection at 210 and 254 nm using the following two methods: 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; and 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. The identity of the peptides was confirmed by MALDI-TOF-MS analysis. Commercially available amino acid derivatives and resins were purchased from Novabiochem, Watanabe Chemical Industries, Peptide Institute, Bachem, AnaSpec, Chem-Impex International and American Peptide Company, while other reagents, such as coupling and de-protection reagents, were purchased from Wako Pure Chemical Industries, Novabiochem, Watanabe Chemical Industries and Nacalai Tesque.

General procedure for synthesis of metastin analogues

All peptides were synthesized in the similar manner as the previous compounds.[16] Detailed synthesis procedure for

Ac-[D-Tyr^{46},Hyp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54) **24** is described as a representative compound (Supporting Information).

Calcium Mobilization Assay

As per the method described in the previous article, the increased activity of the intracellular Ca ion level was measured using FLIPR.[16] A change in the intracellular calcium ion level was measured against the passage of time. The ratio of EC_{50} values of intracellular Ca ion-increasing activity between samples and metastin (45-54) are shown in Table 1. See Supporting Information for the detailed assay protocol.

Evaluation of blood testosterone level decreasing effect of metastin peptide derivatives using mature male rat

A metastin peptide derivative (hereinafter referred to as peptide) was dissolved in 50% DMSO aqueous solution (Otsuka Pharmaceutical Co., Ltd) to prepare a peptide solution with a concentration of 0.1, 0.03, 0.01 or 0.003 mM. Five ALZET osmotic pumps (Model 2001, 0.2 mL in volume, release rate: 0.001 mL/h, DURECT Corporation) were filled with this peptide solution. The ALZET pumps filled with the peptide solution were implanted subcutaneously in five nine-week-old CD (SD) IGS male rats (Charles River Japan, Inc.) on their backs under ether anesthesia by one pump/animal. For negative control, five ALZET osmotic pumps were filled with 50% DMSO aqueous solution, which were similarly implanted in five males CD (SD) IGS rats, respectively. These pump-implanted rats were fed for six days under normal

feeding conditions. After weighing, the animal was decapitated to collect blood. After 0.03 mL/mL blood of aprotinin solution (Trasylol, Bayer) containing 0.1 g/mL EDTA 2Na was added to the blood, the plasma was separated and recovered by centrifugation at 1,800 × g for 25 min. From the plasma obtained, 0.05 mL was used for radioimmunoassay (DPC Total Testosterone Kit, Diagnostic Products Corporation) to measure the plasma testosterone level in each rat.

Pharmacokinetics of metastin analogues in rats

Ten groups of SD rats (three per group) were used to evaluate the pharmacokinetics of metastin analogues as follows. **1**, **24** or **32** was given intravenously or subcutaneously to rats at a dose of 1 mg/kg. Blood samples of 100 μL to determine the concentration of **1**, **24** or **32** were collected at predetermined time intervals, namely 5, 10, 15, and 30 minutes, and 1, 2, 4, 8, and 24 hours after injections. The 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 five minutes. The supernatant obtained was treated with 0.4% aqueous formic acid and centrifuged at 15,000 rpm for five minutes. The plasma concentration of **1**, **24** or **32** was determined by liquid chromatography-mass spectrometry. The pharmacokinetic parameters associated with each group were assessed using noncompartmental analysis. The plasma concentration five minutes after injection (C_{5min}), area under the plasma concentration-time curve up to the last time ($AUC_{0\text{-}24h}$), mean residence time (MRT), volume of distribution at steady state (V_{dss}), and total body clearance (CL_{total}) for each

rat after intravenous administration, were obtained. The maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), area under the plasma concentration-time curve up to last time ($AUC_{0\text{-}24h}$), mean residence time (MRT), and bioavailability (BA) for each rat after subcutaneous administration, were also obtained.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

1. General procedure for synthesis of metastin analogues (PDF)

2. Table S1. Chemical data for nonapeptide metastin analogues (PDF)

3. Calcium mobilization assay (PDF)

4. Molecular formula strings and the associated biochemical and biological data (CSV)

5. Figure S1. Far-UV circular dichroism spectra of metastin analogues (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED[36]

Abz(2), 2-aminobenzoic acid; Ac_2O, acetic anhydride; Aib, 2-aminoisobutylic acid; β-Ala, β-alanine; Arg(Me), N^{ω}-methylarginine; Arg(Boc_2,Me), $N^{\omega,\omega'}$-bis-tert-butoxycarbonyl-N^{ω}-methylarginine; Aze(2), azetidine-2-carboxylic acid; Aze(3), azetidine-3-carboxylic acid; Boc, *tert*-butyloxycarbonyl; azaGly, azaglycine; *t*Bu, *tert*-butyl; CDI, *N*,*N*'-carbonyldiimidazole; DIEA, *N*,*N*'-diisopropylethylamine; DIPCDI, *N*,*N*'-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOAt, *N*-1-hydroxy-7-azabenzotriazole; HOBt, *N*-hydroxybenzotriazole; Hyp, *trans*-4-hydroxyproline; MeAla, *N*-methylalanine; MeGly, *N*-methylglycine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Phe(4Cl), 4-chlorophenylalanine ; Phe(2F), 2-fluorophenylalanine ; Phe(3F), 3-fluorophenylalanine ; Phe(4F), 4-fluorophenylalanine ; Pic(2), piperidine-2-carboxylic acid; Pic(3), piperidine-3-carboxylic acid; RP-HPLC, reversed-phase high performance liquid chromatography; Sar, sarcosine, *N*-methylglycine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Thz, thiazolidine-4-carboxylic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIS: triisopropylsilane; Trt, triphenylmethyl (trityl). Amino acid symbols denote

L-configuration unless indicated otherwise.

REFERENCES

(1) Ohtaki, T.; Shintani, Y.; Honda, S.; Matsumoto, H.; Hori, A.; Kanehashi, K.; Terao, Y.; Kumano, S.; Takatsu, Y.; Masuda, Y.; Ishibashi, Y.; Watanabe, T.; Asada, M.; Yamada, T.; Suenaga, M.; Kitada, C.; Usuki, S.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **2001**, *411*, 613-617.

(2) Kotani, M.; Detheux, M.; Vandenbogaerde, A.; Communi, D.; Vanderwinden, J.M.; De Poul, E.; Brézillon, S.; Tyldesley, R.; Suarez-Huerta, N.; Vandeput, F.; Blanpain, C.; Schiffmann, S.N.; Vassart, G.; Parmentier, M. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J. Biol. Chem.* **2001**, *276*, 34631-34636.

(3) Muir, A.I.; Chamberlain, L.; Elshourbagy, N.A.; Michalovich, D.; Moore, D.J.; Calamari, A.; Szekeres, P.G.; Sarau, H.M.; Chambers, J.K.; Murdock, P.; Steplewski, K.; Shabon, U.; Miller, J.E.; Middleton, S.E.; Darker, J.G.; Larminie, C.G.C.; Wilson, S.; Bergsma, D.J.; Emson, P.; Faull, R.; Philpott, K.L.; Harrison, D.C. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J. Biol. Chem.* **2001**, *276*, 28969-28975.

(4) Roa, J., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive

function. *Front. Neuroendocrinol.* **2008**, *29*, 48-69.

(5) Popa, S.M., Clifton, D.K., Steiner, R.A., The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Annu. Rev. Physiol*. **2008**, *70*, 213-238.

(6) Roseweir, A.K., Millar, R.P., The role of kisspeptin in the control of gonadotrophin secretion. *Hum. Reprod. Update.* **2009**, *15*, 203-212.

(7) Clements, M. K.; McDonald, T. P.; Wang, R.; Xie, G.; O'Dowd, B. F.; George, S. R.; Austin, C. P.; Liu, Q. FMRFamide-related neuropeptides are agonists of the orphan G-protein-coupled receptor GPR54. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 1189-1193.

(8) Lee, D. K.; Nguyen, T.; O'Neill, G. P.; Cheng, R.; Liu, Y.; Howard, A. D.; Coulombe, N.; Tan, C. P.; Tang-Nguyen, A. T.; George, S. R.; O'Dowd, B. F. Discovery of a receptor related to the galanin receptors. *FEBS Lett.* **1999**, *446*, 103-107.

(9) deRoux, N.; Genin, E.; Carel, J. C.; Matsuda, F.; Chaussain, J. L.; Milgrom, E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10972-10976.

(10) Seminara, S. B.; Messager, S.; Chatzidaki, E. E.; Thresher, R. R.; Acierno, J. S., Jr.; Shagoury, J. K.; Bo-Abbas, Y.; Kuohung, W.; Schwinof, K. M.; Hendrick, A. G.; Zahn, D.; Dixon, J.; Kaiser, U. B.; Slaugenhaupt, S. A.; Gusella, J. F.; O'Rahilly, S.; Carlton, M. B.; Crowley, W. F., Jr.; Aparicio, S. A.; Colledge, W. H. The GPR54 gene as a regulator of puberty. *N. Engl. J. Med.* **2003**, *349*,

1614-1627.

(11) Roa, J.; Tena-Sempere, M. KiSS-1 system and reproduction: comparative aspects and roles in the control of female gonadotropic axis in mammals. *Gen. Comp. Endrocrinol*. **2007**, *153*, 132-140.

(12) Felip, A.; Zanuy, S.; Pineda, R.; Pinilla, L.; Carrillo, M.; Tena-Sempere, M.; Gomez, A. Evidence for two distinct KiSS genes in nonplacental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol. Cell. Endocrinol*. **2009**, *312*, 61-71.

(13) Matsui, H.; Takatsu, Y.; Kumano, S.; Matsumoto, H.; Ohtaki, T. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 383-388.

(14) Matsui, H.; Tanaka, A.; Yokoyama, K.; Takatsu, Y.; Ishikawa, K.; Asami, T.; Nishizawa, N.; Suzuki, A.; Kumano, S.; Terada, M.; Kusaka, M.; Kitada, C.; Ohtaki, T. Chronic administration of the metastin/ kisspeptin analog KISS1-305 or the investigational agent TAK-448 suppresses hypothalamic pituitary gonadal function and depletes plasma testosterone in adult male rats. *Endocrinology* **2012**, *153*, 5297-5308

(15) Asami, T.; Nishizawa, N.; Matsui, H; Takatsu, Y.; Suzuki, A.; Kiba, A.; Terada, M.; Nishibori, K.; Nakayama, M.; Ban, J.; Matsumoto, S.; Tarui, N.; Ikeda, Y.; Yamaguchi, M.; Kusaka, M.; Ohtaki, T.; Kitada, C. Design, synthesis, and biological evaluation of novel investigational nonapeptide KISS1R agonists with testosterone-suppressive activity. *J. Med. Chem.* **2013**, *56*, 8298-8307.

(16) Asami, T.; Nishizawa, N.; Matsui, H; Nishibori, K; Ishibashi, Y.; Horikoshi, Y.; Nakayama, M.; Matsumoto, S.; Tarui, N.; Yamaguchi, M.; Matsumoto, H.; Ohtaki, T.; Kitada, C. Physicochemically and pharmacokinetically stable nonapeptide KISS1 receptor agonists with highly potent testosterone-suppressive activity. *J. Med. Chem*. **2014**, *57*, 6105-6015.

(17) Niida, A.; Wang, Z.; Tomita, K.; Oishi, S.; Tamamura, H.; Otaka, A.; Navenot, JM.; Broach, JR.; Peiper, SC.; Fujii, N. Design and synthesis of downsized metastin (45–54) analogs with maintenance of high GPR54 agonistic activity. *Bioorg. Med. Chem. Lett*. **2006**, *16*, 134-137

(18) Tomita, K.; Niida, A.; Oishi, S.; Ohno, H.; Cluzeau, J.; Navenot, JM.; Wang, ZX.; Peiper, SC.; Fujii, N. Structure-activity relationship study on small peptidic GPR54 agonists. *Bioorg. Med. Chem. Lett.* **2006**, *14*, 7595-7603

(19) Tomita, K.; Oishi, S.; Cluzeau, J.; Ohno, H.; Navenot, JM.; Wang, ZX.; Peiper, SC.; Akamatsu, M.; Fujii, N. SAR and QSAR studies on the N-terminally acylated pentapeptide agonists for GPR54. *J. Med. Chem.* **2007**, *50*, 3222-3228.

(20) Tomita, K.; Oishi, S.; Ohno, H.; Peiper, SC.; Fujii, N. Development of novel G-protein-coupled receptor 54 agonists with resistance to degradation by matrix metalloproteinase. *J. Med. Chem*. **2008**, *51*, 7645-7649.

(21) Curtis, AE.; Cooke, JH.; Baxter, JE.; Parkinson, JR.; Bataveljic, A.; Ghatei, MA.; Bloom, SR.; Murphy, KG. A kisspeptin-10 analog with greater in vivo bioactivity than kisspeptin-10. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *298*, E296-303.

(22) Beltramo, M.; Robert, V.; Galibert, M.; Madinier, JB.; Marceau, P.; Dardente, H.; Decourt, C.; De Roux, N.; Lomet, D.; Delmas, AF.; Caraty, A.; Aucagne, V. Rational design of triazololipopeptides analogs of kisspeptin inducing a long-lasting increase of gonadotropins. *J. Med. Chem*. **2015**, *58*, 3459-3470.

(23) Asami, T.; Nishizawa, N.; Ishibashi, Y.; Nishibori, K.; Horikoshi, Y.; Matsumoto, H.; Ohtaki, T.; Kitada, C. Trypsin resistance of a decapeptide KISS1R agonist containing an N^{ω}-methylarginine substitution. *Bioorg. Med. Chem. Lett*. **2012**, *22*, 6328-6332

(24) Asami, T.; Nishizawa, N.; Ishibashi.; Y, Nishibori, K.; Nakayama, M.; Horikoshi, Y.; Matsumoto, S.; Yamaguchi, M.; Matsumoto, H.; Tarui, N.; Ohtaki, T.; Kitada, C. Serum stability of selected decapeptide agonists of KISS1R using pseudopeptides. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6391-6396

(25) Kitada, C.; Asami, T.; Nishizawa, N. Metastin derivatives and use thereof. *WO2006001499*.

(26) Abbruscato, T. J.; Williams, S. A.; Misicka, A; Lipkowski, A.W.; Hruby, V. J.; Davis, T. P. Blood-to-central nervous system entry, and stability of biphalin, a unique double-enkephalin analog, and its halogenated derivatives. *J. Pharmacol. Exp. Ther*. **1996**, *276*, 1049-1057.

(27) Gentry, C. L.; Egleton, R. D.; Gillespie, T. The effect of halogenation on blood-brain barrier permeability of a novel peptide drug. *Peptides* **1999**, *20*, 1229-1238.

(28) Weber SJ, Abbruscato TJ, Brownson EA, Lipkowski AW, Polt R, Misicka A,

Haaseth RC, Bartosz H, Hruby VJ, Davis TP. Assessment of an in vitro blood-brain barrier model using several [Met^5]enkephalin opioid analogs. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 1649–1655.

(29) Weber, S. J.; Greene, D. L.; Sharma, S. D.; Yamamura, H. I.; Kramer, T. H.; Burks, T. F.; Hruby, V. J.; Hersh, L. B.; Davis, T. P. Distribution, and analgesia of [^{3}H][D-Pen^2, D-Pen^5]enkephalin, and two halogenated analogs after intravenous administration. *J. Pharmacol. Exp. Ther*. **1991**, *259*, 1109-1117.

(30) Liu, H-M; Liu, X. F.; Yao, J. L.; Wang, C. L.; Yu, Y.; Wang, R. Utilization of combined chemical modifications to enhance the blood–brain barrier permeability and pharmacological activity of endomorphin-1. *J.Pharmacol. Exp. Ther.* **2006**, *319*, 308-316.

(31) Asami, T.; Nishizawa, N. Metastin derivatives and use thereof. *WO2007072997*.

(32) Kuze, Y.; Jinno, F.; Kondo, T.; Asahi, S. Highly sensitive liquid chromatography-tandem mass spectrometry method for quantification of TAK-448 in human plasma. *J. Pharm. Biomed. Anal*. **2013**, *83*, 75-81.

(33) Yoshida, N.; Nishizawa, N.; Matsui, H.; Moriya, Y.; Kitada, C.; Asami, T.; Matsumoto, H. Development and validation of sensitive sandwich ELISAs for two investigational nonapeptide metastin receptor agonists, TAK-448 and TAK-683. *J. Pharm. Biomed. Anal.* **2012**, *70*, 369-377.

(34) Matsui, H.; Masaki, T.; Akinaga, Y.; Kiba, A.; Takatsu, Y.; Nakata, D.; Tanaka, A.; Ban, J.; Matsumoto, S. I.; Kumano, S.; Suzuki, A.; Ikeda, Y.; Yamaguchi, M.; Watanabe, T.; Ohtaki, T.; Kusaka, M. Pharmacologic profiles of

investigational kisspeptin/metastin analogues, TAK-448 and TAK-683, in adult male rats in comparison to the GnRH analogue leuprolide. *Eur. J. Pharmacol.* **2014**, *735*, 77-85.

(35) MacLean, DB.; Matsui, H.; Suri, A.; Neuwirth, R.; Colombel, M. Sustained exposure to the investigational Kisspeptin analog, TAK-448, down-regulates testosterone into the castration range in healthy males and in patients with prostate cancer: results from two Phase 1 studies. *J. Clin. Endocrinol. Metab.* **2014**, *99*, E1445-1153.

(36) Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. Amino acid symbols denote the L-configuration unless indicated otherwise.

Table 1. Biological activities of metastin (45-54) and nonapeptide metastin analogues substituted at positions 47 and 50

Ac-D-Tyr-AA47-Asn-Thr-AA50-azaGly-Leu-Arg(Me)-Trp-NH_2

			agonist activities EC_{50} nM (95% CI)[a]		testosterone suppressive activity[b] number of rats (T < 0.04 ng/mL, n = 5)			
compound	AA47	AA50	human	rat	0.1 nmol/h	0.03 nmol/h	0.01 nmol/h	0.003 nmol/h
metastin(45-54)			0.96 (0.55–1.7)	4.2 (3.0–6.1)	ND[c]	ND[c]	ND[c]	ND[c]
1	D-Trp	Phe	0.33 (0.25–0.44)	1.3 (1.1–1.6)	5	4	0	ND[c]
2	D-Ala	Phe	2.7 (0.92–8.1)	16 (7.9–33)	3	ND[c]	ND[c]	ND[c]
3	D-Leu	Phe	2.0 (0.71–5.9)	12 (5.7–26)	ND[c]	5	ND[c]	ND[c]
4	D-Lys	Phe	3.6 (1.1–11)	18 (6.5–50)	5	ND[c]	ND[c]	ND[c]
5	D-Glu	Phe	22 (9.7–52)	40 (21–79)	5	2	ND[c]	ND[c]
6	Trp	Phe	0.61 (0.25–1.5)	4.8 (2.5–9.3)	ND[c]	ND[c]	5	ND[c]
7	Lys	Phe	1.6 (1.1–2.3)	6.8 (3.3–14)	ND[c]	5	4	ND[c]
8	Glu	Phe	2.8 (2.1–3.6)	11 (5.9–21)	ND[c]	5	4	ND[c]
9	Gly	Phe	5.2 (2.3–12)	46 (10–200)	ND[c]	5	ND[c]	ND[c]
10	Ala	Phe	1.2 (0.76–2.0)	6.0 (3.2–11)	ND[c]	ND[c]	3	ND[c]
11	Aib	Phe	2.4 (1.6–3.6)	17 (6.4–47)	ND[c]	5	3	ND[c]
12	MeAla	Phe	4.4 (1.5–13)	43 (15–130)	ND[c]	0	ND[c]	ND[c]
13	D-MeAla	Phe	1.8 (1.4–2.4)	7.3 (5.9–9.0)	ND[c]	3	ND[c]	ND[c]
14	Sar	Phe	4.9 (3.0–8.1)	20 (15–30)	ND[c]	ND[c]	ND[c]	ND[c]
15	β-Ala	Phe	3.3 (2.0–5.4)	11 (6.4–18)	ND[c]	0	ND[c]	ND[c]
16	Abz(2)	Phe	5.2 (2.5–11)	47 (11–200)	ND[c]	0	ND[c]	ND[c]
17	Pro	Phe	0.48 (0.20–1.2)	9.3 (7.6–11)	ND[c]	ND[c]	5	0
18	D-Pro	Phe	5.8 (3.0–11)	110 (96–130)	0	ND[c]	ND[c]	ND[c]
19	Aze(2)	Phe	1.9 (1.1–3.4)	16 (9.7–26)	ND[c]	5	1	ND[c]
20	Pic(2)	Phe	2.8 (1.5–5.3)	17 (11–28)	ND[c]	3	ND[c]	ND[c]
21	Aze(3)	Phe	2.3 (1.3–4.3)	15 (10–21)	ND[c]	ND[c]	ND[c]	ND[c]
22	Pic(3)	Phe	5.5 (4.3–7.0)	45 (23–85)	ND[c]	0	ND[c]	ND[c]
23	Tic	Phe	0.49 (0.20–1.2)	7.0 (4.0–12)	ND[c]	4	0	ND[c]
24	Hyp	Phe	5.2 (2.3–12)	36 (15–81)	ND[c]	ND[c]	5	4
25	Thz	Phe	2.3 (1.3–4.3)	23 (10–54)	ND[c]	1	ND[c]	ND[c]
26	D-Trp	Phe(2F)	0.65 (0.31–1.4)	1.8 (0.91–3.6)	ND[c]	ND[c]	0	ND[c]
27	D-Trp	Phe(3F)	1.4 (1.1–1.8)	4.8 (3.3–7.1)	ND[c]	ND[c]	4	0
28	D-Trp	Phe(4F)	1.9 (0.62–6.0)	4.7 (1.8–13)	ND[c]	5	0	ND[c]
29	D-Trp	Phe(4Cl)	0.56 (0.27–1.1)	2.8 (1.7–4.8)	ND[c]	ND[c]	0	ND[c]
30	Trp	Phe(3F)	0.073 (0.043–0.12)	1.2 (0.80–1.9)	ND[c]	ND[c]	1	ND[c]
31	Lys	Phe(3F)	0.43 (0.16–1.1)	7.1 (5.8–8.6)	ND[c]	ND[c]	4	ND[c]
32	Glu	Phe(3F)	0.47 (0.27–0.84)	20 (14–28)	ND[c]	ND[c]	5	0
33	Gly	Phe(3F)	1.1 (0.81–1.6)	7.4 (7.0–8.0)	ND[c]	ND[c]	ND[c]	0
34	Aib	Phe(3F)	1.2 (0.81–1.7)	7.0 (4.7–11)	ND[c]	ND[c]	ND[c]	0
35	Hyp	Phe(3F)	0.75 (0.36–1.6)	8.5 (6.5–11)	ND[c]	ND[c]	5	0

[a] EC_{50} values of agonist activities were determined as concentrations of peptide analogs that gave half-maximum $[Ca^{2+}]$ mobilizing activities. [b] Plasma testosterone suppressive activities of peptide analogs at 0.1, 0.03, 0.01 or 0.003 nmol/h. Peptides were dissolved in 50% DMSO to prepare 0.1, 0.03, 0.01 or 0.003 mM solutions used in ALZET® osmotic pumps implanted subcutaneously in five CD(SD)IGS male rats. After six days, plasma testosterone levels were measured using radioimmunoassay. Testosterone suppression was expressed as the number of rats with plasma testosterone levels below the limit of detection (0.04 ng/mL). [c] Not determined.

Table 2. Plasma testosterone suppressive activities by continuous administration of **1**, **24** and **32**

Ac-D-Tyr-AA47-Asn-Thr-AA50-azaGly-Leu-Arg(Me)-Trp-NH_2

			testosterone suppressive activity (number of rats, T < 0.04 ng/mL, n = 5)[a]							
			dose (pmol/h)							
			3		10		30		100	
compound	AA47	AA50	1 wk	4 wk	1 wk	4 wk	1 wk	4 wk	1 wk	4 wk
1	D-Trp	Phe	0	0	3	2	5	4	5	5
24	Hyp	Phe	5	1	5	3	5	5	ND[b]	ND[b]
32	Glu	Phe(3F)	0	0	5	4	5	3	ND[b]	ND[b]

[a]Plasma testosterone suppressive activities by continuous administration of compound **1**, **24** or **32** in male rats at 3, 10, 30, or 100 pmol/h. Compound **1**, **24** or **32** dissolved in 50% DMSO to prepare 3, 10, 30, or 100 μM peptide solutions was added to ALZET® osmotic pumps which were implanted subcutaneously in 5 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. [b]Not determined.

Table 3. Pharmacokinetic parameters of metastin analogues after intravenous and subcutaneous administration in rats

compound	**1**	**24**	**32**
iv			
C_{5min} (ng/mL)	2509 ± 287	2850 ± 121	2633 ± 165
$AUC_{0\text{-}24h}$ (ng·h/mL)	1225 ± 79	1547 ± 105	1331 ± 275
MRT (h)	0.69 ± 0.02	0.62 ± 0.04	0.53 ± 0.03
V_{dss}(mL/kg)	562 ± 37	403 ± 5	406 ± 58
CL_{total} (mL/h/kg)	819 ± 52	648 ± 45	771 ± 143
sc			
C_{max} (ng/mL)	100 ± 44	342 ± 40	703 ± 97
T_{max} (h)	0.19 ± 0.10	0.33 ± 0.14	0.39 ± 0.19
$AUC_{0\text{-}24h}$ (ng·h/mL)	115 ± 44	679 ± 196	1258 ± 71
MRT (h)	0.86 ± 0.15	1.21 ± 0.34	1.09 ± 0.13
B.A. (%)	9.4 ± 3.6	43.9 ± 13.0	94.5 ± 20.2

Peptides were administered intravenously or subcutaneously to SD rats at a dose of 1 mg/kg. Blood samples were collected at 5, 10, 15, 30 min, 1, 2, 4, 8, 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. The C_{5min}, $AUC_{0–24h}$, MRT, V_{dss}, and CL_{total} were obtained for each rat after intravenous administration and expressed as mean ± standard deviation (n=3). The C_{max}, T_{max}, $AUC_{0–24h}$, MRT, and B.A. were obtained for each rat after subcutaneous administration and expressed as mean ± standard deviation (n=3).

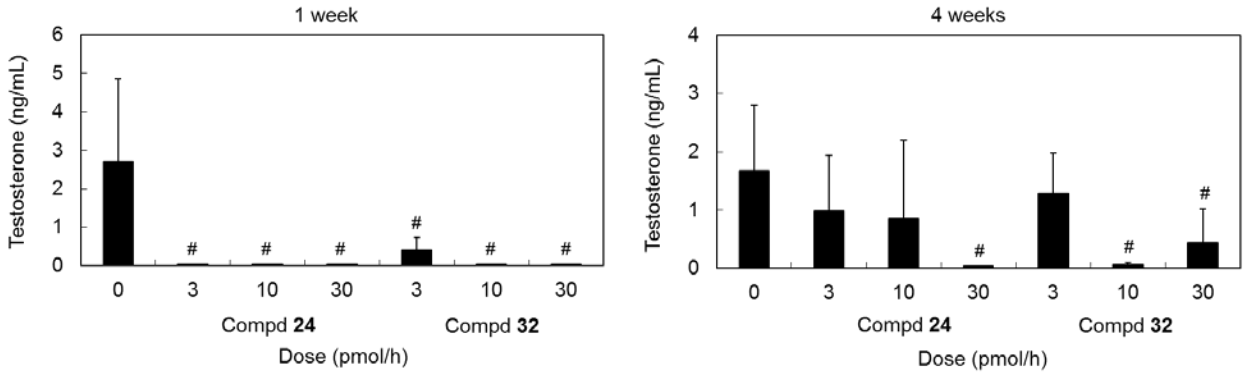
Table 4. Physicochemical profiles of nonapeptide metastin analogues substituted at positions 47 and 50

Ac-D-Tyr-AA47-Asn-Thr-AA50-azaGly-Leu-Arg(Me)-Trp-NH_2

			physicochemical properties[a] aqueous solution (20 mg/mL)	
compound	AA47	AA50	initial appearance	gel-forming ability
1	D-Trp	Phe	soluble	gelation within 3 hr
6	Trp	Phe	poorly soluble	gelation instantly
9	Gly	Phe	soluble	apperance of suspended solid after 40 hr
11	Aib	Phe	soluble	no gelation for 5 days
24	Hyp	Phe	soluble	no gelation for 5 days
31	Lys	Phe(3F)	soluble	no gelation for 5 days
32	Glu	Phe(3F)	poorly soluble	gelation in the presence of liquid phase after 48 hr

[a]Physicochemical properties of metastin analogues were evaluated in terms of water solubility at the concentration of 20 mg/mL and gel-forming ability up to 5 days after dissolution.

Figure 1. Plasma testosterone-suppressive activities by continuous administration of **24** or **32** in male rats at 3, 10, 30 pmol/h. Compound **24** or **32** dissolved in 50% DMSO to prepare 3-, 10- or 30-μM peptide solutions was added to ALZET® osmotic pumps which were implanted subcutaneously in five Crl:CD(SD) male rats. After one or four 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. # indicates $p \leq 0.025$ (one-tailed Shirley-Williams test) vs. vehicle (0 pmol/h) group.



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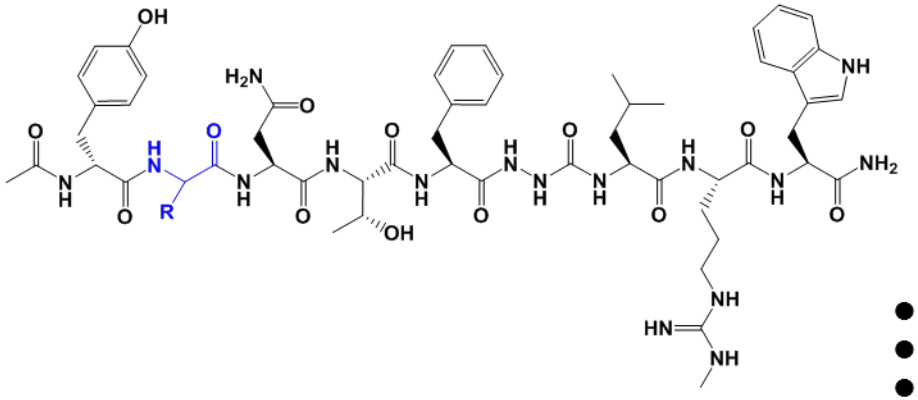
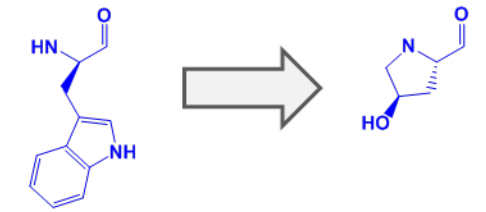
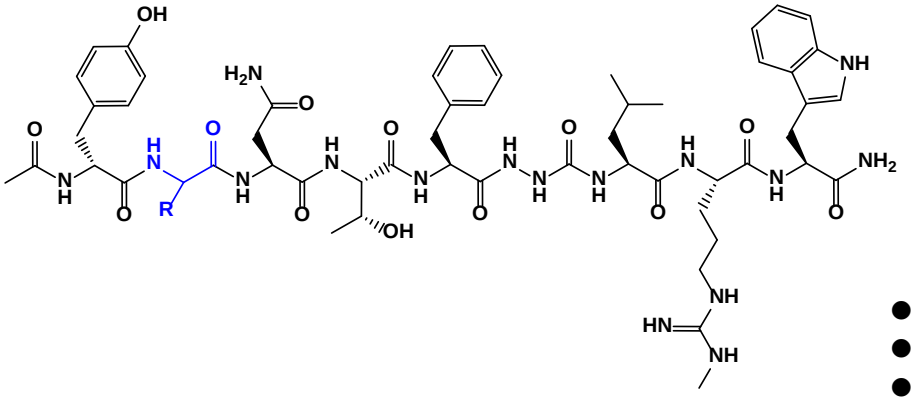
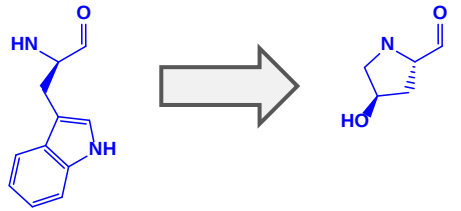




- Testosterone suppression (rats, 10 pmol/h)
- Water solubility (> 500 mg/mL)
- No gelation in water (20 mg/mL, 5 days)





$_{D}$-Trp **1** Hyp **24**

- Testosterone suppression (rats, 10 pmol/h)
- Water solubility (> 500 mg/mL)
- No gelation in water (20 mg/mL, 5 days)