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Structure–activity relationship study on small peptidic GPR54 agonists

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Abstract—Metastin (kisspeptin-54) is an endogenous ligand that modulates gonadotropin-releasing hormone (GnRH) secretion through the interaction with a G protein-coupled receptor (GPCR), GPR54. The short-chain C-terminal decapeptide amide, metastin (45–54) (kisspeptin-10), exerts the identical bioactivities to metastin, such as metastasis suppression of cancer cells and inhibition of trophoblast migration and invasion. In order to understand the structural requirement for GPR54 agonistic activity, structure–activity relationship (SAR) study on pentapeptide-based C-terminal metastin analogues was carried out. As a result, H-Amb-Nal(2)-Gly-Leu-Arg-Trp-NH_2 **34** was identified as a novel GPR54 agonist that possessed the most potent GPR54 agonistic activity reported so far.

1. Introduction

GPR54 (OT7T175, AXOR12) is a G protein-coupled receptor (GPCR) that exclusively transduces the signal of peptides derived from the *KiSS-1* metastasis suppressor gene through $G_{q/11}$-α subunits of heterotrimeric G proteins.[1–4] GPR54 is highly expressed in multiple regions of the brain, including hypothalamus as well as peripheral regions. Although GPR54 shares ~45% homology with galanin receptors, the latter ligand does not bind to GPR54 or induce signaling.

Metastin, also known as kisspeptin-54 (KP-54), is a fragment of the protein encoded by the *KiSS-1* gene[5] that has been identified as an endogenous ligand for GPR54.[2–4] This peptide, which has 54 amino acid residues and a C-terminal amide, has been found to suppress fetal bovine serum (FBS)-induced migration of CHO cells[2–6] transfected with GPR54 and the mobility of thyroid cancer cell lines[7] in vitro. In addition, KP-54 inhibited the formation of lung metastases in an in vivo mouse model using a human melanoma cell line that expresses GPR54.[2] It has recently been demonstrated that activation of GPR54 by KP-54 blocks chemotaxis induced by the CXC chemokine stromal cell derived factor 1 (SDF-1, CXCL12) by inhibiting signaling through CXCR4, a GPCR that programs pro-metastatic activities in many tumor cell types.[8]

Subsequent to the discovery of the role of kisspeptins and GPR54 in the suppression of metastatic behavior, this receptor–ligand pair was revealed to play a major role in reproductive physiology and the onset of puberty.[9–14] Kisspeptins can be detected at high levels in the plasma of pregnant women and mRNA transcripts are present in high levels in placenta and neurons that express gonadotropin-releasing hormone (GnRH).[15] A point mutation that is associated with loss of GPR54 function has been identified as the genetic lesion responsible for familial forms of isolated hypogonadotropic hypogonadism and introduction of this lesion into the germ line of mice reproduces the human disorder.[16–18] Recent investigations suggest that the induction of GPR54 signaling in hypothalamic neurons activates GnRH release, which promotes the onset of puberty.[19,20] Thus, kisspeptins and GPR54 play a critical role in normal endocrine physiology and the pathogenesis of tumor cell mobility and metastatic spread.

KP-54 and shorter peptides, KP-13 and KP-14 isolated from human placenta, contain a common RF-amide motif at the C-terminus that is present on multiple

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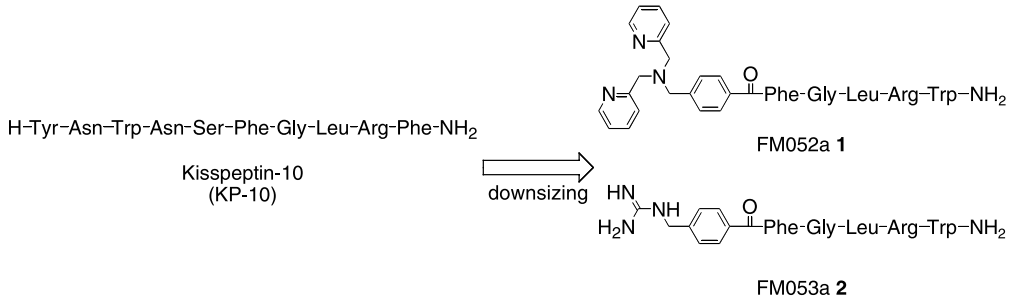


Figure 1. Kisspeptin-10 (KP-10) and its downsized analogues.

Table 1. Sequences and bioactivities of downsized KP-10 analogues

Compound	Sequence	% activity[a]	EC_{50} (nM)	Q[b]
FM052a **1**	BisPy-Amb-Phe-Gly-Leu-Arg-Trp-NH_2	88.9 ± 2.6	3.3	3.1
FM053a **2**	Gmb-Phe-Gly-Leu-Arg-Trp-NH_2	93.7 ± 1.8	1.4	1.6
3	H-Amb-Phe-Gly-Leu-Arg-Trp-NH_2	96.5 ± 0.3	3.1	3.4
KP-10	H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	—	0.18–1.1	1

Abbreviations: BisPy, *N*,*N*-bis(2-picolyl); Amb, 4-(aminomethyl)benzoic acid; Gmb, 4-(guanidinomethyl)benzoic acid.
[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).
[b] Q values are calculated as $Q = EC_{50}(\text{compound})/EC_{50}(\text{KP-10})$.

neuropeptides that are ligands for GPCRs. KP-10, a synthetic decapeptide amide from the C-terminus of KP-54 corresponding to amino acid residues 45–54, has a 10-fold greater binding affinity for GPR54 than KP-54, localizing the critical pharmacophore for receptor binding to the C-terminus.[2] It has been reported that 5- and 7-residue neuropeptides from invertebrates that contain the RW- or RF-amide motif have GPR54 agonist activity in the micromolar range.[21] To extend this approach, we studied on the structure–activity relationship of downsized KP-10 peptides and identified two novel pentapeptides that have high level potency and efficacy for GPR54 (i.e., FM052a **1** and FM053a **2**; Figure 1, Table 1).[22] FM052a and FM053a share the C-terminal motif FGLRW-NH_2 and N-modified 4-(aminomethyl)benzoyl group at the N-terminus, and possess two pyridine rings or a guanidino group at the respective N-termini. These N-terminal basic groups which were newly identified could be involved in the specific interaction with GPR54 receptor as the potential pharmocophores. These compounds had high potency for GPR54 and suppressed the motility of pancreatic cancer cells, although the magnitude of these activities did not correlate perfectly between FM052a **1** and FM053a **2**.[23] A study of structure–activity relationships was performed to clarify the structural requirements for biochemical and biological activity. Herein, we report the identification of minimum functionalities as well as the activity of novel N-terminal structures for GPR54 activation.

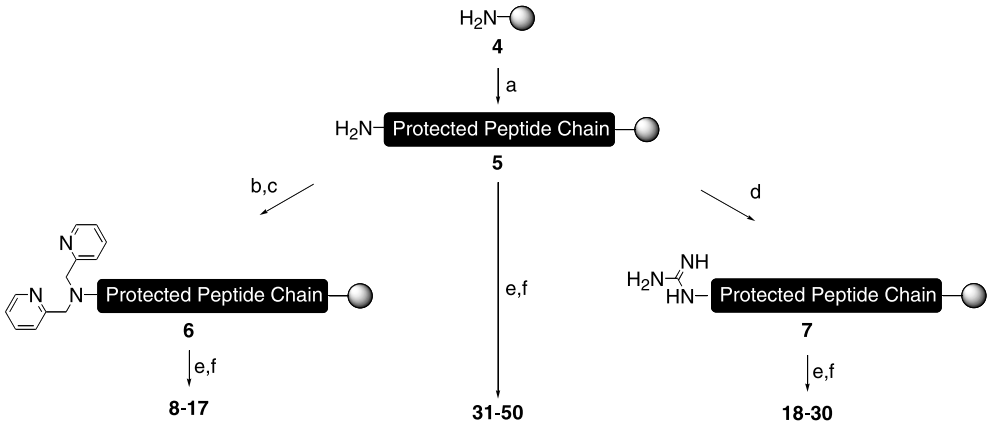
2. Results and discussion

2.1. Synthesis

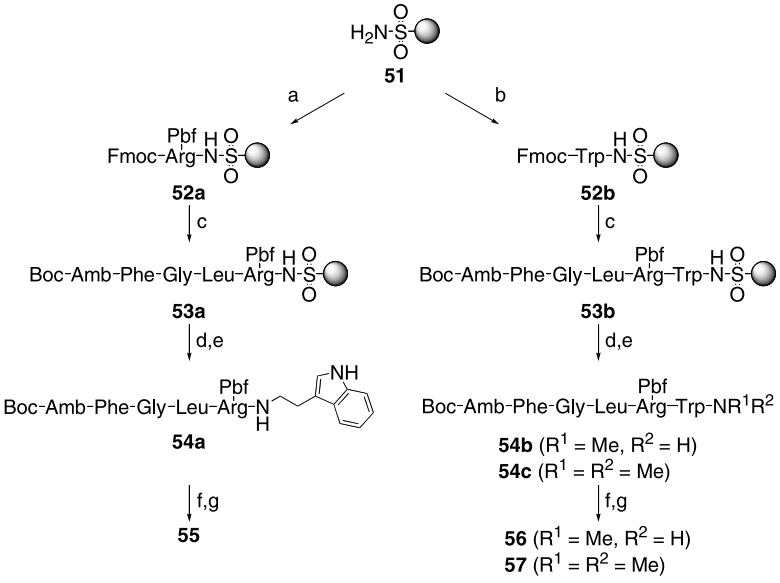
All peptide chains were constructed by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on sulfonamide resin **51** for C-terminally *N*-alkyl amides **55**–**57**, hydrazine resin **58** for peptide hydrazide **60**, and Rink-amide resin **4** for the other peptides. (Schemes 1–3) For the side-chain protection, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, Trt for Gln, *t*-Bu ester for Glu, Boc for Lys and Orn, and *t*-Bu for Tyr were employed. Amino group of 4-(aminomethyl)benzoic acid (Amb) of **5** was modified by reductive amination with 2-pyridinecarboxaldehyde and $NaBH_3CN$ on the resin to give *N*,*N*-bis(2-picolyl) (BisPy) group. The N-terminal guanidino group of protected peptides **7** was prepared by modification of **5** with 1*H*-pyrazole-1-carboxamidine. For the preparation of C-terminally *N*-alkyl amides, Arg or Trp as a C-terminal amino acid was coupled using benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) and *i*-Pr_2NEt on sulfonamide resin **51**. Protected peptides **54a**–**c** were released by the respective amines (tryptamine for **55**, methylamine for **56**, and dimethylamine for **57**) after activation of the sulfonamides **53a**,**b** with ICH_2CN. Final deprotection and/or cleavage from the resin with 1 M TMSBr-thioanisole/TFA in the presence of *m*-cresol and 1,2-ethanedithiol followed by reverse-phase HPLC purification afforded the peptides as TFA salts. All peptides were identified with ion-spray mass spectrometry and the purity was more than 95% by analytical HPLC.

2.2. Biological assay

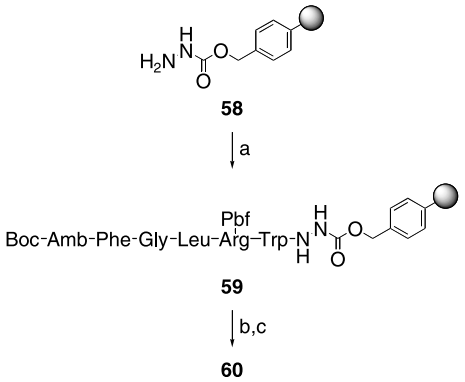
In order to evaluate GPR54 agonistic activity, the ability of candidate synthetic peptides to induce mobilization of intracellular Ca ions in GPR54-CHO cell transfectants loaded with FURA2 was measured using a spectrofluorometer. In the initial round of screening, relative maximum agonistic activity (% activity) induced by 10 nM of each compound was evaluated ($n = 3$) as compared with the maximum signal induced by the addition of 0.1 or 1 μM KP-10 ($n = 3$). For peptides that



Scheme 1. Preparation of peptide amides **8**–**50**. Reagents: (a) Fmoc-based SPPS; (b) 2-pyridinecarboxaldehyde; (c) $NaBH_3CN$; (d) 1*H*-pyrazole-1-carboxamidine hydrochloride, *i*-Pr_2NEt; (e) 1 M TMSBr-thioanisole/TFA, *m*-cresol, 1,2-ethanedithiol; (f) HPLC purification.



Scheme 2. Preparation of C-terminally *N*-alkyl peptide amides **55**–**57**. Reagents: (a) Fmoc-Arg(Pbf)-OH, *i*-Pr_2NEt, PyBOP; (b) Fmoc-Trp-OH, *i*-Pr_2NEt, PyBOP; (c) Fmoc-based SPPS; (d) ICH_2CN, *i*-Pr_2NEt; (e) amines; (f) 1 M TMSBr-thioanisole/TFA, *m*-cresol, 1,2-ethanedithiol; (g) HPLC purification.



Scheme 3. Preparation of peptide hydrazide **60**. Reagents: (a) Fmoc-based SPPS; (b) 1 M TMSBr-thioanisole/TFA, *m*-cresol, 1,2-ethanedithiol; (c) HPLC purification.

induced more than 20% activity in the first assay, the EC_{50} values for GPR54 agonistic activity were determined ($n = 3$). EC_{50} value means the concentration needed for 50% of the full agonistic activity induced by 1 μM KP-10. Since the EC_{50} values of KP-10 varied among the assay plates in the range of 0.18–1.1 nM, we also calculated *Q* values of compounds as EC_{50}(compound)/EC_{50}(KP-10), which show the comparative agonistic activities.

2.3. Structure–activity relationship studies on pentapeptide compounds for GPR54 agonists

2.3.1. Alanine and D-amino acid scanning of FM052a and FM053a. For investigation of the structural requirements for GPR54 agonistic activity and identification of ligand pharmacophores, each amino acid residue of FM052a **1** and FM053a **2** was substituted with alanine residue. Our expectation was that substitution of indispensable residues could lead to significant decrease of the bioactivity due to the absence of a potential interactive functional group or the conformational change. Ala-substitution of Gly residue in **1** and **2** resulted in

moderate decrease of the agonistic activity (EC_{50} (**11**) = 21 nM; EC_{50} (**21**) = 3.2 nM). On the other hand, less than 10% agonistic signal at 10 nM of peptides **8**–**10**, **12**, **18**–**20**, **22** was observed by replacement of the other residues with Ala (Table 2). This indicates that the position of Gly residue could be further optimized using diverse amino acids for the improved agonistic activity, while the other residues may be involved in GPR54 activation through direct or indirect interaction with the receptor.

In order to explore the bioactive conformations of FM052a **1** and FM053a **2**, D-amino acid scanning of these peptides was performed. Since D-amino acids can stabilize some secondary structures such as β-turn conformation, which is often identified as interactive surfaces in bioactive peptides and proteins, we expected that substitution of an amino acid with its D-isomer without changing the side-chain functional group might afford more potent GPR54 agonists. For the Gly position, D-Ala residue was also applied to compare the effect with L-Ala substitution. However, on the contrary to our expectation, all peptides **13**–**17** and **23**–**27** exerted less than 10% agonistic activity at 10 nM. It is of note that the potencies of peptides **16** and **26** having a D-Ala residue at the original Gly position of FM052a **1** and FM053a **2** were significantly lower than the L-Ala-substituted congeners **11** and **21**, respectively. Preference of (*S*)-configuration for all residues of FM052a **1** and FM053a **2** may imply that secondary structures, such as a β-turn conformation induced by D-amino acids, may not contribute to GPR54 agonistic activity.

2.3.2. Optimization of Phe residue. Next, we conducted the optimization of each residue of pentapeptide GPR54 agonists. As reference compounds, FM053a **2** and peptide **3**, which contains 4-(aminomethyl)benzoic acid at the N-terminus, were utilized. Peptide **3** was equipotent to FM052a **1** (EC_{50} = 3.1 nM, Table 1). We speculated that hydrophobic side chains were essential at the Phe position, because of the significantly less bioactivity of Ala-substituted analogues **12** and **22**. In order to determine whether the aromatic groups are required for bioactivity, cyclohexylalanine (Cha), which mimics phenylalanine by a saturated cyclic carbohydrate, was inserted in this position (Table 3). Cha-substituted analogues **31** showed 20% of the potency (EC_{50} = 14 nM) of peptide **3**, suggesting that aromatic groups at this position are important for the agonistic activity. On

Table 3. Bioactivities of Phe-substituted pentapeptide analogues, H-Amb-Xaa-Gly-Leu-Arg-Trp-NH_2

Compound	Xaa	% activity[a]	EC_{50} (nM)	*Q*
3	Phe	96.5 ± 0.3	3.1	3.4
31	Cha	44.4 ± 1.0	14	15
32	Tyr	10.1 ± 0.4	—[b]	—
33	Trp	77.9 ± 2.2	4.5	4.9
34	Nal(2)	88.9 ± 0.4	0.82	0.9

Abbreviations: Amb, 4-(aminomethyl)benzoic acid; Cha, 3-cyclohexylalanine; Nal(2), 3-(2-naphthyl)alanine.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

[b] Not tested.

Table 2. Alanine and D-amino acid scanning of FM052a **1** and FM053a **2**

Compound	Sequence	% activity[a]	EC_{50} (nM)	*Q*
FM052a **1**	BisPy-Amb-Phe-Gly-Leu-Arg-Trp-NH_2	88.9 ± 2.6	3.3	3.1
8	BisPy-Amb-Phe-Gly-Leu-Arg-*Ala*-NH_2	4.1 ± 0.3	—[b]	—
9	BisPy-Amb-Phe-Gly-Leu-*Ala*-Trp-NH_2	4.5 ± 0.1	—[b]	—
10	BisPy-Amb-Phe-Gly-*Ala*-Arg-Trp-NH_2	5.5 ± 0.4	—[b]	—
11	BisPy-Amb-Phe-*Ala*-Leu-Arg-Trp-NH_2	26.6 ± 1.3	21	35
12	BisPy-Amb-*Ala*-Gly-Leu-Arg-Trp-NH_2	5.1 ± 0.1	—[b]	—
13	BisPv-Amb-Phe-Gly-Leu-Arg-D-*Trp*-NH_2	5.5 ± 0.2	—[b]	—
14	BisPy-Amb-Phe-Gly-Leu-D-*Arg*-Trp-NH_2	7.1 ± 0.3	—[b]	—
15	BisPy-Amb-Phe-Gly-D-*Leu*-Arg-Trp-NH_2	6.9 ± 0.4	—[b]	—
16	BisPy-Amb-Phe-D-*Ala*-Leu-Arg-Trp-NH_2	3.6 ± 0.3	—[b]	—
17	BisPy-Amb-D-*Phe*-Gly-Leu-Arg-Trp-NH_2	10.0 ± 1.0	—[b]	—
FM053a **2**	Gmb-Phe-Gly-Leu-Arg-Trp-NH_2	93.7 ± 1.8	1.4	1.6
18	Gmb-Phe-Gly-Leu-Arg-*Ala*-NH_2	5.5 ± 0.2	—[b]	—
19	Gmb-Phe-Gly-Leu-*Ala*-Trp-NH_2	5.7 ± 0.2	—[b]	—
20	Gmb-Phe-Gly-*Ala*-Arg-Trp-NH_2	7.6 ± 0.3	—[b]	—
21	Gmb-Phe-*Ala*-Leu-Arg-Trp-NH_2	84.4 ± 2.4	3.2	5.3
22	Gmb-*Ala*-Gly-Leu-Arg-Trp-NH_2	6.5 ± 0.3	—[b]	—
23	Gmb-Phe-Gly-Leu-Arg-D-*Trp*-NH_2	8.3 ± 0.3	—[b]	—
24	Gmb-Phe-Gly-Leu-D-*Arg*-Trp-NH_2	6.9 ± 0.9	—[b]	—
25	Gmb-Phe-Gly-D-*Leu*-Arg-Trp-NH_2	5.3 ± 0.7	—[b]	—
26	Gmb-Phe-D-*Ala*-Leu-Arg-Trp-NH_2	3.4 ± 0.2	—[b]	—
27	Gmb-D-*Phe*-Gly-Leu-Arg-Trp-NH_2	8.6 ± 0.8	—[b]	—

Abbreviations: BisPy, *N*,*N*-bis(2-picolyl); Amb, 4-(aminomethyl)benzoic acid; Gmb, 4-(guanidinomethyl)benzoic acid.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

[b] Not tested.

the basis of this result, optimization using aromatic amino acids such as Tyr, Trp, and 3-(2-naphthyl)alanine (Nal(2)) was performed. Tyr-substituted analogue **32** showed significantly low agonistic activity in spite of possessing an aromatic group (10% agonistic activity at 10 nM). The low activity may be due to an unfavorable interaction between the 4-hydroxy group and GPR54. Trp-substituted analogue **33** showed slightly less agonistic activity than peptide **3** (EC_{50} = 4.5 nM). In contrast, the Nal(2)-substituted analogue **34** demonstrated four times the potency of peptide **3** (EC_{50} = 0.82 nM). This suggested the potential presence of a large pocket in the receptor for ligand binding. Development of more potent agonists might be possible by further optimization of this position using large aromatic group-containing amino acids.

2.3.3. Structure–activity relationships of Leu residue position. Analysis of structure–activity relationship for the leucine position of **3** was carried out by utilizing amino acids possessing an aliphatic or aromatic side chain. The results are summarized in Table 4. We prepared **35**–**37** for investigation of the relationship between the side-chain length and biological activity. When the side chain was replaced with methyl group (alanine), the analogues showed significantly lower activity (Table 2; **10** and **20**: 6% and 8% agonistic activity at 10 nM, respectively). Among the analogues having straight-chain alkyl groups, peptide **36**, which contains an *n*-propyl group (norvaline; Nva), was more potent than ethyl (2-aminobutyric acid; Abu(2))- and *n*-butyl (norleucine; Nle)-substituted analogues (EC_{50} values: **35**, 19 nM; **36**, 6.1 nM; **37**, 75 nM). The side-chain length of **36** is the same as that of Leu, indicating that straight- or branched-propyl chains may be more appropriate for this side chain. The effect of branched aliphatic side chains was evaluated by comparing *i*-propyl (Val) and (*S*)-*sec*-butyl (Ile) with the original *i*-butyl groups. Val-containing peptide **38** showed 6-fold higher bioactivity as compared with **35**, suggesting a β-methyl group might contribute to increase agonistic activity. In contrast, the (*S*)-*sec*-butyl group in **39** was not compatible with the agonistic activity (EC_{50} > 100 nM). Cha-containing peptide **40** showed higher activity than **37**, although the side chain is bulkier than *n*-butyl group in **37**. Incorporation of a hydroxyl group led to the loss of the agonistic activity in Ser **41**- or homoserine (Hse) **42**-substituted analogues, suggesting that ligands could interact with the receptor by hydrophobic interaction in this site. The retention of agonistic activity following substitution with aromatic amino acids, such as Phe and Nal(2), also supported the preference for hydrophobic interaction for the agonistic activity (EC_{50} (**43**) = 48 nM; EC_{50} (**44**) = 25 nM).

Table 4. Bioactivities of Leu-substituted pentapeptide analogue, H-Amb-Phe-Gly-Xaa-Arg-Trp-NH_2

Compound	Xaa	% activity[a]	EC_{50} (nM)	*Q*
3	Leu	96.5 ± 0.3[b]	3.1	3.4
35	Abu(2)	32.3 ± 1.8[c]	19	79
36	Nva	65.3 ± 1.4[c]	6.1	21
37	Nle	0.3 ± 0.3[c]	75	260
38	Val	101.3 ± 2.5[c]	4.4	12
39	Ile	9.6 ± 0.7[c]	>100	>1400
40	Cha	46.5 ± 1.8[c]	25	70
41	Ser	0.0 ± 0.0[c]	>100	>400
42	Hse	0.1 ± 0.1[c]	>100	>400
43	Phe	22.7 ± 1.2[c]	48	120
44	Nal(2)	49.1 ± 2.4[c]	25	61

Abbreviations: Amb, 4-(aminomethyl)benzoic acid; Abu(2), 2-aminobutyric acid; Nva, norvaline; Nle, norleucine; Cha, 3-cyclohexylalanine; Nal(2), 3-(2-naphthyl)alanine; Hse, homoserine.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%).

[b] Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

[c] Maximum agonistic activity signal at 0.1 μM KP-10 was used as reference (100%).

2.3.4. Importance of Arg guanidino group for GPR54 agonistic activity. Previously, Clements et al. reported that GPR54 agonistic activity was maintained in the case of substitution of Arg with Lys in antho-RW amides derived from sea anemones.[21] On the basis of this report, ornithine (Orn) and Lys were applied to the Arg position in peptide **3** (Table 5). However, loss of the bioactivity was observed in peptides **45** and **46** (1% agonistic activity at 10 nM), although the mechanism for the loss of activity is not clear. In expectation of potential hydrogen bonding with the receptor interactive site, several hydrophilic amino acids such as citrulline (Cit), Lys(Ac), Gln, and Glu were utilized at this position, but it was not possible to identify any active derivatives among **47**–**50**. As such, all of these peptides lacked agonistic activity, suggesting that Arg guanido group could play a critical role for ligand binding and/or receptor activation.

2.3.5. Structure–activity relationships of Trp residue. Since the C-terminal amino acid residue is diverse among species (i.e., Phe for human, Tyr for mouse and rat[24]), this position was a candidate for further optimization. Based on the previous Clements's report,[21] we replaced the C-terminal residue of KP-10 with tryptophan, which resulted in increased agonistic activity.[22] Using these aromatic amino acids, we investigated the appropriate aromatic ring for bioactivity with human GPR54. As a result, the original peptide FM052a **2**

Table 5. Bioactivities of Arg-substituted pentapeptide analogue, H-Amb-Phe-Gly-Leu-Xaa-Trp-NH_2

Compound	Xaa	% activity[a]	EC_{50}(nM)	*Q*
3	Arg	97 ± 0.2	3.1	3.4
45	Orn	1 ± 0.3	—[b]	—
46	Lys	0.9 ± 0.2	—[b]	—
47	Cit	0.7 ± 0.1	—[b]	—
48	Lys(Ac)	0.4 ± 0.2	—[b]	—
49	Gln	0.9 ± 0.2	—[b]	—
50	Glu	1.0 ± 0.2	—[b]	—

Abbreviations: Amb, 4-(aminomethyl)benzoic acid; Orn, ornithine; Lys(Ac), N^{ϵ}-acetyllysine; Cit, citrulline.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

[b] Not tested.

Table 6. Bioactivities of Trp-substituted pentapeptide analogue, Gmb-Phe-Gly-Leu-Arg-Xaa-NH_2

Compound	Xaa	% activity[a]	EC_{50} (nM)	*Q*
FM053a **2**	Trp	93.7 ± 0.9	1.4	1.6
28	Phe	69.6 ± 2.0	2.7	4.0
29	Tyr	60.1 ± 2.9	10	15
30	Nal(2)	68.4 ± 1.7	6.8	10

Abbreviations: Gmb, 4-(guanidinomethyl)benzoic acid; Nal(2), 3-(2-naphthyl)alanine.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

Table 7. Bioactivities of C-terminal-modified pentapeptide analogue, H-Amb-Phe-Gly-Leu-Arg-R

Compound	R	% activity[a]	EC_{50} (nM)	*Q*
3	–Trp-NH_2	96.5 ± 0.3[b]	1.4	1.6
55	–Tryptamine	3.8 ± 0.1[b]	—[d]	—
56	–Trp-NHMe	0.2 ± 0.1[c]	>100	>560
57	–Trp-NMe_2	0.1 ± 0.1[c]	>100	>560
60	–Trp-$NHNH_2$	0.0 ± 0.0[c]	>100	>560

Abbreviation: Amb, 4-(aminomethyl)benzoic acid.

[a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%).

[b] Maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%).

[c] Maximum agonistic activity signal at 0.1 μM KP-10 was used as reference (100%).

[d] Not tested.

having Trp was the most potent ligand among three peptides, although the other peptides containing Phe and Tyr showed a similar level of agonistic activity (Table 6; EC_{50}(**28**) = 2.7 nM; EC_{50}(**29**) = 10 nM). Since naphthalenes can be a mimetic of indole ring of Trp, a peptide with Nal(2) in this position was tested. This substitution resulted in a slight decrease of the agonistic activity (EC_{50} = 6.8 nM). This indicates that the fused aromatic groups may not be required for high level bioactivity.

2.3.6. Importance of C-terminal amide group. The C-terminal carboxylate derivative of KP-10 was reported to be inactive,[2] suggesting that a carboxamide group is a potential functional component of this pharmacophore. To verify the significance of the C-terminal amide group for the agonistic activity, a carboxamide-deficient analogue **55** was prepared (Table 7). *N*-methylamide **56**, *N*,*N*-dimethylamide **57**, and hydrazide **60** were also designed and evaluated as amide derivatives of peptide **3**. All of the variants of peptides **55**–**57** and **60** showed remarkable loss of the agonistic activity. It is interesting that even addition of a methyl group onto the amide was not tolerated. This implies that the amide hydrogens may be involved in direct interactions through hydrogen bonding with GPR54.

3. Conclusion

In conclusion, we performed the SAR study on pentapeptide GPR54 agonists **1**–**3**. In this study, it was demonstrated that limited functional groups within Leu-Arg dipeptide would be tolerable. On the other hand, N-terminal Phe and C-terminal Trp residues can be optimized by using natural and non-naturally occurring aromatic amino acids. In addition, a novel potent GPR54 agonist H-Amb-Nal(2)-Gly-Leu-Arg-Trp-NH_2 **34** was identified by substitution of Phe in peptide **3** with Nal(2). Since we assessed only the agonistic activities by Flipr assay in this study, less potent compounds could be potential partial agonists or antagonists. Based on the results from this SAR study, a series of N-terminal-modified derivatives are now being investigated to develop more potent agonists as well as antagonists or inverse agonists for GPR54.

4. Experimental

4.1. General synthesis

Ion-spray mass (ISMS) spectra were obtained with an API*III*E triple quadrupole mass spectrometer. Optical rotations were measured with a Horiba high-sensitive polarimeter SEPA-200 (Kyoto, Japan). For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min on a Shimadzu LC-l0ADvp (Shimadzu corporation, Ltd, Kyoto, Japan). Preparative HPLC was performed using a Cosmosil 5C18-ARII column (20 × 250 mm, Nacalai Tesque Inc.) on a Shimadzu LC-6AD (Shimadzu corporation, Ltd) in an isocratic mode of CH_3CN solution containing 0.1% (v/v) TFA at a flow rate of 10 mL/min. Fmoc-protected amino acids and resins were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan) or Merck Ltd (Tokyo, Japan). All the other chemicals were purchased from either Nacalai Tesque Inc. (Kyoto, Japan) or Sigma–Aldrich JAPAN (Tokyo, Japan).

4.2. Synthesis of FM052a and FM053a analogues

4.2.1. BisPy-Amb-Phe-Ala-Leu-Arg-Trp-NH_2 11. The protected peptide-resin was manually constructed using Fmoc-based solid-phase synthesis on an Fmoc-Rink amide-resin (0.34 mmol/g, 150 mg, 0.05 mmol). Fmoc-protected amino acid derivatives (0.25 mmol, 5.0 equiv) were successively condensed using 1,3-diisopropylcarbodiimide (DIPCDI) (39 μL, 0.25 mmol, 5.0 equiv) in the presence of *N*-hydroxybenzotriazole (HOBt) (77 mg, 0.5 mmol, 10.0 equiv). After removal of Fmoc protection of Amb, amino group was reacted with 2-pyridinecarboxaldehyde (24 μL, 0.25 mmol, 5.0 equiv) in DMF/MeOH (2 mL, 1:1) for 1 h followed by reduction with $NaBH_3CN$ (16 mg, 0.25 mmol, 5.0 equiv) in DMF/MeOH/AcOH (2 mL, 9:9:2) for 30 min. This operation was repeated twice. The resulting protected resin was treated with 1 M TMSBr-thioanisole/TFA (8 mL) in the presence of *m*-cresol (375 μL, 70 equiv) and 1,2-ethanedithiol (375 μL, 90 equiv) at 4 °C for 1 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry diethyl ether (40 mL). The resulting powder was collected by centrifugation and then washed

three times with ice-cold dry diethyl ether (3× 40 mL). The crude product was purified by preparative HPLC to afford 8 mg of the expected peptide **11** as a colorless powder [5 μmol, 11% yield based on the Fmoc-Rink amide-resin].

4.2.2. Gmb-Phe-Ala-Leu-Arg-Trp-NH_2 21. Protected peptide **21** was prepared by the identical procedure as described in the preparation of **11** except for the N-terminal modification step: after removal of Fmoc protection of Amb, amino group was reacted with 1*H*-pyrazole-l-carboxamidine hydrochloride (73 mg, 0.5 mmol, 10 equiv) in the presence of *i*-Pr_2NEt (170 μL, 1.0 mmol, 20 equiv) in 2 mL of DMF for 10 h. Peptide **21**: yield 11 mg [11 μmol, 20% yield based on the Fmoc-Rink amide-resin].

4.2.3. H-Amb-Phe-Gly-Leu-Arg-Trp-NHMe 56. Fmoc-Trp-OH (2.4 g, 5.5 mmol, 5 equiv) was condensed with sulfonamide resin (1.1 mmol/g, 1.0 g, 1.1 mmol) using PyBop (2.9 g, 5.5 mmol, 5 equiv) in the presence *i*-Pr_2NEt (1.9 mL, 11 mmol, 10 equiv) in 10 mL of $CHCl_3$ at −20 °C for 8 h. This operation was repeated twice. Unreacted amino group was protected with acetyl group

Table 8. Characterization data of the synthetic peptides

Compound	Yield (%)	Optical rotations			Formula	IS-MS	
		$[\alpha]_D$ (H_2O)	*C* (g/dL)	Temperature (°C)		Found	Calculated
1	18	−15.87	0.189	22.2	$C_{54}H_{65}N_{13}O_6$	993.0	993.2
2	44	−16.74	0.209	22.6	$C_{43}H_{58}N_{13}O_6$	853.0	853.0
3	19	−15.38	0.169	24.4	$C_{42}H_{56}N_{11}O_6$	811.0	811.0
8	27	+3.41	0.163	24.7	$C_{46}H_{61}N_{12}O_6$	878.0	878.0
9	16	+21.82	0.110	24.7	$C_{51}H_{59}N_{10}O_6$	908.5	908.1
10	18	+1.20	0.167	24.8	$C_{51}H_{60}N_{13}O_6$	951.0	951.1
11	11	+3.45	0.145	24.9	$C_{55}H_{68}N_{13}O_6$	1007.0	1007.2
12	22	+3.47	0.259	25.0	$C_{48}H_{62}N_{13}O_6$	917.5	917.1
13	22	+15.56	0.212	24.9	$C_{54}H_{66}N_{13}O_6$	993.0	993.2
14	24	+4.61	0.433	24.9	$C_{54}H_{66}N_{13}O_6$	993.0	993.2
15	13	+12.98	0.208	25.0	$C_{54}H_{66}N_{13}O_6$	993.0	993.2
16	25	+11.07	0.316	25.1	$C_{55}H_{68}N_{13}O_6$	1007.5	1007.2
17	11	+13.72	0.204	25.1	$C_{54}H_{66}N_{13}O_6$	993.0	993.2
18	29	−0.52	0.193	25.1	$C_{35}H_{53}N_{12}O_6$	738.0	737.9
19	23	0	0.192	25.1	$C_{40}H_{51}N_{10}O_6$	768.0	767.9
20	23	−0.65	0.154	25.1	$C_{40}H_{52}N_{13}O_6$	811.0	810.9
21	20	+2.24	0.134	25.0	$C_{44}H_{60}N_{13}O_6$	867.0	866.0
22	22	+3.24	0.185	24.9	$C_{37}H_{54}N_{13}O_6$	777.0	776.9
23	28	+0.27	0.364	24.8	$C_{43}H_{58}N_{13}O_6$	853.0	853.0
24	25	−0.74	0.271	24.7	$C_{43}H_{58}N_{13}O_6$	853.0	853.0
25	27	+0.65	0.153	24.6	$C_{43}H_{58}N_{13}O_6$	853.0	853.0
26	44	+2.51	0.558	24.6	$C_{44}H_{60}N_{13}O_6$	867.0	867.0
27	22	−12.56	0.207	24.4	$C_{43}H_{58}N_{13}O_6$	853.0	853.0
28	46	−21.98	0.464	24.0	$C_{41}H_{57}N_{12}O_6$	814.0	814.0
29	44	−22.64	0.424	23.9	$C_{41}H_{57}N_{12}O_7$	830.0	830.0
30	43	−17.50	0.297	23.9	$C_{45}H_{59}N_{12}O_6$	864.0	864.0
31	29	−15.54	0.238	24.3	$C_{42}H_{62}N_{11}O_6$	817.0	817.0
32	23	−18.94	0.359	24.3	$C_{46}H_{58}N_{11}O_6$	861.0	861.0
33	21	−11.64	0.189	24.3	$C_{44}H_{57}N_{12}O_6$	850.0	850.0
34	20	−16.57	0.181	24.2	$C_{42}H_{56}N_{11}O_7$	827.0	827.0
35	41	−46.52	0.155	23.0	$C_{40}H_{52}N_{11}O_6$	783.0	782.9
36	37	−26.10	0.175	23.1	$C_{41}H_{54}N_{11}O_6$	797.0	796.9
37	26	−25.70	0.097	23.1	$C_{42}H_{56}N_{11}O_6$	811.0	811.0
38	38	−15.93	0.251	24.1	$C_{45}H_{60}N_{11}O_6$	851.0	851.0
39	37	−10.94	0.274	24.1	$C_{45}H_{54}N_{11}O_6$	845.0	845.0
40	46	−7.43	0.269	24.1	$C_{49}H_{56}N_{11}O_6$	895.0	895.0
41	24	−27.75	0.418	24.2	$C_{41}H_{54}N_{11}O_6$	797.0	796.9
42	40	−23.93	0.234	24.2	$C_{42}H_{56}N_{11}O_6$	811.0	811.0
43	28	−26.77	0.201	23.1	$C_{39}H_{50}N_{11}O_7$	785.0	784.9
44	37	−47.12	0.161	23.2	$C_{40}H_{52}N_{11}O_7$	799.0	798.9
45	35	−29.41	0.238	24.1	$C_{41}H_{54}N_9O_6$	769.0	768.9
46	36	−26.90	0.301	24.0	$C_{42}H_{56}N_9O_6$	783.0	782.9
47	32	−31.00	0.358	24.0	$C_{42}H_{55}N_{10}O_7$	812.0	811.9
48	39	−25.68	0.405	24.0	$C_{49}H_{58}N_9O_7$	825.0	825.0
49	35	−33.22	0.310	24.0	$C_{41}H_{52}N_9O_7$	783.0	782.9
50	37	−33.33	0.267	24.0	$C_{41}H_{51}N_8O_8$	784.0	783.9
55	36	−7.40	0.135	23.4	$C_{41}H_{55}N_{10}O_5$	768.0	767.9
56	16	−181.74	0.126	21.7	$C_{43}H_{58}N_{11}O_6$	825.0	825.0
57	16	−100.87	0.115	20.5	$C_{44}H_{60}N_{11}O_6$	839.0	839.0
60	16	−27.45	0.168	23.2	$C_{42}H_{57}N_{12}O_6$	826.0	826.0

using Ac_2O (890 μL, 11 mmol, 10 equiv) in the presence of *i*-Pr_2NEt (960 μL, 5.5 mmol, 5 equiv). On this resin (0.53 mmol/g, 1.50 g, 0.80 mmol), amino acids were successively condensed according to the identical procedure as described in the preparation of **11**. The resulting protected peptide resin **53b** (0.41 mmol/g, 250 mg, 0.1 mmol) was washed with *N*-methyl-2-pyrrolidone (NMP). Then it was added ICH_2CN (140 μL, 2.0 mmol, 20 equiv) and *i*-Pr_2NEt (87 μL, 0.5 mmol, 5 equiv) in NMP (1.6 mL) through alumina, and reacted for 24 h under the dark. After the resin was washed with NMP and THF, the resin was reacted with $MeNH_2$ in THF (2 M solution, 2 mL, 4.0 mmol, 40 equiv) for 16 h. After removal of the resin by filtration, the filtrate was concentrated under reduced pressure. Final deprotection and purification by the identical procedure as described in the preparation of **11** gave 17 mg of the title peptide **56** as white powder [16 μmol, 16% yield] (see Table 8).

4.3. Cell line

The stable expression cell line GPR54 was acquired by transduction of expression plasmid pAK-rGPR54 for animal cells into CHO/dhfr cells (obtained from ATCC), using CellPhect Transfection Kit (Amersham Pharmacia Biotech, Inc.). First, 240 μL of buffer A (attached to Cell Phect Transfection Kit) was added to 9.6 μg of plasmid DNA dissolved in 240 μL of distilled water. After settling the mixture for 10 min, 480 μL of buffer B (CellPhect Transfection Kit) was added to the mixture. Then, 4×10^5CHO/dhfr cells were inoculated on a 60 mm Petri dish. After culturing the cells in Ham's F-12 medium (Nissui Seiyaku Co., Ltd) supplemented with 10% fetal bovine serum (FBS; Bio-Whittaker, Inc.) at 37 °C for 2 days in 5% CO_2, 480 μL of the liposomes was added dropwise to the cells. After culturing the cells at 37 °C for 6 h in 5% CO_2, the cells were washed twice with serum-free Ham's F-12 medium and 3 mL of 15% glycerol was added to the cells to treat for 2 min. The cells were then washed twice with serum-free Ham's F-12 medium followed by incubation in 10% FBS/Ham's F-12 medium at 37 °C for 15 h in 5% CO_2. After the trypsin treatment, the recovered cells were inoculated onto a 6-well plate in 1.25×10^4 cells/well and initiated in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku Co., Ltd) containing 10% dialyzed fetal bovine serum (dFBS; JRH Biosciencess, Inc.) at 37 °C under 5% CO_2. The medium was exchanged on days 1 and 2 to remove dead cells. From the cells in approximately 20 isolated colonies of the CHO transformants that kept growing on days 8 –10 after the incubation, cells showing high reactivity with the ligand peptide metastin (hereinafter merely referred to as GPR54/CHO) were selected to provide for the following experiment.

4.4. Measurement of $[Ca^{2+}]_i$ using the Flipr technology

Pigment mixture was prepared by addition of 2 vials of Fluo3-AM (50 μg/vial) in 21 μL of dimethylsulfoxide and 21 μL of 20% pluronic acid to 10 ml of HANKS/HBSS (prepared from 9.8 g of HANKS, 0.35 g of sodium hydrogencarbonate and 20 ml of 1 M HEPES, pH 7.4) containing 2.5 mM probenecid and 1% FBS. GPR54/CHO (3.0×10^4 cells/200 μL/well) was inoculated in 10% dFBS/DMEM on a 96-well plate for FLIPR (Black Plate Clear Bottom, Coster, Inc.), followed by incubation at 37 °C overnight in 5% CO_2. After the medium was removed, the pigment mixture was dispensed onto the cell plate in 100 μL each/well, followed by incubation at 37 °C for an hour in 5% CO_2. 1 mM peptide in dimethylsulfoxide was diluted with HANKS/HBSS containing 2.5 mM probenecid, 0.2% BSA, and 0.1% Chaps. The dilution was transferred to a 96-well plate for FLIPR (V-Bottom plate, Coster, Inc.; hereinafter referred to as a sample plate). After completion of the pigment loading onto the cell plate, the cell plate was washed 4 times with wash buffer (2.5 mM Probenecid in HANKS/HBSS) using a plate washer. After the washing, 100 μL of wash buffer was left. The cell plate and the sample plate were set in FLIPR (Molecular Devices, Inc.) and 0.05 ml of a sample from the sample plate was automatically transferred to the cell plate. A change in intracellular calcium ion level for 40 s was measured with passage of time.

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